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ORIGINAL ARTICLE

New mechanistic insights on *Justicia vahlii* Roth: UPLC-Q-TOF-MS and GC–MS based metabolomics, *in-vivo*, *in-silico* toxicological, antioxidant based anti-inflammatory and enzyme inhibition evaluation

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Abbreviations: BEJv, *n*-butanol extract of *Justicia vahlii*; DPPH, 2,2-diphenyl-1-picrylhydrazyl); ABTS, (2,2-azinobis(3-ethylbenothiazoline)-6-sulfonic acid); CUPRAC, Cupric reducing antioxidant capacity; FRAP, Ferric Reducing Antioxidant Power; GAE, Gallic acid equivalent; RE, Rutin equivalent; ACAE, Acarbose equivalent; TUE, Thiourea equivalent; KAE, Kojic acid equivalent; DE, Dry extract; p.o, Per orally; i.p, Intraperitoneally; r.t, Retention time; SOD, Superoxide dismutase; GSH, Glutathione reductase; UPLC-QTOF-MS, Ultra-performance liquid chromatography- quadrupole time-of-flight-mass spectrometry; GCMS, Gas chromatography-mass spectrometry; TNF, Tumor necrosis factor; IL, Interleukin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide

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KEYWORDS

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Abstract *Justicia vahlii* Roth. (acanthaceae) is an important medicinal food plant used in pain relief and topical inflammation. The present study aimed to evaluate phytochemical composition, toxicity, anti-inflammatory, antioxidant and enzyme inhibition potential of *n*-butanol extract of *J. vahlii* (BEJv). The extract prepared through maceration was found rich in total phenolic contents (TPC) 196.08 ± 6.01 mg of Gallic acid equivalent (mg GAE/g DE) and total flavonoid contents (TFC) 59.08 ± 1.32 mg of Rutin equivalent (mg RE/g DE). The UPLC-Q-TOF-MS analysis of BEJv showed tentative identification of 87 compounds and 19 compounds were detected in GC–MS analysis. The HPLC-PDA quantification showed the presence of 14 polyphenols amongst which kaempferol (3.45 ± 0.21 µg/ mL DE) and ferulic acid (2.31 ± 1.30 µg/ mL DE) were found in highest quantity. The acute oral toxicity study revealed the safety and biocompatibility of the extract up to 3000 mg/kg in mice. There was no effect of BEJv on human normal liver cells (HL 7702) and very low cytotoxic effect on liver cancer cells (HepG2) and breast cancer cells (MCF-7). In anti-inflammatory evaluation, the BEJv treated groups showed significant inhibition ($p < 0.001$) of late phase carrageenan induced paw edema at 400 mg/kg and increased the levels of oxidative stress markers; catalase, superoxide dismutase (SOD) and glutathione (GSH) while decreased the inflammatory markers; interleukin-1beta (IL-β) and tumor necrosis factor alpha (TNF-α) in paw tissue of mice. BEJv displayed highest results in Ferric reducing antioxidant power (FRAP) assay 97. 21 ± 2.34 mg TE (trolox equivalent)/g DE, and highest activity 3.32 ± 0.31 mmol ACAE (acarbose equivalent)/g D.E against α-glucosidase. Docking study showed good docking score by the tested compounds against the various clinically significant enzymes. Conclusively the current study unveiled *J. vahlii* as novel non-toxic source with good antioxidant-mediated anti-inflammatory potential which strongly back the traditional use of the species in pain and inflammation.

1. Introduction

Inflammation is an immune response of the living organism or vascularized tissue(s) against burn, trauma, infection, injury or pathological alterations in the body (Ouda et al., 2021). Over the decades, number of studies revealed the interrelationship of inflammation and oxidative stress (Yesmin et al., 2020). Oxidative stress has been described as the imbalance between the production of reactive oxygen species (ROS) (free radicals) such as hydroxyl, superoxide anion, peroxyl and hydroperoxyl, and the antioxidant defense system (Ahmad et al., 2019). Normally cells produce low concentration of ROS for maintenance of cellular functions. However in pathological conditions, the imbalance of the physiological mechanism causes over production of ROS leading to overexpression of inflammatory mediators which initiate and maintain inflammation (Ansari et al., 2020). The increased ROS production activates the cellular defense mechanism against oxidative stress to eliminate the ROS from the cell. Various enzymes such CAT, SOD and endogenous antioxidant like GSH are the part of the cellular antioxidant defense system (Fukui and Zhu 2010). SOD demolish oxidative stress by converting reactive oxygen (O_2^-) to hydrogen peroxide (H_2O_2), and the H_2O_2 is then converted to water and oxygen molecules by CAT (Ansari et al., 2020). Whereas, the free radical mediated release of inflammatory markers decrease the levels of the endogenous antioxidant markers and devastate the inflammation (Li et al., 2020).

Considerable research studies showed the involvement of oxidative stress mediated inflammation in etiology of many diseases such as diabetes, aging, skin tissue damages and peptic ulcer (Tandon et al., 2004, Babu et al., 2015, Asmat et al., 2016). Therefore, the developing drugs that counter the oxidative stress are considered suitable choice for combating the disease(s) that involve oxidative stress. The therapeutic agents used for the management of diabetes are based on targeting enzymes α-amylase and α-glucosidase. The malfunctioned antioxidant defense mechanism of human body may contribute drastically to the developed insulin resistance. So, there is need of therapeutic agents with maximum desired effects and minimal unwanted effects. Clinically significant enzymes are being considered as one of the suitable drug targets while addressing a pathophysiological condition. For instance tyrosinase can be targeted to avoid the overproduction of melanin, which is the underlying cause of various skin disorders. Similarly through the inhibition of urease, an enzyme responsible for initiation and development of peptic ulcer, the condition can be treated (Bakht et al., 2014, Ullah et al., 2019, Jeon et al., 2021). The intense need of new drug candidates with good safety profile increased the interest of researchers to seek the desired products from natural sources. Because nature has a long history of contribution in provision of functional bioactives (Pieczykolan et al., 2021).

J. vahlii Roth. with local name kodasoori and bhekkar and synonyms *Justicia diffusa* and *Rustellaria vahlii* (https://www.theplantlist.org), is an important medicinal food plant of the family acanthaceae used traditionally in treating tooth pain and various skin and respiratory disorders and as food additive (Begum and Khan 2001, Ignacimuthu et al., 2008, Maheshwari et al., 2012, Farooq et al., 2019, Basit et al., 2021). The use of advance hybrid techniques such UPLC-Q-TOF-MS and GC–MS for the identification of functional bioactives responsible for the pharmacological potential of plant species has been increased in last two decades due to the multiple advantages of the techniques (Qi et al., 2014). To the best of our knowledge and literature search there is very limited literature available to date on pharmacological and phytochemical aspects of *J. vahlii*. Therefore, the current study was designed to evaluate comprehensively phytochemical profile, toxicity, anti-inflammatory, antioxidant and enzyme inhibition potential of *n*-butanol extract of *J. vahlii* (BEJv). In this study the traditional use of the species in inflammation was scientifically explored.

2. Material and methods

2.1. Plant material collection

The whole plant material of *J. vahlii* was collected from District Bannu, Khyber Pakhtunkhwa, Pakistan, which was identified by Prof. Dr. Ghulam Sarwar at the Department of Botany, The Islamia University of Bahawalpur, Punjab, Pakistan, and a specimen was deposited in the herbarium of the university with a voucher number 145/Botany for future record and reference. The plant material was washed thoroughly with distilled water to remove the impurities and dust, followed by shade drying at ambient temperature in a well-ventilated room.

2.2. Preparation of extract

The dried plant material (5 kg) was then subjected to grinding followed by extraction with *n*-butanol (8 L). The soaked material was shaken mechanically with a glass rod occasionally for 15 days, and then it was filtered two times using Whatman No. 1 filter paper. The process of filtration was repeated for three times to get fine filtrate. The filtrate was concentrated under reduced pressure at 45 ℃ using rotary evaporator which yielded a semisolid mass of 67 g of *n*-butanol extract of *J. vahlii* (BEJv). The percent (%) yield was calculated on dry weight basis using the below formula;

$$\textit{Percent yield} = \textit{Weight of the dry extract} / \textit{weight of the dry plant material} * 100$$

2.3. Phytochemical profiling

2.3.1. Total Phenolic (TPC) and flavonoid (TFC) content determination

The total phenolic contents (TPC) and total flavonoid contents (TFC) were determined using Folin-Ciocalteu and aluminium trichloride methods respectively (Uysal et al., 2018). The results of TPC were expressed as milligrams of gallic acid equivalents per gram of dry extract (mg GAE/g DE) and TFC were noted as milligrams of rutin equivalents per gram of dry extract (mg RE/g DE).

2.3.2. UPLC-Q-TOF-MS analysis

The chemical composition of BEJv was evaluated using Triple TOF™ 5,600 (AB Sciex, Foster, USA) LC-MS mass spectrometry with a Duo Spray TM ion source. The chromatographic separation was performed by using a Prominence TM UPLC system (Shimadzu, Japan). The apparatus employed for analysis include CBM-20A lite controller system, Autosampler Shimadzu SIL-30AC. The identification of compounds was carried out in accordance with the Natural Product HR-MS/MS Spectral Library 1.0 software database, which contains approximately 1000 plus Chinese standard reference compounds, previous literature support and Metlin database (Hu et al., 2020, Saleem et al., 2020).

2.3.3. Polyphenolic quantification using HPLC-PDA analysis

HPLC-PDA analysis was applied for quantification of polyphenolic compounds in BEJv using standard described method (Locatelli et al., 2017). The standard compounds; chlorogenic acid, catechin, syringic acid, vanillic acid, p-hydroxy benzoic acid, naringin, ferulic acid, o-coumaric acid, narigenin, cinnamic acid, p-coumaric acid, 2,3-dimethoxy-benzoic acid, rutin, 3-hydroxy-4-dimethoxy-benzoic acid, 3-hydroxy benzoic acid, epicatechin, gallic acid, gentesic acid, kaempferol, quercetin, apigenin, emodin, skullcapeflavone were purchased from sigma Aldrich (Shanghai, China). The results were presented as μg/mg dry extract (DE).

2.3.4. GC–MS analysis

The BEJv was further characterized by employing GC–MS Agilent 7890B equipped with Mass hunter acquisition software. The instrument has an ultra-inert capillary non polar column (HP-5MS) with proportions of 30 mm £ 0.25 mm ID £ 0.25 mm film. The carrier gas used was Helium and flow rate set was 1 ml/min. Temperature set for injector operation was 250 °C and the temperature of oven was set in such a manner; 50 °C for five min, then gradually increased at 100 °C/ min to reach 250 °C, and finally to 3000 °C for 10 min at 70 °C/ min. The sample was dissolved in methanol, added to GC–MS vial placed in auto sampler, and 1uL of sample extract solution was injected and the split ratio was 20:1. The compound identification was performed using NIST-17 MS Library search while the percentage composition was computed from GC peak areas (Achakzai et al., 2019, Hayat and Uzair 2019).

2.4. Effect of BEJv on human normal and tumoral cell lines

2.4.1. Cell culture

The HL 7702 cell line (normal human liver cells), HepG2 (human liver cancer cell line) and MCF-7 (human breast cancer cell lines were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The cells were grown in Dulbecco Modified Eagle's Medium (DMEM) and Roswell Park Memorial Institute Medium (RPMI) culture media at 37 °C with 5 % CO_2 and 95 % humidity in a gas incubator (BB 16 gas incubator, Heraeus Instruments GmbH). The cells were supplemented with 1 % non-essential amino acid (NEAA), 10 % Fetal Bovine Serum (FBS) and 1 % PEST.

