JOURNAL OF NATURAL REMEDIES
DOI: 10.18311/jnr/2025/46249
RESEARCH ARTICLE

Evaluation of Total Phenolic Content, Antioxidant Potential, Cytotoxicity and Anti-inflammatory Activity of *Stylosanthes fruticosa* Leaf Extract

V. Vadivel[1], P. Rajalakshmi[1], M. Karunanithi[1], R. Sivakumar[2], R. Renuka[2] and S. Sriram[1*]

[1]Centre for Advanced Research in Indian System of Medicine (CARISM), SASTRA Deemed To Be University, Thanjavur - 613401, Tamil Nadu, India; sriram@scbt.sastra.edu

[2]Srinivasa Ramanujam Centre (SRC), SASTRA Deemed to be University, Kumbakonam - 612001, Tamil Nadu, India

Abstract

Background: *Stylosanthes fruticosa* is a relatively unexplored fodder shrub from the legume family *Fabaceae*. **Aim:** This study focuses on estimating the phenolic content of various solvent-based extracts from *S. fruticosa* leaves and evaluating their antioxidant, anticancer, and anti-inflammatory potential. **Methods:** The total phenolic content was estimated using a standard method with gallic acid as the positive control. The inhibition of lipid peroxidation was examined using goat liver tissue to assess MDA levels and the *in vitro* cytotoxicity of *S. fruticosa* extracts was studied against $HepG_2$ cells by MTT assay. *In vitro* anti-inflammatory potential of the extracts was assessed using RBC membrane stabilization assay. Screening of phytocompounds in the plant extract was done using LC-MS/MS and the identified compounds were subsequently used for *in silico* docking studies against pro-inflammatory markers IL6 and TNF-α. **Results:** Among various extracts, ethanol-based extraction showed the highest phenol concentration. The ethanol extract of *S. fruticosa* exhibited higher antioxidant potential (85%), followed by hexane extract (46%). The hexane and ethyl acetate extracts showed better lipid peroxidation inhibition than ethanol extract. *In vitro* cytotoxic studies revealed that ethyl acetate extract showed higher level of toxicity (67.59%) against hepatocarcinoma cells when compared to hexane (41.27%) and ethanol extracts (-0.7%). In the membrane stabilization study the ethyl acetate extract of *S. fruticosa* was more effective in preventing membrane damage of RBC. Further analysis with LC-MS/MS identified six flavonoid-based phenolic compounds within the ethyl acetate extract, which were docked against TNF-α and IL6 to evaluate their anti-inflammatory potential.

Major Findings: Datiscin emerged as a compound with the strongest interactions with both TNF-α and IL6, suggesting it to be a key factor in the anti-inflammatory effects observed in *S. fruticosa*. These findings offer a basis for further *in vivo* and molecular mechanistic studies.

Keywords: Antioxidant, Anti-inflammatory, cytotoxicity, IL6, Membrane Stabilization, *S. fruticosa*, TNF-α

1. Introduction

The *Fabaceae*, or *Leguminosae* family, ranks among the largest and most economically significant groups of flowering plants, comprising around 730 genera and nearly 19,400 species, second only to *Asteraceae* and *Orchidaceae*[1]. Known for its rich diversity in morphology and chemical makeup, this family is valued not only for its medicinal phytoconstituents but also for applications in horticulture, agriculture, and industry[2]. Members of *Fabaceae* serve as essential protein sources for humans, livestock, and wildlife.

The genus *Stylosanthes* within *Fabaceae* encompasses roughly 40 species based on morphological characteristics, with many serving as crucial forage legumes across tropical, subtropical, and temperate zones in the Americas, Tropical Africa, and Southeast Asia, thriving in both annual and perennial agricultural systems[3,4]. Traits such as effective phosphorus extraction, drought resistance, and adaptability to

*Author for correspondence

Article Received on: 05.10.2024 Revised on: 07.01.2025 Accepted on: 16.01.2025

warm, arid climates have made *Stylosanthes* a vital pasture legume globally[5].

