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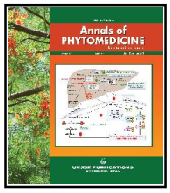

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Evaluation of the phytochemistry of *Gymnostachyum febrifugum* Benth. extract and its hepatoprotective activity using *in vitro* and *in silico* methods

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Abstract

The plant, *Gymnostachyum febrifugum* Benth. (Nelumucchala) of the Acanthaceae family is a herb, significant to traditional medicine practitioners of the South Western India. The dried and powdered plant was subjected to successive Soxhlet extraction using petroleum ether, ethyl acetate, and ethanol. The ethyl acetate extract was chosen for further studies and its antioxidant capacity was evaluated using four different methods; FRAP, DPPH, ABTS, and total antioxidant studies. The extract was further subjected to HR-LCMS, for its chemical characterization. Licoricesaponins A3, AFN911, Austalide A, and Caryoptin were a few of the compounds that were identified in abundance in the extract. These compounds were evaluated for their capacity to bind to various receptors involved in hepatic disorders using the molecular docking approach. *In silico* studies were carried out using the Schrodinger suite. The docking study was done on four receptors PPARα (PDB-2ZNN), PPARγ (PDB-2ATH), 5-LOX (PDB-6N2W), and TGF-β receptor I(PDB-6B8Y). The compound AFN911 exhibited an excellent binding score and stability with all four receptors. Licoricesaponins A3, Austalide A, and Caryoptin exhibited good docking scores and binding stability with the receptor 5-LOX.

1. Introduction

G. febrifugum, is a small scrapigerous, stemless herb, indigenous to the South Western Ghats of India (Silpa and Thomas, 2021). Based on the literature review, the decoction prepared from the root of the plant was used by traditional medicine practitioners, for treating various diseases like purpureal fever, metrorrhagia, and indigestion (Vijayalakshmi and Haridasan, 2017). Despite its significant usage in folklore medicine, the plant has not been extensively studied for its ethnopharmacology. The purpose of the study was to investigate the phytochemistry of the plant, *G. febrifugum* and evaluate its potential in the management of various liver disorders. As per the 2023 updates, 4% of all deaths worldwide are due to liver diseases. The current scenario of liver diseases is alarming and the awareness that, not much effective treatment is still available is shocking. Liver disorders ranging from chronic viral hepatitis, genetic/autoimmune/ drug-induced liver diseases, alcoholic liver diseases (ALD), fatty liver disorders progressing to non-alcoholic fatty liver diseases/ nonalcoholic steatohepatitis (NAFLD/NASH), cirrhosis, hepato-cellular carcinoma (HCC), accounts for 2 million deaths per year worldwide (Devarbhavi *et al*., 2023 ; Bari *et al*., 2022).

The preliminary phytochemical evaluation of the plant was carried out by Vijayalakshmi and Haridasan (2017), and the plant was found to be rich in phenolic compounds. Arunachalam and Parimelazhagan (2011), reported the antioxidant and antimicrobial potential of the methanolic extract of the plant root. Some journals have reported various traditional uses of this plant extract, but no scientific evidence has been produced for its reliability. Hence, the study was oriented towards understanding the phytochemistry of the plant and evaluating its therapeutic potential by *in vitro* and *in silico* methods.

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2. Materials and Methods

2.1 Plant collection and extraction

The fresh, whole plant was collected from its natural habitat, Kakkayam, Kozhikode, Kerala. The plant was identified by Dr. V. B. Sreekumar, Dept. of Botany, KFRI (Kerala Forest Research Institute), Peechi, Thrissur, and a specimen of the authenticated sample (Voucher No. 18126) was deposited in the herbarium. The whole plant samples were rigorously washed under running water to remove soil and other debris sticking to the roots and leaves. The samples were shade-dried for 10 days, powdered, and sieved through a 45-mesh sieve. 2000 g of the powdered plant material was subjected to Soxhlet extraction using 5000 ml of petroleum ether followed by ethyl acetate and ethanol. All the extracts obtained were concentrated at a temperature below 40°C, air dried, and stored at 2-8°C. The qualitative and quantitative preliminary phytochemical evaluation of ethyl acetate extract was found to contain flavonoids, total phenolics, saponins, steroids, and tannins (Hamid *et al*., 2020).

2.2 Antioxidant study

The antioxidant capacity of a plant extract is a preliminary parameter to monitor its therapeutic potency. The capacity of the ethyl acetate extract to scavenge free radicals was evaluated using different *in vitro* assay methods.

