Fractions of Aqueous Extract of *Plumbago zeylanica* (L.) Root Induced Mitochondrial Membrane Permeability Transition Pore Opening in Rats

Olaide K. Olanrewaju [a], Leonard O. Ehigie [a], Sunday Faith Oyelere [a,b], Tolulope A. Oyedeji [c], Fiyinfoluwa D. Ojeniyi [a], Folasade P. Olabinrin [a], Ige. F. Olaoye [d] and Adeola F. Ehigie [a*]

[a] *Department of Biochemistry, Ladoke Akintola University of Technology, Ogbomoso, Nigeria.*
[b] *Institute of Genetics, Department of Biology, HHU, Dusseldorf, Germany.*
[c] *Department of Biochemistry, University of Lagos, Nigeria.*
[d] *Department of Biological Sciences, McPherson University, Nigeria.*

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Mitochondrial membrane permeability pore opening is recognized as a molecular mechanism involved in programmed cell death. The purpose of this study was to evaluate the effects of certain fractions crude aqueous extract of the root bark of *Plumbago zeylanica* (PZ) on the opening of the mitochondrial membrane permeability transition pore in rat liver. Pore opening, mitochondrial ATPase activity, lipid peroxidation and liver function markers were assessed spectrophotometrically while caspase 9 activity and liver morphology were monitored by immunohistochemistry and histology respectively. All fractions of PZ induced pore opening and enhanced the activity of caspase-9. Significant increases were observed in the mitochondrial ATPase activity while the level of malondialdehyde was significantly reduced. Sinusoidal dilation and hypertrophy were observed in liver sections of treated rats. These results suggest that aqueous fractions of PZ have bioactive agents capable of altering mitochondrial bioenergetics and inducing the opening of MMPT pore with mild hepatotoxicity.

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Corresponding author: E-mail: afehigie@lautech.edu.ng;

ABBREVIATIONS

MMPT   : *Mitochondria Membrane Permeability Transition;*
APAF-1 : *apoptotic protease-activating factor-1;*
dATP   : *Deoxyadenosine triphosphate;*
SMAC   : *second mitochondria-derived activator of caspase;*
ATP   : *adenosine triphosphate;*
CAE   : *crude aqueous extract;*
HF   : *n-hexane fraction;*
EF   : *ethylacetate fraction;*
BF   : *butanol fraction;*
AF   : *aqeous fraction;*
TBARS : *thiobarbituric acid reacting substances;*
MDA   : *malondialdehyde;*
γ-GT   : *Gamma glutamyl Transferase;*
SMAC   : *second mitochondria-derived activator of caspase;*
CyP-D : *cyclophillin-D.*

1. INTRODUCTION

Mitochondrial permeability transition is highly involved in the regulation of apoptosis [1]. Mitochondrial membrane permeability transition (MMPT) pore opening is generally accompanied by structural changes in a couple of proteins (Voltage-Dependent Anion Channels and Adenosine Translocase) embedded within the membrane, which in certain conditions seem to play a significant physiological role [2,3]. These proteins oligomerize, leading to the permeabilization of the mitochondrial membrane transition pore. Opening of the MMPT pore is the committed step of intrinsic apoptosis [4]. This opening permits the escape of certain intermembrane proteins such as cytochrome c, second mitochondria-derived activator of caspase (SMAC) and Omi. In response to the emancipation of cytochrome c, the apoptosome is formed from cytochrome c, apoptotic protease-activating factor-1 (APAF-1), dATP, and procaspase-9 [3]. In the apoptosome, the conversion of procaspase-9 into caspase-9 occurs, [5] leading to the activation of the terminating caspases-3 and caspase-7 [6]. The terminating caspases subsequently break down cellular proteins leading to cell death. A breach in mitochondrial membrane integrity leading to the formation of the MMPT pore prompts the cell to develop an apoptotic phenotype triggered by the release of apoptogenic proteins – a process known as mitochondrial permeability transition [7]. At that point, the inner membrane which is always selective in terms of permeability becomes a passage for small molecules less than 1500 Da [8]. Also, hydrolysis of ATP, influx of water, and mitochondrial swelling are part of the molecular consequences that result from MMPT pore opening [9]. Certainly, the intrinsic pathway of apoptosis suffers inhibition in cancers; cancer cells are unyielding to apoptotic control [10]. Thus, they have a high survival rate and accumulate mutations which increase invasiveness, aid angiogenesis, hinder cell proliferation and disturb cell differentiation during tumor progression [3]. A successful non-surgical approach to cancer therapy could be substances that target apoptosis; being effective for all types and forms of cancer [11].

