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ORIGINAL ARTICLE

Isolation and Characterization of Flavonoids from Fraction of *Blepharis persica* (Burm. f.) O. Kuntze Upregulated Testosterone Biosynthesis *in vitro* using TM3 Leydig Cells

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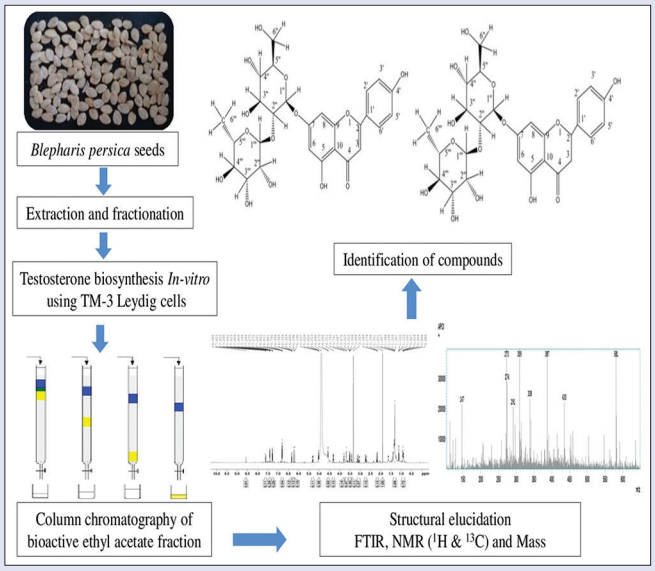
ABSTRACT

Background: Seeds of *Blepharis persica* (Burm. f.) O. Kuntze are mentioned in Ayurveda to treat male reproductive functions debilities. **Objectives:** The present study aimed to evaluate the effect of extract and fraction prepared from seeds of *B. persica, in vitro* using TM3 Leydig cells and isolation of constituents from bioactive fraction. **Materials and Methods:** Methanol extract and ethyl acetate fraction prepared that were tested *in vitro*, using TM3 cell line for the testosterone production. Cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Ethyl acetate fraction of methanol extract was further taken up for detailed chemical investigations, considering biological potential. The fraction was subjected to column chromatography. The column was eluted with a step gradient of the proportion of methanol in chloroform for the separation of compounds. The isolated compounds were characterized using Fourier-transform infrared, nuclear magnetic resonance (^{1}H and ^{13}C), and mass spectroscopy for identification. **Results:** MTT assay performed using TM3 cells revealed that extract and fraction had a half-maximal inhibitory concentration value of more than 300 µg/ml. The methanol extract showed more than half-fold and ethyl acetate fraction of methanolic extract showed a one-fold increase in testosterone at a concentration of 150 µg/ml as compared to nontreated cells. Two constituents isolated from ethyl acetate fraction of methanolic extract were identified as apigenin 7-O-neohesperidoside and naringin using comparative spectral studies. **Conclusion:** The studies suggested that the plant extract and fraction could upregulate testosterone biosynthesis in Leydig cells. Two compounds isolated from the bioactive fraction might serve as a marker for assessment of the quality of plant material.

Key words: Ayurveda, *Blepharis persica*, isolation, Leydig, spectroscopy, testosterone

SUMMARY

- The present study highlighted the potential of methanol extract and ethyl acetate fraction of *Blepharis persica* seeds that could stimulate testosterone biosynthesis *in vitro* using TM3 Leydig cells. Apigenin 7-O-neohesperidoside and naringin were isolated for the first time from the bioactive ethyl acetate fraction using the column chromatography method through the bioactivity-guided separation approach. The isolated flavonoid compounds of flavone and flavanone classes were characterized using Fourier-transform infrared, nuclear magnetic resonance (^{1}H and ^{13}C), and mass spectroscopy for identification. The results could be used to explore further studies of benzopyran in stimulating steroidogenesis in Leydig cells.



Abbreviations used: MTT: 3-(4,5-*dimethylthiazol*-2-yl)-2,5-*diphenyltetrazolium bromide*; FTIR: Fourier-transform infrared spectroscopy; NMR: Nuclear magnetic resonance; TLC: Thin-layer chromatography; NP-PEG: Natural product-polyethylene glycol; DMSO: Dimethyl sulfoxide; LC-MS: Liquid chromatography–mass spectrometry; HSD: Hydroxysteroid dehydrogenase; cAMP: Cyclic adenosine monophosphate; StAR: Steroidogenic acute regulatory protein.

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INTRODUCTION

Blepharis persica (Burm. f.) O. Kuntze. (Syn. *Blepharis edulis* Pers.), belonging to the family Acanthaceae, is commonly known as Utangan in Hindi and Uttingana in Sanskrit language in the Indian system of medicine.[1] *B. persica* is found in Punjab, western Rajasthan, Malwa region of Madhya Pradesh, Kutcha, Saurashtra, Pakistan, Iran, and Egypt.[1,2] Seeds of *B. persica* are used in the treatment of male factor infertility

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as per the Ayurvedic Pharmacopoeia of India.[1,2] Roots are considered diuretic, useful to cure urinary discharges and dysmenorrhea.[1] Seeds are also traditionally used as diuretic, adjuvant, aphrodisiac, expectorant, deobstruent, and in treatment of sexual debility.[1,2] The plant is used to treat asthma, cough, fever, and inflammation of the throat in folk medicine.[2] The literature review revealed the presence of allantoin and blepharin from the seeds of *B. persica*.[2-4] 4-hydroxybenzoic acid, acacetin, naringenin-7-O-(3"-acetyl-6"-E-p-coumaroyl-β-D-glucopyranoside), apigenin-7-O-(6"-E-p-coumaroyl-β-D-glucopyranoside), vanillic acid, naringenin-7-O-(6"-E-p-coumaroyl-β-D-glucopyranoside), apigenin-7-O-(2"-acetyl-6"-E-p-coumaroyl-β-D-glucopyranoside), naringenin, and apigenin-7-O-(4"-acetyl-6"-E-p-coumaroyl-β-D-glucopyranoside) were isolated from ether fraction of the aerial parts of *B. persica*.[5] Verbascoside, cis-verbascoside, isoverbascoside, and leucosceptoside-A (phenylethanoids) were isolated from the methanolic fractions of the aerial parts of *B. persica*.[6] *B. persica* extracts showed antimicrobial and antioxidant activity.[7,8] Methanolic extract of *B. persica* root showed significant antihyperglycemic activity in rats.[9] The hydroalcoholic extract of *B. persica* exhibited antispasmodic, bronchodilator, and antiplatelet aggregation activities.[10] Ethanolic extract prepared from the seeds of *B. persica* showed hepatoprotective activity against acetaminophen-induced hepatotoxicity in rats.[11] Ethanolic extract of the *B. persica* seed showed significant improvement in sexual activity and sexual behavior in male albino mice.[12] Although the plant has been screened for biological potential as well as few chemicals that have been isolated from the plant, there could not be any systematic exhaustive chemical studies reported from bioactive extract or fraction of *B. persica*. The present sets of studies thus were aimed to isolate constituents through the bioactivity-guided separation approach, which could be possibly utilized as a marker for standardization of the plant drug.

