Chemical composition and biological activities of *Umbilicaria crustulosa* and *Umbilicaria cylindrica* extracts

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ABSTRACT

The main compounds of acetone, methanol, ether and ethyl acetate extracts of *Umbilicaria crustulosa* are tridepsides, gyrophoric acid and crustinic acid, and didepside, lecanoric acid. Chemical composition of *Umbilicaria cylindrica* extracts depends on the solvent used for the extraction and the major components are depsidones, salazinic acid and norstictic acid or depsides, gyrophoric acid and atranorin. Extracts of *U. crustulosa* and *U. cylindrica* have shown antibacterial, antioxidant, cytotoxic, antiproliferative and anticlastogenic activity. Acetone extracts of *U. crustulosa* and *U. cylindrica* are promising candidates for *in vivo* experiments considering antioxidant and anticlastogenic activity.

Keywords: Umbilicaria crustulosa, Umbilicaria cylindrica, antioxidant activity, antimicrobial activity, cytotoxic activity, micronucleus test, cholinesterase inhibition

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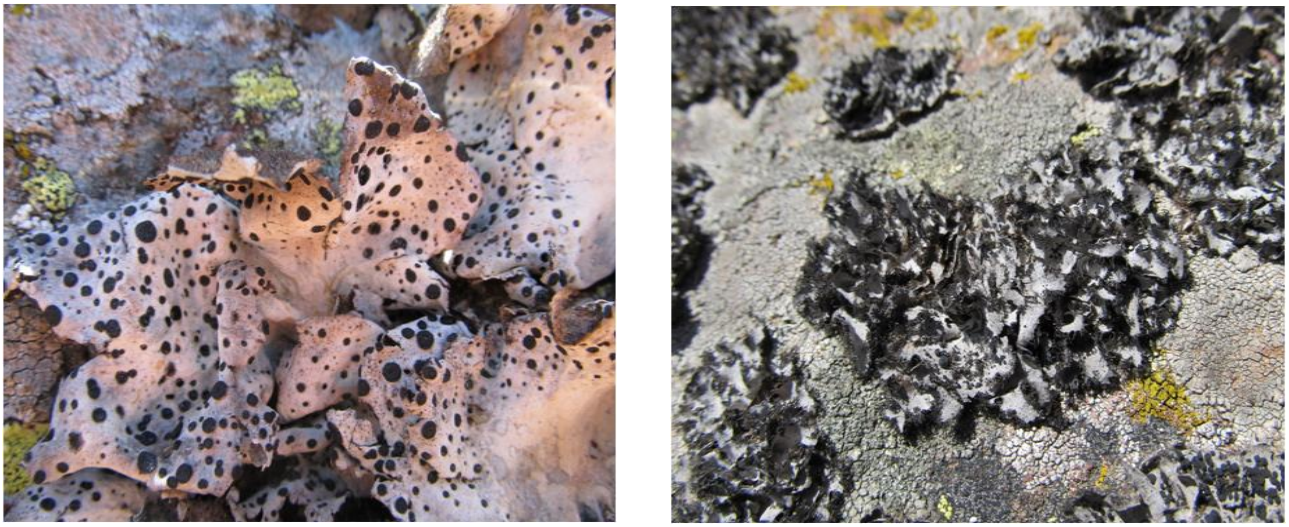
Introduction

Lichen represents symbiotic organisms consisted of algae (photobionts) which are responsible for photosynthesis and fungi (mycobionts) which produce specific secondary metabolites. Lichens are potential natural source of bioactive compounds which are used in folk medicine during centuries (Molnar and Farakas, 2010). *Umbilicaria* is a genus of lichenised fungi in the phylum Ascomycota within the family Umbilicariaceae. The lichen species belonging to genus *Umbilicaria* are commonly used in folk medicine as purgative and as a food during famine. The name of the genus is derived from Latin *umbilicaris*-umbilical and is also known as „rock tripe". *Umbilicaria crustulosa* (Figure 1) represents a foliose type of lichens with a common name crusty navel lichen and synonyms: *Gyrophora depressa* (Ach.) Schaer., *Omphalodiscus crustulosus* (Ach.) Schol., *Gyrophora crustulosa* (Ach.). Upper surface is flat and smooth, gray and lower surface is brown. *Umbilicaria cylindrica* L. Delise ex Duby (Figure 2) also belongs to a foliose type of lichens within light gray upper surface with black margins. These species are growing on siliceous rocks in Northern hemisphere mostly in Alpine vegetation zone (Frey, 1997).



