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Original Article

GC-MS PROFILE OF *IN VIVO* AND *IN VITRO* SHOOTS OF *CLEOME GYNANDRA* L.

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ABSTRACT

Objective: Investigation of the bioactive compounds from the ethanol shoot extracts of *in vivo* and *in vitro* plants of *Cleome gynandra* (*C. gynandra*) through GC-MS analysis.

Methods: The nodal explants were cultured on Murashige and Skoog (MS) medium supplemented with different concentrations of 6-benzyl-aminopurine (BAP), kinetin-6-furfurylaminopurine (Kin) and indole 3 acetic acids (IAA) for shoot induction. In the present study, the phytochemical constituents were analyzed from the ethanol extract of *in vivo* and *in vitro* plants of *C. gynandra* using Gas Chromatography-Mass Spectrometry (GC-MS) analysis. The mass spectrum of the ethanol extract was compared with the available library sources.

Results: In the present study, different concentrations of cytokinins and in the combination of IAA are used to develop regenerated shoots. The maximum number of shoots was obtained 9.2±0.41 with the length of 6.6 cm and highest frequency of (100%) shoot induction was observed on MS medium containing 10 µM BAP with 4 µM IAA. The GC-MS analysis revealed that the shoots of *in vivo* and *in vitro* plants contained 21phytochemicals, of these 3 components were similar in both *in vivo* and *in vitro* plants, 2 phytochemical's are repeated with different RT, 7 components are having biological activity and in the remaining 9 components, biological activities are not reported.

Conclusion: The present study, the *in vitro* regeneration, combinations of hormones (10 µM BAP plus 4 µM IAA) tested showed the best result than individual and also revealed that the synthesis of more number of phytochemicals present in the ethanolic extracts of *in vitro* plants than the *in vivo* plants of *C. gynandra.*

Keywords: GC-MS, *C. gynandra*, *In vivo* and *in vitro* plants, Biological activity

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INTRODUCTION

C. gynandra L. is commonly known as Cat whiskers, Spider flower and Nalvelai in Tamil. *C. gynandra* is used as a medicinal plant and can be found in all over the world [1]. It grows as a weed in paddy fields, roadsides and in open grasslands. In India, it is not at all cultivated but grows unexpectedly all over the place. Different species of *Cleome* can be found in all the states of India [2]. According to the Indian traditional system of Ayurveda medicine, lays prominent on the promotion of health concept of strengthening host defenses against different diseases [3]. In the literature of Pharmacopeia of India and also in other ancient medical texts it is described that the medicinal application of *C. gynandra* is an important medicinal plant in Ayurveda [4]. The plant's nutritional value may vary with soil fertility, environment, plant type, plant age and the production techniques used [5].

Plant tissue culture has been a preferred biotechnology for applications regarding medicinal plants for many years [6]. According to Alferman *et al.* [7], many attempts have been made to use plant cell culture for commercial production of plant secondary metabolites; In addition, Preil [8], blamed the lack of progress specifically on the high cost of bioreactors, slow growth of plants, and low yields of active metabolites. Plant tissue culture is one of the most useful methods for conservation and mass propagation of medicinal plants [9]. Medicinal plants have two types of metabolites; one of them is primary and another is secondary metabolites. Primary metabolites are directly involved in plant activities whereas secondary are not directly involved [10]. Development of *in vitro* procedures will help to establish tissues for germplasm conservation, rapid propagation and for secondary metabolite production to meet the vastly increased demand of therapeutic and other industries [11].

Phytochemical studies of *C. gynandra* have shown antimicrobial activity due to their bioactive constituents such as tannins, flavonoids, alkaloids and saponins [12-15]. The medicinal actions of particular plant species depend upon the secondary metabolites [16-18]. Screening of the bioactive constituents leads to the development of new novel drugs for curing various maladies [19]. To the best of our knowledge and literature survey works on GC-MS analysis of *C. gynandra* is lacking. Hence the present study aims to explore the phytochemical constituents present in the ethanolic shoot extract of *in vivo* and *in vitro* plants of *C. gynandra* by GC-MS technique.

MATERIALS AND METHODS

Chemicals and reagents

All the solvents and other reagents used in the present study are of analytical grade and purchased from Himedia laboratories, India.

