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IN VITRO CYTOTOXIC, ANTIOXIDANT AND GC-MS STUDY OF LEAF EXTRACTS OF *CLERODENDRUM PHLOMIDIS*

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ABSTRACT: *Clerodendrum phlomidis* is a shrub common in India and Sri Lanka. It is one of the highly traded medicinal plants from tropical forests, as the leaves and roots are used in folk-lore, *Ayurveda*, *Siddha* and *Unnani* medicines. In this study preliminary phytochemical screening, cytotoxic, antioxidant and GC - MS study of leaf of *C. phlomidis* was evaluated. Successive extraction of the leaf of *C. phlomidis* was carried out with 5 solvents petroleum ether, chloroform, ethyl acetate, methanol and water. Antioxidant activity was evaluated using DPPH free radical scavenging assay at four different doses of 31.25, 125, 500 and 1000 µg/mL, cytotoxic potential was studied by brine shrimp lethality assay at four different doses of 1, 10, 100 and 1000 µg/mL. GC-MS analysis of methanol leaf extract was carried out on Thermo GC-TRACE ultra version: 5.0, Thermo MS DSQ II. Phytochemical screening analysis of the extracts indicated the presence of secondary metabolites such as steroids, steroidal glycosides, phenols, saponins, coumarins, alkaloids and flavanoids. Methanol leaf extract showed significant cytotoxic potential with the LC_{50} value of 9.62 µg/mL and good DPPH free radical scavenging activity with the IC_{50} value of 71.64 µg/mL. GC-MS analysis of the methanol extract revealed the presence of 17 compounds. Most of the identified compounds possess medicinal values such as antioxidant, antimicrobial, anti inflammatory, vasodilator, analgesic, sedative, dermatitigenic, hypocholesterolemic, antidiabetic, anti-tumor, cancer preventive *etc*. Methanol extract of the *C. phlomidis* leaf can be used as a lead for the isolation of the cytotoxic active chemical constituents in further study.

INTRODUCTION: Natural products have been the backbone of traditional system of healing throughout the globe, and have also been an integral part of history and culture.

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It has been well documented that natural products played critical roles in modern drug development, especially for antibacterial and antitumor agents. A huge number of natural product derived compounds in various stages of clinical development highlighted the existing viability and significance of the use of natural products as sources of new drug candidates [1].

Clerodendrum phlomidis is a shrub common in India and Sri Lanka. It is one of the highly traded medicinal plants from tropical forests, as the leaves

and roots are used in folk-lore, *Ayurveda*, *Siddha* and Unani medicines. Different parts of the *C. phlomidis* plant possess valuable medicinal properties. Leaf is used as remedy to treat diabetes, fever due to sunstroke, malaria, stomach pain, dyspepsia, digestive disorders, eye complaints, lung diseases, rheumatism, asthma, inflammatory diseases, and swellings.

Leaf juice is used to treat mental tensions and mental disturbances in Tamil Nadu. Leaf and leaf juice is used as bitter tonic, alternative and prescribed in syphilitic complaints. Leaf decoction is used for inflammation and is effective in treating bronchitis, headache, weakness, drowsiness and digestive problems [2]. Due to the wide application of the leaf in various diseases, in the present study different leaf extracts of *C. phlomidis* were selected to study for antioxidant activity by DPPH free radical scavenging assay, cytotoxic potential by brine shrimp lethality assay and to identify the chemical constituents by GC-MS study.

MATERIALS AND METHODS:

Chemicals and Reagents: Brine shrimp eggs were purchased from MVR Aqua World, Thuthookudi, Tamil Nadu. DPPH (2, 2-diphenyl-1-picrylhydrazyl) and other chemicals and solvents of analytical grade was obtained from STAR Scientific Chemicals, Salem, Tamil Nadu.

Collection of Plant Material: Leaves of *Clerodendrum phlomidis* were collected from the outskirts of Tirupati, Andhra Pradesh, India. Plant material was authenticated by Dr. K. Madhava Chetty, Taxonomist, Department of Botany, Srivenkateswara University, Tirupati, Andhra Pradesh. Voucher specimen number CP/L/2015/ 1160.

Extraction: The leaves were dried in shade and homogenized to coarse powder and stored in opaque screw tight jars until use. Powdered drug was charged into soxhlet apparatus and successive extraction was carried out with the solvents in the following order petroleum ether (40 - 60 °C), chloroform, ethyl acetate, methanol and water till exhausted. Each time before employing the solvent of higher polarity the marc was dried. Each extract was then concentrated using rotary vacuum evaporator at 50 °C under vacuum and dried residue was stored in an opaque glass bottles for further studies.

