Full Length Research Paper

The obtaining of an antioxidant product based on *Issopus officinalis* freeze: Dried extract

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The aim of this study was to obtain a freeze-dried extract from *Issopus officinalis* with an important antioxidant and antimicrobial effect. The extract was obtained in a fluidized bed solid extractor and the optimal values of phenols, flavonoids and the highest reduction power were determined when using 75% ethanol as solvent, the antioxidant activity ranging between 90 to 95%. In case of the freeze-dried product, the maximum antioxidant activity was registered when using 0.5 mg/ml powder and the results were optimal as well in case of determining flavonoids, phenols and the reducing power. The antimicrobial effect of the extract 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 75% ethanol as solvent. The optimal inhibitory effect of the freeze-dried product of around 1.7 cm was determined by a 0.5 mg/ml concentration.

**Key words:** Extraction, ethanol, 1.1 – diphenyl – 2 – Picrylhydrazyl, bacteria, colony quant.

INTRODUCTION

Hyssop is a plant with non-lignified branched stems, small elongated leaves, a small stalk and glossy leaves, with full edges. The purple, pink or pinky-white flowers are borne in bouquets. They have a scented smell and a soft-stinging aromatic taste. Due to the components from the essential oil, it has expectorant and antiseptic effect. It is used in respiratory tract diseases, particularly chronic bronchitis and asthma. It is seldom used as cicatrizer. It is included in the composition of anti-asthmatic and diaphoretic tea (Ebrahimzadeh et al., 2010). Lately there were pointed out the benefits of the products containing *Issopus officinalis* in treating or preventing diseases of the nervous system, such as anxiety, fatigue, stress-related illnesses, mental pressure or neurosis. They are based on the antioxidant effect of alcoholic extracts from hyssop and on their capacity to inhibit free radicals. Therefore, the negative effects of free radicals on the human body may be prevented (Kazazi et al., 2007; Ghasemi et al., 2009). So far, studies on hyssop properties are very few and it is mostly used by traditional medicine. Due to the wish of finding new sources of treatment for modern diseases, the products based on hyssop extracts are a viable alternative because of their phenolic component and the increased reducting potential, similar to that of known preparations, such as those made of rosemary (Pham-Huy et al., 2008).

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

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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 (Bjelakovic et al., 2008; Carvalho et al., 2005; Knight, 2000). The extraction of bioactive compounds from plant materials is the first step in the utilization of phytochemicals in the preparation of dietary supplements or nutraceuticals, food ingredients and pharmaceutical products. Usually before extraction plant samples are treated by milling, grinding and homogenization, which may be preceded by air-drying or freeze-drying. Generally, freeze-drying retains higher levels of phenolics content in plant samples than air-drying (Abascal et al., 2005). For example, Asami et al. (2003) showed that freeze-dried plant samples consistently had a higher total phenolic content level compared with those air-dried (Dai and Mumper, 2010). Thus, freeze-drying was chosen as a final operation in obtaining the extract from I. officinalis. The purpose of the research was to obtain a freeze-dried product from hyssop. In case of this extract, it was determined and characterized the antimicrobial effect on strains with pathogenic potential for humans (E. coli CBAB 2, B. cereus CMGB 215, L. 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 Issopus officinalis extract

A quantity of 20 g of vegetal material was submitted to hydro alcoholic extraction, in ethanol 50, 75 and 90% (v/v). The vegetal material consisted of dried whole plants, leaves and flowers, being provided by Fares Bio Vital Laboratories, Oraștie, România. The extractions and studies were realized in Biotehnol Center Laboratory, in 2010. 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 a Christ 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. According to the obtained results, the best alcohol concentration was selected to obtain a fluidized bed extract using the extractor faxIKA 200. The extract was submitted to freeze-drying and was analyzed in the optimal concentration which was found, in accordance with the methods described as follows (Vamanu et al., 2010):

Determining the total phenols content

The total phenolic content was determined by the Folin – Ciocâlteu reagent (Sigma Chemical Co., St Louis, MO, USA, catalog no. F5292). The vegetal extract (1 mg/ml) was mixed to 5 ml of Folin – Ciocâlteu reagent (diluted to 1/10 in distilled water). 4 ml of Na carbonate 7.5% (Sigma Chemical Co., St Louis, MO, USA, catalog no. 34277) were also added to the mixture. The mixture was shaken for a few seconds, then incubated for 30 min at 40°C. The absorbance was 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 AlCl₃ 2% (Sigma Chemical Co., St Louis, MO, USA, catalog no. 206911) in ethanol (Chimopar Romania, p.a.) and 0.5 ml of vegetal extract was prepared. It was left for 60 min at room temperature and afterwards the absorbance was measured at 420 nm. The total content of flavonoids 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) (Sigma–Aldrich GmbH. Sterneheim, Germany) free radical scavenging capacity. 100 µl of extract were mixed to 3 ml of ethanol solution of DPPH 0.004%. In 30 min, the absorbance was read at 517 nm (Afolayan et al., 2008; Rahman et al., 2008; Szabo et al., 2007).