2.2.1 By FRAP assay

The ability of the extract to reduce ferric ions to ferrous ions was evaluated by the FRAP method. Concentrations of ascorbic acid at 10 to 50 µg/ml were used as standard and were mixed with FRAP reagent. The extract was diluted to various concentrations in ethanol and mixed with FRAP reagent. All the solution tubes were incubated (30 min, 37°C) in the dark, and absorbances were read at 593 nm. The unknown concentration of the sample was also determined similarly and absorption was extrapolated from the standard calibration graph. The results obtained were reported as AAE/g (ascorbic acid equivalents) (Sudha *et al.*, 2012).

2.2.2 DPPH radical-scavenging activity

A final concentration of DPPH solution (0.1 mM) was prepared by adding 4 ml DPPH to ethanol and volume was made up to 100 ml and mixed well. 40 µl of sample extracts of various concentrations were made up with DMSO, added 2.96 ml of DPPH solution, and mixed well. The final solution was incubated for 20 min in the dark. 3 ml of DPPH was taken as control. The ability of the ethyl acetate extract to scavenge DPPH was evaluated, and the % activity was obtained using the formula:

$$\% \text{ DPPH scavenging activity} = (A_{control} - A_{sample})/A_{control} \times 100,$$

where, $A_{control}$ is the absorbance of the control solution and A_{sample} is the absorbance of the sample solution (Hue *et al*., 2012 ; Baskaran and Subash, 2021**).**

2.2.3 ABTS radical scavenging activity assay

Different concentrations of the extract were prepared in methanol. 2.99 ml of ABTS radical working solution was added to 10 µl of various concentrations of sample solutions and absorbances were taken at 734 nm. A calibration graph was plotted using various concentrations of ascorbic acid prepared similarly. 10 µl of methanol was used as the control solution. The ABTS free radical reducing power of the extract was measured by evaluating the scavenging capacity of the methanolic extract against the free radical, ABTS using the formula:

$$\% \text{ Antioxidant activity} = (\text{Acontrol} - \text{Aspl})/\text{Acontrol} \times 100$$

where, Acontrol is the absorbance of the control, and Aspl is the absorbance of the sample, (Roberta *et al*., 1999).

2.2.4 Determination of total antioxidant capacity assay

The phosphomolybdenum free radical was used to estimate the total antioxidant capacity of the extract. 0.3 ml of the ethyl acetate extract was mixed with 3 ml of phosphomolybdenum reagent solution and a blank solution was prepared using 0.3 ml of solvent. The test tubes were incubated at a temperature of 95°C for 90 min. The absorbances were read at 695 nm, and higher absorbance exhibited higher antioxidant activity. A standard calibration curve for various concentrations of ascorbic acid was plotted and the unknown sample concentration was extrapolated from the graph (Sudha *et al*., 2012).

2.3 Evaluation of phytoconstituents by HR-LCMS

The phytochemical evaluation of the ethyl acetate extract was done using HR-LCMS (Jiji and Muralidharan, 2021; Badraoui *et al*., 2020). Hypersil GOLD C18 column with a dimension of 100 x 2.1 mm-3µ (G1316C) was used under a column temperature of 40°C. Gradient elution technique (Binary pump, G4220B) was used where; ratios of 0.1% formic acid in water and acetonitrile were used as mobile phase, with a flow rate of 0.3 ml. A diode array detector (G4212B) was used to detect the peaks. The mass spectrum was obtained using a TOF/Q-TOF mass spectrometer (MS Q-TOF-G6550A).

2.4 *In silico* studies

The pathophysiology of hepatic disorders is very complex and involves several receptors and genes involved with it. The complexity of this network has made it very difficult to identify a single receptor that could be a potential target in the therapy of hepatic diseases. Hence, four receptors were chosen, which could be potential targets in managing liver diseases (Shareef and Bhavya, 2021).