MATERIALS AND METHODS

Chemicals and reagents

All the chemicals and solvents used for extraction were of analytical grade and procured from Loba Chemie Pvt. Ltd. Materials and media were purchased from Hi-Media, Loba Chemie Pvt. Ltd., India.

Plant material

Seeds of *B. persica* were procured from Yucca Enterprises, Wadala (E), Mumbai, and were authenticated by A. S. Upadhye, Scientist, Plant Drug Authentication, Biodiversity and Palaeobiology Group, Agharkar Research Institute, Pune, and authentication certificate was issued with reference no. 17-218. Seeds were separated and dried under a shed for 15 days. The dried seeds were milled using a laboratory grinder to a coarse powder. The powder was preserved in airtight glass containers.

Preparation of methanol extract and fraction

The powdered seeds of *B. persica* (900 g) were defatted with petroleum ether 60°C–80°C (2000 ml) and dried marc was extracted in a Soxhlet apparatus using methanol (2000 ml) at the boiling point of methanol. The process was continued for 8 h. The methanolic extract was concentrated using a rotary vacuum evaporator at 40°C. The concentrated mass was then dried using a hot water bath to remove methanol. The methanolic extract was preserved in a glass desiccator containing anhydrous silica. Methanolic extract (10 g) was mixed with water (50 ml) and the mixture was partitioned with ethyl acetate (50 ml). The fraction was concentrated using a rotary vacuum evaporator and evaporated to dryness using a hot water bath. The yield of methanol extract and ethyl acetate fraction was 18.5% w/w and 5.56% w/w determined based on the weight of dried seed powder.

Development of thin-layer chromatography fingerprint

The methanol extract and ethyl acetate fraction (100 mg), weighed separately, were dissolved in 5 ml methanol. The mixture was sonicated and volume was made up to 10 ml using methanol. The resultant solution was filtered using a 0.45 µ syringe filter. Ten microliters of this solution was applied as separate bands on precoated silica gel 60 F_{254} aluminum sheets backed thin-layer chromatography (TLC) plate using a Hamilton syringe with the help of Linomat 5 applicator. Development of TLC was carried out using the mobile phase comprising of ethyl acetate: formic acid: glacial acetic acid: water (10:1.1:1.1:2.6, v/v/v/v). The plates after development were derivatized using natural product-polyethylene glycol 4000 reagent (NP-PEG reagent).[13] The plates were observed before and after derivatization in a long wave using the ultraviolet chamber.

Cell culture and maintenance

The *Mus musculus* Leydig cell line TM3 was procured from the National Centre for Cell Science (NCCS), Pune. The *M. musculus* Leydig cell line TM3 was maintained in culture plates containing 5% fetal bovine serum supplemented in Dulbecco's Modified Eagle Medium (pH 7.2) at 37°C in a humidified atmosphere of 5% carbon dioxide in the air. All the subsequent procedures were carried out under these conditions.

Sample preparation

Accurately about 100 mg quantity of methanol extract and ethyl acetate fraction was weighed individually. The weighed quantity was dissolved separately in a minimum amount of dimethyl sulfoxide (DMSO) and diluted up to 10 ml using culture media to prepare the stock solution (10,000 µg/ml). These stock solutions were diluted using culture media to get the concentration covering the range of 10 µg/ml to 1000 µg/ml. The concentration of DMSO was not more than 0.1% v/v in the final solution. Media containing 0.1% DMSO was used as a control for all experiments.

Determination of half-maximal inhibitory concentration value

TM3 cells were seeded at 1.5×10^4 cells/well in 96-well plates and preincubated for 24 h. The cells were then treated with sample solutions ranging from 1000 µg/ml to 10 µg/ml concentration for 24 h. Cell viability was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which is based on the conversion of MTT to MTT-formazan by mitochondrial enzymes.[14] A 20 µl aliquot of MTT solution (5 mg/ml) was added to each well of a 96-well culture plate immediately after completion of the incubation period. The mixture was further incubated for 4 h at 37°C, and the medium containing MTT was removed. The incorporated formazan crystals in the viable cells were solubilized with 150 µl DMSO, and the absorbance was measured at 540 nm using a microplate reader. Nontreated cells were used as control. Six replicates were taken for each concentration. Experimental groups were compared to the control groups for cell viability. The half-maximal inhibitory concentration (IC_{50}) value was determined graphically.

Effect of test solutions on testosterone concentration in culture media

TM3 cells were seeded at 1.5×10^4 cells/well in 96-well plates and incubated for 24 h at 37°C in a humidified atmosphere of 5% carbon dioxide in the air. Further, cells were incubated with various concentrations of the plant extract (50, 100, and 150 µg/ml) and a fraction (50, 100, and 150 µg/ml) for 24 h with appropriate controls. Total testosterone in the culture supernatant was measured using the IMMULITE 1000 system, manufactured by Siemens using testosterone estimation kit (Catalog

number: LKTWI). The estimation was solid-phase, enzyme-labeled, and competitive chemiluminescent immunoassay. The studies were performed for three selected concentrations of each test solution; each experiment was performed six times to get a statistically significant result. The mean concentration of testosterone for each test group was compared to that obtained for the control group to assess statistical significance.

