

GAS CHROMATOGRAPHY-MASS SPECTROMETRY ANALYSIS, *IN VITRO* CYTOTOXIC AND ANTIOXIDANT EFFICACY STUDIES ON *CLEOME GYNANDRA* L. (LEAVES): A TRADITIONAL DRUG SOURCE

RENUKA SARAVANAN¹, BRINDHA PEMAIAH², MAHESH NARAYANAN¹, SIVAKUMAR RAMALINGAM^{1*}

¹Department of Chemistry & Biosciences, SRC, SASTRA University, Kumbakonam - 612 001, Tamil Nadu, India. ²Centre for Advanced Research in Indian System of Medicine, SASTRA University, Thanjavur, Tamil Nadu, India. Email: rsiva@src.sastra.edu

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ABSTRACT

Objective: This study was aimed to assess the phytoconstituents, cytotoxic, and antioxidant efficacy of ethyl extract of *Cleome gynandra* leaves.

Methods: Qualitative phytochemical analysis with different solvent extracts was performed. Quantitative and gas chromatography-mass spectrometry (GC-MS) analysis of the extract was performed with ethyl acetate extract. The cytotoxic effect of the ethyl acetate extract was determined by 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) assay on Michigan Cancer Foundation-7 (MCF-7) cells using taxol as standard and free radical scavenging ability using 1,1-diphenyl-2-picrylhydrazyl (DPPH).

Results: Leaves extracts with different solvents revealed the presence of alkaloids, cardiac glycosides, flavonoids, phenols, and tannins. GC-MS analysis of ethyl acetate of the plant leaves showed the presence of n-hexadecanoic acid. The IC₅₀ value of the ethyl acetate extract was found to be 90.2 µg/ml on MCF-7 cell line, and the extract was found to possess significant DPPH free radical scavenging activity.

Conclusion: From the results, we conclude that the *C. gynandra* extract possesses antioxidant and antiproliferating activity against MCF-7 cells.

Keywords: Anticancer, *Cleome gynandra*, Palmitic acid, Phytol, phenol, 1,1-diphenyl-2-picrylhydrazyl and 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide.

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INTRODUCTION

Cancer is one of the major threats, which is characterized by proliferation of abnormal cells [1]. Chemotherapy is still counted as an important component of cancer therapy, though there are other advances in this therapy. At present, 90% drug failures in metastatic cancers can be attributed to chemoresistance [2]. Mounting evidence has documented two major chemoresistance mechanisms in various human cancers, namely, *de novo* (intrinsic) that refers to cancer cells being resistant to chemotherapeutic drugs from the beginning of the treatment, and acquired (extrinsic) due to genetic and epigenetic alteration of crucial genes in cancer during repetitive chemotherapy [3]. Hence, there is a need to understand the mechanism of chemoresistance by developing novel therapeutic approach to treat cancer.

Michigan Cancer Foundation-7 (MCF-7). It is the breast cancer cell line isolated in 1970 from 69 years old Caucasian woman. It is a malignant tumor that starts in the cells of the breast. It is found mostly in women. MCF7 cells have been extensively used as the model for breast cancer and breast cancer therapy [4].

Breast cancer is one of the most prevalent cancers among women worldwide. Chemotherapy generally leads to drug resistance and severe side effects thus making it crucial to identify and develop highly efficient chemotherapeutic agents [5]. Genetics plays a limited but important role as a risk factor for breast cancer. Only 5-6% of breast cancers are considered hereditary. BRCA-1 and BRCA-2 account for an estimated 80% of hereditary breast cancer, but again, this only represents 5-6% of all breast cancers. BRCA-1 and/or BRCA-2 positive women have a 50-85% lifetime risk of developing breast cancer, and 15-65% risk of developing ovarian cancer beginning at age 25 [6].

Although modern medicine uses synthetic drugs as therapeutic agents, plants still play a prominent role in contemporary pharmacy, as sources of pharmaceutical drugs in the form of isolated molecules or as sources of precursors. It is, however, important to realize that despite the advancement in biomedicine, the progress afforded the residents of first world countries is beyond the reach of the majority of the world's population. The World Health Organization is actively promoting the development of traditional herbal medicines [7].