2.4.2. In vitro MTT assay

The cytotoxicity study against HL 7702 (normal human liver cells), HepG2 (human liver cancer cells) and MCF-7 (human breast cancer cell lines) was carried out by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium (MTT) assay using methodology prescribed (Chavan et al., 2020). Briefly in this method the cell lines were seeded in 96-well plates at a density of 1×10^4 cells/well and incubated for 24 h. After the incubation the cells were then treated with the extract at 25, 50, 100, 200 and 300 μg/mL and time interval of 24 and 48 h. Then MTT reagent (5 mg/mL in 20 ml phosphate buffered-saline (PBS)) was added per well and the cells were further incubated at 37 °C for 4 h with CO_2. The medium was then removed and 150 μL DMSO was added to dissolve the formazan. Then after 10 min the absorbance were recorded at 490 nm by a microplate reader (BioTek, USA) to assess the cell viability.

2.5. In vitro antioxidant potential

Antioxidant potential of the BEJv was evaluated by using DPPH (2,2 diphenyl-1-picrylhydrazyl), ABTS (2,2 azinobis (3-ethylbenothiazoline)-6-sulfonic acid), ferric reducing antioxidant power (FRAP) and cupric reducing antioxidant capacity (CUPRAC) as per the standard protocols described by (Khurshid et al., 2019). Results were expressed in milligrams trolox equivalent per gram of dry extract (mg TE/g DE).

2.6. In vitro enzyme inhibition

The *in vitro* enzyme inhibition studies of the extract were performed against α-amylase, α-glucosidase and tyrosinase, following previously described protocols (Khurshid et al., 2019). The α-amylase and α-glucosidase activities were expressed as milli mole acarbose equivalent (mmol ACAE/g DE), while tyrosinase inhibition was expressed in milligram kojic acid equivalent (mg KAE/g DE). anti-urease assay was performed in accordance with the procedure described (Weatherburn 1967) with slight modifications. Total volume of solution used in the assay was 200 µL. 15 µL of urease, 15 µL of phosphate buffer (1 M, pH: 7), and 15 µL of the test sample (5 mg/mL) were added to a 96-well plate and incubated at 37 °C for 15 min. Then 40 µL of urea as a substrate was mixed and re-incubated in the same conditions. After incubation, pre-read was taken by measuring the absorbance at 630 nm. Then 45 µL of phenol and 70 µL of alkali reagents were added to the mixture. The reaction mixture was incubated again at 37 °C for 50 min. Absorbance was measured again at 630 nm as post-read. Methanol was taken as a negative control and thiourea was taken as a positive control. Results were expressed as milligrams thiourea equivalent per gram of dry extract (mg TUE/g DE).

2.7. In vivo toxicological evaluation

2.7.1. Animals

BALB/c mice weighing 25–30 g were purchased from the animal house of the National Institute of Health Rawalpindi Pakistan and were acclimatized at 25 ± 2 °C under a 12 h dark/light cycle for 7 days. Animals were housed six mice per cage with *ad libitum* access to food and water. The experimental procedures for this study were approved by the Institutional Ethical Committee of Faculty of Pharmacy, The Islamia University of Bahawalpur (Permit number 41-2021/PAEC) which were in accordance with the ethics and guidelines of the UK Animals (Scientific Procedures) Act 1986 and conformed to the Animal Research: Reporting of *In Vivo* experiments (ARRIVE) (Liu et al., 2016).

2.7.2. Acute oral toxicity test

Acute Oral toxicity assessment was conducted according to Test No. 423 of the Organization for Economic Co-operation for Development (OECD) guidelines and standard protocols described (OECD. 1994, Syahmi et al., 2010). The mice were categorized into 3 groups with 6 mice in each group. The control group was treated with normal saline (10 ml/kg p. o.), group 2 and 3 received BEJv *via* oral gavage tube dissolved in normal saline at 1000 mg/kg and 3000 mg/kg respectively. The animals were continuously observed for the first 2 h and then every 24 h post dose administration for 14 days for spontaneous activity, aggressiveness, cyanosis, ataxia, tail pinch response, righting reflex, writhing, convulsions, catalepsy and bizarre behavior. On day 14 all the animals were euthanized by using ketamine 350 mg/kg body weight and blood was collected for biochemical analysis and vital organs for histopathological examination.

2.8. anti-inflammatory potential of BEJv

2.8.1. Carrageenan induced paw edema model

The anti-inflammatory potential of BEJv was evaluated by using carrageenan induced paw edema model in mice according to previously established method (Yang et al., 2020) with minor modifications. The mice were divided into 5 groups (n = 6). Group 1 was control group which received normal saline (10 ml/kg), group 2 was positive control and received dexamethasone subcutaneously (1 mg/kg) while groups 3–5 received orally BEJv at dose of 100 mg/kg and 200 mg/kg and 400 mg/kg. After 60 min of dosing all the mice were injected carrageenan (100 µL, 1 %) subcutaneously into the right hind paw. The change in the volume of the paw was measured as marker of induction of inflammation. The initial volume (V1) was measured before carrageenan administration and after injection volume (V2) was measured at 0.5, 1, 2, 4 and 5 h using the plethysmometer (Ugo Basile) and percent inhibition of edema was calculated by using the below formula:

$$\% \text{ Inhibition} = (V_{control} - V_{treated})/ V_{control} \times 100.$$

All the animals were sacrificed using ketamine 350 mg/kg immediately at the 5th hour of the experiment and the paw tissue portion was separated quickly. Homogenates of paw sample (10 % w/v) of different treatment groups were prepared using tris-HCl buffer (0.1 M and pH 7.4) and sterilized water and used for estimation of oxidative stress markers and inflammatory markers.

2.8.2. Effect of BEJv on oxidative stress markers

CAT levels in the paw tissue homogenate was estimated according to standard protocols (Doherty et al., 2010). Briefly, H_2O_2-phosphate buffer (3 ml) was taken in an experimental cuvette and then enzyme extract (40 µL) was added rapidly followed by thorough mixing. The absorbance of reaction mixture was recorded using UV-spectrophotometer at 240 nm. Results were expressed in U/mg protein. Levels of SOD in the homogenate was quantified using standard protocol (Kakkar et al., 1984) with slight modifications. In this method, the superoxide anion mediated reduction of nitro blue tetrazolium (NBT) to formazan was measured at 540 nm. The SOD addition stopped the reaction and the extent of the inhibition was expressed in U/mg protein as enzyme activity. GSH was measured according to established method (Farombi et al., 2007) with minor modifications. Briefly, the homogenate (0.1 ml) was mixed with 2.4 ml of phosphate buffer. 5, 5′-dithio bis(2-nitrobenzoic acid) (DTNB) solution was added to make the final volume 3 ml. The readings were recorded at 412 nm using spectrophotometer. Results were expressed in nmol/mg protein.

2.8.3. Effect of BEJv on IL-1β and TNF-α expression (using ELISA kit)

The tissue samples from all the treated groups for determination of IL-1β and TNF-α were prepared according to the standard method (Khan et al., 2019) with minor modifications. The sample was then homogenized and centrifuged at 3000 g for 10 min and the supernatant was stored at −20 °C. IL-1β and TNF-α quantification was carried out using commercially available TNF-α and IL-1β ELISA kit (eBioscience, Inc., San Diego, CA).

2.9. In silico molecular docking studies

Three bioactives identified in the UPLC-Q-TOF-MS analysis of BEJv namely skullcapflavone-II, sesaminol and conidendrin were selected for docking studies with α-amylase, α-glucosidase, tyrosinase and urease. Structures of the compounds were downloaded from the zinc database (Irwin et al., 2012). AM1 semi-emperical method using guassian 09 software was applied for optimization of 3D structures to the ground state energy. Structure of the target proteins; urease, tyrosinase and α-amylase were downloaded from protein data bank using PDB ID: 4H9M, 4P6R, and 4GQR respectively (Berman et al., 2000). To date, no crystal structure of α-glucosidase is reported in the protein data bank; hence first homology model was generated using previously described method (Yamamoto et al., 2010). Discovery Studio Client software was used for the preparation of all target protein structures, which removes water molecules, heteroatoms, assign charges and add hydrogens and missing residues (if any). After preparation of targets structures, the active site was defined using co-crystal ligands and centroid on all residues within 10 Å co-crystal ligands for each targeted protein. Autodock4 software (Molinspiration Database) was used to add polar hydrogens, neutralize the protein structures using Kollman united atom charges and to perform the docking calculations. Docking the three inhibitors was performed in a 60 × 60 × 60 grid box with 0.375 Å distance between points using Lamarckian genetic algorithm (LGA) for the prediction of binding free energies. Two hundred fifty conformations were produced for each enzyme-inhibitor complex and the control ligand. The docked conformations were ranked into clusters based on the binding free energy (ΔG). Discovery studio 5.0 visualizer was used to visualize to docked inhibitors at the active site and identify the intermolecular interactions with the active site.

2.10. Statistical analysis

Each assay was performed in triplicate. All the results were presented as mean ± standard deviation of at least 3 experiments in triplicate. The data were analyzed using one-way ANOVA, followed by *post hoc* Tukey's multiple comparison test. The level of $p < 0.05$ was used to determine statistical significance. All calculations were carried out through Graph Pad Prism 7.0 software.

3. Results and discussion

3.1. TPC, TFC and UPLC-Q-TOF-MS analysis

The current study is the first report on comprehensive phytochemical composition, toxicological and pharmacological evaluation of BEJv. Plant secondary metabolites particularly phenols and flavonoids are responsible for the biological functions of plants. Previous investigations have demonstrated the identification, extraction and pharmacological effects of different bioactives (Ahmad et al., 2019, Pervaiz et al., 2020). Solvent selection is an important step in extraction of natural products from plant origin. *n*-butanol is considered as one of the most suitable solvents used in extraction processes (Liste and Alexander 2002).

In the present study BEJv was evaluated for determination of total phenolic and flavonoid contents (Table 1). The quantification revealed the BEJv as rich in TPC 196.08 ± 6.01 mg GAE/g DE and TFC (59.08 ± 1.32 mg RE/ g DE). Higher phenolic contents than flavonoids in the extract were expected as flavonoid is a subclass of phenols. The extract with 100 % *n*-butanol solvent showed higher quantity of phenols and flavonoids that showed the *n*-butanol as suitable choice for preparation of plant extracts with high yield of phenols and flavonoids (Jan et al., 2013, Jing et al., 2015).

Based on rich quantity of phenols and flavonoids observed in the preliminary quantification, BEJv was subjected to chemical profiling using highly sophisticated technique UPLC-QTOF-MS. The present analysis showed the identification of total 87 different phytoconstituents, 48 in positive mode of ionization (Table 2) and 39 in negative mode of ionization (Table 3). Total ionic chromatograms of positive and negative mode are displayed in Figs. 1 & 2 respectively. Peak detection was based on comparing accurate mass with ± 5 error of accuracy and isotope ratio with a tolerance of 10 % difference with the established database using Peakview 1.2 software (ABSciex, USA). Among the identified compounds the number of flavonoids and their derivatives were in abundance, which were tentatively identified in positive mode and in negative mode of ionization. Second highest class of secondary metabolites were phenolic compounds followed by alkaloids. Moreover, hydroxycinnamic acid glycoside, lignans, and coumarine, stilbene and phenylpropaonoid, iridoid glycoside, diterpene, quinic acid, anthocyanin glycoside and anthocyanin flavonoid each were tentatively identified in the current analysis of BEJv. The present analysis showed the presence of number of phytoconsituents which are reported with good antioxidant and anti-inflammatory potential in previous investigations (Maleki et al., 2019, Piva et al., 2021).

Moreover, BEJv was subjected for further evaluation of polyphenolic quantification using HPLC-PDA analysis. The extract was tested for the presence of 23 phenolic compounds (as standards). Quantification of the compounds was done according to their retention time and confirmed by comparing their UV/Vis spectra to the standard compounds. The analysis

Table 1 Extraction and percent yield, Total phenolic (TPC) and flavonoid (TFC) contents of BEJv.

	BEJv
Yield of extract (g)	67
Percent yield	1.34 %
TFC mg RE/g DE	59.08 ± 1.32
TPC mg GAE/g DE	196.08 ± 6.01

All values are expressed as mean ± SD., (n = 3).
RE: rutin equivalent; GAE: gallic acid equivalent; DE: dry extract.