Column chromatography of bioactive ethyl acetate fraction

Column (600 mm L × 40 mm ID) was packed with silica gel 200–400 mesh size (~150 g) as a stationary phase. Glass column was filled with anhydrous silica gel by pouring silica slurry prepared in chloroform. The ethyl acetate fraction (7 g) was dissolved in a minimum amount of methanol and mixed with silica gel for column chromatography. The mixture was dried and loaded over a silica gel column (200–400 mesh). The column was eluted initially with chloroform, and the further polarity was increased with an increase in proportionate of methanol (1%–100%) in chloroform. The procedure yielded four subfractions as subfraction A, subfraction B, subfraction C, and subfraction D separated based on the TLC profile of elutes. Subfraction C was rechromatographed on the column (290 mm L × 15 mm ID) packed with silica gel 200–400 mesh size (~30 g) as a stationary phase. This was eluted initially with chloroform, and the further polarity was increased using methanol (1%–100%) in chloroform to afford subfractions C-1 (1%–15% methanol in chloroform), C-2 (20%–35% methanol in chloroform), and C-3 (40%–100% methanol in chloroform). Sub fraction C-1 was further chromatographed using silica gel column and the column was eluted with methanol: chloroform mixtures (5:95, v/v, 10:90,v/v), as mobile phase, leading to the isolation of two compounds, BP-1 and BP-2.

Thin-layer chromatography

The optimized TLC conditions were used to monitor the column elution as well as for assessing the purity of isolated constituents. The stationary phase was silica gel G 60 F_{254}-coated aluminum sheets. The mobile phase was optimized to resolve the maximum number of constituents on a single run.

Spectral characterization of isolated compounds

Identification and characterization of the isolated compound were carried out using spectroscopy techniques such as Fourier-transform infrared (FTIR), mass, and nuclear magnetic resonance (NMR) spectroscopy. The FTIR spectra of compound BP-1 and compound BP-2 were performed individually using FTIR-Eco-ATR (Bruker, Optic, Model ALPHA II). The mass spectrum was recorded on LC-MS-2020 (Shimadzu, Kyoto, Japan) mass spectrometer with an atmospheric pressure chemical ionization source. ^{1}H and ^{13}C-NMR data were measured on a Bruker Biospin 800 MHz instrument (Avance AV 800, Switzerland) in deuterated methanol (CD_3OD).

Statistical analysis

Results are presented as mean ± standard error of the mean in the cell assays. The statistical significance was measured by comparing the data set of a parameter for each test group with that of the control group using one-way analysis of variance followed by Dunnett's test. The values were considered to be statistically significant if $P < 0.001$. GraphPad Prism software (GraphPad Software, Inc., San Diego, California) was used for the computation of statistical significance.

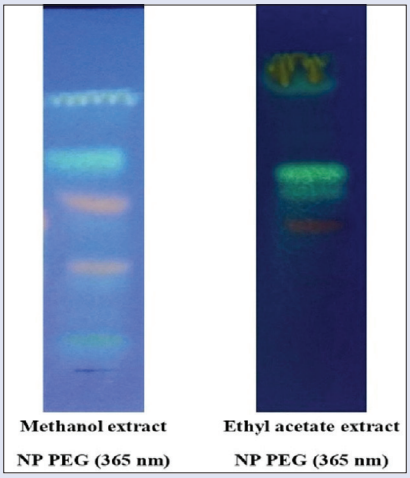

Figure 1: Thin-layer chromatography fingerprint of methanol extract and ethyl acetate fraction in ethyl acetate: Formic acid: Glacial acetic acid: Water (10:1.1:1.1:2.6) at 365 nm after derivatization using natural product-polyethylene glycol reagent

RESULTS

TLC fingerprint of methanol extract and ethyl acetate fraction [Figure 1] revealed the presence of flavonoids after derivatization using NP-PEG 4000 reagent. The developed fingerprint would remain as one of the identifying parameters for the selected extract and fraction.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

The TM3 Leydig cells were exposed to different concentrations of ethyl acetate fraction and methanol extract of *B. persica* seeds. Methanolic extract and ethyl acetate fraction showed IC_{50} at a concentration of more than 300 µg/ml while determined using MTT assay. The concentration of roughly half of the IC_{50} value was selected as the maximum concentration for bioassay performed using TM3 cell line. Empirically three concentrations, lesser than 150 µg/ml, were selected to carry out the assay. The extract and fraction were tested for their ability to alter the content of testosterone in culture media of TM3 cells was carried out at three selected concentrations (50 µg/ml, 100 µg/ml, and 150 µg/ml).

Effect of test extract and fraction on testosterone production

After incubating methanol extract and ethyl acetate fraction with TM3 cells for an optimized duration of time, the supernatant was separated for determining the amount of testosterone. The studies showed an increase in the mean value of testosterone present in culture media as compared to that of the control. The blank solution containing 0.1% v/v DMSO when used as a control, testosterone content was determined to be 21.20 ± 0.52 ng/dL. The cells incubated with methanol extract and ethyl acetate fraction showed a dose-dependent increase in testosterone content [Table 1]. The statistical analysis revealed that the alteration after treatment for each group was statistically significant as compared to control ($P < 0.001$). The comparative effect of extract and fraction on Leydig cells about testosterone content is depicted by showing fold alteration, graphically [Figure 2].

