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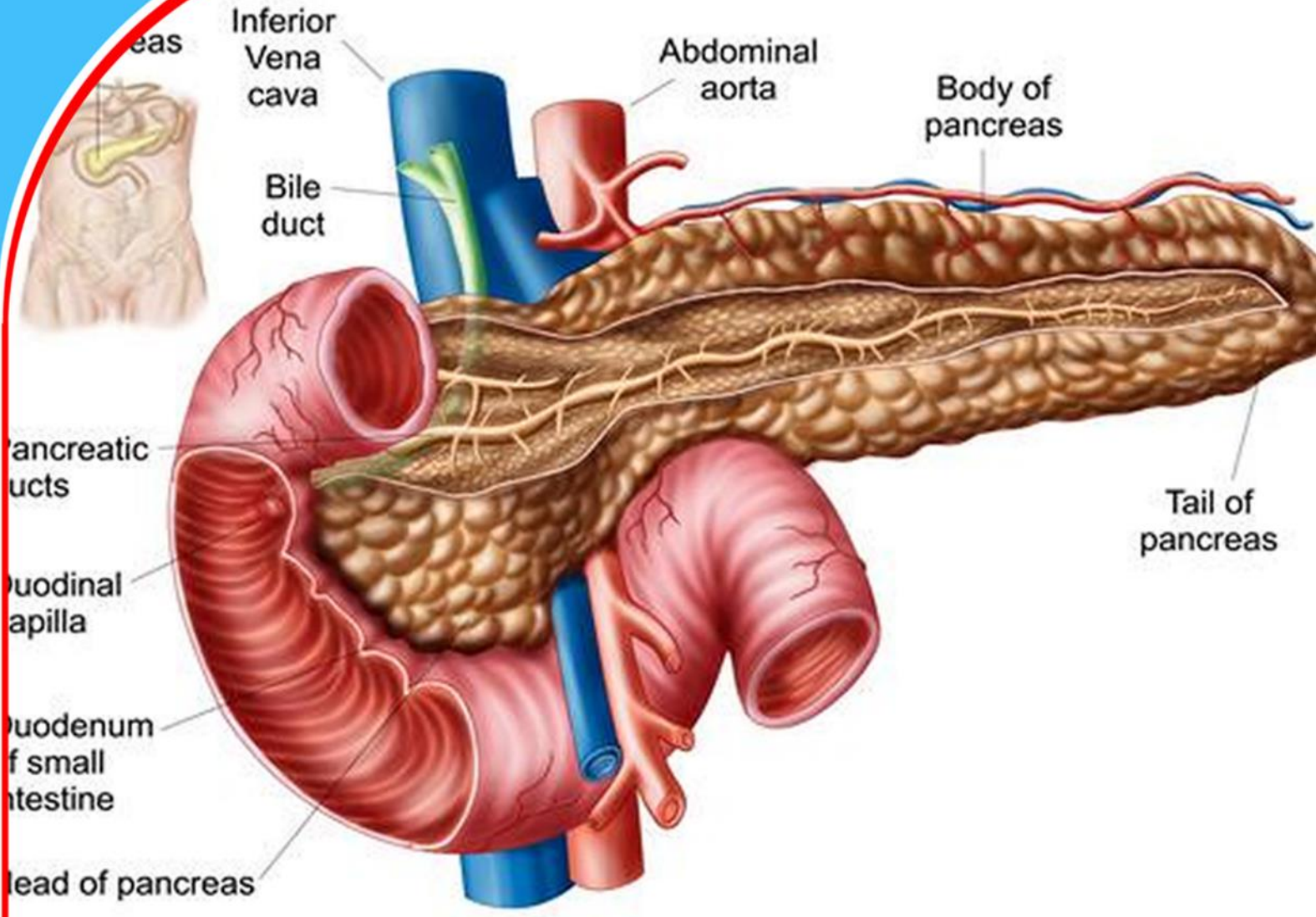

Assessment of the Effect of Ethylacetate Extract of *Plumbago Zeylanica* on Mitochondria Permeability Transition Pore

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Assessment of the Effect of Ethylacetate Extract of *Plumbago Zeylanica* on Mitochondria Permeability Transition Pore

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Abstract

Purpose: This study was therefore conducted in order to investigate the modulatory effects of ethylacetate root extracts of PZ on MPTP using animal model.

