International Journal of Pharmacology

ISSN 1811-7775

science alert

ansinet
Asian Network for Scientific Information

International Journal of Pharmacology 6 (4): 425-430, 2010
ISSN 1811-7775

The Obtaining of an Antioxidant Product Based on a *Rosmarinus officinalis* Freeze-Dried Extract

[1,2]V. Emanuel, [1,2]V. Adrian and [3]Niţă Sultana
[1]Applied Biochemistry and Biotechnology Center,
Biotehnol, Bd. Mărăşti No. 59, Sector 1, Bucharest, Romania
[2]Faculty of Biotechnology, University of Agronomic Sciences and Veterinary Medicine,
Bd. Mărăşti No. 59, Sector 1, Bucharest, Romania
[3]National Institute of Chemical-Pharmaceutical Research-Development,
ICCF Bucharest, Vitan Road No. 112, Sector 3, Bucharest, Romania

Abstract: The aim of this study was to obtain a freeze-dried extract with an important antioxidant and antimicrobial effect from *Rosmarinus officinalis*. The extracts were obtained in a fluidized bed solid extractor and there has resulted that the optimal values of phenols, flavonoids and the highest reduction power were determined when using 70% ethanol as solvent, the antioxidant activity ranging between 90-95%. In case of the freeze-dried product, the maximum antioxidant activity was registered when using 0.2 mg mL^{-1} powder and the results were optimal as well in case of determining flavonoids, phenols and the reduction power. The antimicrobial effect of the extracts and of the freeze-dried product were quantified by using the Colony Quant software on the strains *Escherichia coli* CBAB 2, *Bacillus cereus* CMGB 215, *Listeria innocua* CMGB 218. An inhibition area of maximum 2 cm resulted when using 70% ethanol as solvent. The optimal inhibitory effect of the freeze-dried product, of around 1.8 cm, was determined by a 0.2 mg mL^{-1} concentration.

Key words: Extraction, *Escherichia coli*, *Bacillus cereus*, *Listeria innocua*, colony quant

INTRODUCTION

Rosemary is a shrub originally coming from the Southern Mediterranean regions. It is used as an ornamental plant, for medicinal purpose and as a type of spice. The main use is that for medicinal purposes, by its extracts which may be found in various versions in pharmacies. It is a perennial shrub, usually with blue flowers. Rosemary contains a high quantity of iron, calcium and vitamin B6 (Satoh *et al*., 2008). The maximum height of the plant is of 125 cm. Its benefits are known since ancient times, being mainly used due to its effects on the nervous and circulatory systems. There has been demonstrated that the *Rosmarinus officinalis* extracts have antioxidant and antibacterial properties. Furthermore, they also have a high content of flavonoids, phenols and an important antioxidant activity.

At the brain level, it is considered that the main inhibitor of the free radicals is carnosic acid. It also reduces the risk of stroke and the occurrence of neurodegenerative diseases, mainly Alzheimer (Saber *et al*., 2006). Carnosic acid determines 90% of the antioxidant effect of this plant (Satoh *et al*., 2008). In addition to this compound, the rosemary extracts contain rosemary acid, caffeic acid, ursolic acid, rosmanol, isorosmanol, rosmadial (Caruso *et al*., 2000; Takahashi *et al*., 2009).

Currently, the rosemary products are recommended for digestive, liver and cardiovascular system diseases, for the improvement of the nervous system performance and to combat the effects of ageing. In addition to all these effects, these products have a general tonic effect (Charles *et al*., 2005).

Nowadays, the improvement of the extraction techniques, by using modern methods, represents an important research direction. These methods include the fluidized bed solid extraction and the supercritical fluid extraction. The consequences consist of the extracts improvement, the decrease of solvent quantities, a low material consumption and an optimal final price. The products obtained from these extractions are considered to have the highest antioxidant and antimicrobial activities (Carvalho *et al*., 2005; Knight, 2000).