Preliminary Qualitative Phytochemical Screening: All the extracts were studied for the presence and absence of secondary metabolites such as alkaloids, carbohydrates, glycosides, saponins, proteins and aminoacids, phytosterols, phenolics, flavonoids, and tannins by qualitative chemical tests.

TABLE 1: QUALITATIVE PHYTOCHEMICAL SCREENING DATA OF VARIOUS EXTRACTS OF *CLERODENDRUM PHLOMIDIS*

Successive extracts	Appearance	Odour	Secondary Metabolites Present
Petroleum ether	Yellowish brown sticky mass	No characteristic odour	Steroids, steroidal glycosides
Chloroform	Blackish green resinous mass	Slightly pungent	Steroids, steroidal glycosides
Ethyl acetate	Green sticky mass	Characteristic odour	Phenols, saponins, coumarins
Methanol	Reddish brown sticky mass	Characteristic odour	Alkaloids, steroids, coumarins, flavonoids
Water	Brown shiny powder	Pleasant, tea like odour	Steroids, coumarins, flavonoids, phenolics

DPPH Free Radical Scavenging Activity: Free radical scavenging activity was done by DPPH assay method. DPPH is a stable free radical and accept an electron, or hydrogen radical to become a stable diamagnetic molecule. DPPH reacts with an antioxidant compound that can donate hydrogen and get reduced. The change in colour (from deep violet to light yellow) was measured. The intensity of the yellow colour depends on the amount and nature of free radical scavenger present [3]. DPPH stock solution of 100.0 µM was prepared using methanol as the solvent To 1.0 mL of 100.0 µM DPPH solution in methanol, equal volume of the sample in methanol of different concentrations (31.25, 125, 500 and 1000 µg/mL) were added separately and incubated in dark for 30 minutes. The change in coloration was observed in terms of absorbance using a spectrophotometer at 514 nm. 1.0 mL of methanol instead of test sample was added to the control tube. The different concentrations of ascorbic acid (31.25, 125, 500 and 1000 µg/mL) was used as reference compound to check that the procedures are working correctly [4]. Each concentration was tested in triplicate. Percentage inhibition was calculated from the equation;

[(Absorbance of control - Absorbance of test) / Absorbance of control] × 100

Inhibitory concentration (IC_{50}) values were determined using statistical analysis.

TABLE 2: ANTIOXIDANT ACTIVITY OF VARIOUS EXTRACTS OF *C. PHLOMIDIS* IN DPPH FREE RADICAL SCAVENGING ASSAY

Extracts / Standard	% Inhibition				IC_{50} (µg/mL)
	31.25 (µg/mL)	125 (µg/mL)	500 (µg/mL)	1000 (µg/mL)	
Petroleum ether extract	37.52±0.70	43.15±0.53	56.27±0.97	68.43±0.83	370.44
Chloroform extract	34.15±0.73	47.2±0.68	54.68±0.49	62.95±1.06	423.98
Ethylacetate extract	32.53±0.25	46.15±0.78	54.27±0.37	63.84±0.94	443.21
Methanol extract	46.25±0.89	58.1±0.63	66.81±0.38	70.15±0.15	71.64
Water extract	42.69±0.84	53.15±1.00	68.52±0.72	73.01±0.76	94.17
Ascorbic acid	48.17±0.42	58.62±0.94	69.03±0.61	77.15±0.86	57.64

