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Antioxidant and GC-MS analysis of *Thamnolia subuliformis* (Ehrh.) W.L. Culb. from western Himalaya

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Abstract
Lichens are the unique organism in the plant kingdom and produces different types of secondary metabolites than in higher plants. These different metabolites along with other chemical compounds can be utilized for curing aches and diseases. In the present study, extraction yield (Soxhlet method), qualitative screening of biochemicals (alkaloids, carbohydrates, fats, flavonoids, phenolics, proteins, saponins, steroids and tannins), antioxidant activity using different assays (DPPH, TAA, FCA and Reducing power activity), TPC and TFC in different solvents (acetone, ethanol and methanol) along with GC-MS analysis was performed on a macrolichen, *T. subuliformis.* Among the solvents, the methanol showed comparatively higher activity than in other two solvents. The yield obtained was higher than previous studies. The biochemical screening reveals the presence of all selected biochemicals except alkaloids and steroids. Among all the experiments performed methanol showed comparatively better results than acetone and ethanol. The GC-MS analysis of the extract showed the presence of the 60 different biochemicals out of which, hexadecanoic acid, methyl ester, phytol and evernic acid, etc., are reported to possess anticancer, anti-inflammatory, antimicrobial, antioxidants, diuretic and hypocholesterolemic activities. Thus, *T. subuliformis* is a potent source of antioxidants and nutraceutical as well as functional food applications.

Keywords: lichens, antioxidant activity, GC-MS, *T. subuliformis*

1. Introduction
In aerobic organisms, 5% of total oxygen inhaled, produces harmful intermediates called reactive oxygen species (ROS) which interacts with the other molecules and produces new radicals like superoxide anion (O_2^o), hydroxyl radical (OH), hydrogen peroxide (H_2O_2) and peroxyl radicals (ROO^o) and induces oxidative stress[1]. Oxidative stress caused by these free radicals leads to the toxicity in the biological system damaging the lipid bio-membrane and causes gastric ulcer, cancer, coronary artery disease, Parkinson's disease, etc. For the prevention in biological systems, antioxidants play an important role. Antioxidants are the compounds that delay or inhibit the oxidation of the lipids or other molecules by inhibiting the initiation of oxidative chain reaction [2]. In recent times, the increased use of synthetic antioxidants exhibited harmful effects on human wellness. Presently, a great pile of attention is being devoted to the exploration of natural antioxidants, particularly from the natural resources such as plants, which significantly plays an important role in neutralizing harmful free radicals [3].

Lichens are the unique organisms having a symbiotic association between fungi and photosynthetic alga and/or Cyanobacteria [4]. The use of lichens in medicine is due to their secondary metabolites that are unique compared to those of higher plants [5] which shows impressive biological activities including antioxidant, antibiotic, antimycobacterial, antiviral, anti-inflammatory, analgesic, antipyretic, antiproliferative and cytotoxic activities [6]. While numerous activities of lichen metabolites are now recognized, their therapeutic potential has not been fully explored and remains pharmaceutically unexploited.

Among the lichens a macrolichen, *Thamnolia* sp. locally known as "snow tea" has been used in traditional Chinese medicine for hundreds/thousands of years and is believed to counteract inflammation [7]. The pharmacological research of this macrolichen has suggested that it has antioxidant and anticancer functions and can be used for the treatment of hypertension, cough and neurasthenia [8]. Recently, several phenolic compounds such as vermicularin, squamatic acid, barbatinic acid, *D*-arabitol, mannitol and baeomycesic acid have been reported from *T. vermicularis* [9]. However, there is no information on GC-MS analysis of a macrolichen

Thamnolia subuliformis (Ehrh.) W. L Club. from Western Himalaya.

2. Materials and Methods

2.1 Collection and preparation of samples

Lichen samples were collected from Sri Narayan Ashram (N 29°58.174′, 80°39.321′ E) at an elevation of 2556 m above sea level (established by Gurudev Narayan in 1936) in the district Pithoragarh, Uttarakhand state, India, and identified on the basis of morphological, anatomical and chemical characters by Awasthi (2007) [10] and authenticated by Dr. Yogesh Joshi, Lichenology, Laboratory, S.S.J. Campus, Kumaun University, Almora. The air-dried healthy lichen thalli were grounded and extractions were prepared by taking 5 g powdered material for soxhlet methods in three different solvents *i.e.,* acetone, ethanol and methanol and kept in the freezer and used for further experimentation.

