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Vol. 17(5), pp. 180-200, May, 2023
DOI: 10.5897/JMPR2022.7285
Article Number: 295FACA70745
ISSN 1996-0875
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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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Received 25 November, 2022; Accepted 20 February, 2023

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.

INTRODUCTION

Albizia coriaria raw materials refer to the dried stem barks of *Albizia coriaria* Oliv (Fabaceae) used to manufacture herbal medicinal products. Traditionally, decoctions and infusions of *A. coriaria* have been used for the treatment of several diseases in humans, including menorrhagia, threatened abortion, and postpartum hemorrhage (Kokwaro, 2009), diarrhea, cough, and snake bite (Tabuti et al., 2003), amoebiasis and syphilis (Namukobe et al., 2011), and diarrhoeal diseases caused by protozoa (Orwa et al., 2009) in animals. At the industrial level, A. coriaria is found in local herbal products marketed for the treatment of respiratory tract disorders (Anywar et al., 2020; Kaggwa et al., 2022), These products include Gabogola syrup®, Kwesiima cough mixture®, Muwereza herbal cough remedy®, Phycof cough syrup® which have been authorized by the Uganda National Drug Authority (National Drug Authority, 2021).

In Uganda, raw materials for *A. coriaria* are sourced from the wild in different parts of the country by vendors and sold to manufacturers in general markets. Although the NDA has published some general guidelines for the harvesting and processing of herbal materials (National Drug Authority, 2016) neither good cultivation and harvesting practices (GCHP) nor good manufacturing practices (GMP) are mandatory. In addition, there is no local herbal pharmacopoeia and for *A. coriaria*, no monographs have been published by any other available pharmacopoeia (Kaggwa et al., 2022). Consequently, the safety of consumers of herbal products is at great risk.

According to (Wang et al. 2015), variation in the chemical composition of herbal raw material occurs due to climate, soil type, harvesting season, extraction, or manufacturing procedures. For batch-to-batch consistency, safety, and efficacy of the medicinal product, quality control of herbal raw materials is of paramount importance. This creates the need for phytochemical standardisation, which can be achieved by comparing the chromatographic fingerprints of each batch of raw materials with standard fingerprints. (Wang et al., 2015). The quality of chromatographic fingerprints can be improved by including marker compounds and simultaneous quantification of such markers (Noviana et al., 2022). For this study, catechin, lupeol, and betulinic acid, some of the known bioactive components of *A. coriaria* (Byamukama et al., 2015; Omara et al., 2022) were evaluated for suitability as quality control markers, according to WHO guidelines (World Health Organization, 2016) and the herb marker classification *system (Bensoussan* et al., 2015*).*The most suitable markers are those whose biological activity is directly related to the traditional use of the herb, are present in sufficient quantities (in the material) to enable identity and quantification, and their analytical standards are readily available.

Since A. coriaria containing products are used for the treatment of upper respiratory tract disorders (URTDIs) (Kaggwa et al., 2022), these markers were selected based on scientific evidence indicating they can alleviate symptoms of URTDIs, or have antimicrobial activity against implicated bacteria or viruses (Thomas and Bomar, 2020). Lupeol reduced mucus secretion and overall inflammation in the lungs in a murine model (Vasconcelos et al., 2008; Wal et al., 2011). The antiviral activity of lupenone, an analogue of lupeol, inhibits herpes simplex virus and African swine fever virus (Xu et al., 2018), although these viruses do not cause URTI. Betulinic acid protected mouse lungs against polymicrobial induced sepsis and lipopolysaccharide-induced lung inflammation, by inhibiting the production of inflammatory mediators (Lingaraju et al., 2015). The antiallergenic and anti-inflammatory activity of catechins was demonstrated by Ohmori et al. (1995) while Ide et al. (2014) inhibited influenza virus activity using green tea catechins. Therefore, catechin, lupeol, and betulinic acid score highly on the marker ranking system (MaRS) for suitability as markers (Bensoussan et al., 2015); since the compounds can alleviate major symptoms related to traditional use of the products and their analytical standards are available commercially.

The aim of this study was to evaluate the suitability of lupeol, betulinic acid and catechin as markers for quality control of A. coriaria stembark materials in reference to; stability in analytical solvents, relative abundancies in samples obtained from different agroecological zones and ability to be detected (in TLC and HPLC fingerprints) and quantified by HPLC.

METHODS

Study setting

The study was conducted in Uganda. Samples of *A. coriaria* were collected from seven of the ten agroecol- ogical zones, in order to cater for the effect of climatic variation on the phytochemical composition of plant materials. These zones are classified on the basis of distinct vegetation and farming systems which are determined by soil types, climate, socio-economic and cultural factors (Plan, 2010). Table 1 summarises the geographical characteristics of the agroecological zones and sampling sites (Kajobe et al., 2016).

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Table 1. Characteristics of the agroecological zones of Uganda.

Agroecological zone	Summary of climatic characteristics	Sample collection site and coordinates longitudes latitudes
West Nile	Bimodal average rainfall of 1,270 mm/ year with high variability. Characterised by savanna vegetation with open mixtures of trees and shrubs standing within tall grass	Pakwach; Padyang 31° 25' 20.5"E 2° 26' 42.4"N
Mid Northern	Unimodal low to high rainfall (1000-1,200 mm/year). Characterised with flat terrain covered by thick savannah grassland	Dokolo; Ilong. 33° 3' 14.4"E 1° 52' 18.82"N
Karamoja dry lands	Unimodal low rainfall (400-700 mm/ year). Vegetation is characterized by thorny shrubs, cammiphora, woodlands, occasional small trees, and patches of grassland.	Moroto; Moroto municipality 34° 40' 45.34"E 2° 29' 19.41"N
Lake Albert crescent	Bimodal average rainfall of 1,270 mm/year with high variability. Vegetation ranges from rainforest to savanna grasses	Masindi; Nyabyeya Forestry College 31° 39' 34.88"E 1° 44' 33.67"N
Lake Victoria crescent	Bimodal high rainfall (>1,200 mm/year). Has a forest / savanna mosaic characterised by patches of dense forest in the south and scattered trees with shrubs and grasslands in the north.	Mpigi; Kibibi 32° 5' 48.12"E 0° 5' 51.36"N
Eastern	Bimodal high rainfall >1,200 mm/ year. Vegetation ranges from mountainous forest to high open moorland	Iganga; Kawete community center for traditional medicine 33° 26' 15.36"E 0° 37' 23.45"N
Western highlands	Bimodal average rainfall of 1,270 mm/year with high variability. Natural equatorial forest vegetation and rich natural savannah grasslands in relatively drier areas	Kabarole; Tooro botanic gardens 30° 12' 12.2"E 0° 39' 39.69"N

Source: Kajobe et al., (2016)

Study design

The study was experimental. Different solvents and extraction methods were evaluated to establish appropriate TLC and HPLC fingerprints based on catechin, lupeol, and betulinic acid markers. In addition, validated HPLC methods was developed for simultaneous quantification of lupeol and betulinic acid in *A. coriaria* raw materials.

