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The Nephrotoxicity of Cotton Seed Oil and Gossypol and the Possible Modulatory Role of Grape-seed Proanthocyanidin in Adult Rats

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ABSTRACT

Introduction: Gossypol is a proven cytotoxic yellow pigment obtained from cotton seed oil, while grape seed proanthocyanidin has high free-radical eliminating antioxidants characteristics. Therefore, the effect of grape-seed proanthocyanidin on gossypol induced kidney damage of adult Wistar rats was evaluated.

Method: a total of thirty (30) adult male rats were randomly divided into six (6) groups; Group A: normal control group (received 0.1mls of PBS), Group B: proanthocyanidin control group; received 15 mg/100gm BW of grape seed proanthocyanidin extract, Group C: cotton seed extract group; received 15mg/100gm BW of cottonseed oil only, Group D: Isolated gossypol, ISG; (received 15 mg/100gm BW of isolated gossypol only), and the protection group grape seed, Group E: (received 15 mg/100gm BW of cottonseed oil + 15 mg/100gm BW of grape seed Proanthocyanidin extract and Group F:15 mg/100gm BW of isolated gossypol + 15 mg/100gm BW of grape seed proanthocyanidin. After 56 days of continuous oral administration, animals were euthanized by cervical dislocation and left kidney was excised and fixed in 10% formo-saline for histological preparation, while right kidney was homogenized in 5 % sucrose solution for biochemical assay of malondialdehyde (MDA) and superoxide dismutase (SOD) activities.

Result: animals treated with isolated gossypol (ISG) and cotton seed oil (CSO) showed alterations in the histo-architecture of the renal cortex ranging from the presence of alterations in renal corpuscle, distortion in the epithelial lining of the tubules and significant increase in lipid peroxidation (elevated MDA activity) ($p<0.05$) than that of the control groups.

Immuno-histological expression showed increase expression of cytokeratin-7 (CK-7) among the animals treated with isolated gossypol (ISG) and cotton seed oil (CSO). However, treatment with grape seed proanthocyanidin showed significant decrease in MDA level (lipid peroxidation) relative to control animals, improved histological appearance and moderate expression of cytokeratin-7 (CK-7) along the epithelia lining of the renal tubules. Grape seed proanthocyanidin maintained the antioxidant enzymes concentration and integrity, while cotton seed oil and isolated gossypol treatment caused measurable increase in antioxidant activities imposed by cell injury (reactive oxygen species) during gossypol toxicity

Conclusion: grape-seed proanthocyanidin extract (GSP) decrease the expression of cytokeratin-7 (CK-7), and reduce lipid peroxidation damage incotton seed oil and isolated gossypol induced kidney damage.

Keywords: Gossypol; Proanthocyanidin; Grape, kidney; Cotton seed oil and Wistar rats.

Introduction

Oxidative stress had been implicated in renal failure; inability of the kidney to remove metabolic end products from the blood and regulate the fluid electrolyte and pH balance of the extracellular fluid (Retting, 1996). The highly reactive oxygen intermediates could cause serious chemical damage to DNA integrity, proteins, and unsaturated lipids thereby resulting in cell injury and/or cell death. These reactive oxygen species intermediates have been implicated in several pathologic processes involving reperfusion injury, cancer, inflammatory disease, and aging. Cottonseed oil extracted from the seeds of cotton plants of various species, mainly used as cooking oil, cotton fiber for animal feed (Madhaven, 2001) salad oil, mayonnaise, and salad dressing (O'Brien *et. al.*, 2005 and Katgrada *et. al.*, 2010). Cotton seed oil contains gossypol, a yellow pigment classified as dimeric/bis-naphthaleneobtained from cotton seeds.

Gossypol remains the predominant active component in the cotton seed oil, while other polyphenolic pigments only present in trace

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component (Dare *et. al.*, 2021a).Gossypol pigments are present in two forms; a pigment bound in protein and another pigment relatively in free forms. The free form pigment generates free oxygen radicals and is implicated in cell death (Halliwell *et. al.*, 1992). It could initiate oxidative injuries in soft tissues (Welsch, 1995; Dare *et. al.*, 2021a), and could lead to cell death and altered metabolic activities of cells(Wang *et. al.*, 2013).