2.2 Extraction yield (%)

The yield of the samples was calculated by using the following formula:

$$\text{Yield (\%)} = \frac{\text{Total crude extract (g)}}{\text{Dried powder (g)}} \times 100$$

2.3 Screening of biochemicals

Biochemicals, such as alkaloids, carbohydrates, fats, flavonoids, phenolics, proteins, saponins, steroids and tannins in all the three extracts were performed as suggested by Harborne (1973) [11] and Sofowora (1993) [12].

2.4 2-2-Diphenyl picryl hydrazyl free Radical scavenging activity (DPPH) assay

The free radical scavenging activity of the extracts was determined by the method given Yen *et al.,* (1993) [13] and Cuendet *et al.,* (1997) [14]. Different concentrations of extracts (20, 40, 60, 80 and 100 µg ml^{-1}) were mixed with 1ml of DPPH solution (0.1 mm of DPPH in methanol). The mixture was incubated in a dark for 30 min and the absorbance was measured at 517 nm in a UV – visible spectrophotometer (Thermo). The fresh DPPH solution was prepared and stored in an amber light bottle in a dark room at 4 °C. The standard and control were also subjected to the same procedure, except for the control where there was no addition of sample and for the standard, sample was replaced by BHT. The lower absorbance indicated higher radical scavenging power. DPPH radical scavenging activity was calculated by following equation:

$$\text{DPPH radical scavenging activity (\%)} = \frac{(A_0 - A_1)}{A_0} \times 100$$

Where, A_0 is absorbance of the control and A_1 is the absorbance of the extract at 517 nm.

2.5 Total Antioxidant Activity (TAA)

Phosphomolybdenum method is considered in the evaluation of the total antioxidant activity. The assay is based on the principle of reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of the green phosphate Mo (V) complex at acid pH [15]. The extract (0.3 ml) was taken into test tube containing 3 ml of reagent solution (0.6 M sulfuric acid, 28 mm sodium phosphate and 4 mm ammonium molybdate) and incubated at 95 ºC for 90 min. The absorbance of each sample after cooling at room temperature was measured at 695 nm by UV – visible spectrophotometer (Thermo) against blank expressed as ascorbic acid equivalent (AAE).

2.6 Chelating Effect on Ferrous Ions (FCA)

The chelating of ferrous ions was estimated by the method of [16] and the absorbance of the solution was measured spectrophotometrically at 562 nm. The percentage of ferrozine-Fe^{2+} complex formation is given by the formula:

$$\text{Inhibition (\%)} = \frac{(A_0 - A_1)}{A_0} \times 100$$

Where, A_0 is absorbance of the control and A_1 is the absorbance of the extract at 562 nm.

2.7 Reducing power activity

The reducing power activity was determined according to the method of Oyaizu (1986) [17]. Different concentrations of extract, 1 ml of methyl alcohol was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and 2.5 ml of 1 % potassium ferricyanide [$K_3Fe(CN)_6$]. The mixture was incubated at 50 ºC for 20 min, then 2.5 ml of trichloroacetic acid (10 %) was added and centrifuged for 10 min at 10000 rpm. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1 %) and absorbance was measured at 700 nm.

2.8 Total phenol content (TPC)

The concentration of phenolic compounds in different extracts (expressed as gallic acid equivalents, GAEs) was measured according to the method of Singleton and Rossi (1965) [18] with some modifications. One millilitre of sample was mixed with 1 mL of Folin Ciocalteu's reagent. After 3 min, 1 ml of saturated Na_2CO_3 (30 %) was added to the mixture and it was made up to 10 mL by adding distilled water. The reaction was kept in the dark for 90 min and its absorbance was read at 725 nm. A calibration curve was constructed with different concentrations of gallic acid (0.01 - 0.1 mm) as standard.

