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Ameliorative Effects of the Methanol Extract of *Phyllantus amarus* on Cyclophosphamide-Induced Toxicity in Mice

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Abstract: ***The liver performs some roles in carbohydrate metabolism, protein metabolism which includes the synthesis as well as degradation; production of clotting factors, as well as red blood cell production. It is part of the mononuclear phagocyte system which contains high numbers of Kupffer cells that are involved in immune activity. When toxicity sets in the aforementioned becomes alerted and ultimately affects physiological functions leading to various diseases. About 300g of Phyllantus amarus was collected from Karu, Nasarawa State, Nigeria. The leaves were air dried under shed before homogenized to fine powder using Laboratory Hammer mill. Extraction was done using 100% methanol and the extract obtained using rotatory evaporator. The animals were grouped into 6 with each group having 6 albino mice. Group 1 served as normal control received distill water orally for 14 days, Group 2 received cyclophosphamide 250mg/kg ip only, Group 3 received cyclophosphamide 250mg/kg ip before Sylimarin 100mg/kg after 24h, Group 4 received Cyclophoshamide 250mg/kg ip before MPAM 100mg/kg after 24h, Group 5 received cyclophosphamide 250mg/kg ip before MPAM 300mg/kg after 24h and group 6 received cyclophosphamide 250mg/kg before MPAM 900mg/kg after 24h for 14 days. On day 14, animals were sacrificed and blood samples were collected from the heart in to plained bottles for the determination of biochemical parameters. The methanol extract of Phyllantus amarus at 100mg/kg and 300mg/kg were able to decrease (P<0.01) the level of CRT, 300mg/kg only reduced (P<0.05) the level of ALP, 100mg/kg and 300mg/kg decreased (P<0.05) AST level when compared to the Cyclophosphamide group. The 900mg/kg showed increase in the level of all the biomarkers and there was no significance difference in the T. Pro when compared to the toxic group (Fig 2). There was no significance difference in the level of the biomarkers in both 100mg/kg and 300mg/kg when compared with the Sylimarin group except with AST at 300mg/kg where there was increase P (<0.01) while at 900mg/kg there was increase (P<0.0010) in the level of T. Bil, D. Bil, CRT, ALT.***

Keywords: Phyllantus amarus, Cyclophosphamide, toxicity, mice, methanol extract

1.Introduction

The various functions of the liver are carried out by hepatocytes which are responsible for up to 500 separate functions, mostly in combination with other systems and organs (Saxena *et al.*, 2016. Currently, no artificial organ or device can perform all the functions of the liver. Though some functions can be carried out by liver dialysis which is an experimental treatment for liver failure (Hoekstra *et al.*, 2013). The liver also accounts for about 20% of resting total body oxygen consumption (Schweitzer *et al.*, 2015).

The liver performs some roles in carbohydrate metabolism, protein metabolism which includes the synthesis as well as degradation; production of clotting factors, as well as red blood cell production. It is part of the mononuclear phagocyte system which contains high numbers of Kupffer cells that are involved in immune activity. Bile helps to digest and absorb fats, cholesterol and some vitamins.

Cyclophosphamide is an orally active alkylating agent, used to treat a variety of malignant and nonmalignant disorders (Davis and Reimold, 2017). Although it has some tumour selectivity, it also possesses a wide spectrum of toxicities. The requirement of metabolic activation before cyclophosphamide exerts either its therapeutic or toxic effects is well established, but has not led to effective countermeasures (DeVrieze and Hurley, 2018). Clinically, damage to the bladder (haemorrhagic cystitis), immunosuppression and alopecia are the most significant toxicities associated with cyclophosphamide (Robinson *et al.*, 2016). Its use as antineoplastic drug causes toxicity of normal cells due to its toxic metabolites.

