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Proapoptotic and Antimigratory Effects of *Pseudevernia furfuracea* and *Platismatia glauca* on Colon Cancer Cell Lines

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SUMMARY

The aim of this study is to investigate cytotoxic, proapoptotic, antimigratory and pro-antioxidant effects of methanol, acetone and ethyl acetate extracts of lichens *Pseudevernia furfuracea* and *Platismatia glauca* on colorectal cancer (HCT-116 and SW-480) cell lines. We compared the cytotoxic effects on colorectal cancer cells with the effects obtained from normal human fibroblast (MRC-5) cell line. Tetrazolium (MTT) test evaluated the cytotoxic effects, Transwell assay evaluated cell migration, acridine orange/ethidium bromide (AO/EB) fluorescent method followed the apoptosis, while prooxidant/antioxidant effects were determined spectrophotometrically through concentration of redox parameters. The tested extracts showed considerable cytotoxic effect on cancer cells with no observable cytotoxic effect on normal cells. Ethyl acetate and acetone extract of *P. furfuracea* induced the highest cytotoxicity (IC_{50}=(21.2±1.3) μg/mL on HCT-116, and IC_{50}=(51.3±0.8) μg/mL on SW-480 cells, respectively, after 72 h), with noteworthy apoptotic and prooxidant effects, and antimigratory potential of methanol extract. *P. glauca* extracts induced cytotoxic effects on HCT-116 cells after 72 h (IC_{50}<40 μg/mL), while only methanol and acetone extracts had cytotoxic effects on SW-480 cells after 24 h, with proapoptotic/necrotic activity, as a consequence of induced oxidative stress. In conclusion, lichen extracts changed to a great extent cell viability and migratory potential of colorectal cancer cell lines. HCT-116 cells were more sensitive to treatments, *P. furfuracea* had better proapoptotic and antimigratory effects, and both investigated lichen species might be a source of substances with anticancer activity.

Key words: apoptosis, colorectal cancer, cytotoxic effects, lichen extracts, cell motility, redox status

INTRODUCTION

Colorectal cancer (CRC) is among major causes of mortality in the world, and the second cause of cancer-related death (*1*). Carcinogenesis is a multistage process with progressive changes at the cellular, genetic and epigenetic level. The main characteristics of cancer cells are proliferation, inactivated tumour suppressor genes, suppressed and inactivated genes and pathways responsible for cell death and capacity to migrate to other organs (*2*). Generally, treatment options for CRC patients are surgery, chemotherapy and radiation. Novel anticancer therapeutic strategies find basis in the activation of programmed cell death (apoptosis) and the suppression of cell migration (*2*). The activation of apoptosis is a key molecular mechanism responsible for anticancer activities of most of the currently studied potential anticancer agents (*2*). Oxidative stress and reactive oxygen species (ROS) as signal molecules have a major role in many physiological and pathological processes in cells (*3*). Overproduction of ROS induces the cytotoxic activity and apoptosis induction by various anticancer agents (*e.g.* cisplatin, camptothecin, inostamycin, doxorubicin and mitomycin C) (*4*), and some active compounds isolated from natural sources (*5*). Also, the reduction of cell migration, as a critical process in cancer development, depends on the production of ROS and reactive nitrogen species (RNS) in the treatments with anticancer drugs (*6*).

Natural sources (plants, mushrooms and lichens) contain different active substances with antitumour activity. Great number of drugs isolated from natural sources are approved

for use in traditional medicine (*7*). Lichens have been widely used in the folk medicine as laxatives and antiemetic, which indicates their application in treatments of gastrointestinal disorders (*8*). Lichens synthesize a plenty of secondary metabolites with diverse biological activities (*9*). Also, many studies have shown that extracts of lichen species obtained from different areas in Serbia had antioxidant, antimicrobial and antiproliferative potential (*10-12*). *Pseudevernia furfuracea* (L.) Zopf. demonstrated a notable cytotoxic effect (in acetone extract) on the FemX (human melanoma) and LS174 (human colon carcinoma) cell lines (*11*). In addition, *Platismatia glauca* (L.) W.L. Culb. & C.F. Culb. had considerable cytotoxic effects (in *n*-hexane extracts) on L1210 (murine leukemia), 3LL (murine Lewis lung carcinoma) and U251 (human glioblastoma) (*13*). The literature data confirm that some active substances, such as acids isolated from lichens, can induce apoptosis in murine leukemia L1210 cell (*13*).

Recently, Mitrović *et al*. (*14*) have shown that extracts of *P. glauca* and *P. furfuracea* had antioxidant, antimicrobial and antibiofilm effects, and also gave the chemical profile of these extracts. The goal of this study is to evaluate cytotoxic, proapoptotic, antimigratory and pro-antioxidant impact of the methanol, acetone end ethyl acetate extracts of *P. furfuracea* and *P. glauca* lichen species from Serbia on two colorectal cancer cell lines (HCT-116 and SW-480). For estimation of antitumour effects, we also evaluated cytotoxicity of lichen extracts on normal human fibroblast MRC-5 cell line.

MATERIALS AND METHODS

Lichen samples and extraction

The lichen samples of *Pseudevernia furfuracea* (L.) Zopf. and *Platismatia glauca* (L.) W.L. Culb. & C.F. Culb. were collected from two locations on Tara mountain, Kaludjerske bare, Solotuša, Serbia: 43°53′17.7″N, 19°33′21.2″E and 43°53′32.2″N, 19°33′25.0″E, as described by Mitrović *et al.* (*14*), who also described the procedure of extraction of powdered (A 11 basic analytical mill, IKA, Staufen, Germany) lichen material with different solvents (methanol, acetone and ethyl acetate; Zorka Pharma, Šabac, Serbia) (*14*). During the extraction, the extracts were filtered through the Whatman no. 1 filter paper (Sigma Chemicals Co., Merck, St Louis, MO, USA) and then concentrated in a rotary vacuum evaporator (WU-28710-00; IKA). Lichen extracts were dissolved in 0.5 % dimethyl sulfoxide (DMSO) (*15*) and conditional Dulbecco's modified Eagle medium (DMEM) with 10 % fetal bovine serum (FBS), 100 IU/mL penicillin and 100 μg/mL streptomycin (Gibco, Invitrogen, Thermo Fisher Scientific, New York, NY, USA) in final concentration of 1 mg/mL.

