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Chromatographic Determination of Bioactive Compounds in *Hippophae* Leaf Extracts: A Comparative Study of Three Varieties.

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ABSTRACT

Seabuckthorn plants (*Hippophae Linn*), belonging to the family Elaeagnaceae have shown diverse therapeutic potential and the adaptogenic activity of some of the species have also been established in our previous studies. The present study aims to characterize aqueous and alcoholic leaf extracts of three different varieties of seabuckthorn, namely, *Hippophae salicifolia*, *Hippophae rhamnoides mongolica* and *Hippophae rhamnoides turkestanica* and evaluate their antioxidant potential *in vitro.* An elaborate characterisation of phytochemicals such as volatile organic compounds (VOC) and flavonoids occurring in the concerned extracts has been carried out by GC-MS and HPTLC respectively. GC-MS demonstrated the presence of 35 distinct VOCs in the seabuckthorn leaf extracts which are known to possess substantial pharmacological and antioxidant potential. The most abundant VOCs identified were trimethylsilyl palmitate, methyl octadec-9-enoate, methyl palmitate, methyl stearate and methyl (9E)-9-octadecenoate. HPTLC results revealed variable quantities of quercetin, gallic acid, ascorbic acid and rutin in all the seabuckthorn leaf extracts. HPTLC-centered chemometric analysis using R programming helped to distinguish among the various extracts based on pattern recognition and unsupervised clustering, thus, enabling grouping of the extracts for further studies.

Keywords: Bioactive compounds; Chemometrics; GC-MS; HPTLC; Seabuckthorn

1. INTRODUCTION

Seabuckthorn, *Hippophae* spp. (SBT) is a deciduous, spinescent shrub belonging to family *Elaegnaceae* with narrow, lanceolate leaves that is prevalent in high altitudes (~3500 m above sea level) of Eurasian regions[1], being mainly distributed in the north west Himalayan ranges[2]. This plant has been revered since centuries as a precious medicinal plant on account of various therapeutic properties localised in its different parts. For example, ripe berries contain aplenty vitamins (A, C and E) and organic acids (malic acid and oxalic acid) which exert appreciable detoxifying and anti-inflammatory activities[3], peel of stems is rich in 5-HT which widely acts as a neurotransmitter as well as anticoagulant[4], leaves contain flavonoids[5] that exhibit antioxidant properties, etc. Maximum research documented on therapeutic values of SBT has been centered on berries and pulp. However the current paper aims at characterisation of SBT leaves, considering the reported richness of SBT leaves in phytoconstituents such as phenolics[6], nucleobases[7], glycoprotein and fatty acids. The paper seeks to present a comparative analysis among leaf extracts of three different varieties of SBT, namely *Hippophae salicifolia*, *H. rhamnoides mongolica* and *H. rhamnoides turkestanica*.

Previous studies carried out on the afore mentioned *Hippophae* spp leaves had established the adaptogenic potential against multiple stress factors resulting in high altitudes, viz., cold, hypoxia and restraint (CHR)[8]. In the present study, to further explore the possibility of utilizing these leaf samples as herbal interventions, their antioxidant efficacy was tested by estimating nitric oxide and superoxide free radical scavenging activities.

Leaves from these three SBT varieties were characterised for the first time by gas chromatography-mass spectrometry (GC-MS), with regards to various volatile organic compounds (VOCs). Besides being active components in rendering flavor[9], VOCs are known to play an important role in exerting bacteriostatic and bactericidal actions as well[10]. Inter-relationships between the leaf samples with respect to VOCs detected in all three SBT varieties under study were listed out and these VOCs were represented by a Venn diagram. Similarly, flavonoids, that are known to play important role in lowering cholesterol, reducing the risk of cancer and cardiovascular disease, reversing hyperthyroidism, lessening inflammation[11], neutralizing stress-induced free radicals[12], etc, were identified in the SBT leaf samples by high performance thin layer chromatography (HPTLC). To define correlational patterns among all six SBT leaf extracts, the results obtained from HPTLC analysis were subjected to chemometric analytical tools in the form of heat maps and principal component analysis, thus, accomplishing data reduction in an otherwise larger set of data.

2. MATERIALS AND METHODS

2.1 Preparation of SBT Leaf Extracts

Preparation of aqueous and alcoholic extracts from leaves

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of the three SBT varieties under study, namely, *Hippophae salicifolia* (HS), *Hippophae rhamnoides mongolica* (HRM) and *Hippophae rhamnoides turkestanica* (HRT) were carried out by a method described elsewhere. Leaves from the said SBT varieties were procured from CSK, Palampur, Himachal Pradesh and were kindly authenticated by Dr Virendra Singh, CSK Palampur, Himachal Pradesh where they grow at an altitude of 2730 m. All the extracts were prepared using Accelerated Solvent Extractor (ASE)[8].

The aqueous leaf extracts of HS, HRM and HRT were labeled as SBT-1, SBT-3 and SBT-5 respectively whereas their alcoholic complements were labeled as SBT-2, SBT-4 and SBT-6.

2.2 Chemicals and Reagents

All the standards used throughout the study were purchased from Sigma-Aldrich (USA). All the reagents and solvents used were procured from Sigma-Aldrich (USA) and belonged to HPLC grade. Water used was of Millipore grade (Merck, USA).

2.3 Apparatus and Software

Extraction procedure was carried out using Accelerated Solvent Extractor (Dionex 350, USA). Solvent evaporation and freeze drying of the extracts were achieved using Buchi Rotavapor and Allied Frost FD 5 lyophilizer, respectively. GC-MS analysis was performed on a QP2010 Ultra model (Shimadzu scientific, Japan). Venn diagram was drawn using InteractiVenn tool[13]. HPTLC analysis was carried out using an assembly from CAMAG, Switzerland. Chemometric studies were conducted using the software R: A Language and Environment for Statistical Computing (R Foundation for Statistical Computing, Vienna, Austria (http://cran.r-project.org/).

