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Synergistic Effects of Calcium-Protein Energy Malnutrition and Methanolic Extract of *Plumbago zeylanica* Root on Mitochondria Permeability Transition Pore: An *In Vitro* Study

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Abstract

The purpose of this study was to investigate the synergistic effects of calcium ion-protein energy malnutrition (Ca^{2+}-PEM) and methanolic extract of *Plumbago zeylanica* (Pz) root on mitochondria permeability transition pore (MPTP). Twenty-four male Wistar rats were studied. The Wistar rats were divided into two groups (experimental and control) of 12 each. The experimental rats were fed with protein-deficient diet, and the control rats were fed with normal rat chow and water ad libitum for 42 days. To monitor MPTP induction and inhibition in both experimental and control Wistar rats, 3 mM Ca^{2+}, 1 mM Mg^{2+}, 120, 160, and 200 μg/ml of Pz extract were used. The rats were sacrificed, and mitochondria were isolated from the livers to monitor MPTP. Our study showed that Ca^{2+} and Mg^{2+} induced and inhibited MPTP, respectively. However, PEM drastically increased Ca^{2+} and Mg^{2+} MPTP induction and inhibition, respectively, when compared to control. At varying dose and timing, Pz extracts steadily induce MPTP in both experimental and control Wistar rats. Taken together, the results suggest that Ca^{2+}-PEM increased the MPTP induction, while PEM decreased the MPTP induction of Pz extract in dose- and time-dependent pattern when compared to the control that plausibly suggests that PEM may increase Ca^{2+} induction of MPTP as well mitigate therapeutic effects of Pz extract in diseases related to mitochondria targeting.

Keywords: Induction; Inhibition; Mitochondria permeability transition pore; Malnutrition; *Plumbago zeylanica*.

1. INTRODUCTION

Mitochondria are unique organelles because their structures provide compartmentalization of metabolism and are helpful in the maintenance of electron gradient across the membrane that allows generation of adenosine triphosphate (ATP), which is essential for life sustenance [1-3]. Mitochondria generate reactive oxygen radical (ROR) during electron transport and also play a crucial role in the production and transduction of calcium ion (Ca^{2+}) signals. Hence, it becomes imperative to regulate intracellular Ca^{2+} concentration and RORs generated via mitochondria because of its functional role in mediating several cellular activities such as signaling, proliferation, and cell death [4-6].

Several toxic stimuli convert mitochondria from a life-sustaining organelle to an inducer of cell death [4-6]. However, the role of mitochondria in cell death, in this case, involves the mechanisms adopted by the organelle within cells via induction of mitochondria permeability transition pore (MPTP). MPTP induction is characterized as a major event that precedes cell death. These events involved the activity of noxious stimuli such as Ca^{2+} overload, oxidative stress caused by ROR, and several proapoptotic proteins (BCL-2 family) in the inner membrane of the mitochondria [7-10]. BCL-2 family is also localized in endoplasmic reticulum (ER) membranes where they regulate intracellular Ca^{2+} homeostasis in ER [10-13].

MPTP induction causes reduction/deprivation of ATP that is essential for cell proliferation and other diseases related to mitochondria targeting via uncoupling of oxidative phosphorylation. Cancer cells are good examples of such diseases. Starvation of these cells (cancer) of their energy (ATP) demand can attenuate their proliferation, metastasis, and can eventually lead to their death. However, the apoptotic machinery of cancer cell mitochondria is structurally and functionally different from other normal mitochondria in the cell. Under physiologic condition, MPTP of normal mitochondria is closed because of its inner membrane impermeability to ions and metabolites, whereas in stress/pathological condition—cancer, MPTP becomes induced and opens in response to toxic stimuli such as ROR and Ca^{2+} overload. The opening of MPTP triggers the release of cytochrome c. The release of cytochrome c is considered a point of no return in initiating cell death. It becomes imperative to focus and develop a novel drug that will be able to target mitochondria, induce MPTP, and can eventually cause cell death, and that can act as a therapy against diseases related to mitochondria targeting [14].