(a) **(b)**

Figure 1. (a) *Umbilicaria crustulosa* (Ach.) Frey and (b) *Umbilicaria cylindrica* L. Delise ex Duby (photo by: Ivana Zlatanović, locality: Babin zub, Stara planina)

The majority of morphologically defined lichen species have constant chemical composition described in the literature. Lichens are characterized by the presence of cortical metabolite and one or more medullary metabolites (Culberson, 1969). Lichens metabolites are mostly crystalline compounds deposited on the surface of hyphae and can be isolated from the lichen matrix using different solvents for extraction (Huneck and Yoshimura, 1996).

Many lichen secondary metabolites exhibited antioxidant, antimicrobial, cytotoxic and antiviral properties and could be used as active components of drugs (Molnar and Farakas, 2010; Stojanović et al., 2012). Having in mind the need to find new natural bioactive components the present study reviews bioactivities of *U. crustulosa* and *U. cylindrica* lichens extracts.

Chemical composition

Major secondary metabolites of lichens are depsides which are consisted of two, three or four hydroxybenzoic acid residues linked through ester bond and depsidones with additional ether bond between aromatic rings (Huneck and Yoshimura, 1996). Didepside, lecanoric acid and tridepside, gyrophoric acid, are formed by intermolecular coupling of two or three orsellinic acid units through ester bond in *para*-position (Posner et al., 1992). Meta-depsides appear less frequently similarly to crustinic acid, tridepside metabolite of *U. crustulosa*.

Serina and Arroyo (1996) distinguish two chemotypes of *U. crustulosa*: 1) gyrophoric acid type, with the gyrophoric acid as the main component followed by lecanoric acid and crustinic acid; 2) crustinic acid type, with crustinic acid as the main component followed by lecanoric acid and gyrophoric acid. The samples which have been examined by Zlatanović et al. (2017a) also belong to gyrophoric acid chemotype. The following constituents were also identified in these samples: methyl orsellinate, methyl lecanorate and atranorin (Zlatanović et al., 2016; Zlatanović et al., 2017a)

As well as for *U. crustulosa*, two chemical races of *U. cylindrica* were described in the literature, one without lichen substances and one characterized by different lichens substances (Huneck et al., 1991; Posner et al., 1992). Namely, Posner et al. (1992) were reported the presence of depsidones, norstictic acid and connorstictic acid while recent research of *U. cylindrica* samples from Serbia (Manojlović et al., 2012) were revealed salazinic acid, norstictic acid, methyl β-orcinol carboxylate, ethyl haematommate, atranorin and usnic acid as compounds of chloroform and methanol extracts. Zlatanović et al. (2017b) were detected norstictic acid as a main component followed by salazinic acid and atraric acid in ether and ethyl acetate extracts of *U. cylindrica* while atranorin was predominant component of dichloromethane extract. However, main compound of acetone extract of *U. cylindrica* was gyrophoric acid which was represented in smaller amounts in the other examined extracts (Zlatanović, 2019). The structural formulae of the constituents of *U. crustulosa* and *U. cylindrica* extracts are given in the Figure 2.

