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BIOTECHNOLOGICAL PROCESSES FOR OBTAINING HERBAL ANTIOXIDANTS USEFUL IN FOOD INDUSTRY

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Abstract
A systematic scientific research for useful bioactivities from medicinal plants is now considered to be a rational approach in nutraceutical, drug research and food. A sustainable development of extractive biotechnological processes from herbal species can be achieved by classical method and also by supercritical fluid extraction(SFE). This paper is presenting the obtaining of some selective extracts from Rosmarinus officinalis L. species, with dual function of ingredients and antioxidants for food products. The fresh and dried leaves of Rosmarinus officinalis are frequently used as a food preservative and in traditional Mediterranean cuisine as a flavoring agent. Classical Soxhlet method and supercritical fluid extraction (SFE) method with CO_2 and ethanol cosolvent were applied for obtaining the Rosemary officinalis L. extracts. The herbal extracts have been investigated by UV-Vis spectroscopy for quantitative determination of the total polyphenols and flavonoids content, polyphenolcarboxylic acids and rosmarinic acid, according to Romanian and European Pharmacopoeia. In addition to this, four SFE extracts and four Soxhlet extracts were screened for their radical-scavenging capacities and antioxidant activities by various in vitro, non cellular assays, respectively chemiluminescence method in an luminol hydrogen peroxide system, and DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging. The results revealed excellent correlation between the antioxidant capacity and the amount of active constituents of extracts (rosmarinic acid, total polyphenols and flavonoids content, polyphenolcarboxylic acids).

Key words:*Rosmarinus officinalis, Antioxidant activity; Supercritical CO_2 extraction.*

INTRODUCTION

Natural antioxidants are frequently used in the pharmaceutical and food industry, and obtaining these biocompounds by innovative biotechnological processes is a continuing challenge to replace synthetic food additives. Synthetic antioxidants such as butylhydroxy-anisole (BHA) and dibutylhydroxytoluene (BHT) are often and quite efficiently used in food processing. It is important to consider that there are some restraints to their use because of the evidence that they may be harmful to human health. Due to these considerations, obtaining of natural antioxi-dants represents a priority not only to prevent food degradation, but also to achieve additives and ingredients non toxic for use in the pharmaceutical and cosmetic industries (Kikuzari and Nikatani 1993), (P. F. Leal, et al. 2003).

Rosemary (*Rosmarinus officinalis* L.) is a spontaneous shrub, growing in all Medite-rranean countries. It is a herbal species frequently used as a food preservative and, in traditional Mediterranean cuisine, as a flavoring agent (Angioni, et al. 2004), Sotelo - Félix, et al. 2002). Also, *Rosmarinus officinalis* L. has been used as a medicinal herb due to its pharmacological actions: hepatoprotective (Sotelo - Félix, et al., 2002), antimicrobial (Del Campo, Amiot and Nguyen-The, 2000; Bozin, et al., 2007), antithrombotic (Yamamoto, et al. 2005), diuretic (Haloui, et al. 2000), antidiabetic (Bakirel, et al. 2008), anti-inflammatory (Altinier, et al. 2007), antioxidant (Perez-Fons, Garzon and Micol 2010) and anticancer (Lo, et al. 2002; Dörrie, Sapala and Zunino, 2001; Huang, et al., 2005; Visanji, Thompson and Padfield 2006).

These potent biological activities, including antioxidant properties have been assigned to the presence of many bioactive compounds in its composition. Phenolic acids, flavonoids, hydroxycinnamic acid derivatives and polyphenol-carboxilic acids are considered as the main dietary phenolic compounds. In addition to this diversity, polyphenols may be associated with various carbohydrates and organic acids (Manach, et al. 2004). These compounds exhibit a wide series of therapeutical properties, such as antimicrobial, anti-atherogenic, anti-inflammatory, antithrombotic, anti-allergic, cardioprotective, vasodilatory effects and antioxidant(N. Balasundram 2006).The major types of compounds found in rosemary are phenolic diterpenes including: carnosic acid, carnosol or rosmanol; flavonoids such as genkwanin, cirsimaritin or homoplantaginin; and triterpenes such as ursolic acid (Bai, et al. 2010; Bicchi, Binello and Rubiolo 2000; Del Baño, et al. 2004). The most well-studied bioactive compounds of *Rosmarinus officinalis*L, are carnosic acid (Figure 1a), caffeic acid (Figure 1b) and its derivative, rosmarinic acid (α-o-caffeoyl-3,4-dihydroxyphenyllactic acid) (Figure 1c). These compounds are thought to have biological and antioxidant significant properties and are under investigation as potential therapeutics for different illness. (Perez-Fons, Garzon and Micol 2010).

