Original Research Article

Investigation of Polyphenol Content and Antioxidative Activity of *Cucurbita pepo* L. Leaf Extracts Obtained by Ultrasonic Extraction

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ABSTRACT

In this research, the antioxidant activity and its correlation with the polyphenolic content in pumpkin leaf extracts (*Cucurbita pepo* L.) were examined. Dried and pulverized pumpkin leaves were used as extraction material. Various solvents (water, methanol, ethanol, acetone) and their mixtures, in a ratio of 50:50 (*v/v*) (water: methanol, water: ethanol, water: acetone) were used for extraction. The solid-to-solvent ratio was 1:10. The influence of solvents on phenolic extraction, as well as the effect of ultrasonic extraction was investigated. The samples were subjected to ultrasound for 15 minutes. The total phenolic content was determined by the Folin-Ciocalteu method and the antioxidant activity of the extracts by FRAP and DPPH methods. The obtained results indicate the importance of choosing an adequate extraction solvent for phenolic isolation from plant material. Mixtures of organic solvents and water, especially a mixture of water and acetone, are the most suitable for the extraction of phenolic compounds. At the same time, a positive correlation was established between the content of total phenols and the antioxidant activity of the extracts. This suggests that phenols contribute significantly to the antioxidant properties of pumpkin leaves. The results showed the potential medicinal properties of pumpkin leaves but further studies are needed to identify, characterize and isolate different bioactive components, which could be used as a basis for obtaining new drugs for the treatment of various diseases.

Keywords: pumpkin, extraction, UAE, phenolic compounds, antioxidant activity

1. INTRODUCTION

Cucurbita pepo L., known as pumpkin or gourd, is a plant species from the Cucurbitaceae family, which includes over 90 genera and about 975 species, both wild and cultivated around the world. Pumpkin and its products are traditionally used in many countries, due to their anti-inflammatory, antiviral, analgesic, hypoglycemic, antioxidant and other effects [1].

The official drug is pumpkin seeds (Cucurbitae peponis seed). Pumpkin seeds are important in the treatment of prostate disease and urinary tract disorders, which can be associated with antioxidant and anti-inflammatory properties. The seeds are rich in zinc and have a diuretic effect. In addition, they have been used in the treatment of gastritis, nephritis, bronchitis, hemorrhoids, headaches, and anemia in various parts of the world. Some studies report antihypertensive and cardioprotective effects of seed oil, as well as its inhibitory effect on arthritis in rats, similar to indomethacin [1, 2]. It is important to mention that other plant parts of the pumpkin, such as the fruit, flower and young leaves, and their extracts are also used in nutrition and for therapeutic purposes [3]. The fruit of the plant is used in traditional medicine to treat colds and fatigue, as well as to relieve pain. In addition, beneficial effects have been recorded in the treatment of eye infections, throat infections, coughs, rheumatoid arthritis, hemorrhoids, burns, etc. Analgesic and anti-inflammatory effects were shown by preparations obtained from the pedicle, the part of the pumpkin stem, which is attached to the fruit [1]. It has been suggested that extracts of peel, pulp or flesh of the fruit and pumpkin seed oil can inhibit breast (MCF7) and liver (HEPG2) cancer cells [4]. The leaves exhibit analgesic and antimicrobial effects. They are used for external burns, fever, against nausea and to increase the hemoglobin content in the blood [1, 4, 5]. They can be useful in the treatment of urinary and respiratory system infections, dermatitis, soft tissue infections, etc. The results of some studies suggest that the aqueous extracts of the leaves show good antimicrobial activity against *P. aeruginosa*, but additional studies are needed to determine precisely which substances and at what concentration have antimicrobial activity.

