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GC-MS PROFILING OF TRANSFORMED ROOTS OF *CALOTROPIS PROCERA*

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Abstract

Description of the subject: *Calotropis procera* (Ushaar) is a xerophyte species containing secondary metabolites that confer a number of biological and pharmacological properties to be used as a potential source of disease-treatment drugs. A number of studies have reported on the chemical composition of Ushaar leaves but there are no reports on hairy roots composition.

Objective: The aim of this study was thus to analyze the phytochemical composition of the hairy roots of *C. procera* induced by genetic transformation via *Agrobacterium rhizogenes.*

Methods: Induced hairy roots (4 weeks old) have been dried, grounded into fine powder and solubilized into a solvent solution containing 20% dichloromethane, 50% acetonitrile, 10% ethanol, and 20% hexane. The extracts have been analyzed by Gas chromatography (GC) and mass spectrometry (MS).

Results: Different chemical compounds that belong to different classes of secondary metabolites have been identified. Those identified compounds include fatty acids, sterols, tri-terpenes, alkanes and esters. The most abundant components were; palmitic acid (23.15%); linoleic acid isomers 1 and 2 (14.36%, 12.18% respectively); squalene (8.60%); stigmasterol (12.96%); β-sitosterol (5.07%) and campesterol (4.93%).

Conclusion: The identified compounds have various therapeutic effects as anticancer, anti-inflammatory and antibacterial molecules. The ability to induce hairy roots containing active compounds suggests the possibility of using *Calotropis* as a source of potential therapeutic metabolites through a plant molecular pharming setting.

Keywords: *Calotropis procera*; *Apocynaceae*; hairy roots; GC-MS; secondary metabolites, ushaar

PROFILAGE PAR CPG-SM DES RACINES TRANSFORMÉES DE *CALOTROPIS PROCERA*

Résumé

Description du sujet : *Calotropis procera* (Ushaar) est une espèce xérophyte contenant des métabolites secondaires qui confèrent un certain nombre de propriétés biologiques et pharmacologiques pouvant être utilisé comme source potentielle de médicaments pour le traitement des maladies. Plusieurs études ont rapporté la composition chimique des feuilles de l'Ushaar, mais aucune n'a rapporté la composition chimique des chevelus racinaires.

Objectifs : Le but de cette étude est d'analyser la composition phytochimique des chevelus racinaires de *C. procera* induits par la transformation génétique via *Agrobacterium rhizogenes*.

Méthodes : Les chevelus racinaires induits (âgées de 4 semaines) ont été séchés, broyés en poudre fine et solubilisés dans une solution de solvant contenant 20% de dichlorométhane, 50% d'acétonitrile, 10% d'éthanol et 20% d'hexane. Les extraits ont été analysés par chromatographie en phase gazeuse (CPG) et spectrométrie de masse (SM).

Résultats : Différents composés chimiques appartenant à différentes classes de métabolites secondaires ont été identifiés. Ces composés identifiés comprennent les acides gras, les stérols, les tri-terpènes, les alcanes et les esters. Les composants les plus abondants étaient ; l'acide palmitique (23,15%) ; les isomères 1 et 2 de l'acide linoléique (14,36%, 12,18% respectivement); le squalène (8,60%); le stigmastérol (12,96%); le β-sitostérol (5,07%) et le campestérol (4,93%).

Conclusion : Les composés identifiés ont divers effets thérapeutiques en tant que molécules anticancéreuses, anti-inflammatoires et antibactériennes. La capacité à induire des chevelus racinaires contenant des composés actifs suggère la possibilité d'utiliser *Calotropis* comme source de métabolites thérapeutiques potentiels par le biais des paramètres du molecular pharming des plantes.

Mots clés : *Calotropis procera* ; Apocynaceae ; chevelus racinaires ; CPG-SM ; métabolites secondaires, ushaar

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INTRODUCTION

Plants have been used as a natural source of medication since the dawn of civilization. Plant roots and shoots have been described by ancient practitioners to treat different kinds of diseases without knowledge of precise chemical content or mode of action of the active compounds in plant tissues [1], [2]. *Calotropis procera* (Ait.) W.T. Aiton (Fig. 1) (also known as giant milkweed, ushaar, rubber bush, king's crown or Sodom apple) is a xerophytic perennial desert shrub (Apocynaceae) that contain different types of chemical and medicinal properties. Native to tropical and subtropical Afro-Asian regions, the shrub grows on a variety of soils and under harsh environmental conditions [3], [4], [5].

Figure 1. *Calotropis procera*.

