Viscum album L. & Abies alba borisii regis effects on platelet aggregation and tumor metastasis

Ioannis Zelovitis¹, Dimitrios Peschos¹, Vasilios Ragos², Anna-Maria Vlachou¹, Evangelos Kontargiris¹, Irida Dhima¹, Apostolos Scaltsoyiannes³, Yannis V. Simos⁴*, Maria Chatzidimitriou⁵, Christianna Zachariou⁶, Panagiotis Grivas¹, Angelos Evangelou¹, Spyridon Karkabounas¹

¹Laboratory of Physiology, Medical Department, University of Ioannina, 45110 Ioannina, Greece.
²Clinic of Maxillofacial Surgery, Medical Department, University of Ioannina, 45110 Ioannina, Greece.
³Hellenic Agricultural Organization – Demeter/Dairy Research Department, 45221 Ioannina, Greece.
⁴Department of Nursing, Epirus Institute of Technology (T.E.I. of Epirus), 47100 Ioannina, Greece.
⁵Laboratory of Forest Genetics and Plant Breeding, Department of Forestry and Natural Environment, Aristotle University of Thessaloniki, 541 24 Thessaloniki, Greece.
⁶Medical Laboratory Studies, Alexander Technological Educational Institute, 57400 Sindos, Greece.

ARTICLE INFO
Received on: 15/05/2018
Accepted on: 23/09/2019
Available online: 03/12/2019

Key words:
Viscum album L., Abies alba, platelet aggregation, metastasis, cancer.

ABSTRACT
Viscum album L. is a widely used medicinal plant in cancer treatment, known since ancient times. The aim of this study was to investigate the antimetastatic properties of the ethanolic extracts of synergistic plants V. album (epiphyte) and Abies alba (host) used alone or in combination (mixture). Inhibition of platelet aggregation was evaluated in washed rabbit and human platelets. Levels of thromboxane A2 (TXA²) were estimated by radioimmune assay and natural killer cells (NKCs) cytotoxicity by flow cytometry. The antimetastatic properties of V. album and A. alba were studied in a tumor-bearing Wistar rat model. All extracts inhibited platelet aggregation in a dose-dependent manner as well as TXA² production by three pathways of aggregation (adenosine diphosphate, platelet-activating factor, and arachidonic acid) while the mixture significantly increased NKCs cytotoxicity against cancer cells. In tumor-bearing Wistar rats, the treatment with the mixture of V. album L. and A. alba extract reduced the metastatic locations almost by 77%. These data suggest that V. album L. and A. alba extract reduced metastasis through inhibition of platelet aggregation and amplification of the body defense mechanisms against cancer cells.

INTRODUCTION
Most cancer deaths are caused by metastasis rather than by the primary tumor (Yilmaz et al., 2007). Platelets have a role in cancer progression and metastasis that has largely been attributed to the platelet-mediated enhancement of tumor cell survival, extravasation, and angiogenesis (Gupta and Massague, 2004). Platelets form aggregates with tumor cells in circulation, facilitating their adhesion to leukocytes and to the vascular endothelium, promoting the metastatic cascade (Amo et al., 2014). Moreover, platelet aggregation around tumor cells inhibits in vitro NKCs tumorolytic activity and reduces the cytotoxic activity of tumor necrosis factor α (TNF-α) (Gay and Felding-Habermann, 2011; Nieswandt et al., 1999). Once the tumor cells exit the circulation, factors derived from activated platelets promote neoangiogenesis, thereby enabling cancerous tumor growth at the metastatic site (Gay and Felding-Habermann, 2011; Nieswandt et al., 1999).

Viscum album L. belongs to the family Loranthaceae, commonly known as European mistletoe and it is native to Europe and Western and Southern Asia (Vidal-Russell and Daniel, 2008). Viscum album L. does not have the capacity for autonomous growth. In order to survive and reproduce, it uses by necessity a variety of hosts with which it establishes a synergistic, rather
than a destructive parasitic relationship (as it is generally the case with other parasitic organisms of the plant kingdom). Common hosts of *V. Album* are *Pinus halepensis*, *Abies alba*, *Quercus silicuastrum*, *Malus communis*, and *Flamuria melia* (Böhling et al., 2003).

