EVALUATION OF PROTECTIVE POTENTIAL OF CITRUS AURANTIFOLIA (CHRISTM) SWINGLE PEEL EXTRACT IN ATTENUATING DOXORUBICIN-INDUCED HEPATOTOXICITY IN EXPERIMENTAL MODELS

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ABSTRACT

Citrus aurantifolia is a very common, and widely cultivated and consumed for its antioxidant properties due to its robust flavonoids content. Doxorubicin (DOX) is an antibiotic broadly used in the treatment of different types of solid tumours, but its use also comes at a cost, organ toxicity. The curative and preventive properties of *Citrus aurantifolia* peel extract (CAPE) in doxorubicin-induced hepatotoxicity in Wistar rats were evaluated. Thirty female rats were divided into six groups of five (5) rats each in both curative and preventive studies. In curative study, five groups of rats (II – VI) received Doxorubicin (mixed with normal saline, 15 mg/kg body weight i.p) on day one, 24 hours after, graded doses of CAPE and alpha-lipoic acid (A.L.A.) were administered to groups II- V and VI respectively for 7 consecutive days. For the prophylactic study, groups II – V and VI received graded doses of CAPE and A.L.A. respectively, 24 hours after Doxorubicin was administered to groups II-VI. Groups treated with D.W. and A.L.A. were used as a negative and positive control, respectively Liver enzymes such as A.S.T., A.L.T. and A.L.P., including liver samples, were examined for histopathological changes. A significant reduction ($p< 0.05$) in serum A.S.T. and A.L.T. levels was observed in animals treated with CAPE 200 and 400mg/kg in the preventive study, while in curative, a significant reduction in an expected rise in serum A.S.T., A.L.T. and A.L.P. ($p<0.05$) was observed in animals treated with CAPE 400mg/kg when compared to the group treated with DOX + distilled water. Hepatocellular necrosis was observed in the histology of DOX- distilled water treated group. Besides, the hepatocytes of groups treated with CAPE (200 and 400mg/kg) in this study showed narrow foci of mild vacuolar change as compared with groups treated with the lowest dose of CAPE (100mg.kg) and distilled water, which revealed random foci of mild vacuolar change. This study has provided information that DOX damaged liver tissue due to an increase in liver enzymes and histopathological results showing tissue damaged in groups treated with lower doses of CAPE and distilled water. This study further demonstrates that groups treated with CAPE 200, 400 mg/kg and A.L.A. protect hepatic damage induced by DOX.

Keywords: C*itrus aurantifolia,* Doxorubicin, Toxicity, Liver, Rat

Accepted Date: 12 June 2020

INTRODUCTION

DOX has been described as an anthracyclin egycoside antibiotic that acquires an effective and broad spectrum antitumor activity against a variety of human solid tumors like ovarians, breast, lungs, uterine and cervical cancers (Chang *et al.,* 2011). Previous research postulated that oxidative stress and reactive oxygen species (R.O.S.) were involved in the pathogenesis of DOX- induced hepatotoxicity. It has been recommended that DOX in the form of DOX semiquinone plays the main role in hepatotoxicity (Mohan *et al.,* 2010). Chemotherapeutic drugs used to treat cancer are given to most of the people, which help to sustain them until when the cancer treatment is completed (El-Sayyad *et al.,* 2009) . *Citrus aurantifolia* (Christm) Swingle (Family Rutaceae) fruit peels have been found very useful domestically. In the kitchen, it is used for cooking to add flavor to food, cakes and roasted chicken. It is used to garnish salad and to add flavor to drinking water. It can also

be used for bathing and washing hair, and as a deodorizer to freshen up smelly garbage and composite pile (Nwachukwu *et al.,* 2007). It serves as a natural room freshener and has been found useful as a repellant against mosquitoes from the body, moths from cloths and cats from gardens (Effiom *et al.,* 2012). Citrus fruit peels have been transformed into volatile gases through a high-powered microwave. These gasses are then distilled into liquid that is used for making plastics. The pulp of citrus fruit peels is used as cereal substitute in ruminant feeds due to its high energy content and good digestibility in ruminant species (Nwachukwu *et al.,* 2007).