Table 2 Compounds identified in UPLC-QTOF-MS (positive mode) analysis of BEJv.

Peak no.	RT (min)	Mol. mass	Tentative identification	Mol. Formula	Adduct	B. peak (*m*/*z*)	Error ppm	Class	M/S Product ions	Reference
1	1.01	289.71	Epicatechin	$C_{15}H_{14}O_6$	+H	290.71	1.9	Flavanol	109.05, 151.23, 203.05, 245.08	(Zhuang et al., 2018
2	3.95	189.10	Castanospermine	$C_8H_{15}NO_4$	+H	190.10	−1.4	Alkaloid	74.02, 88.03, 116.03, 144.10, 149.06	(Rajana et al., 2018)
3	5.34	227.09	Flindersine	$C_{14}H_{13}NO_2$	+H	228.10	−1.1	Alkaloid	79.05, 103.05, 107.05, 149.05, 209.87, 228.10	(Cantrell et al., 2005)
4	5.36	195.08	Damascenine	$C_{10}H_{13}NO_3$	+H	196.09	−4.4	Alkaloid	Nil	(Sieniawska et al., 2019)
5	6.03	180.04	Caffeic acid	$C_9H_8O_4$	+H	181.04	0.2	Phenol	65.04, 82.04, 93.03, 120.95, 121.95, 123.00, 181.04	(Wang et al., 2015)
6	6.03	198.05	Syringic acid	$C_9H_{10}O_5$	+H	199.05	−0.9	Phenol	65.04, 77.03, 97.02, 120.03, 136.93, 140.04, 155.06, 180.91	(Li et al., 2022)
7	7.04	159.06	Echinopsine	$C_{10}H_9NO$	+H	160.07	−4.5	Alkaloid	Nil	(Liu et al., 2021)
8	8.45	194.05	Ferulic acid	$C_{10}H_{10}O_4$	+H	195.06	−1.4	Hydroxycinnamic acid	117.03, 145.02, 149.05, 177.05, 195.04	(Li et al., 2022)
9	8.7	192.04	Scopoletin	$C_{10}H_8O_4$	+H	193.04	−0.8	Coumarin	64.84, 65.04, 71.93, 81.07, 93.03, 103.01, 112.96, 121.02, 133.89, 149.02, 151.90, 162.90, 193.09	(Fischer et al., 2011)
10	9.63	354.09	Chlorogenic acid	$C_{16}H_{18}O_9$	+H	355.10	−0.2	Phenolic	59.05, 103.07, 117.03, 145.02, 177.05, 250.96, 266.99, 308.88, 355.06	(Clifford et al., 2007)
11	10.1	138.03	Sesamol	$C_7H_6O_3$	+H	139.03	2.1	Phenolic	60.98, 77.03, 83.04, 102.94, 119.08, 120.95, 121.04, 139.10	(Mekky et al., 2021)
12	10.42	272.06	Naringenin	$C_{15}H_{12}O_5$	+H	273.07	0.6	Flavonoid	119.05, 147.04, 153.01, 273.07	(Mekky et al., 2021)
13	11.23	302.07	Hesperetin	$C_{16}H_{14}O_6$	+H	303.08	−0.2	Flavonoid derivative	153.01, 177.05, 285.03, 303.08	(Mekky et al., 2021)
14	11.48	505.13	Peonidin 3-O-(6′′-acetyl-glycoside)	$C_{24}H_{25}O_{12}$	+H	506.14	3.4	Anthocyanin	nil	(Bennett et al., 2021)
15	11.81	310.10	Cinnamoyl hexose	$C_{15}H_{18}O_7$	+H	311.11	−0.4	Phenolic acid	128.06, 166.06, 167.06, 181.08, 247.09, 293.10, 311.11	(Farag et al., 2022)
16	11.99	666.17	Naringin 6′-malonate	$C_{30}H_{34}O_{17}$	+H	667.18	−1.3	Flavonoid	85.03, 153.01, 255.08, 273.07, 315.09, 521.12	(Alam et al., 2022)
17	12.14	259.08	Skimmianine	$C_{14}H_{13}NO_4$	+H	260.09	−0.5	Alkaloid	87.06, 102.94, 128.05, 156.07, 182.91, 200.92, 218.92, 242.08	(Chang et al., 2021)
18	12.31	544.12	Paeoniflorin sulfonate	$C_{23}H_{28}O_{13}S$	+H	545.13	−4.3	Monoterpene	nil	(Basit et al., 2021)
19	12.34	254.05	Daidzein	$C_{15}H_{10}O_4$	+H	255.06	−1.2	Flavonoid	nil	(Bragagnolo et al., 2021)
20	14.02	340.13	6-Prenylnaringenin	$C_{20}H_{20}O_5$	+H	341.13	−0.8	Flavonoid	137.05, 187.07, 291.10, 323.12, 341.09	(Martinez and Davies 2015)
21	14.12	544.15	3,5-Diferuloylquinic acid	$C_{27}H_{28}O_{12}$	+H	545.16	−1	Phenolic acid	85.03, 291.06, 307.09, 308.10, 324.10, 365.10, 383.11, 526.15, 545.16	(Clifford et al., 2006)
22	15.13	386.10	4-Caffeoylquinic acid	$C_{20}H_{18}O_8$	+H	387.10	−0.3	Phenolic acid	135.04, 137.05, 148.55, 149.02, 208.94, 247.07, 275.06, 351.08	(Chen et al., 2021)
23	15.22	533.12	Pelargonidin 3-O-(6′′-succinyl-hexoside)	$C_{25}H_{25}O_{13}$	+H	534.13	−3.8	Anthocyanin	nil	(Diretto et al., 2019)
24	15.35	330.07	Jaceosidin	$C_{17}H_{14}O_7$	+H	331.08	0.1	Flavonoid	124.05, 139.07, 139.01, 212.04, 255.06, 313.07	(Cui et al., 2021)
25	16.45	496.15	Negundoside	$C_{23}H_{28}O_{12}$	+H	497.16	−5	iridiod glycoside	120.95, 211.08, 220.96, 288.16, 309.05, 346.94, 399.18, 479.22, 496.16	(Meena et al., 2022)

Table 2 (*continued*)

Peak no.	RT (min)	Mol. mass	Tentative identification	Mol. Formula	Adduct	B. peak (*m*/*z*)	Error ppm	Class	M/S Product ions	Reference
26	16.83	344.07	5-O-Galloylquinic acid	$C_{14}H_{16}O_{10}$	+H	345.08	−2.3	Tannin	191.21	(Maldini et al., 2011)
27	17.81	479.11	Petunidin 3-O-glycoside	$C_{22}H_{23}O_{12}$	+H	480.12	1.9	Flavonoid	258.32, 274.04, 302.56, 317.61	(Downey and Rochfort 2008)
28	18.58	154.02	Gentisic acid	$C_7H_6O_4$	+H	155.03	0	Phenolic acid	Nil	(Li et al., 2022)
29	18.88	493.13	Malvidin 3-O-hexoside	$C_{23}H_{25}O_{12}$	+H	494.14	1.6	Anthocyanin	180.01, 243.23, 271.43	(Downey and Rochfort 2008)
30	19.78	402.13	Nobiletin	$C_{21}H_{22}O_8$	+H	403.13	−0.9	Flavonoid	Nil	(Ho and Kuo 2014)
31	21.26	270.05	Apigenin	$C_{15}H_{10}O_5$	+H	271.05	−0.5	Flavonoid	121.02, 149.02, 271.06	(Basit et al., 2021)
32	21.45	331.08	Malvidin	$C_{17}H_{15}O_7$	+H	332.08	1.2	Anthocyanidin	Nil	(Choi et al., 2010)
33	22.01	372.12	Tangeretin	$C_{20}H_{20}O_7$	+H	373.12	−1	Flavonoid	Nil	(Alam et al., 2022)
34	22.02	342.11	Tetramethylkaempferol	$C_{19}H_{18}O_6$	+H	343.11	−1	Flavonoid	Nil	(Ma et al., 2013)
35	22.49	305.12	Rohitukine	$C_{16}H_{19}NO_5$	+H	306.13	−0.6	Alkaloid	176.03, 236.05, 306.13	(Jain et al., 2014)
36	23.57	228.07	Resveratrol	$C_{14}H_{12}O_3$	+H	229.08	−1.1	Polyphenol	Nil	(Tisserant et al., 2016)
37	24.28	452.13	3-Hydroxyphloretin 2′-O-hexoside	$C_{21}H_{24}O_{11}$	+H	453.14	1.9	Phenolic glucoside	Nil	(Leng et al., 2022)
38	28.93	370.10	Sesaminol	$C_{12}H_{18}O_7$	+H	371.09	2.3	Lignan	Nil	(Mekky et al., 2021)
39	29.45	358.10	Gardenin B	$C_{19}H_{18}O_7$	+H	359.11	4.5	Flavonoid	Nil	(Brito et al., 2021)
40	31.35	449.10	Cyanidin 3-O-hexoside	$C_{21}H_{21}O_{11}$	+H	450.11	−0.9	Anthocyanin	Nil	(Mekky et al., 2021)
41	33.43	148.05	Cinnamic acid	$C_9H_8O_2$	+H	149.05	0.1	Phenolic acid	Nil	(Mekky et al., 2021)
42	33.91	521.12	Petunidin 3-O-(6′’-acetyl-hexoside)	$C_{24}H_{25}O_{13}$	+H	522.13	−0.1	Anthocyanin	Nil	(Chen et al., 2021)
43	33.98	561.12	Vitisin A	$C_{26}H_{25}O_{14}$	+H	562.13	−3.8	Proanthocyanin	339	(Gordillo et al., 2012)
44	34.53	300.06	Hispidulin	$C_{16}H_{12}O_6$	+H	301.07	2.4	Flavonoid	nil	(Cui et al., 2021)
45	35.54	535.14	Malvidin 3-O-(6′’-acetyl-hexoside)	$C_{25}H_{27}O_{13}$	+H	536.15	2.8	Anthocyanin	331	(Talpur et al., 2017)
46	35.91	370.10	Sesaminol	$C_{20}H_{18}O_7$	+H	371.11	2.7	Lignan	Nil	(Brigante et al., 2022)
47	36.97	164.04	*p*-coumaric acid	$C_9H_8O_3$	+H	165.04	1.3	Polyphenol	163,159	(Cui et al., 2010)
48	37.59	276.21	Linolenic acid	$C_{18}H_{30}O_2$	+H	277.21	3.7	Fatty acid	Nil	(Basit et al., 2021)

RT: retention time; B. peak: base peak; error pm = ± 5.

Table 3 Compounds identified in UPLC-QTOF-MS (negative mode) analysis of BEJv.

