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COMPARATIVE ANTIOXIDANT POTENTIALS AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF LEAF EXTRACTS OF *CITRUS SINENSIS* AND ITS EPIPHYTES

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Abstract

Citrus sinensis) is known and consumed globally because it is an outstanding source of potent antioxidants. This study investigated the phytochemicals and antioxidants present in methanol extracts of *Citrus sinensis* (CS) leaf and its epiphyte (CSE) *in vitro*. Phytochemicals present in the extracts were determined by HPLC fingerprinting. Antioxidant assays; 1, 1-diphenyl-2-picrylhydrazyl (DPPH), nitric oxide radicals, inhibition of lipid peroxidation and ferric reducing antioxidant power (FRAP) were determined spectrophotometrically. HPLC results revealed the presence of some bioactive phytochemicals such as eriocitrin, quercetogenin, tangeretin, hesperidin, among others in CS and CSE with eriocitrin as the most abundant in CSE (3.43 ± 0.02) and tangeretin in CS (10.78 ± 0.02). CS had better antioxidant potentials than CSE. These suggest that CS is richer in phytochemicals than CSE; the extracts have potent bioactive phytochemicals with good antioxidant potentials which may be responsible for the antioxidant activity so observed. It could be recommended that the extracts would be helpful in ameliorating pathological conditions.

Keywords: *Citrus sinensis, Epiphytes, Antioxidants, Phenolics*.

Introduction

Medicinal plants have been used traditionally in the prophylactic and therapeutic treatment of several human ailments, this use of medicinal plants depends on their pharmacological and therapeutic properties which are being attributed to the constituent phytochemicals. It is believed that phytochemicals may be useful in preventing diseases attributable to their antioxidant effect (Halliwell and Gutteridge, 1992; Akinmoladun *et al.,* 2007). Antioxidants bestow its protective properties on molecules from oxidation when they are exposed to free radicals, this process have been implicated in the aetiology of many diseases (Farombi, 2000).

Sweet orange (*Citrus sinensis*) is consumed globally because it is an excellent source of ascorbic acid (vitamin C) which is a potent antioxidant (Etebu and Nwauzoma, 2014). It has been used ethnobotanically for treatment of ailments like constipation, cramps, colic, diarrhea, bronchitis, tuberculosis, cough, cold, hypertension, anxiety, depression, and stress among others (Milind and Chaturvede, 2012). *C. sinensis* is a rich source of secondary metabolites, which are major players in the pharmacological activities of this plant. Examples of the phytochemicals identified in different parts of the plant (fruits, peel, leaves, juice and roots) include flavonoids (Gattuso *et al.,* 2007), carotenoids (Aschoff *et al.,* 2015), peptides (Matsubara *et al.,* 1991), steroids, hydroxyamides, alkanes and fatty acids (Rani *et al.,* 2009), coumarins (Li *et al.,* 2007), carbohydrates (Kolhed and Karlberg, 2005), carbamates and alkylamines (Soler *et al.,* 2006), volatile compounds (Gómez-Ariza *et al.,* 2004; Selli *et al.,* 2008), and nutritional elements such as potassium, magnesium, calcium and sodium among others (Niu *et al.,* 2009). Hesperidine, a bioflavonoid from *C. sinensis* was reported to lessen the toxicant-induced oxidative stress in *Drosophila melanogaster* (Abolaji *et al.,* 2017).

Epiphytes usually grow on the surface of a plants and derive moisture and nutrients from the nutrient supply of the parent plant, air, rain, water (in marine environments) or from debris accumulating around it. Epiphytes take part in nutrient cycles and add to both the diversity and biomass of the ecosystem in which they occur, like any other organism. Folkloric reports revealed that various epiphytes have been used to treat various ailments. Cocoa epiphytes for example are used in treating heart related diseases, kolanut epiphytes are also used in treatment of diabetes, and moringa epiphytes in the treatment of diabetes and malaria. While many researches had been carried out on the parent plant *C. sinensis,* there is sparse information on the phytochemical composition of epiphytes on *C. sinensis* viz-a-viz its antioxidant capacity. Therefore, this study is designed to unravel the phytoconstituents present in this epiphyte and its antioxidant potentials.

