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Antiangiogenic Potential of *Phellodendron amurense* Bark Extract Observed on Chorioallantoic Membrane

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***Abstract*—**Angiogenesis, a formation of new blood vessels from a pre-existing vasculature, plays an important role in pathologic processes such as the growth and metastasis of tumours. Tumours cannot grow beyond a few millimetres without blood supply from the newly formed blood vessels from the host tissue, a process called tumour-induced angiogenesis. The successful research of antiangiogenic treatment of cancer has focused on nutraceuticals with angiogenesis-modulating properties. Berberine, as a major active component of the bark of *Phellodendron amurense* Rupr., has shown antitumour activity by intervening into different steps of carcinogenesis. The influence of ethanolic extract of *Phellodendron amurese* bark on the angiogenesis was tested *in vivo* on chick chorioallantoic membrane (CAM). The irritancy of the CAM after the application of the crude bark extract dissolved in normal saline (10 mg/mL) was investigated on embryonic day 7. No significant signs of the irritancy, such as vasoconstriction, hyperaemia, haemorrhage or coagulation were observed which indicates the harmless character of the extract. A significant reduction in vessel sprouting and higher percentage of avascular zone was observed in the case of CAM treated with the extract in comparison with non-treated CAM (control), which is a proof of the antiangiogenic potential of the extract. These results could contribute to the development of novel drugs for the treatment of cancer or other diseases, in which angiogenesis plays a significant role.

***Keywords*—**Angiogenesis, berberine, chorioallantoic membrane, *Phellodendron amurense.*

I. Introduction

THE plant *Phellodendrom amurense* (PA) from the family *Rutaceae* is one of the major plants of the traditional Chinese medicine, which have been used traditionally for hundreds of years as an anti-inflammatory, astringent and antidiarrheal agents [1]. The research community has made a significant progress in studying pharmacological properties of this plant. In 2005-2014, the number of published papers dealing with PA was > 15 per year, which is a proof that this plant is constantly of significant interest for the research community (Fig. 1). The dry *Phelodendron amurense* bark (PAB) is the most commonly used for therapeutic purposes at a dose of 3-10 g. The bark contains mainly isoquinoline alkaloids such as berberine, oxyberberine, epiberberine, jatrorrhizine and palmatine [1]. This plant is also the source of limonoids, isovanillin, ferulic acid, ethyl caffeate and other phenolic substances [2], [3]. The plant material containing berberine has an antimicrobial action. The PAB represents a potential alternative to the treatment of inflammation. This effect is associated with the inhibition of NO production and inducible nitric oxide synthase (iNOS) expression via the inactivation of the nuclear factor (NF-κB) and mitogen-activated protein kinase (MAPKs), as well as by the inhibition of cytokines such as IL-6, IL-1β and macrophage chemo-attractant protein (MCP-1) [4]. The *Phellodendron amurense* bark extract (PABE) is also a potential therapeutic agent to protect cartilage against the osteoarthritic progression [5]. PA is able to inhibit the prostatic contractility suggesting that it may be useful in the treatment of urological disorders caused by the prostatic urethral obstruction, such as in the case of benign prostatic hyperplasia [6]. There are a few preparations with PABE used for the anti-inflammatory and analgesic properties available on the market [7]. Scientific studies have confirmed the antiproliferative activity, the increased apoptosis, the inhibition of cell proliferation and metastasis formation [1]. Berberine is also an antioxidant and it has a synergistic effect with other chemotherapeutics [8].

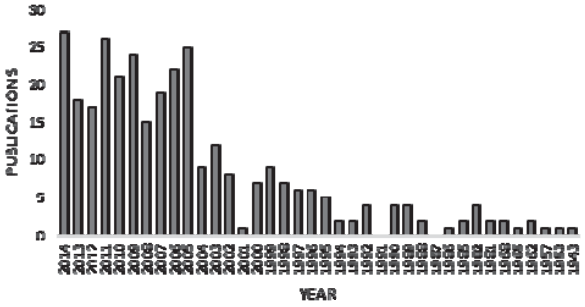

Fig. 1 Number of publications dealing with plant *Phellodendron amurense* in the years 1943-2014

In the initial stage of the carcinogenic process, the nutrition of a tumour tissue depends on the diffusion from the surrounding host tissues. Later, the formation of the vascular system that is existentially important for a tumour follows. In contrast to normal vascular beds, the new tumour vasculature provides an inefficient blood supply to the tumour, characterised by a highly disorganised structure, increased permeability, abnormal spacing, decreased and abnormal pericyte–endothelial cell adhesions, irregular basement membrane structures and the incorporation of bone-marrow-derived endothelial progenitor cells into the new microvasculature [9]. The anti-angiogenic therapy can be

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potentially used for all types of cancer because the tumours need the formation of vasculature in order to grow [10]. It is possible that PABE could exhibit ant-angiogenic activity.

