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Micropropagation and Screening of Phytocompounds Present among *in vitro* Raised and Wild Plants of *Rauvolfia serpentine*

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Abstract

Rauvolfia serpentina, commonly known as Sarpgandha, is a plant well-known for being utilized for several medicinal purposes. Unrestricted collection from natural stands and overexploitation for medicinal and traditional purposes have rendered it endangered, hence there is necessary requirement for the development of cultivation protocols for mass propagation and sustained utilization of the plant. In the present study, *in vitro* culture of the apical parts of *R. serpentina* on MS (Murashige and Skoog) medium enriched with NAA resulted in development of callus, whereas multiple shoot regeneration along with callus development was achieved on medium combination MS + NAA + Kn and MS + NAA + BAP. MS + 4µM NAA + 4µM BAP was found to be most optimum media composition for regeneration of shoots and callus. Among different media combinations utilized for *in vitro* rooting, a maximum of 82.6 % explants developed in vitro roots on ½ MS + 12µM NAA. Gas chromatography-mass spectrometry (GC-MS) analysis of methanolic extract prepared from leaves of wild and micropropagated plants of *R. serpentina* revealed the presence of 38 and 48 phytocompounds, respectively. 9, 12-Octadecadienoic acid, Methyl linoleate, Methyl stearate, Hexadecanoic acid, methyl ester, Linoleic acid, Ergost-4, 7, and 22-trien-3.alpha.-ol were some of the major compounds found to be present in the leaves of wild plants, and Linoleic acid, methyl ester, Cis-Linoleic Acid Methyl Ester, Methyl elaidate, hexadecanoic acid, and methyl ester were major compounds found to be present in *in vitro* raised plants. Many of the compounds detected have been known to possess 1 or more biological or pharmacological activities.

Keywords: *Rauvolfia serpentine*, Conservation, Apical part, Phytochemical analysis

Introduction

Rauvolfia serpentina (L) Benth. Ex. Kurz is a well known medicinal plant and has been utilized for medicinal purposes in the Ayurvedic system of medicine for a long time [1,2]. *R. serpentina* is commonly known as Sarpgandha and is a perennial evergreen shrub. The plant is found in India, China, Indonesia, Pakistan, Nepal, Malaysia, and Sri Lanka. *R. serpentine* is known to inhabit regions with an annual rainfall of 200 - 250 cm, along with an altitude of 1,000 m. The plant possesses several biological and pharmacological activities including lowering of blood pressure, controlling fever, stimulation of uterine contraction, treating anxiety, insomnia and dyspepsia, and has hypolipidemic, antihypertensive, and antidiabetic activities [3]. The plant is specifically utilized against snake and scorpion bites [1,4-6]. The plant is also reported to possess antifungal and antimicrobial activities [7]. Several bioactive

phytocompounds have been reported to be present in *R. serpentine* including flavonoids, alkaloids, glycosides, tannins, etc. Reserpine, ajmalicine, serpentine, yohimbine, deserpidine, and ajmaline constitute different bioactive compounds identified and characterized to possess biological activities [8]. Reserpine, by far, remains the most commonly utilized phytocompound obtained from *R. serpentine* for medicinal purposes [9,10].

Overexploitation for medicinal purposes, poor rate of germination, destruction of habitat, and inadequate commercial cultivation have, together, made the plant endangered [11-13]. *R. serpentine* is a "red" listed plant in India and has been listed as an endangered plant by the International Union for Conservation of Nature (IUCN) [14,15]. Hence, there is an urgent requirement for the development of procedures and strategies for the conservation and mass propagation of this endangered species. Considering the present status of the plant, studies needs to be conducted that are applicable not only for propagating the plant at a laboratory level but which can simultaneously be utilized for rehabilitation of the plant in wild stands. The present study reports an effective and rapid method for mass propagation through *in vitro* culture of the apical parts of *R. serpentina*. Also, mother and *in vitro* regenerated plants were subjected to GC-MS analysis to bring out a comparative account of phytocompounds produced in wild and micropropagated plants.

Materials and methods

Explants

For the present study, plants of *Rauwolfia serpentina* (**Figure 1a**) were procured from the medicinal garden, Patanjali Roorkee, and were authenticated by Dr. Manjul Dhiman, Head of the Department of Botany, Kanahiya Lal DAV (PG) College, Roorkee, India. Apical parts (approximately 1.5 - 2 cm from the tip, possessing meristematic tissue) obtained from mature plants were utilized as explants.

Sterilization of explants

Apical parts were thoroughly washed with running tap water for approximately 10 min to remove any external impurities attached. Explants were then transferred to a beaker containing 1 % solution of Tween 20 for about 5 min with constant stirring, after which explants were again washed with water to remove all traces of detergent. Treatment of the fungicide Bavastin was given for about 3 min, following which the explants were washed with distilled water. Explants were then rinsed with 70 % ethanol for about 45 - 60 sec and washed with distilled water. After treatment with 70 % ethanol, explants were surface sterilized with 0.1 % $HgCl_2$ for about 3 min under aseptic conditions in laminar air flow. After sterilization, explants were washed at least 3 times with sterile distilled water to remove all traces of $HgCl_2$, dried using sterile Whatman paper, excised to appropriate size, and inoculated onto culture medium.

Establishment of cultures

Sterilized apical parts were inoculated on MS (Murashige and Skoog) media [16] fortified with different concentrations of 1-Naphthaleneacetic acid (NAA) (2, 4, 8 and 10 µM) alone or in combination with varying concentrations of Kinetin (Kn) (2, 4 and 8 µM) or 6-Benzylaminopurine (BAP) (2, 4 and 8 µM). Cultures were incubated at 25±2 ºC with 16 h photoperiod of light (15 uE/m^2/s irradiance). Each experimental setup comprised at least 20 cultures, and each experiment was repeated twice in case microbial contamination occurring in the cultures was less than 10 %; however, in any experimental setup, if bacterial or fungal contamination occurred in more than 10 % of cultures, an additional set of the experiment was performed so as to possess an adequate number of replicates of each medium combination. Each experimental setup was supplemented with a control treatment comprising hormone-free media.

***In vitro* rooting**

Well elongated shoots were aseptically transferred to rooting media. MS+NAA (4, 8 and 12 µM); MS + IAA (4, 8 and 12 µM); MS + IBA (4, 8 and 12 µM) were the 3 culture media utilized for development of *in vitro* roots. Both full and half strength media were employed in an independent set of experiments for each concentration of NAA, IAA (Indole-3-acetic acid), and Indole-3-butyric acid (IBA) (**Table 2**). Each experimental setup was supplemented with a control treatment comprising hormone-free media.

