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Biological capacity and chemical composition of secondary metabolites from representatives Japanese Lichens

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ABSTRACT

Lichens represent a well-known symbiotic system. *Brachythecium velutinum* (Brown), *Brachythecium rutabulum, Lepraria incana, Brachythecium velutinum (Green) and Dirinaria applanata* were collected from the tree trunks and rocks in Japanese forests. The alcoholic extracts of these lichens were examined for their antioxidant and cytotoxicity effect. *Dirinaria applanata methanolic extract* (2.5 mg /mL^{-1}) was the most active in scavenging DPPH free radical (75.09±0.37%) and in reducing Fe^{3+}/ferricyanide complex to the ferrous form (1.88±0.03). It was also the most potent in $ABTS^{\bullet+}$ scavenging activity using ABTS assay 89.35%)at a concentration (0.5 mg.mL^{-1}). *Brachythecium rutabulum* and *Dirinaria applanata* extracts demonstrated a significant in vitro cytotoxic effect on 549 human lung carcinoma cell line with IC_{50} values (34.7±2.1 and 38.6±2.8) µg/mL, respectively. Dereplication of these extracts using LC-HRMS was performed to gain insight about secondary metabolism profiles of these lichens and their rule in the antioxidant and cytotoxic activities.

INTRODUCTION

Lichens are considered as a well-known symbiotic relationship between algae and fungi. They can grow on rocks and exist as epiphytes on trees and leaves (Fabian *et al*., 2005).Most of lichens are terrestrial and few are marine with the ability to adapt water and saline stress, extreme temperature and air pollutants (Nash, 2008). Interactions between the symbiotic partners explain this spectacular success of lichens in unusual environments (Bacˇkor and Fahselt, 2008). This symbiotic system was used in food industry and as a drug for different human diseases.

Biological valuation of secondary metabolites from lichens indicated that they were produced as a defense compounds to protect themselves against microbes and other predators.

Lichens are known to produce aliphatic and aromatic compounds of low molecular weight (Türk*et al*., 2003) which are unique to the lichen-forming fungi and accumulate either in the cortex such as atranorin, parietin, usnic acid, and fungal melanins or the medulla such as physodic acid, physodalic acid To the best of our knowledge, the biological potential of many lichens has been unexplored, for this concept we aimed to estimate the antioxidant activity and the cytotoxicity of five different lichens collected from Japanese forests as well as tentative identification of the chemical profile of each species using liquid chromatograph coupled to high resolution mass spectrometer (LC-HRMS)to gain insight about secondary metabolism profiles of these lichens and their rule in the screened biological activities.

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MATERIALS AND METHODS

Collection and extraction of Samples

Lichens were collected from the trees and rocks of Kashiihama Higashi-ku Fukuoka-shi and Hakozaki Higashi-ku Fukuoka-shi forests in Japan and were identified by Lichenological Society of Japan (http://eng.lichenjapan.jp/?page_id=23). Samples were washed with running distilled water and dried. About 500 grams of each specimen were undergone extraction by the use of 80% methanol. The extracts were dried under vacuum by the rotatory evaporator and weighed.

LC-HRMS analysis

High resolution mass spectral data was obtained from a Thermo Instruments MS system (Finnigan LTQ / LTQ Orbitrap) coupled to a Thermo Instruments HPLC system (Accela PDA detector, Accela PDA autosampler and Accela Pump). The following conditions were used: capillary voltage 45 V, capillary temperature 260 °C, auxiliary gas flow rate 10–20 arbitrary units, sheath gas flow rate 40-50 arbitrary units, spray voltage 4.5 kV, mass range 100–2000 amu (maximum resolution 60000). Gradient separation was achieved using a Poroshell EC-C18 RP analytical HPLC column (2.7µm, 2.1 × 100 mm, Agilent, USA) with a mobile phase of 0–100% MeCN over 25 min followed by 100% MeCN over 10 min at a flow rate of 0.5 mL/min.

Cytotoxicity

Cell culture

A human lung carcinoma cell line (A549) was maintained in RPMI-1640 media, supplemented with 10% fetal bovine serum and antibiotic-antimycotic mixture. The cell line was cultured in 95% humidity, 5% CO_2 and 37°C.