The chick chorioallantoic membrane (CAM) is a good model for the assessment of irritancy after the application of the test extract. The CAM is a membrane which surrounds the developing chick embryo and is highly vascularised [11]. The CAM is formed by a fusion of two extra-embryonic membranes, namely the chorion and the allantois. Besides being a respiratory and excretory organ, it provides support underlying extraembryonic blood vessels [12]. The CAM is thin and transparent and therefore, the highly vascular structures can be easily seen.

The aim of our research activity was to determine the vascular irritation and anti-angiogenic potential after the application of various concentrations of PABE (namely 0.1; 1.0; 10.0 mg/mL). To our knowledge, this is the first time when potential inhibition of the formation of blood vessels by PABE is reported.

II. Materials and Methods

A. Preparation of Extracts

The ethanolic extract (1:10 w/v) of the PA bark was prepared by the maceration of the PAB in 70 % ethanol for one week. This extract was used for study of angiogenesis. The prepared tincture was evaporated by a rotary evaporator at 50°C. After the evaporation of solvent, the residues were diluted in saline solution to the final concentration 0.1; 1.0 and 10.0 mg/mL to determined vascular irritation and safety of application.

B. Preparation of Eggs

Freshly laid fertilized white Leghorn chicken eggs from the local hatchery (Slovakia) were cleaned by ethanol (60 %). From each egg, 2 mL of albumen was removed by a syringe with a needle. Then the hole in shell was covered with paraffin. The eggs were then incubated (with the large ends up) under the appropriate conditions (temperature 37°C, humidity 62 %). After 10 days, the shells from the large end and the inner shell membranes of each egg were removed. The vascular chorioallantoic membrane (CAM) was then exposed. The chick embryo is considered a seminal product in the Slovak Republic. Therefore, the approval of the experiments by an appropriate ethics committee is not necessary.

C. Vasoactivity

The test extracts (0.1; 1.0 and 10.0 mg/mL of PABE dissolved in saline solution) were dropped onto the CAM in a volume of 100 µL. After the application of the test extract, the blood vessels and capillary system of CAM were evaluated for irritant effects including hyperaemia, haemorrhages and coagulation after 0.5, 2 and 5 minutes from the application of PABE. As described by Luepke [13], the numerical time-dependent scores for hyperaemia, haemorrhage and coagulation (Table I) can be summed to give a single numerical value which indicates the irritation assessment (Table II). The results for each extract were evaluated as the average of three determinations.

TABLE I
THE NUMERICAL TIME-DEPENDENT SCORES FOR IRRITATION BY LUEPKE [13]

Effect	Score at a specified time		
	0.5 min.	*2 min.*	*5 min.*
Hyperaemia	5	3	1
Haemorrhage	7	5	3
Coagulation	9	7	5

TABLE II
CLASSIFICATION OF CUMULATIVE SCORES IN THE CHORIOALLANTOIC MEMBRANE TEST BY LUEPKE [13]

Cumulative score	Irritation assessment
0 – 0.9	Practically none
1 – 4.9	Slight
5 – 8.9	Moderate
9 – 21	Strong

D. Angiogenesis

The ethanol extract of PABE was put on a sterile 15 mm cover slip and after drying, it was applied to the CAM surface. The holes in the eggshells were immediately covered using an electrical insulation tape as described by Sedmera et al. [14] and the eggs were returned to the incubator with the same humidity and temperature settings. After 3 days, the blood vessels were observed and photographed to measure the anti-angiogenic effect. The photographs were obtained using a digital camera DP 70 and Cell P (Olympus) software. The percentage of avascular zone (area without blood vessels) of the chorioallantoic membrane was evaluated by ImageJ 1.48 software (NIH Image, USA) [15].

III. Results

A. Vasoactivity

The increasing concentration of the PABE causes the irritation of the CAM blood vessels (Table III). The test solution with a concentration of 0.1 mg/mL did not cause a detectable effect after 5 minutes. The administration of the extract with the concentration 1.0 mg/mL may result in slightly irritant effect to vessels after 5 minutes of the application with the risk of hyperaemia. The experiments show that the extract concentration of 10.0 mg/mL causes hyperaemia with a clear haemorrhage. Such high concentration of the extract induces a moderate irritation and the damage of blood vessels (Fig. 2).

B. Angiogenesis

The anti-angiogenic effect of PABE on the treated CAM is shown in Fig. 3. The evaluation of vascularization was done by capturing the images of CAM at a specific area under the cover slip, both with and without the extract (the latter was applied as a negative control). It was observed that the tested PABE inhibited angiogenesis in the CAM, causing the reduction of the vascular density (right part of Fig. 3), whereas the vehicle (cover slip without the extract) alone did not (left part of Fig. 3). The results can be interpreted also in percent-

whereas in the case of control, 27.5% of figure can be considered vessels, this value is only 10.9 % for the CAM treated with PABE extract (Fig. 4).

