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INVESTIGATION OF SELECTED SERBIAN LICHENS FOR ANTIOXIDANT, ANTIMICROBIAL AND ANTICANCER PROPERTIES

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ABSTRACT

The acetone extracts of the lichens *Parmeliopsis ambigua*, *Parmelia pertusa* and *Hypogymnia physodes* were tested for antioxidant, antimicrobial and anticancer potential. Antioxidant activity was evaluated by measuring free radical and superoxide anion scavenging activity and reducing power. These results revealed that the extract from *Parmeliopsis ambigua* had highest free radical scavenging activity (62.12% inhibition). Moreover, the tested extracts have strong reducing power and superoxide anion radical scavenging. Content of phenols and flavonoids in extracts was estimated as pyrocatechol equivalent, and as rutin equivalent, respectively. Between total phenolic and flavonoid contents and the antioxidant effect of tested extracts, the strong relationships were found. The antimicrobial activity was determined by measuring the minimal inhibitory concentration by the broth microdilution method against *Bacillus mycoides*, *Bacillus subtilis*, *Staphylococcus aureus*, *Enterobacter cloaceae*, *Escherichia coli*, *Klebsiella pneumonia*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Botrytis cinerea*, *Candida albicans*, *Fusarium oxysporum*, *Mucor mucedo*, *Paecilomyces variotii*, *Penicillium purpurescens*, *Penicillium verrucosum* and *Trichoderma harsianum*. The extract of *Parmelia pertusa* was found to be the most active with lowest MIC values (0.78 to 12.5 mg/ml). Anticancer activity was tested by using the microculture tetrazolium test on FemX (human melanoma) and LS174 (human colon carcinoma) cell lines. The extracts tested has strong anticancer activity against both cell lines with IC_{50} values ranging from 6.84 to 43.45 µg/ml.

Key words: Anticancer activity; Antimicrobial activity; Antioxidant activity; Lichens.

INTRODUCTION

Nowadays, natural products have very important role in the drug discovery for the curing of human, animal and plant diseases. Numerous side effects and sometimes resistance are common consequence the long-term use of synthetic drugs (Karaman *et al.,* 2003). Different from synthetic drugs, bioactive natural products have positive influence on the whole organism and without side effects. In search for new bioactive natural products, many of research teams have focused attention on lichens.

Lichens are complex symbiotic associations between algae and fungi which are important constituents of many ecosystems. These organisms are used to human and animal nutrition, for obtaining colors, in alcohol and perfumes production. Lichen and lichen products which for centuries have been used in traditional medicines and still have large importance as alternative treatments in many countries in the world (Huneck, 1999). Numerous researches have found various biological activities of lichens, which justified the long-term use of some lichens in the traditional medicine.

Lichen substances are secondary metabolites of lichens, which include dibenzofurans, depsidones, xanthones and terpene derivatives. Lichens and their metabolites have multiple biological activities: antiviral (Esimone *et al.,* 2007), antibiotic (Ranković *et al.,* 2010), antitumor (Manojlović *et al.,* 2010), antiherbivore (Lawrey, 1983), ecological roles (Richardson, 1992) and enzyme inhibitory (Romagni *et al.,* 2000). The therapeutic potential of many lichens and their metabolites has largely remained unexplored, due to a relatively recent resurgence in lichen bioactivity. For that reason, the aim of present investigation is to estimate the antioxidant, antimicrobial and anticancer activities of the lichens *Parmeliopsis ambigua*, *Parmelia pertusa* and *Hypogymnia physodes*.

MATERIALS AND METHODS

Lichen samples: Lichen samples of *Parmeliopsis ambigua* (Wulf.) Nyl., *Parmelia pertusa* (Taylor), and *Hypogymnia physodes* (L) Nyl., were collected from Kopaonik, Serbia, in September of 2010. The voucher specimen of the lichens (Voucher No. 52, 58 and 61) was deposited at the Department of Biology and Ecology, Faculty of Science, University of Kragujevac, Serbia. Identification of the tested lichens was carried out with standard keys from Purvis *et al*. (1992) and Wirth (1995).

Preparation of the lichen extracts: Finely dry ground thalli of the examined lichens (50 g) were extracted using acetone (200 ml) in a Soxhlet extractor. The extracts were filtered then concentrated under reduced pressure in a

rotary evaporator. The dry extracts were stored at -18°C until they were used in the tests. The extracts were dissolved in 5% dimethyl sulphoxide (DMSO) for further testing.

