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GC-MS ANALYSIS OF *PERGULARIA DAEMIA* AND *TERMINALIA CATAPPA L.* LEAF EXTRACTS

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ABSTRACT

The present study was carried out to identify the phytochemicals present in the Pergularia daemia and Terminalia catappa L. leaf extracts. The Hydroalcoholic extracts of Pergularia daemia (HAEPD) and Terminalia catappa L. (HAETC) were used for the GC-MS analysis and molecular docking with selective proteins. Totally fifty-eight fractions were collected from the hydroethanolic leaf extracts of Pergularia daemia and Terminalia catappa by Silica gel column chromatography. Depending on the TLC profile, the above fractions were pooled together and finally five fractions were collected (PDF1-PDF5), (TCF1-TCF5) for each plant extract. The column fractions are used assess the cell viability (Hepatocyte cell) by MTT assay, the column fraction with high cell viability were used to study the bioactive constituents present in the above selected column fractions by GC-MS analysis. The plant extracts were subjected to GC-MS analysis and compounds was matched with data in the library of national Technology (NIST). A total of 12 phytocompounds were identified from leaves of HAEPD and 15 compounds from leaves of HAETC. The nature of the phytochemical compounds in HAEPD and HAETC are Isoflavone, flavones, Carotenoids, fatttyacid methyl ester with various pharmacological activities.

KEY WORDS

HAEPD, HAETC, MTT, GC-MS, NIST.

INTRODUCTION

Plants are the source of most complex organic molecules. These molecules are showing wide structural diversities and are serving as templates for many semi synthetic and synthetic drug molecules. Plant derived drug molecules are frequently used in treating the disease conditions with minimal side effects compared to the synthetic molecules [1].

Medicinal plants play a pivotal role in the health care of ancient and modern cultures. Ayurveda, the Indian system of medicine mainly uses plant based drugs or formulations to treat various human ailments because they contain the components of therapeutic value [2]. In addition, plant based drugs remain an important source of therapeutic agents because of the availability, relatively cheaper cost and non-toxic nature when compared to modern medicine [3].

Medicinal plant parts are commonly rich in phenolic compounds, such as flavonoids, phenolic acids, stilbenes, tannins, coumarins, lignans and lignins. These compounds have multiple biological effects including antioxidant activity [4]. Medicinal plants, as source of remedies, are widely used as alternative therapeutic tools for the prevention or treatment of many diseases. The recent studies have investigated that the antioxidant effect of plant products is mainly attributed to phenolic compounds such as flavonoids, phenolic acids, tannins etc. [5,6).

Pergularia daemia is said to have more magical application than medical application as it possesses diverse healing potential for a wide range of illnesses.

Some of the Folklore people use this plant to treat jaundice, as laxative, anti-pyretic, expectorants and also in infantile diarrhea. The leaf latex is locally used as pain killer killer and for relief from toothache [7], the sap expressed from the leaves are held to cure sore eyes in Ghana. In traditional system of medicine, the whole aerial part of the plant is extensively used for the treatment of jaundice, liver diseases, and inflammatory disorders [8,9].

The phytochemical screening on quantitative analysis showed that the leaves of the *Pergularia daemia* are rich in popular phytochemical constituents such as flavonoids, terpenoids, carbohydrates, steroids and alkaloids. In Indian Ayurvedic system, the various parts of this plant are reported to possess numerous pharmacological properties such as [10], wound healing [11], anti-diabetic [12], hepatoprotective [9], cardioprotective effect [13] and antibacterial activity [14].

Terminalia catappa L. belongs to the family Combretaceae. *T. catappa* is used primarily as an ornamental, shade, and salt-tolerant street tree, but the leaves provide food for the tasar silkworm, and the seeds are edible like almonds with similar oils. On the Malay peninsula and through the Canary Islands this tree is known as the tropical almond. *T. catappa* has been claimed to have therapeutic effects for liver related diseases [15].

