AQUEOUS EXTRACT OF CARICA PAPAYA LINN ROOTS HALTS SODIUM ARSENITE-INDUCED RENAL INFLAMMATION THROUGH INHIBITING ADENOSINE DEAMINASE, 8-HYDROXY-2’-DEOXYGUANOSINE, C-REACTIVE PROTEIN AND INDUCIBLE NITRIC OXIDE SYNTHASE ACTIVITY

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VODENI EKSTRAKT KORENA CARICA PAPAYA L. ZAUSTAVLJA INFLAMACIJU BUBREGA IZAZVANU NATRIJUM-ARSENITOM, INHIBIRAJUĆI ADENOZIN DEAMINAZU, 8-HIDROKSI-2’-DEOKSI-GUANOZIN, C-REAKTIVNI PROTEIN, INOS AKTIVNOST

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Running title: Anti-inflammatory activity of Carica papaya root
Kraći naslov: Antiinflamatorna aktivnost korena Carica papaya

ABSTRACT

Objectives: Inflammation plays a crucial role in many of the metabolic abnormalities. The prototypic marker of inflammation is C-reactive protein (CRP), Nitric Oxide (NO), inducible nitric oxide synthase (iNOS) and their inhibition is considered a promising strategy to combat inflammation. Here, we report the anti-inflammatory mechanism of Carica papaya root aqueous extract in sodium arsenic-induced renal dysfunction.

Methodology: Thirty-five rats were used for the experiments. Griess assay was used to evaluate the inhibitory effect of Carica papaya roots aqueous extract on the overproduction of nitric oxide (NO). ELISA was used to determine the level of pro-inflammatory markers including c-reactive protein (CRP). ELISA was used to analyze 8-OHdG. The inhibitory effect on the enzymatic activity of inducible nitric oxide synthase (iNOS), adenosine deaminase (ADA), malondialdehyde (MDA) was tested by enzyme activity assay kits.

Results: Carica papaya roots aqueous extract suppressed sodium arsenite-stimulated NO production and pro-inflammatory secretion, such as CRP. Carica papaya roots aqueous extract significantly (p < 0.05) decrease the activities of iNOS, 8-OHdG, ADA and MDA.

Conclusion: These results indicated that potent inhibition on CRP, NO, iNOS, ADA, 8-OHdG might constitute the anti-inflammatory mechanism of Carica papaya roots aqueous extract.

Keywords: Carica papaya Linn, roots, anti-inflammatory biomarkers, renal inflammation

SAŽETAK

Ciljevi: Infiamacija ima ključnu ulogu u brojnim metabolickim poremećajima. Smatra se da inhibicija faktora infiamacije kao što su C-reaktivni protein (CRP), azot monoksid (NO), inducibilna azot monoksid sintetaza (iNOS) predstavlja obećavajuću strategiju u borbi sa infiamacijom. U ovom radu smo izvestili o antiinfiamatornom mehanizmu vodenog ekstrakta korena Carica papaya kod natrijum-arsenitom izazvane bubrežne disfunkcije.


Rezultati: Vodeni ekstrakt korena Carica papaya suprimita natrijum-arsenitom stimulisana produkciju NO i sekreciju proinfamloratornih molekula kao što je CRP. Vodeni ekstrakt korena Carica papaya je statistički značajno (p<0,05) smanjio aktivnost iNOS, 8-OHdG, ADA i MDA.

Zaključak: Ovi rezultati pokazuju da jaka inhibicija CRP, NO, iNOS, ADA, 8-OHdG može predstavljati potencijalan mehanizam antiinfamatornog delovanja vodenog ekstrakta korena Carica papaya.