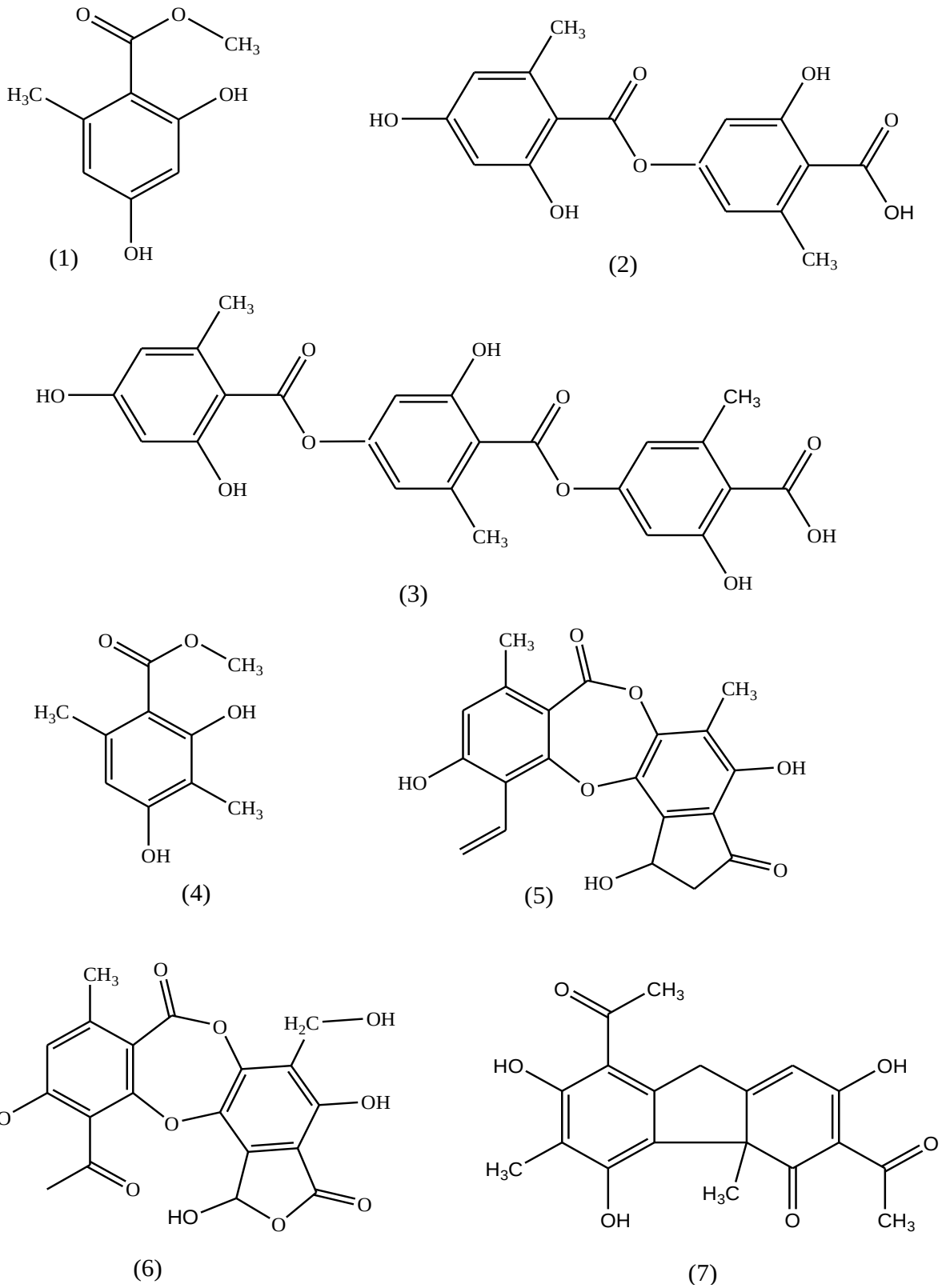


Figure 2. Structural formulae of (1) methyl orsellinate, (2) lecanoric acid, (3) gyrophoric acid, (4) atraric acid, (5) norstictic acid, (6) salazinic acid, (7) usnic acid

Biological activities

Review of published biological activity data is given in the Table 1.

Table 1. Biological activities of acetone and methanol extracts of *U. crustulosa* and *U. cylindrica*

***U. crustulosa* acetone extract**	***U. crustulosa* methanol extract**	***U. cylindrica* acetone extract**	***U. cylindrica* methanol extract**
Antioxidant (Kosanić et al., 2014; Zlatanović et al., 2017c) Antibacterial (Ranković et al., 2009) Anticlastogenic (Zlatanović, 2019)	Antibacterial (Ranković et al., 2009; Kosanić et al., 2014) Cytotoxic (Kosanić et al., 2014)	Antioxidant, Antibacterial, Anticlastogenic (Zlatanović, 2019)	Antioxidant (Manojlović et al., 2012) Cytotoxic (Kosanić et al., 2014) Antimicrobial (Ranković et al., 2009; Buçukoglu et al., 2013)