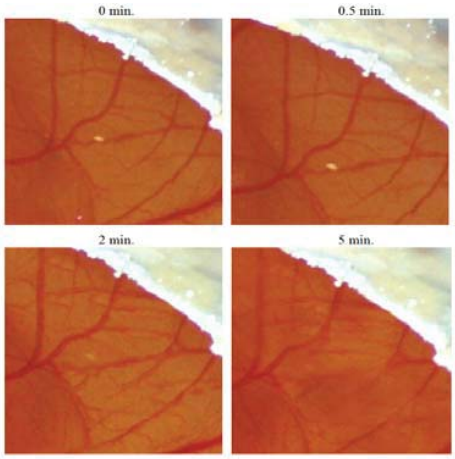

Fig. 2 Photographs of CAM after a specified time (0; 0.5; 2 and 5 minutes) after the application of the extract with the concentration 10 mg/mL

TABLE III
VASOACTIVE EFFECT OF *PHELLODENDRON AMURENSE* BARK EXTRACT TO BLOOD VESSELS OF CHORIOALLANTOIC MEMBRANE

Conc. of extract [mg/ mL]	Irritant effect after a specified time			Irritation by *Luepke*
	0.5 min	*2 min*	*5 min*	
0.10	No effect	No effect	No effect	Practically none
1.00	No effect	No effect	Hyperaemia	Slight
10.00	No effect	Hyperaemia	Haemorrhage	Moderate

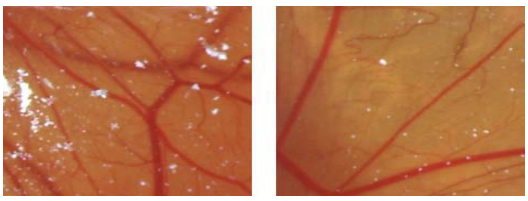

Fig. 3 Photographs of CAM without sample (left) and with sample (right)

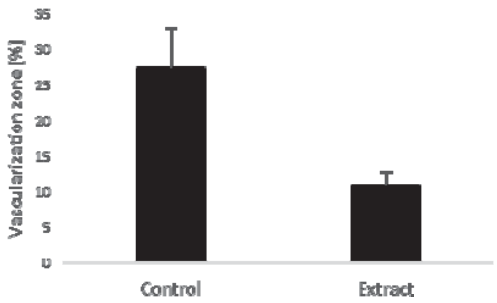

Fig. 4 Anti-angiogenic activity of PABE

IV. DISCUSSION

Although the evidence from the epidemiological and animal studies suggests that the increased consumption of the plant-based diet can reduce the risk of cancer, little information is available concerning the use of botanicals in the anti-angiogenic therapy. As a step toward developing botanicals as anti-angiogenic agents, we examined the effect of *Phellodnendron amurense* on angiogenesis by *in ovo* method.

Angiogenesis is the formation of new blood vessels in physiological and pathological process. It consists of activation, proliferation and migration of endothelial cells, degradation of extracellular matrix by proteolytic enzymes and the formation of capillary vessels [16]. It has a physiological function in the organ regeneration, menstrual cycle (namely ovulation and menstruation), embryonic development and formation of placenta. The utilization of anti-angiogenic activity is one of the promising therapeutic methods against retinopathy, rheumatoid arthritis, chronic inflammatory diseases, diabetic Kaposi's sarcoma, tumour neovascularization and other angioproliferative diseases connected with the uncontrolled endothelia cell proliferation [17].

Recently, bark of PA and its main component berberine were reported to exhibit antitumour properties. Although the mechanism is not fully understood, some of the activity against cancer cells is beginning to be deciphered [18], [19]. According to our best knowledge, no anti-angiogenic activity has been studied for this plant until now.

It was observed that *Phellodendron amurense* inhibits the proliferation of lung and prostate cancer cells by various mechanisms, including the inhibition of cell migration and invasion, induction of apoptosis, cell cycle arrest via a regulation of multiple pathways, such as changes in reactive oxygen species generation, matrix metalloproteinase regulation, p53 activation, NF-kB signal activation and DNA or RNA binding [18]–[20]. It was observed that lower intracellular redox state plays a role in the regulation of angiogenesis and has an anti-angiogenic potential. Free radical scavengers may be useful for the inhibition of angiogenesis.

The present data show no significant signs of the irritancy, such as vasoconstriction, hyperaemia, haemorrhage or coagulation for the concentration 0.10 and 1.0 mg/mL of the PABE, which indicates its harmless character and the possibility of potential application as a therapeutic agent. The moderate irritancy was observed only in the case of the highest applied concentration 10 mg/mL. The harmless character of PABE observed within our study is in correlation with other studies [7], [20]. E.g. in the reference [20] the minimal negative effects of orally administered product containing PABE during the tests on human was observed. Only few subjects reported gentle stomach discomfort, such as nausea. The preparations based on PABE are very well-tolerated and adverse effects were observed only occasionally [7], [20].

V. CONCLUSION

In summary, it appears from the results of this *in ovo* pilot study that *Phellodendrom amurense* bark extract has an anti-angiogenic activity with minimal negative effect. A significant reduction in vessel sprouting and a higher percentage of

avascular zone was observed after the application of the extract, which is a proof of its anti-angiogenic potential. These results could contribute to the development of novel drugs based on the extracts from *Phellodendron amurense* for the treatment of cancer or other diseases, in which angiogenesis plays a significant role.

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