Acclimatization

Plants with well-developed shoots and roots were carefully removed from the culture vessel under aseptic conditions [17]. Roots of the plants were gently washed with sterile water so as to remove any media particles attached to the roots. Each plant was individually transferred to plastic pots (with an approximate size of 5″×5″×8″ (L×B×H) which were filled (1/3 volume) with autoclaved sand and soil in a ratio of 1:1. Pots were covered with transparent poly bags and incubated under similar conditions utilized for *in vitro* culture (**Figures 1N** and **1O**). Pots were irrigated with a solution having ¼ strength MS nutrients to keep the soil sufficiently moist. After about 10 days a minor incision was made onto the polybags. After a period of another 15 days, when new leaves began to emerge, the polybags were completely removed, and pots were transferred to glass chambers. Plants exhibiting normal growth were transferred to natural conditions.

Recording of data and statistical analysis

Cultures were regularly monitored for any morphological changes or growth. Observations made were analyzed to evaluate the percentage of cultures exhibiting *in vitro* response on each media combination. Results were subjected to Duncan's Multiple Range Test (DMRT) so as to evaluate the significance of respective media combinations.

GC-MS analysis

Methanolic extract prepared from dry leaf powder of leaves obtained from mother and *in vitro* regenerated plants were subjected to GC-MS analysis. The plant powder was extracted with methanol using Soxhlet apparatus. GC-MS analysis was done through a Perkin Elmer Auto System XL GC-MS analyzer, which utilized an electron ionization energy system with ionization energy of 70 eV for detection of compounds present in an extract. Helium gas (99.999 %) served as a carrier with a constant flow rate of 1.51 ml/min, and an injection volume of 2µl was employed. Software used, adapted to handle mass spectra and chromatograms was Turbo Mass. Identification of phytocompounds was based on the respective molecular structure and molecular mass and on direct comparison of retention times and mass spectral data with those for standard compounds and computer matching with the library (Wiley library, database NIST 98, NIST data bank). The relative percentage amount of each component was calculated by comparing the average peak area to the total areas. Once identified, the phytocompounds were searched for their documented and reported biological and pharmacological properties and medicinal values.

Results and discussion

Regeneration from apical parts of *R. serpentina*

In vitro culture of the apical parts onto hormone-free (control) media resulted in negligible growth, with a maximum of 44.6 % cultures exhibiting marginal enlargement of explants and induction of growth (**Figure 1B**). However, the apical parts failed to exhibit further growth and proliferation. *In vitro* culture of apical parts onto MS + NAA (2 - 8 µM) resulted in the development of callus on all concentrations of NAA. A moderate extent of callusing (**Figures 1C** and **1D**) was attained onto lower concentration of NAA (2 and 4 µM), and maximum proliferation of callus was achieved onto MS + 8µM NAA.

Regeneration of thin fibrous whitish roots was achieved on all media combinations, irrespective of concentration of NAA. Callus obtained was fragile in nature and creamish or green in color. When apical parts were cultured onto MS media fortified with different concentrations of Kn and NAA, regeneration of callus along with shoot bud induction was attained (**Figure 1F**). All the cultures exhibited callus regeneration, with callus developing predominantly from the basal part of explants present in direct contact with media. Percentage of explants developing shoot buds varied depending upon concentration of PGRs present in the medium, with a maximum of 88.8 % cultures developing shoots onto medium combination MS + 8μMKn + 8μM NAA. On average, about 2.4 ± 0.2 shoots, with a maximum of 5 shoots, developed onto this medium combination. Extensive proliferation of callus and regeneration of adventitious roots from callus was attained after about 3 weeks of induction of callus (**Figure 1J**). Regenerated roots were thin and fibrous onto media combinations containing lower NAA concentrations (2 - 4 μM); however, thick roots were obtained on media containing higher NAA concentrations (8 - 10 μM).

In vitro culture of apical parts onto MS + BAP (2 - 8 μM) + NAA (2 - 10 μM) also resulted in shoot bud induction, callus development, and regeneration of roots from callus. With an increase in the concentration of BAP, a simultaneous increase in number of regenerated shoots was achieved. On average, 4.4 ± 0.2 shoots were obtained on medium combination MS + 8μM BAP + 4μM NAA (**Figure 1G**), with a maximum regeneration of 7 shoots. Extensive callus development was achieved on all media combinations. BAP supplemented media were found to be more effective for the proliferation of *in vitro* developed callus as compared to Kn supplemented media. Callus obtained were greenish brown (**Figure 1E**). Several roots originated from the callus; some of the roots became thick and eventually spread throughout the culture media. *In vitro* regenerated shoots exhibited enhanced elongation and multiple shoot regeneration when transferred onto MS media with low BAP (2 - 4 μM) concentrations (**Figures 1H** and **1I**). Regenerated shoots when subcultured onto basal medium also exhibited elongation (**Figure 1K** and **1L**), Rajasekharan [18] also achieved proliferation of shoots from axillary meristem of *R. serpentina* on MS medium supplemented with IBA and NAA. Rani *et al.* [1] reported axillary shoots of *R. serpentina* exhibited regeneration of maximum number of shoots on MS medium containing IAA and BAP. In another micropropagation study conducted by Khan *et al.* [19] on *R. serpentina*, MS + BAP + NAA was found to be effective enough to induce direct regeneration of shoots from nodal segments and development of callus, followed by shoot regeneration from *in vitro* cultured leaf segments. In their study, regeneration from nodal and leaf segments was also achieved on MS + BAP + Kn and MS + BAP + IAA. Callus regeneration from nodal segments of *R. serpentina* were also reported [20] on MS + 0.5 mg/l BA + 2mg/l IAA. Ilahi *et al*. [21] reported the development of callus along with the regeneration of meristemoid structures from nodal segments of *R. serpentina* onto MS + 2mg/l BAP + 1mg/l IAA.

Table 1 Effect of different PGRs on *in vitro* culture of apical parts of *R. serpentine*.

Plant growth regulator (μM)		% cultures developing shoots	Avg. no. of shoots	Maximum no. of shoots	Degree of callusing	Nature of callus
NAA						
2		-	-	-	++	Creamish
4		-	-	-	++	fragile
8		-	-	-	++++	Green fragile
10		-	-	-	+++	
NAA	**Kn**					
2	2	46.4	1.4 ± 0.2^{d}	3	++	
2	4	48.0	1.2 ± 0.4^{e}	3	++	Crystalline
2	8	52.8	2.4 ± 0.2^{b}	5	++	white compact