Table 1: Measurement of testosterone production after incubation of methanol extract and ethyl acetate fraction in TM3 Leydig cells

Sample details	Dose (µg/ml)	Testosterone level (ng/dL), mean±SEM
Control	-	21.20±0.52
Bp-ME	50	23.66±0.50***
Bp-ME	100	28.73±0.45***
Bp-ME	150	34.09±0.54***
Bp-ME-D-EAF	50	30.48±0.50***
Bp-ME-D-EAF	100	32.79±0.47***
Bp-ME-D-EAF	150	42.52±0.71***

Values represent mean±SEM of six independent experiments (n=6). Significantly different from control at ***P<0.001, ANOVA followed by Dunnett's test. *B. persica: Blepharis persica*; Bp-ME: Methanol extract of *B. persica*; Bp-ME-D-EAF: Direct ethyl acetate fraction from methanolic extract of *B. persica*; SEM: Standard error of mean; ANOVA: Analysis of variance

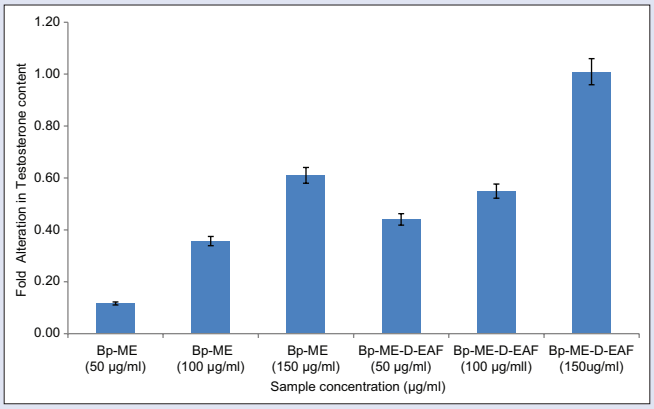

Figure 2: The comparative effect of extract and fraction on TM3 Leydig cells as fold alteration with reference to testosterone content

Column chromatography of bioactive ethyl acetate fraction

Ethyl acetate fraction subjected to column chromatography resulted in four subfractions as subfraction A (0.43 g), subfraction B (0.87 g), subfraction C (0.96 g), subfraction D (1.21 g). Three subfractions C-1 (0.21 g), C-2 (0.16 g), and C-3 (0.18 g) were obtained from column chromatography of subfraction C. Further column chromatography of subfraction C 1 using methanol: chloroform (5:95, v/v, 10:90,v/v), yielded two compounds BP-1 and BP-2. Purity assessment of isolated compound BP-1 and compound BP-2 was performed using TLC.

Spectral characterization of isolated compound BP-1

Fourier-transform infrared spectroscopy

Benzo-γ-pyrone (4*H*-1-benzopyran-4-one) is one of the backbone structures of the flavonoid class of compounds. Benzo-γ-pyrones consist of a benzene ring fused to a pyrone ring. The FTIR spectra of isolated compound BP-1 showed characteristic stretches for the O-H group at 3394.41 cm^{-1} in the region around 3200-3650 cm^{-1}. The aliphatic C-H stretching was observed at 2923.27 cm^{-1} and 2852.93 cm^{-1}. Due to factors such as conjugation, physical state, and ring strain, the ketone C = O stretching vibrations appeared at 1616 cm^{-1} at a lower wavenumber than reported for the carbonyl group. Overtones were observed between 2000-1700 cm^{-1}, confirming the presence of an aromatic ring in the structure. The absorption of the C-O-C band was obtained in the region of 1250-1050 cm^{-1}. The combination of C = C and C = O stretching vibrations around 1420-1400 cm^{-1}, 1465-1445 cm^{-1}, 1570-1540 cm^{-1}, 1535-1525 cm^{-1} attributed to specific bands of γ-pyrone was observed in the recorded IR spectra.[15] Comparative studies of recorded FTIR spectral data of isolated compound BP-1 and the reported FTIR spectral data suggested that the isolated compound had benzo-γ-pyrone ring backbone and it might be apigenin 7-O-neohesperidoside.[16] The hypothesis was further tested through ^{1}H-NMR, ^{13}C-NMR, and mass spectroscopy.

^{1}H-NMR spectroscopy

Recorded ^{1}H-NMR spectra [Figure 3a] of the isolated compound BP-1 were interpreted to ensure the ring structure of benzo-γ-pyrone and substitution at various positions considering apigenin 7-O-neohesperidoside. NMR spectra showed signals for the presence of different individual hydrogens. Proton at C-6 and C-8 occurs as a doublet at chemical shift (δ) value in the range of δ = 6.2–6.9 ppm in ring A of 7-glycosides of flavones. Two doublets at δ = 6.305 ppm and δ = 6.909 ppm were observed and attributed to protons at C-6 and C-8 positions, respectively. The chemical shift (δ) value of protons at C-6 appeared upfield compared to protons at C-8. Four protons at C-2', C-3', C-5', and C-6' appeared doublets as two pairs of ortho coupled doublets in the range of 6.5–7.9 ppm. The chemical shift (δ) value of protons at C-3' and C-5' appeared upfield compared to protons at C-2' and C-6'. Two doublets at δ = 7.833 ppm and δ = 7.214 ppm were observed and attributed to protons at C-2', C-6' and C-3', C-5' position, respectively. The singlet at δ = 6.247 ppm was attributed to proton at the C-3 position. Glucosylation at C-7 position with attachment to oxygen showed noticeable differences in chemical shift (δ) value of ring A. The signal of H-6''' proton for the methyl group appears as a doublet at chemical shift (δ) value of δ = 1.308 ppm and δ = 5.11 ppm at H-1'''. The signal for the proton at H-1'' is attributed to chemical shift (δ) value of δ = 4.8–5.2 ppm. Proton signals for the remaining protons in sugars are attributed with chemical shift (δ) value in the range of δ = 3–5.5 ppm.

^{13}C-NMR spectroscopy

As per ^{13}C-NMR spectra [Figure 3b] of the isolated compound BP-1, the resonances for chemical shift (δ) values at C-6(δ) = 99.83 ppm and C-8(δ) = 94.43 ppm position were attributed, respectively, in ring A, whereas in ring C, chemical shift (δ) values of carbonyl resonances at C-4(δ) = 179.14 ppm. The resonances for chemical shift (δ) values at C-2(δ) = 165.6 ppm, C-3(δ) = 105.67 ppm, C-5(δ) = 161.43 ppm, C-7(δ) = 163.06 ppm, C-9(δ) = 157.6 ppm, and C-10(δ) = 105.67 ppm were also attributed. The resonances for chemical shift (δ) values in ring C were attributed as C-1'(δ) = 125.62 ppm, C-2'(δ) = 129.65 ppm, C-3'(δ) = 116.23 ppm, C-4'(δ) = 160.07 ppm, C-5'(δ) = 115.56 ppm, and C-6'(δ) = 128.18 ppm. The resonances for chemical shift (δ) values of isolated compound BP-1 along with those recorded from ^{13}C-NMR spectra of apigenin 7-O-neohesperidoside are tabulated [Table 2].[17] Data obtained from FTIR, ^{1}H-NMR, and ^{13}C-NMR, it was assumed that compound BP-1 was apigenin 7-O-neohesperidoside, which was further concluded by the fragmentation pattern and fragmented peaks obtained by mass spectra.

