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***In vitro* propagation, callus culture and phytochemical profiling of Manjishtha-A invaluable medicinal species of Leh-Ladakh**

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Ladakh, a high altitudinal Indian state situated in trans-Himalayan region, is well known for its extreme ecosphere, rugged topography and scarce vegetation with peculiar ability to biosynthesize a number of phytochemicals. *Rubia cordifolia* L. is one of such herb known for its phytochemical enriched roots. Since the outbreak of COVID-19, a great emphasize has been given towards the exploration of health beneficial plants. Keeping this in view, in present study, an *in vitro* regeneration protocol has been developed using nodal and leaf explant followed by a comparative determination of phytochemical profile of *in vitro* grown leaves (RURL) and roots (RUMR). MS basal medium supplemented with 1.0 μ M, each of BAP and KN was found optimum for production of maximum number of shoots (42.33 ± 1.43) from nodal explant and 8.33 ± 0.88 in leaf explant. Further, 99.67 ± 0.33 % rooting was achieved in 0.1 mg/l NAA supplemented MS media. GC-MS profiling of methanolic extract of RURL and RUMR revealed a total of 31 phytochemicals in both extracts with 8 compounds, commonly present in both extract describing the medical potential of both leaves as well as roots.

1. Introduction

Phytochemicals, God's boons of healing power in plants describes the scientific base of application of plants as eternal source of food and drugs to mankind. These are the non-nutritive constituents of plant-based diet with disease-preventing or curative capabilities to humans (Surh, 2003). Biosynthesis of these metabolites are carried out mostly through secondary metabolism, resulting in production of chemically and taxonomically varied compounds with diverse range of biological activities, viz., pharmaceutical applications in human therapy, veterinary, agriculture, scientific research and in numeral other areas (Morton, 1981; Ogunmefun, 2018). The plant kingdom is a treasure trove of many potential drugs. In recent decade, usage of plants with medicinal properties are achieving more popularity among public due to their considerable less side effects in comparison to synthetic drugs fascinating people around world to look for the natural resources of new drugs (Johnson and William, 2002). It has been documented that 80% of human public around the globe has a strong belief in traditional medicine, especially in drugs from plant origin for their primary healthcare (Tsay and Agrawal, 2005; Bin-Nyeem and Mannan, 2018). A number of organic compounds belonging to different classes of phytoactive constituents, viz., tannins, alkaloids,

carbohydrates, phenols, terpenoids, anthocyanins, steroids and flavonoids are suggested to be responsible for these therapeutical and physiological actions on the human body (Badyal *et al.*, 2003; Bin-Nyeem and Mannan, 2018). Undoubtedly, plant kingdom is a rich reservoir of numeral medicinal plants species however, their medicinal attributes should be unraveled for better understanding of their safety and efficiency (Arunkumar and Muthuselvam, 2009; Yadav and Agarwala, 2011).

Rubia cordifolia L., also known as Indian Madder or Manjishtha, is a perennial, flowering plant species belonging to Rubiaceae family. Plants of this species are found to have more or less cylindrical four angled stem with sharp recurved prickles on the ridges and white flowers arranged in cyme inflorescence (Warrier *et al.*, 2001; Devi and Siril, 2014). Since ancient times, roots of *R. cordifolia* have been found to be used as ingredient in different herbal formulations like in Chadanasava for treatment of urinogenital disorders, Jatyaadi Ghrita for chronic and septic ulcers; Phala Sarpi Ghrita and Phal Kalyaan Ghrita for amenorrhoea, Manjishthadi Taila (oil) for headaches, ring worm and numeral other fungal infections (Shekokar *et al.*, 2013; Venugopal, 2002). Decoction of manjishtha cooked with ghee and *Moringa oleifera* is useful for bleeding piles, etc. Numerous biologically active compounds belonging to different classes, viz., anthraquinones and their glycosides, naphthoquinones and glycosides, terpenes, bicyclic hexapeptides, iridoids, carboxylic acids (malic, citric, quinic, rosmarinic acids) and saccharides (xylose, ribose, fructose, glucose, sucrose, primverose) have been reported in various parts of *R. cordifolia* (Itokawa *et al.*, 1990; Son *et al.*, 2008; Devi and Siril, 2014).

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Ladakh, an Indian union territory state, is situated in high altitude region of trans-Himalayas at an altitude ranging from 8000 ft to 24000 ft above mean sea level (Kala and Manjrekar, 1999). This region is well known for its rugged ecosystem, scree slopes, low vegetation and for extreme climatic features such as high UV radiation (120000 lux), reduced water precipitation (20-30 mm annual rainfall), subzero temperatures, etc., (Kumar *et al.*, 2013). Barren mountains of Ladakh Himalayas, which otherwise seem parched and devoid of vegetation at first site, are wealthy in medicinally important herbs and approximately 1100 different species have been reported in this region, forming the basis of local Amchi medicinal system (Navchoo and Buth, 1989). *R. cordifolia* is one of such medicinal plant species, found to grow near water source in summer season. It is a well known fact that plant do a lot of changes in their cellular and genetic constitution in order to adapt to existing natural environmental conditions. Biosynthesis and accumulation of phytochemicals is one of such approach which depends on prevailing various external environmental factors, viz., temperature, humidity, light intensity, water stress and CO₂ concentration (Gharibi *et al.*, 2016). However, limited growing season make this plant unavailable throughout the year for extraction of phytochemicals. Plant cell culture is a promising technique for extraction of phytochemicals under controlled conditions of environment irrespective of external conditions. Further, selection of source of explants is necessary for efficient production of phytochemicals as biosynthesis and accumulation of phytochemicals under influence of ectopic environment in tissue/organ specific. Therefore, present study has been carried out to establish an *in vitro* propagation protocol for *R. cordifolia* growing in Leh using two explant types, viz., leaf and nodal explants followed by GC-MS analysis of methanolic extract of *in vitro* grown leaves (RURL) and roots (RUMR) for determining phytochemical profile of both extracts.

2. Materials and Methods

2.1 Chemicals

All components of MS medium and growth regulators were purchased from Sigma Aldrich Pvt. Ltd. Water used for media preparation and various analyses were used from Merck Millipore Academic instrument. Analytical grade methanol was purchased from Merck and Qualigens Fisher Scientific.

2.2 Plant material and source of explants

Healthy, disease-free plantlets and certified seeds of *R. cordifolia* were collected from herbal garden maintained at Defence Institute of High Altitude research (DRDO), Leh of India in the month of June (Figure 1).

2.3 Explants preparation

Two types of explants, viz., leaf and nodal segments were used for establishment of *in vitro* culture of *R. cordifolia*. Nodal explant was prepared by cutting the stem at internodal portion, whereas leaves were used directly by cutting from their petioles. Both of the explants were soaked in 5% Tween-20 solution and bevestein for half an hour followed by thorough washing under running tap water for 2 h 3 min. Further, three step surface sterilization of explants was carried out by using ethanol (70% (v/v)) for 120 secs, HgCl₂ (0.04% (w/v)) for 60 secs accompanied by thorough washing with autoclaved distilled water (4-5 times) to remove the remaining traces of sterilizing agents.

2.4 Establishment of aseptic culture from nodal and leaf explants

Both surface sterilized explants were cut carefully and inoculated on MS basal medium having sucrose (3.00%; w/v), agar (0.78%; w/v) and different combinations of PGRs, i.e., BAP KN and NAA for efficient shoot induction (Tables 1 to 3). pH of the medium was adjusted to 5.8 before addition of agar. Following which, the culture medium was autoclaved for 15 min at 120°C. Inoculations were carried out in aseptic condition and kept at a temperature of 22 ± 2°C and 70-80% relative humidity conditions. Photoperiod cycle of 16/8 h was maintained by using light illumination of 40 µmol/m² s with help of cool white fluorescent lamps. Readings were noted after 45 days of inoculation.

2.5 Rooting of *in vitro* regenerated shoots

Regenerated shoots from both the explants were excised carefully, separated and transferred to root induction medium containing three different concentrations, viz., 0.5 mg/l, 0.1 mg/l and 0.2 mg/l of each of NAA and IBA (auxins) respectively, to get complete plantlets. Inoculated shoots were evaluated for per cent root regeneration after 4 weeks of culturing.

2.6 Preparation of methanol extracts of *in vitro* grown leaves and roots

Leaves and roots of *in vitro* grown plants of *R. cordifolia* were cleaned in sterile distilled water followed by shade drying for 15 days. Further, dried leaves and roots were ground using mortar and pestle. Dried and powdered material was extracted using sonication extraction technique in 100% methanol for 30 min in three cycles. Material, thus obtained was stored at 4°C until further use (Bhardwaj *et al.*, 2014).

