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SCREENING OF THE ANTIOXIDANT CAPACITY AND TOTAL PHENOLIC CONTENT OF THREE NATIVE PLANTS♣

M. BUTU [a], S. RODINO[a,b,*], D. A. GOLEA [a], A. BUTU[a]
[a]*National Institute of Research and Development for Biological Sciences, 060031, Bucharest, Romania*
[b]*University of Agronomic Sciences and Veterinary Medicine, 011464,Bucharest, Romania*

The medicinal herbs, a valuable source of biologically active compounds, have been used in folk medicine for various diseases, but phytochemical exploration is still incomplete. This study presents the extractive technological processes and spectrophotometric characterization of threeindigenous plants in order to obtain anantifungal formula with applications inhorticulture.Therefore, it was evaluated the antioxidant activity, total phenolic (TPC) and flavonoid contents (TFC) and the correlation between them for individual extracts obtained by two different methods: Soxhlet (S) and percolation (P).The obtained data showed a significant difference both between the plant material used, as well as the extraction method applied. Most of the extracts prepared by Soxhlet method had higher antioxidant properties and phenolic contents than the ones obtained by percolation. Based on the results, were elaborated five association formulas, of which *Rosmarinus officinalis*L. combination 1:1 (S: P) showed the best results:TPC - 236.38 mg GAE / g DW, TFC - 247.11 mg rutin / g DW, EC_{50} - 0.030 mg/mL.

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1. Introduction

In a world where the main food source is provided by agriculture, the climate change, pollution and resistance developing microorganisms limit the ability of farmers to produce the raw material necessary for obtaining products that comply with the quality standards.

Even though the conventional methods are the most used methods for control, the intense use of synthetic pesticides started to be restricted because of the requirements for exceeding a certain threshold of residues in the agricultural products, such as the appearance of pathogen resistance to the main active compounds[1, 2].

Even though the pesticides are designed to destroy the pathogen agents, when they are used in an abusive way, they can represent a hazard for the environment through their effect on the soil[3] and the useful entomo-fauna[4]. The issue arisen from the irrational use of pesticides represents one of the challenges faced by the international organizations for environment protection, such as United States Environmental Protection Agency (EPA), The European Environment Network (IMPEL).

The problems caused by the synthetic pesticides and by their residues, led to a need for finding new alternatives for biologic control, such as high selectivity biodegradable formulas, which should reduce the irrational use and to minimize their impact on the environment[5]. Thus, the alternative strategies include searching for new types of bio-products which will degrade in

♣ All authors have equal contributions to this work

* Corresponding authors:steliana.rodino@yahoo.com

non-toxic products, and, at the same time, have to be adequate for usage in integrated control programs for diseases and pests[5,6].

Promising alternatives include the use of compounds that are bio-synthesized by plants that have antimicrobial effect[7,8]. Modern agrochemical researches all around the world showed the viability of such substances obtained from plant extracts, with regard to prevention and treatment the diseases produced by phytopatogens[9-13]. Thus, the use of plant active compounds is an alternative form of phytochemical treatment, having an increasingly higher awareness since the 90's. Literature data showed that there are direct relationships between the type and the concentration of the active substances (volatile oils and phenolic compounds) from plants and their antimicrobial action[14]. These compounds are synthesized and used by plants as means of defense against biotic and abiotic factors[15]. Defense mechanisms include physical and chemical defense barriers that prevent the microbial attack by using both the preformed compounds, as well as those induced by the defense response[4]. The plant defense response is mainly due to the compounds that belong to the secondary metabolites group, which are classified according to the chemical characteristics (alkaloids and phenols, polyphenols, tannins), plant origin and the biosynthetic origin (terpenoids, polyketides, phenilpropanoids)[16].

The paper belongs to an extensive project in which it is sought the exploitation of the antifungal potential of indigenous plants in view of obtaining such natural alternatives, with effect on oomycetes that attack horticultural crops.

