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Effect of *Citrus maxima* (Shaddock) fruit juice on some oxidative stress parameters in CCl_4 induced oxidative stress in wistar rats

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Abstract
This study was designed to evaluate the *in vivo* anti-oxidative stress potentials of *Citrus maxima* juice. Thirty six wistar rats were divided into nine groups. Group 1 served as the normal control. Groups 2-5 which served as the protective study groups were treated from day 1 to 14 of experiment, and administered Carbon tetrachloride on days 13 and 14. Groups 6-9 served as the curative study groups and were administered Carbon tetrachloride on days 0 and 1 of the experiment and treated from day 1 to 14. All animals were sacrificed on the 15th day after a 14-16 hour overnight fast and blood sample collected through ocular bleeding for biochemical analyses. Intoxication with Carbon tetrachloride decreased significantly (p>0.05) the activities of the antioxidant enzymes - Superoxide dismutase, Gluthatione peroxidase and Catalase. Administration of CMJ increased non-significantly (p>0.05) the activities of the antioxidant enzymes.

Keywords: *Citrus maxima*, shaddock, oxidative stress, carbon tetrachloride (CCL_4) wistar rats

Introduction
Humans are continuously exposed to different kinds of chemicals such as food additives, industrial chemicals, pesticides, drugs and other undesirable contaminants in the air, food and soil [1]. Exposure to these toxic chemicals can cause cellular injuries through metabolic activation of reactive oxygen species (ROS) [2]. Reactive oxygen and nitrogen species (RONS), including superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\bullet}$) and nitric oxide ($NO^{\bullet}$) are highly reactive molecules produced by living organisms as a result of normal cellular metabolism and environmental factors. These molecules can damage cell structure components such as carbohydrates, nucleic acids, lipids, and proteins leading to disruption of biomembranes and dysfunction of cells and tissues [3, 4]. In a healthy individual, a well maintained balance exists between production of these free radicals and their rates of removal by various antioxidant defence mechanisms [5]. However, when one gets exposed to adverse physicochemical, environmental or pathological toxins, this delicately maintained balance is shifted in favour of pro-oxidants resulting in oxidative stress [6].

Oxidative stress is a redox disequilibrium in which the pro-oxidant/antioxidant balance is shifted in favour of the pro-oxidants [7]. Oxidative stress is implicated in the pathogenesis of various diseases including hypertension, atherosclerosis, diabetes mellitus, cancer, neurological diseases such as Alzheimer's disease, Parkinson's disease, etc, as well as in the ageing process [8]. Although cells are equipped with an impressive repertoire of antioxidant enzymes (e.g. superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx)) as well as small antioxidant molecules (e.g. glutathione, α-tocopherol, ascorbic acid, bilirubin etc), these agents may not be sufficient enough to normalize the redox status during oxidative stress [9, 10]. Therefore, dietary intake of antioxidants is imperative to protect cells from damage caused by free radicals [4]. Antioxidants are compounds that can delay, inhibit, or prevent the oxidation of oxidizable materials by scavenging free radicals and diminishing oxidative stress [11]. Consumption of antioxidant enriched fruits and vegetables are known to lower the risk of several diseases caused by free radicals. Such health benefits are mainly due to the presence of phytochemicals such as polyphenols, flavonoids, and vitamins A, C, and E.

[4] Carbon tetrachloride (CCl4) is a well-known toxicant, and exposure to this chemical is known to induce oxidative stress by the formation of free radicals [12]. It requires biotransformation by hepatic microsomal cytochrome P-450 to produce toxic metabolites, namely trichloromethyl free radicals (CCl_3) and subsequent derivative Cl_3COO [1].

