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ASSESSMENT OF CYTOTOXIC AND GENOTOXIC ACTIVITY OF ALCOHOL EXTRACT OF *POLYSCIAS FILICIFOLIA* SHOOT, LEAF, CELL BIOMASS OF SUSPENSION CULTURE AND SAPONIN FRACTION

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Abstract: Some medicinal plants are the object of biotechnologists' special interest owing to their content of secondary metabolites, which have a strong pharmacological effect. *Polyscias filicifolia* is a plant known for long in traditional medicine of the Southeast Asia. Literature data suggest that it acts on the endocrine system, has adaptogenic and antiulcerative activity, shows bactericidal and insecticidal properties, restores the activity of the protein synthesis system in the conditions of long- and short-term anoxia, as well as reduces the effect of many mutagens *in vitro*. The purpose of the studies was to assess the cytotoxic and genotoxic effect of ethanol extracts from *Polyscias filicifolia* dry shoots and leaves obtained *in vitro*, as well as cell biomass from suspension culture. Saponin fraction from dried shoots was also tested. Initially, the cytotoxic effect was evaluated using the murine connective tissue cell line C3H/AN – L929. The genotoxic properties of the extracts were assessed using standard screening tests: the Ames test and the micronucleus test. Based on the obtained results it can be concluded that none of the extracts increases the number of revertants, both in tests with and without metabolic activation. The lack of *in vitro* genotoxic and mutagenic activity of tested shoot, dried leaf, cell biomass extracts, as well as the saponin fraction from dried shoots allows us to hope that *Polyscias filicifolia* could be used as a possible pharmaceutical raw material showing therapeutic properties.

Keywords: *Polyscias filicifolia*, cytotoxicity, genotoxicity, Ames test, micronucleus test

Abbreviations: 2AA – 2-aminoanthracene, 9AA – 9-aminoacridine. ATCC – American Type Culture Collection, BAP – benzylaminopurine, EDTA – ethylenediaminetetraacetic acid, FBS – fetal bovine serum, K – kinetin, LS – Linsmaier and Skoog medium, MEM – Minimal Essential Medium Eagle, MMS – methyl methanesulfonate, MS – Murashige and Skoog medium, NPD – 4-nitro-1,2-phenylendiamine, PBS – phosphate buffered saline

Some medicinal plants are the object of biotechnologists' special interest owing to their content of secondary metabolites, which have a strong pharmacological effect. These compounds, due to their complex chemical structure, are obtained from cell, tissue and organ cultures using the technique of *in vitro* culture cloning, allowing more rapid multiplication and intensification of metabolite production.

Polyscias filicifolia is a bush, which in natural conditions grows in subtropical and tropical regions and has been long known in traditional medicine of the Southeast Asia. Literature data suggest that *Polyscias filicifolia* extracts act on the endocrine system (1), have adaptogenic and antiulcerative activity (2), show bactericidal and insecticidal (3, 4) properties, restore the activity of the protein synthesis system in the conditions of long- and short-term anoxia (5), as well as reduce the effect of many mutagens *in vitro* (6).

Extracts of *P. filicifolia* biomass obtained from suspension cell cultures grown in bioreactors at the Institute of Plant Physiology, Academy of Sciences in Moscow, are used in the production of „Vitagmal", which is an adaptogenic and immunostimulating commercial preparation marketed as a dietary supplement (3).

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At the Department of Pharmaceutical Biology and Botany, Medical University of Warsaw, we have developed a method of rapid multiplication of *P. filicifolia* (7) for obtaining plant biomass in an *in vitro* culture, of which ethanol extracts and triterpene saponin fraction were prepared. Extracts were also prepared from biomass of *P. filicifolia* obtained in bioreactor in the Plant Physiology Department of the Moscow State University.

The purpose of the studies was to assess the cytotoxic and genotoxic effect of ethanol extracts from *Polyscias filicifolia* dry shoots and leaves obtained *in vitro*, as well as cell biomass from suspension culture. Saponin fraction from dried shoots was also tested. In the study, standard screening tests were used. Initially the cytotoxic effect was evaluated using the murine connective tissue cell line C3H/AN – L929. The genotoxic properties of the extracts were assessed using the Ames test and the micronucleus test.