T. catappa is a well-recognized herb in Ayurveda. The juice of its fresh leaves is used in the preparation of medicinal lotion for leprosy and scabies, and it is taken internally for stomach ache and headache. Tannins have also been reported to exert other physiological effects, such as the acceleration of blood clotting, a reduction in blood pressure, a decrease in serum lipid levels, liver necrosis, and modulation of the immune response [16, 17]. In the present investigation, we investigated the phytochemicals present in the selective column fraction of HAEPD and HAETC through GC-MS analysis.

MATERIALS AND METHODS

(i) Collection of Plant Sample:

The fully matured leaves *of Pergularia daemia* & *Terminalia catappa* (Red leaves) were collected in August – September 2012 from South Poigainallur Village in Nagapattinam district of Tamilnadu, India and the Plant was taxonomically identified by Dr.P.Jayaraman, Plant Anatomy Research Centre, West Tambaram ,Chennai.The Voucher Specimen of *Pergularia daemia* (PARC/2013/211) *Terminalia catappa* was PARC/2014/2063.

(ii) Preparation of extract:

Matured leaves *of Pergularia daemia* &*Terminalia catappa* (Red leaves) were shade dried at room temperature and were powdered by using mechanical grinder. The hydro alcohol extracts of *Pergularia daemia* &*Terminalia catappa* were obtained by soxhlet apparatus & extracting at 65°C till discoloration. The extract was kept in air tight vials after complete evaporation and was stored in refrigerator till use.

COLUMN CHROMATOGRAPHY

Separation of Active constituents by silica gel – column chromatography [18,19].

Requirements:

Stationary phase – Silica gel (100-200 mesh), Mobile phase - Hexane, Ethyl acetate, Ethanol, Methanol, Distilled water, Charged material – Hexane, Volume of each fraction – 50 ml, Resolving solvents for TLC – Toluene, Chloroform: Methanol: Formic acid (7:3:1,9:1:0.5), Spraying solvent – Vanidilin sulphate, for the Visualization of compounds in TLC.

The Hydroalcoholic extract was subjected to column chromatography using different solvent systems. The fractions collected were pooled. Silica gel G (100-200 Mesh size) was used as stationary phase. Column chromatography was done by using a glass column. The dimension of the column was 4 x15 cm (height) and 4 cm (diameter). The column was packed with silica gel by wet packing method. A cotton layer was placed at the bottom of the column and then it was filled with eluting solvent of the lowest polarity (hexane). Then the required amount of stationary phase (silica gel) was poured into the column to form a bed of silica. The extract was made by 1:3 ratio of silica gel (100-200) and then poured on to the top of silica and a layer of cotton was covered and solvents were poured into it. Then the column was gradually eluted.

Procedure: The column was first eluted with 100% Hexane. The polarity of mobile phase was gradually increased from Hexane followed with ethyl acetate, ethanol, methanol and water. The collected fractions were concentrated. The desired concentrated fractions were screened for hepatoprotective activity by MTT assay. The desired concentrated and dried fractions were kept in suitable container for future use. Fractions

collected from column were pooled together according the TLC profiles. The details of collected fractions are given in the results.

***IN-VITRO* HEPATOPROTECTIVE ACTIVITY**

3.8.1. Isolation of rat hepatocytes

The method developed by Sarkar and Sil was used for the isolation of hepatocytes with slight modifications [20]. The liver were isolated under aseptic conditions and placed in HEPES (N-2-Hydroxyethyl piperazine-N-2-ethanesulphonic acid) buffer I containing HEPES (0.01M), NaCl (0.142M), and KCl (0.0067 M), and 0.5% collagenase type IV,pH 7.6 for about 45 minutes at 37°C in an incubator with constant shaking. Hepatocytes were obtained after filtration and cold centrifugation (4°C, 200 rpm /2 minutes for 3 times) and suspended in HEPES buffer.

3.8.2. Primary culture of rat hepatocytes

The method of Tingstrom **and obrink (1989)** with slight modification was used for the primary culturing of rat hepatocytes [21]. The freshly isolated viable hepatocytes were suspended in RPMI culture medium supplemented with calf serum (10%), HEPES and gentamicin (1µg/ml). These cells (1-1.2 X10 ^{6}/ml) were then seeded into culture bottles and incubated at 37°C in atmosphere of 5% CO_2.