Cytotoxicity test

5000 cells were seeded per well in 96 well plates, left to attach overnight, and then treated with samples for three days. Extracts were tested at 250, 125, 62.5, 31.2, 15.6, 7.8, 3.9 and 1.9 µg/mLfinal concentrations. Staurosporine (2 µM) was used as positive control and 0.5% DMSO was used negative control. Cell cytotoxicity was determined using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay as described by Mosmann 1983.

In brief, medium was aspirated, 40 µl MTT salt (2.5µg/mL) were added to each well and incubated for further 4 h. To stop the reaction and dissolve the formed crystals, 150µLof 10% Sodium dodecyl sulphate (SDS) in deionized water was added to each well and incubated overnight at 37°C. The absorbance was then measured at 595 nm and a reference wavelength of 690 nm.The equation used for calculation of percentage cytotoxicity was:

$$[1-(av(X))/(av(NC))]*100$$

Where: av: average, X: absorbance of test extract, NC: absorbance of negative control.

IC_{50} values were determined by SPSS computer program (SPSS for windows, statistical analysis software package / version 9 / 1989 SPSS Inc., Chicago, USA).

Antioxidant potential Antioxidant Activity (DPPH Assay)

The free radical scavenging activity using the 1.1-diphenyl-2-picryl- hydrazil (DPPH) reagent was determined according to Brand-Williams *et al.* (1995). Several concentrations of the collected Lichens methanol extracts (0.5, 1.0, 1.5, 2.0 and 2.5 mg /mL) were added to1.0 mLof freshly prepared methanolic DPPH solution (20 µg. mL^{-1}) and stirred. The decolorizing process was recorded after 5 min of reaction at 517 nm and compared with a blank control.

Antioxidant activity = [(control absorbance - sample absorbance) / Control absorbance] × 100%

Reducing power Assay

The reducing power of lichen extracts were evaluated according to the method of Oyaizu, (1986). 0.5mLof different concentrations (0.5, 1.0, 1.5, 2.0 and 2.5 mg/ml) methanol extracts were added to Phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and 1% potassium ferricyanide (2.5mL). The mixture was incubated at 50°C for 20 min. Aliquots of trichloroacetic acid (2.5 mL,10%) were added to the mixture, which was then centrifuged at 1000 rpm for 10 min. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and a freshly prepared $FeCl_3$ solution (0.5 mL, 0.1%). The absorbance was measured at 700 nm. Increased absorbance of their action mixture indicated increased reducing power.

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) ABTS radical scavenging assay

The method of Re *et al*. (1999) was investigated. The stock solutions included 7 mM ABTS solution and 2.4 mM potassium persulfate solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 mL $ABTS^{\bullet+}$ solution with 60 mL methanol to obtain an absorbance of 0.706 ± 0.001 units at 734 nm using the spectrophotometer. $ABTS^{\bullet+}$ Solution was freshly prepared for each assay. 0.150 mL of each extract (0.1,0.2,0.3,0.4 and 0.5) mg/mL of sample 1,4 and 5 while (0.5,1.00,1.5,2.00 and 2.5) mg/mL of samples 2 and 3 were allowed to react with 2850 µL of the $ABTS^{\bullet+}$ Solution and the absorbance were taken at 734 nm after 7 min using the spectrophotometer. The $ABTS^{\bullet+}$ scavenging capacity of the extract and isolated compounds percentage inhibition calculated as ABTS radical scavenging activity.

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

Where $Abs_{control}$ the absorbance of ABTS radical + methanol; Abs.sample is the absorbance of ABTS radical + sample extract.

Ferric reducing antioxidant power assay (FRAP)

The FRAP assay was done according to Benzie and Strain (1999) with some modifications. The stock solutions included 300 mM acetate buffer, pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mLTPTZ solution, and 2.5 mL$FeCl_3 \cdot 6H_2O$ solution and then warmed at 37 °C before using. 150 µLof lichens methanol extracts (0.5, 1.00, 1.5, 2.00 and 2.5 mg/mL) were allowed to react with 2850 µLof the FRAP solution for 30 min in the dark condition. Readings of the colored product [ferrous tripyridyltriazine complex] were then taken at 593 nm. Results are expressed in µmol Trolox /100 g dry matter. Additional dilution was needed if the FRAP value measured was over the linear range of the standard curve.

