

The impact of extract of mistletoe on tumour cells and hemostasis of human plasma



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Background: The plant *Viscum album* (Mistletoe) is a known source of biologically active substances, used in traditional medicine in Europe and Asia.

Aim: The goal was to study cytotoxic/cytostatic effect of mistletoe extract on tumour and normal cells and its influences on certain proteins of the blood coagulation system.

Setting: Mistletoe was collected in Ukraine in November 2020.

Methods: Water extract of *V. album*, both leaves and stems, was obtained and fractionated; it was characterised using high-performance liquid chromatography (HPLC) and mass spectrometry and tested on cancer cells and blood plasma.

Results: Extract demonstrated the presence of viscotoxins and carbohydrates and thermolabile compounds that enhance the activity of thrombin and factor X in plasma in the presence of calcium ions and increased the activated partial thromboplastin time (APTT) by 2.7 times. The cytotoxic/cytostatic action of the mistletoe fractions, the total extract and the fraction <10000 Da were higher in relation to Lewis lung carcinoma (LLC) cells than with Vero cells. The IC50 of total extract for LLC cells was 35% ($p < 0.05$) lower than that for Vero cells (1.12 ± 0.2 and 1.71 ± 0.15 mg/ml). Thus, the IC50 of the fraction <10000 Da for LLC cells was more than by 44% ($p < 0.05$) lower than that for Vero cells (2.07 ± 0.26 and 3.67 ± 0.41 mg/ml).

Conclusion: It was shown that both extracts exhibited a cytotoxic/cytostatic effect, more pronounced against tumour cells than normal cells and enhanced the clotting of blood plasma by thermolabile compounds by facilitating plasma coagulation.

Contribution: This research makes it possible to study mistletoe as a light and cheap anticancer therapy plant and cure blood coagulopathy or construct antibleeding bandages.

Keywords: *V. album*; plant extract; cancer cells; hemostasis; plasma coagulation; deficient plasma.

Introduction

Wild plant *Viscum album* (Mistletoe) is a known source of biologically active substances that are used in traditional medicine for preventing or curing of cancer diseases and stopping cessation of bleeding (Singh et al. 2016). The compounds of natural plant origin are being studied as modern agents for antitumour therapy. The growing interest for these compounds is explained by the discovery and detailed study of a number of plant-derived cytostatics and the successful application of anti-blastic drugs. Frequently, such compounds should be named among the compounds of plant origin (Orhan et al. 2006).

This compound is a group of biologically active proteins, peptides and alkaloids. Water extracts of leaves and stems of mistletoe (*V. album*) contain about 22 low-molecular-weight compounds and 6 proteins (Franz, Ziska & Kindt 1981).

Among them, low molecular weight compounds and their polymers are gamma-aminobutyric acid, acetylcholine, choline, histamine, terpenoids: alpha-amyrin, beta-amyrin, betulonic acid, ursolic acid, triterpene saponins, such as emuteraside, alkaloids: tyramine, luparin, flavonoids: isoramnetin, ramentin, quercetin, histamine, organic acids: caffeic acid, chlorogenic acid, sugars and alcohols, mannitol, vitamin E, polysaccharides ramnogalactan and arabinogalactan (Amer et al. 2013; Franz et al. 1981; Nhiem et al. 2013; Orhan et al. 2006).

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Mistletoe stems contain ursolic and oleanolic acids, choline and its derivatives (acetylcholine, propionyl choline), viscibine, viscotoxin. In addition, triterpene saponins, resins, inositol, amines (viscibine, norviscibine, tyramine, phenylethylamine), carotenoids and vitamin C were found in mistletoe stems (Amer et al. 2013 Nhiem et al. 2013).

Carotenoids, vitamin C, fatty oils containing linoleic, oleic and palmitic acids and resinous substances were found in the mistletoe berries and in the bark of the plant – glycoside syringing.

Also, this plant contains bioactive proteins: agglutinin VAA-I, chitin-binding lectin, viscotoxin, lectins ML: ML1p, ML2p and ML3p (Amer et al. 2013; Franz et al. 1981; Nhiem et al. 2013; Orhan et al. 2006).

The most important and the most studied mistletoe substances today are mistletoe lectins I–III. They have been identified as the maximal cytotoxic compounds in aqueous *V. album* L. extracts (Jung et al. 1990).

Toxic lectins of mistletoe belong to a family of proteins that inactivate ribosomes, in which the enzymatic A chain is linked to a disulfide bond with the lectin B chain. A-chain is a specific ribonucleic acid (RNA) N-glycosidase that hydrolytically cleaves the adenine residue from a highly conserved region of 28S ribosomal RNA (rRNA). The ribosome modified in this way is incapable of linking the factors of elongation, which leads to a stop translation and, ultimately, cell death. The B-chain binds to oligosaccharides of glycoproteins and glycolipids of the cell surface containing galactose endpoints, providing endocytosis of the toxin. This family also includes *Ricinus mites* (*Ricinus communis* L.) and