In the medical and pharmaceutical field, natural products are always rich source providers of new chemical units that enhance human health. Many commercialized drugs that help in the management of cancer are derived from natural sources through the process of modifying the structure or by semi-synthetic preparation. Hence, it becomes essential to develop novel anticancer drugs using improved cytotoxic agents which need to be discovered yet. The chemoresistance of the cancer cells has become a key issue, as there is a possibility of domination of the chemoresistant cells that may eventually lead to mortality. Due to this problem, there is a growing need to develop a new drug. Further, due to the cost effectiveness and less toxicity, herbal medicines as cancer therapeutic agents is gaining popularity among the present researchers. Clinically proven anticancer drugs such as camptothecin, taxol, vincristine and vinblastine are phytochemicals derived from plants. Conventional drugs used in the treatment of cancer are often linked with metastatic conditions which indicate a growing need for the discovery of more efficient new agents from phytochemical sources.

C. gynandra a plant with medicinal properties is distributed across the globe. It is common weed that could be spotted in paddy fields, open grass lands or along the roads. In India, it is a common weed and hence, never cultivated. Various species of the plant are found throughout India. Throughout the world, this plant is used to treat diseases ranging

from epilepsy to some ordinary infections such as irritable bowel syndrome due to worm and protozoan infections. Due to its antioxidant properties and nutritional value, it is used in traditional culinary [8].

Botanical description

The selected plant is an erect herb with palmately compound leaves. The presence of glandular hair is a unique feature of this plant [9].

In ancient medicines, this plant is used as a rubefacient and antihelminthic. The leaves of the plant when applied externally on the wound prevent sepsis. The plant is known to cure common ailments such as malaria, piles, and rheumatism to serious problems like tumor. The decoction of the root is used in the treatment of fever and to relieve scorpion sting. The juice of the leaves is used as a remedy for earache [10].

C. gynandra possess enormous therapeutic potential including antitumor activity and is also safe and efficacious. This prompted us to study the antiproliferate and antioxidant activity of *C. gynandra* on MCF-7 cell lines, employing *in vitro* methods and attempts were also made to identify the compounds present in the extracts for the study using gas chromatography-mass spectrometry (GC-MS).

METHODOLOGY AND PROCEDURE

The whole plant sample was collected from Thanjavur, Tamil Nadu, and its adjoining areas. The sample is authenticated by comparing with the voucher specimen in the herbarium (Acc. No. RHT 172) available at Rapinat Herbarium, St. Joseph College, Tiruchirappalli, India. The leaves of the plant were processed by washing and drying in shade. Finally, the powdered leaves are extracted using ethyl acetate.

Preparation of ethyl acetate extract

About 50 g of the powder was soaked in ethyl acetate at room temperature for 72 hrs. It is then filtered through Whatman filter paper and concentrated to 1/8th part and dried to get crude extract.

Preliminary phytochemical screening

The preliminary phytochemical screening was performed for various extracts according to methods [11].

Quantitative analysis

Estimation of phenols

Phenol was estimated by the method [12]. One gram of the powdered was extract weighed and ground with 10-time volume of 80% ethanol. The supernatant was prepared by centrifuging the homogenate at 10,000 rpm for 20 minutes. The supernatant obtained was dried by evaporation and the residue thus obtained was dissolved in 5 ml distilled water. Different aliquots of the extract were pipetted out in test tubes and made up to 3 ml with water. To the tubes, 0.5 ml of Folin's reagent was added followed by the addition of 2 ml of 20% Na₂CO₃ and the contents were mixed well and placed in a boiling water bath for 1 minute. Then, the intensity is measured in a UV spectrophotometer at 650 nm with catechol as standard. The concentration of phenols was expressed as mg phenols/100 g material.

Estimation of alkaloid

Alkaloid was estimated by the method [13]. Using 0.1N HCl, the plant powder was extracted. The aqueous acidified layer obtained was partitioned with chloroform using a separate funnel. The aqueous layer was basified with NaOH and partitioned with the chloroform layer was discarded. Then, the aqueous solution was partitioned with chloroform and the aqueous solution is discarded and the chloroform remaining was evaporated and the residue was treated as total alkaloid and confirmed with dragendorff's reagent. The residue was weighed, dried and calculated.