Hydrophobic degeneration of renal tubules in proximal convoluted tubules has been associated with animals treated with gossypol. Other degenerative features include, scattered tubules, which are dilated and lined by flattened epithelium instead of cuboidal cells and local absence of epithelial cells in some areas (Wang *et. al.*, 2013). Gossypol had been also reported to cause decrease in cellular level of GSH (glutathione peroxidase) and lactate dehydrogenase (Chenc *et. al.*, 2013 and Wang *et. al.*, 2013).

Antioxidants are free radical scavengers that can inhibit initiation, promotion and transformation of tumor in cells and protect cells against oxidative damage. The antioxidant substances neutralize the free oxygen radicals and protectcell membranes from injury and lipid peroxidation (Halliwell *et. al.*, 1992).Several plants have been reported to contain compounds including bioflavonoid and proanthocyanidins, ellipticine and taxol, indole derivatives, dithiolthiones, phytoestrogens that exhibit chemo-preventive or anticancer properties due tothe abilities to mop up free radicals (Dare *et. al.*, 2021b).Vegetables, fruits and seeds are the major reservoir of vitamins C and E, and β-carotene; these protect the cells against tumor as well. Grape seed proanthocyanidin possessed antimicrobial, antioxidant and anti-inflammatory constituents; that has very high amounts of disease-fighting capacities and free-radical eliminating phytonutrients called bioflavonoid (Chang, 1981). In addition to proanthocyanidin antioxidant constituents, grape seed extract shows vitamin C, sterols, tocopherols, citric acid, liminoids, and other trace minerals with anti-cancer potentials (Kaur *et. al.*, 2009). This research work aimed at investigating the protective effects of grape-seed proanthocyanidin extracts on the cotton seed oil and gossypol induced renal damage in adult wistar rats.

Material and Methods

Experimental Animal

A total of thirty (30) adult wistar rats (90-100g) were obtained from National Veterinary Research Institution (NVRI) Vom, Nigeria. The rats were kept in Bingham University Animal house to acclimatize for a period of four (4) weeks and attained weight of 170-240g. The animals were fed on starter mash (vital feeds grand cereals) and water was given ad libitum. The animal room was well ventilated with a temperature range of 25-27 degree Celsius under day/night 12 -24 hr.

Extraction of Grapeseed

12.5g of powdered seed was soaked in 70 % ethanol for 24hours. Filtration was done using funnel and filter paper; the collective filtrates were evaporated to dryness on a water bath. The extract obtained from above was subjected to further extraction by adding acetone water to remove the oil. The acetone water filtrate was again extracted using chloroform, the aqueous layer was then concentrated to dryness to obtain the proanthocyanidin extract.

Extract of cotton seed oil and gossypol

Cotton seed was obtained from Dengi market, Plateau state, Nigeria. The seeds were grinded to powder and the cotton seed oil was extracted using the solvent extraction method described by Chang *et. al.*, 1981.The sample collected was properly cleaned to remove foreign materials. Samples were oven dried in the laboratory at a temperature of 1300C, to a moisture content of 12%. This was done because the lesser the moisture content, the more the oil yield (Schneider, 2005, Taiwo *et. al.*, 2008). The seeds were then crushed into powder, and 12 g of the powder sample was mixed with 5 ml of N-hexane. The mixed sample was placed on a filter paper and the filter paper properly folded and inserted into the assembled Soxhlet apparatus. The weight of the filter paper and sample was recorded. One hundred and fifty milliliters (150ml) of the solvent (N- hexane) was measured and poured into a five hundred milliliters (500ml) round bottom flask which is the lower part of the Soxhlet apparatus. This was heated with a heating mantle at 60oC for 6 hours. As the solvent boiled, it evaporated into the reflux condenser and this hot solvent vapour was cooled by the surrounding water which flowed continuously through the Soxhlet arrangement. The cooled solvent then condensed back into the portion of the Soxhlet containing the folded sample, and this facilitated the extraction of the oil from the sample. The sample left after the oil had been removed was subjected to hot pressing using hydraulic press to remove the bulk of the oil remaining in the press cake.