A number of secondary metabolites have already been identified in this plant, such as sterols [6], cardiac glycosides [7], triterpenes [8], flavonoids [9], alkaloids, coumarins, saponins and tannins [10]. Many biological and pharmacological properties have also been attributed to the plant, such as anticancer activity [11]. Despite some toxic effects, different organs of *Calotropis* have been used in local traditional medicine for the treatment of multiple diseases or injuries, such as rheumatism and asthma, leprosy, elephantiasis, fever, menorrhagia, malaria, snake bite [5], [12], antimicrobial, anti-inflammatory, anti-diarrheal, anthelmintic, spasmolytic, immunomodulatory, antipyretic, antioxidant, wound healing, protective, analgesic, acaricide, antinociceptive, antiulcer and anti-fertility, cheese-making activities and as a source of natural colorants for textile or in the production of nanoparticles [5], [13], [14], [15], [16], [17], [18], [19]. Over the last few decades, the culture of hairy roots induced by Agrobacterium rhizogenes has been largely used for the production of valuable bioactive molecules [20], [21], [22], [23], [24], [25]. Hairy roots cultures offer many advantages related to rapidity of growth in hormone-free culture medium, genotypic and biochemical stability, and high productivity of secondary metabolites compared to cells or callus cultures, or even the roots of the mother plants [26], [27], [28]. Several studies already reported the chemical composition of leaves and roots of *C. procera* [29], [30], [7], but to the best of our knowledge, no research has been reported so far on the chemical analysis of *C. procera* hairy roots.

Our aim was to induce the formation of hairy roots in *C. procera* through *Agrobacterium rhizogenes* (*A. rhizogenes*) and to analyze the chemical composition of the developing hairy roots using gas chromatography (GC) and mass spectrometry (MS). These are powerful techniques routinely used to analyze the content of small molecules in plant, plant genetic and metabolic engineering studies.

MATERIALS AND METHODS

1. Plant material

Healthy plants of *C. procera* grown in the region of Adrar (South Algeria) were used as a source of seeds. Mature fruits have been harvested and undamaged seeds extracted, cleaned from silk fibers, and cultured in vitro. The seeds were first plated into test tubes containing 20 mL of Murashige and Skoog (MS) medium supplemented with 7 g/L of agar and 20 g/L sucrose. In vitro seedlings were then transferred into a growth chamber at a temperature of 26± 1°C and a photoperiod of 16 hours light and 8 hours dark. After 30 days in these culture conditions, the seedlings have been used as a source explants.

2. Culture of transformed roots (hairy roots)

This research was conducted in the Genetic Ressources and Biotechnology laboratory at the Higher National Agronomic School, El Harrach, Algiers, Algeria. *C. procera* plants cultivated in vitro were genetically transformed by *A. rhizogenes strains* (A4 and AR15834) to obtain transformed roots or hairy roots. These latter were cultivated in half strength B5 medium in complete darkness and harvested after 4 weeks for further chromatographic analysis by GC-MS.

3. Sample preparation

Hairy roots (4 weeks old) were dried in an oven at 40° C until a constant dry weight was obtained.

Dried hairy roots were ground into a fine powder in a ceramic mortar and pestle. Two grams of hairy root powder was solubilized in 10 ml of a solvent solution containing 20% dichloromethane, 50% acetonitrile, 10% ethanol, and 20% hexane. The tube containing the sample was agitated in a vortex mixer (vortex VWR) for 1 min, and then homogenized in an ultrasonic bath (Fisher Scientific) at room temperature. The samples were shaken mechanically for 30 min in a rotary shaker (Heidolph), and then centrifuged for 5 min at 3500 rpm (SIGMA). An evaporation step to remove any residual liquids was performed in nitrogen evaporator (Liebisch) until drying the extract. 500 µL of methanol (LC-MS grade) was added, followed by vortexing for 1 hour. The mixes were homogenized in an ultrasonic bath for 20 min and centrifuged for 5 minutes at 3500 rpm. The supernatant was recovered and filtered using a 45 µm microfilter in a vial for GC-MS analysis.

4. Gas chromatography – Mass spectrometry (GC/MS) conditions

Qualitative analysis was carried out by GC-MS. The study was conducted using a Hewlett Packard HP 5890 Series II Gas Chromatograph GC System coupled to an HP 5971A mass selective detector (MSD) manufactured by Agilent (United States), which is a quadrupole mass analyzer. 1-µL samples were injected automatically in splitless mode. Chromatographic separation of the analyzed samples was performed on a HP-5MS capillary column (30 m, 0.25 mm, 0.25µm) in a temperature gradient consisted of five segments. Initial column temperature (90°C) was maintained for 0.5 min, increased linearly at the rate of 20°C/min to 200°C, then to 280°C at 15°C/min and finally to 320°C at 20°C/min, maintained at this temperature for 3.67 min. Helium at a constant flow of 1 mL/min was used as a carrier gas. The spectrometer was operated in electron ionization mode (EI) and the electron beam energy was 70 eV. Positive ions were analyzed. Acquisition was carried out in scan mode, and the entire mass range from 38 to 650 amu was collected.

