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Chemical composition and antioxidant activity of lichen *Toninia candida*

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Abstract: In the present investigation, methanol, chloroform and petrol ether extracts from the lichen *Toninia candida* (Weber) Th. Fr, Catillariaceae, were assayed for their antioxidant activity. The phenolic composition of the extracts was determined by HPLC-UV analysis. The predominant phenolic compound in all the extracts was depsidone, norstictic acid. All the tested extracts of *T. candida* contain, besides norstictic acid, atranorin, stictic, protocetraric and usnic acid, but in different amounts and relations. The lichen extracts showed comparable and strong antioxidant activity, exhibited higher DPPH and hydroxyl radical scavengings, chelating activity and inhibitory activity towards lipid peroxidation. This is the first report of chemical composition and antioxidant antimicrobial activity of the lichen *Toninia candida*.

Article

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Introduction

Lichens are valuable plant resources and are used as food, fodder, medicine, dyes perfume, spice, and for miscellaneous purposes throughout the world. More than one thousand primary and secondary metabolites with identified structures are currently known in lichens (Shukla et al., 2010). The use of lichens in medicine is based on the fact that they contain unique and varied biologically active substances. Lichen metabolites exert a wide variety of biological actions including antibiotic, antimycobacterial, antiviral, anti-inflammatory, analgesic, antipyretic, antiproliferative and cytotoxic effects (Huneck, 1999; Shukla et al., 2010; Manojlovic et al., 2002; Manojlovic et al., 2010a; Manojlovic et al., 2010b; Manojlovic et al., 2010c). Even though these manifold activities of lichen metabolites have now been recognized, their therapeutic potential has not yet been fully explored and thus remains pharmaceutically unexploited.

The most numerous classes of secondary metabolites are depsides and depsidones. Depside molecules consist of 2-4 hydroxybenzoic acid residues linked by ester groups. Many depsides reported in literature have been found to possess important physiological properties. The antioxidant property has been reported in depsides isolated from various lichen species (Hidalgo et al., 1994). More than one hundred compounds are depsidones, which have an additional ether bond between aromatic rings. Depsidones in lichen are believed to arise by oxidative cyclisation of depsides. It has been found that depsidones are more efficient antioxidants than depsides. The higher efficiency of the depsidones could be related to a larger incorporation into lipidic microdomains (Hidalgo et al., 1994). Depsidone and depside compounds such as pannarin, 10-chloropannarin and sphaerophorin, tested in cell cultures of lymphocytes, were shown to have a higher cytotoxic effect than colchicine (Correche´ et al., 2002). The depsidones salazinic acid, stictic acid and psoromic acid were the most apoptotic active derivatives among fifteen lichen compounds evaluated on primary cultures of rat hepatocytes (Correche´ et al., 2002). *Toninia candida* (Weber) Th. Fr, Catillariaceae, is widely distributed in continental areas in the Northern Hemisphere (Timdal, 1991). In Serbia, *T. candida* could be found in Jelasnica gorge (Mt. Suva Planina) on a vertical limestone rocks.

Thus, the aim of the present work was to identify and quantify phenolic acids composition of *Toninia candida* lichen by HPLC-UV and to evaluate the antioxidant capacity of methanol, chloroform and petrol ether extracts from this lichen using different systems, including DPPH and hydroxyl radical scavenging, metal chelating activity, as well as to screen their antimicrobial activity.

Material and Methods

Lichen material

The lichen material of *Toninia candida* (Weber) Th. Fr, Catillariaceae, was collected from Mt. Suva (Jelasnica gorge) in Serbia during October 2010. A voucher specimen (HMN 5459) has been deposited at the Herbarium Moesiacum Nis in the Department of Biology and Ecology, Faculty of Sciences and Mathematics, University of Nis, Serbia.

Preparation of the lichen extracts

The lichen material was air-dried at room temperature (26 °C) for one week, after which it was ground to a uniform powder. Each extract (methanol, chloroform and petrol ether) extract was prepared by soaking 500 g dry powdered lichen material in 2000 mL of solvent at room temperature for three days. The extracts were filtered through a Whatman no. 42 (125 mm) filter paper and concentrated using a rotary evaporator.

