Phytochemical Analysis And Antioxidant Potential Of Ethyl Acetate Extract Of *Stylosanthes Hamata* (L.) Taub.

Mukeshbabu. N[1*] and Mary Kensa V[2]

[1*]Research Scholar, Department of Botany, S.T. Hindu College, Nagercoil, Affiliated to MS University, Tirunelveli, Tamil Nadu, India-629002. *Email: mukeshbabu17598@gmail.com
[2]Associate Professor, Department of Botany, S.T. Hindu College, Nagercoil, Affiliated to MS University, Tirunelveli, Tamil Nadu, India-629002. *Email: surejkensa@gmail.com

ABSTRACT
Plants have been used in traditional medicine since ancient times and are now an integral part of modern scientific therapeutics. This study evaluated the phytochemical constituents, antioxidant capacity, and Gas Chromatography-Mass Spectroscopy (GC-MS) profile of the ethyl acetate extract from *Stylosanthes hamata* (L.) Taub. Qualitative analysis detected the presence of alkaloids, flavonoids, tannins, phenols, terpenoids, glycosides, steroids, carbohydrates, proteins, reducing sugars, and starch, while saponins were absent. Quantitative measurements indicated the extract contained alkaloids (36.3 ± 0.04 mg/g), flavonoids (88.2 ± 0.04 mg/g), tannins (62.46 ± 0.04 mg/g), phenols (38.1 ± 0.01 mg/g), terpenoids (20.23 ± 0.04 mg/g), glycosides (22.86 ± 0.04 mg/g), steroids (2.28 ± 0.02 mg/g), carbohydrates (60.23 ± 0.04 mg/g), proteins (96.6 ± 0.04 mg/g), reducing sugars (2.83 ± 0.009 mg/g), and starch (27.43 ± 0.04 mg/g). The antioxidant evaluation demonstrated significant activity, with the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay showing an IC_{50} value of 106.13 µg/mL and the ferric reducing antioxidant power (FRAP) assay an IC50 value of 133.03 µg/mL, using ascorbic acid as the standard. These findings highlight the potential of *S. hamata* as a valuable source of natural antioxidants.

Keywords: Ethyl acetate extract, *Stylosanthes hamata*; phytochemical analysis, GC-MS analysis, antioxidant activity.

1. Introduction

Free radicals originate from both endogenous and exogenous sources and are capable of causing cellular damage at the macromolecular level, potentially compromising the integrity of the entire cell structure (Badmus *et al*., 2022). Antioxidants are compounds that counteract free radicals by halting their propagation. They inhibit chain oxidation reactions initiated by reactive radicals through several mechanisms, including hydrogen atom donation, radical scavenging, and singlet oxygen quenching (Irivibulkovit *et al*., 2018). These antioxidant molecules are strongly linked to the prevention of various degenerative conditions, including cardiovascular and neurological disorders, oxidative stress-related dysfunctions, and cancer (Gohari *et al*., 2011). Natural antioxidants play a critical role in defending the body against oxidative damage and may help delay the onset and progression of chronic diseases (Ghanimi *et al*., 2021). Owing to their positive effects on the human immune system, natural antioxidants are preferred over synthetic alternatives, which have been shown to pose health risks (Shalini and Ilango, 2021).

The antioxidant potential in plants is largely attributed to the presence of phytochemicals. These naturally occurring bioactive compounds function as both macro- and micronutrients and contribute significantly to human health (Gulo *et al*., 2021). Plants are abundant in secondary metabolites such as phenolic acids, flavonoids, tannins, stilbenes, and lignans, which offer protective effects against ailments like cardiovascular diseases and cancer due to their potent free radical scavenging capabilities. Additionally, antioxidants are widely utilized in the food industry to improve shelf life. Nonetheless, due to the possible health hazards and toxicity of synthetic antioxidants like butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), there is growing interest in replacing them with natural alternatives. In this context, the exploration of antioxidant activity and the search for new natural sources has become increasingly important (Ghanimi *et al*., 2022). Therefore, the current study focuses on evaluating the phytochemical profile (both qualitative and quantitative), identifying bioactive compounds using GC-MS analysis and assessing the antioxidant properties from the ethyl acetate extract of *Stylosanthes hamata* (L.) Taub., for the first time.

2. Materials and Methods

2.1 Plant Collection

The experimental plant, *Stylosanthes hamata* (L.) Taub, belonging to the family Fabaceae, was collected from an S. T. Hindu college at Nagercoil in Kanyakumari District of Tamil Nadu, India. with an elevation of about 460 meters (Mean Sea Level with 8.11 Latitude and 77.53 Longitude).

2.2 Qualitative Phytochemical Analysis

The dry powder of the whole plant of *S. hamata* was extracted with ethyl acetate solvent at 20 % (w/v) using a Soxhlet apparatus. The extract were concentrated and used for qualitative phytochemical analyses following the Harborne (1998)

method.

a) Test for alkaloids
2 ml of extract was measured in a test tube to which 3-4 drops of 2% of picric acid solution was added. An orange colouration indicated the presence of alkaloids (Deshpande *et al*., 2014).

b) Test for flavonoids
2 ml of 2.0% NaOH was mixed with extract; a concentrated yellow colour was developed. This result showed the presence of flavonoids (Gul *et al.*, 2017).

c) Test for tannins
About 2 ml extract was mixed with 5% ferric chloride solution and observed for formation of blue, green or violet colour solution indicates the presence of tannins (Singh and Kumar, 2017).