*Viscum album* L. extracts are often used in complementary and alternative medicine therapies for cancer (Horneber et al., 2008; Kienle et al., 2009; Steele et al., 2015). The anticancer properties of *V. album* L. have been attributed to various biologically active compounds. Among them, *V. album* L. lectins and triterpene acids have been extensively studied in different cancer models and were found to exert predominant cytotoxic activity (Choi et al., 2004; Delebinski et al., 2015; Twardziok et al., 2016). As for the cell death mechanisms triggered, the induction of apoptosis against different cancer cell lines was recorded (Choi et al., 2004; Delebinski et al., 2015; Kim et al., 2003; Lyu et al., 2002; Twardziok et al., 2016; Ucar et al., 2012).

The objective of this study was to investigate the antitumoral properties of Ethanolic extracts of synergetic plants *V. album* L. and its host *A. alba* in a series of *in vitro*, *ex vivo*, and *in vivo* experiments.

**MATERIALS AND METHODS**

**Preparation of *V. album* L. and *A. alba* extracts**

The *V. album* L. and *A. alba* (Abies borisii regis) plants were spotted in various areas of mountain Pindos in the Epirus Region, Greece. The soil where the plants developed was characterized as limestone. The host plant was *Abies borisii regis* which was a hybrid of *A. alba × Abies cephalonica* and the harvesting of *V. album* L. took place between late November and late February covering a period of about 100 days during the winter season. Identification of the plants was performed by Emeritus Professor P. Efthymiadis, Agricultural University of Athens. A voucher specimen for all the plants has been submitted to the Laboratory of Physiology, Faculty of Medicine, University of Ioannina (IZ-2014 380) as a prerequisite of the doctoral dissertation of the main author of this article.

Three types of extracts were prepared: i) *V. album* L. (leaves and stems), ii) *A. alba* (leaves and barks), and iii) 1:1 v/v mixture of *V. album* L. (leaves and stems) and *A. alba* (leaves and barks). Processing of the plant tissues took place entirely in a cold chamber at 4°C–8°C. Leaves, barks, and small green stems were washed thoroughly with tap and deionized water and the moisture was removed by filter paper. All tissues were homogenized in ethanol using an electric blender. The pulverized plants were previously weighed so that each extract contained 30 g plant per 100 ml of pure ethyl alcohol.

The extracts were placed in dark-brown amber flasks, while the air chamber of the bottles was filled with argon, sealed and placed in a cold chamber where they were stirred 1 hour per day with gentle circular agitation (40 rev/minutes for 60 minutes). After 30 days, the flasks were unsealed, the extract was filtered thoroughly with Whatman Grade 40 filters, and placed in airtight cryovials at −80°C, under an argon inert atmosphere. After 30 days, the extracts were dried in a stream of nitrogen under aseptic conditions and inserted into a lyophilizer vacuum for the removal of moisture where they remained for 12 hours. The amount of dry extract was standardized at 180 mg/ml of saline.

**Volunteer blood donors**

Twelve healthy volunteers accepted to donate blood for the completion of the experimental protocols of this study. The volunteers did not consume any kind of medication, food supplements, or consume alcoholic drinks during the experiments. The participation of the volunteers in the study was without monetary compensation and in agreement with the human rights legislation from the Declaration of Helsinki (World Medical Association, 2013). All volunteers provided written consent and were free of medical problems in their history.

**Blood collection from rabbits**

An amount of 30 to 40 ml of blood was withdrawn from the ear veins of five male New Zealand white rabbits weighing 4–6 kg each via free blood flow. The animals were housed in large cages that allowed communication among them with a constant light period of 12 hours. Access to food and water was ad libitum.

**Estimation of platelet aggregation**

Experiments with washed rabbit platelets were performed according to Evangelou et al. (1998), whereas for the human platelets, the procedure described by Simos et al. (2011) was followed. Briefly, blood was collected in test tubes containing citric acid and centrifuged in order to separate the plasma rich in platelets (PRP) fraction. Then, the PRP fraction was then used for the platelet experiments. Platelets concentration was fixed at 2.5 × 10⁵ cells/μl before the addition of the platelet agonists [adenosine diphosphate (ADP), platelet- activating factor (PAF), and arachidonic acid (ARA)]. The agonists’ concentration was adjusted to cause maximum, irreversible accumulation in control platelets. Apart from the agonists (ADP, PAF, and ARA), a leiomyosarcoma cell line (LMS cells, isolated from histological tumors in Wistar rats) (Aydikos et al., 2007) was used for human platelet aggregation (Metsios et al., 2012). The half-maximal inhibitory concentrations (IC50) of the three extracts were then estimated. The Ca-500 aggregometer (Chronolog Co, USA) was used for the evaluation of platelet aggregation.