The purpose of the research was to obtain a freeze-dried product from rosemary. In case of this extract, it was determined and characterized the antimicrobial

Corresponding Author: Vamanu Emanuel, Applied Biochemistry and Biotechnology Center, Biotehnol, Bd. Mărăşti No. 59, Sector 1, Bucharest, Romania

effect on strains with pathogenic potential for humans (*Escherichia coli* CBAB 2, *Bacillus cereus* CMGB 215, *Listeria innocua* CMGB 218). Another aspect of the research was to determine the free radicals scavenging activity, the total phenols and flavonoids contents. All these tests were performed on alcoholic extracts, as well as on the freeze-dried product, with the main goal of determining the optimum concentration of alcohol to obtain an effective antioxidant product. Another aim of the study was to determine the optimal concentration against the microbial strains.

MATERIALS AND METHODS

Obtaining the *Rosmarinus officinalis* extract: A quantity of 20 g of vegetal material was submitted to hydro alcoholic extraction, in ethanol 50, 70 and 90% (v/v) and it was separated in ultrapure water. The extractions and studies were realized in Biotehnol Center Laboratory, in 2009. The extraction was performed in a fluidized bed solid extractor fexIKA 200. To obtain a solid substance, the alcoholic extracts were concentrated in a rotary evaporator Buchi R 210, with vacuum controller, at the following parameters: 40°C, 175 mbar and 200 rpm (Carvalho *et al*., 2005). The elected concentrated solution was freeze-dried in an Alpha 1-2D freeze-drier, to obtain the solid substance. After the obtaining, the liquid and solid extracts were kept in a refrigerator at a temperature of 4°C. The extracts were named as follows: P1-simple extraction in water, P2-P1 concentrate, P3-simple extraction in ethanol 90%, P4-P3 concentrate, P5-fluidized bed extraction, using water, P6-P5 concentrate, P7-fluidized bed extraction, using ethanol 90%, P8-P7 concentrate, P9-fluidized bed extraction with ethanol 70%, P10-P9 concentrate, P11-fluidized bed extraction with ethanol 50%, P12-P11 concentrate.

Determining the total phenols content: The total phenolic content was determined by the Folin-Ciocalteu reagent. The vegetal extract (1 mg mL^{-1}) is mixed to 5 mL of Folin-Ciocalteu reagent (diluted to 1/10 in distilled water). Four mililiter of Na carbonate 7.5% are also added to the mixture. The mixture is shaken for a few seconds, then incubated for 30 min at 40°C. The absorbance is read at 765 nm using a Helios spectrophotometer. The total phenolic content was provided as the equivalent in mg/g to gallic acid (Colin and Bruce, 2003; Afolayan *et al*., 2008).

Determining the total contents of flavonoids: A mixture consisting of 0.5 mL $AlCl_3$ 2% in ethanol and 0.5 mL of vegetal extract is prepared. It is left for 60 min at room temperature and afterwards the absorbance is measured at 420 nm. The total flavonoid content was provided in mg/ml equivalent to quercetin (Afolayan *et al*., 2008; Krishna *et al*., 2010).

Determining the total antioxidant activity: The antioxidant activity was measured by determining the 1.1-diphenyl-2-Picrylhydrazyl (DPPH) free radical scavenging capacity. One hundred microliter of extract are mixed to 3 mL of ethanol solution of DPPH 0.004%. In 30 min, the absorbance is read at 517 nm (Afolayan and Jimoh, 2009; Rahman *et al*., 2008; Szabo *et al*., 2007).

Outlining the antibacterial capacity: *Escherichia coli* CBAB 2, *Bacillus cereus* CMGB 215, *Listeria innocua* CMGB 218 were used for the tests. Each strain was inoculated on a Petri plate on which there was poured LB-agar medium. Twenty microliter of vegetal extract were added and the plate was left to absorb the extract for 30 min. Afterwards, it was incubated at 28 to 30°C, for 24 h. The inhibiting zone was analyzed using the special Colony Quant software (Jazani *et al*., 2008; Puangpronpitag *et al*., 2009; Rozman and Jersek, 2009).

RESULTS

In the first phase of the researches, the extract was obtained by simple extraction in water and ethanol 90%. The results indicate that the sample P4 is the best, with a maximum antioxidant activity. Its value is comparable to the sample P3. At least from the point of view of the antioxidant activity, the values are approximately identical. It is important to note that by fluidized bed extraction, using water as solvent, there doesn't result an increase of the total antioxidant activity. In exchange, if ethanol is used as solvent for the fluidized bed extraction, the antioxidant activity increases simultaneously with the increase of the alcohol concentration. The differences between the results obtained with 70 and 90% ethanol are very small, below 10% (Fig. 1).

