Journal of Scientific & Industrial Research
Vol. 84, August 2025, pp. 879-889
DOI: 10.56042/jsir.v84i8.13776

Integrated Metabolomics and Network Pharmacology to Decipher the Antioxidant and Anti-inflammatory Potential of *Gymnostachyum febrifugum* (Benth) and an *In Vitro* Validation

Spandana K J[1], Wilson Joel Rodrigues[2], Chandrashekar K R[3] & Bhagya N[1]*

[1]Yenepoya Research Centre, Yenepoya Deemed to be University, Mangalore 575 018, Karnataka, India

[2]Department of Biochemistry, JSS Academy of Higher Education and Research, Shivarathreeshwara Nagara, Mysuru 570 015, Karnataka, India

[3]Yenepoya Pharmacy and Ayush Research Centre (YEN PARC), Yenepoya (Deemed to be University), Deralakatte, Mangalore, Karnataka 575 018, India

Received 16 September 2024; revised 10 June 2025; accepted 01 July 2025

Gymnostachyum febrifugum is an endemic species of the Western Ghats of India and is well-mentioned in ethnomedicine to treat fever, ulcers, cough, and metrorrhagia. The present study focuses on the GC-MS/MS analysis to identify the phytochemicals with antioxidant and anti-inflammatory properties followed by integrated metabolomics and network pharmacology approaches to determine the molecular targets of antioxidant and anti-inflammatory potential of the phytochemicals in *G. febrifugum*. Further, the antioxidant and anti-inflammatory activities were validated using *in vitro* experimental methods. A total of 10 antioxidant and anti-inflammatory metabolites were identified by GC-MS/MS analysis. Based on the network pharmacology, ALB, HSP90AA1, PPARG, SOD2, and CASP3 as antioxidant protein targets, and ALB, CD4, MMP9, HMOX1, and EGFR as anti-inflammatory protein targets were identified. Molecular docking also confirmed binding affinities of the detected phytochemicals with these protein targets. The *in vitro* studies validated the *in-silico* results on the antioxidant and anti-inflammatory activities by the *G. febrifugum*. The *in vitro* results showed the potent antioxidant activity of ethyl acetate extract of *G. febrifugum* with SC_{50} values for 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS), and hydrogen peroxide scavenging assay values of 0.17 ± 0.0 mg/mL, 0.23 ± 0.03 mg/mL and 0.110 ± 0.03 mg/mL respectively. The Ferric-Reducing Ability of Plasma (FRAP) assay showed an FRAP value of 0.2108 ± 0.01 mg GAE/mL. The antioxidant activity of gallic acid with SC_{50} for DPPH and ABTS was 0.0028 ± 0.10 and 0.004 ± 0.7, respectively. Similarly, the anti-inflammatory potential was confirmed with the inhibition of heat-induced protein denaturation and proteinase enzyme activity with IC_{50} values for 0.0410 ± 0.003 mg/mL and 0.0721 ± 0.073 mg/mL, respectively. Therefore, the study concludes that integrating *in silico* analysis with *in vitro* results validates the antioxidant and anti-inflammatory potential of *G. febrifugum*. This will further confirm the ethnomedicinal claim of *G. febrifugum* to treat fever, ulcer, cough, and snake bites. Further work on the isolation of individual phytochemicals with antioxidant and anti-inflammatory activities will be beneficial to develop suitable health-benefiting antioxidant and anti-inflammatory agents.

Keywords: Alkaloids, Endemic plant, Molecular docking, Phenolics, Terpenes

Introduction

Gymnostachyum febrifugum (Benth), of the Acanthaceae family, is a stemless, tiny scapigerous herb with woody rootstock, long petiolated ovate leaves, bearing an attractive flower, which makes it a potential wild ornamental plant.[1,2] The plant is endemic to India and is distributed in the South Western Ghats region of India. The root decoction of *G. febrifugum* is used as a febrifuge, for indigestion, headache, metrorrhagia, ulcers, cough, and as an antidote for viper bites by the folklore practitioners of Karnataka and Kerala states of India.[2,3] Scientific reports have proven that plants are rich in phenolics, flavonoids, tannins, and steroids.[2–9] Besides, the pharmacognostic properties of the root ensure the quality, safety, and efficacy of *G. febrifugum* to be considered in therapeutics.[3] Additionally, hexane, dichloromethane, ethyl acetate, methanol, water extract of root, shoot, and whole plant ethanolic extract of *G. febrifugum* are reported with good antioxidant[5–8], anti-inflammatory and cytotoxic[4,8,9] anti-microbial[6] and hepatoprotective[5] properties. Mathew *et al*.[5] reported the presence of 2-(4-Methyl-5-thiazolyl) ethyl decanoate, licorice-saponin A3, avocadynofuran from the ethanolic extracts of the

*Author for Correspondence
E-mail: bhagya163@gmail.com

whole plant by HR-LC/MS. Similarly, Silpa[7] detected imexon, difenoxin, genistin, luteolin, stachyose, and ursocholic acid from methanolic and aqueous extracts of shoot and root of *G. febrifugum* by LC-MS/MS method. Studies also report on the presence of esculin (flavonoid), piperlongumine (alkaloid), ardisiacrispin B (triterpenoid saponin) and berbamine (bisbenzylisoquinoline alkaloid) from methanol[8] and lupeol a high abundant phytochemical from ethyl acetate[9] extracts of *G. febrifugum* roots. Other related species of *Gymnostachyum* showed the presence of hexadecanoic acid, *n*-nonane, *n*-octadecane, sesquiterpene hydrocarbons, phenyl derivatives, and oxygenated monoterpene by the GC-MS method.[10] However, no reports are available on the anti-inflammatory potential of this herb and the possible mode of action of metabolites in regulating the signaling mechanism that is involved in antioxidant and anti-inflammatory pathways. It is difficult to perform all the experiments collectively to identify the major protein targets for the metabolites. Hence, a recent bioinformatics-based approach of network pharmacology, a multidisciplinary field of research discovered by Hopkins in 2007 is useful in studying the interaction of drugs to multiple protein targets or diseases in a short time, acting as a drug screening tool.[11] The network analysis of biological systems, and selection of specific nodes for multi-targeted drugs, emphasizes the success rate of new drugs in clinical trials with increased therapeutics and reduced side effects.[11] Several plant metabolites have been evaluated for their activity against SARS-CoV-2 S proteins, viral infections, and cancer.[11–14] Tetrandrine (*Cyclea peltata*), epigallocatechin gallate (*Camellia sinensis*), kobophenol A (*Glycyrrhiza glabra*), myricetin (*Ampelopsis japonica*) are the alkaloids, phenolics, flavonoids or terpenoids that are experimentally validated using structure-based approaches.[11–14]

