



Antiproliferative activity of various *Uncaria tomentosa* preparations on HL-60 promyelocytic leukemia cells

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Abstract:

The woody Amazonian vine *Uncaria tomentosa* (cat's claw) has been recently more and more popular all over the world as an immunomodulatory, antiinflammatory and anti-cancer remedy. This study investigates anti-proliferative potency of several cat's claw preparations with different quantitative and qualitative alkaloid contents on HL-60 acute promyelocytic human cells by applying trypan blue exclusion and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction assay (MTT). By standardization and statistical comparison of the obtained results pteropodine and isomitraphylline are indicated to be most suitable for standardization of medical cat's claw preparations.

Key words:

Uncaria tomentosa, cat's claw, oxindole alkaloids, cytotoxicity, HL-60, MTT

Abbreviations: B/W₃₇ – *Uncaria tomentosa* bark extracted in water at 37°C, B/50E₃₇ – bark extracted in 50% ethanol at 37°C, B/96E₃₇ – bark extracted in 96% ethanol at 37°C, B/W_b – bark extracted in boiling water, B/E_b – bark extracted in boiling ethanol, B/S_{ri} – bark alkaloid-rich preparation, L/E_b – leaves extracted in boiling ethanol

Introduction

Uncaria tomentosa (Willdenow ex Roemer & Schultes) De Candolle is a large woody vine indigenous to a number of central and South American countries [6]. This species, also known as “una de gato” or “cat's claw”, is widely used in folk healing as an immunomodulatory, anti-cancer and anti-inflammatory

remedy. The list of treated diseases includes, for instance, gastric ulcers, diarrhoea, gonorrhoea, arthritis and rheumatism, acne, diseases of the urinary tract and cancers. The most often method of cat's claw medical administration is drinking its decoctions prepared through boiling in water or by macerating its bark in alcohol [8].

Numerous investigations have been carried out to isolate and determine secondary metabolites of this plant [4]. So far, over fifty identified compounds have already been reported, including oxindole alkaloids (speciophylline, mitraphylline, uncarine F, pteropodine, isomitraphylline, uncarine E), ursane type pentacyclic triterpenes with a variety of ursolic acid derivatives, quinic acid and quinovic acid glycosides, phenolic tannins like kaempferol and dihydrokaempferol, sterols and procyanidins [1, 7, 20, 25]. These compounds

may be responsible for many pharmacological activities described in literature [1, 4, 7, 9, 19]. These activities include, for example, inhibition of nuclear factor kappa B (NF- κ B), which is believed to be a crucial element in regulation of numerous genes in the organism, including target genes of the central nervous system [14].

One of the most interesting properties of cat's claw is inhibiting cell proliferation that has been demonstrated in human leukemic and lymphoma cell lines (K562, HL-60, Raji) [21]. Such an effect of *U. tomentosa* is likely to result from synergic action of many phytochemicals, however, the possibility that the observed anti-proliferation is caused by one or few constituents should also be taken into consideration. Anti-leukemic activity was demonstrated *in vitro* for the individual oxindole alkaloids, particularly uncarine F, triterpenoid saponins and ursolic acid [7].

Therefore, it is necessary to evaluate antimitotic activity on various *U. tomentosa* extracts obtained by employing different methods and techniques. Unfortunately, little has been done in this matter, although, it may be expected that an extraction method and processing of crude material must determine chemical content and a bioactivity profile of the final preparations. This assumption is in agreement with the data provided by Riva et al. [18]. The IC₅₀ values obtained by these authors for some chromatographic fractions of cat's claw and MCF7 breast cancer cells are within the range of 18 μ g/ml to 270 μ g/ml.

This study was designed for further exploration of the cat's claw anti-proliferative potency. We try to optimize chemical processing of the bark and leaves for

obtaining highly efficient extracts against proliferation of cancer cells. The activities of several preparations are evaluated on HL-60 acute promyelocytic human cells by applying trypan blue exclusion and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction assay (MTT).

Materials and Methods

Chemicals

The standards of oxindole alkaloids were purchased from ChromaDex (Santa Ana, USA). Ammonium hydroxide, sodium chloride, sulfuric acid, methanol, acetonitrile, ethyl acetate were from POCh (Gliwice, Poland). Trypan blue, penicillin G, streptomycin, glycine and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide were supplied by Sigma. RPMI was obtained from Institute of Immunology and Experimental Therapy (Wrocław, Poland).