2.7 GC-MS analysis of MEML

GC (Shimadzu Gas Chromatography system GC-2010 Plus, Japan) coupled with MS (GC-MS-QP 2010 Ultra) analysis of RURL and RUMR was carried out to decipher the type of phytochemicals accumulated in leaves and roots, respectively. For this, the temperature of injection port was set at 280°C whereas, oven temperature was programmed as: initial temperature as 80°C with a hold time of 2.0 min which was further increased at the rate of 4°C/min to 236°C with a hold of 5 min. Finally, the temperature was increased at the rate of 5°C/min to 300°C and held for 19.0 mins. Helium gas was used as carrier with the flow rate of 1.10 ml/min and split ratio of 1:5. The mass spectrum was recorded at 70 eV with electron ionization (EI) mode. Identification of various components was carried out exclusively by a comparison of their retention time and mass spectral data with the retention time and mass spectral database of Wiley 8 library. Compounds were quantified on the basis of area under the peak and results were presented in terms of percentage area (Bhardwaj *et al.*, 2020).

2.8 Data analysis

Statistical analysis of results has been represented in the form of mean ± standard error. One-way-ANOVA (analysis of variance) with Duncan's multiple range tests was used to determine the significance of obtained results. The probability (*p*) value ≤ 0.05 was considered significant for ANOVA and marked correlations among different assays. Results were processed with the help of computer programs, viz., MS Excel (USA) and SPSS v. 17.0 (USA).

Table 1: Effect of BAP and kinetin on nodal explant

Sr.No.	Treatment	Medium composition	No. of shoots	Length of shoots
1.	RUBK0A	MS basal medium + 0.0 µM BAP + 0.0 µM KN	NG ^a	NG ^a
2.	RUBK1A	MS basal medium + 0.5 µM BAP + 0.5 µM KN	22 ± 0.577 ⁱ	2.47 ± 0.32 ^f
3.	RUBK2A	MS basal medium + 0.5 µM BAP + 1.0 µM KN	4.67 ± 0.94 ^b	0.6 ± 0.066 ^{b,c,d}
4.	RUBK3A	MS basal medium + 0.5 µM BAP + 2.5 µM KN	8 ± 1.154 ^{c,d}	1.37 ± 0.27 ^{e,f}
5.	RUBK4A	MS basal medium + 0.5 µM BAP + 3.0 µM KN	19.66 ± 1.45 ^{a,h,i}	1.5 ± 0.360 ^f
6.	RUBK5A	MS basal medium + 0.5 µM BAP + 5.0 µM KN	20.33 ± 1.45 ^{h,i}	1.73 ± 0.176 ^f
7.	RUBK6A	MS basal medium + 1.0 µM BAP + 0.5 µM KN	12.33 ± 1.45 ^e	0.33 ± 0.12 ^{a,b,c}
8.	RUBK7A	MS basal medium + 1.0 µM BAP + 1.0 µM KN	42.33 ± 1.45 ^k	1.53 ± 0.29 ^f
9.	RUBK8A	MS basal medium + 1.0 µM BAP + 2.5 µM KN	17.66 ± 1.45 ^{f,g,h}	0.63 ± 0.12 ^{b,c,d}
10.	RUBK9A	MS basal medium + 1.0 µM BAP + 3.0 µM KN	8 ± 1.154 ^{c,d}	0.87 ± 0.088 ^{c,d,e}
11.	RUBK10A	MS basal medium + 1.0 µM BAP + 5.0 µM KN	16.67 ± 0.88 ^{f,g}	0.93 ± 0.088 ^{d,e}
12.	RUBK11A	MS basal medium + 2.5 µM BAP + 0.5 µM KN	C ^a	C ^a
13.	RUBK12A	MS basal medium + 2.5 µM BAP + 1.0 µM KN	6.67 ± 0.88 ^{b,c}	0.2 ± 0.057 ^{a,b}
14.	RUBK13A	MS basal medium + 2.5 µM BAP + 2.5 µM KN	29.33 ± 0.88 ^j	1.53 ± 0.11 ^f
15.	RUBK14A	MS basal medium + 2.5 µM BAP + 3.0 µM KN	16 ± 0.577 ^f	1.67 ± 0.088 ^f
16.	RUBK15A	MS basal medium + 2.5 µM BAP + 5.0 µM KN	18 ± 0.577 ^{f,g,h}	1.67 ± 0.18 ^f
17.	RUBK16A	MS basal medium + 5.0 µM BAP + 0.5 µM KN	0 ^a	0 ^a
18.	RUBK17A	MS basal medium + 5.0 µM BAP + 1.0 µM KN	10.33 ± 0.33 ^{d,e}	0.73 ± 0.12 ^{b,c,d}
19.	RUBK18A	MS basal medium + 5.0 µM BAP + 2.5 µM KN	12.67 ± 0.66 ^e	1.33 ± 0.088 ^{e,f}
20.	RUBK19A	MS basal medium + 5.0 µM BAP + 3.0 µM KN	17 ± 0.577 ^{f,g}	0.433 ± 0.120 ^{a,b,c,d}

Values are means ± SE of three determinations. The values having different superscript (small alphabet) letters within a column were significantly different ($p \leq 0.05$); NG- no growth, C-Callus.

Table 2: Effect of BAP, NAA and kinetin on regeneration from leaf explant

Sr.no.	Treatment	Medium composition	No. of shoots	Length of shoots
1.	RUBNK0A	MS basal medium + 0.0 µM BAP + 0.0 µM NAA + 0.0 µM KN	C ^a	C ^a
2.	RUBNK1A	MS basal medium + 0.5 µM BAP + 0.0 µM NAA + 0.5 µM KN	2.67 ± 0.67 ^c	0.9 ± 0.26 ^{b,c}
3.	RUBNK2A	MS basal medium + 0.5 µM BAP + 1.0 µM NAA + 0.5 µM KN	4.33 ± 0.33 ^d	2.13 ± 0.35 ^f
4.	RUBNK3A	MS basal medium + 0.5 µM BAP + 3.0 µM NAA + 0.5 µM KN	2.33 ± 0 ^{b,c}	1.63 ± 0.12 ^e
5.	RUBNK4A	MS basal medium + 0.5 µM BAP + 5.0 µM NAA + 0.5 µM KN	C ^a	C ^a
6.	RUBNK5A	MS basal medium + 1.0 µM BAP + 0.0 µM NAA + 1.0 µM KN	8.33 ± 0.88 ^f	1.5 ± 0.17 ^e
7.	RUBNK6A	MS basal medium + 1.0 µM BAP + 1.0 µM NAA + 1.0 µM KN	2.67 ± 0.33 ^c	2.17 ± 0.24 ^f
8.	RUBNK7A	MS basal medium + 1.0 µM BAP + 3.0 µM NAA + 5.0 µM KN	C ^a	C ^a
9.	RUBNK8A	MS basal medium + 1.0 µM BAP + 5.0 µM NAA + 1.0 µM KN	C ^a	C ^a
10.	RUBNK9A	MS basal medium + 2.5 µM BAP + 0.0 µM NAA + 2.5 µM KN	1.33 ± 0.33 ^{a,b}	1.37 ± 0.29 ^{d,e}
11.	RUBNK10A	MS basal medium + 2.5 µM BAP + 1.0 µM NAA + 2.5 µM KN	2.67 ± 0.57 ^c	0.467 ± 0.25 ^{a,b}
12.	RUBNK11A	MS basal medium + 2.5 µM BAP + 3.0 µM NAA + 2.5 µM KN	C ^a	C ^a
13.	RUBNK12A	MS basal medium + 2.5 µM BAP + 5.0 µM NAA + 2.5 µM KN	C ^a	C ^a
14.	RUBNK13A	MS basal medium + 3.0 µM BAP + 0.0 µM NAA + 3.0 µM KN	1.33 ± 0.577 ^{a,b}	0.76 ± 0.055 ^{b,c}
15.	RUBNK14A	MS basal medium + 3.0 µM BAP + 1.0 µM NAA + 3.0 µM KN	6.67 ± 0.88 ^e	0.87 ± 0.26 ^{b,c,d}
16.	RUBNK15A	MS basal medium + 3.0 µM BAP + 3.0 µM NAA + 3.0 µM KN	1.66 ± 0.33 ^{b,c}	1.13 ± 0.12 ^{c,d,e}
17.	RUBNK16A	MS basal medium + 3.0 µM BAP + 5.0 µM NAA + 3.0 µM KN	C ^a	C ^a

Values are means ± SE of three determinations. The values having different superscript (small alphabet) letters within a column were significantly different ($p \leq 0.05$); C-Callus.

