The Annals of the University Dunarea de Jos of Galati
Fascicle VI – Food Technology (2015), 39(2), 77-87

ORIGINAL RESEARCH PAPER

Presented at the 7th International Symposium EuroAliment 2015

ANTIOXIDATIVE ACTIVITY AND STABILITY OF THE EXTRACTS OF LIQUORICE ROOT (*GLYCYRRHIZA GLABRA*)

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Received on 3rd September 2015
Revised on5thNovember 2015

The active principles from the aqueous liquorice plant extracts were investigated and quantified by evaluation of bioactive compounds (saponins) through phytochemical reactions. The presence of saponins was evaluated by measuring the foam index, which was around 500. A major component was Glycyrrhizic acid, responsible for the antioxidant activity, found in concentration of 5.82 % at plant maturity. A time-dependent decrease in concentration of the bioactive compounds from aqueous liquorice extracts was observed. The antimicrobial activity of the extracts was tested by the agar diffusion method, showing a moderate inhibitory activity against *Bacillus* sp. and strong inhibitory activity against coliforms. A liquorice syrup was obtained and subsequently could be used as nutraceutical additive in bread with good results, showing characteristic, optical and antimicrobial properties and good stability in time. Adding liquorice syrup in food products could be an alternative to improve nutraceutical potential.

Keywords: *Glycyrrhiza glabra*, liquorice, active principles

Introduction

The glycyrrhizic acid is one of the main biologically active components from liquorice, responsible for the sweet taste of the product and the expectorant, anti-inflammatory, diuretic, bacteriostatic, healing, anti-ulcer actions (Asl *et al.*, 2008, Patil *et al.*, 2009). The roots and rhizomes of liquorice (*Glycyrrhiza*) species have long been used worldwide as a herbal medicine for the treatment of different diseases and as natural sweetener. A large number of components have been isolated from liquorice, including triterpene saponins, flavonoids, isoflavonoids

and chalcones, with glycyrrhizic acid normally considered the main biologically active component (Asl *et al.*, 2008). To contribute to the understanding of the mechanism underlying the beneficial effects of liquorice, the antioxidant, free radical-scavenging and immunostimulating effects of a liquorice infusion were investigated and two major components were identified as liquiritin and glycyrrhizin (Cheel *et al.*, 2010). The roots and rhizomes of *Glycyrrhiza glabra* (*G. glabra*) have been employed clinically for centuries for their anti-inflammatory, antiulcer, expectorant, antimicrobial and anxiolytic activities. Many studies have been focused on the active principles of *G. glabra* and on obtaining pharmaceutical products (Ambawade *et al.*, 2002, Kamei *et al.*, 2005). *G. glabra* and its phytoconstituents have been known to possess widespread pharmacological properties as an anti-inflammatory, anti-viral, antitumour and hepatoprotective drug. *G. glabra* (almost devoid of glycyrrhizin) exhibits anti-inflammatory properties likely through the inhibition of PGE_2, $TXB_2$and LTB_4 in mammalian cell assay system, which could be influenced in part by glabridin and isoliquiritigenin (Chandrasekaran *et al.*, 2011).

The traditional use of liquorice is to treat and prevent diseases in which oxidants or free radicals are involved and suggest that liquorice infusion could be used as a potential non-specific immune stimulator. Many studies were carried out to optimize the conditions for the extraction of antioxidative materials from liquorice root, *Glycyrrhiza glabra*. 95% ethanol gave the highest free radical scavenging activity (Kim *et al.*, 2006).

The effects of aqueous extract of *G. glabra*, popularly known as liquorice, on depression in mice was reported (Dhingra *et al.*, 2006). The dose of 150 mg/kg of the aqueous extract of liquorice significantly improved the learning and memory of mice (Dhingra *et al.*, 2004). Also, the anti-inflammatory and antioxidant properties of liquorice may be contributing favorably to the memory enhancement effect. The antidepressant-like effect of the liquorice extract seems to be mediated by an increase of brain norepinephrine and dopamine, but not by the increase of serotonin. The monoamine oxidase inhibiting effect of liquorice may be contributing favorably to the antidepressant-like activity (Dhingra *et al.*, 2006).

