Pharmacogn. Res.
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ORIGINAL ARTICLE

Kaempferol – A Dietary Flavonoid Isolated from *Blepharis integrifolia*

Anil Kumar K.M, Ramith Ramu[1], Chandan S[1], Shivaraju Harikaranahalli Puttaiah

Department of Water and Health Sciences, Division of Environmental Sciences, Faculty of Life Sciences, JSS Academy of Higher Education and Research, [1]Department of Water and Health Sciences, Division of Biotechnology and Bioinformatics, Faculty of Life Sciences, JSS Academy of Higher Education and Research, Mysore, Karnataka, India

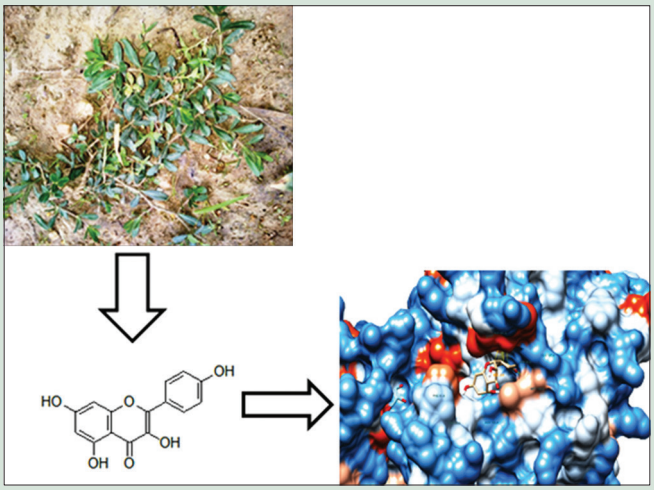
ABSTRACT

Background: *Blepharis integrifolia* is in the preparation of traditional formulation for the treatment of various diseases, including digestive disorders such as flatulence and dysentery. Although a very few studies have assessed the pharmacological properties, nothing is known about the phytochemicals present and the effect of extracts on its pharmacological activities. **Objectives:** Therefore, the objectives of the present study were to assess the role of methanol extract of *B. integrifolia* (MBI) for its anti-inflammatory, antioxidant, and antibacterial activities. **Materials and Methods:** The phytochemicals present in the extract were evaluated using high-resolution liquid chromatography and mass spectrometry (HR-LCMS). The extract and the isolated compound were subjected to antioxidant, antibacterial, and anti-inflammatory studies. Results: MBI exhibited an effective antibacterial, antioxidant, and anti-inflammatory activities at a more significant level than the standards. HR-LCMS analysis of MBI revealed the presence of kaempferol, rutin, and several phytochemicals. The activity-guided repeated fractionation of the methanol extract by silica gel column chromatography yielded a compound that exhibited strong antioxidant activity. Based on various physicochemical and spectroscopic analyses (ultraviolet, infrared, proton nuclear magnetic resonance [NMR], carbon-13 NMR, and MS), the bioactive compound isolated was elucidated as 3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one (kaempferol). Furthermore, the molecular docking studies for kaempferol revealed that it binds β-lactamase at MEG binding site and was found to interact with binding site residues PHE91, TRP94, ASN109, MET148, GLU147, ASN36, and ILE201. **Conclusion:** The results from the present study suggest that the potent antioxidant, antibacterial, and anti-inflammatory activities observed are a result of the presence of these bioactive compounds within the extract. In addition, these results also demonstrate the antioxidant potency of kaempferol which could be the basis for its alleged health-promoting potential.

Key words: Anti-inflammatory, flavonoid, free radicals, kaempferol, methanol extract

SUMMARY

- Antibacterial activity was used as the guide for the isolation of bioactive component from *Blepharis integrifolia*. Sequential extraction with petroleum ether, chloroform, and ethyl acetate led to the isolation of kaempferol. The samples were further tested for its antibacterial activity using minimum inhibitory concentration assay, antioxidant activity using 2,2-diphenyl-1-picrylhydrazyl, ABTS, and superoxide anion scavenging assays. Molecular modeling studies for the inhibition of β-lactamase activity was also performed and showed the competitive inhibition of kaempferol, suggesting this as a mechanism of antibacterial activity.



Abbreviations Used: DPPH: 2,2-diphenyl-1-picrylhydrazyl, PHE: Phenyl alanine, TRP: Tryptophan, ASN: Asparagine, MET: Methionine, GLU: Glutamate, ILE: Isoleucine.

Correspondence:
Dr. Shivaraju Harikaranahalli Puttaiah,
Department of Water and Health Sciences, Division of Environmental Sciences, Faculty of Life Sciences, JSS Academy of Higher Education and Research, Mysore, Karnataka, India.
E-mail: shivarajuenvi@gmail.com
DOI: 10.4103/pr.pr_114_19

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INTRODUCTION

Health-care providers are always on the lookout for newer and safer antibiotics which are useful to treat bacterial infections. The genetic makeup of bacteria is such that they effectively transmit and acquire resistance to drugs which are generally used for preventing their spread.[1] Considering this ever-growing resistance, the presently available drugs have become less effective, and therefore, potentially broad-spectrum alternatives are needed with less toxic effects.[2] In developing countries like India, studies have shown that gross inadequacy of public finance and lack of awareness of the usage of drugs without prescription (self-medication) has been identified as the major cause for the development of this drug resistance among several bacterial

Cite this article as: Anil Kumar KM, Ramu R, Chandan S, Puttaiah SH. Kaempferol – A dietary flavonoid isolated from *Blepharis integrifolia*. Phcog Res 2020;12:250-9.

Submitted: 17-Dec-2019 **Revised:** 09-Jan-2020
Accepted: 11-Mar-2020 **Published:** 14-Aug-2020

strains.[3] Therefore, along with adequate understanding of the genetic basis of bacterial resistance, it is also necessary to create awareness against self-medication to reduce the growing resistance in pathogenic bacteria.[4]

Inflammatory diseases are commonly encountered worldwide. Despite the fact that they are one of the oldest known diseases, the efficacy of the presently available treatment is not satisfactory. The use of plant extract obtained from willow leaves was first documented by Celsius as early as in 30 AD, for the treatment of inflammation and pain.[5] This study constituted the basis for the identification of acetyl salicylic acid, the major active ingredient of aspirin, used as a nonsteroidal anti-inflammatory drug (NSAIDs). Following to this study, several NSAIDs were identified as anti-inflammatory molecules with limited uses and adverse effects such as hemorrhage and ulcers.[6] Therefore, identification of a potential therapeutic agent with anti-inflammatory ability on a wide range of inflammation is essential.