Materials and Methods: Different concentrations of ethylacetate (8µg/ml, 24µg/ml, 40µg/ml, and 56µg/ml) extract of the root of PZ were used *in-vitro*. For *in-vivo* analysis, different doses of 5, 10, 20, and 25 mg/kg bwt of the ethylacetate extract were orally administered to four groups of animals respectively. The control group was fed distilled water given in the same doses as the experimental groups. Hepatic injury was assessed histologically and by the quantification of Alanine transaminase (ALT) and Aspartate transaminase (AST) levels in serum samples. Epididymal sperm samples were analyzed for motility, sperm counts and morphological disorders. Results obtained were statistically analyzed using analysis of variance (ANOVA) at 0.05 significance.

Findings: There was significant induction of the mitochondrial membrane permeability transition pore in a dosage dependent manner. Ethylacetate extract of *P. zeylanica* induces maximally at 24µg/ml and 25mg/*kg invitro* and *invivo* respective. Calcium played an important role in these assays as little or no induction was observed when all assays were carried out without calcium. Haematological, histological and fertility tests revealed no significant difference in all treatment groups when compared with the control.

Implications to Theory, Practice and Policy: These results therefore suggest that bioactive agents present in *P. zeylanica* possess properties that induces MPTP and thus may become promising drug candidate for the treatment of cancer.

Keywords: *Multi-Drug Resistance MDR, Apoptosis, Cell-Cycle, Mitochondria Permeability Transition MPT, Plumbago Zeylannica PZ, Cancer*

1.0 INTRODUCTION

In the treatment of cancer, chemotherapy is the preferred choice of clinical interventions especially in advance cases where other therapeutic alternatives such as irradiation would be ineffectual. Still, chemotherapy cannot with all certainty within the limits of safety, be administered without the possible presentation of its attending side effects. These side effects are always nearly in all cases known, deleterious and overall injurious to the health of the patient. Hence, in a bid to discover drug candidate that is non-aggressively invasive yet potent against cancer growth, survival and proliferation; scientists for the past decades, have expended efforts channeled into unraveling and characterizing natural phytochemicals present in plants and herbs.

Ayurveda; a system of ancient medicine traditionally practiced in India and China (and also Africa) involves the use of natural plants and herbs for the treatment of diverse ailments. A plant that has been vastly employed in Ayurveda medicine for the treatment of diverse range of ailments from simple skin acne to more complex molecular disorders such as cancer is *Plumbago zeylanica* (PZ). Apoptosis, the concomitant opposite of cell division and procreation is a desirable cellular event when fully controlled. It is well documented fact that one of the many ways through which malignant cells continues to divide and hence grow to undesirable mass per volume ratio is through the inhibition of the apoptotic process.

Thus it is most important that research work is directed to unraveling the mechanisms of inhibition of apoptosis in cancer cells. A little respite, so to say, is the fact that in the last two decades, dedicated research works had shed more light on some of these mechanisms. A particularly instructive explanation is that cancer cells requires a consistent and ample supply of energy in the form of ATP enough to support their ability to continuously grow and proliferate. Coincidentally intrinsic apoptosis is triggered by cellular events occurring inside the mitochondria. It does follow that compounds targeting cancerous cells' mitochondria function are promising candidates for drug development. Recently, PZ has been investigated and reported as possessing anticarcinogenic activity (Yin *et al*, 2020), However, much work still needs be done before the true potential of this plant of many phytochemicals can be fully articulated and in which lies a very robust future in cancer therapy.