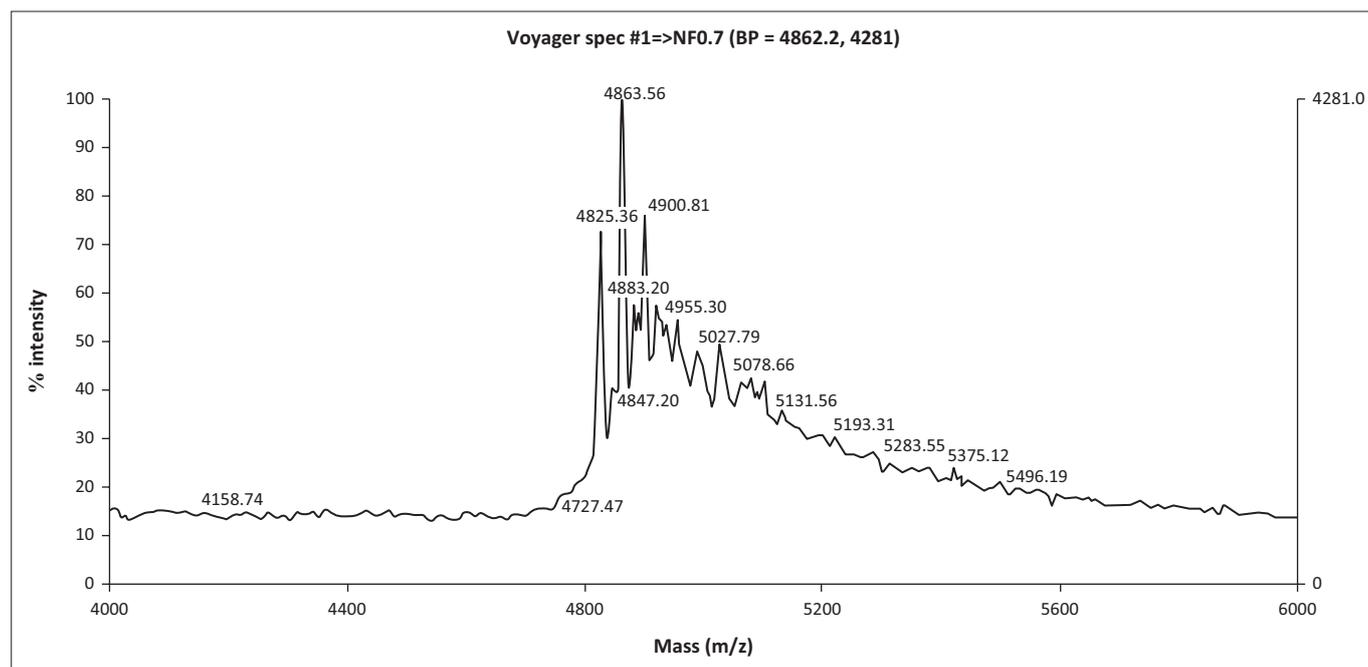
Shigga toxin produced by the bacterium *Shigella dysenteriae* (Sudarkina 2007).

Commercial mistletoe water-based extracts contain hydrophilic cytotoxic and immune-modulatory proteins: mistletoe lectins and viscotoxins (ed. Bussing 2000; Hajto et al. 2011; Schaller et al. 1998; Urech, Schaller & Jaggy 2006). They are known as stimulators of the immune system. The mechanism involves activating leukocytes, cytokine release and induction of apoptosis *in vitro* and *in vivo*, inhibiting cell proliferation (Simon, Simard & Girard 2013; Tabiasco et al. 2002). Apoptosis induced by mistletoe lectins is initiated by PI3K/Akt-, MAPK-, TLR-signaling pathways that lead to the activation of caspases (Bantel et al. 1999; Bussing et al. 1996, 1999; Park et al. 2010).

For solid tumours and leukaemia cell lines, *in vitro* and *in vivo* cytotoxic effects have been demonstrated, also metastatic activity was discontinued (Duong Van Huyen et al. 2006; Klingbeil et al. 2013; Park, Do & Jang 2012; Seifert et al. 2008). The Akt-, MAPK-, ERK-, and JNK-signaling pathways involve inducing apoptosis with oleanolic acid and betulinic acids, similar to lectin-induced apoptosis (Baracchini et al. 2006; Guo et al. 2013; Konopleva et al. 2005; Wang et al. 2013). Also, leukaemia cells demonstrate induction of apoptosis and inhibition of cell growth and cell proliferation (Pan et al. 2015; Urech et al. 2005).

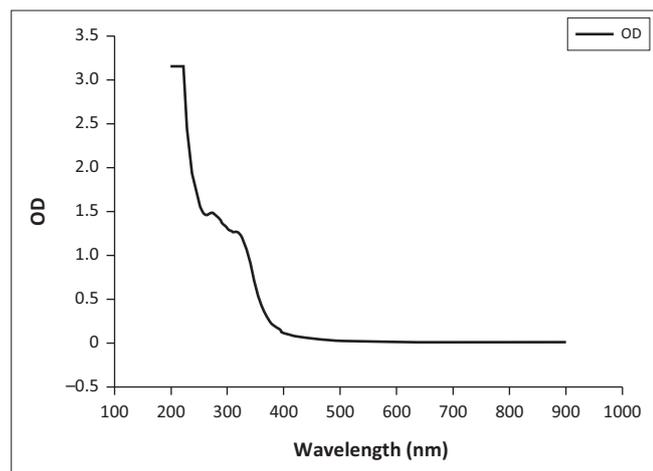
Two antitumour effects, from oleanolic acid and betulinic acids and ursolic acid, are compared *in vitro* and *in vivo* (Gheorghesou et al. 2014; Soica et al. 2014a,b), and new results showed a synergistic effect of combined oleanolic and ursolic acids for several human melanoma cell lines (Soica et al. 2014a,b).