Materials and methods

Reagents and Chemicals

Ascorbic acid, Folin C, Tannic acid, hydrochloric acid (HCl), and other reagents used were of analytical grade and were purchased from Sigma.

Plant material and extraction

The plant materials (*Citrus sinensis* leaves and epiphytes) were collected from Fasdel farm settlement, Ikere-Ekiti, Ekiti State, South-Western Nigeria, and were identified by Mrs Chukwuma at the Department of Plant Science and Biotechnology, Federal University,Oye-Ekiti (FUOYE) with voucher no CS 02 & 03; fresh plant parts were deposited in the herbarium. The leaves were cleaned with distilled water, air dried and powdered. The pulverized sample was extracted by maceration in 100% methanol for 72 h and then filtered. The filtrate was concentrated in a rotary evaporator and freeze-dried to obtain the crude methanol extracts which were preserved in a refrigerator for further use.

Methods

Antioxidant Activity Determination

- **Inhibition of lipid peroxidation**

This assay was carried out in accordance to the methods described by (Ruberto *et al.,* 2000) with slight modification.

Briefly, 0.1mL of egg yolk homogenate (10% v/v) was added to 0.5mL of varying concentrations of the extracts in test tubes. The volume in each test up was made up to 1.0mL with distilled water. Afterwards, 0.05mL of ferrous sulfate was added and incubated at 37oC for 30 minutes. Then, 0.5mL of acetic acid-thiobarbituric acid reagent prepared in

dimethyl sulfoxide was added. The resulting mixture was mixed and incubated at 950C for 1 hour. Afterwards, the test tubes were allowed to cool and centrifuged at 650 × g for 5 minutes and the absorbance of the supernatant was read at 532 nm. The percentage inhibition was calculated as follow:
% Inhibition = (Abs of blank – Abs of sample)/ Abs of blank × 100

- **Ferric Reducing Power Assay (FRAP)**

The Reducing Power Assay was carried out by the method of Oyaizu (1986). About 2.5ml of the extract solution was mixed with 2.5 ml of 0.2M sodium phosphate buffer and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min and 2.5 mL of trichloroacetic acid solution (100 mg/L) was added. The mixture was centrifuged at 650 rpm for 10 min, and 5 mL of the supernatant was mixed with 5 mL of distilled water and 1 mL of 0.1% ferric chloride solution. The absorbance was measured at 700 nm.

- **DPPH Radical Scavenging Capacity**

The DPPH• assay was performed as described by (Shirwaikar*et al*., 2006). This method depends on the reduction of purple DPPH• to a yellow colouredd iphenylpicrylhydrazine and the remaining DPPH• which showed maximum absorption at 517 nm was measured. About 2 ml of various concentrations of each extract were added to 2 ml solution of 0.1 mM DPPH•. An equal amount of methanol and DPPH• served as control. After 20 min of incubation at 300C in the dark, the absorbance was recorded at 517 nm. The experiment was performed in duplicates. The DPPH radical scavenging activity was calculated according to the following equation:
% inhibition = [A of blank – A of sample] / A of blank x 100.

- **Nitric Oxide Scavenging Capacity**

Nitric oxide radical scavenging activity was determined according to the method reported by (Garrat,1964). Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be determined by the use of the Griess Illosvoy reaction. 2 mL of 10 mM sodium nitroprusside in 0.5 mL phosphate buffer saline (pH 7.4) was mixed with 0.5 mL of extract at various concentrations and the mixture incubated at 250C for 150 min. From the incubated mixture 0.5 mL was taken out and added into 1.0 mL sulfanilic acid reagent (33% in 20% glacial acetic acid) and incubated at room temperature for 5 min. finally, 1.0 mL naphthylethylenediaminedihydrochloride (0.1% w/v) was mixed and incubated at room temperature for 30 min before measuring the absorbance at 540 nm was measured with a spectrophotometer. The nitric oxide radicals scavenging activity was calculated.
% inhibition = Ao-A1)/A1 x 100