Estimation of tannin

Tannins were estimated by the method [14]. 2 g of the powder was defatted with petroleum ether for 12 hrs. The residue obtained was

boiled with 300 ml of double distilled water for 2 hrs. Cooled and diluted to 500 ml and filtered. 25 ml of this infusion was put into 2 L porcelain dish; added 20 ml of indigo solution and 750 ml of double distilled water. This was titrated against 0.1N KMnO₄ solution, 1 ml at a time, until blue solution changes to green. Thereafter, potassium permanganate solution was added drop by drop until solution becomes golden yellow in color. Similarly, the mixture of 20 ml of indigo solution and 750 ml of double distilled water was titrated. The difference between the titration in ml was calculated. Each ml of 0.1N potassium permanganate solutions is equivalent to 0.004157 g of tannins.

Estimation of saponins

Saponin was estimated by the method [15]. In a conical flask, 20 g of the powder was taken and 100 ml of 20% aqueous ethanol was added. The samples were heated over a hot water bath with constant stirring at 55°C. After the mixture was filtered, the residue was re-extracted with 20% ethanol. Then, the combined residues were reduced to 40 ml over a water bath at 90°C. The concentrate was transferred into a 250 ml separating funnel and 20 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. 60 ml of n-butanol was added. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation, the samples were dried in an oven to a constant weight; the saponins content was calculated in percentage.

Estimation of flavonoids

Flavonoid was estimated by the method [16]. The plant powder was extracted with ethyl acetate. The extract was dried over anhydrous Na₂SO₄, filtered and concentrated under vacuum up to a concentration of 1 g/ml of extract. The extract was further diluted with ethyl acetate to obtain 0.01 g/ml solution in the experiments. About 10 ml of the solution was transferred into 25 ml volumetric flask, 1 ml of 2% AlCl₃ was added and the solution was filled up to the volume with methanol-acetic acid and was kept aside for 30 minutes, the absorbance was measured at 390 nm. A blank was also maintained. Luteolin was used to construct the calibration curve in the concentration of 1-10 µg/ml.

Antioxidant activity

Radical Scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH)

DPPH was performed by the method [17]. The DPPH assay was performed as per the ethyl acetate extracts of leaves of the plants dissolved in methanol, then 0.5 mM DPPH solution in methanol was prepared and 0.5 ml of this DPPH solution was mixed with 0.1 ml of various amounts of the extract and mixed thoroughly, then 4 ml of methanol was added and allowed to stand in a darkroom for 1 hr. The absorbance was measured at 516 nm in UV spectrophotometer (Perkin Elmer). Decrease in absorbance of the DPPH solution indicates an increase in DPPH radical scavenging activity. The DPPH scavenging of the plant activity was expressed as percentage of inhibition of the DPPH radicals.

GC-MS analysis

Instruments and chromatographic conditions

GC-MS analysis of ethyl acetate extract of *C. gynandra* was performed using the equipment PerkinElmer Clarus 500 GC-MS. The equipment has Elite-5ms column (30 m 0.25 mm 0.25 µm film thickness, coated with 5% diphenyl-95% dimethylpolysiloxane) interfaced with mass detector. Helium was used as carrier gas with a flow rate of 1 ml per minute. Temperature program was 50-150°C hold for a few minute at the rate of 3°C per minute and increased to 290°C (10 minute) at the rate of 8°C/minute. The plant extract was dissolved in methanol and 1 µl of the filtered methanol extract was injected with split ratio as 1:1. Mass spectra were recorded in the EI mode at 70 eV in a scan range of 40-60. Injector and ion sources temperature were maintained at 280

and 200°C, respectively. The resulted spectra were compared with NIST library database [18].

Cell line

The human breast adenocarcinoma cell lines (MCF-7) were procured from National Centre for Cell Science, Pune, India, and grown in Eagle's minimal essential medium containing 10% fetal bovine serum (FBS). The cells were maintained at 37°C, with 5% CO₂, 95% air and 100% relative humidity.