Natural products have long been used in traditional practices as a part of their daily routine without the knowledge of its use in the treatment of several diseases. It has been estimated that more than 80% of the world population rely on the plant extracts or their active components for the treatment of several diseases.[7] A recent report suggests that around 61% of the recently developed drugs are based on the natural products.[8] The last two decades have witnessed growing research in identifying plant-based products for the treatment of several ailments including diabetes, cardiovascular disorders, cancer, and AIDS.[9] The ability of plants to synthesize a wide range of phytochemicals such as phenols, terpenoids, alkaloids, flavonoids, tannins, saponins, and others have been the basis of research to exploit the presence of plant-based compounds for medicinal applications.[10] These secondary metabolites are a rich source of plant-derived antimicrobial substances.[11] Their antioxidant potential is exploited by several food producers in order to render protection against a number of degenerative diseases, thereby enhancing the nutritional value of these functional foods. These antioxidants from natural sources not only exert protective effects against free radicals generated during the disease condition, but also have multifunctional pharmacological attributes and therefore are beneficiary over the synthetic drugs which are targeted for a particular disease.

Traditional Indian community uses folkloric medicine for the treatment of diseases such as common cold, cough, and many of the life-threatening disorders such as diabetes and cardiovascular ailments.[12] These medicines are effective and cheaper than their modern counterparts and thus popular worldwide. Apart from their positive attributes, they possess little or no adverse effects and therefore have resulted in a growing attention on the knowledge of traditional medicine. Based on this knowledge, over 150 plants were screened at the Western Ghats region of India in our previous study and *Blepharis integrifolia* was chosen for the assessment of antimicrobial and anti-inflammatory efficacy and identification of its constituent bioactive principles.[13]

B. integrifolia is a threatened endemic herb belonging to the family Acanthaceae and commonly called as Haridachchu in Kannada. It has several traditional medicinal uses. The crushed leaves are used as a therapy for headache and in the treatment of dysentery.[14] In addition, its leaves are used in the treatment of flatulence.[15] Extracts from the roots can be consumed as an antidote for snakebite[15] and the entire plant is used in the treatment of bone fractures, skin diseases, urinary infections bone settings, and allergies.[16]

Based on this knowledge about the ethnomedicinal applications of this endemic plant, it was selected in the present study. The present study reports the isolation and characterization of the bioactive components from the plant extract. In addition, we also discuss the *in vitro* and *in silco* antimicrobial efficacy of the isolated compound.

MATERIALS AND METHODS

Plant material and extraction

The whole plant of *B. integrifolia* was collected from the Western Ghats of Shimoga district and stored in sterile bags. The specimen was identified by the Department of Horticulture, Government of Karnataka, Mysuru, India. The samples were shade dried, homogenized using a mixer and subjected to extraction using various solvents.

50 g of coarse powder of the plant was subjected to hot solvent extraction using methanol (99%). The resulting filtrate was concentrated under vacuum using a rotary evaporator (Rotavapor R-200, Buchi, Switzerland), and the yield of methanol extract was recorded. The extract was further subjected to phytochemical screening to evaluate the phytoconstituents based on standard protocols.[17]

Isolation of bioactive compound

The dry powder of the whole plant *B. integrifolia* (2 kg) was extracted in a Soxhlet apparatus using methanol twice and filtered. The methanol extract of *B. integrifolia* (MBI) was concentrated in vacuo using a rotary evaporator (Rotavapor R-200, Buchi, Switzerland) and the weight of the residue was noted. The residue (32 g) was suspended in water and then serially extracted twice each with petroleum ether, chloroform, and ethyl acetate, to obtain their respective fractions yielding petroleum ether (7.9 g), chloroform (5.8 g), ethyl acetate (4.6 g), and H_2O-soluble (11 g). The resulting fractions were subjected to the antibacterial assays against food pathogens *in vitro,* which established the chloroform-soluble fraction (CSF) for the most potent antibacterial activity.

This fraction was subjected to silica gel (100–200 mesh, 1.5 kg) column (length 80 cm and 7 cm diameter) chromatography (elution rate of 2 ml/min flow with a total elution of 500 ml) and eluted with a gradient of chloroform: methanol (97:3 [4 l], 95:5 [10 l], 90:10 [7 l], 85:15 [6 l], 80:20 [7 l], 75:25 [6.5 l], 70:30 [7.5 l], 60:40 [9 l], and 50:50 [4 l]) to acquire fractions CSF1 (1.3 g), CSF2 (3.5 g), CSF3 (9.3 g), CSF4 (5.5 g), CSF5 (4.5 g), CSF6 (4.5 g), CSF7 (2.1 g), CSF8 (6.4 g), and CSF9 (10.2 g). Fraction CSF7, CSF8, and CSF6 exhibited potent activity in the antibacterial assessment with the order CSF8 > CSF7 > CSF6.

Fraction CSF8 was yet again subjected to silica gel column (length 50 cm and 3 cm diameter) chromatography (elution rate of 1 ml/min flow with a total elution of 100 ml) and eluted with linear gradients of chloroform: acetone (90:10; 80:20; 70:30; 60:40; 50:50; and 20:80; v/v), to obtain 6 subfractions. Subfraction 3 was further separated by silica gel CC using chloroform–acetone (70:30) followed by re-chromatography on a Sephadex LH-20 column with methanol as the eluting solvent to obtain compound kaempferol (15.1 mg).

Thin-layer chromatography

Thin-layer chromatography (TLC) was performed over precoated silica gel F_{254} plates (25 cm × 25 cm, Merck, Germany). The optimum resolution was attained in the solvent system containing chloroform: acetone: formic acid (74:18:8 v/v/v). To visualize the spot, TLC plate was exposed under multiband UVGL-58 UV-254/366 nm ultraviolet (UV) light and stained with 2,4-dinitrophenylhydrazine. On the chromatogram, the yellow band of isolated kaempferol exhibited a retention factor (R_f) of 0.20, which was similar to that of the standard kaempferol.

Spectroscopic analysis

The nuclear magnetic resonance (NMR) spectrum of the isolated compound wasrecorded on a Bruker DRX-400 spectrometer (Bruker Biospin Co., Karlsruhe, Germany) with ^{1}H NMR at 400 MHz and ^{13}C NMR at 100 MHz. The isolated compound

was prepared by deuterated methanol (99.8 atom % of deuterium) with tetramethylsilane (TMS) as an internal standard in 5 mm NMR tubes. Data were measured in $CDCl_3$ with chemical shifts according to the TMS signal and was expressed in parts per million (δ). The infrared (IR) spectrum was recorded using KBr discs on a NICOLET 380 Fourier-transform IR spectrometer (Thermo Fisher Scientific, France) in the range of 400–4000 nm. The UV spectrum of kaempferol in methanol was recorded on a Shimadzu UV-1800 spectrophotometer. The melting point was determined on an electrically heated VMP-III melting point apparatus and was uncorrected. Elemental analysis of kaempferol was performed on a Perkin Elmer 2400 elemental analyzer.

The mass spectrum was recorded with Q-TOF waters ultima instrument (Q-TOF GAA 082, Waters, Manchester, UK) with an electron spray ionization (ESI) source. The positive ion mode with a spray voltage at 3.5 kV, at a source temperature of 80°C, was set to obtain the spectra. Mass spectra were recorded under electron impact ionization at 70 eV energy. The sample was prepared in the concentration range of 0.25–0.50 mg/ml and injected by flow analysis at a flow rate of 10 µl/min. The recorded mass was in the range of 100–500 m/z.