RESULTS

We aimed to analyze the chemical composition of hairy roots in the medicinal plant *Calotropis procera* after transformation by *A. rhizogenes*. The infection by *A. rhizogenes* results in the transfer of genetic material from bacteria to the plant and the formation of hairy roots [24], [28].

The *C. procera* hairy root culture was grown in half strength B5 medium in complete darkness. Thirty-six bioactive compounds belonging to different classes of metabolites have been identified by GC-MS analysis (Figure 2, 3, 4, 5 and 6). The identification of these compounds was performed based on the interpretation of the corresponding mass spectra compared with data reported in the literature as well as with the spectra of the NIST library. The GC-MS retention time (RT), name, molecular formula, molecular weight and peak area of the compounds are given in Table (1).

The main metabolites that have been found in *C. procera* hairy roots are palmitic acid (23.15%), linoleic acid: isomer 1 and isomer 2 (14.36%, 12.18% respectively), stigmasterol (12.96%), squalene (8.60%), β-sitosterol (5.07%), and campesterol (4.93%) (Figure 7). Other compounds of lower concentrations have also been identified, such as 4.8.A Dimethyl.6(2. methyl oxyranil)-4A,5,6,7,8,8A-hyxahydro-2(1H) naphthalene (2,98%), stigmastadienone (1.69%), stearic acid (1.41%), tocopherol (vitamin E) (1.34%) and egosta-5, 22-dien-3-ol (1.07%). A certain number of compound trace concentrations have also been found, including cholesterol (0.85%), pentadecanoic acid (0.83%), 1,4-Dihydro-9-isopropylidene-5,6,7,8-tetrametoxy-1,4-methanonaphtalene (0,80%), tricosane (0.77%), Delta.6-progesterone (0.75%), cyclotetradecane (0.67%), O-benzo[B]xenthen-12-one,11-(acetyloxy) (0,63%), palmitic acid ethyl ester (0.54%), palmitic acid glycerol ester (0.49%), 4. ((1.E).3 hydroxyl.1.proponyl)-2-methoxyphenol (0,43%), heptadecanoic acid (0.41%), ferulic acid glycine conjugate (0.40%), heptadecane (0.38%), β-progesterone (0.36%), 1,21-Docosadiene (0,30%), brassidic acid methyl ester (0.27%), 4.4'.-Dibutoxy-1,1'biphenyl (0,26%), Elemicin.M (demethyl-) iso-2 (0,24%), 17-Cyclohexyl triacontane (0,17), linolenic acid (0.15%), octadecane (0.15%), pentadecane (0.14%), 2-(octadecyloxy) ethanol (0,04%).