Pumpkin leaves contain 9% protein, 18% fat and 20% vitamins, which are responsible for the high nutritional, medicinal and industrial value of pumpkin. Pumpkin leaf extracts have shown antimicrobial properties, and are also used to increase the hemoglobin content in the blood, relieve nausea and lower the body temperature [6]. Studies conducted in South Africa have shown positive effects of orally administered pumpkin leaves in the treatment of arthritis, and these effects are associated with the anti-inflammatory properties of the leaves. Pumpkin leaves and fruits have also shown neuroprotective effects [7]. Characterization of the plant compounds is necessary to evaluate the biological activity of the extracts. Experimental studies have shown that ethanol extracts of pumpkin leaves and stems contain sugars, saponins, alkaloids, flavonoids, sterols, glycosides, terpenoids and phlobatannins. Flavonoids, phlobatannins and proteins are present in higher concentrations [8].

2. MATERIAL AND METHODS

Dried leaves of pumpkin (*C. pepo* L. subsp. *pepo*) were used as plant material for extraction. The leaves were collected at the end of August 2020, in the locality of Vlasenica, Bosnia and Herzegovina. The leaves were adequately washed and cleaned, and left to dry for ten days. The drying process was carried out at room temperature, in a dry place, protected from direct sunlight. The dried leaves were then ground to a powdery consistency in an electric mill and stored at room temperature.
All chemicals used were of analytical grade and were used as received without any further purification. Chemicals were purchased from Merck (Darmstadt, Germany) and Sigma Chemical Co. (St. Louis, Missouri, USA).

2.1. Preparation of extracts

For testing polyphenol content and antioxidant capacity, extracts were prepared by mixing 2 grams of plant material with 20 mL of solvent. The samples were subjected to extraction in an ultrasonic bath for 15 minutes. After the extraction process was completed, the samples were filtered through blue tape filter paper. The filtrates were stored in a cold and dark place until the beginning of the analysis.

2.2. Determination of total phenolic content (TPC)

Total phenolic content was quantified spectrophotometrically using the Folin-Ciocalteu test according to the protocol by Singleton et al [9], with some modifications. 100 µL of the extract was mixed with 1270 µL of 10% Folin-Ciocalteu reagent. After 5 minutes, 210 µL of 10% sodium carbonate was added. After incubation for an hour, 455 µL of distilled water was added to the incubated solution. Absorbance was measured on a spectrophotometer at a wavelength of 765 nm. Quantitative analysis was performed based on the gallic acid standard calibration curve.

2.3. DPPH radical inhibition test

The 2,2-diphenyl-1-picryl-hydrazyl (DPPH) method was carried out according to the previously described method [10] with certain modifications. 0.5 mL of the extract was added to the test tube and made up to 2 mL with methanol. Then 0.5 mL of 0.5 mM DPPH solution was added and the samples were left to incubate for 30 minutes in a dark room at room temperature. Absorbance was measured at 517 nm with methanol as a blank. The radical scavenging effect (%) or DPPH radical inhibition percentage was calculated according to the equation:

$$[(A_c - A_s) / A_c] \times 100 \, [\%]$$

where A_s is the absorbance of the solution containing the sample at 517 nm and A_c is the absorbance of the control.

2.4. FRAP (Ferric-Reducing Antioxidant Power) Assay

The FRAP (Ferric-Reducing Antioxidant Power) method is based on the ability of the extract to reduce Fe(III) to Fe(II) ions. The test was conducted according to a published protocol (Benzie and Strain, 1999). 3 mL of prepared FRAP reagent was mixed with 100 µL of diluted extracts. Absorbance at 593 nm was recorded after incubation for 30 minutes at 37° C. The FRAP value was calculated from the calibration curve of ferrous sulfate heptahydrate.

3. RESULTS AND DISCUSSION

The aqueous extract (U_1) was cloudy and reddish-brown. Extracts with methanol (U_2), ethanol (U_3) and acetone (U_4) were clear and intensely green. Samples, in which mixture of water and methanol (U_5) or ethanol (U_6) was used as a solvent, were clear and orange. In the orange acetone extract, a slight turbidity was observed. The aforementioned turbidities did not interfere with the spectroscopic measurements.