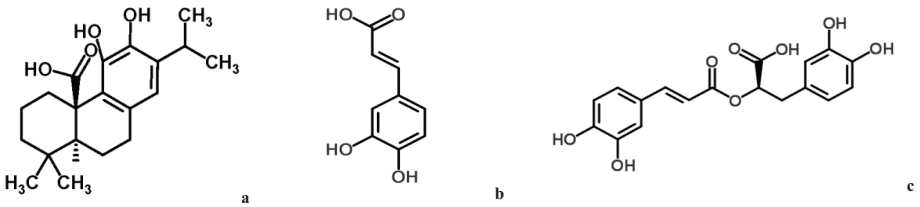

Figure 1. Chemical structure a) carnosic acid, b) caffeic acid. c) rosmarinic acid

In this regards the present paper is focused on key trends in the biotechnological production of antioxidants obtained by processing hydroalcoholic extracts of *Rosmarinus officinalis* L. It presents a new experimental design, applying classical Soxhlet extraction and supercritical fluid extraction SFE, and processing hydroalcoholic extracts, in order to achieve some antioxidants useful in the food industry. Bioactive compounds such as polyphenols, flavonoids and phenolic diterpenes from plant sources have been traditionally extracted by a conventional solid-liquid extraction or Soxhlet. Several studies shows new promising extraction methods are arising, which introduce some form of additional energy in order to facilitate extraction process of chemical compounds in a faster way (Garcia-Salas, et al. 2010). Such a method is the supercritical fluid extraction (SFE) using different types of co solvent at high pressure, or supercritical fluids such as CO_2. SFE has received much attention in the past several years, especially in food, pharmaceutical and cosmetic industries, because it presents an ecological alternative for conventional processes which use toxic organic solvents. An important disadvantage in the use of supercritical CO_2 is its low polarity, making the extraction of polar analytes quite difficult. However this limitation can be solved by adding of solvents such as methanol or ethanol which have the function of polar modifiers, to increase supercritical CO_2 solution power (Jian Bo Xiao 2007.)

MATERIALS AND METHODS

Plant materials. The *Rosmarinus officinalis* L. species are commercial samples, obtained from FARES: S.C. Romania. The dried leaves were stored in dark at 4ºC, for 10 days. Just before the extraction process by Soxhlet and supercritical fluid extraction (SFE) method, the leaves were ground in a blender, to produce a powder with an approximate size of 0.5 mm.

Chemicals. Aluminium chloride, sodium acetate, Folin-Ciocalteu phenol reagent, Arnow reagent, 1-diphenyl-2-picrylhydrazyl, 3-aminophthalhydrazide, hydrogen peroxide, rutin, quercetin, gentisic acid, caffeic acid,

syringic acid, gallic acid, rosmarinic acid, ascorbic acid were purchased from Sigma-Aldrich.

Solvents: Ethanol, Methanol, Acetone, Ethyl acetate, HCl, DMSO (Merck, analytical grade), ultrapure water (Millipore water system). Carbon dioxide 99.8% Linde Group Romania.

Equipments. Soxhlet extraction system, Supercritical Fluid Extraction System SFT-150, (Supercritical Fluid Technology, SUA)

Spectrophotometer UV-Vis, Jasco, Japan V-570 for DPPH method and quantitative determination of flavonoids, polyphenols, polyphenolcarboxylic acids and total hydroxycinnamicderivates, (Romanian Pharmacopoea, edition X 1993; Ciulei, et al. 1995)

Chemiluminometer (Sirius Luminometer Berthelot - GmbH Germany): for *antioxidant activity* measurements by chemiluminescence tehnique (CL).

Soxhlet extraction (SHE)

Plant material was extracted in a Soxhlet extraction apparatus, using 100g of dried and ground herbs (approximate size of 0.5 mm) in 1000 ml solvent1:1 (v/v) mixture of ethanol (99.8%, P.A., Merck) and ultrapure water (Millipore water system). The heating power was set to 50°C and the extraction was achieved within 3 h.

Supercritical fluid extraction (SFE)

Rosmarinic extracts were obtained using CO_2 (99.8%, food grade, Linde Group Romania), 20% [wt] of co-solvent, a 1:1 (v/v) mixture of ethanol (99.8%, P.A., Merck), and ultrapure water (Millipore water system) at 50 °C, and pressures of 200 and 300 bars. The CO_2 was admitted into the system at a flow rate of $6x\ 10^{-5}$ kg/s, up to the point where no solute was observed at the exit of the column (60 min). Considering that CO_2 behaves as a supercritical fluid above its critical temperature (304.25 K) and critical pressure (72.9 atm or 73.8659 bar/ 7.39 MPa), the experiment was run at pressures between ~~of~~ 200 and 300 bars and temperatures of 50 °C (Rodrigues, et al. 2002; Leal, et al. 2003).