CYP is activated by the hepatic microsomal cytochrome P450 mixed functional oxidase system, yielding phosphoramide mustard and acrolein. Acrolein inhibits P-450 by alkylating the sulfhydryl groups (Singh *et al.*, 2016). Acrolein is metabolized principally via rapid modification of glutathione sulfhydryl groups (GSG), forming mercapturic acid that is eliminated in the urine. Through this mechanism, acrolein directly enhances cellular oxidative stress by reducing the levels of glutathione (Monach *et al.*, 2010).

Acrolein and its glutathione adduct (glutathionylpropionaldehyde) are acted upon by xanthine oxidase and aldehyde dehydrogenase. Xanthine oxidase oxidizes acrolein to the acroleinyl radical and O2−. Aldehyde dehydrogenase metabolizes acrolein, with the formation of O2−, but does not act on the acrolein radical. The oxygen radicals produced may play roles in the induction of lipid peroxidation by acrolein (Fuchs, 2015). Glutathionylpropionaldehyde is a toxic metabolite of acrolein and may play a role in the in vivo toxicity of acrolein (Krobichler *et al.*, 2015). Numerous studies have shown that CP has toxic effects on the liver (Huyan *et al.*, 2011).

In the body, molecules termed antioxidants can prevent the damage caused by free radicals. These antioxidants are classified into three groups: antioxidant enzymes, chain-breaking antioxidants, and transition metal-binding proteins.

Vitamins E and C and beta-carotene are enzymatic, non-coupling antioxidants. They block cascade reactions triggered by free radicals by hydrogenating the free radical molecules. The combined use of cytotoxic agents and an antioxidant does not suppress the anti-tumor effects of the cytotoxic agents (Intarauchikul *et al.*, 2019). It induces nephrotoxicity that limits its use (Giraud 2010).

2.Material and Methods

Plant material

About 300g of *Phyllantus amarus* was collected from Karu, Nasarawa State, Nigeria. The leaves were air dried under shed before homogenized to fine powder using Laboratory Hammer mill (Zhen Chang Equipment SFS P66). Extraction was done using 100% methanol and the extract obtained using rotatory evaporator.

Pilot study

This was done using 2000mg/kg of the methanol extract using Lorke 1983 method and animals were observed for changes in behavior and mortality for 4h, 24 and a week.

Experimental Animals and their care

A total of 36 Swiss albino mice of both sexes weighing 19g-24g were obtained from Animal facility center of Department of Pharmacology and Toxicology, NIPRD, Abuja. The animals were handled in accordance with international principles guiding the use and Handling of experimental animals (United States National Institutes for animal health) after ethical approval was obtained from the Animal Care and Use Research Ethics Committee (ACUREC) of the University of Ibadan with number UI-ACUREC/190121. The rats were fed with standard rat feed (Vital Feeds from Grand Cereals Limited, Jos (a subsidiary of UAC Nigeria) during the period of acclimatization and throughout the period of the experiment. The rats were also maintained at an ambient temperature between 28-30^{o}C, humidity of 55±5%, and standard natural photoperiod of approximately 12h of lighting (06: 30h-18: 30h) alternating with approximately 12h of darkness (18: 30-06: 30h)

Cyclophoshaminde induced toxicity in animals

The animals were grouped into 6 with each group having 6 albino mice. Group 1 served as normal control received distill water orally for 14 days, Group 2 received cyclophosphamide 250mg/kg ip only, Group 3 received cyclophosphamide 250mg/kg ip before Sylimarin 100mg/kg after 24h, Group 4 received Cyclophoshamide 250mg/kg ip before MPAM 100mg/kg after 24h, Group 5 received cyclophosphamide 250mg/kg ip before MPAM 300mg/kg after 24h and group 6 received cyclophosphamide 250mg/kg before MPAM 900mg/kg after 24h for 14 days.