Ključne reči: koren Carica papaya L., antiinfamatorni biomarkeri, bubrežna infiamacija

ABBREVIATIONS

CRP – C reactive protein;
NO – Nitric oxide;
iNOS – inducible nitric oxide synthase;
ADA – Adenosine deaminase;
8-OHdG - 8-hydroxy-2’-deoxyguanosine;
MDA - malonaldehyde
INTRODUCTION

Arsenic is a ubiquitous element present in food, soil, water and air, and it is released into the environment from both anthropogenic and artificial sources (1, 2). The foremost inorganic forms of arsenic comprise of the trivalent meta arsenite As$^{3+}$ and the pentavalent arsenate As$^{5+}$. Trivalent arsenic form has a higher affinity for thiol groups and is more cytotoxic and genotoxic than As$^{5+}$ (3, 4). Organisms that store the trivalent intermediates are thought to be at greater risk of arsenic-induced ailments (4). Some of the organic forms contain the methylated metabolites monomethylarsonic acid (MMA), dimethylarsenic acid (DMA) and trimethylarsine oxide (TMAO) as well as arsenocholine, arsenobetaine (AsB) and arsenosugars. Over 80% of commercially employed arsenic compounds are used to manufacture products with agricultural applications for examples herbicides, fungicides, insecticides, algaecides, wood preservatives, dyestuffs, sheep dops and medicines for the eradication of tapeworms in sheep and cattle. Arsenic compounds have been used for at least a century in the treatment of yaws, amoebic dysentery, syphilis and trypanosomiasis (5).

Toxic effects of arsenic accredited to production of reactive oxygen species (ROS) and oxidative stress results in the alteration of the antioxidant defense system, increased oxidative stress and cell death (6, 7). Liver and kidney are target organs for arsenic toxicity in rats, though, concentration of arsenic was found to vary in these organs (8). Arsenic induced nephrotoxicity results in disturbing the alteration of the antioxidant defense system, protein oxidation and lipid peroxidation products. It also leads to renal functional deterioration (9). Though, its high affinity for sulphydryl groups of protein has a tendency to readily react with the sulphydryl groups of proteins and this turn inhibit biochemical pathways and considered to be the biologically active form and the major source to arsenic toxicity (10). Lipid peroxidation leading to decrease glomerular filtration rate and to elevate nitrogenous waste such as urea, uric acid and creatinine (11, 12). 8-OHdG is formed from deoxyguanosine in DNA by hydroxyl free radicals. Because of its stability, 8-OHdG is known as one of the most reliable markers of oxidative DNA damage (13).

Recent studies have implicated the endogenous signaling molecule called adenosine in renal function. Adenosine is produced by enzymatic phosphohydrolysis of its precursor molecules, particularly adenosine triphosphate (ATP) and Adenosine monophosphate (AMP) (14, 15, 16). Hence, inhibition of ADA activity has been proposed to be a good therapeutic approach for the management or prevention of kidney dysfunction.

Carica papaya belongs to the family of Caricaceae. Papaya is a herbaceous succulent plant that possess self-supporting stems (17). It is a large perennial herb with a swift growth rate. The plants are usually short-lived, but can produce fruit for more than 20 years (18). The papaya has a rather complex means of reproduction. The plant was originally derived from the southern part of Mexico. C. papaya is a perennial plant, and it is presently distributed over the whole tropical area. In particular, C. papaya fruit circulates widely, and it is accepted as food. In Nigeria, pawpaw is one of the most popular, cheapest, economically important fruit tree grown and consumed for its nutritional content (19). Papaya is not a tree but an herbaceous succulent plant that possesses self-supporting stems (17). The top ten countries that produces Carica papaya plants worldwide are; Brazil (25%), Nigeria (15%), India (12%), Mexico (11%), Indonesia (10%), Ethiopia (4%), Congo (4%), Peru (3%), Venezuela (3%), and lastly China (2%) (FAO, 2004). Hence, this study investigated the effect of Carica papaya root extract on the sodium arsenite-renal inflammation through inhibition of anti-inflammatory biomarkers.