d) Test for phenols
The extract was diluted to 5 ml with distilled water. A few drops of neutral 5% ferric chloride solution were added to that. A dark green colour indicates the presence of phenolic compounds (Mukherjee, 2002).

e) Test for saponins
The extract was shaken vigorously with water. The formation of persistent foam was indicated by the presence of saponin (Ansari, 2006).

f) Test for Terpenoids
To the test solution, 2 ml of chloroform was added with a few drops of concentrated. Sulphuric acid (3 ml) at the side of the test tube. An interface with a reddish-brown colouration indicated the presence of terpenoids (Harbone, 1998).

g) Test for glycosides
To 2 ml of the extract, glacial acetic acid, one drop of 5% $FeCl_3$ and conc. H_2SO_4 was added. A reddish-brown colour appeared at the junction of two liquid layers and the upper layer turned bluish green, indicating the presence of glycosides (Ansari, 2006).

h) Test for steroids
To two ml of the extract, an equal volume of conc. H_2SO_4 was added carefully along the sides of the tube. The upper layer turned red, and the lower layer turned yellow with green fluorescence, indicating the presence of steroids in the extract (Singh and Kumar, 2017).

i) Test for carbohydrates
One ml of extract and Barfoed's reagent were mixed in a test tube and heated on the water bath for 2 minutes. Red color due to the formation of cupric oxide indicates the presence of monosaccharide (Singh and Kumar, 2017).

j) Tests for protein (Biuret test)
2ml of filtrate was taken to which 1 drop of 2% copper sulphate solution was added; 1ml of 95% ethanol was added. Then it was followed by an excess addition of KOH. The appearance of pink colour indicates the presence of protein (De Silva *et al.*, 2017).

k) Tests for reducing sugar (Biuret test)
500 µl of Benedict's reagent and extract were mixed in a test tube and heated on a water bath for 5-10 minutes. The solution appears green, yellow or red depending on the amount of reducing sugar present in the test solution, which indicates the presence of reducing sugar (Singh and Kumar, 2017).

l) Tests for starch
The 5 ml extract were treated with the iodine reagent. Any shift to blue-violet indicates the presence of starch (Zohra *et al*., 2012).

2.3 Quantitative Phytochemical Analysis
The positive results from the preliminary phytochemicals assay were estimated quantitatively.

a) Total alkaloid content
The total alkaloid content from the extract was quantified according to the method of Devanaboyina *et al*. (2013). Briefly, 1 ml of extract was added with 5 ml of phosphate Buffer (pH 4.7), followed by the addition of 5 ml of BCG solution and a mixture with 4 ml of chloroform. The extracts were collected in a 10 ml volumetric flask and then diluted to adjust volume with chloroform. The absorbance of the complex in chloroform was measured at 470 nm against the blank prepared as above, but without extract. Atropine is used as a standard, and the assay is with Atropine equivalents.

b) Total flavonoid content
Total flavonoid content was determined by the Aluminium chloride method using catechin as a standard (Saeed *et al.*, 2012). 1 ml of the test sample and 4 ml of water were added to a volumetric flask (10 ml volume). After 5 min, 0.3 ml of 5 % Sodium nitrite, and 0.3 ml of 10% Aluminium chloride were added. After 6 min incubation at room temperature, 2 ml

of 1 M Sodium hydroxide was added to the reaction mixture. Immediately the final volume was made up to 10 ml with distilled water. The absorbance of the reaction mixture was measured at 510 nm against a blank spectrophotometrically. Results were expressed as catechin equivalents (mg catechin/g dried extract).

c) Total Tannins content

The tannin contents or Proanthocyanidin were determined by the method of Broadhurst *et al*. (1978) with slight modification, using tannic acid as a reference compound. 1 ml of the extract was mixed with 5 ml of vanillin hydrochloride reagent (mix equal volumes of 8% HCL in methanol and 4% vanillin in methanol). The reaction mixer was allowed to stand for 20 mins and measure the absorbance at 500 nm. The standard graph was plotted for working standard tannic acid solution.

d) Total Phenolic content

The total phenolics content in different solvent extract was determined with the Folin- Ciocalteu's reagent (FCR) (Saeed *et al.*, 2012)**.** In the procedure, different concentrations of the 1 ml of the extract were mixed with 0.4 ml FCR (diluted 1:10 v/v). After 5 min, 4 ml of 7% sodium carbonate solution was added. The final volume of the tubes was made up to 10 ml with distilled water and allowed to stand for 90 min at room temperature. The absorbance of sample was measured against the blank at 750 nm using a spectrophotometer. A calibration curve was constructed using Gallic acid solutions as standard.

e) Total Terpenoid content

Total terpenoid content was determined by the method of Ghorai *et al.* (2012). To 1 mL of the extract, 3 mL of chloroform was added. The sample mixture was thoroughly vortexed and left for 3 min and then 200 µl of concentrated sulfuric acid (H_2SO_4) was added. Then it was incubated at room temperature for 1.5h - 2h in dark conditions and during incubation a reddish-brown precipitate was formed. Then carefully and gently, all supernatant of the reaction mixture was decanted without disturbing the precipitation. 3 mL of 95% (v/v) methanol was added and vortexed thoroughly until all the precipitation dissolved in methanol completely. The absorbance was read at 538 nm using a UV/visible spectrophotometer. The total terpenoid content was calculated by the calibration curve of Linalool and the results were expressed as Linalool equivalent (mg/g).