**Measurement of thromboxane A₂ production by platelets**

Levels of the stable metabolite TXA₂ were estimated by a radioimmune assay using the kit “TXA2/2.3-DINOR-TXA, [125] RIA KIT” (Isotop company, Institute of Isotops Co. Ltd. Budapest, Hungary). Radioactivity of each sample was measured by using a γ-counter (Nucleus Co Model 1600). All samples were stored at −80°C for 10 days (Benedetto et al., 1997).

**Flow cytometry analysis on natural killer lymphocytes**

Twenty ml of whole blood were collected from 12 volunteers and transferred into tubes that contained heparin as an anticoagulant. Isolation of peripheral blood mononuclear cells (PBMCs) was performed as previously described by Neri et al. (2001). NKC's were isolated from PBMC using the RosetteSep
A model of in vivo hematogenous metastatic spread in Wistar rats

Nineteen female Wistar rats aged 3 months and weighing 175 ± 12 g were used in this study. Animals were kept in laboratory cages at room temperature (20°C ± 2°C), with control lighting (12 hours light/12 hours dark). All experiments were handled in accordance with the European legislation (European Union directive for the protection of animals used for scientific purposes, 2010/63/EU) for the care and the use of laboratory animals (Institutional permission number 20E02).

Animals were divided into two groups: the Control Group (CG) composed of 9 rats and the Treatment Group (TG) composed of 10 rats. Inoculation of malignant cells (LMS cells) to the rats was performed according to the procedure described by Verginadis et al. (2011). The extract administered to the rats was the mixture of V. album L. + A. alba and the treatment lasted for 5 weeks. Animals in the TG received intraperitoneally 136 mg/kg body wt of extract in the first week, 271 mg/kg body wt of extract for the 2 week, and 406 mg/kg body wt of extract for the last 3 weeks. The gradual increase of the extract was necessary for the adaptation of animals to the chemical stress, derived from the extract’s components.

After the end of the treatment period, the animals were sacrificed by the administration of Pentothal. An autopsy, a fixation of the organs in 8% formaldehyde, and a histological examination were also performed. More emphasis was given to the detection of metastasis in the lungs, liver, and in oversized and parenchymatous organs, wherein the malignant cells might afford additional metastasis.

Statistical analysis

Data are expressed as mean ± SE. Statistical significance between data means was determined by Student’s t-test and one-way analysis of variance (ANOVA) (SPSS version 17.0, SPSS Inc. Illinois, Chicago). p values <0.05 were considered significant.

RESULTS

Inhibition of platelet aggregation

The three extracts exerted a dose-dependent inhibition of washed rabbit platelet aggregation. Viscum album L. extract achieved total inhibition at higher doses than the other two (Fig. 1). The sensitivity of V. album L. extract was higher to the PAF pathway compared to the ADP and ARA pathways (Fig. 1B). The A. alba extract, as well as the mixture of the two plants, achieved total platelet aggregation inhibition (caused by all three agonists used) in doses lower than 5 mg/ml PRP. A similar effect but slightly less potent was observed to the inhibition of human platelet aggregation by the three extracts (Fig. 2). Maximum platelet aggregation was observed at higher concentrations for all three extracts. Again, V. album L. extract sensitivity was higher to the PAF pathway (Fig. 2B). When LMS cells were used as an agonist, a dose-dependent inhibition of human platelet aggregation was recorded for all three types of extracts regardless of the agonist used (Fig. 3). The IC50 of the three extracts for the washed rabbit and human platelets is presented in Table 1.

The platelets significantly decreased TXA2 production when the extracts were added (10 mg/ml PRP). The three main agonists for platelet aggregation enhanced TXA2 production, with PAF being the most potent. A higher sensitivity was revealed for the ADP and ARA pathways (8-fold reduction) in comparison to the PAF pathway (4.6-fold reduction) (Fig. 4).