Plumbago zeylanica L., is an abundantly branched perennial herb with alternate leaves [12]. It is an annual plant that can grow up to the height of 3-4 ft. Leaves are thick, fleshy, sessile, oval, and lance-elliptic in shape. Flowers of this plant are 10-25 cm long and arranged in terminal and axillary elongated spikes [13]. Different parts of *P. zeylanica* L. have been utilized for centuries for various medicinal purposes. The root and root bark of the plant has been utilized in the preparation of varied ayurvedic medicines [12]. In ethnomedicine, it has been indicated for its significant protective role in enlarged liver and spleen [12]. It is a bitter tonic and suggested as a rejuvenator, well known for its use in chronic colds and cough. It also finds its use in correcting chronic menstrual disorders, viral warts, and chronic diseases of the nervous system. Extracts of *P. zeylanica* are reported as anticancer drugs. Root bark is additionally considered beneficial in obesity [12]. Notably, extracts of *P. zeylanica* and

certain phytochemicals isolated from the plant has been reported to have an apoptotic effect [13].

This study investigated the apoptotic effects of HF, EF, BF, and AF fractions of crude-water-soluble extract of *P. zeylanica* in healthy male Wistar rats by determining the effect of the plant on MMPT pore opening, mitochondrial caspase-9 expression, mitochondrial ATPase activities, mitochondrial lipid peroxidation level, serum level of liver enzymes and liver histology.

2. MATERIALS AND METHODS

2.1 Plant Material

P. zeylanica root was bought from Akoda, Ede, Osun State, and authenticated at the Department of Plant Biology, University of Ilorin, Ilorin, Nigeria. The voucher number is UTLH/001/1374. The preparation of the roots was done by the modification of the method described by Aribigbola et al. [14]. Briefly, the fresh root of *P. zeylanica* was washed with distilled water, drained, and air-dried. Five hundred (500 g) of the air-dried *P. zeylanica* root was pulverized and macerated in 5000 mL distilled water for 24 hr. The macerated mixture was filtered using a muslin cloth, and the filtrate, crude aqueous extract (CAE) was collected.

2.2 Partitioning of Aqueous *P. zeylanica* Crude Fraction with Organic Solvents

The crude aqueous extract of *P. zeylanica* was mixed with various organic solvents (n-hexane, ethylacetate, butanol, and water) for 1 hr and separated using a separating funnel to obtain the HF, EF, BF, and AF fractions of the crude aqueous extract. The separated organic portions were concentrated using rotary evaporator at 50°C and weighed to calculate the percentage yield. The fractions obtained were orally administered to the animals to assess their effect on rat liver MMPT pore.

2.3 Experimental Design

Sixty male Wistar rats weighing between 100 g and 150 g were randomly allocated into four major groups (n=15) which are further divided into three subgroups. Each subgroup is fed with 0.40mg/kg body weight (BW), 0.05 mg/kg BW and 0.06 mg/kg BW of HF, EF, BF and AF fractions of *P. zeylanica* respectively for 28 consecutive days. The control animals (n=5) are fed with normal saline. All animals were sacrificed by cervical dislocation after 28days, and the liver was excised.

2.4 Mitochondrial Fraction Isolation

Overnight-fasted animals were sacrificed by cervical dislocation and liver mitochondria were isolated according to the method reported by Lapidus and Sokolov 1993. Rat liver was rapidly excised, trimmed (to remove excess tissue) and washed in a buffer containing 210 mM Mannitol, 70 mM Sucrose, 5 mM HEPES, 1 M KOH, and 1 mM EGTA, pH 7.4. Subsequently, the liver was weighed, chopped, and suspended in the same buffer to make a 10% homogenate and homogenized. The mitochondrial fraction of the liver was obtained at 13000 rpm for 10 min and washed three times at 12000 rpm for 10 min with a washing buffer which contained 210 mM Mannitol, 70 mM Sucrose, 5 mM HEPES-KOH and 0.5% BSA, pH 7.4. The mitochondrial pellets were suspended in swelling buffer (210 mM Mannitol, 70 mM Sucrose, and 5 mM HEPES-KOH, pH 7.4) and immediately dispensed in 1 ml Eppendorf tubes [15].

2.5 Mitochondrial Swelling Assay

Mitochondrial membrane permeability transition pore opening was determined according to the method of Lapidus and Sokolov 1993. This was monitored by measuring the changes in absorbance of mitochondria at 540 nm in a 752N UV/Visible spectrophotometer. Mitochondria (0.4 mg protein/ml) were preincubated in the presence of 8 μM rotenone in a medium containing 210 mM mannitol, 70 mM sucrose, 5 mM HEPES-KOH (pH 7.4) for 3 min at 30°C before the addition of 300 μM $CaCl_2$, while 50 μM sodium succinate was added 30 sec later and MMPT pore opening was measured at 540 nm for 12 min at 30 sec interval. The inhibitory effect of cyclosporine on the induction of the pore was carried out before the addition of $CaCl_2$. The inductive effects of fractions were monitored when the fractions were replaced with $CaCl_2$ [15].

2.6 Determination of ATPase Activity

Mitochondrial ATPase assay was done by modifying the method of Lardy and Wellman [16]. Each test medium contained 65 mM Tris-HCL (pH 7.4), 0.5 mg protein (mitochondria), 0.5 mM KCl, 1 mM ATP and 25 mM sucrose. The final assay volume was 2 mL. Each dosage of *P.*

zeylanica of fractions (HF, EF, BF and AF) were included as required. The reaction started on the addition of the ATP and continued for 30 min with consistent shaking at 37°C. 1 mL of 10% sodium dodecyl sulfate was added to the mixture in each test tube to stop the reaction. After which, 4 mL of distilled water was added to each test tube and then 1 mL of the resulting solution was put into fresh test tubes where 1 mL of 1.25% Ammonium molybdate in 6.5% Sulphuric acid was added. 1 mL of 9% ascorbic acid was further added for colour development and estimated at 660 nm. All analyses were performed in triplicates.

