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RESEARCH ARTICLE

Protective effects of *Sphaeranthus indicus* floral extract against BPS-induced testicular damage in rats occurs through downregulation of RIPK1/3-MLK-driven necroptosis and Fas-FasL-mediated apoptosis

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Abstract

Bisphenol S (BPS) is one of the monomers preferred in the manufacturing of polycarbonate plastics. Unfortunately, its estrogenic and genotoxic effects are similar to those of bisphenol A. The protective effects of *Sphaeranthus indicus* floral extract (SFE) against reprotoxic effects of BPS (50 µg/kg per body weight) in rats exposed to it via drinking water was investigated. Different SFE doses (25, 50, and 100 mg/kg) were administered via oral gavage for 10 weeks. High-performance liquid chromatography (HPLC) results indicated that SFE was rich in polyphenols, with rutin and quercetin being important bioactive molecules modulating BPS-induced necroptosis and apoptosis. Biochemical analyses unveiled that rats administered BPS only exhibited considerable elevation of biomarkers of nitro-oxidative stress, necroptotic (RIPK1/RIPK3 and MLKL), and apoptotic mediators (Fas/FasL and caspase 3/caspase-8). These events caused changes in sperm characteristics (sperm motility, sperm head, and sperm viability), sperm count, and hormonal profile (thyroid stimulating hormone, luteinizing hormone, and follicle-stimulating hormone) of the rats. Histological analysis suggested that there was pronounced sloughing of Sertoli cells, reduced spermatogenic cell density, increased levels of seminiferous tubules, and disorganized morphometric parameters related to seminiferous tubules. The SFE supplementation in rats with BPS-containing water restored nitro-oxidative stress biomarkers, which led to the reduction of necroptosis and apoptosis. Reinstatement of all the biomarkers of oxidative stress, inflammation, necroptosis, and apoptosis after SFE supplementations restored the hormonal profile and normal histoarchitecture of the testes. Virtual screening elucidated that the key regulators of the necroptosis are RIPK3-rutin and RIPK1-quercetin complexes. Further studies are needed to assess its pharmacodynamics, kinetics, and effective concentration for daily use in humans.

Keywords Bisphenol · *Sphaeranthus Indicus* · Testicular damage · Oxidative stress · Inflammation

Introduction

Reproductive dysfunction is one of the health issues that affect human society. More than 186 million people of reproductive age worldwide and 48 million couples are affected by infertility, which is a multifactorial problem (Akalewold et al. 2022). The major cause of male reproductive dysfunction is environmental pollutants that directly or indirectly affect the normal spermatogenic cycle. Bisphenol compounds are used for hardening plastics and are commonly found in the packaging of baby bottles, baby formula, container linings, and dental implants. When food is microwaved in plastic food containers that have been exposed to harsh chemicals, monomers are broken down and released into the food or drink (Thoene et al. 2020). However, bisphenol polymers

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may leach into drinks and food and then affect endocrine pathways by mimicking estrogen (Hafezi and Abdel-Rahman 2019). Bisphenol A (BPA) is one of the most well-known endocrine-disrupting compounds, with significant effects on metabolic disorders, endocrine disorders, obesity, the reproductive system, and the nervous system, as well as being linked to oxidative stress and DNA damage (Beronius et al. 2010; Birnbaum et al. 2012; Vandenberg et al. 2013). The European Food Safety Authority recently reduced the tolerable daily intake of BPA to 0.2 ng/kg bw/day (Prueitt and Goodman 2024). Nonetheless, few studies were conducted to evaluate the reference dose or the safety of BPA substitution levels as healthier BPA-free.

Bisphenol S (BPS) is a chemical compound that bears structural similarity to BPA, featuring two phenol moieties flanking a sulfonyl group. To date, BPS has been shown to exhibit superior thermal and photostability when compared to BPA. As a result, BPS has been widely adopted as a primary substitute for BPA in various industrial applications (Herrero et al. 2018). Considering this, it is plausible that the concentration of BPS in food items may surpass that of BPA, exposing humans to hazardous health risks (Siracusa et al. 2018). Furthermore, plants have recently gained widespread attention as a source of medication due to their availability in local communities, lower costs, simplicity of administration, and fewer side effects. Herbal medicine may also be an effective alternative treatment for a variety of adverse effects and medication resistance (Wang et al. 2023).

Sphaeranthus indicus L. (*S. indicus* hereafter) is an aromatic herb in the family Asteraceae which is commonly known as East Indian globe-thistle (English), Koṭṭaikkarantai (Tamil), and Mundi (Hindi). It occurs in humid tropical zones of the Garhwal Himalaya and Indian plains (Agrawal 1997; Sala 1997; Kumar 1998). The herb *S. indicus* possess intriguing in vivo bioactivities including immunomodulatory, anti-inflammatory, nephroprotective, and antioxidant effects which lend credence to its claimed ethnomedicinal uses (Galani et al. 2010).

For the study of biological systems at the molecular level, in silico methods have rapidly become an indispensable complement to traditional laboratory experimentation (Smith et al. 2013; Lu et al. 2014; Neto 2014). Molecular docking studies provide insights into the degree to which two or more molecular structures such as a drug and an enzyme or a protein receptor interact. The pharmaceutical industry makes extensive use of this capability to develop new drugs. The most crucial use of docking software is in virtual screening wherein potentially bioactive molecules of interest are further explored using a computer simulation. This places constraints on the computational approach, which must be both efficient and trustworthy (Yang et al. 2011).

Asian countries like Pakistan are facing environmental distress owing to industrial waste because of violations of regulations by the Environmental Protection Agency. One of the pollutants is BPS, which leaches into drinking water and may cause infertility following long-term exposure and subsequent disturbance of the normal reproductive cycle. Its antidote must be found and may be used regularly as a daily supplement to reduce BPS-induced reproductive damage. Various plants have been found to demonstrate protective mechanisms against some of the aforementioned hazards to human bodily functions. Hence, the current study investigated *S. indicus* as a possible daily supplement to mitigate BPS-induced reproductive damage to lower the risk of male infertility due to BPS contamination in drinking water. BPS exposure was imitated in Sprague Dawley rats and target nitro-oxidative, anti-inflammatory, and apoptotic biomarkers that induce cellular apoptosis and testicular disruption were assessed. The study focused on the molecular coupling of the polyphenols in the methanolic extract of *S. indicus* flowers (SFE) identified by high-performance liquid chromatography (HPLC) to unravel the molecular interaction with various target receptors.

Materials and methods

Chemicals

The chemicals and reagents used in the assessment of sperm characteristics, antioxidant determination, and ATPase activity were of analytical grade and purchased from Sigma Aldrich (USA). Reagents used for HPLC analyses were of HPLC grade. ELISA kits used for assessing the biomarkers of nitrosative stress (iNOS/NO) were purchased from R&D Systems Inc., Minneapolis, USA. Hematoxylin and eosin stain kit-H-3502 for histological analysis was obtained from a vector laboratory (USA).

Plant material

The herb, *S. indicus* (voucher code: AB2036), was purchased from a local market of Faisalabad and was authenticated by a taxonomist. The voucher specimen has been submitted to the herbarium of the Department of Zoology, Government College University Faisalabad (GCUF), Faisalabad, Pakistan. The flowers of *S. indicus* were removed and cleaned to eliminate dust particles. They were dried and 1 kg was ground to obtain fine powder which was stored in an airtight container until required for analysis.

Floral extract preparation

Extraction of phytochemicals in the powder employed maceration, with methanol (1:3 w/v) for 15 days in the dark at room temperature. The extract (SFE) was sieved using gauze, filtered through Whatman filter paper no. 1, concentrated on a rotary evaporator under vacuum at 45°C to remove methanol, and then freeze-dried. The dried SFE was prepared with deionized water for dose preparation.

Quantitative characterization of *S. indicus* by HPLC

The analysis proceeded on a Flexar HPLC system (Perkin Elmer, Waltham, MA 02451 USA) equipped with an FX-UV/Vis detector (N2920013). HPLC elution used acetonitrile: water (9:1, v/v) at a pH of 3.0, and a flow rate of 1 mL/min. The prepared SFE sample (4 µL) was injected and analyzed at 270 nm to determine the polyphenols in it. In HPLC, a high pressure supplied by a pump forces a liquid SFE sample kept in a tube through a column (stationary phase). In comparison to components that interact less with the column, which showed shorter retention durations, components with a high interaction level with the column were slowly eluted and hence had longer retention times. SFE compounds were separated using either a gradient elution or an isocratic elution. While the composition of the solvent system changes throughout a gradient elution, the solvent system in an isocratic elution stays constant. HPLC control computer program with data for analysis collected and saved the signal detections that matched the quantity of each eluted compound, using the appropriate detectors and preferred wavelengths. Using standard solutions against the target analytes allowed for quantification. The same HPLC settings were applied to the standard solutions as well as the target analytes to enable quantification. Each compound was separated based on the affinity of compounds within the analyte to the mobile phase and stationary phase. Each signal peak represents an analyte that has been transported by a mobile phase through the column (Zu et al. 2006; Petrova and Sauer 2017).