EXPERIMENTAL

Plant material

A method of *P. filicifolia* multiplication *via* somatic embryogenesis was elaborated previously (7) and shoots and plantlets growing *in vitro* were the source of plant material tested in the present study.

The shoots developed within 6 weeks of culture on Linsmaier and Skoog (LS) medium (8) with 2.0 mg/L benzylaminopurine (BAP), 0.5 mg/L kinetin (K), 30 g/L sucrose and 5.5 g/L agar (Plant Propagation LAB-AGAR™ – Biocorp).

The leaves were collected from plantlets growing *in vitro* on modified Murashige and Skoog (MS) medium (9) containing one third of macro- and microelements and full-strength of MS vitamins, 10 mg/L sucrose and 7.5 g/L agar (Agar Bacto, Difco). The cultures were kept in a culture room at 24 ± 2°C under 12-h photoperiod with a light intesity of 40 mol m^{-2} s^{-1}.

Apart from shoots and leaves, *P. filicifolia* cell biomass from suspension culture grown in bioreactor was tested (4). The biomass was obtained from prof. A. Nosov from the Plant Physiology Department of the Moscow State University.

Alcohol extract preparation

The ground dry shoots, leaves and the cell biomass cell suspension culture (10 g) were extracted with 70% (v/v) ethanol (1:5 w/v) for 7 days in darkness on a rotary shaker at 100 rpm. The extracts were evaporated to dryness on a rotary evaporator. The dry residue was 2.28 g, 1.75 g and 4.15 g, respectively.

Saponin fraction preparation

The ground dry shoots (350 g) were refluxed 4 times for 1 h with methanol (500 mL). The methanol extracts were combined and evaporated to dryness under reduced pressure, the residue (90.72 g) was suspended in 500 mL of water was extracted 4 times with n-hexane (4:1 v/v), 4 times with dichloromethane (4:1 v/v), 4 times with ethyl acetate (4:1 v/v) and 4 times with butanol saturated with water (4:1 v/v), successively. The butanol layers were combined and evaporated to dryness under reduced pressure. The 5.75 g of the dry residue (12.57 g) were dissolved in 50 mL of methanol and the crude saponins were obtained by successive precipitation with diethyl ether (50 mL), acetone (50 mL) and ethyl acetate (50 mL). The precipitates were collected by filtration and dried in the air (0.64 g).

Bacteria

Salmonella typhimurium strains: TA97 (CIP 108115), TA98 (CIP 103798), TA100 (CIP 103799), TA102 (CIP 104406) from the Pasteur Institute culture collection. The strains are nutritional auxotrophic mutants, requiring histidine and biotin for growth.

Cells

L929 cell line (C3H/AN murine connective tissue) was from the ATCC collection. Cells were cultured in the MEM growth medium supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic.

Bacterial culture media

Liquid broth medium (Nutrient Broth Oxoid); minimal agar medium (Bacto Agar – Difco with Vogel and Bonner salt and 40% glucose); semi-liquid surface agar (Bacto Agar – Difco).

Cell culture media and reagents

MEM medium (Lonza); fetal bovine serum FBS (Lonza); 0.5% trypsin-EDTA (Gibco); calcium and magnesium ion-free PBS (IITD, Wrocław); Antibiotic-antimycotic (Sigma).

Compounds used as positive controls in the Ames test without metabolic activation

4-Nitro-1,2-phenylenediamine (NPD) (Merck), in an amount of 20 µg/plate for strains TA97, TA98 and TA100, methyl methanesulfonate

(MMS) (Merck), in an amount of 1.0 μg/plate for strain TA102.

Compounds used as positive controls in the Ames test with metabolic activation

2-Aminoanthracene (2AA) (Fluka), in an amount of 10 μg/plate for strains TA97, TA98, TA100, 9-aminoacridine (9AA) (Sigma), in an amount of 50 μg/plate for strain TA102.

Fraction S9

Many substances show their mutagenic properties only after metabolic activation. Fraction S9 (MP Biomedicals, Inc.) contains enzymes able to cause biochemical changes of substances.