Collection and processing of plant materials

The plant materials were harvested in January 2020 following guidelines on good agricultural and collection practices for medicinal plants (American Herbal Products Association, 2017) under the guidance of a botanist, who identified the trees. About 2 kg of *A. coriaria* stembarks were cut using pangas from at least three different trees available in the same district or different districts in the same agroecological zone; the three samples were then combined to make one composite sample. Samples were collected in the morning between 9:00AM and 11:00Am. The sample was assigned voucher No AG636 and deposited in the Makerere University herbarium. After three weeks of air drying, the stembark materials were ground into a coarse powder packed in three layers of aerated polyethylene bags and then transferred to the laboratory for chromatographic work. The extraction of the plant samples was done by maceration, decoction and soxhlet after blending into fine powders. Maceration involved soaking about 50g of stembark powders in 300ml of ethanol or ethyl acetate in solvent bottles. The mixture was left to stand for up to 7 days with occasional shaking. For decoction, approximately 50g of stembark powders were added to 500ml of distilled water, boiled on a heater for 45 min and then freeze dried. For Soxhlet extraction, stembark powders (50g) were refluxed with 95% ethanol or ethyl acetate (200mL) until exhausted; the extracts were rotary evaporated and air dried.

Chemicals

All solvents used (N-hexane, trifluoroacetic acid, acetonitrile, methanol, ethyl acetate, dichloromethane,

ethanol and sulphuric acid), reagents (iodine, vanillin,) and analytical standards (lupeol and betulinic acid) were bought from M/s. Sigma-Aldrich® through a local agent, Kobian Scientific®. Whenever available, analytical or HPLC grade materials were used as required.

Chromatographic equipment and setup

TLC: Chromatographic fingerprints were developed on 5cm x 10cm aluminium TLC sheets (tests) and 10cm x 20cm glass plates (final fingerprints), coated with 60Å silica gel matrix; with fluorescent indicator 254; the plates were sourced from Sigma Aldrich® and Fisher Scientific®, respectively.

HPLC: HPLC analysis was performed on a UPLC; equipped with a Phenomenex Luna C18 column (250 × 4.6 mm. 5 µm), and a UV-visible detector

Development of TLC fingerprints

Preparation of standard and test solutions

Standard solutions were prepared by dissolving lupeol, catechin, and betulinic acid (1 mg) in methanol (2 mL) to make 0.5 mg/mL solution. *A. coriaria* test solutions were prepared by dissolving the powdered stembark material (50 mg) in methanol (5 mL) to make 10mg/mL solutions.

Preparation of mobile phase solutions

The solvent systems were optimised according to the PRISMA system (Nyiredy, 2002). Various proportions of n-hexane, ethyl acetate, methanol, and dichloromethane were tested until the best fingerprints were obtained. The optimized solvent system consisted of 4, 2, 1, 1 parts of n-hexane, ethyl acetate, dichloromethane and methanol respectively.

Stability of extracts during the experiment

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.

Spotting, development, and visualization

Spotting was done manually by introducing about 4µL of the test and standard solutions using a capillary tube. Development was carried out in a saturated glass chamber: the TLC plates were removed after the solvent front had moved three quarters of the plate length. Once the plates were dry, they were visualised in day light, UV light (254 and 366 nm), after derivatization with vanillin sulfuric acid reagent

HPLC fingerprinting and quantification of markers

Preparation of standard solutions and test solutions

Albizia coriaria solutions were prepared by dissolving, with sonication, the powdered stembark material in methanol to make 1 mg/mL solutions. Standard solutions containing 10, 20, 40, 80, 140 and 160 µg/mL were prepared by dissolving lupeol and betulinic acid in methanol and diluting serially.

Mobile phase solutions and HPLC conditions

To obtain the mobile phase solutions, we experimented with solvent systems developed earlier for quantification of lupeol (Strzemski et al., 2016; Yanaso et al., 2021) and betulinic acid (Felföldi-Gáva et al., 2009; Holonec et al., 2012) in other plant materials. The final solvent system composed of acetonitrile (99.99%) and 1% trifluoroacetic acid (0.01%). The optimal mobile phase flow rate was 1mL/min, while the column temperature was 25^{o}C.

Fingerprint development, visualization, and identification of markers

Fingerprints were developed by injecting 10µL of the sample solutions and varying mobile solvent phase systems (isocratic elution) into the chromatograph and visualizing with a UV detector at 205 nm. Lupeol and betulinic acid were identified based on their retention times.

Stability of the extracts solutions was assessed by computing the similarity indices (c and r) of the fingerprints of a sample solution stored for up to three days.

Quantification of markers in *Albizia coriaria* test solutions

The concentrations of lupeol and betulinic acid were based on standard calibration curves and the corresponding peak areas. A preliminary quantification of the markers in one type of sample (Iganga) to determine the best extraction method was done; then all the other samples were extracted by this best method, and the markers quantified.

Validation of quantitative HPLC methods

The accuracy was determined by computing the percentage recovery of markers from three spiked samples, while the intra-day and inter-day repeatability were obtained from the percentage relative standard deviation of nine repeated measurements of samples of different concentrations on the same day and after three days respectively. The linearity (sensitivity) and range were obtained from the standard calibration curve. The limit of detection (LOD) and the limit of quantification (LOQ) were the 3.3*SD/slope and 10*SD/slope products, respectively.

Data analysis

The fingerprints were qualitatively analysed by visualization and semi-quantitatively by computing similarity indices. Those fingerprints were looked for with the best resolution of the components indicated by the number of peaks and in which the standards were well present. For fingerprint similarity analysis, we identified peaks that were common in all samples according to R_F and colour (TLC) after derivatization and retention time and peak area (HPLC). HPLC fingerprint peaks were further compared by similarity indices (correlation coefficient [r], congruence coefficient or cosine [c]), as well as by clustering and principal component analysis using PAST 4® software. With clustering, the level of similarity was indicated c and number of clusters in the Dendrogram. For PCA, similarity was evaluated by the minimum spanning tree distances of the sample fingerprints from the(median) reference fingerprint in a scatter plot of the first two components. Additionally, loading plots demonstrated the peaks responsible for the variation of fingerprints.

Quantitative data for lupeol and betulinic acid were recorded for the different samples in Microsoft Excel 2016®. The effect of

Table 2. Summary of peak properties for TLC fingerprints.

