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Tamarindus indica seed extracts inhibit mouse microsomal 5α-reductase *in vitro*

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ABSTRACT: *Tamarindus indica* seeds have important roles in the field of health sciences. The seeds are used in traditional medicine due to their beneficial effects on urologic disorders, including benign prostatic hyperplasia. The present study evaluates the inhibitory activity of *T. indica* seed extracts against 5α-reductase (5αR). This enzyme is responsible for the conversion of testosterone to dihydrotestosterone, which stimulates prostatic enlargement. Seeds were separately extracted with ethanol, ethyl acetate, and hexane. The inhibitory activity of the extracts was assessed in the presence of testosterone and 5αR from mouse liver. Unchanged testosterone was quantified by high-performance liquid chromatography with photodiode array detector. Inhibition was calculated based on the remaining testosterone and was expressed as dutasteride equivalent 5αR inhibitory activity (DE; μg dutasteride equivalent per mg extract). The most active extract underwent phytocompound profiling by ultra-performance liquid chromatography quadruple time-of-flight mass spectrometric analysis. The results showed that ethyl acetate extract was the most active 5αR inhibitor with a DE value of 153.31 ± 10.60. Fifteen phytocompounds from the classes of tocopherol, fatty acid, phytosterol, saponin, terpenoid, and phenolic compounds were identified, which may be partly responsible for the inhibitory activity of the extract. The findings indicate that *T. indica* seeds can be a source of 5αR inhibitors.

KEYWORDS: 5α-reductase; benign prostatic hyperplasia; dihydrotestosterone; *Tamarindus indica*; testosterone.

1. INTRODUCTION

Benign prostatic hyperplasia (BPH) is a common condition in middle-aged to elderly men, characterized by an enlargement of the prostate gland as a result of an increase in epithelial and stromal cells [1]. This causes the urethra to be compressed, causing several symptoms in the lower urinary tract [2]. Although the precise molecular etiology of BPH is complex and not fully understood, androgens have been linked to this condition because the growth and development of the prostate are androgen-dependent [3, 4]. In men, testosterone accounts for approximately 90% of the circulating form of androgen [5]. It is irreversibly converted to dihydrotestosterone (DHT) by the action of microsomal enzyme 5α-reductase (5αR), which is intrinsically more potent in stimulating the growth of the prostate [6]. The potency may be related to the superior affinity and selectivity of DHT over testosterone for androgen receptors [7]. The accumulation of DHT within the prostate acts as the hormonal mediator of hyperplasia [8]. Studies have concluded that individuals with low DHT levels due to reduced expression of 5αR have small prostates. Moreover, those with 5αR deficiency do not have palpable prostates, and the occurrence of BPH has not been reported [9, 10].

With the knowledge that 5αR plays a pivotal role in the production of DHT, and that DHT is affirmatively linked to the pathologic growth of the prostate, therapeutic inhibitors of the enzyme have been identified [11, 12]. Dutasteride is a 5αR inhibitor drug that markedly suppresses DHT serum levels by effectively preventing the conversion of testosterone to DHT [13]. However, its use is limited by its multiple side effects such as impotence, erectile dysfunction, and decreased libido. To answer this problem, plant-based alternatives are being stPudied because they are believed to be more effective and well-tolerated by the body than their synthetic counterparts [14, 15].

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Tamarindus indica seeds have been used in healthcare since time immemorial [16]. The seeds are considered to be agri-industrial by-products but were found to be applicable as food additives and pharmaceutical excipients. The plant is also one of the most widely used herbal medicines against urologic problems due to its wide geographic distribution [17]. For instance, the decoction of *T. indica* seeds has been traditionally used in the treatment and management of BPH [18]. With the view of providing scientific support for its use in BPH, we evaluated the *in vitro* 5αR inhibitory activity of *T. indica* seed extracts and qualitatively analyzed the phytocompounds present in the active extract.

2. RESULTS AND DISCUSSION

Three *T. indica* crude seed extracts, namely, *T. indica* ethanol extract (TIEE), *T. indica* ethyl acetate extract (TIEAE), and *T. indica* hexane extract (TIHE) were obtained via maceration. All extracts were dark green, with sticky consistency and semisolid appearance. The TIEE showed the highest yield of 2.35% followed by TIEAE (0.95%) and TIHE (0.82%). This indicates that polar compounds occur abundantly in the seeds of *T. indica*.