Table 3: Rooting of *in vitro* regenerated shoots

Sr.No.	Auxins	Percent rooting
1.	0.05 NAA	85 ± 1.73
2.	0.01 NAA	99.67 ± 0.33
3.	0.02 NAA	95 ± 1.154
4.	0.05 IBA	74 ± 1.45
5.	0.01 IBA	81 ± 0.577
6.	0.02 IBA	73.66 ± 0.88

Values are means ± SE of three determinations.

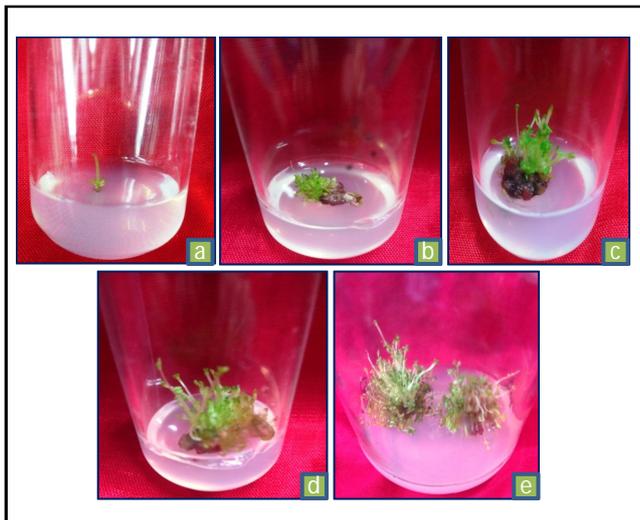
**Figure 1: *Rubia cordifolia* growing in its natural habitat.**

Figure 2: (a) Nodal explant inoculated on MS media
 (b) Initiation of shooting (15 days old)
 (c) Multiple shoot induction
 (d) Regeneration of new shoots
 (e) *In vitro* proliferated shoots and roots (40 days).

3. Results

3.1 Multiple shoots induction from nodal explants

A total of 20 different combinations of BAP and KN were investigated for effective shoot regeneration from nodal explant keeping basal MS media as control (Table 1). Equal concentrations of both hormones were found optimum for effective shoots regeneration. Maximum number of shoots (42.33 ± 1.43) were found to develop on RUBK7 media containing $1.0 \mu\text{M}$ concentration of each of hormone whereas shoots with maximal length (2.47 ± 0.32) were obtained in RUBK1 medium ($2.5 \mu\text{M}$ BAP and $2.5 \mu\text{M}$ KN) (Table 1, Figure 2).

3.2 Multiple shoots induction from leaf explants

Surface sterilized leaves were cut with sterile scalpel blade and placed horizontally on medium containing 16 different concentration of NAA (μM) with BAP (μM) and KN (μM) for effective organ differentiation. All the explants were found to induce callus, however duration of callus formation varied in different concentrations used (Table 2). Maximum number of shoots (8.33 ± 0.88) were found to grow in RUBNK5 medium containing MS media supplemented with $1.0 \mu\text{M}$ concentration of each of BAP and NAA. Whereas shoots with maximum length were observed in MS medium containing $1.0 \mu\text{M}$ of each of BAP, KN and NAA. Further, a higher concentration of NAA in comparison to both KN and BAP were found to show no effect on organ differentiation in callus (Table 2, Figure 3).

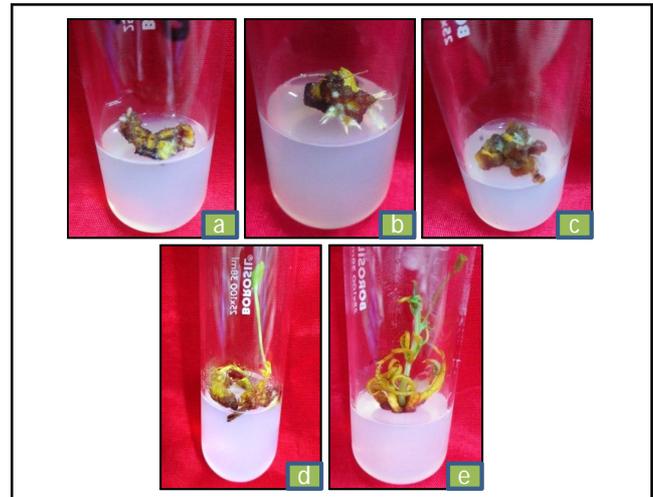


Figure 3: (a) Leaf explant inoculated on MS media, (b) *In vitro* root proliferation (25 days old), (c) *In vitro* shoot proliferation, (d) Regeneration of new shoots from nodal explant and (e) *In vitro* development of complete plant (40 days).

3.3 Rooting of *in vitro* grown shoots

In vitro grown shoots were cultured on MS medium supplemented with different concentrations of NAA and IBA (Table 3). Maximum healthy and vigorous root system was found to develop after two weeks of inoculation after which no noticeable changes in development was observed. Maximum per cent root regeneration (99.67 ± 0.33) was observed in 0.1 mg/l NAA, whereas in case of NAA, $81 \pm 0.577 \%$ root regeneration was found to develop in 0.1 mg/l IBA concentration (Table 3).

Table 4: Compounds identified in GC-MS analysis of RUML

Sr. No.	Name	Formula	SI	% Area	Medicinal use	References
1.	Octyl 2-Methylpropanoate	C ₁₂ H ₂₄ O ₂	84	0.95	Antiinflammatory and antibacterial	Hussein <i>et al.</i> , 2016
2.	Neophytadiene	C ₂₀ H ₃₈	95	11.82	Antibacterial, skin diseases, rheumatism	Singh <i>et al.</i> , 2012
3.	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	C ₂₀ H ₄₀ O	90	4.31	Anxiolytic, antioxidant, autophagy-anti-inflammatory, immune-modulating, and antimicrobial effects	Islam <i>et al.</i> , 2016
4.	Heptadecanoic Acid, methyl Ester	C ₁₈ H ₃₆ O ₂	91	1.00	Hypoglycaemic, antioxidantactivities	Babu <i>et al.</i> , 2017
5.	6-Octadecenoic acid, methyl ester	C ₁₉ H ₃₆ O ₂	84	3.64	Antioxidant, anticancer	Belakhdar <i>et al.</i> , 2015
6.	Phytol	C ₂₀ H ₄₀ O	87	5.61	Antioxidant, hypocholesterolemia, nematicide, hemolytic,	Sharma <i>et al.</i> , 2015.
7.	1,2-Benzenedicarboxylic Acid, Dioctyl ester	C ₂₄ H ₃₈ O ₄	82	0.86	Antimicrobial activity	Osuntokun and Gamberini, 2019.
8.	9-Octadecenamide	C ₁₈ H ₃₅ NO	91	18.85	Anti-inflammatory activity and antibacterial activity	Hussein <i>et al.</i> , 2016
9.	Squalene	C ₃₀ H ₅₀	95	9.96	Antioxidant and anti tumouractivities	Haung <i>et al.</i> , 2009
10.	Pentadecanone	C ₁₅ H ₃₀ O	83	2.05	Antioxidant and antibacterial activity	Bhardwaj <i>et al.</i> , 2014
11.	alpha-Tocopherol	C ₂₉ H ₅₀ O ₂	88	7.18	Antioxidant and anti-inflammatory activities	Cassano <i>et al.</i> , 2015
12.	Stigmast-5-en-3-ol	C ₂₉ H ₅₀ O	81	4.87	Antidiabetic agent	Sujatha <i>et al.</i> , 2015
13.	Gamma-Sitosterol	C ₂₉ H ₅₀ O	81	3.80	Antihypercholesterolemic, antiviral (influenza), antiinflammatory, antiacne, antiprotozoal (leishmania), antibacterial	Adnan <i>et al.</i> , 2019
14.	[1,1'-Bicyclohexyl]-2,2'-diol	C ₁₂ H ₂₂ O ₂	77	0.97	No reported biological activity	
15.	Cyclopenta[C]Pyran-3(1H)-one, Hexahydro-4,7-Dimethyl-	C ₁₀ H ₁₆ O ₂	74	1.40	No reported biological activity	
16.	4-Tridecanol	C ₁₃ H ₂₈ O	79	1.52	Esters for synthetic lubricants; detergents; antifoam agent	Lewis, 2001
17.	3,3-Dimethylbutyric acid 2,2,3,3,4,4,4-heptafluorobutyl ester	C ₁₀ H ₁₃ F ₇ O ₂	67	0.67	No reported biological activity	
18.	N-[2-(4-Benzenesulfonyl-Piperazin-1-YL)-Ethyl]-N'-(4-Methyl-Benzyl)-Oxalamide	C ₂₂ H ₂₈ N ₄ O ₄ S	68	1.27	No reported biological activity	
19.	Erythro-9,10-dibromopentacosane	C ₂₅ H ₅₀ Br ₂	65	1.40	No reported biological activity	
20.	2,3,4-trimethyl-1-pentanol	C ₈ H ₁₈ O	75	0.29	No reported biological activity	