The triterpene acids extract from *V. album*-enhanced apoptosis, and all types of extracts are able to induce



BP, base peak in massspectromrtry.

FIGURE 1: Mass-spectrometry analysis of *V. album* water extract showed the presence of protein with a molecular mass of 4.8 kDa – similar to viscotoxin.



OD, optical density in spectrophotometry.

FIGURE 2: Adsorption spectra of water mistletoe extract 200–900 nm.

apoptosis via caspase-8 and -9-dependent pathways with downregulation of members of the inhibitor of apoptosis and BCL-2 families of proteins.

A mouse model of acute myeloid leukaemia demonstrated the therapeutic efficiency of viscum triterpene acid treatment that caused tumour weight reduction in comparison to the effect in cytarabine-treated mice. These results showed that the *Viscum* triterpenes treatment may cause a therapeutic impact on acute myeloid leukaemia (Delebinski et al. 2012, 2015; Struh et al. 2013).

The aim of this study was to determine and measure the effects of whole mistletoe extract and its fractions on cancer and normal cells and the effect of the whole extract on plasma coagulation.

Research methods and design

Chemicals

In this study, we used acetonitrile, ammonium sulfate high-performance liquid chromatography (HPLC) grade, HCl HPLC-grade, Sinapine acid (Sigma-Aldrich), cultural medium RPMI 1640 (Sigma, USA), DMEM (Sigma, USA), foetal bovine serum (FBS, Sigma, USA), heparin sodium salt, gentamicin, trypan blue dye (Thermo Fisher Scientific, USA), W.B.C. diluting fluid (Qualigen, India). Tris and sodium chloride HPLC grade (Fluka, Monte Carlo), APTT-reagent firms Renam (Russia) and CaCl_2 0.025M solution, chemical pure, titrated by the manufacturer.

Viscum album extraction

Water extracts of leaves of medical plant mistletoe were obtained from dried plants. Leaves and stems were used to prepare water extract. Mistletoe was grown on an apple tree and harvested in the period of *V. album* fruiting in November, drying at +20°C in the shadow. To prepare water extract with the volume of 200 ml, 4 g of milled and dried plant were taken. It was washed by bubbling up water and infused for 30 min under a closed lid. Next, extracts with cropped

plant parts were stored in a plastic thermosaving box for 12 h. Then it was filtered through Whatman paper filter and 0.22- μm teflon filters. Then it was frozen and used if necessary.

For obtaining fractions <3500, <10000, <30000 Da, the researchers used centrypreps with mesh with micrometre diameter holes, through which extract was separated during rotation in a centrifuge in the lower part of the test tube, using the so-called grid analysis method. Centrypreps for 3.5 kDa, 10 kDa and 30 kDa were used to obtain fractions <3500, <10000, <30000 Da, respectively. Whole unfiltered fraction was separated with this centrypreps on the centrifuge. Centrifugation was conducted on two Eppendorf centrifuges: for 2 ml tubes (50 min, 0 °C, 2500 rpm) and 10–50 ml plastic tubes (50 min, 0 °C, 3500 rpm).

Determination of dry pellet of extract

3.5 cm in diameter dry Petri plates were used, which were marked with marker and weighed for the first time. Then 1 ml of each extract was added to each plate. They were weighed and placed into a drying oven for 24 h at +37 °C. Then Petri plates were weighed for the third time, and the mass of dry pellet was calculated. Further, it was used as a concentration of dry pellet per 1 ml of extract's fraction.

Obtaining blood plasma for turbidimetry and amidase assay

A total of 1 volume of 3.8% sodium citrate solution was added to 9 volumes of whole blood, gently mixed. Then it is centrifuged at 3000 rpm (300 g) for 40 min two times. Each time after centrifugation we carefully took all the plasma that is above the blood cells that are deposited on the bottom of the tube.

Plasmas deficient in blood coagulation factors VIII, X, IX were obtained from Renam (Russia); plasma with concentrated vitamin-K dependent enzymes (factor IX) was obtained in the researchers laboratory from whole blood plasma by precipitation (salting out) with 30% BaSO_4 .

Cell cultures

As tumour cells were used murine Lewis lung carcinoma cells (LLC) and as conditionally normal cells were used Vero cells, both obtained from the National Bank of Cell Lines and Tumour Strains of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of the National Academy of Sciences of Ukraine. LLC cells were kept in RPMI 1640 medium (Sigma, USA) supplemented with 10% foetal bovine serum (FBS, Sigma, USA) and 40 mg/ml gentamicin at 37°C in a humidified atmosphere with 5% CO_2 .

VERO cells were cultured in medium with 4500 mg/l glucose (DMEM, Dubecco's Modified Eagle Medium, Sigma, USA) and supplemented with 10% FBS and 40 mg/ml gentamicin as described above.

