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STUDY AND PURPOSE OF USNIC ACID MOTHER LIQUOR AS POTENTIAL ANTISEPTIC IN SOME PRODUCTS

Goran S. Nikolić[1], Saša Ž. Zlatković[2], Tatjana D. Kundaković[3],
Dragiša S. Đokić[1], Aleksandra S. Djordjević[4], Ivana M. Savić[1]
[1] Faculty of Technology, University of Niš, 16000 Leskovac, Serbia
[2] Actavis Trading Limited, 11000 Beograd, Serbia
[3] Faculty of Pharmacy, Department of Pharmacognosy, 11000 Beograd, Serbia
[4] Faculty of Science, Department of Chemistry, 18000 Niš, Serbia
E-mail: goranchem_yu@yahoo.com

Abstract

The antimicrobial and antiviral activity, antiseptic and other properties of usnic acid mother liquor (Usnea barbata), as well as dry residue of mother liquor, in terms of its application for the production of pharmaceutical and cosmetic preparations, were investigated. The composition of the mother liquor was monitored by HPLC. Correlation of the data with the activities of crystal usnic acids isolated from Usnea barbata and sodium usniate found that investigated mother liquor have qualitatively and quantitatively preserved antiseptic properties. The activity of mother liquor to the following test microorganisms was confirmed: Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermidis, Micrococcus flavus, Sarcina lutea, Micrococcus pyogenes, Micrococcus luteus, Klebsiella pneumoniae and Candida albicans. Therefore, the development of new antiseptics and antimicrobial agents for the treatment of skin infections is interest in this study.

Key words: *usnic acid, mother liquor, antimicrobial activity, antiseptics*

1. INTRODUCTION

Plant: Lichens are representatives of symbiotic union of fungi and algae. Constitutional components of many lichen species show some antibiotic activities. Of many studied, lichens containing usnic acid as an active principle are of significance (Frankos, 2006). Usnic acid is one of the most abundant characteristic secondary metabolite of lichens (Fig. 1). Lichens containing usnic acid have been used as traditional medicines and crude drugs in other countries. The most common source is the lichen *Usnea barbata* and related species that is promoted for its antibacterial and antifungal properties in complementary and alternative medicine practices. It is also promoted for consumption as a brewed tea for its effects as a natural metabolic stimulant. These ingredients are also used in deodorants, cosmetic products, lotions, balms and air fresheners based on their antibacterial and antifungal properties. Many patents exist for usnic acid in these types of products. It is unknown what the exposure level or risk may be associated with the use of these common products.

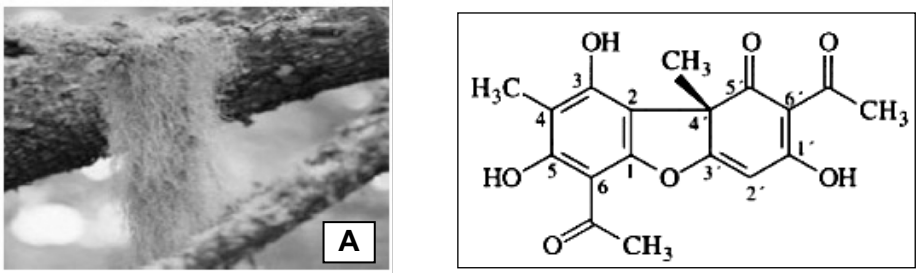


Figure 1. Usnea barbata lichen (A) and structure of usnic acid (B)

Usnic acid chemistry: Usnic acid is a yellow crystal substance of natural origin, isolated by Knop from the lichen *Usnea barbata*, having significant antibacterial activity. Usnic acid is a dibenzofuran derivative, a complex polycyclic, phenolic chemical compound (Fig. 1B). It occurs as a (+) or D-usnic and a (-) or L-usnic enantiomer. In addition, two other natural isomers, (+) and (-) isousnic acids are also found in lichens (Frankos, 2006).

Usnic acid biology: A number of biological roles such as antibiotic, antimycotic, antiefedent, phytotoxic, photobiont regulator, and UV filter etc. have been linked to usnic acid lichens rich in usnic acid. However, it must be mentioned that neither of these effects have been substantiated nor formally attributed to usnic acid. These indications have simply served as a guide for pharmacological studies of these substances. The compound probably does not have a single major biological role, but can play different species-specific roles. Furthermore, both the enantiomers have been known to exhibit different biological activities. For instance, (-) usnic acid specifically inhibits the activities of arginase and urease, whereas (+) form was found to be a more effective antimicrobial agent (Proska et

al, 1996; Lauterwein et al, 1995; Ghione et al, 1988). Even usnic acid's well known toxic effect allergic contact dermatitis is also enantioiner-specific. For instance, both forms of usnic acid sensitized lichen pickers, while only its d- form sensitized lumberjacks (Benezra, 1987). In an acute study in larvae of a herbivorous insect, the (-) usnic acid proved to be 10 times more toxic than its (+)-enantiomer (Emmerich et al, 1993).

Commercial availability and use: A highly purified form of usnic acid isolated from genus Usnea is commercially available. It is also prepared in tissue culture using tiny segments of thalli from Usnea and Ramalina species. In its purified form, usnic acid has been extensively used in medicinal products, creams, toothpaste, mouthwash, powders, hair shampoo, perfumery, deodorants, sunscreen products and in ecological applications, either as an active principle or a preservative (Reynolds, 2000; Rafanelli et al, 1995; Correch et al, 1998). FDA has not received any certification from industry on the safety or use of this substance in cosmetic products. *Usnea barbata*, usnic acid, and copper usnate have been produced as antimicrobial preparations. It has also been indicated that usnic acid could also be used as a biomarker to assess pollution, as its concentration in lichens increases with the increased exposure to toxicants. In recent years, usnic acid and its salt form, sodium usniate, have been marketed as an ingredient in dietary supplement products (mostly with claims as weight-loss aides, though some as antimicrobial agents). However, to date, no sound non-clinical or clinical data have been presented to support any such claims.

