Uncovering the Anticancer Potential of Lichen Secondary Metabolites

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Abstract: Lichens produce a plethora of primary and secondary metabolites. Secondary metabolites have several biological functions that can be used for human health. Recent studies have described their antioxidant, anti-inflammatory, antimycotic, and antibiotic/antiviral activities. However, attention has mainly been focused on their antiproliferative, cytotoxic, and anticancer effects. Because there are many publications describing the molecular mechanisms leading to the anticancer effects of lichen secondary metabolites, the aim of this review is to summarize results from current research with the main emphasis on atranorin, usnic and gyrophoric acid.

Keywords: Lichens, secondary metabolites, cancer, *in vitro*, *in vivo*, atranorin, usnic acid, gyrophoric acid.

INTRODUCTION

Lichens are symbiotic organisms that consist of mycobionts (fungal partner) and photobionts (photoautotrophic partner) [1]. These unique organisms produce many chemical compounds known as primary and secondary metabolites. While primary metabolites are essential for proper growth of lichens, secondary metabolites do not play a role in development or other functions, but rather mediate plant–environment interactions [2-5]. Secondary metabolites function to protect plants against predators and microbes, against changing abiotic factors, and they mediate communication with other organisms [6, 7]. Lichens contain 800 - 1,050 secondary metabolites belonging to various groups, including aliphatic acids, anthraquinones, phenolic compounds, quinones, pulvinic acid derivatives, steroids, terpenes, and xanthones [8]. Most of them are localized in the cortex part of the lichen body and form specific crystals on the surface of the fungal hyphae [9].

LICHENS SECONDARY METABOLITES

Lichens secondary metabolites represent a large group of chemically different compounds. They comprise many classes of compounds, including amino acid derivatives, sugar alcohols, aliphatic acids, macrolytic lactones, monocyclic aromatic compounds, quinines, chromones, xanthones, dibenzofurans, depsides, depsidones, depsones, terpenoids, steroids, carotenoids, and diphenyl ethers [10].

Mycobiont part of the lichen organism produces secondary metabolites and accumulates them in the cortex, such as atranorin (ATR), parietin, or usnic acid (UA) or in the medullary layer (physodic or physodalic acid) in the form of crystals [9]. The solubility of these compounds in water is very poor, but organic solvents can be used for their isolation [10].

The secondary metabolites can be classified according to their biosynthetic origin and chemical structure:

> the acetyl-malonate pathway is characterized by the main intermediate in the biosynthesis of depsides and depsidones – orsellinic acid. These metabolites are depsides, depsidones, ibenzofurans, chromones, xanthrones, and anthraquinones [9]. Depsides include evernic acid [11], lecanoric acid [12], gyrophoric acid [13] and ATR [14]. Physodic acid is best-known representative of depsidones [15]. One of the most studied lichen secondary metabolite is UA, a dibenzofuran [16].

The mevalonate pathway forms terpenoids derived from naturally produced C5 isoprene units. Classes of lichen substances derived from the mevalonate pathway are terpenes (such as limonene or zeorin), steroids (such as brassicasterol, ergosterol, and lichesterol), and carotenoids (such as beta-carotene, lutein and zeaxanthin) [9].

The shikimate pathway provides a route for aromatic compounds, particularly aromatic amino acids and their derivatives. This pathway is used by microorganisms and plants, but not by animals. The central intermediate in this pathway is shikimic acid [17].

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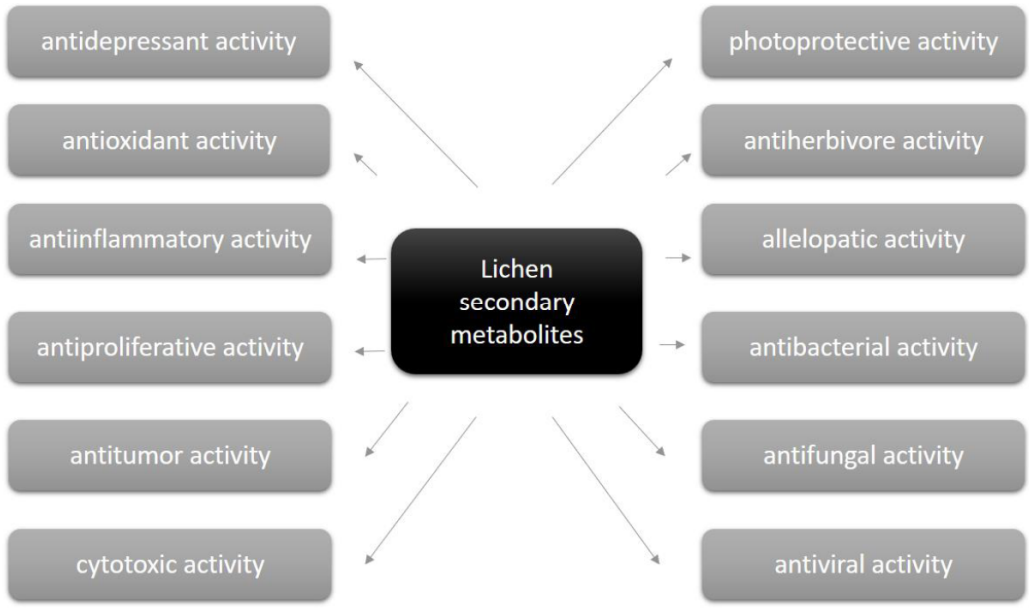