MATERIALS AND METHODS

Plant material

Roots of C. papaya Linn. was freshly collected in May, 2016 from Ijadu farm, Ado-Ekiti, Ekiti State, Nigeria. The plant was identified and authenticated by a senior taxonomist at the herbarium unit of the Department of Biological Science, Afe Babalola University, Ado-Ekiti, Nigeria and a voucher specimen number ABUAD/040 was deposited accordingly. All plant names in this manuscript are formatted according to the latest revision in “The Plant List”, and correspond to the good practices in publishing studies on herbal materials, as described by (20).

Preparation of aqueous extract of C. papaya root

The aqueous extract of the powdered C. papaya root was prepared using method described previously (12). The roots were air-dried in the laboratory at ambient temperature (30 ± 2 °C) for 28 days, minced using a laboratory mechanical grinder and the powders obtained stored until further use. 50 g of the powdered sample were extracted with distilled water (via maceration) for 48 h. The percentage yield extraction calculated as follows:

\[
\text{% yield} = \frac{\text{Weight of the dry extract}}{\text{Weight of powdered leaves}} \times 100\%
\]

Experimental Animals

Eight weeks old male albino rats of Wistar stain with initial mean body weight (bw) (155.25 ± 11.52 g) were used in this study. The animals were obtained from Animal house, Afe Babalola University, Ado-Ekiti, Ekiti State, Nigeria. The animals were housed in large clean spacious cages and were given food and water ad libitum. The animal room was well ventilated with a 12 h light/dark cycle, throughout the period of the experiment. They were fed ad libitum on rat pellets (Top Feeds, Nigeria) and water. The handling of animals was conformed to the standards...
of National Institute of Health (NIH publication 85-23, 1985) for experimental animal maintenance. Animals were maintained according to the rules and regulations of the Experimental Animal Research Ethics Committee of Afe Babalola University, Ado-Ekiti, Ekiti State, Nigeria (Ethical approval number: ABUAD/ACA/121).

**Animal distribution**

Animals were randomly divided into five groups as follows: Group I Rats that received vehicles alone (served as control), Group II Rats that received arsenic as sodium arsenite in drinking water at a concentration of 100 ppm. Group III Rats that were treated with arsenic along with ascorbic acid (200 mg/kg body wt. dissolved in water) given by oral gavage once a day. Group IV Rats that were given aqueous extract of *C. papaya* roots (150 mg/kg/bw) by oral gavage once a day. Group V Rats that were administered arsenic along with aqueous extract of *C. papaya* roots (150 mg/kg/bw) by oral gavage once a day for 21 days.

**Isolation of blood and organs**

At the end of the experimental period, animals were euthanized by diethyl ether and blood and organ samples were collected. The whole blood of each animal was collected via cardiac puncture and immediately preserved in a refrigerator until further processing. The blood samples were centrifuged at 3000 rpm for 15 min and serum from each blood sample was separated and preserved at -30 °C for further analysis. The kidney was collected from each animal, washed with normal saline, wiped with filter paper, weighed and preserved at -30 °C until subsequent analysis.

**Estimation of Plasma 8-hydroxy-2′-deoxyguanosine (8-OHdG)**

Blood Plasma samples from each group were diluted twice and filtered through 20 μm filters and subsequently filtered sample was used for 8-OHdG analysis. The 8-OHdG analysis was performed using the NWLSTML 8-OHdG ELISA kit (Northwest Life Science Specialties, LLC, Vancouver, WA). The protein content in each sample was estimated using DC protein Assay kit. The result was expressed as 8-OHdG (ng/mg of protein).

**Estimation of Adenosine deaminase**

ADA activity determination was performed as method described previously (21) which is based on the direct measurement of the formation of ammonia, produced when adenosine deaminase acts in excess of adenosine. 50 μL of kidney homogenates reacted with 21 mmol/L of adenosine, pH 6.5, and was incubated at 37 °C for 60 min. The protein content used for the platelet experiment was adjusted to between 0.7 and 0.9 mg/mL. Results were expressed in units per liter (U/L). One unit (1 U) of ADA is defined as the amount of enzyme required to release 1 mmol of ammonia per minute from adenosine at standard assay conditions.