Plant growth regulator (µM)		% cultures developing shoots	Avg. no. of shoots	Maximum no. of shoots	Degree of callusing	Nature of callus
4	2	56.4	1.2 ± 0.2^{e}	3	++++	
4	4	68.4	2.4 ± 0.2^{b}	5	+++	Crystalline
4	8	68.2	1.4 ± 0.2^{d}	5	+++	white fragile
8	2	58.6	1.6 ± 0.2^{c}	4	++++	
8	4	70.5	2.8 ± 0.2^{a}	5	+++	Green fragile
8	8	88.4	2.4 ± 0.2^{b}	5	++++	
NAA	**BAP**					
2	2	62.8	1.9 ± 0.2^{h}	5	++	
2	4	100	2.6 ± 0.2^{e}	6	+++	
2	8	100	3.2 ± 0.4^{d}	6	+++	Crystalline
4	2	70.0	2.3 ± 0.6^{fg}	5	+++	white fragile
4	4	100	4.0 ± 0.5^{b}	8	++++	
4	8	100	4.4 ± 0.2^{a}	7	++++	
8	2	76.4	2.4 ± 1.2^{fg}	5	+++	
8	4	100	3.2 ± 0.2^{d}	6	++++	Green
8	8	100	3.4 ± 0.4c	6	++++	crystalline

- NAA (Naphthalene acetic acid), Kn (Kinetin), BAP (Benzyl aminopurine).
- Values are means of 3 replicates. Mean values followed by same letters are not significantly different at $p \geq 0.05$ DMRT.
- ++ (Moderate callusing), +++ (Good callusing), ++++ (Extensive callusing)

***In vitro* rooting**

Earlier studies achieved *in vitro* rooting from regenerated shoots of *R. serpentina* onto MS medium containing either NAA and IAA or NAA and IBA. In the present study, comparative analysis of 3 auxins was conducted so as to evaluate their efficacy to induce the formation of *in vitro* roots. One of the most prominent findings was enhanced *in vitro* rooting onto half strength MS medium as compared to full strength. An earlier study conducted by the authors [13] also reported half MS medium to be far superior than full strength medium for the development of *in vitro* roots in *R. serpentina*. Besides *R. serpentina,* several studies conducted on other plant species have also implicated enhanced efficiency of ½ strength MS medium to induce development of *in vitro* roots as compared to full strength medium. Among the different PGR utilized, NAA was found to be the most appropriate for the development and proliferation of roots from regenerated shoots.

In the present study, 38.4 % of explants developed *in vitro* roots on MS + 4µM NAA, with an average number of 3.4 ± 1.2 roots per explants. When the strength of the medium was reduced to half with the same concentration of NAA, *in vitro* rooting was achieved in 58.4 % of cultures, with an average and maximum number of roots being 3.8 ± 0.2 and 5, respectively. On increasing the concentration of NAA to 8 µM, *in vitro* rooting was achieved in 42.8 and 72.8 % of cultures onto full and half strength medium, respectively. Average and maximum number of roots onto ½ MS + 8 µM NAA were 4.2 ± 0.6 and 8, respectively. When the concentration of NAA was further increased to 12 µM, 68.6 % of cultures developed roots on full strength medium and 82.6 % of cultures exhibited development of roots onto half strength medium. In another study [13], *in vitro* rooting was achieved in about 88.6 % of cultures onto MS + 20 µM NAA. The roots obtained in the present study exhibited limited branching (**Figure 1M**), which supported the easy handling of regenerated plants while removing media attached to roots before acclimatization.

When IAA was utilized as a rooting hormone, 42 % of cultures developed roots onto MS + 4 µM IAA. The rooting response was enhanced to 48.4 % when the strength of the medium was reduced to half. Onto higher concentration of IAA (8 µM), 44.8 and 66 % of cultures exhibited *in vitro* rooting onto full and half strength medium, respectively. Average and maximum number of roots onto half MS + 8 µM IAA were 4.2 ± 0.6 and 8, respectively. On further increasing the concentration of IAA to 12µM, the overall percentage of cultures developing roots were enhanced to 58.2 % onto full strength medium and 74.2 % onto half strength medium.

In full strength MS medium enriched with 4 µM IBA, about 32.4 % cultures developed *in vitro* roots, with an average number of 3 ± 0.6 roots per explant. On reducing the strength of medium to half, *in vitro* rooting was achieved in 52 % when the concentration of IBA was enhanced to 12 µM, and a maximum of 62.6 and 72.6 cultures developed *in vitro* roots onto full and half strength medium, respectively. Average and maximum number of roots onto half MS + 12 µM IBA were 5.6 ± 0.4 and 8, respectively. Earlier studies [4,12] achieved development of *in vitro* roots onto IBA supplemented MS medium. In another study [22], *in vitro* rooting was achieved in 72 % of cultures onto MS + 25µM IBA, with an average number of 6.0 ± 0.6 roots per explant. Medium containing both IBA and IAA has also been reported to be a suitable medium for development of *in vitro* roots in *R. serpentine* [23]. A maximum of 62.4 % *in vitro* regenerated plants survived during the process of transplantation and exhibited normal growth and development.

Table 2 *In vitro* rooting from regenerated shoots of *R. serpentine*.

MS	PGR	% Culture developing roots	Avg. no. of roots	Max. no. of roots
Full strength	Control	-	-	-
	4µM NAA	38.4	3.4 ± 1.2^{j}	05
	8µM NAA	42.8	3.2 ± 0.4^{k}	04
	12µM NAA	68.6	2.8 ± 0.6^{l}	04
	4µM IAA	42.0	3.6 ± 1.2^{i}	05
	8µM IAA	44.8	3.8 ± 0.6^{h}	06
	12µM IAA	58.2	4.8 ± 0.4^{e}	06
	4µM IBA	32.4	3.2 ± 0.6^{k}	05
	8µM IBA	38.8	4.2 ± 0.2^{f}	07
	12µM IBA	62.6	4.0 ± 0.6^{g}	06
Half strength	Control	06.4	1.4 ± 0.2^{i}	02
	4µM NAA	58.4	3.8 ± 0.2^{h}	05
	8µM NAA	72.8	4.2 ± 0.6^{f}	08
	12µM NAA	82.6	6.0 ± 0.4^{a}	09
	4µM IAA	48.4	4.0 ± 0.2^{g}	07
	8µM IAA	66.0	5.2 ± 0.4^{d}	08
	12µM IAA	74.2	4.8 ± 1.2^{e}	07
	4µM IBA	52.0	5.2 ± 0.4^{d}	08
	8µM IBA	62.8	5.4 ± 0.4^{c}	09
	12µM IBA	72.6	5.6 ± 0.4^{b}	08

- NAA (Naphthaline acetic acid), IAA (Indole butyric acid), IBA (Indole butyric acid).
- Values are means of 3 replicates. Mean values followed by same letters are not significantly different at $p \geq 0.05$ DMRT