Table 5: Compounds identified in GC-MS analysis of RUMR

Sr.No.	Name	Formula	SI	% Area	Medicinal use	References
1.	Neophytadiene	C ₂₀ H ₃₈	84	1.79	Antibacterial, skin diseases, rheumatism	Singh <i>et al.</i> , 2012
2.	2-Decen-1-ol	C ₁₀ H ₂₀ O	83	0.70		
3.	Tetradecanoic acid, 12-methyl-, methyl ester	C ₁₆ H ₃₂ O ₂	89	1.95	No reported biological activity	
4.	Cyclobutene, 1-Methyl-3-Propyl	C ₈ H ₁₄	81	0.31	No reported biological activity	
5.	7-Hexadecenoic acid, methyl ester	C ₁₇ H ₃₂ O ₂	85	2.98	Antibacterial and antifungal	Chandrasekaran <i>et al.</i> , 2011
6.	9-Octadecenoic acid (Z)-, methyl ester	C ₁₉ H ₃₆ O ₂	83	26.41	Hypocholesterolemic, 5-alpha reductase inhibitor, anemiagenic insectifuge, flavor	Gnana <i>et al.</i> , 2018
7.	Heptadecanoic Acid, Methyl Ester	C ₁₈ H ₃₆ O ₂	89	1.09	Hypoglycaemic, antioxidantactivities	Babu <i>et al.</i> , 2017
8.	1,2-Benzenedicarboxylic Acid, Dioctyl Ester	C ₂₄ H ₃₈ O ₄	88	1.09	Antibacterial	Ajoke <i>et al.</i> , 2015)
9.	Squalene	C ₃₀ H ₅₀	95	11.22	Antioxidant and antitumour activities	Haung <i>et al.</i> , 2009
10.	dl-.alpha.-Tocopherol	C ₂₉ H ₅₀ O ₂	88	1.87	Antioxidant and antiinflammat oryactivities	Cassano <i>et al.</i> , 2015
11.	Adenosine, N6-phenylacetic acid	C ₁₈ H ₁₉ N ₅ O ₆	74	33.05		
12.	Cyclohexaneethanol	C ₈ H ₁₆ O	79	1.02	Perfuming and flavouring agents	Larranaga <i>et al.</i> , 2016
13.	[1,1'-Bicyclohexyl]-2,2'-Diol	C ₁₂ H ₂₂ O ₂	78	0.92	No reported biological activity	
14.	2H-Pyran, tetrahydro-4-methylene-	C ₆ H ₁₀ O	69	0.36	No reported biological activity	
15.	Phosphinous chloride	C ₅ H ₁₁ C ₁₂ P	70	0.65	No reported biological activity	
16.	Cyclopentaneethanol, 2-(Hydroxymethyl)-,	C ₈ H ₁₆ O ₂	75	0.84	No reported biological activity	
17.	1H-Imidazole, 4,5-dihydro-2,4-dimethyl	C ₅ H ₁₀ N ₂	71	0.97	No reported biological activity	
19.	3,3-dimethylbutyric acid, 2,2,3,3,4,4,4-heptafluorobutyl ester	C ₁₀ H ₁₃ F ₇ O ₂	72	0.63	No reported biological activity	
20.	N-[2-(4-Benzenesulfonyl-Piperazin-1-YL)-Ethyl]-N'-(4-Methyl-Benzyl)-Oxalamide	C ₂₂ H ₂₈ N ₄ O ₄ S	73	0.84	No reported biological activity	

Table 6: Compounds identified in both extracts along with CAS no and structures

Sr. No.	CAS	Retention time	Name	Structure
1.	109-15-9	20.229	Octyl 2-methylpropanoate	CCCCCCCCOC(=O)C(C)C
2.	504-96-1	21.084	Neophytadiene	CC(C)CCCC(C)CCCC(C)CCCC(=C)C=C
3.	102608-53-7	21.507	3,7,11,15-tetramethyl-2-hexadecen-1-ol	CC(C)CCCC(C)CCCC(C)CCCC(=CCO)C
4.	1731-92-6	22.604	Heptadecanoic acid, methyl ester	CCCCCCCCCCCCCCCC(=O)OC
5.	2777-58-4	25.379	6-Octadecenoic acid, methyl ester	CCCCCCCCCCCC=CCCCC(=O)OC
6.	150-86-7	25.560	Phytol	CC(C)CCCC(C)CCCC(C)CCCC(=CCO)C
7.	117-84-0	32.756	1,2-Benzenedicarboxylic acid, dioctyl ester	CCCCCCCCC1=C(C(=CC=C1)C(=O)OCCCCCCCC)C(=O)OCCCCCCCC
8.	301-02-0	37.453	9-Octadecenamide	CCCCCCCC=CCCCCCCC(=O)N

9.	111-02-4	37.805	Squalene	<chem>CC(=CCCC(=CCCC(=CCCC=C(C)CCC=C(C)CCC=C(C)C)C)C)C</chem>
10	2765-11-9	38.203	Pentadecanal	<chem>CCCCCCCCCCCCCCC=O</chem>
11.	59-02-9	41.399	alpha-tocopherol	<chem>CC1=C(C2=C(CCC(O2)(C)CCCC(C)CCCC(C)CCCC(C)C(=C1O)C)C</chem>
12.	17385-36-3	24.825	[1,1'-bicyclohexyl]-2,2'-diol	<chem>C1CC(CCC1C2CCC(CC2)O)O</chem>
13.	485-43-8	26.748	Cyclopenta[C]pyran-3(1H)-one, hexahydro-4,7-dimethyl-,	<chem>CC1CCC2C1COC(=O)C2C</chem>
14.	26215-92-9	28.274	4-tridecanol	<chem>CCCCCCCCCCC(CCC)O</chem>
15.	0-00-0	29.064	3,3-dimethylbutyric acid 2,2,3,3,4,4,4-heptafluorobutyl ester	<chem>CCCCCOC(=O)C(C(C(F)(F)F)(F)F)(F)F</chem>
16.	0-00-0	36.449	N-[2-(4-benzenesulfonyl-piperazin-1-yl)-ethyl]-N'-(4-methyl-benzyl)-oxalamide	<chem>CCNS(=O)(=O)C1=CC=CC(=C1)C(=O)NC2=CC=C(C=C2)CN3CCCC(C3)C(=O)N</chem>
17.	59907-02-7	41.625	Erythro-9,10-dibromopentacosane	<chem>CCCCCCCCCCCCCCCC(C(CCCCCC)Br)Br</chem>
18.	83-48-7	42.827	Stigmasterol	<chem>CCC(C=CC(C)C1CCC2C1(CCC3C2CC=C4C3(CCC(C4)O)C)C)C(C)C</chem>
19.	83-47-7	43.610	Gamma-sitosterol	<chem>CCC(CCC(C)C1CCC2C1(CCC3C2CC=C4C3(CCC(C4)O)C)C)C(C)C</chem>
20.	2438-20-2	29.747	1-Butanol,2-methyl,propanoate	<chem>CCC(C)COC(=O)CC</chem>
21.	22104-80-9	21.508	2-Decen-1-ol	<chem>CCCCCCC=CCO</chem>
22.	5129-66-8	22.604	Tetradecanoic acid, 12-methyl-, methyl ester	<chem>CCC(C)CCCCCCCCCCC(=O)OC</chem>
23	122699-82-5	25.263	Cyclobutene, 1-methyl-3-propyl	<chem>CCCC1CCCC(C1)C</chem>
24.	56875-67-3	25.375	7-Hexadecenoic acid, methyl ester	<chem>CCCCCCCCC=CCCCCCC(=O)OC</chem>
25.	112-62-9	25.541	9-Octadecenoic acid (Z)-, methyl ester	<chem>CCCCCCCCC=CCCCCCCC(=O)OC</chem>
26.	96760-57-5	14.593	Adenosine, N6-phenylacetic acid	<chem>C1=CC(=CC=C1CC(=O)O)NC2=C3C(=NC=N2)N(C=N3)C4C(C(C(O4)CO)O)O</chem>
27.	4442-79-9	21.822	Cyclohexane ethanol	<chem>C1CCC(CC1)CCO</chem>
28.	36838-71-8	25.472	2H-Pyran, tetrahydro-4-methylene-	<chem>CC1CCOCC1</chem>
29.	78055-63-7	26.107	Phosphinous chloride	<chem>CCCC(C)P(Cl)Cl</chem>
30	15773-82-7	26.739	Cyclopentaneethanol, 2-(hydroxymethyl)-,	<chem>CCCCCCC(=O)O</chem>
31.	28.288	930-61-0	1H-Imidazole, 4,5-dihydro-2,4-dimethyl	<chem>CC1CN=C(N1)C</chem>