Peak no.	R_F	Compound			Visualisation in day light after derivatization	Identity of compound	Relative retardation factor (reference to betulinic acid)
		Betulinic acid	Lupeol	Catechin			
1	0.986				Purple	Unknown	0.722
2	0.956				Purple	Unknown	0.745
3	0.890		0.890		Purple	Lupeol	0.800
4	0.795				Purple	Unknown	0.896
5	0.712	0.712			Purple	Betulinic acid	1.000
6	0.548				Purple	Unknown	1.300
7	0.425				Purple	Unknown	1.675
8	0.397				Purple	Unknown	1.793
9	0.268				Purple	Unknown	2.657
10	0.219				Purple	Unknown	3.251
11	0.178				Purple	Unknown	4.000
12	0.11			0.110	Brown	Catechin	6.414

R_F-Retardation Factor; this is determined by diving the distance moved by the samples component peak by the distance moved by the solvent front, measured from the sample application point. Relative retardation factor is obtained by dividing the R_F of the reference (betulinic acid) by the R_F of the peak.
Source: Authors

extraction systems and source of raw materials were evaluated by two-way ANOVA grouped analysis, then Tukey's multiple comparison tests in GraphPad Prism 9® at the 5% significance level (p=0.005).

RESULTS

TLC fingerprint

The resultant TLC fingerprint was achieved using a solvent system comprising 4 parts of n-hexane, 2 parts ethyl acetate, 1 part of methanol and 1 Dichloromethane mobile phase system. Up to 12 peaks common for all samples were identified (Figure S1-supplementary materials).

The retardation factors (R_F) of the peaks and their relative retardation factors (with reference to the betulinic acid peak) are summarized in (Table 2).

Stability of extracts under TLC conditions

Stability during chromatography is indicated by a two-dimensional TLC (Figure 1).

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

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

HPLC fingerprinting

Stability of the extracts in solution

The extracts were stable in methanol over three days of refrigeration at 2-8°C, as shown by the similarity indices of the three-day fingerprints in Table 3.

Similarity of sample fingerprints

Pattern recognition

The typical fingerprint of the *Albizia coriaria* stembark showed 10 similar peaks as shown in Figure 2. Although the different sample fingerprints showed a similar pattern on visual inspection (Figure S3-supplementary materials), the number of peaks varied greatly, ranging from 37 (Pakwach) to 55 (Iganga).

Fingerprint similarity indices

The similarity indices for the entire chromatogram (all peaks) fingerprints (Table S1-supplementary materials and Table 4) were lower than those calculated for only common peaks (Table S2- supplementary materials and Table 5). The p values (α=0.05) from the correlation

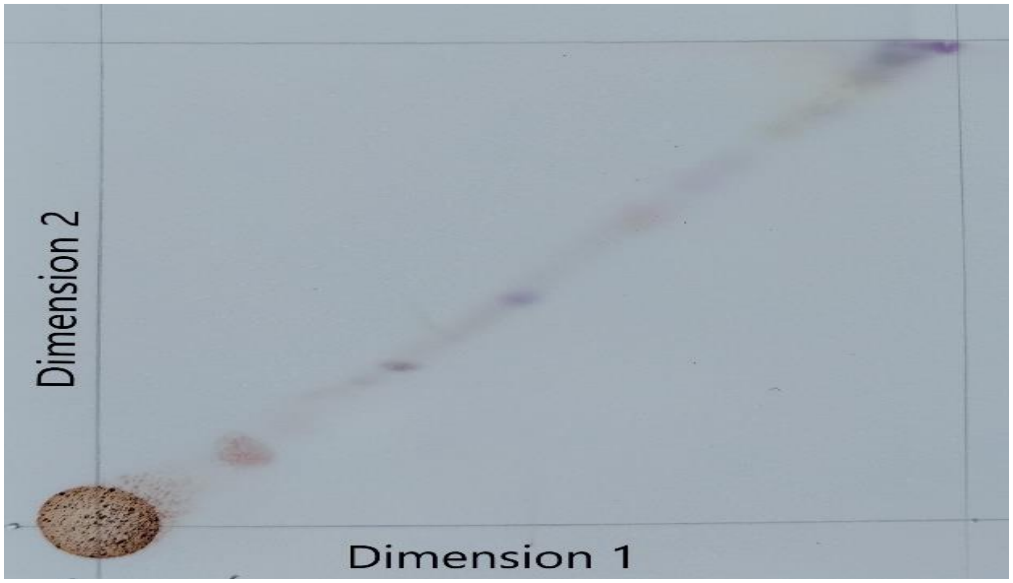


Figure 1. Two-dimensional (2D) TLC; Stability of *Albizia coriaria* stembark raw materials extracts during chromatography. Solvent system; 4n-hexane: 2ethylacetate:1dichloromethane:1methanol. Derivatization: Vanillin sulfuric acid reagent. Visualisation; day light and the sample from Iganga were evaluated.
Source: Authors

Table 3. Similarity indices showing stability of *Albizia coriaria* stembark raw materials in methanol.

	Day 1		Day 2		Day 3	
	r	**c**	**r**	**c**	**r**	**c**
Day 1	1	1	0.9991	0.9991	0.9995	0.9995
Day 2	0.9991	0.9991	1	1	0.9998	0.9998
Day 3	0.9995	0.9995	0.9998	0.9998	1	1

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 be approximate.
Source: Authors

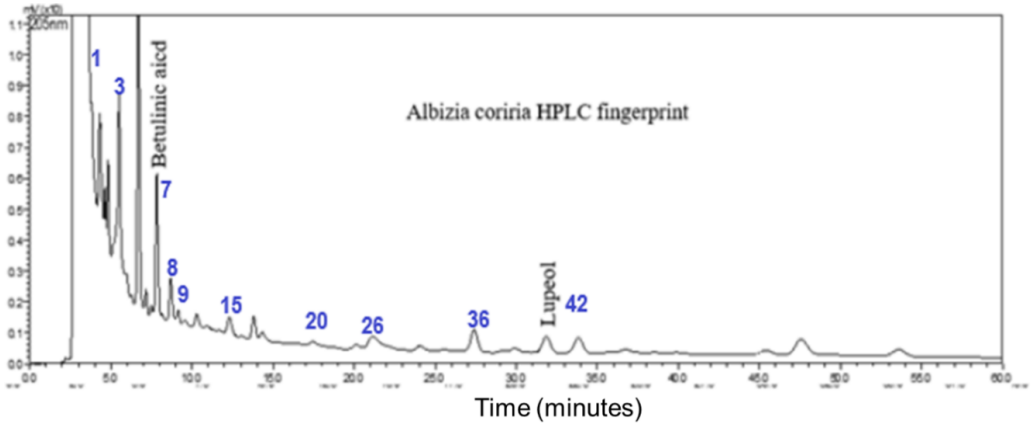


Figure 2. Typical HPLC-UV fingerprint of raw materials from *A. coriaria* raw materials; lupeol and betulinic acid were reference markers. The number and relative positions of the peaks constitute the fingerprint. The peaks common to all samples are numbered.
Source: Authors

Table 4. 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

Source: Authors

Table 5. 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)
	r	p	
Masindi	0.4941	0.1880	0.584
Pakwach	0.985	<0.0001	0.988
Kabarole	0.961	<0.0001	0.959
Moroto	0.991	<0.0001	0.993
Iganga	0.872	0.0024	0.895
Mpigi	0.926	0.0004	0.935
Dokolo	0.993	<0.0001	0.994

Source: Authors

matrices, of both types of fingerprints indicated that the samples were significantly similar, most even at α=0.01.

Common fingerprints constitute peaks with similar retention time (±0.05 min) 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 5, all fingerprints were significantly similar to the median 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.

Multivariate analysis of common fingerprint peaks

Hierarchical cluster analysis

Classical cluster analysis of HPLC fingerprints based on the common peaks gave four clusters: Samples in group 1 (Mororto, Pakwach, and Dokolo) were more than 99% similar (congruence coefficient>0.99) to each other and to the reference, while samples in group 2 (Iganga and Kabarole) were approximately 96% similar (congruence coefficient >0.96); cluster 3 (Mpigi) and cluster 4 (Masindi). All fingerprints, apart from those for Masindi were at least 85% similar (Figure 3). The Dendrogram Cophenetic correlation coefficient was 0.860, implying a good preservation of the original un-modelled data.

Principal component analysis (PCA)

PCA yielded 7 components, with the first two contributing more than 98.9% of the total variance and having the highest Eigen values $3.99*10^{12}$ and $9.91*10^{11}$ respectively. These PCAs were validated by scree testing; the plot formed an elbow after the first two components (Figure S4-supplementary materials).

From the scatter plot, the minimum spanning tree distance confirmed that samples from Mororto were closest to the reference, followed by those from Dokolo, Mpigi, Packwach and then Kabarole and Iganga. Masindi samples were the most distant (Figure 4).

Contribution of the peaks to fingerprint variations

The loading plots showed that the peaks 1, 7 (betulinic

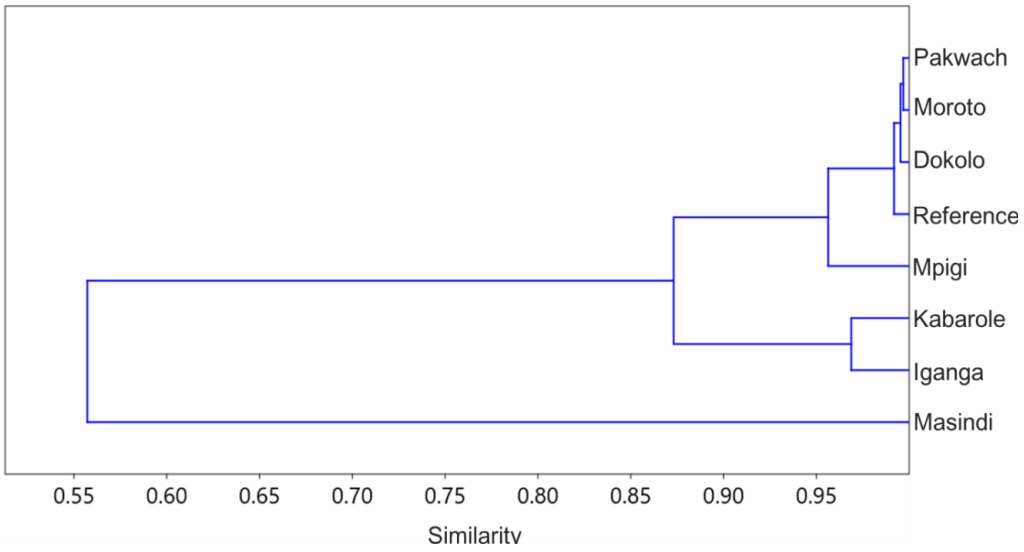


Figure 3. Dendrogram for the classical hierarchical cluster analysis of *A. coriaria* stembark samples obtained from seven different geographical sources: Algorithm, Unweighted pair group method with arithmetic mean (UPMGA); similarity index, congruence coefficient.
Source: Authors

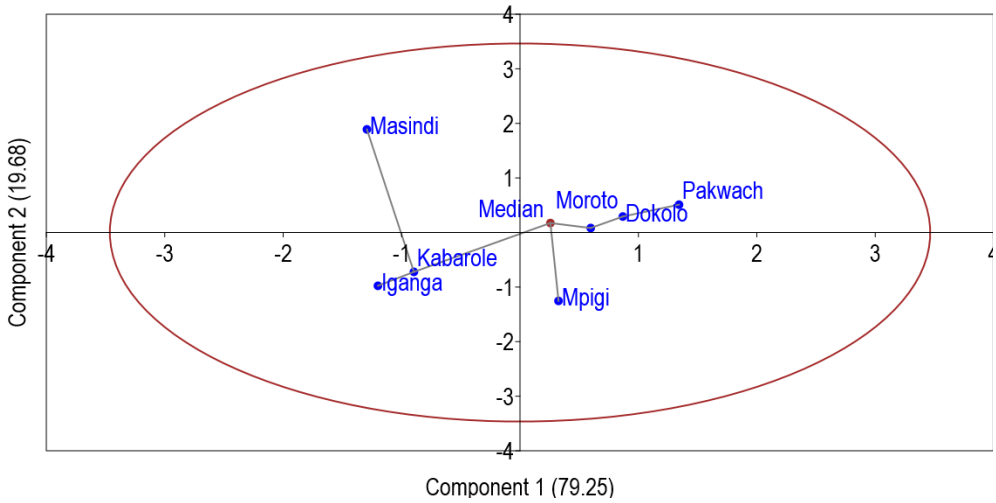


Figure 4. PCA fingerprint characteristics: factor scores of observations plotted on the first two components; with minimum spanning tree (MST) showing the closeness of sample fingerprints to the reference).
Source: Authors

acid), 8, 9, 15, 20, 26, and 43 (lupeol) contribute to PC 1 (Figure S5), while the peaks 1, 3, 7 (betulinic acid), 15, 20 and 26 contribute to PC 2 (Figure S6)- supplementary materials.

HPLC quantification methods

Identification of markers was based on their retention times while quantification on the peak's areas in reference to the standard calibration curves. Figure 5 shows the chromatogram for the standards mixture.

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 (Table 6).

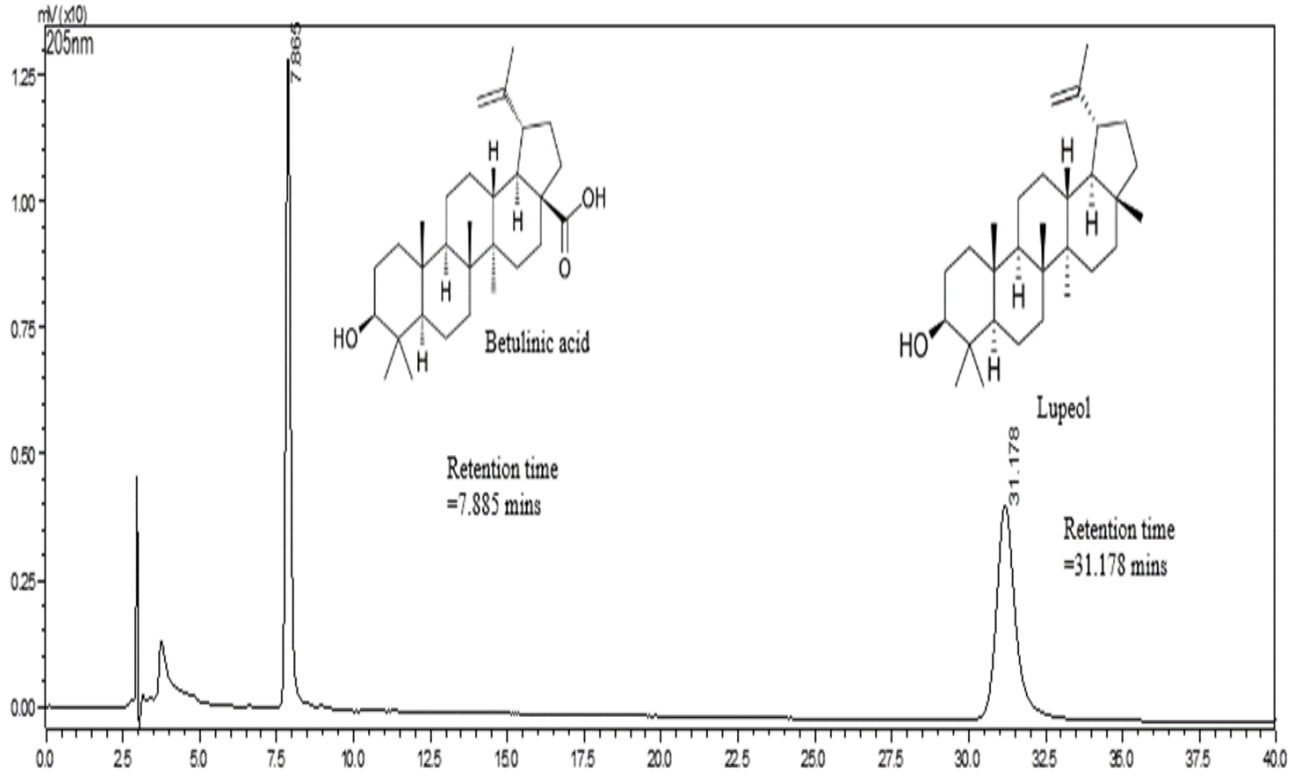


Figure 5. HPLC chromatogram for lupeol and betulinic acid mixture; average retention time, betulinic acid (7.86), lupeol (31.18). Source: Authors

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 6).

The two-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).

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 7).

The two-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. Further analysis (Tukey's multiple comparison tests) showed a marked variation in the concentrations of both lupeol and betulinic acid among districts. The Tukey's multiple comparison test results are in Tables S3 and S4 in the supplementary materials.

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

Table 6. 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	
Sample 2	2.34	1.50	3.65	95	99.0±0.22
Sample 3	3.30	2.50	5.84	100.70	
Lupeol					
Sample 1	3.76	2.50	5.92	94.60	
Sample 2	2.86	2.00	4.80	98.80	97.2±0.02
Sample 3	3.02	2.00	4.95	98.50	

Repeatability (determined to pre-analysed samples based on the calibration curve					
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)					
R^2		0.9999			0.9979
Regression equations		y = 3551.8x - 6396.2			y = 4730.1x - 17101

Source: Authors

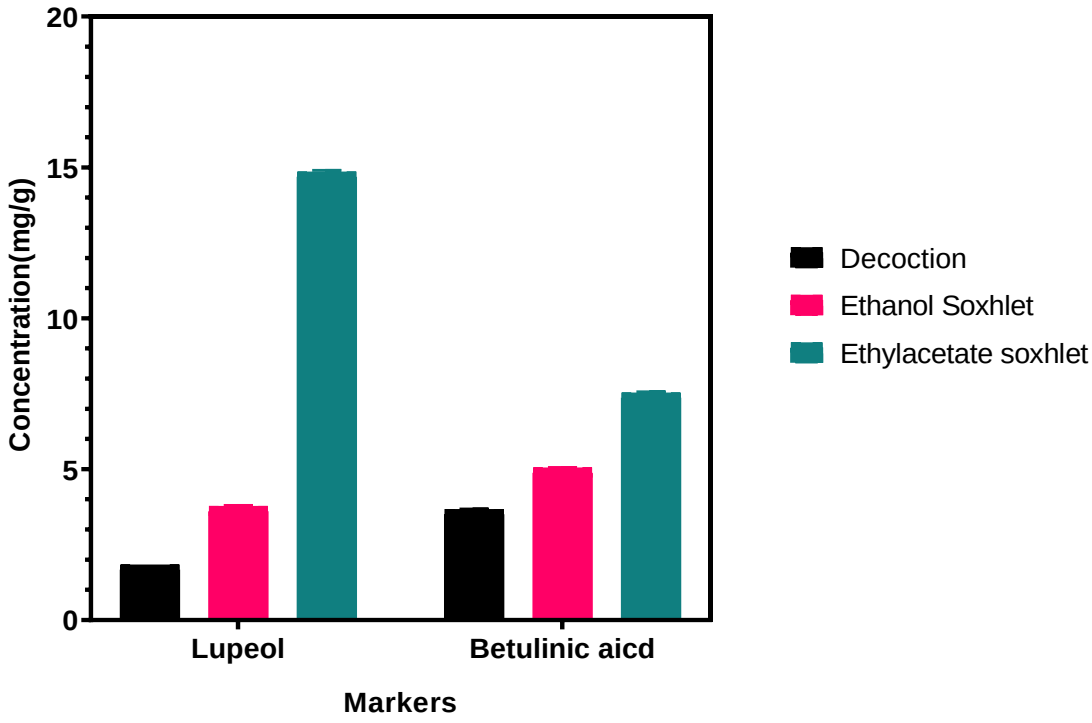


Figure 6. Effect of the extraction method on the concentration of betulinic acid and lupeol from the stembark materials of *A. coriaria.* Iganga samples were analysed.
Source: Authors

Table 7. 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

Source: Authors

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). 12 peaks common to all samples were identified, by comparing their relative RFs and their colours after derivatization (Table 2). The concentration variations of the chemicals (size of peaks) are also evident from the fingerprint (Figure S1).

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-ethyl acetate extraction; 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 min and development time 15 min; 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 1), 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, 2007; Indrayanto, 2012).

Effect of solvent and extraction method on markers and other peaks

The authors 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 S2).

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 et al., 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

Stability of the extracts in solution

The extracts were stable in methanol over three days of refrigeration at 2-8°C, as shown in the by the similarity indices of the three-day fingerprints in Table 3. Samples are considered similar and, therefore, stable if the similarity indices are close to 1 (Noviana et al., 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., 2006). 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 S3), 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, the whole sample fingerprints was compared 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±0.05min), 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 et al., 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 (Mororto, Packwach, and Dokolo) were more than 99% similar (congruence coefficient>0.99) to each other and to the reference, while the samples in group 2 (Iganga and Kabarole) were approximately 96% similar (congruence coefficient >0.96);

group 3 (Mpigi) (c>0.95), and group 4 (Masindi) (c<0.9). The Dendrogram Cophenetic correlation coefficient was 0.860, implying a good preservation of the original un-modelled data. From the PCA results, the minimum spanning tree distance confirmed that samples from Mororto were closest to the reference, followed by those from Dokolo, Mpigi, Packwach and then Kabarole and Iganga. The samples from Masindi were the most distant.

The results of similarity and multivariate analysis show that samples from Moroto, Pakwach, and Dokolo are identical. Practically, they can be substituted by the manufacturer without affecting the consistence of the herbal products.

Samples from Kabarole and Iganga follow 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. It was recommended that for practical applications, Albizia coriaria stembark material HPLC fingerprints should have similarity coefficients of at least 0.90 to be considered phytoequivalent (Sahoo et al., 2010).

To identify the peaks that contribute to the most fingerprint variation, loading plots came in handy (Figure S5 and Figure S6): peaks 1, 7 (betulinic acid), 8, 9, 15, 20, 26, and 43 (lupeol) contributed to PC 1, while the peaks 1, 3, 7 (betulinic acid),15, 20, and 26 contribute to PC 2. In general, peaks 1 and 3 caused more variation than lupeol and betulinic acid; 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; 1, 3, 8, 9, 15, 20, 26, at the observed retention times. 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°C.

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 curves. 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 et al., 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 extract (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±0.06 mg/g) and lupeol (10.10±0.02 mg/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 7). 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, materials were collected 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±0.06 mg/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.coriaira 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., 2009). 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; that is 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 et al., 2022). From the 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).

Conclusion

The current work has provided critical information on analytical methodology and potential sources for good quality materials. Tentatively, the chromatographic methods that have been developed can be incorporated into routine quality control and standardization of materials obtained from various wild sources to ensure batch to batch consistency.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Figure S1. TLC fingerprint showing the distribution of catechin, lupeol, and betulinic acid and other common peaks, in *Albizia coriaria* stembark materials obtained from different agroecological zones (representative districts included here). Peaks identified by similarity of retardation factor. The appropriate marker compounds should be present in all samples.
Source: Authors

Table S1. Common peaks used to analyze similarity of *Albizia coriaria* stembark fingerprints.

	Peak numbers and areas									
	1	**3**	**Betulinic acid**	**8**	**9**	**15**	**20**	**26**	**36**	**Lupeol**
Retention time										
Sample										
Median	3698481	1604104	117491	131253	231096	69211	59554	51117	38306	51038
Masindi	790400	3601931	17288	12103	36540	28097	5197	2555	4829	14226
Pakwach	5831523	1722501	670706	275670	334374	207507	309225	313887	47035	88499
Kabarole	1294021	942819	260910	194587	238923	38213	104976	26917	63810	55720
Moroto	4350349	1437307	449070	71247	130647	69211	44338	50232	24804	32108
Iganga	680733	749294	51378	86085	231096	89537	53778	51117	36764	44407
Mpigi	3698481	174030	77461	131253	195967	46386	59554	80601	38306	51038
Dokolo	4913329	1604104	117491	271609	263056	178772	253168	152437	39843	95678

Source: Authors

Table S2. Peaks used to analyze similarity of *Albizia coriaria* stembark fingerprints whole chromatogram fingerprints.

Peak number	sample and peak area							
	Iganga	**Dokolo**	**Kabarole**	**Masindi**	**Moroto**	**Mpigi**	**Pakwach**	**Median**
1	2336	1976486	1294021	524632	4350349	3698481	31438	1294021
2	680733	4913329	806375	790400	1437307	1714030	2075997	1437307
3	693833	1604104	942819	1033008	449070	179477	5831523	942819
4	749294	819564	327986	3601931	85262	77461	1722501	749294
5	201534	264990	260910	17288	221272	131253	1428833	221272
6	345925	117491	289612	38803	71247	77867	275670	117491
7	146959	271609	317709	3503	130647	195967	670706	195967
8	51378	263056	194587	12103	71783	115316	334374	115316
9	86085	63875	238923	2327	255153	260384	143916	143916
10	231096	105254	161126	36540	151881	60998	97504	105254
11	46551	255634	351872	2923	236712	39258	207507	207507
12	33211	110698	78692	102077	69211	46386	137814	78692
13	166131	80702	49660	9876	43360	36407	271892	49660
14	39266	178772	38213	2170	142440	90637	309225	90637
15	29459	109595	50756	28097	105251	44321	232313	50756
16	89537	109550	103916	16035	44338	58675	145459	89537
17	25417	119335	40901	2724	87629	59554	313887	59554
18	66257	253168	72112	1492	85747	64534	263844	72112
19	35700	217269	77532	5197	116923	29914	95195	77532
20	49866	140624	104976	5951	91011	67733	119113	91011
21	53778	152437	75867	4865	50232	31072	5414	50232
22	54717	44510	47694	21968	60339	44120	222826	47694
23	28611	87703	55846	2555	59311	80601	102264	59311
24	57329	245731	78432	1557	46907	41431	129997	57329
25	30303	217021	26257	3181	25979	5110	77936	26257
26	40586	17985	26917	1675	34187	8988	97369	26917
27	51117	203122	22892	1028	68719	54098	88499	54098
28	21314	98532	23264	9144	66292	39773	97397	39773
29	14007	125046	62462	4829	24804	8029	373533	24804
30	33750	57382	66280	2077	104139	23927	47035	47035
31	50157	110881	5006	23401	21227	111827	59505	50157
32	22768	95678	31917	1330	34641	38306	98424	34641
33	4187	77537	138192	1640	41904	29317	72792	41904
34	28271	30832	63810	1427	61712	7493	14335	28271
35	75660	93049	43679	14226	25232	37979	47713	43679
36	16881	4829	23944	28700	32128	19257	9720	19257
37	36764	56474	78051	7758	32108	22538	16627	32108
38	25961	10850	30382	2186	34200	51038		28171.5
39	9883	32532	23260	24248	18631	24179		23719.5
40	53405	39843	18458	32645	21262	7997		26953.5
41	17629	5499	55720	9949	12480	4455		11214.5
42	30479	10942	3329	1827	8133	23625		9537.5
43	44401	10831	53478		8332	10212		10831
44	46870	91935	69710		17350	16901		46870
45	52520	98152	30763		29534	7410		30763
46	18752	63378	24896		13688	16036		18752
47	44440	15953	122952		20493	41381		41381
48	10164	2127	31184		10220	31269		10220
49	26591		40520					33555.5
50	64444		7006					35725

Table S2. Cont'd

51	1332	4774	3053
51	6289		6289
53	21568		21568
54	5636		5636
55	3386		3386

Source: Authors

Table S3. 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

Source: Authors

Table S4. 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.	Summary	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	ns	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

Table S4. Cont'd

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

Source: Authors



Figure S2. Effect of solvent and extraction method on the number of extracted compounds (seen as peaks).; solvent system; 4n-hexane:2ethylacetate:1dichloromethane:1methanol. Derivatization: Vanillin sulfuric acid reagent. Visualisation; day light. Tracks: 1. SOX-Soxhlet ethyl acetate, 2. MAC-maceration (ethanol), 3. DEC-decoction, 4. Betulinic acid, 5. Catechin, 6. Lupeol and SOX-Soxhlet ethanol. The number and intensity of the peaks were indicative of the extraction power of the method.
Source: Authors

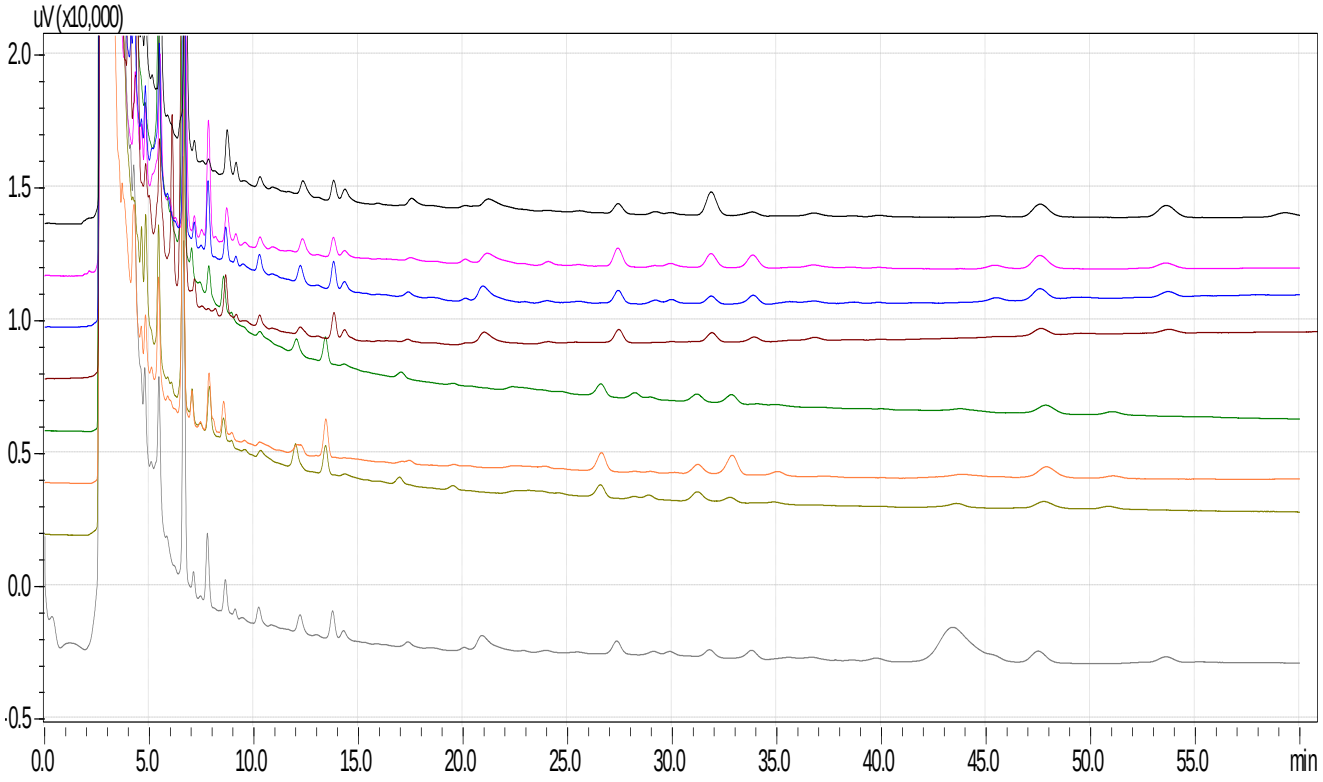


Figure S3. *Albizia coriaria* HPLC fingerprint overly; shows similarities of samples obtained from different agroecological zones.
Source: Authors

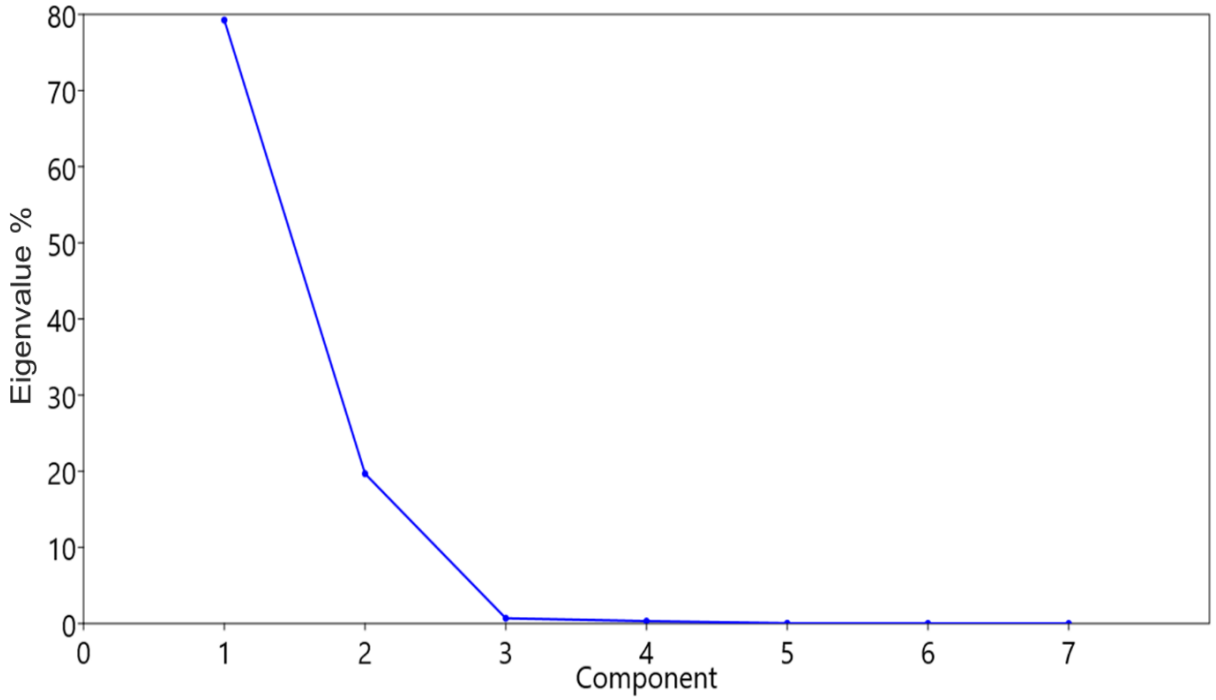


Figure S4. A scree plot to identify the components to use in PCA of *Albizia coriaria* stembark HPLC fingerprints. The first two components on the left side of the elbow were selected and plotted.
Source: Authors

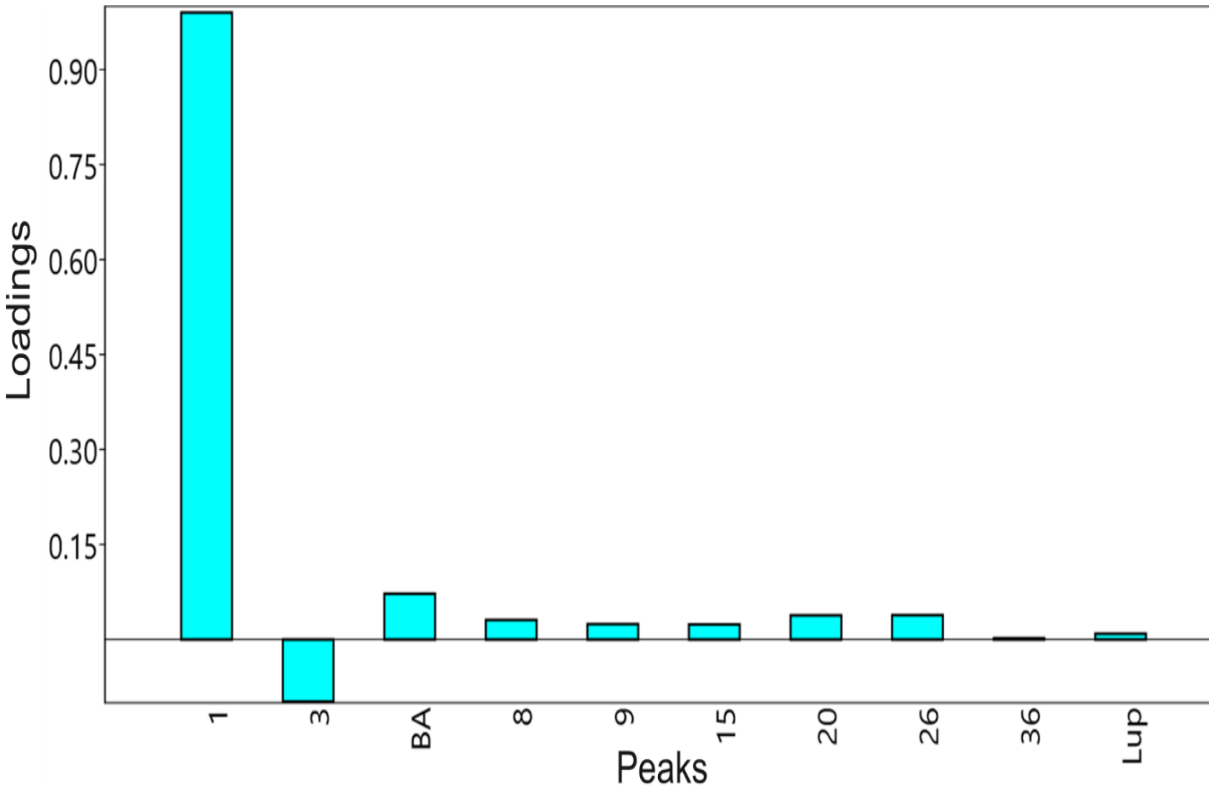


Figure S5. Loading plots for *A. coriaria* fingerprints showing the contribution of the peaks to the variance of PC 1 variance; BA-betulinic acid, lup-lupeol. Loadings in this case show the proportion of the fingerprint that's contributed to by each peak.
Source: Authors

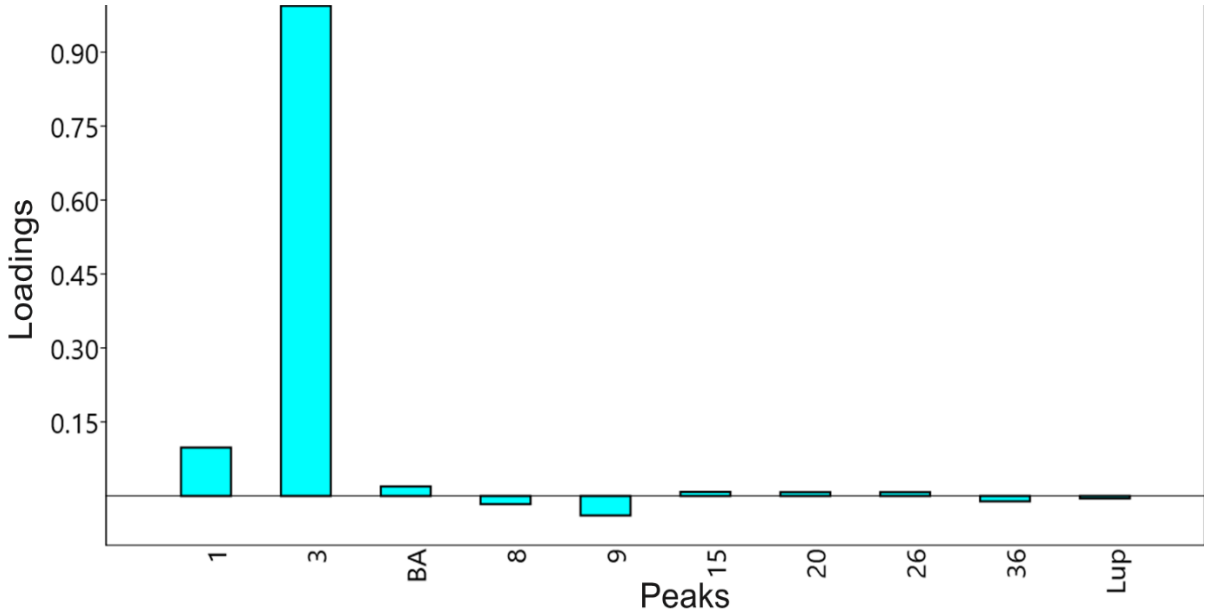


Figure S6. Loading plots for *A. coriaria* fingerprints showing the contribution of the peaks to the variance of PC 2 variance; BA-Betulinic acid, lup-lupeol. Loadings in this case show the proportion of the fingerprint that's contributed to by the peaks.
Source: Authors

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