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Phytochemical Screening and *in vivo* Antioxidant Assessment of *Citrus sinensis* Peel Hexane Extract in Albino Rats

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ABSTRACT

Background and Objective: Oxidative stress plays a key role in many chronic diseases, and safer natural antioxidants are in demand. This study aimed to evaluate the *in vivo* antioxidant potential of *Citrus sinensis* (L.) peel N-Hexane extract in albino rats. **Materials and Methods:** The extract was screened for phytochemical compounds and its acute toxicity and antioxidant properties. Eighteen adult albino rats of both sexes were used for *in vivo* analysis, divided into six groups with three rats each. Groups 1, 2, and 3 received distilled water, carbon tetrachloride (CCl_4), and silymarin, serving as normal, negative, and positive controls, respectively. Groups 4, 5, and 6 received 100, 200, and 400 mg/kg of the extract, respectively, and served as the extract-treated groups. Oxidative stress was induced intraperitoneally using CCl_4 in all groups except the normal control group. Blood samples were collected seven days after oxidative induction and analyzed for MDA, GSH, SOD, and CAT. Data were analyzed using One-way ANOVA with Duncan's multiple comparison test in SPSS (v25), considering $p<0.05$ as significant. **Results:** The phytochemical analysis revealed the presence of alkaloids, glycosides, steroids, and saponins, while flavonoids, tannins, and volatile oils were absent. Acute toxicity tests showed that the extract was well-tolerated at 5000 mg/kg b.wt. The results of the *in vivo* study showed that the extract significantly ($p<0.05$) decreased MDA and GSH levels and increased SOD and CAT activities compared to the negative control group. **Conclusion:** This study suggests that the *C. sinensis* peel hexane extract has antioxidant potential in albino rats and could contribute to existing literature on its pharmacological properties.

KEYWORDS

Antioxidants, *Citrus sinensis*, oxidative stress, albino rats, Carbon Tetrachloride (CCl_4)

INTRODUCTION

Oxidative stress, stemming from an imbalance between reactive oxygen species (ROS) production and the body's neutralization capacity, is implicated in various diseases[1]. Mitochondrial respiration, enzymatic reactions, and external factors contribute to ROS generation[2]. Antioxidants, both enzymatic (e.g., SOD, catalase, glutathione peroxidase) and non-enzymatic (e.g., vitamins C and E, glutathione, flavonoids), counteract oxidative stress[3]. Research explores the roles of specific antioxidants in diseases, highlighting the significance of oxidative stress and antioxidants in health management[4].

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Citrus sinensis (L.), known for its medicinal properties, has been historically used to address various ailments, such as digestive issues, respiratory conditions, and immune enhancement[5]. The peel's high fiber and nutrient content contribute to weight management and heart health[6,7]. The fruit itself is low in calories, cholesterol-free, and a good source of vitamin C, potassium, and calcium. Overall, *C. sinensis* presents a holistic approach to health, addressing a range of conditions from digestive issues to cancer prevention through its diverse bioactive components[6,7].

Indeed, there are several studies that have investigated the antioxidant potential of *C. sinensis in vitro*. However, the *in vitro* studies have little significance unless supported by *in vivo* studies. Therefore, the present study aimed at investigating the *in vivo* antioxidant effect of *C. sinensis* peel extract in albino rats.

MATERIALS AND METHODS

Study area: The research was conducted at the Biochemistry Research Laboratory, Department of Biochemistry, Faculty of Life Sciences, Kebbi State University of Science and Technology, Aliero, Nigeria from April 6 to August, 24, 2023, spanning four months.

Experimental animals and ethical approval: Adult Wister albino mice of both sexes aged 2-4 months and weighing between 20-30 g were used for the experiments. They were purchased from National Veterinary Research Institute, Vom, Nigeria, and kept under standard laboratory conditions (22-24°C; 12:12 hrs dark/light cycle). The animals were allowed free access to both food (commercial rodent pellets) and water *ad libitum*[8] and allowed to acclimatize for 2 weeks. The weight of each mouse was taken before the commencement of the experiment. All animal experiments were conducted per the guidelines for the use and care of experimental animals[8].

Collection and identification of plant sample: The peel of *C. sinensis* (L.) was collected in April 2023 from Aleiro, Aleiro Local Government Area of Kebbi State, Nigeria. The plant was authenticated by a Taxonomist from the Department of Plant Science and Biotechnology, Kebbi State University of Science and Technology, Aleiro where the Voucher specimen Number (KSUSTA/PSB/H/Voucher NO:285 A) was deposited.