Gossypol was extracted from the cotton seed oil using 70% cold acetone; Gossypol decompose poorly in acetone compared to methanol, chloroform, ethanol, and acetonitrile according to (Benbouza *et. al.*, 2002; Villacorta *et. al.*, 2003)

Mode of Administration:

Group A: (PBS-Control Group) received 0.1 ml of phosphate buffer solution (vehicle for the administration)

Group B: (Proanthocyanidin Control Group) received 15 mg/100gm BW of grape seed proanthocyanidin extract (Liu *et. al.*, 2020)

Group C: (Cotton seed extract group); received 15mg/100gm BW of cottonseed oil only(Akinola *et. al.* 2006)

Group D: (Isolated gossypol, ISG); received 15

mg/100gm BW of isolated gossypol only(Weinbauer *et. al*., 1983)

Group E: (Protection group: cottonseed + grape seed); received 15 mg/100gm BW of cottonseed oil + 15 mg/100gm BW of grape seed proanthocyanidin extract

Group F: (Protection group: Isolated gossypol + grape seed); received 15 mg/100gm BW of isolated gossypol + 15 mg/100gm BW of grape seed proanthocyanidin (Abdel Hafez *et. al*., 2017)

The administration was done daily for the period of eight (8) weeks; administration of the extract was done orally using oro-gastric cannula.

Animal Sacrifice

Animals were euthanized by cervical dislocation and kidney was excised following abdominal incision. Left kidney were fixed in formo-saline for histological analysis, while the right kidney was homogenized in 5% sucrose solution for enzyme assay.

Routine Histological Preparation

The left kidney were cut into slabs of about 0.5cm thick transverse sections and fixed in formol-saline. The fixed samples were allowed through graded alcohol for dehydration, andcleared in xylene. Infiltration was carriedout in two changes of molten paraffin wax for 1 hour each at 65oC, and subsequently embedded in molten paraffin wax. Serial sections were cut using rotatory microtome at six microns. The cut ribbon was flattened out in hot water and transferred onto albumenized slide. The slides were stained in haematoxylin and eosin for histological observations (Dare *et. al*., 2021b).

Immuno-histochemical Staining

Immunoperoxidase technique was performed using monoclonal mouse anti-body for CK7 (dilution 1:100 each). The positive controls consisted of samples previously shown to be positive to CK7. Trisbuffered saline in place of the primary antibody was used as a negative control. Cells were considered positive for CK7 when distinct cytoplasm and/or cell membrane yellow to brown staining was identified (Al-Maghrabi *et. al*., 2018).

Enzyme Histochemistry

Excised right kidney tissues were put in homogenizer with 5% sucrose which was added according to weight of organ and homogenized properly. Tissue homogenates were collected in sample bottle for enzyme assay; MDA was measuredas described by Zingg and Azzi (2004), and SOD activity was determined by the method of Misra and Fridovich (1972) as modified by Atikson and Epand (2008).

Statistical Analysis

The calculations were done using the Med Calc software package for analysis of the data. The data were presented as Mean± Standard Error of Mean. Means were compared using student's t test. Differences were considered to be of statistically significant at an error probability of less than 0.05 ($P<0.05$).

Results

Histological Findings

Both Control groups (group A &B) showed the same histological appearance of the renal cortex. The renal corpuscle exhibits the glomerular capillaries, parietal and visceral epithelium of the glomerular (Bowman's) capsule, and the capsular space. The capsular space is observed to be continuous with the lumen of the proximal convoluted tubule. The proximal convoluted tubules are lined with acidophilic pyramidal cells with distinct brush borders, and narrow lumen, the distal convoluted tubules DCT are seen lined with cubical cells with indistinct brush borders and wider lumen (figure 1).