Protein-energy malnutrition (PEM) is a form of malnutrition capable of generating RORs within cells. RORs have been reported to play a crucial role in MPTP. However, the ROR generated in the cell stimulates excess influx of Ca^{2+} to the cytosol that can eventually find its way to the mitochondria matrix through Ca^{2+} uniporter. This increases the level of Ca^{2+} in the matrix that can further induce MPTP opening [15]. In this case, the free radicals induced via PEM become a factor contributing to mitochondria ROR and may greatly enhance the sensitivity of the MPTP to Ca^{2+} that can eventually lead to MPTP induction. A key

factor in the regulation of MPTP induction is matrix Ca^{2+} content interchange between Ca^{2+} influx and efflux [16]. Matrix Ca^{2+} acts as a permeable factor for most pore inducers, and its activity may be competitively inhibited by Mg^{2+}, Mn^{2+}, and Sr^{2+} [17].

Plumbago zeylanica (Pz) is popularly known as Ceylon leadwort. Indians call it Chitrak, Germans call it Bleiwurz, and in African vernacular (Yoruba) it is called "Ina buru." The root is 30 cm or more in length, 6 mm or more in diameter, blackish red in color, light yellow cultured when fresh, and reddish brown when dry. It has characteristic odor with acrid and bitter taste. *Inter alia,* Pz have been reported to have anticancer activity [18, 19]. The anticancer activity of Pz is attributed to its bioactive compounds and antioxidants activity that greatly aid in induction of MPTP [19].

From the aforementioned description, we investigated the synergistic effects of Ca^{2+}-PEM and methanolic extract of Pz root on MPTP in Wistar rats.

2. METHODS

2.1. Chemicals and Reagents

All chemicals used were of analytical grade supplied by BDH Chemicals Limited, Poole, UK.

2.2. Plant Materials

Pz roots were obtained fresh from a private-owned farm in Ede, Osun State, and authenticated in Plant Biology Department, University of Ilorin with voucher number U.I.H/ 001/1137.

2.3. Extraction Procedure

Fresh roots of Pz were oven-dried at 40°C and pulverized. The pulverized Pz was extracted using Soxhlet extraction apparatus. Briefly, 1000 ml methanol was used in the extraction process of 100 g of pulverized Pz roots. The solvent fraction obtained was concentrated with rotary evaporator. The yield of the crude extract of Pz obtained was 4.25 g. The concentrate was air-dried at room temperature. Then, an aliquot of the concentrate was dissolved in distilled water and was used for analysis.

2.4. Qualitative Phytochemical Screening

The qualitative phytochemical screening of the methanolic extract of Pz was carried out using the procedures highlighted by Ravikumar and Sudha [20].

2.4.1. Alkaloids

The extract of 1 ml was mixed with 5 ml of diluted hydrochloric acid and was filtered. Subsequently, 1 ml of Wagner's reagent was added to 1 ml of the filtrate, and turbid brown color was observed.

2.4.2. Tannins

The extract of 1 ml was stirred with 1 ml of ferric chloride, and greenish-black precipitate was obtained.

2.4.3. Steroids

The extract of 0.5 ml was dissolved in 2 ml of acetic anhydride and cooled in ice before 1 ml of conc. H_2SO_4 was added, and brown color was observed in the reaction mixture.

2.4.4. Flavonoids

Diluted NaOH of 0.2 ml was added to 0.2 ml of the extract, and after shaking gently, a dirty yellowish-brown precipitate was obtained.

2.4.5. Saponin

The extract of 0.2 ml was mixed with 5 ml of distilled water and kept on a shaker for 20 min, and the foam was observed for a while.

2.4.6. Glycosides

The extract of 1 ml was mixed with 2 ml of Fehling's solution and boiled for 5 min, and red color precipitate was obtained.

2.5. Formulation of Protein-Deficient Feed

This was done according to modified methods of Adelusi and Olowookere [21]. Briefly, the compositions of the isocaloric protein diet (per 1000 g) are as follows: fishmeal (30 g), maize shaft (50 g), sucrose (100 g), cornstarch (755 g), vegetable oil (60 g), and vitamin and mineral mix (5 g). The formulated diet was oven-dried at 40°C.

