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Ripe fruit *Carica papaya* administration attenuates testicular connective tissue alterations in experimental alcohol toxicity model

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Abstract:

BACKGROUND: The use of fruits as natural remedies to mitigate cellular and tissue induced alterations caused by exogenous toxins like alcohol have been widely documented, and the ethnopharmacological potentials of the *Carica papaya* have also been reported.

AIM AND OBJECTIVES: The study aims to investigate if ripe fruit *carica papaya* administration attenuates testicular connective tissue alterations in experimental alcohol toxicity model.

MATERIALS AND METHODS: Thirty (30) animals weighing between 150 to 220 g were used for this study, divided into 6 groups as follows; group 1 received distilled water 2 ml per kilogram body weight, group 2 received 40 % ethanol 5 ml per kilogram body weight, group 3 received 40 % ethanol 5 ml + clomiphene citrate 50 mg per kilogram body weight, groups 4, 5 and 6 received 40 % ethanol 5 ml + 500, 1000 and 1500 mg *C. papaya* per kilogram body weight respectively. Rats were weighed and thereafter sacrificed by anaesthetizing with chloroform, thoraco-abdominal wall dissected to access the heart and the testes. Harvested testes were fixed and sent to the laboratory for processing and histological analysis.

RESULTS: Result of histological analysis showed ethanol treatment induced testicular atrophy, spermatogenic cell degeneration, interstitial tissue vacuolations, connective tissue disruptions, conditions which were ameliorated by *C. papaya*. This ameliorative potential of *C. papaya* appeared to be effective and dose dependent.

CONCLUSION: While it is attributable that *C. papaya* may confer protective capabilities against ethanol induced testicular connective tissue damages, this study provides evidence that moderate intake of *C. papaya* ameliorated injuries induced by ethanol to the testicular connective tissue of rats with a mechanism believed to be antioxidant mediated.

Keywords:

Alcohol, *Carica papaya*, connective tissue, rat, testis

Introduction

Excess alcohol consumption is a most frequent and expensive form of drug abuse, which gives a worldwide concern considering its socioeconomic consequences, and a major factor contributing to many disease categories (Akhila and Vijayalakshmi 2015; Akomolafe *et al*. 2015). Reproductive impairment in males by drug influence factors is a major disease burden in the developed countries and will continue to be so (Aliyu 2006). Testicular toxicity caused by ethanol toxins is said to cause fertility abnormalities and suppressed sexual behaviors associated with impairment in sperm motility and low sperm count both in humans and in laboratory animals (Amanvernez and Agara 2006; Apte *et al*. 2005). Testicular atrophy is induced by free radicals from the alcohol metabolism, which are harmful molecules

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triggering oxidative damages to the testis through tissue degeneration and cell death (Awobajo *et al*. 2010; Azu *et al*. 2011).

Most countries consider alcohol as having neuropsychological effect when one exceeds a limit of 0.08 g in 100 ml of the human blood (Bhardwaj and Pandey 2011). It is also the leading risk factor for death among males aged 15–59 years, particularly in Eastern Europe (Bogue 1923). Gross pathological changes associated with alcohol drinking include: fat accumulation (steatosis), inflammation, necrosis, and fibrosis (Borden and Fergusson 2011). Alcohol highly diffuses through membrane of cells and is metabolized by most tissues, hence causes toxicity to these organs, namely, the liver, gut, testis, and brain (Bogue 1923; Chiang *et al*. 1992; David 2006). Excess alcohol intake causes impaired fertility, premature and low-birth-weight and fetal alcoholic syndrome spectrum disorder of the testis in males (Bogue 1923), and poor semen production and sperm quality (Dosumu 2006). However, herbal treatment causes good recovery tendencies to testicular degeneration, even to normalcy after alcohol exposure (Gabriela 2014).