3.8.3. Preparation of Rosewell park memorial institute medium (RPMI)

The powdered medium (RPMI) was dissolved in 900 ml of sterile Millipore water in an autoclaved glass conical flask under sterile condition. The antibiotics were added and stirred well with 3.7 gm of sodium carbonate until it gets dissolved completely. 10% calf serum was added and mixed well. The liquid was slowly poured into the upper portion of a media sterilization unit and filtered through a 0.2µ filter under negative pressure. The medium was immediately stored at 4 °C.

3.8.4. Treatment with extracts

The column fractions of TCF 2,3,4 and PDF 1,2,3 were tested for their hepatic cytotoxicity at 25,50,100,250,500 µg/ml on isolated rat hepatocytes. The selected extracts were dissolved in dimethyl sulfoxide (DMSO) (sigma, USA) and used for MTT assay.

3.8.5. Procedure for MTT (3-(4,5–dimethyl-2-thiazolyl) -2,5-diphenyl – tetrazolium bromide) assay.

The cytotoxicity of sample on hepatocytes cells was determined by MTT assay [22]. The cells (1X10 ^{5}/ well) were plated in 100µl of medium /well in 96 well plates. After 48 hours of incubation the cell reaches the confluence. Then, the cells were incubated in the presence of various concentrations of plant extracts (*Pergularia daemia* and *Terminalia catappa* leaf extracts) in 0.1% DMSO for 48 hours at 37 °C. After removal of the sample solution and washed with phosphate buffered saline (pH 7.4), 20µl (0.5 mg /ml) of 0.5% (3-(4,5 – dimethyl -2-thiazolyl) -2,5-diphenyl – tetrazolium bromide (MTT) phosphate – buffered saline was added. After four hours incubation, 0.04 M HCl /Isopropanol was added. The viable cells were determined by the absorbance at 570 nm with reference at 655 nm. The absorbance were measured with a microplate reader (Bio-Rad Richman,CA),using wells without samples as blank. All the experiments were performed in triplicate. The effect of the sample on the proliferation of rat hepatocyte cells was expressed as the % cell viability, using following formula:

% Cell viability = A_{570} of treated cells / A_{570} of control cells X 100

Data analysis

The Percentage of cell viability is reported as mean ± standard deviation of three independent experiments.

Gas Chromatography-Mass spectrometry (GC-MS) analysis

The experiment was carried out in Sophisticated analytical instrumentation facility (SAIF), IIT, Chennai, India.

The selected column fractions from HAEPD and HAETC were used for GC-MS analysis. 2 µl of the column extracts of leaves of *Pergularia daemia* and *Terminalia catappa* were employed for GC-MS analysis [23]. These extracts were dissolved in HPLC grade methanol and subjected to GC and MS JEOL GC mate equipped with secondary electron multiplier. The column (HP5) with fused silica 50 m x 0.25 mm I.D was used. Analysis conditions were 20 min. at 100°C, 3 min at 235°C for column temperature, 240°C for injector temperature, helium was the carrier gas and split ratio was 5:4. The sample (1 µl) was evaporated in a split less injector at 300°C. Run time was 22 min [24]. The compounds were identified by gas chromatography coupled with mass spectrometry. The molecular weight and structure of the compounds of test materials were ascertained by interpretation on mass spectrum of GC-MS using the database of National Institute Standard and Technology (NIST)

Identification of Compounds Interpretation of mass spectrum of GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the known components was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials was ascertained [25].

Results and discussion

Table -1 and 2 shows the number of column fractions obtained from silica gel column chromatography. The silica gel column chromatography was used to separate the hydro alcholic extract of *Pergularia daemia* and hydro alcholic extract of *Terminalia catappa L*. Leaf samples. The column was first eluted with 100% hexane and gradually with different solvents in ratio of hexane: ethyl acetate (75 :25,50 :50,25 :75%). This was followed by 100% of ethyl acetate and different ratio of ethyl acetate: ethanol (as above ratio). Then the column was eluted by the same ratio of ethanol, ethanol: methanol, methanol, methanol: water and finally with water. Totally fifty-eight column fractions were collected from each of the plant extracts. Above fractions were pooled together depending on the TLC profile of each fraction. Finally, we collected five fractions from each plant extracts.