The optimized method for the assay of testosterone is summarized in Table 1. The α,β-unsaturated ketone in the A-ring of testosterone absorbs maximum UV light at 240 nm [19], thus this wavelength was used. Previous studies reported an isocratic elution of the mobile phase composed of methanol and water for the analysis of testosterone. The use of a binary solvent mixture has been well reported to be efficient in the identification of analytes [20] and the appropriate solvent ratio was probed to obtain a chromatogram that presents a better separation of analyte and internal standard (IS). For the assay of testosterone, a suitable IS was selected from a pool of compounds, namely propylparaben, ursodeoxycholic acid, and cholecalciferol. Individually, all three compounds absorb UV at 240 nm, with peaks eluting at around 2 min. However, with testosterone, clear peak separation was only observed using propylparaben. In addition, propylparaben was stable and non-reactive with testosterone, hence it was chosen as the IS for the study. A flow rate of 1.00 ml/min provided early elution of testosterone and propylparaben. In the study, a high injection volume of 10 µl produced broad peaks, resulting in bad separation. This was improved by lowering the injection volume to 5 µl. An elevated column temperature at 50°C was also set to improve column efficiency, lessening the effects of peak broadening [21]. Given these conditions, propylparaben and testosterone gave retention times of 2.1729 ± 0.0037 and 3.1045 ± 0.0061 min, respectively. A representative chromatogram is presented in Figure 1.

Table 1. Optimized HPLC-PDA method for the detection and quantification of testosterone.

Parameter	Optimized Condition
UV detection	240 nm
Mode of elution	Isocratic
Mobile phase composition	Methanol:Water (90:10)
Internal standard	Propylparaben
Flow rate	1.00 ml/min
Injection volume	5 µl
Column temperature	50°C

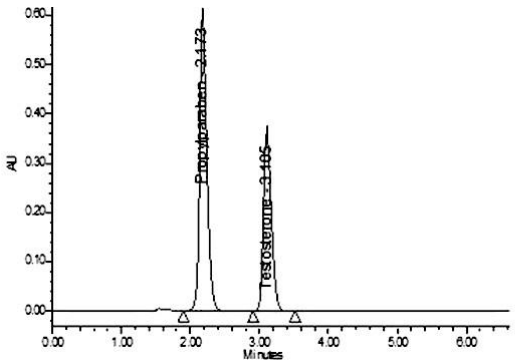

Figure 1. Representative chromatogram of propylparaben (IS) and testosterone.

The use of the mouse liver as a source of 5αR in conjunction with high-performance liquid chromatography with photodiode array detector (HPLC-PDA) for the detection and quantification of unchanged testosterone was recognized to be useful for the analysis of 5αR inhibitory activity of various compounds. Prior to the HPLC-PDA analysis, the protein content of the resulting pink microsomal pellets was quantified using the modified Lowry method. It is a combination of reactions from the Biuret method where copper ions react with peptide bonds along the protein chain and the Folin-Ciocalteu reagent reacting with the ring structure of aromatic amino acids within the protein [22]. These reactions produced a stable, dark blue complex. Based on the bovine serum albumin curve, the microsomal pellet had a protein concentration of 840 µg/ml.

The microsomal pellets isolated were used for the determination of the 5αR inhibitory activity of the extracts. Dutasteride gave a half-maximal inhibitory activity (IC_{50}) of 109.75 ± 4.53 µg/ml. The inhibitory equation (Eq. 1) of dutasteride was:

(Eq. 1) $y = 0.1856x + 22.622$ ($R^2 = 0.9975$)

with y representing the % inhibition and x representing the concentration of dutasteride. This equation was used to calculate the 5αR inhibitory activities of the extracts expressed as dutasteride equivalent 5αR inhibitory activity (DE; µg dutasteride equivalent per mg extract). The DE values of extracts are summarized in Table 2. A high DE value signifies a strong 5αR. The varied inhibitory potential of the extracts may be attributed to the amount and classes of phytocompouds present. Based on the study, the highest activity shown by TIEAE suggests that moderately polar compounds may actively play in the 5αR inhibition.

Table 2. 5α-reductase inhibitory activity of *T. indica* seed extracts.

