

The anemonin content of four different *Ranunculus* species

Teodora Neag¹, Neli-Kinga Olah^{1,2*}, Daniela Hanganu^{3*}, Daniela Benedec^{3*},
Flavia Furtuna Pripon², Aurel Ardelean¹ and Claudia Crina Toma¹

¹Vasile Goldi Western University of Arad, Faculty of Pharmacy, 86 L. Rebreanu St., Arad, Romania

²SC PlantExtrakt SRL, Radaia, Cluj, Romania,

³Iuliu Haieganu University of Medicine and Pharmacy, Faculty of Pharmacy, 12 I. Creangă St., Cluj-Napoca, Romania

Abstract: The *Ranunculus* species are poorly known as medicinal plants. They have potential toxicity given by the ranunculin and its enzymatic degradation compounds: protoanemonin and anemonin. This paper aims to evaluate the anemonin content of four species: *R. bulbosus*, *R. ficaria*, *R. sardous* and *R. sceleratus*. The evaluation was performed by TLC and HPLC. There were evaluated two types of extracts hydroalcoholic (HA) and glycerol-ethanol (GE). The most concentrated extract in anemonin was found to be the *R. sardous* aerial part HA extract: 2.66 mg/ml. The lowest anemonin content is in *R. sceleratus*: 0.13-0.19 mg/ml. In *R. bulbosus* aerial part the anemonin content is less than the used HPLC method detection limits (7.68 mg/ml). In all cases the GE extracts are less concentrated in anemonin, being more safely for human administration.

Keywords: *Ranunculus ficaria*, *Ranunculus bulbosus*, *Ranunculus sardous*, *Ranunculus sceleratus*, TLC, HPLC-DAD, anemonin.

INTRODUCTION

It is known that natural products have been used since ancient times to treat or prevent certain diseases. These traditional uses of medicinal plants could lead to future phytochemical and pharmacological studies that may give rise to new medicinal resources.

The *Ranunculaceae* family groups about 1500 species spread under very diverse ecological conditions, especially in the temperate and cold zone of the northern hemisphere (Antal and Coste, 2004). Most *Ranunculus* species synthesize toxic glycosides, some of them with therapeutic value if administered in small amounts. Anemonin and anemonol or protoanemonin are toxic, revulsive and bladder substances that cause irritations and rashes at the contact with skin or mucous, being specifically bio-synthesized by the *Ranunculus* species. The bio-synthetic pathway of the enzymatic stabilization of ranunculin is given below (fig. 1).

Ranunculus bulbosus is used traditionally in gout pain, arthritic pain and neuralgia. The entire plant, but especially the sap, is astringent, calming, antispasmodic, diaphoretic and rubefiant. The chemical constituents present in *Ranunculus bulbosus* L. are hexadecanoic acid, β -sitosterol, anemonine and protoanemonine (Aslam *et al.*, 2012). In homeopathy, the *Ranunculus bulbosus* remedy has action on articular, dermatological, respiratory and neural disorders. Protoanemonin isolated from the aerial parts of *Ranunculus bulbosus* has marked inhibitory effects and broad spectrum of activity against aerobes and anaerobes including multiresistant pathogenic strains

(Didry *et al.*, 2006). Protoanemonin also called anemonol or ranunculol, a component of *Ranunculus bulbosus*, was tested as an antifungal agent on selected strains of dermatophytes and yeasts (Mares, 1987).

Leaves and roots of *Ranunculus ficaria* L. are used in ethnopharmacology in Romania as an infusion or decoct for astringent, trophic and anti-inflammatory effects in case of varicose veins, hemorrhoids and skin disorders. This traditional indications, knew by the great number of rural population, in Romania is justified by the blood circulation stimulation made from the macerate and tinctures obtained from *Ranunculus* ssp (Tita *et al.*, 2009), being mostly used in the treatment of hemorrhoids (Toma, 2008). The tubers of *Ranunculus ficaria* contain saponosides with hederagenin and oleanolic acid aglyca (Bruneton, 1993). In the fresh plant, ranunculin and products of its enzymatic decomposition have been observed (Bonora *et al.*, 1988). It has also been identified flavonoids such as quercetin and rutoside (Tomczyk *et al.*, 2002). The plant product used is the aerial part, called *Ficariae herba*.