Instrumentation and conditions

HPLC analysis was carried out on an Agilent 1200 Series HPLC instrument with C18 column (C18; 25 cm x 4.6 mm, 10 m) and a UV spectrophotometric detector with methanol-water-phosphoric acid (80:20:0.9, v/v/v) solvent. Methanol was of HPLC grade and was purchased from Merck (Darmstadt, Germany). Phosphoric acid was analytical-grade reagent. Deionized water used throughout the experiments was generated by a Milli-Q academic water purification system (Milford, MA, USA). The sample injection volume was 10 mm^3. The flow rate was 1.0 mL/min. The standards used were obtained from the following sources: norstictic acid (2, t_R=4.01±0.20 min) was isolated from lichen *Ramalina furinacea*, stictic acid (1, t_R=3.31±0.01 min) from lichen *Xanthoparmelia conspersa*, protocetraric acid (3, t_R=4.28±0.05 min) from lichen *Cetraria islandica*, usnic acid (5, t_R=14.54±0.30 min) and atranorin (6, t_R=16.47±0.30 min) from lichen *Evernia prunastri.* The standard samples were isolated in our laboratory and their structures were confirmed by spectral data. The retention times and UV spectra of these standards are shown in Table 2 and Figure 1.

Reference solutions of decreasing concentrations were obtained by dilution with eluent of the requisite standard solution. These solutions were analyzed and the corresponding peak areas plotted against the concentration of acid injected. The concentrations of the components in the analyzed samples (were calculated from the chromatogram peak areas using the normalization method. The identification of the different compounds was achieved by comparison of both tR and the absorption spectra obtained for each diluted peak with those obtained for the standards. The data obtained were statistically processed by using a standard non-parametric variance analysis method (ANOVA) for determining significant intergroup differences. Data are presented as the mean±standard deviation (SD) of three separate experiments performed on different samples.

LC-MS/MS analysis was carried out using an Agilent 1100 LC system consisting of degasser, binary pump, auto sampler, and column heater. The column outlet was coupled to an Agilent MSD Ion Trap XCT mass spectrometer equipped with an ESI ion source. For the chromatographic separation, a Zorbax 300Ĺ Extend-C-18 Column (2.1×150 mm, 1.8 µm) was used. The mobile phase was pumped at 1 $mLmin^{-1}$ flow rate and consisted of methanol-water-formic acid (80:20:0.9, v/v/v).

Determination of the total phenolics

The total phenolics content was determined using the Folin-Ciocalteau method (Singleton et al.,1999). Extract was diluted to the concentration of 1 mg/mL, and aliquots of 0.5 mL were mixed with 2.5 mL of FC reagent (previously diluted 10-fold with distilled water) and 2 mL of $NaHCO_3$ (7.5%). After 15 min of staying at the 45 °C the absorbance was measured at 765 nm on spectrophotometer versus blank sample. Total phenols were determined as gallic acid equivalents (mg GA/g extract), and the values are presented as means of triplicate analyses.

Determination of total antioxidant capacity

The total antioxidant activity of the *Toninia candida* extract was evaluated by the phosphomolybdenum method (Prieto et al., 1999). The assay is based on the reduction of Mo (VI)-Mo (V) by the antioxidant compounds and subsequent formation of a green phosphate/Mo (V) complex at acid pH. 0.3 mL of sample extract was combined with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95 °C for 90 min. Then the absorbance of the solution was measured at 695 nm using spectrophotometer against blank after cooling to room temperature. Methanol (0.3 mL) in the place of extract was used as the blank. Ascorbic acid (AA) was used as standard and the total antioxidant capacity is expressed as milligrams of ascorbic acid per gram of the dry extract.