Table 1 Details of the experimental design used in the study

Labeling of rat cohort	Treatments (per body weight)	Route of administration
C	Deionized water as a negative control group	Oral
B-G1	50 µg/kg of BPS	Oral (intake of BPS-contaminated water)
B-SI-G2	50 µg/kg of BPS + 25 mg/kg of SFE	Oral gavage
B-SI-G3	50 µg/kg of BPS + 50 mg/kg of SFE	Oral gavage
B-SI-G4	50 µg/kg of BPS + 100 mg/kg of SFE	Oral gavage

Acute toxicity test for SFE

Oral acute toxicity of SFE was determined in twenty Sprague Dawley rats (150–200 g) following OECD guideline 423 as described previously (Iqbal and Omara 2024). Briefly, rats were fasted overnight. The animals were randomly allocated into three groups ($n = 5$). Group 1, the negative control, was given water and treated groups (2nd and 3rd) were given SFE at doses of 300 and 2000 mg/kg/BW.

Experimental approach

In total, 25 healthy postnatal (21-day old) male Sprague Dawley rats (~30 g initial weight and ~250 g final weight) followed the guidelines of the National Institutes of Health. The animals were kept in an animal house with a 12-hour light/dark cycle and relative humidity of 50%. Throughout the trial, rats had unrestricted access to commercial rodent meal pellets (Al-Hafiz Animal Feed, Faisalabad, Pakistan) and water. According to the principle of Laboratory Animal Care, the experiment was approved and the protocol was adhered to and followed the rules of the Ethical Committee of the University on Animal Experimentation (GCUF/EC/22/899).

Randomization and group allocation of animals

Male Sprague Dawley rats were randomly divided into 5 groups ($n = 5$ in each case). The treatments given to each experimental group are given in Table 1. The experiment was conducted for 10 weeks as the laboratory rats are sexually mature at 10–12 weeks. BPS doses were based on previous reports (Rezg et al. 2018, 2019; Mornagui et al. 2019; Darghouthi et al. 2022) and their non-observed adverse effect level (den Braver-Sewradj et al. 2020). The doses of SFE were selected based on acute and sub-acute toxicity test results and given via oral gavage.

Protein-ligand docking

The PyRx Virtual screening program (version 0.9), which evaluates the appropriate binding alignments of the ligands and the targeted proteins was used. Briefly, data from docking was used to identify the ligand orientations that best fit the protein active sites in terms of binding affinities. To display the ligands and the proteins binding with the corresponding amino acid residues, Discovery Studio Visualizer 2021 (BIOVIA Dassault Systèmes, USA) was used. The ideal positions were chosen based on the lowest binding affinities required for the ligand to bind to the protein.

Optimization of protein for docking

The BIOVIA Discovery Studio Visualizer was used for the molecular docking studies of the synthesised compounds. The coordinated crystal structure of the protein (RIPK1: PDB ID; 7mxk, RIPK3: ID; 4BTF, Fas: PDB ID; 1DDF) was obtained using the Protein Data Bank (http://www.rcsb.org/pdb/home/home.do) and saved in PDB format and optimized for protein by adding polar hydrogen and removing ligand.

Ligand for docking

HPLC-identified compounds were downloaded from the PubChem database as SDF files to dock in protein active sites (Table 2).

Normal mode analysis for molecular dynamics

The docking complex was validated through Normal Mode Analysis using the iMODS web server (Lopez-Blanco et al. 2014). The iMODS was used to calculate the molecular motion and examine the structural dynamics of the docking complex. In addition, this web server produces complicated deformability, covariance maps, eigenvalues, and elastic networks.

Hormonal profile

Serum was detected for male hormone levels including testosterone (TSH), luteinizing hormone (LH), and follicle-stimulating hormone (FSH) by a commercially available ELISA kit, following the manufacturer's instructions.

Table 2 Ligands used for molecular docking and their PubChem identification

S/*N*	Ligands	PubChem ID
1.	Hydroxybenzoic acid	135
2.	Coumarin	323
3.	Salicylic acid	338
4.	Gallic acid	370
5.	Vanillic acid	8468
6.	Ferulic acid	445,858
7.	Sinapic acid	637,775
8.	Caffeic acid	689,043
9.	Chlorogenic acid	1,794,427
10.	Quercetin	5,280,343
11.	Rutin	5,280,805
12.	Kaempferol	5,280,863

Evaluation of sperm characteristics of the epididymis

Sperm motility

The sperm suspension was coated onto a slightly larger microscope slide. A sperm count was done to observe motile and nonmotile sperm. The spermatozoa with progressive motility were counted as motile (Anderson et al. 1983; Organisation 1999; Kvist and Björndahl 2002).

Sperm morphology

The sperm suspension was put on a sheet and left to dry. Eosin was used to stain the slide at 1%. Using an optical microscope with x100 and oil immersion lenses, morphological defects of the sperm were analyzed and studied (Eliasson 1977). Under a light microscope, morphological anomalies (damaged head, bent tail, without tail, tailhead, and total damaged sperm) in spermatozoa were enumerated and their percentage was estimated as reported previously (Seed et al. 1996; Organisation 1999).

Sperm viability

The eosin/nigrosin stain was used to determine sperm viability. One drop of semen suspension (10 µL) was combined with 20 µL of eosin and stained for 3 min. Then nigrosin (40 µL) was added to the solution and used to make the smears right away. Live spermatozoa were left undamaged, but dead spermatozoa were discolored. The fraction of intact cells was correlated with sperm viability (Eliasson and Treichl 1971).

Sperm count

A hemocytometer was used to count the sperm. The epididymis was crushed and suspended in normal saline for five minutes at 37 °C in a separate Eppendorf tube. It was shown that counting sperm in a counting chamber with two or three drops of the sample increased the accuracy of the procedure. A light microscope at 20× magnification was used to count the sperm heads in ten squares (millions of sperm per mL) and calculated mean of sperm count (Soni et al. 2016).

Morphometric histological analysis

The height-to-diameter ratio of the seminiferous tubule was evaluated by measuring the diameter and epithelial height. The evaluation of the testis was conducted on a transverse section of the organ. Epithelial height and seminiferous tubule diameter were assessed in 10 transverse slices of seminiferous tubules per experimental animal. This was accomplished through the use of an optical microscope and ZEN lite 2012 software, with a magnification of 400x, as the means of examination. The findings are exhibited in the form of the proportionality between the average of five measurements of epithelium height and the average of five measurements of seminiferous tubule diameter. (Garcia et al. 2021). The injury level of testis damage was scored according to the percentage of seminiferous tubules with necrosis: 0; normal, 1; ≤10%, 2;10–25%, 3; 26–75%, and 4;≥75% (Soni et al. 2016).

Johnsen scoring

The presence of spermatogenic cells in the seminiferous tubules was graded using Johnsen's score, which ranges from 1 to 10. The following are the specifics of the histology score: 1; degenerated seminiferous tubules, 2; without germ cell, 3; spermatogonia only, 4; few spermatocytes, without any spermatozoa, 5; very less spermatozoa and numerous spermatocyte, 6; without any spermatozoa or very less early spermatids, 7; without any spermatids or numerous early spermatids, 8; <5 spermatozoa and few early spermatids, 9; slightly reduced spermatogenesis, late spermatids, and distorted epithelium, 10; complete spermatogenesis (Soni et al. 2016).

Sertoli cell count

The number of Sertoli cells was counted in twenty seminiferous tubules as the mean number of sterile cells per seminiferous tubule per rat (Garcia et al. 2021).

Evaluation of antioxidant activity

By converting hydrogen peroxide to water and oxygen, catalase protects cells from oxidative stress and lowers reactive oxygen species according to the method of Aebi (Aebi 1974). The linked enzyme technique was used to determine glutathione peroxidase (GPX) activity according to Paglia and Valentine (Paglia and Valentine 1967). At 560 nm, the developed blue color of the reaction was determined. Superoxide dismutase (SOD) activity was expressed in units as the quantity of enzyme necessary to block the decrease of NBT by 50% and in units per milligram of protein (Durak et al. 1993).