3.4 GC-MS analysis

GC-MS chromatogram of methanolic extract of *in vitro* grown leaves (RUML) recorded a total of 20 peaks corresponding to bioactive phytoconstituents by relating their retention time, peak area and height with Wiley 8 library (Figure 4A-B, Table 4). Phytoconstituents identified in RUML were Octyl 2-methyl propanoate, neophytadiene, 3,7,11,15-tetramethyl-2-hexadecen-1-ol, heptadecanoic acid, methyl ester, 6-octadecenoic acid, methyl ester, phytol, 1,2-benzenedicarboxylic acid, dioctyl ester, 9-octadecenamamide, squalene, pentadecanone, alpha-tocopherol, stigmast-5-en-3-ol, gamma-sitosterol, [1,1'-bicyclohexyl]-2,2'-diol, cyclopenta[c]pyran-3(1h)-one, hexahydro-4,7-dimethyl-,4-tridecanol, 3,3-dimethylbutyric acid 2,2,3,3,4,4,4-heptafluorobutyl ester, n-[2-(4-benzenesulfonyl-piperazin-1-yl)-ethyl]-n'-(4-

methyl-benzyl)-oxalamide, erythro-9,10-dibromopentacosane, and 2,3,4-trimethyl-1-pentanol (Table 4). Nine identified phytoconstituents were found with S.I. score > 80. Further, GC-MS analysis of methanolic extract of *in vitro* grown roots (RUMR) also recorded characteristic 19 peaks corresponding to different phytocomponents with various reported biological activities (Table 5; Figures 5A-B). It included neophytadiene, 2-decen-1-ol, tetradecanoic acid, 12-methyl-, methyl ester, cyclobutene, 1-methyl-3-propyl, 7-hexadecenoic acid, methyl ester, 9-octadecenoic acid (Z)-, methyl ester, heptadecanoic acid, methyl ester, 1,2-benzenedicarboxylic acid, dioctyl ester, squalene, dl-alpha-tocopherol, adenosine, N6-phenylacetic acid, cyclohexane ethanol, [1,1'-bicyclohexyl]-2,2'-diol, 2H-pyran, tetrahydro-4-methylene-, phosphinous chloride, cyclopentaneethanol, 2-(hydroxymethyl)-, 1H-imidazole, 4,5-dihydro-2,4-dimethyl, 3,3-dimethylbutyric acid,

2,2,3,3,4,4,4-heptafluorobutyl ester and N-[2-(4-benzenesulfonyl-piperazin-1-yl)-ethyl]-n'-(4-methyl-benzyl)-oxalamide. Out of 19 compounds observed in RUMR, 10 were found with S.I. value >

80% (Table 5). Further, 8 compounds were observed common in both samples. Structure of the all detected 40 compounds are given in Table 6 with their CAS no.

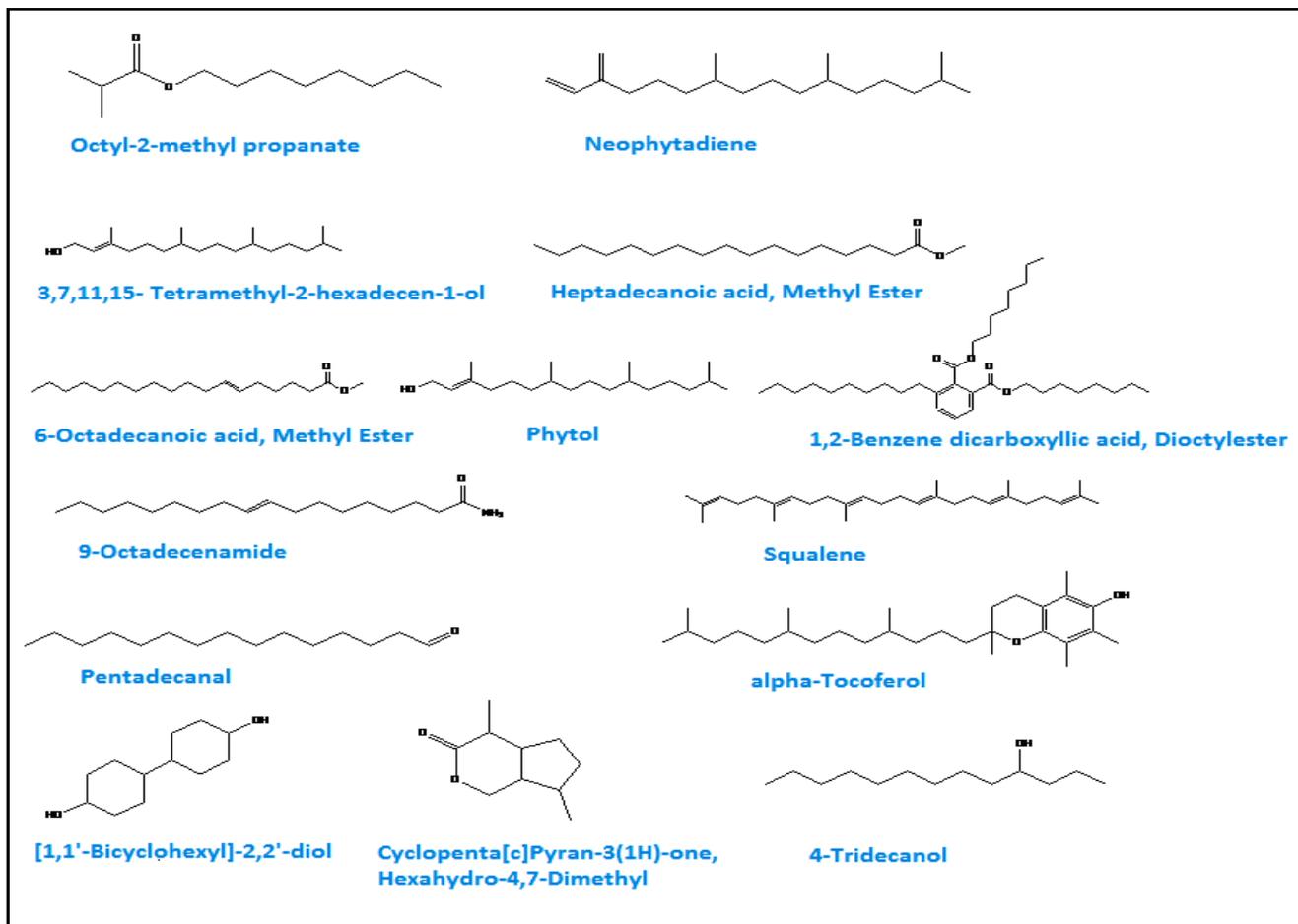


Figure 4A: Identified compounds in GC-MS profile of *in vitro* grown leaves (RUMR).

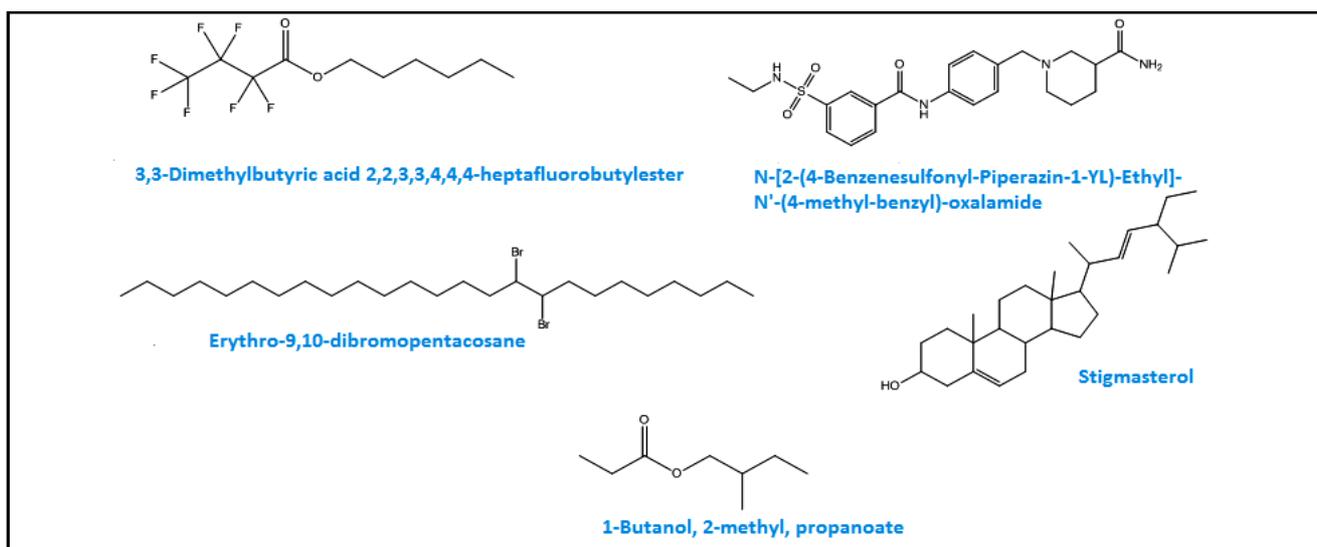


Figure 4B: Identified compounds in GC-MS profile of *in vitro* grown leaves (RUMR).

