

DEVELOPMENT OF CHROMATOGRAPHIC METHODS FOR PHYTOCHEMICAL STANDARDIZATION OF SELECTED HERBAL RAW MATERIALS USED IN UGANDA

(A thesis submitted by)

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DECLARATION OF AUTHORSHIP

The work presented in this thesis is my own. Where I have used the works of other persons, due acknowledgements are clearly stated. No portion of this work has been submitted in support of an application for a degree or qualification to any other University or Institute of higher learning.

I present it without any reservations for examination.

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DEDICATION

Without support and patience of family and friends, this work would be only a dream. The work is dedicated to Maama Kaggwa, who had a vision and sacrificed to see it pass. Dr. Dalton Segawa, PhD; gave birth to the academician in me by rewarding the small victories in high school.

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ACRONYMS AND ABREVIATIONS

NDA	National Drug Authority
WHO	World Health Organization
U.S. FDA	United States Food and Drug Authority
WHO	World Health Organization
AHPA	American Herbal Products Association
AOAC	The Association of Official Analytical Chemists
HPLC	High Performance Liquid Chromatography
HPLC/UV	High Performance Liquid Chromatography/Ultra Violet
TLC	Thin Layer Chromatography
HPTLC	High Performance Thin Layer Chromatography
GC/MS	Gas Chromatography/Mass Spectrometry
LC/MS	Liquid Chromatography/Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification
R^2	Square of the correlation coefficient (coefficient coefficient)
IC_{50}	Inhibitory concentration, 50%
TPC	Total Phenolic Concentration
R_F	Retardation Factor
tR	Retention time
r	correlation coefficient
c	congruence coefficient
d	Euclidean distance
PCA	Principal Component Analysis
HCA	Hierarchical

DEFINITIONS

Chemical markers

Chemically defined constituents of an herbal material utilized for control purposes. They may or may not contribute to the clinical efficacy. Markers are generally employed when constituents of known therapeutic activity are not found or are uncertain, and may be used to identify the herbal material or preparation or calculate their quantity in the finished product (World Health Organization 2016).

Chromatographic fingerprint of an herbal medicine/material

A characteristic pattern of peaks that is obtained from a chromatographic separation of a herbal product or plant material. The fingerprint can be used to identify the individual components of the mixture and to assess the overall quality and consistency of the sample (Xie et al. 2006a).

Herbal materials/herbal raw materials

Herbal materials include, in addition to herbs, other crude plant materials. These other plant materials can be, gums, resins, balsams, exudates, and other such materials. Herbal materials may also be referred to as medicinal plant materials (World Health Organization 2016).

Herbal medicines/herbal medicinal products

Finished, labelled medicinal products that contain as active ingredients aerial or underground parts of plants, or other plant material, or combinations thereof, whether in the crude state or as plant preparations. Plant material includes juices, gums, fatty oils, essential oils, and any other substances of this nature. Herbal medicines may contain excipients in addition to the active ingredients. Medicines containing plant material combined with chemically defined active substances, including chemically defined, isolated constituents of plants, are not considered to be herbal medicines (World Health Organization 2016)

Herbal materials

Herbal materials include, in addition to herbs, fresh juices, gums, fixed oils, essential oils, resins and dry powders of herbs. In some countries, these materials may be processed by various local procedures, such as steaming, roasting or stirbaking with honey, alcoholic beverages or other plant materials (Karlberg 2001).

Quality assurance

Quality assurance is the totality of the arrangements made with the object of ensuring that pharmaceutical products are of the quality required for their intended use (World Health Organization 2007a).

Standardization

The establishment of reproducible pharmaceutical quality by comparing a product with established reference substances, and by defining minimum and maximum amount of one or more compounds, or groups of compounds that are necessary in the product for efficacy and safety (World Health Organization 2016).

Chemometrics

An interdisciplinary field that involves the application of statistical and mathematical methods to chemical data. It has its roots in the field of multivariate analysis, which is concerned with the analysis of data sets that have more than one variable. Chemometrics extends multivariate analysis techniques to chemical data and is widely used in many areas of chemistry, including spectroscopy, chromatography, and mass spectrometry (Smilde, Geladi, and Bro 2005).

Analytical standards

An analytical standard is a well-defined and characterized material that is used as a reference for the quantitative and qualitative analysis of other substances. It is a known substance of high purity, identity, and concentration that is used to calibrate instruments, validate methods, and ensure the accuracy and precision of analytical measurements (AOAC 2012) .

Reference materials

Reference materials refer to materials other than substances appropriate for intended uses in standardization or quality control of herbs and herbal materials. Reference materials include, among others, herbarium samples, authentic specimens of herbal materials (such as extracts and their fractions), herbal reference preparations and authentic spectra or fingerprints (World Health Organization 2016).

Phytoequivalence

Phytoequivalence is a term used in the evaluation of herbal medicines and refers to the equivalence of two or more herbal products with respect to their bioactive constituents.

Specifically, phytoequivalence refers to the extent to which two or more herbal products contain the same or similar bioactive compounds, and in the same or similar amounts, and exhibit comparable pharmacological effects when administered at the same dose (Pace and Martinelli 2022).

Pharmacopoeia and monographs.

This is a formulary, usually having legal force in all pharmacies of a given country, containing a description of drugs in current medical practice and noting their formulae, analytical composition if known, physical constants, main chemical properties useful in identification and mode of preparation of compound or combination products. Details may also include specifications of assay methods to regulate purity, content of active constituents, preservation of quality, and, where appropriate, biological potency. Monographs are descriptions of different herbal medicinal formulae which can either be included in a pharmacopoeia or exist separately (World Health Organization 2019).

Good Manufacturing Practices (GMPs)

GMPs are codes of practice designed to reduce to a minimum the chance of procedural, instrument or manufacturing plant problems that could adversely affect a manufactured product. They specify “many requirements for quality control of starting materials, including correct identification of species of medicinal plants, special storage and special sanitation and cleaning methods for various materials” (World Health Organization 2019).

ABSTRACT

Background

The quality assurance of herbal materials is challenging because of the wide variation in phytochemical composition. This variation occurs naturally due to genetic difference and also because of the different climatic and soil characteristics of the plant cultivation sites. Furthermore, compound variation can also be introduced by agronomic practices, as well as post-harvest handling and processing of the plant materials. This necessitates reliable standardization methods to demonstrate phytoequivalence of the plant materials and their products. Due to the multicomponent nature of plant drugs, analyzing all compounds in a sample is not only expensive but also not practical. To solve this challenge, the use of markers and fingerprinting comes in handy. This work was aimed at establishing markers, and developing chromatographic fingerprints for the quality control of commercially important herbal materials. It specifically aimed to answer the following questions: (i) What analytical markers are suitable for quality control of selected medicinal plant materials used in Uganda? (ii) What are the TLC and HPLC fingerprint characteristics for the medicinal plant materials? (iii) What are the optimum HPLC methods parameters for quantification of markers in the medicinal plant materials? (iv) Are herbal materials harvested from the different agroecological zones of Uganda phytoequivalent?

Methods

This work employed the Herb Marker ranking system (HerbMaRS) to establish quality control markers for the most commercially used herbal materials in Uganda. Using a modified Herb MaRS, compounds were given scores from 0 to 8; where 8 indicated the most suitable chemical marker. Evidence of biological activity, was divided into three levels based on the number of symptoms of the disease condition a compound can treat or alleviate; (i) one symptom (1 point), two symptoms (2 points), 3 and more symptoms, with well elucidated mechanisms of action. The reported concentrations of the compounds were scored; concentration ≥5ppm (1 point), (concentration ≥50ppm, 2 points); then availability of analytical standards (1 point); and availability of an analytical method (1 point).

HPLC methods were developed to quantify the identified markers in different batches of the plant materials. TLC and HPLC fingerprints were developed and, in conjunction with chemometric data analysis techniques, including similarity indices, Principal component analysis, hierarchical cluster analysis and analysis of variance, used to evaluate the

phytoequivalence of different batches of the plant materials. The batches were defined as materials of the same plant harvested from different agroecological zones of Uganda.

Results

Using the Herb MaRS ranking system, this study has identified suitable markers for the quality assurance of herbal materials and phytochemical standardization of six out of the seven most commonly used medicinal plants in commercial products in Uganda, namely, *Eucalyptus globulus* Labill, *Aloe barbadensis* (L.) Burm.f., *Albizia coriaria* Oliv., *Mangifera indica* L., *Azadirachta indica* A.Juss., *Zingiber officinalis* Roscoe. The suitable markers for each plant are as follows: aromadendrine, α-terpineol, globulol, and 1,8,- cineol (for *Eucalyptus globulus* Labill.); aloin, aloe emodin, acemannan (for *Aloe barbadensis* (L.) Burm.f.); lupeol, lupenone, betulinic acid, betulin, and catechin (for *Albizia coriaria* Oliv.); mangiferin, catechin, quercetin, and gallic acid (for Mangifera indica L.); azadirachtin, nimbin, nimbidin (for Azadirachta indica A.Juss.); and 6, 8, 10-gingerols, 6-shogaol (for Zingiber officinalis Roscoe). For *W. ugandensis*, the compounds with known biological activity scored poorly as chemical markers because they lack analytical standards and/or analytical methods.

By comparing the TLC fingerprint images and calculating the jaccard indices of the bands, it was demonstrated that samples from different agroecological zones are phytoequivalent. While HPLC analysis of markers demonstrated marked variability among samples harvested from different agroecological zones., The chemometric analysis of HPLC fingerprints showed that samples of *M. indica* leaf materials and *A. coriaria* stembark materials were largely phytoequivalent. *W. ugandensis* stembark materials varied greatly. The possible sources of botanical reference materials were also highlighted: Kabarole, Pakwach and Ntungamo (*M. indica* leaves); Kabarole, Moroto, Mpigi (*A. coriaria* stembark); Kabarole, Kabale, Mpigi (*W. ugandensis* stembark).

Recommendations

The National Drug Authority and manufacturers of herbal medicines should adopt the identified chemical markers for quality assurance and standardization of products containing *Eucalyptus globulus* Labill, *Aloe barbadensis* (L.) Burm.f., *Albizia coriaria* Oliv., *Mangifera indica* L., *Azadirachta indica* A.Juss., *Zingiber officinalis* Roscoe. The identified markers should be evaluated for suitability at the various stages of the production chain of the herbal medicines in Uganda i.e., from authentication and quality control of raw materials to evaluating reproducibility in efficacy, safety, and stability of finished products notified by the National Drug

Authority. For *Albizia coriaria*, and *Mangifera indica* the developed HPLC quantitative method can be used to monitor the levels of markers in both the herbal materials and the finished herbal materials. The information about marker evaluation can be included in the future Ugandan medicinal plant monographs and or product data bases to guide their quality assurance. In addition, the developed chromatographic fingerprints should be used to control the quality of the *Albizia coriaria*, *Mangifera indica* and *Warburgia ugandensis* materials.

CHAPTER ONE: INTRODUCTION

1.1 Background

Medicinal plants have been used for centuries to treat ailments. They have also been a major source of modern medicines; plants contain diversity of secondary metabolites, hence offer an unlimited source of novel compounds. Plant based medicinal products are consumed all over the world and form a major component of traditional and alternative systems of medicine (World Health Organization 2019). Herbal remedies are particularly popular in the developing world, where more than 80% of the population relies on traditional medicines for primary health care. These medicines are readily available, affordable and perceived to be safe (World Health Organization 2019).

Traditionally, medicinal plant products have been prescribed by traditional healers within communities or self-prescribed. In this regard, assessment of efficacy and safety was based on history of use and informal knowledge passed on from generation to generation (Vanherweghem and Degaute, 1998). With commercialization however, there is need for scientific proof that the products are effective and safe. In fact, various studies have demonstrated deficiencies in the quality of herbal medicines; from adulteration with conventional medicines (Dumba et al. 2021), to substitution with ineffective or harmful plant materials and contamination with soil, microorganisms, pesticides, aflatoxins among others (Cosyns et al., 1999; Ernst, 2002). Furthermore, the phytochemical composition of plant materials varies from batch to batch due to a number of factors; plant species or cultivar, geographical source of the material, cultivation practices, harvesting and post-harvest handling, and manufacturing processes (Xie et al. 2006a).

To ensure phytoequivalence of herbal materials and products, selected marker compounds must be quantified coupled with fingerprint profiles. Markers are chemical compounds that are characteristic of a particular plant or group of plants, these might be biologically active or inactive. The quantitative analysis of active markers is used as a measure of the material's potency and the quality of the materials. Since the pharmacological activity of plants is due to many phytochemicals, some of which might not be known, most pharmacopoeias recommend augmentation of quantitative marker analysis with fingerprinting of the plant materials. Fingerprints are unique patterns representing chemicals present in the herbal material, as detected by the analytical technique such as chromatography, spectroscopy, DNA bar coding, among others. By analyzing the quantitative data and fingerprint profiles with chemometric

techniques, it is possible to classify materials in quality grades and to determine consistency in sources of raw materials and manufacturing processes thereby ensuring standardized materials and products.

Quality assurance of herbal medicines is complicated by the lack of harmonized guidelines and therefore the requirements for market authorization vary greatly; regulatory authorities in developed countries tend to be stricter than in developing countries. For instance, the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) require the manufacturer to demonstrate the efficacy and safety of the product through clinical trials, present a quality assurance plan for cultivation and harvesting of the plant materials as well as protocols for good manufacturing practices (Anquez-Traxler C, 2011; U.S. Food and Drug Administration, 2012). In contrast, traditional medicines are hardly regulated in developing countries; only 43% of countries in the WHO African region have a medicines regulation system, compared to 91%, 86% and 85% for the South-East Asia, Eastern Mediterranean, and European regions, respectively (World Health Organization 2019).

To facilitate standardization of herbal medicines in resource limited countries, the WHO has published a number of guidelines for quality assurance of herbal medicines, including the guidelines for registration of traditional medicines in the African region (WHO, 2010), guidelines for assessing the quality of herbal medicines (WHO, 2017) and the guidelines for good herbal processing practices for herbal medicines (WHO, 2018). Despite these guidelines, in many African countries, traditional medicines are only notified but not registered. This is because the medicines have not been evaluated fully for quality, safety and efficacy owing to regulatory, scientific and manufacturer inadequacies (World Health Organization 2019). In addition, due to lack of financial support for training and research on T&CM, most African countries lack locally relevant scientific data on efficacy and safety of traditional medicines (World Health Organization 2019).

1.2 Problem statement

Despite the high consumption of herbal medicines in Uganda, their regulation is still inadequate. All locally manufactured herbal medicinal products authorized for marketing in Uganda are only notified and not registered, due to lack of evidence for safety, efficacy and quality (National Drug Authority 2021). The only requirements for notification of products are phytochemical screening, botanical and organoleptic evaluation of the plant raw materials, and absence of microbial contamination. One of the major challenges is that Uganda lacks

herbal medicines pharmacopoeia or local plant monographs (World Health Organization 2019). While it is possible to validate and adopt plant monographs published elsewhere, these are rare for the majority of plant materials used for industrial production of herbal products in Uganda. Of the 33 plant materials used commercially in Uganda, only 30% (10) have monographs by the WHO, 30% (10) by the African Pharmacopoeia and 20% (6) by the West African Herbal Pharmacopoeia. More than 55% (18) of the plants do not have a monograph in any of these pharmacopoeia (Kaggwa, Kyeyune, Munanura, Anywar, Lutoti, Aber, Bagoloire, Weisheit, Tolo, Kamba, et al. 2022).

Even for plant materials with monographs published elsewhere, adoption is a challenge. Unlike for conventional medicines for which the chemical constituents are the same, such that monographs developed by authorities in innovator countries such as the British Pharmacopoeia, can be easily implemented in other countries, herbal medicines differ in composition due to differences in climate, cultivation, harvesting and manufacturing practices. In addition, the plants are used to treat different ailments by different cultures. This means that evidence of efficacy and safety of plant materials established in one country might not apply in another. Further still, the choices of analytical marker compounds might differ especially if active markers are required, and the recommended analytical methods recommended in rich countries might not be affordable in most African countries.

Therefore, countries struggling to regulate herbal medicines such as Uganda need to establish their own reference literature, including monographs to guide the production and use of herbal medicines; evidence of efficacy and safety of the plants, appropriate cultivation sites, harvesting and processing practices, and quality evaluation of the herbal materials and their products. In addition, there is need to establish, technical protocols, reference botanical materials and fingerprints to facilitate standardization of herbal products.

1.3 Significance of the study

In this study, TLC and HPLC fingerprints were developed. In addition, a database of markers for the most used herbal materials was established. Last but not least analysis of materials gave insight into the best geographical sources of raw materials. The reference fingerprints and quantitative methods established will be used by the drug regulatory agency, herbal product manufacturers and quality control labs to assess the quality of herbal raw materials at various stages in the production chain including; selection of planting materials, cultivation sites, monitoring the consistency in the quality of materials, detection of adulterated and poor-quality materials, calculation of dosages for product formulation. Furthermore, the knowledge

generated by the study will be applied to develop protocols for quality assurance of other plant materials and herbal medicinal products, which can contribute to a future Ugandan pharmacopeia for herbal medicines. The information generated and protocols so developed have been published to the scientific community, in four manuscripts, as a contribution to evidence based safe and effective use of herbal medicines (Kaggwa, Munanura, et al. 2023; Kaggwa, Nakayita, et al. 2023; Kaggwa, Anywar, et al. 2023; Kaggwa, Kyeyune, Munanura, Anywar, Lutoti, Aber, Bagoloire, Weisheit, Tolo, and Kamba 2022); the abstracts are shown in Appendix 1, Appendix 2, Appendix 3 and Appendix 4.

1.4 Objectives

1.4.1 Main objective

To establish analytical markers and chromatographic methods for standardization of selected medicinal plant materials used in Uganda.

1.4.2 Specific objectives

i. To establish analytical markers for quality control of *Eucalyptus globulus, Aloe barbadensis, Mangifera indica, Azadirachta indica, Zingiber officinalis, and Albizia coriaria, and W. ugandensis materials* used in Uganda.
ii. To develop TLC and HPLC fingerprints for the qualitative standardization of *A. coriaria stembark, M. indica leaves* and *W. ugandensis* stembark materials used in Uganda.
iii. To develop HPLC methods for quantitative standardization of *A. coriaria stembark, M. indica leaves* and *W. ugandensis stembark* materials used in Uganda.
iv. To determine if *A. coriaria stembark, M. indica leaves* and *W. ugandensis stembark materials* obtained from different agroecological zones in Uganda are phytoequivalent.

CHAPTER TWO: LITERATURE REVIEW

2.1 Phytochemical standardization of herbal materials; why standardize?

Standardization of herbal materials is required to limit the variability of the chemical compounds in herbs in order to produce products with reliable safety, quality and efficacy (American Herbal Products Association 2003). The phytochemical composition of plant materials varies depending on the species, climatic and soil properties of the cultivation site, cultivation and harvesting practices, as well as manufacturing processes. Furthermore, variation in herbal composition can result from deliberate or accidental use of the wrong plant species or part of the plant (Sidhu, Kumar, and Behl 2004; World Health Organization 2016). In order to produce a consistent product, controls must be implemented over both the raw material supply and the manufacturing process. Controls over raw materials include specifying the plant species, variety, and plant part used; use of consistent cultivation conditions; and adherence to appropriate harvesting and post-harvest processing practices. To confirm batch to batch consistency in the chemical composition of the plant materials, qualitative and quantitative analyses should be performed. The qualitative evaluation of plant materials involves screening and quantification of the major phytochemical groups, and the establishment of fingerprint profiles., on the other hand, quantitative evaluation involves the determining the concentration of selected chemical markers (American Herbal Products Association 2003). Once a chemical profile is established, the data are analyzed by use chemometric methods to determine the phytoequivalence of the different plant batches (Cai, Ong, and Liu 2012) (Figure 1). Phytoequivalence is a term used in the evaluation of herbal medicines and refers to the equivalence of two or more herbal products with respect to their bioactive constituents. Specifically, phytoequivalence refers to the extent to which two or more herbal products contain the same or similar bioactive compounds, and in the same or similar amounts, and exhibit comparable pharmacological effects when administered at the same dose (Pace and Martinelli 2022).

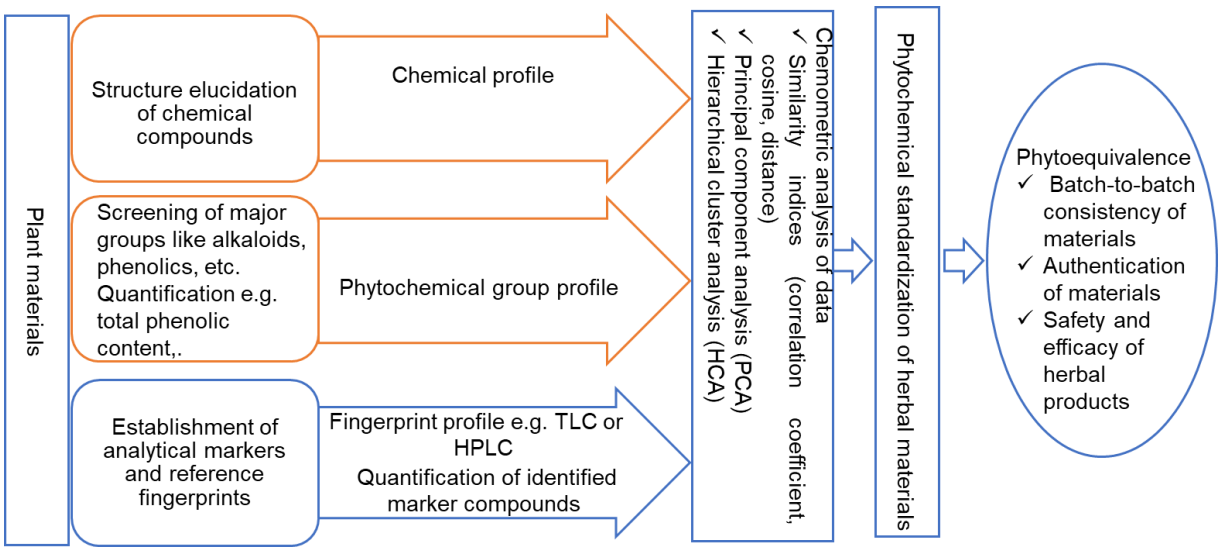


Figure 1: Theoretical framework for phytochemical standardization of plant materials; the text in blue outlines shows the scope of this work. The figure was drawn by the Author.

2.4 Quantification of phytochemical groups

Once the presence of particular phytochemical groups is confirmed, the quantification of the major chemical groups of interest follows. This is especially so when the phytochemicals are responsible for the efficacy of the drug. This approach is sometimes preferred to analysis of a few marker compounds and particularly when the activity of a particular plant extract cannot be attributed to a single or few compounds (American Herbal Products Association 2003) . Such tests include total alkaloids, total flavonoid content, total phenolic content, total tannins, total saponins among others. Most of these studies are spectrophotometric methods measuring the concentration of complexes formed by reacting chemical reagents with phytochemicals (American Herbal Products Association 2003). In this study, only the total phenolic content (TPC) was applied and thus it is discussed in details.

Phenolics are compounds that have one or more hydroxyl groups on the aromatic skeleton. These include flavonoids, tannins, phenolic acids, stilbenes and lignans. They possess a number of biological activities, notably anti-inflammatory and analgesic (Evans 2009). These activities are attributed to the ability of the phenolic to; (i) scavenge free radicals and reactive oxygen species (ROS), thereby reducing oxidative stress and protecting cells and tissues from damage, and (ii) inhibit the production or activity of inflammatory mediators, such as cytokines and enzymes like cyclooxygenase (COX) and lipoxygenase (LOX) (Rice-Evans, Miller, and Paganga 1996). The TPC of a herbal material can be determined by the popular Folin-Ciocalteu (FC) method developed by Singleton and Rosi in 1965 (Pungot et al. 2020). The FC

reagent, a mixture of phosphomolybdic/ phosphotungstic acid complexes is reduced by phenolic compounds in alkaline medium to form a blue complex which absorbs light in the range of 690 to 765 nm. The absorbance of the complex is proportional to the concentration of phenolic compounds in the plant material. Gallic acid is used as the reference standard compound and results are expressed as gallic acid equivalents (mg/g). While the Folin-Ciocalteu method is fast and simple, it has got a number of limitations: First, the method measures the reducing capacity of the plant sample; this reaction is not specific and so non-phenolic reducing compounds in the sample, such as ascorbic acid and reducing sugars, can also contribute to the measured absorbance, leading to an overestimation of the phenolic content (Amorati and Valgimigli 2015). Second, the Folin-Ciocalteu method is also sensitive to interference from other components in the sample matrix, such as proteins, pigments, and organic acids. These substances can result in false positive or negative responses, affecting the accuracy of the quantification. Since different phenolic compounds can exhibit varying reactivities towards the Folin-Ciocalteu reagent, some phenolics may not be detected or may be underestimated using this method, while others may be overestimated due to their higher reactivity (Peterson 1979) .

2.5 Use of chemical markers for quality control of herbal medicines

The European Medicines Agency (EMEA) defines chemical markers as chemically defined constituents or groups of constituents of a herbal medicinal product which are of interest for quality control purposes regardless whether they possess any therapeutic activity or not (The European Medicines Agency, 2008). Ideally, chemical markers should be unique components that contribute to the therapeutic effects of a herbal medicine. As only a small number of chemical compounds were shown to have clear pharmacological actions, other chemical components are also used as markers. The quantity of a chemical marker can be an indicator of the quality of a herbal medicine. Markers may be measured in both raw materials and finished products to obtain useful information for various applications. These include; the identification and selection of raw materials where concentration limits are set, identification of adulterants and toxicants, assessing batch-to-batch uniformity of materials from different sources, controlling of the manufacturing process, assessing the suitability of packaging and storage, standardizing physiological activities; and calculation of dosage of raw materials to include in the product formula (Figure 2)

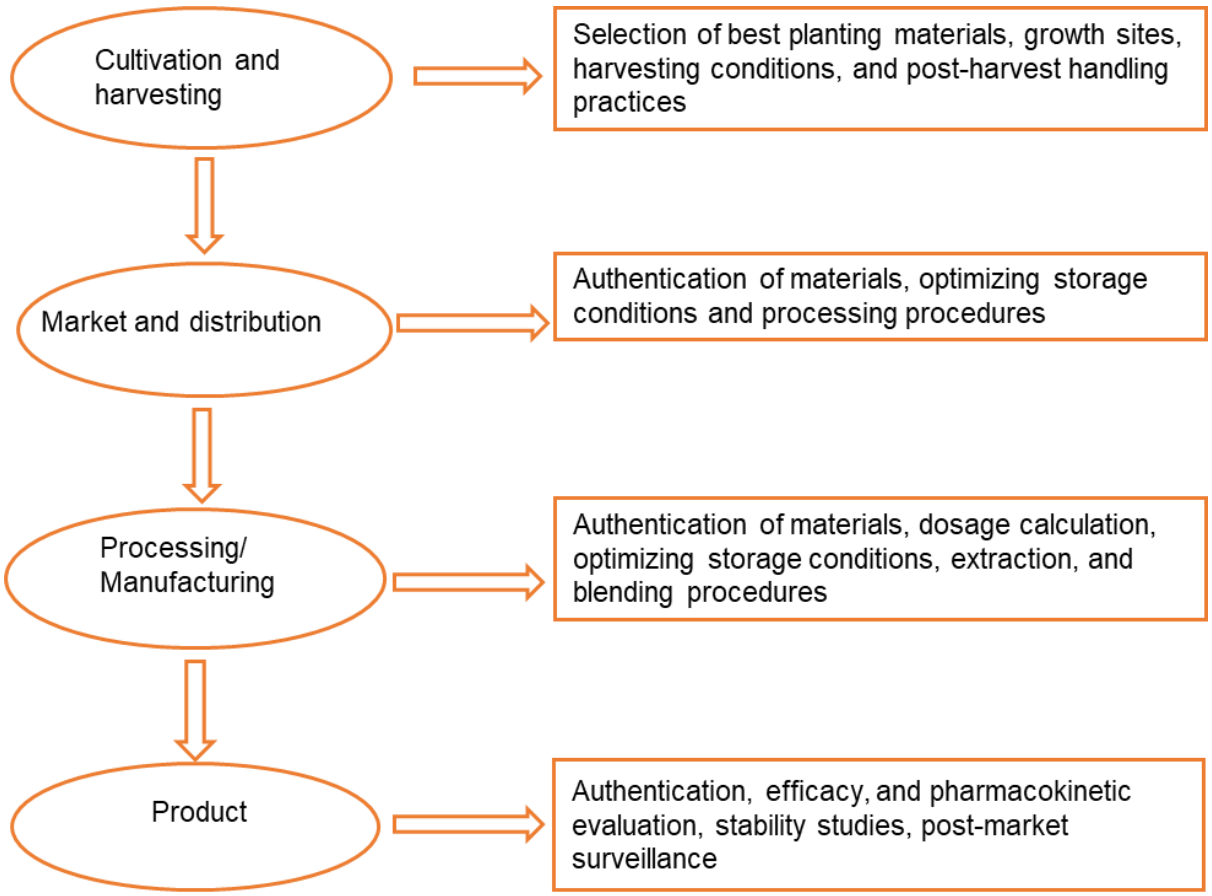


Figure 2: Applications of markers at different stages of the herbal medicines production chain. The figure was drawn by the Author.

According the WHO, the selection of reference substances for quality control of herbal medicines should consider that various ingredients may have different levels of influence on the final quality, safety and efficacy. The order of selection of markers compounds for identification and quantification of herbal materials or products should follow the following rules: 1) If constituents with known therapeutic activity (activities), relevant to the traditional use of the plant, have been identified, they should be used as markers. However, for most plant extracts, clinical efficacy data is not available for the potential marker compounds. Furthermore, it is known that the observed pharmacological activity of plants is contributed by several compounds. Therefore, it is hard to assign the observed biological activities to a few compounds. 2) in this case, constituent(s) with some recognized pharmacological activity (activities) can be used be used as markers. For such compounds, evidence of biological activity has been demonstrated by invitro and/or in-vivo bioassays. Such evidence is not sufficient to confidently attribute the pharmacological activity exclusively to these compounds.

3) If these compounds are also not available, as is the case for more herbal materials, the identity and quantity of herbal materials, can be established by the production process and by analyzing marker substance(s) containing other characteristic constituent(s), whose contribution to the activity of the plant is not known-such compounds are for analytical purposes only and the results of analysis will not relate to the activity of the plant material (World Health Organization 2016).

Markers used as chemical reference substances should be international chemical or pharmacopeial reference substances. If others are used, markers for quantitative determination should be of high purity as required by national regulations, determined by validated analytical methods, including physical and chemical ones. These analytical methods may be different from those employed for quantifying herbal materials. For markers used for identification, lower purity may be suitable. The general requirements for markers are: identity, specificity and selectivity using the specified analytical method(s), should be present in traceable quantity for identification or sufficient quantity for assay; should be easily obtained, stable under specified storage conditions; should be easily detected and quantified analytically (World Health Organization 2016).

In line with the WHO and other regulatory guidelines, researchers at the National Institute of Complementary Medicine, University of Western Sydney, proposed a Herbal Chemical Marker Ranking System (Herb MaRS) for selecting markers for quality control of complex herbal products. The Herb MaRS was aimed at providing a uniform and comprehensive guide for selection of markers compounds for quality control of polyherbal products. The authors validated this system using a herbal product made from seven herbs. To determine the suitability of phytochemicals as markers, the Herb MaRS ranks the compounds on a scale of 0 to 5. A compound scoring 5 is the most suitable; this is a compound with the highest pharmacological activity related to major symptoms of the disease as claimed by the manufacturer; present in relatively high concentration in the herb or finished product (at least 5µg/mL); and bioavailable. In addition, it is mandatory to screen toxic compounds and so they are scored 5 by default. (Bensoussan et al. 2015). The Herb MaRS criterion is summarized in Table 1 (Bensoussan et al. 2015).

Table 1: Herbal Chemical Marker Ranking System (Herb MaRS) criteria for identification of QA/QC in complex herbal medicines

Rank	Details	Notes
X	There are no bioactivity studies currently available for this compound.	A comprehensive literature search does not reveal evidence to support relevant bioactivity. (Bioactivity studies refer to studies which are designed to determine physiological or pharmacological effects of the relevant compound using in vitro and/or in vivo models.)
0	Compound's activity is not related to indication of the disease of interest or no pure reference standard is available.	Indication of disease refers to principal indicative symptoms or biological markers of disease.
1	Compound's bioactivity is indirectly related to symptoms of disease OR Compound is present in too low a concentration in the herb for it to be screened OR Compound's activity is not supported by traditional and therapeutic use of herb	The compound may address a minor or less common symptom of the disease, but not the major clinical presentation. Usually, concentration of a constituent in a herbal medicine < 5ppm is generally regarded as too low. Biological activity of the compound is not related to the current use of the herb and/or its traditional use in the context of the formula.
2	Compound has bioactivity related to a symptom (major or minor) of the disease, however there is only 1 good quality study to support this action AND	Minor symptoms include those less clinical troublesome or less common presentations.

	Compound is present in low concentration (5–50 ppm or < 1 µg/mL) in the herb AND Traditional or current use of the herb is consistent with the bioactivity or pharmacological effects of the compound
3	Compound has bioactivity related to a symptom (major or minor) of the disease, however there is only 1 good quality study to support its action AND Compound is present in relatively high concentration (> 50 µg/mL) (or >10mg/g) in the herb or finished product AND Traditional or current use of the herb is consistent with the bioactivity or pharmacological effects of the compound
4	Compound has high bioactivity related to major symptom(s) of the disease for which there is sufficient evidence to support its activity (≥2 reported good quality studies) AND

	Compound is present in relatively high concentration in the herb or finished product AND Traditional or current use of the herb is consistent with the bioactivity or pharmacological effects of the compound	
5	Compound may be toxic and needs to be screened to comply to safety limits OR (all the following apply) Compound has highest bioactivity related to major symptoms of disease for which there is sufficient evidence to support its activity (≥2 reported good quality studies) AND Compound is present in relatively high concentration in the herb or finished product? AND Traditional or current use of the herb is consistent with the bioactivity or pharmacological effects of the compound AND Compound and/or its metabolites are bioavailable	Potentially toxic compounds are recommended to be monitored if used at one 50th or more the anticipated delivery dose in a final product.

5’ represents the most suitable marker for monitoring; ‘0’ represents the least suitable; ‘x’ means the marker is currently unsuitable. **Source:** (Bensoussan et al. 2015)

Once the suitable marker is established, quality assurance of herbal materials follows the steps outlined in the (Figure 3) below (American Herbal Products Association 2003).

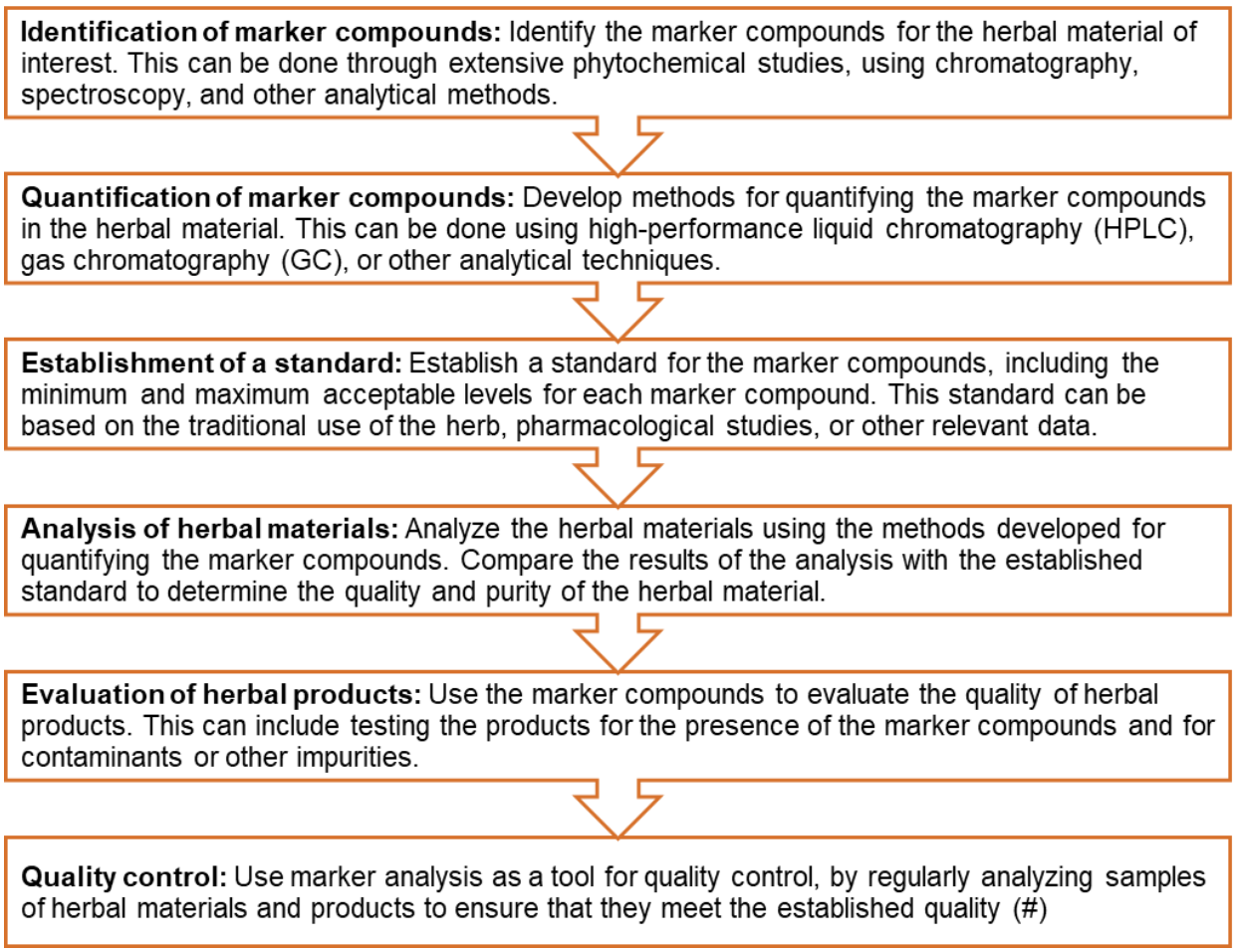


Figure 3: Flowchart of activities that constitute quality assurance of herbal materials using marker compounds.

The U. S. Food and Drug Administration (FDA) has established two different minimum levels that label claims must meet depending on the type of ingredient. These two guidelines are commonly known as “the 80% rule” and “the 100% rule.” The 80% rule applies to constituents that occur naturally in an ingredient and whose level in the ingredient is not adjusted by the manufacturer. The 100% rule applies to components whose content is controlled directly by the manufacturer (American Herbal Products Association 2003).

Limitations of using markers for quality assurance of herbal materials

Marker analysis is a useful tool for standardizing herbal materials and medicines, but it has some limitations that should be considered (American Herbal Products Association 2003; World Health Organization 2016): Herbal materials are complex mixtures of many compounds, and the identification and quantification of marker compounds can be challenging. Some compounds may be present in low concentrations, or may not be easily extracted or quantified using the methods available. Furthermore, herbal materials can vary in their composition, depending on factors such as the growing conditions, harvesting methods, and processing techniques (Bensoussan et al. 2015). This variability can impact the presence and concentration of marker compounds, making it difficult to establish a consistent standard. It can be difficult to select appropriate marker compounds, as some compounds may not be specific to the herbal material, or may not be the compounds responsible for the therapeutic effects of the herb (World Health Organization 2016). The choice of markers should be based on a thorough understanding of the phytochemistry of the herb, and should be validated through pharmacological studies or other relevant data. One of the major challenges in analysis of markers is lack of standardized methods for the analysis of marker compounds in herbal materials, which can lead to variability in the results obtained by different laboratories (American Herbal Products Association 2003). This variability can impact the reliability of the results and make it difficult to compare the quality of herbal products from different sources. Even when markers are successfully analysed, there is limited applicability of the results. This is because this kind of analysis is only able to assess the presence and concentration of specific marker compounds. It may not provide a comprehensive assessment of the quality of the herbal material or product, and may not identify contaminants or other impurities that are not present as marker compounds (American Herbal Products Association 2003; Noviana, Indrayanto, and Rohman 2022).

Because of these limitations, controlling of the concentration of marker compounds does not always ensure batch to batch phytoequivalence. This necessitates the use of complementary methods such as chemical fingerprints to provide a more comprehensive assessment of the herbal material.

2.6 Use of chemical fingerprints in quality assurance of herbal materials

Chemical fingerprints of a herbal material refer to specific patterns or profiles of chemical constituents present in the medicinal plant (Xie et al. 2006a). They can be generated using

various analytical techniques including chromatographic methods (e.g., high performance liquid chromatography (HPLC), gas chromatography (GC), thin layer chromatography (TLC), spectroscopic techniques (e.g., nuclear magnetic imaging (NMR), mass spectroscopy (MS), infra-red (IR), capillary electrophoresis, deoxyribonucleic acid (DNA) profiling, among others. Chromatographic fingerprinting is the main form of fingerprinting used by manufacturers and researchers because of the strong separating power and adaptability. The chromatographic fingerprints visually represent peaks or bands of known and unknown compounds present in the herbal material (Noviana, Indrayanto, and Rohman 2022). These fingerprints provide valuable information about herbal materials and can be applied to solve a number of analytical questions: (i) Identity of the material; this is achieved by is comparing the fingerprint of the unknown material to that of the reference botanical material and or by investigating for presence of some specified marker compounds. (ii) Purity of the material (freedom from adulterants or substitutes); the fingerprint of the authentic material is run alongside that of the suspect sample, the likely adulterant plant material and investigated for presence of specified marker compounds. (iii) stability of the herbal material; the fingerprints collected at different times of storage or during stress tests are examined for change in the number, color and intensity of bands (Xie et al. 2006a; American Herbal Products Association 2003). This enables establishment of the shelf life of the materials. In addition to these applications, chromatographic fingerprinting can be employed during (iv) process development to assess the effect of variation in process parameters; and (v) to determine the best practices for cultivation and harvesting of plant materials such as seed selection, time for planting and harvesting, post-harvest handling, among others. When chromatographic fingerprints are correlated with pharmacological activity, the therapeutic equivalence of herbal materials can be modeled, this gives rise to the so-called bioactive fingerprints (Xie et al. 2006a); these can guide isolation of active compounds and development of modern medicines

Selection of a chromatographic technique to develop the fingerprints is determined by the type of sample, the required performance characteristics and the availability of the equipment. TLC is easy to operate, uses affordable and readily available equipment and can be used when a high throughput screening of samples is required, for instance, to identify herbal materials (Tistaert, Dejaegher, and Vander Heyden 2011). Several samples can be analyzed in parallel on the same plate under the same conditions. In addition, optimization of parameters such as sample application type, mobile phase composition, and plate development is quite easy. Once developed, the fingerprints can be detected by several methods including normal light, UV light, and as colored bands formed by spraying the TLC plate with chemical reagents

(Reich and Schibli 2007a). The visual images facilitate data analysis and storage for further reference. However, TLC is hindered by its low reproducibility and resolution and requires use of concentrated solutions of samples and reference substances (Ettre 2002). The poor reproducibility is due to the open nature of the TLC system. The plate is exposed to environmental factors such as light, air, fumes and climatic conditions including humidity and temperature. Also, manual application of samples is also hard to reproduce. The reproducibility and resolution of TLC can be improved by upgrading to an HPTLC system; In HPTLC, samples are spotted on high performance plates by an automatic sampler, plate development is carried out in a closed chamber whose temperature and humidity can be controlled, and derivatization, visualization, as well as recording of data are all computerized. By scanning the plates with a densitometer, quantitative information can be generated unlike in ordinary TLC. Of course, the improved performance comes at a cost. The HPTLC equipment is expensive to buy, and maintain (Reich and Schibli 2007a) and so cannot be afforded by most manufacturers and laboratories in developing countries.

HPLC is the most popular analytical technique for construction of herbal materials fingerprints. It is easy to operate and automate, and has high resolution, selectivity, sensitivity and reproducibility (Snyder, Kirkland, and Dolan 2011). Furthermore, several detectors for identification of the separated compounds and evaluation of fingerprints are available, including; ultra violet (UV) detectors , diode array detectors (DAD) , evaporative light scattering detectors (ELSD) mass spectrometers (MS) , among others (Kazakevich and Lobrutto 2007). Unlike TLC, HPLC is a closed-system. This ensures a good stability of the samples during analysis. However, HPLC equipment is expensive to procure and maintain and the high volumes of waste chemicals produced can damage the environment (Tistaert, Dejaegher, and Vander Heyden 2011).

GC shares the positive attributes of HPLC i.e., high resolution, sensitivity and reproducibility. However, the technique is limited to analysis of volatile plant samples (and compounds) such as essential oils. Nonvolatile components need to be derivatized, which increases the analysis costs; besides, derivatization is not possible for some compounds. Due to the high temperatures applied, thermolabile compounds might be degraded (Tistaert, Dejaegher, and Vander Heyden 2011). Just like HPLC, procurement and maintenance of GC equipment is costly. This study was focused on development of TLC and HPLC fingerprints for herbal materials, which are discussed in the next section.

2.6.1 Development of chromatographic fingerprinting methods

A method is a detailed description of the steps to follow in analysis of a material, from sample preparation to reporting of results, for a stated purpose (AOAC 2002). The development of chromatographic fingerprints involves collection of a representative sample of the plant material, optimization and validation of chromatographic separations and characterization of the fingerprints (Cai, Ong, and Liu 2012). These activities are summarized in Figure 4 below.

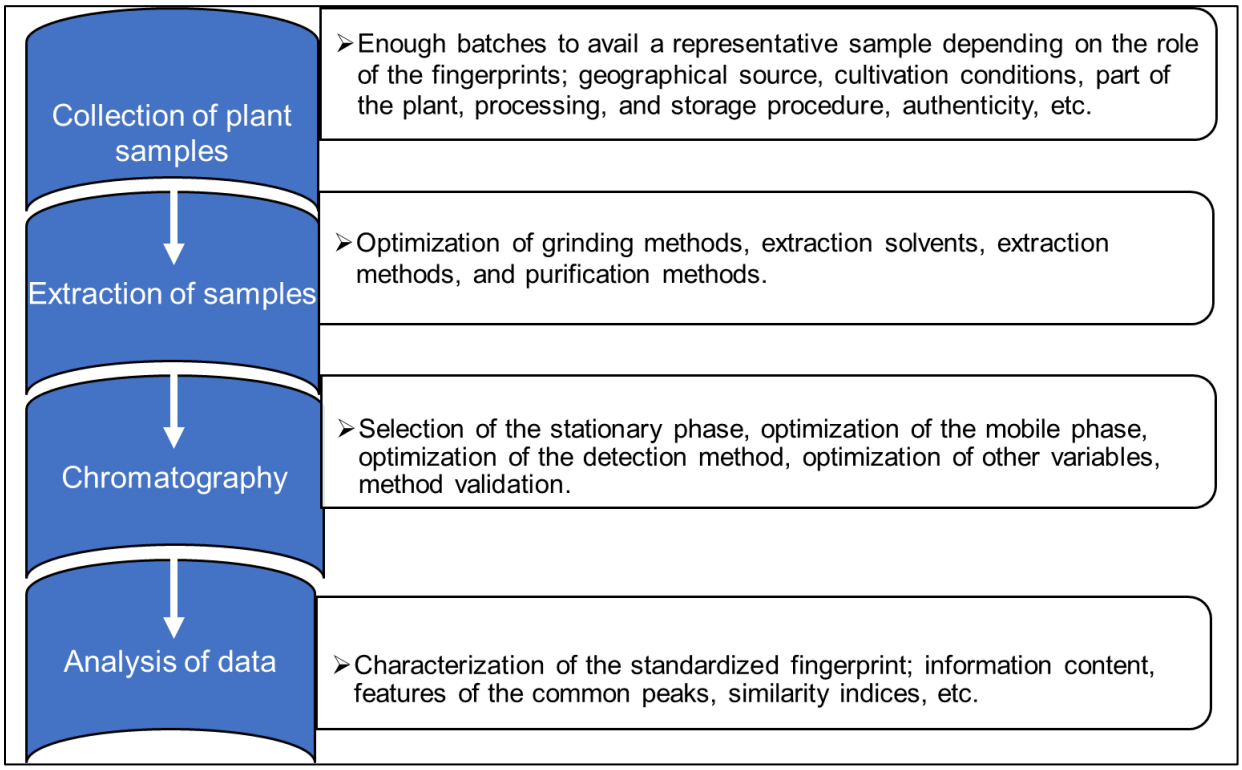


Figure 4: Flowchart showing steps involved in developing chromatographic fingerprints for quality assurance of herbal materials.

Collection of pant materials

Sample collection is an essential step in the development of chromatographic fingerprints. The samples collected must be representative to enable collection of adequate data for studying the variables at hand (Noviana, Indrayanto, and Rohman 2022). The variables include; the plant cultivar or species, the part of the plant used to produce the medicine, geographical source of the material, cultivation conditions, season of collection, processing and storage procedures. These are the factors that influence the distribution of secondary

metabolites within medicinal plants (Cai, Ong, and Liu 2012). The pharmacopoeia of the people's republic of China recommends collection of at least ten "batches", where a batch refers to "mutually independent samples, that is, samples from the same location, same channel or same collection time should not be considered as separate sample batches, so as to ensure that the test results are representative (Xie et al. 2006a)". Examples of different batches can be obtained as follows; collecting samples from different cultivation sites, collecting samples of different grades from the same place of origin, collecting samples from different parts of the plant, collecting samples from different cultivars or varieties of the same plant, among others. The ultimate number of samples is also determined by the number of sample categories available for example number of cultivars of the plant species, the different geographical cultivation areas, weather seasons within the year, and so on (Cai, Ong, and Liu 2012; Alaerts et al. 2010). Each category should be represented (Goodarzi, Russell, and Vander Heyden 2013).

Extraction of the samples

Sample preparation is a critical step in fingerprint development. The extent and nature of extraction is dependent on the type or purpose of the fingerprint. Fingerprints might target particular functional groups such as alkaloids, flavonoids, terpenoids etc. or they might be intended to evaluate the whole range of compounds within the herbal materials, for which a general fingerprint is required (Liang, Xie, and Chan 2004). Thus, to select a suitable extraction and separation method, one ought to pay attention to the chemical composition of the medicinal material, the resultant extract, and the requirements of the fingerprints. To optimize sample preparation, experiments are carried out on selected solvents, and extraction methods. Broad spectrum solvents like methanol and ethanol and their aqueous mixtures (70-80%) are preferred for general fingerprints, to maximize the number of components extracted (Goodarzi, Russell, and Vander Heyden 2013). Specific solvent compositions to target particular components can be prepared based on the polarity of their solubility. For instance, hexane and toluene are the solvents of choice for lipophilic compounds like terpenes, while hydrophilic constituents like flavonoids and tannins are better extracted by polar solvents such as ethyl acetate, alcohols, water, formic acid, and acetic acid. Whenever available, continuous reflux extraction (soxhlet) is preferred to cold maceration because it is more efficient. However, the method is not ideal for thermolabile compounds. In that case, maceration should be used; it efficiency can be improved by sonication. Sometimes decoction is favored so that the fingerprint gives an indication of the quality of the native extract (herbal decoction

product). For essential oils, which are volatile, distillation is the method of choice (Cai, Ong, and Liu 2012; Zhang, Lin, and Ye 2018; Tistaert, Dejaegher, and Vander Heyden 2011).

Selection of chromatographic technique

The factors that influence the selection of chromatographic techniques have been discussed in the previous section 2.6.

Chromatography method development

Chromatography is a separation of components of a mixture based on their different affinities for the stationary and mobile phases. As the mobile phase moves through the stationary phase, it 'drags' with it those components with high affinity for it. The components with higher affinity for the stationary phase follow on in a rather slower fashion, hence the separation. This section describes the optimization of various parameters to obtain a standard TLC or HPLC fingerprint.

Selection of the stationary phase

The stationary phase in TLC is a thin layer of material supported on glass, aluminium or plastic plate, while for HPLC the material is coated on the walls, or packed inside a stain-less steel column (Poole 2015). The material is made of, most commonly silica and its derived products. the popularity of silica based stationary phases arises from the facts that silica is made from readily available and inexpensive raw materials, silica stationary phases allow higher sample loadings, and are inactive to sample components. Other stationary phase materials include; aluminium oxide, kieselguhr, cellulose, starch, among others (Tistaert, Dejaegher, and Vander Heyden 2011). The nature of the stationary phase determines the types of interactions with the components of the sample mixture, as well as the with the mobile phase. Polar stationary phases, used in normal phase chromatography, adsorb molecules of the analytes, the more polar the components, the more they are retained. The polarity of silica is due to free silanol hydroxyl (-OH) and siloxane groups. These interact with polar analyte molecules through proton donor or acceptor (amines, acids, phenols, alcohols) and, dipole-dipole (carbonyls, ethers, halogenated compounds) interactions (Ahuja and Dong 2005; Kazakevich and Lobrutto 2007). Depending on the nature of functional groups present in the component molecules as well as their stereochemistry, other interactions are possible with silica. For instance, aromatic compounds and unsaturated compounds can interact through dipoles induce by the stationary phase, while nonpolar compounds interact based on dispersion forces. The polarity and types of interactions can be modified by forming bonded silane

derivatives, aimed at separation of specific phytochemicals, as shown in Figure 5 (Figure drawn by author).

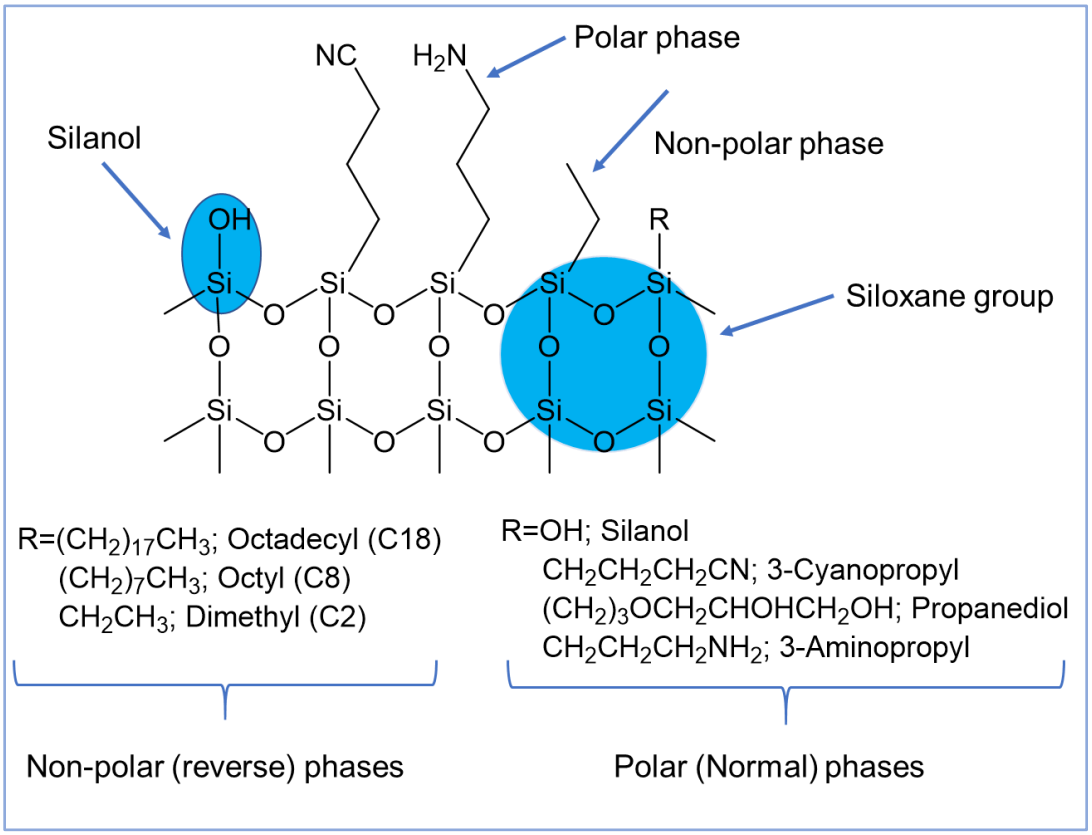


Figure 5: Types of stationary phases derived from silica

Non-polar bonded phases, used for reverse phase chromatography, are alkyl silane derivatives. The longer the chains, the more hydrophobic the stationary phase. The commonly used reverse phase materials are shown in Figure 5. The main mode of retention on non-polar stationary phases is partition. For TLC, some of the mobile phase is adsorbed on to the stationary phase during chromatography, resulting in the so-called solvent generated stationary phase; analytes distribute between this layer of the mobile phase and that in transit (Reich and Schibli 2007a). In HPLC, a liquid stationary phase is immobilized on a solid support by chemical bonds or adsorption. Besides the chemistry of the stationary phase, the resolution of the mixture is also affected by the size of the particles. According to Van Deemter (Equation 1 and Figure 6), the constant A, a measure the eddy diffusion, depends solely on the average particle size and homogeneity of the stationary phase. The larger the particle sizes, the higher the A term and thus the broader the peaks (higher H=lower column efficiency). Therefore, to

improve the quality of the peaks, the particle size needs to be kept at an ideal size (Kazakevich and Lobrutto 2007).

$$H = A + {}^{B}/_{u} + Cu \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots Equation\ 1$$

Where H is the height equivalent of a theoretical plate, A term expresses eddy diffusion (Eddy diffusion refers to the process of molecular diffusion in turbulent fluids), B is the longitudinal diffusion constant, C is constant expressing the stagnant mobile phase, and stationary phase mass transfer, while u is the flow velocity of the mobile phase.

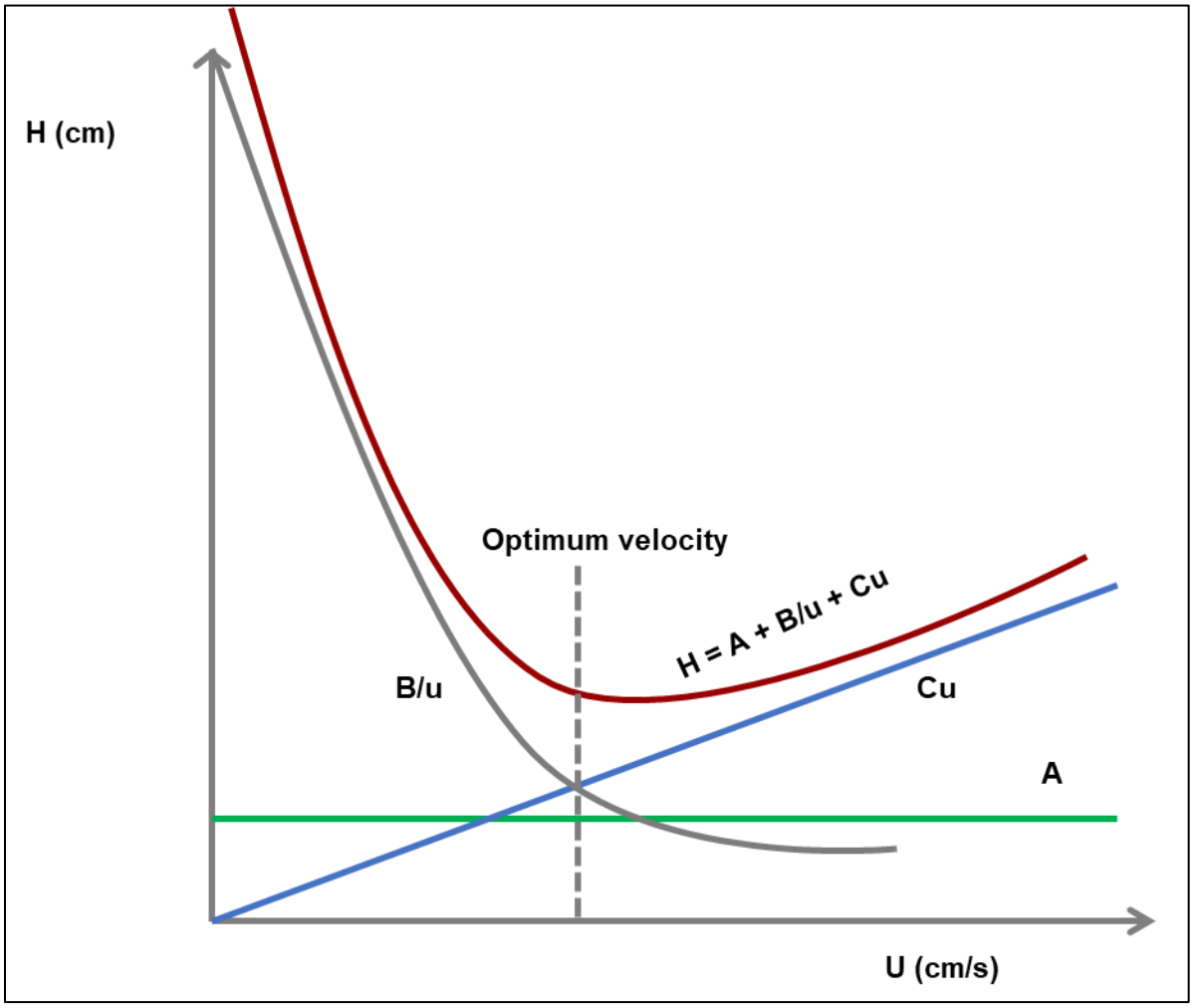


Figure 6: Plot of the Van Deemter equation. H is the height equivalent of a theoretical plate The A term expresses eddy diffusion, B is the longitudinal diffusion constant, C is constant expressing the stagnant mobile phase, and stationary phase mass transfer; while u is the flow velocity of the mobile phase (Reich and Schibli 2007a).

Below a certain size, the particles become too compacted, leaving little space for the mobile phase to pass through. Besides, the backpressure is too high. Thus, for TLC and HPTLC systems, where the mobile phases move through the stationary phase solely by capillary attraction, the particle sizes are limited to 15 µm and 5 µm respectively. On the other hand, the use of pressure to pump the mobile phase through the column affords HPLC stationary phases pore sizes as low as 1um. This partly explains why HPLC has better resolution than both TLC and HPLC (Poole 2015; Kazakevich and Lobrutto 2007).

Optimization of the mobile phase

The composition of the mobile phase is determined by the stationary phase used in the HPLC or TLC system. Polar solvent systems are used with reverse phases while non-polar solvents are used with normal phases (Ramis-Ramos, García-Álvarez-Coque, and Navarro-Huerta 2017). To optimize selectivity (ability of different solvents or solvent mixtures to interact differently with analytes) and resolution of components in a mixture, solvents of varying eluotropic strength (ε^o) or polarity (P') (Table 2) (Reich and Schibli 2007a)

Table 2: Solvents strength of solvents that are commonly used in chromatography. The eluotropic values and polarity are based on silica as stationary phase .

Solvent strength			
Solvent	**Eluotropic value (ε°)**	**Polarity (P')**	**Selectivity group**
Pentane, Hexane, Heptane	0.00	0.1	-
Xylene	0.22	2.5	VII
Toluene	0.22	2.4	VII
Diethyl ether	0.29	2.8	I
Dichloromethane	0.30	3.1	V
Chloroform	0.31	4.1	VIII
Acetone	0.43	5.1	VI
Dioxane	0.43	4.8	VI
Tetrahydrofuran	0.48	4.0	III
Ethyl acetate	0.48	4.4	VI
Dimethyl sulfoxide	0.50	7.2	III
Acetonitrile	0.52	5.8	VI
Isopropanol	0.60	3.9	II
Ethanol	0.68	4.3	II
Methanol	0.73	5.1	II
Acetic acid	0.73	6.2	IV
Water	-	10.2	VIII

Normal-phase chromatography range

Reverse-phase chromatography range

The solvent selectivity values (measured by basicity X_e, acidity X_d and dipole attraction X_n) are summarized in Figure 7 (Ramis-Ramos, García-Álvarez-Coque, and Navarro-Huerta 2017).

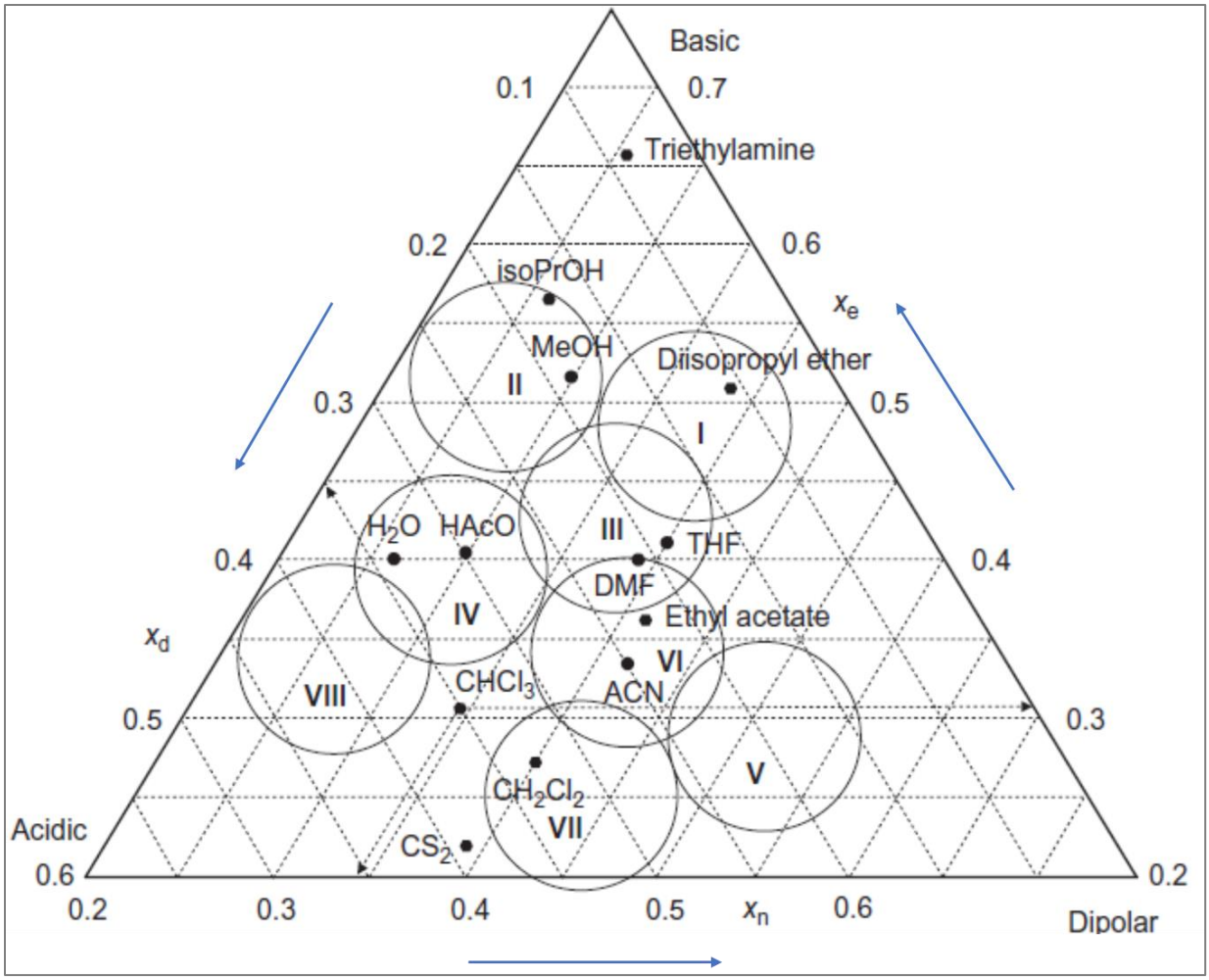


Figure 7: Snyder solvent selectivity triangle Snyder's solvent-selectivity triangle, indicating the eight solvent families (large circles).

The solvent selectivity families are: (I) aliphatic ethers and amines; (II) aliphatic alcohols; (III) pyridine and THF; (IV) glycols and acetic acid; (V) dichloromethane and dichloroethane; (VI) aliphatic ketones, esters, 1,4-dioxane, and nitriles; (VII) aromatic hydrocarbons and nitro compounds; and (VIII) phenols and water. The arrows starting from chloroform illustrate how to read the scales. X_d-solvent acidity, X_e-solvent basicity, X_n-solvent bipolar attraction. Adopted from (Ramis-Ramos, García-Álvarez-Coque, and Navarro-Huerta 2017; Reich and Schibli 2007a)

Solvent strength (eluotropic strength) describes the ability of the solvent to elute the solutes from the stationary phase. In normal phase chromatography, the solvent strength increases with the solvent polarity and so it is common to use the polarity indices to optimize resolution. Conversely, in reverse phase chromatography, non-polar solvents are stronger eluants that

polar ones. The resultant solvent strength (polarity), ε_T° of the solvent mixture is easily obtained from the individual solvent polarities and the fractions of each solvent in the mixture as shown in equation **2.**

$$\varepsilon_T^\circ = \sum_i^n f_i \, \varepsilon_i^\circ \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots Equation\ 2$$

Where *i* is *i*th solvent, f_i is its fraction in the solvent mixture and ε_i° is its eluotropic strength. The elution strength of mobile phase determines the retention time (HPLC) or retardation factor (TLC), while the selectivity affects the separation and sequence of spots or peaks.

The mobile phase selectivity describes the chemical interaction of the solvent with different sample molecules; its optimization is crucial for selectivity (separation and elution sequence) among analytes in a sample mixture. While selectivity of the solvent mixture cannot be calculated easily like its solvent strength, the interactions with analytes, and therefore elution time can be deduced from the character (basicity, acidity and bipolarity) of the constituent solvents in reference to the Snyder solvent selectivity triangle. For example, substituting a Methanol-water mixture with an iso-eluotropic acetonitrile-water mixture will reduce the ability of the mobile phase to accept hydrogen ions (methanol is a stronger proton acceptor than acetonitrile); so, the interaction with acidic solute will be reduced and their retention time will increase. Simultaneously, the dipolar character of the mobile phase will increase (acetonitrile is more dipolar than methanol) so that polarizable compounds will elute earlier (Ramis-Ramos, García-Álvarez-Coque, and Navarro-Huerta 2017). Since the selectivity and polarity of a mixture changes with the constituent solvents, sometimes, it is hard to derive an optimum mobile phase. To solve this challenge, (Nyiredy et al. 1985) proposed the PRISMA model for optimizing solvent systems in TLC. The model combines principles from the Snyder solvent selectivity triangle and solvent polarity (Reich and Schibli 2007a; Nyiredy et al. 1985). It involves preparation of equi-eluotropic solvent mixtures, from which mobile phases of varying selectivity can be obtained. Initially, eight to ten solvents representing the eight selectivity groups are each used to elute the sample on a TLC plate. Solvents that are able to separate the mixture to give R_Fs of at least between 0.3 to 0.5 are selected and optimized further; as mixtures of three solvents, as shown in planes A, B and C (Figure 8)(Mulyaningsih et al. 2011)

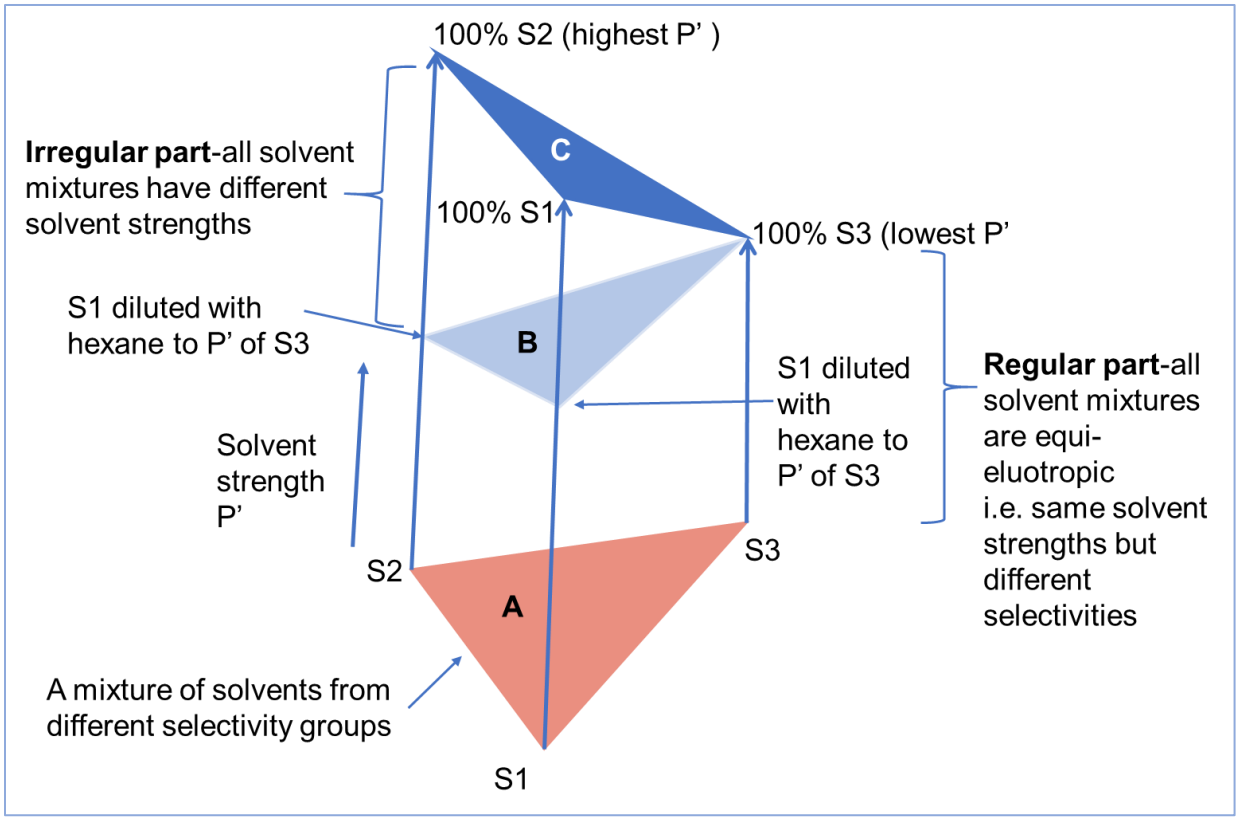


Figure 8: Schematic for optimization of mobile phases for TLC using the Prisma model

First, the polarity (P') or solvent strength of the mixture is set at the polarity of the weakest solvent (S3)(plane B) by diluting the other solvents (S1 and S2) using hexane (p=0). Then solvent systems of varying selectivity are obtained by mixing different fractions of solvents S1, S2 and S3, until well resolved peaks are obtained. If the R_Fs are still high, the polarity can be lowered further by selecting planes between A and B (regular part of the pyramid). This is achieved by diluting all the solvents with hexane. Alternatively, the plate can be deactivated with small amounts of water or other modifiers. Relatively polar samples can be separated by strong solvent mixtures in plane C, but solvent optimization is harder in this plane since different solvent compositions will have different strengths and selectivities.

Another approach to optimizing mobile phase system is based on their adsorption interactions with the stationary phase, such as silica, (Figure 9)(Reich and Schibli 2007a).

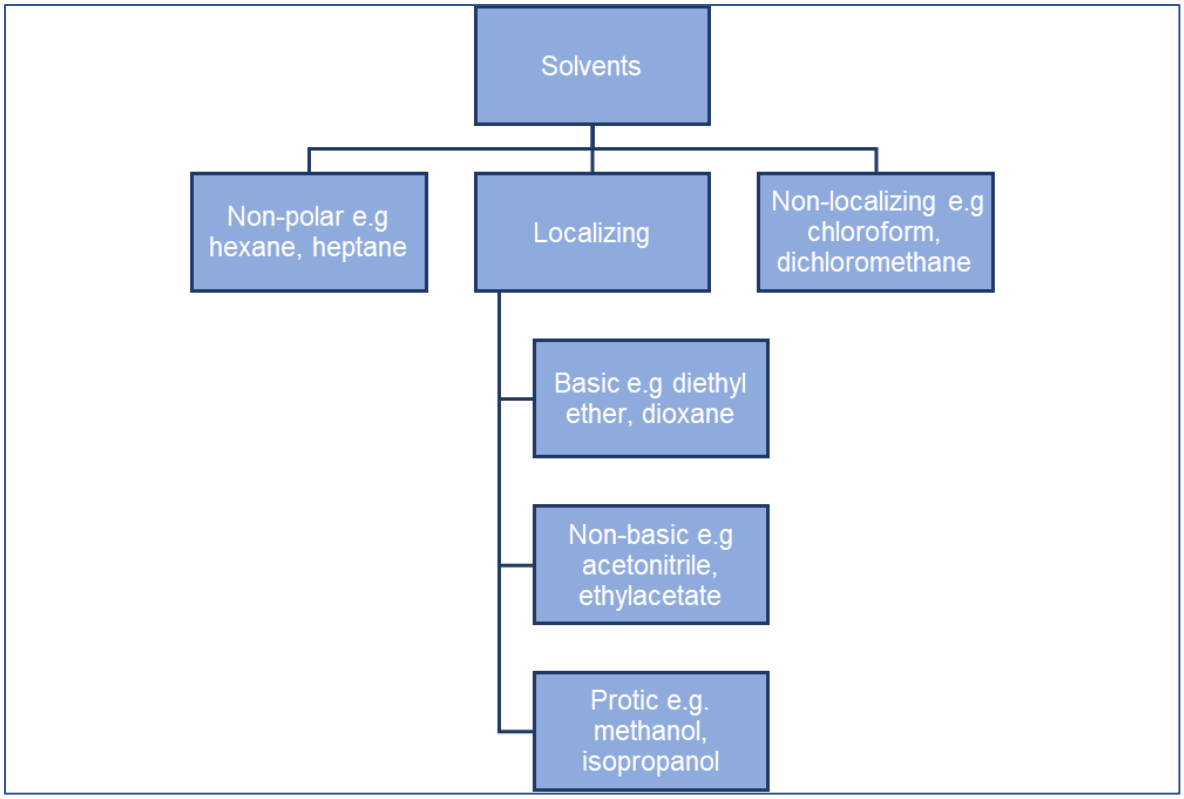


Figure 9: Classification of solvents based on adsorption interactions with silica

Non-localizing solvents lack polar groups and so their position on the stationary phase is irrelevant. Non-polar solvents are weakly adsorbed. Localizing solvents are polar, with either basic, non-basic, or protic functional groups. They therefore interact with specific functional groups in the stationary phase which influences the way they traverse it, as well as their interaction with sample components. “The four-solvent approach” recommends the initial selection of three solvents each from the localizing basic, localizing non-basic or protic and non-localizing to constitute mixtures that give appropriate strengths (based on R_Fs). The appropriate solvent strengths are achieved by diluting with non-polar solvents (forth solvent) such as hexane, while the selectivity is adjusted by substituting solvents with the same solvent strength but different localizing behavior (Geiss 1987; Reich and Schibli 2007a).