2.7 Determination of Mitochondrial MDA Level

The level of thiobarbituric acid reacting substances (TBARS) was performed as described previously (Aeschbach et al., 1994) and the level of malondialdehyde (MDA) was calculated by using

$$MDA(units/mg\ of protein) = \frac{Absorbance \times volume\ of\ mixture}{E532 \times volume\ of\ sample \times mg\ of\ protein}$$

2.8 Determination of Serum Levels of Liver Enzymes

Aspartate aminotransferase (AST), alkaline phosphatase (ALP), alanine aminotransferase (ALT) and γ-glutamyltransferase (γ-GT) levels in the serum were determined using Randox kits following the manufacturer specifications.

2.9 Tissue Processing and Histopathology

Rats were anesthetized intramuscularly with 0.5 ml/ kg ketamine, and subjected to transcardial perfusion with normal saline, followed by 4% paraformaldehyde (PFA). After perfusion, rat liver was excised and fixed in 4% PFA for 48 hr. The tissues were fixed in 10% buffered formalin processed, embedded in paraffin, and sectioned at 5 μm thick with the aid of a rotary microtome. Tissue staining was carried out using hematoxylin and eosin stains and photomicrographs were taken using an Olympus microscope, Japan.

2.10 Immunohistochemistry

The liver section was processed and embedded in paraffin. They were deparaffinized and the primary and secondary antibodies were added followed by the addition of horseradish peroxidase. The sections were reacted with horseradish peroxidase for 10 min, followed by the addition of immunoperoxidase diaminobenzidine (DAB) (chromogenic agent). Tissue sections were counterstained in hematoxylin, viewed under a microscope then the photomicrographs were taken and analysed using "Image J" software.

2.11 Statistical Analysis

The results are expressed as mean + standard deviation. Comparison of the variables was determined by one-way analysis of variance using Graph Pad Prism 6.0 statistical software. Significant values are determined at $P < 0.05$.

3. RESULTS

3.1 Fractions of *P. zeylanica* Induced Mitochondrial Membrane Permeability

Swelling of the mitochondrial membrane permeability transition pore is the first step in the intrinsic pathway of apoptosis. The effects of the HF, EF, BF and AF fractions of *P. zeylanica* plant on the permeabilization of the mitochondrial membrane of male Wistar rats were determined at 40 mg/100 g BW, 50 mg/100 g BW and 60 mg/100 g BW after 28 days of oral administration of treatment.

No significant changes were observed in the volume of intact respiring mitochondria energized on succinate in the absence of calcium, while calcium ion induced significant opening of MMPT pore up to about 4.07 folds (Fig. 1). This observed induction was reversed by spermine, the standard inhibitor of the pore.

Treatment of intact mitochondria with varying doses of AF fraction of *P. zeylanica* induced opening of mitochondrial membrane permeability transition pore at all tested dosages (Fig. 2a); 3.09-, 4.65- and 3.94-fold increases were observed in the group treated with 40 mg/100 g BW, 50 mg/100 g BW and 60 mg/100 g BW respectively. All observed openings were reversed by spermine.

Also, swelling of the mitochondrial membrane was observed in intact mitochondria treated with the dosages of BF fraction of *P. zeylanica* (Fig. 2b). Fold increases of 6.36, 5.65 and 5.17 were observed in the groups treated with 40 mg/100 g

BW, 50 mg/100 g BW and 60 mg/100 g BW respectively. All observed openings were reversed by spermine.

The HF fraction of *P. zeylanica* induced the opening of MMPT pore at all tested dosages (Fig. 2c); 4.89-, 5.32- and 3.79-fold increases were observed in the group treated with 40 mg/100 g BW, 50 mg/100 g BW and 60 mg/100 g BW respectively. All observed openings were reversed by spermine.

Treatment of intact mitochondria with dosages of EF fraction of *P. zeylanica* induced the opening of MMPT pore, with 2.36-, 4.11- and 1.96-fold increases were observed in the group treated with 40 mg/100 g BW, 50 mg/100 g BW and 60 mg/100 g BW respectively (Fig. 2d). All observed openings were reversed by spermine.

3.2 Fractions of *P. zeylanica* Increase the Expression of Caspase-9 in Rat Liver

To determine whether the observed permeabilization of the mitochondrial membrane caused the activation of caspase 9, an initiator of apoptosis, the expression of caspase-9 was assessed immunohistochemically in the liver sections of the rats treated with *P. zeylanica* fractions. Caspase-9 is a key protein that triggers the activation of downstream caspases, culminating in cell death. Arrows and brown coloration indicate high immunoreactive cells in the photomicrograph. Caspase 9 expression is

observed in all sections of the livers treated with the different doses of HF, EF, BF and AF fractions of *P. zeylanica* and magnification is x100. The quantitative immunohistochemical representation of apoptotic marker of liver sections labelled with anti-caspase 9 is shown in Fig. 3. Caspase 9 expression is significantly increased in all the groups administered with HF, EF, BF and AF fractions of *P. zeylanica*. Increase in caspase-9 expression was observed to be dose-dependent (Fig. 4).