Natural treatment of human ailments has been based on local plants since prehistoric time, and the use of medications practically dates back to onset of human civilization (Gao and Bartaller 2011). *Carica papaya* is a tropical fruit plant, in the family *Caricaceae*, native to South Mexico and/or in Central America (Gelse 2003). The whole-plant parts have been documented to have various medicinal properties (Khattab 2007). *Papaya* fruit phytochemically contains carotenoids, polyphenols, folate, and the essential vitamins (A, C, and E) (Kiernan and 2008). Other active components identified in fruits and leaves such as papain, chymopapain, cystatin, α-tocopherol, ascorbic acid, flavonoids, cyanogenic glucosides, and glucosinolates are observed to increase antioxidant potency in the blood and cause a reduction in the lipid peroxidation level (Lasheen 2015). Antibacterial and urinary tract infection treatment of flowers (Lohiya *et al*. 2002; Mahmood *et al.* 2005), wound healing, reduction in cardiovascular disease risk, anti-inflammatory activity, antitumor activity, immune adjuvant for vaccine therapy, antimalarial and antiplasmodial activities of leaves (Mahmood *et al.* 2005; Mahmuda 2014; Malik *et al*. 2017; Maneesh *et al*. 2006; National Institute of Health 2011; Nimse and Pal 2015), skin protective and digestive tract disease treatments of papaya milky juice (Oremusu and Akang 2014), and contraceptive effect of seeds (Otsuki *et al*. 2010) have been investigated and documented.

Connective tissues in mammals are best studied using staining procedure which differentiates its different structural components. Masson's trichrome staining protocol is a histochemical technique, which simultaneously permits the study of muscle fibers, collagen, and blood vessels as well as cellular nuclei by a varying color staining property (Owoyele *et al.* 2008). Studies have demonstrated the use of the Masson's Trichrome staining technique in the study of the role of collagen in testicular connective tissue developments and in wound healing (Paranko and Pelliniemi 1992; Patricia *et al*. 2014).

Studies on the testicular protective effect of ripe fruit *C. papaya* by histochemical assessments have not been carried out; hence, this study aims at investigating the attenuative effect of ripe fruit of *C. papaya* administration on the testicular connective tissue in experimental alcohol toxicity model.

Materials and Methods

Fruit collection, authentication, and extract preparation

Ripe *C. papaya* fruits harvested from a local farm in Nduetong Oku, in Uyo municipality in April 2017, then authenticated by the department of botany, and specimen voucher number (herbarium number UUPH18a) was obtained from the Faculty of Pharmacy herbarium, University of Uyo. The fruit pulp was prepared fresh after washing, the epicarp peeled off, seeds removed, and blended with a fruit blender (Ralf *et al*. 2002). Thereafter, the blended fruit was wet macerated with 95% ethanol and kept for 72 h, and then first filtered with cheese cloth and second with filter paper (Whatman no. 4). The extract was subsequently concentrated to dryness in warm water bath at 45°C, and the yield obtained was kept in a closed glass container in a refrigerator at 2°C–8°C until required.

Animal care and use

Thirty adult male Wistar rats were purchased and housed within the Faculty of Basic Medical Sciences, Animal House of the University of Uyo. Animals weighing 150–220 g were acclimatized for 2 weeks and given humane care in accordance with the principle of laboratory animal care and use (Rivera-Pastrama *et al.* 2010). Five animals were randomly selected into six groups and kept in well-ventilated wooden cages at temperature 25°C ± 5°C and 12 h light/dark cycle. The rats were fed with standard rat chow (vital feed) and water provided *ad libitum* for 28 days. Treatments were by oral intubation and grouped as follows: the Group 1 (control) received distilled water 2 ml/kg body weight, Group 2 received 40% ethanol 5 ml/kg body weight, Group 3 received 40% ethanol 5 ml and clomiphene citrate 50 mg/kg body weight, and Groups 4, 5, and 6 received 40% ethanol 5 ml and *C. papaya* 500, 1000, and 1500 mg/kg body weight, respectively.

Ethical approval

This study received approval of the Department of Anatomy and Faculty of Basic Medical Sciences Research and Ethics Committees of the University of Uyo.

Sample collection

Twenty-four hours after the last administration, animals were sacrificed under chloroform inhalation, and a longitudinal laparoscopy extending to the median septum was employed to assess the testes.