Liquorice root is a traditional medicine used mainly for diseases and experimental studies suggest that it has several other useful pharmacological properties such as antiinflammatory, antiviral, antimicrobial, antioxidative, anticancer activities, immunomodulatory, hepatoprotective and cardioprotective effects (Fiore *et al.*, 2005, Kamei *et al.*, 2005). Several studies indicate that *Glycyrrhiza liquorice* can be used as ingredient in food products to improve their nutraceutical potential due to the presence of various antioxidant compounds in the liquorice roots (Isbrucker *et al.*, 2006, Tohma *et al.*, 2010).

The aim of our work is to study the active principles of *Glycyrrhiza glabra* responsible for the antioxidant properties of liquorice. Moreover, the possibility of obtaining a liquorice syrup to be used in food products as a source of antioxidant compounds was also investigated. This syrup could be used as an additive in bread to improve its nutraceutical potential.

Materials and methods

Plant material

The liquorice sample (A) was the powder of its own rearing. The plant material of liquorice (*Glycyrrhiza glabra*) powder involved roots of this plant collected from a greenhouse in Galati, in dry and sunny time (March 2015). Initially, the roots were dried and grinded using an electric grinder. They were triturated until a homogenous powder was obtained and it was passed through a fine sieve. 5 g of homogenous powder were dried in a drying stove at 100±5°C for 3 hours to evaluate the humidity of the samples. The liquorice powder was calcined at 800°C for 1 hour to measure the total ash. The soluble substances from the liquorice roots were evaluated by extracting 5 g of plant material through maceration for 24 hours in distilled water. Afterwards, the solvent was evaporated to dryness and the obtained residue was dried in a drying stove at 100±5°C for 3 hours (European Pharmacopoeia, 2007). The dry roots powder was kept at constant temperature (15°C) for further chemical and physico-chemical evaluation, antimicrobial activity test and to obtain a liquorice syrup. The second sample (obtained from a medicinal herb shop-Plafar-B), used only for glycyrrhizic acid determination, was a commercial product with a specified liquorice content (80 g of liquorice roots).

Chemical and physico-chemical analysis of the aqueous liquorice extract

A sample was prepared from 2 g of homogenous powder dissolved in 100 mL distilled water to evaluate the stability in time of the aqueous liquorice extract. The sample was filtered through a filter paper in a dry container. The filtrate was analyzed for pH and conductivity with CONSORT C-862 equipment. The spectral measurements were performed using a UV-VIS spectrophotometer (T+90) with dual and quartz optical prisms.

To detect the foam index of liquorice extract, 5 g of homogenous powder was extracted with 100 mL distilled water. In a series of 10 test tubes, 1÷10 mL of aqueous extract was introduced and the tube was filled up to 10 mL with distilled water. The content of each test tube was shaken vertically for 15 seconds and let to rest for 15 minutes. Finally, the height of the column of the foam persistence was measured (European Pharmacopoeia, 2007).

Active principles analysis

The qualitative chemical analysis of the active principles of the aqueous liquorice extract was performed from aqueous extraction of the homogenous powder following soluble constituents. The identification of the bioactive compounds was developed by Shibata, Styassny, Fehling, Molisch and Lugol reactions (Monciu *et al.*, 2001; Sandulescu *et al.*, 2007). All colour and precipitation reagents for quantification of the bioactive compounds were purchased from Merck.

The glycyrrhizic acid content was analyzed using 2 g of homogenous powder mixed with 3.25 mL nitric acid (250 g/L) and 96.75 mL acetone and vigorously stirred for 1 hour. Finally, it was filtered. Afterwards, the solution was extracted with acetone and mixed with 40 mL alcohol and concentrate ammonia until a yellow, abundant and flocculent precipitate was obtained. The glycyrrhizic acid

precipitate obtained was dissolved in 50 mL distilled water. The concentration of glycyrrhizic acid was measured by spectrophotometric method using 30 mL of precipitate diluted in 470 mL distilled water.

