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Evaluation of anti-inflammatory, antibacterial and antioxidant properties of ethanolic extracts of *Citrus sinensis* peel and leaves

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ABSTRACT

The objectives of this study was to evaluate the antioxidant, anti-inflammatory and antibacterial properties of ethanolic extracts of Citrus sinensis peels and leaves. Free radical scavenging activity was evaluated by using nitric oxide inhibition and anti-lipid peroxidation models. Both peels and leaves extracts produced a dose–dependent inhibition in vitro. Highest inhibition was seen in the extracts of highest concentration 65.7% for peels and 57.4% for leaves in nitric oxide (NO) scavenging assay, 66.5% and 54.7% for peels and leaves extract respectively in anti-lipid peroxidation assay. Leaves extract also exhibited anti-inflammatory property at different time intervals that was dose-dependent. Extracts of both peels and leaves showed moderate antibacterial activity against Staphylococcus aureus, Streptococcus pneumoniae, Escherichia coli, Proteus mirabilis and Pseudomonas aeruginosa. Results clearly indicate that free radical scavenging, antibacterial and anti-inflammatory properties of ethanolic extracts of Citrus sinensis are comparable to ascorbic acid, ciprofloxacin and aspirin used as standards respectively.

Keywords: *Citrus sinensis*, peel, leaves, anti-inflammatory, antioxidant, antibacterial, ethanolic extract, zone of inhibition

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INTRODUCTION

Citrus sinensis (L) OSbeck belongs to *Rutaceae* family and it is commonly known as sweet orange [1] It is the most commonly grown tree fruit in the world [2]The sweet orange is an evergreen flowering tree generally growing to 9–10m in height. Its fruit is strengthening, cardiotonic, Laxative, anthelmintic and removes fatigue [3]It possesses anti-inflammatory,antibacterial and antioxidant properties [4]Its leaves are shiny and leathery, arranged alternatively. Oranges are said to lower cholesterol and aid in the digestion of fatty foods [5]). The vitamin C in Oranges is concentrated mainly in the peel and the white layer just under the peel. The peel contains citral, an aldehyde that antagonizes the action of vitamin A. Therefore, anyone eating quantities of orange peel should make certain that their dietary intake of vitamin A is sufficient [6]

Sweet orange oil is a by-product of the juice industry produced by pressing the peel. It is used as a flavouring in food and drink and for its fragrance in perfumes and aromatherapy. It consists of about 90% d-limonene [7]. Limonene now is known as a significant chemopreventive agent [8]with potential value as a dietary anti-cancer agent in humans [9]

Increased production or deficient elimination of reactive oxygen species (ROS), and other oxidizing agents, leads to oxidative stress and damage of cells and tissues. There is evidence that oxidative damage is an important contributor to aging and various chronic diseases such as cancer and neurodegeneration [10,11,12]. Both dietary antioxidant and those endogenous to the body are involved in controlling oxidative damage. In the context of nervous system, antioxidants have been shown to improve motor and cognitive functions in experimental animals and prevent ROS-medicated neuronal death [13, 14 10,15]

From a public health perspective, there is much evidence that increased consumption of plant foods, including flavonoid–rich Citrus fruits may decrease the risk of cognitive impairments and neurodegenerative disorders [16,17,18,19,20]

Citrus sinensis

Consumption of fruits such as Citrus sinensis is beneficial to health and contributes to decrease of the mortality rate of Cardiovascular and other diseases [21].This positive influence is attributed to some natural antioxidant phytonutrients [22]. The majority of antioxidant capacity of Citrus sinensis has been attributed to the presence of vitamin C and flavonoids This study is aimed at investigating the anti-inflammatory, antibacterial and antioxidant potential of ethanolic extracts of peels and leaves of *Citrus sinesis*.

EXPERIMENTAL SECTION

Plant material:
Citrus sinensis peels and leaves were harvested from a tree in Olokoro Village, Umuahia. The authentication of the plant specimen was done at the Botany Department of Michael Okpara University of Agriculture, Umudike by a taxonomist; Dr. Omosun Garuba.

Chemical used:
All reagents and chemicals used in the experiments were of analytical grade, purchased from His grace Chemicals Nigeria Limited, Aba, Abia State:

Ascorbic acid, Sodium nitroprusside, Sulphanilamide α-napthyl–ethylene, diaminedihydrochloride, Monosodium phosphate, Disodium phosphate, Phosphoric acid ,Ethanol, Ferric chloride ,Trichloroacetic acid (TCA), Thiobarbituric acid (TBA, Ciprofloxacin, Aspirin.

Animal Material:
Goat liver which was used for anti–lipid peroxidation assay was collected from slaughter house in Umuahia main market immediately after slay. Experiment was conducted within one hour after collection. Albino wistar rats used for anti-inflammatory study were obtained from the Veterinary Medicine department of National Root Crops Research Institute, Umudike, and kept in cages.

Bacteria Strains:
Clinical strains of micro organism used for the antimicrobial study were *Staphylococcus aureus*, *Streptococcus pneumoniae, Escherichia coli, Proteus mirabilis* and *Pseudomonas aeruginosa*, were obtained from the microbiology laboratory of Federal Medical Centre (FMC) Umuahia, Abia State.