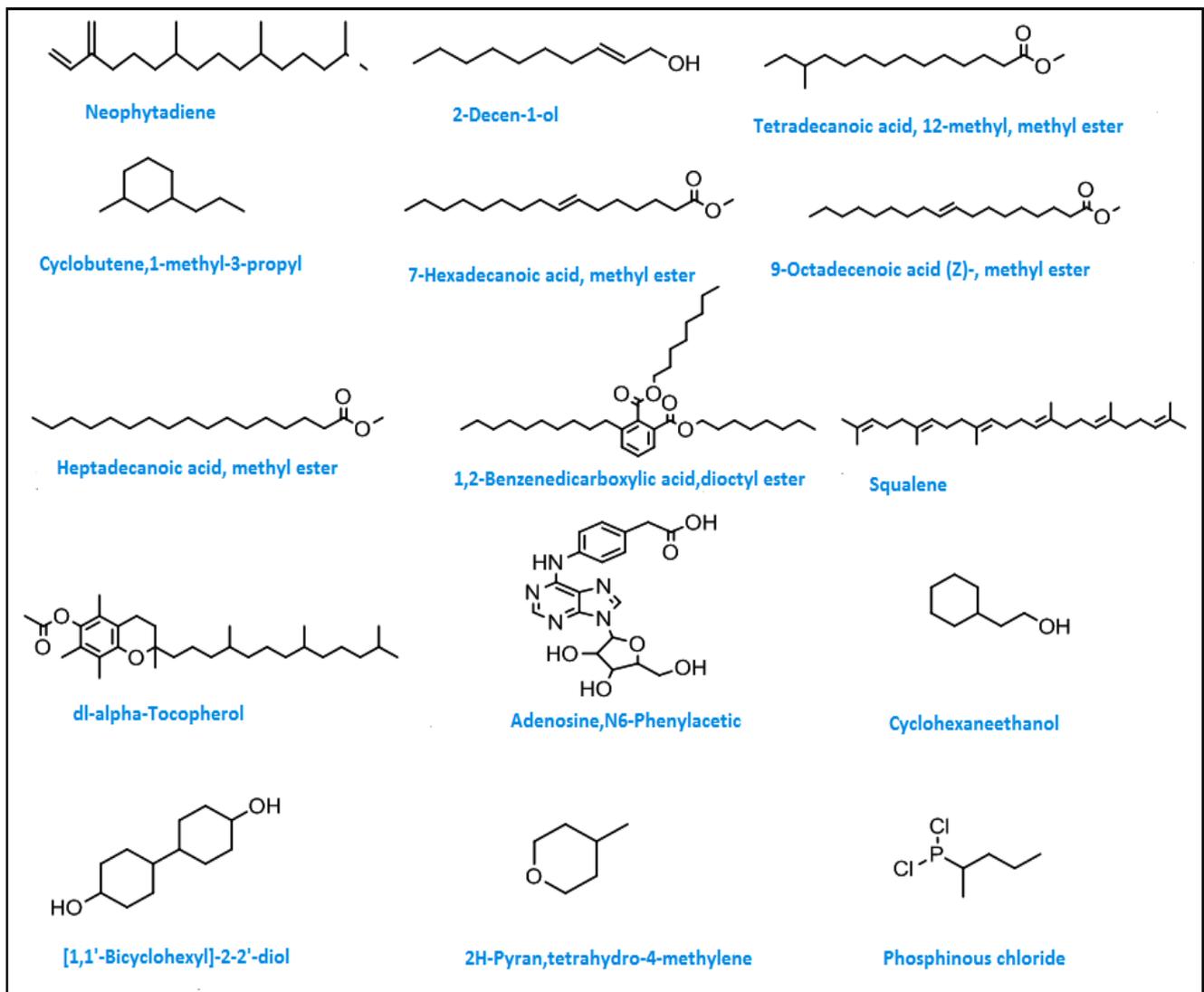


Figure 5A: Compounds identified in GC-MS profile of *in vitro* grown leaves (RUMR).

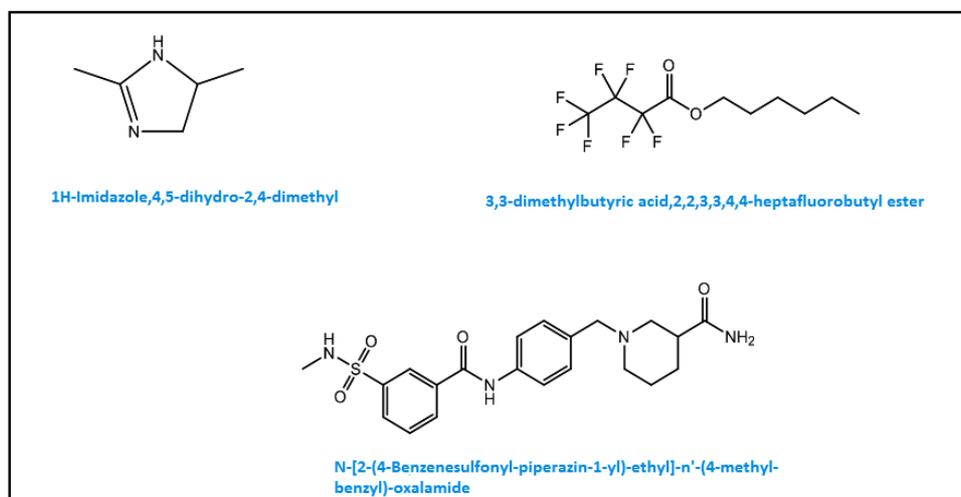


Figure 5B: Compounds identified in GC-MS profile of *in vitro* grown leaves (RURL).

4. Discussion

Members of plant kingdom produce amazing diversity of low molecular weight organic compounds which have been classified into three prominent groups: primary (required directly for plant growth); secondary (mediate plant-environment interactions) and hormones (regulate physiological functions and metabolism) (Matthias and Kliebenstein, 2020). Among these, secondary metabolites are unique and more of scientific interest as these help the plants to adapt and defend themselves in various biotic and abiotic stressed environmental conditions (Ramakrishna and Ravishankar, 2011). Phytochemicals with ability to display therapeutical effects are fortuitous blessings for human health. However, biosynthesis and accumulation of these secondary metabolites are strongly influenced by various environmental factors, viz., temperature, humidity, light intensity, supply of water, minerals, and CO₂, etc. This ability of biosynthesizing various types of secondary compounds and in different concentrations has been achieved by the plants in the course of evolution in order to shield themselves from the selective notions of nature (Pichersky and Gang, 2000). Ladakh, also known as 'land of high passes' is an Indian state known for its rugged ecosystem and limited vegetative period of 4-5 months only (Kumar *et al.*, 2013). However, a number of medicinal plant species (1100) have been found to grow on these steep and rocky mountains (Navchoo and Buth, 1989). Local medical practitioner combines these herbs with various other indigenous ingredients like mineral salts and animal products to develop traditional therapeutic formulations. These tremendous health promoting benefits of Himalayan medicinal plants has been traced to their unique bioactive secondary metabolites which in turn attributes the plants with tolerance to harsh climatic conditions of high altitude habitat (Kumar *et al.*, 2017). *R. cordifolia* is one of perennial herbaceous medicinal plant species, found to grow actively in close proximity to water sources. In traditional medicinal system, different parts of this plant species are used for treatment of various ill conditions like skin diseases, diabetes, rheumatism, jaundice, stomach and urinary disorders (Gaurav *et al.*, 2017). This plant species has been reported to contain different phytoconstituents like xanthopurpurin, manjishthin, nephthoquinones, pseudo-purpurin, etc. (Devipriya and Siril, 2014). However, various regional and environmental restrictions, limit the commercial extraction of these phytoconstituents. Plant cell culture technologies have been effective tools for both studying and producing desired plant secondary metabolites under *in vitro* conditions along with improvement of plants for large scale production of secondary metabolites. Plant tissue culture is the science of growing plant cells, tissue or organs independently from mother plant on artificial nutrient media under controlled environmental conditions (Li *et al.*, 2017). In present study, an *in vitro* regeneration method for *R. cordifolia* has been developed using leaf and nodal explant collected from Leh region followed by GC-MS analysis to determine the phytochemical profile. Biosynthesis and accumulation of bioactive phytoconstituents are species specific and tissue specific which are further influenced by the environmental conditions, in which the source of explant is growing (Nielsen *et al.*, 2019). Among the tested combinations of two cytokinins, i.e., BAP and KN, maximum shoot induction and proliferation (42.33 ± 1.43) was observed in 1.0 µM concentration of each of two cytokinin tested. A low concentration of BAP with high concentration of KN was found more effective for efficient shoots development. Further, numerous concentrations of three different hormones, viz., KN, BAP and NAA