Cell culture

The human colon cancer HCT-116, SW-480 and normal human fibroblast MRC-5 cell lines were obtained from the American Tissue Culture Collection, Manassas, VA, USA. The cells were maintained and seeded in a 96-well plate as described by Šeklić *et al.* (*16*). MRC-5 cell line was seeded only for tetrazolium (MTT) assay. Untreated cells served as negative control. The results for MTT, acridine orange/ethidium bromide (AO/EB) staining, nitroblue tetrazolium (NBT), Griess and glutathione (GSH) tests were obtained after 24 and 72 h. For Transwell migration assay the results were measured 24 h after incubation.

Cytotoxicity assay

Cell viability was determined by MTT assay as described by Šeklić *et al*. (*16*). The concentrations of the three used lichen extracts (methanol, acetone and ethyl acetate) were 1, 10, 50, 100, 250 and 500 μg/mL. The results were presented as percentage of viability (ratio between the absorbance of treated and control cells). IC_{50} values (the extract dose that inhibited cell growth by 50 %) served as the measure of cytotoxicity and were calculated by CalcuSyn v. 2.1 (*17*).

Fluorescence microscopic analysis of cell death

For the analysis of the stages of cell death in control and treated cells, we used AO/EB and double staining fluorescent microscopic assays according to the procedure described by Mitrović *et al.* (*12*). For this method, HCT-116 and SW-480 cells were treated with 100 μL of each extract at two selected concentrations (10 and 100 μg/mL). The cells were observed under the fluorescence microscope Eclipse Ti (Nikon Instruments Inc., Melville, NY, USA) at 400× magnification using Nikon NIS-Elements Advanced Research software v. 3.2 (*18*).

Transwell migration assay

Transwell migration assay (*19*) measured the effects of lichen extracts on the cell migration. Šeklić *et al.* (*16*) described a detailed protocol. The HCT-116 and SW-480 cells were treated with two selected concentrations (10 and 50 μg/mL) of methanol lichen extracts, and the results were presented as index of migration/absorbance (*A*) (*19*), and (index of migration/*A*)/*N*(viable cells), respectively.

Determination of redox status parameters

Monitoring the concentrations of superoxide anion radical, nitrites and reduced glutathione (GSH) following detailed protocols described by Šeklić *et al.* (*16*) helped determine the prooxidant/antioxidant status. These parameters were determined in the four selected concentrations of each extract (1, 10, 50 and 100 μg/mL).

The colour reaction in MTT, Transwell, NBT, nitrites and GSH tests was measured spectrophotometrically on RT-2100C ELISA microplate reader (Hamburg, Germany). The results for NBT, nitrites and GSH tests were expressed in μM per number of viable cells on the basis of data given in MTT test according to Živanović *et al.* (*20*).

Statistics

All data are presented as the mean value±standard error (S.E.) of three independent experiments, in triplicate for each dose. Student's *t*-test and one-way ANOVA for multiple comparisons evaluated statistical significance using SPSS for Windows, v. 17 (*21*).

RESULTS AND DISCUSSION

Cytotoxic activity

Fig. 1 shows the effects of treatments with methanol, acetone and ethyl acetate extracts of *Pseudevernia furfuracea* and *Platismatia glauca* on colorectal cell viability, while **Table 1** shows cytotoxic activities of the tested extracts, expressed *via* IC_{50} values. All treatments considerably decreased cell viability in dose- and time-dependent manner (except the *P. glauca* extracts in lower dose on SW-480 cells) of tested cancer cell lines (**Fig. 1**) and had moderate effect on normal MRC-5 cells (**Table 1**).

The most significant cytotoxic effect was observable of ethyl acetate extract of *P. furfuracea* on HCT-116 cells (IC_{50}=(21.2±1.3 IC_{50} μg/mL)) after 72 h. Acetone extract of *P. furfuracea* after 72 h had the highest cytotoxic effect on SW-480 cells. All extracts of *P. glauca* demonstrated significant cytotoxic effect (IC_{50}<40 μg/mL) on HCT-116 cell line after 72 h, while methanol and acetone extracts of *P. glauca* had significant cytotoxic effects on SW-480 cells after 24 h. The literature data defined criteria for cytotoxicity of the extracts as IC_{50}<30 μg/mL (*22*) and according to this criterion, ethyl acetate extract of *P. furfuracea* expressed significant cytotoxicity on HCT-116 cell line. As our results showed, all tested samples demonstrated cytotoxic effect on both cell lines, while HCT-116 cells were more sensitive to treatments.

