The study of polyphenolic compounds profile of some *Rosmarinus officinalis* L. extracts

Neli-Kinga Olah[1,2], Gyongyi Osser[1], Ramona Flavia Câmpean[2], Flavia Roxana Furtuna[2], Daniela Benedec*[3], Lorena Filip[3], Oana Raita[4] and Daniela Hanganu[3]

[1]Vasile Goldi Western University of Arad, Faculty of Pharmacy, Rebreanu Street, Arad, Romania
[2]SC PlantExtrakt SRL, 407059, Rădaia, Cluj, Romania
[3]Iuliu Haieganu University of Medicine and Pharmacy, Cluj-Napoca, Faculty of Pharmacy, Creangă Street, Cluj-Napoca, Romania
[4]National Institute for Research and Development of Isotopic and Molecular Technologies, 1 Donath str, Cluj-Napoca, Romania

Abstract: The purpose of this study was to compare the polyphenol profile of different Rosemary (*Rosmarinus officinalis*) extracts obtained from dry and fresh herb and the evaluation of their antioxidant effect. There were studied the polyphenols from hydroalcoholic extracts (1:5- dry plant:solvent) obtained from fresh respectively dry plant and the gemmotherapic extract obtained from fresh plant (1:20- dry plant: solvent). The polyphenol profile was evaluated by UV-Vis spectrophotometry, TLC and HPLC. The total polyphenol respectively flavonoids content were determined by UV-Vis spectrophotometry. The antioxidant effect was evaluated by DPPH, ABTS, FRAP, CUPRAC and silver nanoparticle (SNP) methods. The hydroalcoholic extract obtained from fresh plant contains the highest concentration of total polyphenols, (0.601mg/ml rosmarinic acid), total flavonoids, (0.270mg/ml luteoline) and rosmarinic acid (0.350 mg/ml). The less concentrated is the gemmotherapic extract, but also the extraction ratio is higher than the hydroalcoholic extracts. The high content in polyphenols of the mentioned hydroalcoholic extract was confirmed by highest values of antioxidant activity: 39.1ml (DPPH), 7.7 ml (ABTS), 698mM ET/100 ml (FRAP), 1947 mM ET/100 ml (CUPRAC), 4570mM ET/100 ml (SNP). These differences in the polyphenols profiles show the importance of use the fresh plants for obtaining the good quality extracts.

Keywords: *Rosmarinus officinalis,* polyphenols profile, HPLC, TLC, antioxidant effect, dry and fresh plants.

INTRODUCTION

The Rosemary is a well-known culinary and aromatic herb, used by centuries for its aromatic property in preparing foods, but also for its well being and hepato-protective effects. In phytotherapy is used the dry Rosemary leaves, but culinary and in homeopathy are also used the fresh, young shoots. Today the Rosemary was re-discovered by food industry being used as food supplements, as preservative or aromatic herb. The re-discover of this herb is due also by the pharmacological studies performed on vegetal material and extracts that proved the Rosemary beneficial effects on the human's health, mainly on liver.

Being an aromatic herb the first bioactive compound class studied at this plant was the terpenes contained by volatile oil fraction: 1,8-cineole, alpha-pinene, camphor, camphene, borneol, bornyl acetate etc. (Jordan *et al*., 2013; Erkan *et al*., 2008; Zaouali *et al*., 2010). The studies demonstrate that the volatile oil from Rosemary is responsible for its antimicrobial activity (Bernardes *et al*., 2010), but also other bioactive compounds were identified in the leaves and herb. The Rosemary leaves contains diterpenes (carnisolic acid, isorosmanol) and triterpenes (ursolic and oleanolic acid) (Kontogianni *et al*., 2013).