Biochemical Analysis

On day 14, animals were sacrificed using chloroform inhalation and blood samples were collected from the heart in to plained bottles for the determination of biochemical parameters such as Alkaline phosphatase (ALP), Aspartate transaminase (AST), Alanine transaminase (ALT), Blood urea (BUN), Creatinine (CRT), Total bilirubin (T. bil) and Total protein (T. Pro). well labeled eppendurff bottles were used to collect the blood samples, allowed for 4 hours before centrifuging using Uniscope Laboratory Centrifuge (Model SM 112, Surgifreind Medicals, England) at 2000 revolution per minute for 15 minutes to separate the sera from clotted blood cells. Each serum was carefully seperated in the eppendurff bottles that were well label accordingly at room temperature of 23-26^{0}C. The activies of AST, ALP, and ALT were estimated as described by Reitman and Frankel (1957). The activites of T. bil were determined by colorimetric method using a kit supplied by Randox test kit. The blood urea was determined using urease-Berthelot (enzymatic) colorimetric method and serum total protien was evaluated based on the Gornall *et al., (*1949) method.

Gross and Histopathology

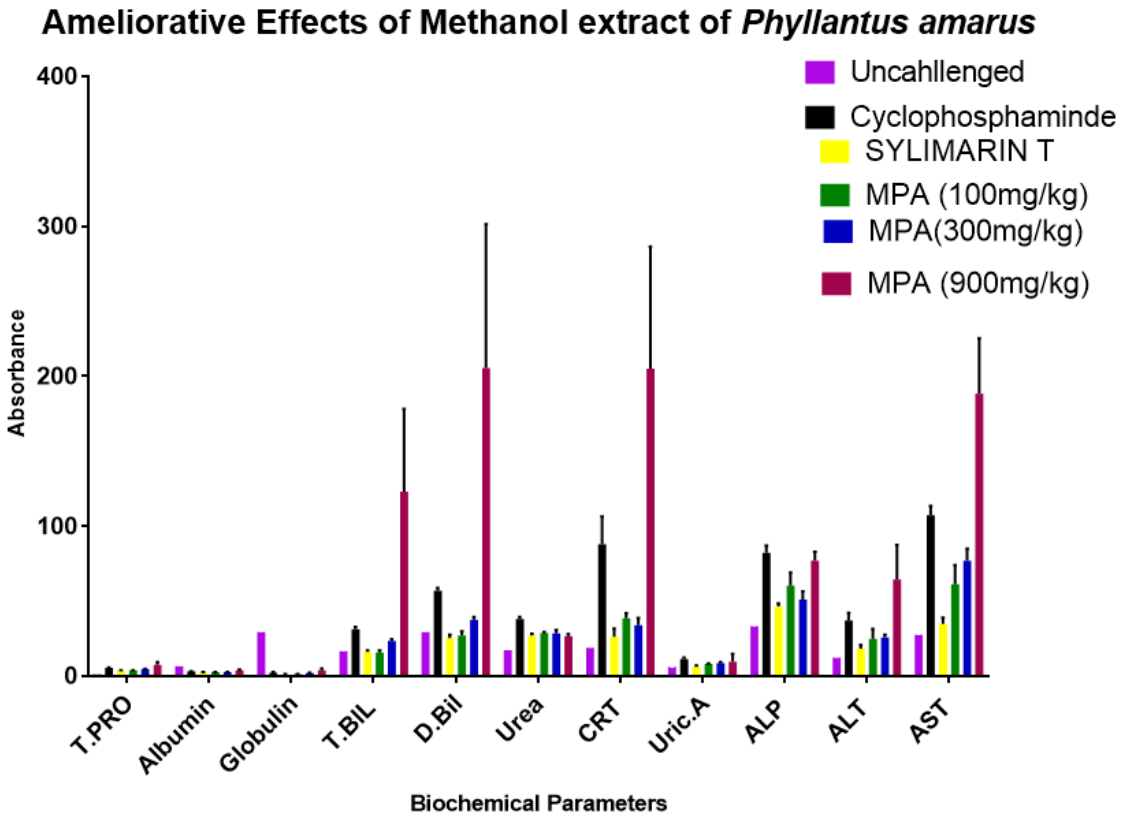
The organs were identified (kidney and liver), removed whole and rinsed in normal saline before weighing on the weighing machine. The whole was later sectioned and fixed in 10% formaline, dehydrated with ethanol solution and embedded in paraffin and sectioned to 5µm, stained using H & E method, cleared in xylene and monted in a mountant (Akanbi & Taiwo 2014).