Another important factor to consider while selecting solvents for HPLC is the UV cutoff. This is the wavelength at which the solvent absorbs a significant portion of the irradiated light, such that very little light reaches the sample. Samples should always be eluted with solvents with significantly lower or higher UV cutoffs than their UV absorption maxima if known, or the detection wavelength. For general chromatographic fingerprints, where most of the components are unknown, solvents with low cutoffs such as acetonitrile (190nm), methanol

(205nm), and isopropyl alcohol (205nm) are preferred to high absorbing ones like tetrahydrofuran (215nm), ethyl acetate (256nm) and DMSO (268nm)(Ahuja and Dong 2005; Kazakevich and Lobrutto 2007).

Optimization of the detection method

Detection techniques enable visualization of the eluted compounds and further analysis of the fingerprint. They work by measuring some physical or chemical property of the analytes to produce a signal that can be used to identify and/or quantify the components of the sample. In TLC, compounds with strong chromophores can be visualized under day light (for those that absorb above 400nm and so are colored), or by use of fluorescence indicators (Reich and Schibli 2007a). For the latter, the plate is irradiated by ultraviolet light of 366nm and 254nm to cause fluorescence of the indicators embedded within the stationary phase layer (Reich and Schibli 2007a). This fluorescence is quenched by sample analytes that absorb at 254nm and 366nm to produce dark zones on a green or blue background. Further still, the plate can be treated with chemical reagents to produce distinct colors, a process termed derivatization (Reich and Schibli 2007a; Geiss 1987). Reagents specific to certain functional groups enable their identification e.g., ninhydrin for amino acids and secondary amines; Mayer's reagent for alkaloids, among others. Non-specific reagents such as conc. sulphuric acid, anisaldehyde/sulphuric acid, vanillin/sulphuric acid, are preferred in cases where the sample components are unknown and to maximize the number of bands visible, as is the case for most plant fingerprints (Poole 2015).

Detection of compounds in HPLC is most commonly achieved by ultra violet detectors. Either the detector is set at a single wavelength or it scans the entire spectrum (DAD-diode array detector) to find a suitable wavelength for the different components in the eluate (Ahuja and Dong 2005). The DAD is preferable to the single wavelength detector for samples with several analytes such as plant extracts. In addition, the DAD is able to differentiate co-eluting peaks (this ensures peak purity). This condition is crucial especially in quantitative analysis of markers (Kazakevich and Lobrutto 2007). For analytes that do not absorb electromagnetic radiation, several other detectors can be used. The mass detector offers universality, high selectivity and sensitivity. It measures mass to charge ratios of ionized compounds (Ahuja and Dong 2005). However, LC-MS systems are quite expensive to buy and maintain. Fluorescence detectors are selective for compounds that fluoresce, Reflective index detectors (RID) measure the change in reflective index of the eluate due to eluting components. Others include evaporative light scattering detector (ELSD), and conductivity detector: Conductivity

detectors are used for detecting ions in the eluent. They are commonly employed in ion chromatography and for the analysis of ionic species (Ahuja and Dong 2005).

Fine tuning the method; optimizing other method parameters

Other parameters that affect the quality of TLC fingerprints include; sample application technique and development chamber conditions. Application of samples can be done manually or by automatic applicators where available. As expected, automatic sample application is more reproducible and gives more refined spots. Manual application is done by spotting appropriate sample volumes (1-25µL) using micropipettes or syringes. While it is possible to apply bands manually, the procedure requires more skill, is tedious and time-consuming (Reich and Schibli 2007a). Besides, it is hard to ensure uniformity of sample concentration along the bands. Therefore, bands are better applied by autosamplers. The automatic sampler is fast and is capable of applying many smaller zones; for instance, with the ATS4 (CAMAG), up to 66 samples can be applied on a 20cm plate by either contact spotting or spray on technique. The samples are applied at least 10mm from the lower edge of the plate such that they are not in contact with the mobile phase before developments starts. In addition, the samples should be separated from one another by at least 10mm, to avoid interaction of the components. Whatever method of application is used, samples should be applied parallel to the lower edge of the plate, since the separated components are evaluated based on the retardation factor (R_F)., R_F is ratio of the distance travelled by the analyte spot to that travelled by the solvent front (Equation 3).

$$R_F = {}^{Da}/_{Ds} \text{.....equation 3}$$

Where *Da* is the distance moved by the analyte band and *Ds* is the distance moved by the solvent front.

Besides sample application, plate development conditions can also affect the quality of the TLC fingerprints. The chamber should be shaped in such a way as to allow for uniform movement of mobile phase over all the samples-they must be flat at all edges and corners. While unsaturated chambers produce sharp zones and reduce analysis time, reproducibility is lower, and the solvent fronts are not straight (Reich and Schibli 2007a). On the other hand, saturated chambers give more reproducible results. Saturation is obtained by lining one wall of the development chamber with a filter paper (or saturation pad) soaked in the mobile phase. The chamber is then tightly closed and left to stand for about 20 to 30 minutes to allow for the vapor to build up. Partially saturated chambers are obtained in a similar fashion; the only

difference is that there is no filter paper. Since TLC is greatly affected by the environmental conditions, the temperature and humidity should be always recorded and perhaps studied for system suitability (Reich and Schibli 2007a; Poole 2015). The following parameters affect the quality of HPLC fingerprints; velocity (flow rate of the mobile phase), column temperature, and elution type. The optimum velocity for maximum efficiency can be obtained from the Van Deemter equation (equation 2) and plot (Figure 6) already explained. at the optimum mobile phase velocity, the number of theoretical plates is at the lowest (maximum efficiency). If the column has already chosen (meaning the length and particle size are constant), the velocity of the mobile phase can only be changed by adjusting the column pressure (Kazakevich and Lobrutto 2007). The Velocity (v) of the mobile phase is related to the pressure of the HPLC system by equation 4.

$$P = {}^{vL\eta\Phi}\Big/_{d_p^2} \quad \text{.....equation 4}$$

Where L is the length of the column, η is the viscosity and Φ is the resistance factor. Of course, the amount of pressure applied is limited by the capability of the machine. The viscosity of the mobile phase can be reduced by heating the column, hence reducing the C term in Van Demeter's equation and Φ above equation. This enables operation at higher velocities without affecting the column efficiency, hence short analysis time. The reduced viscosity also improves diffusion of the analytes between the mobile phase and the stationary phase, resulting in sharper peaks. For some analytes, the rise in temperature leads to change in selectivity especially for polar and ionic compounds, for which the temperature change gives rise to different chemical species. That said, it is often necessary to operate the HPLC analysis at temperatures above ambient (usually above 40°C), to avoid influences of temperature variations in the laboratory. This ensures long term retention reproducibility of the HPLC system as long as the analytes are thermostable (Kazakevich and Lobrutto 2007).

The type of elution can be isocratic or gradient. Isocratic elution involves the use of a constant concentration of premixed solvents to elute the sample components. Hence, the selectivity and strength of the solvent system is independent of the column length. A constant solvent composition gives rise to constant analyte- eluent and analyte–stationary-phase interactions, leading to more reproducible separations. However, due to a lower separation power, fewer peaks are produced and for the strongly retained components, peaks are flat and broad and therefore hard to recognize. Conversely, gradient elution utilizes a variable composition of the mobile phase along a single run. By use of different pumps, the HPLC system adjusts the

amounts of the strong and weak solvents as programmed. Since the solvent strength and selectivity change along the column, gradient elution affords better resolution that isocratic elution, especially for complex samples, and, later peaks are sharper. However, the solvent gradients are hard to reproduce and more time is spent on rinsing the column in-between runs. While isocratic elution can be achieved with simple HPLC systems having a single pump, gradient elution requires more sophiscated equipment, with at least two pumps and programming software (Kazakevich and Lobrutto 2007; Ahuja and Dong 2005).

2.6.2 Validation of chromatographic fingerprint methods

Validation of chromatographic fingerprints ensures that they meet the requirements for their applications. This is achieved by demonstrating that the developed fingerprints are stable and reproducible under the stated conditions. The stability of HPLC fingerprints is evaluated by analyzing samples at time intervals, to cover the duration of time the analysis is expected to last; for example; at 0, 2, 4, 6, 8, 24 hours (Cai, Ong, and Liu 2012). The relative standard deviation (RSD) of the relative retention times and peak areas of common peaks (or marker if any) should be not exceed 3%. For TLC, the stability of the analytes under the chromatographic conditions is determined by performing a two-dimension TLC, using the same mobile phase. If stable, all the analyte spots should lie on the diagonal line joining the sample application point to the intersection of the solvent fronts (Reich and Schibli 2007a). Additionally, the stability of samples on the plate and in the extraction solvent can be demonstrated by comparing fingerprints of samples that have been in solution and on the TLC plate for different periods, determined by the estimated time the whole analysis will take (Reich and Schibli 2007a).

HPLC instrumental precision is determined by injecting at least six (6) replicates of a single sample into the machine. The RSD of the relative retention times and the peak areas of the common peaks (or markers) should not exceed 3%. If samples were collected from different batches, e.g., varieties, geographical sources, 3 replicates of samples from at least three different batches should be studied. The repeatability of the method is evaluated from 6 replicate analyses of samples from the same batch or 3 replicate analyses from at least 3 samples from different batches (Xie et al. 2006a; Cai, Ong, and Liu 2012).

Besides the RSD, fingerprints can be evaluated by calculating similarity indices of the peak areas under the method validation conditions, such as the correlation coefficient. In each case, the higher the correlation coefficient, the higher the stability, instrumental precision and method repeatability; the Chinese pharmacopoeia recommends a correlation or congruence

coefficient of 0.9 for reliable methods (Xie et al. 2006a; Cai, Ong, and Liu 2012). Apart from stability and reproducibility, the developed herbal fingerprints may be demonstrated to be different from those of other herbs ("specificity"). This can be done by analyzing samples of different but commonly used plants or those that are morphologically similar, along the target plant samples (Reich and Schibli 2007a).

2.7.0 Development of HPLC methods for quantification of marker compounds

2.7.1 Method development

The process of developing HPLC quantitative methods is similar to that of fingerprinting methods described above. However, emphasis is put on optimizing extraction methods, and selecting chromatographic conditions to maximize resolution of the markers to be quantified. This enables establishment of accurate and precise methods. The resolution (R) of two analytes 1 and 2, (Figure 10) can be calculated from equation 5.

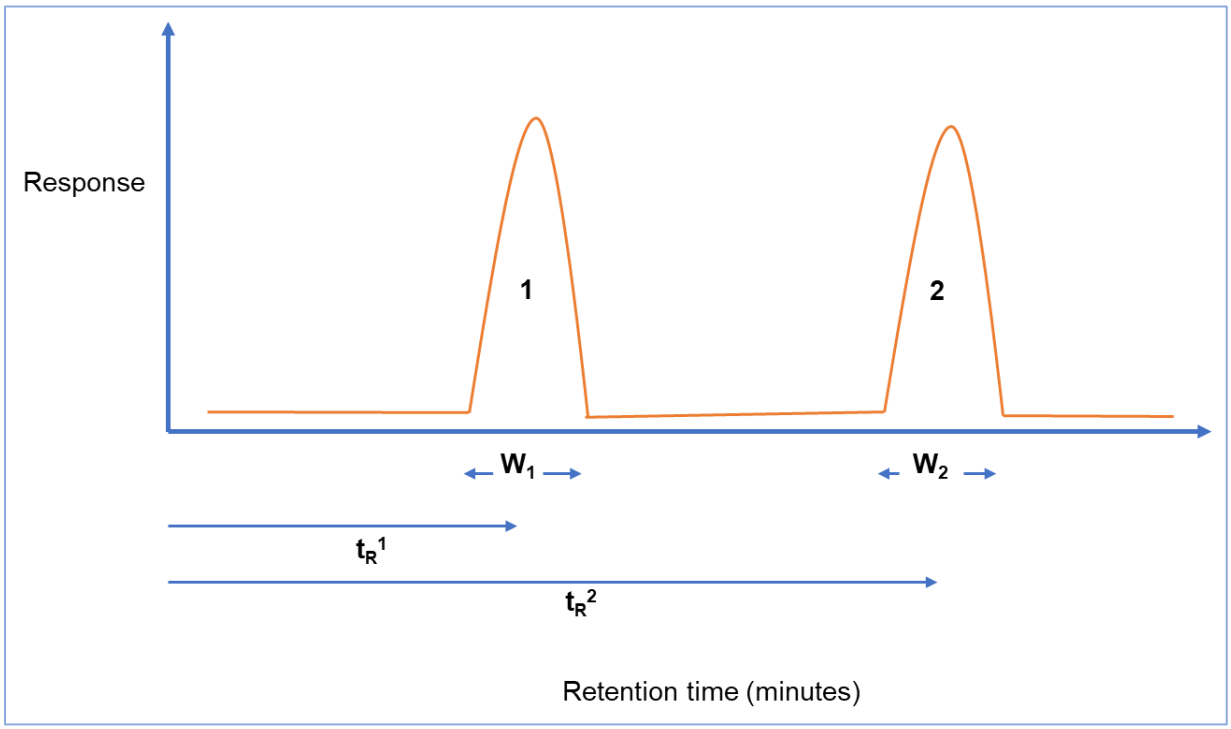


Figure 10: HPLC chromatogram showing characteristics of two separated peaks.

$$R = 2\left(\frac{t_R^2 - t_R^1}{w_2 + w_1}\right)$$----------equation 5

Where t_R^1 and t_R^2 are the retention times of the analytes whose peak widths are w_1 and w_2. The expression (t_R^2 - t_R^1) relates to the selectivity (α) of the HPLC system while (w_2 + w_1) reflects its efficiency (N) (Kazakevich and Lobrutto 2007).

The selectivity (α) between two analytes obtained from their retention factors k1 and k2, or their retention times t_R^1 and t_R^2, according to equation 6.

$$\alpha = \frac{k_2}{k_1} = \frac{t_R^2 - t_0}{t_R^1 - t_0}$$ ----------equation 6

While the efficiency (N), of the HPLC system for an analyte is related to its retention time and peak width according to equation 7.

$$N = 16\left(\frac{t_R}{w}\right)^2$$ ----------equation 7

For the resolution of a critical pair of analytes (analytes with minimal distance between them), it is assumed that their peak widths are equal, then equation 5 reduces to equation 8

$$R = 2\left(\frac{t_2 - t_1}{w}\right)$$ ----------equation 8

Combining equations 6,7 and 8 gives the Master resolution formula (equation 9)

$$R = \frac{\alpha - 1}{\alpha} x \frac{k_2}{1 + k_2} x \frac{\sqrt{N}}{4}$$ ----------equation 9

From equation 9, it is clear that the resolution can be improved by improving the efficiency and selectivity of the chromatographic system. Efficiency can be improved by using columns with smaller and uniformly distributed particles, increasing the flow rate of the mobile phase to the optimum velocity, and a longer column length, as well as optimizing instrument parameters that modify molecular diffusion and mass flow dynamics of the mobile phase such as temperature. On the other hand, the selectivity can be improved by choosing a column with the appropriate solid phase chemistry, and a solvent system that influences/modifies the interactions of the analytes with the stationary phase e.g., through ionization, solvation, tautomerization, among others (Kazakevich and Lobrutto 2007; Ahuja and Dong 2005).

A resolution of 1.5 is ideal for sufficiently separated analytes. However, this level of resolution is hard to obtain for complex matrices like herbal materials and products, because they contain several compounds with similar physical chemical properties. For such samples, a resolution of 1.0 is acceptable for the markers being quantified (AOAC 2002).

2.7.2 Method validation

As with chromatographic fingerprint methods, validation of quantitation methods ensures quality, consistency and reliability of the analytical results. This is achieved by demonstrating that the performance parameters of the method are acceptable for the intended purpose(s). Such parameters include; selectivity, linearity, sensitivity, recovery, and reproducibility (AOAC 2002; Agency 1995).

The selectivity of a chromatographic method is measured by its resolution (equation 5) i.e., the ability of the method to identity and measure the analyte of interest in presence of many others. The purity of the peaks is assessed by changing chromatographic conditions, scanning at different wavelengths (DAD) or by enhancing the peak due to the analyte of interest by spiking the samples with the analytical standard. Selectivity is also checked by analyzing blank samples; there should be no signal corresponding to the analyte.

To establish the relationship between the analyte parameter (such as concentration) and the response of the equipment (determined by the detector), such absorbance or area under the curve, a calibration curve is constructed from a series (at least 6) of standard solutions of different concentrations. The correlation coefficient (R^2) obtained by linear regression analysis should be close to 1 (practically, $R^2 \geq 0.990$). If a single marker is used to quantify multiple compounds, its calibration curve should be accompanied by the structures and the response factors of the compounds (AOAC 2002).

Accuracy is the degree of agreement between the measured quantity and the true value, expected or reference value. The accuracy of a method is often determined by quantifying the amount of a marker in the standard material, with different attributes such as geographical source, season of harvesting or variety of the plant species (AOAC 2012). At least three specimens are measured in duplicate or more replicates. The measurements should be made at different concentrations levels of the marker. The mean value should be within acceptable limits. Most often, the accuracy is expressed as the recovery of the analyte from the botanical reference material or sample. In this case, the already characterized material is spiked with varying amounts of the analytical standard. The materials are extracted and analyzed to determine the total amount of marker present. This value is expressed as the fraction of the original amount of marker in the sample, or the recovery (Konieczka and Namiesnik 2016). The recovery for materials should be within acceptable limits as shown in table 3. Whenever available, blank matrices should be used to evaluate the recovery. Otherwise, the standard reference samples with known marker concentration should be used. If these are also not

available, the characterized samples can be used. The recovery in this case is calculated either as the total recovery-marker in sample + added marker standard, or as marginal recovery-based on only the added marker standard. In the latter case, the marker concentration in the spiked sample must be subtracted from both the numerator (amount of marker found on analysis) and denominator (amount of marker in sample + amount of marker standard spiked) (AOAC 2002). The total recovery is calculated from equation 10.

$$\%\ Recovery = \frac{(C_s - C_0)}{C_m}\ x\ 100 \text{----------equation 10}$$

Where C_s and C_0 are the concentration of the marker in the spiked and un-spiked samples respectively, and C_m is the concentration of the marker standard added. Accepted limits of recovery are shown in Table 3 (AOAC 2002).

Table 3: Examples of acceptable limits for recovery and repeatability of analytical methods for quantification of markers compounds in herbal materials.

Concentration of marker	Recovery limits	Repeatability limits
100 %	98-101%	1 %
10 %	95-102%	1.5%
1 %	92-105%	2 %
0.1 %	90-108%	3 %
0.01%	85-110%	4 %
10 ug/g (ppm)	80-115%	6 %
1 ug/g	75-120%	8 %
10 ug/kg (ppb)	70-125%	15 %

Precision measures the degree of scatter among measurements obtained from multiple sampling of the same homogenous sample under the stated conditions. It can be determined at three levels i.e., (i) under the same laboratory conditions (analyst, reagents, equipment, and instruments) for a short period of time (repeatability), (ii) under varying conditions such as different analysts and chemical sources, but in in the same laboratory (intermediate precision) and under completely different laboratory conditions including the HPLC equipment, chemicals and analysts (reproducibility) (AOAC 2002). Repeatability is evaluated by injecting at least 9 replicates of the same batch of the herbal material into the HPLC machine or three replicates of three different batches. The RSD for the quantity of selected

markers should be within acceptable limits as shown in table 3 above. The repeatability RSD can also be calculated as a function of the concentration range C, according to equation 11.

$$RSD = C^{-0.15}$$----------equation 11

The acceptable RSD values of should lie between 0.5 and 2 times the calculated value.

Limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection and the limit of quantification are the minimum amounts of the marker compound that the method can identify or quantify with acceptable accuracy and precision, respectively. These parameters are not usually prescribed for assay methods since active compounds are only useful in relatively high amounts (Agency 1995). However, some phytochemicals are toxic (negative markers) and so are not tolerated. Such negative markers are then supposed to be kept to a minimum in any preparation (AOAC 2002). Therefore, a method developed to control such a constituent must exhibit an LOD and LOQ below the acceptable concentration (toxicity) limits of the compound. Furthermore, when evaluating phytochemicals for suitability as identity or quantification markers, such compounds should be present in concentrations above 5ppm and 50ppm respectively, for reliable measurement. (Bensoussan et al. 2015). Consequently, the accompanying method must perform above these limits. Since the LOD and LOQ are very small values, they are majorly determined by the capability of the equipment, in this case the HPLC system. Several techniques are available for measuring the LOD and LOQ, namely; signal to noise ratio (S/N), determination from blank samples, and determination from the standard calibration curve. The LOD and LOQ are equivalent to 3S/N and 10S/N respectively. To determine the signal due to noise, the response due to a blank sample, or one containing a very low analyte concertation is measured at the retention time of the analyte. The signal is then converted into concentration. Alternatively, the signals due to at least 10 blank samples are obtained; the mean (X_{blank}) and standard deviation (SD) are calculated (Konieczka and Namiesnik 2016; AOAC 2002). Then the LOD and LOQ are obtained according to equations 12 and 13.

$$LOD = X_{blank} + 3\ x\ SD$$----------equation 12

$$LOQ = X_{blank} + 10\ x\ SD$$----------equation 13

To determine the LOD and LOQ from the standard calibration graph, the equations 14 and 15 are utilized.

$$LOD = \frac{3.3\ x\ SD}{b}$$----------equation 14

$$LOD = \frac{10\ x\ SD}{b}$$----------equation 15

Where SD is the either the residual standard deviation of the calibration curve or the standard deviation of the Y-intercept of the calibration curve, and b its slope.

2.8.0 Evaluation of chromatographic fingerprints

The fundamental reason of quality control of herbal materials is based on the concept of phytoequivalence; this implies that two or more materials or products contain similar bioactive compounds in comparable amounts (Pace and Martinelli 2022). These bioactive compounds are responsible for the medicinal properties and therapeutic actions of the herbal materials. Phytoequivalence is often sought after in herbal medicine to ensure consistency and predictability in the therapeutic effects of different products or preparations. It helps ensure the quality, and safety of herbal formulations by establishing standards for the active ingredients and their concentrations (Liang, Xie, and Chan 2004). Regulatory bodies often require evidence of phytoequivalence to support the approval, registration, or licensing of herbal medicines and products in order to ensure their efficacy and safety for consumers. Phytoequivalence is assessed by comparing the similarities of the (chromatographic) fingerprints i.e., the separation and concentration distribution of the components (e.g., peaks or bands), by qualitative, semi-quantitative and quantitative methods.

2.8.1 Qualitative evaluation of chromatographic fingerprints

This is based on direct visual evaluation of the characteristic appearance of the samples in comparison to the reference fingerprints. This method is suited for rapid qualitative identification of herbal materials. It is often used when there is little data in the fingerprint. Features such as the number of peaks, shape of peaks, relative retention times and relative peak area, number of strong peaks, are considered (Tistaert, Dejaegher, and Vander Heyden 2011).

2.8.2 Semi-quantitative and quantitative evaluation of chromatographic fingerprints

Calculation of information content of the fingerprint

Information content of chromatographic fingerprints can be used to evaluate the quality of fingerprints developed under different conditions such as extraction methods, stationary phases, mobile phases, among others. It is thus beneficial during method development and optimisation. If the chromatogram is considered a continuous signal, representing a concentration distribution curve, due to several peaks, then its information content ϕ is defined by equation 16 (Ru 2004).

$$\phi = -\int P_x log\ P_x dx$$----------equation 16

Where P_x is the probability or concentration of the components in the distribution function. Φ is maximum is P_x is normally distributed. This is synonymous to complete resolution of the chromatographic peaks with the fingerprints. Since practically, it is hard to achieve complete resolution of peaks in an herbal material or product, due to the enormous number of peaks which causes overlapping, the areas of the peaks are obtained by vertical splitting. This is followed by an assumption that both peaks in the overlapping cluster are pure. Of course, some information is lost. Because the shape of the chromatogram depends on both the separation of peaks and their concentration distribution, some normalization is needed before calculating the information content. One simple way is to divide the peak areas with the total peak area; such normalization also gives the chromatogram a probability property. Then equation 16 is modified as shown in equation 17 (Gong et al. 2003; Goodarzi, Russell, and Vander Heyden 2013).

$$\phi = -\int \left(\frac{P_x}{\sum P_x}\right) log\ \left(\frac{P_x}{\sum P_x}\right) dx$$----------equation 17

Where P_x is the peak area of a component, and sum $\sum P_x$ is the total area of all the peaks in the fingerprint. The information content obtained this way can also be used to compare the quality of different batches of a herbal material (Gong et al. 2003)

2.8.3 Evaluation of chromatographic fingerprints using chemometric methods

Chemometrics is the science of relating chemical measurements made on a chemical system to the property of interest, such as concentration, through the application of mathematical or statistical methods (Smilde, Geladi, and Bro 2005). During analysis of herbal materials, a lot of data is generated which records small differences and similarities among samples. These variations can be evaluated by means of chemometrics and the samples classified into some important categories like cultivar, grade, source, authentic vs adulterated etc.,(Cai, Ong, and Liu 2012; Wagner et al. 2011). Chemometric techniques include unsupervised and supervised data analysis. Unsupervised methods are particularly useful when exploring unknown or complex datasets, identifying outliers, or discovering new patterns or relationships within the chromatographic fingerprints. Such methods include; similarity indices, principal component analysis (PCA), and Hierarchical cluster analysis (HCA). On the other hand, supervised methods rely on prior knowledge or class labels associated with the samples. In the context of evaluating chromatographic fingerprint similarity of herbal materials, supervised methods involve using reference standards or known chemical profiles to guide the analysis. Examples of supervised methods include; linear discriminant analysis (LDA) or partial least squares

discriminant analysis (PLS-DA), Soft Independent Modeling of Class Analogy (SIMCA), among others (Cai, Ong, and Liu 2012; Noviana, Indrayanto, and Rohman 2022; Xie et al. 2006a).

Calculation of similarity indices

Similarity indices are numerical values that provide a measure of the similarity between two (chromatographic) fingerprints (Xie et al. 2006a). There are several methods for calculating similarity indices of (chromatographic) fingerprints of herbal materials, including: the euclidean distance (d), included angle cosine (c), the correlation coefficient (r), jaccard index**,** among others.

Included angle Cosine (c)

This is a measure of the cosine of the angle between two vectors (fingerprints), with a value of 1 indicating perfect similarity and a value of 0 indicating no similarity. It is a correlation calculated with respect to the origin. It is also called the congruence coefficient (Equation 18) (Cai, Ong, and Liu 2012).

$$c_{ir} = \frac{\sum_{k=1}^{m} x_{ik} * x_{rk}}{\sqrt{(\sum_{k=1}^{m} {x_{ik}}^2)(\sum_{k=1}^{m} {x_{rk}}^2)}} \ldots\ldots\ldots\ldots\ldots\ldots\ldots Equation\ 18$$

This kind of degree of similarity is related to the changing patterns of characteristic variables in fingerprints.

Correlation coefficient (r)

The Pearson's correlation coefficient (or simple correlation coefficient) is used to measure the degree of similarity among test samples in cluster analysis. It is the scalar product of the normed mean-centered fingerprints (Equation 19) (Tistaert, Dejaegher, and Vander Heyden 2011; Cai, Ong, and Liu 2012).

$$r_{ir} = \frac{\sum_{k=1}^{m}(x_{ik} - \bar{x}_i)(x_{rk} - \bar{x}_r)}{\sqrt{\sum_{k=1}^{m}(x_{ik} - \bar{x}_i)^2 \sum_{k=1}^{m}(x_{rk} - \bar{x}_r)^2}} \ldots\ldots\ldots\ldots\ldots\ldots Equation\ 19$$

Where X_{ik} represents the *k*th characteristic variable of the *i*th sample, $\bar{X}_i$ represents the mean value of all variables of test samples, X_{rk} represents the *k*th characteristic variable of a common pattern, and $\bar{X}_r$ represents the mean value of all variables of a common pattern. The closer r is to 1, the more similar the fingerprints. The correlation coefficient is not related to variable units and is not sensitive to the values of different characteristic variables. The different values of different variables, which have little effect on the changing patterns of characteristic variables, are ignored.

The similarity evaluation system for chromatographic fingerprints of Traditional Chinese Medicines (Chinese Pharmacopoeia Committee, 2004) uses the r parameter while the Computer Aided Similarity Evaluation (CASE) software integrates both the congruence coefficient (but refers to it as the similarity index) and the correlation coefficient (Alaerts et al. 2010).

Euclidean distance (d)

A measure of the straight-line distance between two fingerprints (Equation 20), with a value of 0 indicating perfect similarity and a larger value indicating greater dissimilarity. This method is good for the quality control of herbal medicinal materials (Cai, Ong, and Liu 2012).

$$d_{ir} = \sum_{k=1}^{m} (x_{ik} - x_{rk})^2 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots Equation\ 20$$

Where X_{ik} represents the kth characteristic variable (k =1, 2, …, m) of the ith sample, X_{rk} represents the kth characteristic variable (k = 1, 2, …, m) of the mean vector of a common pattern. During the calculation of the Euclidean distance, the absolute value of the absolute distance is replaced by a square function, which makes the calculation easier and can emphasize the effects from bigger characteristic variables. These indices are also included in Hierarchical clustering and Principal component analysis, which are discussed later (Alaerts et al. 2010).

When comparing fingerprints to evaluate their quality, a suitable reference should be available. However, this is not the case for most herbal materials. In such a situation, the mean or median fingerprint of the samples is considered as the reference (Noviana, Indrayanto, and Rohman 2022).

Jaccard index (J)

The Jaccard index of chromatographic fingerprints is the size of their intersection divided by the size of their union. It can be applied to evaluate the similarity of chromatographic fingerprints of herbal materials when the fingerprints are represented as binary data (Cai, Ong, and Liu 2012). To generate binary data, a threshold for the presence or absence of a peak or component in the chromatogram is it, if a peak is above the threshold, it is considered present (1), otherwise absent (0). The binary data sets of the two chromatographic fingerprints x and y are compared to determine the number of peaks present in both fingerprints, known as the intersection (∩) and to determine the total number of peaks in the two chromatograms, called the union (∪). Then the Jaccard index of the fingerprints J_{ir} is calculated from equation 21.

$$J_{xy} = {|x \cap y|}\Big/{|x \cup y|} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots Equation\ 21$$

The Jaccard index value of 0 indicates no similarity between the chromatographic fingerprints, while a value of 1 indicates complete similarity, meaning that the fingerprints have the same set of peaks (Ahmad, Tamboli, and Parveen 2013).

Principal components analysis (PCA)

It involves a mathematical method of reducing the dimensionality of complex data while retaining the most important information. The main idea behind PCA is to identify and extract the underlying variables or "principal components" that explain the majority of the variability in the data. In the context of evaluating the quality of herbal medicines, PCA can be applied to various types of data such as chemical composition, biological activity, or physical characteristics. Using PCA makes it possible to identify relationships between the variables and patterns among the samples. This information can be used to classify different batches of herbal medicines into different quality groups and to determine the most important variables that contribute to the quality of the herbal medicines (Tistaert, Dejaegher, and Vander Heyden 2011; Xie et al. 2006a).

In chromatographic fingerprint analysis of herbal materials (such as HPLC), PCA can be applied to identify relationships between the peaks in the chromatograms and to identify patterns among the fingerprints. This information can be used to classify different batches of herbal materials into different quality groups and to determine the most important peaks that contribute to the quality and variability of the materials (Cai, Ong, and Liu 2012).

When included, scatter plots enable visualising the relationships between the peaks in the chromatograms and between the samples. A scatter plot can be created for each pair of principal components, with each sample represented by a point in the plot. The position of the point in the plot represents the scores of the sample on the two principal components. By plotting the samples in this way, it is possible to visualize the relationships between peaks and the samples (Noviana, Indrayanto, and Rohman 2022).

In addition to scatter plots, loading plots are also often employed to visualize the relationships between the peaks in the chromatograms and the principal components. A loading plot represents the relationship between each peak and the principal component. The magnitude and direction of the loadings indicate the contribution of each peak to the principal component. A peak with a high loading in a principal component has a large contribution to the variability in that component, while a peak with a low loading has a small contribution. Loading plots can be used to identify which peaks are the most important for a particular principal component and to explain the differences among the samples (Goodarzi, Russell, and Vander Heyden 2013).

Hierarchical clustering analysis

Hierarchical clustering analysis is a technique used to classify objects or data points into groups based on their similarities or dissimilarities. It creates a hierarchical structure of clusters, also known as a dendrogram, which illustrates the relationships and grouping of the data points (Tistaert, Dejaegher, and Vander Heyden 2011). Hierarchical clustering analysis can be applied to evaluate chromatographic fingerprints of herbal materials in several ways: By calculating the distances between pairs of fingerprints, the clustering algorithm can identify clusters of herbal materials that exhibit similar chemical profiles. This helps in organizing and classifying herbal materials into distinct groups based on their chromatographic characteristics. This information can be useful for identifying materials that are likely to contain similar active compounds or chemical markers (Cai, Ong, and Liu 2012). Hierarchical clustering can be employed to assess the consistency and quality of herbal materials across different batches or sources. By clustering fingerprints from different batches, it is possible to identify clusters representing materials from the same source or batch. This helps in evaluating batch-to-batch consistency and detecting potential variations or adulteration in herbal products (Noviana, Indrayanto, and Rohman 2022). In addition, by comparing the chromatographic fingerprints of suspect samples with those of authentic materials, hierarchical clustering can reveal if the samples belong to different clusters or if they are significantly dissimilar. This can help in detecting the presence of adulterants or identifying substituted materials. Furthermore, by analysing the peaks that contribute most to the clustering, using a two-way dendrogram, it is possible to identify key chromatographic features that distinguish different materials. These features can serve as marker compounds for quality control, authentication, or standardization purposes (Wu et al. 2020). These fingerprint evaluation statistics have been successfully applied by researchers to evaluate the quality of herbal materials (Chen, Ozcan, and Harnly 2007; Alaerts et al. 2012; Cai, Ong, and Liu 2012; Wu et al. 2020; Viapiana et al. 2016) and products (Wang, Nie, and Zang 2015; Yang et al. 2017; Ren et al. 2011; Xu et al. 2009; Yang et al. 2016)

CHAPTER THREE: METHODS AND MATERIALS

3.1 Summary of methods

The initial phase of the work involved a preliminary study of notified local herbal products, to establish the main plant materials used; this was followed by establishment of analytical markers for the top five plant species. Then, among these, three plants with limited published information regarding their quality control (without monographs and analytical methods published), were selected for further study. TLC and HPLC fingerprints were developed for qualitative evaluation, while HPLC quantitative methods were developed to quantify markers in the plant materials. Lastly, the methods so developed were used to evaluate the phytoequivalence of plant samples harvested from different geographical sources. The chronology of activities carried out to achieve the objectives of this PhD work is summarized in the concept map below (Figure 11)

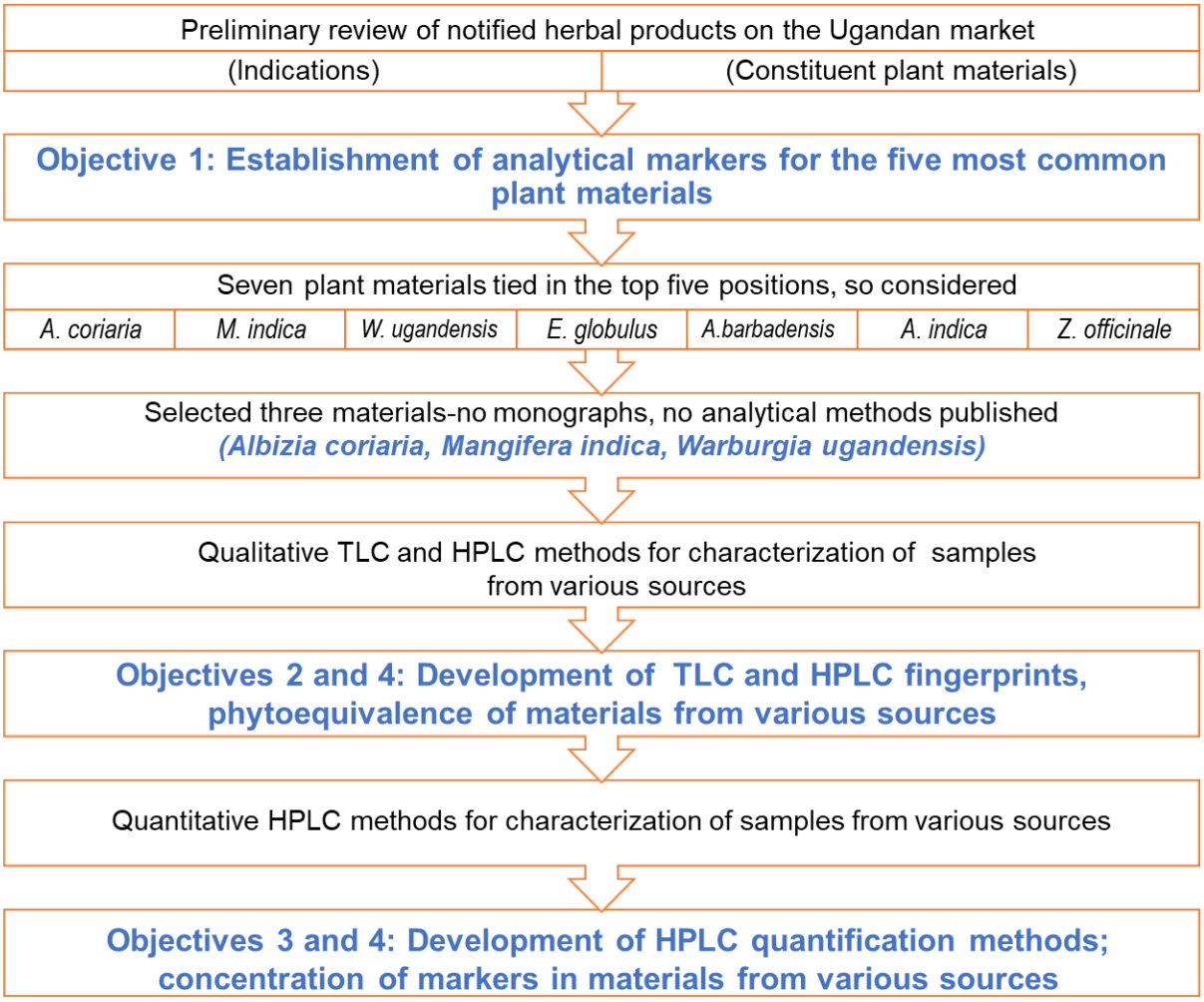


Figure 11: Concept map for the PhD work.

3.2 Method 1: Establishment of analytical markers for phytochemical standardization of herbal materials used for manufacture of herbal products in Uganda

3.2.1 Study design & setting

This was a cross-sectional study of local herbal products registered and sold in Uganda in 2019, and a review of published literature on the safety and efficacy of the herbal raw materials between 1960 and 2021. The products were bought from the busy cities of Mbarara, Jinja, Wakiso, and Kampala. In addition, most of the manufacturers are located this area. The pharmacies were accessed conveniently, and the only important factor was the presence of the products of interest.

3.2.2 Data collection methods

Data were collected mainly by desk review. Drug registers, product labels, and journal articles were considered. The data were collected as follows:

3.2.3 Identification of the most common plant raw materials and indicated use of the products

The most current drug register (August 2019) of herbal medicinal products was obtained from the NDA website (https://www.nda.or.ug/drug-register-downloads/). Then a list of the notified (partial registration to permit marketing) local herbal medicinal products was compiled. A sample of each of the products on the notified list was purchased from local pharmacies.

After the products were purchased, their labels were studied to identify the indications (use of the drug) and the active ingredients (medicinal plant materials). A database of all the herbal plant materials was then developed, and the materials ranked according to their popularity (frequency of appearance) in the herbal medicinal products.

3.2.4 Establishment of evidence for efficacy and safety of plant materials

We conducted a literature review to obtain evidence of biological activity concerning the current traditional use (according to the manufacturers' labels) and safety of all the plant species used. We searched databases and search engines such as PubMed, Google Scholar, ResearchGate, Web of Science, and Scopus. The search terms consisted of the scientific plant names, pharmacological/therapeutic activity of interest and toxicity. Only full-length articles published in English were reviewed.

3.2.5 Search for plant monographs already available.

Since there are no Ugandan monographs or pharmacopeia, we were interested in finding out if monographs of the plants used in Uganda have been published in other African countries. The uses and composition of the plants were considered to be similar due to their similarity in culture and climate. We reviewed the African Pharmacopoeia 2014 (Inter African Committee on Medicinal et al. 2014), and the West African Pharmacopoeia 2013 (West African Health Organization 2013). Due to the absence of the monographs for most of the plants in the African pharmacopeia, we also searched WHO monographs on selected medicinal plants volumes I-IV published between 1999 and 2009 (World Health Organization 2007b, 2009); (World Health Organization 1999; Murray and Shaw 2000).

3.2.6 Identification of potential marker compounds for the most five most used plant materials

The suitability of analytical markers was determined based on the WHO guidelines for selecting marker substances of herbal origin for quality control of herbal medicines (World Health Organization 2016) and also on the Herbal Chemical Marker Ranking System (Herb MaRS) (Bensoussan et al. 2015). Both the WHO guidelines and the Herb MaRS give priority to a compound whose biological activity is related to the traditional use of the plant and can be identified and quantified by the analytical methods available. Also, the compound chosen should be available commercially in pure form. The following steps were followed:

Establishment of active compounds and evidence of biological activity

Once the most consumed herbal materials were established, an extensive literature search to identify the bioactive compounds and compile the evidence of their biological activity relevant to the industrial application of the plant materials ensued. Such evidence included studies reporting on the ability of the compound (s) to treat or ameliorate one or more symptoms of the disease condition as indicated by the manufacturer. We browsed search engines like Google, and indexes including PubMed, Google Scholar, ResearchGate, and Web of Science. The search terms consisted of the chemical name, pharmacological or therapeutic activity of interest; such as “anti-inflammatory activity of 6-gingerol”; plant botanical name and bioactive compounds thereof, such as; “bioactive compounds in *Mangifera indica* leaves”, “mechanism of action of mangiferin” and so on. Only full-length articles published in English were reviewed.

Availability of analytical methods for the potential marker compounds

In addition to evidence of biological activity of the identified compounds, we also searched for information regarding quality control methods recommended by existing pharmacopeial monographs, particularly the WHO monographs on selected medicinal plants (Murray and Shaw 2000; World Health Organization 1999, 2007b, 2009; Inter African Committee on Medicinal et al. 2014), the African Pharmacopoeia, the West African Herbal Pharmacopoeia(West African Health Organization 2013) and the Pharmaceutical monographs for South African plants species (Scott and Springfield 2004). Further still, we searched for availability of assay methods for quantification of markers in the respective plant materials. We focused on high performance liquid chromatography (HPLC) methods because they are highly sensitive, specific, and versatile. Where HPLC methods were not available or not suitable such as for essential oils, high performance thin layer chromatography (HPTLC), gas chromatography (GC) or ultra violet/visible (UV/VIS) spectrophotometric methods were considered.

Availability of analytical reference standards for the potential marker compounds

We assessed for the availability of analytical standards and the prices for the smallest units. For this we preferably searched the Sigma Aldrich ® website (https://www.sigmaaldrich.com/UG/en) for two main reasons: (i) from experience, they are the most reliable supplier of high-quality chemicals, and (ii) they willingly display the prices for various grades and quantities of the same analytical standard. If the compounds were not available from Sigma Aldrich ®, we did a general google such to establish other potential sources. Finally, the cost of 1mg or mL of the marker was computed with an assumption this amount is sufficient for a single analysis-to construct calibrations curves.

Selection of the most suitable marker compounds

Using a modified Herb MaRS, compounds were given scores from 0 to 8; where 8 indicates the most suitable chemical marker. The Herb MaRS (see details here (Bensoussan et al. 2015)) does not elaborate on how the individual attributes of the compound are scored but rather gives a lumpsum mark after the overall assessment. Therefore, for more objective scoring, we modified the ranking system as follows: Evidence of biological was divided into three levels based on the number of symptoms of the disease condition a compound can treat or alleviate; (i) one symptom (1 point), two symptoms (2 points), 3 and more symptoms, with well elucidated mechanisms of action (3 points). We also scored the reported concentrations of the compounds (concentration not determined (0 points), concentration ≥5ppm (1 point),

(concentration ≥50ppm, 2 points) and availability of analytical standards (1 point); lastly, we scored for availability of an analytical method (1 point).

3.3 Methods for objectives 2, 3 and 4

Scope: Development of TLC and HPLC fingerprints for the qualitative standardization of the selected medicinal plant materials- *Mangifera indica, Warburgia ugandensis and Albizia coriaria.* In addition to the qualitative methods, HPLC quantitative methods were developed for quantification of markers in *M. indica* and *A. coriaria* materials. Finally, the characterization of materials obtained from different agro-ecological zones using the developed methods. The activities involved during method development are practically intertwined and so are presented together. Where specific experiments were done for a plant material, these have been highlighted.

3.3.1 Study design

The studies were experimental. Different solvents and extraction methods were evaluated to establish appropriate TLC and HPLC fingerprints, and to maximize the concentration of markers extracted. Also, the effect of geographical source or plant variety, on the fingerprint profiles and concentration of markers in the plant materials were evaluated. Additional experiments for *M. indica* aimed at generating quality grades from the different varieties growing at the National Crops Resources Research Institute (NaCCRI). These cultivars were purposely bred in 2007 to increase fruit yield and have since been distributed to farmers countrywide (Ddamulira et al. 2019). They include; Apple mango, Sejjembe, Kawanda green, Kate, Asante, Kawanda wide, Suu, Koona, Kagoogwa, MPI, Takataka, Boribo, Ngoogwe, Bire, and Doodo red. To characterize the varieties, selected parameters were determined namely; the extractive index, total phenolic content, antioxidant activity, and mangiferin concentration. Then field samples were placed in the quality grades generated, based on their HPLC fingerprint characteristics.

3.3.2 Study setting

The study was conducted in Uganda. Samples of *A. coriaria* stembark, *Warburgia ugandensis* stembark and *Mangifera indica* leaves were collected from the ten agro ecological zones of Uganda, to cater for effect of climatic variation on phytochemical composition of plant materials. These zones are classified on the basis of distinct vegetation and farming systems which are determined by determined by soil types, climate, and socio-economic and cultural factors (Ministry of Agriculture Animal Industry & Fisheries 2010). While I intended to collect

samples of each plant material from all the ten zones, I could not easily obtain *A. coriaria* and W. ugandensis from some zones; most of the trees had been already debarked. In addition, inhabitants said such trees are commonly cut down for charcoal, or to create land for agriculture. This practice is quite common for wildly growing hard wood trees (World Bank 2013). However, I did not search all villages in these agro-ecological zones because of time and logistical limitations. Table 4 summarizes the geographical characteristics of the agro ecological zones (Ministry of Agriculture Animal Industry & Fisheries 2010; Kajobe et al. 2016), while Table 5 and Figure 12 shows the sampling sites.

Table 4: Characteristics of the Agro ecological zones of Uganda

Ago-ecological zone	Summary of climatic characteristics
West Nile (North Western Savanah grasslands)	Bimodal average rainfall of 1,270 mm/ year with high variability. Characterized by savanna vegetation with open mixtures of trees and shrubs standing within tall grass
Mid Northern (Northern Eastern Savanah Grasslands)	Uni-modal low to high rainfall (1000-1,200 mm/year). Characterized by flat terrain covered by thick savannah grassland
Karamoja dry lands (North Eastern Dry Lands)	Uni-modal low rainfall (400-700mm/ year). The Vegetation is characterized by thorny bushes, cammiphora woodlands, occasional small trees and patches of grassland.
Lake Albert crescent (Western Savanah Grasslands)	Bimodal average rainfall of 1,270mm/ year with high variability. Vegetation ranges from rainforest to savanna grasses.
Lake Victoria crescent	Bimodal high rainfall (>1,200mm/ year). Has forest / savanna mosaic characterized by patches of dense forest in the south and scattered trees in shrubs and grassland of the north.
Eastern (Highland ranges)	Bimodal high rainfall >1,200mm/ year. Vegetation ranges from montane forest to high open moorland.
South Eastern (Kioga Plains)	Bimodal high rainfall 1,200-1440mm/ year. Characterized by savanna vegetation with open mixtures of trees and shrubs standing within tall grass.

Southern dry lands (Pastoral dry lands)	Uni-modal low to high rainfall (1000-1,200 mm/year). Characterized by hills covered by moderate savannah grassland.
Southern highlands (Highland ranges)	Bimodal high rainfall 1,200-1440mm/ year. Characterized by high hills and low temperatures. Vegetation is mainly equatorial forest vegetation.
Western highlands (Highland ranges)	Bimodal average rainfall of 1,270 mm/ year with high variability. Natural equatorial forest vegetation and rich natural savannah grasslands in the relatively drier areas.

Table 5: Sampling sites for plant materials

Agro-ecological zone	District(s)	Village	Plant species
1. West Nile	Pakwach	Padyang	*Mangifera Indica* *Albizia Coriaria*
2. Mid northern	Lira	Wiodyek	*Mangifera Indica*
	Dokolo	Ilong	*Warburgia Ugandensis* *Albizia coriaria*
3. Karamoja drylands	Moroto	Moroto municipality	*Mangifera indica* *Albizia coriaria*
4. Lake Albert crescent	Masindi	Nyabyeya Forestry college	*Mangifera indica* *Albizia coriaria*
5. Lake Victoria crescent	Mpigi	Kibibi	*Mangifera indica* *Albizia coriaria*
6. Eastern	Iganga	Kawete community center for traditional medicine	*Mangifera indica* *Albizia coriaria* *Warburgia ugandensis*
7. South Eastern	Buikwe	Najjembe (Mabira forest)	*Mangifera indica*
8. Southern drylands	Ntungamo	Rwentobo	*Mangifera indica*

9. Southern highlands	Kabale	Maziba	*Mangifera indica* *Warburgia Ugandensis* *Albizia coriaria*
10. Western highlands	Kabarole	Tooro botanic gardens	*Mangifera indica* *Albizia coriaria*
	Bushenyi	Ishaka	*Warburgia Ugandensis*

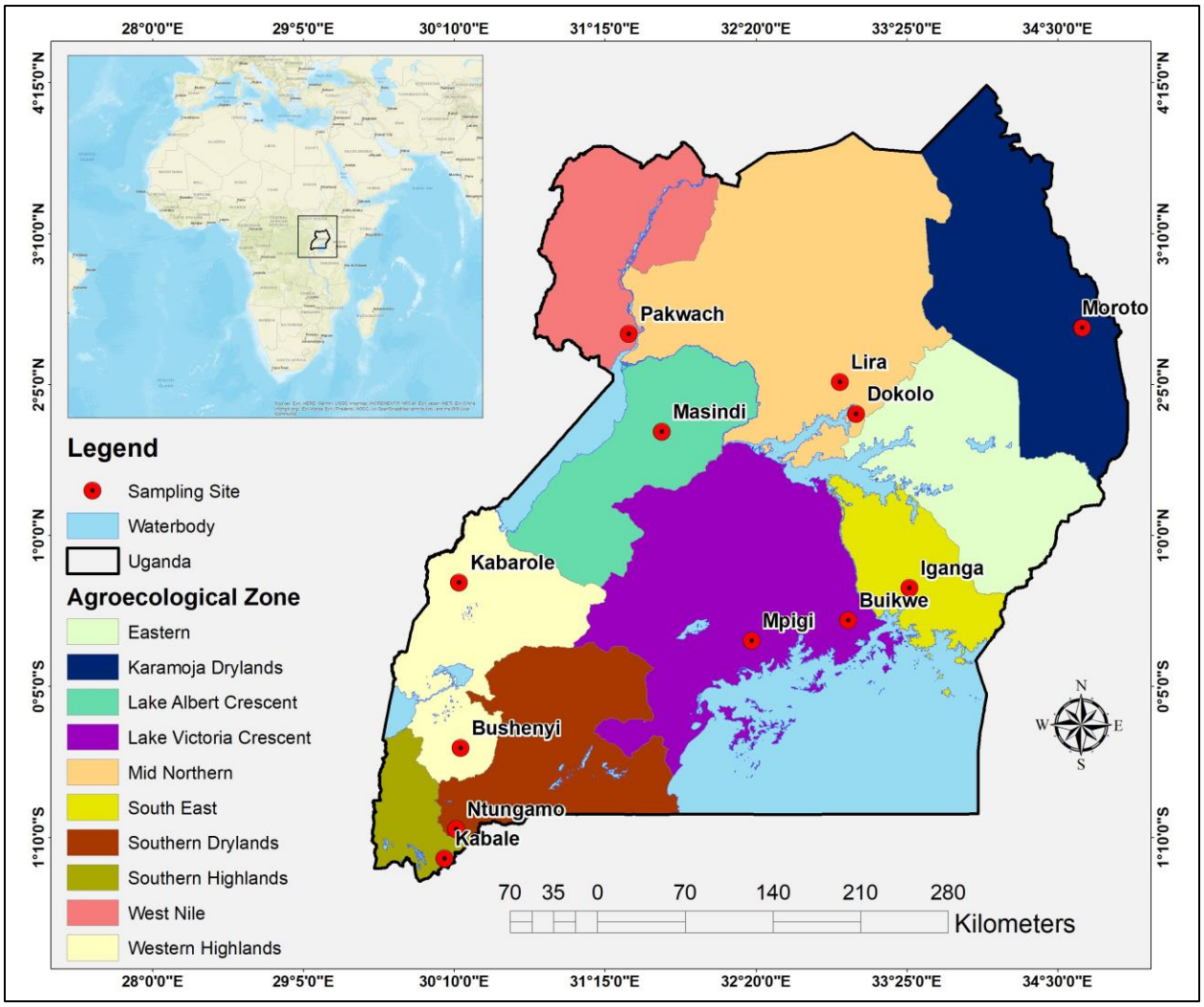


Figure 12: Map of Uganda showing the sampling sites for A. coriaria from the different agro-ecological zones (Kajobe et al. 2016) (created using Esri-ArcGIS® 10.8)

Mangifera indica leaves of local varieties were collected from NaCRRI**.** The NaCRRI was established in Namulonge village, Wakiso district, 19 km North of Kampala, Uganda. Namulonge lies at 1150 meters above sea level, within the agroecological zone of Lake Victoria Crescent at GPS coordinates 0.5288°N, 32.6123°E. It is characterized with a mean daily temperature of 20.8^{o}C, 258.89 millimeters of rainfall annually, and red sandy clay loam soils of pH 4.9 - 5.0. These climatic conditions favor the proliferation of *M. indica* (Ddamulira et al. 2019). Field samples were collected from different districts around Uganda.

3.3.4 Collection and processing of plant materials

The plant materials were harvested in January 2020 following WHO guidelines on good agricultural and collection practices for medicinal plants (World Health Organization 2003) under the guidance of the botanist, Dr. Godwin Anywar, on the team who identified the trees. About 2 kg of *A. coriaria* and *W. ugandensis* stembarks were cut using pangas, from at least three different trees in the same district where available or different districts in the same agro-ecological zone; the three samples were then combined to make one composite sample. Mature *M. indica* leaves (about 2kg) of all the *M. indica* cultivars were collected from NaCRRI in June 2022, with the aid of a taxonomist. Field samples were picked from different districts of the country as for *A. coriaria* and *W. ugandensis* above. The samples were labelled with appropriate codes and stored in perforated gunny sacks until they were finally delivered to the pharmaceutical chemistry laboratory at Makerere University for further processing. Voucher specimens of each sample were collected and deposited at the Makerere University herbarium for confirmation. After three weeks of air drying at room temperature, the stembark materials and leaves were ground using a motor and pestle into coarse powders, then into fine powders using an electric sample grinder. The powders were then packed in three layers of aerated polyethylene bags, and transferred to the pharmaceutical sciences laboratory at Mbarara University of Science and Technology, for chromatographic work and further analysis. Extraction of *M. indica* samples was done by maceration; that of *W. ugandensis* samples was done by decoction, and maceration while *A. coriaria* samples were extracted by all the three methods. For decoction, about 50g of the stembark powders were added to 500mL distilled water and boiled on a heater, at 100^{o}C, for 45 minutes and the extracts freeze dried. For Maceration, about 50g of the stembark or 30g of the leaf powders were added to 300 to 500mL of 70% ethanol in solvent bottles, stored in a cool dark place, with occasional shaking, for seven days. For Soxhlet extraction, stembark powders (50g) were refluxed with either 70% ethanol and ethyl acetate (200mL) until exhausted; the extracts were then rotary evaporated and air dried (Zhang, Lin, and Ye 2018).

3.3.5 Chemicals

All solvents used for extraction and for chromatographic analysis (N-hexane, trifluoroacetic acid, acetonitrile, methanol, ethyl acetate, dichloromethane, ethanol, acetic acid, phosphoric acid) were HPLC grade. The reagents for derivatization of TLC plates including sulphuric acid, iodine, vanillin, were of reagent grade. Reference markers; lupeol, betulinic acid, catechin and mangiferin were of analytical standard grade. Reagents for the quantification of total phenolic compounds and antioxidant activity including ascorbic acid, DPPH, magnesium carbonate, Folin–Ciocalteu reagent, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), gallic acid, sodium bicarbonate and ascorbic acid were either reagent or analytical grade. All the chemicals were bought from Sigma Aldrich® through a local agent Kobian Scientific®. Whenever available, analytical grade materials were used. Distilled water was prepared in the laboratory.

The TLC sheets and plates were sourced from Sigma Aldrich® and Fisher Scientific®.

3.3.6 Chromatographic setup

TLC: The chromatographic fingerprints were developed on 5cm x 10cm aluminium TLC sheets (trials) and 10cm x 20cm glass plates (final fingerprints), coated with 60Å silica gel matrix of particles size between 8.0 and 12.0 µm, and impregnated with indicators that fluoresce at 254nm.

HPLC analysis was performed on a UPLC Prominence Shimadzu chromatograph (Japan). The HPLC machine comprised a communicator (CBM-20A), a UV/visible detector (SPD-20A), SIL-20AC HT auto-sampler, LC 20 AD pumps, column oven (CTO-20AC), a Phenomenex Luna C18 column (250mm × 4.6 mm, 5 µm), and an online degassing unit (DGU-20A).

3.3.7 Development of TLC fingerprints

3.3.7.1 Preparation of standard solutions and test solutions

Standard solutions were prepared by dissolving about 1mg of the reference markers; lupeol, catechin, and betulinic acid (for *A. coriaria*) and Mangiferin (for *M. indica*) in about 2mL of methanol to make 500µg/mL solutions. Test solutions *A. coriaria, W. ugandensis and M. indica* were prepared by dissolving the powdered stembark or leaf material 50mg) in methanol (5mL) to make 1000µg/mL solutions.

3.3.7.2 Preparation of mobile phase solutions

The mobile phase solvent systems were optimized according to the PRISMA system (Nyiredy et al. 1985). Various proportions of n-hexane, ethyl acetate, methanol, and dichloromethane (for *A. coriaria,* and *W. ugandensis)* and ethyl acetate, acetic acid, methanol and water (*M. indica)* were tested until the best fingerprints were obtained. Solvents were chosen to represent different selectivity groups and polarities (solvent strengths) according to Snyder selectivity triangles and polarity tables (Snyder, Kirkland, and Dolan 2011). The final optimization of the solvent systems was achieved using solvent optimization prism model ((Nyiredy et al. 1985) The final solvent systems were as follows: *A. coriaria* (4 n-hexane: 2 ethyl acetate: 1 dichloromethane: 1 methanol), *W. ugandensis* (8 n-hexane: 2 ethyl acetate: 1 dichloromethane: 1 methanol) and M. indica 8 ethyl acetate: 2 acetic acid: 1 methanol:1 water). The solvent prisms are shown in the results sections.

3.3.7.3 Spotting, development, and visualization

Spotting was done by introducing about 4µmL of the test solutions and standards using a micropipette. Development was done into a saturated (30 minutes) glass chamber, with about 12 mL (10cm x 10cm chamber) or 30mL (20cm x 20cm chamber) of mobile phase solvent. The TLC plates were removed after the solvent front had moved three quarters of the plate length. The developed plates were air-dried for about 5 minutes. Once the plates were dry, they were visualized in day light, under UV (254nm and 366nm) and their photos taken. The plates were then derivatized using vanillin sulphuric acid reagent and heated in an oven at 105^0C for 15-30 minutes until the reaction of the components and reagents was complete (no further change in color intensity of the spots), and visualized in day light. Photos of the derivatized plates were again taken using a Nokia 6.1 ® mobile phone camera. The experiments were conducted in the July 2021. This month is the driest and so has least fluctuation in the daily temperature and humidity. While daily temperatures range between 17 and 28^0C and the average humidity in Mbarara is 64% (https://weather-and-climate.com/average-monthly-Rainfall-Temperature-Sunshine,Mbarara,Uganda), the experiments were conducted in the midmornings and afternoons at temperatures between 25 and 28^0C and humidity of 48 to 60%.

3.3.8 Validation of the TLC fingerprints

To determine whether the extracts were stable during the development of the spotted plate, two-dimensional TLC was employed. This involved developing the plate in two perpendicular

directions with the same solvent system. If stable, all the analyte spots should lie on the diagonal line joining the sample application point to the intersection of the solvent fronts. Additionally, the stability of samples on the plate and in the extraction solvent were confirmed by comparing fingerprints of samples that had been in solution and on the TLC plate for different periods (Reich and Schibli 2007a). To demonstrate ability of the fingerprint to distinguish between different plant species, samples of *W. ugandensis* and *A. coriaria* were spotted alongside each other.

3.3.9 HPLC fingerprinting and quantification of markers

3.3.9.1 Preparation of standard solutions and test solutions

A. coriaria, W. ugandensis and M. indica test solutions were prepared by dissolving, with sonication, the powdered stembark or leaf material in methanol to make 1mg/mL solutions. Standard solutions containing 10, 20, 40, 80, 140 and 160µg/mL were prepared by dissolving the appropriate reference markers; lupeol and betulinic acid (*A. coriaria*) and mangiferin (*M. indica*) in methanol and diluting serially. The calibration curves are shown in appendix 24 (*M. indica* and appendix 25 and 26 (*A. coriaria*).

3.3.9.2 Mobile phase solutions and HPLC conditions

To obtain the mobile phase solutions for *A. coriaria*, I experimented, with the aid of Snyder solvent selectivity triangles (Snyder, Kirkland, and Dolan 2011), the solvent systems developed earlier for quantification of lupeol (Yanaso et al. 2021; Strzemski et al. 2016) and betulinic acid (Holonec et al. 2012; Felföldi-Gáva et al. 2009) in other plant materials. In addition, to visual inspection for separation of peaks, the information contents of the fingerprints were calculated to determine the best solvent systems. The final solvent system composed of acetonitrile (99.99%) and 1% aqueous trifluoroacetic acid (0.01%). The optimal mobile phase flow rate was 1mL/min, while the column temperature was 25^{o}C while the detection was done at 205nm. To obtain the mobile phase solutions for *M. indica*, various proportions of solvent fractions developed earlier for quantification of mangiferin and other phenolic compounds were compared (Zhang et al. 2014; Pan et al. 2018; Wu et al. 2020). The final solvent system composed of methanol (31%) and 0.01% orthophosphoric acid (69%) and detection was done at 258nm. The optimal mobile phase flow rate was 1mL/min, while the column temperature was 25^{o}C. For *W. ugandensis*, the solvent systems established by (ONDORA 2016) were evaluated. The final chromatographic conditions were as follows: the solvent system composed of methanol (30%) and water (70%) and detection was done at

230nm. The optimal mobile phase flow rate was 1mL/min, while the column temperature was 30^0C,the fingerprint acquisition time was 50 minutes.

3.3.9.3 Fingerprint development, visualization and identification of markers

Fingerprints were developed by injecting 10µL of the sample solutions, and varying mobile solvent phase systems as described above, with isocratic elution, into the chromatograph and visualizing at the appropriate wavelengths. The reference markers, Lupeol betulinic acid and mangiferin were identified based on their retention times. The standard peaks were confirmed from selective enhancement after spiking the samples with the reference materials. Fingerprints with the highest information content were identified and used to characterize plant samples from different batches.

3.3.9.4 Validation of fingerprint methods

The stability of HPLC fingerprints was evaluated by analyzing samples prepared and stored at laboratory conditions for up to three days. The correlation coefficients of the common peak areas were then calculated. HPLC instrumental precision was determined by injecting at least six (6) replicates of a single sample into the machine. The correlation coefficients of the common peak areas were then calculated. The repeatability of the methods was evaluated by analysis of 6 samples from the same batch prepared by the same procedure. The correlation coefficients were then calculated. Correlation coefficients should be close to 0.9 for acceptable method performance (Cai, Ong, and Liu 2012).

3.3.10 Quantification of markers in the *A. coriaria* and *M. indica* test solutions

The concentrations of lupeol and betulinic acid (*A. coriaria),* and mangiferin *(M. indica*) were based on external standard calibration curves constructed using the respective reference markers and the corresponding peak areas.

3.3.11 Validation of the HPLC quantitative methods

The accuracy was determined by computing the percentage total recovery of three replicate measurements of markers from three spiked samples from different batches at different concentrations. The intra-day and inter-day repeatability were obtained from the percentage relative standard deviation of three repeated measurements of three samples of different concentrations (batches) on the same day and after three days respectively.

The linearity (sensitivity) and range were obtained from the standard calibration curve (Appendix 24, 25 and 26). The limit of detection (LOD) and the limit of quantification (LOQ)

were obtained using the calibration graph method. The values were calculated according to equations 14 and 15.

3.3.12 Additional experiments for *M. indica* leaves

To characterize the *M. indica* varieties in different quality grades, their extractive indices (%yields), total phenolic contents, antioxidant activities, and mangiferin concentrations were determined.

3.3.12.1 Determination of the ethanolic extraction index of *M. indica* leaves

About 30g of leaf powder for each cultivar was macerated in 150ml of 70% v/v ethanol in darkness for 7 days at room temperature with occasional shaking. The extracts were filtered using a filter paper and solvent evaporated to dryness using an oven. The extractive index was calculated as the percentage yield following extraction of the plant materials according to equation 22.

$$Extraction\ index\ \% = \frac{M_r}{M_s}\ x\ 100 \text{----------equation 22}$$

where M_r denotes the mass of the dried residue and M_s the mass of the extracted leaf powder (World Health Organization 2011).

3.3.12.2 Determination of total phenolic content of the *M. indica* leaf extracts

The total phenolic content (TPC) of the extracts was determined using the Folin–Ciocalteu method as applied by (Núñez Sellés et al. 2002a). The sample powders (100mg) were dissolved in 10mL of distilled water. A volume of 0.5mL of the test solutions were transferred into test tubes and then 0.5mL of Folin–Ciocalteu reagent. After about 10 minutes, 1.5mL of 2% (w/v) sodium carbonate solution and 4.5mL of distilled water were added. The reaction mixture was incubated in the dark at room temperature (28^{o}c) for 30 minutes. The TPC of the samples were determined from their visible electromagnetic energy absorbances at 755nm, in comparison to Gallic acid standard solutions (Appendix 27) using a UV/Visible spectrophotometer (JENWAY 6705 UV/Vis, Bibby Scientific, United Kingdom).

3.3.12.3 Determination of antioxidant activity

The antioxidant activity was determined using the DPPH scavenging activity as applied by (Pungot et al. 2020). Sample solutions were prepared by dissolving the powdered leaf materials (0.1 g) in 10 mL of 99.9% methanol, by orbital shaking at 200 rpm for 30 minutes. The solutions were then filtered and made up to 10mL with methanol. To prepare the standard solutions of ascorbic of ascorbic acid, 0.01g were dissolved in 10ml of 99.9% methanol; then 1mL of this solution was further diluted to 10 mL with methanol. To determine the scavenging

activity, 20, 30, 40, and 50µL of ascorbic acid solutions were each added to a solution of 3mL DPPH (0.0039mg/mL) and 1mL methanol and shaken to mix. The absorbance of each solution was obtained at 517nm., Methanol was used as the negative control while DPPH (0.0039mg/mL) solution was the blank. The activity of the test solutions was determined similarly by adding 20, 30, 40 and 50µL of each sample solution to a solution containing 3 ml of DPPH and 1mL of methanol. The decrease in absorbance of the ascorbic acid and samples was calculated in comparison to a blank sample containing only methanol and the DPPH.
The percentage decrease in absorbance (hereby referred to as the percentage inhibition) was calculated according to equation 23.

$$\%\ inhibition\ = \left(\frac{Abs\ blank - Abs\ S}{Abs\ blank}\right) x\ 100$$----------equation 23

Where Abs blank denotes the absorbance of the blank sample and Abs S denotes the absorbance of either the test solution or standard ascorbic acid solutions. The % inhibition data were plotted against concentration to determine the amount of the sample and ascorbic that inhibits 50% of the DPHH (IC_{50}), the measure of antioxidant capacity. The calibration curve for ascorbic acid is shown in Appendix 28.

3.4 Data analysis

The fingerprints were analyzed qualitatively and semi-quantitatively. Qualitatively, fingerprints were examined for good separation of components, number peaks/spots, and presence of standard peaks or spots. Semi-quantitatively, fingerprints were evaluated for information content to optimize solvent systems (HPLC). To evaluate the phytoequivalence of samples obtained from different batches, their fingerprints were assessed for similarity, by use of similarity indices, based on the constituent peaks or bands. For TLC, common bands are those with similar retardation factors (R_F) and color (TLC) after derivatization. For HPLC, common peaks have similar retention times and shape. The degree of similarity of the peaks were established by calculating the Jaccard index (for TLC fingerprints) or the correlation coefficient, congruent coefficient and Euclidian distances (for HPLC fingerprints). Samples are said to be phytoequivalent if their similarity index value is ≥0.9.

HPLC fingerprint peaks for different samples batches were further compared by cluster analysis and principal component analysis in GraphPad Prism 8®. Similarity was indicated by the samples being in the same clusters of the dendrograms or scatter plots., These techniques were also used to classify samples of *M. indica* based on the selected parameters.

Quantitative data for the markers, lupeol, betulinic acid, and mangiferin, as well as the peak areas were recorded for the different samples in Microsoft Excel 2016®. Additional quantitative data for *M. indica* included that from total phenolic content, antioxidant activity, and extraction index. Analysis of difference in concentrations of the samples due to different source districts (agro-ecological zones) and different plant varieties (*M. indica*), different extraction and solvent systems *(A. coriaria and W. ugandensis*) were performed by running ANOVA at significance level (p=0.05), in GraphPad Prism 9®. Further analysis to identify significantly different groups was performed using Tukey's Comparison test.

3.5 Ethical Approval

Approval to conduct the studies were obtained from the Uganda National Council for Science and Technology (UNCST) and the Mbarara University of Science and Technology Institutional Review Board (Appendix 29). All findings have been published in peer reviewed journals. The methods developed will be availed to the National Drug Authority and the manufacturers, as well as other researchers on request.

CHAPTER FOUR: RESULTS, ANALYSIS, AND DISCUSSION

4.1.0 Analysis and discussion of results for objective one: Establishment of analytical markers for phytochemical standardization of herbal materials used for manufacture of local products

4.1.1 Identification of the most common plant raw materials and indications in commercial herbal products

By 31st March 2019, the NDA drug register had 4,148 conventional products and 214 herbal medicinal products All the herbal products were not fully registered but rather notified, and the majority (130) were imported, mainly from India and China. 84 products were locally manufactured, of which, 82% (n=69) were syrups or solutions indicated for systemic treatment of upper respiratory tract disorders, such as cough, asthma, and flu; about 14% (n=12) were ointments, creams, balms, or soaps for treatment of skin infections and promoting wound healing. Three products were indicated for the treatment of peptic ulcers (Table 7).

Although the NDA register contained 84 locally manufactured products, only 18 products could be found in the drug outlets within the study areas. We evaluated these products to obtain the most commonly used medicinal plant species in the formulations. Two products that whose labels did not indicate the active ingredients were excluded from analysis.

Desk review of the product labels and manufacturer's leaflets revealed 33 plant species used as raw materials. Whereas various scientific names of the plant species and/or their synonyms were used on the product information labels, we adopted the approved scientific names and their authorities according to the Kew database; Plants of the World http://www.plantsoftheworldonline.org accessed on 19th March 2021. This ensured uniformity and maintained scientific standards. We did not verify from the manufacturers if the plants indicated on the labels were the ones used to manufacture the products. The products found during the survey are shown in Table 6.

Table 6: Notified local herbal products found in pharmacies at the time of the survey, their indications and plant ingredients as listed on the labels.

No.	Product	Indication (s) as per product label	Verified scientific names of plant constituents (Family)

1	Nana herbal mouth wash®	Toothache Bad odor Sensitivity Bleeding gums Cavities Tooth decay Antibacterial	i. ii. iii. iv. v. vi. vii. viii. ix. x.	*Calendula officinalis* L. (Asteraceae) *Krameria lappacea* (Dombey) Burdet & B.B. Simpson (Krameriaceae) *Camellia sinensis* (L.) Kuntze (Theaceae). *Carica papaya* L. (Caricaceae) *Eucalyptus globulus* Labill. (Myrtaceae) *Aloe vera* (L.) Burm.f. Synonym: *Aloe barbadensis (*Asphodelaceae) *Terminalia catappa* L. (Combretaceae). *Salvadora persica* L. (Salvadoraceae) *Syzygium aromaticum* (L.) Merr. & L.M.Perry (Myrtaceae) *Mentha* × *piperita* L. (Lamiaceae)
2	Gabogola syrup®	Whooping cough, Catarrh Sore throat Congestion from asthma and bronchitis	i. ii. iii.	*Mangifera indica* L. (Anacardiaceae) *Eucalyptus globulus* Labill. (Myrtaceae) *Albizia coriaria* Oliv. (Fabaceae)
3	Kwesiima cough mixture®	Cough Catarrh Sore throat Congestion from asthma and bronchitis	i. i. ii. iii.	*Albizia coriaria* Oliv. (Fabaceae) *Mangifera indica* L. (Anacardiaceae) *Eucalyptus globulus* Labill. (Myrtaceae) *Syzygium aromaticum* (L.) Merr. & L.M.Perry (Myrtaceae)
4	Lucas syrup®	Cough Flu Mouth sores Measles symptoms	i. ii.	*Eucalyptus globulus* Labill. (Myrtaceae) *Warburgia ugandensis* Sprague (Canellaceae)
5	Yeco cough doctor®	Allergic cough Smokers cough Whooping cough Productive cough Flu Lung cleaning	i. ii. iii. iv. v. vi.	*Zanthoxylum chalybeum* Engl. (Rutaceae) *Zingiber officinale* Roscoe (Zingiberaceae) *Combretum molle* R.Br. ex G.Don (Combretaceae) *Morella kandtiana* (Engl.) Verdc. & Polhill (Myricaceae) Synonym: *Myrica kandtiana* *Mangifera indica* L. (Anacardiaceae) *Allium sativum* L. (Amaryllidaceae)
6	Damaleo vera tablets®	Cleanses GIT Promotes kidney function Boost immune system	i.	*Aloe vera* (L.) Burm.f. (Asphodelaceae) Synonym: *Aloe barbadensis*

7	Muwereza herbal cough remedy	Cough Flu Sore throat Sinusitis	i. ii. iii. iv. v.	*Azadirachta indica* A.Juss. (Meliaceae) *Aloe vera* (L.) Burm.f. (Asphodelaceae) Synonym: *Aloe barbadensis* *Zingiber officinale* Roscoe (Zingiberaceae) *Dracaena steudneri Engl.* (Asparagaceae). *Albizia coriaria* Oliv. (Fabaceae)
8	Rezalin for ulce®	Gastric ulcers Stomach ulcers Flatulence Constipation	i. ii. iii. iv. v.	*Kigelia africana* (Lam.) Benth. (Bignoniaceae). *Tamarindus indica* L. (Fabaceae) *Azadirachta indica* A.Juss. (Meliaceae) *Entada abyssinica* A.Rich. (Fabaceae) *Salvia hispanica* L. (Lamiaceae).
9	Sacco syrup®	Common colds Flu Cough Sinusitis Rhinitis Asthma Catarrh Whooping cough Allergic conditions	i. ii. iii. iv.	*Morella kandtiana* (Engl.) Verdc. & Polhill (Myricaceae) Synonym: *Myrica kandtiana* *Eucalyptus globulus* Labill. (Myrtaceae) *Musa x paradisiaca* (Musaceae). *Warburgia ugandensis* Sprague (Canellaceae)
10	Replenish capsules®	Immune booster Antioxidant	i.	*Moringa oleifera* Lam. (Moringaceae)
11	Focus herbal cough syrup®	Bronchial congestion Flu associated with cough Painful coughing Dry/irritating cough Mouth sores Hiccups	i. ii. iii.	*Warburgia ugandensis* Sprague (Canellaceae) *Eucalyptus globulus* Labill. (Myrtaceae) *Fragaria* × *ananassa* (Duchesne ex Weston) Duchesne ex Rozier (Rosaceae)
12	Jenacid negus®	Ulcers	i. ii. iii. iv. v.	*Zanthoxylum fagara* (L.) Sarg. (Rutaceae). *Solanum spp (Solanaceae).* *Warbugia ugandensis* Sprague (Canellaceae) *Curcuma longa* L. (Zingiberaceae) *Allium sativum* L. (Amaryllidaceae)
13	Witch hazel®	Relief of minor skin irritations due to; insect bites Minor cuts, minor scrapes	i.	*Hamamelis virginiana* L. (Hamamelidaceae).