Preparation and extraction of plant extracts:
Fully matured leaves and unripe peels of *Citrus sinensis* were collected, washed and shade dried. The dried samples were ground to coarse powder form using a milling machine and extracted by crude extraction using ethanol for 18 hrs with a mass to volume ratio of 1: 4 (g/ml). The extracts were evaporated to dryness on a rotary evaporator.

Phytochemical Investigation:
The extracts were subjected to qualitative and quantitative chemical analysis. The phytochemical components were determined using the methods of Harbone (1984)[23] and Trease and Evans (1989)[24].

FREE RADICAL SCAVENGING ACTIVITY
Nitric oxide scavenging activity:
This was determined according to the method described by [25]. The nitric oxide scavenging activity was conducted based on the Greiss assay method. 2.0ml of 10mM sodium Nitroprusside and 5.0 ml of phosphate buffer were mixed with 0.5ml of different concentrations of the plant extracts and incubated at 25^{o}C for 150 mins.

The samples were run as above but the blank was replaced with the same amount of water, after the incubation period, 2 ml of the above incubated solution was added to 2ml of Greiss reagent (1% sulphanilamide, 0.1% α-napthyl-ethylenediamine Dihydrochloride and 3 % phosphoric acid) and incubated at room temperature for a period of 30 minutes.

The absorbance of the pink chromophore formed by the diazotization of nitrite with α-napthyl–ethylene diamine dihydrochloride was measured at 540 nm. Ascorbic acid was used as positive control and results were expressed as percentage inhibition of nitric oxide. All determinations were performed in triplicates.

$$\% \text{ Inhibition} = \frac{\text{A control} - \text{A sample}}{\text{A control}} \times 100$$

Anti-lipid Peroxidation Activity:
This was determined according to the method described by [26]. Ethanolic extracts of *C. sinensis* were used in various concentrations (2500, 2000, 1250, 1000 and 500 µg/ml) individually. 3ml of liver homogenate was added with 100µl of 15 mM ferric chloride and was shaken for 30mins. From collected mixture, 100µl was added with 1ml of different concentration of plants extracts individually in different tests tubes. The same procedure was followed for control and blank. Water was used as a control and ascorbic acid (1000 µg/ml) as standard. All the test tubes were incubated for 4hrs at 37^{o}c. After incubation 20% trichloroacetic acid (TCA) was added to all test tubes containing the mixture in 1:1 ratio and centrifuged for 30 mins. The supernated liquid was collected and 0.6% thiobarbituric acid (TBA) was added in 1:1 ratio and heated for 1 hour in a water bath, cooled and absorbance measured at 530nm. The percentage of anti-lipid peroxidation activity was calculated by using the formula

$$\% \text{ Inhibition} = \frac{(\text{Acontrol} - \text{Asample})}{\text{Acontrol}} \times 100$$

ANTI-INFLAMMATORY ACTIVITY
A total of 24 adult albino wistar rats of both sexes were used. They were placed in cages and grouped into six (A – F) of 4 per group. They were then left to acclimatize for four days. The animals were deprived of feed for 12 hrs prior to the experiment but were allowed access to pure drinking water. They were not allowed access to both feed and pure drinking water during the experiment.

The crude extract and aspirin were separately administered intra-peritoneally. Group A was used as negative control thus, received neither aspirin nor the crude extract; group B received 250 mg/ml of the extract. Group C received 200mg/ml, group D received 100 mg/ ml while group E received 50 mg/ml of the extract. Group F was used as positive control, thus, received 100mg/ml of aspirin. The animals were left for 30mins after which 1 ml of fresh egg albumen was injected into the sub-plantar of the right hind paw of each of the rats. Using a vernier caliper, the diameter of the paw was measured and recorded at 30mins intervals for 3hrs. Aspirin (100mg/ml) was used as standard. Percentage inflammation and inhibition of inflammation were calculated with the formulas below:

$$\% \text{ Inflammation} = \frac{Ct}{Co} \times 100$$

$$\% \text{ Inhibition} = \frac{Co - Ct}{Co} \times 100$$

Where;
Ct = Average inflammation of the treated group
Co = Average inflammation of the negative control group.

ANTIBACTERIAL ACTIVITY

The disc agar diffusion method was used in this study. The test organisms (1:100 dilution of an 18h broth cultures) were inoculated onto nutrient agar plates with sterile cotton swabs soaked in the Inocula. Disc of different extract concentrations were placed firmly on the surface of the inoculated agar plates and incubated at 37^{o}c for 18hrs under aerobic conditions. Zones of inhibition were measured and recorded in millimeters.

Statistical Analysis:

Data generated from antioxidant activity were subjected to analysis of variance (ANOVA). The data (mean ± standard deviation) shown are mean values andthe significant differences were compared by using Dunnett's multiple comparison test at the $P < 0.05$ probability level.

RESULTS

Phytochemical Investigation:

Ethanolic extracts of *Citrus sinensis* peels and leaves were subjected to qualitative and quantitative phytochemical screening. Table 1 and table 2 show the results respectively.