Commonly referred to as Wild Lucerne, *S. fruticosa* is a dense, woody herb or shrub from the *Stylosanthes* genus introduced into India from Australia and South America in the early 1970s[6]. It is well-distributed across southern India and has been valuable in enhancing soil fertility, stabilizing wastelands, and providing highly nutritive forage for animals[7,8].

While much is known about the nutritional and economic benefits of *S. fruticosa*, scientific documentation of its medicinal potential remains scarce. Thus, this study investigates the antioxidant, anticancer, and anti-inflammatory activities of *S. fruticosa*.

2. Materials and Methods

2.1 Extract Preparation

Leaves of *S. fruticosa* were collected from areas around Sastra University, Tamil Nadu, India. The plant material was thoroughly washed, dried, ground into powder, and sifted for uniformity.10-gram of sample was treated with 50 ml of various solvents and extracted at room temperature for 12 hours. Filtration, removed the solvent, yielding a dry extract. To calculate extract yield, a 10 ml portion was heated in a pre-weighed crucible at 105°C for one hour, then cooled, and weighed again. The final yield was noted as a percentage. Each dry extract was dissolved in water (10 mg/ml) for further testing.

2.2 Total Phenolic Content

Total phenolic content was estimated using the modified method of Singleton *et al*[9]. A 10 µl extract sample was mixed with 25 µl of Folin reagent and 230 µl of 4.4% NaCO in a 96-well plate, then incubated in the dark for half an hour. Absorbance was measured at 750 nm using an ELISA reader (Biotek Epoch). Results were standardized with gallic acid calibration (100-1000 mg/L, $R^2 = 0.9978$) and expressed in Gallic Acid Equivalents (GAE).

2.3 Antioxidant Activity

100 µl extract sample was added to 3.9 ml of 0.025 g/L DPPH solution and incubated for 30 minutes at 25°C. Absorbance at 515 nm was recorded with a spectrophotometer. Ethanol served as the control solvent, while ferulic acid was the positive standard. Radical scavenging was expressed as a percentage of inhibition[10].

2.4 Lipid Peroxidation Inhibition

The inhibition of lipid peroxidation was examined using goat liver tissue to assess MDA levels, following the method of Yen and Hsieh[11]. Liver slices were treated with 2 ml PBS, 0.5 ml of each extract for 2 hours, homogenized, then reacted with TBA in a boiling water bath. The absorbance of the supernatant was recorded at 532 nm. TBARS values were then calculated using the standard curve and expressed as mg MDA equivalents per 100 g of sample.

2.5 *In Vitro* Cytotoxicity

In vitro cytotoxicity of *S. fruticosa* extracts in HepG2 cells was studied by MTT assay[12]. DMEM medium containing 10% FBS, amino acids, and antibiotics were used for growing cells. After reaching 90% confluence, cells were harvested and transferred to a 96-well plate (10,000 cells per well) and exposed to extract concentrations of 1-5 mg/ml. After 12 hours, 5% MTT reagent was added, incubated, and dissolved with acidic isopropanol. Optical density was measured at 590 nm, and cytotoxicity percentages were calculated.

2.6 Compound Screening using LC-MS/MS

The phytocomponent composition of the ethyl acetate extract was analysed using LC-HR-ESI-S/MS (UHPLC Dionex C18 RP Acclaim 120 Å, 2.1 × 150 mm, 3.0 µm column) and MS/MS Bruker Q-II TOF).

LC settings included UV detection at 355 nm with a gradient flow of 0.2 ml/min. Mobile phase A consisted of acetonitrile, and mobile phase B was water acidified with 1% acetic acid. MS settings included an ESI nebulizer (30.5 psi with 6.0 l/min N flow). The spectrum was recorded in negative ion mode over an m/z range of 50-1000[13].

2.7 Anti-Inflammatory Activity

The anti-inflammatory potential of the extracts was assessed using an RBC membrane stabilization assay[14]. Blood samples were collected in heparinized tubes, washed with PBS, and centrifugated at 3000 rpm for 10 minutes. RBCs were suspended in phosphate buffer and

incubated with extracts and hypotonic solution for 30 minutes at room temperature. Samples were centrifuged at 1500 rpm for 10 minutes, and supernatant absorbance was measured at 560 nm. Membrane stabilization was calculated based on absorbance differences between extract and control samples.

