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Antioxidant Activity of Watermelon Seeds Determined by DPPH Assay

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Abstract

The purpose of this study was to assess the antioxidant activity of the fresh extracts of Croatian and Greek watermelon seeds. Samples were prepared using two extraction methods: Soxhlet and maceration. Ethanol was used as a solvent in the Soxhlet, and methanol in the maceration method. The mass fractions of the extracts obtained by maceration were lower compared to those obtained by the Soxhlet method.

The antioxidant capacity of fresh watermelon-seed extracts was determined by DPPH (2,2-diphenyl-1-picrylhydrazyl radical) method. The antioxidant capacity of the extracts was also expressed *via* IC_{50} (half maximal inhibitory concentration). Based on the obtained IC_{50} values (1.41 to 2.60 mg ml^{-1}), all tested extracts showed antiradical activity but antioxidant capacity was better in extracts obtained by the maceration method. The reason for this may be the use of methanol as a solvent, which was to be expected, since methanol is a more polar solvent than ethanol.

The neutralisation capacity of DPPH radicals of analysed extracts was compared to the neutralisation capacity of ascorbic acid as standard. The concentrations of tested extracts required to neutralize 50 % of DPPH radicals were significantly higher than the required concentration of ascorbic acid (0.25 mg ml^{-1}).

Keywords

Watermelon seeds, Soxhlet extraction, maceration, DPPH

1 Introduction

Watermelon, *Citrullus lanatus (Thunb.) Matsum. & Nakai*, a tropical plant, is an annual herbaceous vegetable belonging to the *Cucurbitaceae* family. It is cultivated almost all over the world for its edible, large, round fruits with juicy pulp.[1,2] Although the juicy pulp of watermelon is used for dietary purposes, the seeds, peels, and rind are usually discarded or used as a feed supplement for animals. Still, in countries where watermelon cultivation is significant, seeds have widespread use. They can be used to prepare different products such as flour, snacks, beauty products, and as an ingredient for sauces. Therefore, the seeds should not be discarded.[3] Most studies on the medical use of watermelon have used its leaves, fruit, roots, or flowers.[4] However, the seeds, which are an often-neglected part of watermelon, have attracted great attention due to their richness in different chemical compounds and health benefits. Watermelon seeds are rich in essential nutrients such as vitamins, minerals, carbohydrates, crude protein, various amino acids, fats, fatty acids, and crude fibre.[5] In recent years, research is shifting attention toward the search for antioxidant and antimicrobial compounds from natural sources. Natural sources have become the centre of attention because pathogens have become resistant to the activity of certain synthetic drugs. Studies have shown that the seeds contain the following phytochemicals: phenols, saponins, alkaloids, tannins, glycosides, terpenoids, steroids, phytates, flavonoids, oxalates, phenols, and phytosterols. Most of these phytochemicals have antioxidative activity and present potentially effective biological compounds.[6] In addition to the antioxidant effect, the seeds also possess anti-ulcer, anti-inflammatory, antifungal, antimicrobial, and hepatoprotective activity.[7] There are several methods for determining the antioxidant activity of a substance, the most commonly used being spectrophotometric methods, such as DPPH (2,2-diphenyl-1-picrylhydrazyl) assays,[8] Foline-Ciolteau assays,[9] and many others. Bioamperometry and cyclic voltammetry can also be used.[10] Common methods to extract antioxidants from watermelon seed are solvent extraction (ethanol, methanol, hexane, *etc*.) sometimes assisted by sonication.[11]

In this study, two types of extraction were performed: Soxhlet extraction and maceration, and the antioxidant activity of watermelon-seed extracts were determined by DPPH assay because the total antioxidant scavenging activity by this method can be investigated quickly and easily.

2 Materials and methods

2.1 Samples

Two varieties of fresh watermelon fruits originating from Croatia and Greece were selected. The watermelon seeds were removed from the pulp (juicy part of the watermelon mostly pink or red). The cleaned seeds were stored at 4–6 °C until analysis.