Antimicrobial activity

The antimicrobial activity of liquorice roots was tested from both extracts (aqueous and hydroalcoholic) by the agar diffusion method using three test strains: *Bacillus* sp. (Gram positive bacteria), coliforms (Gram negative bacteria) and *Aspergillus niger* (fungi), respectively. To obtain the aqueous extract, 5 g of homogenous powder were dissolved in 50 mL distilled water. Meanwhile, the hydroalcoholic extract was got using 5 g of liquorice powder dissolved in 25 mL ethanol 70%. The culture media used in our study were malt's worth modified agar (MMA) for *Aspergillus niger* and meat broth with agar (BCA) for *Bacillus* sp. and coliforms. 1mL of each test strain was transferred in three sterile Petri plates. About 15 mL of thinned culture medium were introduced into each Petri plate. After a subsequent cooling and solidification of the plates, the surface of the preinoculated culture medium was filled with three different samples: aqueous and hydroalcoholic extracts and drugs taken as reference. The references were: Amoxicillin for *Bacillus* sp. and coliforms and the antimycotic drug, Fluconazole in case of *Aspergillus niger*. The Petri plates with the two liquorice extracts and *Aspergillus niger* were incubated at 25°C for 72 hours and the ones with coliforms and *Bacillus* sp. at 37°C for 24 hours. The antibacterial potential of each extract on microbial growth was evaluated by measuring the diameter of the inhibition zone.

Obtaining a liquorice syrup from this plant product

The forms of this plant product involved two kinds of syrup: a soak of liquorice roots and simple syrup. Each form of the plant product was obtained in triplicate. The soak was obtained by grinding 3 g of liquorice roots, placing them on a piece of gauze and passing them under water flush to remove impurities. Then, they were introduced in a beaker with 50 mL distilled water, stirring the content of this container for 30 minutes. The final solution was decanted and filtered through a cotton piece. The filtrate was brought to the provided mass by washing the residue with distilled water without squeeze. The simple syrup was achieved by dissolving 64 g of sugar in 100 mL water. Afterwards, it was boiled for 1-2 minutes and stirred continuously. Usually, a part of the water can evaporate. In this case, distilled water heated to 70°C is added to obtain the desired mass. The hot solution is homogenized and filtered [European Pharmacopoeia, 2007]. The final product consisted of 10 g soak and 90 g of simple syrup. To evaluate the quality and stability of liquorice syrup, various physico-chemical parameters were analyzed using certain devices: density (pycnometer), refractive index and sugar concentration (Abbé refractometer), pH and conductivity measurements (CONSORT C-862 equipment), respectively. All experimental data are shown as average values.

Results and discussion

Qualitative and quantitative chemical analysis

Qualitative and quantitative analyses were developed by performing various preliminary tests such as humidity and total ash quantification, dosage of the soluble substances and the foam index measurement.

Humidity and total ash quantification

Any plant product consists of water and dry matter in certain proportions. The presence of water in the plant product determines the quality and influences the stability in time of the product. Humidity refers to the amount of water remaining in the plant product after drying. Meanwhile, the quantity of dry plant is a reference value for the expression of the active principles content. Commonly, the results of the analysis performed on plant materials are expressed as percentage of dry matter filtered. The humidity of dry powder of the liquorice extract was around 9.48 %, while the dry matter content of powder was 4.52%. This result is in accordance with European Pharmacopoeia, 2007, no more than 14%.

The mineral substances are quantitatively expressed as ash. The ash content is an important quality characteristic for many products, especially for those of the plant origin. Ash is the residue obtained after sample calcinations and is expressed as the percentage of minerals and mineral impurities from a product. The total ash represents all mineral substances regardless of origin and mineral impurities in the sample; in dry liquorice samples it was found to be 4.98 %. Therefore, in the case of ash content from liquorice powder, there has been a percentage no more than 6% complying with the accepted previsions of European Pharmacopoeia, 2007.

Dosage of the soluble substances

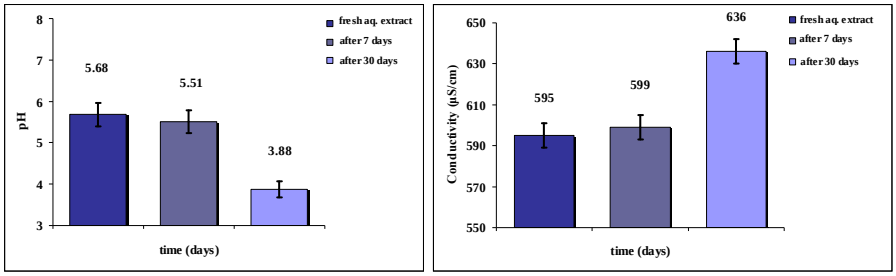
The assessment of the soluble substances from the plant products depicts those compounds that can be extracted with solvents in certain conditions provided in the relative monograph. The obtained extractive solution is subjected to calcinations and the residue represents the soluble substances being related to 100 g plant product. The content of soluble substances in dry liquorice samples was 25.83%. Thus, the content of soluble compounds in liquorice powder was at least 25% according to predictions specified in European Pharmacopoeia, 2007.