Plant material and establishment of explants

The plant material *C. gynandra* was collected from Arugampalayam, near Thanthonimalai, Karur of Tamil Nadu, India. The nodal segments with shoot tips were selected for the source of explants in the present study. The explant of nodal segments was excised and washed with running tap water for 30 min and followed by liquid detergent (Teepol) for 5 min for surface sterilization. They were rinsed with distilled water for 2-3 times to remove all the surface sterilizing agents. The surface sterilized materials were kept inside the laminar air flow chamber, where they were disinfected with 70% alcohol for 45 seconds followed by 0.1% mercuric chloride for 3 min. Finally, the material was thoroughly rinsed with sterile double distilled water for 4-5 times to remove all the sterilizing agents.

Procedure for preparing culture media

MS media was used as the basal medium with 3% of sucrose and respective growth regulators for shoot induction and multiplication. The medium was solidified with 0.8% of agar and the pH of the medium was adjusted to 5.8 using 0.1N HCl or NaOH. The culture

media were sterilized by autoclaving at 1.06 kg cm^{-3} and 121 °C for 15-18 min [20].

Plant growth regulators

MS medium containing different concentrations of Kin (5-25 µM), BAP (5-25 µM) either alone or in combination with IAA (2-10 µM) were utilized for shoot induction and multiplication from nodal explants segments. Each experiment comprises of a minimum of 50 cultures and the experiments were repeated at least twice. The cultures were maintained in a culture room at 25±2 °C under 16 hr photoperiod with a light intensity of 30-40 µM mm^{-2} s^{-1} supplied by cool white fluorescent tubes. The relative humidity (RH) within the culture room was maintained at 55±5%. The media was refreshed at 4W intervals.

Statistical analysis

The design of all the experiments was a randomized complete block design and each experiment consisted of ten tubes with one explant in each and ten replicates. The parameters recorded were the number of shoots per explant, shoot length and number of shoots.

Extraction of samples for GC-MS analysis

The *in vivo* and *in vitro* shoots were cleaned, shade dried and powdered in a mechanical grinder. 6g of dry powder of each plant was soaked in 60 ml ethanol [21]. The flask was shaken, this process repeated for 3 d. Then it was filtered and evaporated at room temperature. Now the prepared sample is subjected to analysis [22].

Gas chromatography-mass spectrometry analysis

The GC-MS analysis of *in vivo* and *in vitro* plants of ethanol extracts of *C. gynandra* were performed using a GC-MS (Model; Thermo GC–Trace Ultra) equipped with a VF 5ms fused silica capillary column of 30m length, diameter 0.25 µm film thickness. Injection temperature was set at 260 °C. The oven temperature was programmed from 70 °C with the increase of 6 °C/min-raised to 260 °C. Ionization of the sample components was performed in electron impact mode (EI, 70 eV). Helium (99.9995% purity) was the carrier gas fixed with a flow rate of 1.0 ml min^{-1}. The mass range from 40-1000 m/z was scanned at a rate of 3.0 scans/s 1.0 µl of the ethanol extract of *C. gynandra* was injected with a Hamilton syringe to the GC-MS manually for total ion chromatographic analysis in split injection technique. Total running time of GC-MS is 30 min. The relative percentage of each extract constituents was expressed as a percentage with pack area normalization.

Identification of components

The relative percentage amount of each component was calculated by comparing its average peak area to the total area. The detection employed the NIST (National Institute of Standards and Technology) Ver.2.0-Year 2005 library. The compound prediction is based on Dr. Duke's Phytochemical and Ethnobotanical Databases by Dr. Jim Duke of the Agricultural Research Service/USDA. Interpretation of GC-MS was conducted using the database of NIST having more than 62,000 patterns. The spectrum of the unknown component was compared with the ADMET of the known components stored in the NIST library [23].

RESULTS

Shoot induction and multiplication

The nodal explants of *C. gynandra* were inoculated on MS medium supplemented with different concentrations of BAP and Kin ranging from 5 to 25 µM alone or in combination with IAA for shoot bud induction (table 1; fig. 1). Among the two cytokinins tested, BAP was found to be induced the maximum number of shoots than Kin. The highest frequency of shoot induction (100%), a maximum number of shoots (8.2) and the shoot length of 6.36 cm were observed on MS medium containing 10 µM BAP. However, Kin has induced 5.25 shoots with the shoot length of 4.62 cm at 15 µM.