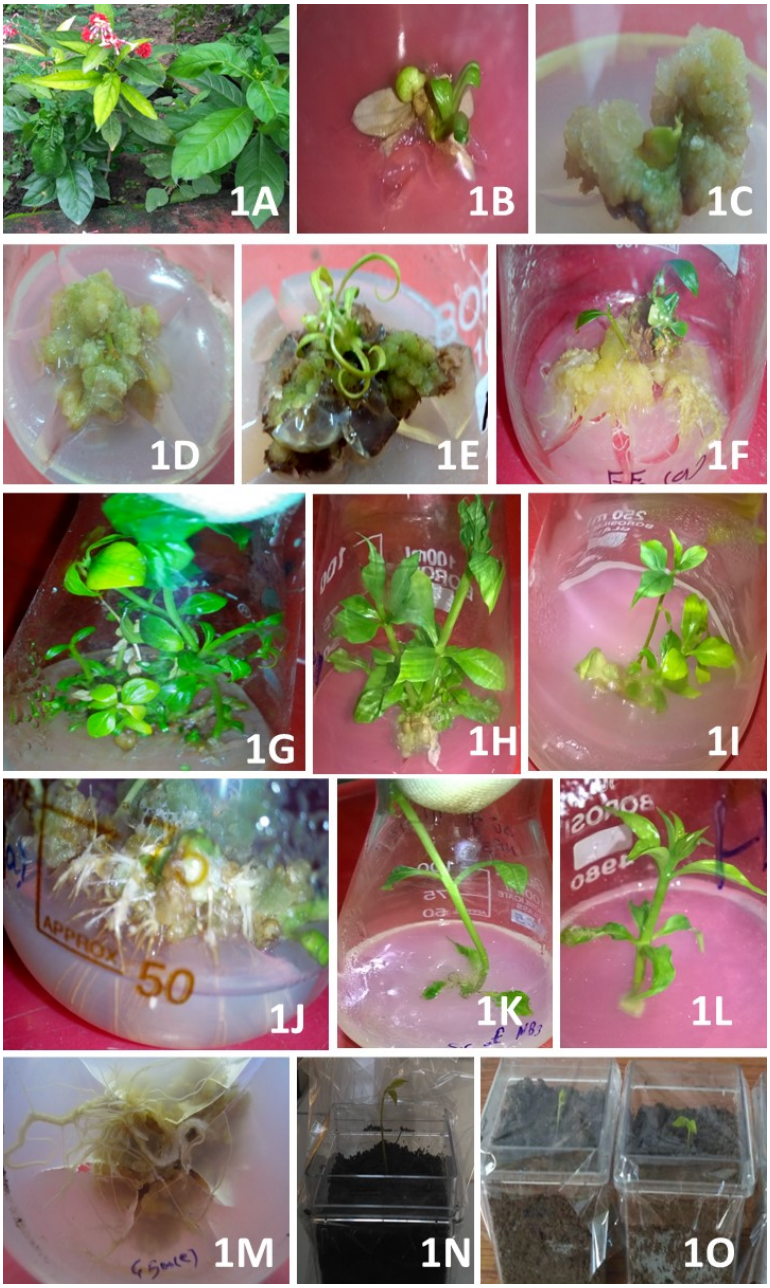

Figure 1A-Mature plant of *R. serpentina*; **B-**Induction of growth (no further proliferation) on hormone-free medium; **C**, **D**-Moderate extent of callusing onto lower concentration of NAA 2 and 4 µM respectively; **E-**Regeneration of callus and shoot bud induction on MS + Kn + NAA; **F-**Callus and shoot bud induction on MS + BAP + Kn; **G-**Multiple shoot regeneration on MS + 8µM BAP + 4µM NAA; **H**, **I**-Enhanced elongation and multiple shoot regeneration from subculture of regenerated shoots onto MS medium with low BAP (2 - 4 µM) concentration; **J-**Extensive proliferation of callus and regeneration of adventitious roots from callus on MS + 8µMKn + 8µM NAA; **K**, **L**-Elongation of shoots onto basal medium; **M-***In vitro* rooting on MS + 20 µM NAA; **N**, **O** -*In vitro* regenerated plants under the process of acclimatization.

GC-MS analysis of leaves of micropropagated and mother plants of *R. serpentina*

Methanolic extract of leaves of the mother plant and *in vitro* regenerated plant when subjected to GC-MS analysis revealed the presence of a range of phytocompounds. A total of 38 compounds were found to be present in methanolic extract prepared from the leaves of the mother plant (**Table 3**, **Figure 2**), among which the major compounds found to be present were linoleic acid (24.42 %), 9, 10 octadecadienoic acid (20.4 %), 9 - 10 octadecanoic acid methyl ester (14.11 %), and hexadecanoic acid methyl ester (7.03 %). Similarly, the presence of 41 phytocompounds were detected in the methanolic extract of the leaves of a micropropagated plant (**Table 4**, **Figure 3**) of *R. serpentine*. **Figures 4** and **5** depicts mass spectra of some of the compounds identified through GC-MS in the leaves of the mother plant and the micropropagated plant, respectively. Major compounds found to be present include Cis-linoleic acid methyl ester (24.35 %), methyl elaidate (15.34 %), linoleic acid (15.28 %), hexadecanoic acid, and methyl ester (11.89 %). Many of the compounds were found to be present in the mother as well as in the *in vitro* raised plant; these compounds include 1-pentadecanamine, n, n-dimethyl, hexadecanoic acid, methyl ester, methyl stearate, linoleic acid, Linoleoyl chloride, eicosanoic acid, methyl ester, 9, 12-octadecadienoyl chloride, (z, z)-, 15-hydroxypentadecanoic acid, cis-11-eicosenoic acid, methyl ester, glycidol stearate, docosanoic acid, methyl ester, Ethyl linoleate, tetracosanoic acid, methyl ester, ergost-4, 7, 22-trien-3.alpha.-ol, and Tridecanedial. However, a significant amount of variation was observed in their concentrations. The percentage of hexadecanoic acid methyl ester in the extract of the tissue cultured plant was 11.89 %, which decreased to 7.03 % in the mother plant. Similarly, the percentage of linoleic acid in the micropropagated plant was found to be 15.28 %, which was enhanced to 24.42 % in the extract of the mother plant. The percentage of tetracosanoic acid methyl ester in the extract of the micropropagated and the mother plants was found to be 0.15 and 0.31 %, respectively. In the micropropagated plant, the ethyl linoleate percentage was 2.43%, which decreased to 0.21% in the mother plant. Besides these common phytocompounds, compounds including eiosane methyl elaidate, Bormyl chloride, and cholesteryl benzoate were found to be present only in the leaves of the micropropagated plant and were absent in the extract prepared from the leaves of mother plants. The presence of compounds of methyl linoleate, 1-glyceryl linoleate, and glycerol 2 palmitate was confined only to mother plants. Concentrations of compounds 9-hexadecenoic acid, methyl ester (z)-, linoleic acid, Linoleoyl chloride, 9-tricosene (z)-, glycidol stearate, 9, 12-octadecadienoyl chloride (z, z)-, tetracosanoic acid methyl ester, ergost-4, 7, 22-trien-3 alpha.-ol, and Tridecanedial were found to be higher in the extract of the mother plant as compared to their concentrations in the tissue cultured plants. On the contrary, concentrations of 1-pentadecanamine n, n-dimethyl, methyl stearate, Ethyl linoleate, cis-11-eicosenoic acid, and methyl ester were found to be higher in extracts of leaves of micropropagated plants as compared to the mother plant. Another study [24] reported n-hexane extracts of roots of *R. serpentine* to possess 18 phyto compounds, among which cis-1, 3-dimethyl cyclohexane; 2 methyl pentane-2, 4-diol; 3 methyl heptan-2-ol; and 1, 1, 2, 3-tetramethylcyclopropane were the major compounds found to be present. In another study conducted by Sivaraman *et al.* [25], GC-MS analysis of ethanolic extracts of roots of *R. serpentina* was performed and the presence of 23 phytochemical compounds was reported. Phenol 1, 2, 4-bis (1, 1-dimethylethyl); Hexadecanoic acid 2-hydroxy-1-(hydroxyl methyl) ethyl ester; octadecanoic acid, 2, 3-dihydroxy propyl ester; and diethyl phthalate, eicosane comprised the major compounds found to be present in the extract. Most of the earlier studies conducted, pertaining to GC-MS analysis of *R. serpentine*, were restricted to the roots of the plant (owing to the immense medicinal potential of the roots); however, the present study reports the presence of phytocompounds with either medicinal properties or biological activities in the leaves of the plant as well. GC-MC analysis has been recognized as an extremely effective tool for screening or analyzing for the presence of various phytocompounds in different parts of plants. GC-MS analysis of an ethanolic extract of the roots of *Coleus froshpotei* revealed the presence of 19 phytocompounds [26]. Similarly, Patel *et al*. [27] reported 14 phytocompounds to be present in methanolic extracts of leaves of *Terminalia coriacea*. Rukshana *et al*. [28], in their study, analysed ethanolic extracts of leaves of *Pergalaria daemia* through GC-MS and