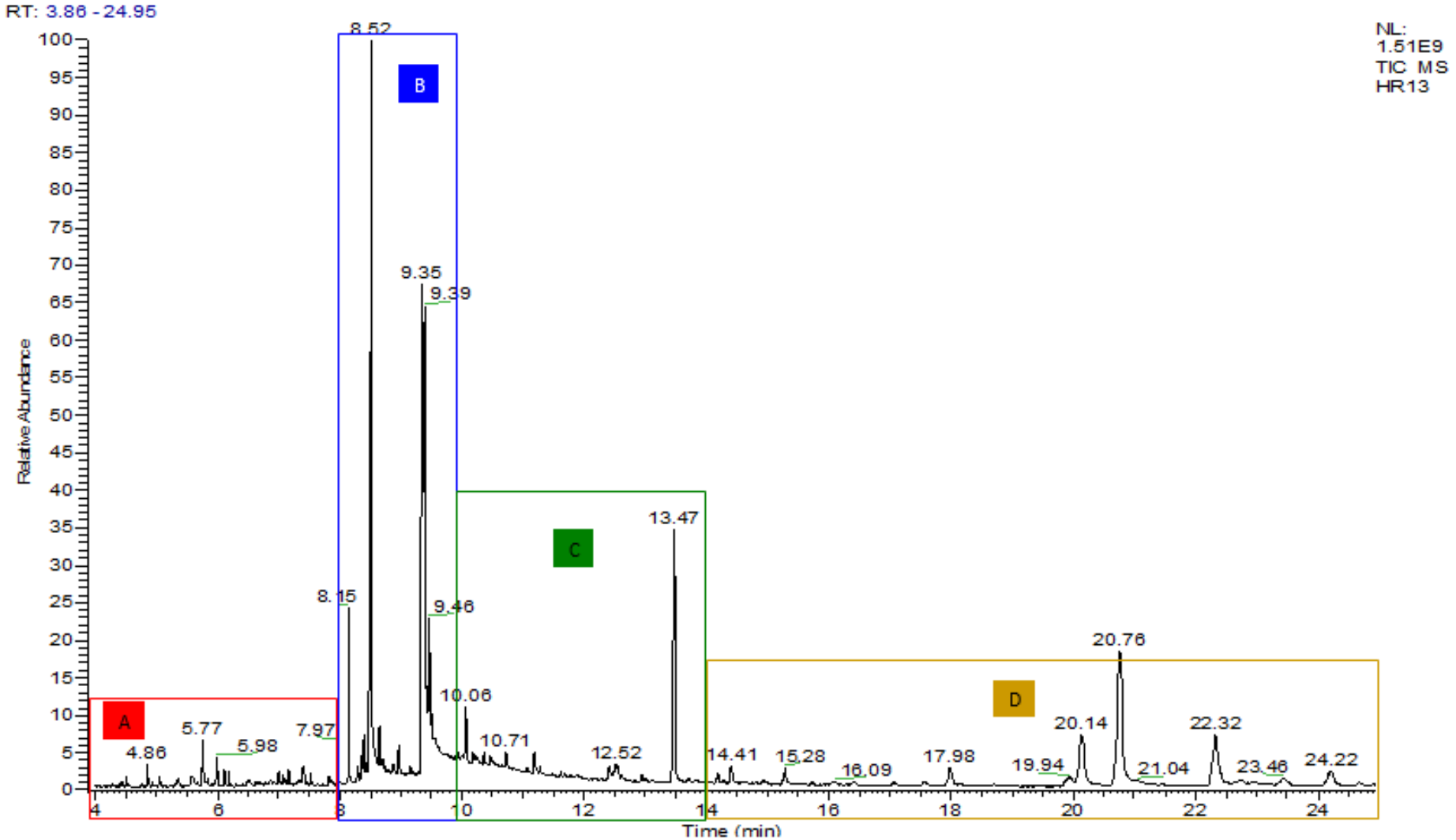

Figure 2: GC-MS chromatogram (TIC: Total Ion Chromatogram) of hairy roots of *C. procera Ait.*