were investigated for multiple shoot regeneration from leaf explant. Equal concentration of BAP and KN without NAA was found more effective for shoot proliferation by indirect organogenesis. Synergistic effects of KN and BAP hormones were observed more efficient for multiple shoot induction whereas higher concentration of NAA in comparison to other two hormones was found to develop callus. Development of organs in *in vitro* culture depends on the concentration and balance of exogenously supplied phytohormones which either directly leads to organogenesis or induce the already present hormones with in cells for organ development (Mercier *et al.*, 2003). Previously, 1.0 mg/l BAP in combination with 0.02 mg/l IAA was found optimum for effective shoot induction (5.2 shoots/explant) (Radha *et al.*, 2011). In another study carried out by Gurav *et al.* (2017), only bud sprouting was achieved in MS media supplemented with 3.0 mg/l BAP and 0.1 mg/l IAA from nodal explant, whereas maximum number of shoots development (20-25) was observed in TDZ (0.5 mg/l) with 0.1% PVP containing MS media (Gaurav *et al.*, 2017). Further, triumph of clonal propagation study depends on effective rooting of *in vitro* regenerated shoots (Faisal *et al.*, 2006). It has been described that requirement of external supply of different concentrations of auxins for root induction depend upon inherent concentrations of auxins in plant tissue. In addition to this, plant genotype, age and type of explant, concentration and kind of auxin further influence the induction of roots (Li *et al.*, 2017) Therefore, supplementation of correct concentration of specific auxin in culture medium is detrimental for adequate roots development (Mahendran and Bai, 2013). In present study, IBA was found more effective for efficient root regeneration in comparison to NAA where a percent root regeneration of 99.67 ± 0.33 was achieved in 0.1 mg/l NAA. Similar efficiency of IBA for effective root regeneration has also been reported in *R. cordifolia* (Gaurav *et al.*, 2017). Further, GC-MS analysis was carried out to determine the phytochemical profile of methanolic extract of both *in vitro* grown leaves (RURL) and roots (RUMR). Thirty nine chemical phytochemicals including 8 common bioactive constituents with various biological activities were identified in both extracts (Tables 1, 2). These compounds were found to show different biological activities, viz., anti-inflammatory (octyl 2-methylpropanoate, 9-octadecenamide), antimicrobial (neophytadiene; 1, 2-benzenedicarboxylic acid, dioctyl ester; gamma-sitosterol), antioxidant (heptadecanoic acid, methyl ester; 6-octadecenoic acid, methyl ester); pentadecanone; alpha-tocopherol, antidiabetic (stigmast-5-en-3-ol), antitumour (squalene), etc., along with other compounds were identified in both RUMR and RURL, suggesting the therapeutical potential of *in vitro* grown plants of *R. cordifolia* for various pharmaceutical applications. Previously, Gazi *et al.* (2015) identified rubiadin and purpurin in ethanolic extract of roots of *R. cordifolia*. To the best of our knowledge, this is the first report of establishment of *in vitro* culture of *R. cordifolia* through leaf explant. Further, for the first time, GC-MS analysis has been performed to determine the phytochemical profile of *in vitro* grown leaves and roots.

5. Conclusion

Present investigation has been focused on determination of phytochemical potential of *in vitro* grown plants of *R. cordifolia* grown in Leh-Ladakh. For the first time, this study has been carried out to establish an effective *in vitro* regeneration protocol using leaf explant which could be applied to aid in producing uniform

propagules throughout the year for industrial purpose without destructing its natural habitat. A total of 20 compounds were identified in methanolic extract of *in vitro* grown leaves and 19 from roots depicting the profound potential of *in vitro* grown plants for pharmaceutical utility. Therefore, this work paves way for mass propagation and cultivation of this high value medicinal plant using biotechnological approaches. Also, a scope of *in vitro* synthesis of bioactive phytochemicals and their plausible elicitation using biotic and abiotic stresses is being suggested, further.

Conflict of interest

The authors declare that there are no conflicts of interest relevant to this article.