Total phenolic content and antioxidant activity

Reactive oxygen species could cause oxidative stress in lichen thallus. However, secondary metabolites of lichens provide protection due to high ability to scavenge free radicals (Molnar and Farakas, 2010). Antioxidant activity of *U. crustulosa* and *U. cylindrica* extracts has been the focus of numerous studies (Buçukoglu et al., 2013; Kosanić et al., 2014; Manojlović et al., 2012; Zlatanović et al., 2017a). Namely, Manojlović et al. (2012) were found that methanol and chloroform extracts of *U. cylindrica* were free radical scavengers (IC_{50} (μg mL^{-1}) 34.45 ± 1.15 and 31.34 ± 1.10, respectively). Also, Zlatanović et al. (2017a) found that DPPH scavenging activity of *U. crustulosa* and *U. cylindrica* acetone extracts was high and amounted 88.7% and 77%, respectively. In the same experiment, the assessment of ABTS scavenging activity showed that acetone extracts reduce the concentration of ABTS radicals for 96.2% and 78.4%, respectively (Zlatanović et al., 2017a; Zlatanović, 2019). Buçukoglu et al. (2013) found that umbilicaric acid exhibited stronger activity than gyrophoric and lecanoric acid at concentrations of 5 mg mL^{-1} (68.14%, 50.96% and 32.48%, respectively). Also, DPPH scavenging activity of mentioned lichen acids was higher than DPPH values of *U. cylindrica* methanol extract

(21.07%) (Buçukoglu et al., 2013). Kosanić et al. (2014) reported that methanol extracts of *U. crustulosa* have shown good scavenging activity on DPPH radical (79.85%).

Total phenolic content of acetone extracts of *U. crustulosa* and *U. cylindrica* were expressed as gallic acid equivalents (GAE) and amounted 350.4188±14.587 and 267.9710 ±8.011 μg GAE mg^{-1} of dry extract weight (Zlatanović et al., 2017a; Zlatanović, 2019). Kosanić et al. (2014) expressed the amount of total phenolic content of *U. crustulosa* methanol extract as the pyrocatechol equivalents (PE), and amounted 55.03 ± 1.096 μg PE mg^{-1} of dry extract weight.

The results of the total reducing power ability (TRP) for *U. crustulosa* and *U. cylindrica* acetone extracts were 0.6197±0.0166 and 0.6515±0.1846 μg ascorbic acid equivalents per mg dry extract weight (Zlatanović et al., 2017a; Zlatanović, 2019). Kosanić et al. (2014) have reported that methanol extract of *U. crustulosa* shows the weakest reducing power among the tested extracts. Measured values for absorbance of methanol extract were 0.066.

Obtained result for cupric reducing capacity (CUPRAC) of *U. crustulosa* acetone extract was 19.7641±0.01 μg TE mg^{-1} of dry extract. The higher CUPRAC value is observed for *U. cylindrica* acetone extract and amounted 21.9521 ± 0.23 μg TE mg^{-1} of dry extract (Zlatanović et al., 2017a; Zlatanović, 2019).

Antioxidant activity of *U. crustulosa* and *U. cylindrica* extracts assessed by the different systems could be attributed to their high total phenolic content. Acetone extract of *U. cylindrica* consisted of monoaromatic compound (atraric acid), depsidones (salazinic acid and norstictic acid) and depsides (gyrophoric acid and atranorin). On the other hand, acetone extract of *U. crustulosa* is characterised by the presence of depsides (lecanoric acid, crustinic acid, gyrophoric acid and atranorin) and monoaromatic compound methyl orsellinate (Zlatanović et al., 2017a, Zlatanović, 2019). Hidalgo et al. (1994) have reported stronger antioxidant activity of depsidones than depsides. This fact could be the reason why acetone extract of *U. cylindrica* was found to be more effective antioxidant in free radical scavenging as well as in reducing power assays. Salazinic acid possesses four hydroxyl groups (two phenolic groups) in the molecule while norstictic acid possesses two phenolic groups, and they, therefore, might contribute to the antioxidant activity of the tested extracts. Some authors believe that the higher efficiency of the depsidones was related to a larger incorporation into lipidic microdomains (Hidalgo et al., 1994).