Usnic acid clinical pharmacology: Prior to the discovery of penicillin, usnic acid was under active investigation for its broad spectrum antibiotic activities. Activity of usnic acid is approximately the same against Gram-positive bacteria (*Enterococcus faecalis, Enterococcus faecium Staphylococcus aureus, Streptococcus mutans, Streptococcus pyogenes*), anaerobic bacteria (*Bacteroides fragilis,*

Bacteroides ruminicola ssp. brevis, Bacteroides thetaiotaomicron, Bacteroides vulgatus, Clostridium perfringens, Propionibacterium acnes) and mycobacteria (*Mycobacterium aurum, M. avium, M. smegmatis, M. tuberculosis var. bovis, M. tuberculosis var. hominis*).

Antimicrobial activity: Interest in usnic acid was renewed because of increasing experience of multi-drug resistance caused by overuse of synthetic antibiotics (Cocchietto et al, 2002). Earlier it was demonstrated that both the optical antipodes of usnic acid were active against Gram positive bacteria and mycobacterium (Shibata et al, 1948; Stoll et al, 1950). In recent years, the antibacterial properties of usnic acid have been confirmed. In preliminary clinical trials, a mouthwash containing 1% (+) usnic acid was administered to volunteers, and at regular intervals the samples of oral bacterial flora were examined. It was reveled that the growth of *Streptococcus mutans* involved in the etiology of dental caries, was selectively suppressed (Ghione et al, 1988). A number of usnic acid (+) containing preparations are marketed (Cocchietto et al, 2002). Using standardized assays, the *in vitro* susceptibility of pathogenic Gram positive and anaerobic bacteria towards usnic acid has been confirmed (Ingolfsdottir, 2002). Usnic acid has been shown to suppress the growth of Gram positive organisms mainly responsible for body odor. Ethoxydiglycol extracts of lichens containing 10% usnic acid on wet weight basis have been demonstrated to have preservative potential in moisturizing cream (Scifert and Bertram, 1995). Usnic acid was found to be effective against *Mycobacterium aureum* (Ingolfsdottir et al, 1998). *In vitro* assays, usnic acid and its salt inhibited the growth of *Mycobacterium tuberculosis* at relatively low concentrations (Krishna and Venkataramana, 1992).

Antimycotic activity: During a short-term treatment with usnic acid salt, 65 patients with *Tinea pedis* exhibited a significant improvement in clinical condition (De Battisti et al, 1991).

Antiprotozoal activity: Usnic acid (-) exhibited significant inhibitory effect against the pathogenic protozoan *Trichomonas vagina/is* at comparatively lower concentrations than metronidazole (Wu et al, 1995). The compound also showed leishmanicidal properties both *in vitro* and *in vivo* studies; intralesional administration produced a reduction in lesion weight as well as in body burden of parasite (Fourner et al, 1997).

Antiviral activity: In a cancer chemoprevention assay, (+) usnic acid isolated from *Usna longissima* was found to be significantly effective against tumor-promoter-induced Epistein-Barr virus with an ED50 of 1.0 µg/mL (Yamamoto et al, 1995). (+) usnic acid also inhibited the cytopathic effects of Herpes simplex type 1 and polio type 1 viruses in the infected kidney cells of African green monkey (Perry et al, 1999). In a clinical trial, the effect of an intravaginal formulation containing usnic acid and zinc sulfate as an adjuvant therapy to radio surgical treatment was evaluated in 100 females infected with genital human papilloma virus. The treatment significantly improved the time of reepithilization one month after the radio surgery (Scrippa et al, 1999).

Antiproliferative activity: (-) Usnic acid caused moderate inhibition in the murine P388 leukemia assay, and also exhibited cytotoxic activity against cultured L1210 cells; it was inferred that p-triketone moiety was essential for the optimum activity (Takai et al, 1979). On the other hand, (+) usnic acid (50µg/mL) reduced the cell counts of leukemic (K-562) and endometrial carcinoma cell culture (Ishikawa HEC-50) (Cardarelli et al, 1997; Kristmundsdottir et al, 2002). (+) usnic acid exhibited cytotoxic activity against human keratinocyte cell cultures (Kumar and Muller, 1999).

Anti-inflammatory activity: In an acute rat paw edema and a chronic rat cotton pellet assays at 100 mg/kg oral dose level, the anti-inflammatory action of (+) usnic acid was comparable to ibuprofen at the same dose level (Vijayakumar et al, 2000).

Analgesic and antipyretic activity: In two mice studies, the analgesic and antipyretic effects of usnic acid were evaluated (Okuyama et al, 1995). At 100 mg/kg oral dose level, usnic acid exhibited a significant analgesic effect as indicated in acetic acid-induced writhing- and tail pressure tests. At oral

dose levels up to 300mg/kg, usnic acid also expressed significant antipyretic activity determined through lipopolysachharide-induced hyperthermia.

Usnic acid toxicology: Irrespective of a long history of usnic acid containing products, only a few animal studies were conducted to evaluate the clinical safety of usnic acid. There is a total lack of systemic sub chronic and chronic general toxicity studies. The conduct and quality of the some of the available studies is also questionable.