Characteristics of plant material

The bark of *U. tomentosa* originated from Peru and was supplied by A-Z Medica Company, Gdańsk, Poland. The general characteristics of this material are shown in Table 1. Additionally, some evaluations were carried on dried and powdered *U. tomentosa* leaves produced by Andean Medicine Center, London, Great Britain. The voucher materials are deposited at the Laboratory of Phytochemistry, Institute of Bioor-

Tab. 1. Characteristics of the *Uncaria tomentosa* bark and leaves according to the Laboratorios Induquímica S.A. (Peru)

Feature	Bark	Leaves
Description	Fine reddish or chestnut brown powder with acrid-bitter taste	Greenish powder with acid-bitter taste
Foreign bodies	Up to 0.3%	Up to 0.2%
Particle size	40 mesh	50 mesh
Microorganisms		
Aerobic	140 UFC/g	No data
Yeast and moulds	10 UFC/g	No data
Salmonella	Not present	Not present
Pseudomonas aeruginosa	Not present	Not present
Staphylococcus aureus	Not present	Not present
Escherichia coli	Not present	Not present

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HPLC-fingerprint analysis of alkaloids

According to the standardization protocols developed for cat's claw [22, 24], the bark was analyzed for oxindole alkaloid content. To 100 mg of the bark, 15 ml 2% sulfuric acid solution were added and sonified for 15 min in an ultrasonic bath (Bandelin Sonorex RK 103H). The mixture was then centrifuged at 3000 rpm for 10 min and extracted three times with 10 ml of ethyl acetate. The aqueous phase was separated and adjusted to pH = 10 with 10% ammonium hydroxide and extracted three times with 10 ml of ethyl acetate each. The organic extracts were combined, evaporated to dryness and the residue was dissolved in 1 ml of methanol. The qualitative and quantitative content of alkaloids were determined by the HPLC fingerprint analysis [HPLC: L-7100 Intelligent Pump (Merck-Hitachi), L-7200 Autosampler (Merck-Hitachi), L-7450 Diode Array Detector (Merck-Hitachi); Software: D-7000 Chromatography Data Station Software ver. 4.0; Column: LiChrospher® 100 RP-18 (250 mm × 4 mm, Merck); Precolumn: LiChrospher® 100 RP-18 (4 mm × 4 mm, Merck); Solvents: A – phosphate buffer solution (10 mM, pH = 6.6), B – methanol : acetonitrile (1:1); Gradient: (60% A and 40% B) to (30% A and 70% B); Time: 30 min; Washing: 20% solvent A and 80% B; Temperature: 21°C; Flow Rate: 1.0 ml/min; Detection: 245 nm]. See Figure 1 and Table 2 for results of this analysis.

Preparation of extracts

One gram of the bark was extracted in 10 ml of water, 25%, 50%, and 96% ethanol for 8 h at 37°C. For water and 96% ethanol extractions were also performed at boiling temperature of these solvents for 8 h. One of the cat's claw leaves was extracted in 10 ml 96% ethanol for 8 h at 37°C. Then, the extracts were centrifuged (MLW K70D) for 15 min at 4000 rpm. Supernatants were evaporated on Speed-Vac and next exsiccated with P₂O₅. All these extracts were analyzed by HPLC according to the procedure described above. For cytotoxic studies 10 mg of each preparation were dissolved in 1 ml of 96% ethanol and adjusted with 333 µl of water. The control was prepared in the similar way by adjusting 1 ml of 96% ethanol with 333 µl

of water. All of the preparations were sterilized by filtration through 0.22 µm filter.

Obtaining alkaloids-rich bark preparation (B/S_{rt})

Ten grams of the bark of *U. tomentosa* were extracted with 50 ml of water (6 h, 37°C). Next, the sample was centrifuged at 35000 rpm. To the water supernatant 50 ml of dichloromethane was added and the mixture was intensively shaken. The organic layer was evaporated under vacuum at 40°C to dryness. The residue (150 mg) was dissolved in 1.5 ml of 96% of ethanol and filtered through 0.22 µm filter. The obtained filtrate was stock solution of EOA preparation used for further analysis.

Cytotoxicity evaluation

HL-60 cells were grown in RPMI medium, containing 10% fetal calf serum and 1% penicillin G and streptomycin, at 37°C in a controlled humidified incubator in 5% CO₂. Then cells after, a few passages were centrifuged, resuspended in fresh medium at a concentration of 0.30 × 10⁶ cells per millilitre and transferred onto several plates in 2 ml volumes. The cells were exposed to 100 µg/ml of the cat's claw preparations and to control for 72 h. For obtaining these concentrations, 26.7 µl of the prepared stock solutions were added to each plate.