2.4 Evaluation of Antioxidant Activity

2.4.1 Superoxide Radical Scavenging Activity

The superoxide radical ($O_2^{\cdot-}$) scavenging potential for the six seabuckthorn leaf extracts under study was assessed using the method as described elsewhere[14]. Superoxide radical was produced by adding sodium hydroxide to dimethyl sulfoxide (DMSO). To 100 µL of alkaline DMSO, 10 µL of nitrobluetetrazolium (NBT) chloride salt and 30 µL of respective seabuckthorn extract in various concentrations (10-100 µg/mL) were added, resulting in a final reaction mixture of 140 µL. The absorbance of this reaction mixture was measured at 560 nm using a micro plate reader (Bio-Tek Power Wave XS2, USA). The superoxide radical scavenging activity was expressed as corresponding IC_{50} values in units of µg/mL. Absorbances of different concentrations of ascorbic acid ranging from 10-100 µg/mL were taken to generate the standard curve. The chemical reaction of superoxide anion radical with NBT forms a chromophore formazan[15].

2.4.2 Nitric Oxide Radical Scavenging Activity

The nitric oxide radical ($NO^{\cdot}$) scavenging activity of the seabuckthorn leaf extracts was estimated by a method reported elsewhere[16]. To the wells of a 96-well microplate, 60 µL of the respective seabuckthorn extract in various concentrations (10 µg/mL - 50 µg/mL) was added, followed by 60 µL of 10 mM sodium nitroprusside dissolved in 1X phosphate-buffered saline (PBS). Then the microplate was incubated at room temperature for 150 m, after which 60 µL of Griess reagent was added, resulting in formation of a chromophore complex[17] whose absorbance was recorded at 577 nm. The nitric oxide scavenging activity was expressed as corresponding IC_{50} values in units of µg/mL. The absorbances of different concentrations of gallic acid (10 µg/mL - 50 µg/mL) were taken to plot the standard curve.

2.5 GC-MS Analysis

2.5.1 Sample Preparation

All sample solutions were prepared by dissolving 1 mg of each extract (SBT 1-6) in 1 ml methanol. The samples were filtered through 0.22 µm syringe filters (Merck Millipore).

2.5.2 GC-MS Characterisation

For each of the six SBT leaf extracts, 1 µl of sample solution was injected into a gas chromatograph coupled with mass spectrometer. Characterisation was performed following parameters similar to those described elsewhere[18]. Fused silica capillary column (*Rtx-5MS,* 30 m x 0.25 mm x 0.25 x 2 µm) was used for separation of components. The injection temperature was 260 °C and the flow rate of septum purge was 3 ml/min. Gas flow rate through the column was maintained at 1.21 ml/min. The initial temperature of column was maintained at 60 °C for 2 min. Then there was an increase in temperature from 60 °C to 200 °C at a rate of 6 °C/min and then the temperature was further increased from 200 °C to 280 °C at a rate of 10 °C/min. The total run time was kept at 27 mins. An electron beam of 70 eV was used for ionization of samples.

All volatile organic compounds (VOCs) detected in the extracts were characterised by comparing their MS spectra and retention indices with those in libraries like NIST and Wiley.

2.6 HPTLC Analysis

2.6.1 Preparation of Standard Stock Solutions

Stock solutions of the flavonoid standards namely quercetin, gallic acid, ascorbic acid, hesperidin and rutin were prepared by dissolving 1 mg of each standard in 1 ml methanol. A mixture comprising equal volumes from each of these standards was prepared.

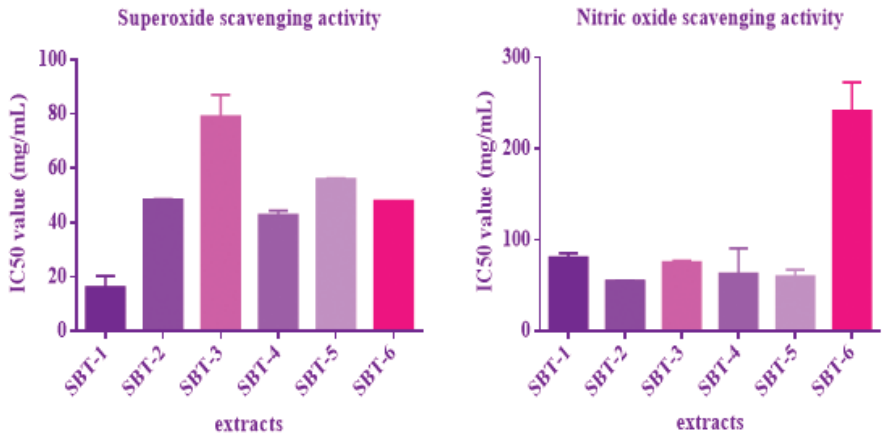
2.6.2 Preparation of Sample Solutions

Twenty mg of each seabuckthorn extract (SBT-1-6) was weighed and dissolved in 10 ml of methanol, thus resulting in a sample concentration of 2 mg/ml. These sample solutions were kept at 4 ºC for further analysis.

2.6.3 Chromatography Analysis

Samples (60 µl) were applied on a 20 cm x 10 cm glass backed silica gel 60 F_{254} HPTLC plate (Merck) as 6 mm wide bands, using a CAMAG Linomat 5 sample applicator equipped with a 100 µL syringe (Hamilton). Also, three different volumes (5 µl, 6 µl, 7 µl) of the solution containing mixture of flavonoid standards were applied as shown in Fig.1. These

different volumes of the standards mixture corresponded to three different concentrations of the standards. The plate was then developed at room temperature in a CAMAG twin-trough vertical development chamber containing appropriate mobile phase (ethyl acetate: dichloromethane: formic acid: glacial acetic acid: methanol in a ratio of 10 v/v: 10 v/v: 1 v/v: 1 v/v: 2 v/v)[19]. The migration distance was maintained at 85 mm. Following this, the plate was exposed to densitometry scanning at a wavelength of 254 nm using a CAMAG TLC Scanner 3 with deuterium as the light source.