Mass spectroscopy

The recorded mass spectra of isolated compound BP-1 [Figure 3c] revealed a molecular ion peak (M + H) at 579 *m/z*. The peak at 271 *m/z* showed the base peak for the aglycone part of the molecule present due to the removal of both sugar units (rhamnose [*m/z* = 146] and glucose [*m/z* = 162]), respectively. Fragmentation of isolated compound BP-1 showed the presence of carbohydrates as sugars. In isolated compound BP-1, loss of one sugar unit (rhamnose-146 units) from the

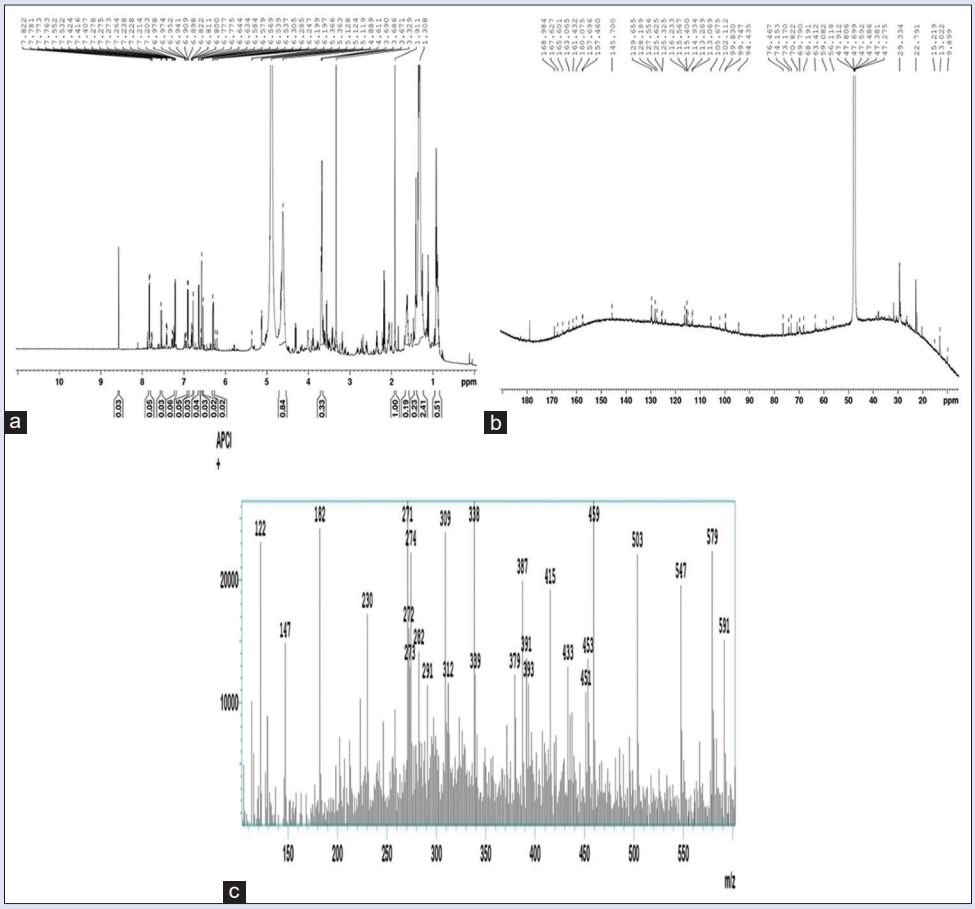

Figure 3: (a) ^{1}H-NMR spectrum of isolated compound BP-1 (b) ^{13}C-NMR spectrum of isolated compound BP-1 (c) Mass spectra of isolated compound BP-1

Table 2: Chemical shift values of isolated compound BP-1 and its comparison with the reported chemical shift values of ^{13}C-nuclear magnetic resonance spectra (apigenin 7-O-neohesperidoside)

Carbon position	Recorded chemical shift (ppm) values of isolated compound BP-1	Reported chemical shift (ppm) values of apigenin 7-O-neohesperidoside[17]
C-2	165.6	164.4
C-3	105.67	103.3
C-4	179.14	181.4
C-5	161.43	161.2
C-6	99.83	99.6
C-7	163.06	162.6
C-8	94.43	94.8
C-9	157.6	157
C-10	105.67	105.6
C-1’	125.62	121.2
C-2’	129.65	128.4
C-3’	116.23	116.1
C-4’	160.07	161.2
C-5’	115.56	116.1
C-6’	128.18	128.4
C-1”	99.74	98.5
C-2”	74.15	77
C-3”	74.15	77
C-4”	70.82	70.9
C-5”	76.46	77.3
C-6”	59.08	61
C-1”’	102.11	100.6
C-2”’	69.79	70.6
C-3”’	68.19	70.2
C-4”’	73.17	72.3
C-5”’	63.41	68.4
C-6”’	22.79	17.9

molecular ion peak (M + H) 579 *m/z* generated fragments (M-146) 433 *m/z* in positive ion mode of mass spectra. Further loss of second sugar unit (glucose-162 units) generated fragments (M-308) 271 *m/z* in positive ion mode, indicating the aglycone present.

Data obtained from FTIR, ^{1}H-NMR, ^{13}C-NMR, and mass spectroscopy, it was concluded that the isolated compound BP-1 was apigenin 7-O-neohesperidoside.

