Anti-microbial and Anti-oxidant Activity of Watermelon (*Citrullus lanatus)* Fruit and Watermelon Seed

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Abstract

Watermelon (*Citrullus lanatus)* flesh and seeds were dried and pulverized, and their extracts were diffused to sterile discs for the evaluation of anti-microbial activity. The disc-diffusion technique was used to assess anti-bacterial activity against *Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Pasteurella multocida, Yersinia enterocolitica, Serratia marcescens, Klebsiella pneumoniae, Xanthomonas campestris, Staphylococcus aureus*. Anti-fungal activity against the yeasts *Candida albicans* and *Rhodotorula glutinis* was also examined. Standard anti-biotics were also tested as controls.

Watermelon flesh and seed extracts were found to be effective against gram-positive and gram-negative bacteria and yeasts. The extracts were also screened for anti-oxidant activity using the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) free-radical-scavenging assay, total reducing ability using the Fe^{3+}–Fe^{2+} transformation method and ferrous ion (Fe^{2+})-chelating activity. Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), ethylenediaminetetraacetic acid and α-tocopherol were used as reference anti-oxidant radical scavenger compounds.

The most potent anti-bacterial activity was demonstrated by watermelon–ethanol extract (inhibition zone 30 mm) against *K. pneumoniae,* and the most potent anti-fungal activity was demonstrated by watermelon–acetone extract (inhibition zone 26 mm) against *R. glutinis.* Watermelon–ethanol and watermelon seed–ethanol extracts both demonstrated marked anti-oxidant activity.

These results highlight that watermelon fruit and seed extracts have potential for the development of efficient, safe and cost-effective natural anti-oxidant compounds for application in the functional food industries.

Key Words: anti-microbial, anti-oxidant, *Citrullus lanatus,* watermelon, watermelon seed.

1 Introduction

Although the concept of anti-microbial activity became popular in the 1930s, anti-microbial resistance has emerged after the introduction of newer anti-microbial compounds.[1] This has prompted a search for alternative anti-microbial agents in the hope that novel products will trigger a decrease in anti-microbial resistance. Research into the anti-oxidant and anti-microbial activity of various common foods has already been conducted; for example, rich sources of proteins, such us; date, grape seed, pumpkin seed, hawthorn and absinthe have been analysed.[2-6] However, the analysis of watermelon fruit and seeds for new anti-microbial agents has not been reported. Therefore, the work presented here is a novel study.

The importance of controlling antioxidant activity has increased because of the consumption of foods containing significant amounts of unsaturated fatty acids. The interest in and use of anti-oxidants has

also increased as they can extend the shelf-life of food items, particularly those with high fat content. The use of synthetic anti-oxidants such as butylated hidroksiyanosil (BHA) and butylated hydroxytoluene (BHT), is limited because of their suspected carcinogenic properties.[7] Therefore, there has been a substantial increase in studies on natural plant-based anti-oxidants.[8]

There is a growing interest in substances possessing natural anti-bacterial activity and oxidation inhibition effects such as plants, herbs and spices. They have characteristic flavours and show anti-oxidant activity in addition to anti-microbial activity. [9] It is now well known that certain plant extracts (e.g., grape seed and green tea) have anti-microbial properties and contain catechins. Thus, the search for natural anti-oxidants and anti-microbial products is ongoing.[10]

In this study, as part of the search for alternative herbal anti-oxidants, we aimed to compare the anti-oxidant activity of freshly squeezed fruit juices and commercial fruit juices.[11] Anti-oxidant activity has been investigated in flax seed, bulbous plants, tomato (*Solanum betaceum* Cav.), salvia (*Salvia officinalis*), plants with leaves consumed as either salad or spices.[12-16]

Watermelon is one of the substantially underutilized fruits grown in the warmer parts of the world. Watermelon seeds are high in protein content and contain the complete range of essential amino acids. They are said to also contain considerable amounts of minerals which assist in the growth and development of a healthy body.[17] Watermelon seeds are used for oil production in India and some African countries.[18] Watermelon is cultivated in Turkey, and watermelon seeds are dried and eaten as a snack in South Turkey.

