Antioxidant Activity in Some *Citrus* Leaves and Seeds Ethanolic Extracts

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***Abstract*---** This study was conducted on selected leaves and seeds of Citrus species such as *(C. aurntium* L., *C. limon* (L.) Burm. f., *C. paradisi* Macfad, *C. reticulata* Blanco, and *C. sinensis* (L.) Osbeck to investigate antioxidant activity. The extracts of the leves and seeds were analyzed for total phenolic content, total flavonoides, Antioxidant activities, Reducing power, chelating ions and scavenging hydrogen peroxide. The ethanoic extracts of grapefruit was 373.2 GAE/ g dw shown superior amount of total phenolic compounds, scavenging of Hydrogen peroxide 95.1% , chelating of ferrous ion 70.8%, sour orange seeds extract had higher flavonides (371.3 mg/100g dw) compering with other four species, antioxidant activity of orange seeds was 70.5 mg/ml, and ethanol extracts of lemon seeds had maximum priority in reducing power was (220.2 %) Also the Increasing the concentration of the extracts led to increase of exhibited inhibition of peroxidation in linoleic acid system.

***Keywords*---** Antioxidant activity, Citrus seeds and leaves, H_2O_2

I. Introduction

PLANTS such as fruits have a lot of biological effective compounds, that have ability to attack radical free and work as anti-natural oxidative stress such as phenolic compounds (phenolic acids, flavonoids and tannins) make them play an important role in reducing the risk of many diseases like cancers, cardiovascular, and neurological diseases. [1]-[2]

The auto fatty oxidation happened when they interact direct with oxygen this led to have negative effects on the quality of the food and the loss of value in terms Foods [3] caused by a short chain fatty acids, alcohols, ketones and aldehydes as a final outputs of the process of auto fatty oxidation -service that is responsible for a flavor that unacceptable smell [4].

There is a known method of protection from the risk of oxidative stress the use of certain substances that have the ability to suppress, or reduce, or delay the oxidative stress defined as antioxidant compounds [5].

This compounds act as a widespread in nature and have diversity mechanisms via their interaction with radical free in fat and composition of stable and inactive output [6] like (BHT) Butylated hydroxyl toluene, (BHA) Butylated hydroxyl anisole, and (PG) Propyl gallate.

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There is an increasing interest in the antioxidant effects of compounds derived from plants, which could be relevant in relation to their role in health and disease beside their nutritional value. Different aromatic herbs and spices have been investigated for their antioxidant activity. Some, particularly those belonging to the Rutaceae family have been found to be very effective with regard to natural antioxidants.

Many doubts about the suitability of this compounds for antidepressants in terms of health and their use has become controversial as carcinogens or toxic effects or which has toxic effect [7].

Thus recently raised focusing on the potential of natural resources in plants especially edible one as phenolic compounds – as natural antioxidant- which is aromatic compounds bearing one or more groups of hydroxylated groups exist in almost all plant parts like leaves [8]-[9].

Citrus is one of the important plants economically but attention leaves, and seeds the role of citrus not given importance in comparison fruits despite the presence of phenols quantity that varies among spices [10]. Goal of this study to know the effectiveness of plant extracts and antioxidants that have a role in the food manufacturing.

II. Materials And Methods

A. Sample Preparation

fresh leaves and seeds of five spices of Citrus, *C. aurntium* L., *C. limon* (L.) Burm. f., *C. paradisi* Macfad, *C. reticulata* Blanco, and *C. sinensis* (L.) Osbeck were collected from local markets in Dayala city. The specimens were identified and deposited at Baghdad, Iraq: college of education herbarium, university of Baghdad (BUE). These samples were dried in liquid nitrogen, smashed to powder, and storage at 4C° till used.

B. Extraction technique

An accurately weighed sample of Citrus powder 100 gm from each species and add 500 ml ethanol (95 %), as [11] recorded and mixed via magnetic mixer, after this put it at room temperature, and filtered with filter papers what man No.1 the resulting extract was dried under vacuum on a rotary evaporator at 45 ºC to remove the solvent, put filter – as sticky substance in dark bottles at 4ºC till used [12].