2.0 MATERIALS AND METHODS

Experimental Animals

Infant albino rats (Wister) each weighing between (90-125g), were commercially purchased from a local farmer in Ibadan. The animals so obtained were housed and acclimatized to condition at the university's small animal house for a period of five weeks before the commencement of the work. The animals were fed with normal, commercially available and partly pelletized rat chow obtained from CAPS Feed, Airport area, old Ife road Ibadan. Tap water *ad libitum* was freely and adequately made available to the animal's needs.

Experimental Design

This experiment was carried out for five weeks post animal sacrifice. Method of administration of test substance was intubation *per os*. A total of thirty animals were used in this study; divided in equal population for both *in-vitro* and *in-vivo* work respectively. Fifteen animals of the total animal population were further divided into four dosage test groups and a control group, each group comprising of three animals each.

For the *in-vivo* analysis, the test animals were administered (*per os*) with the test sample by intubating a dosage regime of 5mg/kg, 10mg/kg, 20mg/kg and 25mg/kg body weights of the animals. For the *in-vitro* analysis; the effect of PZ was investigated on isolated and processed rat liver mitochondria. The doses used (*p.o*) were 8µg/ml, 20µg/ml, 40µg/ml and 56µg/ml.

Preparation of Crude Plant Extract

The plant investigated was *Plumbago zeylanica. Linn.* The root bark of the herb was freshly obtained from native herbal medical practitioners in part of Osun State of Nigeria. The bark of the root so obtained was carefully peeled and air dried for a period of two weeks at the end of which it was milled to a fine pulp. Subsequent solvent extraction of phytochemicals followed standard protocols.

Preparation of Rat Liver Mitochondria

Animals were sacrificed by cervical dislocation, and dissected. Vital organs including the liver were immediately excised and temporarily stored on ice at 20^{o}C. The rat livers were weighed in a sterilized beaker and then washed with buffer C, (*see appendix*) homogenized in a 10% suspension buffer (*described in appendix*). The mitochondria obtained were immediately re-suspended in an appropriate solution of ice cold MSH Buffer (Mannitol, Sucrose, HEPES, pH 7.4-KOH), it was then dispensed in Eppendorf tubes in aliquot and kept on ice and used fresh. All experiments were carried out in an ice cold medium in order to preserve and retain the mitochondrial integrity. Subsequent mitochondria isolation from hepatic cells was carried in a low ionic salt environment by employing standard differential centrifugation as described by Johnson and Lardy *(1967)*

Assessment of Mitochondrial Transition Permeability Pore (MPTP)

Mitochondria loaded with calcium can be induced to undergo a permeability transition in which the inner membrane becomes non-selectively permeable to small (<1500Da) solutes *(Lapidus and Sokolove, 1994).* Isolated mitochondria undergoing permeability transition (PT) show colloidal osmotic behaviour, with large amplitude swelling which results in a decrease photometric absorption at 540nm. Several experimental permeability transitions were assessed by measuring the swelling of mitochondria by monitoring the associated decrease in light scattering.

Principle

The basis of this method is that when mitochondria swells, their refractive index changes and thus light passing through the cuvette resulted in decrease in absorbance measured with a spectrophotometer. To avoid any complications that changes in the redox state of respiratory chain components might cause, the wavelength of the incident light should be at the isosbestic point for the cytochromes (520nm or 540nm) as used in several studies on isolated mitochondria.

Reagents

All reagents used for this study are of analytical grade purchased from academically renown vendors. For detailed information on how the different buffers used in the study were prepared, see the index section.

Procedure

Mitochondrion swelling was determined according to the method of Lapidus and Sokolove *(1994).* Mitochondria (4mg/ml) were incubated in the presence of 0.8µM Rotenone for 3.5 minutes, prior to the addition of 5mM sodium succinate. When Ca^{2+} was used as a triggering agent, mitochondria were

pre-incubated in the presence of 0.8uM rotenone for 3 minutes. Ca^{2+} was added after the 3 minutes of mitochondria incubation and 30 seconds later, sodium succinate was added.