2 Materials and Methods

All chemicals and solvents used were of analytical purity. BHT was purchased from Merck. Ferrous chloride, α-tocopherol, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 3-(2-pyridyl)-5,6-bis(phenylsulfonic acid)-1,2,4-triazine (ferrozine), BHA, ethylenediaminetetraacetic acid and trichloroacetic acid were bought from Sigma. The anti-microbial activity of watermelon flesh and seeds was evaluated on seven different microorganisms obtained from the microbiology laboratories of Atatürk Health Services Vocational School, Kafkas University (Kars, Turkey) and the Biology Department, Atatürk University (Erzurum, Turkey). Anti-bacterial activity was tested against two gram-positive bacteria (*Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 25213), and seven gram-negative bacteria (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, *Serratia marcescens* ATCC 14756, *Yersinia enterocolitica* ATCC 27729, *Klebsiella pneumoniae* ATCC 4352, *Xanthomonas campestris* ATCC 33913 and *Pasteurella multicoda* ATCC 6538). Two species of yeast, *Candida albicans* ATCC 10231 and *Rhodotorula glutinis* ATCC 28052 were used as indicator microorganisms to detect anti-fungal activity.

2.1. Preparation of extracts

Watermelons were purchased from a local market. The fruit was separated from seeds and peel and was then washed thoroughly with distilled water. Watermelon fruit and seeds were dried at 50°C until constant weight was reached, and they were powdered. Seperate preparations of fruit and seed were made using a Waring blender. Different solutions were made by adding petroleum ether, water, ethanol and acetone solvents in a powder: solvent ratio of 1:20. The solutions were incubated for 8 h in a turbulent water bath at 50°C. Then they were centrifuged for 15 mins at 5000 rpm before filtering through Watman filter paper no 1. Solvents were evaporated at 60°C and the extracts obtained were loaded onto empty, sterile, filter-paper discs.

2.2. Preparation of microorganism culture and anti-microbial disc-diffusion assays

We used the agar-disc-diffusion method described to screen the extracts for anti-microbial activity.[19, 20] Bacterial stock cultures were prepared by inoculating Mueller–Hinton agar plates with commercial bacteriological loops containing the test organisms; these cultures were then incubated the plates at 37°C for 24 h. Fungal stock cultures were similarly prepared, using Sabouraud Dextrose Agar and incubating at 25°C for 24–48 h. Bacterial suspensions were prepared in nutrient

broth and fungal suspensions in Sabouraud dextrose broth, then incubated at 37°C for 24 h and at 25°C for 48 h, respectively. The turbidity of each culture was adjusted to obtain a similar optical density to that of McFarland 0.5 standard. To perform the disc-diffusion assays, a sterile filter-paper disc (6 mm in diameter) was impregnated with an extract and placed onto an inoculated agar plate under slight pressure. Ampicillin (20 μg/disc) and erythromycin (10 μg/disc) were used as positive controls, whereas sterilized distilled water, ethanol, petroleum ether and acetone were used as negative controls. Bacteria-inoculated plates were incubated at 37°C for 24 h, and yeast-inoculated plates were incubated at 25°C for 48 h. Following incubation, anti-microbial activity was evaluated by measuring the diameter (mm) of any observed inhibition zone, including that of the disc. Three independent experiments were performed for each assay, and data was collected for each in order to determine averages, which are presented here.

2.3. Determination of Reducing Power

The reducing power of the watermelon extracts was determined according to the method of Oyaizu (1958).[21] Different concentrations of the samples (20-100 μg/mL) in 1 mL of distilled water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH=6.6) and potassium ferricyanide (2.5 mL, 1%). The mixture was incubated at 50°C for 20 min and afterwards a portion (2.5 mL) of trichloroacetic acid (10%) was added to the mixture, which was centrifuged for 10 min at 1000xg. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%), and then the absorbance at 700 nm was measured in a spectrophometer. Higher absorbance of the reaction mixture indicated greater reducing power.

2.4. Determination of DPPH Free Radical Scavenging Activity

Free radical scavenging activity of the watermelon extracts was measured via $DPPH^{\cdot}$ by using the method of Blois (1986).[22] Briefly, 0.1 mM solution of DPPH. in ethanol was prepared, and this solution (1 mL) was added to 1,5mL of extracts at different concentrations (50-50 μg/mL). The mixture was shaken vigorously and allowed to remain at the room temperature for 30 min. Then, the absorbance was measured at 517 nm in a spectrophometer. The lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The DPPH concentration (mM) in the reaction medium was calculated from the following calibration curve and determined by linear regression (R: 0.997):

$$\text{Absorbance} = (0.0003 \times DPPH^{\cdot}) - 0.0174$$

The capability to scavenge the DPPH radical was calculated by using the following equation: $DPPH^{\cdot}$ scavenging effect (%) = $(A_0 - A_1/A_0) \times 100$, where A_0 is the absorbance of the control reaction, and A_1 is the absorbance in the presence of the samples or standards.