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2.2 Materials

The materials used for this analysis included: ethanol, methanol, toluene, butanol, and ethyl acetate purchased from Sigma Aldrich (Germany). Acetic acid (99.00–100.00 %), formic acid, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Merck (Germany). All other reagents used were of high analytical grade.

2.3 Maceration extraction

Six grams of fresh watermelon seeds were weighed and transferred to a mortar and pestle. Maceration was performed with methanol (80 ml). After maceration, the samples were transferred to a laboratory beaker, and left at room temperature with constant stirring for 24 h. After soaking and stirring, the methanolic solutions were filtered through Whatman filter paper, Grade 1 to remove coarse impurities. The methanol extract was then concentrated on a rotary vacuum evaporator at 40 °C. The resulting extract was stored at 4–6 °C in the dark.

2.4 Soxhlet extraction

Six grams of fresh watermelon seeds were pulverised using a pestle and mortar. The pulverised seeds were transferred in a thimble (Cellulose, 43 × 123 mm). The thimble was placed in the centre of the Soxhlet apparatus. Extraction with ethanol as solvent was performed for 24 h. The ethanol extract was concentrated on a rotary vacuum evaporator at 40 °C. The resulting extract was stored at 4–6 °C in the dark.

2.5 2,2-Diphenyl-1-picrylhydrazyl assays

Preparation of DPPH solution

10 mg of DPPH was dissolved in 10 ml of ethanol (1 mg ml^{-1}).

Preparation of acetate buffer solution

6.80 g of sodium acetate and 500 ml of distilled water were added to the volumetric flask. Acetic acid solution, which was a mixture of 2.88 ml of concentrated acetic acid and 500 ml of distilled water was added to the same flask. The pH of the prepared buffer solution was 4.80.

Determination of antioxidant activity

Prepared samples of watermelon-seed extracts (1.00 mg ml^{-1}) were diluted to concentrations 0.05, 0.20, 0.40, 0.60, and 0.80 mg ml^{-1}. In the first six tubes labelled "blank", 0.10 ml of extract solutions of the indicated concentrations were pipetted, then 1 ml of acetate buffer and 1.90 ml of ethanol were added. In the other six tubes labelled as "sample", 0.10 ml of diluted extract solutions of the indicated concentrations were pipetted. To the prepared solutions were added 1 ml of acetate buffer and 1.90 ml of 130 mM DPPH solution. An amount of 1 ml of acetate buffer, 1.90 ml of DPPH, and 0.10 ml of ethanol were pipetted into one tube labelled "control". After the mentioned preparation, the tubes were mixed on a Vortex apparatus, and then left in the dark for 90 min. The absorbance was measured on a spectrophotometer (model UV-1280 – Shimadzu) at 517 nm.

The antioxidant activity determined by the DPPH method is expressed by the RSC value (Radical Scavenging Capacity), which is calculated according to Eq. (1).[12]

$$\mathrm{RSC}(\%) = 100 - \left[\left(A_S - A_B \right) \cdot \frac{100}{A_C} \right] \quad (1)$$

A_S, A_B, and A_C are absorbances of the sample, the "blank", and the "control", respectively, all measured at 517 nm.

3 Results and discussion

3.1 Yield of extracts

Watermelon-seed extracts were obtained using two extraction methods: Soxhlet and maceration. Two extraction methods were used to gain insight into the extraction power of these methods, and the influence of different conditions under which the extraction was carried out. Some of these conditions include the type of solvent used and the temperature. Methanol was used as a solvent in the maceration process, ethanol for Soxhlet extraction. One of the major differences between these two extraction methods was that the maceration took place at room temperature, while Soxhlet extraction is performed by heating. Another important difference between these two methods is that Soxhlet extraction is one of the continuous extraction methods.

The advantage of continuous methods is that less solvent is required, as the solvent circulates through the system and comes back into contact with the material to be extracted. This lower solvent consumption is reflected in the lower solvent/solid component ratio, and the same result can be achieved if larger amounts of solvent are used in other methods.[13] The mass fractions of fresh watermelon-seed extracts are shown in Table 1.