Spectral characterization of isolated compound BP-2

Fourier-transform infrared spectroscopy

The FTIR spectra of isolated compound BP-2 showed characteristic stretches for the typical O-H group at 3365.64 cm^{-1} in the region around 3200-3650 cm^{-1}. The aliphatic C-H stretching vibration is observed at 2923.16 cm^{-1} and 2853.21 cm^{-1}. Ketone (C=O) stretch appeared at 1646 cm^{-1}, appeared at a lower wavenumber than reported, due to the factors such as conjugation, physical state, and ring strain. Overtones were observed between 2000-1700 cm^{-1}, confirming the presence of an aromatic ring in the structure. The stretching for C-O-C was observed in the region of 1170-1087 cm^{-1}. Stretches corresponding to a combination of C = O and C = C vibrations were seen around 1570-1540 cm^{-1}, 1535-1525 cm^{-1}, 1465-1445 cm^{-1} and 1420-1400 cm^{-1}. This might be attributed to the presence of γ-pyrone.[15] A comparison of data obtained from the recorded FTIR spectrum of compound BP-2 with the reported FTIR spectrum of naringin had similarities in terms of peak size, shape, and wavenumber.[18] It suggested that the isolated

compound BP-2 might possess a backbone structure of benzo-γ-pyrone and it might be naringin. The hypothesis was further tested through ^{1}H-NMR, ^{13}C-NMR, and mass spectroscopy.

^{1}H-NMR spectroscopy

^{1}H-NMR spectra of the isolated compound BP-2 [Figure 4a] showed signals for the presence of different hydrogens. Proton at C-6 and C-8 occurs as a doublet at chemical shift (δ) value in a range of δ = 5.9–6.4 ppm. Two doublets at δ = 6.198 ppm and δ = 6.334 ppm were observed and attributed to protons at C-6 and C-8 positions, respectively. The chemical shift (δ) value of protons at C-6 occurred upfield compared to protons associated with C-8. Four protons at C-2', C-3', C-5', and C-6' appeared doublets as two pairs of ortho coupled doublets in the range of 6.8–7.2 ppm. The chemical shift (δ) value of protons at C-3' and C-5' appeared upfield compared to protons at C-2' and C-6'. Two doublets at δ = 7.286 ppm and δ = 6.822 ppm were observed and attributed to protons at C-2', C-6' and C-3', C-5' position, respectively. H-3 proton appears as a signal of each quartet around δ = 2.74–2.73 ppm (H-3a) and δ = 2.712–2.72 ppm (H-3b) due to spin-spin coupling with H-2 proton. Glucosylation at C-7 position with attachment to oxygen showed distinguishable differences in chemical shift (δ) value of ring A. The signal of H-6''' proton for the methyl group appears as a doublet at chemical shift (δ) value of δ = 1.307 ppm and δ = 5.3 ppm at H-1'''. The signal for proton at H-1'' was attributed to chemical shift (δ) value of δ = 5.03 ppm. Proton signals for the remaining protons in sugars are attributed with chemical shift (δ) value in the range of δ = 3–5.5 ppm.

^{13}C-NMR spectroscopy

As per ^{13}C-NMR spectra [Figure 4b] of the isolated compound BP-2, the resonances for chemical shift (δ) values at C-6(δ) = 96.49 ppm and C-8(δ) = 95.65 ppm position were attributed, respectively, in ring A, whereas in ring C, chemical shift (δ) values of carbonyl resonances at C-4(δ) = 197.09 ppm and characteristic chemical shift (δ) values of C-3(δ) = 42.64 ppm and C-2(δ) = 79.14 ppm. The resonances for chemical shift (δ) values at C-5(δ) = 165.43 ppm, C-7(δ) = 163.58 ppm, C-9(δ) = 160.59 ppm, and C-10(δ) = 103.62 ppm were also attributed. The resonances for chemical shift (δ) values in ring C were attributed as C-1'(δ) = 129.85 ppm, C-2'(δ) = 129.39 ppm, C-3'(δ) = 115.65 ppm, C-4'(δ) = 157.8 ppm, C-5'(δ) = 114.97 ppm, and C-6'(δ) = 129.31 ppm. The resonances for chemical shift (δ) values of isolated compound BP-2 and its comparison with the recorded resonance value for carbons in reported ^{13}C-NMR spectra of naringin are tabulated [Table 3].[17]

Data obtained from FTIR, ^{1}H-NMR, and ^{13}C-NMR, it was assumed that compound BP-2 was naringin, which was further concluded by the fragmentation pattern and fragmented peaks obtained by mass spectra.

Mass spectroscopy

The recorded mass spectra of isolated compound BP-2 [Figure 4c] showed a molecular ion peak (M + H) at 581 *m/z* with the highest intense peak. The peak at 273 *m/z* showed the base peak for the aglycone part of the compound due to the removal of both sugar units (rhamnose-146 [M-146] and glucose 162 [M-162]). Fragmentation of isolated compound BP-2 showed the presence of carbohydrates as sugars. In isolated compound BP-2, loss of one sugar unit (rhamnose-146 units [M-146]) from the molecular ion peak (M + H) 581 *m/z* of compound BP-2 generated fragments 435 *m/z* in positive ion mode of mass spectra. Further loss of second sugar unit (glucose-162 units [M-162]) generated fragments 273 *m/z* in positive ion mode, indicating the aglycone present.