Data Analysis

All values were presented as the mean ± standard error of mean (SEM). Differences between means was assessed by one-way or two-way analysis of variance (ANOVA) followed by Dunnett's post statistically hoc test where appropriate using Graph Pad Prism version 7.0 (Graph Pad Software, San Diego, CA, USA).

3.Results

The methanol extract of *Phyllantus amarus* at 100mg/kg and 300mg/kg were able to decrease ($P<0.01$) the level of CRT, 300mg/kg only reduced ($P<0.05$) the level of ALP, 100mg/kg and 300mg/kg decreased ($P<0.05$) AST level when compared to the Cyclophosphamide group. The 900mg/kg showed increase in the level of all the biomarkers and there was no significance difference in the T. Pro when compared to the toxic group (Fig 2). There was no significance difference in the level of the biomarkers in both 100mg/kg and 300mg/kg when compared with the Sylimarin group except with AST at 300mg/kg where there was increase P (<0.01) while at 900mg/kg there was increase ($P<0.0010$) in the level of T. Bil, D. Bil, CRT, ALT, AST and no significance difference in others biomarkers when compared to the toxic group (Fig 1).



The weight of the organs relative to the body weight in the cyclophosphamide group showed inflammation compared to the unchallenged group, there was reduction in 300mg/kg group both in the kidney and liver with no significance difference when compared to sylimarin group. The 900mg/kg group show inflammation and there was no significance difference when compared to the toxic group as described in Fig 2.