RESULTS AND DISCUSSION

Lichens used in this study were collected from different Japanese forest as presented in Table 1.

Table 1: The collected and identified lichens.

Number	Lichen
1	*Brachythecium velutinum* (Brown)
2	*Brachythecium rutabulum*
3	*Lepraria incana*
4	*Brachythecium velutinum*(Green)
5	*Dirinaria applanata*

Cytotoxicity

The cytotoxic effect of the five lichen extracts was evaluated on A549 human lung carcinoma cell line. Staurosporine (2 µM) was used as positive control and 0.5% DMSO was used negative control. Our results showed that these extracts had a cytotoxic potential at all concentrations and the sensitivity of the treated lung cells was maximum at the highest concentration. Significant cytotoxic effect was clearly obtained by treatment with *Brachythecium reticulum* extract which exhibited IC_{50}value of34.7±2.1 µg/mL Followed by that of *Dirinaria applanata* which showed IC_{50}value of 38.6±2.8µg/mL, table(2). This could be attributed to the presence of terpenes, flavonoids and alkaloids, chemical classes of natural products which had proven record of strong cytotoxic effects based on the LCMS analysis of these lichens.

Table 2: Cytotoxic effect of lichens alcoholic extract.

Lichen name	IC_{50} (µg/mL)
1.Brachythecium velutinum(Brown)	132.4±5.7
2.Brachythecium rutabulum	34.7±2.1
3. Lepraria incana	43.7±3.9
4.Brachythecium velutinum(Green)	57.3±4.5
5.Dirinaria applanata	38.6±2.8

Antioxidant capacity

DPPH free radical scavenging activity

The antioxidant activity of the methanol extract of these lichens was measured in terms of hydrogen-donating or radical-scavenging ability, using the stable DPPH as reagent. The results, presented in figure 1 revealed the variability in antioxidant capability of the examined samples .Of the five tested samples, only sample 2 and 3 did not demonstrate good antioxidant activity. All the other samples exhibited good antioxidant activity specially sample 5 which showed about 75.09±0.37% at the concentration 2.5 mg/mL.This activity was gradually decreased to 40.74±1.04% at a concentration of 0.5 mg /mL. All the samples exhibited significant dose dependent inhibition of DPPH activity. Many human diseases such as neurodegenerative, cardiovascular and diabetes are developed pathologically by reactive oxygen species. Due to their antioxidant behavior, terpenes have been shown to provide relevant protection under oxidative stress conditions in different diseases. The identified terpenes such as Bufotalin, Dantaxusin A, moreollic acid, taxuspine B Suberixanthin, Gaigrandin, ergosteryl acetate and dichrostachine Fin *Dirinaria applanata, Brachythecium velutinum* (Green) and *Brachythecium velutinum* (Brown) could be related to this strong antioxidant potential through free radical scavenging mechanisms(González-Burgos and Gómez-Serranillos 2012).

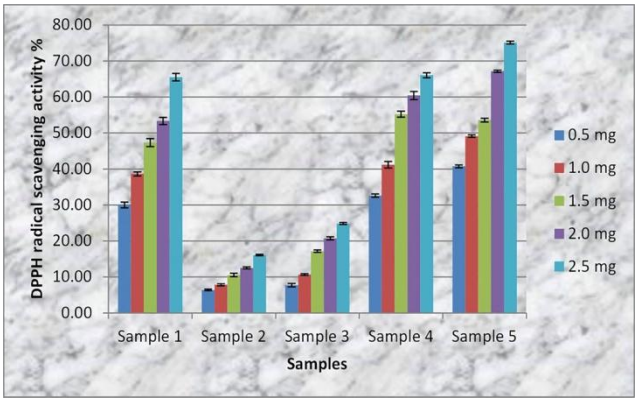

Fig. 1: DPPH radical scavenging activity of methanol extracts of lichens at different concentrations.Each value represents the mean of three replicates (Mean ±SD).