Peak no.	RT (min)	Mol. mass	Tentative identification	Mol. Formula	Adduct	B. peak (*m/z*)	Error ppm	Class	M/S product ions	Reference
49	1.01	290.07	Catechin	$C_{15}H_{14}O_6$	-H	289.07	−1	Polyphenol	205.12, 246.43	(Brito et al., 2021)
50	2.64	295.06	Caffeoyl aspartic acid	$C_{13}H_{13}NO_7$	-H	294.06	3.9	Phenolic acid	132.76, 175.34,	(Farag et al., 2016)
51	2.8	316.07	Protocatechuic acid 4-O-glucoside	$C_{13}H_{16}O_9$	-H	315.07	−0.2	Phenolic glycoside	108.04, 109.03, 152.01, 153.03, 198.91, 216.91, 260.86, 278.87, 296.87, 315.07	(Basit et al., 2021)
52	2.86	519.11	Pelargonidin 3-O-(6′'-malonyl-hexoside)	$C_{24}H_{23}O_{13}$	[M−2H]	517.10	−1.7	Anthocyanin glycoside	151.09, 470.74, 481,34	(Tian et al., 2017, Nowicka et al., 2019, Peniche-Pavía and Tiessen, 2020
53	5.23	342.09	Caffeic acid 4-O-hexoside	$C_{15}H_{18}O_9$	-H	341.08	−0.2	Phenolic acid	89.02, 119.03, 134.04, 135.04, 161.04, 179.03, 248.88, 322.83, 341.10	(Dong et al., 2016, Zhang et al., 2016)
54	5.33	326.10	p-Coumaric acid 4-O-hexoside	$C_{15}H_{18}O_8$	-H	325.09	0	Phenolic acid	119.05, 163.04, 238.89, 288.82, 306.84, 325.01	(Zhang et al., 2016, Qian et al., 2020)
55	6.12	356.11	Feruloyl hexose	$C_{16}H_{20}O_9$	-H	355.10	−0.4	Hydroxycinnamic acid glycoside	149.06, 134.03, 178.02, 193.05, 300.86, 318.86, 355.09	(López-Velázquez et al., 2020)
56	6.93	431.09	Kaempferol 3-O-deoxyhexoside	$C_{21}H_{19}O_{10}$	-H	430.09	−0.6	Flavonoid glycoside	255.07, 285.78	(Ning et al., 2019)
57	8.55	368.11	4-Feruloylquinic acid	$C_{17}H_{20}O_9$	-H	367.10	−2.4	Quinic acid	155.31, 173.21, 193.08	(Cheiran et al., 2019)
58	8.98	374.10	Skullcapflavone-II	$C_{19}H_{18}O_8$	-H	373.09	−2.2	Flavonoid	229.34, 257.29, 272.31, 285.43, 300.09, 328.41	(Zheng et al., 2012)
59	9.63	636.16	Kaempferol 3-O-(6′'-acetyl-glycoside) 7-O-deoxyhexoside	$C_{29}H_{32}O_{16}$	-H	635.16	−0.6	Flavonoid	Nil	(Kitahiro et al., 2019)
60	9.88	208.07	Caffeic acid ethyl ester	$C_{11}H_{12}O_4$	-H	207.06	4	Hydroxycinnamic acid	181.43, 199.06	(Sáenz-Navajas et al., 2010)
61	10.27	390.13	Polydatin	$C_{20}H_{22}O_8$	-H	389.12	−1.8	Polyphenol	134.04, 143.05, 165.05, 178.02, 193.05, 257.11, 389.13	(Zhu et al., 2012)
62	10.36	624.16	Isorhamnetin 3-O-hexoside 7-O-deoxyhexoside	$C_{28}H_{32}O_{16}$	-H	623.15	−4.4	Flavonoid	255.02, 299.07, 314.12, 315.41	(Lee and Shaari 2022)
63	11.01	488.13	6′'-O-Acetylglycitin	$C_{24}H_{24}O_{11}$	-H	487.12	−3.8	Isoflavonoid	337.07, 372.77, 487.13	(Zhu et al., 2022)
64	11.24	610.18	Hesperidin	$C_{28}H_{34}O_{15}$	-H	609.18	−2.6	Flavonone	301.07, 489.14, 609.18	(Hu et al., 2015)
65	11.48	597.14	Delphinidin-3-O-sambubioside	$C_{26}H_{29}O_{16}$	[M−2H]	595.13	0	Anthocyanin flavonoid	66.98, 169.09, 549.76, 523.13,	(Tian et al., 2017, Majdoub et al., 2019)
66	12.04	420.14	Apigenin 7-O-hexoside	$C_{21}H_{24}O_9$	-H	419.13	−1.6	Flavonoid	151.07, 269,78, 311.23	(Lee and Shaari 2022)
67	12.59	338.10	4-p-Coumaroylquinic acid	$C_{16}H_{18}O_8$	-H	337.09	−1.3	Phenolic acid	96.97, 160.01, 161.05, 175.04, 198.90, 220.90, 272.83, 300.85, 337.09	(Kiselova-Kaneva et al., 2022)
68	12.65	772.20	Quercetin 3-O-hexosyl-deoxyhexosyl-hexoside	$C_{33}H_{40}O_{21}$	-H	771.20	4.4	Flavonoid glycoside	Nil	(Kelebek 2016)

Table 3 (*continued*)

Peak no.	RT (min)	Mol. mass	Tentative identification	Mol. Formula	Adduct	B. peak (*m*/*z*)	Error ppm	Class	M/S product ions	Reference
69	13.92	610.15	Rutin	$C_{27}H_{30}O_{16}$	-H	609.14	−0.7	Flavonoid	Nil	(Ferracane et al., 2010)
70	14.12	564.14	Apigenin 7-O-apiosyl-hexoside	$C_{26}H_{28}O_{14}$	-H	563.14	0.1	Flavonoid glycoside	193.05, 207.03, 369.08, 457.06, 563.23	(Tang et al., 2019)
71	14.21	930.27	1,2′-Disinapoyl-2-feruloylgentiobiose	$C_{44}H_{50}O_{22}$	-H	929.26	−4.9	Phenolic acid	481.23, 499.08, 511.46, 529.03	(Lin et al., 2011)
72	15.46	474.07	Chicoric acid	$C_{22}H_{18}O_{12}$	-H	473.07	−1.4	Phenylpropanoid	121.03, 225.05, 313.06, 401.08, 418.77, 454.82	(Khoza et al., 2016)
73	15.55	388.15	Trachelogenin	$C_{21}H_{24}O_7$	-H	387.14	−3.5	Lignan	Nil	(Basit et al., 2021)
74	15.57	286.04	Kaempferol	$C_{15}H_{10}O_6$	-H	285.04	0.9	Flavonoid	93.41, 117.23, 143.25, 227.68, 255,46, 268.73, 285.05	(Fathoni et al., 2017)
75	15.63	516.12	3,4-Dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	-H	515.11	−1.5	Phenolic acid	293.08, 321.07, 424.79, 478.79	(Parejo et al., 2004)
76	16.63	550.16	Isoliquiritin apioside	$C_{26}H_{30}O_{13}$	-H	549.16	−0.6	Flavonoid	119.044, 450.825, 549.159	(Basit et al., 2021)
77	19.71	356.12	Conidendrin	$C_{20}H_{20}O_6$	-H	355.11	1.2	Lignan	Nil	(Eklund et al., 2004)
78	20.95	358.14	Matairesinol	$C_{20}H_{22}O_6$	-H	357.13	−0.4	Lignan	Nil	(Dias et al., 2017)
79	21.23	406.12	Piceatannol 3-O-hexoside	$C_{20}H_{22}O_9$	-H	405.11	−3.8	Stilbene	121.03, 249.03, 322.85, 337.15, 405.10	(Goufo et al., 2020)
80	23.56	423.26	Senbusine B	$C_{23}H_{37}NO_6$	-H	422.25	−1.1	Diterpene alkaloid	Nil	(Jaiswal et al., 2013)
81	27.04	284.06	Oroxylin A	$C_{16}H_{12}O_5$	-H	283.06	2.5	Flavonoid	240.23, 268.05	(Fong et al., 2014)
82	28.45	212.08	Pinosylvin	$C_{14}H_{12}O_2$	-H	211.07	4.2	Stilbene	Nil	(Fu et al., 2019)
83	29.06	284.16	Stearic acid	$C_{18}H_{36}O_2$	-H	283.09	1	Fatty acid	283.12	(Basit et al., 2021)
84	31.53	408.19	6-Geranylnaringenin	$C_{25}H_{28}O_5$	-H	407.18	0.9	Flavonoid	183.32, 274.56, 339.24	(Basit et al., 2021)
85	33.03	342.34	Caffeic acid-O-hexoside	$C_{15}H_{18}O_9$	-H	341.34	1.6	Phenolic glycoside	135.89, 179.56	(Álvarez-Fernández et al., 2015)
86	36.51	256.43	Palmitic acid	$C_{16}H_{32}O_2$	-H	255,45	2.7	Fatty acid	255.12	(Basit et al., 2021)
87	38.61	299.05	Kaempferide	$C_{16}H_{11}O_6$	-H	298.04	−2	Flavonoid	Nil	(Zehl et al., 2011)

RT: retention time; B. peak: base peak; error pm = ± 5.

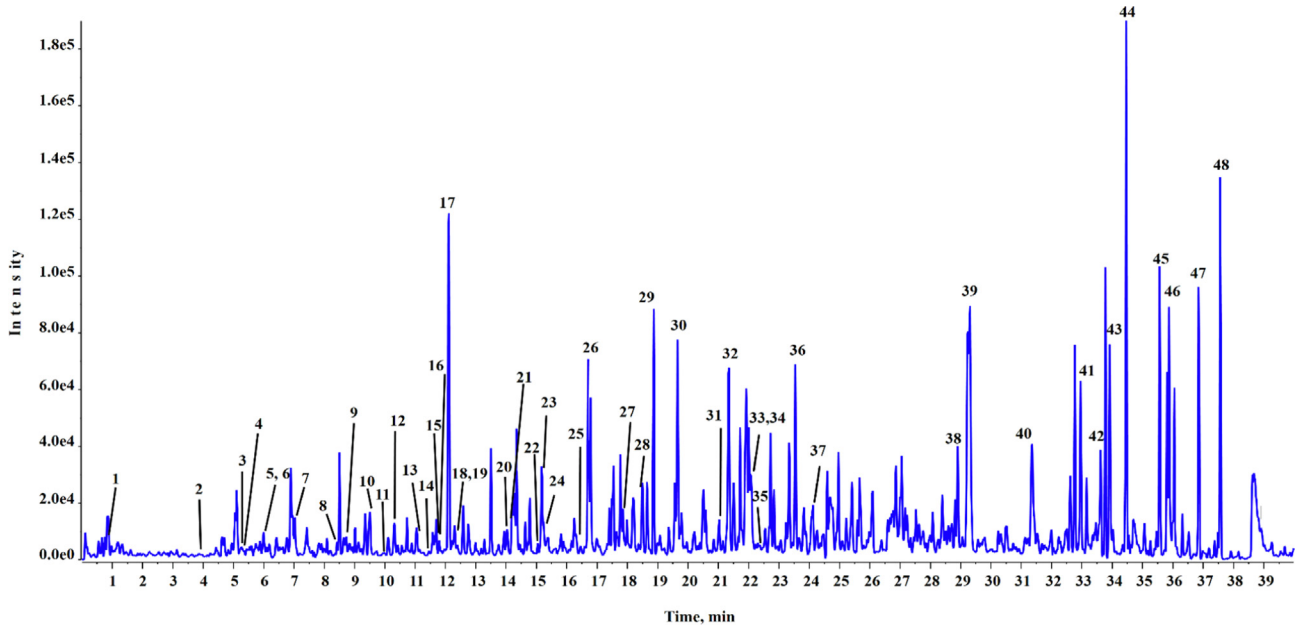

Fig. 1 UPLC-Q-TOF-MS chromatogram of BEJv in positive mode of ionization.