Plant secondary metabolites such as alkaloids, phenolics, and terpenes are low molecular weight compounds[15] having their role in cellular metabolic processes, to prevent or cure diseases by acting as an antioxidant and/or anti-inflammatory agent. Recent scientific studies focus on the health benefits of naturally derived compounds in the prevention of diabetes, obesity, cancer, and cardiovascular diseases due to their increased efficacy with minimal side effects. The free radicals (ROS/RNS) formed as by-products of cellular metabolism under normal physiological conditions or in response to pathological conditions need to be maintained in equilibrium by the redox homeostasis process.[15] However, the overproduction of reactive oxygen species will generate oxidative stress inside the cells, leading to chronic inflammatory diseases. The plant metabolites such as phenolics (e.g. caffeic acid, coumaric acid, gallic acid, ellagic acid), flavonoids (e.g. quercetin, kaempferol, luteolin, epicatechin, and resveratrol) and alkaloids (e.g. piperidine, isoquinoline, tropane and pyrrolidine) are known to scavenge free radicals by donating their hydrogen atoms or electrons.[16] The dietary composition including these molecules has shown slowed progression of many oxidative stress-related diseases by improving the lipid profile, reducing the generation of ROS and improving the antioxidant capacity of the body in *in vivo* studies as well as clinical research.[15] Furthermore, the phytochemicals will reduce inflammation by inhibiting prostaglandin production, nuclear factor kappa B (NF- *k*B) activity, and increased cytokine production, thus acting as good antioxidant/anti-inflammatory agents.[16,17] Therefore, in the present study, an attempt is made to unravel the anti-inflammatory and anti-oxidant metabolites in *G. febrifugum* roots. An attempt is also made to understand the molecular targets for metabolites using network pharmacology and molecular docking. Further, the study involves the experimental validation of antioxidant and anti-inflammatory activities of *G. febrifugum* using *in vitro* methods. The overall workflow of the study is given in Fig. 1.

Materials and Methods

Chemicals and Reagents

The 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS), 2,4,6-tripyridyl-S-triazine (TPTZ), potassium persulfate, ferric chloride ($FeCl_3$), Bovine Serum Albumin (BSA) were purchased from SRL (India). The organic solvents used for extraction were of analytical grade purchased from SRL (India). Hydrogen peroxide, tris base was purchased from Hi Media (Mumbai, India). All the plastic and glassware were purchased from Tarsons and Borosil India respectively.

Plant Material, Extraction, and Identification of Metabolites

G. febrifugum roots were extracted by following the protocol described by Spandana *et al.*[8] Briefly,

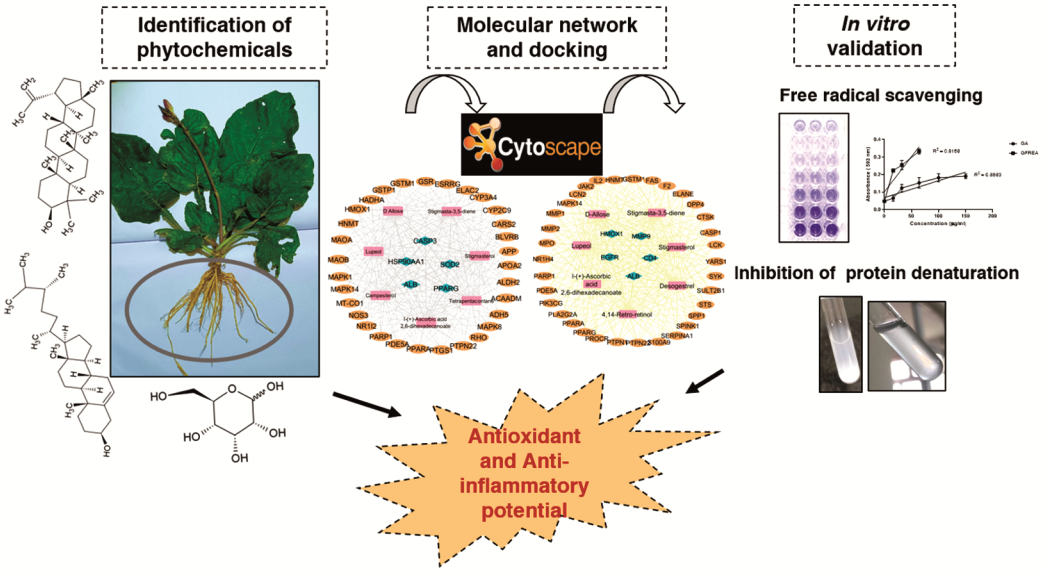

Fig. 1 — Workflow showing the identification of antioxidant and anti-inflammatory phytochemicals from *G. febrifugum*, understanding the mechanism of action by network pharmacology and molecular docking followed by experimental validation of antioxidant and anti-inflammatory activities

G. febrifugum plants were collected from the Aivaranadu village (12° 39' 59.99" N and 75° 21' 59.99" E) of Sullia taluk, Karnataka, India and authenticated using the Flora of Madras Presidency.[18] A voucher specimen (MU/AB/BN/05) is deposited at the Department of Applied Botany, Mangalore University, India. The roots were separated from the aerial parts of the plants, washed, shade-dried, and powdered using a lab blender. Later, 25 g of the powdered sample was extracted in Soxhlet using ethyl acetate. The extract was lyophilized, weighed, and stored at 4ºC until further use. The metabolites present in *G. febrifugum* were identified using GC-MS/MS (QP2020, Shimadzu, Japan). Briefly, 0.5 µL of ethyl acetate extract dissolved in DMSO was injected into SH Rxi 5Sil MS column (30 m × 0.25 mm ID × 0.25 µm) with helium (99.999) as the carrier gas and temperature varying from 70°C for 2 min, then increased to 180°C for 2 min and finally held at 290°C for 10 min. The chromatogram was analysed using GC solutions and the National Institute Standard and Technology 17 (NIST 17) library and PubChem database were used to identify the metabolites.

Network Pharmacology and Molecular Docking

The metabolites identified in *G. febrifugum* roots in the current study were listed for the reported antioxidant and anti-inflammatory potential based on the already published reports. The protein targets of antioxidant and anti-inflammatory metabolites were later predicted using PharmMapper[19], Drug Bank[20], and already published articles.[21–23] The genes linked with antioxidant and anti-inflammatory activities were obtained from GeneCards[24], DisGeNET[25] (relevance score > 0.1), Online Mendelian Inheritance in Man (OMIM)[26] databases using the keywords 'oxidative' and 'inflammatory'. A Venn diagram (using the Venny 2.1 tool) was used to determine the common protein targets of metabolites and diseases.[27] Following the intersection, protein-protein interaction (combined score > 0.4) was established using STRING[28], and the component-target network was visualized using Cytoscape software (version v3.10.1) to identify the targets. For molecular docking, the 3D structure of active metabolites was retrieved from PubChem[29] and saved as PDB files using Open Babel software.[30] Similarly, the structures of target proteins were downloaded from the PDB database[31], and water molecules were removed and hydrogenated using Discovery Studio (BIOVIA Discovery Studio 2021). The PyRx software (v.0.9.2) and BIOVIA discovery studio were used to explore and visualize the binding potential of metabolites with target proteins respectively.