Reducing power capability

Reducing agents provide electrons to the reduced species. The presence of these molecules such as antioxidant substances in the screened samples causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. The yellow color of the test solution changes to various shades of green and blue depending on the reducing power of antioxidant samples. Therefore, Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm (Chung et al., 2002). Figure 2 depicted the reducing activity of the methanol extracts of lichens. Reducing power capability increased gradually by increasing the concentration of each extract. The reducing power capability of sample 2 found to be the least among all tested samples and ranged from 0.16±0.02 to 0.61±0.05 at the concentrations 0.5 and 2.5 mg/mL, respectively. On the contrary sample 5 exhibited the highest reducing capability in an ascending manner with values of 0.97±0.02, 1.17±0.01, 1.39±0.02, 1.72±0.09 and 1.88±0.03 at the concentrations 0.5, 1.00, 1.5, 2.00 and 2.5 mg/mL, respectively.

This could be attributed to the presence of tannins in *Dirinaria applanat*a extract such asellagic acid 3, 3'-di-O-methyl ether, 5, 5'-dehydrodiferulic acid, and 3, 3', 4-tri-O-methylellagic acid. Tannins do not function as primary antioxidants (i.e., they donate hydrogen atom or electrons), but they could be considered as secondary antioxidants. Tannins are able to chelate the metal Fe(II) and interfere with one of the reaction steps and thereby impede oxidation (Karamac´*et al.*, 2006).

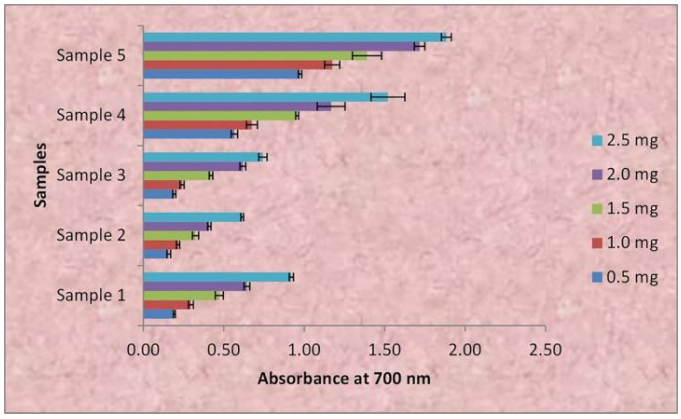

Fig. 2: Reducing power capability of the methanol extracts of lichens at different concentrations. Each value represents the mean of three replicates (Mean ±SD).

ABTS Radical scavenging activity

Excessive formation of free radicals accelerates the oxidation of lipids in foods and decreases food quality and consumer acceptance (Min, 1998). Hence, radical scavenging activities are very important due to the deleterious role of free radicals in foods and in biological systems. A more appropriate format for the assay is a decolonization technique in that the radical is generated directly in a stable form prior to reaction with putative antioxidants. The improved technique for the generation of $ABTS^{\bullet+}$ described here involves the direct production of the blue/green $ABTS^{\bullet+}$ chromophore through the reaction between $ABTS^{\bullet+}$ and potassium persulfate. As seen in figure 3, several concentrations were used foreach methanol extract of lichens to determine the radical scavenging activity using ABTS assay.

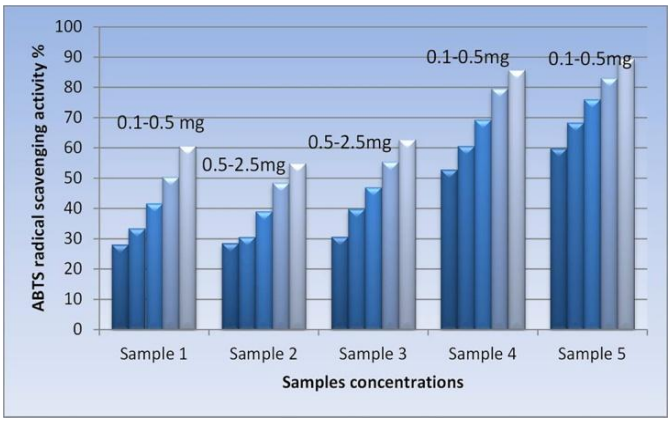

Fig. 3: Scavenging effect of the methanol extracts of lichens on the $ABTS^{\bullet+}$ at different concentrations.

