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Phytochemical and biological characterization of acetone extracts from the lichen *Cladonia coniocraea* and *Cladonia ramulosa* growing in Serbia

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A comparative study of aromatic secondary metabolites and antioxidant, antimicrobial, and anticancer potential of two lichen species *Cladonia coniocraea* and *Cladonia ramulosa* is presented in this paper. HPLC-UV analysis revealed the presence of depsidone fumarprotocetraric acid and depside sekikaic acid as major aromatic metabolites in both tested species but in different amounts. The antioxidant activity was evaluated by free radical scavenging and reducing power assays. In both assays the extracts showed weak activity (IC50>1000 μg/ml, while absorbances for reducing power were from 0.0118 to 0.1675). The total content of phenol and flavonoid in extracts was examined using Folin–Ciocalteu reagent and aluminium chloride method and the obtained values were expressed as pyrocatechol equivalents, and as rutin equivalents, respectively. Further, the antimicrobial activity was estimated by determination of the minimal inhibitory concentration by the broth microdilution method against five bacterial and 10 fungal species. Tested extracts showed similar antimicrobial activity with minimum inhibitory concentration values ranging from 0.156 to 20 mg/ml. Finally, the cytotoxic activity was tested using MTT method on the human epithelial carcinoma (Hela), human lung carcinoma (A549) and human colon carcinoma (LS174) cells. The strongest cytotoxic activity with IC_{50} value of 185.59 μg/ml expressed extract of *C. ramulosa* toward Hela cells. Obtained results indicate that these lichens showed the potential for further investigation and possible biopharmaceutical application.

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Keywords: Cladonia coniocraea, Cladonia ramulosa, HPLC-UV, Lichen metabolites, Bioactivity

1. Introduction

Lichens are symbiotic organisms consisting of a fungus partner and a photosynthetic organism (either an alga or Cyanobacteria). Interactions between the symbiotic partners allow lichens facilitate life in unusual environments. They grow practically everywhere, on and within rocks, on soil and tree barks, on almost any inanimate object. Lichens are able to survive in extreme ecological conditions; they can adapt to extreme temperatures, drought, inundation, salinity, high concentrations of air pollutants, and nutrient-poor, highly nitrified environments [1-3].

It is well known that many lichens have been widely used by people for different purposes such as nutrition, decoration, for getting colors, perfumes, alcohol, etc. [4]. Furthermore, lichens also have beneficial effects to human health. They produce a wide range of secondary metabolites, called lichen substances, which have high therapeutic values. Nowadays, various studies show that

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lichens and their metabolites have many important health benefits, including anticancer, immunomodulatory, antiinflammatory, antidiabetic, antiviral, antioxidant, antimicrobial, antineurodegenerative properties as well as antihypertensive and cholesterol-lowering properties [3,5-7].

Since there is a lot of interest in new bioactive products of natural origin that do not cause side effects, the present study represents lichens as very interesting natural source of bioactive substances that have great biotechnological application. In that respect, this report focuses on the lichens *Cladonia coniocraea* and *C. ramulosa*.

Cladonia is a genus of moss-like lichens belongs to Cladoniaceae group. Representatives of the genus *Cladonia* are fruticose lichens, which as a rule consist of two kinds of thallus: horizontal primary thallus (squamulose or seldom crustose, sometimes disappearing) and vertical secondary thallus (podetia). *C. coniocraea* forms a green to gray-green, narrow, often bent podetia, sometimes with very small narrow cups. *C. ramulosa* have green-brown podetia with many squamules on the lower parts, small cups present on the tips and large apothecia. Considering that these two species occurs in abundance, it is important to recognize their bioactive properties. Since the data about their bioactivity is limited and very scarce, in this research we examined the phytochemical analysis of *C. coniocraea* and *C. ramulosa* acetone extracts with their antimicrobial, antioxidative and anticancer potentials.

2. Materials and Methods

2.1. Collection of lichen samples and preparation of the extract

Lichen samples of *Cladonia coniocraea* (Flörke) Spreng., and *Cladonia ramulosa* (With.) J.R. Laundon were collected from Serbia, during May 2014. The identification was performed using relevant monographs [8,9]. The voucher specimen of the lichens (Voucher No. 84 and 88) was deposited in herbarium at the Department of Biology and Ecology, Faculty of Science, University of Kragujevac.