2.6. Animal Management and Experimental Design

Twenty-four male Wistar albino rats of average weight 250 ± 2.44 were housed in a controlled temperature (20-25°C), controlled humidity (40-60%), clean, and disinfected animal facility, with a 12-h light and dark cycle. The rats had unlimited access to normal rat chow and water ad libitum and were utilized after one week of acclimatization. All animal procedures were conducted under an animal protocol approved by the Institutional Animal Care Ethics Committee of the University of Ilorin, Ilorin, Nigeria. The individual weights of the rats were taken after fasting for 24 h prior to the commencement of the experiment. The Wistar rats were divided into two groups (experimental and control) of 12 each. The experimental group was fed with isocaloric protein diet, and the control group was fed with normal rat chow and water ad libitum for six weeks. Data obtained for the average weight changes within groups were recorded on a weekly basis for six weeks in total. The animals were sacrificed 24 h after feeding one at a time by cervical dislocation, and the livers were harvested, immediately washed in ice-cold 0.25 M sucrose solution, and stored in an ice-cold buffer (210 mM mannitol/70 mM sucrose/5 mM HEPES/1 mM EGTA, pH 7.4) at −4°C for further analysis.

2.7. Protein Concentration Determination

The protein level of the rat liver homogenate was measured by the Biuret method [22]. Briefly, 0.1 ml of the diluted liver homogenate was added to a solution containing Biuret reagent (0.4 ml), 3% deoxycholate (0.4 ml), and distilled water (0.5 ml). Subsequently, the contents were gently mixed and incubated for 20 min. The absorbance was measured at 540 nm by using a 752S UV spectrophotometer. Bovine serum albumin was used as the protein standard.

2.8. Mitochondria Isolation

Mitochondria were isolated from the livers according to the method of Johnson and Lardy [23]. Briefly, the 10% liver homogenates prepared in ice-cold medium were centrifuged twice using refrigerated centrifuge at 2300 × g for 5 min. The supernatant was centrifuged at 13,000 × g for 10 min to sediment the mitochondria in the form of pellets. Then, the supernatant was discarded, and the mitochondria pellets were washed twice by suspending in ice-cold isolation buffer (210 mM mannitol/70 mM sucrose/5 mM Tris, pH 7.4) and centrifuged at 12,000 × g for 10 min. The mitochondria were resuspended in ice-cold mannitol sucrose HEPES (MSH) buffer (210 mM mannitol/70 mM sucrose/5 mM HEPES/1 mM EGTA/5 mM Tris/1 mM KH_2PO_4, pH 7.4) and later dispensed into ice-cold Eppendorf tubes in aliquots and stored on ice. Mitochondria were used immediately after preparation for the assay.

2.9. Mitochondria Permeability Transition Pore Assay

MPTP was assayed according to the method described by Lapidus and Sokolove [24] with modification. Briefly, mitochondria (0.4 mg/ml) were preincubated in MSH buffer (210 mM mannitol/70 mM sucrose/5 mM HEPES/1 mM EGTA/5 mM Tris/1 mM KH_2PO_4, pH 7.4) for 3 min 30 s at 30°C in the absence of 3 mM Ca^{2+}, 1 mM Mg^{2+}, and in the presence of 5 mM succinate before the assay was started. The assay was conducted interchangeably with TA—triggering agent ($+Ca^{2+}$), I—inhibitor (Mg^{2+}), NTA—nontriggering agent ($-Ca^{2+}$), and different concentrations (120, 160, and 200 μg/ml) of methanolic extract of Pz. Mitochondria swelling caused by an influx of solutes across the inner membrane was measured at an interval of 10 s for 5 min by recording the decrease in absorbance at 520 nm on a 752S UV spectrophotometer.