**Corresponding author:* e-mail: dani_67ro@yahoo.com

Using the chromatographic methods ITLC, HPLC) in Rosemary leaves were identified polyphenols like: caffeic acid derivatives, e.g. rosmarinic acid; flavonoids, e.g. hesperidin, luteolin, cirsimarin, etc. The rosmarinic acid specific to Lamiaceae family, was for the first time identified in Rosemary. The studies demonstrates that the total polyphenol content of Rosemary is 2.19mg GAE/g fresh plant (Zheng and Wang, 2001) or 1.71mg GAE/100 g dry plant (Wojdylo *et al*., 2007) to 3.4 -5.8 until 162 mg GAE/g dry plant (Jordan *et al*., 2013; Erkan *et al*., 2008). The rosmarinic acid content was found to be from 116 to 367mg/100 g of dry plant and until 8% respectively 32.8 mg/100g fresh plant (Jordan *et al*., 2013; Erkan *et al*., 2008; Zheng and Wang, 2001). Other studies reveal a content of 12.45-406mg/100 kg dry plant of caffeic acid, 19-1081.5mg/100g dry plant of ferulic acid respectively 5.52-616mg/100g dry plant of luteolin and 6.11-43.8 mg/100 g dry plant of apigenin (Wojdylo *et al*., 2007; Jordan *et al*., 2013).

The high content of Rosemary in different polyphenols can be linked with its significant antioxidant potency that contributed to the preservative property and also to its hepato-protective effect. The antioxidant potency of Rosemary was evaluated by different chemical, spectral methods: DPPH, ABTS, FRAP, ORAC. For dry plant were obtained the following values: an IC_{50} value of 54mM to 151.5-597.9mg/ml determined by DPPH, 15.5-

540.4mM TE/g determined by ABTS respectively 6.62-20.6mM TE/g determined by FRAP (Zheng and Wang, 2001; Wojdylo *et al*., 2007; Jordan *et al*., 2013; Erkan *et al*., 2008).

The purpose of this study was to compare the polyphenol profiles of different Romanian Rosemary extracts to highlight the similarities and the differences between the extracts content respectively the differences between the fresh plant extracts and dry plant extracts.

MATERIAL AND METHOD

The vegetal raw material and the preparation of extracts

The Rosemary dry leaves were acquired by the Fares Bio Vital Laboratories Orastie (Hunedoara, Romania), being harvested from the organic cultures of the company. The Rosemary fresh young shoots were harvested from an organic culture near Oradea, Romania in 2013. The fresh vegetal material was botanical identified in the quality control laboratory of the company Plant Extrakt, Radaia, Cluj, Romania. A voucher sample was retained (Voucher no. 5679).

The tincture (T) from dry plant and the mother tincture (MT) from fresh plant were obtained by cold extraction (maceration) with 100 ml of 70% ethanol (1:5 - dry plant: solvent) according to method described in European Pharmacopoeia (Eur. Ph., 2015).

The glycerol macerate (GM) was obtained from the cut fresh young shoots with 96% ethanol and glycerol, according to method described in European Pharmacopoeia (1:20- dry plant: solvent) (Eur. Ph., 2015).

TLC analysis

The polyphenols were determined by thin layer chromatography using a silica chromatographic plate with fluorescence indicator at 254 nm. The mobile phase was dichloromethane (Merck) -acetone (Merck) - formic acid (Merck), in proportion of 85:25:8,5 v/v, saturated for 60 minutes. The used standards were rosmarinic acid, caffeic acid, luteolin and luteolin 7-glucoside, each having a concentration of 1mg/mL in methanol. It was applied 25µL from each sample and 10µL from each standard. The plate after development was dried at 100-105ºC, and then sprayed with a solution of 1% of aminoethanol diphenylborate in methanol (Neu Reagent) and then with a solution of 5% of polyethylene glycol 400in ethanol (PEG reagent). After 30 minutes, are observed the separated polyphenols in fluorescence at 365nm. By reaction with the spraying reagents the flavonoids are orange or yellow, the caffeic acid derivatives are blue or blue-green (Romanian Pharmacopoeia, 1993).

