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Assessment of Antioxidant, Antimicrobial, Cytotoxic Activities and Isolation of Some Chemical Constituents from Different Extracts of *Pergularia daemia*

Saleh B. Alghamdi[1], Walid E. Abdallah[2], Khaled A. Abdelshafeek[2,3]*

[1]*Biology Department, Faculty of Science, Albaha University, Albaha, KSA*
[2]*Chemistry of Medicinal Plants Department, Pharmaceutical Industries Division, National Research Centre, El Bohouth st. Dokki, Giza, P.O. 12622, Egypt*
[3]*Chemistry Department, Faculty of Science and Arts, AlMukhwah, Albaha University, KSA*

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ABSTRACT

Pergularia daemia is a wild plant growing in Albaha region. This study aimed to investigate some chemical constituents of *P. daemia* and their biological activities. The aerial parts of *P. daemia* were extracted with water and organic solvents (chloroform, ethyl acetate, butanol, methanol, and petroleum ether). The chemical constituents of the unsaponifiable and the fatty acid fractions were identified using GC-MS analyses. The ethyl acetate and *n*-butanol extracts were subjected to chromatographic techniques. Total phenolic and flavonoid contents in the extracts were determined using colorimetric assay. The antioxidant activity was determined using 1,1-diphenyl-2-picrylhydrazyl free radical scavenging assay. The antimicrobial activity was evaluated using the modified Kirby-Bauer disc-diffusion method while the cytotoxic activity was evaluated using HepG-2 and HCT-116 cancer cell lines. Chromatographic separation of the ethyl acetate and *n*-butanol extracts afforded five flavonoidal compounds; apigenin, chyrsoeriol, quercetin, vitexin and lucenin-2.The total phenolic content of the extract ranged from 15.14 ± 0.02 to 39.33 ± 0.17 mg GAE/g. The ethyl acetate extract had the highest total flavonoid content (76.11 ± 0.16 mg QE/g) and the highest antioxidant activity (93.95%). The extracts showed varied degrees of inhibition against tested strains with the ethyl acetate extract having the maximum inhibition zone (17 to 23 mm against *Staphylococcus aureus* and *Bacillus subtilis*). The ethyl acetate extract showed a strong cytotoxicity activity against HEPG2 and HCT-116 cancer cell lines with IC_{50} values of 1.62 µg/mL and 2.48 µg/mL, respectively. This is the first report of the isolation of the five flavonoidal compounds from *P. daemia*. The plant has potential as antioxidant, antibacterial, and cytotoxic agents.

Keywords: Asclepiadaceae, *Pergularia daemia*, Antioxidant, Antimicrobial, Cytotoxic activity, Flavonoids.

Introduction

Traditional medicine is an area of practice and skill utilizing a vast knowledge, beliefs and experiences for the treatment and prevention of human pathological conditions and mental ailments.[1,2] About 80% of the human population, in some Asian and African regions, depends on traditional medicine and many countries are working on stating regulations for using herbal drugs in therapy. Nearly 50% of licensed drugs registered in the last three decades are natural products.[3,4]

Pergularia daemia (Forssk.) Chiov. (Family *Asclepiadaceae*) is a perennial climber, which is used as an anthelmintic, laxative, antipyretic, and expectorant. It is also used in the treatment of infantile diarrhea, malarial intermittent fevers, biliousness and sore eyes.[5,6] Other reported pharmacological activities include its antifertility,[7] antidiabetic,[8] hepatoprotective,[9] anti-inflammatory and antipyretic,[10] cardio-vascular,[11] and anti-arthritic effects.[12] Daily administration of different callus extracts of *P. daemia* could result in decreased blood glucose level in Streptozotocin(STZ) induced diabetic rats.[13] The curative properties of the medicinal plants could be attributed to the presence of different chemical compounds and constituents of therapeutic importance. Previous results of phytochemical screening of *P. daemia* indicated the presence of alkaloids, terpenoids, flavonoids, and steroids.[14] The Liquid Chromatography-Electron Spray Ionization with Mass Spectrometric (LC-ESI/MS) analysis resulted in the identification of major flavonoids including; formononetin, quercetin, chrysoeriol, taxifolin and naringenin.[12]

The present study aims to isolate some chemical constituents and to investigate some of the biological activities of the different extracts of *Pergularia daemia* including their antimicrobial, antioxidant, and cytotoxic activities.

Materials and Methods

Plant material

The plant, *P. daemia* was collected from Albaha region, KSA, in April 2018. The plant was authenticated by Dr. Hedir Abdelkader, a taxonomist at Biology Department, Faculty of Science, Albaha University, KSA. A voucher specimen (pd 1135, SA) was kept at the herbarium of Biology Department, Faculty of Science, Albaha University. After complete drying, the aerial parts were finely powdered and kept at 5 ºC for extraction and analyses.

Preparation of lipid fraction

About 100 g of dried plant powder were extracted with petroleum ether (40-60 ºC) in a Soxhlet apparatus for two days till exhaustion to obtain a greenish coloured extract which was then divided into two portions. A portion of this extract was passed over fuller's earth to remove the coloured pigments to give a clear yellowish extract. Then

*Corresponding author. E mail: khabdelhady@gmail.com
Tel: 00201155470568

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the solvent was evaporated under reduced pressure using rotatory evaporator. The petroleum ether extract was then obtained and was further analyzed using GC/MS. The second portion of the petroleum ether extract was evaporated to give a semisolid oily residue (6PL) which was used for biological tests. The obtained residue of the first portion was saponified to produce the unsaponifiable matter and the fatty acid methyl esters (FAMEs) were both analyzed by GC/MS.

GC-MS analysis of unsaponifiable fraction

The GC-MS analysis of the unsaponifiable fraction of *P. daemia* was carried out using gas chromatography-mass spectrometry instrument at the Department of Medicinal and Aromatic Plants Research, National Research Centre with the following specifications, Instrument: a TRACE GC Ultra Gas Chromatographs (THERMO Scientific Corp., USA), coupled with a thermo mass spectrometer detector (ISQ Single Quadrupole Mass Spectrometer). The GC-MS system was equipped with a TG-5MS column (30 m x 0.25 mm i.d., 0.25 µm film thickness). Analyses were carried out using helium as carrier gas at a flow rate of 1.0 mL/min and a split ratio of 1:10 using the following temperature program: 50°C for 3 min; rising at 5.0°C/min to 300°C and held for 20 min. The injector and detector were held at 280°C. Diluted samples (1:10 hexane, v/v) of 0.2 µL of the mixtures were always injected. Mass spectra were obtained by electron ionization (EI) at 70 eV, using a spectral range of m/z 40-450. The mass spectra were interpreted using the reference library of the National Institute of Standards and Technology (NIST), US, along with Willey 5 and mass finder, as well as data reported by Adams.[15] The constituent percentages were measured based on the peak area.

GC/MS conditions for FAMEs:

The GC/MS analysis of the fatty acidsmethyl ester of *P. daemia* was carried out using the same conditions for the unsaponifiable fraction. The only difference was the temperature program which was 80°C for 1 min; rising at 4.0°C/min to 300°C and held for 1 min. The injector and detector were held at 240°C. Diluted samples (1:10 hexane, v/v) of 0.2 µL of the mixtures were always injected. Mass spectra were obtained by electron ionization (EI) at 70 eV, using a spectral range of m/z 40-450.

Preparation of different plant extracts:

Dried plant samples (50 g each) were placed in five different conical flasks. The samples were extracted by maceration with 100 mL of the different solvent types (each in a separate flask); chloroform, ethyl acetate, n-butanol, methanol and water for two days with shaking from time to time. The mixture was filtered to remove saturated solvents. The process was repeated three times till complete extraction. The solvents were partly evaporated using a rotary evaporator at 45°C and any residual solvents were removed from the resulting residue under high vacuum, to afford the chloroform extract (1PL), ethyl acetate extract (2PL), n-butanol extract (3PL), methanol extract (4PL) and water extract (5PL), respectively. The different extracts were investigated for biological activities. All extracted material was stored at - 18°C until use.