Trypan blue exclusion

Trypan blue was added to cell cultures at a ratio 1:1 and left for 10 min. Approximately 20 µl of cell suspension was loaded by a micropipette to the Bürker chamber. The cells were counted under a microscope at 100 × magnification. The ratios of live and dead cells were determined. The assay was performed in duplicate 24, 48 and 72 h after starting exposure.

Tetrazolium reduction assay (MTT)

The tetrazolium reduction assay was conducted as described by Denizot and Lang [3]. Briefly, 100 µl volumes of the medium with cells were transferred into Eppendorf tubes and centrifuged for 5 min at 1600 rpm. The supernatants were removed and cells resuspended in 100 µl of fresh media. The control containing fresh medium without cells was also prepared.

A portion of 20 μl of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide) solution (5 mg/ml in PBS) were added to each sample. All plates were incubated for 4 h at 37°C in a humidified incubator in 5% CO₂. Next, 120 μl of DMSO was added for dissolving formazan crystals. After 3 h of incubation at 37°C, all samples were centrifuged and 25 μl of Sørensen buffer (0.1 M glycine, 0.1 M sodium chloride, pH = 10.5) were added to each supernatant. Absorbance of the formazan product was measured at 570 nm. The assay was performed in duplicate 24 h after starting exposure.

Results and Discussion

General characteristics of the *U. tomentosa* materials used for obtaining the preparations are presented in Table 1. The oxindole-alkaloid profiles of the used bark and leaves determined by HPLC are shown in Figure 1.

Under the applied chromatographic conditions, isomitraphylline and pteropodine were not separated what was previously observed in chromatograms of the oxindole-alkaloid standards (unpublished data). However, the used method of standardization was sufficient for achieving aims of this experiment. The authenticities of the used bark and leaves were confirmed by the presence of oxindole alkaloids that are unique to *Uncaria* genus. Total alkaloid contents were 725.25 mg/100 g of bark and 1082.00/100 g of leaves. These results are generally difficult to compare due to very little literature data. Some previously reported determinations [12] carried out on leaves and roots of different *U. tomentosa* plants indicate higher total alkaloid contents (2159.80 \pm 1068 mg/100 g of young leaves, 1514.00 \pm 404.76 mg/100 g of mature leaves and 1682.00 \pm 361.83 mg/100 g of roots; recalculated results for specimen no. 1–5). These data are also different taking into account particular oxindole-alkaloid proportions.

For example mitraphylline and isomitraphylline were not detected in leaves and they were only observed in roots with respective 13.67% and 12.25% participations in total alkaloid content. Most alkaloids, were dependent on the analyzed material. In young leaves uncarine F was in the 50.75% majority, whereas in mature leaves and roots speciophylline

was the most abundantly represented alkaloid (44.78% and 30.44%, respectively). This is surprising considering that uncarine F and speciophylline in isolated forms are unstable isomers of D/E *cis*-fused group of alkaloids which easily undergo isomerization [11, 12]. On the contrary, our results indicate mitraphylline and isomitraphylline / pteropodine as main alkaloid compounds in leaves (44.07% and 40.46%, respectively). The results obtained for bark are also different showing predominance of isomitraphylline/pteropodine (51.21%), speciophylline (15.89%) and isopteropodine (14.40%).

TLC analyses of the used bark and leaves did not show the presence of any tetracyclic oxindole alkaloids (e.g. rynchophylline) in both of the investi-