Figure 1: Biological activities of lichen secondary metabolites.

SELECTED BIOLOGICAL PROPERTIES OF LICHEN SECONDARY METABOLITES

Many biological acitivities of Lichens and their metabolites have been described. Some of the most intensively studied activities include antioxidant and anti-inflammatory activities; antibacterial, antiviral, and antifungal effects, cytotoxic and anticancer features, antiherbivore, photoprotective, and allelopathic activities (Figure **1**) [10].

In 1980, the antimicrobial activities of different species of the *Aspergillus glaucus* group (genus *Eurotium*) were investigated. The presence of emodin, erythroglaucin, physcion, physcion-9-anthrone, questin, catenarin, and catenarin-8-methyl ether was described and antimicrobial properties of all the substances were examined. Gram-positive bacteria are the most sensitive organisms and catenarin is the most active naturally occurring substance [18]. In *Bacillus brevis*, catenarin and emodin preferentially inhibited the incorporation of uracil and leucine. *In vitro*, the inhibition of the growth of *Escherichia coli* by catenarin and emodin was observed [18]. Strong antimicrobial activity has been reported by Rankovic and Misic (2008) for lichen secondary metabolites such as ATR, fumarprotocetraric acid, gyrophoric acid, lecanoric acid, physodic acid protocetraric acid, stictic acid, and UA against six bacteria and ten varrious fungi species [19]. Antifungal properties of *Protousnea poeppigii* extracts against fungal pathogens such as *Microsporum gypseum*, *Trichophyton mentagrophytes*, and T*. rubrum*, as well as against some yeasts, such as *Candida albicans*, *C. tropicalis*, *Saccharomyces cerevisiae* and the filamentous fungi *Aspergillus niger*, *A. flavus* and *A. fumigates* were examined. The extract consisted of metabolites such as isodivaricatic acid, divaricatinic acid and UA. Activity against *Microsporum gypseum*, *Trichophyton mentagrophytes* and *T. rubrum* [20] was observed.

Extract of several lichens, such as *Umbilicaria antarctica*, *Cladonia furcata*, *Sphaerophorus globosus* and *Usnea antarctica,* were found to have strong *in vitro* antioxidant properties [21]. ATR is known for its antioxidant properties *in vitro* [22] and *in vivo* [23]. UA has been shown to exhibit antioxidant properties in various models [24, 25]. In addtion, parietin possesses antioxidant properties [26]. The acetone extract from *Hypogymnia physodes* (physodic acid) showed significant antioxidant properties [27].

Inflammation has long been recognized as a major cause of different diseases. Dysregulation of immune responses causes chronic inflammatory and/or autoimmune diseases, and contributes to degenerative pathologies and even organ failure [28]. The main organ systems associated with acute or chronic inflammation-mediated tissue injury are the heart, pancreas, kidneys, lung, brain, gastrointestinal tract, and reproductive system [29]. The potential anti-inflammatory effects of ATR (100 and 200 mg/kg) were tested in a model of carrageenan-induced paw edema in Wistar rats. The results showed that ATR exhibited significant anti-inflammatory activity (paw edema and leukocyte migration), with no significant acute or

subchronic toxicity and cytotoxicity in other organs [30]. The anti-inflammatory effects of ATR, (+)-iso-UA, methyl orsellinate, and parietin were evaluated on RAW 264.7 macrophages at different concentrations. ATR and (+)-iso-UA showed a high anti-inflammatory potential, whereas methyl orsellinate showed moderate anti-inflammatory activity [31]. Organic extracts of *Parmotrema hypoleucinum*, *Roccella phycopsis*, and *Xanthoria parietina* exhibited increased anti-inflammatory activity on nitric oxide (NO) levels in lipopolysaccharide (LPS)-stimulated macrophages [31].