In the Ames test with metabolic activation, bacterial cells and tested extracts were incubated in the presence of fraction S9.

In the micronucleus test with metabolic activation, tested extracts underwent an additional step of preincubation with fraction S9.

Cytotoxicity test

To evaluate the cytotoxic effect, the EZ4U Cell Proliferation Assay kit (the 4th generation non radioactive cell proliferaton & cytotoxicity assay, Biomedica) was used. The EZ4U test is based on transformation of tetrazolium salts into water-soluble, intensely colored formazan derivatives, caused by succinate dehydrogenase, the enzyme present in living cells. Spectrophotometric measurement allows the evaluation of cell viability and proliferation, as color intensity is proportional to the number of living cells.

The test was performed in 96-well plates, according to the test manufacturer's protocol.

Cell suspension of density of 3×10^5 cells/mL was prepared from 48-hour culture of L929 cells and added into wells in an amount of 100 μL/well. The cells were exposed to solutions of tested extracts at concentrations from 10.0 μg/mL to 20 mg/mL. The solutions were prepared in the growth MEM medium with 10% FBS and antibiotics. Cell cultures were incubated with tested extracts for 48 hours at 37°C in 5% CO_2 atmosphere.

After the incubation period, 10 μL of the EZ4U reagent, prepared according to the manufacturer's protocol, was added to each well on the plate. Absorbance was measured using the microplate reader at 492 and 620 nm wavelength. Measurement at 620 nm wavelength was performed to adjust the color background of the tested substances. When calculating the results, the absorbance value measured at $\lambda = 620$ wavelength was subtracted from that measured at $\lambda = 492$ wavelength.

Cell survival was presented as % of living cells compared to the control culture.

Results are the mean measurement values obtained from 2 independent experiments.

Genotoxicity tests

Ames test

The mutagenic effect of the extracts was evaluated using reference Ames test. The test was performed according to the procedure proposed by Maron and Ames (10). The test consists in the assessment of the tested substance's ability to cause reversion of nutritional mutation in auxotrophic *Salmonella typhimurium* strains. The bacteria in which reversion of mutation has occurred, contrary to the *S. typhimurium* mutants, show the ability to multiply and form colonies on the minimal medium without the growth factor (histidine).

The tested strains were cultured in a liquid broth medium for 18 h at 37°C in water bath with shaking. After incubation, 0.1 mL of bacterial culture and 0.1 mL of tested extract solution were added to 2 mL of semi-liquid surface agar containing 0.05 mM biotin and 0.05 mM histidine, and poured on a plate with the minimum agar medium. In tests with metabolic activation also 0.5 mL of fraction S9 mix was added. After 48–72 h of incubation at 37°C, the colonies of his^+ revertants on the plates were counted.

The test results were presented as the mean number of colonies (from two independent experiments performed in triplicates) of histidine-independent revertants, for each concentration of the tested extract. The mutagenic effect of the extract is confirmed by showing a dose-response relationship, i.e., when the number of his^+ revertants on the plate increases with increasing concentration of the tested extract.

According to the generally accepted procedure for this test, the substance shows mutagenic activity, when it causes doubling of the number of his^+ revertants comparing to control (11).

***In vitro* micronucleus test**

The micronucleus test is a cytogenetic method for detecting chromosome breaking or damage of the karyokinetic spindle (12–14).

The micronuclei are formed from acentric fragments of the chromosomes, which remain behind during cell nucleus division, and at the completion of telophase they are not included into daughter nuclei, or from complete chromosomes remaining outside the nucleus as a result of karyokinetic spindle damage. Any population of dividing cells can be used for the test.