Non-clinical usnic acid acute toxicity studies [Martindale, Monographs 1982-2003]: mouse LD50 oral = 838 mg/kg, LD50 intravenous = 25mg/kg, LD50 subcutaneous = 75 mg/kg; rabbit: LD50 oral > 500 mg/kg. In an acute study where larvae of a herbivore insect *(Spodoptera littoralis)* received injections of both enantiomers of usnic acid in the hemolymph, (-)-form was found to be 10 times more toxic than its (+)-form (LD50 8.6 versus 90.8 μmol) (Emmerich et al, 1993).

Reproductive and developmental toxicity studies: In a 35-day oral study in 5-6 weeks old male Swiss mice, no adverse effects of 200 mg/kg/day of (+) usnic acid on the number, motility and structure of epididymal spermatozoa, were observed. Additionally, no quantitative differences in the content of testicular protein, RNA and DNA were recorded (AI-Bekairi et al, 1991).

Antiseptics: Antiseptics are antimicrobial substances which inhibit the growth and development of microorganisms, and that are applied to living tissue/skin to reduce the possibility of infection, sepsis, or putrefaction. For practical purposes, antiseptics are routinely thought of as topical agents, for application to skin, mucous membranes, and inanimate objects, although a formal definition includes agents which are used internally, such as the urinary tract antiseptics. Antiseptics are a diverse class of drugs which are applied to skin surfaces or mucous membranes for their anti-infective effects. This may be either bacteriocidal or bacteriostatic. Their uses include cleansing of skin and wound surfaces after injury, preparation of skin surfaces prior to injections or surgical procedures, and routine disinfection of the oral cavity as part of a program of oral hygiene. Antiseptics are also used for disinfection of inanimate objects, including instruments and furniture surfaces. Antiseptics are generally distinguished from antibiotics by the latter's ability to be transported through the lymphatic system to destroy bacteria within the body, and from disinfectants, which destroy microorganisms found on non-living objects. Some antiseptics are true germicides, capable of destroying microbes (bacteriocidal), whilst others are bacteriostatic and only prevent or inhibit their growth. Antibacterials are antiseptics that have the proven ability to act against bacteria. Microbicides which kill virus particles are called viricides or antivirals. Antiseptics are not known to interact with any other medicines. However, they should not be used together with any other topical cream, solution, or ointment (McDonnell et al, 1999).

The aim of study: The study of lichen extracts (usnic acid and its mother liquor) is of particular importance due to their practical application in the production of pharmaceutical and cosmetic preparations. Actually, the substances own pronounced antibacterial activity especially in the area of mouth cavity and upper respiratory organs. Na-usninate coats mucous membrane like a protective film and as a surface antibiotic selectively inhibits causes of infection. The formed film covers lesions caused by the infection or mechanical injury and helps their healing, without damaging the healthy microflora of the mucous membrane. Having in mind very low solubility of usnic acid (Ingolfsdottir, 2002), which is one of its greatest disadvantages for the application, it is transformed into more readily soluble sodium usniate. Procedures for isolation and characterization of usnic acid, as well as the procedures of sodium usniate synthesis are described in detail in scientific and patent literature (Cocchietto et al, 2002; Huneck et al, 1996; Ingolfsdottir, 1998; Cakic et al, 1998; Shibata et al, 1948; Stankovic et al, 1989). Usnic acid can be produced synthetically and a broad spectrum of derivatives and condensation products based on usnic acid are also known (Sladic et al, 1998). Bearing in mind

certain pharmacodynamical activity (antiseptic and anesthetic effects) (Konschai, 1977; Wunderer, 1986), and the fact that usnic acid can be taken, without any side effects, in daily doses of 3-6 g *per os*, the investigated substance finds application in the production of pharmaceutical (septolete-lingualete, tooth paste) and cosmetic preparations (preservative in hydrated creams, inhibitor of Gram-positive microorganisms in deodorant sprays). However, from the technological point of view, there is a permanent problem of solubility of lichen acids. This is the limiting factor for the application of usnic acid in pharmaceutical-cosmetic industry, so its sodium salt is used more often.

Taking into account former experience and great interest for the application of lichen extracts, in this work first was made the selection of optimal solvents for usnic acid and sodium usniate. Consequently, criteria for the selection of solvent were defined: good solubility of tested substances in the range of working concentration for microbiological analysis and inertness of solvents to selected test-microorganisms. On the other hand, extended number of patented and described procedures for the isolation of usnic acid from various lichens, and synthesis of sodium usniate is described in literature. These procedures are, for the most part, characterized by insufficient consumption of reactants and solvents, longer reaction times and lower degree of purity of the obtained usniate. All data of structural and antibiotic tests shown in this work refer to chemically pure or crystallized compounds. However, there is no data in literature about investigation of usnic acid mother liquor, obtained as a by product in the production process.

A report by Russian investigators from VILAR institute (personal paper, collaboration Zdavlje Leskovac – Vilar Moskva), asserting that usnic acid kept for 24 years under inadequate conditions has lost very little of its antibiotic activity compared to freshly isolated substance, opened a new idea for the use of mother liquor from the practical point of view.

Because, the aim of this work is the investigation of antimicrobial activity of usnic acid mother liquor and its dry residue, with respect to its application in pharmaceutical and cosmetic industry. Similarly, it is interesting to compare activities of mother liquor, sodium usniate and their dry residues, in view of its better solubility. By correlation of the obtained data with the activity of crystal usnic acid and the synthesized sodium usniate, the possibility of practical application of mother liquor for the production of pharmaceutical and cosmetic products could be opened.