Total phenolic content

MBI and CFS were subjected for the evaluation of total phenolics according to Folin–Ciocalteu method.[18] The standards were set using the gallic acid equivalent and the total phenolic content (TPC) of the samples was expressed as gallic acid equivalents in mg per gram dry weight (mg GAE/g).

Total flavonoid content

The $AlCl_3$ method was used to determine the flavonoids present in MBI and CSF fractions as per the method given by Ordon-Ez *et al*.[19] In brief, 20 µl of the extract was treated with 2% of $AlCl_3.6H_2O$, shaken vigorously for 2 min and diluted with water to a total volume of 10 ml. The samples were incubated for 10 min and the absorbance was read at 440 nm. The total flavonoid content (TFC) of MBI was expressed as quercetin equivalents in mg per gram dry weight (mg QE/g).

Total proanthocyanidin content

Proanthocyanidin content of MBI and CSF were determined according to Sun *et al*.[20] with minor modifications. 0.1 mg/ml of the extract was mixed with 3 ml of 4% vanillin–methanol solution and 1.5 ml hydrochloric acid was incubated at room temperature for 15 min. The absorbance was measured at 500 nm and the proanthocyanidin content (TCC) was expressed as mg GAE/g.

High-resolution liquid chromatography and mass spectrometry analysis

The plant sample was extracted with methanol and then subjected to high-resolution liquid chromatography and mass spectrometry (HR-LCMS) analysis. The HR-LCMS of MBI was carried out at sophisticated analytical instrument facility (SAIF), IIT Bombay, Mumbai. Chemical fingerprints of the selected medicinal plant extracts were prepared by Agilent HR-LCMS model-G6550A with 0.01% mass resolution.

The acquisition method was set to be MS – minimum range 50 (m/z) and maximum 1000 Dalton (m/z) with scanning rate each spectrum per second. Gas chromatography was maintained at 250°C with gas flow 13 psi/min. Chromatographic separations were performed on column 18 (100 mm × 1.0 mm, particle size 1.8 µm; waters), 100 µl/min, ejection speed with flush out factor 5 µl and 8 µl injection volume. The solvent system used for HR-LCMS was 100% water in A pump and 100% acetonitrile in B pump. The identification of components in the extract and interpretation on mass spectrum HR-LCMS was performed using the database of SAIF (IIT Bombay), which has more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the SAIF library. The name, molecular weight, and structure of the components of the test materials were determined.

Antioxidant assays

In the present study, three principle methods of antioxidant estimation, namely, 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical, 2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid (ABTS) cation radical, and superoxide anion radical scavenging activity, were evaluated.

2,2-Diphenyl-1-picrylhydrazyl radical scavenging assay

The plant samples were subjected to radical scavenging assay according to the method described by Williams-Brand *et al*.[21] with minor modifications. The radical solution was prepared by dissolving 2.4 mg of DPPH in methanol. 5 µl aliquots of the test samples (with diverse concentrations) were added to the DPPH solution to obtain a final volume of 4 ml. The reaction mixture was shaken well and incubated for 30 min in dark conditions. The absorbance was measured at 515 nm using Hewlett-Packard 8453 UV/Vis spectrometer. The antioxidant butylated hydroxyanisole (BHA) was used as a positive control in all the antioxidant assays. The radical scavenging activity was measured as a decrease in the absorbance of DPPH and calculated using the following equation:

Scavenging effect (%) = $([A_{control} - A_{sample}]/A_{control}) \times 100$.

Where A = absorbance.

ABTS radical scavenging assay

The test samples (MBI, diverse CSFs, and isolated kaempferol) were assessed for their radical scavenging potential using ABTS radical cation decolorization assay as per the methods of Re *et al*.[22] A reaction with 7 mM ABTS in water and 2.45 mM potassium persulfate (1:1) stored in dark at room temperature for 12–16 h produced the ABTS + cation radical. This cation solution was diluted using methanol until an absorbance of 0.700 at 734 nm. Further, using this radical cation, the antioxidant ability of the test samples was evaluated by incubating a diverse concentration of the test sample (5 µl volume) with 3.995 ml of diluted cation for 30 min. The absorbance was measured at 734 nm against blank. The scavenging effect was calculated using the equation as described for DPPH.

Superoxide anion scavenging activity

The superoxide anion scavenging activity was performed as per the method of Yen and Chen[23] In brief, the reaction mixture consisted of 1 ml of each of test sample with diverse concentrations, 1 ml of 60 µM phenazine methosulfate prepared in 0.1 M phosphate buffer (pH 7.4) and 1 ml of Nicotinamide adenine dinucleotide (NADH) prepared in phosphate buffer (pH 7.3). Further, the mixtures were incubated at 25°C for 5 min and the absorbance was measured at 230 nm using phosphate buffer as blank. The scavenging effect was calculated using the equation as described for DPPH. Radical scavenging potential was expressed as EC_{50} values for all the antioxidant assays. An EC_{50} value represents 50% of free, cation, and anion radicals scavenged by the test samples.

Antibacterial activity

Gram-positive bacteria, namely, *Bacillus cereus*, *Micrococcus luteus*, and *Staphylococcus aureus*, and Gram-negative bacteria, namely, *Klebsiella*

pneumoniae, *Enterobacter aerogenes*, *Escherichia coli*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, and *Salmonella* Enteritidis, were selected for the study. The bacterial strains were obtained from the Institute of Microbial Technology, Chandigarh, (MTCC). The bacterial stock cultures were incubated for 24 h at 37°C on nutrient agar and stored in the refrigerator at 4°C.

To evaluate the antibacterial activity of MBI, fractions, and kaempferol, the antibacterial agar well diffusion assay was employed following the methods described by Ramu *et al.*[24] with slight modifications. The nutrient agar medium was inoculated with nine food pathogenic bacterial strains. Subsequently, sterile discs (6 mm diameter) comprising samples (1 mg/ml) were placed on the inoculated nutrient agar media. The impregnated discs with diverse samples (prepared with methanol) were dried, placed on inoculated plates, and incubated for 24–48 h at 37°C. The diameter of the zone of inhibition around the disc was measured in millimeters. The lowest concentration required to inhibit the growth of the organism was estimated by minimum inhibitory concentration (MIC). Amoxicillin (1 mg/ml) and methanol were used as positive and negative control, respectively.

Anti-inflammatory activity

Inhibitory potential of albumin denaturation

The MBI and kaempferol were assessed for their inhibitory role on the denaturation of albumin using the method given by Sakat *et al.*[25] Briefly, the test samples were taken along with 1% aqueous bovine albumin and incubated at 37°C for 20 min. Further, the samples were heated to 51°C for 20 min and cooled allowing the samples to turn turbid. The turbidity was then measured at 660 nm using a spectrophotometer. The protein denaturation inhibitory activity was expressed as percentage inhibition using the formula given below:

Inhibition (%) = $[(A_{control} - A_{sample})/A_{control}] \times 100$.

where A = absorbance.

IC_{50} values were determined from the curve using % inhibition of the individual sample to that of the concentration of sample.