Table 1 – Mass fractions of fresh watermelon-seed extract
Tablica 1 – Maseni udjeli ekstrakta svježih sjemenki lubenice

Samples (Watermelon-seed extracts)	Extract mass / mg g$^{-1}_{\text{fresh sample}}$
S1	73.43 ± 1.70
S2	49.76 ± 2.10
S3	90.39 ± 2.30
S4	46.07 ± 1.90

Data are shown as means with ± SD of three replicates. S1 – ethanol extract of watermelon seeds from Croatia; S2 – methanol extract of watermelon seeds from Croatia; S3 – ethanol extract of watermelon seeds from Greece; S4 – methanol extract of watermelon seeds from Greece.

The mass fractions of methanol extracts of watermelon seeds (S2 and S4) obtained by the maceration method are lower than the mass fractions of ethanol extracts (S1 and S3) obtained by the Soxhlet method. Possible differences in mass may be due to the use of different extraction methods and different solvents.[14]

3.2 Determination of antioxidant activity

The antiradical or antioxidant activity of S1–S4 extract samples was determined using the DPPH method. With this method, the resulting redox reaction between the extract sample and the free DPPH radical was followed by a change in absorption to 517 nm, *i.e.*, the total free radical scavenger capacity (RSC) was determined spectrophotometrically by measuring the disappearance of the radical DPPH•. The RSC results of samples S1–S4 are shown in Table 2. It is well known that the DPPH method is based on the reduction of free DPPH radicals by an antioxidant acting as a donor of hydrogen atoms or electrons (Fig. 1.).[9]

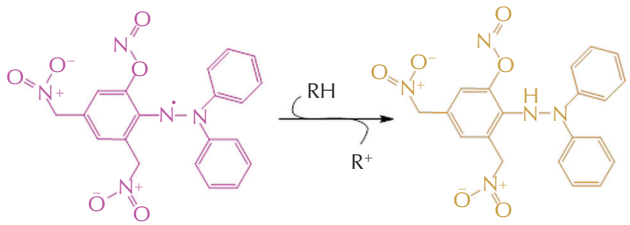

Fig. 1 – Reaction between the radical DPPH• (violet) and an antioxidant yields the neutralised DPPH molecule (yellow-brown)[15]

Slika 1 – Reakcija između radikala DPPH• (ljubičasta) i antioksidansa daje neutralnu molekulu DPPH (žuto-smeđa)[15]

The DPPH• has a violet colour due to the presence of an unpaired electron, and after reaction with an oxygen atom from the phenolic compounds present in the samples, it forms a yellow coloured 2,2′-diphenyl-1-picrylhydrazine (DPPH-H) compound.

Table 2 – Total free radical scavenger capacity (RSC) of samples S1–S4

Tablica 2 – Ukupni kapacitet uklanjanja slobodnih radikala (RSC) uzoraka S1 – S4

Concentration / mg ml^{-1}	RSC / %			
	S1	S2	S3	S4
0.10	42.70	46.85	43.32	39.80
0.20	42.95	47.36	43.58	41.56
0.40	43.70	47.48	44.08	42.82
0.60	44.96	47.86	44.71	44.08
0.80	45.72	48.11	44.96	45.72
1.00	45.97	48.61	45.84	46.73

Another way to express the antioxidant capacity of a sample is the IC_{50} (half maximal inhibitory concentration). The IC_{50} value (mg ml^{-1}) has been defined as the extract concentration required to react with 50 % DPPH radicals (RSC = 50 %) under previously defined experimental conditions.[9] The IC_{50} was calculated using the equation from the curves that describe the relation between RSC and concentration. Fig. 2 shows the calibration curves for calculating the IC_{50} values, while the obtained IC_{50} concentrations for samples S1–S4 are shown in Fig. 3.