2.9 Total flavonoid content (TFC)

The total flavonoid content was estimated by the method suggested by Choi *et al.,* (2006) [19]. 250 mL of distilled water was mixed with 75 µL of 5 % $NaNO_2$ solution and incubated for 5 min and 150 µL of 10 % aluminium chloride was added. After another 6 min, 500 µL of 1 M of NaOH and 275 µL of distilled water was added. The solution was mixed well and intensity of pink colour was measured at 510 nm by spectrophotometer. Quercetine was used to prepare standard curve at different concentrations and the results were expressed as mg/g of extract.

2.10 Gas chromatography – mass spectrometry analysis (GC-MS)

The GC-MS analysis was performed in a GCMS-QP2010 Ultra Shimadzu system comprising a gas chromatograph interfaced to a mass spectrophotometer (GC-MS) at Jawahar Lal Nehru University, New Delhi. Instrument employing the following conditions: Plunger Speed (suction): High Viscosity Comp. Time: 0.2 sec, Plunger Speed (Injection): High

Syringe Insertation Speed: High, Injection Mode: Normal, Pumping Times: 5, Inj. Port Dwell Time: 0.0 sec, Terminal Air Gap: No Plunger Washing Speed: High, Washing Volume; 6 µL, Syringe Suction Position: 0.0 mm, Syringe Injection Position: 0.0 mm, Solvent Selection: Column Oven Temp.: 80.0 ºC, Injection Temp: 260.0 ºC, Injection Mode: Split, Flow Control Mode: Linear Velocity, Pressure: 819 kPa, Total Flow: 16.3 mL/min, Column Flow: 1.21 mL/min, Linear Velocity: 40.5 cm/sec, Purge Flow: 3.0 mL/min, Split Ratio: 10:0, I on Source Temp.: 230.0 ºC, Interface Temp.: 270.0 ºC, Solvent Cut Time: 3.50 min, Detector Gain Mode Relative Detector Gain: +0.00 kV, Threshold: 1000, Start Time: 4.00 min, End Time; 50.24 min ACQ Mode: Scan Event Time: 0.20 sec, Scan Speed: 333 Start m/z: 40.00 End m/z:650.00. National Institute of Standards Technology (NIST) library, Possessing 62,000 plus patterns and mass spectral database of Wiley (New York) were used for identification of unknown bioactive compounds.

2.11 Statistical analysis
All the experiments were performed in triplicates and statistical analysis was performed by STPR 2. The significant differences between the means were determined by DMRT using SPSS 16.0 version at 5 % level of significance.

3. Results and Discussion
3.1 Extraction yield (%)
The yield (%) of extract is an essential aspect for determining its biological activity. In the present study, the yield was extracted by using acetone, ethanol and methanol by soxhlet technique. The yield varied greatly from solvent to solvent. Paudel *et al*., (2008) [20] reported the yield in the aqua - methanol extract in *Stereocaulon alpinum* Laur. (0.2 %), *Ramalina terebrata* Hook and Taylor (16 %), *Caloplaca* sp. (0.3 %), *Lecanora* sp. (0.4 %) and *Caloplaca regali* (Vain.) Zahlbr. (26.30 %) by cold extraction which clearly indicates variation in the yield due to variation in the species. However, it was 55.63±0.920, 46.03±0.888 and 60.50±0.208 % in acetone, ethanol and methanol respectively which was much greater than the earlier study.

3.2 Biochemical analysis
In the present study, the qualitative screening of the biochemicals in acetone, ethanol and methanol extracts reveals the presence of carbohydrates, fats, flavonoids, phenols, saponins and tannins in all the three solvents however, proteins were not detected in methanol solvent. Moreover, alkaloids and steroids were completely absent in all the three solvents (Table 1).

Table 1: Biochemicals of *T. subuliformis* in three different solvents.

Biochemicals	Acetone	Ethanol	Methanol
Alkaloids	-	-	-
Carbohydrates	+	+	+
Fats	+	+	+
Flavonoids	+	+	+
Phenols	+	+	+
Proteins	+	+	-
Saponin	+	+	+
Steroids	-	-	-
Tannins	+	+	+

'+'indicates the presence and '-'indicates the absence of the biochemical.