2.5. Determination of Ferrous Ions Chelating Activity

The chelation of ferrous ions by the watermelon extracts and standards was estimated by the method of Dinis *et al* (1994).[23] Shortly, the extracts (20-100 μg/mL) were added to a 2 mM solution of $FeCl_2$ (0.05 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL), and then the mixture was shaken vigorously and left remaining at the room temperature for 10 min. After the mixture had reached equilibrium, the absorbance of the solution was measured at 562 nm in a spectrophotometer. All tests and analyses were carried out in triplicate and averaged. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was given by the formula: Inhibition% = $(A_0 - A_1/A_0) \times 100$, where A_0 is the absorbance of the control, and A_1 is the absorbance in the presence of the samples or standards. The control did not contain compound or standard.

2.6. Statistical Analysis

The antioxidant and antimicrobial tests were conducted in triplicate and the results were expressed as mean ± standard error (SD) of the mean. Analysis of variance (ANOVA), tests were applied using Statistical Package for the Social Sciences (SPSS version 20.0) for comparisons of variance and means, and differences were considered significant at $P \leq 0.05$.

3 Results and Discussion

3.1. Anti-microbial disc-diffusion assays

The anti-microbial activities of the watermelon and seed extracts against the various bacteria and fungi were assessed by observing the presence or absence of inhibition zones and their diameters.

The diameters of the inhibition zones (including the disc diameter) are displayed in Table 1. The results show various levels of anti-microbial activity and degrees of germ sensitivity amongst the eight extracts. They reveal that the extracts inhibited microorganism growth, creating inhibition-zone diameters ranging 10–18 mm for gram-positive bacteria and 10–30 mm for gram-negative bacteria.

All watermelon fruit extracts obtained using ethanol, water and acetone displayed anti-microbial activity against test bacteria and yeast, but only the petroleum-ether extract demonstrated anti-microbial activity against *B. subtilis* and *R. glutinis* yeast (inhibition zone 8–30 mm). Only the water extract of watermelon and the ethanol extract of watermelon seed showed anti-microbial activity against *X. campestris.* The anti-bacterial and anti-fungal effect of the watermelon–petroleum-ether extract and watermelon seed–acetone extract were too low for determination.

The results from the anti-bacterial assays involving gram-positive bacteria reveal that the strongest activity was demonstrated by the watermelon–ethanol extract against *K. pneumoniae* (inhibition zone 30 mm), followed by the activity of the same extract against *E. coli* (inhibition zone 26 mm) and then the activity of the watermelon–acetone extract, which was similar in magnitude to the activity against *R. glutinis* (inhibition zone 26 mm) and *K. pneumoniae* (inhibition zone 25 mm).

For watermelon seed extracts, the maximal activity was observed for the seed–water extract against *E. coli* (inhibition zone 22 mm), followed by the same extract against *K. pneumoniae* (inhibition zone 20 mm).

The watermelon–petroleum-ether extract displayed the lowest overall level of activity, with inhibition zones on

B. subtilis and *R. glutinis* of just 10 mm. The inhibition zone shown by the watermelon–acetone extract on *S. marcescens* was just 10 mm. The same result was observed for the watermelon–ethanol extract on *S. aureus* and the water extract on *B. subtilis* (inhibition zone 10 mm). The lowest activity for watermelon seed extracts was demonstrated by the water extract against *S. marcescens,* with an inhibition zone of just 8 mm.

The results presented in Table 1 clearly show that watermelon and its seed extracts demonstrated anti-microbial activity against the tested bacteria and yeasts.