found 15 phytocompounds to be present. Rautela *et al*. [29], in their study, implicated the types and number of phytocompounds to vary depending upon the solvent system utilized for preparing the plant extracts. In their study, 35 and 49 phytochemical compounds were reported to be present in methanolic and ethanolic extracts of leaves of *Withania somnifera*, respectively. The utility of GC-MS analysis becomes more pronounced in reference to plant tissue culture studies when considering comparative phytochemical analysis of micropropagated and wild plants. Sharma *et al.* [30], in their study on *Withania coagulans*, reported the presence of 44 and 56 phytocompounds to be present in methanolic extracts of leaves of wild and micropropagated plants, respectively. Karthikeyan and Sudan [31] also reported variation in a number of phytocompounds found to be present in *in vitro* cultured and in *in vivo* cultivated plants of *Cleome gynandra.*

Biological activities of phytocompounds found to be present in leaves of *R. serpentine* have been reported in the available literature [32-40]. **Table 5** shows the pharmacological and biological activities of some of the phytocompounds identified in extracts of leaves of *R. serpentine.* Hence, the leaves of the plant can serve as efficient ingredients of medicinal preparations and be effectively utilized in the treatment of various diseases. Presently, the roots of *R. serpentine* are the part of the plant mainly utilized for medicinal purposes. However, the validation of leaves (extract) for their medicinal potential will not only provide an alternate source of raw material for medicinal formulation but, simultaneously, aid in the conservation of the species. Hence, further studies are recommended for evaluating the effective utilization of the leaves of *R. serpentine* in the treatment of specific diseases.

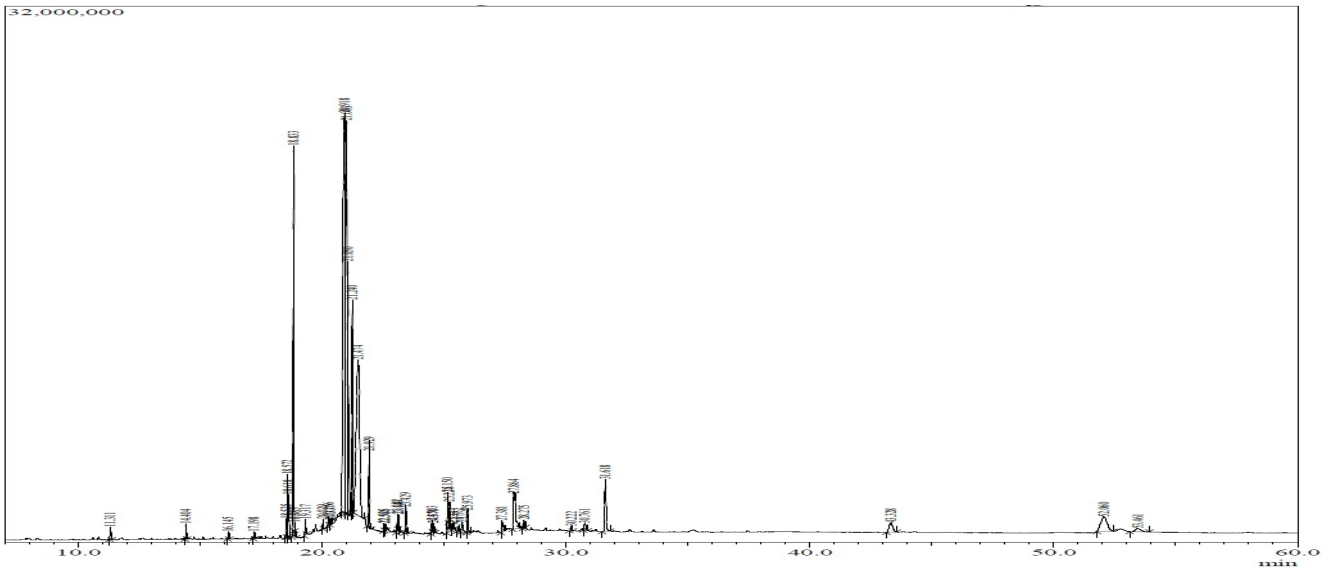

Figure 2 GC-MS analysis of methanolic extract of leaves of mother plant of *R. serpentine*.

Table 3 GC-MS analysis of methanolic extract of leaves of mother plant of *R. serpentine*.