C. Determination of total phenols

High performance liquid chromatography (HPLC) [12] as stated in the [13] with some modification, the leaves and seeds powder were separated by a central chapter quickly and 7500 r

/ min for 15 minutes. Column was composed of column: Zorbaxclips XDB-C-18, 3µm particle size (50 x 4.6 mm Mobile phase: water: methanol: acetonitrile (50:40:10) gradient program from 0% B to 100% B for 8 minutes Detection UV set at 280 nm. Temperature: ambient Flow rate: l ml / ml Sample Injection volume: 20µl was calculated concentration of phenolic material in the sample according to the following equation: Conc. of Sample Mg / m = (Area of sample) / (Area of standard) × conc.of standard × dilution factor.

D. Determination of total flavonoid contents

Followed by [5] to determination total flavonoid in extracts. Solved 1 gm extract in 1.5 ethanol (95%) with $AICI_3.6H_2O$ (2%) , the absorbance at 367 nm was analyzed with a spectrophotometer and the flavonoid content was calculated based on concentrations analyzed relationship between con. of acid and absorption.

E. Total Antioxidative assay

by thiocyanate method, Different amounts of samples dissolved in 100 [A of chloroform were put736 into a solution of linoleic acid 0.13 ml, 10 ml ethanol(99.5 %), and 10 ml phosphate buffer PH = 7 (0.05 M), the total volume was adjusted to 25 ml with distilled water. The mixed solution in a conical flask was kept in a constant temperature oven at 40°C. At intervals during incubation, using thiocyanate by added this above solution to ethanol 9.7 ml |(75%) , thiocyanate ammonium 0.1 ml (30%) and after 3 min added Fe_3 Cl_2 0.1 ml (20µM) that prepared in HCL (3.5%) as a coloring reagent and the absorbance at 500 nm. the colored solution was measured with percentage of inhabitation of peroxidase of linoleic acid according to equation: Antioxidative activity % = 1- (**A** sample / **A** control) x 100. Where AControl is the absorbance of the control, and ASa mple is the absorbance in the presence of the sample of aqu eous and alcoholic extracts. The outcome data compared with BHT and E vitamin were used as standard

F. Reducing power

The reducing power was determined according to the method of [14]. Each extract in ethanol (2.5 ml) was mixed with 2.5 ml of 200 mM sodium phosphate buffer (pH 6.6, and 2.5 ml of 1% potassium ferricyanide, and the mixture was incubated in water bath for 20 min at 50 °C. After incubation, 2.5 ml of 10% trichloroacetic acid (w/v) were added; the mixture was centrifuged at 3000 rpm for 10 min. The upper layer (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% ferric chloride, and the absorbance was measured at 700 nm against a blank in spectrophotometer. The reducing power percentage was measured via equation: reducing power % = 100- (A sample / **A** control) x 100., the outcome data compared with BHT and Ascorbic acid were used as standard andphosphate buffer as blank solution. The absorbance of the f inal reaction mixture Increased absorbance of the reaction mixt ure indicates stronger reducing power. The outcome data compared with BHT and Ascorbic acid were used as standard

G. H_2O_2 scavenging activity

H_2O_2 scavenging ability of aqueous and alcoholic extracts of Citrus species was determined according to the method of [15].as reported [16] A solution of H_2O_2 (40mM) was prepared in phosphate buffer (pH 7.4). The alcoholic extract at the 30µg/mL concentration in 3.4mL phosphate buffer were ad ded to H_2O_2 solution (0.6mL, 40mM). The absorbance value of the reaction mixture was recorded at 230 nm. Blank solution was containing the phosphate buffer without H_2O_2.