Table 1. Results of anti-microbial disc-diffusion assays for the eight watermelon extracts

Test microorganism	İnhibition zones (mm)									
	Extracts									
	W. Wat.	W. Eth.	W. Ace.	W. Pet.	WS. Wat.	WS. Eth.	WS. Ace.	WS. Pet.	A1	A2
B. subtilis	10c	15a	15a	10c	–	10c	–	13b	15a	10c
S. marcescens	12c	15b	10d	–	8e	–	–	–	12c	20a
E.coli	20c	26b	22c	–	22c	–	–	–	30a	30a
Y. enterocolitica	20a	–	21a	–	14b	–	–	–	–	20a
K. pneumoniae	13e	30a	25b	–	20c	–	–	–	12e	16d

P. aeruginosa	18b	23a	20b	–	–	10d	–	–	13c	10d
P. multocida	12b	15a	12b	–	–	10c	–	–	13b	12b
S. aureus	18a	10c	13b	–	–	–	–	–	15a	12bc
X. campestris	–	12b	–	–	15a	–	–	–	–	15a
C. albicans	18b	18b	14c	–	–	12d	12d	14c	30a	14c
R. glutinis	12d	20c	26b	10d	10d	–	–	10d	30a	20c

Wat.: Water; Eth.: Ethanol; Ace.:Aceton Pet.: Petroleum ether;

W.: Watermelon fruit; WS.: Watermelon seed

A1: Erythromycin10 mg; A2: Ampicillin 20 mg

a,b,c,d,e means in the same column followed by the same letter are not significantly different at the $p<0.05$ level.

The extracts obtained from watermelon fruit and seed demonstrated anti-fungal effects. The strongest activity was demonstrated by the watermelon–acetone extract towards *R. glutinis* (inhibition zone 26 mm), followed by that of the watermelon–ethanol extract (inhibition zone 20 mm). Against *C. albicans*, the watermelon–water and watermelon–ethanol extracts produced the greatest inhibition zones (inhibition zone 18 mm).

All bacteria and fungi in this study, except for *X. campestris* and *Y. enterocolitica*, which showed resistance to erythromycin, demonstrated sensitivity to erythromycin and ampicillin anti-biotics. Unlike the extracts, ampicillin was able to penetrate all the gram-positive and gram-negative bacteria. This may be attributed to the fact that the watermelon and seed extracts represented a complex mixture of components, in comparison to the purity of the anti-biotic.[24,6]

B. subtilis was as sensitive to the watermelon–ethanol and watermelon–acetone extracts as erythromycin (inhibition zones 15 mm for both), and these zones were larger than those produced by ampicillin (inhibition zone 10 mm). The watermelon–ethanol extract produced a greater inhibition zone against *S. marcescens* (inhibition zone 15 mm) than erythromycin. The watermelon–ethanol extract produced a greater inhibition zone than that of either of the anti-biotics, on *K. pneumoniae.* In addition, *P. aeruginosa* was more sensitive to watermelon–ethanol, watermelon–acetone and watermelon–water extracts than the tested anti-biotics. Erythromycin produced the greatest inhibition zone against both species of fungi (30 mm). Results for *C. albicans* revealed that the watermelon–ethanol and water extracts created wider inhibition zones than ampicillin, and the watermelon–acetone extract zone was of the same diameter on *R. glutinis* was more sensitive to the watermelon–acetone extract than to ampicillin.

In this context, anti-microbial activity appears not to be dependent on whether the tested bacteria are gram-negative or gram-positive or have a particular shape. It is demonstrated here that extracts of watermelon fruit and seed are anti-bacterial against both gram-positive bacteria (*B. subtilis and S. aureus*) and gram-negative bacteria (*Y. enterocolitica, P. multocida, P. aeruginosa, X. campestris, K. pneumoniae, E. coli and S. marcescens*). The extracts also demonstrated anti-fungal properties.[25, 6] The extracts generated even higher inhibition zones for the tested fungi (*C. albicans and R. glutinis*) than standard erythromycin anti-biotic. The reason for this could be that polyphenols exhibit a strong anti-fungal effect.[26, 27] Other studies have demonstrated the anti-microbial effect of flavonoids against gram-positive or gram-negative bacteria, particularly the flavones, flavonols, flavanones, anthocyanidins and flavins, which possess significant anti-bacterial activities.[28]

According to the results we obtained, watermelon extracts were more effective than watermelon seed extracts against the test bacteria and yeast. The extracts of watermelon and its seeds in this study

were both anti-bacterial and anti-fungal. These findings provide useful knowledge about the value of what we consume in our daily life, and of the fruits grown in our country.