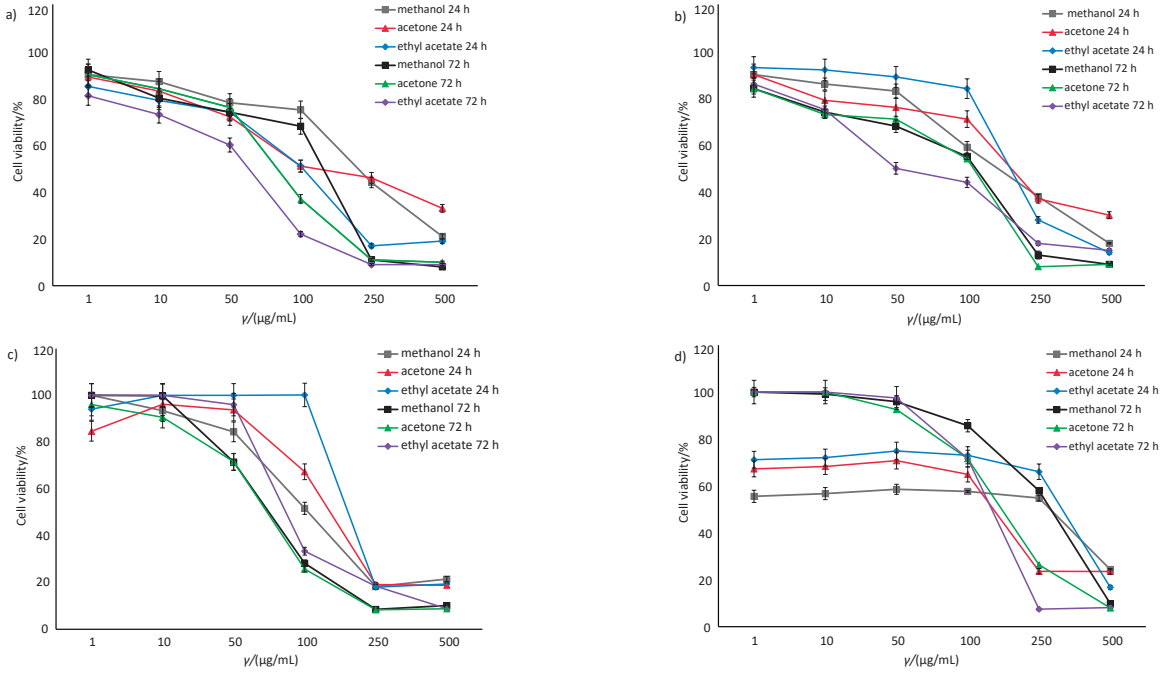

Fig. 1. The effects of methanol, acetone and ethyl acetate extracts of *Pseudevernia furfuracea* and *Platismatia glauca* on HCT-116 and SW-480 colorectal cancer cell viability: a) HCT-116 cells, *P. furfuracea*, b) HCT-116 cells, *P. glauca*, c) SW-480 cells, *P. furfuracea*, and d) SW-480 cells, *P. glauca*. All values are mean±S.E., *N*=3

Table 1. Growth inhibitory effects, expressed as IC_{50} values (μg/mL), of methanol, acetone and ethyl acetate extracts of *Pseudevernia furfuracea* and *Platismatia glauca* extracts on HCT-116, SW-480 and MRC-5 cell lines after 24 and 72 h of exposure

Extract		*t*/h	IC_{50}/(μg/mL)		
			HCT-116	SW-480	MRC-5
P. furfuracea	methanol	24	215.7±12.9	105.0±2.6	226.8±0.6
		72	57.8±9.1	77.9±1.1	371.8±0.5
	acetone	24	176.4±9.1	86.6±1.0	225.7±0.8
		72	41.9±6.6	51.3±0.8	201.4±1.1
	ethyl acetate	24	62.4±5.5	294.46±0.05	394.3±0.9
		72	21.2±1.3	142.0±0.8	256.0±0.1
P. glauca	methanol	24	115.3±12.9	56.4±1.5	414.3±0.1
		72	38.2±4.5	197.1±0.5	389.1±0.6
	acetone	24	205.0±12.0	60.2±0.3	246.1±1.0
		72	34.3±2.5	206.9±0.1	380.93±0.06
	ethyl acetate	24	184.9±12.9	421.2±2.5	442.2±0.8
		72	37.7±4.1	173.8±1.6	216.6±0.4

Results are expressed as the mean value±S.E. of three independent experiments

Concerning the results obtained with *P. glauca*, the present study revealed significant cytotoxicity of all tested extracts on HCT-116 cells after 72 h, while the highest effect of methanol and acetone extracts was on SW-480 cells after 24 h. Based on the obtained results, we can conclude that the *P. glauca* extract induces a significantly acute cytotoxic effect. The literature data show that extracts of lichen may have significant cytotoxic effects on colon cancer cells after 24 h (*23*). The investigated treatment with *P. glauca* in low concentrations activated proliferation in survivor cells and the effects of proliferation were measured after 72 h (higher IC_{50} values after 72 h).

In our earlier study, chemical profiling of *P. glauca* extract revealed dimethyl caperate, atraric acid, chloratranol and atranol as major components (*14*). We must be aware of the presence of other minor components (5,7-dihydroxy-6-methylphtalide, isoadiantone, olivetol, methyl and ethyl haematommate) and their hidden contribution to the detected cytotoxic activity.

However, normal human fibroblast MRC-5 cell line is modestly sensitive to treatments (IC_{50}>200 μg/mL) (**Table 1**), thus indicating cell-specific activities of tested lichen extracts. According to the data presented in our study, *P. furfuracea* and *P. glauca* extracts might be considered a possible source of anticancer agents.

Proapoptotic effects

Our results show that the tested extracts induced cell death mainly by apoptosis. The treated cells showed morphological changes typical for apoptosis (reduction of cell size, blabbing effects with condensation and fragmentation of nuclear material, and formation of apoptotic bodies) (**Fig. 2**). **Table 2** shows the percentages of viable, early apoptotic, late apoptotic and necrotic cells in HCT-116 and SW-480 cell lines, respectively. The number of viable cells detected with MTT assay (**Fig. 1**) correlates with the number of viable and early apoptotic cells observed by microscopic AO/EB double staining, as a more sensitive assay. The literature data (*23*) confirm that cells in the early apoptosis still have the capacity to metabolize the tetrazolium salt (the main component of the MTT test), and according to the MTT test, they are detected as living. It is also known that cellular adhesion is not impaired in the early apoptosis, so the cells at this stage are still attached to the plate, *i.e.* they are detected as alive (*23*). The changes of molecules responsible for