Citrus aurantifolia in its natural state is widely used in West Africa, particularly in Nigeria where it is employed in herbal medicine to treat several illnesses. It forms an essential ingredient in the preparation of most herbal concoctions. Different parts of the tree have been used traditionally to cure some illness (Effiom *et al.,* 2012). In Nigeria, C*itrus aurantifolia* juice is added to sugar and palm oil or honey to relief cough. The diluted form of the C*itrus aurantifolia* fruit juice is used for mouth wash to treat sore mouth and sore throat. The juice has been found useful to treat irritation, diarrhea and swelling due to mosquito bite and sometimes mixed with oil and used as vermifuge and also incorporated into weight management diet (Yano *et al.,*1999). Liver toxicity has been an inherent problem for people taking anticancer medications and has not been resolved till date. It is critical to introduce new drugs to cure or prevent the hepatotoxic effects of anticancer medications.

Several mechanisms are responsible for either inducing hepatic injury or worsening the damage process, certain chemicals damage mitochondria, releases excessive amount of oxidants that, in turn, injure hepatic cells and activation of some enzymes in the cytochrome p-450 system such as CYP2E1 also leads to oxidative stress (Jaeschke *et al.,* 2002). Research has shown that *Citrus aurantifolia* peel has high level of antioxidant properties (Boshtam *et al.,* 2011).These prompted the scientific investigation of the hepatoprotective potentials of CAPE to determine if better results will be obtained when compared to that of presently available hepatoprotective drugs.

MATERIALS AND METHODS

Plant Collection and Identification

Citrus aurantifolia fruits were collected in Ibadan, Southwest of Oyo State, Nigeria. The plant was identified at the Herbarium, Department of Botany, University of Ibadan, Ibadan by a taxonomist, D.P.O Esimekhuai in February, 2018. A sample of the plant was deposited in the same Herbarium and given a voucher number of UIH-22957

Experimental Animals

Sixty female Wistar rats weighing 140 to 150 g were obtained from the Animal House Facility of the Institute for Advance Medical Research and Training (IAMRAT), College of Medicine, University of Ibadan, The animals were maintained on standard animal feed and tap water ad libitum, and allowed to adapt to the laboratory environment for two weeks in order to acclimatize. All animal experimentation was carried out according to the guidelines of Institutional Animal Ethics Committees. (IAEC)

Drugs, Chemicals and Reagents

Enzopak biochemical kits (Reckon Diagnostics P. L.T.D., India), alpha lipoic acid capsules (UniPharma, Cairo Egypt), doxorubicin (Bhoomi marketing CO. India), Folin-Ciocalteu reagent (B.D.H. chemicals, U.S.A.) and methanol (Sigma Chemical Co. U.S.A.) were used. Drugs and Chemicals Formalin, ethylene diamine tetra acetic acid (EDTA) and sodium dihydrogen phosphate were purchased from Sigma (St. Louis, MO, U.S.A.).

Laboratory Methods

Preparation of plant material

The whole peels of Citrus aurantifolia were collected and shade dried at room temperature (27 – 30ºC) to constant weight over a period of 7 days. Dried whole peels were coarsely powdered using a mortar and pestle and further pulverized using an electric blender.

Extraction of Citrus aurantifolia peel material

The pulverized plant materials were separately extracted with aqueous methanol (80%v/v) for 72 hours at room temperature by maceration. The residues were filtered and concentrated over a water bath at 60°C. The solutions of extracts were freshly prepared for each study.

Experimental Design

Prophylactic Study

Thirty female rats were divided into six (6) groups (n=5). Distilled water only, Distilled water and CAPE (100, 200, 400 mg/kg) were administered orally respectively to all the groups for seven (7) days while and Alpha lipoic acid (150 mg/kg) was given to only group VI also for seven days. On day 8, group II, III, IV V, and VI received Doxorubicin (mixed with normal saline, 15 mg/kg p.o.)