Plant sample preparation and extraction: The *C. sinensis* (L.) peels were washed with clean water and allowed to dry in the shade for two weeks. It was then ground to coarse powder using a grinding machine.

About 200 g of orange peel was placed into the thimble and placed in the Soxhlet chamber[8]. A 1500 mL of N-hexane were placed in a round-bottom flask and assembled for the Soxhlet extractor, then the distillation process was begun. After completing the extraction process, the solvent and extractor were placed in a water bath to evaporate the solvent. The extracted orange peel oil was weighed by using the following equation:

$$\text{Oil yield (\%)} = \frac{W_1 - W_2}{W_1} \times 100$$

Where:

W_1 = Sample weight initially placed in the thimble

W_2 = Sample weight after dried in the oven

Qualitative phytochemical screening of *Citrus sinensis* (L.) peel hexane extract

Detection of alkaloids: The extract (0.2 g) was boiled with 5 mL of 2% HCl on a steam bath for 20 min. The mixture was then filtered, and 1 mL portion of the filtrate was treated with two drops of Wagner's reagent. A reddish-brown precipitate was formed, and this indicates the presence of alkaloids[9].

Detection of flavonoids: The extract (0.2 g) was dissolved in 1 mL of 10% Sodium Hydroxide (NaOH) a yellow colour developed which indicates the presence of a flavonoid[9].

Detection of tannins: Ferric chloride solution 5% was added drop by drop to 2 mL of the extract and the colour produced was noted. A dark green colour was observed, which indicates the presence of tannins[10].

Detection of steroids: The extract (0.5 g) was dissolved in 2 mL of chloroform, and 1 mL of concentrated sulphuric acid was carefully added to form a lower layer. A reddish-brown interface showed the presence of steroids[9].

Detection of saponins: To 0.5 g of the extract, 2.5 mL of distilled water was added, and sharked strong appearance of froth that lasts for several minutes indicates the presence of saponins[9].

Detection of glycosides: Exactly 2.5 mL of 50% H_2SO_4 was added to 0.5 g of the extract in a test tube. The mixture is heated in boiling water for 15 min. Cool and neutralized with 10% NAOH, then 5 mL of Fehling solution was added, and the mixture was boiled. A brick-red precipitate was observed, which indicates the presence of glycosides[9].

Detection of volatile oils: To 1 g of the extract, 1 mL of water was added, and the solution was then mixed with dilute HCL. Formation of white precipitate indicated the presence of volatile oils[10].

Acute toxicity profile of *Citrus sinensis* (L.) peel hexane extract: The acute oral toxicity studies of *C. sinensis* (L.) peel hexane extract were undertaken as per the Organization for Economic Co-operation and Development[11] guidelines for testing of chemicals by fixed dose procedure. The rats were fasted overnight, and the weight of each rat used was recorded just before use. Animals were divided randomly into one treatment group consisting of five Albino rats. The animals were administered with an extract 5000 mg/kg *C. sinensis* (L.) peel hexane. Animals were kept under close observation for 1 hr, 4, 6 and 12 hrs after administering the extracts, and then they were observed daily for 14 days for any change in general behaviour and other physical activities.

***In vivo* antioxidant evaluation of *Citrus sinensis* (L.) hexane extract**

Experimental design: Oxidative stress was induced in the rats by intraperitoneal injection of Carbon Tetrachloride (CCl_4) in a dose of 0.25 mL of CCl_4 in liquid paraffin (1:1) 1.25 mL/kg[12]. Then the rats were randomly divided into 6 groups (n = 4) and treated as follows (Table 1).

Determination of lipid peroxidation (malondialdehyde) level: Lipid peroxidation was determined by measuring spectrophotometrically the level of the lipid peroxidation product, Malondialdehyde (MDA), as described by Wallin *et al.*[13]. Briefly, 0.1 mL of the serum was mixed with 0.9 mL of distilled water in a tube. About 0.5 mL of 25% Trichloroacetic Scid (TCA) and 0.5 mL of 1% thiobarbituric acid (TBA) in 0.3% NaOH were also added to the mixture. The mixture was boiled for 40 min in a water bath and then cooled in cold water. Then 0.1 mL of 20% sodium dodecyl sulfate (SDS) was also added to the cooled solution and mixed properly. Thereafter, the absorbance was measured at the wavelengths of 532 and 600 nm against a blank:

$$\text{MDA coneentration (nmoL / L)} = \frac{\text{Abosrbance}}{1.56} \times 100$$

where, 1.56 is the conversion factor used to convert the absorbance measurement to the desired concentration unit (nmoL/L).

