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Phytochemical Profiling and GC-MS Analysis of Ethanolic Extract of Phyllanthus Acidus Leaves

[1]Nimya Jayaprasad, [1]Anjaly Nair G, [1]Akshaya C.S, [1]Anagha K.O, [1]Aneeta Paul
ELIMS College of Pharmacy, Ramavarmapuram,Villadam,Thrissur,Kerala

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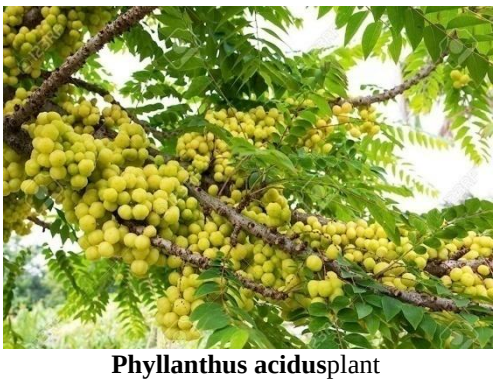
ABSTRACT: This study aim to determine the phytochemical profile of ethanolic extract of leaves of phyllanthus acidus using GC-MS. Phyllanthus acidus L. Skeels is a tropical tree belonging to the Phyllanthaceae family.Phytochemical screening, total bioactive content, and GC-MS analysis were used to determine its phytoconstituents profile. The plant extract exhibited the maximum total phenolic (35.61 µg of GAE/g) and total flavonoid contents (305.5 µg of QE/g), which may be related to the higher antioxidant potential of the extract. The GC-MS analysis of the extract provided the preliminary identification of 21 phytocompounds.

Keywords:GC-MS, Phyllanthus acidus, Phytochemical screening

I. I.INTRODUCTION

Plants are put to medicinal use all over the world since time immemorial. The importance of medicinal plants and traditional health systems in solving the health care problems of the World as gaining increased attention. Due to this resurgence of interest, the research on plants of medicinal importance is growing tremendously. Historically, all medicinal preparations were derived from plants, whether in the simple form of raw plant materials or in the refined form of crude extracts and mixtures[1].Phyllanthus acidus L. Skeels is a tropical tree belonging to the Phyllanthaceae family.Commonly known as Otaheite/Tahitian/star/country/West Indian/Malay gooseberry, the tree of Madagascan origin reaches 5-9 m high and is distributed in south eastern Asian countries. Yellow, waxy, crisp, juicy, acrid, single-seeded fruits with six to eight ribs are borne in thick clusters and are edible. The bark is rough and gray producing small, dense, pink flowers. Flowering and fruiting occur between January-May. Fruits are densely clustered having a diameter of 2 cm. The tree is raised in home gardens in India, Thailand, Taiwan, Philippines, Malaysia, Brazil, Indonesia, SriLanka, Australia, and Venezuela; it is grown with mango in Trinidad and Tobago.The tree prefers moist soil and is propagated by seeds or by budding, greenwood cuttings, and air layering[2].Extraction of leaves of phyllanthus acidus was performed by soxhlet extraction using ethanol as solvent.

Gas chromatography –mass spectrometry is an analytical method that combines the features of gas chromatography and mass spectrometry to identify different substances within a test sample.Gas chromatography Mass spectroscopy analysis of ethanolic extract was performed using Shimadzu GC-MS Model number: QP2010S equipped with Column - ELITE-5MS,30 meter length,0.25mm ID,0.25µm thickness.The oven temperature was programmed from 70.000C .Electron ionization system was used.Helium gas was used as the carrier gas. GC-MS Software: GCMS Solutions, Libraries used: NIST 11& WILEY 8.17[3].Phenolic compounds are important plant constituents with redox properties responsible for antioxidant activity . The hydroxyl groups in plant extracts are responsible for facilitating free radical scavenging.Phenolic content was measured using the Folin–Ciocalteu reagent in each extract.Flavonoids are secondary metabolites with antioxidant activity, the potency of which depends on the number and position of free OH groups. Flavonoid contents in selected plant extracts were determined using aluminium chloride in a colorimetric method[4].



Phyllanthus acidusplant

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II. MATERIALS AND METHODS

Plant collection, authentication and drying

The leaves of Phyllanthus acidus (L) Skeels were collected from rural areas ofThrissur, Kerala, India. The plant material was then dried under shade for about 20 days, powdered withmechanical grinder and stored in an air tight container.