2.8 Molecular Docking Studies with IL6 and TNF-α

2.8.1 Ligand Preparation

Ligands were prepared with the POAP pipeline, which uses Open Babel for structure optimization. A 3D conformer search produced minimized structures, which were converted to 'pdbqt' format with the MGL Tools package for docking.

The POAP method was employed to carry out virtual screening of multiple protein docking *vs* various ligands. During the docking process, the POAP input data comprised receptors along with their respective grid, docking parameter files and the prepared receptor structure. Finally, the protein-ligand complex files and docking scores generated were analysed. The protein-ligand interactions in 2D were mapped using Discovery Studio Visualizer. Automation of ligand dataset optimization and multithreaded virtual screening was achieved through POAP (Parallelised OpenBabel and Autodock Pipeline).

2.8.2 Docking with IL6

IL6, co-crystallized with its receptor (PDB ID: 1P9M), was used after receptor coordinates were removed, leaving IL6 for docking. Hydrogen atoms and Gasteiger charges were added, and the protein was saved in 'pdbqt' format. Blind docking was performed with a grid covering the entire protein at 0.375 Å spacing.

2.8.3 Docking with TNF-α

For TNF-α, the reference ligand-bound structure (PDB ID: 2AZ5) was selected, and chains A and B were retained (among A, B, C and D) for docking excluding water molecules and other chains. The rationale for selection of chains were adopted based on earlier reports. Similar to IL6, the structure was prepared for docking using MGLTools-1.5.6. The binding cavity was centered on the ligand site with 0.375 Å grid spacing. For both the proteins, docking parameters included a population size of 300, 20 million evaluations, and 50 docking runs with a 1 Å RMSD cut-off for clustering.

2.8.4 Statistical Analysis

All the data was represented as Mean ± SEM. Statistical analysis was carried out using Graph Pad Prism 10. t-test and One-way ANOVA with Bonferroni's multiple comparison test was performed among the groups for different experiments.

3. Results and Discussion

3.1 Phenolic Composition

Phenolic compounds in plants serve as defence against environmental stress that include UV radiation and oxidative image[15]. Human diets are rich in polyphenols, whose antioxidant and free radical scavenging potential may be attributed to hydroxyl group substitution in their structure[16]. In this study, maximum phenol composition was observed in the ethanol extract of *S. fruticosa* which is significantly greater than the other extracts (Figures 1a and 1b).

Generally, phenolic compounds are highly polar in nature and soluble in high polar solvents like ethanol. In addition, phenolic compounds contain hydroxyl groups in their structure that form hydrogen bonds with ethanol, thus enhancing their solubility in the solvent. Thus, the phenolic compounds from leaf materials of *S. fruticosa* have been extracted efficiently in ethanol. The abundance of phenol in the plant is in accordance with

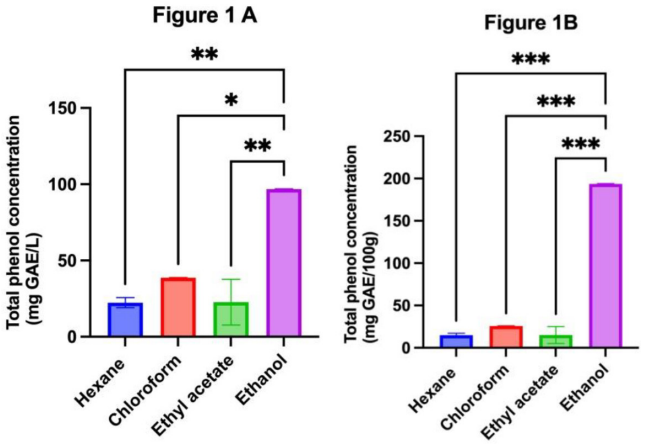


Figure 1a and 1b. Total phenolic content in various solvent extracts of *Stylosanthes fruticosa*.