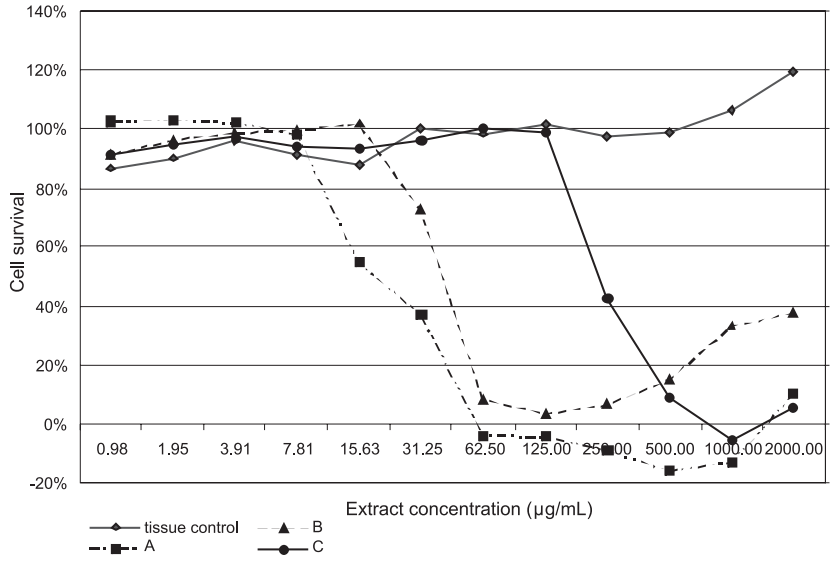

Figure 1. The effect of *Polyscias filicifolia* dry shoot (A), leaf (B) and cell biomass (C) extract on L929 cell survival

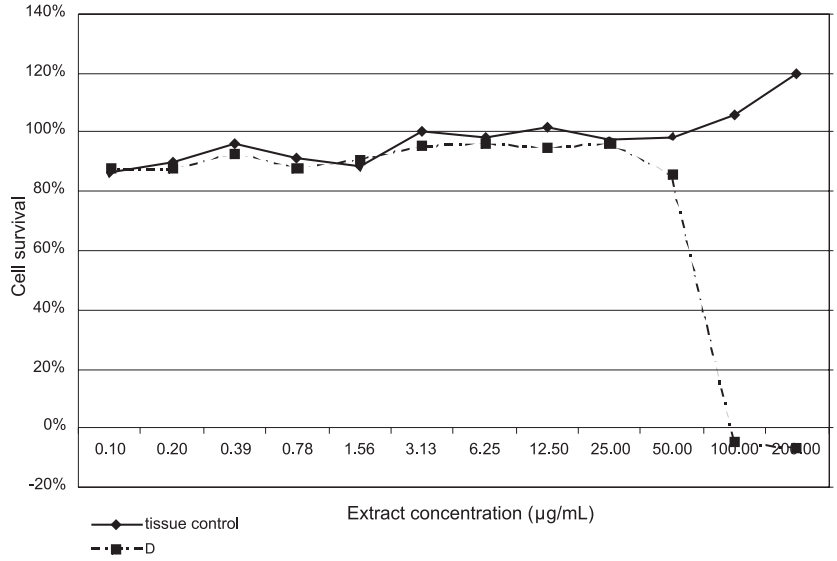

Figure 2. The effect of triterpene saponin fraction obtained from *Polyscias filicifolia* dry shoots (D) on L929 cell survival

To assess the genotoxic activity of the extracts, the *in vitro* micronucleus test with cytokinesis blocking technique with cytochalasin B (15), using C3H/AN murine connective tissue cell line (L929) was performed.

Forty-eight-hour cell cultures were trypsinized, diluted in MEM growth medium with 10% FBS and 1% antibiotics to get 10^5 cells/mL, inoculated onto 4-well plates in the amount of 1 mL/well, and incubated for 24 h at 37°C in 5% CO_2 atmosphere. Then, the cells were exposed to the tested extracts at selected concentrations diluted in growth medium for 2 h at 37°C in 5% CO_2 atmosphere. After this time, medium with tested extracts was removed. Fresh medium with cytochalasin B was added to the cell culture. The cells were incubated for 24 h at 37°C in 5% CO_2 atmosphere. When the incubation period was completed, the cultures were rinsed twice with PBS, dried, fixed with methanol for 20 min and stained for 20 min with 10% Giemza dye solution in 0.05 M phosphate buffer at pH 6.8. Dried slides underwent microscopic evaluation.