The percentage of H_2O_2 scavenging of alcoholic extract and standard compounds were calculated using the formula: [(AControl- ASample) /AControl] × 100. The outcome data compared with Rutine and Ascorbic acid were used as standard

H. Measurement of ferrous ion chelating activity

The abilities of iron-chelating of the Citrus extracts and standards was estimated by the method of [17] as [18] with minor changes. Four dilutions in (DMSO) Dimethyl sulfoxide $(CH_3)_2SO$. (20 mg/mL, 10 mg/mL, 5 mg/mL and 2.5 mg/mL) were prepared from the dried extracts. Briefly, 0.05 mL of each dilution was added to a 2.7 mL TRIS buffer (pH=7.4). Thereafter, 0.05 mL of 2 mM $FeCl_2$ were added and vortexed for 15 sec. At 30 sec, the reaction was initiated by the addition of 5 mM ferrozine (0.2 mL), the mixture was shaken at vortex for 10 sec. After 1 min beyond addition of $FeCl_2$ solution, absorbance of the solution was measured spectrophotometrically at 562 nm. The ability of extracts to chelate ferrous ion was calculated relative to the control (consisting of TRIS buffer, iron and ferrozine only) using the formula: chelating activity (%) = 100 x [(A Control x A Sample)/A Control], the outcome data compared with Citric acid and EDTA were used as standard.

III. Results And Discussion

A. Determination of total phenols

Figure 1 shown the total phenols of the different cultivar citrus seeds and leaves extracts of their concentrations of phenols, in general the phenols in seeds were higher more than leaves, particularity seeds and leaves of grapefruit in maximum range of phenols were (373.2, 292.1 GAE/ g dw), while relatively equal percentage of phenols in seeds and leaves of sour orange were (262.7, 242.8 GAE/ g dw), and mandarin were(123.6, 122.5 GAE/ g dw). According to [19], the total polyphenols ranges between 36.9 and 75.9 mg GAE/ g dw) of eight different citrus fruit, while in white grapefruit and his hybrid, the phenolic content are 63.0 and 69.6 (mg gallic acid equivalent /g dw) [20]). Phenols in our result of in citrus species were much higher than that of Algerian date [21], our results agree with [22] that they studded mandarin cultivars. As far as we know, there is no such investigation of these citrus species in Iraq. The difference among these species back up to several factors such as the genetic differences amongst different taxa, the tissue analyzed, as well as the geographical origin and the clones [23], maturation stages [24], extraction time, temperature and solvent [25] plant height [26], and vegetative rootstocks [27] could influence the phenolic content.

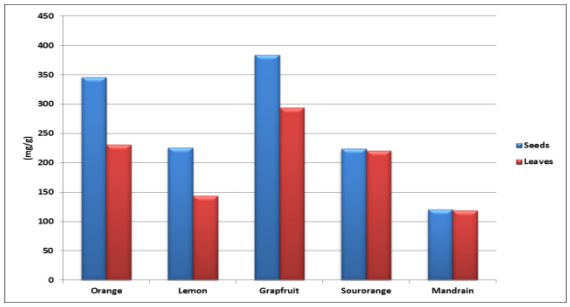

Fig 1 total phenols content in extract seeds and leaves citrus species

B. Determination of total flavonoid contents

Sour orange was recorded maximum range of total flavonoid contents in its seeds extract was(371.3 mg/100g dw) compering with other four species, while graduate the seeds extract of mandarin, orange, lemon, and grapefruit in total flavonoids contents were (156.7, 151.0, 126.2, 125.1 mg/100g dw). Despite the extract leaves of sour orange not has so different with other species, but it had the highest levels was(123.5 mg/ 100 g dw), while grapefruit had the lowest (85.3 mg/100 g dw) respectively.as figure 2 shows. [19] referred that flavonoid content in different studied citrus fruits ranges from 8.41 to 21.6 mg Rutin Equivalent/ g dw, whereas the content of total flavonoids in peeled hybrids (mg Catechin equivalent/100 g fresh weight) in white grapefruits are 47.12 ± 4.1 and 37.7 ± 3.2 [20]. In this study, the total flavonoid levels were within the range of [19] study, citrus flavonoid composition appears based on their genetic origin, the age of leaves, time of collection, and the different parts of the used fruit (peel, and edible parts) [28]. The presence and/or not of flavonoids in seeds can be affected by the fruit development stage [29]-[30]. Furthermore, [31] reported that naringin content of grapefruit juice from the same trees fluctuates during a season and varies considerably between crop years. The difference between phenol s and flavanoids was found Because of the diversity and complexity of the natural mixtures of phenolic compounds, measuring methods are using for determination, occurrence of other compounds in the sample, The amount of phenols was also dependent on the extraction solvent, Characterize every compound and elucidate its structure, the utilization of the molecule in the complex phytochemical factory of the plant. etc. [32]-[33]