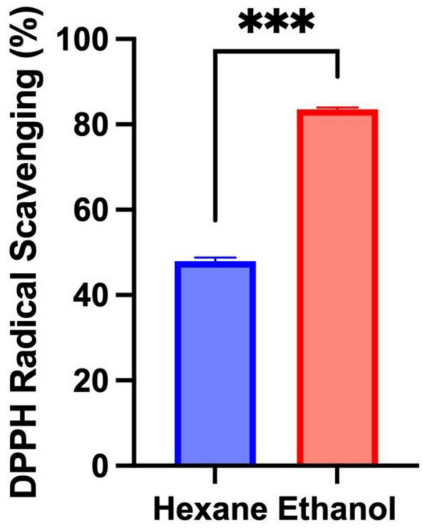
previous reports on abundance of phenolic compounds in the *Fabaceae* family[17,18].

3.2 Antioxidant Capacity

Incomplete reduction of oxygen during biochemical reactions in the body can result in excessive production of free radicals[19]. Prolonged exposure to ROI can result in DNA mutation, tissue injury and can also lead to cardiovascular diseases, diabetes and cancer[20,21]. Maintaining a healthy balance between free radicals and antioxidants (both endogenous and exogenous) is essential for overall well-being. In some cases, the body's own enzymatic antioxidant system may be insufficient to combat oxidative stress, making it necessary to regularly consume external antioxidants through diet or supplements. Many species of *Fabaceae* have always been a regular part of our staple diets and plant derived medicines mainly due to the high antioxidant content of their phytoconstituents[22]. Accordingly, in the present work, ethanol extract of *S. fruticosa* exhibited significantly higher level of radical scavenging activity (85%), when compared to the hexane extract (46%) whereas the ethyl acetate and chloroform extracts did not show any antioxidant potential (Figure 2). The rich phenolic content of the ethanolic extract could be responsible for its strong antioxidant potential[17,18].

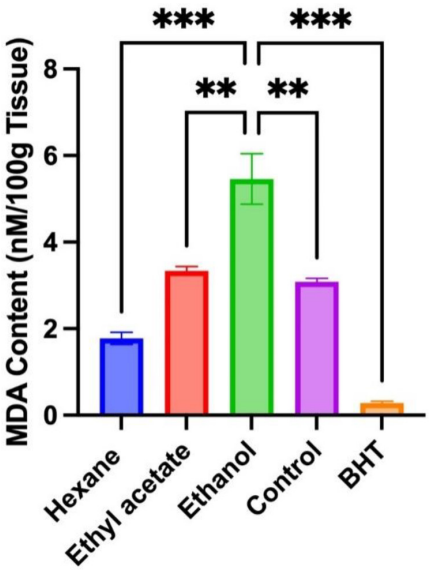
3.3 Lipid Peroxidation Inhibition

Lipid peroxidation caused by free radicals has been linked to various diseases, including cancer, diabetes mellitus, and inflammatory disorders[23]. In this study we have analyzed the malondialdehyde (MDA, a biomarker of lipid peroxidation) content of goat liver treated with different solvent extracts of *S. fruticosa*. Interestingly, in contrast to DPPH assay, the ethanol extract treated liver homogenate exhibited the highest content of MDA level indicating excessive lipid peroxidation (Figure 3). But the hexane and ethyl acetate extracts showed low levels of MDA respectively and were found to be effective in preventing lipid peroxidation. The ethanol extract showed a highly significant difference in MDA levels when compared with Butylated Hydroxy Toluene (BHT) and significant difference when compared with the ethyl acetate and hexane extracts. This variation could be attributed to the interaction of low-polarity compounds in the hexane extract with the liver.



The data was represented as Mean±SEM . *** P < 0.001

Figure 2. Antioxidant potential of different solvent extracts of *S. fruticosa.*



The data was represented as Mean±SEM . * P < 0.05, ** P < 0.01, *** P < 0.001

Figure 3. Lipid peroxidation level (MDA) in goat liver slice treated with solvent extracts of *S. fruticosa*.

