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Research Article

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Phytochemical Screening, Free Radical Scavenging and Phenolic Content Evaluation of Aqueous- Ethanol Extract of Seeds of *Citrus sinensis* (L) Osbeck (Rutaceae)

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ABSTRACT

Citrus by-products are a major source of phenolic compounds which could serve as natural antioxidants. This study was carried out to determine the free radical scavenging potential and the phenolic content of the aqueous-ethanol extract of the seeds of Citrus sinensis. Preliminary phytochemical screening of the crude extract was also investigated to find out the different secondary metabolites present. The seeds of C. sinensis were obtained from local source, dried and pulverized using mechanical grinder. The resulting powder was subsequently extracted by cold maceration in 70% ethanol. Preliminary phytochemical screening was carried out employing standard procedures. Free radical scavenging and phenolic content was determined, utilizing DPPH and Folin-ciocalteu's phenolic reagent respectively. Phytochemical screening revealed the presence of tannins, carbohydrates, alkaloids, flavonoids, cardiac glycosides and steroids. The per cent free radical scavenging activity of the extract was found to be 89.30%, with IC_{50} of 0.136 mg/mL. The standard substance (ascorbic acid) gave 86.66% inhibition of the DPPH radical activity, with IC_{50} of 0.332 mg/mL. The total phenolic content of the extract expressed as gallic acid equivalent was 8.54 mg/g of dry mass at 1mg/mL concentration. There was a positive correlation between radical scavenging activity and phenolic content of the extract. The high free radical scavenging activity demonstrated by the aqueous-ethanol extract of the seeds of C. sinensis is traceable to the copious amount of total phenol found in the extract. There is need for further phytochemical study in order to isolate the particular compounds that could be responsible for this activity.

Keyword: Free radical; Phenolic content; *Citrus sinensis;* Aqueous-ethanol extract; Flavonoids

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INTRODUCTION

Free radicals and reactive oxygen species (ROS), which are formed under normal physiological condition could lead to oxidative stress when not being eliminated by the endogenous antioxidant system. In fact, oxidative stress results from an imbalance between the generation of reactive oxygen species and endogenous antioxidant systems, and this is the fundamental mechanism underlying a number of human neurodegenerative disorders; diabetes, inflammation, viral infections, autoimmune pathologies and digestive system disorders [1]. There are some synthetic antioxidants in use such as vitamin C and E, which could prevent cellular damage resulting from free radicals and ROS; but these are known to have several side effects, such as risk of liver damage and carcinogenesis in laboratory animals [2]. Accumulated evidence suggests that ROS can be scavenged through chemoprevention utilizing natural antioxidant compounds present in foods and medicinal plants. The secondary metabolites like phenolics and flavonoids from plants have been reported to be potent free radical scavengers [3]. They are found in all parts of plants such as leaves, fruits, seeds, roots and bark. *C.sinensis* (sweet orange) fruits and juice are an important source of bioactive compounds which is attributed to the presence of antioxidants such as ascorbic acid, flavonoids, phenolic compounds, hydroxycinnamic acids, carotenoids and pectins [4]. Citrus is a large genus that includes several major cultivated species, including *C. sinensis*, *C. reticulate*, *C. lemon*, *C. grandis*, and *C. paradise.* Orange trees were

found to be the most cultured fruit in the world and are widely grown in tropical and subtropical climate [5]. Citrus by products such as the seeds and peels have been reported to have the highest concentration of flavonoids [6]. Although the citrus fruits and by products are believed to have several health benefits, empirical findings to validate such belief are scanty. For this reason we have undertaken in this work, to find out by *in vitro* experiments the free radical scavenging activity of the seed extract of *C. sinensis* using 2, 2-Diphenyl-1-picrylhydrazyl (DPPH).

EXPERIMENTAL SECTION

Plant material

Sweet orange (*C. sinensis*) was collected at the ripening stage from an orange tree located at Sagbama town, Bayelsa State (South of Nigeria). It was identified and authenticated by Dr. T. Oladele, Department of Pharmacognosy and Herbal Medicine, Faculty of Pharmacy, Niger Delta University. The seeds were collected by cutting open the edible parts of the fruit into two slices, squeezing out the juice. The seeds obtained were oven dried at 50 - 60^{o}C, then pulverized into fine powder using a grinder.

Chemicals

All solvents used in the experiment (n-hexane, dichloromethane, ethyl acetate, absolute ethanol) were purchased from Loba Chemie Pvt. Ltd (India). Ascorbic acid, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), Folin ciocalteu's reagent, gallic acid, sodium carbonate were purchased from Sigma Chemicals (USA). All other chemicals and reagents used were of analytical grade.