3.3 DPPH radical scavenging activity
The extract of *T. subuliformis* in all the three solvents at different concentrations showed DPPH radical scavenging activity and the values ranged from 35.58 ±0.493 % (20 µl conc.) in acetone to 93.43±0.075 % (100 µl conc.) in methanol extracts. Among all the extracts, methanol showed highest DPPH radical scavenging activity (93.43±0.075%), followed by ethanol (48.67 ±0.278^{e} %) and acetone (44.13 ±0.238 %) at 100 µl µl (Table 1). The methanolic extracts of *Parmotrema reticulatum* and *Usnea* sp. was 10 % to 31.5 % of DPPH activity, respectively reported by Sharma *et al*., (2012) [21], which is much lower than the present study. Similar results were also reported by Verma *et al.*, (2008) [22] in methanolic extract of *Usnea ghattensis* G. Awasthi (89.6 %). However, Rankovic *et al*., (2011) reported that the DPPH radical scavenging activity in *Lecanora atra* (94.7 %), followed by *Lecanora muralis* (52.35 %) and *Cladonia furcata* (44.83 %) in acetone extract which is higher than the present study.

3.4 Total Antioxidant Activity (TAA)
The total antioxidant activity (µg ascorbic acid equivalents, AAE/mg) of *T. subuliformis* in acetone, ethanol and methanol, extracts at different concentrations (20, 40, 60, 80 and 100 µg/ml) varies greatly µl (Table 1). The highest TAA was obtained in ethanol (106.90±0.155), followed by acetone (100.00± 0.010) and methanol (96.35±0.872) (Fig. 2) extract. Similar results were also observed by Manojlovic *et al.* (2012) [23] in *Toninia candida* (78.45±0.58, 56.67±0.30 and 51.45.0.31 µg AA/g in methanol, chloroform and petroether extract, respectively). However, Sharma *et al.* (2012) [21] studied TAA (µg vitamin E equivalent /mg) in two lichen species and reported 0.781 ±.00057 and 1.58±.00577 in *Parmotrema reticulatum* and 2.025±.001 and 0.690±.001 in *Usnea* sp. in ethanolic and methanolic extracts, respectively which were lower than the present study.

3.5 Ferrous Chelating Activity (FCA)
The metal ion chelating activity (FCA) of *T. subuliformis* in acetone, ethanol and methanol extracts at different concentrations (20, 40, 60, 80 and 100 µg/ml) varies greatly µl (Table 1). The highest FCA was observed in the methanol extract ranged from 32.19 ± 0.495 to 58.24 ± 0.248 %, followed by 39.79 ± 0.378 to 65.75 ± 0.283 % in ethanol and 83.08 ± 0.129 to 91.70 ± 0.005 % in acetone at different concentrations (Fig. 3). Kekuda *et al.*, (2011) [24] studied FCA in *Everniastrum cirrhatum* at different concentrations (2.5,5,10,25,50 and 100 µg/ml) in methanol and the values ranged from 40 % at 2.5 µg/ml to 75 % at 100 µg/ml which are more or less equal to present study.

3.6 Reducing power activity (RPA)
The extract of *T. subuliformis* in all the three solvents at different concentrations showed the reducing power activity (nm) and the values ranged from 0.018±0.008 (20 µl) in acetone to 0.062±0.009 (100 µl) in methanol extract. Among all the extracts, methanol showed highest reducing power activity (nm) (0.062±0.009), followed by acetone (0.330±0.021) and ethanol (0.027±0.009^{d}) at 100 µl (Table 1). Kosanic *et al*., (2011) [25] reported 0.0890 nm and 0.0324 nm at 1000 µg/ml for *Toninia candida* and *Usnea barbata* respectively which is more or less equal to the absorbance measured in the present study. In a similar study, Odabasoglu (2005) [26] reported reducing power activity of *Bryoria*

fuscescens, *Dermatocarpon intestiniformis*, *Peltigera rufescens* and *Pseudevernia furfuracea* was 0.215±0.005, 0.119±0.006, 0.542±0.048 and 0.229±0.003 nm, respectively which were much higher than the present study.