Table 1: Qualitative phytochemical screening of ethanolic extracts of *Citrus sinensis* peels and leaves

Plant constituent	Peels	Leaves
Alkaloids	+	+
Flavonoids	+	+
Tannins	+	+
Saponins	+	+
Carbohydrates	-	-
Steroid	+	+
Hydrogen cyanide	-	-

- *Absent* + *present*

Table 2: Quantitative phytochemical screening of ethanolic extracts of *Citrus sinensis* peels and leaves

Plant constituent	Peels (%)	Leaves (%)
Alkaloids	4.50 ± 0.02	4.00 ± 0.03
Flavonoids	2.50 ± 0.04	1.00 ±0.15
Tannins	1.69 ± 0.00	1.83 ± 0.00
Saponins	0.05 ± 0.01	0.33 ±0.01
Steroid	0.13 ± 0.01	0.17 ± 0.01

Antibacterial activity:

The antibacterial activities of ethanolic extracts of *Citrus sinensis* peels and leaves were compared to that of Ciprofloxacin, a broad spectrum antibiotic used as standard. Results are shown in Tables 3 – 5.

Table 3: Antibacterial activity of *Citrus sinensis* peels

Pathogen	Zone diameter (mm) of growth inhibition at:					
	250 mg/ml	125 mg/ml	62.5 mg/ml	31.25 mg/ml	15.5 mg/ml	MIC mg/ml
Staphylococcus aureus	31.5±0.71	25.5±0.71	9±1.41	2.5±0.71	0	31.25
Streptococcus pneumonia	30.5±0.71	23±1.41	9.5±0.71	1.5±0.71	0	31.25
Escherichia coli	28.5±0.71	21.5±0.71	7.5±0.71	0	0	62.5
Proteus mirabilis	32±1.41	26.5±0.71	10.5±0.71	2.5±0.71	0	31.25
Pseudomonas aeruginosa	26.5±0.71	17.5±0.71	6.5±0.71	0	0	62.5

Table 4: Antibacterial activity of *Citrus sinensis* leaves

Pathogen	Zone diameter (mm) of growth inhibition at:					
	250 mg/ml	125 mg/ml	62.5 mg/ml	31.25 mg/ml	15.5 mg/ml	MIC mg/ml
Staphylococcus aureus	37±1.41	27.5±0.71	10.5±0.71	3.5±0.71	0	31.25
Streptococcus pneumonia	31.5±0.71	25±1.41	8.5±0.71	2.5±0.71	0	31.25
Escherichia coli	29±1.41	20.5±0.71	7±1.41	1.5±0.71	0	31.25
Proteus mirabilis	33±1.41	25.5±0.71	16.5±0.71	0	0	62.5
Pseudomonas aeruginosa	28.5±0.71	15.5±0.71	7.5±0.71	0	0	62.5

Table 5: Antibacterial activity of Ciprofloxacin

Pathogen	Zone diameter (mm) of growth inhibition at:					
	250 mg/ml	125 mg/ml	62.5 mg/ml	31.25 mg/ml	15.5 mg/ml	MIC mg/ml
Staphylococcus aureus	43±1.41	30.5±0.71	17.5±0.71	10.5±0.71	2.5±0.71	15.5
Streptococcus pneumoniae	39.5±0.71	29±1.41	18±1.41	11.5±0.71	3.5±0.71	15.5
Escherichia coli	39±1.41	25±1.41	18.5±0.71	8.5±0.71	2±0.00	15.5
Proteus mirabilis	40.5±0.71	38.5±0.71	21.5±0.71	9±1.41	3.5±0.71	15.5
Pseudomonas aeruginosa	28.5±-.71	26.5±0.71	17±1.41	5.5±0.71	1.5±0.71	15.5

Anti-Inflammatory Activity:

Egg albumen was used to induce inflammation in hind paws of rats divided into six groups. The activity of leaves extract was compared to that of aspirin used as standard. Table 6 – 8 and figure 1 show the results of calculated % Inflammation and % Inhibition of inflammation per time interval.