Foam index measurement

A lot of medicinal plants contain saponins that can cause persistent foam formation when an aqueous decoction is stirred. The ability of an aqueous decoction from plant materials or their extracts to form the foam is measured by quantification of the foam index which represents the minimum dilution of a solution containing saponins. By vertical vigorous stirring of the solution for 15 seconds in a test tube 160 mm high and 16 mm in diameter, a foam column of 1 cm appears, persistent for at least 15 min. By measuring the height of the persistent foam column corresponding to a series of 10 test tubes, it was observed that the foam is greater than 1 cm in all tubes. In conclusion, the foam index of the aqueous liquorice extract is greater than 100, namely 500, in agreement with the requirements of European Pharmacopoeia, 2007.

Stability of the aqueous liquorice extract

The stability of the aqueous liquorice extract was evaluated by pH, conductivity and spectral measurements. The analyses were developed from fresh aqueous extract and also at two different periods of time, after 7 days and 30 days, respectively (Figure 1). A slight decrease of pH in time of the aq. liquorice extract has been identified. After 7 days, a decrease in pH from 5.68 to 5.50 was identified, while after 30 days it reached 3.88. The low pH values showed the instability of the extract and its trend over time toward more acidity, around 2 pH units. The conductivity measurements showed the opposite trend, namely, the conductivity remains almost constant in time (from 595 µS/cm to 599 µS/cm) and a decrease at 636 µS/cm after 30 days. This fact explains that the extract dissociates in time, more ions in the transfer of electric charges being involved.



(A) **(B)**

Figure 1. Variation in time of aq. liquorice extract for pH (A) and conductivity (B)

The stability of the aqueous extract was also emphasized by spectrometric analysis from the fresh extract and after 30 days. The absorption spectra in UV of liquorice extracts analyzed are shown in Figure 2.

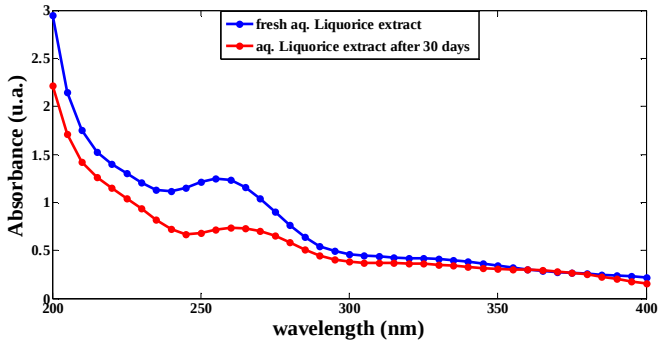


Figure 2. UV spectra of the aq. liquorice extract

The fresh extract showed an absorbance of 1.287 u.a. at λ of 264 nm. After 30 days the extract showed an absorbance of 0.744 u.a. and a hypochromic effect due to modification of λ to 258 nm. The UV absorbance decreases in both samples for the

fresh extract after 30 days and it proves the degradation in time of some compounds solubilized in the aqueous liquorice extract.

Evaluation of active principles from aqueous liquorice extract

The active principles from the aqueous liquorice extract were quantified by evaluation of the bioactive compounds through phytochemical reactions and the presence of Glycyrrhizic acid. The main bioactive compounds found in the aqueous extract were saponins, flavonoids, and tannins, reducing substances, oze and poliholozide according to data reported (Asl *et al.*, 2008). The glycyrrhizic acid is one of the main biologically active component from liquorice, responsible for the sweet taste of the product and the expectorant, anti-inflammatory, diuretic, bacteriostatic, healing, anti-ulcer actions (Asl *et al.*, 2008; Parvaiz *et al.*, 2014). The aqueous liquorice extract from two separate samples (sample A – powder of its own rearing, and sample B – powder from a commercial product) was tested for glycyrrhizic acid by spectrometric method according to European Pharmacopoeia, 2007 and data reported (Tohma *et al.*, 2010, Kim *et al.*, 2006). Both samples showed maximum absorbance in the UV domain at 258 nm: sample A (a value of absorbance of 0.39 u.a.) and sample B (a value of absorbance of 0.09 u.a.) (Figure 3) and it confirms the presence of glycyrrhizic acid.