Peak	R. time	Area %	Name of compound	Mol formula	Mol weight
1	18.604	0.7	1-Pentadecanamine, N,N-dimethyl-	$C_{17}H_{37}N$	255
2	18.797	7.03	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270
3	19.274	0.41	Pentadecanoic Acid	$C_{15}H_{30}O_2$	242
4	20.242	0.54	Hexadecanoic acid, trimethylsilyl ester	$C_{19}H_{40}O_2Si$	328
5	20.866	20.4	9,12-Octadecadienoic acid	$C_{19}H_{34}O_2$	294
6	20.943	14.11	9-Octadecenoic acid, methyl ester,(E)-	$C_{19}H_{36}O_2$	296
7	20.999	3.23	Methyl linoleate	$C_{19}H_{34}O_2$	294
8	21.062	0.21	Ethyl linoleate	$C_{20}H_{36}O_2$	308
9	21.209	3.49	Methyl stearate	$C_{19}H_{38}O2$	298
10	21.438	24.42	Linoleic acid	$C_{18}H_{32}O_2$	280
11	21.91	1.23	Methyl 10-trans,12-cis octadecadienoate	$C_{19}H_{34}O_2$	294
12	22.517	0.26	Linoleoyl chloride	$C_{18}H_{31}ClO$	298
13	22.58	0.25	9-Tricosene, (Z)-	$C_{23}H_{46}$	322
14	23.075	0.47	15-Hydroxypentadecanoic acid	$C_{15}H_{30}O_3$	258
15	23.142	0.22	cis-11-Eicosenoic acid, methyl ester	$C_{21}H_{40}O_2$	324
16	23.418	0.49	Eicosanoic Acid, Methyl Ester	$C_{21}H_{42}O_2$	326
17	23.714	0.17	1-Glyceryl linoleate	$C_{21}H_{38}O_4$	354
18	24.49	0.47	9,12-Octadecadienoic acid (Z, Z)-, 2-hydroxy-1-(hydroxymet	$C_{21}H_{38}O_4$	354
19	24.563	0.34	Oleoyl chloride	$C_{18}H_{33}ClO$	300
20	25.141	1.37	9,12-Octadecadienoyl chloride,(Z, Z)-	$C_{18}H_{31}ClO$	298
21	25.214	1.02	9-Octadecenal, (Z)-	$C_{18}H_{34}O$	266
22	25.329	0.41	9,12-Octadecadienoyl chloride,(Z, Z)-	$C_{18}H_{31}ClO$	298
23	25.568	0.26	Glycidol stearate	$C_{21}H_{40}O_3$	340
24	25.721	0.52	Glycerol, 2-palmitate	$C_{19}H_{38}O_4$	330
25	25.961	0.64	Docosanoic acid, methyl ester	$C2_3H_{46}O_2$	354
26	27.367	0.38	.beta.-Eudesmol, trimethylsilyl ether	$C_{18}H_{34}OSi$	294
27	27.849	1.79	Butyl 9,12-octadecadienoate	$C_{22}H_{40}O_2$	336
28	27.895	1.61	Oleoyl chloride	$C_{18}H_{33}ClO$	300
29	28.268	0.31	Tetracosanoic Acid, Methyl Ester	$C_{25}H_{50}O_2$	382
30	30.211	0.29	Cholesta-3,5-diene	$C_{27}H_{44}$	368
31	30.746	0.85	9,12-Octadecadien-1-OL	$C_{18}H_{34}O$	266
32	31.607	4.72	Ergost-4,7,22-trien-3.alpha.-ol	$C_{28}H_{44}O$	396
33	32.604	0.18	Cholesta-2,8-dien-6-ol, 14-methyl-, acetate, 5.alpha., 6.alpha.	$C_{30}H_{48}O_2$	440
34	33.582	0.21	Cholesterol	$C_{27}H_{46}O$	386
35	35.729	0.44	Yohimban-16-Carboxylic Acid, 17-Hydroxy-,	$C_{21}H_{26}N_2O_3$	354
36	43.29	1.88	14-Methyl-8-Hexadecyn-1-Ol	$C_{17}H_{32}O$	:252
37	51.989	3.69	Tricyclo[20.8.0.0(7, 16)]triacontane, 1(22), 7(16)-diepoxy-	$C_{30}H_{52}O_2$	444
38	53.385	0.97	Tridecanedial	$C_{13}H_{24}O_2$	212

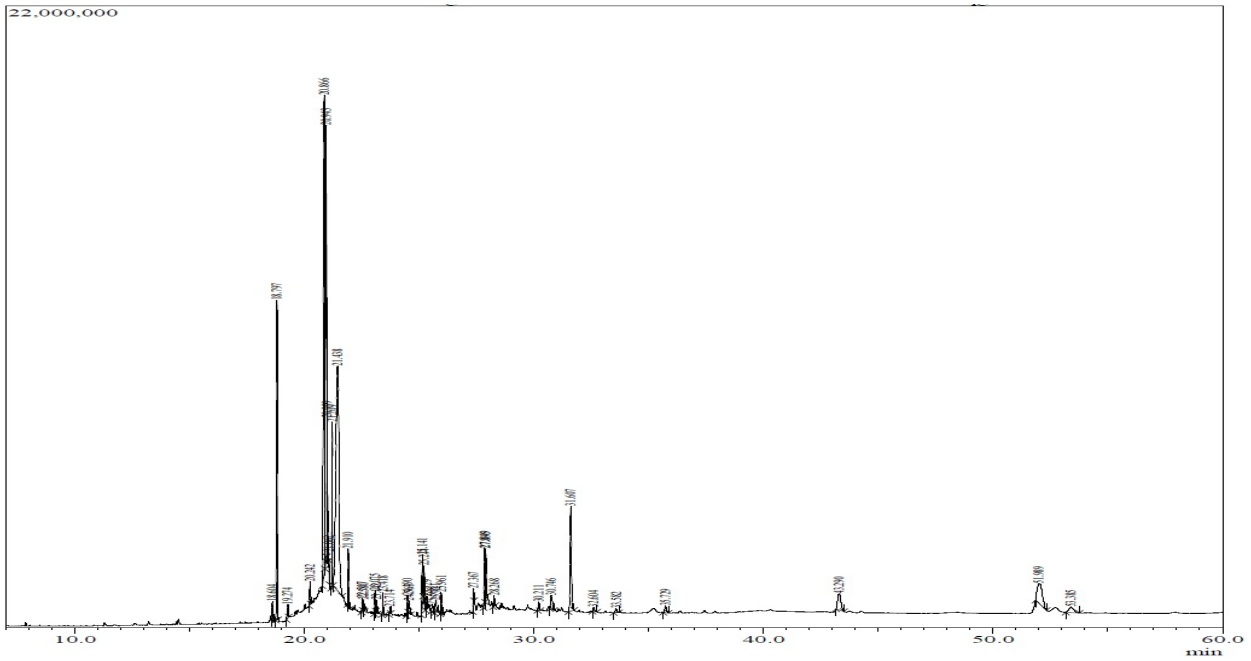

Figure 3 GC-MS analysis of methanolic extract of leaves of *in vitro* regenerated plant of *R. serpentine*.

