PHYTOCHEMICAL CHARACTERIZATION AND PHARMACOLOGICAL EVALUATION OF *RHODIOLA IMBRICATA* EDGEW. ROOT FROM TRANS-HIMALAYAN COLD DESERT REGION OF LADAKH, INDIA

By

AMOL BAPURAO TAYADE

A THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR

THE DEGREE OF DOCTOR OF PHILOSOPHY

IN

PHARMACEUTICAL CHEMISTRY

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY
WAKNAGHAT

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CERTIFICATE

This is to certify that the thesis entitled, **"Phytochemical characterization and pharmacological evaluation of *Rhodiola imbricata* Edgew. root from trans-Himalayan cold desert region of Ladakh, India"** which is being submitted by **Amol Bapurao Tayade** in fulfillment for the award of degree of Doctor of Philosophy in **Pharmaceutical chemistry** by the Jaypee University of Information Technology, is the record of candidate's own work carried out by him under our supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

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DECLARATION

I certify that

a. The work contained in this thesis is original and has been done by me under the guidance of my supervisor.
b. The work has not been submitted to any other organization for any degree or diploma.
c. Whenever, I have used materials (data, analysis, figures or text), I have given due credit by citing them in the text of the thesis.

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(Amol Bapurao Tayade)

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LIST OF ABBREVIATIONS

Abbreviations	Full form
% RSD	Percent Relative standard deviation
DPPH	1,1-Diphenyl-2-picrylhydrazyl radical
ABTS	2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
TPTZ	2,4,6-Tripyridyl-*s*-triazine
DCFHDA	2′,7′-Dichlorofluorescein diacetate
FMOC-Cl	9-fluorenylmethyl-chloroformate
ANOVA	Analysis of variance
ARRIVE	Animal Research: Reporting *In Vivo* Experiments
ARP	Antiradical power
C20:0	Arachidic acid methyl ester
C20:4n6	Arachidonic acid methyl ester
AA	Ascorbic acid
ALS	Automatic Liquid Sampler
C22:0	Behenic acid methyl ester
BDL	Below detection limit
B_7	Biotin
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
C4:0	Butyric acid methyl ester
CE	Capillary electrophoresis
CE-MS	Capillary electrophoresis-mass spectroscopy
C10:0	Capric acid methyl ester
C6:0	Caproic acid methyl ester
C8:0	Caprylic acid methyl ester
CAS Number	Chemical Abstracts Service No
D_3	Cholecalciferol
C17:1	*cis*-10-Heptadecenoic acid methyl ester
C15:1	*cis*-10-Pentadecenoic acid methyl ester
C20:3n3	*cis*-11,14,17-Eicosatrienoic acid methyl ester

Abbreviations	Full form
C20:2	*cis*-11,14-Eicosadienoic acid methyl ester
C20:1	*cis*-11-Eicosenoic acid methyl ester
C22:2	*cis*-13,16-Docosadienoic acid methyl ester
C22:6n3	*cis*-4,7,10,13,16,19-Docosahexaenoic acid methyl ester
C20:5n3	*cis*-5,8,11,14,17-Eicosapentaenoic acid methyl ester
C20:3n6	*cis*-8,11,14-Eicosatrienoic acid methyl ester
CoA	Coenzyme A
C-H-R	Cold, hypoxia and restraint
CID	Collision-induced decomposition
CAM	Complementary alternative medicine
B_{12}	Cyanocobalamin
DIPAS	Defence Institute of Physiology & Allied Sciences
DI	Deionised
DAD	Diode array detector
dw	Dry weight
DB	Durabond
C18:1n9t	Elaidic acid methyl ester
EI	Electron impact OR Electron ionization
EI^+	Electron ionisation positive
ESI	Electrospray ionization
EPA	Environmental Protection Agency
D_2	Ergocalciferol
C22:1n9	Erucic acid methyl ester
FID	Fame ionization detection
FAME	Fatty acid methyl ester
FRAP	Ferric reducing antioxidant power
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
B_9	Folic acid
fw	Fresh weight
GAE	Gallic acid equivalent

Abbreviations	Full form
GC	Gas chromatography
GC-FID	Gas chromatography coupled with flame ionization detector
GC/MS	Gas chromatography mass spectrometry
GC/MS MS	Gas chromatography tandem mass spectrometry
EC_{50}	Half maximal efficiency concentration
IC_{50}	Half maximal inhibitory concentration
C21:0	Heneicosanoic acid methyl ester
C17:0	Heptadecanoic acid methyl ester
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
HILIC	Hydrophilic interaction liquid chromatography
H+D	Hypoxia + drug treated group
H+V	Hypoxia + vehicle group
ICP-OES	Inductively coupled plasma optical emission spectrometer
IAEC	Institutional Animal Ethical Committee
IUPAC	International Union of Pure and Applied Chemistry
Abu	L-2-amino-n-butyric acid
Ala	L-Alanine
Arg	L-Arginine
Asp	L-Aspartic Acid
C12:0	Lauric acid methyl ester
Cys	L-Cystine
Cys HCl	L-Cystine HCL
Glu	L-Glutamic Acid
GSSG	L-glutathione oxidized
GSH	L-glutathione reduced
Gly	L-Glycine
His	L-Histidine
C24:0	Lignoceric acid methyl ester
LOD	Limits of detection
LOQ	Limits of quantification
C18:2n6c	Linoleic acid methyl ester

Abbreviations	Full form
C18:2n6t	Linolelaidic acid methyl ester
LC	Liquid chromatography
LC/MS	Liquid chromatography mass spectrometry
LC/MS MS	Liquid chromatography tandem mass spectrometry
Ile	L-Isoleucine
Leu	L-Leucine
Lys	L-Lysine
Met	L-Methionine
Nor Leu	L-Nor Leucine
Orn	L-Ornithine
Phe	L-Phenylalanine
Pro	L-Proline
Ser	L-Serine
Thr	L-Threonine
Trp	L-Tryptophan
Val	L-Valine
MS	Mass spectrometry
m/*z*	mass-to-charge
Mpa	Megapascal
K_2	Menaquinone
MDL	Minimum detection limit
Mol. Formula	Molecular Formula
Mol. Wt.	Molecular Weight
MUFA	Monounsaturated fatty acid
MRM	Multiple Reaction Monitoring
C14:0	Myristic acid methyl ester
C14:1	Myristoleic acid methyl ester
Ng	Nanogram
NAAS	National Academy of Agricultural Sciences
NIST	National Institute of Standards and Technology
NIST MS	National Institute of Standards and Technology Mass spectrometry

Abbreviations	Full form
NIST/EPA/NIH Mass Spectral Database	National Institute of Standards and Technology/Environmental Protection Agency/ National Institutes of Health Mass Spectral Database
NIH	National Institutes of Health
C24:1	Nervonic acid methyl ester
B_3	Niacin
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
N+V	Normoxia + vehicle
N+D	Normoxia + drug treated group
ND	Not determined or Not detectable
C18:1n9c	Oleic acid methyl ester
OPA	*o*-phthalaldehyde
C16:0	Palmitic acid methyl ester
C16:1	Palmitoleic acid methyl ester
B_5	Pantothenic acid
PBS	Phosphate buffer saline
C15:0	Pentadecanoic acid methyl ester
PITC	Phenylisothiocyanate
H_3PO_4	Phosphoric acid
K_1	Phylloquinone
PUFA	Polyunsaturated fatty acid
$K_3Fe(CN)_6$	Potassium ferricyanide
PPS	Potassium persulfate
$K_2S_2O_8$	Potassium persulphate
B_6	Pyridoxine
QE	Quercetin equivalent
RRLC	Rapid Resolution Liquid Chromatography
RRLC/MS	Rapid Resolution Liquid Chromatography mass spectrometry
ROS	Reactive oxygen species
RSD	Relative standard deviation

Abbreviations	Full form
RT	Retention time
A	Retinol
RP	Reversed phase
RP-HPLC	Reversed phase high performance liquid chromatography
B_2	Riboflavin
SFA	Saturated fatty acid
Na_2HCO_3	Sodium bicarbonate
Na_2HPO_4	Sodium hydrogen phosphate
SD	Standard deviation
SPSS	Statistical Program for Social Sciences
C18:0	Stearic acid methyl ester
TCC	Thermostated Column Compartment
B_1	Thiamine
TTP	Thiamine pyrophosphate
TLC	Thin layer chromatography
E	Tocopherol
C23:0	Tricosanoic acid methyl ester
C13:0	Tridecanoic acid methyl ester
TEA	Triethylamine
QQQ	Triple Quadrupole
QqQ	Triple quadrupole mass spectrometer
UPLC	Ultra-performance liquid chromatography
C11:0	Undecanoic acid methyl ester
UFA	Unsaturated fatty acid
w/v	Weight by volume
C18:3n3	α-Linolenic acid methyl ester
C18:3n6	γ-Linolenic acid methyl ester

ABSTRACT

Rhodiola imbricata Edgew., belonging to the family *Crassulaceae*, is an important and widely used food crop and traditional medicinal plant distributed in trans-Himalayan cold desert regions in India, Pakistan, Tibet, China, Nepal and many other countries. In traditional medicinal system as well as in various *in vivo* and *in vitro* studies, its pharmacological potentials are well documented in different root extracts of the plant. However, the active principle such as volatile, semi-volatile and polar compounds in these extracts still remains unidentified. Moreover, the root of the plant is used as an adaptogen but it's used as to increase physical endurance remains untested. Beside these, plant is widely used as edible plant, but its nutritional quality with respect to its vitamins, fatty acids, amino acids, and mineral content still remained unexplored except the species was tested for the heavy metals such as As, Pb, Hg, Cd, Zn, Cu, and Cr. The volatile, semi-volatile, polar compounds, nutritional quality (amino acid, vitamin, minerals and fatty acid) along with physical endurance test need to be analysed to ascertain the bioactivity and pharmacological potential of the plant.

In light of the aforementioned ongoing research and present research gaps in *R. imbricata* this work was carried out to ascertain *in vitro* antioxidant radical scavenging capacities such as DDPH radical scavenging capacity, ABTS radical scavenging capacity, FRAP radical scavenging capacity, and reducing power capacities in methanolic and aqueous root extracts, their correlation among the content of total phenolic compounds such as total polyphenols, phenolic acids, flavanoids and flavonols; to optimize RRLC-MS/MS technique for the sequential determination of fat- and water-soluble vitamins in *R. imbricata* root with liquid chromatography/tandem mass spectrometry; to determine fatty acid composition in root using GC-FID technique.; to study free amino acid profiling in root using RP-HPLC; and to determine distribution of phyto-constituents in various root extracts with hyphenated gas chromatography mass spectrometric technique; Pharmacological evaluation (Physical endurance) of hydro-alcoholic (60% ethanolic,v/v) root extract on rats following exposure to hypobaric hypoxia.

Phytochemical profile of the n-hexane, dichloroethane, chloroform, ethyl acetate, methanol, and 60% ethanol root extracts of *R. imbricata* were performed by hyphenated gas chromatography mass spectrometry (GC/MS) technique. Phytochemical profile of root

extracts revealed the presence of 63 phyto-constituents, among them, 1-pentacosanol; stigmast-5-en-3-ol, (3*β*,24*S*); 1-teracosanol; 1-henteracontanol; 17-pentatriacontene; 13-tetradecen-1-ol acetate; methyl tri-butyl ammonium chloride; bis(2-ethylhexyl) phthalate; 7,8-dimethylbenzocyclooctene; ethyl linoleate; 3-methoxy-5-methylphenol; hexadecanoic acid; camphor; 1,3-dimethoxybenzene; thujone; 1,3-benzenediol, 5-pentadecyl; benzenemethanol, 3-hydroxy, 5-methoxy; cholest-4-ene-3,6-dione; dodecanoic acid, 3-hydroxy; octadecane, 1-chloro; ethanone, 1-(4-hydroxyphenyl); *α*-tocopherol; ascaridole; campesterol; 1-dotriacontane; heptadecane, 9-hexyl were found to be present in major amount. Eventually, in the present study we have found phytosterols, terpenoids, fatty acids, fatty acid esters, alkyl halides, phenols, alcohols, ethers, alkanes, and alkenes as the major group of phyto-constituents in the different root extracts of *R. imbricata.* All these compounds identified by GC/MS analysis were further investigated for their biological activities and it was found that they possess a diverse range of positive pharmacological actions. In future, isolation of individual phyto-constituents and subjecting them to biological activity will definitely prove fruitful results in designing a novel drug.

Study of antioxidant capacities and phenolic constituents of methanol and aqueous extracts of *R. imbricata* Edgew. root from Trans-Himalayan cold desert of Ladakh showed that DPPH and ABTS radical scavenging capacity of the root extracts increased in a dose dependent manner (upto 0.1 mg/ml) and root extract concentrations required for 50% inhibition of radical scavenging effect (IC_{50}) were recorded as 0.013 and 0.014 mg/ml (for DPPH) and 0.016 and 0.017 mg/ml (for ABTS) for methanol and aqueous extracts respectively. The total antioxidant power of the extract was determined by FRAP assay. Total polyphenol and phenolic acid content of methanol and aqueous extracts were 112.24, 59.06, 39.02, and 16.95 mg GAE/g of extract respectively. Total flavonoid and flavonol contents were estimated to be 30.2, 17.67, 20.68, and 7.38 mg QE/g of extract respectively. In all antioxidant capacity assays, the methanol extract exhibited significantly higher antioxidant capacity than that of aqueous extract due to the presence of significantly higher amount of vital phyto-constituents, *viz.* polyphenol, phenolic acid and flavonol. GC/MS analysis showed that phytosterols, alkyl halide, phenols and fatty acid esters were major phytochemical cluster. On the other hand, monoterpenes, fatty acids, tocopherols, aliphatic hydrocarbons and ethers were found to present in comparatively less amount in the methanol extract. Hence, our study signifies that this high-altitude medicinal herb could be used as the natural source

of antioxidants and supports its use in traditional system of medicine to ameliorate oxidative stress and high altitude maladies.

A rapid method was developed to determine both types of vitamins in *R. imbricata* root for the accurate quantification of free vitamin forms. Rapid resolution liquid chromatography/tandem mass spectrometry (RRLC-MS/MS) with electrospray ionization (ESI) source operating in multiple reactions monitoring (MRM) mode was optimized for the sequential analysis of nine water-soluble vitamins (B_1, B_2, two B_3 vitamins, B_5, B_6, B_7, B_9, B_{12}) and six fat-soluble vitamins (A, E, D_2, D_3, K_1, K_2). Both types of vitamins were separated by ion-suppression reversed-phase liquid chromatography with gradient elution within 30 min and detected in positive ion mode. Deviations in the intra- and inter-day precision were always below 0.6% and 0.3% for recoveries and retention time. Intra- and inter-day RSD values of retention time for water- and fat-soluble vitamin were ranged between 0.02-0.20% and 0.01-0.15%, respectively. The mean recoveries were ranged between 88.95-107.07%. Sensitivity and specificity of this method allowed the limits of detection (LOD) and limits of quantitation (LOQ) of the analytes at ppb levels. The linearity was achieved for fat- and water-soluble vitamins at 100-1000 ppb and 10-100 ppb. Vitamin B-complex and vitamin E were detected as the principle vitamins in the root of this adaptogen which would be of great interest to develop novel foods from the Indian trans-Himalaya.

Quantification of fatty acids from root sample of *R. imbricata* using gas chromatography coupled with flame ionization detector (GC-FID) with fatty acid methyl esters (FAMEs) derivatization was studied. Data on the fatty acid profile of *R. imbricata* root is very scarce. The present study revealed the presence of 10 fatty acids in the plant root. The total lipid was found to be a rich source of saturated fatty acids such as capric acid (19.98%), caproic acid (10.85%), palmitic acid (9.37%), lignoceric acid (6.17%), and behenic acid (5.67%), which together constituted ~52% of the total lipid. Among the unsaturated fatty acids (UFAs), linoleic acid (15.04%), oleic acid (12.33%), arachidonic acid (8.39%), linolelaidic acid (6.17%), and docosadienoic acid (6.04%) were prominent. MUFA and PUFA were 35.64% and 12.33% of the total lipid content respectively in the root sample. The study represents the first report of quantification of fatty acids from roots of *R. imbricata*.

Reversed phase high performance liquid chromatography (RP-HPLC) with precolumn derivatization is a reliable and reproducible technique for the identification and quantification of amino acids in biological matrices. Amino acid content of *R. imbricata* root still remains to

be studied. We quantified amino acids content in *R. imbricata* root by RP-HPLC with precolumn phenylisothiocyanate (PITC) derivatization. Nine essential and twelve non essential amino acids were quantified and the contents were ranged between 214.67 and 1640.67 µg/g. Among the essential amino acids, histidine (1434.33 µg/g) and lysine (1329.33 µg/g) were found to be dominant, while glycine (1640.67 µg/g), proline (1263.67 µg/g), alanine (1142.33 µg/g), cystine HCl (239.33 µg/g) and leusine (928.67 µg/g) were major non essential amino acids. This is the first ever study on the amino acid profiling of *R. imbricata* root by RP-HPLC that validates its potential as a good source of natural amino acids.

Data on the mineral profile of *R. imbricata* root is inadequate. The mineral elements were determined using an inductively coupled plasma optical emission spectrometer (ICP-OES). *R. imbricata* root mineral composition analysis showed the presence of various minerals. Heavy metal load in the root sample was below the maximum permissible limit as set by WHO. Thus *R. imbricata* root was found to be safe for human consumption.

The pharmacological evaluation of the *R. imbricata* root extract on rats following exposure to hypobaric hypoxia showed increased body weight, food intake and physical endurance. It also found to upregulate the production of endogenous antioxidant enzymes and thereby showing positive pharmacological potential in minimizing oxidative stress due to the presence of antioxidants which were previously confirmed.

DEDICATED

To

my beloved parents,

mother Sau. Tai Bapurao Tayade and

father Bapurao Dauji Tayade,

Who always picked me up on time and

motivate me to go on every adventure,

especially this one

through their endless love, support,

encouragement and patience.

CHAPTER 1

INTRODUCTION

Abstract

Rhodiola imbricata Edgew., belonging to the family *Crassulaceae*, is an important and widely used food crop and traditional medicinal plant distributed in trans-Himalayan cold desert regions in India, Pakistan, Tibet, China, Nepal and many other countries. In Amchi and Tibetian system of traditional medicine, the roots are used against lung problems, cold, cough, fever, loss of energy and pulmonary complaints. Review of literature revealed that the extracts, particularly those from roots/rhizomes aqueous, alcoholic and hydro-alcoholic extracts of the plant were extensively studied and found to possess useful pharmacological activities such as immuno-stimulatory, anticancer, cytoprotective, radioprotective, antioxidant, free radical scavenging, metal chelating, anti-lipid peroxidation, anti-hemolytic activity, dermal wound healing activity, anti-hypoxic, anti-inflammatory and antitumour activity. Besides, the root of the plant is used as an adaptogen but it's efficacy in increasing physical endurance remains untested. Although, various *in vivo* and *in vitro* pharmacological potentials are well documented in different root extracts of the plant, the active principle such as volatile, semi-volatile and polar compounds in these extracts still remain unidentified. Besides, the plant is widely used as an edible plant, but its nutritional quality with respect to its vitamins, fatty acids, amino acids, and mineral content still remain unexplored, the species having been tested only for the heavy metals such as As, Pb, Hg, Cd, Zn, Cu, and Cr. Thus the volatile, semi-volatile, polar compounds, nutritional quality need to be analysed to ascertain the bioactivity and pharmacological potential of the plant.

1.1 Origin and distribution of *Rhodiola* species

The stone crop family, Crassulaceae consists of over 1400 species distributed in 33 genera including *Rhodiola* (Rose root, Golden root or Arctic root) and is commonly distributed worldwide especially in the Northern Hemisphere and South Africa (Gupta et al., 2007). The descriptions, distribution and collection sites of the various species of the family are available in different literatures (Kumar et al., 2007). The species of this family grows in drained soil at an altitude of 2700-5000 m and has been known for long to possess immense medicinal potential (Mishra et al., 2007). The Rhodiola, consisting of about 90 species, are widely distributed in the high altitude cold desert region of the Northern Hemisphere (Yidong Lei, et al., 2003). In China, there are 73 species, mainly growing in the Qinghai-Tibet Platean (Fu and Fu, 1984). Many *Rhodiola* species have been used in traditional medicines for the treatment of long term illness and weakness due to infection in Tibet and other regions for over 1000 years (Xiong, 1995; Rohloff, 2002).

In India, there are six species of *Rhodiola*, namely *R. heterodonta, R. imbricata, R. quadrifida, R. sinuate, R. tibetica,* and *R. wallichiana* (Chaurasia and Gurmet, 2003). *Rhodiola imbricata* Edgew. previously known as *Sedum roseum* (Linn.) Scop., belonging to the plant family Crassulaceae. It is a succulent perennial herb locally known as rose root due to the rose-like attar (fragrance) of the fresh cut rootstock (Chaurasia et al., 2007). In India it is commonly known as Rose root, Golden root, Arctic root, Shrolo, Stone crop and/or Himalayan stone crop (Chaurasia et al., 2007; Chaurasia and Singh, 1996). Roots and rhizomes are the used parts of the plant. The root of *R. imbricata* has been getting a lot of attention lately. *R. imbricata* is a cold tolerant, medicinal plant with adaptogenic properties.

1.2 *Rhodiola imbricata* Edgew.

1.2.1 Origin and distribution of *Rhodiola imbricata*

R. imbricata Edgew. [(Synonyms: *Rhodiola imbricatum* Edgew.; *Sedum imbricatum* Walp.; *Sedum imbricatum* Hook.f. & Thomson; *Sedum rhodiola* auct. Non DC.; *Sedum roseum*); (Common names: Golden root, rose root, arctic root or Shrolo)] is a dioecious, herbaceous perennial plant, originating in the mountain regions of South West China and native around whole of the Northern hemisphere (Chaurasia et al., 2007; Chaurasia and Singh, 1996). It is a slow growing, perennial garden plant that extends from the Pakistan, India, Nepal to China. In India, the plant grows wildly primarily on rocky slopes, wet places, higher passes at high altitudes (14000-18500 ft above mean sea level) in the trans-Himalayan cold desert and in high Arctic latitudes and mountain regions of Eurasia (Khanum et al., 2005). It is commonly found in Indus and Leh valley of Indian trans-Himalaya (Chaurasia et al., 2007; Chaurasia and Singh, 1996).

1.2.2 Taxonomy and botany of *Rhodiola imbricata*

Around, 80% of the inhabitants in developing countries depend mostly on traditional medicine for health care needs, of which a bulk portion involves the utilization of plant extracts or their bioactive principles. One of the disparagements of herbal medicine is requirement of standardization and quality control profiles for the accurate recognition of the species concerned, whether in the fresh, dried or powdered state (Springfield et al, 2005). The misclassification of species and the erroneous changeover is a real threat in the preparation and administration of herbal medicine (Opara, 2004). Some herbs are so strikingly similar in appearance to the untrained eye that they are normally mistaken for one another. The misclassification of species and the mistaken substitution of herbs have also given rise to serious adverse effects (Chan and

Critchley, 1996). Thus, minute examination of medicinal plant materials is essential for the correct recognition of fresh, broken or powdered materials (WHO, 1998). Therefore, the taxonomic and botanical classification and recognition for the accurate species from its natural habitat of *R. imbricata* the information below is quite useful.

Figure 1.1 The plant *Rhodiola imbricata* from trans-Himalayan cold desert of Ladakh region, India

R. imbricata (Figure 1.1) is a succulent herb with a thick rhizome, golden outside, pink inside, 10-35 cm with rose scented massive rootstock; leaves 1.3-3 cm long, oblong to narrow

elliptic, nearly entire; flowers pale yellow in congested cluster, surrounded by an involucres of leaves; fruits are 4-5, many seeded. Flowering and fruiting occurs in July-September (Chaurasia et al., 2007; Chaurasia and Singh, 1996). Classification of *R. imbricata* is given below.

Kingdom	Plantae
Phylum	Magnoliophyta
Class	Magnoliopsida
Order	Rosales
Family	Crassulaceae
Genus	*Rhodiola*
Species	*imbricata* EDGEW

1.2.3 Cultivation and propagation of *Rhodiola imbricata*

According to cultivation experiences, Rhodiola sp. can be successfully grown for its root yield in South Finland using organic growing methods (Galambosi et al, 1999). For seedling production, seeds have to be sown in autumn, for natural winter stratification. The seedlings should be kept in pots for one year before transplantation, since the growth of the plants during the first 2-3 years is quite slow. The first root yield is harvested after four years from sowing and the root weight and root yield strongly depends on age of the plant.

Cultivation and propagation practices indicate that *R. imbricata* plant is propagated through seeds as well as rootstocks cuttings. 65% of seed germination was achieved in field conditions while in case of rootstocks plantation 86% of the survival rate has been achieved. Hence, it was found to be easily propagated through division of rootstocks and 3-4 years plants

are ideal for suitable rootstocks plantation. Three-five years old plants are considered ideal for rootstocks plantation (Chaurasia et al., 2007).

1.2.4 Phytochemistry of *Rhodiola imbricata*

Analysis of different species of genus Rhodiola, chemical composition revealed six groups of active principles (Khanum et al., 2005). Phenylpropanoids: rosavin, rosin, rosarin. The name rosavin includes these three. Phenylethanol derivatives: rhodioloside (salidroside, rhodosin), tyrosol. Flavonoids: rhodiolin, rhodionin, rhodiosin, acetylrhodalgin, tricin. Monoterpenes: rosiridol. Triterpenes: daucosterol, β-sitosterol. Phenol acids: chlorogenic, hydroxycinnamic and gallic acids. The presence of triandrine, p-coumaric alcohol and its glucosides (vimalin), p-cumaric acid, caffeic acid, β-sitosterol, daukosterol and salidroside (in trace amounts) has also been detected in callus tissues cultures (Dreger et al., 2007). Hydrodistillation air dried root of Rhodiola gives 0.05% to 1% of essential oil. The volatile compounds in this plant can be grouped into monoterpene hydrocarbons, oxygenated monoterpenes, aliphatic compounds, aromatic compounds, phenols, and heterocyclic compounds.

However, in case of *R. imbricata*, aqueous, alcoholic and hydro-alcoholic root/rhizome extract have been reported to have wide medicinal potential but, the active principle of the plant in these extract still remains unexplored. Scientific literature revealed that the different species of Rhodiola, root/rhizomes were found to contain 0.05 to 1% of essential oil but the *R. imbricata* has not yet been studied for essential oil content. The volatile, semi-volatile, polar compounds along with nutritional quality need to be analysed to ascertain the bioactivity and pharmacological potential of this vital plant.

1.2.5 Pharmacological properties of *Rhodiola imbricata*

Rhodiola species have been used as traditional medicines for the treatment of long term illness and weakness due to infection in Tibet and other regions for over 1000 years (Xiong, 1995; Rohloff, 2002). Recent pharmacological studies have showed that *R. imbricata* aqueous, ethanolic and hydroalcoholic root extracts reported to possess immuno-stimulatory, anticancer, cytoprotective, radioprotective, antioxidant, free radical scavenging, metal chelating, anti-lipid peroxidation, anti-hemolytic activity, dermal wound healing activity, anti-hypoxic, anti-inflammatory, antitumour, and adaptogenic activity. These pharmacological properties have been depicted in detail in the following sections.

1.2.5.1 Anti-cellular and immunomodulatory potential

Mishra et al. (2012) studied an aqueous extract of *R. imbricata* rhizome which showed anti-cellular and immunomodulatory potential which inhibits the proliferation of human T cell lymphoma cell line EL-4 and erythroleukemic cell line HL-60. The number of TNF-α spots was found to increase in *R. imbricata* rhizome treated human peripheral blood mononuclear cells (hPBMCs). The up regulation of TLR-4 mRNA expression was confirmed through *R. imbricata* rhizome treated rat splenocytes in reverse transcriptase polymerase chain reaction (RT-PCR) analysis. Thus, it is concluded from the present study that potent immune boosting activity of *R. imbricata* rhizome might be useful for immunocompromised individuals.

1.2.5.2 Immunostimulatory activity

Mishra *et al.* (2009) investigated the effect of aqueous extract of *R. imbricata* rhizome on Toll like receptor-4 (TLR-4) and intracellular granzyme-B expression in mouse splenocytes. It has been suggested from the study that *R. imbricata* rhizome stimulates the innate immune

pathway and has potent immunostimulatory activity, which can be used in modulating the immune system of immunocompromised individuals.

Mishra *et al.* (2006) evaluated the immunomodulatory activity of aqueous extract of *R. imbricata* rhizome in human peripheral blood mononuclear cells (PBMCs) and mouse macrophage cell line RAW 264.7. The extract was found to stimulate production of interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-alpha) in human PBMCs as well as RAW 264.7 cell line. The aqueous extract of *R. imbricata* rhizome can be used for upregulation of immune response in patients with inadequate functioning of the immune system.

1.2.5.3 Adjuvant activity

Anti-inflammatory or immunosuppressive effect of *R. imbricata* rhizome in adjuvant-induced arthritis model (AIA) has been evaluated. The study suggests that *R. imbricata* rhizome has adjuvant/immunopotentiating activity in terms of humoral as well as cell-mediated immune response against strong antigen like TT and weak antigen like OVA (Mishra *et al.*, 2010).

1.2.5.4 Safety and adaptogenic activity

It has been reported from the studies that 250 and 500 mg/Kg aqueous extract of *R. imbricata* increases body weight in both the sexes of rats but without a change in their organ/body weight. Similarly, increase in plasma glucose and protein level was recorded, which were restored to normal after a 2-week withdrawal of treatment (Tulsawani *et al.*, 2011).

A dose dependent adaptogenic activity aqueous extract of *R. imbricata* root was orally administered in rats at different doses, 30 min prior to cold (5°C)-hypoxia (428 mm Hg)-restraint (C-H-R) exposure was studied. The acute and sub-acute toxicity of the extract was also studied in rats. No significant changes in any of the parameters examined in treated group's animal, in comparison to control animals, were observed in biochemical and hematological analysis. Oral

LD_{50} of the extract was observed to be >10 g/Kg, indicating an adequate margin of safety. No histopathological changes were observed in the vital organs studied of the treated animals. These results suggest that aqueous extract of *R. imbricata* root possess potent adaptogenic activity with no acute and sub-acute toxicity (Gupta *et al.*, 2008).

1.2.5.5 Radioprotective efficacy

Chawla *et al.* (2010) studied the comparison of pro-oxidant/antioxidant activities of three fractionated extracts of *R. imbricata*. The aqueous fraction showed significant ($P < 0.05$) pro-oxidant activity (up to 100 µg/ml) under metal ion-induced stress ± flux [transition metal (Fe/Cu) ± 0.25 kGy]. A decrease in the dielectric constant of the solvent system utilized for extraction, exhibited a significant ($P < 0.05$) negative correlation (-0.955) with mean protection potential of lipid against radiation flux. Solvent extraction and dose are crucial in bioactivity modulation and *R. imbricata* could be developed as a potential prophylactic radiation countermeasure for use in nuclear and radiological emergencies.

An investigation done on protection against whole-body lethal gamma irradiation (10 Gy)-induced mortality in Swiss albino strain "A" mice. The maximum tolerance dose values for aqueous (RD-I) and aqua-alcoholic (RD-II) extracts were 1,100 and 1,300 mg/Kg of body weight, respectively. Pre-irradiation administration of RD-I produced >90% survival, while RD-II produced >83% survival beyond the 30-day observation period. The studies show that *R. imbricata* is a suitable radioprotector of herbal origin (Goel *et al.*, 2006).

It has been reported that radioprotective properties of a hydro-alcoholic rhizome extract of *R. imbricata* (code named REC-7004). REC-7004 (1 µg/ml) exhibited significant anti-hemolytic capacity by preventing radiation-induced membrane degeneration of human

erythrocytes. It concludes the *R. imbricata* rhizome renders *in vitro* and *in vivo* radioprotection via multifarious mechanisms that act in a synergistic manner (Arora *et al.*, 2005).

1.2.5.6 Radiomodulatory and Free-Radical Scavenging Activity

Arora *et al.* (2008) studied the radio modifying effects of a fractionated extract and electron-donation potential, super-oxide ion scavenging ($IC_{50} \leq 0.025$ mg/ml), nitric oxide (NO) scavenging potential ($IC_{50} = 0.5$ mg/ml), and antihemolytic activity. Reducing power, superoxide ion, and nitric oxide scavenging ability of the fractionated extract increased in a dose-dependent manner. The present study shows that *R. imbricata* has immense potential for alleviation of biological damage in a radiation environment.

1.2.5.7 Cold, hypoxia and restraint (C-H-R) exposure and post-stress recovery

Gupta *et al.* (2010) examined the anti-oxidative potential of *R. imbricata* root aqueous extract in rats, administered orally at a dose of 100 mg/Kg both in single and multiple doses, 30 min prior to cold (5°C)-hypoxia (428 mmHg)-restraint (C-H-R) exposure. Multiple doses treatment of the extract further increased blood, liver and muscle GSH and GST levels; restricted increase in LDH on attaining $T_{(rec)}$23°C and recovery; increased CAT during recovery. Results suggested the anti-oxidant potential of *R. imbricata* root extract during C-H-R exposure and post-stress recovery and it also maintained cell membrane permeability.

1.2.5.8 Cold, hypoxia and restraint (C-H-R) stress induced hypothermia and post stress recovery: Mechanism and action

Gupta *et al.*, 2009 orally administered in rats (100mg/Kg body weight), 30 min prior to cold (5°C)-hypoxia (428 mmHg)-restraint (C-H-R) exposure up to fall of $T_{(rec)}$23°C and recovery $T_{(rec)}$37°C from hypothermia. The single and five doses extract treatment decreased or better maintained tissue glycogen and enzyme activities, *viz.* HK, PFK, CS and G-6-PD, in blood, liver

and muscle, on attaining $T_{(rec)}$23°C and recovery. The results suggest that *R. imbricata* extract treatment in rats shifted anaerobic metabolism to aerobic, during C-H-R exposure and post stress recovery.

1.2.5.9 Anti-proliferative effects

Mishra *et al.* (2008) studied the anti-proliferative effects of *R. imbricata* aqueous extract on human erythroleukemic cell line K-562 using MTT cell proliferation assay and found that the proliferation of K-562 significantly decreased after 72h incubation with RAE at 100 and 200 µg/ml. *R. imbricata* root extract also reduced induced intracellular reactive oxygen species (ROS) in K-562 cells at 200 µg/ml when incubated overnight. The observations suggest that *R. imbricata* root extract has very potent anti-cancer activities, which might be useful in leukemia cancer treatment.

1.2.5.10 Dermal wound healing potential

Gupta *et al.* (2007) investigated the wound healing efficacy of *R. imbricata* rhizome ethanol extract using a rat excision wound model. The extract was found to be rich in polyphenols which possess a significant wound healing activity. The results were also supported by histological examinations.

1.2.5.11 Cytoprotective and antioxidant potential

Kanupriya *et al.* (2005) reported that cytoprotective and antioxidant activity of aqueous and alcoholic extracts of *R. imbricata* rhizome on *tert*-butyl hydroperoxide (*tert*-BHP) induced cytotoxicity in U-937 human macrophages. Significant increase in cytotoxicity and apoptosis was observed in the presence of *tert*-BHP over control cells, however there was significant decrease in mitochondrial potential and increase in apoptosis and DNA fragmentation. It can be

concluded from the study that alcoholic and aqueous extracts of *R. imbricata* rhizome have marked cytoprotective and antioxidant activities.

1.2.5.12 Analysis of heavy metals

Saggu *et al*. (2006) showed that the heavy metal concentrations in aqueous extracts of *R. imbricata*, except chromium, were below the maximum permissible ranges as per the maximum permissible values proposed by World Health Organization (WHO).

1.2.5.13 Free radical scavenging property and antiproliferative activity of extracts in HT-29 human colon cancer cells

The *in vitro* free radical scavenging property and antiproliferative activity of acetone and methanol rhizome extracts in HT-29 human colon cancer cells have also been reported (Senthilkumar et al., 2013). The extracts was further reported to show DPPH, ABTS, FRAP superoxide anion, hydroxyl radical scavenging activities and metal chelating ability due to the presence of the natural antioxidants such as phenolics, tannins and flavonoid. Moreover, the extracts also inhibited the proliferation of HT-29 human colon cancer upon treatment at higher concentration (200 μg/mL) (acetone and methanol, 84% and 84%, respectively). On assessment acetone extract reveled antiproliferative potential in a dose dependent manner while, methanol extract showed both dose dependent and time dependent inhibitory potential.

1.2.5.14 Hepatoprotective effect of rhizome against paracetamol-induced (2 g/kg) liver toxicity in rats

Senthilkumar et al. (2014) also tested hepatoprotective effect of administration of acetone rhizome extract (200 and 400 mg/kg) against paracetamol-induced (2 g/kg) liver toxicity in rats. The oral administration of rhizome acetone extract considerably protected the hepatic cells from injury. The hematological and biochemical parameters were also compared with control and

reported to inhibit oxidative stress with increasing bodily antioxidant status. The HPLC analysis showed the presence of phenolic compounds which could be responsible for the hepatoprotective activity.

1.3 Research highlights and gaps in *R. imbricata*

1.3.1 Research highlights in *R. imbricata*

R. imbricata is an important and widely used food crop and traditional medicinal plant distributed in trans-Himalayan cold desert regions. In Amchi and Tibetian system of traditional medicine, the roots are used against lung problems, cold, cough, fever, loss of energy and pulmonary complaints (Chaurasia et al., 2007). Modern pharmacological studies have showed that *R. imbricata* aqueous, ethanolic and hydro-alcoholic root extracts possess anti-cellular and immunomodulatory potential (Mishra, Ganju, Singh, 2012), immunostimulatory activity (Mishra et al., 2006; Mishra et al., 2009), adjuvant activity (Mishra et al., 2010), adaptogenic activity (Tulsawani et al., 2011; Gupta et al., 2008), radioprotective efficacy (Arora et al., 2005; Goel et al., 2006; Chawla et al., 2010), radiomodulatory (Arora et al., 2008), cytoprotective (Kanupriya et al., 2005), antioxidant potential (Kanupriya et al., 2005), free-radical scavenging activity and metal chelating activity (Arora et al., 2008), cold, hypoxia and restraint (C-H-R) exposure and post-stress recovery (Gupta et al., 2010), cold, hypoxia and restraint (C-H-R) stress induced hypothermia and post stress recovery: mechanism and action (Gupta et al., 2009), anti-proliferative effects (Mishra et al., 2008), anti-cancerous (Mishra et al., 2008), dermal wound healing potential (Gupta et al., 2007), hepatoprotective effect (Senthilkumar et al., 2014), radical scavenging and antiproliferative activity of extracts in HT-29 human colon cancer cells (Senthilkumar et al., 2013), and found to be safe (Saggu et al., 2006; Gupta et al., 2008; Tulsawani et al., 2011).