(a) (b)

Figure 1. Superoxide (a) and nitric oxide (b) radical scavenging activities of seabuckthorn leaf extracts*

2.7 Chemometric Analysis

Chemometric analysis comprising heat maps, cluster dendrograms and Principal Component Analysis (PCA) was achieved using the HPTLC-generated R_f values of the various flavonoid standards detected in all the six SBT leaf extracts. This analysis was achieved using R console: A Language and Environment for Statistical Computing (http://cran.r-project.org/). The R package pvclust[20] was used for sample set redistribution and construction of cluster dendrograms, using average linkage method. Heat maps were plotted using Heatplus package to specify the occurrence of a particular compound in the extract[21]. Principal component analysis (PCA) aided to generate the variables factor map[19] by utilizing peak area values of the constituent flavonoids identified in the extracts, for further data reduction while keeping the relationship between the various extracts intact.

3. RESULTS AND DISCUSSION

3.1 Free Radical Scavenging Activity of BT Leaf Extracts

Superoxide anion radical is one of the reactive oxygen species (ROS) that can get converted into other harmful ROS like hydroxyl free radical (OH^{-}) and peroxide free radical (O_2^{2-}) thus, causing damage to biological molecules[18]. Another harmful free radical is nitric oxide radical ($NO^{\cdot}$) which gets produced in biological systems by the enzyme nitric oxide synthase. An increased concentration of $NO^{\cdot}$ can enhance nitrosylation reactions that adversely alter structure and functions of protein molecules[14].

All the seabuckthorn extracts studied here were found to be capable of scavenging both superoxide and nitric oxide radicals thereby, minimizing the effect of ROS. The IC_{50} values observed for both superoxide and nitric oxide radical scavenging activities are given in Figs. 1(a) and 1(b) (in that order). The superoxide radical scavenging activity for aqueous seabuckthorn extracts in decreasing order was SBT-1>SBT-5>SBT-3 and that for alcoholic extracts was SBT-4>SBT-2>SBT-6. Similarly, for nitric oxide radical, the order of scavenging potential in aqueous extracts was observed to be SBT-5>SBT-3>SBT-1 and for alcoholic extracts, the order was SBT-2>SBT-4>SBT-6. The better antioxidant abilities of some extracts as indicated above could be attributed to their higher content of polyphenolic compounds[22].

3.2 Characterisation and Quantification of VOCs by GC-MS

VOCs belonging to different classes viz. alcohols, esters, fatty acids, terpenes etc. and possessing varied bioactivities, were identified in the extracts as shown in *Appendix 'A'*. Venn diagram as depicted in Fig. 2 demonstrates the distribution of the 35 most abundant VOCs in all the six SBT extracts. It also brings out the presence of three