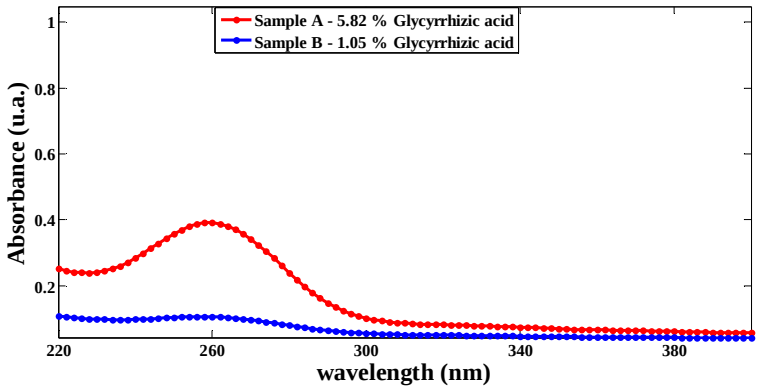
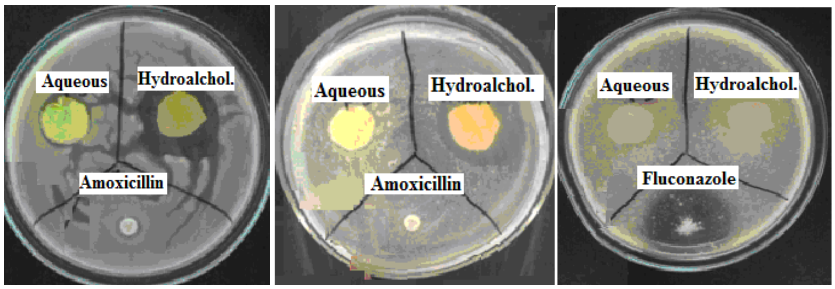


Figure 3. UV spectra of sample A (powder of its own rearing) and sample B (powder from a commercial product)

The glycyrrhizic acid content, reported at the absorbance maximum indicated, is 5.82% for sample A and 1.05% for sample B. Thus, the commercial product is characterized by a small concentration of glycyrrhizic acid which is not within the limits of at least 1.5% imposed European Pharmacopoeia, 2007 and it should not be commercialized because no standards imposed by Pharmacopoeia in use are achieved. But due to its antioxidant potential, the liquorice could be used as additive in bread and as a source of antioxidant components useful in some food products (Isbrucker *et al.*, 2006, Aoki *et al.*, 2007, Hayashi *et al.*, 2009).

Antimicrobial activity

The evaluation of the antimicrobial activity of liquorice roots was performed by the agar disc diffusion method. Usually, liquorice (*Glycyrrhiza glabra*) roots exhibited antimicrobial activity against various Gram-positive and Gram-negative bacteria (Gupta *et al.*, 2008, Patil *et al.*, 2009). A comparative study was developed between the inhibitory activity of liquorice extracts (aqueous and hydroalcoholic) and the two drugs (Amoxicillin and Fluconazole) against the following strains: Gram-positive bacteria (*Bacillus* sp.), Gram-negative bacteria (coliforms) and fungi (*Aspergillus niger*) (Figure 4).



(A) **(B)** **(C)**

Figure 4. Antimicrobial activity of aqueous and hydroalcoholic liquorice extracts comparative with Amoxicillin against *Bacillus* sp. (A), coliforms (B) and Fluconazole against *Aspergillus niger* (C)

The diameters of the inhibition areas were measured to quantify the antimicrobial activity. The qualitative results of measurement conversion of inhibition areas diameters in nm (Table 1) are shown in Table 2.

Table 1. Interpretation of the inhibition areas

Diameter (mm)	Symbol
0	-
1	±
2-3	+
3-5	++
>5	+++

Antimicrobial activity against Bacillus sp.

Both the aqueous extract and Amoxicillin as reference showed a moderate inhibitory activity while the hydroalcoholic extract achieved a strong antimicrobial activity corresponding to 5 mm halo.

Antimicrobial activity against coliforms

The antimicrobial activity of hydroalcoholic extract against coliforms bacteria strain was strong compared to the aqueous extract and Amoxicillin. The liquorice

extracts were characterized by a 2-3 mm halo equivalent for a moderate inhibitory activity.