Group I = Distilled water (only)
Group II = Distilled water + DOX 15mg/kg)
Group III = CAPE 100mg + DOX 15mg/kg
Group IV = CAPE 200mg + DOX 15mg/kg
Group V = CAPE 400mg + DOX 15mg/kg
Group VI = Alpha lipoic acid (150mg/kg) + DOX 15mg/kg

Curative Study

Thirty female rats were divided into six (6) groups (n=5).Distilled water, Doxorubicin (mixed with normal saline (normal saline is used because it is a physiological solution which acts like a buffer solution that prevents dissociation or ionization of Doxorubicin), 15 mg/kg p.o.) were administered to rats in groups II, III, IV and V, group VI. Twenty four (24) hours after, groups I to VI received Distilled water, CAPE (100, 200, 400 mg/kg) and Alpha lipoic acid (150 mg/kg) for a period of seven (7) days respectively.

Group I = Distilled water (only)
Group II = DOX 15mg/kg + Distilled water
Group III = DOX 15mg/kg + CAPE 100mg
Group IV = DOX 15mg/kg + CAPE 200mg
Group V = DOX 15mg/kg + CAPE 400mg
Group VI = DOX 15mg/kg + Alpha lipoic acid (150mg/kg)

Twenty four (24) hours after Doxorubicin and CAPE administration in prophylaxis and curative studies respectively, all animals were anesthetized. Blood samples were collected from rats of all groups on the ninth day of the experiment, into heparinized tubes for spectrophotometrical analyses of A.S.T. and A.L.T. as described by Bergmeyeret al. (1985), A.L.P. as described by Babson et al. (1962). Livers removed were blotted of blood and preserved in 10% buffered formalin (pH 7.2) and dehydrated through a series of ethanol solutions, embedded in paraffin and routinely processed for histological analysis. Sections of 2 µm thickness were cut and stained with haematoxylin-eosin for examination. The stained tissues were observed through an Olympus microscope (BX-51) and photographed by a chare-couple device (C.C.D.) camera. Histopathological analysis was done on the fixed livers.

Statistical Analysis:

All experimental results were expressed as Mean ± S.E.M. Data was analyzed using one way analysis of variance (ANOVA) and Student t-test. The values of $p<0.05$ were considered statistically significant.

Results

(a) Prophylactic results

A significant reduction ($p< 0.05$) in serum A.S.T. and A.L.T. was observed in animals pretreated with CAPE (200mg/kg and 400mg/kg) when compared to the group treated with DOX + distilled water (Table 1). A.L.T. and A.L.P. in serum increased significantly ($p< 0.05$) in group treated with distilled water compared to group administered with distilled water only. Photomicrograph of the liver for prophylactic results showed that the hepatic plates are closely-packed. There are locally extensive foci of mild vacuolar change of hepatocytes with random foci of single-cell hepatocellular necrosis (arrows) (Figures 1 and 2). Figure 3 shows random foci of hepatocellular necrosis (black arrow). Furthermore, there are a few foci of mild thinning of hepatic cords (yellow arrow) with scanty foci of mild vacuolar change of hepatocytes (black arrow) (Figure 4). Also, the result of the liver shows that there are a few foci of mild thinning of hepatic cords (black arrows) with scanty foci of mild vacuolar change of hepatocytes (arrow heads) as shown in Figure 5 while there is widespread mild thinning of hepatic cords (black arrow) with scanty foci of single-cell hepatocellular necrosis (arrow heads) (Figure 6)

(b) Curative results

Doxorubicin administered rats treated with distilled water showed raised serum activities of A.S.T., A.L.T. and A.L.P. levels when compared to the group taking distilled water only as shown in Table 2. A significant reduction ($p< 0.05$) in serum A.S.T., A.L.T. and A.L.P. levels was observed in animals treated with graded doses of CAPE(100mg/kg, 200mg/kg and 400mg/kg) when compared to the group taking DOX + distilled water.

For curative results, the photomicrograph of the liver for prophylactic results showed that the hepatic plates of the liver are closely-packed with normal general appearance of the Hepatocytes including mild K.C.H. (Figure7). Hepatic plates are closely-packed while there are locally extensive foci of mild vacuolar change of hepatocytes with random foci of single-cell hepatocellular necrosis (arrows) (Figure 8). In Figure 9, there is widespread marked vacuolar change of hepatocytes (yellow arrows). There are random foci of single-cell hepatocellular necrosis (black arrows). Furthermore, the liver shows that the hepatic cords are closely-packed with scanty foci of single-cell hepatocellular necrosis (black arrows) as shown in Figure10 while in Figure 11, there are multiple foci of mild thinning of hepatic cords (yellow arrows) with resultant dilated sinusoids with moderate foci of single-cell hepatocellular necrosis (black arrows). Finally, on the liver there are a few foci of mild thinning of hepatic cords (yellow arrows) as shown in Figure12.