Thus, there were chosen three species of indigenous plants: ivy (*Hedera helix* L.), rosemary (*Rosmarinus officinalis* L.) and sage (*Salviaofficinalis* L.), whose therapeutic value has been previously demonstratedas cytotoxic, antipyretic, analgesic[17], disinfectant[18-20], etc.

In the frame of the experiment, these plants have been exploited from the biologic point of view with the purpose of emphasizing the species that have a maximum accumulation of biologically active compounds, in view of their association and further use as natural fungicide in horticulture.

Taking all these into account, the objectives of this investigation were as follows: the biochemicalcharacterization of the plant extracts in terms of quantitative determination of the total phenolic content, total flavonoids content, free radical scavenging activity and the correlation betweenthese, depending on the obtaining methods used for the plant extracts.

According to the obtained results, there were elaborated and characterized several association formulas using the individual plant extracts, which will be the subject of the *in vitro* research regarding their effect on the phytopatogen *Phytophtora infestans*.

2. Materials and methods

2.1 Chemical reagents and materials

1,1-diphenyl-2-picrylhydrazyl free radical (DPPH), Folin-Ciocalteu reagent, gallic acid, rutin and quercetin reagentswere obtained from Sigma Chemicals, while the sodium carbonate, sodium nitrite, aluminum chloride, hidroxid solution were purchased from Fluka Chemical. All of the reagents and solvents used in the experiment were of adequate analytical grade.

2.2 Plant materials and extraction

The material plants were obtained from SC VITAPLANT SRL, Mureş. The plants that were investigated include: *H. helix*L., *R. officinalis*L. and *S.officinalis*L.. The dried aerial parts were milled using a blender, and the hydroalcoholic extracts were obtained by two different methods: percolation (P) and Soxhlet (S). For both extractions, the ratio between the plant material and solvent was 1:10, and the solvent was ethanol 50%. Experimental studies have shown that the highest extraction yields were achieved with polar alcohol-based solvents, furthermore, the addition of water in appropriate amounts exerting an improvement for the extraction efficiency[21,22]. For the percolation method, the plant material was continuously flushed with solvent for 30 h. For the Soxhlet extraction, there were performed 6 recirculation cycles. The obtained hydroalcoholic extracts were first filtered through Whatman No.1 filter paper and then centrifuged at 4025 X g for 15 min. The supernatant was used for analysis without any further treatment.The concentration of the extract was expressed as mg DW (dry weight)/mL.

2.3 The total phenolic content (TPC)

The total phenol contentpresent inhydroalcoholicextractswas determinedspectrophotometricallyaccording to theFolin-Ciocalteu method[23]. The reaction mixture was prepared by mixing 0.025 mL of hydroalcoholic extract, 1.975 mL of distilled water, 0.125 mL of Folin-Ciocalteu reagent and 0.375 mL of 20 % sodium carbonate. After 2 h since the reaction started, the optical density (OD) was measured at the wavelength of 750 nm, against the control that was prepared by replacing the extract with distilled water. The results were calculated based on the calibration curve for gallic acid prepared in various concentrations (300 ÷ 3000 µmol/L). The TPC of the extracts was expressed as mg of gallic acid equivalents (GAE)/g DW.

2.4 Total flavonoid content (TFC)

The total flavonoid content of the hydroalcoholic extracts were determined by the aluminum chloride spectrophotometric methodof Erel et al. with some modifications[19]. An appropriate amount of 0.25 mL of extract was added to 1 mL of distilled water. After that, to the flask, there were added 0.075 mL of sodium nitrite(5 %) and 5 min later, 0.075 mL of aluminum chloride(10 %). After 6 min of rest, 0.5 mL of 1 mol/L sodium hydroxide solution was added and the total volume was filled to 2.5 mL with distilled water. The solution was mixed well and the optical density was measured at 510 nm against the control containing distilled water instead of the extract. The standard curve was prepared using rutin in various concentrations (100 ÷ 1000 µmol/L) by the same method. The total flavonoid content was expressed as mg of rutin equivalents (RE)/g DW.