Determination of DPPH free radical scavenging activity

The method used by (Takao et al., 1994) was adopted with suitable modifications from (Kumarasamy et al., 2007). DPPH (8 mg) was dissolved in MeOH (100 mL) to obtain a concentration of 80 µg/mL. Serial dilutions were carried out with the stock solution (1 mg/mL) of the extract. Solutions (2 mL each) were then mixed with DPPH

(2 mL) and allowed to stand for 30 min for any reaction to occur, and the absorbance was measured at 517 nm. Ascorbic acid (AA), gallic acid (GA) and butylated hydroxytoluene (BHT) were used as reference standards and dissolved in methanol to make the stock solution with the same concentration (1 mg/mL). Control sample was prepared containing the same volume without test compounds or reference antioxidants. Methanol 95% was used as blank. The DPPH free radical scavenging activity (%) was calculated using the following equation:

$$\% \text{ inhibition} = \frac{Ac - As}{Ac} \times 100$$

The IC50 value, which is the concentration of the test material that reduces 50% of the free radical concentration, was calculated as µg/mL through sigmoidal dose-response curve.

Determination of the inhibitory activity toward lipid peroxidation

The antioxidant activity was determined by the thiocyanate method (Hsu et al., 2008). Serial dilutions were carried out with the stock solution (1 mg/mL) of the extracts, and 0.5 mL of each solution was added to linoleic acid emulsion (2.5 mL, 40 mM, pH 7.0). The linoleic acid emulsion was prepared by mixing 0,2804 g linoleic acid, 0.2804 g Tween-20 as emulsifier in 50 mL 40 mM phosphate buffer and the mixture was then homogenized. The final volume was adjusted to 5 mL with 40 mM phosphate buffer, pH 7.0. After incubation at 37 °C in the dark for 72 h, a 0.1 mL aliquot of the reaction solution was mixed with 4.7 mL of ethanol (75%), 0.1 mL $FeCl_2$ (20 mM) and 0.1 mL ammonium thiocyanate (30%). The absorbance of this mixture was measured at 500 nm, after it was stirred for 3 min. Ascorbic acid, gallic acid, α-tocopherol and BHT were used as a reference compounds. To eliminate the solvent effect, the control sample, which contained the same amount of solvent added to the linoleic acid emulsion in the test sample and reference compound, was used. Inhibition percent of linoleic acid peroxidation was calculated using following formula:

$$\% \text{ inhibition} = \frac{Ac - As}{Ac} \times 100$$

Measurement of ferrous ion chelating ability

The ferrous ion chelating ability was measured by the decrease in absorbance at 562 nm of the iron (II)-ferrozine complex (Carter, 1971; Yan et al., 2006). One milliliter of 0.125 mM $FeSO_4$ was added to 1.0 mL sample (with different dilutions), followed by 1.0 mL of 0.3125 mM ferrozine. The mixture was allowed to equilibrate for 10 min before measuring the absorbance. The ability of the sample to chelate ferrous ion was calculated relative to the control (consisting of iron and ferrozine only) using the formula:

$$\text{Chelating effect (\%)} = \frac{Ac - As}{Ac} \times 100$$

Determination of hydroxyl radical scavenging activity

The ability of *Toninia candida* to inhibit non site-specific hydroxyl radical-mediated peroxidation was carried out according method described by Hinneburg et al. (2006). The reaction mixture contained 100 µL of extract dissolved in water, 500 µL of 5.6 mM 2-deoxy-D-ribose in KH_2PO_4-NaOH buffer (50 mM, pH 7.4), 200 µL of premixed 100 µM $FeCl_3$ and 104 mM EDTA (1:1 v/v) solution, 100µL of 1.0 mM H2O2 and 100 µL of 1.0 mM aqueous ascorbic acid. Tubes were vortexed and incubated at 50 °C for 30 min. Thereafter, 1 mL of 2.8% TCA and 1 mL of 1.0% TBA were added to each tube. The samples were vortexed and heated in a water bath at 50 °C for 30 min. The extent of oxidation of 2-deoxyribose was estimated from the absorbance of the solution at 532 nm. The percentage inhibition values were calculated from the absorbance of the control (Ac) and of the sample (As), where the controls contained all the reaction reagents except the extract or positive control substance. The values are presented as the means of triplicate analyses.

