Evaluation of Citrus Paradisi Antioxidant Potential in Carbon Tetrachloride Induced Hepatotoxic Damage in Experimental Wistar Rats

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Abstract:
Objective: This project aimed to evaluate the effect of carbon tetrachloride induced hepatotoxicity i.e. liver damage on the status of antioxidants enzymes and metabolites in rat liver and possible antioxidant ameliorative effect of Citrus paradisi on various biomarker of oxidative stress.

Method: Animals were weighed and divided into six groups consisting of five rats per group with Group I consisting of rats pretreated with 1ml/kg of 0.9% normal saline, Group II consisting of rats treated with 3ml/kg of 30% carbon tetrachloride before oral treatment with 10ml/kg of normal saline, Group III consists of rats treated with 3ml / kg of 30 % carbon tetrachloride before oral treatment with 10ml / kg of ascorbic acid in 0.9% normal saline. Group IV consisting of rats treated with 3ml/kg of 30% carbon tetrachloride before 125mg/kg of aqueous seed extract of Citrus paradisi in 0.9% normal saline, Group V consisting of rats treated with 3ml/kg of 30% carbon tetrachloride before 250mg/kg of aqueous seed extract of Citrus paradisi in 0.9% normal saline, Group VI consisting of rats treated with 3ml/kg of 30% carbon tetrachloride before 500mg/kg of aqueous seed extract of Citrus paradisi in 0.9% normal saline. The effect of carbon tetrachloride induced hepatotoxicity was examined in liver antioxidant enzymes such as serum malondialdehyde, liver malondialdehyde, serum glutathione and liver glutathione.

Results: Single intraperitoneal treatment of a rat with carbon tetrachloride causes significant elevation/reduction in liver antioxidant enzymes when the Groups are compared with controls (Group I). Treatment with graded dose of Citrus paradisi (125mg-500mg/kg body weight) caused a significant reduction in serum malondialdehyde in a dose-dependent manner (Group IV-VI). In the same reaction, aqueous seed extract of Citrus paradisi (250mg-500mg/kg body weight) significantly reduce the liver malondialdehyde level (Group IV-VI) but not dose-dependently. Oral graded dose of Citrus paradisi (125p-500mg/kg body weight). (Group IV-VI) also significantly elevates reduced serum glutathione dose-dependently. Results of the liver reduced glutathione also followed the same trend as that of the serum.

Conclusion: The treatment showed that aqueous seed extract of Citrus paradis has an antioxidant activity with potential free radical scavenging attributes. Therefore, Citrus paradisi can be recommended for the prevention of liver damage caused by carbon tetrachloride toxins.

Keywords: Antioxidant, Carbon tetrachloride, Cytochrome P-450, Hepatotoxicity, Hypersensitivity, Phytochemical, Metabolism, Oxidative stress.
1. INTRODUCTION

Various environmental toxicants and clinically useful drugs can cause severe organ toxicities through the metabolic activation to highly reactive free radicals including the superoxide and reactive oxygen species (ROS) [1]. Aside from reactive oxygen species, some compounds could also be metabolized to reactive nitrogen species (RNS). One of the most extensively studied of the environmental toxicants is carbon tetrachloride (CCl₄).

Carbon tetrachloride, which is a tetramethylmethane, had all the hydrogen atoms in methane substituted by chlorine, a member of the halogen series. It is therefore a haloalkane. Because it has no C-H bond, carbon tetrachloride does not easily undergo free radical reaction. This compound is known to undergo reductive metabolism by cytochrome 2E1 (CYP2E1) into a highly reactive trichloromethyl radical (·CCI₃) and phosgene that initiates lipid peroxidation, disrupts membrane integrity and causes cell death [5]. There are pieces of evidences that various enzymatic and non-enzymatic systems have been developed by the cell to ameliorate the effect of the oxidative stress that is associated with high level of reactive oxygen species that may result from the imbalance between the oxidant and antioxidant status of any organism [16]. It is also common knowledge that the aetiopathogenesis of various organ injuries arising from degenerative diseases are traceable to reactive oxygen/reactive nitrogen species. Degenerative diseases such as cancer, Alzheimer’s disease, etc. have their causative agents in most cases rooted in excessive production of reactive species such as reactive oxygen species and reactive nitrogen species [14].

