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Polyphenols Content, Antioxidant and Antimicrobial Activity of Ethanol Extracts from the Aerial Part of Rock Rose (*Helianthemum nummularium*) Species

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Abstract: This study aimed to evaluate polyphenols content, antioxidant and antimicrobial activities of ethanol extract from rock rose (*Helianthemum nummularium* (L.) Mill.). Plant material has been harvested in July from Romanian Carpathian Mountains. The ethanolic extract (70%, v/v) was prepared from the aerial part of rock rose species. High performance thin layer chromatography (HPTLC) analysis of ethanol extract and subsequently hydrolysed sample indicated that quercetin glycosides were the major polyphenolic compounds, and kaempferol derivates, chlorogenic and gallic phenylcarboxylic acids are also being present in the polar extracts. Chemiluminescence assay in luminol/H_2O_2 system indicated very high antioxidant activity of the ethanolic type of extract (IC_{50} = 1.27 μg/mL), while microbiological studies (cylinder method in plates) indicated certain antimicrobial activity (measuring from 12.5 mm to 21.5 mm of the diameter of the inhibition zone) of the propylene glycol (20%, v/v) standardized extract (5 mg gallic acid equivalents/mL sample) against several standard bacterial strains, *Staphylococcus aureus* ATCC 6538 and *Escherichia coli* ATCC 8739, *Salmonella typhimurium* ATCC 14028 and *Salmonella enterritidis* ATCC 13076, respectively. Therefore, based on the augmented antioxidant effect of the rock rose polar extracts, there can be found numerous applications in the pharmaceutical industry, but also in non-pharmaceutical fields, for example in cosmetic and hygiene products areas, due to certain antimicrobial properties.

Key words: *Helianthemum nummularium* (L.) Mill., polyphenols content, antioxidant and antimicrobial activity.

1. Introduction

There are very little data about chemical composition and biological effects of rock rose *Helianthemum nummularium* (L.) Mill. species (Cistaceae family). Literature data show that amongst the 24 species known, *H. nummularium* is mainly found in the north of Europe, while all others are spread mainly in the Mediterranean area [1].

Concerning the rock rose chemical composition, based on their special attentions in perfumery and aroma industries, analytical studies mainly focused on the essential oils content. Such as, studies of Viuda-Martos et al. [2] on Moroccan species *Cistus ladanifer* L. and *Cistus monspeliensis* L. have reported high contents of 1,8-cineole (19.27%) and viridiflorol (16.38%), bornyl acetate (9.14%) and a-pinene (5.84%), respectively. Similarly, Nicoletti et al. [3] studied on Tunisians' *Cistus monspeliensis*, *Cistus libanotis* and *Cistus villosus* rock rose species and indicated diterpenes compounds as the main constituents of the labdanum, the resin used in perfumery obtained from the leaves of Mediterranean rock rose species. Also, De Freitas et al. [4] reported 2,2,6-trimethylcyclohexanone and ethyl dihydrocinnamate as being the two flavour and fragrance agents of rock rose *Cistus ladaniferus* species, and both of them were identified in young fortified wines from the Douro Demarcated region.

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Concerning polyphenols' content, studies on Algerian *Helianthemum kahiricum* (Del.) species indicated the prevalence of kaempferol derivates, kaempferol-3-β-D-(-6-O-trans-p-coumaroyl) glucopyranoside and kaempferol-3-β-D-(3"-6"-di-O-trans-p-coumaroyl) glucopyranoside, respectively [1]. This was further confirmed by Viuda-Martos et al. [2] on Moroccan *Cistus ladanifer* L. and *Cistus monspeliensis* L. rock rose species, indicating the presence of 3,7'-di-O-methyl-kaempferol and 3-O-methyl-kaempferol compounds.