The optimum level of BAP (10 µM) was further tested with different concentrations of IAA ranging from 2 to 10 µM. Among the combinations tested, a maximum number of shoots (9.2) with shoot length of 6.6. cm was obtained on MS medium supplemented with 10 µM BAP along with 4 µM IAA.

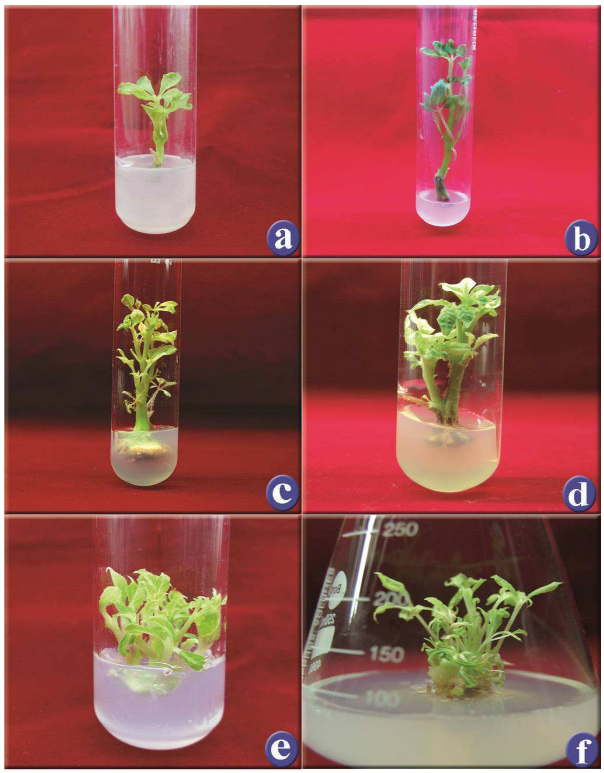

Fig. 1: *In vitro* regeneration of shoots from the nodal explants of *C. gynandra* a. Shoot initiation; b and c. Shoot elongation; d, e and f. Shoot multiplication

Table 1: Effect of different concentration of cytokinins and auxin on shoot induction and multiplication of *C. gynandra* from the nodal explants

Concentration of cytokinins		Concentration of auxin	Shoot induction frequency (%)	Number of shoots	Shoot length (cm)
BAP	Kin	IAA			
5 µM	---	---	80.0	5.6±0.57	4.86±1.37
10 µM	---	---	100	8.2±0.41	6.36±1.66
15 µM	---	---	66.6	4.0±0.40	4.24±0.65
20 µM	---	---	63.3	3.75±0.47	4.28±0.88
25 µM	---	---	53.3	3.8±0.41	3.64±1.21
---	5 µM	---	66.6	2.5±0.28	4.14±0.98
---	10 µM	---	73.3	3.75±0.47	5.66±1.50
---	15 µM	---	70	5.25±0.75	4.62±0.62
---	20 µM	---	50	3.6±0.57	3.88±1.06
---	25 µM	---	46.6	3.4±0.44	2.82±1.02
10 µM	---	2 µM	95	8.0±0.57	5.86±1.37
10 µM	---	4 µM	100	9.2±0.41	6.6±1.66
10 µM	---	6 µM	76.6	5.0±0.40	4.74±0.65
10 µM	---	8 µM	73.3	4.35±0.47	4.68±0.88
10 µM	---	10 µM	66.3	3.8±0.41	4.02±1.21

Values are mean±SE. Ten replicates repeated 3 times recorded after 30 d of culture.

Phytochemical analysis by GC-MS

The GC-MS analysis revealed that various bioactive compounds were identified in the ethanol extract of *in vivo* and *in vitro* shoots of *C. gynandra* (table 2, fig. 2 and 3). The active principle components with their retention time (RT), molecular formula, molecular weight (MW) and concentration (%) in the ethanol extract of *in vivo* and *in vitro* shoots were tabulated in table 2.