3. RESULTS

3.1. Phytochemical Results of Methanolic Extract of *Plumbago zeylanica* Root

The qualitative photochemical screening of methanolic extract of Pz root showed that the plant possessed secondary metabolites excluding steroids that are highlighted in Table 1.

3.2. MPTP Induction and Inhibition in Isolated Liver Mitochondria of Control

In the control group, the biological induction and inhibition of MPTP in the presence and absence of Ca^{2+} and Mg^{2+} in isolated liver mitochondria were examined. In the results, MPTP induction by Ca^{2+} was observed and decreased Ca^{2+} MPTP induction in the presence of Mg^{2+}. The mean values and percentage of MPTP induction and inhibition in the presence of Ca^{2+}, Mg^{2+}, and NTA ($-Ca^{2+}$) are shown in Table 2A. The pronounced effects of MPTP induction and inhibition in the presence of Ca^{2+} and Mg^{2+} *and in the absence of* Ca^{2+} *(NTA)* are graphically illustrated in Figure 1A.

MPTP induction of methanolic extract of Pz was tested at different doses in comparison to MPTP induction of Ca^{2+} as shown in Table 2B. The results showed absolute reduction in the MPTP induction of the extract at 120 and 160 μg/ml when compared to Ca^{2+}. However, at 200 μg/ml, there was pronounced MPTP inductive effect of the extract that overshoots MPTP induction by Ca^{2+} as shown in Figure 1B.

3.3. MPTP Induction and Inhibition in Isolated Mitochondria of Malnourished Animals

In the experimental (protein energy malnourished) group, we also investigated the effects of PEM on induction of MPTP in the presence and in the absence of Ca^{2+}, methanolic extract of Pz, and Mg^{2+}. The results showed increased MPTP induction of Ca^{2+}

Table 1: Qualitative phytochemical results of methanolic extract of *Plumbago zeylanica* root.

Phytochemicals	*Plumbago zeylanica*
Alkaloids	++
Tannins	++
Steroids	−−
Flavonoids	++
Saponine	+
Glycosides	++

KEY: ++ (present); − (absent); + (moderately present).

Table 2A: Percentage MPTP induction and inhibition of control in isolated mitochondria.

Group	Mean ± SEM	Induction/ Inhibition (%)
NTA	−0.019 ± 0.062	
TA (Ca^{2+})	−0.069 ± 0.065	72 (0)
I (Mg^{2+})	−0.037 ± 0.064	49 (32)

Values are expressed as means of thirty-one (31) replicates ± SEM, whereas values in parentheses indicate percentage inhibition.

Figure 1A: Time-dependent course of MPTP induction of TA (+Ca^{2+}), inhibition (Mg^{2+}), and NTA (−Ca^{2+}) in isolated rat liver mitochondria of control.