It has also been well reported that when the oxidative stress is overwhelming, the various inherent defense mechanisms such as the antioxidant defense mechanisms, intracellular concentration of glutathione, superoxide dismutase and catalase activities becomes impaired and insufficient and diseased conditions progress, exacerbate and consequently lead to death [15]. This situation that can enhance mortality and morbidity arising from disease conditions traceable to cellular oxidative stress calls for search for solution in substances that could attenuate oxidative stress. Such a search had over the years been extended to natural products as a result of certain toxicities associated with synthetic ones.

Citrus paradisi, popularly known as grape fruit, is widely cultivated in most countries of the world. Its tree grows up to 3-5 meters high and its fruit is big, globular, nippled at its apex and bright yellow or lemon coloured [3]. Its juice is mildly acidic and has a slight bitter taste. In humans, grape fruit seed extract has been reported to possess antibacterial, antifungal, wound healing and antioxidant properties [11]. Clinical studies have also documented the effectiveness of grapefruit seeds in the treatment of genitourinary tract infections caused by pathogenic organisms such as Pseudomonas aeruginosa, Klebsiella spp, Staphylococcus aureus and Escherichia coli [2]. In Nigerian folkloric medicine, ethanolic decoction prepared from this seed is employed in the treatment of throat/chest and genital infections, diarrheal diseases, stomach ulcer, poisonings, diabetes mellitus, obesity, blood deficiency and as an immunostimulant [3].

Certain phytochemicals with potent antioxidant capabilities have been isolated from Citrus paradisi. One of such is a bioflavonoid known as aringenin [9]. Aringenin is the aglycone of the natural glycoside which constitutes its bitter principle. This principle is believed to act in concert with other phytochemicals to exert antioxidant property.

Herbal treatment is an essential component of traditional medicine that is practiced worldwide because it is believed to be cheap, easily accessible and the confidence reposed in it due to ancestral experience [13]. In spite of the successes recorded in the past years, there is still a surge in the use of herbal remedies in the treatment of human and animal diseases due to perceived untoward effects of synthetic drugs. Also, the increasing rise in the incidence of degenerative diseases such as cancers, neurological diseases of various forms, diabetes, etc., and the realization of the fact that oxidative stress have been implicated in the aetiology of these diseases, aside from the fact that there is a renewed concerted efforts to channel energy of researchers to drug discovery, pharmacological and toxicological evaluation of medicinal herbs with good therapeutic and economic prospects, it is also imperative to use available local resources to solve urgent public health concerns such as the growing wave of degenerative diseases many of which are arising as a result of growing number of toxicants in our environment.
In view of the beautiful reports about *Citrus paradisi* in literature, it will be a worthwhile venture to take a critical look into the health benefits that could result from exploring the possible therapeutic effects.

**Hepatotoxicity** can be defined as injury to the liver that is associated with impaired liver function caused by exposure to a drug or another non-infectious agent. The distinction between injury and function is important, because it is mainly when function is impaired that symptoms and clinically significant disease follow. Agents with recognized hepatotoxicity include carbon tetrachloride, alcohol, dantrolene sodium, valproic acid, and isonicotinic acid hydrazide.

### 2. MATERIALS AND METHODS

#### 2.1 Materials

1. **Plant Material**
   
   Dried seeds of *Citrus paradisi* were used in this study. They were collected during the months of February to April 2015 from various vendors in Lagos. The identification of the plant was based on earlier botanical identification and voucher specimen referencing as done by Adeneye (2008).

2. **Animals**
   
   Wistar rats of both sexes weighing between 150 and 250g were used in this study. They were procured from the breeding stock maintained at the Animal House of the College of Medicine, University of Lagos and housed in well ventilated plastic cages in the Animal House of the Lagos State University College of Medicine, Ikeja. The rats were maintained under a standard natural photoperiodic condition of 12 hr. of light alternating with 12 hr. of darkness with room temperature of between 25 and 26°C and humidity of 65±5%. They were fed with rat chow and allowed access to drinking water until they were grouped into the various experimental groups. Before experimentation, the animals were allowed to grow and acclimatized for a period of three months. Throughout the period of experimentation, experimental procedures involving the animals and their care were conducted in conformity with the guidelines for the care of Laboratory Animals in Biomedical Research as promulgated by Canadian Council of Animal Care (1985) [7].