Cytotoxicity assay

When the cells are living, they are adherent to the cultural plastic and when the cells die, they detach from it. This property can be used for determination number of dead and surviving cells under the influence of death-inducing agents.

The method with crystal violet helps to detect the maintained adherence of cells using crystal violet dye, which binds to proteins and DNA. Cells that undergo cell death lose their adherence and are subsequently lost from the population of cells, reducing the amount of crystal violet staining with a culture. Then cells on the culture plastics undergo ethanol extraction after straining by crystal violet and washing. Then the amount of crystal violet is measured on spectrophotometer with a specific crystal violet extinction wave length. This method suits to indirect quantification of cell death and to determine differences in proliferation upon stimulation with death-inducing agents. This method has proven itself well for the examination of the impact of chemotherapeutics or other compounds on cell survival and growth inhibition. Common method is well described at Feoktistova, Geserick & Leverkus (2016).

The cytotoxic and/or cytostatic effects of fractionated and whole water extract of *V. album* were assessed by the IC_{50} index, the concentration of the agent, which causes a 50% reduction in the number of viable cells as compared to control because of its cytotoxic and/or cytostatic action.

For that, LLC and VERO cells were seeded into 96-well plates in 0.1 ml medium at a density of 10^5 cells/ml and incubated overnight under standard conditions.

At the end of the preincubation period, 0.1 ml of fresh medium was added to the cells, which contained the test agents in progressively decreasing concentrations (with a factor of 2) and incubated under standard conditions for 24 h.

As a solvent control (positive control) were cells, to which was added Tris-HCl buffer (pH = 7.6), diluted with nutrient medium according to the dilution of the test agents. Cells, to which nutrient medium was added without any agents, were used as negative control. Each agent concentration was evaluated in three replicates. At the end of the incubation period, after one day, the number of viable cells/well was determined using crystal violet and read using a multiwell spectrophotometer at a wavelength of 595 nm.

The total number (N) of viable cells (in % of control) as a function of the concentration (C) of the studied agent was analysed using the following exponential model:

$$N = N_R + N_S \cdot \exp\left(-\frac{C - C_0}{\alpha}\right), \quad [\text{Eqn 1}]$$

where

N_R – the portion of cells that are resistant to studied agent

N_S – the portion of cells that are sensitive to studied agent

C_0 – the maximum of the non-cytotoxic concentrations of the studied agent

α – index of cell sensitivity to agent cytotoxicity.

Model parameters were determined from the best fit of the model (Eqn 1) to corresponding experimental data using nonlinear regression analysis (Origin Pro, v.9.5).

High-performance liquid chromatography

Chromatographic system Agilent 1100 was used for the analysis of extract with column Dupont Instrument (250 mm long and 4.7 mm over) with Zorbax Silicogel (20 μ m) with C18 inoculation at a pressure of 140 bar and flow of 1.5 ml per minute. Solvents gradient decreasing 0.1% TFA was used (trifluoroacetic acid) in bi-distillate water and incising 100% acetonitrile in 70 min long (0 min–20 min – 100% ddH₂O, 0.1 % TFA; 20 min–40 min – gradient; 40 min–70 min – 100% acetonitrile). For characterisation, HPLC extract was dissolved in deionised distilled water at a concentration of 1 mg per ml and filtered through a syringe filter of 0.22 μ m.

The chromatogram was taken at a wavelength of 280 nm with an adsorption detector at a temperature of +30 °C. After analysis, the system was washed with 30% acetone and then several times with 30% isopropanol.

Xanthoproteic test

Xanthoproteic test was used for the characterisation of the protein content of the extract. One volume of extract was mixed with an equal volume of nitric acid. The mixture was heated and then cooled, and then it was slowly mixed with 40% sodium hydroxide until the mixture becomes alkaline and a colour change is noted. Yellow or orange colour indicates the presence of an aromatic amino acid.

Steady-state absorption spectra

The differential absorbance spectra were recorded in the 200–900 nm range using spectrophotometer Optizen-POP (Optizen, Korea) at room temperature.

MALDI-TOF analysis

Mass spectrometric analysis of *V. album* extract was performed on a MALDI-TOF Voyager DE Pro mass spectrometer manufactured by Applied Biosystems (USA), serial number 6393. The samples were mixed with a solution of sinapic acid as a matrix (1 mg was dissolved in a mixture of 70 μ l of a 0.1% aqueous solution of trifluoroacetic acid and 30 μ l of acetonitrile) in a ratio of 1:1 (by volume), so-called H⁺-matrix for ionisation of polypeptides. Mass spectra were obtained in the linear mode, applied voltage of 25 kV, with positive ionisation of the studied samples.

Results were analysed by Data Explorer 4.0.0.0 (Applied Biosystems) (ed Chapman 2000).