3.1. Polyphenol content

Figure 1 presents the calibration curve of gallic acid. Table 1 shows the results of polyphenol content in pumpkin leaf extracts.

The results indicate the highest content of phenolic compounds in U_7, followed by samples U_5 and U_6. It is significant that all three extracts were obtained using a mixture of organic solvents and water, and this confirms the results of numerous studies, in which aqueous mixtures of organic solvents were shown as the most suitable for the extraction of phenolic compounds [11, 12, 13]. The high content of phenol was also confirmed in U_1, and it is comparable to the recorded content in U_6. These results are consistent with the fact that phenolic compounds, which are most often responsible for the antioxidant activity of the samples, are hydrophilic antioxidants. A better solvation of antioxidant molecules is achieved, as a result of interactions (hydrogen bonds) between the polar parts of these molecules and the solvent [11]. The low content of phenolic compounds was confirmed in U_2, U_3 and U_4, i.e.samples with pure organic solvents, of which acetone gave the weakest results. Ethanol proved to be less effective in extracting bioactive compounds than methanol, although their polarity is similar. The reason for this may be the low solvation of the molecule by ethanol, which is probably due to the presence of the non-polar ethyl part, which is longer than the methyl part. This deficiency can be overcome by adding water to ethanol. Studies show that 50% ethanol is one of the most commonly used solvents for extracting polyphenols from plants. However, for the isolation of polyphenols from these extracts, purification is required, since the water-ethanol mixture also extracts other compounds (carbohydrates, organic acids), which contribute to the high content of TPC. The sample with acetone showed the lowest content of antioxidant compounds, probably due to their weaker solvation, since acetone molecules are only proton acceptors, while the other solvents, methanol, ethanol and water are also proton donors. Phenols are usually extracted using water-alcohol mixtures, which are polar protic solvents, but it appears that an aprotic solvent such as acetone combined with water can extract more polyphenols. This is consistent with the TPC results obtained for sample U_7. This effect can be explained by better solvation of polar molecules, after the addition of water, which increases the polarity [13]. It is suggested that a water:acetone mixture is effective for the extraction of polar molecules, specifically higher molecular weight flavanols. Earlier studies suggested that acetone with 50% water could extract the highest TPC from plant species, such as *Camellia sinensis* [12]. In addition, the advantage of the mixture of acetone and water is that it is considered safe for use in food products.

Figure 1. Calibration curve of gallic acid (mg GAE/L)

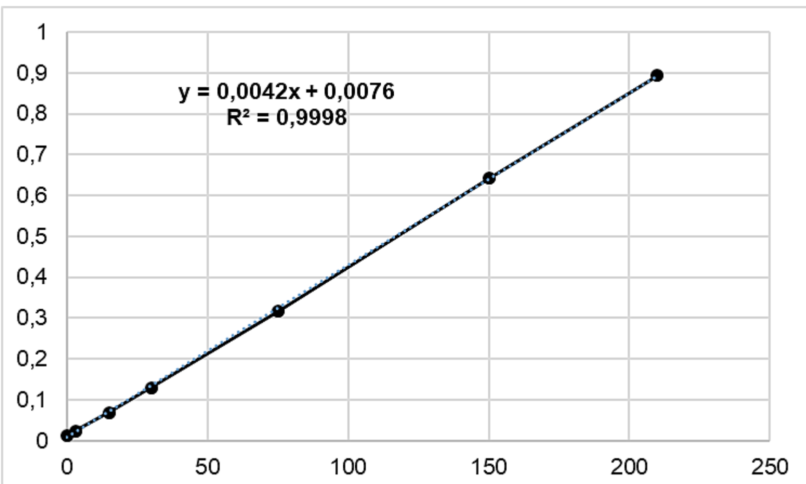

Table 1. Polyphenol content in pumpkin leaf extracts

Sample	TPC [mg GAE/g]
U_1	10,85
U_2	6,355
U_3	4,585
U_4	2,570
U_5	12,50
U_6	11,31
U_7	13,03