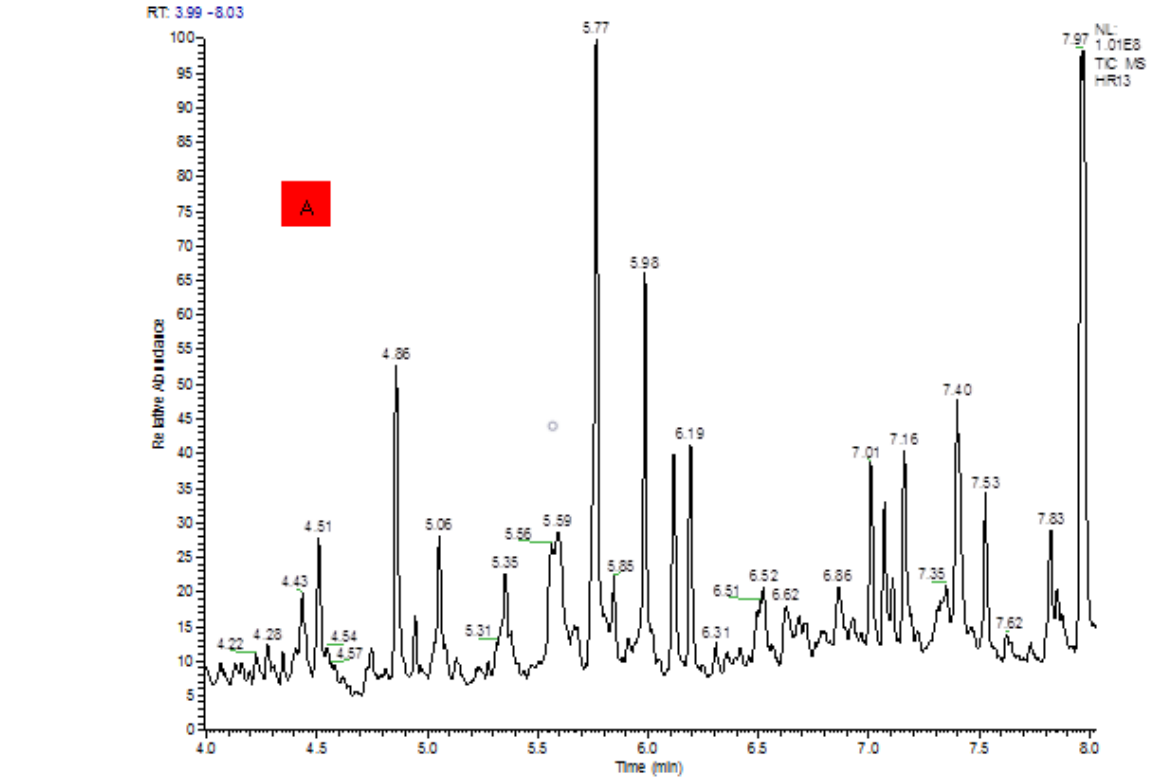

Figure 3: Part A: GC-MS chromatogram of hairy roots of *C. procera Ait.* (Retention time: 4.00-8.00 min)

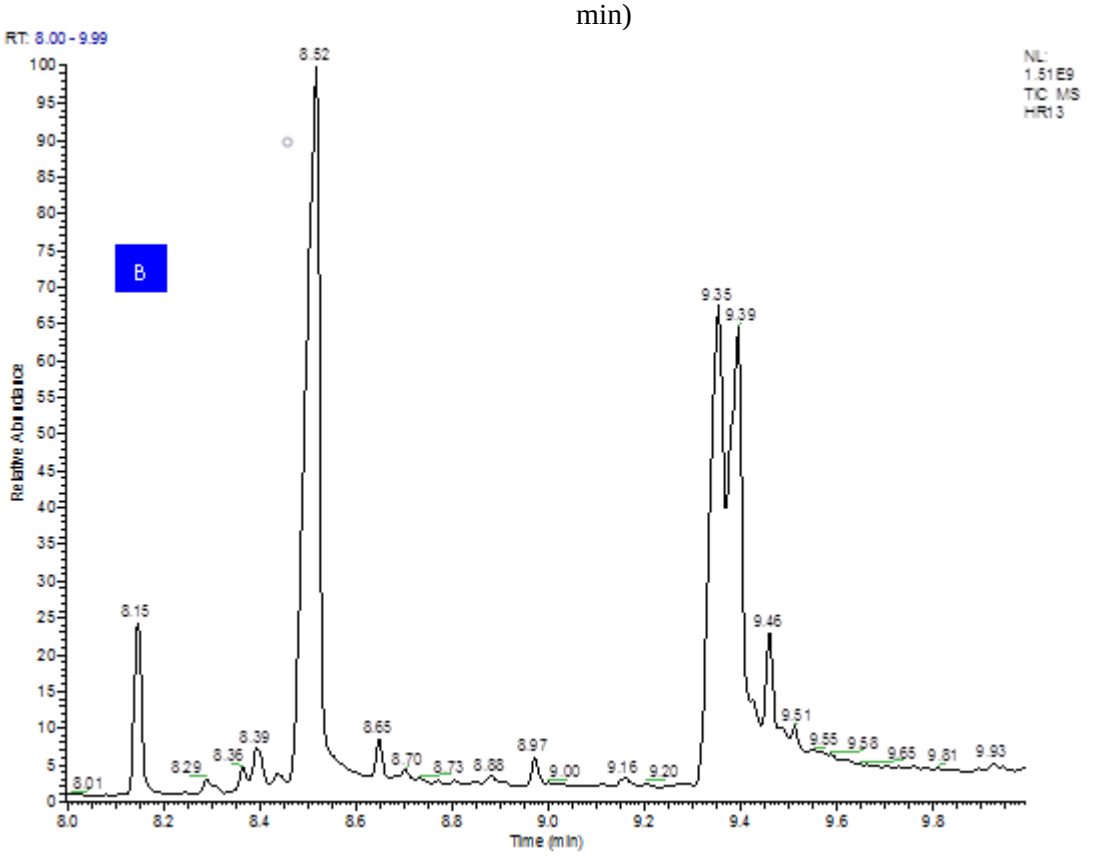

Figure 4: Part B: GC-MS chromatogram of hairy roots of *C. procera Ait.* (Retention time: 8.00-10.00 min)