HPLC analysis

The HPLC analysis was performed in a Varian Star HPLC system with autosampler, tertiary pump and DAD detector. It was used a silica column C18-Luna 150 mm x 4.6 mm x 5 mm. The mobile phase was a tertiary gradient prepared from solution of water-acetic acid (90:10) (v/v), acetonitrile and water. The elution started with a linear gradient, beginning with 10% water-acetic acid (90:10) (v/v), 15% acetonitrile and ending at 75% water; isocratic elution followed for the next 10 minutes with 10% water-acetic acid (90:10) (v/v), 45% acetonitrile and 45% water, and at the end for the 15 minutes with 10% water-acetic acid (90:10) (v/v), 45% acetonitrile and 45% water. The flow rate was 1mL/min, the detection was performed at 330 nm and the injection volume was 10µL (Daraban *et al*., 2015). As standard was used rosmarinic acid and the calibration curve was drawn from 25 to 100 mg/mL.

UV-Vis spectrophotometric analysis

The total flavonoids were determined using aluminum chloride 2.5% at 430nm, according to Romanian Pharmacopoeia (Romanian Pharmacopoeia, 1993; Benedec *et al*., 2015; Mocan *et al*., 2015). As standard was used a solution of luteolin 0.05mg/mL in methanol. There was build a calibration curve from 0.004 to 0.016 mg/mL range.

The polyphenols were determined using sodium molybdate reagent at 505 nm (Romanian Pharmacopoeia, 1993; Benedec *et al*., 2015; Mocan *et al*., 2015). As standard was used the rosmarinic acid 0.05mg/mL in methanol. There was build a calibration curve from 0.05 to 0.3mg/mL range.

The antioxidant potency by DPPH method was performed at 517nm (Thaipong *et al*., 2006). At 5mL of 25mM DPPH solution in methanol is added 5mL of samples having different antioxidant (Rosemary extracts) quantities: 40-240mL. All mixtures were incubated for 30 minutes at 40ºC. There was prepared in the same manner also a reference solution using 5mL 25mM DPPH solution and 5mL methanol. As blank solution was used methanol. For each antioxidant quantity were determined the free DPPH radical inhibition percentage and from the curves built for each sample, quantity in function of inhibition percentage, were determined also the IC_{50} values. There was used the following formula to determine the inhibition percentage: $\%I = (A_r - A_s)*100/A_r$ where A_r is the absorbance of reference solution and A_s is the absorbance of the solutions with samples.

The antioxidant potency by ABTS method was performed at 734 nm (Arnao *et al*., 2001; Thaipong *et al*., 2006). At 1 part of 7.5mM ABTS solution in methanol is added 1 part of 2.6mM potassium persulfate solution in water and left to stand 12 hours at dark. Than 1mL of this mixture was mixed with 60mL of methanol and used for determinations, as ABTS reagent solution. At 15, 30 and 45mL from each extract was added 6mL ABTS reagent solution and incubated at room temperature, at dark for 2 hours. There was prepared in the same manner also a

Table 1: The TLC analysis

Samples	R_f value	Appearance fluorescence band
Luteolin 7-glucoside - standard	0,05	Pink-orange
Rosmarinic acid - standard	0,48	Blue-greenish
Caffeic acid - standard	0,78	Blue-greenish
Luteolin - standard	0,79	Pink-orange
T	0,05	Pink-orange
	0,08	Yellowish
	0,12	Pink-orange
	0,15	Bluish
	0,30	Yellow-bluish
	0,78	Blue
	0,82	Pink-orange
	0,93	Blue
	0,95	Pink-orange
	0,96	Blue
	0,99	Brown
MT	0,05	Pink-orange
	0,08	Bluish
	0,15	Yellowish
	0,29	Week blue
	0,48	blue-greenish
	0,77	Blue
	0,81	Pink-orange
	0,83	Pink-orange
	0,93	Green-bluish
	0,96	Yellow
GE	0,05	Pink-orange
	0,12	Pink-orange
	0,48	Blue-greenish
	0,75	Blue
	0,78	Brown
	0,85	Blue
	0,89	Blue
	0,93	Brown
	0,96	Brown

reference solution using methanol in place of the samples. As blank solution was used methanol. For each extract were determined the free ABTS radical inhibition percentage and from the curves built for each sample, quantity in function of inhibition percentage, were determined also the IC_{50} values. There was used the same formula as at DPPH method.