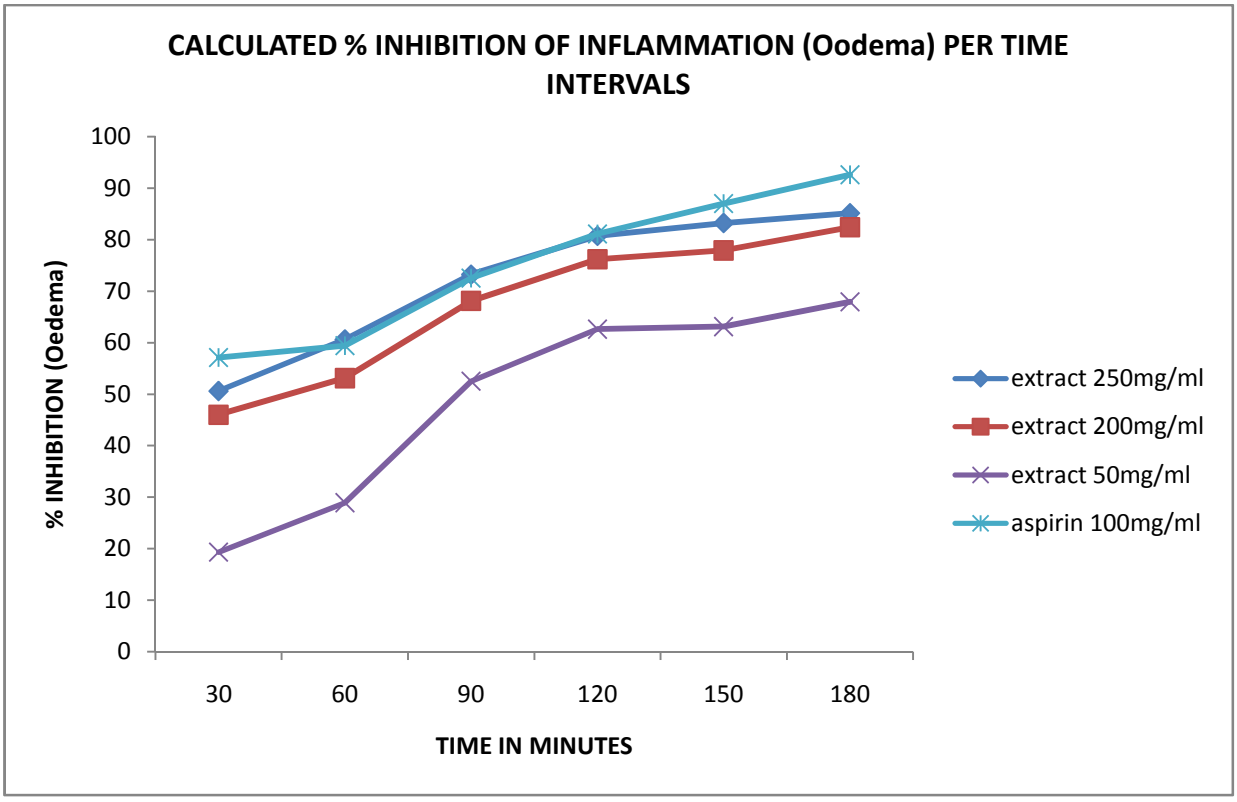

Figure 1: % Inhibition of Inflammation against Time in minutes

Table 6: Average Inflammation Of Hind Paw (Oedema) Using 2mg/ml Of Fresh Egg Albumen In Diameter (mm)

Dose	30mins	60mins	90mins	120mins	150mins	180mins
Normal saline (control)	14.91±0.41	15.38±0.08	19.28±0.45	22.02±0.29	18.81±0.27	18.2±0.28
250 mg/ml	7.36±0.06	6.06±0.08	5.16±0.08	4.26±0.23	3.16±0.08	2.71±0.13
200 mg/ml	8.05±0.07	7.21±0.01	6.15±0.07	5.25±0.21	4.15±0.07	3.2±0.28
100 mg/ml	10.95±0.07	9.05±0.07	7.95±0.07	6.52±0.41	5.71±0.16	4.5±0.14
50 mg/ml	12.03±0.15	10.93±0.10	9.15±0.07	8.23±0.11	6.94±0.06	5.85±0.07
Aspirin 100 mg/ml	6.4±0.42	6.25±0.07	5.3±0.14	4.16±0.08	2.44±0.02	1.35±0.07

Table 7: Calculated % Inflammation (oedema) per time intervals using non-treated animals as control

Dose	30mins	60mins	90mins	120mins	150mins	180mins
Extract 250mg/ml	49.4±0.06	39.4±0.08	26.8±0.08	19.3±0.23	16.8±0.08	14.9±0.13
Extract 200mg/ml	54.0±0.07	46.9±0.01	31.9±0.07	23.8±0.21	22.1±0.07	17.6±0.28
Extract 100mg/ml	73.4±0.07	58.8±0.07	41.2±0.07	29.6±0.41	30.4±0.16	24.7±0.14
Extract 50 mg/ml	80.7±0.15	71.1±0.10	47.5±0.07	37.4±0.11	36.9±0.06	32.1±0.07
Aspirin 100mg/ml	42.9±0.42	40.6±0.07	27.5±0.14	18.9±0.08	13.0±0.02	7.4±0.07

Table 8: Calculated % Inhibition of Inflammation (oedema) per time intervals

Dose	30mins	60mins	90mins	120mins	150mins	180mins
Extract250mg/ml	50.6±0.06	60.6±0.08	73.2±0.08	80.7±0.23	83.2±0.08	85.1±0.13
Extract 200mg/ml	46.0±0.07	53.1±0.01	68.1±0.07	76.2±0.21	77.9±0.07	82.4±0.28
Extract 100mg/ml	26.6±0.07	41.2±0.07	58.8±0.07	70.4±0.41	69.6±0.16	75.3±0.14
Extract 50 mg/ml	19.3±0.15	28.9±0.10	52.5±0.07	62.6±0.11	63.1±0.06	67.9±0.07
Aspirin 100mg/ml	57.1±0.42	59.4±0.07	72.5±0.14	81.1±0.08	87.0±0.02	92.6±0.07

Antioxidant activity:

Free radical scavenging activity of extracts of peels and leaves were investigated. Results of nitric oxide scavenging activity are shown in Table 9 and figures 2-3while Table 10and figures 4-5 show the result of anti-lipid peroxidation activity.