Antioxidant activity: Antioxidant activity was evaluated by free radical scavenging, superoxide anion radical scavenging and reducing power. The free radical scavenging activity of lichen extracts was measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH) according to the Ranković *et al's* method (2012). Oyaizu method (1986) was used to determine the reducing power. The superoxide anion radical scavenging activity was detected according to the Nishimiki *et al's* method (1972).

Determination of total phenolic and flavonoid compounds: The amount of total phenols in the lichen extracts was determined as pyrocatechol equivalent using Folin-Ciocalteu reagent according to Slinkard and Singleton's method (1997). Dowd method (Meda *et al.,* 2005) was used for the quantification of total flavonoid in the extracts with rutin as standard.

Antimicrobial activity: The following bacteria were used as test organisms in this study: *Bacillus mycoides* (IPH 197), *Bacillus subtilis* (IPH 189), and *Staphylococcus aureus* (IPH 221) (gram-positive bacteria); *Enterobacter cloaceae* (IPH 241), *Escherichia coli* (IPH 246), and *Klebsiella pneumoniae* (IPH 251) (gram-negative bacteria). All the bacteria used were isolates of the Institute for Protection of Health in Kragujevac (IPH). The fungi used as test organisms were: *Aspergillus flavus* (ATCC 9170), *Aspergillus fumigatus* (DBFS 310), *Botrytis cinerea* (DBFS 133), *Candida albicans* (IPH 1316), *Fusarium oxysporum* (DBFS 292), *Mucor mucedo* (ATCC 52568), *Paecilomyces variotii* (ATCC 22319), *Penicillium purpurescens* (DBFS 418), *Penicillium verrucosum* (DBFS 262), and *Trichoderma harsianum* (DBFS 379). Among the fungi were agents of human, animal, and plant diseases; mycotoxin producers; and food spoilage agents. They were from the mycological collection maintained by the Mycological Laboratory within the Department of Biology of Kragujevac University's Faculty of Science (DBFS).

Bacterial inoculi were obtained from bacterial cultures incubated for 24 h at 37°C on Müller-Hinton agar substrate and brought up by dilution according to the 0.5 McFarland standard to approximately 10^8 CFU/ml. Suspensions of fungal spores were prepared from freshly mature (3- to 7-day-old) cultures that grew at 30°C on a potato dextrose agar substrate. The spores were rinsed with sterile distilled water, used to determine turbidity spectrophoto metrically at 530 nm, and were then further diluted to approximately 10^6 CFU/ml according to the procedure recommended by NCCLS (1998).

Broth microdilution method using 96-well micro-titer plates (Sarker *et al.,* 2007), was used to determinate the minimal inhibitory concentration (MIC). Two-fold dilutions of extracts were prepared in Müller-Hinton broth for bacterial cultures and SD broth for fungal cultures. The MIC was determined with resazurin.

Anticancer activity: The human melanoma FemX and human colon carcinoma LS174 cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). Both cancer cell lines were maintained in the recommended RPMI-1640 medium supplemented with 10% heat-inactivated (56ºC) fetal bovine serum, l-glutamine (3mM), streptomycin (100 mg=ml), penicillin (100 IU=ml), and 25mM HEPES and adjusted to pH 7.2 by bicarbonate solution. Cells were grown in a humidified atmosphere of 95% air and 5% CO_2 at 37ºC.

Stock solutions (100mg/ml) of extracts, made in dimethylsulfoxide (DMSO), were dissolved in corresponding medium to the required working concentrations. Neoplastic FemX cells (5000 cells per well) and neoplastic LS174 cells (7000 cells per well) were seeded into 96-well microtiter plates. Twenty-four hours later, after the cell adherence, five different concentrations of investigated extracts were added to the wells, except for the control cells to which a nutrient medium was added only. Final concentrations achieved in treated wells were 200, 100, 50, 25, and 12.5µg/ml. Nutrient medium was RPMI 1640 medium, supplemented with l-glutamine (3mM), streptomycin (100 lg/ml), and penicillin (100 IU/ml), 10% heat inactivated (56˚C) fetal bovine serum (FBS) and 25 mM Hepes, and was adjusted to pH 7.2 by bicarbonate solution. The cultures were incubated for 72 hrs.

