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EFFECTS OF EXTRACTION SOLVENT AND TECHNIQUE ON THE ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY OF SPRING SAFFRON (CROCUS VERNUS (L.) HILL)

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ABSTRACT

Spring saffron (lat. Crocus vernus (L.) Hill) is a perennial plant species from the Iridaceae family. Purple or white coloured flowers bloom in the early spring. Saffron is commonly used in cookery, but because of its healing properties, saffron is also used for improving blood circulation, treating the cardiovascular diseases, preventing tumour and healing skin diseases. This research aimed to determine the influence of extraction solvents (water, ethanol and acetone) and technique (Soxhlet extraction, ultrasonic extraction and maceration) on antioxidative and antimicrobial activity and the content of total phenols. FRAP and DPPH methods were used to examine antioxidative activity. Antibacterial activity was analysed by using diffusion technique on bacterial strains of Escherichia coli, Enterococcus faecalis, Staphylococcus aureus, Bacillus subtilis, Listeria monocytogenes, and Pseudomonas aeruginosa. Antifungal activity was examined on Candida albicans. The results showed that the ethanol extracts have the highest antioxidant activity and the aqueous extracts have the lowest one. None of the tested extracts showed antibacterial activity, but aqueous extracts and ethanolic extract of saffron, obtained by maceration, showed antifungal activity.

Keywords: spring saffron, DPPH, FRAP, total phenols, antimicrobial activity

INTRODUCTION

Crocus vernus (L.) Hill, commonly known as Dutch saffron or spring crocus, is an attractive spring-flowering species. [1] The majority of taxa (species and subspecies) are restricted to Turkey and the Balkan Peninsula. [2] Because of its high protein level, pollen of this taxa belongs to the first class pollens. Saffron is a wild plant and it is highly valued for colour, taste, aroma and healing properties, if used in small doses. [3] Stigma is rich in carotenoids such as crocin, but many other carotenoid compounds derived by carotenoid cleavages, such as safranal, can be found in essential oils. [4-6] Latest studies showed that saffron has significant bioactivity, such as prevention of PC-12 cells death, chemopreventive and tumoricidal properties. [7,8] The pharmacological activity of saffron is mostly associated with its stigmas and their main compounds - carotenoids. Crocetin and its esters, crocins, are responsible for the colour of saffron.

[9] Lycopene, α- and β-carotenes and zeaxanthins are also contributing to biological activities of saffron. [10] The purpose of this study was to examine the bioactivity of the Crocus vernus (L.) Hill extract, as a species that is less represented in scientific papers, and to compare the results with the published results for Crocus sativus L. In recent times, several studies have been conducted in order to evaluate the antioxidative capacity of different reproductive parts of Crocus sativus. Results of these studies reveal that saffron and its active compounds, such as crocin and carotenoids, have high antioxidative activity. [11] The use of herbal extracts and phytochemicals, with well-known antimicrobial properties, can be very significant in therapeutic treatments. [12] Approximately, 20% of known plants were used in pharmaceutical studies, where their positive effect, regarding to the treatment of cancer and other diseases, were proven. [13] In order to use a phytochemicals for dietary supplements or food additives, pharmaceutical or cosmetic products, the very first step is the extraction of bioactive compounds from herbal materials. [14] Generally, plants produce many secondary metabolites, such as phenols, flavonoids, alkaloids, saponins, kinins and sterols. [15] Herbal extracts are known for their antihyperglycemic, antioxidative, antimutagenic, antifungal,

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anti-inflammatory, antiviral and antibacterial properties. [16]

MATERIAL AND METHODS

All chemicals were of reagent grade, purchased from Aldrich, Germany and used without further purification.

2.1. Preparation of extracts

A sample of spring saffron was collected in Northeastern Bosnia. The extractions were done with a fresh, unchopped sample. Distilled water, ethanol and acetone were used as solvents. The content of total phenols and antioxidant activity were examined on the collected extracts, without prior solvent removal. To determine antimicrobial activity, the solvent residue was removed on a rotavapor (Buchi R-210).

Extraction in the Soxhlet extractor: A paper thimble was filled with 45 g of saffron, 150 mL solvent was added and exctracted in Soxhlet apparaturs for 6 hours. The extracts were collected and analyzed.

Ultrasonic extraction: 50 g of sample was transferred to a flat-bottomed flask and 200 mL of solvent was added. The flask was inserted into the ultrasonic bath at 40° C for 2 hours. The resulting extracts were filtered and used for analysis.

Maceration: 50 g of saffron was transferred to a flat-bottomed flask, 200 mL solvent was added and continuously mixed on vibromix at 300 rpm for 5 hours, after which the extracts were filtered and analyzed.