The largest quantity of phenols is obtained by fluidized bed extraction, using 70% ethanol as solvent. This time, it can be noticed that ethanol, notwithstanding the concentration, is a better solvent than water. Using water for extraction, the smallest quantity of phenols is obtained. Furthermore, due to the increased P9 concentration, the quantity of phenols increases as well (Fig. 2).

The largest quantity of flavonoids results in case of using 70% ethanol in fluidized bed extraction (Fig. 3). The obtained results are identical for the concentrated sample and for the one taken out of the extractor. For all samples,

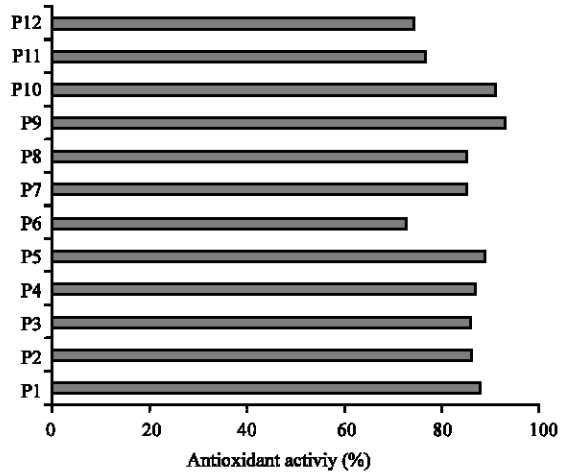

Fig. 1: Antioxidant activity of the *Rosmarinus officinalis* extracts

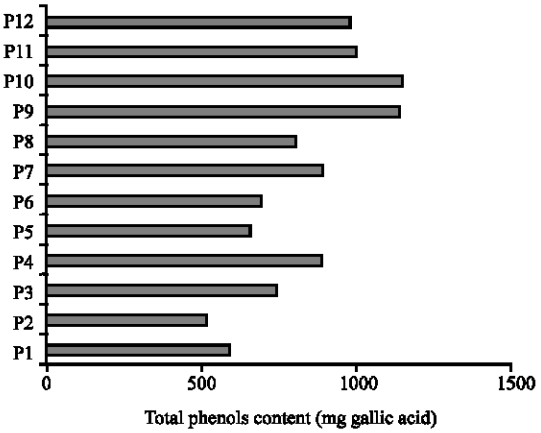

Fig. 2: Total phenols content of *Rosmarinus officinalis* extracts

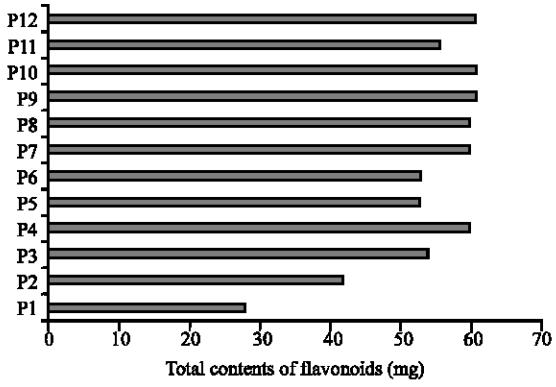

Fig. 3: Total contents of flavonoids of the *Rosmarinus officinalis* extracts

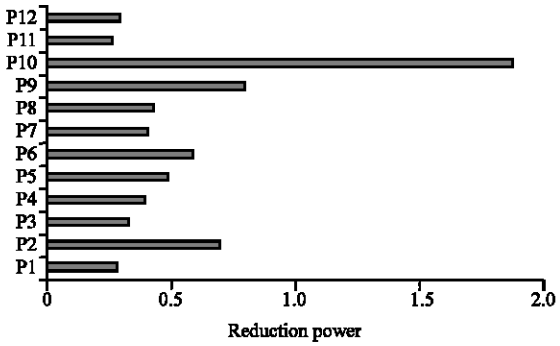

Fig. 4: Reduction power of the *Rosmarinus officinalis* extracts

it can be noticed that the concentration operation determines an increase of the flavonoid quantity or at least a maintenance thereof.