1.3.2 Research gaps in *R. imbricata*

In light of the aforementioned ongoing research, following research gaps in *R. imbricata* research have been noticed.

1. Although, various *in vivo* and *in vitro* pharmacological potentials are well documented in polar extracts, the active principle of the plant in these extracts still remains a myth.
2. Moreover, the plant is widely used as an edible plant in India, Nepal, Tibet, and China, its nutritional quality with respect to its vitamins, fatty acids, amino acids, and mineral content still remained unexplored in *R. imbricata* root except the species was tested only for the heavy metals such as As, Pb, Hg, Cd, Zn, Cu, and Cr.
3. The volatile, semi-volatile and polar compounds in different extracts need to be analyzed to ascertain the bioactivity and pharmacological potential of the plant.
4. Recent ongoing research in the plant revealed vital bioactivity in root/rhizome of the plant tested in different solvents. However, the active principles in these extract still remained unrevealed.
5. Pharmacological evaluation (physical endurance) of *R. imbricata* root hydro-alcoholic extract on rats under normoxic and hypobaric hypoxic environmental conditions have not yet been tested beside the fact that the plant extract reported to show adaptogenic potential and oxidative stress neutralizer.

1.4 Proposed work

1.4.1 Objectives of the research work

In light of the aforementioned ongoing research and present research gaps in *R. imbricata* following objectives were selected to have

1. Determination of phyto-components in different solvent extracts of *R. imbricata* root with hyphenated gas chromatography mass spectrometry (GC-MS MS) technique.
2. To ascertain *in vitro* antioxidant radical scavenging capacities and their correlation among the content of total phenolic compounds *viz.* Total polyphenols, phenolic acids, flavonoids and flavonols in methanolic and aqueous root extracts of *R. imbricata*.
3. Nutritional and mineral profiling of *R. imbricata*
    a. Optimization of reversed phase rapid resolution liquid chromatography/tandem mass spectrometry (RRLC-MS/MS) technique for the sequential determination of fat- and water-soluble vitamins in *R. imbricata* root.
    b. Estimation of fatty acid composition in root of *R. imbricata* using gas chromatography coupled with flame ionization detector (GC-FID) with fatty acid methyl esters (FAMEs) derivatization technique.
    c. Free amino acid profiling in root of *R. imbricata* using reversed phase high performance liquid chromatography (RP-HPLC) with precolumn phenylisothiocyanate (PITC) derivatization.
    d. Mineral profiling of *R. imbricata* root by inductive coupled plasma optical emission spectrometer (ICP-OES).

4. Pharmacological evaluation (physical endurance) of *R. imbricata* root hydro-alcoholic extract on rats under normoxic and hypobaric hypoxic environmental conditions.

1.4.2 Significance of the research work under study

The present work has thus been undertaken in view of the significance of *R. imbricata* to help pharmaceuticals, researchers, nutritionalists and phytochemist to explore the potential utility of the plant for the benefit of mankind. The possible significance of the work under study is discussed bellow.

1. Evaluation of *in vitro* antioxidant capacities will be useful in determining the free radical scavenging potential of the root extracts of *R. imbricata*. Furthermore, it will give an insight into the major phyto-components responsible for the antioxidant capacities of the plant.
2. Assessment of nutritional quality such as vitamins, amino acids and fatty acids in roots of *R. imbricata* will be useful in developments of nutraceutical herbal preparations from this plant.
3. The phytochemical profile of *R. imbricata* root will give the range of bioactive principles and their distribution with respect to the polarity of the solvent. The knowledge of these phyto-constituents might be useful in developing a new drug from this plant.
4. The present study will also be useful in extending the pharmaceutical utility of the plant as a physical performance enhancer.

1.4.3 Outline of the research works

Each of these four pieces of work has distinct characteristics. At the same time they are related to one another. To clearly and coherently demonstrate the goal, results and conclusion of each piece of work, we have arranged each work chapter wise in a publishing format. The format will benefit readers to understand the idea of development, conclusion, coherence and full significance as each chapter will be a full manuscript from background to conclusion at publication stage.

CHAPTER 2

PHYTOCHEMICAL PROFILE OF ROOT EXTRACTS OF *RHODIOLA IMBRICATA* EDGEW. WITH HYPHENATED GAS CHROMATOGRAPHY MASS SPECTROMETRIC TECHNIQUE

Abstract

Rhodiola imbricata Edgew. (Rose root or Arctic root or Golden root or Shrolo), belonging to the family Crassulaceae, is an important food crop and medicinal plant in the Indian trans-Himalayan cold desert. Phytochemical profile of the n-hexane, dichloroethane, chloroform, ethyl acetate, methanol, and 60% ethanol root extracts of *R. imbricata* were performed by hyphenated gas chromatography mass spectrometry (GC/MS) technique. GC/MS analysis was carried out using Thermo Finnigan PolarisQ Ion Trap GC/MS MS system comprising of an AS2000 liquid autosampler. Interpretation on mass spectrum of GC/MS was done using the NIST/EPA/NIH Mass Spectral Database, with NIST MS search program v.2.0g. Phytochemical profile of root extracts revealed the presence of 64 phyto-constituents, among them, 1-pentacosanol; stigmast-5-en-3-ol, (3*β*,24*S*); 1-teracosanol; 1-henteracontanol; 17-pentatriacontene; 13-tetradecen-1-ol acetate; methyl tri-butyl ammonium chloride; bis(2-ethylhexyl) phthalate; 7,8-dimethylbenzocyclooctene; ethyl linoleate; 3-methoxy-5-methylphenol; hexadecanoic acid; camphor; 1,3-dimethoxybenzene; thujone; 1,3-benzenediol, 5-pentadecyl; benzenemethanol, 3-hydroxy, 5-methoxy; cholest-4-ene-3,6-dione; dodecanoic acid, 3-hydroxy; octadecane, 1-chloro; ethanone, 1-(4-hydroxyphenyl); *α*-tocopherol; ascaridole; campesterol; 1-dotriacontane; heptadecane, 9-hexyl were found to be present in major amount. Eventually, in the present study we have found phytosterols, terpenoids, fatty acids, fatty acid esters, alkyl halides, phenols, alcohols, ethers, alkanes, and alkenes as the major group of phyto-constituents in the different root extracts of *R. imbricata.* All these compounds identified by GC/MS analysis were further investigated for their biological activities and it was found that they possess a diverse range of positive pharmacological actions. In future, isolation of individual phyto-constituent and subjecting them to biological activity will definitely prove fruitful results in designing a novel drug.

2.1 Introduction

To identify and evaluate the therapeutic potential of medicinal herbs, isolation of active components and structural elucidation of these compounds is very essential in medicinal chemistry and natural product research. In recent years, a lot of attention has been given towards the study of organic compounds from medicinal herbs and to elucidate their pharmacological activities. Numerous extraction techniques and analytical systems like spectrophotometry, capillary electrophoresis, high performance liquid chromatography (HPLC), high performance thin layer chromatography (HPTLC), gas chromatography (GC) with flame ionization detection (FID), gas chromatography/mass spectrometry (GC/MS) have been developed for the analysis and characterization of active compounds from medicinal plants. GC/MS has become an ideal technique for qualitative and quantitative analysis of volatile and semi-volatile compounds of plant origin. It has the unique combination of a perfect separation system (GC) with the excellent identification and confirmation technique (MS) which has made it the best suited analytical system for plant compound characterization. Additionally, for rapid extraction and precise analysis of these active phytochemicals, the experimental design should also be optimized to obtain enhanced recoveries, low solvent consumption, and reduced extraction time [Gherman et al., 2000; Carro et al., 1997; Robards and Antolovich, 1997; Pallado et al., 1997; Iordache et al., 2009].

Rhodiola imbricata Edgew. (Rose root/ Arctic root/ Golden root/ Shrolo), belonging to the family Crassulaceae, is an important food crop and medicinal plant in the high altitude region of Indian trans-Himalayan cold desert. It is a popular medicinal plant in Pakistan, Nepal, India, Tibet, China, and many other countries and is widely used as food and traditional medicine around the world. A number of metabolites like phenylpropanoids, phenylethanol derivatives, flavanoids, terpenoids, and phenolic acids have been found in good quantity from these *Rhodiola* species and extracts of these plant species, particularly

those from roots, have been shown to possess pharmacological activities. A survey of literature showed that *Rhodiola* species influence a number of physiological functions including neurotransmitter levels, central nervous system activity, and cardiovascular function. It is being used to stimulate the nervous system, decrease depression, enhance work performance, eliminate fatigue, and prevents high-altitude sickness. Most of these effects have been ascribed to constituents such as salidrosides (rhodiolosides), rosavins, and p-tyrosol. Many pharmacological studies on *R. imbricata* have demonstrated that this plant exhibits cardioprotective, anti-inflammatory, antistress, dermal wound healing, and adaptogenic activities. It has also been found to possess antioxidant, antiaging, immuno-stimulant, radioprotective, and anticarcinogenic properties [Mishra et al., 2012; Tulsawani et al., 2011; Chawla et al., 2010; Gupta et al., 2010; Mishra et al., 2010; Kumar et al., 2010a; Kumar et al., 2010b; Gupta et al., 2009; Mishra et al., 2009; Gupta et al., 2008; Mishra et al., 2008; Arora et al., 2008; Gupta et al., 2007; Kumar et al., 2007; Mishra et al., 2006; Goel et al., 2006; Kanupriya et al., 2005; Arora et al., 2005; Khanum et al., 2005]. All these reports validate its use in traditional system of medicine.

However, the phytochemistry of the most important plant part having the medicinal and therapeutic potential, the root of *R. imbricata* has not been studied in considerable details. Hence, aim of the present investigation was to identify and quantify the phytochemicals extracted successively in different solvents such as n-hexane, dichloroethane, chloroform, ethyl acetate, methanol, and 60% ethanol, from roots of *R. imbricata* from trans-Himalayan cold desert of Ladakh, India, by hyphenated GC/MS technique.

2.2 Materials & methods

2.2.1 *Chemicals and reagents*

n-Hexane, dichloroethane, chloroform, ethyl acetate, methanol, ethanol, and water CHROMASOLV HPLC grade and all other chemicals used were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2.2 *Ethics statement*

All necessary permits were obtained for the described field studies. The permit was issued by Dr. B. Balaji (IFS), Divisional Forest Officer, Leh Forest Division, Jammu & Kashmir, India.

2.2.3 *Plant materials and extraction*

R. imbricata roots were collected from the trans-Himalayan region (Chang-La Top, altitude = 5330 m above mean sea level, Indus valley, Ladakh) of India in the month of October, 2011 after the period of senescence, with prior permission from the local authorities. The plant roots were washed thoroughly and cut into small pieces and shade dried at room temperature for 15 days. Then they were finely powdered and used for extraction. The root powder (20 gm) was taken for the sequential extraction in six solvent systems with increasing polarity *viz.* n-hexane, dichloroethane, chloroform, ethyl acetate, methanol, and 60% ethanol by Soxhlet apparatus (Borosil GlassWorks Limited, Worli, Mumbai, India). The extracted fractions were concentrated under vacuum and reduced pressure (BUCHI Rotavapor R-205, BUCHI Labortechnik AG CH-9230, Flawil, Switzerland) at 40°C by circulation of cold water using thermostat maintained at 4°C in order to minimize the degradation of thermolabile compounds. The dry extracts were then stored in a -80°C freezer till further analysis.

2.2.4 ***Preparation of sample for GC/MS analysis***

Twenty five milligrams of concentrated n-hexane, dichloroethane, chloroform, ethyl acetate, methanol, and 60% ethanol root extracts were redissolved in the respective solvents, vortexed properly and filtered through 0.22 µm syringe filter (Millipore Corp., Bedford, MA, USA). One microlitre aliquot of the sample solution was injected into the GC/MS MS system for the requisite analysis.

2.2.5 ***Instrumentation and chromatographic conditions***

GC/MS analysis was carried out on a Thermo Finnigan PolarisQ Ion Trap GC/MS MS system comprising of an AS2000 liquid autosampler (Thermo Finnigan, Thermo Electron Corporation, Austin, TX, USA). The gas chromatograph was interfaced to a mass spectrometer instrument employing the following conditions *viz.* Durabond DB-5ms column (30 m × 0.25 mm × 0.25 µm), operating in electron impact [electron ionisation positive (EI^+)] mode at 70 eV, helium (99.999%) was used as carrier gas at a constant flow of 1ml/min, an injection volume of 0.5 EI was employed (split ratio of 10:1), injector temperature 280°C, and transfer line temperature 300°C. The oven temperature was programmed from 50°C (isothermal for 2 min), with gradual increase in steps of 10°C/min, to 300°C. Mass spectra were taken at 70 eV, a scan interval of 0.5 s, and full mass scan range from 25 m/z to 1000 m/z. The data acquisition was performed on Finnigan Xcalibur data acquisition and processing software version 2.0 (ThermoQuest, LC and LC/MS Division, San Jose, California, USA).

2.2.6 ***Identification of components***

Interpretation of mass spectrum of GC/MS was done using the NIST/EPA/NIH Mass Spectral Database (NIST11), with NIST MS search program v.2.0g [National Institute Standard and Technology (NIST), Scientific Instrument services, Inc., NJ, USA]. The mass

spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight, and structure of the components of the test materials were ascertained.

2.3 Results

Gas chromatograms of n-hexane (Figure 2.1), dichloroethane (Figure 2.2), chloroform (Figure 2.3), ethyl acetate (Figure 2.4), methanol (Figure 2.5), and 60% ethanol (Figure 2.6) root extracts of *R. imbricata* as per the experimental procedure discussed above, showed various peaks indicating the presence of different phytochemicals in the respective extracts.

2.3.1 *GC/MS* phytochemical *profile*

2.3.1.1 *n-Hexane root extract*

The n-hexane root extract revealed the presence of 22 different phytochemicals which were characterized and identified (Table 2.1, Figure 2.1) by comparison of their mass fragmentation patterns with the similar in NIST database library. Of these 22 phytochemicals, 1-pentacosanol (28.21%), stigmast-5-en-3-ol, (3*β*,24*S*) (13.40%), 1-teracosanol (9.23%), 1-henteracontanol (8.53%), 17-pentatriacontene (7.01%), and 13-tetradecen-1-ol acetate (6.40%) were found to be major constituents whereas 1-hentriacontane (3.66%), 1-heptacosane (3.47%), 1-tericosanol (2.51%), 13-docosan-1-ol, (*Z*) (2.12%), eicosen-1-ol, *cis*-9 (1.99%) 1,30-triacontanediol (1.49%), stigmast-4-en-3-one (1.28%), bis(2-ethylhexyl) phthalate (1.20%), hexadecanoic acid (1.16%), 1-tetrateracontane (0.90%), campesterol (0.90%), *α*-Tocopherol-*β*-D-mannoside (0.73%), stigmastanol (0.71%), 1-pentatriacontane (0.48%), and 3-methoxy-5-methylphenol (0.46%) were found to be present in trace amount.

2.3.1.2 *Dichloroethane root extract*

GC/MS phytochemical profile of dichloroethane root extract illustrated the presence of 25 different phytochemicals (Table 2.2, Figure 2.2). Among these, camphor (17.78%), stigmast-5-en-3-ol, (3*β*,24*S*) (15.42%), ethyl linoleate (9.95%), 1,3-dimethoxybenzene

(8.15%), hexadecanoic acid (6.55%), and thujone (4.73%) were present in major amount, whereas, benzene sulfonic acid, 4-amino-3-nitro (3.96%), campesterol (3.88%), methanol, (4-carboxymethoxy) benzoyl (3.27%), stigmast-4-en-3-one (2.84%), 1-hentetracontanol (2.74%), oleic acid (2.18%), bis(2-ethylhexyl) adipate (2.16%), bacteriochlorophyll-*c*-stearyl (2.11%), eucalyptol (1.95%), ethanone, 1-(2,6-dihydroxy-4-methoxyphenyl) (1.66%), 1-dotriacontane (1.58%), linalyl isovalerate (1.49%), 3-methoxy-5-methylphenol (1.47%), 1-chloro-2,4-dimethoxybenzene (1.44%), borneol (1.22%), 4-chlorothiophenol (1.02%), phenol, 2,4-bis(1,1-dimethylethyl) (0.94%), fenchyl alcohol (0.91%), and stigmast-3,5-dien-7-one (0.60%) were found to be present in traces.

2.3.1.3 ***Chloroform root extract***

GC/MS phytochemical profile of chloroform root extract showed the presence of 18 different phytochemicals (Table 2.3, Figure 2.3). Amongst these, stigmast-5-en-3-ol, (3*β*,24*S*) (24.30%), methyl tri-butyl ammonium chloride (14.64%), bis(2-ethylhexyl) phthalate (11.50%), 7,8-dimethylbenzocyclooctene (7.97%), ethyl linoleate (4.75%), 3-methoxy-5-methylphenol (4.16%), and hexadecanoic acid (4.13%) were found to constitute major amount while, campesterol (3.94%), 1-pentacosanol (3.82%), 17-pentariacontene (3.38%), benzene sulfonic acid, 4-amino-3-nitro (3.21%), orcinol (2.93%), benzenemethanol, 3-hydroxy, 5-methoxy (2.62%), 1-hentetracontanol (2.54%), 1-tetracosanol (1.86%), stigmast-4-en-3-one (1.82%); and *α*-tocopherol (1.31%), and eicosen-1-ol, *cis*-9 (1.13%) were found to be present in trace quantity.

2.3.1.4 ***Ethyl acetate root extract***

Nineteen different phytochemicals were identified in ethyl acetate extract (Table 2.4, Figure 2.4). Amongst these 19 phytochemicals, 1,3-dimethoxybenzene (27.61%), 1,3-benzenediol, 5-pentadecyl (16.90%), 3-methoxy-5-methylphenol (10.11%), 1,3-benzenediol, 5-methyl (8.40%), benzenemethanol, 3-hydroxy, 5-methoxy (5.75%), cholest-4-ene-3,6-

dione (5.75%), and dodecanoic acid, 3-hydroxy (4.46%) were found to constitute major amount, whereas, 7,8-dimethylbenzocyclooctene (3.57%) 3,5-dimethoxyphenyl acetate (3.44%), *α*-D-glucopyranoside, O-*α*-D-glucopyranosyl-(1.fwdarw.3)-*β*-D-fructofuranosyl (2.95%), stigmast-5-en-3-ol, (3*β*,24*S*) (2.12%), eicosen-1-ol, *cis*-9 (2.12%), hexadecanoic acid (1.84%), oleic acid (1.34%), bacteriochlorophyll-*c*-stearyl (1.14%), phenol, 2,4-bis(1,1-dimethylethyl) (0.85%), 1-pentatricontene (0.72%), 1-dodecanol, 3,7,11-trimethyl (0.61%), and stigmast-4-en-3-one (0.32%) were found to be present in trace.

2.3.1.5 ***Methanol root extract***

The methanol root extract revealed the presence of 18 different phytochemicals (Table 2.5, Figure 2.5). Among the identified phytochemicals, stigmast-5-en-3-ol, (3*β*,24*S*) (21.91%), octadecane, 1-chloro (17.01%), ethanone, 1-(4-hydroxyphenyl) (11.07%), *α*-tocopherol (8.42%), ascaridole (5.92%), and campesterol (4.98%) were found to be present in major amount, while, linolein, 2-mono (3.99%), hexadecanoic acid (3.67%), 1,3-dimethoxybenzene (3.57%), ethyl linoleate (3.35%), 1-dotriacontane (2.21%), linolein, 1-mono (1.74%), methyl palmitate (1.73%), stigmast-4-en-3-one (1.55%), 1-dodecane (0.66%), *δ*-tocopherol (0.56%), and 3-methoxy-5-methylphenol (0.43%) were found to be present in traces.

2.3.1.6 ***60% Ethanol root extract***

GC/MS phytochemical profile of 60% ethanol root extracts illustrated the presence of 12 different phytochemicals (Table 2.6, Figure 2.6). Amongst the identified phytochemicals, dotriacontane (5.69%), and heptadecane, 9-hexyl (5.44%) were found to be present in major amount, whereas, bis(2-ethylhexyl) phthalate (3.58%), hexadecanoic acid, methyl ester (2.27%), and dibutyl phthalate (1.23%) were found to be present in traces.

2.4 Discussion

We have conducted the present investigation to identify the major volatile and semivolatile components in the root of *R. imbricata*. The presence of various bioactive compounds justifies the use of the plant by traditional practitioners of 'Amchi' system of medicine in trans-Himalayan Ladakh region. Also, extensive pharmacological studies have been conducted by different researchers with the plant root extracts [Mishra et al., 2012; Tulsawani et al., 2011]

Table 2.1: Phyto-constituents identified in the n-hexane root extract of *R. imbricata* by GC/MS.

S. No.	Peak RT (min)	Peak area	Peak area (%)	Compound detected	CAS No	Mol. Formula	Mol. Wt.
1	13.66	3489208	0.46	3-Methoxy-5-methylphenol	3209-13-0	$C_8H_{10}O_2$	138
2	22.84	9266063	1.16	Hexadecanoic acid	57-10-3	$C_{16}H_{32}O_2$	256
3	24.91	34516141	4.16	Ethyl linoleate	544-35-4	$C_{20}H_{36}O_2$	308
4	26.69	6791237	0.9	1-Tetratetracontane	7098-22-8	$C_{44}H_{90}$	618
5	27.74	3769798	0.48	1-Pentatriacontane	630-07-9	$C_{35}H_{72}$	492
6	28.77	30375043	3.66	1-Hentriacontane	630-04-6	$C_{31}H_{64}$	436
7	29.07	9600422	1.2	Bis(2-ethylhexyl) phthalate	117-81-7	$C_{24}H_{38}O_4$	390
8	29.82	20564432	2.51	1-Tricosanol	05-01-3133	$C_{23}H_{48}O$	340
9	30.08	16467246	1.99	Eicosen-1-ol, *cis*-9	112248-30-3	$C_{20}H_{40}O$	296
10	30.7	56408242	7.01	17-Pentatriacontene	6971-40-0	$C_{35}H_{70}$	490
11	31.95	74961756	9.23	1-Tetracosanol	506-51-4	$C_{24}H_{50}O$	354
12	33.31	16764245	2.12	13-Docosen-1-ol, (*Z*)	629-98-1	$C_{22}H_{44}O$	324

13	33.17	68435403	8.53	1-Hentetracontanol	40710-42-7	$C_{41}H_{84}O$	592
14	35.01	229932016	28.21	1-Pentacosanol	26040-98-2	$C_{25}H_{52}O$	368
15	35.5	12157246	1.49	1,30-Triacontanediol	36645-68-8	$C_{30}H_{62}O_2$	454
16	36.4	29211487	3.47	1-Heptacosane	593-49-7	$C_{27}H_{56}$	380
17	36.74	5914760	0.73	α-Tocopherol-β-D-mannoside	CID 597057	$C_{35}H_{60}O_7$	592
18	37.95	54630744	6.4	13-Tetradecen-1-ol acetate	56221-91-1	$C_{16}H_{30}O_2$	254
19	38.08	6914671	0.9	Campesterol	474-62-4	$C_{28}H_{48}O$	400
20	39.39	107745880	13.4	Stigmast-5-en-3-ol, (3β,24*S*)	83-47-6	$C_{29}H_{50}O$	414
21	39.62	5803953	0.71	Stigmastanol	19466-47-8	$C_{29}H_{52}O$	416
22	41.64	10789041	1.28	Stigmast-4-en-3-one	1058-61-3	$C_{29}H_{48}O$	412

Table 2.2: Phyto-constituents identified in the dichloroethane root extract of *R. imbricata* by GC/MS.

S. No.	Peak RT (min)	Peak area	Peak area %	Compound detected	CAS No	Mol. Formula	Mol. Wt.
1	8.19	1187393	1.95	Eucalyptol	470-82-6	$C_{10}H_{18}O$	154
2	9.64	2910856	4.73	Thujone	546-80-5	$C_{10}H_{16}O$	152
3	10.44	11188301	0.85	Camphor	76-22-2	$C_{10}H_{16}O$	152
4	10.91	742204	1.22	Borneol	464-45-9	$C_{10}H_{18}O$	154
5	11.32	552901	0.91	*β*-fenchyl alcohol	470-08-6	$C_{10}H_{18}O$	154
6	13.06	631861	1.02	Benzenethiol, 4-chloro	106-54-7	C_6H_5ClS	144
7	13.66	898266	1.47	3-Methoxy-5-methylphenol	3209-13-0	$C_8H_{10}O_2$	138
8	15.88	2126709	3.27	Methanol, (4-carboxymethoxy) benzoyl	80099-44-1	$C_{10}H_{10}O_5$	210
9	16.5	571000	0.94	Phenol, 2,4-bis(1,1-dimethylethyl)	96-76-4	$C_{14}H_{22}O$	206
10	17.36	885243	1.44	1-Chloro-2,4-dimethoxybenzene	7051-13-0	$C_8H_9ClO_2$	172
11	17.56	926151	1.49	Linalyl isovalerate	50649-12-2	$C_{15}H_{26}O_2$	238
12	19.33	953645	1.58	1-Dotriacontane	544-85-4	$C_{32}H_{66}$	450

13	20.49	975679	1.66	Ethanone, 1-(2,6-dihydroxy-4-methoxyphenyl)	7507-89-3	$C_9H_{10}O_4$	182
14	22.81	4154976	6.55	Hexadecanoic acid	57-10-3	$C_{16}H_{32}O_2$	256
15	23.18	1089256	2.18	Oleic Acid	112-80-1	$C_{18}H_{34}O_2$	282
16	24.28	1364685	2.11	Bacteriochlorophyll-*c*-stearyl	CID5367801	$C_{52}H_{72}MgN_4O_4$	840
17	24.94	6245279	9.95	Ethyl linoleate	544-35-4	$C_{20}H_{36}O_2$	308
18	27.61	1330635	2.16	Hexanedioic acid, bis(2-ethylhexyl) ester	103-23-1	$C_{22}H_{42}O_4$	370
19	28.74	2480706	3.96	Benzene sulfonic acid, 4-amino-3-nitro	616-84-2	$C_6H_6N_2O_5S$	218
20	29.1	5070293	8.15	1,3-Dimethoxybenzene			
21	33.12	1747914	2.74	1-Hentetracontanol	40710-42-7	$C_{41}H_{84}O$	592
22	38.04	2440601	3.88	Campesterol	474-62-4	$C_{28}H_{48}O$	400
23	39.36	9725435	15.42	Stigmast-5-en-3-ol, (3***β***,24*S*)	83-47-6	$C_{29}H_{50}O$	414
24	40.9	366122	0.6	Stigmast-3,5-dien-7-one	2034-72-2	$C_{29}H_{46}O$	410
25	41.65	1869647	2.84	Stigmast-4-en-3-one	1058-61-3	$C_{29}H_{48}O$	412

Table 2.3: Phyto-constituents identified in the chloroform root extract of *R. imbricata* by GC/MS.

S. No.	Peak RT (min)	Peak area	Peak area %	Compound detected	CAS No	Mol. Formula	Mol. Wt.
1	11.69	9872792	14.64	Methyl tri-butyl ammonium chloride	56375-79-2	$C_{13}H_{30}ClN$	235
2	13.75	2364627	4.16	3-Methoxy-5-methylphenol	3209-13-0	$C_8H_{10}O_2$	138
3	15.14	1927265	2.93	1,3-Benzenediol, 5-methyl	504-15-4	$C_7H_8O_2$	124
4	18.35	1520584	2.62	Benzenemethanol, 3-hydroxy-5-methoxy	30891-29-3	$C_8H_{10}O_3$	154
5	20.55	5041042	7.97	7,8-dimethylbenzocyclooctene	99027-76-6	$C_{14}H_{14}$	182
6	22.89	2762363	4.13	Hexadecanoic acid	57-10-3	$C_{16}H_{32}O_2$	256
7	25.01	3042142	4.75	Ethyl linoleate	544-35-4	$C_{20}H_{36}O_2$	308
8	28.8	2066650	3.21	Benzene sulfonic acid, 4-amino-3-nitro	616-84-2	$C_6H_6N_2O_5S$	218
9	29.11	7589941	11.5	Bis(2-ethylhexyl) phthalate	117-81-7	$C_{24}H_{38}O_4$	390
10	30.12	670458	1.13	Eicosen-1-ol, *cis*-9	112248-30-3	$C_{20}H_{40}O$	296
11	30.74	2150315	3.38	17-Pentariacontene	6971-40-0	$C_{35}H_{70}$	490

12	31.98	1129772	1.86	1-Tetracosanol	506-51-4	$C_{24}H_{50}O$	354
13	33.23	1665168	2.54	1-Hentetracontanol	40710-42-7	$C_{41}H_{84}O$	592
14	34.99	2475797	3.82	1-Pentacosanol	26040-98-2	$C_{25}H_{52}O$	368
15	36.83	898525	1.31	α-Tocopherol	59-02-9	$C_{29}H_{50}O_2$	430
16	38.17	2267712	3.94	Campesterol	474-62-4	$C_{28}H_{48}O$	400
17	39.51	16343303	24.29	Stigmast-5-en-3-ol, (3β,24*S*)	83-47-6	$C_{29}H_{50}O$	414
18	41.82	1236875	1.82	Stigmast-4-en-3-one	1058-61-3	$C_{29}H_{48}O$	412

Table 2.4: Phyto-constituents identified in the ethyl acetate root extract of *R. imbricata* by GC/MS.

S. No.	Peak RT (min)	Peak area	Peak area %	Compound detected	CAS No	Mol. Formula	Mol. Wt.
1	10.11	18961417	4.16	3-Methoxy-5-methylphenol	3209-13-0	$C_8H_{10}O_2$	138
2	14.63	17158116	8.4	1,3-Benzenediol, 5-methyl	504-15-4	$C_7H_8O_2$	124
3	16.5	1721777	0.85	Phenol, 2,4-bis(1,1-dimethylethyl)	96-76-4	$C_{14}H_{22}O$	206
4	17.78	1213849	0.61	1-Dodecanol, 3,7,11-trimethyl	6750-34-1	$C_{15}H_{32}O$	228
5	18.07	11340896	5.75	Benzenemethanol, 3-hydroxy-5-methoxy	30891-29-3	$C_8H_{10}O_3$	154
6	19.54	7034263	3.44	Phenol, 3,5-dimethoxy acetate	23133-74-6	$C_{10}H_{12}O_4$	196
7	20.4	7323033	3.57	7,8-Dimethylbenzocyclooctene	99027-76-6	$C_{14}H_{14}$	182
8	20.6	4129209	2.12	Eicosen-1-ol, *cis*-9	629-96-9	$C_{20}H_{40}O$	296
9	21.22	6328149	2.95	α-D-glucopyranoside, O-α-D-glucopyranosyl-(1.fwdarw.3)-β-D-fructofuranosyl	597-12-6	$C_{18}H_{32}O_{16}$	504

10	22.8	3545343	1.84	Hexadecanoic Acid	57-10-3	$C_{16}H_{32}O_2$	256
11	23.17	2659100	1.34	Oleic Acid	112-80-1	$C_{18}H_{34}O_2$	282
12	24.97	9179686	4.46	Dodecanoic acid, 3-hydroxy	1883-13-2	$C_{12}H_{24}O_3$	216
13	25.52	2261098	1.14	Bacteriochlorophyll-*c*-stearyl	CID5367801	$C_{52}H_{72}MgN_4O_4$	840
14	27.68	1352685	0.72	17-Pentatriacontene	6971-40-0	$C_{35}H_{70}$	490
15	29.08	76996880	27.61	1,3-Dimethoxybenzene	151-10-0	$C_8H_{10}O_2$	138
16	30.51	29595091	16.9	1,3-Benzenediol, 5-pentadecyl	3158-56-3	$C_{21}H_{36}O_2$	320
17	32.06	11993388	5.75	Cholest-4-ene-3,6-dione	984-84-9	$C_{27}H_{42}O_2$	398
18	39.36	4575754	2.12	Stigmast-5-en-3-ol, (3*β*,24*S*)	83-47-6	$C_{29}H_{50}O$	414
19	41.64	684162	0.32	Stigmast-4-en-3-one	1058-61-3	$C_{29}H_{48}O$	412

Table 2.5: Phyto-constituents identified in the methanol root extract of *R. imbricata* by GC/MS.

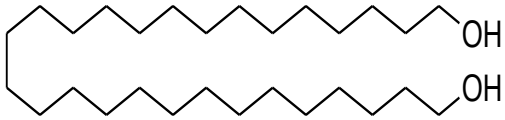
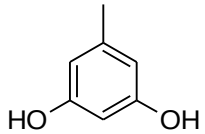
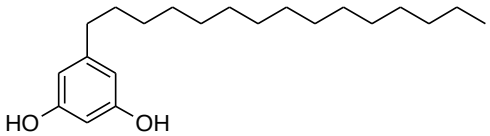
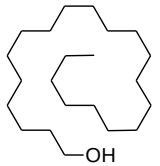
S. No.	Peak RT (min)	Peak area	Peak area %	Compound detected	CAS No	Mol. Formula	Mol. Wt.
1	13.73	224745	0.43	3-Methoxy-5-methylphenol	3209-13-0	$C_8H_{10}O_2$	138
2	15.78	5548712	11.07	Ethanone, 1-(4-hydroxyphenyl)	99-93-4	$C_8H_8O_2$	136
3	17.87	345721	0.66	1-Dodecane	112-40-3	$C_{12}H_{26}$	170
4	19.33	1157289	2.21	1-Dotriacontane	544-85-4	$C_{32}H_{66}$	450
5	20.69	8405167	17.01	Octadecane, 1-chloro	386-33-2	$C_{18}H_{37}Cl$	288
6	22.32	905705	1.73	Hexadecanoic acid, methyl ester	112-39-0	$C_{17}H_{34}O_2$	270
7	22.89	1901742	3.67	Hexadecanoic acid	57-10-3	$C_{16}H_{32}O_2$	256
8	24.36	1592833	3.99	9,12-Octadecadienoic acid (*Z*,*Z*)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester	3443-82-1	$C_{21}H_{38}O_4$	354
9	24.44	821776	1.74	9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester, (*Z*,*Z*,*Z*)	18465-99-1	$C_{21}H_{36}O_4$	352
10	24.96	1495805	3.35	Ethyl linoleate	544-35-4	$C_{20}H_{36}O_2$	308

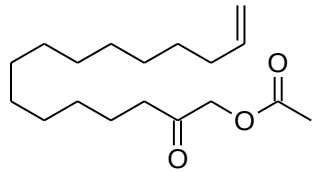
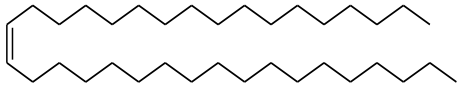
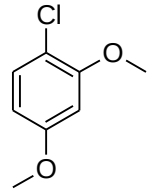
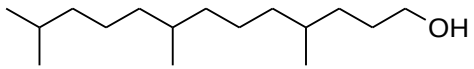
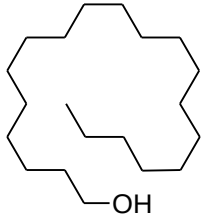
11	29.06	1614826	3.57	1,3-Dimethoxybenzene	151-10-0	$C_8H_{10}O_2$	138
12	30.91	3109377	5.92	Ascaridole	512-85-6	$C_{10}H_{16}O_2$	168
13	31.92	3533697	7.23	Unknown	-	-	-
14	33.77	174824	0.56	δ-Tocopherol	119-13-1	$C_{27}H_{46}O_2$	402
15	36.71	4419092	8.42	α-Tocopherol	59-02-9	$C_{29}H_{50}O_2$	430
16	38.04	2615057	4.98	Campesterol	474-62-4	$C_{28}H_{48}O$	400
17	39.36	11245680	21.91	Stigmast-5-en-3-ol, (3β,24*S*)	83-47-6	$C_{29}H_{50}O$	414
18	41.38	902689	1.55	Stigmast-4-en-3-one	1058-61-3	$C_{29}H_{48}O$	412

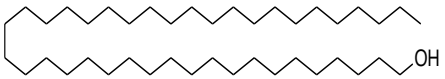
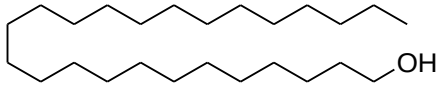
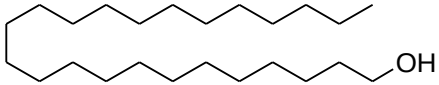
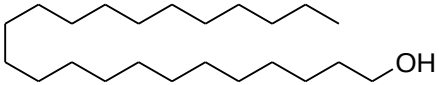
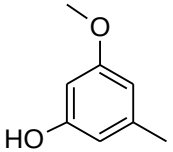
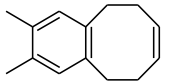
Table 2.6: Phyto-constituents identified in the 60% ethanol root extract of *R. imbricata* by GC/MS.