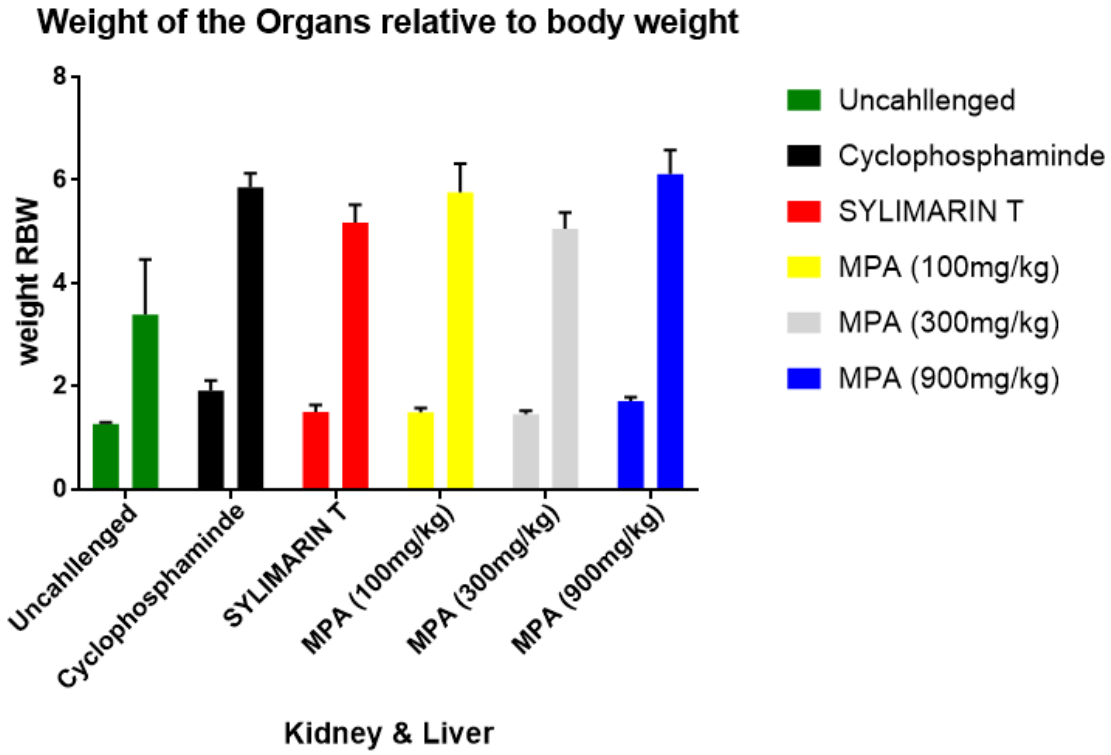
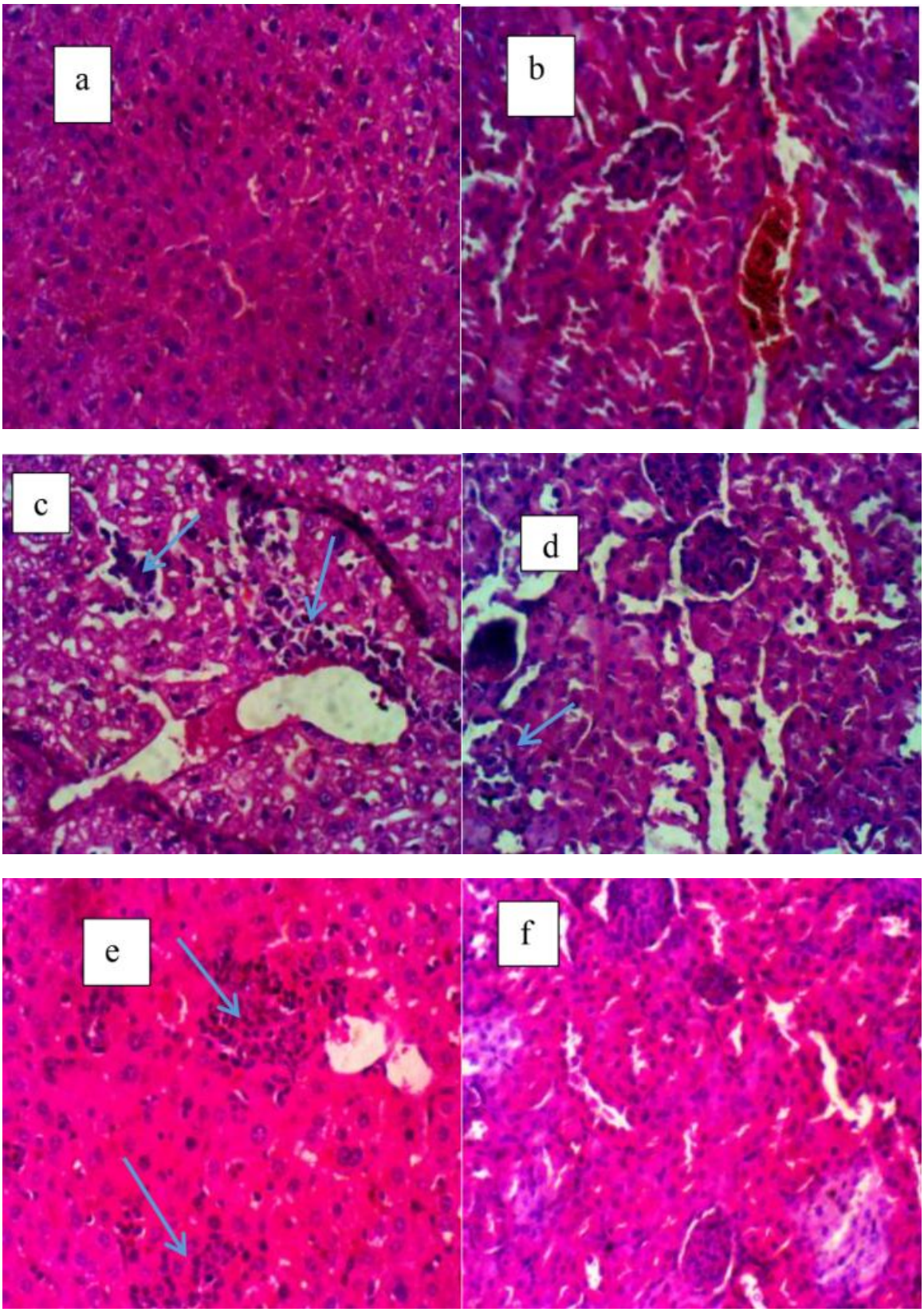