In case of the reduction power (Fig. 4), a clear difference is observed for the sample P10. It results the increase of more than 50% of the reduction power, proportional to the increase of the quantity of alcohol. However, a concentration of more than 70% ethanol doesn't generate the increase of the obtained values. In case of water, the use of the extractor increases the values.

Once the optimal ethanol concentration for the extraction, of 70%, is determined, a quantity of 250 mL of *Rosmarinus officinalis* extract is obtained in the extractor. The extract is inserted in a freeze-drying balloon of 500 mL and in 72 h the process is stopped, resulting freeze-dried *Rosmarinus officinalis* powder. In order to be used for obtaining pharmaceutical products, within a preliminary stage, the optimum concentration of the values of the chemical species will be determined. The powder is dissolved in pure ethanol, with a concentration of 0.1 and 0.2 mg mL^{-1}.

The maximum antioxidant activity, of 91%, is registered in case of using 0.2 mg mL^{-1} of freeze-dried powder. This is valid for the quantity of flavonoids and phenols as well. Furthermore, the reduction power has an increase of approximately 75%, namely it reaches 2.8, when using a concentration of 0.2 mg mL^{-1}, as compared to 0.1 mg mL^{-1} of freeze-dried product. The quantity of phenols and flavonoids increases by 50% when the quantity of freeze-dried product is doubled.

The antimicrobial effect of the obtained preparations was demonstrated against three microbial strains: *Escherichia coli* CBAB 2, *Bacillus cereus* CMGB 215, *Listeria innocua* CMGB 218, in parallel, by determining the diameter of the inhibition zone (cm) (Table 1).

Table 1: Antimicrobial effect of the *Rosmarinus officinalis* products

Sample	Diameter of the inhibition zone (cm)
Products effect against *Escherichia coli* CBAB 2	
P1	0.0
P2	0.0
P3	1.0
P4	1.0
P5	0.0
P6	0.8
P7	2.0
P8	1.9
P9	1.8
P10	2.0
P11	1.2
P12	1.0
Products effect against *Bacillus cereus* CMGB 215	
P1	0.7
P2	0.9
P3	1.7
P4	1.7
P5	0.9
P6	0.95
P7	1.2
P8	1.8
P9	1.4
P10	2.15
P11	0.9
P12	1.4
Products effect against *Listeria innocua* CMGB 218	
P1	0.8
P2	0.8
P3	1.6
P4	1.6
P5	0.0
P6	0.0
P7	1.5
P8	1.4
P9	1.3
P10	1.7
P11	0.8
P12	0.8

Generally speaking, the extracts (aqueous or alcoholic) of *Rosmarinus officinalis* have a powerful antimicrobial effect. The use of the fluidized bed extractor generates extracts with a higher antimicrobial effect. Vacuum concentration mainly leads to the increase of the inhibition zone. The extract with the highest antimicrobial effect is P9 and respectively, P10. The concentration causes an inhibition zone of at least 2 cm against *Escherichia coli* CBAB 2 and *Bacillus cereus* CMGB 215, as well as 1.7 cm for *Listeria innocua* CMGB 218. The ultrapure water used for the extraction is not an appropriate solvent. Furthermore, the alcohol concentration increased to 90% doesn't generate automatically more effective extracts.

The most pronounced antimicrobial effect is noticed against the *Bacillus cereus* CMGB 215 strain. The minimum diameter was of 0.7 cm, for simple extraction with ultrapure water. The fluidized bed extraction in 70% alcohol generates the tripling of the inhibition zone. Against *Listeria innocua* CMGB 218, under the same conditions, for simple extraction, the diameter is approximately similar, of 0.8 cm. In exchange, the extraction by 70% alcohol generates an increase of the diameter of the inhibition zone by approximately 55%. When using *Escherichia coli* CBAB 2, simple water extraction doesn't generate an inhibition zone. The extraction by 70% alcohol, in the extractor, generates a maximum diameter of approximately 2 cm. There is to be noted the fact that, upon extraction with 90% alcohol in the extractor, there result inhibition zones with identical diameters as when using 70% alcohol.