14	Phycof cough syrup®	Dry, wet, and whooping cough Sore throat Bronchial asthma Relieving fever Nasal congestion	ii. iii. iv. v. vi. vii. viii. ix. x. xi.	*Zingiber officinale* Roscoe (Zingiberaceae) *Curcuma longa* L. (Zingiberaceae) *Eucalyptus globulus* Labill. (Myrtaceae) *Mentha* × *piperita* L. (Lamiaceae) *Cymbopogon citratus* (DC.) Stapf (Poaceae) *Citrus limon* (L.) Osbeck *Tinospora sinensis* (Lour.) Merr. (Menispermaceae). Synonym: *Tinospora cordifolia* *Albizia coriaria* Oliv. (Fabaceae) *Acorus calamus* L. (Acoraceae). *Xanthium strumarium* L. (Astaraceae)
15	Princess aloe lip balm®	Dry, cracked, and painful lips	i. ii.	*Aloe vera* (L.) Burm.f. (Asphodelaceae) Synonym: *Aloe barbadensis* *Azadirachta indica* A.Juss. (Meliaceae)
16	Princess pain balm®	Pain relief	i. ii.	*Eucalyptus globulus* Labill. (Myrtaceae) *Cinnamomum verum* J.Presl (Lauraceae) Synonym: *Cinnamomum zeylanicum*

Where wrongly spelled scientific plant names were found on the product labels, corrected and verified names were used instead. Of the 33 plants listed, *Eucalyptus globulus* was by far the most used material. It was mentioned in 50% (f=9) of the products. *Aloe barbadensis* and *Albizia coriaria* were the second most used plant materials, being used in 22.2% of the products. *Mangifera indica*, *Warburgia ugandensis, Azadirachta indica* and *Zingiber officinalis* tied in third place, and were used in 16.7% of the products (Figure 13).

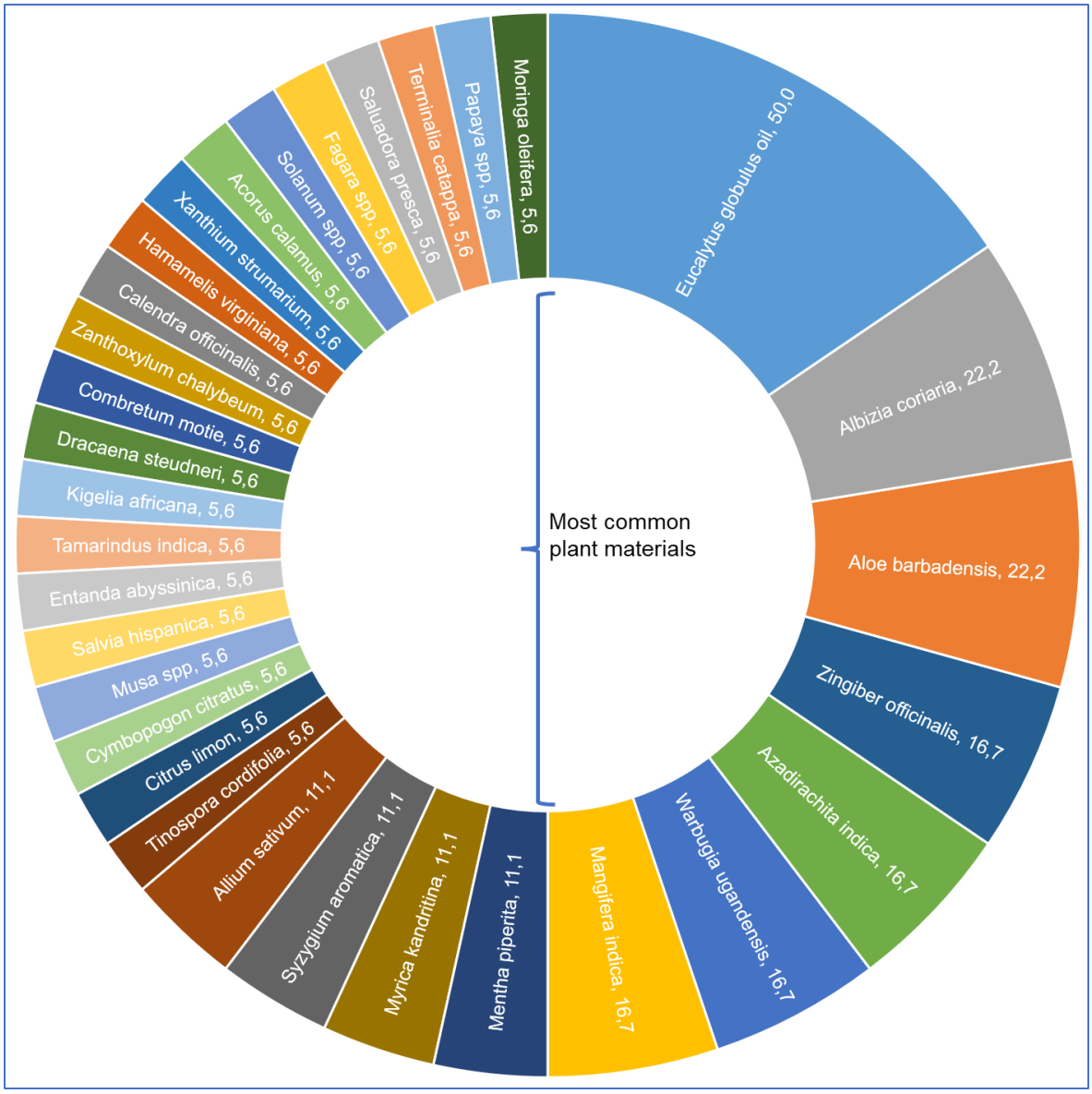


Figure 13: Popularity of the plant raw materials for manufacture of herbal products. The plants were ranked based on the number of times they appeared on the product label as active ingredients.

Most of the herbal products are used to manage self-limiting conditions. For simplification, the product indications, as written on the product label, were classified into eight major disease categories as shown in (Figure 14).

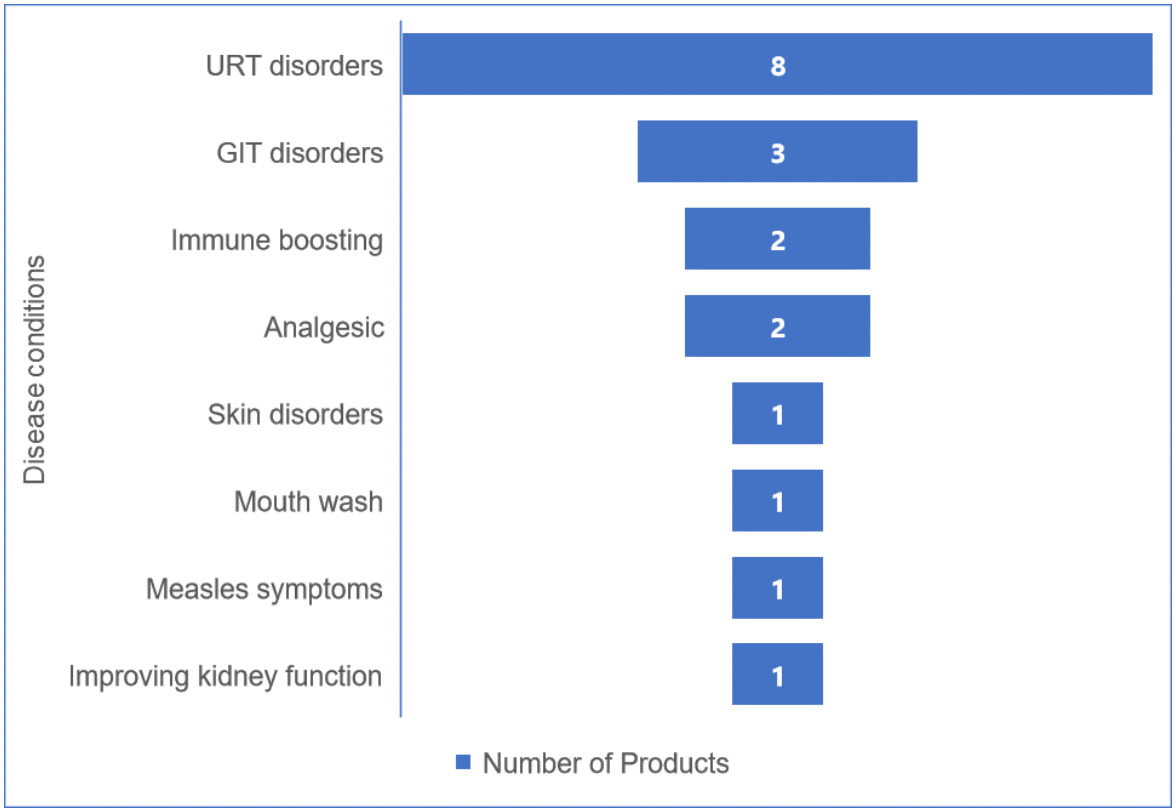


Figure 14: Disease conditions managed by Ugandan made herbal products. GIT-gastro intestinal tract; URT-upper respiratory tract.

Over 40% of the medicines are used to treat upper respiratory tract disorders (whooping cough, catarrh, sore throat, asthma, bronchitis, cough, flu, measles symptoms, allergic cough, smokers cough, productive cough, lung cleaning, sinusitis, common colds, rhinitis, allergic conditions, bronchial congestion, painful coughing, dry/irritating cough, nasal congestion). The rest of the products are indicated for gastrointestinal disorders (cleanses GIT, gastric ulcers, stomach ulcers, flatulence, constipation, ulcers); immune boosting (boost the immune system, antioxidant); analgesic (pain relief, painful lips); skin disorders (relief of minor skin irritations due to; insect bites, minor cuts, and minor scrapes); mouth wash (toothache, bad odor, sensitivity, bleeding gums, cavities, tooth decay).

4.1.2 Establishment of evidence for efficacy and safety of the most used plant materials

Published literature on seven of the most frequently used plant materials were reviewed in detail, for evidence of safety and efficacy in managing the conditions on the label claim. These

were; *A. coriaria, M. indica, A. indica, A. barbadensis, W. ugandensis, Z. officinale,* and *E. globulus.* The indications of the products are summarized in Table 7 below.

Table 7: Disease conditions managed by the most industrially consumed herbal materials in Uganda.

No.	Plant material Source of plant Part of plant used	Dosage form of product containing the plant material	Indication(s) of the product containing the plant material
1	*Eucalyptus globulus* Labill. (Myrtaceae) Wild, cultivated Leaf oil	Mouth wash	Toothache, bad odor, sensitivity, bleeding gums, (tooth) cavities, tooth decay, antibacterial, mouth sores
		Cough syrups	Cough, common cold, catarrh, sore throat, congestion from asthma, bronchitis, whooping cough, allergic conditions- sinusitis, rhinitis, mouth sores, hiccups, relieving fever, measles symptoms
		Pain balm	Pain relief
2	*Aloe vera* Burm.f. (Asphodelaceae) Cultivated Whole leaf	Mouth wash	Toothache, bad odor, sensitivity, bleeding gums, (tooth) cavities, tooth decay, antibacterial,
		Aloe tablets	Cleanses GIT
		cough syrup	Cough, flu, sore throat, sinusitis
		Lip balm	Dry, cracked, and painful lips
3	*Albizia coriaria* Oliv. (Fabaceae*)* Wild Stem bark	(Cough) syrups	Whooping cough, catarrh, sore throat, congestion from asthma and bronchitis, relieving fever, sinusitis
4	*Mangifera indica* L. (Anacardiaceae) Wild, cultivated Stembark, leaves	(Cough) syrups	Whooping cough, catarrh, sore throat, congestion from asthma and bronchitis
5	*Warburgia ugandensis* Sprague (Canellaceae) Wild, cultivated Stem bark, leaves	(Cough) syrups	Cough, flu, mouth sores, measles symptoms, common colds, sinusitis, rhinitis, asthma, catarrh, whooping cough,

			bronchial congestion, mouth sores, hiccups
		(antiulcer) syrups	(GIT) Ulcers
6	*Azadirachta indica* A. Juss. (Meliaceae) Wild, cultivated Stem bark, leaves	Cough syrup	Cough, flu, sore throat, sinusitis
		(antiulcer) syrups	Gastric ulcers, stomach ulcers, flatulence, constipation
		Lip balm	Dry, cracked, and painful lips
7	*Zingiber officinale* Roscoe (Zingiberaceae) Cultivated Rhizome	cough syrups	Allergic cough, smokers cough, whooping cough, productive cough, flu, lung cleaning, sore throat, sinusitis, bronchial asthma, relieving fever

4.1.2.1 *Albizia coriaria* Oliv. (Fabaceae)

Albizia coriaria (light wood) is used in many local herbal preparations to manage upper respiratory tract (URTI) and other infections in Uganda (Anywar et al. 2020; Schultz, Anywar, Wack, et al. 2020). The plant is locally known as mugavu (Luganda), musisa (runyakitara), itek (Luo), among others (Orwa et al. 2009b). From the survey, the products containing *A. coriaria* included Gabogola syrup®, Kwesiima cough mixture®, Muwereza herbal cough remedy®, Phycof cough syrup®. Upper respiratory tract infections are typically caused by the rhinovirus, adenovirus, influenza, enterovirus, and respiratory syncytial virus (Thomas and Bomar 2020). Bacteria cause about 15% URTI and the most implicated bacteria is the *Streptococcus pyogenes*. URTI causes a variety of diseases with overlapping presentations, including; acute bronchitis, influenza, rhinitis, pharyngitis, tonsillitis, laryngitis, and other respiratory distress syndromes. Symptoms of URTI commonly include; cough, sore throat, runny nose, nasal congestion, headache, low-grade fever, facial pressure, sneezing, malaise, myalgias. Most viral infections are self-limiting (Thomas and Bomar 2020). The goal of treatment is symptom relief, and in case of bacterial infections, anti-infectives can be used (Irwin et al. 2006). Therefore, for the effective management of URTI, the medication should possess one or more of the following activities; antiviral, antibacterial, anti-inflammatory, expectorant, and decongestant activity.

Bioactive compounds, such as lupeol, lupenone, betulinic acid, and catechin extracted using ethyl acetate, from the stem back of *A. coriaria* had activity against *Pseudomonas aeruginosa*

and *Escherichia coli (Byamukama et al. 2015)*. These bacteria are, however not commonly implicated in the causation of URTI. No specific studies were evaluating the activity of the bark of *A. coriaria* against gram-positive cocci, which are the causative agents of URTI. Lupeol has anti-inflammatory activity (Wal et al. 2011). Lupeol reduced mucus secretion and overall lung inflammation in a murine model (Vasconcelos et al. 2008). The antiviral activity of lupenone is selective. It exhibits a strong viral plaque inhibitory effect against herpes simplex virus (HSV)-1 and HSV-2 and inhibits African swine fever virus (Wu et al. 2017), but none of these viruses cause URTI. When studied in experimentally induced polymicrobial sepsis in mice, betulinic acid exhibited a protective effect on the lungs by inhibiting the production of inflammatory mediators. In another study, betulinic acid showed anti-inflammatory and antioxidant properties that protect the lungs against lipopolysaccharide-induced lung inflammation in rats (Nader and Baraka 2012).

Herbalists use *A. coriaria* in very small quantities in polyherbal preparations and boil it for several hours because of its known toxicity. In a previous study,, the DMSO and ethanol extracts of *A. coriaria* were highly cytotoxic to the human glioblastoma U87.CD4.CXCR4 cells (CC_{50} = 6.4 and < 4µg/ml respectively) (Anywar et al. 2020). *Albizia spp*. has a toxic compound, 4-Methoxypyridoxine, which antagonizes vitamin B_6 (Botha and Penrith 2008). Other *Albizia* species have shown different toxicities, including several central nervous system disturbances and other effects on the cardiovascular and respiratory systems. For instance, seed methanolic extracts of *Albizia greveana* and *Albizia bernieri* are toxic to mice with LD50 of 1.13-2.3 and 52 mg/kg body weight, respectively, the former being the more toxic one (Randrianarivo et al. 2014). However, the methanol and aqueous extracts of *A. coriaria* have been shown to have very low cytotoxicity (CC_{50} >500µg/ml) against human embryonic lung fibroblast cells (HELF)(Kigondu et al. 2009),. and the methanol, ethanol, ethyl acetate, and diethyl ether extracts of *A. coriaria* were not toxic to the human keratinocyte cell line (HaCaT) IC_{50} > 512 (Schultz, Anywar, Tang, et al. 2020).

There is preclinical evidence for the usefulness of *A. coriaria* in the symptomatic management of respiratory tract conditions. However, studies need to be done to assess activity against the causative organisms of upper respiratory tract infections. Toxicity studies on the bark of *A. coriaria* need to be done to conclude on the safety of the plant.

4.1.2.2 *Mangifera indica* L. (Anacardiaceae)

From the survey, *Mangifera indica* (mango) is used in many preparations for URTI management, including Gabogola syrup®, Kwesima cough mixture®, Yeco cough doctor®.

The plant is locally known as muyembe (among the Bantu ethnic group) (Orwa et al. 2009a), Previous reports indicate that *M. indica* is widely used to manage various infections and as an immune booster in Uganda (Anywar et al. 2020). Various pharmacological investigations have been carried out to explore the therapeutic potential of the phytochemicals in different plant parts of *M. indica* (Shah et al. 2010). For the management of URTI, the antiviral, antibacterial, anti-inflammatory, expectorant, and decongestant activity of *M. indica* is important. Extracts from the fruit pulp of *M. indica* possess anti-viral activity against influenza virus H9N2.(Amin 2019). Mangiferin, extracted from the leaves of *M. indica* inhibited Herpes simplex virus 2 (HSV-2) replication. (Zhu et al. 1993). Extracts from the stem bark of *M. indica* have some bacteriostatic activity against S. *aureus* albeit lower than the activity of the positive control used, ampicillin (Mushore and Matuvhunye 2013). (Alok et al. 2013) demonstrated the antibacterial effects of the aqueous kernel extracts of two *M. indica* varieties (Bagnapalli and Senthura) from Tamil Nadu (India) against *Staphylococcus aureus* and *Pseudomonas aeruginosa.* Acetone, methanol, and water extracts of the leaves on *M. indica* exhibited lower antibacterial activity against *S. aureus, S. pyogenase, S. pneumoniae, Bacillus cereus, E. coli, P. aeruginosa, Proteus mirabilis, Salmonella typhi* and *Shigella flexnerri* using the agar well (cup plate) diffusion method than gentamicin and erythromycin (Doughari and Manzara 2008).

A toxicological evaluation of *M. indica* leaf extract containing 60% Mangiferin revealed a no-observed-adverse-effect level in male and female rats of 2000 mg/kg body weight/day. Additionally, no evidence of genotoxicity was found in a bacterial reverse mutation test (Reddeman et al. 2019). *In vivo* acute toxicological studies of the stem bark aqueous extract of *M. indica* in rats and mice showed no lethality at the limit doses of 2,000 mg/kg body weight and no adverse effects when given by oral or dermal administration. Deaths occurred when the extract was administered intra-peritoneally at 200mg/kg in mice (Garrido et al. 2009). *M. indica* extracts were non-irritating on rabbits' skin, ocular and rectal mucosa (Garrido et al. 2009).

There were no specific studies found evaluating the expectorant and decongestant activities of *M. indica*. Overall, the antiviral and antibacterial activity of various parts of the plant supports its use in URTI management.

4.1.2.3 *Azadirachta indica* A. Juss. (Meliaceae)

From the survey, *Azadirachta indica,* commonly known as neem tree is used in the following products: Muwereza herbal cough remedy®, Princess aloe lip balm®; a lip balm®, and Rezalin for ulcer®, a syrup for management of gastric ulcers, flatulence, and constipation. To

support its use in a cough remedy, *A. indica* should possess expectorant, decongestant, antiviral and/or antibacterial activity (Irwin et al. 2006). The bark extract of *A. indica* significantly blocked HSV-1 entry into cells at concentrations ranging from 50 to 100 µg/mL (Tiwari et al. 2010). The methanolic extract fraction of leaves of *A. indica* is virucidal against coxsackie virus B-4, by interfering viral replication (Badam, Joshi, and Bedekar 1999). Thus, the antiviral activity has been demonstrated but not in the viruses known to commonly cause URTI, such as the rhinovirus, adenovirus, influenza virus, enterovirus, and respiratory syncytial virus.

In other studies, the bark, leaf, seed, and fruit extracts of *A. indica* showed a concentration-dependent antibacterial activity against bacteria isolated from an adult mouth (Yerima et al. 2012). In addition, the ethanolic extract of neem twigs, barks, and leaves are bactericidal against *streptococcus mutans* (Kumari et al. 2019).

The methanol fraction of neem oil possesses bactericidal activity against 9 strains of *Helicobacter pylori*, the causative agent of gastric ulcers (Blum, Singh, and Merrell 2019). The extract's minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against the *H. pylori* strains ranged between 25 and 51 µg/mL and 43-68 µg/mL, respectively. The bactericidal activity was time- and concentration-dependent (Blum, Singh, and Merrell 2019). This evidence supports the use of neem in Rezalin® syrup to manage ulcers.

The wound-healing effect of neem could be beneficial in the lip balm. The water extract of the stem bark of *A. indica* promoted wound healing in mice by increasing the rate of wound contraction and levels of hydroxyproline, DNA and protein content, and nitric oxide. These effects promote wound healing through increased inflammatory response and neovascularization (Maan, Yadav, and Yadav 2017).

Neem oil poisoning is rare in adults. However, one such case was reported in an adult male who presented with vomiting, seizures, metabolic acidosis, and toxic encephalopathy (Mishra and Dave 2013). The patient had a complete recovery from symptomatic management. *In-vivo* evaluation of acute toxicity of water extracts of *A. indica* leaves and seeds in rats revealed a LD_{50} of 6.2 and 9.4 ml/ kg, respectively (Bakr 2013).

There is evidence to support the use of *A. indica* in URTI management, gastric ulcers associated with H. pylori infection, and wound healing. The use of the oil has the potential to cause adverse events.

4.1.2.4 *Aloe barbadensis* (L.) Burm.f. (Asphodelaceae)

Aloe barbadensis, commonly known as Aloe vera, is a constituent of Nana herbal mouth wash®, Damaleo vera tablets® (for cleansing the GIT, promotion of kidney function and boosting the immune system), Muwereza herbal cough remedy® and princess aloe lip balm®.

A wound dressing gel containing acemannan, extracted from the internal leaf aloe gel, reduced radiation-induced skin reactions in mice when applied daily for at least 2 weeks, beginning immediately after irradiation (Roberts and Travis 1995). Furthermore, the extracts of crude *A. barbadensis* gel suppresses the induction of T cell-mediated contact and delayed-type hypersensitivity caused by cutaneous exposure to ultraviolet light (Byeon et al. 1998). This effect is attributed to Aloe oligosaccharides. The above evidence supports the use of aloe extracts in lip balm.

Acemannan from *A barbadensis* extract stimulated macrophage production of monokines, including interleukin-1 and tumor necrosis factor resulting in the initiation of immune attack, necrosis, and regression of implanted sarcomas in mice (Peng et al. 1991). In another study, *A. barbadensis* stimulated cellular and humoral immune responses in rabbits following immunization against myxomatosis (Vahedi et al. 2011). Furthermore, Aloe extracts enhanced the ability of broiler chickens to mount a humoral immune response when challenged with Newcastle disease virus (Ojiezeh and Eghafona 2015). These studies support the use of *A. barbadensis* extract to boost the immune system.

Emodin, an anthraquinone prepared from aloin, was shown to inactivate herpes simplex virus type 1 and type 2, varicella-zoster virus, pseudorabies virus, and influenza virus and rhinovirus. Electron microscopic examination of the anthraquinone-treated herpes simplex virus demonstrated that the anthraquinones work by disrupting the viral envelopes. (Sydiskis et al. 1991). This antiviral activity against rhino viruses is important in the management of URTI.

Aloe emodin is a known stimulant laxative (Lombardi et al. 2020) and this could be the basis of the use of *A. barbadensis* in Damaleo vera tablets for cleansing the GIT.

Several single case reports have been published on the toxicity of *A. barbadensis* in humans. Some include massive intraoperative bleeding after consumption of *A. barbadensis* tablets (Lee et al. 2004), acute renal failure (Luyckx et al. 2002), severe vomiting (Wang et al. 2003), acute hepatitis (Rabe et al. 2005), Henoch-Schonlein purpura (Evangelos, Spyros, and Spyros 2005). (Ernst 2000) reported on the hypersensitivity and allergic reactions due to *A.*

barbadensis. In another study, rats fed aloe whole-leaf powder, administered for 90 days showed that adverse effects started at a doses above 2 g/kg BW (Zhou et al. 2003). Aloe inner leaf gel rich in high molecular weight fibers and soluble polysaccharides is cytotoxic to the human intestinal cell line Caco-2 (CC_{50} = 1 g/l), even at concentrations below the recommended dose for human consumption (López et al. 2019). Processing of aloe inner leaf gel or aloe leaf extract by filtration through diatomaceous earth and activated carbon reduces their cytotoxicity and improves their immunomodulatory activity (López et al. 2019).

Literature supports the use of aloe extracts for cosmetic purposes, boosting the immune system, management of URTI and constipation. However, studies also show that aloe extracts can be toxic although processing can reduce such toxicities.

4.1.2.5 *Warburgia ugandensis (*Sprague. (Canellaceae)

W. ugandensis is commonly known as East African greenheart or pepper-bark tree. In Uganda, is locally called muwiya, or mukuzanume (Luganda) (Orwa et al. 2009b). From the study, the following products contained the plant material: Lucas syrup®, Sacco syrup® and Focus herbal cough syrup®, used in the management of URTI, and Jenacid negas® indicated for the management of gastric ulcers.

The water extracts of *W. ugandensis* exhibited antimicrobial activity against *E. coli* and *S. aureus* in the agar well assay, with higher activity against *S. aureus* than *E. coli.* This activity was only detectable at high concentrations of the extract i.e., up to 50 mg/ml of crude extract (Olila and Opuda-Asibo 2001). In another study, the crude and petroleum ether extracts of *W. ugandensis* leaves exhibited antimicrobial activity against *S. boydii* and *S. aureus* (Merawie et al. 2013). The biological activities of the plant are attributed to the drimane and colorotane sesquiterpenes, including polygodial, warburganal, muzigadial, mukaadial and ugandensial, as well as flavonoids and other compounds (Leonard and Viljoen 2015; Maroyi 2014). The sesquiterpene muzigadial isolated from the ethyl acetate extract of the stem bark of *W. ugandensis* was the main antibacterial agent (IC = 12.5 µg/ml) against both *S. aureus* and *Bacillus subtilis* (Rabe and Van Staden 2000).

Other reported pharmacological activities of *W. ugandensis,* that could be relevant in managing upper respiratory tract disorders include, antimycobacterial activity, anti-inflammatory and antioxidant properties, (Maroyi 2014). The anti-inflammatory effects of *W. ugandensis* was demonstrated by (Schultz et al. 2021). The diethyl ether extract inhibited

both COX-2 and COX-1 *in vitro* with IC_{50} values of 13.33 ± 4.36 and 11.05 ± 1.43 µg/ml respectively.

While *W. ugandensis* is widely used by herbalists in Uganda for treating various infections, it is also known to be toxic if used in high doses (Anywar et al. 2020). The acute toxicity evaluation of the aqueous stem bark extract of *W. ugandensis* in mice gave a LD_{50} > 5000 mg/kg body weight with no mortalities recorded for all dose levels used.

Antiviral activity studies against viruses that cause URTIs and evidence of anti-ulcer activity were not found in literature. Thus, the scientific evidence to support the efficacy of *W. ugandensis* in treatment of URTIs and ulcers is weak, although some benefits may accrue from the anti-bacterial and anti-inflammatory activities reported above. The drug can be toxic when used in high doses.

4.1.2.6 *Zingiber officinale* (Rosc.) (Zingiberaceae)

Zingiber officinale, commonly known as ginger is a household product in Uganda. The plant is known locally as Ntungawuzi (Luganda). The survey results showed that ginger is a constituent of syrups for the management of URTI including Yeco cough doctor® and Muwereza herbal cough remedy®.

Z. officinale contains various phytochemicals including phenolic compounds, terpenes, polysaccharides, lipids, organic acids, and raw fibers. The major pharmacological activities of ginger have been attributed to phenolic compounds such as gingerols and shogaols (Mao et al. 2019). Various studies have demonstrated the anti-inflammatory activity of ginger and its bioactive compounds. Orally administered ginger reduced the pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β), and increased the anti-inflammatory cytokines (IL-10 and IL-22) in mouse colitis models (Zhang et al. 2016). *Z. officinale* attenuated post exercise induced elevation in several pro-inflammatory cytokines including interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) in well trained male endurance runners (Zehsaz, Farhangi, and Mirheidari 2014).

Intravenous administration of 6-gingerol (at 1.75-3.5 mg/kg) and 6-shogaol (at 1.75-3.5 mg/kg); and oral administration of both at (at 70-140 mg/kg) produced an inhibition of spontaneous motor activity, as well as antipyretic and analgesic effects, the latter producing more intense effects than the former. 6-Shogaol also showed an intense antitussive effect in comparison with dihydrocodeine phosphate (Suekawa et al. 1984). These activities support the use of *Z. officinale* in herbal cough remedies for their cough suppressant activity.

The ethanolic extract of *Z. officinale* rhizome was active against common respiratory tract pathogens; *S. aureus, S. pyogenes, S. pneumoniae* and *Hemophilus influenza (Akoachere et al. 2002).* Furthermore, a herbal extract of *Z. officinale* inhibited biofilm formation and affected the membrane integrity of multidrug resistant *P. aeruginosa*, both of which play a role in the mechanism of infection and antimicrobial resistance (Chakotiya et al. 2017). The crude and methanolic fraction of ginger reduced biofilm formation, inhibited glucan synthesis and adhesion, and downregulated the virulence genes in *S. mutans* (Saffari et al. 2015) .

The antiviral activity of ginger has been demonstrated in some viruses not specifically implicated in the causation of common URTI. Fresh *Z. officinale* dose-dependently inhibited human respiratory syncytial virus (HRSV), and induced plaque formation in human respiratory tract cell lines (San Chang et al. 2013). Fresh ginger also dose-dependently inhibited viral attachment and internalization and at high concentration, stimulated mucosal cells to secrete interferon β (IFN-β) that possibly contributed to counteracting viral infection. Other studies have demonstrated antiviral activity of *Z. officinale* in hepatitis C virus (HCV) (Abd El-Wahab, El-Adawi, and El-Demellawy 2009) and Chikungunya virus which causes an arthritogenic febrile illness (Kaushik et al. 2020).

Studies on the safety of different *Z. officinale* rhizome extracts showed no treatment-related signs of toxicity or mortality in any animals at tested doses (Plengsuriyakarn et al. 2012; Rong et al. 2009; Shalaby and Hamowieh 2010; Weidner and Sigwart 2000). One such study revealed a no-observed-adverse-effect-level for a crude ethanolic extract of 5000 mg/kg body weight (Plengsuriyakarn et al. 2012).

There is scientific evidence in support of the use of *Z. officinale* extracts in preparations for the management of upper respiratory tract diseases. The rhizome has been established to be safe.

4.1.2.7 *Eucalyptus globulus* Labill. (Myrtaceae)

Eucalyptus globulus is locally called eucalyptus. This survey established that it is a constituent of several products namely; Nana herbal mouth wash®, Princess pain balm®, Phycof cough syrup®, focus herbal cough syrup® Lucas syrup®, Kwesiima cough mixture®, Gabogola syrup® and Sacco syrup®.

An essential oil blend of *E. globulus* with other plant oils was effective against fourteen gram-positive and gram-negative bacteria strains tested, including some antibiotic-resistant strains (Brochot et al. 2017). The organisms tested in the study included those implicated in causation

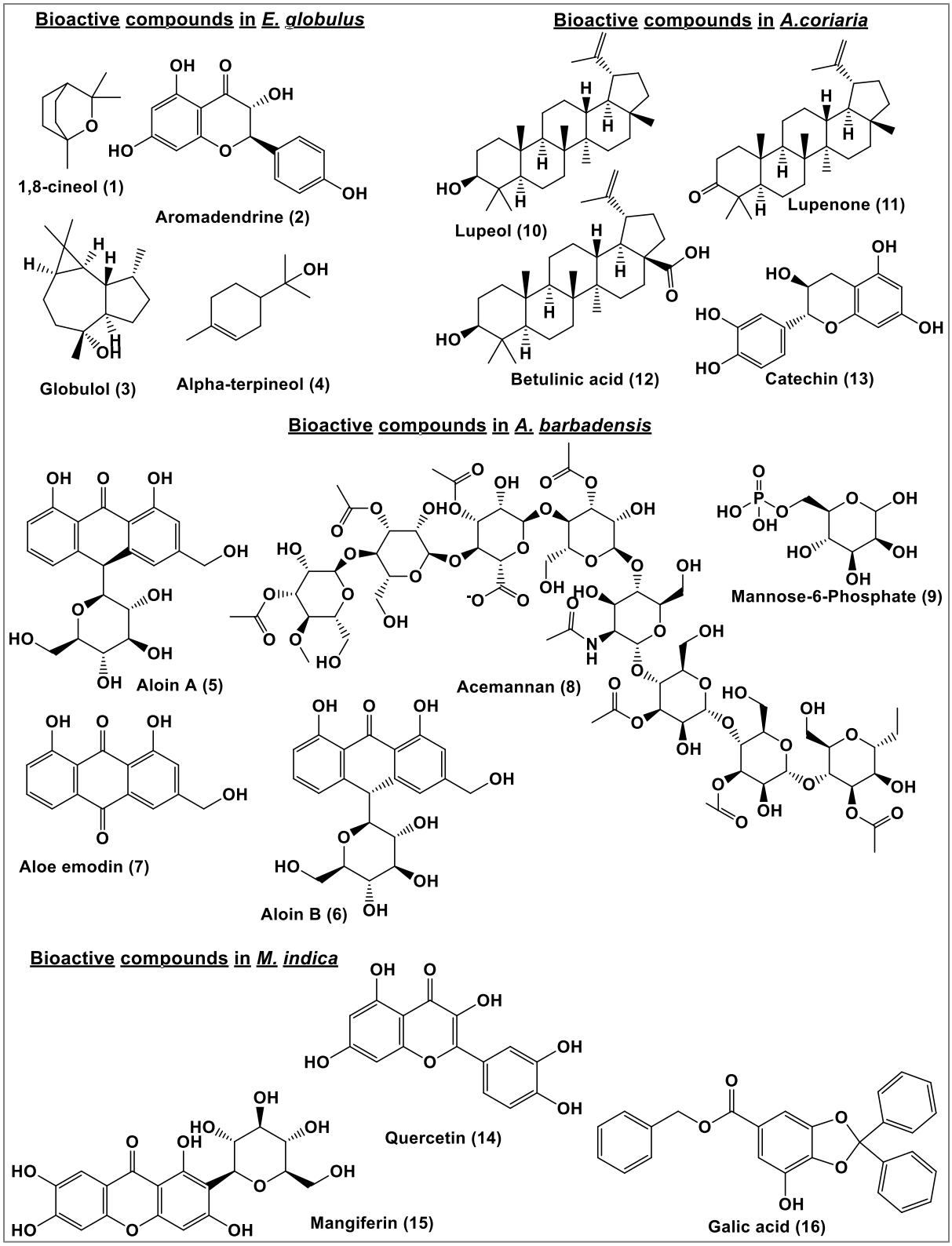
of URTI for example *S. pyogenes*, *S. pneumoniae*, *Listeria monocytogenes* and *S. aureus.* MICs ranged from 0.01% to 3%. The study also found the blend effective against H1N1 influenza virus making it suitable for management of influenza and post-influenza bacterial pneumonia infections (Brochot et al. 2017). The antibacterial effect of *E. globulus* has also been demonstrated in the organisms causing dental carries especially *S. mutans* (Goldbeck et al. 2014) and *Lactobacilli* (Ambrosio et al. 2018; Khozeimeh et al. 2015).

The essential oil of *E. globulus* exhibited analgesic and anti-inflammatory effects in mice and rat models (Silva et al. 2003). The essential oil of *E. globulus* possesses central and peripheral analgesic effects as well as neutrophil-dependent and independent anti-inflammatory activities (Silva et al. 2003).

An eucalyptus oil water emulsion had an LD_{50} of 3811.5 mg/kg in rats. The oil slowed growth of the male rats and damaged the liver and the kidneys at 792 and 1188 mg/kg BW after oral administration (Hu et al. 2014). In another study, the a LD_{50} of 1650 mg/kg in mice was determined. However, there was no abnormal skin reaction observed when a 5% ointment formulation was administered on the rats' skin indicating the safety of *E. globulus* essential oil at a relatively lower concentration (Mengiste et al. 2020). *E. globulus* is safe for use, at low concentrations, for the management of URTI and dental conditions.

4.1.3 Bioactive compounds supporting the industrial applications of plants used in local products

For most of the plant materials, there are compounds with biological evidence relevant to the industrial application of the products in which the medicinal plant is contained. However, most of the compounds in *Warburgia ugandensis* have not been individually evaluated. The well studied bioactive compounds important to the industrial applications of these plants are shown in Figure 15. In *E. globulus, they are; (*1,8-cineol (1), aromadendrin (2), globulol (3) and α-terpineol (4)). In *A. barbadensis*; (aloin A (5), aloin B (6), aloe emodin (7), acemannan (8) and mannose 6 phosphate (9)). In *A. coriaria*; (lupeol (10), lupenone (11), betulinic acid (12), catechin (13)). In *M. indica*; (catechin (13), quercetin (14), mangiferin (15) and gallic acid (16)). In *Azadirachta indica*; (azadirachtin (20), mahmoodin (21), nimbin (22), and nimbolide (23). In *Zingiber officinalis*; (gingerols (17), shogaols (18) and zingerone (19)). In *Warburgia ugandensis*; (bemadienolide (24), polygodial (25), warbuganal (26), ugandensolide (27), and muzigadial (28).


Bioactive compounds in E. globulus
1,8-cineol (1)
Aromadendrine (2)
Globulol (3)
Alpha-terpineol (4)
Bioactive compounds in A.coriaria
Lupeol (10)
Lupenone (11)
Betulinic acid (12)
Catechin (13)
Bioactive compounds in A. barbadensis
Aloin A (5)
Acemannan (8)
Mannose-6-Phosphate (9)
Aloe emodin (7)
Aloin B (6)
Bioactive compounds in M. indica
Quercetin (14)
Mangiferin (15)
Galic acid (16)

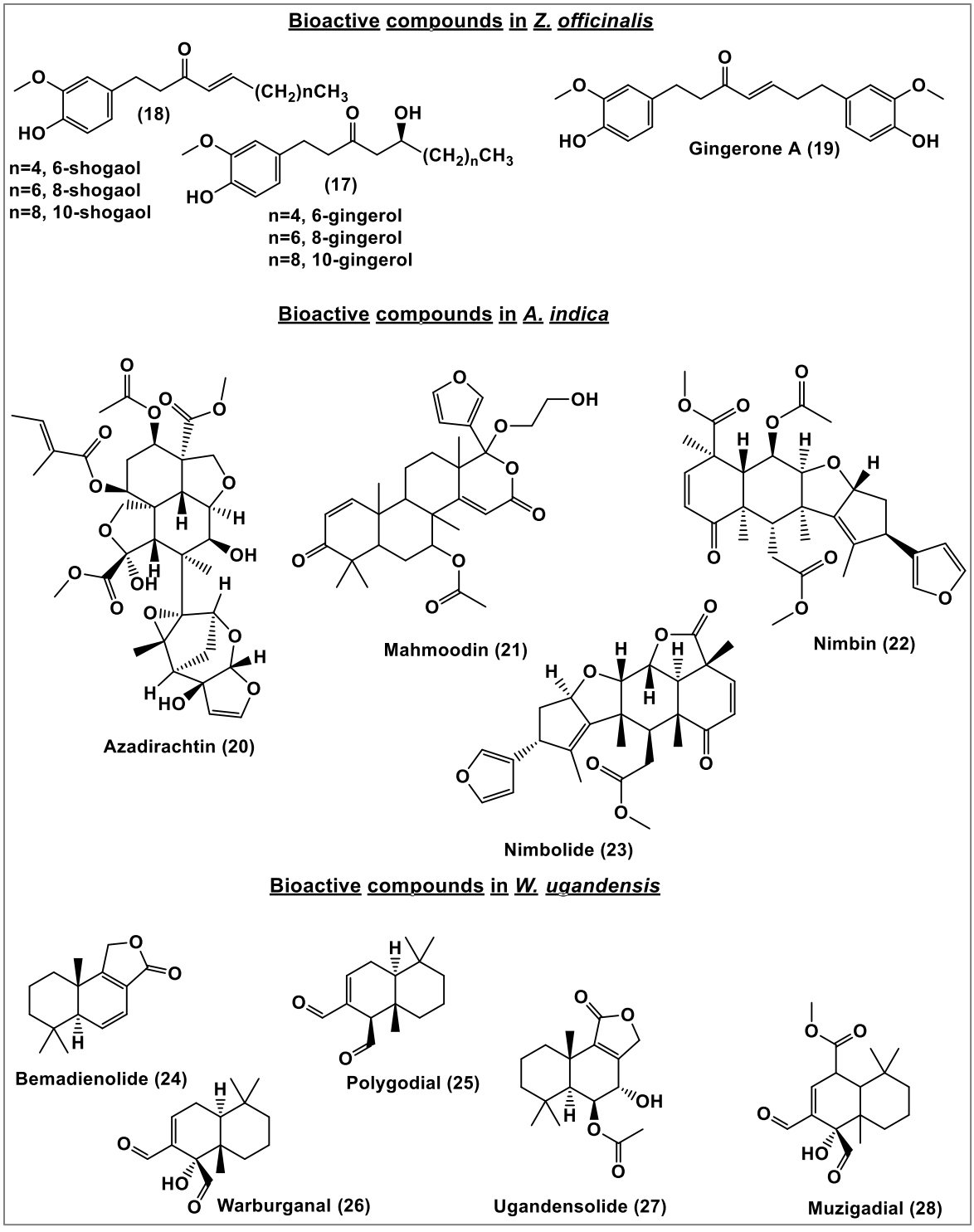


Figure 15: Bioactive compounds in the studied medicinal plants relevant to industrial application (The structures were generated with ChemDraw® software).

The biological activities of these compounds and their mechanisms of action are summarized in Table 8 below.

Table 8: Evidence of biological activity of the compounds found in the plant materials, relevant for industrial use

Plant species and manufacturer therapeutic claim	Bio-active compound	Evidence of Biological activity of bioactive compound related to traditional use of the plant	Mechanism of action
Eucalyptus globulus For pain relief, URT disorders, mouth wash, GIT disorders	Aromadendrine (dihydrokaempferol)	Anti-inflammatory Ani-oxidant	Scavenging of reactive oxygen species, chelation of metal ions (Amić et al. 2021)
		Antibacterial; *Staphylococcus aureus, Streptococcus mutans*	Inhibits biofilm formation (Zeng et al. 2019)
	1,8-cineol	Antibacterial-against *K. pneumoniae*, *S. aureus*, *P. aeruginosa*	Disruption of bacterial cell membrane & loss of intracellular materials (Moo et al. 2021)
		Antiasthma, anti-bronchitis, anti COPD, Anti-influenza	Down-regulation of inflammation cytokines such as interleukin- 1b (IL-1b) and tumor necrosis factor-a resulting in bronchial muscle relaxant and reduction in mucus secretion (Juergens et al. 2004)
		Anti-inflammatory Ani-oxidant	Regulates nuclear factor-kappa B (NF- κB) and nuclear factor erythroid-2-related factor 2 (Nrf2) pathways (Cai et al. 2021; Jiang et al. 2019)
		Analgesic/sedative	Suppression on the CNS by modulating glutamatergic & dopaminergic systems, activates transient receptor potential melastatin 8 (Luedtke et al. 2012; Takaishi et al. 2012)

		Antispasmodic and antisecretory, gastroprotective, antidiarrheal	Promotes regeneration of the gastric cells, increases gastric mucus, antioxidant anti-inflammatory effects (Cai et al. 2021)
	globulol	Antibacterial-against *K. pneumoniae*, *S. aureus*, *P. aeruginosa* (Bachheti 2015; Mulyaningsih et al. 2011)	
	α-terpineol	Anti-inflammatory & antioxidant mainly analgesic	Suppresses superoxide production by monocytes; inhibits release of inflammatory mediators including serotonin, histamine, bradykinin, & prostaglandins (Brand et al. 2001; Quintans-Júnior et al. 2011)
		Antibacterial-against *K. pneumoniae*, *S. aureus*, *P. aeruginosa*	As 1,8-cineole
		Anti-gastric ulcer	As 1,8-cineole
Aloe barbadensis for cracked lips, mouth wash, URT disorders, GIT disorders	Aloin A and B Aloe emodin	Laxative	Inhibition of Na+/K+ pump & Cl-channels increase gastric motility; stimulate secretion of mucus and chloride ions (de Witte 1993; Ishii, Tanizawa, and Takino 1990)
		and immune-modulatory effects	Inhibition of histamine release from mast cells -reduced production of Tumor Necrosis Factor (TNF)-α (Arosio et al. 2000)
		anti-inflammatory-anti-oxidant	Cyclooxygenase pathways and reducing prostaglandin

			E2 production (Im et al. 2005; Park, Kwon, and Sung 2009)
		anti-bacterial activity-H pylori	Activation of phagocytic leukocytes; inhibition of the N-acetyltransferase activity of *H. pylori* (Sánchez-Machado et al. 2017; Wang et al. 1998)
	Acemannan	Wound healing anti-inflammatory	Activates macrophages to release fibrogenic cytokines; inhibits thromboxane A_2 (Roberts and Travis 1995; Karaca, Sharma, and Nordgren 1995) Cyclooxygenase pathways and reducing prostaglandin E2 production (Arosio et al. 2000)
	Mannose 6 phosphate	Wound healing	Enhances activity of fibroblasts (Davis, Donato, et al. 1994; Davis, DiDonato, et al. 1994)
	Lupeol	Anti-inflammatory-anti-oxidant Wound healing effect	Lupeol reduces TNF-α, IL-1, and IL-6 cytokine production. This lowers the infiltration macrophage to damaged tissues, hence reducing inflammation. (Rathinavel, Ammashi, and Shanmugam 2021). It also chelates toxins such as heavy metal ions (Sunitha, Nagaraj, and Varalakshmi 2001) Stimulates the production, and migration of keratinocytes and

			fibroblasts to injured tissues, by activating the PI3K-PKB/Akt and p38/ERK/MAPK pathways (Pereira Beserra et al. 2018).
Albizia coriaria for URT disorders	Lupeol and lupenone	Anti-inflammatory Analgesic	Decreases PGE_2, TNF-α, interleukin-1b production (Ciurlea et al. 2010) (Thirumalaisamy et al. 2020)
		Immunomodulating; anti allergen, anti-asthmatic,	Reduces the production eosinophils, thus interleukins-reduced fluid production in the bronchoalveolar pathway (Lee, Lee, et al. 2016)
		Antibacterial-against *S. aureus*, *K. pneumoniae*, *P. aeruginosa* (Okusa et al. 2014; Ahamed et al. 2007)	
		Anti-viral-herpes simplex (Mutai et al. 2012)	Inhibits virus plaque formation (Tanaka et al. 2004)
	Betulinic acid/betulin	Anti-inflammatory Analgesic	Inhibits production of nitric oxide & PG_2 (cyclooxygenase-2 activity); also decreases production of pro-inflammatory cytokines including IL-1β, IL-6, IL-8, IL-12, & TNF (Yun et al. 2003; Oyebanji, Saba, and Oridupa 2014)
		Antibacterial-*P. aeruginosa*, *S. aureus*, *Mycobacterium tuberculosis* (Maghraby and Abdelfattah 2022)	Enhance the rate of electron transport chain activity, which results in excess production of ROS, which damage DNA, and cause bacterial death (Oloyede et al. 2017).

		Anti-viral-against herpes simplex (Kurokawa et al. 1999)	Inhibits viral plaque formation (Tanaka et al. 2004)
	(+/-) Catechin	Anti-inflammatory	Radical scavenging; activates production of erythroid-derived factor 2 which regulates antioxidant enzymes (Chiou et al. 2016)
		Immunomodulatory; anti-allergenic	Reduces production & infiltration of lung tissue by inflammatory cytokines such as TNF-α, IL-1β (Kim et al. 2021)
		Anti-viral-against Influenza A, SARS-CoV-2 (Mishra et al. 2021; Onishi et al. 2020)	Inhibits viral receptor binding (Song, Lee, and Seong 2005)
Mangifera indica For URT disorders	Mangiferin	Antioxidant, anti-inflammatory, antipyretic, analgesic,	Scavenging of ROS, chelation of toxic metal ions; down-regulates phosphorylation of NF- κB pathways-reduces production of proinflammatory cells (Pardo-Andreu, Sánchez-Baldoquín, Ávila-González, et al. 2006; Li et al. 2017)
		Antiallergic, anti-asthmatic, immunomodulatory	Reduces tracheal contraction by inhibiting the nitric oxide-cyclic GMP pathway (Vieira et al. 2013); Inhibits production of nitric oxide and PG2 (cyclooxygenase-2 activity); also decreases production of pro-inflammatory cytokines including (Rivera et al. 2011b)

		Antibacterial-activity against *S. aureus* (Biswas et al. 2015)	Increased antibody titers; increases cell mediated immunity. Stimulates lysozyme activity, (Sahu et al. 2007)
		Antiviral-against Herpes simplex (Du et al. 2018)	Inhibits viral replication (Zheng and Lu 1990)
	(+/-) Catechin, epicatechin	Anti-viral, anti-inflammatory and anti-allergenic	Activity against influenza A and B; catechins inhibit receptor binding and sialidase activities (Song, Lee, and Seong 2005). Catechins regulate the production of proinflammatory agents such as TNF-α, NF-κB, COX-2 in lung tissue. They also scavenge noxious metal ions and ROS (Oh, Ambigaipalan, and Shahidi 2019; Wang et al. 2020).
	Gallic acid	Antioxidant, anti-inflammatory	Scavenging of ROS, chelation of toxic metal ions; down-regulates phosphorylation of NF- κB pathways-reduces production of proinflammatory cells [50-52]
		Antimicrobial- *P. aeruginosa, S. aureus*	Interferes with colonization by inhibiting motility & adherence; disrupts cell membrane leading to leakage of cell nutrients; inhibits dihydrofolate reductase, topoisomerase IV (Kahkeshani et al. 2019; Kang et al. 2008)

		Antiviral - *Haemophilus* influenza A & B	Disruption of the viral particles (Lee, Oh, et al. 2016)
	Quercetin	Antioxidant, anti-inflammatory,	Scavenging of ROS, chelation of toxic metal ions; down-regulates phosphorylation of NF- κB pathways-reduces production of proinflammatory cells, inhibits cyclooxygenase & lipoxygenase enzymes (Oh, Ambigaipalan, and Shahidi 2019; Tang et al. 2014; Granado-Serrano et al. 2012; Boesch-Saadatmandi et al. 2011)
		Immune-modulatory; anti-allergic	Inhibits IL 8 & 6, TNF-α (Li et al. 2016)
		Antibacterial; *P. aeruginosa*, *S. aureus*, *B. subtilis*, *M. tuberculosis*, *K. pneumoniae*	Inhibits nucleic acid synthesis, disrupts plasma membrane, inhibits glutamine synthetase, inhibits biofilm formation (Hossion et al. 2011; Safwat et al. 2018)
		Antiviral - *Influenza-A* virus (Fan et al. 2011)	Interacts with Hemagglutinin (HA) glycoprotein to prevent entry into the host cell, thereby inhibiting viral replication. It also inhibits the M2 protein and neuraminidase (NA) glycoprotein interfering with packaging of genome segments into influenza virus particles (Di Petrillo et al. 2022).

Azadirachta indica For URT disorders, cracked lips (wound healing)	Tetranortriterpenes-Azadirachtin	Anti-inflammatory, antipyretic, antioxidant Wound healing, Anti-gastric ulcer	Inhibits cyclooxygenase (COX), & lipoxygenase (LOX) enzymes. Modulates transcription factors NF-κB, radical scavenging
		Immunostimulant	Inhibits TNF-induced biological responses (Thoh et al. 2011)
		Antibacterial- against *S. aureus* & MRSA	Inhibits biofilm formation (Sarmiento, Maramba, and Gonzales 2011)
	Nimbidin, Nimbin,	Antipyretic, anti-inflammatory and antioxidant (Bhargava et al. 1970; Pillai and Santhakumari 1981)	Suppresses production of inflammatory cytokines from neutrophils & macrophages (Hossain et al. 2013)
		Anti-gastric ulcer	Reduces secretion of gastric acid by inhibiting histamine (H2) receptors and muscarinic receptors (Pillai and Santhakumari 1985)
		Immunomodulatory; anti-allergic	Inhibits macrophage migration (Alam et al. 2012)
	Nimbolide	Antibacterial (Rojanapo et al. 1985)	
	Mahmoodin	Anti-inflammatory, Antibacterial (Ara et al. 1989)	
	Diterpens-Margolone, margolonone, margolonone	Antibacterial-against *Klebsiella, Staphylococcus* & *Serratia* Species (Ara et al. 1989)	
Zingiber officinalis For URT disorders, GIT disorders	Gingerols 8- gingerol 10-gingerol 12-gingerol 6-gingerol	Antioxidant activity, Anti-inflammatory, analgesic: 6-gnigerol and 6-shogaol most studied	Scavenging of ROS, chelation of metal ions; oxygenation arachidonic acid, a substrate for cyclooxygenase enzymes, thus inhibiting production of

	Shogaols 6-shogaol		prostaglandins; reduced activation of macrophages; inhibit nitrite oxide (NO) production (Mustafa, Srivastava, and Jensen 1993; Suekawa et al. 1986)
		Anti-asthmatic, anti-allergen	Reduced contraction of smooth respiratory muscles by reduction in Ca2+ influx & β2 receptor activation; reduced production of proinflammatory cytokines (Aimbire et al. 2007)
		Antibacterial- *S. aureus, Mycobacteria, Streptococcus pyogenes, Streptococcus pneumoniae, Hemophilus influenzae*	Inhibition of biofilm formation, inhibition of hydroxymethyl-7, 8- dihydro pterin pyrophosphokinase, 6-gingero>8- gingerol>10-gingerol>12-gingerol (Hiserodt, Franzblau, and Rosen 1998; Park, Bae, and Lee 2008)
		Anti-gastric-ulcer activities -anti- *H. pylori,* 10-gingero>8-gingerol>6-gingerol>6-shogaol(Mahady et al. 2003) -anti-emetic-	 Inhibit 5-hydroxytryptamine to increase gastric motility and emptying (Yamahara et al. 1989; HUANG et al. 1991)
	Zingerone (major pungent compound in ginger)	Anti-inflammatory antioxidant activity	Reducing ROS production; chelation of metal ions (Aeschbach et al. 1994)
		Antibacterial activity	Dihydro pterin pyrophosphokinase inhibition

Warburgia ugandensis For URT disorders	Drimane sesquiterpenes Muzigadial, Muzigadiolide Warburganal, warburgadione, warburgadial, warburgin Ugandensidial, Ugandensolide polygodial	Anti-inflammatory and anti-allergic – polygodial	inhibition of phospholipase A2 and neuropeptide release (da Cunha et al. 2001)
		Antimicrobial-antimycobacterial (muzigadial & muzigadiolide) (Wube et al. 2005)	
		Antibacterial (against K. pneumoniae, P. aeruginosa, S. aureus (warburganal, ugandensidial, and polygodial)(Khumalo et al. 2019)	

URT-upper respiratory tract, GIT-gastro-intestinal, ROS-reactive oxygen species.

4.1.4 Availability of analytical standards and assay methods

For *Eucalyptus globulus*, *Aloe barbadensis*, *Zingiber officinalis*, and *Azadirachta indica,* monographs with well elaborated quality control methods have been published. In addition, analytical standards for the selected compounds are available commercially and their assay methods have been developed. On the other hand, no monographs for *Albizia coriaria*, *Mangifera indica*, and *Warburgia ugandensis* exist., for *W. ugandensis*, there are even no analytical standards or assay methods to quantify the individual compounds. The cost per mg or mL of analytical standard ranged from €0.6 to 498 for α-terpineol and azadirachtin respectively, with an average cost of €62.5±101.5. The results are summarized in Table *9*.

Table 9: Availability of analytical standards and assay methods for selected medicinal plant compounds

Plant material	Identified Analytical markers	Commercial sources	Smallest commercial unit available, purity and /cost (Euros-€) in 2022	Cost (Euros-€) per mg/mL of marker (a single analysis)	Analytical methods for the plant material mentioned in local or WHO Pharmacopoeia	Assay methods already developed for the compounds in same or other materials
Eucalyptus globulus oil	Aromadendrine (dihydrokaempferol)	Sigma-Aldrich/Supelco	Analytical standard-530/5mg ≥95% HPLC grade-221/10mg ≥95% LC/MS-ELSD-421/1mg	106.0 22.1 421	WHO monographs &African pharmacopoeia-identification tests for 1,8-cineole in oil & leaf materials. TLC fingerprint for leaf materials with 1,8-cineole as reference (World Health Organization	Gas chromatography FID & MS method for quantification of oil components (Mulyaningsih et al. 2011; Apel et al. 2017)
	α-terpineol	Sigma-Aldrich/Supelco	Analytical standard (≥95%)-63.9/100mg	0.6		

	globulol	Sigma-Aldrich	≥98.5% (sum of enantiomers, GC)-247/100mg	2.5	1999; Inter African Committee on Medicinal et al. 2014).	
	1,8-cineol (eucalyptol)	Sigma-Aldrich/Supelco	Analytical standard-48.3/1mL	48.3		
Aloes - whole leaf products	Aloin A	Sigma-Aldrich	Analytical standard-440/10mg	44.0	WHO monographs-Thin-layer chromatography, microchemical analyses to identify anthracenes, Spectrophotometry-to determine total glycosides as aloin (World Health Organization 1999)	HPLC-DAD/MS quantification methods for anthracenes in aloe vera samples (Elsohly et al. 2007)
	Aloin B	Sigma-Aldrich	Phyproof® Reference Substance-605/10mg	60.5		
	Aloe emodin	Sigma-Aldrich	Analytical standard 311/10mg	31.1		
Aloe vera gel	Acemannan	Toronto Research chemicals	Technical grade-208.3/10mg	20.8	Chemical test for carbohydrates; Polysaccharide composition analysis by gas–liquid chromatography (World Health Organization 1999)	Molecular Exclusion Chromatography (Cuevas et al. 2016) UV-Vis Spectrophotometry (Metcalfe 2019)
	D-Mannose 6 phosphate	Sigma-Aldrich	≥98% (HPLC) (sodium salt)-354.0/100mg	3.5		None found
Albizia coriaria stem bark	Lupeol	Sigma-Aldrich	Analytical standard-130/10mg	13.0	No monographs for *Albizia coriaria* stem bark materials	HPLC-DAD quantification method for triterpenoids (Somwong and Theanphong 2021; Kaggwa, Munanura, et al. 2023)

	lupenone	Toronto Research chemicals	Analytical standard-170/2.5mg	68.0		HPLC-DAD quantification method for triterpenoids in *Albizia inundata* (Andrade et al. 2020)
	Betulinic acid	Sigma-Aldrich/Supelco	Analytical standard 95.1/10mg ≥98% (HPLC)-70/5mg	9.5 14.0		HPLC-DAD quantification method for betulinic acid in *Albizia lebbeck* (Tatke, Desai, and Gabhe 2014)
	Catechin	Sigma-Aldrich/Supelco	Analytical standard - 307/10mg	30.7		HPLC-DAD quantification method *Albizia lebbeck* (Desai et al. 2020),
Mangifera indica leaf or stem bark	Mangiferin	Sigma-Aldrich/Supelco	Analytical standard-108/10mg	10.8	No monographs for *Mangifera indica* leaf or stem bark materials	HPLC-UV quantification method for mangiferin (Zhang et al. 2012)
	Catechin	Sigma-Aldrich/Supelco	Analytical standard - 307/10mg	30.7		HPLC-UV quantification method for phenolic compounds (Núñez Sellés et al. 2002b) HPLC methods for quantification of epicatechin (Gottumukkala et al. 2014; Kim et al. 2022)
	Epicatechin	Not found	N/A			
	Gallic acid	Sigma-Aldrich	Phyproof® Reference Substance - 345/100mg	34.5		
	Quercetin	Sigma-Aldrich	United States Pharmacopeia (USP) Reference			

			Standard-357/200mg phyproof® Reference Substance-253/20mg ≥95% (HPLC)-68.3/10g	1.8 12.7 6.8		
Azadirachta indica stem bark or leaf	Azadirachtin	Sigma-Aldrich	Phyproof® Reference Substance-403/5mg ~95%-249/0.5mg	80.6 498.0	WHO monographs-high-performance liquid chromatography quantification of tetranortriterpenes in oil and leaf materials (World Health Organization 2007b)	HPLC-UV quantification method for Azadirachtins (Ramesh and Balasubramanian 1999)
	Nimbin	Toronto Research chemicals	Analytical standard-105/1mg	105.0		HPLC-UV quantification method for nimbin (Sidhu, Kumar, and Behl 2004)
	Nimbidin	Not found	N/A			None found
	Nimbolide	Sigma-Aldrich	≥98%-624/5mg	124.8		HPLC-UV quantification method for nimbolide (Suttiarporn and Choommongkol 2020)
	Mahmoodin	Not found	N/A			None found
Zingiber officinalis rhizome	8-gingerol	Sigma-Aldrich/Supelco	Analytical standard 489.0/10mg	48.9	WHO monographs-Thin-layer chromatography fingerprinting to identify gingerols	HPLC-MS quantification methods for gingerols and
	10-gingerol	Sigma-Aldrich	Analytical standard-			

			546/10mg ≥98% (HPLC)-276/5mg phyproof® Reference Substance-472/10mg	54.6 55.2 47.2	and shogaols; quantitative gas chromatography and high-performance liquid chromatography analyses of ginger oils for gingerols, Shogaols (World Health Organization 1999)	related compounds (Ashraf et al. 2014) (Tao et al. 2009; Park and Jung 2016)
	12-gingerol	Not found	N/A			
	6-gingerol	Sigma-Aldrich	Analytical standard- 448 /10mg ≥98% (HPLC)- 358 /5mg phyproof® Reference Substance-472/10mg	44.8 71.6 47.2		
	6-shogaol	Sigma-Aldrich	Analytical standard-538/10mg Phyproof® Reference Substance-657/10mg	53.8 65.7		
	Zingerone	Sigma-Aldrich	Analytical standard-84.50/50mg	1.7		None found
Warburgia ugandensis stem bark	Polygodial	Sigma-Aldrich	≥97% (HPLC)-216/10mg	21.6	No monograph for *Warburghia ugandensis* stem bark	None found
	Bemadienolide	Not available	N/A			None found
	Muzigadial	Not available	N/A			None found
	Warbuganal	Not available	N/A			None found
	Warbugadione	Not available	N/A			None found
	Warbugadial	Not available	N/A			None found

	Warbugin	Not available	N/A		None found
	Ugandensolide	Not available	N/A		None found
	Ugandensidial	Not available	N/A		None found

N/A-not applicable

4.1.5 Selection of markers

Most of the compounds scored at least 5 points out of 8 except those for *Warburgia ugandensis* which scored only 1 to 3 points. The scores of the markers for each plant are summarized in Table 10.

Table 10: Ranking of the potential analytical markers in the plant materials

Plant material and manufacturer therapeutic claim	Bio-active Compound	Evidence of Biological activity of bioactive compound	Level of evidence of biological activity related to traditional use of the material	Relative Concentration in plant or extract	Mean Concentration above 5 ppm %ppm	Mean concentration above 50ppm Yes (1)/no (0) E	Reference Standard available Yes (1)/No (0) F	Analytical methods available? Yes (1)/No (0)	Herb MaRS score
Score		**Yes-1 No-0 (A)**	**1 symptom-1 2 symptoms-2 3 symptoms-3 (B)**		**Yes (1) No (0) (C)**	**Yes (1) No (0) (D)**	**Yes (1) No (0) (E)**	**Yes (1) No (0) (F)**	**/8 (A+B+C+D+E+F)**
Eucalyptus globulus leaf/oil	aromadendrine	1	2	10-30% of essential oil (Mulyaningsih et al. 2011)	1	1	1	1	7
	α-terpineol	1	3	0.50% of essential oil	1	1	1	1	8

				(Mulyaningsih et al. 2011)					
	Globulol	1	1	10-11% of essential oil (Mulyaningsih et al. 2011)	1	1	1	1	6
	1,8-cineol	1	3	97.32% of essential oil (Aldoghaim, Flematti, and Hammer 2018)	1	1	1	1	8
Aloe barbadensis whole leaf/gel	Aloin A and B	1	3	0.1-0.6% of leaf, 10-30% of latex (Kanama et al. 2015; Reynolds 1985)	1	1	1	1	8
	Aloe emodin	1	2	0.09- 0.29 mg/g of whole leaf (Kumar et al. 2017)	1	1	1	1	7
	Acemannan	1	2	109-135ppm of gel (Cuevas et al. 2016)	1	1	1	1	8
	Mannose 6 phosphate	1	1	ND	0	0	1	0	3
Albizia coriaria stem bark	Lupeol	1	3	1-6mg/g of stembark (Kaggwa, Munanura, et al. 2023)	1	1	1	1	8

	Lupenone	1	1	19-200 ppm of stem bark (Andrade et al. 2020)	1	1	1	1	6
	Betulinic acid	1	3	1.2-10mg/g of stem bark (Tatke, Desai, and Gabhe 2014; Kaggwa, Munanura, et al. 2023)	1	1	1	1	8
	Catechin	1	3	0.2-12mg/g of stem bark (Desai et al. 2020)	1	1	1	1	8
Mangifera indica leaves/stem bark	Mangiferin	1	3	5-20mg/g of leaves (Zhang et al. 2012)	1	1	1	1	8
	Catechin	1	3	71.4mg/g of stem bark (Núñez Sellés et al. 2002b)	1	1	1	1	8
	Epicatechin	1	1	8.07mg/g of stem bark (Núñez Sellés et al. 2002b)	1	1	0	1	5
	Gallic acid	1	3	2.08mg/g of stem bark (Núñez					

				Sellés et al. 2002b)					
	Quercetin	1	3	0.76 to 1.16 mg/g of leaves (Fitmawati et al. 2020)	1	1	1	1	8
Azadirachta indica leaves/stem bark/seeds	Azadirachtin	1	3	3.8 to 4.8mg/g of seeds (Song et al. 2018) 0.1-1mg/g of leaves (Forim et al. 2010)	1	1	1	1	8
	Nimbin,	1	3	0.018 to 0.64mg/g of oil (Sidhu, Kumar, and Behl 2004)	1	1	1	1	8
	Nimbolide	1	2	0.9-6.7mg/g of leaf (Suttiarporn and Choommongkol 2020)	1	1	1	1	7
	Mahmoodin	1	2	Not determined	1	1	0	0	5
Zingiber officinalis rhizome	12-gingerol	1	2	0.01-0.02mg/g of rhizome (Park and Jung 2016)	1	1	0	1	6
	10-gingerol	1	2	0.2-0.4mg/g of rhizome	1	1	1	1	7

				(Ashraf et al. 2014)					
	8-gingerol	1	1	0.4-0.5mg/g of rhizome (Ashraf et al. 2014)	1	1	1	1	6
	6-gingerol	1	3	1.1-2.0mg/g of rhizome (Ashraf et al. 2014)	1	1	1	1	8
	6-shogaol	1	3	0.01-0.02mg/g of rhizome (Park and Jung 2016)	1	1	1	1	8
	Zingerone	1	2	ND	0	0	1	1	5
Warburgia ugandensis stem bark	Bemadienolide	1	0	ND	0	0	0	0	1
	Muzigadial,	1	1	ND	0	0	0	0	2
	Polygodial	1	2	ND	0	0	1	0	3
	Warbuganal,	1	1	ND	0	0	0	0	2
	warbugadione,	1	0	ND	0	0	0	0	1
	warbugadial,	1	0	ND	0	0	0	0	1
	Warbugin	1	0	ND	0	0	0	0	1
	Ugandensidial,	1	1	ND	0	0	0	0	2
	Ugandensolide	1	0	ND	0	0	0	0	1

Key: ND=Not determined

4.1.5 Discussion

Standardization is a key step in the quality assurance of herbal materials and their products; it is essential to ensure reproducibility of the biological activity and quality of the product. In this study I established a list of compounds that can be used as markers for seven of the most commonly used plant materials in commercial products in Uganda. The emphasis was on compounds that are known to be active such that their determination informs both the quality and efficacy of the materials.

With the use of a modified Herbal marker ranking system, compounds were identified that can be utilized to control the quality of the herbal materials. A good chemical marker should have evidence of biological activity, availability of the analytical standard and availability of an analytical method. Thus, a compound should score at least 5 points to be suitable; that is 3 points for biological activity, and one point each for analytical standard and analytical method availability. The minimum concentrations of the markers in the plant material, if not already known, can be established by the manufacturer, and the compound assigned a qualitative (≤50ppm) or quantitative (≥50ppm) role depending on the concentrations in the plant material (Bensoussan et al. 2015). Another important factor to consider in quantitative analysis is the cost of the marker compounds. In this study we highlight unit costs and the costs of the smallest packs for each compound. It is important to note, however, that the final acquisition costs will include vendor or agent markups and so might be significantly higher. While 1 mg or 1 mL is considered sufficient for external calibration, other forms of calibration such as standard addition will require higher amounts of the marker. To show relevance of the selected markers, we list situations for which standardization of each plant material is required.

4.1.5.1 Markers for *Eucalyptus globulus* oil

Eucalyptus globulus is known for the essential oils obtained from the leaves of the plant. In Uganda, the oil is used to manufacture products such as syrups used to manage symptoms of respiratory tract disorders (cough, common cold, catarrh, sore throat, congestion from asthma, bronchitis, allergic conditions- sinusitis, rhinitis, hiccups), fever and measles; pain balms applied topically to manage pain and inflammation; and as mouth washes for conditions such as toothache, bad odor, sensitive teeth, bleeding gums, tooth cavities, tooth decay, and mouth sores (Kaggwa, Kyeyune, Munanura, Anywar, Lutoti, Aber, Bagoloire, Weisheit, Tolo, Kamba, et al. 2022). Among the bioactive compounds of *E. globulus* relevant to its industrial applications, four have a sufficient score to be used as chemical markers for its products, namely, aromadendrine, α-terpineol, globulol, and 1,8-cineol, with scores of 7, 8, 6, and 8,

respectively. All the four compounds are available in pure form and several quantitative methods have been established. Aromadendrine is the most expensive, with a unit cost of €183) while α-terpineol is the cheapest, with a unit cost of €0.6. The WHO and the African pharmacopoeia recommend the use of 1,8-cineol as a standard for both analytical chemical reactions and TLC fingerprint identification methods (World Health Organization 1999; Inter African Committee on Medicinal et al. 2014). However, some studies have shown aromadendrine to be the major component, and perhaps a multi-marker approach is more appropriate than determining only cineol (Mulyaningsih et al. 2011). These markers can be used to authenticate, determine phyto-equivalence and monitor the consistency in quality of oils obtained from different subspecies and geographical locations.

4.1.5.2 Markers for *Aloe barbadensis*

Aloe vera is used as the gel, latex or the whole leaf extract. In Uganda, the whole leaf and latex products are used to treat constipation, to “cleanse” the GIT, and to treat wounds (Kaggwa, Kyeyune, Munanura, Anywar, Lutoti, Aber, Bagoloire, Weisheit, Tolo, Kamba, et al. 2022). The main active ingredients are anthraquinone glycosides Aloin (barbaloin A and B) and aloe emodin- (Sánchez-Machado et al. 2017) The efficacy of these compounds is well established, their analytical standards are available and many analytical methods have been published. In the Herb Mars, both aloin A and B passed the cut-off for being suitable chemical markers as they scored 8- and 7 points, respectively. The unit cost of analytical standards for aloin A is €44.0, aloin B is €60.5, and aloe emodin is €31.1. The WHO monograph recommends chemical and TLC methods for identification of anthracenes, and spectrophotometric determination of total anthracene glycosides as barbaloin equivalence for quantitative analysis (World Health Organization 1999). These markers can be employed in identifying aloes obtained from different geographical regions and to determine the best geographical sources of aloe vera gel products (Kumar et al. 2017) , and also to standardize aloe products marketed for treatment of constipation (World Health Organization 1999).

Because of suspected carcinogenicity (Boudreau et al. 2017), some regulatory authorities have banned the inclusion of aloe (whole leaf and latex) in oral over the counter nutraceuticals and laxative products. For instance, the International Aloe Science Council set a limit of 10 ppm total anthraquinone glycoside concentration (as aloin) while the European Medicines agency and Food and Drug Administration set the limit at 0 ppm (Bayne 2002; European Commission Directorate-General for Health and Food Safety 2021). In this case aloin and aloe emodin are negative markers and can be used to assess the quality of over-the-counter

products. However, the Uganda National Drug Authority and Uganda Bureau of standards have not published any regulations on the use of aloes.

The main components of the gel are carbohydrates such as glucomannans and sugars (Cuevas et al. 2016)). One of the main compounds, acemannan scored 8 points. Its biological activity is well studied, analytical markers are available and analytical methods have been developed. The main sugar, mannose 6 phosphate scored only 3 points since its bioactivity is not well studied, there are no analytical methods, and thus its concentration in the gel has not been reported. The unit cost of acemannan is about €20.8. For quality assurance of *Aloe vera* gel, the WHO monograph recommends a chemical test for carbohydrates and polysaccharide analysis by GC/MS. A molecular exclusion chromatographic method and a UV-Vis spectrophotometric method for polysaccharides have also been published and are more affordable.

Assay methods for acemannan can be used to select the high yielding plant varieties, the best cultivation sites, suitable agronomic practices, and harvest seasons (Lucini, Pellizzoni, and Molinari 2013; JIANG et al. 2014)

4.1.5.3 Markers for *Albizia coriaria*

The dried stem bark of *Albizia coriaria* is the plant material of interest for medicinal purposes. The Ugandan commercial products containing *A. coriaria* are used for managing symptoms of respiratory tract disorders (whooping cough, catarrh, sore throat, congestion from asthma bronchitis, fever, sinusitis) (Kaggwa, Kyeyune, Munanura, Anywar, Lutoti, Aber, Bagoloire, Weisheit, Tolo, Kamba, et al. 2022). Some pharmacologically active compounds such as the triterpenoids; lupeol, lupenone, betulinic acid and betulin (Byamukama et al. 2015; Omara, Kiprop, and Kosgei 2022) have been elucidated with scores of 8, 6, 8, 8 respectively. These compounds possess the biological activities relevant to the commercial uses of the products, are available in pure form, and have been quantified in many Albizia species although assay methods specific to *A. coriaria* are scarce. The unit costs range between €68, for betulinic and €9.5 for betulinic acid.

There are no pharmacopeial methods or monographs for *A. coriaria* materials. The markers identified can be used to monitor the batch to batch consistency of raw materials (Omara, Kiprop, and Kosgei 2021; Anywar et al. 2020) and to evaluate efficiency of extraction methods.

4.1.5.4 Markers for *Mangifera indica*

The pharmacologically active compounds are obtained from extracts of the stem bark and leaves of *Mangifera indica*. The commercial products produced in Uganda, containing these extracts are used for management of respiratory tract disorders (whooping cough, catarrh, sore throat, congestion from asthma and bronchitis) (Kaggwa, Kyeyune, Munanura, Anywar, Lutoti, Aber, Bagoloire, Weisheit, Tolo, Kamba, et al. 2022). Several phenolic compounds have been characterized and shown to possess biological activity relevant to the medicinal use of the materials. Mangiferin, catechin, quercetin and gallic acid scored 8. The unit costs of these markers range from €1.8 for quercetin, to €34.5 for gallic acid. While epicatechin scored 5 points, its analytical standard is not readily available.