All values are Mean ± SD, n = 3

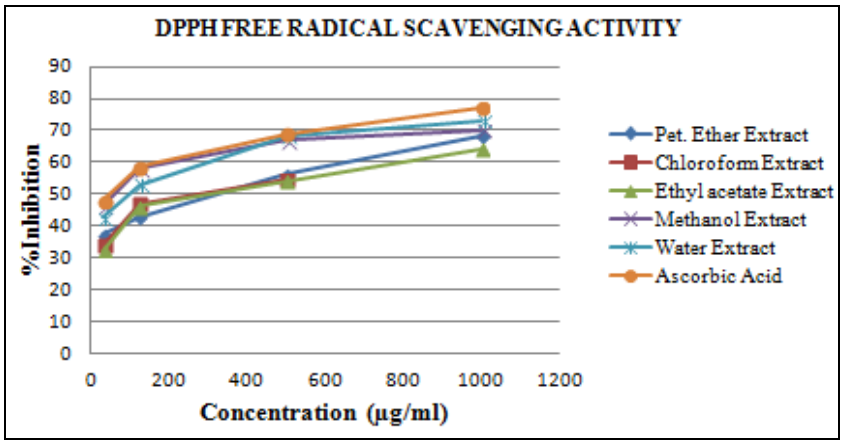


FIG. 1: PERCENTAGE INHIBITION OF VARIOUS EXTRACTS OF *C. PHLOMIDIS* IN DPPH FREE RADICAL SCAVENGING ASSAY

Brine Shrimp Lethality Assay (BSLA): Brine shrimp eggs, Artemia salina, were hatched in artificial seawater prepared by dissolving 38 g of sea salt in 1 lit of distilled water. After 24 hours of incubation period at room temperature, the shrimps hatched as nauplii (*Artemia salina*) and a lamp was placed above the open side of the tank to attract the hatched shrimps close to the tank wall. The brine shrimp lethality bioassay was carried out on the different extract using the standard procedure [5 - 8]. Twenty milligrams of the various extract were dissolved in 1 mL of Propyleneglycol: Tween 80: water (4:1:4) to give a crude extract concentration of 20 mg/mL. A two-fold serial dilution was carried out with salt water to obtain a test solution in the range of 1 - 1000 µg/mL. Each concentration was tested in triplicate. A test tube containing propyleneglycol: Tween 80: water (4:1:4) in 5 mL of salt water was used as the drug free control or negative control (blank). Ten milligrams of reference standard potassium dichromate (positive control) was dissolved in prophyleneglycol: Tween 80: water (4:1:4) and serially diluted, to obtain test concentrations ranging from 1 to 1000 µg/mL. A suspension of 0.1 mL, containing 10 larvae was counted and added into each test tube of different concentrations and incubated for 24 hours. The test tubes were then examined, and the number of live nauplii in each test tube was counted and recorded. The percentage mortality and lethal concentration (LC_{50}) were determined using statistical analysis.

Percentage Mortality = (Total nauplii - live nauplii) x 100 / Total nauplii

TABLE 3: CYTOTOXIC ACTIVITY OF VARIOUS EXTRACTS OF *C. PHLOMIDIS* IN BRINE SHRIMP LETHALITY ASSAY

Extracts / Standard	% Mortality				LC_{50} (µg/mL)
	1 (µg/mL)	10 (µg/mL)	100 (µg/mL)	1000 (µg/mL)	
Petroleum ether extract	13.33±5.77	33.33±5.77	46.66±5.77	96.66±5.77	313.74
Chloroform extract	16.66±5.77	26.66±5.77	43.33±5.77	100±0.00	322.23
Ethylacetate extract	16.66±5.77	26.66±5.77	50.00±10.0	93.33±5.77	100.00

Methanol extract	36.66±5.77	50.07±10.0	53.33±5.77	100±0.00	9.62
Water extract	23.33±5.77	43.33±5.77	53.33±5.77	100±0.00	92.29
Potassium dichromate	10.00±0.00	23.33±5.77	46.66±5.77	93.33±5.77	371.20