***In Vitro* Validation of Antioxidant Activities**

The antioxidant capacity of the extract was assessed by four different assays such as 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis-3-

ethylbenzothiazoline-6-sulphonic acid (ABTS), ferric reducing ability of plasma (FRAP) assay, hydrogen peroxide (H_2O_2) radical scavenging assays.

2, 2-diphenyl-1-picrylhydrazyl Radical (DPPH) Assay

The assay was carried out following Xiao *et al.*[32] Briefly, 1 mL of methanolic DPPH solution (0.4 mM) was added to the tubes containing 1 mL of varying concentrations of extract (10–1000 µg/mL) and standard gallic acid (1–10 µg/mL) separately. Following 30 min of incubation in the dark at room temperature, the reduction of DPPH was measured at 517 nm. Methanolic DDPH solution was taken as the blank. The percentage radical scavenging activity of the extract and standard was calculated using the following formula, and the concentration required to scavenge 50% (SC_{50}) of free radicals was calculated from the calibration curve by linear regression analysis using Microsoft Excel.

$$Radical\ scavenging\ activity\ (\%) = [\frac{Ac-As}{Ac}]\times 100 \quad \ldots (1)$$

where, Ac is the absorbance of the control, As is the absorbance of the sample or standard gallic acid.

2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic Acid (ABTS) Assay

The ABTS reaction mixture was prepared by mixing 1 mL of ABTS in acetic acid buffer (pH 4.5), 5 mL of potassium persulfate in acetic acid buffer (pH 4.5) and incubated for 12–16 hrs in dark Xiao *et al*.[32] Further, 2.80 mL of ABTS reaction solution was diluted to 65 mL in acetic acid buffer (pH 4.5) to obtain the ABTS working solution and kept at room temperature for 30 min in the dark. The assay was carried out by taking 200 µL of ABTS working solution and 10 µL of varying concentrations of extract (500–2000 µg/mL) and standard gallic acid (2–150 µg/mL). The reaction mixture was mixed well and incubated in the dark for 7 min, and the absorbance was measured at 734 nm against distilled water as a blank.

Ferric-Reducing Ability of Plasma (FRAP) Assay

The assay was carried out as described by Xiao *et al*.[32] To 100 µL of FRAP working solution in a 96 well plate, acetic acid buffer, TPTZ solution, $FeCl_3$ (10:1:1), and 10 µL of extract (500–2000 µg/mL) or standard gallic acid (2–200 µg/mL) was added. The reaction mixture was incubated at 37°C for 15 minutes in the dark. Distilled water served as a blank. The absorbance was measured at 593 nm. The results were expressed in terms of gallic acid equivalent antioxidant capacity in mg GAE/mL by plotting a standard curve of gallic acid.

Hydrogen Peroxide (H_2O_2) Radical Scavenging Assay

The H_2O_2 radical scavenging assay was carried out according to Sroka and Cisowski.[33] Three millilitres of 50 mM phosphate buffer solution (pH 7.4) containing 2 mM hydrogen peroxide was mixed with 1 mL of extract (50–100 µg/mL) or standard gallic acid (3.125–100 µg/mL) and vortexed. After 10 min of incubation, the absorbance was measured at 230 nm. Phosphate buffer without hydrogen peroxide served as a blank, and gallic acid as a positive control. The ability to scavenge hydrogen peroxide was calculated using the following equation:

$$Hydrogen\ peroxide\ scavenged\ (\%) = \left[\frac{Ac-As}{Ac}\right]\times 100 \quad \ldots (2)$$

where, Ac and As are the absorbances of the positive control and sample respectively.

***In Vitro* Validation of Anti-inflammatory Activity**

Inhibition of Protein Denaturation

The inhibition of protein denaturation was evaluated as described by Gunathilake *et al.*[34] Briefly, varying concentrations of extract (50–1000 µg/mL) and 1% BSA were mixed, heated at 37°C for 20 min followed by 57°C for 20 min, and allowed to cool. The turbidity formed in the samples was measured at 660 nm. Aspirin served as the positive control, and 1% BSA without samples served as the blank. The percentage inhibition of protein denaturation was calculated using Eq. 3.

$$Inhibition\ (\%) = [\frac{Ac-As}{Ac}] \times 100 \quad \ldots (3)$$

where, Ac and As are the absorbances of the control and sample respectively.

Proteinase Inhibition Assay

The proteinase inhibitory assay was carried out as described by Gunathilake *et al.*[34] by taking 2 mL of 0.06 mg of trypsin, 1 mL of Tris-HCl buffer (20 mM, pH 7.4) and 1 mL of the test sample at different concentrations (2.5–200 µg/mL) and incubated at 37°C for 5 min. Further, 1% BSA was added and incubated for an additional 20 min. To this, 70% of perchloric acid (2 mL) was added to stop the reaction, the cloudy suspension obtained was centrifuged and the absorbance of the suspension was measured at

210 nm. Tris-HCl buffer (20 mM) served as blank and the percentage inhibition was calculated using Eq. 3.

Statistical Analysis

The antioxidant and anti-inflammatory assays were carried out in triplicates and data were represented as mean ± standard deviation (SD).

Results and Discussion

Plant Material, Extraction, and Identification of Metabolites

The use of different purification methods for the phytochemicals from a crude mixture, such as column chromatography and preparative High-Performance Liquid Chromatography (prep-HPLC), followed by spectroscopic methods to characterize the isolated phytochemicals with potent bioactivities, is gaining increased interest in recent days.[35] In the present study, the root samples of *G. febrifugum* extracted in ethyl acetate resulted in a total yield of 4.8% of the dried sample. GC-MS/MS analysis of the *G. febrifugum* ethyl acetate extract showed the presence of various metabolites such as D-allose, l-(+)-ascorbic acid 2,6-dihexadecanoate, tetrapentacontane, campesterol, stigmasterol, lupeol, 4,14-retro-retinol, desogestrel, and stigmasta-3,5-diene (Fig. 2).