The markers identified can be used to monitor the batch to batch consistency of raw materials, to select the most suitable plant cultivars to source from (Núñez Sellés et al. 2002b), and to control the extraction and processing methods. There are no monographs for *M. indica* materials (Kaggwa, Kyeyune, Munanura, Anywar, Lutoti, Aber, Bagoloire, Weisheit, Tolo, Kamba, et al. 2022).

4.1.5.5 Markers for *Azadirachta indica*

The seed oil, leaves and stem bark of *Azadirachta indica* are used as the herbal materials. The commercial Ugandan products containing these herbal materials are used to manage respiratory tract disorders (Cough, flu, sore throat, sinusitis), gastrointestinal disorders (gastric ulcers, flatulence, constipation) and as lip balms (dry, cracked, and painful lips) (Kaggwa, Kyeyune, Munanura, Anywar, Lutoti, Aber, Bagoloire, Weisheit, Tolo, Kamba, et al. 2022). The most important compounds are the limonoid, azadirachtin and the tetranortriterpenes (Hossain et al. 2013) nimbin, nimbidin, nimbolide and mahmoodin. Most compounds scored 7 and above and are therefore suitable markers. The unit costs of these markers range from €105 to 289 for nimbin and azadirachtin respectively. Although nimbolide and mahmoodin scored 5 points, they lack analytical standards and assay methods. According to the WHO monographs, high-performance liquid chromatography quantification of oxidized tetranortriterpenes in oil and leaf materials can be used for quality control (World Health Organization 2007b). Assay of these markers can be applied to select habitats for cultivation of neem, determine the best harvesting season as well as to ensure consistency of materials obtained from various sources (Sidhu, Kumar, and Behl 2004; Sidhu and Behl 1996; Gupta et al. 2010).

4.1.5.6 Markers for *Zingiber officinalis* (Ginger)

The herbal material for *Zingiber officinalis* is obtained from the rhizome and the products manufactured in Uganda are used to manage symptoms of respiratory tract disorders (cough, flu, sore throat, sinusitis, bronchial asthma, and fever) (Kaggwa, Kyeyune, Munanura, Anywar, Lutoti, Aber, Bagoloire, Weisheit, Tolo, Kamba, et al. 2022). Gingerols and their dehydration products, the shogaols have been extensively studied (Mustafa, Srivastava, and Jensen 1993). The compounds possess several pharmacological activities relevant to the application of the products; their analytical standards are readily available (apart from 12-gingerol) and analytical methods have been published. Thus, all compounds scored between 6 and 8 points and are thus suitable as markers. The unit cost of the analytical standards ranges between 50 and 60 euros. The WHO monographs recommend TLC fingerprinting with gingerols and shogaols as standards; and GC and HPLC assay methods (World Health Organization 1999). Since gingerols are dehydrated to form shogaols during storage and on exposure to heat (Jung et al. 2018), the ratio of gingerols to shogaols can be used to determine the freshness of the ginger samples and optimize storage conditions. The quantities of the markers can be applied to optimize extraction processes and to study phyto-equivalence of gingers obtained from different sources (Pace and Martinelli 2022).

4.1.5.7 Markers for *Warburgia ugandensis*

The bark of the stem is used as the drug for the treatment of respiratory tract disorders (cough, measles symptoms, common colds, sinusitis, rhinitis, asthma, catarrh, bronchial congestion, and hiccups) and gastric ulcers (Kaggwa, Kyeyune, Munanura, Anywar, Lutoti, Aber, Bagoloire, Weisheit, Tolo, Kamba, et al. 2022). While many compounds have been elucidated, the most important being the drimane sesquiterpenes bemadienolide , muzigadial, polygodial, warburganal, ugandensolide, muzigadial (Wube et al. 2005) , specific bioactivity studies are rare.

Only polygodial, muzigadial muzigadiolide, warburganal, and ugandensidial have been shown to possess some antimycobacterial activity (Wube et al. 2005). Besides limited pharmacological evidence, most of these compounds are not available in pure form for use as analytical standards and no assay methods have been published. Thus, the compounds scored between only 1 and 3 points and are therefore not suitable quality control markers according to the Herb MaRS. In such cases, the WHO recommends use of other constituents, whose biological activities are known even though the relevance of such activities to the traditional use of the plant may not be well established (World Health Organization 2016).

Thus, compounds like linoleic acid, myrcene, linalool which are known components of *W. ugandensis* (Kioy, Gray, and Waterman 1990) can be used for its quality control-such evaluation will not be relevant to pharmacological standardization of the plant materials. This lack of pharmacological data, analytical methods and standards is common to plants that are exclusively used in Africa (Kaggwa, Kyeyune, Munanura, Anywar, Lutoti, Aber, Bagoloire, Weisheit, Tolo, and Kamba 2022).

4.4.6 Conclusions

Using the Herb MaRS ranking system, this study has identified suitable markers for the quality assurance of herbal materials and phytochemical standardization of six out of the seven most commonly used medicinal plants in commercial products in Uganda, namely, *Eucalyptus globulus* Labill, *Aloe barbadensis* (L.) Burm.f., *Albizia coriaria* Oliv., *Mangifera indica* L., *Azadirachta indica* A.Juss., *Zingiber officinalis* Roscoe. The suitable markers for each plant are as follows: aromadendrine, α-terpineol, globulol, and 1,8,- cineol (for Eucalyptus globulus Labill.); aloin, aloe emodin, acemannan (for *Aloe barbadensis* (L.) Burm.f.); lupeol, lupenone, betulinic acid, betulin, and catechin (for *Albizia coriaria* Oliv.); mangiferin, catechin, quercetin, and gallic acid (for *Mangifera indica* L.); azadirachtin, nimbin, nimbidin (for *Azadirachta indica* A.Juss.); and 6, 8, 10-gingerols, 6-shogaol (for *Zingiber officinalis* Roscoe). For *W. ugandensis*, the compounds with known biological activity scored poorly as chemical markers because they lack analytical standards and/or analytical methods.

4.4.7 Recommendations

The National Drug Authority of Uganda and manufacturers of herbal medicines should adopt the identified chemical markers for quality assurance and standardization of products containing *Eucalyptus globulus* Labill, *Aloe barbadensis* (L.) Burm.f., *Albizia coriaria* Oliv., *Mangifera indica* L., *Azadirachta indica* A.Juss., *Zingiber officinalis* Roscoe. The identified markers should be evaluated for suitability at the various stages of the production chain of the herbal medicines in Uganda i.e., from authentication and quality control of raw materials to evaluating reproducibility in efficacy, safety, and stability of finished products notified by the National Drug Authority. The information about marker evaluation can be included in the future Ugandan medicinal plant monographs and or product data bases to guide their quality assurance. Besides quantification of marker compounds, construction of fingerprint databases for the various plants is encouraged. The standardized fingerprints can then be used for routine quality assessment of the plant materials. On the other hand, further studies to develop analytical standards and their analytical methods for bioactive compounds in *W. ugandensis*

are needed to produce suitable chemical markers. It is recommended that the Herb MaRS ranking system is applied to the other twenty-five (25) medicinal plants that are found in commercial products in Uganda, namely, *Syzygium aromaticum, Allium sativum, Morella kandtiana, Curcuma longa, Mentha piperita, Moringa oleifera, Carica papaya, Terminalia catappa, Salvadora persica, Zanthoxylum fagara, Solanum spp, Acorus calamus, Xanthium strumarium, Hamamelis virginiana, Calendula officinalis, Zanthoxylum chalybeum, Combretum mole, Dracaena steudneri, Kigelia africana, Tamarindus indica, Entada abyssinica, Salvia hispanica, Musa paradisiaca, Cymbopogon citratus, Citrus limon,* and *Tinospora sinensis.*

4.2.0 RESULTS AND DISCUSSION FOR OBJECTIVES 2, 3 AND 4

The results are presented together for each plant, because the activities involved were intertwined. The work involved the development of qualitative TLC and HPLC methods (objective 2) and quantitative HPLC methods (objective 3) and finally characterization of different batches of plant materials, using the developed methods, to determine their phytoequivalence. Three plants, *A. coriaria, M. indica* and *W. ugandensis* were studied, and the results are presented in this respective order.

4.2.1.0 *Albizia coriaria* stembark materials

HPLC and TLC fingerprints, as well as HPLC quantitative methods were developed and validated. These methods were then applied to evaluation of the phytoequivalence of *A. coriaria* stembark samples harvested from the different agroecological zones of Uganda.

4.2.1.1 Development of TLC fingerprint methods

The best TLC fingerprints were obtained using mobile phase system comprising 4,2,1, and 1 volumes of n-hexane, ethyl acetate, methanol, dichloromethane. The optimized solvent mixture is represented by point d, in plane D of the solvent optimization prism (Figure 16).

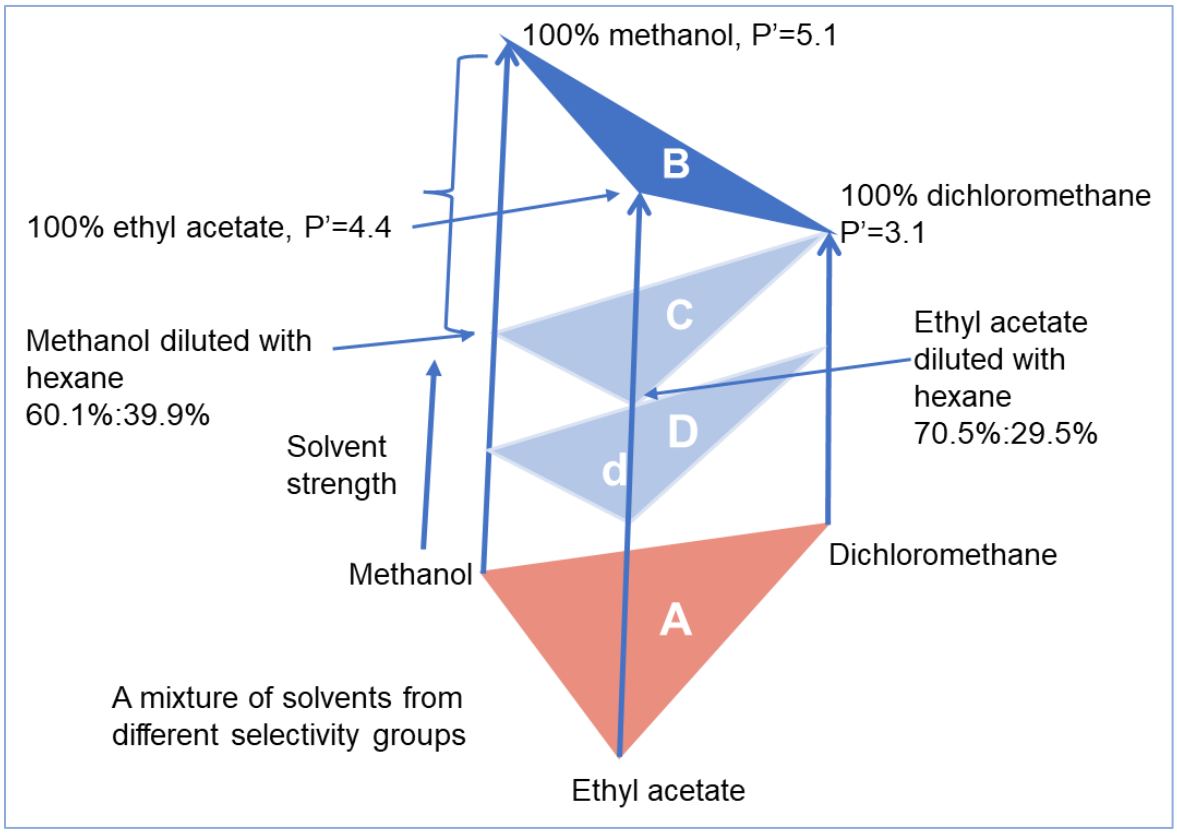
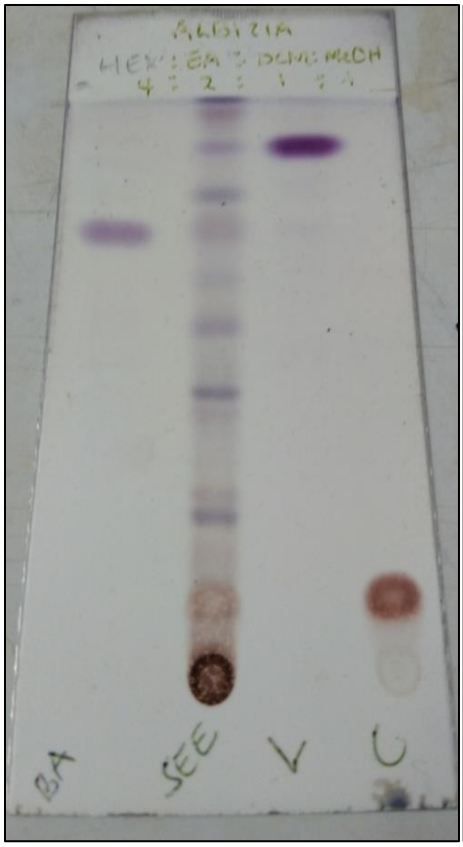


Figure 16: Solvent optimization prism for the mobile phase for TLC fingerprinting of A. coriaria stembark materials. Solvent mixtures in plane B have different polarities and selectivities; those in plane C the same polarity but different selectivities. Plane D shows the optimum solvent polarity-point d is an estimate of the compositions of the solvents.

The polarity P', of the optimized solvent system is 2.13, as calculated from equation 2. The solvents represent selectivity groups II, V and VI for methanol, dichloromethane and ethyl acetate. Since hexane is not selective and its P' = 0, it is only used to dilute other solvents, during adjusting of the solvent strength (Reich and Schibli 2007a). This solvent system strength is sufficient to elute moderately none polar compounds like lupeol, betulinic acid and related terpenoid compounds in *A. coriaria*. The wide range of selectivity also increases the spectrum of the solvent system, enabling several types of interaction with the different phytoconstituents of the samples (Ramis-Ramos, García-Álvarez-Coque, and Navarro-Huerta 2017). At about 28^0C, and humidity of 48%, the plates were developed within only 20 minutes.

The derivatization of the TLC plates was achieved by vanillin/sulphuric acid reagent. A typical fingerprint is shown in Figure 17.



Top of plate	
Lupeol: a purple zone	A purple zone
	A purple zone
Betulinic acid: a purple zone	A light purple zone
	A strong purple zone
	A strong purple zone
	A faint purple zone
Catechin: a brown zone	A brown zone
Reference solution	Test solution

Figure 17: Left: Typical fingerprint for Albizia coriaria stembark raw material; Solvent system; n-hexane, ethyl acetate, dichloromethane, methanol (4:2:1:1). Tracks: BA-Betulinic acid (RF=0.712), SEE-Sample Soxhlet extracted with ethyl acetate; L-lupeol (RF=0.0.89), C-catechin ((RF=0.11). Right: Schematic of the fingerprint as presented in Pharmacopoeia.

The spots developed to full color in only 4 minutes, when the plate was heated at 105 minutes in an oven. Fingerprints visualized under UV 254nm and 366nm, for the underivatized plates are shown in Appendix 5.

4.2.1.2 Stability of the TLC fingerprints

The samples were stable chromatography is indicated by a two-dimensional TLC (Figure 18)

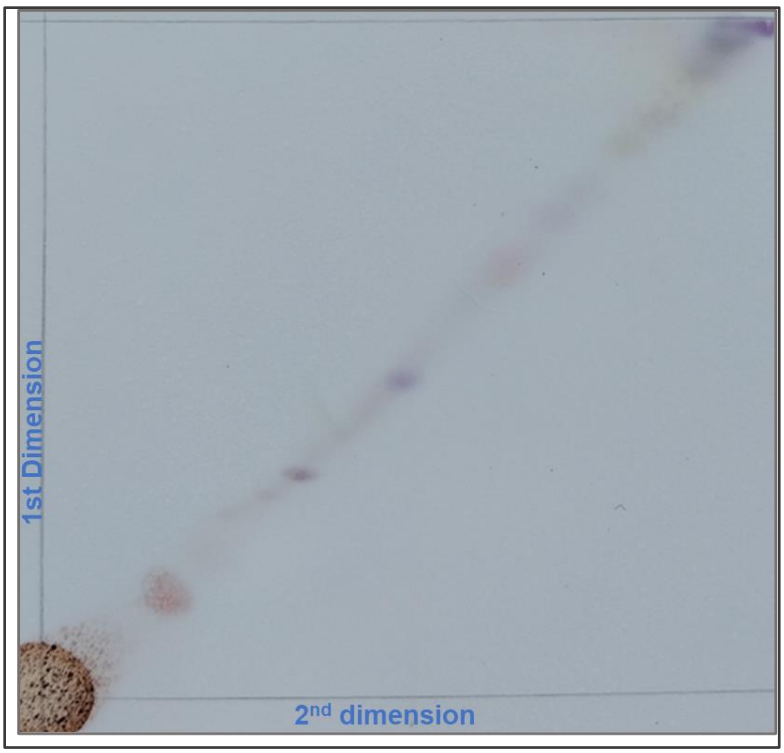


Figure 18: Two-dimensional (2D) TLC of Albizia coriaria stembark raw materials extracts. Solvent system; n-hexane, ethyl acetate, dichloromethane, methanol (4:2:1:1)

Since all spots lie on the diagonal line joining the sample application point to the intersection of the solvent fronts, the samples are stable under the chromatographic conditions used (Reich and Schibli 2007a).

4.2.1.3 Effect of solvent and extraction method on the quality of fingerprints

Samples extracted with a soxhlet in ethyl acetate exhibited the same number but more intense peaks as those extracted with a soxhlet in ethanol; but more peaks than the samples extracted by maceration. Decoctions showed the least number and intensity of peaks; with the only marker detected being catechin (Figure 19).



Figure 19: TLC fingerprinting; effect of solvent and method of extraction; decoction also depicts the effect of processing (boiling) on the concentration of markers lupeol and betulinic acid. Tracks: SOX-Soxhlet ethyl acetate, MAC-maceration (ethanol), DEC-decoction, Betulinic acid, catechin, lupeol, SOX-Soxhlet ethanol. Solvent system; n-hexane, ethyl acetate, dichloromethane, methanol (4:2:1:1)

4.2.1.4 Evaluation of phytoequivalence of samples harvested from different agroecological zones

Evaluation of sample similarity using the developed TLC fingerprints

We identified 12 peaks common to all samples (Figure 20), by comparing their relative RFs and their colors after derivatization (Table 11). The concentration variations of the chemicals (size of peaks) are also evident from the fingerprint. Spots 4, 8,9 were as prominent as the markers while x, y and z were only visible in samples from Kabarole district.

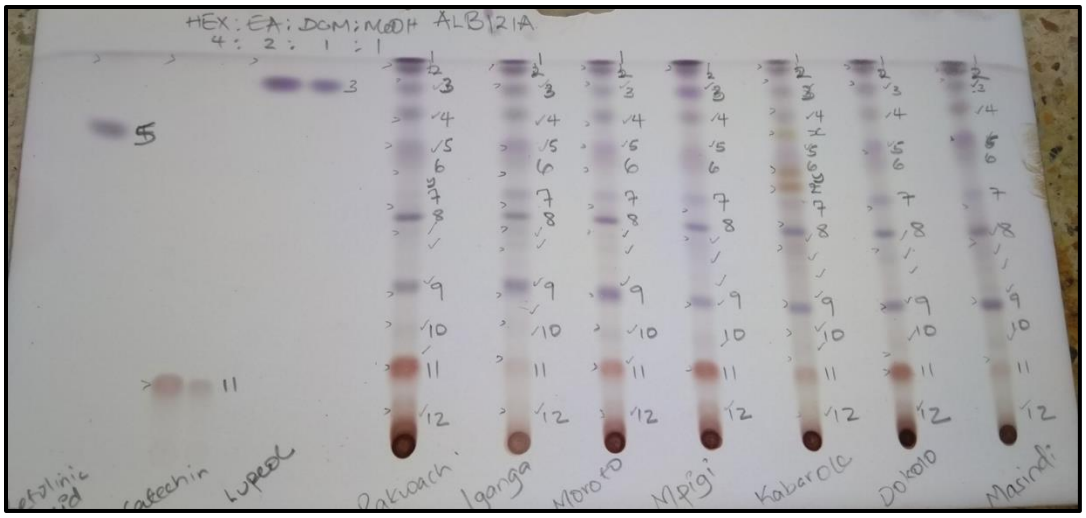


Figure 20: Distribution of catechin, lupeol and betulinic acid in Albizia coriaria materials obtained from different agro-ecological zones (representative districts included here). Solvent system; n-hexane, ethyl acetate, dichloromethane, methanol (4:2:1:1)

Table 11: Summary of peak properties for TLC fingerprints

Peak no.	R_F	Visualization			Identity of compound	HRF RF*100	Relative R_F (betulinic acid)
		366nm	254nm	Daylight after derivatization			
1	0.99	-	Pink	purple	unknown	99	1.39
2	0.97	-	-	purple	unknown	97	1.36
3	0.89	-	-	purple	lupeol	89	1.25
4	0.80	-	-	purple	unknown	80	1.12
5	0.71	-	-	purple	betulinic acid	71	1.00
6	0.55	Fluorescence	Pink	purple	unknown	55	0.77
7	0.43	-	-	purple	unknown	43	0.61
8	0.40	Fluorescence	Pink	purple	unknown	40	0.56
9	0.27	-	-	purple	unknown	27	0.38
10	0.22	Fluorescence	Pink	purple	unknown	22	0.30
11	0.19	-	-	purple	catechin	19	0.27
12	0.11	-	-	brown	unknown	11	0.15

R_F-Retardation Factor.

The presence x, y and z were in samples from Kabarole district reduced their similarity to other samples as shown by the lower Jaccard index (Table 12)

Table 12: The Jaccard indices between TLC fingerprints of samples of A. coriaria sampled from different districts.

Sample	Jaccard index between samples						
	Dokolo	Iganga	Masindi	Kabarole	Moroto	Mpigi	Pakwach
Dokolo	1,0						
Iganga	1,0	1,0					
Masindi	1,0	1,0	1,0				
Kabarole	0.8	0.8	0.8	1,0			
Moroto	1,0	1,0	1,0	0.8	1,0		
Mpigi	1,0	1,0	1,0	0.8	1,0	1,0	
Pakwach	1,0	1,0	1,0	0.8	1,0	1,0	1,0

4.2.1.5.0 Development of HPLC fingerprint methods

4.2.1.5.1 Optimization of experimental conditions

The best solvent system was acetonitrile 99.99% and aqueous trifluoroacetic acid (0.01). This solvent system produced fingerprints with the most information content. While previous studies had reported the use of methanol and water for quantification of related terpenoids (Holonec et al. 2012; Tatke, Desai, and Gabhe 2014), this solvent system was not suitable because lupeol and betulinic acid markers, as well several other constituents of *A. coriaria* absorb UV radiation at the same wavelength as methanol (205-210nm). Thus, acetonitrile, which absorbs at a much lower wavelength (190nm), would cause less detection interference (Kazakevich and Lobrutto 2007). The optimal mobile phase flow rate was 1mL/min, with isocratic elution, while the column temperature was 25^{o}C.

4.2.1.5.2 Validation of the HPLC fingerprinting method

The repeatability of the fingerprints was acceptable, since similarity indices of the six runs of the test samples (Dokolo 1) were approximately 0.9. Similarity, the extracts were stable in methanol over three days under laboratory conditions (Table 13).

Table 13: Similarity indices showing stability of Albizia coriaria stembark raw materials HPLC fingerprints

Repeatability validation for six runs-similarity index-correlation coefficients (r)						
	run 1	*run 2*	*run 3*	*run 4*	*run 5*	*run 6*
run 1	1,000					
run 2	0,973	1,000				
run 3	0,970	0,960	1,000			
run 4	0,886	0,933	0,960	1,000		
run 5	0,995	0,967	0,977	0,905	1,000	
run 6	0,970	0,960	1,000	0,960	0,977	1,000
	Day 1	*Day 2*	*Day 3*			
Day 1	1	1	0.9991			
Day 2	0.9991	0.9991	1			
Day 3	0.9995	0.9995	0.9998			

r- correlation coefficient, c-congruence coefficient; the samples from Iganga were evaluated. Day 1, 2 and 3 show the same sample analysed on different days for three days. For stability, the coefficients r and c should approximate 1.

The typical fingerprint of the *Albizia coriaria* stembark showed 10 common peaks as shown in Figure 21.

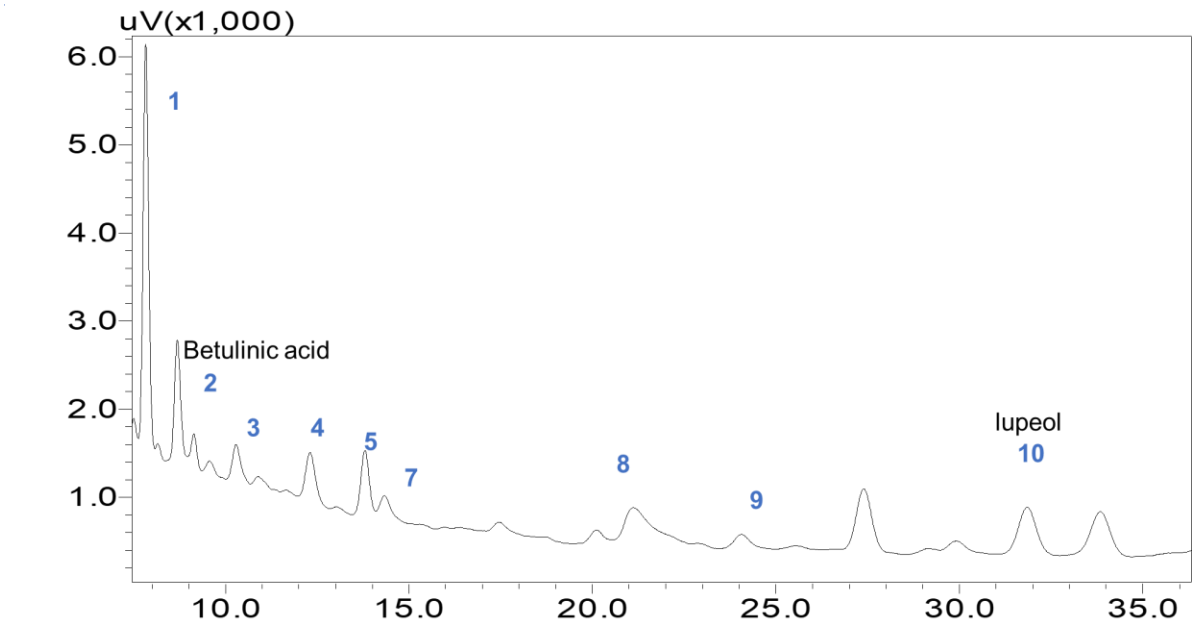


Figure 21: Typical HPLC-UV fingerprint of raw materials from A. coriaria raw materials; lupeol and betulinic acid were reference markers. The peaks common to all samples are numbered.

4.2.1.6 Evaluation of the phytoequivalence of samples harvested from different agroecological zones

Although the different sample fingerprints showed a similar pattern on visual inspection (Figure 22), the number of peaks varied greatly, ranging from 37 (Pakwach) to 55 (Iganga).

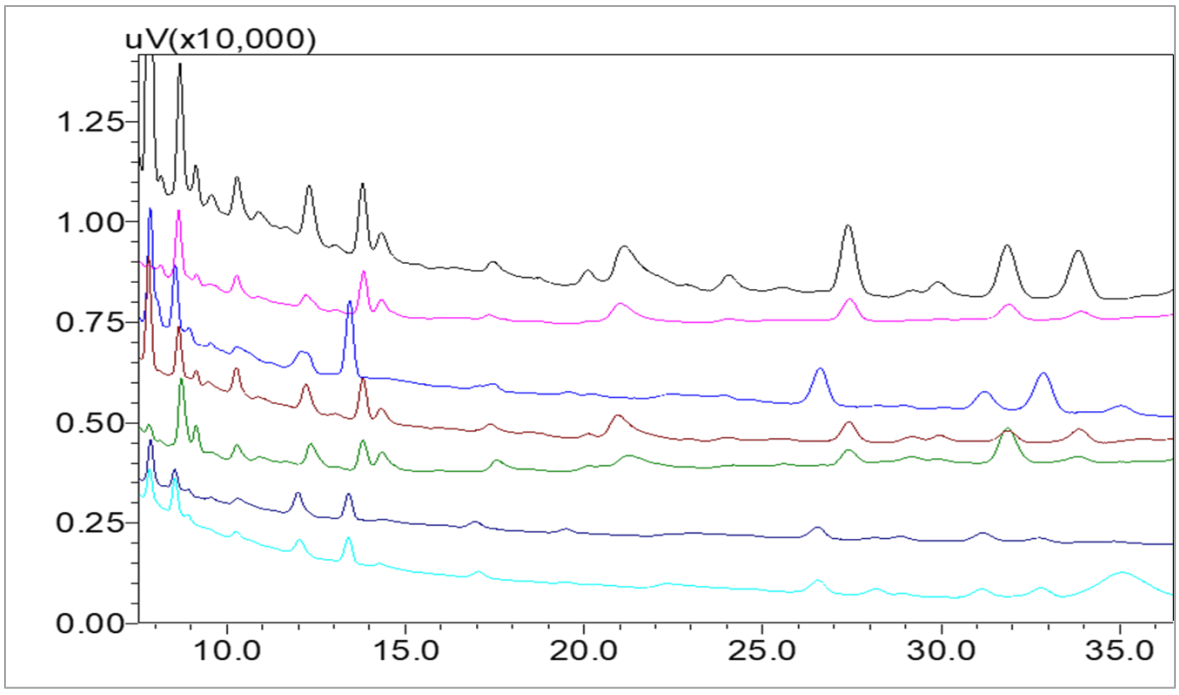


Figure 22: Albizia coriaria HPLC fingerprint overly; shows similarities of samples obtained from different agroecological zones.

Similarity indices of fingerprints for general chromatograms and common peak fingerprints

The similarity indices for general fingerprints (Table 14) were lower than those calculated for only common peaks fingerprints (Table 15), although the p values (α=0.05) from the correlation matrices, of both types of fingerprints indicated that the samples were significantly similar, most even at α=0.01.

Table 14: Similarity indices of Albizia coriaria stembark material samples of HPLC-UV fingerprints of whole chromatograms to the median fingerprint.

Sample source	correlation coefficient (r) and p value		congruence coefficient (c)
	r	p	
Masindi	0.574	<0.0001	0.611
Pakwach	0.604	<0.0001	0.678
Kabarole	0.926	<0.0001	0.938
Moroto	0.761	<0.0001	0.781
Iganga	0.711	0.0024	0.611
Mpigi	0.787	0.0004	0.801
Dokolo	0.907	<0.0001	0.918

Table 15: Similarity indices of Albizia coriaria stembark material samples of HPLC-UV fingerprints based on 10 common peaks to the median fingerprint.

Sample source	correlation coefficient (r) and p value		congruence coefficient (c)	Euclidean distance (d)
	r	p		
Masindi	0.4941	0.1880	0.584	71.43
Pakwach	0.985	<0.0001	0.988	11.14
Kabarole	0.961	<0.0001	0.959	24.16
Moroto	0.991	<0.0001	0.993	6.07
Iganga	0.872	0.0024	0.895	31.33
Mpigi	0.926	0.0004	0.935	25.66
Dokolo	0.993	<0.0001	0.994	6.462

Common fingerprints constitute peaks with similar retention time (±0.05 minutes) in the different sample chromatograms; the similarity indices were obtained by comparing the sample fingerprints to a median fingerprint, which was obtained by calculating the medians of the peaks of all sample fingerprints. In Table 16, all fingerprints were significantly similar to the median fingerprint at 99% confidence ($p<0.01$) except for those for samples from Iganga ($p=0.0024$) and Masindi (not significantly similar even at 95% confidence ($p=0.147$). The samples of Moroto and Dokolo were the most similar to the reference. Generally, the

congruence coefficients were slightly higher than the correlation coefficients, although the trend was the same. The Euclidean distances were in agreement with the other indices.

Multivariate analysis of common fingerprint similarity

Hierarchical cluster analysis.

Classical cluster analysis of HPLC fingerprints based on the common peaks gave four clusters: Samples in group 1 (Pakwach, and Dokolo) were more than 90% similar (congruence coefficient>0.85), while samples in group 2 (Moroto and Kabarole) were approximately 90% similar (congruence coefficient >0.90); samples in cluster 3 (Mpigi and Iganga) were about 90% similar while cluster 4 samples (Masindi) were less than 75% similar to the rest. All fingerprints, apart from those for Masindi were at least 80 % similar: (Figure 23). The Dendrogram Cophenetic correlation coefficient was 0.85, implying a good preservation of the original un-modelled data.

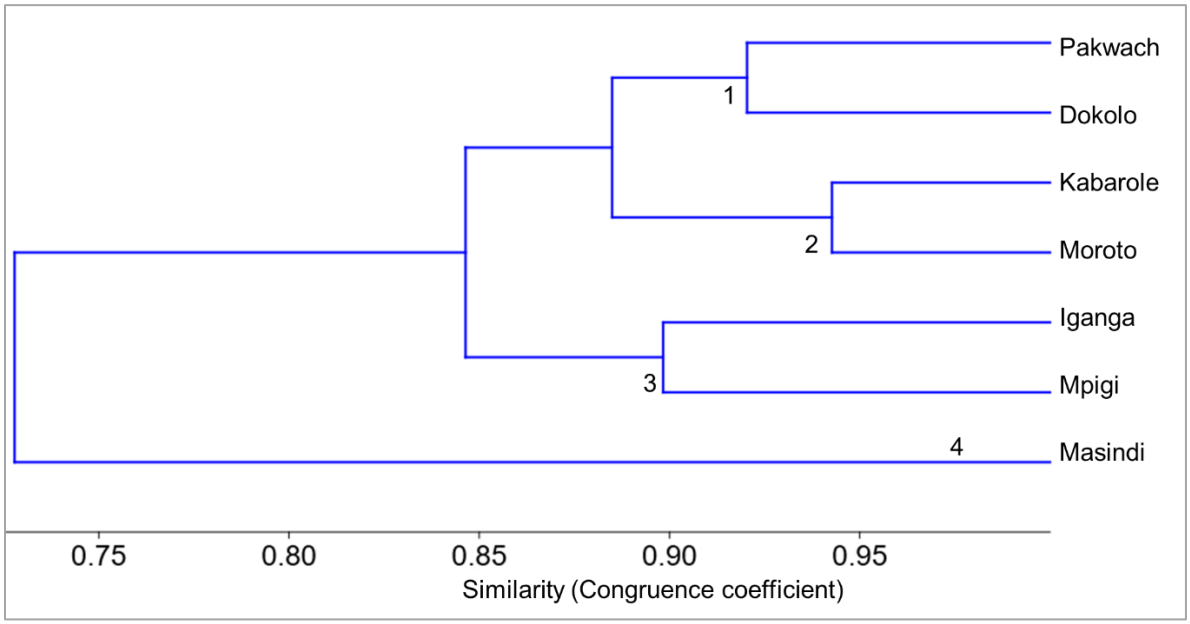


Figure 23: Dendrogram for the classical hierarchical cluster analysis of A. coriaria stembark samples obtained from seven different geographical sources: Algorithm, Unweighted pair group method with arithmetic mean (UPMGA); similarity index, congruence coefficient.

A two-way dendrogram showed two main groups for peak variation: The peaks due to lupeol, betulinic acid, co-vary with peaks at 8.6, 13.0, and 24.1 minutes while peaks at 9.1, 10.3, 10.9, 14.3 and 20.1 also co-vary (
Figure *24*)

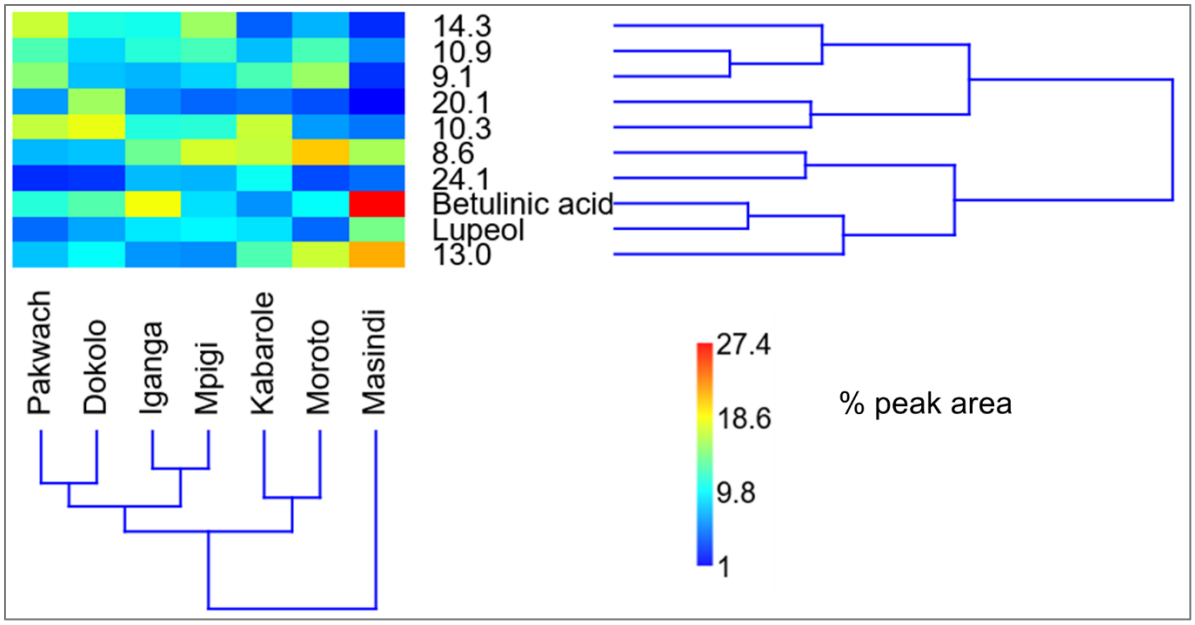


Figure 24: Two-way dendrogram for A. coriaria stembark samples showing similarity of samples and covariance of peaks. Algorithm: Ward's method; similarity index, congruence coefficient.

The figure also shows that samples from Masindi had a very high concentration of betulinic acid, which would explain it different from the rest of the samples.

Principal Component Analysis (PCA)

PCA yielded 7 components, with the first two contributing more than 74.6% of the total variance. These PCs were validated by scree testing; the plot formed an elbow after the first two components (Figure p4-appendix). From the scatter plot, the minimum spanning tree distance confirmed that samples from Kabarole were closest to the reference, followed by those from Kabarole, and Mpigi. As expected, samples from Masindi were the most distant (Figure 25).

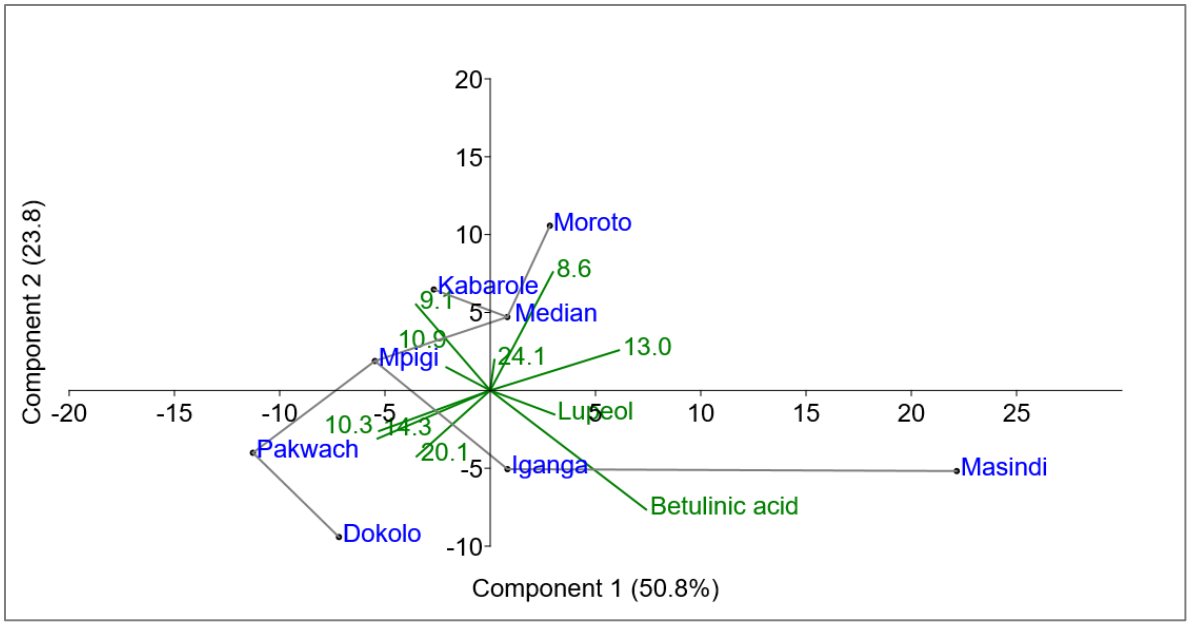


Figure 25: PCA of HPLC fingerprints of A. coriaria stembark materials collected from different districts: factor scores of observations plotted on the first two components; with minimum spanning tree (MST) showing the closeness of sample fingerprints to the reference fingerprints

From the figure, it can be seen that the variance in samples from Masindi is caused by betulinic acid.

4.2.1.7 Contribution of the peaks to characteristic fingerprint variations.

From figure 24, peaks due to lupeol and betulinic acid are among the most important explaining the variance among the samples (Figure 26).

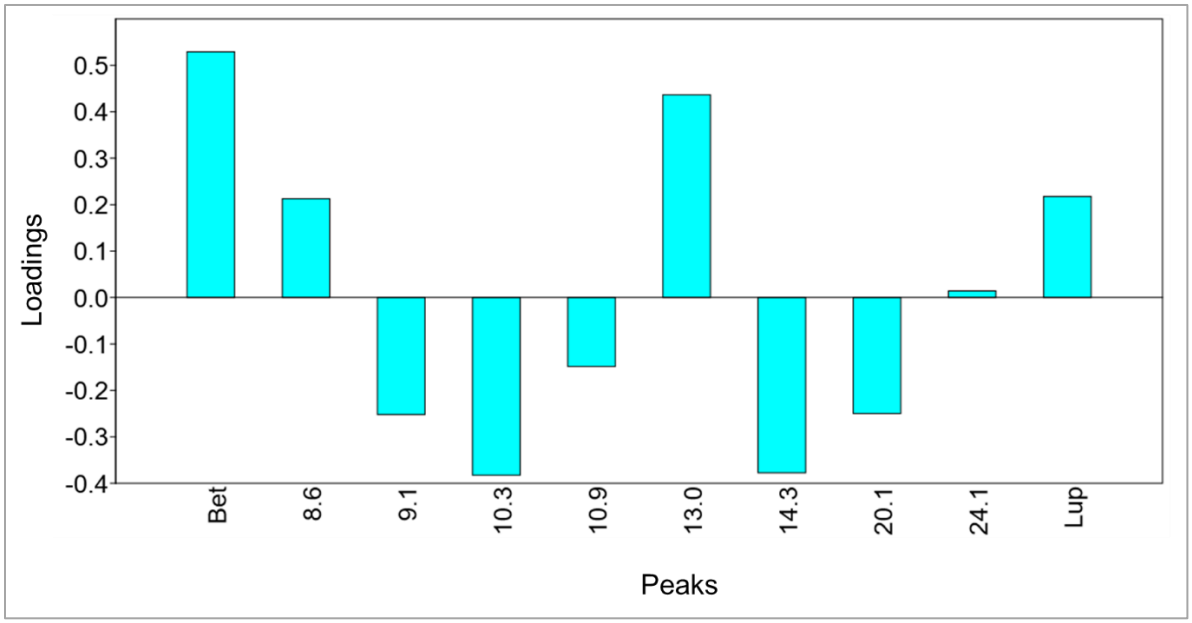


Figure 26: Loading plots for A. coriaria fingerprints showing the contribution of the peaks to the variance of PC 1; Bet-betulinic acid, Lup-lupeol. Loadings in this case show the proportion of the fingerprint that's contributed to by each peak.

The loading scores for betulinic acid and lupeol for PC1 were the highest, at 0.52 and 0.22 respectively. Implying a good choice of markers. The peaks with the highest scores for PC2 were those at 8.6 and 9.1 minutes, with socres of 0,52 and 0.39 respectively (Figure 27)

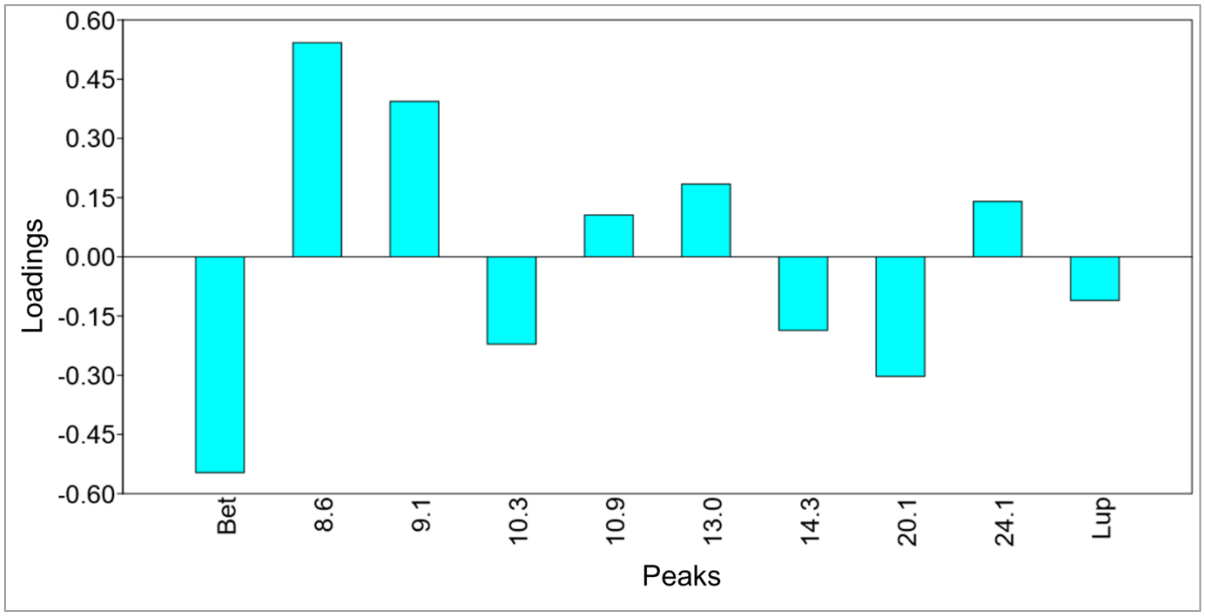


Figure 27: Figure 26: Loading plots for A. coriaria fingerprints showing the contribution of the peaks to the variance of PC 2; Bet-Betulinic acid, Lup-lupeol. Loadings in this case show the proportion of the fingerprint that's contributed to by the peaks.

4.2.1.8.0 Development of HPLC quantification methods

The optimized method parameters were as described above for the fingerprints method.

Identification of markers was based on their retention times while quantification on the peak's areas in reference to the standard calibration curve. Figure 28 shows the chromatogram for the standards mixture.

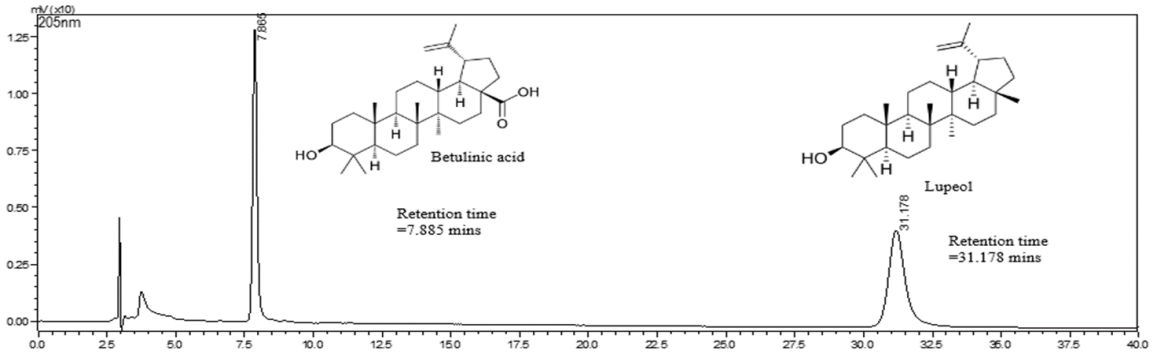


Figure 28: HPLC chromatogram for lupeol and betulinic acid mixture; average retention time, betulinic acid (7.86 minutes), lupeol (31.18 minutes).

4.2.1.8.1 Method Validation

The method was linear, accurate and precise for the quantification of both betulinic acid and lupeol, in accordance with the Association of Official Analytical Chemists (AOAC 2012). The validation parameters are summarized in Table *16*.

Table 16: Method validation parameters for an HPLC quantification method for lupeol and betulinic acid in Albizia coriaria stembark raw materials.

Recoveries*					
Sample ID	***Amount present in extract (µg/mL)***	***Amount added (µg)***	***Mean amount found (µg/mL***	***Recovery (%)***	***Mean***
					Recovery (%) ±SD (n=9)
Betulinic acid					
Sample 1	*7.45*	*5.00*	*12.57*	*101%*	99.0±0.22
Sample 2	*2.34*	*1.50*	*3.65*	*95%*	
Sample 3	*3.30*	*2.50*	*5.84*	*100.70%*	
Lupeol					
Sample 1	*3.76*	*2.50*	*5.92*	*94.60%*	*97.2±0.02*
Sample 2	*2.86*	*2.00*	*4.80*	*98.80%*	
Sample 3	*3.02*	*2.00*	*4.95*	*98.50%*	
Repeatability					

Intra-day			***Inter-day***		
Sample ID	***Mean amount present in extract in 3 runs (µg/mL)***	***RSD%***	***Mean amount present in extract (µg/mL) in 3 days***		***RSD***
Sample 1					
betulinic acid	*14.84*	*0.43*	*14.77*	*0.45*	*0.45*
lupeol	*7.519*	*0.56*	*7.47*	*0.64*	*0.64*
Sample 2					
betulinic acid	*6.59*	*1.78*	*6.55*	*1.82*	*1.82*
lupeol	*6.039*	*1.69*	*6.20*	*1.80*	*1.80*
Sample 3					
betulinic acid	*2.52*	*0.58*	*2.61*	*0.86*	*0.86*
lupeol	*10.105*	*0.21*	*10.09*	*0.28*	*0.28*
mean RSD %	***betulinic acid***		***0.93±0.74***		***1.04±0.70***
	lupeol		*0.82±0.77*		*0.91±0.79*
LOD and LOQ	***betulinic acid***			***lupeol***	
LOD (µg/mL)	*4.4*			*1.5*	
LOQ (µg/mL)	*11.4*			*3.8*	
linearity (sensitivity)					

R2	0.9999	*0.9979*
Regression equations	*y = 3551.8x - 6396.2*	*y = 4730.1x - 17101*

4.2.1.8.3 Effect of extraction method on the amount of marker extracted.

For both lupeol and betulinic acid, soxhlet extraction in ethyl acetate gave the highest yields, followed by soxhlet extraction in ethanol. Decoction extracted the lowest amounts of markers (Figure 29).

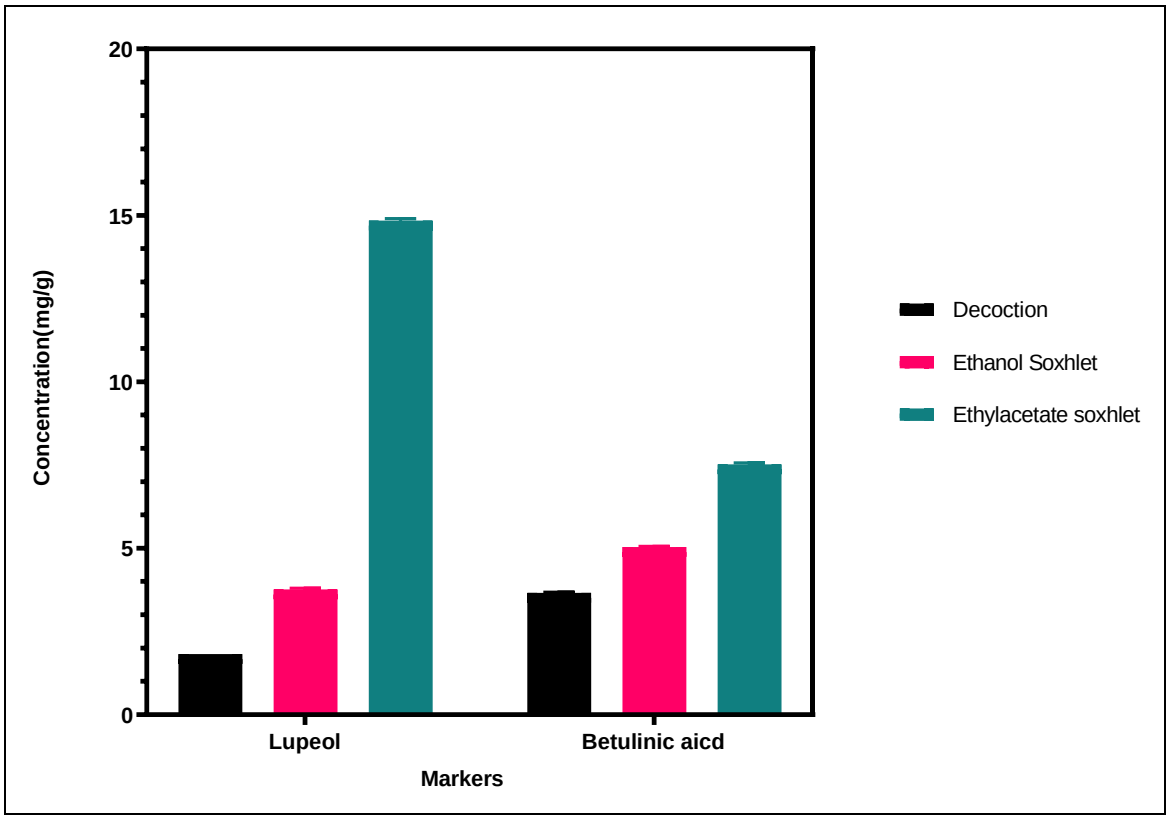


Figure 29: Effect of the extraction method on the concentration of betulinic acid and lupeol from the stembark materials of A. coriaria. Iganga samples were analyzed.

The one-way ANOVA showed that the extraction method significantly affected the amount of marker extracted (P<0.0001). Furthermore, the Turkey's multiple comparison test revealed that for both lupeol and betulinic acid, soxhlet extraction in ethyl acetate was significantly better than decoction (p<0.0001, 95% CI (-3.95 to -3.78) and soxhlet extraction in ethanol (p<0.0001, 95% CI (-2.57 to 2.40). Soxhlet extraction in ethanol was better than decoction (p<0.0001, 95% CI (-2.03 to -1.86).

4.2.1.8.4 Quantification of markers in samples harvested from different agroecological zones

The raw materials from seven different agroecological zones showed significant differences in the concentration of lupeol and betulinic acid (Table *17*).

Table 17: Concentration of betulinic acid and lupeol in A. coriaria stembark materials obtained from different agroecological zones.

Agroecological zone	Source district	Concentration of markers (mg/g)		Ratio of concentration of betulinic acid to Lupeol
		Betulinic acid (n=3)	Lupeol (n=3)	
Mid-Northern	Dokolo	2.52±0.01	6.37±0.02	0.4
Eastern	Iganga	14.84±0.06	7.52±0.02	2.0
Western highlands	Kabarole	9.1±0.11	6.09±0.01	1.5
Lake Albert crescent	Masindi	1.9±0.01	6.04±0.02	0.3
Karamoja dry lands	Morotto	4.68±0.06	5.52±0.02	0.8
Lake Victoria crescent	Mpigi	6.59±0.12	10.1±0.01	0.7
West Nile	Packwach	6.77±0.10	5.72±0.02	1.2
Average amount of markers and ratio		6.63±4.41	6.77±1.61	1.0

The one-way ANOVA showed that the source of *A. coriaria* materials significantly affects the concentration of both markers ($p<0.0001$), with an average ratio of 1. Tukey's multiple comparison tests (Appendix 6 and Appendix 7) showed a marked variation in the concentrations of both lupeol and betulinic acid among districts.

4.2.1.9 Discussion

This study developed TLC and HPLC methods that can be used for routine qualitative (TLC and HPLC fingerprints) and quantitative (HPLC-UV) standardisation of *Albizia coriaria* stembark raw materials. To streamline the methods for applicability, three known bioactive active compounds; lupeol, catechin and betulinic were evaluated for suitability as analytical markers. The qualitative evaluation was based on the presence of the three markers and the similarity of the entire fingerprints among samples sourced from different geographical locations in the country. The HPLC quantification of lupeol and betulinic acid would offer a method for quantitative standardisation. Acceptability of analytical methods is premised on acceptable performance parameters such as accuracy and reproducibility.

Composition of the reference fingerprint and comparison of different samples

The TLC fingerprint was achieved by a solvent system comprising of 4 parts of n-hexane, 2 parts ethyl acetate,1 part of methanol and 1 Dichloromethane mobile phase system; the solvents were strategically selected from different polarity groups (Snyder 1978) to constitute a mobile phase with an elution power that maximizes separation of components in a complex herbal matrix. Vanillin/sulfuric acid derivatization gave the better fingerprints than iodine or anisaldehyde; sulphuric acid is a non-selective reagent that works by charring organic material, while acidified vanillin forms colourful complexes with many organic compounds (Merck 1980). The TLC fingerprints were generally similar; all had jaccard indices of 1, except those of samples from Kabarole. These had three uncommon bands which lowered their jaccard indices with other samples to 0.8.

We identified 12 peaks common to all samples, by comparing their relative RFs and their colours after derivatization (Table 11). The concentration variations of the chemicals (size of peaks) are also evident from the fingerprint (Figure 20). Since peaks 4, 8 and 9 were as prominent as the marker spots, we propose to add them to catechin, betulinic acid and lupeol to constitute the reference fingerprint. Since R_F values for the identification of compounds by TLC had low reproducibility; the European Pharmacopoeia recommends the inclusion of relative retardation factors of the principal spots to show the relative positions of the peaks with respect to the reference marker. In addition, the colour of the peaks can be easily reproduced (Wagner et al. 2011). The proposed reference fingerprint can be reproduced under the following conditions: soxhlet extraction with ethyl acetate solvent; solvent system: 4, 2, 1, and 1 parts of n-hexane, ethyl acetate, dichloromethane, and methanol respectively; stationary phase; aluminium TLC sheets or glass plates,10cm x 20cm, coated with 60Å silica gel 254 fluorescent matrices; chamber saturation 15 minutes and development time 15 minutes; vanillin/sulphuric acid spray reagent.

Stability of extracts under TLC conditions.

Since TLC analysis is open to the environment, the sample can decompose due to exposure to air, light, moisture, variable temperatures among others. These decomposition reactions are enhanced by active adsorbents such as silica gel. As seen in the two-dimensional TLC (Figure 18), all peaks are located on the diagonal line connecting the application position with the intersection of the two solvent fronts. This demonstrated that the extracts were stable during chromatography (Reich and Schibli 2007b; Indrayanto 2012).

Effect of solvent and extraction method on markers and other peaks

We evaluated the suitability of the solvents (water, ethanol, and ethyl acetate) and extraction methods (decoction, maceration, and Soxhlet). Samples extracted with soxhlet/ethyl acetate exhibited the same number, but more intense peaks than those extracted with soxhlet/ethanol; but more peaks than ethanol maceration samples. Decoctions showed the least number and intensity of peaks; with the only marker detected being catechin (Figure 19). These results imply that, as with betulinic acid and lupeol, most of the components are nonpolar, and perhaps decoction is not an appropriate extraction method. In fact, most known compounds have been isolated in ethanol and ethyl acetate extracts (Byamukama et al. 2015; Omara, Kiprop, and Kosgei 2022). Because of heat and continuous change in concentration gradient, soxhlet extraction gives higher yields than cold maceration. In addition, less solvent is used. On the other hand, cold maceration preserves heat labile phytochemical constituents, which would otherwise degrade under soxhlet or decoction conditions. Decoction offers an advantage of using the safest, cheapest and most easily accessible solvent, water (Handa 2008).

HPLC fingerprinting methods

Stability of the Extracts in solution

The extracts were stable in methanol over three days of refrigeration at 2-8^{o}C, as shown in the by the similarity indices of the three-day fingerprints in Table 13. Samples are considered similar and, therefore, stable if the similarity indices are close to 1 (Noviana, Indrayanto, and Rohman 2022).

Similarity of fingerprints of samples obtained from different climatic zones

Pattern recognition

The similarity analysis of HPLC fingerprints assumes that ‘similar peaks’ (the same retention time and peak area) belong to the same compounds. Therefore, one can compare the chemical composition of different samples without necessarily identifying the compounds (Xie et al. 2006b). HPLC fingerprints were developed for seven samples obtained from different agroecological regions. These fingerprints were then compared for similarity. Although the different sample fingerprints showed a similar pattern on visual inspection (Figure 22), the number of peaks varied greatly, ranging from 37 (Pakwach) to 55 (Iganga). These results show that the phytochemical concentration varies significantly with the source of the material; missing peaks mean that the concentrations of the responsible compounds are too low to be detected.

Similarity indices and multivariate analysis.

Since there are no botanical reference materials for *Albizia coriaria* yet, we compared the whole sample fingerprints to a median ("reference") fingerprint, generated by calculating the medians of the sample peak areas at the respective retention times. Furthermore, we compared fingerprints containing only the 10 common peaks (retention time±0.05minutes), including those for betulinic acid and lupeol, after aligning the chromatograms with the HPLC software. The similarity indices for whole chromatogram fingerprints were lower than those calculated for only common peaks. Interestingly, the p values (α=0.05) from the correlation matrices, of both types of fingerprints indicated that the samples were significantly similar, most even at (α=0.01). Of course, for the entire chromatogram fingerprints, these p-values are meaningless for practical applications, since r or c should approximate 1 for botanical samples to be considered identical (Alaerts et al. 2012). This is one of those cases of "frequent insignificance of a significant p-value" pointed out by (McGiffin, Cumming, and Myles 2021) For similarity indices based on only common peaks, all fingerprints were significantly similar to the reference at 99% confidence ($p<0.01$) except for samples from Iganga ($p=0.0024$) and Masindi ($p=0.147$). The samples of Moroto and Dokolo were the most similar to the reference. On hierarchical cluster analysis, the common peaks fingerprints gave four clusters: The samples in group 1 (Packwach, and Dokolo) were more than 90% similar (congruence coefficient>0.90) to each other, while the samples in group 2 (Moroto and Kabarole) were approximately 95% similar (congruence coefficient >0.94); group 3 (Mpigi and Iganga) (c>0.90), and group 4 (Masindi)(c<0.75). The Dendrogram Cophenetic correlation coefficient was 0.85, implying a good preservation of the original un-modelled data. From the PCA results, the minimum spanning tree distance confirmed that samples from Kabarole and Mororto were closest to the reference, followed by those from, Mpigi, Iganga, Packwach and Dokolo. The samples from Masindi were the most distant.

The results of similarity and multivariate analysis show that samples from Moroto and Kabarole were most identical. Practically, they can be substituted by the manufacturer without affecting the consistence of the herbal products. Samples from Pakwach, and Dokolo followed closely and can be improved by minimal manipulation. On the other hand, samples obtained from Masindi bear little similarity to the rest of the samples; thus, these samples will require significant dilution or concentration by standard materials before they can be used; otherwise, should be discarded (Eisner 2001). Since the similarity analysis is based on peak areas, the observed differences are due to differences in phytochemical concentration of the

phytochemicals (Alaerts et al. 2012) the differences in lupeol and betulinic acid concentrations were demonstrated by quantitative methods for samples sourced from different agroecological zones. We recommend that for practical applications, *Albizia coriaria* stembark material HPLC fingerprints should have similarity coefficients of at least 0.90 to the median to be considered phytoequivalent (Sahoo, Manchikanti, and Dey 2010).

To identify the peaks that contribute to the most fingerprint variation, loading plots came in handy (Figure 26 and Figure 27): betulinic acid, lupeol and peaks at retention times 8.6, 13.0, and 24.1 minutes contributed to PC 1, while the peaks at retention times 8.6, 9.1, 10.9, 13.0 and 24.1 minutes contributed to PC 2. In general, peaks at 8.6, 13.0 minutes and those due to lupeol and betulinic acid caused the most variation; Identifying the responsible compounds and adding them to the list of quality control markers would be highly beneficial. The standardized fingerprint for *Albizia coriaria* stembark raw materials should consist of peaks for lupeol and betulinic acid as well as peaks at 8.6, 9.1, 10.9, 13.0 and 24.1 minutes. This fingerprint can be achieved by the following chromatographic conditions: Soxhlet-ethyl acetate extraction; UV/visible detector 205nm; C18 (250mm × 4.6 mm x 5 µm), solvent system; acetonitrile (99.99%) and 1% trifluoroacetic acid (0.01%); flow rate 1mL/min; and column temperature, 25^0C.

HPLC quantification methods

An HPLC method was developed to simultaneously determine betulinic acid and lupeol after soxhlet extraction in ethyl acetate. Identification of markers was based on their retention times while quantification on the peak's areas in reference to the standard calibration curve. The respective peaks were confirmed by spiking; selective enhancement of the peaks makes them more conspicuous (Indrayanto 2018). The method was linear, accurate and precise for the quantification of both betulinic acid and lupeol, in accordance with the Association of Official Analytical Chemists (AOAC 2012); at the marker concentration levels found in the tested samples, accuracy of 90–107% and reproducibility of 8-5.3% are acceptable.

Effect of the extraction method on marker concentration

Three methods, namely, decoction, soxhlet extraction in ethanol and soxhlet extraction with ethyl acetate were compared to select the one which extracts the highest quantity of makers. *A coriaria* samples from Iganga were extracted. The solvents, ethanol and ethyl acetate were chosen based on previous work on the isolation of compounds from *A. Coriaria* (Byamukama et al. 2015; Omara, Kiprop, and Kosgei 2022). From TLC fingerprinting results, it was clear

that soxhlet was superior to maceration, therefore we did not consider the latter further. Decoction, the secondary processing method used by most manufacturers, was included to establish the suitability of these markers to monitor the quality of the processed materials (or products in case of native mono-component extracts (Eisner 2001). In factories, the herbal materials are boiled in water for several hours to extract the active ingredients (World Health Organization 2018). The analysis results showed that soxhlet extraction in ethyl acetate was superior to both soxhlet extraction in ethanol and decoction. This can be explained by the fact that both lupeol and betulinic acid are triterpenoid compounds with just one polar functional group and, thus, are more soluble in the least polar ethyl acetate. Although the TLC fingerprint showed almost no peak for decoctions (implying no compounds or very low amounts extracted), the more sensitive HPLC was able to quantify both betulinic acid and lupeol in decoctions. Even then, in some samples including those from Moroto, Masindi and Pakwach, betulinic acid and lupeol concentrations were below the LOQ. Perhaps lupeol and betulinic acid are not suitable markers for monitoring the quality of the *A. Coriaria* raw decoctions; but if these markers are important for activity, then decoction is not a suitable method for secondary processing as most of the active ingredients are not extracted or are degraded by the heat (World Health Organization 2018). Referring to the herb marker ranking system, analytical markers should be present in the herb, at concentrations above 10mg/g (Bensoussan et al. 2015); These amounts were achieved for both betulinic acid (14.84±0.06mg/g) and lupeol (10.10±0.02mg/g) in soxhlet-ethyl acetate extracts, although in samples from only one district each; Iganga and Mpigi, respectively.

Effect of the source of *A. Coriaria* materials on the concentration of markers

The raw materials from seven different agroecological zones showed significant differences in the concentration of lupeol and betulinic acid (Table *17*). The source of plant materials is influenced by factors such as (i) the options available in the marketplace, that is, cultivated or wild; and (ii) the required properties of the raw material and/or finished product including; content of particular markers, the absence of environmental contaminants, among others (American Herbal Products Association 2003). In this study, we collected materials from different agroecological regions and studied their variation in the concentration of lupeol and betulinic acid markers. The highest yield of betulinic acid (14.84±0.06mg/g) ($p<0.001$, compared to all other districts), was obtained from raw materials obtained from Iganga district (Eastern), while the lowest (1.90±0.01mg/g) were from Kabarole district (Western highlands); significantly lower than the rest of the rest ($P<0.001$). On the other hand, the highest yield of

lupeol (10.10±0.02mg/g) was found in raw materials from Mpigi district (Lake Victoria crescent) ($p<0.001$, compared to all the other districts), while the lowest (5.52±0.02mg/g) were from Moroto district (Karamoja drylands) and was significantly lower than the rest ($P<0.001$). The western highlands (Kabarole district), Lake Victoria crescent (Mpigi district), and Eastern receive the highest amounts of rainfall and have fertile soils compared to the Karamoja drylands and the rest of the agroecological zones (Plan 2010; Kajobe et al. 2016). However, both the lake Victoria crescent and the eastern agroecological zones are located at lower altitudes, close to Lake Victoria, with warm temperatures, unlike the cold western highlands and the hot Karamoja drylands (Plan 2010). Perhaps altitude and temperature are better determinants of the concentrations of lupeol and betulinic acid in *A. coriaria* than the amount of rainfall and soil fertility. In fact, the plant is known to thrive on low lands, with a variety of soil types (Orwa et al. 2009a). In addition to agroecological factors, the amount and nature of phytochemicals are affected by other factors such as; growth stage, harvesting time, the weather conditions at harvesting time, and minor differences in post-harvest handling, among others. Establishment and observance of good cultivation and harvesting practices can ameliorate such effects (American Herbal Products Association 2017).

Despite the geographical variation of marker concentrations, both dilute and concentrated materials are important for phytochemical standardization during manufacturing. Thus, dilute ones can be employed as diluents, while the concentrated ones as concentrators. In fact, this approach is preferred to diluting concentrated materials with fillers or concentrating dilute ones by adding pure active compounds (Eisner 2001). The final concentration of markers in the materials is determined by factors such as concentration of the same markers in other plant ingredients, effect on physical attributes of the drug such as colour, taste, etc., and the role of markers in the material or products; i.e. efficacy, stability, toxicity or batch-to-batch consistency indicating (American Herbal Products Association 2003).

Furthermore, materials of different obtained from the various climatic regions are used to obtain a representative sample, the botanical reference materials (Noviana, Indrayanto, and Rohman 2022). From the botanical reference materials (BRM), specified superior test materials (SSTM) and specified inferior test materials (SITM) are then prepared. Once these are in place, a validated botanical identification method (BIM) is then developed (AOAC 2012). In reference to the current study, the SSTM samples can be obtained from Moroto, Dokolo, Pakwach, Kabarole, Iganga, and Mpigi, while the SITM can be derived by mixing the established SSTM with inferior samples like those from Masindi, and other macroscopically

similar materials such as other Albizia species which can be mistakenly or scrupulously used by manufacturers. This approach requires analysis of many samples (at least 15 batches from each region) to maximize the observed variability (Indrayanto 2022).

4.2.1.10 Conclusions

The current work has provided critical information on analytical methodology and potential sources for good quality materials. The materials of *A. coriaria* harvested from different regions of the country are generally phytoequivalent. Catechin, lupeol and betulinic are ideal markers for chromatographic quality control of *A. coriaria* herbal raw materials.Tentatively, the chromatographic methods developed can be incorporated into routine quality control and standardization of materials obtained from various wild sources to ensure batch to batch consistency.

4.2.2.0 RESULTS AND DISCUSSION FOR *MANGIFERA INDICA* LEAF MATERIALS

This work aimed at establishing standards for classification of *Mangifera indica* leaf materials obtained from different varieties. To classify the materials, selected parameters relevant to the management of respiratory disorders were quantified and analyzed by chemometric techniques to generate different quality grades. *M. indica* extracts alleviate symptoms of respiratory tract disorders by reducing inflammation of the airways, chelating and neutralizing of harmful substances thereby reducing irritation and damage of the respiratory mucosa, as well as regulation of immune responses. These activities have been demonstrated in *M. indica* extracts rich in phenolic compounds, and particularly mangiferin. Since the antioxidant activity results from the combined effects of many compounds in addition to phenolics, including vitamins, terpenoids and minerals, it is logical to consider it as a separate phytochemical parameter. In addition to these parameters, the use of chemometric methods to analyze the data enabled generation of distinct patterns (grades) of *M. Indica* leaves and assigning a variety to the field samples. TLC and HPLC fingerprints were developed and used to evaluate the phytochemical equivalence of indica leaves from different varieties and field samples.

4.2.2.1.0 Classification of *Mangifera indica* leaves from different cultivars

4.2.2.1.1 Extraction index (EI), total phenolic content (TPC), mangiferin concentration (MC) and antioxidant activities (AOA) of *Mangifera indica* leaves.

The mean EI was 9.39±1.64%; and it varied significantly among the varieties (p=0.001). Similarly, the TPC and the MC varied significantly (P<0.0001) among the leaf extracts. The TPC ranged from 183.29±2.36 mg/g in Takataka variety to 79.47±0.58 mg/g Apple mango variety, with an average value of 131.22±32.03 mg/g. The MC was highest in Kate variety (105.75±0.60 mg/g) and lowest in Asante (39.53±0.30 mg/g), with an average of 59.19±18.09 mg/g. The highest AOA was 16.81±2.85µg/mL for Doodo red while the lowest was

67.67±20.19µg/mL for Asante; the average was 35.98±18.31 ug/mL. The results are summarized in Table 18 below, and thereafter, each parameter is explained in details.

Table 18: Extraction index, total phenolic content, mangiferin concentration and antioxidant activities of Mangifera indica leaves obtained from fifteen different varieties.

No.	*Mangifera indica* variety	Extraction index (EI) (%) (n = 3)	Total phenolic content (TPC) of extract (mg/g GAE) (n = 3)	Mangiferin concentration of extract (MC) (mg/g) (n = 3)	Antioxidant activity (AOA) (IC_{50} ug/mL) (n = 3)
1	**Apple Mango**	9.90±0.56^{ab}	79.47±0.58^{m}	44.60±0.34^{k}	55.41±2.05^{abc}
2	**Sejjembe**	9.01±0.59^{ab}	92.54±0.23^{l}	53.52±0.21^{g}	37.97±2.55^{bcde}
3	**Kawanda green**	9.45±0.19^{ab}	171.74±1.43^{c}	46.61±0.07^{j}	53.66±22.10^{abcd}
4	**Kate**	11.85±0.00^{ab}	163.59±0.21^{d}	105.75±0.60^{a}	18.35±17.95^{e}
5	**Asante**	8.02±4.39^{b}	112.94±0.42^{i}	39.53±0.30^{l}	67.67±20.19^{a}
6	**Kawanda wide**	12.96±0.42^{a}	141.58±0.55^{f}	46.36±0.07^{j}	53.80±13.58^{abcd}
7	**Suu**	9.85±0.20^{ab}	124.63±0.46^{h}	47.56±0.67^{i}	33.69±10.98^{cde}
8	**Koona**	7.84±0.32^{b}	144.33±0.70^{e}	92.66±0.18^{b}	23.08±9.00^{e}
9	**Kagoogwa**	11.49±0.08^{b}	177.13±1.23^{b}	51.10±0.15^{h}	19.74±3.36^{e}
10	**MPI**	7.74±1.35^{b}	114.98±0.40^{i}	55.70±0.25^{f}	65.88±8.85^{ab}
11	**Takataka**	10.01±0.89^{ab}	183.29±2.36^{a}	65.55±0.26^{c}	22.82±2.26^{e}
12	**Boribo**	7.77±1.58^{b}	108.79±0.61^{j}	58.30±0.02^{e}	25.17±4.30^{de}
13	**Ngoogwe**	8.14±0.76^{a}	130.83±0.06^{g}	65.94±0.06^{c}	26.93±4.60^{cde}
14	**Bire**	7.95±1.76^{b}	126.41±0.31^{h}	61.40±0.39^{d}	18.71±3.18^{e}
15	**Doodo red**	8.88±0.73^{ab}	96.06±0.06^{d}	53.29±0.27^{g}	16.81±2.85^{e}
16	**Mean values**	9.36±1.64	131.22±32.03	59.19±18.09	35.98±18.31
17	**Ascorbic acid**	NA	NA	NA	2.94±0.12

GAE-Galic acid equivalent; compact letter display-means that do not share a letter are significantly different; (α=0.05).

Extraction index (EI)

The extraction index determines the non-structural proportion of the drug biomass that is extracted by solvents. Thus, the extractable matter contains primary metabolites including proteins, lipids and carbohydrates and their building units and, secondary metabolites such as waxes, terpenes, gums, resins, phenolics, alkaloids, essential oils and inorganic compounds (Roy, Pakdel, and Brouillard 1990). For native extracts (for which no excipients

or other substances are added) the extractable matter is also the final drug, thus the extraction index exhibits the efficiency of the processing method (Commonwealth of Australia 2011). Therefore, plant materials with high yields are desirable for profitability of the herbal medicines business. In this study, the EI of *M. indica* leaf materials was highest for Kawanda wide variety at12.96±0.60% and lowest for MPI variety at7.74±1.91% (*Table 18*). These values were similar to those obtained by other researchers (Ndoye et al. 2018).