3.7 Total Phenolic Content (TPC)

The TPC of *T. subuliformis* in all the three solvents varied significantly ($p<0.05$) (Table 2). Among all the solvents, the highest TPC value (mg GA/g) is found in methanol (109.99±0.00), followed by ethanol (75.99±0.02) and acetone (53.89±0.01). Odabasoglu *et al*., (2005) [26] reported 75.1 mg GA/g phenolic content in methanolic extract of *Pseudevernia glauca* which is equal to the present study. However, Mitrovic *et al*., (2011) [27] reported 78.12 and 141.59 mg GAE/g TPC in the methanol extracts of *Cladonia foliacea* and *Hypogymnia physodes*, respectively. Among these, *C. foliacea* showed almost equal value to the the present study whereas *H. physodes* showed greater value than the present study, indicating that the present species of lichen has potent total phenolic content.

3.8 Total Flavonoid Content (TFC)

Ethanolic extract of *T. subuliformis* showed the higher total flavonoid content (µg QE/mg) (26.45±0.01) than in both methanol (18.92±0.06) and acetone (14.913±0.01) extracts (Table 2). Kosanic *et al*., (2011) [25] reported the higher amount of flavonoid in acetone (49.62 µg of rutin equivalence), followed by methanol (28.67 µg of rutin equivalence) in *Lasallia pustulata* which is higher than the present results. In another study, Plaza *et al*., (2014) [28] reported flavonoid content in the ethanol extract of the *Thamnolia vermicularis* as 12.37 ± 0.07 µg QE/mg which is lower than the present study.

Table 2: Antioxidant activities of *T. subuliformis* in three different solvents.

Activities	Extracts	Concentrations (µl/ml)				
DPPH (%)		20	40	60	80	100
	Acetone	35.58 ±0.493^{abc}	37.58 ± 0.265^{de}	39.82 ±0.123^{f}	41.52 ±0.411^{g}	44.13 ±0.238^{h}
	Ethanol	41.40 ± 0.007^{a}	44.58 ±0.000^{b}	45.46 ±0.128^{c}	47.53 ±0.098^{d}	48.67 ±0.278^{e}
	Methanol	82.71±0.202^{ab}	86.57±0.196^{c}	89.10±0.028^{d}	92.78±0.075^{e}	93.43±0.075^{fg}
TAA (AAE/mg)	Acetone	20.00 ± 0.003^{a}	40.00 ± 0.669^{b}	59.87 ± 0.006^{c}	80.84 ± 0.005^{d}	100.00± 0.010^{e}
	Ethanol	20.00±1.205^{a}	41.23±0.650^{bc}	63.86±0.733^{de}	85.79±1.381^{e}	106.90±0.155^{fg}
	Methanol	25.00±0.949^{a}	42.56±1.322^{bc}	60.21±1.584^{de}	79.23±2.065^{e}	96.35±0.872^{f}
FCA (%)	Acetone	32.19 ±0.495^{ab}	38.95 ±0.806^{cd}	48.94 ±1.237^{ef}	55.05 ±0.553^{gh}	58.24 ±0.248^{ij}
	Ethanol	39.79 ± 0.378^{ab}	41.73 ± 0.550^{bc}	46.53 ± 0.773^{ef}	62.57 ± 0.583^{ghi}	65.75 ± 0.283^{ij}
	Methanol	83.08 ± 0.129^{a}	84.73 ± 0.401^{b}	89.16 ± 0.190^{c}	91.59 ± 0.096^{def}	91.70 ± 0.005^{efg}
RPA (nm)		50	100	150	200	250
	Acetone	0.018±0.008^{a}	0.024±0.027^{bc}	0.029±0.001^{cd}	0.037±0.030^{e}	0.043±0.007^{f}
	Ethanol	0.023±0.003^{a}	0.024±0.004^{ab}	0.025±0.004^{bc}	0.026±0.006^{cd}	0.027±0.009^{d}
	Methanol	0.0146±0.003^{b}	0.0254±0.004^{c}	0.035±0.029^{de}	0.056±0.006^{f}	0.062±0.009^{g}

Table 3: Total Phenolic and Flavonoid content of *T. subuliformis* in three different solvents.