Turbidimetric studies

The hemostatic potential was determined by the turbidimetric method in accordance with Storozhuk et al. (2018). The total unfractionated extract of the *V. album* was used for this purpose. As a control of proteins influence onto blood coagulation system, one part of extract was used after boiling under closed lid for 1 h in the water bath, and another part was not boiled. Hemostatic potential of blood plasma was determined spectrophotometrically, the scattering of light by a fibrin clot at 350 nm, formed in the 10 mm spectrophotometric cuvette, where following reagents were consecutively placed: 100 µl of blood plasma and 100 µl dissolved APTT reagent and incubated for 3 min at +37 °C, then 0.05 M TRIS-HCl buffer, pH 7.4, containing 0.15 M NaCl (400–600 µl) and 100 µl 0.025 M CaCl₂ were added to start reaction. Final concentration of total mistletoe extract was 0.019 mg/ml. Analysis of turbidity curves was performed using a specialised computer program.

Amidase activity assay of thrombin

Using chromogenic substrate S2238 (H-D-Phe-Pip-Arg-pNA), the influence of *V. album* total extract on thrombin activity and activation of the coagulation system of blood plasma was studied. The analysis was done in 0.05 M Tris-HCl buffer of pH 7.4 solution, 37 °C. Chromogenic substrate was the final concentration of 30 µM. The final concentration of *V. album* extract was 0.019 mg/ml (Lottenberg et al. 1981). To detect proteins and enzymes that influence the blood coagulation system there was used a boiled and intact extract of the mistletoe too. The boiled extract was prepared intact by boiling in a water bath for 1 h.

Statistics

MS Excel 2016 and Origin 9 programs were used for statistical data processing.

Ethical considerations

Ethic committee of Palladin Institute of Biochemistry analysed ethical issues in the article entitled 'The Effect of Aqueous Extract of Mistletoe and Its Fractions on Tumor Cells and Hemostasis of Human Plasma' that was prepared for the publication in the *Journal of Medical Plants for Economic Development* by Dr. Rostyslav Marunych and co-authors. Plasma samples were obtained from the blood of healthy donors. Volunteers signed informed consent prior to blood sampling according to the Helsinki Declaration.

On the committee assembly on 07 March 2021 was decided:

- the presented article was not face with any ethical problems that can occur during the investigation, writing and preparing to the publication; all donors signed the informed and voluntary consent documents in accordance to The Declaration of Helsinki, 1964. Signed forms were properly prepared and sent to Committee by authors.

- use of animal-cultivated cell lines (LLC and Vero cells) obtained from the National Bank of Cell Lines and Tumor Strains of Ukraine (IEPOR) does not require the ethical approval.

In the light of all mentioned above, Ethic Committee of Palladin Institute of Biochemistry has no objections against the publication of article 'The Effect of Aqueous Extract of Mistletoe and Its Fractions on Tumor Cells and Hemostasis of Human Plasma' that was prepared for the publication in the *Journal of Medical Plants for Economic Development*. The deputy director of Palladin Institute of Biochemistry, vice head of Ethic Committee, Dr Sc. Volodymyr Chernyshenko. Members of committee: Dr Sc. Velykyj Mykola Mykolajovych Ivanova Rayisa Borysivna Ph.D. Grygoryeva Majya Volodymyrivna.

Results and discussion

Biochemistry characterisation of *Viscum album* extract

We conducted chemical characterisation of *V. album* extract using mass spectrometry, spectrophotometry and HPLC. In the total water extract, some proteins and other compounds were found. In Figure 1 proteins presented are viscotoxine (4.8 kDa), there was possibly, peptides (Figure 1 and Figure 2) and some peaks may correspond to polysaccharides and pigments. There was no possibility to determine such high-molecular weight compounds as chitin or cellulose-binding domain and lectins ML1P, ML2P, ML3P with the MALDI – TOF.

Also, the same sample of mistletoe extract was analysed by spectrophotometry and showed spectra with maximum of adsorption at UV diapason. This absorption may correspond to proteins and plant pigments.

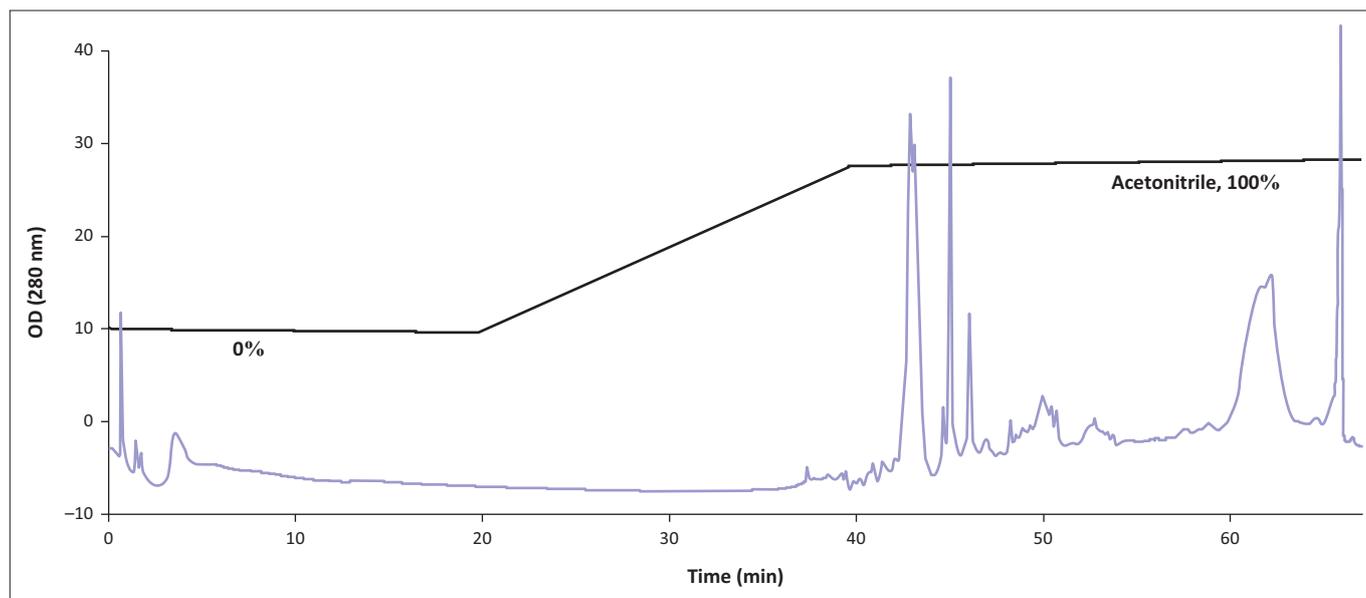
The HPLC analysis of water mistletoe extract was performed with HPLC Agilent 1100, using water solvent and acetonitrile gradient. Most details of chromatographic conditions were shown under Figure 3 and in the section of Materials and Methods, HPLC-chromatography.