In Asian traditional medicine *Ranunculus sceleratus* is used in blood stasis, internal abscess, malaria, scrofula, snake or scorpion bite and acute icteric hepatitis (Aslam *et al.*, 2012). The plant bio-synthesizes ranunculin, protoanemonin and anemonin (Mei *et al.*, 2012). The aerial part of *Ranunculus sceleratus* has antiinflammatory and antibacterial activity (Aslam *et al.*, 2012).

From the pollen harvested from *Ranunculus sardous*, a lipophilic flavonoid, 7-O-methylherbacetin 3-O-E-feruloyl- β -d-glucoside, was isolated (Markham *et al.*, 1997). The plant exhibits irritant action and is not used in therapy.

*Corresponding author: e-mail: neliolah@yahoo.com

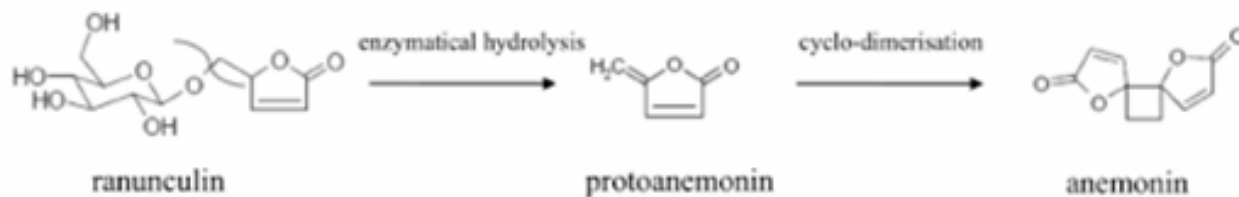


Fig. 1: The enzymatic stabilization of ranunculin into toxic protoanemonin and anemonin (Seigler, 1995)

The purpose of this study was to evaluate the anemonin content of four *Ranunculus* species. There were evaluated the tinctures and the glycerol macerates obtained from different part of these plants. The aim of this study was to establish which extracts type obtained from these species can be more safely administrated as therapeutic agents.

MATERIALS AND METHODS

Materials

The pharmaceutical grade extraction solvents, 96% vol. ethanol respectively 100% glycerol were purchased from Coman Prod, Ilfov, Romania and KLK Oleo, Germany. The TLC plates and SPE extraction cartridges were obtained from Merck, Germany. The silicagel-C18 HPLC column was produced by Phenomenex, USA.

All solvents and reagents used for perform this study were of analytical grade: ethyl acetate, ethyl-methyl ketone, methanol (HPLC), acetonitrile (HPLC), formic acid, hydrochloric acid, sulfuric acid and phosphoric acid were purchased from Merck, Germany. The anemonin standard was prepared by Phytolab, Germany.

The vegetal raw material and the preparation of extracts

The vegetal materials were harvested from Arad County, Romania, in June 2015. There were collected the aerial parts of *Ranunculus bulbosus*, *Ranunculus ficaria*, *Ranunculus sardous* and *Ranunculus sceleratus* respectively the roots of *Ranunculus bulbosus*. The plant material was identified in the laboratories of Vasile Goldi Western University and from all species were retained voucher specimens at herbarium. There were prepared two types of extracts: hydroalcoholic (HA) and glycerol-ethanol (GE) according to European Pharmacopoeia monographs.

The mother tinctures (HA) were prepared from fresh plant by maceration with 70% vol. ethanol. It was kept at room temperature for 10 days, shaking 3-4 times a day. The extractive liquid was decanted and the residue pressed.

The glycerol-ethanol extracts (GE) were obtained by maceration of the fresh vegetal material with a 1:1 mixture of 96% vol. ethanol and glycerol, using an extraction ratio of 1:20 (m/m). They were kept at room temperature for 20 days with periodical shaking, after which they were filtered.

TLC analysis

The analyses were carried out on Silica F254 plates, purchased from Merck, Germany.

The mother tinctures were applied directly on the plates, the GE extracts were extracted on SPE-silicagel C18 columns (Merck, Germany) and the retained compounds were eluted using methanol at 4 pH with hydrochloric acid (Cobzac *et al.*, 1999). The eluted compounds were brought to the same volume as the initial GE extract volume. There were applied bands of 20 mm at 15 mm from the edge of the plates using a dispenser pipette, 20 μ l from each sample.

As standard was used an anemonin methanolic solution (0.35 mg/ml) from that was applied 10 μ l as 20 mm long band.