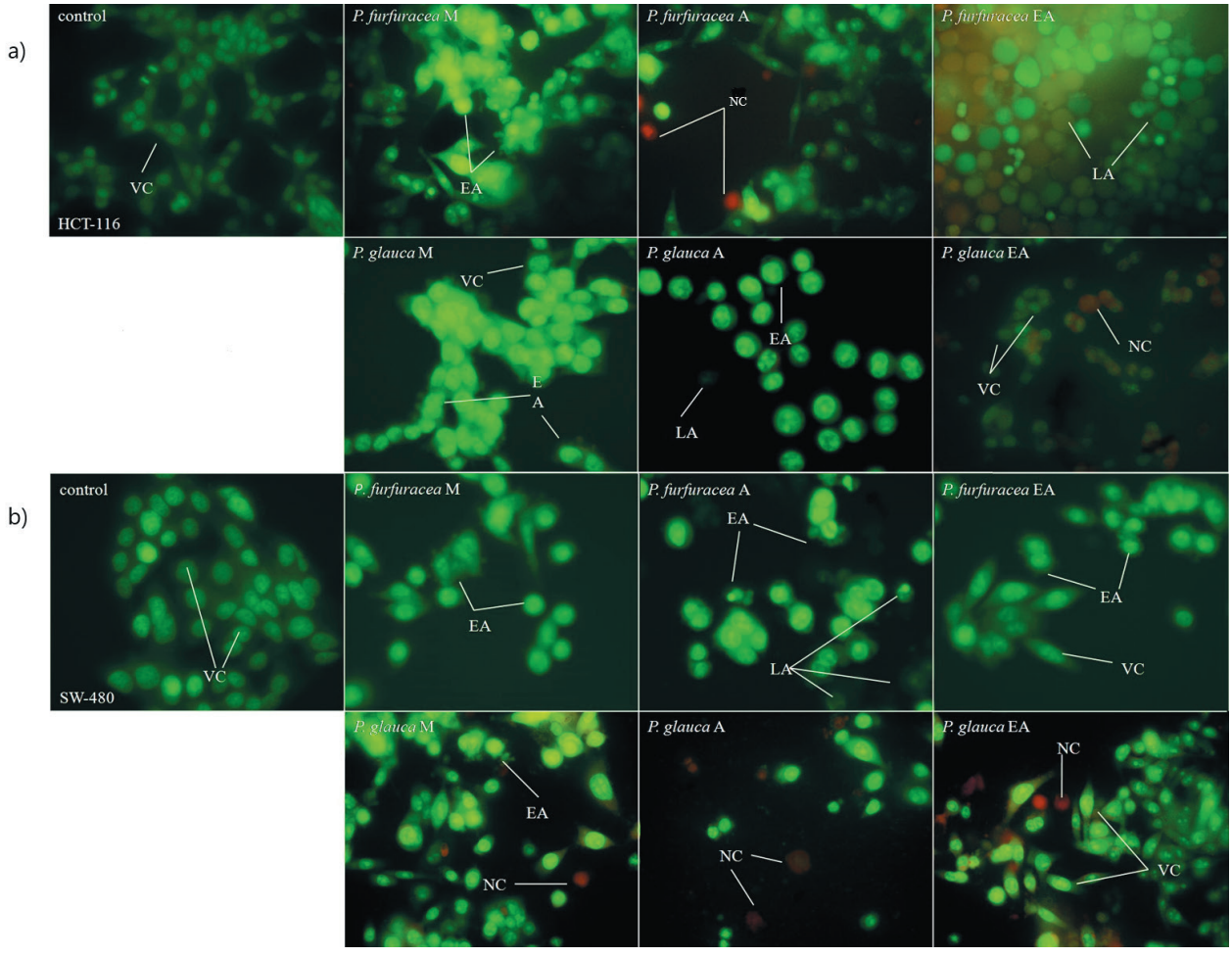

Fig. 2. Acridine orange/ethidium bromide (AO/EB) staining of: a) HCT-116, and b) SW-480 colorectal cancer cells for determination of the stages of cell death induced by methanol, acetone and ethyl acetate extracts of *Pseudevernia furfuracea* and *Platismatia glauca*. Untreated cells were observed as a negative control. VC=viable cells, EA=early apoptosis, LA=late apoptosis, NC=necrotic cells

Table 2. Effects of methanol, acetone and ethyl acetate extracts of *Pseudevernia furfuracea* and *Platismatia glauca* on the stage of cell death in HCT-116 and SW-480 cells

			t/h	γ/(μg/mL)	Viable cells/%	Early apoptosis/%	Late apoptosis/%	Necrosis/%
HCT-116	Control		24	0	97.4±3.6	2.6±0.4	0	0
			72	0	95.4±1.4	4.56±0.03	0	0
	P. furfuracea	methanol	24	10	79.1±1.1	10.5±0.1	10.3±0.1	0
				100	65.2±2.1	16.5±0.5	18.3±1.0	0
			72	10	73.6±2.7	16.1±0.7	10.1±0.9	0.2±0.0
				100	31.7±2.0	39.5±0.5	28.84±0.05	0
		acetone	24	10	66.5±1.0	13.6±1.0	19.9±0.2	0
				100	28.8±1.6	21.6±0.3	49.6±0.1	0
			72	10	61.4±0.4	18.0±0.4	20.5±0.5	0.1±0.0
				100	18.4±0.1	18.1±0.9	58.47±0.06	5.06±0.03
		ethyl acetate	24	10	62.8±0.4	17.8±0.8	18.6±0.7	1.17±0.02
				100	28.3±0.5	21.12±0.01	50.07±0.03	0.48±0.05
			72	10	38.5±1.1	41.4±0.1	20.1±0.0	0.28±0.01
				100	8.0±0.1	10.9±0.4	75.4±0.2	5.62±0.01
	P. glauca	methanol	24	10	73.7±0.1	15.3±0.0	11.0±0.2	0
				100	57.1±0.1	11.9±0.3	38.1±0.4	0
			72	10	60.1±0.4	31.5±0.7	18.3±1.0	0
				100	19.7±0.7	37.9±0.1	42.4±0.8	0
		acetone	24	10	68.0±0.4	12.5±0.1	15.2±0.5	4.2±0.1
				100	40.0±1.4	26.8±0.5	26.17±0.08	7.4±1.1
			72	10	46.7±2.0	22.4±0.1	22.0±0.2	8.8±0.1
				100	20.1±0.1	37.0±0.3	32.7±0.5	10.2±0.5
		ethyl acetate	24	10	76.5±1.8	13.2±0.5	9.02±0.08	1.21±0.01
				100	74.15±0.01	30.73±0.01	13.1±0.3	12.0±0.8
			72	10	45.5±0.7	21.5±0.7	22.2±0.8	10.78±0.03
				100	20.62±0.01	28.2±1.0	29.5±0.7	21.6±0.3
SW-480	Control		24	0	96.8±1.8	3.2±0.0	0	0
			72	0	94.4±1.1	5.6±0.1	0	0
	P. furfuracea	methanol	24	10	75.9±1.5	14.1±1.0	9.99±0.01	0
				100	26.6±0.6	17.5±0.4	55.8±0.5	0
			72	10	82.1±0.8	15.3±1.1	8.66±0.01	0
				100	18.0±0.1	13.9±0.1	60.6±0.9	7.49±0.04
		acetone	24	10	83.1±1.0	8.2±1.3	8.77±0.09	0
				100	40.4±0.1	24.8±0.5	34.78±0.09	0
			72	10	70.2±1.2	18.8±0.8	10.9±0.0	0
				100	12.0±0.0	14.2±0.8	69.6±1.7	4.1±0.4
		ethyl acetate	24	10	86.4±1.1	8.3±0.8	5.28±0.02	0
				100	81.0±0.1	10.5±0.8	8.5±0.3	0
			72	10	80.73±0.09	11.0±0.8	8.24±0.01	0
				100	24.82±0.08	5.6±0.9	66.1±0.5	3.49±0.04
	P. glauca	methanol	24	10	31.3±0.4	22.2±1.0	33.15±0.09	13.3±0.1
				100	22.6±0.1	31.58±0.4	27.4±0.6	18.4±1.0
			72	10	87.5±0.7	9.67±0.05	5.59±0.02	2.81±0.02
				100	64.2±0.8	16.8±0.7	8.7±0.4	10.3±0.8
		acetone	24	10	44.1±0.5	25.4±1.0	15.15±0.04	15.3±0.1
				100	18.8±0.1	32.3±0.4	28.4±0.6	20.4±1.0
			72	10	77.8±0.6	12.9±0.9	6.70±0.01	2.6±0.0
				100	49.0±0.1	24.4±0.8	20.58±0.05	6.0±0.4
		ethyl acetate	24	10	61.5±0.9	8.8±0.01	24.60±0.04	5.1±0.1
				100	50.5±0.7	18.4±1.0	15.6±0.7	15.52±0.09
			72	10	78.8±1.0	9.03±0.07	7.10±0.05	5.1±0.6
				100	49.0±0.7	22.1±0.3	10.5±1.0	18.4±0.1