Quantitative determination of Total Phenolic Content (TPC) and Total Flavonoidal Content (TFC):

Total phenolic content in the extracts were determined using the Folin-Ciocalteu method and gallic acid was used as standard.[16] Aluminum chloride colorimetric method wasused for total flavonoid content determination using quercetin as standard.[17]

Since, the ethyl acetate and the *n*- butanol fractions gave the highest biological activity, they were selected for further phytochemical investigation.

Extraction and fractionation of flavonoids:

The air-dried powdered plant material of *P. daemia*(1 kg) was exhaustively extracted with 80% methanol by percolation (9 L). The methanol extract was concentrated under reduced pressure.The residue of the methanol extract (120 g) was dissolved in hot distilled water (650 mL), left overnight, filtered and the filtrate was partitioned successively with petroleum ether (3 x 500 mL) and chloroform (3 x 500 mL), followed by ethyl acetate, then *n*-butanol (3x 500 ml).

Isolation of the ethyl acetate and butanol fractions of P. daemia

The residue of the ethyl acetate fraction (2.8 g) was chromatographed on a column (100 x 6 cm) packed with sephadex LH-20. Elution was carried out using solvent system 90% MeOH. Twenty-five fractions of 50 mL each were collected and concentrated under reduced pressure. Similar fractions were pooled together to give three main sub-fractions (P1 (3-8), P2 (10-17) and P3 (18-23).

The fraction P1(3-8, 0.4 g), was chromatographed on silica gel column (50 x 3 cm), and eluted with chloroform and chloroform-methanol gradients. The main fraction obtained was rechromatographed on sephadex LH-20 column (50 x 3 cm) to yield 20 mg of **compound1**. The fraction P2 (10-17, 0.5 g) was further purified using silica gel column (50 x 3 cm) eluted with chloroform: methanol (90 : 10) to yield 35 mg of **compound 2**. While the fraction P3(18-25, 0.2 g) was subjected to preparative paper chromatography with solvent *n*-butanol: acetic acid: water (3:1:1) to yield 40 mg of **compound 3**. Purity of the three isolated compounds was ascertained by TLC and PC.

About 5 g of the *n*-butanol fraction was loaded on a column (100 x 8 cm) packed with 350 g polyamide. Elution was carried out using distilled water followed by the addition of methanol with increasing concentrations till 100% methanol. Fractions of 250 mL were collected and concentrated under reduced pressure, then subjected to paper chromatographic investigation on Whatmman 1 mm using BAW(3:1:1) and 15% acetic acid as solvent systems. The chromatograms were examined under UV light, before and after spraying with $AlCl_3$. The fractions exhibiting the same chromatographic pattern were pooled together.

The main fraction (16-22) (4% MeOH), 1.5 g was further rechromatogaphed on a column (100 x 3 cm) packed with sephadex LH-20, 85% aqueous methanol giving two compounds which were further purified on a sephadex LH-20 column to afford 30 mg of **compound P4** and 25 mg of **compound P5**.

Antioxidant activity assay

The radical scavenging activity of different plant extracts was estimated by DPPH (2, 2-diphenyl-1-picryl hydrazyl hydrate), based on previously reported procedure [18]using quercetin as a standard.

Antimicrobial Activity test

The antimicrobial activity was evaluated using different organisms as shown in table 6, using a modified Kirby-Bauer disc-diffusion method.[19] Six microorganisms (bacterial and fungal strains acquired from Micro Analytical Center, Faculty of Science, Cairo University) were investigated. *Staphylococcus aureus* (ATCC 12600) and *Bacillus subtilis* (ATCC 6051) were used as Gram-positive bacteria; *Escherichia coli* (ATCC 11775) and *Pseudomonas aeruginosa* (ATCC 10145) were used as Gram-negative; *Candida albicans* (ATCC 7102) and *Aspegillus flavus*(ATCC 9643) was used as fungal strains. Ampicillin and Amphotericin B (Bristol-Myers Squibb, Switzerland) were used as standard antibacterial and antifungal drugs, respectively. The filter discs impregnated with 10 µL of solvent (distilled water, chloroform, DMSO) were used as negative control. The region with no growth in the region of the disc was designated as zone of inhibition or clear zone and 100 µL of microbial suspension have been spread on to agar plates.

Approved standard (M38-A) Disc diffusion technique for filamentous fungi[20] for investigating susceptibilities of filamentous fungi to antifungal agents was used. Approved standard method (M44-P) for yeasts and chronological lifespan (CLS) were also used.[21] Plates inoculated with *A. flavus* were incubated at 25°C for 48 hours; *S. aureus, B. subtilis, E. coli* and *P. aeuroginosa* were incubated at 35-37°C for 24-48 hours. The *C. albicans* was incubated at 30°C for 24-48 hours and finally the diameters of the inhibition zones were measured.[22]

Evaluation of cytotoxicity on HepG-2 and HCT-116 cell lines

This work was done at Al-Azhar University, the Regional Center for Mycology &Biotechnology. The Mammalian cell lines HepG-2 cells

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(human hepatocellular cancer cell line) and HCT-116 (colon carcinoma) were obtained from VACSERA Tissue Culture Unit.

Cytotoxicity assay;

For cytotoxicity assay, the cells were seeded in 96-well plate at a cell concentration of 1×10^4 cells per well and 100 µL of growth medium was kept in each well. Fresh medium containing different test samples were added after 24 h. Two-fold dilutions of samples were added to confluent cell monolayers dispensed into 96-well, flat-bottomed microtiterplates (Falcon, NJ, USA). The microtiter plates were incubated at 37ºC in a humidified incubator with 5% CO_2 for a period of 48 h. Three wells have been dedicated for each concentration of the test sample. Control cells were incubated without test samples and with or without DMSO. The little percentage of DMSO present in the wells (maximal 0.1%) wasfound to be unable to affect the experiment. The viability of cells was determined by a colorimetric method as reported earlier.[23] Media were aspirated, and the crystal violet solution (1%) was added to each well and kept for 30 minutes.[24]

Statistical Analysis.

The results of the study were expressed as mean ± SEM, n = 6. ANOVA [25] was used to analyze and compare the data.

Results and Discussion

Identification of chemical constituents in pet. ether extract

The results of GC/MS analysis of the volatile constituents of *P. daemia*(Figure 1 and Table 1) revealed the presence of 31 compounds belonging to different chemical classes. Esters constituted the highest percentage (68.51%) and methyl oleate represented the major compound (20.2%). Hydrocarbons (saturated and unsaturated) were up to 11.22% with n-pentadecane ($C_{15}H_{32}$) representing the highest portion, followed by monoterpenes (8.30%) which included carvacrol methyl ether as the main component, then Sesquiterpenes (4.41%) and finally Steroids (2.71%). These findings are partially in agreement with the results reported for the analysis of the ethanol extract of *P. daemia* leaves using GC/MS, where it, proved the presence of methyl palmitate (33.42%), 14-methyl-Pentadecanoic methyl ester (36.23%), and Ethyl 9,12,15-octadecatrienoate (33.12%) in addition to some steroids and phenols.[26] So, the most common compounds are esters as presented in the obtained results.