Membrane stabilization test

Red blood cell (RBC) suspension preparation: The RBC suspension was prepared according to the procedure given by Sakat *et al.*[25] and Sadique *et al.*[26] Fresh whole human blood (10 ml) was collected from healthy volunteers into a centrifuge tube. The tubes were centrifuged at 3000 rpm for 10 min and washed thrice with equal volume of saline. The blood volume was then measured and made up to 10% v/v suspension with normal saline.

Hemolytic assay: The samples were taken in aliquots of 1 ml along with 1 ml of 10% RBC suspension. The control tube was taken with saline instead of the test sample and aspirin was used as the standard. The reaction mixture was incubated at 56°C for 30 min and cooled under running tap water. Further, the reaction mixture was centrifuged at 2500 rpm for 5 min and supernatant was collected. The absorbance of the supernatant was measured at 560 nm to assess the percentage membrane stabilization activity. The ability of the sample to stabilize the membrane was calculated using the equation described for protein denaturation activity.

Proteinase inhibitory activity

The inhibitory potential of the test samples on the enzyme proteinase was assessed according to the method of Oyedapo and Famurewa.[27] Briefly, the reaction mixture included 0.06 mg trypsin, 20 mM Tris HCl buffer (pH 7.4), and 1 ml test sample. The resulting mixture was incubated at 37°C for 5 min followed by the addition of 0.8% w/v casein. This was further incubated for 20 min and the reaction was terminated by the addition of 2 ml of 70% perchloric acid leading to a cloudy supernatant. This was centrifuged and the absorbance of the supernatant was measured at 210 nm against buffer blank. Based on the optical density, the percentage inhibition was calculated as described for protein denaturation activity.

Molecular modeling study

The binding site of the procured protein structure was analyzed through ligand explorer of RCSB PDB server. The two-dimensional (2D) structure of kaempferol was generated using ChemSketch tool. The generated 2D structure was saved as.Mol file and the same was used to generate 3D structure where the hydrogens were added and 3D coordinates were generated using open-babel tool and saved in PDB format. The procured β-lactamase structure from RCSB PDB was further refined by removing water residues, followed by addition of Gasteiger (-Marsili) charges, and was further refined by merging nonpolar hydrogens using AutoDock V.4.0. Upon refinement of protein, its structure was selected for rigid molecule and the 3D structure of the ligand was selected for map type. Based on the binding site residues, the grid box was set such that all the binding site residues fit inside the grid box and hence this box was set with X: 48, Y: 48, and Z: 50 dimensions. Upon saving the grid, its parameter file (gpf) was generated and saved. The saved grid parameter file was run using Autogrid4. On successful completion of Autogrid, molecular docking of the ligand was carried out using genetic algorithm using AutoDock4, followed by generation of docking parameter file (dpf). The saved docking parameter file was used to run Autodock4.

Statistical analysis

The experiments were performed in triplicates. Results were expressed as mean ± standard error. Statistical comparisons between the treatment groups and control were performed by one-way analysis of variance, followed by Duncan's multiple range test using SPSS Software (version 21.0, Chicago, USA). The results were considered statistically significant if the *P* values were 0.05 or less. Pearson's correlation was performed to indicate the relationship between total phenolic, flavonoid, and proanthocyanidin content and radical scavenging activities of test samples. GraphPad PRISM software (version 4.03, Graph Pad Software Inc., San Diego, CA) was used for calculating IC_{50} values.

RESULTS AND DISCUSSION

In the present study, MBI exhibited a wide range of antibacterial activity by inhibiting the growth of foodborne pathogens (Gram-positive and Gram-negative) at a more effective level than the standard antibiotic amoxicillin (positive control), with a higher free radical scavenging activity in all the assays tested. Furthermore, MBI inhibited albumin denaturation, hemolysis of RBC, and proteinase activity better than the positive control. These results indicate the presence of prospective inhibitory compounds in the extract. Hence, an effort was made to identify the bioactive compounds in MBI responsible for its biological activity.

Isolation and identification of kaempferol from methanol extract of *Blepharis integrifolia*

The primary qualitative analysis of MBI revealed a high presence of tannins, flavonoids, coumarins, anthraquinones, terpenoids, saponins, and reducing compounds. Furthermore, moderate presence of steroids, carotenoids, and alkaloids was observed with no traces of phlobatannins and cardiac glycosides. This was in agreement with several previous

studies which suggest that the methanol extract of the plant is rich in tannins, flavonoids, and terpenoids.[28]

To characterize the bioactive component responsible for pharmacological properties, the methanol extract of the plant was fractionated by sequential extraction with petroleum ether, chloroform, and ethyl acetate. The antibacterial fraction and the CSF were separated by repeated column chromatography over silica gel and Sephadex LH-20 to obtain kaempferol [Figure 1]. The structural elucidation of the kaempferol was determined on various physicochemical and spectroscopic methods (UV, IR, ^{1}H NMR, ^{13}C NMR, and MS). The elucidation of the structures is as follows.

Kaempferol: 3,5,7- Trihydroxy-2-(4- hydroxyphenyl)-4H-chromen-4-one

Pale yellow crystalline solid, melting point: 270–272°C; IR (KBr): 1140 (C-O-C), 1200 (phenolic C-O), 1750 (C=O), 3650 cm^{-1} (phenolic-OH); ^{1}H NMR ($CDCl_3$): δ 4.8 (bs, 4H, 4OH), 6.15 (s, 2H, Ar-H), 6.85 (d, J = 7 Hz, 2H, Ar-H), 7.25 (d, J = 7 Hz, 2H, Ar-H). ^{13}C NMR (DMSO-d6): δ 98.0, 98.2, 105.6, 115.9, 115.5, 123.0, 127.8, 127.8, 136.5, 157.7, 160.1, 160.3, 163.8, 166.4, 178.4. LC-MS: m/z 287 (M + 1), analytical calculated data for $C_{15}H_{10}O_6$ (286): C, 62.94; H, 3.52. Found: C, 62.99; H, 3.51%. The identity of the compound was deciphered on the basis of the above results as well as in comparison with the NMR and MS data in the literatures.[29,30]

Antibacterial activity

In this study, MBI was assessed for *in vitro* antibacterial activity against nine food pathogens which were qualitatively and quantitatively assessed by the presence/absence for zone of inhibition and MIC values. The standard antibiotic amoxicillin was used as positive control. Impregnated paper discs containing only methanol (negative control) did not exhibit zone of inhibition. Table 1 reveals that MBI, diverse CFS, and isolated compound kaempferol exhibited significant antibacterial activity against all the selected strains of micro-organisms tested, among which MBI was remarkably better than the positive control. Results obtained from the agar well diffusion method were used to determine the MIC [Table 2]. In terms of MIC (mg/ml) values, it is evident that MBI, isolated kaempferol, and all the fractions tested possessed a strong inhibition on *S. aureus* (Gram-positive) and *E. coli* and *E. aerogenes* (Gram-negative) and were significantly higher ($P < 0.05$) than amoxicillin. On the whole, the MIC values ranged from 0.75 mg/ml up to 6.75 mg/ml. Our results exhibited that MBI, diverse CSFs, and kaempferol possess a broad range of antibacterial activity by inhibiting the growth of foodborne pathogens (Gram-positive and Gram-negative), thereby providing a baseline for future studies on potentials of both the extract and the compound as antibacterial contributors. It is well established that the polyphenolic compounds interfere with the structural properties and reduce the nutrition availability and digestibility in bacteria.[31] Our results are supported by several previous studies which suggest that secondary metabolites belonging to the class of alkaloids, flavonoids, and phenolic compounds exhibit significant antibacterial properties.[32-35] In this study, the extracts showed stronger inhibition over the isolated compounds, which are in accordance with several other studies, suggesting that a synergistic action of the various phytoconstituents responsible for the observed effect.[36-38]