The chromatogram of the extract shows some separate peaks at 280 nm, which may correspond to cellular plant's proteins and enzymes. Xanthoproteic test demonstrated the presence of proteins too. It was shown that these components are mostly hydrophobic and separately eluted only in acetonitrile.

Cytotoxicity of *Viscum album* extract fractions

It was found that total extract and all the investigated fractions of mistletoe extract exhibited a cytotoxic and/or cytostatic effect on both tumour and normal cells.

However, the sensitivity of tumour cells to the action of the investigated agents was significantly higher than that of normal cells. The data obtained are presented in Table 1.



OD, optical density in spectrophotometry.

FIGURE 3: High-performance liquid chromatography chromatogram of mistletoe water extract, using chromatographic system Agilent1100 with column Dupont Instrument (250 mm long and 4.7 mm over) with Zorbax Silicogel (20 μ m) with C18 inoculation at a pressure of 140 bar and flow of 1.5 ml per minute. The buffer gradient decreased from 0.1% TFA (trifluoroacetic acid) in bi-distillate water and incising 100% acetonitrile in 70 min long (0 min–20 min – 100% bidistilled H₂O, 0.1 % TFA; 20 min–40 min – gradient; 40 min–70 min – 100 % acetonitrile). The chromatogram was taken at a wavelength of 280 nm with adsorption detector and temperature of +30 °C.

TABLE 1: The concentration of the studied agents that result in 50% inhibition of Lewis lung carcinoma cells and Vero cell growth and viability (IC_{50}).

Fraction	IC_{50} (mg/ml)	
	LLC cells	Vero cells
Total extract	1.12 \pm 0.2	1.71 \pm 0.15*
<30 000 Da	1.69 \pm 0.5	2.04 \pm 0.15
<10 000 Da	2.07 \pm 0.26	3.67 \pm 0.41*
<3500 Da	1.04 \pm 0.04	0.77 \pm 0.07

Da, Daltons molecular mass; LLC, Lewis lung carcinoma.

* $p < 0.05$ compared to LLC cells.

TABLE 2: Model parameters of Lewis lung carcinoma cells cell viability as a function of concentrations for total extract.

Model parameters	Parameter units	LLC cells	Vero cells
N_R	%	28.5 \pm 3.1	39.0 \pm 1.9*
N_S	%	75.6 \pm 3.9	60.6 \pm 2.3*
C_0	mg/ml	0	0.36 \pm 0.04
α	mg/ml	0.89 \pm 0.13	0.79 \pm 0.08
IC_{50}	mg/ml	1.12 \pm 0.2	1.71 \pm 0.18*

* $p < 0.05$ compared to LLC cells.

LLC, Lewis lung carcinoma; N_R , the portion of cells that are resistant to studied agent; N_S , the portion of cells that are sensitive to studied agent; C_0 , the maximum of the non-cytotoxic concentrations of the studied agent; α , index of cell sensitivity to agent cytotoxicity.

Comparative analysis showed that the most pronounced differences between IC_{50} for tumour and normal cells were recorded for total extract and fraction <10000 Da. Thus, the total extract concentration leading to a 50% decrease in the number of viable LLC cells because of its cytotoxic and/or cytostatic action (IC_{50}) was 35% ($p < 0.05$) lower than that for Vero cells. Thus, the IC_{50} itself of the fraction < 10000 Da for LLC cells was more than 44% ($p < 0.05$) lower than that for Vero cells. For total extract and fraction < 10000 Da, a detailed analysis of the dependence of their cytotoxic effect on concentration was carried out using the model (Table 2, Figure 4). This analysis showed