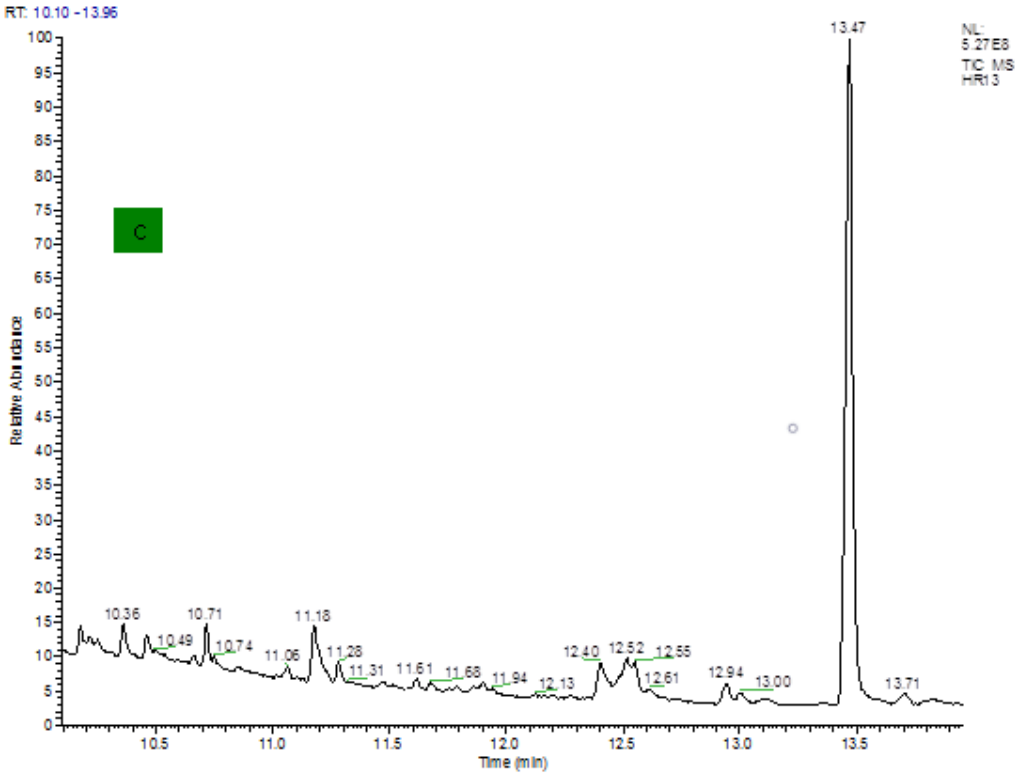

Figure 5: Part C: GC-MS chromatogram of hairy roots of *C. procera Ait.* (Retention time: 10.00-14.00 min)

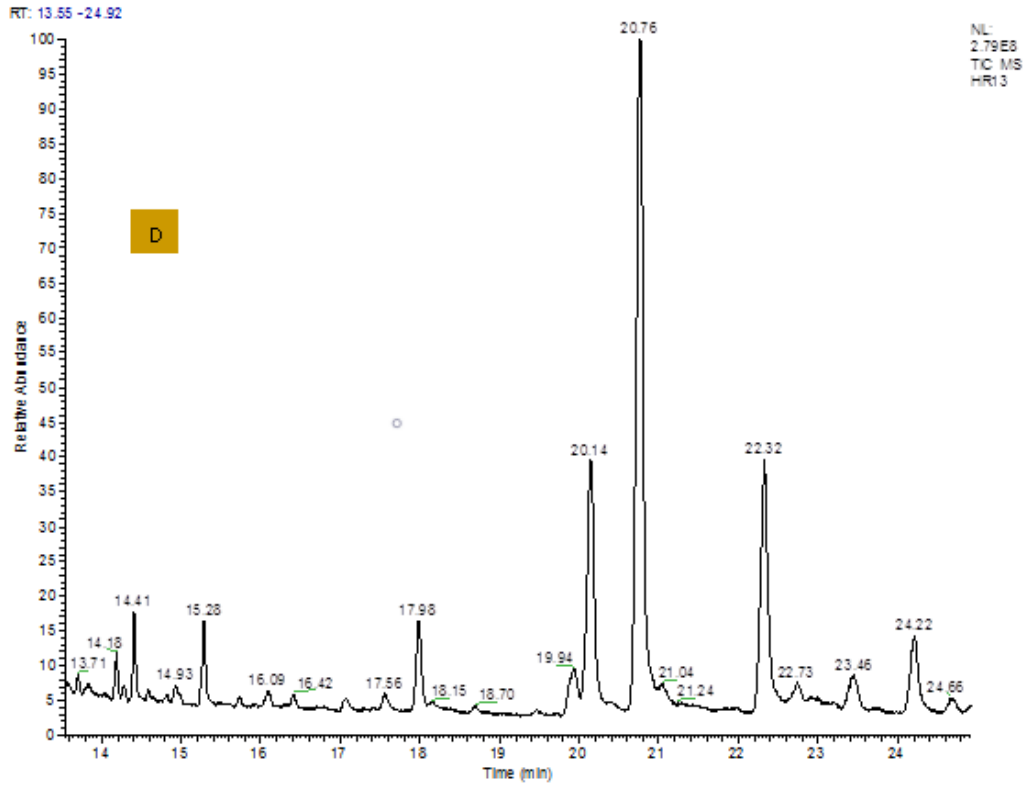

Figure 6: Part D: GC-MS chromatogram of hairy roots of *C. procera Ait.* (Retention time: 14.00-25.00 min)

