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GC-MS Based Comparative Phytochemical Profiling of Rhodiola Imbricata Roots Collected from Different High Mountain Passes of Ladakh (India) and a First Report of Apocynin from Genus Rhodiola

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ABSTRACT

Rhodiola imbricata is a high value medicinal plant of trans-Himalayan mountain passes in Ladakh. This plant is a highly sought after in national and international herbal product market due to its unique phytochemical composition and resultant medicinal properties. However, compositional variation in the raw material from different geographical locations results in variation in quality as well as efficacy of the final products. The current study was designed to generate the comparative GC-MS profiles of hydro-methanolic extracts of *Rhodiola imbricata* root samples collected from various locations in Ladakh i.e., Chang La (17605 ft), Khardung La (18,379 ft), and Shashi La (13908 ft) mountain passes. The study highlighted variations in volatile phytochemical composition in root samples collected from different locations, especially with respect to phenols, terpenes and fatty acids. Samples from Chang La had maximum amount of phenolic compounds (96.78 %), followed by samples from Khardung La (77.05 %) while they were undetected in samples from Shashi La pass. Specifically, comparative GC-MS profiling revealed that peak area percentage of two important bioactive compounds (i.e. piceol and apocynin) varied amongst samples. In the samples collected from Chang La, the piceol covered (94 %) and apocynin covered (2.78 %) peak area whereas in samples collected from Khardung La, piceol covered (73.8 %) and apocynin covered (3.25 %) peak areas respectively, however, samples collected from Shashi La showed none of these compounds. Interestingly, in the present study, apocynin (a potent NADPH oxidase inhibitor) is being reported for the first time from *Rhodoila* genus. On the basis of present findings, samples collected from Chang La and Khardung La passes showed better phytochemical composition of pharmacological active phenolic compounds than Shashi La sample.

Keywords: Rhodiola imbricata; GC-MS; Piceol; Apocynin; Ladakh

1. INTRODUCTION

Owing to its extreme environmental conditions of trans-Himalayan region the cold dessert of Ladakh is known for its ethano-botanical wealth especially unique high value medicinal plants[1]. Ladakh, the northern most part of trans-Himalaya, is one of the largest cold deserts of India. It is a land of valleys and high mountain passes varying in altitudes, thus encompassing unique micro-climatic conditions that uniquely affect the phytochemicals contents of native medicinal plants. Among these high value medicinal plants, *Rhodoila imbricata* is an important medicinal herb also known as Himalayan stone crop or *golden arctic root* belongs to Crassulaceae family (stonecrops) with plethora of established medicinal properties. Most of the *Rhodoila* species are dispersed in cold area of northern hemisphere which includes Asia and Europe whereas, distribution of *Rhodoila imbricata* is only restricted to trans-Himalayan region of Leh-Ladakh in India[1,2]. Extracts of *R. imbricata* has been studied for many pharmacological activities like neurogenerative disorder, antioxidant, radioprotective, immunomodulatory, and antiproliferative due to the presence of bioactive constituents like phenylpropanids and phenylethanoids. The cultivation of *R. imbricata* has been very tough due to challenging environment or habitat, low seed viability and embryo abortion etc. besides this its average growing time is expected to be 5 to 7 years for the development of the active constituents[3-7]. Since, wild resources are the major source of raw material for *Rhodiola* based products, selection of quality raw material is imperative for optimum efficacy of the finished product. However, despite such extensive folk usage of this plant in Amchi medicinal system of Ladakh, there is scanty reports on comparative phytochemical composition of plants growing in different locations in the region.

Earlier reports of GC-MS chemo profiling of different extract (n-hexane, ethyl acetate, chloroform, methanol, ethanol and aqueous) of *R. imbricata* roots have revealed the presence of different phytochemicals like fatty ester,

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flavonoids, terpenoids, phytosterols, alkanes, alkylaldheydes etc[8]. Thus, the present study was undertaken to generate comparative GC-MS chemometric profiles of *R. imbricata* (a 50:50 hydromethanolic extract) from samples collected from three different locations in Ladakh (viz. Chang La, Khardung La and Shashi La passes). An interesting outcome of this study is that it is for the first time that a potent ROS down regulator compound[9], *i.e.* Apocynin, is being reported across genus *Rhodiola,* specifically from *R. imbricata* in the present study. Further, the study brings out variations in volatile photochemical compound compositions in samples collected from different locations in Ladakh region, emphasising the importance of site of sample collection as well as quality assurance markers for commercial product development.