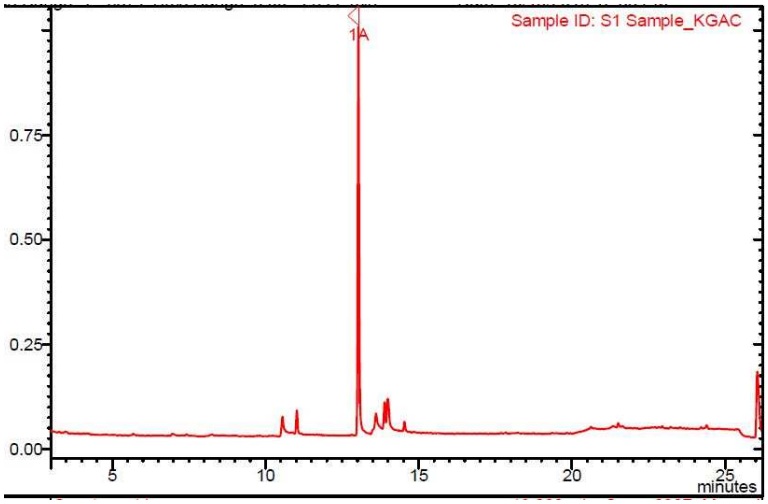

Fig. 2: GC-MS chromatogram of ethanol extract of *in vivo* shoots of *C. Gynandra*

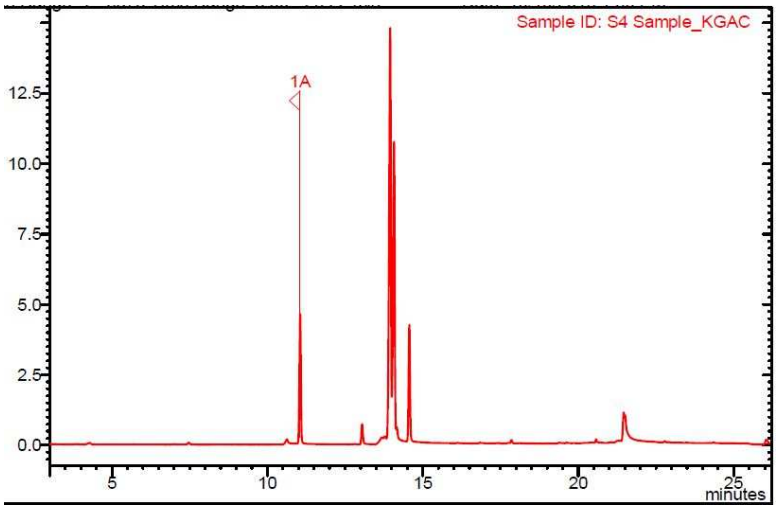

Fig. 3: GC-MS chromatogram of ethanol extract of *in vitro* shoots of *C. Gynandra*

The mass spectrum of leaves of *in vivo* and *in vitro* plants revealed the presence of 21 phytochemicals. Of these 3 components were similar in both *in vivo* and *in vitro* plants. They are namely n-hexadecanoic acid, hexadecanoic acid, ethyl ester, 3,7,11,15-tetramethyl-2-hexadecen-1-ol but the RT is slightly increased in *in vitro* plants when compared to *in vivo* plants. 2 phytochemical's are repeated with different RT, namely 9,12,15-octadecatrienal and eicosanoic acid ethyl ester. Among the 16 different phytochemicals, 7 components are having biological activity and in the remaining 9 components the activities are not reported (table 3).

The shoots of ethanolic extracts of *in vivo* plants showed 10 different phytochemicals. They are n-hexadecanoic acid, hexadecanoic acid, ethyl ester, 3,7,11,15-tetramethyl-2-hexadecen-1-ol, 9-octadecynoic acid, 9,12,15-octadecatrienal, 8,11,14-eicosatrienoic acid, methyl ester, (Z,Z,Z), acetamide, N-methyl-N-[4-(3-hydroxypyrrolidinyl)-2-butynyl], 1-hexadecanol, 2-methyl, methylene chloride. Similarly, the shoots of ethanolic extracts of *in vitro* plants showed 11 different components.

They are acetic acid, 3-cyano-2-cyclohexyl-[1,2]oxazinan-6-ylmethyl ester, eicosanoic acid, ethyl ester, n-hexadecanoic acid, hexadecanoic acid, ethyl ester, 3,7,11,15-tetramethyl-2-hexadecen-1-ol, Ethyl 9. cis.,11. Trans.-octadecadienoate, (E)-9-octadecenoic acid ethyl ester, eicosanoic acid, ethyl ester, ethyl 14-methyl-hexadecanoate, docosanoic acid, ethyl ester, 2-methyl-Z, Z-3,13-octadecadienol.