- **Total Flavonoids Content**

Flavonoid content was performed as described by (Shisha *et al*.1999) with slight modifications. The flavonoids were measured by aluminium chloride colorimetric assay based on the development of flavonoid-aluminium complex with a maximal absorption at 510 nm. Exactly, 1.0 mL of the extract (105 µg/mL) was mixed with 1.0 mL of AlCl3 (5%). The mixture was allowed to stand at room temperature (29 °C) for 5 minutes after which 2.0 mL of $NaNO_2$ (7%) was added. Afterwards, 1.0 mL of sodium hydroxide (1%) was added to the reaction mixture. The absorbance was determined at 510 nm against a blank which contained all reagents without the samples. The results were calculated from standard curve obtained using varying concentrations of quercetin (5-20 µg/mL) in methanol and expressed in mg/100g quercetin equivalents (QE).

- **Total Phenolic Content Determination**

The total phenol content of methanolic extracts was determined using the Folin-Ciocalteu spectrophotometric method (Singleton *et al.,* 1999). Folin-Ciocalteu spectrophotometric method or calometric method is based on a chemical reaction of the reagent, a mixture of tungsten and molybdenum oxides. The products of the metal oxide reduction have a blue colour that exhibits a broad light absorption with a maximum at 765nm. The intensity of the light absorption at that wavelength is proportional to the concentration of phenols. 0.12g Ascorbic acid was dissolved in 2.4mL ethanol and then diluted to 100mL with water to

give a final concentration of 0.5mg/mL Ascorbic acid stock solution. Sodium Carbonate Solution: 200g anhydrous sodium carbonate was dissolved in 300mL water and the solution was boiled. After cooling, few crystals of sodium carbonate were added. The solution was then filtered through Whatman no. 1 filter paper and diluted with water to 1 litre. The solution stored immediately at room temperature.

Quantification of compounds by HPLC-DAD

Reverse phase chromatographic analyses were carried out under gradient conditions using C_{18} column (4.6 mm x 250 mm) packed with 5µm diameter particles. The mobile phase was water containing 1% formic acid (A) and acetonitrile (B), and the composition gradient was: 13% of B until 10 min and changed to obtain 20%, 30%, 50%, 60%, 70%, 20% and 10% B at 20, 30, 40, 50, 60, 70 and 80 min, respectively (Boligon *et al.,* 2012) with slight modifications. The methanolic extracts were analyzed dissolved in ethanol at a concentration of 20 mg/mL. The presence of thirteen (13) antioxidant compounds was investigated, namely, eriocitrin, sinensetin, poncirin, quercetin, rutin, apigenin, deosmin, quercetogetin, tangeretin, hesperidine, luteolin, rhoifolin, and nobiletin. Identification of these compounds was performed by comparing their retention time and UV absorption spectrum with those of the commercial standards. All the samples and mobile phase were filtered through 0.45 µm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standards references were prepared in the HPLC mobile phase at a concentration range of 0.030 – 0.250 mg/ml for all the standards. All chromatography operations were carried out at ambient temperature and in triplicate. (Boligon *et al.,* 2013; Falode *et al.,* 2019)

Results

- **Antioxidant Parameters**

The antioxidant assays carried out demonstrated the free radical scavenging potentials of the extracts under study. Figure 1 showed the DPPH radical scavenging power of the extracts; which revealed *C. sinensis* (CS) as the most potent followed by *C. sinensis* epiphytes extract (CSE). This trend was the same in lipid peroxidation inhibition assay; Figure 2. The result for nitric oxide radical scavenging capacity; Figure 3 also demonstrated the same fashion as Figure 1, where CS had the most potent scavenging capacity followed by CSE. Figure 4 showed ferric reducing antioxidant potential (FRAP), total phenolics and total flavonoids contents of the extracts; it revealed that the parent leaf extract had better phytoconstituents than the epiphytes. Values for CS and CSE are significantly different from each other in these assays.

- **HPLC Fingerprinting**

Table 1 revealed the components identified from *C. sinensis* leaf and epiphyte extracts through HPLC DAD. This profile revealed that CS is rich in phytochemicals as exemplified by eriocitrin, sinensetin, poncirin, quercetin, rutin, apigenin, deosmin, quercetogenin, tangeretin, hesperidin, luteolin, rhoifolin and nobiletin; while CSE only had eriocitrin, quercetin, apigenin, deosmin, quercetogenin, tangeretin.

