Original Article

Effect of Carica papaya bark extract on oxidative stress parameters in testes of male albino rats

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Summary: Carica papaya bark has contraceptive benefits when given at a high dose in male rats the mechanism at which it achieves this is yet to be fully understood. Forty male Wistar rats were used for the study. They were randomized into 8 groups Ia, IIa, IIIa, Ib, IIb, IIIb, IV and V respectively (n=5). Groups Ia-IIIa and Ib-IIIb were treated for 4 and 8 weeks with Carica papaya bark extract at doses 50 and 100 mg ml⁻¹day⁻¹ comparing to 2-5 ml distilled water baseline control. Group IV rats were observed for reversibility treated with alternating bark extract and distilled water for 16 weeks (8 weeks each). They were compared to Group V treated with distilled water alone for similar duration. The rats were sacrificed under chloroform anesthesia. The estimated parameters were testes volumes biochemical activities of testicular antioxidant enzymes and non-enzymes [superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH) and malondialdehyde (MDA)] as well as total protein (TP). The extract treated groups (Groups Ia, IIa, Ib & IIb) showed a decrease in TP and testicular volume. Also in these groups were significant increases in testicular MDA levels compared to control (Groups IIIa & IIIb). At both durations for the two doses, the extract resulted in a significant decrease in antioxidant enzymes. Animals in the reversibility study group showed values similar to baseline control. In conclusion, Carica papaya leaf extract essentially perturbed the testicular oxidative system this may be responsible for the contraceptive effect seen.

Industrial relevance: The contraceptive benefits of aqueous extract of Carica papaya bark in orally treated male rats had been confirmed. This present study was tailored at reviewing variations in the gonadal proteins and stressors index in male rats administered established contraceptive doses of the extract. This would be helpful to industries intending to develop this herbal preparation as a contraceptive adjunct because it provides base line biochemical information on the testes that explains the likely possible mechanism of action of the herb.

Keywords: Carica papaya; testes; superoxide dismutase; catalase; glutathione; malondialdehyde

INTRODUCTION

Contraception is a subset of birth control technique and literally means the prevention of fertilization. In centuries past, herbal potions and pessaries have been concocted with the main aim of averting, and or disrupting pregnancy with many yet to be evidence based. Carica papaya is a plant amid few whose fractions have been exploited and documented for its antifertility properties (Lohiya et al., 1999; Lohiya et al., 2005). In Nigeria, Carica papaya (pawpaw, in the English language) is called Ibepe amongst the Yoruba speaking populace; Popo among the Igbos and Gwanda among the Hausas. Though the plant is tropical American in origin, it is now widely spread throughout tropical Africa. It is extensively grown for its fruits and is widely cultivated throughout the world and used as a food supplement and as traditional medicine, particularly as an antiseptic (Mehdipour et al., 2006).
Little is known about the exact mechanism by which *Carica papaya* extract affects the testes negatively to bring about infertility in the male rats. Although the testis is known to be highly sensitive to a number of physical stresses including oxidative stress which may be an important mediator of testicular injury (Zini and Schlegel, 1997). With the exception of trauma, death at cellular levels is mediated by oxidation. The malondialdehyde (MDA) levels have been shown to associate positively with tissue damage and therefore widely used as a marker for lipid peroxidation (Duru et al., 2008) and oxidative stress. A number of investigators have suggested that oxidative stress may be an important mediator of testicular injury and the implicating pathway has been pointed at a free radical mechanism which either kill or harm them. The by-products of oxygen reaction called reactive oxygen species (ROS) are responsible for producing the damaging effects. ROS-mediated damage to sperm is a significant contributing pathology in 30–80% of cases (Agarwal et al., 2006). Numerous antioxidants are related to the ROS detoxification, including superoxide dismutase, catalase and glutathione peroxidase.

We have confirmed the qualitatively contraceptive prospective of the aqueous extract of *Carica papaya* bark in orally treated male rats (Kusemiju et al., 2002). In the study, the contraceptive ‘model’ revealed a resounding inclusive loss of fertility attributed to a decline in sperm motility and alteration in their testicular morphologies (Kusemiju et al., 2002; Kusemiju et al., 2010). In another of our study with the bark extract (at similar doses), we demonstrated that hematological indicators in the rats which assessed organ-specific toxicity showed no apparent damage except for relative variations in the leukocytes count and indices (Kusemiju et al., 2011). This present study (a replica of the erstwhile), was modified to appraise parallel variations in the testicular proteins and oxidative stressors as well as testicular morphometry in the Wistar rats administered these contraceptive doses of *Carica papaya* bark extract. The knowledge gained from this study would provide clues to the cell types primarily affected and aid understanding of possible mechanism of action and functioning of the male reproductive tract.