Determination of biomarkers of nitrative stress

An ELISA kit was used to measure the serum levels of iNOS/NO. Using an automatic plate reader, the treated microplates were evaluated (Multiskan FC microplate photometer).

Assessment of malondialdehyde

Malondialdehyde (MDA) concentrations were measured using the same procedures as those used in the earlier Ohkawa et al. 1979 research. After centrifuging the reaction mixture at 4000 rpm for 10 min, the absorbance of the top layer was measured at 532 nm. The data were represented in nanomolar concentrations per gram of tissue (Ohkawa et al. 1979).

cDNA synthesis

After assessing the amount and integrity of the entire RNA sample, a solution was prepared of 5 µg of total RNA with 10 µL volume, 1 µL of random hexamer primer, and 1 µL of oligo (dT) primer. Subsequently, the aforementioned solution was incubated at a temperature of 70 °C for 300 s then augmented with 0.5 µL of RNase inhibitor (40 U/µL), 1 µL of deoxyribonucleoside triphosphates (10 mM each), 1 µL of reverse transcriptase (Moloney Murine Leukemia Virus), and 4 µL of quintuple first-strand buffer. Bahari et al. (2017) reported that the cDNA synthesis reaction was stopped by heating at 70 °C for 5 min, after incubation at a temperature of 42 °C for 60 min.

Real-time PCR techique

The experimental procedure involved adding 0.5 µL of primers of the genes MLKL, RIPK1, RIPK3, caspase3, caspase 8, Fas, FasL, and β-actin (Table 3), nuclease-free water (8 µL), SYBR Green qPCR Mix 2×(10 µL), and cDNA (1 µL) in 0.2 mL PCR microtube. In the cycling process, the initial holding phase is at 95 °C, denaturation at 95 °C, annealing at 60 °C, extension at 72 °C, and final extension at 72 °C. After the amplification of each PCR product, a melting curve analysis was attained, which generated a single peak (Mohammadi et al. 2014; Rassouli et al. 2022).

Statistical analysis

The biochemical analysis parameters were subjected to analysis of variance using the general linear model. Tukey's post hoc test was employed to determine the pairwise differences of the means within the experimental groups at a 5% level of significance. The statistical software Minitab version 21 (Minitab Inc., USA) was utilized for the analysis.

Table 3 Primer sequences for the real-time quantitative reverse transcription-polymerase (RT-qPCR)

Target gene	Primer	Primer Sequence 5′-3′	Expected size (bp)	Accession number
MLKL	MLKL-F MLKL-R	TCCAGTCACCATCAAAGTATTCA TCCTTGTCTCTATCCAGCAGTTC	225	NM_001401077.1
RIPK1	RIPK1-F RIPK1-R	CTTAAGCCCAAGTGCAGTCA ATAGCCCAACAAGGAGGATG’	166	XM_039095696.1
RIPK3	RIPK3-F RIPK3-R	TAGTTTATGAAATGCTGGACCGC GCCAAGGTGTCAGATGATGTCC	145	NM_139342.2
Fas	Fas-F Fas-R	TCTAGTTGGAAAGAACCGAAGG CCACAAACGAGATGCAATCAC	302	NM_139194.3
FasL	FasL-F Fas-R	CAAAGACCACAAGGTCCAACA TCAGGAACAGTCTTCTTCCCA	108	NM_012908.1
Caspase- 3	Caspase-3-F Caspase-R	GGAGCTTGGAACGCGAAGAA ACACAAGCCCATTTCAGGGT	169	NM_012922.2
Caspase-8	Caspase-8-F Caspase-8-R	TAAGACCTTTAAGGAGCTTCATTTT GA AGGATACTAGAACCTCATGGATTTG AC	92	XM_039084166.1
β-actin	β-actin-F β-actin-R	TTGCTGATCCACATCTGCTG-3 GACAGGATGCAGAAGGAGAT	146	NM_031144.3

Results

Phytochemical profile of SFE

According to the HPLC profile, SFE has a chlorogenic acid content of 114.81 ppm, 100.05 ppm of vanillic acid, 100.70 ppm of gallic acid, 100.79 ppm of caffeic acid, 100.00 ppm of kaempferol, 100.06 ppm of sinapic acid, 99.98 ppm of ferulic acid, 99.97 ppm of salicylic acid, 99.85 ppm of coumarin, 99.91 ppm of quercitin, 99.91 ppm of benzoic acid, 99.87 ppm of rutin (Fig. 1; Table 4).

Acute toxicity results

The animals were observed for any toxic effects after 24 h and 14 days following the treatment period, and no drug-related changes in breathing, hematology parameters, histopathological analysis, behaviour, body temperature food intake, skin, and water consumption were found. The finding suggested at a dose of 2000 mg/kg, the extract appears to be safe, and the LD_{50} was determined to be > 2,000 mg/kg (OECD 2001).

SFE improved the sperm characteristics of BPS-exposed semen

All morphological parameters such as the percentage of the bent tail, damaged head, without a head, without tail, and total damaged sperm were significantly ($P<0.001$) higher on BPS exposure for 14 days as compared to the control and SFE-treated groups. In the rats who drank BPS after SFE treatments, their sperm characteristics were significantly ($P<0.001$) reinstated when compared to only the BPS-exposed rats for 14 days. The maximum improvements were observed at the dose of 100 mg/kg per BW (Table 5).

SFE improved the viability and mobility of BPS-exposed sperm cells

Sperm viability and mobility were observed in only the BPS-treated groups, untreated, and the SFE-treated groups and found significant ($P<0.05$) mortality and less mobility after exposure to BPS than in the control, as well as SFE-treated groups. This alteration of semen indicated the presence of hypospermatogensis. In addition, SFE treatments for 14 days improved sperm viability and mobility compared to the group that received BPS only (Table 6).

Effects of SFE on BPS-induced oxidative stress/nitrative stress

The SFE reduced the levels of oxidative biomarkers (MDA, SOD, CAT, and GPx) and nitrative stress (iNOS and NO). Treatment of rats with 50 µg/kg of BPS for 10 weeks significantly increased serum level of MDA ($P<0.001$) and decreased the activities of GPx, SOD, CAT, NO, and iNOS ($P<0.001$) than the control group treated for 10 weeks, while groups treated with SFE doses for 10 weeks were showed the optimal parameter level of parameters (Figs. 2 and 3).

SFE improved male hormonal profile

The general linear model for data analysis showed a significant decrease in the reproductive hormonal profile compared to the control (Fig. 4). The serum levels of testosterone (TSH), luteinizing (LH), and follicle-stimulating