2.5 DPPH free radical scavenging assay

The DPPH radical-scavenging activity is one of the most frequently used test for the determination of the antioxidant capacity[24] and show a positive correlation with another such methods as the trolox equivalent antioxidant capacity (TEAC)[25], the total radical trapping antioxidant parameter assay (TRAP)[26], the photo-chemiluminescence assay (PCL)[27], and the ferric reducing antioxidant potential assay (FRAP)[28].

The DPPH free radical scavenging assay of the hydroalcoholic extracts was determined spectrophotometrically[23].A quantity of 0.5 mL of the extract was added to 1 mL of 0.1 m mol/L DPPH solution (prepared daily and protected from light). The mixture was shaken vigorously and incubated at room temperature for 30 min, than the optical density was measured against methanol at 515 nm. The control contained all components except the extract. The optical density of the DPPH in methanol did not undergo major changes throughout the period of the assay. The capability to scavenge the DPPH radicals was calculated using the following equation:

$$AA_{DPPH}(\%) = \frac{OD_{control} - OD_{sample}}{OD_{control}} \times 100$$

where, $OD_{control}$ is the optical density of the control (containing all reagents except for the extract) and OD_{sample} is the optical density in the presence of the extract.

The antioxidant properties were expressed as mg quercetin equivalents/g DW. The parameter EC_{50} (efficient concentration value) (mg/mL) was also calculated. This coefficient, which measures the effective concentration at which the DPPH radicals were scavenged by 50 %, was obtained by extrapolation from linear regression analysis.

2.6 Statistical analysis

All the analyses were carried out in triplicate. For the statistical evaluations there was applied the one – way analysis of variance (ANOVA) followed by t-Test. The data resulted are expressed as mean values ± standard deviation. All the values of probability were less than 0.05 and were considered statistically significant. Microcal Origin was used for graph plotting.

3. Results and discussion

3.1 The total phenolic (TPC) and total flavonoid contents (TFC)

Total phenolic contents and total flavonoid contents of different three plant materials, processed by two extracting techniques (Soxhlet and percolation) are presented in table 1.

The total phenolic contentexpressed as mg GAE/g DW was determined spectrophotometric based on the calibration curve achieved for gallic acid with the regression equation $\text{Optical density} = -0.00487 + 0.000216 \times C_{\text{gallic acid}}$, (R = 0.9990, SD = 0.0088, N = 8, $p<0.05$). The concentration of TPC ranged from a minimum of 60.44 ± 0.06 mg GAE/g DW for *H. helix* L. extract obtained by percolation, to a maximum of 255.13±0.55 mg GAE/g DW for *R. officinalis* L.extract obtained by Soxhlet method (Table 1). The content of total phenolic was higher for Soxhlet extraction compared to the samples obtained by percolation.

Table 1. The total phenolic content (TPC) and the total flavonoid content (TFC) of the individual extracts

Plant material	Extraction method**	Concentration (mg/mL)	Total phenolic content* (mg GAE / g DW)	Total flavonoid content* (mg RE / g DW)
H. helix	S	34	92.93 ± 0.06	141.57 ± 0.30
	P	25	60.44 ± 0.06	116.34 ± 0.42
R. officinalis	S	23	255.13 ± 0.55	337.97± 0.50
	P	7	243.63 ± 0.17	297.36± 0.55
S. officinalis	S	17	228.65 ± 0.18	279.78± 1.52
	P	17	150.05 ± 0.289	250.75 ± 0.955

*Each value is the mean of three replicate determinations ± standard deviation
**S = Soxhlet and P = percolation method

There are diverse reports regarding the TPC for the *R. officinalis*L. extract. Our findings are in agreement with previous investigation of S. Moreno[29]. They have reported for the acetone extracts of rosemary a total phenol content of 19 g GAE/100 g DW, for the methanol extract a concentration of 12 g GAE/100 g DW and for the water extract 3 g GAE/100 g DW. Similar values were reported by Tavassoli[30], analysis which was carried out on methanolic extract from rosemary obtained by Soxhlet method, showing a content of 4.99 g/100 g dry leaves. As a result, the solvent used in the extraction process and all the parameters used in the extraction methods (the time, the ratio between the plant material and the solvent) are very important.