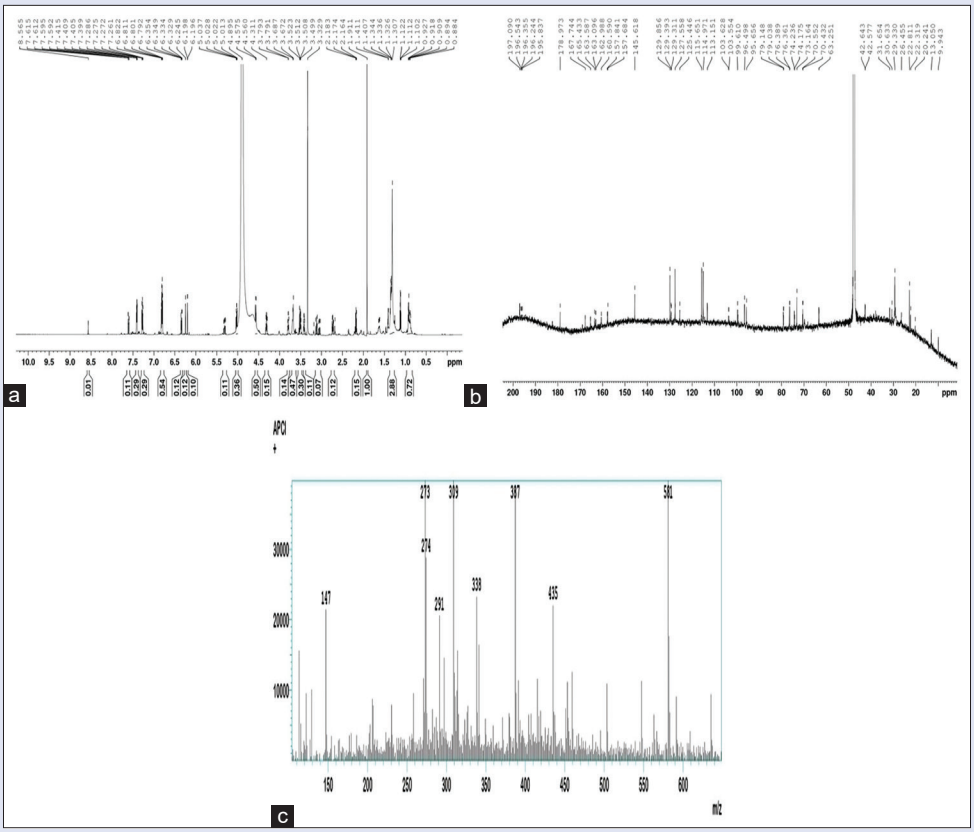

Figure 4: (a) ^{1}H-NMR spectrum of isolated compound BP-2 (b) ^{13}C-NMR spectrum of isolated compound BP-2 (c) Mass spectra of isolated compound BP-2

Data obtained from FTIR, ^{1}H-NMR,^{13} C-NMR, and mass spectroscopy, it was concluded that the isolated compound BP-2 was naringin.

DISCUSSION

The present work represents the process from the collection and authentication of plant material to its extraction by different solvents to obtain different extracts and fractions from crude drugs. Preliminary phytochemical screening and development of TLC fingerprint were used for the detection of secondary metabolites. Flavonoids and their derivatives are used as disease preventive and therapeutic agents in traditional medicine and their occurrence is reported to be in several parts of the plants.[19] Flavonoids belong to the class of secondary metabolites and are characterized by the C6-C3-C6 group. Beneficial effects of flavonoids have been reported to have anticholinesterase activity, anti-inflammatory activity, anticarcinogenic activity, antioxidant activity, and steroidogenesis modulators.[19-22] The effect of natural flavonoid chrysin reported the increasing testosterone production mainly by enhancing cAMP-induced StAR gene expression in Leydig cells.[23,24] Similarly, the studies on apigenin showed increase steroidogenesis in Leydig cells mainly by enhancing StAR protein expression.[25] It is also reported that abyssinones and related flavonoids can be used as potential steroidogenesis modulators against three enzymes 3β-hydroxysteroid dehydrogenase (HSD), 17β-HSD, and aromatase of the steroidogenesis pathway.[19,26] Considering the ability of flavonoids to alter the testosterone biosynthesis, ethyl acetate fraction of methanol extract containing flavonoids was selected for biological screening using TM3 cell line.

Table 3: Chemical shift values of isolated compound BP-2 and its comparison with the reported chemical shift values of ^{13}C-nuclear magnetic resonance spectra (naringin)

Carbon position	Recorded chemical shift (ppm) values of isolated compound BP-2	Reported chemical shift (ppm) values of Naringin[17]
C-2	79.14	78.5
C-3	42.64	42
C-4	197.09	196.7
C-5	163.58	162.9
C-6	96.49	96.5
C-7	165.43	164.9
C-8	95.65	95.4
C-9	160.59	162.7
C-10	103.62	103.5
C-1'	129.85	128.7
C-2'	129.39	128
C-3'	115.65	115.3
C-4'	157.8	157.7
C-5'	114.97	115.3
C-6'	129.31	128
C-1"	99.61	98
C-2"	79.03	77.2
C-3"	76.38	77
C-4"	73.16	70.1
C-5"	76.36	76.7
C-6"	63.21	60.8
C-1"'	103.5	100.4
C-2"'	70.55	70.7
C-3"'	70.43	70.4
C-4"'	74.23	72.1
C-5"'	73.16	68.2
C-6"'	22.81	17.8

Methanolic extract and ethyl acetate fraction were tested on the TM3 Leydig cells for IC_{50} value and measurement of testosterone production at different concentrations.[14] The MTT assay is a colorimetric assay for measuring cell metabolic activity.[14] This helped in determining the inhibition of the growth of cells incubated with test solutions. The dose selection was initially based on the IC_{50} value of test solutions. The dose roughly half of IC_{50} concentration was the highest dose used to test the efficacy of the solutions. Other doses to be tested were selected by adopting a trial and error approach. The graph showed that ethyl acetate fraction was potent than methanolic extract in exerting action on TM3 cells. Data obtained from the *in vitro* studies revealed that methanol extract, as well as ethyl acetate fraction, could stimulate testosterone content in culture media derived from the cells incubated with test extract and fraction. The results of *in vitro* studies indicated that the experimental concentrations of the extract and the fraction at a dose of 50 µg/ml, 100 µg/ml, and 150 µg/ml showed a significant increase in the testosterone production than in control. It also revealed that ethyl acetate fraction was more potent than methanol extract in stimulating testosterone synthesis.

The bioactive ethyl acetate fraction when subjected to repetitive chromatography yielded compounds BP-1 and compound BP-2 using silica gel column chromatography. The identification and characterization of compound BP-1 and compound BP-2 were performed using spectroscopy techniques such as FTIR, ^{1}H-NMR, ^{13}C-NMR, and mass spectroscopy.