Table 1: - Prophylactic effects of *Citrus aurantifolia* on liver biomarkers in Doxorubicin induced hepatotoxicity in Wistar rat

Groups	A.S.T. **(U/L)**	A.L.T. **(U/L)**	A.L.P. **(U/L)**
DW	25.45±1.95	42.85±5.45	46.95±2.05
DW + DOX	97.40±1.58	227.8.5[b]	106.23±9.45[b]
CAPE 100 mg/kg + DOX	60.47±16.22	116.20±4.16	75.95±5.05
CAPE 200 mg/kg + DOX	41.35±3.65[a]	84.17±4.13[a]	63.4±3.29
CAPE 400 mg/kg +DOX	38.50±2.04[a]	71.60±12.7[a]	68.70±1.80
α-Lipoic acid +DOX	35.23±1.54[a]	111.50±4.44	73.40±5.50

a= $p<0.05$ significant against DW + DOX
b = $p<0.05$ significant against DW

Table: 2 - Curative effects of *Citrus aurantifolia* on liver biomarkers in Doxorubicin induced hepatotoxicity in Wistarrat

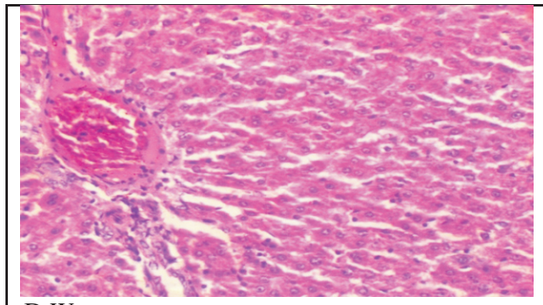
Groups	A.S.T.**(U/L)**	A.L.T.**(U/L)**	A.L.P.**(U/L)**
DW	35.45±1.95	21.85±5.45	16.95±2.05
DOX + DW	157.40±1.58[b]	97.80±23.50[b]	80.23±9.45[b]
DOX + CAPE 100 mg/kg	107.20±1.70	67.75±1.75	60.33±7.90
DOX + CAPE 200 mg/kg	78.20±8.90[a]	40.85±7.35[a]	50.95±6.45
DOX + CAPE 400 mg/kg	71.97±1.47[a]	29.77±1.79[a]	29.43±1.23[a]
DOX +α-Lipoic acid	85.47±1.89[a]	28.90±5.17[a]	37.57±13.8[a]

a= $p<0.05$ significant against DOX + DW
b = $p<0.05$ significant against DW

+α-Lipoic acid: Alpha-lipoic acid wasused because it has the potential to act as either the lipid and water-soluble phase in the cell unlike ascorbic acid which just acts on the water-soluble phase in the cell

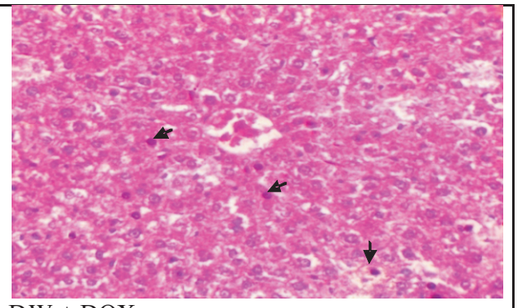
Histopathology Results

Prophylactic group (H**aematoxylin and Eosin stain (H&E)**



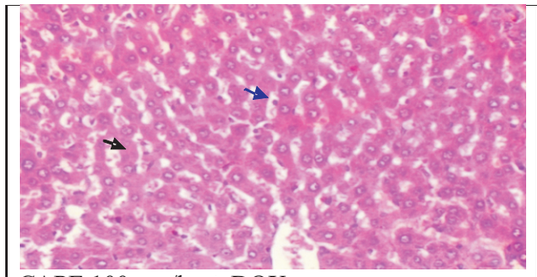
D.W.