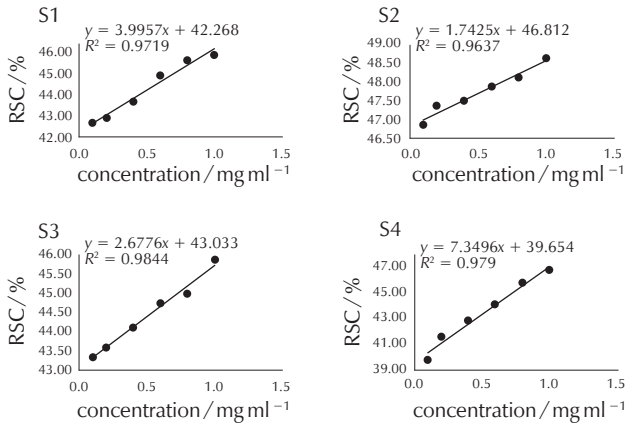

Fig. 2 – Calibration curves for determined IC_{50} values of samples S1–S4

Slika 2 – Kalibracijske krivulje za određene vrijednosti IC_{50} uzoraka S1 – S4

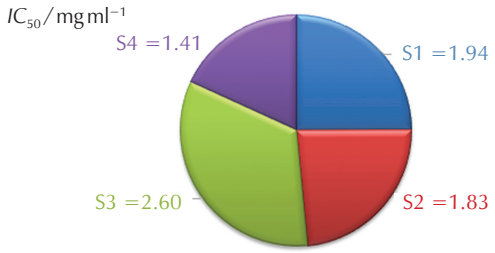

Fig. 3 – Graphic representation of the obtained IC_{50} values for the tested samples (S1–S4)

Slika 3 – Grafički prikaz dobivenih vrijednosti IC_{50} za ispitivane uzorke (S1 – S4)

Higher IC_{50} values mean a lower antioxidant capacity of the sample. Reasons why this method is frequently used are its simplicity and speed, as well as the favourable equipment for its implementation.[9,16] Based on the IC_{50} values shown, it is obvious that samples S1–S4 showed antiradical activity. Sample S3 had the highest antioxidant capacity and S4 the lowest.

Concentration dependence in relation to expressed activity was observed in all samples. Based on the IC_{50} values, it can be said that sample S1 has a lower antioxidant capacity than sample S2, the same applies to samples S3 and S4. Thus, extracts prepared by maceration with methanol as a solvent showed better antioxidant capacity.

Thus, the aforementioned suggested that the antioxidant capacity was affected by the extraction method. Possible reason for more successful maceration extraction is the use of methanol as solvent. It is known that the type of extraction solvent used can further affect the efficiency of the method.[13,14]

The capacity for neutralising DPPH radicals of samples S1–S4 was compared with the capacity for neutralising ascorbic acid as standard, *i.e.*, the obtained IC_{50} values of samples S1–S4 were compared with the obtained IC_{50} value (0.25 mg ml^{-1}) of ascorbic acid (Fig. 4), which is used as commercial antioxidant.

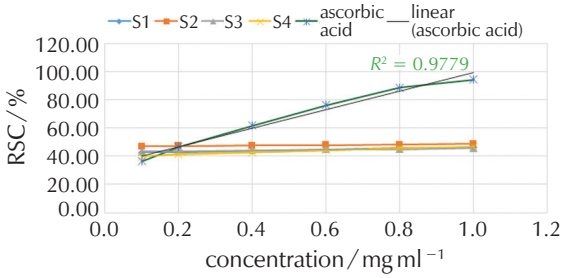

Fig. 4 – Graphic representation of the dependence of DPPH• inhibition on the concentration of samples S1–S4 compared to a commercial antioxidant, ascorbic acid

Slika 4 – Grafički prikaz ovisnosti inhibicije radikala DPPH• o koncentraciji uzoraka S1 – S4 u usporedbi s komercijalnim antioksidantom, askorbinskom kiselinom

Based on the results obtained, it can be concluded that the concentrations of samples S1–S4, which were required to neutralize 50 % of DPPH radicals, were significantly higher than the required concentration of ascorbic acid.