As about pharmacological properties, previous studies demonstrated the antimicrobial effects of some non-polar extracts (petroleum ether, chloroform and butanol extracts) isolated from *H. kahiricum* species [1]. Studies by Nicoletti et al. [3] on Tunisians *C. monspeliensis*, *C. libanotis* and *C. villosus* indicated antioxidant potency of the rock rose species by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, conversion of the Fe (3+) ferricyanide complex and inhibition of β-carotene bleaching tests. On the studies of essential oils extracted from *Cistus creticus*, *C. salvifolius*, *C. libanotis*, *C. monspeliensis* and *C. villosus* [5], important antioxidant effects have also been revealed by DPPH and 2'-azino-bis(3-ethyl-benzothiazoline-6-sulphonic) acid (ABTS), ferric reducing ability of plasma (FRAP) and β-carotene bleaching assays, but also acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activity concluding their potential use for the prevention and treatment of Alzheimer's disease. Referring punctual *H. nummularium* rock rose species, data indicate its presence in an herbal medicine called Bach flower essences described as a remedy that comes to the rescue when we are faced with stressful, fearful situations [6]. It must be also noted that, as directed, there is no enough information to know if rock rose is safe or what the possible side effects might be [7].

Given these, this work aimed to study polyphenols content, antioxidant and antimicrobial activities of an ethanolic extract (70%, v/v) prepared from the aerial part of *H. nummularium* harvested from the Romanian Carpathians, and thus to hypotesise its potential application in the pharmaceutical field.

2. Materials and Methods

2.1 Plant Materials

The aerial part of rock rose *H. nummularium* (L.) Mill. species was collected in July 2015 from Romanian Carpathian Mountains, Sinaia region, at about 1,400 m altitude. Taxonomic identification has been done by the botanist's team of National Institute of Chemical-Pharmaceutical Research and Development (ICCF), Bucharest and a voucher specimen (codified Hmu.scr1) is deposited in ICCF plant material storing room.

2.2 Extracts Preparation

The plant material has been air-dried, and then ground in a cross beater mill equipped with a 2 mm sieve. Two samples of plant powder of 10 g each were separately extracted with 100 mL of ethanol solvent (70%, v/v), two times consecutively, for 1 h, at reflux temperature and continuously agitation state. The two ethanolic extracts were submitted to vacuum filtration (medium filter paper), and the extracts resulted were quantified as concerning quantitative (total flavones content expressed as rutin (R) equivalents and total phenols content expressed as gallic acid (GAE) equivalents) and qualitative (HPTLC method) aspects. The ethanolic extracts resulted were set as follows:

(1) 50 mL of ethanolic extract has been concentrated at spiss residue (using a rotary flash evaporator), and then the *spiss* (sticky brown) residue was dissolved into 20% (v/v) propylene glycol (PEG) solvent so as to achieve the exactly content of 5 mg GAE/1 mL sample. The resulted standardized product (codified HnPEG20) has been used for microbiological studies.

(2) Other 50 mL of ethanolic extract has also been concentrated at *spiss* residue, and then the *spiss* residue was solved into 50 mL 4 N HCl and

hydrolysed for 30 min. The resulting hydrolysed sample has been extracted with ethyl acetate (100 mL each, three times consecutively). After that, the combined ethyl acetate fractions were evaporated to dryness and redissolved in 96% (v/v) ethanol to a final volume of 25 mL. The resulting (hydrolysed) ethanolic sample (codified HnH) has been subjected to qualitative HPTLC study in order to complete studies on the origin rock rose (70%, v) ethanolic extract (Hnet70).

2.3 Chemicals

Chemicals ($AlCl_3$, CH_3COONa, H_2O_2, luminol, 0.2 M Tris-HCl with pH 8.5 and dimethyl sulfoxide/DMSO), Folin-Ciocalteau and natural products (2-aminoethyl diphenylborinate, diphenylboric acid 2-aminoethyl ester; natural product reagent A—polyethylene glycol and natural product reagent B—NP/PEG) reagents, solvents (methanol, ethanol, ethyl acetate, formic acid, chloroform, glacial acetic acid), as well as reference compounds (rutin, hyperoside, cosmosiin, vitexin, kaempferol, apigenin, chlorogenic, caffeic, gallic, protocatechuic and rosmarinic acids) products were purchased from Sigma-Aldrich Co., Bucharest, Romania.