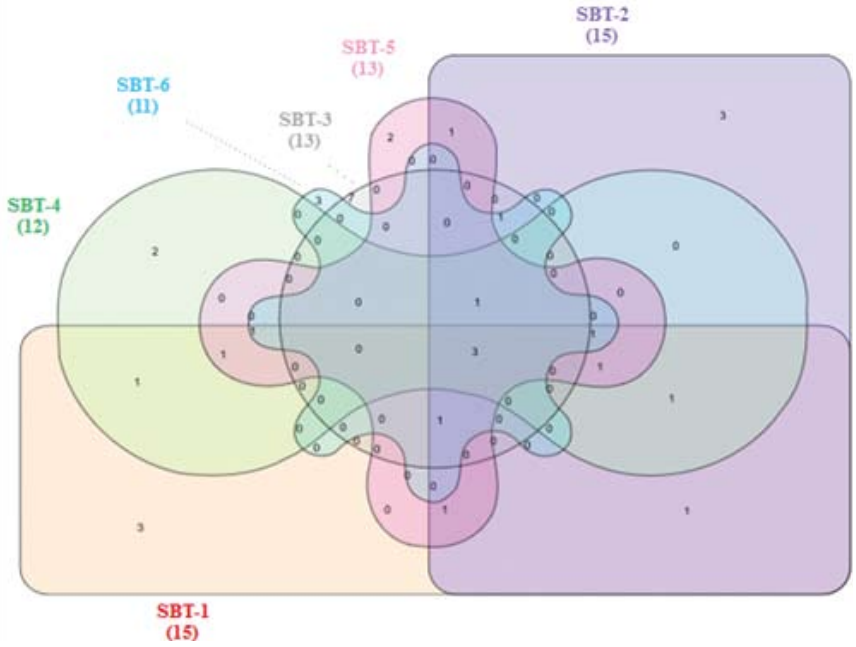
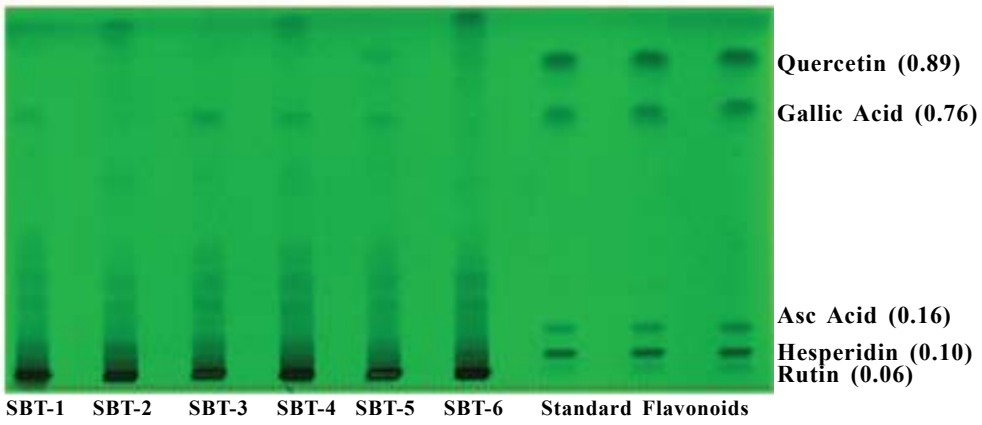
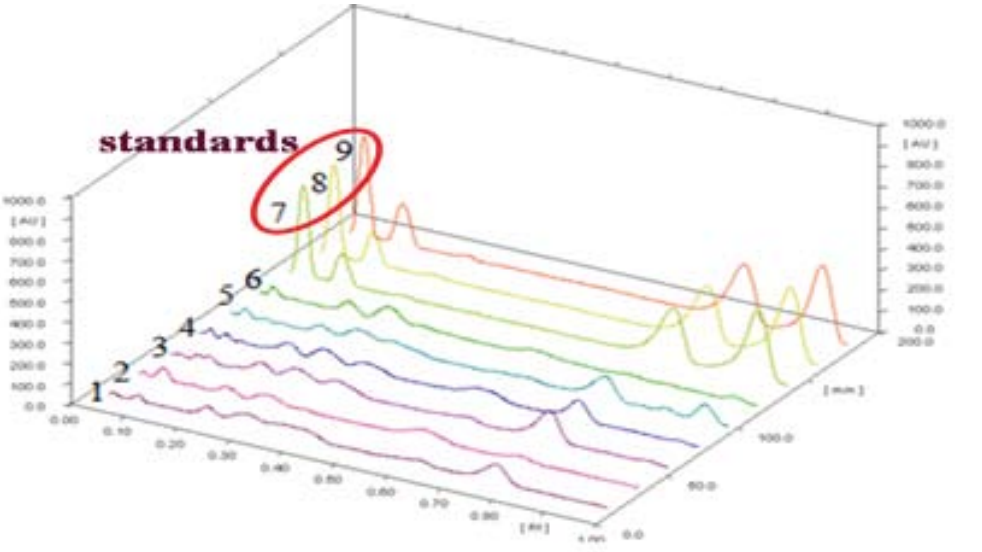


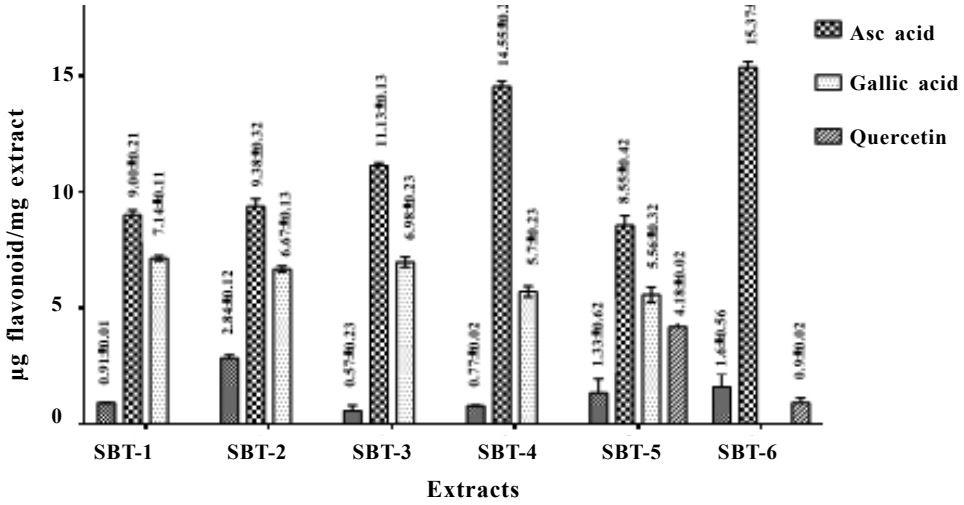
Figure 2. Venn diagram showing distribution of volatile organic compounds (VOCs) detected in seabuckthorn leaf extracts*



(a)



(b)



(c)

Figure 3. (a) HPTLC fingerprint showing presence of flavonoids in seabuckthorn leaf extracts*, (b) 3D spectra of flavonoids detected in seabuckthorn leaf extracts* by HPTLC, and (c) Quantities of flavonoids detected in various seabuckthorn leaf extracts*.

(*SBT-1, SBT-3, SBT-5 represent aqueous extracts of *Hippophae salicifolia, Hippophae rhamnoides mongolica and Hippophae rhamnoides turkestanica*; SBT-2, SBT-4, SBT-6 represent alcoholic extracts of *Hippophae salicifolia, Hippophae rhamnoides mongolica and Hippophae rhamnoides turkestanica*)

common compounds namely methyl palmitate, trimethylsilyl palmitate and trimethylsilyl (9e)-9-octadecenoate in all SBT extracts. Among the compounds detected, the ones having ≥ 1 per cent peak area were considered as significant. Overall, SBT-1, SBT-2, SBT-3, SBT-4, SBT-5 and SBT-6 contained 15 VOC_S, 15 VOC_S, 13 VOC_S, 12 VOC_S, 13 VOC_S and 11 VOC_S respectively.

Among these compounds, two VOCs were common between SBT-4 and SBT-5; three were common in SBT-1 and SBT-2 and seven VOCs were similar in SBT-6 and SBT-3 (Fig. 2; *Appendix 'A'*).