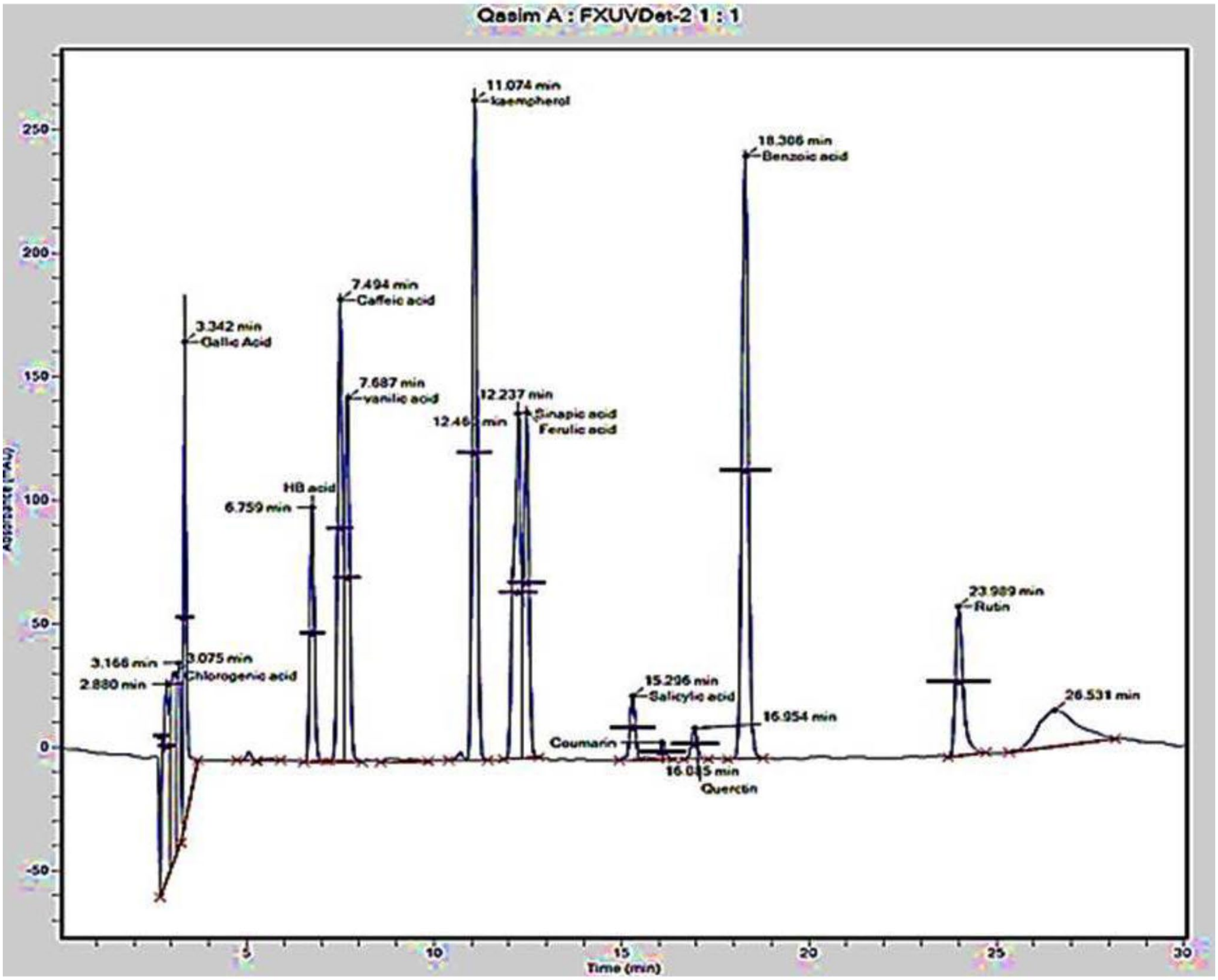

Fig. 1 HPLC chromatogram of *S. indicus* methanolic floral extract and compounds were detected by comparing the retention time with standards

Table 4 Phytochemical profile of methanolic floral extract of S. *indicus*

Retention time (min)	Area	Amount (ppm)	Compound
7.494	1,708,360.2	100.79	Caffeic acid
11.074	2,451,101.1	100.00	Kaempferol
12.237	1,764,878.2	100.06	Sinapic acid
12.460	1,303,526.2	99.98	Ferulic acid
15.296	265,192.1	99.97	Salicylic acid
16.085	83,211.5	99.85	Coumarin
16.954	143,558.6	99.91	Querctin
18.306	2,896,116.4	99.91	Hydroxybenzoic acid
23.989	883,827.4	99.87	Rutin
2.880	883,651.6	114.81	Chlorogenic acid
7.687	1,287,656.7	100.05	Vanillic acid
3.342	1,144,413.3	100.70	Gallic acid

hormones (FSH) in the SI-treated groups compared to the control group at doses (25, 50, and 100 mg/kg) were significantly ($P < 0.001$) improved and Tukey's comparisons showed that there were no significant differences between treatment results and after 10 weeks found within optimal parameter levels.

SFE downregulated target mediators of apoptosis and necroptosis

The mediators of extrinsic apoptosis (Fas/FasL and Caspase 3/-8) and necroptosis (RIPK1/-3 and MLKL) were significantly ($P < 0.001$) elevated in rats who only drank BPS-containing water. SI was significantly ($P < 0.001$) downregulated target elements of necroptosis and apoptosis (Fig. 5).

Table 5 Morphological characteristics of sperm from rats exposed to BPS and *Sphaeranthus indicus* extracts

Groups	Bent tail (%)	Without head (%)	Damaged head (%)	Without tail (%)	Total damaged sperm (%)
C (control)	12.0 ± 2.4	1.0 ± 1.45	1.0 ± 1.15	1.0 ± 2.15	25.0 ± 1.43
B-G1	23.0 ± 1.4###	3.0 ± 1.23###	5.0 ± 1.35###	4.0 ± 1.25###	50.0 ± 2.13###
B-S-G2	16.0 ± 3.3***	2.0 ± 2.1***	2.0 ± 2.45***	2.0 ± 1.37***	33.0 ± 1.73***
B-S-G3	14.0 ± 1.9***	1.0 ± 2.34***	1.0 ± 1.75***	2.0 ± 1.98***	29.0 ± 1.89***
B-S-G4	13.0 ± 3.1***	1.0 ± 1.45***	1.0 ± 2.25***	1.0 ± 1.57***	27.0 ± 2.11***

Note: Values are means ± standard deviations of quintuplicates. **Groups**: B-G1 = 50 μg/kg BW of BPS, B-SI-G2 = 50 μg/kg BW of BPS + 25 mg/kg BW of SFE, B-SI-G3 = 50 μg/kg BW of BPS + 50 mg/kg BW of SFE, B-SI-G4 = 50 μg/kg BW of BPS + 100 mg/kg BW of SFE. ###$P < 0.001$ vs. Control, *** $P < 0.001$ vs. B-G1

Table 6 Sperm viability and mobility after BPS and *Sphaeranthus Indicus*

Groups	Sperm count (× 10^6 cells)	Sperm viability (%)	Mobility (%)
Control (C)	31.2 ± 2.29	95.0 ± 1.29	30.0 ± 2.4
B-G1	21.4 ± 3.29###	23 ± 1.19###	18.0 ± 3.19###
B-S-G2	24.0 ± 1.33***	65.0 ± 2.2***	25.0 ± 1.31***
B-S-G3	27.6 ± 2.19***	85.0 ± 2.12***	27.0 ± 1.23***
B-S-G4	30.0 ± 2.09***	92.0 ± 1.31***	28.0 ± 1.46***

Note: Values are means ± standard deviations of quintuplicates. **Groups**: B-G1 = 50 μg/kg body weight (BW) of BPS, B-SI-G2 = 50 μg/kg BW of BPS + 25 mg/kg BW of SFE, B-SI-G3 = 50 μg/kg BW of BPS + 50 mg/kg BW of SFE, B-SI-G4 = 50 μg/kg BW of BPS + 100 mg/kg BW of SFE.###$P < 0.001$ vs. Control, ***$P < 0.001$ vs. B-G1.

Effects of SFE on histopathological and morphometric parameters of the testes

Histopathological examination of the testes of rats revealed normal histoarchitecture in the control group (Fig. 6; A 400×). In cross-section, the seminiferous tubules of only BPS-treated rats (B-G1) showed atrophy of seminiferous tubules due to the absence of spermatozoan (Fig. 6; B 400×), reduced Sertoli cells/ST and height/diameter of ST, increased injury level, and decreased spermatogenic cell density (Fig. 7). SFE treatments (25, 50, and 100 mg/kg per BW for each rat) have thickened the seminiferous tubules. The internal tissue or connective tissue were endocrine cells (Leydig cells), and the germ cells of spermatogenesis (spermatid, primary spermatocyte, and sperm) along with morphometric parameters were improved after SFE treatments. The findings of morphometric evaluation revealed a reduction of ST cells and the ratio of seminiferous tubule height and diameter was significantly reduced in the group that received only BPS containing drinking water, while SFE doses restored the morphometric parameters in SFE treated groups.

Protein-ligand interaction for in silico approach

The findings of docking studies indicated maximum binding affinity of RIPK3 with rutin (-9.7 kcal/mole), and RIPK1-quercetin (-9.1 kcal/mole) (Table 7). The docking analysis depicted RIPK1/RIPK3 are key regulators of necroptosis and apoptosis.

The eigenvalue was substantially greater, which shows the stability of RIPK1-quercetin 1/ and RIPK3-rutin complex (**Fig. S1 & Fig. S2; b and e**). **Figures S1c & S2c** show the elastic network model and depict that docked protein molecule (Cα) atoms are interconnected with "springs" of certain strengths and the darker greys of the graph represent the stiffer spring. The covariance matrix expressed the protein correlations; red motions were identified as correlated, white motions as uncorrelated, and blue motions as anti-correlated (**Figures S1d & S2d**). The deformability map elucidates that residues formed a coiled structure and shows that the peaks were correlated to deformable regions in the RIPK3, with the largest peaks denoting most deformable regions, which illustrated the flexibility of RIPK1-quercetin and RIPK3-rutin complex (Figures S1e & S2e).

Discussion

Characterization of SFE indicated the presence of chlorogenic acid, quercetin, gallic acid, quercetin, kaempferol, coumarin, ferulic acid, vanillic acid, rutin, salicylic acid, and sinapic acid. Of these, chlorogenic acid occurred in the highest quantity.