**MATERIALS AND METHODS**

**Collection and preparation of *Carica papaya* bark:** The bark of the *Carica papaya* plant was obtained from a forest in Lagos and authenticated by a taxonomist in the Department of Botany, University of Lagos. A voucher specimen (code: LUH 2151) was deposited in the institutions herbarium. The bark of the plant was dried in an electric oven at 40°C for 4 days. The extract preparation was done according to methods described by Kusemiju et al., 2011. Briefly; 50 g of the bark material was crushed to obtain a coarse powder that was used for the extraction in the Pharmacognosy Department of the Faculty of Pharmacy, University of Lagos. It was kept in 500 ml of distilled water for 24 h at the same temperature. The filtrate was thereafter obtained from the solution using the whatman no 1 filter paper and evaporated to dryness in an oven at 60°C. The residue of the extract obtained in the form of paste was stored in a capped bottle and kept in desiccators (Kusemiju et al., 2011).

**Investigational animals and experimental procedure:** Forty mature male Wistar rats (7-10 weeks old) weighing between 115-150 g domiciled in 8 well-ventilated synthetic cages were used for the study. Each cage designating a particular group comprised 5 randomized rats. These groups were identified clearly as: Ia, IIa, IIIa, Ib, IIb, IIIb, IV and V respectively. The rats were validated by a taxonomist (Malaka 2005) at the Zoology Department University of Lagos Nigeria. Their ambient temperatures range between 29–30°C and a relative humidity of 50–55%.

Accordingly, groups Ia-IIIa and Ib-IIIb were orogastrically treated for 4 and 8 weeks with *Carica papaya* bark extract at doses 50 and 100 mg ml⁻¹day⁻¹ comparing to 2-5 ml distilled water baseline control. Group IV rats were observed for reversibility. They were treated with 50 mg ml⁻¹day⁻¹ of the bark extract for 8 weeks, discontinued and treated for another 8 weeks with 2-5 ml distilled water. They were compared to Group V (normative control) treated with 2-5 ml distilled water alone for similar duration. Throughout the research periods the animals were nurtured in the animal holding division of Anatomy department, College of Medicine, University of Lagos, Nigeria and exposed to a rhythm that permitted a photoperiod of 12 h light, alternating with 12 h darkness. Rat chow (Livestock feeds Plc. Ikeja, Lagos, Nigeria), and clean tap water were provided *ad libitum*.

**Necropsy and testicular volume estimation:** The rats were sacrificed after by placing them in a stopped jar containing cotton wool sucked with chloroform anesthesia. The abdominal cavity was opened up through a midline abdominal incision to expose the reproductive organs (Saalu et al., 2011). The testes were excised and trimmed of all fat. The testes volumes were measured by water displacement method. The two testes of each rat were measured and the average value obtained for each of the two parameters was regarded as one observation (Yama et al., 2010).

**Biochemical assessments:** *Testicular antioxidant enzymes activities:* Superoxide dismutase (SOD) activity was determined according to the method of Winterbourn et al., 1975 as described by Rukmini et al., 2004. The principle of the assay was based on the ability of SOD to inhibit the reduction of nitro-blue tetrazolium (NBT). Briefly, the reaction mixture contained 2.7 ml of 0.067M phosphate buffer, pH 7.8, 0.05 ml of 0.12mM riboflavin, 0.1 ml of 1.5mM NBT, 0.05 ml of 0.01M methionine and 0.1 ml of enzyme samples. Uniform illumination of the tubes was ensured by placing it in air aluminum foil in a box with a 15W fluorescent lamp for 10 minutes. Control without the
enzyme source was included. The absorbance was measured at 560nm. One unit of SOD was defined as the amount of
ezyme required to inhibit the reduction of NBT by 50% under the specific conditions.