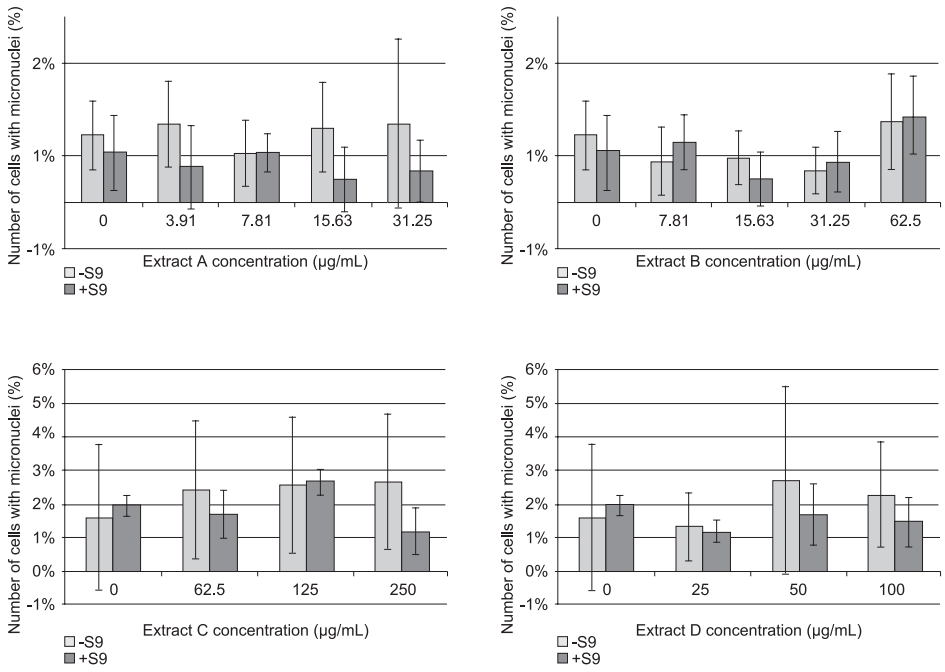

Figure 3. Assessment of the genotoxic effect of *Polyscias filicifolia* dry shoot extract (A), dried leaf extract (B), cell biomass extract (C) and fraction of triterpene saponines from dry shoots (D) in the micronucleus test with and without the metabolic fraction S9

Table 1. IC_{50} values for *Polyscias filicifolia* dry shoot (A), dry leaf (B), cell biomass (C) extracts and triterpene saponin raction from dry shoots (D).

Extracts studied	IC_{50} values (µg/mL)*
A	23.85
B	41.91
C	234.33
D	69.66

* The IC_{50} value was calculated from the straight line equation for the selected segment of the cell survival curve in the presence of extracts at concentrations causing survival reduction. Extract A, 7.8 to 31.25 µg/mL; extract B, 15.62 to 62.50 µg/mL; extract C, 125 to 500 µg/mL; extract D, 50 to 100 µg/mL

In micronucleus test with metabolic activation, the extracts were initially preincubated for 1 h with S9 fraction mix. After preincubation, solutions of extracts and S9 were filtered and added to the cell cultures. Further steps were performed as described above.

The genotoxic effect is defined as the percentage of cells with micronuclei in population of not less than 1000 binucleate cells.

The following criteria can be used to assess the test results (16): The result is positive when:

– there is a dose-response relationship, i.e., statistically significant relationship between the number of cells with micronuclei and the concentration of tested substance;

– there is a repeatable and statistically significant positive response, in at least one of the concentrations tested.

Statistical analysis

The Medistat System (microcomputer statistical system for medicinal purposes, version 2.1; 1992) was used to check the statistical significance of the results.

RESULTS AND DISCUSSION

The assessment of the cytotoxic and genotoxic activity of *Polyscias filicifolia* extracts was performed based on the results obtained in cytotoxicity and genotoxicity tests with L929 cells, and those obtained in the Ames test using *Salmonella typhimurium* strains.

The results are presented in Figures 1–3 and in Tables 1–5.

The results of cytotoxicity testing of the extracts of *Polyscias filicifolia* dried shoots (A), leaves (B) and cell biomass (C) are presented in Figure 1, and those of the saponin fraction obtained

Table 2. The effect of *Polyscias filicifolia* dried shoot extract (A) on the number of his+ revertants in *Salmonella typhimurium* strains in the tests with and without the metabolic fraction S9 mix.

Strain	-S9 mix					+S9mix				
	Spontaneous reversion*	Concentration of studied compound (µg/plate)				Spontaneous reversion*	Concentration of studied compound (µg/plate)			
		15.63	31.25	62.5	125		15.63	31.25	62.5	125
TA97	112 ± 14	115 ± 20	105 ± 23	115 ± 18	108±7	105 ± 4	94 ± 10	111 ± 18	100 ± 11	107 ± 4
TA98	38 ± 7	47 ± 5	47 ± 9	52 ± 6	50±7	30 ± 1	30 ± 9	27 ± 1	37 ± 12	44 ± 1
TA100	150 ± 27	140 ± 30	166 ± 46	177 ± 32	182±32	161 ± 21	145 ± 3	146 ± 10	108 ± 13	151 ± 16
TA102	266 ± 30	300 ± 40	300 ± 50	245 ± 10	279±54	297 ± 9	296 ± 30	301 ± 11	344 ± 58	350 ± 35

*cultures without studied compound

Table 3. The effect of *Polyscias filicifolia* dried leaf extract (B) on the number of his+revertants in *Salmonella typhimurium* strains in the tests with and without the metabolic fraction S9 mix.

Strain	-S9 mix					+S9mix				
	Spontaneous reversion*	Concentration of studied compound (µg/plate)				Spontaneous reversion*	Concentration of studied compound (µg/plate)			
		15.63	31.25	62.5	125		15.63	31.25	62.5	125
TA97	112 ± 14	107 ± 20	109 ± 15	116 ± 20	112 ± 14	105 ± 4	112 ± 1	115 ± 14	115 ± 14	107 ± 4
TA98	38 ± 7	41 ± 12	38 ± 7	39 ± 6	40 ± 5	30 ± 1	32 ± 3	39 ± 4	39 ± 4	44 ± 1
TA100	150 ± 27	191 ± 30	158 ± 25	158 ± 18	165 ± 20	161 ± 21	141 ± 17	146 ± 5	146 ± 5	151 ± 16
TA102	266 ± 30	282 ± 12	208 ± 51	299 ± 19	280 ± 42	297 ± 9	287 ± 7	229 ± 18	229 ± 18	350 ± 35

*cultures without studied compound

Table 4. The effect of *Polyscias filicifolia* cell biomass extract (C) on the number of his+revertants in *Salmonella typhimurium* strains in the tests with and without the metabolic fraction S9 mix.

Strain	-S9 mix					+S9mix				
	Spontaneous reversion*	Concentration of studied compound (µg/plate)				Spontaneous reversion*	Concentration of studied compound (µg/plate)			
		62.5	125	250	500		62.5	125	250	500
TA97	105 ± 9	105 ± 11	120 ± 8	125 ± 23	114 ± 18	143 ± 25	139 ± 30	129 ± 22	136 ± 30	136 ± 34
TA98	39 ± 7	36 ± 6	38 ± 6	39 ± 4	30 ± 8	35 ± 9	26 ± 4	26 ± 4	26 ± 5	32 ± 5
TA100	139 ± 23	136 ± 20	137 ± 28	137 ± 20	147 ± 13	121 ± 1	105 ± 1	120 ± 2	142 ± 3	155 ± 21
TA102	315 ± 29	289 ± 54	277 ± 53	290 ± 52	362 ± 48	235 ± 9	219 ± 19	254 ± 26	239 ± 24	201 ± 16

*cultures without studied compound

Table 5. The effect of *Polyscias filicifolia* fraction of triterpene saponines from dry shoots extract (D) on the number of his^+ revertants in a *Salmonella typhimurium* strain in the tests with and without the metabolic fraction S9 mix.

Strain	-S9 mix					+S9mix				
	Spontaneous reversion*	Concentration of studied compound (µg/plate)				Spontaneous reversion*	Concentration of studied compound (µg/plate)			
		15.63	31.25	62.5	125		15.63	31.25	62.5	125
TA97	114 ± 2	100 ± 2	102 ± 5	101 ± 6	102 ± 2	150 ± 26	139 ± 4	137 ± 8	119 ± 7	105 ± 12
TA98	48 ± 7	36 ± 82	38 ± 11	29 ± 4	33 ± 1	55 ± 9	37 ± 7	38 ± 2	38 ± 5	36 ± 6
TA100	162 ± 21	105 ± 7	127 ± 15	100 ± 5	111 ± 1	138 ± 8	139 ± 3	141 ± 3	122 ± 8	113 ± 14
TA102	342 ± 25	295 ± 45	281 ± 17	343 ± 18	270 ± 24	378 ± 8	333 ± 21	359 ± 26	326 ± 21	318 ± 15

*cultures without studied compound

from *Polyscias filicifolia* dry shoots (D) are presented in Figure 2.

Based on the obtained results, it can be concluded that extract A shows the strongest cytotoxic activity among all tested extracts. A reduction in numbers of living cells in cultures containing extract A was already seen at the concentration of 15.63 μg/mL, and in case of extract B, at the twice higher concentration of 31.25 μg/mL. At the concentration of 62.50 μg/mL, extracts A and B show a strong cytotoxic effect, manifesting itself in practically no living cells in the culture (Fig. 1). A similar cytotoxic effect was seen when testing the saponin fraction from dried shoots. The number of living cells is reduced to 85% in cultures containing saponins at the concentration of 50.0 μg/mL, whereas at 100 μg/mL in the medium, the cultures were practically completely destroyed (Fig. 2).

The results obtained in the EZ4U test show that the cell biomass extract (C) has the weakest cytotoxic effect of all tested extracts. Reduction in numbers of living cells to about 50% was observed at the concentration of 250 μg/mL. Extract from the cell biomass destroys all living cells only at the concentration of 1000 μg/mL.

Based on the results obtained in the cytotoxicity tests, the IC_{50} value for each extract was calculated (Table 1). One can risk a conclusion that the presence of saponins is not the only cause of the observed cytotoxic activity. It has been confirmed in cytotoxicity tests, where IC_{50} value of saponin fraction from dry shoots (D) was 3 times higher than the dry shoot extract's (A) and 1.5 times higher than the leaf extract's (B).

The results obtained in the Ames test are presented in Tables 2–5. Due to lower sensitivity of the bacterial cells to the cytotoxic effect of chemicals, the extract concentrations above the IC_{50} value were selected for the Ames test. Based on the obtained results it can be concluded that none of the extracts increases the number of revertants, both in tests with and without metabolic fraction. *Polyscias filicifolia* shoots (A), dried leaf (B), cell biomass (C) extracts, as well as the saponin fraction from dried shoots (D) have shown no mutagenic activity in the Ames test.

The results of the genotoxic activity assessment of the tested extracts using the micronucleus test are presented in Figure 3. The test was performed using the L929 cells, which, due to their susceptibility to the cytotoxic effect, are recommended for these tests. The genotoxic activity assessment of the extracts was performed to control culture without the extracts. The range of tested concentrations was limited by cytotoxicity values. The extracts were tested at concentrations below and above the IC_{50} value. For each tested concentration, about 1000 binucleate cells were counted, i.e., about 250 cells from a single well on the 4-well plate. In the population of binucleate cells, the micronucleus-containing cells were counted. The percentage of cells with micronuclei among binucleate cells was calculated. The charts show the mean results obtained in two independent experiments.

None of the concentrations of the tested extracts induced an increase in the number of micronuclei, both in the experiments with and without metabolic fraction.

Based on the results obtained in the micronucleus test, it was concluded that the tested extracts show no genotoxic effect. No statistically significant increase in the number of micronuclei was seen *versus* the control.

The lack of *in vitro* genotoxic and mutagenic activity of all tested extracts allows us to hope that *Polyscias filicifolia* could be used as a possible pharmaceutical raw material showing therapeutic properties.

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