Collected lichen species was dried on air, at room temperature for two weeks, and then ground. Dry ground lichens thalli (100 g) were extracted with acetone in a Soxhlet extractor. The extract was filtered and then concentrated by a rotary evaporator under reduced pressure. The samples were stored at -18°C until analysis. For further experimental assays, 5% dimethyl sulphoxide (DMSO) was used for dissolving extract.

2.2. High-performance liquid chromatography (HPLC) analysis

The extracts of *C. coniocraea* and *C. ramulosa* and standards (fumarprotocetraric acid, norstictic acid and sekikaic acid) were dissolved in 1 ml of methanol and analyzed on an Agilent 1200 System HPLC (Agilent Technologies) instrument with C18 column (C18; 25 cm × 4.6 mm, 10 m). UV spectrophotometric detector with methanol–water–phosphoric acid (75:25:0.9, v/v/v) solvent was used. Methanol was of HPLC grade and was purchased from Merck (Darmstadt, Germany). Deionized water used throughout the experiments was generated by a Milli-Q academic water purification system (Milford, MA, USA). Phosphoric acid was analytical-grade reagent. The sample injection volume was 10 µl with a flow rate of 1.0 ml/min. Identification of the aromatic compounds was conducted by comparison of their retention times with those of standards. The standards have been previously isolated in our laboratory from the following sources and identified on the basis of UV, NMR and MS spectra: fumarprotocetraric acid from *Cladonia rangiferina*; norstictic acid from *Toninia candida*; sekikaic acid from *Ramalina fraxinea*.

2.3. Antioxidant activity

Antioxidant activity of *C. coniocraea* and *C. ramulosa* acetone extracts was evaluated applying free radical scavenging and reducing power assays. The free radical scavenging activity was measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH) method, according to Kosanić *et al.* [10]. The Oyaizu method [11] was used to determine the reducing power of the extract. In both antioxidant assays, ascorbic acid was used as a positive control. In addition, content of phenol and flavonoid in extracts was examined using Folin–Ciocalteu reagent with the method of Slinkard &

Singleton [12] and aluminium chloride method [13] and the obtained values were expressed as pyrocatechol equivalents (PE), and as rutin equivalents (RE), respectively.

2.4. Antimicrobial activity

Antimicrobial activity of investigated lichens was tested by determining the minimal inhibitory concentration (MIC) by the broth microdilution method with using 96-well micro-titer plates [14] against five species of bacteria: *Bacillus cereus* (ATCC 11778), *B. subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Proteus mirabilis* (ATCC 12453) and 10 species of fungi: *Aspergillus flavus* (ATCC 9170), *A. niger* (ATCC 16888), *Candida albicans* (ATCC 10231), *Mucor mucedo* (ATCC 20094), *Trichoderma viride* (ATCC 13233), *Cladosporium cladosporioides* (ATCC 11275), *Fusarium oxysporum* (ATCC 62506), *Alternaria alternata* (ATCC 11680), *Penicillium expansum* (ATCC 20466), *P. chrysogenum* (ATCC 10106). All the microorganisms were from the American Type Culture Collection (ATCC). Bacterial inoculi were obtained from bacterial cultures incubated at 37°C for 24 h on Müller-Hinton agar substrate. The turbidity of suspension was adjusted by being compared with 0.5 McFarland's standard (bio- Mérieux, Marcy 10Etoile, France) to approximately 10^8 CFU/mL. Fungal suspensions were obtained from cultures (3-to 7-day-old) growing at 27°C on potato dextrose (PD) agar substrate. Spores were rinsed with sterile distilled water, their turbidity was measured spectrophotometrically (Jenway, Bibby Scientific Limited, Stone, UK) at 530 nm and then additionally diluted to about 10^6 CFU/mL. Standard antibiotics (streptomycin for bacteria and ketoconazole for fungi) were used as positive controls, while DMSO was used as a negative control.

2.5. Cytotoxic activity

Human epithelial carcinoma Hela cells, human lung carcinoma A549 cells and human colon carcinoma LS174 cells were obtained from American Type Culture Collection (Manassas, VA, USA). All cancer cell lines were cultured as a monolayer in the RPMI 1640 nutrient medium, with 10% FBS (inactivated at 56ºC), 3 mM of L-glutamine, and antibiotics, at 37ºC in humidified air atmosphere with 5% CO2.