The antioxidant potency by FRAP method was performed at 593 nm (Benzie and Strain, 1996). At 2.5mL 10mM TPTZ solution in 40mM hydrochloric acid is added 2.5mL 20mM ferric chloride solution and 25mL acetate buffer at pH = 3.6. This mixture is the FRAP reagent. At 4mL from each extract were added water until 0.8mL and 6mL FRAP reagent. There was prepared in the same manner also a blank solution using water in place of the samples. In same manner was determined a curve for Trolox using 10 to 40mg of substance. It was determined the antioxidant capacity by calculate the mM Trolox equivalent/100mL extract.

The antioxidant capacity by CUPRAC method was performed at 450 nm (Ozyurek *et al*., 2012). At 1mL 7.5 mM neocupreine solution is added 1mL 10mM copper chloride solution and 1mL ammonium acetate buffer at pH = 6.8. This mixture is the CUPRAC reagent. To 4mL from each extract was added water until 1.1mL and 3mL CUPRAC reagent. The mixtures were incubated at room temperature for 30 minutes. There was prepared in the same manner also a blank solution using water in place of the samples. In same manner was determined a curve for Trolox using 11.4 to 45.6mg of substance. It was determined the antioxidant capacity by calculate the mM Trolox equivalent/100mL extract.

The antioxidant capacity by silver nanoparticle (SNP) method was performed at 423 nm (Ozyurek *et al*., 2012). At 2ml of SNP reagent obtained from 10mM silver nitrate solution and 1% trisodium citrate solution at boiling is added 4mL from each extract diluted with water until 0.8mL. The mixtures were incubated at room temperature