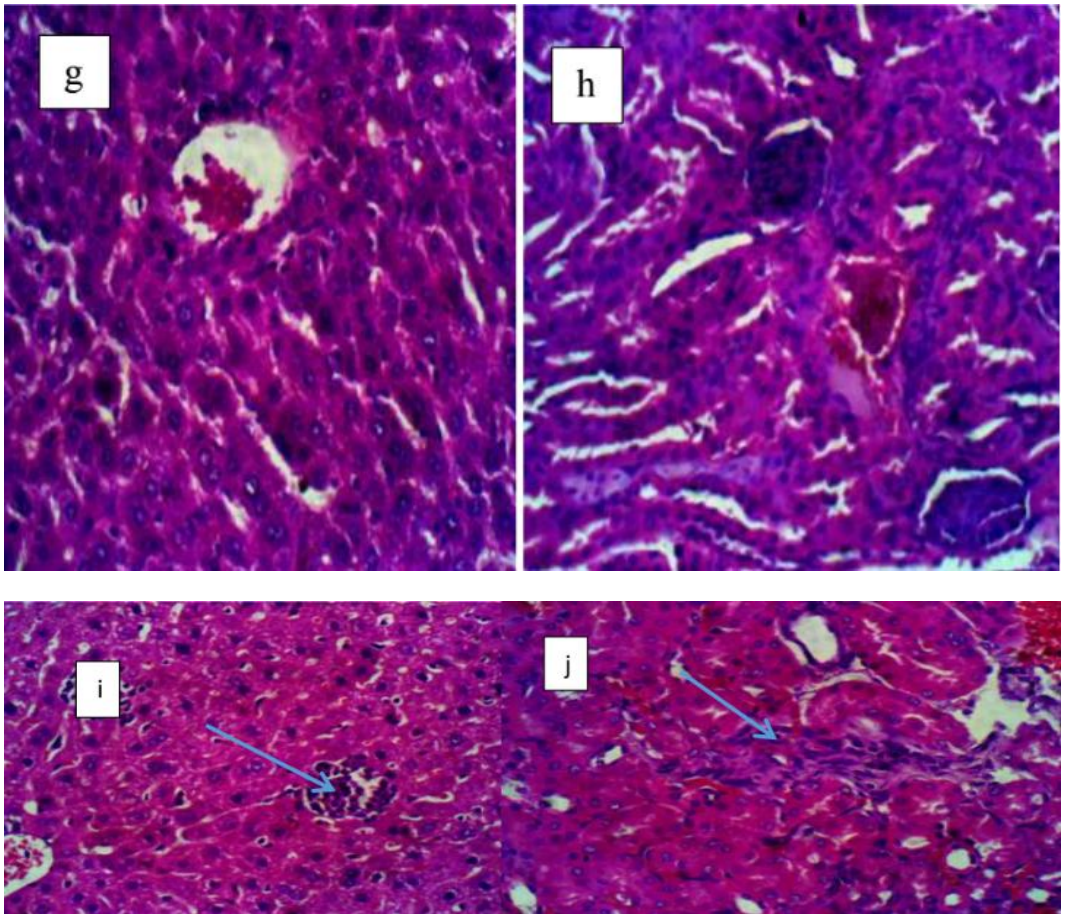