2. MATERIAL AND METHODS

2.1. Tested material

Experimental investigation included the following samples: usnic acid freshly isolated from *Usnea barbata* (sample I), crystallized usnic acid (sample II), mother liquor of usnic acid (sample III), dry residue of usnic acid mother liquor (sample IV), crystal sodium usniate (sample V), mother liquor of sodium usniate (sample VI) and dry residue of sodium usniate mother liquor (sample VII).

The usnic acid and its mother liquor, which were used in this study, were obtained from the lichen *Usnea barbata* L. The plant material, cutted lichen *Usnea barbata* (20 ± 0.1 g), granulation 0.6-1.0 mm and moisture content 4.82 %, was extracted with 96 % ethanol, under reflux, during 15-30 min. Usnic acid was isolated by recrystallization. The remaining ethanol solution (mother liquor) was evaporated under reduced pressure. The total yield of mother liquor dry residue was 1.54 g. Sodium usniate was obtained by synthesis from isolated and recrystallized usnic acid in accordance with literature data (Stankovic, 1989).

2.2. Tested solutions

Solutions of tested usnic acid were prepared with the following concentrations: 0.0125; 0.025; 0.050; and 0.100 mg/ml. The solutions were prepared from the basic solution of usnic acid of 1 mg/ml by adding required quantity of solvent mixture dimethyl formamide-buffer pH 6 (Ph. Jug. V) in 1:1 volume ratio. Working solutions of sodium usniate were prepared in the same way, but with optimized solvent mixture ethanol-water 1:2 v/v. By the same method, the solutions of dry residues of mother liquors of usnic acid and sodium usniate were prepared, and corresponding mother liquors were used after dilution to selected working concentrations.

2.3. Antimicrobial activity

The antimicrobial activity of the ethanol extracts was examined using the nutrient agar broth disc diffusion method and the microdilution method. For testing the antimicrobial activity of samples I-VII a diffusion method with paper disc was used (Ph. Jug. V).

The disc diffusion method was performed using a 24 h culture which was reseeded on the nutrient broth at a temperature of 37 °C. The concentrations of the cultures were adjusted with sterile water. One cm^3 of suspension was added over the plates containing nutrient agar broth in order to achieve a uniform microbial growth on both the control and test plates. The ethanol extracts was sterilized. Under aseptic conditions, empty sterilized discs (Whatman No. 5, 14 mm diameter) were impregnated with different concentrations of the respective extract, and placed on the agar surface. The plates were left for 30 min at room temperature to allow diffusion of the extract and then incubated at 37 °C. After the incubation period (48 h), the zone of inhibition was measured and is presented in mm. A paper disc of the solvent (ethanol) was used as the control for comparison. Each test was performed in triplicate and repeated three times.

For MIC determination microdilution method was applied. The minimal inhibitory concentration of the ethanol extract was determined by the microdilution two-fold serial technique. A series of two-fold dilutions of the extract was prepared in Mueller-Hinton broth with the addition 0.1 cm^3 of a suspension of the bacterial spores. The results were determined after 24 h and the MIC values were determined as the lowest concentration of the extract which inhibited visible growth of each organism.

drug.

2.4. Used microorganisms

The following test microorganisms were used: *Escherichia coli* ATCC 25922, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538 and 25923, *Staphylococcus epidermidis* ATCC 12228, *Micrococcus flavus* ATCC 10240, *Micrococcus luteus* ATCC 10240, *Sarcina lutea* ATCC 9341, *Micrococcus pyogenes* ATCC 6538P, *Klebsiella pneumoniae* NCIMB 9111, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853 and *Candida albicans* ATCC 10259 and 24433.

2.5. GC-MS analysis

Sample analysis was performed essentially as described in literature, with the exception of the column used (suitable for fraction analysis). Briefly, sample volumes of 1 µl were injected with a split ratio of 1:200 using a hot-needle technique. The GC-MS system consisted of an autosampler, a GC 8000 gas chromatograph and a Voyager quadrupole mass spectrometer (ThermoQuest). Gas chromatography was performed on a 0.32 mm x 30 m capillary column Rtx-5Sil MS of 0.25 mm inner diameter with integrated guard column and a 0.25 µm film (Restek GmbH). Injection temperature was 230 °C, the interface set to 250 °C and the ion source adjusted to 200 °C. The carrier gas used was helium set at a constant flow rate of 1 $ml \cdot min^{-1}$. The temperature program was 5 min isothermal heating at 70 °C, followed by a 5 °C min^{-1} oven temperature ramp to 310 °C and a final 1 min heating at 310 °C. The

system was then temperature equilibrated for 6 min at 70 °C prior to injection of the next sample. Mass spectra were recorded at two scans per sec with an *m*/*z* 50–600 scanning range. The chromatograms and mass spectra were evaluated using the Masslab software (ThermoQuest).

3. RESULTS AND DISCUSSION

Usnic acid, which is a key secondary metabolite of lichen *Usnea barbata*, is known to exhibit different types of biological activity. In order to obtain new biologically active compounds, we investigated the remaining ethanol extract after the isolation of usnic acid, i.e. mother liquor of usnic acid. Namely, it is known that the conventional extraction process of lichens for isolation of usnic acid from plant produces large volumes of mother liquor. This residual liquid represents a potential value because it contains the soluble antimicrobial substances. Furthermore, solubility problems and chemical constituents are key factors that explain samples behavior for an eventual use of these natural products.

3.1. Choice of solvents

In scientific and patent literature diverse information on antimicrobial activity of usnic acid and its derivatives are reported. These variations are not only a function of origin and quality of tested substance but are also results of their low solubility, impeding reproduction and accuracy of testing methods. Therefore, we made the selection of the optimal solvent for usnic acid and its sodium salt, in the first place. The solvent should be selected in such a way to enable reaching of working concentration of the solution and, at the same time, avoid its being toxic to test-microorganisms.

In the experiment, as solvents for sodium usniate, the following were tested: distilled water, buffer pH 6 and ethanol-water mixture 1:1 v/v and 1:2 v/v. Test results showed that optimal mixture was ethanol-water 1:2 v/v. The mixture of dimethyl formamide-buffer pH 6, 1:1 v/v was selected as the optimal solvent for usnic acid. This mixture was advantageous compared to tetrahydrofuran and mixture chloroform-methanol 1:1 v/v.

3.2. Antimicrobial activity

Antimicrobial activity of the tested samples and the solvent was monitored by measuring the diameters of inhibition zones (Table 1), and recorded semi-quantitavely using marks "+" for the lowest activity and "++++" for the highest activity. Correlation of inhibition zone diameters and the corresponding marks is given in Table 1. Results of tested antimicrobial activity for the samples I-IV are shown in Table 2 and for the samples V-VII in Table 3.

Test results of antimicrobial activities of various samples of usnic acid (Table 2) indicate that tested samples I-III have the same inhibiting activities against the selected microorganisms. Usnic acid from mother liquor retained the same activity as the crystallized form. The activity of sample IV (dry residue of mother liquor) is slightly lower against all tested test-microorganisms (Table 2). The reason for this could be that mother liquor and its dry residue do not contain pure usnic acid but also traces of other lichen acids and other impurities. However, its antimicrobial activity was not changed significantly from qualitative or quantitative point of view or with respect to the selected test-microorganisms. The most resistant microorganism was *Sarcina lutea*.

Test results of antimicrobial activity of various samples of Na-salts of usnic acid (V-VII, Table 3) show that the samples of Na-usninate V and VI practically have the same activity, whereas the activity of the sample VII is lower. Generally speaking, all tested samples I-VII show good antimicrobial activity against all tested test-microorganisms, but the samples I-IV containing usnic acid are superior. Significant antimicrobial activity was found in mother liquors of usnic acid and Na-usninate, creating

possibility for their application in pharmaceutical and cosmetic preparations, first of all for external use.

Table 1. Correlation of inhibition zone diameters and antimicrobial activity of usnic acid and its sodium salt samples ("+"-active, "0"-inactive)

Working concentrations (mg/cm^3)	Antimicrobial activity	Diameter of inhibition zone (mm)
From 0.0125 to 0.1000	+	14 - 16
	++	16 - 19
	+++	19 - 22
	++++	22 - 26
	+++++	26 - 30
Solvent	o	o

Table 2. Test results of antimicrobial activity of various usnic acid samples: I-usnic acid; II-sodium salt of usnic acid; III- usnic acid mother liquor; IV-dry residue of mother liquor

Microorganism	Sample	Concentration (mg/cm^3)				Pure solvent
		0.0125	0.025	0.050	0.100	
Escherichia coli ATCC 25922	I	++	+++	++++	+++++	o
	II	++	+++	++++	+++++	o
	III	++	+++	++++	+++++	o
	IV	+	++	+++	++++	o
Bacillus subtilis *ATCC 6633*	I	++	+++	++++	+++++	o
	II	++	+++	++++	+++++	o
	III	++	+++	++++	+++++	o
	IV	+	++	+++	++++	o
Staphylococcus auerus ATCC 6538	I	++	+++	++++	+++++	o
	II	++	+++	++++	+++++	o
	III	++	+++	++++	+++++	o
	IV	+	++	+++	++++	o
Micrococcus flavus ATCC 10240	I	++	+++	++++	+++++	o
	II	++	+++	++++	+++++	o
	III	++	+++	++++	+++++	o
	IV	+	++	+++	++++	o
Micrococcus pyogenes ATCC 6538P	I	++	+++	++++	+++++	o
	II	++	+++	++++	+++++	o

	III	++	+++	++++	+++++	o
	IV	+	++	+++	++++	o
Sarcina lutea ATCC 9341	I	+	++	+++	+++++	o
	II	++	+++	++++	+++++	o
	III	+	++	+++	++++	o
	IV	o	+	++	+++	o
Candida albicans ATCC 10259	I	++	+++	++++	+++++	o
	II	++	+++	++++	+++++	o
	III	++	+++	++++	+++++	o
	IV	+	++	+++	++++	o

Table 3. Test results of antimicrobial activity of various samples of sodium usniate: V- crystal sodium usniate; VI- sodium usniate mother liquor; VII-dry residue of sodium usniate mother liquor

Microorganism	Sample	Concentration (mg/cm^3)				Pure solvent
		0.0125	0.025	0.050	0.100	
Escherichia coli ATCC 25922	V	+	++	+++	++++	o
	VI	+	++	+++	++++	o
	VII	o	+	++	+++	o
Bacillus subtilis *ATCC 6633*	V	+	++	+++	++++	o
	VI	+	++	+++	++++	o
	VII	o	+	++	+++	o
Staphylococcus auerus ATCC 6538	V	+	++	+++	++++	o
	VI	+	++	+++	++++	o
	VII	o	+	++	+++	o
Micrococcus flavus ATCC 10240	V	+	++	+++	++++	o
	VI	+	++	+++	++++	o
	VII	o	+	++	+++	o
Micrococcus pyogenes ATCC 6538P	V	+	++	+++	++++	o
	VI	+	++	+++	++++	o
	VII	o	+	++	+++	o
Sarcina lutea ATCC 9341	V	+	++	+++	++++	o
	VI	+	++	+++	++++	o
	VII	o	+	++	+++	o
Candida albicans ATCC 10259	V	+	++	+++	++++	o
	VI	+	++	+++	++++	o
	VII	o	+	++	+++	o