According to percentage areas as mentioned in Appendix 'B', 'trimethylsilyl palmitate' was the most abundant compound in both SBT-1 and SBT-2, with peak areas of 20.67 per cent and 22.47 per cent, correspondingly. In SBT-3, maximal concentration of 'methyl palmitate' was found, with a peak area percentage of 21.79 per cent. SBT-4 had 'methyl (9e)-9-octadecenoate' as the most frequently occurring compound (peak area % = 19.16). In SBT-5 and SBT-6, 'methyl octadec-9-enoate' and 'methyl palmitate' were observed to be the most abundant compounds with peak area percentages of 21.93 and 25.2, respectively.

From the Venn diagram given in Figure 2, it can be perceived that all the six extracts individually had certain VOCs which were unique to each of them. SBT-1, SBT-2 SBT-6 had three such unique VOCs each; SBT-4 and SBT-5 had two exclusive VOCs each and finally, SBT-3 had seven distinctive VOCs, depicting that each individual extract had distinct characteristics in terms of bioactivity.

Appendix 'A' lays down some of the established biological and/or pharmaceutical applications of the VOCs identified by GC-MS in all the six SBT leaf extracts under study and it has been found that some of these VOCs have active role to play in oxidative stress management[23-61].

3.3 HPTLC Analysis

The high throughput, less complexity and efficient automation of HPTLC procedure makes it a preferred tool for identification and quantification of bioactive molecules[62]. Flavonoids represent a class of phytochemicals obtained from plants that are widely distributed in nature and have got potential benefits for human consumption. They occur naturally in fruit, vegetables, and beverages such as tea and wine[63]. Among several flavonoids, quercetin, gallic acid, ascorbic acid, hesperidin and rutin were chosen as the standard flavonoids for quantification in seabuckthorn leaf extracts owing to their diverse biological activities as described in Appendix 'B'[64-73]. These flavonoids possess anti-carcinogenic, anti-mutagenic and anti-oxidative modes of actions[74] . Animal and human studies suggest that seabuckthorn flavonoids are capable of scavenging free radicals, lowering blood viscosity and enhancing cardiac function[10]. In the current study, HPTLC fingerprinting (Fig. 3(a)) proved the presence of rutin and ascorbic acid in all the six extracts, i.e., SBT 1-6. Gallic acid was detected in extracts SBT 1-5 but in SBT-6, it was below detection limit. Quercetin was detected only in SBT-5 and SBT-6, i.e., aqueous and alcoholic leaf extracts of *Hippophae rhamnoides turkestanica*. Thus, SBT-5, bearing four of the

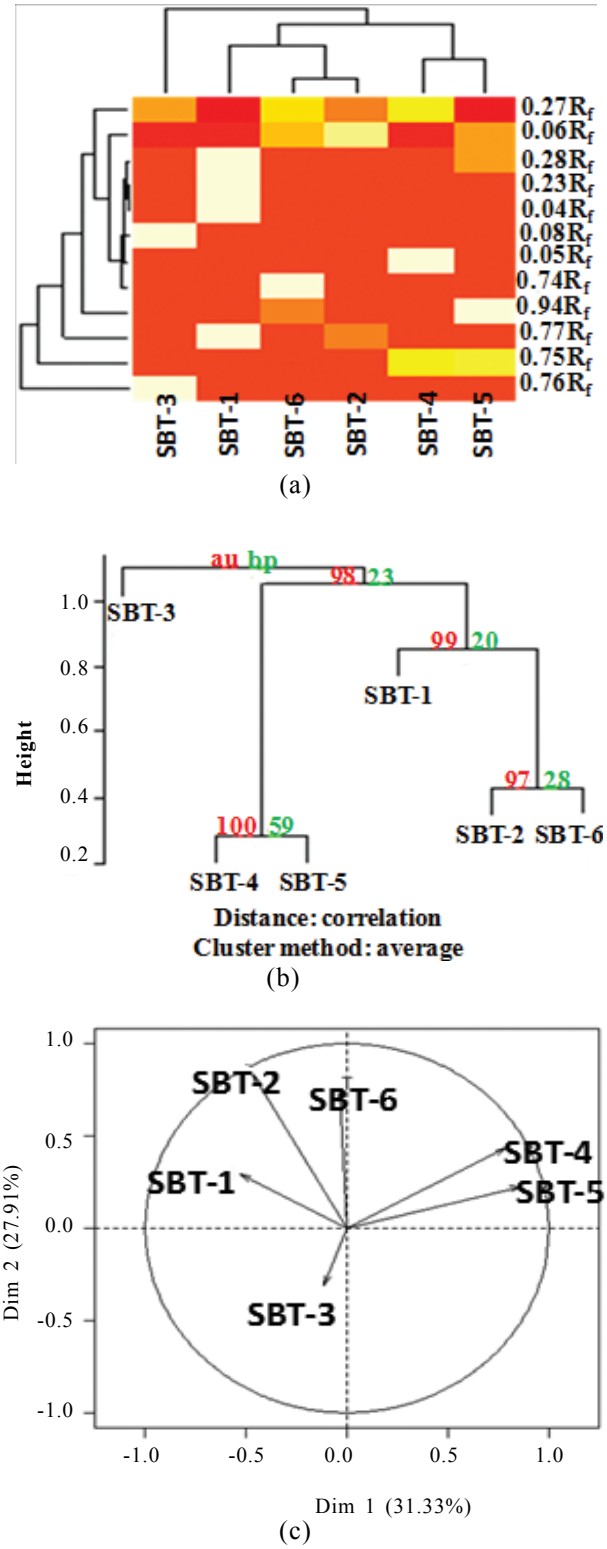


Figure 4. (a) Heat maps depicting the intensity of various metabolites occurring in different seabuckthorn leaf extracts*, (b) Cluster dendrograms showing hierarchical relationships among seabuckthorn leaf extracts*, and (c) Variables factor map showing correlations among seabuckthorn leaf extracts*.