Besides quantification of the drug, the WHO recommends the use of extractable matter as a quality control method especially for plants without a suitable chemical or biological assay method (World Health Organization 2011). As such, the extraction index can be used to monitor consistency in the quality of raw materials, extraction process or monitor the effect of changes in the manufacturing process and plant source (variety or species). Furthermore, the extract strength is also relevant for calculating of the dosages of the individual materials to include in the product formula (Commonwealth of Australia 2011).

Total phenolic content (TPC)

The TPC of *M. indica* is a summation of phenolic acids, flavonoids and xanthones, the main phytochemicals implicated for antioxidant activity. Therefore, the TPC is an indicator of the quality of materials intended for use as antioxidants or indications based on antioxidant activity: this approach is easier, cheaper and more relevant than quantifying the individual compounds. The TPC and/or individual phenolic compounds are known to vary among *M. indica* cultivars (Romero et al. 2015; Berardini et al. 2005). In this study, the TPC of the materials varied significantly with the *M. indica* variety ($P<0.0001$), as demonstrated by Tukey's multiple comparisons (Appendix 8). Takataka variety had the highest content followed by Kagoogwa while Apple mango and Sejjembe varieties had the lowest (*Table 18*). The TPC values were similar to those reported earlier (Jhaumeer Laulloo et al. 2018). Since there are no established limits for TPC of *M. indica* materials or products for the treatment of respiratory tract disorders; it is incumbent upon the manufacture to establish the minimum acceptable

TPC of raw materials after establishing its relevance to bioactivity (and indication) of their materials and/or products. This can be done by designing dose-response experiments to establish the relationship between the TPC of materials and/product and the ability to ameliorate the symptoms of the disease.

Anti-oxidant activity (AOA) of *Mangifera indica* leaves.

Anti-oxidant activity (AOA) is one of the major biological activities of *M. indica* extract. Actually, some researchers have postulated that many of the other pharmacological activities of the plant are secondary to its ability to scavenge ROS involved in the pathogenesis of the diseases. As such brain-protective (Pardo-Andreu, Sánchez-Baldoquín, Avila-González, et al. 2006), antidiabetic (Rolo and Palmeira 2006), cardio-protective, anti-inflammatory, hepato-protective (Valtuena et al. 2008; Prasad, Kalra, and Shukla 2007), reno-protective, and anticancer activities (Manna, Kuo, and Aggarwal 1999) have been demonstrated. This implies that a measure of the AOA of the raw materials is a direct measure of their potency and so ensure pharmacological reproducibility (Vlietinck, Pieters, and Apers 2009). This approach is more appropriate than measuring quantities of individual compounds or groups of compounds (e.g. TPC); besides, it is not cost effective to determine all the active compounds. In addition, it is more practical for the manufacturer to measure the AOA of the plant materials than determining the therapeutic effect (in this case several activities relevant to treating respiratory tract disorders), as a quality assurance measure. Given the fact that the composition of antioxidant phytochemicals varies with the variety or plant species, the AOA of *M. indica* leaf materials is expected to vary with the source cultivar. In this study, it varied significantly ($p<0.0001$) (Appendix 9). Doodo red and Kate varieties had the highest AOA (lowest IC_{50}) i.e. 16.81±2.85µg/mL and 18.35±1.49 µg/mL respectively. On the other hand, the AOA of Asante (IC_{50}=67.67±20.19µg/mL), and MPI (IC_{50}=65.88±8.85µg/mL) were the lowest (highest IC_{50}) (*Table 18*). The values of AOA obtained in this study are similar to those reported elsewhere

(Sferrazzo et al. 2022). The performance parameters of the spectrophotometric method are summarized in Table P7-appendix.

Mangiferin concentration (MC)

Mangiferin is one of the most studied phenolic compounds of *Mangifera indica* with several pharmacological activities as outlined in the introduction. For the respiratory tract, mangiferin reduces inflammation of the airway, inhibits cytokine production and protects against lipopolysaccharide induced allergy (Rivera et al. 2011a; Li et al. 2021). This makes it a favorable candidate for use as an activity marker (Bensoussan et al. 2015). In addition to a diverse biological profile, mangiferin is found in only a few other plant species such as *Iris unguicularis*, *Anemarrhena asphodeloides, Bombax ceiba, Salacia species, Cyclopia species,* and *Crocus species,* (Khare and Shanker 2016), which are morphologically distinct from *M. indica.* This makes it ideal as a bioanalytical marker. Besides, the analytical standard is readily available commercially, and can be easily isolated in high amounts from several parts of the plant using common solvents like methanol, and ethanol. In addition, mangiferin can be quantified by basic spectroscopic and HPLC methods (the method used in this study). For use as pharmaceutical raw materials, it is only logical that cultivars with high mangiferin concentrations are desirable. In this study, Kate variety had the highest mangiferin concentration, followed by Koona and Ngoogwe while Asante, Apple mango and Kawanda wide had the lowest (*Table 18*). The MC varied significantly with *M. indica* variety (P<0.0001), as demonstrated by ordinary one ANOVA and Tukey's multiple comparisons (Appendix 10). The performance parameters of the HPLC method are summarized in Appendix 11.

Relationships among the phytochemical parameters

The Pearson correlation analysis of the parameters showed a direct (positive) relationship, as expected, with the correlation between the mangiferin concentration and antioxidant activity being statistically significant at α=0.05 (Table *19*).

Table 19: Pearson correlation for the studied phytochemical parameters

Parameters	Total Phenolic content r (p value)	Anti-oxidant activity r (p value)	Mangiferin content r (p value)
Anti-oxidant activity	-0.292 (0.291)		
Mangiferin content	0.363 (0.184)	-0.567 (0.028) *	
Extraction index	0.443 (0.098)	-0.053 (0.853)	0.043 (0.879)
* Significant at the 95% confidence level (p < 0.05)			

The TPC positively correlated with the extraction index, although not significantly (r=0.443, p=0.098). In addition, the TPC/EI ratio, an indicator of how much of the extracted matter is active drug (where activity due to phenolic compounds is of primary interest) ranged between 8.0 to 18.4 with a mean of 14.2. Koona, Kawanda green and Takataka varieties had the highest TPC for a unit percentage yield of the extract, while Apple mango, Doodo red and Sejjembe had the lowest (Appendix 12). This observation can be explained by the fact 70% ethanol extracts a variety of relatively polar compounds besides phenolic compounds, the concentration of which could also vary in the different varieties. Some of these compounds , like terpenoids and minerals augment the bioactivity of phenolics and so are desirable (Ribeiro and Schieber 2010).

A correlation analysis revealed that the AOA of *M. indica* materials increases proportionately with increase in on the TPC although not statistically significant (r=-0.292, p=0.291). Actually, some samples with high TPC had low antioxidant activity such as Asante, Kawanda green and Kawanda wide. This notwithstanding, most varieties showed a direct relationship between the TPC and AOA; that is Kate, Koona, Kagoogwa and Takataka. Some varieties such as Doodo red, had comparatively high AOA despite lower TPC (Table 18). The low correlation between TPC and AOA can be explained by the fact that *M. indica* contains (i) other non-phenolic antioxidant compounds such as terpenoids, carotenoids, vitamins E and C, and minerals and (ii) phenolic compounds with low or no AOA such as amino acids (Ribeiro et al. 2008; Rocha Ribeiro et al. 2007). However, these components are known to concentrate mainly in the fruits. Their role as antioxidants in other parts of the plant are yet to be

elaborated; (iii) another factor is the variation in the concentration of the specific phenolic compounds with varying activity. Structure activity relationship analysis of different phenolic compounds indicates that anti-oxidant activity is affected by the number of aromatic and hydroxyl groups (Figure 30) as well as their relative positions in the structure (Rice-Evans, Miller, and Paganga 1996), thus tannins (A), flavonoids (B) and xanthones (C) exhibit different levels of activity.

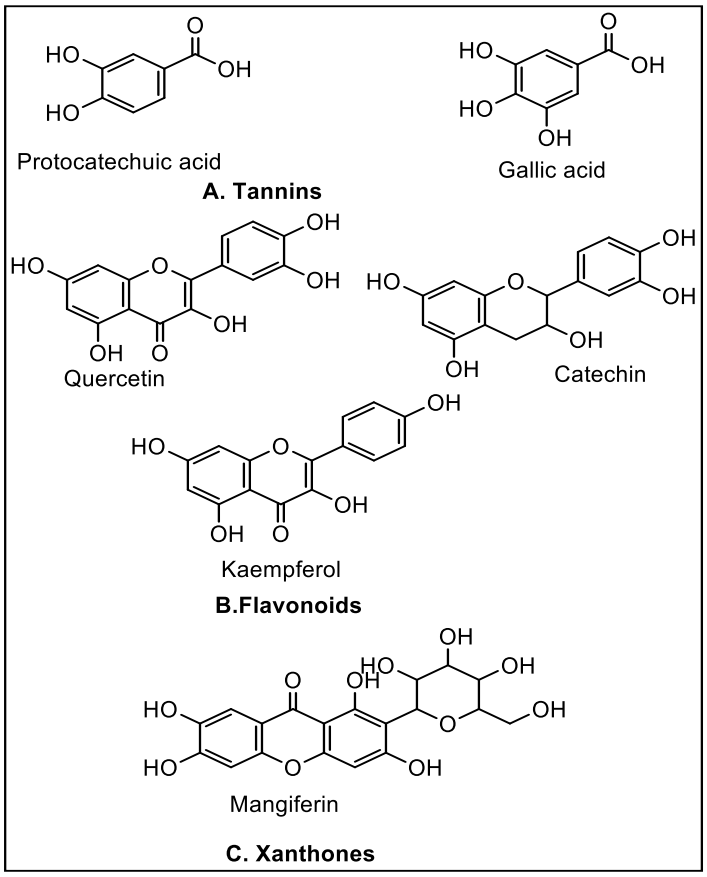


Figure 30: Examples of phenolic compounds in Mangifera indica leaves, showing the different nature of the phenolic nucleus, number and distribution of hydroxyl groups-structures drawn by ChemBioDraw Ultra® version 14.

Thus a *M. indica* variety may only produce a low of TPC but contain a high concentration of the compound (s) with high activity and vice versa. In addition, some phenolic compounds , might work additively, synergistically or antagonistically as demonstrated by (Palafox-Carlos et al. 2012). While the correlation between the TPC and AOA was low, the AOA/TPC ratio should be established and utilized to monitor the consistency in the composition of the herbal materials. The AOA/TPC ranged from 1.3 to 8.9 and an average of 4.2 (Appendix 12).

There was a significant correlation between the mangiferin concentration (MC) of the *Mangifera indica* leaves and their anti-oxidant activity (AOA) (r=-0.567, p=0.028). This shows, as expected, that the AOA increases (reducing IC_{50}) with an increase in the MC. This correlation was higher than that seen with TPC and AOA. The results demonstrate the

importance of mangiferin as an antioxidant component of *M. indica* leaves, qualifying it as a marker for AOA. Since r<1, this validates the fact that the observed total AOA is a result of synergism among the various phytochemicals. Actually, some samples with relatively lower MC such as Takataka had relatively high AOA; such samples are likely to be rich in non-mangiferin antioxidant compounds. The AOC/MC ratios ranged from 1.2 to 6.9, with an average value of 2.8 (Table P8-appendix). The manufacturer can set a minimum acceptable ratio depending on the relevance of mangiferin to the application of the materials or products. Generally, there was a positive although not significant correlation between the mangiferin concentration (MC) of the *M. indica* leaves and the total phenolic content (TPC) (r=0.363, p=0.184). Samples with the highest MC per TPC were Ngoogwe, Bire and Kate while Asante, Kawanda wide and Kawanda green had the lowest. The MC/TPC ranged from 0.3 to 0.6, with a mean of 0.5. These results show that mangiferin is just one of the phenolic compounds in *M. indica*.

Classification of the *Mangifera indica* leaf varieties based on extraction index, total phenolic content, mangiferin concentration and antioxidant activity

4.2.2.1.2 Clustering analysis

Clustering is a multivariate analysis tool that groups samples based on the similarity of the measured parameters. In this study, we used hierarchical clustering and Ward's method-algorithm (measures similarity by Euclidean distances) to classify *Mangifera indica* leaf cultivars depending on the variation of four parameters namely; extraction index, total phenolic content, mangiferin concentration and antioxidant activity. Four main groups, A, B, C and D, with several subgroups, were obtained (Figure 31).

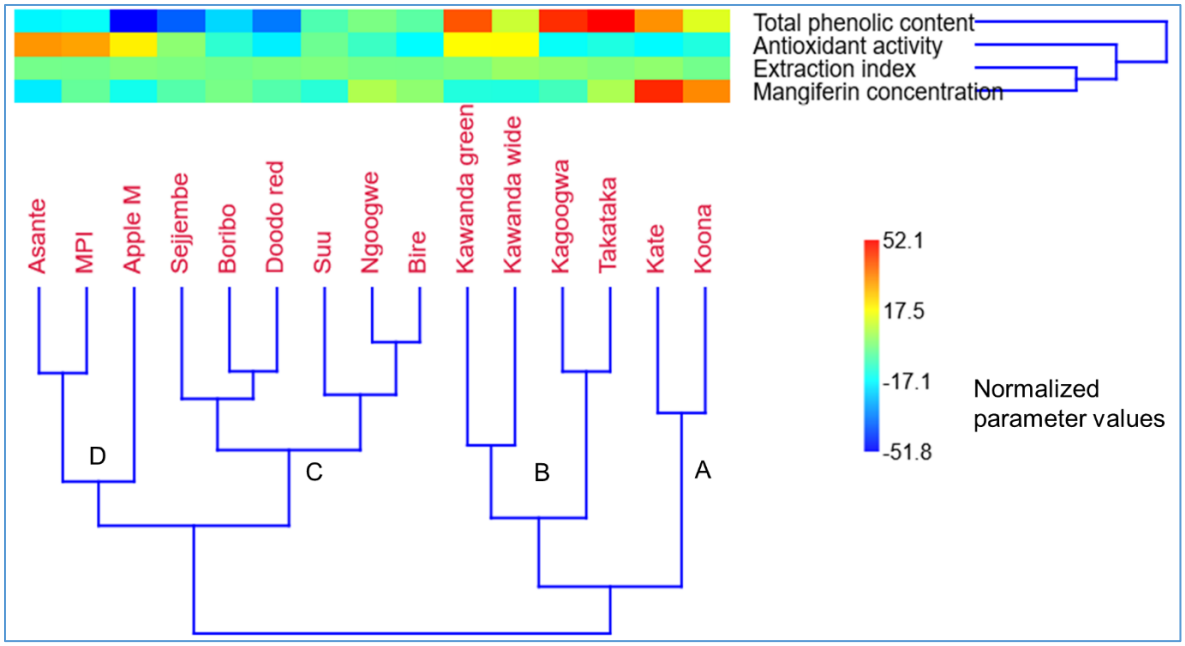


Figure 31: Two-way dendrogram for the classical hierarchical cluster analysis of M. indica leaf varieties. Algorithm: Ward's method; similarity index, Euclidean distance.

Group A contains varieties with the highest total phenolic contents, mangiferin concentrations and antioxidant activities. These parameters reduce from group B to group D, to below average values. Based on these clusters and on the average quantities of the studied parameters, we generated four grades of *M. indica* leaf materials. These are summarized in (Table 20).

Table 20: Classification of Mangifera indica leaf varieties based on the extraction index, total phenolic content, mangiferin concentration and antioxidant activity.

Group	Group members	Characteristics	Average quantity of parameters of groups compared to the overall average values			
			TPC (131.2)	AOA (35.0)	MC (59.2)	EI (9.4)
Grade A A	Koona, Kate,	Above average total phenolic content, mangiferin concentration, antioxidant activity, and	153.9	20.7	99.2	9.8

		extraction index.				
Grade B	Kagoogwa, Takataka, Kawanda green, Kawanda wide	Above average total phenolic content and extraction index, below average mangiferin concentration and antioxidant activity.	168.4	37.5	52.4	11.0
Grade C	Bire, Ngoogwe, Suu, Doodo red, Boribo, Sejjembe	Below average extraction index, mangiferin concentration, and total phenolic content but above average antioxidant activity.	113.2	26.4	56.7	8.6
Grade D	Apple mango, MPI, Asante	Below average extraction index, total phenolic content, mangiferin concentration, and very low antioxidant activity.	102.5	63.0	46.6	8.5

The contribution of the parameters to the observed clusters are well illustrated by a PCA scatter biplot (Figure 32).

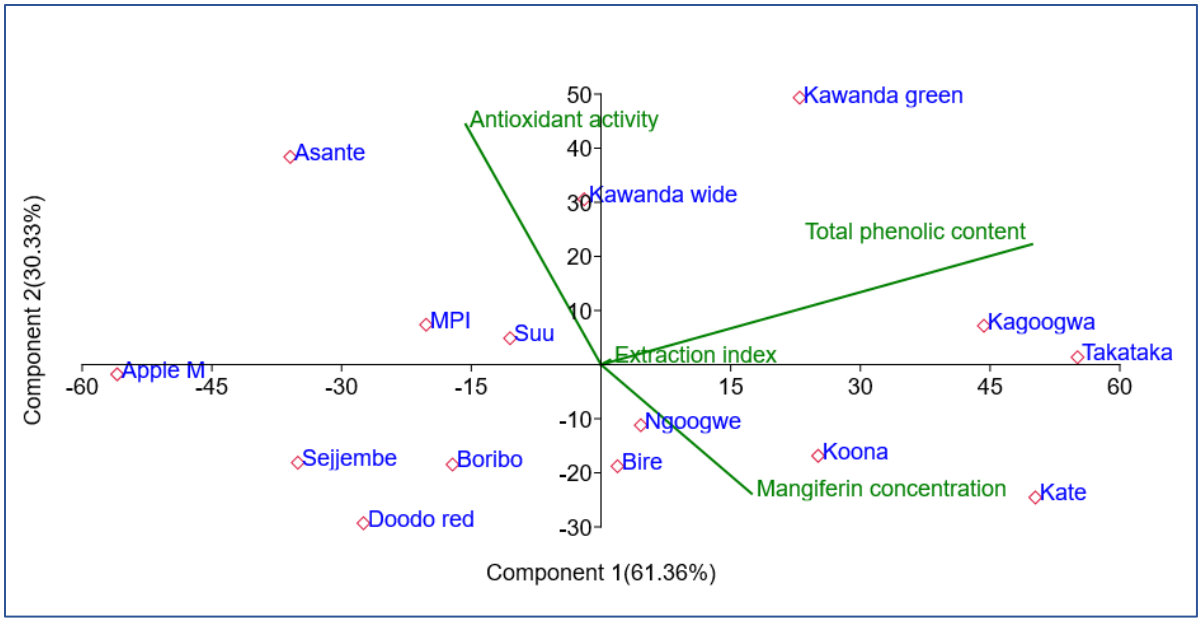


Figure 32: PCA scatter biplot of various Mangifera indica leaf cultivars showing contribution to variation of different phytochemical parameters; factor scores of observations were plotted on the first two components- Drawn by PAST software version 4.1

From Figures 29 and 30, it is clear that the total phenolic content varies most, followed by the mangiferin concentration and anti-oxidant activity and. The extraction index is the least variable parameter. These grades generated can guide manufacturers and botanist to select the best varieties for use as pharmaceutical raw materials. For therapeutic applications for which the studied parameters are relevant, such as respiratory tract disorders (as it is in Uganda), samples with high parameters are preferred. However, the manufacturer might need to determine the minimum amounts of each parameter that provides optimum potency of the product; this was beyond the scope of this work.

4.2.2.1.2 Classification of the *Mangifera indica* leaf varieties based on fingerprint characteristics

The classification of Mangifera indica leaf varieties as based on the common fingerprints pattern recognition and multivariate analysis of common peak (common fingerprints) and whole chromatogram peak areas (whole fingerprint). The typical fingerprint is shown in Figure 33.

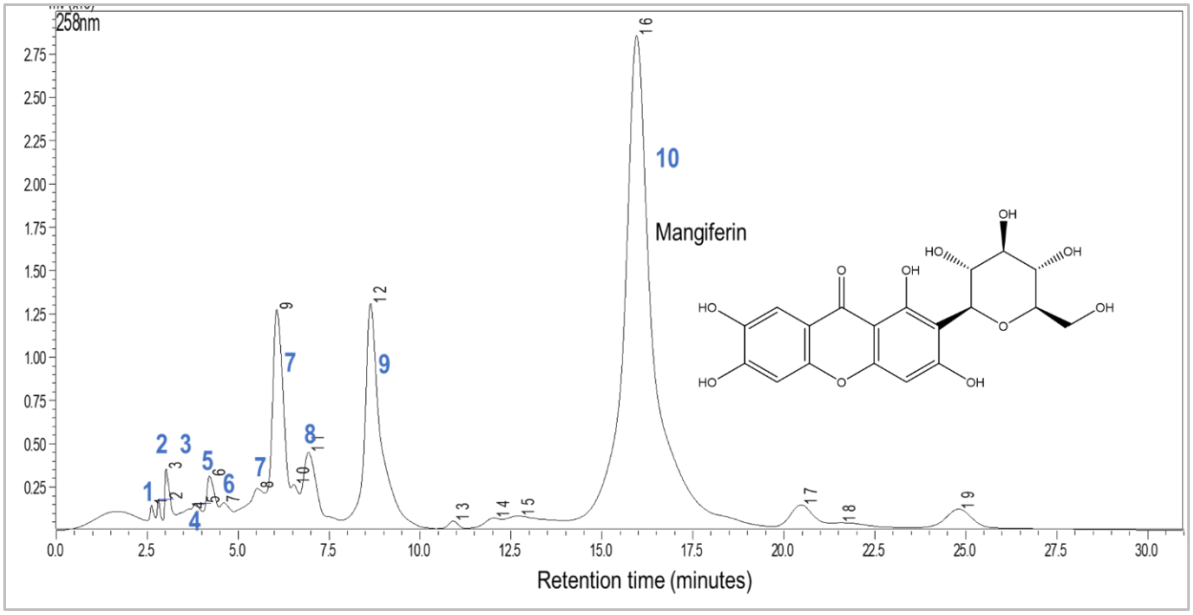


Figure 33: Representative HPLC fingerprint of M. indica leaf ethanolic extract showing common peaks (blue numbers).

Visual analysis for pattern recognition of HPLC fingerprints

Visual inspection of the 30 minutes whole fingerprints showed high similarity (Figure *34*). However, individual fingerprints varied greatly in the number of peaks; from 19 in Kawanda green to 29 in Kawanda wide, with an average of 23.5±2.9 peaks.The total peak areas ranged from 5,863,448 mVmins for the Kate variety to 1,568,633mVmins for the Asante variety, with an average area of 2,457,451±1,026,790 mVmins (Appendix 14).

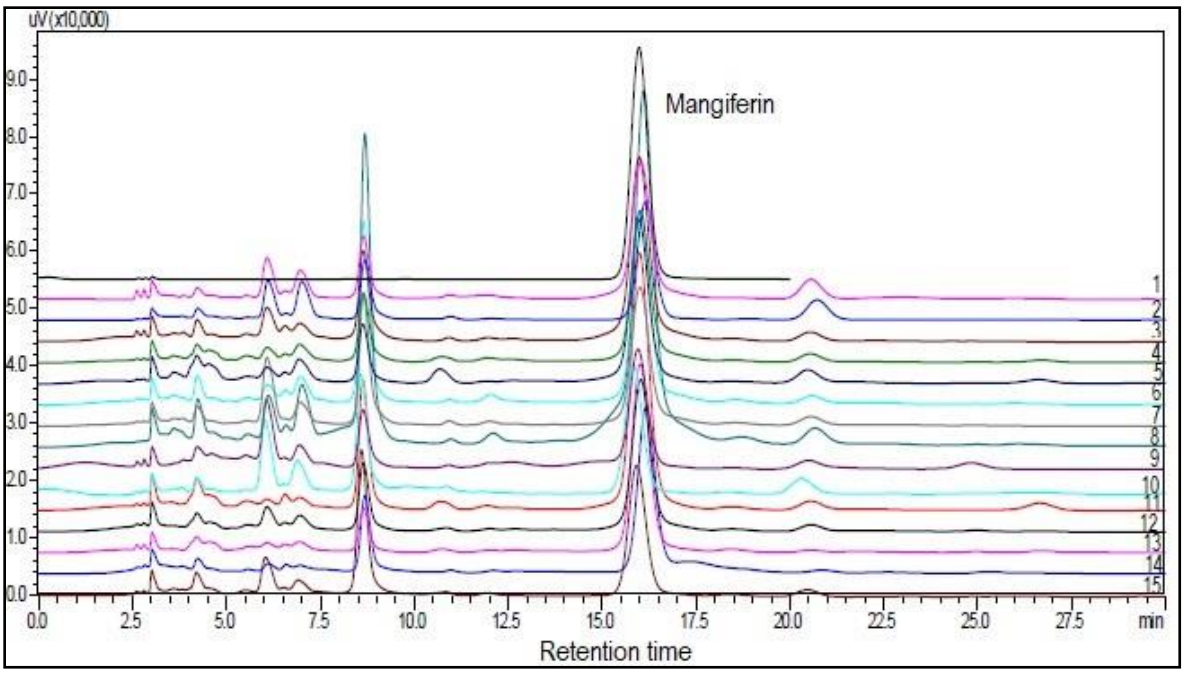


Figure 34: HPLC Fingerprint overlay showing the similarities of the different varieties of Mangifera indica leaf extracts.1-Apple mango, 2-Asante, 3-Bire, 4-Boribo, 5-Doodo, 6-Takataka, 7-Kagoogwa, 8-Kate, 9-Kawanda green, 10-Kawanda wide, 11-Koona, 12-MPI, 13-Ngoogwe, 14-Sejjembe, 15-Suu.

These results demonstrate marked phytochemical variability of the *M. indica* varieties. This can be explained by the fact that the genetic makeup of plants determines the nature and amounts of plant metabolites by influencing the nature and number of enzymes and co-factors produced by a particular cultivar or subpsecies(Iannucci, Suriano, and Codianni 2021). Also, certain cultivation of plants in non-natural habitats may affect their metabolic rate because of unfavaorable climatic conditions in the new environments(Suyal et al. 2021). Thus a project whose aim is to domesticate medicial plants needs prior investigation of the suitability of the agroecological factors in the new habitat. In absence of specific markers, whole fingerpints can be used to demonstrate similarity phyto-equivalnce of medicinal plant varieites, and also study the effect of changes in cultivation, harvesting and post harvest handling practices-(American Herbal Products Association 2017).

To reduce the complexity and cost of analyzing many markers in plant materials, the chinese pharmacopoeia recommends analysis of common fingerprints, which are

constructed peaks that are common to all samples (same retention times)(Xie et al. 2006a). Common fingerprints are also easier to reproduce than whole fingerprints. For this study, we obtained ten common peaks (Figure 31); their retetnion times and peak areas are shown in (Table P 10-appendix). The variety “Kawanda wide”, which had the highest number of peaks was used as the reference in selection and matching of peaks. The total area of common fingerprint peaks ranged from 3,710,796 mVmins to 961,454 mVmins with an average of 1,652,214±652910 mVmins. The variations in peak areas are proportional to the variations in concentration of the compunds resposible for the peaks; and thus shows the variability of the samples. The pattern of the peaks is characteristic of the plant material for the specifed anlysis conditions and so can be used identify and autheticate the materials. The fingerprints we developed were reproducible (Appendix 16 and Appendix 17). In absence of markers or if the chemical composition of the material is not known, strong peaks (peak area more than 10% of the total area) are used as reference in describing relative positions and areas of other peaks (Wagner et al. 2011). For this study, only peak 10 (Mangiferin, Rt=15.828mins), was a strong peak, making up more than 70% of total peak area (Appendix 15).

4.2.2.1.3 Fingerprint similarity analysis of fingerprints using correlation indices

Besides visual and descriptive evaluation, fingerprints can also be compared for similarity by calculating similarity indices such as correlation coefficients (r), cosines (c) Euclidean distances (ED), among others. We calculated the r of the whole and common fingerprints to determines the similarity of the leaves from different M. indica varieties. (Note: The whole fingerprints consist of all peaks in the chromatogram of each sample, while the common fingerprints constitute only peaks that are “common” to all the chromatograms of different samples.)

The whole fingerprints showed low correlation, with only a few fingerprints having r>0.8 (Figure 35).

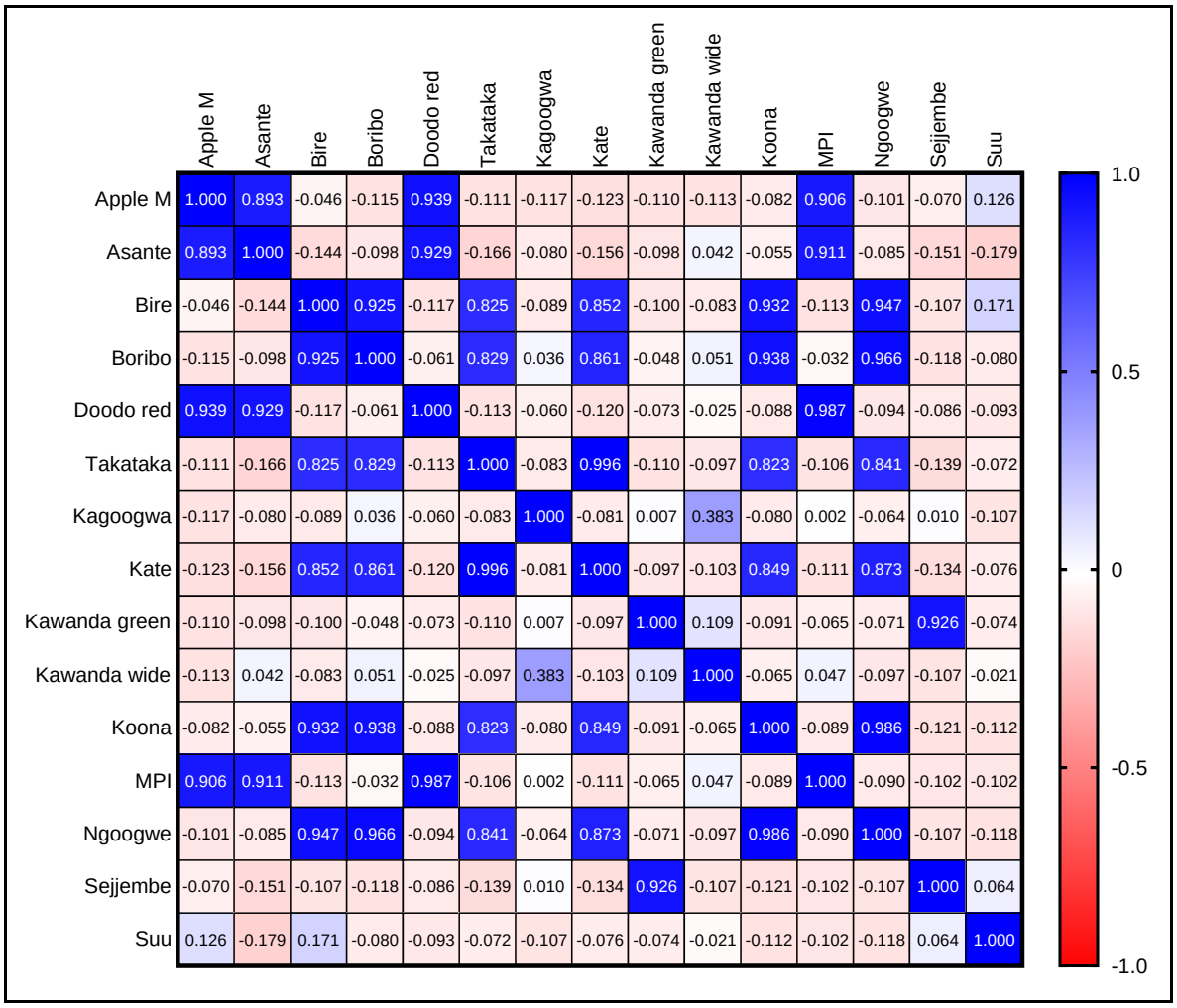


Figure 35: Heat map of the Pearson correlation matrix of Mangifera indica leaf variety fingerprints based on whole chromatogram peak areas; the intensity of blue is proportional to the degree of similarity of the fingerprints.

On the other hand, the Pearson correlation analysis of common fingerprints showed that all the *Mangifera indica* leaf varieties were significantly similar ($r>0.9$, $p<0.001$), as shown in Figure 36.

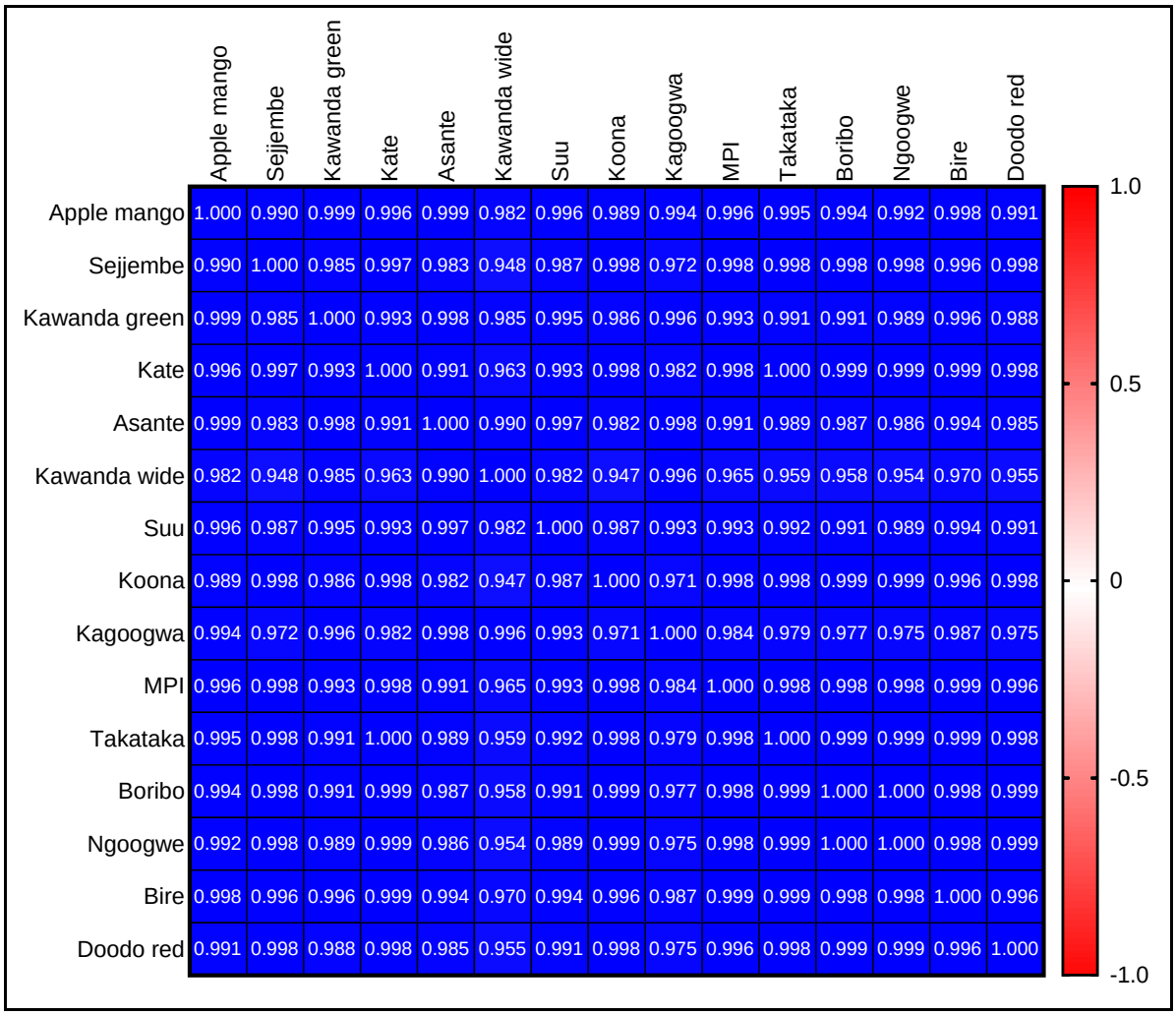


Figure 36: Heat map of the Pearson correlation matrix of Mangifera indica leaf variety fingerprints based on common peak areas; the intensity of blue is proportional to the degree of similarity of the fingerprint- Drawn by GraphPad Prism version 9.

This information can be used to guide the manufacturer in selecting phyto-equivalent materials. According to the Chinese pharmacopoeia, only samples with r≥0.9 are considered identical and therefore phyto-equivalent; such materials can be substituted without significantly altering the chemical composition and thus the potency of product (Xie et al. 2006a). This approach is more accurate than analyzing just a few markers(Kamboj 2012), Hence, from Figure 32, the following varieties are equivalent: Apple mango=Doodo red=MPI; Asante=MPI=Doodo red; Bire=Boribo=Doodo red=Koona=Ngoogwe; Takataka=Kate; Kawanda green=Sejjembe; Kawanda wide and Suu varieties have no substitutes. Although

common fingerprint analysis gives higher r values (Figure 33), these fingerprints are based on only a few compounds. Therefore, it is crucial to study the plant material extensively to ensure that the selected peaks represent the most important active phytochemicals, in order to generate an accurate bioactivity-fingerprint (Noviana, Indrayanto, and Rohman 2022). Nevertheless, common peak fingerprints are valuable in authenticating herbal raw materials or products (Wang, Nie, and Zang 2015).

4.2.2.1.4 Principal component analysis (PCA) of common fingerprints

In the PCA scatter plot, all the *Mangifera indica* leaf varieties lay within the 95% similariy ellipse apart from Kate (Figure 37) and (Figure 38).

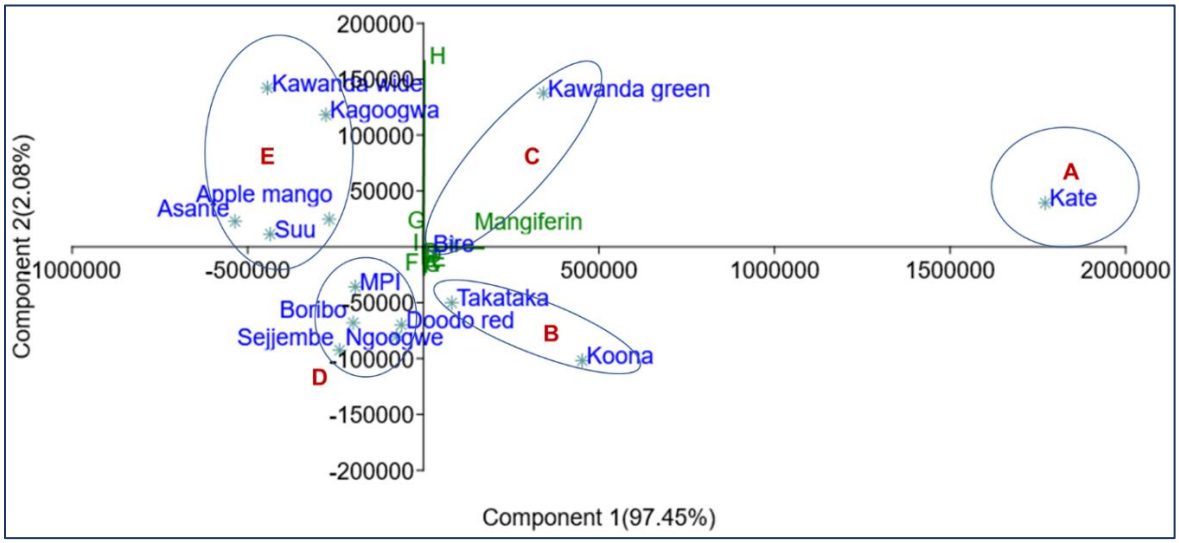


Figure 37: PCA scatter biplot of Mangifera indica leaf fingerprints and the contributing peaks; factor scores of observations plotted on the first two components- Drawn by PAST software version 4.12.

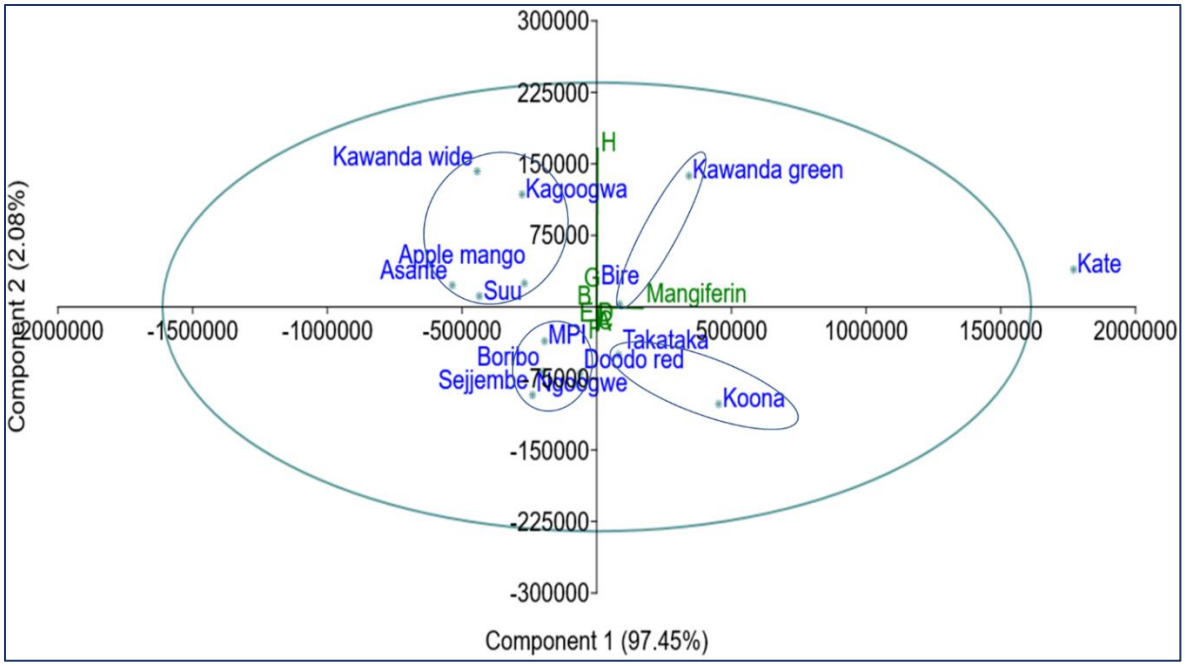


Figure 38: PCA scatter biplot of Mangifera indica leaf fingerprints and the contributing peaks; the 95% confidence ellipse showing the Kate variety as an outlier- Drawn by PAST software version 4.12.

Since it is not an undesirable to have high a concentration of mangiferin, we did not eliminate Kate variety from the classification, but rather assigned it a separate group. Thus, from Figure 37, five major groups are noticeable: A (Kate), B (Koona and Takataka), C (Bire, and Kawanda green), D (MPI, Doodo red, Boribo, Sejjembe, and Ngoogwe) and E (Asante, Suu, Kawanda wide, Kagoogwa, and Apple mango). These arise due to the variations in the concentration of the chemical constituents of the samples, as illustrated by the he biplot (in green), and by Figure 39 and Figure 40: Here, the loading plots for principal components 1 and 2 revealed that most of the sample variance is caused by two compounds, one at Rt=15.828 minutes (mangiferin) and the other at 6.021 minutes (unidentified-compound 8 (H)).

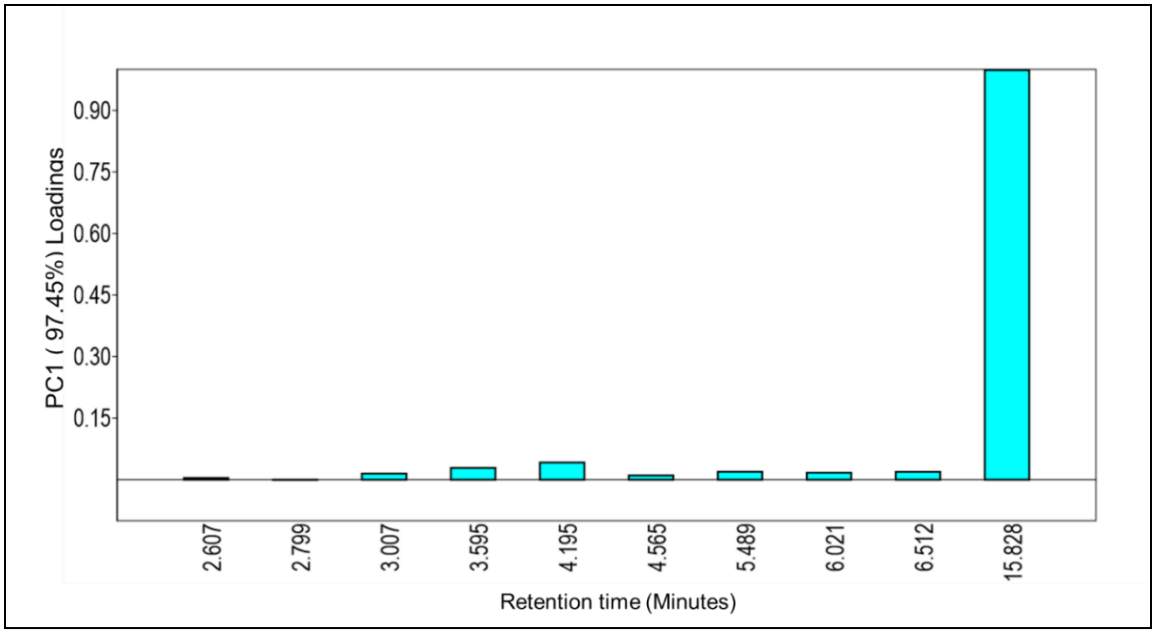


Figure 39: Loading plots Mangifera indica leaf fingerprints showing the contribution of the peaks to the variance of PC1 (97.45%). Peak 10-mangiferin (Rt=15.828 minutes) contributed the most to PC1 variance (loading score >0.9)- Drawn by PAST software version 4.12.

Since all the peaks contribute to PC1, they should be part of the reference fingerprints. For PC2, only the peak due to Mangiferin contributes to the variance (Figure 40)

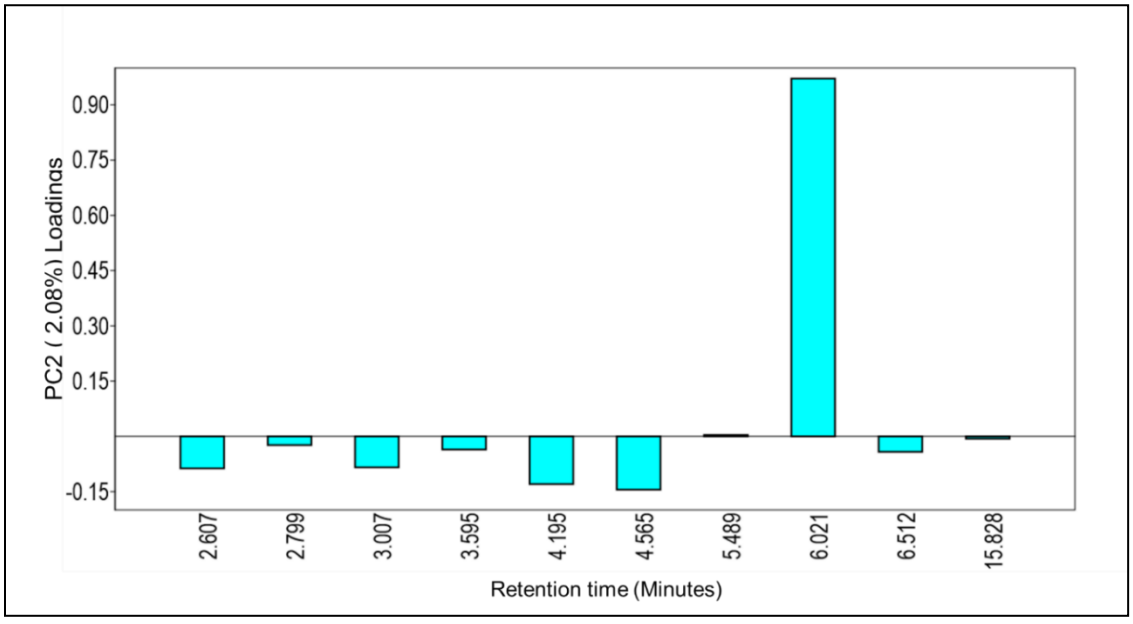


Figure 40: Loading plots of Mangifera indica leaf fingerprints showing the contribution of the peaks to the variance of PC2 (2.08%). Peak 8 (Rt=6.021 minutes) contributed the most to the variance of PC2 (loading score >0.9)- Drawn by PAST software version 4.12.

Thus, group A and B consist of varieties with the highest mangiferin concentration but about average of compound 8.Group C; above average mangiferin and compound 8 concentrations. Group D; average mangiferin concentration and low compound 8 concentration. Group D; lowest mangiferin concentration and above average compound 8 concentration).

4.2.2.1.5 Comparison of the HPLC fingerprints of the *M. indica* cultivars with those of unidentified samples collected from various parts of the country (field samples).

To identify the groups of materials to which the field samples relate to, hierarchical clustering of the common fingerprints of unknown samples collected from ten different districts in Uganda were compared to the standardized fingerprints of the 15 cultivars. The results indicated that most of the field samples were close to Koona, Kate and Kagoogwa green varieties. The others were close to Takataka and Bire varieties as illustrated by Figure 41.

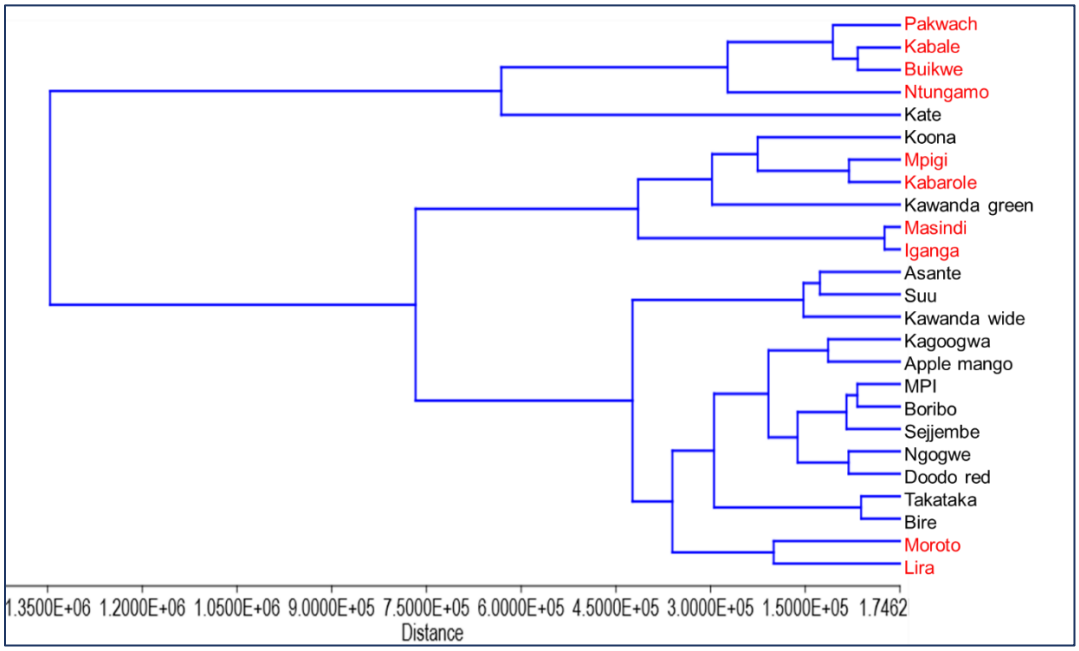


Figure 41: Dendrogram for the classical hierarchical cluster analysis of M. indica leaf varieties and field samples. Algorithm: Ward's method; similarity index, Euclidean distance. The labels in red indicate the samples collected from different districts in Uganda.

While the actual variety to which the field samples belong can only be confirmed with genetic studies such as DNA bar coding, the results obtained give an insight into the most cultivated *Mangifera indica* varieties, and thus the sources of the herbal materials. According to

(Ddamulira et al. 2019), Kate, Koona, and Kawanda green, Takataka and Bire are among the high fruit yielding varieties, hence grown by most households. Coincidentally, Kate, Koona, and Takataka varieties belong to group A (high in TPC, MC, and AOA), according to our grading, while Kawanda green belongs to group B. However, Bire belongs to the lowest group D. Perhaps more samples are needed to further test the method and validate these results.

4.2.2.2.0 Evaluation of phytoequivalence of *Mangifera indica* samples collected from different agroecological zones.

4.2.2.2.1 Development of the thin layer chromatography fingerprint method

The best TLC fingerprints were obtained using mobile phase system comprising 8,2,1, and1 volumes of ethyl acetate, acetic acid, methanol and water. The optimized solvent mixture is represented by point c, on plane C of the solvent optimization prism (Figure 42).

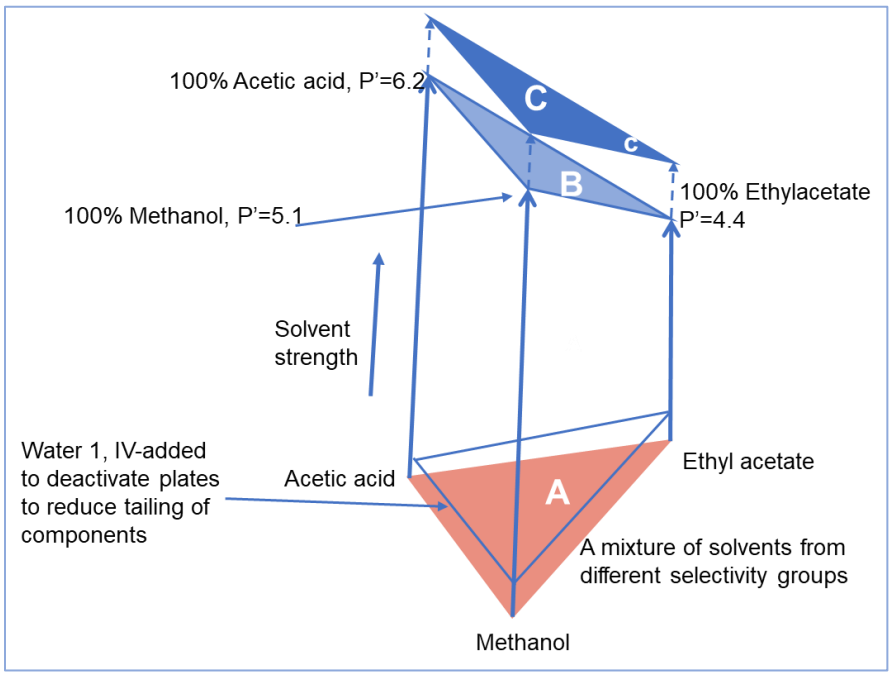


Figure 42: Solvent optimization prism for the mobile phase for TLC fingerprinting of M. indica leaf materials. Solvent mixtures in plane B, all have different polarities and selectivities. Plane C solvent mixtures have higher polarity that the original solvents due to addition of water.

The polarity P', of the solvent system is 6.3, as calculated from equation 1. The solvents represent selectivity groups II, IV, VI and VIII for methanol, acetic acid, ethyl acetate and water respectively (Ramis-Ramos, García-Álvarez-Coque, and Navarro-Huerta 2017). This is quite a strong solvent system and it stresses the fact the main constituents of the *M. indica* extracts are polar, majorly phenolic compounds. The wide selectivity of the solvent mixture enables a wide spectrum of interaction with the components in the sample, to enable separation (Snyder 1978). Using this solvent system, the plates were developed in 35 minutes; the laboratory temperature was 27^0C and humidity, 56%. This duration was longer than seen for *A. coriaria* (20 minutes) because the more polar solvent system used interacts more with silica gel, thus moving slowly through the stationary phase (Reich and Schibli 2007b)

The derivatization of the TLC plates was achieved by vanillin/sulphuric acid reagent. A typical fingerprint is shown in Figure 43.

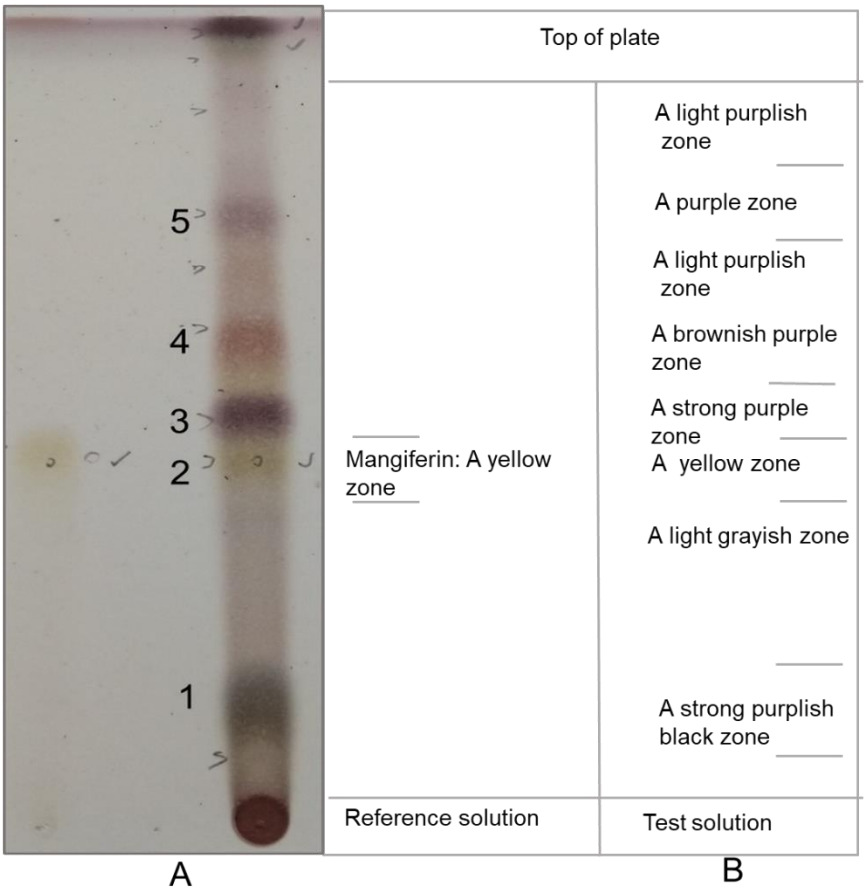


Figure 43: A: Typical TLC fingerprint for M. indica ethanolic leaf extract: Solvent system; ethyl acetate, acetic acid, methanol, water (8:2:1:1). Tracks: 1-5-strong peaks, 2-mangiferin standard. B: Schematic of the fingerprint as presented in the Pharmacopoeia.

The derivatization was complete in about 15 minutes, on heating the plate at 120^{o}C in an oven. Images of fingerprints observed before derivatization under normal light and after illumination with UV light are shown in Appendix 13.

Validation of the TLC fingerprints

Stability of the TLC fingerprints

The samples were stable under the chromatographic conditions used, as indicated by a two-dimensional TLC (Figure 44)

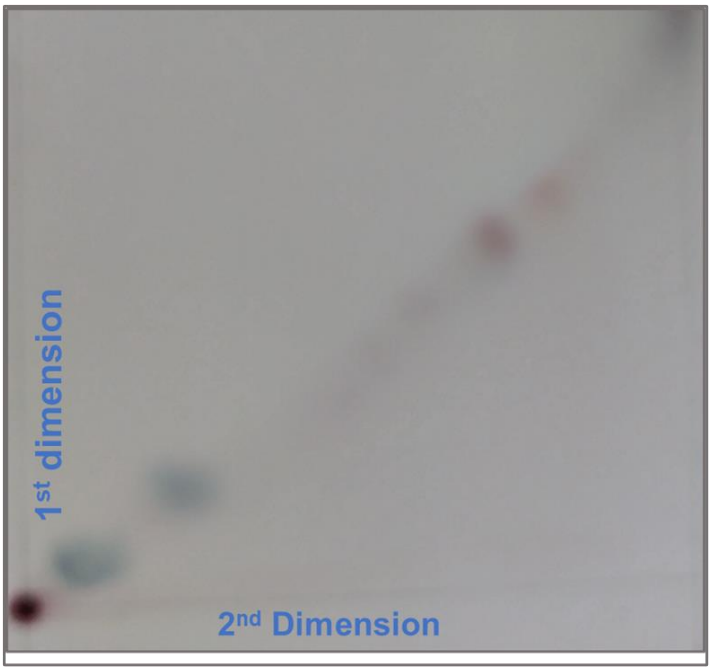


Figure 44: Two-dimensional (2D) TLC of M. indica ethanolic extracts. Solvent system; ethyl acetate, acetic acid, methanol, water (8:2:1:1).

Since all spots lie on the diagonal line joining the sample application point to the intersection of the solvent fronts, the samples are stable under the chromatographic conditions.

Thus, the solvent (methanol), the mobile phase, and the stationary phase (silica gel) did not affect the composition of the sample. In addition, the environmental conditions within the laboratory did not cause any decomposition of the sample.

4.2.2.3.0 Evaluation of phytoequivalence of *M. indica* leaf samples harvested from different agroecological zones.

Similarity of TLC fingerprints

There were 13 peaks common to all samples (Figure 45), by comparing their relative R_Fs and their colors after derivatization (Table 21) . The concentration variations of the chemicals (size of peaks) are also evident from the fingerprint. Spots 1, 3, 4 and 5 (Figure 43) were as prominent as the mangiferin spot (2).

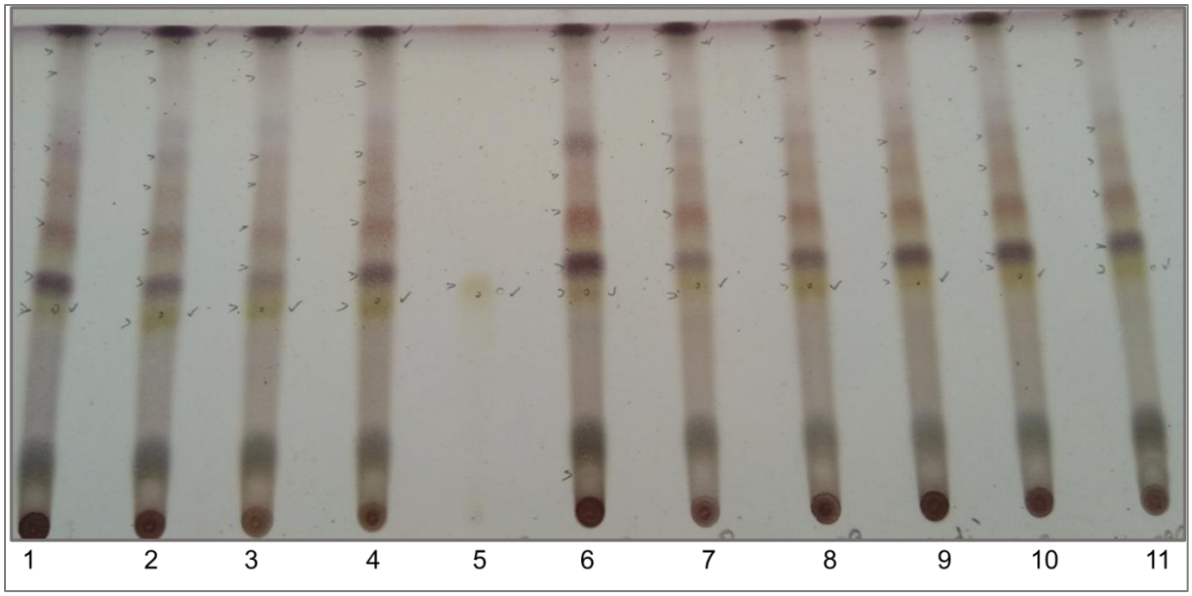


Figure 45: Comparison of TLC fingerprints for M indica leaf samples collected from different agroecological zones (represented by sampling districts); Tracks 1-Mpigi, 2-Kabarole, 3-Buikwe, 4-Pakwach 5-Mangiferin standard, 6-Kabale, 7-Moroto, 8-Iganga, 9-Masindi, 10-Lira, 11-Ntungamo. Solvent system; ethyl acetate, acetic acid, methanol, water (8:2:1:1).

Table 21: Summary of peak properties for TLC fingerprints

Peak no.	R_F	Visualization			Identity of compound	HRF RF*100	Relative R_F (Mangiferin)
		366nm	254nm	Daylight after derivatization			
1	0.97	Bluish green	Grey black	Grayish	Unknown	97	2.43
2	0.95	Bluish green	Grey black	Grayish	Unknown	95	2.38
3	0.94	-	Pink	Purplish	Unknown	94	2.35
4	0.89	-	Pink	Grayish	Unknown	89	2.23
5	0.80	-	-	Grayish	Unknown	80	2.00
6	0.73	-	Pink	Dark grey	Unknown	73	1.83
7	0.67	-	Pink	Grayish	Unknown	67	1.68
8	0.60	-	-	Grayish	Unknown	60	1.50
9	0.55	-	Pink	Dark gray	Unknown	55	1.38
10	0.51	-	-	Brownish	Unknown	51	1.28
11	0.41	-	Pink	Purple	Unknown	41	1.03
12	0.40	Bluish green	Greyish black	Yellowish	Mangiferin	40	1.00
13	0.12	-		Purplish Black	Unknown	12	0.30

R_F-Retardation Factor.

The fingerprints were similar, as shown in Table 22, all the peaks being present in the different samples.

Table 22: Distribution of bands in fingerprints of samples harvested from different districts in Uganda. 1 means the band is present, while 0 means it is absent.

Peak no.	Presence of peak in fingerprints of *Mangifera indica* leaf samples from different districts									
	Lira	Iganga	Masindi	Kabarole	Moroto	Mpigi	Pakwach	Ntungamo	Buikwe	Kabale
1	1	1	1	1	1	1	1	1	1	1
2	1	1	1	1	1	1	1	1	1	1
3	1	1	1	1	1	1	1	1	1	1
4	1	1	1	1	1	1	1	1	1	1
5	1	1	1	1	1	1	1	1	1	1
6	1	1	1	1	1	1	1	1	1	1
7	1	1	1	1	1	1	1	1	1	1
8	1	1	1	1	1	1	1	1	1	1
9	1	1	1	1	1	1	1	1	1	1
10	1	1	1	1	1	1	1	1	1	1
11	1	1	1	1	1	1	1	1	1	1
12	1	1	1	1	1	1	1	1	1	1
13	1	1	1	1	1	1	1	1	1	1

Since all the visible bands were present in all samples, the jaccard indices were all equal to 1 (Table 23).

Table 23: Jaccard indices of TLC fingerprints of M. indica samples collected from different agroecological zones.

Samples	Jaccard indices								
Lira	Lira	Iganga	Masindi	Kabarole	Moroto	Mpigi	Pakwach	Ntungamo	Buikwe
Iganga	1,0								
Masindi	1,0	1,0							
Kabarole	1,0	1,0	1,0						
Moroto	1,0	1,0	1,0	1,0					
Mpigi	1,0	1,0	1,0	1,0	1,0				
Pakwach	1,0	1,0	1,0	1,0	1,0	1,0			
Ntungamo	1,0	1,0	1,0	1,0	1,0	1,0	1,0		
Buikwe	1,0	1,0	1,0	1,0	1,0	1,0	1,0	1,0	
Kabale	1,0	1,0	1,0	1,0	1,0	1,0	1,0	1,0	1,0

The TLC fingerprint similarity analysis results show that all the *M. indica* leaf samples obtained from different agroecological zones are phytoequivalent. Because some bands are too faint,

meaning their concentrations are too low, they are less reproducible and thus not suitable for routine quality assessment. Therefore spots 1, 3, 4 and 5, which were more intense, were selected as principal peaks and they should be considered as mandatory for the reference fingerprint.

4.2.2.4.0 HPLC fingerprint methods

The fingerprints for field samples were similar to those obtained for different cultivars, already discussed above. Thus the same common peaks were used for evaluating the phytoequivalence. Hierarchical cluster analysis of HPLC fingerprints based on the common peaks gave four very close clusters : cluster i composed of only samples from Lira, and was the least similar to the rest of the sample (correlation coefficient = 0.992). Samples in cluster ii (Moroto and Kabarole) were more than 99.9% similar (congruence coefficient >0.999); samples in cluster iii (Mpigi, Masindi and Iganga) were 99.8% similar while those in cluster iv (Kabale, Buikwe, Pakwach and Ntungamo) were at least 99.7% similar (Figure 46).

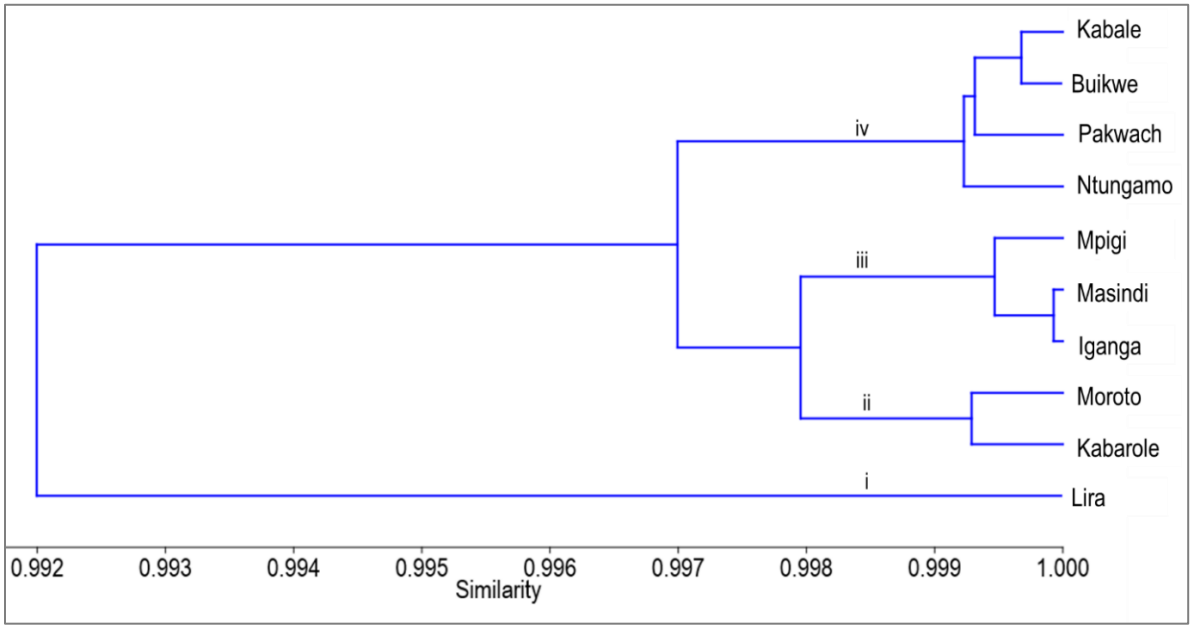


Figure 46: Dendrogram for the classical hierarchical cluster analysis of M. indica leaf samples obtained from ten different geographical sources: Algorithm, Unweighted pair group method with arithmetic mean (UPMGA); similarity index, correlation coefficient.

The Dendrogram Cophenetic correlation coefficient was 0.854, implying a good preservation of the original un-modelled data. These results imply that the samples are phytoequivalent. Fingerprints of samples collected from Kabarole were closest to the reference fingerprints, followed by those of samples from Pakwach and Ntungamo (Figure 47).

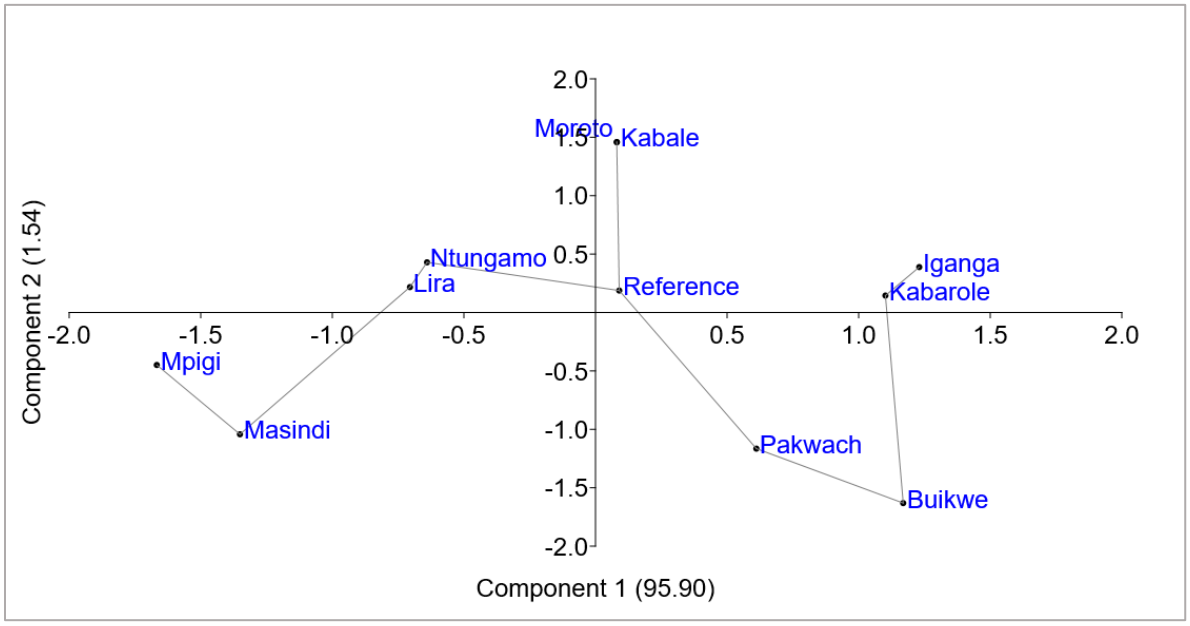


Figure 47: PCA of HPLC fingerprints of M. indica samples collected from different districts: factor scores of observations plotted on the first two components; with minimum spanning tree (MST) showing the closeness of sample fingerprints to the reference fingerprints.

On the other hand, the general fingerprints were largely not similar. Only a few sample pairs had correlation coefficients higher than 0.9 (Figure 48).

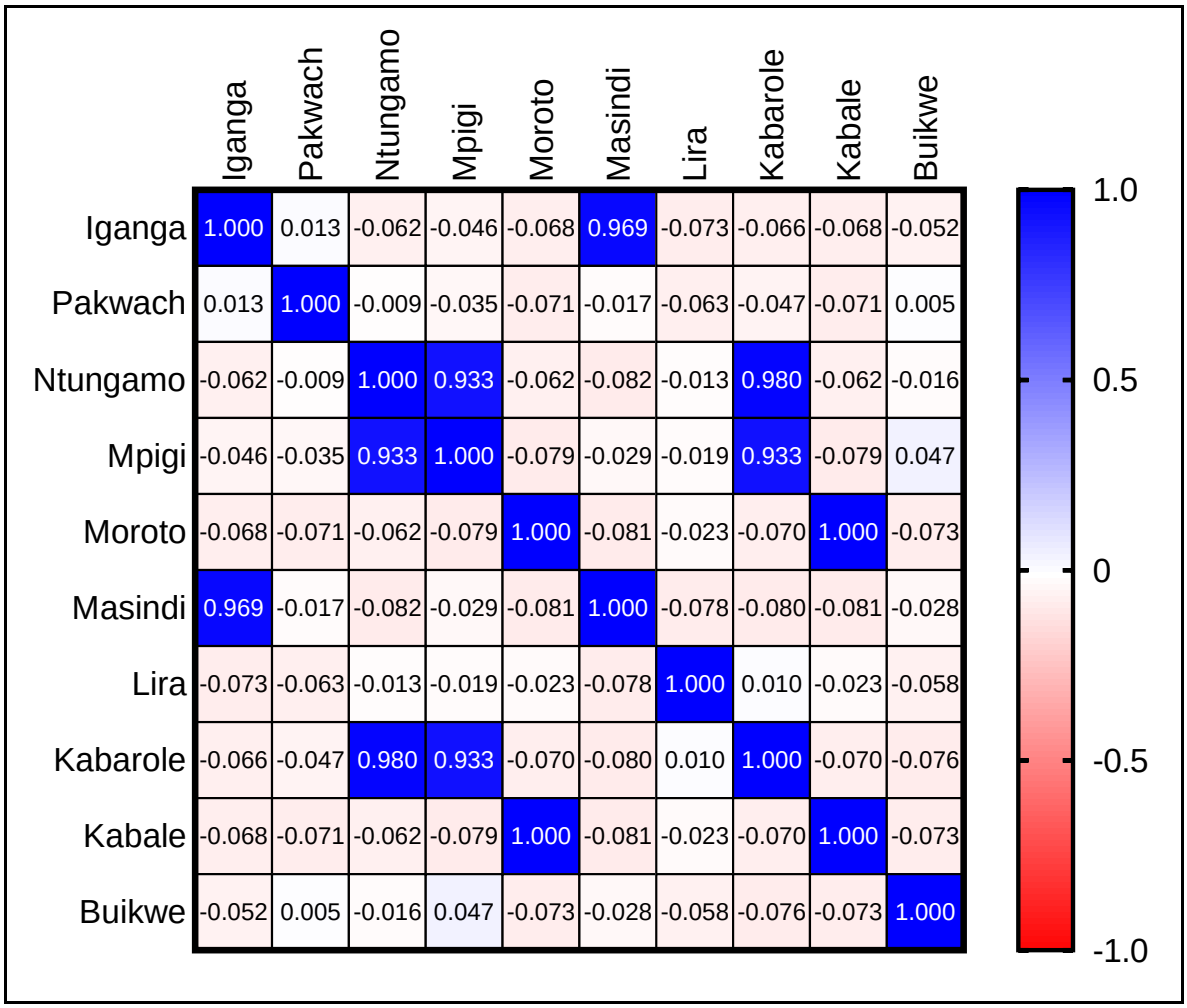


Figure 48: Heat map of the Pearson correlation matrix of M. indica leaf whole fingerprints of samples collected from different districts; the intensity of blue is proportional to the degree of similarity of the fingerprints.