Compared to other studies, in which the content of total phenols in aqueous and ethanolic extracts is about 40 mg GAE/g [3], the total phenolic content of the extracts determined by our investigation is significantly lower. This may be due to differences in the extraction and defined experimental conditions. Unconventional extraction methods, such as ultrasonic extraction with "green" solvents, can significantly contribute to a higher content of extracted antioxidants from pumpkin. Ultrasound accelerates the disintegration of plant cells, breaking the chemical bonds between macro- and micromolecules, thus facilitating the release of phenol from the cell. Ultrasonic waves have a better penetration through the cell than microwaves, which can also lead to the degradation of phenolic compounds by raising the temperature. In the case of pumpkin, studies suggest that its fruit is the richest source of phenols. Some studies have shown the total phenolic content of the fruit to be around 90 mg GAE/100 g of fresh fruit, i.e.0.9 mg GAE/g [14]. The results of determining the content of phenols vary, since in another study a content of about 5 mg GAE/g was proven in fresh pumpkin fruit, as well as in the peel [15, 16]. It is interesting that the mentioned content is significantly lower than the content of total phenols in leaf extracts, determined by this investigation. This is consistent with the results of the study conducted by Kim et al., where it was shown that the ethanolic extract of *C. moschata* leaf has the highest content of total phenols, the best DPPH radical inhibition capacity and the highest reducing activity, compared to other pumpkin parts [17].

3.2. Antioxidant activity

The FRAP and DPPH methods were used to determine the antioxidant capacity. The obtained FRAP values of the prepared extracts, shown in Table 2, are consistent with the results that reflect the total phenolic content in the samples. The highest efficiency in reducing activity is observed in sample U_7, which also shows the highest content of phenolic compounds. This can confirm the results of previous studies, which indicate a positive correlation between the phenolic content and the antioxidant activity of plant extracts. The difference in the reducing activity of the water-methanol and water-ethanol samples is very small, and these samples also show high FRAP values. The sample with acetone has the lowest total phenolic content and thus the weakest reducing activity. The sample with water has twice the reducing activity, which is associated with a higher polarity of water and, consequently, a higher content of polar phenols. In addition to a good reducing activity, the advantage of the aqueous extract is that water is the safest for use.

Table 2. Results of the reduction potential of pumpkin leaf extracts

Sample	FRAP value [μmol/g]
U_1	119,75
U_2	68,80
U_3	58,70
U_4	23,45
U_5	142.0
U_6	141,95
U_7	183,70

Better extraction of phenolic compounds means better reducing properties of the extract. The reducing capacity of a compound can be a significant indicator of its potential antioxidant activity. The antioxidant activity of phenolic compounds is mainly the result of their redox properties, which can play an important role in the adsorption and neutralization of free radicals, the quenching of singlet and triplet oxygen or the decomposition of peroxides. Fe (III) reduction is often used as an indicator of electron donating activity, which is an important mechanism of phenolic antioxidant activity. The antioxidant capacities of the extracts have a strong relationship with the solvent used, mainly due to the different antioxidant potential of compounds with different polarities [11]. Many existing studies clearly state that the use of aqueous mixtures of organic solvents such as ethanol, methanol, acetone, isopropanol increases the antioxidant efficiency of most herbal products. In earlier research, *C. pepo* fruit extracts obtained using a mixture of water and organic solvents (methanol, ethanol, acetone), in different proportions, showed very high reducing activity. FRAP values ranging from 4295.8 μmol Fe(II)/g to 5164.2 μmol Fe(II)/g of dry sample of different varieties of *C. pepo* were recorded. Based on our results, it can be concluded that the leaf extracts have a weaker reducing activity.