Our results agree with the previous studies by Arunachalam[6], Vijayalakshmi[3], Mathew[5] wherein, they reported the presence of various classes of metabolites such as phenolics, alkaloids and terpenes in *G. febrifugum.* The major metabolites from *G. febrifugum* such as 2-(4-methyl-5-thiazolyl) ethyl decanoate, licorice-saponin A3, avocadynofuran were detected from the whole plant ethanolic extract by HR-LC/MS method.[5] Similarly, Silpa detected imexon, difenoxin, genistin, luteolin, stachyose, and ursocholic acid from methanolic and aqueous extracts of shoot and root of *G. febrifugum* by LC-MS/MS method.[7] Spandana *et al*.[8] culin (flavonoid), piperlongumine (alkaloid), ardisiacrispin B (triterpenoid saponin) and berbamine (bisbenzylisoquinoline alkaloid) from methanolic extract of *G. febrifugum* roots by HR-LCMS/MS. The compounds such as hexadecanoic acid, *n*-nonane, *n*-octadecane, sesquiterpene hydrocarbons, phenyl derivatives, and oxygenated monoterpene were detected in the other related species of *Gymnostachyum* by GC-MS method.[9] β-sitosterol, stigmasterol, lupeol from the aerial parts of *Justicia acuminatissima*[36] 1-carboethoxy-1-cyano-1,2-dihydro-[1,2]- cyclopropcholest-3-one a steroid from *J. gendarussa*[37], stigmasterol, and β-sitosterol from *Odontonema callistachyum*[38] are similar metabolites detected in other plant species of Acanthaceae family with reported anti-inflammatory, antioxidant, and antimicrobial potential. In the current study, among the 10 metabolites detected in *G. febrifugum*, D-allose, l-(+)-ascorbic acid 2,6-dihexadecanoate, tetrapentacontane, campesterol, stigmasterol, lupeol, and stigmasta-3,5-diene probably contribute to antioxidant activities as reported.[21–23] Similarly, D-allose, l-(+)-ascorbic acid 2,6-dihexadecanoate, 4,14-retro-retinol, desogestrel, stigmasterol, lupeol and stigmasta-3,5-diene might be contributing to the anti-inflammatory potential.[21–23,29]

Network Pharmacology and Molecular Docking

A total of 548 antioxidant metabolite target proteins, 429 antioxidant genes (Supplementary Table S1), 511 anti-inflammatory metabolite target proteins and 443 anti-inflammatory genes (Supplementary Table S2) were selected in the present study**.** After removing the duplicates from the pooled targets, Venn diagrams showed 36 and 39 common targets for antioxidants (Fig. 3a) and anti-inflammatory (Fig. 3b) pathways respectively. The metabolite-target network diagram obtained using Cytoscape showed 43 nodes and 246 edges for antioxidants (Fig. 3c) and 46 nodes and 273 edges for anti-inflammatory molecules (Fig. 3d), which revealed the synergistic multicomponent and multitargeted effects of *G. febrifugum* contributing to their antioxidant and anti-inflammatory activities. Further identification of hub genes from the Protein-Protein Interaction (PPI) using the STRING database with a combined score of > 0.4 was selected. Based on the maximum neighbourhood component (MNC) algorithm and CytoHubba plugin, the top 5 target proteins of antioxidants such as ALB, HSP90AA1, PPARG, SOD2, and CASP3 were identified (Fig. 3b). These targets were reported to exhibit antioxidant potential by scavenging the reactive free radicals.[40–42] The molecular docking of antioxidant metabolites detected in *G. febrifugum* (D-allose, l-(+)-ascorbic acid 2,6-dihexadecanoate, tetrapentacontane, campesterol, stigmasterol, lupeol, and stigmasta-3,5-diene) against the top 5 targets was conducted to understand their binding capacity. The details on binding energy, type of interaction, and amino acids involved in the binding are given in Table 1. Docking results showed the highest binding affinity of ALB (−9.9 kcal/mol) (Fig. 4a) and SOD2 (−9.5 kcal/mol) (Fig. 4b) to stigmasta-3,5-diene, respectively. Probably, stigmasta-3,5-diene detected in *G. febrifugum* might have regulated the antioxidant