Unsaponifiable fraction

The unsaponifiable fraction of *P. daemia* was analyzed using GC/MS. Figure 2 showed the total ion chromatogram and Table 3 displayed the different constituents of the unsaponifiable fraction. The results of GC/MS analysis of unsaponifiable fraction proved to be a mixture of hydrocarbons, sterols and triterpenes. Hydrocarbons ranged from C_4 to C_{44} in which C_{15} (8.81%) was the main hydrocarbon. Stigmasterol and taraxasterol were also present, in which taraxasterol was the main sterol (2.50%). The GC/MS chromatograms of leaves of *P. daemia* were found to contain *β*-sitosterol, *α*-amyrin, and lupeol as compared with the standard.[27]

Fatty acid methyl esters

The analysis of fatty acid methyl esters using GC/MS (Figure 3 and Table 4) revealed the presence of 12 fatty acids which represented 87.79% of the total acid. Palmitic acid methyl ester($C_{16:0}$) was the main fatty acid detected (34.08%), followed by oleic acid methyl ester(30.57%).

Identification of flavonoidal compounds

P1 (apigenin) was obtained as a yellow amorphous powder, showing a purple colour spot under UV light that changes to yellowish green with $AlCl_3$. R_f values were 0.13 in 15% acetic acid, and 0.94 in butanol: acetic acid: water (BAW,3:1:1), indicating an aglycone. All the obtained data (UV, MS and ^{1}H-NMR) were compactable with that reported for apigenin.[28]

P2 (chrysoeriol) with R_f 0.80 in BAW, indicate the presence of an aglycone, and gave the same fluorescence under UV light before and after exposure to ammonia vapour upon spraying with 2.5% alc. $AlCl_3$.

The UV spectra appeared to be identical to that reported for chrysoeriol and the mass spectrum showed the molecular ion (M^+) at m/z 300 and fragments at m/e 285 (M^+ -CH_3), 272 (M^+ -CO), 269 (M^+ OCH_3), 152 A_1 , 148 B_1 which were found to be identical with that reported for chrysoeriol.[28]

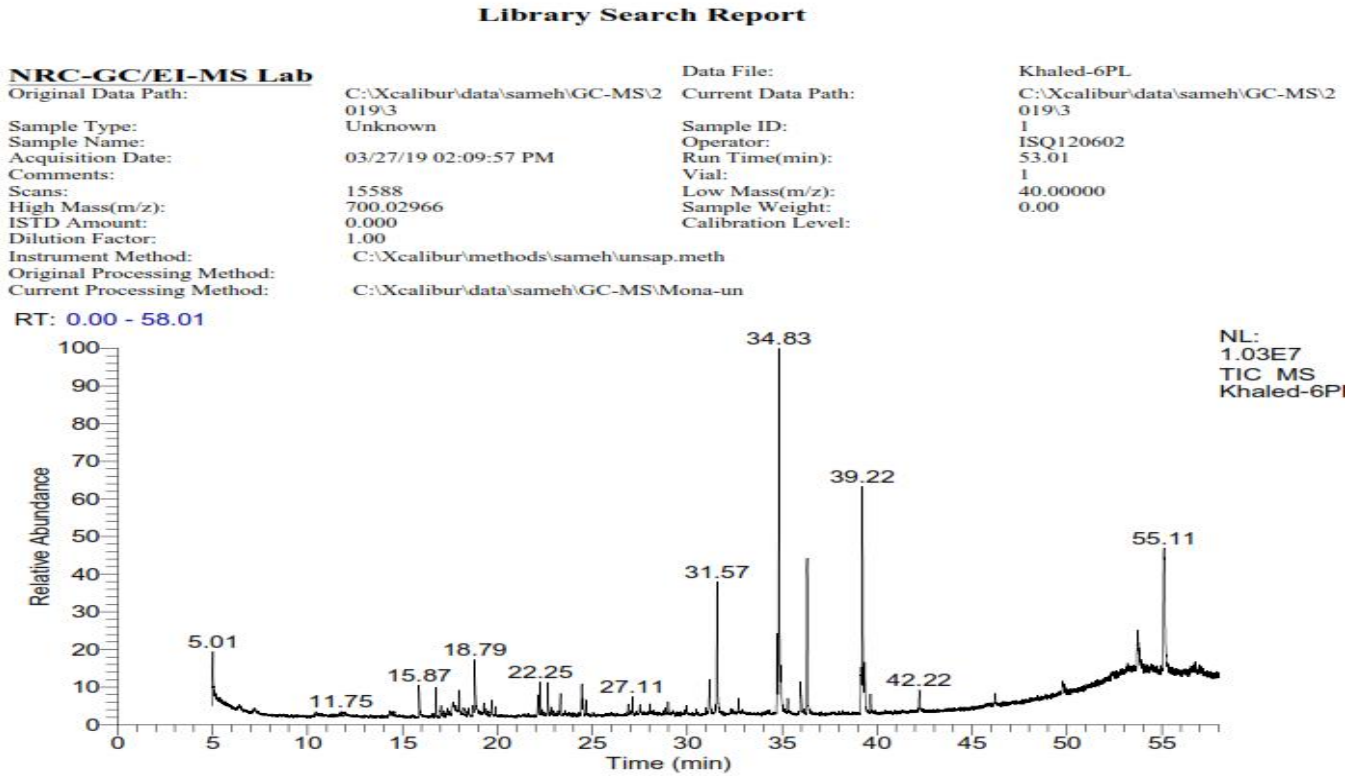

Figure 1: GC chromatogram of pet. ether extract of *P. daemia*

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Table 1: GC/MS data of pet. ether extract of *P. daemia*

Peak no.	Compound	R_t (min.)	Area (%)	M. wt.	Chemical formula
1	Carvacrol methyl ether	15.87	3.8	164	$C_{11}H_{16}O$
2	Bornyl acetate	17.02	0.85	196	$C_{12}H_{20}O_2$
3	(-)-à-Selinene	17.35	0.32	204	$C_{15}H_{24}$
4	Aromadendrene	17.69	1.24	204	$C_{15}H_{24}$
5	pentadecane	17.99	3.1	212	$C_{15}H_{32}$
6	Triacetin	18.79	4.5	218	$C_9H_{14}O_6$
7	2-methyl-,3-hydroxy-2,4,4-trimethylpentyl Propionate	19 .28	1.52	216	$C_{12}H_{24}O_3$
8	Caryophyllene oxide	19.89	0.87	220	$C_{15}H_{24}O$
9	cis-Farnesol	19.70	1.98	222	$C_{15}H_{26}O$
10	Cetene	22.14	1.43	224	$C_{16}H_{32}$
11	5,8-Diethyldodecane	22.66	2.51	226	$C_{16}H_{34}$
12	1-Nonadecene	23.32	1.23	266	$C_{19}H_{38}$
13	Methyl palmitoleate	24.44	1.55	268	$C_{17}H_{32}O_2$
14	Nonadecane	24.51	0.98	268	$C_{19}H_{40}$
15	Cetal	24.68	0.89	242	$C_{16}H_{34}O$
16	n-Heneicosane	27.11	1.97	296	$C_{21}H_{44}$
17	Methyl octadec-17-enoate	31.17	3.44	296	$C_{19}H36O_2$
18	Methyl palmitate	31.57	7.02	270	$C_{17}H_{34}O_2$
19	n-Butyl myristate	32.70	0.98	284	$C_{18}H_{36}O_2$
20	Methyl undecenoate	34.73	3.55	294	$C_{19}H_{34}O_2$
21	Methyl oleate	34.83	20.20	296	$C_{19}H_{36}O_2$
22	Methyl stearate	34.95	3.34	298	$C_{19}H_{38}O_2$
23	isoPropyl 9-hexadecenoate	35.96	2.2	296	$C_{19}H_{36}O_2$
24	n-Butyl palmitate	36.31	7.95	312	$C_{20}H_{40}O_2$
25	n-Propyl 9-octadecenoate	39.22	13.89	324	$C_{21}H_{40}O_2$
26	butyl Oleate	39.34	2.54	338	$C_{22}H_{42}O_2$
27	Butyl stearate	39.65	1.06	340	$C_{22}H_{44}O_2$
28	Dioctyl phthalate	42.22	1.2	390	$C_{24}H_{38}O_4$
29	3,3-Ethylenedioxy-5-à-cholestane	46.21	0.85	430	$C_{29}H_{50}O_2$
30	(5á)Pregnane-3,20á-diol, 14à,18à-[4-methyl-3-oxo-(1-oxa-4-azabutane-1,4-diyl)]-, diacetate	49.77	1.86	489	$C_{28}H_{43}NO_6$
31	9-Desoxo-9-acetoxy-3,8,12-tri-acetylingol	53.73	3.34	536	$C_{28}H_{40}O_{10}$