Figure 2

Histopathological Results





a. Liver of normal control group showed no visible lesion
b. Kidney of normal control group with no visible lesion. (HE X400)
c. Liver of CYP group showed multifocal hepatocellular necrosis and inflammation
d. Kidney of CYP group showed patchy tubular epithelial necrosis and inflammation. HE x400
e. Liver of MPAM 100mg/kg showed multifocal hepatocellular necrosis and inflammation. HE x400
f. Kidney of MPAM 100mg/kg showed no observable lesion.
g. Liver of MPAM 300mg/kg showed no observable lesion. HE x400
h. Kidney of MPAM 300mg/kg showed no observable lesion.
i. Liver of MPAM 900mg/kg showed multifocal hepatocellular necrosis and inflammation. HE x400
j. Kidney of MPAM 900mg/kg showed congestion of interstitial capillaries and inflammation

4.Discussion

Natural and herbal products have shown potential for prevention and treatment of diseases due to the presence of their phytochemicals constituents (Altermimi *et al.,* 2017). The need to explore these potentials with pharmacological evaluations is the driving force in this study. We investigate the ameliorative effects of the methanol extract of *Phyllantus amarus* on the liver and kidney at three different doses. *P. amarus* has been used by herbal practitioners to treat some health challenges and management of liver diseases (Patel *et al.,* 2011). Work has been done to evaluate its claim to prevent liver diseases; its hepatoprotective effects was evaluated using the aqueous extract on ethanol-induced hepatic injury in rats (Pramyothin *et al.,* 2007), the hepatoprotective potential of another specie *P. niruri* was confirmed in carbontetrachloride induced liver injury in rats (Harish *et al.,* 2006); the protective effects of ethanol extract of *P. amarus* has also been evaluated in aflatoxin B_1-induced liver damage in mice (Naaz *et al.,* 2007) and they were found the reduce the elevated liver biochemical parameters due to the toxicants thus confirming its protective effects on the liver.

The aetiology for liver and kidney damage are multi-factorial but in this study we consider drug-induced injury to both organs using cyclophosphaminde. We determine the effect of *P. amarus* in managing liver and kidney damaged by cyclophosphamide which is an anticancer agent.

From this work, 100mg/kg of the methanol extract of *P. amarus (*MPAM) was able to manage the damage caused by CYP on the kidney as shown in the histopathological analysis (fig3) and reduced the biomakers elevated by cyclophosphamide (fig1). The weight of the kidney is lower compared to the CYP group while that of the liver show no significant difference when compared to the CYP group (fig 2). MPAM 100mg/kg therefore has better ameliorative effects on the kidney compared to the liver.

The 300mg/kg of MPAM was able to reduce the biomakers of both liver and kidney better than the 100mg/kg compared to the CYP group (fig1); the histopathological analysis confirmed this with no lesion in both organs (fig2) and the

weight of the organs were lowered compared to 100mg/kg and CYP group but no significance difference when compared to the sylimarin group (fig3).

MPAM at 900mg/kg is an overdose that causes more damage to both organs compared to the toxic group. There was elevation in all the biochemical parameters (fig1), the histopath analysis showed injury in both organs (fig2) and the weight of each organs were heavier compared to the toxic group (fig3).

Cyclophosphamide has been associated with oxidative stress which is the release of super-oxide free radicals, increase lipid per-oxidation and glutathione depletion (Nafees *et al.,* 2015), its use at therapeutic dose has been limited by onset of liver and kidney toxicity (Kocahan *et al.,* 2017).

P. amarus has been shown to have phytochemicals like alkaloids, flavonoids, tannins, Lignins, polyphenols and tetracylic triterpenoids (Verma *et al.,* 2014) which are involved in anticancer (Tan *et al.,* 2013), anti-inflammatory, nephroprotective and cardioprotective activities (Kiran *et al.,* 2011). The phytochemicals that have the ability to scavenge free radicals as documented by Obanime *et al.,* 2008 are responsible for the protective and therapeutic properties of the plant (Pramythin *et al.,* 2007).

5.Conclusion

The investigation carried out in this work indicates that the methanol extract of *P. amarus* at 100mg/kg can ameliorates the toxic effects of cyclophosphamide in the kidney while 300mg/kg has therapeutic effects on liver and kidney injured by cyclophosphamide; the 900mg/kg dose is toxic on both organs and therefore should be avoided in further studies for therapeutic effects.

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