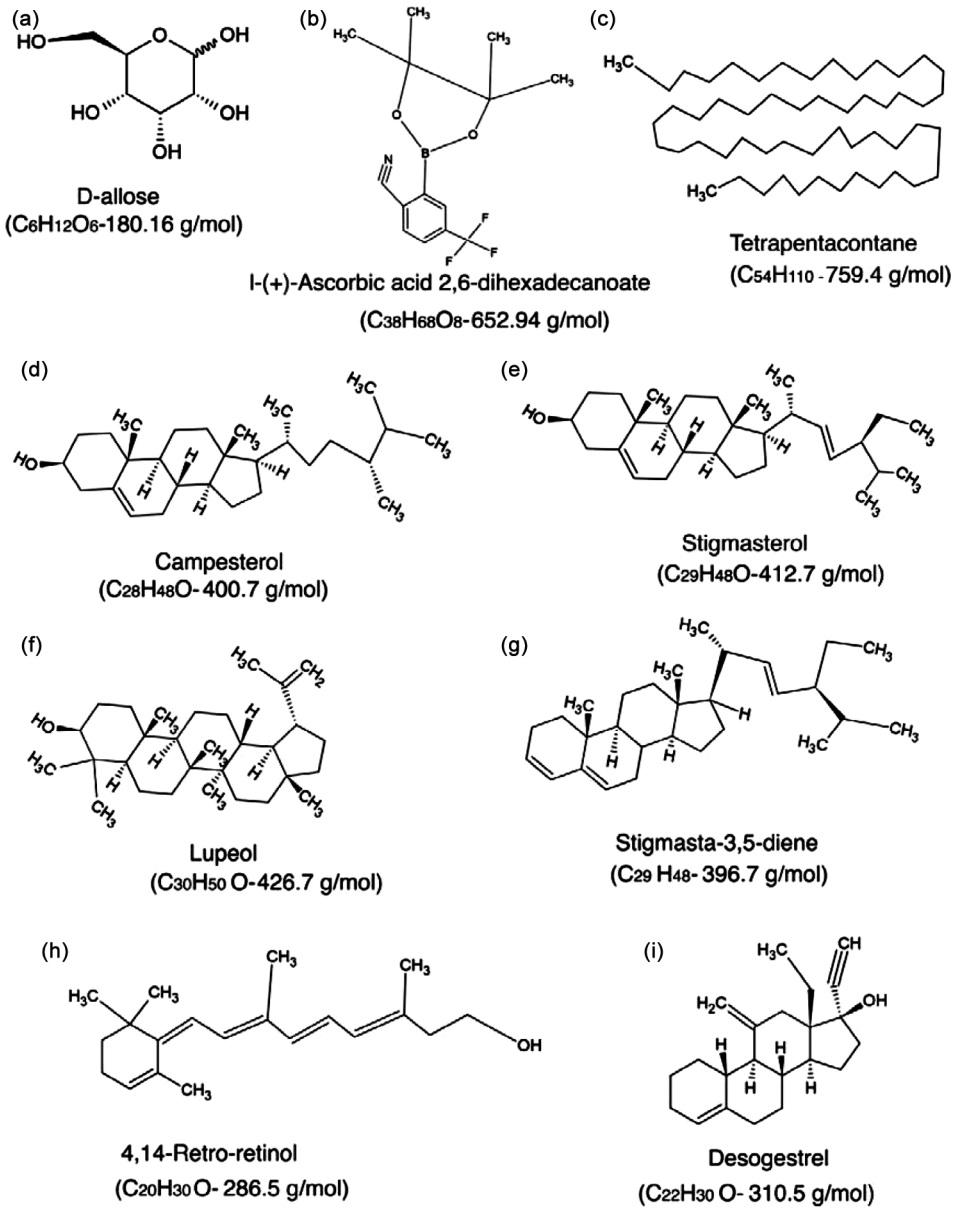

Fig. 2 — Chemical structure, molecular formula and molecular weight of antioxidant and anti-inflammatory metabolites in *G. Febrifugum*: (a) D-allose, (b) l-(+)-ascorbic acid 2,6-dihexadecanoate, (c) tetrapentacontane, (d) campesterol, (e) stigmasterol, (f) lupeol, (g) stigmasta-3,5-diene, (h) 4,14-retro-retinol, (i) desogestrel

mechanism by upregulating the ALB activity, or PPARG-mediated activation of SOD2 as reported by Kim and Yang.[40] Similarly, ALB, CD4, MMP9, HMOX1, and EGFR are the top 5 protein targets obtained from the PPI network analysis of inflammatory conditions (Fig. 3d). All these protein targets are known to actively contribute to inflammatory responses.[42,45]

The details on binding energy, type of interaction, and amino acids obtained after molecular docking of anti-inflammatory metabolites detected in *G. febrifugum* (D-allose, l-(+)-ascorbic acid 2,6-dihexadecanoate, 4,14-retro-retinol, desogestrel, stigmasterol, lupeol, and stigmasta-3,5-diene) against the top 5 target proteins are given in Table 1. Docking results showed the highest binding affinity of ALB to 4,14-retro-retinol (−9.8 kcal/mol) (Fig. 4c) and ALB to stigmasta-3,5-diene (−9.9 kcal/mol) (Fig. 4a). The current study showed that the selected bioactive metabolites can efficiently bind to the receptors and exhibit the anti-inflammatory potential as reported by Eitsuka *et al.*[43]

The stimulation of pro-inflammatory signaling pathways activates and releases leukocytes,

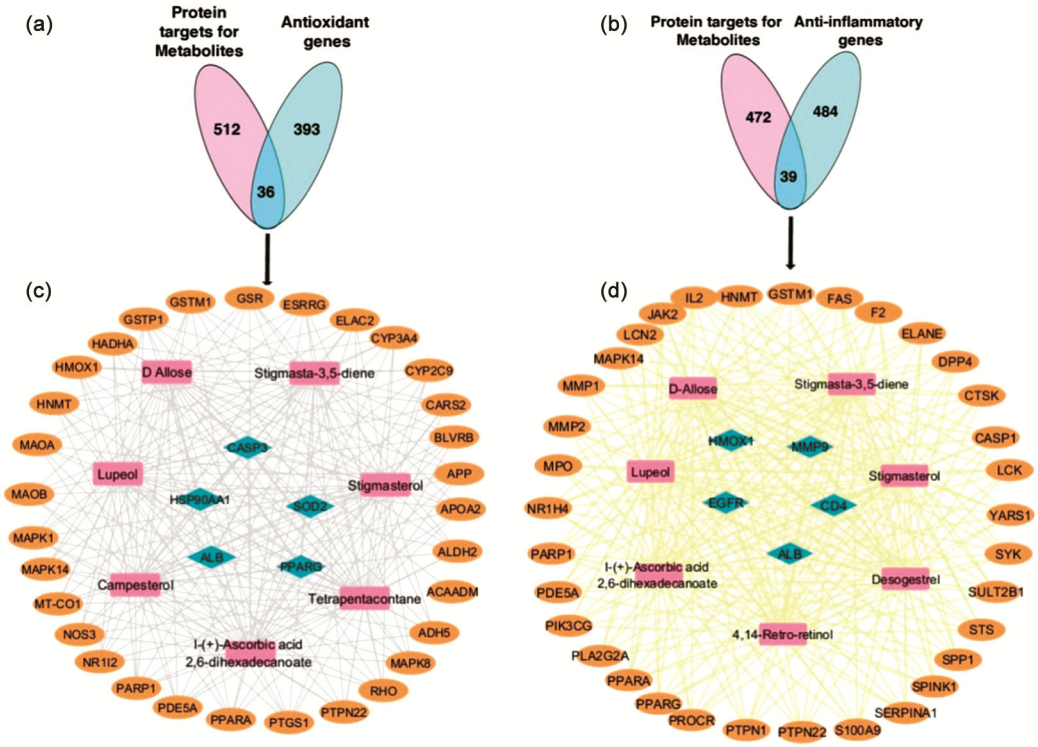

Fig. 3 — Venn diagram showing the protein targets for metabolites and (a) antioxidant genes, (b) anti-inflammatory genes; Protein-protein Interaction (PPI) network analysis between metabolites and common genes of: (c) antioxidant and (d) anti-inflammatory genes [In figures (c) and (d), orange coloured circles represent intersection genes among which blue coloured diamond shapes represent the hub genes and pink coloured squares represent the interacting metabolites]

Table 1 — Molecular docking of metabolites with target protein

Metabolite	Proteins	Biding Affinity (kcal/mol)	Type of interaction	Interacting amino acid residues
Stigmasta-3,5-diene	ALB	−9.9	Van der Waals, Alkyl, Pi-Alkyl	Arg, Met, Pro, Phe, Lys, Ala, Val,Tyr, Leu, Ile, Glu
	SOD2	−9.5	Van der Waals, Alkyl, Pi-Alkyl, Pi-sigma	Glu, Phe, Ala, Gln, Val, Ile, Tyr, His, Trp, Leu, Asn, Lys,
4,14-retro-retinol	ALB	−9.8	Van der Waals, Alkyl, Pi-Alkyl	Lys, Phe, Leu, Pro, Met, Arg, Ile, Tyr

macrophages, and reactive oxygen/nitrogen species that lead to oxidative stress.[37] On the other hand, oxidative stress, in turn, activates NF-*k*B which enhances the pro-inflammatory gene expression. Hence, the anti-inflammatory and antioxidant are linked via two transcription factors NF-*k*B and nuclear factor erythroid-related factor 2 (NRF2), which regulate the cellular response to inflammation and oxidative stress, respectively. The major antioxidant target proteins, such as HSP90 and PPARG, identified in the present study might suppress the NF-*k*B inflammatory response under cellular stress.[45] The studies also proved that oxidative stress-mediated release of NRF2 will bind to the antioxidant response elements (ARE) in the nucleus and increase the transcriptional activation of anti-inflammatory protein - HMOX1.[42,45]