Discussion

The use of plants as food and medicine is an age-long practice which is gaining more attention globally because of numerous phytochemicals present in them; hence, the use of medicinal plants and their isolated bioactive principles as part of chemopreventive sand chemotherapeutic agents (Fulda *et al.,* 2010; Falode *et al.,* 2017). Phytochemicals in medicinal plants perform their functions through several mechanisms which comprise antioxidative, hepatoprotective, neuroprotective, nephroprotective, immunomodulatory effects, induction of apoptosis, scavenging of reactive oxygen species (Falode *et al.,* 2017).

Epiphyte are organisms that grow on the surface of a plant and derive moisture and nutrients from the nutrient supply of the parent plant, air, rain, water (in marine environments) or from debris accumulating around it. Epiphytes take part in nutrient cycles and add to both the diversity and biomass of the ecosystem in which they occur, like any other organism. Folkloric reports revealed that various epiphytes have been used to treat various ailments. Cocoa epiphytes for example are used in treating heart related diseases, kolanut epiphytes

are also used in treatment of diabetes, and moringa epiphytes in the treatment of diabetes and malaria.

This research revealed that the antioxidant parameters of the extracts exhibited an outstanding radical scavenging capacity, as demonstrated from their power to scavenge DPPH radical, nitric oxide radicals, inhibit lipid peroxidation and reduce ferric radicals (Figures 1-4). CS showed the most potent antioxidant power followed by CSE. Since epiphytes derive water and phytonutrients from the parent plant, it is logical and reasonable to see the trend of results as normal and scientific; as the results revealed that CS better than CSE.

HPLC is a chromatographic technique used to assess and quantify the organic composition of biological matter (Falode *et al.,* 2016). HPLC fingerprinting also revealed the existence of many phytoconstituents in the extracts. Tables 1 revealed the components identified from *C. sinensis* leaf and *C. sinensis* epiphyte extracts through HPLC DAD. The result revealed that CS had phytochemicals as exemplified by eriocitrin, sinensetin, poncirin, quercetin, rutin, apigenin, deosmin, quercetogenin, tangeretin, hesperidin, luteolin, rhoifolin and nobiletin; while CSE only had eriocitrin, quercetin, apigenin, deosmin, quercetogenin, tangeretin. Almost all these phytoconstituents are examples of already established antioxidative plant phytochemicals, such as quercetin, rutin, apigenin, hesperidin, luteolin. Hesperidine for example has been reported to lessen the toxicant-induced oxidative stress in *Drosophila melanogaster* (Abolaji *et al.,* 2017).

Conclusively, the indication of these results is that the extracts are very rich in phytochemicals, and it could be inferred that the presence of these compounds is responsible for the outstanding activities observed so far.

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Conflict of Interest Disclosure
The authors declare no conflict of interest.

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Table 1 – Phytochemical composition of *Citrus sinensis* leaf and epiphyte extracts as identified by HPLC DAD.

Compounds	*Citrus sinensis* leaf (mg/g)	*Citrus sinensis* epiphyte (mg/g)
Eriocitrin	10.55 ± 0.01[a]	3.43 ± 0.02[a]
Sinensetin	2.46 ± 0.01[b]	ND
Poncirin	0.15 ± 0.03[c]	ND
Quercetin	2.79 ± 0.02[b]	0.77 ± 0.01[b]
Rutin	0.21 ± 0.01[c]	ND
Apigenin	0.13 ± 0.03[c]	0.18 ± 0.02[b]
Deosmin	0.16 ± 0.01[c]	0.17 ± 0.05[b]
Quercetogetin	2.75 ± 0.01[b]	0.80 ± 0.02[b]
Tangeretin	10.78 ± 0.02[a]	3.23 ± 0.03[a]
Hesperidine	0.78 ± 0.03[c]	ND
Luteolin	0.34 ± 0.01	ND
Rhoifolin	0.28 ± 0.03	ND
Nobiletin	0.21 ± 0.01	ND