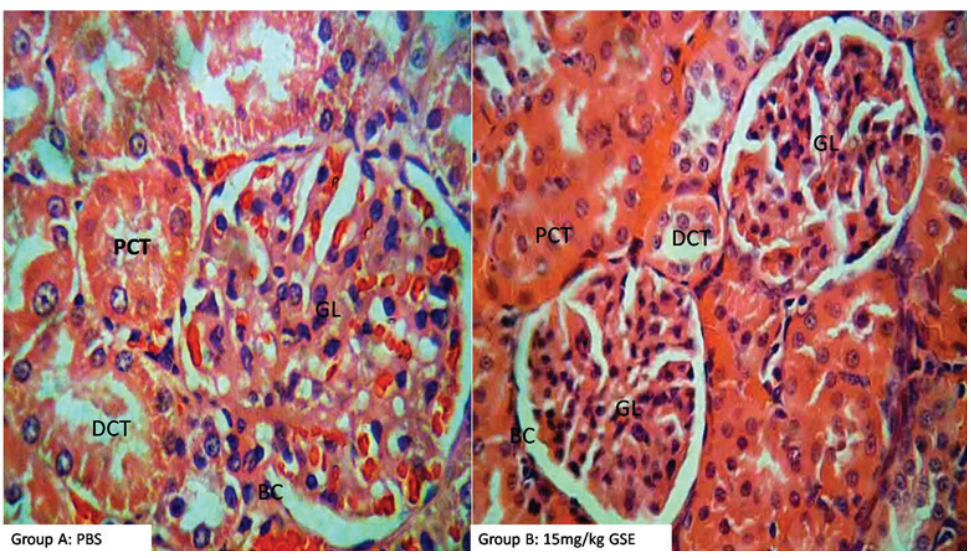

Figure 1. Photomicrograph of the renal cortex of wistar rat in control group A; showed expression of normal BC-bowman's capsule, DCT-Distal convoluted tubule, GL-Glumerulus and proximal convoluted tubule (PCT).Group B;Renal cortex of wistar rat in grape seed oil only, normal Bowman's capsule-BC, Distal convoluted tubule-DCT, Proximal convoluted tubule-PCTand GL- Glumerulus.H/E stain MgX400.

Animals of group C (exposed to CSO only) (fig. 2) showed vacuolation of the epithelial cells lining PCTs and flattening of the cells lining the DCTs (result of degeneration of the epithelia lining) and widening and distention of Bowman's capsule-BC. Animals of Group D, (isolated gossypol) revealed reduced Bowman's space, loss of the brush border of PCTs, flat epithelial lining and vacuolation of the cells lining the proximal and distal tubules with widened lumen of some of the tubules (figure 2).

Treatment with CSO and GSP (Group E) , showed improvement in the histology of the renal cortex as compared to groups C. The renal corpuscle, with their glomerular capillaries appeared similar to the control group with preserved Bowman's space. The cells of both proximal and distal tubules appear like those of the control group with preserved brush border. Treatment with Gossypol and GSP extract (Group F) showed preserved Bowman's space and cells of both proximal and distal tubules appear with mild and reduced cell loss and degeneration as compared to group D (Figures 3).

Cytokeratin (CK-7) expression in the control groups (groups A & B) showed No to little expression of CK-7 in the epithelial lining of the renal corpuscle (both along the glomerular capillaries and Bowman's capsule) and also along both PCTs & DCTs (fig 4).