Cytotoxic assay (in vitro)

To make single cell suspension, the monolayers were detached using trypsin-EDTA. The viable cells were counted using hemocytometer. To get a final density of 1×10⁵ cells/ml, the cells were suspended in 5% FBS medium. In a 96 well plate, wells were seeded with cell suspension of 100 µl at a density of 10,000 cells per well. Then, incubated at 37°C for cell attachment with 5% CO₂, 95% air 100% relative humidity. After 24 hr, the cells were treated with serial concentrations of the test samples; they were initially dissolved in dimethyl sulfoxide (DMSO). An aliquot of the sample solution was diluted to twice to get the desired final maximum test concentration with serum-free medium. Additional four serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100 µl of these different sample dilutions were added to the appropriate wells already containing 100 µl of medium, resulting in the required final sample concentrations. Following sample addition, the plates were incubated for an additional 48 hr with desired conditions. The medium without plant extract served as control and triplicates were maintained for all concentrations. 5 mg of ethyl acetate extract was dissolved in 100 µL of DMSO, and then diluted with culture media to get a series of concentration (200, 100, 50, 25, and 12.5 µg/ml). The culture media was used as negative control and taxol was used as positive control with the concentrations of 0.001, 0.01, 0.1, 1, and 10 µg/ml. The assay was performed in triplicate [19].

Cytotoxic assay with 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide [MTT] method

MTT is a yellow water-soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells.

After 48 hrs of incubation, 15 µl of MTT (5 mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37°C for 4 hrs. The medium with MTT was then flicked off and the formed formazan crystals formed were solubilized in 100 µl of DMSO and absorbance was measured at 570 nm using microplate reader. The % of cell inhibition was determined using the following formula:

$$\% \text{ cell inhibition} = 100 - \text{Abs (sample)} / \text{Abs (control)} \times 100$$

Nonlinear regression graph was plotted between % cell inhibition and log concentration, and IC₅₀ was determined using GraphPad Prism software [20].

RESULT AND DISCUSSION

Large scale screening of the crude plant extracts is a crucial and basic step to establish the efficacy in clinical application. Several reports show that plant crude extracts replicate at tumor sites under hypoxic conditions and stimulate the host immune response and gene expression and leads to the inhibition of tumor growth [21]. In this research, *C. gynandra* a common medicinal plant used in the treatment of tumor was selected and evaluated for its cytotoxic potential employing *in vitro* methods. Attempts were also made by employing GC-MS to identify the compounds present in the active fractions. Different extracts of *C. gynandra* leaves revealed the presence of alkaloids, flavonoids, steroids, tannins, cardiac glycosides, terpenoids, and phenols (Table 1).

The phytoconstituents present in the extract may be responsible for the various pharmacological actions of the plant although their specific roles remain to be unexplored. Quantitative analysis of the ethyl acetate extract of the plant was performed and the results obtained were presented in Table 2.

From the quantitative analysis of phytoconstituents, we observed that the plant extract found to possess a significant amount of steroid, flavonoids, phenol and terpenoid and lesser amount of tannins, saponins, and alkaloids.

Data obtained in DPPH radical scavenging efficacy of *C. gynandra* leaves extract are presented in Fig. 1. The previous studies showed that ethyl acetate extract of *C. gynandra* found to possess antioxidant, anti-inflammatory, and lysosomal protection properties in adjuvant induced arthritic rats [22]. The antioxidant property of biomolecules, in particular, the plant extract is determined by its ability to scavenge DPPH radicals [23]. The results are expressed as an IC₅₀ value (the amount of antioxidant necessary to decrease the initial DPPH concentration by

Table 1: Phytoconstituents in ethyl acetate extract of *Cleome gynandra* leaves

| Phytoconstituents | Methanol extract | Aqueous extract | Chloroform extract | Ethyl acetate extract |
|-------------------|------------------|-----------------|--------------------|-----------------------|
| Alkaloids | - | - | - | + |
| Flavonoids | + | + | + | + |
| Tannins | + | + | + | - |
| Glycosides | - | + | + | - |
| Phenols | + | + | - | + |
| Saponins | + | + | + | + |
| Terpenoids | + | + | + | + |
| Carbohydrate | + | + | + | + |
| Protein | + | + | + | + |
| Phlobatannins | - | - | - | - |
| Anthraquinones | - | - | - | - |
| Cardiac glycoside | + | - | - | + |

Table 2. Quantitative analysis of phytoconstituents in ethyl acetate extract of *Cleome gynandra* leaves

| S. No. | Phytoconstituents | (%) |
|--------|-------------------|-------|
| 1. | Phenol | 13.9 |
| 2. | Flavonoid | 11.94 |
| 3. | Alkaloid | 0.064 |
| 4. | Steroid | 16.32 |
| 5. | Terpenoid | 7.4 |
| 6. | Tannins | 5.0 |
| 7. | Saponins | 2.3 |