Exposure to BPA and its analogues causes reproductive damage. The relatively low dose of BPS has an impact on the reproductive system. There is an urgent need to develop an antidote to BPA and its analogues that has fewer side effects. In the current study, we focused on BPS-induced male reproductive toxicity through drinking water and focused on targets related to oxidative stress, necroptosis, apoptosis, and mitigation by SFE to find out whether it can

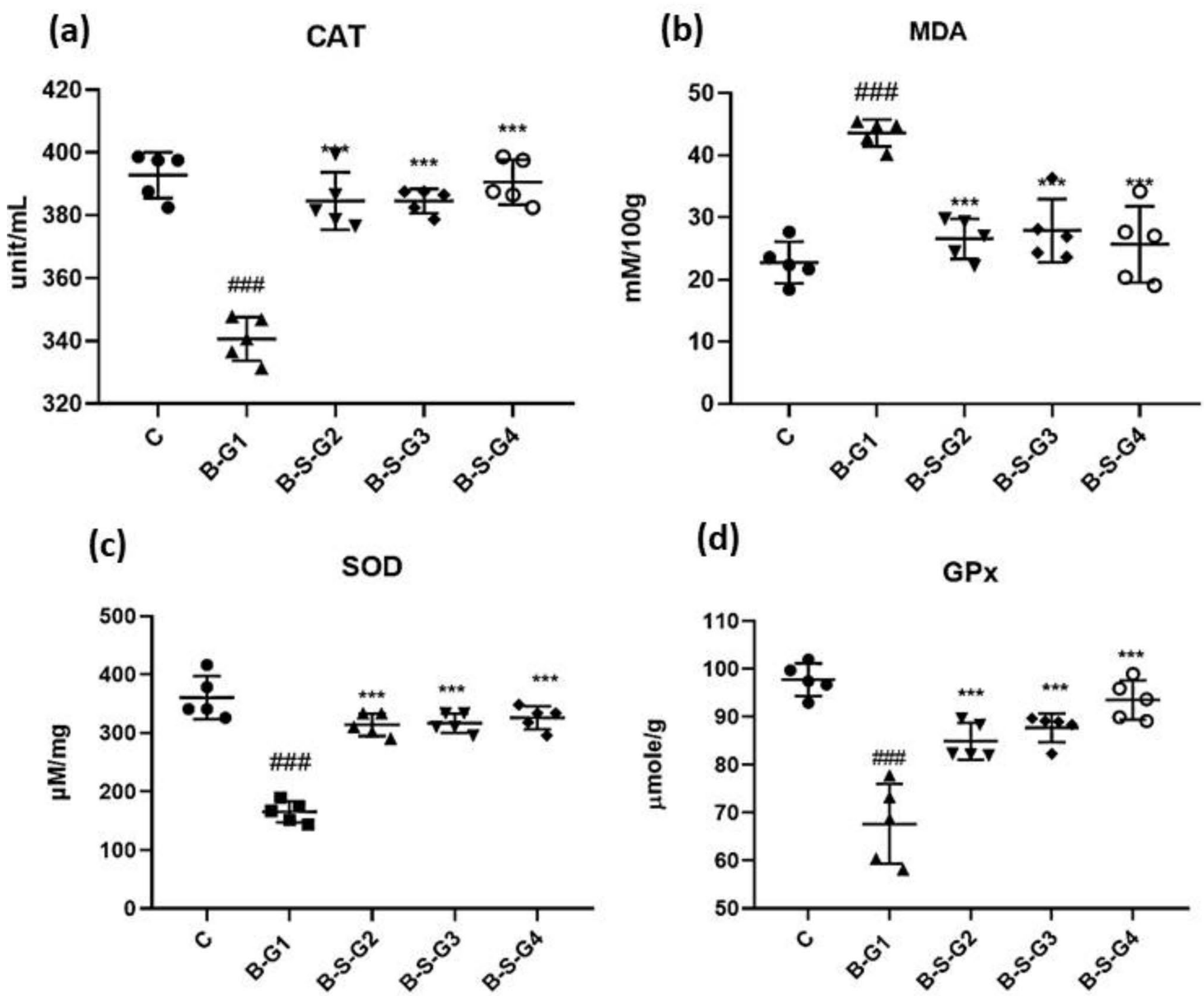

Fig. 2 BPS in contaminated water reduced levels of SOD, CAT, and GPx and increased MDA levels in B-G1 group rats after 10 weeks than control. SFE assuaged the levels of MDA, SOD, CAT, and GPx in B-SI-G2, B-SI-G3, and B-SI-G4 groups than B-G1. Values are means ± standard deviations of quintuplicates. **Groups**: C = control group, B-G1 = 50 µg/kg BW of BPS, B-SI-G2 = 50 µg/kg BW of BPS + 25 mg/kg BW of SFE, B-SI-G3 = 50 µg/kg BW of BPS + 50 mg/kg BW of SFE, B-SI-G4 = 50 µg/kg BW of BPS + 100 mg/kg BW of SFE. Abbreviations: (**a**) Catalase (CAT); (**b**) Malondialdehyde (MDA); (**c**) Superoxide dismutase (SOD); (**d**) Glutathione peroxidase (GPx)

downregulate target biomarkers associated with the onset of reproductive toxicity.

Morphological parameters (related to head and tail), sperm viability, motility, and sperm count were significantly ($P < 0.001$) altered in rats that received BPS-contaminated water than in the control. Administration of SFE (at 25, 50, and 100 mg/kg per BW) considerably improved sperm deformities including said parameters ($P < 0.001$). The present finding was in line with previous studies where male rats exposed to low doses of the BPA analogues in drinking water from early to mature sexual age harmed their spermatogenic cycle and produced various morphological deformities in normal reproductive activities (Wu et al. 2018; Thoene et al. 2020). Exposure to BPA in rats at a low dose is consistent with human exposure in the vicinity of a low dose through byproducts of items having BPA that leach into the environment and indirectly affect humans (Darghouthi et al. 2022). The spermatogenic deformities were improved after 10 weeks of SFE (25, 50 and 100 mg/kg/BW) administration. Previous authors hinted that chlorogenic acid, which is the major constituent of SFE in this study, is repro-protective through inhibition of testosterone degradation and caused an increase in the number of sperm (43–37%) in rats (Park and Han 2013). It was also shown to reinstate the performance of interstitial cells, and consequently increase testosterone synthesis in arsenic-exposed mice (Banihani 2019; El-Khadragy et al. 2021).

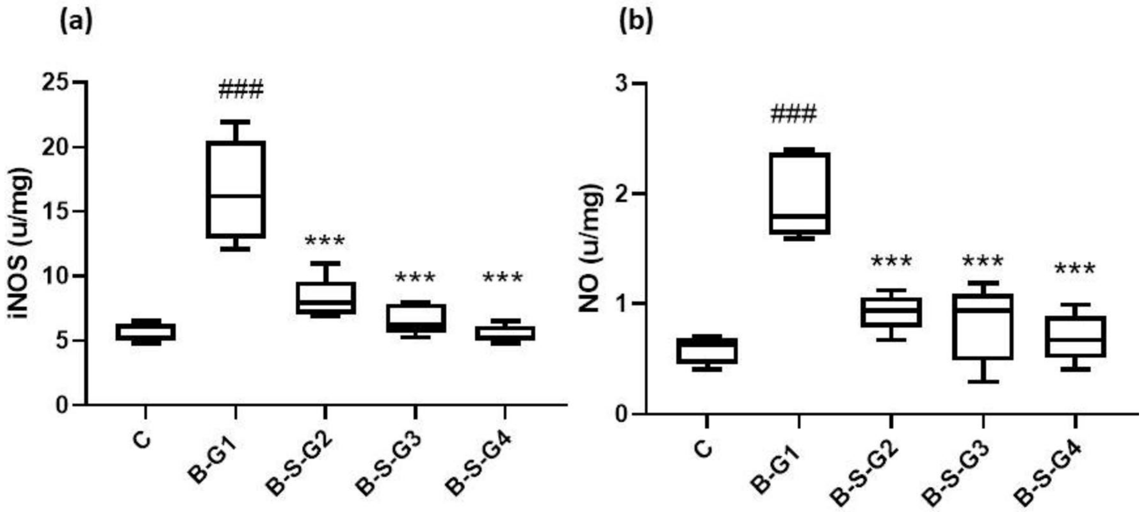

Fig. 3 BPS containing water was promoted nitrative stress as levels of (**a**) nitric oxide (iNOS) and (**b**) nitric oxide (NO) increased from the normal limit in the B-G1 group than the control. *S. indicus* flower extract was reduced the levels of iNOS and NOS in B-SI-G2, B-SI-G3, and B-SI-G4 groups. Values are means ± standard deviations of quintuplicates. **Groups**: C = control group, B-G1 = 50 µg/kg BW of BPS, B-SI-G2 = 50 µg/kg BW of BPS + 25 mg/kg BW of SFE, B-SI-G3 = 50 µg/kg BW of BPS + 50 mg/kg BW of SFE, B-SI-GM = 50 µg/kg BW of BPS + 100 mg/kg BW of SFE. ###$P < 0.001$ vs. Control, *** $P < 0.001$ vs. B-G1