Reducing power assay

Various concentrations of freeze-dried ethanolic extract (2.5 ml) were mixed with 2.5 ml of 200 mmol/l sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide (Sigma Chemical Co., St Louis, MO, USA, catalog no. 702587). The mixture was incubated at 50°C for 20 min. After 2.5 ml of 10% trichloroacetic acid (w/v) (Sigma Chemical Co., St Louis, MO, USA, catalog no. T6399) were added, the mixture was centrifuged at 650 rpm for 10 min. The upper layer (5 ml) was mixed with 5 ml deionised water and 1 ml of 0.1% ferric chloride (Sigma Chemical Co., St Louis, MO, USA, catalog no. 236489) and the absorbance was measured at 700 nm: higher absorbance indicated higher reducing power (Ferreira et al., 2007).

Outlining the antibacterial capacity

E. coli CBAB 2, B. cereus CMGB 215, L. innocua CMGB 218 were used for the tests. Each strain was inoculated on a Petri plate on which there was poured LB-agar medium (Sigma Chemical Co., St Louis, MO, USA, catalog no. L3022). 20 µl 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 (Vamanu et al., 2011; Jazani et al., 2008; Puangpranpitag et al., 2009; Rožman et al., 2009).

Statistical analysis

Experimental results were mean ± S.D. of three measurements. The values p < 0.05 were regarded as significant.

RESULTS

During the first stage of the research, the hyssop extract
Table 1. Antioxidant activity of the *Issopus officinalis* simple extracts.

<table>
<thead>
<tr>
<th>Sample concentration (mg/ml)</th>
<th>Antioxidant activity (%)</th>
<th>50% ethanol</th>
<th>75% ethanol</th>
<th>90% ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.1</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>0.1</td>
<td>46.54±0.02</td>
<td>56.14±0.02</td>
<td>0±0.01</td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>68.1±0.03</td>
<td>91.4±0.01</td>
<td>2.58±0.01</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>88.4±0.02</td>
<td>96.04±0.01</td>
<td>75.64±0.02</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>88.56±0.01</td>
<td>94.46±0.02</td>
<td>54.19±0.02</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>87.08±0.01</td>
<td>94.57±0.02</td>
<td>75.43±0.01</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. Total contents of flavonoids of the *Issopus officinalis* simple extracts.

was obtained by simple extraction in 50, 75 and 90% ethanol. The antioxidant effect of freeze-dried extracts, in various concentrations, is provided in (Table 1). Note should be made that an extraction with 75% alcohol determined a maximal antioxidant activity, notwithstanding the used concentration. The extraction with 90% alcohol was the one determining the minimal antioxidant activity. A concentration of 0.5 mg/ml of extract determined a maximal value, equal to that of vitamin E (96.15%) and ascorbic acid (96%) at the same concentration. An approximately equal value, of 94%, was obtained as well for 1 and 2 mg/ml hyssop extract, but also when using 75% alcohol. For the content of flavonoids (Figure 1), the doubling of the extract quantity from 0.5 to 1 mg/ml, for instance, did not mean the automatic increase or doubling of the obtained value. For the 90% ethanol extract, the obtained values were even lower, of 2 mg/ml. The maximum quantity of flavonoids was obtained when using 75% ethanol. For 1 mg/ml of extract there resulted a higher value, by approximately 4% and for 2 mg/ml, by approximately 23%.