f) Total Glycoside content

Take 10 ml of the extract and 10 ml of Baljet's reagent were taken and allowed to stand for one hour. Then dilute the solution with 20 ml distilled water and mix. Read the intensity of the colour obtained against blank at 495 nm using a spectrophotometer. The difference between test and control is taken for calculation. The standard graph can be prepared using standard digitoxin (Solich *et al.*, 1992).

g) Total Steroid content

1 ml of test extract of steroid solution was transferred into 10 ml volumetric flasks. Sulphuric acid (4N, 2 ml) and iron (III) chloride (0.5% w/v, 2 ml), were added, followed by potassium hexacyanoferrate (III) solution (0.5% w/v, 0.5 ml). The mixture was heated in a water bath maintained at 70±20° C for 30 minutes with occasional shaking and diluted to the mark with distilled water. The absorbance was measured at 780 nm against the reagent blank. The total steroid content was calculated by the calibration curve of Gallic acid and the results were expressed as Gallic acid equivalent (mg/g) (Devanaboyina *et al.*, 2013).

h) Total Carbohydrate content

The total sugar content was estimated by the anthrone method (Roe, 1955). A known amount of the sample was taken, ground well with 80% ethanol and was centrifuged at 4000 rpm. From the supernatant, 0.5 ml was taken and 5 ml of anthrone reagent was added. The tubes were kept in a boiling water bath for 15 min. After that, they were kept in a dark room for another 15 minutes. The colour intensity developed was read in a spectrophotometer at 650 nm.

i) Total protein content

Protein content was estimated by the method of Lowry *et al.* (1951). 1 ml of sample was mixed with 0.5 ml of 0.1 N sodium hydroxide and 5 ml of alkaline copper reagent. The mixture was incubated in room temperature for 30 minutes. Folin– Ciocalteau reagent, 0.5 ml was added and incubated again for 10 minutes at room temperature. The absorbance was read at 660 nm against a reagent blank. The estimation was done in triplicates and the results were expressed mg/g sample.

j) Total Reducing sugar content

The total Reducing sugar content was estimated by the method of Jain *et al.*, (2020). Take 3 ml of sample in a screw-capped tube and 1 mL of DNSA reagent was added. Blank solution is prepared by adding 1 mL of DNS reagent to 3 mL of distilled water. Place the tubes in a boiling water bath for 5 min and cool at room temperature. A red-brown colour develops. Add 1 mL of sodium potassium tartrate solution to obtain a stable colour. Read the absorbance of the tube at 540 nm against blank. The standard curve was prepared by using glucose as a standard and used to determine the concentration of reducing sugar in an unknown sample**.**

k) Total starch content

To evaluate the starch content, the residue from extraction of soluble carbohydrate was dried. The residue was suspended in 5 ml of distilled water and 6.5 ml of 52% perchloric acid. After 15 min, the mixture was centrifuged at 2000 g for 10 min. the procedure was repeated three times and the supernatants were gathered anddiluted to the final volume of 100

ml. 2.5 ml of extract was analyzed for starch content following the same procedure as that of soluble carbohydrate (McCready *et al.*, 1950)

2.4 GC-MS analysis of phytocomponents

GC-MS analysis of the ethyl acetatet extract of whole plant dry powdered material of*S. hamata* was performed using a Perkin Elmer GC Clarus 500 system comprising AOC-20i auto-sampler and a Gas Chromatograph interfaced to a Mass spectrometer (GC-MS) equipped with an Elite – 5MS (5 % Diphenyl/95 % Dimethyl Poly Siloxane) fused capillary column (30 x 0.25 µm IDx0.25 µm df). For GC-MS detection, an electron ionization system was operated in electron impact mode with ionization energy of 70 eV. Helium gas (99.999 %) was used as carrier gas at a constant flow rate of 1.491 ml/min, and an injection volume of 2 µl was employed (split ratio of 10:1). The injector temperature was maintained at 250˚C, the ion- source temperature was 200ºC, and the oven temperature was programmed from 110˚C with an increase of 10˚C/min to 200˚C, then 5˚C/min to 280˚C, ending with a 9 min isothermal at 280˚C. Mass spectra were taken at 70eV, a scanning interval of 0.5 seconds, and fragments from 45-450 Da. The solvent delay was 0 to 2 min and the total GC-MS running time was 36 min. The relative percentage amount of each component was calculated by comparing its average peak area to the total area. The mass detector used in this analysis was Turbo-Mass- Gold-Perkin Elmer and the software adopted to handle mass spectra and chromatograms was GC-MS solution ver-2.53.

2.5 Assessment of *in vitro* antioxidant activity

The whole plant, dried powder of *S. hamata* was extracted successively with , ethyl acetate solvents. The ethyl acetate extract was evaporated to dryness, stored at 4ºC, and used for analysing free scavenging activity such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Blois, 1958) and Ferric reducing antioxidant power FRAP (Pulido *et al.* 2000).

a) DPPH assay

Ethyl acetate extract of *S. hamata* at various concentrations (50 - 250 mg) were added separately to 5 ml of a 0.1 mM methanol solution of DPPH and allowed to stand for 20 min at 27˚C. The absorbance of the sample was measured at 517 nm using a spectrophotometer. Radical scavenging activity was expressed as the inhibition percentage of free radical by the sample and was calculated using the formula: DPPH radical scavenging activity (%) = (Control OD – Sample OD / Control OD) x 100. Half-maximal inhibitory concentration (IC50) value was calculated using linear regression analysis.

b) FRAP assay

The FRAP assay of ethyl acetate extract of *S. hamata* was determined by Gashaye and Birhan (2023) with slight modification. Each extract sample 2.5 mL of 0.2 M sodium phosphate buffer (pH = 6.6) and 2.5 mL of 1% potassium ferricyanide [K3Fe(CN)6] solution was added. The reaction mixture was vortexed well and then incubated at 50°C for 20 min. Then 2.5 mL of trichloroacetic acid was added and the solution was centrifuged for 10 min at 3000 rpm. The supernatant (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride solution. Moreover, 2.5 mL of distilled water was used as a blank solution. The absorbance of the colored solutions was recorded at 700 nm against the blank using a UV– vis spectrophotometer. Herein, AA was used as a reference standard and the reducing power of each sample was compared with the reference standard solution (prepared in the same way as the extract samples). The percentage inhibition of FRAP was calculated as follows.