Table 2: Components detected in the *in vivo* and *in vitro* shoots in ethanol extract of *C. gynandra*

S. No.	R. T.	Name of the compound	Peak area %		Molecular Weight
			In vivo plants	*In vitro* Plants	
1.	10.587	n-hexadecanoic acid	4.080	0.52	256
2.	11.021	Hexadecanoic acid, ethyl ester	5.192	9.91	284
3.	13.021	3,7,11,15-tetramethyl-2-hexadecen-1-ol	61.893	1.68	296
4.	13.645	9,12,15-octadecatrienal	7.036	--	262
5.	13.867	9-octadecynoic acid	0.084	---	280
6.	13.931	9,12,15-octadecatrienal	15.777	--	262
7.	14.561	8,11,14-Eicosatrienoic acid, methyl ester, (Z,Z,Z)-	1.443	--	320
8.	21.540	Acetamide, N-methyl-N-[4-(3-hydroxypyrrolidinyl)-2-butynyl]-	0.050	--	210
9.	24.404	1-hexadecanol, 2-methyl-	2.790	--	256
10.	26.050	methylene chloride	0.054	--	84
11.	4.529	Acetic acid, 3-cyano-2-cyclohexyl-[1,2]oxazinan-6-ylmethyl ester	--	0.26	266
12.	7.475	Eicosanoic acid, ethyl ester	--	0.21	340
13.	13.962	Ethyl 9. cis.,11. trans.-octadecadienoate	--	35.424	308
14.	14.083	(E)-9-octadecenoic acid ethyl ester	--	32.28	310
15.	14.563	Eicosanoic acid, ethyl ester	--	9.52	340
16.	17.876	Ethyl 14-methyl-hexadecanoate	--	0.49	298
17.	20.582	Docosanoic acid, ethyl ester	--	0.41	368
18.	21.524	2-methyl-Z,Z-3,13-octadecadienol	--	6.84	280

Table 3: Biological activity of phytochemical identified in the ethanol extracts of *in vivo* and *in vitro* shoots of *C. gynandra*

S. No.	Name of the compound	Nature of compound	Biological activity
1.	n-hexadecanoic acid	Palmitic acid	Antioxidant, hypocholesterolemic nematicide, pesticide, lubricant, antiandrogenic, flavor, hemolytic, 5-alpha-reductase inhibitor
2.	Hexadecanoic acid, ethyl ester	Palmitic acid ester	Antioxidant, hypocholesterolemic nematicide, pesticide, lubricant, antiandrogenic, flavor, hemolytic 5-alpha reductase inhibitor
3.	3,7,11,15-tetramethyl-2-hexadecen-1-ol	Terpene alcohol	Antimicrobial, anti-inflammatory
4.	9,12, 15-octadecatrienal	Linoleic acid	Cancer preventive, hypocholesterolemic, anti coronary activity
5.	11,14,17-eicosatrienoic acid, methyl ester	Unsaturated fatty acid ester	Antiarthritic, anti-coronary Anti-inflammatory
6.	9-octadecenoic acid (Z) ethyl ester	Fatty acid ethyl ester	Steroids and primer pheromone
7.	8,11,14-eicosatrienoic acid, methyl ester, (Z,Z,Z)-	Ester compound	No activity reported
8.	Acetamide, N-methyl-N-[4-(3-hydroxypyrrolidinyl)-2-butynyl]-	Alcoholic compound	No activity reported
9.	1-hexadecanol, 2-methyl-	Alcoholic compound	No activity reported
10.	Methylene chloride	Acetic compound.	No activity reported
11.	Acetic acid, 3-cyano-2-cyclohexyl-[1,2]oxazinan-6-ylmethyl ester	Ester compound.	No activity reported
12.	Ethyl 9. cis.,11. trans.-octadecadienoate	Bronsted acid	No activity reported
13.	(E)-9-octadecenoic acid ethyl ester	Ester compound	Antioxidant, anti-inflammatory
14.	Eicosanoic acid, ethyl ester	Ester compound	No activity reported
15.	Ethyl 14-methyl-hexadecane	Alcoholic compound	No activity reported
16.	Docosanoic acid, ethyl ester	Ester compound	No activity reported