3.4 Cytotoxicity in HepG2 Cells

Free radical induced lipid peroxidation and its association with cancer have already been well documented[23]. Lipid peroxidation and malondialdehyde levels in goat liver treated with different solvents have been already discussed in the previous section and the results indicated that bioactive compounds in plant extracts could be effective in alleviating cancer. Hence, in this

study, the cytotoxic potential of various *S. fruticosa* solvent extracts was evaluated using the HepG2 human liver cancer cell line, with the results presented in Figure 4a-4c.

From Figure 4 it is evident that the ethyl acetate extract demonstrated a higher level of toxicity against hepatocarcinoma cells compared to the hexane and ethanol extracts. At a high concentration of 5 mg/mL, ethyl acetate extract exhibited 67.59% cytotoxicity, which was followed by hexane (41.27%) and ethanol extracts (-0.7%). In the case of ethyl acetate and hexane extracts, a dose dependent increase in cytotoxicity levels was observed, while the ethanol extract showed an inverse trend and there was no toxicity at 5 mg/mL. Interestingly, the absence of cytotoxic activity in the ethanolic extract despite its rich antioxidant activity may imply that phytoingredients like phenolic compounds exhibit selective activity. These compounds are known more as effective antioxidants than as anticancer agents. Also, the concentration of phenolic compounds in the ethanol extract might have been sufficient for exhibiting antioxidant activity but not for cytotoxic activity.

Based on the observed results, it could be noted that mid-polar phytochemicals from ethyl acetate are more effective against human liver cancer cells when compared to non-polar hexane and high polar ethanolic extracts. Hence, ethyl acetate extract could be considered for further studies to elucidate anti-cancer compounds for developing novel and safe natural chemotherapeutic agents. The extract could also be subjected to *in vivo* studies to validate the effectiveness of ethyl acetate extract or pure active compound (s) isolated from this extract.

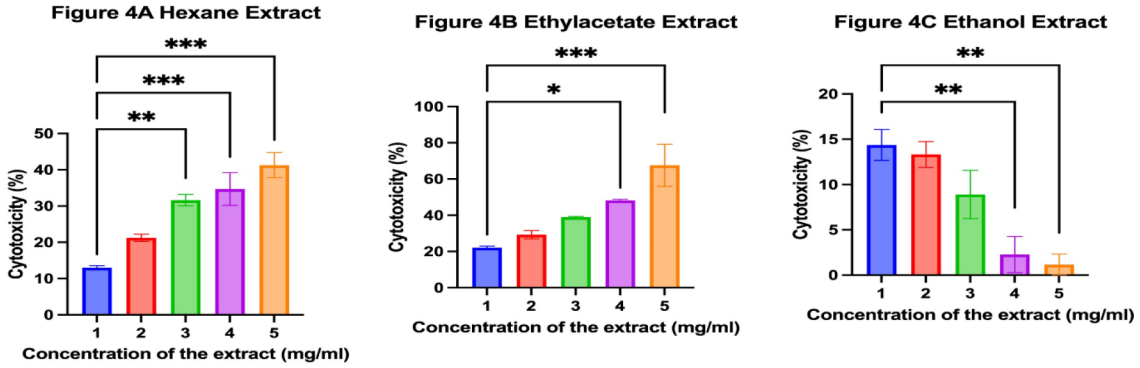
3.5 LC-MS/MS

LC-MS/MS results identified six polar phenolic compounds: Datiscin, Isorhametin-3-O-rutinoside, Rutin, Hisrutin, Isorhametin-3, 4'-diglycoside, and Quercetin-3,4'-O-diglycoside. The detection of these phenolic compounds in the specified plant extract aligns with well-established documentation regarding the occurrence of phenolic compounds in the *Fabaceae* family and also supports the findings of total phenol content in *S. fruticosa*[17,18].