References

- Adnan, M.; Chy, N.U.; Mostafa Kamal, A.T.M.; Azad, M.O.K.; Paul, A.; Uddin, S.B. and Cho, D.H. (2019). Investigation of the biological activities and characterization of bioactive constituents of *Ophiorrhiza rugosa* var. *Prostrata* (D. Don) and Mondal leaves through *in vivo*, *in vitro*, and *in silico* approaches. *Molecules*, **24**(7):1367.
- Ajoke, F.P.; Kaita, H. and Ilyas, M. (2014). Antibacterial activity of 1,2-benzenedicarboxylic acid, dioctyl ester isolated from the ethyl acetate soluble sub-portion of the unripe fruits of *Nauclea latifolia*. *Int. J. Pure App. Biosci.*, **2**:223-230.
- Arunkumar, S. and Muthuselvam, M. (2009). Analysis of phytochemical constituents and antimicrobial activities of *Aloe vera* L. against clinical pathogens. *World Journal of Agricultural Sciences*, **5**(5): 572-576.
- Babu, A.; Anand, D. and Saravanan, P. (2017). Phytochemical analysis of *Ficus arnottiana* (Miq.) leaf extract using GC-MS analysis. *IJPPR*, **9**(6):775-4.
- Badyal, D.K.; Lata, H. and Dadhich, A.P. (2003). Animal models of hypertension and effect of drugs. *Indian J. Pharmacol.*, **35**(6): 349-362.
- Belakhdar, G.; Benjouad, A. and Abdennebi, E.H. (2015). Determination of some bioactive chemical constituents from *Thesium humile* Vahl. *J. Mater Environ. Sci.*, **6**(10):2778-5.
- Bhardwaj, A.; Shakil, N.A.; Jha, V. and Gupta, R.K. (2014). Screening of nutritional, phytochemical, antioxidant and antibacterial activity of underutilized seeds of *Scirpus articulatus*: The basis of Khubahi Ramdana industry. *J. Pharmacogn. Phytochem.*, **3**(4):311-320.
- Bhardwaj, P.; Naryal, A.; Thakur, M.S.; Aggarwal, N.K.; Saxena, S.; Chaurasia, O.P. and Kumar, R. (2020) Comparative antioxidant, antibacterial, and GC-MS analysis of methanol extract's fractions and isolation of luteolin from leaves of trans-Himalayan *Codonopsis clematidea*. *Ind. Crops Prod.*, **144**:112046.
- Bin-Nyeem, M.A.B. and Mannan, M.A. (2018). *Rubia cordifolia*-phytochemical and pharmacological evaluation of indigenous medicinal plant: A review. *Int. J. Physiol. Nutri. Phys. Edu.*, **3**(1): 766-771.
- Cassano, R. (2012). Vitamin E chemistry, biological activity and benefits on the skin. In handbook of diet, nutrition and the skin, Wageningen Academic Publishers, pp:144-163.
- Chandrasekaran, M.; Senthilkumar, A. and Venkatesalu, V. (2011). Antibacterial and antifungal efficacy of fatty acid methyl esters from leaves of *Sesuvium portulacastrum* L. *Eur. Rev. Med. Pharmacol. Sci.*, **15**:775-780.
- Devi Priya, M. and Siril, E. A. (2014). Traditional and modern use of indian madder (*Rubia cordifolia* L.): An overview. *Int. J. Pharm. Sci. Rev. Res.*, **25**(1):154-164.
- Devi P, M. and Siril, E. A. (2014). Traditional and modern use of indian madder (*Rubia cordifolia* L.): An overview. *Int. J. Pharm. Sci. Rev. Res.*, **25**(1):154-164.
- Gaurav, A.M.; Sinha, R.; Mhase, A.G.; Rao, G.; Mangal, A.K and Srikanth, N. (2017). Conservation of Manjishtha - *Rubia cordifolia* L. through nodal culture. *Journal of Drug Research in Ayurvedic Medicine*, **2**(4):267-273.
- Gazi, S.; Sadath, A.; Luqman, S. and Rizwan, S.H. (2016). Gas chromatography mass spectrometry analysis of ethanolic extract of roots of *Rubia cordifolia* (MANJISTHA). *Asian J. Pharm Clin. Res.*, **9**(1):191-93.
- Gharibi, S.; Sayed, B.E.; Saeidi, T.G.; Talebi, M. and Matkowski, A. (2019). The effect of drought stress on polyphenolic compounds and expression of flavonoid biosynthesis related genes in *Achillea pachycephala* Rech.f. *Phytochem.*, **162**:90-98.
- Gnana, G.; Beulah, P.; Soris, P.T. and Mohan, V.R. (2018). GC-MS determination of bioactive compounds of *Dendrophthoe falcata* (L.F) Ettingsh: An epiphytic plant. *IJHSR.*, **8**:261-269.
- Guurav, A.M.; Sinha, R.; Mhase, A.G.; Rao, G.; Mangal, A.K. and Srikanth N. (2017). Conservation of Manjishtha - *Rubia cordifolia* L. through nodal culture. *J. Drug Res. Ayurvedic Sci.*, **2**(4):263-273.
- Huang, Z.R.; Lin, Y.K. and Fang, J.Y. (2009). Biological and pharmacological activities of squalene and related compounds: potential uses in cosmetic dermatology. *Molecules*, **14**(1):540-554.
- Hussein, H. M.; Hameed, L.H. and Ibraheem, O.A. (2016). Antimicrobial activity and spectral chemical analysis of methanolic leaves extract of *Adiantum capillus-Veneris* using GC-MS and FTIR spectroscopy. *IJPPR*, **8**(3):369-416.
- Islam, M.T.; Ali, E.S.; Uddin, S.J.; Shaw, S.; Islam, M.A. and Ahmed M.I. (2018). *Phytol*: A review of biomedical activities. *Food Chem. Toxicol.*, **121**:82-12.
- Itokawa, H.; Qiao, Y.F. and Takeya, K. (1990). New arborane type triterpenoids from *Rubia cordifolia* var. *pratensis* and *R. oncotricha*. *Chem. Pharm. Bull.*, **38**(5):1435-1437.
- Johnson, W.C. and William, O.W. (2002). Warfarin toxicity. *J. Vasc. Surg.*, **35**:413-421.
- Kala, C.P. and Manjrekar, N. (1999). Ethnomedicobotany of Indian trans-Himalaya: A case study from Spiti. *J. Econ. Taxon. Bot.*, **23**:177-183.
- Kumar, J.; Dhar, P.; Tayade, A.B.; Gupta, D. and Chaurasia, O.P. (2013). Antioxidant capacities, phenolic profile and cytotoxic effects of *saxicolous* lichens from trans-Himalayan cold desert of Ladakh. *PLoS One*, **2**:259-289.
- Kumar, R.; Thakur, M.S.; Bhardwaj, P.; Bhardwaj, G.; Raghuvanshi, R. and Chaurasia, O.P. (2017). GC-MS profiling and bioactivity study of trans-Himalayan plant *Centaurea depressa*. *Der. Pharma. Chem.*, **9**:63-69.
- Larranaga, M. D. and Lewis, R. J. (2016). *Hawley's condensed chemical dictionary* 14th edition. John Wiley and Sons, Inc., New York, pp: 1002.
- Lewis, R. J. and Hawley, G.G. (2001). *Hawley's condensed chemical dictionary* 14th edition. John Wiley and Sons, Inc., New York, pp:1124.
- Li, S.; Huang, P.; Ding, G.; Zhou, L.; Tang, P.; Sun, M. and Lin, S. (2017). Optimization of hormone combinations for root growth and bud germination in Chinese fir (*Cunninghamia lanceolata*) clone leaf cuttings. *Sci. Rep.*, **7**(1):1-10.

- Mahendran, G. and Bai, V.N. (2014).** Micropropagation, antioxidant properties and phytochemical assessment of *Swertia corymbosa* (Griseb.) Wight ex CB Clarke: A medicinal plant. *Acta Physiol. Plan.*, **36**(3):589-603.
- Matthias, E. and Kliebenstein, D.J. (2020).** Plant secondary metabolites as defenses, regulators, and primary metabolites: the blurred functional trichotomy. *Plant Physiol.*, **184**:39-52.
- Mercier, H.; Souza, B.M.; Kraus, J.E.; Hamasaki, R.M. and Sotta, B. (2003).** Endogenous auxin and cytokinin contents associated with shoot formation in leaves of pineapple cultured *in vitro*. *Braz. J. Plant Physiol.*, **15**(2):107-112.
- Morton, A.G. (1981).** History of Botanical Science. An account of the development of botany from ancient times to the present day, Academic Press, London, pp:474.
- Navchoo, I.A.; and Buth, G.M. (1989).** Medicinal system of Ladakh, India. *J. Ethnopharmacol.*, **26**:137-146.
- Nielsen, E.; Temporit, M.E.E. and Cella, R. (2019).** Improvement of phytochemical production by plant cells and organ culture and by genetic engineering. *Plant Cell. Rep.*, **38**(10):1199-1215.
- Ogunmefun, O. T. (2018).** Phytochemicals, God's Endowment of Curative Power in Plants. In phytochemicals-source of antioxidants and role in disease prevention. *Intech. Open*, pp:8-23.
- Osuntokun, O.T. and Gamberini, M.C. (2019).** Bioguided isolation, chemical purification, identification, antimicrobial and synergistic efficacy of extracted essential oils from stem bark extract of *Spondias mombin* (Linn). *Int. J. Mol. Biol.*, **4**(4):135-138.
- Radha, R.K.; Shreena, S.R.; Divya, K.; Krishnan, P.N. and Seeni, S. (2011).** *In vitro* propagation of *Rubia cordifolia* Linn., a medicinal plant of the Western Ghats. *Int. J. Botany*, **7**(1):90-96.
- Ramakrishna, A. and Ravishankar, G.A. (2011).** Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signal Behav.*, **6**(11):1720-1731.
- Sharma, A.; Singh, T. and Vijayvergia, R. (2015).** GC-MS analysis of bioactive phytocomponents from *Rumex vesicarius*. *Int. Res. J. Pharm.*, **6**(9):269-3.
- Shekokar, A.V.; Borkar, K.M.; Pandao, P.O. and Londe, S.S. (2013).** A Case Study of Manjistadi Taila with Kadalipatra and Bactigauze for local application in the Management of Burn. *Int. J. Adv. Ayurveda, Yoga, Unani, Siddha and Homeopathy*, **2**:112-118.
- Singh, R.; Dar, S.A. and Sharma, P. (2012).** Antibacterial activity and toxicological evaluation of semi purified hexane extract of *Urtica dioica* leaves. *J. Med. Plant Res.*, **6**:123-135.
- Son, J.K.; Jung, S.J.; Jung, J.H.; Fang, Z.; Lee, C.S.; Seo, C.S. and Woo, M.H. (2008).** Anticancer constituents from the roots of *Rubia cordifolia* L. *Chem. Pharm. Bull.*, **2**:213-216.
- Sujatha, S.; Anand, S.; Sangeetha, K.N.; Shilpa, K.; Lakshmi, J.; Balakrishnan, A. and Lakshmi, B.S. (2010).** Biological evaluation of (3 β)-STIGMAST-5-EN-3-OL as potent antidiabetic agent in regulating glucose transport using *in vitro* model. *Int. J. Diabetes Mellit.*, **2**(2):101-109.
- Surh, Y.J. (2003).** Cancer chemoprevention with dietary phytochemicals. *Nat. Rev. Cancer*, **3**(10):768-780.
- Tsay, H. S. and Agrawal, D. C. (2005).** Tissue culture technology of Chinese medicinal plant resources in Taiwan and their sustainable utilization. *Int. J. App. Sci. Eng.*, **3**:215-223.
- Venugopal, S. N. (2002).** Simple formulations for primary health care uses based on ayurveda, foundation for revitalisation of local health traditions, Andra Pradesh Forest Department, pp:26-27.
- Warrier, P.K.; Nambiar, V.P.K. and Ganapathy, P.M. (2001).** Some important medicinal plants of the Western Ghats, India. A Profile, International Dev. Res. Cent., New Delhi, pp:329-342.
- Yadav, R. N. S. and Agarwala, M. (2011).** Phytochemical analysis of some medicinal plants. *J. Phytol.*, **3**(12):10-14.

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