A similar trend was noticed in case of using a concentration of 50% ethanol, but the values were significantly lower. The same observation was valid in case of phenols quantity (Figure 2). By doubling the extract quantity from 0.5 to 1 mg/ml there resulted an increase of the phenols quantity of merely 25% and for an increase from 0.2 to 0.5 mg/ml there resulted an increase by 43% of the phenols quantity. In exchange, for the other extractions, with 50 and 90% alcohol, the doubling of freeze-dried extract quantity determined the increase of the phenol quantity by only 13%. The reducing potential (Figure 3) had a maximal effect in case of 0.5 mg/ml of extract obtained with 75% ethanol. By
doubling the quantity, only an increase of 2% was obtained and, therefore, it was not justified to use such a quantity. For 50 and 90% alcohol extracts, larger variations were obtained, by 13% on average. The maximum antimicrobial activity was obtained against *E. coli* CBAB 2, *B. cereus* CMGB 215 and the lowest against *L. innocua* CMGB 218, with the extract in 75% ethanol. For the last strain, the inhibition zones were lower in proportion of 25 to 50%, the maximum inhibition zone being determined for a concentration of at least 0.5
mg/ml. Against *E. coli* CBAB 2 and *B. cereus* CMGB 215, the maximum inhibition zone, of 2.5 cm, was obtained for a concentration of 0.5 mg/ml freeze-dried extract. At an extract concentration of 1 or 2 mg/ml, the inhibition zone remained constant. For extracts in 50 and 90% ethanol, the maximum inhibition was maintained against *E. coli* CBAB 2 and *B. cereus* CMGB 215, also at a concentration of at least 0.5 mg/ml freeze-dried extract.

Once the optimal ethanol concentration of 75%, was established in relation to the extraction, a volume of 250 ml *I. officinalis* extract was obtained in the fluidized bed extractor. The extract was placed in a freeze-drying flask of 500 ml and in 72 h the process was stopped, thus resulting the freeze-dried *I. officinalis* powder. In order to use it in obtaining pharmaceutical products, as a preliminary stage, the optimal concentration of the chemical species values shall be determined for this extract as well. The powder was dissolved in pure ethanol, in a concentration of 0.1, 0.2, 0.5, 1 and 2 mg/ml (Figure 4). The determinations proved that a concentration of 0.5 mg/ml could be sufficient to use such an extract. The antioxidant activity of approximately 93% was obtained in case of using 0.5 mg/ml freeze-dried powder. The observation was valid as well for the flavonoid and phenol quantity. Even if there were increases of the obtained values, they were not in agreement with the increase of the extract concentration. When doubling the extract quantity of 1 mg/ml, a phenol quantity by just 6% higher was obtained and the flavonoid quantity was only increasing by 25% that is 92 µg/ml quercitin. In exchange, the reducing potential increased by approximately 59%, when using a concentration of 1 mg/ml, as opposed to 0.5 mg/ml of freeze-dried product. If 2 mg/ml of extract were used, there resulted an increase of the reducing potential of 76%. When testing the antibacterial effect of freeze-dried powder, a diameter of at least 1.5 cm was noticed against the three strains.

The maximum antibacterial effect, of 2 cm, was manifested against *E. coli* CBAB 2 and *B. cereus* CMGB 215, at a concentration of at least 0.5 mg/ml. This determined the same diameter of the inhibition zone as in case of a concentration of 1 or 2 mg/ml of freeze-dried powder. In case of *L. innocua* CMGB 218, the inhibiting effect was of maximum 1.9 cm at a concentration of 2 mg/ml (Figures 5 to 7). For 0.5 mg/ml, the inhibition diameter was of 1.7 cm by only 10% less.
Figure 5. Inhibition of *Escherichia coli* CBAB 2 by the freeze-dried powder.

Figure 6. Inhibition of *Bacillus cereus* CMGB 215 by the freeze-dried powder.
DISCUSSION

The fluidized bed extraction in the extractor, using 75% ethyl alcohol as solvent, represented the optimal variant. It determined 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 reducing power were obtained. The antioxidant activity, even if it did not have a maximum value, was high, ranging between 90 to 95%. These data are supported by the previous researches of Bjelakovic et al. (2008), Knight et al. (2000) and Oguntibeju et al. (2009). A maximum value was 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 depended on the fact that certain strains may be sensitive to certain chemical compounds which were included in the extract. Others, such as L. innocua CMGB 218, were less sensitive. The findings of Rune et al. (2005), Sang et al. (2008), Scartezzini et al. (2000) and Stuckey and Osborne (2007) are in support of this result. The maximum antioxidant effect in case of using freeze-dried powder of 0.5 mg/ml was of 93%, which is a high value.

Very good results were obtained for the other chemical species which have been determined, resulting that this was the optimal concentration which can be used to obtain functional antioxidant products. The value was confirmed by the antimicrobial effect of the product, with an inhibiting effect of more than 1.7 cm against the three strains. The maximum inhibiting effect was manifested against E. coli CBAB 2 and B. cereus CMGB 215.

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REFERENCES