Table 9: Nitric oxide scavenging activity of *Citrus sinensis* peels and leaves

Concentration (mg/ ml)	% inhibition activity			
	Peels	Standard	Leaves	Standard
2.5	65.7 ± 1.789*		57.4 ± 1.212*	
2.0	49.0 ± 1.732		45.2 ± 0.519*	
1.25	31.8 ± 1.500*		28.9 ± 0.231*	
1.0	14.6 ± 2.335*	50.5±0.866	13.2 ± 2.425*	50.5 ± 0.866
0.5	9.1 ± 1.500*		8.6 ± 0.808*	
	IC_{50}=1100µg/ml		IC_{50}=1100µg/ml	

The values are mean ±SD. Means with superscripts (*) within a column are significantly different from each other at P < 0.05 as determined by Dunnett's multiple comparison test.

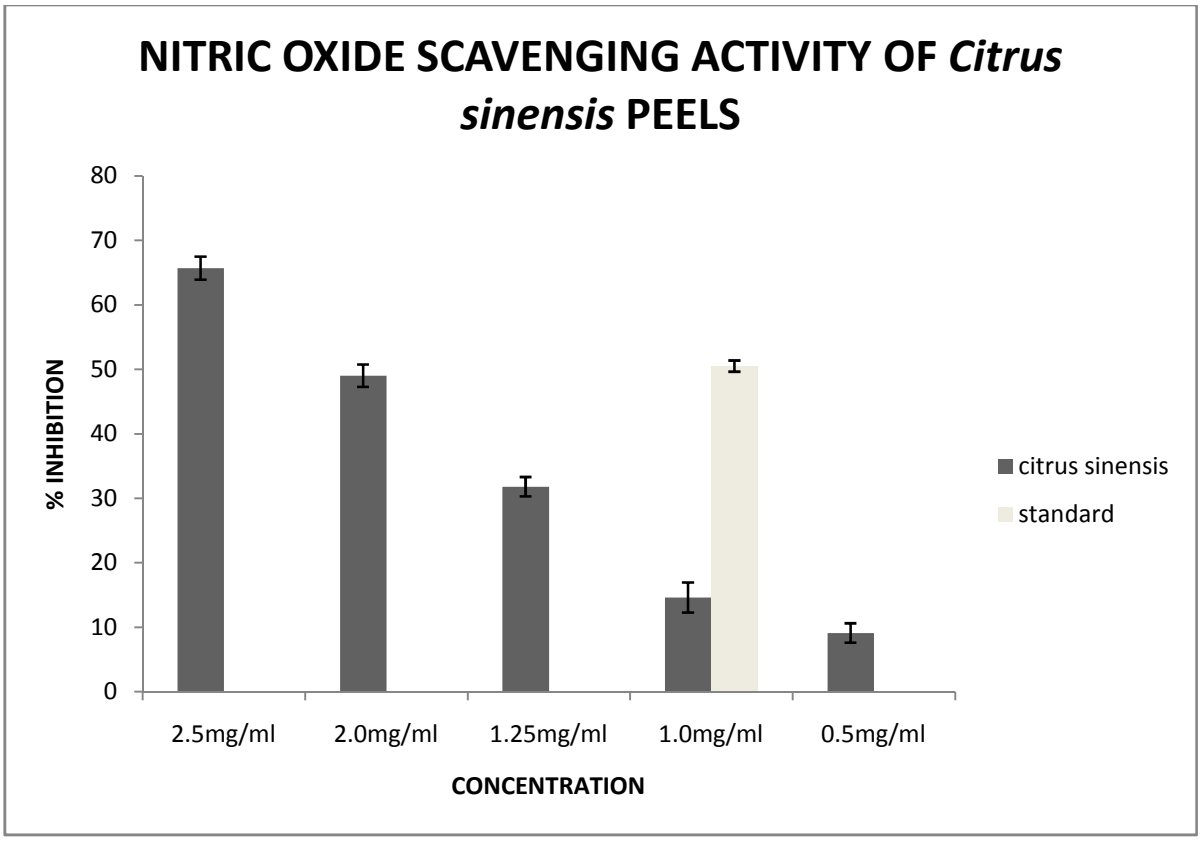

Figure 2: Nitric oxide scavenging activity