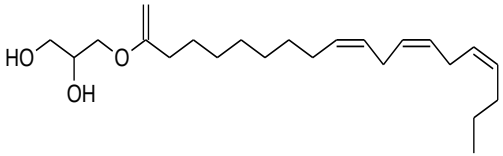
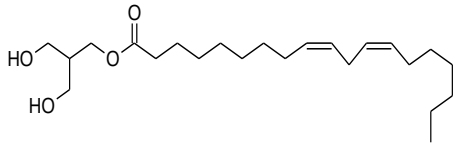
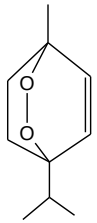
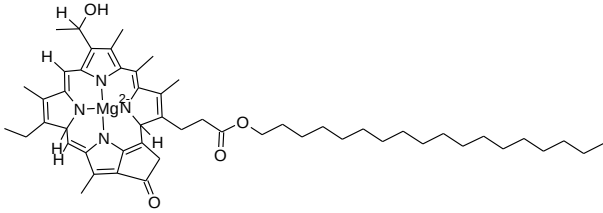
S. No.	Peak RT (min)	Peak area	Peak area %	Compound detected	CAS No	MF	MW
1	19.37	258681	5.69	1-Dotriacontane	544-85-4	$C_{32}H_{66}$	450
2	20.72	276232	5.44	Heptadecane, 9-hexyl	55124-79-3	$C_{23}H_{48}$	324
3	21.56	62567	1.23	Dibutyl phthalate	84-74-2	$C_{16}H_{22}O_4$	278
4	22.36	115053	2.27	Hexadecanoic acid, methyl ester	112-39-0	$C_{17}H_{34}O_2$	270
5	23.22	296422	5.84	Unknown	-	-	-
6	24.48	194215	3.83	Unknown	-	-	-
7	25.41	56168	1.11	Unknown	-	-	-
8	29.1	181582	3.58	Bis(2-ethylhexyl) phthalate	117-81-7	$C_{24}H_{38}O_4$	390
9	34.21	644518	12.7	Unknown	-	-	-
10	39.51	1832781	35.53	Unknown	-	-	-
11	39.95	550771	10.86	Unknown	-	-	-
12	40.92	604576	11.92	Unknown	-	-	-

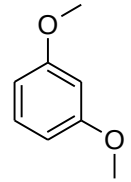
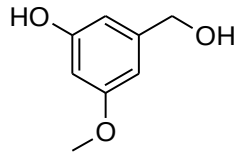
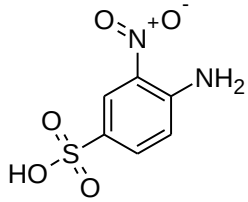
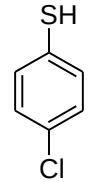
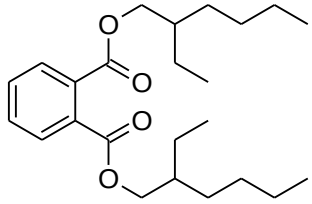
Table 2.7: Phyto-constituents in different root extracts of *R. imbricata*

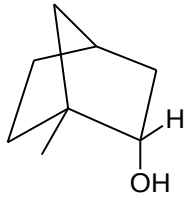
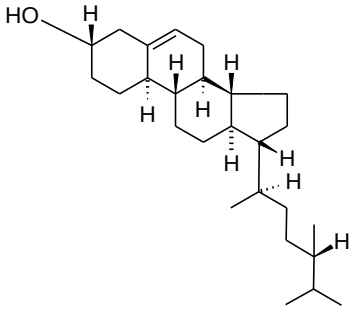
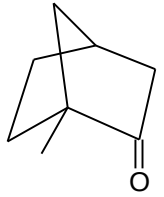
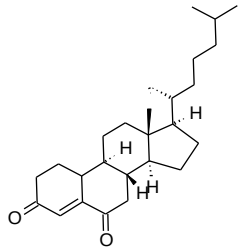
Phyto-constituents	Root extracts						Structure
	n-Hexane	Dichloroethane	Chloroform	Ethyl acetate	Methanol	60% Ethanol	
1,30-Triacontanediol	√	–	–	–	–	–	
1,3-Benzenediol, 5-methyl	–	–	√	√	–	–	
1,3-Benzenediol, 5-pentadecyl	–	–	–	√	–	–	
13-Docosen-1-ol, (*Z*)	√	–	–	–	–	–	

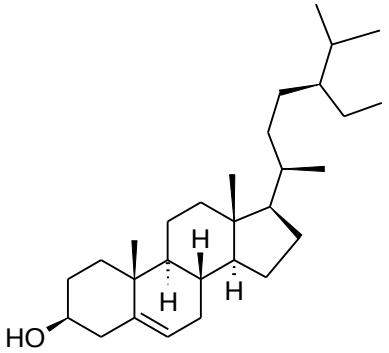
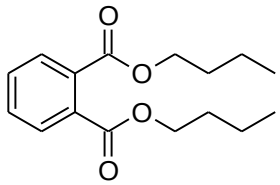
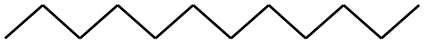
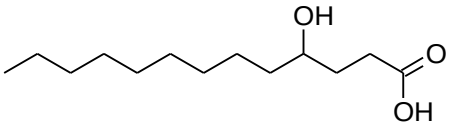
13-Tetradecen-1-ol acetate	√	–	–	–	–	–	
17-Pentatriacontene	√	–	√	√	–	–	
1-Chloro-2,4-dimethoxybenzene	–	√	–	–	–	–	
1-Dodecanol, 3,7,11-trimethyl	–	–	–	√	–	–	
Eicosen-1-ol, *cis*-9	–	–	–	√	–	–	

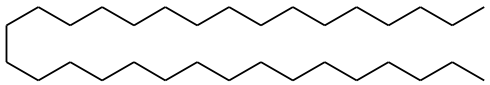
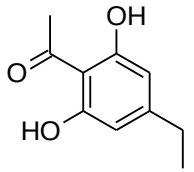
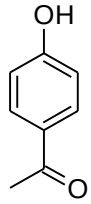
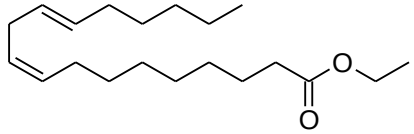
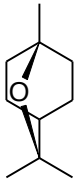
1-Hentetracontanol	√	√	–	–	–	–	
1-Pentacosanol	√	–	√	–	–	–	
1-Tetracosanol	√	–	√	–	–	–	
1-Tricosanol	√	–	–	–	–	–	
3-Methoxy-5-methylphenol	√	√	√	√	√	–	
7,8-dimethylbenzocyclooctene	–	–	√	√	–	–	

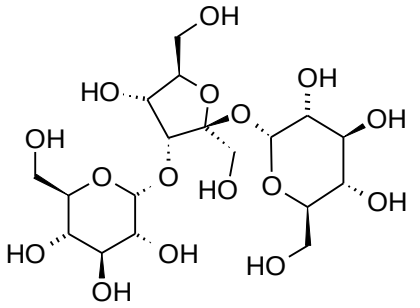
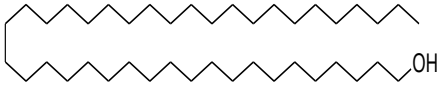
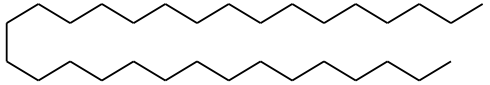
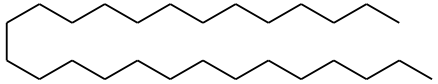
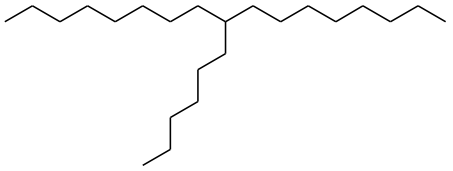
9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester, (*Z*,*Z*,*Z*)	–	–	–	–	√	–	
9,12-Octadecadienoic acid (*Z*,*Z*)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester	–	–	–	–	√	–	
Ascaridole	–	–	–	–	√	–	
Bacteriochlorophyll-*c*-stearyl	–	√	–	√	–	–	

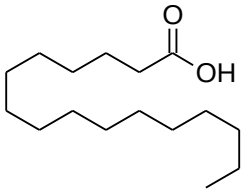
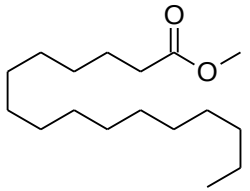
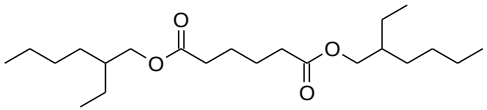
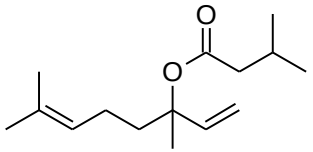
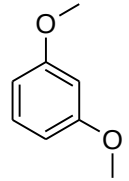
Benzene, 1,3-dimethoxy	–	–	–	√	√	–	
Benzenemethanol, 3-Hydroxy-5-Methoxy	–	–	√	√	–	–	
Benzene sulfonic acid, 4-amino-3-nitro	–	√	√	–	–	–	
Benzenethiol, 4-chloro	–	√	–	–	–	–	
Bis(2-ethylhexyl) phthalate	√	–	√	–	–	√	

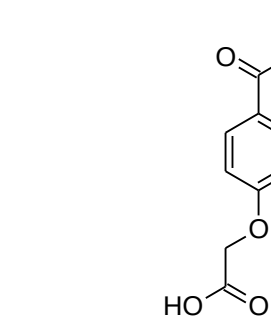
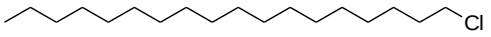
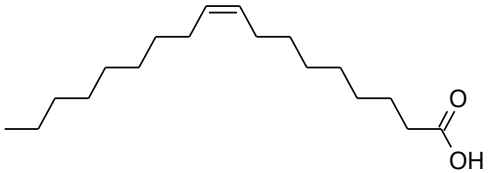
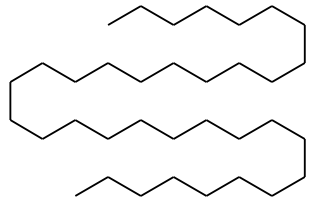
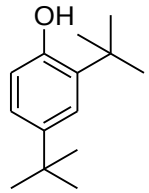
Borneol	–	√	–	–	–	–	
Campesterol	√	√	√	–	√	–	
Camphor	–	√	–	–	–	–	
Cholest-4-ene-3,6-dione	–	–	–	√	–	–	

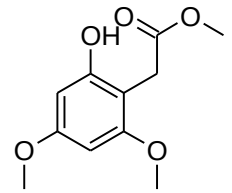
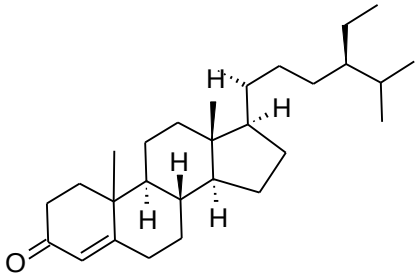
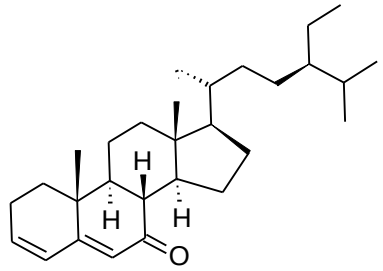
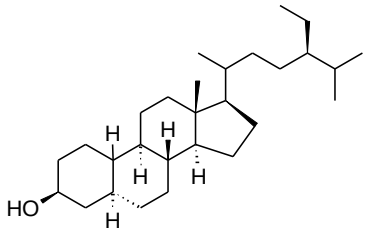
Stigmast-5-en-3-ol, (3*β*,24*S*)	√	√	√	√	√	–	
Di-butyl phthalate	–	–	–	–	–	√	
1-Dodecane	–	–	–	–	√	–	
Dodecanoic acid, 3-hydroxy	–	–	–	√	–	–	
1-Dotriacontane	–	√	–	–	√	√	

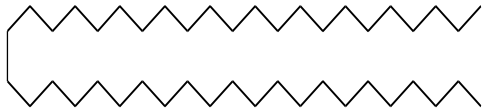
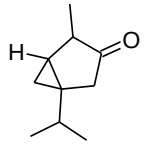
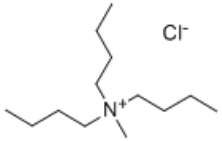
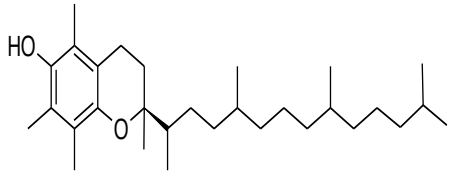
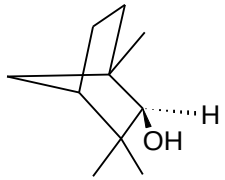
							
Ethanone, 1-(2,6-dihydroxy-4-methoxyphenyl)	–	√	–	–	–	–	
Ethanone, 1-(4-hydroxyphenyl)	–	–	–	–	√	–	
Ethyl linoleate	√	√	√	–	√	–	
Eucalyptol	–	√	–	–	–	–	

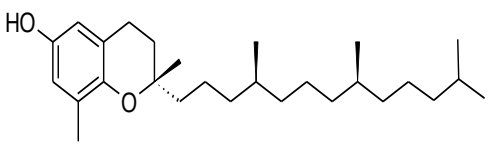
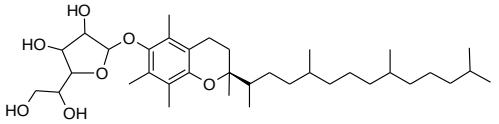
α-D-glucopyranoside, O-*α*-D-glucopyranosyl-(1.fwdarw.3)-*β*-D-fructofuranosyl	–	–	–	√	–	–	
1-Hentetracontanol	–	–	√	–	–	–	
1-Hentriacontane	√	–	–	–	–	–	
1-Heptacosane	√	–	–	–	–	–	
Heptadecane, 9-hexyl	–	–	–	–	–	√	

Hexadecanoic acid	√	√	√	√	√	–	
Hexadecanoic acid, methyl ester	–	–	–	–	√	√	
Hexanedioic acid, bis(2-ethylhexyl) ester	–	√	–	–	–	–	
Linalyl isovalerate	–	√	–	–	–	–	
1,3-Dimethoxybenzene	–	√	–	–	–	–	

Methanol, (4-carboxymethoxy)benzoyl	–	√	–	–	–	–	
Octadecane, 1-chloro	–	–	–	–	√	–	
Oleic Acid	–	√	–	√	–	–	
1-Pentatriacontane	√	–	–	–	–	–	
Phenol, 2,4-bis(1,1-dimethylethyl)	–	√	–	√	–	–	

Phenol, 3,5-dimethoxy-, acetate	–	–	–	√	–	–	
Stigmast-4-en-3-one	√	√	√	√	√	–	
Stigmast-3,5-dien-7-one	–	√	–	–	–	–	
Stigmastanol	√	–	–	–	–	–	

1-Tetratetracontane	√	–	–	–	–	–	
Thujone	–	√	–	–	–	–	
Methyl tri-butyl ammonium chloride	–	–	√	–	–	–	
α-Tocopherol	√	–	√	–	√	–	
β-Fenchyl alcohol	–	√	–	–	–	–	

δ-Tocopherol	–	–	–	–	√	–	
α-Tocopherol-*β*-D-mannoside	√	–	–	–	–	–	

√ Present; – Absent

Table 2.8: Biological activities of active principles present in different root extracts of *R. imbricata*

Phyto-constituents	**Biological activity**
Eicosen-1-ol, *cis*-9	Antimalarial, antifungal, antioxidant
1-Tricosanol	Antibacterial, antifungal
9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester, (*Z*,*Z*,*Z*)	5-Alpha-reductase inhibitor, antiMS, antiacne, antialopecic, antianaphylactic, antiandrogenic, antiarteriosclerotic, antiarthritc, anticoronary, antieczemic, antifibrinolytic, antigranular, antihistaminic, antiinflammatory, antileukotriene-D4, antimenorrhagic, antiprostatitic, cancer-preventive, carcinogenic, comedolytic, hepatoprotective, hypocholesterolemic, immunomodulator, insectifuge, metastatic, nematicide, propecic
9,12-Octadecadienoic acid (*Z*,*Z*)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester	Antiinflammatory, hypocholesterolemic, cancer preventive, hepatoprotective, nematicide, insectifuge, antihistaminic, antieczemic, antiacne, 5-alpha reductase inhibitor antiandrogenic, antiarthritic, anticoronary, insectifuge
Ascaridole	Analgesic, ancylostomicide, anthelmintic, antiflatulent, antimalarial, carcinogenic, carminative, fungicide, nematicide, pesticide, plasmodicide, sedative, transdermal, trypanocide, vermifuge

Borneol	(-)-Chronotropic, (-)-inotropic, allelochemic, analgesic, antiacetylcholine, antibacterial, antibronchitic, antiescherichic, antifeedant, antiinflammatory, antiotitic, antipyretic, antisalmonella, antispasmodic, antistaphylococcic, antiyeast, CNS-stimulant, CNS-toxic, candidicide, choleretic, flavor; fungicide, hepatoprotective, herbicide, herbicide, inhalant, insect-repellent, insectifuge, irritant, myorelaxant, nematicide, perfumery, pesticide, sedative, tranquilizer
Campesterol	Antioxidant, hypocholesterolemic
Camphor	Allelopathic, analgesica, anesthetic, antiacne, antidiarrheic, antidysenteric, antiemetic, antifeedant, antifibrositic, antineuralgic, antipruritic, antiseptic, antispasmodic, CNS-stimulant, cancer preventive, carminative, convulsant, cosmetic, counterirritant, decongestant, deliriant, ecbolic, emetic, epileptigenic, expectorant, fungicide, herbicide, insect-repellent, insectifuge, irritant, nematicide, occuloirritant, P450-2B1-inhibitor, pesticide, respirainhibitor, respirastimulant, rubefacient, stimulant, transdermal, verrucolytic, vibriocide
Stigmast-5-en-3-ol, (3*β*,24*S*)	Androgenic, angiogenic, anorexic, antiadenomic, antiandrogenic, antibacterial, anticancer (breast), anticancer (cervix), anticancer (lung), antiedemic, antiestrogenic, antifeedant, antifertility, antigonadotrophic, antihyperlipoproteinaemic, antiinflammatory, antileukemic, antilymphomic, antimutagenic, antiophidic, antioxidant, antiprogestational, antiprostaglandin, antiprostatadenomic, antiprostatitic, antipyretic, antitumor (breast), antitumor (cervix), antitumor (lung), antiviral, apoptotic, artemicide, cancer-preventive, candidicide, caspase-8-inducer, estrogenic, febrifuge, gonadotrophic, hepatoprotective, hypocholesterolemic,

	hypoglycemic, hypolipidemic, pesticide, spermicide, ubiquiot, ulcerogenic
Di-butyl phthalate	Antimicrobial, Antifouling
Dodecanoic acid, 3-hydroxy	Flavor
Eucalyptol	Anesthetic, anthelmintic, antibacterial, antihalitosic, antiseptic, antitussive, decongestant, expectorant, hypotensive, insectifuge, irritant, pesticide, vermicide
α-D-glucopyranoside, O-α-D-glucopyranosyl-(1.fwdarw.3)-β-D-fructofuranosyl	Preservative
Hexadecanoic acid	Antioxidant, hypocholesterolemic, nematicide, pesticide, lubricant, antiandrogenic, flavor, hemolytic 5-alpha reductase inhibitor
Hexadecanoic acid, methyl ester	Antioxidant, nematicide, pesticide, lubricant, antiandrogenic, flavor, hemolytic 5-alpha reductase inhibitor, hypocholesterolemic
Linalyl isovalerate	Fragrance

Oleic Acid	5-Alpha-reductase-inhibitor, allergenic, anemiagenic, antialopecic, antiandrogenic, antiinflammatory, antileukotriene-D4; cancer-preventive, choleretic, dermatitigenic, flavor, hypocholesterolemic, insectifuge, irritant, percutaneostimulant, perfumery, propecic
1-Pentatriacontane	Herbistat
Stigmast-4-en-3-one	Antiprostatitic
Stigmast-3,5-dien-7-one	Antifertility
Thujone	Abortifacient, anthelmintic, antibacterial, antiseptic, antispasmodic, cerebrodepressant, convulsant, counterirritant, emmenagogue, epileptigenic, hallucinogenic, herbicide, neurotoxic, perfumery, pesticide, respirainhibitor, toxic
β-Fenchyl alcohol	Antimicrobial, antioxidant, flavor

δ-Tocopherol	5-HETE-inhibitor, allergenic, analgesic, antiMD, antiMS, antiPMS, antiaggregant, antiaging, antialzheimeran, antianginal, antiarteriosclerotic, antiarthritic, antiatherosclerotic, antibronchitic, anticancer (breast), anticariogenic, anticataract, antichorea, antichoreic, anticonvulsant, anticoronary, antidecubitic, antidementia, antidermatitic, antidiabetic, antidysmenorrheic, antiepitheleomic, antifibrositic, antiglycosation, antiherpetic, antiinfertility, antiinflammatory, antiischemic, antileukemic, antileukotriene, antilithic, antilupus, antimaculitic, antimastalgic, antimelanomic, antimyoclonic, antineuritic, antineuropathic, antinitrosaminic, antiophthalmic, antiosteoarthritic, antioxidant, antiparkinsonian, antiproliferant, antiradicular, antiretinopathic, antirheumatic, antisenility, antisickling, antispasmodic, antisterility, antistroke, antisunburn, antisyndrome-X, antithalassemic, antithrombotic, antithromboxane-B2, antitoxemic, antitumor; antitumor (breast), antitumor (colorectal), antitumor (prostate), antitumor (stomach), antiulcerogenic, apoptotic, calcium-antagonist, cancer-preventive, cardioprotective, cerebroprotective, circulatory-stimulant, circulotonic, hepatoprotective, hypocholesterolemic, hypoglycemic, immunomodulator, immunostimulant, insulin-sparing, lipoxygenase-inhibitor, NO-inhibitor, ornithine-decarboxylase-inhibitor, P21-inducer, phospholipase-A2-inhibitor, protein-kinase-C-inhibitor, vasodilator

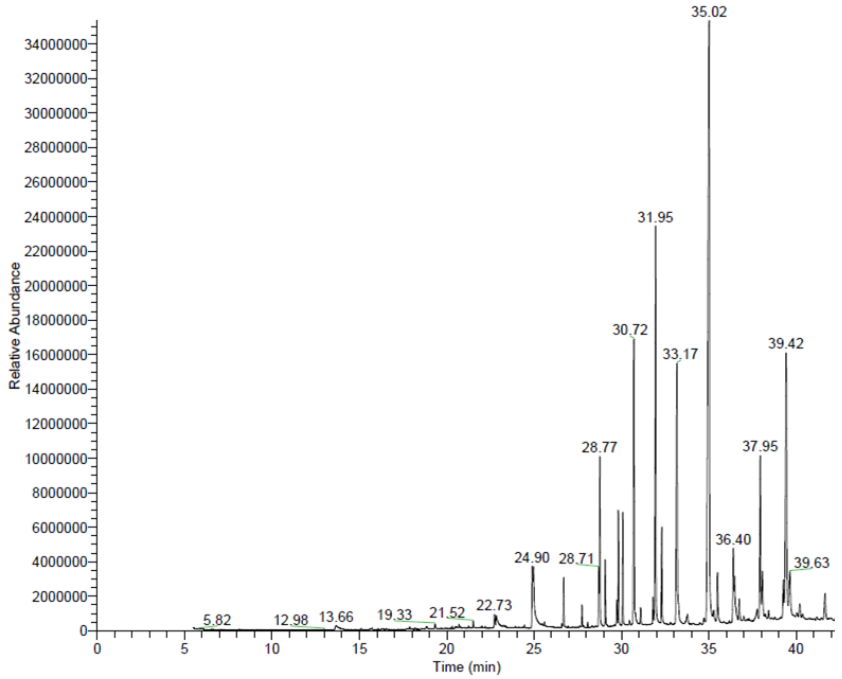


Figure. 2.1 Gas chromatogram of n-hexane root extract of *R. imbricata.*

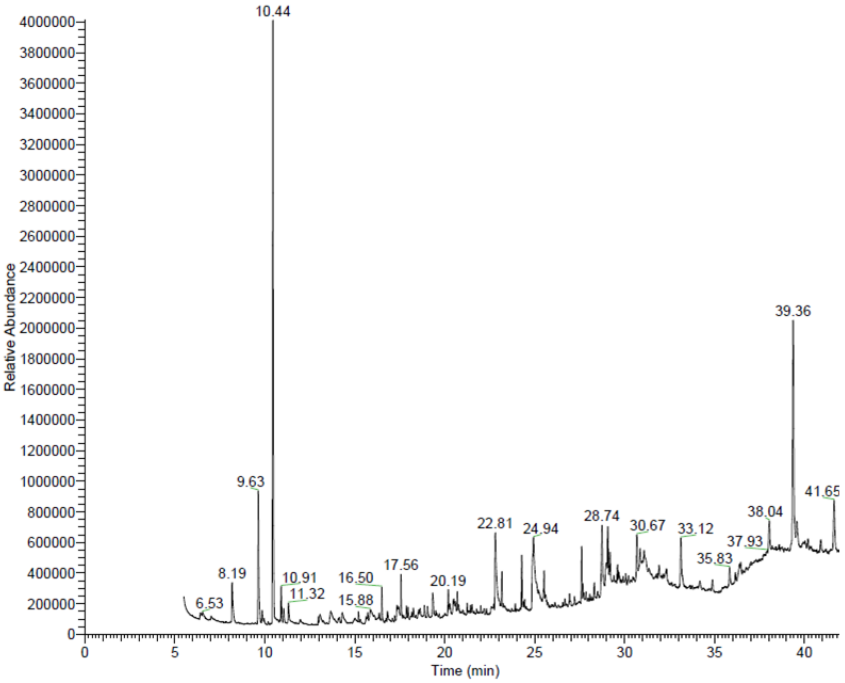


Figure. 2.2 Gas chromatogram of dichloroethane root extract of *R. imbricata*.

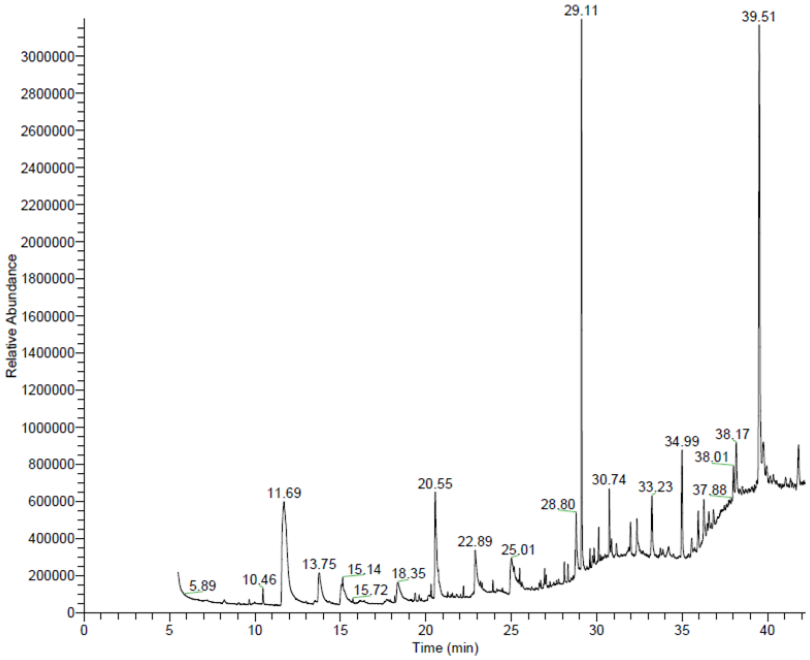


Figure. 2.3 Gas chromatogram of chloroform root extract of *R. imbricata.*

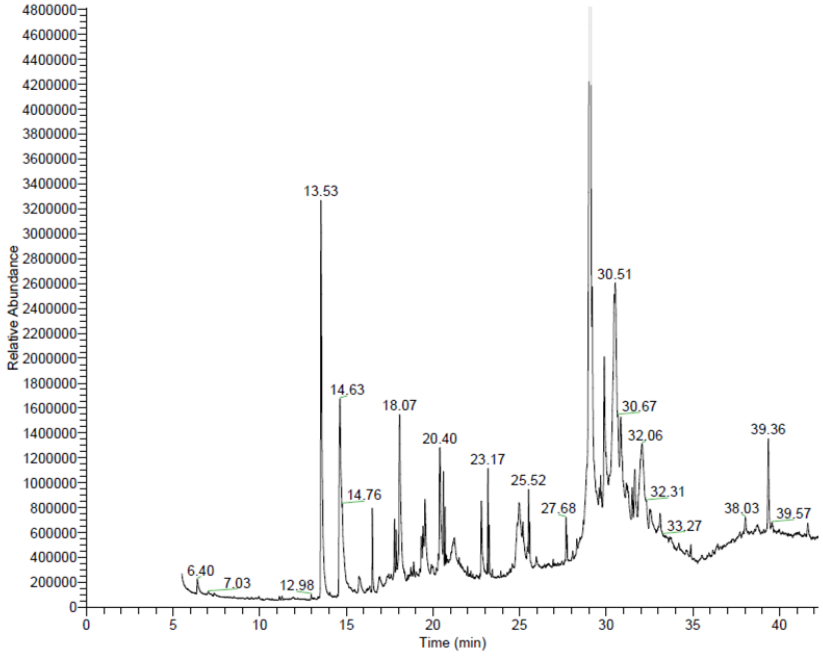


Figure. 2.4 Gas chromatogram of ethyl acetate root extract of *R. imbricata.*

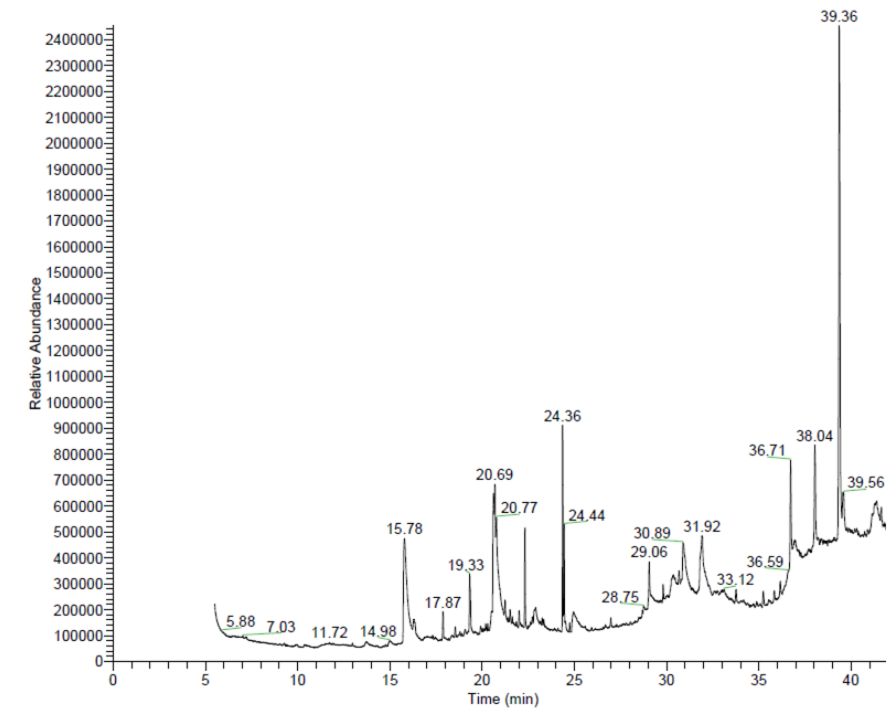


Figure. 2.5 Gas chromatogram of methanol root extract of *R. imbricata.*

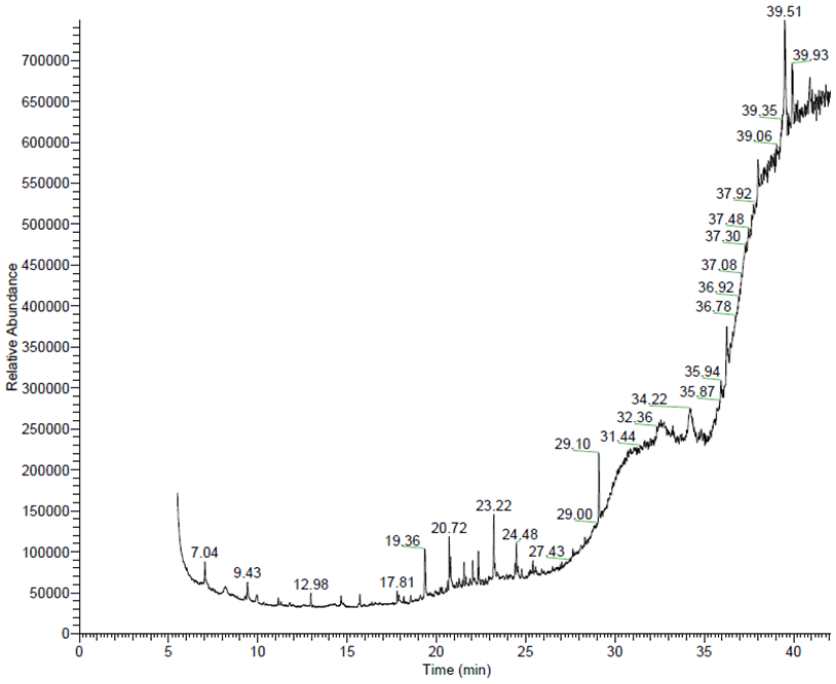


Figure. 2.6 Gas chromatogram of 60% ethanol root extract of *R. imbricata.*

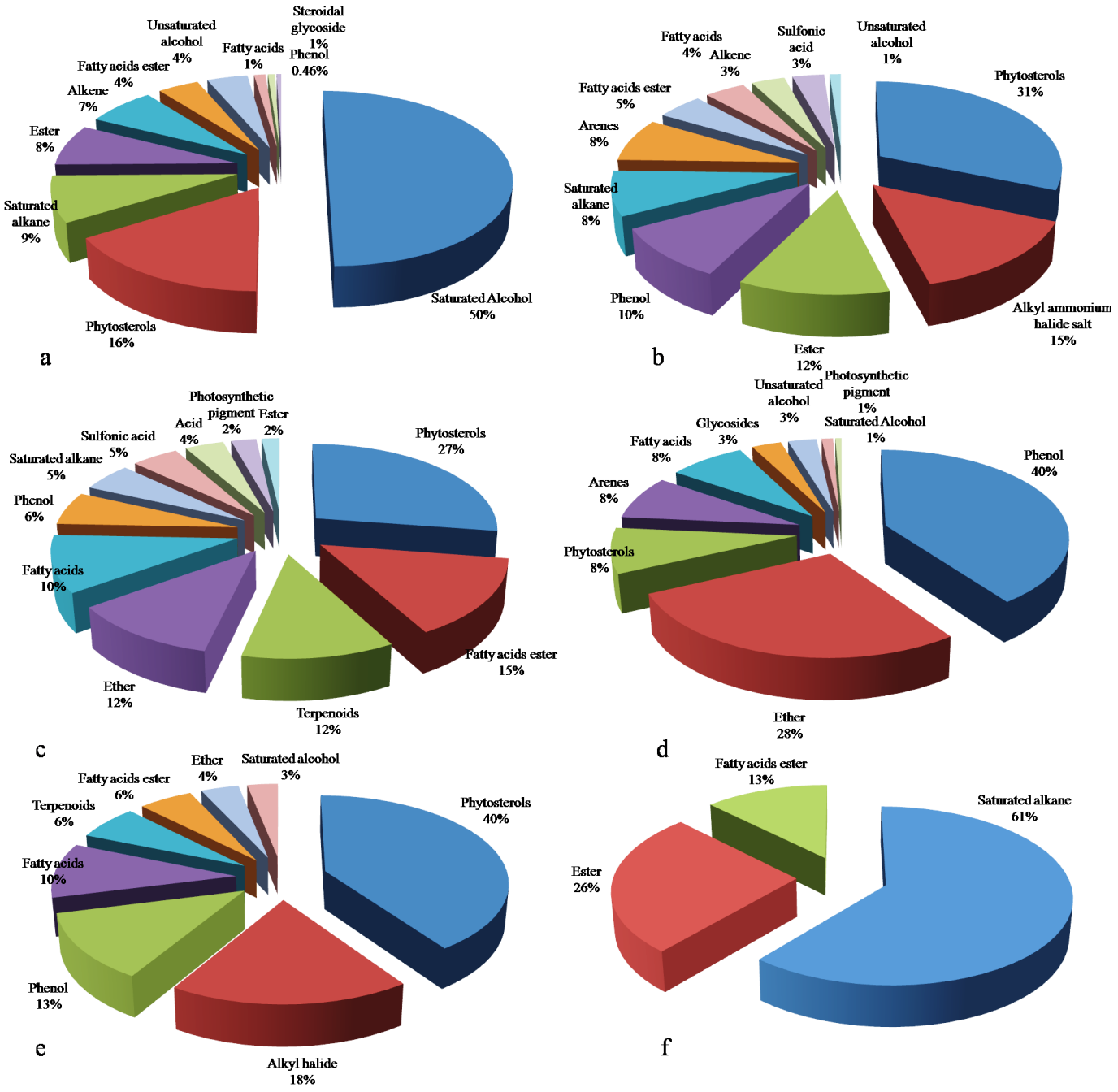


Figure. 2.7 Estimation of major phytochemical groups in different root extracts of *R. imbricata*, a) n-hexane extract, b) chloroform extract, c) dichloroethane extract, d) ethyl acetate extract, e) methanol extract, f) 60% ethanol extract.

Chawla et al., 2010; Gupta et al., 2010; Mishra et al., 2010; Kumar et al., 2010a; Kumar et al., 2010b; Gupta et al., 2009; Mishra et al., 2009; Gupta et al., 2008; Mishra et al., 2008; Arora et al., 2008; Gupta et al., 2007; Kumar et al., 2007; Mishra et al., 2006; Goel et al., 2006; Kanupriya et al., 2005; Arora et al., 2005] and the results were very promising to justify the use of this plant as therapeutic agent.

However, the phytochemical profiling of the plant root still remains unexplored and to the best of our knowledge, this is the first ever study of its kind on the GC/MS phytochemical profiling of the root extracts. In medicinal chemistry, it is very essential to ascertain the phytochemical profiling of medicinal plant parts that are responsible for its numerous pharmacological properties and by this technique we may be able to scientifically determine and validate the traditional uses, pharmacological activities, and therapeutic potential of these plant parts. Profiling of metabolites in plant extracts permits the complete phenotyping of genetically or environmentally adapted plant systems and such investigations draw on simple extraction procedures that have been shown to be very robust and have permitted broad range of high-throughput applications in plant metabolomics [Shellie et al., 2009; Roessner et al., 2001; Gullberg et al., 2004].