Results are expressed as the mean value±S.E. of three independent experiments

cell adhesion and mitochondrial dysfunction, and for the metabolism of tetrazolium, are characteristic of cells in late stages of apoptosis (formation of apoptotic bodies) and necrosis, so that these cells are detected as dead by the MTT test (*23*). Besides, cytotoxic effects of the extracts tested in this study, evaluated by MTT test, were the consequence of late apoptotic and necrotic cells, recognized as dead cells, because only nonviable cells accumulated ethidium bromide and emitted red fluorescence in AO/EB double staining.

According to the data presented in **Table 2**, apoptotic effects depend on the concentration of treatments and time of exposure. Stronger effects were observed after longer exposure time (72 h), except for *P. glauca* extract tested on SW-480 cells, which showed better results after 24 h (**Table 2**). The ethyl acetate extract of *P. furfuracea,* with the strongest cytotoxic effects at HCT-116 cells after 72 h, showed a significant apoptotic effect at both tested doses (10 μg/mL caused 20.12 % of late apoptotic cells, while 100 μg/mL induced 75.39 % of late apoptotic cells and 5.62 % of necrotic cells; **Table 2**). A significant percentage of viable cells detected by MTT were in the early apoptosis stage (41.37 and 10.94 % in the presence of 10 and 100 μg/mL ethyl acetate extract of *P. furfuracea*, respectively), indicating that this lichen extract possessed very strong proapoptotic potential against HCT-116 cells. The extracts of *P. glauca* induced significant, dose- and time-dependent proapoptotic effects on HCT-116 cells. However, the acetone and ethyl acetate extracts of *P. glauca* induced modest necrotic effects on the same cells. Regarding the induced apoptosis on HCT-116 cells, the extracts of *P. furfuracea* could be ranged as follows: ethyl acetate>acetone>methanol, and of *P. glauca*: methanol>ethyl acetate>acetone (**Table 2**).

The results presented in **Table 2** show that methanol and acetone extracts of *P. furfuracea* had the highest proapoptotic effects on SW-480 cells after 72 h, which was in accordance with the results of the cytotoxicity (IC_{50} values in **Table 1**). The methanol and acetone extracts of *P. glauca* induced higher apoptotic effects on SW-480 cells after 24 h of exposure (**Table 2**). Regarding the induced apoptosis in SW-480 cells, *P. furfuracea* extracts could be ranged as follows: acetone>methanol>ethyl acetate, and of *P. glauca*: methanol>acetone>ethyl acetate (**Table 2**). All *P. furfuracea* extracts had modest necrotic effects on SW-480 cells (3.49–7.49 %), but only in higher dose (100 μg/mL) after 72 h. However, *P. glauca* extracts demonstrated the strongest necrotic effects on the same cells, in both tested doses and treatment periods (**Table 2**). The methanol and acetone extracts of *P. glauca* had the highest necrotic potential after 24 h, and together with induced apoptosis, these data clearly coincided with the cytotoxic activity (IC_{50} values in **Table 1**) and may be a consequence of oxidative stress (*24*). Generally, the cytotoxic effects of *P. furfuracea* extracts were the consequence of induced apoptosis in HCT-116 and SW-480 cells, while *P. glauca* extracts induced apoptosis and necrosis in both cell lines, especially 72 h after treatments. According to the presented data, lichen extracts with the highest cytotoxic effects had the strongest proapoptotic effects on the tested cancer cell lines.

A few studies (*12,13,23*) demonstrate the induction of apoptosis in cancer cells by lichen metabolites. Apoptosis, as a strongly regulated process of programed cell death, can be induced *via* two main pathways, the extrinsic (death receptor pathway) and the intrinsic (mitochondrial pathway) (*25*). The literature data show that the treatment with lichen extracts induces the genomic DNA fragmentation with significant elevation of caspase 3 activities in cancer cells (*26*). All tested extracts, especially *P. furfuracea* extracts, induce nuclear condensation and cell damage in apoptotic process, indicating that the tested extracts have a potential to eliminate tumour cells by apoptosis. According to the obtained results, we can conclude that HCT-116 cells are more sensitive to proapoptotic effects of lichen extracts.