Activity	Acetone	Ethanol	Methanol
Total Phenolic Content (TPC) (mg GA/g)	54.32±1.360^{a}	73.11±0.435^{b}	109.99±0.149^{c}
Total Flavonoid Content (TFC) (mg QE/g)	14.91±0.218^{a}	26.45 ±0.281^{c}	18.93 ±1.428^{b}

3.9 GC-MS Analysis

The GC-MS analysis of the methanolic extract of the *T. subuliformis* revealed the presence of 60 different chemical compounds with the highest value of 1,3-Benzenediol, 2,5-dimethyl (23.52 %), followed by a unique chemical of lichens Evernic acid (18.41 %) and 3-Methoxy-5-methylphenol (14.03 %) (Table 3). The analysis also reveals the presence of trace amounts of some biologically active compounds such as eicosanoic acid, methyl ester (0.13 %), hexadecanoic acid, methyl ester (0.22 %), L-(+)-ascorbic acid 2,6-dihexadecanoate (3.05 %), octadecanoic acid (1.14 %), phytol(1.53 %), squalene (0.74 %), α,β.-d-ribopyranose (1.27 %) etc. (Table 2). The chemical compounds presented in Tab. 3 are important in terms of their activities like 5-α reductase inhibitor, anti-aging, anti-androgenic, anti-bacterial, anti-coronary, anti-eczema, anti-inflammatory, anti-microbial, anti-oxidant, chemoprotective, flavouring agents, hepatoprotective, hypocholesterolemic, immune system stimulating and nematicide [29, 30, 31, 32].

In the present study, the *T. subuliformis* showed higher antioxidant activity in methanolic extract than in acetone and ethanol indicating that methanol is more efficient solvent for the extraction of antioxidants from this species. The antioxidant activity in all assays was comparatively higher than the earlier reports and it may be due to the presence of different phenolic compounds and particularly presence of L-ascorbic acid-2, 6-dihexadecanoic acid. However, differences may also be due to the chemical agents present in different lichen species, collection of time, age and methods. The GC-MS analysis reveals the presence of 60 different compounds and some of these compounds possess several biological activities and can be used in the pharmaceutical industries. GC-MS analysis also showed the presence of several chemical compounds which are used as flavouring agents which support its use as spice worldwide and also by local people. Further, the compounds such as stigmata 5,22-dien-3-ol, which is used as synthetic progesterone in the modern medicine and others in dietary supplements as flavouring agent, cosmetics, and pharmaceutical formulations as an antioxidant agent or as a source of important nutraceutical (Table 4).