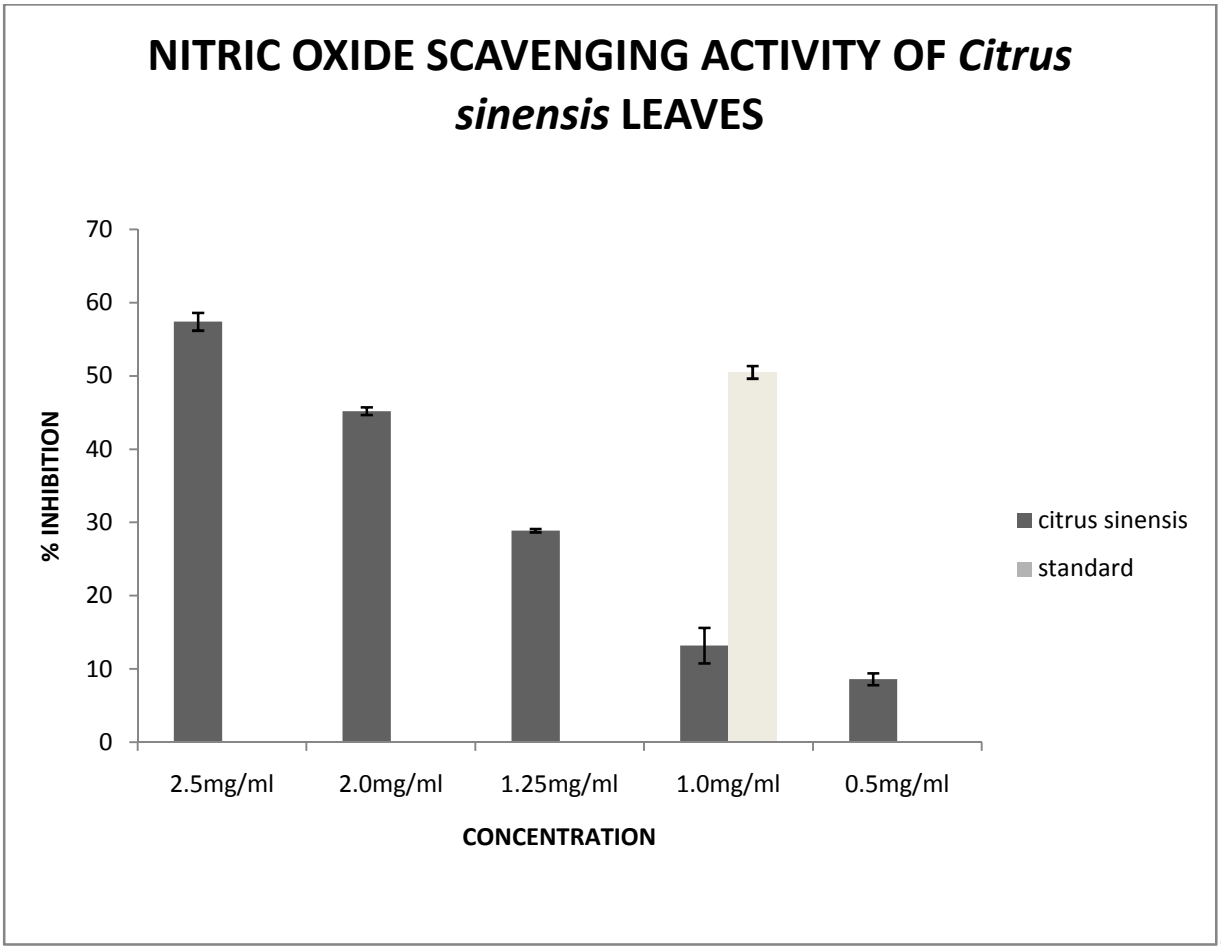

Figure 3: Nitric oxide scavenging activity

Table 10: Anti-lipid peroxidation activity of *Citrus sinensis* peels and leaves

Concentration (mg/ ml)	**% inhibition activity**			
	Peels	Standard	Leaves	Standard
2.5	66.5 ± 0.346*		54.7 ± 0.404*	
2.0	56.0 ± 0.346*		52.3 ± 0.346*	
1.25	51.7 ± 2.425*		47.1 ± 0.346*	
1.0	46.7 ± 0.600*	37.4±0.346	40.2 ± 0.346*	37.4 ± 0.346
0.5	39.4 ± 0.000		23.0 ± 0.600*	
	IC_{50}=1000µg/ml		IC_{50}=1100µg/ml	

The values are mean ±SD. Means with superscript (*) within a column are significantly different from each other at P<0.05 as determined by Dunnett's multiple comparison test.