3.6 Anti-Inflammatory Activity

While inflammation serves as a protective mechanism, the complex processes and mediators involved in the inflammatory response can induce, sustain, or exacerbate various diseases, including asthma, arthritis, and other autoimmune disorders[24]. Over the years, significant attention has been given to the impact of inflammation in the pathogenesis of atherosclerosis and cancer[25,26]. Increasingly, the literature indicates that inflammation not only plays a part in the pathogenesis of cancer but also impacts the efficacy and resistance of chemotherapy[27].

Widely used Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) can injure the gastric and duodenal mucosa by suppressing prostaglandin production, which serves as a protective agents for the gastric mucosa. This suppression has consequently resulted in significant morbidity and mortality[28]. Under the circumstances, traditional medicine involving medicinal plants with their active principles is being increasingly considered as the alternative and ideal anti-inflammatory medicines due to their increased efficacy, best potency, and lowest or no side effects.



The data was represented as Mean±SEM. * P < 0.05, ** P < 0.01, *** P < 0.001

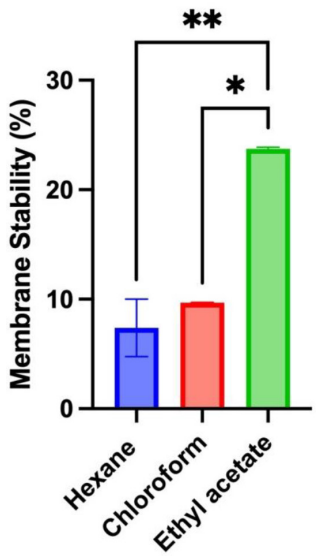
Figure 4a, 4b, and 4c. Cytotoxic effect of solvent extracts of *S. fruticosa* in HepG1 cell line.

Several researchers have utilized erythrocytes as a model to study drug interactions with membranes. In our investigation, the ethyl acetate extract of *S. fruticosa* demonstrated significantly higher effectiveness (23%) in preventing membrane damage in red blood cells compared to the hexane and chloroform extracts. There was no significant difference between the hexane and chloroform extracts (Figure 5).

Lipid peroxides are mostly responsible for membrane destabilization. Several earlier literature evidences demonstrated that flavonoids and phenolic compounds found in plant extracts are highly effective at scavenging free radicalsand are also know to exhibit appreciable analgesic and anti-inflammatory effect through their membrane stabilizing ability[29-32]. So, either single or group of compounds present in the ethyl acetate extract of *S. fruticosa* might have inhibited the membrane damage of RBC induced by oxidative stress under *in vitro* conditions.

3.7 Docking with IL6 and TNF-α

Pro-inflammatory cytokines such as TNF-α and IL-6 are well-established as key biomarkers of systemic inflammation and have also been linked to various other diseases[33]. The results revealed that among all phytocompounds of *S. fruticose*, Datiscin showed interaction with residues of IL6 which were earlier reported to be involved in IL6-IL6 receptor interactions as visualized in the crystal structure of 1P9M.pdb and also demonstrated in earlier studies[34]. It exhibited a binding energy of -6.26 Kcal/mol while interacting with Q175, S176, and R179 through hydrogen bonds. While all the other ligands did not interact in other regions (Figures 6a-f; Table 1), Datiscin showed two strong pi-cation interactions with K66.