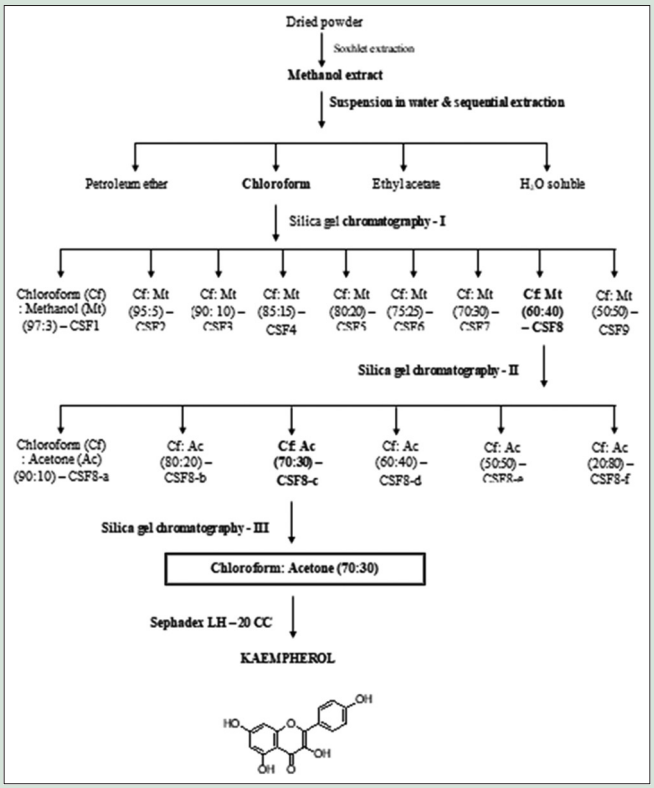

Figure 1: Separation scheme of kaempferol from methanol extract of *Blepharis integrifolia* and its structure

Antioxidant ability

The free radical scavenging ability of MBI and its constituent was studied by employing an array of *in vitro* assays, namely, DPPH, ABTS, and superoxide, whereas BHA was used as a positive control. Results were expressed as EC_{50} values (μg of tests per ml) as described in Table 3, revealing that the isolated kaempferol was relatively higher ($P < 0.05$) than MBI in radical scavenging activities. In all the assays used in this study, MBI and CSF and CSF8 were effective than BHA and the activities ascended in the order MBI > CSF > CSF8 > BHA > CSF8-c > kaempferol. The results revealed that MBI and kaempferol possess strong antioxidant ability, with significantly lower and higher ($P < 0.05$) EC_{50} values, respectively, than the positive control. Enormous literature is available on the antioxidant potential of various plant extracts, and in most of the studies, methanolic extracts have exhibited the optimal results among all the solvent extracts.[39-42] In all the studies, it was also observed that the extracts containing high phenolic compounds and flavonoids are the most powerful antioxidants which exert protection against the oxidative

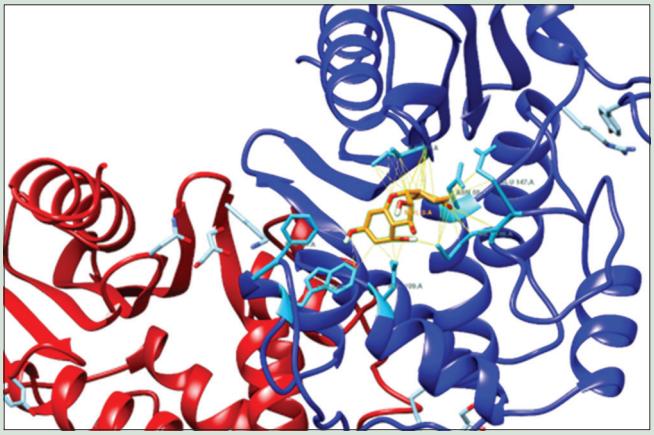

Figure 2: Image showing kaempferol bound to β-lactamase at MEG binding site

damage induced by free radicals in several diseases such as carcinoma, diabetes, asthma, dementia, Parkinson's, and others.[43] Likewise, in our study, the strong antioxidant potential exerted by MBI and kaempferol upholds the enormous beneficiary potential of these as therapeutic agents.

As shown in Table 3, the study indicated that MBI was found to have high phenolic content (365 mg GAE/g). The content of total phenols was concentrated in the MBI, and CSFs showed high TPC in the same order: CSF (308) > CSF8 (190) > CSF8-c (96) (56 mg GAE/g) as that of the antioxidant assays. Wang *et al.*[44] reported that the TPC is a significant indicator of the strength of the antioxidants in plant extracts and therefore fall in line with the results obtained in our study. TFC of the test samples (MBI and diverse CSF) ranged from 26 to 156 mg QE/g and the ranking was as follows: MBI (156) > CSF (111) > CSF8 (73) > CSF8-c (32) (26 mg QE/g respectively) as shown in Table 3. Total proanthocyanidin content was higher in all samples tested ranging from 155 to 786 mg GAE/g and the ranking was similar to TPC and TFC. Reasonably high concentration of TFC and proanthocyanidins are known to possess multiple therapeutic applications as antioxidant, antimicrobial, and anti-inflammatory agents, and thus, in our study, the therapeutic applications of the extract have been exploited.

Table 1: Antibacterial activity by disc diffusion for methanol extract of *Blepharis integrifolia*, diverse chloroform-soluble fractions, and isolated compound kaempferol

	Zone of inhibition* (mm)					
	Std.	MBI	CSF	CSF8	CSF8-c	Kf
Gram positive						
Bacillus cereus	11.02±0.09	20.13±0.62	16.62±0.50	15.06±0.42	13.35±0.24	10.42±0.36
Micrococcus luteus	14.25±0.06	18.06±0.36	15.23±0.90	13.09±1.33	12.05±0.69	9.79±1.01
Staphylococcus aureus	18.67±0.09	13.03±1.07	10.06±0.48	10.00±1.47	8.52±1.36	8.83±0.22
Gram negative						
Klebsiella pneumoniae	11.08±0.15	15.08±0.93	15.05±0.09	14.67±0.36	9.98±0.42	9.45±0.33
Enterobacter aerogenes	15.25±0.88	27.07±0.11	20.66±1.02	16.66±0.60	14.44±1.54	13.08±1.00
Escherichia coli	32.04±0.53	22.53±0.36	18.09±0.86	17.13±0.56	15.38±0.70	15.10±0.91
Pseudomonas fluorescens	18.09±0.31	14.48±0.60	8.98±1.13	6.75±0.59	5.00±0.01	4.61±0.85
Pseudomonas aeruginosa	25.06±0.80	11.11±0.34	9.56±0.38	8.56±1.88	6.00±2.50	5.08±0.94
Salmonella Enteritidis	18.40±0.32	10.75±0.77	7.86±0.75	7.00±0.33	3.52±0.92	2.50±0.02