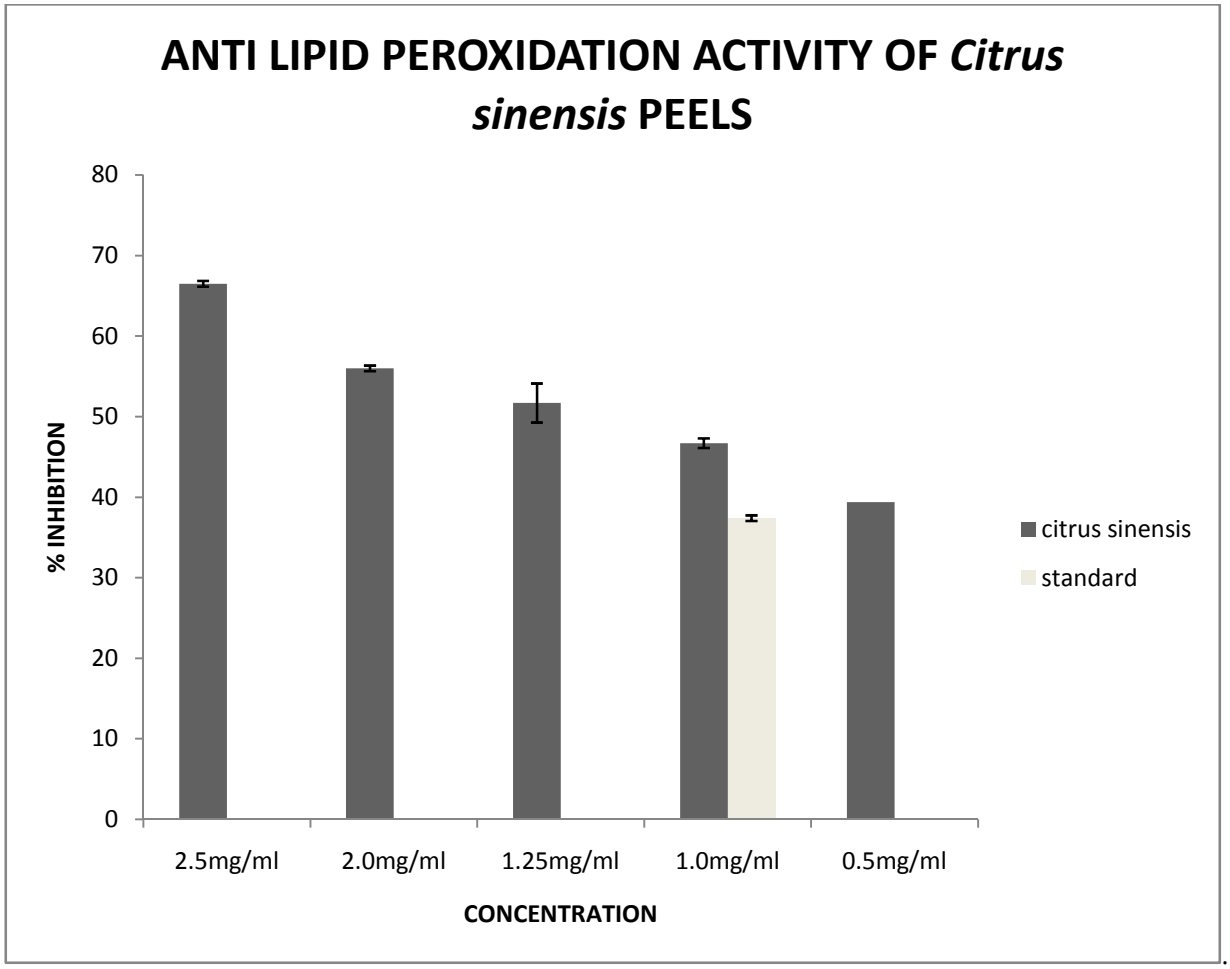

Figure 4: Anti-lipid Peroxidation Activity

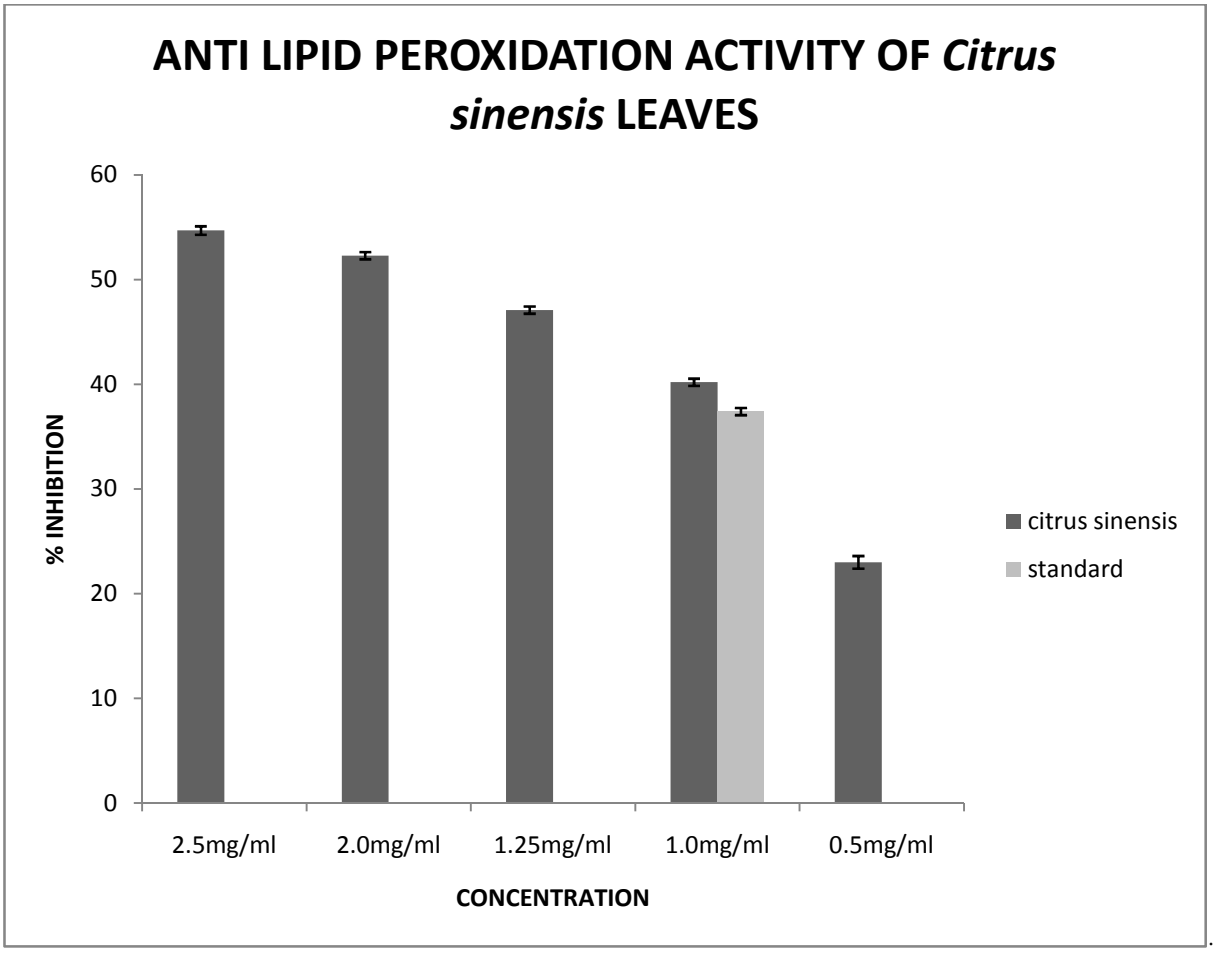

Figure 5: Anti-lipid Peroxidation Activity

DISCUSSION

Phytochemical screening of extracts of *Citrus sinensis* peels and leaves showed the presence of alkaloids, flavonoids, tannins, phenols, saponins and steroid. Ethanolic extracts at different concentrations (250, 125, 62.5, 31.25, and 15.5 mg/ml) exhibited moderate antibacterial activity against *Staphylococcus aureus, Streptococcus pneumoniae, Escherichia coli, Proteus mirabilis* and *Pseudomonas aeruginosa*, in a dose-dependent manner. Zone inhibition diameter was found to decrease with decreasing concentration. Highest inhibitions was seen in Proteus mirabilis (32 mm) by peels extract and in *Staphylococcus aureus* (37mm) by leaves extract at concentration of 250mg/ml. Both extracts showed no antibacterial activity at concentration of 15.5mg/ml.

Since the Minimum Inhibitory Concentration (MIC) of *Proteus mirablilis* and *Pseudomonas aeruginosa* for leaves extract is 62.5mg/ml, like-wise for *Escherichia coli* and *Pseudomonas aeruginosa* MIC is 62.5mg/ml for peel extract, it means that these organisms require a higher dose of the test drug for significant effect.[27,28]

Egg albumen was used to activate the mast cells to secrete inflammatory mediators. Figure 1 clearly shows that leaves extract administered at different concentrations demonstrated good anti-inflammatory activity that was dose-dependent. The % Inflammation decreased steadily as time intervals increased while the % Inhibition of Inflammation increased steadily, thereby establishing a trend. The anti-inflammatory property may be due to the higher concentration of tannins present in the leaves. Tannins have been reported to possess anti-inflammatory properties [29,30].

The ethanolic extracts of *Citrus sinensis* peels and leaves showed good antioxidant activity in nitric oxide (NO) inhibition and anti-lipid peroxidation models as shown in tables 9–10 and figure 2 – 5.