Histopathological analysis

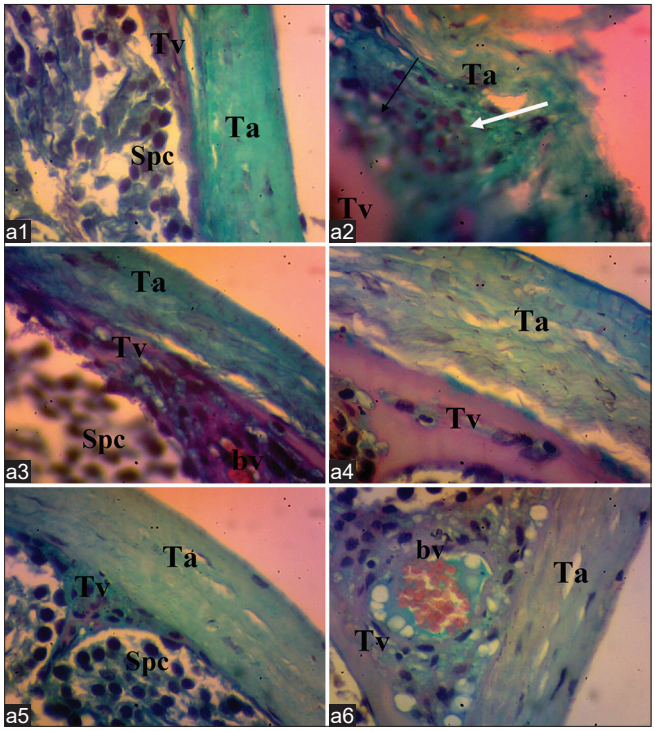
The testes were histologically processed after fixation for 1 week, then dehydrated through ascending grades of alcohol, cleared, embedded in paraffin, mounted, and sectioned at 5-µm thickness. Staining procedures for Masson's trichrome as described by Kiernan (2008) and employed by Lasheen *et al*. (2015) were then carried out for histochemical evaluation using light microscopy (Olympus-CX31).

Results

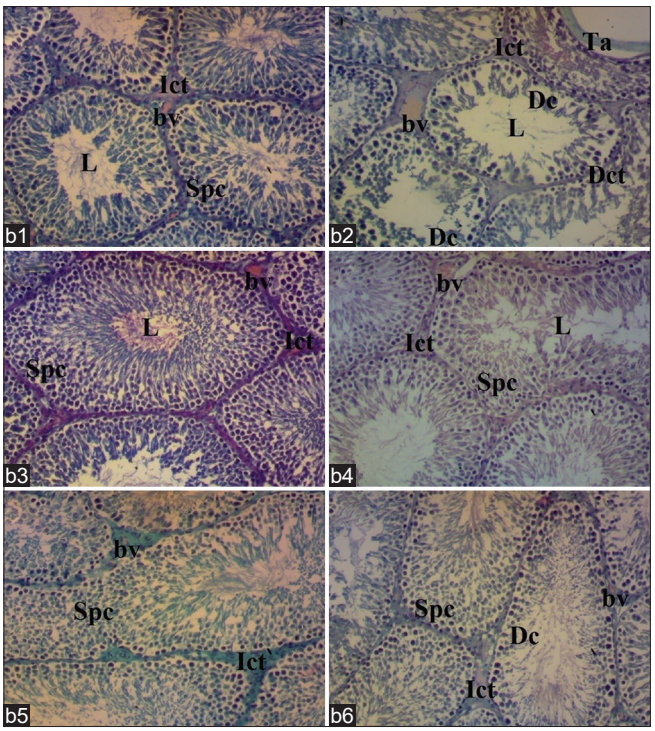
Testicular tissues showed a well-structured interstitium with well-arranged histoarchitectural representation of spermatogenic cells in the various groups except Group 2. Basement membrane was visibly seen to show the sequential maturation of the germ cells. Rays of maturing spermatogenic cells toward the tubular lumina were well presented with good staining intensity of the nuclei and connective tissues. Numerous blood vessels were seen within the interstitial tissues in all the groups except Group 2. Seminiferous tubules were observed to be clearly separated by interstitial connective tissues which showed an even distribution of Leydig cells in the control testis and the extract-treated groups.