2.4 Qualitative Analysis for Polyphenols

Polyphenols analysis was performed by HPTLC method according to Wagner and Bladt [8] and Reich and Schibli [9], and the standard setting for polyphenols (1, ethyl acetate: formic acid: glacial acetic acid: water/100:11:11:26) and saponins (2, chloroform: glacial acetic acid: methanol: water /64:32:12:8) assay, as described in previous work [10].

2.5 Estimation of Total Flavones Content

Total flavones content was appraised by using $AlCl_3$ in sodium acetate medium according to the standard method of "Romanian Pharmacopoeias (FRX)" [11], which was also described in the authors' previous work [10]. The results were expressed as rutin (R) equivalents.

2.6 Estimation of Total Phenols Content

Total phenols were evaluated by using Folin-Ciocalteau reagent according to the standard method of "Romanian Pharmacopoeias (FRX)" [11], which was also described in previous work [10]. The results were expressed as gallic acid equivalents (GAE).

2.7 Antioxidant Activity Assay

Antioxidant activity was determined by using chemiluminescence (CL) method [12] and Turner BioSystems $20/20^n$ (SUA) luminometer equipment.

Briefly, the test sample is vacuum filtered (on slow filter paper), and (n) aliquots of each 50 μL test sample are mixed with 200 μL 1 mM luminol (prepared in DMSO), 700 μL 0.2 M Tris-HCl with pH 8.6 and 50 μL 1 mM H_2O_2 (prepared in bi-distilled water). The reference sample consists of 50 μL test sample solvent (in this case 70%, v/v, ethanol) mixed with identical 200 μL 1 mM luminol, 700 μL 0.2 M Tris-HCl with pH 8.6 and 50 μL 1 mM H_2O_2. The intensity of the chemiluminescence reaction (expressed as activity units/a.u.) of the reference sample of the (n) aliquots at each 5 s, with total 60 s are measured. After that, the antioxidant activity (AA%) of the test sample is calculated by using the bellow Eq. (1), and the final result is the mean value of the n aliquots:

$$AA\% = \frac{RI_r - RI_t}{RI_r} \times 100 \quad (1)$$

where, RI_r is the chemiluminescence reaction intensity of the reference sample (a.u.), while RI_t is the chemiluminescence reaction intensity of the tested sample (a.u.).

2.8 Microbiological Tests

Microbiological tests were performed using cylinder method in plates according to "Romanian

Pharmacopoeias" [11] standard method, which was also detailed in the authorts' previous work [10]. Four standard microbial strains, three gram-negative (*Escherichia coli* ATCC 8739, *Salmonella typhimurium* ATCC 14028 and *Salmonella enterritidis* ATCC 13076) and one gram-positive (*Staphylococcus aureus* ATCC 6538) bacteria were used. The test organisms were purchased from Mecconti (Merck Romania SRL).

Tests were done on the rock rose *H. nummularium* standardized extract (HnPEG20 with exactly 5 mg GAE/1 mL sample).

3. Results and Discussion

3.1 Polyphenols Analysis

Two series of rock rose (70%, v/v) ethanolic extracts (Hnet70) were assessed for polyphenols by HPTLC qualitative analysis, respectively, i.e., (1) ethyl acetate: formic acid: glacial acetic acid: water in 100:11:11:26 and (2) chloroform: glacial acetic acid: methanol: water in 64:32:12:8, as shown in Fig. 1.

In series one of rock rose ethanolic extracts (Hnet70), as seen from chromatogram A in Fig. 1, the quercetin glycosides was predominated, and the yellow-orange fluorescent spots distributed along the entire chromatogram A (s1: $Rf \approx 0.18$, s5: $Rf \approx 0.46$, s6: $Rf \approx 0.53$, s8: $Rf \approx 0.59$, s9: $Rf \approx 0.69$, s10: $Rf \approx 0.83$, s11: $Rf \approx 0.86$). Based on the current reference compounds Rf values and literature data [9, 10] as well, s5, s9, s10 and s11 spots have been attributed to quercetin 3-rutinoside, quercetin 3-galactoside, quercetin 3-rhamnoside and quercetin 3-arabinoside compounds, respectively.