Results are expressed as mean ± standard deviations (SD) of three determinations. Averages followed by different letters differ by Tukey test at $p < 0.05$

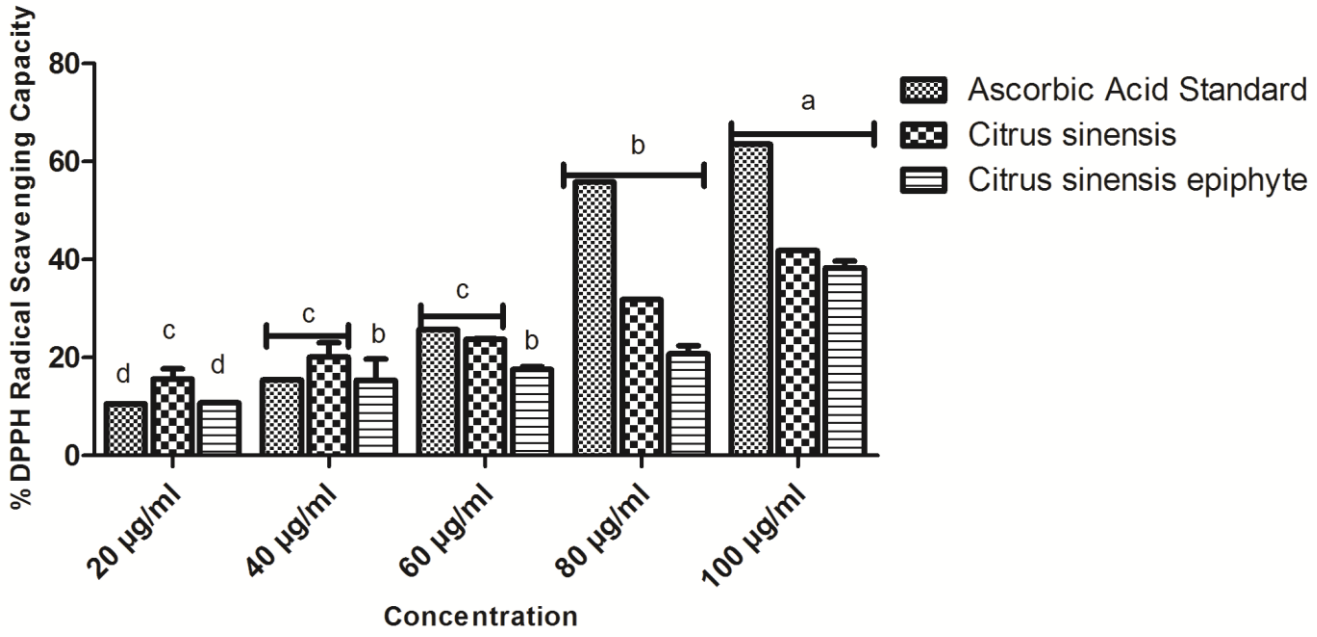

Figure 1: The percentage DPPH radical inhibition of Methanol extract of *Citrus sinensis* and epiphyte leaf.
Results are expressed as mean ± standard deviations (SD) of three determinations.
Averages followed by different letters differ by Tukey test at $p < 0.05$