The results of the antioxidant activity obtained by the DPPH method are consistent with the results of the FRAP method, and also with the certain content of total phenols in the extracts. Thus, the highest percentage of DPPH radical inhibition is shown by sample U_7, which corresponds to a high content of phenolic compounds. The aqueous extract shows a higher percentage of inhibition than the ethanolic and methanolic extracts. However, mixtures of ethanol and methanol with water gave significantly better results and greater DPPH radical inhibition, thus a better antioxidant capacity. The weakest antioxidant capacity is shown by the leaf extract obtained with acetone, which has a significantly weaker inhibition compared to the other extracts. The results of DPPH radical inhibition are shown in Table 3.

Table 3. Inhibition of DPPH radicals

Sample	Inhibition [%]

U_1	42,36
U_2	30,43
U_3	27,51
U_4	19,51
U_5	51,97
U_6	48,22
U_7	57,98

Different extraction solvents affect the phenolic content and antioxidant properties of extracts. Dar et al. proved in their research that the highest percentage of radical inhibition, at a concentration of 500 mg/mL, was shown by pumpkin leaf extracts with ethyl acetate (79.44%) and *n*-butanol (68.9%). These extracts also have the highest content of total phenols. Phenolic compounds can be non-polar or polar in nature, so their solubility in solvents also depends on this. Non-polar, ethyl acetate and n-butanol proved suitable for the extraction of non-polar or less polar phenols and flavonoids [3]. In our research, all pumpkin leaf extracts, at a concentration of 25 g/L, showed significantly higher antioxidant potential than the extracts of the previously mentioned research. Namely, the highest measured percentage of DPPH radical inhibition is 57.98% for U_7. In another study, a similar result was obtained by the aqueous extract, but only at a concentration of 500 mg/mL, which is significantly higher than the concentration of our extracts. The antioxidant potential of the aqueous extract, determined by our test, is similar to the potential of the aqueous leaf extract, whose concentration is 10 times higher than in our case. In our research, solvents of higher polarity were used, and these extracts, at significantly lower concentrations, gave better results of antioxidant activity. From this it can be concluded that polar solvents are the most suitable for the extraction of pumpkin leaves. In addition to phenolic compounds, antioxidant activity can be attributed to other compounds identified in the leaf, such as ascorbic acid, carbohydrates, vitamins, fatty acids, phospholipids and others. It has been proven that water and methanol extracts of pumpkin seeds contain a high proportion of carbohydrates, and a better effect of inhibiting free radicals. However, no clear correlation can be established between carbohydrate content and antioxidant activity, since the concentrations of carbohydrates and phenols are proportional to each other. This is connected with the fact that a higher phenolic content corresponds to a higher proportion of phenolic glycosides [18]. Compared to vitamin C, which is one of the most well-known antioxidants, with a DPPH radical inhibition percentage of 99.8%, the calculated antioxidant activity of pumpkin leaf samples is weaker. The highest recorded inhibition percentage of 57.98% is almost twice lower than that of vitamin C. However, the obtained values are not negligible, as they prove the presence of antioxidant molecules with the potential to remove free radicals, and that can be the basis for further studies.

4. CONCLUSION

For the extraction of phenolic compounds from pumpkin leaves, mixtures of organic solvents and water proved to be more suitable than pure solvents, which is explained by the greater polarity of the solvent mixtures, and thus the greater solubility of polar phenolic compounds. By adjusting the optimal solvent volume ratio of the mixture, the extraction yield can be influenced. The FRAP and DPPH method proved that extracts with the highest content of total phenols showed the best reducing activity and inhibition of DPPH radicals, i.e. antioxidant activity, which leads to the conclusion that there is a positive correlation between the antioxidant activity of pumpkin leaf extracts and the content of total phenolic compounds. Optimizing the mentioned parameters can improve the extraction yield, that is, the quality and quantity of antioxidant molecules in plant extracts. Further research can be directed to the identification of the nature of bioactive molecules, with the aim of more detailed evaluation of the biological activity of the extracts.

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