Table 1: Chemical compounds identified in *C. procera* hairy roots extract using GC-MS analysis

TIC Part	N°	Retention Time (RT) (minute)	Compound's Name	Molecular formula	Molecular Weight (MW)	Peak Area %
A	1	4.43	Pentadecane	$C_{15}H_{32}$	212	0,14
	2	5.06	Octadecane	$C_{18}H_{18}$	254	0,15
	3	5.59	Heptadecane	$C_{17}H_{36}$	240	0,38
	4	5.77	Cyclotetradecane	$C_{14}H_{28}$	196	0,67
	5	5.98	Ferulic acid glycine conjugate	$C_{14}H_{17}NO_5$	279	0,40
	6	6.31	2-(octadecyloxy) ethanol	$C_{20}H_{42}O_2$	314	0,04
	7	6.52	Linolenic acid	$C_{19}H_{32}O_2$	292	0,15
	8	7.16	Elemicin.M (demethyl-) iso-2	$C_{13}H_{16}O_4$	236	0,24
	9	7.40	4. ((1.E).3 hydroxyl.1.proponyl)-2-methoxyphenol	$C_{10}H_{12}O_3$	180	0,43
	10	7.97	Pentadecanoïc acid	$C_{15}H_{30}O_2$	242	0,83
B	11	8.15	4.8.A Dimethyl.6(2. methyl oxyranil)-4A,5,6,7,8,8A-hyxahydro-2(1H)naphthalene	$C_{15}H_{22}O_2$	243	2,98
	12	8.52	Palmitic acid	$C_{16}H_{32}O_2$	256	23,15
	13	8.65	Palmitic acid ethyl ester	$C_{18}H_{36}O_2$	284	0,54
	14	8.97	Heptadecanoic acid	$C_{17}H_{34}O_2$	270	0,41
	15	9.35	Linoleic acid isomer 1	$C_{18}H_{32}O_2$	280	14,36
	16	9.39	Linoleic acid isomer 2	$C_{18}H_{32}O_2$	280	12,18
	17	9.46	Stearic acid	$C_{18}H_{36}O_2$	284	1,41
C	18	10.06	Tricosane	$C_{23}H_{48}$	324	0,77
	19	10.36	Brassidic acid methyl ester	$C_{23}H_{44}O_2$	352	0,27
	20	10.46	Linoleic acid isomer 3	$C_{18}H_{32}O_2$	280	0,20
	21	10.71	1,21-Docosadiene	$C_{22}H_{42}$	306	0,30
	22	11.18	Palmitic acid glycerol ester	$C_{19}H_{38}O_4$	330	0,49
	23	11.28	17-Cyclohexyl triacontane	$C_{39}H_{78}$	546	0,17
	24	12.40	O-benzo[B]xenthen-12-one,11-(acetyloxy)	$C_{19}H_{12}O_4$	304	0,63
	25	12.94	4.4'.-Dibutoxy-1,1'biphenyl	$C_{20}H_{26}O_2$	298	0,26
	26	13.47	Squalene	$C_{30}H_{50}$	410	8,60
D	27	14.18	β-Progesterone	$C_{21}H_{30}O_2$	314	0,36
	28	14.41	Delta.6-progesterone	$C_{21}H_{28}O_2$	312	0,75
	29	15.28	1,4-Dihydro-9-isopropylidene-5,6,7,8-tetrametoxy-1,4-methanonaphtalene	$C_{18}H_{22}O_4$	302	0,80
	30	17.98	Tocopherol	$C_{29}H_{50}O_2$	430	1,34
	31	19.94	Ergosta-5, 22-dien-3-ol	$C_{28}H_{46}O$	398	1,07
	32	20.14	Campesterol	$C_{28}H_{48}O$	400	4,93
	33	20.76	Stigmasterol	$C_{29}H_{48}O$	412	12,96
	34	22.32	β-sitosterol	$C_{29}H_{50}O$	414	5,07
	35	23.46	Cholesterol	$C_{27}H_{46}O$	386	0,85
	36	24.22	Stigmastadienone	$C_{29}H_{46}O$	410	1,69