Table 2: Classes of the volatile constituents of *P. daemia*

	Compounds	%
1	Monoterpenes	8.3
2	Sesquiterpenes	4.41
3	Hydrocarbons(saturated and unsaturated)	11.22
4	Esters	68.51
5	Steroids	2.71

P3 (*quercetin*) the compound was isolated as yellow powder (40 mg). It is an aglycone in nature according to its chromatographic behavior on PC in different solvent systems (R_f = 0.69 in BAW, and R_f= 0.21 in 15% acetic acid). All the measured spectra of the compound P3 were the same as reported for quercetin.[28]

P4 (apigenin 8-C-glucoside (vitexin)): This compound was isolated as yellow amorphous powder (30 mg). It is glycosidic in nature according to its chromatographic behavior on PC in different solvent systems (R_f = 0.40, in BAW, and R_f= 0.48 in 15% acetic acid).
The UV absorption spectra of the compound P4 displayed two major peaks in the region 335 nm and 271 nm which can identify the flavonoidal structure of this compound. The EI-MS of the compound showed a molecular ion peak M^+ at *m/z* = 433 which is compatible with the molecular formula $C_{21}H_{20}O_{10}$. All the NMR data (^1H and ^{13}C) were in accordance with that reported for apigenin 8-*C*-glucoside (vitexin).[29] Both compounds; P4 and P5 were found to resist the acid hydrolysis and responded to the enzymatic hydrolysis with *β*-glucosidase affording glucose and apigenin as an aglycone in compound P4, glucose as a sugar, and luteolin as an aglycone in compound P5. **P5** (*luteolin 6,8-di-C glucoside*(*Lucenin-2*), was isolated as a yellowish powder, appeared as a purple spot which changed to greenish yellow by ammonia vapour and became yellow with Naturstoff reagent (NA).

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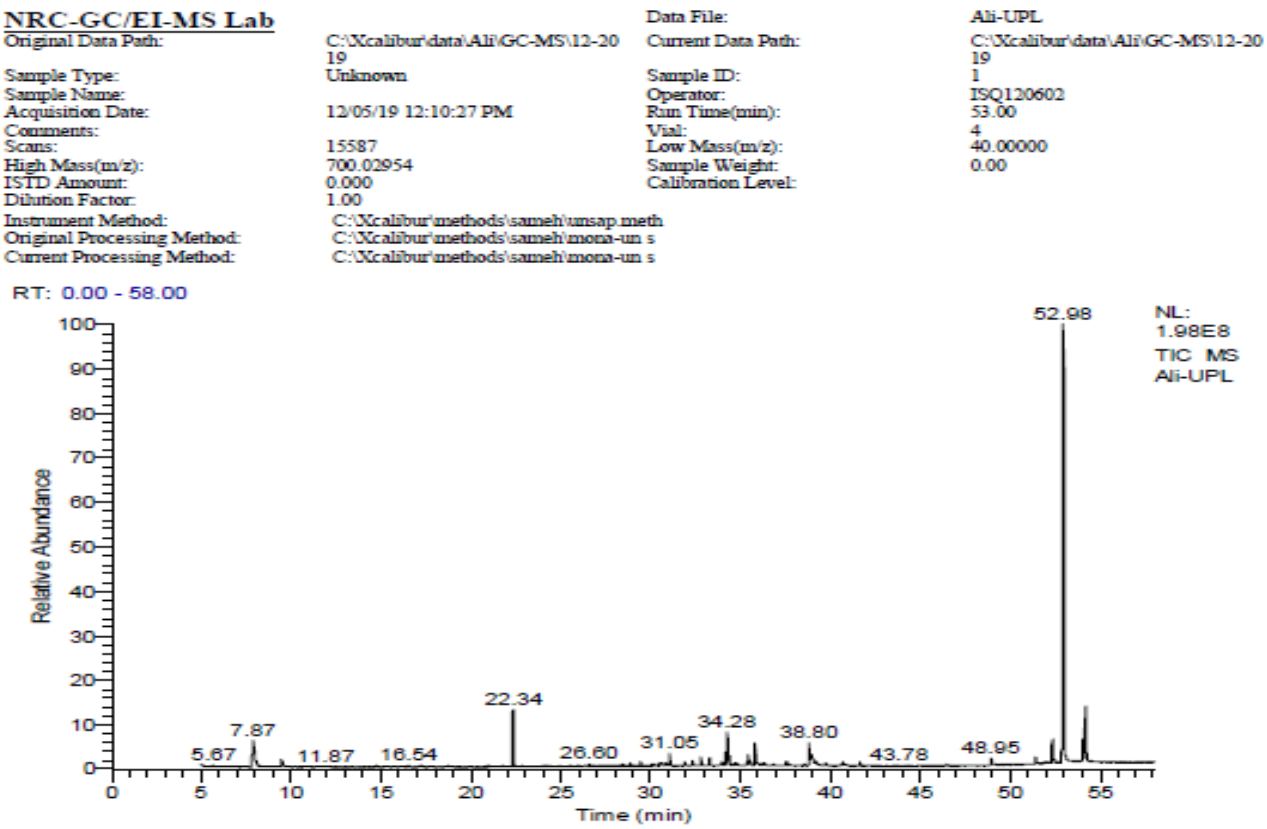

Figure 2: GC Chromatogram of unsaponifiable fraction of *P. daemia*

Table 3: GC/MS data of unsaponifiable fraction of *P. daemia*

Peak No.	Rt(min)	Rel.%	Mol.wt.	Molecular formula	Compounds
1	7.87	6.23	130	$C_8H_{18}O$	1-Hexanol,2-ethyl
2	9.44	1.04	176	$C_4H_7C_{l3}O$	2-Propanol,1,1,1Trichloro-2-methyl
3	22.34	8.81	220	$C_{15}H_{24}O$	Butyl hydroxy toluene
4	26.60	0.51	310	$C_{22}H_{46}$	Docosane
5	28.87	0.34	266	$C_{18}H_{34}O$	16-Octadecenal
6	29.44	0.41	268	$C_{18}H_{36}O$	2-Pentadecanone,6,10,14 trimethyl
7	30.20	0.49	324	$C_{23}H_{48}$	Tricosane
8	30.57	0.36	252	$C_{18}H_{36}$	9-Octadecene
9	31.05	2.25	268	$C_{18}H_{36}O$	Octadecanal
10	34.28	6.23	294	$C_{21}H_{42}$	10-Heneicosene
11	35.80	2.83	255	$C_{16}H_{33}NO$	Hexadecanamide
12	38.81	0.92	490	$C_{35}H_{70}$	17-Pentatriacontene
13	37.57	0.75	408	$C_{29}H_{60}$	Nonacosane
14	48.95	0.90	380	$C_{27}H_{56}$	Heptacosane
15	51.43	0.94	618	$C_{44}H_{90}$	Tetratetracontane
16	51.94	0.38	400	$C_{29}H_{48}O$	Stigmasterol
17	52.37	2.82	426	$C_{30}H_{50}O$	α-Amyrin
18	52.98	50.87	426	$C_{30}H_{50}O$	Lupeol
19	54.05	2.50	426	$C_{30}H_{50}O$	Taraxasterol
20	54.20	6.73	468	$C_{32}H_{52}O2$	Lupeyl acetate
		96.4			