The technological processing for antioxidants obtaining

The crude extract solutions obtained by Soxhlet and SFE method were processed, through a succession of technological steps consisting in vacuum concentration until obtaining a residue which was passed through successive precipitations with polar and non-polar solvents, centrifugation, filtering at low pressure and purification. Operational parameters specific to each stage mentioned above are presented in Table 1,2.

Table 1. Processing the extract of *Rosmarinus officinalis* L. obtained by the Soxhlet method (SHE)

Samples	RH1	RH1	RH3	RH4
Temperature of concentration (°C)	30	40	50	60
Speed of concentration (rpm)	100	200	300	400
Solvent of precipitation	Acetone	Ethanol	Ethyl acetate	Acidulated ethanol
Ratio of precipitation (w/v)	1:10	1:15	1:2	1:10
Time of centrifugation (min)	20	30	40	45
Speed of centrifugation (rpm)	3000	6000	3000	6000

Table 2. Processing the extract of *Rosmarinus officinalis* L. obtained by Supercritical fluid extraction method (SFE)

Samples	RS1	RS2	RS3	RS4
Temperature of concentration (°C)	30	40	50	60
Speed of concentration (rpm)	100	200	300	400
Solvent of precipitation	Acetone	Ethanol	Ethyl acetate	Acidulated ethanol
Ratio ofprecipitation (w /v)	1:10	1:15	1:2	1:10
Time of centrifugation (min)	20	30	40	45
Speed of centrifugation (rpm)	3000	6000	3000	6000

It may be noted that manufacturing processes are similar and the corresponding operational parameters of each stage are identical, the only difference between the two sets (RH and RS) of samples is the extraction method: SHE respectively SFE.

Powders obtained in the processing steps were dried at room temperature, washed successively with ethanol, to remove toxic compounds and then allowed to completely dry. The washing operation was repeated three times. Each of the eight obtained yellowish - white powders, was milled to a very fine consistency (RH1, RH2, RH3, RH4 – Soxhlet method and RS1, RS2, RS3, RS4- Supercritical fluid extraction method)

Chemical analysis of the samples

The quantitative determinations of the flavonoids, polyphenols, polyphenolcarboxilic acids and total hydroxycinnamic derivativeswere done according to the FR X and the

European Pharmacopoeia Ed. 6.0, Rosemary leaf Monography (Romanian Pharmacopoea, Edition X, 1993). All measurements were repeated three times.

Antioxidant activities

CL method. The antioxidant activity (AA%) of the samples (RH1, RH2, RH3, RH4 – Soxhlet method and RS1, RS2, RS3, RS4- Supercritical fluid extraction method) has been determined and compared with that of pure standards: rutin, quercetin, gentisic acid, caffeic acid, syringic acid, gallic acid, rosmarinic acid, ascorbic acid. Chemiluminescence method (CL) was applied using luminol-H_2O_2 as generator system, in TRIS-HCl, buffer pH= 8.4, using Sirius Luminometer Berthelot - GmbH Germany. The antioxidant activity of samples was calculated using the following relation (Iftimie Badea N., 2004; Del Baño, et al. 2004)

$$AA\,\% = \frac{I_0 - I}{I_0} \cdot 100$$

where: I_0 = the maximum CL for standard at t=5 s; I = the maximum CL for sample at t =5 s.

DPPH Method. Quantitative evaluation of radical scavenging abilities (SR%) of the samples was carried out by an adapted DPPH method (Sanja Matić 2013). Sample stock solutions (1.0mg/mL) were diluted to final concentrations of 250, 125, 50, 25, 10 and 5 g/mL, in methanol. One mL of a 0.3 mM solution of DPPH in methanol was added to 2.5 mL of sample solutions of different concentrations, and allowed to react at room temperature. After 30 min the absorbance values were measured at 518 nm and converted into the percentage of radical-scavenging activity (SR%) using the following formula:

$$SR\% = 100\left(1 - \frac{Abs_{sample} - Abs_{blank}}{Abs_{control}}\right)$$

Methanol (1.0 mL) plus herbal extract solution (2.5 mL) was used as a blank. DPPH solution (1.0 mL; 0.3 mM) plus methanol (2.5 mL) was used as a negative control.