The major phytochemical groups in n-hexane, ethyl acetate, and 60% ethanol extracts were saturated alcohol (50%), phenols (40%), and alkanes (61%) respectively. On the other hand, phytosterols were the major group in chloroform (31%), dichloroethane (27%), and methanol (40%) extracts. The total of various volatile and semi volatile groups present in different root extracts of *R. imbricata* had the following distribution order: phytosterols (122%), alkanes (83%), phenols (69.46%), esters (48%), ethers (44%), fatty acid esters (43%), fatty acids (33%), terpenoids (18%), arenes (16%), alkyl ammonium halide salt (15%), alkenes (10%), sulfonic acid (8%), unsaturated alcohols (8%), organic acids (4%), saturated alcohols (4%), glycosides (3%), photosynthetic pigments (3%), steroidal glycoside

(1%). The order of extraction capacities of different polarity solvents for phytosterols, phenols, fatty acids, alkanes, esters, fatty acid esters, ethers, unsaturated alcohols, arenes, terpenoids, alkenes, sulfonic acid, photosynthetic pigment, and saturated alcohols was as follows:

1. Phytosterols: methanol (40%), chloroform (31%), dichloroethane (27%), n-hexane (16%), ethyl acetate (8%)
2. Phenols: ethyl acetate (40%), methanol (13%), chloroform (10%), dichloroethane (6%), n-hexane (0.46%)
3. Fatty acids: dichloroethane (10%) = methanol (10%), ethyl acetate (8%), chloroform (4%), n-hexane (1%)
4. Alkane: 60% ethanol (61%), n-hexane (9%), chloroform (8%), dichloroethane (5%)
5. Esters: 60% ethanol (26%), chloroform (12%), n-hexane (8%), dichloroethane (2%)
6. Fatty acid esters: dichloroethane (15%), 60% ethanol (13%), methanol (6%), chloroform (5%), n-hexane (4%)
7. Ethers: ethyl acetate (28%), dichloroethane (12%), methanol (4%)
8. Unsaturated alcohols: n-hexane (4%), ethyl acetate (3%), chloroform (1%)
9. Arenes: chloroform (8%) = ethyl acetate (8%)
10. Terpenoids: dichloroethane (12%), methanol (6%)
11. Alkenes: n-hexane (7%), chloroform (3%)
12. Sulfonic acid: dichloroethane (5%), chloroform (3%)
13. Photosynthetic pigment: dichloroethane (2%), ethyl acetate (1%)
14. Saturated alcohols: methanol (3%), ethyl acetate (1%)

The steroidal glycoside, alkyl ammonium halide salt, organic acids, and glycoside were found only in n-hexane (1%), chloroform (15%), dichloroethane (4%), and ethyl acetate (3%), respectively (Table 2.7, Figure. 2.7). Eventually, in the present study we have found

phytosterols, terpenoids, fatty acids, fatty acid esters, alkyl halides, phenols, alcohols, ethers, alkanes, and alkenes as the major group of Phyto-constituents in the different root extracts of *R. imbricata.* All these compounds identified by GC/MS analysis were further investigated for their biological activities [Dr. Duke's Phytochemical and Ethnobotanical Databases, 2012] and most of them were found to possess a diverse range of positive pharmacological actions (Table 2.8).

Most of the pharmacological studies were conducted with aqueous, ethanol, and hydro-alcoholic root extracts of this plant and it was found to have numerous biological activities such as anti-stress, adaptogenic, anti-hypoxic, immune-stimulatory, anti-cancer, cytoprotective, radioprotective, anti-hemolytic, anti-inflammatory, and wound healing potential [Mishra et al., 2012; Tulsawani et al., 2011; Chawla et al., 2010; Gupta et al., 2010; Mishra et al., 2010; Kumar et al., 2010a; Kumar et al., 2010b; Gupta et al., 2009; Mishra et al., 2009; Gupta et al., 2008; Mishra et al., 2008; Arora et al., 2008; Gupta et al., 2007; Kumar et al., 2007; Mishra et al., 2006; Goel et al., 2006; Kanupriya et al., 2005; Arora et al., 2005]. Our investigations conclude that the compounds present in the ethanol and water extracts have the potential to perform these functions. Though, the root extracts of the plant obtained by polar solvent extraction have been investigated for their pharmacological actions in considerable detail, non polar root extracts have not been studied till date. Hence, our primary objective in the present work was to find the bioactive constituents present in the non polar extraction of root of this herb. These findings will definitely usher in new directions in pharmacological and therapeutic investigations with the root extracts obtained from non polar solvent extraction such as n-hexane, dichloroethane, chloroform, and ethyl acetate.

2.5 Conclusion

In the present study, sixty four phyto-constituents have been identified from n-hexane, dichloroethane, chloroform, ethyl acetate, methanol, and 60% ethanol root extracts of *R. imbricata* by GC/MS analysis. It showed the existence of various bioactive principles that confirm the application of *R. imbricata* for various ailments in traditional system of medicine. However, isolation of individual phyto-constituents and subjecting them to biological activity will definitely give fruitful results to find a novel drug. It could be concluded that *R. imbricata* contains various bioactive phyto-constituents having phyto-pharmaceutical importance. However, further studies will need to be undertaken to ascertain its bioactivity, toxicity profile, effect on the ecosystem, and agricultural products.

CHAPTER 3

ANTIOXIDANT CAPACITIES, PHENOLIC CONTENTS AND GC/MS ANALYSIS OF *RHODIOLA IMBRICATA* EDGEW. ROOT EXTRACTS FROM TRANS-HIMALAYA

Abstract

Our aim was to assess the antioxidant capacities and phenolic constituents of methanol and aqueous extracts of *Rhodiola imbricata* Edgew. root from trans-Himalayan cold desert of Ladakh. The DPPH and ABTS radical scavenging capacity of the root extracts increased in a dose dependent manner (upto 0.1 mg/ml) and root extract concentrations required for 50% inhibition of radical scavenging effect (IC_{50}) were recorded as 0.013 and 0.014 mg/ml (for DPPH) and 0.016 and 0.017 mg/ml (for ABTS) for methanol and aqueous extracts respectively. The total antioxidant power of the extract was determined by FRAP assay. Total polyphenol and phenolic acid content of methanol and aqueous extracts were 112.24, 59.06, 39.02, and 16.95 mg GAE/g of extract respectively. Total flavonoid and flavonol contents were estimated to be 30.2, 17.67, 20.68, and 7.38 mg QE/g of extract respectively. In all antioxidant capacity assays, the methanol extract exhibited significantly higher antioxidant capacity than that of aqueous extract due to the presence of significantly higher amount of vital phyto-constitiuents, *viz.* polyphenol, phenolic acid and flavonol. GC/MS analysis showed that phytosterols, alkyl halide, phenols and fatty acid esters were major phytochemical cluster. On the other hand, monoterpenes, fatty acids, tocopherols, aliphatic hydrocarbons and ethers were found to be present in comparatively less amount in the methanol extract. Hence, our study signifies the potential of this high-altitude medicinal herb as a natural source of antioxidants and supports its use in traditional system of medicine to ameliorate oxidative stress and high altitude maladies.

3.1 Introduction

Reactive oxygen species (ROS) are involved in a variety of cellular processes and they are responsible for the progression of many diseases [Bland, 1995]. Intake of bioadsorbable dietary antioxidant could prevent a large number of diseases by inhibiting or delaying the oxidation of susceptible cellular substrates [Surh, 2002]. The biological and pharmacological activities, including antioxidative, anti-inflammatory, and antiviral effects of natural products have been reported from epidemiological evidences and these activities result from the active phytochemical constituents present in these products [Larson, 1988; Stanner et al., 2004; Ali et al., 2008].

Rhodiola imbricata (rose root or arctic root), belonging to the family Crassulaceae, is an important food crop and medicinal plant in the high altitude region of Indian trans-Himalayan cold desert. It is a popular medicinal plant in India, Pakistan, Nepal, Tibet, China, and many other countries and is widely used as food and folk medicine around the world. A number of metabolites like phenylpropanoids, phenylethanol derivatives, flavanoids, monoterpernes, triterpenes, and phenolic acids were found in good yield from *Rhodiola* sp. and these have been shown to activate a number of physiological functions including neurotransmitter levels, central nervous system activity, and cardiovascular function. Many pharmacological studies have demonstrated that this plant exhibits cardioprotective, antiinflammatory, antipyretic, antistress, and adaptogenic activities. It has also been found to possess antioxidant, antiaging, immuno-stimulant, anti-depressant, radioprotective, and anticarcinogenic properties [Kumar et al., 2007; Mishra et al., 2007; Chawla et al., 2010; Gupta et al., 2010; Kumar et al., 2010a, b]. It is being used to stimulate the nervous system, decrease depression, enhance work performance, eliminate fatigue, and prevent high-altitude sickness. Most of these effects have been ascribed to phenolics

such as salidrosides (rhodiolosides), p-tyrosol and glycosides like rosavins [Khanum et al., 2005]. These reports support its use in traditional system of medicine and are the basis of developing a number of plant based products from our institute in recent years. Many of them contain the roots of this plant species and were found to possess high antioxidant capacities [Ballabh et al., 2007; Dhar et al., 2012]. However, the *in vitro* antioxidant capacities of the methanolic and aqueous extracts of *R. imbricata* were not studied in detail with respect to its antioxidant potential. Hence, aim of the present investigation was to determine the antioxidant capacities, total phenolic, and flavonoid contents of methanol and aqueous extracts of *R. imbricata* roots from trans-Himalayan cold desert of Ladakh. Additionally, the root methanol extract was selected for gas chromatography-mass spectroscopy (GC/MS) study to validate its prominent antioxidant capacity.

3.2 Materials & methods

3.2.1 *Chemicals and reagents*

1,1-diphenyl-2-picrylhydrazyl radical ($DPPH^{\cdot}$), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,4,6-tripyridyl-*s*-triazine (TPTZ), potassium ferricyanide [$K_3Fe(CN)_6$], trichloroacetic acid, $FeSO_4.7H_2O$, $AlCl_3$, sodium acetate, sodium bicarbonate (Na_2HCO_3), potassium persulphate ($K_2S_2O_8$), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), ascorbic acid (AA), quercetin, β-carotene, and $FeCl_3$ were purchased from Sigma-Aldrich (St. Louis, MO, USA). Folin-Ciocalteu's phenol reagent, mercuric chloride, potassium iodide, iodine, copper sulphate, potassium sodium tartarate, sodium hydroxide, potassium hydroxide, ferric chloride, sodium citrate, lead acetate, hydrochloric acid, sulphuric acid, methanol, chloroform, ethanol, and sodium carbonate were from Merck Chemical

Supplies (Merck KGaA, Darmstadt, Germany). All the other chemicals used including solvents, were of analytical grade.

3.2.2 *Ethics statement*

All necessary permits were obtained for the described field studies. The permit was issued by Dr. B. Balaji (IFS), Divisional Forest Officer, Leh Forest Division, Jammu & Kashmir, India.

3.2.3 *Plant materials and extraction*

Rhodiola imbricata roots were collected from the trans-Himalayan region (Chang-La Top, altitude = 5330 m above mean sea level) of India in the month of October, 2011 after the period of senescence. The plant roots were washed thoroughly, and cut into small pieces and shade dried at room temperature for 15 days. Then it was finely powdered and used for extraction. The root powder (20000 mg) was taken for extraction in methanol and water by Soxhlet apparatus (Borosil GlassWorks Limited, Worli, Mumbai, India). The methanol and aqueous fractions were concentrated under vacuum and reduced pressure at 40°C by circulation of cold water using thermostat maintained at 4°C in order to minimize the degradation of thermolabile compounds, lyophilized and stored at −80°C freezer.

3.2.4 *DPPH and ABTS radical scavenging assay*

The effect of root extracts on DPPH radical was determined using the method of Liyana-Pathiranan and Shahidi [2005]. A solution of 0.135 mM DPPH in methanol was prepared and 1 ml of this solution was mixed with 1 ml of root extracts. Plant root extracts contained 0.02–0.10 mg of the dried extract. The reaction mixture was vortexed thoroughly and left in dark at room temperature for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. The ABTS assay was performed as described by Re et al. [1999]. The stock solutions

included 7 mM ABTS solution and 2.4 mM potassium persulfate (PPS) solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12h at room temperature in dark. The solution was then diluted by mixing 1 ml $ABTS^{\cdot+}$ solution with 60 ml of methanol/water to obtain an absorbance of 0.706 ± 0.001 units at 734 nm using spectrophotometer (Spectramax M2^{e}, Molecular Devices, Germany). Plant root extracts (1 ml) were allowed to react with 1 ml of the $ABTS^{\cdot+}$ solution and the absorbance was taken at 734 nm after 7 min. The scavenging capacity of the extract was compared with that of quercetin and BHT. The percentage inhibition was calculated as follows:

Radical scavenging capacity (%) = $[(Abs_{control} - Abs_{sample})] / (Abs_{control})] \times 100$

Where, $Abs_{control}$ is the absorbance of DPPH/ABTS radical + methanol/water; Abs_{sample} is the absorbance of DPPH/ABTS radical with sample extract or standard. The half maximal inhibitory concentration (IC_{50}), the amount of antioxidant required to decrease the initial DPPH concentration by 50%, termed as efficiency concentration (EC_{50}), and the effectiveness of antioxidant and radical scavenging capacity demonstrated as antiradical power (ARP) were calculated as described by Prakash et al. [2007], Kroyer [2004], and Dajanta et al. [2011]. The IC_{50} value was calculated from the regression equation between sample concentration and rate of inhibition. The EC_{50} was calculated from the following formula:

$EC_{50} = IC_{50}$ / [DPPH] in mg/ml.

The ARP was also determined as follows:

$ARP = 1 / (EC_{50} \times 100)$

3.2.5 ***Total antioxidant capacity (FRAP Assay) and reducing power assay***

A modified method of Benzie and Strain [1996] was adopted for the ferric reducing antioxidant power (FRAP) assay. The stock solutions included 300 mM acetate buffer (3.1 g

CH_3COONa and 16 ml CH_3COOH), pH 3.6, 10 mM TPTZ (2,4,6-tripyridyl-*s*-triazine) dissolved in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. The fresh working FRAP solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ, and 2.5 ml $FeCl_3 \cdot 6H_2O$. Plant extracts (150 µl) were allowed to react with 2850 µl of the FRAP solution for 30 min in dark. Observations of the colored product (ferrous tripyridyltriazine complex) were measured as absorbance at 593 nm. The calibration curve was prepared from the equation $y = 0.058x + 0.048$, $R^2 = 0.913$, where x was absorbance and y was $FeSO_4$ concentration (µM). The standard curve was linear between 200 and 1000 µM $FeSO_4$. Results were expressed in µM Fe (II)/g of extract and compared with that of AA, BHA and BHT.

The reducing power of root extracts was estimated by a method described by Yen and Chen [1995]. Plant root extracts (100-1000 µg/ml) were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide [$K_3Fe(CN)_6$] (1%, w/v). The mixture was incubated at 50^oC for 30 min. After that 2.5 ml of trichloroacetic acid (10%, w/v) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, 2.5 ml of upper layer solution was mixed with 2.5 ml distilled water and 0.5 ml $FeCl_3$ (0.1%, w/v), and the absorbance was measured at 700 nm.

2.2.6 ***Phytochemical screening***

Phytochemical screening of methanol and aqueous extracts of *R. imbricata* roots were conducted by standard qualitative analytical methods for alkaloids [Wagner, 1993; Evans, 1997; Harborne, 1998], carbohydrates [Deb, 2001], saponins [Kokate, 1999], proteins [Gahan, 1984], phenolics [Mace, 1963; Raaman, 2006] and flavonoids [Shinoda, 1928; Harborne 1998].

3.2.7 ***Total polyphenol, phenolic acid, flavonoid, and flavonol assay***

Total polyphenol content of plant root extracts were measured using Folin-Ciocalteu colorimetric method as described previously by Gao et al. [2000]. The root extracts (100 µl) were mixed with 0.2 ml of Folin-Ciocalteu's reagent and 2 ml of H_2O, and incubated at room temperature for 3 min. Following the addition of 1 ml of 20% (w/v) sodium carbonate to the mixture, total polyphenol was determined after 1h of incubation at room temperature. The absorbance of the resulting blue color was measured at 765 nm. Quantification was done with respect to the standard curve of gallic acid. Polyphenol contents were expressed as gallic acid equivalent (GAE) (mg/g) using the following equation based on the calibration curve: $y = 0.003x + 0.058$, $R^2 = 0.986$, where x was absorbance and y was GAE mg/g of extract at a final concentration of 0.1 mg/ml. The standard curve was linear between 0.02 and 0.1 mg gallic acid.

Total phenolic acid assay was carried out using Folin-Ciocalteu's reagent as depicted by Marinova et al. [2005]. One milliliter of root extract was added into a flask containing 9 ml of distilled water. Then 1 ml of Folin-Ciocalteu's phenol reagent was added and the mixture was thoroughly mixed. After 5 min of incubation, 10 ml of 7% (w/v) Na_2CO_3 were added. Then the mixture was diluted to 25 ml with the addition of 4 ml of distilled water. Then the mixture was incubated at room temperature for 90 min. Finally, the absorbance was measured using spectrophotometer at 750 nm. Phenolic acid contents were expressed as GAE (mg/g) using the following equation based on the calibration curve: $y = 0.003x + 0.058$, $R^2 = 0.986$, where x was absorbance and y was GAE mg/g of extract at a final concentration of 0.1 mg/ml. The standard curve was linear between 0.02 and 0.1 mg gallic acid.

Estimation of the total flavonoid in the root methanol and aqueous extracts was carried out using the method of Ordon et al. [2006]. Briefly, to 0.5 ml of sample, 0.5 ml of 2% (w/v)

$AlCl_3$ ethanol solution was added. The contents were incubated for 1h at room temperature and the absorbance was measured at 420 nm. Total flavonoid content was calculated as quercetin equivalent (QE) (mg/g) using the following equation based on the calibration curve: y = 0.009x + 0.137, R^2 = 0.992, where x was absorbance and y was QE (mg/g of extract) at a final concentration of 0.1 mg/ml. The standard curve was linear between 0.02 and 0.1 mg quercetin.

Total flavonol in the plant extracts were estimated using the method of Kumaran and Karunakaran [2007]. To 2 ml of root extract, 2 ml of 2% $AlCl_3$ ethanol and 3 ml (50 g/l) sodium acetate solutions were added. The absorption at 440 nm was measured after 2.5h at 20°C. Total flavonol content was also calculated as QE (mg/g) using the following equation based on the calibration curve: y = 0.009x + 0.137, R^2 = 0.992, where x was absorbance and y was QE (mg/g of extract) at a final concentration of 0.1 mg/ml. The standard curve was linear between 0.02 and 0.1 mg quercetin.

3.2.8 ***GC/MS analysis***

Methanol extract (25 mg) was dissolved in 25 ml of the solvent, vortexed properly and filtered through 0.22 µm syringe filter (Millipore Corp., Bedford, MA, USA). One microliter aliquot of the sample solution was injected into the Thermo Finnigan PolarisQ Ion Trap GC/MS MS system comprising of an AS2000 liquid autosampler (Thermo Finnigan, Thermo Electron Corporation, Austin, TX, USA) and the peaks in the chromatogram were identified on the basis of their mass spectra. The gas chromatograph interfaced to a mass spectrometer (GC/MS) instrument employing the following conditions *viz.* Durabond DB-5ms column (30 m × 0.25 mm × 0.25 µm), operating in electron impact [electron ionisation positive (EI^+)] mode at 70 eV, helium (99.999%) was used as carrier gas at a constant flow of 1 ml/min and an injection volume of 0.5 EI was employed (split ratio of 10:1), injector temperature was 280°C, transfer line

temperature was 300°C. The oven temperature was programmed from 50°C (isothermal for 2 min), with gradual increase of 10°C/min, to 300°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 s; and full mass scan range from 25 m/z to 1000 m/z. The data acquisition was performed on Finnigan Xcalibur data acquisition and processing software version 2.0 (ThermoQuest, LC and LC/MS Division, San Jose, California, USA). Interpretation on mass spectrum of GC/MS was done using the database of National Institute of Standards and Technology (NIST) database. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight, and structure of the components of the test material were finally ascertained.

3.2.9 *Statistical analysis*

All the experimental results are expressed as mean ± standard deviation (SD) using statistical analysis with SPSS (Statistical Program for Social Sciences, SPSS Corporation, Chicago, IL) version-17. Analysis of variance (ANOVA) in a completely randomized design, Duncan's multiple range test and Pearson's correlation coefficients were performed to compare the data. Post hoc analysis was performed using Neumen Keul's Test, and values with $p < 0.05$ were considered statistically significant.

3.3 Results and Discussion

3.3.1 *DPPH radical scavenging capacity*

The DPPH radical has been used as a stable free radical to determine the antioxidant capacity of natural compounds [Shimada et al., 1992; Ramnik et al., 2008]. The free radical scavenging capacity of methanol and aqueous extracts of *R. imbricata* root and the two positive controls *viz.* quercetin and BHT were compared. The IC_{50} values were found to be 0.013, 0.014, 0.016 and 0.013 mg/ml for methanol extract, aqueous extract, quercetin, and BHT respectively.

The DPPH scavenging capacities of the root methanol extract, aqueous extract, quercetin and BHT expressed as EC_{50} value, were 0.24, 0.26, 0.30 and 0.24 mg/ml respectively. The ARP values of the methanol and aqueous extracts were also calculated and were found to be 409.49 and 380.24 respectively. The DPPH activity of root methanol and aqueous extracts were found to increase in a dose dependent manner. The root extracts at the used concentrations exhibited potential effect of DPPH activity as percentage of free radicals inhibition (Table 3.1). A higher DPPH radical scavenging capacity is associated with a lower IC_{50} value. These results suggested that the root methanol and aqueous extracts contained strong free radical scavenging capacity. The methanol extract showed significantly higher radical scavenging capacities than that of aqueous extract at 0.01–0.1mg/ml concentrations.

3.3.2 ***ABTS radical scavenging capacity***

The $ABTS^{\cdot+}$ radical scavenging capacity (%) of the methanol and aqueous extracts of *R. imbricata* root compared to quercetin and BHT is depicted in Table 3.1. The methanol and aqueous extracts of *R. imbricata* root scavenged the ABTS radical in a dose dependent manner being similar to that of the positive controls *viz.* quercetin and BHT at concentration range of 0.02–0.1 mg/ml. The quantities of methanol and aqueous root extracts of *R. imbricata* required to produce 50% inhibition (IC_{50}) of ABTS radical were found to be 0.016 and 0.017 mg/ml respectively analogous to the IC_{50} by quercetin and BHT (Table 3.1). The EC_{50} and ARP values of root extracts were also comparable to the standards. EC_{50} of root methanol and aqueous extracts were found to be 0.25 and 0.27 mg/ml respectively. Quercetin and BHT were found to produce EC_{50} at concentrations of 0.27 and 0.25 mg/ml respectively. The ARP value of the root methanol and aqueous extracts were 400.08 and 376.55 respectively (Table 3.1). The ABTS and

DPPH radical scavenging capacities of the methanol extract were significantly higher than that of aqueous extract at 0.02–0.1mg/ml concentrations.

3.3.3 ***Total antioxidant power***

The total antioxidant power assay [Benzie and Strain 1996] was used in the present study to determine the ability of the root extracts to reduce ferric ions. An antioxidant capable of providing a single electron to the ferric-TPTZ [Fe(III)-TPTZ] complex would cause the reduction of this complex into the blue ferrous-TPTZ [Fe(II)-TPTZ] complex which absorbs strongly at 593 nm. The FRAP values of root methanol and aqueous extracts were found to be 280.41 and 200.1 µM Fe (II)/g of extract respectively. The FRAP values for the extracts were significantly lower than that of AA, BHA, and BHT (Table 3.2).

3.3.4 ***Reducing power***

We examined Fe^{3+}–Fe^{2+} transformation in the presence of crude extracts and AA for measurements of reducing power. The reducing power increases with increasing concentrations of the extract that was similar to the antioxidant activity. The reducing ability of root extracts increased with an increase in concentration when compared with the positive standard, AA (Figure 3.1). In general, the reducing ability of a compound depends on the presence of reductones [Duh, 1998], which exhibit antioxidative capacity, by breaking the free radical chain and donating a hydrogen atom [Gordon, 1990]. The presence of reductants (i.e. antioxidants) in the extract fractions causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, the Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. The methanol and aqueous extracts of *R. imbricata* root at all concentrations exhibited higher antioxidant activities when compared with control, which were found to be statistically significant ($p < 0.05$). The methanol extract at all concentrations exhibited higher reducing

power in comparison to aqueous extract which were found to be statistically significant ($p < 0.05$).

3.3.5 ***Phytochemical screening***

The phytochemical screening revealed the presence of carbohydrtates, saponins, proteins, phenols, and flavonoids in the aqueous root extract. Carbohydrates, proteins, and saponins were found to be absent in the methanol extract. The methanol extract was found to contain phenols and flavonoids as major phyto-components.

3.3.6 ***Total polyphenol, phenolic acid, flavonoid, and flavonol contents***

It has been well documented that the phenolic content of plant materials is strongly associated with their antioxidant capacity [Zheng and Wang, 2001; Skerget et al., 2005]. Plant derived phenolics are well-known natural antioxidants and can contribute directly to the antioxidative action [Duh et al., 1999]. Applications of phenolics are increasing rapidly in the food industry to enhance the nutritional value and quality of food [Aneta et al., 2007]. In the present study, the content of polyphenol in methanol and aqueous extracts of *R. imbricata* root was found to be 112.24 and 59.06 mg GAE/g of extract respectively. Total phenolic acid content in methanol and aqueous root extracts were 39.02 and 16.95 mg GAE/g of extract respectively (Table 3.3). The total polyphenol and phenolic acid contents were significantly higher in the methanolic extract when compared with the aqueous extract. Results obtained from the current investigation strongly recommend that phenolics are vital components of the root extracts of *R. imbricata* and the pharmacological antioxidant properties of this plant root may possibly be due to these constituents.

Flavonoids are natural phenolic compounds and recognized antioxidants. Several studies revealed that antioxidant capacities of plant extracts which were rich in flavonoids were

moderately high [Cakir et al., 2003]. The concentration of flavonoid in the methanol and aqueous extracts of *R. imbricata* root were found to contain 30.2 and 20.68 mg QE/g of extract respectively.

Flavonols are the active constituents in a variety of fruits and vegetables and possess high antioxidant and antiradical capacity with various therapeutic applications [Peterson and Dwyer,

Table 3.1 DPPH and ABTS radical-scavenging capacities of root extracts of *R. imbricata*.

Concentration (mg/ml)	Inhibition (%)							
	DPPH radical-scavenging capacity				ABTS radical-scavenging capacity			
	Methanol extract	Aqueous extract	Quercetin	BHT	Methanol extract	Aqueous extract	Quercetin	BHT
0.002	7.09 ± 0.47	6.84 ± 0.55	6.49 ± 0.31	7.32 ± 0.81	6.42 ± 0.37	5.81 ± 0.75	5.62 ± 0.56	5.92 ± 0.86
0.005	17.66 ± 0.023	16.98 ± 0.63	15.12 ± 0.58$^{*\#}$	17.45 ± 0.86$^{\$}$	15.75 ± 0.16	14.88 ± 0.56	13.65 ± 0.73$^{*\#}$	14.63 ± 0.73
0.01	35.58 ± 0.29	34.69 ± 0.37*	31.77 ± 0.32$^{*\#}$	36.97 ± 0.45$^{*\#\$}$	30.24 ± 0.44	29.78 ± 0.68	28.12 ± 0.71$^{*\#}$	29.43 ± 0.28$^{\$}$
0.02	72.24 ± 0.65	70.44 ± 0.48*	65.04 ± 1.06$^{*\#}$	74.58 ± 0.56$^{*\#\$}$	65.52 ± 0.65	60.42 ± 0.31*	57.39 ± 0.88$^{*\#}$	60.27 ± 1.14$^{*\$}$
0.04	76.69 ± 0.54	74.22 ± 0.73*	75.95 ± 0.66$^{\#}$	79.85 ± 0.98$^{\$}$	68.23 ± 0.32	64.45 ± 0.35*	63.58 ± 0.59$^{*\#}$	67.78 ± 0.35$^{\#\$}$
0.06	82.21 ± 0.76	78.46 ± 0.75*	78.77 ± 0.65	80.69 ± 0.86$^{\#\$}$	72.21 ± 0.56	69.63 ± 0.97*	68.25 ± 1.05*	71.28 ± 0.45$^{\#\$}$
0.08	85.44 ± 0.58	81.45 ± 0.48*	80.45 ± 0.23*	81.68 ± 0.78$^{*\$}$	76.08 ± 0.86	73.65 ± 0.68*	75.29 ± 0.44$^{\#}$	79.82 ± 0.66$^{*\#\$}$
0.1	89.82 ± 0.63	86.62 ± 0.64*	86.61 ± 0.74*	89.76 ± 1.32	82.24 ± 0.74	77.88 ± 0.32*	79.21 ± 0.87*	82.19 ± 0.84$^{\#\$}$
IC_{50} (mg/ml)	0.013	0.014	0.016	0.013	0.016	0.017	0.017	0.016
EC_{50} (mg/ml)	0.24	0.26	0.30	0.24	0.25	0.27	0.27	0.25
ARP values	409.49	380.24	332.71	409.49	400.08	376.55	376.55	400.08

$p < 0.05$: * compared with methanol extract; # compared with aqueous extract; $ compared with quercetin

BHT: Butylated hydroxytoluene

Table 3.2 Total antioxidant capacity of *R. imbricata* root extracts.

Sample	FRAP (μM Fe (II)/g of extract)
Methanol extract	280.41 ± 33.08
Aqueous extract	200.1 ± 4.4*
AA	4421.44 ± 402.86*#
BHA	4118.11 ± 706.07*#
BHT	3918.14 ± 496.62*#

$p < 0.05$: * compared with methanol extract; # compared with aqueous extract

AA: Ascorbic acid

BHA: Butylated hydroxyanisole

BHT: Butylated hydroxytoluene

Table 3.3 Polyphenol, phenolic acid, flavonoid and flavonol contents of *R. imbricata* root extracts.

Phyto-components	**Methanol extract**	**Aqueous extract**
Total polyphenol (mg gallic acid/g of extract)	112.24 ± 6.62	59.06 ± 3.67*
Total phenolic acid (mg gallic acid/g of extract)	39.02 ± 2.13	16.95 ± 0.97*
Total flavonoid (mg quercetin/g of extract)	30.2 ± 1.29	20.68 ± 0.93*
Total flavonol (mg quercetin/g of extract)	17.67 ± 1.17	7.38 ± 0.56*

$p < 0.05$: * compared with methanol extract

Table 3.4 GC/MS phytochemical profile of methanolic root extract of *R. imbricata*.

S. No.	Peak RT (Min)	Peak area	Peak area (%)	Compound detected	Other Name	Mol. Formula	Mol. Wt.
1	13.73	224745	0.43	3-Methoxy-5-methylphenol	Phenol, 3-methoxy-5-methyl	$C_8H_{10}O_2$	138
2	15.78	5548712	11.07	Ethanone, 1-(4-hydroxyphenyl)	4-Hydroxyacetophenone	$C_8H_8O_2$	136
3	17.87	345721	0.66	Dodecane	n-Dodecane	$C_{12}H_{26}$	170
4	19.33	1157289	2.21	Dotriacontane	n-Dotriacontane	$C_{32}H_{66}$	450
5	20.69	8405167	17.01	1-Chlorooctadecane	Octadecane, 1-chloro	$C_{18}H_{37}Cl$	288
6	22.32	905705	1.73	Hexadecanoic acid, methyl ester	Methyl palmitate	$C_{17}H_{34}O_2$	270
7	22.89	1901742	3.67	Hexadecanoic acid	Palmitic acid	$C_{16}H_{32}O_2$	256
8	24.36	1592833	3.99	2-mono Linolein	9,12-Octadecadienoic acid (*Z*,*Z*)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester	$C_{21}H_{38}O_4$	354
9	24.44	821776	1.74	1-mono Linolein	9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester, (*Z*,*Z*,*Z*)	$C_{21}H_{36}O_4$	352
10	24.96	1495805	3.35	Ethyl linoleate	Linoleic acid, ethyl ester	$C_{20}H_{36}O_2$	308
11	29.06	1614826	3.57	1,3-Dimethoxybenzene	Benzene, 1,3-dimethoxy	$C_8H_{10}O_2$	138

12	30.91	3109377	5.92	Ascaridole	2,3-Dioxabicyclo[2.2.2]oct-5-ene, 1-methyl-4-(1-methylethyl)	$C_{10}H_{16}O_2$	168
13	31.92	3533697	7.23	Unknown	–	–	–
14	33.77	174824	0.56	*δ*-Tocopherol	(2*R*)-2,8-Dimethyl-2-[(4*R*,8*R*)-4,8,12-trimethyltridecyl]-6-chromanol	$C_{27}H_{46}O_2$	402
15	36.71	4419092	8.42	*α*-Tocopherol	(2*R*)-2,5,7,8-Tetramethyl-2-[(4*R*,8*R*)-(4,8,12-trimethyltridecyl)]-6-chromanol	$C_{29}H_{50}O_2$	430
16	38.04	2615057	4.98	Campesterol	Ergost-5-en-3-ol, (3*β*,24*R*)	$C_{28}H_{48}O$	400
17	39.36	11245680	21.91	Clionasterol / *τ*-sitosterol	Stigmast-5-en-3-ol, (3*β*,24*S*)	$C_{29}H_{50}O$	414
18	41.38	902689	1.55	4-Stigmasten-3-one	Stigmast-4-en-3-one	$C_{29}H_{48}O$	412

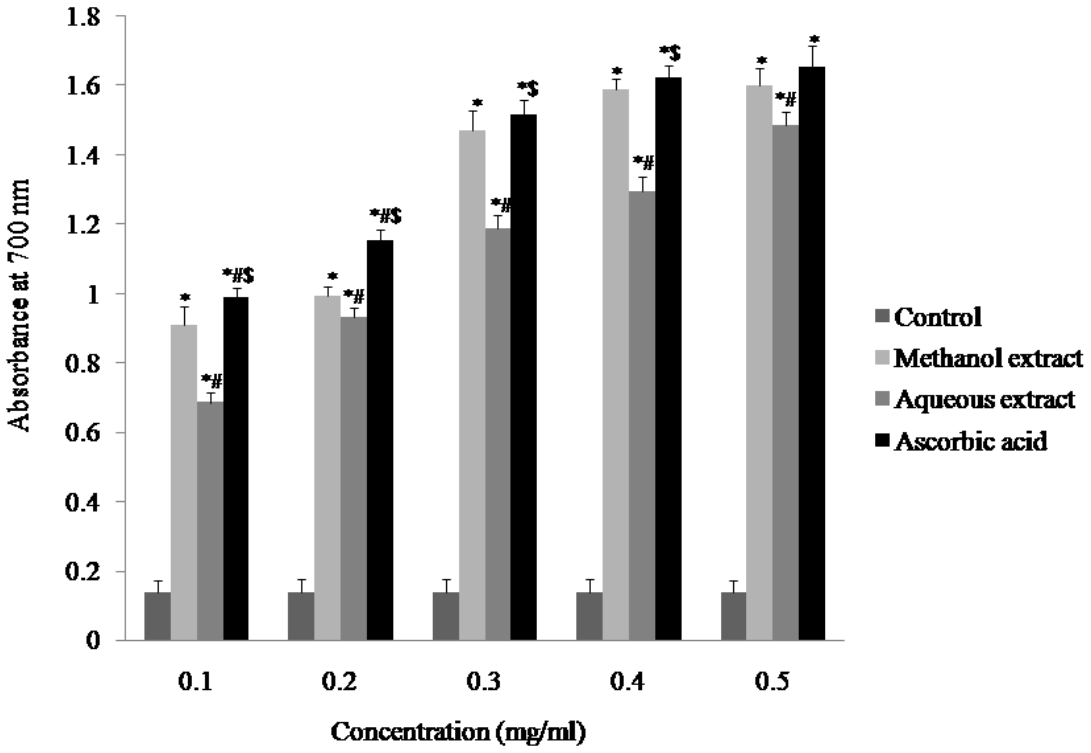


Figure 3.1 Reducing power activity of *R. imbricata* root extracts.

$p < 0.05$: * compared with control; # compared with methanol extract; $ compared with aqueous extract

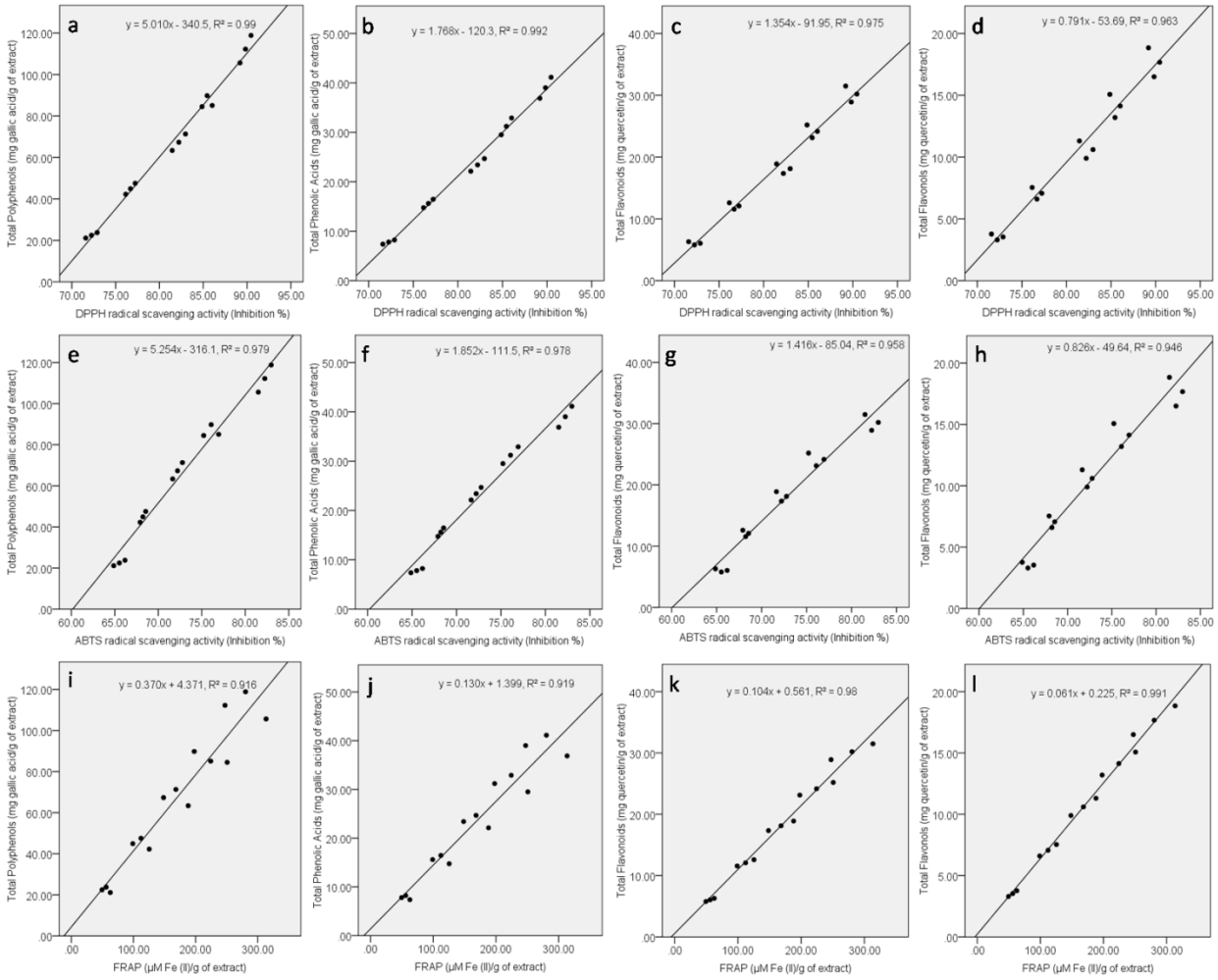


Figure 3.2 Correlations between the parameters determined in methanol extract: (a) DPPH vs. Total Polyphenol, (b) DPPH vs. Total Phenolic Acid, (c) DPPH vs. Total Flavonoid, (d) DPPH vs.Total Flavonol, (e) ABTS vs. Total Polyphenol, (f) ABTS vs. Total Phenolic Acid, (g) ABTS vs. Total Flavonoid, (h) ABTS vs.Total Flavonol, (i) FRAP vs. Total Polyphenol, (j) FRAP vs. Total Phenolic Acid, (k) FRAP vs. Total Flavonoid, (l) FRAP vs.Total Flavonol.