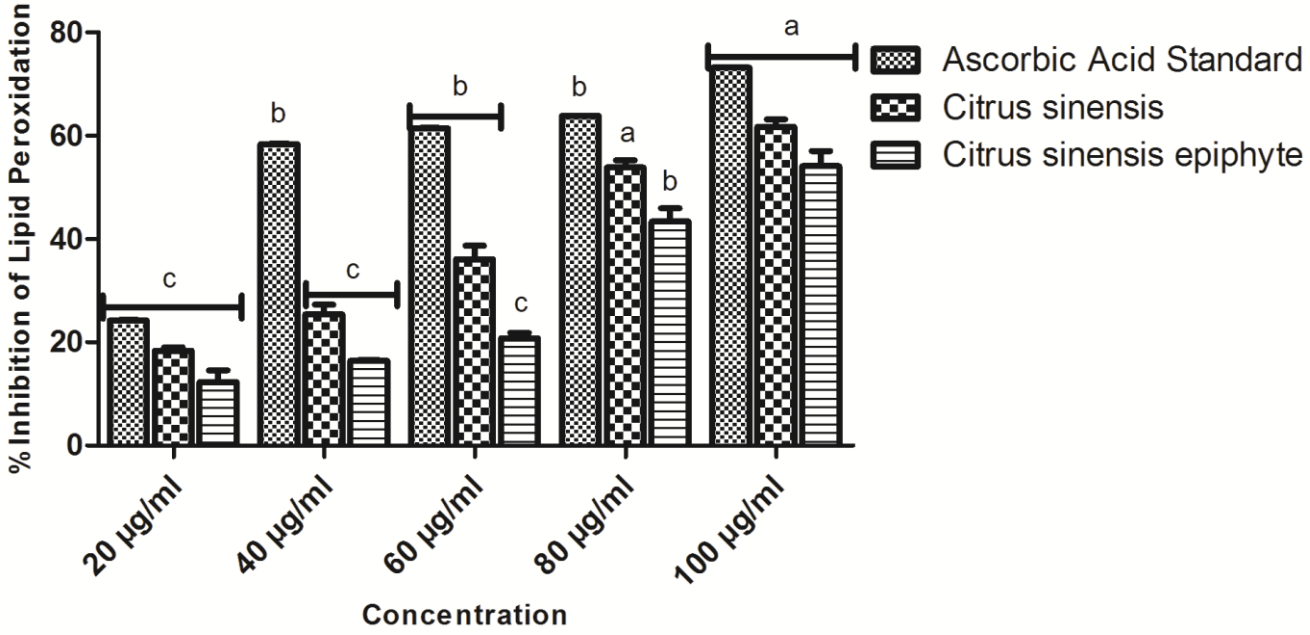

Figure 2: The percentage inhibition of lipid peroxidation of Methanol extract of *Citrus sinensis* and epiphyte leaf.

Results are expressed as mean ± standard deviations (SD) of three determinations.
Averages followed by different letters differ by Tukey test at $p < 0.05$