#### 2.2 Methods

1. **Preparation of Aqueous Seed Extract of *Citrus paradisi***
   
   Mature, ripe grapefruits (*Citrus paradisi*) were cut into pieces and the seeds were mechanically separated out. The seeds were thoroughly and gently rinsed in distilled water several times at room temperature. The washed seeds were drained and water films adhered to the seeds were blotted out with the aid of kitchen towel. The seeds were completely dried in an oven set at 45°C and 300g of the dried seeds were reduced to a powdered substance by grinding. The grounded seeds were soaked in distilled water at 4°C for 48 hrs. Following which it was filtered through a Whatman No. 1 filter paper. The filtrate was evaporated *in vacuo* to give a dark yellowish solid crude mass which was stored in a dessicator until it was ready for use.

2. **Experimental Design and Grouping of Animals**
   
   I= rats pretreated with 1ml/kg of 0.9% normal saline.
   II= rats treated with 3ml/kg of 30% carbon tetrachloride before oral treatment with 10ml/kg of normal saline.
   III= rats treated with 3ml/kg of 30% carbon tetrachloride before 125mg/kg of aqueous seed extract of *Citrus paradisi* in 0.9% normal saline.
   IV= rats treated with 3ml/kg of 30% carbon tetrachloride before 250mg/kg of aqueous seed extract of *Citrus paradisi* in 0.9% normal saline.
   V= rats treated with 3ml/kg of 30% carbon tetrachloride before 500mg/kg of aqueous seed extract of *Citrus paradisi* in 0.9% normal saline.
2.2.3 Induction of Carbon Tetrachloride-Induced Hepatotoxicity/Nephrotoxicity

Drug-induced hepatic/renal toxicity model used in the conduct of this study was done using 30% carbon tetrachloride dissolved in olive oil according to the modified method of Lu et al. (2002)[10]. Animals were injected intraperitoneally (i.p) 30% carbon tetrachloride dissolved in olive oil at a dose 3ml/kg body weight to induce hepatotoxicity/nephrotoxicity. This was followed by appropriate treatments.

2.2.4 Grouping of Animals and oral treatment with Aqueous Seed Extract of Citrus paradisi

The animal model employed in this study conformed to the guiding principles for research involving animals as recommended by the Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals [4]. In this curative model of hepatotoxicity/nephrotoxicity, rats were randomly divided into five groups (I-VI) of five rats each such that the weight differences within and between groups did not exceed ±20%. Group I rats consist of rats pretreated with 1 ml/kg of 0.9% normal saline given intraperitoneally (i.p) while group II rats were intraperitoneally treated with 3 ml/kg of 30% CCl₄ 1 hour before oral treatment with 10ml/kg of normal saline, Group III rats are pretreated within 3ml/kg of 30% carbon tetrachloride before oral treatment with 10ml/kg of ascorbic acid in 9% normal saline. While groups IV-VI rats were intraperitoneally treated 125 mg/kg, 250 mg/kg, and 500 mg/kg of aqueous seed extract of Citrus paradisi, respectively, all dissolved in 0.9% normal saline. (Table 1). All treatment lasted 7 days. Twenty-four hours after the last treatment on day 7, the rats were sacrificed humanely under diethyl ether anesthesia. Ascorbic acid, being a known potent antioxidant was used as a standard reference drug.