Table 1: Experimental design

Groups	Treatment
Group A	Served as the normal control. Neither induced nor treated
Group B	CCl_4 induced rats, but untreated and served as the negative control
Group C	CCl_4 induced rats and treated with silymarin (0.2 mg/kg) and served as the standard control
Group D	CCl_4 induced rats and treated with extracts (100 mg/kg) and served as the treatment group
Group E	CCl_4 induced rats and treated with extracts (200 mg/kg) and served as the treatment group
Group F	CCl_4 induced rats and treated with extracts (400 mg/kg) and served as the treatment group

The extract was administered to the animals orally. The rats were sacrificed on the seventh day of the experiment. Blood samples were collected in heparinized bottles for antioxidant analysis

Determination of reduced glutathione (GSH): This was based on the method of Jollow *et al.*[14]. Briefly, a volume of the sample (0.1 mL) was mixed with 0.9 mL of the distilled water in a beaker. The 1 mL of 2% sodium sulphate was also added. This was followed by the addition of 0.02 mL of 20% lithium sulphate, 0.2 mL of 20 % Na_2CO_3 and shaken vigorously. Then, 0.2 mL of phospho-18-tungstic acid was added inside. The absorbance was measured at 450 nm:

$$\text{Glutahione conentration} = \frac{\text{Abs of sample}}{\text{Abs of std}} \times \text{Conc. of std (100 mg/dL)}$$

Assay for superoxide dismutase (sod) activity: This was determined using the method of Xin *et al.*[15]. Briefly, 0.01 g of adrenaline was dissolved in 17 mL of distilled water, and 0.1 mL of serum and 0.9 mL of phosphate buffer (pH 7.8) were taken in triplicate in 2.5 mL buffer. A volume (0.3 mL) of adrenaline solution was added and mixed inside the cuvette. The absorbance was taken at 480 nm at 30 sec intervals five times.

The changing rate of absorbance was used to determine superoxide dismutase activity using this equation:

$$\text{SOD inhibiton (U/mL) (\%)} = \frac{\text{Absorbance of the blank - Absosrbance of the sample}}{\text{Absorbance of the blank}} \times 100$$

Assay for catalase activity: The activity of catalase was assayed according to the method of Aebi[16]. Briefly, 2 mL of hydrogen peroxide and 2.5 mL of phosphate buffer were added to a beaker. Subsequently, 0.5 mL of the sample was also added and mixed. Then, 1 mL portion of the reaction mixture was added to 2 mL of dichromate acetic acid reagent. The absorbance was read at 570 nm at a minute interval into 4 places. Catalase activity was calculated using the following equation:

$$\text{Catalaseaactivity (U/mL)} = 0.23 \times \frac{\log \text{Abs 1}}{0.00693}/\text{Abs2}$$

Where:

0.23 and 0.00693 = Constant value used
Abs1 = Absorbance of the sample
Abs2 = Absorbance of the standard

Data analysis: The data collected were subjected to statistical analysis. The significance of the difference between the means was determined using One-way Analysis of Variance (ANOVA), and $p<0.05$ was considered as significant. Duncan Multiple Comparison Test was used to test the differences using SPSS (Version 25) statistical software.

RESULTS

Qualitative phytochemical screening: The qualitative phytochemical screening of *C. sinensis* (L.) peel hexane extract is presented in Table 2. The results revealed the presence of alkaloids, steroids, saponins, and glycosides, while flavonoids, tannins, and volatile oils were not detected.

Acute toxicity (LD_{50}) profile of *Citrus sinensis* (L.) peel hexane extract: There were no obvious signs of toxicity such as bulging of eyes, restlessness, convulsion or mortality after administering *C. sinensis* (L.) peel hexane extract (5000 mg/kg). Hence, the LD_{50} was considered to be greater than 5000 mg/kg b.w.