**Assay of Serum C-reactive Protein**

Serum C-reactive protein (CRP) was measured according to the method described previously (22) by ELISA technique using kits purchased from DIA MED (Belgium).

**Quantification of Nitric Oxide (NO)**

Nitric Oxide content in kidney homogenates was estimated in a medium containing 400 mL of 2% vanadium chloride (VCl₃) in 5% HCl, 200 mL of 0.1% N-(l-naphthyl) ethylene-diaminedihydrochloride, and 200 mL of 2% sulfanilamide (in 5% HCl). After incubating at 37 °C for 60 minutes, nitrite levels, which correspond to an estimative of levels of NO, was determined spectrophotometrically at 540 nm, based on the reduction of nitrate to nitrite by VCl₃ using method described previously (23). Kidney nitrite and nitrate levels were expressed as nanomoles of NO per milligram of protein.

**Determination of Pro-Antioxidant Marker**

The non-antioxidant markers such as malonaldehyde (MDA) was measured via adopting the method described previously (24).

**Estimation of arsenic**

Tissue/blood samples were digested according to the method described previously (25). To 100 mg of tissues/1 ml of blood, 1 ml of concentrated nitric acid was added, followed by 1ml of perchloric acid. The sample was then digested over a sand bath until the solution turned yellow in color. If the color of the digest was brown, more nitric acid and perchloric acid were added and the oxidation was repeated. The digest was made up to known volume with deionized water. Aliquots of this were used to estimate arsenic by using the atomic absorption spectrophotometer. The concentration of arsenic was expressed as μg/ dl blood or μg/g tissue.

**Data analysis**

Data were presented as the mean ± SEM (n= 7). Data were analyzed by using a statistical software package (SPSS for Windows, version 20.0, IBM Corporation, NY, USA) using One-Way ANOVA, Duncan multiple range post-hoc test (DMRT). Values were considered significantly different at p < 0.05.

**RESULTS**

The result for the marker of DNA in serum are presented in Fig 1. The 8-OHdG level was significantly (p <
0.05) increased in arsenic treated rats compared to normal control rats. However, treatment with aqueous extract of *C. papaya* root at 100 mg/kg body weight and 150 mg/kg body weight significantly decreased the level of 8-OHdG in serum compared with arsenic untreated rats. Vitamin C administration significantly decreased the levels of 8-OHdG (*p* < 0.05) (Fig. 1). These effects were found to be pronounced in the arsenic + 150 mg/kg body weight *C. papaya* root aqueous extract.

The data for adenosine deaminase (ADA) in serum are presented in Fig 2. As seen from the (Figure 2), ADA activity was significantly higher in arsenic only treated rats (*p* < 0.05) compared to normal control rats. Treatment with aqueous extract of *C. papaya* root at 100 mg/kg body weight and 150 mg/kg body weight all exerted significant decrease inhibitions (*p* < 0.05) on the ADA activity compared with arsenic untreated rats. Vitamin C administration significantly decrease ADA activity (*p* < 0.05).

The data for serum C-reactive protein (CRP) are presented in Fig 3. The serum C-reactive protein concentration was significantly higher in arsenic untreated rats compared to normal control rats (*p* < 0.05). Treatment with aqueous extract of *C. papaya* root at 100 mg/kg body weight and 150 mg/kg body weight significantly decreased (*p* < 0.05) the concentration of serum C-reactive protein compared with arsenic untreated rats. Vitamin C administration significantly decreased concentration of serum C-reactive protein (*p* < 0.05) (Fig.3).

The levels of nitric oxide (NO) as a measure of enhanced oxidative stress has been measured and presented in Fig 4. Rat exposed to arsenic exhibited significant decrease in nitric oxide levels compared to normal control (*p* < 0.05). Simultaneous treatment with aqueous extract of *C. papaya* root at 100 mg/kg body weight and 150 mg/kg body weight and vitamin C significantly increases the nitric oxide levels (*p* < 0.05) in serum compared to those treated with arsenic alone (Fig. 4).