### Table 1: Curative effect of Aqueous Seed Extract of Citrus paradisi against Carbon Tetrachloride-induced hepatic and renal toxicities in Wistar rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>1 ml/kg of 0.9% normal saline i.p. 1 hour before 10 ml/kg of 0.9% normal saline p.o.</td>
</tr>
<tr>
<td>Group II</td>
<td>3 ml/kg of 30% CCl₄ in olive oil i.p. 1 hour before 10 ml/kg of 0.9% normal saline p.o.</td>
</tr>
<tr>
<td>Group III</td>
<td>3 ml/kg of 30% carbon tetrachloride before oral treatment with 10 ml/kg of ascorbic acid in 0.9% normal saline.</td>
</tr>
<tr>
<td>Group IV</td>
<td>3 ml/kg of 30% CCl₄ in olive oil i.p. 1 hour before 125 mg/kg of aqueous seed extract of Citrus paradisi in 0.9% normal saline p.o.</td>
</tr>
<tr>
<td>Group V</td>
<td>3 ml/kg of 30% CCl₄ in olive oil i.p. 1 hour before 250 mg/kg of aqueous seed extract of Citrus paradisi in 0.9% normal saline p.o.</td>
</tr>
<tr>
<td>Group VI</td>
<td>3 ml/kg of 30% CCl₄ in olive oil i.p. 1 hour before 500 mg/kg of aqueous seed extract of Citrus paradisi in 0.9% normal saline p.o.</td>
</tr>
</tbody>
</table>

2.2.5 Animal Sacrifice and Collection of Blood Samples

Prior to sacrifice, animals were fasted for 24 hr. The fasted animals were anesthetized by placing them in a closed jar containing cotton wool soaked with diethyl ether anesthetic. Following anesthesia, blood samples were collected through cardiac puncture into plain sample bottles (for serum preparation) and heparinized tubes (for plasma preparation).

2.2.6 Preparation of Serum

Blood samples collected in plain sample bottles were allowed to clot at room temperature for 6 hrs before they were centrifuged using a Uniscope Laboratory Centrifuge (Model SM 902V, Surgifriend Medicals, England) at 3000 rpm for 20 min to allow the sera to separate. The clear sera portions were aspirated off and the residues discarded. The separated sera were stored wet-frozen until ready for use.
2.2.7 Preparation of Plasma

Blood samples collected in heparinized tubes were centrifuged using a Uniscope Laboratory Centrifuge (Model SM 902V, Surgifriend Medicals, England) at 3000 rpm for 1 hr. to sediment the blood cells. The clear plasma was aspirated off and the residue discarded. The plasma produced was stored wet-frozen until ready for use.

2.2.8 Excision of Organs

Following bleeding the animals by cardiac puncture, the entire bodies of the animals were swabbed with cotton wool soaked with ethanol. After this, the abdominal cavities of the animals were opened up through a midline abdominal incision to expose the abdomen in order to excise the kidney/liver. The excised kidney/liver samples were washed thoroughly in ice-cold normal saline several times to remove gross fat and blood. The thoroughly washed kidney/liver samples were then stored wet-frozen at -80°C in a Revco freezer until ready to use.

3. RESULTS

3.1 Effect of serum malondialdehyde in Treated, Normal and Untreated in hepatotoxic rats.

Figure 3.1 summarizes the effect of serum malondialdehyde in treated, normal and untreated in hepatotoxic rats. Single intraperitoneal treatment of a rat with carbon tetrachloride causes significant elevation in serum malondialdehyde (Group II) when compared with controls (Group I). Treatment with graded dose of *Citrus paradisi* (250mg-500mg/kg body weight) caused a significant reduction in serum malondialdehyde in a dose-dependent manner (Group IV-VI).

3.2 Effect of liver malondialdehyde in Treated, Normal and Untreated in hepatotoxic rats.

Figure 3.2 summarizes the effect of liver malondialdehyde in treated, normal and untreated in hepatotoxic rats. Treatment with carbon tetrachloride caused a significant increase in liver malondialdehyde (Group II) when compared with the control group (Group I). There was significant reduction in level of liver malondialdehyde (Group III) when treated with ascorbic acid. In the same reaction, aqueous seed extract of *Citrus paradisi* (250mg-500mg/kg body weight) significantly reduce the liver malondialdehyde level (Group IV-VI) but not dose-dependently.