The total flavonoid content expressed as mg rutin equivalents/g DW (Table 1) was determined based on the calibration curve done for rutin with the regression equation $\text{Optical density} = 0.00329 + 0.000665 \times C_{rutin}$, (R = 0.9992, SD = 0.0088, $p<0.05$). The TFCranged from a minimum of 116.34±0.42 mg RE/g DW for *H. Helix*L.extractobtained by percolation, to a maximum of 337.97±0.50 mg RE/g DW for *R. officinalis*L.extractobtained by Soxhlet method. The average amount determined for both TPC and TFC in the case of *R. officinalis* L.extractswas almost 3-fold higher compared with *H. Helix* L. extracts.Also, the extracts obtained with Soxhlet extractor have a higher content of total flavonoids than the samples obtained by percolation.

The TPC and TFC contents varied considerably (statistically significant (P <0.05)) both between the various plant extracts and between the samples obtained using the two extraction methods. In various studies[31,32] it was shown the influence of different extraction solvents (methanol, ethanol, acetone, propanol and ethyl acetate) as well as the techniques used on both qualitative and quantitative content of the interest compounds.

3.2 DPPH free radical scavenging assay

In this study, the antioxidant activity was expressed both as the EC_{50} factor and the mg quercetin equivalent/g DW (Table 2). The DPPH radicals were reduced in all the cases. The results provide a direct comparison between thesetwo analyses.The hydroalcoholic extract of *R. officinalis* L. scavenged 91.47 % of DPPH free radicals and is significantly different ($p < 0.05$) as compared with the *H. helix* L. extract which scavenged 78.55 %.

Table 2. Antioxidant activity results for the individual extracts

Plant material	Extraction method**	EC_{50}* (mg/mL)	Quercetin equivalent* (mg/g DW)
H. helix	S	0.233 ± 0.005	2.75 ± 0.5331
	P	0.198 ± 0.005	3.93 ± 0.3046
R. officinalis	S	0.035 ± 0.002	16.77 ± 0.622
	P	0.037 ± 0.003	4.77 ± 2.242
S. officinalis	S	0.038 ± 0.005	15.37 ± 0.622
	P	0.049 ± 0.002	6.19 ± 0.762

*Each value is the mean of three replicate determinations ± standard deviation
**S = Soxhlet and P = percolation method

The antioxidant potential is inversely proportional to the EC_{50} value, which was calculated from the linear regression of the antioxidant activity versus the concentrations of the extracts. The antioxidant activity expressed as mg quercetin equivalent/g DW was calculated based on the calibration curve done for quercetin with the regression equation$DPPH\% = 27.8235 + 1.6385 \times C_{quercetin}$,R = 0.9984, SD = 1.20434, N = 8, $p < 0.05$.

The EC_{50}values for the extracts prepared both by Soxhlet and percolation were in the following decreasing order: *H. helix* L.>*S. officinalis*L.>*R. officinalis*L.. Lower EC_{50} values mean higher radical scavenging capacity. Also,as in the case of the total phenolic and flavonoid contents, it was observed that most of the samples obtained by Soxhlet method had higher antioxidant capacity than those prepared by percolation.

3.3 Correlation between DPPH % free radical scavenged activity and TPC, TFC

Specializedexperimental studies[33]reported that the phenols and flavonoids can be responsible for the antioxidant activity of medicinal plants. Therefore, in order to determinate the relative importance, the DPPH free radical scavenging activity was correlated with the content of these compounds, for each plant and extraction method, respectively.

The Pearson correlation coefficient (R^2) represents the statistical technique for measuringanddescribing the degree oflinearregressionbetween twocontinuous quantitative variables that are normally distributed. In our study, the R^2 value of the correlation between DPPH % free radical scavenged and the total phenolic content (*0.81036*) and total flavonoid content (*0.84229)*, for both extraction methods, are shown in Fig. 1and Fig. 2 respectively. The two values are approximately the same. Thiscorrelationis statistically significantat $p < 0.05$.Appreciable antioxidant potential of all the extracts was determined. These results suggest that a great part of the antioxidant capacity of these plant extracts is attributed to the total phenolic and flavonoid contents in the extract, which have the hydrogen-donor ability to scavenge the free radicals.Similar

studies[26,33,34]suggest a linear relationship between antioxidant capacity and the total phenolic and flavonoid contents of the plant extracts.