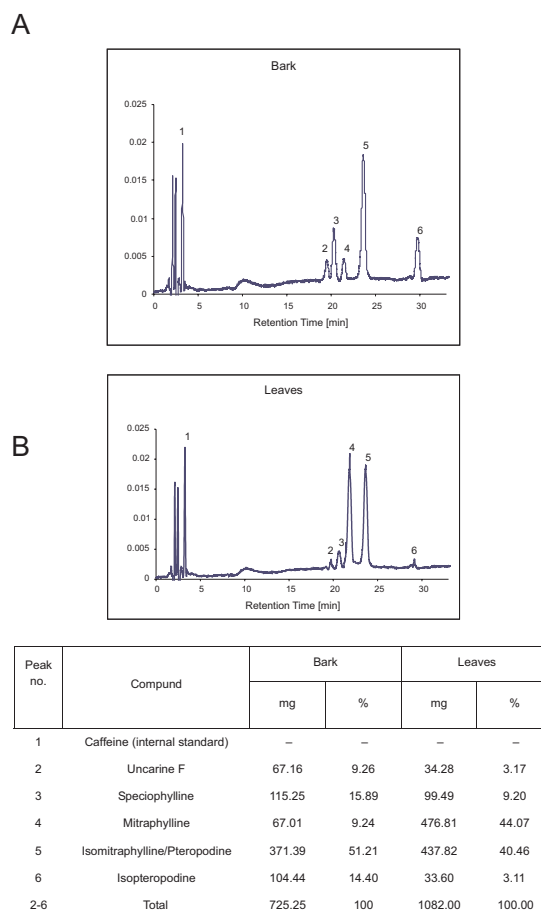


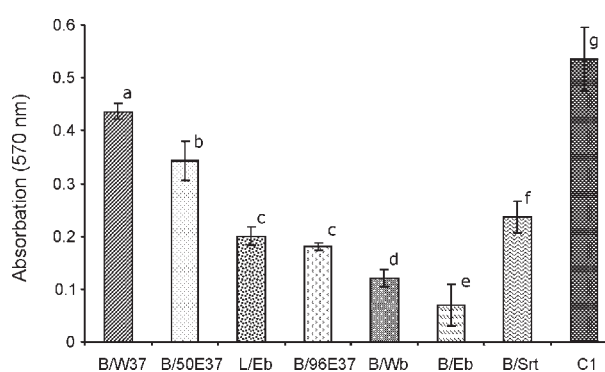
Fig. 1. The oxindole-alkaloid profile of the studied bark and leaves: (A) HPLC-chromatograms (B) Content of alkaloids expressed in percents and mg/100 mg

Tab. 2. Contents of alkaloids in different cat's claw preparations expressed in percents and mg/100 g

Alkaloid	B/W ₃₇		B/W _b		B/50E ₃₇		B/E _b		B/96E ₃₇		L/E _b		B/S _{rt}	
	mg	%	mg	%	mg	%	mg	%	mg	%	mg	%	mg	%
Uncarine F	28	6.59	39	7.89	131	7.12	303	9.21	322	9.26	68	1.74	2712	5.38
Speciophylline	68	15.89	32	6.60	217	11.79	78	2.38	553	15.89	66	1.70	10937	21.7
Mitraphylline	33	7.74	13	2.58	151	8.22	223	6.79	322	9.24	1648	42.16	8014	15.9
Pteropodine & Isomitraphylline	230	53.44	249	50.88	987	53.62	1855	56.38	1782	51.21	1686	43.12	19303	38.3
Isopteropodine	70	16.34	157	32.05	354	19.25	830	25.24	501	14.40	441	11.28	9435	18.72
Total	430	100	490	100	1840	100	3289	100	3480	100	3909	100	50401	100

gated materials (unpublished data). Several studies have shown that *U. tomentosa* occurs in nature in two different chemotypes characterized by pentacyclic or tetracyclic pattern of indole and oxindole alkaloids (POA and TOA chemotypes). These patterns seem to be changeable under seasonal conditions, because a number of observed cat's claw plants shifted their chemotypes in the course of the years [13]. It is well known that pentacyclic and tetracyclic oxindole alkaloids appear antagonistic, therefore, mixtures of these alkaloids are less suitable for medicinal use [17]. Asháninka Indians who have been familiar with *U. tomentosa* for over two thousand years use only plants with POA chemotype. They believe that only these plants are inhabited by good *Manincaarite* beings and can be applied for healing purposes [8]. Thus, the lack of TOA in our samples may be very advantageous from a pharmacological point of view.