Statistical analysis

All the results are presented as mean±standard deviations of three determinations. Statistical analyses were performed using Student´s t-test and one way analysis of variance. Multiple comparisons of means were done by LSD (least significant difference) test. A probability value of 0.05 was considered significant. All computations were made by employing the statistical software (SPSS, version 11.0). IC50 values were calculated determined by nonlinear regression analysis from the sigmoidal dose-response inhibition curve.

Results and Discussion

A representative chromatograms for standards and *Toninia candida* methanol, chloroform and petrol ether extracts eluted by HPLC are represented in Figure 1 and Figure 2. As it is evidenced in the chromatograms, there were the presence of depsidones as the most abundant substance class in the extracts examined. As the most abundant depsidone, norstictic acid (NOR) (t_R=4.01±0.2 min, **2**) was identified. This compound was previously reported from the family Umbilicariaceae (Narui et al.,

1996) and had been reported to demonstrate antimicrobial effect on *Bacillus subtilis, Listeria monocytogenes, Proteus vulgaris, Staphylococcus aureus, Streptococcus faecalis, Aeromonas hydrophila, Candida albicans* and *Candida glabrat* (Tay et al., 2004).

As can be seen in chromatograms, beside norstictic acid (**2**), stictic acid (t_R=3.31±0.01 min, **1**), protocetraric acid (t_R=4.28±0.05 min, **3**), usnic acid (t_R=14.54±0.30 min, **5**) and atranorin (t_R=16.47±0.30 min, **4**) were also identified. Four detected compounds belonging to the depsidones. Atranorin is depside and usnic acid is antibiotic with dibenzofurane structure. The UV spectra of depsidones have three absorption maxima and are dissimilar from those of depsides and monocyclic compounds. The UV spectra of protocetraric acid are very similar to those of norstictic acid. Absorbance maxima at 234 and 282 nm are characteristic for usnic acid. Except norstictic acid, other compounds were found in the extracts in small amounts. Identification of these compounds was achieved by comparison of their tR values with the standard substances previously isolated from lichens. The UV absorbance spectral data (200-400 nm) also corresponded with those of standards and found in Refs. (Yoshimura at al., 1994; Huneck & Yoshimura, 1996). LC-MS analysis was also used to confirm the structures of detected molecules. Values of molecular and fragmentation ions were consistent with those published in literature (Huneck & Yoshimura, 1996). Table 2. shows the retention time of the detected lichen substances, their absorbance maxima (nm) and fragmentation ions. All compounds are identified for the first time in this lichen.