MTT assay is the most sensitive method for detecting cytotoxic events, MTT assay is mainly based on mitochondrial respiratory activity or on the enzymatic conversion of MTT in the mitochondria. It is thought that the inhibition of the mitochondrial respiration induces active oxygen related cell death. Reactive oxygen species can be generated within the mitochondria which can also damage the mitochondrial components [26].

Figure - 1 shows the Cell viability percentage of different column fractions of *Pergularia daemia* and *Terminalia catappa L.* leaf extracts at different concentrations (25,50,100,200,250 and 500 µg /ml). The cell (Rat hepatocyte cell) viability percentage was significantly decreased with concentrations of different column fractions of HAEPD (PDF1,2,3) and HAETC (TCF2,3,4). The cell viability was significantly higher in PDF2 and TCF4 than the other fractions.

The cell viability percentage of PDF2 ranges from 95.18 ± 0.15, 93.59 ± 0.12, 89.88 ± 0.10 and 86 ± 0.18, 77.4 ± 0.03, 67.62 ± 0.05 and TCF4 range were 99.66 ± 0.11, 93.89 ± 0.14, 89.53 ± 0.27 and 82.46 ± 0.10, 76.7 2 ± 0.10, 64.44 ± 0.11 respectively at 25 to 500µg/ml concentration of fractions.

From MTT assay the Maximum cell viability was observed in PDF2 and TCF4 column fractions of *Pergularia daemia* and *Terminalia catappa*. These fractions were used to determine the phytochemical compounds by GC-MS analysis.

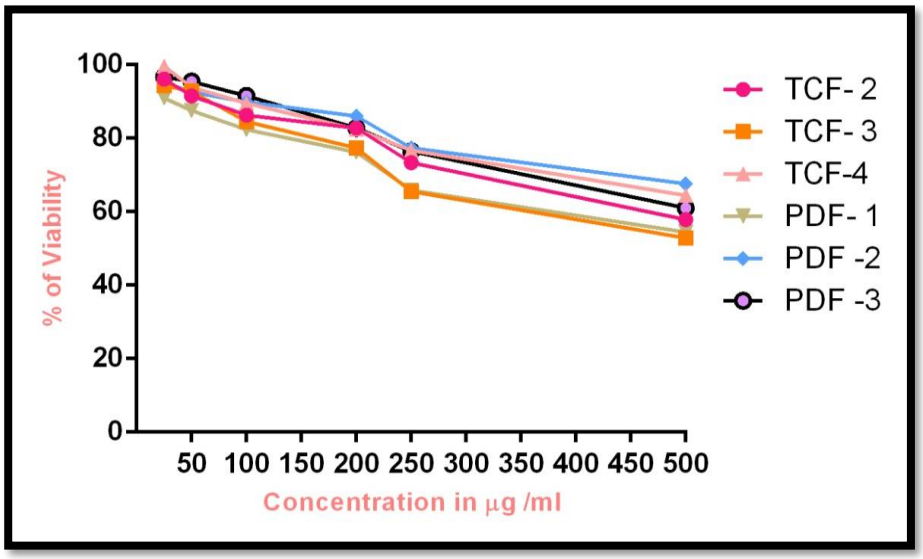
Table 1: **Shows the column fractions prepared from Pergularia *daemia* leaf extract**

S.No	Column fractions	Weight (Gms)	Yield (%)	Colour of the fraction
PDF1	Fractions 1- 21	6.8	13.6	Pale green
PDF2	Fractions 22-33	2.2	4.5	Green
PDF3	Fractions 34 -42	2.2	4.5	Dark green
PDF4	Fractions 43 -50	7.9	16	Dark green
PDF5	Fractions 51 -58	11.4	22.7	Dark brown

Table 2: Shows the column fractions prepared from Terminalia *cataapa L.*leaf extract