*Values are expressed as mean±SE. Std.: Amoxicillin; -: Inactive; MBI: Methanol extract of *Blepharis integrifolia*; CSF: Chloroform-soluble fraction; Kf: Kaempferol; SE: Standard error

Table 2: The minimum inhibitory concentration for methanol extract of *Blepharis integrifolia*, diverse chloroform-soluble fractions, and isolated compound kaempferol

	MIC* (mg/ml)					
	Std.	MBI	CSF	CSF8	CSF8-c	Kf
Gram positive						
Bacillus cereus	1.34±0.61	0.75±0.55	1.15±0.20	1.45±0.36	2.04±0.26	2.42±0.33
Micrococcus luteus	2.50±0.26	0.82±0.24	1.25±0.40	1.95±1.54	2.85±0.12	2.85±1.78
Staphylococcus aureus	3.10±0.12	0.93±1.05	1.60±0.84	1.98±1.47	2.52±1.00	2.75±0.01
Gram negative						
Klebsiella pneumoniae	4.05±0.38	0.80±0.15	0.88±0.70	0.89±0.43	1.98±0.58	2.45±0.33
Enterobacter aerogenes	1.75±0.25	0.50±0.10	0.69±0.32	0.77±0.40	0.94±1.41	1.01±0.65
Escherichia coli	1.00±0.20	0.65±0.45	0.74±6.01	0.89±0.67	1.25±0.79	1.50±0.21
Pseudomonas fluorescens	3.08±0.95	1.05±0.50	2.82±1.13	2.95±0.93	3.00±1.07	4.19±2.45
Pseudomonas aeruginosa	2.50±0.04	0.94±0.22	1.63±0.41	2.34±2.04	4.00±1.82	5.32±0.46
Salmonella Enteritidis	1.95±0.45	0.99±0.77	1.86±2.23	3.25±0.89	6.47±0.68	6.71±3.02

*Values are expressed as mean±SE. Std.: Amoxicillin; MIC: Minimum inhibitory concentration; MBI: Methanol extract of *Blepharis integrifolia*; CSF: Chloroform-soluble fraction; Kf: Kaempferol; SE: Standard error

Table 3: Total phenolic, flavonoid, proanthocyanidin contents, and antioxidant activity of methanol extract of *Blepharis integrifolia*, diverse chloroform-soluble fractions, and isolated compound kaempferol

Sample	TPC (mg GAE/g)	TFC (mg QE/g)	TCC (mg GAE/g)	EC_{50}[x,y] (µg/ml) Radical scavenging activities		
				DPPH	ABTS	Superoxide
MBI	365.68±0.98[d]	156.05±2.23[d]	786.01±1.90[d]	17.02±1.62[a]	12.06±0.77[a]	44.44±2.05[a]
CSF	308.58±0.15[c]	111.22±0.74[c]	636.25±0.36[c]	20.80±0.90[b]	16.49±0.24[b]	49.09±1.81[b]
CSF8	190.12±0.90[b]	73.43±1.07[b]	420.55±1.58[b]	33.17±4.07[c]	26.67±1.90[c]	64.31±1.02[c]
CSF8-c	96.46±1.70[a]	32.05±0.86[a]	299.00±0.50[a]	41.50±1.21[e]	34.96±0.35[e]	74.00±0.01[e]
Kf	-	-	-	44.14±1.54[f]	35.05±0.08[e]	76.67±0.14[f]
BHA	-	-	-	35.55±0.01[d]	30.16±1.28[d]	66.57±0.34[d]

[x]Values are expressed as mean±SE. Means in the same column with distinct superscripts are significantly different ($P \leq 0.05$) as separated by Duncan multiple range test; [y]The EC_{50} value is defined as the effective concentration of the test samples to show 50% of antioxidant activity under assay conditions. TPC: Total phenolic content; TFC: Total flavonoid content; TCC: Total proanthocyanidin content; DPPH: 2,2-diphenyl-1-picrylhydrazyl; MBI: Methanol extract of *Blepharis integrifolia*; CSF: Chloroform-soluble fraction; Kf: Kaempferol; SE: Standard error; BHA: Butylated hydroxyl anisole; ABTS: 2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid

Correlation between antioxidant activity and total phenolic content, total flavonoid content, and TCC

A correlation study was performed to explore the relationships between total phenolic, flavonoid, and proanthocyanidin content and the diverse antioxidant assays measured in MBI, diverse CSF, and Kf [Table 4]. There was a significant linear correlation between diverse free radical scavenging activities performed and total polyphenolic compounds (phenolic, flavonoid, and proanthocyanidin). The strongest correlative value was obtained with ABTS and total phenolic compounds in the MBI (R = 0.998) fraction, followed by that of DPPH and total flavonoid in the MBI (R = 0.989) fraction. These results indicate that total polyphenolics in MBI resulted in stronger antioxidant activity. Previous studies carry out on the mulberry fruits by Natić *et al.*[45] suggest that the high levels of polyphenols were indeed responsible for the strong antioxidant–antiradical scavenging activity and the observed superoxide radical scavenging potential. Furthermore, a significant correlation between TFC and hydroxyl radical scavenging activity was also observed in our study which was also support by the study carried out by Metrouh-Amir *et al.*[46] In addition, the strong association of total phenols and hydrogen peroxide radical scavenging ability was also observed. The results therefore suggest that the concentration of proanthocyanidins and flavonoids is a direct indicator of the hydroxyl and hydrogen peroxide radical scavenging potential of the extracts, respectively.

Anti-inflammatory activity

It is a general understanding that inflammation is associated with denaturation of proteins and therefore assessed in our study. The test samples (MBI, diverse CSF, and Kf) with diverse concentrations (25–1000 µg/mL) inhibited albumin denaturation, hemolysis of RBC, and proteinase activity [Table 5]. The inhibitory effect of MBI and CSF on protein denaturation was found to be 1.8- and 1.3-fold higher than aspirin (positive control), respectively. MBI inhibited protein denaturation with an IC_{50} of 44 µg/ml [Table 5]. Kaempferol (IC_{50}: 96 µg/ml) isolated from MBI was found to be a potent inhibitor, but nonetheless, the inhibition was slightly lower than MBI, CSF, and aspirin. In addition, the potential of MBI and its constituents on RBC membrane modification was investigated in the present experimental conditions. Treatment with MBI and its constituents provided a strong shield from denaturation (i.e., significant improvement). Furthermore, similar studies were conducted to evaluate whether MBI and its constituents also inhibited proteinase enzyme. The 50% inhibition of proteinase by MBI and its active