Table 4: Bioactive compounds present in methanolic extract of *T. subuliformis.*

Compound Name	R.Time	Molecular formula	Molecular weight	Area%
4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl	7.819	$C_6H_8O_4$	144	0.70
Trans-1,2-Diethyl cyclopentane	8.719	C_9H_{18}	126	0.67
2,5-Monomethylene-l-rhamnitol	11.028	$C_7H_{14}O_5$	178	2.73
3-Methoxy-5-methylphenol	12.421	$C_8H_{10}O_2$	138	14.03
α,β.-d-Ribopyranose	12.998	$C_9H_{14}O_7$	234	1.27
1-Deoxy-1-nitroheptitol	13.473	$C_7H_{15}NO_8$	241	1.35
2,6-dimethoxytoluene	13.692	$C_9H_{12}O_2$	152	0.64
1-tridecanol	13.895	$C_{13}H_{28}O$	200	0.05
Butane, 2-propoxy	14.359	$C_7H_{16}O$	116	0.60
5-oxo-pyrrolidine-2-carboxylic acid meth	14.560	$C_6H_9NO_3$	143	0.31
2-Deoxy-2-fluoro-1,6-anhydro-.beta.-d-glucopyranose	14.701	$C_6H_9FO_4$	164	1.15
1,3-Benzenediol, 2,5-dimethyl	15.272	$C_8H_{10}O_2$	138	23.52
Benzoic acid, 2-hydroxy-4-methoxy-6-methyl-, methyl est	17.799	$C_{10}H_{12}O_4$	196	0.42
Everninic acid	18.755	$C_{12}H_{16}O_4$	224	18.41
Succinic acid, cyclohexylmethyl 2-chlorophenyl ester	19.098	$C_{17}H_{21}ClO_4$	324	0.39
Propyl everninate	20.203	$C_{12}H_{16}O_4$	224	0.60
Benzoic acid, 3-formyl-2,4-dihydroxy-6-met	20.442	$C_{10}H_{10}O_5$	210	0.07
1,13-tetradecadiene	20.519	$C_{14}H_{26}$	194	0.76
Benzoic acid, 3-formyl-2,4-dihydroxy-6-methyl-, methyl ester	20.767	$C_{10}H_{10}O_5$	210	0.85
Benzoic acid, 2,4-dihydroxy-3,6-dimethyl-, methyl ester	21.618	$C_{10}H_{12}O_4$	196	1.22
1-nonadecene	23.188	$C_{19}H_{38}$	266	0.13
Benzoic acid, 3-formyl-4,6-dihydroxy-2,5-dimethyl-, methyl ester	23.924	$C_{11}H_{12}O_5$	224	0.85
2,6,10-trimethyl,14-ethylene-14-pentadecne	24.085	$C_{20}H_{38}$	278	0.47
2-Pentadecanone, 6,10,14-trimethyl-	24.205	$C_{18}H_{36}O$	268	0.30
Benzoic acid, 2-hydroxy-4-methoxy-6-methyl-, ethyl ester	24.357	$C_{11}H_{14}O_4$	210	0.32
Phthalic acid, isobutyl undec-2-en-1-yl ester	24.584	$C_{23}H_{34}O_4$	374	0.20
{3,3-dimethyl-2-[3-methyl-1,3-butadienyl]cyclohexyl} methanol	24.970	$C_{14}H_{24}O$	208	0.33
9-hexadecenoic acid, methyl ester, (z)-	25.775	$C_{17}H_{32}O_2$	268	0.07
Hexadecanoic acid, methyl ester	25.887	$C_{17}H_{34}O_2$	270	0.88
L-(+)-Ascorbic acid 2,6-dihexadecanoate	26.886	$C_{38}H_{68}O_8$	652	3.05
Linoleic acid, methyl ester	29.076	$C_{19}H_{34}O_2$	294	3.42
6-Octadecenoic acid, methyl ester, (Z)-	29.198	$C_{19}H_{36}O_2$	296	3.27
11-Octadecenoic acid, methyl ester	29.298	$C_{19}H_{36}O_2$	296	0.22
Phytol	29.395	$C_{20}H_{40}O$	296	1.53
Methyl stearate	29.686	$C_{19}H_{38}O_2$	298	0.50
Cyclodecene	30.160	$C_{10}H_{18}$	138	3.72
2H-Pyran, 2-(2-heptadecynyloxy)tetrahydro-	30.272	$C_{22}H_{40}O_2$	336	2.02
Octadecanoic acid	30.572	$C_{18}H_{36}O_2$	284	1.14
Cyclopentanecarboxylic acid, 1-cyclopentylethyl ester	30.741	$C_{13}H_{22}O_2$	210	0.35
10-Heptadecen-8-ynoic acid, methyl ester, (E)-	32.054	$C_{18}H_{30}O_2$	278	0.12
2-methyltetracosane	32.714	$C_{25}H_{52}$	352	0.07
13-octadecenal, (z)-	33.031	$C_{18}H_{34}O$	266	0.32
Eicosanoic acid, methyl ester	33.182	$C_{21}H_{42}O_2$	326	0.13
Tetradecylcyclohexane	33.425	$C_{20}H_{40}$	280	0.58
3-Cyclopentylpropionic acid, 2-dimethylaminoethyl ester	35.203	$C_{12}H_{23}NO_2$	213	0.07
5-[2-(dimethylamino)ethyl]-2-(4-methoxyphenyl)-4-oxo-2,3,4,5-tetrahydro-1,5-benzothiazepin-3-yl a	35.306	$C_{22}H_{26}N_2O_4S$	414	0.17
1-eicosanol	35.850	$C_{20}H_{42}O$	298	0.09
Dicyclohexyl phthalate	36.108	$C_{20}H_{26}O_4$	330	0.01
Hexadecanoic acid, 2-hydroxy-1(hydroxymethyl)ethyl est	36.177	$C_{19}H_{38}O_4$	330	0.22
1,2-benzenedicarboxylic acid	36.428	$C_{24}H_{38}O_4$	390	0.31
Ethyl linoleate	39.500	$C_{20}H_{36}O_2$	308	0.15
Cyclopentanecarboxylic acid, 2,2-dimethylpropyl ester	40.423	$C_{11}H_{20}O_2$	184	0.66
Squalene	42.192	$C_{30}H_{50}$	410	0.74
Methyl 2-hydroxy-tetracosanoate	42.996	$C_{25}H_{50}O_3$	398	0.12
Ergosta-5,7,9(11),22-tetraen-3-ol, (3.beta.,22E)-	44.156	$C_{28}H_{42}O$	394	0.40
2,5,7,8-tetramethyl-2-(4,8,12-trimethyltride)	47.093	$C_{35}H_{60}O_7$	592	0.43
Ergosta-5,8,22-trien-3-ol, (3.beta.,22E)	47.853	$C_{28}H_{44}O$	396	0.82
Ergosta-5,7,22-trien-3-ol, (3.beta.,22e)	48.538	$C_{28}H_{44}O$	396	1.06
Stigmasta-5,22-dien-3-ol, (3.beta.,22e)	49.646	$C_{29}H_{48}O$	412	0.26
Phytyl tetradecanoate	59.893	$C_{34}H_{66}O_2$	506	0.77
				100.00