Assay for spermine inhibition of swelling involved addition of spermine prior to mitochondria incubation with rotenone and allowed to stay for 3 minutes after which Ca^{2+} was added and lastly 30 seconds later, sodium succinate was finally added to the reaction tube. Absorbance was taken at a wavelength of 540nm in a camspec spectrophotometer, every 30 seconds for 12 minutes. The temperature was maintained at 30^{o}C and swelling rate quantified as an A540/min/mg.zw

3.0 FINDINGS

Invivo effects of varying doses of ethylacetate extracts of PZ on mitochondria permeability transition pore in the presence and absence of *Ca2+* From a body of knowledge that can be obtained from vast volumes of literatures available on the medical and health promoting applications of PZ, it is safe to affirm the utilitarian capability of the plant. In the present study effort was made to investigate antimutagenic ability of PZ by studying its effects on the induction of the opening and closing of MPTP. Mitochondrion is a very important organelle in this study because of the fact that besides its calcium regulatory and homeostasis function; mitochondrion is also responsible for the production of the day to day energy need of the cell in form of ATP production. It is also interesting to note that for any living cell to continue to survive, it must have access to ample, untruncated supply of energy and nourishment; both functions are directly and indirectly saddled to the mitochondrion.

Cancerous cells are known to possess mechanisms that synthesize both their protein and energy need autonomously. The theory therefore is that compounds that effectively compromise mitochondria membrane integrity would be promising drug candidates. A very effective way of compromising the mitochondrion integrity is to create a permeability tension in the inner membrane; this could be achieved by creating a high calcium tension.

Calcium is a known inductor of the permeability transition pore of the mitochondria membrane. At a high intracellular Calcium load, cytochrome c, a protein which is normally sequestered within the mitochondrion is released resulting in the opening of MPTP pore which is sufficiently large enough to accommodate the passage of small solutes into the interior of the mitochondrion. This leads to increase in the volume of the mitochondrion; a situation termed ‘swelling’ in membrane biophysics

The results obtained from this experiment revealed a positive inhibition of MPTP pore by ethylacetate extracts in a dosage dependent manner when the experiment was conducted with the triggering agent (Ca^{2+}), except for 5mg/kg (bwt) which showed a slight induction. However, this trend was not repeated when the experiment was monitored in the absence of the triggering agent. All dosage treatment groups of ethylacetate extract in this case exhibited a positive induction of the MPT pore opening. These results are depicted as mean ± standard deviation.

The effects of orally administered ethylacetate extract of PZ was investigated on liver function in order to ascertain hepatic injury, if any, as a direct result of the experiment. This was carried out by quantifying the Aspartate Transaminase (AST) and Alanine Transaminase (ALT) content present in blood serum as biomarkers. Although these enzymes are present in tissues throughout the body, they are most often elevated in patients with liver diseases and may reflect liver injury. Decrease activity of these enzymes were observed in experimental animals treated with ethylacetate extract of *P. zeylanica* as there was no significant difference in a treatment groups when compared with control ($p<0.05$)

Table 1: In-Vivo Effects of Varying Dosages of Ethylacetate Extract on MPT in the Presence of $Ca2^{+}$

Test	Mean ± SD	Induction (fold)
TA (control)	-0.411 ±0.154	26.37
5mg/kg bwt	-0.416± 0.160	26.84
10mg/kg bwt	0.232±0.141[a*]	23.21
20mg/kg bwt	-0.328 ±0.196	29.16
25mg/kg bwt	0.297±0.181[a*]	30.05