Citrus fruit species are the most popularly consumed fruits in the world today are usually consumed as fresh produce or juice. *Citrus maxima* commonly known as pomelo or shaddock, is a medium sized tree, with large leaves, flowers, and fruits. [13] *Citrus maxima* fruit is the largest of all citrus varieties. It is globose, pear-shaped with 11-14 segments. The pulp is white or pinkish red spindle-shaped juice sacks that may separate easily from one another, with a sweetish-acidic flavor, and its juice contains nutrients as well as many phytochemicals [14, 15].

Intake of foods/fruits with antioxidant properties aids in managing oxidative stress and hence ameliorating the onset and progress of some diseases. Therefore the assessment of such properties is imperative.

Material and methods

Materials

Animals

The animals used in this study were female Wistar rats with a weight range of 70-140 g. They were purchased from the animal house of the Department of Zoology and Environmental Biology, University of Nigeria, Nsukka, and were acclimatized at the animal house of the Department of Biochemistry, University of Nigeria, and Nsukka for one week prior to the experiment. They were maintained on growers mash (feed) and water *ad libitum*.

Plant Material

Citrus maxima (Shaddock) fruits (white variety) were obtained from Ngwo town in Enugu State and were identified by Mr. Onyeukwu, C.J. of the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka.

Chemicals/Reagents

All chemicals used in this study were of analytical grade and products of Sigma Aldrich (USA), British Drug House (BDH) (England), Burgoyne (India), Hopkins and Williams (England), Qualikems (India), Fluka (Germany) and May and Baker (England). Reagents used for the assays were commercial kits and products of Randox, (USA), QCA (Spain) and Teco (TC) (USA).

Methodology

Preparation of plant material

Citrus maxima (Shaddock) fruits were washed with clean water, peeled and juice extracted with a manual screw juice extractor. Juice obtained was called 100% *Citrus maxima* juice (CMJ). Juice administered to animals was prepared by diluting 100% juice with distilled water in the ratio 1:1 (v/v) to obtain 50% CMJ.

Experimental design

Thirty six (36) rats were distributed into nine (9) groups of four rats each. The study phases were divided into two (2); Preventive (Groups 2-5) and curative (Groups 6-9) and administered as follows: Group 1: Normal control (Feed and water only).

Preventive study

Group 2: 5 ml/kg b. w. of 50% *Citrus maxima* juice (CMJ) + CCl_4 Group 3: 10 ml/kg b. w. of 50% *Citrus maxima* juice (CMJ) + CCl_4 Group 4 (Standard control): 100 mg/kg b. w. of Ascorbic acid (AA) + CCl_4 Group 5: CCl_4 only.

Curative study

Group 6: CCl_4 + 5 ml/kg b. w. of 50% *Citrus maxima* juice (CMJ) Group 7: CCl_4 + 10 ml/kg b. w. of 50% *Citrus maxima* juice (CMJ) Group 8 (Standard control): CCl4 + 100 mg/kg b. w. Ascorbic acid (AA) Group 9: CCl_4 only for the preventive study, animals were administered CMJ from day 1 to 14 of experiment, and were injected a double dose of carbon tetrachloride (CCl_4) intra-peritoneally on days 13 and 14 with the last dose given 14-16 hours before the end of experiment. For the curative study, CCl_4 was injected as a double dose intra-peritoneally on days 0 and 1 of the experiment, and fruit juice administered from day 1 to day 14. On the 15^{th} day, the animals were sacrificed after an overnight fast and blood samples collected by ocular bleeding into sample tubes. After about 1 hour, blood samples were centrifuged for 10 minutes at 3000 rpm and serum carefully pipetted into clean sample tubes for subsequent biochemical analyses.

Biochemical assay

The effect of *Citrus maxima* juice on serum biochemical parameters of rats intoxicated with carbon tetrachloride (CCl_4) was studied. Superoxide dismutase (SOD) activity was assayed using the method described by Xin *et al.* [16]. Glutathione peroxidase activity was assayed according to the method of Palgia and Valentine [17]. Catalase (CAT) activity was assayed according to the method described by Aebi [18]. Glutathione (GSH) Concentration was determined according to method described by King and Walton [19].