Many studies have shown that the difference in the antioxidant activity of seeds of different watermelon varieties may be due to the presence of phenolic components in different extracts, as phenols have a high antioxidant activity compared to other phytochemicals.[17,18]

O. A. Oseni and *V. I. Okoye*[19] reported a 56.93 % ability to remove free DPPH radicals from watermelon seeds. This corresponds to the percentage of the free radical scavenging ability of seeds Charleston Gray watermelon varieties shown by *A. Ramazan et al*.[20] in their study. These studies agree with our results. However, a study conducted by *B. Tabiri*[17] shows different antioxidant activity in three watermelon varieties. Crimson Sweet watermelon seed showed the highest percentage of DPPH removal ability with 94.46 %, followed by Black Diamond with 70.06 % removal ability, and Charleston Gray 59.88 %.[17]

Free radicals are a constant threat to our bodies and are known to be destroyed by antioxidants. Antioxidants are therefore essential components in the fight against aging. Antioxidants also offer protection against tissue damage caused by harmful environmental factors and other active ingredients, and stimulate collagen growth by fighting harmful free radicals.[21] Consuming watermelon seeds can reduce the likelihood of cardiovascular disease and cancer due to the significant amount of phenols contained in the seeds.[17]

4 Conclusion

The present study investigated the effect of two extraction methods on the yield of watermelon-seed extract from two watermelon varieties, *Citrullus lanatus (Thunb.) Matsum, & Nakai,* grown in Croatia and Greece. Watermelon seeds contain many chemical compounds, like phenolic compounds such as flavonoids, tannins, and lactones, as well as alkaloids, lectins, *etc.*

Comparison of the extraction methods used revealed that the maceration method had lower extraction yields than the Soxhlet method.

The extract yield was probably influenced by solvent, extraction time, and temperature.

However, the antioxidant capacity varied significantly from technique to technique, and in this case, was higher for the extract obtained by maceration. The values obtained contribute to the fact that the consumption of watermelon seeds could reduce the risk of cancer and cardiovascular disease due to their antioxidant activity.

List of abbreviations and symbols
Popis kratica i simbola

RSA – radical scavenging activity
– antiradikalna aktivnost

DPPH – 1,1-diphenyl-2-picrylhydrazyl
– 1,1-difenil-2-pikrilhidrazil

IC_{50} – half maximal inhibitory concentration
– polovica maksimalne inhibitorne koncentracije

A_S – absorbance of sample
– apsorbancija uzorka

A_B – absorbance of "blank"
– apsorbancija slijepe probe

A_C – absorbance of "control"
– apsorbancija kontrole

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SAŽETAK

Antioksidativna aktivnost sjemenki lubenice određena DPPH testom

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Svrha ovog istraživanja bila je procijeniti antioksidativno djelovanje ekstrakata svježih sjemenki lubenice uzgojenih u Hrvatskoj i Grčkoj. Ekstrakti su pripremljeni dvjema metodama: Soxhletovom metodom i maceracijom. Etanol je upotrijebljen kao otapalo u Soxhletovoj metodi, a metanol u maceraciji. Maseni udjeli ekstrakta dobiveni maceracijom niži su od onih dobivenih Soxhletovom metodom. Antioksidacijski kapacitet ekstrakta svježih sjemenki lubenice određen je metodom DPPH (2,2-difenil-1-pikrilhidrazilni radikal).

Antioksidacijski kapacitet ekstrakata također je izražen s IC_{50} (polovica maksimalne inhibitorne koncentracije). Na temelju dobivenih vrijednosti IC_{50} (1,41 do 2,60 mg ml^{-1}), svi ispitani ekstrakti pokazali su antiradikalno djelovanje, ali je antioksidacijski kapacitet bio bolji u ekstraktima dobivenim metodom maceracije. Razlog tome može biti upotreba metanola kao otapala, što se moglo očekivati budući da je metanol polarnije otapalo od etanola.

Kapacitet neutraliziranja DPPH radikala analiziranih ekstrakata uspoređen je sa standardom za neutraliziranje askorbinske kiseline. Koncentracije svih ispitnih ekstrakata potrebne za neutraliziranje 50 % radikala DPPH bile su znatno veće od potrebne koncentracije askorbinske kiseline (0,25 mg ml^{-1}).

Ključne riječi
Sjemenke lubenice, Soxhletova ekstrakcija, maceracija, DPPH

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