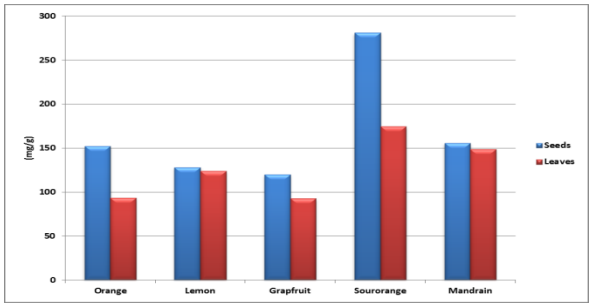

Fig 2 total flavonoids content in extract seeds and leaves citrus species

C. Total Antioxidative assay

Reached maximum with percentage of inhabitation of peroxidase of linoleic acid was shown to be the most active orange seeds was (70.5 mg/ml) with concentration 120 mg/ml, followed by the sour orange leaves and orange leaves were(66.7, 65.3 mg/ml), while lemon seeds (35.1 mg/ml) clearly showed a lower range, as figure 3 indicated all species under study were appeared antioxidant activity lower than BHT, and E vitamin. Raised the percentage of inhabitation of peroxidase of linoleic acid with increased the concentration. The author suggest there are another compounds effect on the antioxidant activity [20]-[24]- [34]-[35]- [36]-[37]. However, not only the qualitative characterization of different constituents was important but also the knowledge of their amounts was so important. Thus, the action of the different compounds, which might comprise synergic and antagonist effects, present in the citrus seeds and leaves extract result in the total antioxidant activity.

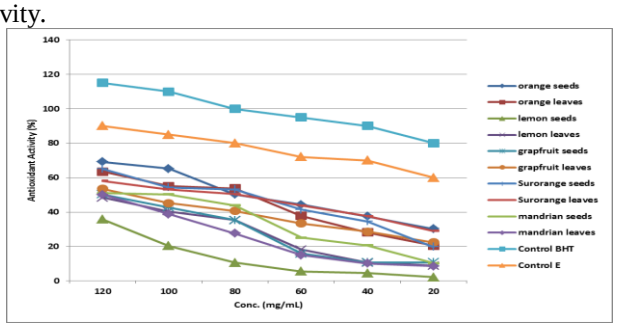

Fig 3 antioxidant activity in extract seeds and leaves citrus species

D. Reducing power

Figure 2 shows the reducing power of the different citrus species alcohols extracts. The reducing power of the extracts increased with their concentrations in the medium. The values of absorbance at 700 nm for the seeds and leaves revealed that all samples had a capacity to reduce iron (III) and had electron donor properties for neutralizing free radicals by forming stable products [22]. The present work has been done to the reducing reaction is to terminate the radical chain reactions that may otherwise be very damaging [38], In this assay the reducing power of the citrus species extracts had lemon seeds extract compete with vitamin E was (220.2%), followed by orange seeds and lemon leaves extract were (175.3, 175 %) but grapefruit had minimum rang was (50.5%). An increase in absorbance of the reaction mixture would indicate an increase in reducing capacity [39]-[40]. The yellow color of the test solution changes to various shades of green and blue depending on the reducing power of each compound [41]-[42]. Many studies focused on the relationship between reducing power values the antioxidant activity of the phenolic compounds, as[43]-[44]-[45]. The results showed that omission of chloroform fraction reports could lead to a significant correlations between antioxidant activities, phenolic and flavonoid contents in polar and semi-polar fractions [46].