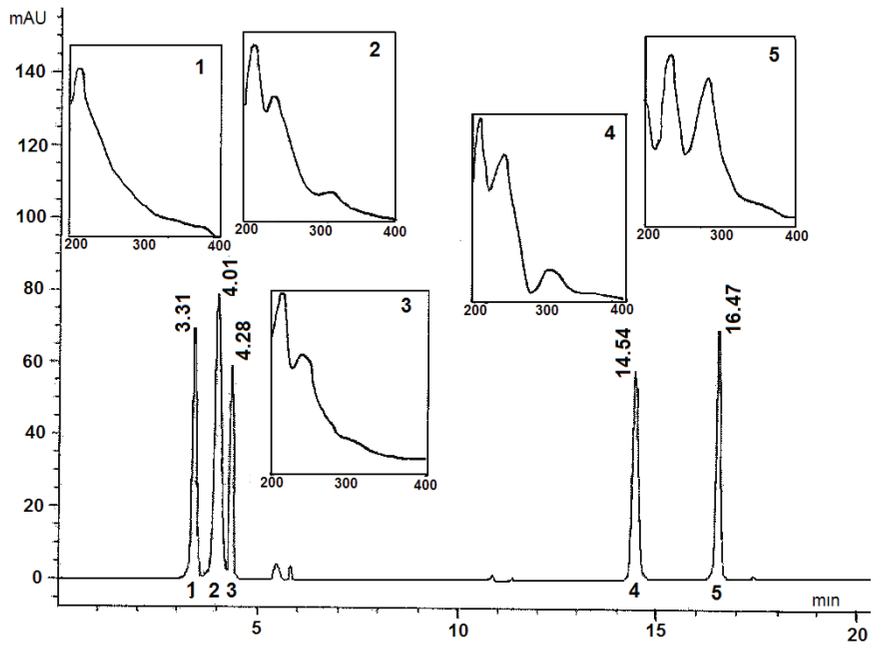


Figure 1. Chromatogram of the standards used for identification of the compounds present in the *Toninia candida*. In detel can be observed the UV spectra of these compounds (200-400 nm).

Table 1. The amounts of presented compounds in the lichen *Toninia candida.*

Compound	Substance class	µg/g DW		
		MET	CHL	PET
Stictic acid (**1**)	Depsidone	2.0±0.2	5.2±0.5	4.7±0.2
Norstictic acid (**2**)	Depsidone	51.5±1.0	53.6±0.5	64.3±1.0
Protocetraric acid (**3**)	Depsidone	0.6±0.4	2.5±0.2	2.1±0.4
Usnic acid (**5**)	Dibenzofurane	-	0.6±0.1	-
Atranorin (**4**)	Depside	-	0.8±0.1	-

Table 2. Retention time of the examined lichen substances and their absorbance maxima (nm) and fragmentation ions.

Peaks N°	Compound	Retention time (t_R±SD)* (min)	Absorbance maxima (nm) UV spectrum	MS ions (*m/z*)
1	Stictic acid (**1**)	3.31±0.01	212, 236, 310^m	386, 368, 341, 314, 193, 191
2	Norstictic acid (**2**)	4.01±0.20	212, 239, 310^m	372, 354, 327, 298, 179, 177
3	Protocetraric acid (**3**)	4.28±0.05	212, 240, 320^m	374, 356, 312, 258, 230, 179, 151
4	Usnic acid (**5**)	14.54±0.30	234, 282	344, 328, 260, 233, 217
5	Atranorin (**4**)	16.47±0.30	210, 252, 321^m	374, 196, 179, 164, 136