Extraction

The plant material was shade dried and coarsely powdered. Around 100g of driedpowder was first soaked and defatted with petroleum ether. The dried marc wasmoistened with the solvent and packed in the Soxhlet extractor and was thenextracted by using 1000 ml ethanol for 5 hours. The extract was then filteredthrough Whatman No. 1 filter paper and concentrated. The extract obtained was thensubjected to qualitative and quantitative phytochemical analysis. The percentageyield of extract was found.

Qualitative phytochemical analysis

The ethanolic extract ofPhyllanthus acidus (EEPA) was subjected to standard phytochemical screening tests for establishing different constituents present in it.

1.Test for alkaloids

Small amount of EEPA was mixed with few ml of dilute Hydrochloric acid. Shakenwell and filtered. Following tests were performed with the obtained filtrate.

- Mayer's test: A few drops of Mayer's reagent (Potassium mercuric iodide solution) were added to 2-3 ml of filtrate. Cream (dull white) precipitate indicates the presence of alkaloids.
- Dragendorff's test: A few drops of Dragendorff's reagent (Potassium bismuth iodide solution) were added to 2-3 ml of filtrate. Orange red precipitate indicates the presence of alkaloids.
- Hager's test: A few drops of Hager's reagent (Picric acid) were added to 2-3 ml of filtrate. Yellow precipitate indicates the presence of alkaloids.
- Wagner's test: A few drops of Wagner's reagent (solution of Iodine in Potassium iodide) were added to 2-3 ml of filtrate. Reddish brown precipitate indicates the presence of alkaloids.

2.Test for carbohydrates

- Molisch's test: Few drops of Molisch's reagent were added to 2-3 ml of filtrate, followed by addition of concentrated Sulphuric acid along the sides of the test tube. Formation of violet colour at the junction of two liquids indicates the presence of carbohydrates.
- Benedict's test: Few ml of filtrate was mixed with equal volume of Benedict's reagent (alkaline solution containing cupric citrate complex) and heated in boiling water bath for 5 minutes. Formation of reddish brown precipitate infers the presence of reducing sugars.
- Fehling's test: 1 ml Fehling's-A (Copper sulphate in Distilled water) was added to 1 ml of Fehling's-B (Potassium tartarate and Sodium hydroxide in Distilled water) solution, boiled for one minute. To this added 1 ml of filtrate and heated gently. Formation of brick red precipitate indicates the presence of reducing sugars.

3.Test for steroids and sterols

- Salkowskireaction:A small quantity of the EEPA was mixed with 2 ml Chloroform and 2 ml concentrated Sulphuric acid. Shake it well. Chloroformlayer appears red and acid layer shows greenish yellow fluorescence.
- Liebermann- Burchardreaction:A small quantity of the EEPA was mixedwith Chloroform. To that mixture added 1-2 ml of acetic anhydride and 2drops of concentrated Sulphuric acid along the sides of the test tube. Thesolution becomes red, then blue and finally bluish green color.

4.Test for saponins

- Froth test: 5 ml of test sample was added to Sodium bicarbonate solution.After vigorous shaking the mixture, kept it for 3 minutes. A honey comb likefroth formation indicates the presence of saponins.
- Foam Test: A small quantity of the extract was diluted with 20 ml ofdistilled water and shaken it in a graduated cylinder for 3 minutes. Foam of1cm after 10 minutes indicates the presence of saponins.

5.Test for glycosides

- Keller-Killiani test: Glacial acetic acid was added to 2 ml extract, followedby the addition of trace quantity of Ferric chloride and 2 to 3 drops ofconcentrated Sulphuric acid. Reddish brown color appears at the junction oftwo liquid indicates the presence of cardiac glycosides.
- Legal's test: 1 ml of Pyridine and 1 ml of Sodium nitroprusside was addedto a small

International Journal of Pharmaceutical Research and Applications
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quantity of the extract. Pink to red color indicates the presence ofglycosides.

- Baljet test: A small quantity of the extract was added to Sodium picratesolution. Yellow to orange color formation indicates the presence ofglycosides.

6.Test for flavonoids

- Alkaline reagent test: A few drop of Sodium hydroxide solution wassupplemented to the extract. Development of an intense yellow color, whichturns to colorless on addition of few drops of dilute Hydrochloric acid,indicates the presence of flavonoids.

7.Test for proteins and aminoacids

- Ninhydrin test: A mixture of 3 ml test solution and 3 drops of 5% Ninhydrinsolution was heated in a boiling water bath for 10 minutes. Formation ofpurple or bluish color indicates the presence of free amino acids.
- Biuret test: 3 ml of test solution was added to 4% Sodium hydroxide andfew drops of 1% Copper sulphate solution. Formation of violet colorindicates the presence of proteins.