The newest information is that secondary metabolites may also have antidepressant/anxiolytic effects, as has been investigated using ATR [23].

ANTICANCER ACTIVITY OF LICHEN SECONDARY METABOLITES

Cancer is the leading cause of death in up to 60 countries including China [32]. Nature has been inspiring the search for new possibilities to cure diseases. Therefore, natural products with remarkable chemical diversity have been intensively studied for their anticancer potential. There is an ever-growing role of natural products as lead structures for the treatment of cancer and other diseases as well [33]. Collective efforts from the community have contributed to tremendous progress, putting natural products in clinical use and finding new therapeutic possibilities, but the challenges are still ahead [34].

One of the most intensively studied groups of natural products with anticancer acitivities are lichen secondary metabolites. Growing evidence in last five years supports the right direction of research. After 2000, interest in lichen secondary metabolites increased when evidence of their *in vitro* antitumor activities increased. In 2004, the anticancer potential of UA was evaluated. It displays cytotoxic activity in various cancer cell lines (L1210, 3LL, DU145, MCF7, K-562, and U251) [35]. In 2010, Bogo *et al*. tested the cytotoxic activity of lecanoric acid and its orsellinate derivatives in various cancer and normal cell lines (HEp-2 larynx carcinoma, MCF7 breast carcinoma, 786-0 kidney carcinoma, B16-F10 murine melanoma, and normal Vero cell line) [36]. In 2011, Backorova *et al*. tested the sensitivity of up to nine human cancer cell lines (A2780, HeLa, MCF-7, SK-BR-3, HT-29, HCT-116 p53(+/+), HCT-116 p53(-/-), HL-60 and Jurkat) to the anti-proliferative/cytotoxic effects of four lichen secondary metabolites (parietin, ATR, UA, and gyrophoric acid – GA). It has been shown that lichen metabolites possess cytotoxic activity in a time- and dose-dependent manner [37]. In a subsequent study, the cytotoxic activity of these four metabolites was demonstrated in A2780 and HT-29 cancer cell lines [38]. ATR, caperatic acid, physodic acid, squamatic acid, salazinic acid, and lecanoric acid were studied using two- and three-dimensional GBM cell line models. The tested compounds generated oxidative stress, interfered with the cell cycle, and induced apoptosis in T98G cells. They also inhibited the Wnt/β-catenin pathway, and this effect was stronger in the case of co-treatment with temozolomide [39]. It has been shown that ATR together with lecanoric and evernic acid reduce viability of tested cell lines (HCT-116, HEK293T, HeLa, NIH3T3, RAW246.7) in a cell line-dependent manner, with lecanoric acid being the most effective [12]. The antitumor activity of evernic acid against HeLa cancer cell line has also been reported [40].

ATR and UA are the most intensivelly studied lichen secondary metabolites. Interest in the antiproliferative effects of GA has increased markedly. This review focuses on these three compounds.

USNIC ACID

UA is one of the most studied lichen secondary metabolits. Owing to the wide biological and ecological activities, it is used in cosmetics, deodorants, toothpastes, and medical creams [9]. It exhibits many biological activities, including anti-inflammatory [41, 42], analgesic [43], and anticancer activity.

The anticancer activity of UA was tested in various cancer cell lines (A2780, HeLa, MCF-7, SK-BR-3, HT-29, HCT-116 $p53^{+/+}$, HCT-116 $p53^{-/-}$, HL-60, and Jurkat). UA was the most effective metabolite of all tested, with a concentration of 50 μM, proving to be effective against almost all cell lines. Higher concentrations (100, 150 and 200 μM) increase the effects of the metabolite [37]. UA causes a massive loss of mitochondrial membrane potential with concomitant activation of caspase-3 in HT-29 cells. Moreover, it induces ROS and RNS production, which could partially explain the cytotoxic effects of UA. UA has been described as an activator of programmed cell death in A2780 and HT-29cells, based on the protein expression of PARP, p53, Bcl-2/Bcl-xL, Bax, and p38 (Figure **2**) [38]. Using other cancer cell lines (CaCo2, RD, Hep2C, HepG2, and Wehi), UA (6.25, 12.5, 25, 50, 100, and, 200μM) was observed to iduce apoptosis by affecting p53 and Bcl-2 expression [44]. Another study