Also, rock rose 70% ethanolic extract (Hnet70) indicated the presence of three kaempferol derivates, the intense green fluorescent spots situated in the bottom part of chromatogram A (s2: $Rf \approx 0.28$, s3: $Rf \approx 0.36$ and s4: $Rf \approx 0.38$), likely owing to kaempferol 3-O-coumaroyl glycosides, as reported by Bouzergoune et al. [1]. Chlorogenic acid and gallic acid has also been evidenced in Hnet70 sample by the intense blue fluorescent spot s7 at $Rf \approx 0.55$, and the

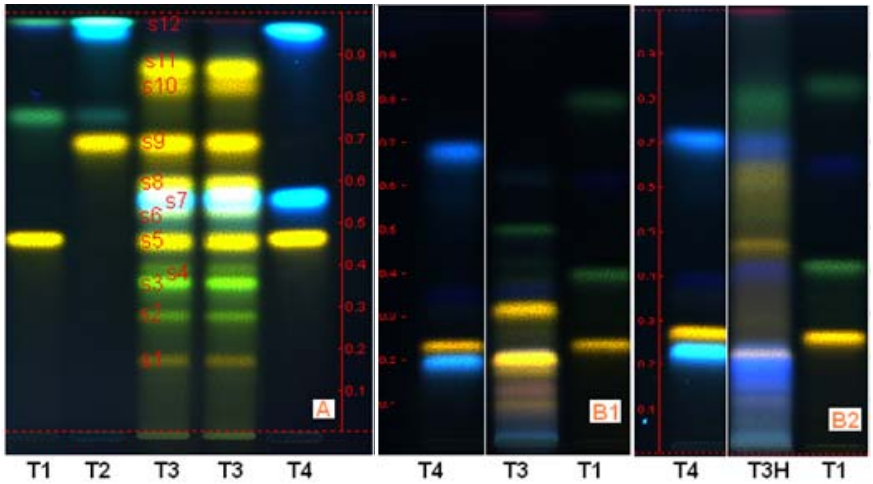

Fig. 1 HPTLC aspects of two series of rock rose 70% ethanolic extract (Hnet70) and related hydrolysed sample (HnH).
In chromatogram A1:
T1—rutin, vitexin, protocatechuic acid and apigenin (ref.); T2—hyperoside, cosmosiin, rosmarinic acid and kaempferol (ref.); T3—rock rose 70% ethanolic extract/Hnet70 duplicate samples; T4—rutin, chlorogenic acid, gallic acid and caffeic acid (ref.).
In chromatogram B1:
T4—chlorogenic acid, rutin, gallic acid and caffeic acid (ref.); T3—rock rose 70% ethanolic extract/Hnet70 single sample; T1—rutin, vitexin, protocatechuic acid and apigenin (ref.).
In chromatogram B2:
T4—chlorogenic acid, rutin, gallic acid and caffeic acid (ref.); T3H—rock rose hydrolyzed sample/HnH single sample; T1—rutin, vitexin, protocatechuic acid and apigenin (ref.).

intense blue-indigo fluorescent spot s12 at *Rf* ≈ 0.95 situated at the front of chromatogram A, respectively.

The saponins system (series 2) was demonstrated as very useful for polyphenols aglycones separation, too [10]. In series 2 of rock rose ethanolic extracts (Hnet70), as shown in chromatograms B in Fig. 1, gallic acid has been confirmed occurrence in both Hnet70 sample (see T3 track from chromatogram B1, the blue-indigo fluorescent spot at *Rf* ≈ 0.38) and related hydrolysed sample HnH (see T3H track from chromatogram B2, the blue-indigo fluorescent spot at *Rf* ≈ 0.42). Furthermore, the hydrolysed sample HnH (T3H track, chromatogram B2) have revealed important quantities of kaempferol aglycone (green fluorescent spot at *Rf* ≈ 0.88) [10], supporting s2, s3 and s4 spots attribution to kaempferol derivates (sub) class as well.

Overall, HPTLC qualitative study have revealed quercetin glycosides as being the major polyphenols compounds in the polar (ethanolic) samples, kaempferol derivates, chlorogenic and gallic pheylcarboxylic acids also being present in *H. nummularium* aerial part derived products.