FRAP (%) = (Sample OD – Blank OD / Sample OD) x 100

3 Result

3.1 Qualitative Phytochemical Analysis

The qualitative phytochemical analysis of the ethyl acetate extract of *S. hamata* shows the presence of alkaloids, flavonoids, tannin, phenol, saponin, terpenoids, glycosides, steroids, carbohydrates, protein, reducing sugars and starch except saponin (Table 1).

3.2 Quantitative Phytochemical Analysis

The quantitative estimation of ethyl acetate extract of *S. hamata* showed the presence of alkaloid (36.3±0.04 mg/g), flavonoid (88.2±0.04 mg/g), tannin (62.46±0.04 mg/g), phenol (38.1±0.01 mg/g), terpenoids (20.23±0.04 mg/g), glycoside (22.86±0.04 mg/g), steroids (2.28±0.02 mg/g), carbohydrates (60.23±0.04 mg/g), protein (96.6±0.04 mg/g), reducing sugars (2.83±0.009 mg/g) and starch (27.43±0.04 mg/g) (Table 2).

3.3 Gas chromatography-mass spectrometry (GC-MS) analysis

GC-MS analysis of the ethyl acetate extract of *S. hamata* demonstrates the presence of the various phytoconstituents having different biological activities such as anti-oxidant, anti- proliferative, anti-bacterial, anti-fungal, analgesic, anti-leukemic, anti-hepatoprotective, anti- hypertensive, anti-inflammatory, anti-nociceptive and anthelmintic. The peaks in the chromatogram were integrated and compared with the database of a spectrum of known compounds available in the GC-MS library. The details of the results of GC-MS analysis of ethyl acetate extract were given in Table 3 and Fig. 1, such as molecular formula, molecular weight, structure and biological activity are reported. The phytocompounds present in the GC- MS analysis of ethyl acetate extract of *S. hamata* were acetic acid ethyl ester, azulene, 1-(1,5-dimethyl-4-hexenyl)-4-met, diethyl phthalate, 2,6,10-trimethyl,14-ethylene-14-p, dibutyl phthalate, l-(+)-ascorbic acid 2,6-dihexadecanoate, octadecanoic acid, phthalic acid, di(2- propylpentyl) ester, 1,3-benzene dicarboxylic acid, bis (2-

ethylhexyl), Octadecanoic acid, 2- (hexadecyloxy)ethyl ester.

3.4 Antioxidant activity of ethyl acetate extract of *S. hamata* using various assays

The free radical scavenging activity of the ethyl acetate extract of the *S. hamata* is carried out using DPPH and FRAP

a) DPPH assay

DPPH free radical scavenging activity of ethyl acetate extract of S. *hamata*, while ascorbic acid was used as a standard, and the recorded data were presented in Table 3.4. The result concluded that the percentage of scavenging activity was concentration-dependent the half maximal inhibitory concentration (IC_{50}). The IC_{50} value of ethyl acetate extract scavenging activity (106.13 μg/ml).

b) FRAP assay

FRAP free radical scavenging activity of ethyl acetate of *S. hamata*, while ascorbic acid is used as a standard, and the recorded data are presented in Table 3.5. has the highest IC_{50} value of ethyl acetate extract scavenging activity (133.03 μg/ml).

Table 1 Qualitative phytochemical analysis of Ethyl acetate extract of *S. hamata*

S. No.	Tests	Ethyl acetate extract of *S. hamata*
1.	Alkaloid	+
2.	Flavonoid	+
3.	Tannin	+
4.	Phenol	+
5.	Saponin	-
6.	Terpenoids	+
7.	Glycoside	+
8.	Steroids	+
9.	Carbohydrate	+
10.	Protein	+
11.	Reducing sugars	+
12.	Starch	+

Present (+); Absent (-)

Table 2 Quantitative phytochemical analysis of Ethyl acetate extract of *S. hamata*

S. No.	Tests	Ethyl acetate extract of S. hamata (mg/g)
1.	Alkaloid	36.3±0.04
2.	Flavonoid	88.2±0.04
3.	Tannin	62.46±0.04
4.	Phenol	38.1±0.01
5.	Terpenoids	20.23±0.04
6.	Glycoside	22.86±0.04
7.	Steroids	2.28±0.02
8.	Carbohydrate	60.23±0.04
9.	Protein	96.6±0.04
10.	Reducing sugars	2.83±0.009
11.	Starch	27.43±0.04