The oxindole alkaloid profiles of all preparations used in cytotoxicity evaluations were presented in Table 2. The majority of these preparations (B/W₃₇, B/W_b, B/50E₃₇, B/E_b, B/96E₃₇, B/S_{rt}) were produced from the same bark material, however, it was assumed *a priori* that using a different concentration of ethanol significantly differentiated their chemical content and probably their pharmacological activities. It was also presumed that the L/E_b should be characterized by higher alkaloid content mainly caused by mitraphylline elevation. As shown, the alkaloid content in preparations increases with the ethanol concentrations reaching the highest value at 96% ethanol while elevation of temperature is rather insignificant, but changes alkaloids proportions. Generally, the extracts obtained by extraction in boiling solvents contain lower contents of speciophylline and mitraphylline

**Fig. 2.** Results of MTT assay performed on HL-60 cells exposed on B/W₃₇, B/50E₃₇, B/96E₃₇, B/W_b, B/E_b, B/S_{rt}, L/E_b and control (C1). Different letters mean significant difference ($p < 0.05$)

and higher contribution of isopteropodine. For instance, using boiling ethanol over 6 times decreased the speciophylline content in total alkaloids in comparison to the extract obtained at 37°C. This observation is in agreement with literature in which non-stability of speciophylline was reported [8, 10, 11]. The above-presented changes in alkaloid profiles are probably a result of isomerization by retro-Manich ring opening, rotation and Mannich ring closure. Such behavior of oxindole alkaloids is accelerated by low protonation and increasing polarity of solvents [8].

The null hypothesis about the same antiproliferative activity of the preparations was tested by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. This method, first described by Mosmann [15], is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form

dark blue formazan crystals, which are largely unable to cross cell membranes, thus resulting in its accumulation within healthy cells. Solubilisation of the cells by the addition of a detergent results in the liberation

of the crystals, which are solubilized. A number of surviving cells are directly proportional to the level of the formazan product created. The color can then be spectrophotometrically quantified. The obtained re-

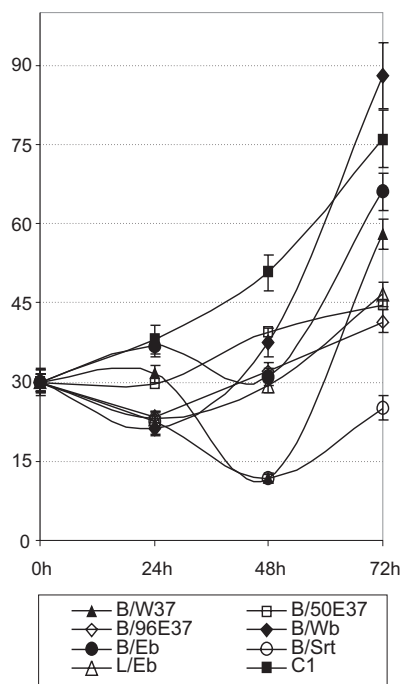


Fig. 3. Proliferation of HL-60 exposed to B/W₃₇, B/50E₃₇, B/96E₃₇, B/W_b, B/E_b, B/S_{rt}, L/E_b and control (C1). Number of cells [× 10000 per ml]

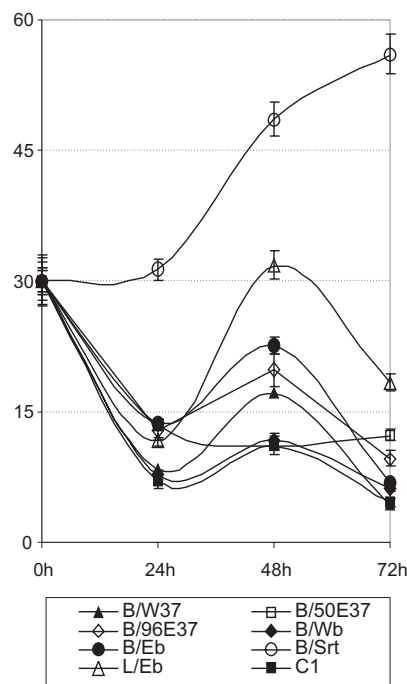


Fig. 4. Cell viability of HL-60 exposed to B/W₃₇, B/50E₃₇, B/96E₃₇, B/W_b, B/E_b, B/S_{rt}, L/E_b and control (C1). Number of cells [× 10000 per ml]

Tab. 3. Standardized and total anti-proliferative potency of B/W₃₇, B/50E₃₇, B/96E₃₇, B/W_b, B/E_b, B/S_{rt}, L/E_b and control (C1)

Preparation	MTT	Proliferation	Cell Viability	Total Activity
B/Srt	1.22	1.50	2.38	1.70
L/Eb	0.36	0.45	0.20	0.34
B/96E37	0.55	0.69	-0.30	0.32
B/50E37	-0.18	0.55	-0.14	0.07
B/Eb	0.96	-0.50	-0.46	0.00
B/W37	-0.67	-0.11	-0.61	-0.46
B/Wb	0.65	-1.58	-0.49	-0.48
C1	-1.19	-0.99	-0.59	-0.92