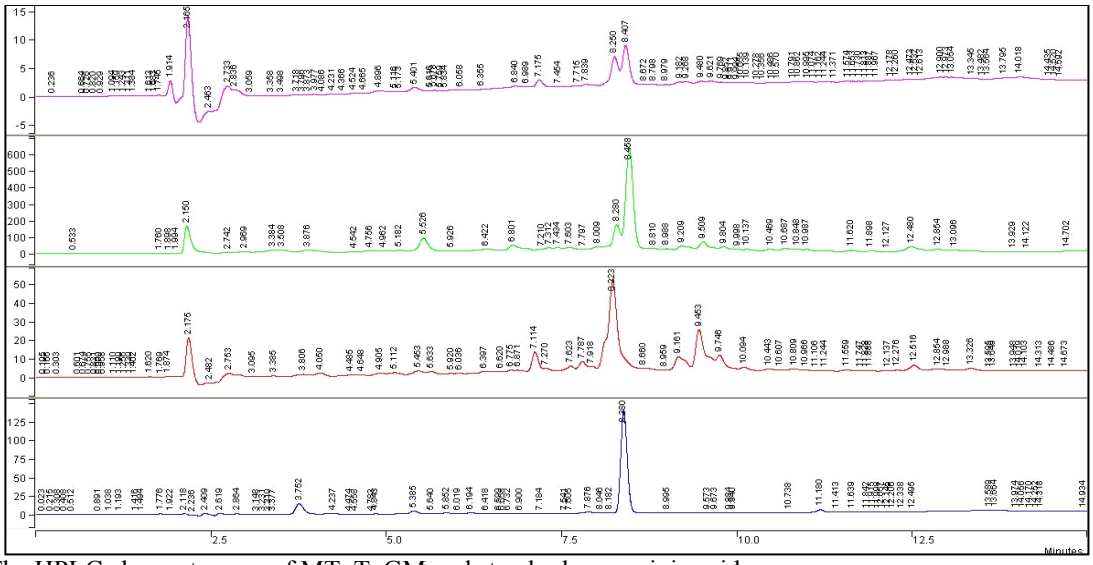

Fig. 1: The HPLC chromatogram of MT, T, GM and standard rosmarinic acid

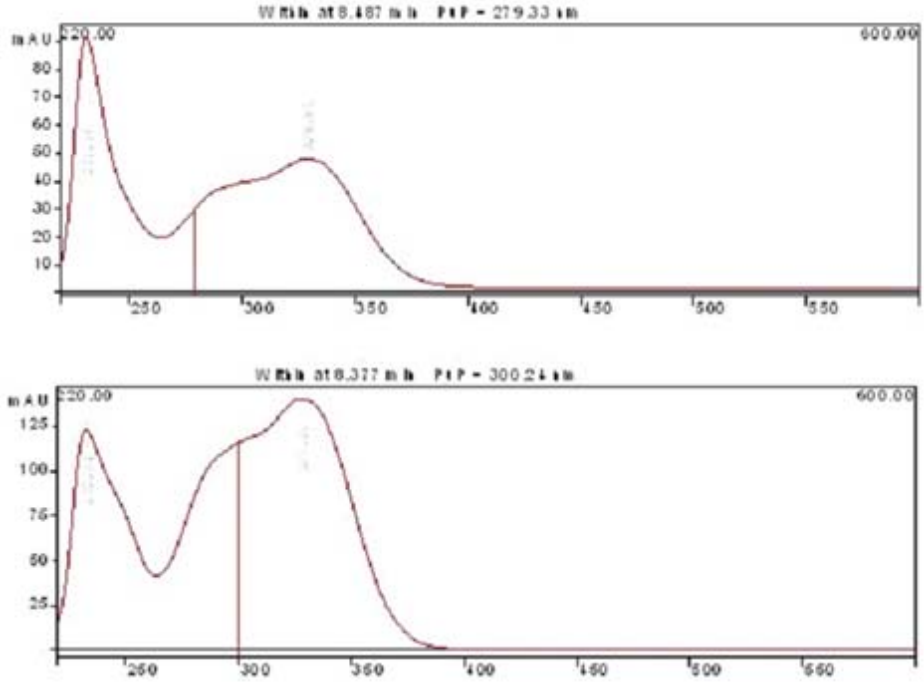

Fig. 2: The UV-Vis spectra of GM and standard rosmarinic acid.

for 30 minutes. There was prepared in the same manner also a blank solution using water in place of the samples. In same manner was determined a curve for Trolox using 22.8 to 182.4 mg of substance. It was determined the antioxidant capacity by calculate the mM Trolox equivalent/100mL extract.

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 perfomed using the Excel software program.

RESULTS

In table 1 are presented the TLC analysis data and the fig. 1-2 present the HPLC chromatograms and the obtained UV-Vis spectras.

The TLC analysis gives the possibility to compare the chromatographic fingerprint of the 3 studied extracts. It could be observed similarities, but also differences. There are similar bands on the upper part of chromatogram at the two hydroalcoholic extracts, the GM having different aspect. It could be identify by comparison with standards the luteoline, luteoline -7- glucoside and caffeic acid in all