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Table 4: GC/MS data of fatty acid methyl esters of *PL*

Peak no.	Rt (min)	Rel.%	Mol. Wt.	Molecular formula	Compound
1	8.67	0.67	264	$C_{17}H_{28}O_2$	Hexadecatrienoicacid,methyl ester($C_{18:3}$)
2	10.48	0.45	242	$C_{15}H_{30}O_2$	Myristic acid methyl ester($C_{14:0}$)
3	12.40	0.31	268	$C_{17}H_{32}O_2$	Palmitoleic acid methyl ester ($_{16:1}$)
4	12.60	3.64	296	$C_{19}H_{36}O_2$	6-Octadecenoic acid methyl ester($C_{18:1}$)
5	13.09	34.08	270	$C_{17}H_{34}O_2$	Palmitic acid methyl ester ($C_{16:0}$)
6	14.76	0.31	296	$C_{19}H_{36}O_2$	Elaidic acid methyl ester($C_{18:1}$)
7	16.94	30.57	296	$C_{19}H_{36}O_2$	Oleic acid methyl ester($C_{18:1}$)
8	17.05	5.88	296	$C_{19}H_{36}O_2$	8-Octadecenoic acid methyl ester($C_{18:1}$)
9	17.48	9.31	298	$C_{19}H_{38}O_2$	Stearic acid methyl ester ($C_{18:0}$)
10	21.24	0.79	354	$C_{23}H_{46}O_2$	Behenic acid methyl ester($C_{22:0}$)
11	21.63	1.37	326	$C_{21}H_{42}O_2$	Arachidic acid methyl ester ($C_{20:4}$)
12	29.12	0.32	382	$C_{25}H_{50}O_2$	Lignoceric acid methyl ester($C_{24:0}$)
		87.79			

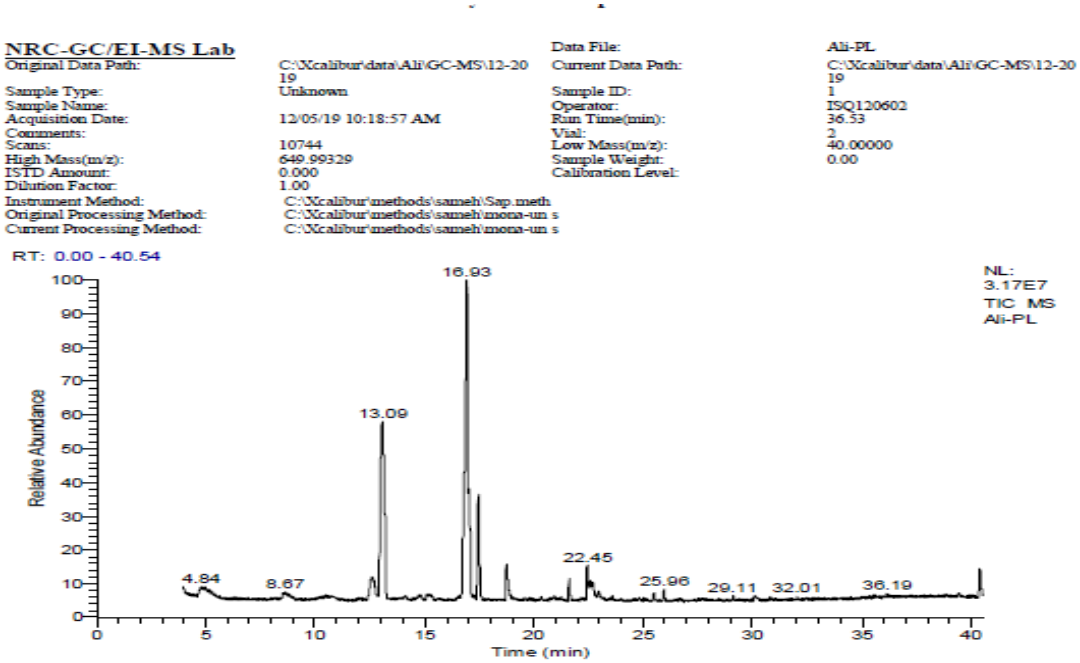

Figure 3: GC Chromatogram of fatty acid methyl ester of *P. daemia*

Its behavior on paper proved its glycosdic nature where it has R_f = 0.63 in 15% AcOH and 0.31 in BAW (3:1:1). The UV spectrum in methanol substantiated that it is a flavone type structure (band-I at $\lambda_{max\ (MeOH)}$ = 345 nm). The presence of dihydroxy groups at C-3' and C-4' was proved through $AlCl_3$/HCl and NaOAc/H_3BO_3 spectra and NaOAc spectrum displayed band-II at λ_{max} = 270 nm with a bathochromic shift from methanol spectrum = 15 nm which means the presence of a free hydroxy group at C-7. MeOH 257sh (shoulder),271, 350; λ_{max} NaOMe 272, 337sh, 415 (inc.); λ_{max} AlCl3 278, 426; λ_{max} $AlCl_3$/HCl 263sh, 277, 299,359, 386sh; λ_{max} NaOAc 270sh, 282, 404;λ_{max} NaOAc/H_3BO_3 270, 287sh, 430.
The EI-mass spectrum displayed M^+ at *m/z* = 610 which fitted with the molecular formula $C_{27}H_{30}O_{15}$[30]. From the above data and in comparison with what is mentioned in the literature, compound 5, was identified as uteolin 6,8-di-*C* glucoside(Lucenin-2).

Quantitative analysis of the phytochemicals
The total phenolic content of the *P. daemia* different extracts is expressed in terms of gallic acid equivalent, using Folin-Ciocalteu's reagent (table 5). The total phenolic content in the tested extracts ranged from 15.14 ± 0.02 mg GAE/g to 39.33 ± 0.17 mg GAE/g in the examined extracts. The ethyl acetate extract (2PL) showed the highest total phenolic content (39.33 ± 0.17 mg GAE/g), followed by methanol extract (4PL), chloroform extract (1PL), water extract (5PL) and the least was that of the butanol extract (3PL) (30.11 ± 0.02, 18.44 ± 0.07, 16.55 ± 0.20 and 16.15 ± 0.15 mg GAE/g, respectively). The petroleum ether extract (6PL) had the least total phenolic content (15.14 ± 0.02 mg GAE/g). The polar solvents give better extraction than non-polar solvents and the extraction efficiency is directly related to the polarity of solvents.[30] The variation values in the total flavonoid content of the different extracts presented in table 5, showed that the ethyl acetate extract (2PL) gave the highest total flavonoid content (76.11 ± 0.16 mg QE/g), followed by the methanol extract (4PL) (70.13 ± 0.15 mg QE/g). The least total flavonoid contentwas detected with petroleum ether extract (6PL) (18.27 ± 0.0.21 mg QE/g). These results are in agreement with that previously reported,[29] where it was found that the polarity of the extraction solvent increases the quantities of the flavonoids extracted.