2.4.1 Peroxisome proliferator-activated receptors (PPARs)

PPARα (PDB-2ZNN) and PPARγ (PDB-2ATH) are two receptors actively involved in the metabolism of fatty acids and carbohydrates. The docking results of ligands were compared with that of the standard, Orlistat, for both receptors (Mandal *et al*., 2022). In the liver, PPARα enhances the β oxidation of fatty acids and the metabolism of sphingolipids. From various studies, it is evident that with the progression of fibrosis, expression of PPARα decreased; hence an induction of these receptors could control fibrosis. The possible mechanism by which PPARα ligands decrease steatosis, inflammation, and fibrosis might be, by counteracting nuclear factor kappa B (NF-κB) and enhancing the fibroblast growth factor 21 (FGF21). Activation of PPARγ is steatogenic but, based on various studies conducted on mice, (genetically obese or NAFLD/NASH induced), the hepatic triacylglycerol (TAG) levels decreased with activation of PPARγ. The enhanced glucose uptake and fatty acid oxidation in hepatocytes by PPARγ reduces liver steatosis by activating AMPK (5' AMP-activated protein kinase) an enzyme responsible for cellular energy homeostasis. PPARγ can regulate the proliferation of hepatic stellate cells (HSC), by the apoptosis of activated HSC and hence, exhibits significant anti-inflammatory and antifibrotic properties (Wang *et al*., 2020). An agonist binding with PPARα and PPARγ could thus be promising in ameliorating liver disorders ranging from fatty liver/steatosis/fibrosis/hepatitis progressing into cirrhosis or hepatocarcinoma.

2.4.2 5-lipoxygenase (5-LOX)

The metalloenzyme, 5-LOX (PDB-6N2W) can activate the biosynthesis of leukotrienes (LTs) from arachidonic acid (AA) which is released during an injury (Khayat *et al*., 2022). Activation of the 5-LOX activating protein (Flap) oxidizes arachidonic acid to the 5-hydroperoxy eicosatetraenoic acid, which is further converted to leukotriene A4 (LTA4) by dehydration. By enzymatic reactions, LTA4 is further converted to LTB4 or LTC4, the inflammatory lipid mediators that can promote the activation of HSC by an extracellular signal-regulated kinase signaling pathway. Activation of HSC promotes liver fibrosis. HSC stays quiescent in the normal liver (Ogawa *et al*., 2006), but an injurious stimulus, can convert the quiescent HSC to an active state (Puche *et al*., 2013), making it proliferative and thus trigger the production of large amounts of collagens and α-smooth muscle actin (α-SMA). This excessive accumulation of extra cellular matrix results in fibrosis and causes scars in the liver. Thus, an antagonistic ligand binding on 5-LOX could inhibit the activation of HSC, preventing inflammation and fibrosis. From the studies on mouse fibrotic models induced with

hepatic fibrosis, it was evident that ablation or inhibition of the 5-LOX enzyme in HSC could reverse hepatic fibrosis and injury. Based on the studies conducted on the liver sections of patients with NASH and hepatic fibrosis, an increased expression of the enzyme 5-LOX was observed (Pu *et al*., 2021). 5-LOX is involved in the production of interleukins (IL), which could potentially increase the inflammation of the hepatic cells which could later develop into the formation of stellate cells. An antagonist action on this receptor could reduce the continuous inflammation and development of fibrosis. Nordihydroguaiaretic acid (NDGA), the co-crystallized ligand was used as the standard, to compare the docking score of ligands with 5-LOX.

2.4.3 Transforming growth factor β receptor type 1 (TGF-βR1)

TGF-βRI (PDB-6B8Y) also known as, ALK5, is a gene that binds to the TGF-β family of cytokine signaling ligands. Linagliptin, the co-crystallized ligand was used as the standard to compare the docking scores (Rahman *et al*., 2022). TGF-β plays an important role in regulating tumor suppression in epithelial cells, but in advanced cancer cells, it can reverse the tumor suppressive role (Ahmed and Magid, 2022). TGF-β is also a major mediator for fibrogenesis (Biernacka *et al*., 2022). TGF-β1 and the receptor gene, TGF-βR1, can promote tumor cell proliferation, its migration, and can cause epithelial-mesenchymal transitions. Mutated cells without the gene TGF-βR1, cannot bind to TGF-β1, resulting in the inhibition of the TGF-β signaling pathway (Wang *et al*., 2021). Hence, TGF-β receptor I (PDB 6B8Y) was also identified as a suitable target to prevent fibrosis and tumor genesis. The inhibitory capacity of ligands was evaluated using *in silico* methods. Inhibition of this receptor in the later stages of hepatic disorders could curb the progression of fibrosis and tumors. Glide docking software was used for the evaluation and calculation of the docking score and Prime MM-GB/SA (Schrodinger suit 2019-4) was used for the calculation of binding energy.

3. Results

3.1 Extraction and preliminary phytochemical evaluation

The Soxhlet extraction using ethyl acetate yielded 4.95%w/w crude extract which was used for further studies. A preliminary phytochemical evaluation of the extract was performed. Total flavonoid content was evaluated using UV spectroscopy and was quantified to be 5.39%. The gravimetric saponin content evaluation yielded 9.17%. The total phenolic content was estimated by the Folin-Ciocalteau reagent and was found to be 1.60%. Steroids and tannin content were quantified to be 1.93% and 0.77%, respectively.