Migratory potential

Cell migration is a key problem during the development and progression of cancer. Thus, we evaluated antimigratory potential of methanol extracts of *P. furfuracea* and *P. glauca* on HCT-116 and SW-480 cells. The methanol extract was selected because it had the weakest cytotoxicity on the tested cell lines after 24 h (except the extracts of *P. glauca* on SW-480 cells). The 10 and 50 μg/mL methanol extracts of tested lichens considerably decreased migration of HCT-116 and SW-480 cells (**Fig. 3**). After the evaluation of absorbance related to the number of viable cells, all methanol extracts of *P. furfuracea* and *P. glauca* showed noteworthy antimigratory effects on HCT-116 cells. Further, the tested extracts showed antimigratory effects on the SW-480 cells, except the *P. glauca* extract at the lower concentration (10 μg/mL), thus demonstrating promigratory effect. As the literature data confirmed, a significant change in the prooxidant/antioxidant balance could have an impact on the stimulation of cellular mobility (*27,28*). On the other hand, *P. glauca* extract induced multiple increase in GSH in SW-480 cells, indicating an antioxidant effect. A high level of GSH in cancerous cells allowed them to survive oxidative stress by favouring the survival of cells with high invasive and metastatic potential (*28,29*).

P. furfuracea extract (50 μg/mL) revealed the best antimigratory effects in both cell lines (**Fig. 3**). In the literature, there are a few studies that focus on the effects of whole polyphenol extracts on migration and metastatic potential of cancer *in vitro* (*30,31*). To the best of our knowledge, there are no data about the effects of the tested lichen species extracts on cell migratory potential, and our study is the first to show the antimigratory potential of *P. furfuracea* and *P. glauca* on colorectal cancer HCT-116 and SW-480 cell lines. Our results suggest stronger antimigratory capacity of *P. furfuracea* than *P. glauca* probably according to their chemical profile. The comparison of the obtained results revealed that methanol extract of *P. furfuracea* was richer in phenols than the same extract of *P. glauca* (*14*), thus suggesting that the phenols participated in antimigratory effects on the tested cancer cells. The literature data confirmed that polyphenolic compounds could suppress cell migration and reverse metastatic cells (*31,32*).

The results presented in this work indicate that HCT-116 cells are more sensitive to antimigratory effects of the investigated lichen species than SW-480 cells, which agrees with our earlier findings on migratory potential of these cell lines (*33*).

Redox status

As a very potent and significant member of reactive oxygen species (ROS), the superoxide anion radical indicates the occurrence of oxidative stress in supraphysiologically high concentration. Fig. 4. shows the effects of methanol, acetone and ethyl acetate extracts of *P. furfuracea* and *P. glauca* on $O_2^{\bullet-}$ level in HCT-116 and SW-480 cells, expressed per number of cells. Generally, all treatments were prooxidative, with increased $O_2^{\bullet-}$ level, mainly in dose- and time-dependent way. Higher concentrations of extracts (50 and 100 μg/mL) considerably increased $O_2^{\bullet-}$ levels in both cell lines, and the highest prooxidative effect was observed in *P. furfuracea* (Fig. 4).

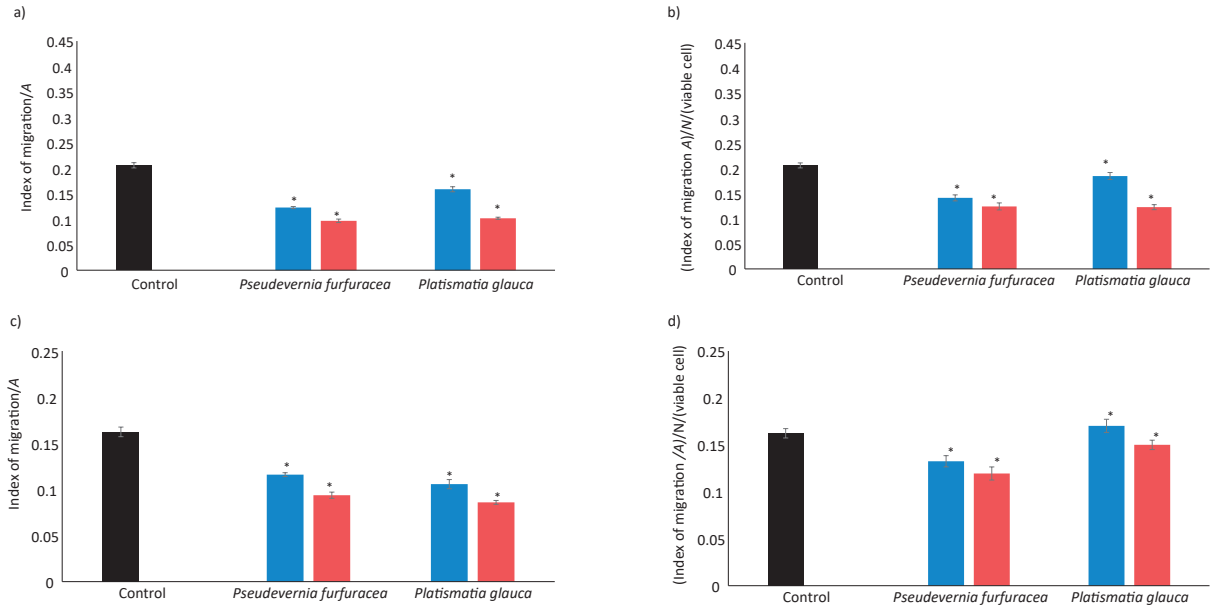

Fig. 3. Effects of *Pseudevernia furfuracea* and *Platismatia glauca* methanol extracts on migratory potential of: a and b) HCT-116, and c and d) SW-480 colorectal cancer cells. The results measured after 24 h of exposure represent index of migration/absorbance (*A*) and (index of migration/*A*)/*N*(viable cells). All values are mean±S.E., *N*=3; * p<0.05 as compared with control