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The results presented in Table 5 showed that the ethyl acetate extract (2PL) of the different extracts of *P. daemia* exhibited very high antioxidant activity towards DPPH (93.95 %), followed by methanol extract (4PL) (88.87 %), butanol extract (3PL) (84.26 %) and chloroform extract (1PL) (83.24 %). The least antioxidant activity is represented in the petroleum ether extract (6PL). These results are in agreement with that reported for *P. daemia*,[29] as it proved that, the ethanol extract followed by ethyl acetate shows a very close antioxidant activity when compared with the standard, and the least antioxidant activity was exhibited in the n-hexane, and humans can eliminate the free radicals and get relief from oxidative stress effectively by consuming *P. daemia* even at low concentration.
The redox properties of the phenolic compounds play a key role as antioxidants or free radical scavengers which can absorb and neutralize free radicals, triplet oxygen or decomposing peroxides.[32]

Antimicrobial activity
Screening of six extracts for their antimicrobial activity has been conducted using six various microorganisms; 2 Gram- positive, 2 Gram-negative bacteria and fungal species. The diameters of zone inhibition were determined and illustrated in table 6. The results revealed that the different extracts of *P. daemia* showed different degrees of antimicrobial activity against all tested isolates. The ethyl acetate extract (2PL) showed a wide range of activity, being effective with a larger inhibition zone that ranged from 17 to 23 mm, against Gram-positive bacteria *St. aureus* and *B. subtilis*, and was less active against Gram-negative bacteria *E. coli* and *Ps. aeruginosa* showing a clear zone diameter of 15 mm. These findings are in agreement with Jogi and Akkewar (2014),[33] where they reported that ethyl acetate and methanol extracts were found to be highly sensitive against Gram positive *Staphylococcus aureus* and gram -negative *E. coli* (with zone of inhibition above 13 mm). The ethyl acetate extracts of *P. daemia* exhibited more antibacterial activity compared to petroleum ether and aqueous extracts.[34] Also, the extract (2PL), showed a weak antimicrobial activity against *C. albicans* and no inhibitory activity against *A.flavus*.
The methanol extract (4PL), chloroform extract (1PL) and n-butanol extract (3PL), showed good antimicrobial activity against Gram-positive having clear zone diameter that ranged from 12 to 14 mm against Gram-positive bacteria, and ranged from 12 to 13 mm against Gram-negative bacteria. The methanol extract (4PL), showed a weak antimicrobial activity against *C. albicans* and no inhibitory activity against *A. flavus*. The inhibitory activity of two extracts; water (5PL) and petroleum ether (6PL), was weak with inhibition zone diameters ranging from 9 to 10 mm against Gram-positive and Gram-negative bacteria. No inhibitory activity of the two extracts was recorded against fungal strains *C. albicans* and *A. flavus*.

Cytotoxic activity
From Figures 4 - 15 and Tables 7 - 9, it could be noticed that the different extracts of *P. daemia* were tested for cytotoxic activity against liver (HEPG2) and colon (HCT-116) cancer cell lines. The IC_{50} values, showed that these different extracts had an apparent inhibitory activity on the viability of the two used cell lines. The tested extracts showed that this activity appears only at the specified concentrations and with these cell line types where the activity increases with decreasing IC_{50} values. Thus, it was found that the ethyl acetate extract (2PL) had a strong cytotoxicity activity against both (HEPG2) and (HCT-116) cancer cell lines, represented by the lowest IC_{50} value that were shown to be 1.62 µg/mL and 2.48 µg/mL, respectively.

Table 5: Total phenolic contents, total flavonoid content and antioxidant activities of different extracts of *P. daemia*

Sample	Total phenolic contents (TPC) (mg/g gallic acid eq.)	Total flavonoid content (TFC) (mg/g quercetin eq.)	Antioxidant activity (%)
Chloroform extract (1PL)	18.44 ± 0.07	55.17 ± 0.12	83.24
Ethyl acetate extract (2PL)	39.33 ± 0.17	76.11 ± 0.16	93.95
Butanol extract (3PL)	16.15 ± 0.15	65.32 ± 0.15	84.26
Methanol extract (4PL)	30.11 ± 0.02	70.13 ± 0.15	88.87
Water extract (5PL)	16.55 ± 0.20	22.55 ± 0.06	75.15
Pet. ether extract (6PL)	15.14 ± 0.02	18.27 ± 0.0.21	61.22

Table 6: Antimicrobial activity of different extracts of *P. daemia*

Samples		Inhibition zone diameter (mm/mg Sample)					
		Bacterial species				Fungal species	
		G^+		G^-			
		B. subtilis	***St. aureus***	***E. coli***	***Ps. aeruginosa***	***A.flavus***	***C.albicans***
Standards	**Ampicillin**	31	24	30	28	-	-
	Amphotericin B	-	-	-	-	16	
1PL		13	13	13	12	0.0	0.0
2PL		17	23	15	15	0.0	0.0
3PL		12	13	13	13	0.0	21
4PL		13	14	12	12	0.0	9
5PL		10	0.0	10	9	0.0	0.0
6PL		9	10	0.0	9	0.0	0.0

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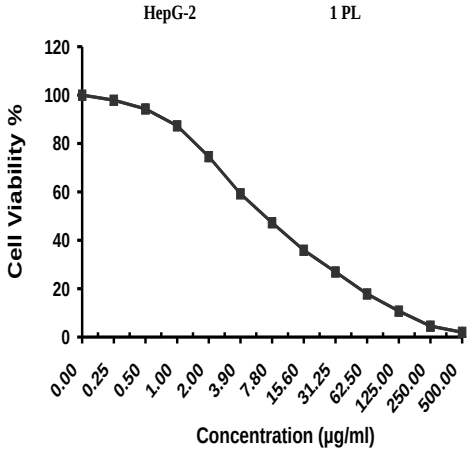

Figure 4: Inhibition of HepG-2 cells by chloroform extract (1PL) of *P. daemia*

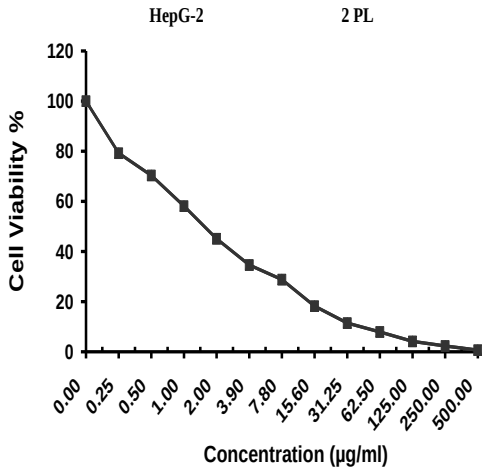

Figure 5: Inhibition of HepG-2 cells by ethyl acetate extract (2PL) of *P. daemia*