revealed that UA inhibits the transition into the S-phase of cell cycle and reduces cell size of the breast cancer cell line T-47D and the pancreatic cancer cell line Capan-2 [45]. The tumor-suppressive molecular mechanism of UA in RKO colorectal cancer cells transfected with miRNA18a-5p was evaluated. UA can upregulate ATM via miR-18a to activate the DNA damage signaling pathway and inhibit the proliferation and migration of RKO cells in a concentration-dependent manner [46]. The rates of cell proliferation and cell apoptosis were significantly increased following ectopic expression of miR-185-5p in breast cancer BT-474 cells. Furthermore, UA induced G1/S phase arrest [47]. Differential expression of 74 apoptosis-related genes in breast cancer after SK-BR-3 cells was observed after UA (7.21 μM) treatment for 48 h, following the analysis of Bcl-2, Bax, caspase-3, and caspase-9 proteins. These results suggest that UA has cytotoxic properties mediated via the activation of the mitochondrial apoptotic pathway [48]. However, the antineoplastic activity of UA was not related to alterations of microtubules in the breast cancer cell line MCF7 or the lung cancer cell line H1299 [49]. In H1650 and H1975 lung cancer cell lines, β-catenin-mediated TOPFLASH activity and KITENIN-mediated AP-1 activity decreased in a dose-dependent manner after UA treatment. UA concomitantly decreased mRNA levels of CD44, Cyclin D1 and c-myc, which are downstream target genes of both β-catenin/LEF and c-jun/AP-1. When combined with cetuximab, even stronger inhibitory effects of UA on cell invasion have been reported [50]. The anticancer activity of UA against human gastric cancer cells was tested. Human gastric carcinoma cell lines (BGC823 and SGC7901) were used *in vitro*. UA inhibited cell proliferation in a dose- and time-dependent manner (BGC823: 100, 200, and 400 μM; SGC7901: 300, 600, and 1200 mM; 24 and 48h). UA significantly arrests the cell cycle, and promotes apoptosis in cancer cells [51]. At concentrations of 10 – 25 μM, UA causes significant apoptosis in human gastric adenocarcinoma AGS and gastric carcinoma SNU-1 cells, accompanied by an increase in the ratio of Bax:Bcl-2 expression and cleaved-PARP, and DNA damage [52]. UA at concentrations of 3, 10, 30 and 100 μM decreased the expression of PD-L1 in HeLa cells and enhanced cytotoxicity of co-cultured T cells by cooperatively inhibiting STAT3 and RAS pathways [53]. Furthermore, UA inhibits cell proliferation, angiogenesis, migration, and invasion of HCT-116, HeLa, HcerEpic, A549, and Hep3B cells [53].

In a model of gastric cancer using female BALB/C nude mice, UA at a dose of 100 mg/kg body weight suppresed tumor growth without affecting body weight, and regulated the amount of Bax and Bcl2 proteins in tumor tissues [51]. In female BALB/C nu/nu mice, UA at concentrations of 25, 50, or 100 mg/kg of body