2.2. Determination of total phenolic content

The content of polyphenols was determined according to the procedure published by Tawaha et al. [17] Appropriate dilution was prepared for each of the extracts. Absorbance of the resulting blue colored liquids was measured at 765 nm, using a Shimadzu UV-mini-1240 UV/Vis Spectrophotometer. Quantitative analysis was performed based on the standard calibration curve of gallic acid. The concentrations of gallic acid in the solution from which the curve was prepared were 3, 15, 30, 75 and 150 mg/mL ($y = 0.0038x + 0.0094$; $R^2 = 0.9995$).

2.3. DPPH radical scavenging activity

2,2-diphenyl-1-picryl-hydrazyl (DPPH) method was performed according to the method described earlier. [18] The radical scavenging effect (%) or percent inhibition of DPPH radical was calculated according to the equation: $[(A_{control}-A_{sample})/A_{control}] \times 100$, where A_{sample} is the absorbance of the solution containing the sample at 517 nm and $A_{control}$ is the absorbance of the DPPH solution. The results are expressed as the IC_{50} value (mg/mL) or the concentration of extract that caused 50% neutralization of DPPH radicals.

2.4. Ferric Reducing Antioxidant Power Assay (FRAP)

The determination of ferric reducing antioxidant power or ferric reducing ability (FRAP assay) was performed as described earlier. [19] To prepare the calibration curve, solutions of $FeSO_4 \times 7H_2O$ were prepared in the concentration range of 200-1000 µmol/L ($y = 0.001x + 0.0615$; $R^2 = 0.9907$). In each tube, 0.1 mL of extract and 3 mL of FRAP reagent were added. The samples were incubated in an aqueous bath for 30 minutes at 37 °C, and the absorbance was measured at 593 nm.

2.5. Determination of antimicrobial activity

Antibacterial activities were investigated by diffusion method on reference bacterial strains Escherichia coli, Enterococcus faecalis, Staphylococcus aureus, Bacillus subtilis, Listeria monocytogenes and Pseudomonas aeruginosa. Antifungal activity of the complex was tested on Candida albicans. From the microorganisms strains of overnight cultures, suspensions of 0.5 McFarland turbidity were prepared (density 10^7-10^8 CFU/mL, depending on soy). The strains were then placed on the surface of the nutrient substrate-Mueller-Hinton agar (MH), dispersed in sterile Petri dishes. Substrate thickness was 4 mm. In the agar sterile drill-shaped holes were made ("wells"), into which 100 µL of extract. After the plates were left at room temperature for 15 minutes, the substance was diffused into agar, incubated at 37^0C/24 h. After the incubation period, the size of the inhibitory zone was measured and the sensitivity of the microorganisms was expressed as follows: if the inhibitory zone of the microorganism growth was greater than 20 mm, it was marked with three pluses (+++), which is the highest sensitivity of microorganisms. If the inhibitory zone was in the range of 16-20 mm it was marked with two pluses (++). Very low sensitivity is indicated with one plus (+), if the inhibitory zone is 10-15 mm in diameter. The minus (-) mark is used for an inhibitory zone of less than 10 mm or if it's absolutely absent. [20]

RESULTS AND DISCUSSION

The extraction yield from saffron, using various solvents and extraction techniques, is shown in Table 1. In the case of all methods, ethanol extracts and then acetone extracts have the highest yield. The most effective extraction method in terms of yield is Soxhlet extraction, then ultrasonic extraction and maceration in a yield of 1.11 to 2.33%.

Table 1. Results of extraction

Method	Solvent	Yield (%)	Extract colour
Soxhlet extraction	water	2.68	Brown
	ethanol	15.38	Orange
	acetone	11.14	Yellow
Ultrasonic extraction	water	1.62	Red
	ethanol	9.41	Orange
	acetone	6.97	Yellow
Maceration	water	1.11	Orange
	ethanol	2.33	Orange
	acetone	1.87	Yellow

3.1. The content of total phenols

The content of total phenols in the spring saffron extracts is shown graphically in Figure 1. Ethanol has proved to be the most efficient solvent for the extraction of these biologically important compounds, with the content of 0.983 mg GAE/g obtained by Soxhlet extraction, and 0.772 mg GAE/g obtained by maceration. The previously published results show that the various parts of Crocus sativus L. have a higher content of phenolic compounds compared to our research [21-23]. The reason for this can primarily be the difference in species, parts of the plant, used solvents, location from which the sample was taken, the sample treatment before analysis and the method used to extract the phenolic compounds. Studies have shown that drying can affect the increase or decrease in the content of bioactive components and antioxidant capacity. [24,25] The method of drying is extremely important since the plant material contains temperature labile compounds which can be dissolved by heating.