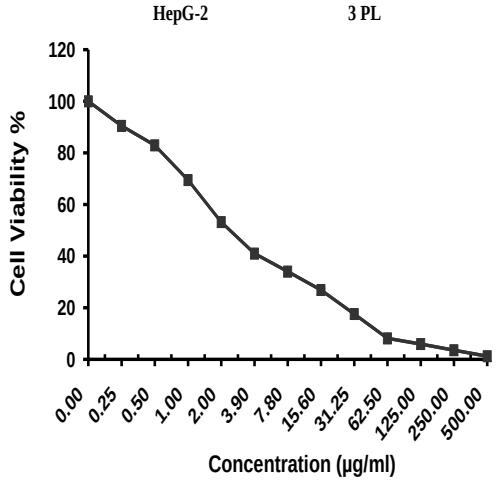

Figure 6: Inhibition of HepG-2 cells by butanol extract (3PL) of *P. daemia*

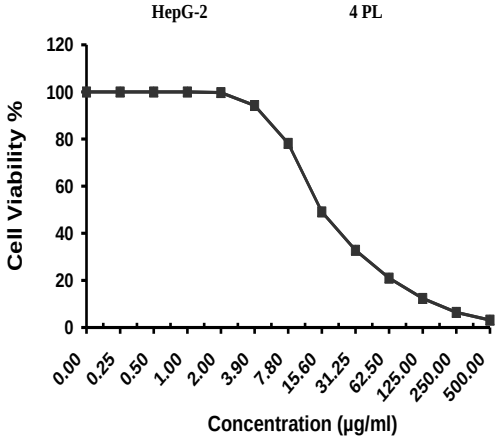

Figure 7: Inhibition of HepG-2 cells by methanol extract (4PL) of *P. daemia*

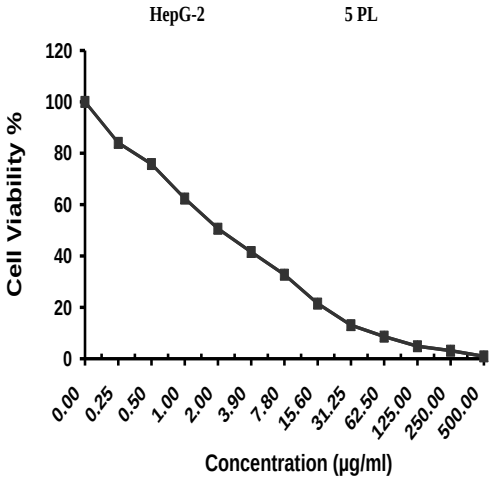

Figure 8. Inhibition of HepG-2 cells by water extract (5PL) of *P. daemia*

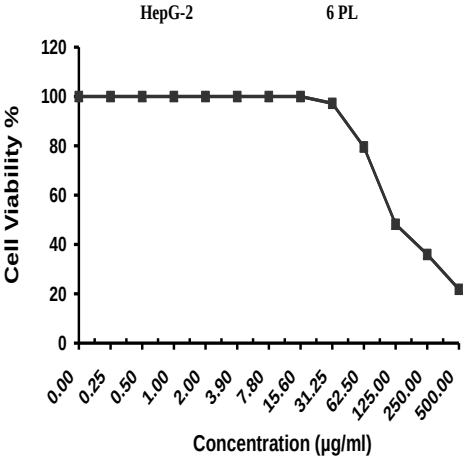

Figure 9. Inhibition of HepG-2 cells by pet. ether extract (6PL) of *P. daemia*

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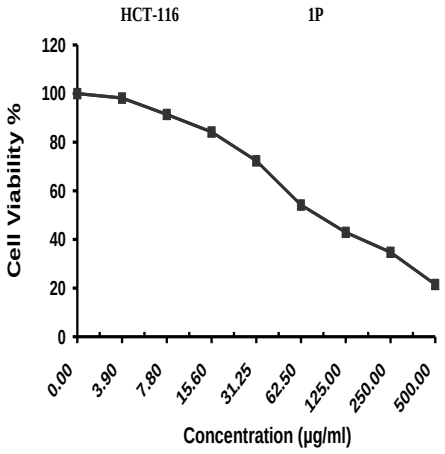

Figure 10: Inhibition of HCT-116 cells by chloroform extract (1PL) of *P. daemia*

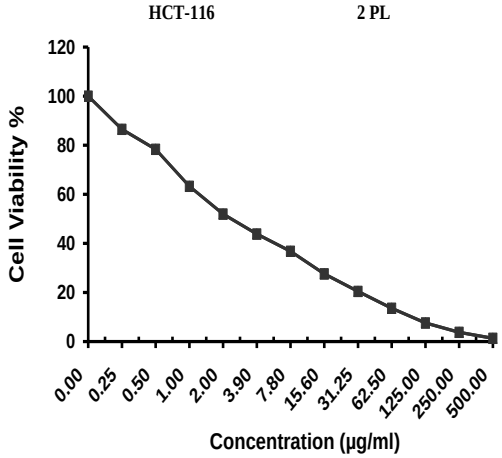

Figure 11: Inhibition of HCT-116 cells by ethyl acetate extract (2PL) of *P. daemia*

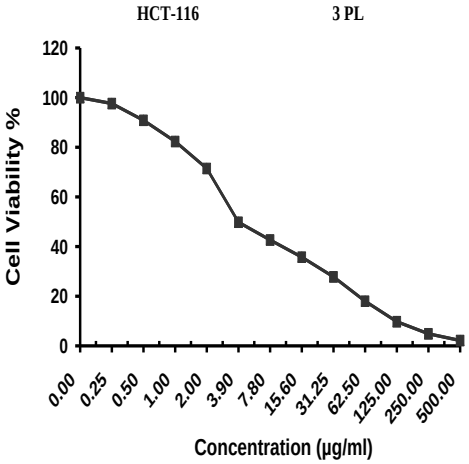

Figure 12: Inhibition of HCT-116 cells by butanol extract (3PL) of *P. daemia*

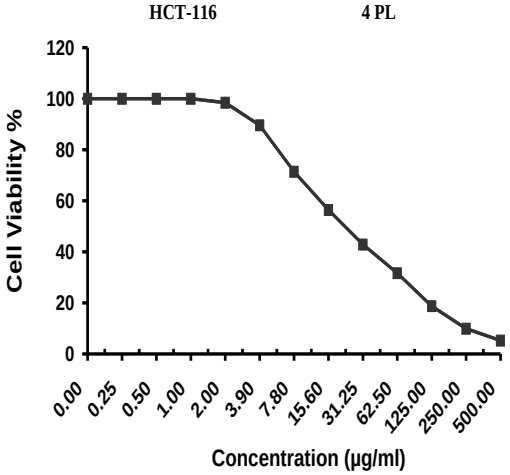

Figure 13: Inhibition of HCT-116 cells by methanol extract (4PL) of *P. daemia*

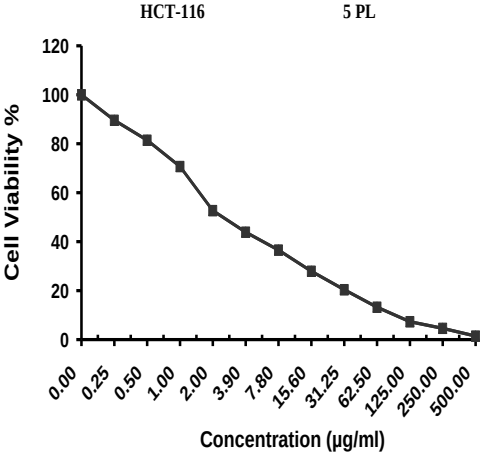

Figure 14. Inhibition of HCT-116 cellsby water extract (5PL) of *P. daemia*

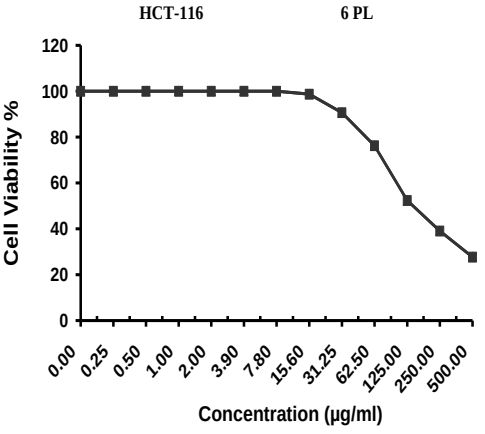

Figure 15. Inhibition of HCT-116 cells by pet. ether extract (6PL) of *P. daemia*

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Table 7: Cytotoxicity activity of different extracts of *P. daemia* against HepG-2 cell line