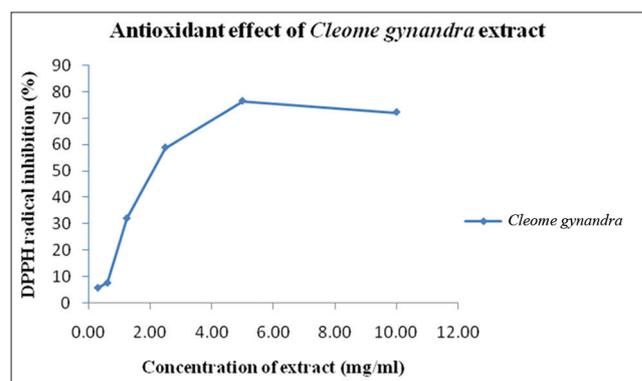


Fig. 1: 1,1-diphenyl-2-picrylhydrazyl radical scavenging of ethyl acetate leaf extract of *Cleome gynandra*

50%) as 72.24%. Our results of the DPPH free radical scavenging assay suggested the antioxidant nature of the extract (Fig. 1).

The cytotoxic effect of ethyl acetate extract of *C. gynandra* leaves is represented in Fig. 2 and Plate 1. The potent inhibitory activity (IC_{50}) of the plant extract was found to be 90.2 $\mu\text{g/ml}$ against MCF-7 cancer cell line.

Fragmentation pattern of few compounds identified in the selected plant extracts through GC-MS analysis is given in Table 3. Maximum % of n-hexadecanoic acid has been detected in the leaf extracts of *C. gynandra* and followed by the other compounds were recorded (Figs. 3 and 4, Table 3).

Palmitic acid or hexadecanoic acid in IUPAC nomenclature is the most common fatty acid (saturated) found in animals, plants, and microorganisms [24]. As the name indicates, it is a major component of the oil from palm trees (palm oil), but can also be found in meats, cheese, butter, and dairy products. In this study, we observed the significant cytotoxic effect of the extract on MCF-7 cells. This efficacy might be due to the presence of palmitic acid (n-hexadecanoic acid). Among the chemical phytoconstituents, n-hexadecanoic acid or palmitic acid was found to be more in the leaf extract. Fatty acids can be attributed to the antioxidant activity. Palmitic acid was reported to be a more effective free radical scavenger than β -carotene [25] besides the palmitic acid, n-hexadecanoic acid present in essential oil may also contribute to the antioxidant activity.

This *in vitro* cytotoxic observation is in agreement with earlier reports states that palmitic acid (n-hexadecanoic acid) induced apoptosis in the human leukemic cell line MOLT-4. Palmitic acid also showed *in vivo* antitumor activity in mice. One of the molecular target of palmitic acid in tumor cells is DNA topoisomerase I, [26] however, interestingly, it does not affect DNA topoisomerase II, suggesting that palmitic acid could be a lead compound to develop anticancer drugs. Palmitic acid ethyl esters also possess antioxidant, hypocholesterolemic, nematocidal, pesticide, antiandrogenic flavor, hemolytic, and alpha reductase inhibiting property [27]. The phenolic compounds such as gallic acid, protocatechuic acid, p-hydroxybenzoic acid, chlorogenic acid, vanillic acid, syringic acid, catechol, 4-methyl catechol, syringol and pyrogallol, and fatty acids such as linoleic acid, myristic acid, and palmitic acid from the fractions of *Polygonum bistorta* L. and the fractions were evaluated for their cytotoxic activity on human hepatocellular carcinoma cell line (HCCLM3). These fractions showed good to strong cytotoxicity in the range of 200-800 $\mu\text{g/ml}$ [28].

It has been previously reported that phytol, an acyclic diterpene alcohol, plays a key role in the control of tumor growth by reducing the level of cytokine production and also by preventing the formation of free radicals [29]. Phytol also exhibits antimicrobial, anticancer, diuretic, and anti-inflammatory [30]. In the plant selected for this study phytol was also detected. Further evidence prove that phytol is found to have cytotoxic, anticancer, antidiabetic, lipid-lowering, and antiteratogenic activities [31].