It was concluded from FTIR spectroscopy that compound BP-1 and compound BP-2 showed characteristic stretches for O-H group, aliphatic C-H stretching vibration, ketone C = O stretching vibrations, aromatic ring structure, C-O-C band, bands of γ-pyrone, and one of the backbone structure of benzo-γ-pyrones (benzene ring fused to a pyrone ring) that is flavonoid class of compounds.[15,16,18]

As per ^{1}H-NMR spectroscopy [Table 4], 7-glycosides of flavone and 7-glycosides of flavanone compounds were identified.[27] In 7-glycosides

Table 4: Chemical shift values of isolated compound BP-1 and compound BP-2 and its comparison with the reported chemical shift values of ^{1}H-nuclear magnetic resonance spectra (apigenin 7-O-neohesperidoside and naringin)

Hydrogen position	Recorded chemical shift (ppm) values of isolated compound BP-1	Reported chemical shift (ppm) values of apigenin 7-O-neohesperidoside[27]	Recorded chemical shift (ppm) values of isolated compound BP-2	Reported chemical shift (ppm) values of naringin[27]
H-3	6.24	6.3	2.72-2.74	2.8
H-6	6.28-6.30	6.2-6.4	6.196-6.198	5.9-6.1
H-8	6.89-6.90	6.5-6.9	6.32-6.33	6.1-6.4
H-2'	7.82-7.83	7.7-7.9	7.26-7.28	7.1-7.3
H-3'	6.63-6.64	6.5-7.1	6.79-6.82	6.5-7.1
H-5'	6.63-6.64	6.5-7.1	6.79-6.82	6.5-7.1
H-6'	7.82-7.83	7.7-7.9	7.26-7.28	7.1-7.3
H-1"	5.36	4.8-5.2	5.03	4.8-5.2
H-1"'	5.11	4.9-5.0	5.3	4.9-5.0
H-6"'	1.30	1.1-1.3	1.30	1.1-1.3

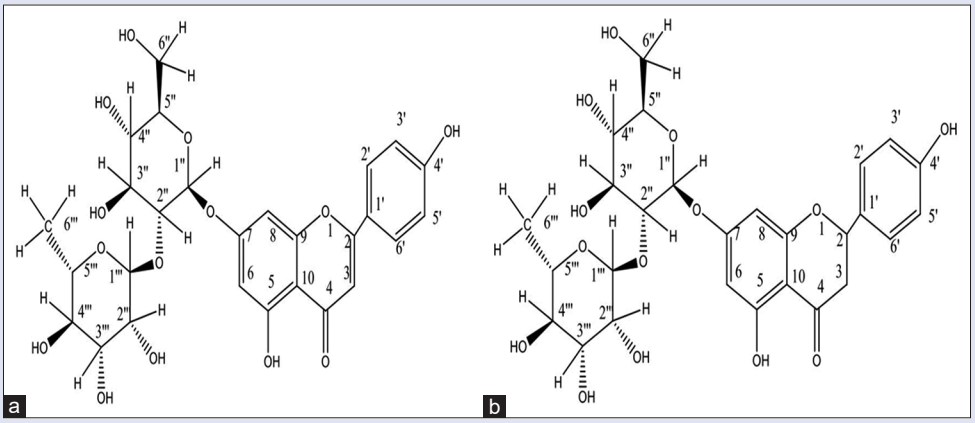

Figure 5: (a) Apigenin 7-O-neohesperidoside structure (Compound BP-1) (b) Naringin structure (Compound BP-2)

of flavones, H-3 proton appears as a singlet, and H-6 and H-8 protons occur as a doublet.[16,28] H-6 and H-8 signals are deshielded at higher (δ) values as an attachment of sugar to the oxygen at the C-7 position. In contrast, in flavanones, the signals for the A ring proton appear at a higher field than in the flavones. In flavanones, H-3 proton appears as a signal of each quartet around 2.8–3.0 ppm due to spin-spin coupling with H-2 proton.[27] In ring B, H-3' proton and H-5' protons are shielded and appear up-field as compared to H-2' and H-6' proton due to C-4' oxygen substituted.[29,30] In flavonoid rhamnoglycosides, C-1" proton of the β-linked sugar has a diaxial coupling with the C-2" proton showing an attributed C-1 proton signal in the range of δ = 4.8–5.2 ppm and it was associated in both isolated compound with a chemical shift value of δ = 5.1 ppm in 7-glycosides of flavones and δ = 5.3 ppm in 7-glycosides of flavanones.[27-29] Flavonoid rhamnoglycosides contain either the rutinosyl or hesperidosyl moieties differing only in the point of attachment of the rhamnose to the glucose. In flavonoid 7-O-neohesperidoside, the signal for the rhamnose H-6'" proton for the methyl group appears as a doublet at chemical shift (δ) value of δ = 1.308 ppm confirming the presence of neohesperidoside.[27-29]

As per the obtained data from ^{13}C-NMR spectroscopy [Tables 2 and 3], the resonances for chemical shift (δ) values are characteristic at C-3 (methylene group) with δ = 42.64 ppm, C-2 (δ) = 79.14 ppm, and C-4 (δ) = 197.09 ppm in 7-glycosides of flavanones as compared to 7-glycosides of flavones.[17,28,29] Downfield shifts were observed at C-5, C-7, C-9, and C-1' resonances and upfield shifts at C-6 and C-4' in 7-glycosides of flavanones as compared to 7-glycosides of flavones.[17,28,29]

In mass spectroscopy, fragmentation of diglycosyl flavonoids shows cleavage at glycosidic O-linkages with loss of sugar bonds, i.e., the loss of 162 units (glucose) and 146 units (rhamnose), allowing the determination of the carbohydrate sequence.[31,32] A significant peak of the aglycon part was observed with high intensity. Glycoside bond was feasible with a hydroxyl group attached to C-7 and C-5, however, the hydroxyl group substituted at C-5 position shows a very low reactivity towards glycosylation attributed to its involvement in the formation of intramolecular hydrogen bond with the carbonyl group present on C-4. This led to propose glycosylation of the hydroxyl group attached to the C-7 position in both the compounds BP-1 and BP-2.[32]

The spectroscopic data were thus initially interpreted independently for both the compounds. The data for both compounds were found in full agreement with those reported compounds. The studies revealed that compound BP-1 was apigenin 7-O-neohesperidoside and BP-2 was naringin [Figure 5].

CONCLUSION

The studies suggested that seeds of *B. persica* could act on Leydig cells and upregulated testosterone biosynthesis. This could justify the traditional indication of the seeds in the treatment of debilities of the male reproductive system. Two compounds isolated from the bioactive fraction might serve as a marker for the assessment of the quality of plant material. These compounds were isolated and reported for the first time from the seeds of *B. persica*. The studies also highlighted the potential of benzopyran in stimulating steroidogenesis in Leydig cells, which is needed to be explored.

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Conflicts of interest

There are no conflicts of interest.

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