Extract	Dutasteride equivalent 5αR inhibitory activity (µg DE per mg of crude extract)
TIEE	50.11 ± 3.65
TIEAE	153.31 ± 10.60
TIHE	9.77 ± 3.82

Accurate mass screening was carried out using the UNIFI data analysis software. The base peak ions of distinct peaks were subjected to library matching using the Traditional Chinese Medicine Library. The base peak ion chromatograms of the TIEAE and blank are shown in Figures 2A and 2B, respectively. Blank peaks were discriminated from the chromatogram of the sample and were not annotated. Annotation of the candidate masses was based on the accurate mass match, isotopic ratio match, and precursor ion intensity counts. Peak annotations in the chromatogram are the retention time of the most intense peak in each spectrum. Each peak corresponds to one component sample. A total of 92 peaks were observed, 15 of which have putative identification based on the library search (Table 3). The 15 phytocompounds identified belonged to various classes such as phenolic compounds, tocopherols, fatty acids, phytosterol, saponins, and terpenoids.

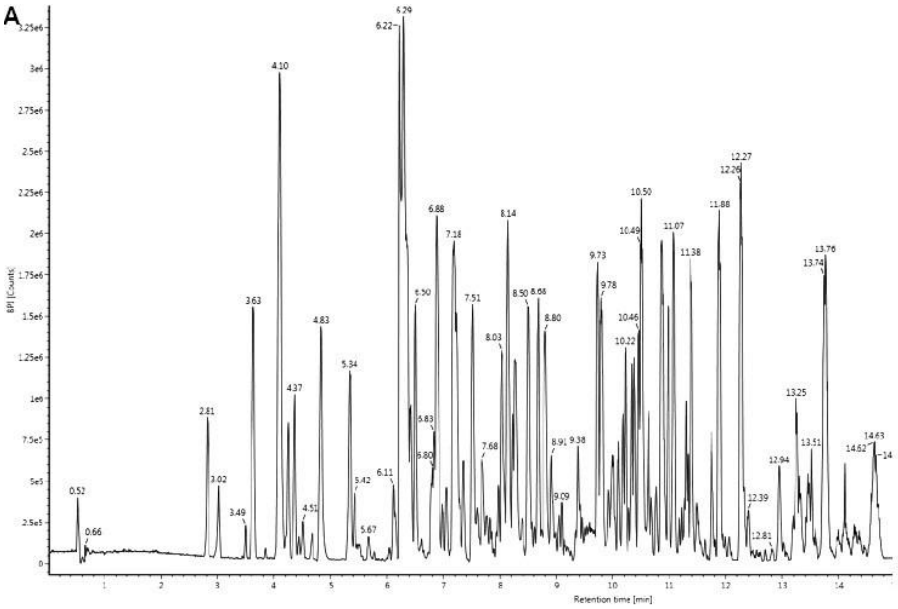

Figure 2. Base peak ion chromatogram of TIEAE (A) and methanol, as blank (B).

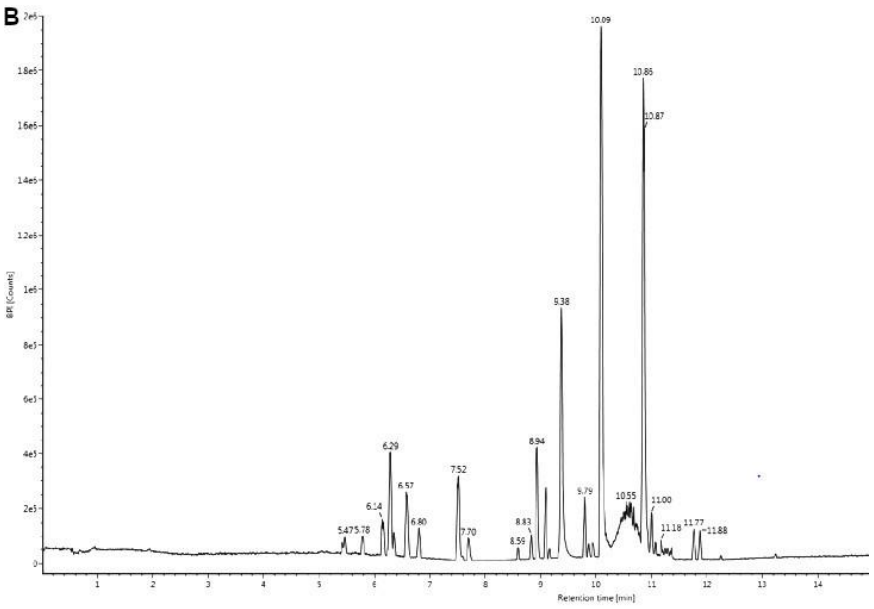

Figure 2. (Cont.) Base peak ion chromatogram of TIEAE (A) and methanol, as blank (B).