3.2. Total reductive capability using the potassium ferricyanide reduction method

The reductive capabilities of extracts were assessed by the extent of conversion of the Fe^{3+}/ferricyanide complex to the Fe^{2+}/ferrous form. Therefore, Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm.[29] The reducing powers of the samples were observed at different concentrations, and results were compared with BHA, BHT and α-tocopherol. As shown from Figure 1, watermelon-ethanol and watermelon-water extracts had effective and powerful reducing power using the potassium ferricyanide reduction method when compared to the standard antioxidants. The reducing power of all extracts (except watermelon-petroleum ether, watermelon seed-petroleum ether) BHA, BHT and α-tocopherol increased steadily with increasing concentration of samples. Reducing power of the extracts and the standards were found as following order: BHA > watermelon-ethanol > watermelon-water > BHT > α-tocopherol > watermelon seed-ethanol > watermelon seed-acetone > watermelon-acetone > watermelon seed-water, at the highest concentration.

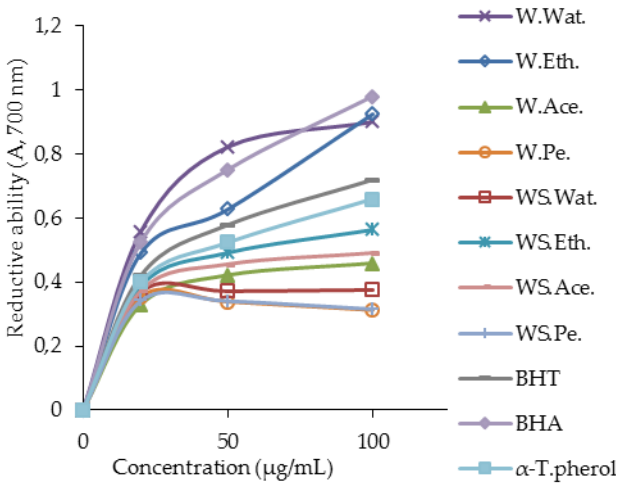

Figure 1. Total reductive potential of different concentrations of extracts, BHT, BHA and α-tocopherol.

3.3. DPPH· radical scavenging activity

Antioxidant properties, especially radical scavenging activities, are very important due to the deleterious role of free radicals in foods and in biological systems. Excessive formation of free radicals accelerates the oxidation of lipids in foods and decreases food quality and consumer acceptance.[30, 31] The model of scavenging the stable DPPH radical is a widely used method to evaluate antioxidant activities in a relatively short time compared with other methods. The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen donating ability.[32] DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule.[33] The reduction capability of DPPH radicals was determined by decrease in its absorbance at 517 nm induced by antioxidants. The absorption maximum of a stable DPPH radical in ethanol was at 517 nm. The decrease in absorbance of DPPH radical was caused by antioxidants because of reaction between antioxidant molecules and radical, progresses, which resulted in the scavenging of the radical by hydrogen donation. It is visually noticeable as a discoloration from purple to yellow.

In the study, antiradical activities of extracts and standard antioxidants such as BHA, BHT and α-tocopherol were determined by using DPPH· method. Scavenging effect values of extracts, BHA, BHT and α-tocopherol at different concentrations are given Figure 2, which illustrates a decrease in the concentration of DPPH radical due to the scavenging ability of extracts, except watermelon-water, and standards. The scavenging effect of extracts and standards on the DPPH· decreased in the order of α-tocopherol > BHA = watermelon-ethanol > watermelon seed-ethanol > watermelon-acetone > BHT > watermelon seed-water > watermelon seed-acetone, at the highest concentration.

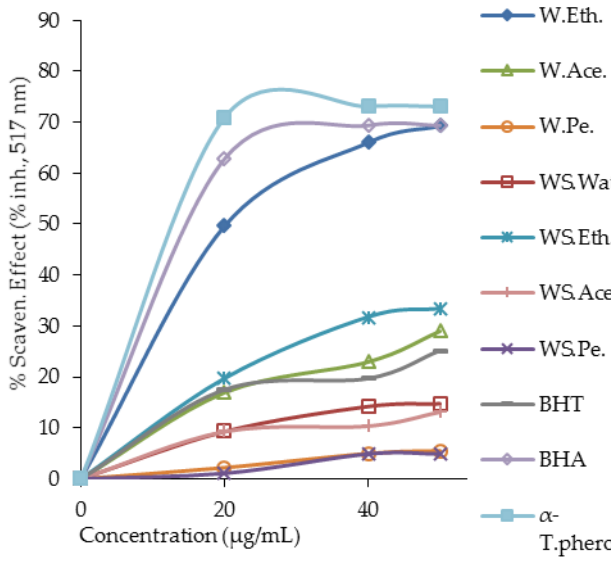

Figure 2. Scavenging effect of extracts, BHT, BHA and α-tocopherol at different concentrations (20-40-50 µg/mL).