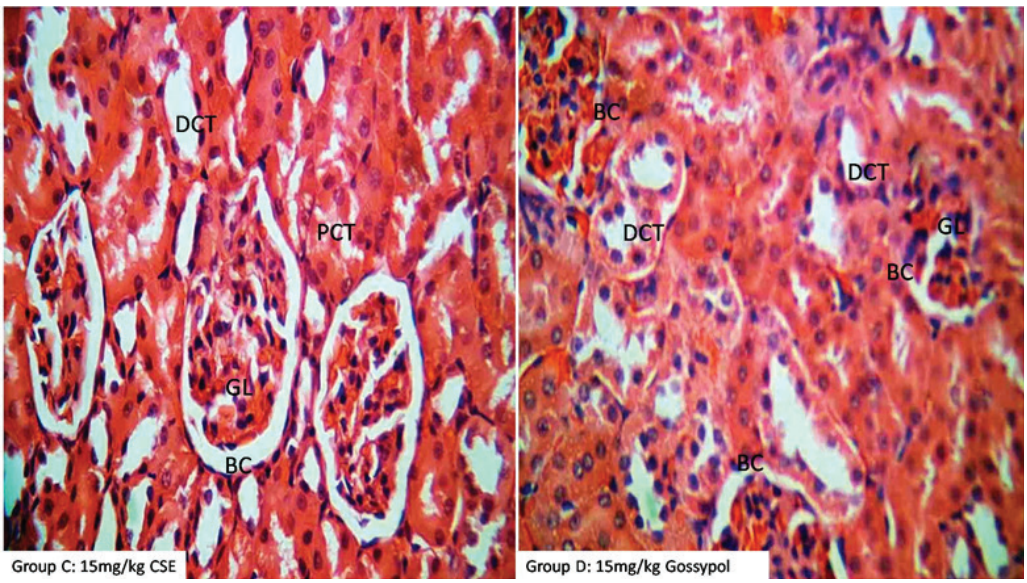

Figure 2. Photomicrograph of the renal cortex of wistar rat in Group C; cotton seed extracts treated group, and Group D; isolated gossypol treated group. Group C; Renal cortex of Wistar rat in cotton seed oil only showing vacuolation of the epithelial cells lining the proximal convoluted tubules (PCT) and Distal convoluted tubules (DCT) and distention of Bowman's capsule-BC. Group D; Renal Cortex of wistar rats in gossypol only, showing shrunken reduced glumerulus (gl) with narrow Bowman's space (BC), the cells lining the tubules are vacuolated and flattened with loss of brush border and widened lumen of some of the tubules (↑). H/E stain MgX400.

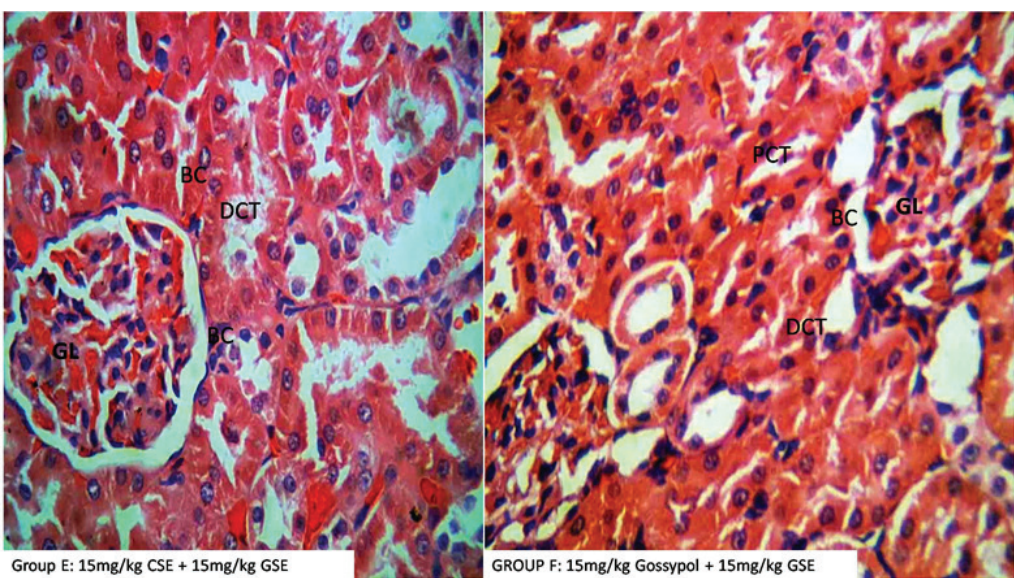

Figure 3. Renal Cortex of Wistar Rats in Group E; cotton seed oil treated with grape seed proanthocyanidin; the renal corpuscle, with their glumerulus capillaries appeared similar to the control group with preserved bowman's space. The cells of both proximal and distal tubules appear like those of the control group with preserved brush border. Group F; Renal cortex of Wistar rats in gossypol treated with grape seed proanthocyanidin; revealed BC (bowman's capsule), and DCT-distal convoluted, mild and reduced cellular loss as well as reduced vacuolation in Bowman's capsule-BC, Proximal convoluted tubule-PC,Distal convoluted tubule-DC.

While in groups C & D there is increased expression (moderate) of the CK-7 protein in the renal tubular lining (proximal and distal)(figure 5), as compared to the control groups (groups A & B).

Animals that were treated with grape-seed oil, (group E and F)showedno evidence of epithelia lining damage, as well as negative tomild expression of CK-7 in the renal tubular lining (proximal and distal) (figure 6).