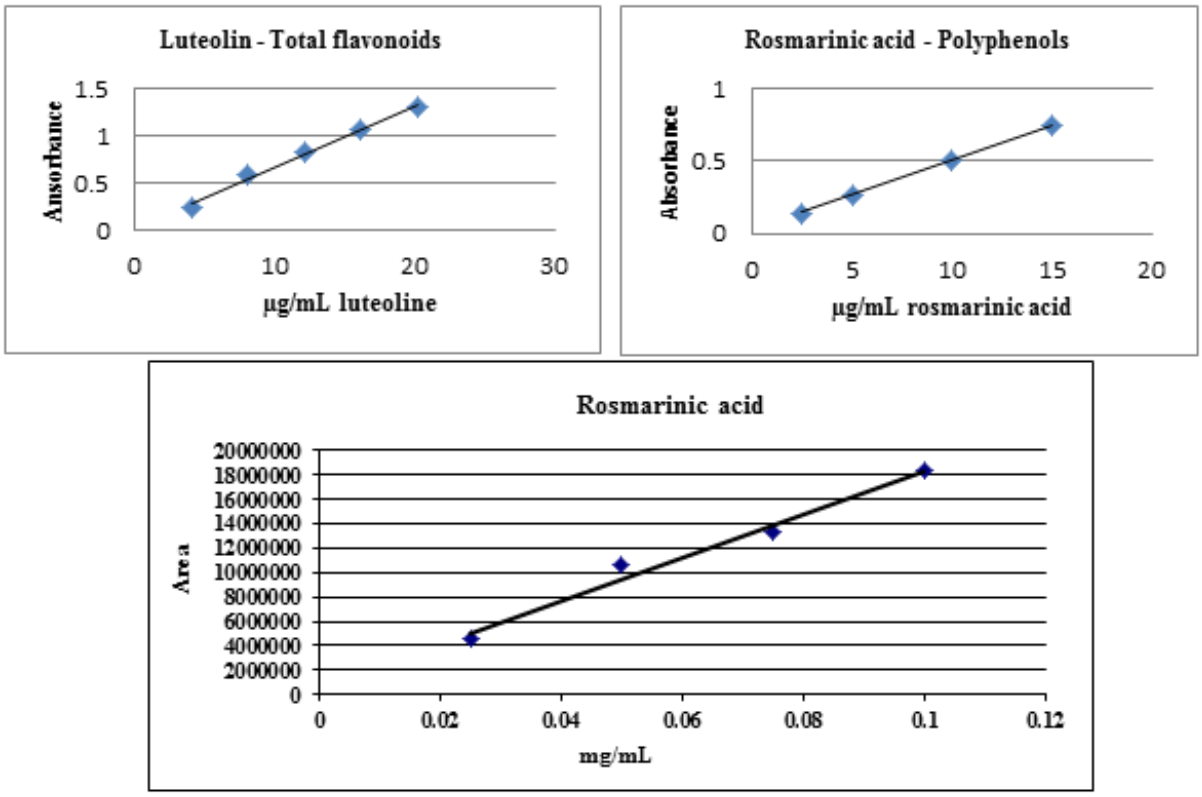

Fig. 3: The calibration curves for luteolin, rosmarinic acid (UV-Vis) and rosmarinic acid (HPLC).

Table 2: The quantitative determination of total flavonoids, polyphenols and Rosmarinic acid contents

Extract	Total flavonoids (mg LE/mL)	Polyphenols (mg RAE/mL	Rosmarinic acid (mg/mL)
Calibration curves	A = 0.0638*c + 0.033	A = 0.0483*c + 0.0231	A = $2*10^8$*c + 633444
Correlation factor	0.9934	0.9999	0.9804
T	0.133 ± 0.013	0.199 ± 0.021	0.180 ± 0.011
MT	0.270 ± 0.035	0.601 ± 0.035	0.350 ± 0.015
GM	0.013 ± 0.003	0.054 ± 0.005	0.013 ± 0.001

Notes: LE: Luteolin equivalents; RAE: Rosmarinic acid equivalents

Table 3: The antioxidant capacity determined by different methods

Extracts	DPPH IC_{50}, µL	ABTS IC_{50}, µL	FRAP, µM TE/100 mL	CUPRAC, µM TE/100 mL	SNP,µM TE/100 mlL
T	66	10.8	314	1260	785
MT	39	7.7	698	1947	4570
GM	103	113.8	310	1320	370

Note: TE: Trolox equivalents

extracts respectively the rosmarinic acid mostly in fresh plant extracts, MT and GE.

The HPLC analysis was used to identify with more accuracy the rosmarinic acid. This phenolic acid was identified in all extracts based on comparison of retention times and UV-Vis spectra of standard substance and the compound separated from each extract. The retention time of the standard rosmarinic acid is 8.4 min, the separated compounds from extracts being at 8.2-8.5min. The spectra maximal absorbances are at 233 and 328 nm for both standard and compound separated from extracts respectively the shape is characteristic and very similar.