8.Test for tannins

- Lead acetate test: A few drop of Lead acetate was added to 5 ml of aqueousextract. Formation of yellow or red color precipitate indicates the presence of tannins[5] .

Estimation of Total Phenols

Reagents and chemicals

- Folin-ciocalteu reagent
- 7% Na2CO3
- Gallic acid
- De-ionized water

Method

Total phenols assay was conducted by mixing 2.7ml of de-ionized water 0.3ml of sample (EEPA) and standard (gallic acid),0.3ml of 7% of Na2CO3 and 0.15ml Folin-ciocalteu reagent. Absorbance of mixture was measured at 725nm.The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of Gallic acid and the calibration line was constructed. Based on the measured absorbance, the concentration of phenolics was read (µg/ml) from the calibration line; then the content of phenolics in sample was expressed in terms of gallic acid equivalent (mg of GA/g of sample).

Estimation of Total Flavanoids

Reagents and chemicals.

- Quercetin
- 10% AlCl3
- 1M NaOH
- 5% NaNO2
- Methanol
- Distilled water

Method

400 µl of EEPA was taken in a test tube and to this added 1600µl of deionized waterand 120 µl of sodium nitrite solution (5% w/v). After that, the mixture wasincubated for 6 min at room temperature. After incubation, 120 µl of aluminiumnitrate solution (10% w/v) was added and allowed to stand for another 6 min. Then added 800µl of NaOH solution (4% w/v) and made up the volume to 960µl with deionized water. The reaction mixture was kept in dark at room temperature for 15min. The intensity of the yellow colour developed indicated the concentration of the flavonoid content in the EEPA. The intensity of the developed pink colour was measured at 510 nm using a spectrophotometer. Total flavonoid content was calculated with the help of a standard curve with quercetin and the flavonoid content was expressed as mg quercetin equivalent (mg of QE/g of sample)[4]

GC-MS analysis

The plant material was shade dried, coarsely powdered and subjected toSoxhlet extraction by using solvent ethanol for 5 hours**.**The extract was filtered through Whatman No. 1 filter paper and concentrated. The extract obtained was then subjected to GC-MS analysis.Gas chromatography Mass spectroscopy analysis of ethanolic extract was performed using Shimadzu GC-MS Model number:QP2010S equipped withColumn - ELITE-5MS,30 meter length,0.25mm ID,0.25µm thickness.The oven temperature was programmed from 70.00˚C. Electron ionization system was usedand helium gas was used as the carrier gas. GCMS Software: GCMS Solutions, Libraries used: NIST 11& WILEY 8.17[3].

For the detailed phytochemical investigation of ethanolic extract, GC-MS analysis was performed. GCMS analysis of ethanolic extract of leaves of Phyllanthus acidus was carried out and a group of 21 compounds were identified.

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III. RESULTS AND DISCUSSION

Extractive yield

Percentage yield of extraction of leaves of Phyllanthus acidus obtained as tabulated below in thetable

Extract	Method of extraction	Extractive yield (%w/w)
Petroleum ether	Cold maceration	0.65
Ethanol	Soxhlet extraction	2.86

Table 5.Percentage yield of extract

The extractive yield of petroleum ether and ethanolic solvent extraction of the plant material was performed by cold maceration and soxhlation and a yield obtained was 0.65and2.86 % w/w respectively.

Qualitative phytochemical analysis

Tests	Petroleum ether extract	Ethanolic extract
Alkaloids	+	+
Carbohydrates	+	+
Steroids and sterols	+	+
Saponins	-	-
Glycosides	+	-
Phenols	+	+
Flavonoids	+	+
Proteins and aminoacids	-	-
Tannins	+	-

Table 6.Qualitative phytochemical analysis of extracts

Determination of Total Phenols

Total phenols present in the EEPA was determined by Folin -Ciocaltechu method using Standard Gallic acid (100 µg/ml)

Concentration (µg/ml)	Absorbance
30	0.67
60	0.91

International Journal of Pharmaceutical Research and Applications
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90	1.2
120	1.6
150	1.8
EEPA	0.7

Table 7.OD of Gallic acid at different concentrations

Concentration (µg/ml)	**Absorbance**
100	0.05
200	0.103
300	0.217
400	0.304
500	0.450
EEPA	0.230