Antimicrobial activity against Aspergillus niger

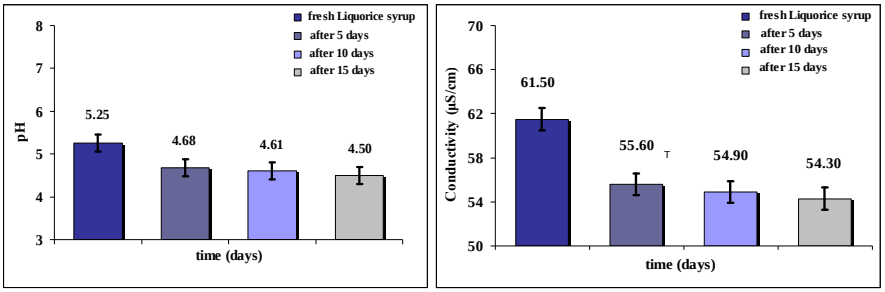
The lower inhibitory activity was recorded for aqueous and hydroalcoholic extracts against *Aspergillus niger*. Meanwhile, Fluconazole as reference showed a strong antimicrobial activity against this strain (5 mm). As a conclusion, Fluconazole inhibited the growth and multiplication of the fungi but in the case of liquorice extracts no activity could be identified. These results are according to the antimicrobial potential evaluation of various liquorice species against certain microorganisms using the agar diffusion method (Patil *et al.*, 2009, Bassyouni *et al.*, 2012).

Table 2. Evaluation of antimicrobial and antifungal activity of liquorice extracts

Sample	Gram-positive bacteria (*Bacillus* sp.)	Gram-negative bacteria (coliforms)	Fungi *(Aspergillus niger)*
Aqueous extract	++	+	–
Hydroalcoholic extract	+++	+++	–

Quality and stability control of the liquorice cough

A liquorice cough was extracted and clear, viscous, slightly opalescent syrup with sweet taste and characteristic odor was obtained. The syrup was characterized by the following physico-chemical parameters: relative density (d_{20}^{20} = 1.279), refractive index (n_D^{20} = 1.338) and sugar concentration of 51.8%. The syrup stability was carried out in the range of 5, 10 and 15 days (Figure 5).



(A) **(B)**

Figure 5. Variation in time of liquorice syrup for pH (A) and conductivity (B)

From pH and conductivity measurements a decrease in time of these parameters was detected, which highlights the instability in time of the syrup extracted from liquorice roots. The slight diminution of pH values can be explained as a result of

the acid pH of soak and as a consequence of higher value of the reaction rate in acid syrup due to sugar inversion after hydrolysis. Therefore, the essential properties of syrup are not entirely changed, but these changes accelerate the alteration of syrup. As the syrup has a concentration of 51.8% sugar, as European Pharmacopoeia, 2007 provides for the addition of antimicrobial preservatives (1.5% mixture of nipagin and nipasol) or other preservatives which are to be mentioned on the label. Potential biologically actions of syrup are: expectorant, mucolytic (Glycyrrhizin), anti-inflammatory, antibacterial (Glycyrrhizic acid), antispasmoclic (coumarines, glycyrretic) and estrogen (sterols) (Fiore *et al.*, 2005). Our study clearly showed that liquorice syrup could be used as an ingredient for bread, but also that it is recommended as fresh product.

Conclusions

The active principles from liquorice extracts vary according to parasitic host and harvesting age. For the plant material, some characteristic analyses were developed, such as humidity (9.48%), ash (4.96%) and total soluble substances (25.83%). The presence of saponins (bioactive compounds) was quantified by measuring the foam index, which was around 500. A time-dependent decrease in concentration of the bioactive compounds from aqueous liquorice extracts was observed. The Glycyrrhizic acid, 5.82% in the extract, responsible for the antioxidant activity, confirms that the plant has reached maturity. A liquorice syrup was obtained and subsequently could be used as nutraceutical additive in bread with good results, showing characteristic, optical and antimicrobial properties and good stability in time. Adding liquorice syrup in food products could be an alternative to improve nutraceutical potential.

Acknowledgments

This work was supported by a grant of the Romanian National Authority for Scientific Research, CNCS-UEFISCDI project number PN-II-ID-PCE-2011-3-0226.

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