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Polyphenolic content and toxicity assessment of *Anthriscus sylvestris* Hoffm.

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Abstract

Polyphenols are bioactive compounds that naturally occur in small quantities in plant and food products. On basis of their therapeutic properties, in the last years, numerous studies focused on finding new sources, especially in the food and pharmaceutical industry. Anthriscus sylvestris Hoffm. - wild chervil (Apiaceae family) is used in human nutrition, the root being intensively studied in anticancer therapy due to the presence of deoxypodophyllotoxin. The present study investigated the obtaining of rich phenolic crude extracts from the aerial parts of A. sylvestris. Three extracts were obtained using the following solvents: water, ethanol 50% and ethanol. The amount of phenolic compounds was evaluated using UV/VIS spectrophotometry and FT-IR spectroscopy. The toxicity of the extracts was evaluated using an alternative method with high degree of correlation with the methods on rodents and cell cultures, the Daphnia magna bioassay. Highest total flavonoid content was registered for the ethanolic extract and the highest total phenolic content in the hydroethanolic extract. Although the aqueous extract has a moderate content of phenolics, it induced the lowest toxicity of A. sylvestris extracts. We conclude that aqueous extract from A. sylvestris can be used as a source of polyphenols, whereas the ethanolic and hydroethanolic extracts should be purified in order to remove the lignans responsible of the toxicity.

Keywords: wild chervil, plant extracts, *Apiaceae*, phenolic acids, flavonoids, *Daphnia magna* bioassay, alternative toxicity assessment.

1. Introduction

Anthriscus sylvestris (L.) Hoffm. is a wild plant common in most of the temperate regions and belongs to the *Apiaceae* family, tribe *Scandiceae* Drude, section *Cacosciadium* Rchb [1]. Related members of *Apiaceae* or *Umbelliferae* include angelica, anise, carrot, celery, chervil, coriander, fennel, hemlock, parsley and parsnip [2]. It is also known as wild chervil or cow parsley and is most characteristic of hedgerows and road verges, but also found on woodland edges, neglected pastures and hay meadows. *A. sylvestris* is sed as antipyretic, analgesic, diuretic, and cough remedy in popular medicine [3]. The chemical composition of *A. sylvestris* was analyzed in several phytochemical studies thus revealed terpenoid compounds, phenolic compounds and flavonoid lignans as the major components [4]. Deoxypodophyllotoxin, also known as anthricin, was identified as the main lignan and is considered the plant's most

important constituent because of the antimicrobial, anti-inflammatory and antitumor effects [5,6]. It is also important as starting material in the synthesis of the cytostatic agents etoposide and teniposide, considering the podophyllotoxin's scarcity in natural sources and very difficult and expensive synthesis procedures [7]. Deoxypodophyllotoxin binds directly to tubulin and inhibits its polymerization resulting in the suppression of microtubule assembly, leading the cell cycle to stop at the G2/M checkpoint and to accumulate the cells in sub-G1 division phase, followed by apoptosis [8]. Other major lignans found in the plant's root are yatein and anhydropodorhizol [9]. The research on *A. sylvestris* was focused on its anti-proliferative properties and on the lignans responsible for it [10], and less on the study of its polyphenol content and their antioxidant potential. Polyphenols are one of the largest secondary plant metabolites present in plants and consist of a wide variety of chemical structures [11]. Mounting evidence suggests that polyphenols are health promoting phytochemicals and their dietary intake is correlated with a reduced risk of various illnesses, including cardiovascular disease, diabetes, cancers, and gastrointestinal diseases [12]. The phenolic compounds are responsible for many plants' chemopreventive properties, functioning as antioxidants, anticarcinogenic, or antimutagenic agents [13]. *A. sylvestris* is a common, fast-growing plant, and highly adaptability to grow in almost any type of soil and therefore a highly valuable resource for phytochemicals. Based on this consideration we analyzed the phenolic content and the plant's toxicity using alternative approaches.

2. Materials and Methods

2.1. Plant material

Anthriscus sylvestris (L.) Hoffm. was cultivated at SC Hofigal Export Import SA, Bucharest, Romania and the plant material (stems and leaves) was harvested during the flowering period (July 2015). The identity of the plant material was established based on the macroscopic and microscopic characteristics described in the literature [14-16] and by comparing with herbarium specimens from "Dimitrie Brandza" Botanical Garden, Bucharest. A voucher specimen is available in the drug collection of the Department of Botany and Cell Biology, Carol Davila University of Medicine and Pharmacy, Bucharest, Romania, and at "Dimitrie Brandza" Botanical Garden, Bucharest, Romania (no. 403536). After harvesting, the raw material was sorted and dried in the absence of the light at room temperature (25±4°C), and stored in paper bags until further processing.

2.2. Extracts preparations

The plant material (15 g) was grounded and passed through a 1.41 mm sieve (mesh 14). Grounded material was extracted twice for 30 min with solvent (2 x 150 mL) by heating under reflux. The plant material was extracted with water (W), water/ethanol 1/1 (*v*/*v*) (WE) and ethanol (E). After cooling, the extractive solutions were separated by filtration on Whatman paper, under vacuum, in Büchner funnel. The combined extractive solutions were concentrated at 40°C under vacuum using a rotary evaporator (RVO 004; Ingos, Czech Republic) and then lyophilized at -55°C (CoolSafe ScanVac 55; LaboGene, Denmark). Total dried extracts AS_W, AS_{WE} and AS_E were stored in the absence of light at room temperature in a desiccator 4°C until use.

2.3. Phytochemical characterization

2.3.1. Determination of total phenolic content

Total phenolic content (TCP) was determined according to the Folin & Ciocalteu method described by GONZÁLES & al. [17] with some modifications [18, 19]. Aliquots of 100 µL of extract solutions were mixed with 2000 µL distilled water, 600 µL of diluted Folin & Ciocalteu's phenol reagent (Sigma-Aldrich) (with distilled water), 2.0 mL of 15% Na_2CO_3

aqueous solution and completed with distilled water to 10 mL. Samples were incubated at 50±0.5°C for 15 min in the dark using a water bath (WNB10, Memmert, Germany). After cooling, the absorbance of the samples was measured at λ=750 nm using a UV/VIS spectrophotometer (Halo DB-20-220 Dynamica Precisa, Germany). The calibration curve was prepared using gallic acid (Scharlau Co.) in the range of concentrations from 0.5 to 10.0 µg/mL using the condition described above. The TCP was calculated using regression parameters of the calibration curve. All determinations were performed in triplicate. The results were expressed as the means ± standard error of the mean (SEM) of the experiments in milligrams gallic acid equivalents (GA equiv.) per gram of dry material (DM).

2.3.2. Determination of total flavonoid content

Quantitative determinations of total flavonoid content (TFC) was performed using the method described by CHANG & al. [20] and BAZYLKO & al. [21] with some modifications [18]. Accurately weighted 20 mg of each plant extract were dissolved in 10 mL of 50% methanol. 600 µL of each solution were mixed with 2000 µL distilled water, 200 µL 10% $AlCl_3$ (Scharlau Co.) aqueous solution, 200 µL of 1M CH_3COOK (Sigma-Aldrich) aqueous solution, and then completed with distilled water to 10 mL. Samples were incubated at room temperature for 45 min, protected from light, and measured at λ=430 nm. The calibration curve was prepared using quercetin (Sigma-Aldrich) according to the procedure described above. Total flavonoid content was calculated using regression parameters of the calibration curve. All determinations were performed in triplicate. The results were expressed as the means ± SEM of the experiments in milligram quercetin equivalents (Q equiv.) per gram of DM.

2.3.3. Statistical analysis

Statistical significance of differences between means was assessed by ANOVA with Tukey's post-hoc tests. P values below 0.05 were considered statistically significant. The 95% confidence intervals (CI95%) of the average values were also determined. All calculations were performed using GraphPad Prism version 5.0 software (USA).

2.3.4. Infrared analysis

Qualitative evaluation of the three lyophilized extracts was performed using the Fourier Transform Infrared (FT-IR) spectra according to the method described in the literature [22, 23]. The FT-IR spectra were recorded using a JASCO FT/IR-4200 spectrometer with an ATR PRO450-S accessory, on a spectral range of 4000-400 cm^{-1} and a resolution of 4 cm^{-1}. The extracts were analysed in duplicate.

2.4. Acute toxicity assay

2.4.1. Daphnia magna bioassay

Daphnia magna bioassay, an alternative method for toxicity assessment, was used for the evaluation of the biological toxic effects of the *A. sylvestris* extract. *Daphnia magna* Straus organisms were selected from a culture maintained parthenogenetically at 'Carol Davila' University (Department of Pharmaceutical Botany and Cell Biology) since 2012. Prior to the assay, *Daphnia magna* young organisms were selected according to their size and kept in fresh water under continuous aeration for 24 h in a climatic chamber (Sanyo MLR-351H, USA). The biological assessment was performed according to the protocol described by FAN & al. [24] and NITULESCU & al. [25] with modifications [18]. Ten daphnids were inserted in graduated test tubes with the plant extracts dissolved in 0.5% DMSO (Sigma-Aldrich) in synthetic media in order to obtain solutions of 1500, 1000, 500, 200, 100 and 10 g extract/mL. Synthetic medium with 0.5% DMSO was used as a negative control. Due to the absence of specific information about the photo-stability of the plant extracts, the bioassay was performed in the absence of light in a climatic chamber (Sanyo MLR-351H, USA). Each sample was performed in duplicate. After 24 h, the number of surviving organisms was

counted and recorded. The daphnids were considered dead only if they did not move their appendages for 30 s during observations.

2.4.2. Statistical analysis

Lethality was calculated for each sample and the lethal concentrations which cause the death of 50% of organisms (LC_{50}) were determined by interpolating the concentrations on lethality - logarithm of concentration curves. The upper and lower limits of the 95% confidence interval of LC_{50} (CI95%) and the correlation coefficient (r2) were also calculated. All calculations were performed using GraphPad Prism version 5.0 software (USA).

3. Results and discussions

3.1. Extraction yield

In this research we obtained three extracts from *Anthriscus sylvestris* by reflux extraction, followed by concentration and freeze drying. Extraction yields range from 10.95 and 32.19% (w/w) (table 1). AS_W exhibited the highest yield compared to all other extracts. The lowest extraction yield was obtained with ethanol (table 1).

3.2. Phytochemical characterization

Phenolics are ubiquitous compounds in plant species, being found in all organs. These compounds have different functions in plants such as: defence against the different environmental and stress factors, antimicrobial, prevention of UV-B radiation penetration into deeper tissues, the activation of genes from nodule bacteria and protection against heavy metal intoxication [26, 27]. For humans and animals, phenolics, as dietary components, are thought to have health-promoting properties, mainly due to their antioxidant capacity [28]. Thus, the regular intake of phenolics have protective properties on enzyme systems and prevent numerous disorders, the most important being cardio-vascular diseases and cancers [29, 30]. Isolated or in purified extracts, polyphenols have demonstrated antitumor, neuroprotective, estrogenic, anti-inflammatory and antimicrobial properties [31-35]. We determined the TCP and TFC for the obtained extracts from *A. sylvestris*, using UV/VIS spectrophotometry. The results of quantitative determinations of the polyphenols are presented in table 1. The TFC was recorded between 18.57 to 41.63 mg Q equiv./g DM, ethanol being the best solvent for the extraction of flavonoids. TPC ranged from 25.76 to 85.45 mg GA equiv./g DM. In this case, the best solvent for the extraction of all phenolic compounds was ethanol 50%. All results are statistically significant (ANOVA, $p<0.0001$). The results indicate that the amount of phenolic compounds is moderate in extracts obtained from *A. sylvestris*. The infrared spectra is a rapid and nondestructive investigation to fingerprint plant powders and extracts and were used to evaluate the different solvent extracts of *A. sylvestris*. The statistically relevant differences between the IR spectra indicate the importance of the solvent on the chemical composition of each extract. The large band close to 3100 cm^{-1} was registered for each extract and is considered an indicator of polyphenols content.

Table 1. Yield extraction, TFC and TPC for the *Anthriscus sylvestris* extracts.

Extract	Yield of crude extract (%)	TFC (mg Q equiv./g DM)	CI95% of TFC (mg Q equiv./g DM)	TPC (mg GA equiv./g DM)	CI95% of TPC (mg GA equiv./g DM)
AS_W	32.19	28.99 ± 0.0357	28.66 - 29.31	55.51 ± 3.0722	47.88 - 63.14
AS_{WE}	26.79	18.57 ± 3.0681	13.06 - 23.01	85.45 ± 1.1626	82.56 - 88.33
AS_E	10.95	41.63 ± 3.1070	33.91 - 49.35	25.76 ± 0.5809	24.32 - 27.20

Values are mean± standard deviation of triplicate analysis. GA: gallic acid; TFC: total flavonoid content; Q: quercetin; TPC: total phenolic content; DM: dry plant material; CI95%: confidence interval (α=0.05) of average; ASw: *A. sylvestris* aqueous extract; AS_{WE}: *A. sylvestris* hydroethanolic 50% extract; AS_E *A. sylvestris* ethanolic extract.

3.2. Acute toxicity assay

Lately, the evaluation of toxicity by alternative methods is widely used. The use of the invertebrate organisms in the toxicity assessment has considerable advantages over the pre-clinical methods. Thus, these methods are inexpensive, time saving and have a high degree of correlation with the acute toxicity performed on rodents and other mammalian models [36-38]. In the past ten years, methods which are based on assays with snail, worms and crustaceous organisms were run for several classes of chemicals with impact over the environment and pharmaceutical substances [36]. These methods are also largely used for the rapid assessment of the toxicity induced by plant extract or compounds isolated from botanicals [38]. Toxicity of the three extracts of *A. sylvestris* was performed using *Daphnia magna* bioassay. The results of the determination are presented in figure 1. Accordingly, it has been revealed that the ethanolic and hydroalcoholic extracts induced 100% lethality at highest concentration (1500 µg/mL) and a significant lethality at low concentrations. The aqueous extract induced about 70% lethality at highest concentration and significant lower by comparing with the other two extracts on lower concentrations.

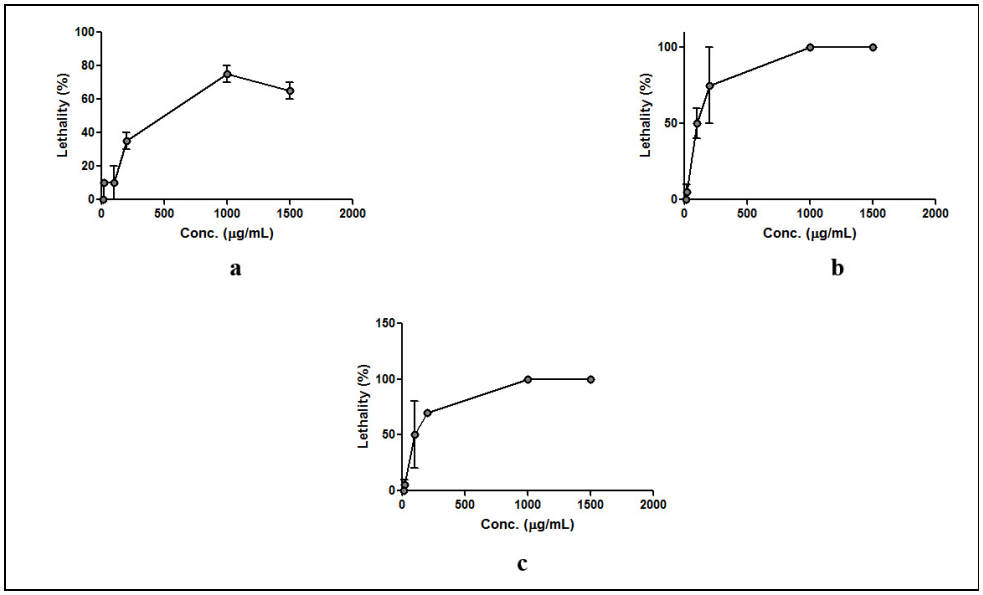

Figure 1. Lethality versus concentration of aqueous extract (a), hydroalcoholic extract (b) and ethanolic extract (c)

The results of the LC_{50} determination and of correlation between logarithm of concentrations and lethality on *D. magna* are presented in table 2 and the lethality – logarithm of concentration curves in the figure 2. Good correlation between concentration and lethality was found for all three *A. sylvestris* extracts ($r^2 > 0.90$) and no significant differences were found ($p < 0.05$) between the replicates of the experiment.

Table 2. Acute toxicity of the Antriscus sylvestris extracts on *D. magna*.

Extract	LC_{50} (µg/mL)	CI95% of LC_{50} (µg/mL)	Goodness of fit (r^2)
AS_W	483.70	321.50 - 727.70	0.9093
AS_{WE}	102.40	70.53 - 148.80	0.9293
AS_E	106.90	69.42 - 164.70	0.9108

LC_{50}: lethal concentration 50%; CI95%: confidence interval (α=0.05) of LC50; r^2 - correlation coeficient. ASw: *A. sylvestris* aqueous extract; AS_{WE}: *A. sylvestris* hydroethanolic 50% extract; AS_E *A. sylvestris* ethanolic extract.

AS_{WE} and AS_E present low LC_{50} values with a narrow 95%CI, results which indicate that these two extracts have high toxicity. The high toxicity induced by ethanolic and hydroethanolic extracts could be due to the amounts of deoxypodophyllotoxin and related lignans. These substances are high toxic for animal cell and have cytotoxic properties.

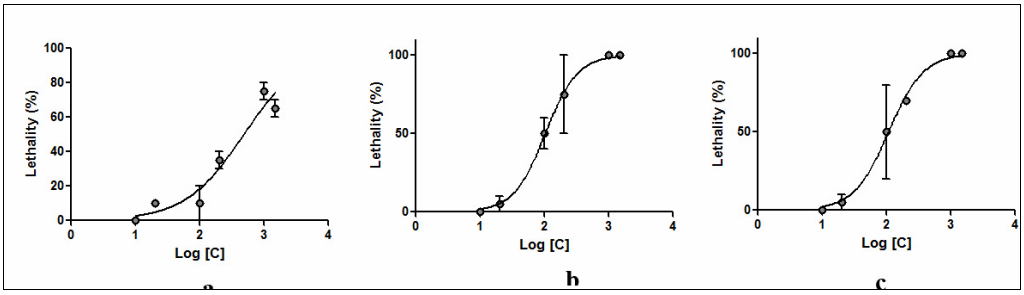

Figure 2. Lethality-logarithm of concentration curves for the tested; a - aqueous extract, b - hydroalcoholic extract, c - ethanolic extract.

AS_W has the lower toxicity among tested extracts, with a LC_{50} about 4.5-fold higher than both, ASW_E and AS_E. Although the CI95% is relatively wide, its lower limit is higher than the toxicity threshold reported by GUILHERMINO & al. [36] for toxic substances.

4. Conclusion

In the present work we obtained three extracts from aerial parts from *Anthryscus sylvestris* L. (*Apiaceae*) using three "friendly" environment solvents. The phenolic compounds from the extracts were characterized by UV/VIS and FT-IR spectrometry. The extracts were also tested for acute toxicity using an alternative method - *Daphnia magna* bioassay. All extracts presents moderate quantities of phenolic compounds. Although the ethanolic and hydroethanolic extracts shown the higher content in flavonoids and total phenols, both extracts induced high toxicity on *D. magna*, even at moderate and low concentrations. The toxicity is most likely induced by deoxypodophyllotoxin and other lignans which are more soluble in ethanol and practically insoluble in water. Therefore we conclude that aqueous extracts from *A. sylvestris* can be used as a source of polyphenols and that the ethanolic and hydroethanolic extracts should be purified in order to remove the lignans responsible of the toxicity.

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