In fig. 3 are presented the calibration curves obtained for luteolin and rosmarinic acid by UV-Vis spectrophotometry and used for quantitative determinations of total flavonoids and polyphenols respectively the calibration curve for rosmarinic acid obtained by HPLC. The results of quantitative determinations are presented in table 2.

From quantitative determinations can be observed that the highest concentration of total flavonoids, polyphenols and rosmarinic acid was found in the MT hydroalcoholic extract obtained from fresh plant, being with 2 or 3 times higher than the dry plant hydroalcoholic extract. These can lead to conclude that also the polyphenolic compounds suffer changes or degradations during the drying process.

The ratio of rosmarinic acid from the polyphenols was found to be different: 90% in the MT, 57% in the T and 24% in the GM. These differences could be due by the degradations, hydrolysis during the drying process and also to the different composition of extraction solvent. The extraction power of polyphenols done by glycerol is probably lower than the ethanol, and for this reason the GM has lower concentration of polyphenols than the hydroalcoholic extracts.

The polyphenol content of studied extracts can be associated to their antioxidant capacity. The antioxidant capacity was evaluated by 5 different methods and the results are presented in the table 3.

It can be observed a better antioxidant capacity for the fresh plant hydroalcoholic extract. The GM extract has a very good antioxidant capacity if we consider also the extraction ratio that is 1:20 in comparison with the hydroalcoholic extracts that have a ratio of 1:5. The antioxidant capacity is in direct relation with the flavonoids, polyphenols and rosmarinic acid contents. The significant antioxidant capacity of GM extract lead us to conclude that also other bioactive compounds, near the polyphenols, contribute to this effect.

DISCUSSION

The obtained values are comparable with those obtained in other studies. The total polyphenols are around 1mg/g dry plant in T, lower that those obtained by the researcher teams of Wojdylo (1.71 mg GAE/100 g dry plant), Jordan or Erkan -3.4 -5.8 until 162mg GAE/g dry plant (Wojdylo *et al*., 2007; Jordan *et al*., 2013; Erkan *et al*., 2008). Also the rosmarinic acid content is lower in case of dry plant 90mg/100g dry plant (T) in comparison with 116 to 367mg/100g of dry plant obtained by Jordan's and Erkan's teams (Jordan *et al*., 2013; Erkan *et al*., 2008).

In MT were found 3mg/g total polyphenols and 175 mg/100g plant rosmarinic acid. These values are higher than 2.19mg/g polyphenols respectively 8-32.8mg/100 g rosmarinic acid obtained by Zheng and Wang (Zheng and Wang, 2001).

The antioxidant capacity determinations shown higher effect in the studied extracts like those obtained by other researchers using DPPH method. The T, MT and GM has an IC_{50} determined by DPPH method of 39 to 103 ml in comparison with 151.5-597.9 mg/ml found in references (Zheng and Wang, 2001; Wojdylo *et al*., 2007; Jordan *et al*., 2013; Erkan *et al*., 2008). These differences could be due by the different sources of the vegetal raw material used in studies.

CONCLUSION

The study compared the polyphenols profile of 3 different Rosemary extracts, obtained by cold extraction, with different solvents, from fresh respectively dry plant. The results lead us to conclude that the more concentrated extract in polyphenols compounds is the hydroalcoholic extract (mother tincture) obtained at cold extraction, with 90 % vol. ethanol, from fresh plant. This good result was confirmed also by high antioxidant potency. Good results from this point of view presented also the gemmotherapic extract (GM) obtained at cold extraction, with a mixture of 96% ethanol and glycerol, also from fresh plant.

The use of cold extraction and the fresh plant lead us to obtain better Rosemary extracts from quality point of view, with higher antioxidant capacity that proposes the fresh plant extract for a better therapeutic effect. This study highlight the importance of choose the fresh Rosemary extracts for potential use as therapeutic more effective extracts.

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