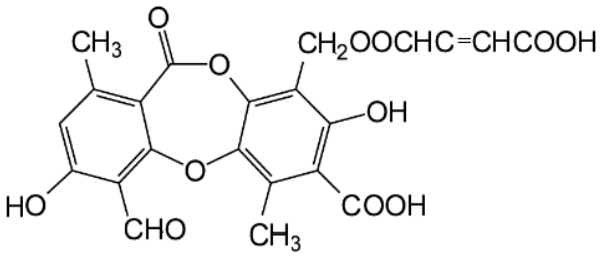
The effect on cancer cell survival was determined 72 hours after the addition of the extract, by the MTT test (microculture tetrazolium test) according to Mosmann [15].

2.6. Statistical analysis

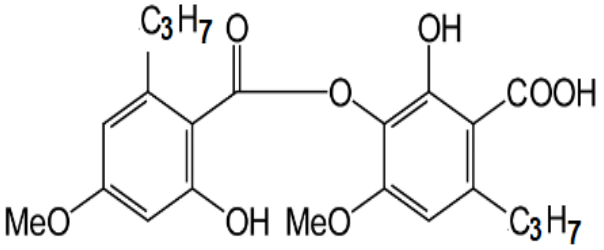
All results were presented as means ± standard deviations (mean ± SD) of three measurements. Data were analyzed using Microsoft Excel and SPSS software package. Student's t-test was used to determine the statistical significance of antioxidant activity.

3. Results and Discussion

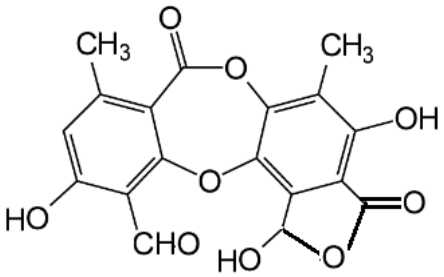
Continuing our research on *Cladonia* lichens [16,17], in this paper we presented the results of phytochemical analyses and biological activity of two lichens, *Cladonia coniocraea* and *Cladonia ramulosa*. HPLC-UV analysis was used for identification the major aromatic metabolites in the acetone extracts of the lichens *Cladonia coniocraea* and *Cladonia ramulosa* growing in Serbia. The structures of the detected compounds are shown in Fig. 1.



Fumarprotocetraric acid



Sekikaic acid



Norstictic acid

Fig. 1. Chemicals structures of the identified compounds.

The chromatograms of a mixture of three reference compounds (fumarprotocetraric acid, norstictic acid and sekikaic acid) and the lichen acetone extracts eluted by HPLC are presented in Figs. 2 and 3.

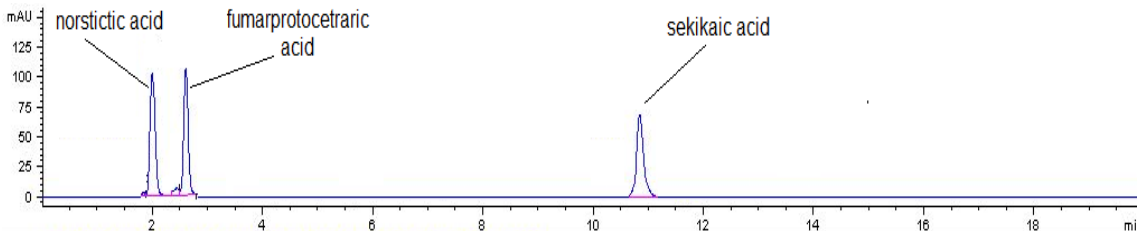


Fig. 2. HPLC chromatograms acquired at 254 nm of the standards used for identification of the aromatic compounds present in Cladonia coniocraea and Cladonia ramulosa.