(*SBT-1, SBT-3, SBT-5 represent aqueous extracts of *Hippophae salicifolia, Hippophae rhamnoides mongolica and Hippophae rhamnoides turkestanica*; SBT-2, SBT-4, SBT-6 represent alcoholic extracts of *Hippophae salicifolia, Hippophae rhamnoides mongolica and Hippophae rhamnoides turkestanica*)

five standard flavonoids under study was the richest extract among all. Fig. 3(b) depicts the corresponding 3D spectra of the flavonoids as detected at 254 nm. The quantification of these flavonoids are shown in Fig. 3(c) in terms of micrograms (µg) of flavonoid standard per milligram (mg) of extract (µg/mg). Difference in quantities of these selected flavonoids in the six extracts under current study could be attributed to their variable solubilities in various solvents[27].

3.4 Chemometric Analysis

Chemometric analysis has recently been adopted as a means for sampling and clustering of data in order to find similarities and variations among samples under study. Here for the first time, we associated the techniques of HPTLC and chemometrics to estimate the flavonoids content in the SBT leaf extracts. The heat maps generated using R programming (as shown in Fig. 4(a) depicted the grouping of the six seabuckthorn leaf extracts according to their flavonoid contents. The cluster dendrograms as shown in Fig. 4(b) were constructed for grouping of extracts having similar R_f values. The variables factor map obtained after principal component analysis for all the SBT leaf extracts is given in Fig. 4(c).

The heat maps generated from R programming served the purpose of grouping the extracts according to their functional similarity. The color intensity of a specific grid corresponded to the concentration of a certain compound occurring at a unique R_f value. White through red colors indicated the concentrations of the components from highest to lowest value respectively[21]. From the cluster dendrograms, it was seen that with respect to the flavonoid contents, SBT-4 and SBT-5 were included in one cluster whereas SBT-2 and SBT-6 comprised another cluster to which SBT-1 was fairly similar. SBT-3 was an altogether unique cluster in itself. The variables factor map obtained after PCA was helpful in compressing data and defining only certain 'principal components' (Dim 1 and Dim 2) as can be seen in Fig. 4(c). These principal components represented the entire dataset, while at the same time conserving the uniqueness of the clusters among all the six SBT leaf extracts, in terms of flavonoid contents. It can be observed that there is a direct correlation between the results obtained from PCA and those from heat map analysis, thus, conforming the grouping of SBT leaf extracts as explained above.

4. CONCLUSIONS

Based on the results obtained from the current study, it could be deduced that aqueous and alcoholic leaf extracts of the three seabuckthorn varieties, viz, *Hippophae salicifolia*, *Hippophae rhamnoides mongolica* and *Hippophae rhamnoides turkestanica* had appreciable free radical scavenging potential. There was an abundance of bioactive volatile organic compounds in all the six seabuckthorn extracts as confirmed by GC-MS analysis. Outcomes from HPTLC analysis clearly brought out the presence of bioactive flavonoids (quercetin, gallic acid, ascorbic acid and rutin) in the aforementioned extracts. Finally, chemometric analysis demonstrated clear distinction of metabolites occurring in the various seabuckthorn leaf extracts, thus, establishing their putative efficiency for use in drugs as well as nutraceuticals.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest concerning this article.

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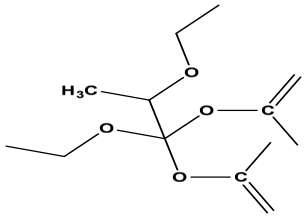
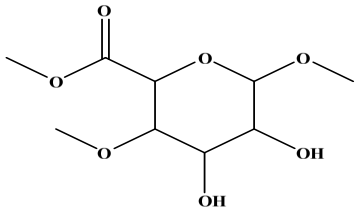
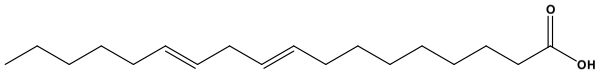
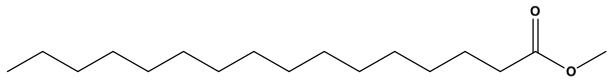
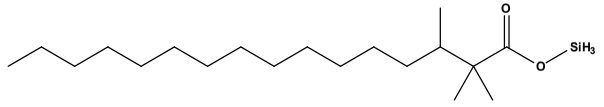
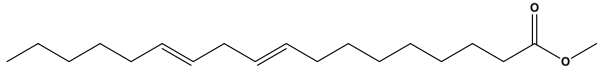
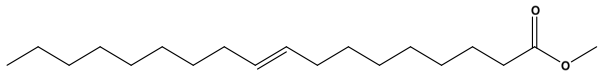
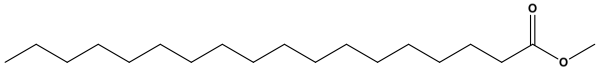
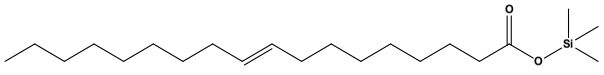
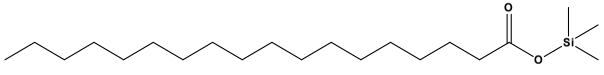
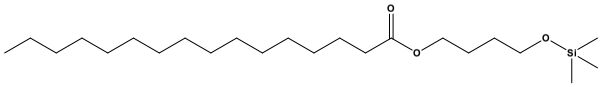
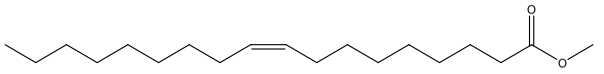
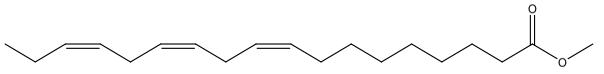
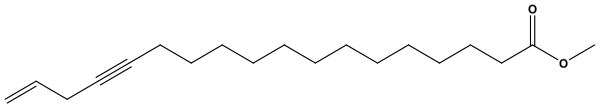
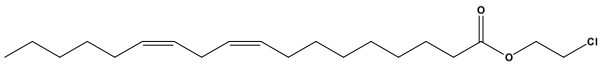
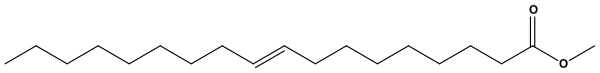
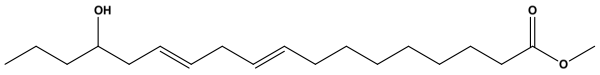
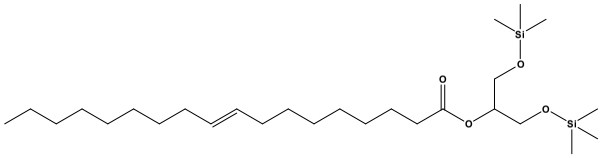
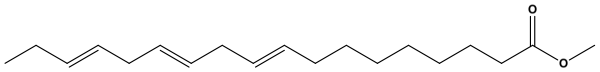
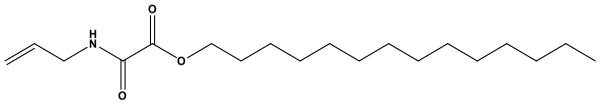
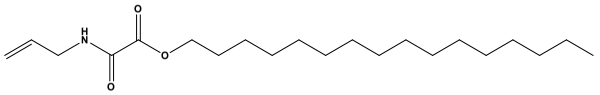
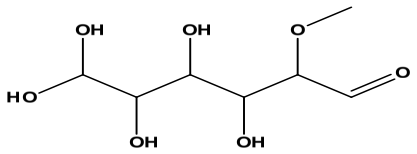
In the current study, she was involved in the conceptualisation of the entire study, data analysis and overall supervision encompassing experimentation to manuscript preparation.