The elution was performed with a mixture of ethyl acetate – ethyl-methyl ketone – formic acid (75:20:5, v/v) in standard chromatography tanks, according to German Homeopathic Pharmacopoeia monograph for *Ranunculus bulbosus* mother tincture. The elution distance was 10 cm from the application level.

The eluted plates were dried and were sprayed with 10% sulfuric acid in methanol. After 10 minutes at 100°C heating the plates were visualized in visible light using a Camag Reprostar II documentation system with digital photo camera.

HPLC analysis

The analyses were performed on a ProStar Varian HPLC system (Varian, USA), with autosampler, tertiary pump system and diode array UV-Vis detector.

The separations were carried out on silicagel C18 column (Luna 5u, 100A, 150 x 4.60 mm, Phenomenex, USA). The column was maintained at 25°C, using the mobile phase presented in table 1. It was used 1 ml/min flow rate and the detection was performed at 260 nm. All solvents were of HPLC grade, purchased from Merck, Germany.

As standard was used anemonin (Phytolab, Germany). It was built a calibration curve with solutions of anemonin in methanol having the concentration from 0.1 to 1 mg/ml. The curve is linear in all this range of concentration. The HA extracts were injected without

diluting, meanwhile the GE extracts were diluted 1 to 5 with methanol. There were injected 10 μ l from each sample and standard solution (Dărăban *et al.*, 2015).

STATISTICAL ANALYSIS

For all samples were made three individual determinations and the final results are the mean value of those three determinations. The graph and the calculations were performed using the Excel software program.

RESULTS

The chromatograms obtained at initial screening with TLC are presented in figs. 2 and 3.

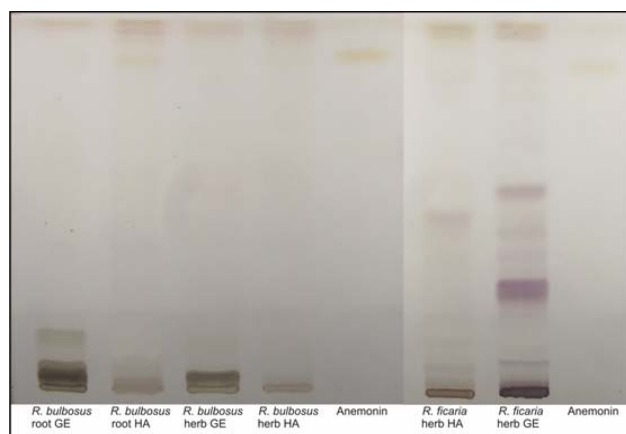


Fig. 2: The TLC chromatograms of *R. bulbosus* and *R. ficaria* extracts.



Fig. 3: The TLC chromatograms of *R. sardous* and *R. sceleratus* extracts.

In fig. 4 is presented the anemonin calibration curve obtained at HPLC analysis. The curve equation is: Area = $2 \cdot 10^7 \cdot \text{concentration [mg/ml]} + 76848$. The correlation factor for linearity is 0.9958. The detection limit is 7.68 mg/ml.

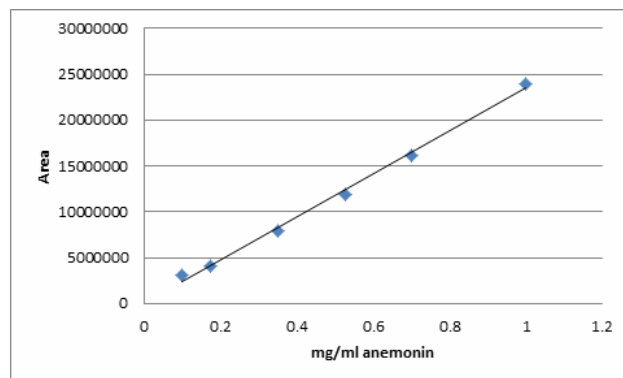


Fig. 4: The HPLC calibration curve of anemonin.

The HPLC analysis results are presented in figs. 5 and 6. In table 2 are presented the retention times, the UV-Vis spectra maximum absorbance respectively the anemonin content of each studied extract. In fig. 7 are presented the UV-Vis spectra of standard anemonin and those of the compounds identified as anemonin in extracts.