Concerning quantitative aspects, results showed that the Hnet70 extract contains 1.13 g total flavones expressed as rutin equivalents (R) and 3.95 total phenols expressed as gallic acid equivalents (GAE) per 100 g dried plant powder (± 5%).

3.2 Antioxidant Activity

The antioxidant activity assay is measurement of the activity units/a.u. at 5 s after chemiluminescence reaction initiation at different concentration points/dilution series (μg/mL) and corresponding IC_{50} values. Fig. 2 shows the antioxidant activity assay of rutin and gallic acid references samples. Rutin and gallic acid reference samples were prepared as 10^{-3} M ethanol solutions (70%, v/v), with eight dilution series (1×, 2×, 5×, 6×, 7×, 8×, 9× and 10×, respectively).

Fig. 3 shows antioxidant activity assay and IC_{50} value of the test sample Hnet70, which was prepared as six dilution series (2×, 5×, 10×, 50×, 100× and 200×, respectively).

As the chemiluminescence measurements shown in Fig. 2, the important antioxidant effect of reference compound rutin has been appraised with IC_{50} = 2.54 μg/mL, while gallic acid indicated IC_{50} = 0.85 μg/mL. By comparison, rock rose ethanolic extract Hnet70 has been estimated with IC_{50} = 1.27 μg/mL, suggesting very high antioxidant potency of the polar extracts isolated from *H. nummularium* species (Fig. 3).

3.3 Microbiological Results

Table 1 demonstrate certain antimicrobial activity of HnPEG20 extract on *E. coli* ATCC 8739 strain, as well as weak to moderate activity on *S. aureus* ATCC 6538, *S. typhimurium* ATCC 14028 and *S. enterritidis* ATCC 13076 strains. Also, the results in the present study demonstrate antimicrobial potency of the polar extracts (e.g., ethanolic and propylene glycol extracts) prepared from rock rose *H. nummularium* species. Differently, studies on Algerian rock rose *H. kahiricum*

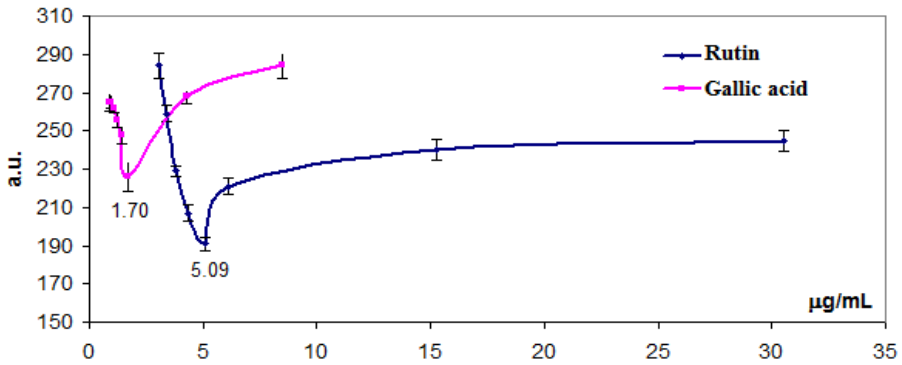

Fig. 2 Chemiluminescence assay on the rutin and gallic acid phenolics reference samples.

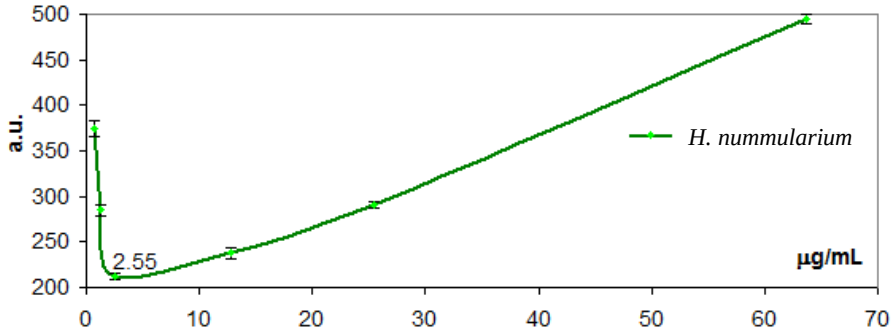

Fig. 3 Chemiluminescence assay on the rock rose *H. nummularium* ethanolic extract Hnet70.