Stress Maker Molecules Analysis

Expression of the activities of Malondiadehydes (MDA) revealed the level of lipid peroxidation and activities of Superoxide dismutase (SOD) antioxidant enzymes as shown in Table I.

Malondiadehydes (MDA) activities are significantly reduced in control group B (GSP treated animals); when compared with the normal control animals; group A.

Animals treated with gossypol (group D) revealed significant increased activities of the enzyme MDA compared to control group A, while group C (CSO treated) had non-significant increase than the control group A.

Animals of groups E (treated with GSP with CSO) had significantly lowered MDA activities compared with the animals that received only CSO(group C), while animals of group F that received GSP with gossypol

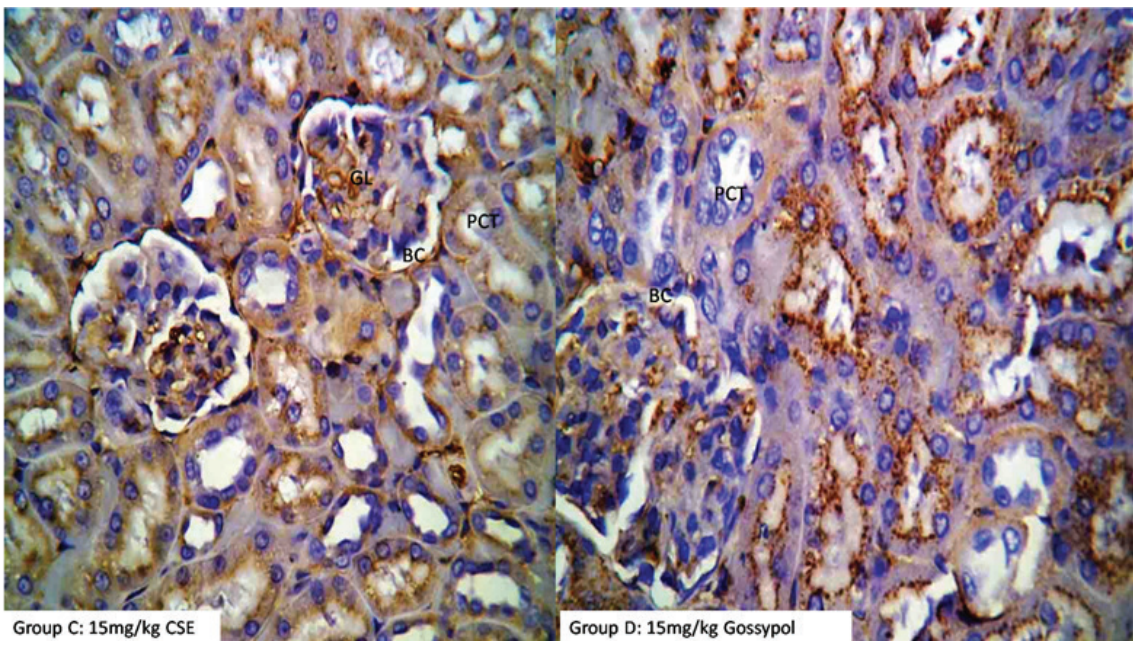

Figure 5. Photomicrograph of both renal cortex of wistar rats in Group C; cotton seed extracts treated group, and Group D; isolated gossypol treated group. Both groups showed increased (moderate) expression of the Cytokeratin-7 (CK-7) along the epithelia lining of some of the tubules.