3.2 Antioxidant capacity evaluation reports

The antioxidant capacity of the ethyl acetate extract was evaluated by four different methods using ascorbic acid as standard. The efficiency of the extract to reduce ferric ion (Fe^{3+}) was evaluated and was estimated as 205 µg of AAE (ascorbic acid equivalents) per gram. The IC_{50} values for the DPPH scavenging capacity of the extract and standard were calculated to be 65 µg/ml and 37 µg/ml, respectively. The IC_{50} value for the ABTS radical scavenging activity was quantified to be 67.3 µg/ml for the extract when compared to 38.1 µg/ml of the standard. The total antioxidant activity of the extract was quantified to be 295 µg/g, as obtained from the calibration graph of ascorbic acid.

3.3 Phytochemical evaluation using HR-LCMS

The evaluation of phytochemical constituents of the ethyl acetate extract was carried out using HR-LCMS. Electron spray ionization data was obtained in the positive and negative modes and the spectra and molecular weights obtained were compared with the database available at SAIF, IIT, Mumbai. From the positive mode of ESI (electron spray ionization) data obtained, 59 compounds were detected; out of which 42 compounds were identified by comparing with the available database of mass spectra. The chromatogram is provided in Figure 1. Some of the most abundant compounds identified are listed in Table 1.

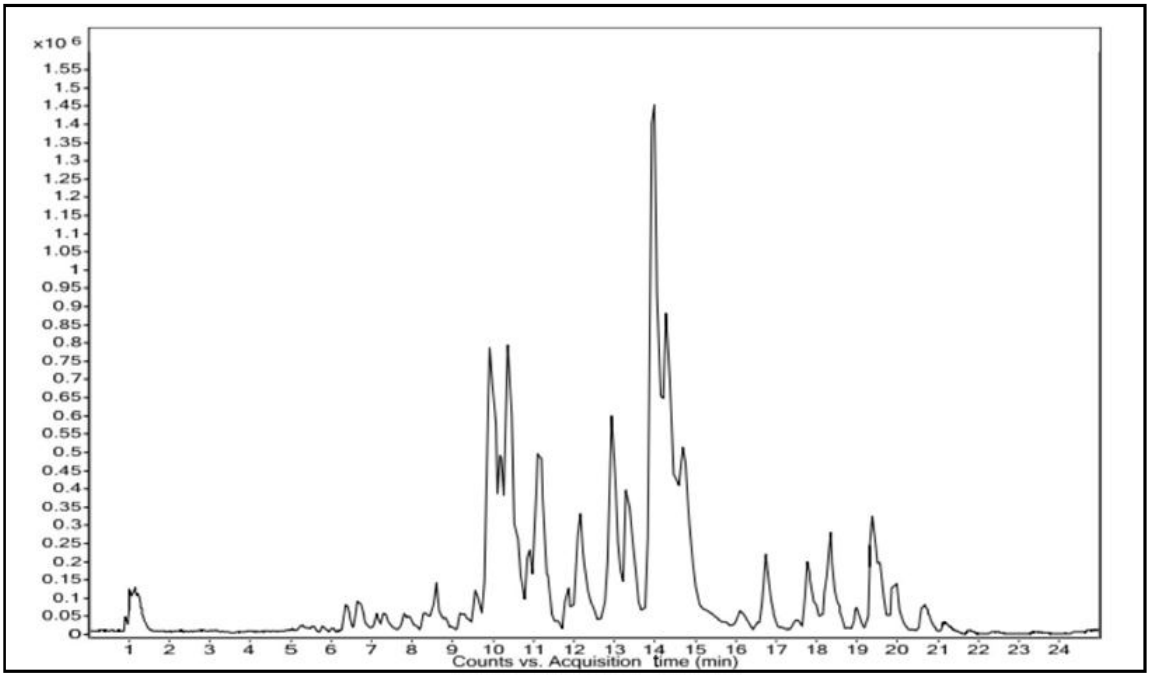


Figure 1: HR-LCMSchromatogram positive ESI.

Table 1: Compounds from ethyl acetate extract of *G. febrifugum* (ESI data in positive mode)

S.No.	Name	Formula	Mass	Mass (DB)	RT	Abund^
1	Austalide A	$C_{28}H_{36}O_9$	516.2299	516.2359	13.855	714599
2	Licoricesaponin A3	$C_{48}H_{72}O_{21}$	984.465	984.4566	9.985	464868
3	Loganin pentaacetate	$C_{27}H_{36}O_{15}$	600.2124	600.2054	10.374	402209
4	Flumethasone pivalate	$C_{27}H_{36}F_2O_6$	494.2457	494.248	10.204	385126
5	AFN911[$]	$C_{29}H_{33}N_7O_2$	511.2723	511.2696	10.206	274216
6	1alpha,3beta,22R-trihydroxyergosta-5,24E-dien-26-oic acid 3-O-b-D-glucoside 26-O-[b-D-glucosyl-(1->2)-6-acetyl-b-D-glucosyl] ester	$C_{48}H_{76}O_{21}$	986.4842	988.4879	10.252	267337
7	28-Glucosyl-30-methyl-3b,23-dihydroxy-12-oleanene -28,30-dioate 3-[arabinosyl-(1->3)-glucuronide]	$C_{48}H_{74}O_{21}$	986.4779	986.4723	10.172	237800
8	Caryoptin	$C_{26}H_{36}O_9$	492.2304	492.2359	10.173	218272
9	Sterebin A	$C_{18}H_{30}O_4$	310.2126	310.2144	10.208	191141
10	Avocadynofuran	$C_{17}H_{26}O$	246.1976	246.1984	10.209	152134
11	6-Hydroxysandoricin	$C_{31}H_{40}O_{12}$	604.2605	604.252	16.888	112784
12	Ganoderic acid F	$C_{32}H_{42}O_9$	570.2802	570.2829	17.973	100404

[$] -N-[4-(hydroxymethyl)-3-{[4-(pyridin-3-yl)pyrimidin-2-yl]amino}cyclohexa-1,5-dien-1-yl]-4-[(4-methylpiperazin-1-yl)methyl]benzamide

^ - Abundance

From the negative mode of ESI data obtained; 38 compounds were detected, out of which 35 compounds were compared with the database and identified. The chromatogram is provided in Figure 2, and the most abundant compounds identified are listed in Table 2.

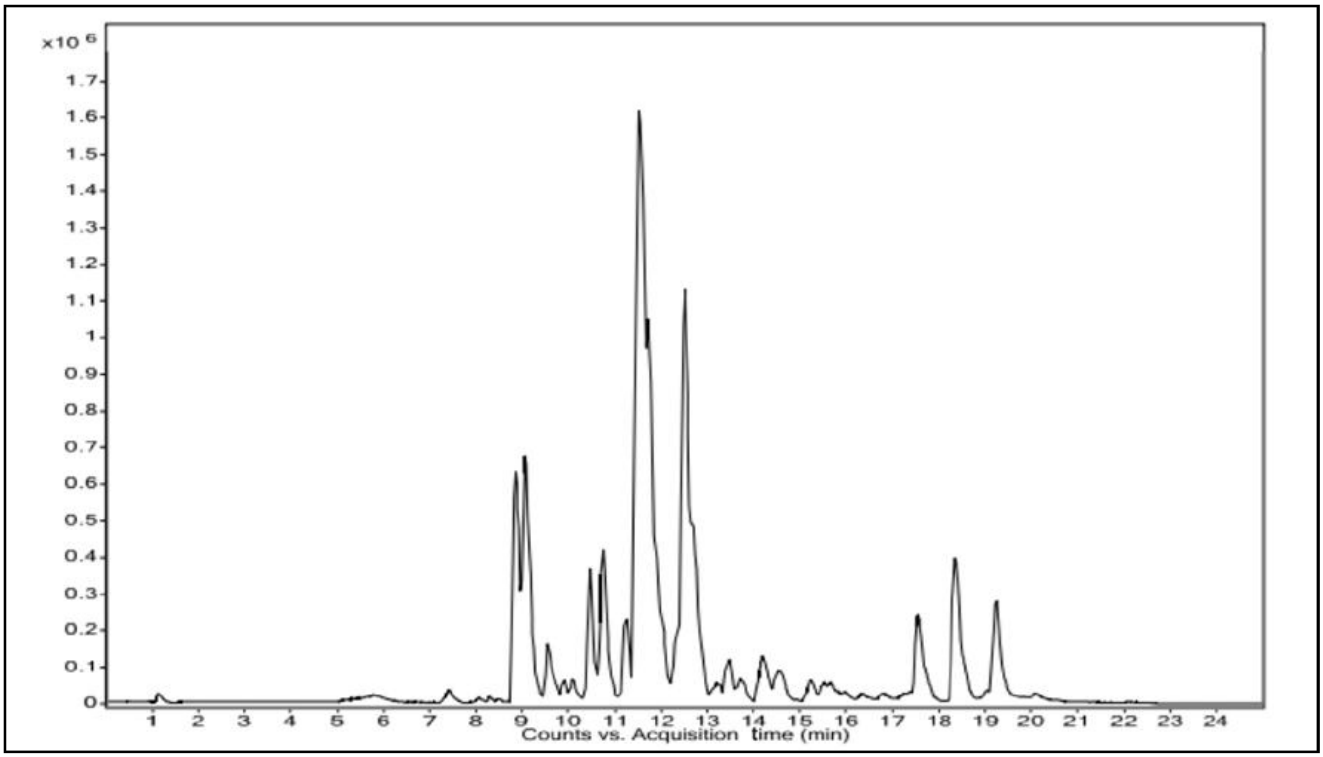


Figure 2:HR-LCMSchromatogram negative ESI.

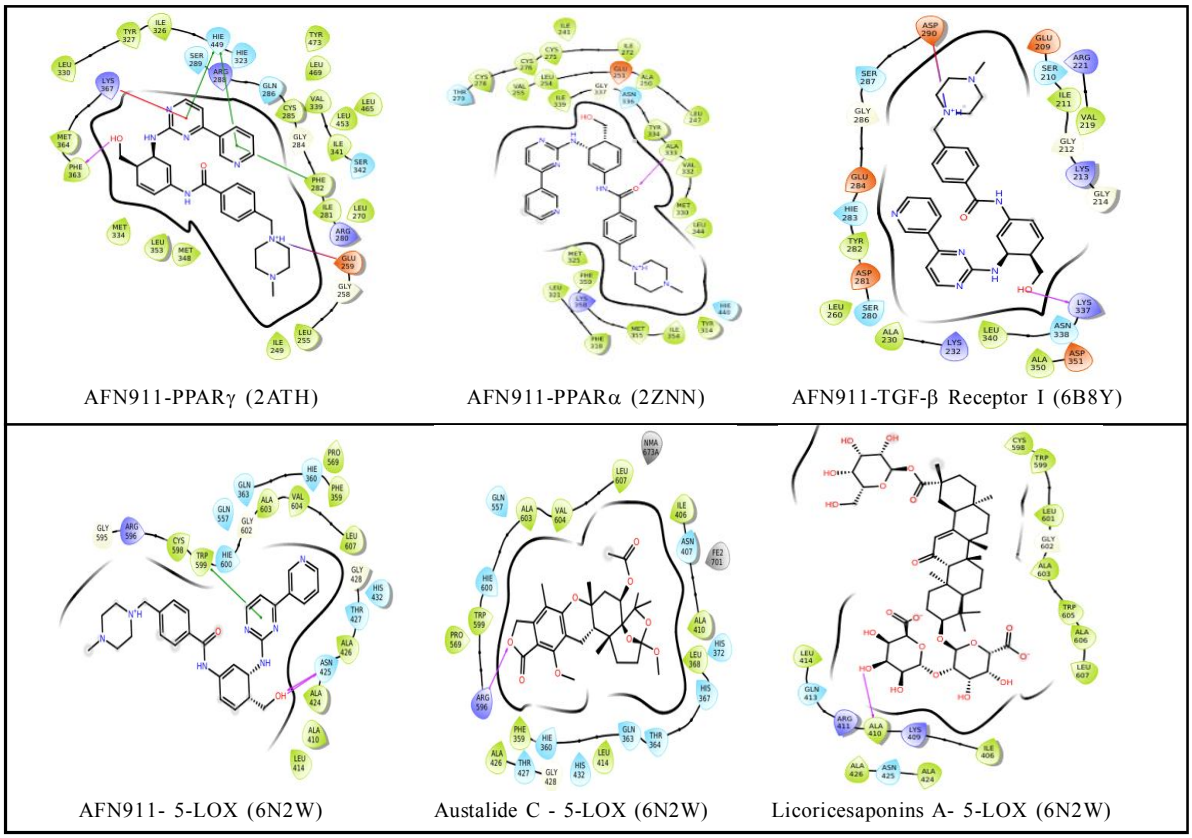
Table 2: Compounds from ethyl acetate extract of *G. febrifugum* (ESI data in negative mode)

S. No.	Name	Formula	Mass	Mass (DB)	RT	Abund^
1.	Ethyl 2E,4Z-hexadecadienoate	$C_{18}H_{32}O_2$	280.2403	280.2402	18.346	201040
2.	Tetrahydro aldosterone-3-glucuronide	$C_{27}H_{40}O_{11}$	540.2589	540.2571	11.932	199341
3.	3-α(S)-strictosidine	$C_{27}H_{34}N_2O_9$	530.2296	530.2264	11.785	193145
4.	Glaucarubin	$C_{25}H_{36}O_{10}$	496.2319	496.2308	10.527	127796
5.	Raucaffricine	$C_{27}H_{32}N_2O_8$	512.2194	512.2159	12.831	103309
6.	Clocortolone pivalate	$C_{27}H_{36}ClFO_5$	494.222	494.2235	9.596	70261
7.	9-HOTE[&]	$C_{18}H_{30}O_3$	294.2192	294.2195	14.498	52606
8.	Linalyl caprylate	$C_{18}H_{32}O_2$	280.2402	280.2402	18.728	47116
9.	Tetrahydrofolyl-[Glu](2)	$C_{24}H_{30}N_8O_9$	574.2107	574.2136	13.738	46374
10.	10-Oxo-11-octadecen-13-olide	$C_{18}H_{30}O_3$	294.2194	294.2195	14.532	44895
11.	8S-HODE[#]	$C_{18}H_{32}O_3$	296.2353	296.2351	15.2	38108
12.	Lucidenic acid J	$C_{27}H_{38}O_8$	488.2471	490.2567	12.109	26820

[&](9E,11E,15Z)-9-Hydroxy-9,11,15-octadecatrienoic acid, [#] 8-Hydroxyoctadeca-9Z,12Z-dienoic acid, ^-Abundance

3.4 Molecular docking

A few compounds identified in the ethyl acetate extract were evaluated for their binding capacity with various receptors involved in the pathophysiology of hepatic diseases. The receptors chosen were PPARα, PPARβ, 5-LOX, and TGF-β receptor I (TGF-βR1), which could be the most suitable targets involved in the development of a damaged liver. The target IDs were collected from the PDB data bank. The binding affinity and glide energy of the four abundant compounds in the extract were docked against each of the PDB structures using Schrodinger docking software (Glide). The binding postures, interactions, and distances of ligand-receptor complexes in 2D forms are presented in Figure 3 (Farag *et al*., 2022).



AFN911-PPARγ (2ATH) | AFN911-PPARα (2ZNN) | AFN911-TGF-β Receptor I (6B8Y)

AFN911- 5-LOX (6N2W) | Austalide C - 5-LOX (6N2W) | Licoricesaponins A- 5-LOX (6N2W)

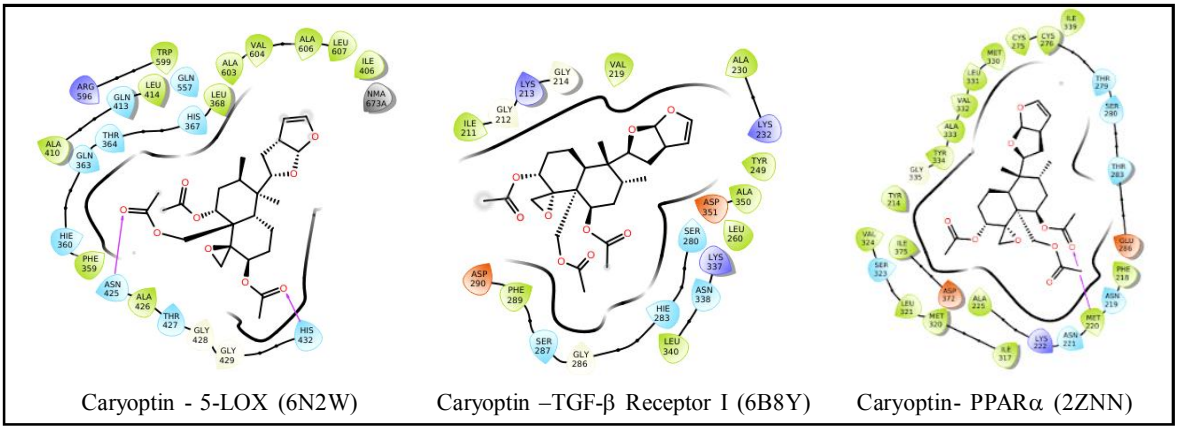


Figure 3: Molecular docking of HR-LCMS-identified compounds.

The compound AFN911 exhibited the best docking score and binding energy with all four receptors. The other three compounds had good docking scores and MMGBSA values with 5-LOX nuclear receptors. The docking scores for each ligand-receptor and standard-receptor complexes and MMGBSA values for ligand-receptor complexes are provided in Table 3.

Table 3: Docking score and MMGBSA values for each ligand-receptor complex

S.No.	Ligand	Docking study results							
		PDB							
		2ATH-PPARγ		2ZNN-PPARα		6N2W- 5-LOX		6B8Y-TGF-β Receptor I	
		Dock Score	MMGBSA	Dock Score	MMGBSA	Dock Score	MMGBSA	Dock Score	MMGBSA
1	AFN911	-10.180	-36.645	-6.145	-61.867	-6.713	-144.674	-5.103	-55.424
2	AustalideA	*	*	*	*	-5.877	-131.245	*	*
3	Caryoptin	*	*	0.462	-54.366	-4.422	-93.601	1.328	-29.605
4	LicoricesaponinsA3	*	*	*	*	-7.314	-75.970	*	*
5	Linagliptin	*	*	*	*	*	*	-4.487	*
6	Nordihydroguaiaretic acid	*	*	*	*	-8.411	*	*	*
7	Orlistat	-7.642	*	-9.917	*	*	*	*	*

***No docking scores**

4. Discussion

Various hepatic disorders and their possible therapeutic strategies have been a topic of extensive research around the world for a few years, as they account for approximately 2 million deaths worldwide annually. Despite all efforts, no molecule has been identified yet, that could be promising in the management of liver disorders or hepatic regeneration from hepatic fibrosis/cirrhosis/carcinoma.

Our effort was to search and identify phytoconstituents from the plant *G. febrifugum* that could be potential therapeutic agents for hepatic disorders. The preliminary phytochemical and antioxidant studies of the ethyl acetate extract revealed that the plant has several secondary metabolites with potential activity. The phytochemistry of the extract was studied by subjecting it to HR-LCMS, and some of the secondary metabolites were identified by comparing it with the available database. Four compounds identified, AFN911, austalide C, caryoptin, and licoricesaponins A3, were subjected to *in silico* studies by docking them with four nuclear receptors that played a significant role in the progression of liver diseases. The docking score and binding energies were calculated. AFN911 exhibited good docking scores and low binding energy with all four receptors. The ligand also exhibited better docking scores with receptors PPARγ and TGF-βR1 than their respective co-crystallized Ligands, Orlistat and Linagliptin. The compound, AFN911 could bind agonistically with PPARα and PPARγ, hence can increase the fatty acid and glucose metabolism in the body thus reducing the fatty acid deposition in the liver.

All four ligands bound antagonistically with 5-LOX, but the docking scores were less when compared to the standard drug Nordihydroguaiaretic acid. The receptor, 5-LOX is very important for the inflammatory response and synthesis of leukotrienes. Sustained injury and inflammation can develop into scarring and the formation of stellate cells in liver cells leading to fibrosis. Hence, inhibition of this receptor could prevent the development of fibrosis from continuous inflammation and might also be useful in treating various inflammatory disorders. As per the literature, TGF-βRI is directly involved in the generation of fibrosis in the lungs (Mishra *et al*., 2015). TGF-β1 has a pivotal role in liver repair and fibrosis, mediated *via* progenitor cells-(HPCs) (Harikrishnan *et al*., 2018). The compound AFN911 is an antagonist for the gene TGF-βR1 and hence may inhibit the formation of fibrosis. Compounds, austalide C, caryoptin, and Licoricesaponins A3 are possible inhibitors of the receptor 5-LOX and could prevent hepatic inflammation and fibrosis as identified from *in silico* studies.

5. Conclusion

Phytoconstituents are considered safer options for therapy when compared to synthetic drugs, but the usage of natural medicines without proper characterization might also be detrimental. The inconsistent concentrations of phytoconstituents and anthropogenic or environmental factors that affect the secondary metabolites are all factors that can adversely affect the patient's health. Hence, it is significant that proper characterization and phytochemical screening of herbal medicines is done to ensure the safe and effective usage of natural sources of medicine. *In vitro* studies proved that the plant, *G. febrifugum* has good antioxidant potential. The study sheds light on the numerous chemical constituents of the ethyl acetate extract of the plant. Based on *in silico* studies the extract has a potent constituent, AFN911 that can target multiple receptors in the pathway of liver disorders. Further evaluation of the compound for its therapeutic activity or development of synthetic derivatives similar to AFN911 could be promising efforts in the management of hepatic disorders. *G. febrifugum* is a plant that has been less studied but is a profusion of secondary metabolites that could have immense potential in managing hepatic disorders. Extensive studies on plant chemistry can undermine enormous data that could be beneficial to mankind.

Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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