Sample conc. (µg/ml)	1PL		2PL		3PL		4PL		5PL		6PL	
	Viab.%	Inhib.% (±) S.D.	Viab.%	Inhib.% (±) S.D.	Viab.%	Inhib.% (±) S.D.	Viab.%	Inhib.% (±) S.D.	Viab.%	Inhib.% (±) S.D.	Viab.%	Inhib.% (±) S.D.
500	1.98	98.02±0.24	0.69	99.31±0.04	1.23	98.77±0.15	3.18	96.82±0.06	0.92	99.08±0.24	21.84	78.16±0.92
250	4.52	95.48±0.19	2.35	97.65±0.12	3.54	96.46±0.08	6.42	93.58±0.26	3.16	96.84±0.37	35.93	64.07±1.51
125	10.73	89.27±0.65	4.17	95.83±0.09	5.92	94.08±0.73	12.36	87.64±0.49	4.89	95.11±0.23	48.21	51.79±2.88
62.5	17.84	82.16±0.36	7.92	92.08±0.48	8.13	91.87±0.62	20.97	79.03±0.31	8.64	91.36±0.42	79.53	20.47±1.95
31.25	26.91	73.09±0.73	11.45	88.55±0.21	17.59	82.41±0.35	32.81	67.19±0.63	13.08	86.92±0.36	97.24	2.76±0.12
15.6	35.89	64.11±0.96	18.23	81.77±0.39	26.87	73.13±0.29	49.06	50.94±1.72	21.49	78.51±0.37	100	0
7.8	47.26	52.74±1.48	28.79	71.21±0.37	34.02	65.98±0.16	78.15	21.85±0.97	32.76	67.24±0.84	100	0
3.9	59.21	40.79±2.53	34.60	65.40±1.25	40.98	59.02±0.84	94.23	5.77±0.19	41.57	58.43±0.95	100	0
2	74.58	25.42±1.26	45.07	54.93±1.34	53.21	46.79±1.23	99.70	0.30±0.06	50.64	49.36±1.72	100	0
1	87.34	12.66±0.19	58.12	41.88±.097	69.47	30.53±0.95	100	0	62.38	37.62±0.91	100	0
0.5	94.27	5.73±0.31	70.34	29.66±0.48	82.93	17.07±0.34	100	0	75.81	24.19±0.35	100	0
0.25	97.88	2.12±0.06	79.23	20.77±0.31	90.46	9.54±0.48	100	0	84.02	15.98±0.64	100	0
0	100	0	100	0	100	0	100	0	100	0	100	0

Table 8: Cytotoxicity activity of different extracts of *P. daemia* against HCT-116 cell line

Sample conc. (µg/ml)	1PL		2PL		3PL		4PL		5PL		6PL	
	Viab.%	Inhib.% (±) S.D.	Viab.%	Inhib.% (±) S.D.	Viab.%	Inhib.% (±) S.D.	Viab.%	Inhib.% (±) S.D.	Viab.%	Inhib.% (±) S.D.	Viab.%	Inhib.% (±) S.D.
500	2.65	97.35±0.41	1.34	98.66±0.24	2.15	97.85±0.21	5.27	94.73±0.19	1.43	98.57±0.117	27.69	72.31±0.35
250	7.31	92.69±0.23	3.79	96.21±0.06	4.89	95.11±0.37	9.94	90.06±0.62	4.58	95.42±0.24	39.08	60.92±1.46
125	13.27	86.73±0.54	7.61	92.39±0.14	9.76	90.24±0.28	18.75	81.25±0.59	7.34	92.66±0.68	52.37	47.63±0.98
62.5	21.49	78.51±0.17	13.59	86.41±0.25	18.02	81.98±0.14	31.64	68.36±1.38	13.26	86.74±0.52	76.25	23.75±0.73
31.25	30.62	69.38±0.53	20.47	79.53±0.39	27.81	72.19±0.35	42.87	57.13±2.45	20.41	79.59±0.19	90.63	9.37±0.11
15.6	39.58	60.42±0.74	27.58	72.42±0.64	35.76	64.24±0.9	56.39	43.61±1.87	27.92	72.08±0.34	98.72	1.28±0.04
7.8	51.73	48.27±0.91	36.79	63.21±1.25	42.65	57.35±0.71	71.42	28.58±1.36	36.58	63.42±1.26	100	0
3.9	68.04	31.96±0.68	43.85	56.15±0.78	49.83	50.17±0.92	89.57	10.43±0.29	43.89	56.11±1.75	100	0
2	81.36	18.64±0.72	51.94	48.06±1.42	71.48	28.52±1.78	98.43	1.57±0.15	52.71	47.29±1.53	100	0
1	90.65	9.35±0.19	63.27	36.73±0.91	82.32	17.68±1.21	100	0	70.69	29.31±0.34	100	0
0.5	98.78	1.22±0.06	78.36	21.64±0.68	90.87	9.13±0.62	100	0	81.43	18.57±0.62	100	0
0.25	100	0	86.49	13.51±0.23	97.62	2.38±0.16	100	0	89.57	10.43±0.19	100	0
0	100	0	1.34	0	100	0	100	0	100	0	100	0

Table 9: The effect of different *P. daemia* extracts on HepG-2 (liver carcinoma cell line) and HCT-116 (colon carcinoma cell line)

Sample	HepG-2 (IC_{50}) ± S.D. µg/mL	HCT-116 (IC_{50})±S.D. µg/mL
Chloroform extract (1PL)	6.91±0.5	8.91±1.2
Ethyl acetate extract (2PL)	1.62±0.1	2.48±0.3
Butanol extract (3PL)	2.52±0.1	3.98 ±0.4
Methanol extract (4PL)	15.35±1.3	22.9±1.9
Water extract (5PL)	2.14±0.2	2.61±0.3
Pet. Ether extract (6PL)	121±5.7	147±7.4

It was stated that the methanol extract of *P. daemia* significantly inhibited cancer development to a greater extent than the ethyl acetate on DMBA-induced buccal pouch carcinogenesis in hamsters.[34] The suggested mechanism is the modulation of lipid peroxidation and enhancement of antioxidant status. The water extract (5PL), showed higher cytotoxic activity on both the HEPG2 and HCT-116 cancer cell lines represented by the lower IC_{50} values; 2.14 µg/ml and 2.61µg/mL, respectively, followed by the butanol extract (3PL) on both the HEPG2 and HCT-116 cancer cell lines represented by lower IC_{50} values; 2.52µg/mL and 3.98 µg/mL, respectively[35].

Ibrahim et al. (2018),[29] proved that vitexins are lignan compounds that induce apoptosis and suppress tumor growth. They have got potent anti-oxidant effect and hence can be effectively used for the prevention of UV-induced adverse skin reactions such as free radical production and skin cell damage.

The chloroform extract (1PL) exhibited high cytotoxic activity on both the HEPG2 and HCT-116 cancer cell lines that is represented by the lower IC_{50} values; 6.91 µg/mL and 8.91 µg/mL, respectively.

On the other hand, the methanol extract (4PL), showed moderate cytotoxic activity on both the HEPG-2 and HCT-116 cancer cell lines that is represented by the IC_{50} values; 15.35µg/mL and 22.9µg/mL, respectively.

The petroleum ether extract (6PL) showed low cytotoxic activity against both cancer cell lines (HEPG-2 and HCT-116) as represented by the IC_{50} values 0121 µg/mL and 147 µg/mL, respectively. It was reported that the phenolic compounds consumed daily from vegetable and fruit diet over a short period of time, exhibited antimutagenic and anticarcinogenic properties in humans at approximately 1.0 g of mixed antioxidant compounds.[36]

Conclusion

The present study led to the isolation and identification of the lipid constituents in petroleum ether extract (unsaponifiable matter and fatty acids methyl esters) using GC-MS and five flavonoidal compounds were identified for the first time from *Pergularia daemia*. The plant had significant amount of phenolics and flavonoids. Of all the extracts, the ethyl acetate extract exhibited the highest antioxidant, antibacterial and cytotoxic activities.

Conflict of Interest

The authors declare no conflict of interests.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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