Sample 5 had the highest radical scavenging activity which showed 59.92, 68.36, 76.07, 83.11 and 89.35% at the concentrations of 0.1, 0.2, 0.3, 0.4 and 0.5 mg/mL, respectively. Similar results were obtained in sample 4, it possessed high radical scavenging activity of 85.78 % at the concentration 0.5 mg/mL. Moderate radical scavenging activities of 60.65 and 62.77% were found at the high concentration of sample 1 and 3 (0.1 mg/mL), respectively.

Ferric reducing antioxidant power (FRAP)

The FRAP test was used to measure the total antioxidant capacity of lichens. Method is based on electron transfer and is regarded as accurate indicators of total antioxidant power, since total reducing power is defined as the sum of the reducing powers of the individual compounds contained in a particular sample (Tezcan *et al*., 2011). FRAP activity of samples is given in figure 4.All the investigated samples exhibited ferric reducing antioxidant power and their activity increased in a dose dependent manner with the increase in the concentration. Sample 5 and 4 exhibited the highest ferric reducing antioxidant power which ranged from 2105 to 3830µM TEAC/100g at 0.5 to 2.5 mg/mL and from 2242 to 3424µM TEAC/100g at 0.5 to 2.5 mg /mL for samples 5 and 4, respectively. Results also indicated that sample 2 had the lowest ferric reducing antioxidant power at 1543 µM TEAC/100g at the highest concentration (2.5 mg/mL).

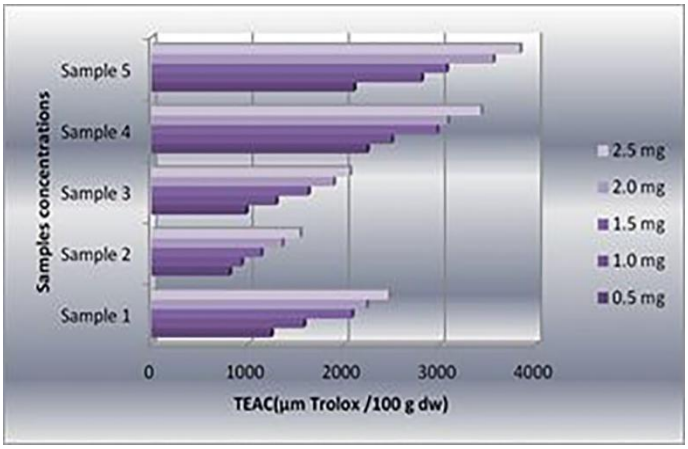

Fig. 4: Ferric Reducing antioxidant property (FRAP) of the methanol extracts of lichens at different concentrations.

LC-HRESIMS analysis of the methanolic extract of lichens

The LC-HRMS analysis of the methanolic extract of Lichens (Table 3 and Figure 5) indicated the presence of a range of secondary metabolites with different chemical classes ranging fromnon-polar steroids and terpenes to the polar flavonoids and tannins. Most of the identified metabolites previously showed moderate to strong cytotoxic and antioxidant activities.