Figure 1: Photomicrographs of the liver shows that the Hepatic plates are closely-packed. Hepatocytes generally appear normal. There is mild KCH.(H & E) X 400



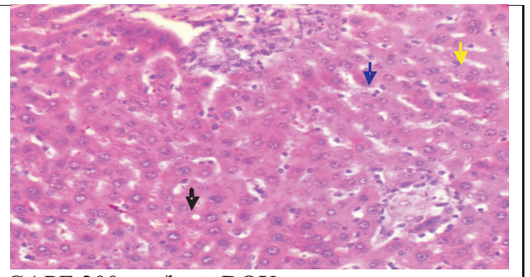
DW + DOX

Figure 2; Photomicrograph of the liver shows that the Hepatic plates are closely-packed. There are locally extensive foci of mild vacuolar change of hepatocytes. There are random foci of single-cell hepatocellular necrosis (arrows). There is mild K.C.H. (Haematoxylin and Eosin stain (H & E) X 400



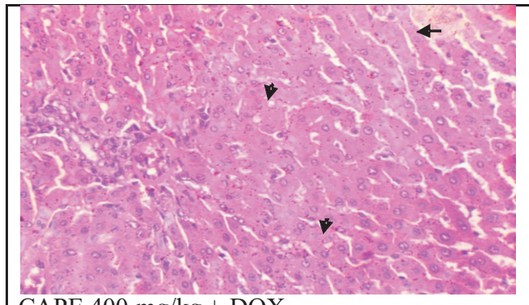
CAPE 100 mg/kg + DOX

Figure 3: Photomicrograph of the liver shows that the hepatic cords are tightly packed. There are random foci of hepatocellular necrosis (black arrow). There is moderate K.C.H. (blue arrow). (H & E) X 400



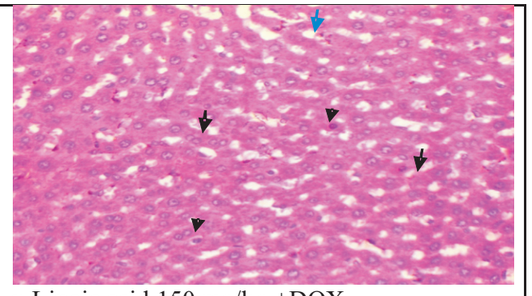
CAPE 200 mg/kg + DOX

Figure 4: Photomicrographs of the liver shows that there are a few foci of mild thinning of hepatic cords (yellow arrow). There are narrow foci of mild vacuolar change of hepatocytes (black arrow). There is mild K.C.H. (blue arrow). (Haematoxylin and Eosin stain (H & E) X 400



CAPE 400 mg/kg + DOX

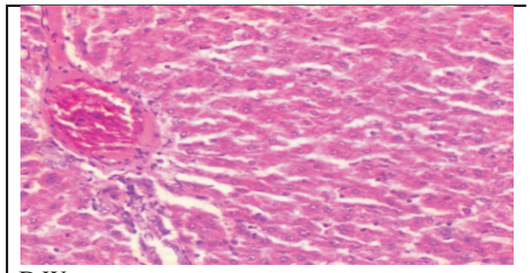
Figure 5: Photomicrograph of the liver shows that there are a few foci of mild thinning of hepatic cords (black arrows). There are narrow foci of mild vacuolar change of hepatocytes (arrow heads). There is mild K.C.H. (Haematoxylin and Eosin stain (H & E) X 400



α-Lipoic acid 150 mg/kg +DOX

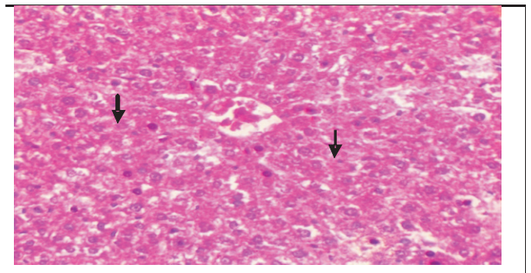
Figure 6: Photomicrographs of the liver shows that There is widespread mild thinning of hepatic cords (black arrow). There are scanty foci of single-cell hepatocellular necrosis (arrow heads). There is moderate K.C.H. (blue arrows) (Haematoxylin and Eosin stain.
(H & E) X 400

Curative group



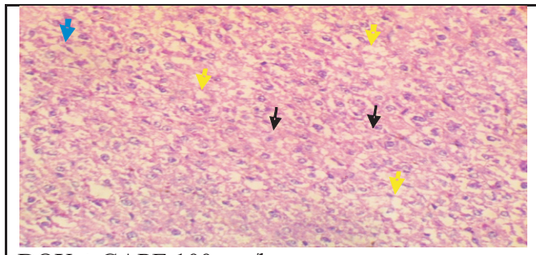
D.W.