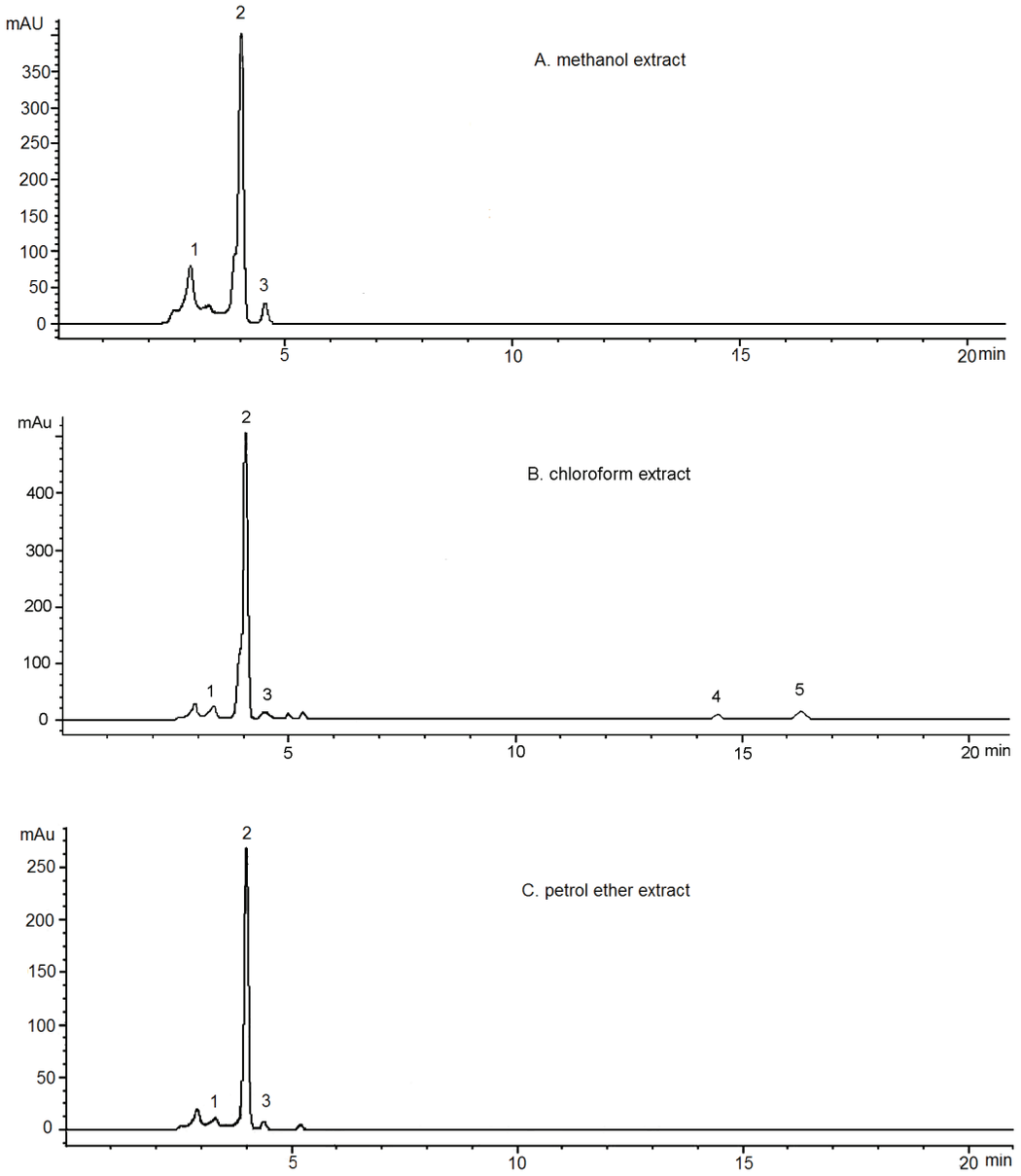


Figure 2. HPLC hromatograms acquired at 256 nm of the methanol (A), chloroform (B) and petrol etar (C) extracts of *Toninia candida*. Chromatographic peaks identities are reported in Table 2.

Phenolic compounds have been reported to be associated with antioxidative action in biological systems, mainly due to their red-ox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Saha et al., 2008). The results of determination of total phenolic and antioxidant capacity are given in Table 3. Total phenolic contents were determined and amounted to 76.26±0.32 mg GA/g, 45.25±0.72 mg GA/g and 42.98±0.15 mg GA/g, for methanol, chloroform and petrol ether extracts, respectively. The results showed that the methanolic, chloroform and petroleum ether extracts possess antioxidant activity, with total antioxidant capacity of 78.45±0.58 µg AA/g, 56.67±0.30 µg AA/g and 51.45±0.31 µg AA/g, respectively.

The assessment of antioxidant activity showed that all tested extracts were able to scavenge this radical (Table 4). The chloroform extract displayed a higher activity than petrol ether and methanol extracts (48.98±1.45, 50.10±0.95 and 51.45±1.78 µg/mL, respectively). Although this scavenging effect was lower than that of BHT (Table 4), it was stronger than the antioxidant activity reported in many other lichen species (Gulluce et al., 2006). Results demonstrated also

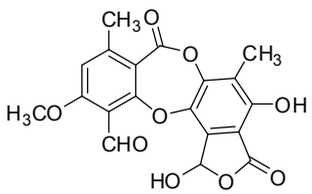
that all tested extracts exhibited significant inhibitory activity towards lipid peroxidation (from 21.45±1.55 µg/mL to 46.46±1.68 µg/mL). The results of metal chelating activity are also shown in the Table 4 and these values were very similar for all tested extracts.