In Vitro Validation of Antioxidant and Anti-inflammatory Potential

In vitro evaluation of the antioxidant and anti-inflammatory activities of ethyl acetate extract validates the results obtained in network pharmacology and docking studies. In the present study, 50% scavenging (SC_{50}) of DPPH and ABTS free radicals by the ethyl acetate extract containing antioxidant metabolites was observed at a concentration of 0.17 ± 0.01 mg/mL and 0.23 ± 0.03 mg/mL, respectively (Table 2). The extract also showed the

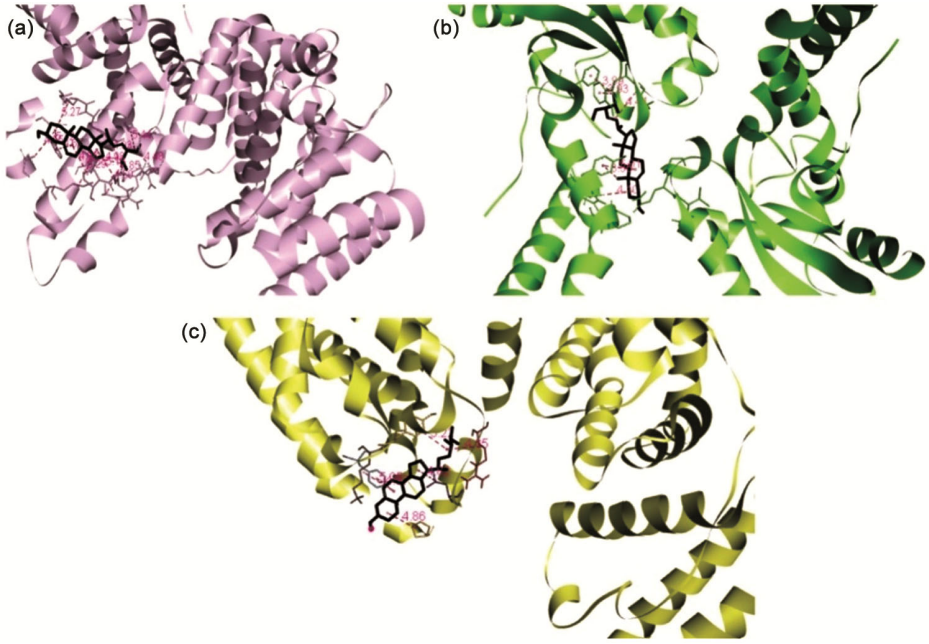

Fig. 4 — Molecular docking of Stigmasta-3,5-diene with (a) ALB and (b) SOD2, (c) 4,14-retro-retinol with ALB

Table 2 — Antioxidant and anti-inflammatory potential of *G. febrifugum*

Pharmacological activities	Assay	Root Ethyl acetate extract (mg/mL)	Standard (mg/mL)
Antioxidant	DPPH (SC_{50})	0.17 ± 0.01	0.0028 ± 0.10 *
	ABTS (SC_{50})	0.23 ± 0.03	0.004 ± 0.7*
	H_2O_2 Scavenging (SC_{50})	0.110 ± 0.03	—
	FRAP Value (mg GAE/ml) $	0.2108 ± 0.01	—
Anti-inflammatory (IC_{50} in mg/mL)	Inhibition of protein denaturation	0.0410 ± 0.003	—
	Protease inhibitory activity	0.0721 ± 0.073	—

Results are shown as mean ± standard deviation of experiments in triplicates; * represents gallic acid; $ represents in terms of gallic acid equivalence per milli litre

capacity to scavenge 50% of H_2O_2 free radicals at a concentration of 0.110 ± 0.03 mg/mL (Table 2). The reducing ability of the antioxidants in the extract was correlated with an increased absorbance with the increasing concentrations of the sample by FRAP assay, and the observed FRAP value was 0.2108 ± 0.01 mg/mL. The dose-dependent antioxidant activity observed in the present study is in accordance with the earlier reports of Mathew *et al*.[5], Silpa[7] and Spandana *et al*.[8] from *G. febrifugum* and Nair *et al*. from *G. warriearanum* leaf.[45] Evidences from epidemiological studies and clinical trials have shown that exogenous supplementation of antioxidants and antioxidant-rich foods slows down or delays the onset and progression of many chronic age-related diseases.[16] Phytochemicals, including gallic acid, hesperidin, catechin, syringic acid, and hydroxybenzoic acid, are the naturally occurring compounds with antioxidant activities reported from citrus species, mushroom varieties, viz, *Cordyceps sinensis*, *Ganoderma lucidium*, and *Cyclocybe cylindracea*.[17] Similarly, allantoin, a natural antioxidant molecule having its presence in plants including *Plantago lanceolate*, *P. major*, *Robinia pseudoacacia*, *Platanus orientalis*, and *Aesculus hippocastanum*, is being used in cosmetic industries, topical pharmaceutical preparations for skin-related diseases.[17] In plants, allantoin is involved in nitrogen metabolism for plant growth and development. However, in animals, serum allantoin or the allantoin/uric acid ratio is elevated in various chronic diseases and suggested as a biomarker for superoxide anion-associated oxidative stress. The presence of keto-carotenoid spheroidenone in callus cultures of *Cylea peltata*

exhibited antioxidant potential[11]. Probably, in the present study, the antioxidant metabolites in *G. febrifugum* roots might have induced the antioxidant potential.

The present study forms the first report on the anti-inflammatory potential of *G. febrifugum*. The metabolites present in *G. febrifugum* were effective in inhibiting the heat-induced albumin denaturation with a 50% inhibitory value of 0.0410 ± 0.36 mg/mL (Table 2). Similarly, the 50% protease inhibitory activity of the extract was observed at a concentration of 0.0721 ± 0.07 mg/mL (Table 2). Probably, the presence of D-allose, l-(+)-ascorbic acid 2,6-dihexadecanoate, 4,14-retro-retinol, desogestrel, stigmasterol, lupeol, and stigmasta-3,5-diene in *G. febrifugum* might have contributed to the anti-inflammatory activities. Similar studies on the role of flavonoids, tannins, phenolic compounds, and phytosterols, either alone or in combination, to exhibit analgesic and anti-inflammatory effects have been reported.[35] The antioxidant and anti-inflammatory activities detected in our study probably support the claims on the ethnomedicinal uses of *G. febrifugum* to treat ulcers, cough, snake bites, headache, and fever.