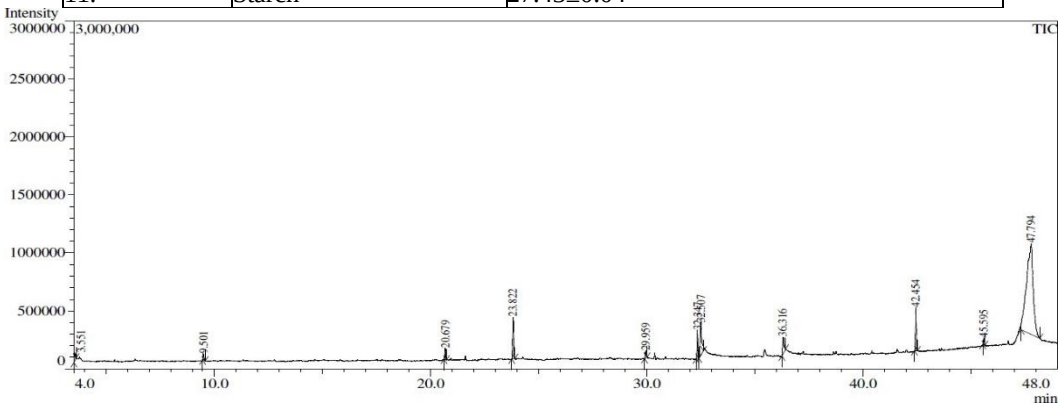
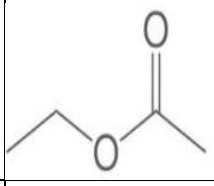
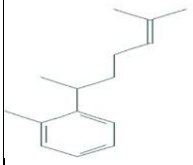
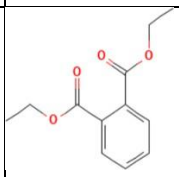
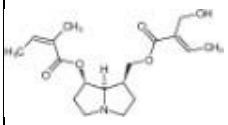
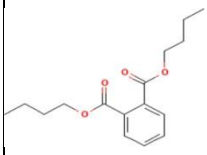
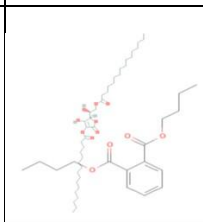
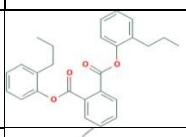
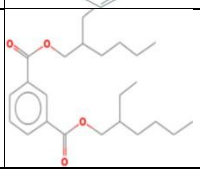
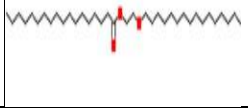


Figure 1 Chromatogram for GC-MS analysis of ethyl acetate extract of *S. hamata*

Table 3. 3 GC-MS analysis of ethyl acetate extrct of *S. hamata*

S. No	Name	Retention time	Area %	Molecular formula	Molecular weight (g/mol)	Structure	Biological activity Reported
1	Acetic acid ethyl ester	3.551	1.01	C4H8O2	88.11		-
2	Azulene	9.501	0.76	C10H8	128.17		Anti- inflammatory, Antimicrobial and Anticancer (Leino et al., 2022)
3	1-(1,5-Dimethyl-4-hexenyl)-4-methylbenzene	20.679	1.37	C15H22	202.33		Anti- inflammatory and Antimicrobial (Sultana et al., 2021)
4	Diethyl Phthalate	23.822	4.47	C12H14O4	222.24		Antimicrobial (Premjanu, 2014)
5	2,6,10-Trimethyl,14-Ethylene-14-pentadecane	29.959	0.84	C18H27NO5	337.41		Antiproliferative (Arora and Meena, 2017)
6	Dibutyl phthalate	32.347	2.76	C16H22O4	278.34		Antimicrobial (Roy et al., 2006)
7	l-(+)-Ascorbic acid 2,6-dihexadecanoate	32.507	6.45	C38H68O8	652.9		Antioxidant, anticancer and anti-infertility (Hadi et al., 2016)
8	Octadecanoic acid	36.316	2.73	C18H36O2	284.5		Antimicrobial and Antioxidant (Sudharsan et al., 2015)
9	Phthalic acid, di(2-propylpentyl) ester	42.454	4.58	C26H26O4	402.5		Antimicrobial and Anti- inflammatory (Cherian and Vadivel, 2023)
10	1,3-Benzenedicarboxylic acid, bis(2-ethyl hexyl)	45.595	0.64	C24H38O	390.55		Anticancer (Krishnan et al., 2014)

11	Octadecanoic (hexadecyloxy)ethyl ester	47.794	74.41	$C_{36}H_{72}O_3$	553.0		Antioxidant and Anti-inflammatory (Ganesh and Mohankumar, 2017)

\

Table 30. 4 DPPH free radical scavenging activity of ethyl acetate extract of *S. hamata*

Successive extract used	Concentrations			IC50Value
	100 µg/ml	200 µg/ml	300 µg/ml	
Ethyl acetate	47.30 ± 0.86	74.99 ± 0.87	95.12 ± 0.95	106.13
Standard (Ascorbic acid)	59.59 ± 0.97	86.25 ± 0.97	119.54 ± 0.98	71.69

The values are means of 3 independent analyses ± standard deviation (n=3). *-Significance at **1% level**

Table 3.5 FRAP free radical scavenging activity of ethyl acetate extract of *S. hamata*

Successive extract used	Concentrations			IC50Value
	100µg/ml	200µg/ml	300µg/ml	
Ethyl acetate	45.58±0.94 [a]	59.816 ± 087 [c]	70.83 ± 0.97 [e]	133.03 [a]
Standard (Ascorbic acid)	52.51 ± 0.96 [a]	70.17± 0.95 [a]	92.16 ± 0.94 [h]	93.35 [a]

The values are means of 3 independent analysis ± standard deviation (n=3). *-Significance at **1% level**

4 DISCUSION

The present study revealed that the qualitative analysis of ethyl acetate extract of *S. hamata* revealed the presence of several secondary metabolites, including alkaloids, flavonoids, tannins, phenols, terpenoids, glycosides, steroids, carbohydrates, proteins, lipids, reducing sugars, and starch.