In recent years, plant-derived bioactive substances especially anticancer ones have gained considerable attention [32]. Moreover, many plants

containing phenolic compounds have been found to possess good anticancer activity [33,34] and based on this fact plants rich in phenolic content have been considered as interesting in developing anticancer drug [35,36]. Phenolic compounds are known to be strong antioxidant compounds with the ability to scavenge free radicals [37,38] and the antioxidant potential of *Lonicera japonica* essential oil might be due to the presence of high levels of phenolic compounds [39,40]. Phenols are present to the tune of 13.9% in the plant selected for this study. Hence, the antiproliferative activity of *C. gynandra* against MCF-7 cells could also be attributed to the presence of palmitic acid, phytol and phenol probably through apoptosis and free radical scavenging mechanism.

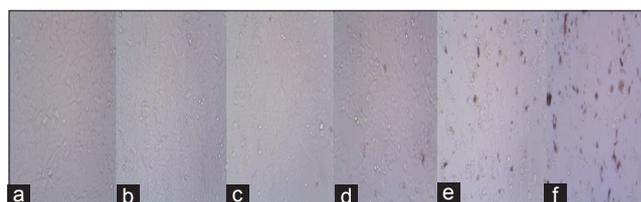


Plate 1: Effect of *Cleome gynandra* ethyl acetate extract on Michigan Cancer Foundation-7 cell line. (a) Cells with no extract, from (b-f) cells with treated with 12.5, 25, 50, 100, and 200 $\mu\text{g/ml}$

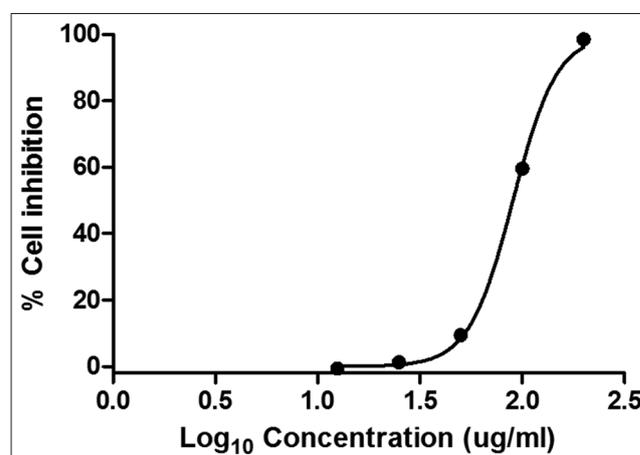


Fig. 2: Cytotoxicity of *Cleome gynandra* leaf extract on Michigan Cancer Foundation-7 cells

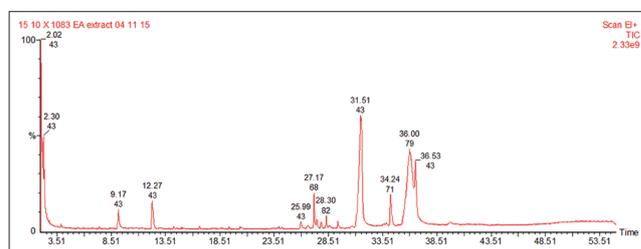


Fig. 3: Gas chromatography-mass spectrometry chromatogram of ethyl acetate extract of *Cleome gynandra*

Table 3: Identified molecules from the leaves extract of in ethyl acetate extract of *Cleome gynandra*

| Peak name | M.F | M.W | Retention time | Peak area | Peak area (%) |
|---|-------------------|-----|----------------|-----------|---------------|
| 1,2,3-propanetriol, monoacetate | $C_5H_{10}O_4$ | 134 | 9.17 | 36455736 | 3.3089 |
| 1,2,3-propanetriol, 1 acetate | $C_5H_{10}O_4$ | 134 | 12.27 | 46973656 | 4.2636 |
| 3,7,11,15-tetramethyl - 2 hexadecen-1-ol | $C_{20}H_{40}O$ | 296 | 27.17 | 42732324 | 3.8786 |
| n-hexadecanoic acid | $C_{16}H_{32}O_2$ | 256 | 31.51 | 468538720 | 42.5274 |
| Phytol | $C_{20}H_{40}O$ | 296 | 34.24 | 60032340 | 5.4489 |
| 9,12,15-octadecatrienoic acid | $C_{18}H_{30}O_2$ | 278 | 36 | 296011136 | 26.8678 |
| 2,3,3-trimethyl-2(4-methylpentanoyl)-cyclopentanone | $C_{14}H_{24}O_2$ | 224 | 39.8 | 11550140 | 1.0484 |