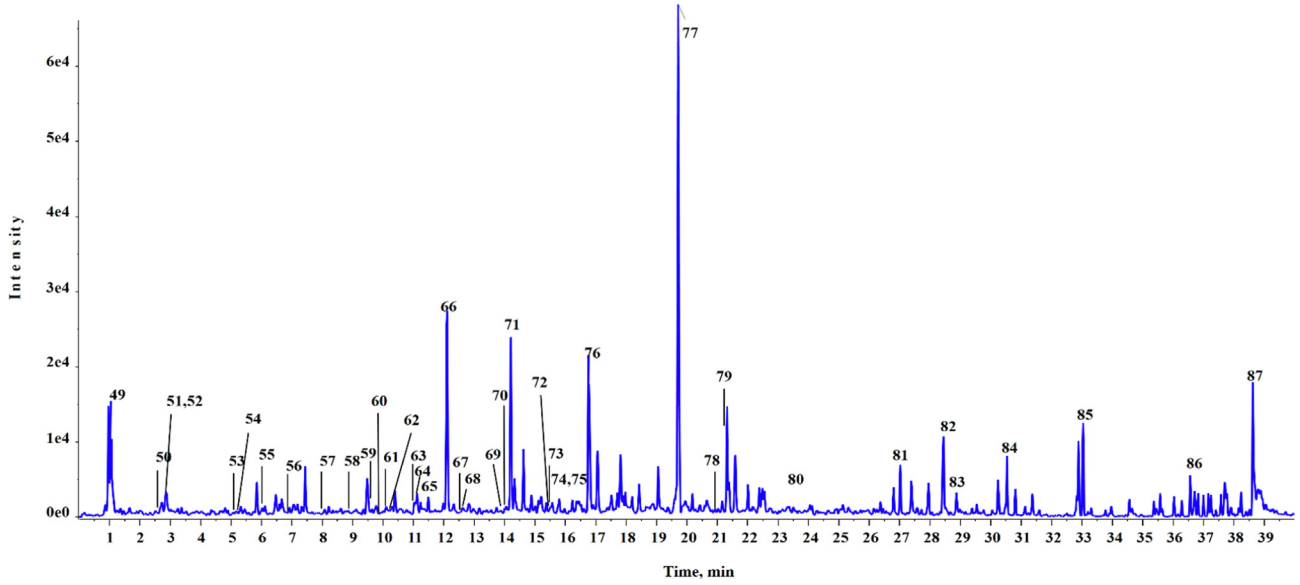

Fig. 2 UPLC-Q-TOF-MS chromatogram of BEJv in negative mode of ionization.

revealed presence of 14 compounds in BEJv (Table 4). Among the quantified compounds kamepferol was found with highest quantity (3.45 ± 0.21 µg/mg DE) followed by ferrulic acid (2.78 ± 0.31 µg/mg DE), whereas, apigenin, vannilic acid and catechin were found below limit of detection (< 0.01 µg/mg DE). Compounds p-hydroxy benzoic acid, o-coumaric acid, p-coumaric acid, 2,3-dimethoxy-benzoic acid, 3-hydroxy-4-dimethoxy-benzoic acid, 3-hydroxy benzoic acid, epicatechin, gallic acid, emodin were not detected in BEJv. The results of the quantification were expressed in µg/mg dry extract (DE). The yield of dry extract was 67 g.

GC–MS is considered highly reliable and widely used spectroscopic technique for the analysis of volatile components in plant extracts (Vieira et al., 2020). To extend the phytocochemical evaluation of BEJv to volatile compounds GC–MS technique was applied in the present study. The analysis depicted tentative presence of 19 compounds (Table 5. and Fig. 3). The tentative identification of the compounds was carried

Table 4 Polyphenols quantified in BEJv.

S. No.	Standard name	Polyphenols quantified (μg/mg DE)
1	Kaempferol	3.45 ± 0.21
2	Syringic acid	0.45 ± 1.2
3	Caffeic acid	0.98 ± 0.03
4	Cinnamic acid	1.07 ± 0.26
5	Vannilic acid	BLD
6	Gentesic acid	1.87 ± 0.08
7	Rutin	BLD
8	Ferrulic acid	2.31 ± 1.30
9	Skullcapflavone	1.78 ± 0.27
10	Apigenin	BLD
11	Conidendrin	2.78 ± 0.31
12	Chlorogenic acid	1.65 ± 0.06
13	Naringenin	0.18 ± 0.05
14	Catechin	BLD

All the values are presented as mean ± SD., (n = 3). nd: not detected; BLD: below limit of detection (< 0.1 μg/ g DE.); DE: dry extract.

out according to NIST library database search. Major class observed in the analysis was fatty acid and their derivatives. In detail methyl linolenate (Rt = 11.26 min) was found in highest quantity (3.69 %) followed by Piperonylcyanoacetic acid hydrazide (Rt = 29.40 min, 2.80 %), methyl linoleate (RT = 11.18 min, 2.14 %), methyl palmitate (Rt = 9.41 mi n, 1.64 %) and linoleic acid (Rt = 11.73 min, 1.16 %). A potent phenolic antioxidant sesamol (Zhou et al., 2021) was observed at (Rt = 4.83) with 0.17 % peak area. The good antioxidant and pharmacological potential of BEJv might be due to the presence of potent secondary metabolites found in GC–MS characterization of the extract.

3.2. Effect of BEJv on human normal and tumoral cell lines

Moreover, MTT assay was used to assess and evaluate the toxicity of BEJv against normal human liver cells (HL 7702), human liver cancer cells (HepG2) and human breast cancer cell (MCF-7) at various concentrations (25, 50, 100, 200 and 300 μg/mL). The biocompatibility and cell viability evaluation of BEJv were first time reported against the aforementioned cell lines. The extract showed no statistically different percent cell viability compared to the control group after 24 h and 48 h against human normal liver cells (Fig. 4A and B). These findings confirm the safety profile of BEJv with no deteriorating effect on the normal liver cells and also rectify the results of *in vivo* acute oral toxicity test. In case of liver cancer cells the extract showed moderate toxicity ($p < 0.05$) after 24 h at 200 and 300 μg/mL while lower cell viability was observed after 48 h at 300 μg/mL compared to control. Similarly BEJv showed moderate toxicity ($p < 0.05$) against breast cancer cell lines after 24 h and relatively higher toxicity ($p < 0.01$) at 100 μg/mL and $p < 0.001$ at 200 and 300 μg/mL after 48 h as compared to the control group. These findings suggest very low cytotoxicity effect of BEJv.

3.3. In vitro antioxidant

The involvement of free radicals in the etiology of various diseases is well understood. The free radicals induce toxic reactions such as peroxidation of the membrane lipids which leads to fragmentation and cross linking of macromolecules that causes cell death (Umamaheswari et al., 2008). Antioxidants scavenge the free radicals and minimize their toxic reactions in the pathogenesis of diseases (Fang et al., 2002). There are two categories of antioxidants; endogenous which include CAT, SOD and GSH and exogenous which include the bioac-

Table 5 Compounds identified in GC–MS analysis of BEJv (compound search based on NIST Library).

Peak no.	RT (min)	Pct. Area	Compound name	Mol. Formula	Mol. Mass	Quality
1	4.83	0.67	Sesamol	C7H6O3	138.1	95
2	6.15	0.35	2,4-di-*tert*-butylphenol	C14H22O	206.3	97
3	8.02	0.53	Coniferol	C10H12O3	180.2	97
4	8.75	0.22	Phytone	C18H36O	268.5	90
5	9.41	1.64	Methyl palmitate	C17H34O2	270.5	99
6	9.72	0.87	Palmitic acid	C16H32O2	256.4	99
7	11.18	2.14	Methyl linoleate	C19H34O2	294.5	99
8	11.26	3.69	Methyl linolenate	C19H32O2	292.5	99
9	11.40	0.46	Phytol	C20H40O	296.5	91
10	11.52	0.24	Methyl stearate	C19H38O2	298.5	99
11	11.64	0.51	Linoleic acid	C18H32O2	280.4	99
12	11.73	1.16	Linolenic acid	C18H30O2	278.4	99
13	12.62	0.81	Heliotropine	C8H6O3	150.1	79
14	19.61	0.27	Di-n-2-propylpentylphthalate	C24H38O4	390.6	62
15	25.75	0.65	Cubebinolide	C20H18O6	354.4	83
16	27.69	1.16	2-ethylacridine	C15H13N	207.2	65
17	29.40	2.80	Piperonylcyanoacetic acid hydrazide	C11H11N3O3	233.2	60
18	30.50	0.76	4-Methyl-2-trimethylsilyloxy-acetophenone	C12H18O2Si	222.3	68
19	36.43	0.84	Diethyl bis(trimethylsilyl) silicate	C10H28O4Si3	296.5	70

R.t: retention time; pct area: percent area.

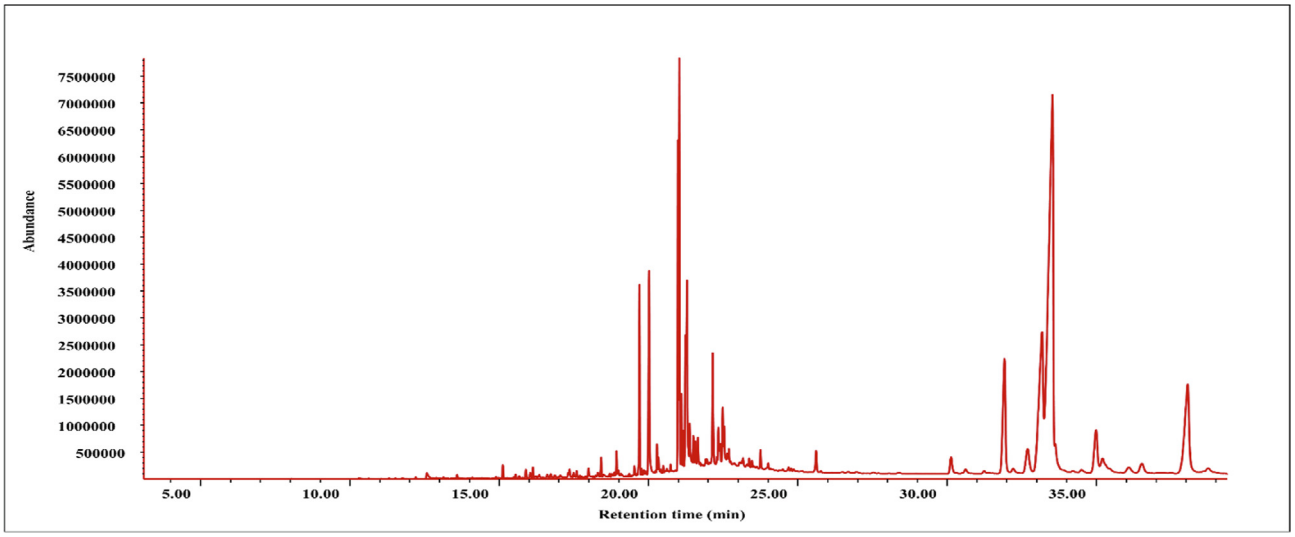

Fig. 3 GC–MS chromatogram of BEJv.

tive contents such as flavonoids and phenols (Lee et al., 2004). Role of nature is immense in provision of antioxidant agents. In the present study the antioxidant potential of BEJv was studied using four different methods. BEJv showed highest power reducing activity in the FRAP assay (97.21 ± 2.34 mg TE/g DE) followed by CUPRAC (89.32 ± 3.21 mg TE/g DE), while in free radical scavenging assay BEJv showed higher results in DPPH (45.46 ± 1.8 mg TE/g DE) than ABTS (41.13 ± 1.3 mg TE/g DE) as shown in Table 6. The antioxidant activity may be due to the presence of flavonoids and phenols in the BEJv as previous studies correlated the antioxidant activity of plant extracts to the presence of phenols and flavonoids. The results also ratify the presence of functional phytoconstituents with antioxidant potential tentatively identified in the chemical profiling of BEJv (Santos et al., 2013, Ruankham et al., 2021). (Saleem et al., 2020). Some other species of genus *Justicia* have also been reported with good antioxidant potential (Mondal et al., 2019).

3.4. In vitro enzyme inhibition

α-glucosidase and α-amylase inhibitors are used for the treatment of diabetes mellitus. Researchers are employing natural inhibitors of the enzymes for better outcomes in terms of promising results with minimal side effects as the synthetic sources of the enzyme inhibitors lack surety of efficacy and safety (Zengin et al., 2018). In *in vitro* enzyme inhibition, BEJv was tested to evaluate their inhibition potential against α-glucosidase and α-amylase (Table 6). The BEJv showed moderate to high inhibition potential against the tested enzymes. The BEJv showed comparatively higher inhibition of α-glucosidase (3.32 ± 0.31 mmol ACAE/g DE) compared to α-amylase inhibition (1.23 ± 0.6 mmol ACAE/g DE). The higher α-glucosidase activity of BEJv might be due to the presence of potent α-glucosidase inhibitors of phenol and flavonoids revealed in UPLC-Q-TOF-MS analysis of BEJv (Elmazar et al., 2013, Singh et al., 2021). The detailed *in silico* molecular docking revealed and confirmed the enzyme inhibition potential by docking three bioactives identified in UPLC-Q-TOF-MS analysis of BEJv.