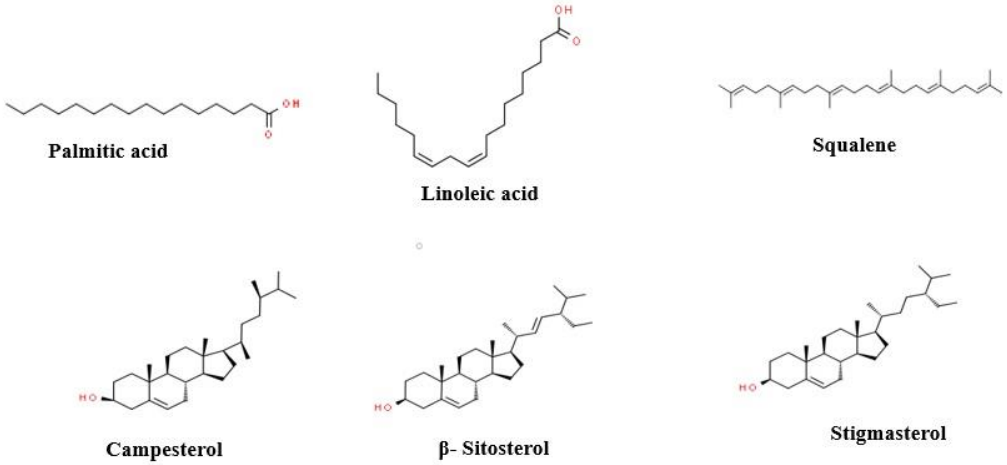

Figure 7. Structure of major constituents identified from *C. procera* hairy root cultures: palmitic acid, linoleic acid, squalene, campesterol, β-sitosterol and stigmasterol.

DISCUSSION

Previous studies reported chemical composition of leaves, latex and fruits of *C. procera* by GC-MS analysis [31], [32], [33]. In this research, a variety of bioactive molecules was identified in hairy root culture extracts, ranging from fatty acids, sterols, terpenes, alkanes, esters and others. Some of these molecules were already detected in different parts of *C. procera* plants.

Palmitic acid, linoleic acid and other fatty acids were previously detected in *C. procera* [32], [34], [35]. These fatty acids and their derivatives are known for their antibacterial, antiviral, anti-carcinogenic and anti-atherosclerotic proprieties [36], [37]. Previous studies indicated the presence of sterols in *C. procera*, such as stigmasterol, β-sitosterol and campesterol [6], [31]. Several activities of stigmasterol have been described as antioxidant, anti-tumor and anti-inflammatory molecules [38]. Paniagua-Pérez et *al*. [39] have also investigated immunostimulant potential of β-sitosterol. Antifungal activities of these three sterols have already been proved [40]. Naser et *al*. [32] and Alhazmi et *al.* [41] have also reported the presence of squalene in *C. procera* leaf extract. This compound is widely used as a principal component of parenteral emulsions for drug and vaccine delivery [42]. Squalene seems also to reduce inflammation when used as an adjuvant during a chemotherapy in tumor-bearing mice [43].

Some studies report the presence of tocopherol (Vitamin E), tricosance, esters and alkanes [32], [33], [41]. Tricosane and alkanes have antibacterial effects [44].

The presence of all these molecules indicates that *C. procera* hairy roots can be used as a source of anti-inflammatory, antibacterial, antifungal, anti-ulcerative and anti-tumoral agents [45], [46].The multiple therapeutic properties of the chemical compounds present in this plant suggests that the use of *C. procera* roots in traditional medicine is somewhat supported by scientific evidence. However, further research would be required to enhance the overall yield of bioactive molecules produced in hairy roots at economic and pharmaceutical levels, which would require advanced research to understand their biosynthetic pathways and mechanisms and then to enhance their production for biopharmaceutical purposes.

CONCLUSION

This study aims to analyzing the chemical composition of the extracts of genetically transformed roots of *C. procera*, a high medicinal valued plant widely spread across Algeria. The analysis has been carried out using GC-MS method as a direct and fast analytical approach for the identification of fatty acids, sterols, terpenes, alkanes and esters. Thirty-six compounds of different chemicals and metabolites classes have been identified that the predominant molecules were palmitic acid, linoleic acid, stigmasterol, β-sitosterol, campesterol and squalene. Some of these compounds have anti-cancerous, antibiotic anti-inflammatory, antifungal and antipyretic effects, suggesting the high potential of *C. procera* hairy roots to produce preventive and therapeutic biopharmaceuticals.

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