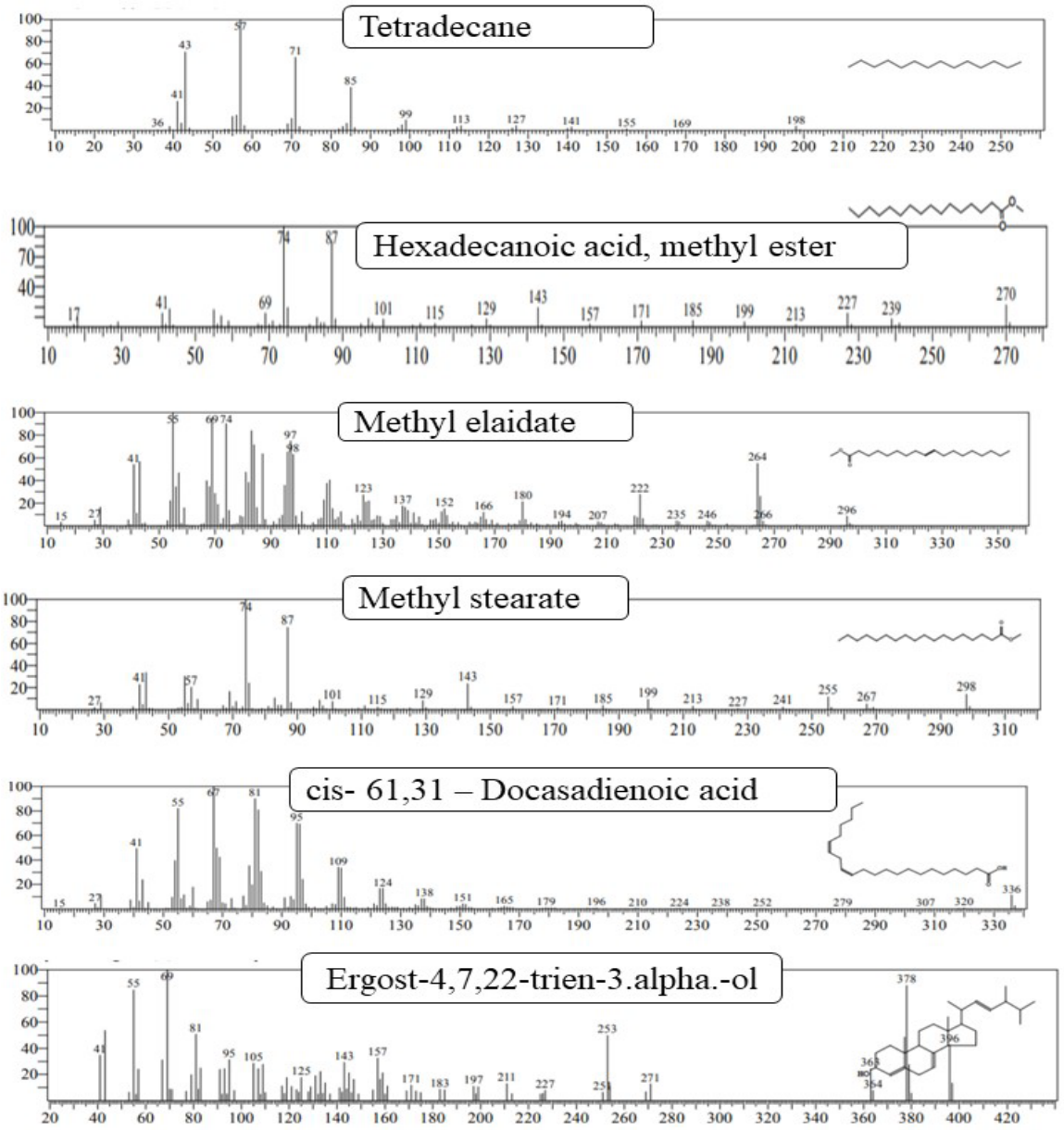

Figure 4 Mass spectra of selected compounds identified to be present in leaves of mother plant.

Table 4 GC-MS analysis of methanolic extract of leaves of *in vitro* regenerated plant of *R. serpentine*.

Peak	R. time	Area %	Name of compound	Mol formula	Mol weight
1	11.311	0.32	Tetradecane	$C_{14}H_{30}$	198
2	14.404	0.27	Heptadecane	$C_{17}H_{36}$	240
3	16.145	0.13	Methyl tetradecanoate	$C_{15}H_{30}O_2$	242
4	17.198	0.12	Eicosane	$C_{20}H_{42}$	282
5	18.525	0.32	9-hexadecenoic acid, methyl ester, (z)-	$C_{17}H_{32}O_2$	268
6	18.572	1.29	1-pentadecanamine, n,n-dimethyl-	$C_{17}H_{37}N$	255
7	18.618	1.32	1-pentadecanamine, n,n-dimethyl-	$C_{17}H_{37}N$	255
8	18.833	11.89	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270
9	18.883	0.16	1,3-dimesityl-2-propen-1-one	$C_{21}H_{24}O$	292
10	19.317	0.38	Pentadecanoic acid	$C_{15}H_{30}O_2$	242
11	20.029	0.17	Heptadecanoic acid, methyl ester	$C_{18}H_{36}O_2$	284
12	20.266	0.21	Hexadecanoic acid, trimethylsilyl ester	$C_{19}H_{40}O_2Si$	328
13	20.337	0.08	Hexahydrocyclobuta[c]pentalen-3-one	$C_{10}H_{14}O$	150
14	20.918	24.35	Cis-Linoleic Acid Methyl Ester	$C_{19}H_{34}O_2$	294
15	21.003	15.34	Methyl elaidate	$C_{19}H_{36}O_2$	296
16	21.05	7.66	Linoleic acid, methyl ester	$C_{19}H_{34}O_2$	294
17	21.24	4.5	Methyl stearate	$C_{19}H_{38}O_2$	298
18	21.474	15.28	Grape seed oil/ linoleic acid	$C_{18}H_{32}O_2$	280
19	21.929	1.77	9,12-Octadecadienoic Acid, Methyl Ester	$C_{19}H_{34}O_2$	294
20	22.525	0.12	Linoleoyl chloride	$C_{18}H_{31}ClO$	298
21	22.586	0.14	9-tricosene, (z)-	$C_{23}H_{46}$	322
22	23.082	0.27	15-hydroxypentadecanoic acid	$C_{15}H_{30}O_3$	258
23	23.149	0.25	cis-11-eicosenoic acid, methyl ester	$C_{21}H_{40}O_2$	324
24	23.429	0.56	Eicosanoic acid, methyl ester	$C_{21}H_{42}O_2$	326
25	24.503	0.28	Linoleoyl chloride	$C1_8H_{31}ClO$	298
26	24.57	0.11	Oleoyl chloride	$C_{18}H_{33}ClO$	300
27	25.15	0.79	9,12-octadecadienoyl chloride, (z, z)-	$C_{18}H_{31}ClO$	298
28	25.224	0.6	9-octadecenal, (z)-	$C_{18}H_{34}O$	266
29	25.344	0.19	9,12-octadecadienoyl chloride, (z, z)-	$C_{18}H_{31}ClO$	298
30	25.577	0.11	Glycidol stearate	$C_{21}H_{40}O_3$	340
31	25.736	0.42	1-Glycerol hexadecanoate	$C_{19}H_{38}O_4$	330
32	25.973	0.61	Docosanoic acid, methyl ester	$C_{23}H_{46}O_2$	354
33	27.381	0.51	Bornyl cinnamate	$C_{19}H_{24}O_2$	284
34	27.864	2.43	Ethyl linoleate	$C_{20}H_{36}O_2$	308
35	28.275	0.15	tetracosanoic acid, methyl ester	$C_{25}H_{50}O_2$	382
36	30.222	0.13	Cholesteryl benzoate	$C_{34}H_{50}O_2$	490
37	30.761	0.38	9,12-octadecadien-1-ol	$C_{18}H_{34}O$	266
38	31.618	2.05	Ergost-4,7,22-trien-3.alpha.-ol	$C_{28}H_{44}O$	396
39	43.328	1.03	(14Z)-14-Tricosenyl Formate	$C_{17}H_{32}O$	252
40	52.06	2.75	Tricyclo[20.8.0.0(7,16)] triacontane, 1(22),7(16)-diepoxy-	$C_{30}H_{52}O_2$	444
41	53.461	0.54	Tridecanedial	$C_{13}H_{24}O_2$	212