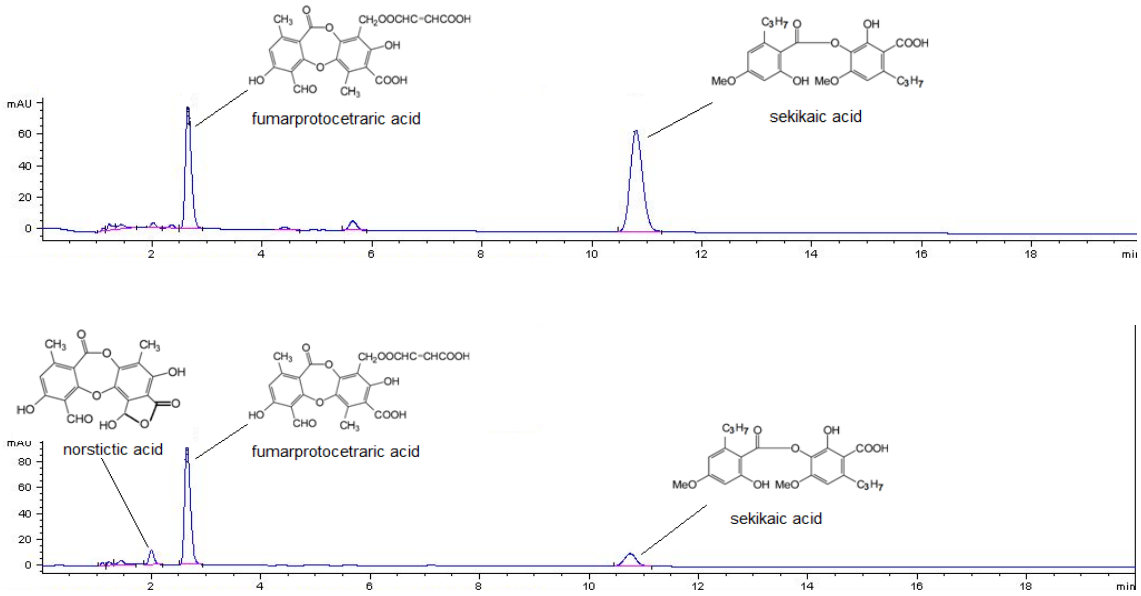


Fig. 3. HPLC chromatograms acquired at 254 nm of the acetone extracts of Cladonia coniocraea and Cladonia ramulosa.

Identification of the aromatic lichen metabolites was achieved by comparison of their retention times (t_R) and UV spectra (200 - 400 nm) from HPLC-UV with the standard substances previously isolated from lichens in our laboratory (Table 1).

Table 1. The examined lichen substances: retention time, absorbance maxima and relative abundance.

Compound	**Retention time (t_R±SD)*(min)**	**Absorbance maxima (nm)**	*Cladonia coniocraea*	*Cladonia ramulosa*
Fumarprotocetraric acid	2.65 ± 0.10	212, 240, 318	37.13	80.51
Sekikaic acid	10.80 ± 0.20	217, 264,303	57.92	12.82
Norstictic acid	1.95 ± 0.10	212, 239, 310	/	11.69

**Values are the means of three determinations ± SD*

Two dominant peaks can be observed in the *C. coniocraea* chromatogram. The first dominant peak (37.13%) in the chromatogram (t_R = 2.65 ± 0.10 min) originates from fumarprotocetraric acid (4-[[(E)-3-carboxyprop-2-enoyl]oxymethyl]-10-formyl-3,9-dihydroxy-1,7-dimethyl-6-oxobenzo[b][1,4]benzodioxepine-2-carboxylic acid). The UV spectrum of fumarprotocetraric acid has 3 absorption maxima (212, 240, 318 nm). This acid belongs to the depsidones and can be found in other *Cladonia* species [18]. Previous research has shown that this acid has a wide range of biological activity, including imunostimulatory, antioxidant, antimicrobial and anticarcinogenic activities [19,20]. The second dominant peak (57.92 %) in the chromatogram (t_R = 10.80 ± 0.10 min) originates from sekikaic acid (2-hydroxy-3-(2-hydroxy-4-methoxy-6-propylbenzoyl)oxy-4-methoxy-6-propyl benzoic acid. This acid is depside characterized by two propyl groups bonded to the aromatic rings and is less commonly represented in lichens than fumarprotocetraric acid. The UV spectrum of sekikaic acid has 3 absorption maxima (217, 264 and 303 nm). This acid has been found in other lichen genera such as *Ramalina* and *Cladonia* and showed antioxidant activity [21]. Dies & Urban [22] reported the antibacterial and antitumor properties of the mixture of sekikaic and its chlorine derivative, 5-chlorosekikaic acid. The HPLC chromatogram of the lichen *Cladonia ramulosa* shows the dominant peak of fumarprotocetraric acid (80.51 %) and the second, less intense peak of sekikaic acid (12.82 %). *C. ramulosa* extract also contains norstictic acid (t_R = 1.95 ± 0.20 min) not found in the chromatogram of *C. coniocraea* extract. Norstictic acid (absorbance maxima: 212, 239, 310 nm) has very small peak

(11.69%) and present satellite substances in the chromatogram. This is the first report of norstictic acid in *C. ramulosa* and sekikaic acid in *C. coniocraea*.