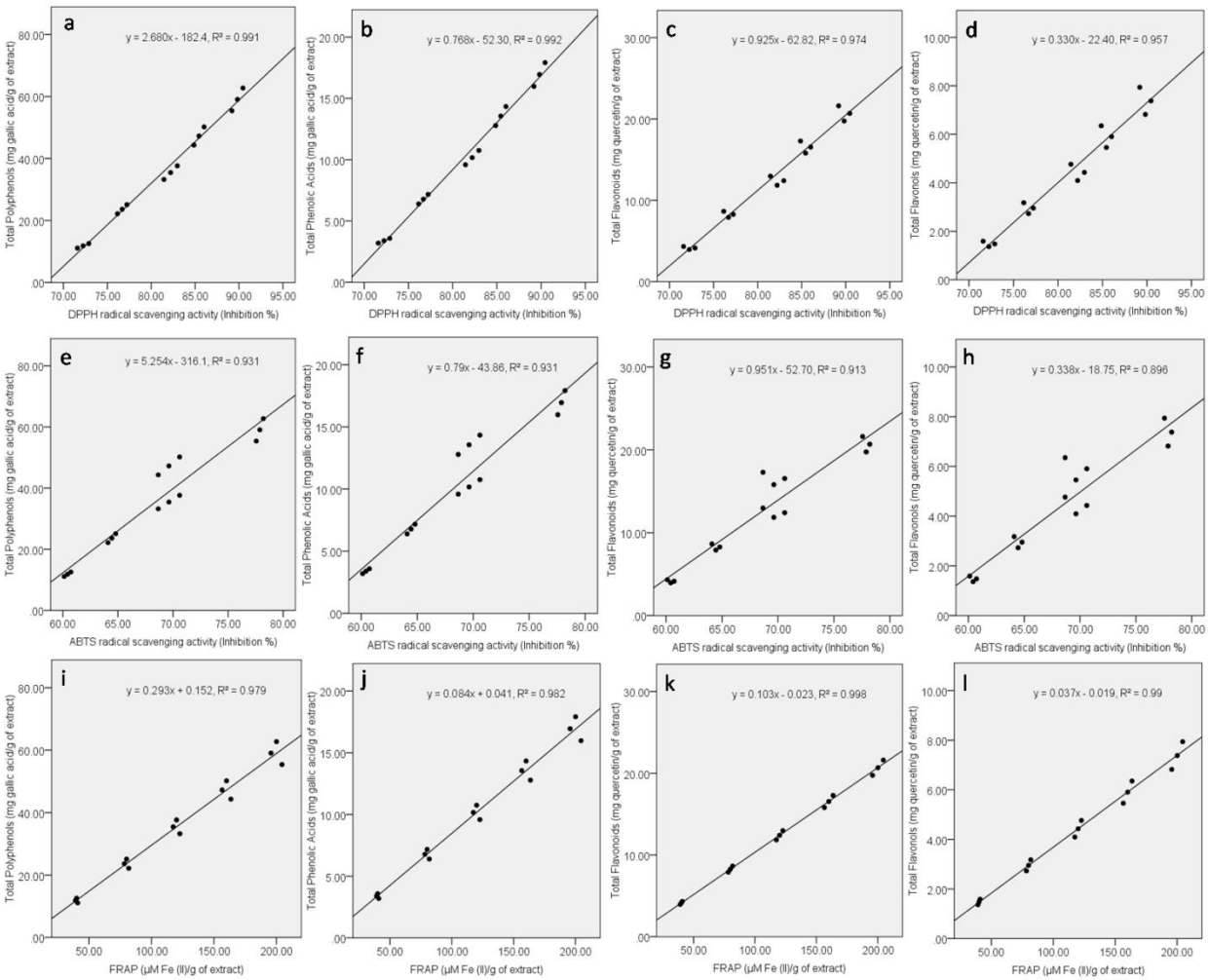


Figure 3.3 Correlations between the parameters determined in aqueous extract: (a) DPPH vs. Total Polyphenol, (b) DPPH vs. Total Phenolic Acid, (c) DPPH vs. Total Flavonoid, (d) DPPH vs.Total Flavonol, (e) ABTS vs. Total Polyphenol, (f) ABTS vs. Total Phenolic Acid, (g) ABTS vs. Total Flavonoid, (h) ABTS vs.Total Flavonol, (i) FRAP vs. Total Polyphenol, (j) FRAP vs. Total Phenolic Acid, (k) FRAP vs. Total Flavonoid, (l) FRAP vs.Total Flavonol.

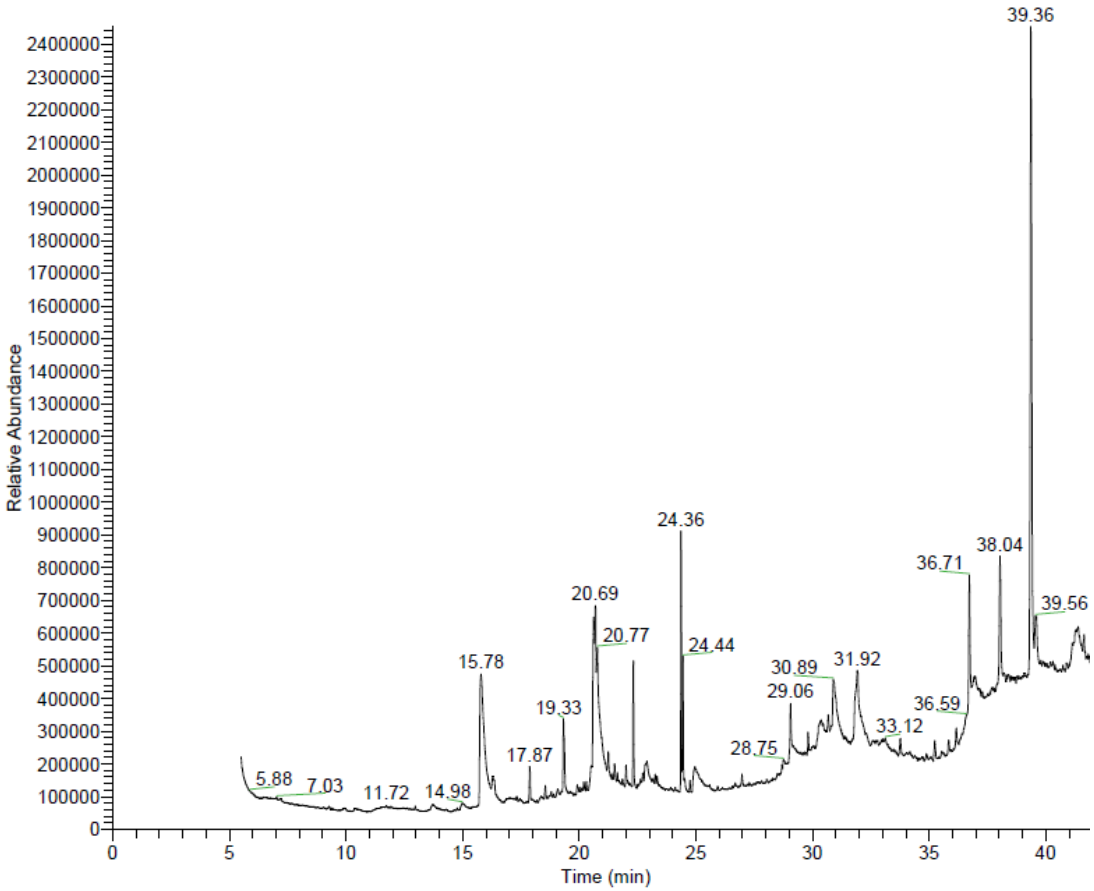


Figure 3.4 Gas chromatogram of methanol root extract of *R. imbricata*.

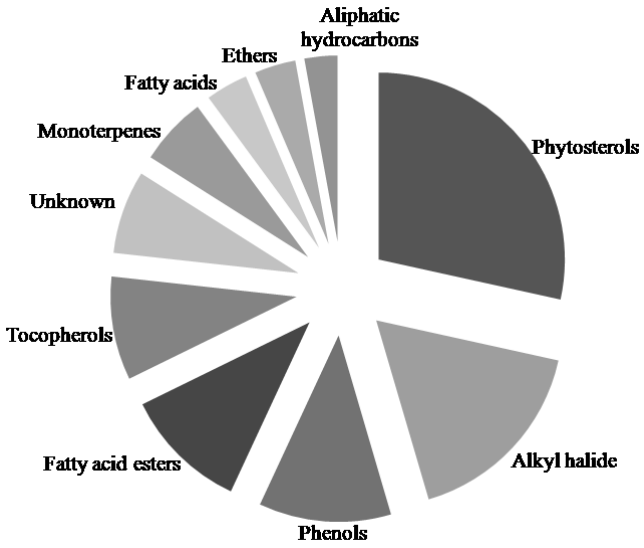


Figure 3.5 Major phytochemical groups from GC-MS analysis in methanol root extract of *R. imbricata*.

1998]. In the present study, the content of flavonol in *R. imbricata* root methanol and aqueous extracts has been depicted in Table 3.3 and it was found to be 17.67 and 7.38 mg QE/g of extract respectively. The methanol extract exhibited significantly higher flavonol concentration in comparison to aqueous extract.

Previous studies by Kumar et al. [2010a] have reported the IC_{50} of DPPH assay in *R. imbricata* root methanol extract as 0.33 mg/ml which is much higher (i.e. low antioxidant capacity) than that of our present finding. On the other hand, total phenolic content was reported to be 185 mg GAE/g extract which is significantly lower than that of our results. There were a large number of discrepancies in the previous report on the antioxidant capacities of *R. imbricata* root hydro-alcoholic extract [Kumar et al., 2010b]. In this study, the total phenol content was reported to be 49.8 mg GAE/g extract whereas the total flavonoid content was found to be 167.4 mg QE/g extract which is surprisingly higher than phenol content. Kumar et al. [2010b] reported the DPPH radical scavenging capacity and the reducing power activity of the hydro-alcoholic extract of *R. imbricata* which were significantly lower than that of our results. Interestingly, our results exhibited significantly high antioxidant capacities and the phenolic contents in our extracts showed significantly higher values than the previous reports. The reason behind these findings could be attributed to the time of collection of plant material from natural condition and their extraction process. We have collected the plant material after senescence, in the month of October, 2011 while in the previous studies the month for the collection of plant material was June (before the period of senescence). During the life cycle, plant synthesizes and utilizes various metabolites for flowering and fruiting and after senescence, plant undergoes dormancy and the bioactive constituents are deposited in the root system. Hence, it is recommended to collect the well matured root after the period of senescence to have optimum bioactivities.

Plant roots are one of the most important sources of natural antioxidants responsible for inhibiting the harmful consequences of oxidative stress. The secondary metabolites of plants include the phenolic compounds that play a crucial role in the regulation of plant growth and development in stressful environmental conditions. *R. imbricata* grows in the extreme climatic conditions of the high altitude cold desert of trans-Himalaya, with severe cold, aridity, and water scarcity. These stressful conditions in turn could result into the up-regulation of secondary metabolites synthesis pathways and the increased production of polyphenolics in the plant root system. A number of scientific reports signify that certain plant compounds *viz.* terpenoids, steroids, and phenolic compounds such as tannins, coumarins, and flavonoids have protective effects against oxidative stress owing to their antioxidant capacities [Chandrasekhar et al., 2006]. The most wide spread secondary metabolites in the plant kingdom reported so far are the phenolics and they have received much attention as potential natural antioxidant in terms of their ability to act as both efficient radical scavenger and metal chelator. Strong positive correlations were established among the antioxidant capacities (DPPH, ABTS, FRAP) and phenolic contents (total polyphenol, phenolic acid, flavonoid and flavonol) in both methanol and aqueous extracts (Figure 3.2 and 3.3). In agreement with previous reports, our results on correlations of different antioxidant assays and total phenolic contents also have shown that, the higher antioxidant capacity associated with this medicinal herb is attributed to the total phenolic compounds [Hong et al., 2008]. Thus, the outcome of the present study highlights the antioxidant capacities rendered by the root methanol and aqueous extracts of *R. imbricata* under oxidative stress conditions.

3.3.7 *GC/MS phytochemical fingerprinting of methanolic root extract*

In the present investigation the methanol extracts have shown significantly higher antioxidant capacities when compared with the aqueous extract. Hence, we decided to go for further analysis of this extract by GC/MS phytochemical fingerprinting. The chromatogram of GC/MS analysis of methanol root extract of *R. imbricata*, in accordance with the above discussed experimental conditions with a total run time of 43 min and the comparison of mass fragmentation pattern of compounds to that of in NIST library revealed the presence of 18 phytocomponents of different groups (Figure 3.4 and 3.5, Table 3.4). Among the 18 phytocomponents, clionasterol (21.91%), 1-chlorooctadecane (17.01%), 4-hydroxyacetophenone (11.07%), α-tocopherol (8.42%), unknown (7.23%), ascaridole (5.92%), and campesterol (4.98%) were found to present in major amount. On the other hand, linolein, 2-mono (3.99%), hexadecanoic acid (3.67%), 1,3-dimethoxybenzene (3.57%), ethyl linoleate (3.35%), dotriacontane (2.21%), linolein, 1-mono (1.74%), methyl palmitate (1.73%), 4-stigmasten-3-one (1.55%), dodecane (0.66%), δ-tocopherol (0.56%), and 3-methoxy-5-methylphenol (0.43%) were found to be minor components present in methanolic root extract of *R. imbricata*. These phytochemicals are well recognized for their antioxidative action [Walton and Brown, 1999; Dimitrios, 2006; Deng, 2012) and we assume that these components could also be the contributing factor for antioxidant capacity of methanol extract of *R. imbricata* root.

3.4 Conclusion

The antioxidant capacities of the methanol and aqueous extracts from the roots of *R. imbricata* were assessed in an effort to validate the medicinal potential of the underground root part of this multipurpose medicinal plant. The methanol and aqueous root extracts indicated the presence of vital phytoconstituents *viz.* polyphenol, flavonoid, and flavonol that contributed

significantly to their antioxidant capacities. A significant correlation existed between the phenolic content and antioxidant capacities of the extracts. Our statistical analysis has clearly shown that, in the DPPH, ABTS, FRAP, and reducing power assay, the methanol extract exhibited significantly higher antioxidant capacity than that of aqueous extract. The phytoconstitiuents, *viz.* polyphenol, phenolic acid, flavonoid, and flavonol were present in significantly higher amount in the methanol extract as compare to aqueous extract accounting for the significant difference in the antioxidant capacities of these two extracts. From GC/MS analysis it was revealed that phytosterols, alkyl halide, phenols, and fatty acid esters were present as the major phytochemical cluster and monoterpenes, fatty acids, tocopherols, aliphatic hydrocarbons, and ethers were found to be present in comparatively less amount in the methanol extract. Hence, from the results of our study, it can be concluded that the methanol and aqueous root extracts of *R. imbricata* may have potential health benefits and could be used as a source of natural antioxidants for pharmacological preparations. Further studies should be conducted with *in vitro* and *in vivo* systems for novel product development and human consumption to combat stressful environmental conditions.

Chapter 4

SEQUENTIAL DETERMINATION OF FAT- AND WATER-SOLUBLE VITAMINS IN *RHODIOLA IMBRICATA* EDGEW. ROOT FROM TRANS-HIMALAYA WITH LIQUID CHROMATOGRAPHY/TANDEM MASS SPECTROMETRY

Abstract

It is very challenging to develop a method for simultaneous analysis of water- and fat-soluble vitamins in complex biological matrices. We have developed a practical and rapid method to determine both types of vitamins in *Rhodiola imbricata* root which could be effective in providing an accurate quantification of all the free vitamin forms in this plant root. A simple, sensitive, low-cost and time-efficient method based on liquid chromatography/tandem mass spectrometry (LC/MS/MS) with electrospray ionization (ESI) source operating in Multiple Reaction Monitoring (MRM) mode was optimized for the sequential analysis of nine water-soluble vitamins (B_1, B_2, two B_3 vitamers, B_5, B_6, B_7, B_9, B_{12}) and six fat-soluble vitamins (A, E, D_2, D_3, K_1, K_2) in *R. imbricata* root from trans-Himalaya. Both types of vitamins were separated by ion-suppression reversed-phase liquid chromatography with gradient elution within 30 min and detected in positive ion mode. There was a minute deviation in the intra- and inter-day precision but they were always below 0.6% and 0.3% for recoveries and retention time respectively, which signifies the excellent precision for the retention time and recoveries of analytes. The retention time precision for intra- and inter-day analysis ranged between 0.009–0.172% and 0.022–0.201% respectively. The mean recoveries ranged between 88.95–107.07%, 97.67–103.55%, and 97.71–102.33% for recoveries of three different. Sensitivity and specificity of this method allowed the limits of detection (LOD) and limits of quantification (LOQ) of the analytes at ng g^{-1} levels. The linearity was achived for fat- and water-solule vitamins at 100–1000 µg L^{-1} and 10–100 µg L^{-1} respectively. Vitamin B-complex and vitamin E were detected as the principle vitamins in the root of this wonder adaptogen which would be of great interest to develop novel phyto-foods from the Indian trans-Himalaya.

4.1 Introduction

Plant-derived foods for example fruits, vegetables, grains, oils, nuts etc have become an essential part of the human diet owing to their beneficial health promoting effects (Millward, 1999; Kushi et., 1995). The Indian sub-continental diets, in particular, also consist of a diverse array of phytofoods such as cereals, pulses, green leafy vegetables, roots, tubers, fruits, oil seeds, spices and condiments (Gopalan, 2000). These botanical foods provide a large number of vital dietary components such as vitamins, antioxidant compounds, amino acids, and fatty acids, etc that are essential for human health. Vitamins are characterized by a cluster of both chemically and analytically heterogeneous compounds that may exist in numerous chemically diverse but biologically inter convertible form. These are one of the most important essential micronutrients for human nutrition and a significant number of world populations are still prone to the health threats linked with low micronutrient ingestions even after taking balanced diet (Flynn et al., 2003; Gilpin and Pachla, 2003, Southon, 1994; DellaPenna, 1999; Ball, 2006). Therefore, intake of dietary supplements with optimum vitamin contents is a useful strategy in maintaining proper health.

Vitamins can be broadly classified in two major groups, water-soluble and fat-soluble vitamins. Water-soluble vitamins include B group vitamins *viz*. thiamine (B_1), riboflavin (B_2), niacin (B_3), pantothenic acid (B_5), pyridoxine (B_6), biotin (B_7), folic acid (B_9), cyanocobalamin (B_{12}) and ascorbic acid (C) while the fat-soluble vitamins are retinol (A), ergocalciferol (D_2) and cholecalciferol (D_3), tocopherol (E), and phylloquinone (K_1) and menaquinone (K_2). These vitamins play a number of vital functions in metabolism, and can cause health problems when they are either deficient or in surplus in human body (Table 4.1).

Numerous analytical techniques such as spectrometric assays (Morelli, 1995; Ortega-Barrales et al., 1998; Monferrer-Pons et al., 2003; Tesfaldet et al., 2004), volumetric assays (Kwon et al., 2000), fluorimetry (Li and Chen, 2000; Garcia et al., 2001), chemiluminiscence (Zhou, 1991; Song and Hou, 2003), microbiological assays (Guilarte, 1991; Kall, 2003; Han and Tyler, 2003), capillary electrophoresis (Schiewe et al., 1995; Delgado-Amarreño et al., 2002; Okamoto et al., 2003), thin-layer chromatography (Diaz, 1993), high-performance liquid chromatography (HPLC) (Klejdus et al., 2004; Zafra-Goamez et al., 2006; Li and Chen, 2001; Fallon, 1987), and HPLC–mass spectrometry (LC–MS) and LC MS/MS (Leporati et al., 2005; Chen et al., 2006; Luo et al., 2006; Santos et al 2012; Gilliland et al., 2012; Gentili and Caretti, 2011) have been reported for the analysis of vitamins in biological samples. Among these techniques LC MS/MS is considered to be a powerful method for the concurrent determination of multiple vitamins in different foods and food products, nutraceuticals, and pharmaceutical preparations.

In recent time, the ultra-performance liquid chromatography (UPLC) separation strategy coupled with tandem (in space) mass spectrometric detection (MS/MS) has emerged as a more efficient separation technology as it has certain advantages over the traditional LC–MS based techniques. UPLC based separation is a well evolved strategy for a number of technological improvements such as system optimization, development of miniature particle-packed columns with small diameter, design of detector, data acquisition, and processing. These factors contribute to the system efficiency, sensitivity, reduction of analysis time, increased resolution, and higher sample throughput (Swartz,, 2005; Nováková et al., 2006). The UPLC system operates in high pressure with high mobile phase flow rate and must be coupled to an exceptionally high specificity detector with high response rate in order to instantly capture and

ionize the separated samples to obtain appropriate quantification. Triple quadrupole mass spectrometer (QqQ) operated at rapid scan rate can suitably meet this condition with high specificity and sensitivity to produce optimum results (Barcelo-Barrachina et al., 2006; Kovalczuk, 2006; Hampel et al., 2012; Stevens and Dowell, 2012; Chandra-Hioe et al., 2011; Lu et al., 2008).

Rhodiola imbricata (rose root or arctic root), belonging to the family Crassulaceae, is an important food crop and medicinal plant in the high altitude region of Indian trans-Himalayan cold desert. A number of metabolites like phenylpropanoids, phenylethanol derivatives, flavanoids, monoterpenes, triterpenes, and phenolic acids were found in good yield from *Rhodiola* sp. and some have been shown to possess useful biological activities (Kelly, 2001; Brown et al., 2002; Khanum et al., 2005; Yousef et al., 2006). Many pharmacological studies have demonstrated that *R. imbricata* exhibits cardioprotective, antiinflammatory, antipyretic, antistress and adaptogenic activities. It has also been found to possess antioxidant, antiaging, immuno-stimulant, anti-depressant, radioprotective, and anticarcinogenic properties (Chawla et al., 2010; Gupta et al., 2010; Mishra et al., 2012). All these reports support its use in traditional system of medicine and in recent times, a number of phyto products have been developed from our institute, using this plant root that was found to possess high nutritive properties and antioxidant capacities (Ballabh et al., 2007; Dhar et al., 2012). However, there is a dearth of information regarding the vitamin profile of *R. imbricata* root which could play a vital role not only in the bio-activity and pharmacological properties but also could validate its use in the preparation of numerous pharmaceutical and nutritional preparations. Hence, in the present investigation, our aim was to separate, identify and quantify the fat- and water-soluble vitamins in *R. imbricata* root by UPLC-MS/MS hyphenation.

4.2 Materials & methods

4.2.1 *Chemicals and reagents*

Reference standards of fat soluble vitamins: *trans*-retinol (A), ergocalciferol (D_2), cholecalciferol (D_3), D-α-tocopherol (E), phylloquinone (K_1), and menaquinone (K_2); water soluble vitamins: thiamine hydrochloride (B_1), riboflavin (B_2), nicotinic acid (B_3), nicotinamide (B_3), D-pantothenic acid (B_5), pyridoxine hydrochloride (B_6), D-Biotin (B_7), folic acid (B_9), and cyanocobalamin (B_{12}) were procured from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). Structures of the vitamins under analysis along with their active form, regulatory, activity biological function, and recommended daily dose (mg/day) are shown in Table 4.1. The solvents, acetonitrile, methanol, triethylamine were of gradient grade for liquid chromatography (MERCK KGaA, Darmstadt**,** Germany) and ultra pure water was prepared by a Milli-Q purification system (Millipore Corp., Bedford, MA, USA) and deionised (DI) water were used. Potassium dihydrogen phosphate and potassium hydroxide were of analytical grade purchased from MERCK (MERCK, KGaA, Darmstadt**,** Germany). The chemicals and reagents used were of HPLC and/or analytical grades. All solutions were passed through 0.45 µm Teflon membrane filters (MetaChem, Torrance, CA, USA) prior to analyses. The pH value of the solution was equilibrated with HI 8424 Portable. pH/mV/°C meter (HANNA Instruments Inc., RI, USA) regularly calibrated with NBS buffer solutions.

4.2.2 *Ethics statement*

All necessary permits were obtained for the described field studies. The permit was issued by Dr. B. Balaji (IFS), Divisional Forest Officer, Leh Forest Division, Jammu & Kashmir, India.

4.2.3 ***Plant materials***

R. imbricata roots were collected from the trans-Himalayan region (Chang-La Top, altitude = 5330 m above mean sea level, Indus valley, Ladakh) of India in the month of October, 2011 after the period of senescence, with prior permission from the local authority. The plant roots were washed thoroughly, and cut into small pieces and shade dried at room temperature for 15 days. Then they were finely powdered and used for further study.

4.2.4 ***Chromatographic apparatus***

Fat and water soluble vitamin detection and quantification were performed using the Agilent 1200 Series Rapid Resolution LC (RRLC) Binary modules interfaced to the ultra-performance Agilent 6410 Triple Quadrupole (QQQ) LC-MS/MS (G6410A, Agilent Technologies, Santa Clara, CA, USA) with HPLC-Chip Cube. The RRLC system was incorporated with Agilent 1200 Series ultra-high performance liquid chromatographic (UPLC) system which was equipped with Agilent 1200 series vacuum micro degasser (G1322A), Agilent 1200 series binary pump system SL (G1312B), Agilent 1200 series High Performance Autosampler (ALS) SL (G1367C), and Agilent 1200 series Thermostated Column Compartment (TCC) SL (G1316B). The Triple Quadrupole Mass Spectrometer was equipped with an electrospray ionization (ESI) probe. The RRLC system was controlled by Agilent ChemStation module. MassHunter versions B.02.01, B.02.01, and B.03.01 were used for data acquisition, qualitative, and quantitative analysis respectively. The separation of analytes was carried out on Aligent Poroshell 120, narrow bore EC-C18 (2.1 x 100mm, 2.7 µm particle sizes) analytical column.

4.2.5 ***LC/MS/MS method details***

The LC conditions are given in Table 4.2, while the MS conditions are given in Table 4.3. Table 4.4 lists the RRLC gradient elution conditions.

4.2.6 ***Standard solutions***

4.2.6.1 ***Water-soluble vitamins***

4.2.6.1.1 ***Water-soluble vitamins stock solution***

Standards of vitamin B_1, B_3 (nicotinamide and nicotinic acid), B_5, B_6, B_7, and B_{12} were prepared by accurately weighing 1 mg of the respective vitamin powder and adding 7 mL of DI water to form stock solutions of 1.0 mg/mL of each vitamin. Vitamin B_2 and B_9 had limited solubility in water. Hence, the stock solution of vitamin B_2 was prepared in 5 mM KOH. Stock solution of vitamin B_9 was prepared in 20 mM $KHCO_3$ instead of DI water (DIONEX, 2010).

4.2.6.1.2 ***Water-soluble vitamin mix standard preparation***

A standard mix containing nine water soluble vitamins *viz*. thiamine (B_1), riboflavin (B_2), nicotinic acid (B_3), nicotinamide (B_3), D-Pantothenic acid (B_5), pyridoxine (B_6), D-biotin (B_7), folic acid (B_9), and cyanocobalamin (B_{12}) was diluted in water : methanol (90:10, v/v) with 10 mM ammonium formate and 0.1% formic acid to the following concentrations: 10, 50, and 100 and μgL^{-1} (Mohsin, 2008).

4.2.6.2 ***Fat-soluble vitamins***

4.2.6.2.1 ***Fat-soluble vitamins stock solution***

One milligram each of vitamins A, D_2, D_3, and E was accurately weighed and dissolved in 4 mL of methanol to form 1.0 mg/mL stock solution of each vitamin. Vitamin K_1 and K_2 were prepared in acetone. The stock standard solutions were stored at 4°C for further analysis.

4.2.6.2.2 *Fat-soluble vitamin mix standard preparation*

A standard mix containing six fat soluble vitamins *viz*. retinol (A), ergocalciferol (D_2), cholecalciferol (D_3), E (α-tocopherol), phylloquinone (K_1), and menaquinone (K_2) was diluted in methanol : water (90:10, v/v) with 10 mM ammonium formate and 0.1% formic acid to the following concentrations: 100, 500, and 1000 μgL^{-1} (Mohsin, 2008).

4.2.7 *Sample preparation and extraction of vitamins*

4.2.7.1 *Extraction of water soluble vitamins*

Ground and homogenized root sample (1 g) was transferred into a 50 mL vial, 25 mL of 0.1 N HCl was added to it and heated in water bath at 100°C for 20 min in order to dissolve water soluble vitamins. Then it was cooled to ambient temperature, and the volume was adjusted to 1 L with DI water, followed by filtration through a 0.45 µm glass microfiber membrane (Whatman® GD/X) and the filtrate was collected for UPLC–MS–MS analysis (Mohsin, 2008).

4.2.7.2 *Extraction of fat-soluble vitamins*

Extraction of fat soluble vitamins was carried out with 1 g of ground and homogenized root sample. It was transferred into 10 mL volumetric flask and 8 mL of methanol-dichloromethane (1:1, v/v) was added to it. After 15 min of ultrasonic extraction, methanol-dichloromethane was added to the mark and filtered through a 0.45 µm glass microfiber membrane. The prepared sample solution was stored in the dark till further analysis (DIONEX, 2010).

4.3 Results and discussion

Estimation of free forms of fat- and water-soluble vitamins in biological samples can be achieved by two different approaches. The first is to separate the entire vitamin forms in a single analysis, while the second is to optimize two individual methods for a superior and precise

measurement of the diverse vitamins (Santos et al., 2012). Previous reports on the simultaneous determination of numerous fat- and water-soluble vitamins in a single chromatographic run with diode array detector (DAD) and MS detectors to analyze the vitamin profile of functional drinks, nutritional beverages, pharmaceutical preparations, foodstuff and parenteral nutrition admixtures have already been published and amongst them, only a few methods are related to complex food sample analysis (Santos et al., 2012; Klejdus et al., 2004; Li and Chen, 2001; Mendiola et al., 2008). In addition to that, very limited literature is available for the sequential estimation of water- and fat-soluble vitamins with QQQ-MS detector and there is no previous report on the vitamin profile of *R. imbricata* root which is used as an important ingredient in several nutraceutical, pharmacological and therapeutic preparations developed from our institute (Ballabh et al., 2007; Dhar et al., 2012). In the present study, we have developed a practical and rapid method with an objective to determine both types of vitamins in *R. imbricata* root which could be effective in providing an accurate quantification of all the free vitamin forms in this plant root. The plant root sample is a real complex biological material that increases the level of difficulties in determining the fat- and water-soluble vitamins in a single chromatographic run. The major constraints in this investigation were, firstly, the hindrance of co-elutions among the different vitamins and with other bio-active components like organic acids, phenolics, and flavonoids, selection of the most excellent MS detection conditions along with ionization technique for two different classes of vitamins etc. These issues were taken into consideration with minute details and we have ultimately developed a sequential method that could be applicable in parallel for the precise and rapid quantification of both fat- and water-soluble vitamins. Hence, the UPLC–MS/MS conditions, and the extraction protocol to recover both types of vitamins were optimized to produce the accurate results.

4.3.1 *UPLC–MS/MS determination of water- and fat-soluble vitamins*

4.3.1.1 *Optimization of LC conditions*

Sequential analysis of water- and fat- soluble vitamins depends largely on the selection of a suitable chromatographic column for one run separation with enhanced resolution. The smaller system volume, high throughput, smaller particle-size and ultra-high pressure in UPLC column gives the advantage of separating more peaks in shorter run time. A number of columns such as Nova-Pack C18 (150 × 3.9 mm, 4 μm particle size), Separon C18 (150 × 3 mm, 7 μm particle size), and MetaChem Polaris C18-A (150 × 4.6 mm, 3 μm) were used to separate the water- and fat-soluble vitamins with HPLC (Klejdus et al., 2004; Scalia, 1995; Moreno and Salvado, 2000). In the present study, the performance of two different reversed phase (RP) columns was tested in the optimization process for the separation of the 9 water-soluble vitamins and 6 fat-soluble vitamins studied. A short UPLC C18 column (2.1 × 50 mm, 1.9 μm particle diameter) and a longer C18 column (2.1 × 100 mm, 2.7 μm particle size) of Agilent Poroshell 120 EC-C18 were compared for analysis. The longer column was found to have a much better separation among the analytes, including B_1 and both types of B_3 vitamins that were weakly retained in the C18 stationary phase along with short duration of analysis (9 min), and D_2 and D_3 that had very close retention time. In our study, the selection of column was in agreement with previous reports in which the long C_{18} column with small particle size was found to produce optimized peak separation (Klejdus et al., 2004; Li and Chen, 2001; Santos et al., 2012; Lu et al., 2008; Jin et al., 2012; Goldschmidt and Wolf, 2010). The complete chromatograms of the 9 water-soluble and 6 fat-soluble vitamins and the detailed compound chromatograms describing the quantifier, qualifier, and MRM transitions of blank, standard, and all individual vitamins are shown in Figure 4.1, 4.2, 4.3, and 4.4. The separation of all the studied compounds was successfully

achieved. Only a few water-soluble vitamins such as B_1, $B_{6,}$ and B_3 can also be separated by hydrophilic interaction liquid chromatography (HILIC) where the RP column is replaced by column of other type that retain the more hydrophilic vitamins longer than RP chromatography (Goldschmidt and Wolf, 2010). Hence, with regard to our present study with a wider group of water-soluble vitamins, the validation of the HILIC approach would be of limited significance.

The selection of the mobile phase and the composition of organic solvents are extremely critical in LC separation as it has significant effect on the retention time of analytes, peak shapes, sensitivity and resolution and consequently on the overall chromatographic separation efficiency of the developed process. It is recommended that the mobile phases should contain comparatively low concentrations of organic, water miscible solvents and aqueous solution of inorganic components as buffering agents to assure precise chromatographic separation of water-soluble vitamins. The pH-values of mobile phases should also be adjusted by addition of acid (Klejdus et al., 2004; Li and Chen, 2001; Lazzarino et al., 2003). The highly polar water-soluble vitamins have poor retention on RP columns and thus presence of ion pair reagents such as acetic acid, trifluoroacetic acid, pentafluoropropionic acid, formic acid, or heptafluorobutyric acid in the mobile phase have been shown to improve the separation and retention of these compounds. Of these reagents, formic acid is a convenient, contamination-free alternative for preparing elution solvents for LC separations and after comparing these reagents, formic acid was found to be suitable. . However, the ion pair reagents often produce high background levels inside the MS-system and to neutralize this negative effect we have developed a rapid and sensitive method using ammonium formate in the mobile phase solvent to retain hydrophilic components. After taking lead from the previous reports (Klejdus et al., 2004; Santos et al., 2012; Goldschmidt and Wolf, 2010; Chen et al., 2006; Heudi et al., 2005), we have developed a

gradient elution method using 0.1% formic acid in water (mobile phase A) and in methanol (mobile phase B) with the addition of 10 mM ammonium formate as the buffering agent. Other alternative mobile phases including acetonitrile were also evaluated but in the current study the separation was not enhanced. Thus, the use of acidified water and methanol was maintained to restrain the dissociation of acidic vitamins like nicotinic acid, pyridoxine, pantothenic acid, folic acid, to promote improved ionization of the basic sites of all vitamins, and moreover, to improve the peak shapes with higher resolution (Gentili, 2008). Different concentrations of formic acid and acetic acid (0.1% – 0.3%) were tested, and in contrast to the previous study (Santos et al., 2012), 0.1% formic acid provided better peak shapes than acetic acid. Satisfactory resolution between peaks and excellent peak shapes were obtained for all the studied compounds under these LC-analytical conditions that can be observed in Figure 4.3 and 4.4.

4.3.1.2 ***Fragmentation study and optimization of MS/MS conditions***

After achieving optimum separation, the optimization of different MS/MS detection parameters was carried out to confirm the structural identities of analytes and instrumental sensitivity. It is critical to optimize the quantitative ion and impact energy for determination of multiple vitamins by MRM. Positive ESI ionization modes were selected for the production of typical precursor and product ions of each compound with the direct infusion of standard solutions. Selection of precursor ions was based on the most abundant mass-to-charge (*m*/*z*) values and subsequently, two product ions (qualifier ion and quantifier ion) for each precursor were selected. Quantification was achieved using the most intense product ion while the other was employed for authentication of compound identity. The precursor and product ions chosen for each studied compound, the collision energies, fragmentor voltage, and dwell time values employed for their detection have been summarized in Table 4.6. All vitamins were detected as

$[M+H]^+$ except cyanocobalamin where the doubly charged ion $[M+H]^{2+}$ at m/z 678.3 was selected as the parent ion. Thiamine and pyridoxine were observed as the loss of associated chloride, whereas calcium pantothenate was determined as pantothenic acid by the loss of calcium. Ergocalciferol, and both K vitamins (K_1 and K_2) lost one molecule of water and phytyl chain respectively to produce the product ions in the collision-induced decomposition (CID) while, fragmentation of D-*α*-tocopherol induced the cleavage of the chromanol ring that led to the production of product ions. All other parameters in the ESI detection of the studied water- and fat-soluble vitamins, *viz.* capillary temperature, drying gas flow, nebulizer pressure, capillary voltage, delta EMV, resolution and scanning mode (MRM) were optimized and the values that offered the most excellent response for all vitamins are described in Section 2.4 (Table 4.2).