Table 4: Correlation between EC_{50} of radical scavenging activities and total phenolic, flavonoid, proanthocyanidin content of methanol extract of *Blepharis integrifolia*, diverse chloroform-soluble fractions, and isolated compound kaempferol

	Correlation (R)*		
	Phenolic	Flavonoid	Proanthocyanidin
DPPH			
MBI	0.987	0.989	0.955
CSF	0.946	0.965	0.930
CSF8	0.876	0.899	0.895
CSF8-c	0.786	0.803	0.750
Kf	0.645	0.780	0.745
ABTS			
MBI	0.998	0.920	0.956
CSF	0.956	0.899	0.934
CSF8	0.856	0.739	0.911
CSF8-c	0.801	0.721	0.756
Kf	0.756	0.680	0.701
Superoxide			
MBI	0.856	0.789	0.800
CSF	0.783	0.754	0.708
CSF8	0.721	0.652	0.695
CSF8-c	0.698	0.568	0.658
Kf	0.650	0.550	0.574

*Values are expressed as mean±SE. DPPH: 2,2-diphenyl-1-picrylhydrazyl; MBI: Methanol extract of *Blepharis integrifolia*; CSF: Chloroform-soluble fraction; Kf: Kaempferol; SE: Standard error; ABTS: 2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid

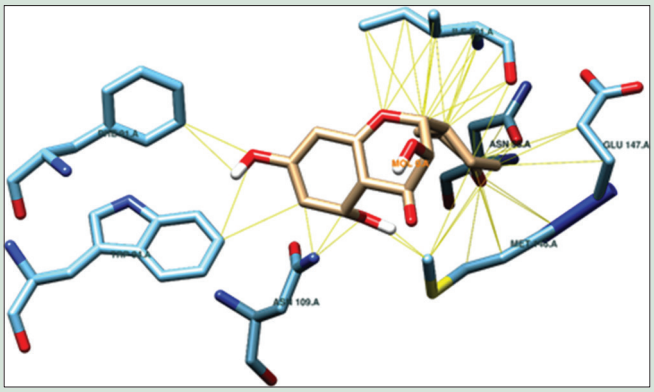

Figure 3: Image showing interaction of kaempferol with the binding site residues PHE91, TRP94, ASN109, MET148, GLU147, ASN36, and ILE201

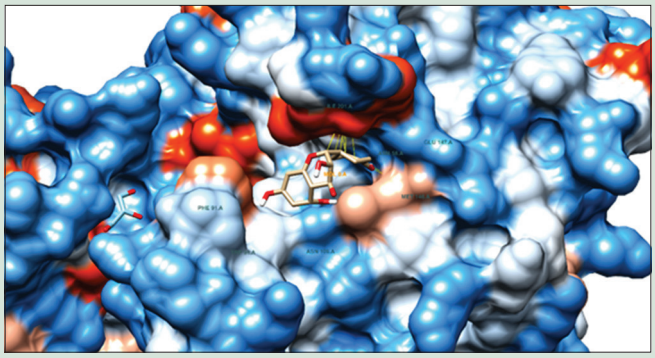

Figure 4: Image showing hydrophobic interaction of kaempferol with β-lactamase

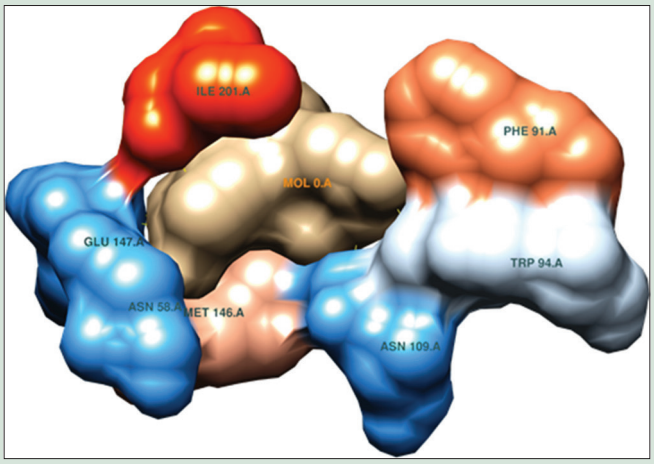

Figure 5: Image showing hydrophobic interaction of kaempferol with MEG binding site residue of β-lactamase

compound is detailed in Table 5. Results showed that MBI (IC_{50}: 63 μg/ml) possessed the highest inhibitory activity as compared to aspirin (IC_{50}: 100 μg/ml), whereas kaempferol (IC_{50}: 127 μg/ml) had a slightly lower inhibitory effect. The proteinase inhibitory effect (based on IC_{50} values) of CSF (IC_{50}: 111 μg/ml), CSF8 (IC_{50}: 121 μg/ml), and CSF8-c (IC_{50}: 123 μg/ml) was comparatively lower ($P < 0.05$) than the therapeutic drug aspirin ($IC_{50:}$ 100 μg/ml).

The present study provides a strong basis for the understanding of anti-inflammatory potential of MBI and kaempferol. The possible mechanism of action could be via the inhibition of release of lysosomal contents by the neutrophils at the site of inflammation. The major component of lysosomal content includes proteases and bactericidal enzymes and their release augments to the inflammatory site by enhancing the damage. In several studies, the high TPC has been indicative of anti-inflammatory potential via the inhibition of proteinase activity.[25,47] Consequently, our findings are in agreement with the previous reports, suggesting a strong association of TPC with anti-inflammatory potential of the MBI and kaempferol.

High-resolution liquid chromatography and mass spectrometry profile study for the active principles in methanol extract

To characterize the bioactive components responsible for pharmacological activities, MBI was subjected to HR-LCMS analysis. The HR-LCMS chromatogram (+ESI and − ESI) revealed the presence of diverse bioactive compounds with the highest concentrations. The active principles with their molecular formula are presented in Tables 6 and 7. The HR-LCMS analysis revealed the presence of 3-methylsuberic acid, Galbeta1-4GlcNAcbeta-Sp, undecanedioic acid, deoxyloganin tetraacetate, tridecanal, kaempferol, rutin, and several bioactive principles. Earlier studies have demonstrated that plant extracts are rich in phenolics, terpenoids, and flavonoids which have a strong correlation with increased biological and pharmacological activities. Gohari *et al.*[48] suggested antibacterial modulating activity of undecanedioic acid, whereas tridecanal is used as a food additive with potential antioxidant ability. Similarly, kaempferol, first isolated from saffron, is known for its potential in reducing risks of cancer whereas rutin has been used to strengthen blood vessels as well as to prevent stroke.[49] Therefore, the observed antibacterial and anti-inflammatory effects observed in our study could be a synergistic effect of these compounds working in tandem.