3.3 Effect of serum glutathione in Treated, Normal and Untreated hepatotoxic rats.

Figure 3.3 summarizes the effect of serum glutathione in treated, normal and untreated in hepatotoxic rats. Serum reduced glutathione was significantly reduced by carbon tetrachloride intoxication (Group II) while vitamin C caused a significant increase (group III) when compared with the controls (Group I). Oral graded dose of *Citrus paradisi* (125-500mg/kg body weight). (Group IV-VI) also significantly elevates reduced serum glutathione dose-dependently.

3.4 Effect of liver glutathione in Treated, Normal and Untreated in hepatotoxic rats.

Figure 3.4 summarizes the effect of liver glutathione in treated, normal and untreated in hepatotoxic rats. Results of the liver reduced glutathione also followed the same trend as that of the serum.
FIGURE 3.1: serum malondialdehyde in Treated, Normal and Untreated in hepatotoxic rats.

Each value was represented as Mean ± SEM
I= rats pretreated with 1ml/kg of 0.9% normal saline.
II= rats treated with 3ml/kg of 30% carbon tetrachloride before oral treatment with 10ml/kg of normal saline.
III= rats treated with 3ml/kg of 30% carbon tetrachloride before oral treatment with 10ml/kg of ascorbic acid in 0.9% normal saline.
IV= rats treated with 3ml/kg of 30% carbon tetrachloride before 125mg/kg of aqueous seed extract of Citrus paradisi in 0.9% normal saline.
V= rats treated with 3ml/kg of 30% carbon tetrachloride before 250mg/kg of aqueous seed extract of Citrus paradisi in 0.9% normal saline.
VI= rats treated with 3ml/kg of 30% carbon tetrachloride before 500mg/kg of aqueous seed extract of citrus paradisi in 0.9% normal saline.

FIGURE 3.2: of liver malondialdehyde in Treated, Normal and Untreated in hepatotoxic rats.
Each value was represented as Mean ± SEM
I= rats pretreated with 1ml/kg of 0.9% normal saline.
II= rats treated with 3ml/kg of 30% carbon tetrachloride before oral treatment with 10ml/kg of normal saline.
III= rats treated with 3ml/kg of 30% carbon tetrachloride before oral treatment with 10ml/kg of ascorbic acid in 0.9% normal saline.
IV= rats treated with 3ml/kg of 30% carbon tetrachloride before 125mg/kg of aqueous seed extract of *Citrus paradisi* in 0.9% normal saline.
V= rats treated with 3ml/kg of 30% carbon tetrachloride before 250mg/kg of aqueous seed extract of *Citrus paradisi* in 0.9% normal saline.
VI= rats treated with 3ml/kg of 30% carbon tetrachloride before 500mg/kg of aqueous seed extract of *Citrus paradisi* in 0.9% normal saline.

![FIGURE 3.3: serum Glutathione in Treated, Normal and Untreated in hepatotoxic rats.](image)

Each value was represented as Mean ± SEM
I= rats pretreated with 1ml/kg of 0.9% normal saline.
II= rats treated with 3ml/kg of 30% carbon tetrachloride before oral treatment with 10ml/kg of normal saline.
III= rats treated with 3ml/kg of 30% carbon tetrachloride before oral treatment with 10ml/kg of ascorbic acid in 0.9% normal saline.
IV= rats treated with 3ml/kg of 30% carbon tetrachloride before 125mg/kg of aqueous seed extract of *Citrus paradisi* in 0.9% normal saline.
V= rats treated with 3ml/kg of 30% carbon tetrachloride before 250mg/kg of aqueous seed extract of *Citrus paradisi* in 0.9% normal saline.
VI= rats treated with 3ml/kg of 30% carbon tetrachloride before 500mg/kg of aqueous seed extract of *Citrus paradisi* in 0.9% normal saline.
FIGURE 3.4: liver Glutathione in Treated, Normal and Untreated in hepatotoxic rats.

Each value was represented as Mean ± SEM

I= rats pretreated with 1ml/kg of 0.9% normal saline.
II=rats treated with 3ml/kg of 30% carbon tetrachloride before oral treatment with 10ml/kg of normal saline.
III=rats treated with 3ml/kg of 30% carbon tetrachloride before 125mg/kg of aqueous seed extract of Citrus paradisi in 0.9% normal saline.
IV= rats treated with 3ml/kg of 30% carbon tetrachloride before 250mg/kg of aqueous seed extract of Citrus paradisi in 0.9% normal saline.
V= rats treated with 3ml/kg of 30% carbon tetrachloride before 500mg/kg of aqueous seed extract of Citrus paradisi in 0.9% normal saline.

4. DISCUSSION

Reactive oxygen species (such as hydrogen peroxide, hydroxide, oxygen, collectively known as ROS) play important physiological functions and can also cause extensive cellular damage. Cells are provided with efficient molecular strategies to strictly control the intracellular ROS level and to maintain the balance between oxidant and antioxidant molecules. Oxidative stress, resulting from an imbalance between the generation of ROS and the antioxidant defense capacity of the cell [17], affects major cellular components, including lipids, proteins, and DNA. This phenomenon is closely associated with a number of human disorders such as many degenerative diseases, including cardiovascular disease, diabetes, cancer and neurodegenerative disorders [6]and with almost all liver pathologies [8]. All these conditions appear to be mostly related to chronic oxidative stress. However, the acute exposure to high levels of ROS seems also to be responsible for the development of different damage such as during ischemia/reperfusion (I/R) in liver [12]. Besides to produce cell damage, ROS can also be considered as molecular second messengers within the cell as they can be generated during triggering of particular cellular responses by cytokines, hormones, growth factors and other soluble mediators such as extracellular ATP. Therefore, ROS represent a double face medal and could act either positively or negatively on cell functioning depending on the intensity and duration of the oxidative stress produced on a cell. It is therefore not surprising the role of ROS either as apoptotic molecules or stimulators of cell proliferation, depending on the cell type and on the intensity of the stress produced.

Inflammation begins when tissues react to a local irritation usually caused by a physical injury, by infection or by exposure to a toxicant. Fluid and accompanying white blood cells traverse the vascular barrier leading to swelling, erythema, further inflammation and attraction of further white blood cells. The
resolution phase of inflammation involves repair and cell proliferation ultimately leading to tissue regeneration. During the inflammatory phase, there is a burst of respiration leading to the creation and release of free radicals, including production of both reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS and RNS utilize three main pathways of signaling which results in damage to DNA, proteins and lipids. Lipid peroxidation triggers the arachidonic-acid cascade, with the production of cell proliferation-stimulating eicosanoids. While free radicals induce DNA damage directly, by-products of the arachidonic-acid pathway such as malondialdehyde (MDA) and 4-hydroxynonenol (4-HNE) also are DNA-damaging agents.

In the present study, the effects of carbon tetrachloride intoxication on liver and serum malondialdehyde and reduced glutathione was evaluated and the ability of aqueous seed extract of *Citrus paradisi* to ameliorate the deleterious effect of carbon tetrachloride was also evaluated. All the effects of reactive oxygen species highlighted above are applicable to carbon tetrachloride as its deleterious effects are mediated through reactive oxygen species. In the present study, carbon tetrachloride intoxication caused a significant decrease in both serum and liver reduced glutathione (Figs. 3.3 and 3.4) while both liver and serum malondialdehyde levels were significantly elevated (Figs. 3.1 and 3.2). This is expected in an organism that had been stressed oxidatively.

However, treatment with graded oral dose of aqueous seed extract of *Citrus paradisi* attenuated the toxic effects of carbon tetrachloride at all the dose tested. While the level of reduced glutathione was significantly increased, that of malondialdehyde was significantly reduced. The reduction observed may not be unconnected with the presence of active principles in the extract that possess antioxidants or agents that are capable of scavenging the free radicals.

5. CONCLUSION

The present study has established that *Citrus paradisi* posses antioxidant properties and can also scavenge reactive oxygen species in the face of mounting oxidative stress. Previous reports has indicated the presence of active phytochemical component in *Citrus paradisi* that exhibits antioxidant attributes responsible for the attenuating effects in carbon tetrachloride toxicity as reported in this study. It is hoped that with more work, it may be possible for this extract to be useful in the management /treatment of oxidative stress to prevent degenerative diseases or to manage them.

6. REFERENCES


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