* = p < 0.05 a = mean difference is significant when compared with TA

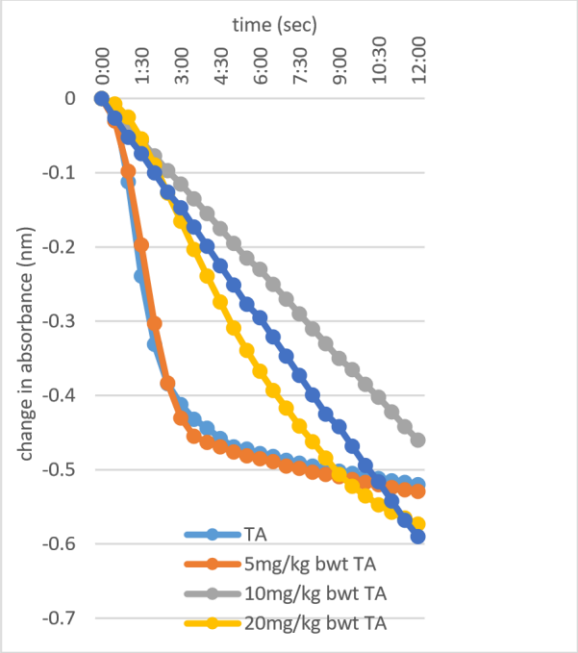

Figure 1: In-Vivo Effects of Varying Dosages of Ethylacetate Extract on MPT in the Presence of Ca^{2+}

Table 2: In-Vivo Effects of Varying Dosages of Ethylacetate Extract on MPT in the Absence of Ca2+

16	Mean ± SD	Induction (fold)
TA (control)	-0.116 ± 0.107	6.21
8µg/ml TA	-0139 ± 0.107	6.56
24µg/ml TA	-0.306 ± 0.177a*	9.67
40µg/ml TA	-0.153 ± 0.118	7.70
56µg/ml TA	-0.220 ± 0.141a*	8.86

* = p < 0.05 a = mean difference is significant when compared with TA

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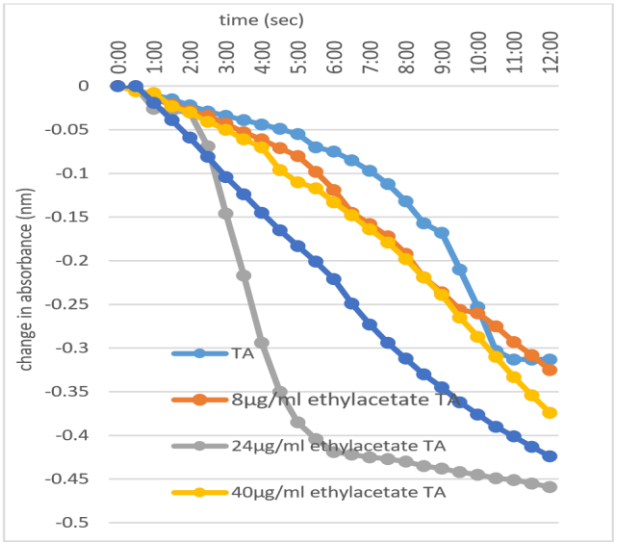

Figure 2: In-Vivo Effects of Varying Dosages of Ethylacetate Extract on MPT in the Presence of Ca2+

Assessment of Histological Parameters

The effect of ethylacetate extract of PZ was investigated on some tissue and organ damage biomarkers such alanine transaminase, (ALT) aspartate transaminase (AST). The result of the experiment showed that there was no significant difference in the activity of AST and ALT assayed in the serum. The results are expressed as mean of the pulled values.

Table 3: Effect of PZ on AST and ALT

U/l	Control	5mgkg	10mg/kg	20mg/kg	25mg/kg
Mean AST	23	16	21	25	31
Mean ALT	51	41	51	48	50