Thus, from Figure 48, the following samples can be considered phytoequivalent; Iganga = Masindi, and Ntungamo = Mpigi = Kabarole. Compared the results of different *M. indica* varieties, there seems to be more variability among the samples due to geographical source of the materials. This is perhaps because the field samples belong to only a few or the same variety. If this were true, then most of the variability would be due to climatic and soil characteristics of the sample source, as seen with *A. coriaria*.

4.2.2.5.0 Concentration of mangiferin in samples harvested from different agroecological zones

The samples were compared to determine the mangiferin concentration, as a way of determining the variation caused by the geographical source. The concentration of mangiferin varied significantly among the samples (Table 24 and Table P7-appendix), with an average of 101.69±27.55 mg/g). Samples from Buikwe had the highest concentration (136.32±0.23 mg/g), while samples from Masindi had the lowest mangiferin concentration (67.98±0.06 mg/g).

Table 24: Concentration of mangiferin in M. indica ethanolic leaf extracts of samples obtained from different agroecological zones of Uganda. Compact letter display-samples that share a letter are not significantly different.

No.	Agroecological zone	Source district	Concentration of mangiferin mg/g of dry extract (n = 3)
1	West Nile	Pakwach	127.09±0.48^{a}
2	Southern drylands	Ntungamo	89.73±1.00^{b}
3	Lake Victoria crescent	Mpigi	65.09±0.18^{c}
4	Karamoja drylands	Moroto	77.88±0.60^{d}
5	Lake Albert crescent	Masindi	67.98±0.06^{e}
6	Mid northern	Lira	88.25±0.04^{f}
7	Western highlands	Kabarole	124.44±0.19^{g}
8	Southern highlands	Kabale	107.77±0.01^{h}
9	Eastern	Iganga	134.31±0.19^{i}
10	South Eastern	Buikwe	136.32±0.23^{j}

Materials with the highest concentration of mangiferin could be preferred for preparing drugs, if there are no resources to develop fingerprints or to determine other parameters as discussed in the previous sections. In addition, high yielding plants for sourcing pure mangiferin should be cultivated in favorable agroecological zones.

4.2.3.0 Conclusions

This study has demonstrated that the *Mangifera indica* leaf materials have relatively similar ethanolic extractive indices, but differ in the total phenolic content and mangiferin

concentration and thus antioxidant activity. Based on these parameters, we graded the raw materials and showed the varieties that can be substituted (those in the same quality grade) for production of medicines for respiratory tract disorders. These parameters and the ratios of their quantities can also be utilized to monitor the consistency in the quality of materials, and the production process. In addition to the quality grades, we also developed TLC and HPLC fingerprints which can be utilized to authenticate the materials by demonstrating phytoequivalence at correlation coefficients greater than 0.9. For standardization purposes, where minimum required mangiferin marker concentrations are set, materials of different concentrations are used to standardize each other i.e., dilution or concentration. This approach is preferred to addition of pure markers (e.g., mangiferin) to dilute samples or addition of inactive substances (excipients) to concentrated materials. Also, to develop a GMP compliant Botanical Identification Method (BIM), both “dilute” and “concentrated” materials are needed. That said, more sampling and testing is necessary to cater for as much variability as possible. There is also need to test materials growing in different agroecological regions of the country to cater for climatic influence and generalize the application of the analytical parameters.

4.3.0 RESULTS AND DISCUSSION FOR *WARBURGIA UGANDENSIS* STEMBARK MATERIALS

Warburgia ugandensis is used for the treatment of respiratory tract disorders (cough, measles symptoms, common colds, sinusitis, rhinitis, asthma, catarrh, bronchial congestion, and hiccups) and gastric ulcers. While many compounds have been elucidated, the most important being the drimane sesquiterpenes bemadienolide, muzigadial, polygodial, warburganal, ugandensolide, muzigadial, none of them is available in pure form for use as markers. For such plants, general chromatographic fingerprints are used for their identification and evaluation of the batch-to-batch phytoequivalence.

4.3.1 Thin layer chromatography fingerprint method

The best TLC fingerprints were obtained using mobile phase system comprising 8,2,1, and1 volumes of n-hexane, ethyl acetate, methanol, dichloromethane. The optimized solvent mixture is represented by point d, in plane D of the solvent optimization prism (Figure 49).

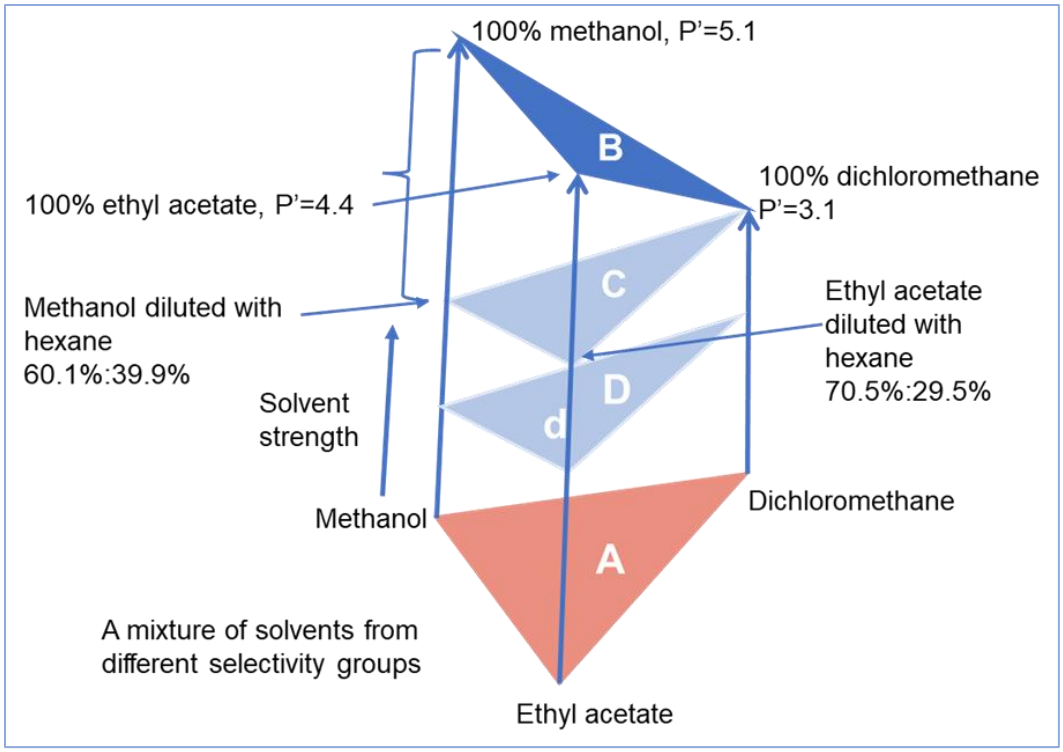


Figure 49: Solvent optimization prism for the mobile phase for TLC fingerprinting of W. coriaria stembark materials. Solvent mixtures in plane B have different polarities and selectivities; those in plane C the same polarity but different selectivities. Plane D shows the optimum solvent polarity-point d is an estimate of the compositions of the solvents.

The polarity P', of the solvent system is 1.42, as calculated from equation 2. The solvents represent groups II, V and VI for methanol, dichloromethane and ethyl acetate. Since hexane is not selective and its P' = 0, it is only used to dilute other solvents, during adjusting of the solvent strength (Ramis-Ramos, García-Álvarez-Coque, and Navarro-Huerta 2017). The low solvent strength demonstrates the low polarity of the constituents. In fact, the most important biologically active compounds in *W. ugandensis* extracts are all sesquiterpenes (Leonard and Viljoen 2015). The wide selectivity of the solvent system improves separation of the components. At a laboratory room temperature of 260C, and humidity of 60%, the plates were developed within 20 minutes.

The derivatization of the TLC plates was achieved by vanillin/sulphuric acid reagent. A typical fingerprint is shown in Figure 50. The derivatization reaction was complete within 15 minutes of heating the plate at 120^0C.

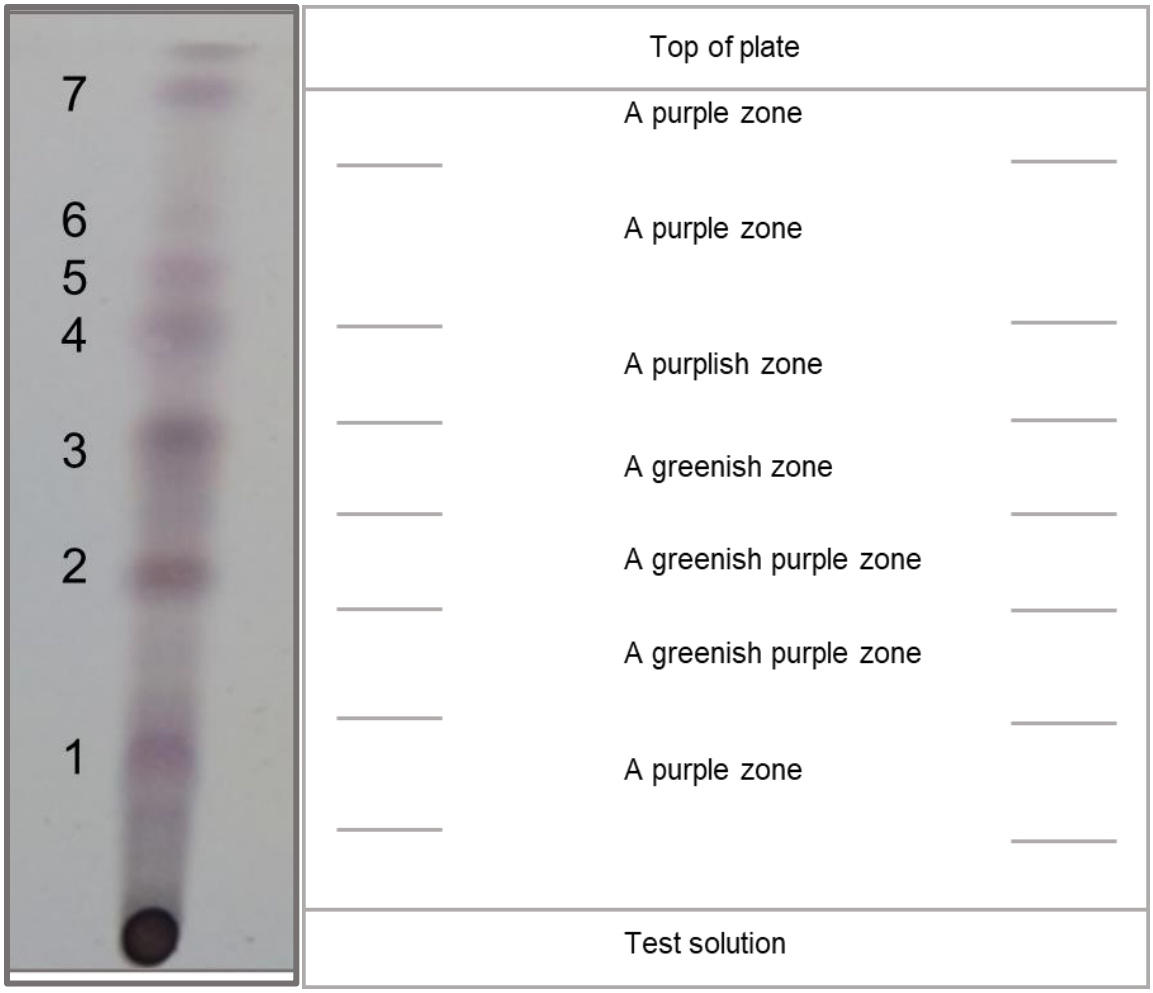


Figure 50: Left: Typical TLC fingerprint for W. ugandensis ethanolic stembark extract: Solvent system; n-hexane, ethyl acetate, dichloromethane, methanol (8:2:1:1). Right: Schematic of the fingerprint, as presented in the Pharmacopoeia.

The images of fingerprints for the derivatized plates are shown in Appendix 18.

4.3.1.2 Stability of the TLC fingerprints

The samples were stable under the conditions of the chromatography as indicated by a two-dimensional TLC (Figure 51);

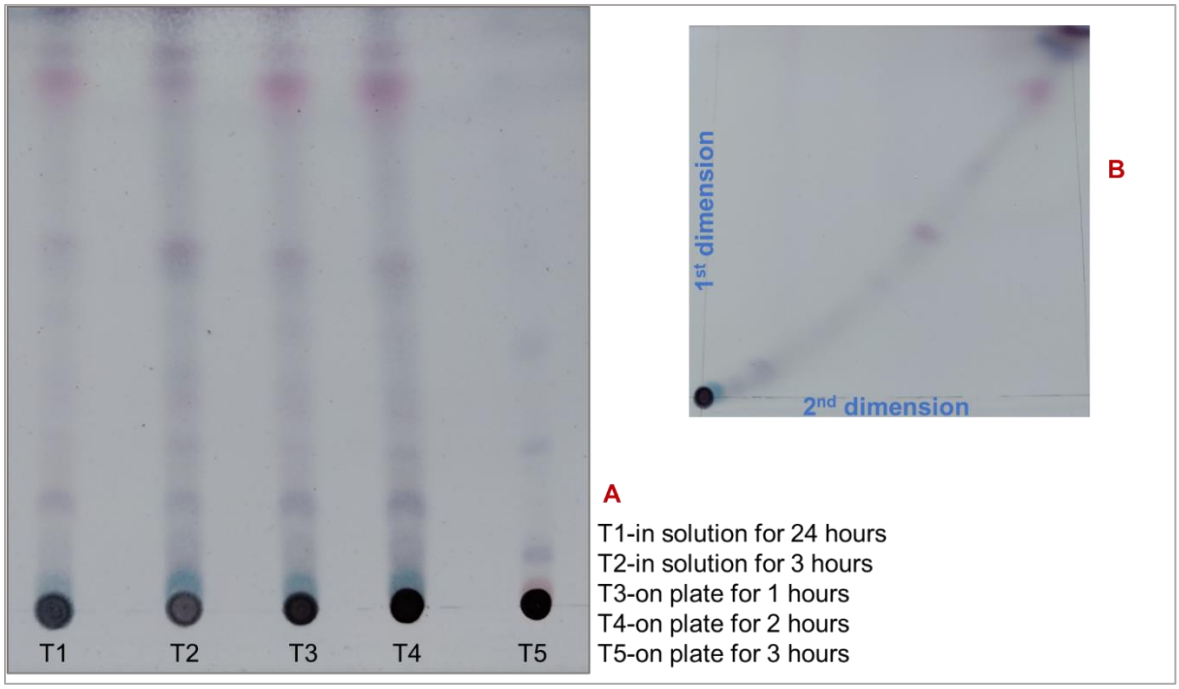


Figure 51: stability of W. ugandensis stembark materials in methanol and on the TLC plate (A): Two-dimensional (2D) TLC showing stability of W. ugandensis stembark raw materials during chromatographic development. Solvent system; n-hexane, ethyl acetate, dichloromethane, methanol (8:2:1:1).

Since all spots lie on the diagonal line joining the sample application point to the intersection of the solvent fronts, the samples are stable under the chromatographic conditions (Reich and Schibli 2007a). Because this line was slightly curvilinear, samples were further evaluated for stability on the plate and in solution as shown in figure 51 A. Only samples that had stayed on the plate for three hours were not stable. This could be due to degradation of sample component after reacting with gases or other chemical contaminants within the laboratory environment (Reich and Schibli 2007a) Thus the samples should be analyzed within two hours to ensure reproducible results.

4.3.1.3 Effect of solvent and extraction method on the quality of the fingerprint

Maceration in ethanol gave a higher number of peaks, which were also more intense than decoction (Figure 52).

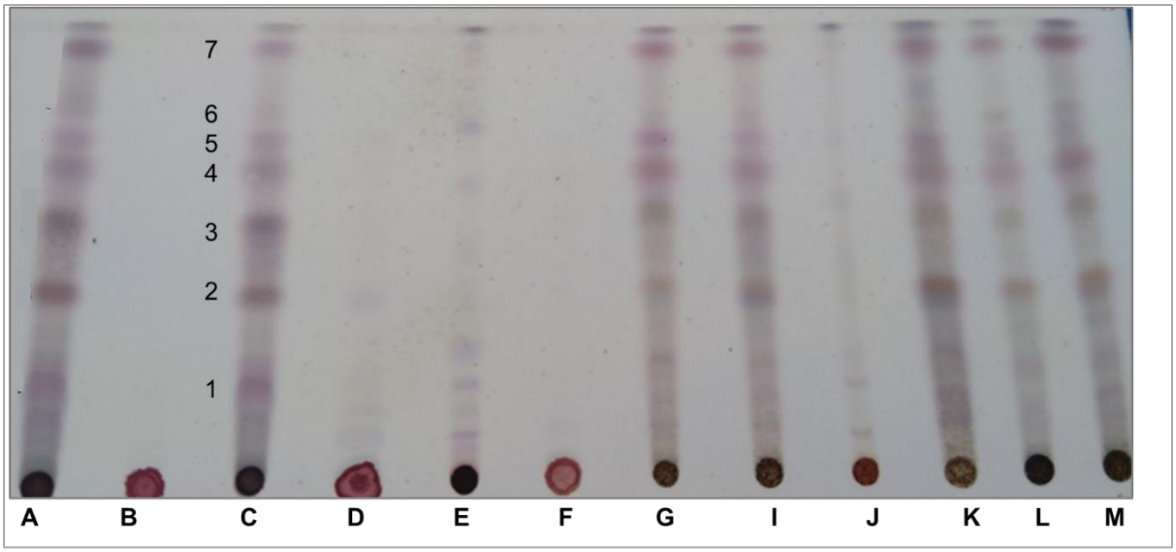


Figure 52: TLC fingerprinting; effect of solvent and method of extraction; decoction also depicts the effect of processing (boiling) on the concentration of components. Tracks A, C, G, I, K, L, M- W. ugandensis samples from Iganga, Ishaka, Kabale, Buikwe, Dokolo, Kabarole and Mpigi prepared by maceration in ethanol; B, D and F-samples prepared by decoction; E and J-Albizia coriaria stembark samples to determine selectivity of fingerprints. Solvent system; n-hexane, ethyl acetate, dichloromethane, methanol (8:2:1:1).

Figure 52 shows that there were very few peaks for samples extracted by decoction, implying this extraction method is not suitable for quality control of processed materials, as used traditionally. This situation was also observed with *A. coriaria*. Perhaps a more polar mobile phase solvent system, as in a reverse phase TLC system is more suited for evaluation of such samples. Nevertheless, the medicinally reported compounds in both plants are non-polar to moderately polar and so the extractions should be performed using solvents of moderate polarity such as ethyl acetate, ethanol, among others (Byamukama et al. 2015; Kioy, Gray, and Waterman 1990). As seen in figure 52, the ethanol extracts give fingerprints with more spots, which are also more intense. Ethanol has a hydroxyl group that interacts with polar functional groups and an ethyl group that interacts with nonpolar functional groups; these properties give it a high extraction power (Handa 2008).

4.3.1.4 Evaluation of phytoequivalence of *W. ugandensis* stembark materials harvested from different agroecological zones

Similarity of TLC fingerprints

Up to 13 peaks were identified (Figure 53), by comparing their relative RFs and their colors after derivatization (Table 25). The concentrations variations of the chemicals (size of peaks) are also evident from the fingerprint.

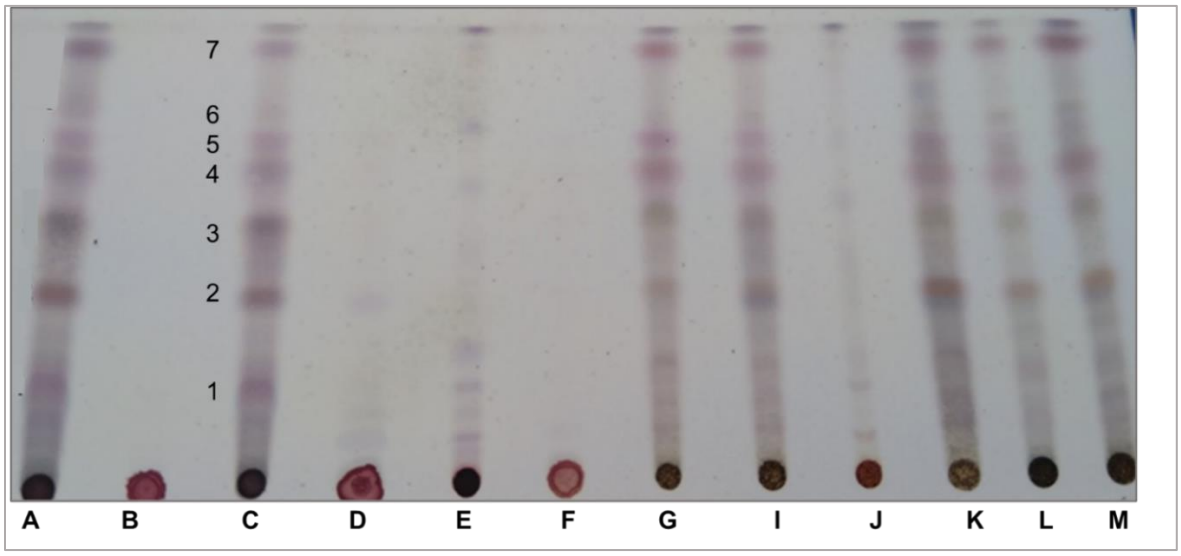


Figure 53: Comparison of W. ugandensis stembark materials obtained from different agro-ecological zones (representative districts included here). Tracks A, C, G, I, K, L, M- W. ugandensis samples from Iganga, Ishaka, Kabale, Buikwe, Dokolo, Kabarole and Mpigi prepared by maceration in ethanol; B, D and F-samples prepared by decoction; E and J-Albizia coriaria stembark samples to determine selectivity of fingerprints. The peaks labelled 1-7 are the strong or principal peaks chosen. Solvent system; n-hexane, ethyl acetate, dichloromethane, methanol (8:2:1:1).

Table 25: Summary of peak properties for W. ugandensis TLC fingerprints.

Peak no.	R_F	Visualization			Identity of compound	HRF RF*100
		366nm	254nm	Daylight after derivatization		
1	0.97	Bluish green	Grey black	Dark green	Unknown	97
2	0.96	Bluish green	Grey black	Green	Unknown	95
3	0.93	-	Pink	Grey	Unknown	94
4	0.84	-	Pink	Grey	Unknown	89
5	0.78	-	-	Grey	Unknown	80
6	0.71	-	Pink	Dark grey	Unknown	73

7	0.67	-	Pink	Dark grey	Unknown	67
8	0.63	-	-	Grey	Unknown	60
9	0.60	-	Pink	Dark grey	Unknown	55
10	0.54	-	-	Grey	Unknown	51
11	0.53	-	Pink	Purple	Unknown	41
12	0.51	Bluish green	Greyish black	Yellowish green	Mangiferin	40
13	0.47	-	-	Black	Unknown	12

R_F-Retardation Factor.

The observed peaks were present in all the samples (Table 26)

Table 26: Distribution of peaks in fingerprints of W. ugandensis stembark samples from different districts

Peak no.	Distribution of peaks					
	Ishaka	Iganga	Dokolo	Kabarole	Buikwe	Mpigi
1	1	1	1	1	1	1
2	1	1	1	1	1	1
3	1	1	1	1	1	1
4	1	1	1	1	1	1
5	1	1	1	1	1	1
6	1	1	1	1	1	1
7	1	1	1	1	1	1

Consequently the Jaccard were all equal to 1(Table 27); implying high similarity among the samples (Cai, Ong, and Liu 2012) .

Table 27: The Jaccard indices among fingerprints of W. ugandensis stembark samples from different districts.

Samples	Jaccard index				
Ishaka	Ishaka	Iganga	Dokolo	Kabarole	Buikwe
Iganga	1,0				
Dokolo	1,0	1,0			
Kabarole	1,0	1,0	1,0		
Buikwe	1,0	1,0	1,0	1,0	
Mpigi	1,0	1,0	1,0	1,0	1,0

Spots labelled 1-7 were the most intense, and so were selected as the principal peaks of the fingerprints. Such peaks are easily reproducible (Cai, Ong, and Liu 2012). The fingerprints of all samples were similar as shown by the jaccard index scores of 1. Thus based on these results alone, the samples can be considered phytoequivalent.

4.3.2.0 HPLC fingerprint methods

4.3.2.1 Optimization of the method

The best solvent system consisted of Methanol (70%) and water (30%), with the highest information content among the solvent systems tried. Elution was done isocratically, at a velocity of 1.0mL/minute, while the optimum temperature was 30^0C. Methanol preferred to acetonitrile since it widely interacts with different sample components, thus better selectivity. This is because it has both acidic and basic properties due to the hydroxyl group. This property also enables methanol to dissolve a wider range of compounds than acetonitrile (Ramis-Ramos, García-Álvarez-Coque, and Navarro-Huerta 2017). Furthermore, the analyte retention on reverse phase columns varies linearly with the concentration of methanol and non-linearly with acetonitrile. So, it is easier to predict the effect on component resolution, of changing the solvent composition, with methanol based mobile phases (Snyder 1978). However, methanol is quite viscous and so requires high operation pressures and long analysis time. Thus the column was heated slightly from 25^0C to 30^0C to lower the viscosity of methanol, thereby allowing relatively faster flow rates, without degradation of the sample components (Kazakevich and Lobrutto 2007).

4.3.2.2 Validation of the HPLC fingerprints

The repeatability of the fingerprints was acceptable, since similarity indices of the six runs of the test samples (Dokolo 1) were approximately 0.9 (Cai, Ong, and Liu 2012). Similarly, the extracts were stable in methanol over three days under laboratory conditions (Table 28),

Table 28: Validation of the HPLC fingerprinting method for W. ugandensis stembark materials

Repeatability validation for six runs-similarity index-correlation coefficients (r)						
	run 1	*run 2*	*run 3*	*run 4*	*run 5*	*run 6*
run 1	1,000					
run 2	0,984	1,000				
run 3	0,993	0,979	1,000			

run 4	0,984	1,000	0,979	1,000		
run 5	0,984	1,000	0,979	1,000	1,000	
run 6	0,993	0,979	1,000	0,979	0,979	1,000
Stability of samples analysed on three different days-similarity index-r						
	Day 1	*Day 2*	*Day 3*			
Day 1	1,000					
Day 2	0,999	1,000				
Day 3	0,926	0,925	1,000			

4.3.2.3 Fingerprint characteristics

The representative HPLC fingerprint is shown in Table 28; while the characteristics of the general fingerprints are summarized in Appendix 19.

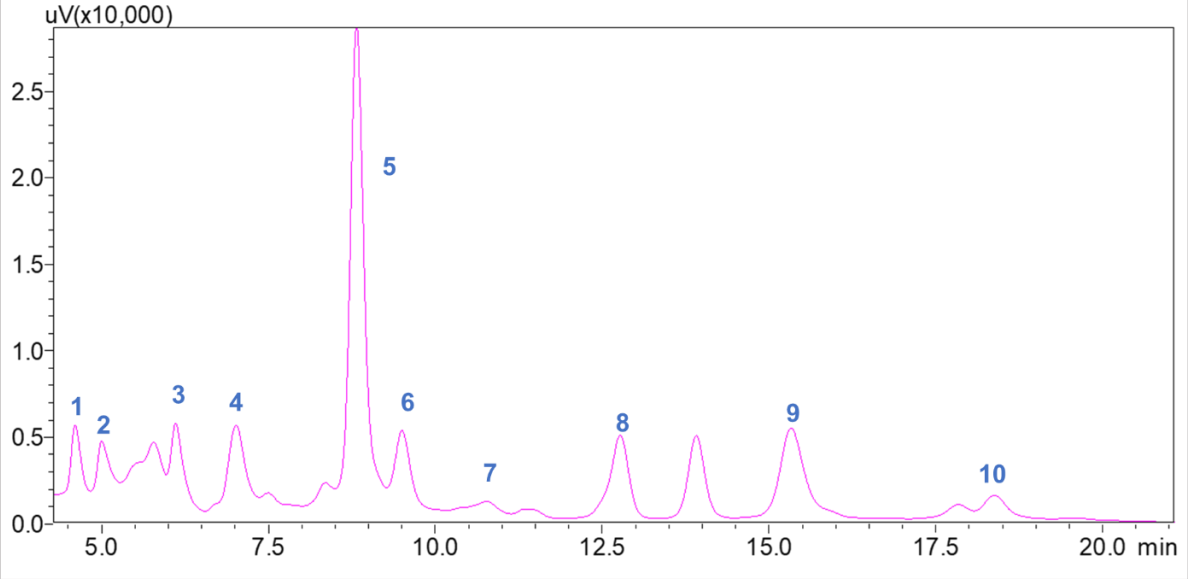


Figure 54: Characteristic HPLC fingerprint of W. ugandensis ethanol stembark extracts; the peaks numbered are those common to all samples.

Pattern recognition

The similarity analysis of HPLC fingerprints assumes that 'similar peaks' (the same retention time and peak area) belong to the same compounds. Therefore, one can compare the chemical composition of different samples without necessarily identifying the compounds (Xie et al., 2006). HPLC fingerprints were developed for sixteen samples obtained from six different

agroecological regions. Within the same agroecological zone district, samples were collected from different plots (villages) to determine if they materials similar. Although the different sample fingerprints showed a similar pattern on visual inspection (Figure 55), the number of peaks varied greatly, ranging from 28 (Iganga 1) to 48 (Iganga 2), with an average of 39±8 peaks.

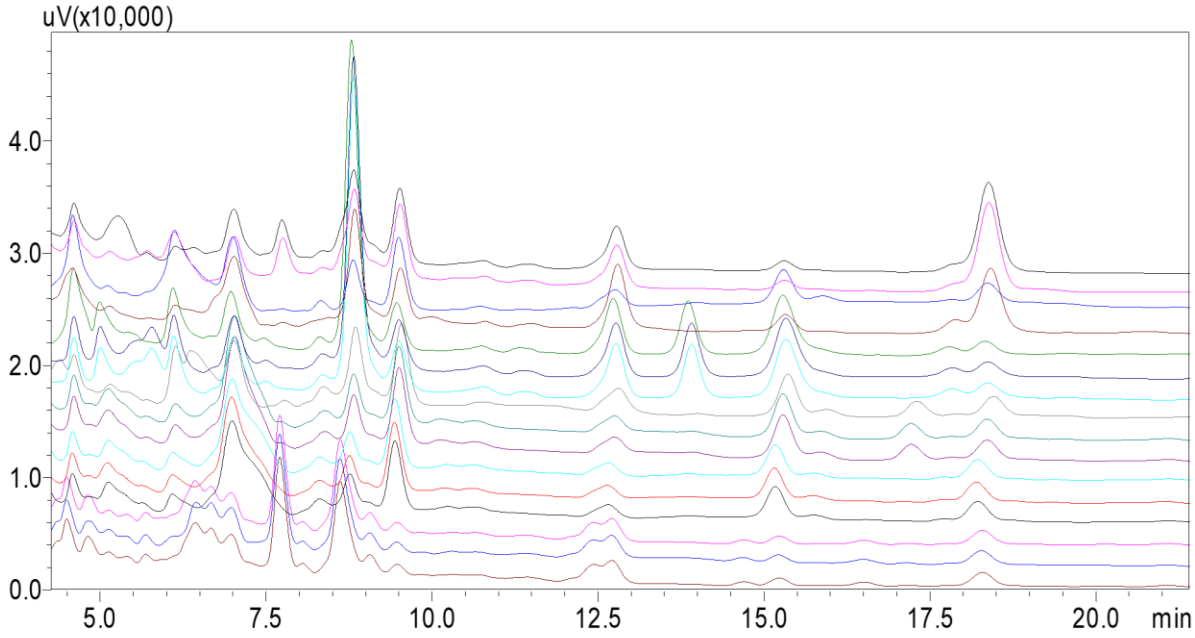


Figure 55: Overly of W. ugandensis stembark HPLC fingerprints of samples obtained from different agroecological zones.

These results show that the phytochemical concentration varies significantly with the source of the material. The missing peaks mean that the concentrations of the responsible compounds are too low to be detected such samples, although the compounds might be missing if the geographical factors have a great impact on the secondary metabolism of the plant species; this can only be proved by genetic studies (Iannucci, Suriano, and Codianni 2021).

Similarity indices of general fingerprints and characteristic fingerprints

Since there are no botanical reference materials for W. *ugandensis*, sample fingerprints were compared to a median ("reference") fingerprint, generated by calculating the medians of the sample peak areas at the respective retention times. General fingerprints were generated from whole chromatograms while characteristic fingerprints were constructed from 10 common peaks (retention time±0.1min), after aligning the chromatograms with the HPLC software. Generally, the similarity indices for the whole general fingerprints were lower than those calculated for only common peaks (Table 29 and

Table *30*).

Table 29: Similarity indices for comparison of general fingerprints to the median fingerprint for W. ugandensis stembark ethanolic extracts.

Source of samples	r	c	d
Iganga 2	0,683	0.821	13.62
Dokolo 1	0,500	0.747	19.44
Dokolo 2	0,394	0.783	20.00
Iganga 1	0,441	0.677	37.05
Ishaka 2	0,448	0.719	18.48
Ishaka 3	0,178	0.638	22.09
Kabale 3	0,538	0.692	20.38
Ishaka 1	0,445	0.772	18.10
Kabale 1	0,613	0.769	20.22
Mpigi 1	0,395	0.692	18.38
Mpigi 2	0,767	0.885	10.74
Mpigi 3	0,787	0.891	9.790
Kabarole 1	0,673	0.849	14.72
Kabarole 2	0,703	0.824	13.99
Kabarole 3	0,620	0.781	15.68
Kabale 2	0,515	0.715	23.67

However, even for the characteristic fingerprints, none of them had r equal to or higher than 0.9 and only a few had c above 0.9, namely; Iganga 1, Iganga 2, Ishaka 1, Kabale 3, and Kabarole 1. Nonetheless, most of the characteristic fingerprints had c close to 0.8, and small distances (d).

Table 30: Similarity indices for comparison of characteristic fingerprints to the median fingerprint for W. ugandensis stembark ethanolic extracts.

Comparison of sample fingerprints to median fingerprint			
	r	c	d
Iganga 2	0,798	0.956	10.43
Dokolo 1	0,230	0.846	19.95
Dokolo 2	0,050	0.799	23.04
Iganga 1	0,597	0.910	15.27
Ishaka 2	0,780	0.891	18.67
Ishaka 3	0,815	0.912	16.48
Kabale 3	0,812	0.983	6.09
Ishaka 1	-0,200	0.811	21.31
Kabale 1	0,373	0.883	17.17
Mpigi 1	0,557	0.895	16.75
Mpigi 2	0,360	0.872	18.08
Mpigi 3	0,336	0.845	20.50
Kabarole 1	0,505	0.904	15.57
Kabarole 2	0,345	0.845	20.48
Kabarole 3	0,331	0.850	19.96
Kabale 2	0,479	0.866	19.28

4.3.3.0 Similarity of samples collected from different agroecological zones

Evaluation based on general fingerprints

Unlike *M. indica* for which some samples were very similar (r > 0.9), correlation of general fingerprints of *W. ugandensis* samples indicates that the samples were not similar, even for those collected from different plots within the same district (Figure 56).

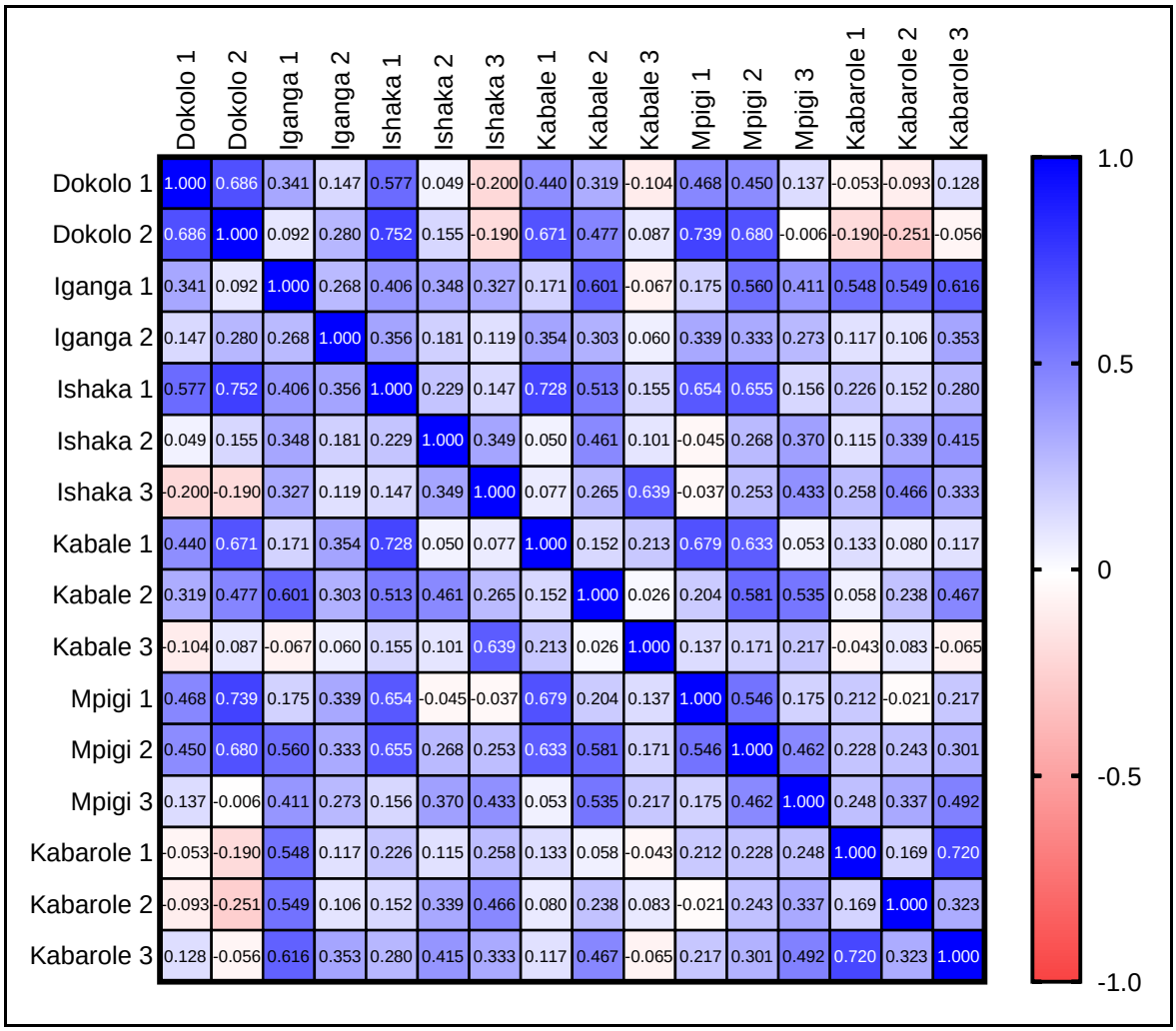


Figure 56: Heat map of the Pearson correlation matrix of W. ugandensis stembark HPLC general fingerprints; the intensity of blue is proportional to the degree of similarity of the fingerprints.

Thus at the phytoequivalence threshold of 0.9, none of the samples can be considered phytoequivalent. The marked could be arise due to climatic and soil differences within the sampling sites, which affect the metabolism of the plants (American Herbal Products Association 2017; Omara, Kiprop, and Kosgei 2021). Alternatively, there could be several chemotypes of *W. ugandensis* growing in the different agroecological zones (Sidhu and Behl 1996). Since the peaks were matched without guidance of a marker compound, the errors arising due to matching the wrong peaks could reduce the similarity of peaks (Goodarzi, Russell, and Vander Heyden 2013; Cai, Ong, and Liu 2012).

Evaluation of samples based on characteristic (common) fingerprints

There were 10 peaks common to all fingerprints that had relatively high areas. Peak 5, being the strongest was used as the reference peak for retention time shift adjustment, to enable peak matching. the average retention time shift was 0.1 minutes. The relative areas of other peaks relative that of peak 5 are shown in Appendix 20. A reference peak was chosen because no reference marker compound was available. While the use of other characteristic markers such as betulinic acid and 1,8-cineole is recommended (World Health Organization 2016), this was not possible because these compounds lacked the specific physical chemical characteristics requisite for the method. For instance, betulinic acid has a poor UV absorption spectrum, with a λmax of 205nm, yet a general fingerprint should be developed at 254nm and above, to cater for more compounds. On the other hand, cineole, besides its poor absorption of electromagnetic energy, it is only slightly soluble in methanol, the solvent used to dissolve the samples and also a component of the mobile phase. Thus these compounds were not ideal standards. In such a scenario, it is advisable to identify the strongest peak, among the common peaks and use it as the reference peak (Cai, Ong, and Liu 2012).

Hierarchical cluster analysis of samples based on characteristic fingerprints

Classical cluster analysis of the characteristic peaks gave four major clusters (Figure 57).

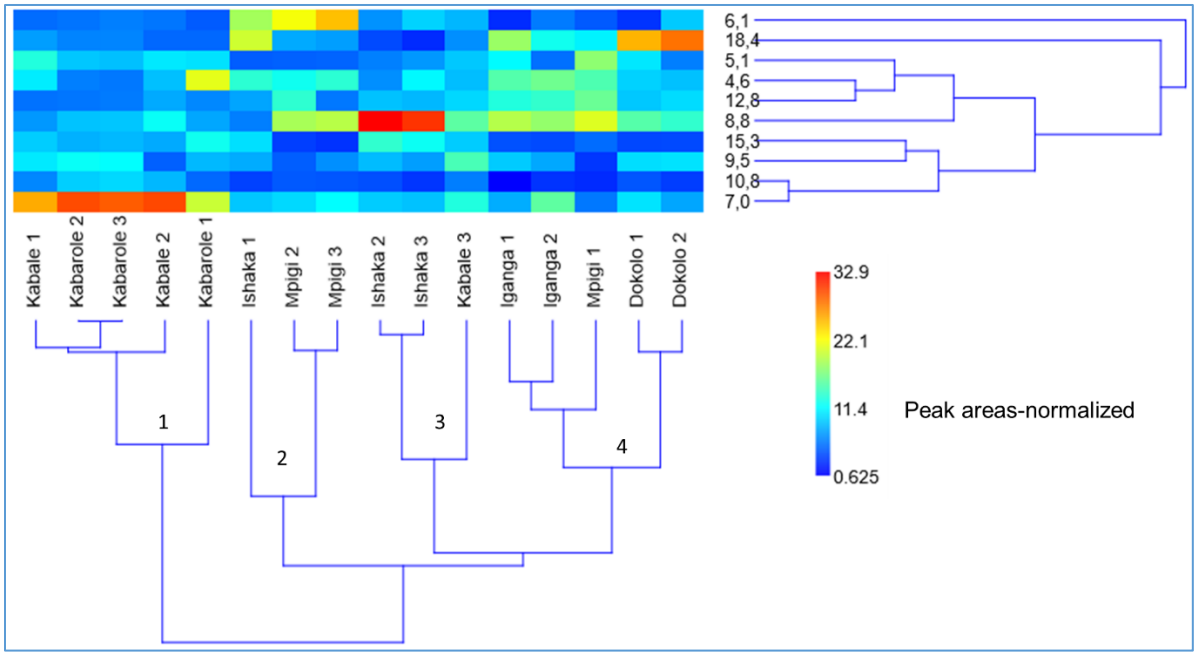


Figure 57: Two-way dendrogram for the classical hierarchical cluster analysis of W. ugandensis stembark samples. Algorithm: Ward's method; similarity index, Euclidean distance.

Cluster 1(Kabale 1, Kabale 2 and Kabarole 2 and Kabarole 3), cluster 2 (Ishaka 1, Mpigi 2, Mpigi 3), cluster 3 (Ishaka 2 and Ishaka 3, Kabale 3), cluster 4 (Dokolo 1, Dokolo 2, Iganga 1, Iganga 2, Mpigi 1 and Kabarole 1). These groups represent variability in the quantities of phytochemicals responsible for the common peaks.

From Figure 57, it is evident that Peaks at 7.0, 8.8, and 18.4minutes cause the highest variability among the samples (clearer in the loading plots below). These correspond to peaks no. 4, 5 and 10 (Figure 54). Their areas relative to the total peak area were 7.7%, 7.9% and 5.7% respectively. In addition, samples from plots within the same district generally cluster together, which is a good indication of overall similarity. However, at a threshold of r = 0.9, only a few samples can be considered identical (Figure 58).

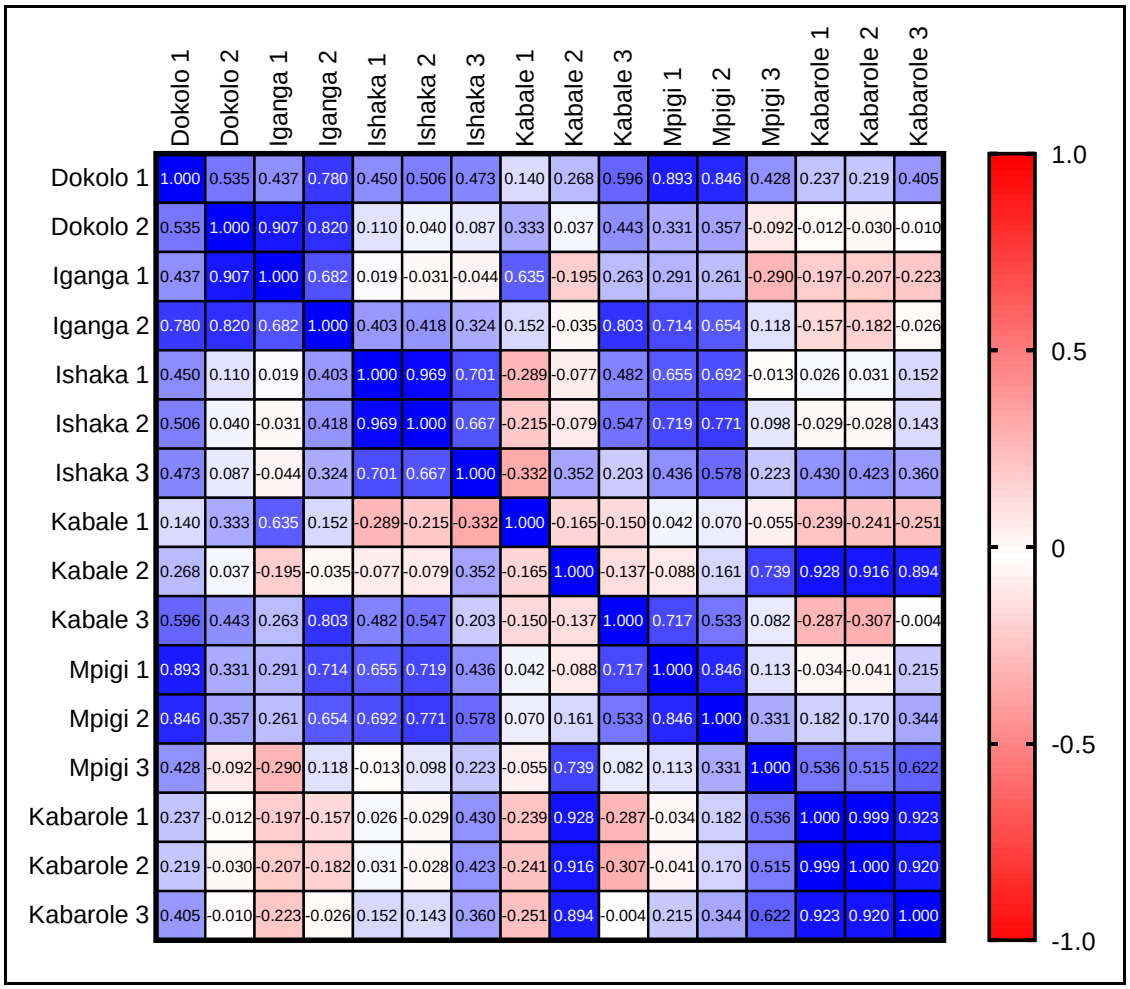


Figure 58: Heat map of the Pearson correlation matrix of W. ugandensis stembark HPLC common peak fingerprints; the intensity of blue is proportional to the degree of similarity of the fingerprints.

Thus only Dokolo 1 = Dokolo 2, Ishaka 2 = Ishaka 3, Iganga 2 = Mpigi 2 and Kabale 1 = Kabale 2 = Kabarole 2 =Kabarole 3. Nevertheless, several samples had correlations between 0.70 and 0.89, such that if the threshold is lowered to r = 0.7, then Iganga 1 = Iganga 2 = Dokolo 1 = Dokolo 2 = Mpigi 1 = Mpigi 2 = Mpigi 3, Ishaka 1 = Dokolo 1 = Dokolo 2, Ishaka 2 = Ishaka 3 = Kabale 3 = Mpigi 2 = Mpigi 3, Kabale 1 = Kabarole 1. Of course, such adjustment requires more studies to the reliability of such results (Cai, Ong, and Liu 2012).

Principal Component Analysis (PCA) of samples based on characteristic fingerprints

PCA yielded 10 principal components, with the first two contributing more than 68.3% of the total variance. From the scatter plot, the minimum spanning tree distance confirmed that samples from Kabale 3, Kabarole 1, Mpigi 1 and Iganga 1 were closest to the reference (Figure 59).

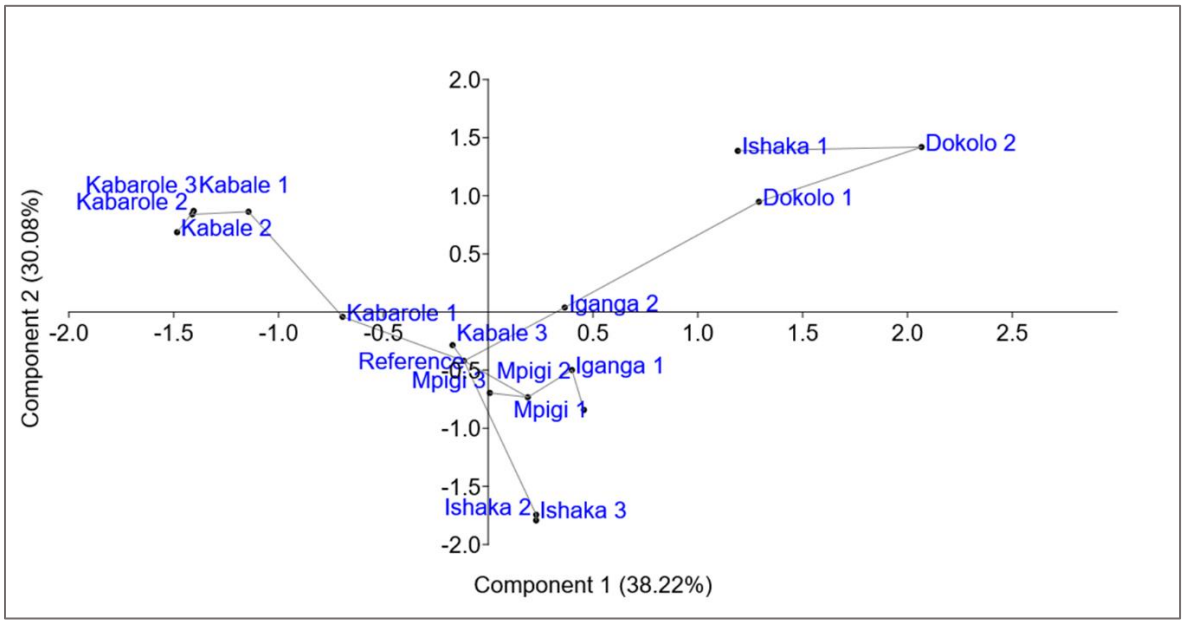


Figure 59: PCA of HPLC fingerprints of W. ugandensis samples collected from different districts: factor scores of observations plotted on the first two components; with minimum spanning tree (MST) showing the closeness of sample fingerprints to the reference.

The figure shows that samples are far from each other, indicating great variability.

4.3.4.0 Important peaks to consider for the reference fingerprints.

PC1 was contributed to by peaks at 5.1, 7.0, 9.5, 10.8, and 15.3 minutes (Figure 60)

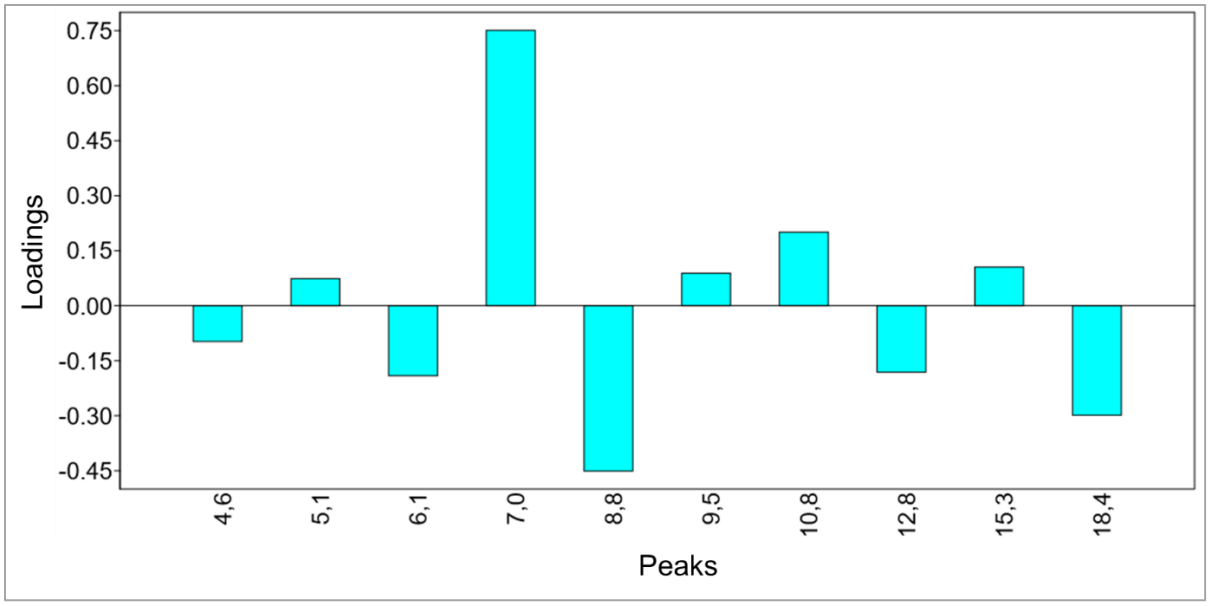


Figure 60: Loading plots W. ugandensis stembark HPLC fingerprints showing the contribution of the peaks to the variance of PC1 (38.22%)

On the other hand, the peaks that contributed to PC2 are those at 6.1, 7.0, 8.8, 10.8 and 15.3 minutes (Figure 61)

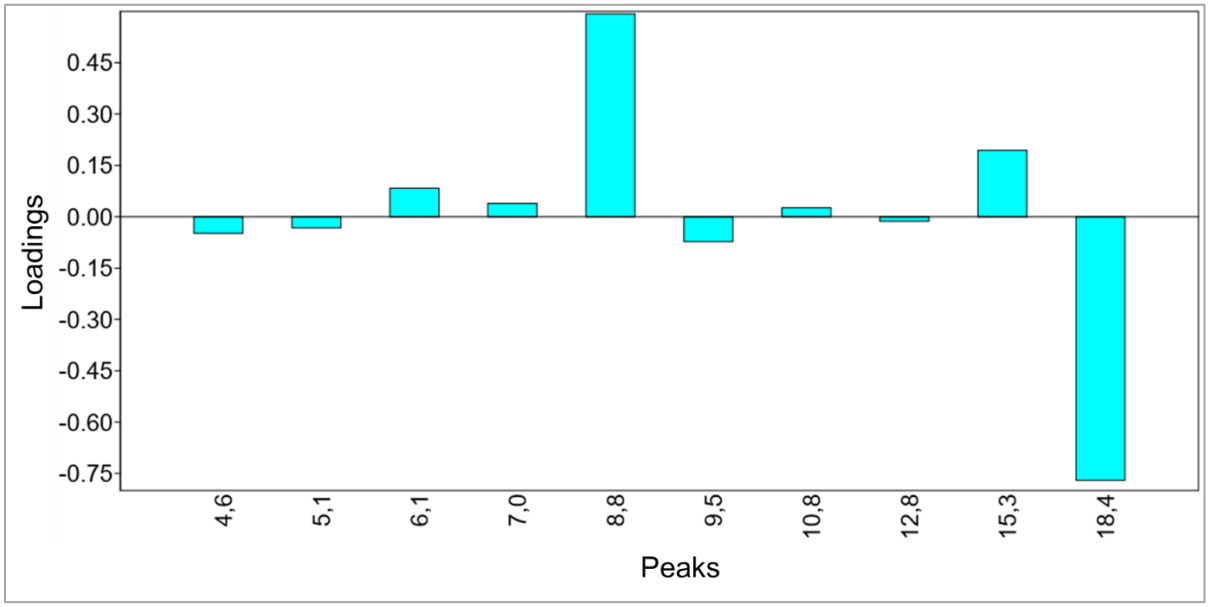


Figure 61: Loading plots W. ugandensis stembark HPLC fingerprints showing the contribution of the peaks to the variance of PC2 (30.08%)

Thus the following peaks should constitute the reference fingerprints: peak 2 , 3, 4, 5, 6, 7, and 9, with retention time 5.1, 6.1, 7.0, 8.8, 9.5, 10.8 and 15.3 minutes.

4.3.4.0 Conclusions

The developed fingerprints can be used to evaluate the quality of *W. ugandensis* raw materials. However more sampling is required to further study the reasons for the marked variability phytochemical variability.

CHAPTER FIVE: GENERAL DISCUSSION

The quality assurance of herbal materials is challenging because of the wide variation in phytochemical composition. This variation occurs, majorly, naturally due to genetic difference and also because of the different climatic and soil characteristics of the plant cultivation sites. Compound variation can also be introduced by agronomic practices, as well as post-harvest handling and processing of the plant materials (American Herbal Products Association 2003). Whatever the source of variation, standardization of plants drugs is complicated by the multicomponent nature; which makes it both expensive and impractical to analyze all the compounds in a sample. To solve this challenge, the use of markers and fingerprinting have come in handy. This work employed the Herb Marker ranking system (Bensoussan et al. 2015) to establish quality control markers for herbal materials used in Uganda. This approach was applied successfully to *E. globulus*, *A. barbadensis*, *M .indica, A. indica, Z. officinalis*, and *A. coriaria*. For these plants, there is significant literature available for their bioactive compounds; such that the mechanism of action of the constituents are well elaborated. In addition, these materials are used world over and so pure compounds, which can be used as analytical standards, are widely available. Besides, several analytical techniques have been developed. However, the HerbMaRs is not applicable to plant materials like *W. ugandensis* whose literature is limited. For such plants, it is hard to get information about the activity and/or mechanism of action of the compounds. Also, their analytical standards are not readily available. While the WHO recommends the use of characteristic compounds in absence of biomarkers (World Health Organization 2016), these may not be ideal internal standards if they lack the required analytical physical and chemical properties. For such plants, general fingerprinting becomes the most viable option. Another challenge observed is that even for plants whose analytical standards exist, they are quite expensive and are not available locally. An order for supply of chemicals will take at least 2 months to be fulfilled. For plant samples

in which the markers were quantified (*A. coriaria* and *M. indica*), there was marked concentration variation among the different sources of the materials. Besides different soil and climatic factors, these changes can also be due slight differences in the harvesting conditions. These were minimized by harvesting the plant materials in the same dry season (January to December), and the same time period of the day (between 9;00Am and 12:00pm). Noteworthy, is the fact that the materials were growing wildly, especially for *W. ugandensis* and *A. coriaria*; for such plants, the growing conditions and thus phyto-composition, are only influenced by nature; although human activity, such as deforestation and pastoralism can influence the environmental conditions around the plant (Gafna et al. 2017).

The fingerprinting methods developed by this work included thin layer (TLC) and high-performance liquid chromatography (HPLC). TLC is quite affordable and the results are easy to interpret. Thus, the method is easily applicable to industry in countries lacking quality control methods like Uganda. While the reproducibility of TLC-based analytical methods for pure compounds is low, that of fingerprints is acceptable since it relies on the whole sample; such that the number of spots, their color and shapes are relatively easy to reproduce (Geiss 1987; Ettre 2002). By comparing the fingerprint images and calculating the jaccard indices of the bands, it was demonstrated that samples from different agroecological zones are phytoequivalent. Perhaps, due to the low sensitivity of TLC, small differences in the samples could not be detected. One wonders whether this is a good or bad property, especially when the effect of such variations on the efficacy of materials is negligible or not known. In addition to assessment of phytoequivalence of plant materials, it was possible to study the effect of solvents and extraction methods on the quality of the fingerprints. Thus TLC can be an affordable technique in evaluating extraction methods. In all cases, decoction produced fingerprints with fewer compounds, and with less intense colors, than either maceration or soxhlet extraction in organic solvents. While this shows that decoction extracts less

components, it could also be due to the fact that the normal phase system is less suited to evaluation of very polar components. Probably, a reverse phase system would produce better results for such samples.

HPLC fingerprints yielded more peaks than TLC because of increased sensitivity and number of theoretical plates. Consequently, HPLC is able to detect and quantify minor differences among samples of different batches (Wagner et al. 2011). The effect of such differences on the efficacy of the plant materials should evaluated, to avoid rejection of rather good materials. For *M. indica* leaf materials, there was no correlation between the HPLC fingerprints and the antioxidant activity. To study the phytoequivalence of the plant, various chemometric methods were used; similarity indices, principal component analysis and hierarchical cluster analysis (Cai, Ong, and Liu 2012). In all cases, general fingerprints were less similar than fingerprints based on common peaks. Even then, some samples of *A. coriaria* and *M. indica* were phytoequivalent when evaluated with at a threshold of r = 0.9. With common peak fingerprints, all samples for *M. indica* and most of *A. coriaria* were phytoequivalent. Only a few samples of *W. ugandensis* were phytoequivalent. The results for *M. indica* can be explained by the fact that most of the samples were picked from moderately controlled environments (general gardens or specific plantations). In fact, even samples collected from different cultivars were phytoequivalent. The marked variation in *W. ugandensis* samples could be due to unknown genetic variation, since even samples collected from different plots but in the same district varied greatly. Nevertheless, the effect of environmental and physiological conditions and small differences in harvesting activities cannot be overlooked. Another factor, is that while peak marking for *M. indica* and *A. coriaria* were based on observed variation in retention times of actual reference markers, that of *W. ugandensis* was based on the reference peak, whose purity in the several samples could not be proved.

In assessing similarity and therefore phytoequivalence of plant materials, the threshold is at a congruence coefficient (c) or correlation coefficient (r) of 0.9. This seems to be too high for materials that are collected from uncontrolled environments. This threshold was set by countries, such as China, in which plants are grown in botanical gardens by licensed growers. In such countries, good agricultural and harvesting practices are well streamlined and are mandatory. That said, there are no thresholds set for similarity of plant materials based on general fingerprints. A general observation from this study is that values of c are always higher than those of r. One wonders which one to consider in case one index fails and the other passes the sample. The Chinese Pharmacopoeia recommends the use of two fingerprint evaluation softwares; (i) the Fingerprint Analysis System of Chinese Medicine and (ii) the Computer-assisted Similarity Evaluation System; the former system uses the correlation coefficient while the later uses the congruence coefficient.

Limitations of the study

The HPLC system used in this study is equipped with a single mode UV detector, as such only components that absorb electromagnetic radiation at the selected wavelength are detected. This results in a lower number of peaks in the fingerprint, than would be produced by either a diode array or mass spectrum detector (Kazakevich and Lobrutto 2007). Furthermore, the samples were eluted by the isocratic rather than gradient method, which reduced the number of peaks further. Nevertheless, this work capitalized on generating fingerprints with a few peaks (up to 10), that are easily reproducible. Afterall, most studies employing DAD detectors and gradient elution report between 10 to 15 common peaks (Viapiana et al. 2016; Cai, Ong, and Liu 2012). It is also a fact that although gradient elution affords more separation of components, the fingerprints are more variable due to low reproducibility of the solvent concentration gradients (Kazakevich and Lobrutto 2007).

Another limitation of the study was that sampling of plant materials was limited to a single season, due to logistic and time constraints. The development of botanical reference materials (BRMs) requires collection of large numbers of samples in such as way as to maximize the variability. Thus, samples should be collected from various geographical sources, during different climatic seasons and agronomic practices (Noviana, Indrayanto, and Rohman 2022). The data from large samples also gives more valid models of quality control. Due to limited resources, the materials were collected only during the dry season of December to February. The results might not be reproducible to other seasons. Nevertheless, the study established methodologies that can be employed to study the phytochemical variation of plant materials.

CHAPTER SIX: CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

This work established markers that can be used to control the quality of commercially used herbal materials produced in Uganda namely; *E. globulus* leaf oil, *A. barbadensis* leaf and gel, *A. coriaria* stembark, Azadirachta indica leaves, *Zingiber officinalis* rhizome, and *M. indica* stembark and leaves. The markers are as follows: aromadendrine, α-terpineol, globulol, and 1,8,- cineol (for *Eucalyptus globulus* Labill.); aloin, aloe emodin, acemannan (for *Aloe barbadensis* (L.) Burm.f.); lupeol, lupenone, betulinic acid, betulin, and catechin (for *Albizia coriaria* Oliv.); mangiferin, catechin, quercetin, and gallic acid (for *Mangifera indica* L.); azadirachtin, nimbin, nimbidin (for *Azadirachta indica* A.Juss.); and 6, 8, 10-gingerols, 6-shogaol (for *Zingiber officinalis* Roscoe).

In addition, TLC and HPLC qualitative and quantitative methods, coupled with chemometric data analysis models were developed. These can be used to assess the quality of herbal *A. coriaria* stembark, *M. indica* leaves and *W. ugandensis* stembark raw materials at various levels of the supply chain. Using the developed methods, it was demonstrated that while there is marked variation in the concentration of specific marker compounds, *M. indica* leaf materials and *A. coriaria* harvested from different geographical sites are largely phytoequivalent. *W. ugandensis* stembark varied greatly. Possible geographical sources of botanical reference materials were also highlighted: Kabarole, Pakwach and Ntungamo (*M. indica* leaves); Kabarole, Moroto, Mpigi (*A. coriaria* stembark); Kabarole, Kabale, Mpigi (*W. ugandensis* stembark).

6.2 RECOMMENDATIONS

Using the HerbMaRS, markers should be established for all the 33 herbal materials used in manufacturing commercial herbal products in Uganda. These should be quantified and concentration limits set based on the relevance of the compounds to biological activity. There

is need to build local capacity to isolate and purify phytoconstituents to establish markers for the most important plants, especially those whose markers are not available in the international market, such as for *W. ugandensis*. Further sampling of *W. ugandensis* should be done to establish the source of phytochemical variability. The markers established in this study should be added to the routine quality control of *E. globulus leaf oil, A. barbadensis leaf and gel, A. coriaria stembark, Azadirachta indica leaves, Zingiber officinalis rhizome, and M. indica* stembark and leaves.

In addition, a database of chromatographic fingerprints for *A. coriaria stembark*, *M. indica* leaves and *W. ugandensis* stembark should be developed and a quality control framework established. In future, plant monographs should be compiled.

REFERENCES

Abd El-Wahab, A, H El-Adawi, and M El-Demellawy. 2009. 'In vitro study of the antiviral activity of Zingiber officinale', *Planta Medica*, 75: PF7.

Aeschbach, R, J Löliger, BC Scott, A Murcia, John Butler, B Halliwell, and OI Aruoma. 1994. 'Antioxidant actions of thymol, carvacrol, 6-gingerol, zingerone and hydroxytyrosol', *Food and Chemical Toxicology*, 32: 31-36.

Agency, European Medicines. 1995. 'ICH Topic Q 2 (R1) validation of analytical procedures: text and methodology', *Prescrire Int*, 20: 278.

Ahamed, B, V Krishna, HB Gowdru, H Rajanaika, HM Kumaraswamy, S Rajshekarappa, CJ Dandin, and KM Mahadevan. 2007. 'Isolation of bactericidal constituents from the stem bark extract of Grewia tiliaefolia Vahl', *Res. J. Med. Plant*, 1: 72-82.

Ahmad, S, ET Tamboli, and R Parveen. 2013. 'Determination of metabolic and genetic diversity in Coleus forskohlii Briq. using HPTLC and RAPD analysis', *Planta medica*, 79: P103.

Ahuja, Satinder, and Michael Dong. 2005. *Handbook of pharmaceutical analysis by HPLC* (Elsevier).

Aimbire, F, SC Penna, M Rodrigues, KC Rodrigues, Rodrigo Alvaro Brandão Lopes-Martins, and Jayme Antonio Aboin Sertié. 2007. 'Effect of hydroalcoholic extract of Zingiber officinalis rhizomes on LPS-induced rat airway hyperreactivity and lung inflammation', *Prostaglandins, leukotrienes and essential fatty acids*, 77: 129-38.

Akoachere, JF TK, RN Ndip, EB Chenwi, LM Ndip, TE Njock, and DN Anong. 2002. 'Antibacterial effects of Zingiber Officinale and Garcinia Kola on respiratory tract pathogens', *East African medical journal*, 79: 588-92.

Alaerts, Goedele, Bieke Dejaegher, Johanna Smeyers-Verbeke, and Yvan Vander Heyden. 2010. 'Recent developments in chromatographic fingerprints from herbal products: set-up and data analysis', *Combinatorial chemistry & high throughput screening*, 13: 900-22.

Alaerts, Goedele, Jurgen Van Erps, Sigrid Pieters, Melanie Dumarey, Anne-Marie Van Nederkassel, Mohammad Goodarzi, J Smeyers-Verbeke, and Yvan Vander Heyden. 2012. 'Similarity analyses of chromatographic fingerprints as tools for identification and quality control of green tea', *Journal of Chromatography B*, 910: 61-70.

Alam, Athar, Saikat Haldar, Hirekodathakallu V Thulasiram, Rahul Kumar, Manish Goyal, Mohd Shameel Iqbal, Chinmay Pal, Sumanta Dey, Samik Bindu, and Souvik Sarkar. 2012. 'Novel anti-inflammatory activity of epoxyazadiradione against macrophage migration inhibitory factor: inhibition of tautomerase and proinflammatory activities of macrophage migration inhibitory factor', *Journal of Biological Chemistry*, 287: 24844-61.

Aldoghaim, Fahad S, Gavin R Flematti, and Katherine A Hammer. 2018. 'Antimicrobial activity of several cineole-rich Western Australian Eucalyptus essential oils', *Microorganisms*, 6: 122.

Alok, Prakash, V Keerthana, Jha Chandan Kumar, Kumar Ratan, and Agrawal Dinesh Chand. 2013. 'Antibacterial property of two different varieties of Indian mango (Mangifera indica) kernel extracts at various concentrations against some human pathogenic bacterial strains', *Research Journal of Biological Sciences*, 2: 28-32.

Ambrosio, Carmen MS, Severino M de Alencar, Andrea M Moreno, and Eduardo M Da Gloria. 2018. 'Evaluation of the selective antibacterial activity of Eucalyptus globulus

and Pimenta pseudocaryophyllus essential oils individually and in combination on Enterococcus faecalis and Lactobacillus rhamnosus', *Canadian journal of microbiology*, 64: 844-55.

American Herbal Products Association. 2003. "Standardization of botanical products: White paper." In *Starting Materials of Herbal Origin. London: European Agency for the Evaluation of Medicinal Products, 2002.* London.

———. 2017. 'Good Agricultural and Collection Practices and Good Manufacturing Practices for Botanical Materials', *American Herba l Products Association*.

Amić, Ana, Dejan Milenković, Zoran Marković, Denisa Cagardová, Jaime Rodríguez-Guerra Pedregal, and Jasmina M Dimitrić Marković. 2021. 'Impact of the phenolic O–H vs. C-ring C–H bond cleavage on the antioxidant potency of dihydrokaempferol', *New Journal of Chemistry*, 45: 7977-86.

Amin, ASAL. 2019. 'AL Dulaimi and Marwa AA AL Rawi, Antiviral Activity of Mangifera Extract on Influenza Virus Cultivated in Different Cell Cultures', *J Pure Appl Microbiol*, 13: 455-58.

Amorati, Riccardo, and Luca Valgimigli. 2015. 'Advantages and limitations of common testing methods for antioxidants', *Free radical research*, 49: 633-49.

Andrade, Eberson MJ, Jéssica S Teixeira, Dahara KC Silva, Tatiana B dos Santos, Maria GA Korn, Hugo N Brandão, Caline G Ferraz, Cássio S Meira, Milena BP Soares, and Elisava T Guimarães. 2020. 'Phytochemical analysis, multi-element composition and biological activities of extracts and lupenone from Albizia inundata (Mart.) Barneby & JW Grimes', *Journal of the Brazilian Chemical Society*, 31: 1825-37.

Anywar, G, E Kakudidi, R Byamukama, J Mukonzo, A Schubert, and H Oryem-Origa. 2020. 'Indigenous traditional knowledge of medicinal plants used by herbalists in treating opportunistic infections among people living with HIV/AIDS in Uganda', *Journal of ethnopharmacology*, 246: 112205.

AOAC. 2012. 'Guidelines for Single-laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals, Official Methods of Analysis, AOAC', *INTERNATIONAL, Gaithersburg, MD*.

AOAC, AOAC. 2002. 'Guidelines for single laboratory validation of chemical methods for dietary supplements and botanicals', *Obtenido de https://doi. org/10.1063/1.4915424*.

Apel, Miriam Anders, Rodney AF Rodrigues, Luiz Alberto Lira Soares, and Amélia Terezinha Henriques. 2017. 'Quantification of the components in commercial essential oil of Eucalyptus globulus labill. by gas chromatography–GC-FID and GC-MS', *Drug Analytical Research*, 1: 9-14.

Ara, Iffat, Bina Shaheen Siddiqui, Shaheen Faizi, and Salimuzzaman Siddiqui. 1989. 'Structurally novel diterpenoid constituents from the stem bark of Azadirachta indica (Meliaceae)', *Journal of the Chemical Society, Perkin Transactions 1*: 343-45.

Arosio, Beatrice, Nicoletta Gagliano, Lorena Maria Pia Fusaro, Luciano Parmeggiani, Jacopo Tagliabue, Paola Galetti, Diego De Castri, Claudia Moscheni, and Giorgio Annoni. 2000. 'Aloe-emodin quinone pretreatment reduces acute liver injury induced by carbon tetrachloride', *Pharmacology & toxicology*, 87: 229-33.

Ashraf, Kamran, Mohd Mujeeb, Altaf Ahmad, Sayeed Ahmad, Niyaz Ahmad, and Mohd Amir. 2014. 'Determination of gingerols in ginger by ultra-high performance liquid chromatography-tandem mass spectrometry', *Analytical Letters*, 47: 2120-28.

Bachheti, RK. 2015. 'Chemical composition and antibacterial activity of the essential oil from the leaves of Eucalyptus globulus collected from Haramaya University, Ethiopia', *Der Pharma Chemica*, 7: 209-14.

Badam, Lalita, SP Joshi, and SS Bedekar. 1999. "In vitro'antiviral activity of neem (Azadirachta indica. A. Juss) leaf extract against group B coxsackieviruses', *The Journal of communicable diseases*, 31: 79-90.

Bakr, Shori Amal. 2013. 'Evaluation of acute toxicity of water extract of Azadirachta indica leaves and seeds in rats', *Pakistan journal of biological sciences: PJBS*, 16: 697-700.

Bayne, Holly J. 2002. 'FDA issues final rule banning use of aloe and cascara sagrada in OTC drug products', *HerbalGram*, 56: 56.

Bensoussan, Alan, Samiuela Lee, Christine Murray, Suzannah Bourchier, F Van Der Kooy, Jarryd L Pearson, Juanxun Liu, Dennis Chang, and CS Khoo. 2015. 'Choosing chemical markers for quality assurance of complex herbal medicines: Development and application of the herb MaRS criteria', *Clinical Pharmacology & Therapeutics*, 97: 628-40.

Berardini, Nicolai, Ramona Fezer, Jürgen Conrad, Uwe Beifuss, Reinhold Carle, and Andreas Schieber. 2005. 'Screening of mango (Mangifera indica L.) cultivars for their contents of flavonol O-and xanthone C-glycosides, anthocyanins, and pectin', *Journal of agricultural and food chemistry*, 53: 1563-70.

Bhargava, KP, MB Gupta, GP Gupta, and CR Mitra. 1970. 'Anti-inflammatory activity of saponins and ot-her natural products', *The Indian journal of medical research*, 58: 724-30.

Biswas, Taniya, Argha Sen, Rini Roy, Sushomasri Maji, and Himangshu Sekhar Maji. 2015. 'Isolation of mangiferin from flowering buds of Mangifera indica L and its evaluation of in vitro antibacterial activity', *J Pharm Anal*, 4: 49-56.