2. METHODOLOGY

2.1 Sample Collection

Samples of *R. imbricata* plants were collected from different locations of Leh-Ladakh i.e., Shashi La- 34°33'53"N 76°23'29"E (4239 m=13908 ft), Chang La- 34°02'44"N 77°55'51"E (5366 m=17605 ft), Khardung La-34°16'38"N 77°36'14"E (5297.1192 m=17379 ft) respectively. A specimen sample (accession number: 351) was preserved in DIHAR-DRDO, Leh collected by Manoj Kumar Patel and Qasim further authenticated by Dr. OP Chaurasia (Director, DIHAR).

2.2 Preparation of Extracts

Briefly, the dried and powdered (5 gm) plant material was extracted with (50 per cent) methanol and (50 per cent) water with the help of orbital shaker by soaking in the solvent overnight at room temperature for 24 hrs to afford 455.2 mg (R.I.C), 460.9 mg (R.I.K) and 470 mg (R.I.S) of extracts with extractive value (9.1 per cent, 9.2 per cent and 9.4 per cent). Further, all three extracts (R.I.C, R.I.K, and R.I.S) were collected and dried with the help of rotary vapour under reduced pressure at 40°C. Further, the brown colored extracts were analysed for GC-MS analysis.

2.3 GC-MS Analysis

Different samples were analysed using Shimadzu (GC-2030) series GC-MS equipped with a Headspace (HS-20) and column SH-Rxi-5 SILMS (0.25 X 30 X 0.25). Helium was used as the carrier gas with flow rate of 1.69 ml/min. The column temperature was initially programmed at 50 ^{0}C held for 4 min then increased to 130 ^{0}C at the rate of 7 ^{0}C held for 4 min then increased to 240 ^{0}C at the rate of 7 ^{0}C held for 4 min, through split ratio (50:50) mode. Injector temperature was 250 ^{0}C, Ion source temperature was 220 ^{0}C & interface temp was 260 ^{0}C. Sample was diluted in ethyl acetate 10:100 v/v and 1 µl injected with a constant temperature of 250^{0}C through a auto sampler injector. The ionisation energy was 70 eV and mass range of 40–500 AMU. The management of the GC-MS system, parameter settings for GC and mass spectrometry, and data receipt and processing were performed using Shimadzu Real-time Analysis. The compounds were identified by using NIST library[10].

2.4. Statistical Analysis

Results have been represented in the form of mean ± standard deviation in Fig. 1(d). Data were analysed by using Graph Pad Prism 8 software. Statistical difference was calculated by the two-tailed unpaired t-test.

3. RESULTS

In general, variations were observed in phytocomponent compositions in *R. imbricata* samples collected from these different locations, viz. phenols (96.78 per cent) were the main volatile compound in samples from Chang La followed by terpenes (0.29 per cent) and fatty acids (0.25 per cent), samples from Khardung La showed phenols (77.05 per cent) and fatty acid/ester/alkane/ alkyl alchol (4.24 per cent) whereas in samples from Shashi La, alkanes (44.3 per cent) were the main phytocomponent followed by fatty acids/esters (15.9 per cent) etc. These samples were analysed using gas chromatography combined with mass spectrometry, which is a sensitive method for the analysis of volatile and semi-volatile phytochemicals. It provides information of percentage yield, molecular weight, and structures of the compound matched with NIST library.

A total of thirteen phyto-compounds (S.I. ≥ 80 per cent) were detected in the samples of *R. imbricata* collected from various locations in Ladakh and among these eleven compounds are being reporting for the first time (Table 1.). The phyto-compounds in different samples of *R. imbricata* were identified on the basis of molecular weight (MW), retention time (RT) and structures of the bioactive compounds. The top two major bioactive compounds present in the hydro-methanolic extracts of *R. imbricata* roots collected from Chang La were piceol or 4-hydroxyacetophenone (94 per cent) and apocynin (2.78 per cent) followed by alpha-altantone (0.29 per cent) and eicosanoic acid (0.25 per cent) with similarity index above (80 per cent), like wise in extracts of samples collected from Khardung La, the two top major bioactive compounds were piceol (73 per cent) and apocynin (3.25 per cent) followed by heneicosane (2.37 per cent), tridecanol, 2-ethyl- 2-methyl (0.64 per cent), phytone (0.62 per cent), n-hexadeconoic acid (0.61 per cent), where as in the extract of samples collected from Shashi La, only fatty acid, ester, alcohol and aldehydes have been detected in acceptable percentange i.e., ocatcosane (30.4 per cent), heneicosane (12.54 per cent), eicosyl acetate (10.9 per cent), n-hexadeconoic acid (3.21 per cent), tetracosanal (5.14 per cent), ethanol, 2-(9,12-octadecadienyloxy) (1.84 per cent), hexacosane (1.45 per cent). The GC-MS chromatograms of the three extracts shown in Fig. 1 (a-c) with the retention time in the column followed by detected peaks which correspond to the bioactive compounds present in the extract. Statistical difference in percent peak areas of two