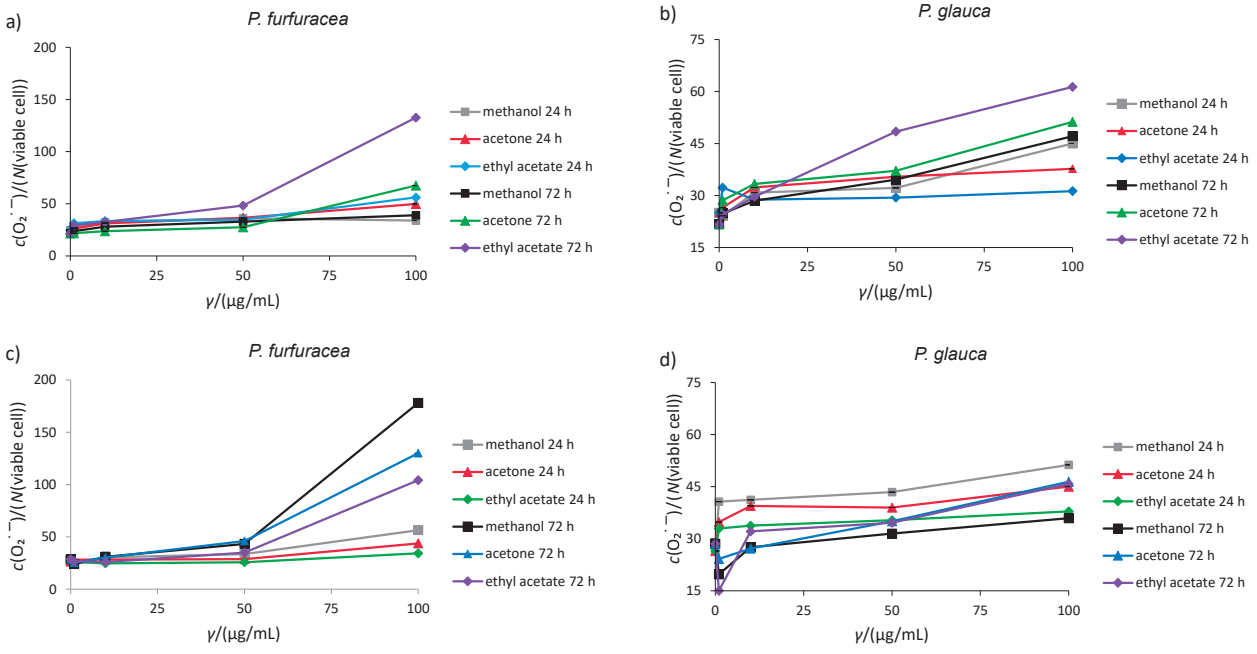

Fig. 4. The concentration of $O_2^{\bullet-}$ in: a and b) HCT-116, and c and d) SW-480 colorectal cancer cells treated with methanol, acetone and ethyl acetate extracts of *Pseudevernia furfuracea* and *Platismatia glauca*. The parameters are expressed as $c(O_2^{\bullet-})$/μM per *N*(viable cells). All values are mean±S.E., *N*=3

High concentrations of nitrites (as an indicator of nitric oxide) could be a parameter of nitrosative stress in cells. We investigated the nitrite level and showed the obtained data in **Fig. 5**. Generally, all tested lichen extracts showed dose- and time-dependent increase of nitrite level in HCT-116 cells. In lower doses, this increase of nitrites was not as prominent as in higher doses. *P. furfuracea* extracts induced greater nitrite increase than *P. glauca* extracts. On the other hand, we also observed nitrite increase in SW-480 cells, but in a slightly different way. Lower concentrations of *P. furfuracea* induced very slow nitrite elevation with the increase of applied extract doses, and only the highest dose induced extreme nitrite rise, especially after 72 h at treatment. On the other hand, after 24 h of treatment, *P. glauca* induced greater increase at lowest concentrations, thus reaching the plateau of effect with almost no change in the increase of extract doses. After 72 h, the increase of nitrites was dose-dependent. *P. glauca* induced greater nitrite levels after 24 h than after 72 h. We conclude that *P. furfuracea* had greater impact on the tested cell lines than the lichen species.

The reduced glutathione (GSH) content is considered a very important parameter of cell antioxidative capacity. In our experiments, all treatments predominantly induced decrease of GSH at low concentrations and increase in higher concentrations, depending on a lichen species, type of extract, concentration and treatment period (**Fig. 6**). *P. furfuracea* induced greater increase of GSH level in the investigated cells, especially in SW-480 after 72 h of treatment. *P. glauca* induced greater increase in HCT-116 cells, while the investigated extracts induced notable GSH increase in SW-480 cells, in low doses and after 24 h of treatment.

Considering the given parameters of redox status in relation to the number of viable cells, we concluded that the treated cancer cells were in oxidative stress. All the measured parameters ($O_2^{\bullet-}$, NO_2^- and GSH) considerably increased at higher concentrations, mainly 72 h after treatments. *P. furfuracea* induced higher oxidative stress in both tested cell lines than *P. glauca* extracts.

It is known that lichen extracts and purified components could change oxidative characteristics of cancer cells and that the ROS overproduction has important role in the cytotoxicity, the initiation and execution of apoptosis (*4,26*). According to our results, the investigated cells were under great oxidative/nitrosative stress, which induced enhanced cytotoxicity through apoptosis. These effects were the most prominent with *P. furfuracea* extracts. *De novo* generated nitrites spontaneously combine with $O_2^{\bullet-}$ and generate peroxynitrites, which are considered very reactive and toxic reactive nitrogen species (RNS) with notable cytotoxic and proapoptotic effects (*33*). Our results showed that prooxidative capacity (increased $O_2^{\bullet-}$) of *P. furfuracea* and *P. glauca* methanol extracts directly correlated with their antimigratory effects on the treated colon cancer cell lines. The literature data (*6*) confirmed that ROS, in the first instance $O_2^{\bullet-}$ and H_2O_2, could reduce migratory potential of cancer cells.

Glutathione as a parameter of antioxidative protection of cells is the most abundant redox pool of cells, and depletion of reduced glutathione content could be the consequence of antioxidant response of the tested cells to the increased oxidative stress (*34*). Our results show that GSH content increased in HCT-116 and more evidently in SW-480 cells. These findings suggest that the tested colorectal cell lines had different response to the same treatment. As a consequence of