Furthermore, the inhibitory effect of aqueous extract of *C. papaya* root on the activity of iNOS enzyme was examined. As shown in Fig. 5, arsenic untreated rats caused a significant (*p* < 0.05) increment of iNOS enzymatic activity compared to normal control. Administration of aqueous extract of *C. papaya* root at 100 mg/kg body weight and 150 mg/kg body weight and vitamin C strongly inhibited (*p* < 0.05) the iNOS enzymatic activation in arsenic treated rats compared to arsenic alone.

As shown in Figure 6, compared with the control group, arsenic significantly increased (*p* < 0.05) the levels of MDA in arsenic untreated rats compared to normal control. When aqueous extract of *C. papaya* root at 100 mg/kg body weight and 150 mg/kg body weight and vitamin C was administered to arsenic, the levels of MDA were significantly reduced (*p* < 0.05) compared to arsenic untreated rats.

The arsenic concentrations in blood and kidney tissue are present in Figure 7. Results showed that arsenic accumulated preferentially in the kidney and serum. Arsenic concentrations in the blood and kidney tissues were significantly higher (*p* < 0.05) in arsenic untreated rats compared to normal control rats. Administration with 100 mg/kg body weight of *C. papaya* root aqueous extract and 150 mg/kg body weight of *C. papaya* root aqueous extract groups and vitamin C significantly (*p* < 0.05) decreased the concentrations.
DISCUSSION:

At the moment, therapeutic options to prevent or manage acute renal injury associated with heavy metal poisoning are tremendously restricted, hence, the look for novel therapeutic interventions is an area of intense investigation. 8-OHdG, may be a novel biomarker for arsenic-induced renal inflammation and oxidative stress. It was reported that elevated levels of 8-OHdG in plasma will cause DNA damage in arsenic exposed rats (26). In this study, levels of 8-OHdG in plasma from arsenic only treated rats were significantly increased compared to control rats, which is similar to the reports of (26). Increased levels of 8-OHdG could also be a result of oxidative stress induced by the arsenic (27). Treatment with *C. papaya* root aqueous extract at 150 and 100 mg/kg body weight respectively, or vitamin C, to arsenic-induced rats considerably reduced the 8-OHdG levels in rat plasma compared to arsenic only rats which might be due to the antioxidant activities of *C. papaya* root and its ability to ameliorate oxidative stress induced by arsenic as well as present phytochemical components such as phenolic, flavonoids and saponins (28).

Of recent, studies involve the endogenous signal molecule adenosine in renal function or protection. Thus, enzymatic production of nucleoside adenosine from its precursor molecules adenosine triphosphate and adenosine monophosphate, and regulation of its level by ADA play a vital role in decreasing renal damage and conserving the functionality of the kidney during incidents of renal heavy metal poisoning (15, 16). The results of this study revealed that ADA activity was considerably increased in the kidney of arsenic only treated rats compared with the control (*p* < 0.05). Earlier researches have confirmed upregulation in the ADA activity in renal injury (29, 30, 31). Hence, increase ADA activity found in this study might lead to a reduction in the level of adenosine, a renoprotector molecule (15,16). Numerous studies have linked the levels of adenosine in hypoxia, inflammation, or acute renal injury (15, 32). Treatment with aqueous *C. papaya* roots was able to prevent an increase in ADA activity in arsenic-treated rats (Figure 2). This implies that aqueous *C. papaya* roots...
Cytokines synthetized by neutrophils and macrophages stimulate the production of acute-phase proteins, like C-reactive protein (CRP). CRP is synthesized by the hepatocytes. CRP levels in serum increase during infection and inflammation (33). C-reactive protein is a marker, which appears during the late phase of infection (34, 35). In the present study rats chronically exposed to a wide range of arsenic concentrations through drinking water, arsenic exposure was associated with increased serum CRP levels in arsenic-treated rats compared with the control ($p < 0.05$) (Figure 3). Arsenic exposure has been proven to cause inflammation; this may be a mechanism for arsenic-induced diseases, as inflammation is involved in the pathogenesis of many chronic diseases, including cardiovascular disease, metabolic syndrome (36), chronic kidney disease (37), and cancer (38). Chronic exposure of rats to 100 ppm sodium arsenite in drinking water resulted in an increase in the acute-phase protein C-reactive protein (CRP) in the kidney (39).