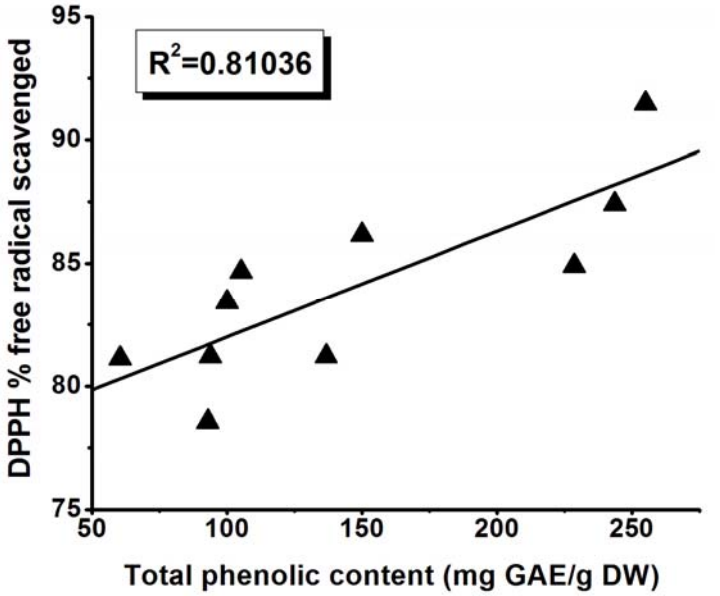

Fig. 1. Correlation between DPPH % free radical scavenged activity and the total phenolic content (mg GAE/g DW), (R^2=0.81036, SD = 2.33755, p< 0.05)

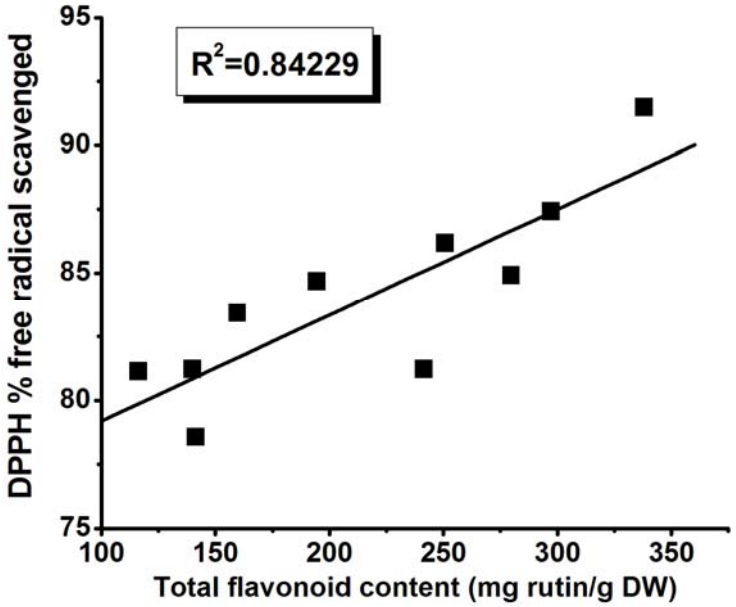

Fig. 2. Correlation between DPPH % free radical scavenged activity and the total flavonoid content (mg rutin/g DW), (R^2=0.84229, SD = 2.15787, p< 0.05)

3.4 Biochemical characterization of the biopreparations

Based on the above results, several association formulas of the individual plant extracts with high efficiency have been elaborated, in order to obtain bio-preparations which have to respond to the target purpose.