Statistical Analysis

The data were analyzed using Statistical Product for Social Sciences (SPSS) version 16 and the results were expressed as mean±standard deviation. Significant differences were established by one-way analysis of variance (ANOVA). Mean values with $p<0.05$ were considered statistically significant.

Results

Effect of *Citrus maxima* juice on some oxidative stress parameters in rats

Table 7 shows the activity of SOD, GPx and CAT, and concentration of GSH in the serum of experimental rats in the various groups of the study. Compared to the normal control, animals administered CCl_4 without treatment showed a decrease in SOD ($p>0.05$), GPx ($p>0.05$) and CAT ($p>0.05$) activity, except for CAT and GSH of the protective study which showed a non-significant ($p>0.05$) increase. Compared to the untreated group, animals in both study phases treated *C. maxima* juice showed a non-significant ($p>0.05$) increase in the activities of SOD, GPx, and CAT except for the GPx activity in the curative study animals which showed a non-significant decrease. Glutathione concentration in CMJ treated animals of both protective and curative studies showed a non-significant ($p>0.05$) decrease compared to the untreated group.

Table 1: Effect of *Citrus maxima* juice on some oxidative stress parameters in rats

Groups	Parameters			
	SOD (U/L)	GPX (U/L)	CAT (IU/L)	GSH (mg/dl)
Preventive Study				
Normal control CCl_4	8.13±2.69[b] 1.95±1.48[a]	26.54±20.00[a] 7.92±4.82[a]	5.50±0.42[a] 6.08±0.52[ab]	2.93±0.77[a] 4.27±1.41[a]
5ml/Kg b. w. CMJ + CCl_4	8.26±2.49[b]	28.68±20.9[a]	5.58±0.62[a]	3.77±1.60[a]
10ml/Kg b. w. CMJ + CCl_4	5.70±2.68[ab]	28.01±25.94[a]	7.55±2.29[b]	3.78±0.58[a]
100mg/Kg b. w. AA + CCl_4	4.68±2.49[ab]	8.08±4.41[a]	5.85±0.31[ab]	3.44±3.11[a]
Curative Study				
Normal control CCl_4	8.13±2.69[b] 4.17±0.34[a]	26.54±20.00[b] 18.53±12.08[ab]	5.50±0.42[a] 4.70±1.03[a]	2.93±0.77[a] 2.75±0.94[a]
CCl_4 + 5ml/Kg b. w. CMJ	5.88±1.56[ab]	7.79±3.58[a]	5.44±0.53[a]	2.74±1.16[a]
CCl_4 + 10ml/Kg b. w. CMJ	5.61±1.59[ab]	5.85±4.20[a]	5.27±0.29[a]	2.40±1.00[a]
CCl_4 + 100mg/kg b. w. AA	5.14±3.40[ab]	11.21±5.45[ab]	5.00±0.26[a]	2.97±0.85[a]

CMJ= Citrus maxima juice; AA= Ascorbic acid; SOD= Superoxide dismutase; GPx= Glutathione peroxidase; CAT= Catalase; GSH= Reduced glutathione; b. w. = body weight. Values are Mean ± SD. (n=4)

Down the groups, numbers with different superscript letters are significantly different at $p>.05$.