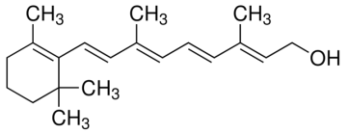
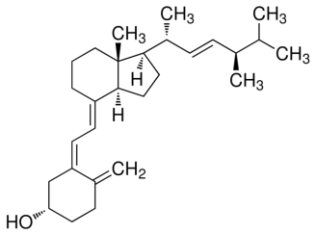
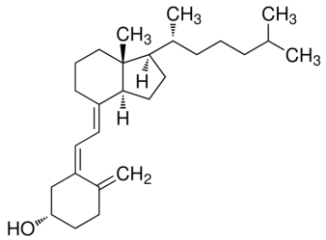
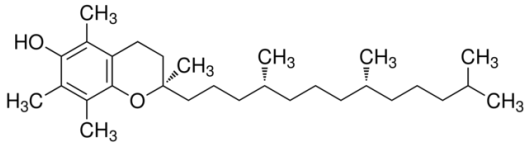
4.3.2 ***Validation results***

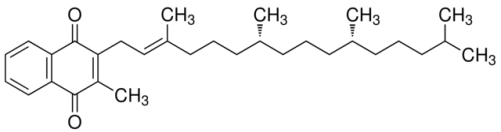
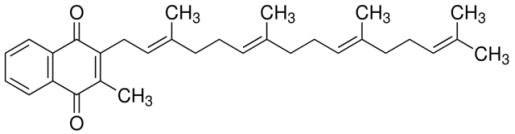
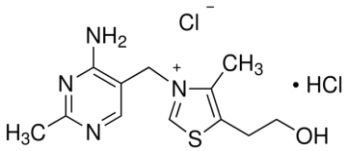
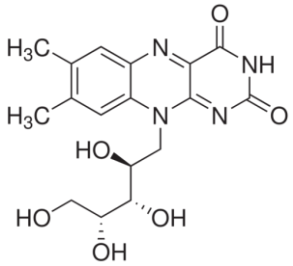
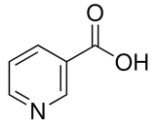
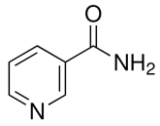
4.3.2.1 ***Recovery and precision***

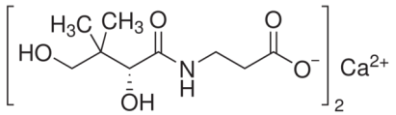
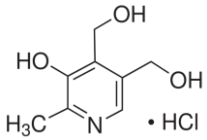
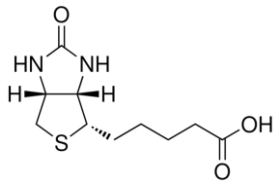
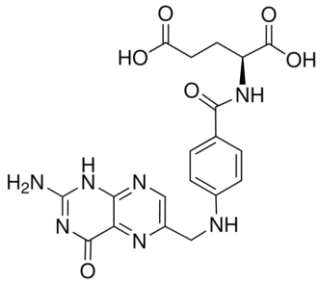
The UPLC-separation conditions and MS/MS detection parameters were selected and optimized and we then moved forward to the evaluation of instrumental intra-day and inter-day precision. Intra-day precision was evaluated through the successive injection (n = 5) of standards that were injected consecutively in the same day, and this was repeated in four different days for assessment of inter-day precision (n = 20). The obtained results of intra- and inter-day precision (% RSD values) for retention time and recoveries are depicted in Table 4.7. The intra- and inter-day precisions of response are illustrated in Table 4.8. For water-soluble vitamins, the intra- and inter-day RSD values of retention time ranged between 0.024 – 0.172% and 0.085 – 0.201% respectively. The intra- and inter-day RSD values of retention time for fat-soluble vitamins ranged between 0.009 – 0.85% and 0.022 – 0.152% respectively. For water-soluble vitamins, the recovery precision for the same day, ranged between 0.17 – 0.29%, 0.22 – 0.27%, and 0.22 –

0.29% for recoveries of three different concentrations of standards. The RSD values, for inter-day, were slightly higher and ranged between 0.39 – 0.52%, 0.40 – 0.47%, and 0.32 – 0.44%. The mean recoveries were ranged between 88.95 – 107.07%, 98.35 – 103.27%, and 98.77 – 102.33%. On the other hand, for the fat-soluble vitamins, the intra-day precision of recovery ranged between 0.16 – 0.37%, 0.18 – 0.28%, and 0.19 – 0.29% for three concentrations studied. Again, the inter-day RSDs were somewhat higher and ranged between 0.43 – 0.57%, 0.31 – 0.44%, and 0.30 – 0.37%. The mean recoveries for fat-

Table 4.1 Chemical structure, biological activities, recommended daily doses of fat- and water-soluble vitamins.

Vitamins	Structure	Active form	Regulatory activity	Biological function	Recommended daily dose (mg/day)
Fat-soluble vitamin					
Retinol (A)		11-cis-Retinal	Visual cycle	Formation and maintenance of skin and mucous membranes, increasing resistance to infections, essential for night vision, promotes bones and tooth development	0.8–1.0
Ergocalciferol (D_2)				Supports hardening of bones and teeth, facilates the absorption of calcium	
Cholecalciferol (D_3)		1,25-Dihydroxycholecalciferol	Calcium and phosphate metabolism	Supports hardening of bones and teeth, facilates the absorption of calcium	0.08
D-α-Tocopherol (E)		-	Antioxidant	Protects vitamins A and C and fatty acids, prevent damage to cell membranes, antioxidant	8–10

Phylloquinone (K_1)		-	Prothrombin biosynthesis	Helps blood to clot	
Menaquinone (K_2)		-	Prothrombin biosynthesis	Helps blood to clot	0.07–0.08
Water-soluble vitamin					
Thiamine (B_1)		Thiamine pyrophosphate (TTP)	Aldehyde group transfer	Releases energy from foods; promotes normal appetite; improve functions of nervous system	1.0-1.5
Riboflavin (B_2)		Flavin mononucleotide (FAD), flavin adenine dinucleotide (FAD)	Electron transfer	Releases energy from foods; promotes good vision, healthy skin	1.3-1.8
Nicotinic acid (B_3)		Nicotinamide adenine dinucleotide (NAD, NADP)	Electron transfer	Releases energy from foods; aides digestion, promotes normal appetite; promotes healthy skin, nerves	15-20
Nicotinamide (B_3)		Nicotinamide adenine dinucleotide (NAD, NADP)	Electron transfer, coenzyme maintaining redox state of a cell	Releases energy from foods; aides digestion, promotes normal appetite; promotes healthy skin, nerves	

D-Pantothenic acid (B_5)		Coenzyme A (CoA)	Acyl group transfer	Involved in energy production; aids in formation of hormones	10–100
Pyridoxine (B_6)		Pyridoxal phosphate	Amino group transfer	Aids in protein metabolism and absorption; aids in red blood cell formation; helps body use fats	2
D-Biotin (B_7)		-	Carbon dioxide transfer	Helps release energy from carbohydrates; aids in fat synthesis	0.3
Folic acid (B_9)		Tetrahydrofolic acid	One carbon group transfer	Aids in protein metabolism; promotes red blood cell formation; prevents birth defects of spine, brain; lowers homocystein levels thereby lowers coronary heart disease risk	0.4

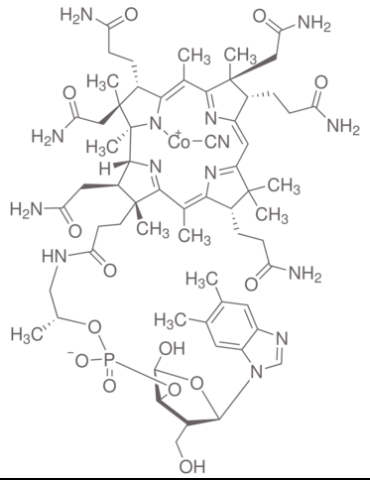
Cyanocobalamin (B_{12})		Coenzyme cyanocobalamin	1,2 shift of hydrogen atoms	Aids in building of genetic material; aids in development of normal red blood cells; maintenance of nervous system	2.4

Table 4.2 LC conditions for chromatographic separation of fat and water-soluble vitamins.

Parameters	**Fat-soluble vitamins**	**Water-soluble vitamins**
Column	Aligent Poroshell 120 EC-C18 Narrow bore, 2.1 x 100 mm, 2.7 µm particle size (p/n 695775-902)	Aligent Poroshell 120 EC-C18 Narrow bore, 2.1 x 100 mm, 2.7 µm particle size (p/n 695775-902)
Column temperature	35°C	35°C
Mobile phase	A = 0.1% HCOOH in water + 10 mM ammonium formate B = 0.1% HCOOH in methanol + 10 mM ammonium formate	A = 0.1% HCOOH in water + 10 mM ammonium formate B = 0.1% HCOOH in methanol + 10 mM ammonium formate
Flow rate	0.3 mL/min	0.3 mL/min
Elution	Gradient elution	Gradient elution
Total run time	25 min	20 min
Post run time	5min	5min
Sample Thermostat	5°C	5°C
Injection volume	5 µL	5 µL
Pressure	550 bar	550 bar
Detector	QQQ MS	QQQ MS
Needle wash	20 s in wash port with 90:10 methanol: water mix	20 s in wash port with 10:90 methanol: water mix

Table 4.3 MS conditions for quantification of fat- and water-soluble vitamins.

Parameters	**Fat-soluble vitamins**	**Water-soluble vitamins**
Ionization mode	Positive ESI using Agilent G1948B ionisation source	Positive ESI using Agilent G1948B ionisation source
Capillary temperature	325°C	350°C
Drying gas flow	8 L/min	8 L/min
Nebulizer pressure	45 psi	50 psi
Capillary voltage	2500 V	2500 V
Delta EMV	0 V	0 V
Resolution (FWHM)	Q1 = Unit, Q3 = Unit	Q1 = Unit, Q2 = Unit
Scan mode	Multiple Reaction Monitoring (MRM)	Multiple Reaction Monitoring (MRM)

Table 4.4 RRLC (UPLC) gradient[a] elution program for the separation of fat- and water-soluble vitamins on Aligent Poroshell 120 SB-C18 reverse phase column.

Time (min:s)	Flow rate (mL/min)	Solvent A[b] (%, v/v)	Solvent B[c] (%, v/v)
Fat-soluble vitamins			
0	0.3	10	90
3	0.3	10	90
4	0.3	0	100
17	0.3	0	100
18	0.3	10	90
25	0.3	10	90
Water-soluble vitamins			
0	0.3	90	10
8	0.3	45	55
10	0.3	45	55
11	0.3	90	10
18	0.3	90	10

[a] Total run time = 25 and 20 min, for fat- and water- soluble vitamins, respectively; post time = 5 min for fat- and water-soluble vitamins.
[b] A = 0.1% HCOOH in water + 10 mM ammonium formate.
[c] B = 0.1% HCOOH in methanol + 10 mM ammonium formate.

Table 4.5 Validation data for determination of fat- and water-soluble vitamins (*n=3*).

Analyte	Molecular weight	Ionisation mode	RT (min)[a]	Regression equation	$R^{2,b}$	LOD (µg/L)[c]	LOQ (µg/L)[d]	RT RSD (%)[e]
Fat-soluble vitamins								
Retinol (A)	286.45	ESI+	2.9387 ± 0.0091	y = 40.0574x + 339.2849	0.9991	0.18	0.61	0.3088
Ergocalciferol (D_2)	396.65	ESI+	8.6523 ± 0.0225	y = 0.0923x + 11.8327	0.9980	47.82	159.41	0.2598
Cholecalciferol (D_3)	384.64	ESI+	8.7463 ± 0.0117	y = 19.5077x + 408.9163	0.9961	0.35	1.17	0.1335
D-*α*-Tocopherol (E)	430.71	ESI+	9.6253 ± 0.0131	y = 41.8311x − 139.1714	0.9996	0.18	0.59	0.1356
Phylloquinone (K_1)	450.70	ESI+	11.7927 ± 0.0220	y = 14.0609x − 412.1498	0.9911	0.41	1.37	0.1862
Menaquinone (K_2)	444.65	ESI+	9.3223 ± 0.0107	y = 28.8488x − 391.4693	0.9973	0.21	0.68	0.1147
Water-soluble vitamins								
Thiamine (B_1)	337.3	ESI+	1.1310 ± 0.0017	y = 201.1694x − 429.8190	0.9969	0.04	0.13	0.1531
Riboflavin (B_2)	376.36	ESI+	8.7147 ± 0.0035	y = 8.2510 x + 175.5517	0.9861	0.86	2.87	0.0403
Nicotinic acid (B_3)	123.11	ESI+	1.3417 ± 0.0067	y = 7.4249x + 57.4607	0.9678	0.86	2.88	0.4963
Nicotinamide (B_3)	122.12	ESI+	1.6220 ± 0.0017	y = 51.5908x + 315.5337	0.9775	0.14	0.46	0.1068
D-Pantothenic acid (B_5)	219.23	ESI+	2.9730 ± 0.0276	y = 1.9568x + 5.3674	0.9948	2.85	9.51	0.9291
Pyridoxine (B_6)	169.18	ESI+	1.3747 ± 0.0023	y = 71.7554x + 399.2698	0.9906	0.08	0.27	0.1680
D-Biotin (B_7)	244.31	ESI+	7.7440 ± 0.0060	y = 30.9773x + 73.1416	0.9883	0.20	0.66	0.0775

Folic acid (B_9)	441.41	ESI+	7.3167 ± 0.0225	y = 1.6740x + 6.3379	0.9994	3.30	11.00	0.3075
Cyanocobalamin (B_{12})	1355.38	ESI+	7.7023 ± 0.0168	y = 3.6046 x + 22.2344	0.9733	1.27	4.22	0.2178

[a] Retention time in min.
[b] Regression coefficients.
[c] Limits of detection (3.S/N).
[d] Limits of quantitation (10.S/N).
[e] Relative standard deviations.

Table 4.6 Retention times (min) and optimised MS/MS transition parameters.

Analytes	RT (min)	Precursor ion (m/z)	MRM Transition		Fragmentor voltage (V)	Dwell time (ms)
			Quantifier ion (m/z) (collision energy, eV/V)	Qualifier ion (m/z) (collision energy, eV/V)		
Fat-soluble vitamins						
Retinol (A)	2.9387	269.1 $[M+H]^+$	269.1 ➔ 93.0 (20)	269.1 ➔ 213.1 (13)	100	100
Ergocalciferol (D_2)	8.6523	397.2 $[M+H]^+$	397.2 ➔ 379.1 (10)	397.2 ➔271.1 (11)	120	50
Cholecalciferol (D_3)	8.7463	385.3 $[M+H]^+$	385.3 ➔ 367.2 (10)	385.3 ➔ 259.1 (12)	120	50
D-α-Tocopherol (E)	9.6253	431.4 $[M+H]^+$	431.4 ➔ 165.0 (18)	431.4 ➔ 137.0 (45)	120	50
Phylloquinone (K_1)	11.7927	451.3 $[M+H]^+$	451.3 ➔ 187.0 (23)	451.3 ➔ 57.1 (36)	140	50
Menaquinone (K_2)	9.3223	445.3 $[M+H]^+$	445.3 ➔ 81.1 (36)	445.3 ➔ 187.1 (15)	130	50
Water-soluble vitamins						
Thiamine (B_1)	1.1310	265.0 $[M+H]^+$	265.0 ➔ 122.0 (10)	265.0 ➔ 144.0 (8)	80	100
Riboflavin (B_2)	8.7147	377.1 $[M+H]^+$	377.1 ➔ 243.1 (22)	377.1 ➔ 172.0 (41)	140	100
Nicotinic acid (B_3)	1.3417	124.0 $[M+H]^+$	124.0 ➔ 80.0 (10)	124.0 ➔ 53.0 (8)	80	100
Nicotinamide (B_3)	1.6220	123.0 $[M+H]^+$	123.0 ➔ 80.0 (20)	123.0 ➔ 53.0 (32)	100	100
D-Pantothenic acid (B_5)	2.9730	220.1 $[M+H]^+$	220.1 ➔ 90.1 (9)	220.1 ➔ 202.1 (6)	70	100
Pyridoxine (B_6)	1.3747	170.0 $[M+H]^+$	170.0 ➔ 134.0 (19)	170.0 ➔ 152.0 (8)	80	100
D-Biotin (B_7)	7.7440	245.1 $[M+H]^+$	245.1 ➔ 227.1 (9)	245.1 ➔ 97.0 (30)	70	100
Folic acid (B_9)	7.3167	442.1 $[M+H]^+$	442.1 ➔ 295.0 (15)	442.1 ➔ 120.0 (40)	60	100
Cyanocobalamin (B_{12})	7.7023	678.3 $[M+H]^{2+}$	678.3 ➔ 147.0 (41)	678.3 ➔ 359.0 (20)	140	100

Table 4.7 Intra- and inter-day precision of retention time, recovery, and linearity of fat- and water-soluble vitamins (*n=5*).

Analyte	RT precision (% RSD)		Recovery precision (% RSD) and mean recovery (%)									Linearity range (µg/L)
	Intra-day	Inter-day	Intra-day	Inter-day	Mean recovery	Intra-day	Inter-day	Mean recovery	Intra-day	Inter-day	Mean recovery	
Fat-soluble vitamins			$\mu gmL^{-0.1}$			$\mu gmL^{-0.5}$			μgmL^{-1}			
Retinol (A)	0.054	0.146	0.28	0.54	99.16	0.28	0.43	103.55	0.22	0.31	99.72	100–1000
Ergocalciferol (D_2)	0.085	0.152	0.16	0.43	97.60	0.20	0.31	100.30	0.23	0.35	99.30	100–1000
Cholecalciferol (D_3)	0.048	0.136	0.24	0.50	101.53	0.27	0.43	100.72	0.23	0.35	100.75	100–1000
D-α-Tocopherol (E)	0.033	0.134	0.20	0.46	103.36	0.19	0.44	97.67	0.19	0.37	97.71	100–1000
Phylloquinone (K_1)	0.009	0.022	0.37	0.46	101.91	0.18	0.35	97.74	0.29	0.35	97.79	100–1000
Menaquinone (K_2)	0.040	0.116	0.25	0.57	104.14	0.26	0.42	99.67	0.20	0.30	99.93	100–1000
Water-soluble vitamins			$\mu gmL^{-0.01}$			$\mu gmL^{-0.05}$			$\mu gmL^{-0.1}$			
Thiamine (B_1)	0.137	0.140	0.17	0.41	107.07	0.22	0.41	98.74	0.29	0.40	98.77	10–100
Riboflavin (B_2)	0.024	0.085	0.25	0.39	90.93	0.23	0.47	103.27	0.25	0.41	101.56	10–100
Nicotinic acid (B_3)	0.172	0.196	0.29	0.52	88.95	0.27	0.42	100.63	0.28	0.42	100.30	10–100
Nicotinamide (B_3)	0.078	0.125	0.26	0.45	90.58	0.26	0.45	101.62	0.26	0.36	101.68	10–100
D-Pantothenic acid (B_5)	0.139	0.201	0.23	0.41	92.24	0.24	0.40	99.41	0.29	0.44	99.38	10–100
Pyridoxine (B_6)	0.141	0.153	0.25	0.48	91.80	0.24	0.41	99.71	0.25	0.44	99.61	10–100

D-Biotin (B_7)	0.041	0.110	0.23	0.48	99.23	0.23	0.42	98.35	0.28	0.32	98.77	10–100
Folic acid (B_9)	0.049	0.092	0.24	0.52	95.81	0.24	0.43	102.88	0.29	0.32	102.33	10–100
Cyanocobalamin (B_{12})	0.162	0.192	0.25	0.51	89.53	0.23	0.40	99.71	0.22	0.41	99.84	10–100

Table 4.8 Intra- and inter-day precision of response of fat- and water-soluble vitamins.

Analyte	Precision of response					
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
Fat-soluble vitamins	$\mu gmL^{-0.1}$		$\mu gmL^{-0.5}$		μgmL^{-1}	
Retinol (A)	0.05	0.07	0.01	0.03	0.03	0.04
Ergocalciferol (D_2)	5.02	8.70	3.41	5.04	2.14	3.20
Cholecalciferol (D_3)	0.08	0.11	0.02	0.03	0.05	0.06
D-α-Tocopherol (E)	0.05	0.07	0.01	0.02	0.04	0.05
Phylloquinone (K_1)	0.16	0.22	0.04	0.06	0.12	0.15
Menaquinone (K_2)	0.05	0.10	0.02	0.03	0.06	0.09
Water-soluble vitamins	$\mu gmL^{-0.01}$		$\mu gmL^{-0.05}$		$\mu gmL^{-0.1}$	
Thiamine (B_1)	0.13	0.18	0.03	0.04	0.06	0.08
Riboflavin (B_2)	0.82	1.06	0.32	0.43	0.93	1.56
Nicotinic acid (B_3)	1.44	2.04	0.57	0.69	0.69	1.02
Nicotinamide (B_3)	0.20	0.41	0.07	0.09	0.15	0.23
D-Pantothenic acid (B_5)	7.26	8.52	2.21	2.85	1.86	3.22
Pyridoxine (B_6)	0.13	0.23	0.06	0.07	0.12	0.20
D-Biotin (B_7)	0.44	0.69	0.16	0.21	0.25	0.36
Folic acid (B_9)	7.68	8.33	2.24	2.68	2.02	3.22
Cyanocobalamin (B_{12})	2.32	3.27	1.04	1.39	1.76	2.28

Table 4.9 Results of determination of fat- and water-soluble vitamins in *R. imbricata* root (*n=5*).

Analyte	Content in original sample (µg/Kg)
Fat soluble vitamin	
Retinol (A)	ND
Ergocalciferol (D_2)	ND
Cholecalciferol (D_3)	ND
D-α-Tocopherol (E)	4314.294 ± 131.74
Phylloquinone (K_1)	ND
Menaquinone (K_2)	ND
Water soluble vitamins	
Thiamine (B_1)	63.614 ± 5.42
Riboflavin (B_2)	78.464 ± 7.31
Nicotinic acid (B_3)	635.964 ± 21.04
Nicotinamide (B_3)	253.922 ± 10.59
D-Pantothenic acid (B_5)	397.614 ± 14.64
Pyridoxine (B_6)	286.672 ± 13.11
D-Biotin (B_7)	ND
Folic acid (B_9)	ND
Cyanocobalamin (B_{12})	ND

ND: Not detected.

soluble vitamins ranged between 97.60 – 104.14%, 97.67 – 103.55%, and 97.71 – 100.75% for the three said concentrations. Hence, there was a minute deviation in the intra- and inter-day precision but they were always below 0.6% and 0.3% for recoveries and retention time respectively, that signifies the excellent precision for the retention time and recoveries of analytes studied in the present investigation.

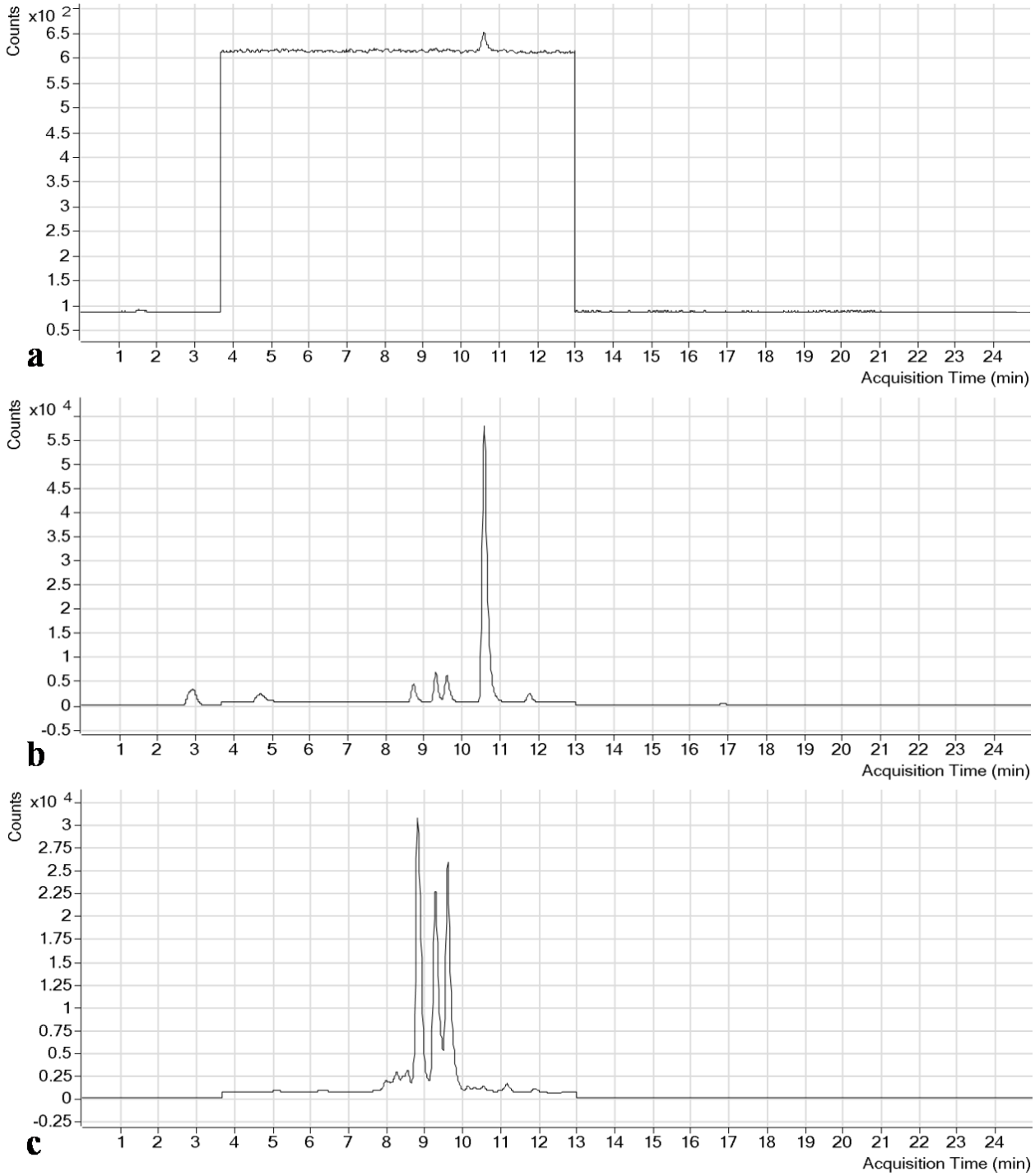


Figure. 4.1 RRLC MS/MS chromatogram of a. blank, b. six fat-soluble vitamin standards, and c. *R. imbricata* root sample under the optimum analysis conditions.

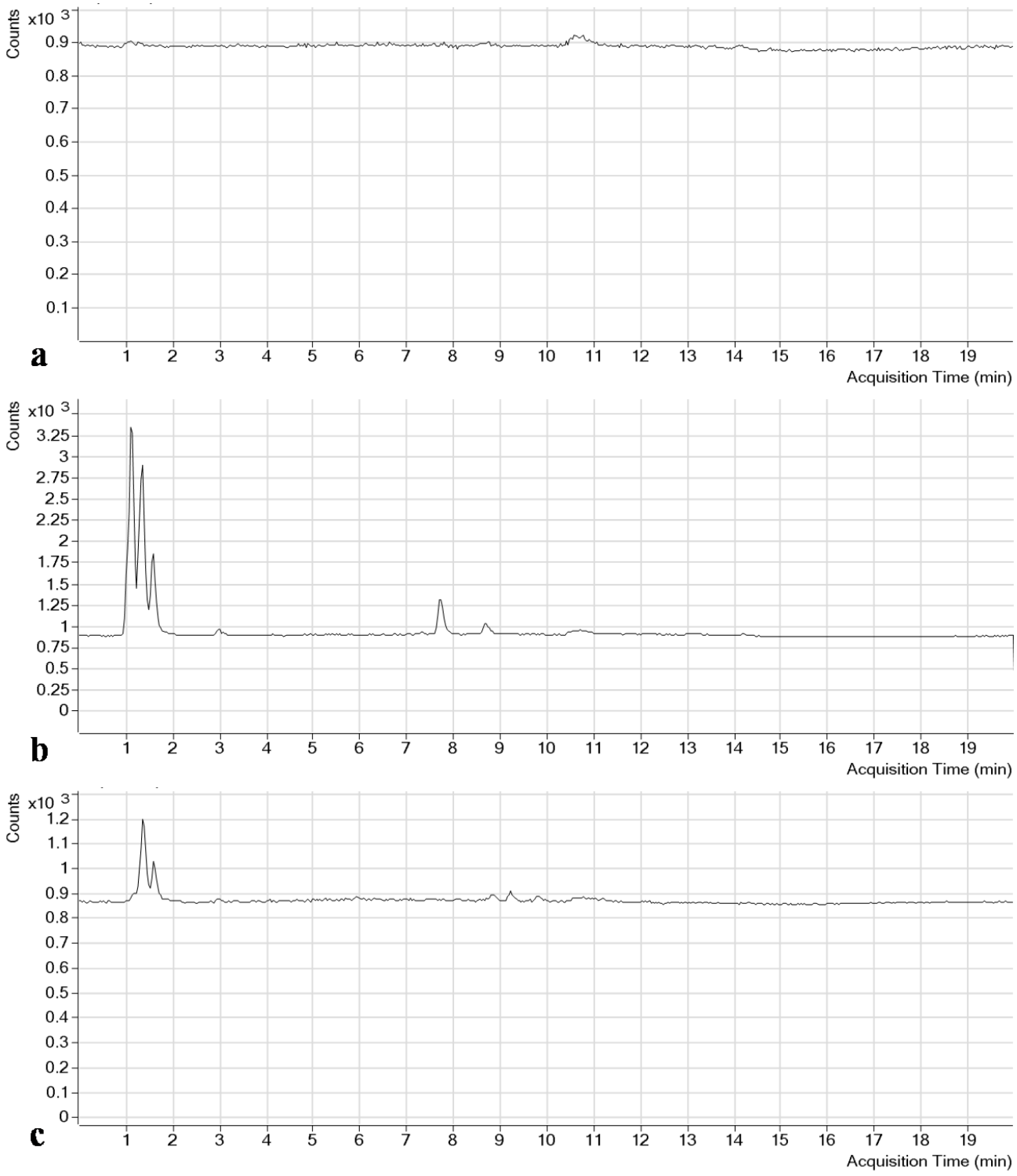


Figure. 4.2 RRLC MS/MS chromatogram of a. blank, b. nine water-soluble vitamin standards, and c. *R. imbricata* root sample under the optimum analysis conditions.

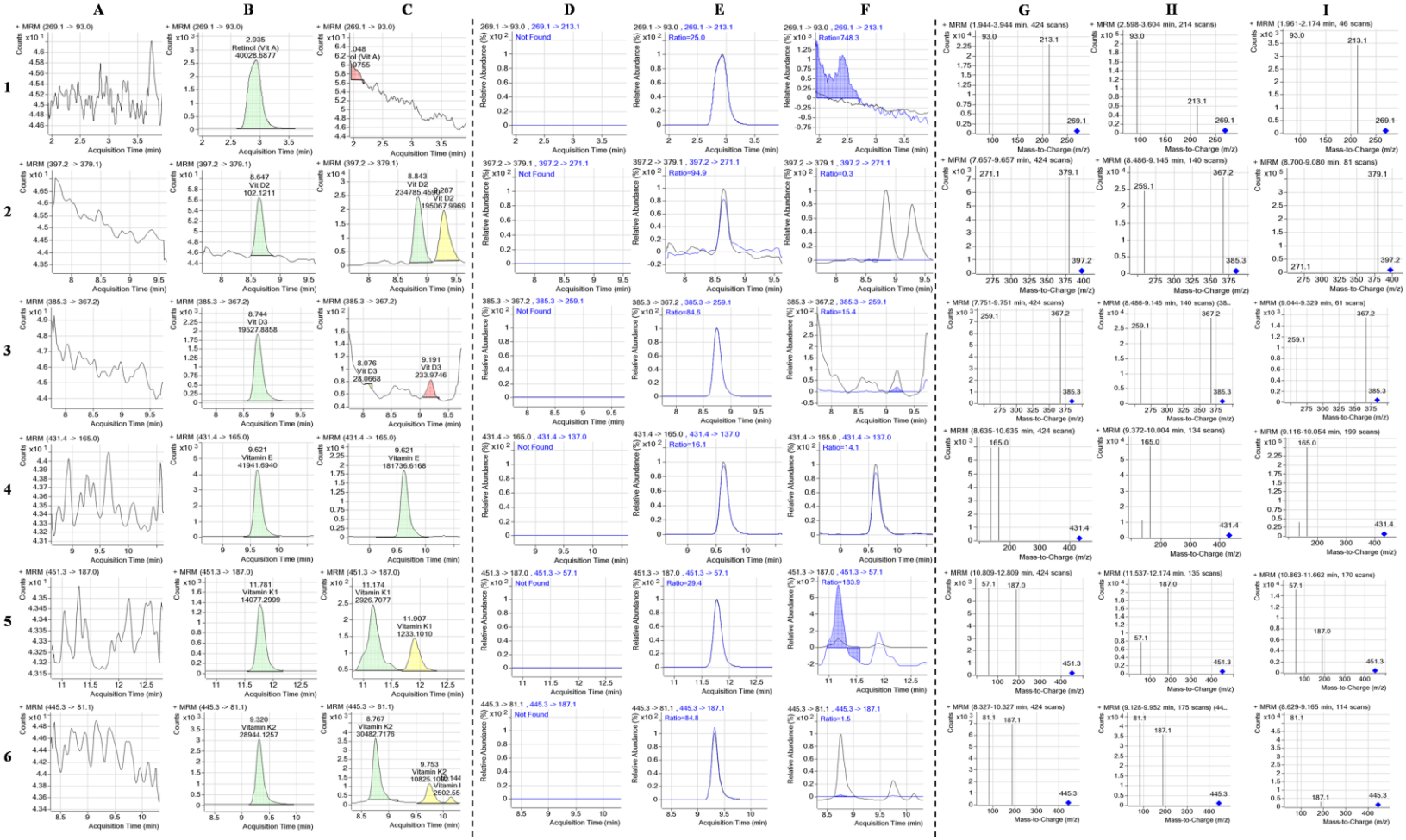


Figure. 4.3 Extracted LC-MRM ion chromatogram of the analytes. The quantifier, qualifier, and MRM transitions of extracted ions for blank, six fat-soluble standards and *R. imbricata* root sample are shown for 1A–1I: vit A, 2A–2I: vit D_2, 3A–3I: vit D_3, 4A–4I: vit E, 5A–5I: vit K_1, and 6A–6I: vit K_2 respectively.

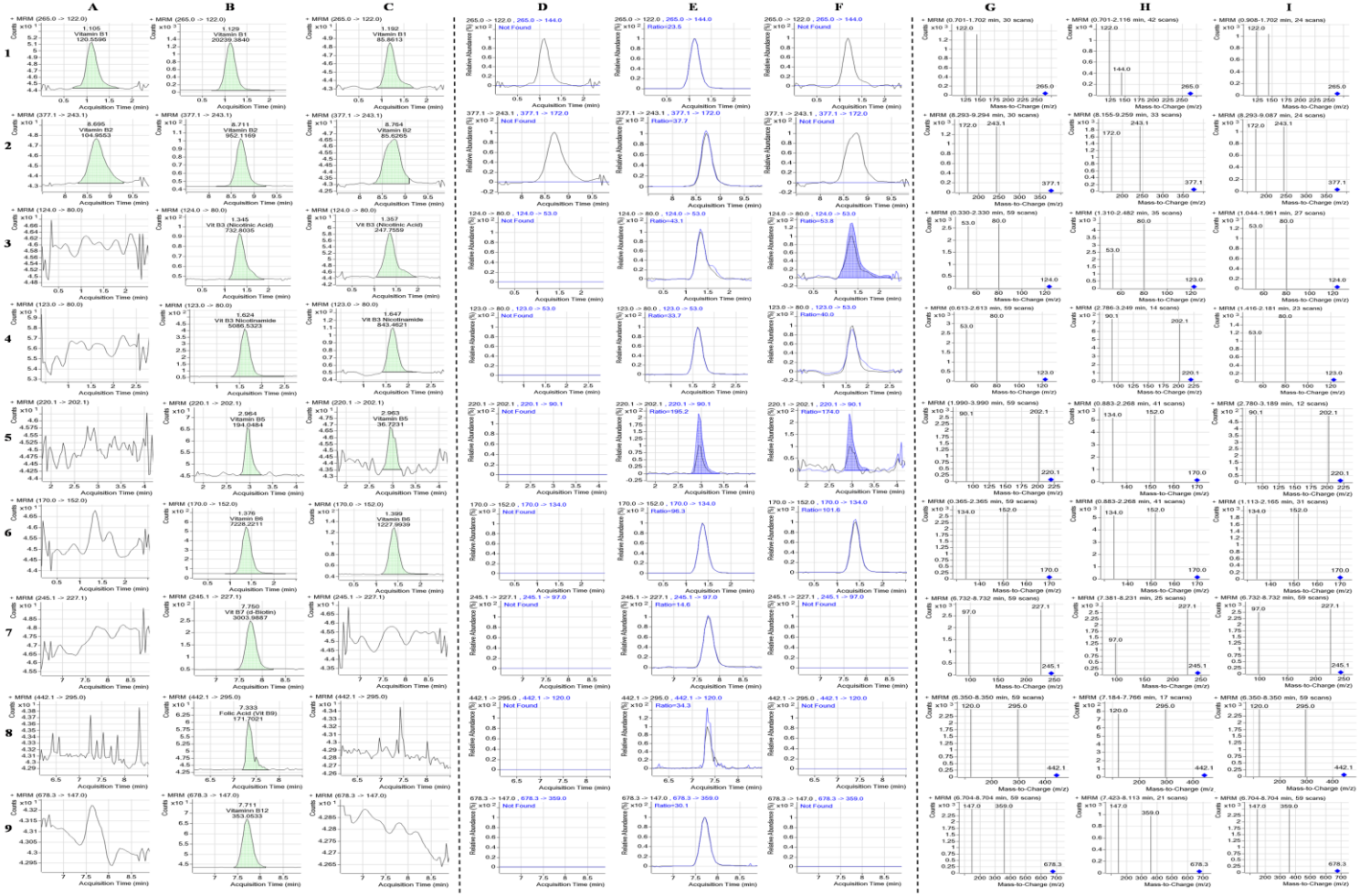


Figure. 4.4 Extracted LC-MRM ion chromatogram of the analytes. The quantifier, qualifier, and MRM transitions of extracted ions for blank, nine water-soluble standards and *R. imbricata* root sample are shown for 1A–1I: vit B_1, 2A–2I: vit B_2, 3A–3I: vit B_3, (nicotinic acid) 4A–4I: vit B_3, (nicotinamide), 5A–5I: vit B_5, 6A–6I: B_6, 7A–7I: B_7, 8A–8I: B_9, and 9A–9I: vit B_{12} respectively.