Tyrosinase is an enzyme with therapeutic significance, involved in the melanogenesis process and its inhibitors prevent skin hyperpigmentation, a common cause of the skin darkening (Ciganović et al., 2019). In the current study the melanin reducing potential of BEJv was evaluated through tyrosinase inhibition. BEJv presented inhibition (193.21 ± 0.76 mg KAE/g DE) (Table 6). The *in silico* study showed significant docking score of the compounds against tyrosinase. The anti-tyrosinase potential of *J. vahlii* might be attributed to the presence of bioactives reported with good tyrosinase inhibition activities in literature (Farasat et al., 2020). The antityrosinase results proved the traditional use of *J. vahlii* in skin disorders.

Urease is the enzyme secreted by bacteria *H.pylori* in the human stomach, initiating and exaggerating the severity of peptic ulcer (Mobley 1996). In the current investigation BEJv showed significant inhibition (217.05 ± 4.5 mg TUE/g DE) of urease,. The phytochemical profiling has revealed the presence of phenols and flavonoids which were also confirmed by UPLC-Q-TOF-MS, which have a promising role in urease inhibition. The pyrogallol and catechol derivatives have been reported with *H. pyloric* urease inhibition (Xiao et al., 2010). Therefore, the results of the *in vitro* urease inhibition assay can be correlated to the diverse phytochemical profile of the species. Moreover, the three compounds skullcapeflavone, sesaminol and conidendrin showed good docking score against urease in *in silico* molecular docking study.

3.5. In vivo toxicity evaluation

The therapeutic applications of herbal drugs without scientific evidence may be associated with serious concerns regarding their toxicity profiles. Therefore, toxicity assessment of herbal drugs are carried out in different animal models to ensure their safety for human use (Liu et al., 2019). In the present study, oral administration of BEJv at the dose of 1000 mg/kg and 3000 mg/kg, exhibited no morbidity and mortality in mice

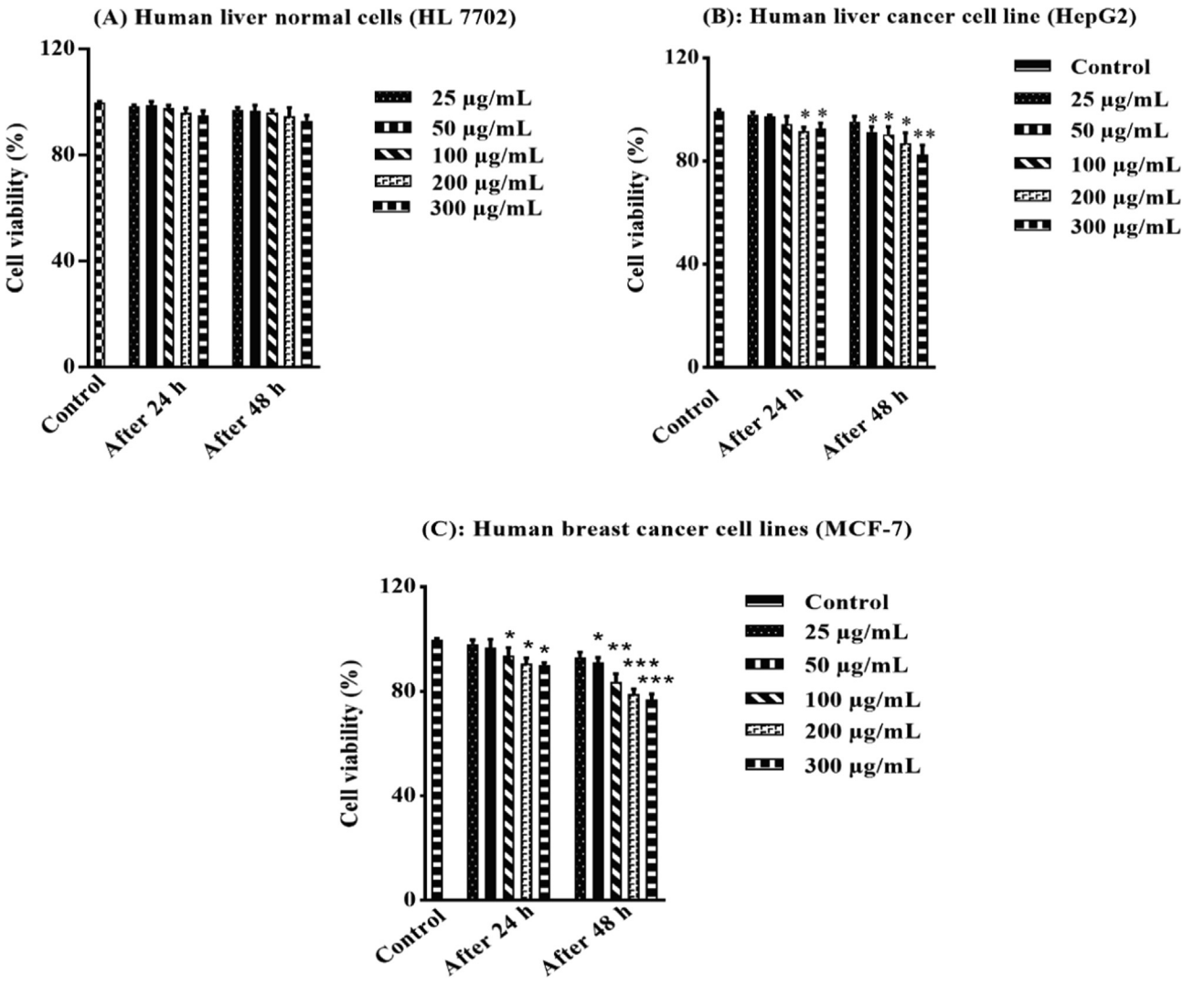

Fig. 4 Cytotoxicity using MTT assay. Percent cell viability against human liver normal cells (HL 7702) after 24 h and 48 h **(A),** percent cell viability against human liver cancer cell line (HepG2) after 24 h and 48 h **(B),** percent cell viability against human breast cancer cell lines (MCF-7) after 24 h and 48 h **(C)**. All the value are expressed as mean ± SD (n = 3). One-way ANOVA (with post hoc analysis-Tukey s multiple comparison test) was applied and the symbols used for significance were shown as *, ** and *** when p-value was < 0.05, 0.01 and < 0.001 respectively.

Table 6 Antioxidant and enzyme inhibition potential of BEJv.

	BEJv
DPPH mg. TE/g DE	45.46 ± 1.8
ABTS mg TE/g DE	41.13 ± 1.3
FRAP mg TE/g DE	97. 21 ± 2.34
CUPRAC mg TE/g DE	89.32 ± 3.21
α- glucosidase mmol ACAE/g DE	3.32 ± 0.31
β-amylase mmol ACAE/g DE	1.23 ± 0.6
Tyrosinase mg KAE/g DE	193.21 ± 0.76
Urease mg TUE/g DE	217.05 ± 4.5

All the values are expressed as mean ± SD (n = 3). TE: trolox equivalent; ACAE: acarbose equivalent; TUE: Thiourea equivalent; DE: Dry extract.

compare to the control group. Although, there was a slight change observed in the color of stool at 3000 mg/kg. This change might be due to excretion of some ingredients of the extracts *via* stool. Biochemical and hematological parameters were found normal in both treated and control group. No gross lesions, distortion, deformation and toxicity related signs and symptoms in vital organs were observed after administration of BEJv for 14 days in comparison to the control group (Fig. 5 and Table 7 & 8). These findings suggest that BEJv is non-toxic, biocompatible, and the species is safe for use as food additive and therapeutic applications.

3.6. Effect of BEJv on carrageenan induced paw edema, oxidative stress parameters and inflammatory mediators

The carrageenan induced paw edema model is a widely employed preliminary model for evaluation of anti-

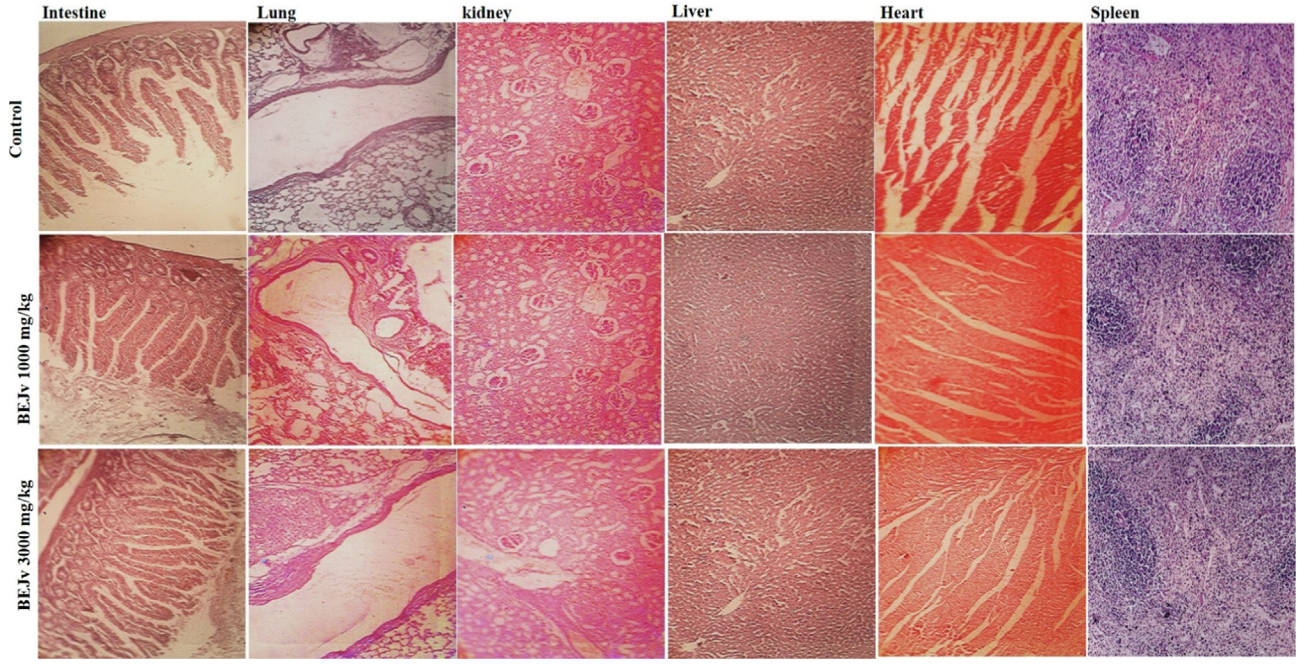

Fig. 5 Representative photomicrographs of histopathological examination of vital organs of mice in acute oral toxicity assessment of BEJv at 1000 mg/kg and 3000 mg/kg.

inflammatory potential of plant extracts (Piva et al., 2021). The carrageenan induced edema occurs in two phases. The initial phase is predominantly non-phagocytic edema which lasts from 1 to 1.5 h followed by later phase from 2 to 6 h with increase edema induction. The first phase is characterized by excessive secretion of inflammatory mediators such as histamine and serotonin while in later phase, there is overproduction of cyclooxygenase takes place which cause an increase in the formation of prostaglandins and free radicals, initiating inflammation by further secretion of inflammatory mediators such as interleukins and cytokines (Ayal et al., 2019). In the current study, the subcutaneous administration of carrageenan (1 %) induced inflammation by increasing volume of mice paw in control group (Table 9). The results were in accordance with the previously published reports (Piva et al., 2021). The BEJv treated groups showed inhibition of the edema in dose dependent manner with relatively lower inhibition at 100 mg/kg and highest percent inhibition ($p < 0.001$) at 400 mg/kg compared to the control group. At the end of 0.5 h BEJv showed highest inhibition (19.43 %) at 400 mg/kg. Similarly at the end of 1 h the BEJv showed inhibition; 11.37 %, 17.53 % and 35.98 % ($p < 0.05$) at 100, 200 and 400 mg/kg respectively. After 2 h of carrageenan injection the inhibition showed by BEJv was 13.08 %, 30.37 % ($p < 0.05$) and 37.91 % ($p < 0.01$) at 100, 200 and 400 mg/kg. Significant inhibition was shown by BEJv 34.40 % ($p < 0.05$) at 200 mg/kg and 43.57 % ($p < 0.01$) at 400 mg/kg after 4 h compared to control group. Highest inhibition 52.01 % ($p < 0.001$) at 400 mg/kg was observed at 5 h. Animals treated with standard dexamethasone 5 mg/kg showed significant inhibition 28.06 % ($p < 0.05$), 49.28 % ($p < 0.01$), 56.54 % ($p < 0.001$), 72.59 %

Table 7 Effect of BEJv on weight (g) of organs of mice.