DISCUSSION

These TLC chromatograms allow the identification of anemonin in the studied extracts by comparison of the bands color and position of the standard and separated compounds from extracts. The TLC chromatograms indicate, in the given experimental conditions, the separation of the anemonin on the upper side, at an R_f value of 0.85 as a grey-yellow band. The bands separated from extracts are of same color and at same level with the standard. The TLC analyzes show us the presence of anemonin in *R. bulbosus* roots, *R. ficaria* herb, *R. sardous* herb and possibly in *R. sceleratus* herb. The bands intensity are different, the most intensive being those from *R. sardous*. The bands separated from HA extracts are more intensive than those from GE extracts, indicating that the ethanol will better extract the potential toxic anemonin.

The TLC analyzes results were confirmed also by the HPLC analyzes. The identification of anemonin by HPLC is based on the comparison of retention time and UV-Vis spectra maximum absorbance respectively spectra shape between standard and separated compounds. It could be observed the separation at 4.40-4.70 minutes of the anemonin, at a very similar retention time as the standard. The correct identification was confirmed also by the UV-Vis spectra's maximum (262-263 nm) and shape (fig. 7), the 1 nm difference at some extracts being in the accepted deviation value according to the European Pharmacopoeia.

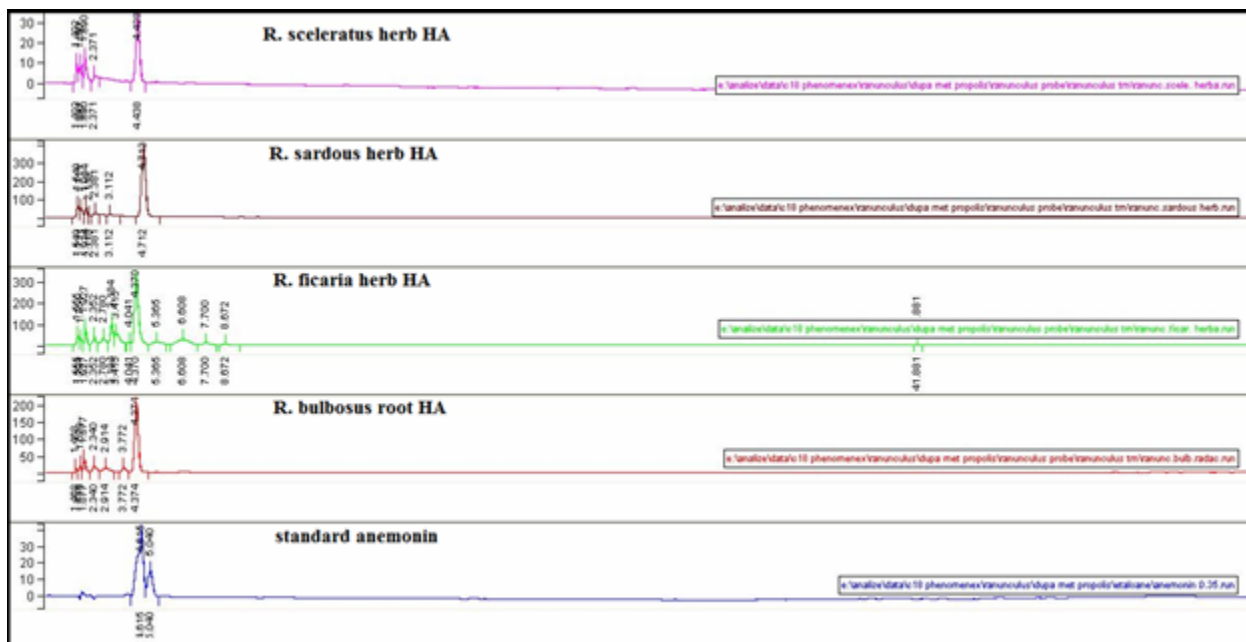


Fig. 5: The HPLC chromatograms of HA extracts.