Extraction

The powdered material (156.449 g) was extracted by maceration with 70% ethanol (3 x 200mL). The soaked material was allowed to stand for 72 hours with occasional shaking and filtered subsequently using Whatman No.4 filter paper at room temperature. The filtrate was concentrated by evaporating on the water bath set at 40^{o}C. The dark brown extract obtained (24.331 g) was stored in an air tight container until utilized.

Phytochemical screening

Using standard procedure, several tests were carried out to detect the presence of some phytochemical components in the ethanolic crude extract as prescribed by Sofowora [7].

DPPH radical-scavenging activity

The stable 2, 2-diphenyl-1-picryl hydrazyl radical (DPPH) was used for determination of free radical-scavenging activity of the extract [8]. Different concentrations of extract were added, at an equal volume, to methanolic solution of DPPH (100µM). After 15 minutes at room temperature, the absorbance was recorded at 517 nm. The experiment was repeated three times. Vitamin C was used as standard control. Per cent DPPH radical-scavenging effect was calculated using the following equation:

$$\% \text{ DPPH radical scavenging effect} = (A_o - A_1) / A_o \times 100$$

Where A_o is the absorbance of the control (blank) after 15min and A_1 is the absorbance of the sample after 15min. Ascorbic acid was used as the positive control. IC_{50} values represent the concentration of sample, which is required to scavenge 50% of DPPH free radicals.

Determination of total phenol content

Total phenolic content was determined using Folin-ciocalteu's reagent [8] with slight modifications. Different concentrations of gallic acid (standard phenolic compound) were prepared ranging from 1- 0.0625 mg/mL. A 0.5 mL of the different gallic acid dilutions and 1mL of a 1 mg/mL extract solution were mixed respectively with Folin-ciocalteu's reagent (2mL, 1:10 dilution) and aqueous Na_2CO_3 (2mL, 7.5%;) was added. The mixture was allowed to stand for 15 minutes and the absorbance recorded using Jenway 6300 spectrophotometer at the wavelength of 765nm. The total phenolic concentration was calculated from a calibration curve obtained by plotting the absorbance against concentration of the gallic acid and results were expressed as gallic acid equivalent (GAE) of the fresh weight (dry mass of the sample; mg/g). The formula: T = C X V/M was used to calculate the total phenolic content expressed as gallic acid equivalent (GAE) in mg/g of dry powdered extract which is a common reference compound. T = Total phenolic content (mg/g) of extract; C = concentration of gallic acid established from the calibration curve (mg/mL); V = volume of extract solution (mL); M = Weight of extract (powdered dry mass) in g.

Statistical analysis

Statistical analysis was performed with Graph pad prism 6 demo. Differences were tested for significance by linear regression procedure, using a significance level of $p \leq 0.05$.

RESULTS AND DISCUSSION

The result for the preliminary phytochemical screening of the crude extract is shown in Table 1.
The extract showed strong free radical scavenging activity (IC_{50} = 0.136 $mgmL^{-1}$). The IC_{50} value for Ascorbic acid was 0.332 $mgmL^{-1}$. The per cent DPPH radical scavenging activity of the various concentrations of the extract is shown in Table 2.

Table 1: Phytochemical screening of the crude ethanol-water extract of *C. sinensis*

Phytoconstituents	Test performed	Result Ethanolic Extract
Tannins	Ferric chloride Test	+
Saponins	Froth Test	–
Carbohydrates	Molisch Test	+
Anthraquinones	Borntrager's Test	–
Alkaloids	Dragendorff's Test	+
Flavonoids	Shinoda test	+
Cardiac glycosides	Legal test	+
Steroids	Libermann burchard Test	+

Table 2: Per cent (%) DPPH Scavenging effect of the water-ethanol extract of the seed of *C. sinensis* and ascorbic acid

Concentration (mg/mL)	Extract (C. sinensis)	Ascorbic acid
1	89.3	86.66
0.8	83.9	80
0.6	75	68.88
0.4	73.2	66.66
0.2	44.6	31.11

Total phenol compounds, as determined by Folin Ciocalteu's method, is reported as gallic acid equivalent by reference to standard curve (y = 0.8138x, r^2 = 0.8936). The total phenolic content was found to be 8.54 mg gallic acid equivalent/g^{-1}. The correlation between the DPPH radical scavenging activity and phenol content is shown in Figure 1.