Considering the fact that the extracts obtained from aerial parts of *R. officinalis* L. and *S. officinalis* L., both prepared by the two extraction methods, showed an increased antioxidant content as well as a greater content of interest compounds than the other extracts analyzed, the following association formulas (AF) were created:

- AF 1 → *R. officinalis* L. (S)–*R. officinalis* L. (P), ratio1:1;
- AF 2 → *S.officinalis* L. (S)–*S.officinalis* L. (P), ratio1:1;
- AF 3 → *R. officinalis* L. (S) –*S.officinalis* L. (S), ratio1:1;
- AF 4→ *R. officinalis* L.(P) – *S.officinalis* L.(P), ratio1:1;
- AF 5→ *R. officinalis* L.(S) – *R. officinalis* L. (P) –*S.officinalis* L.(S) –*S.officinalis* L.(P), ratio1:1:1:1;

where(S)and(P)represent the extraction methods – Soxhlet and percolation respectively.

The total phenolic (TPC) and total flavonoid contents (TFC)

The total phenolic content expressed as mg GAE/g DW was spectrofotometrically determined using the same calibration curve achieved for gallic acid, previously presented, while the total flavonoid content expressed as mg rutin/g DW was determined based on the same calibration curve performed for rutin.

Following the analysis of experimental data the highest content of phenols and flavonoids was observed for the association formula 1 (AF 1). That is followed by the AF 3, AF 2, AF 4 and AF 5 (Table 3).

Table 3. The total phenolic content (TPC) and the total flavonoid content (TFC) of the association formulas

Association formulas	**Concentration (mg/mL)**	**Total phenolic content* (mg GAE / g DW)**	**Total flavonoid content* (mg RE / g DW)**
AF 1	15	236.38 ± 0.65	247.11 ± 0.79
AF 2	17	180.62 ± 2.44	190.31 ± 0.31
AF 3	20	212.07 ± 0.26	218.39 ± 0.73
AF 4	12	152.83 ± 2.01	151.27 ± 1.48
AF 5	16	136.78 ± 0.46	144.63 ± 1.25

*Each value is the mean of three replicate determinations ± standard deviation

DPPH free radical scavenging assay

The antioxidant activity expressed as mg quercetin equivalents / g DW, calculated based on the same calibration curve made for quercetin, is presented in the Table 4.The antioxidant activity expressed both as EC_{50}, as well as mg quercetin equivalents / g DW for the five association formulas is in the following descending order: AF 1, AF 3, AF 2, AF 4, AF 5.

Table 4.Antioxidant activity results for the association formulas

Association formulas	**EC_{50}* (mg/mL)**	**Quercetin equivalent* (mg/g DW)**
AF 1	0.030 ± 0.001	9.61 ± 0.013
AF 2	0.066 ± 0.002	7.10 ± 0.07
AF 3	0.061 ± 0.006	7.78 ± 0.04
AF 4	0.075 ± 0.001	6.94 ± 0.02
AF 5	0.082 ± 0.001	5.58 ± 0.01

*Each value is the mean of three replicate determinations ± standard deviation

As in the case of the total phenolic and total flavonoid contents, as well as in the case of the antioxidant activity values, the results confirm the data obtained for the individual extracts.

4. Conclusions

The study provides useful information with respect tothe antioxidant properties, the TPC and TFC and their correlation for three native medicinal plant species, processed by two methods: percolation and Soxhlet. Thescavenging activities of both types of extracts varied significantly and had a good correlation with the total phenolic and flavonoid contents. In conclusion, after analyzing allthe data obtainedfor the three plants, it can be noted that the *R. officinalis* extract obtained by Soxhlet method has the highestoxidationcapacity and the highest contentof phenolsandflavonoids. In general, the Soxhlet method used for the extraction of the interest compounds hashigherefficiency compared to the percolation process.

Based on the above results, several association formulas of the individual plant extractshave been elaborated, in order to obtain bio-preparations which have to respond to the target purpose.

Following their analysis in terms of the biochemically, it was found that association formula between *R. officinalis* L.extracts (S:P 1:1) presents enhanced antioxidant activity in comparison with other formulas.

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