DISCUSSION

Micropropagation techniques allow being created millions of genetically identical plants. Several plant growth regulators are available for shoot induction and multiplication. However, BAP, Kin, thidiazuron (TDZ) and Isopentenyl adenine (2-iP) are widely used. In the present study, BAP and Kin are used individually or in the combination of both. As far as the individual hormone concerned 10 µM BAP was found to be the optimum concentration for shoot multiplication from the nodal explants. Similar results are also reported in several plants such as *Mentha piperita* [24], *Swertia chirayita* [25], *Andrographis paniculata* [26], *Sarcostemma brevistigma* [27]. The results are in agreement with earlier findings as observed in *Sesamum indicum* [28], *Enicostema axillare* [29], same as the concentration [30], whereas, the combination of 10 µM BAP plus 4 µM IAA increases the shoot induction and more number of shoots. The results also are in agreement with earlier findings as observed in *Mentha viridis* [31], *Bacopa monneiri* [32], *Lobelia nicotianaefolia* [33] and *Solanum nigrum* [34] where BAP and KIN resulted in marked increase in shoot multiplication.

For the detection and identification of plant metabolites analysis, a new discipline known as plant metabolites developed. Metabolite profiling and metabolite fingerprinting are recently developing technology for phenotypic diagnosis of plant analysis [35-36]. They help us for the identification of most important compounds (or groups of compounds) present between genotypes or phenotypes.

For *in vivo* and *in vitro* plant, n-hexadecanoic acid was identified as the first component with the RT of 10.587 min. This compound has the molecular formula of $C_{16}H_{32}O_2$ and comprised in *in vivo* plants in 4.08 % and *in vitro* plants in 0.52% of the ethanolic shoots extract of *C. gynandra* (table 2). It may have the role of antioxidant, hypocholesterolemic, nematicide, pesticide, lubricant, anti-androgenic (table 3) [37-38].

The 3,7,11,15-tetramethyl-2-hexadecen-1-ol ($C_{20}H_{40}O$) also called as phytol was the second compound having a molecular weight of 296 with the RT of 13.021 min. It was present in *in vivo* plant in 61.893% and *an in vitro* plant in 1.68 % this compound has antimicrobial and anti-inflammatory activity [39-40]. The third compound was (E)-9-octadecenoic acid ethyl ester ($C_{20}H_{38}O_2$) which was determined at the RT of 13.867 min and it has the molecular weight of 280 (table 2). This compound was absent in *in vitro* regenerated shoots of *C. gynandra* and it has the property of antimicrobial and anti-inflammatory activity (table 3) [34-36]. 9,12,15-octadecatrienal is also one of the chemical constituents that were identified from plantlets. It is a linoleic acid ($C_{18}H_{30}O$) present in the RT of 13.645 min (table 2). It has a molecular weight of 262, and it has anti-inflammatory, hypocholesterolemic, cancer preventive [41-42] hepatoprotective, nematicide, insectifuge, antihistaminic, antieczemic, antiacne, 5-alpha-reductase inhibitor, anti-androgenic, antiarthritic, anti-coronary and insecticidal activity [43-45].

The compound (E)-9-octadecenoic acid ethyl ester was present only in *in vitro* regenerated shoots of *C. gynandra* with the RT of 14.083 and has a molecular weight of 310. It was an ester compound and has an antioxidant, anti-inflammatory activity. The percentage of the peak area was 32.28% [46].

CONCLUSION

In vitro regeneration system provides disease-free plant material that is free from external contamination. In the present study, nodal explants were cultured on MS medium fortified with various concentrations of BAP or Kin. BAP was comparatively better than Kin for shoot regeneration. The addition of IAA along with BAP significantly increased the number of shoots per explant and average shoot length. This protocol could be used for large-scale production of this important medicinal plant. The *in vitro* regenerated shoots and *in vivo* shoots were further investigated for the presence of phytochemical constituents through GC MS. From the GC-MS study, 16 different compounds have been identified from the ethanol extract of *in vivo* and *in vitro* shoots of *C. gynandra*. Therefore the presence of various bioactive compounds in the plants of *C. gynandra* justifies the use of the plant in the treatment of different ailments by the traditional practitioners and it further holds promise for the production of novel pharmaceuticals.

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AUTHORS CONTRIBUTION

Most of the experimental part of the work was done by the Research scholar I. Sudan. Dr. A. V. P. Karthikeyan, Guide and Principal Investigator of the UGC project, guided and monitored the experimental design, data compilation, and statistical analysis and corrected the manuscript.

CONFLICT OF INTERESTS

The authors do not have any conflict of interest to declare.

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