AST and ALT values $p < 0.05$

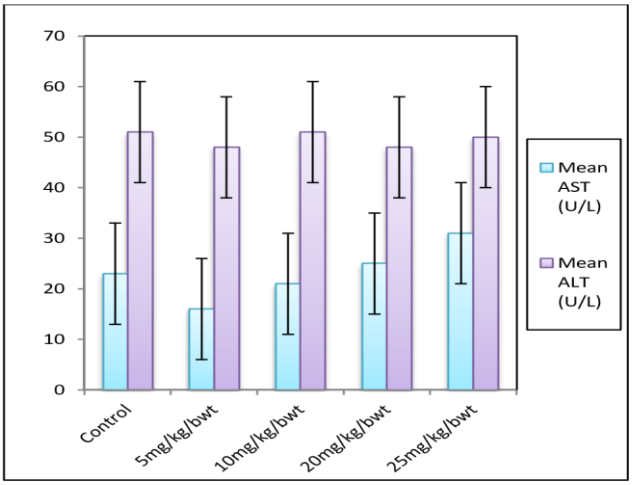

Figure 3: Effect of PZ on AST and ALT

Assessment of Lipid Peroxidation

The effect of ethylacetate extract of PZ was investigated on Lipid peroxidation (LPD) as a biomarker of oxidative damage using thiobarbituric reactive substances assay (TBARS). Result shows an insignificance difference compared to the control.

Table 4: Assessment of LPD

U/l	Control	5mg/kg	10mg/kg	20mg/kg	25mg/kg
LPD	0.195	0.218	0.177	0.152	0.166

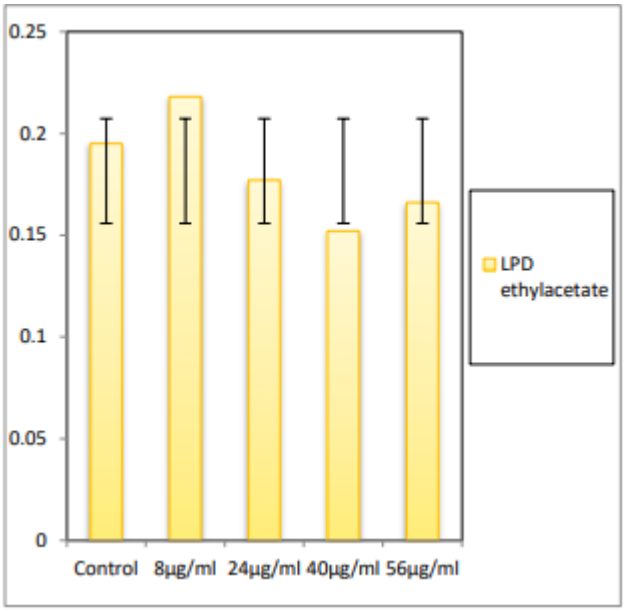

Figure 4: Assessment of LPD

4.0 DISCUSSION, CONCLUSION AND RECOMMENDATIONS

Plumbago zeylanica (PZ) has been described as a multifarious potent herb (Sharma & Singh, 2015). Many of the health benefits of this plant have been scientifically identified and clearly elucidated as far back as three decades ago or even perhaps possibly predating this time period (Mandavkar & Jalalpure, 2011, Shukla *et al*, 2021) Recent works however, have taken a holistic and more in-depth look at the many health promoting activities of PZ using not only the traditional *invitro* but also computer base phytochemical (*insilico*) screening (Krishnan *et al*, 2017; Jenifer *et al*, 2020; Jenifer *et al*; 2021). The results from these works catalogued diverse and comprehensive properties and uses of PZ including action against cancer.

The mitochondrion is perhaps the singular most important organelle in any living organism (of course excluding innate viruses). Even the brain which is the coordinating center of eukaryotes activities cannot survive without ample supply of ATP. ATP is the energy currency of the cell and needed for all cellular activities. Mitochondrion on the other hand is solely responsible for ATP production. Therefore, by the share important functions relative to the functions of all other organelles that makes up the framework of existence of any living cell, tissue or organ; the proper functioning of the mitochondrion is an infallible factor in the equation of metabolism. Due to this summation, it is safe

to rationalize that the mitochondrion is a very important molecular target for drug development (more importantly in combatting cancer). For the mitochondrion to maintain proper functioning, membrane integrity of the mitochondrion must be maintained at all cost. However, it is known that cancerous cells exhibit a hyper-activity in mitochondrion function sustained by intact membrane integrity. Many scholars have reasoned that comprising mitochondrion membrane integrity would lead to induction of membrane permeability, leading to apoptosis i.e. induced death of the cell (Borutaite, 2010, Jeena *et al*, 2019; Liu & Shi, 2020, Jin *et al*, 2022).