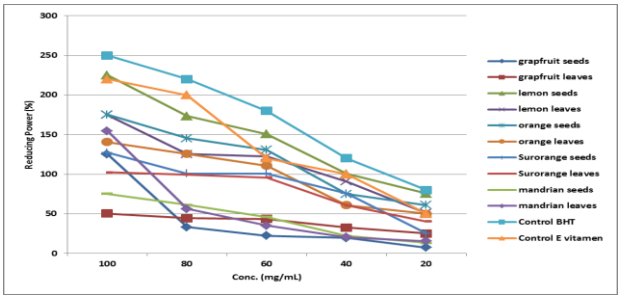

Fig 4 reducing power in extract seeds and leaves citrus species

E. H_2O_2 scavenging activity

Antioxidant compounds present in the extracts/standard can donate electrons to H_2O_2 and converted to H_2O. Extracts of Citrus spices showed promising antioxidant activity 95.1, 92.1, 78.1, 60.4, 35.3 % for seeds alcoholic to grapefruit, orange, lemon, sour orange, and mandarin) while the leaves extract were 80 % for orange and grapefruit, followed by 78.2 % in lemon, 76.3% in sour orange, and was the minimum presents in mandarin was 50.1% . Reference standard yielded 79.1 %, and 77.5 %H_2O_2 scavenging activity (Hydrogen peroxide inactivates a few enzymes directly, usually by oxidation of essential thi ol group (-SH). responsible for various toxic effects can cross membranes and reacts with Fe^{2+} and Cu2+ ions to form hydroxy radical.

Antioxidant compounds react with H_2O_2 and converted into H_2O. Quantitative phytochemical indicated that the plant co ntains significant amounts of phenolic compounds were res ponsible for antioxidant andfree radical scavenging effect of pl ant material[47].

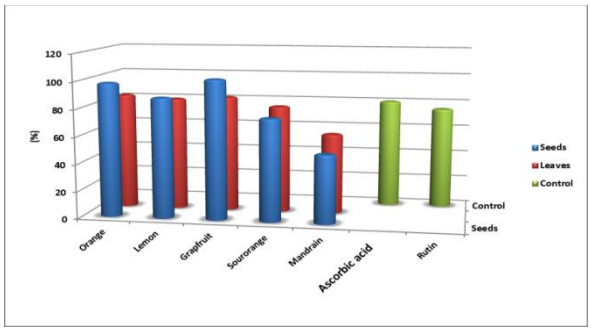

Fig 5 H_2O_2 scavenging activity in extract seeds and leaves citrus species

F. The chelating of Fe^{2+}

The presence of chelating of Fe^{+2} in the figure 6. Measurement of color reduction, therefore, allows the estimation of the chelating activity of the coexisting cheater. All extracts showed a variety of increasing activity, grapefruit seeds approach with lemon seeds were (70.8, and 68.7 %) at 5 conc. mg/ml, while clearly the orange leaves was had minimum range was(23.5%) mg/ml. The transition metal ion, Fe^{2+} possess the ability to move single electrons by virtue of which it can allow the formation and propagation of many radical reactions, even starting with relatively non-reactive radicals [48].

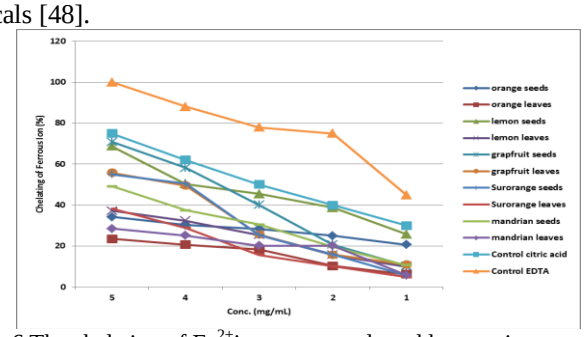

Fig 6 The chelating of Fe^{2+}in extract seeds and leaves citrus species

IV. Conclusion

The results on the basis it is concluded that the extracts of seeds and leaves o Citrus contain high quantities of phenolic compounds, which exhibit antioxidant and free radic al scavenging activity, total flavonoides, Antioxidant activities, Reducing power, chelating Fe^{+2}ions, and scavenging hydrogen peroxide practically in seeds. all these constituents helps extracts of Citrus to be as effectiveness natural antioxidants as leaves and seeds to have a role in the food manufacturing.

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