Catalase (CAT) activity was determined according to the method of Beers and Sizer as illustrated by Saalu et al., 2011, by measuring the decrease in absorbance at 240 nm due to the decomposition of H₂O₂ in a UV recording spectrophotometer. The reaction mixture (3ml) contained 0.1 ml of testicular homogenate in phosphate buffer (50 mM, pH 7.0) and 2.9 ml of 30 mM H₂O₂ in phosphate buffer pH 7.0. An extinction coefficient for H₂O₂ at 240 nm of 40.0 M⁻¹cm⁻¹ (Aebi 1984) was used for the calculation. The specific activity of CAT was expressed as moles of H₂O₂ reduced. **Non-enzymatic antioxidants and Lipid peroxidation:** Assay of testicular reduced glutathione (GSH) concentration was determined by the method of Ellman (1959) as presented by Saalu et al., 2011. 1.0 ml of supernatant was treated with 0.5 ml of Ellmans reagent (19.8 mg of 5, 5’-dithiobisnitro benzoic acid (DTNB) in 100 ml of 0.1% sodium nitrate) and 3.0 ml of phosphate buffer (0.2 M, pH 8.0). 0.4 ml of distilled water was added. The mixture was thoroughly mixed and the absorbance was read at 412 nm. Lipid peroxidation in the testicular tissue was estimated colorimetrically by thiobarbituric acid reactive substances (TBARS) method of Buege and Aust (1978); A principle component of TBARS being malondialdehyde (MDA), a product of lipid peroxidation (Yama et al., 1978); A principle component of TBARS being malondialdehyde (MDA), a product of lipid peroxidation (Yama et al., 2011a). In brief, 0.1 ml of tissue homogenate (Tris-Hcl buffer, pH 7.5) was treated with 2 ml of (1:1:1 ratio) TBA-TCA-HCl reagent (thiobarbituric acid 0.37%, 0.25 N HCl and 15% TCA) and placed in water bath for 15 min, cooled. The absorbance of clear supernatant was measured against reference blank at 535 nm. Concentration was calculated using the molar absorptivity of malondialdehyde which is 1.56 x 10⁵ M⁻¹ cm⁻¹. **Determination of total protein:** This was determined using Biuret method (Gonall et al., 1949). 5.0 ml of blank Biuret reagent prepared by dissolving CuSO₄·5H₂O crystal in 500 ml of distilled water was added to sample blank. These were mixed well arid allowed to stand for 20 min at room temperature 25 - 27°C. Absorbance was read for one test and standard against a blank at 540 nm. The concentration of protein was calculated using: optical density for standard x concentration of standard.

**Statistical analysis and Animal ethics:** The data obtained were expressed as mean (µ) ± Standard Deviation (SD). Test of statistical significance was done by analysis of variance (ANOVA), and unpaired one t tail Student’s t-test, p-value taken as < 0.05 or otherwise stated.

All procedures involving animals 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 (American Physiological Society, 2002) and were approved by the Departmental Committee on the Use and Care of Animals in conformity with international acceptable standards.

**RESULTS**

**Testicular volumetric analysis:** The mean testicular volume (in milliliters) of the control group (Groups IIIa, IIIb and V) 4, 8 and 16 weeks were: 1.0± 0.0, 0.8± 0.1 and 1.00± 0.2 ml. The extract treated groups (Groups Ia, Iia, Ib & IIb) showed a decrease in volume which was significant in Group IIb treated with the extract for 8 weeks (p < 0.05). The testicular volume of the rats after extract withdrawal 1.0± 0.0 ml at week 16 was not statistically different from control (Group V at week 16; p > 0.05; Table 1).

**Effect on lipid peroxidation and testicular protein content:** There were both concentration and duration dependent significant increase in testicular MDA levels in the group that received the extract (Groups Ia, Iia, Ib & IIb) compared to control (Groups IIIa & IIIb). At 4 weeks 50 and 100 mg ml⁻¹day⁻¹ extract resulted in a significant increase (p < 0.01) in testicular MDA levels to 12.1±1.4 and 18.3±0.8 compared to 0.4±0.0 of control (Table 1). Also observed was a converse decrease in TP content of 20.5±1.2 (p < 0.001) and 13.9±3.2 (p < 0.01) more marked with the higher dose as against 69.1±1.6 baseline control. In the rats treated (50 and 100 mg ml⁻¹day⁻¹ extract) for longer durations (8 weeks), MDA concentrations were significantly elevated to 11.3±1.1 (p < 0.01) and 26.0±5.2 (p < 0.05) compared to 0.5±0.0 of control. Conversely the TP levels declined from 73.9±6.13 of control to 20.1±0.6 (p < 0.01) and 12.1±1.4 (p < 0.01) in the extract treated. The reversibility study showed both MDA levels and TP values similar to control rats (Table 1).