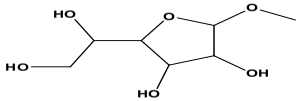
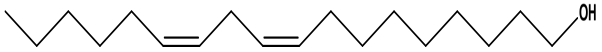
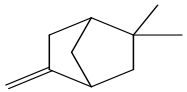
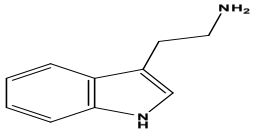
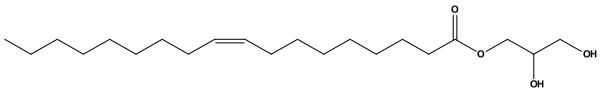
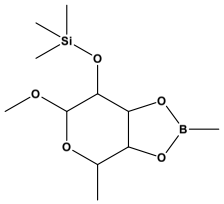
Appendix 'A'

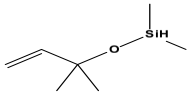
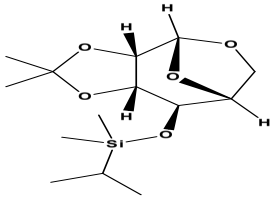
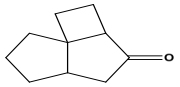
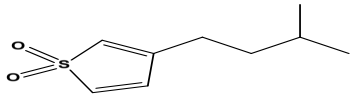
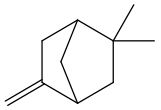
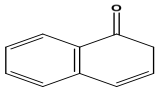
Volatile compounds (VOCs) in all six SBT extracts (numbers in subscripts indicate references)

S. No	Compound(s)	Area%						Properties
		SBT-1	SBT-2	SBT-3	SBT-4	SBT-5	SBT-6	
	ESTERS							
1	Diethyl 1,2-dioxypropyldiacetate 	–	–	–	–	1.01	–	Anti-cholinergic and anti- emetic activities[23]
2	Dimethyl 4-o-methylhexopyranosiduronate 	–	2.8	–	–	–	–	Used as a drug for osteoporosis[24]
	FATTY ACID							
3	(9e,12e)-9,12-octadecadienoic acid 	–	1.4	–	–	1.04	–	Fatty acid therapeutic and/or prophylactic treatment of cartilage degenerative conditions,reduction of blood pressure,prevent hypothyroidism[25,26]
	FATTY ACID ESTERS							
4	Methyl palmitate 	18.04	17.73	21.79	17.05	19.47	25.2	Pharmaceutical, cosmetic and industrial applications[27]
5	Trimethyl silyl palmitate 	20.67	22.47	13.37	18.81	16.97	15.03	Cosmetic use[28]
6	Methyl octadeca-9,12-dienoate 	2.02	–	–	–	–	–	Beneficial effects against oxidative stress[29]

S. No	Compound(s)	Area%						Properties
		SBT-1	SBT-2	SBT-3	SBT-4	SBT-5	SBT-6	
7	Methyl (9e)-9-octadecenoate or Methyl elaidate 	20.65	_	_	19.16	_	_	Fatty acid therapeutic[30]
8	Methyl stearate 	8.46	8.02	8.46	_	8.63	9.8	Increased herbicide penetration[31]
9	Trimethylsilyl (9E)-9-octadecenoate 	5.23	4.03	2.49	3.19	2.51	1.82	Bio adhesive substances[32]
10	Trimethylsilyl stearate 	1.11	_	_	_	_	_	Biodiesel fuel properties[33]
11	4-[(Trimethylsilyl)oxy]butyl palmitate 	1.7	1.69	_	1.09	_	_	Biodiesel fuel properties[33]
12	Methyl cis-9-octadecenoate or oleic acid ester 	_	2.76	3.16	_	_	4.26	Help in Ketogenesis[34]
13	Linolensaeuremethylester 	_	1.82	_	_	_	_	Cardiovascular-related diseases[35]
14	Methyl 17-octadecen-14-ynoate 	_	_	2.23	_	_	_	Determine biodiesel impurities[36]
15	2-Chloroethyl linoleate 	_	_	1.03	_	_	_	French dressing[37]