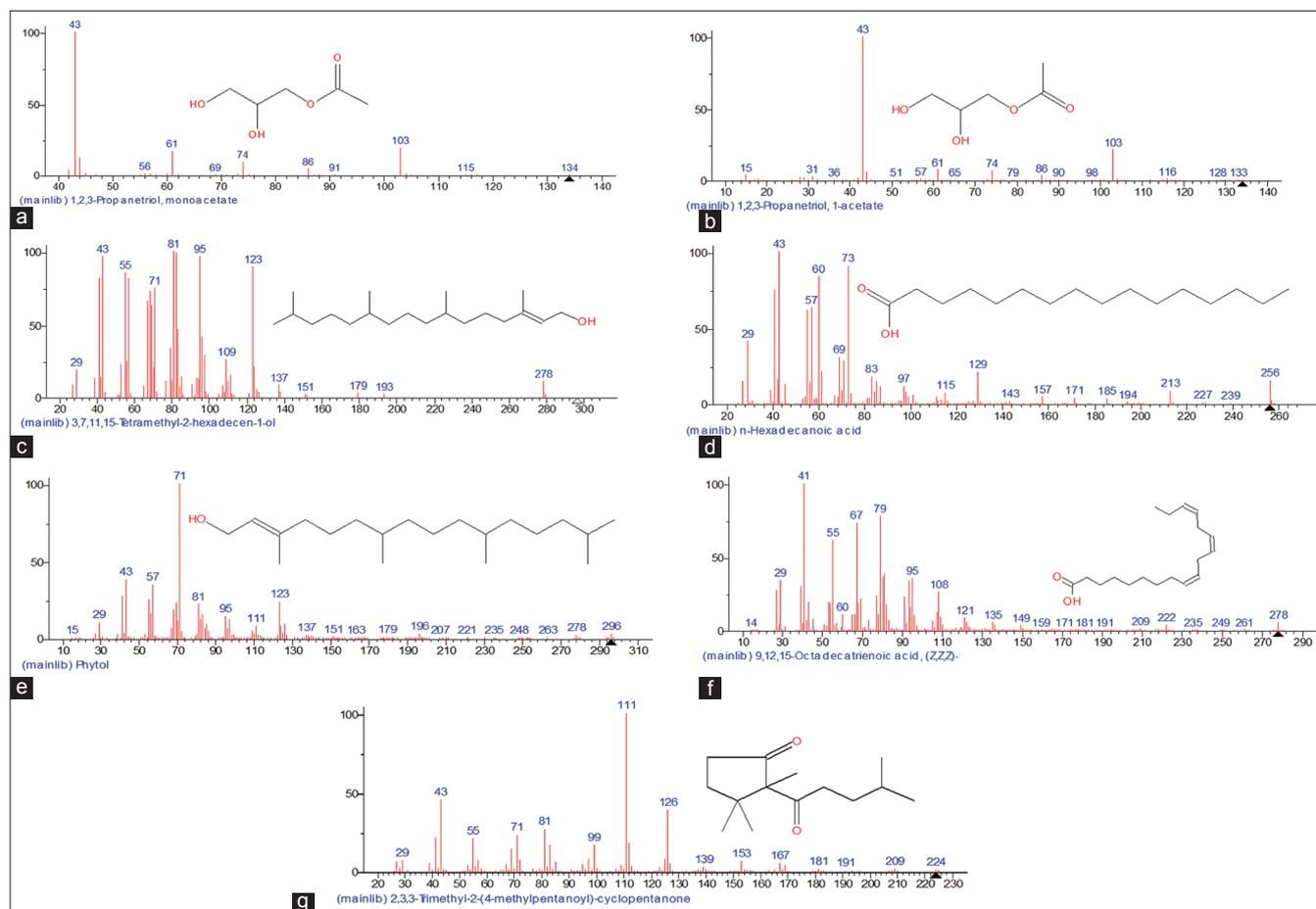


Fig. 4: (a-g) Gas chromatography-mass spectrometry spectrum of various compounds identified in *Cleome gynandra*

CONCLUSION

The data obtained through *in vitro* studies depicted that *C. gynandra* possess significant anticancer and antioxidant activities. Chemical moieties detected in the GC-MS analysis such as palmitic acid, phytol, and phenol further provided chemical evidence for anticancer action of the selected extract. Further studies in this area might help in developing a novel anticancer drug for the betterment of cancer population.

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