Conclusions

The current study shows the presence of potent bioactive molecules in *G. febrifugum,* which could be used as potent antioxidants and anti-inflammatory agents. Molecular docking analysis showed a good affinity of stigmasta-3,5-diene metabolites towards ALB and SOD2 antioxidant proteins. Similarly, 4,14-retro-retinol and stigmasta-3,5-diene phytochemicals showed good affinity towards ALB anti-inflammatory proteins. The detected molecular targets are part of NF-*k*B and NRF2 signaling pathways, suggesting the possibility of the phytochemicals having their role in the said pathways and thereby acting as antioxidant and anti-inflammatory agents. Furthermore, all these signaling pathways are triggered in diseases such as fever, ulcer, and cough, as well as in snake bites. Probably, the action of phytochemicals in *G. febrifugum* on the protein molecules of these pathways is responsible for the health benefits which are mentioned in the ethnomedicinal claims. However, further in-depth studies to isolate the individual phytochemicals from *G. febrifugum* and evaluation of the bioactivities will be beneficial to isolate the health-benefiting antioxidant and anti-inflammatory agents. Furthermore, the plant extracts may be used in pharmaceutical and cosmetic industries as a supplement to synthetic antioxidants and anti-inflammatory agents.

Supplementary Matter

Supplementary data of this article is available at https://nopr.niscpr.res.in/handle/123456789/46

Declaration of Interest

The authors declare no conflict of interest.

Acknowledgement

The senior author is grateful to Yenepoya (Deemed to be University) for the financial support in the form of a Junior Research Fellowship (Y/REG/ ACA/JRF/Appointment/2021). All the authors acknowledge Yenepoya (Deemed to be University) for bearing the consumable cost in the form of a Seed grant (No. YU/Seed grant/097-2021). The authors acknowledge Mahatma Gandhi University, Kottayam for providing the GC-MS/MS facility.

References

1 Gopalakrishna Bhat K, Flora of South Kanara, *Taxonomy Research Centre, Department of Botany, Poomaprajna College, Udupi*, 782 (2014).

2 Silpa P & Thomas T D, High-frequency shoot regeneration from flower bud derived callus of *Gymnostachyum febrifugum* Benth., an endemic medicinal plant to the Western Ghats, *Plant cell, Tissue and Organ Cult (PCTOC)*, **147 (2)** (2021) 221–228,DOI: 10.1007/s11240-021-02118-y

3 Vijayalakshmi P B & Haridasan A P, Pharmacognostic and preliminary phytochemical evaluation of Nelamuchchala (*Gymnostachyum febrifugum* Benth.), *Int J Res*, *AYUSH Pharm*, *Res*, **1(1) (**2017) 1–6.

4 Divya N & Mallya S, Pharmacognostic, phytochemical, and high-performance thin-layer chromatography studies of aerial vegetative parts of *Gymnostachyum febrifugum* Benth., *J Res Siddha Med*, **7 (2)** (2024) 85–92, DOI: 10.4103/jrsm.jrsm_16_24

5 Mathew L, Suresh R, & Babu A A, Investigation on the phytochemistry, antioxidant, and hepatoprotective properties of the ethanolic extract of *Gymnostachyum febrifugum* Benth, *Ind J Pharm Edu Res*, **58(2) (**2024) 496–502, DOI: 10.5530/ijper.58.2.55

6 Arunachalam K & Parimelazhagan T, Antioxidant and antimicrobial potential of methanolic extract of Indian sacred grove *Gymnostachyum febrifugum* Benth. root, *Pharma innov*, **3 (2)** (2011) 67–71