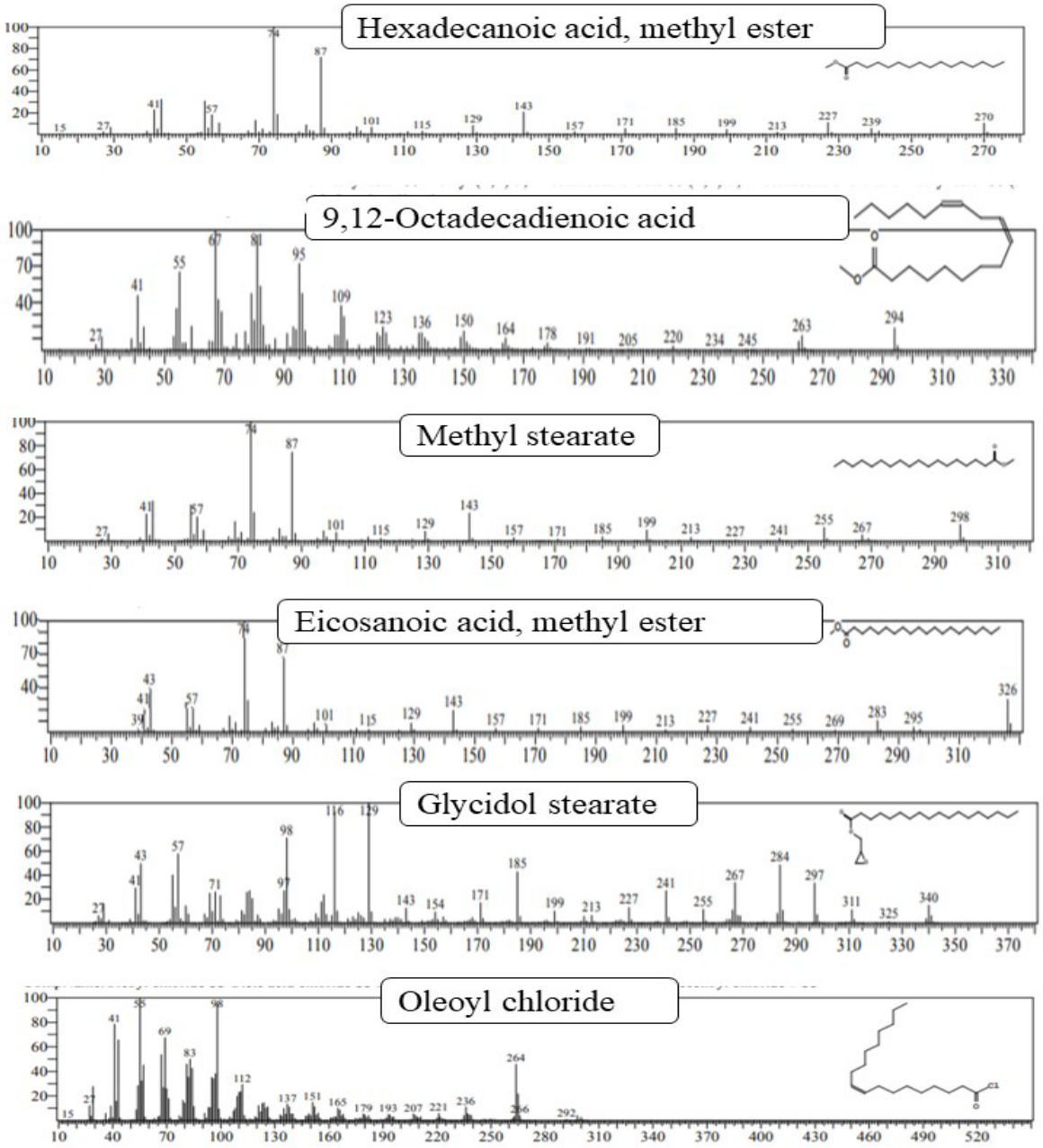

Figure 5 Mass spectra of selected compounds identified to be present in leaves of micropropagated plant.

Table 5 Biological activities (reported in literature) of selected phytocompounds found to be present in leaves of *R serpentina.*

Name of phytocompound	Biological properties	Author / Reference
Methyl stearate	Antidiarrheal, cytotoxic, antiproliferative activities	[32,33]
Eicosane	Anti-tumor, antimicrobial activities	[34,35,36]
Esters of octadecanoic acid	Antimicrobial, antioxidant activities	[32]
Methyl elaidate	Potential apoptotic agent	[37]
Methyl linoleate	Antifungal, antiproliferative properties	[38]
Methyl tetradecanoate	Antioxidant, cancer preventive, nematicidal, hypercholesterolemic activities	[39]
9, octadecanoic acid methyl ester	Anti-inflammatory, anti-cancer, antiandrogenic, 5-alpha reductase activities	[39]
Linoleic acid	Antibacterial and antifungal activity	[40]
Linoleic acid, methyl ester	Antibacterial, anti-inflammatory, hepatoprotective activities	[36]
Hexadecenoic acid, methyl ester	Anti-inflammatory, antioxidant, antidiabetic, hypocholesterolemic activities	[35]

Conclusions

The present study reports a simple and effective method for the *in vitro* propagation of *R. serpentine* through micropropagation of apical parts, considering the apical part to possess meristematic tissue. Moreover, the presence of biologically active phytocompounds in the leaves of *R. serpentine*, as revealed by GC-MS analysis, supports the utilization of leaves for medicinal purposes, for which further studies are required for authentication and validation.

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