Mardina *et al*. (2020) conducted a qualitative phytochemical screening of the ethyl acetate extract derived from the entire plant of *Wedelia biflora* (L.) DC. Their study identified the presence of alkaloids, flavonoids, and steroids as the primary secondary metabolites. while tannins were absent which was similar to the results of the present study. Phytochemical qualitative analysis of the whole plant ethyl acetate of *Ruellia tuberosa* L. showed the presence of secondary metabolites such as flavonoid, tannin, phenol, steroids absent of alkaloids (Arirudran *et al*., 2011). Murugan and Mohan (2014) conducted a qualitative phytochemical analysis of ethyl acetate derived from the entire *Aerva lanata* (L.) Juss. Their findings revealed the presence of various secondary metabolites such as alkaloids, flavonoids, terpenoids, tannins, phenols, glycosides, and steroids. A comparison with our study indicates that the ethyl acetate extract of the *A. lanata* plant lacks tannins, phenols, and glycosides.

The present study revealed a quantitative analysis of ethyl acetate extract of *S. hamata*. The ethyl acetate extract demonstrated that proteins were the most quantified, followed by flavonoids, tannins, carbohydrates, phenols, alkaloids, starch, glycosides, terpenoids, reducing sugars, and steroids. Roghini and Vijayalakshmi (2018) conducted a quantitative phytochemical analysis of three distinct extract from the *Citrus paradisi* plant. The findings indicated that the ethyl acetate extract of *C. paradisi* demonstrated the highest quantification of phenols, followed by alkaloids, tannins, flavonoids, and saponinsUpon comparing these findings to our present study, it is evident that the quantity of alkaloids, tannins, and flavonoids in our current research is notably higher. however, Saponin is absent in ethyl acetate extract of *S. hamata*.

In the present study, the GC-MS analysis of the entire plant ethyl acetate extract of *S. hamata* revealed the presence of eleven compounds. In general, the identified compounds have biological properties such as antimicrobial, anti-inflammatory, anti-ulcer, and anti-oxidative properties. In a study conducted by Perumal *et al*. (2021), the GC-MS analysis of the ethyl acetate extract of *Flueggea leucopyrus* revealed the presence of eleven bioactive phytocompounds. This finding is consistent with our current study of the ethyl acetate extract of *S. hamata*, which also identified a similar number of phytocompounds. The analysis by gas chromatography-mass spectrometry (GC-MS) of the ethyl acetate extract of *Justicia carnea* identified the presence of bioactive compounds, including 2-butyne-1,4-diol diformate and Bicycle [2.2.1] heptan-2-ol,1,7,7-trimethyl-, (1 S-endo) (Okocha et al., 2023). The number of bioactive compounds detected was lower compared to our present study. Al-Malki *et al*. (2015) conducted a GC-MS analysis of the ethyl acetate extract of *Balanites aegyptiaca*. Their findings revealed the presence of three known bioactive components: Vanillic acid, Syringic acid, and β-sitosterol. It was observed that the number of bioactive compounds detected in their study was lower in comparison to our current research. Baeshen *et al*. (2023) conducted a GC-MS analysis of the ethyl acetate extract of *Rhazya stricta* and reported the presence of ten bioactive compounds. Notably, the quantity of bioactive compounds identified in their study was fewer compared to our present study.

In the present study, ethyl acetate extract of *S. hamata* showed the antioxidant activity in dose dose-dependent manner. In the DPPH assay, the ethyl acetate showed IC_{50} value of 106.13 µg/mL. Similarly, the IC_{50} value in the FRAP assay was found to be 133.03 µg/mL. The antioxidant activity of *Citrus sinensis* (L.) Osbeck ethyl acetate extract was tested using DPPH radical method, with concentrations ranging from

50 to 1000 µg/ml. The DPPH method showed 53.37±0.90% inhibition at 1000 µg/ml for the ethyl acetate extract. The ethyl acetate extract showed an IC_{50} value of 511.63 µg/ml (Gulo *et al.*, 2022). Ghanimi *et al*., (2022) used the FRAP assay to assess the antioxidant activity of ethyl acetate extract of *Lavandula mairei* Humbert aerial part of the plant. The extract demonstrated the maximum antioxidant activity of 0.786±0.07 mg/ ml.

5. Conclusions

In conclusion, this study represents the first comprehensive evaluation of the antioxidant potential of the ethyl acetate extract derived from *Stylosanthes hamata* (L.) Taub. Our results revealed a rich profile of phytochemicals, with markedly higher concentrations of flavonoids ($P < 0.05$) compared to other bioactive constituents. Notably, the abundant presence of flavonoids, along with significant levels of tannins and phenols, suggests that these compounds play a crucial role in mediating the extract's potent antioxidant activity. *S. hamata* shows promise as a natural source of high-value antioxidants, meriting further research on purification and biological activities for potential applications in nutraceuticals and therapeutics.