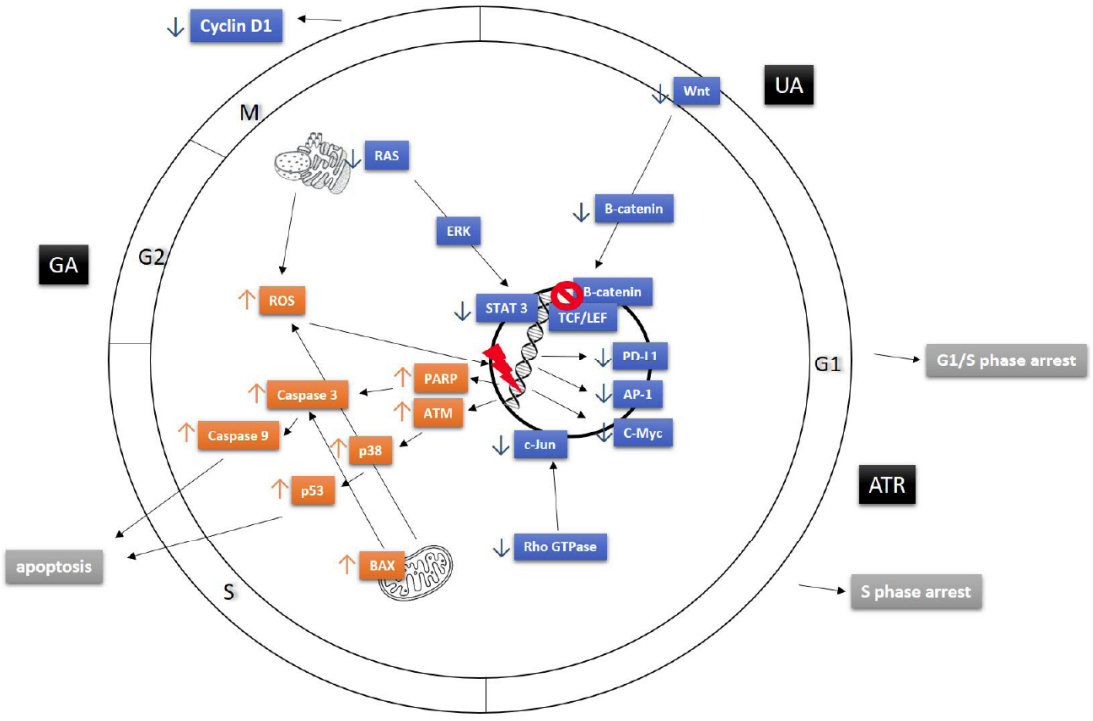

Figure 2: Molecular pathways leading to apoptosis of cancer cells mediated by lichen secondary metabolites.

weight, markedly inhibites tumor growth in a dose-dependent manner without inducing significant toxicity [54].

ATRANORIN

ATR is a compound with a depside structure and belongs to one of the most common lichen secondary metabolites, characteristic to numerous lichen families,; however, it is rarely found in mosses and higher plants [55].

Numerous research teams have studied anticancer effects of ATR. Backorova *et al*. (2011) discovered that already at concentration of 50 μM, ATR is effective against HL-60 cells. Other tested cell lines (A2780, MCF-7, SK-BR-3, HT-29, HCT-116 $p53^{+/+}$, HCT-116 $p53^{-/-}$, HL-60, and Jurkat) are sensitive to higher ATR concentrations [37]. ATR causes an increase in caspase-3 activation in a dose-dependent manner (Figure **2**) [38]. To directly analyze the effects of ATR on hepatocellular carcinogenesis, the authors Jeon *et al*. (2019) selected various cell lines, SKHep1 and Huh-7, as representatives of epithelial metastatic carcinomas and the SNU-182 cell line as a primary cancer cell line. Cell proliferation and cell cycle analyses showed the inhibitory effects of ATR in a dose-dependent manner (5, 10, 20, 40, and 80 μM). ATR also induces cell death via necrosis [56]. ATR suppresses β-catenin-mediated TOPFLASH activity by inhibiting the nuclear import of β-catenin and downregulating β-catenin/LEF and c-jun/AP-1 downstream target genes such as CD44, cyclin-D1 and c-myc in the lung carcinoma cell line A549. Moreover, it decreases KAI1 C-terminal interacting tetraspanin (KITENIN)-mediated AP-1 activity and the activity of the KITENIN 3′-untranslated region. ATR inhibits tumorigenesis by affecting AP-1, Wnt, and STAT signaling and suppressing RhoGTPase activity [57]. In MDA MB-231 and MCF-7 breast cancer cells, ATR inhibits cell growth in a dose-dependent manner with IC50 of 5.36 ± 0.85 μM and 7.55 ± 1.2 μM respectively [58]. Moreover, ATR inhibits ROS production and downregulates anti-apoptotic Akt compared to Bcl-2, Bcl-xL and Bcl-w proteins, with a significant increase in the Bax protein level and caspases-3 activity in the breast cancer cells when compared with the Akt inhibitor ipatasertib [58]. The total cell number of mouse breast carcinoma 4T1 cells decreases after ATR treatment in a time- and concentration-dependent manner, with a significant decline after incubation with 50 and 75 μM of ATR [59]. Moreover, ATR reduced the clonogenicity of 4T1 cells without affecting that of normal NMuMG cells [59].

In vivo, ATR at a dose of 30 mg/kg body weight prolonged the survival time of BALB/c mice inoculated with 4T1 cells and reduced tumor size. Importantly, ATR shows protective effects against oxidative stress in the liver of tumor-bearing mice [59]. The hepatoprotective effects of ATR at a dose of 10mg/kg body weight were also demonstrated by Simko *et al*. [14]. ATR reduces the tumor volume and weight, and Ki-67 immunoreactivity. The main target genes, such as KITENIN, STAT, c-myc, CD44, and/or cyclin-D1 are suppressed *in vivo* [57].