3.3 Fractions of *P. zeylanica* Increased Rat Mitochondrial ATPase Activities

Mitochondrial ATPase activities were significantly increased in the groups treated with the HF, EF, BF and AF fractions of *P. zeylanica* at all tested doses; hence, the hydrolysis of ATP was enhanced by the fractions of the plant.

3.4 Fractions of *P. zeylanica* Increased Rat Mitochondrial Malondialdehyde Level

The mitochondrial levels of malondialdehyde (MDA) were quantified in rat livers treated with the fractions of *P. zeylanica* to determine the effects of the plant fractions on the generation of free radicals. The results obtained showed that there were significant reductions ($P<0.05$) in the levels of mitochondrial MDA at all tested doses of the fractions compared with the untreated group (Fig. 5).
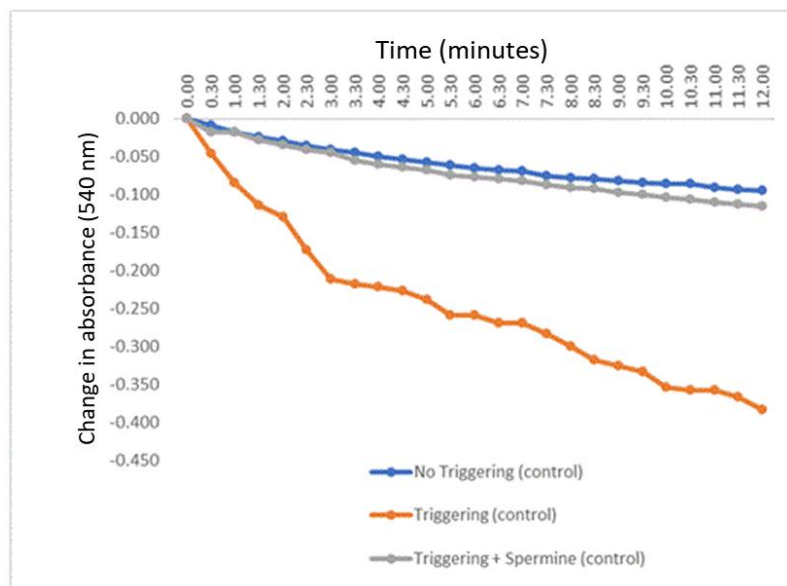

Fig. 1. The *In-vivo* effect of calcium ion on the opening of MMPT pore and reversal with spermine
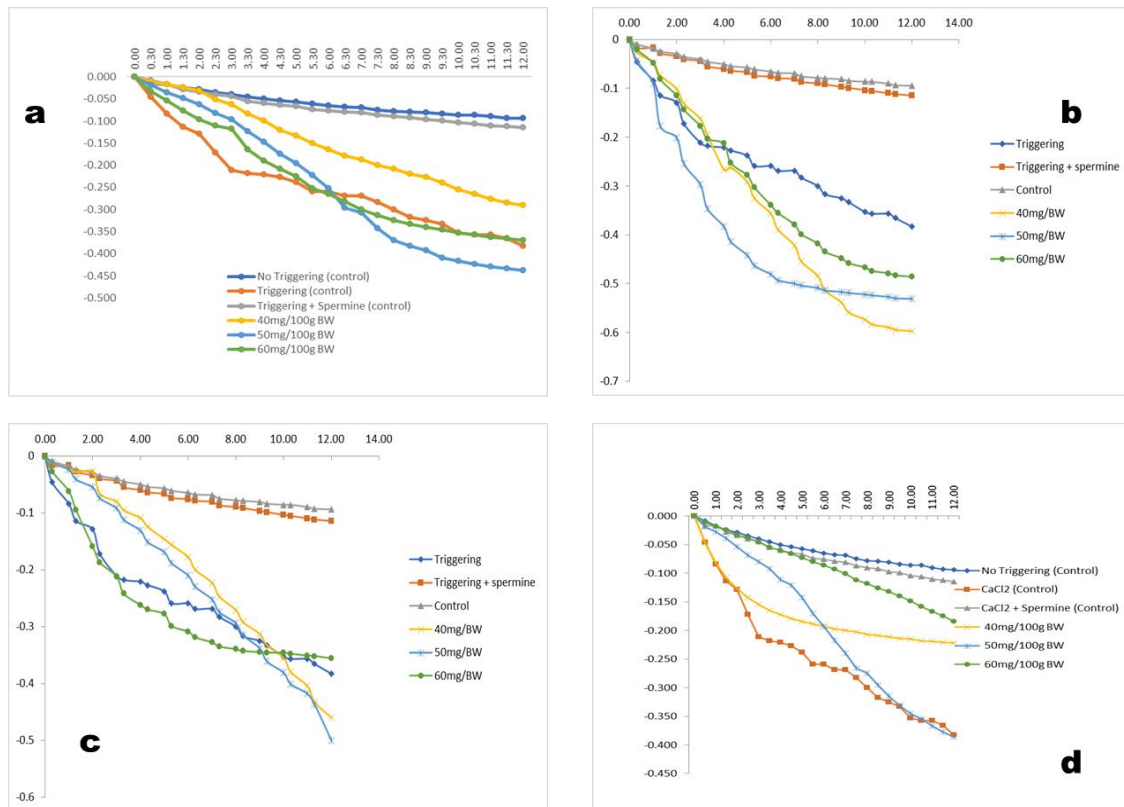
Fig. 2. Varying doses of root fractions *of P. zeylanica* induced MMPT pore opening (a) aqueous (AF) (b) butanol (BF) (c) n-hexane (HF), and (d) ethylacetate (EF) fractions
X axis – Time (minutes), Y axis – change in absorbance