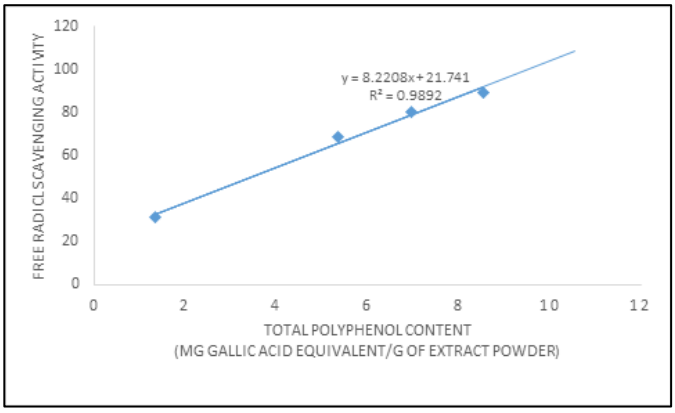

Figure 1: Correlation between free radical scavenging activity (DPPH method) and polyphenol content of ethanol-water extract of C. *sinensis*

The antioxidant and free radical scavenging activity of the extract of the seed of *C. sinensis* was determined using DPPH method, a stable free radical which is widely used to assess the radical scavenging activity of antioxidant compounds [8]. The antioxidant effect is proportional to the disappearance of the purple colour of DPPH. The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples [9]. It was found that the radical scavenging activity of the extract (*C. sinensis*) and standard (ascorbic acid) increased with increasing concentrations. High absorbance is an indication of high concentration of formed peroxides. Therefore, low concentration indicates high antioxidant activity [10]. The high content of phenol and flavonoids of the seed of *C. sinensis* is responsible for the high antioxidant activity of this fruit. Phenols and polyphenols compounds such as flavonoids found in food products derived from plant sources such as *C. sinensis*, have been shown to possess significant antioxidant activities [11].

The highest radical scavenging activity was shown by the extract of *C. sinensis* (89.3% free radical inhibition and the standard (ascorbic acid) produced 86.6% inhibition at 1mg/mL concentration. From linear regression analysis, extract indicated ($y = 50.05x + 43.17$, $r^2 = 0.8386$) and ascorbic acid ($y = 62.22x + 29.33$, $r^2 = 0.8385$). The IC_{50} (concentration required to cause a 50% DPPH inhibition) for the seed extract was obtained from linear regression curve as 0.136 mg/mL and 0.332 mg/mL for the standard. Lower $1C_{50}$ indicates greater antioxidant activity [12]. This means that the extract has more potential antioxidant activity than ascorbic acid. It is known that only flavonoids with a certain structure; particularly hydroxyl group in certain positions in their molecule can act as proton donating and show radical scavenging activity. Similarly the extract contains complex mixtures of many different compounds with varying activities [13]. The antioxidant activities of putative antioxidants have been attributed to various mechanisms; among these are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction and radical scavenging [14,15]. In addition, it has been reported that the free –OH groups in phenolic compounds are mainly responsible for antioxidant activity [16]. Thus these results may suggest that DPPH radical scavenging activity is an indication of potential antioxidant activity for the seed extract. This result is comparable with what was obtained for the antioxidant activity of the peels and tissues of some citrus species [17].

Total phenolic content can be estimated using Folin-Ciocalteu's reagent as described by Singleton [18] using gallic acid as standard. As reported, antioxidant activity of fruits and vegetables significantly increase with the presence of high concentration of total polyphenol content. Positive correlations between total phenolic and antioxidant capacity have been reported [19]. A direct correlation between radical scavenging activity and phenolic content of the seed extract was demonstrated by Pearson correlation. The correlation between total phenolic content and antioxidant activity was demonstrated from the plot as depicted in figure I. There is a positive correlation between the antioxidant activity and total phenolic content. However, the relationship between the antioxidant activity and phenolic compounds depend on several factors such as chemical structure of individual component, synergistic interaction among them and specific conditions applied in different assay [20]. It seems that the antioxidant activity of *C. sinensis* was not attributed to be the property of a single phytochemical compound but is widely distributed between vitamin C and phenolic constituents. According to Rice-Evans and Miller, [21] ascorbic acid could exert a synergistic effect with phenolic components. Thus the high phenolic content resulted in a high antioxidant activity. In general, extracts or fractions with a high radical scavenging activity showed a high phenolic content as well. Therefore antioxidant activity correlated strongly with total phenolic content in *C. sinensis.*

CONCLUSION

The ethanol-water extract of the seed of *C. sinensis* showed a significant inhibition of the DPPH free radical even more than the control agent (ascorbic acid); which can be attributed to the high phenolic content of the seed extract. This result validates the popular belief that the citrus by products have several health benefits.

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