Table 3. Putatively identified phytocompounds present in *T. indica* ethyl acetate extract based on the library search.

Component Name	Retention Time	Observed m/z	Error	Adducts
Catechin	3.02	291.0889	2.595	+H
Catechin-(4α→8)-catechin	3.49	579.1483	-1.3544	+H, +Na, +K
Chrysin	4.51	255.0670	1.8247	+H
3-Hydroxy baicalein	4.83	287.0547	-0.2938	+H
Darutigenol	6.11	361.2051	-8.8839	+K
Nigakilactone I	7.83	399.1784	0.6304	+H, +Na, +K

24-Methylcholesta-7,22-dien-3β-ol	8.40	399.3594	-2.7619	+H, +Na
Kushenol I	8.57	455.2041	-2.2984	+H
4,8,12-Trimethyl-tridecanoic acid	8.68	279.232		+Na
(20S)-3α,29-dihydroxylupan-27-oic acid	9.79	497.3618	1.6218	+Na, +K
Abrisapogenol B	9.99	497.3596	-0.4973	+Na
ar-Abietatriene	10.22	309.1947	-0.5379	+K
12-Hydroxyhydromethyl abietate	10.38	337.2724	-1.3166	+H
Vitamin Eβ	10.77	439.3529	-1.7232	+Na
3-O-[β-D-Glucopyranosyl-(1→2)]-β-D-glucopyranosyl-kaempferol	12.27	633.1488	6.1792	+H, +Na, +K

Studies have identified the use of *T. indica* in the management of BPH [18, 23]. In the present study, 5αR which is important in the progression of BPH was found to be inhibited by TIEAE. The activity of *T. indica* against 5αR may be attributed to the phytocompounds present. The seeds of *T. indica* are abundant in procyanidin [24], wherein the procyanidin B3 dimer, known as catechin-(4α→8)-catechin [25] was putatively identified in TIEAE. In the study of Hiipakka et al. [26], procyanidin along with other phenolic compounds such as catechin, chrysin, baicalein, and kaempferol, possess various enzyme inhibitory effects against 5αR. The same study showed that the number and position of B-ring hydroxyl groups in phenolic compounds appear to be related to the varied bioactivity. The present study was also able to putatively identify Vitamin Eβ or β-tocopherol in TIEAE. In the molecular dynamic study of Khantam et al. [27], tocopherols including β-tocopherol were found to bind in multiple residues of 5αR, affecting the enzyme activity. Long-chain saturated fatty acids also elicit inhibitory activity for 5αR, with C12-C16 chains possessing enhanced inhibition [28]. Although no literature was found about the 5αR inhibitory potential of 4,9,12-trimethyl-tridecanoic acid, its carbon chain number is within the 5αR inhibitory criteria of long-chain saturated fatty acids reported by Liu et al. [28]. Furthermore, TIEAE was found to contain phytosterol, which may possess the potential to inhibit 5αR [29, 30]. However, a high oral dose of phytosterol is needed to inhibit the enzyme as seen in the animal study of Awad et al. [31]. Lastly, terpenoids were also identified in TIEAE which may partly contribute to the bioactivity against 5αR.

3. CONCLUSIONS

This study demonstrated the inhibitory potential of various extracts of *T. indica* seeds against 5αR, TIEAE being the most active. Phenolic compounds, tocopherols, fatty acids, phytosterols, saponins, and terpenoids were putatively identified in the extract which may be the responsible phytocompounds in the enzyme inhibition. It is recommended, however, to conduct further studies on the specific phytocompounds putatively identified such as *in vivo* studies using animal models of BPH, to fully characterize its application in the urologic condition.