Figure 7: Photomicrographs of the liver shows that the Hepatic plates are closely-packed. Hepatocytes generally appear normal. There is mild K.C.H. (Haematoxylin and Eosin stain (H & E) X 400



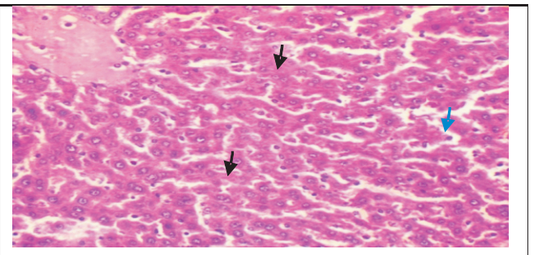
DOX + DW

Figure 8: Photomicrograph of the liver shows that the Hepatic plates are closely-packed. There are locally extensive foci of mild vacuolar change of hepatocytes. There are random foci of single-cell hepatocellular necrosis (arrows). There is mild K.C.H. (Haematoxylin and Eosin stain (H & E) X 400



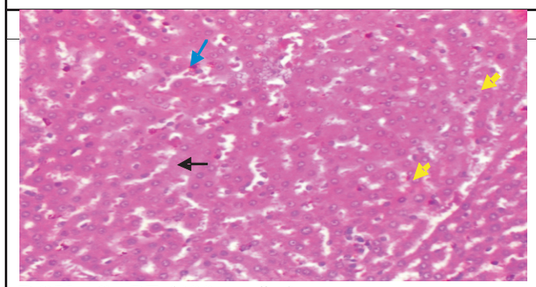
DOX + CAPE 100 mg/kg

Figure 9: Photomicrographs of the liver shows that the Hepatic plates are closely-packed. There is widespread marked vacuolar change of hepatocytes (yellow arrows). There are random foci of single-cell hepatocellular necrosis (black arrows). There is mild K.C.H. (blue arrows) (Haematoxylin and Eosin stain.(H & E) X 400



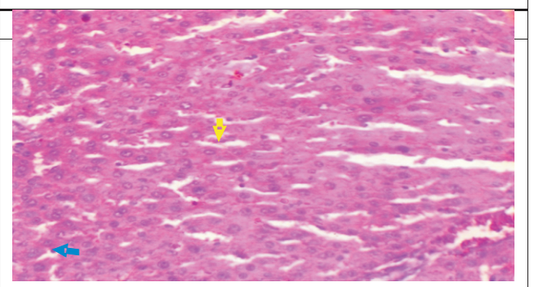
DOX + CAPE 200 mg/kg

Figure 10: Photomicrographs of the liver shows that the Hepatic cords are closely-packed. There are scanty foci of single-cell hepatocellular necrosis (black arrows). There is moderate K.C.H. (blue arrow). (Haematoxylin and Eosin stain (H & E) X 400



DOX +CAPE (400 mg/kg)

Figure 11: Photomicrograph of the liver shows that There are multiple foci of mild thinning of hepatic cords (yellow arrows) with resultant dilated sinusoids. There are moderate foci of single-cell hepatocellular necrosis (black arrows). There is moderate K.C.H. with Kupffer cells containing dark brown pigments (blue arrows) (Haematoxylin and Eosin stain.(H & E) X 400



DOX + Alphslipoicscid

Figure 12: Photomicrographs of the liver shows that There are a few foci of mild thinning of hepatic cords (yellow arrows). There is mild K.C.H. (blue arrow). (Haematoxylin and Eosin stain (H & E) X 400