All values are Mean ± SD, n = 3

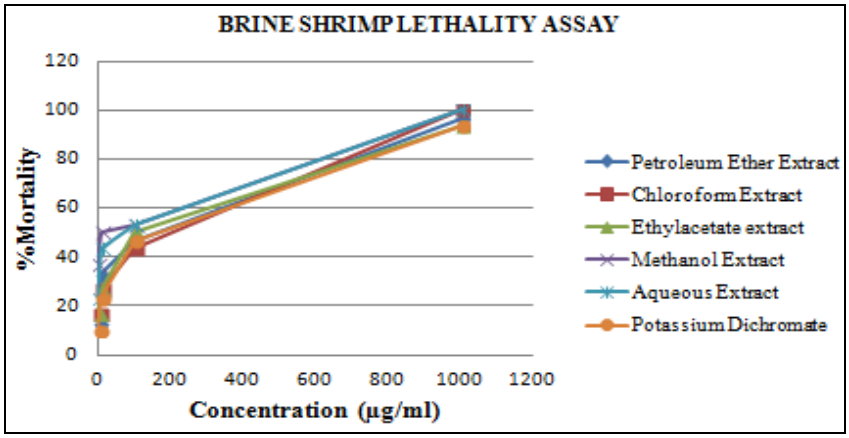


FIG. 2: CYTOTOXIC EFFECT OF THE VARIOUS EXTRACTS OF *C. PHLOMIDIS* AFTER 24 HOURS USING BRINE SHRIMP LETHALITY ASSAY

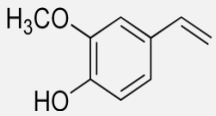
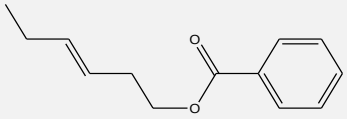
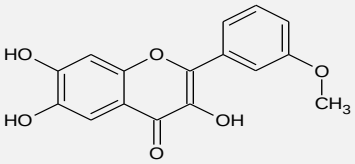
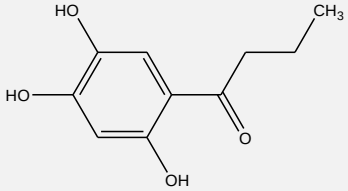
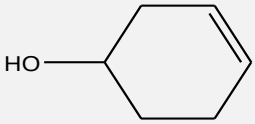
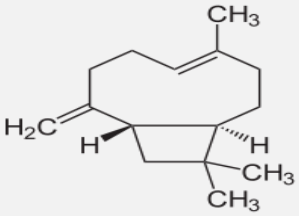
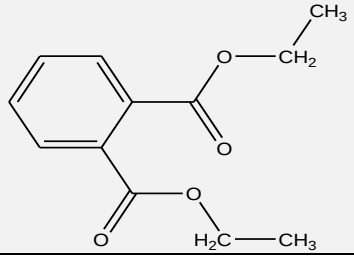
Gas Chromatography-Mass Spectrometry (GC-MS) Analysis: Mass spectrometry, coupled with gas chromatography (GC-MS) is normally used for direct analysis of components existing in traditional medicines and medicinal plants. In recent years GC-MS studies have been increasingly applied for the analysis of medicinal plants as this technique has proved to be a valuable method for the analysis of non polar components and volatile essential oil, fatty acids, lipids and alkaloids [9, 10]. Methanol leaf extract of *C. phlomidis* showed significant cytotoxic and antioxidant activity compared to other extracts. Hence GC-MS Study was carried out with methanol leaf extract to determine the possible chemical components present.

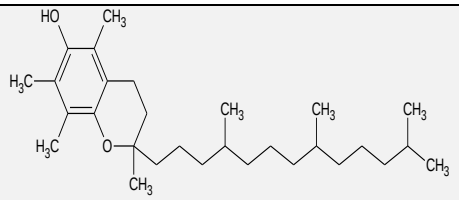
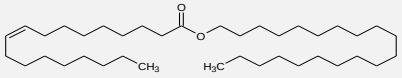
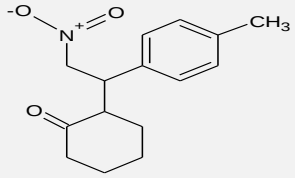
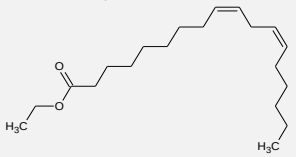
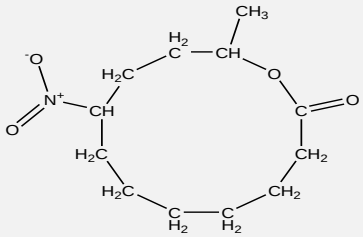
GC-MS analysis of methanol leaf extract was carried out on Thermo GC-TRACE ultra version: 5.0, Thermo MS DSQ II. Experimental conditions of GC-MS system were as follows: TR 5-MS capillary standard non-polar column, dimension: 30Mts, ID: 0.25 mm, Film thickness: 0.25 µm. Flow rate of mobile phase (carrier gas: He) was set at 1.0 mL/min. In the gas chromatography, temperature programme (oven temperature) was 40°C raised to 250 °C at 5 °C/min and injection volume was 1.0 µl. A scan interval of 0.5 seconds with scan range of 40 - 600 m/z. Total GC running time was 50 minutes and the results were compared by using Wiley Spectral library search programme.