3.5 Effects of Fractions of *P. zeylanica* on the Level of Liver Enzymes in the Serum

The effects of HF, EF, BF and AF fractions of *P. zeylanica* on the level of liver enzymes – alanine transaminase (ALT), aspartate transaminase (AST), alanine phosphatase (ALP) and γ-glutamyl transferase (GGT) in the serum was assessed to determine the possible hepatotoxic effect of the plant. Significant ($P<0.05$), dose-dependent increases were observed in the level of GGT in the group treated with aqueous and ethylacetate fractions of *P. zeylanica* as compared with the untreated group. However, GGT serum level significantly decreased in both butanol and n-hexane fractions of *P. zeylanica* as compared with the control group (Fig. 6a). Significant decreases were observed in the serum level of ALP (Fig. 6b) in the group administered with EF and AF fractions of *P. zeylanica* when compared to the control group. However, significant increases were observed in the level of serum ALP in the group treated with doses of butanol and n-hexane fractions as compared with the control. Significant increases

were observed in the serum level of AST (Fig. 6c) in the group administered with all fractions of *P. zeylanica* compared with the control group. Although a significant decrease was observed in the group treated with 40mg/100g BW of n-hexane extract. Compared to control group, significant increases were observed in the groups treated with doses of HF (except at 40 mg/100 g BW), EF, and AF fractions of *P. zeylanica*.

3.6 Fractions of *P. zeylanica* Caused Sinusoidal Dilation and Hypertrophy of the Liver Section

The effect of the fractions of *P. zeylanica* on rats' liver morphology were assessed to determine how the extract affects the structure of liver tissue. General morphological presentation of liver across the groups are shown by Haematoxylin and Eosin staining. Sinusoidal dilation (red arrow) and hypertrophy (white arrow of the central vein were observed in the groups treated with HF, EF, BF and AF fractions of *P. zeylanica* at all doses (Fig. 7).
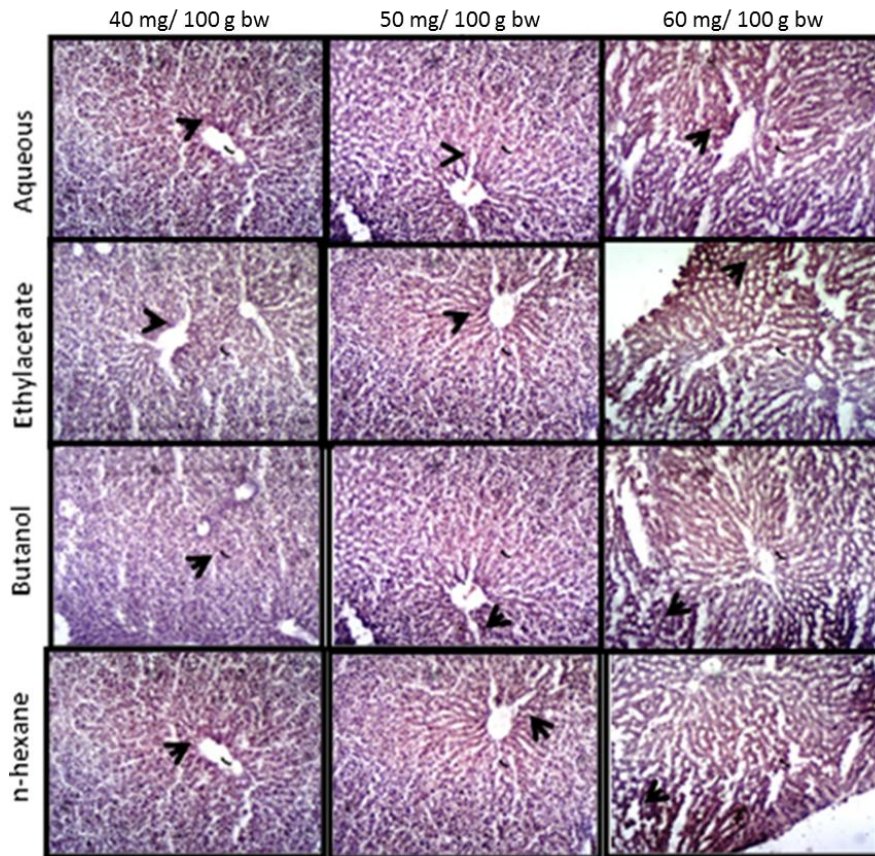
Fig. 3. Representative photomicrographs of apoptotic marker of liver sections labelled with anti-caspase 9 of animals treated with varying doses of HF, EF, BF and AF fractions of *P. zeylanica* (x100 magnification)
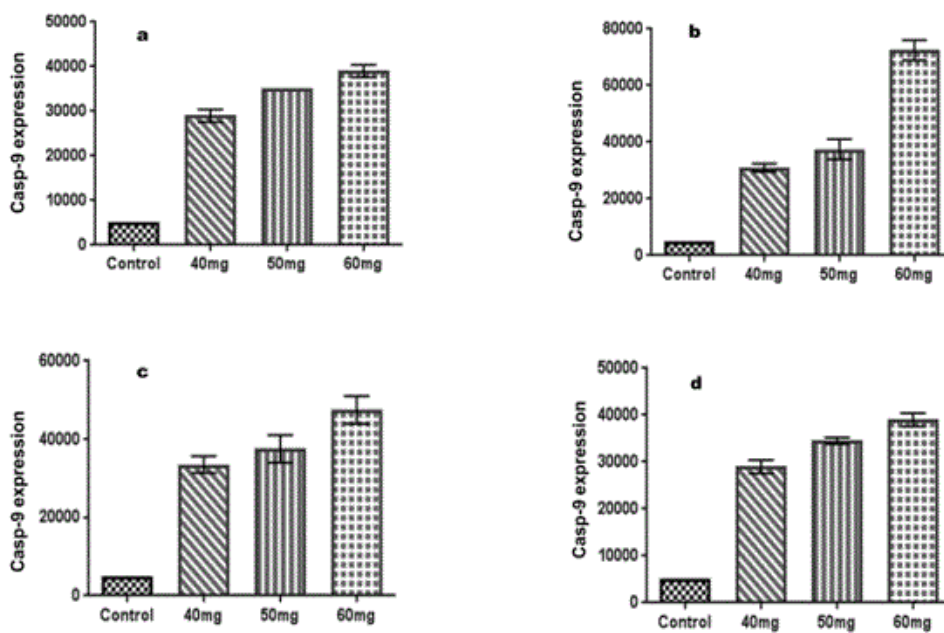