NKCs functionality

Incubation of the K562 cells with V. album L. + A. alba extract resulted in an increase of cancer cell deaths by 9.1% ± 0.7%. Incubation with 10 mg/ml of the extract resulted in a significant increase of NKCs cytotoxicity in all three ratios. The extract was able to significantly stimulate NKCs up to 340% at the...
lowest ratio (12.5:1). Stimulation of the NKCcs at the highest ratio (50:1) was 74% (Table 2).

**Antimetastatic activity of the *V. album L. + A. alba* extract**

The *V. album L. + A. alba* extract showed potent antimetastatic properties against tumor-bearing animals (Table 3). There were 60 metastatic locations observed in the CG: 36 in the liver, 17 in the lungs, and 7 in the rest of the organs. Fifteen from the 17 lung metastasis were obvious and large. On the contrary, fewer metastatic locations were observed in the TG. There were totally 14 metastatic locations: eight in the liver, one in the lungs, and five in the rest of the organs (Fig. 5). Only mild side effects were observed in a small number of TG animals which subsided after the first 2 weeks (Fig. 6).

**DISCUSSION**

Metastasis is the spread of cancer cells to new areas of the body (often via the lymphatic system or the bloodstream). A metastatic cancer is one that has spread from the primary site of origin (where it started) into different area(s) of the body. Depending on their tissue of origin, cancer cells subsequently spread to distinct target organs where they seed secondary tumors (Yilmaz et al., 2007). The treatment for metastatic cancer along with other factors depends on the type of cancer, the primary site of origin, the size, and the location of the metastasis and aims to slow the cancer growth or spread.

In a recent prospective randomized open-label study on overall survival, it was shown that patients with locally advanced or metastatic pancreatic cancer supplemented with a commercial form of the *V. album L.* extract (the drug Iscador® Qu spezial) for 12 months had no liver metastasis (Tröger et al., 2013). There are several *in vitro* and *in vivo* studies supporting that the ability of *V. album L.* to inhibit metastasis results from the inhibition of angiogenesis. Specifically, Korean *V. album L.* extract induces TNF-α that results in the inhibition of endothelial cells growth and to the suppression of tumor-induced angiogenesis (Yoon et al., 1995). The antiangiogenic activity of Korean *V. album L.* extract was verified by chorioallantoic membrane assay in C57BL6 mice inoculated with B16-BL6 melanoma cells and treated with the extract (Park et al., 2001). *In vitro* angiogenesis assay (matrigel) showed that the European *V. album L.* extract antiangiogenic activity is correlated to its high cytotoxic effect towards endothelial cells (EA-hyp926 cells) (Elluru et al., 2009). The findings from these studies support that *V. album L.* extracts strongly inhibit metastasis. We observed that treatment with the *V. album L.* and *A. alba* extracts not only resulted in a significant reduction in the number of metastatic locations but also in the total regression of 30% of the animals’ tumors.

Coagulation of platelets to cancer cells is essential for metastasis. It has been shown that surface shielding by platelet aggregates protects tumor cells from NKC lysis (Nieswandt et al., 1999) and reduces the cytotoxic activity of TNF-α (Neri et al., 2001). Thus, substances with antiplatelet properties might be of...
great importance in the defense of the organism against the spread of cancer cells and the formation of metastatic cancer tumors. We used washed rabbit platelets to exclude coagulation factors from the plasma in order to study cell-to-cell interaction. All three extracts inhibited platelet aggregation in a dose-dependent manner and these results were also confirmed in human platelets. These results might indicate that the extracts contain substances that act directly on the platelets rather than indirectly through the coagulation factors of plasma. Moreover, the ability of these three extracts to inhibit platelet aggregation induced by all platelet stimulators used (ADP, PAF, and ARA) suggests that they acted as non-selective anticoagulation agents.