Microculture tetrazolium test (MTT), according to Mosmann (1983) with modification by Ohno & Abe (1991), 72 h upon addition of the compounds, was used to determining the effect of extracts on cancer cell survival. IC_{50} concentration was defined as the concentration of an agent inhibiting cell survival by 50%, compared with a vehicle-treated control.

Statistical analyses: Statistical analyses were calculated using Excel software (Microsoft 2007) and SPSS version 16.0 for Windows 2007 (Ranković *et al.,* 2012). Statistical significance was determined by using student's t-test. Correlation coefficients (r) were determined by using Pearson's bivariate correlation test. All the results are shown as mean ± standard deviation (SD) of three parallel measurements.

RESULTS AND DISCUSSION

Antioxidant activity: The scavenging DPPH radicals, superoxide anion radical scavenging and reducing power of the studied lichen extracts are represented in Figure 1, Figure 2 and Figure 3.

Largest DPPH radical scavenging activity (62.12%) showed extract from lichen *Parmeliopsis*

ambigua. The extract of the lichen *Parmelia pertusa* demonstrated strongest reducing power, while maximum superoxide anion scavenging activity (55.09%) was also in *Parmelia pertusa* extract. In different antioxidant activities, statistically significant difference was found between extracts and control ($P<0.05$). Antioxidant activities were compared to ascorbic acid, butylated hydroxyanisole (BHA) and α-tocopherol. The results showed that tested extracts had a slightly weaker activity than standard antioxidants.

Total phenolic and flavonoid content of the tested extracts are given in Table 1. Phenolic content for tested extracts is obtained from the regression equation of calibration curve of pyrocatechol (y = 0.0021 x total phenols [µg PE/mg of dry extracts] - 0.0092, R^2 = 0.9934). Highest phenolic components were identified in acetone extract of *Parmeliopsis ambigua* at a 45.86 µg PE/mg. The concentrations of the flavonoids were calculated according to the equation that was obtained from the standard rutin graph (y = 0.0144 x total flavonoid [µg RE/mg of dry extracts] + 0.0556, R^2 = 0.9992). Flavonoid content for acetone extracts of *Parmeliopsis ambigua*, *Parmelia pertusa* and *Hypogymnia physodes* were 31.52, 20.09 and 25.81 µg RE/mg, respectively.

Correlation coefficient between free radical scavenging activity and phenolic and flavonoid compounds of the tested extracts were r=0.93 and r=0.92, respectively.

The antioxidative nature of the tested extracts might depend on its phenolics. We found that the tested extracts with the greatest amount of phenolic content exhibited the highest antioxidant activity. These results agree with numerous other studies for the antioxidant activity of extracts with high content of phenolic compounds (Ranković *et al.*, 2010; Odabasoglu *et al.*, 2004). In most lichens, phenols are important antioxidants because of their ability to donate hydrogen to free radicals and so stop the chain reaction of lipid oxidation at the initial stage (Ranković *et al.*, 2010). However, some researchers have found that the antioxidant activity of extracts did not always correlate with the content of phenolics, suggesting that the antioxidant activity of different lichens may also depend on other, non-phenol components. (Odabasoglu *et al.*, 2004).

Antioxidant activity of some other lichens has been studied by other researchers (Manojlović *et al.*, 2010; Praveen Kumar *et al.*, 2010). In comparison with the results obtained in experiments with other lichens, we noticed that the tested samples showed a relatively powerful antioxidant activity.

Antimicrobial activity: The antimicrobial activity of the tested lichen extracts against the tested microorganisms was shown in the Table 2. The MIC for the extracts in the tested bacteria was 0.78 to 6.25 mg/ml, while MIC for fungi ranged from 1.56 to 25 mg/ml. The strongest antimicrobial activity showed extracts from lichen *Parmelia pertusa*, which inhibited the tested microorganisms in relatively low concentrations. The antimicrobial activity was compared with streptomycin (antibiotic) and ketoconazole (antimycotic). The results showed that standard antibiotics had stronger activity than tested extracts.

The extracts used in this study show a relatively strong antimicrobial activity. The tested extracts had a stronger antibacterial than antifungal activity, and Gram-positive bacteria are more sensitive to the antibacterial activity than the Gram-negative bacteria. These results could be expected due to the fact that numerous tests proved that bacteria are more sensitive to the antibiotic compared to fungi (Sarnthima & Khammuang, 2012), The reason for different sensitivities between fungi and bacteria can be found in different permeabilities of the cell wall.

Numerous taxonomic similar lichens have been screened for antimicrobial activity in a search for new antimicrobial agents (Ranković *et al.*, 2010; Goel *et al.*, 2011). Compared with their results, the results of this research suggest that the tested samples showed a relatively showed relatively strong antimicrobial activity.

Anticancer activity: The anticancer activity of the lichen extracts against the cell lines tested is shown was in Table 3. The IC_{50} against FemX and LS174 cell lines varied from 6.84 to 43.45 µg/ml. The best anticancer activity was exhibited the *Parmelia pertusa* lichen. Positive control (Cis-DDP) had slightly better anticancer activity than tested lichen extracts.

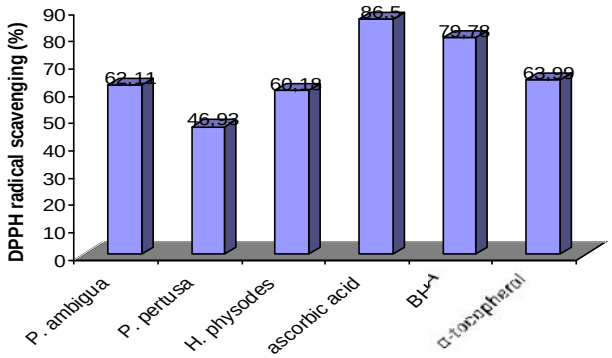

Figure 1. DPPH radical scavenging of the acetone extracts of the lichens *Parmeliopsis ambigua, Parmelia pertusa* and *Hypogymnia physodes* and standards.

Until now, only few researchers have proved that lichen extracts have some anticancer activity (Manojlović *et al.*, 2010; Trigiani *et al.*, 2009). In comparison with their results, the results of our research

suggest that the tested lichen showed strong anticancer activity.

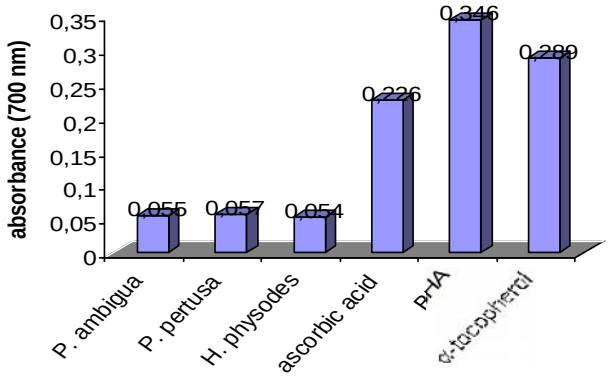

Figure 2. Reducing power of the acetone extracts of the lichens *Parmeliopsis ambigua, Parmelia pertusa* and *Hypogymnia physodes* and standards.

The obtained data indicates that *P. pertusa* extract has remarkable cytotoxic activity, since they display very low IC_{50} values. Finally, *P. pertusa* extract are considered as agent with potential antitumor activity, and can therefore be candidate for further stages of screening *in vitro* and/or *in vivo.*

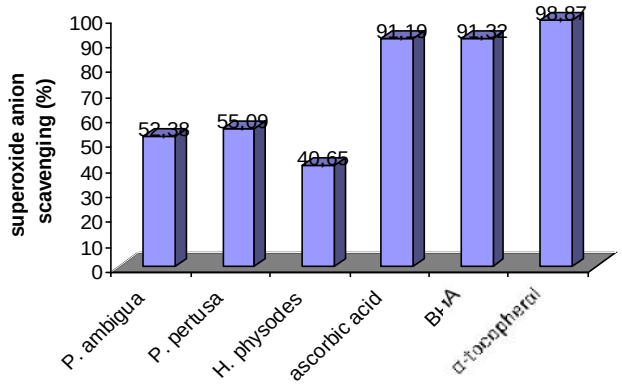

Figure 3. Superoxide anion scavenging of the acetone extracts of the lichens *Parmeliopsis ambigua, Parmelia pertusa* and *Hypogymnia physodes* and standards.

Table 1. Total phenolics and flavonoid content of acetone extracts of *Parmeliopsis ambigua, Parmelia pertusa* and *Hypogymnia physodes*

Lichen species	Phenolics content (µg PE/mg of extract)	Flavonoid content (µg RE/mg of extract)
P. ambigua	45.86 ± 1.191	31.52 ± 1.066
P. pertusa	30.00 ± 1.269	20.09 ± 1.113
H. physodes	38.22 ± 1.211	25.81 ± 1.195

Table 2. Minimum inhibitory concentration (MIC) of acetone extracts of *Parmeliopsis ambigua, Parmelia pertusa* and *Hypogymnia physodes*

Lichen species	*P. ambigua*	*P. pertusa*	*H. physodes*	S	K
B. mycoides	1.56[a]	0.78	3.12	7.81	-
B. subtilis	1.56	0.78	6.25	7.81	-
E. cloacae	1.56	0.78	1.56	1.95	-
E. coli	-	-	6.25	31.25	-
K. pneumoniae	1.56	0.78	3.12	1.95	-
S. aureus	1.56	0.78	3.12	31.25	-
A. flavus	25	6.25	25	-	3.9
A. fumigatus	25	6.25	12.5	-	3.9
B. cinerea	12.5	1.56	3.12	-	1.95
C. albicans	25	1.56	3.12	-	1.95
F. oxysporum	25	6.25	12.5	-	3.9
M. mucedo	25	6.25	12.5	-	31.25
P. variotii	25	3.12	6.25	-	1.95
P. purpurescens	25	12.5	12.5	-	3.9
P. verrucosum	25	12.5	12.5	-	3.9
T. harsianum	25	6.25	25	-	7.81

[a]Minimum inhibitory concentration (MIC); values given as mg/ml for lichen extract and as µg/ml for antibiotics. Values are the mean of three replicate Antibiotics: K – ketoconazole, S – streptomycin

Table 3. Growth inhibitory effects of acetone extracts of *Parmeliopsis ambigua, Parmelia pertusa* and *Hypogymnia physodes* on FemX and LS 174 cell lines

Lichen species	FemX	LS 174
	IC_{50} (µg/ml)	
P. ambigua	10.25 ± 1.62	9.82 ± 1.77
P. pertusa	6.84 ± 0.18	8.59 ± 0.96
H. physodes	43.45 ± 2.58	38.08 ± 0.66
Cis-DDP	0.94 ± 0.35	2.3 ± 0.31

Conclusion: Our experiment aims to justify the traditional use of lichens as complementary and alternative medicines. Based on the results in used tests, it can be stated that studied lichen extracts have different and relatively strong antioxidant, antimicrobial, and anticancer activities. The presence of different bioactive components in tested extracts is probably a cause of differences in biological activities of different extracts. Further investigation is required to identify active compounds in these lichen extracts and isolate and characterize the individual bioactive. Identification of the active compounds of lichen will lead to their evaluation in considerable commercial potential in medicine, food production and the cosmetic industry.

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REFERENCES

Dorman, H.J., O. Bachmayer, M. Kosar and R. Hiltunen (2004). Antioxidant properties of aqueous extracts from selected Lamiaceae species grown in Turkey. J. Agr. Food. Chem. 52: 762–770.

Esimone, C.O., K.C. Ofokansi, M.U. Adikwu, E.C. Ibezim, D.O. Abonyi, G.N. Odaibo and D.O. Olaleye (2007). In vitro evaluation of the antiviral activity of extracts from the lichen Parmelia perlata (L.) Ach. against three RNA viruses. J. Infect. Dev. Ctries. 1: 315-320.

Goel, M., P. Dureja, A. Rani, P.L. Uniyal and H. Laatsch (2011). Isolation, Characterization and Antifungal Activity of Major Constituents of the Himalayan Lichen *Parmelia reticulata Tayl.*†. J. Agr. Food Chem. 59: 2299-2307.

Huneck, S. (1999). The significance of lichens and their metabolites. Naturwissenschaften. 86: 559-570.

Karaman, I., F. Sanin, M. Güllüce, H. Ogutcu, M. Sengul and A. Adigüzel (2003). Antimicrobial activity of aquaeos and methanol extracts of *Juniperus oxicedrus* L. J. Ethnopharmacol. 85: 231-235.

Lawrey, J.D. (1983). Vulpinic and pinastric acids as lichen antiherbivore compounds: contrary evidence. Bryologist 86: 365-369.