Fig. 4. Effect of (a) n-hexane, (b) ethylacetate, (c) butanol and (d) aqueous fractions of the aqueous extract of the root bark of P. zeylanica on Caspase 9 expression levels. Increase in caspase-9 expression was observed to be dose-dependent
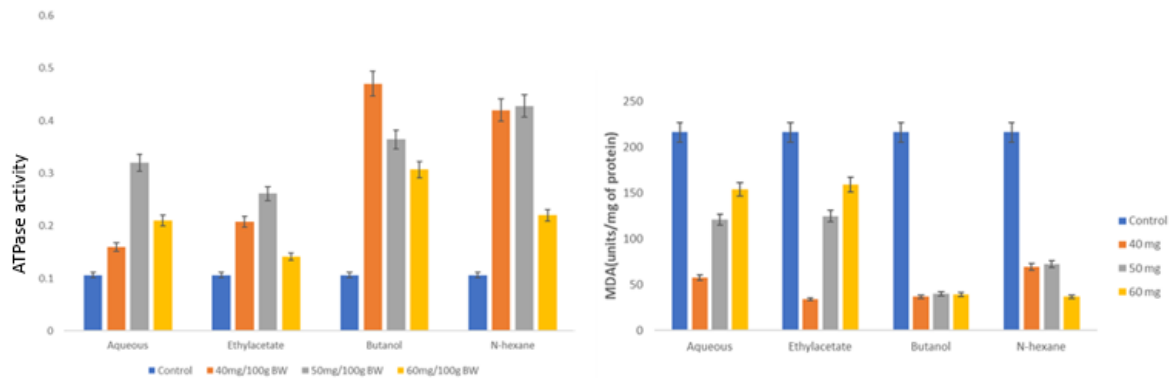
Fig. 5. (a)The effects of varying doses of fractions of *P. zeylanica* on mitochondria ATPase activities (b) MDA levels in the liver mitochondria of male Wistar rats intubted with varying doses of root aqueous, ethyl acetate, butanol and n-hexane fractions of *Plumbago* zeylanica