RESULTS AND DISCUSSIONS

The application of Soxhlet extraction is ordinary, but supercritical solvent extraction for the preparation of natural antioxidants has, until now, been limited. The procedure was used for the extraction of rosemary and sage leaves. Propane, butane, methanol, ethanol may be used as co-solvents for improving yield or selectivity. Supercritical fluid extraction allows a continuous modification of dissolution power and selectivity by changing the solvent density. An essential drawback in the use of supercritical CO_2 is its low polarity, making the extraction of polar analytes difficult. This limitation may be overcame by adding small amounts of polar modifiers, such as methanol or ethanol to the supercritical CO_2, in order to increase its solution power. In the present study, the modifier ethanol enhanced the solubility of solutes in supercritical CO_2 and thus the efficiency of extraction increased, as demonstrated by the phytochemical characterization of the obtained bioproducts. (Mühlnickel T 1992; Djarmati Z 1991; Jian Bo Xiao 2007; Mühlnickel 1992).

Chemical analysis

Phytochemical analysis showed that SFE method is more efficient for extraction of the compounds of therapeutic interest. Thus, there is a significant increase (46.2%-57%) of the amount of flavonoids, polyphenols, polyphenolcarboxilic acids and total hydroxycinnamic derivatives (Figure 2,3,4,5). All the extract samples obtained by SFE, and further processed in the same conditions as the extract samples obtained by Soxhlet method, led to much higher amounts of biological active compounds, comparing to those obtained from the Soxhlet extracts.

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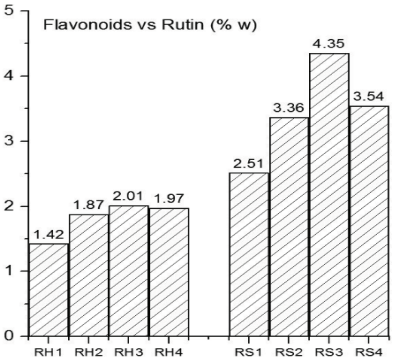

Figure 2. Flavonoids content, mass % (as rutin)

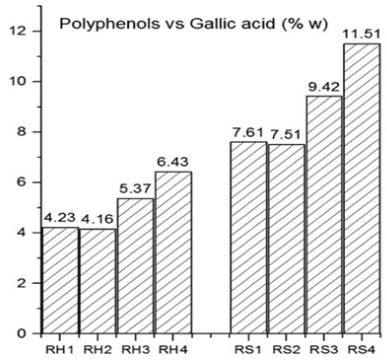

Figure 3. Polyphenols content, mass % (as gallic acid)

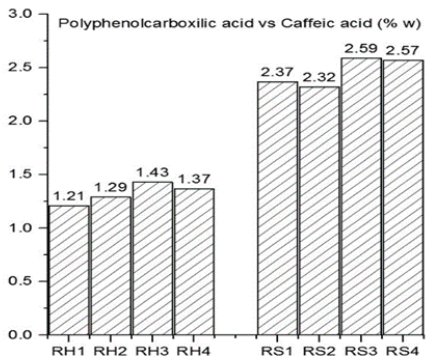

Figure 4. Polyphenolcarboxilic acids content, mass % (as caffeic acid)

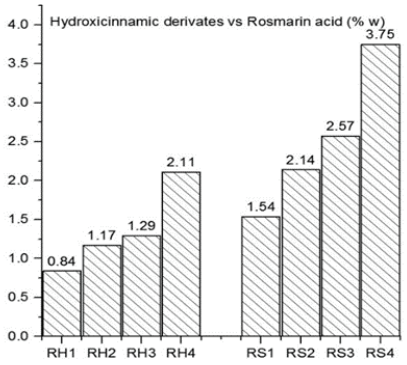

Figure 5.Hydroxycinnamic derivatives content, mass % (as rosmarinic acid)

Antioxidant activities

Antioxidant activity for the two sets of analyzed samples (RH set - Soxhlet extraction and RS set - supercritical fluid extraction with CO_2 solvent), demonstrated by both methods, DPPH and CL, reached high values, ranging from 92.78% - 97.01% for DPPH and 94.23% - 98.94% for CL.

The choice of optimal processing parameters, in conjunction with the method of extraction (SFE), led finally to obtaining of selective extracts, enriched in active principles (flavonoids, polyphenols, polyphenolcarboxilic acids and total hydroxycinnamic derivatives) which present a very high antioxidant activity (98.94% $_{CL}$, 97.01% $_{DPPH}$).