In this present study, it revealed a significant decrease in the levels of nitric oxide (NO) in arsenic-induced renal injury (Figure 4). Though, treatment with aqueous C. papaya roots, restores the level of NO in arsenic treated rats. This increase in nitric oxide could be a result of the fact that C. papaya roots exhibited an inhibitory effect on the activity (40). However, an increasing evidence that nitric oxide, a potent vasodilator, is one amongst the foremost crucial paracrine modulators and mediators in the regulation of renal functions, for examples total and regional renal blood flow, renal autoregulation, glomerular filtration rate, renin secretion, and salt excretion (41). Nitric oxide also plays a vital role in the pathogenesis of several renal syndromes, for instance diabetic nephropathy, inflammatory glomerular disorders, acute renal failure, and nephrotoxicity of drugs or heavy metals, assigning both beneficial effects through its hemodynamic functions (41).

Aqueous C. papaya roots inhibits arsenic induced expression of inflammation markers inducible nitric oxide synthase (iNOS) activity. In this study, arsenic untreated rats caused a significant ($p < 0.05$) increment of iNOS enzymatic activity compared to normal control (Figure 5). Though, treatment with aqueous extract of C. papaya root at 100 mg/kg body weight and 150 mg/kg body weight and vitamin C strongly inhibited ($p < 0.05$) the iNOS enzymatic activation in arsenic treated rats compared to arsenic alone. This result is in agreement with Figure 4, where we observed a decrease in the levels of nitric oxide in arsenic treated rats (42).

The mechanism of arsenic-induced oxidative stress involves an imbalance between generation and removal of reactive oxygen species in tissues and cellular elements, inflicting harm to membranes, DNA, and proteins (43). Reactive oxygen species generated by arsenic initiate lipid peroxidation of the membrane-bound polyunsaturated fatty acids, resulting in impairment of the membrane structural and functional integrity, an interaction between free radicals of numerous pedigrees and unsaturated fatty acids that are typical in membrane lipids. Degradation of polyunsaturated fatty acids in cell bio-membranes by reactive oxygen species, induced by arsenic, ends up in the destruction of bio-membranes and therefore the formation of thiobarbituric acid reactive species, MDA, or conjugated dienes as indicators of lipid peroxidation (44, 45). In this study, the level of MDA was measured as an indicator of lipid peroxidation (Figure 6). The level of MDA was considerably increased in the kid-
ney tissue of arsenic treated rats. The experimental findings suggest that oxidative stress plays a very crucial role in arsenic-induced renal injuries. Our result is in accordance with other findings (44, 45, 46,47). The increased MDA levels in renal tissue is a sign of over accumulation of lipid peroxides in tissue, causing overconsumption.

Statistics generated revealed that arsenic accumulated preferentially in the kidney tissues. Arsenic concentrations in the kidney tissues (Figure 7) were considerably higher ($p < 0.05$) in arsenic treated rats alone. However, treatment with C. papaya root aqueous extract at 150 and 100 mg/kg body weight considerably decrease the levels of arsenic in the tissues. Vitamin C administration also reduced the levels of arsenic in the tissues compared to the arsenic-only exposed rats. These results are in accordance with results reported by (48) which showed that arsenic dispersal in tissues depends on the route of administration and its form.

CONCLUSION

In conclusion, C. papaya roots halts arsenic-induced renal inflammation by inhibiting anti-inflammatory biomarkers in the kidney. Hence, these activities could suggest some probable mechanisms of action for its renoprotective activity.

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Declaration of Conflict of Interest

We declare that we have no conflict of interest.

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