Characterisation of Polyphenolic Compounds in Viscum album L. and Allium sativum L. extracts

SVETLANA TRIFUNSIHI, MELANIA F. MUNTEANU*, ELENA POGURSCHI**, RAMONA GLIGOR

1 Vasile Goldis Western University of Arad, Department of Pharmaceutical Sciences, 86th Liviu Rebreanu Str., 310045, Arad, Romania
2 Vasile Goldis Western University of Arad, Department of General Medicine, 86th Liviu Rebreanu Str., 310414, Arad, Romania
3 University of Agronomic Sciences and Veterinary Medicine of Bucharest, Department of Formative Sciences, 53 Marasti Str., 011463, Bucharest, Romania

There are numerous phytochemicals (isoflavones, flavonoids, catechins, anthocyanins, carotenoids, polyphenols, phenolic acids), which are largely used in the pharmaceutical industry. This research describes the process through which, one can obtain medicinal extracts from mistletoe (Viscum album L.) and garlic (Allium sativum L.) while giving an exact characterisation of the two extracts. The HPLC/MS analysis was employed in order to identify and evaluate the presence of phenolic acids (gentisic acid, chlorogenic acid, p-coumaric acid, ferulic acid and sinapic acid) and of flavonoids (hyperoside, isoquercitrin, rutin and luteolin). The results suggest that these medicinal extracts could replace the chemical compounds used in various therapies and which unfortunately have side effects on patients.

Keywords: mistletoe, garlic, HPLC/MS, phenolic acids, flavonoids

Medicinal plants have always been a viable alternative for the treatment of different diseases. Viscum album L. and Allium sativum L. have been used from ancient times to cure a wide variety of health conditions. The mistletoe, either by itself or in combination with other plants, is used for the treatment of high blood pressure, neuralgia, chronic rheumatism and osteoporosis. The garlic can successfully control blood pressure, being at the same time a highly efficient anti-platelet, a pulmonary antiseptic and an important element in the treatment of diabetes [1, 2]. Moreover, it can be used for its antithrombotic, antimicrobial and anti-inflammatory properties [3, 4].

Viscum album is an evergreen, semi parasitic plant, belonging to the Santalaceae family. It is usually found on a variety of tree hosts but mainly on apple, pear, sour-cherry, lime, beech, oak, etc. The branched system of suckers allows the mistletoe to fix itself in the hylum of its host. The mistletoe has a short, thick and polidicotomic branched root, while its leaves are displayed in opposite coriacea or lanceolate pairs, having a yellow-greenish colour. Its dicazzi, small flowers bloom from March until May, while its fruit is a white-yellowish berry [5].

Allium sativum is a perennial, eatable plant, belonging to the Alliaceae family. It can reach a height of 60-90 cm, with the flower located on top. Its bulb has several, white cloves [6].

The chemical composition of Viscum album, belonging to the Santalaceae family, consists of flavonoids, mucilage and alkaloids. The Allium sativum L. (Alliaceae family) bulbs contain allicin and ajoene [7, 8]. Recent studies in this area pointed out the presence of polyphenols in the Allium sativum bulbs [9, 10]. Studies done on Viscum album [11,12] pointed out the presence of polyphenols, as well as their antioxidant properties. The studies carried out by Farrukh et al.[13] and by Roman et al. [14] showed that the antioxidant properties of the mistletoe result from combined action of the phenolic acids and flavonoids, which are part of its chemical structure.

The main objective of this study is to present and analysis of the polyphenols found in unhydrolysed and hydrolysed extracts of Viscum album and Allium sativum. This research is the beginning of a deeper study, which aims to identify the antioxidant properties of these extracts. The research will be followed by the preparation of eye drops embedded in nanocapsules, which will be tested on the melanoma of the choroid.

Experimental part
Preparation of the vegetal material

The vegetal material used to make the extracts consisted of leaves of Viscum album parasiting the pear tree and Allium sativum bulbs. The leaved twigs were harvested from the perimeter of the Groseni village, located in Arad County (Romania), in July 2014. The Allium sativum bulbs were harvested from the same perimeter, in August 2014. After being harvested, the vegetal material was dried at a temperature of 90°C for 48 h. Then, it was kept in paper bags and boxes, in total darkness, at bellow ambient temperature [15,16].

Preparation of extracts necessary to determine the total polyphenols

In order to identify the polyphenolic content of the vegetal material, there were used two extraction procedures: extraction under reflux and ultrasound-assisted extraction. These extraction procedures are largely used for extracting various active, biological compounds from different types of vegetal material. The ultrasound-assisted extraction (UAE) is one of the most modern and efficient methods employed for this purpose. The UAE procedure is particularly useful because it lowers considerably the extraction temperature, reduces the solvent quantity and shortens the extraction time. Therefore, it is recommended for extracting instable and thermo-sensitive compounds. This procedure is employed for the extraction of geniposide from Gardenia [17], of syringin from flex rotunda [18], polyphenols from Inula helenium [19], phenolic compounds from Equisetum arvense L [20], and from Mesembryanthemum edule L. [21].

Two types of extracts were obtained: a) Methanol extracts – 1.00 g of dry vegetal material (Viscum album and Allium sativum) were extracted with 10 mL 70% methanol solution, for 100 min, at a temperature of 70°C, while being shaken [10]. After cooling

* email: anaionescu@yahoo.com; elena.pogurschi@gmail.com
down and reaching the ambient temperature, the extracts were centrifuged at 200 rpm and filtered. The process consisted by taking 1 mL from each filter and adding to it 100 mL of H2O, thus resulting an extract of Viscum album (V1) and an extract of Allium sativum (U1) [22].

b) Aqueous extracts - 100 g of fresh vegetal material (Viscum album and Allium sativum) was mixed with 20 mL of H2O, in an ultrasonic cleaning bath for a minute. The ultrasound equipment operated at a frequency of 40 KHz, 300 W of power, and at a temperature of 25°C [19]. Then, the mixture was centrifuged at 10000 rpm for 5 min and the resulting extract was filtered. The filters were labelled as follows: aqueous extract of Viscum album (AVA) and aqueous extract of Allium sativum (AUA) [11].

Preparation of extracts for the HPLC analysis
The crushed vegetal material, weighing 1.0 g, underwent a shaking extraction procedure, with 70% ethanol solution (the solvent ratio for the vegetal material was equal to 1:10), for a period of 24 h, in total darkness. Then, the extract was filtered and centrifuged at 3000 rpm for 5 min. A rotavapor was used to evaporate the solvent. This process allowed us to obtain the following extracts: unhydrolysed mistletoe extract (EV1) and unhydrolysed garlic extract (EU1). The two extracts were concentrated, until they almost reached their dry state. The process was repeated with 50 mL of distilled water, while the vegetal material underwent a process of acid hydrolyisation, with HCl 1N, at a temperature of 80°C, for 2 h. The resulting products were: hydrolysed mistletoe extract (EV2) and hydrolysed garlic extract (EU2) [7].

The Total polyphenol content according to the Folin-Ciocalteu method
The analysis of the total polyphenols was done using the spectrophotometric procedure described by Singleton et al. [23], but with some minor modifications. 1.0 mL of each extract were diluted with 5.0 mL of Folin-Ciocalteu reactive (Merck KGaA, Germany) (1:10) and then enriched with 4.0 mL of carbonate. The resulting solutions were kept at an ambient temperature, for 60 min. The absorbance was measured at 765 nm. The control solution was prepared in a similar manner, but the extract was replaced with H2O. The total polyphenols was expressed in the equivalent (Sigma Aldrich) (GAE) g. of gallic acid/100 g vegetal material. Gallic acid was used as standard for tracing the calibration curve.

The HPLC Analysis
The polyphenol compounds, in the vegetal extracts were determined through the chromatography of high performance liquids, coupled with mass spectrometry (HPLC/MS) [12,11]. The system had: a HP 1100 Series binary pump, a HP 1100 Series auto-sampler, a HP 1100 Series thermostat, an Agilent Ion Trap 1100 VL mass spectrometer. Analytical column: Zorbax SB-C18 100 mm x 3.0 mm i.d., 5 µm. Mobile phase: methanol mixture: 0.1% acetic acid solution (V/V), gradient elution (beginning with 5% methanol, after 35 min with 42% methanol, after 38 min with 42% methanol, and after 45 min with 5% methanol-rebalancing). Debit: 1 mL/min, temperature: 48°C, Ultraviolet detection: 330 nm until 17 min, 70 nm until 38 min. Injection volume: 5µL. The following standards were used: gentisic acid, p-coumaric acid, sinapic acid, hyperoside, isouqueritin, rutin and luteolin. For quantification purposes, the calibration curve of each compound had a concentration interval between 0.5-5.0 µg/mL. The compounds of vegetal extracts can be identified by making an analysis of the MS spectra.

Results and discussions
The total polyphenol content according to the Folin-Ciocalteu method
The total polyphenol content was determined using the reactive Folin-Ciocalteu, expressed in mg as gallic acid equivalent on dry vegetal material (mg GAE/g vegetable material). The total polyphenols content of the alcoholic and aqueous extracts of Allium sativum and Viscum album are presented in figure 1.

Fig. 1. The polyphenol content of alcoholic (EU1, EV1) and aqueous (AVA, AUA) extracts of Allium sativum and Viscum album

It can be observed that for the Allium sativum extracts, the total polyphenol content has the highest values for the aqueous extract, ranging from 5.87 to 16.04 mg GAE/g dry material. The total polyphenol content, determined using the Folin-Ciocalteu method and expressed as gallic acid equivalent on fresh vegetal material (GAE/FW), has higher values in the Viscum album extracts obtained by Vicas et al. [11].

Allium sativum has similar values of its polyphenol content, which are in accordance with its medicinal uses. The ethanolic extract of Allium sativum has a low value of 11.57 mg GAE/g, when compared to the one determined by Gramza-Michalowska et al. [24], whose garlic ethanolic extract had a value of 41.1 mg GAE/g.

HPLC Analysis
The HPLC/MS analysis of the EU1 hydrolysed extract of Allium sativum, points out that polyphenols can not be quantified. In the EU2 hydrolysed extract of garlic, it can be observed the presence of two phenolic acids. After analysing the Allium album extract with the HPLC method, it was observed a higher concentration of ferulic acid, equal to 0.254 µg/mL.

The presence of both flavonoids and phenolic acids can be observed in the unhydrolysed extract of Viscum album.

The hydrolysed extract of Viscum album, has only one phenolic acid (chlorogenic acid) and one flavonoid (rutin). The chlorogenic acid has higher concentration values in the unhydrolysed extract (15.803 µg/mL), while in the
hydrolysed extract the concentration values are much lower, being equal to 13.236 µg/mL. The flavonoids are represented by luteolin, in the unhydrolysed extract, having a concentration of 0.260 µg/mL, while rutin is present only in the hydrolysed extract, having a concentration of 0.115 µg/mL. The gentisic acid and the p-coumaric acid have lower values than in the extracts analysed by Luczykiewicz [25]. The gentisic acid was not present in Lucykiewicz’s extracts while in our extracts was found in little quantity, with values lower than 0.713 µg/mL; the chlorogenic acid in our extracts had higher values than in Lucykiewicz’s extracts, having values of 15.803 µg/mL and of 13.236 µg/mL, respectively [25].

Conclusions
The polyphenol content determined with the Folin-Ciocalteu method for the Allium sativum and Viscum album...
extracts, decreased in the following order: AVA>U1>V1>AUA. For the alcoholic extracts, the highest content was observed in the V1 extract of Viscum album (16.04 mg GAE/g veg. mat.), while for the Allium sativum extracts the highest concentration was identified in the AUA extract (32.22 mg GAE/g vegetal material).

The HPLC/MS identification was done using 9 compounds. Five of them were phenolic acids (gentisic acid, chlorogenic acid, p-coumaric acid, ferulic acid and sinapic acid) and four were flavonoids (hyperoside, isoquercitrin, rutin and luteolin).

No compound was identified in the EU1 unhydrolysed extract of Allium sativum, while in the EU2 hydrolysed extract of EU2 were identified only phenolic acids (p-coumaric acid and ferulic acid). Four phenolic acids (gentisic acid, chlorogenic acid, p-coumaric acid and sinapic acid) and one flavonoid (luteolin) were identified in the EV1 unhydrolysed extract of Viscum album. Chlorogenic acid and rutin were identified in the EV2 hydrolysed extract of Viscum album.

The study of the polyphenolic compound of the analysed vegetal material, Viscum album and Allium sativum, is just the first phase of our future researches in the field. The polyphenols are compounds which have a high antioxidant potential, being considered the most efficient active compounds, which are used as natural ingredients in the pharmaceutical industry and medicine [26].

Acknowledgement: This work was financially supported by Vasile Goldis Western University of Arad, internal competition grant (contract no. Pi5/21.07.2014). We would like to thank to our colleague Professor L. Vlase at Iuliu Hatieganu University of Medicine and Pharmacy of Cluj-Napoca, for the HPLC/MS analyses.

References

Manuscript received: 11.12.2016