The data was represented as Mean±SEM . * P < 0.05, ** P < 0.01, *** P < 0.001

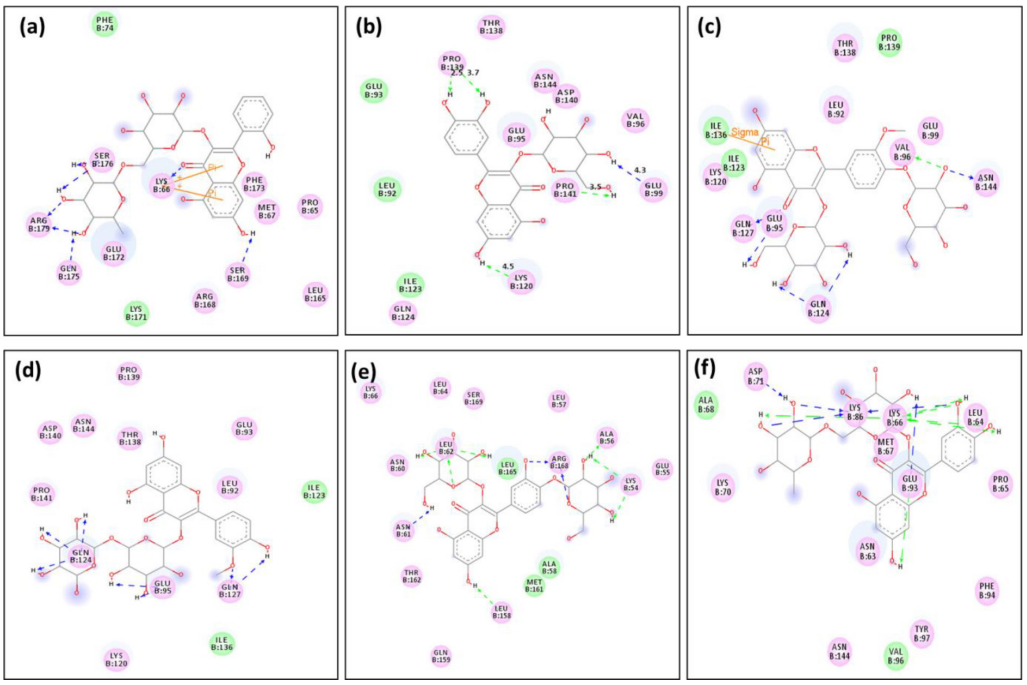
Figure 5. Anti-inflammatory activity of different solvent extracts of *S. fruticosa* in human RBC membrane stabilization model.

Among all the ligands studied Datiscin exhibited a better binding energy of -7.24 (Kcal/mol) with TNF-α showing interactions with active site residues forming H-bond with Y151B, S60B, Y119B and pi-pi interaction with Y59A (Figure 7a). These residues were also found to be important for TNF-alpha Dimerization inhibition, as inferred from the TNF-α co-crystallized ligand complex (PDB ID:2AZ5) and also from previously reported docking studies[35,36]. Other compounds were also found to interact with critical regions for the TNF-α inhibition and their corresponding energy and interaction plots are given above (Figure 7b-f).

Numerous previous studies have documented the anti-inflammatory potential of different plant extracts and compounds by modulating different inflammatory pathways and inhibiting or interacting with the pro-inflammatory cytokines TNF-α and IL-6. The docking results from the present study regarding Datiscin and its interaction with TNF-α and IL-6 are comparable to earlier findings involving compounds such as rosmarinic acid, capsaicin, and carnosic acid[37-39].

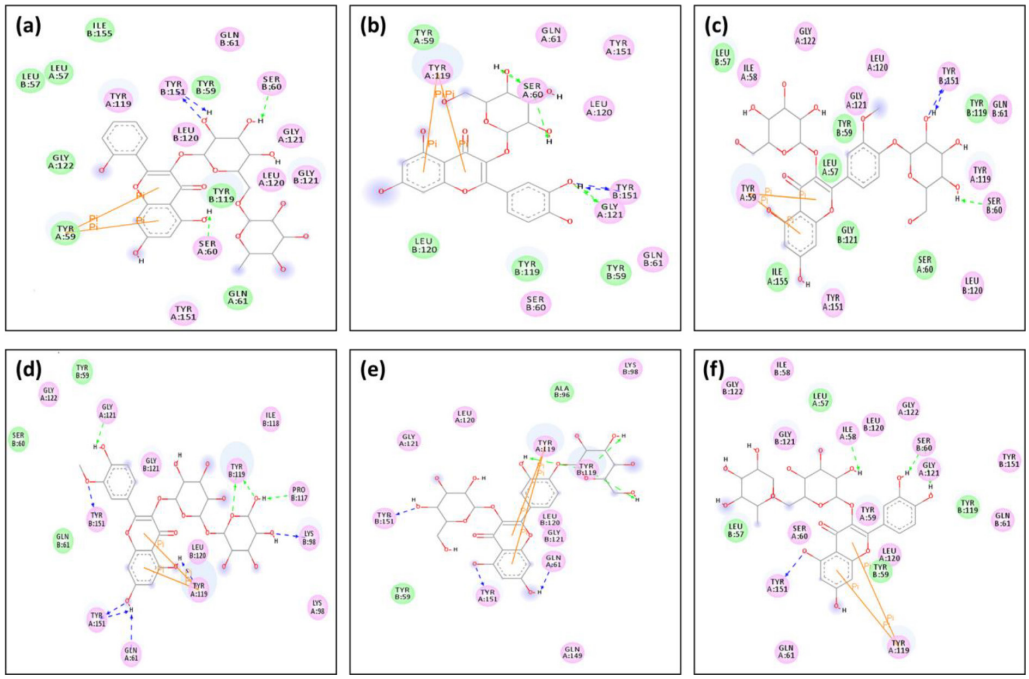
Table 1. Docked ligands and their respective binding energy