4. MATERIALS AND METHODS

4.1. Chemicals and reagents

Ethanol, ethyl acetate, and hexane used for extraction were of analytical grade (Ajax Finechem Pty Limited, New South Wales, Australia). Sucrose, testosterone, NADPH, NaOH, and Folin-Ciocalteu phenol were obtained from Sigma Aldrich (St. Louis, Missouri, USA) while dithiothreitol was procured from Promega (Madison, Wisconsin, USA). Solvents used for the HPLC-PDA analysis were of HPLC grade (Duksan, Ansan, South Korea), while solvents used for the ultra-performance liquid chromatography quadruple time-of-flight mass spectrometry (UPLC-qTOF-MS) analysis were of LC-MS grade (Duksan, Ansan, South Korea), including the additive formic acid (Scharlab, Sentmenat, Spain). Additionally, type I Milli-Q® water (Millipore, Merck, Germany) was utilized. All other solvents and reagents used were of analytical grade and were obtained from

local suppliers (Belman Laboratories, Quezon City, Philippines, and Harnwell Chemicals, Makati, Philippines).

4.2. *T. indica* crude seed extracts preparation

Fruits of *T. indica* were collected in the province of Abra in the Cordillera Administrative Region, Philippines. A sample was submitted to the Bureau of Plant Industry Manila under a voucher specimen number PLT-ID-CRPSD-2246-19 and was certified to be *T. indica* L. Fruit pods were manually cracked, and the seeds were separated from the fruit pulp. The seeds were washed, air-dried, and powdered using RT-12 pulverizing machine (Rong Tsong Precision Technology Co., Taichung, Taiwan). The resulting powdered *T. indica* seeds were separately macerated using three different solvents of varying polarities, namely, ethanol, ethyl acetate, and hexane. Maceration of seeds was performed for 48 h followed by 30 min sonication using Powersonic 410 sonicator (Human Lab Inc., Hwaseong, South Korea). The mixtures were filtered, and the filtrates were evaporated to dryness to obtain TIEE, TIEAE, and TIHE. The percent yield of the crude extracts was calculated. Afterward, they were placed in wide-mouth amber-colored bottles and stored under 2-8°C prior to the *in vitro* 5αR inhibitory assay.

4.3. HPLC-PDA method

An HPLC-PDA method was used for the detection and quantification of testosterone in the *in vitro* 5αR inhibitory assay. Waters Alliance e2695 HPLC system and 2998 PDA detector (Waters, Milford, Connecticut, USA) were used, equipped with HiQsil C18 column (4.6 x 150 mm, 5 µm). Empower® 3 software was employed for data acquisition. Chromatographic conditions were optimized in terms of mobile phase composition, mode of elution, flow rate, UV detection, column temperature, and injection volume. For the selection of IS, triplicate runs were first conducted on testosterone with the candidate IS (i.e. propylparaben, ursodeoxycholic acid, and cholecalciferol) for their suitability in the method.

4.4. Mouse liver enzyme preparation

The 5αR inhibitory activity of the three crude extracts was screened following the methods of Lee et al. [32] with modifications. Procedures for animal use were approved by the Institutional Animal Care and Use Committee of the University of the Philippines Manila (protocol number: 2019-034). Three healthy ICR mice were fasted overnight to decrease the concentration of liver glycogen and then anesthetized with 30 mg/kg of tiletamine-zolazepam (Zoletil®, Virbac, Carros, France) followed by cervical dislocation. The livers were excised and washed with ice-cold homogenizing buffer (0.32 M sucrose, 0.001 M dithiothreitol, and 0.02 M potassium phosphate, pH 6.5). The livers were minced in a beaker with a pair of pre-sterilized scissors. The minced tissue was homogenized in the homogenizing buffer followed by centrifugation (MRC Laboratory-Instruments, Holon, Israel) at 3,940 xg for 10 min. The pellet produced was washed twice with 2 volumes of homogenizing buffer. The supernatants were combined from the two centrifugations and were centrifugated further at 3,940 xg for 1 h. The resulting pellet (microsome) was suspended in the homogenizing buffer and centrifugated at 3,940 xg for an additional 1 h. The microsome suspension was divided into small aliquots and was stored in an ultralow freezer (ThermoFisher Scientific, Asheville, North Carolina, USA) at -80°C prior to its use.