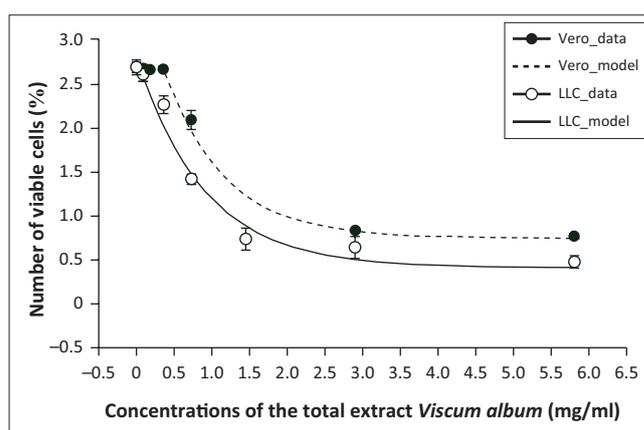


FIGURE 4: Number of viable Lewis lung carcinoma cells and Vero cells as function of total extract concentration. Symbols – experimental data. Lines – fitting curve.

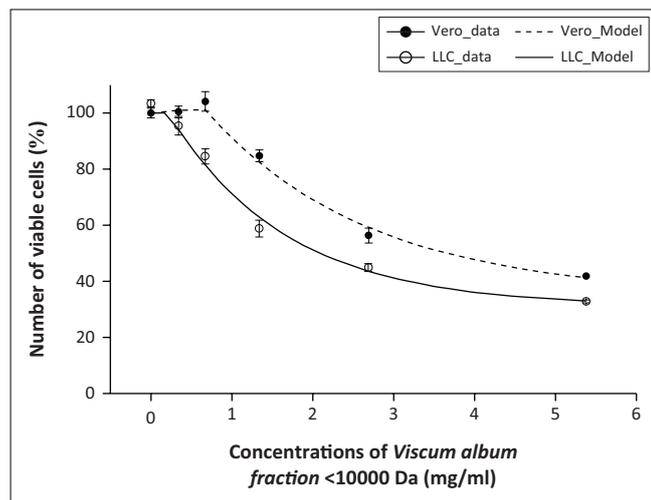
that the higher sensitivity of tumour cells (as compared to normal cells) to the action of total extract was because of a statistically smaller volume of LLC subpopulation of LLC cells resistant to this fraction of mistletoe and a larger proportion of the sensitive subpopulation. As can be seen from Table 2, the volume of the sensitive subpopulation of cells among LLC cells was 15% higher ($p < 0.05$), and the volume of the resistant subpopulation was almost 10.5% lower ($p < 0.05$) than the corresponding subpopulations among Vero cells.

A detailed analysis of the sensitivity of cells to the action of the fraction <10000 Da showed that the volume of sensitive and resistant subpopulations among tumour cells practically did not differ from those among normal cells (Table 3, Figure 5). Meanwhile, statistically significant ($p < 0.05$) differences in the α parameter indicated that the cytotoxic effect of the

TABLE 3: Model parameters of Lewis lung carcinoma cell viability as a function of concentrations for fraction < 10 000 Da.

Model parameters	Parameter units	LLC cells	Vero cells
N_R	%	31.0 ± 1.8	35.0 ± 1.2
N_S	%	71.1 ± 2.2	66.2 ± 2.5
C_0	mg/ml	0.17 ± 0.02	0.67 ± 0.04
α	mg/ml	1.45 ± 0.13	2.0 ± 0.19*
IC_{50}	mg/ml	2.07 ± 0.26	3.67 ± 0.41*

* $p < 0.05$ compared to LLC cells.

**FIGURE 5:** Number of viable Lewis lung carcinoma and Vero cells as function of < 10 000 Da fraction concentrations. Symbols – experimental data. Lines – fitting curve.

fraction < 10000 Da on the sensitive subpopulation is significantly higher in tumour cells than in normal cells (Table 3).

The obtained data indicate a significantly higher efficiency of the cytotoxic/cytostatic action of the studied mistletoe fractions in relation to LLC cells as compared to Vero cells.

Influence of *Viscum album* extract onto blood coagulation system

The influence of mistletoe water extract onto human blood coagulation system was studied.

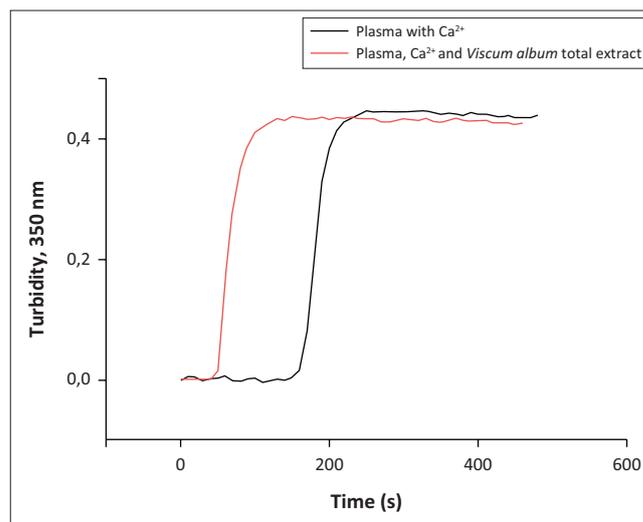
It was demonstrated that *V. album* extract in concentration 0.019 mg/ml facilitates plasma coagulation initiated by Ca^{2+} addition in 2.7 times (Figure 6).