Molecular modeling

As shown in Figures 2-5, kaempferol was found to bind β-lactamase at MEG binding site and was found to interact with binding site residues PHE91, TRP94, ASN109, MET148, GLU147, ASN36, and ILE201. Kaempferol was found to have good affinity toward MEG binding site

Table 5: Albumin denaturation, membrane protection/stabilization and proteinase inhibition potential of methanol extract of *Blepharis integrifolia*, diverse chloroform-soluble fractions, and isolated compound kaempferol

Anti-inflammatory	$IC_{50}^{x,y}$ (μg/ml)					Aspirin#
	MBI	CSF	CSF8	CSF8-c	Kf	
Albumin denaturation	44.44±0.80[a]	65.22±0.18[b]	92.08±2.02[d]	94.71±0.51[e]	96.90±2.18[f]	80.11±0.11[c]
Membrane protection	86.05±1.24[a]	97.78±0.75[c]	117.18±1.29[d]	119.60±0.42[e]	123.18±0.11[f]	90.80±0.40[b]
Proteinase inhibition	63.19±0.27[a]	111.32±0.22[c]	121.38±1.82[d]	123.42±0.14[e]	127.59±0.15[f]	100.42±0.17[b]

[x]Values are expressed as mean±SE. Means in the same row with distinct superscripts are significantly different ($P \leq 0.05$) as separated by Duncan multiple range test; [y]The IC_{50} value is defined as the inhibitor concentration to inhibit 50% under assay conditions; #Aspirin was used as positive control. MBI: Methanol extract of *Blepharis integrifolia*; CSF: Chloroform-soluble fraction; Kf: Kaempferol; SE: Standard error; IC_{50}: Half maximal inhibitory concentration

Table 6: Chemical profile of the methanol extract of *Blepharis integrifolia* by high-resolution liquid chromatography and mass spectrometry in + electron spray ionization mode

Compound detected	Molecular formula	DB difference (ppm)
+ESI mode		
Caffeoylputrescine	$C_{13}H_{18}N_2O_3$	8.57
Tridecanal	$C_{13}H_{26}O$	16.98
3-n-decyl acrylic acid	$C_{13}H_{26}O_2$	15.74
p-Acetamidophenyl glucuronide	$C_{14}H_{17}NO_8$	7.07
Kaempferol	$C_{15}H_{10}O_6$	5.53
3"-HydroxyPravastatin	$C_{23}H_{36}O_8$	11.74
Prostaglandin I3	$C_{20}H_{30}O_5$	12.05
Thromboxane A2	$C_{20}H_{32}O_5$	12.95
9S,11R,15S-trihydroxy-2,3- dinor-13E-prostaenoic acid cyclo [8S,12R]	$C_{18}H_{32}O_5$	6.34
1alpha, 25-dihydroxy26,26,26,27,27,27-hexafluoro16,17,23,23,24,24- hexadehydro-19-norvitamin D3/1a	$C_{26}H_{32}F_6O_3$	15.69
12-oxo-9-octadecynoic acid	$C_{18}H_{30}O_3$	6.0
2E,6E,8E,10Edodecatetraenoic acid	$C_{12}H_{16}O_2$	5.63
12-oxo-9-octadecynoic acid	$C_{18}H_{30}O_3$	6.01
GPEtn (18:1 (11Z)/18:1 (9Z))[U]	$C_{41}H_{78}NO_8P$	12.66
N-Acetylsphingosine	$C_{20}H_{39}NO_3$	5.64
12beta-Hydroxy-3-oxo-5betacholan-24-oic Acid	$C_{24}H_{38}O_4$	6.74
Methyl 9,10-epoxy-12,15- octadecadienoate	$C_{19}H_{32}O_3$	4.45
Oleamide	$C_{18}H_{35}NO$	4.33
Stearamide	$C_{18}H_{37}NO$	4.61
12beta-Hydroxy-3-oxo-5betacholan-24-oic Acid	$C_{28}H_{38}O_4$	6.74

Compounds were identified by referring to the METLIN database from SAIF, IIT Bombay. ESI: Electron spray ionization; SAIF: Sophisticated Analytical Instrument Facility

Table 7: Chemical profile of the methanol extract of *Blepharis integrifolia* by high-resolution liquid chromatography and mass spectrometry in –electron spray ionization mode

Compound detected	Molecular formula	DB difference (ppm)
–ESI mode		
Phenylacetylglycine methyl ester	$C_{11}H_{13}NO_3$	24.47
Tuberonic acid	$C_{12}H_{18}O_4$	24.26
Bromopride	$C_{14}H_{22}BrN_3O_2$	15.64
3-Methylsuberic acid	$C_9H_{16}O_4$	24.94
Rutin	$C_{27}H_{30}O_{16}$	13.78
Galbeta1-4GlcNAcbeta-Sp	$C_{16}H_{28}N_4O_{11}$	24.86
Undecanedioic acid	$C_{11}H_{20}O_4$	24.07
9,12,13-trihydroxy-10,15- octadecadienoic acid	$C_{18}H_{32}O_5$	18.14
13S-hydroxy-9E,11Zoctadecadienoic acid	$C_{18}H_{32}O_3$	20.98
Deoxyloganin tetraacetate	$C_{25}H_{34}O_{13}$	23.67
Isoacitretin	$C_{21}H_{26}O_3$	8.65
Embelin	$C_{17}H_{26}O_4$	8.96
Prostaglandin E2 Ethanolamide	$C_{22}H_{37}NO_5$	18.52
2-Hydroxyethinylestradiol	$C_{20}H_{24}O_3$	8.12
9,13-dihydroxy-11- octadecenoic acid	$C_{18}H_{34}O_4$	20.46
Tetranor Iloprost	$C_{18}H_{26}O_4$	20.20
8,13-dihydroxy-9,11- octadecadienoic acid	$C_{18}H_{32}O_4$	20.02
Gemfibrozil M1	$C_{15}H_{22}O_4$	8.08

Compounds were identified by referring to the METLIN database from SAIF, IIT Bombay. ESI: Electron spray ionization; SAIF: Sophisticated Analytical Instrument Facility

with binding energy of – 4.9 Kcal. Kaempferol acts as a competitive inhibitor of β-lactamase by binding to MEG binding site. Kaempferol prevents MEG a natural substrate of β-lactamase, thereby inhibiting the action of β-lactamase enzyme. Inhibition of β-lactamase limits the organism ability to degrade penicillin class of antibiotics. Hence, antibiotics fortified with kaempferol can aid in treating drug resistant micro-organisms.

CONCLUSION

The results from the present study suggest that there was a strong correlation between the TPC and their antioxidant effects. The presence of high amount of phenolics, flavonoids and proanthocyanidins in the MBI proved to possess remarkable antibacterial and anti-inflammatory potential as well. The activity-guided isolation of the bioactive compound kaempferol suggested its role in the observed therapeutic effects. The docking studies also provide a strong basis for the use of this isolated compound as a potent antibacterial agent with β-lactamase inhibition. Further studies are required to establish the effectiveness of the extract and the isolated compound through additional toxicity and other potential pharmacological properties.

Acknowledgements

All the authors thank the chancellor, JSS AHER, Mysuru, for his blessings and encouragement. We are also grateful to SAIF – IIT Bombay, for providing HR-LCMS facility.

Financial support and sponsorship

This research was supported by a Senior Research Fellowship (45/5/2018-Nan/BMS) provided to Anil Kumar from ICMR India.

Conflicts of interest

There are no conflicts of interest.

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