Table 8.OD of Quercetin at different concentrations

ESTIMATION OF TOTAL PHENOLS

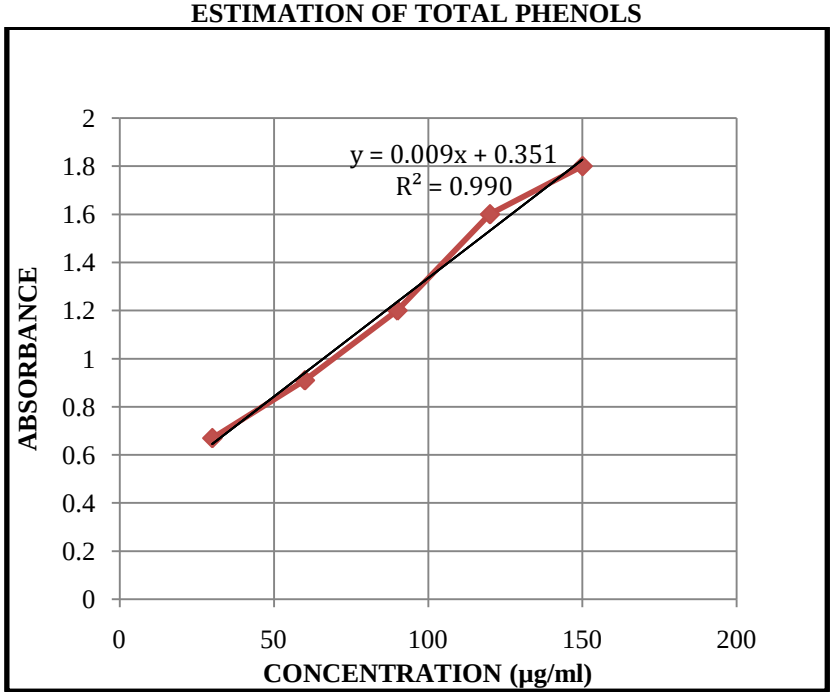

Fig 4.Standard graph of gallic acid

Antioxidants, derived from plant origin, especially flavonoids and polyphenols have been used to treat various disease such as aging, diabetic, cancer and prevention of cardiovascular diseases. The FolinCiocalteu method is a routine assay in studying phenolic antioxidants as it is rapid, convenient, simple and reproducible. A calibration curve was prepared as shown in **Figure 4**. The total phenolic content of EEPA was found to be **35.61 µg** of GAE/ mg of sample.

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Determination of Total Flavanoid

Total flavanoids present in the EEPA was determined by aluminium chloride colorimetric method using Standard -Quercetin (1mg/ml stock)

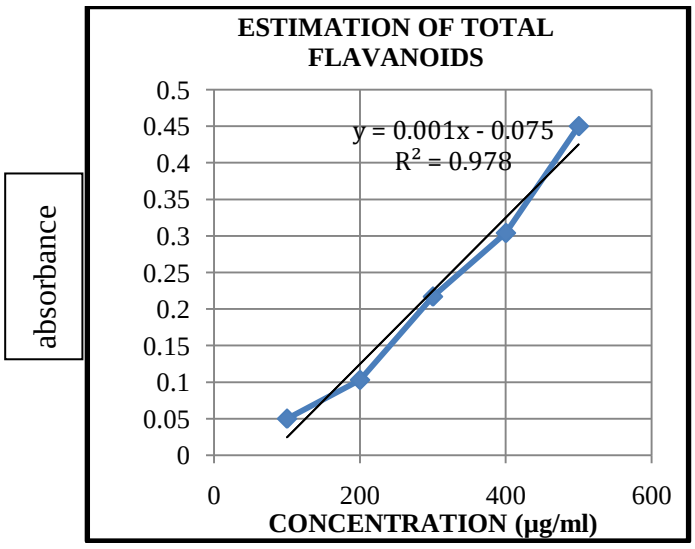

Fig 5.Standard graph of quercetin

Flavonoids are potent antioxidants and have aroused considerable interest recently because of their potential beneficial effects on human health in fighting degenerative diseases. The capacity of flavonoids to act as antioxidants depends upon their molecular structure.A calibration curve was prepared using quercetinas standard as shown in **Figure 5**. The total flavanoid content of EEPA was found to be 305.5 of QE/ mg of sample

GC-MS ANALYSIS

GCMS chromatogram of EEPA was obtained as follows:

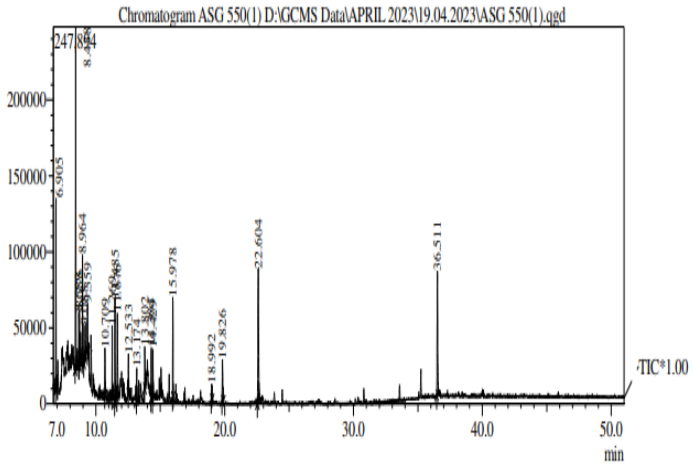

Fig 5.GC-MS chromatogram of EEPA

For the detailed phytochemical investigation of ethanolic extract, GC-MS analysis was performed. GCMS analysis of ethanolic extract of leaves of Phyllanthus acidus was carried out and a group of 21 compounds were identified. The identified compounds were found to be

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pharmacologically and biologically active. Naphthalene(19.36%), Ethylmethylmaleimide (5.92%) ,methylvinylmaleimide (2.73%), 1-Tridecene (2.35%) ,1-Tetradecene (3.96%),Isosorbide Dinitrite (2.88%) are the compounds identified.

Ethylmethylmaleimide , methylvinylmaleimide 1-Tridecene and 1-tetradecene are known to posses anti diabetic activity[6].Isosorbide Dinitrate is known to possess thrombolytic activity[7]. Naphthalene have both antidiabetic[8] and thrombolytic activity and antioxidant activity[9].Maleimide is known to posses antidiabetic activity[10].

Peak	R.Time	Area %	Height%	Name	Base m/z
1	6.905	8.27	10.79	1,1,3-TRIETHOXYBUTANE	73.10
2	8.438	19.07	19.36	NAPHTHALENE	128.10
3	8.683	2.47	2.97	PENTANOIC ACID	60.05
4	8.726	3.09	3.14	2-tert-Butyl-1,3-dioxaspiro[4.4]nonane-4,6-dione	70.10
5	8.964	4.19	5.92	ETHYLMETHYLMALEIMIDE	139.10
6	9.088	1.98	2.13	2-Ethyl-5-methyl-1,3,2-dioxaborolan-4-one	84.10
7	9.359	1.92	2.73	METHYLVINYLMALEIMIDE	66.00
8	10.709	2.02	2.65	4-Methyl-2-vinyldioxolane	87.05
9	11.269	2.51	3.96	1-TETRADECENE	55.05
10	11.485	4.90	5.82	2,3-Dihydroxystearic acid	76.05
11	11.676	5.73	4.78	GUANOSINE	57.00
12	12.533	1.25	2.28	OCTADECANE	57.10
13	13.174	1.12	1.82	(2,6,6-Trimethyl-2-hydroxycyclohexylidene)acetic acid lactone	111.05
14	13.802	1.51	2.35	1-TRIDECENE	83.10
15	14.304	2.81	2.88	IsosorbideDinitrate	109.10
16	14.429	3.03	2.82	Sucrose	73.10
17	15.978	4.95	5.68	(-)-LOLIOLIDE	111.05
18	18.992	1.05	0.90	PENTACHLOROBROMOBENZENE	73.10
19	19.826	3.34	2.35	ETHYL PALMITATE	88.05
20	22.604	13.78	7.66	3,7,11,15-TETRAMETHYL-2-HEXADECEN-1-OL	71.10
21	36.511	11.01	7.02	2-methyloctacosane	57.05

Table 9. Peakreport TLC

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CONCLUSION

In the present study the ethanolic extract of *Phyllanthus acidus* leaves have shown to have various phytochemicals which possesses many pharmacological properties. The GC-MS analysis showed the presence of 21 phytochemical constituents which contribute the activities like anti-diabetic, anti-oxident, anti-cancer, anti-infecvtiven and thrombolytic activities. Hence the presence of phytoconstituents are responsible for their more therapeutic effects.The present research will enhance the existing knowledge of *Phyllanthus acidus* and also pave the way for more research work to be conducted to unravel the hidden properties of the pant.

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