GYROPHORIC ACID

Interest in GA has increased in recent years. This depside has attracted the attention of researchers owing to its anticancer effects. The therapeutic potential of this compound is associated with its chemical versatility as a polyaromatic depside with functional carboxyl and hydroxyl groups [60].

GA at a concentration of 100 μM induces antiproliferative effects in HL-60, A2780, and Jurkat cells [37]. After 48 and 72h, caspase-3 activation was observed following GA treatment [38]. Caspase-3 activation followed by PARP cleavage, PS externalization, and cell cycle changes was observed in HeLa cells treated with GA (150 – 350 μM) (Figure **2**). The production of ROS leads to DNA damage and changes in the stress/survival pathways activation [13]. GA (300 – 500 μg/mL) reduces the viability of breast cancer MCF-7 cells by 98% [61]. GA showed mild cytotoxic activity in MM98 malignant mesothelioma cells, and A431 vulvar carcinoma cells. However, GA has unique qualities for wound healing and tissue regeneration [62].

Until now, no *in vivo* study studied the potential anticancer activity of GA.

FUTURE CHALLENGES

Even despite the fact that there is a growing evidence about the use of different licehn secondary metabolites in various areas, there are many shortcomings that should be resolved.

First, only a limited number of *in vivo* studies have been conducted to date. There is a need to comprehensively examine the activity of each significant metabolite and its potential adverse effects. To date, only a few adverse effects have been investigated, such as temporary anemia caused by ATR [23] or harmful effects of UA on hepatic metabolism [63].

Lichen metabolites are known to be poorly soluble in water. They are soluble in polar solvents, which are used for their isolation [9]. This is the reason why there is a lack of pharmacological/pharmacodynamic studies *in vivo*. Intravenous application of such insoluble compounds presents a significant problem. There are two possible solutions to this problem. One is to improve the solubility and concomitant use of the lichen secondary metabolites via the use of their derivatives. Potassium usnate (derivative of UA) has increased bioavailability as compared to UA in tumor tissue, liver and blood plasma in CT26 syngeneic mouse tumor xenograft model after oral administration [64]. It also has shown anticancer effects in colorectal cancer and inhibits liver metastasis [64]. A series of UA derivatives was synthesized, and their antiproliferative activity against various cancer cells was tested. Compounds 2a and 2b were more active than UA and inhibited the survival of MCF-7, PC-3, and HeLa cells in a dose- and time-dependent manners. All active UA derivatives induce G0/G1 arrest and decrease the fraction of HeLa cells in the S and G2/M phases [65]. Pyrczak-Felczykowska *et al*. (2022) tested *in vivo* the molecular mechanisms underlying their activity. The derivative 2b induces vacuolization originating from the endoplasmic reticulum. This stress leads to apoptosis of cancer cells, but not of healthy ones. When applied to nude mice with xenografted breast cancer cells, 2b inhibits tumor growth [66].

Another way to increase the bioavailability of the tested lichen metabolites is to use nanocarriers. Herbal medicine is often used with nanocarriers to boost their therapeutic impact [67]. Diverse nanostructures such as lipid nanoparticles, nanoliposomes, nanomicelles, and carbon nanotubes or different delivery systems, including lactoferrin, can significantly increase the bioavailability, effectiveness, enhance stability, and improve pharmacokinetics of many compounds [68]. Lichen-based nanoparticles are also becoming increasingly popular owing to their biocompatibility, eco-friendliness, and cost-effectiveness [69]. The green synthesis of silver nanoparticles (AgNPs) is a method in which compounds from plants are used for the reduction of $AgNO_3$ instead of toxic chemicals. Different bacterial strains were tested for the antibacterial activity of AgNPs-containing products, showing that the nanoparticles exhibit strong antibacterial properties against all strains [70, 71]. Many other reports have described the synthesis of nanoparticles from various types of lichens [72-76]. For the list of publications on lichen-based nanoparticles see the review by Rattan *et al*. [69]. However, none of these nanoparticles have been tested in an *in vivo* cancer model.

CONCLUSION

The lichen secondary metabolites represent a valuable source of substances with promissing antitumor properties. However, because of their low water solubility and low bioavailability, there is a need to develop derivatives and/or nanoparticles that can reach targeted tumor tissues.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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