S.No	Column fractions	Weight (Gms)	Yield (%)	Colour of the fraction
TCF1	Fractions 1-3	3.5	7.1	Pale yellow
TCF2	Fractions 4- 15	5.35	10.7	Yellow
TCF3	Fractions 16 -24	16	32	Honey
TCF4	Fractions 25 -36	7.1	14.2	Brown
TCF5	Fractions37 - 58	8.9	17.8	Dark brown

Figure 1 : Impact of HAEPD and HAETC on cell viability



PDF – *Pergularia daemia* Fractions, TCF – *Terminalia catappa L* Fractions

The chromatogram obtained from GC –MS of column fractions of HAEPD (PDF2) and HAETC (TCF4) is shows in **figure - 2,3**. The GC-MS analysis of column fractions of HAEPD and HAETC leaf samples revealed the presence of twelve and fifteen phytochemical compounds in the above column fractions with various biological activities. The phytochemical compounds and their retention time, molecular formula and molecular weight, peak area (%) are given in **table -3,4.**

From PDF2 chromatogram, the higher percentage of peak area was observed in 4',5,7 Trihydroxy isoflavone (Genistein) – 37.46% and heptadecanoic acid 16 methyl methyl ester (18.48%) followed by 11- Octadecenoic acid methyl ester (16.28%), Mitoflaxone (10.17%). In TCF4 chromatogram, the percentage of peak area was higher in 4',5,7 trihydroxy isoflavone (Genistein) - 33.11% and α-keto stearic acid (22.55%) ,3 Phenyl 6,7 dicarboxy indane 1one (14.13%).

Genistein is an isoflavone found primarily in the soy protein [27]. It has an estrogenic and antioxidant activity [28]. It was shown in previous studies the beneficial effects of genistein were associated with its antioxidant effect [29]. Administration of oral genistein was established to reduce lipid peroxidation in the liver and to increase total antioxidant capacity in hamsters [29,30].

The isoflavone genistein is one of the most abundant polyphenolic compounds naturally present in soybeans, soy products and cereals [31,32]**.** It has been proposed that the protective effect exerted by genistein against chronic vascular diseases and early atherogenic events could be related to its antioxidant properties [33]**.** It has also been demonstrated that genistein inhibits lipid peroxidation induced *in vitro* by several pro-oxidant agents on model and natural membranes [34]**,** in cultured cells [35,36] and in low density lipoproteins [37,38]. **Record *et al.,*** provided the evidence that genistein was an effective scavenger of H_2O_2 in liposomes [39]. **Matsuda *et al.,*** reported that the Isoflavone (Genistein) from stem of *Erycibe expansa* Wall would act as a hepatoprotective agent [40].