Ligand Name	IL6 (Kcal/mol)	TNF-aplha (Kcal/mol)
Daticin	-6.26	-7.24
Isorhametin_3_O_rutinoside	-5.33	-6.42
Rutin	-6.44	-6.26
Hirsutrin	-6.16	-6.04
Isorhametin_3,4'_di_glycoside	-4.88	-5.1
Quercetin_3,4'-O-_di_glycoside	-6.41	-4.91



(Hydrogen bond interactions between amino acid side chain and ligand atoms are shown in blue dotted lines, hydrogen bond interaction of amino acid main chain with ligands are shown in green dotted lines, pi-pi and pi-cation interactions are of amino acids with ligands are shown solid yellow lines.)

Figure 6. IL6 interaction with: **(a)** Datiscin **(b)** Hisrutin **(c)** Isorhametin_3,4′_di_glycoside **(d)** Isorhametin_3_O_ rutinoside **(e)** Quercetin_3,4′-O-_di_glycoside **(f)** Rutin.



(Hydrogen bond interactions between amino acid side chain and ligand atoms are shown in blue dotted lines, hydrogen bond interaction of amino acid mainchain with ligands are shown in green dotted lines, pi-pi and pi-cation interactions are of amino acids with ligands are shown solid yellow lines).

Figure 7. TNF-α interaction with **(a)** Datiscin **(b)** Hisrutin **(c)** Isorhametin_3,4′_di_glycoside **(d)** Isorhametin_3_O_ rutinoside **(e)** Quercetin_3,4′-O-_di_glycoside **(f)** Rutin.

4. Conclusion

This study demonstrates the remarkable antioxidant, anticancer, and anti-inflammatory potential of *S. fruticosa* leaf extract. While the ethanol extract contained a higher phenolic composition exhibiting better antioxidant activity than other solvent extracts, the ethyl acetate extract displayed the highest levels of lipid peroxidation inhibition and maximum cytotoxicity against hepatocarcinoma cells. Additionally, mass spectrometric analysis of the ethyl acetate extract identified six flavonoid-based compounds, which were subsequently docked against TNF-α and IL-6 to evaluate their anti-inflammatory activity. Datiscin showed the strongest interaction with TNF-α and IL-6, suggesting it could be a significant contributor to the anti-inflammatory effects of *S. fruticosa*. The promising outcome of this study could serve as solid preliminary data for further *in vivo* and molecular mechanistic investigations of this plant.

5. Acknowledgement

The authors are thankful to Sastra University for providing the necessary infrastructure and facilities to carry out this research work.

6. Author Contribution

V. Vadivel: *In vitro* antioxidant analysis, phenolic content evaluation; P. Rajalakshmi: Selection of plant, manuscript preparation; M. Karunanithi: Anti-inflammatory analysis, results interpretation, manuscript preparation; R. Sivakumar and R. Renuka: *In vitro* cytotoxicity studies, Statistical analysis; S. Sriram: LC-MS/MS analysis, *In silico* docking analysis, overall manuscript writing, statistical analysis. All authors have read and agreed to the published version of the manuscript.

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