Manojlović, N., P. Vasiljević, M. Jusković, S. Najman, S. Janković and A. Milenkovic-Andjelković (2010). HPLC analysis and anticancer potential of extracts from the lichen, *Thamnolia vermicularis var. Subuliformis*. J. Med. Plants Res. 4: 817-823.

Manojlović, N., P. Vasiljević, W. Gritsanapan, R. Supabphol and I. Manojlović (2010). Phytochemical and antioxidant studies of *Laurera benguelensis* growing in Thailand. Biol. Res. 43: 169-176.

Meda, A., C.E. Lamien, M. Romito, J. Millogo and O.G. Nacoulma (2005). Determination of the total phenolic, flavonoid and proline contents in burkina fasan honey, as well as their radical scavenging activity. Food. Chem. 91: 571–577.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and Anticancerity assays. J. Immunol. Methods. 65: 55-63.

NCCLS (National Commitee for Clinical Laboratory Standards), (1998). Reference Method for Broth Dilution Antifungal Susceptibility Testing of Conidium-forming Filamentous Fungi: Proposed Standard M38-P. Wayne, PA, USA.

Nishimiki, M., N.A. Rao and K. Yagi (1972). The occurrence of super-oxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. Biochem. Biophys. Res. Commun. 46: 849-853.

Odabasoglu, F., A. Aslan, A. Cakir, H. Suleyman, Y. Karagoz, M. Halici and Y. Bayir (2004). Comparison of antioxidant activity and phenolic content of three lichen species. Phytother. Res. 18: 938-941.

Ohno, M and T. Abe (1991). Rapid colorimetric assay for the quantification of leukemia inhibitory factor (LIF) and interleukin-6 (IL-6). J. Immunol. Methods. 145: 199-203.

Oyaizu, M. (1986). Studies on products of browning reaction prepared from glucoseamine. Jpn. J. Nutr. 44: 307-314.

Praveen Kumar, S.V., T.R. Prashith Kekuda, K.S. Vinayaka and S.J. Sudharshan (2010). Anthelmintic and Antioxidant efficacy of two Macrolichens of Ramalinaceae. Pharmacogn. J. 1: 4.

Purvis, O.W., B.J. Coppins, D.L. Hawksworth, P.W. James and D.M. Moore (1992). The Lichen Flora of Great Britian and Ireland. Natural History Museum Publications in association with the British Lichen Society, London

Ranković, B., D. Ranković, M. Kosanić and D. Marić (2010). Antioxidant and antimicrobial properties of the lichen *Anaptychya ciliaris*, *Nephroma parile*, *Ochrolechia tartarea* and *Parmelia centrifuga*. Cent. Eur. J. Biol. 5: 649-655.

Ranković, B., M. Kosanić, T. Stanojković, P. Vasiljević and N. Manojlović (2012). Biological Activities of *Toninia candida* and *Usnea barbata* Together with Their Norstictic Acid and Usnic Acid Constituents. Int. J. Mol. Sci. 13: 14707-14722.

Richardson, D.H.S. (1992). Pollution Monitoring with Lichens. *Naturalists' Handbook 19*. Richmond Publishing Co. Ltd., England.

Romagni, J.G., G. Meazza, N.P.D. Nanayakkara and F.E. Dayan (2000). The phytotoxic lichen metabolite, usnic acid, is a potent inhibitor of plant p-hydroxyphenylpyruvate dioxygenase. FEBS Lett. 480: 301–305.

Sarker, S.D., L. Nahar and Y. Kumarasamy (2007). Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the *in vitro* antibacterial screening of phytochemicals. Methods. 42: 321-324.

Sarnthima, R. and S. Khammuang (2012). Antibacterial activities of *Solanum stramonifolium* seed extract. Int. J. Agric. Biol. 14: 111–115.

Slinkard, K and V.L. Slingleton (1997). Total phenolic analyses: automation and comparison with manual method. Am. J. Enol. Viticult. 28: 49-55.

Triggiani, D., D. Ceccarelli, A. Tiezzi, T. Pisani, S. Munzi, C. Gaggi and S. Loppi (2009). Antiproliferative activity of lichen extracts on murine myeloma cells. Biologia. 64: 59-62.

Wirth, V. (1995). Die Flechten Baden-Württembergs-Teil 1 und 2. Vergal Ulmer, Stuttgart.