Antioxidant activities of tested extracts are shown in Table 2. The results of this assays present that tested lichens showed weak DPPH radical scavenging activity (IC_{50} values for both extracts were >1000). As shown in Table 2, the reducing power was concentration-dependent. Measured values of absorbance varied from 0.0118 to 0.1675. Among the tested lichen species, *C. coniocraea* give higher reducing power. In addition, the amount of total phenolic compounds was determined as the PE using an equation obtained from a standard pyrocatechol graph ($y = 0.0057x - 0.1646$, $R^2 = 0.9934$), while the amount of total flavonoid compounds was determined as the RE using an equation obtained from a standard rutin graph ($y = 0.0144x + 0.0556$, $R^2 = 0.9992$). Result show that total phenolics contents in *C. coniocraea* and *C. ramulosa* acetone extracts were 33.53 and 33.19 μg PE/mg of extract, while total flavonoids contents were 24.54 and 20.86 μg RE/mg of extract.

Table 2. DPPH radical scavenging activity, reducing power and phenolics and flavonoids content of examined lichens.

		Cladonia coniocraea	*Cladonia ramulosa*	Ascorbic acid
DPPH radical scavenging IC_{50} (μg/mL)		>1000	>1000	9.42 ± 0.07
Reducing power Absorbance (700 nm)	1000 μg/mL	0.1675 ± 0.04	0.0815 ± 0.03	2.1158 ± 0.03
	500 μg/mL	0.1521 ± 0.02	0.0683 ± 0.02	1.6509 ± 0.02
	250 μg/mL	0.1118 ± 0.02	0.0672 ± 0.03	0.9609 ± 0.01
	125 μg/mL	0.0734 ± 0.03	0.0118 ± 0.02	0.4155 ± 0.01
Phenolics content (μg PE/mg of extract)		33.53 ± 0.98	33.19 ± 0.86	
Flavonoids content (μg RE/mg of extract)		24.54 ± 0.89	20.86 ± 0.93	

**Values are the means of three determinations ± SD*

Data concerning the antioxidant capacity of the *C. coniocraea* and *C. ramulosa* is very scarce, but a great number of reports concerning the other *Cladonia* species have appeared in the literature. Prior this research, antioxidant effects of *C. fimbriata, C. furcata, C. subulata, C. foliacea* and *C. rangiferina* were evaluated by free radical scavenging and reducing power [17]. As a result of the study, all tested extracts showed relatively strong antioxidant activity, but *C. furcata* had the largest effect for both assays. In another study, the same authors compared antioxidant activities between three *Cladonia* species, among which *C. pyxidata* showed the highest activity [20]. Our tested lichens contain fumarprotocetraric acid, norstictic acid and sekikaic acid for which have been shown to exhibit powerful antioxidant activity [20,23,24]. However, in this study, extracts showed less antioxidant activity. This suggests that the components containing the extracts may interact antagonistically leading to a decrease in the overall activity of the extracts. Also, examined lichen extracts have a high content of phenols and flavonoids which means that antioxidant activity of extracts may not be necessarily correlated with the content of polyphenolics, but it may also depend on other, non-phenol components [25].

In our experiments, it was recorded relatively strong antimicrobial activity of tested lichen extracts (Table 3). Both extracts inhibited all tested bacteria and fungi. The MIC values varied from 0.156 to 10 mg/mL for bacteria and from 0.625 to 20 mg/mL for fungi. The lowest measured MIC value (0.156 mg/mL) was against *P. mirabilis* (for both tested species) and *B. subtilis* (in regard to *C. coniocraea*). The antimicrobial activity of the lichens was compared to standard antibiotics, streptomycin (for bacteria) and ketoconazole (for fungi). The results showed that standard antibiotics were more active than the tested samples. DMSO, the negative control, had no effect on the growth of microorganisms.

Table 3. Minimum inhibitory concentration (MIC) of acetone extracts of examined lichen species.

Lichen species Test microbes	*Cladonia coniocraea*	*Cladonia ramulosa*	Streptomycin	Ketoconazole
S. aureus	0.625	0.625	0.031	/
B. subtilis	0.156	0.625	0.016	/
B. cereus	5	10	0.016	/
E. coli	5	5	0.062	/
P. mirabilis	0.156	0.156	0.062	/
M. mucedo	20	20	/	0.156
T. viride	20	20	/	0.078
C. cladosporioides	10	10	/	0.039
F. oxysporum	20	20	/	0.078
A. alternata	20	20	/	0.078
A. flavus	20	20	/	0.312
A. niger	20	20	/	0.078
C. albicans	5	5	/	0.039
P. expansum	5	0.625	/	0.156
P. chrysogenum	10	20	/	0.078

Values given as milligram per milliliter.