3.4. Ferrous ion chelating activity

The chelating effect towards ferrous ions by the extracts and standards was determined. Ferrous ion can form quantitatively complexes with Fe^{2+}. In the presence of chelating agents, the complex formation is disrupted with the result that the red color of the complex is decreased. Measurement of color reduction, therefore, allows estimation of the chelating activity of the coexisting chelator.[34] Transition metals have pivotal role in the generation oxygen free radicals in living organism. The ferric iron (Fe^{3+}) is a relatively biological inactive form of iron. However, it can be reduced to the active Fe^{2+}, depending on condition, particularly pH [35] and oxidized back through Fenton type reactions with the production of hydroxyl radical or Haber-Weiss reactions with superoxide anions. The production of these radicals may lead to lipid peroxidation, protein modification and DNA damage. Chelating agents may not activate metal ions and potentially inhibit the metal-dependent processes.[36] Also, the production of highly active ROS such as $O_2^{\cdot-}$, H_2O_2 and $OH^{\cdot}$ are catalyzed by free iron through Haber-Weiss reactions:

$$O_2^{\cdot-} + H_2O_2 \rightarrow O_2 + OH^- + OH^{\cdot}$$

Among the transition metals, iron is known as the most important lipid oxidation pro-oxidant due to its high reactivity. The ferrous state of iron accelerates lipid oxidation by breaking down the hydrogen and lipid peroxides to reactive free radicals via the Fenton reactions:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot}$$

Fe^{3+} ion also produces radicals from peroxides, even though the rate is tenfold less than that of Fe^{2+} ion, which is the most powerful pro-oxidant among the various types of metal ions.[37]

Ferrous ion chelating activities of extracts and standard antioxidants such as EDTA and α-tocopherol at different concentrations are shown in Figure 3, which illustrates a decrease in the concentration of metal-chelate complex due to the metal chelating effect of only for watermelon seed-ethanol, watermelon seed-water and watermelon-water extracts. Low absorbance at 562 nm indicates high metal chelating activity. The metal chelating effect of extracts and standards decreased in the order of EDTA > α-tocopherol > watermelon seed-ethanol > watermelon seed-water > watermelon-ethanol, which were 87.3%, 74.2%, 62.7%, 48.7%, 25.8%, at the highest concentration, respectively.

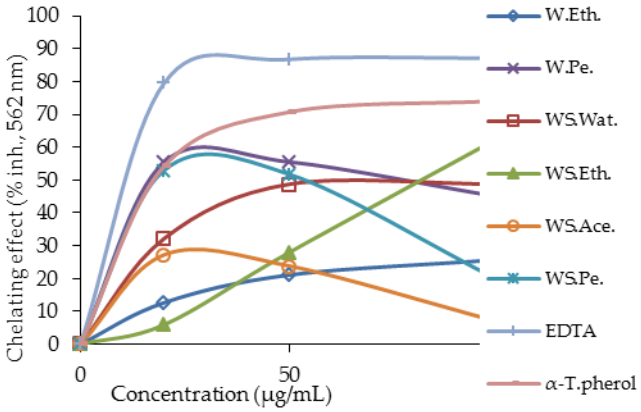

Figure 3. Metal chelating effect of different amount of the extracts, EDTA and α-tocopherol on ferrous ions.

4 Conclusion

The present study is the first to report the anti-microbial activities and anti-oxidant properties of watermelon fruit and seed extracts. The extracts demonstrated a wide range of anti-microbial activity against various pathogens, generating inhibition-

zone diameters of up to 30 mm.

According to data obtained from the anti-oxidant evaluation, the ethanolic extract of watermelon fruit and seed demonstrated marked reducing power, DPPH-scavenging activity and iron-binding ability. Furthermore, watermelon seed–water extract possessed moderate activity for all three methods.

In conclusion, these results highlight that watermelon fruit and seed extracts have potential for the development of efficient, safe and cost-effective natural anti-oxidant compounds for application in the functional food industries. However, further studies to isolate the putative biochemical compounds from the extracts are needed.

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