3.3. Minimal inhibitory concentration

Because of the economic reasons, as well reported liver toxicity, the antimicrobial property of *Usnea barbata* ethanol extract after the isolation of usnic acid (mother liquor) was studied. In this sense, the lichen was extracted with 96% ethanol, during 15 and 30 min, and usnic acid was isolated by recrystallization. The remaining solution was evaporated under reduced pressure in order to obtain the dry residue of mother liquor. The other biological method is with serial dilutions to detect the size of minimal inhibitory concentrations (MIC). The antimicrobial investigations of this samples have shown higher activity of ethanol extract without usnic acid (mother liquor) against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Micrococcus luteus*, *Bacillus subtilis* with MIC of 0.78 μg/cm^3, *Klebsiella pneumoniae* with MIC of 1.56 μg/cm^3, and lower antifungal or antiviral activity than usnic acid (Table 4).

Table 4. Minimal inhibitory concentration of isolated usnic acid ans its mother liquors

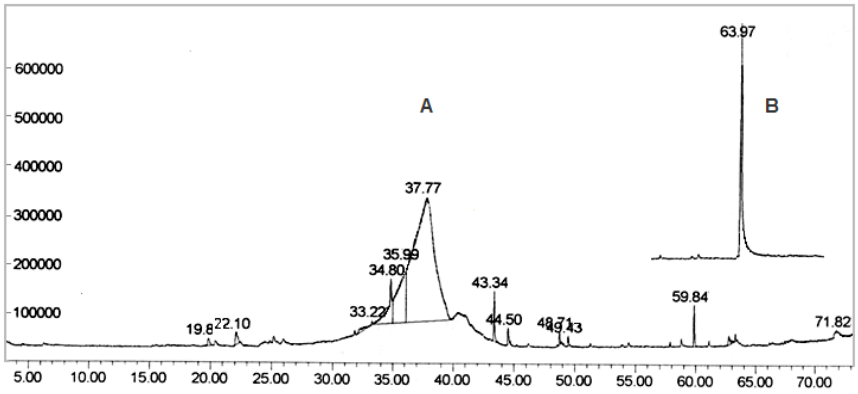
Minimal inhibitory concentration, MIC (μg/cm^3)					
	Usnic acid	* DR 15	* ML 15	* DR 30	* ML 30
Staphylococcus aureus ATCC 25923	1.17	0.78	0.78	0.78	0.78
Staphylococcus epidermidis ATCC 12228	1.17	0.78	0.78	0.78	0.78
Micrococcus luteus ATCC 10240	1.17	0.78	0.78	0.78	1.56
Enterococcus faecalis ATCC 29212	2.34	50	50	1.56	50
Bacillus subtilis ATCC 6633BB	1.17	0.78	0.78	0.78	0.78

Table 4. Continued

Escherichia coli ATCC 25922	0.58	0.78	0.78	0.78	1.56
Klebsiella pneumoniae NCIMB 9111	2.34	1.56	1.56	1.56	1.56
Pseudomonas aeruginosa ATCC 27853	4.68	50	50	50	50
Candida albicans ATCC 10259	37.5	25	50	25	50
Candida albicans ATCC 24433	18.78	25	25	25	50
* ML - mother liquor after 15 or 30 min; DR - dry residue of mother liquor after 15 or 30 min.					

3.4. Identification of mother liquor constituents

The lichen extracts were subject to GC-MS analysis, leading to peak resolution and signal responses that were acceptable for both qualitative identification and quantification purposes. A typical example of a GC-MS chromatogram from the polar phase of a ethanol of lichen is shown in Fig. 2.



Signal : TIC: MLMEOH.D\data.ms

peak #	R.T. min	first scan	max scan	last scan	PK TY	peak height	corr. area	corr. % max.	% of total
1	19.835	2630	2724	2764	BB	15985	1923362	0.64%	0.421%
2	22.109	3053	3094	3139	BB 3	26151	3143811	1.05%	0.687%
3	33.223	4696	4901	4914	BB 3	7116	2787441	0.93%	0.609%
4	34.803	4932	5158	5184	BV 8	90217	20791780	6.93%	4.546%
5	35.998	5184	5352	5370	VV 8	120731	53366154	17.79%	11.668%
6	37.777	5370	5642	5984	VV 8	251472	300040275	100.00%	65.603%
7	43.349	6514	6548	6602	BB 5	102440	5045251	1.68%	1.103%
8	44.504	6714	6736	6755	BV 2	32674	1838691	0.61%	0.402%
9	48.714	7406	7421	7436	BV 9	28195	1249034	0.42%	0.273%
10	49.438	7512	7539	7555	BB 9	20269	877091	0.29%	0.192%
11	59.847	9200	9231	9266	BB	84838	4048911	1.35%	0.885%
12	62.719	9677	9698	9726	BV 5	18704	1536977	0.51%	0.336%
13	63.261	9726	9787	9806	VB 5	16402	1397008	0.47%	0.305%
14	71.823	11070	11179	11242	BB 2	13041	2805868	0.94%	0.613%
1	63.979	9859	9903	10054	BB 2	647297	56503321	18.83%	12.354%

Sum of corrected areas: 457354974

Figure 2. GC-MS total (5-75 min) chromatogram of *Usnea* lichen ethanol extract without usnic acid (mother liquor) (A) and ethanol extract of usnic acid (B), with the corresponding data table. Identification of lichen constituents was performed by demonstration of co-chromatogram spectrum to the standard. Peak identification: xylitol (37.77 min), palmitic acid (43.34 min), stearic acid (48.71 min), and usnic acid (63.97 min)