Mitochondria permeability transition pore is a molecular channel that when created across the mitochondrion membrane facilitate the release of cytochrome c from the mitochondrion matrix and into the intracellular milieu. This event leads to the activation of other proapoptotic proteins of the Bcl-2 super family and thus culminating in cell death. The present study was therefore conducted to investigate the ability of PZ to induce apoptosis transduced via the intrinsic pathway which involves the mitochondrial membrane integrity. From results obtained the divalent cation Ca^{2+} plays an indispensable role in opening of the mitochondrion membrane via creation of MPTP. In all instances of experiments carried out, there was near none induction by PZ in the absence of Ca^{2+}. However, when PZ was administered in the presence of Ca^{2+}, there was approximately doublefold induction compared to when Ca^{2+} alone was administered both *in-vivo* and *in-vitro* in all dose regimes. This result therefore, is suggestive that PZ maybe playing an attenuation role regards to the Ca^{2+} ability to induce MPTP.

Also, the fact that this study was conducted using ethylacetate as the extraction solvent should not be overlooked. Different solvent extracts of the same plant had been reported to exhibit discrepancies in their various effects (De Paiva *et al*, 2003)). From literatures, aqueous, ethanol or methanol solvents extracts were mostly used to carry out similar experiments (Olagunju *et al*, 1999; Arunachalam *et al* 2010; Ittiyavirah, & Ruby, 2014). It is indeed possible that PZ may have yielded more positive induction of MPTP if this experiment was carried out using different solvent extract.

Dosage regimes used for this study were arrived at after carrying out LD_{50} study. It has been reported that PZ indeed possesses cell cytotoxicity effect. This report informed the doses that were used for the study. However, it is safe to assume that higher doses of PZ extract may indeed potentiate higher induction of MPTP and probably without Ca^{2+}. Also, this study returned no significant effect of ethylacetate extract of PZ on haematological, histological and fertility parameters in all cases of the experiment. Howbeit, these observations may be dependent on dose as the study was conducted within the threshold of dose tolerance dictated by LD_{50} (cut off = 5000mg/kg bwt). It is probable that higher doses may initiate significant cytotoxic effect on some of these parameters.

Conclusion

The results from the experiment conducted revealed that ethylacetate extract of PZ induces the MPTP in a dose dependent manner as expected when all tests were assayed in the presence of the triggering agent, Ca^{2+} (Baumgartner *et al*, 2009; Bauer, & Murphy, 2020; NavaneethaKrishnan *et al*, 2020). This result was not replicated when assays were carried out in the absence of Ca^{2+}. The induction fold calculated as: *[(change in absorbance of test – change in absorbance of control)/change in absorbance of control]* shows positive maximum induction of 9.76 and minimum of 6.56 for 24µg/ml and 8µg/ml doses respectively *invitro* study. For *invivo* study, maximum and minimum induction was 30.05 and 26.84 folds for doses 25mg/kg and 5mg/kg body weight respectively. These results were significantly different ($p<0.05$) to the result obtained for the control in all instances of the experiment.

These results have shown that PZ does indeed possess properties capable of modulating membrane permeabilization of the mitochondria leading to the lowering of membrane potential which may facilitate a sequence of events that may lead to the transduction of apoptosis. However, cannot conclude that PZ acting independently can induce MPTP without the synergistic action of Ca^{2+} (Kerr & Willie, 1972). It is suggested that further work is carried out to assess the comparative of PZ using different solvent extracts and probably in higher doses. It is also suggested that "comet" assay for the assessment of apoptotic bodies should be run as to determine the ability of in transducing apoptosis via MPTP.

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