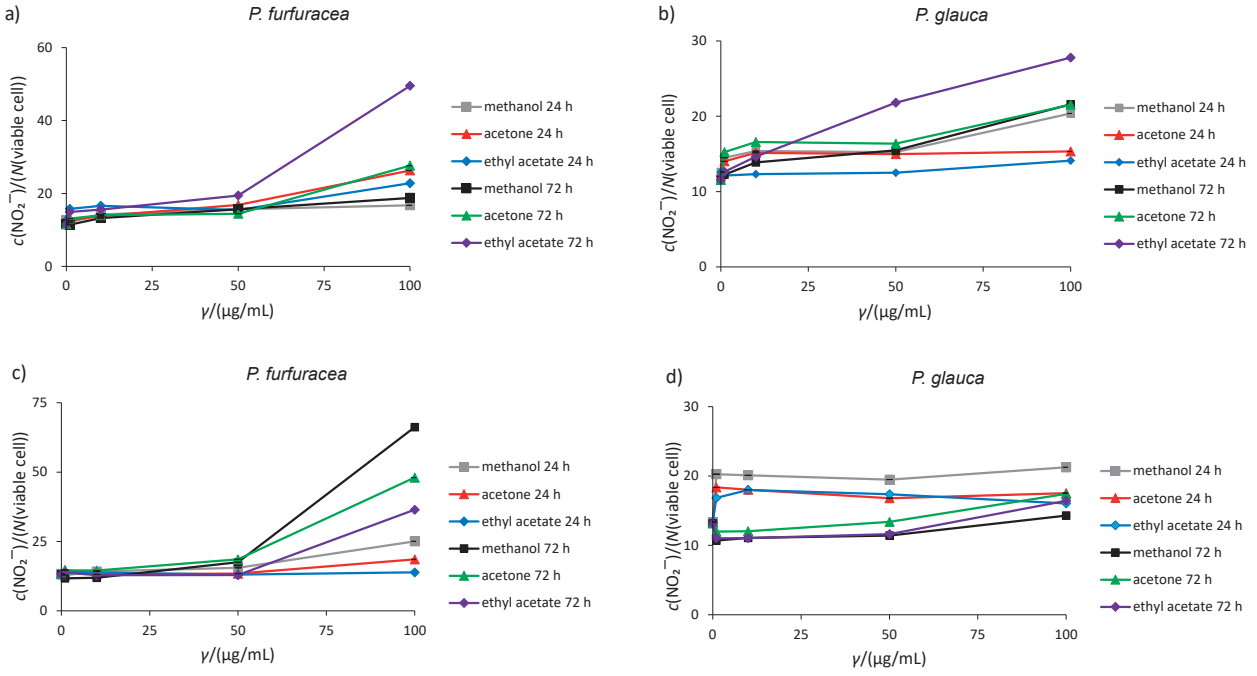

Fig. 5. The concentration of NO_2^- in: a and b) HCT-116, and c and d) SW-480 colorectal cancer cells treated with methanol, acetone and ethyl acetate extracts of *Pseudevernia furfuracea* and *Platismatia glauca*. The parameters are expressed as $c(NO_2^-)$/μM per *N*(viable cells). All values are mean±S.E., *N*=3

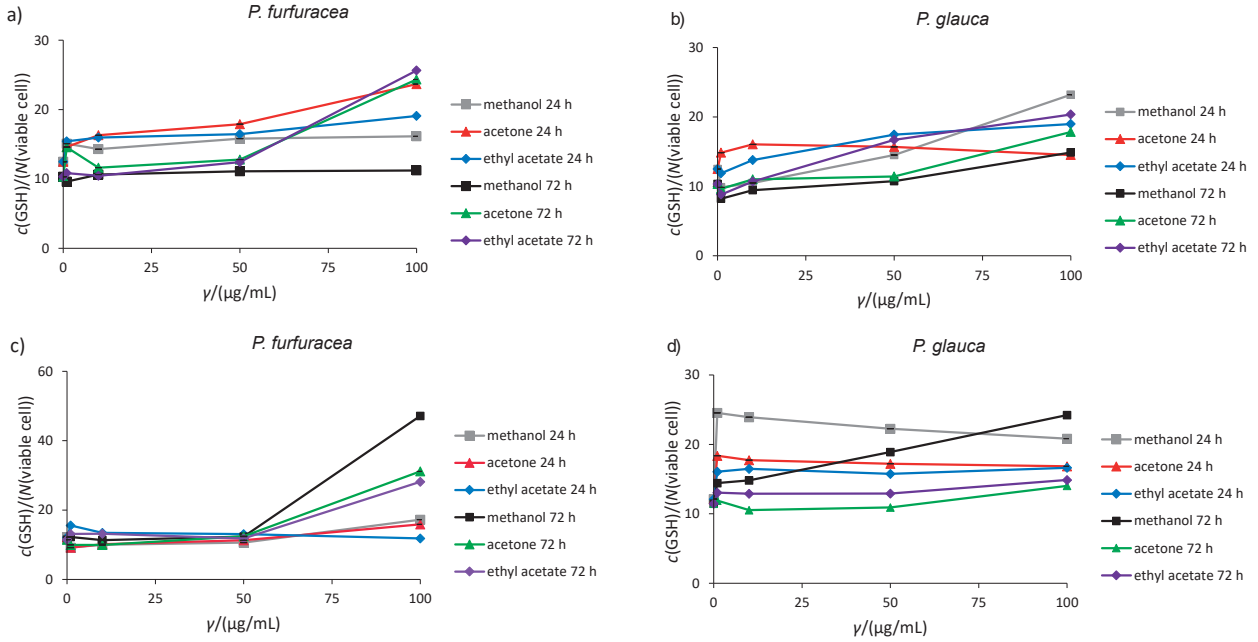

Fig. 6. The concentration of glutathione (GSH) in: a and b) HCT-116, and c and d) SW-480 colorectal cells treated with methanol, acetone and ethyl acetate extracts of *Pseudevernia furfuracea* and *Platismatia glauca*. The parameters are expressed as *c*(GSH)/μM per *N*(viable cells). All values are mean±S.E., *N*=3

the genetic defect in the hMLH1 gene and deficiency in DNA mismatch repair (MMR) of HCT-116 cells, this phenotype becomes more prone to oxidative stress than SW-480 cells (*35*).

CONCLUSIONS

Lichen species *Pseudevernia furfuracea* and *Platismatia glauca* demonstrated relevant cytotoxic, proapoptotic, prooxidative and antimigratory effects on colorectal cancer cells (HCT-116 and SW-480), without observable cytotoxic effects on normal MRC-5 cells lines. The ethyl acetate extract of *P. furfuracea* showed the strongest antitumour activity and this species might be considered for deeper investigation into potential anticancer action. Further studies will elucidate specific factors of these activities within *P. furfuracea* and explain molecular mechanisms of their action.

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