DISCUSSION

This study found that the DOX (15 mg/kg body weight i.p. single dose) induced pathological changes in the liver tissues and increase serum transaminases enzymes in groups treated with distilled water. This result is in concordance with what has been previously reported by Liu *et al*. (2007). According to Mohan *et al*. (2010), serum transaminases have long been used as sensitive markers of liver damage. Membrane permeability and transport function are altered by injured hepatocytes, which lead to the leakage of enzymes from the cells that cause reduction in the levels of A.L.T., A.S.T. and A.L.P. in serum Mohan *et al*., 2010). Varieties of drugs used for cancer chemotherapy are known to produce toxic side effects in multiple organ systems including the liver. Doxorubicin (DOX) has long been one of the most extensively used chemotherapeutic agents for treatment of various cancers (El-Sayyad *et al.,* 2009). The clinical application of this drug is, however, complicated by its potential toxicity to

the liver (Sundaram & Sangavai, 2009). The DOX has been found to generate free radicals either by the enzymatic pathway of redox cycling between a semiquinone form and a quinone form or by the non-enzymatic pathway of forming a DOX-Fe3+ complex (Liu *et al*., 2007) .The increase in serum A.S.T., A.L.T. and A.L.P. levels in curative study suggests an increased leakage of these enzymes from mitochondria as a result of toxicity induced by DOX (Girish *et al*., 2009). Administrations of graded doses *Citrus aurantifolia* peel extract (100, 200 and 400 mg/kg/day, p. o. for 7 days) in DOX-induced hepatotoxicity have prevented increased serum marker enzymes A.S.T., A.L.T. and A.L.P. level, which may be related to its antioxidant and scavenging activities against free radicals (Baratto *et al*., 2003; Kumaran *et al*., 2007). This is in agreement with similar study investigated on serum marker enzymes in relation to antioxidant and scavenging activities against free radicals (Thabrew & Joice, 1987). The prophylactic results of the present study showed DOX-induced elevation in serum A.S.T. and A.L.T. levels are significant in the groups treated with distilled water and CAPE 100mg/kg, which has been attributed to the damaged structural integrity of the liver, because these enzymes are normally located in the cytoplasm of hepatocytes and are released into circulation after cellular damage (El-Sayyad *et al.,* 2009; Ojokuku *et al*., 2017)**.** The significant decreased concentrations of liver enzymes (A.S.T. and A.L.T.) as a result of CAPE administered at 200 and 400mg/kg, which might have been due to prophylactic support of hepatocytes, antioxidant and membrane stabilizing activities of *Citrus aurantifolia* peel extract at the two doses (in the testing groups) as described by Ojokuku *et al.* (2017). Plasma A.L.P. is also related to the status and function of hepatic cells. Increase in serum A.L.P. indicates increased biliary obstruction (Willianson *et al.,* 1996). This study further indicates that *CAPE* plant extract at the three doses (in the testing groups) slightly decreases plasma A.L.P. levels when compared to DOX-induced group (without extract pretreatment). The histology of liver tissues in control groups showed normal architectural examination, whereas random foci of single-cell and hepatocellular necrosis were observed in groups treated with distilled water and CAPE 100mg/kg. The groups treated with CAPE 200 and 400mg/kg revealed scanty and moderate foci of single-cell hepatocellular necrosis, the histology results obtained from this study was similar work done by El-Sayyad., *et al*. (2009) .

CONCLUSION

This study provides firm evidence that Doxorubicin can adversely damage the Liver tissue by inducing significant biochemical and histopathological changes. The results of this study showed that test extract was found effective as hepatoprotective agents, as evidenced by liver biochemical parameters and histological examinations. This indicated that *CAPE* can cure and prevent the liver against DOX-induced and the hepatoprotective effects might be correlated with its antioxidant and free radical scavenger effects.. Therefore the results from this study raise the hope that co-administration of *Citrus aurantifolia* peel extract and Doxorubicin may be a promising solution to complication of Doxorubicin induced hepatotoxicity.

Further Research

A broad-based research involving more subjects should be carried out to further evaluate the potential capability of the *Citrus aurantifolia* peel extractin attenuating doxorubicin-induced hepatotoxicity in higher experimental animal models in order to provide public health advantage.

Acknowledgments

The authors wish to express their gratitude to the management and staff of the Institute for Advanced Medical Research & Training (IAMRAT), College of Medicine, University College Hospital, Ibadan, for providing support and assess to research facilities and Institutional Animal Ethics Committees (IAEC) for granting the approval for the study.

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