4.5. Microsomal protein content determination

The protein content of the microsome was determined using the modified Lowry method. The assay was carried out by diluting the sample to 1 ml with water and adding 0.9 ml of solution A (2 g/l $KNaC_4H_4O_6$ and 100 g/l Na_2CO_3 in 0.5 M NaOH) prior to a 10 min incubation at 50°C. The sample was cooled down to room temperature and 1 ml of solution B (0.2 g/l $KNaC_4H_4O_6$ and 0.1 g/l $CuSO_4$ in 0.1 M NaOH) was added. The mixture was left to stand for 10 min. Three ml of solution C (Folin-Ciocalteu phenol reagent in water, 1:16 v/v) was added and the mixture was incubated at 50°C for 10 min. A standard curve was made using bovine serum albumin, and the absorbance was read using a spectrophotometer (Hitachi High-Tech, Tokyo, Japan) at 600 nm [33].

4.6. Microsomal and sample solution preparation

The microsome was diluted immediately before use with 0.04 M potassium phosphate, pH 6.5, to achieve a 30 µg protein concentration. The three crude extracts were dissolved separately in pure DMSO. From this stock solution, aliquots were diluted with 0.04 M potassium phosphate, pH 6.5, to obtain four concentrations (30, 60, 150, and 300 µg/ml) of the sample solutions in 2% DMSO.

4.7. 5αR assay

The method was based on the studies of Chaiyana et al. [29], Koseki et al. [34], and Morikawa et al. [35] with minor modifications in the determination of the 5αR inhibitory activity of the extracts. Briefly, the reaction solution was preincubated without or with sample solution (50 µl) in 0.001 M dithiothreitol (25 µl), 1 mg/ml testosterone (75 µl), and 1.54 mg/ml NADPH (125 µl). The reaction was started with the addition of microsomal solution (250 µl). The mixture was vortexed (Scientific Industries, Inc., Bohemia, New York, USA) for 3 s and was incubated for 10 min at 37°C. Afterward, the reaction was stopped by the addition of 1 ml ethyl acetate, followed by the addition of IS, 1 mg/ml propylparaben (125 µl). Samples were vortexed for 60 s. The organic layer was collected and evaporated to dryness. The residue was dissolved in 3 ml methanol, passed through a 0.45 µm syringe filter, and transferred to amber-colored HPLC vials for the quantification of testosterone using the HPLC-PDA method described above. Two additional reactions were prepared [36], namely, a solvent blank (sb) containing 2% DMSO instead of the sample solution and an enzyme blank (eb) that was mixed with ethyl acetate before the addition of the microsomal solution causing the non-conversion of testosterone to DHT. The % inhibition was calculated using the testosterone content defined in Eq. 2:

$$\text{(Eq. 2) \% inhibition} = [(T_{ext} - T_{sb})/(T_{eb} - T_{sb})] \times 100$$

wherein T_{ext}, T_{sb}, and T_{eb} correspond to the unchanged testosterone levels in the sample, solvent blank, and enzyme blank solutions, respectively. Dutasteride, a well-known 5αR inhibitor was used as the positive control. The IC_{50} of dutasteride was calculated. Using the inhibitory equation of dutasteride, the DE values of extracts were calculated. Dilution factors for each concentration were considered. Triplicate measurements were performed. The extract with the highest inhibitory activity was subjected to phytocompound identification.

4.8. UPLC-qTOF-MS analysis

The extract with the highest inhibitory activity was subjected to phytocompound analysis using UPLC-qTOF-MS. The extract was dissolved in methanol, diluted to a final concentration of 0.5 mg/ml, and then passed through a 0.2 µm syringe filter. The analysis was performed on Waters Acquity UPLC Class I (Waters, Milford, Connecticut, USA). Five µl of the sample were injected and separated on Acquity HSS T3 C18 column (2.1 x 100 mm, 1.8 µm) at 40°C. The mobile phase was comprised of water + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B). A 0.4 ml/min gradient was initiated. The Waters Xevo G2-XS qTOF, MS^E mode was run in a positive ion mode, with the capillary voltage set at 1.0 kV, cone voltage at 40 V, cone gas flow of 40 l/h, desolvation temperature at 550°C, desolvation gas flow of 950 l/h and source temperature at 120°C. The samples were run at a range of 50-1,200 m/z with a scan time of 0.15 s. Leucine-enkephalin was used as a reference for correction. Data were processed using the Waters UNIFI Scientific Information System v1.8.1.073 software and Waters Traditional Chinese Medicine Library.

4.9. Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) 27.0. The data gathered were recorded as mean ± standard deviation (SD). Data were analyzed with one-way analysis of variance (one-way ANOVA) followed by Tukey's post hoc test. Mean values were considered to be statistically significant when $p<0.05$.

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