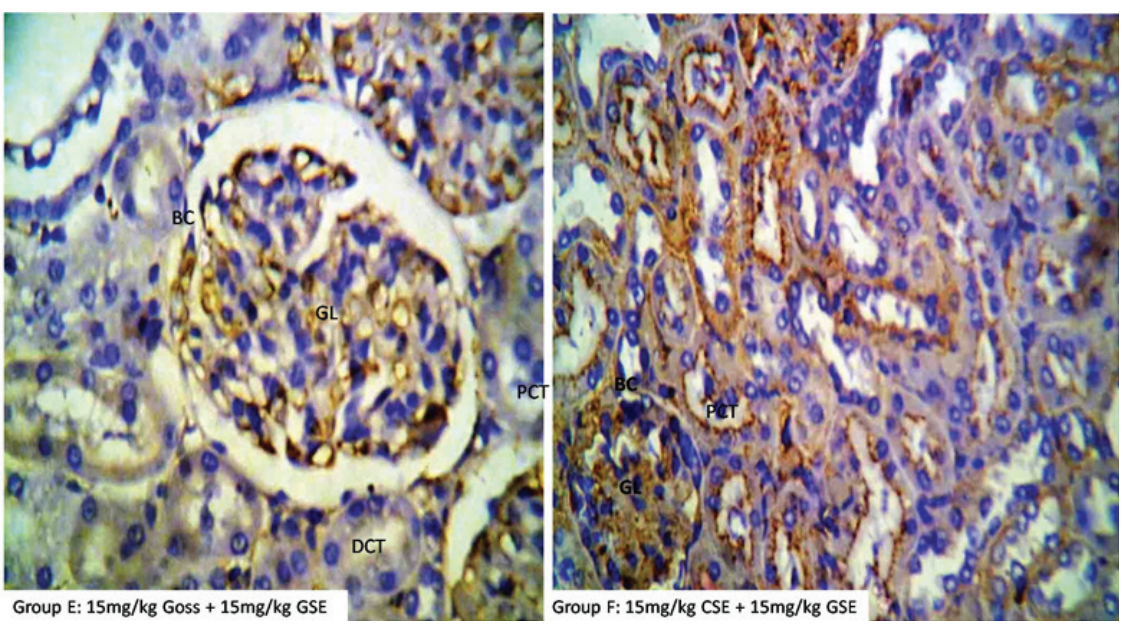

Figure 6. Revealed negative/mild expression of the molecular maker of epithelia tumour using Cytokeratin-7 (CK-7) in Group E and F; CK7 stain Mgx400.

Table 1. Biochemical assay for MDA and SOD activities.

GROUP	MDA(µmol/L X10-7)	SOD(IU/L X 10-7)
A	4.20±0.00	6.7±0.20
B	2.80±0.00*	1.3±8.30*
C	4.73±3.60	7.5±13.60
D	6.16±0.00*	8.0±3.00*
E	2.70±0.00β	4.6±10.40*
F	2.23±2.11€	1.8±0.00*

Values are presented in Mean± SEM
*Significant difference in mean at p< 0.05 than the control group A
βsignificant difference than the treated group C.
€ significant difference than the treated group D.

had significant decrease in MDA activity compared with group D (gossypol alone).

Similar trend of observation in SOD enzymes activities as revealed in table I. Administration of grape seed proanthocyanidin significant maintained the activities of SOD enzymes compared with the control animals. However, cotton seed oil administration and treatment with isolated gossypol significantly elevated the SOD activities in the kidney tissues compared with the control animals in group A. Grape seed proanthocyanidin significantly lowered the SOD enzymes activities in the kidney damage by the administration of cotton seed oil; group E and isolated gossypol; group F compared with the animals that received only cotton seed oil; group C and isolated gossypol; group D without combined treatment with grape seed proanthocyanidin.) SOD activities tend to increase during cellular injuries, increased SOD enzymes required for the first line of defense against oxygen free radicals.

Discussion

The present results confirmed that gossypol induced alterations in renal histology in the form reduced Bowman's space, degeneration and vacuolation of the lining epithelium of the convoluted tubules, and damaged brush border of the PCT with widening of the lumen of the tubules.

Similarly, Herrera and Barbas, (2001) observed morphological changes including; dilatation of the bowman's capsules, epithelial vacuolation and degeneration with calcification and dystrophy in the kidneys of rats treated with cotton seed oil.

Xue *et. al*., 1988 confirmed that gossypol inducesmild proximal tubule vacuolization and proteinuria by causing alteration in the glomerular permeability and damages the brush border membrane of proximal renal tubules and impairs tubular re-absorption function.

Similar results were explained by Song *et. al*., (2012) due to proteins that enter Bowman's capsule instead of being filtered out, due to lack of podocytes and mesangial cells- and due to accumulation of blood clot due to damage of capillaries.