This effect disappeared after *V. album* extract was boiled for 1 h at 100 °C. Boiled extract lost its activating ability.

For the determination of which links of cascade of blood coagulation were involved and activated with *V. album* extract, additional studies with chromogenic substrate S2238 for thrombin were performed.

We measured thrombin generation in control plasma obtained from a healthy donor and in commercially obtained plasma deficient in one of the factors: IX, VII and X.

A study with plasma with the concentrate of factor IX was performed separately. This concentrate contains only vitamin

**FIGURE 6:** Reduction of the time of plasma coagulation by calcium in the presence of mistletoe extract measured at 350 nm.

K-dependent proteins of blood coagulation cascade but did not contain proteins of contact phase of activation of plasma coagulation.

In this variant of the experiment, APTT reagent activates the contact system of coagulation activation, activate, in the presence of calcium ions, a cascade of enzymatic reactions, that leads to thrombin generation. Thrombin cleavages chromogenic substrate with paranitroaniline release.

Concentration of paranitroaniline could be measured using a reader with a wavelength of 405 nm. It was confirmed that components of *V. album* extract were able to facilitate thrombin generation under influence of APTT in control donor's plasma and in plasma deficient in factor VII.

Instead of this, extract had no ability to facilitate plasma coagulation without factor Xa. We can suppose that this effect of stimulation fibrin generation in blood plasma under influence of components of extract of *V. album* can be hang together with address influence onto factor Xa. This will be a subject of further research. A moderate influence on plasma deficient in IX factor and on plasma without factor IX was also observed.

All this data explained that *V. album* includes some thermolabile protein nature compound that interacts with factor X and thrombin and facilitates cleavage of fibrinopeptides of fibrinogen or cleavage of chromogenic substrate.

Thus, from Figure 7, we can conclude that mistletoe extract restores the ability of deficient in VII, IX, factors plasma to coagulate, and the strongest effect is observed on plasma deficient in factor VII.

Limitations

The study was performed with *in vitro*-isolated cellular and plasma components of the hemostasis system and cultivated

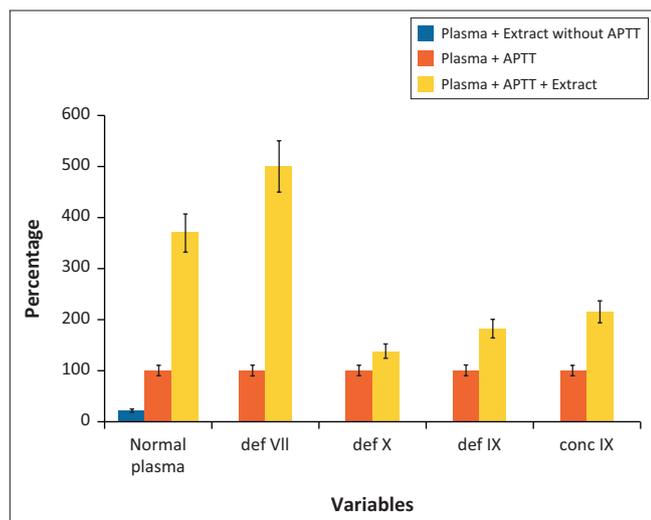


FIGURE 7: Acceleration of different plasma coagulation by *Viscum album* extract.

cells. This is a reduced model of the blood coagulation system and cancer cells; the effects on the whole organism were not studied. Also in this study, we determined some compounds in the plant's extract to characterise it, but the goal of identification and isolation of active compounds was not performed.

Conclusions

Mistletoe water extract was prepared from leaves and stems. In accordance to MALDI-TOF and HPLC analysis, it contained viscotoxin (4.8 kDa) and peptides, polysaccharides and pigments. Crude extracts showed moderate cytotoxic and/or cytostatic activity towards normal Vero cells and pronounced activity towards cancer LLC cells. Crude extract also stimulated blood coagulation, possibly activation of coagulation factor X.

We found that mistletoe extract has a cytotoxic and/or cytostatic effect on tumour cells and exhibits hemostatic properties. Most anticancer activity was demonstrated by total extract and fraction less than 10000 Da. In addition, mistletoe extract has influence on blood plasma coagulation activity. In absence of APTT *V. album* extract showed very little activity, but in the presence of APTT, it demonstrated high activity in stimulation of coagulation activity of normal plasma and plasma deficient in VII factor. High activity *V. album* extract demonstrates in plasma deficient in IX factor and in plasma with concentrate of IX factor and vitamin-K-dependent proteins. Also, effect of extract on coagulation was calcium dependent. This is thermo-dependence of effect on plasma pointed to protein nature of activator.

This data indicate that *V. album* extract has promising properties as source for developing of procoagulant agents.

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Competing interests

The authors declare that they have no financial or personal relationships that may have inappropriately influenced them in writing this article.

Authors' contributions

All authors contributed to the investigation, validation, data analysis, data plotting and writing discussion too.

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Data availability

The data that support the findings of this study are available from the corresponding author, R.Y.M., upon reasonable request.

Disclaimer

The views and opinions expressed in this article are those of authors and do not necessarily reflect the official policy or position of any affiliated agency of the authors.

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