Blum, Faith C, Jatinder Singh, and D Scott Merrell. 2019. 'In vitro activity of neem (Azadirachta indica) oil extract against Helicobacter pylori', *Journal of ethnopharmacology*, 232: 236-43.

Boesch-Saadatmandi, Christine, Agnieszka Loboda, Anika E Wagner, Anna Stachurska, Alicja Jozkowicz, Jozef Dulak, Frank Döring, Siegfried Wolffram, and Gerald Rimbach. 2011. 'Effect of quercetin and its metabolites isorhamnetin and quercetin-3-glucuronide on inflammatory gene expression: role of miR-155', *The Journal of nutritional biochemistry*, 22: 293-99.

Botha, CJ, and M-L Penrith. 2008. 'Poisonous plants of veterinary and human importance in southern Africa', *Journal of ethnopharmacology*, 119: 549-58.

Boudreau, Mary D, Greg R Olson, Volodymyr P Tryndyak, Matthew S Bryant, Robert P Felton, and Frederick A Beland. 2017. 'From the cover: aloin, a component of the aloe vera plant leaf, induces pathological changes and modulates the composition of microbiota in the large intestines of f344/N male rats', *Toxicological Sciences*, 158: 302-18.

Brand, C, A Ferrante, RH Prager, TV Riley, CF Carson, JJ Finlay-Jones, and PH Hart. 2001. 'The water-soluble components of the essential oil of Melaleuca alternifolia (tea tree oil) suppress the production of superoxide by human monocytes, but not neutrophils, activated in vitro', *Inflammation Research*, 50: 213-19.

Brochot, Amandine, Angèle Guilbot, Laïla Haddioui, and Christine Roques. 2017. 'Antibacterial, antifungal, and antiviral effects of three essential oil blends', *Microbiologyopen*, 6: e00459.

Byamukama, Robert, Ganza Barbara, Jane Namukobe, Matthias Heydenreich, and Bernard T Kiremire. 2015. 'Bioactive compounds in the stem bark of Albizia coriaria (Welw. ex Oliver)', *International Journal of Biological and Chemical Sciences*, 9: 1013-24.

Byeon, Son Won, Ronald P Pelley, Stephen E Ullrich, Todd A Waller, Corazon D Bucana, and Faith M Strickland. 1998. 'Aloe barbadensis extracts reduce the production of interleukin-10 after exposure to ultraviolet radiation', *Journal of investigative dermatology*, 110: 811-17.

Cai, B., S. P. Ong, and X. Liu. 2012. *High performance liquid chromatography fingerprinting technology of the commonly-used traditional Chinese medicine herbs*.

Cai, Zi-Min, Jian-Qing Peng, Yi Chen, Ling Tao, Yan-Yan Zhang, Ling-Yun Fu, Qing-De Long, and Xiang-Chun Shen. 2021. '1, 8-Cineole: a review of source, biological activities, and application', *Journal of Asian natural products research*, 23: 938-54.

Chakotiya, Ankita Singh, Ankit Tanwar, Alka Narula, and Rakesh Kumar Sharma. 2017. 'Zingiber officinale: Its antibacterial activity on Pseudomonas aeruginosa and mode of action evaluated by flow cytometry', *Microbial pathogenesis*, 107: 254-60.

Chen, Pei, Mustafa Ozcan, and James Harnly. 2007. 'Chromatographic fingerprint analysis for evaluation of Ginkgo biloba products', *Analytical and Bioanalytical Chemistry*, 389: 251-61.

Chiou, Yi-Shiou, Qingrong Huang, Chi-Tang Ho, Ying-Jan Wang, and Min-Hsiung Pan. 2016. 'Directly interact with Keap1 and LPS is involved in the anti-inflammatory mechanisms of (-)-epicatechin-3-gallate in LPS-induced macrophages and endotoxemia', *Free Radical Biology and Medicine*, 94: 1-16.

Ciurlea, Sorina, Daniela Ionescu, LC Redes, and Codruta Soica. 2010. 'Lupeol, a pentacylcic triterpene that reduces the lesions and irritability on murine skin and is effective on in vitro tumor models', *Journal of Agroalimentary Processes and Technologies*, 16: 427-32.

Commonwealth of Australia. 2011. 'Guidance on equivalence of herbal extracts in Complementary Medicines', *Therapeutic Good Administration*.

Cuevas, Berenice Aranda, Carlos Hernán Herrera Méndez, Ignacio Islas Flores, Sara Solís-Pereira, Luis Cuevas-Glory, Gerardo Rivera Muñoz, María de Lourdes Vargas y Vargas, and Jorge Tamayo Cortez. 2016. 'Main Polysaccharides Isolated and Quantified of Aloe vera Gel in Different Seasons of the Year', *Food and Nutrition Sciences*, 7: 447.

da Cunha, Fernanda M, Tânia S Fröde, Gilboé L Mendes, Angela Malheiros, Valdir Cechinel Filho, Rosendo A Yunes, and João B Calixto. 2001. 'Additional evidence for the anti-inflammatory and anti-allergic properties of the sesquiterpene polygodial', *Life sciences*, 70: 159-69.

Davis, Robert H, Joseph J DiDonato, RW Johnson, and Christopher B Stewart. 1994. 'Aloe vera, hydrocortisone, and sterol influence on wound tensile strength and anti-inflammation', *Journal of the American Podiatric Medical Association*, 84: 614-21.

Davis, Robert H, JJ Donato, Glenn M Hartman, and Richard C Haas. 1994. 'Anti-inflammatory and wound healing activity of a growth substance in Aloe vera', *Journal of the American Podiatric Medical Association*, 84: 77-81.

Ddamulira, G, I Ramathani, T Sebikejje, R Naluyimba, A Otim, A Pariyo, and M Maphosa. 2019. 'Mango Yield Performance in Lake Victoria Cresent Region of Uganda', *American Journal of Plant Sciences*, 10: 1141.

de Witte, Peter. 1993. 'Metabolism and pharmacokinetics of anthranoids', *Pharmacology*, 47: 86-97.

Desai, Sonal, Pratima Tatke, Vinay Gaitonde, and Satish Gabhe. 2020. 'Fast HPLC-DAD Method for Estimation of Catechin for Standardization of Extracts and Antiasthmatic Polyherbal Formulations Containing Albizia lebbeck', *INDIAN JOURNAL OF PHARMACEUTICAL EDUCATION AND RESEARCH*, 54: 550-55.

Di Petrillo, A., G. Orrù, A. Fais, and M. C. Fantini. 2022. 'Quercetin and its derivates as antiviral potentials: A comprehensive review', *Phytother Res*, 36: 266-78.

Doughari, JH, and S Manzara. 2008. 'In vitro antibacterial activity of crude leaf extracts of Mangifera indica Linn', *Afr J Microbiol Res*, 2: 67-72.

Du, Suya, Huirong Liu, Tiantian Lei, Xiaofang Xie, Hailian Wang, Xia He, Rongsheng Tong, and Yi Wang. 2018. 'Mangiferin: An effective therapeutic agent against several disorders', *Molecular medicine reports*, 18: 4775-86.

Dumba, Keneth, Wilberforce Kwiringira, Jane Namukobe, and Muhammad Ntale. 2021. 'Identification and analysis of adulterants in aphrodisiac herbal medicines sold by private herbal clinics, pharmacies and herbal drug shops in Kampala, Uganda', *East and Central African Journal of Pharmaceutical Sciences*, 24: 3-14.

Eisner, S. 2001. 'Guidance for Manufacture and Sale of Bulk Botanical Extracts', *Silver Spring, MD, American Herbal Products Association*.

Elsohly, M. A., W. Gul, B. Avula, and I. A. Khan. 2007. 'Determination of the anthraquinones aloe-emodin and aloin-A by liquid chromatography with mass spectrometric and diode array detection', *J AOAC Int*, 90: 28-42.

Ernst, Edzard. 2000. 'Adverse effects of herbal drugs in dermatology', *British Journal of Dermatology*, 143: 923-29.

Ettre, LS. 2002. 'Milestones in the Evolution of Chromatography (ChromSource', *Portland, Oregon*.

European Commission Directorate-General for Health and Food Safety. 2021. "amending Annex III to Regulation (EC) No 1925/2006 of the European Parliament and of the Council as regards botanical species containing hydroxyanthracene derivatives." In *COMMISSION REGULATION (EU) 2021/468 of 18 March 2021* Official Journal of the European Union.

Evangelos, Cholongitas, Katsoudas Spyros, and Dourakis Spyros. 2005. 'Henoch-Schonlein purpura associated with Aloe vera administration', *European journal of internal medicine*, 16: 59-60.

Evans, William Charles. 2009. *Trease and Evans' pharmacognosy* (Elsevier Health Sciences).

Fan, Dongsheng, Xin Zhou, Chao Zhao, Huaguo Chen, Yang Zhao, and Xiaojian Gong. 2011. 'Anti-inflammatory, antiviral and quantitative study of quercetin-3-O-β-D-glucuronide in Polygonum perfoliatum L', *Fitoterapia*, 82: 805-10.

Felföldi-Gáva, A, Béla Simándi, Sz Plánder, Sz Szarka, Éva Szőke, and Ágnes Kéry. 2009. 'Betulaceae and Platanaceae plants as alternative sources of selected lupane-type triterpenes. Their composition profile and betulin content', *Acta Chromatographica*, 21: 671-81.

Fitmawati, F., E. Resida, S. N. Kholifah, R. M. Roza, M. Almurdani, and E. Emrizal. 2020. 'Phytochemical screening and antioxidant profiling of Sumatran wild mangoes (Mangifera spp.): a potential source for medicine antidegenerative effects', *F1000Res*, 9: 220.

Forim, Moacir Rossi, Quezia Bezerra Cass, João Batista Fernandes, and Paulo Cezar Vieira. 2010. 'Simultaneous quantification of azadirachtin and 3-tigloylazadirachtol in Brazilian seeds and oil of Azadirachta indica: application to quality control and marketing', *Analytical Methods*, 2: 860-69.

Gafna, Dikko J, Klara Dolos, Ishmael O Mahiri, Joseph G Mahiri, and Joy A Obando. 2017. 'Diversity of medicinal plants and anthropogenic threats in the Samburu central sub-county of Kenya', *African Journal of Traditional, Complementary and Alternative Medicines*, 14: 72-79.

Garrido, Gabino, Idania Rodeiro, Ivones Hernández, Gastón García, Gema Pérez, Nelson Merino, Alberto Núñez-Sellés, and René Delgado. 2009. 'In vivo acute toxicological studies of an antioxidant extract from Mangifera indica L.(Vimang)', *Drug and Chemical Toxicology*, 32: 53-58.

Geiss, Friedrich. 1987. 'Fundamentals of thin layer chromatography'.

Goldbeck, Júlia Coswig, José Edmilson do Nascimento, Raquel G Jacob, Ângela Maria Fiorentini, and Wladimir Padilha da Silva. 2014. 'Bioactivity of essential oils from Eucalyptus globulus and Eucalyptus urograndis against planktonic cells and biofilms of Streptococcus mutans', *Industrial Crops and Products*, 60: 304-09.

Gong, Fan, Yi-Zeng Liang, Pei-Shan Xie, and Foo-Tim Chau. 2003. 'Information theory applied to chromatographic fingerprint of herbal medicine for quality control', *Journal of Chromatography A*, 1002: 25-40.

Goodarzi, Mohammad, Paul J Russell, and Yvan Vander Heyden. 2013. 'Similarity analyses of chromatographic herbal fingerprints: a review', *Analytica chimica acta*, 804: 16-28.

Gottumukkala, Raju VSS, Nareshraju Nadimpalli, Kannababu Sukala, and Gottumukkala V Subbaraju. 2014. 'Determination of catechin and epicatechin content in chocolates by high-performance liquid chromatography', *International Scholarly Research Notices*, 2014.

Granado-Serrano, Ana Belén, María Angeles Martín, Laura Bravo, Luis Goya, and Sonia Ramos. 2012. 'Quercetin modulates Nrf2 and glutathione-related defenses in HepG2 cells: Involvement of p38', *Chemico-Biological Interactions*, 195: 154-64.

Gupta, VK, SP Ahlawat, RV Kumar, Ajit, and A Datta. 2010. 'Effect of season and year on azadirachtin A and oil content in neem (Azadirachta indica A. Juss) seeds and relationship of azadirachtin A and oil content with rainfall, temperature and humidity', *Current science*: 953-56.

Handa, SS. 2008. 'An overview of extraction techniques for medicinal and aromatic plants', *Extraction technologies for medicinal and aromatic plants*, 1: 21-40.

Hiserodt, RD, SG Franzblau, and RT Rosen. 1998. 'Isolation of 6-, 8-, and 10-Gingerol from Ginger Rhizome by HPLC and Preliminary Evaluation of Inhibition of Mycobacterium a vium and Mycobacterium t uberculosis', *Journal of Agricultural and Food Chemistry*, 46: 2504-08.

Holonec, Liviu, Floricuta Ranga, Diana Crainic, Alina Truta, and Carmen Socaciu. 2012. 'Evaluation of betulin and betulinic acid content in birch bark from different forestry

areas of Western Carpathians', *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, 40: 99-105.

Hossain, Mohammad A, Wafa AS Al-Toubi, Afaf M Weli, Qasim A Al-Riyami, and Jamal N Al-Sabahi. 2013. 'Identification and characterization of chemical compounds in different crude extracts from leaves of Omani neem', *Journal of Taibah University for Science*, 7: 181-88.

Hossion, Abugafar ML, Yoshito Zamami, Rafiya K Kandahary, Tomofusa Tsuchiya, Wakano Ogawa, Akimasa Iwado, and Kenji Sasaki. 2011. 'Quercetin diacylglycoside analogues showing dual inhibition of DNA gyrase and topoisomerase IV as novel antibacterial agents', *Journal of medicinal chemistry*, 54: 3686-703.

Hu, Zhiqiang, Ruizhang Feng, Fa Xiang, Xu Song, Zhongqiong Yin, Chao Zhang, Xinghong Zhao, Renyong Jia, Zhenzhen Chen, and Li Li. 2014. 'Acute and subchronic toxicity as well as evaluation of safety pharmacology of eucalyptus oil-water emulsions', *International journal of clinical and experimental medicine*, 7: 4835.

HUANG, Qirong, Masatsugu IWAMOTO, Shunji AOKI, Naomi TANAKA, Kiyoko TAJIMA, Johji YAMAHARA, Yoshihisa TAKAISHI, Masaaki YOSHIDA, Toshiaki TOMIMATSU, and Yoshin TAMAI. 1991. 'Anti-5-hydroxytryptamine3 effect of galanolactone, diterpenoid isolated from ginger', *Chemical and pharmaceutical bulletin*, 39: 397-99.

Iannucci, Anna, Serafino Suriano, and Pasquale Codianni. 2021. 'Genetic diversity for agronomic traits and phytochemical compounds in coloured naked barley lines', *Plants*, 10: 1575.

Im, Sun-A, Sun-Tack Oh, Sukgil Song, Mi-Ran Kim, Dong-Seon Kim, Sung-Sick Woo, Tae Hyung Jo, Young In Park, and Chong-Kil Lee. 2005. 'Identification of optimal molecular size of modified Aloe polysaccharides with maximum immunomodulatory activity', *International immunopharmacology*, 5: 271-79.

Indrayanto, Gunawan. 2012. 'Validation of analytical methods—update 2011', *Profiles of drug substances, excipients and related methodology*, 37: 439-65.

———. 2018. 'Validation of chromatographic methods of analysis: application for drugs that derived from herbs', *Profiles of drug substances, excipients and related methodology*, 43: 359-92.

———. 2022. 'The importance of method validation in herbal drug research', *Journal of Pharmaceutical and Biomedical Analysis*: 114735.

Inter African Committee on Medicinal, Plants, Medicine African Traditional, Unity Organization of African, Technical Scientific, and Commission Research. 2014. *African pharmacopoeia* (Organization of African Unity, Scientific, Technical, & Research Commission: Lagos, Nigeria).

Irwin, Richard S., Michael H. Baumann, Donald C. Bolser, Louis-Philippe. Boulet, Sidney S. Braman, Christopher E. Brightling, Kevin K. Brown, Brendan J. Canning, Anne B. Chang, and Peter V. Dicpinigaitis. 2006. 'Diagnosis and management of cough executive summary: ACCP evidence-based clinical practice guidelines', *Chest*, 129: 1S-23S.

Ishii, Yasuko, Hisayuki Tanizawa, and Yoshio Takino. 1990. 'Studies of Aloe. III.: Mechanism of Cathartic Effect.(2)', *Chemical and pharmaceutical bulletin*, 38: 197-200.

Jhaumeer Laulloo, S., M. G. Bhowon, S. Soyfoo, and L. S. Chua. 2018. 'Nutritional and Biological Evaluation of Leaves of Mangifera indica from Mauritius', *Journal of Chemistry*, 2018: 6869294.

JIANG, Chao-Qiang, QUAN Ling-Tong, SHI Feng, YANG Na, WANG Chang-Hai, YIN Xiao-Ming, and Qing-Song ZHENG. 2014. 'Distribution of mineral nutrients and active ingredients in Aloe vera irrigated with diluted seawater', *Pedosphere*, 24: 722-30.

Jiang, Zhihui, Xiao Guo, Kunpeng Zhang, Ganesh Sekaran, Baorui Cao, Qingqing Zhao, Shouquan Zhang, Gordon M Kirby, and Xiaoying Zhang. 2019. 'The essential oils and eucalyptol from Artemisia vulgaris L. prevent acetaminophen-induced liver injury by activating Nrf2–Keap1 and enhancing APAP clearance through non-toxic metabolic pathway', *Frontiers in pharmacology*, 10: 782.

Juergens, UR, T Engelen, M Stöber, K Racké, A Gillissen, and H Vetter. 2004. 'Inhibitory activity of 1, 8-cineol (eucalyptol) on cytokine production in human mononuclear phagocytes in vitro', *Pulm Pharmacol Ther*, 17: 281-87.

Jung, Mun Yhung, Min Kyoung Lee, Hee Jeong Park, Eun-Bi Oh, Je Young Shin, Ji Su Park, Su Young Jung, Jung-Hee Oh, and Dong-Seong Choi. 2018. 'Heat-induced conversion of gingerols to shogaols in ginger as affected by heat type (dry or moist heat), sample type (fresh or dried), temperature and time', *Food Science and Biotechnology*, 27: 687-93.

Kaggwa, B., H. Kyeyune, E. I. Munanura, G. Anywar, S. Lutoti, J. Aber, L. K. Bagoloire, A. Weisheit, C. U. Tolo, P. F. Kamba, and P. E. Ogwang. 2022. 'Safety and Efficacy of Medicinal Plants Used to Manufacture Herbal Products with Regulatory Approval in Uganda: A Cross-Sectional Study', *Evid Based Complement Alternat Med*, 2022: 1304839.

Kaggwa, Bruhan, Godwin Anywar, Edson Ireeta Munanura, Raphael Wangalwa, Henry Kyeyune, Hedmon Okella, Fadhiru Pakoyo Kamba, and Ogwang Patrick Engeu. 2023. 'Application of the Herbal chemical Marker Ranking System (Herb MaRS) to standardization of herbal pharmaceutical raw materials-a case study'.

Kaggwa, Bruhan, Henry Kyeyune, Edson Ireeta Munanura, Godwin Anywar, Stephen Lutoti, Jacqueline Aber, Lynn K Bagoloire, Anke Weisheit, Casim Umba Tolo, and Pakoyo Fadhiru Kamba. 2022. 'Safety and Efficacy of Medicinal Plants Used to Manufacture Herbal Products with Regulatory Approval in Uganda: A Cross-Sectional Study', *Evidence-Based Complementary and Alternative Medicine*, 2022.

Kaggwa, Bruhan, Edson Ireeta Munanura, Henry Kyeyune, Godwin Anywar, Hedmon Okella, Clement Olusoji Ajayi, Raphael Wangalwa, John Mulangwa, Crispin Duncan Sesaazi, and Lynn K Bagoloire. 2023. 'Evaluation of catechin, lupeol, and betulinic acid as markers for the chromatographic quality Control of Albizia coriaria raw materials; an experimental study', *Journal of Medicinal Plants*

Research,, 17: 180-200.

Kaggwa, Bruhan, Maria Gloria Nakayita, Edson Ireeta Munanura, Henry Kyeyune, Clement Olusoji Ajayi, Raphael Wangalwa, Daniel Pillah Walakira, Godwin Anywar, Lynn K Bagoloire, and Teddy Kakazi. 2023. 'Chemometric Classification of Mangifera indica L. Leaf Cultivars, Based on Selected Phytochemical Parameters; Implications for Standardization of the Pharmaceutical Raw Materials', *Evidence-Based Complementary and Alternative Medicine*, 2023.

Kahkeshani, Niloofar, Fatemeh Farzaei, Maryam Fotouhi, Seyedeh Shaghayegh Alavi, Roodabeh Bahramsoltani, Rozita Naseri, Saeideh Momtaz, Zahra Abbasabadi, Roja Rahimi, and Mohammad Hosein Farzaei. 2019. 'Pharmacological effects of gallic acid in health and diseases: A mechanistic review', *Iranian journal of basic medical sciences*, 22: 225.

Kajobe, R, EK Kato, SA Otim, P Kasangaki, and PP Abila. 2016. 'The status of honeybee pests in Uganda', *Bulletin of Animal Health and Production in Africa*, 64: 105-17.

Kamboj, Anjoo. 2012. 'Analytical evaluation of herbal drugs', *Drug discovery research in pharmacognosy*, 3: 23-55.

Kanama, Sowesa K, Alvaro M Viljoen, Guy PP Kamatou, Weiyang Chen, Maxleene Sandasi, Hamid-Reza Adhami, and Ben-Erik Van Wyk. 2015. 'Simultaneous quantification of anthrones and chromones in Aloe ferox ("Cape aloes") using UHPLC–MS', *Phytochemistry Letters*, 13: 85-90.

Kang, Mi-Sun, Jong-Suk Oh, In-Chol Kang, Suk-Jin Hong, and Choong-Ho Choi. 2008. 'Inhibitory effect of methyl gallate and gallic acid on oral bacteria', *The Journal of Microbiology*, 46: 744-50.

Karaca, K, JM Sharma, and R Nordgren. 1995. 'Nitric oxide production by chicken macrophages activated by Acemannan, a complex carbohydrate extracted from Aloe vera', *International journal of immunopharmacology*, 17: 183-88.

Karlberg, Johan P. E. 2001. "General Guidelines for Methodologies on Research and Evaluation of Traditional Medicine." In.

Kaushik, Sulochana, Ginni Jangra, Vaibhav Kundu, Jaya Parkash Yadav, and Samander Kaushik. 2020. 'Anti-viral activity of Zingiber officinale (Ginger) ingredients against the Chikungunya virus', *Virusdisease*, 31: 270-76.

Kazakevich, Yuri V, and Rosario Lobrutto. 2007. *HPLC for pharmaceutical scientists* (John Wiley & Sons).

Khare, Puja, and Karuna Shanker. 2016. 'Mangiferin: A review of sources and interventions for biological activities', *BioFactors*, 42: 504-14.

Khozeimeh, Faezeh., Zahra. Golestannejad, Saeedeh. Seifi, Armita. Pourarian, Shahin. Gavanji, and Farnaz. Farhad. 2015. 'Chemical composition and in vitro antibacterial activities of Cuminumcyminum L. and Eucalyptus globulus essential oil extracts against three lactobacillus strains', □□□ □□□□□□ □□□□ □□□□.

Khumalo, GP, NJ Sadgrove, S Van Vuuren, and B-E Van Wyk. 2019. 'Antimicrobial activity of volatile and non-volatile isolated compounds and extracts from the bark and leaves of Warburgia salutaris (Canellaceae) against skin and respiratory pathogens', *South African Journal of Botany*, 122: 547-50.

Kigondu, Elizabeth VM, Geoffrey M Rukunga, Joseph M Keriko, Willy K Tonui, Jeremiah W Gathirwa, Peter G Kirira, Beatrice Irungu, Johnstone M Ingonga, and Isaiah O Ndiege. 2009. 'Anti-parasitic activity and cytotoxicity of selected medicinal plants from Kenya', *Journal of ethnopharmacology*, 123: 504-09.

Kim, Jong Min, Jin Yong Kang, Seon Kyeong Park, Jong Hyun Moon, Min Ji Kim, Hyo Lim Lee, Hye Rin Jeong, Jong Cheol Kim, and Ho Jin Heo. 2021. 'Powdered Green Tea (Matcha) Attenuates the cognitive dysfunction via the regulation of systemic inflammation in chronic PM2. 5-exposed BALB/c mice', *Antioxidants*, 10: 1932.

Kim, Tae Jin, Ye Jin Kim, Woo Duck Seo, Sang Un Park, and Jae Kwang Kim. 2022. 'Improved quantification of catechin and epicatechin in red rice (Oryza sativa L.)

using stable isotope dilution liquid chromatography-mass spectrometry', *Applied Biological Chemistry*, 65: 85.
Kioy, Deborah, Alexander I Gray, and Peter G Waterman. 1990. 'A comparative study of the stem-bark drimane sesquiterpenes and leaf volatile oils of Warburgia ugandensis and W. stuhlmannii', *Phytochemistry*, 29: 3535-38.
Konieczka, Piotr, and Jacek Namiesnik. 2016. *Quality assurance and quality control in the analytical chemical laboratory: a practical approach* (CRC Press).
Kumar, Sandeep, Manila Yadav, Amita Yadav, Pooja Rohilla, and Jaya Parkash Yadav. 2017. 'Antiplasmodial potential and quantification of aloin and aloe-emodin in Aloe vera collected from different climatic regions of India', *BMC Complementary and Alternative Medicine*, 17: 369.
Kumari, P Divya, Shilpa M Shenoy, Shahnawaz Khijmatgar, Avidyuti Chowdhury, Edward Lynch, and Chitta R Chowdhury. 2019. 'Antibacterial activity of new atraumatic restorative treatment materials incorporated with Azadirachta indica (Neem) against Streptococcus mutans', *Journal of oral biology and craniofacial research*, 9: 321-25.
Kurokawa, Masahiko, Purusotam Basnet, Mizue Ohsugi, Toyoharu Hozumi, Shigetoshi Kadota, Tsuneo Namba, Takashi Kawana, and Kimiyasu Shiraki. 1999. 'Anti-Herpes Simplex Virus Activity of Moronic Acid Purified fromRhus javanica In Vitro and In Vivo', *Journal of Pharmacology and Experimental Therapeutics*, 289: 72-78.
Lee, Anna, Po Tong Chui, Cindy ST Aun, Tony Gin, and Angel SC Lau. 2004. 'Possible interaction between sevoflurane and Aloe vera', *Annals of Pharmacotherapy*, 38: 1651-54.
Lee, Changhyun, Jung Won Lee, Ji Yeon Seo, Sung Wook Hwang, Jong Pil Im, and Joo Sung Kim. 2016. 'Lupeol inhibits LPS-induced NF-kappa B signaling in intestinal epithelial cells and macrophages, and attenuates acute and chronic murine colitis', *Life sciences*, 146: 100-08.
Lee, J. H., M. Oh, J. H. Seok, S. Kim, D. B. Lee, G. Bae, H. I. Bae, S. Y. Bae, Y. M. Hong, S. O. Kwon, D. H. Lee, C. S. Song, J. Y. Mun, M. S. Chung, and K. H. Kim. 2016. 'Antiviral Effects of Black Raspberry (Rubus coreanus) Seed and Its Gallic Acid against Influenza Virus Infection', *Viruses*, 8.
Leonard, Carmen M, and Alvaro M Viljoen. 2015. 'Warburgia: A comprehensive review of the botany, traditional uses and phytochemistry', *Journal of ethnopharmacology*, 165: 260-85.
Li, H, Q Wang, Y Ding, C Bao, and W Li. 2017. 'Mangiferin ameliorates Porphyromonas gingivalis-induced experimental periodontitis by inhibiting phosphorylation of nuclear factor-κB and Janus kinase 1–signal transducer and activator of transcription signaling pathways', *Journal of periodontal research*, 52: 1-7.
Li, N., R. Xiong, R. He, B. Liu, B. Wang, and Q. Geng. 2021. 'Mangiferin Mitigates Lipopolysaccharide-Induced Lung Injury by Inhibiting NLRP3 Inflammasome Activation', *J Inflamm Res*, 14: 2289-300.
Li, Y., J. Yao, C. Han, J. Yang, M. T. Chaudhry, S. Wang, H. Liu, and Y. Yin. 2016. 'Quercetin, Inflammation and Immunity', *Nutrients*, 8: 167.
Liang, Yi-Zeng, Peishan Xie, and Kelvin Chan. 2004. 'Quality control of herbal medicines', *Journal of chromatography B*, 812: 53-70.
Lombardi, Niccolò, Alessandra Bettiol, Giada Crescioli, Valentina Maggini, Eugenia Gallo, Francesco Sivelli, Francesco Sofi, Gian Franco Gensini, Alfredo Vannacci, and Fabio

Firenzuoli. 2020. 'Association between anthraquinone laxatives and colorectal cancer: protocol for a systematic review and meta-analysis', *Systematic reviews*, 9: 1-6.
López, Zaira, Antoni Femenia, Gabriela Núñez-Jinez, Michelle N Salazar Zúñiga, M Eduardo Cano, Teresa Espino, and Peter Knauth. 2019. 'In vitro immunomodulatory effect of food supplement from Aloe vera', *Evidence-based complementary and alternative medicine*, 2019.
Lucini, Luigi, Marco Pellizzoni, and Gian Pietro Molinari. 2013. 'Anthraquinones and β-polysaccharides content and distribution in Aloe plants grown under different light intensities', *Biochemical Systematics and Ecology*, 51: 264-68.
Luedtke, Kerstin, Alison Rushton, Christine Wright, Benjamin Geiss, Tim Patrick Juergens, and Arne May. 2012. 'Transcranial direct current stimulation for the reduction of clinical and experimentally induced pain: a systematic review and meta-analysis', *The Clinical journal of pain*, 28: 452-61.
Luyckx, Valerie A, Robert Ballantine, Magda Claeys, Filip Cuyckens, Hilde Van den Heuvel, Richard K Cimanga, Arnold J Vlietinck, Marc E De Broe, and Ivor J Katz. 2002. 'Herbal remedy-associated acute renal failure secondary to Cape aloes', *American journal of kidney diseases*, 39: e13. 1-e13. 5.
Maan, Preeti, Kuldeep Singh Yadav, and Narayan Prasad Yadav. 2017. 'Wound healing activity of Azadirachta indica A. juss stem bark in mice', *Pharmacognosy magazine*, 13: S316.
Maghraby, Sarah T Sakna Yasmin R, and Mohamed S Abdelfattah. 2022. 'Phytochemical diversity and pharmacological effects of triterpenes from genus Ziziphus: a comprehensive review'.
Mahady, Gail B, Susan L Pendland, Gina S Yun, Zhi-Zhen Lu, and Adina Stoia. 2003. 'Ginger (Zingiber officinale Roscoe) and the gingerols inhibit the growth of Cag A+ strains of Helicobacter pylori', *Anticancer research*, 23: 3699.
Manna, S. K., M. T. Kuo, and B. B. Aggarwal. 1999. 'Overexpression of gamma-glutamylcysteine synthetase suppresses tumor necrosis factor-induced apoptosis and activation of nuclear transcription factor-kappa B and activator protein-1', *Oncogene*, 18: 4371-82.
Mao, QQ., XY. Xu, SY. Cao, RY. Gan, H. Corke, T. Beta, and H. Li. 2019. "Bin.(2019). Bioactive compounds and bioactivities of ginger (zingiber officinale roscoe)." In.: Foods.
Maroyi, Alfred. 2014. 'The genus Warburgia: A review of its traditional uses and pharmacology', *Pharmaceutical Biology*, 52: 378-91.
McGiffin, David C, Geoff Cumming, and Paul S Myles. 2021. 'The frequent insignificance of a “significant” p-value', *Journal of Cardiac Surgery*.
Mengiste, Berhan, Tizazu Zenebe, Kassahun Dires, Ermias Lulekal, Awol Mekonnen, Nigus Zegeye, and Yoseph Shiferaw. 2020. 'Safety Evaluation of Eucalyptus globulus Essential Oils through Acute and Sub-acute Toxicity and Skin Irritation in Mice and Rats', *Current Chemical Biology*, 14: 187-95.
Merawie, Yibeltal, Samuel Sahile, Feleke Moges, and Azamal Husen. 2013. 'Antimicrobial activity of crude and semi-purified fractions of Warburgia ugandensis against some pathogens', *J Coastal Life Med*, 1: 184-91.
Merck, E. 1980. *Dyeing reagents for thin layer and paper chromatography* (E. Merck).

Metcalfe, Charles. 2019. 'Quantitation of aloe vera polysaccharides by O-Acetyl and UV-Vis spectrophotometry: First action 2018.14', *Journal of AOAC International*, 102: 1091-94.
Ministry of Agriculture Animal Industry & Fisheries. 2010. "Agriculture for Food and Income Security, Agriculture Sector Development Strategy and Investment Plan 2010/11-2014/15." In.: Uganda.
Mishra, Ajay, and Nikhil Dave. 2013. 'Neem oil poisoning: Case report of an adult with toxic encephalopathy', *Indian journal of critical care medicine: peer-reviewed, official publication of Indian Society of Critical Care Medicine*, 17: 321.
Mishra, Chandra Bhushan, Preeti Pandey, Ravi Datta Sharma, Md Zubbair Malik, Raj Kumar Mongre, Andrew M Lynn, Rajendra Prasad, Raok Jeon, and Amresh Prakash. 2021. 'Identifying the natural polyphenol catechin as a multi-targeted agent against SARS-CoV-2 for the plausible therapy of COVID-19: an integrated computational approach', *Briefings in Bioinformatics*, 22: 1346-60.
Moo, Chew-Li, Mohd Azuraidi Osman, Shun-Kai Yang, Wai-Sum Yap, Saila Ismail, Swee-Hua-Erin Lim, Chou-Min Chong, and Kok-Song Lai. 2021. 'Antimicrobial activity and mode of action of 1, 8-cineol against carbapenemase-producing Klebsiella pneumoniae', *Scientific reports*, 11: 1-13.
Mulyaningsih, Sri, Frank Sporer, Jürgen Reichling, and Michael Wink. 2011. 'Antibacterial activity of essential oils from Eucalyptus and of selected components against multidrug-resistant bacterial pathogens', *Pharmaceutical biology*, 49: 893-99.
Murray, Virginia, and Debbie Shaw. 2000. 'WHO monographs on selected medicinal plants, volume 2', *Health and Hygiene*, 21: 129.
Mushore, J, and M Matuvhunye. 2013. 'Antibacterial properties of Mangifera indica on Staphylococcus aureus', *African journal of clinical and experimental microbiology*, 14: 62-74.
Mustafa, T, KC Srivastava, and KB Jensen. 1993. 'Pharmacology of ginger, Zingiber officinale', *J. Drug Develop*, 6: 25-39.
Mutai, C, L Keter, L Ngeny, and P Jeruto. 2012. "Effects of Triterpenoids on Herpes Simplex Virus Type1 (Hsv-1)." In.: Vitro.
Nader, Manar A, and Hany N Baraka. 2012. 'Effect of betulinic acid on neutrophil recruitment and inflammatory mediator expression in lipopolysaccharide-induced lung inflammation in rats', *European journal of pharmaceutical sciences*, 46: 106-13.
National Drug Authority. 2021. "Drug Register." In.: National Drug Authorty.
Ndoye, Samba Fama, Didier Fraisse, Blandine Akendengué, Mbayediaw Dioum, Rokhayasylla Gueye, Cheikh Sall, Insa Seck, Catherine Felgines, Matar Seck, and François Senejoux. 2018. 'Antioxidant and antiglycation properties of two mango (Mangifera indica L.) cultivars from Senegal', *Asian Pacific Journal of Tropical Biomedicine*, 8: 137-41.
Noviana, Eka, Gunawan Indrayanto, and Abdul Rohman. 2022. 'Advances in fingerprint analysis for standardization and quality control of herbal medicines', *Frontiers in Pharmacology*, 13.
Núñez Sellés, A. J., H. T. Vélez Castro, J. Agüero-Agüero, J. González-González, F. Naddeo, F. De Simone, and L. Rastrelli. 2002a. 'Isolation and quantitative analysis of phenolic antioxidants, free sugars, and polyols from mango (Mangifera indica L.)

stem bark aqueous decoction used in Cuba as a nutritional supplement', *J Agric Food Chem*, 50: 762-6.

Núñez Sellés, Alberto J, Herman T Vélez Castro, Juan Agüero-Agüero, Johanes González-González, Fabio Naddeo, Francesco De Simone, and Luca Rastrelli. 2002b. 'Isolation and quantitative analysis of phenolic antioxidants, free sugars, and polyols from mango (Mangifera indica L.) stem bark aqueous decoction used in Cuba as a nutritional supplement', *Journal of Agricultural and Food Chemistry*, 50: 762-66.

Nyiredy, Sz, CAJ Erdelmeier, B Meier, and O Sticher. 1985. 'The "PRISMA" Mobile Phase Optimization Model in Thin-Layer Chromatography-Separation of Natural Compounds', *Planta medica*, 51: 241-46.

Oh, Won Young, Priyatharini Ambigaipalan, and Fereidoon Shahidi. 2019. 'Preparation of quercetin esters and their antioxidant activity', *Journal of agricultural and food chemistry*, 67: 10653-59.

Ojiezeh, T I., and N. Eghafona. 2015. 'Humoral responses of broiler chickens challenged with NDV following supplemental treatment with extracts of Aloe vera, Alma millsoni, Ganoderma lucidum and Archachatina marginata', *Central-European journal of immunology*, 40: 300.

Okusa, Philippe N, Caroline Stévigny, Marie Névraumont, Michel Gelbcke, Pierre Van Anrwerpen, Jean Claude Braekman, and Pierre Duez. 2014. 'Ferulaldehyde and lupeol as direct and indirect antimicrobial compounds from Cordia gilletii (Boraginaceae) root barks', *Natural product communications*, 9: 1934578X1400900506.

Olila, D, and J Opuda-Asibo. 2001. 'Antibacterial and antifungal activities of extracts of Zanthoxylum chalybeum and Warburgia ugandensis, Ugandan medicinal plants', *African Health Sciences*, 1: 66-72.

Oloyede, H. O. B., H. O. Ajiboye, M. O. Salawu, and T. O. Ajiboye. 2017. 'Influence of oxidative stress on the antibacterial activity of betulin, betulinic acid and ursolic acid', *Microb Pathog*, 111: 338-44.

Omara, Timothy, Ambrose K Kiprop, and Viola J Kosgei. 2021. 'Intraspecific variation of phytochemicals, antioxidant, and antibacterial activities of different solvent extracts of Albizia coriaria leaves from some agroecological zones of Uganda', *Evidence-Based Complementary and Alternative Medicine*, 2021.

——. 2022. 'Isolation and characterization of compounds in ethanolic extract of Albizia coriaria (Welw ex. Oliver) leaves: a further evidence of its ethnomedicinal diversity', *Bulletin of the National Research Centre*, 46: 1-15.

ONDORA, ONYAMBU MESHACK. 2016. 'DEVELOPMENT OF CHROMATOGRAPHIC FINGERPRINTING AND OTHER QUALITY EVALUATION METHODS FOR WARBURGIA UGANDENSIS SPRAGUE HERBAL MATERIALS', KENYATTA UNIVERSITY.

Onishi, Shintaro, Takuya Mori, Hidetoshi Kanbara, Taichi Habe, Noriyasu Ota, Yuki Kurebayashi, and Takashi Suzuki. 2020. 'Green tea catechins adsorbed on the murine pharyngeal mucosa reduce influenza A virus infection', *Journal of Functional Foods*, 68: 103894.

Orwa, C, A Mutua, R Kindt, R Jamnadass, and A Simons. 2009a. "Agroforestree database: a tree reference and selection guide, version 4.0." In.

Orwa, C, A Mutua, R Kindt, R Jamnadass, and A Simons. 2009b. 'Agroforestree Database: a tree reference and selection guide. Version 4', *Agroforestree Database: a tree reference and selection guide. Version 4.*

Oyebanji, Bukola Olanike, Adebowale Bernard Saba, and Olayinka Ayotunde Oridupa. 2014. 'Studies on the anti-inflammatory, analgesic and antipyrexic activities of betulinic acid derived from Tetracera potatoria', *African Journal of Traditional, Complementary and Alternative Medicines*, 11: 30-33.

Pace, Roberto, and Ernesto Marco Martinelli. 2022. 'The phytoequivalence of herbal extracts: A critical evaluation', *Fitoterapia*, 162: 105262.

Palafox-Carlos, H., J. Gil-Chávez, R. R. Sotelo-Mundo, J. Namiesnik, S. Gorinstein, and G. A. González-Aguilar. 2012. 'Antioxidant interactions between major phenolic compounds found in 'Ataulfo' mango pulp: chlorogenic, gallic, protocatechuic and vanillic acids', *Molecules*, 17: 12657-64.

Pan, Jing, Xiaomin Yi, Shujuan Zhang, Jun Cheng, Yihai Wang, Chunyu Liu, and Xiangjiu He. 2018. 'Bioactive phenolics from mango leaves (Mangifera indica L.)', *Industrial Crops and Products*, 111: 400-06.

Pardo-Andreu, Gilberto L, Carlos Sánchez-Baldoquín, Rizette Ávila-González, René Delgado, Zeki Naal, and Carlos Curti. 2006. 'Fe (III) improves antioxidant and cytoprotecting activities of mangiferin', *European Journal of Pharmacology*, 547: 31-36.

Pardo-Andreu, Gilberto L, Carlos Sánchez-Baldoquín, Rizette Avila-González, Edgar T Suzuki Yamamoto, Andrés Revilla, Sérgio Akira Uyemura, Zeki Naal, René Delgado, and Carlos Curti. 2006. 'Interaction of Vimang (Mangifera indica L. extract) with Fe (III) improves its antioxidant and cytoprotecting activity', *Pharmacological Research*, 54: 389-95.

Park, Mi-Young, Hoon-Jeong Kwon, and Mi-Kyung Sung. 2009. 'Evaluation of aloin and aloe-emodin as anti-inflammatory agents in aloe by using murine macrophages', *Bioscience, Biotechnology, and Biochemistry*, 73: 828-32.

Park, Miri, Jungdon Bae, and Dae-Sil Lee. 2008. 'Antibacterial activity of [10]-gingerol and [12]-gingerol isolated from ginger rhizome against periodontal bacteria', *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives*, 22: 1446-49.

Park, Su Yeon, and Mun Yhung Jung. 2016. 'UHPLC-ESI-MS/MS for the quantification of eight major Gingerols and Shogaols in ginger products: Effects of ionization polarity and mobile phase modifier on the sensitivity', *Journal of Food Science*, 81: C2457-C65.

Peng, SY, J Norman, G Curtin, D Corrier, HR McDaniel, and D Busbee. 1991. 'Decreased mortality of Norman murine sarcoma in mice treated with the immunomodulator, Acemannan', *Molecular biotherapy*, 3: 79-87.

Pereira Beserra, Fernando, Meilang Xue, Gabriela Lemos de Azevedo Maia, Ariane Leite Rozza, Cláudia Helena Pellizzon, and Christopher John Jackson. 2018. 'Lupeol, a Pentacyclic Triterpene, Promotes Migration, Wound Closure, and Contractile Effect In Vitro: Possible Involvement of PI3K/Akt and p38/ERK/MAPK Pathways', *Molecules*, 23: 2819.

Peterson, Gary L. 1979. 'Review of the Folin phenol protein quantitation method of Lowry, Rosebrough, Farr and Randall', *Analytical biochemistry*, 100: 201-20.

Pillai, NR, and G Santhakumari. 1981. 'Anti-arthritic and anti-inflammatory actions of nimbidin', *Planta Medica*, 43: 59-63.
──. 1985. 'Effect of nimbidin on gastric acid secretion', *Ancient Science of Life*, 5: 91.
Plan, Investment. 2010. 'Agriculture Sector Development Strategy and Investment Plan: 2010/11-2014/15'.
Plengsuriyakarn, Tullayakorn, Vithoon Viyanant, Veerachai Eursitthichai, Smarn Tesana, Wanna Chaijaroenkul, Arunporn Itharat, and Kesara Na-Bangchang. 2012. 'Cytotoxicity, toxicity, and anticancer activity of Zingiber officinale Roscoe against cholangiocarcinoma', *Asian Pacific Journal of Cancer Prevention*, 13: 4597-606.
Poole, Colin F. 2015. 'Milestones, core concepts, and contrasts.' in, *Instrumental Thin-Layer Chromatography* (Elsevier).
Prasad, Sahdeo, Neetu Kalra, and Yogeshwer Shukla. 2007. 'Hepatoprotective effects of lupeol and mango pulp extract of carcinogen induced alteration in Swiss albino mice', *Molecular nutrition & food research*, 51: 352-59.
Pungot, Noor Hidayah, Nur Nazihah Shamsuri, Nor Akmalazura Jani, and Najwa Amira Mohamad Latif. 2020. 'Phytochemical Screening, Total Phenolic Content and Antioxidant Activity of Leaves Extracts from Mangifera indica L. cv. Apple'.
Quintans-Júnior, Lucindo J, Makson GB Oliveira, Michele F Santana, Marilia T Santana, Adriana G Guimarães, Jullyana S Siqueira, Damião P De Sousa, and Reinaldo N Almeida. 2011. 'α-Terpineol reduces nociceptive behavior in mice', *Pharmaceutical Biology*, 49: 583-86.
Rabe, Christian, Annemarie Musch, Peter Schirmacher, Wolfgang Kruis, and Robert Hoffmann. 2005. 'Acute hepatitis induced by an Aloe vera preparation: a case report', *World Journal of Gastroenterology: WJG*, 11: 303.
Rabe, T, and J Van Staden. 2000. 'Isolation of an antibacterial sesquiterpenoid from Warburgia salutaris', *Journal of ethnopharmacology*, 73: 171-74.
Ramesh, A., and M. Balasubramanian. 1999. 'Rapid preconcentration method for the determination of azadirachtin-A and -B, nimbin and salannin in neem oil samples by using graphitised carbon solid phase extraction', *Analyst*, 124: 19-21.
Ramis-Ramos, Guillermo, María Celia García-Álvarez-Coque, and José Antonio Navarro-Huerta. 2017. 'Solvent selection in liquid chromatography.' in, *Liquid Chromatography* (Elsevier).
Randrianarivo, Hanitra Ranjàna, Anjarasoa Ravo Razafindrakoto, Holy Christiane Ratsimanohatra, Lovarintsoa Judicaël Randriamampianina, Clara Fredeline Rajemiarimoelisoa, Lolona Ramamonjisoa, David Ramanitrahasimbola, Danielle Aurore Doll Rakoto, and Victor Louis Jeannoda. 2014. 'Toxic effects of seed methanolic extracts of endemic Albizia species (Fabaceae) from Madagascar on animals', *Journal of life Sciences*, 8.
Rathinavel, T., S. Ammashi, and G. Shanmugam. 2021. 'Analgesic and anti-inflammatory potential of Lupeol isolated from Indian traditional medicinal plant Crateva adansonii screened through in vivo and in silico approaches', *J Genet Eng Biotechnol*, 19: 62.
Reddeman, Robin A., Róbert. Glávits, John R. Endres, Amy E. Clewell, Gábor. Hirka, Adél. Vértesi, Erzsébet. Béres, and Ilona Pasics. Szakonyiné. 2019. 'A toxicological evaluation of mango leaf extract (Mangifera indica) containing 60% mangiferin', *Journal of toxicology*, 2019.

Reich, Eike, and Anne Schibli. 2007a. 'High-performance thin-layer chromatography for the analysis of medicinal plants', *(No Title)*.

──. 2007b. *High-performance thin-layer chromatography for the analysis of medicinal plants* (Thieme).

Ren, Yongshen, Ping Zhang, Dan Yan, Jiabo Wang, Xiaoxi Du, and Xiaohe Xiao. 2011. 'A strategy for the detection of quality fluctuation of a Chinese herbal injection based on chemical fingerprinting combined with biological fingerprinting', *Journal of pharmaceutical and biomedical analysis*, 56: 436-42.

Reynolds, T. 1985. 'The compounds in Aloe leaf exudates: a review', *Botanical Journal of the Linnean society*, 90: 157-77.

Ribeiro, SMR, LCA Barbosa, JH Queiroz, Matthias Knödler, and A Schieber. 2008. 'Phenolic compounds and antioxidant capacity of Brazilian mango (Mangifera indica L.) varieties', *Food chemistry*, 110: 620-26.

Ribeiro, Sonia Machado Rocha, and Andreas Schieber. 2010. 'Bioactive compounds in mango (Mangifera indica L.).' in, *Bioactive foods in promoting health* (Elsevier).

Rice-Evans, C. A., N. J. Miller, and G. Paganga. 1996. 'Structure-antioxidant activity relationships of flavonoids and phenolic acids', *Free Radic Biol Med*, 20: 933-56.

Rivera, D. G., I. Hernández, N. Merino, Y. Luque, A. Álvarez, Y. Martín, A. Amador, L. Nuevas, and R. Delgado. 2011a. 'Mangifera indica L. extract (Vimang) and mangiferin reduce the airway inflammation and Th2 cytokines in murine model of allergic asthma', *J Pharm Pharmacol*, 63: 1336-45.

Rivera, Dagmar García, Ivones Hernández, Nelson Merino, Yilian Luque, Alina Álvarez, Yanet Martín, Aylin Amador, Lauro Nuevas, and René Delgado. 2011b. 'Mangifera indica L. extract (Vimang) and mangiferin reduce the airway inflammation and Th2 cytokines in murine model of allergic asthma', *Journal of Pharmacy and Pharmacology*, 63: 1336-45.

Roberts, Dianna B, and Elizabeth L Travis. 1995. 'Acemannan-containing wound dressing gel reduces radiation-induced skin reactions in C3H mice', *International Journal of Radiation Oncology* Biology* Physics*, 32: 1047-52.

Rocha Ribeiro, Sônia Machado, José Humberto Queiroz, Maria Eliana Lopes Ribeiro de Queiroz, Flávia Milagres Campos, and Helena Maria Pinheiro Sant'Ana. 2007. 'Antioxidant in mango (Mangifera indica L.) pulp', *Plant foods for human nutrition*, 62: 13-17.

Rojanapo, W, S Suwanno, R Somaree, T Glinsukon, and Y Thebtaranonth. 1985. 'Mutagenic and antimicrobial testing activity of nimbolide and nimbic acid', *J Sci Thailand*, 11: 177-88.

Rolo, Anabela P, and Carlos M Palmeira. 2006. 'Diabetes and mitochondrial function: role of hyperglycemia and oxidative stress', *Toxicology and applied pharmacology*, 212: 167-78.

Romero, Jacqueline A, Roberto Vandama, Marilin López, Maricela Capote, Carlos Ferradá, Caridad Carballo, René Delgado, Wim Vanden Berghe, and Sandra Apers. 2015. 'Study of physicochemical parameters of different cultivars of** Mangifera indica** L. leaves for their use as a source of Mangiferin', *International journal of pharmacognosy and phytochemical research*, 7: 608-12.

Rong, Xianglu, Gang Peng, Takuya Suzuki, Qinglin Yang, Johji Yamahara, and Yuhao Li. 2009. 'A 35-day gavage safety assessment of ginger in rats', *Regulatory Toxicology and Pharmacology*, 54: 118-23.

Roy, Christian, Hooshang Pakdel, and Dominique Brouillard. 1990. 'The role of extractives during vacuum pyrolysis of wood', *Journal of Applied Polymer Science*, 41: 337-48.

Ru, YQ. 2004. 'Information-Theoretical Fundamentals of Modern Analytical Chemistry', *Changsha, PR China: Hunan Science and Technology (in Chinese)*.

Saffari, Fereshteh, Mohammad Danesh Ardakani, Hengameh Zandi, Hamed Heidarzadeh, and Mohammad Hassan Moshafi. 2015. 'The effects of chlorhexidine and persica mouthwashes on colonization of Streptococcus mutans on fixed orthodontics O-rings', *Journal of Dentistry*, 16: 54.

Safwat, Nesreen A, Mona T Kashef, Ramy K Aziz, Khadiga F Amer, and Mohammed A Ramadan. 2018. 'Quercetin 3-O-glucoside recovered from the wild Egyptian Sahara plant, Euphorbia paralias L., inhibits glutamine synthetase and has antimycobacterial activity', *Tuberculosis*, 108: 106-13.

Sahoo, Niharika, Padmavati Manchikanti, and Satyahari Dey. 2010. 'Herbal drugs: standards and regulation', *Fitoterapia*, 81: 462-71.

Sahu, Swagatika, Basanta Kumar Das, Jyotirmayee Pradhan, BC Mohapatra, BK Mishra, and Niranjan Sarangi. 2007. 'Effect of Magnifera indica kernel as a feed additive on immunity and resistance to Aeromonas hydrophila in Labeo rohita fingerlings', *Fish & shellfish immunology*, 23: 109-18.

San Chang, Jung, Kuo Chih Wang, Chia Feng Yeh, Den En Shieh, and Lien Chai Chiang. 2013. 'Fresh ginger (Zingiber officinale) has anti-viral activity against human respiratory syncytial virus in human respiratory tract cell lines', *Journal of ethnopharmacology*, 145: 146-51.

Sánchez-Machado, Dalia I, Jaime López-Cervantes, Raquel Sendón, and Ana Sanches-Silva. 2017. 'Aloe vera: Ancient knowledge with new frontiers', *Trends in Food Science & Technology*, 61: 94-102.

Sarmiento, Wendy C, Cecilia C Maramba, and Ma Liza M Gonzales. 2011. 'An in-vitro study on the antibacterial effect of neem (Azadirachta indica) leaf extract on methicillin-sensitive and Methicillin-resistant Staphylococcus aureus', *PIDSP J*, 12: 40-45.

Schultz, Fabien, Godwin Anywar, Huaqiao Tang, François Chassagne, James T Lyles, Leif-Alexander Garbe, and Cassandra L Quave. 2020. 'Targeting ESKAPE pathogens with anti-infective medicinal plants from the Greater Mpigi region in Uganda', *Scientific reports*, 10: 1-19.

Schultz, Fabien, Godwin Anywar, Barbara Wack, Cassandra Leah Quave, and Leif-Alexander Garbe. 2020. 'Ethnobotanical study of selected medicinal plants traditionally used in the rural Greater Mpigi region of Uganda', *Journal of ethnopharmacology*, 256: 112742.

Schultz, Fabien, Ogechi Favour Osuji, Barbara Wack, Godwin Anywar, and Leif-Alexander Garbe. 2021. 'Antiinflammatory medicinal plants from the Ugandan greater mpigi region act as potent inhibitors in the COX-2/PGH2 pathway', *Plants*, 10: 351.

Scott, G, and EP Springfield. 2004. "Pharmaceutical monographs for 60 South African plant species used as traditional medicines. South African National Biodiversity Institute (SANBI)." In.

Sferrazzo, Giuseppe, Rosa Palmeri, Cristina Restuccia, Lucia Parafati, Laura Siracusa, Mariarita Spampinato, Giuseppe Carota, Alfio Distefano, Michelino Di Rosa, and Barbara Tomasello. 2022. 'Mangifera indica L. Leaves as a Potential Food Source of Phenolic Compounds with Biological Activity', *Antioxidants*, 11: 1313.

Shah, KA, MB Patel, RJ Patel, and PK Parmar. 2010. 'Mangifera indica (mango)', *Pharmacognosy reviews*, 4: 42.

Shalaby, MA, and AR Hamowieh. 2010. 'Safety and efficacy of Zingiber officinale roots on fertility of male diabetic rats', *Food and Chemical Toxicology*, 48: 2920-24.

Sidhu, Om P., Vishal Kumar, and Hari M. Behl. 2004. 'Variability in triterpenoids (nimbin and salanin) composition of neem among different provenances of India', *Industrial Crops and Products*, 19: 69-75.

Sidhu, OP, and HM Behl. 1996. 'Seasonal variation in azadirachtins in seeds of Azadirachta indica', *Current Science*: 1084-86.

Silva, Jeane, Worku Abebe, SM Sousa, VG Duarte, MIL Machado, and FJA Matos. 2003. 'Analgesic and anti-inflammatory effects of essential oils of Eucalyptus', *Journal of ethnopharmacology*, 89: 277-83.

Smilde, Age K, Paul Geladi, and Rasmus Bro. 2005. *Multi-way analysis: applications in the chemical sciences* (John Wiley & Sons).

Snyder, Lloyd R, Joseph J Kirkland, and John W Dolan. 2011. *Introduction to modern liquid chromatography* (John Wiley & Sons).

Snyder, LR. 1978. 'Classification off the solvent properties of common liquids', *Journal of Chromatographic Science*, 16: 223-34.

Somwong, P., and O. Theanphong. 2021. 'Quantitative analysis of triterpene lupeol and anti-inflammatory potential of the extracts of traditional pain-relieving medicinal plants Derris scandens, Albizia procera, and Diospyros rhodocalyx', *J Adv Pharm Technol Res*, 12: 147-51.

Song, Jae-Min, Kwang-Hee Lee, and Baik-Lin Seong. 2005. 'Antiviral effect of catechins in green tea on influenza virus', *Antiviral research*, 68: 66-74.

Song, Li, Jin Wang, Quan Gao, Xiaojiang Ma, Yuwei Wang, Yaoyao Zhang, Hang Xun, Xi Yao, and Feng Tang. 2018. 'Simultaneous determination of five azadirachtins in the seed and leaf extracts of Azadirachta indica by automated online solid-phase extraction coupled with LC–Q-TOF–MS', *Chemistry Central Journal*, 12: 1-9.

Strzemski, Maciej, Magdalena Wójciak-Kosior, Ireneusz Sowa, Ewelina Rutkowska, Wojciech Szwerc, Ryszard Kocjan, and Michał Latalski. 2016. 'Carlina species as a new source of bioactive pentacyclic triterpenes', *Industrial Crops and Products*, 94: 498-504.

Suekawa, M, K Yuasa, M Isono, H Sone, Y Ikeya, I Sakakibara, M Aburada, and E Hosoya. 1986. 'Pharmacological studies on ginger. IV. Effect of (6)-shogaol on the arachidonic cascade', *Nihon Yakurigaku zasshi. Folia Pharmacologica Japonica*, 88: 263-69.

Suekawa, Mamoru., Atsushi. Ishige, Kazunori. Yuasa, Kazuhiko. Sudo, Masaki. Aburada, and Eikichi. Hosoya. 1984. 'Pharmacological studies on ginger. I. Pharmacological actions of pungent constituents,(6)-gingerol and (6)-shogaol', *Journal of pharmacobio-dynamics*, 7: 836-48.

Sunitha, S., M. Nagaraj, and P. Varalakshmi. 2001. 'Hepatoprotective effect of lupeol and lupeol linoleate on tissue antioxidant defence system in cadmium-induced hepatotoxicity in rats', *Fitoterapia*, 72: 516-23.

Suttiarporn, P., and V. Choommongkol. 2020. 'Microwave-Assisted Improved Extraction and Purification of Anticancer Nimbolide from Azadirachta indica (Neem) Leaves', *Molecules*, 25.

Suyal, R., A. K. Jugran, R. S. Rawal, and I. D. Bhatt. 2021. 'Morphological, phytochemical and genetic diversity of threatened Polygonatum verticillatum (L.) All. populations of different altitudes and habitat types in Himalayan region', *Physiol Mol Biol Plants*, 27: 1795-809.

Sydiskis, RJ, DG Owen, JL Lohr, KH Rosler, and RN Blomster. 1991. 'Inactivation of enveloped viruses by anthraquinones extracted from plants', *Antimicrobial agents and chemotherapy*, 35: 2463-66.

Takaishi, Masayuki, Fumitaka Fujita, Kunitoshi Uchida, Satoshi Yamamoto, Maki Sawada, Chihiro Hatai, Mayumi Shimizu, and Makoto Tominaga. 2012. '1, 8-cineole, a TRPM8 agonist, is a novel natural antagonist of human TRPA1', *Molecular pain*, 8: 1744-8069-8-86.

Tanaka, Tomonori, Tsuyoshi Ikeda, Miho Kaku, Xing-Hua Zhu, Masafumi Okawa, Kazumi Yokomizo, Masaru Uyeda, and Toshihiro Nohara. 2004. 'A new Lignan glycoside and phenylethanoid glycosides from Strobilanthes cusia B REMEK', *Chemical and pharmaceutical bulletin*, 52: 1242-45.

Tang, Yuhan, Yanyan Li, Haiyan Yu, Chao Gao, Liang Liu, Mingyou Xing, Liegang Liu, and Ping Yao. 2014. 'Quercetin attenuates chronic ethanol hepatotoxicity: implication of “free” iron uptake and release', *Food and Chemical Toxicology*, 67: 131-38.

Tao, Y., W. Li, W. Liang, and R. B. Van Breemen. 2009. 'Identification and quantification of gingerols and related compounds in ginger dietary supplements using high-performance liquid chromatography-tandem mass spectrometry', *J Agric Food Chem*, 57: 10014-21.

Tatke, Pratima, Sonal Desai, and SY Gabhe. 2014. 'Estimation of betulinic acid in extracts and formulations containing Albizialebbeck by HPLC', *World Journal of Pharmaceutical Research*, 3: 1277-86.

Thirumalaisamy, R, Fuad Ameen, A Subramanian, T Selvankumar, SS Alwakeel, and M Govarthanan. 2020. 'In-vitro and in-silico anti-inflammatory activity of Lupeol isolated from Crateva adansonii and its hidden molecular mechanism', *International Journal of Peptide Research and Therapeutics*, 26: 2179-89.

Thoh, Maikho, Banaganapalli Babajan, Pongali B Raghavendra, Chitta Sureshkumar, and Sunil K Manna. 2011. 'Azadirachtin interacts with retinoic acid receptors and inhibits retinoic acid-mediated biological responses', *Journal of Biological Chemistry*, 286: 4690-702.

Thomas, Micah, and Paul A Bomar. 2020. 'Upper respiratory tract infection', *StatPearls [Internet]*.

Tistaert, Christophe, Bieke Dejaegher, and Yvan Vander Heyden. 2011. 'Chromatographic separation techniques and data handling methods for herbal fingerprints: a review', *Analytica chimica acta*, 690: 148-61.

Tiwari, Vaibhav, Nissar A Darmani, Beatrice YJT Yue, and Deepak Shukla. 2010. 'In vitro antiviral activity of neem (Azardirachta indica L.) bark extract against herpes simplex virus type-1 infection', *Phytotherapy Research*, 24: 1132-40.

Vahedi, Ghasem, Mehdi Taghavi, Amin Kheirollahzade Maleki, and Reza Habibian. 2011. 'The effect of Aloe vera extract on humoral and cellular immune response in rabbit', *African Journal of Biotechnology*, 10: 5225-28.

Valtuena, Silvia, Nicoletta Pellegrini, Laura Franzini, Marta A Bianchi, Diego Ardigo, Daniele Del Rio, PierMarco Piatti, Francesca Scazzina, Ivana Zavaroni, and Furio Brighenti. 2008. 'Food selection based on total antioxidant capacity can modify antioxidant intake, systemic inflammation, and liver function without altering markers of oxidative stress', *The American journal of clinical nutrition*, 87: 1290-97.

Vasconcelos, Juliana Fraga, Mauro Martins Teixeira, José Maria Barbosa-Filho, Ana Silvia Suassuna Carneiro Lúcio, Jackson Roberto Guedes da Silva Almeida, LP De Queiroz, R Ribeiro-dos-Santos, and Milena Botelho Pereira Soares. 2008. 'The triterpenoid lupeol attenuates allergic airway inflammation in a murine model', *International immunopharmacology*, 8: 1216-21.

Viapiana, Agnieszka, Wiktoria Struck-Lewicka, Pawel Konieczynski, Marek Wesolowski, and Roman Kaliszan. 2016. 'An approach based on HPLC-fingerprint and chemometrics to quality consistency evaluation of Matricaria chamomilla L. commercial samples', *Frontiers in plant science*, 7: 1561.

Vieira, Aline B, Luciana P Coelho, Daniella BR Insuela, Vinicius F Carvalho, Marcelo H Dos Santos, Patricia MR Silva, and Marco A Martins. 2013. 'Mangiferin prevents guinea pig tracheal contraction via activation of the nitric oxide-cyclic GMP pathway', *PLoS One*, 8: e71759.

Vlietinck, Arnold, Luc Pieters, and Sandra Apers. 2009. 'Legal requirements for the quality of herbal substances and herbal preparations for the manufacturing of herbal medicinal products in the European Union', *Planta Medica*, 75: 683-88.

Wagner, Hildebert, Rudolf Bauer, Dieter Melchart, Pei-Gen Xiao, and Anton Staudinger. 2011. *Chromatographic fingerprint analysis of herbal medicines* (Springer).

Wal, P, A Wal, G Sharma, and A Rai. 2011. 'Biological activities of lupeol', *Systematic Reviews in Pharmacy*, 2: 96-96.

Wang, Hwang H, Jing G Chung, Chen C Ho, Lii T Wu, and Shih H Chang. 1998. 'Aloe-emodin effects on arylamine N-acetyltransferase activity in the bacterium Helicobacter pylori', *Planta Medica*, 64: 176-78.

Wang, Pei, Lei Nie, and Hengchang Zang. 2015. 'A useful strategy to evaluate the quality consistency of traditional Chinese medicines based on liquid chromatography and chemometrics', *Journal of analytical methods in chemistry*, 2015.

Wang, Wu, Filip Cuyckens, Hilde Van den Heuvel, Sandra Apers, Luc Pieters, Vanessa Steenkamp, Michael J Stewart, Valerie A Luyckx, and Magda Claeys. 2003. 'Structural characterization of chromone C-glucosides in a toxic herbal remedy', *Rapid communications in mass spectrometry*, 17: 49-55.

Wang, Yafei, Yuqing Tang, Zhou Li, Qihang Hua, Li Wang, Xin Song, Baobo Zou, Min Ding, Jinshun Zhao, and Chunlan Tang. 2020. 'Joint toxicity of a multi-heavy metal mixture and chemoprevention in sprague dawley rats', *International Journal of Environmental Research and Public Health*, 17: 1451.

Weidner, Morten Sloth, and Katrin Sigwart. 2000. 'Investigation of the teratogenic potential of a Zingiber officinale extract in the rat', *Reproductive Toxicology*, 15: 75-80.
West African Health Organization. 2013. "West African Herbal Pharmacopoeia " In. BOBO-DIOULASSO (BURKINA FASO).
World Bank. 2013. "Turn down the heat: Climate extremes, regional impacts, and the case for resilience." In.: The World Bank.
World Health Organization. 2019. *WHO global report on traditional and complementary medicine 2019* (World Health Organization).
World Health Organization. 1999. *WHO monographs on selected medicinal plants* (World Health Organization).
——. 2003. *WHO guidelines on good agricultural and collection practices [GACP] for medicinal plants* (World Health Organization).
——. 2007a. *WHO guidelines on good manufacturing practices (GMP) for herbal medicines* (World Health Organization).
——. 2007b. *WHO monographs on selected medicinal plants : volume 3* (World Health Organization: Geneve).
——. 2009. *WHO monographs on selected medicinal plants : volume 4* (World Health Organization: Geneve).
——. 2011. *Quality control methods for herbal materials* (World Health Organization).
——. 2016. "WHO guidelines for selecting marker substances of herbal origin for quality control of herbal medicines." In, edited by WHO Expert Committee on Specifications for Pharmaceutical Preparations, 72-85.
——. 2018. 'WHO guidelines on good herbal processing practices for herbal medicines', *WHO Technical Report Series*.
World Health Organization, . 2019. *WHO global report on traditional and complementary medicine 2019* (World Health Organization).
Wu, HM, F Xu, YM Wang, HB Qian, and XP Wang. 2017. 'Influence of general situation, glucose tolerance and insulin tolerance for lupenone in insulin resistance of type 2 diabetes rats', *Lishizhen Med Mater Med Res*, 5: 1035-37.
Wu, Lingfeng, Wenhui Wu, Yanzi Cai, Congfa Li, and Lu Wang. 2020. 'HPLC fingerprinting-based multivariate analysis of phenolic compounds in mango leaves varieties: Correlation to their antioxidant activity and in silico α-glucoidase inhibitory ability', *Journal of Pharmaceutical and Biomedical Analysis*, 191: 113616.
Wube, Abraham Abebe, Franz Bucar, Simon Gibbons, and Kaleab Asres. 2005. 'Sesquiterpenes from Warburgia ugandensis and their antimycobacterial activity', *Phytochemistry*, 66: 2309-15.
Xie, P., S. Chen, Y. Z. Liang, X. Wang, R. Tian, and R. Upton. 2006a. 'Chromatographic fingerprint analysis--a rational approach for quality assessment of traditional Chinese herbal medicine', *J Chromatogr A*, 1112: 171-80.
Xie, Peishan, Sibao Chen, Yi-zeng Liang, Xianghong Wang, Runtao Tian, and Roy Upton. 2006b. 'Chromatographic fingerprint analysis—a rational approach for quality assessment of traditional Chinese herbal medicine', *Journal of chromatography A*, 1112: 171-80.
Xu, Lina, Xu Han, Yan Qi, Youwei Xu, Lianhong Yin, Jinyong Peng, Kexin Liu, and Changkai Sun. 2009. 'Multiple compounds determination and fingerprint analysis of

Lidanpaishi tablet and keli by high-performance liquid chromatography', *Analytica chimica acta*, 633: 136-48.
Yamahara, Johji, Huang Qi Rong, Masatsugu Iwamoto, Goro Kobayashi, Hisashi Matsuda, and Hajime Fujimura. 1989. 'Active components of ginger exhibiting anti-serotonergic action', *Phytotherapy Research*, 3: 70-71.
Yanaso, Suthira, Darunee Hongwiset, Sirivipa Piyamongkol, Aekkhaluck Intharuksa, and Ampai Phrutivorapongkul. 2021. 'Quantitation of lupeol from stem bark extract of Betula alnoides Buch.-Ham. ex D. Don by two validated RP-HPLC and TLC-densitometric methods', *Journal of Liquid Chromatography & Related Technologies*: 1-11.
Yang, Bin, Yuan Wang, Lanlan Shan, Jingtao Zou, Yuanyuan Wu, Feifan Yang, Yani Zhang, Yubo Li, and Yanjun Zhang. 2017. 'A novel and practical chromatographic "Fingerprint-ROC-SVM" strategy applied to quality analysis of traditional Chinese medicine injections: Using KuDieZi injection as a case study', *Molecules*, 22: 1237.
Yang, Na, Aizhen Xiong, Rui Wang, Li Yang, and Zhengtao Wang. 2016. 'Quality evaluation of traditional Chinese medicine compounds in Xiaoyan Lidan tablets: fingerprint and quantitative analysis using UPLC-MS', *Molecules*, 21: 83.
Yerima, MB, SM Jodi, K Oyinbo, HM Maishanu, AA Farouq, AU Junaidu, MN Al-Mustapha, and AL Shinkafi. 2012. 'Effect of neem extracts (Azadirachta indica) on bacteria isolated from adult mouth', *Nigerian Journal of Basic and Applied Sciences*, 20: 64-67.
Yun, Yunha, Shinha Han, Eunjung Park, Dongsool Yim, Sookyeon Lee, Chong-Kil Lee, Kyunghae Cho, and Kyungjae Kim. 2003. 'Immunomodulatory activity of betulinic acid by producing pro-inflammatory cytokines and activation of macrophages', *Archives of pharmacal research*, 26: 1087-95.
Zehsaz, Farzad, Negin Farhangi, and Lamia Mirheidari. 2014. 'The effect of Zingiber officinale R. rhizomes (ginger) on plasma pro-inflammatory cytokine levels in well-trained male endurance runners', *Central-European journal of immunology*, 39: 174.
Zeng, Yan, Anna Nikitkova, Hossam Abdelsalam, Jiyao Li, and Jin Xiao. 2019. 'Activity of quercetin and kaemferol against Streptococcus mutans biofilm', *Archives of oral biology*, 98: 9-16.
Zhang, Mingzhen, Emilie Viennois, Meena Prasad, Yunchen Zhang, Lixin Wang, Zhan Zhang, Moon Kwon Han, Bo Xiao, Changlong Xu, and Shanthi Srinivasan. 2016. 'Edible ginger-derived nanoparticles: A novel therapeutic approach for the prevention and treatment of inflammatory bowel disease and colitis-associated cancer', *Biomaterials*, 101: 321-40.
Zhang, Qing-Wen, Li-Gen Lin, and Wen-Cai Ye. 2018. 'Techniques for extraction and isolation of natural products: A comprehensive review', *Chinese medicine*, 13: 1-26.
Zhang, Xiejun, Benwei Su, Jing Li, Yonghua Li, Dong Lu, Kaixin Zhu, Hehuan Pei, and Minghui Zhao. 2012. 'Analysis by RP-HPLC of Mangiferin Component Correlation between Medicinal Loranthus and their Mango Host Trees', *Journal of Chromatographic Science*, 52: 1-4.
——. 2014. 'Analysis by RP-HPLC of mangiferin component correlation between medicinal loranthus and their mango host trees', *Journal of chromatographic science*, 52: 1-4.
Zheng, M. S., and Z. Y. Lu. 1990. 'Antiviral effect of mangiferin and isomangiferin on herpes simplex virus', *Chin Med J (Engl)*, 103: 160-5.

Zhou, Yuhong, Yongquan Feng, Huiling Wang, and Hua Yang. 2003. '90-day subchronic toxicity study of aloe whole-leaf powder', *Wei sheng yan jiu= Journal of hygiene research*, 32: 590-93.

Zhu, XM, JX Song, ZZ Huang, YM Wu, and MJ Yu. 1993. 'Antiviral activity of mangiferin against herpes simplex virus type 2 in vitro', *Zhongguo yao li xue bao= Acta Pharmacologica Sinica*, 14: 452-54.

APPENDICES

Appendix 1: Paper 1

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Research Article

Safety and Efficacy of Medicinal Plants Used to Manufacture Herbal Products with Regulatory Approval in Uganda: A Cross-Sectional Study

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Introduction. The Uganda National Drug Authority requires phytochemical screening, freedom from microbial contamination, and evidence of safety and efficacy of the constituent plants to register herbal products. Since Uganda has no pharmacopeia, safety, efficacy, and plant processing information are not readily available. We documented the plant materials used to manufacture products in Uganda and established evidence of their safety and efficacy and availability of monographs. *Methods*. The NDA register of herbal products was reviewed, and a product list was extracted. The herbal products were purchased from local pharmacies, and their labels were studied to identify plant ingredients and drug use. Literature was reviewed to document evidence of the safety and efficacy of the plant materials concerning manufacturer's claims. Also, the WHO and available African Pharmacopeia were searched to establish the availability of the plant monographs. *Results*. Of the 84 NDA-registered local products, only 18 were obtained from the market; 82% were indicated for respiratory tract disorders. Thirty-three plant materials were listed with *Eucalyptus globulus* Labill, being the commonest. Several *in vitro* and *in vivo* studies demonstrate efficacy, thus supporting the use of the selected plant species for empirical treatment as stated on the product label. While most plants were safe, some species such as *Albizia coriaria* Oliv. had dose-dependent toxicities that cannot be predicted in combinations. The WHO, African Pharmacopoeia, and West African Herbal Pharmacopoeia had only 16 plant monographs of the 33 plants of interest. Nevertheless, *Aloe vera* (L.) Burm.f., *Azadirachta indica* A.Juss., *Zingiber officinale* Roscoe, and *Allium sativum* L. monographs were published by all three pharmacopoeias. *Conclusions*. Preclinical evidence of safety and efficacy exists in the literature for most of the plants used to manufacture registered herbal products in Uganda. More specific bioassays and clinical trials are required for the products to provide conclusive evidence of safety and toxicity. Monographs are urgently needed for the Ugandan plants.

Appendix 2: Paper 2

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Journal of Medicinal Plants Research

Full Length Research Paper

Evaluation of catechin, lupeol, and betulinic acid as markers for the chromatographic quality Control of Albizia coriaria raw materials; an experimental study

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Albizia coriaria **stembark is among the most common raw materials used to manufacture herbal products in Uganda. While the plant material is sourced from the wild, there are neither monographs nor chemical analytical methods for its standardisation. In addition, good cultivation, harvesting, and manufacturing practices are not mandatory. This leads to inconsistent quality of raw materials and products. This work developed TLC and HPLC methods and evaluated the suitability of lupeol, catechin, and betulinic acid markers for standardization of stembark from *A. coriaria.* Samples were extracted by decoction, maceration and soxhlet reflux; in water, ethanol and ethyl acetate. Catechin, lupeol and betulinic acid markers were found in all batches of *A. coriaria* from different agroecological zones, at concentrations higher than 6mg/g. Ethyl acetate fractions gave the highest marker quantities, while decoction gave the lowest. The optimised TLC fingerprint conditions were solvent system: 4, 2, 1 and 1 parts of n-hexane, ethyl acetate, dichloromethane, and methanol, respectively; stationary phase; TLC glass plates, 10 cm × 20 cm, coated with fluorescent 60Å silica gel matrices and vanillin / sulfuric acid spray reagent. The resulting fingerprints comprised of twelve principal peaks. The optimized conditions of the HPLC fingerprint and quantitation method were: C18 column (250 × 4.6 mm x 5 µm), solvent system; acetonitrile (99.99%) and 1% trifluoroacetic acid (0.01%); flow rate, 1mL/min; column temperature, 25°C; and UV/visible detector 205 nm. The common HPLC fingerprints had correlation coefficient >0.99, while quantitative methods were accurate (>97% recovery) and reproducible (RSD < 0.38%). Lupeol and betulinic acid are suitable markers for the quality control of *Albizia coriaria* raw materials. decoctions are better controlled by catechin.**

Key words: Markers, fingerprints, *Albizia coriaria*, betulinic acid, lupeol, quality control, standardisation.