In the chromatogram, presented on Fig. 2, a complex pattern of major, minor and trace peaks can be observed. If the amount of a specific compound exceeds the column capacity or the linear response range of the detector then a subsequent analysis should be performed with a higher sample dilution. As shown in Fig. 2 the total chromatogram displays the peak profile of a complex mixture. In order to

identify the chemical nature of as many peaks as possible, two strategies were followed. First, the spectra of all identifiable peaks were compared with commercially available electron impact mass spectrum libraries such as NIST for Masslab (Fisons). Secondly, GC-MS analysis was performed using several hundred standard compounds which we assumed to be present in detectable amounts in plant tissues. When these approaches were applied to the investigated plant extracts we could identify a total of 14 compounds (Fig. 2). However, there were still a large number of peaks which were not found either in any of the commercial libraries or in the several hundred compounds that we tested directly. These peaks will require significant further efforts before a chemical name and structure can be assigned to them.

As shown in Fig. 2, we were able to detect only a few main components. The following constituents were identified in the ethanol extract of *Usnea* lichen: xylitol (37.77 min), palmitic acid (43.34 min), stearic acid (48.71 min), and usnic acid (63.97 min). Unidentified components include: mucus, bitter substances, and other lichen acids (probably evernic and ursolic acid). On the basis the present experimental results (Fig. 2), it was concluded that the mother liquor of usnic acid from *Usnea* lichen ethanol extract contained cca. 65.60 % xylitol as the main active component (summary cca. 82.48 % of sugar compounds), cca. 12.38 % some lichen acids and cca. 2 % fatty acids.

3.5. Properties of mother liquor with xylitol

Based on the present experimental results, it was concluded that the mother liquor of usnic acid from *Usnea* lichen ethanol extract contained xylitol (over 60 %) as the main active component, with high antimicrobial activity.

Xylitol is an organic compound with the formula $(CHOH)_3(CH_2OH)_2$. This achiral species is one of four isomers of 1,2,3,4,5-pentapentanol. Pure xylitol is a white crystalline substance that looks and tastes like sugar. Xylitol is a sugar alcohol sweetener used as a naturally occurring sugar substitute. Xylitol is roughly as sweet as sucrose with only two-thirds the food energy. Xylitol is widely distributed throughout nature in small amounts. It is found in the fibers of many fruits and vegetables, including various berries, corn husk, corn fiber, birch, raspberries, plums, oats, corn and mushrooms (Gare F, 2003; Sreenivas-Rao et al, 2006). Nevertheless, xylitol can be produced by hydrogenation of xylose, which converts the sugar (an aldehyde) into a primary alcohol.

Xylitol has no known toxicity in humans. The participants consumed a diet containing a monthly average of 1.5 kg of xylitol with a maximum daily intake of 430 g with no apparent ill effects. Like most sugar alcohols, it has a laxative effect because sugar alcohols are not fully broken down during digestion; albeit one-tenth the strength of sorbitol. Xylitol has virtually no aftertaste, and is advertised as "safe for diabetics and individuals with hyperglycemia." This tolerance is attributed to the lower impact of xylitol on a person's blood sugar, compared to that of regular sugars and also has a very low glycemic index of 13 (glucose has a GI of 100). As most sugar alcohols, initial consumption can result in bloating, diarrhea, and flatulence, although generally rather less so than other sugar alcohols like sorbitol.

It is widely used. Virtually all chewing gum is sweetened with xylitol (Tanzer, 1995). Xylitol is a "tooth-friendly," non-fermentable sugar alcohol (Edwardsson, 1977). A systematic review study (Maguire, 2003) on the efficacy of xylitol has indicated dental health benefits in caries prevention, showing superior performance to other polyols (poly-alcohols). Recent research (Tanzer, 1995) confirms a plaque-reducing effect and suggests that the compound, having some chemical properties similar to sucrose, attracts and then "starves" harmful microorganisms, allowing the mouth to remineralize damaged teeth with less interruption. A recent report suggests that consumption of xylitol may help control oral infections of *Candida* yeast; in contrast, galactose, glucose, and sucrose may increase proliferation. Xylitol also has potential as a treatment for osteoporosis (Mattila, 2002). When

bacteria enter the body they hold on to the tissues by hanging on to a variety of sugar complexes. The open nature of xylitol and its ability to form many different sugar-like structures appears to interfere with the ability of many bacteria to adhere. Xylitol has been found to increase the activity of neutrophils, the white blood cells involved in fighting many bacteria. This effect seems to be quite broad, acting even in cases such as general sepsis (Renko et al, 2008). Another exciting benefit from xylitol is its role in reversing bone loss.

3.6. Purpose of mother liquor with xylitol

On the basis of study, given the high content and antimicrobial activity of xylitol in lichen ethanol extract, can be concluded that mother liquor may be used for the production of different dermatological or oral antiseptic preparation. It can say that due to antimicrobial effects on the tested microorganisms, mother liquor may be used for the topical treatment of oral and skin disorders like acne vulgaris and seborrhoic eczema.

Namely, refined mother liquor of *Usnea* lichen can be conveniently delivered to teeth via chewing gum, tablets, or even candy. In the amounts needed to prevent tooth decay (less than 15 grams per day), xylitol is safe for everyone. On the other hand, chewing gum can be replaced with breath mints, or breath spray with comparable xylitol products. It is not necessary to replace all sweeteners to get the dental benefits of xylitol. Look for xylitol sweetened products that encourage chewing or sucking to keep the xylitol in contact with your teeth. The best items use xylitol as the principal sweetener. Studies show that 4 to 12 grams of xylitol per day are very effective. It is not necessary to use more than 15 grams per day as higher intakes yield diminishing dental benefits.