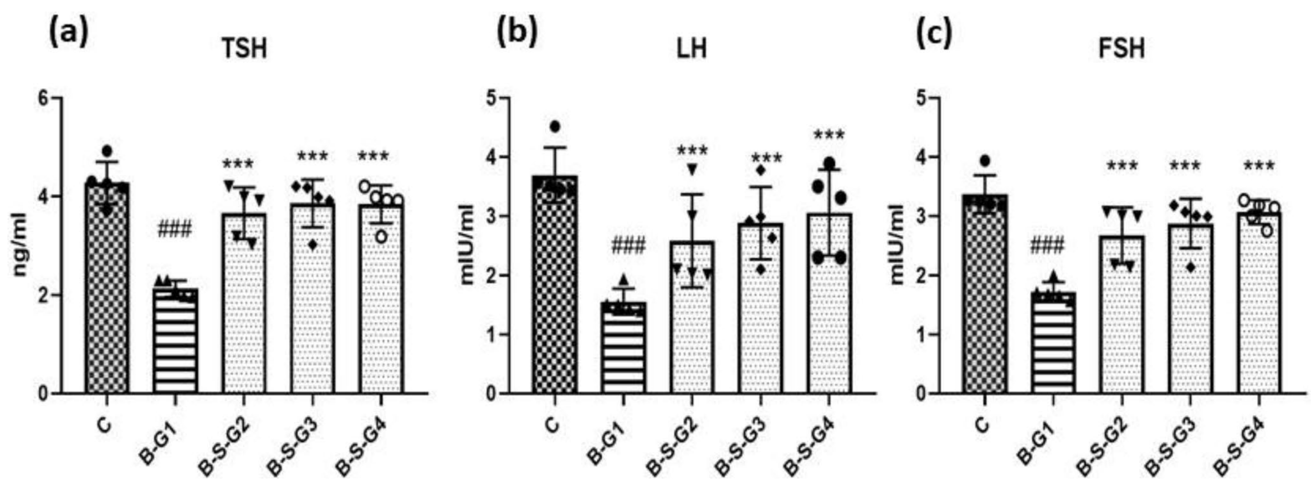

Fig. 4 BPS containing water after 10 weeks was disrupted normal Testosterone (TSH), Luteinizing hormone (LH) and Follicle stimulating hormone (FSH) levels in B-G1 group than the control. *Sphaeranthus indicus* flower extract was restored normal levels of LH, TSH, and FSH in B-SI-G2, B-SI-G3, B-SI-G4 groups. Values are means ± standard deviations of quintuplicates. **Groups**: C = control group, B-G1 = 50 µg/kg BW of BPS, B-SI-G2 = 50 µg/kg BW of BPS + 25 mg/kg BW of SFE, B-SI-G3 = 50 µg/kg BW of BPS + 50 mg/kg BW of SFE, B-SI-G4 = 50 µg/kg BW of BPS + 100 mg/kg BW of SFE. ###$P < 0.001$ vs. Control, *** $P < 0.001$ vs. B-G1

Since oxidative stress is the underlying mechanism of BPA-induced reproductive toxicity (Qi et al. 2014), raising antioxidant levels may be a logical approach to alleviate testicular damage. Herein, we characterized nine polyphenols that have antioxidant efficacy (Mohammed et al. 2020). Thus, it was not surprising to observe significant reductions ($P < 0.001$) in the levels of nitro-oxidative biomarkers (MDA, iNOS, and NO) and lower antioxidant levels (CAT, GPx, and SOD) on BPS exposure for 10 weeks. These are concordant with a preceding report in which BPA analogues induced reactive oxygen species generation and decreased levels of antioxidants in human peripheral blood mononuclear cells (in vitro) and in male rats (in vivo) (Geens et al. 2011; Michałowicz et al. 2015; Usman and Ahmad 2016; Maćczak et al. 2017; Ullah et al. 2019). Rats that received water contaminated with BPS and SFE (25, 50, and 100 mg/kg/BW) for 10 weeks exhibited reduced levels of nitro-oxidative biomarkers (NO, iNOS, and MDA), and reciprocally improved levels of antioxidant biomarkers (CAT, GPx and SOD) (Figs. 2 and 3). Earlier efforts unraveled that phenolic compounds exert their antioxidant effect by blocking the attack of reactive oxidants on DNA, lipids,

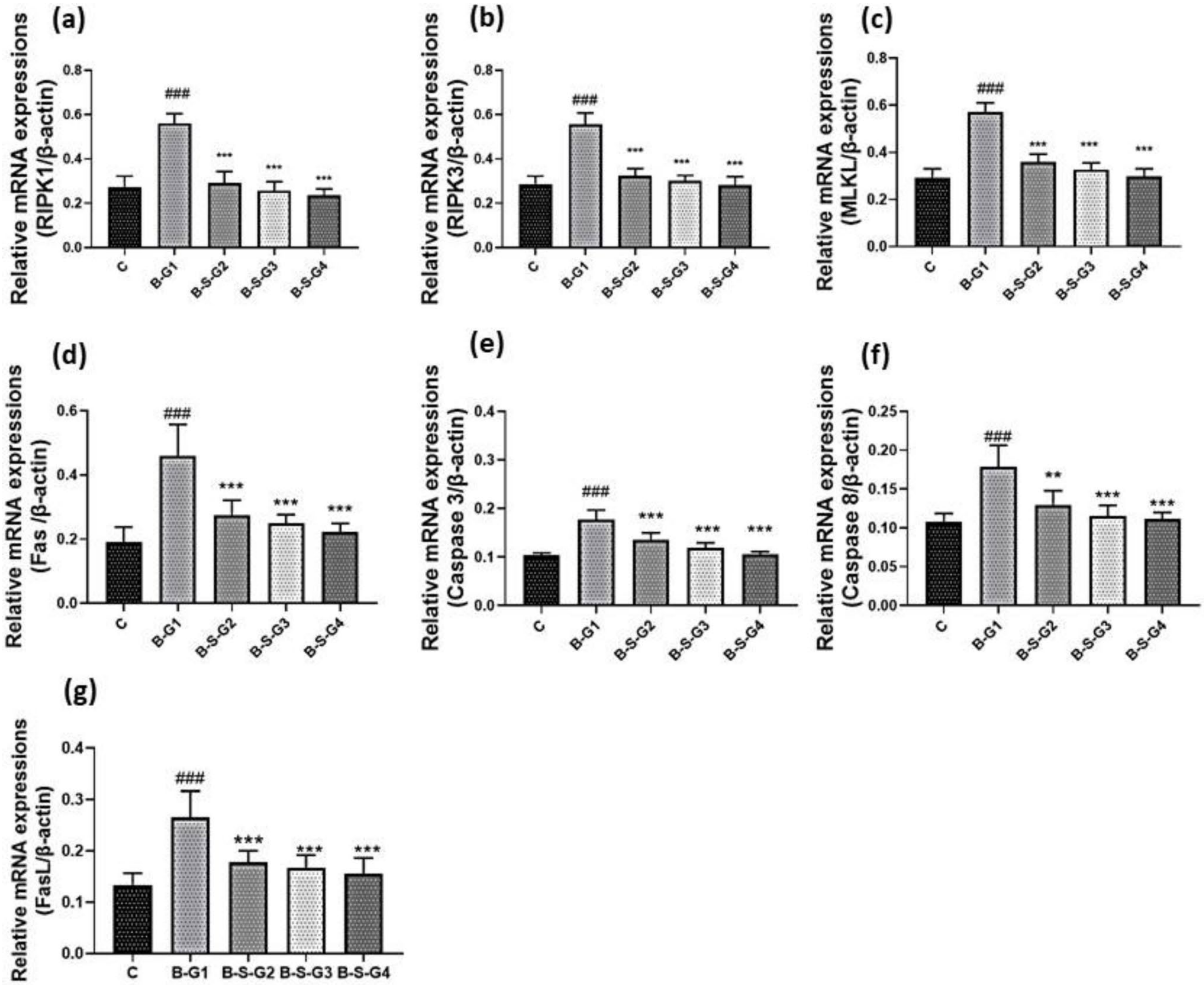

Fig. 5 BPS containing water after 10 weeks was upregulated (**a**) RIPK1, (**b**) RIPK3, (**c**) MLKL (**d**) Fas (**e**) caspase 3, (**f**) caspase 8, and (**g**) FasL in B-G1 group than control. *S. indicus* flower extract was downregulated the target genes in B-SI-G2, B-SI-G3, B-SI-G4 groups. Values are means ± standard deviations of quintuplicates. **Groups**: C = control group, B-G1 = 50 µg/kg BW of BPS, B-SI-G2 = 50 µg/kg BW of BPS + 25 mg/kg BW of SFE, B-SI-G3 = 50 µg/kg BW of BPS + 50 mg/kg BW of SFE, B-SI-G4 = 50 µg/kg BW of BPS + 100 mg/kg BW of SFE. $^{###}P < 0.001$ vs. Control, *** $P < 0.001$ vs. B-G1