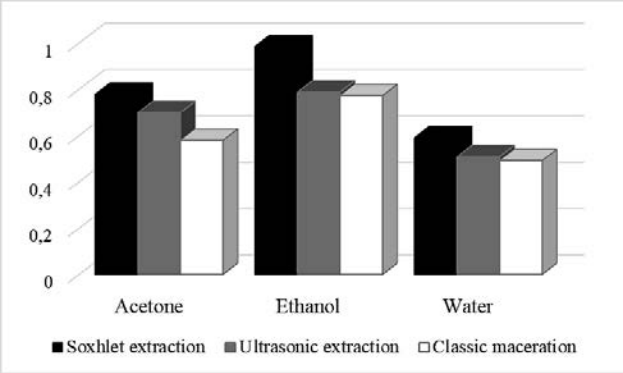

Fig. 1. Content of total phenols in spring saffron extracts [mg GAE/g]

3.2. Antioxidative activity

The results of the antioxidative capacity of spring saffron extracts, obtained by DPPH and FRAP method, are shown in Fig. 2 and 3. The highest antioxidant capacity reflected in lowest IC_{50} value was measured for the ethanolic extract obtained by Soxhlet extraction. The aqueous extracts showed the weakest antioxidant capacity with an IC_{50} value ranging from 9.98-11.13 mg/mL. A vitamin C solution, which was used as a positive control, showed significantly better antioxidant activity with an IC_{50} value of 0.00528 mg/mL. The FRAP value for vitamin C is 285 μmol/L for a concentration of 0.02 mg/mL. Compared to previously published results, spring saffron extracts have a lower antioxidant capacity compared to Crocus sativus L. The results obtained by the FRAP method confirm those obtained by the DPPH method.

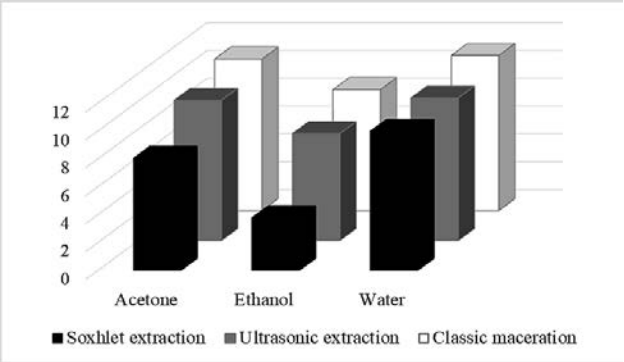

Fig.2. Results od antioxidative capacity obtained by DPPH method (mg/mL).

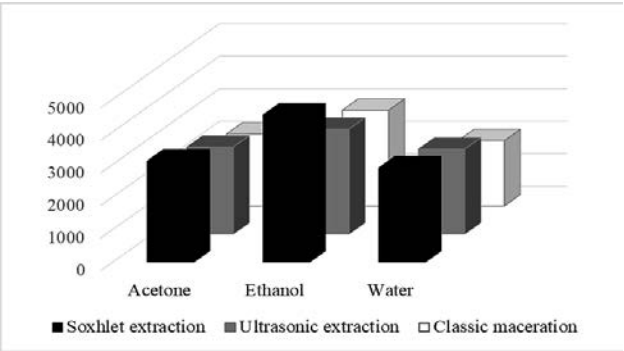

Fig. 3. Results of antioxidative capacity obtained by FRAP method [μmol/L Fe^{2+}]

3.3. Antimicrobial activity

Antimicrobial "screening" has established a complete lack of antibacterial activity of spring saffron extracts against all tested bacterial strains. On the contrary, concentrated aqueous extracts and the ethanol extract, obtained by maceration, showed antifungal activity in the case of Candida albicans. The ethanol extract showed the largest inhibition zone of 25 mm (+++). An inhibition zone of 20 mm was recorded for nystatin, which was used as a control and that is less than the inhibition zone caused by the ethanol extract. The aqueous extract obtained by Soxhlet extraction, showed slightly weaker activity, with an inhibition zone of 20 mm (++). The remaining two aqueous extracts showed lower activity against C. albicans with inhibition zones of 12 and 15 mm (+). In a study carried out by Muzaffar et al. for light petroleum and methanol extracts of C. sativus stigmas [26], certain antimicrobial activity was recorded against B. subtilis, P. aeuroginosa, S. aureus, E. coli and C. albicans, especially at 750 and 1000 μg/disc. The antimicrobial activity of extracts of C. sativus parts (stigma, stamen, leaves and colora) was confirmed by a study conducted by Vahidi et al. [27]. In this study, the antimicrobial effect of ethanol, ethyl acetate and petroleum ether extracts of various parts of C. sativus was investigated, whereby ethyl acetate extracts showed a greater effect against tested microorganisms, compared to the extracts obtained by other solvents.

CONCLUSIONS

Through this study it has been shown that the most efficient solvent for the extraction of total phenols is ethanol, while Soxhlet extraction is considered to be the most appropriate technique. The results showed that Crocus vernus has a lower content of biologically active components and less antioxidant activity than C. sativus, which is also reflected in antimicrobial activity.

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