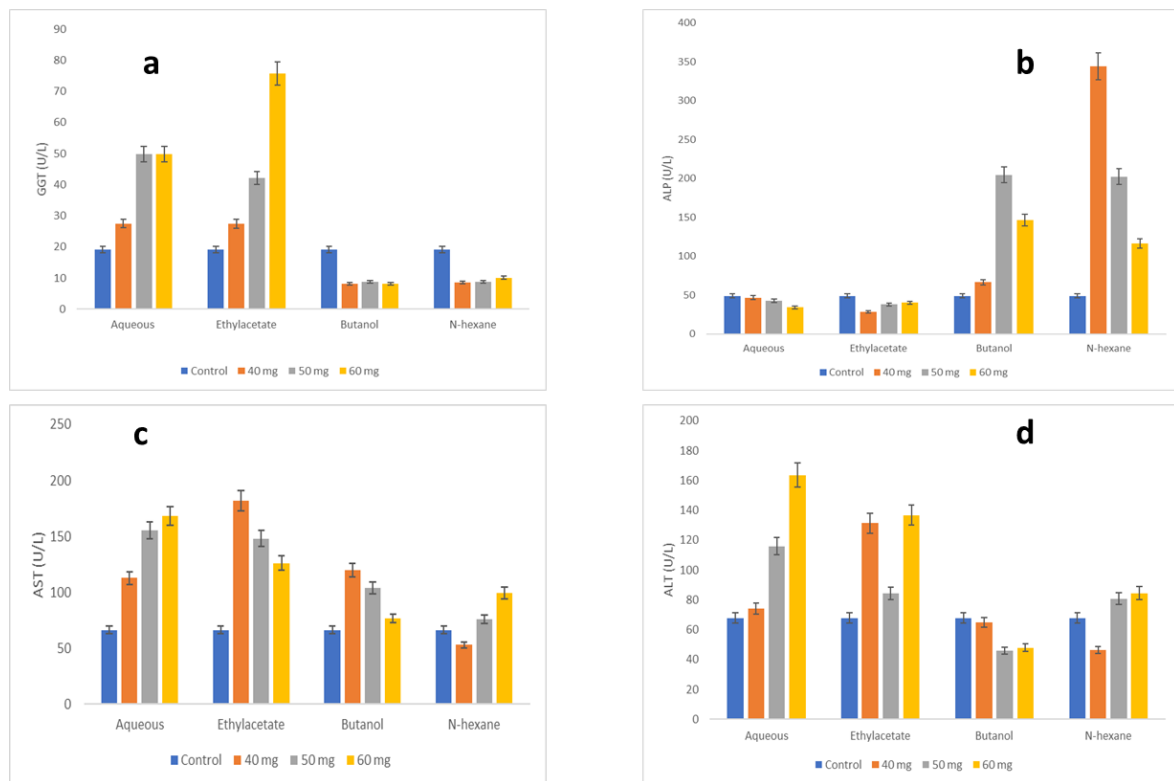**Fig. 6. (a) The effects of fractions of *P. zeylanica* on serum γ-glutamyl transferase activities (b) The effects of fractions of *P. zeylanica* on serum alanine phosphatase activities (c) The effects of fractions of *P. zeylanica* on serum aspartate transferase activities (d) The effects of fractions of *P. zeylanica* on serum alanine transaminase activities**

4. DISCUSSION

Mitochondrial permeability transition is highly involved in the regulation of apoptosis [17]. The permeabilization of mitochondrial membrane permits the release of intermembrane proteins like cytochrome c, second mitochondria-derived activator of caspase (SMAC) and Omi [18]. Upon the release of cytochrome c, the apoptosome is formed from cytochrome c, apoptotic protease-activating factor-1 (APAF-1), dATP and procaspase-9 [3]. Within the apoptosome, procaspase-9 is converted into caspase-9, which activates the terminating caspases-3 and caspase-7 [5]. The terminating caspases subsequently break down proteins leading to cell death [6]. In this study, we showed that fractions of crude-water-soluble extract of *P. zeylanica* are
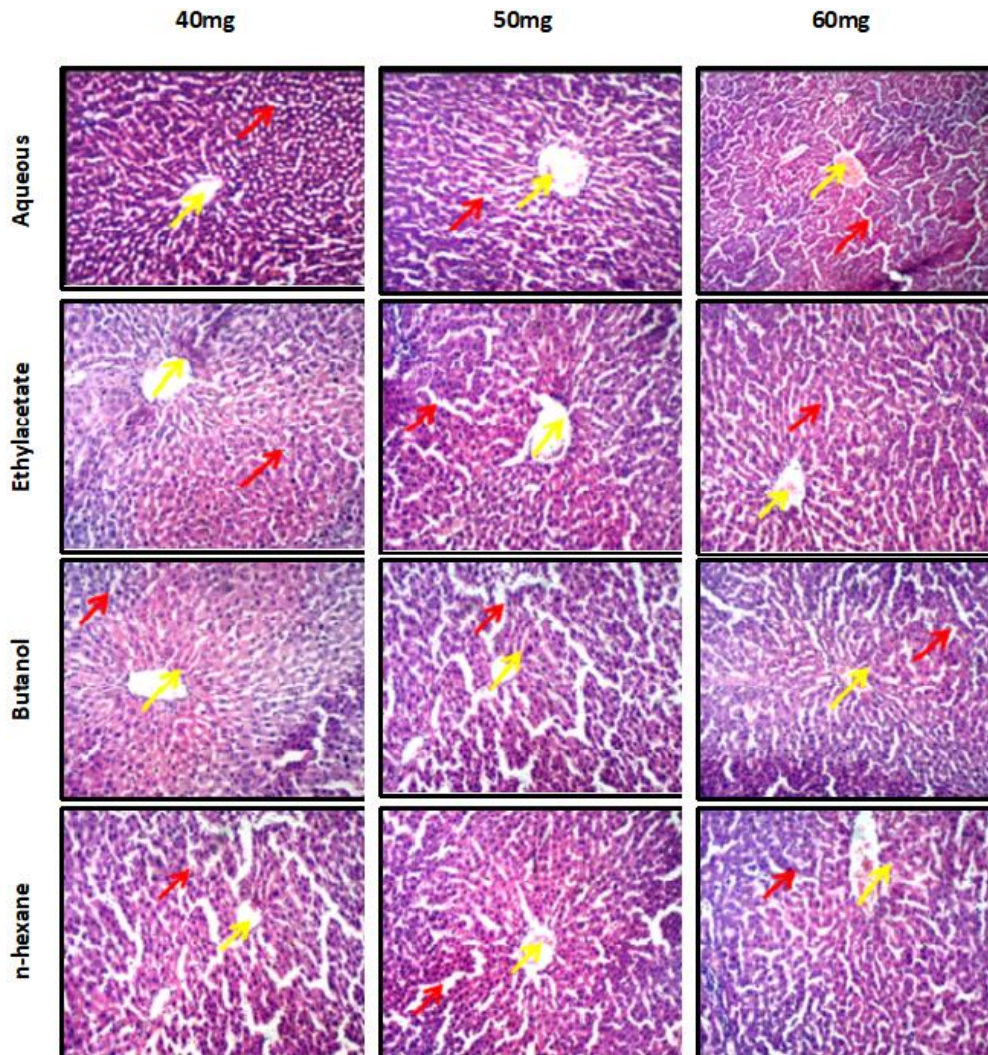
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Fig. 7. Photomicrographs of Liver section of rats treated with aqueous, ethylacetate, butanol and n-hexane fractions of *Plumbago zeylanica*