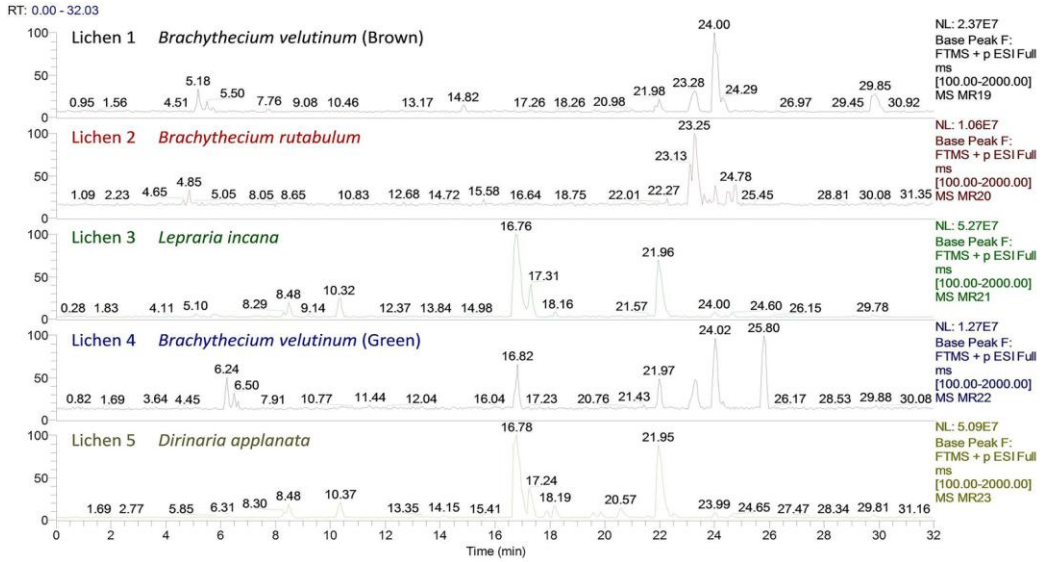

Fig. 5: LC-HRESIMS analysis of the methanolic extract of lichens.

Table 3: LC-HRESIMS analysis of the methanolic extract of lichens.

Rt min	*m/z* Experimental	Selected ion	Formula[a]	Suggested Compound[b]
Lichen 1: *Brachytheciumvelutinum* (Brown)				
5.18	465.1016	$[M+H]^+$	$C_{21}H_{20}O_{12}$	Hyperoside (Flavonoid glycoside)
5.50	303.0488	$[M+H]^+$	$C1_5H_{10}O_7$	Robinetin (Flavonoid aglycone)
5.70	287.0542	$[M+H]^+$	$C_{15}H_{10}O_6$	Scutellarein (Flavonoid aglycone)
14.82	445.2548	$[M+H]^+$	$C_{26}H_{36}O_6$	Bufotalin (Terpene)
21.85	621.2695	$[M+H]^+$	$C_{35}H_{40}O_{10}$	Dantaxusin A (Terpene)
21.98	593.2745	$[M+H]^+$	$C_{34}H_{40}O_9$	moreollic acid (Terpene)
23.28	623.2853	$[M+H]^+$	$C_{35}H_{42}O_{10}$	taxuspine B (Terpene)
24.00	607.2901	$[M+H]^+$	$C_{35}H_{42}O_9$	taxuspine C (Terpene)
24.29	547.2692	$[M+H]^+$	$C_{33}H_{38}O_7$	Dihydroisomorellin (Terpene)
Lichen 2: *Brachytheciumrutabulum*				
4.85	325.0698	$[M+H]^+$	$C_{18}H_{12}O_6$	Sterigmatocystine (xanthene)
23.13	639.2798	$[M+H]^+$	$C_{35}H_{42}O_{11}$	propinquanin B (polyketide)
23.25	623.2853	$[M+H]^+$	$C_{35}H_{42}O_{10}$	taxuspine B (Terpene)
24.00	607.2901	$[M+H]^+$	$C_{35}H_{42}O_9$	taxuspine C (Terpene)
24.50	653.2955	$[M+H]^+$	$C_{36}H_{44}O_{11}$	schisantherin I (polyketide)
24.78	661.4631	$[M+H]^+$	$C_{39}H_{64}O_8$	Tetrahydroxyoleanenoic-acid 28-O-β-D-glucopyranosyl ester (Terpene)
Lichen 3: *Lepraria incana*				
8.29	331.0438	$[M+H]^+$	$C_{16}H_{10}O_8$	ellagic acid 3,3'-di-O-methyl ether (tannin)
8.48	387.1065	$[M+H]^+$	$C_{20}H_{18}O_8$	5,5'-dehydrodiferulic acid (tannin)
10.32	345.0596	$[M+H]^+$	$C_{17}H_{12}O_8$	3,3',4-tri-O-methylellagic acid (tannin)
16.76	389.1587	$[M+H]^+$	$C_{21}H_{24}O_7$	5'-methoxydehydrodiconiferyl alcohol (tannin)
17.31	620.4150	$[M+H]^+$	$C_{35}H_{57}NO_8$	pavoninin-2 (terpene)
20.57	571.3073	$[M+H]^+$	$C_{36}H_{42}O_6$	terpecurcumin Q (terpene)
21.95	439.3565	$[M+H]^+$	$C_{30}H_{46}O_2$	ergosteryl acetate (terpene)
24.00	607.2903	$[M+H]^+$	$C_{35}H_{42}O_9$	taxuspine C (Terpene)
24.60	567.4009	$[M+H]^+$	$C_{36}H_{54}O_5$	lantadene A methyl ester (Terpene)
Lichen 4: *Brachytheciumvelutinum*(Green)				
6.24	365.2061	$[M+H]^+$	$C_{24}H_{28}O_3$	ugonstilbene A (Phenolic)
6.50	366.1903	$[M+H]^+$	$C_{19}H_{27}NO_6$	harzianic acid (Alkaloid)
16.82	389.1587	$[M+H]^+$	$C_{21}H_{24}O_7$	5'-methoxydehydrodiconiferyl alcohol (tannin)
21.97	593.2745	$[M+H]^+$	$C_{34}H_{40}O_9$	moreollic acid (Terpene)
23.25	623.2853	$[M+H]^+$	$C_{35}H_{42}O_{10}$	taxuspine B (Terpene)
24.02	607.2903	$[M+H]^+$	$C_{35}H_{42}O_9$	taxuspine C (Terpene)
25.80	593.4369	$[M+H]^+$	$C_{42}H_{56}O_2$	Suberixanthin (Terpene)
Lichen 5: *Dirinariaapplanata*				
8.30	331.0438	$[M+H]^+$	$C_{16}H_{10}O_8$	ellagic acid 3,3'-di-O-methyl ether (tannin)
8.48	387.1065	$[M+H]^+$	$C_{20}H_{18}O_8$	5,5'-dehydrodiferulic acid (tannin)
10.37	345.0596	$[M+H]^+$	$C_{17}H_{12}O_8$	3,3',4-tri-O-methylellagic acid (tannin)
16.78	389.1587	$[M+H]^+$	$C_{21}H_{24}O_7$	5'-methoxydehydrodiconiferyl alcohol (tannin)
18.19	425.2134	$[M+H]^+$	$C_{22}H_{32}O_8$	Gaigrandin (terpene)
20.57	571.3073	$[M+H]^+$	$C_{36}H_{42}O_6$	terpecurcumin Q (terpene)
21.95	439.3565	$[M+H]^+$	$C_{30}H_{46}O_2$	ergosteryl acetate (terpene)
23.99	607.2903	$[M+H]^+$	$C_{35}H_{42}O_9$	taxuspine C (Terpene)
24.65	621.3057	$[M+H]^+$	$C_{36}H_{44}O_9$	dichrostachine F (Terpene)