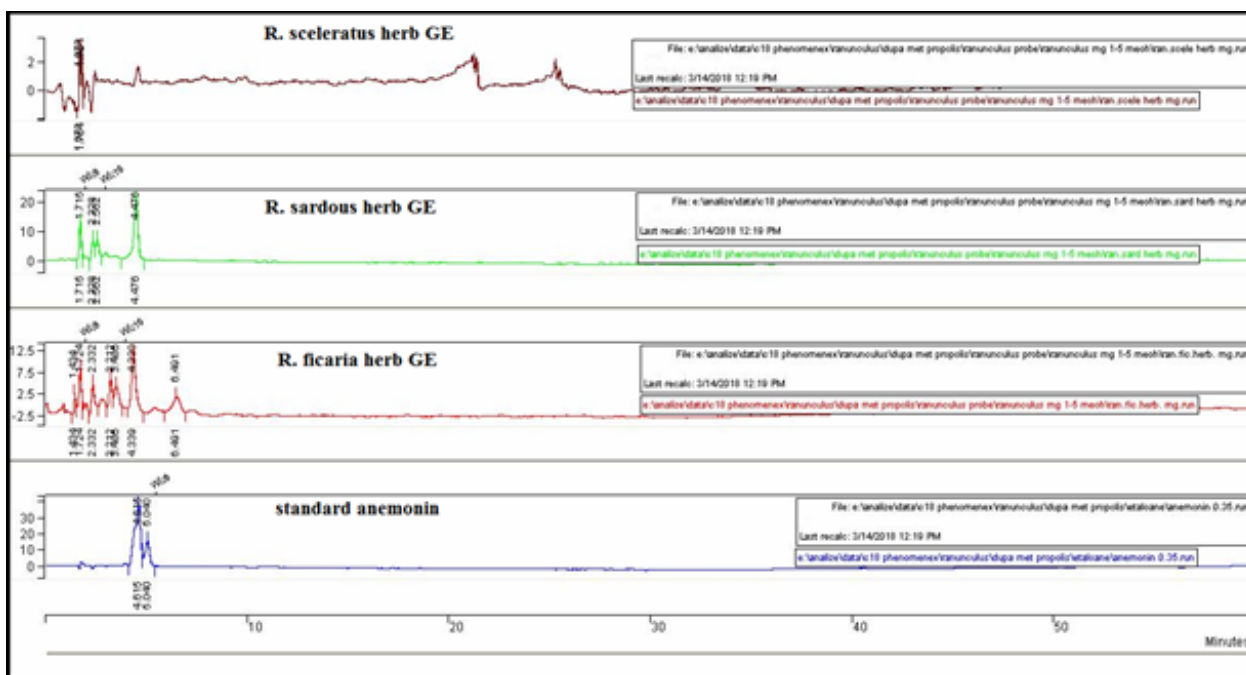


Fig. 6: The HPLC chromatograms of GE extracts.

Table 1: The mobile phase composition for HPLC separation

Time, min.	Water – phosphoric acid, pH = 2.5	Methanol	Acetonitrile
0	75	10	15
30	75	10	15
35	69	12	19
40	67	12	21
60	54	15	81