Table 1 Antimicrobial assay on *H. nummularium* 70% ethanolic extract.

Microbial strain tested	Diameter (mm) of inhibition zone ± SD
Staphylococcus aureus ATCC 6538	15.5 ± 0.57
Escherichia coli ATCC 8739	21.5 ± 0.86
Salmonella typhimurium ATCC 14028	13.0 ± 1.73
Salmonella enterritidis ATCC 13076	12.5 ± 1.73

Values are mean inhibition zone (mm) ± SD of three replicates.

Diameter < 10 mm means no activity, diameter in 10-15 mm means weak activity, diameter in 16-20 mm means moderate activity and diameter > 20 mm means certain antimicrobial activity.

species indicated antimicrobial potency of the non-polar extracts, petroleum ether, chloroform and butanol extracts, respectively [1]. The solvent sample, 20% (v/v) propylene glycol (PEG), does not present any antimicrobial activity.

Therefore, besides augmented antioxidant potency, the ethanolic extract (Hnet70) and the subsequent propylene glycol standardized extract (HnPEG20) from *H. nummularium* L. plant species may also be regarded as a potential source of antimicrobial agents that can be used in managing bacterial infections. The results are more important, as the excessive use of antibiotic leading to antibiotic resistance has become an increasingly hard to control health problem. In support of these, the Center for Disease Control and Prevention (CDC) has reported 23,000 deaths per year in the USA caused by drug-resistant bacteria, which shows the scale of this issue.

Concerning the probable compounds involved in antimicrobial activity of rock rose polar extracts, the quercetin monoglycosides combined with gallic and/or caffeic pheylcarboxylic acid derivates were suggested as being the most active compound [10]. These findings are in accordance with previous study on quercetin and its arabino glycosides from leaves extracts of *Psidium guajava* L. The best results were shown by quercetin-3-O-β-D-arabinopyranoside and quercetin-3-O-α-L-arabinofuranoside, respectively; these compounds have antibacterial effects against *S. aureus*, *E. coli*, *P. aeruginosa* and *C. albicans* [13].

Also, Pepeljnjak et al. [14] reported particular antimicrobial activity of isoquercitrin (quercetin 3-glucoside) against *S. aureus*, *P. rettgeri*, *M. Gypseum*, *C. tropicalis* and also *C. lusitaniae*, as well as of hyperoside (quercetin 3-galactoside) [15] upon *S. aureus*, *S. epidermitis*, *S. pyogenes*, *B. subtilis*, *E. faecalis*, *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *P. mirabilis*, *N. gonorrhoeae* and *C. albicans* strains. Both of them are present in rock rose *H. nummularium* polar extracts. Given these results, rock rose extract can be used in pharmaceutical and non-pharmaceutical applications, for example cosmetic or hygiene products areas.

4. Conclusions

Analytical studies on the ethanolic extract (Hnet70) from *H. nummularium* (aerial part) plant product indicated a high content of biologically active

compounds—flavonoids derivates, such as quercetin and kaempferol glycosides, and chlorogenic and gallic phenylcarboxylic acid derivates. It also indicated that Hnet70 augmented radical oxygen scavenging activity was similar to gallic acid reference product, one of the most active natural compounds in terms of antioxidant potency. Microbiological studies on the propylene glycol standardized extract (HnPEG20) showed certain antimicrobial activity on *E. coli* ATCC 8739 strain and *S. aureus* ATCC 6538, *S. typhimurium* ATCC 14028 and *S. enterritidis* ATCC 13076 strains (12.5 mm to 21.5 mm of diameter of the inhibition zone).

Further studies on the polar extracts from rock rose *H. nummularium* plant species will be carried in order to verify potential anti-inflammatory activity (on *in vitro* and *in vivo* experiments), as well as antimicrobial properties on standard and wild microbial strains, and corresponding minimum inhibitory concentrations (MICs) values.

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