S. No	Compound(s)	Area%						Properties
		SBT-1	SBT-2	SBT-3	SBT-4	SBT-5	SBT-6	
16	Methyl octadec-9-enoate 	–	19.97	21.24	2.75	21.93	24.37	Pathways leading to formation of SOA in the atmosphere are of interest due to their high potential to affect human health and the environment[38]
17	Methyl 15-hydroxy-9,12-octadecadienoate 	–	–	1.87	–	–	–	Fatty acid esters are responsible for pharmacological activities, antioxidant properties[39,40]
18	9-Octadecenoic acid, 2-[(trimethylsilyl)oxy]-1-[[(trimethylsilyl) oxy]methyl]ethyl ester 	–	–	3.21	–	–	–	Endogenous cannabinoid receptor ligand[41]
19	Methyl (9e,12e,15e)-9,12,15-octadecatrienoate 	–	–	–	1.8	1.83	2.1	Act as a stress metabolite of wounded plants[42]
20	Oxalic acid, monoamide, N-allyl-, tetradecyl ester 	–	–	–	1.58	–	–	Antimicrobial activity[43]
21	Oxalic acid, monoamide, N-allyl-, hexadecyl ester 	–	–	–	–	1.35	–	Antihypercholesterolemic as well as antihyperlipidemic activities[44]
	ALCOHOL							
22	Mome inositol 	4.78	-	-	9.32	12.08		Anti-alopecic, anti-cirrhotic, anti-neuropathic, cholesterolytic, lipotropic and a sweetener[45]

S. No	Compound(s)	Area%						Properties
		SBT-1	SBT-2	SBT-3	SBT-4	SBT-5	SBT-6	
23	10,12-hexadecadien-1-ol 	1.05	-	-	-	-	-	Sex pheromone receptor[46]
24	Methyl hexofuranoside 	-	-	-	-	-	5.92	Antimycobacterial agents, cancer therapy, inhibitors of hydrolases[47]
25	(9Z,12Z)-9,12-Octadecadien-1-ol or Linoleyl alcohol 	-	-	-	-	-	1.08	Dermopharmaceutical[48]
	TERPENE							
26	2,2-dimethyl-5-methylenebicyclo[2.2.1] heptane 	1.99	1.83	_	_	_	_	Skin penetration enhancers, cancer suppression,cardiovascular diseases[49,50]
	OTHERS							
27	1h-indole-3-ethanamine 	1.89	1.97	_	_	_		Anticancer therapy, suppression of topoisomerase I , Selective alpha 1A-adenoceptor antagonist[51,52,53]
28	2-monooleoylglycerol trimethylsilyl ether or Monoolein 	2.02	3.41	_	4.86	1.24	1.4	Mucoadhesive properties[54]
29	[(6-Methoxy-2,4-dimethyltetrahydro-4H-[1,3,2]dioxaborolo[4,5-c] pyran-7-yl)oxy](trimethyl)silane 	_	_	4.96	_	_	_	Rapid bio degrader[55]

S. No	Compound(s)	Area%						Properties
		SBT-1	SBT-2	SBT-3	SBT-4	SBT-5	SBT-6	
30	3-(Dimethyl siloxy)-3,3-dimethyl-1-propene 	_	_	1.51	_	_	_	Toxic substances control[56]
31	1,6-Anhydro-2,3-O-isopropylidene-.beta.-D-mannopyranose,tert-butyldimethylsilyl ether 	_	_	4.65	_	_	_	Capillary production of (^{18}F) FDG[57]
32	Hexahydro-1h-cyclobuta[c]pentalen-3(4h)-one 	_	_	_	1.72	_	2.25	Pesticide[58]
33	3-(3-Methylbutyl)thiophene-1,1-dioxide 	1.45	2.43	_	2.25	1.66	_	Removal of H_2S, CO_2 and mercaptans from natural gas[59]
34	2,2-dimethyl-5-methylenebicyclo[2.2.1] heptane 	1.99	_	_	_	2	_	Essential oils[60]
35	1(2h)-naphthalenone 	_	1.16	_	_	_	_	Anti-neoplastics used to reduce toxicity of cells[61]

Appendix 'B'

Reported bioactivities of the selected flavonoids

Flavonoids	Reported bioactivities
Quercetin	a. Inhibits growth of malignant tumor cell lines (e.g.: P-388 leukemia cells, HGC-27 gastric cancer cells, 320-DM colon cancer cells)[64]
	b. Acts as antiviral agent against DENV-2 (dengue virus type-2) in its replication cycle[65]
Gallic Acid	a. Induces caspase dependent apoptosis in prostate cancer cells[66]
	b. Is beneficial in treating cardiovascular diseases[67]
Ascorbic acid	a. Reduces blood pressure and arterial stiffness in Type-2 diabetes[68]
	b. Inhibits hypoxia-induced damages in cardiomyocytes[69]
Hesperidin	a. Possesses analgesic effects[70]
	b. Inhibits azoxymethanol induced colon and mammary cancers[71]
Rutin	a. Acts as strong radical scavengers and inhibitors of lipid peroxidation in vitro[72]
	b. Demonstrates significant antibacterial, antifungal and antihelmintic properties[73]