The test drug was compared with a low concentration of ascorbic acid and the scavenging effect was found to increase with increasing concentration of extracts. Highest inhibition was seen in extract of highest concentration. 65.7 % for peels and 57.4% for leaves in NO scavenging assay, 66.5% and 54.7% for peels and leaves respectively

in anti-lipid peroxidation assay. IC_{50} was obtained as 1100µg/ml for peels and 1100µg/ml for leaves extracts in nitric oxide scavenging assay while for anti-lipid peroxidation assay IC_{50} was 1000µg/ml for peels and 1100µg/ml for leaves extracts. The higher scavenging effect observed in peels extract is because of the higher concentration of flavonoids present in them as revealed by phytochemical screening shown in table 2. It could also be due to the fact that vitamin C in oranges is concentrated mainly in the peel and in the white layer, just under the peel. At probability value of $P<0.05$, there was no significant difference between the mean values of peels extract at 2.0mg/ml and the standard in nitric oxide inhibition assay, while at 0.5mg/ml of peel extracts in anti-lipid peroxidation assay, there was no significant difference between the mean value at that concentration and that of the standard. Hence, it could be said that at those concentrations, the scavenging effect of the extracts were comparable to that of 1mg of ascorbic acid used as standard.[31,32]

CONCLUSION

From the present investigation it may be concluded that ethanolic extracts of *Citrus sinensis* peels and leaves are potent antibacterial,anti- inflammatory and antioxidant agent.This study has shown that sweet orange peels which are considered as waste materials of the fruit, and leaves could serve as potential antibacterial, anti-inflammatory and antioxidant agents.Further works may however be carried out to characterize the specific active component responsible for these activities.

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