4.3.2.2 ***Linearity, Limits of detection and limits of quantitations***

The linearity was also examined through the calibration curves that were obtained by plotting concentration against peak area. A series of three concentration points as described earlier was prepared and each solution was injected ten times for each analyte. The linearity was excellent ($R^2 > 0.99$) for most of the water-soluble vitamins, except nicotinic acid, nicotinamide, and cyanocobalamin ($R^2 = 0.97 – 0.98$), while, for all the fat-soluble vitamins the linearity was found to be very good ($R^2 > 0.99$) within the selected range of concentrations. Gradual dilution of the sample solution was carried out to determine the LOD and LOQ at signal-to-noise (S/N) ratio = 3 and 10 respectively. LODs and LOQs are depicted in Table 4.5 as mean of three replicates. These results indicate that the optimized UPLC–MS/MS method was accurate, precise, reproducible and suitable. for a rapid and sensitive detection of the 9 water-soluble vitamins and 6 fat-soluble vitamins in sequential chromatoghaphic run.

4.3.3 ***Application of the method for real sample analysis: vitamin profiling of R. imbricata root***

In accordance with the aforementioned sections, the complete method of UPLC-MS/MS conditions for the analysis of water- and fat-soluble vitamins was optimized, and afterwards, we aimed at studying the vitamin profile of the *R. imbricata* root sample with this method. The root powder could be considered as a complex matrix that increased our difficulties in estimating its complete vitamin profile. The results obtained from the analysis are summarized in Table 4.9, and expressed as µg of vitamin per kg fresh weight (fw) of root powder. The data presented in Table 4.8 illustrate the average values of five replicate measurements.

It was observed that among the fat soluble vitamins, α-tocopherol was present in good amount where as the other fat-soluble vitamins such as retinol, ergocalciferol, cholecalciferol, phylloquinone, and menaquinone were not detected. Amongst the water-soluble B-group vitamins, nicotinic acid and nicotinamide, pantothenic acid, and pyridoxine were found to be present in higher quantity as compared to thiamine and riboflavin. Nicotinic acid was the richest in all the studied water-soluble vitamin samples. Fat-soluble vitamins such as retinol, ergocalciferol, cholecalciferol, phylloquinone, and menaquinone and water-soluble vitamin cyanocobalamin were not detected as these vitamins are found only from animal sources or animal derived foods. Whereas, water-soluble vitamin folic acid was not detected due to the fact that vitamin is present mainly dark green leafy vegetables. There are very limited numbers of publication on the vitamin composition of plant root, because in comparison to other bio-active phyto-constituents, the plant root possesses very low amount of vitamins. The plant root exudates contain a complex and diverse cluster of compounds among which vitamins function to promote the rhizosphere microbial growth as the valuable source of nutrients that ultimately leads to plant growth in stressful environment with low micronutrient availability conditions (Dakora and Phillips, 2002; Marschner, 1995; Rovira and Harris, 1961; Rovira, 1969; Uren and Reisenauer, 1988). A number of possible explanations from our results could be- on one hand, the presence of highly abundant B-group vitamins in the fresh root signifies the mechanism of nutrient production in the rhizosphere by the root system in response to the highly stressful, fragile and nutrient deficient soil of the trans-Himalyan high altitude; on the other hand, the richness in vitamin E could possibly be due to the up-regulation of the antioxidant defense mechanism of plants to combat the severe environmental stresses that are responsible for the production of several secondary metabolites and antioxidant compounds. From previous reports

it was also revealed that accumulation of α-tocopherol can stimulate tolerance to chilling, water deficit, and salinity in different plants (Guo et al., 2006; Sharma et al., 2012; Yamaguchi-Shinozaki and Shinozaki, 1994; Munné-Bosch et al., 1999; Bafeel and Ibrahim, 2008) and it, therefore, may induce production of vitamin E. As described earlier, most of the research was afforded towards the evaluation of pharmacological and adptogenic properties of *R. imbricata* and several studies were carried out on other *Rhodiola* sp. in different parts of the world (Bai et al., 2005; Pooja et al., 2006; Ruan et al., 2001). Hence, there is lack of information on the vitamin composition of *R. imbricata,* found only in the trans-Himalayan region of India that limits our scope to compare the present study with previous reports.

4.4. Conclusion

It is very challenging to develop a method for the analysis of water- and fat-soluble vitamins in a complex biological matrix mainly due to a number of difficulties such as chemical heterogeneity, linkage of vitamins with the matrix components and the lack of standards and that resulted in the limited number of publications in this particular field of research. To the best of our knowledge, this is the first attempt towards extensive characterization of 15 water- and fat-soluble vitamins with UPLC-tandem mass spectrometry in the root of *R. imbricata* from the trans-Himalaya. The proposed process was simple, low-cost and rapid, with high sensitivity, reproducibility, robustness, time-efficiency and can be useful in the determination of vitamins in a variety of plant samples, food supplements, pharmaceutical preparations, fortified beverages and complicated food matrices. Finally, it can be concluded that, considering the harsh climatic conditions, difficult terrain, logistic constraints at high altitude that together lead to the scarcity of fresh fruits and vegetables, the source of vitamin B-complex and vitamin E from the root of

this wonder adaptogen would be of great interest to develop novel phyto-foods and beverages in the stressful environment of the Indian trans-Himalayan cold desert.

CHAPTER 5

FREE AMINO ACID COMPOSITION, FATTY ACID PROFILE AND MINERAL ANALYSIS IN ROOT OF *RHODIOLA IMBRICATA* EDGEW. FROM INDIAN TRANS-HIMALAYA USING RP-HPLC, GC-FID AND ICP-OES TECHNIQUE

Abstract

Our aim was to separate, identify and quantify the amino acids and fatty acids in *Rhodiola imbricata* Edgew. root by reversed phase high performance liquid chromatography (RP-HPLC) and gas chromatography coupled with flame ionization detector (GC-FID) technique. We analysed the amino acids and fatty acids in the plant root with precolumn phenylisothiocyanate (PITC) and fatty acid methyl esters (FAMEs) derivatization respectively. Nine essential and twelve non essential amino acids were quantified and the contents ranged between 214.67 and 1640.67 µg/g. Among the essential amino acids, histidine (1434.33 µg/g) and lysine (1329.33 µg/g) were found to be dominant, while glycine (1640.67 µg/g), proline (1263.67 µg/g), alanine (1142.33 µg/g), cystine HCL (239.33 µg/g) and leusine (928.67 µg/g) were major non -essential amino acids. The present study revealed the presence of 10 fatty acids in the plant root. The total lipid content was found to be rich in saturated fatty acids such as capric acid (19.98%), caproic acid (10.85%), palmitic acid (9.37%), lignoceric acid (6.17%), and behenic acid (5.67%), which together constituted 52% of the total lipid. Among the unsaturated fatty acids (UFAs), linoleic acid (15.04%), oleic acid (12.33%), arachidonic acid (8.39%), linolelaidic acid (6.17%), and docosadienoic acid (6.04%) were prominent. MUFA and PUFA were 35.64% and 12.33% of the total lipid content respectively in the root sample. The mineral profiling was carried out by inductively coupled plasma optical emission spectrometer (ICP-OES) and it was found to contain thirteen important dietary mineral elements. This is the first ever study on the amino acid, fatty acid and mineral profiling of *R. imbricata* root by RP-HPLC, GC-FID and ICP-OES that validates it as a good source of natural amino acids, fatty acids and dietary minerals.

5.1 Introduction

Natural products are the most dynamic repository of bio-active compounds and have immense potential in drug discovery and development (Mishra et al., 2008). In developed countries, there has been a prominent increase in the use of herbal plants in complementary alternative medicine (CAM) all over the world (WHO, 1998; UNCTD, 2000). The flora of trans-Himalayan India was studied in considerable details (Singh and Chaurasia, 2000; Chaurasia and Singh, 1996–2001; Chaurasia and Gurmet, 2003; Chaurasia et al., 2007). However, lots of efforts are needed for the detailed phytochemical investigation of the native medicinal plants of this remote region for the scientific validation of their uses in traditional system of medicine and therapeutic utilities.

Rhodiola imbricata Edgew. (roseroot or arctic root) belonging to the family Crassulaceae is an important food crop and medicinal plant in the high altitude region of Indian trans-Himalayan cold desert. A number of metabolites like phenylpropanoids, phenylethanol derivatives, flavanoids, monoterpernes, triterpenes, and phenolic acids were found in good yield from *Rhodiola* sp. and some have been shown to possess useful biological activities (Brown et al., 2002; Panossian et al., 2010). Many pharmacological studies have demonstrated that this plant exhibits cardioprotective, antiinflammatory, antipyretic, antistress and adaptogenic activities. It has also been found to possess antioxidant, antiaging, immuno-stimulant, anti-depressant, radioprotective, and anticarcinogenic properties (Chawla et al., 2010; Gupta et al., 2010; Mishra et al., 2012). All these reports support its use in traditional system of medicine and in recent times, a number of phytoproducts has been developed from our institute using this plant root. They were found to possess high nutritive properties and antioxidant capacities (Ballabh et al., 2007; Dhar et al., 2012; Dhar et al., 2013a,b). In our recent study, we have reported the antioxidant

properties, various volatile and semivolatile bioactive phyto-constituents and vitamins from the underground part of this valuable medicinal plant (Tayade et al., 2013a,b,c) and currently plenty of research efforts are focused towards the identification and isolation of polar compounds from this plant root. In the present work we investigated the free amino acid composition, fatty acid profile and mineral element content of *R. imbricata* root by standard analytical technique.

The amino acids found in nature occur either in free form or as linear chains in peptides and proteins. Analysis of amino acids plays a significant role in the study of the composition of proteins, foods, foodstuffs and other materials of biological origin. In multicellular organisms, most of the proteins are based on L-amino acids that have a great influence in both human and animal nutrition, health maintenance, and possess potent therapeutic applications (Chaitow, 1985; Ambrogelly et al., 2007). Plants subjected to different environmental and physiological stresses can accumulate amino acids in their system that play pivotal role in combating the stress. The amino acids produced in plant systems act as osmolyte, regulate ion transport, modulate stomatal opening, activate phytohormones and growth substances, generate chelating effect on micronutrients and play a vital role in the detoxification of heavy metals. They are also responsible for the synthesis and functional properties of specific enzymes, gene expression, and redox-homeostasis (Rai, 2002). Most importantly, in higher plants the amino acids serve as precursors for secondary metabolism (Zhao et al., 1998). Thus, the amino acids are directly related to plant stress physiology and have diverse preventive and recovery effects.

Estimation of amino acids in biological systems could be carried out by a number of advanced techniques *viz.* capillary electrophoresis-mass spectroscopy (CE-MS) (Poinsot et al., 2010), gas chromatography-mass spectroscopy (GC-MS) (Wood et al., 2006), liquid

chromatography-mass spectroscopy (LC-MS) (Nimbalkar, 2012) and many more complex methods. Among these methods, enantiomeric separation of amino acids has received a lot of interest within the pharmaceutical industry for the past two decades (Sarwar and Botting, 1993). Analysis of amino acids present in foods is usually performed after their derivatization. Reversed-phase HPLC (RP-HPLC) technique with precolumn derivatization is universally preferred because of the short time, simple instrumentation and cost-effectiveness. The distinct reagents used for precolumn derivatization are phenylisothiocyanate (PITC); *o*-phthalaldehyde (OPA); 9-fluorenylmethyl-chloroformate (FMOC-Cl); 1-fluoro-2,4-dinitrobenzene; 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide; and dansyl-chloride (Ilisz et al., 2012). Each of these reagents has particular advantages and restrictions. Only PITC and OPA are extensively used for amino acid analysis (Fabiani et al., 2002).

Fatty acids are referred to as aliphatic monocarboxylic acids that function as the building blocks of lipids. Depending on the number of double bonds fatty acids are classified into two broad groups: saturated fatty acids (SFA, without any double bond) and unsaturated fatty acids (UFA, with double bonds). The UFAs are further grouped into monounsaturated (MUFA, with one double bond) or polyunsaturated (PUFA, with more than one double bond). Individual fatty acids within these groups have discrete biological activities (Department of Health, 1994; Rochforta et al., 2008). Fatty acids influence a broad range of cellular processes such as functional ingredients of food and dietary supplements (Elias, 1983) and provide numerous health benefits that include growth promoting effect, nutrition, metabolic functions, and many more (Sargent et al., 1994; Al-Jassir et al., 1995; Tapiero et al., 2002; SanGiovanni and Chew, 2005; Carvalho et al., 2006; Huang, 2007; Berquin et al., 2008; Elvevoll et al., 2009; Galli, 2008; Morisco et al., 2008; Weaver et al., 2008;

Burlingame et al., 2009). All higher organisms including plants contain lipids that are the chief source of substantial quantities of PUFA. The omega-6 (n-6) and omega-3 (n-3) fatty acids are the two principal group of PUFA derived from α-linolenic acids (18:3 n3) and linoleic acid (18:2 n6), respectively. These two parent fatty acids can only be naturally synthesized in plants and therefore, have become the essential dietary components for animal and human diet for maintaining optimum health (Anil, 2007; Din et al., 2004). In addition, the fatty acid composition of plants is influenced by different environmental as well as genetic factors that have been inadequately investigated. Lipids composed of fatty acids are crucial for the functioning of cell. They are the major structural components of cell membrane and alterations in fatty acid composition induced by environmental stress can result in membrane damage and other harmful effects (Kuiper, 1980; Neffati and Marzouk, 2008; Ben Taârit, 2010; Bourgou, 2011).

Determination of fatty acid profiles in nutritional, epidemiological and clinical research with accuracy and swiftness has become very popular for human health and basic research. A variety of techniques have been employed for fatty acid profiling in biological materials. Of these, gas chromatography coupled with flame ionization detection (GC-FID) is a widely used rapid and efficient method for the analysis of complex mixtures of biological samples with compounds of diverse molecular weights (Horning, 1964; Seppänen-Laakso, 2002). Separation, identification and quantification of long-chain fatty acid mixtures by GC-FID have been extensively used to acquire information about various biological systems. In this process, the less volatile fatty acids are converted into more volatile derivatives [such as fatty acid methyl esters (FAMEs)] prior to GC analysis.

Minerals are essential micronutrients that are needed in small amounts to maintain proper health and optimum physical performance (Lukaski, 2004; Huang et al., 2007; Soetan

et al., 2010). There are a number of methods to analyze the mineral content in plant samples and among these techniques inductively coupled plasma optical emission spectrometer (ICP-OES) has become one of the most convenient analytical tools for the determination of trace elements in a wide variety of sample types (Hou and Jones, 2000).

The dearth of information regarding the amino acid composition, fatty acid profile and mineral content of *R. imbricata* root, which could play a vital role not only for the bio-activity and pharmacological properties but also provide new insights into the physiological adaptation aspects of the plant in the severe stressful environment of trans-Himalaya, motivated us towards the extensive analysis of amino acid composition, fatty acid profiling and mineral content analysis in this plant root. In the present investigation, our aim was to separate, identify and quantify the amino acids by RP-HPLC technique comprising precolumn PITC derivatization, fatty acids with FAMEs derivatization by GC-FID system and estimation of dietary mineral content by ICP-OES in *R. imbricata* root.

5.2 Materials & Methods

5.2.1 *Chemicals and reagents*

5.2.2 *Ethics Statement*

All necessary permits were obtained for the described field studies and plant collection. The permit was issued by Dr. B. Balaji (IFS), Divisional Forest Officer, Leh Forest Division, Jammu & Kashmir, India.

5.2.3 *Plant materials*

R. imbricata roots were collected from the trans-Himalayan region (Chang-La Top, Changthag valley, altitude 5330 m above sea level, latitude 34°2'49.81" N, longitude 77°55'49.78" E) of India in the month of October, 2011 after the period of senescence. All

necessary permits were obtained from local authorities for the described field studies. The plant roots were washed thoroughly and cut into small pieces and shade dried at room temperature for 15 days.

5.2.4 *Amino acid analysis*

The amino acid content of the root samples was determined using RP-HPLC with precolumn PITC derivatization according to our recent report with minor modifications (Dhar et al., 2013b; Bidlingmeyer et al., 1984; Cohen et al., 1988; Khan et al., 1994).

5.2.4.1 *Chemicals and reagents*

HPLC grade acetonitrile, ethanol, methanol, sodium acetate trihydrate and glacial acetic acid were obtained from Merck (Merck KGaA, Darmstadt, Germany). Triethylamine (TEA), phenylisothiocyanate (PITC) and amino acid standards, sodium hydrogen phosphate (Na_2HPO_4) and phosphoric acid (H_3PO_4) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All the other chemicals including solvents were of analytical grade. The Milli-R/Q water purification system (Millipore, Bedford, MA, USA) was used for the water used in all assays.

5.2.4.2 *RP HPLC System*

The RP-HPLC system equipped with a Shimadzu Class VP Binary pump LC-10AT_{VP}, DGU-14A On-Line Degasser, 2 µl in-line precolumn filters, 100 µl Semi-Micro Gradient Mixer, SIL-10A_{VP} Auto sampler, CTO-10A_{VP} column temperature Oven, and SPD-10Avp UV/VIS Detector was used in the present study. All the components of the system were controlled using SCL-10Avp System Controller with PC-55N A/D option board for SCL, and a Model 3394A integrator (Hewlett-Packard, Avondale, PA). Reversed phase C-18 column (5 µm, 150 x 4.6 mm) (Pickering Laboratories, Inc., Mountain View, California, USA) was used

for the separation of amino acids. A 30 × 4.6 mm i.d. guard column of the same material was also employed. Data acquisitions were done using Windows® 2000 Data Station and CLASS-VP™ Version 6.13 software with 0.005% minimum detection limit (MDL).

5.2.4.3 ***Preparation of standards***

Individual 21 amino acid standards (10 mg) were prepared by dissolving in 10 ml distilled water. A working standard mixture was prepared by diluting the intermediate stock standard solution to 100 µg/ml. Two hundred microliter of this standard solution was PITC derivatized as discussed in the next section. The derivatized standard solution was further diluted with distilled water up to 500 µl to prepare a final concentration of each amino acid 40 µg/ml in the working standard solution. The solution was filtered and stored at −20°C for further HPLC analysis.

5.2.4.4 ***Extraction of total amino acids through hydrolysis of sample***

A homogeneous 1.0 g sample was weighed into a 25×150 mm hydrolysis tube, aliquot (15 ml) of 6 N HCl was added, purged with nitrogen for 30 sec and the tube was sealed immediately with teflon coated cap. The tube was placed in the oven at 110°C for 24h to hydrolyze the protein completely (Roach and Gehrke, 1970; AOAC, 1990), removed and allowed to cool. The contents of the tube were quantitatively transferred to 25 ml volumetric flask and volume was adjusted with HPLC grade water. Five milliliter of this solution was filtered through 0.45 µm Millipore membrane filter for derivatization. This protein content in glass vial was stored in freezer at −20°C.

5.2.4.5 ***Derivatization procedure***

Aliquots of extract and working amino acid standard solution prepared in section 2.4 were concentrated and dried under vacuum (37°C, 20 mmHg), then a coupling reagent

(methanol/water/TEA, 2:2:1, v/v) was added and the solution was mixed and dried immediately under vacuum. After this, PITC reagent (methanol/TEA/water/PITC, 7:1:1:1, v/v) was added and allowed to stand at room temperature for 20 min before drying in vacuum. PITC derivatives were dissolved in sodium acetate buffer (mobile phase A) (Table 5.1).

5.2.4.6 ***Analytical chromatographic conditions***

The injection volume of the sample and standard was 20 µl. Separation of amino acids was performed on reverse phase C-18 column (5 µm, 150 x 4.6 mm) (Pickering Laboratories, Inc., Mountain-View, California, USA), with sodium acetate buffer, pH 6.4 (mobile phase A) and ACN : H_2O :: 6 : 4 (mobile phase B) with gradient mode of operation. The detector setting was**:** Gain = 5, Temperature = 39°C and Pressure = 250 kPa. The absorbance at 254 nm was recorded and used for calculations. The actual chromagraphic conditions in the present investigation have been depicted in Table 5.1.

5.2.5 ***Fatty acid analysis***

Fat and fatty acids were extracted from the sample by hydrolytic method and analyzed by GC-FID technique with FAMEs derivatization following the method described in a recent report from our institute (Dhar et al., 2013b). Pyrogallic acid was added to minimize oxidative degradation of fatty acids during analysis. The fat was extracted into ether, methylated to FAMEs using BF_3 in methanol, 14% (w/w). FAMEs were quantitatively measured by GC. Total fat was calculated as sum of individual fatty acids. Saturated and monounsaturated fats were calculated as sum of respective fatty acids. Monounsaturated fat includes only *cis* form (AOAC, 2002).

5.2.5.1 ***Chemicals and reagents***

HPLC grade acetonitrile, acetyl-chloride, methanol, 2-propanol and n-hexane were purchased from Merck Chemical Supplies (Merck KGaA, Darmstadt, Germany). Commercial reference FAMEs standard mixture (Supelco 37-Component FAME Mix, 47885-U, Supelco, Bellefonte, PA, USA) containing methyl esters of fatty acids ranging from C4 to C24, including key monounsaturated and polyunsaturated fatty acids was used. Nitrogen (industrial grade) was obtained from Sigma Gases & Services (Delhi, India) and used without further purification.

5.2.5.2 ***Extraction of fat from sample***

Accurately ground and homogenized 1 g root sample was weighed into a Mojonnier flask (Cole-Parmer, Mumbai, India). Then 100 mg of pyrogallic acid and a few boiling granules were added to the flask. After that 2 ml ethanol was added and mixed well. The flask was placed in a shaking water bath at 80°C set at moderate agitation speed for 40 min. Then the contents of the flask were mixed on a vortex mixer for 10 min and cooled to room temperature. An adequate amount of ethanol was added to fill bottom reservoir of flask and mixed gently. Diethyl ether (25 ml) was then added to the Mojonnier flask, sealed with stopper and placed in centrifuge basket. The basket was placed in wrist action shaker and shaken for 5 min. After that, 25 ml petroleum ether was added, sealed with stopper, shaken for 5 min, and centrifuged at 600 rpm for 5 min. The ether (top) layer was decanted into 150 ml beaker. This extraction procedure was repeated thrice and the extracts were combined. Then the ether was evaporated slowly on steam bath, using nitrogen stream to aid evaporation. The remaining residue in the beaker contained extracted fat.

5.2.5.3 ***Fatty acid methyl esters (FAMEs) preparation***

Extracted fat residue was dissolved in 3 ml each of chloroform and diethyl ether. Then the mixture was transferred to glass vial and evaporated to dryness at 40°C in water bath under nitrogen stream. Then 2 ml BF_3-methanol (14%, w/w) and 1 ml toluene were added and sealed. The vial was heated in an oven at 100°C for 45 min with gentle shaking with 10 min interval and cooled to room temperature. After that 5 ml water, 1 ml hexane, and 1 g Na_2SO_4 were added into the vial, caped and shaken for 1 min. The layers were allowed to separate and the top layer (containing FAMEs) was carefully transferred to another vial containing 1 g Na_2SO_4 and finally filtered through 0.22 µ Millipore membrane filter. The filtrate (extracted FAMEs) was then used for further GC-FID analysis.

5.2.5.4 ***Preparation of standard and GC-FID analysis***

A mixture of 37 FAMEs standard solution of varied concentration of 2 and 4% as aforementioned was diluted with 10 times of hexane, filtered, and stored at –20°C for GC-FID analysis. FAMEs were identified by direct comparison with the standard mixture. The percentage of individual FAMEs was made in relation to total area of the chromatogram. As different FAMEs have similar carbon chain length, it was assumed that they have the same response factor and volatility, allowing a direct comparison of the peak areas to determine the sample composition.

5.2.5.5 ***Instrumentation and chromatographic conditions***

A GC-4000A system (East & West Analytical Instruments, Beijing, China) configured with flame ionization detector, split/split-less mode injector (5 ml/min), column oven temperature programming sufficient to implement a hold-ramp-hold sequence, Hewlett Packard capillary column HP-88, 100 m × 0.25 mm × 0.20 µm film (Agilents Technologies

Ltd., Santa Clara, CA, USA) was used in the present investigation. Data acquisition was performed on A5000 Chromatogram Data Processing Workstation (Workstation Software, Version 1.6 with an interface board). GC-FID was employed for analysis of FAMEs. Injector port and FID detector temperature were programmed at 250°C and 280°C, respectively. The oven temperature was programmed as follows: 80°C hold 5 min; 80°C to 140°C @ 8°C/ min (7.5 min) hold 10 min; 140°C to 220°C @ 3°C/ min (26.5 min); and 220°C to 240°C @ 2°C/ min (10 min) hold10 min. Nitrogen, hydrogen, and zero air was used as the carrier gas, reaction gas, and detector gas at pressure of 0.25 MPa, 0.05 MPa, and 0.020 Mpa respectively at a flow rate of 1 ml/min. The injection split ratio was 1:50. The injection volume was 1 µl in the split/split-less injection mode.

5.2.6 ***Determination of mineral elements***

5.2.6.1 ***Chemicals and reagents***

HPLC grade acetonitrile, acetyl-chloride, methanol, 2-propanol and n-hexane were purchased from Merck Chemical Supplies (Merck KGaA, Darmstadt, Germany). Commercial reference FAMEs standard mixture (Supelco 37-Component FAME Mix, 47885-U, Supelco, Bellefonte, PA, USA) containing methyl esters of fatty acids ranging from C4 to C24, including key monounsaturated and polyunsaturated fatty acids was used. Nitrogen (industrial grade) was obtained from Sigma Gases & Services (Delhi, India) and used without further purification.

5.2.6.2 ***Mineralization of root sample***

The hot block digestion method was employed for digestion of the root sample with QBlock (Questron Technologies Corp., Mississauga, Germany) equipment. An amount of 0.5 g of the dry root powder was added in a polypropylene vial and placed inside a fume hood.

After that, 10 ml HNO_3 and 2 ml H_2O_2 were added and then the samples were allowed to outgas before loading them on QBlock. The digestion program was set for digestion of plant sample and reagents were added according to the manufacturer's instructions. It was then diluted to volume with deionized water and was stored in a clean polypropylene bottle.

5.2.6.3 ***Analysis of mineral elements***

The mineral elements *viz.* calcium, potassium, iron, magnesium, phosphorous, sodium, manganese, zinc, chromium, nickel, copper, cobalt and molybdenum were determined using an Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) (Varian, VISTA-MPX, CCD Simultaneous ICP-OES, United States) (Dhar et al., 2013b). The plasma conditions were as follows: RF power 1000 W, nebulizer flow 0.5 l/min, auxiliary flow 1 l/min, plasma flow 15 l/min and sample flow 1.5 ml/min.

5.3 Results and Discussion

5.3.1 *Amino acid analysis*

Amino acid content obtained from the root sample of *R. imbricata* using RP-HPLC with precolumn derivatization is summarized in Table 5.2. The peaks were identified on the basis of comparison between the retention time of the standard of the amino acids and those in the sample and were confirmed by a fortification technique (spiking) (Figure 5.1). Quantitation was achieved through the external standard method using calibration curves fitted by linear regression analysis (Statistica 5.1, StatSoft, Tulsa, OK).

The quantification of amino acids in the root sample revealed the amino acid profile with contribution of 21 amino acids including 9 essential and 12 non essential amino acids (Table 5.2). The content of 21 amino acids quantified by RP-HPLC ranged between 214.67 ± 7.09 and 1640.67 ± 11.85 µg/g. The highest content was of Gly 1640.67 ± 11.85 µg/g

followed by His 1434.33 ± 10.02 µg/g, Lys 1329.33 ± 11.55 µg/g, Pro 1263.67 ± 10.50 µg/g, Ala 1142.33 ± 11.02 µg/g, Lys HCL 1136.33 ± 11.72 µg/g, and Nor Leu 1038.67 ± 10.21 µg/g. Among the essential amino acids, His and Lys were found to be the dominant contributors, while Gly, Pro, Ala, Cys HCL and Leu were found to be the major non essential amino acids in the root sample. L-2-amino-n-butyric acid, Trp, and Orn were not detected. Whereas, Val is not detected as being present below the limit of detection.

Table 5.1 Gradient program employed for the separation of PITC derivatized amino acids[a].

Run time[b] (min)	Flow rate (ml/min)	% Buffer A[c]	% B (60% acetonitrile in water)
0	1	100	0
0.1	1	95	5
5	1	90	10
14	1	90	10
25	1	60	40
30	1	50	50
35	1	40	60
40	1	10	90
52	1	10	90
62	1	95	5
65	1	100	0

[a] Column temperature was maintained at 39°C.

[b] Run time was 62 min with 3 min column regeneration time.

[c] Sodium acetate buffer [19 g of sodium acetate trihydrate was dissolved in 1 liter of HPLC grade water. To this 0.5 mL of TEA was added and the contents were mixed properly. The pH of the solution was adjusted to 6.4 with glacial acetic acid. To the filtrate (940 mL) acetonitrile (60 mL) was added, mixed and filtered through a 0.22 µm Millipore membrane].

Table 5.2 Content, type of amino acid, retention time (RT) and peak area as quantified by RP-HPLC.

Peak no.	Amino acid	Abbreviation	Type	RT (min)	Peak area	Content (µg/g)
1	L-Arginine	Arg	Non essential	3.992	216927	214.67 ± 7.09
2	L-Aspartic Acid	Asp	Non essential	4.475	324956	434.67 ± 8.74
3	L-Glutamic Acid	Glu	Non essential	6.317	273840	320.67 ± 7.77
4	L-Serine	Ser	Non essential	8.500	1210077	839.67 ± 10.97
5	L-Glycine	Gly	Non essential	9.250	3064675	1640.67 ± 11.85
6	L-Histidine	His	Essential	10.442	1154401	1434.33 ± 10.02
7	L-Threonine	Thr	Essential	12.042	1232241	1015.67 ± 8.02
8	L-Alanine	Ala	Non essential	12.850	1517814	1142.33 ± 11.02
9	L-Proline	Pro	Non essential	13.367	1269920	1263.67 ± 10.50
10	L-Methionine	Met	Essential	24.642	434764	736.67 ± 8.02
11	L-Cystine HCL	Cys HCl	Non essential	26.383	1450843	1136.33 ± 11.72
12	L-Cystine	Cys	Non essential	27.500	267474	239.33 ± 8.39
13	L-Isoleucine	Ile	Essential	28.600	66781	91.33 ± 7.77
14	L-Leucine	Leu	Essential	29.842	1084489	928.67 ± 10.79
15	L-Nor Leucine	Nor Leu	Non essential	30.158	1569530	1038.67 ± 10.21
16	L-Phenylalanine	Phe	Essential	31.767	689754	855.33 ± 9.02
17	L-Lysine	Lys	Essential	33.550	2147649	1329.33 ± 11.55
18	L-2-amino-n-butyric acid	Abu	Non essential	ND	ND	ND
19	L-Valine	Val	Essential	21.192	27197	BDL
20	L-Tryptophan	Trp	Essential	ND	ND	ND
21	L-Ornithine	Orn	Non essential	ND	ND	ND

ND: Not detectable; BDL: Below detection limit

Table 5.3 Fatty acid composition of *R. imbricata* root.

Type of fatty acid	IUPAC name	CAS Number	Retention time (min)	Peak area	Peak height	Peak width	% Content in total lipid	Content in root (mg/g)
Saturated fatty acid (SFA)								
Caproic acid (C6:0)	Hexanoic acid	142-62-1	13.58	49613	11702	0.247	10.87	8.8
Capric acid (C10:0)	Decanoic acid	334-48-5	22.60	90878	13856	0.392	19.91	16.2
Lignoceric acid (C24:0)	Tetracosanoic acid	557-59-5	59.30	28127	6418	0.742	6.16	5
Palmitic acid (C16:0)	Hexadecanoic acid, (9*Z*)-	57-10-3	40.70	42991	9095	0.348	9.42	7.6
Behenic acid (C22:0)	Docosanoic acid	112-85-6	55.25	26055	955	1.164	5.71	4.6
Unsaturated fatty acid (UFA)								
Monounsaturated fatty acid (MUFA)								
Oleic acid (C18:1 n9c)	(9*Z*)-Octadec-9-enoic acid	112-80-1	47.46	56480	11686	0.39	12.38	10
Poly unsaturated fatty acid (PUFA)								
Linolelaidic acid (C18:2 n6t)	(9*E*,12*E*)-Octadeca-9,12-dienoic acid	506-21-8	48.86	27903	4221	0.383	6.11	5
Linoleic acid (C18:2 n6c)	*cis, cis*-9,12-Octadecadienoic acid	60-33-3	49.51	68738	14473	0.342	15.06	12.2
Arachidonic acid (C20:4 n6)	(5*Z*,8*Z*,11*Z*,14*Z*)-Eicosatetraenoic acid	506-32-1	57.33	38262	2985	0.424	8.38	6.8
cis-13,16-Docosadienoic acid (C22:2)	*cis*-13,16-Docosadienoic acid	7370-49-2	59.05	27314	5478	0.265	5.99	4.9
∑ SFA							52.034	
∑ UFA							47.966	
∑ MUFA							12.331	
∑ PUFA							35.635	

Table 5.4 Mineral content of *R. imbricata* root as estimated by ICP-OES.

Sr. no.	Minerals	Symbol	Content (mg/kg)
1.	Calcium	Ca	11034.17
2.	Potassium	K	2143.25
3.	Iron	Fe	1441.17
4.	Magnesium	Mg	581.99
5.	Phosphorous	P	376.72
6.	Sodium	Na	109.75
7.	Manganese	Mn	75.78
8.	Zinc	Zn	16.27
9.	Chromium	Cr	7.27
10.	Nickel	Ni	4.89
11.	Copper	Cu	3.49
12.	Cobalt	Co	2.98
13.	Molybdenum	Mo	2.65

In general, the major amino acids are present in elevated concentrations and typically associated with the primary carbon metabolism and nitrogen assimilation. On the other hand, the minor amino acids consist of essential amino acids in human diets which are generally less abundant (Noctor et al., 2002). Conversely, the present analysis revealed a higher concentration of essential amino acids especially His and Lys than non essential amino acids. In agricultural and food sciences, precise identification of edible plants and cataloguing of food products and nutraceutical botanicals have become important issues with the ever increasing alertness in consumers. Free amino acids correspond to a constant fraction of numerous natural foods, and on occasion, their quantity has been exploited for differentiation rationales (Maro, 2011).

The recent advances in amino acid profiling techniques provide a good alternative approach by converting enantiomers to diastereomers through chemical reactions with chiral derivatizing reagents, and by this strategy chromatographic separation can be easily achieved. In general, it is a very tricky task in this chromatographic approach to select the appropriate chiral

reagent, rather than the development of a chromatographic method for separating the

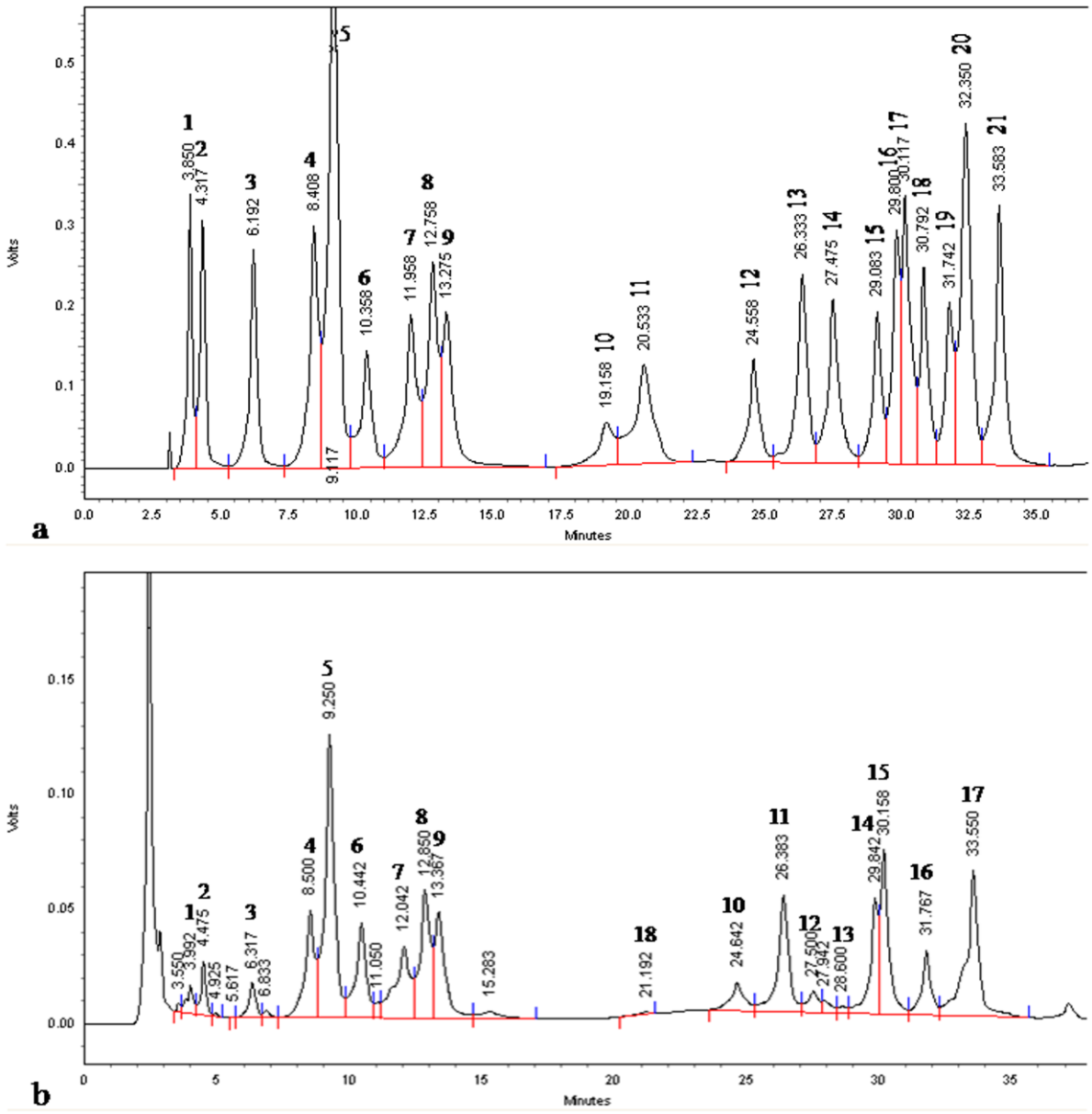


Figure. 5.1. RP-HPLC chromatogram of **a.** 21 amino acid standards, **b.** Amino acid profile of *R. imbricata* root.