Group 2 testicular section showed distortion of seminiferous tubules with areas of ruptured basement membrane and reduction of interstitial tissue between them. It also revealed marked areas with loss of spermatogenic cells and degenerated cells with vacuolated cytoplasm, small dark stained nuclei, and wider tubular lumina. Eroded interstitial cells and reduced number of blood vessels were also observed.

Group 3 (ethanol and clomiphene citrate-treated) showed a well-structured interstitial connective tissue with numerous blood vessels. Groups 4 and 5 (ethanol and *C. papaya* 500 and 1000 mg/kg) showed good histoarchitectural presentation, but Group 6 (40% ethanol + *C. papaya* 1500 mg/kg) showed a distortion of the basal membrane of the seminiferous tubules and eroding of interstitial connective tissue but with numerous blood vessels as in the control.



a1-a6. Photomicrographs of transverse section of the testicular tunica layer Masson trichome x400. Spc=Spermatogenic cells, Ta=Tunica albuginea, bv=Blood vessels, White arrow=Abnormally proliferated cells, Black arrow=Vacoulation of cells



b1-b6. Photomicrographs of transverse section of the testis Masson trichome stained at x100. Spc=Spermatogenic cells, lct=Interlobular connective tissue, bv=blood vessel, Dc=Degenerated cells, Dct=Degenerating connective tissue, Ta=Tunica albuginea, L=Lumen of testicular lobule, bv=blood vessels

The protective connective tissue layers (tunica albuginea and tunica vasculosa) in all groups showed varying thickness and staining intensity. Group 1 (control) section shows a well compact fibrous tissue arrangement of the tunica albuginea and the tunica vasculosa which was well stained confirming its high collagen fiber content in the loose connective tissue and blood vessels sparsely distributed. Tunica albuginea of the Group 2 showed loosely connected and scattered fibrous tissues with no clear distinctions with the tunica vasculosa. Cells were observed to be proliferated and widely scattered within the tunica vasculosa which thickness is comparable to that of the tunica albuginea. Collagen fiber staining was observed to be less intense in this group with scanty blood vessels comparative to the control. Drug- and extract-treated groups (3, 4, 5, and 6) showed thickened tunica albuginea, which consists of collagen fibers with closely packed spindle-shaped fibroblasts and thinner tunica vasculosa which were formed in less intensely stained loose connective tissue and blood vessels compared to control.

Discussion

The testis is a cardinal organ of reproduction with an endocrine functions in males with a great susceptibility to damage because of it highly proliferative nature (Runnie *et al*. 2004). This study was to investigate the histochemical effect of the ripe fruit of *C. papaya* administration on the testicular connective tissue of ethanol toxicity model in adult Wistar rats. The reproductive tissues of rats were chosen for this study due to it comparative similarity to that of humans (Seigler *et al*. 2010).

In the present study, significant increase in thickness of the tunica albuginea is seen across all groups, which contrasts with the thickness of the tunica vasculosa layer. This difference in thickness may be attributed to the age of the rats used in the study (Patricia *et al*. 2014).

Germinal epithelium of the testis of the various groups where well arranged with spermatogenic and supportive cells except in the ethanol alone group, which presented the testicular disturbance induced by ethanol toxicity. Staining accurately showed distortions of the cytoarchitecture within the seminiferous tubules by ethanol which correlates with the findings of Dosumu *et al*., 2006 and Awobajo *et al*., 2010 who demonstrated that ethanol affects testicular tissue by oxidative stress. Necrotic effect on the reproductive cells by ethanol may be caused by impairing mitochondrial function (Setchell *et al*. 1994) and/or damages induced on the polyunsaturated fatty acid of the plasma membrane of germinal cells (Setchell *et al*. 1994; Shaha *et al*. 2010). Protection of the testicular epithelium by *C. papaya* as observed in this study may be a function of its phytochemical contents and the bioavailability of flavonoids to scavenge radicals which cause oxidative stress to testicular tissue as supported by the findings of Nimse and Pal (Sharma and Agarwal 1996) on natural antioxidants and their mechanisms.