Antibacterial activity

One of the possible roles of lichens secondary metabolites is to protect lichen from the pathogens in nature (Lawrey, 1989). It has been shown that lichens and their metabolites are quite effective against a

wide variety of microorganisms in many experiments conducted *in vitro* (Buçukoglu et al., 2013; Manojlović et al., 2012; Ranković et al., 2009; Turk et al., 2006). According to Manojlović et al. (2012) methanol and ethyl acetate extracts of *U. cylindrica* manifested strong antibacterial effect against *Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli, Proteus vulgaris, Proteus mirabilis* and *Bacillus subtilis.* Also, the extracts were efficient against the yeast *Candida albicans* and *Aspergillus niger*. The MIC values were within the concentration range from 15.62 μg mL^{-1} to 62.50 μg mL^{-1}. Ranković et al. (2009) have reported that methanol extract of *U. cylindrica* did not show activity against the majority of tested microorganism except *S. aureus* and *C. albicans* whereas acetone extract showed activity only towards *C. albicans.* However, methanol extract of *U. cylindrica* tested by Bacukoglu et al. (2013) at concentration of 5% and 10% inhibited only *P. aeruginosa* among the five Gram-negative bacteria and *B. cereus,* among the three Gram-positive bacteria. In the same experiment, gyrophoric acid at concentration of 5% inhibited all tested Gram-negative bacteria, except *E. coli* and among Gram-positive bacteria only *B. subtilis* was resistant. Also, methanol extract of *U. cylindrica* and gyrophoric acid did not demonstrated activity towards fungi (Buçukoglu et al., 2013). On the other hand, Zlatanović (2019) was found that acetone extract of *U. cylindrica* possesses moderate activity against Gram-positive bacteria, such as *S. aureus* and *Bacillus subtilis subsp. spizizenii* and no activity on tested Gram-negative bacteria.

Manojlović et al. (2012) were reported that methanol and chloroform extracts of *U. cylindrica* consisted of salazinic acid, norstictic acid, methyl β-orcinol carboxylate, ethyl haematommate, atranorin and usnic acid while acetone extract of *U. cylindrica* analyzed by Zlatanović (2019) consisted mainly of gyrophoric acid (83.5%) followed by small amount of methyl β-orcinol carboxylate (2.8%), norstictic acid (1.7%), salazinic acid (2%), atranorin (5%) and usnic acid (0.2%). The significant antimicrobial activity of *U. cylindrica* extracts is probably due to the presence of depsidones.

Methanol extract of *U. crustulosa* was found to have moderate antimicrobial activity. Namely, Kosanić et al. (2014) reported no activity toward *E. coli*, *Botrytis cinerea* and *C. albicans*. The MIC values for *U. crustulosa* methanol extracts were 6.25 mg mL^{-1} against four species of bacteria and 12.5 mg mL^{-1} against three species of fungi. Ranković et al. (2009) examined the antimicrobial properties of acetone, methanol and aqueous extracts of *U. crustulosa*. Acetone and methanol extracts of *U. crustulosa* were inactive against *E. coli, B. cinerea* and *C. albicans* although both extracts were active against *Bacillus mycoides, Bacillus subtilis, Enterobacter cloacae, Klebsiella pneumoniae, Staphylococcus aureus, Aspergillus flavus, Aspergillus fumigatus, Fusarium oxysporum, Mucor mucedo, Paecilomyces variotii, Penicillium purpurescens, Penicillium verrucosum***,** *Saccharomyces cerevisiae, Trichoderma harzianum* (Ranković et al., 2009). However, acetone extract of *U. crustulosa* did not exhibit activity against tested Gram-positive and Gram-negative bacteria at concentration of 1 mg per disk (Zlatanović et

al., 2017a). Gyrophoric acid isolated from several species of the genus *Umbilicaria* did not show activity towards *E. coli*, *K. pneumoniae*, *P. aeruginosa* i *S. typhimurium* (Candan et al., 2006). Since gyrophoric acid showed no activity against tested bacteria and this compound represents 68.5% of the analyzed acetone extract of *U. crustulosa* these results can be explained due to its inactivity (Zlatanović et al., 2017a).