The results of determination of hydroxyl radical scavenging activity (Table 4) showed that IC50 values were 67,11±0.23, 53.23±0.51 and 50.57±0.75 µg/mL for metanolic, chloroform and petrol ether extracts, respectively. These results revealed that the methanol, chloroform and petrol ethar extracts of *T. candida* organs were free radical scavengers, acting possibly as primary antioxidants. The strong antioxidant activity of *T. candida* assessed by the different systems could be attributed to their high total polyphenolic contents. Moreover, the high yield of the different phenolic compounds (depsidones) found in *T. candida thallus* (Table 1) might contribute to the potent antioxidant activity of the tested extracts, since a positive correlation between phenolic composition and antioxidant activity was proved (Katalinic et al., 2006). Thus, antioxidant property of the lichen could be attributed to the significant amount of depsidones, especially norstictic acid, present in our study with the high amounts of 51.5±2.0, 53.6±1.0 and 64.3±1.5 µg/g DW, respectively in the methanol, chloroform and petrol ether extracts. Other minor phenolic compounds should not be neglected, since synergy of the different chemicals with each other should be taken into consideration for the biological activity. The presence of the phenolic groups in the lichen metabolites is considered to be key element for the antioxidative efficiency (Markovic & Manojlovic, 2010). Norstictic acid posesses two phenolic groups in the molecule which probably play an important role in expression of their antioxidant activity.

In conclusion, this is the first study focused on the chemical composition and biological activities of *T. candida.* The methanol, chloroform and petrol ether extracts of the lichen showed significant antioxidant activity. Four depsidones, one depside and usnic acid were identified, and norstictic acid was the dominant phenolic compound in the lichen. The present study provides data for supporting the use of *T. candida* extracts as natural antioxidant agents, and confirms that these extracts represent a significant source of phenolic compounds. Future investigation will be focused on isolation of phenolic compounds and determination of their biological activities *in vitro* and *in vivo*.

Table 3. Total phenolic and total antioxidant capacity of the examined *Toninia candida* extracts

Total phenolic content (mg GA/g)			Total antioxidant capacity (µg AA/g)		
methanol extract	chloroform extract	petrol ether extract	methanol extract	chloroform extract	petrol ether extract
76.26±0.32	45.25±0.72	42.98±0.15	78.45±0.58	56.67±0.30	51.45±0.31



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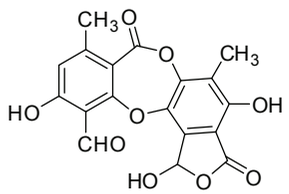

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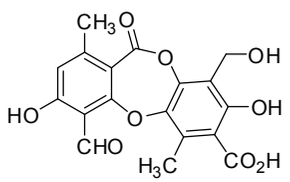

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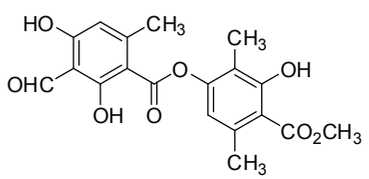

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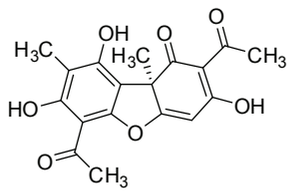

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Table 4. The antioxidant activity of the examined extracts.

Lichen extracts/ standards	[a]IC50 (μg/mL)			
	DPPH scavenging activity	Inhibitory activity toward lipid peroxidation	Metal chelating activity	Hydroxyl radical scavenging activity
Toninia candida methanol extract	51.45±1.78	46.46±1.68	41.91±0.88	67,11±0.23
Toninia candida chloroform extract	48.98±1.45	41.68±1.09	40.45±0.45	53.23±0.51
Toninia candida petrol ether extract	50.10±0.95	21.45±1.55	39.50±0,53	50.57±0.75
Gallic acid	3.79±0.69	255.43±11.68	-	59.14±1.10
Ascorbic acid	6.05±0.34	> 1000	-	160.55±2.31
BHT	15.61±1.26	1.00±0.23	-	33.92±0.79
α-Tocopherol	-	0.48±0.05	-	-

[a]IC50 values were determined by nonlinear regression analysis. Results are mean values±SD from three experiments.

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