1: L-Arginine; **2:** L-Aspartic Acid; **3:** L-Glutamic Acid; **4:** L-Serine; **5:** Glycine; **6:** L-Histidine; **7:** L-Threonine; **8:** L-Alanine; **9:** L-Proline; **10:** L-2-amino-n-butyric acid; **11:** L-Valine; **12:** L-Methionine; **13:** L-Cystine HCl; **14:** L-Cystine; **15:** L-Isoleucine; **16:** L-Leucine; **17:** L-Nor Leucine; **18:** L-Tryptophan; **19:** L-Phenylalanine; **20:** L-Ornithine; **21:** L-Lysine

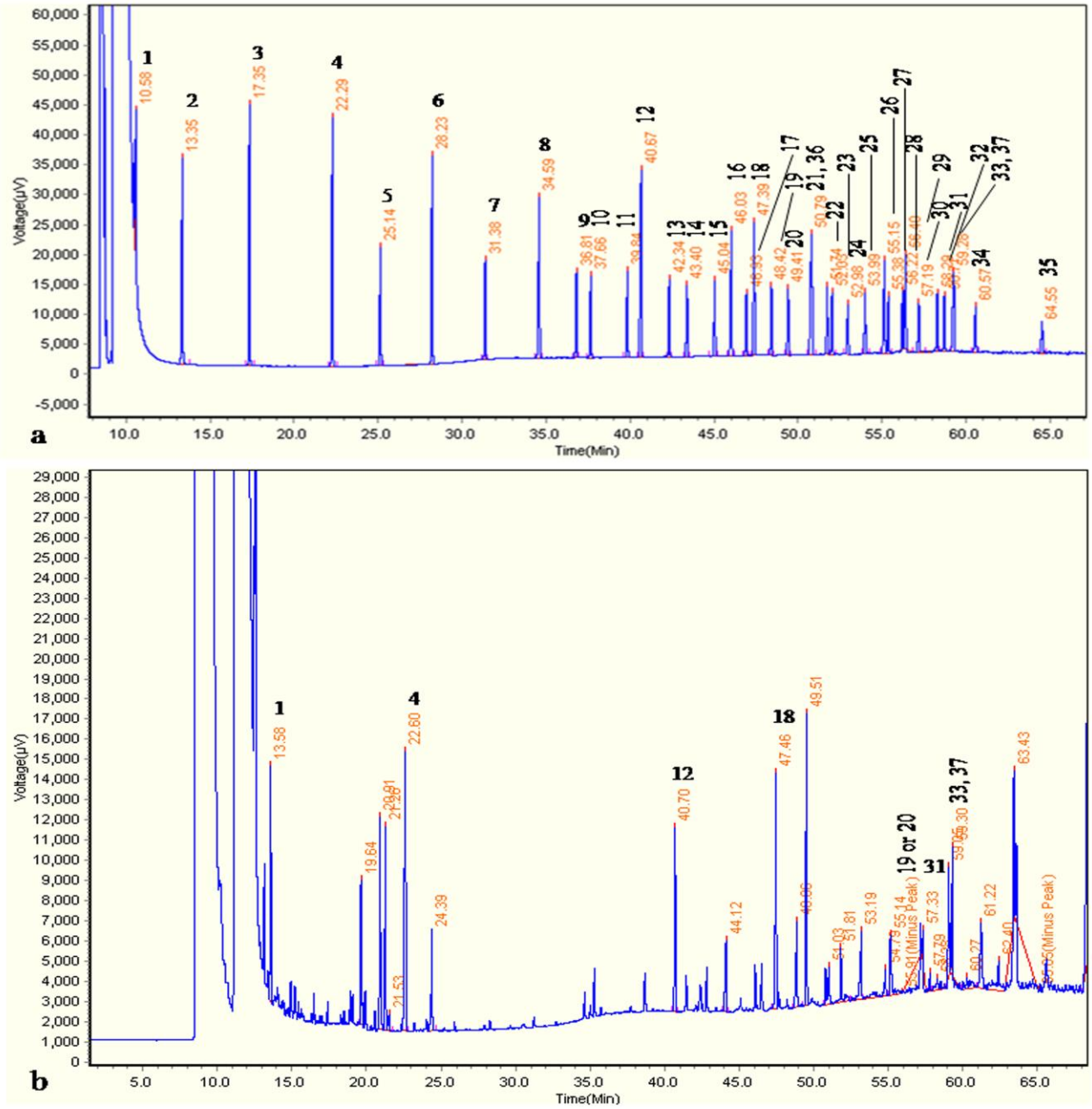


Figure. 5.2. GC-FID chromatogram of **a.** 37 FAMEs standards, **b.** FAMEs of *R. imbricata* root.

1: Butyric acid methyl ester (C4:0); **2:** Caproic acid methyl ester (C6:0); **3:** Caprylic acid methyl ester (C8:0); **4:** Capric acid methyl ester (C10:0); **5:** Undecanoic acid methyl ester (C11:0); **6:** Lauric acid methyl ester (C12:0); **7:** Tridecanoic acid methyl ester (C13:0); **8:** Myristic acid methyl ester (C14:0); **9:** Myristoleic acid methyl ester (C14:1); **10:** Pentadecanoic acid methyl ester (C15:0); **11:** *cis*-10-Pentadecenoic acid methyl ester (C15:1); **12:** Palmitic acid methyl ester (C16:0); **13:** Palmitoleic acid methyl ester (C16:1); **14:** Heptadecanoic acid methyl ester (C17:0); **15:** *cis*-10-Heptadecenoic acid methyl ester (C17:1); **16:** Stearic acid methyl ester (C18:0); **17:** Elaidic acid methyl ester (C18:1n9t);

18: Oleic acid methyl ester (C18:1n9c); **19:** Linolelaidic acid methyl ester (C18:2n6t); **20:** Linoleic acid methyl ester (C18:2n6c); **21:** Arachidic acid methyl ester (C20:0); **22:** *cis*-11-Eicosenoic acid methyl ester (C20:1); **23:** α-Linolenic acid methyl ester (C18:3n3); **24:** Heneicosanoic acid methyl ester (C21:0); **25:** *cis*-11,14-Eicosadienoic acid methyl ester (C20:2); **26:** Behenic acid methyl ester (C22:0); **27:** *cis*-8,11,14-Eicosatrienoic acid methyl ester (C20:3n6); **28:** Erucic acid methyl ester (C22:1n9); **29:** *cis*-11,14,17-Eicosatrienoic acid methyl ester (C20:3n3); **30:** Arachidonic acid methyl ester (C20:4n6); **31:** Tricosanoic acid methyl ester (C23:0); **32:** *cis*-13,16-Docosadienoic acid methyl ester (C22:2); **33:** Lignoceric acid methyl ester (C24:0); **34:** Nervonic acid methyl ester (C24:1); **35:** *cis*-4,7,10,13,16,19-Docosahexaenoic acid methyl ester (C22:6n3); **36:** γ-Linolenic acid methyl ester (C18:3n6); **37:** *cis*-5,8,11,14,17-Eicosapentaenoic acid methyl ester (C20:5n3)

derivatized diastereomers (Chandrul and Srivastava, 2010). Precolumn derivatization following separation of amino acids by RP-HPLC is a method well recognized for its simplicity, sensitivity and speed of separation (Fürst, 1990). In the present investigation, we have used the Pico-Tag method with PITC derivatizing reagent which has the advantage of being highly sensitive and capable of detecting nanogram (ng) amounts of amino acids (Dhar et al., 2013b).

Very little information is available on the amino acid profile of *Rhodiola* sp. from all over the world. In the last decade, only six kinds of *Rhodiola* L. from Xingjiang region of China were studied to estimate the amino acids in the root and rootstalk and they were reported to contain from 8 to 18 kinds of amino acid including 3 to 7 kinds of essential amino acid respectively. *Rhodiola rosea* L. contains most classes of amino acids among the six *Rhodiola* sp. in Xingjiang, which is approved to be included as a herbal drug in the traditional medicinal system of China (Ruan et al., 2001). Although the root of *R. imbricata* has been studied extensively for its pharmacological and therapeutic potentials, data on its free amino acid content determination is very scarce and therefore limits the scope for comparison of present analysis with other reports. From our study, it is also established that *R. imbricata* will be a good source of the natural amino acids that are beneficial to human consumption. This is the first ever study on the amino acid profiling of *R. imbricata* root that will definitely provide metabolomic insights in the near future.

5.3.2 Fatty acid analysis

The analysis of fatty acid obtained from *R. imbricata* roots revealed the presence of 10 fatty acids contributing 99.99% of the total lipid (Table 5.3). The GC-FID chromatogram of the 37 FAMEs standards and the root sample were shown in Figure 5.2. The total lipid was found to be a rich source of SFAs viz. capric acid (19.98%), caproic acid (10.85%), palmitic acid (9.37%), lignoceric acid (6.17%) and behenic acid (5.67%), which together constituted 52% of the total lipid (Table 5.3). Among the UFAs, linoleic acid (15.04%), oleic acid (12.33%), arachidonic acid (8.39%), linolelaidic acid (6.17%) and docosadienoic acid (6.04%) were prominent. MUFA and PUFA were 35.64% and 12.33% of the total lipid content respectively in the root sample.

In general, the nutritional value of fat is evaluated by the ratio of PUFA/SFA. The effects of MUFA and ratio of sum of PUFA and MUFA to SFA (PUFA+MUFA/SFA) on plasma and liver lipid concentrations in rats were investigated and it was revealed that the requisites for maintenance of low plasma and liver lipid concentration are, low MUFA/SFA ratio, low PUFA/SFA ratio, high PUFA/MUFA ratio, and PUFA+MUFA/SFA ratio < 2 (Chang and Huang, 1998). In our present investigation, the value of MUFA/SFA was 0.24, PUFA/SFA was lower (0.69), PUFA/MUFA was high (2.89) and PUFA+MUFA/SFA was 0.92 as the MUFA content was very low in the root of *R. imbricata*.

Previous studies by Mensink et al. (2003) established the fact that even though virgin coconut oil contains < 90% saturated fats and very low MUFA and PUFA, it has cholesterol and triacylglycerol lowering properties which may be accredited to small chain fatty acids (Mensink et al., 2003) and polyphenolic antioxidants (Nevin and Rajamohan, 2004). Conversely, high

MUFA containing diets were also reported to have cholesterol lowering capacities (Jenkins et al., 2010). It was also found that Palm oil containing elevated saturated fats (50%) is suitable for food and food products development, in soap manufacture and production of bio-diesel (Che Man et al., 1999; Soh et al., 2006). Therefore, the high yields of lipid from *R. imbricata* root deserves attention towards its utilization either for food, nutraceutical, dietary botanical supplements, soap or bio-diesel, if cultivated in large areas.

In this current study, PUFAs were found to be a major contributor and it is well established that they are an important health-promoting nutrient that have strong influence in alleviating cardiovascular, inflammatory, heart diseases, atherosclerosis, autoimmune disorder, diabetes and other diseases (Finley and Shahidi, 2001). Especially linoleic acid has the antioxidant effect of natural phenols (Peyrat-Maillard et al., 2003), anti-inflammatory, acne reductive, and moisture retentive properties (Darmstadt et al., 2002; Letawe et al., 1998) and most importantly, are indispensable since they cannot be synthesized by humans. Therefore, it is recommended that further research should be performed on the isolation of these bio-active compositions which will have the proper applications in human health. Arachidonic acid, oleic acid, linolelaidic acid, and docosadienoic acid, was also found to be present in considerable amount in the root. It was reported that arachidonic acid may be associated with improved insulin sensitivity and heart functions (Borkman et al., 1993; Harris et al., 2009). Oleic acid possesses blood pressure reducing effects along with reducing harmful effects of bad cholesterol (Perricone, 2002; Terés et al., 2008).

There is lack of information on the fatty acid composition of R. imbricata from the trans-Himalayan region of India. Most of the studies were carried out on the essential oil composition of

other Rhodiola sp. (Bai et al., 2005). A previous study on Rhodiola rosea was carried out with an objective to determine the phytoconstituents and antioxidant potential of the plant (Pooja et al., 2006). Hence, it limits our scope in comparing the present study with previous reports and this will be the first report in literature on the fatty acid composition of R. imbricata from Indian trans-Himalayan cold desert.

5.3.3 ***Mineral analysis***

The mineral composition of the plant root has been depicted in Table 5.4. It was found to contain thirteen important dietary mineral elements required for human nutrition. Calcium was found to be present in highest concentration followed by potassium, iron, magnesium, phosphorous, sodium, manganese, zinc, chromium, nickel, copper, cobalt and molybdenum. These micronutrients could be beneficial for growth and development.

5.4 Conclusion

To the best of our knowledge, this is the first report of use of RP-HPLC method for analyzing amino acid of *Rhodiola imbricata* root from trans-Himalayan cold desert. It was a reliable and reproducible technique for quantification of amino acids in plant root with pre-column derivatization along with significant accuracy. Current analysis revealed high concentration of essential amino acids in the plant root. The fatty acid analysis by GC-FID with FAMEs derivatization also revealed presence of valuable fatty acids in the plant root for the first time. The mineral elements were estimated by ICP-OES and a number of important dietary minerals were detected. Taking together the whole results, it can be concluded that *Rhodiola imbricata* roots contain different essential amino acids, fatty acids and mineral elements beneficial for human nutrition and it could be used as an ingredient to develop herbal supplements and functional foods to combat health issues associated with nutrition deficiency.

CHAPTER 6

PHARMACOLOGICAL EVALUATION (PHYSICAL ENDURANCE) OF *R. IMBRICATA* ROOT HYDRO-ALCOHOLIC EXTRACT ON RATS FOLLOWING EXPOSURE TO HYPOBARIC HYPOXIA

Abstract

High altitude (HA) exposure is described by hypobaric hypoxic environmental conditions that provoke changes in both physiological and psychological responses. It is the hypoxia at HA that becomes a restraining feature for normal physical and mental performance (Schoene, 1996; Shukitt-Hale B, Lieberman, 1996). Hypobaric hypoxia is known to trigger oxidative stress for which antioxidants are required as scavengers. *Rhodiola imbricata* Edgew. (Family: *Crassulaceae*), an important and widely used food crop and traditional medicinal plant, distributed in trans-Himalayan cold desert regions, has been reported to have wide medicinal potential conformed especially to aqueous, alcoholic and hydro-alcoholic root/rhizome extract due to the presence of vital metabolites including natural antioxidants. In the present study, effect of administration of 60% ethanol root extract (drug: 250 mg/kg of body weight of animal) of the plant on normoxic and hypobaric hypoxia induced (7600 m above mean sea level) oxidative stress and physical endurance of rat (180 + 10 g) was evaluated. At the end of the stipulated period, treadmill test was performed at a speed of 24 m/min at 45°C inclination till the time of exhaustion of the animal. The percentage of endogenous antioxidant status such as GSH, GSSG, and GSH/GSSG were determined for normoxia + vehicle, normoxia + drug, 7 day hypoxia + vehicle and 7 day hypoxia + drug. The effect of root extract supplementation to rat on treadmill exhaustive exercise showed enhance physical endurance as compared to the control group of animals. This fact was also supported by the fact that the drug supplemented animal groups showed an up regulation of endogenous antioxidant which is one of the requisite parameter and known to decrease in oxidative stress. These findings validate its traditional and modern pharmacological potential as a strong adaptogen, oxidative stress regulator and physical performance enhancer.

6.1 Introduction

With the unvarying increase in human populace at high altitude due to tactical or military reasons, athletic training, there is a requirement to examine the consequence of prolonged exposure to high altitude on cognitive and physiological health. Studies on effect of ascent to high altitude on mountaineers confirmed decline in physical and mental performance even on short durations of exposure lasting from few days to <03 months that is significantly influenced by rate of ascent and extent of stay at high altitude (Singh et al., 2004; Sharma et al., 2014).

All mamalian organs need adequate and steady supply of oxygen to fuel a range of biometabolic processes during mitochondrial respiration. Oxidative stress during hypoxia may sound paradoxical. Yet, it may be stimulated not only by increase but also by a fall in oxygen tension. The hypoxic cell relies on anaerobic glycolysis to produce ATP, whereas its outstanding low oxygen delivery supports some level of oxidative production of ATP through the tricarboxylic acid cycle and electron transport chain (ETC). Electrons leaking from the mitochondrial ETC generate an excess of reactive oxygen species (ROS), i.e., oxidative stress. Reoxygenation or high oxygen levels following harsh hypoxia further exaggerate ROS production. This concept is validated by the clinical benefits accruing from the use of agents able to scavenge ROS or preventing their development in hypoxic lesions (Kulkarni et al., 2007).

Plant based natural antioxidants are known to exert a potential role against stress-induced oxidative damage resulting due to the increased production of highly reactive oxygen species (ROS) within the cellular milieu, or due to various diseases and ageing processes (Prasad et al., 1994; Dai et al., 2009; Kohen and Nyska, 2002; Brieger et al., 2012). Consequently, such compounds reflect potential in promoting overall health and well-being, and hold potential for medicinal use, particularly for treating a broad range of ailments and maladies confronting humans.

The Indian trans-Himalayan cold desert region of Ladakh represents a valuable source of large number of natural bio-resources. The diverse floral and faunal composition of this remote Himalayan land provides a vast number of natural products beneficial for the armed forces as well as civil population (Dhar, 2013). This high altitude region is one of the most difficult and hostile terrains for human survival due to the extreme environmental factors and scarcity of fresh

foods (Hota et al., 2011). It is believed that plants growing in high altitude environment produce natural bioactive substances or secondary metabolites, which help them survive under stressful conditions. The high-altitude plant species of Ladakh region have been reported to possess rich medicinal properties and play major role in the traditional systems of medicine since ancient time (Singh and Chaurasia, 2000; Chaurasia et al., 2007) and could be used as prophylactic and therapeutic agent for high altitude maladies and other stress induced disorders (Ballabh and Chaurasia, 2007; Ballabh et al., 2008; Ballabh and Chaurasia, 2009).

R. imbricata is an important and widely used food crop and traditional medicinal plant distributed in trans-Himalayan cold desert regions. In Amchi and Tibetian system of traditional medicine, the roots are used against lung problems, cold, cough, fever, loss of energy and pulmonary complaints (Chaurasia et al., 2007). Modern pharmacological studies have shown that *R. imbricata* aqueous, ethanolic and hydro-alcoholic root extracts possess anti-cellular and immunomodulatory potential (Mishra, Ganju, Singh, 2012), immunostimulatory activity (Mishra et al., 2006; Mishra et al., 2009), adjuvant activity (Mishra et al., 2010), adaptogenic activity (Tulsawani et al., 2011; Gupta et al., 2008), radioprotective efficacy (Arora et al., 2005; Goel et al., 2006; Chawla et al., 2010), radiomodulatory (Arora et al., 2008), cytoprotective (Kanupriya et al., 2005), antioxidant potential (Kanupriya et al., 2005), free-radical scavenging activity and metal chelating activity (Arora et al., 2008), cold, hypoxia and restraint (C-H-R) exposure and post-stress recovery (Gupta et al., 2010), cold, hypoxia and restraint (C-H-R) stress induced hypothermia and post stress recovery: mechanism and action (Gupta et al., 2009), anti-proliferative effects (Mishra et al., 2008), anti-cancerous (Mishra et al., 2008), dermal wound healing potential (Gupta et al., 2007), hepatoprotective effect (Senthilkumar et al., 2014), radical scavenging and antiproliferative activity of extracts in HT-29 human colon cancer cells (Senthilkumar et al., 2013), and found to be safe (Saggu et al., 2006; Gupta et al., 2008; Tulsawani et al., 2011). We previously (Tayade et al., 2013a,b,c) reported the presence of vital metabolites especially from the polar extract that can be used as an alternative to synthetic antioxidants that could be of use in oxidative stress mediated diseases. We also reported the presence of fat and water soluble vitamins in the root of the plant.

Although, aforementioned review of literature revealed that the extracts particularly those from roots/rhizomes aqueous, alcoholic and hydro-alcoholic extracts of the plant were

extensively studied and found to possess useful pharmacological potentials and widely used as an adaptogen and oxidative stress regulator, it's used as to increase physical endurance remains untested. In the present study, effect of 60% ethanol extract of the plant on hypobaric hypoxia induced oxidative stress and physical endurance on rat animal model was evaluated.

6.2 Materials & methods

6.2.1 *Chemicals and reagents*

The chemicals *viz.* heparin, 2′,7′-dichlorofluorescein diacetate (DCFHDA), PBS buffer, meta-phosphoric acid, L-glutathione reduced (GSH) and L-glutathione oxidized (GSSG) were procured from Sigma-Aldrich (St. Louis, MO, USA) for the animal studies. Ethanol was from Merck Chemical Supplies (Merck KGaA, Darmstadt, Germany). Rests of the chemicals were of analytical grade procured from Sigma-Aldrich.

6.2.2 *R. imbricata root sample collection and extraction*

R. imbricata roots were collected from the trans-Himalayan region (Chang-La Top, altitude = 5330 m above mean sea level) of India in the month of October, 2011 after the period of senescence. The plant roots were washed thoroughly, and cut into small pieces and shade dried at room temperature for 15 days to constant weight. The dried root material was ground separately to powder. Finely ground dried sample (1000 g) was taken for extraction in 60% ethanol (v/v) in a Soxhlet apparatus (Borosil GlassWorks Limited, Worli, Mumbai, India) at 40°C and concentration of the extract under reduced pressure in a rotary (BUCHI Rotavapor R-205, BUCHI Labortechnik AG CH-9230, Flawil, Switzerland) at 40°C with circulation of cold water (4°C) using thermostat in order to minimize degradation of thermolabile metabolites. This concentrated ethnaolic extract was further lyophilized (Christ Alpha 1–4 LSC Freeze Dryer, Martin Christ Freeze Dryers GmbH, Osterode am Harz) and concentrated to yield powder at −90°C. This extract was finally stored at −80°C freezer for further analysis.

6.2.3 ***Effect of R. imbricata 60% ethanol extract on hypobaric hypoxia induced oxidative stress***

6.2.3.1 ***Administration root extract***

Lyophilized 60% ethanol extract powder was dissolved in distilled water to have a concentration of the desired dose of 250 mg/kg body weight of the animal and administered orally to animal in a maximum volume of 1 ml through gastric cannula. In vehicle treated groups animals were orally administered with 1 ml distilled water as a vehicle.

6.2.3.2 ***Animals***

The study protocols were approved by the Institutional Ethics Committee and proper care was taken in order to minimize the sufferings of the animals during entire experimental procedure. Adult male inbred Sprague-Dawley rats (180 ± 10 g) were housed under controlled environment in the animal house of our sister laboratory Defence Institute of Physiology & Allied Sciences (DIPAS), New Delhi, India in hygienic conditions with day and night cycle of 12 h (0600 AM to 0600 PM) each with temperature maintained at 30 ± 2°C and humidity at 55 ± 5%. The animals were fed standard animal food pellets (Lipton Private Limited, New Delhi, India) and water *ad libitum.* Animal handling was performed between 0900 to 1000 hrs to avoid experimental deviation. All the experiments were carried out in accordance with the regualtions specified by the Institutional Animal Ethical Committee (IAEC) and conformed to national guidelines 'Committee for the purpose of control and supervision of experiments on animals' of Government of India on the care and use of laboratory animals.

6.2.3.3 ***Altitude simulation***

The animals selected to be exposed to hypobaric hypoxia were placed in a specially designed animal decompression chamber (Seven Star, New Delhi, India) to simulate altitude of 7,600 m (25,000 ft, 282 mm Hg). The environmental conditions in the chamber were maintained by precisely reducing the ambient barometeric pressure, temperature and humidity. Fresh air was continuously flushed at a rate of 8 L/min to avoid accretion of carbon dioxide inside the chamber. The environmental conditions similar to that in animal house were kept for the animals in the chamber. The rate of ascent and descent to hypobaric conditions was maintained at 300 m/min. The hypobaric exposure was continuously provided for the stipulated period except for 15–20 min interval each day (between 0900 to 1000 hrs) for drug administration, food and water

replenishment, measuring food, water and body weight gain or loss, and for cage changing purpose.

6.2.3.4 *Experimental design*

Male adult Sprague-Dawley rats (n = 10 animals/group) were randomly divided into the following four groups:

Group I: served as normoxia + vehicle (N+V: normoxia group).

Group II: served as normoxia + drug treated group (N+D: normoxia group with orally administration of 1 ml of plant extract).

Group III: served as hypoxia + vehicle group (H+V: rats were exposed to 7,600 m stipulated period with orally administration of 1 ml of vehicle once in a day for 7 days).

Group IV: served as hypoxia + drug treated group (H+D: rats were exposed to 7,600 m stipulated period with orally administration of 1 ml of plant extract once in a day for 7 days).

Four animals per cage of rats were housed for all study groups for 2 days habituation before the start of experimental procedures. The hypoxia groups were allowed to habituate and acclimatize to the experimental conditions for 2 days during the initialization of experimental procedures. Animal body weight, food consumption and water intake were measured and recorded on a daily basis. During the entire experiment, animals were allowed excess food and water freely. For reporting the animal research investigations ARRIVE guidelines were followed (Kilkenny et al., 2010). At the end of the stipulated period of hypoxic exposure, fasting experiments were carried out for 24 h and animals were sacrificed at 0900 AM. Then blood samples were obtained from orbital sinus in heparin coated blood collection vial (Riley, 1960).

6.2.3.5 *Physical endurance test (Treadmill Test)*

Before the experiment, all rats were familiarized with the speed and gradient of treadmill training under normal pressure and normal oxygen. Rats were trained to walk on a treadmill at a speed of 12 m/min at 45°C inclination for 5 min, 2 days before exposure to hypoxia. During the training and the test, rats were given a weak electrical stimulation (3V, 2 Hz) to initiate movement. Treadmill test were performed at a speed of 24 m/min at 45°C inclination till the time of exhaustion of the animal. The rats in the control group stayed on static treadmills beside the exercise treadmills.

6.2.3.6 *GSH and GSSG*

Reduced glutathione (GSH) and oxidized glutathione (GSSG) levels were determined fluorimetrically from the sample homogenate as per the procedure depicted by Hissin and Hilf (1976) with slight modifications. In brief, to 250 µl of the sample homogenate was added to an equal volume of 10% meta-phosphoric acid. The reaction mixture was then centrifuged at 10,000 g for 30 min at 48°C. The supernatant was collected and used for analysis of GSH and GSSG spectrofluorimetrically at 350 nm excitation and 420 nm emissions.

6.2.3.7 *Statistical analysis*

All experiments were performed in triplicate. The values are represented as mean ± SD. Statistical difference between groups was calculated using one-way analysis of variance followed by the Neumann-Keuls test for post hoc analysis. The p value < 0.05 was considered to be statistically significant.

2.3 Results

High altitude (HA) exposure is described by hypobaric hypoxic environmental conditions that provoke changes in both physiological and psychological responses. It is the hypoxia at HA that becomes a restraining feature for normal physical and mental performance (Schoene, 1996; Shukitt-Hale B, Lieberman, 1996).

The results of the pharmacological evaluation of *R. imbricata* ethanolic root extract on rats exposed to hypobaric hypoxia has been depicted in Table 6.1 and 6.2 and Figure 6.1, 6.2 and 6.3.

Table 6.1 Effect of *Rhodiola* root hydro-alcoholic aqueous extract oral administration (250 mg/kg body weight, single dose/day for 7 days) on body weight, food or water intake.

Parameter	Days	Normoxia		Hypoxia	
		N + V	N + D	H + V	H + D
Body weight	0	184 ± 4.23	185 ± 4.32	186 ± 3.38	185 ± 3.01
	7	200 ± 9.27	204 ± 8.89	168 ± 12.07	174 ± 9.12
Food intake	0	12.54 ± 2.64	12.33 ± 3.22	13.03 ± 3.11	13.54 ± 2.86
	7	14.75 ± 2.46	15.63 ± 2.65	7.65 ± 2.96	8.30 ± 2.52
Water intake	0	20.5 ± 2.01	20.9 ± 2.79	19.6 ± 2.58	20.5 ± 2.54
	7	24.6 ± 2.97	25.0 ± 2.30	22.3 ± 2.38	23.1 ± 2.34

Table 6.2 Effects of *Rhodiola* root hydro-alcoholic extract oral administration (250 mg/kg body weight, single dose/day for 7 days) on rat Treadmill exhaustive exercise time in min

Parameter	Days	Normoxia		Hypoxia	
		N + V	N + D	H + V	H +D
Treadmill run	0	55.42 ± 5.76	54.42 ± 3.62	58.76 ± 6.96	57.23 ± 4.65
time (min)	7	60.34 ± 4.43	85.76 ± 4.65	31.54 ± 6.47	49.37 ± 5.83

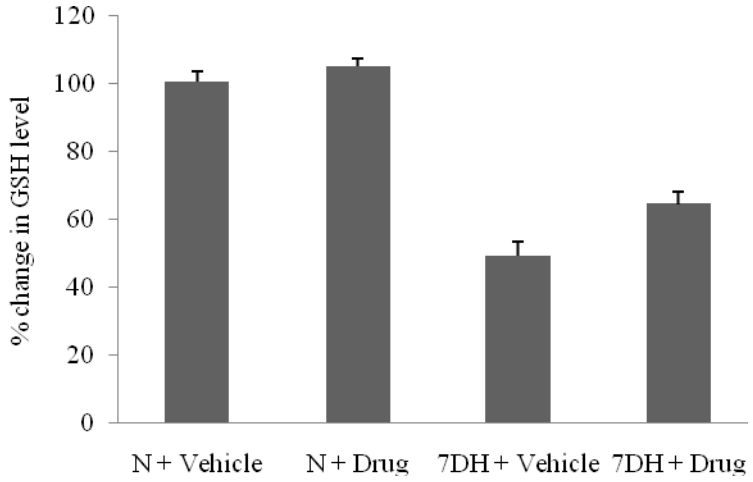


Figure 6.1 Percent change in GSH level in the experimental groups.

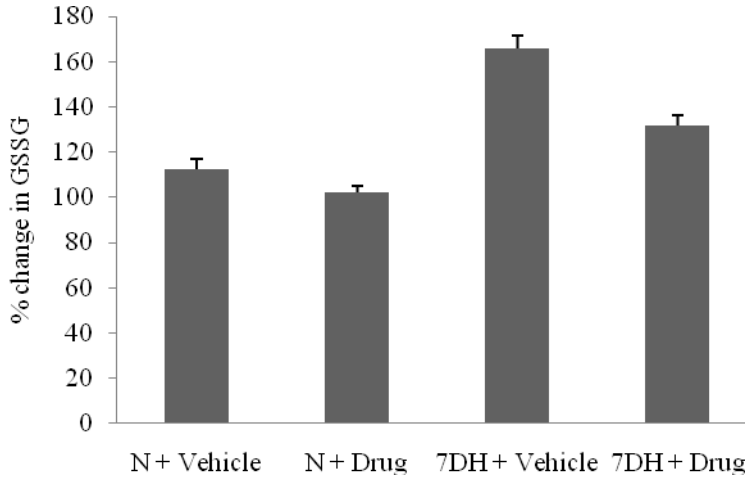


Figure 6.2 Percent change in GSSG level in the experimental groups.

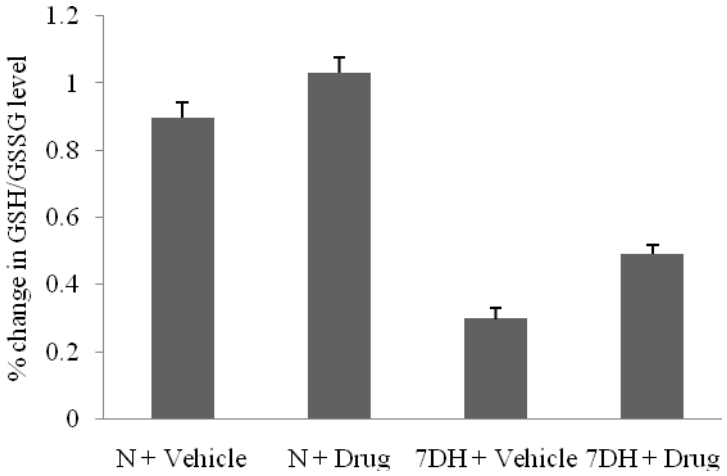


Figure 6.3 Percent change in GSH/GSSG level in the experimental groups.

6.4. Conclusion

The pharmacological evaluation of the *R. imbricata* root extract on rats following exposure to hypobaric hypoxia showed increased body weight, food intake and physical endurance. It was also found to upregulate the production of endogenous antioxidant enzymes and thereby showed positive pharmacological properties in minimizing oxidative stress in drug treated animal groups to those in non treated groups.

CONCLUSIONS

Phyto-constituents in different solvent extracts of *R. imbricata* root with hyphenated gas chromatography mass spectrometry (GC-MS/MS) technique

From GC/MS analysis, various **Phyto-constituents** have been identified which shows the existence of various bioactive principles that confirm the application of *R. imbricata* for numerous ailments in traditional system of medicine. However, isolation of individual **Phyto-constituents** and subjecting them to biological activity will definitely give fruitful results to find a novel drug.

***In vitro* antioxidant radical scavenging capacities of *R. imbricata* root extracts, their correlation among the content of total phenolic compounds *viz.* total polyphenols, phenolic acids, flavanoids and flavonols.**

The methanol and aqueous root extracts of *R. imbricata* came up as a source of natural antioxidants. The presence of vital phyto-constituents *viz.* polyphenol, flavonoid, and flavonol may contribute significantly to their antioxidant capacities. From The results of our study, it can be concluded that the methanol and aqueous root extracts of *R. imbricata* may have potential health benefits and could be used as a source of natural antioxidants for pharmacological preparations.

Sequential determination of fat- and water-soluble vitamins in *R. imbricata* root with reversed phased rapid resolution liquid chromatography/tandem mass spectrometry (RRLC-MS/MS)

According to available literature on the subject this is the first attempt towards extensive characterization of 15 water- and fat-soluble vitamins with RRLC-MS/MS in the root of *R. imbricata* from the trans-Himalaya. The process was simple, low-cost and rapid with high sensitivity, reproducibility, robustness, time-efficiency and can be useful in the determination of vitamins in a variety of plant samples, food supplements, pharmaceutical preparations, fortified beverages and complicated food matrices. Considering the harsh climatic conditions, difficult terrain and logistic constraints at high altitude, which together lead to the scarcity of fresh fruits

and vegetables, the source of vitamin B-complex and vitamin E from the root of this wonder adaptogen would be of great value in developing multivitamin foods and beverages.

Fatty acid profiling in root of *R. imbricata* using gas chromatography coupled with flame ionization detector (GC-FID) with fatty acid methyl esters (FAMEs) derivatization technique

Data on the fatty acid profile of *R. imbricata* root is very scarce and it was studied for the first time. This study revealed the presence of 10 fatty acids in the plant root. MUFA and PUFA were 35.64% and 12.33% of the total lipid content respectively. Capric acid (19.98%) was the major constituent of the saturated fatty acid while among the unsaturated fatty acids, linoleic acid (15.04%) was prominent. In view of the results of the present study, it can be concluded that the roots R *imbricata* may be the one substitute of these fat ratios in maintenance of low plasma and liver lipid concentration particularly in high altitude areas as the desired trend were achieved. Similarly the low fat and lipid content present in the roots *R imbricata* will also serve the purpose of cholesterol and triacylglycerol lowering properties as well.

Free amino acid profiling in root of *R. imbricata* using reversed phase high performance liquid chromatography (RP-HPLC) with precolumn phenylisothiocyanate (PITC) derivatization

The present study revealed that majority of essential and non-essential amino acids are available in good quantity in the roots of *R. imbricata*. Amino acids play a significant role in protein synthesis and many vital activities of the body. The high content of amino acids makes the plant more useful as a dietary supplement. Particularly in high altitude areas, this plant may be supplemented with various foods and health products. From our study, it is established that *R. imbricata* will be a good source of the natural amino acids that are beneficial to human consumption as nutritional dietary supplements.

Mineral profiling of *R. imbricata* root by ICP-OES.

The present study revealed that various minerals were present in good quantity that could be of use to develop a dietary supplement from this plant.

Pharmacological evaluation (physical endurance) of *R. imbricata* root hydro-alcoholic extract on rats following exposure to hypobaric hypoxia.

The pharmacological evaluation (physical endurance) of the *R. imbricata* root extract on rats following exposure to hypobaric hypoxia showed increased body weight, food intake and physical endurance. It was also found to upregulate the production of endogenous antioxidant enzymes and thereby showed positive pharmacological properties in minimizing oxidative stress.

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1. Om Prakash Chaurasia, Shashi Bala Singh, Raj Kumar, Sunil Kumar Hota, **Amol Bapurao Tayade.** A soft-gel capsule formulation for nutraceutical value from Seabuckthorn (**Patent Filed No. 1430/DEL/2011**), The Patent Office Journal, issue 47/2012, 23/11/2012, pp. 23074

2. Om Prakash Chaurasia, Shashi Bala Singh, Basant Ballabh, Tsering Stobdan, **Amol Bapurao Tayade**, Surendra Kumar Saurav, Divakar Sharma. A formulation of a novel herbal antioxidant supplement for nutraceutical value (**Patent Filed No. 635/DEL/2009**), The Patent Office Journal, issue 02/2011, 14/01/2011, pp. 738

Trade Marks Granted from Indian Patent office

3. **HIM RAKHSA** for herbal antioxidant supplement using Seabuckthorn pulp and extracts of high altitude medicinal plants (Trade Mark No. 1832892, J.N. 1444)

4. **SINDHU PRASH** for herbal antioxidant supplement using Seabuckthorn pulp and extracts of high altitude medicinal plants (Trade Mark No. 1832893, J.N. 1444)

5. **SINDHU SANJIWINI** for herbal antioxidant supplement using Seabuckthorn pulp and extracts of high altitude medicinal plants (Trade Mark No. 1832894, J.N. 1444)

6. **BERRY VITA** for herbal antioxidant supplement using Seabuckthorn pulp and extracts of high altitude medicinal plants (Trade Mark No. 1832895, J.N. 1444)

Transfer of Technology

7. Formulation of herbal antioxidant supplement to Indian Medicinal Pharmaceutical Corporation limited (IMPCL), Ministry of Health, Government of India. IMPCL launched this product with the brand name AAU OJAS

Oral paper presentation in Indian Science Congress under Young Scientist Awards Programme 2012-13 under Plant Science section (100th Indian Science Congress, Kolkata, 3-7 Jan 2013)

8. **Amol Bapurao Tayade**, Basant Ballabh, Divakar Sharma, Surendra Kumar Saurav, Tsering Stobdan, Om Prakash Chaurasia, Shashi Bala Singh, Ravi Bihari Srivastava. Formulation and utility of a novel herbal antioxidant supplement.

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