**Testicular antioxidant enzymes and non-enzyme defense:** At both durations for the two doses, the extract clearly resulted in a significant decrease in antioxidant enzymes defense line as shown (Table 2). By the end of the 4th week extract treatment with 50 mg ml⁻¹day⁻¹ dose the values of GSH, SOD and CAT levels were respectively: 5.9±0.5 (p < 0.01), 19.7±5.7 (p < 0.05), 52.1±3.5 (p < 0.01); while and 100 mg ml⁻¹day⁻¹ dose 7.4±0.9 (p < 0.05), 9.4±2.7 (p < 0.01), 17.0±2.4 (p < 0.001) compared to 15.0±1.0, 48.6±3.5, 98.6±0.5 of control. At 8 weeks the concomitant values for GSH, SOD, and CAT were: 3.8±1.7 (p < 0.05), 15.6±5.7, 38.8±9.0 (p < 0.05) for 50 mg/ml and 2.7±1.1 (p < 0.05), 5.1±0.9 (p < 0.05), 8.5±0.5 (p < 0.01) for 100 mg/ml compared to 11.3±0.5, 49.1±12.9, 85.2±5.0 of control. Animals in the reversibility study group showed an appreciable recovery as the antioxidant enzymes mirrored the normative baseline values (Table 2).
ββγ

4 weeks Ia 50 mg ml⁻¹ day⁻¹  12.1±1.4β 20.5±1.2α  0.9±0.1
     IIA 100 mg ml⁻¹ day⁻¹  18.3±0.8β 13.9±3.2β  0.7±0.2
     IIIa 2-5 ml  0.4±0.0  69.1±1.6  1.0±0.0

8 weeks Ib 50 mg ml⁻¹ day⁻¹  11.3±1.1β 20.1±0.6β  0.6±0.2
     IIb 100 mg ml⁻¹ day⁻¹  26.0±5.2γ 12.1±1.4β  0.5±0.2γ
     IIIb 2-5 ml  0.5±0.0  73.9±6.13  0.8±0.1

16 weeks IV 50 mg ml⁻¹ day⁻¹→2-5 ml  1.1±0.6  62.2±8.3  0.7±0.2
     V 2-5 ml  0.5±0.2  64.6±4.6  1.0±0.2

REV: Reversal group (that is treatment with 50 mg ml⁻¹ day⁻¹ extract for 8 weeks and then 2-5 ml of distilled water for another 8 weeks); MDA: Malondialdehyde; TP: Total protein; TV: Testicular volume; α (p < 0.001); β (p < 0.01); γ (p < 0.05).

Table 2. Effect of *Carica papaya* bark extract on testicular antioxidant enzymes

<table>
<thead>
<tr>
<th>Duration</th>
<th>Group (n=5)</th>
<th>Dose: Extract/Distilled water</th>
<th>GSH (μmol/ml)</th>
<th>SOD (nmol/ml)</th>
<th>CAT (μmol/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 weeks</td>
<td>Ia</td>
<td>50 mg ml⁻¹ day⁻¹</td>
<td>5.9±0.5β</td>
<td>19.7±5.7β</td>
<td>52.1±3.5β</td>
</tr>
<tr>
<td></td>
<td>IIA</td>
<td>100 mg ml⁻¹ day⁻¹</td>
<td>7.4±0.9γ</td>
<td>9.4±2.7β</td>
<td>17.0±2.4α</td>
</tr>
<tr>
<td></td>
<td>IIIa</td>
<td>2-5 ml</td>
<td>15.0±1.0</td>
<td>48.6±3.5</td>
<td>98.6±0.5</td>
</tr>
<tr>
<td>8 weeks</td>
<td>Ib</td>
<td>50 mg ml⁻¹ day⁻¹</td>
<td>3.8±1.7γ</td>
<td>15.6±5.7γ</td>
<td>38.8±9.0γ</td>
</tr>
<tr>
<td></td>
<td>IIb</td>
<td>100 mg ml⁻¹ day⁻¹</td>
<td>2.7±1.1γ</td>
<td>5.1±0.9γ</td>
<td>8.5±0.5β</td>
</tr>
<tr>
<td></td>
<td>IIIb</td>
<td>2-5 ml</td>
<td>11.3±0.5</td>
<td>49.1±12.9</td>
<td>85.2±5.0</td>
</tr>
<tr>
<td>16 weeks</td>
<td>IV</td>
<td>50 mg ml⁻¹ day⁻¹→2-5 ml</td>
<td>11.7±1.2</td>
<td>25.6±1.0β</td>
<td>70.0±15.6</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>2-5 ml</td>
<td>14.3±1.0</td>
<td>38.7±3.6</td>
<td>96.2±2.9</td>
</tr>
</tbody>
</table>