References

1. Badmus, J. A., Rafiu, M. A., & Fatoki, J. O. (2022). The protective effect of ethanol leaf extract of Annona muricata against doxorubicin toxicity via modulations of hematological, serum biochemical, antioxidant enzymes, and lipid peroxidation. *Phytomedicine Plus*, *2*(3), 100328.
2. Irivibulkovit, K. S., Ouanthavong, S. N., & Ameenoi, Y. S. (2018). Paper-Base DPPH Assay Antioxidant Activity Analysis. *Analytical Sciences*, *34*(July), 795–800.
3. Gohari, A. R., Hajimehdipoor, H., Saeidnia, S., Ajani, Y., & Hadjiakhoondi, A. (2011). Antioxidant activity of some medicinal species using FRAP assay.
4. Mahdi-Pour, B., Jothy, S. L., Latha, L. Y., Chen, Y., & Sasidharan, S. (2012). Antioxidant activity of methanol extract of different parts of Lantana camara. *Asian Pacific journal of tropical biomedicine*, *2*(12), 960-965.
5. Shalini, K., & Ilango, K. J. P. J. (2021). Preliminary phytochemical studies, GC-MS analysis and in vitro antioxidant activity of selected medicinal plants and its polyherbal formulation. *Pharmacognosy Journal*, *13*(3).
6. Gulo, K. N., Saragih, A. D., Raif, M. A., & Ikhtiari, R. (2021, April). Antioxidant Activity of flavonoid compounds in ethanol and ethyl acetate extract from Citrus sinensis. In *2021 International Conference on Artificial Intelligence and Mechatronics Systems (AIMS)* (pp. 16). IEEE.
7. Ghanimi, Ridwane, Ahmed Ouhammou, and Yassine El Atki. "Antioxidant and Antibacterial Activities of Essential Oils from Three Moroccan Species (Lavandula Mairei Humbert , Lavandula Dentata L . and , Lavandula Stoechas L .)." *Journal of Pharmaceutical Research International*. 2021;33(Mic):64–71.
8. Harborne, J. B. (1998). Phytochemical methods: a guide to modern techniques of plant analysis. Chapman and Hall.
9. Deshpande, P. K., Gothalwal, R., & Pathak, A. K. (2014). Phytochemical analysis and evaluation of antimalarial activity of Azadirachta indica. *The Pharma Innovation*, *3*(9, Part A), 12.
10. Gul, R., Jan, S. U., Faridullah, S., Sherani, S., & Jahan, N. (2017). Preliminary phytochemical screening, quantitative analysis of alkaloids, and antioxidant activity of crude plant extract from Ephedra intermedia indigenous to Balochistan. *The Scientific World Journal*, *2017*(1), 5873648.
11. Singh, V., & Kumar, R. (2017). Study of phytochemical analysis and antioxidant activity of Allium sativum of Bundelkhand region. *International Journal of Life- Sciences Scientific Research*, *3*(6), 1451-1458.
12. Mukherjee, P. K. 2002. Quality control of herbal drugs, business horizons pharmaceutical publishers, New Delhi. 356 - 358.
13. Ansari, S. H. 2006. Essentials of Pharmacognosy, 1st edition, Birla publications, New Delhi. pp. 357-359, 588-590.
14. Horbone, J.B., In: Phytochemical methods, 2nd edition. Chapman and Hall, New York,1984.
15. Ansari, S. H. 2006. Essentials of pharmacognosy, 1st edition, Birla publications, New Delhi. pp. 357-359, 588-590.
16. De Silva, G. O., Abeysundara, A. T., and Aponso, M. M. W. (2017). Extraction methods, qualitative and quantitative techniques for screening of phytochemicals from plants. American Journal of Essential Oils and Natural Products, 5(2), 29-32.
17. Zohra, S. F., Meriem, B., Samira, S., and Muneer, M. A. (2012). Phytochemical screening and identification of some compounds from mallow. J Nat Prod Plant Resour, 2(4), 512-6.
18. Devanaboyina N et al., "Preliminary Phytochemical Screening, Quantitative Estimation and Evaluation of Antimicrobial Activity of Alstoniamacrophylla Stem Bark" IJSIT, 2013, 2(1), 31-39.
19. Saeed, N., Khan, M. R., and Shabbir, M. (2012). Antioxidant activity, total phenolic and total flavonoid contents of whole plant extract Torilis leptophylla L. *BMC complementary and alternative medicine*, *12*, 1-12.
20. Sim, E. W. (2011). *Isolation and determination of anti-nutritional compunds from root and shells of Arachis Hypogaea* (Doctoral dissertation, UTAR).
21. Ghorai, N., Chakraborty, S., Gucchait, S., Saha, S. K., & Biswas, S. (2012). Estimation of total Terpenoids concentration in plant tissues using a monoterpene, Linalool as standard reagent.
22. Solich, P., Sedliakova, V., & Karlíček, R. (1992). Spectrophotometric determination of cardiac glycosides by flow-injection analysis. *Analytica chimica acta*, *269*(2), 199-203.
23. Narendra Devanaboyina, RamaLakshmi N, Satyanarayana B, sudeepthi P, Hemachakradhar K, PavankumarRaju N. Preliminary phytochemical screening, quantitative estimation and evaluation of antimicrobial activity of Alstoniamacrophylla stem bark. International Journal of Science Inventions today.2013; 2(1): 31-39