In present research, the results indicate that studied extracts induced relatively strong antibacterial and antifungal activity. Since there is no data for antimicrobial potential of *C. coniocraea* and *C. ramulosa* lichens, we compared our data to antimicrobial effects of other *Cladonia* species. For example, Mitrović *et al*. [26] studied antibacterial and antifungal activity of methanol extracts of five lichen species including *C. foliacea* and they found that *C. foliacea* manifested the strongest antimicrobial activity among tested lichens. Similarly, five lichen species of *Cladonia* genus were examined by Kosanić *et al*. [17] for their antimicrobial potential. Among tested lichens, *C. fimbriata* showed the strongest antibacterial activity while *C. subulata* showed the strongest antifungal activity. Relatively strong antimicrobial effect against numerous bacteria and fungi was also found for lichen components (fumarprotocetraric acid, norstictic acid and sekikaic acid) that constitute tested *Cladonia* species [23,27,28], so that these components are probably responsible for the detected antimicrobial activity in *C. coniocraea* and *C. ramulosa* acetone extracts. In this experiment, antimicrobial effect was observed against both bacteria and fungi, with fungi being the more resistant one. Fungi were assumed to be more resistant to the tested extract than bacteria due to more complex structure of the cell wall. This observation is in accordance with many other studies focused on antimicrobial activity [17,29], which have demonstrated that the structure and the permeability of the cell wall are main reasons for different sensitivities in bacteria and fungi.

The data obtained for cytotoxic effect of *C. coniocraea* and *C. ramulosa* acetone extracts on Hela, A549 and LS174 cells are shown in Table 4. The results of this study show that *C. coniocraea* acetone extract only reduce the cell viability of Hela cells with IC_{50} value of 185.59 mg/mL, while *C. ramulosa* acetone extract should be considered as non-toxic to all three investigated cells.

Table 4. Growth inhibitory effects of acetone extracts of examined lichen species on Hela, A549 and LS174 cell lines.

Cell lines	Hela	A549	LS174
Lichen species	IC_{50} (μg/mL)		
Cladonia coniocraea	>200	>200	>200
Cladonia ramulosa	185.59 ± 1.27	>200	>200
cis-DDP	0.83 ± 0.19	3.56 ± 0.23	2.58 ± 0.16

IC_{50} values are expressed as the mean ± SD determined from the results of MTT assay in three independent experiments

In this study, *C. coniocraea* and *C. ramulosa* have been tested in terms of cytotoxic activity for the first time. Prior our research, cytotoxic effect of some other *Cladonia* lichens was explored. Bessadottir *et al*. [30] emphasized that *C. arbuscula* induced the formation of autophagosomes in human cancer cells, but had minimal effects on normal human fibroblasts. Brisdelli *et al*. [31] reported about anticarcinogenic activity of *C. lepidophora* which is manifested antiproliferative effect against MCF-7, HeLa and HCT-116 cells. Kosanić *et al*. [17] reported significant growth inhibitory effects for *C. fimbriata, C. furcata, C. subulata, C. foliacea* and *C. rangiferina* lichens toward Hela, A549 and LS174 cell lines with IC_{50} values ranging from 11.69 to 140.13 μg/mL. In the available literature data [20,32,33] was confirmed that compounds contained in *C. coniocraea* and *C. ramulosa* also can be active against some cancer cells including human melanoma Fem-x, human colon carcinoma LS174 cell line, human breast cancer cell lines (MDA-MB-231, MDA-MB-468, MCF-7, T-47D, BT-474, SK-BR-3), etc.

4. Conclusion

It can be seen that there is not much literature data on the biological activity of the *C. coniocraea* and *C. ramulosa* lichens, but a great number of reports concerning the antimicrobial, antioxidant and cytotoxic screening of some other lichens have appeared in the literature. Compared with other results, the results of this research suggest that the tested samples showed a moderate antioxidant and cytotoxic activities and relatively strong antimicrobial effect. Further, this is the first report of norstictic acid in *C. ramulosa* and sekikaic acid in *C. coniocraea*. These results can have a great medical, pharmaceutical and chemotaxonomic importance. Further investigations should be focused to isolation, characterization and testing of individual bioactive compounds from lichens, with stronger biological activity.

Acknowledgments

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