***In vivo* antioxidant effect of *Citrus sinensis* (L.) peel hexane extract in albino rats:** Figure 1 revealed that the level of MDA (Malondialdehyde) and reduced GSH exhibited a significant increase ($p>0.05$) in the negative control group when compared to the normal control group. Administration of the extract significantly ($p<0.05$) decreased the concentrations of the MDA and GSH levels between negative and normal control, and all the extract-treated groups and standard are significantly different compared to negative and normal control. The activity of Superoxide Dismutase (SOD) exhibited a significant decrease ($p>0.05$) in the negative control group in comparison to the normal control group. Notably, only the group treated with the standard drug showed a significant increase ($p>0.05$) when compared to the negative control group. All the extract-treated groups (100, 200, and 400 mg/kg) displayed SOD activities that were significantly similar ($p>0.05$) to those in both the negative and normal control groups. The activity of Catalase (CAT) showed significant similarity ($p>0.05$) in all the extract-treated groups and the standard control group when compared to both the negative and normal control groups.

Table 2: Qualitative phytochemical compositions of *Citrus sinensis* (L.) peel hexane extract

Phytochemicals	Observation
Alkaloids	+
Flavonoids	-
Tannins	-
Steroids	+
Saponin	+
Glycoside	+
Volatile oil	-

+: Detected and -: Not detected

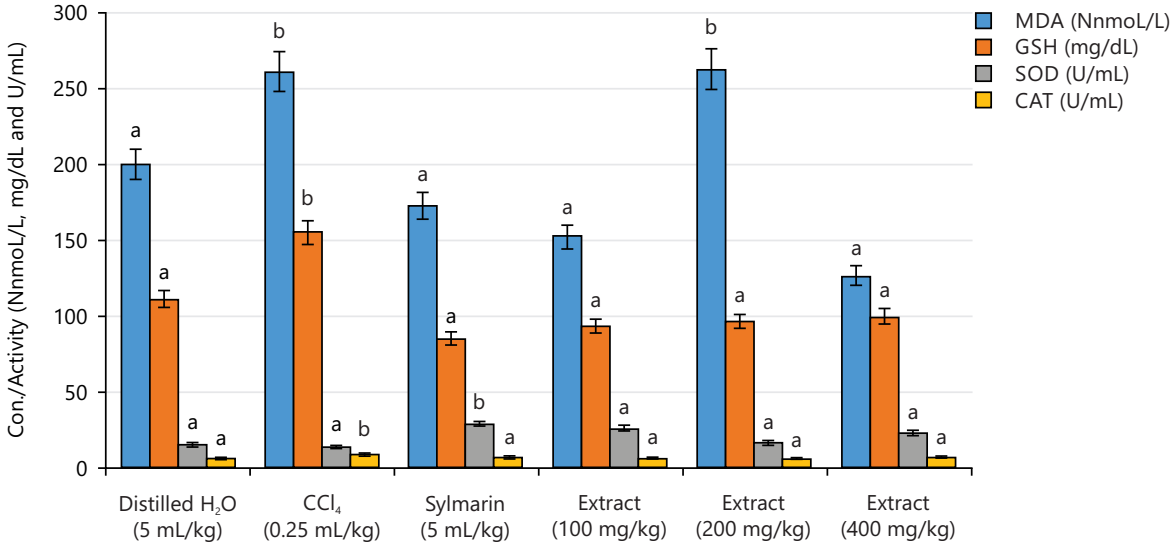

Fig. 1: *In vivo* antioxidant effect of *Citrus sinensis* (L.) peel hexane extract in albino rats

The results are represented as Mean±SD. Bars having similar letters are not significantly difference at $p>0.05$, CCl_4: Carbon Tetrachloride, MDA: Malondialdehyde, GSH: Reduced glutathione, SOD: Superoxide dismutase and CAT: Catalase