24. Roe, J. H. (1955). The determination of sugar in blood and spinal fluid with anthrone reagent.
25. Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent.
26. Jain, A., Jain, R., and Jain, S. (2020). *Basic techniques in biochemistry, microbiology and molecular biology* (pp. 9-10). New York, NY, USA:: Springer.
27. McCready, R. M., Guggolz, J., Silviera, V., & Owens, H. S. (1950). Determination of starch and amylose in vegetables. *Analytical chemistry*, *22*(9), 1156-1158.
28. Blois, M. S. (1958). Antioxidant determinations by the use of a stable free radical. *Nature*, *181*(4617), 1199-1200.
29. Pulido, R., Bravo, L., & Saura-Calixto, F. (2000). Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. *Journal of agricultural and food chemistry*, *48*(8), 3396-3402.
30. Leino, T. O., Sieger, P., Yli-Kauhaluoma, J., Wallén, E. A., & Kley, J. T. (2022). The azulene scaffold from a medicinal chemist's perspective: Physicochemical and in vitro parameters relevant for drug discovery. *European Journal of Medicinal Chemistry*, *237*, 114374.
31. Sultana, A.; Hasan, M.; Rahman, M.; Alam, M.M. 2021.Healing potentials of Marigold flower (*Tagetes erecta*) on full thickness dermal wound in caprine model. *Eur. Respir. J.*, *7*, 332–339.
32. Premjanu, N., & Jaynthy, C. (2014). Antimicrobial activity of diethyl phthalate: An insilico approach. *Asian J Pharm Clin Res*, *7*(4), 141-142.
33. Arora, S., & Meena, S. (2017). GC-MS Profiling of Ceropegia bulbosa Roxb. var. bulbosa, an endangered plant from Thar Desert, Rajasthan. *The Pharma Innovation Journal*, *6*(11), 568-573.
34. Roy, D., Rowe, B. H., Stiell, I. G., Coutu, B., Ip, J. H., Phaneuf, D., ... & Craft Investigators. (2004). A randomized, controlled trial of RSD1235, a novel anti- arrhythmic agent, in the treatment of recent onset atrial fibrillation. *Journal of the American College of Cardiology*, *44*(12), 2355-2361.
35. Hadi, I., & Hussein, H. M. (2016). Antimicrobial Activity and spectral chemical analysis of methanolic leaves extract of Adiantum Capillus-Veneris using GC-MS and FT-IR spectroscopy. *Int J Pharmacogn Phytochem Res*, *8*(3), 369-385.
36. Sudharsan, S., Subhapradha, N., Seedevi, P., Shanmugam, V., Madeswaran, P., Shanmugam, A., & Srinivasan, A. (2015). Antioxidant and anticoagulant activity of sulfated polysaccharide from Gracilaria debilis (Forsskal). *International Journal of Biological Macromolecules*, *81*, 1031-1038.
37. Cherian, A., Vadivel, V., Thiruganasambandham, S., & Madhavankutty, S. (2023). Phytocompounds and their molecular targets in immunomodulation: a review. *Journal of Basic and Clinical Physiology and Pharmacology*, *34*(5), 577-590.
38. Krishnan, K., Mathew, L. E., Vijayalakshmi, N. R., & Helen, A. (2014). Anti- inflammatory potential of β-amyrin, a triterpenoid isolated from Costus igneus. *Inflammopharmacology*, *22*, 373-385.
39. Ganesh, M., & Mohankumar, M. (2017). Extraction and identification of bioactive components in Sida cordata (Burm. f.) using gas chromatography–mass spectrometry. *Journal of food science and technology*, *54*, 3082-3091.
40. Mardina, V., Harmawan, T., Ilyas, S., Tanjung, M., Aulya, W., & Nasution, A. (2020). Preliminary phytochemical screening of different solvent extract of flower and whole plant of Wedelia biflora. In *IOP Conference Series: Materials Science and Engineering* (Vol. 725, No. 1)..
41. Arirudran, B., Saraswathy, A., & Krishnamurthy, V. (2011). Pharmacognostic and preliminary phytochemical studies on *Ruellia tuberosa* L.(whole plant). *Pharmacognosy Journal*, *3*(22), 29-34.
42. Murugan, M., & Mohan, V. R. (2014). Phytochemical, FT-IR and antibacterial activity of whole plant extract of *Aerva lanata* (L.) Juss. Ex. Schult. *Journal of Medicinal Plants Studies*, *4*(3), 51-57.
43. Roghini, R., & Vijayalakshmi, K. (2018). Phytochemical screening, quantitative analysis of flavonoids and minerals in ethanolic extract of *Citrus paradisi*. *International Journal of Pharmaceutical Sciences and Research*, *9*(11), 4859- 4864.
44. Perumal, G. M., Prabhu, K., Janaki, R. M., Kalaivannan, J., & Kavimani, M. (2021). The GC MS Analysis of Ethyl Acetate Extract Of '*Flueggea Leucopyrus*. *nveo-natural volatiles & essential oils Journal. NVEO*, 4035-4040.
45. Okocha, B. I., Orie, K. J., Duru, R. U., & Ngochindo, R. L. (2023). Analysis of the active metabolites of ethanol and ethyl acetate extract of *Justicia carnea*. *African Journal of Biomedical Research*, *26*(1): 109-117.
46. Baeshen, Nabih A., Yaaser Q. Almulaiky, Mohamed Afifi, Ammar Al-Farga, Haytham Ali, Naseebh N. Baeshen, Mosleh M. Abomughaid, Aaser M. Abdelazim, and Mohammed N. Baeshen. 2023. "GC-MS analysis of bioactive compounds extracted from plant *Rhazya stricta* using various solvents." Plants 12, no. 4: 960.
47. Ghanimi, R., Ouhammou, A., El Atki, Y., Bouchari, M. E. H., & Cherkaoui, M. (2022). The antioxidant activities of ethanolic, methanolic, ethyl acetate, and aqueous extract of the endemic species, Lavandula mairei Humbert (a comparative study between cold and hot extraction). Ethiopian journal of health sciences, 32(6).