Figure 2: Chromatogram of Hydro alcoholic Leaf Extract of *Pergularia daemia*

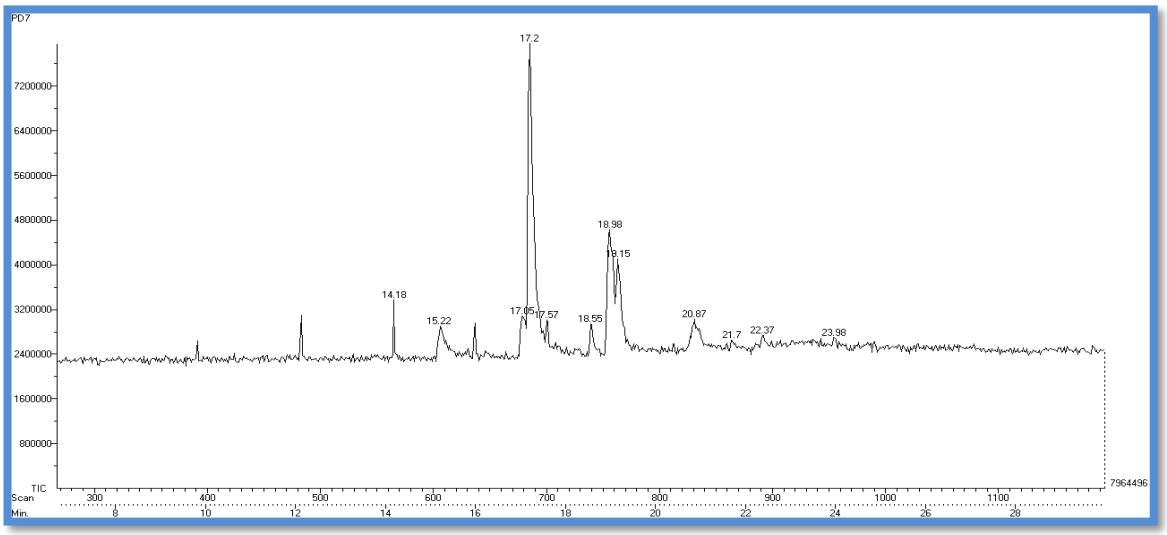


Figure 3 : Chromatogram of Hydroalcoholic Leaf extract of *Terminalia catappa L.*

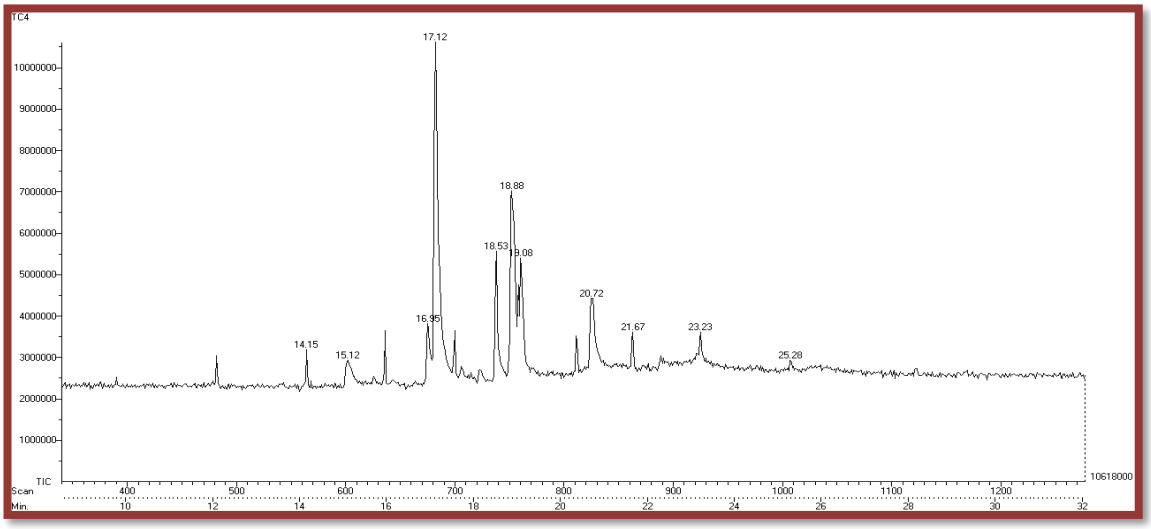


Table 3: Phytochemical compounds present in *Pergularia daemia* –GC-MS

S.NO	Name of the phytochemical compounds	RT(Min)	Molecular formula &Molecular weight	% of Peak area
1.	11 Octadecanoicacid methyl ester	18.98	$C_{19}H_{36}O_2$(296.48)	16.28
2.	Rhodopin	23.98	$C_{40}H_{58}O$ (554.88)	0.93
3.	4'5,7 Trihydroxyisoflavone	17.2	$C_{15}H_{10}O$ (270.24)	37.46
4.	α- Carotene	22.37	$C_{40}H_{56}$(536.87)	1.78
5.	Mitoflaxone	17.57	$C_{17}H_{12}O_4$(280.27)	10.17
6.	3 Azabicyclo (3.3.0) octane 2,4 dionebenzylidine 3 phenyl	18.55	$C_{20}H_{17}NO_2$(303.35)	3.11
7.	Heptadecanoic acid 16 methyl methyl ester	19.15	$C_{19}H_{38}O_2$(298.50)	18.48
8.	Dasy carpidan methanol acetate ester	20.87	$C_{20}H_{26}N_2O_2$(326.43)	4.85
9.	Flavone	15.23	$C_{15}H_{10}O_2$ (222.23)	4.37
10	Isoflavone	14.18	$C_5H_{10}O_2$ (222.23)	2.57
11.	PsiBapigenin	21.7	$C_{16}H_{10}O_5$(282.24)	-
12.	4H1Benzopyran 4one7hydroxy 2phenyl	17.05	$C_{15}H_{10}O_3$ (238.23)	-