This study reveals an obvious distortion of the normal histological structure of the testis treated with ethanol alone. Both spermatogenic cells and supporting cells close to the basement membrane of the testicular parenchyma were highly affected. The degenerated or eroded interstitial tissue implied the destruction of the Leydig cells, which implicates the decrease in production of the reproductive hormone, testosterone (Sirkorski 2001), and reduced the promotion of myoid cell differentiation in the testis (Subramanian *et al*. 2006). The toxicity effects observed could give reasons to the abundance of myofibrils smooth muscle cells within the seminiferous tissue areas, reducing the tubules and spermatogenic cells within it (Subramanian *et al*. 2006 and Suvik and Effendy 2012). Azu *et al*., (2011) highlighted that relatively elevated blood gonadotropin level is best used to respond to decreased primary spermatogenesis which may be induced by toxicity. The histological presentation of Group 2 treated with ethanol alone showed the germinal epithelial cells having cytoplasmic vacuolation, a degeneration reported in a similar testicular toxicological study using aluminum chloride (Talabi *et al*. 2011).

The complex fibrous structure, the tunica capsule consists of various distinct layers of fibroblasts interspersed in collagen fibers (Sirkorski 2001; Titanji *et al*. 2008). Studies, however, suggested that the tunica albuginea contraction may act as the propelling force to transport spermatozoa away from the testis, maintain interstitial pressure, and control adequate blood circulation through the testis (Titanji *et al*. 2008). The connective tissues of the testis protect the testicular interstitium from effects of environmental pressure and hence, the presence of a high content of fibroblast within its tissue. Distortion to the tunica capsule as seen in the ethanol-treated group of this study may suggest that the dense connective tissue has lost its integrity and the ability to compress the blood vessels to aid blood flow for the reproductive tissue nourishment, hence cell death. Poor testicular nourishment, as well as low contractile tendency of the tunica layer leading to inadequate transport of spermatogenic cells, may implicate infertility. However, the integrity of the tunica tissue is seen to be protected by *C. papaya* with a mechanism believed to be phytochemically mediated.

Collagen staining intensity was observed to be low in the ethanol-treated group, comparative to control and *C. papaya*-treated groups, which may indicate a reduction

of the contractile and structural protein through ethanol effect. Collagen is the most dominant structural protein in mammalian connective tissues, made of coiled amino acids in the form of elongated fibrils, and is produced by the fibroblast (World Health Organization 2012; World Health Organization 2011), to contribute to the stability of tissue and organs, and maintaining their integrity (World Health Organization 2011). It also functions in giving the necessary rigidity to connective tissues such as muscle fibers, strengthens blood vessels, and contributes to tissue development Yusha'u *et al*. 2009). Biochemical aberration of the tunica albuginea by the absence of some type of collagen might interfere with the normal function in the reproductive drainage system (Zakaria *et al*. 2006). Reduced collagen may have resulted in the loss of connective tissue integrity, as shown in ethanol alone treated group, and may have affected the contractile activity of the interstitial tissue between seminiferous tubules and the associated blood vessels, thereby altering the physiological system within the testis. The testicular function is directly enhanced by the contents and coordinating functions of the connective tissue materials making up the tunica capsule of the testes. It is, therefore, seen that the structural components of the testis are well protected by *C. papaya* as demonstrated in this study.

Conclusion

Alterations to testicular connective tissues following the administration of 40% ethanol at 5 ml/kg body weight of Wistar rats in a 28 days study can be to a certain extent ameliorated by ripe fruit *C. papaya* supplementation at 500 and 1000 mg, which can be attributed to the antioxidant potency accredited to the bioavailability and bioactivity of its phytochemicals, especially the bioflavonoids.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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