TABLE 4: COMPOUNDS IDENTIFIED FROM METHANOL EXTRACT OF *C. PHLOMIDIS* BY GC-MS ANALYSIS

S. no.	Compound Name	RT	Molecular Formula	Molecular Weight
1	2,3-dihydroxy propanal	6.62	$C_3H_6O_3$	90.03
2	2-methoxy-4-vinylphenol	8.67	$C_9H_{10}O_2$	150.18
3	Isopropyl Linoleate	10.87	$C_{21}H_{38}O_2$	322.53
4	Hexadecane	11.71	$C_{16}H_{34}$	226.45
5	(11*E*)-trideca-1,11-diene-3,5,7,9-tetrayne	12.93	$C_{13}H_8$	164.20
6	Tetradecanoic acid	14.28	$C_{14}H_{28}O_2$	228.38
7	3-Hexen-1-yl benzoate	15.66	$C_{13}H_{16}O_2$	204.27
8	3,6,7- trihydroxy-2- (3-methoxy phenyl)- 4H- chromen-4-one	18.13	$C_{16}H_{12}O_6$	300.27
9	1-(2,4,5 trihydroxy phenyl)-1-butanone	23.08	$C_{10}H_{12}O_4$	196.20
10	3- cyclohexen-1-ol	25.92	$C_6H_{10}O$	98.15
11	Caryophyllene	26.71	$C_{15}H_{24}$	204.36
12	1,2-benzenedicarboxylic acid, diethyl ester, Ethyl phthalate	28.32	$C_{12}H_{14}O_4$	222.24
13	DL-alpha tocopherol	30.20	$C_{29}H_{50}O_2$	430.72
14	Oleic acid, eicosyl ester	32.18	$C_{38}H_{74}O_2$	563.01
15	2-(2-nitro-1-*p*-tolyl-ethyl)-cyclohexanone	35.95	$C_{15}H_{19}NO_3$	261.32
16	Ethyl linoleate	37.05	$C_{20}H_{36}O_2$	308.51
17	8-nitro-11-dodecanolide	38.39	$C_{12}H_{21}NO_4$	243.30

TABLE 5: COMPOUNDS IDENTIFIED FROM METHANOL EXTRACT OF *C. PHLOMIDIS* BY GC-MS ANALYSIS ALONG WITH THEIR STRUCTURE AND BIOLOGICAL ACTIVITY

S. no.	Compound Name	Class	Structure	Biological Activity
1	2,3-dihydroxypropanal	Aldehyde compound		Inhibition of cancer growth [11]
2	2-Methoxy-4-vinylphenol	Phenolic compound		Flavouring agent, antimicrobial, antioxidant, anti inflammatory, analgesic [12]
3	Isopropyl Linoleate	Linoleic acid ester		Formulation of face and skin care products, hair care products, eye and facial makeup [13]
4	Hexadecane	Saturated alkane		Activity not reported
5	Tetradecanoic acid	Myristic acid		Antimicrobial, antispasmodic and anti-inflammatory effects[14] Acidifier, acidulant, inhibit the production of uric acid [15]
6	3-Hexen-1-yl benzoate	Aromatic ester		Activity not reported
7	3,6,7- trihydroxy-2- (3-methoxy phenyl)- 4H-chromen-4-one.	Flavanoids		Activity not reported
8	1-(2,4,5 trihydroxy phenyl)-1-butanone	Phenolic compound		Activity not reported
9	3- cyclohexen-1-ol	Cyclic alcohol		Activity not reported
10	Caryophyllene	Sesquiterpene		Anti-tumor, analgesic, antibacterial, antiinflammatory sedative, fungicide [12]
11	1,2-benzenedicarboxylic acid, diethyl ester, Ethyl phthalate	Phthalate ester		Plasticizer [16]

12	DL-Alpha Tocopherol	Vitamin compound		Antiageing, analgesic, antidiabetic, anti inflammatory, antioxidant, antidermatitic, antileukemic, antitumor, anticancer, hepatoprotective, hypocholesterolemic, antiulcerogenic, vasodilator, antispasmodic, antibronchitic, anticoronary [17]
13	Oleic acid, eicosyl ester	Oleic acid ester		Antiinflammatory, cancer preventive, dermatitigenic hypocholesterolemic, anemiagenic, insectifuge [18]
14	2-(2-nitro-1-*p*-tolyl-ethyl)-cyclohexanone	Aromatic nitro compound		Activity not reported
15	Ethyl linoleate	Linoleic acid ester		Antimicrobial activity [16]
16	(11*E*)-trideca-1,11-diene-3,5,7,9-tetrayne	Polyacetylenic compound		Activity not reported
17	8-nitro-11-dodecanolide	Lactone		Activity not reported