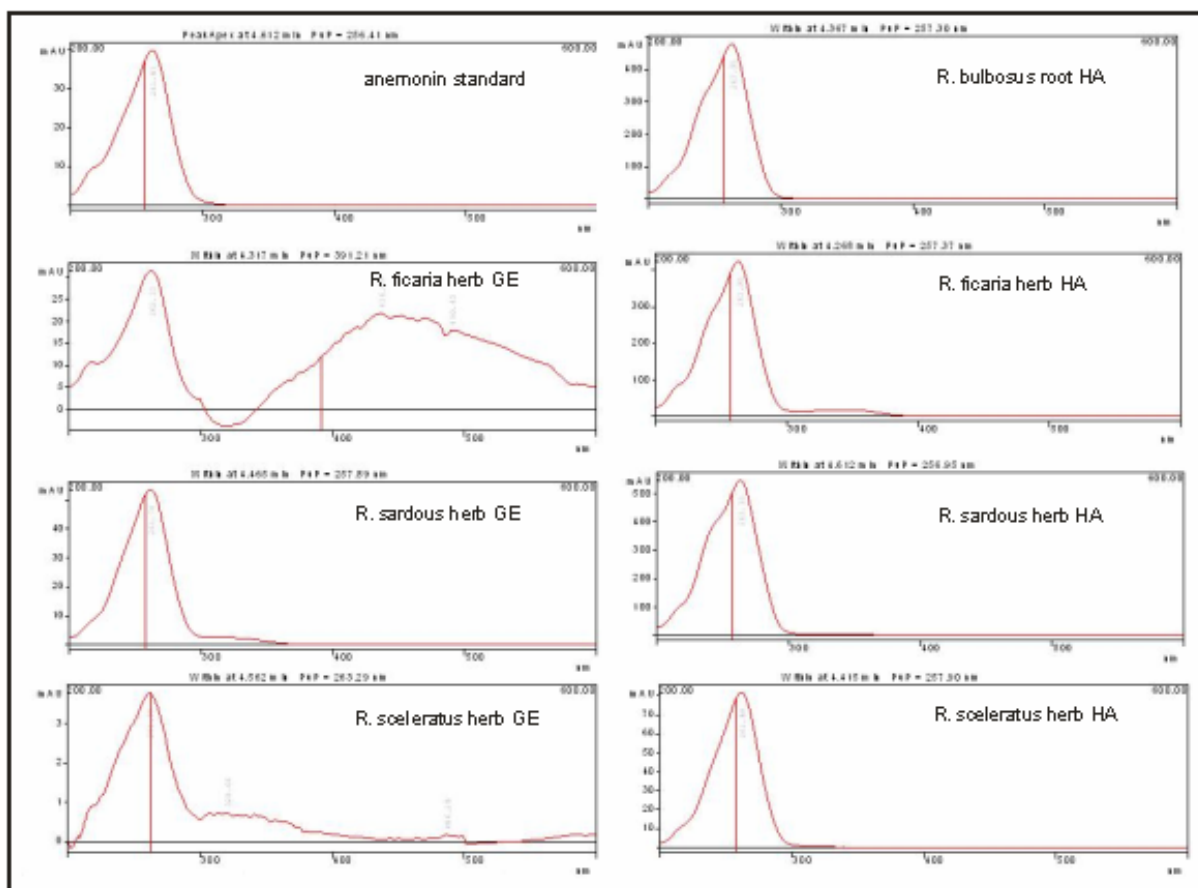


Fig. 7: The anemoinin UV-Vis spectra

Table 2: HPLC analyses results

Standard/Extract	Retention time, min.	UV-Vis spectra maximum absorbance wavelength, nm	Anemoinin content, mg/ml extract
Anemoinin - standard	4.60	262	-
<i>R. bulbosus</i> root HA	4.37	263	1.27 ± 0.005
<i>R. ficaria</i> herb HA	4.37	263	2.14 ± 0.007
<i>R. ficaria</i> herb GE	4.34	262	0.47 ± 0.003
<i>R. sardous</i> herb HA	4.71	263	2.66 ± 0.009
<i>R. sardous</i> herb GE	4.48	262	0.77 ± 0.005
<i>R. sceleratus</i> herb HA	4.44	262	0.19 ± 0.001
<i>R. sceleratus</i> herb GE	4.56	262	0.13 ± 0.001

The HPLC chromatograms shown us also differences in phytochemical profile of the extracts obtained from different *Ranunculus* species. At HA extracts the number of separated compounds increasing from 5 at *R. sardous* and *R. sceleratus* to 6 at *R. bulbosus* roots and 8 at *R. ficaria*. From GE extracts were separated 5 compounds in case of *R. sardous* and *R. sceleratus* respectively 10 in case of *R. ficaria*. These lead us to conclude that this HPLC method could be used also to differentiate the *Ranunculus* species.

The quantitative determinations confirm again the initial TLC screening results'. The most concentrated

Ranunculus species in anemoinin is *R. sardous* and its HA extract shown the highest content. The *R. sceleratus* HA and GE extracts contain the less quantity of anemoinin. The anemoinin content in *R. bulbosus* herb is under the evaluation method's detection limit, only in roots and just in HA extract was identified a higher anemoinin content. This indicates again that the ethanol extracts better the anemoinin like the glycerol-ethanol solvent mixture. Generally the GE extracts have with 31.5-78% less anemoinin like the HA extracts. These information are for first time evaluated and published in this paper. Only in 1968 were published a study regarding the protoanemoinin, anemoinin and ranunculidin content of *R.*

sceleratus by a simple spectral method (Mahran *et al.*, 1968).

Considering the potential toxic effect of anemonin these information lead us to conclude that the *Ranunculus* species GE extracts will be more safely at administration to humans. From this point of view the most safely *Ranunculus* species is *R. bulbosus*, if is used the herb, then *R. sceleratus*. These two species have also traditional use in European (*R. bulbosus*) and Asian (*R. sceleratus*) ethnopharmacology (Aslam *et al.*, 2012). The ethnopharmacological use of *R. ficaria* is restricted to Romania (Toma, 2008), meanwhile *R. sardous* is not known in traditional medicines, probably due by its higher toxic potential.

Considering the results presented in this paper these information could be useful for the future development of some plant based products from *Ranunculus* species, but also for farmers to avoid the poisoning of farm animals by feeding them with grass including *Ranunculus* species.