Standardization was performed according to following formula:

$$Z = \frac{X - \mu}{\sigma}$$

MTT assay – absorbance of A₅₇₀ (X = 0.306, SD = 0.193); proliferation – a number of cells after 72 h (X = 55.700, SD = 20.430); cell viability – a number of cells after 24 h (X = 14.78, SD = 17.291)

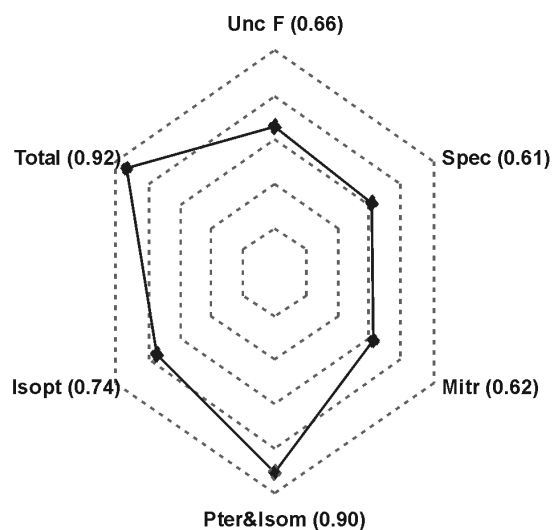


Fig. 5. Standardized antiproliferative activities of uncarine F (Unc F), speciophylline (Spec), mitraphylline (Mitr), pteropodine/isomitrphylline (Pter/Isom), isopteropodine (Isopt) and total alkaloids (Total)

sults only partially indicate ethanolic preparations to have more antiproliferative potency in comparison to the aqueous ones (Fig. 2). Apart from enriched alkaloid fraction B/Srt, the highest cytotoxicity was shown for B/E_b ethanolic preparation, whereas the lowest one for B/W₃₇ aqueous extract. On the other hand, the low absorbation observed for B/W_b sample is surprising. Similar results were obtained for hemacytometric evaluation of the cell number at 24th, 48th and 72nd hour after starting exposure to the cat's claw preparations (Fig. 3). In this case, proliferation rate of HL-60 was more suppressed by B/96E₃₇ than by B/50E₃₇ and particularly B/W₃₇. Finally, these observations may be confirmed by trypan blue exclusion results that show the supreme mortality of cells treated with ethanolic extracts L/E_b, B/E_b, B/96E₃₇ (Fig. 4).

Taking into account some divergences in the above-presented evaluations (especially for B/96E₃₇, B/E_b and B/W_b) and dependence between cell viability and proliferation determinations, we decided to standardize all results and calculate average total activity for each preparation (Tab. 3). The obtained sequence of activities is quite similar to the total alkaloid content in the preparations (Tab. 2). The hypothesis whether more cytotoxic activity of ethanolic preparations is caused equally by all alkaloids was statistically verified. Total activities of each preparation were compared with particular alkaloid determinations by calculating Pearson's correlation factors (Fig. 5). Higher correlations of pteropodine/isomitraphylline and isopteropodine contents than speciophylline and mitraphylline were detected ($r = 0.91$ and $r = 0.74$ vs. $r = 0.61$ and $r = 0.62$, respectively). These alkaloids seem to be the most appropriate for standardization of *U. tomentosa* extracts that is, in the case of pteropodine, already practised by many European manufacturers [4]. However, the possible conclusion about the highest activity of pteropodine/isomitraphylline and isopteropodine should be taken very carefully. Mainly because it would be derived from the activities of extracts containing not only oxindole alkaloids, but also other active phytochemicals. Furthermore, there is no full agreement with previous determinations of oxindole alkaloids cytotoxicity. Some of these determinations show uncarine F as the most anti-proliferative alkaloid toward leukemic cells [23] and speciophylline to be the most cytotoxic on SK-MEL, KB, BT-549, SK-OV-3 and VERO lines [16]. Others indicate proapoptotic potency of pteropodine and uncarine F

on proliferating G0/G1-arrested and bcl-2-expressing acute lymphoblastic leukemia cells (ALL) [2]. Contrary, García Prado et al. [5] demonstrated high antiproliferative effect of mitraphylline on human glioma (GAMG) and neuroblastoma (SK-N-BE2) lines. Summing up the above data, there is a need for carefully designed study on cytotoxicity of pure oxindole alkaloids. Our further investigations will be designed in this way.

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