Organ	Control	BEJv 3000 mg/kg
Spleen	0.201 ± 0.19	0.189 ± 0.18*
Kidney	1.450 ± 0.23	1.427 ± 0.17
Lung	0.636 ± 0.34	0.745 ± 0.13*
Heart	0.417 ± 0.10	0.432 ± 0.21
Liver	3.913 ± 0.67	3.941 ± 0.61

All values are expressed as mean ± SD., (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 compared with control group. ANOVA (one way) followed by Tukey's test.

Table 8 Effect of BEJv on biochemical parameters.

Biochemical parameter	Control group	Treated group (BEJv 3000 mg/kg)
Liver Function Tests		
ALT (U/L)	16.34 ± 1.1	17.41 ± 3.1
AST (U/L)	74.31 ± 1.3	81.87 ± 1.9
ALP (U/L)	73.51 ± 2.3	75.61 ± 2.5
Hematological parameters		
Hemoglobin (g/100 ml)	13.16 ± 2.1	13.1 ± 1.3
Total RBC (mL/cu. mm.)	7.35 ± 1.3	7.27 ± 1.9
PCV (%)	39.73 ± 1.2	40.63 ± 3.2
MCV (cu. micron)	51.03 ± 1.3	48.41 ± 1.3
MCH (Pico gram)	15.43 ± 1.03	15.90 ± 4.1
MCHC (%)	37.31 ± 2.2	36.42 ± 1.2
Total WBC (per cu. mm.)	4875 ± 1132	4989 ± 1076
Neutrophils (%)	31.21 ± 3.5	29.52 ± 4.5
Lymphocytes (%)	70.15 ± 3.7	71.65 ± 3.5
Eosinophils (%)	1.13 ± 0.19	1.23 ± 0.15

All values are expressed as mean ± SD., ($n = 3$).

Table 9 Effect of BEJv on carrageenan induced paw edema volume (mm).

The paw volume (mm), mean ± SD						
Groups	**Initial volume**	**0.5 h**	**1 h**	**2 h**	**4 h**	**5 h**
Normal saline (1 ml/kg)	0.194 ± 0.01	0.196 ± 0.10	0.211 ± 0.02	0.214 ± 0.12	0.218 ± 0.003	0.223 ± 0.06
Dexa	0.173 ± 0.02	0.141 ± 0.02 *	0.107 ± 0.003 **	0.093 ± 0.01***	0.061 ± 0.15 ***	0.058 ± 0.23 ***
BEJv 100 mg/kg	0.187 ± 0.01	0.181 ± 0.01	0.187 ± 0.05	0.186 ± 0.13	0.173 ± 0.01	0.178 ± 0.03
BEJv 200 mg/kg	0.183 ± 0.01	0.182 ± 0.01	0.174 ± 0.03	0.149 ± 0.02 *	0.143 ± 0.02 *	0.129 ± 0.21 **
BEJv 400 mg/kg	0.193 ± 0.10	0.161 ± 0.03	0.137 ± 0.17 *	0.137 ± 0.21 **	0.123 ± 0.14 **	0.107 ± 0.14 ***

All values are expressed as mean ± SD., (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 compared with control group. *ANOVA* (one way) followed by post hoc analysis- Tukeys multiple comparison test.

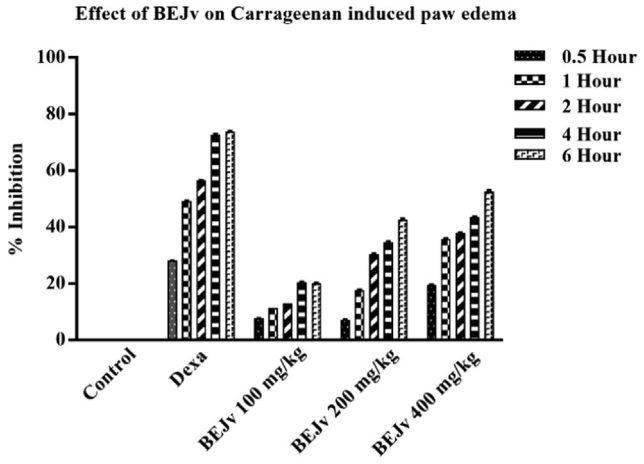

Fig. 6 Percent inhibition of carrageenan induced paw edema by BEJv at 100, 200 and 400 mg/kg after 0.5, 1, 2, 4 and 6 h. All values are expressed as mean ± SD., (n = 3) *p < 0.05 compared with control group; ** p < 0.01, *** p < 0.001 compared with control group. ### p < 0.001 compared with normal saline group One-way ANOVA (with post hoc analysis- Tukey s multiple comparison test).

(p < 0.001) % and 73.99 % (p < 0.001) after 0.5, 1, 2, 4 and 5 h respectively compared to the control group (Fig. 6) which is consistent with the previous reports (Piva et al., 2021). The results showed that BEJv significantly inhibited edema in later phase. The later phase edema inhibition by BEJv might be due to the suppression of prostaglandins by inhibiting cyclooxygenase and so its related products.

The prostaglandins initiate production of ROS and down regulate the oxidative stress markers, that further develop and maintain inflammation (Ansari et al., 2020, Ouda et al., 2021). Therefore, in the present study the effect of BEJv on oxidative stress markers; CAT, SOD and GSH was studied to know the possible anti-inflammatory mechanism of BEJv. The BEJv treated groups showed significant increase in CAT levels 45.31 ± 1.6 U/mg protein (p < 0.01) at 200 mg/kg and 46.67 ± 1.6 U/mg protein (p < 0.01) at 400 mg/kg. Similarly SOD and GSH levels were found higher in BEJv treated animals at 200 mg/kg (p < 0.01) and 400 mg/kg (p < 0.01) as compared to control group. The control group showed a decrease in CAT, SOD and GSH levels (Table 10) that are consistent with previous reports (Ammar et al., 2018). These findings suggest that the increasing effect on oxidative stress markers might be one of the possible mechanisms involved in the anti-inflammatory effect of BEJv.

The inflammatory mediators such as IL-1β and TNF-α play a key role in carrageenan induced edema by down regulating the levels of oxidative stress markers that contribute in the maintenance of inflammation (Ansari et al., 2020). In the present study, a significant increase in the levels of IL-1β and TNF-α was observed after carrageenan injection in the paw tissue of mice, these alterations are consistent with previous reports (Mansouri et al., 2015). BEJv treated groups showed a significant decrease in the levels of IL-1β at 200 mg/kg (p < 0.05) and 400 mg/kg (p < 0.001) while non-significant decrease was observed in mice treated with 100 mg/kg of BEJv compared to the control group (Fig. 7A). Similarly TNF-α levels were also decreased by BEJv at 200 mg/kg (p < 0.05) and 400 mg/kg (p < 0.01) compared to the control group. The standard dexamethasone (1 mg/kg) produced same but high yield effect compared to the control group (Fig. 7B). Con-

Table 10 Effect of BEJv on CAT, SOD and GSH levels in paw edema.

Treatment and dose	**CAT (U/mg protein)**	**SOD (U/mg protein)**	**GSH (nmol/mg protein)**
BEJv			
Carrageenan + Normal saline (10 ml/kg)	21.19 ± 2.1	22.17 ± 1.1	19.41 ± 2.5
Carrageenan + Dexa (75 mg/kg)	51.37 ± 2.4**	44.32 ± 1.2**	43.31 ± 1.9***
Carrageenan + BEJv (100 mg/kg)	37.64 ± 2.2	32.13 ± 2.1	31.23 ± 4.1*
Carrageenan + BEJv (200 mg/kg)	45.31 ± 1.6**	38.29 ± 3.1**	44.34 ± 1.9**
Carrageenan + BEJv (400 mg/kg)	46.67 ± 1.6**	39.13 ± 3.1**	44.84 ± 1.9**

All values are expressed as mean ± SD., (n = 3) *p < 0.05 compared with control group; * p < 0.05, ** p < 0.01, *** p < 0.001 compared with control. One-way ANOVA (with post hoc analysis- Tukey s multiple comparison test) using graph pad prism 7.0 was applied.

clusively it is suggested that the anti-inflammatory effect of BEJv might be due to increase in oxidative stress markers and decrease in inflammatory mediators. These findings support the traditional use of *J. vahlii* in pain and respiratory disorders.

Various investigations revealed the role of phytoconstituents particularly flavonoids, phenols, alkaloids and lignans in attenuation of inflammation by demolishing ROS and free radicals (Ammar et al., 2018). The UPLC-Q-TOF-MS analysis of BEJv showed tentative identification of number

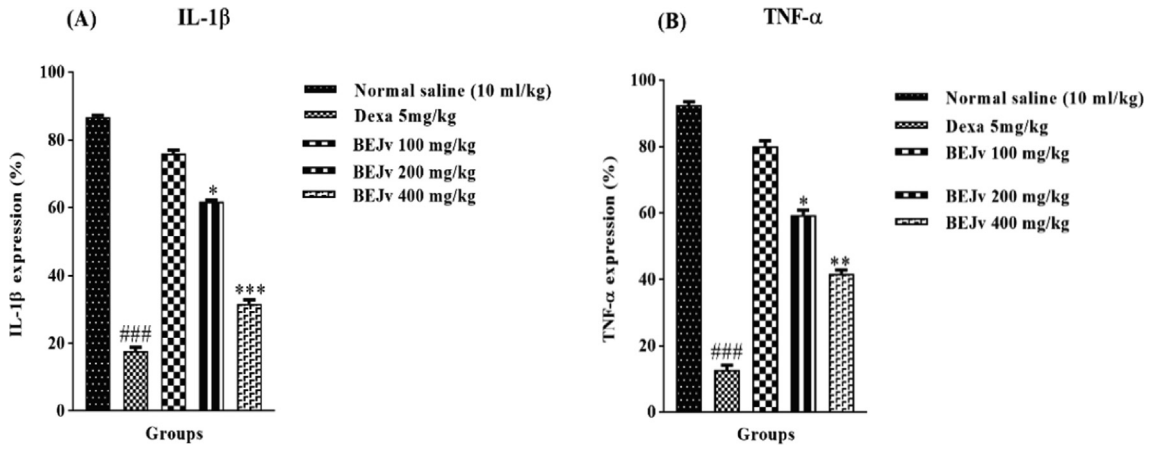

Fig. 7 Effect of BEJv on; IL-1β **(A)** and TNF-α **(B)** expression. All the value are expressed as mean ± SD (n = 3). The software "GraphPad Prism-5" was used and one-way ANOVA (with post hoc analysis- Tukey's multiple comparison test) was applied. *p < 0.05, **p < 0.01, ***p < 0.001 compared with standard. ### Significance p < 0.001 compared with normal saline group.

Table 11 Binding energy (kcal/mol), Inhibition constant Ki, interaction sites of the studied compounds with AChE, BChE, tyrosinase, α-amylase and α-glucosidase enzymes.