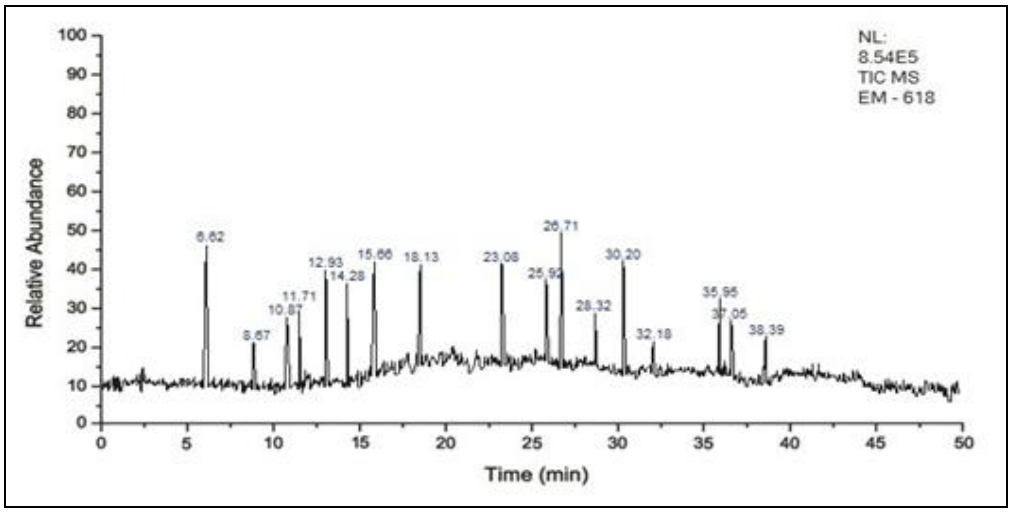


FIG. 3: GC - MS SPECTROGRAM OF METHANOL LEAF EXTRACT OF *C. PHLOMIDIS*

RESULTS AND DISCUSSION: The use of phytoconstituents as drug therapy to scavenge free radicals and to treat disorders due to oxidative stress has proved to be clinically effective and relatively less toxic than the existing drugs. Therefore it is the demand of time to use drugs from plant sources or phytoconstituents to prevent and/or treat oxidative stress [19]. To test the

antioxidant potential of the leaf extracts of *C. phlomidis*, DPPH free radical scavenging assay was used. In DPPH Free radical scavenging assay, methanol and water leaf extract of *C. phlomidis* showed good antioxidant activity with an IC_{50} value of 71.64 µg/mL and 94.17 µg/mL respectively **Table 2**.

Brine shrimp lethality bioassay is a simple, high throughput cytotoxicity test of bioactive chemicals. It is based on the killing ability of test compounds on a simple zoological organism-brine shrimp (*Artemia salina*) [20]. In brine shrimp lethality assay, methanol leaf extract of *C. phlomidis* showed significant cytotoxic potential with the LC_{50} value of 9.62 µg/mL **Table 3**. GC-MS analysis of the methanol leaf extract of *C. phlomidis* revealed the presence of seventeen compounds. The identification of the chemical constituents was confirmed based on the retention time, molecular formula and molecular weight by matching with the Wiley spectral library search programme. The active principles with their retention time (RT), molecular formula and molecular weight are presented in **Table 4**.

The first compound identified was 2, 3-dihydroxypropanal with less retention time of 6.62 min, whereas the last compound identified was 8-nitro-11-dodecanolide with the longest retention time of 38.39 min. The chemical constituents identified through GC-MS analysis possess various biological activities relevant to this study such as 2, 3-dihydroxypropanal-inhibits cancer growth, 2-methoxy- 4- vinylphenol-antioxidant, 3, 6, 7-trihydroxy- 2-(3-methoxy phenyl)- 4H- chromen-4-one- generally flavanoids are good antioxidants, caryophyllene anti-tumour activity, DL-Alpha tocopherol-antioxidant, antitumor, anticancer activity, oleic acid, eicosyl ester-cancer preventive which are listed in **Table 5**.

CONCLUSION: Preliminary phytochemical screening of leaf of *C. phlomidis* indicated the presence of the secondary metabolites such as steroids, steroidal glycosides, phenols, saponins, coumarins, alkaloids and flavanoids. *C. phlomidis* methanol leaf extract has significant cytotoxic and antioxidant potential; also indicating the presence of chemical constituents responsible for the activity which are being identified by the GC-MS analysis. Methanol extract of the *C. phlomidis* leaf can be used as a lead for the isolation of the cytotoxic active chemical constituents in further studies.

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CONFLICT OF INTEREST: The authors declared that there is no conflict of interests.

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