capable of inducing apoptosis in healthy male Wistar rats. The inductive effect of HF, EF, BF and AF fractions of *P. zeylanca* on MMPT pore opening was assessed in this study using the mitochondrial swelling assay as a predictive measurement. Opening of the MMPT pore was observed at all the tested dosages (40 mg/100 g, 50 mg/100 g and 60 mg/100 g) of the plant fractions, suggesting that *P. zeylanica* root contain bioactive ingredients with potential to open the MMPT pore and commits cells to apoptosis. This result is in alignment with the observation of Aribigbola [19] who demonstrated that methanolic fraction of contains certain phytochemicals that is capable of inducing the opening of the MMPT pore albeit, *in-vitro*. Interestingly, the observed opening of the MMPT pore is accompanied by high expression of caspase-9; expression of caspase-9 leads to the

activation of the terminating caspases-3 and caspase-7 and commits the cell to apoptosis [6]. Also, ATP hydrolysis was also enhanced by the fractions of *P. zeylanica* (Fig. 5). This was quantified by the release of inorganic phosphate. ATP synthase, an enzyme responsible for the synthesis of ATP in an intact mitochondrion is also responsible for its hydrolysis when the electrochemical gradient of the inner mitochondrial membrane is breaded [20]. This switch in role is mediated possibly by the binding of the cyclophilin D (cypD), to the ATP synthase; it changes its activity from the synthesis of ATP to that of hydrolysis [21]. Meanwhile, inorganic phosphate is a necessary co-factor for this event [22]. The results obtained in Fig. 5 show that there is reduction in the level of mitochondrial MDA levels in the treated group compared to the control group. This observed reduction in the

treated group may probably means that the fractions of *P. zeylanica* possess high quantities of bioactive compounds with free radical scavenging activity which scavenge free radicals produced in the mitochondria. Although, the antioxidant properties of the fractions of *P. zeylanica* used is not determined in this study; however, previous studies have reported the high antioxidant activities of *P. zeylanica* [23]. In respiring mitochondria, generation of ATP by the ATP synthase enzyme leads to concomitant release of superoxide radicals which are capable of causing oxidative stress, if not scavenged [24]. This may potentiate lipid peroxidation and consequent disruption of the mitochondrial membrane potential [25]. Oxidative stress can also the switch of the ATP synthase enzyme from to that of hydrolysis [26]. The serum level of liver enzymes (AST, ALT, ALP and GGT) were increased in animals administered fractions of *P. zeylanica* (Fig. 6), an indication of mild toxic effects to the rat's liver [27]. This is further confirmed by sinusoidal dilation and hypertrophy observed in the liver section, which is also suggestive of a mild injury to the liver or probably an adaptive response [27].

5. CONCLUSION

The foregoing results show that the fractions of *P. zeylanica* root may be capable of inducing intrinsic cell death with mild hepatotoxic effects in normal male Wistar rats and could thus be a promising therapeutic option in the treatment and management of pathological conditions that can benefit from induced cell death caused primarily by mitochondrial swelling, especially if the hepatoxic effect is ameliorated; hence, more study on the hepatotoxicity of the plant is encouraged.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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