Citotoxic activity

Numerous studies have shown that lichen metabolites possess cytotoxic activity (Kosanić et al., 2014; Kumar and Müller, 1999b). Among the tested lichen extracts methanol extract of *U. crustulosa* manifested the weakest cytotoxic activity against colon cancer adenocarcinoma cell line HTC (Kosanić et al., 2014). Gyrophoric acid significantly inhibited the light-dependent synthesis of ATP and uncoupled electron transfer on the reducing side of photosystem II in freshly lysed illuminated spinach chloroplasts (Rojas et al., 2000), but it was inactive in the inhibition of leukotriene B4 biosynthesis (Kumar and Müller, 1999a). Also, it was found that gyrophoric acid possesses antiproliferative and cytotoxic activity and inhibited the growth of the human keratinocyte cell line HaCaT (Kumar and Müller, 1999b). Some depsidones and depsides (pannarin, 10-chloropannarin and sphaerophorin) were reported to have a higher cytotoxic effect than colchicine (Correché et al., 2002). Among fifteen lichen compounds, depsidones salazinic acid, stictic acid and psoromic acid were the most apoptotic active derivatives on primary cultures of rat hepatocytes. One of the most explored secondary metabolites of lichens is usnic acid which has manifested antitumor activity against Lewis Lung carcinoma (Kupchan and Kopperman, 1975) and antiproliferative effect against human HaCaT keratinocytes (Bezivin et al., 2004; Kumar and Müller, 1999b).

Anticlastogenic activity

The analysis of micronuclei (MN) in cultured lymphocytes is applied as a method to monitor human exposure to genotoxic agents (Fenech and Morley, 1993). The cytokinesis block micronuclei assay (CBMN) is used to test the impact of acetone extracts of *U. crustulosa* and *U. cylindrica* (at concentrations of 1.0, 2.0 and 3.0 μg mL^{-1}) for *in vitro* protective effect on chromosome aberrations in peripheral human lymphocytes. The cell cultures treated with amifostine WR-2721 (positive control) at concentration of 1 μg mL^{-1} gave a significant ($P < 0.01$) decrease in the MN frequency of 11.4% comparing to the control cell cultures. The *U. crustulosa* extract at concentration of 2 μg mL^{-1} gave a

decrease in the MN frequency of 16.3%, which was higher than amifostine, while at concentration of 1 μg mL^{-1} and 3 μg mL^{-1} the effect was lower than amifostine (Zlatanović et al., 2017a). Acetone extract of *U. cylindrica* at concentration of 2 μg mL^{-1} exhibited the most prominent effect of decreasing the MN frequency (11%) while at concentration of 1 and 3 μg mL^{-1} decreasing of the MN frequency was lower (5.3% and 1.6%, respectively) (Zlatanović, 2019). Secondary metabolites isolated from lichens such as atranorin, evernic acid and usnic acid showed significant anticlastogenic activity, reducing the frequency of MN to the same or greater extent than amifostine (11.1, 32.9 and 48.9%, respectively) (Stojanović et al., 2014). All tested extracts and metabolites showed the highest activity at concentration of 2 μg mL^{-1}.

Cholinesterase activity

Synthetic cholinesterase inhibitors represent the treatment of choice for Alzheimer's disease but finding of natural inhibitors of cholinesterase is the subject of many studies (Giacobini, 2004). Results obtained from the screening of the interaction of extracts with cholinesterase from pooled human serum, have shown that acetone extracts of *U. crustulosa* and *U. cylindrica* possess weak activation effect on cholinesterase activity (1.6% and 4.4%, respectively). In the same experiment gyrophoric acid isolated from *U. crustulosa* acetone extract has manifested weak inhibition effect on cholinesterase activity, -18.4% (Zlatanović, 2019). In conducted experiment neostigmine bromide (commercial cholinesterase inhibitor) inhibited cholinesterase to extent of -96.6%.

Conclusion

Two chemotypes of *U. crustulosa* acetone extracts are distinguished: 1) gyrophoric acid type with gyrophoric acid as the main constituent followed by lecanoric acid and crustinic acid; and 2) crustinic acid type with crustinic acid as the main constituent followed by lecanoric acid and gyrophoric acid. Chemical composition of *U. cylindrica* extracts depends on the solvent used for the extraction and the major constituents of methanol, chloroform, ether and ethyl acetate extracts are depsidones, salazinic acid and norstictic acid while main constituents of acetone and dichloromethane extracts are depsides, gyrophoric acid and atranorin, respectively. The present review has shown that *U. crustulosa* and *U. cylindrica* extracts possess antibacterial, antiproliferative, cytotoxic, antioxidant and anticlastogenic activity. Due to significant antioxidant activity and protective effect on human lymphocytes, acetone extracts of *U. crustulosa* and *U. cylindrica* are promising candidates for *in vivo* experiments.

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Conflict-of-Interest Statement

Authors declare no conflict of interest.

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