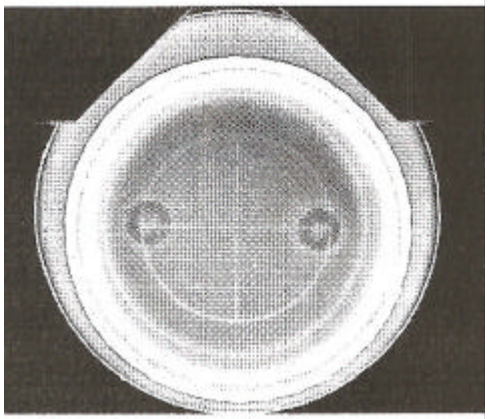

Fig. 5: Antimicrobial effect of the freeze-dried extracts of *Rosmarinus officinalis* (0.1 mg mL^{-1}-freeze-dried on the left and 0.2 mg mL^{-1}-freeze-dried on the right) against *Escherichia coli* CBAB 2

When testing the antibacterial effect of the freeze-dried powder, a diameter of at least 1.1 cm against the three strains was noted. The maximum antibacterial effect was determined against *Listeria innocua* CMGB 218, at a concentration of 0.2 mg mL^{-1} and it was of 1.8 cm. This determines an increase of the inhibition zone by approximately 30% higher than for the concentration of 0.1 mg mL^{-1} of freeze-dried powder. In case of *Escherichia coli* CBAB 2, the inhibiting effect is identical for both concentrations of freeze-dried powder (Fig. 5). For *Bacillus cereus* CMGB 215, the inhibiting effect increases by only 0.1 cm by doubling the concentration of freeze-dried powder to 0.2 mg mL^{-1}.

DISCUSSION

The fluidized bed extraction in the extractor, using 70% ethyl alcohol as solvent, represents the optimal variant. It determines the obtaining of an effective extract, both from the point of view of the antioxidant effect and from the point of view of the antimicrobial effect. In this

case, the largest quantity of phenols and flavonoids and the highest reduction power are obtained. The antioxidant activity, even if it doesn't have a maximum value, is high, ranging between 90 to 95%. These data are supported by the previous researches of Bjelakovic *et al.* (2008), Knight (2000) and Oguntibeju *et al.* (2009).

A maximum value is noticed for the antimicrobial effect as well, against all three bacterial strains used for the tests. The differences between the inhibition zones among the bacterial strains depend, thus, on the fact that certain strains may be sensitive to certain chemical compounds which are included in the extract. Others, such as *Listeria innocua* CMGB 218, are less sensitive. The findings by Rune (2005), Sang *et al.* (2008), Scarterzzini and Speroni (2000) and Stuckey and Osborne (2007) are in support of this result.

The maximum antioxidant effect in case of using freeze-dried powder of 0.2 mg mL^{-1} was of 91%, which is a high value. Very good results were obtained for the other chemical species which have been determined, resulting that this is the optimal concentration which can be used to obtain functional antioxidant products. The value is confirmed by the antimicrobial effect of the product, with an inhibiting effect of more than 1 cm against the three strains. The maximum inhibiting effect was manifested against *Listeria innocua* CMGB 218.

ACKNOWLEDGMENT

The researches were financed through a project PNCDI II No. 62-069/2008.

REFERENCES

Afolayan, A.J. and F.O. Jimoh, 2009. Nutritional quality of some wild leafy vegetables in South Africa. Int. J. Food Sci. Nutr., 5: 424-431.

Afolayan, A.J., O.M. Aboyade and M.O. Sofidiya, 2008. Total phenolic content and free radical scavenging activity of *Malva parviflora* L. (Malvaceae). J. Biol. Sci., 8: 945-949.

Bjelakovic, G., D. Nikolova, L.L. Gluud, R.G. Simonetti and C. Gluud, 2008. Antioxidant supplements for prevention of mortality in healthy participants and patients with various diseases. Cochrane Database Syst. Rev., 2: 71-76.

Caruso, J.L., J. Callahan, C. de Chant, K. Jayasimhulu and G.D. Winget, 2000. Carnosic acid in green callus and regenerated shoots of *Rosmarinus officinalis*. Plant Cell Rep., 19: 500-503.