	Binding energy (kcal/mol)	Interaction site
Skullcapflavone-II		
α-amylase	−7.2	Asp 197 (HB), Glu 233, Ala 198, Leu 162, Lys 200, Ile 235, His 201 (HB), Leu 165, Thr 163, Tyr 62, Gln 63, Trp 59, Trp 58, His 305 Arg 195
α-glucosidase	−8.1	Ser 311 (HB), Pro 312, Asp 307, Asp 242, His 280 (HB), Phe 303, Gln 353, Phe 159, Glu 411, Tyr 158 Arg 315, Phe 314, Thr 310
Urease	−8.2	Lys 445 (HB), Phe 441, Pro 446, Asn 447, Gln 459, Tyr 32 (HB), Val 473, Gln 471 (HB), Val 33, Ala 37, Ile 568
Tyrosinase	−7.8	Thr 69 (HB), Asn 439 (HB), Thr 448, Gln 437, Arg 97, Glu 451, Arg 114, Gly 107, Ser 106, Pro 445, Thr 98 (HB), His 100, Val 68
Sesaminol		
α-amylase	−9.3	Tyr 62 (HB), His 101, Asp 197, Ala 198, Glu 233, Val 234, Lys 200, Leu 162, His 201, Ile 235, Asp 300, Trp 58, Thr 163, Trp 59, Gln 63 (HB), Leu 165
α-glucosidase	−9.6	Ser 298, Lys 13, Thr 290, Cys 341, Asp 341, Trp 343, Lys 16 (HB), Ala 292, His 295, Arg 270, Val 266, Asn 259
Urease	−8.4	Lys 445 (HB), Val 36, Phe 566, Tyr 32, Ala 150, Tyr 475
Tyrosinase	−10.2	Cys 101, His 100, Asn 102, Glu 66, Arg 64, Gly 63, Cys 99, Gly 107 (HB), Ser 106, Tyr 226, Pro 446 (HB)
Conidendrin		
α-amylase	−8.3	Leu 162, Thr 163, Gln 63, Leu 165, Tyr 62, Trp 58, Asp 300 (HB), His 299, Asp 197, Arg 195, Glu 233, Ala 198, His 305 (HB)
α-glucosidase	−7.3	Asp 144, Ile 150, Pro 151, Phe 173, Phe 166 (HB), Thr 165, Ser 162, Arg 176, Gly 160, Trp 238, Lys 148
Urease	−7.7	Glu 159, Leu 215, His 216, Ser 228, Asp 229, Tyr 232, Asp 230, Val 233, Lys 212, Lys 211
Tyrosinase	−7.5	Glu 34, Pro 80, His 81, Ser 38, Arg 55, Ala 35, Met 40, Cys 42 (HB), Pro 43, Gln 29, Pro 51, Asp 82 (HB), His 75

HB*: Hydrogen bonding.

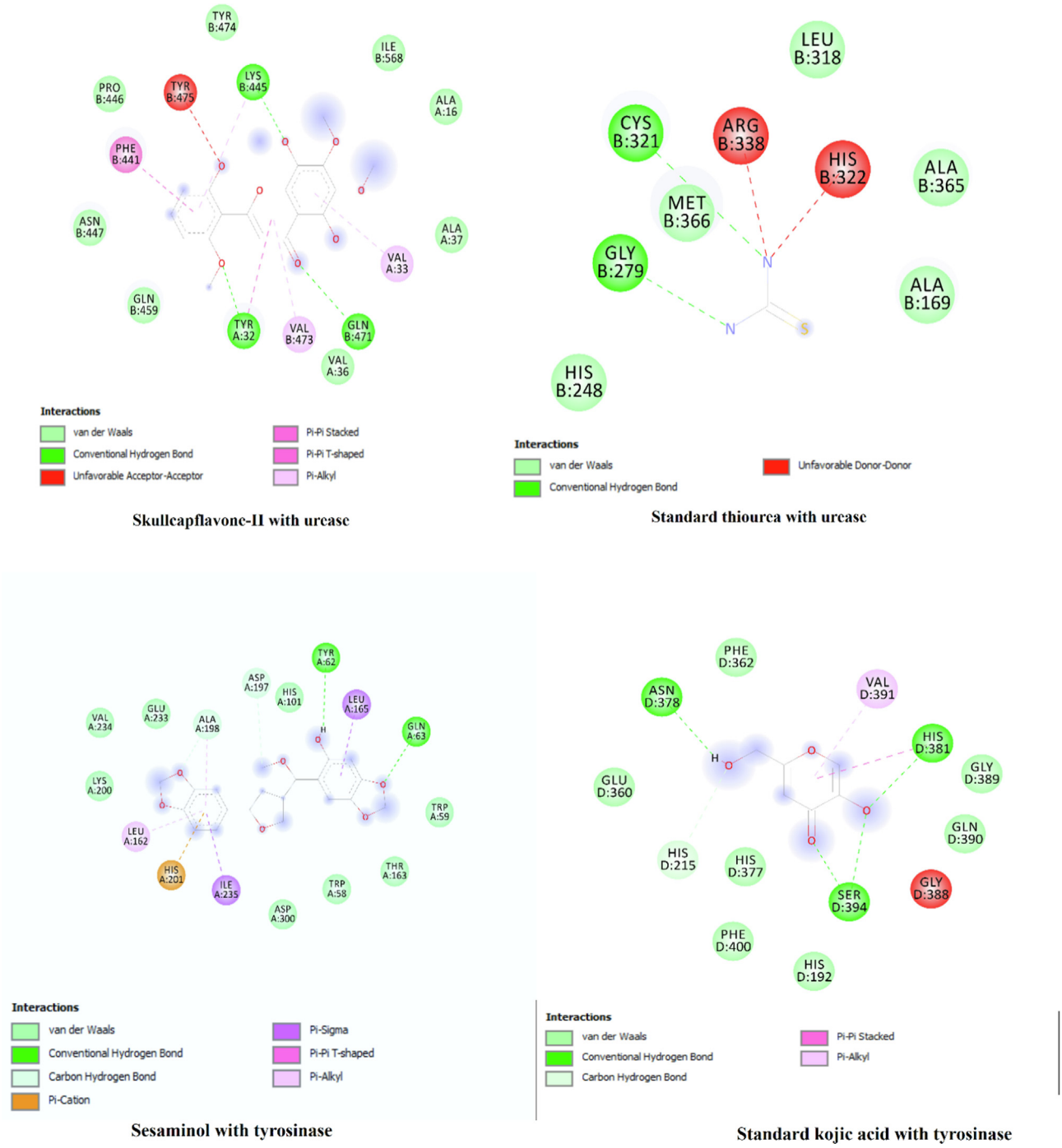

Fig. 8 The docked compounds at the active site of the enzyme and their interactions.

of anti-inflammatory flavonoids such as Naringenin, Hesperetin, Daidzein, Jaceosidin, Apigenin, Hispidulin and Rutin and phenols such as Caffeic acid, Syringic acid and Chlorogenic acid as well as alkaloids and lignans which have been proved as significant anti-inflammatory agents in previous studies (Moon et al., 2006, Agrawal 2011, Conti et al., 2013). Various species of *justicia* were also reported with anti-inflammatory potential (Corrêa and Alcântara, 2012) that ratify our results. The current study validate, the traditional use of *J. vahlii* in pain, skin and respiratory disorders correlated with inflammation (Tandon et al., 2004, Babu et al., 2015, Asmat et al., 2016).

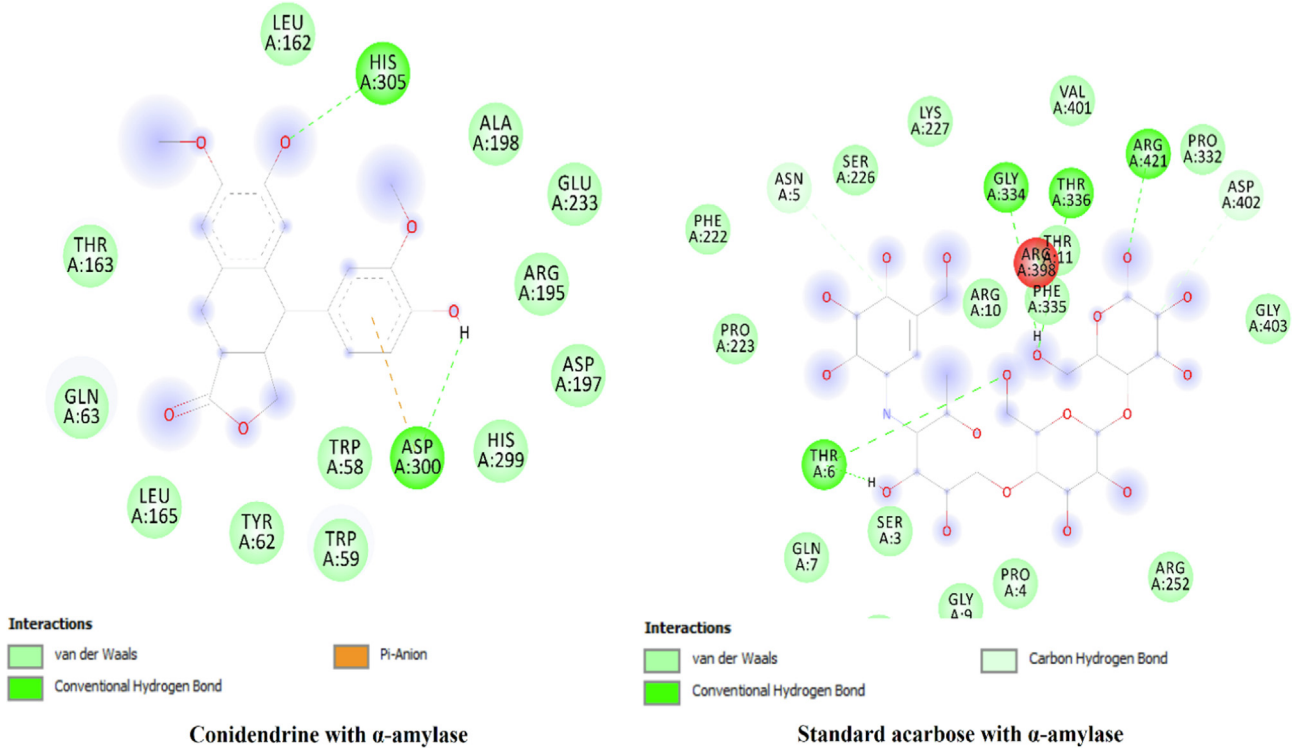

Fig. 8 (*continued*)

3.7. In silico studies

Recently molecular docking is considered as reliable and efficient tools used for prediction of binding free energy and correlation of experimental pharmacological activities. In the present study-three selected compounds as the major compounds were tested via *in silico* approaches against four enzymes namely α-amylase, α-glucosidase, tyrosinase and urease against which the extract and s were evaluated. The active site of the tested enzymes was detected and authenticated through docking the control ligands. Results of the docking are displayed in Table 11 and Fig. 8. The binding affinities of the compounds docked with three enzymes are tabulated in Table 11 with the predicted inhibition constants. More over details of the residual amino acids possibly involved in the interactions with the compounds at the active sites are also listed in the Table 11. Skullcapflavone has shown highest binding free energy (-8.2 kcal/mol) against urease in comparison to other enzymes studied. The inhibition might be due to high number of hydrogen bonds formed with Lys 445, Phe 441, Tyr 32 and Gln 471 at the active site of the enzyme as shown in Fig. 8. Similarly, sesaminol displayed highest binding free energy (-10.2 kcal/mol) against tyrosinase in comparison to other enzymes studied. Conidendrin showed highest results against α-amylase with binding energy of −8.3 kcal/mol. Hydrogen bonding and pi-pi interactions are observed as the dominant interactions between the compounds and the studied enzymes (Fig. 8).

4. Conclusion

The studies conducted suggested that BEJv presented an anti-inflammatory potential *via* modulating release of oxidative stress markers and inflammatory mediators which strongly support the traditional use of the species in inflammation and pain. Acute oral toxicity study and MTT assay suggest that the species is safe, non-toxic and biocompatible, can be consumed as food additive. The UPLC-Q-TOF-MS and GC–MS profiling revealed the tentative identification of functional bioactives with abundance of flavonoids and phenolic compounds in BEJv. The extract presented strong to moderate *in vitro* antioxidant and enzyme inhibition potential. Theoretically, docking study has confirmed the inhibition ability of the three selected compounds against α- amylase, α- glucosidase, tyrosinase and urease and their interactions with the active site are elucidated. Further studies can be done on the isolation of potentially bioactive compounds and sub-acute toxicity of the species.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.arabjc.2022.104135.

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