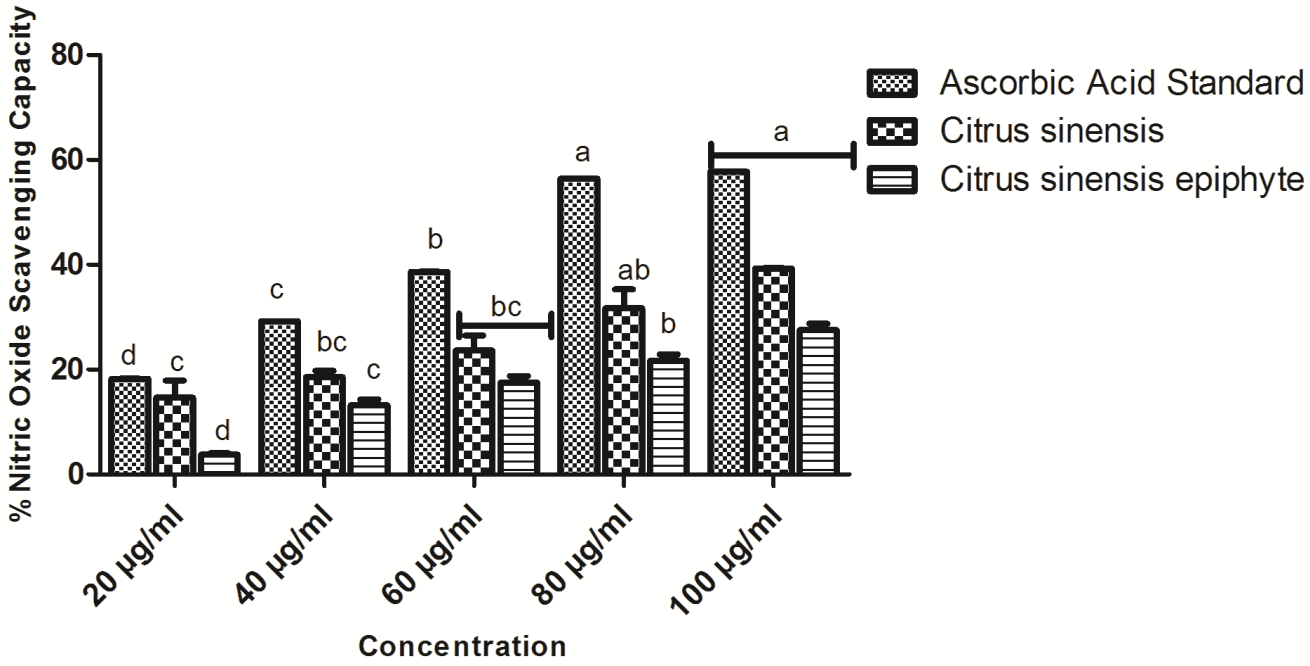

Figure 3: The percentage NO radical inhibition of Methanol extract of *Citrus sinensis* and epiphyte leaf.
Results are expressed as mean ± standard deviations (SD) of three determinations.
Averages followed by different letters differ by Tukey test at $p < 0.05$

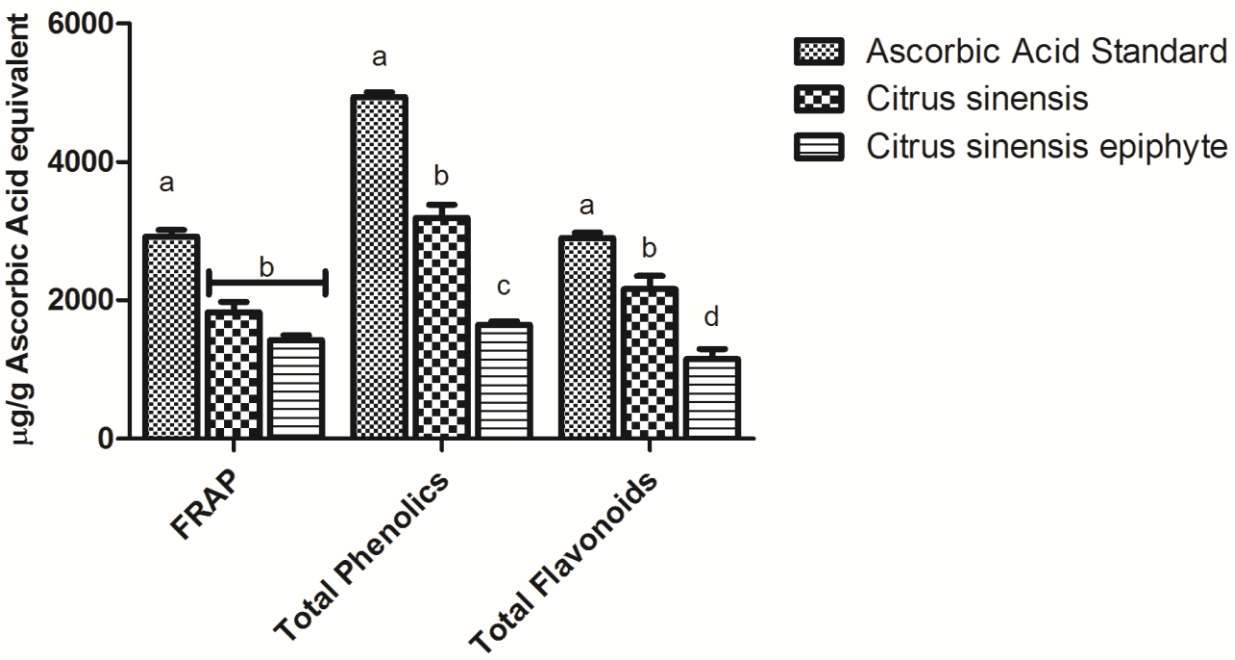

Figure 4: The Ascorbic acid equivalence of FRAP, Total Phenolics and Total Flavonoids of Methanol extract of *Citrus sinensis* and epiphyte leaf.

Results are expressed as mean ± standard deviations (SD) of three determinations.
Averages followed by different letters differ by Tukey test at $p < 0.05$