Table 5: Bioactive properties methanolic extract of *T. subuliformis.*

Compound	Nature	Biological activity	References
4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl	Flavonoid	Antimicrobial, antiproliferative, antioxidative and anti- inflammatory	29, 30, 32
Hexadecanoic acid, methyl ester	Methyl palmitate	Antifungal, antioxidative, hypocholesterolemic, flavouring agent, antiandrogenic, nematicide, 5-α- reductase inhibitor and antibacterial.	34, 35, 31, 32
Octadeconoic acid	Fatty acid	Anti-inflammatory, anticancerous, hypocholesterolemic, nematicide, hepatoprotective, antieczemic, 5-α- reductase inhibitor, anti-coronary, antiarthritic, insectifuge, antipsychotic, lubricant, cosmetic, perfumery and antibacterial.	36, 31, 32
Evernic acid		Anticancerous and antibacterial.	37
Benzoic acid	Carboxylic acid.	Antibacterial	38
Phytol	Diterpene	Antioxidative, antimicrobial, hypocholesterolemic, anticancerous, anti-inflammatory, diuretic and immunostimulatory.	39, 32
Squalene	Triterpene	Antioxidative, antibacterial, pesticide, antitumor, anticancerous, anti-inflammatory, chemoprotective, immune system stimulant, antiaging.	40, 30, 41, 32
Linoleic acid, methyl ester	Fatty acid (methyl linoleate)	Antifungal, antiproliferative.	42
L-ascorbic acid-2,6-dihexadecanoic acid	Vitamin	Wound healer, anti-nociceptive, antioxidant and anti-inflammatory.	43
Stigmasta-5,22-dien-3-ol,	Sterol	Synthetic progesterone	31

4. Conclusion

Lichens are traditionally used in Chinese as well as in Indian Ayurveda for treating many ailments (Hu *et al*., (1980) [33] as they possess some unique secondary chemical compounds, which are lacking in higher plants. Among the three solvents, methanolic extract possessed maximum antioxidant potential in all assays (DPPH, TAA, FCA and Reducing Power) along with TPC and TFC values. GC-MS analysis showed the presence of 60 different biochemicals including 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl, squalene, phytol, octadecanoic acid, evernic acid, which are biologically active and possess activities like anticancer, anti-inflammatory, antimicrobial, antioxidants, diuretic, hypocholesterolemic, etc. are potent. Hence, *T. subuliformis* is a potent source of antioxidants and nutraceutical and can be used for functional food applications.

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