and protein and alleviating the progression of testicular dysfunction (Abd-Elkareem et al. 2021). Notably, chlorogenic acid improved GPx, catalase, and SOD activities in male mice after one week of triptolide-induced oxidative stress (Wang et al. 2018). Similarly, recent research strides have highlighted the antioxidant effects of rutin (against deltamethrin-induced toxicity in rats)(Kucukler et al. 2021) and quercetin in the Achilles tendons of rats (Yoshikawa et al. 2022).

FasL-mediated apoptosis of spermatogenic cells is attributed to the interaction between FasL secreted by Sertoli cells and Fas receptors expressed in the spermatogenic cells (Lee et al. 1997; Nair and Shaha 2003). According to Li et al. (2009) the administration of high doses of BPA resulted in the apoptosis of Leydig and germ cells in the testes of mice. This was achieved through the upregulation of Fas and FasL, as well as the activation of caspase-3 and −8 (D'Alessio et al. 2001; Li et al. 2009). Necroptosis is a form of programmed necrotic cell death that is initiated by cytokines belonging to the tumor necrosis factor family (Christofferson and Yuan 2010; Vandenabeele et al. 2010). Upon activation of TNF receptor family members, the cytosolic aspect of the receptor recruits receptor-interacting kinase 1 (RIPK1), which subsequently undergoes activation of its kinase activity (Holler et al. 2000). Upon interaction with RIPK1, RIPK3 undergoes phosphorylation and subsequent activation (Degterev et al. 2008; He et al. 2009; Zhang et al. 2009). MLKL protein exists in a quiescent monomeric form

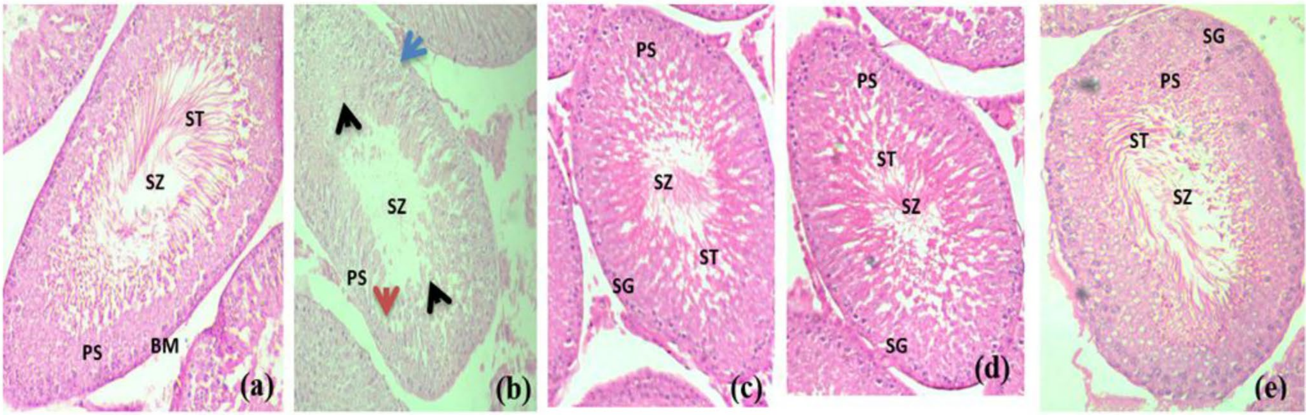

Fig. 6 Cross section of testis tissue (400×) stained by hematoxylin-eosin. The micrograph of the control group (**a**) shows normal histo-architecture arrangements of the seminiferous lumen, spermatogenic cells, basement membrane, spermatids, spermatozoans, and primary spermatocytes; (**b**) histomicrograph of rats that received only BPS (50 µg/kg/BW) shows atrophy of the seminiferous tubules that indicates the absence of spermatozoans, black arrow indicates degeneration of primary spermatocytes and spermatids, blue arrow shows disintegration of the basement membrane, and red arrow marks Slough of Sertoli cells occurred around the seminiferous tubules; **(c)** photomicrograph (400×) of the low dose of SFE (25 mg/kg/BW) shows mild reduction of spermatozoa, spermatids, and primary spermatocytes and also detected sloughing of spermatogenic cells and Sertoli cells. The basement membrane is also altered; **(d)** The histomicrograph of the SFE median dose (50 mg/kg/BW) also exhibited a mild reduction in primary spermatocyte, spermatids, and sperm; **(e)** histomicrograph of SFE (100 mg/kg/BW) indicates increased spermatid, primary spermatocytes, spermatozoan density, and normal spermatogonia cells and basement membrane. **Legend**: BM = basement membrane, ST = spermatids; PS = primary spermatocyte, SZ = spermatozoan, SG = spermatogonia cells

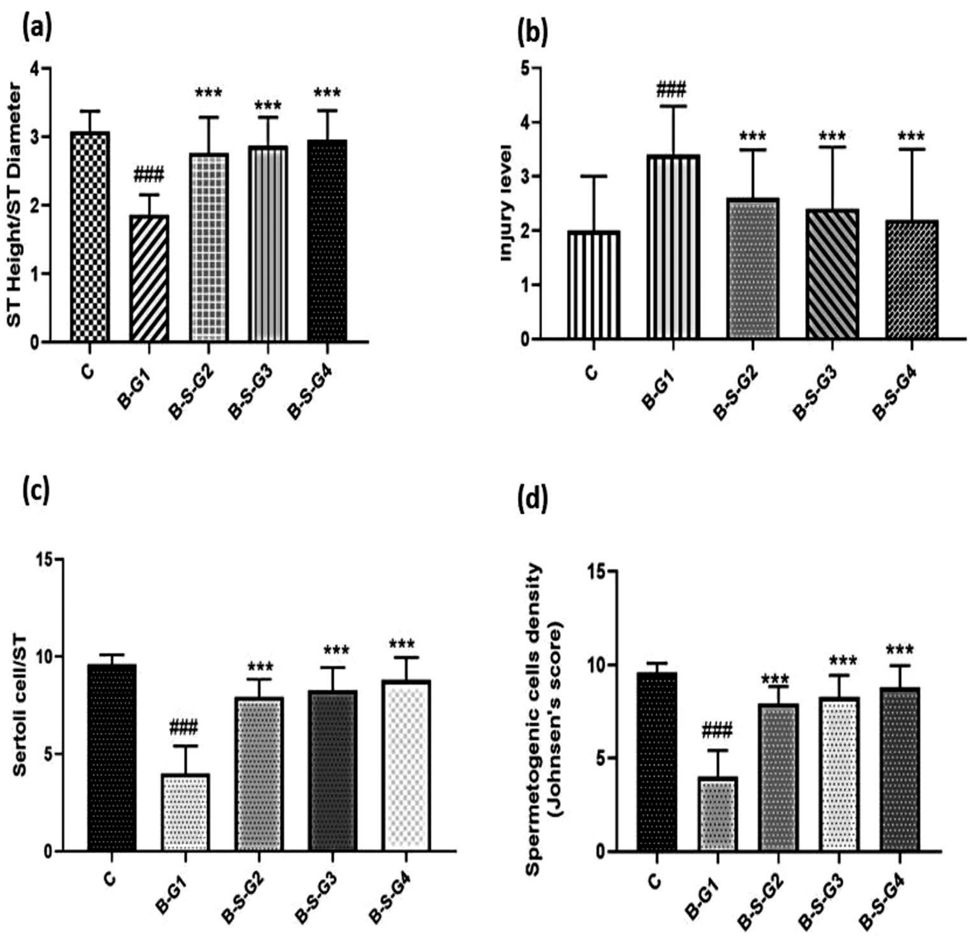

Fig. 7 Sertoli cells and seminiferous tubule (ST) height/ST diameter, as well as spermatogenic cell density, were decreased and the injury level of seminiferous tubules increased in B-G1 rats after 10 weeks. SFE improved Sertoli cells/ST and ST height/ST diameter as well as spermatogenic cell density but injury level decreased in the B-SI-G2,-G3,-G4 groups. Values are means ± standard deviations of quintuplicates. Groups: C = Negative control group, B-G1 = 50 µg/kg BW of BPS, B-SI-G2 = 50 µg/kg BW of BPS + 25 mg/kg BW of SFE, B-SI-G3 = 50 µg/kg BW of BPS + 50 mg/kg BW of SFE, B-SI-G4 = 50 µg/kg BW of BPS + 100 mg/kg BW of SFE. ###$P < 0.001$ vs. Control, *** $P < 0.001$ vs. B-G1