compounds (piceol/4-OH-AP and apocynin) in samples from different valleys was calculated. Difference was considered to be significant at p-value ≤0.001*. It was observed that the concentration of compound 4-OH-AP was significantly different in plant samples from Chang La and Khardung La passes.

(Annexure 1) Chemical composition of the volatile compounds from R.I.C (1), R.I.K (2) and R.I.S (3)

[a]R.I.C : R. *imbricata* Chang La extract; R.I.K: R. *imbricata* Khardung La extract; R.I.S: R. *imbricata* Shashila extract; [b]Reported for the first time; R.I: Retention index: R.T: retention time; S.I: Similarity Index; M.F: Molecular formula; M.W: Molecular weight

4. DISCUSSION

Rhodoila imbricata is one of the important and unique medicinal herbs of trans-Himalayan region and has been used in Amchi medicinal system as key ingredient of folk herbal formulations since long. A number of therapeutic properties has been shown by R. *imbricta* due to presence of a class of bioactive phyto-compounds like, phenylpropanoids and phenylethanol derivatives. Present study gives a comparative account of various phyto-chemicals identified by GC-MS analysis in hydro-methanolic extract of roots of *R. imbricata* plant samples collected from three different locations in Ladakh (Chang La, Khardung La and Shashi La). Results revealed variation in composition as well as concentration of some of the phytochemicals in samples from different locations. The compounds being reported have exhibited various biological activities in earlier studies by other researchers. Piceol (4-OH-AP) has been reported to posses antimycobacterial activities as well as a key anti-hepatitis B virus effect followed by some anti-inflammatory effect. It has also showed the hepatoprotective and choleretic activity in *Artemisia capillaris* and *A. morrisonensis*[11,12]. In earlier reports, piceol or 4-hydroxyacetophenone has been reported from methanol extract of *R. imbricata* but with very less percentage (11.07 per cent)[8]. Apocynin, which is an established plant-derived drug and a potent selective inhibitor of NADPH oxidase-dependent production of ROS has shown efficacy against neurogenerative disorder, ischemia and reperfusion diseases, asthma, etc. in earlier studies[13-16]. Most interestingly, till date, acetovanillone or apocynin has not been identified in any species of the genus Rhodiola. Thus, to best of our knowledge this is the first report of its presence in genus Rhodiola and specifically in *R. imbricata* plants growing in Chang La and Khardung La passes of Ladakh in India. Another phytocompound, (E)-altanone, belongs to terpenoids class of compounds and is responsible for antifungal activities in various essential oils[17-19]. Similarly, all the fatty acids,

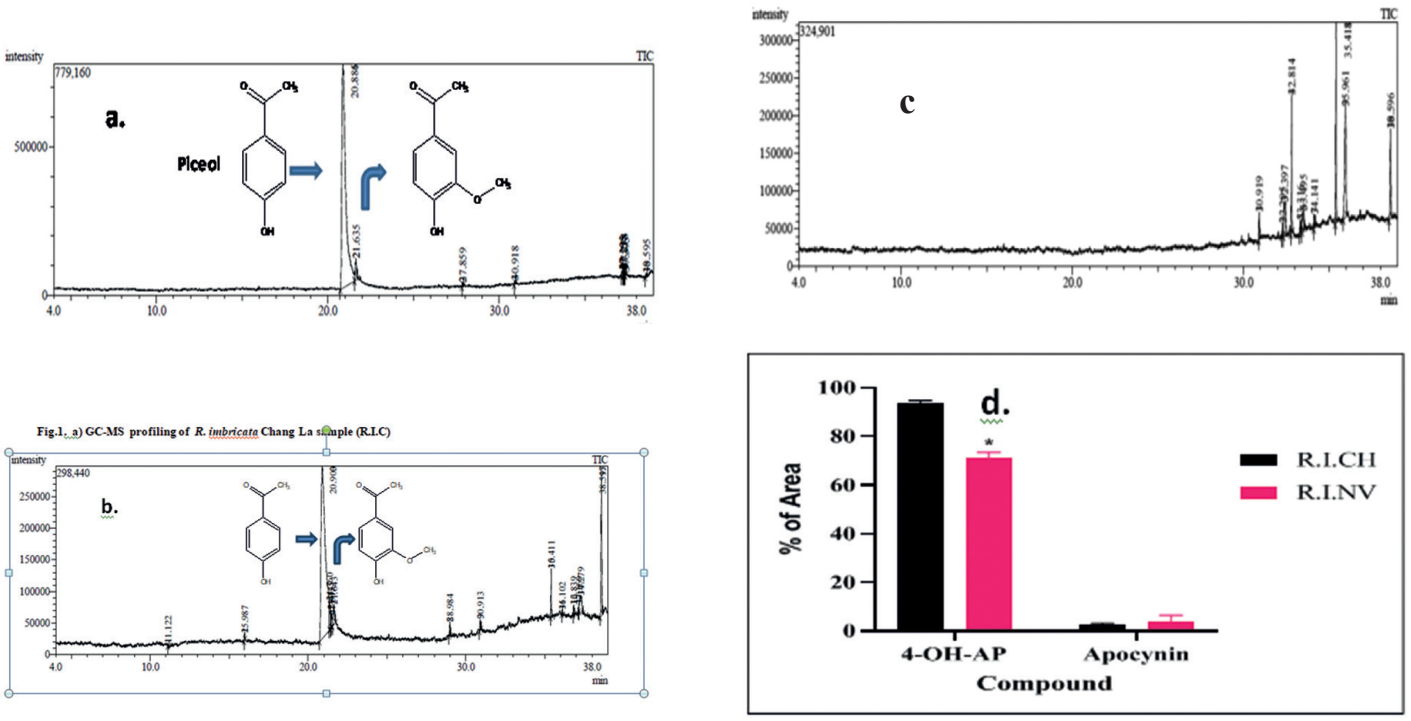

Figure 1. **a) GC-MS profiling of *R. imbricata* Chang La sample (R.I.C) R.**
b) GC-MS profiling of *R. imbricata* Khardung La sample (R.I.K)
c) GC-MS profiling of *R. imbricate* Shashi La sample (R.I.S)
d) Comparative profile of 4-Hydroxyacetophenone and Apocynin peak areas in R.I.C and R.I.K based on statistical analysis.

ester, aldheydes and alcohol (phyton, n-hexadecanoic acid, heneicosane, Tridecanol, 2- ethyl- 2-methyl-, eicosyl acetate, hexacosane, octacosane, tetracosanal and ethanol, 2(9,12-octadecadienyloxy) have been reported to posses antimicrobial activity, antioxidant and other activities (Annexture 1).

5. CONCLUSION

Thus, it can be concluded that the composition and concentration of the bioactive phytocomponents being reported in samples of *R. imbricata* is uniquely regulated by plant location and micro-environment. Literature stated that natural compounds carrying phenols moiety has always been a great pharmacological important candidate for various diseases, so here Chang La sample has shown the maximum percentage of these phenolic or actephenones compounds as compared to other sample which can be considered a good sources of quality raw materials for food and pharmaceutical industries. A novel finding apocynin in the tested samples is an interesting observation via a GC-MS analysis and further validates the anti-oxidant potency of roots of *R. imbricata* observed in several earlier studies. It is being proposed that, considering the biological efficacies pharmaceutical merits of 4-hydroxyl acteophenone and acetovanillone (apocynin), these compounds may serve as potential phytochemical marker substances for quality control of *R. imbricata* raw material collected from various resources. The study also paves way for researchers to investigate the presence of these important phytochemicals in other species of genus Rhodiola.

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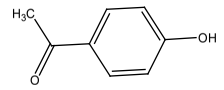
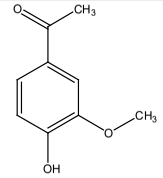
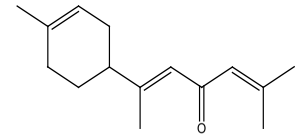
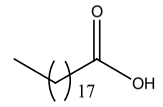
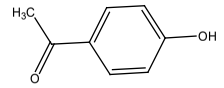
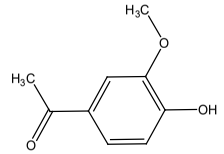
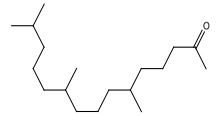
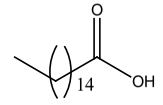
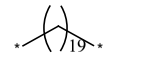
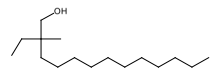
She has drafted the manuscript along with critically reviewing the contents.

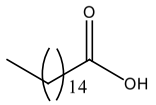
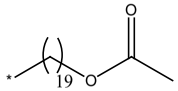
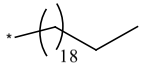
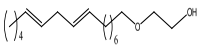
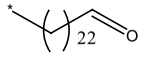
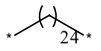
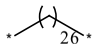
Annexure I

Chemical composition of the volatile compounds from R.I.C (1), R.I.K (2) and R.I.S (3)

Ext	Comp	R.I	R.T	Area per cent	S.I	M.F	M.W	Nature of compound	Biological activities	Structures
	Piceol	1250	20.88	94.0	97	$C_8H_8O_2$	136	Phenol	Antimycobacterial[11] antioxidative, antinociceptive, and anti-inflammatory effects[12]	
1	Apocynin[b]	1439	21.63	2.78	94	$C_9H_{10}O_3$	166.17	Phenol	Immunomodulatory[13]neurogenerative disorder [14], cardioprotective[15], anti-inflammatory[16], anti-asthmatic [16]	
	(E)-Altantone[b]	1654	27.85	0.29	86	$C_{15}H_{22}O_3$	218.33	sesquiterpenes	Antifungal[17,19], Antioxidant[18]	
	Eicosanoic acid[b]	2366	30.91	0.25	75	$C_{20}H_{40}O_2$	312.5	Fatty acid	Antifungal[20]	
	Piceol	1250	20.90	73.80	97	$C_8H_8O_2$	136	Phenol	See above	
	Apocynin[b]	1439	21.6	3.25	91	$C_9H_{10}O_3$	166	Phenol	See above	
2	Phytone[b]	1754	28.9	0.62	90	$C_{18}H_{36}O$	268.5	Fatty ester	Antibacterial[21]	
	N-Hexadecanoic acid	1968	30.9	0.61	87	$C_{16}H_{32}O_2$	256.4	Fatty acid	Anti-inflammatory`[22], Antioxidant, nematicide, pesticide, anti androgenic flavor hemolytic hypocholesterolemic, 5-Alpha reductase Inhibitor[23]	
	Heneicosane[b]	2109	35.4	2.37	95	$C_{21}H_{44}$	296.6	Alkane	Antimicrobial [24]	
	Tridecanol,2-ethyl-2-methyl[b]	1770	37.1	0.64	85	$C_{16}H_{34}O$	242.44	Alkyl alcohol	Antimicrobial[25]	

Annexure I

Chemical composition of the volatile compounds from R.I.C (1), R.I.K (2) and R.I.S (3)

Ext	Comp	R.I	R.T	Area per cent	S.I	M.F	M.W	Nature of Compound	Biological Activities	Structures
	N-Hexadecanoic acid	1968	30.9	3.2	93	$C_{16}H_{32}O_2$	256.4	Fatty acid	See above	
	Eicosyl acetate[b]	2375	32.3	10.9	92	$C_{22}H_{44}O_2$	340.6	Fatty esters	Mosquito repellent [26], antidiabetic, anticancer [27]	
	Heneicosane[b]	2109	32.8	12.5	97	$C_{21}H_{44}$	296.6	Alkane	See above	
3	2-(9,12-octadecadieny loxy - Ethanol[b]	2344	33.3	1.84	87	$C_{22}H_{38}O_2$	310.5	Alkyl alcohol	Antibacterial [28]	
	Tetracosanal[b]	2595	33.4	5.1	81	$C_{24}H_{48}O$	352.6	Alkyl ester	Antibacterial[29]	
	Hexacosane[b]	2606	34.1	1.4	89	$C_{26}H_{54}$	366.7	Alkane	Antimicrobial[30]	
	Octacosane [b]	2804	35.9	30.4	96	$C_{28}H_{58}$	394.8	Alkane	Antimicrobial and antioxidant[31]	

[a] **R.I.C : *R. imbricata* Chang La extract; R.I.K: *R. imbricata* Khardung La extract; R.I.S: *R. imbricata* Shashila extract;** [b] **Reported for the first time; R.I: Retention index: R.T: retention time; S.I: Similarity Index; M.F: Molecular formula; M.W: Molecular weight;**

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