CONCLUSION

The present study is the first evaluating by modern analytical methods of the anemonin content of some *Ranunculus* species, showing that for human and animal use are more safely the *R. bulbosus* and *R. sceleratus* herbs, meanwhile *R. ficaria* and *R. sardous* can lead to poisoning due by their significant anemonin content. Despite of this, *R. ficaria* and *R. sardous* herbs could be a very good anemonin source, a compound with demonstrated antibacterial and anti-inflammatory properties.

The study highlights also that using a glycerol-ethanol mixture for extraction the anemonin content will be lower, but there will be extracted also other valuable bioactive compounds from *Ranunculus* species. In this way can be obtain more safely extracts and the people can benefit of the therapeutic value of these species.

These results can confirm also the traditional uses of these species. *Ranunculus* species are used in traditional Romanian medicine: *R. ficaria* in hemorrhoids and circulatory disorders as local washes or ointments; *R. sceleratus* after drying in skin diseases; *R. bulbosus* in sciatic and arthritic pain. The *R. sardous* is not used in ethnopharmacology, that can be explained by its high content in anemonin and this may be a challenge for future studies, for use the anemonin as therapeutic agent.

REFERENCES

Anonymous (2017). European Pharmacopoeia. 9th edition, EDQM.
Anonymous (2017). German Homeopathic Pharmacopoeia. Deutsche Apotheker Verlag, Stuttgart.

Antal D and Coste I, (2004). *Pharmaceutical botany. Systematic plant*. Ed. Orizonturi Universitare, Timișoara, 81.
Ardelean A and Mohan G (2008). The medicinal flora of Romania, Ed. ALL, Bucuresti, p.33.
Aslam S, Bashir A, Uzair M and Subhan A (2012). The genus *Ranunculus*: A phytochemical and ethnopharmacological review. *International Journal of Pharmacy and Pharmaceutical Sciences*, **4**(5): 15-22.
Bonora A, Botta B, Menziani E and Bruni A (1988). Organ specific distribution and accumulation of protoanemonin in *Ranunculus ficaria* L., *Biochem. Physiol. Pflanzen*, **183**: 451-455.
Bruneton J (1993). Pharmacognosie: Phytochimie, Plantesmédicinales, 2nd edition, Paris, p.558.
Cobzac S, Cimpan G, Olah N and Gocan S (1999). The Quantitative Determination of Rutin in Different Glycerinic Plant Extracts by Solid-Phase Extraction and Thin-Layer Chromatography with Densitometry, *JPC-J Planar Chromat.*, **12**(1): 26-29.
Dărăban A, Olah NK, Câmpean RF, Furtuna F, Cobzac C, Dehelean Gh, Bojiță M, Hanganu D, (2015). Comparative study of polyphenols from propolis extracts of different origin, *Studia UBB Chemia*, **60**(2): 125-136.
Didry N, Dubreuil L and Pinkas M (2006). Microbiological properties of protoanemonin isolated from *Ranunculus bulbosus*. *Phytotherapy Research*, **7**(1): 21-24.
Mahran GH, Saber AH and el-Alfy T (1968). Spectrophotometric determination of protoanemonin, anemonin and ranunculin in *Ranunculus sceleratus* L. *PlantaMedica*, **16**(3): 323-328.
Mares D (1987). Antimicrobial activity of protoanemonin, a lactone from ranunculaceous plants. *Mycopathologia*, **98**(3): 133-140.
Markham K, Mitchell K and Campos M (1997). An unusually lipophilic flavonol glycoside from *Ranunculus sardous* pollen. *Phytochemistry*, **45**(1): 203-204.
Mei H, Zuo S, Ye L, Wang J and Ma S (2012). Review of the application of the traditional Chinese medicinal herb, *Ranunculus sceleratus* Linn. *J. Med. Plant Res.*, **6**(10): 1821-1826.
Seiger DS (1995). Plant Secondary Metabolism. Springer Science + Business Media, LLC, New York, p.267.
Tita I, Mogoșanu G and Tiță M (2009). Ethnobotanical Inventory of Medicinal Plants from South-west of Romania. *Farmacia.*, **57**(2): 141-156.
Toma C (2008). Pharmacognosy. Ed. Mirton, Timisoara, p.193.
Tomczyk M, Gudej J and Sochacki M (2002). Flavonoids from *Ficaria verna* Huds. *Z. Naturforsch.* **57c**: 440-444.