Table 7 Binding affinities of detected compounds of *S. indicus* with proteins related to necroptosis and apoptosis

Compound	Proteins (binding energies in kcal/mol)			
	RIPK1	RIPK3	MLKL	FAS
Hydrobenzoic acid	-5.8	-6.2	-5.9	-4.9
Chlorogenic acid	-7.1	-8.4	-6.9	-6.2
Coumarin	-6.8	-6.8	-6.7	-5.1
Salicylic acid	-5.9	-6.1	-6.2	-5.0
Gallic acid	-6.3	-6.2	-5.7	-5.2
Ferulic acid	-6.8	-6.4	-6.2	-5.4
Quercetin	-9.1	-9.0	-8.0	-6.4
Rutin	-7.8	-9.7	-8.4	-7.8
Kaempferol	-8.7	-8.9	-7.8	-6.5
Sinapic acid	-6.9	-6.8	-6.0	-5.4
Caffeic acid	-6.7	-6.6	-6.1	-5.3
Vanillic acid	-6.0	-6.0	-6.0	-4.9

within the cytosolic compartment. Following the phosphorylation of particular residues on human MLKL or its mouse homolog, MLKL underwent oligomerization and relocated to the plasma membrane, resulting in the eventual breakdown of membrane integrity and necrotic cell demise (Cai et al. 2014; Chen et al. 2014; Hildebrand et al. 2014). In the current study, nitro-oxidative stress-activated apoptotic mediators (Fas/FasL, Caspase 3/8, RIPK1/3, and MLKL) induced apoptosis and inflammation of the testes after 10 weeks of BPS (50 µg/kg/BW) exposure in rats. The levels of apoptotic mediators (Fsas/FasL and caspase 3/8) in SFE-treated groups were downregulated but were not statistically different ($P > 0.001$) for the different groups. In An earlier study, adult rats were given either estradiol-3-benzoate (EB) (0.075 mg/rat/5th day) alone or EB + quercetin (QC) (15 mg/kg bw/alternate day) simultaneously for 30 days. The QC treatments were restored the testicular damage in rats by downregulating the expression of apoptotic markers caspase-3 and poly-ADP-ribose polymerase (PARP), caspase-8 and −9, Fas, FasL, Bax, and p53, (Bharti et al. 2014), these finding justify present study.

Leydig cell testosterone secretion is essential for normal spermatogenesis and normal histoarchitecture of the testes (Sharpe 2010). The current investigation examined the impact of BPS on the male endocrine system, specifically the levels of luteinizing, follicle-stimulating, and thyroid-stimulating hormones in rats that received BPS-contaminated water. The findings revealed a deviation from the typical levels. This observation is strongly linked to a previous conclusion that BPA and its analogues can act as either an agonist or antagonist to the estrogen receptor alpha (ERα) or estrogen receptor beta (ERβ) (Matsushima et al. 2010) and its administration to adult male rats for 14 days induced dysregulation of the hypothalamic-pituitary-testicular axis which decreased LH, FSH levels, serum testosterone (TSH) and a significant drop in testicular mRNA levels of luteinizing hormone receptor (LHR), inhibin B and estrogen receptor alpha (ERα) (Feng et al. 2012).

Mitigation of these effects in treated rats by SFE for 10 weeks was investigated to find a BPS antidote to reinstate the hormonal profile along with all parameters considered in this study. Chlorogenic acid co-treatment with tamoxifen improved the reproductive toxicity of rats and the underlying mechanism involved in the downregulation of NF-kB/NLRP3 inflammasome and activation of Nrf2/HO-1 pathway that activate antioxidant enzymes, deactivating inflammatory cytokine, and restored the Sertoli and spermatogenic cells. The restoration of sperm cells optimized the level of LH, testosterone, and FSH (Owumi et al. 2021). Administration of quercetin was found to possess the capability of inducing androgen production, reinstating redox equilibrium, and averting germ cell apoptosis, thereby resulting in the revival of testicular function in rats (Badr et al. 2019).

The initiation of reactive oxygen species generation, lipid peroxidation, antioxidant depletion, deformities of the spermatogenic cycle, and the disturbance of the male hormonal profile are all alterations that lead to atrophy of seminiferous tubules and the absence of spermatogenesis in the testis of a rat having only BPS-contained drinking water. The morphometric parameters were also found to reduce spermatogenic cell density, Sertoli cells as well as seminiferous tubule height/diameter ratio while the injury level of tubules was elevated. These findings are consistent with previous studies in which exposure to BPA subsequently damaged germinal epithelia, blocked some areas around seminiferous tubules, and accumulated eosinophilic material in the testes of rats (Hassan et al. 2013; Kamel et al. 2018). The pathological condition of the testes was owing to xenoestrogen characteristics of BPA which may prevent normal growth (Tohei et al. 2001; Hassan et al. 2013). These histopathological changes are attributed to the reduction of testosterone levels because of the inhibition of 5-reductase activity in the epididymal tissues (Tohei et al. 2001). Rats that received BPS and SFE were observed to have normal spermatogenesis in seminiferous tubules, and their histoarchitecture was reinstated along with their considered morphometric parameters. The characterized compounds in SFE in this study could have contributed to the restoration of testicular histoarchitecture via inhibition of nitro-oxidative stress-mediated inflammatory and apoptotic mediators. The most relevant study by Abdallah et al. (2012) found the repro-protective effect of caffeic acid to mitigate lambda cyhalothrin-induced testicular damage and played a significant role in improving sperm quality (Abdallah et al. 2012). Quercetin is the scavenger of ROS, such as RNS, and superoxide, such as NO, and alleviates ischemia and reperfusion injury in the testes in rats (Aldemir et al. 2012). Rutin is a derivative of flavone glycoside and exerts different pharmacological activities, such as anti-inflammatory and antioxidant, and a previous study demonstrated alleviation of testicular ischemia perfusion injury in rats by depletion of ROS generation that leads to enhanced endogenous antioxidant enzymes (La Casa et al. 2000; Shen et al. 2004; Wei et al. 2011a). Medicinal plant extracts containing coumarin and kaempferol mitigated testicular injuries after treatments against testicular torsion in rats (Kanter 2011; Wei et al. 2011a, b).

The docking results elucidated that RIPK1/3 is a key regulator of necroptosis and apoptosis as has maximum binding affinities with rutin and quercetin as well as these components are the main bioactive compounds of SFE that modulated the elements of necroptosis.

Conclusion

Taken together, bioactive molecules such as rutin, chlorogenic acid, quercetin, kaempferol, and other phenolic compounds in SFE could be responsible for its repro-protective effects against BPS-induced reproductive impairments via upregulation of inflammatory cytokines and apoptotic mediators. These results pave the way for the future development of SFE as a safe natural resource against BPS-induced testicular damage. However, for human use, in vivo studies are needed to study its pharmacodynamics and kinetics as well as the effective daily dosage.

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Author contributions SI: Design of experiment and drafting of manuscript; TO: Editing, review, and proofreading, IK: Proofreading, Statistical analysis; UMK: Discussion of results. All authors approved the final version of the manuscript.

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Declarations

Conflict of interest Shabnoor Iqbal, Timothy Omara, Ivan Kahwa, and Usma Mir Khan declared no conflict of interest.

Ethical statement The Ethical Committee for Animal Research of Government College University, Faisalabad, Pakistan approved with Approval no. GCUF/EC/22/899. The experiment protocols were carried out following the National Institutes of Health guide (NIH Publications No. 8023, reviewed 1978) for precautions and use of laboratory animals.

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