Carvalho, R.N., S.M. Lucinewton, P.T.V. Rosa and M.A.A. Meireles, 2005. Supercritical fluid extraction from rosemary (*Rosmarinus officinalis*): Kinetic data, extract's global yield, composition and antioxidant activity. J. Supercritical Fluids, 35: 197-204.

Charles, L.C., L.R. Steven, M.N. Gillian, K.S. Brad and T.B. David, 2005. Seco-hinokiol, a new abietane diterpenoid from *Rosmarinus officinalis*. J. Nat. Prod., 68: 98-100.

Colin, D.K. and J.H. Bruce, 2003. The postprandial effects of dietary antioxidants in humans. Curr. Atheroscler. Rep., 5: 452-458.

Jazani, N.H., M. Zartoshti and S. Shahabi, 2008. Antibacterial effect of Iranian *Cuminum cyminum* essential oil on burn isolates of *Pseudomonas aeruginosa*. Int. J. Pharmacol., 4: 157-159.

Knight, J.A., 2000. Review: Free radicals, antioxidants and the immune system. Ann. Clin. Lab. Sci., 30: 145-158.

Krishna, K.L., K. Mruthunjaya and J.A. Patel, 2010. Antioxidant and hepatoprotective potential of stem methanolic extract of *Justicia gendarussa* burm. Int. J. Pharmacol., 6: 72-80.

Oguntibeju, O.O., A.J. Esterhuyse and E.J. Truter, 2009. Cardiovascular disease and the potential protective role of antioxidants. Afr. J. Biotechnol., 8: 3107-3117.

Puangpronpitag, D., N. Nianısa and C. Sittiwet, 2009. Anti-microbial properties of clove (*Eugenia caryophyllum* bullock and harrison) aqueous extract against food-borne pathogen bacteria. Int. J. Pharmacol., 5: 281-284.

Rahman, A., M.M. Rahman, M.M.I. Sheik, M.M. Rahman, S.M. Shadli and M.F. Alanı, 2008. Free radical scavenging activity and phenolic content of *Cassia sophera* L. Afr. J. Biotechn., 7: 1591-1593.

Rozman, T. and B. Jersek, 2009. Antimicrobial activity of rosemary extracts (*Rosmarinus officinalis* L.) against different species of *Listeria*. Acta Agric. Slovenica, 93: 51-58.

Rune, B., 2005. Dietary antioxidants and cardiovascular disease. Curr. Opin. Lipidol., 16: 47-54.

Saber, M., R. Bradbury and B.I. Murray, 2006. Comparative toxicity of *Rosmarinus officinalis* L. essential oil and blends of its major constituents against *Tetranychus urticae* Koch (Acari: Tetranychidae) on two different host plants. Pest Manage. Sci., 62: 366-371.

Sang, U.P., M.R. Uddin, H. Xu, Y.K. Kim and Y.L. Sook, 2008. Biotechnological applications for rosmarinic acid production in plant. Afr. J. Biotechnol., 7: 4959-4965.

Satoh, T., K. Kosaka, K. Itoh, A. Kobayashi and M. Yamamoto *et al*., 2008. Carnosic acid, a catechol-type electrophilic compound, protects neurons both *in vitro* and *in vivo* through activation of the Keap1/Nrf2 pathway via S-alkylation of targeted cysteines on Keap1. J Neurochem., 104: 1116-1131.

Scarterzzini, P. and E. Speroni, 2000. Review on some plants of Indian traditional medicine with antioxidant activity. J. Ethnopharmacol., 71: 23-43.

Stuckey, B.N. and C.E. Osborne, 2007. A review of antioxidant analysis in food products. J. Am. Oil Chem. Soc., 43: 228-232.

Szabo, M.R., C. Iditoiu, D. Chambre and A.X. Lupea, 2007. Improved DPPH determination for antioxidant activity spectrophotometric assay. Chem. Pap., 61: 214-216.

Takahashi, T., T. Tabuchi, Y. Tamaki, K. Kosaka, Y. Takikawa and T. Satoh, 2009. Carnosic acid and carnosol inhibit adipocyte differentiation in mouse 3T3-L1 cells through induction of phase2 enzymes and activation of glutathione metabolism. Biochem. Biophys. Res. Commun., 8: 549-554.