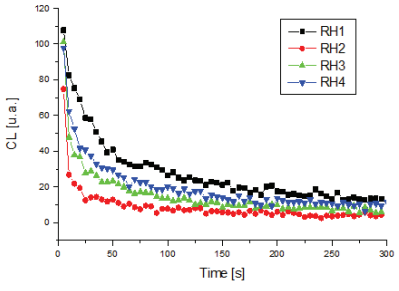

Figure 6. CL evolution in time of RH1, RH1, RH1, RH4 samples

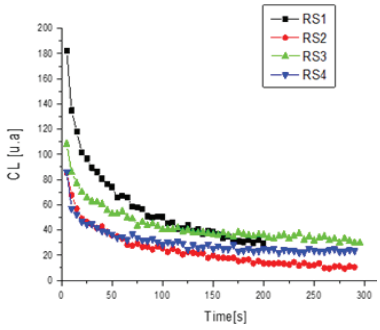

Figure 7. CL evolution in time of RS1, RS2, RS3, RS4 samples

Table 3.Antioxidant activity and kinetics parameters

No.crt	Samples code	Extraction method	$k_i(s^{-1})$ [1]	$v_i\ (s^{-1})$ [2]	AA%
1	RH1	SHE	0.089	243.87	94.23
2	RH2	SHE	0.101	119.15	97.22
3	RH3	SHE	0.097	115.72	96.14
4	RH4	SHE	0.064	480.27	96.92
5	RS1	SFE	0.098	432.74	97.28
6	RS2	SFE	0.115	342.96	98.94
7	RS3	SFE	0.086	427.64	96.81
8	RS4	SFE	0.109	456.11	98.17
9	R[a]	-	0.114	291.80	78.61
10	Q[b]	-	0.107	114.83	96.36
11	GE[c]	-	0.079	398.42	58.93
12	CA[d]	-	0.067	317.14	83.17
13	SA[e]	-	0.112	609.18	64.31
14	GL[f]	-	0.057	169.27	85.60
15	RS[g]	-	0.098	283.42	89.11
16	AS[h]	-	0.094	467.18	99.50

[1] k_i: rate constant, [2] v_i: reaction rate indexed from the chemiluminescence curves; a: Rutin, b:Quercetin, c:Gentisic acid, d:Caffeic acid, e:Syringic acid, f:Gallic acid, g:Rosmarinic acid, h:Ascorbic acid

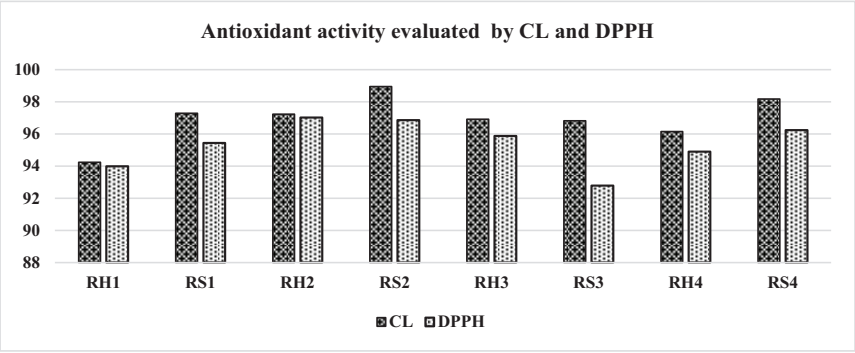

Figure 8. Evaluation of antioxidant activity by CL and DPPH method

CONCLUSIONS

In this work, two extraction methods were applied, SFE and Soxhlet, and the obtained extracts were processed by identical successive steps, obtaining herbal antioxidants with multiple functions: ingredients, flavors, additives and preservatives.

The study led to the development of an obtaining procedure of an antioxidant herbal product, including extraction and further processing steps, as a cost-effective, cleaner and more environmental friendly biotechnological processes than those based on conventional extraction methods. The antioxidants properties of the selective extracts, useful as food additives, are also emphasized. Investigation by CL and DPPH has revealed very high values of antioxidant capacity and phytochemical analysis revealed greater amounts of flavonoids, polyphenols, polyphenolcarboxilic acids and total hydroxycinnamic derivates, when extraction is achieved by SFE method, which is a cost-effective technique in laboratory scale, with possibility to be extended at industrial scale. The advantages of SFE with CO_2, including: avoidance of toxic solvents, low operating temperature (no thermal degradation of most of the labile compounds), high selectivity and fast extraction (Jian Bo Xiao 2007), were demonstrated. Last, but not least, it should be pointed out the excellent correlation between the antioxidant capacity and the amount of active constituents of extracts (rosmarinic acid,

total polyphenol and flavonoids content, polyphenol carboxylic acids).

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