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Research Article

Chemometric Classification of *Mangifera indica* L. Leaf Cultivars, Based on Selected Phytochemical Parameters; Implications for Standardization of the Pharmaceutical Raw Materials

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Introduction. *Mangifera indica* leaves are among the most common materials employed in manufacturing herbal medicinal products. Despite the phytochemical variation of *M. indica* cultivars, there are no monographs to guide the cultivation, processing, and authentication of the materials. *Methods*. This study characterized 15 Ugandan *M. indica* leaf varieties, with reference to extraction index (EI), total phenolic content (TPC), antioxidant activity (AOA), and mangiferin concentration (MC). In addition, HPLC fingerprints were established to evaluate the overall phytoequivalence of the materials. Then, using hierarchical clustering (HC) and principal component analysis (PCA), the materials were assigned quality grades. *Results*. The mean EI was $9.39 \pm 1.64\%$ and varied among the varieties ($P = 0.001$); the TPC varied significantly ($P < 0.0001$), from 183.29 ± 2.36 mg/g (Takataka) to 79.47 ± 0.58 mg/g (Apple mango). AOA ranged from 16.81 ± 2.85 μg/mL (Doodo red) to 87.85 μg/mL (Asante). MC varied significantly ($P < 0.0001$), from 105.75 ± 0.60 mg/g (Kate) to 39.53 ± 0.30 mg/g (Asante). HC gave four major grades: A to D (A, varieties with the highest TPC, MC, and AOA). These parameters reduced to below average from group B to group D. The chromatographic fingerprints were visually similar, but the number of peaks varied, from 19 (Kawanda green) to 29 (Kawanda wide), with 23.5 ± 2.9 average peaks. Whole fingerprints were less similar ($r < 0.8$) than common peak fingerprints ($r > 0.9$, $P < 0.001$). PCA grouped the fingerprints into five clusters; loading plots for PC 1 and 2 revealed two important compounds, one at Rt = 15.828 minutes (mangiferin) and the other at 6.021 minutes. Using the standardized common fingerprints, unknown field samples clustered closely with Koona, Kate, and Kawanda green varieties. *Conclusions*. The EI, TPC, MC, and AOA values can be utilized to monitor consistency in the quality of materials and the production process. The grades generated can be used to select materials for cultivation and manufacturing. Where minimum concentrations are set, materials of different concentrations are used to dilute or concentrate each other. The HPLC fingerprints can be utilized to authenticate the materials. More samples from different agroecological regions of the country should be tested to cater to climatic variations in order to develop GMP-compliant botanical identification methods.

Appendix 4: Paper 4

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Application of the herbal chemical marker ranking system (Herb MaRS) to the standardization of herbal raw materials: a case study

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Abstract

Introduction Phytochemical standardization of herbal materials involves establishing consistent levels of one or more active ingredients or markers. It ensures the authenticity and quality of herbal materials, extracts, and their products. This research aimed to apply the herbal chemical marker ranking system (Herb MaRS) originally proposed for quality assurance of complex herbal products to establish markers for controlling the quality of herbal raw materials.

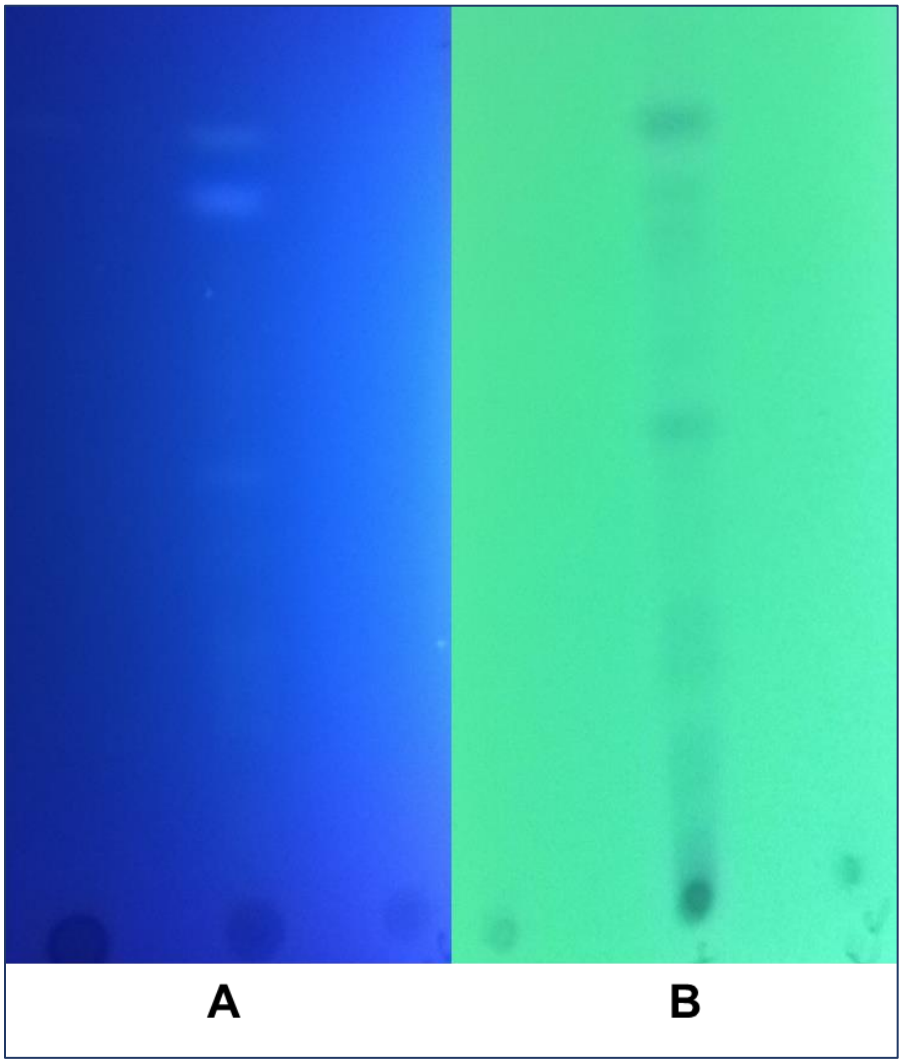
Methods The assessment of compounds for suitability as markers was based on the Herb MaRS, with minor modifications as follows: for more objective scoring, evidence of biological activity of the potential marker compound(s) was determined at three levels based on the number of symptoms of the disease condition a compound can treat or alleviate: (i) one symptom (1 point), two symptoms (2 points), and 3 or more symptoms (3 points). The reported concentrations of the compounds were also scored as follows: concentration not determined (0 points), concentration ≥ 5 ppm (1 point), concentration ≥ 50 ppm (2 points) and availability of analytical standards (1 point). Finally, the compounds were scored for the availability of an analytical method (1 point). The compounds were scored from 0 to 8, where 8 indicated the most suitable chemical marker.

Results The selected markers were as follows: aromadendrine, α-terpineol, globulol, and 1,8-cineol (in *Eucalyptus globulus* Labill.); aloin, aloe emodin, acemannan (in *Aloe barbadensis* (L.) Burm.f.), lupeol, lupenone, betulinic acid, betulin, and catechin (in *Albizia coriaria* Oliv.); mangiferin, catechin, quercetin, and gallic acid (in *Mangifera indica* L.); polygodial (in *Warburgia ugandensis* Sprague); azadirachtin, nimbin, nimbidin (in *Azadirachta indica* A. Juss.); and 6,8,10-gingerols, and 6-shogaol (in *Zingiber officinalis* Roscoe).

Conclusions Herb MaRS can be efficiently applied to select marker compounds for quality control of herbal materials. However, for herbs whose phytochemicals have not been sufficiently researched, it is difficult to establish evidence of activity, and there are no analytical standards and/or methods; this is the case for plants exclusively used in Africa. The markers identified should be incorporated into chromatographic fingerprints, their quantitative methods developed, and evaluated for applicability at the various stages of the production chain of herbal medicines; then,

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Appendix 5: Typical fingerprint for Albizia coriaria stembark raw material; Solvent system; 4n-hexane:2ethylacetate:1dichloromethane:1methanol. Visualization A: UV 254nm, B: UV 366nm.

Appendix 6: Tukey's multiple comparisons test for concentration of lupeol in Albizia coriaria stembark raw materials from different agroecological sources.

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Adjusted P Value
Dokolo vs. Iganga	-1.152	-1.351 to -0.9524	<0.0001
Dokolo vs. Kabarole	0.2753	0.07611 to 0.4746	0.0047
Dokolo vs. Masindi	0.3290	0.1298 to 0.5282	0.0009
Dokolo vs. Moroto	0.8447	0.6454 to 1.044	<0.0001
Dokolo vs. Mpigi	-3.737	-3.936 to -3.538	<0.0001
Dokolo vs. Pakwach	0.6473	0.4481 to 0.8466	<0.0001
Iganga vs. Kabarole	1.427	1.228 to 1.626	<0.0001
Iganga vs. Masindi	1.481	1.281 to 1.680	<0.0001
Iganga vs. Moroto	1.996	1.797 to 2.196	<0.0001

Iganga vs. Mpigi	-2.585	-2.785 to -2.386	<0.0001
Iganga vs. Pakwach	1.799	1.600 to 1.998	<0.0001
Kabarole vs. Masindi	0.05367	-0.1456 to 0.2529	0.9630
Kabarole vs. Moroto	0.5693	0.3701 to 0.7686	<0.0001
Kabarole vs. Mpigi	-4.012	-4.212 to -3.813	<0.0001
Kabarole vs. Pakwach	0.3720	0.1728 to 0.5712	0.0003
Masindi vs. Moroto	0.5157	0.3164 to 0.7149	<0.0001
Masindi vs. Mpigi	-4.066	-4.265 to -3.867	<0.0001
Masindi vs. Pakwach	0.3183	0.1191 to 0.5176	0.0013
Moroto vs. Mpigi	-4.582	-4.781 to -4.382	<0.0001
Moroto vs. Pakwach	-0.1973	-0.3966 to 0.001889	0.0530
Mpigi vs. Pakwach	4.384	4.185 to 4.584	<0.0001

Appendix 7: Tukey's multiple comparisons test for concentration of Betulinic acid in Albizia coriaria stembark raw materials from different agroecological sources.

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Adjusted P Value
Dokolo vs. Iganga	-8.064	-8.289 to -7.840	<0.0001
Dokolo vs. Kabarole	4.878	4.653 to 5.102	<0.0001
Dokolo vs. Masindi	0.1837	-0.04051 to 0.4078	0.1443
Dokolo vs. Moroto	-2.336	-2.561 to -2.112	<0.0001
Dokolo vs. Mpigi	4.251	4.027 to 4.475	<0.0001
Dokolo vs. Pakwach	2.092	1.868 to 2.316	<0.0001
Iganga vs. Kabarole	12.94	12.72 to 13.17	<0.0001
Iganga vs. Masindi	8.248	8.024 to 8.472	<0.0001
Iganga vs. Moroto	5.728	5.504 to 5.952	<0.0001
Iganga vs. Mpigi	12.32	12.09 to 12.54	<0.0001
Iganga vs. Pakwach	10.16	9.932 to 10.38	<0.0001
Kabarole vs. Masindi	-4.694	-4.918 to -4.470	<0.0001
Kabarole vs. Moroto	-7.214	-7.438 to -6.990	<0.0001
Kabarole vs. Mpigi	-0.6267	-0.8508 to -0.4025	<0.0001
Kabarole vs. Pakwach	-2.786	-3.010 to -2.561	<0.0001
Masindi vs. Moroto	-2.520	-2.744 to -2.296	<0.0001
Masindi vs. Mpigi	4.067	3.843 to 4.292	<0.0001
Masindi vs. Pakwach	1.908	1.684 to 2.133	<0.0001
Moroto vs. Mpigi	6.587	6.363 to 6.812	<0.0001
Moroto vs. Pakwach	4.428	4.204 to 4.653	<0.0001
Mpigi vs. Pakwach	-2.159	-2.383 to -1.935	<0.0001

Appendix 8: Tukey's multiple comparisons of leaf TPC with source variety of Mangifera indica.

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Adjusted P Value
Apple M vs. Sejjembe	-10.02	-19.94 to -0.09368	0.0468
Apple M vs. Kawanda green	-13.63	-23.55 to -3.704	0.0035
Apple M vs. Kate	-35.54	-45.46 to -25.62	<0.0001

Apple M vs. Asante	-2.380	-12.30 to 7.541	0.9992
Apple M vs. Kawanda wide	-12.56	-22.48 to -2.639	0.0075
Apple M vs. Suu	-19.37	-29.29 to -9.449	<0.0001
Apple M vs. Koona	-38.66	-48.58 to -28.74	<0.0001
Apple M vs. Kagoogwa	-21.34	-31.26 to -11.42	<0.0001
Apple M vs. MPI	9.360	-0.5613 to 19.28	0.0740
Apple M vs. Takataka	-13.79	-23.71 to -3.869	0.0031
Apple M vs. Boribo	-22.66	-32.58 to -12.73	<0.0001
Apple M vs. Ngoogwe	8.455	-1.466 to 18.38	0.1362
Apple M vs. Bire	-2.055	-11.98 to 7.866	0.9998
Apple M vs. Doodo red	-0.5750	-10.50 to 9.346	>0.9999
Sejjembe vs. Kawanda green	-3.610	-13.53 to 6.311	0.9662
Sejjembe vs. Kate	-25.53	-35.45 to -15.60	<0.0001
Sejjembe vs. Asante	7.635	-2.286 to 17.56	0.2282
Sejjembe vs. Kawanda wide	-2.545	-12.47 to 7.376	0.9984
Sejjembe vs. Suu	-9.355	-19.28 to 0.5663	0.0743
Sejjembe vs. Koona	-28.65	-38.57 to -18.72	<0.0001
Sejjembe vs. Kagoogwa	-11.33	-21.25 to -1.404	0.0183
Sejjembe vs. MPI	19.38	9.454 to 29.30	<0.0001
Sejjembe vs. Takataka	-3.775	-13.70 to 6.146	0.9534
Sejjembe vs. Boribo	-12.64	-22.56 to -2.719	0.0071
Sejjembe vs. Ngoogwe	18.47	8.549 to 28.39	0.0001
Sejjembe vs. Bire	7.960	-1.961 to 17.88	0.1869
Sejjembe vs. Doodo red	9.440	-0.4813 to 19.36	0.0700
Kawanda green vs. Kate	-21.92	-31.84 to -11.99	<0.0001
Kawanda green vs. Asante	11.25	1.324 to 21.17	0.0194
Kawanda green vs. Kawanda wide	1.065	-8.856 to 10.99	>0.9999
Kawanda green vs. Suu	-5.745	-15.67 to 4.176	0.5965
Kawanda green vs. Koona	-25.04	-34.96 to -15.11	<0.0001
Kawanda green vs. Kagoogwa	-7.715	-17.64 to 2.206	0.2174
Kawanda green vs. MPI	22.99	13.06 to 32.91	<0.0001
Kawanda green vs. Takataka	-0.1650	-10.09 to 9.756	>0.9999
Kawanda green vs. Boribo	-9.030	-18.95 to 0.8913	0.0928
Kawanda green vs. Ngoogwe	22.08	12.16 to 32.00	<0.0001
Kawanda green vs. Bire	11.57	1.649 to 21.49	0.0154
Kawanda green vs. Doodo red	13.05	3.129 to 22.97	0.0053
Kate vs. Asante	33.16	23.24 to 43.08	<0.0001
Kate vs. Kawanda wide	22.98	13.06 to 32.90	<0.0001
Kate vs. Suu	16.17	6.249 to 26.09	0.0006
Kate vs. Koona	-3.120	-13.04 to 6.801	0.9894
Kate vs. Kagoogwa	14.20	4.279 to 24.12	0.0023
Kate vs. MPI	44.90	34.98 to 54.82	<0.0001
Kate vs. Takataka	21.75	11.83 to 31.67	<0.0001
Kate vs. Boribo	12.89	2.964 to 22.81	0.0060
Kate vs. Ngoogwe	44.00	34.07 to 53.92	<0.0001

Kate vs. Bire	33.49	23.56 to 43.41	<0.0001
Kate vs. Doodo red	34.97	25.04 to 44.89	<0.0001
Asante vs. Kawanda wide	-10.18	-20.10 to -0.2587	0.0416
Asante vs. Suu	-16.99	-26.91 to -7.069	0.0004
Asante vs. Koona	-36.28	-46.20 to -26.36	<0.0001
Asante vs. Kagoogwa	-18.96	-28.88 to -9.039	<0.0001
Asante vs. MPI	11.74	1.819 to 21.66	0.0136
Asante vs. Takataka	-11.41	-21.33 to -1.489	0.0172
Asante vs. Boribo	-20.28	-30.20 to -10.35	<0.0001
Asante vs. Ngoogwe	10.84	0.9137 to 20.76	0.0261
Asante vs. Bire	0.3250	-9.596 to 10.25	>0.9999
Asante vs. Doodo red	1.805	-8.116 to 11.73	>0.9999
Kawanda wide vs. Suu	-6.810	-16.73 to 3.111	0.3641
Kawanda wide vs. Koona	-26.10	-36.02 to -16.18	<0.0001
Kawanda wide vs. Kagoogwa	-8.780	-18.70 to 1.141	0.1098
Kawanda wide vs. MPI	21.92	12.00 to 31.84	<0.0001
Kawanda wide vs. Takataka	-1.230	-11.15 to 8.691	>0.9999
Kawanda wide vs. Boribo	-10.10	-20.02 to -0.1737	0.0442
Kawanda wide vs. Ngoogwe	21.02	11.09 to 30.94	<0.0001
Kawanda wide vs. Bire	10.51	0.5837 to 20.43	0.0330
Kawanda wide vs. Doodo red	11.99	2.064 to 21.91	0.0114
Suu vs. Koona	-19.29	-29.21 to -9.369	<0.0001
Suu vs. Kagoogwa	-1.970	-11.89 to 7.951	0.9999
Suu vs. MPI	28.73	18.81 to 38.65	<0.0001
Suu vs. Takataka	5.580	-4.341 to 15.50	0.6351
Suu vs. Boribo	-3.285	-13.21 to 6.636	0.9837
Suu vs. Ngoogwe	27.83	17.90 to 37.75	<0.0001
Suu vs. Bire	17.32	7.394 to 27.24	0.0003
Suu vs. Doodo red	18.80	8.874 to 28.72	0.0001
Koona vs. Kagoogwa	17.32	7.399 to 27.24	0.0003
Koona vs. MPI	48.02	38.10 to 57.94	<0.0001
Koona vs. Takataka	24.87	14.95 to 34.79	<0.0001
Koona vs. Boribo	16.01	6.084 to 25.93	0.0007
Koona vs. Ngoogwe	47.12	37.19 to 57.04	<0.0001
Koona vs. Bire	36.61	26.68 to 46.53	<0.0001
Koona vs. Doodo red	38.09	28.16 to 48.01	<0.0001
Kagoogwa vs. MPI	30.70	20.78 to 40.62	<0.0001
Kagoogwa vs. Takataka	7.550	-2.371 to 17.47	0.2401
Kagoogwa vs. Boribo	-1.315	-11.24 to 8.606	>0.9999
Kagoogwa vs. Ngoogwe	29.80	19.87 to 39.72	<0.0001
Kagoogwa vs. Bire	19.29	9.364 to 29.21	<0.0001
Kagoogwa vs. Doodo red	20.77	10.84 to 30.69	<0.0001
MPI vs. Takataka	-23.15	-33.07 to -13.23	<0.0001
MPI vs. Boribo	-32.02	-41.94 to -22.09	<0.0001
MPI vs. Ngoogwe	-0.9050	-10.83 to 9.016	>0.9999
MPI vs. Bire	-11.42	-21.34 to -1.494	0.0172
MPI vs. Doodo red	-9.935	-19.86 to -0.01368	0.0495

Takataka vs. Boribo	-8.865	-18.79 to 1.056	0.1037
Takataka vs. Ngoogwe	22.25	12.32 to 32.17	<0.0001
Takataka vs. Bire	11.74	1.814 to 21.66	0.0136
Takataka vs. Doodo red	13.22	3.294 to 23.14	0.0047
Boribo vs. Ngoogwe	31.11	21.19 to 41.03	<0.0001
Boribo vs. Bire	20.60	10.68 to 30.52	<0.0001
Boribo vs. Doodo red	22.08	12.16 to 32.00	<0.0001
Ngoogwe vs. Bire	-10.51	-20.43 to -0.5887	0.0329
Ngoogwe vs. Doodo red	-9.030	-18.95 to 0.8913	0.0928
Bire vs. Doodo red	1.480	-8.441 to 11.40	>0.9999

Appendix 9: Tukey's multiple comparisons of leaf Antioxidant activity with source variety of Mangifera indica.

Number of families	**1**		
Number of comparisons per family	105		
Alpha	0,05		
Tukey's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Adjusted P Value
Apple M vs. Sejjembe	17,44	-12,11 to 46,99	0,6787
Apple M vs. Kawanda green	1,747	-27,80 to 31,30	>0,9999
Apple M vs. Kate	37,06	7,508 to 66,61	0,0049
Apple M vs. Asante	-12,26	-41,81 to 17,29	0,9613
Apple M vs. Kawanda wide	1,610	-27,94 to 31,16	>0,9999
Apple M vs. Suu	21,72	-7,835 to 51,27	0,3467
Apple M vs. Koona	32,33	2,775 to 61,88	0,0219
Apple M vs. Kagoogwa	35,67	6,118 to 65,22	0,0076
Apple M vs. MPI	-10,47	-40,02 to 19,08	0,9896
Apple M vs. Takataka	32,59	3,035 to 62,14	0,0202
Apple M vs. Boribo	30,24	0,6885 to 59,79	0,0409
Apple M vs. Ngoogwe	28,48	-1,075 to 58,03	0,0678
Apple M vs. Bire	36,70	7,145 to 66,25	0,0055
Apple M vs. Doodo red	38,60	9,045 to 68,15	0,0029
Sejjembe vs. Kawanda green	-15,69	-45,24 to 13,86	0,8065

Sejjembe vs. Kate	19,62	-9,932 to 49,17	0,5028
Sejjembe vs. Asante	-29,70	-59,25 to -0,1485	0,0479
Sejjembe vs. Kawanda wide	-15,83	-45,38 to 13,72	0,7974
Sejjembe vs. Suu	4,277	-25,27 to 33,83	>0,9999
Sejjembe vs. Koona	14,89	-14,66 to 44,44	0,8559
Sejjembe vs. Kagoogwa	18,23	-11,32 to 47,78	0,6154
Sejjembe vs. MPI	-27,91	-57,46 to 1,642	0,0793
Sejjembe vs. Takataka	15,15	-14,40 to 44,70	0,8408
Sejjembe vs. Boribo	12,80	-16,75 to 42,35	0,9466
Sejjembe vs. Ngoogwe	11,04	-18,51 to 40,59	0,9835
Sejjembe vs. Bire	19,26	-10,29 to 48,81	0,5320
Sejjembe vs. Doodo red	21,16	-8,395 to 50,71	0,3857
Kawanda green vs. Kate	35,31	5,762 to 64,86	0,0086
Kawanda green vs. Asante	-14,01	-43,56 to 15,54	0,9009
Kawanda green vs. Kawanda wide	-0,1367	-29,69 to 29,41	>0,9999
Kawanda green vs. Suu	19,97	-9,582 to 49,52	0,4751
Kawanda green vs. Koona	30,58	1,028 to 60,13	0,0370
Kawanda green vs. Kagoogwa	33,92	4,372 to 63,47	0,0133
Kawanda green vs. MPI	-12,22	-41,77 to 17,33	0,9623
Kawanda green vs. Takataka	30,84	1,288 to 60,39	0,0343
Kawanda green vs. Boribo	28,49	-1,058 to 58,04	0,0675
Kawanda green vs. Ngoogwe	26,73	-2,822 to 56,28	0,1088
Kawanda green vs. Bire	34,95	5,398 to 64,50	0,0096
Kawanda green vs. Doodo red	36,85	7,298 to 66,40	0,0052
Kate vs. Asante	-49,32	-78,87 to -19,77	<0,0001
Kate vs. Kawanda wide	-35,45	-65,00 to -5,898	0,0082
Kate vs. Suu	-15,34	-44,89 to 14,21	0,8288
Kate vs. Koona	-4,733	-34,28 to 24,82	>0,9999

Kate vs. Kagoogwa	-1,390	-30,94 to 28,16	>0,9999
Kate vs. MPI	-47,53	-77,08 to -17,98	0,0001
Kate vs. Takataka	-4,473	-34,02 to 25,08	>0,9999
Kate vs. Boribo	-6,820	-36,37 to 22,73	0,9999
Kate vs. Ngoogwe	-8,583	-38,13 to 20,97	0,9985
Kate vs. Bire	-0,3633	-29,91 to 29,19	>0,9999
Kate vs. Doodo red	1,537	-28,01 to 31,09	>0,9999
Asante vs. Kawanda wide	13,87	-15,68 to 43,42	0,9070
Asante vs. Suu	33,98	4,425 to 63,53	0,0131
Asante vs. Koona	44,59	15,04 to 74,14	0,0004
Asante vs. Kagoogwa	47,93	18,38 to 77,48	0,0001
Asante vs. MPI	1,790	-27,76 to 31,34	>0,9999
Asante vs. Takataka	44,85	15,30 to 74,40	0,0004
Asante vs. Boribo	42,50	12,95 to 72,05	0,0008
Asante vs. Ngoogwe	40,74	11,19 to 70,29	0,0014
Asante vs. Bire	48,96	19,41 to 78,51	<0,0001
Asante vs. Doodo red	50,86	21,31 to 80,41	<0,0001
Kawanda wide vs. Suu	20,11	-9,445 to 49,66	0,4644
Kawanda wide vs. Koona	30,72	1,165 to 60,27	0,0356
Kawanda wide vs. Kagoogwa	34,06	4,508 to 63,61	0,0128
Kawanda wide vs. MPI	-12,08	-41,63 to 17,47	0,9655
Kawanda wide vs. Takataka	30,98	1,425 to 60,53	0,0329
Kawanda wide vs. Boribo	28,63	-0,9215 to 58,18	0,0650
Kawanda wide vs. Ngoogwe	26,87	-2,685 to 56,42	0,1050
Kawanda wide vs. Bire	35,09	5,535 to 64,64	0,0092
Kawanda wide vs. Doodo red	36,99	7,435 to 66,54	0,0050
Suu vs. Koona	10,61	-18,94 to 40,16	0,9883
Suu vs. Kagoogwa	13,95	-15,60 to 43,50	0,9033
Suu vs. MPI	-32,19	-61,74 to -2,635	0,0228
Suu vs. Takataka	10,87	-18,68 to 40,42	0,9855

Suu vs. Boribo	8,523	-21,03 to 38,07	0,9986
Suu vs. Ngoogwe	6,760	-22,79 to 36,31	0,9999
Suu vs. Bire	14,98	-14,57 to 44,53	0,8506
Suu vs. Doodo red	16,88	-12,67 to 46,43	0,7221
Koona vs. Kagoogwa	3,343	-26,21 to 32,89	>0,9999
Koona vs. MPI	-42,80	-72,35 to -13,25	0,0007
Koona vs. Takataka	0,2600	-29,29 to 29,81	>0,9999
Koona vs. Boribo	-2,087	-31,64 to 27,46	>0,9999
Koona vs. Ngoogwe	-3,850	-33,40 to 25,70	>0,9999
Koona vs. Bire	4,370	-25,18 to 33,92	>0,9999
Koona vs. Doodo red	6,270	-23,28 to 35,82	>0,9999
Kagoogwa vs. MPI	-46,14	-75,69 to -16,59	0,0002
Kagoogwa vs. Takataka	-3,083	-32,63 to 26,47	>0,9999
Kagoogwa vs. Boribo	-5,430	-34,98 to 24,12	>0,9999
Kagoogwa vs. Ngoogwe	-7,193	-36,74 to 22,36	0,9998
Kagoogwa vs. Bire	1,027	-28,52 to 30,58	>0,9999
Kagoogwa vs. Doodo red	2,927	-26,62 to 32,48	>0,9999
MPI vs. Takataka	43,06	13,51 to 72,61	0,0007
MPI vs. Boribo	40,71	11,16 to 70,26	0,0015
MPI vs. Ngoogwe	38,95	9,395 to 68,50	0,0026
MPI vs. Bire	47,17	17,62 to 76,72	0,0002
MPI vs. Doodo red	49,07	19,52 to 78,62	<0,0001
Takataka vs. Boribo	-2,347	-31,90 to 27,20	>0,9999
Takataka vs. Ngoogwe	-4,110	-33,66 to 25,44	>0,9999
Takataka vs. Bire	4,110	-25,44 to 33,66	>0,9999
Takataka vs. Doodo red	6,010	-23,54 to 35,56	>0,9999
Boribo vs. Ngoogwe	-1,763	-31,31 to 27,79	>0,9999
Boribo vs. Bire	6,457	-23,09 to 36,01	>0,9999
Boribo vs. Doodo red	8,357	-21,19 to 37,91	0,9989
Ngoogwe vs. Bire	8,220	-21,33 to 37,77	0,9990

Ngoogwe vs. Doodo red	10,12	-19,43 to 39,67	0,9924
Bire vs. Doodo red	1,900	-27,65 to 31,45	>0,9999

Appendix 10: Tukey's multiple comparisons of leaf Mangiferin concentration with the Mangifera indica variety.

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Adjusted P Value
Apple M vs. Sejjembe	-9.870	-17.81 to -1.929	0.0089
Apple M vs. Kawanda green	-13.61	-21.55 to -5.669	0.0003
Apple M vs. Kate	-35.15	-43.09 to -27.21	<0.0001
Apple M vs. Asante	-2.270	-10.21 to 5.671	0.9954
Apple M vs. Kawanda wide	-12.36	-20.30 to -4.419	0.0010
Apple M vs. Suu	-20.63	-28.57 to -12.69	<0.0001
Apple M vs. Koona	-38.30	-46.24 to -30.36	<0.0001
Apple M vs. Kagoogwa	-21.08	-29.02 to -13.14	<0.0001
Apple M vs. MPI	8.970	1.029 to 16.91	0.0199
Apple M vs. Takataka	-13.72	-21.66 to -5.779	0.0003
Apple M vs. Boribo	-22.38	-30.32 to -14.44	<0.0001
Apple M vs. Ngoogwe	8.020	0.07920 to 15.96	0.0466
Apple M vs. Bire	0.01000	-7.931 to 7.951	>0.9999
Apple M vs. Doodo red	-0.5200	-8.461 to 7.421	>0.9999
Sejjembe vs. Kawanda green	-3.740	-11.68 to 4.201	0.8308
Sejjembe vs. Kate	-25.28	-33.22 to -17.34	<0.0001
Sejjembe vs. Asante	7.600	-0.3408 to 15.54	0.0674
Sejjembe vs. Kawanda wide	-2.490	-10.43 to 5.451	0.9896
Sejjembe vs. Suu	-10.76	-18.70 to -2.819	0.0040
Sejjembe vs. Koona	-28.43	-36.37 to -20.49	<0.0001
Sejjembe vs. Kagoogwa	-11.21	-19.15 to -3.269	0.0027
Sejjembe vs. MPI	18.84	10.90 to 26.78	<0.0001
Sejjembe vs. Takataka	-3.850	-11.79 to 4.091	0.8045
Sejjembe vs. Boribo	-12.51	-20.45 to -4.569	0.0009
Sejjembe vs. Ngoogwe	17.89	9.949 to 25.83	<0.0001
Sejjembe vs. Bire	9.880	1.939 to 17.82	0.0088
Sejjembe vs. Doodo red	9.350	1.409 to 17.29	0.0142
Kawanda green vs. Kate	-21.54	-29.48 to -13.60	<0.0001
Kawanda green vs. Asante	11.34	3.399 to 19.28	0.0024
Kawanda green vs. Kawanda wide	1.250	-6.691 to 9.191	>0.9999
Kawanda green vs. Suu	-7.020	-14.96 to 0.9208	0.1105
Kawanda green vs. Koona	-24.69	-32.63 to -16.75	<0.0001
Kawanda green vs. Kagoogwa	-7.470	-15.41 to 0.4708	0.0754
Kawanda green vs. MPI	22.58	14.64 to 30.52	<0.0001
Kawanda green vs. Takataka	-0.1100	-8.051 to 7.831	>0.9999
Kawanda green vs. Boribo	-8.770	-16.71 to -0.8292	0.0239
Kawanda green vs. Ngoogwe	21.63	13.69 to 29.57	<0.0001
Kawanda green vs. Bire	13.62	5.679 to 21.56	0.0003

Kawanda green vs. Doodo red	13.09	5.149 to 21.03	0.0005
Kate vs. Asante	32.88	24.94 to 40.82	<0.0001
Kate vs. Kawanda wide	22.79	14.85 to 30.73	<0.0001
Kate vs. Suu	14.52	6.579 to 22.46	0.0002
Kate vs. Koona	-3.150	-11.09 to 4.791	0.9382
Kate vs. Kagoogwa	14.07	6.129 to 22.01	0.0002
Kate vs. MPI	44.12	36.18 to 52.06	<0.0001
Kate vs. Takataka	21.43	13.49 to 29.37	<0.0001
Kate vs. Boribo	12.77	4.829 to 20.71	0.0007
Kate vs. Ngoogwe	43.17	35.23 to 51.11	<0.0001
Kate vs. Bire	35.16	27.22 to 43.10	<0.0001
Kate vs. Doodo red	34.63	26.69 to 42.57	<0.0001
Asante vs. Kawanda wide	-10.09	-18.03 to -2.149	0.0073
Asante vs. Suu	-18.36	-26.30 to -10.42	<0.0001
Asante vs. Koona	-36.03	-43.97 to -28.09	<0.0001
Asante vs. Kagoogwa	-18.81	-26.75 to -10.87	<0.0001
Asante vs. MPI	11.24	3.299 to 19.18	0.0026
Asante vs. Takataka	-11.45	-19.39 to -3.509	0.0022
Asante vs. Boribo	-20.11	-28.05 to -12.17	<0.0001
Asante vs. Ngoogwe	10.29	2.349 to 18.23	0.0061
Asante vs. Bire	2.280	-5.661 to 10.22	0.9952
Asante vs. Doodo red	1.750	-6.191 to 9.691	0.9997
Kawanda wide vs. Suu	-8.270	-16.21 to -0.3292	0.0374
Kawanda wide vs. Koona	-25.94	-33.88 to -18.00	<0.0001
Kawanda wide vs. Kagoogwa	-8.720	-16.66 to -0.7792	0.0250
Kawanda wide vs. MPI	21.33	13.39 to 29.27	<0.0001
Kawanda wide vs. Takataka	-1.360	-9.301 to 6.581	>0.9999
Kawanda wide vs. Boribo	-10.02	-17.96 to -2.079	0.0077
Kawanda wide vs. Ngoogwe	20.38	12.44 to 28.32	<0.0001
Kawanda wide vs. Bire	12.37	4.429 to 20.31	0.0010
Kawanda wide vs. Doodo red	11.84	3.899 to 19.78	0.0015
Suu vs. Koona	-17.67	-25.61 to -9.729	<0.0001
Suu vs. Kagoogwa	-0.4500	-8.391 to 7.491	>0.9999
Suu vs. MPI	29.60	21.66 to 37.54	<0.0001
Suu vs. Takataka	6.910	-1.031 to 14.85	0.1211
Suu vs. Boribo	-1.750	-9.691 to 6.191	0.9997
Suu vs. Ngoogwe	28.65	20.71 to 36.59	<0.0001
Suu vs. Bire	20.64	12.70 to 28.58	<0.0001
Suu vs. Doodo red	20.11	12.17 to 28.05	<0.0001
Koona vs. Kagoogwa	17.22	9.279 to 25.16	<0.0001
Koona vs. MPI	47.27	39.33 to 55.21	<0.0001
Koona vs. Takataka	24.58	16.64 to 32.52	<0.0001
Koona vs. Boribo	15.92	7.979 to 23.86	<0.0001
Koona vs. Ngoogwe	46.32	38.38 to 54.26	<0.0001
Koona vs. Bire	38.31	30.37 to 46.25	<0.0001
Koona vs. Doodo red	37.78	29.84 to 45.72	<0.0001
Kagoogwa vs. MPI	30.05	22.11 to 37.99	<0.0001
Kagoogwa vs. Takataka	7.360	-0.5808 to 15.30	0.0829
Kagoogwa vs. Boribo	-1.300	-9.241 to 6.641	>0.9999

Kagoogwa vs. Ngoogwe	29.10	21.16 to 37.04	<0.0001
Kagoogwa vs. Bire	21.09	13.15 to 29.03	<0.0001
Kagoogwa vs. Doodo red	20.56	12.62 to 28.50	<0.0001
MPI vs. Takataka	-22.69	-30.63 to -14.75	<0.0001
MPI vs. Boribo	-31.35	-39.29 to -23.41	<0.0001
MPI vs. Ngoogwe	-0.9500	-8.891 to 6.991	>0.9999
MPI vs. Bire	-8.960	-16.90 to -1.019	0.0201
MPI vs. Doodo red	-9.490	-17.43 to -1.549	0.0125
Takataka vs. Boribo	-8.660	-16.60 to -0.7192	0.0264
Takataka vs. Ngoogwe	21.74	13.80 to 29.68	<0.0001
Takataka vs. Bire	13.73	5.789 to 21.67	0.0003
Takataka vs. Doodo red	13.20	5.259 to 21.14	0.0005
Boribo vs. Ngoogwe	30.40	22.46 to 38.34	<0.0001
Boribo vs. Bire	22.39	14.45 to 30.33	<0.0001
Boribo vs. Doodo red	21.86	13.92 to 29.80	<0.0001
Ngoogwe vs. Bire	-8.010	-15.95 to -0.06920	0.0470
Ngoogwe vs. Doodo red	-8.540	-16.48 to -0.5992	0.0293
Bire vs. Doodo red	-0.5300	-8.471 to 7.411	>0.9999

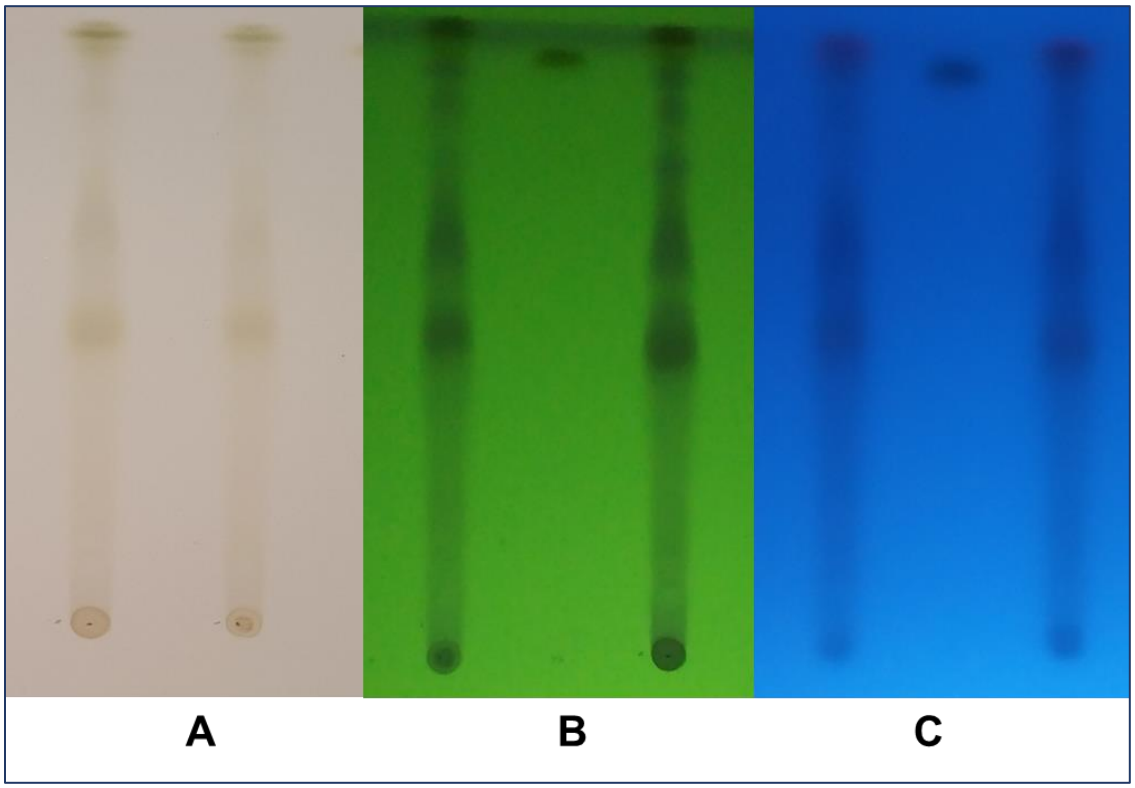
Appendix 11: Validation of the HPLC method for quantification of mangiferin in Mangifera indica leaves

Method validation parameters for the HPLC method	n	Value of parameter	Reference values (AOAC)
Recovery (%)	9	99.4±4.16	92–105
Repeatability (RSD %)			
Intraday	12	0.16±0.04	1.5-2
Inter-day	12	0.32±0.22	3-4
Linearity			
Regression equation		Y=18709.81X-99470.48	
R^2		0.998	
Sensitivity			
LOD (µg/mL)		7.36	
LOQ (µg/mL)		22.3	

Appendix 12: Relationship among the parameters for M. indica leaves from different plant varieties

MF/TPC	TPC/AOA	TPC/EI	AOA/MC
0.6	1.4	8.0	6,9
0.6	2.6	10.3	3,7
0.3	2.3	18.2	3,0
0.6	8.2	14.4	1,3
0.4	1.3	14.1	4,8

0.3	2.1	10.9	4,9
0.4	2.8	12.7	2,7
0.6	4.5	18.4	1,3
0.3	7.7	15.4	1,3
0.5	2.0	14.9	4,4
0.4	8.9	18.3	1,2
0.5	3.7	14.0	1,8
0.5	4.1	16.1	1,7
0.5	5.8	15.9	1,2
0.6	4.9	10.8	1,6
Mean values 0.5	4.2	14.2	2,8



Appendix 13: Typical fingerprint for M. indica leaf materials; Solvent system; 4n-hexane:2ethylacetate:1dichloromethane:1methanol. Visualization conditions without derivatization: A-normal light; B: UV 254nm, C: UV 366nm.

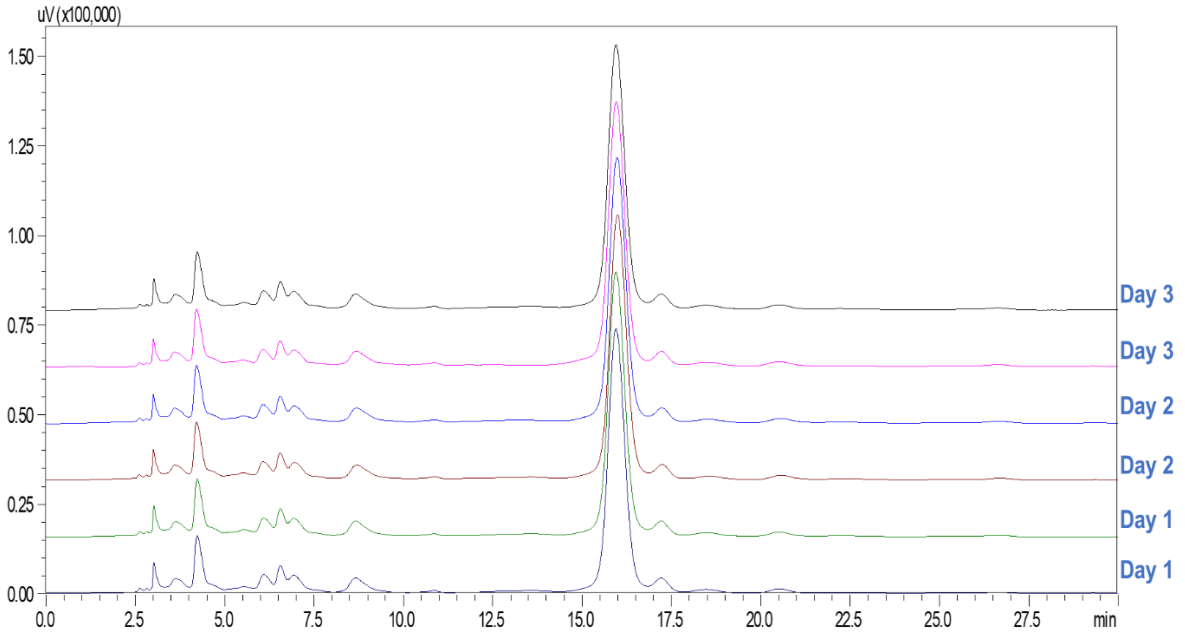
Appendix 14:Characteristics of whole chromatogram fingerprints of Mangifera indica leaves of different cultivars

Variety	Apple M	Asante	Bire	Boribo	Doodo red	Takataka	Kagoogwa	Kate	Kawanda green	Kawanda wide	Koona	MPI	Ngoogwe	Sejjembe	Suu	Averages	SD
No of peaks	25	20	23	24	25	25	20	23	19	29	25	28	23	22	21	23.5	2.9
Total area	1948744	1568633	2478665	1937371	2355795	2833600	2193084	5863448	2678994	2301693	3043927	2000401	1811403	1883593	1962421	2457451	1026790

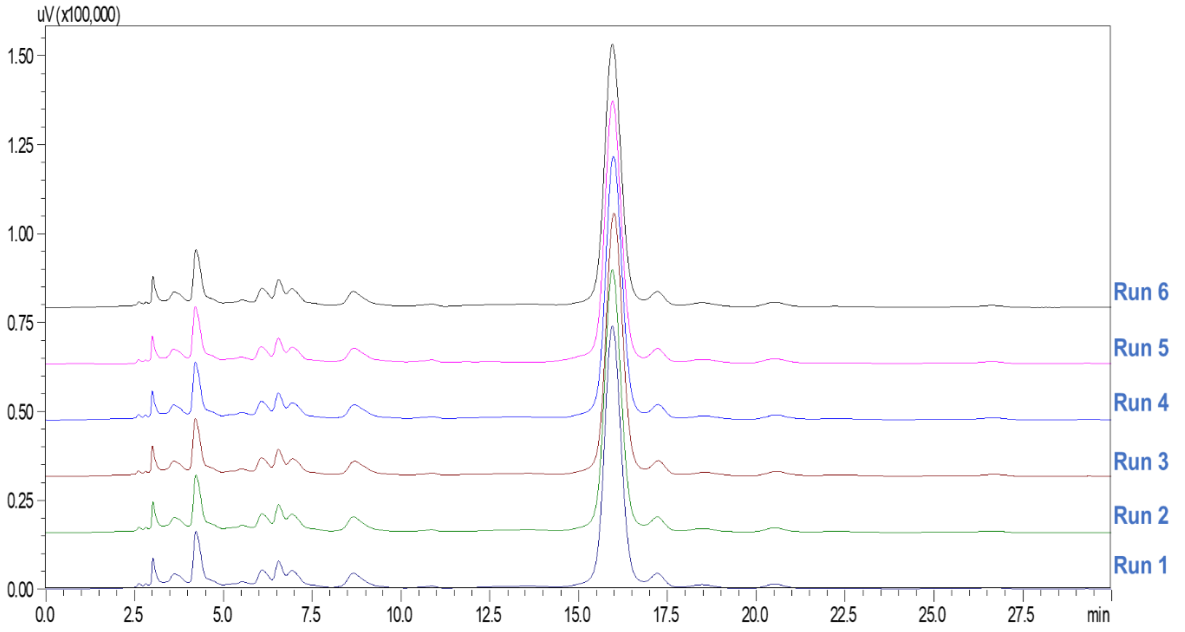
Appendix 15: Common peaks used to calculate fingerprints for M. indica leaves collected from different agroecological zones

Peak number	1	2	3	4	5	6	7	8	9	10	
Retention time	2.607	2.799	3.007	3.595	4.195	4.565	5.489	6.021	6.512	15.828	Total area
Peak areas											
Kawanda wide (reference)	6724	5855	48257	17665	51060	31838	20775	268237	19451	787799	1257661
Apple mango	11323	11040	39909	16507	35181	14750	14717	144109	20125	966630	1274291
Sejjembe	29622	14466	88357	22785	61338	61338	16834	36615	25146	996217	1352718
Kawanda green	5500	6295	28006	12679	41926	20702	62572	263930	28235	1575647	2045492
Kate	14941	5579	86596	90976	150532	48181	57651	200670	56134	2999536	3710796
Asante	1883	2978	34802	6106	37878	13250	11917	137923	15641	699076	961454
Suu	13580	7709	51652	24357	102538	28808	28808	141148	19332	795323	1213255
Koona	64858	13146	72174	25001	123771	63799	51950	46229	48480	1684417	2193825
Kagoogwa	30072	8664	53353	23317	56311	17056	29099	245416	12952	955005	1431245
MPI	53206	16293	71632	16998	67737	39567	40880	96508	24293	1039838	1466952
Takataka	12388	9393	52808	54529	81609	21984	32062	79492	39765	1314151	1698181
Boribo	11080	6295	45009	29931	59765	55201	34609	58715	21996	1034962	1357563
Ngoogwe	16089	10816	38678	13343	57562	46335	33694	44917	23486	1159149	1444069

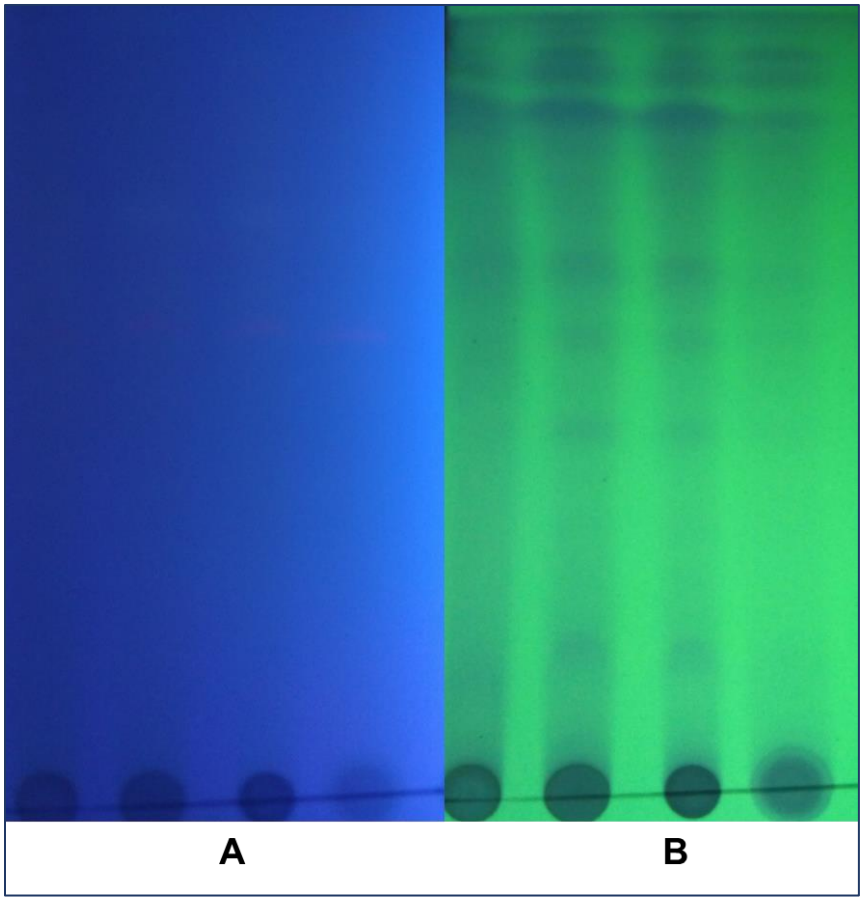
Bire	23852	15584	50710	33913	63670	25552	35720	132430	44361	1318352	1744144
Doodo red	9921	8982	56657	48822	106002	88008	43946	70803	29476	1168948	1631565
Mean	20335.93	9539.667	54573.33	29128.6	73125.33	38424.6	34348.93	131142.8	28591.53	1233003	1652214
STD	17795.01	3974.215	17920.02	21519.35	33464.23	21720.18	15188.3	80609.42	12766.5	561943.8	652910.2
(%) of total peaks area	1.2	0.6	3.3	1.8	4.4	2.3	2.1	7.9	1.7	74.6	



Appendix 16: Inter day repeatability of Mangifera indica leaf fingerprints, samples for Koona variety were used.



Appendix 17: Intraday repeatability of Mangifera indica leaf fingerprints, samples for Koona variety were used.



Appendix 18: Typical fingerprint for W. ugandensis stembark raw material; Solvent system; 8n-hexane:2ethylacetate:1dichloromethane:1methanol. Visualization A: UV 254nm, B: UV 366nm.

Appendix 19: Characteristics of general HPLC fingerprints for W. ugandensis stembark materials collected from different agroecological zones of Uganda.

	Dokolo 1	Dokolo 2	Iganga 1	Iganga 2	Ishaka 1	Ishaka 2	Ishaka 3	Kabale 1	Kabale 2	Kabale 3	Mpigi 1	Mpigi 2	Mpigi 3
	5024	6088	7042	19579	15922	10584	6591	13235	7093	1038	9465	10459	8279
	439011	91748	28334	14592	12296	127683	115972	109885	2927	7969	13921	15930	22132
	222995	397405	511101	17235	20418	189856	840882	780058	9842	3806	2218	3271	105137
	258467	328104	429401	233857	93177	65155	154057	163534	244797	10397	155730	167637	165585,5
	160437	169628	306315	131395	49857	119806	166027	632243	106773	240371	158284	147076	159360,5
	237976	180746	115753	241692	251173	183950	421115	51813	131503	109762	154320	156000	168373
	213930	116781	87425	135867	188436	139620	128874	104838	346430	130615	313371	188242	137743,5
	146560	194718	19201	242742	146585	102921	154032	107257	79063	353092	133485	133527	140043,5
	161235	153883	61714	81223	152780	23792	39868	112294	28911	77681	76200	138797	79452
	33236	105030	1688	31304	34174	157822	82782	24387	118875	31173	52674	80422	43424
	43087	99021	147874	87799	93989	99645	440918	103531	33645	119201	43788	50985	96505
	46586	160894	71584	232072	84997	53476	90875	172374	72964	36516	49519	47982	72274
	158613	133737	4866	27091	148390	134435	138735	158559	392139	81543	241283	46239	136585
	116855	127202	6364	266555	95351	21396	173672	48298	69344	396484	108218	260780	112536,5
	34113	42815	99597	116589	130177	124209	69349	81548	116825	72513	253090	119299	108093
	231888	236523	31518	71151	50072	46242	152304	189731	154111	122364	204868	215121	153207,5
	154465	178865	3027	41965	52249	36748	124072	179453	51198	156550	51783	201061	88160,5
	65968	41797	25313	47979	463790	434348	49123	19958	123496	58270	51004	54011	52507,5
	40380	56477	139179	18067	120580	103182	115252	19989	18659	133591	29468	70078	63277,5
	140376	57605	7873	204081	56005	40347	35221	74071	74211	18874	39574	41364	48684,5
	25310	174187	14684	21107	28188	31900	55319	92342	81124	79459	48901	44844	46872,5

	10379	27965	19109	57013	124120	118659	13278	120935	106742	81035	65063	48203	61038
	55925	16020	55839	18356	105508	97583	74353	32184	60211	115339	148008	60119	60165
	17337	65439	41113	16246	189074	167468	14304	114732	16105	36676	18629	59925	38894,5
	391844	23020		46936	35894	20615	35399	38527	18292	28619	50807	38089	35894
	1838	493191		182751	52795	33824	16665	59962	81688	15871	28246	20481	33824
	3252	37379		48443	21058	7660	16737	15135	16153	19275	35939	28965	19275
	11956	43141		13897	6052	4232	17136	82631	21553	85159	13709	10920	13897
	10536			17322	2583	5773	48197	20739	33447	16697	36330	27586	19030,5
	2988			21535	2620	2376	34890	19314	2560	25839	17587	14521	16054
	2813			4002	4796	10565	18721	19924	11827	39814	86621	76731	15274
	1978			3104	6179	9121	54953	3521	20559	10366	15748	21722	9743,5
	1720			7632	3018	93466	43830	22804	22230	28256	22320	24814	22562
	2119			4091	4242	35133	6316	1930	21448	25940	12472	21814	9394
	10960			10122	9695	334219	1692	19800	29456	20340	11902	15078	13490
	13856			18250	2969			22124	15720	34570	9863	13017	14788
				3155	12813			16324	11446	14264	18504	12681	12813
				21768	13502			11507	21984	15248	59156	3398	15248
				28549	88990			25482	30426	4755	39872	3697	28549
				17547	44625			17880	17778	15981	17717	4473	17717
				12201				33305	2232	16306	7410	4643	9805,5
				12590				14956	8548	5240	45544	1854	10569
				8756				13320	8909	1550	56716	6326	8832,5
				12469				8127	8011	3738		49406	8127
				2711				10829	14656	2022		43224	10829

				2669				10277	7329			14162	8803
								30085				1709	15897
Total area	3476013	3759409	2235914	2876057	3019139	3187811	3951511	4025752	2873240	2904169	3009327	2820683	3178252
number of peaks	36	28	24	46	40	35	35	47	46	45	43	47	39

Appendix 20: Characteristics of common peak HPLC fingerprints for W. ugandensis stembark materials

Retention time	4,58	5,133	6,134	7,011	8,832	9,52	10,795	12,793	15,321	18,411	Total area
Reference	141876	102337,5	82162,5	155239,5	195396	118584,5	56241	119797	110737	85890	1168261
Iganga 2	242742	81223	87799	232072	266555	116589	41965	204081	57013	182751	1512790
Dokolo 1	146560	161235	43087	158613	231888	154465	65968	140376	55925	391844	1549961
Dokolo 2	153883	105030	160894	133737	236523	178865	56477	174187	65439	493191	1758226
Iganga 1	115753	87425	19201	61714	147874	71584	4866	99597	31518	139179	778711
Ishaka 2	93989	84997	95351	130177	463790	120580	56005	124120	189074	52795	1410878
Ishaka 3	157822	99645	134435	124209	434348	103182	40347	118659	167468	33824	1413939
Kabale 3	107257	112294	103531	158559	189731	179453	74071	120935	114732	82631	1243194
Ishaka 1	222956	76766	308197	151920	101113	133022	57048	127818	169616	337789	1686245
Kabale 1	144955	174813	70685	351355	96253	143443	86552	74674	126713	99550	1368993
Mpigi 1	151477	179379	45575	58842	213693	30073	25076	163943	53658	112339	1034055
Mpigi 2	133485	52674		108218	204868	51004	48901	148008	35939	86621	869718
Mpigi 3	138797	50985		119299	201061	70078	44844	59925	28965	76731	790685
Kabarole 1	204239	98675	43346	192556	74380	81751	48992	61940	116824	48667	971370
Kabarole 2	79063	118875	72964	392139	116825	154111	123496	74211	106742	81688	1320114
Kabarole 3	77681	119201	81543	396484	122364	156550	133591	79459	115339	85159	1367371
Kabale 2	128874	154032	82782	440918	173672	69349	124072	115252	115252	74353	1478556

Total areas	2299533	1757249	1349390	3210812	3274938	1814099	1032271	1887185	1550217	2379112	20554806
Average areas	143720,8	109828,1	96385	200675,8	204683,6	113381,2	64516,94	117949,1	96888,56	148694,5	1296724
% of the total area	5,5	4,2	3,7	7,7	7,9	4,4	2,5	4,5	3,7	5,7	50
relative area to reference peak	0,702161	0,536575	0,470897	0,980419	1	0,553934	0,315203	0,576251	0,473358	0,72646	



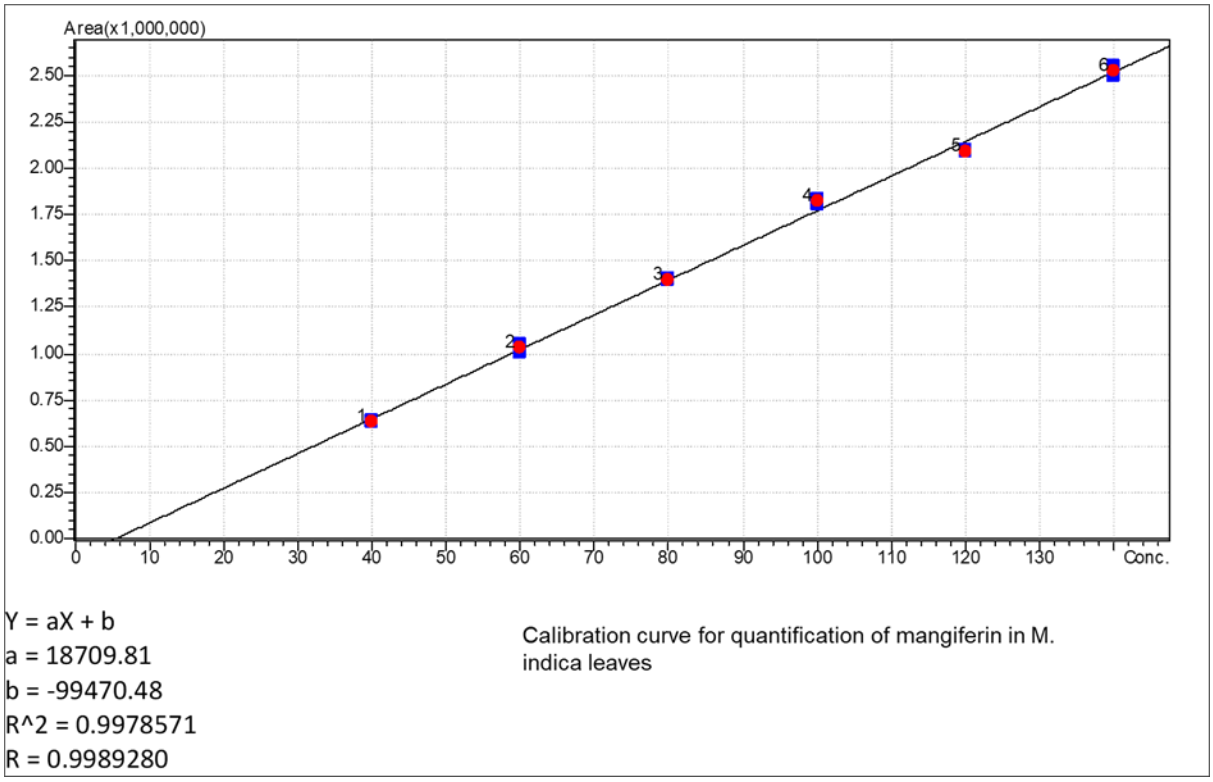
Appendix 21: A. coriaria plant and materials: A-tree, B-fresh stembark, C-dried stembark, D-ethanol macerated powder extract, E-ethanol soxhlet powder extract, F-decoction powder.



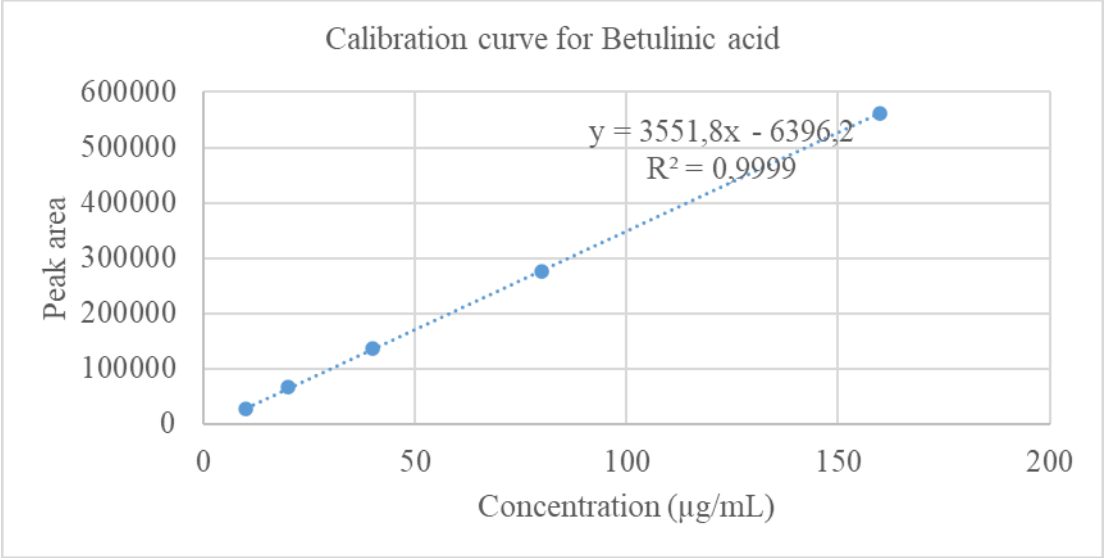
Appendix 22: M. indica plant and materials: A-tree, B-fresh leaves, C-dried leaves, D-ethanol macerated powder extract



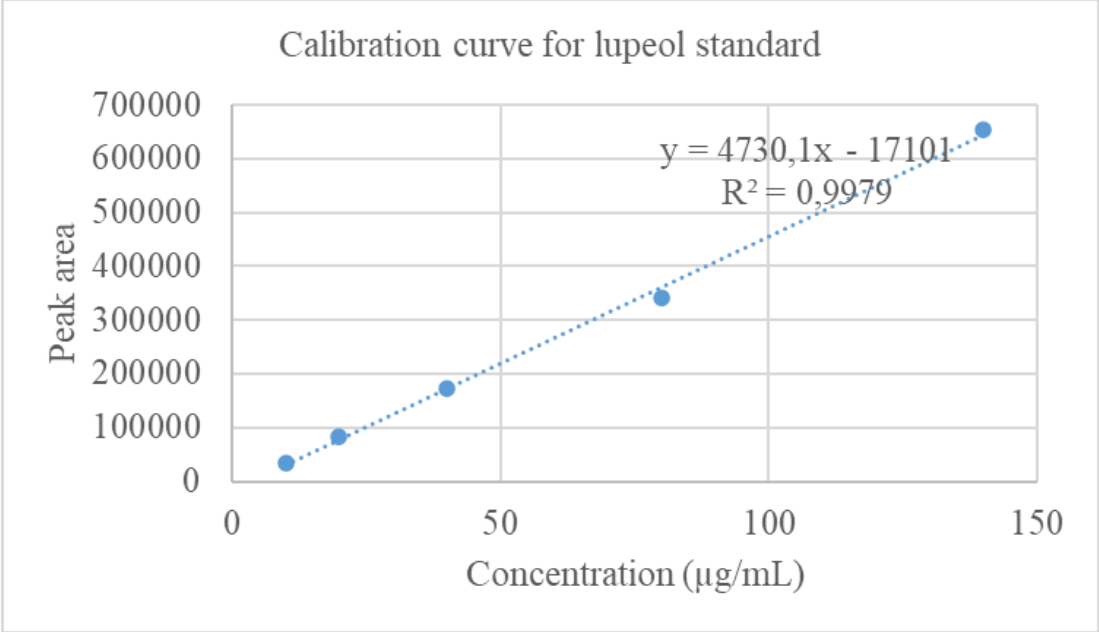
Appendix 23: W. ugandensis and materials: A-tree, B-fresh stembark, C-dried stembark, D-ethanol macerated powder extract, E-ethanol soxhlet powder extract, F-decoction powder.



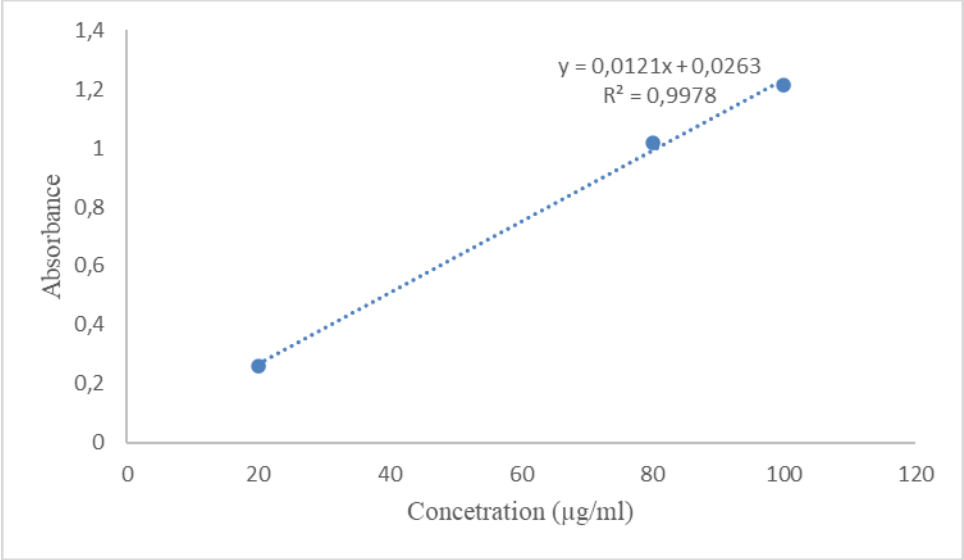
Appendix 24: Calibration curve for quantification of mangiferin in M. indica leaf materials.



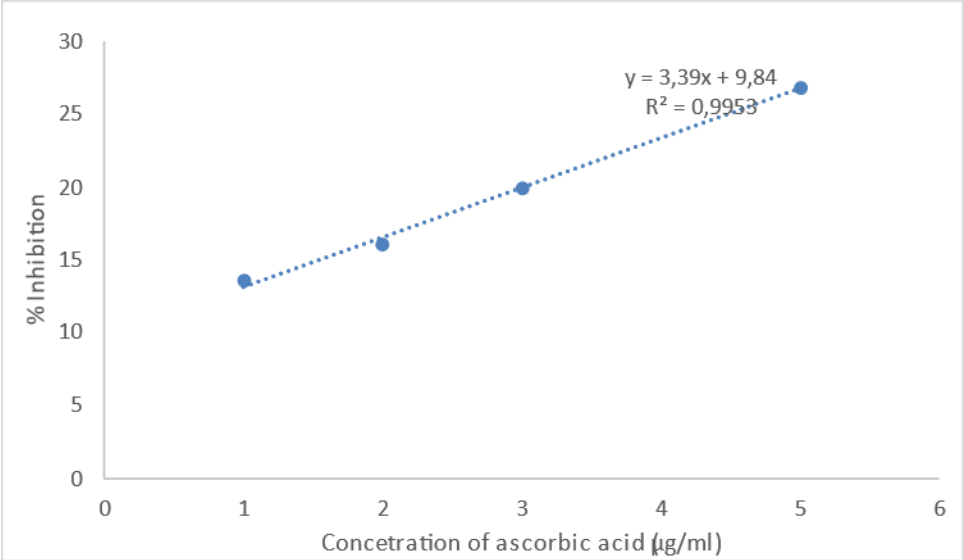
Appendix 25: Calibration curve for quantification of betulinic acid in A. coriaria stembark materials.



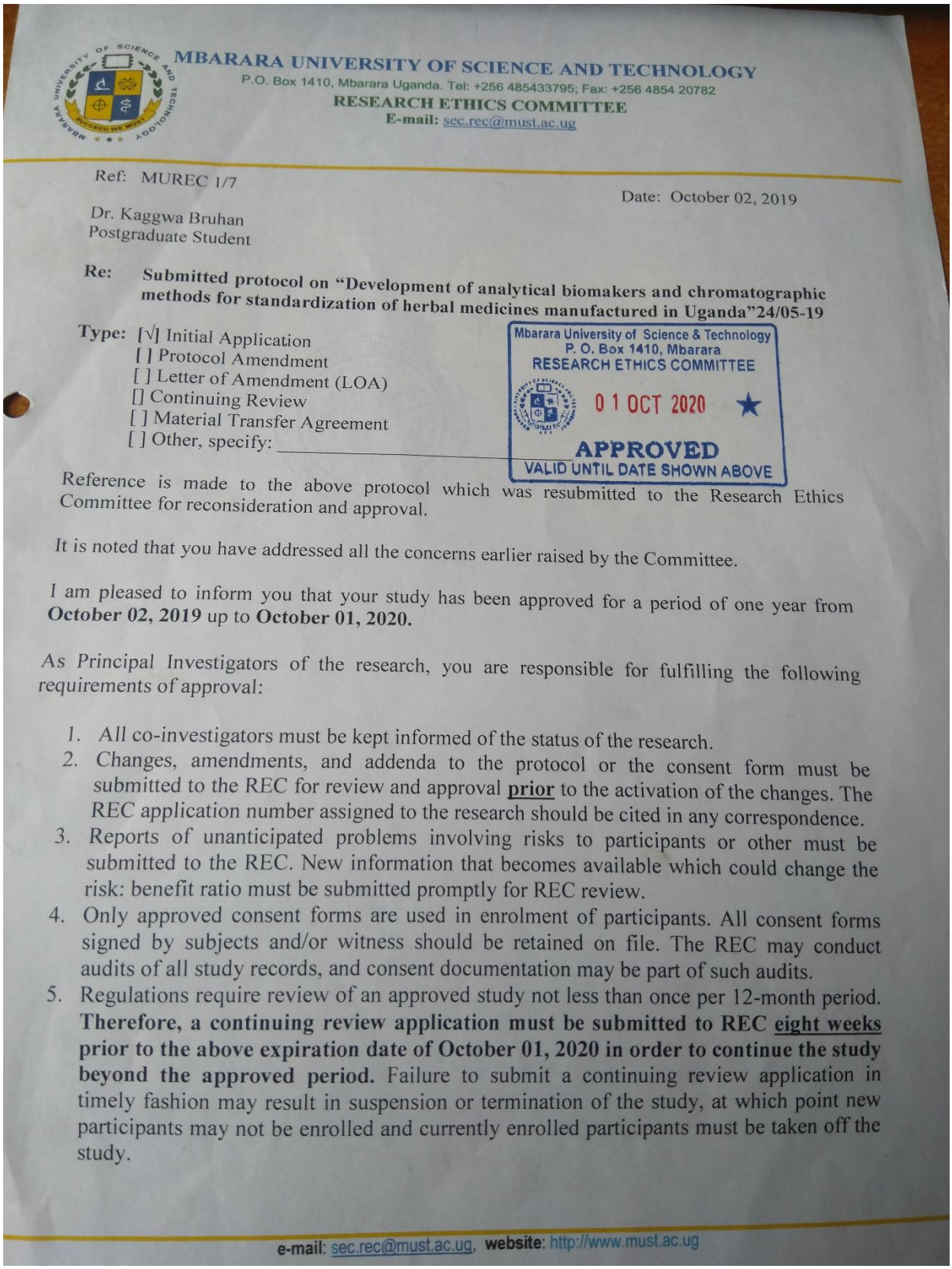
Appendix 26: Calibration curve for quantification of lupeol in A. coriaria stembark materials.



Appendix 27: Calibration curve of gallic acid, for determining the total phenolic content (TPC) of M. indica leaf materials



Appendix 28: Calibration curve of ascorbic acid, for determining antioxidant activity of M. indica leaf materials



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RESEARCH ETHICS COMMITTEE
E-mail: sec.rec@must.ac.ug

Ref: MUREC 1/7

Date: October 02, 2019

Dr. Kaggwa Bruhan
Postgraduate Student

Re: **Submitted protocol on "Development of analytical biomakers and chromatographic methods for standardization of herbal medicines manufactured in Uganda"24/05-19**

Type: [√] Initial Application
[ ] Protocol Amendment
[ ] Letter of Amendment (LOA)
[] Continuing Review
[ ] Material Transfer Agreement
[ ] Other, specify: _____

Mbarara University of Science & Technology
P. O. Box 1410, Mbarara
RESEARCH ETHICS COMMITTEE
01 OCT 2020
APPROVED
VALID UNTIL DATE SHOWN ABOVE

Reference is made to the above protocol which was resubmitted to the Research Ethics Committee for reconsideration and approval.

It is noted that you have addressed all the concerns earlier raised by the Committee.

I am pleased to inform you that your study has been approved for a period of one year from **October 02, 2019** up to **October 01, 2020.**

As Principal Investigators of the research, you are responsible for fulfilling the following requirements of approval:

1. All co-investigators must be kept informed of the status of the research.
2. Changes, amendments, and addenda to the protocol or the consent form must be submitted to the REC for review and approval **prior** to the activation of the changes. The REC application number assigned to the research should be cited in any correspondence.
3. Reports of unanticipated problems involving risks to participants or other must be submitted to the REC. New information that becomes available which could change the risk: benefit ratio must be submitted promptly for REC review.
4. Only approved consent forms are used in enrolment of participants. All consent forms signed by subjects and/or witness should be retained on file. The REC may conduct audits of all study records, and consent documentation may be part of such audits.
5. Regulations require review of an approved study not less than once per 12-month period. **Therefore, a continuing review application must be submitted to REC eight weeks prior to the above expiration date of October 01, 2020 in order to continue the study beyond the approved period.** Failure to submit a continuing review application in timely fashion may result in suspension or termination of the study, at which point new participants may not be enrolled and currently enrolled participants must be taken off the study.

e-mail: sec.rec@must.ac.ug, website: http://www.must.ac.ug

Appendix 29: Ethical approval