Table 4: **Phytochemical compounds present in *Terminalia catappa L.*–GC-MS**

S.NO	Name of the phytochemical compounds	RT(Min)	Molecular formula &Molecular weight	% of Peak area
1.	3 Phenyl 6,7 dicarboxy indane 1one	18.88	$C_{17}H_1O_5$(292.27)	14.13
2.	4Hydroxy2,2,7,7tetra methyl octa hydro 2H dibenzo furan 1,8 dione	18.53	$C_{16}H_{24}O_4$(280.35)	9.09
3.	4'5,7 Trihydroxyisoflavone	17.12	$C_{15}H_{10}O$ (270.24)	33.10
4.	α- Carotene	22.37	$C_{40}H_{56}$(536.87)	3.30
5.	Flavone	15.12	$C_{15}H_{10}O_2$ (222.23)	3.68
6.	Isoflavone	14.15	$C_5H_{10}O_2$ (222.23)	1.88
7.	Apigenin 6C glucoside	18.53	C21H20O10(432.37)	-
8.	α-Ketostearicacid	19.08	$C_{18}H_{34}O_3$(289.46)	22.55
9.	Lycophyll	25.28	$C_{40}H_{56}O_2$(568.87)	1.006
10	11-Hexadecenoic acid methyl ester	16.95	$C_{17}H_{32}O_2$ (268.43)	0.899
11.	11-Eicosenoic acid methyl ester	20.72	$C_{21}H_{40}O_2$(324.54)	8.583
12.	2(E)Hepatenoic acid 4(s)-4([t-butoxy carbonyl (R)phenyl alanyl(s)alanylamino 6 methyl ethyl ester	21.67	$C_{27}H_{41}N_3O_6$(503.63)	1.76
13	4'5,7 Trihydroxyisoflavone	17.12	$C_{15}H_{10}O$ (270.24)	33.10
14	Apigenin 6C glucoside	18.53	C21H20O10(432.37)	-
15	5,7 Dihydroxy 3',4',5' trimethoxy flavone	22.32	$C_{18}H_{16}O_7$(344.31)	-

Carotenoids can be broadly classified into two classes, carotenes (alpha-carotene, beta-carotene, lycopene) and xanthophylls (beta-crytoxanthin, lutein and zeaxanthin). The carotenoids have the important antioxidant function of quenching (deactivating) singlet oxygen, an oxidant formed during photosynthesis [41]. Carotenoid may function as chian breaking antioxidant reducing lipid peroxidation of such vulnerable membrane. The antioxidant properties of carotenoids are primarily associated with their ability to quench singlet oxygen [42] and scavenge free radicals [43,44].

Biochanin- A is an isoflavone found in red clover, soy, alfalfa sprouts, peanuts, and chickpea. It has beneficial pharmacological actions. Its flavonoidal nucleus is perhaps responsible for its antioxidant and anti-inflammatory activities, as confirmed by experimental data [45,46, 47]**.** These properties, in addition to the fact that Biochanin –A inhibits phase I and induces phase II metabolizing enzymes of xenobiotic metabolism [48]**.**

From the above results, the HAEPD and HAETC have several phytochemicals like Genistein, α – carotene, 6-hydroxy isoflavone, 7 hydroxy 3',4'methylene dioxy isoflavone, biochanin A, apigenin -6 - Cglucoside. The peak area percentage was higher for 4',5,7 trihydroxy isoflavone (Genistein) in *Pergularia daemia* (37.46%) and *Terminalia catappa* (33. 11%).Genistein possess antioxidant [49], antimicrobial [50], anti-inflammatory [51], hepatoprotective [52], anti hypercholestermic activities [53]. The phytoconstituents present in HAEPD and HAETC are responsible for various biological properties like, antioxidant and free radical scavenging activity and hepatoprotective activity.

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