[a] The molecular formula was deducted from the accurate mass analysis of the metabolite profile using Xcalibur 3.0 software. [b] The suggested compound based on dereplication using the Dictionary of Natural Product database Version 23.1 on DVD and by comparison with the fragmentation pattern with literature data when applicable.

CONCLUSION

Lichens represent a well-known symbiotic relationship between algae and fungi which is successfully used in food industry and for different human diseases. Lichens tend to produce secondary metabolites as a defense mechanism to protect themselves against external predators. In this study, lichens were collected from Japanese forests and their alchoholic extracts were screened for their potential antioxidant and cytotoxic effects. The methanolic extract of *Dirinaria applanata* was the most potent which exhibited a significant antioxidant effect towards different radicals by the application of different assays. Additionally, the methanolic extract of *Brachythecium rutabulum* and *Dirinaria applanata* considered the most cytotoxic among the five screened lichens against 549 human lung carcinoma cell line. This strong antioxidant activity could be attributed to reducing the prooxidant processes and enhancing the antioxidant ones by participating in the synthesis of prostaglandins and phospholipids components of cell membrane thus which could be applicable in skin protection mechanisms (Butnariu and Giuchici, 2011). Dereplication using LC-HRMS indicated the presence of a lot of known secondary metabolites belonging to different chemical classes which explains the strong antioxidant activity exhibited by these extracts (Rashed and Butnariu, 2014). Based on the LCMS analysis, these lichens were rich in terpenes, flavonoids and alkaloids, chemical classes of natural products which had proven record of strong cytotoxic effects.

This study shed the light of using such lichens as safe and effective preservatives in canned food industry and as a topical application for UV skin protection. Moreover, formulation of these extracts for nanoparticle for drug delivery (Butu *et al.*, 2015) could be a potential point of future research which could allow in-depth study of their cytotoxic effect.

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