The present study demonstrated that administration of grape seed oil maintained the histological architecture of the renal cortex and reduced the damage occurred as a result of gossypol administration. Animals treated with gossypol showed hydrophobic degeneration of renal tubules, however, treatment with grape seed showed restoration of the cells lining the tubules, so they appeared with mild vacuolation than Gossypol only group.

The results are consistent with the study of Abdel-Hafez *et. al*., 2017 who noticed the protective effects of grape seed oil on the renal cortex damaged by the administration of paracetamol.

Similarly, Ashtiyani *et. al*.,(2013) and Traber, (2011) reported that antioxidant acts as a peroxyl radical scavenger, preventing the propagation of free radicals in tissues. This conclusion suggests that grape seed proanthocyanidin extract act as a good free radical scavenger.

Malondialdehyde (MDA) is a reactive aldehyde; this aldehyde is used as a biomarker to measure level of oxidative stress in an organism. MDA results from lipid peroxidation of polyunsaturated species and the degree of lipid peroxidation product that accumulates in much of the pathophysiological process can be estimated by the amount of malondialdehyde in tissue.

Therefore, analysis of MDA in the present work revealed increased activities in gossypol and cotton seed oil treated animals. Increase in MDA activities causes increase in oxygen reactive species, like peroxide and superoxide, which goes into cytosol and into cell membrane and consequently indicating lipid peroxidation and causes cell injuries. However, treatment with grape seed showed significant decrease in lipid peroxidation level as indicated by the reduced activities of MDA relative to the groups treated with cotton seed oil and isolated gossypol respectively.

Superoxide dismutase (SOD) is an antioxidant enzyme that catalyzes the destruction of oxygen free radicals. It protects oxygen metabolizing cells against harmful effect of superoxide free radicals (Azzi, 2007).

(There was also significant increase in SOD activities in animals treated with cotton seed oil and isolated gossypol. This is because in a damaged cell antioxidant enzyme is decreased; it is overwhelmed by reactive oxygen species. Free radicals are generated normally but in low amount and are usually mopped out by antioxidant enzymes like catalase, glutathione, and superoxide dismutase, but when it increased oxidative stress occurs, it overwhelms antioxidant and their activity decreases (Coutinho, 2002). High natural proanthocyanidin present in grape-seed (hesperedin, tocopherol, vitamin C, citric acid and liminoids) has been reported to possess very potent antioxidant abilities as shown by Baiges *et. al*., 2010.

SOD is the first enzyme that combines with active

oxygen free radicals, specifically combines with superoxide anions and prevents cell membrane lipid peroxidation and damaging formation of metabolites (Dare *et. al.*, 2021b). Grape seed proanthocyanidin, are antioxidant that decrease superoxide dismutase by stimulating copper dismutase in the presence of bioflavonoid that causes stability of SOD.

Cytokeratin 7 (CK7) protein expression in the bowman's capsules, brush borders cells along the proximal convoluted tubules and distal convoluted tubules as well as in the tissue parenchymal marked the tumor effect and renal damages capacities of the administration of gossypol and cotton seed oil extracts. This agreed with the previous findings by Zuo *et. al.*, 2011, Song *et. al.*, 2012, Olaku *et. al.*, 2015, Gupta *et. al.*, 2020, and Barbe *et. al.*,2020.

Dare *et. al.*, 2021b noted Cytokeratin 7 (CK7) protein expression in the tumor hepatic cells and had proven Cytokeratin 7 (CK7) protein negatively expressed in normal epithelia while the epithelial tumors characterized by increase expression of Cytokeratin 7 (CK7).

However, administration of grape seed proanthocyanidin showed negative expression of CK7, preserving the epithelia lining integrity as agreed by Gorinstein *et. al.*, 2005 and Azzi, 2007, Athanazio *et. al.*, 2021, Oue *et. al.*, 2012, Xu *et. al.*, 2018 and Hrudka et. al., 2021

Conclusion

This study has established that gossypol induced renal toxicity; characterized by increased level of MDA activities, increased the SOD enzyme activities, increased expression of CK-7 protein and renal cortex vacuolation resulting from degeneration of the lining cells along tubules. Grape-seed proanthocyanidin extract regulate expression of CK-7, maintained histological architecture of the renal cortex and modulate the damages from free radicals substances by its antioxidant potentials.

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