The refined mother liquor, appropriate dosing forms, can be use for ear, nose and throat infections. Recurring middle ear infections, language infections and nose infections pose a great health threat. One of xylitol's versatile benefits is its ability to inhibit the growth of bacteria that cause middle ear infections and language infections. Regularly washing the nose with a spray containing xylitol can decreases the number of harmful bacteria and stimulates normal defensive washing of this area. A clean nose reduces problems with allergies and asthma that originate from nasal irritants and pollutants. Xylitol has been shown to be effective in inhibiting *Candida albicans*, a serious systemic yeast problem, and other harmful gut bacteria including *H. pylori*, implicated in periodontal disease, bad breath, gastric and duodenal ulcers and even stomach cancer.

The mother liquor concentrate would be suitable not only for the treatment of microbial skin diseases, such as acne and impure skin. Owing to the pronounced antiinflammatory effect independent thereof, the extracts would be also outstandingly suitable for the treatment of inflammatory skin diseases, such as eczema, lichen rubber and psoriasis, in particular if the face and the hair-covered head are affected. Preparations comprising mother liquor concentrate thus provide a basis for the treatment of acne and similar skin diseases having a natural basis. In the mother liquor concentrate used, both an excellent effect against acne bacteria and an excellent effect against pityrosporon yeast fungi are simultaneously displayed. The extract would have an anti-inflammatory effect without irritating the skin.

In addition to the mother liquor concentrate, the potential pharmaceutical or dermatological composition would also contain additives which will be added depending on the chosen pharmaceutical or dermatological formulation. In addition to the base substances for gels, ointments, lotions, tinctures, etc., the pharmaceutical or dermatological compositions can optionally contain preservatives, antioxidants, odorous substances, colorants and the like. It is self-evident that all individual components must sum to 100% by weight of the final preparation.

The mother liquor concentrate according to the study can also be provided in the form of an aqueous gel which acquires a solid structure by addition of suitable substances so that transparent pens which contain the pharmaceutical composition and can be used for covering special skin impurities are

preferably provided. Such pens preferably contain polyhydric aliphatic alcohols having preferably 2-6 carbon atoms, such as ethylene glycol, propylene glycol, trimethylene glycol, glycerol and mixtures of these compounds. For stiffening the aqueous gel, it is also possible to use saturated or unsaturated higher fatty acids having preferably 14 to 22 carbon atoms. Examples of these are myristic acid, palmitic acid, stearic acid, oleic acid, linolenic acid or mixtures of these fatty acids.

In a further embodiment, the mother liquor concentrate according to the study can be incorporated into creams. These are semisolid emulsions which are more fluid than ointments and are easier to distribute over the skin. The base constituents of the creams may absorb relatively small amounts of water and can be processed to give emulsions. As a rule, such creams can be washed off with water and do not block the pores of the skin. They are not greasy and, on application to the skin, have an acceptable appearance. Usually, creams consist of two phases, namely an oil-like internal phase which preferably consists of hydrocarbons and high molecular weight alcohols or fatty acids. Furthermore, the creams contain an aqueous phase which frequently contains preservatives, humectants and buffers. Finally, the creams usually comprise one or more emulsifiers. Frequently, anionic, surface-active agents, such as sodium laurylsulphate or triethanolamine stearate, and nonionic surfactants, such as, for example, ethylene oxide derivatives, are used as emulsifiers. Cationic surfactants, such as quaternary ammonium salts, are less preferred since they can frequently lead to skin irritations.

In a preferred embodiment, the pharmaceutical compositions according to the study can be incorporated into aqueous gels. In the case of the aqueous gels, a liquid, aqueous internal phase is immobilised in a three-dimensional matrix. The system is provided by colloidal dispersion of small inorganic or large organic molecules in an aqueous medium. Nonionic or anionic cellulose derivatives, such as, for example, sodium carboxymethylcellulose, acidic carboxyvinyl polymers or other hydrophilic colloids, such as magnesium aluminium silicate, sodium alginate or tragacanth gum, are used as gel-forming substances.

3. CONCLUSION

There is cumulative resistance against antibiotics of many bacteria. Therefore, the development of new antiseptics and antimicrobial agents for the treatment of oral and skin infections is of increasing interest. In the sense, the ethanol extracts of *Usnea* lichen as mother liquor were screened in the paper. The antimicrobial effects against gram-positive, gram-negative bacteria, fungus and viruse of extracts were compared with isolated antisepical plant compound, usnic acid, and its sodium salt. The antimicrobial activities of analysed extracts were tested using agar broth disc diffusion method and the microdilution method. The extracts were active against tested bacteria: *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermidis, Micrococcus flavus*, *Micrococcus luteus*, *Sarcina lutea*, *Micrococcus pyogenes*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Candida albicans*. The extract was highly active especially against gram-positive bacteria with MIC 0,78 μg/cm^3. It is concluded that due to their antimicrobial effects the tested extracts may be used for the topical treatment of oral and skin disorders like acne vulgaris and seborrhoic eczema.

The both mother liquors of usnic acid and sodium usniate, arising as by-products in the process of isolation and production of these substances, retained a range of antimicrobial activity characteristic for chemically pure substances (i.e. usnic acid and its sodium salt). Although the intensity of their activity is slightly lower, there is a possibility of their use in the pharmaceutical and cosmetic industry as active ingredients of antimicrobial phytopreparations. For the investigation of this possibility, further chemical, pharmaceutical and biomedical studies are required. The use of mother liquors of

usnic acid and sodium usniate would increase the yield of bioactive content of plant raw material *Usnea barbata* by 10-15%.

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