DISCUSSION

The results revealed the presence of alkaloids, steroids, saponins, and glycosides, while flavonoids, tannins, and volatile oils were not detected. The presence of alkaloids, steroids, saponins, and glycosides is consistent with previous findings reported in the Caffrey *et al.*[17]. Alkaloids are known for their various medicinal properties, including antimicrobial and analgesic effects[17]. Saini *et al.*[18] reported that Saponins possess potential antioxidant and anticancer activities in plant extracts, and this could be by lowering the Phospholipase A_2 activity, which causes a decrease in the hydrolysis of membrane *phospholipids* and thereby decreasing membrane fluidity. Glycosides are also commonly found in citrus fruits, and they have been reported to have antioxidant, antimicrobial, and cardiovascular protective effects[19]. Steroids, on the other hand, have been associated with anti-inflammatory and immunomodulatory activities[20]. The absence of flavonoids, tannins, and volatile oils in the hexane extract of *C. sinensis* (L.) peel in this study may be due to differences in extraction methods and solvent polarity. Previous studies have reported the presence of flavonoids and volatile oil in *C. sinensis* (L.) peel using different extraction techniques such as methanol or ethanol[21]. Tannins, which are often present in various plant parts, may not have been extracted efficiently using hexane. It is important to note that the presence or absence of phytochemicals may vary depending on factors such as plant species variety, geographical location, and extraction methods. Therefore, it is crucial to compare the findings with other studies conducted on *C. sinensis* (L.) peel using similar extraction methods to draw accurate conclusions.

The present study demonstrated that there were no obvious signs of toxicity, such as bulging of eyes, restlessness, convulsion, or mortality, after administering *C. sinensis* (L.) peel hexane extract (5000 mg/kg). Hence, the LD_{50} was considered to be greater than 5000 mg/kg b.wt. The acute toxicity profile of *C. sinensis* (L.) peel hexane extract was determined using OECD guidelines (OECD Test No. 425). As reported by Nurul Husna *et al.*[22], when test animals show no visible signs of toxicity or death at a specific dose, it indicates that the lethal dose (LD_{50}) exceeds that tested amount. Another study has demonstrated that harmful secondary compounds in plants are the primary cause of adverse effects when plant materials are consumed[23]. Consequently, these findings indicate that the examined plant extracts either do not contain poisonous compounds or contain them at levels too minimal to produce detectable toxic effects. Nevertheless, since the LD_{50} exceeds 5000 mg/kg, the extract can be considered safe for therapeutic use.

The *in vivo* assay showed that *C. sinensis* (L.) peel has antioxidant potential. The level of MDA (Malondialdehyde) and reduced GSH exhibited a significant increase ($p>0.05$) in the negative control group when compared to the normal control group. Administration of the extract significantly ($p<0.05$) decreased the concentrations of the MDA and GSH levels between negative and normal control while all the extract-treated groups and the standard control group were significantly different compared to the negative and normal control groups. This suggests that the hexane extract of *C. sinensis* (L.) peel possesses significant *in vivo* antioxidant potential in terms of reducing MDA and GSH levels. Some studies have reported significant antioxidant effects of *C. sinensis* peel hexane extracts, while others have found no or limited antioxidant activity. Schneider *et al.*[19] demonstrated that a methanol extract of *C. sinensis* (L.) peel had significant antioxidant activity in various *in vitro* assays. The authors attributed this activity to the presence of phenolic compounds such as flavonoids and phenolic acids, which are known to possess potent antioxidant properties. On the other hand, the extract of *C. sinensis* (L.) peel exhibited moderate antioxidant activity *in vitro,* it had limited effects *in vivo* in terms of reducing oxidative stress markers. The extract increased the enzymatic activity of SOD compared to the standard drug, and all extract-treated groups were significantly similar to both the negative and normal control groups. Additionally, the catalase (CAT) activity is increased across all the extract-treated groups and are similar to standard, negative, and normal control. This could be due to an adaptive response of the antioxidant defense system in the untreated animals, as they may have experienced increased oxidative stress.

CONCLUSION

The *C. sinensis* L. peel N-hexane extract exhibited notable antioxidant effects in albino rats by lowering MDA and GSH levels while enhancing SOD and CAT activities. The extract was well-tolerated in acute toxicity tests, indicating its safety at high doses. Its phytochemical profile supports its observed bioactivity. These results highlight its potential as a natural antioxidant for further pharmacological research.

SIGNIFICANCE STATEMENT

This study discovered the antioxidant potential of *Citrus sinensis* L. peel N-hexane extract that can be beneficial for mitigating oxidative stress-related damage in biological systems. The extract's ability to reduce MDA and GSH levels while enhancing SOD and CAT activities demonstrates its role in protecting against free radical-induced cellular injury. Its favorable safety profile further supports its potential for therapeutic use in managing chronic diseases linked to oxidative stress. This study will help researchers to uncover the critical areas of plant-based antioxidant pharmacology that many researchers were not able to explore. Thus, a new theory on the medicinal exploitation of citrus peel bioactive compounds for oxidative stress management may be arrived at.

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