Several studies have been conducted in order to identify the active compounds of *V. album* L. and *A. alba* extracts have been found to contain various classes of terpenoids fractions (Yang et al., 2008). It has been reported that terpenoids could act as peroxisome proliferator-activated receptor (PPARs) agonists (Fuentes and Palomo, 2013). PPARs are present in human platelets and their activation inhibits platelet functions (Akbiyik et al., 2004). The phytochemical profile of *V. album* L. includes compounds such as lectins, viscotoxins, triterpenes, flavonoids, and phenolic acids (Nazaruk and Orlikowski, 2015). We have previously shown that flavonoids like quercetin, found in the *V. album* L., increase the susceptibility of tumor cells to NKCs by decreasing platelet aggregation and stimulating the NK lymphocyte activity (Theoharis et al., 2011). Ascorbic acid, also a constituent of *V. album* L., inhibits platelet aggregation by reducing TXB2 levels and GpIIb/IIIa receptor’s expression and enhances NKCs cytotoxicity (Toliopoulos et al., 2011).

Although the mixed extract (*V. album L. + A. alba*) caused a relatively small degree of direct apoptosis to cancer cells, it successfully stimulated NKCs cytotoxicity. One of the most important findings of this study was that the immunomodulating action of the mixed extract was more profound on the ratio 12.5:1 where a small number of NKCs was involved against TC. NKCs cytotoxicity is significantly reduced in cancer (Johann et al., 2010) and thus, the potential

Table 1. Platelet aggregation IC50 (mg/ml PRP) of the three extracts.

<table>
<thead>
<tr>
<th></th>
<th>Washed rabbit platelets</th>
<th>Human platelets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ADP</td>
<td>PAF</td>
</tr>
<tr>
<td><em>V. album</em> L.</td>
<td>3.2 ± 0.3</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td><em>A. alba</em></td>
<td>2.4 ± 0.3</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td><em>V. album</em> L. + <em>A. alba</em></td>
<td>2.7 ± 0.2</td>
<td>2.1 ± 0.2</td>
</tr>
</tbody>
</table>

ADP, adenosine diphosphate; PAF, platelet activating factor; ARA, arachidonic acid; LMS, leiomyosarcoma cells; PRP, platelet rich plasma.

Table 2. Mean percentages (%) of cytotoxicity of NKCs against K562 TC after incubation with 10 mg/ml of the *Viscum album L. + A. alba* extract.

<table>
<thead>
<tr>
<th>NKCs:TC ratio</th>
<th>12.5:1</th>
<th>25:1</th>
<th>50:1</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Viscum album L. + A. alba</em></td>
<td>340 ± 21</td>
<td>165 ± 25</td>
<td>74 ± 16</td>
</tr>
</tbody>
</table>

Table 3. Tumor locations in the main organs of Wistar rats.

<table>
<thead>
<tr>
<th></th>
<th>Control Group</th>
<th>Treatment Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. animals</td>
<td>No. locations</td>
<td>No. animals</td>
</tr>
<tr>
<td>Liver</td>
<td>6</td>
<td>36</td>
</tr>
<tr>
<td>Upper respiratory system and lungs</td>
<td>5</td>
<td>17</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Pericardium</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Gastrointestinal-urogenital system</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Thyroid gland</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Spleen</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Pectoralis major</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Upper clavicle fossa (injection area)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>No tumor</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Total locations</td>
<td>60</td>
<td>14</td>
</tr>
</tbody>
</table>

Figure 4. Inhibition of TXA2, platelet production by the *Viscum Album L. + A. alba* extract. The baseline levels of TXA2 from resting platelets were used as control. *p < 0.05.
The ability of the extract to enhance their activity could be of great importance for these patients.

CONCLUSION

The V. album L. and A. alba extract exerted potent antimetastatic properties a) by inhibiting platelet aggregation and enabling NKCs to attack and destroy cancer cells and b) by enhancing NKCs cytotoxicity. Moreover, a direct anticancer activity is possible but further research is needed in order to clarify the specific compounds of these plants as well as the specific mechanism of anticancer activity.

CONFLICT OF INTEREST

The authors have declared no conflict to disclose.

ACKNOWLEDGMENT

The author gratefully acknowledges the assistance of Emeritus Professor Panagiotis Efthimiadis, Agronomist, for his assistance on the identification of the plants.

REFERENCES


Fuentes E, Palomo I. Relationship between Platelet PPARs, cAMP Levels, and P-Selectin Expression: Antiepileptoid Activity of Natural Products. Evid Based Complement Alternat Med, 2013; 861786.


How to cite this article: