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Full Length Research Paper

Antioxidant potential of *Citrus maxima* fruit juice in rats

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In the etiology of many pathological conditions increased levels of oxidative stress may be implicated. The protective antioxidant action imparted by many plant extracts and plant products make them promising therapeutic drugs for free radical induced pathologies. In this study the antioxidant potential of *Citrus maxima* fruit juice was assessed. Experimental rats were divided into two groups: Control and *Citrus maxima* treated. Treated rats received *C. maxima* fruit juice at a dose of 200 mg/kg body weight/day for 8 weeks. After the treatment period of 8 weeks, lipid peroxidation (LPO), vitamin C, uric acid and reduced glutathione (GSH) were estimated in plasma and antioxidant enzymes: Glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD) were also assayed. Genotoxicity of *Citrus maxima* fruit juice was assessed by single cell gel electrophoresis (SCGE) of lymphocytes under both in vitro and in vivo conditions. The protective role of *Citrus maxima* juice against hydrogen peroxide (H_2O_2), streptozotocin (STZ) and nitric oxide generating system induced lymphocyte DNA damage was also assessed by SCGE. *Citrus maxima* treated rats showed a significant decrease in plasma LPO and a significant increase in plasma vitamin C, uric acid, GSH levels and GPx, CAT and SOD activities. SCGE experiment reveals that *Citrus maxima* fruit juice was devoid of genotoxicity and had a significant protective effect against induced lymphocyte DNA damage by H_2O_2, STZ and nitric oxide (NO). The results suggest that consumption of *Citrus maxima* juice can be linked to improved antioxidant status and reduction in the risk of oxidative stress.

Keywords: Antioxidants, *Citrus maxima*, lymphocytes, Single Cell Gel Electrophoresis (SCGE)

INTRODUCTION

Oxidative stress occurs in a cellular system when the production of reactive oxygen species (ROS) exceeds the antioxidant capacity of the system. Oxidative stress plays an important contributory role in the process of aging and pathogenesis of numerous diseases like diabetes, cancer, neurodegenerative diseases, and respiratory tract disorder [Anderson *et al*., 2000]. To counteract oxidative stress, the body produces an armoury of antioxidants to defend itself. It is the job of antioxidants to neutralise or 'mop up' free radicals that can harm the cells. Improved antioxidant status helps to minimize the oxidative damage, and thus can delay or decrease the risk for developing many chronic age related, free radical induced diseases. The body's internal production of antioxidants is not enough to neutralize all the free radicals. The body can be helped to defend itself by increasing dietary intake of antioxidants (Rattan, 2006).

Citrus maxima fruit also known as Shaddock is the largest of all Citrus. It is globose, oblate or moderately pear-shaped with 11-14 sections. The pulp is light colored or pink and coarse with large, spindle-shaped juice sacks that separate easily from one another. This tree is a native of southeastern China, where it's grown for its fruit. The Shaddock has also been introduced to the West African countries where it is cultivated as an ornamental tree and is interesting because of its very large fruits. It is closely related to the Grapefruit, but much less cold resistant. The pulp is antitoxic, appetizer, cardiac stimulant and stomach tonic (Ontengco et al., 1995). The essential oil of the Shaddock shows in vitro activity against *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922, and was found to have significant potential as a broad-spectrum antibacterial raw material for galenic preparations (Ontengco et al., 1995).

The generation and subsequent involvement of free radicals in a large number of diseases prompted this study of the antioxidant potential of *Citrus maxima* juice. For any substrate or plant extract to be considered as an effective antioxidant, it should act as antioxidant under both *in vivo* and *in vitro* conditions by decreasing the levels of oxidative damage to biomolecules, and it should render lymphocytes more resistant to oxidative challenges. Therefore, the present study was designed to examine the effect *Citrus maxima* juice treatment on plasma antioxidant status and to evaluate the antigenotoxic potential of *Citrus maxima* juice against STZ, NO and H_2O_2 induced DNA damage in lymphocytes.

MATERIALS AND METHODS

Materials

The chemicals used in the current study were procured from Sigma Chemical Co. (St. Louis, MO, USA) and Koch-Light Laboratories (Huntingdon, Cambridgeshire, England). Shaddock (*Citrus maxima*) fruits were obtained locally. *Citrus maxima* juice was prepared according to the method described by Ani, 2006. The shaddock fruits were thoroughly washed with potable water to remove dirt, soil and other foreign bodies in order to remove the microbial load. They were peeled; the epicarp and seeds were removed. They were also sliced, crushed and about 3 ½ litres of must/juice were obtained using Phillips Juice extractor. The phytochemical screening of the *Citrus maxima* juice was carried out by the modified method of Das and Bhattacharjee [1970].

Animals

Two- to three-month-old male albino Wistar rats weighing 130-150g, procured from University of Ibadan, Ibadan, Nigeria were acclimatized for 7 days to the animal house, and maintained at standard conditions of temperature and relative humidity, with a 12h light/12h dark cycle. Water and standard pellet diet were provided ad libitum.

Experimental design

A total of 16 rats were used in this experiment. The rats were divided into two groups (control and *C. maxima* treated) of 8 animals each. *C. maxima* treated animals received 2 ml of *Citrus maxima* juice (200 mg/kg body weight) orally through gastric tube daily. The dose of *Citrus maxima* juice in the current study is based on preliminary experiments on anti-hyperglycemic effect in alloxan-induced diabetic rats. After 8 weeks of treatment, 1-2 ml of blood was collected from rats by means of a capillary tube through orbital sinus into heparinized tubes. The plasma was then separated and used for biochemical analysis.

Biochemical analysis

Glucose was estimated by glucose oxidase peroxidase method using the Span Diagnostic kit. Vitamin C was estimated by the method of Omaye *et al.* [1979]. Uric acid was estimated by enzymatic method using a Liquid GOLD kit while reduced glutathione was estimated by Ellmans method [1959]. The extent of lipid peroxidation was determined by the method of Utley *et al* [1967].

Enzyme assays

Plasma GPx (EC 1.11.1.9), CAT (EC 1.11.1.6) and SOD (1.15.1.1) were assayed by following the methods of Rotruck *et al.,* [1973], Beers *et al.*[1952] and Soon and Tan[2000] respectively. Protein was estimated by the method of Lowery *et al*., using bovine serum albumin as standard [Lowry et al., 1951]

Single cell gel electrophoresis (SCGE)

Experiments were carried out on the lymphocytes isolated from the blood of rats using histopaque-1077 technique, according to established methods [Boyum, 1983]. After the separation, lymphocytes were suspended in RPMI 1640 medium and counted over a hemocytometer and adjusted to nearly 5 × 105 cells/ml of medium. The basic procedure for the comet assay was applied according to the modified method of Singh *et al*. [1988]. This modification includes a silver staining [Silvina, Laura and Peng, 2001] of comets instead of ethidium bromide. The slides were coded for each sample and cells were screened on each slide under a standard transmission binocular microscope and DNA migration length was measured using an ocular micrometer.

In vitro genotoxicity studies of *Citrus maxima* Juice

Lymphocytes isolated from the blood of normal rats were incubated with *Citrus maxima* Juice in RPMI medium (0.2, 0.4 and 0.6 mg/ ml medium) at 37ºC in CO_2 humidified incubator for one hour. After incubation the cells were centrifuged at 3000 rpm for 5 minutes to pellet the lymphocytes. The pellet was re-suspended in RPMI

Table 1. Effect of *C. maxima* Juice on plasma antioxidants

Parameters	Control rats n = 8	*C. maxima* treated rats n = 8
LPO (nmol of MDA formed/dl)	33.15 ± 0.13	27.29* ± 0.22
Vitamin C (mg/dl)	1.25 ± 0.43	1.68* ± 0.01
Uric acid (mg/dl)	3.37 ± 0.05	5.22* ± 0.08
GSH (mg/dl)	2.38 ± 0.06	3.49* ± 0.02
GPx (µg of GSH consumed/ min/mg protein)	0.33 ± 0.04	0.38* ± 0.09
CAT (mmoles of H_2O_2 decomposed/min/mg protein)	0.50 ± 0.06	0.66** ± 0.08
SOD (U/min/mg protein)	54.22 ± 0.21	68.02** ± 3.2

*Values are mean ± SE from 8 rats in each group; *P < 0.05; **P < 0.001*

medium and the lymphocytes were subjected to SCGE.

Effect of *Citrus maxima* Juice against in vitro oxidative stress caused by nitric oxide generating system/ STZ

The nitric oxide generating system consists of a freshly prepared mixture of 10 mM Tris-HCl buffer pH 7.4 prepared in saline and 30 mM sodium nitroprusside in 1.5:1 ratio. *In vitro Citrus maxima* Juice pretreated (0.2 mg/ml RPMI medium) lymphocytes from normal rats were exposed to nitric oxide generating system (100 µl/1ml medium) or 10 µl of freshly prepared STZ in PBS (to get a final concentration 80 µg/ml medium) for 25 min and centrifuged at 3000 rpm for 5 min to pellet the lymphocytes and then the lymphocytes were subjected to SCGE.

In vivo effect of *Citrus maxima* Juice against H_2O_2 induced lymphocyte DNA damage

Lymphocytes separated from the blood of normal and *C. maxima* juice treated rats were processed for SCGE. DNA damage was induced *in vitro* by exposing lymphocytes to H_2O_2. Two slides were prepared for each sample, one was immersed in a solution of H_2O_2 in PBS (200 µmol/L) for 5 minutes and the other was immersed in PBS alone. The slides were washed twice with PBS and then subjected to SCGE. The protective effects of the plant extract against H_2O_2, NO/STZ induced oxidative stress were screened by measuring comet tail length against controls.

Statistical analysis

All the results are expressed as means ± SE. Statistical analysis of the data was performed by students' t-test and *P* value <0.05 was considered statistically significant.

RESULTS

Phytochemicals

The phytochemical screening of *Citrus maxima* Juice revealed the presence of alkaloids, anthocyanins, flavonoids, limonene, polyphenols, steroids/triterpenoids and tannins. This agrees with the work of Swapnil et al. (2011) who worked on the pharmacognostic and phytochemical characterization of Leaves of *Citrus Maxima*.

General observations

During the experimental period of eight weeks, control rats showed no significant change in blood glucose level (66.20 ± 0.7 mg/dl). However there was a gradual increase in body weight from 144 ± 1.8 to 229 ± 3.6 g. *Citrus maxima* treated rats showed the same trend as control rats regarding plasma glucose (66.57 ± 2.44 mg/dl) and gain in body weight (from 148 ± 4.2 to 240 ± 4.3 g during experimental period. There were no visible side effects (respiratory distress, abnormal locomotion and catalepsy) observed in *C. maxima* treated animals throughout the experiment.

Plasma antioxidants

Table 1 shows the levels of plasma LPO, vitamin C, uric acid and GSH in the two groups of animals. A significant decrease (17.7%) in plasma LPO was observed in treated rats compared to control rats, whereas significant increase in plasma vitamin C (34.4%), uric acid (54.9%) and plasma GSH (46.6%) levels was observed in *C. maxima* treated rats compared to normal rats. In addition, the activities of plasma GPx, CAT and SOD of control and *C. maxima* treated rats are also presented in Table 1. A significant enhancement in the activities of plasma GPx (15.2%), CAT (32%) and SOD (25.5%) was

Table 2. Effect of *in vivo* treatment of *C. maxima* aqueous extract against H_2O_2 induced lymphocyte DNA damage

Group	Control rat Lymphocytes		C. maxima treated rat lymphocytes	
	H_2O_2	Basal Treatment	H_2O_2	Basal Treatment
Percentage of cells showing migration	1.50 ±0.23	58.73** ±0.22	1.33 ±0.67	9.20**f ±0.13
Tail length (µm)	0.90 ±0.14	5.12** ±0.11	0.80 ±0.05	1.24*f ±0.06

*Values are mean ± SE from 400 cells from 8 rats in each group; Values not sharing common superscript letter differ significantly from control group basal level and *P < 0.05; **P < 0.001; A superscript letter .f . indicates a significant difference (P < 0.0001) between H_2O_2 challenged control and C. maxima treated group*

Table 3. Protective ability of *C. maxima* juice against STZ and NO induced lymphocyte DNA damage

Group	Control lymphocytes			C. maxima pretreated lymphocytes		
	Basal	STZ	NO	Basal	STZ	NO
Percentage of cells	0.50	97.44***	98.22***	0.50	0.5***f	8.27***f
Showing migration	± 0.87	± 1.67	± 0.42	± 0.33	± 0.76	± 0.43
Tail length	0.85	4.31***	5.14***	0.85	1.20*f	1.17**f
(µm)	± 0.18	± 0.34	± 0.12	± 0.04	± 0.04	± 0.03

*Values are mean ± SE from 400 cells from 8 rats in each group; Values not sharing common superscript letter differ significantly from control lymphocytes basal level and *P < 0.05; **P < 0.01; ***P < 0.001; A superscript letter .f . indicates a significant difference (P < 0.0001) between STZ/NO challenged control lymphocytes and C. maxima treated lymphocytes.*

observed in *C. maxima* treated animals compared to control animals.

DNA damage

An *in vitro* exposure of lymphocytes at different doses of *C. maxima* did not result in comet formation in SCGE indicating non-genotoxic nature of *C. maxima*. Results presented in Table 2 indicate the effect of supplementation of *C. maxima* 8 weeks on peripheral lymphocyte DNA damage and their ability to resist exogenous H_2O_2 induced damage.

In vivo treatment of *C. maxima* did not result in lymphocyte DNA damage. A significant protection against H_2O_2 induced DNA damage was observed in the lymphocytes of *C. maxima* treated rats compared to normal rats (84.3% decrease in number of damaged cells and 75.8% decrease in tail length of comets).

The *in vitro* antigenotoxic effects of *C. maxima* against STZ and NO induced DNA damage are presented in Table 3. *C. maxima* pretreated lymphocytes showed decrease in the number of damaged cells (89.2% and 91.1%) and length of comets (72.4% and 77.3%) against STZ and NO induced DNA damage respectively.

DISCUSSION

The non-toxic nature of *C. maxima* Juice was evident with the unaltered trend of body weight and plasma glucose levels in *C. maxima* treated rats compared to controls. The antioxidant potential of *C. maxima* juice was confirmed by a significant decrease in the plasma LPO and a significant increase in plasma non-enzymatic antioxidants, vitamin C, uric acid and GSH in *C. maxima* treated rats compared to control.

Recently, free radical induced LPO has gained much importance because of its involvement in several pathologies such as aging, atherosclerosis, diabetes, wound healing, liver disorder, inflammation etc. Protection of the cell membrane from LPO could prevent, cure or delay the aforesaid pathologies. The enhanced plasma GSH, vitamin C, uric acid levels of *C. maxima* treated rats may be responsible for the observed decrease in the extent of plasma LPO.

Glutathione, SOD and CAT protect the cell constituents from oxidative damage. Despite these extensive defense systems, biomolecule damage may still occur and persist within the cell. The significant increase in the activities of SOD and CAT suggests a greater level of endogenous antioxidant associated with the *C. maxima* juice treatment resulting in an enhanced free radical scavenging activity. Plants are the sources for a wide variety of compounds like flavonoids and polyphenols. These compounds may be responsible for increasing antioxidant status.

The first screening of any compound, drug or potential nutraceutical starts with the genotoxicity test [Yen, Chen and Peng, 2001]. Endogenous levels of DNA damage remained unchanged under *in vivo* and *in vitro* treatment of *C. maxima* juice revealing that *C. maxima* juice is devoid of genotoxic and pro-oxidant property. The

decreased levels of H_2O_2 induced DNA damage in *C. maxima* treated rat lymphocytes is attributed to the increased scavenging of H_2O_2 derived ROS by enhanced antioxidants.

Recent studies by Trivendra and Zafar (2010)suggested that ROS, including superoxide (O_2°.), H_2O_2, hydroxyl radical (°OH) and °NO play a central role in the mechanism of DNA damage and cytotoxicity of STZ. Nitric oxide, generated from sodium nitroprusside in aqueous solution at physiological pH, interacts with oxygen to produce nitrite ions.

The decreased levels of STZ/NO induced DNA damage in *C. maxima* juice pretreated lymphocytes (*in vitro*) may be attributed to the possible direct antioxidant capacity of *C. maxima* juice. Phytochemical constituents of *C. maxima* may be responsible for scavenging ROS and protecting the DNA from ROS induced DNA damage. The concept of synergy is central to the holistic approach. The popular modern concept trend to isolate pure compounds may not achieve the desired results as observed in the natural version. Once an active principle is isolated from the natural product without its synergical colleagues to support and/or balance its action, it may lose its character as present in its natural form.

CONCLUSION

This study has shown that *C. maxima* juice lacks genotoxicity and pro-oxidant property. The enhanced antioxidant status observed in *C. maxima* treated rats and its protective role against H_2O_2, STZ and nitric oxide generating system induced DNA damages might be due to the effect of different types of active principles acting individually or synergistically, each with a single or a diverse range of biological activities against oxidative stress. This is a widely recognized factor in many degenerative diseases. The above therefore indicates that the fruit juice of *Citrus maxima* possess antioxidant and free radical scavenging properties and thus continual consumption of the fruit juice could offer health benefits in terms of prevention of diseases caused by oxidative stress.

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