Discussion

Superoxide dismutase (SOD) is the first enzyme involved in the antioxidant defenses against reactive oxygen species (ROS) by dismutation of superoxide anion ($O^{\cdot}_2$) to hydrogen peroxide (H_2O_2) [1]. Intoxication with CCl_4 decreased significantly ($p>0.05$) its activity compared to the normal control, while treatment with CMJ increased its activity compared to the untreated groups. According to Liu *et al.* [20] if the balance between ROS production and antioxidant defense is broken, the enzyme may be exhausted and its concentration depleted. Elevated enzyme activity in CMJ treated groups could be a result of the extract acting either by directly scavenging the reactive oxygen metabolites due to the presence of various antioxidant compounds, or by increasing the synthesis of antioxidant molecules. The increased SOD activity may also be due to the effects of some metal ions present in the juice - zinc and copper, which serve as cofactors for SOD isoenzymes. When copper is not present in sufficient quantities, the activity of superoxide dismutase is diminished and the damage to cell membranes caused by superoxide radicals is increased [21]. Glutathione peroxidase (GPx) is important for extra-peroxisomal inactivation of hydrogen peroxide generated by superoxide dismutase [22]. Intoxication with CCL_4 decreased non-significantly its activity compared to the normal control. Treatment with CMJ non-significantly ($p>0.05$) increased its activity in the protective study, but decreased it in the curative study groups. The increased antioxidant capacity in the system of animals pre-treated with *Citrus maxima* juice could have played a role reducing the concentration of hydrogen peroxide (H_2O_2) in the system and hence prevent increased depletion of this enzyme. However, decrease in enzyme activity observed in the CMJ treated group of the curative study could partly be attributed to the decrease in H_2O_2 concentration, which has been reported to serve as redox signals bringing about increased synthesis of some antioxidant enzymes [23]. In other words, the supply of exogenous antioxidants by *Citrus maxima* juice after CCl_4 administration could have probably spared" the increased synthesis of this antioxidant enzyme. Oyedepo [24] also reported significant increase in SOD and GPx activities in normal rats administered *Citrus maxima* juice for eight (8) weeks compared to the normal control (untreated group). The non-significant increase observed in our study could be as a result of the shorter duration of study which was for two (2) weeks. Catalase (CAT) is a peroxisomal enzymatic antioxidant which decomposes H_2O_2 to oxygen and water, and protects the tissue from highly reactive hydroxyl radicals [8]. Compared to the normal control, the untreated groups showed a non-significant ($p>0.05$) increase and decrease in CAT activity of the protective and curative study groups respectively. Animals treated CMJ showed a higher ($p>0.05$) CAT activity compared to the untreated group. The observed increase ($p>0.05$) in the CAT activity of the untreated protective study group could be attributed to a compensatory increase in enzyme activity to overcome raised oxidative stress/directly inhibit the effects of ROS. A key feature of cellular responses to oxidative stress is the increased expression of certain genes, some of which encode antioxidant proteins [25, 26]. This enables cells to survive oxidant exposures that would normally be lethal [27]. The GSH molecule is a nonenzymatic antioxidant capable of scavenging free radicals, and also serves as cofactor for glutathione peroxidase [28]. Compared to the normal control, the untreated groups showed a non-significant ($p>0.05$) increase and decrease in GSH concentration of the protective and curative study groups respectively. The increase observed in the protective study could be attributed to the reported *de novo* synthesis of glutathione from its amino acid constituents, which is required for the elevation of glutathione as an adaptive response to oxidative stress[10]. Glutathione concentration in CMJ treated animals in both protective and curative studies showed a non-significant ($p>0.05$) decrease compared to the untreated group. This could be attributed to an increased antioxidant level in the system due to an intake of CMJ, hence sparing increased synthesis of glutathione. The results obtained substantiate the fact that oxidative stress affects the activity and concentration of the body's endogenous antioxidant system. The modulatory effect observed in the animal's pre/post-treated with *Citrus maxima* juice could be attributed to the various antioxidant constituents of the fruit, such as vitamin C, vitamin E, phenols, flavonoids, and tannins, which scavenge reactive oxygen species (ROS) and hence prevent ROS accumulation and oxidative damage. Their antioxidant activity has been attributed to various mechanisms such as prevention of chain initiation, the binding of transition metal ion catalysts, decomposition of peroxides, the prevention of continued hydrogen abstraction, the reductive capacity and radical scavenging activity [29].

Conclusion

This study showed that *Citrus maxima* juice possesses relative antioxidant potentials and its intake may be useful in managing oxidative stress.

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