7 Silpa P, *In vitro* conservation phytochemical and pharmacological analysis in *Gymnostachyum febrifugum*

Benth. an important medicinal plant endemic to Western Ghats, *Plant Cell, Tissue and Organ Cult* (PCTOC), **147(2)** (2021) 221–228.
8 Spandana K J, Fathimathul Lubaba K P, Sujina A, Rodrigues W J, Chandrashekar K R & Bhagya N, Evaluation of antioxidant, anti-inflammatory and cytotoxic potential of phytochemicals from *Gymnostachyum febrifugum*-an *in vitro* study, *Plant Biosyst*, **158(6)** (2024) 1415–1427, doi: 10.1007/s12010-024-05173-1.
9 Spandana K J, Rodrigues W J, Ghate S D, Rao R S, Chandrashekar K R & Bhagya N, *In Vitro* cytotoxic potential and integrated network pharmacology, molecular docking and molecular dynamic approaches to decipher the mechanism of *Gymnostachyum febrifugum* Benth., in the treatment of breast cancer, *Appl Biochem Biotech*, **97(5)** (2025) 2887–2909, doi: 10.1007/s12010-024-05173-1.
10 Joshi R K, Chemical composition of *Gymnostachyum glabrum* flowers, *Chem Nat Compd*, **20 (53)** (2017) 1184–1185, DOI: 10.1007/s10600-017-2234-6
11 Bhagya N, Network pharmacology based investigation on the mechanism of tetrandrine against breast cancer, *Phytomed Plus*, **3 (1)** (2023) 100381, https://doi.org/10.1016/j.phyplu.2022.100381
12 Henss L, Auste A, Schürmann C, Schmidt C, von Rhein C, Mühlebach M D & Schbierle B S, The green tea catechin epigallocatechin gallate inhibits SARS-CoV-2 infection, *J Gen Virol*, **102 (4)** (2021) 001574, doi: 10.1099/jgv.0.001574
13 Yu S, Zhu Y, Xu J, Yao G, Zhang P, Wang M, Zhao Y, Lin G, Chen H, Chen L, Zhang J, Glycyrrhizic acid exerts inhibitory activity against the spike protein of SARS-CoV-2, *Phytomedicine*, **85** (2021) 153364, doi: 10.1016/j.phymed.2020.153364
14 Xiao T, Cui M, Zheng C, Wang M, Sun R, Gao D, Bao J, Ren S, Yang B, Lin J & Li X, Myricetin inhibits SARS-CoV-2 viral replication by targeting mpro and ameliorates pulmonary inflammation, *Front Pharmacol*, **12** (2021) 669642, doi: 10.3389/fphar.2021.669642
15 Rodríguez-Negrete, Elda Victoria, Ángel Morales-González, Eduardo Osiris Madrigal-Santillán, Karina Sánchez-Reyes, Isela Álvarez-González, Eduardo Madrigal-Bujaidar, Carmen Valadez-Vega, German Chamorro-Cevallos, Luis Fernando Garcia-Melo & José A, Morales-González, Phytochemicals and their usefulness in the maintenance of health, *Plants* **13(4)** (2024) 523, doi: 10.3390/plants13040523.
16 Muscolo A, Mariateresa O, Giulio T, & Mariateresa R, Oxidative stress: The role of antioxidant phytochemicals in the prevention and treatment of diseases, *Int J Mol Sci*, **25(6)** (2024) 3264, doi: 10.3390/ijms25063264.
17 Selamoglu Z, Sevindik M, Bal C, Ozaltun B, Sen İ & Pasdaran A, Antioxidant, antimicrobial and DNA protection activities of phenolic content of *Tricholoma virgatum* (Fr.) P. Kumm, *Biointerface Res Appl Chem*, **10(3)** (2020), DOI: 10.33263/BRIAC103.500506
18 Gamble J S, *Flora of the Presidency of Madras*, (West, Newman and Adlard), (1928).
19 https://www.lilab-ecust.cn/pharmmapper/ (Accessed: 26.03. 2024)
20 https://go.drugbank.com/(Accessed: 27.03.2024)
21 Liu K, Zhang X, Xie L, Deng M, Chen H, Song J, Long J, Li X & Luo J, Lupeol and its derivatives as anticancer and anti-inflammatory agents: Molecular mechanisms and therapeutic efficacy, *Pharmacol Res*, **164** (2021) 105373, doi: 10.1016/j.phrs.2020.105373.
22 Gao D, Kawai N & Tamiya T, The anti-inflammatory effects of D-allose contribute to attenuation of cerebral ischemia–reperfusion injury, *Medical Hypotheses*, **76(6)** (2011) 911–913, https://doi.org/10.1016/j.mehy.2011.03.007
23 Natarajan P, Singh S & Balamurugan K, Gas chromatography-mass spectrometry (GC-MS) analysis of bio active compounds presents in *Oeophylla smaragdina*, *Res J Pharm Technol*, **12 (6)** (2019) 2736–2741.
24 https://www.genecards.org/(Accessed: 27.03.2024)
25 https://www.disgenet.org/(Accessed: 27.03.2024)
26 https://www.omim.org/(Accessed: 27.03.2024)
27 https://csbg.cnb.csic.es/BioinfoGP/venny.html (Accessed: 16.04.2024)
28 https://string-db.org/ (Accessed: 16.04.2024)
29 https://pubchem.ncbi.nlm.nih.gov/ (Accessed: 26.03.2024)
30 https://sourceforge.net/projects/openbabel/ (Accessed: 26.03.2024)
31 https://www.rcsb.org/ (Accessed: 18.06.2024)
32 Xiao F, Xu T, Lu B, & Liu R, Guidelines for antioxidant assays for food components, *Food Front*, **1(1)** (2020) 60–69, https://doi.org/10.1002/fft2.10
33 Sroka Z & Cisowski W, Hydrogen peroxide scavenging, antioxidant and anti-radical activity of some phenolic acids, *Food and Chem Toxicol*, **41(6)** (2003) 753–758, doi: 10.1016/s0278-6915(02)00329-0
34 Gunathilake K D P P, Ranaweera K K D S & Rupasinghe H P V, *In Vitro* anti-inflammatory properties of selected green leafy vegetables, *Biomedicines*, **6 (4)** (2018) 107, doi: 10.3390/biomedicines6040107.
35 Altemimi A, Lakhssassi N, Baharlouei A, Watson D G & Lightfoot D A, Phytochemicals: extraction, isolation, and identification of bioactive compounds from plant extracts, *Plants (Basel)*, **6(4)** (2017) 42, doi: 10.3390/plants6040042
36 Corrêa G, Abreu V G C, Martins D A, Takahashi J, Fontoura H, Carmona Cara D, Pilo-Veloso D & Alcantara A D, Anti-inflammatory and antimicrobial activities of steroids and triterpenes isolated from aerial parts of *Justicia acuminatissima* (Acanthaceae), *Int J Pharm Pharm Sci*, **6** (2014) 75–81.
37 Bhagya N, Chandrashekar K R & Kalluraya B, Identification of a rare phytosteroid from *Justicia gendarussa*, *Chem Nat Compd*, **49(5)** (2013) 972–973, DOI: 10.1007/s10600-013-0798-3
38 Pierre L L & Moses M N, Isolation and characterisation of stigmasterol and β-sitosterol from *Odontonema strictum* (acanthaceae), *J Innov Pharm Biol Sci*, **2(1)** (2015) 88–95, DOI: 10.13140/RG.2.1.3689.7365
39 Vidor Morgan L, Petry F, Scatolin M, Oliveira P, Alves B, Zilli GAL, Volfe C R B, Oltramari A R, de Oliveira D, Scapinello J & Müller L G, Investigation of the anti-inflammatory effects of stigmasterol in mice: insight into its mechanism of action, *Behav Pharmacol*, **32** (2021) 640–651, doi: 10.1097/FBP.0000000000000658.
40 Kim T & Yang Q, Peroxisome-proliferator-activated receptors regulate redox signaling in the cardiovascular

system, *World J Cardiol*, **5(6)** (2013) 164–174, doi: 10.4330/wjc.v5.i6.164

41 Corona J C & Duchen M R, PPARγ as a therapeutic target to rescue mitochondrial function in neurological disease, *Free Radic Biol Med*, **100** (2016) 153–163, doi: 10.1016/j.freeradbiomed.2016.06.023.

42 Lin C-C, Hsiao L-D, Cho R-L, & Yang C-M, CO-releasing molecule-2 induces Nrf2/ARE-dependent heme oxygenase-1 expression suppressing TNF-α-induced pulmonary inflammation, *J Clin Med*, **8 (4)** (2019) 436, doi: 10.3390/jcm8040436.

43 Eitsuka T, Tatewaki N, Nishida H, Nakagawa K & Miyazawa T, Synergistic anticancer effect of tocotrienol combined with chemotherapeutic agents or dietary components: a review, *Int J Mol Sci*, **17(10)** (2016) 1605, doi: 10.3390/ijms17101605

44 Shmarina G V, Orlova M D, Ershova E S, Jestkova E M, Martynov A V, Veiko N N, Konkova M S, Dolgikh O A, Filev A D & Kostyuk S V, NRF2 and HMOX1 gene expression against the background of systemic oxidative stress in patients with acute psychosis, *Russ J Genet*, **56(1)** (2020) 96–102, DOI 10.1134/s102279542001010x

45 Nair N G, Muhammed S P & Krishnan M, Free radical scavenging activity of *Gymnostachyum warriearanum* leaves extracts, *Pharma Innov*, **5(9)** (2016) 120–121