REV: Reversal group (that is treatment with 50 mg ml⁻¹ day⁻¹ extract for 8 weeks and then 2-5 ml of distilled water for another 8 weeks); GSH: Glutathione (reduced); SOD: Superoxide dismutase; CAT: Catalase; α (p < 0.001); β (p < 0.01); γ (p < 0.05).

DISCUSSION

In the past researchers have shown that the testicular volume to correlate positively with testicular function (Yama et al., 2011b; Takihara et al., 1987) as well as the testosterone level (Mahmoud et al., 2003). In our findings, the volume of all the testicular samples harvested from rats fed *Carica papaya* extract were reduced compared to control group, representative of a wide spread injury which could be ascribed to a reduced or inhibition of protein contents in the testes as also shown by our findings. This therefore means an altered testicular function as supportive findings of decreased sperm quality and testosterone level in previous study (Yama et al., 2011c). Similar results have been observed on the testes with *Semecarpus anacardium* fruits (Sharma et al., 2003), *Momordica charantia* seed extract in the testes of male rats (Yama et al., 2011c).

Testicular MDA levels were significantly increased in animals treated with *Carica papaya*. Conversely, all control animals experienced no elevation in MDA levels. The increased MDA level suggests testicular oxidative stress induced by *Carica papaya* through the generation of free radicals and or a disturbance in the antioxidant status of the testes. The lipid composition of the sperm membrane exerts a significant effect upon the functional quality of spermatozoa (Zalata et al., 1998). The contraceptive effect seen in our previous study (Kusemiju et al., 2002) could be connected to the increase in MDA levels as the latter is an indirect indicator of oxidative stress hence influence sperm quality and function (Morakinyo et al., 2008; Suzuki and Sofikitis, 1999).

It is a known fact that SOD protects dehydratases against free radical, superoxide (Lenzi et al., 1993) and also a low CAT activity closely relates to low motility of ejaculated spermatozoa (Kawakami et al., 2007). The extract treated rats in this present study displayed consistently poor SOD and CAT enzyme activities compared to the normative control. Also in concordance is the fact that GSH, an imperative substrate for glutathione-related enzymes that act as a free radical scavenger (Rudin et al., 2003), were also significantly decreased following *Carica papaya* treatment.

The major antioxidants that protect cells from oxidative stress induced by reactive oxygen species are GSH, SOD and CAT. A succinct fundamental biochemistry in this study of these antioxidant enzymes/ non-enzymes within the testes would be the rapid conversion of superoxide anion (O₂⁻) to hydrogen peroxide (H₂O₂) in the presence of SOD in order to prevent the former from participating in the formation of highly pernicious hydroxyl radicals. The H₂O₂ generated in this manner is a powerful membrane permeant oxidant in its own right that has to be rapidly eliminated from the cell in order to prevent the induction of oxidative damage to lipids, proteins and DNA. The
elimination of H₂O₂ is either effected by catalase or glutathione peroxidase, with the latter predominating in the case of the testes (Zini and Schlegel 1996; Peltola et al., 1992). This activity is critical in the detoxification of peroxidised lipids. An alteration in any of these sequences would lead to oxidative stress and a compromise in male fertility as the effect of our extract in the present study strongly supports.

In conclusion the contraceptive effect of Carica papaya leaf extract may hinge essentially on perturbation or the breakdown of the testicular oxidative system. This is contributed by increased oxidative stress either due to increased free radical release or the decreased antioxidant defense system as well as an associated derangement of protein content of the testes. There are yet no specific data showing that the increased oxidative stress in male rats fed Carica papaya leaf extract is localized only to the testes. A further study is thus required to evaluate other possible systemic oxidative toxicity.

REFERENCES