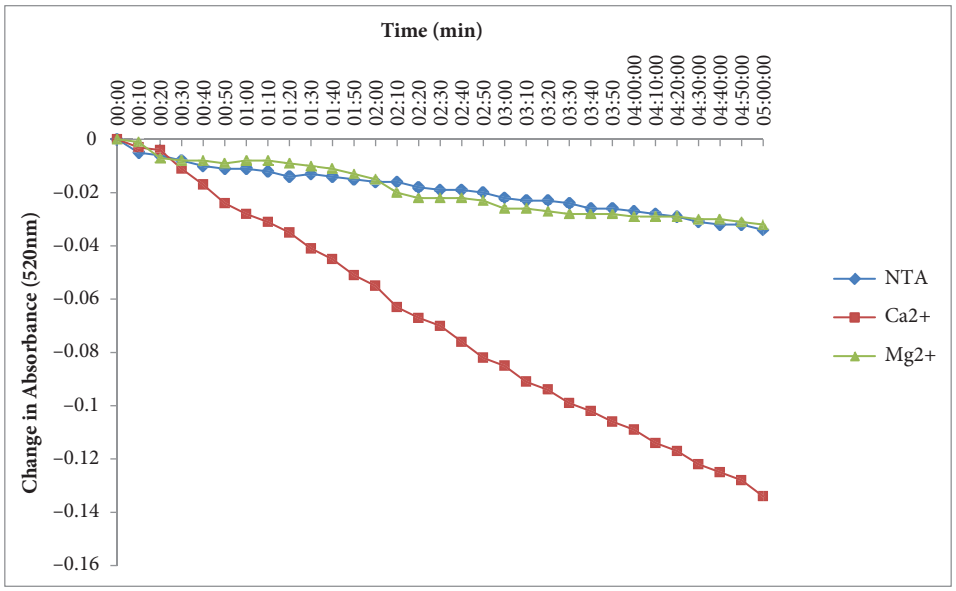


Table 2B: Percentage MPTP induction of methanolic extract of *Plumbago zeylanica* in control.

Group	Mean ± SEM	Induction (%)
NTA	−0.019 ± 0.062	
120 µg/ml	−0.038 ± 0.066	50
160 µg/ml	−0.040 ± 0.066	53
200 µg/ml	−0.125 ± 0.065	85

Values are expressed as means of thirty-one (31) replicates ± SEM.

Figure 1B: Dose- (μg/ml) and time-dependent course of MPTP induction of methanolic extract of *Plumbago zeylanica* (Pz), TA (Ca^{2+}), inhibition (Mg^{2+}), and NTA ($-Ca^{2+}$) in isolated rat liver mitochondria of control.

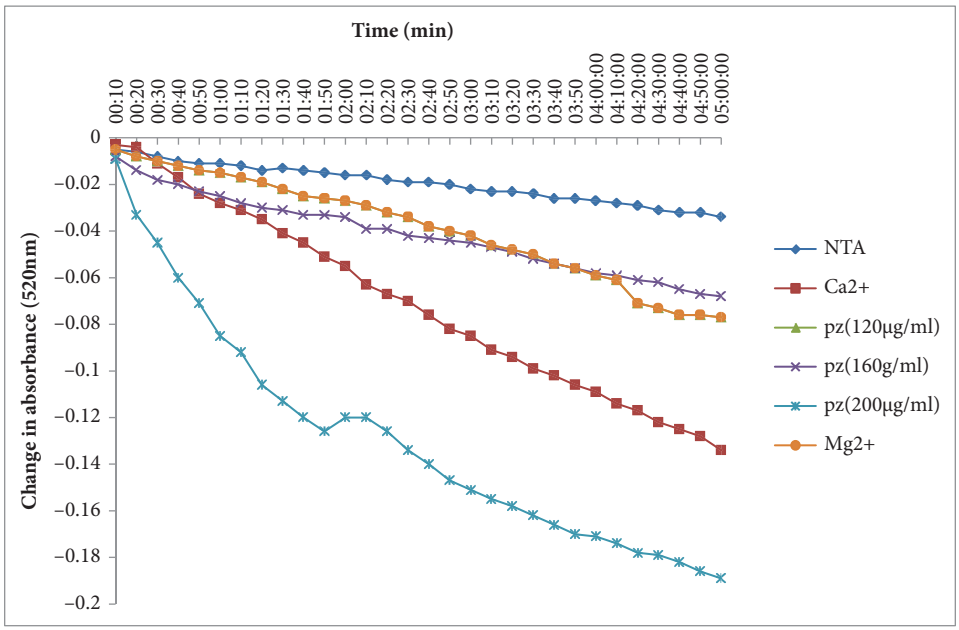


Table 3A: Percentage MPTP induction and inhibition in protein-energy malnourished rats.

Group	Mean ± SEM	Induction/ Inhibition (%)
NTA	−0.013 ± 0.001	
TA (Ca^{2+})	−0.150 ± 0.010	91(0)
I (Mg^{2+})	−0.018 ± 0.002	28 (69)

Values are expressed as means of thirty-one (31) replicates ± SEM, whereas values in parentheses indicate percentage inhibition.

Figure 2A: Time-dependent course MPTP induction of TA ($+Ca^{2+}$), inhibition (Mg^{2+}), and NTA ($-Ca^{2+}$) in isolated rat liver mitochondria of protein-energy malnourished rats.

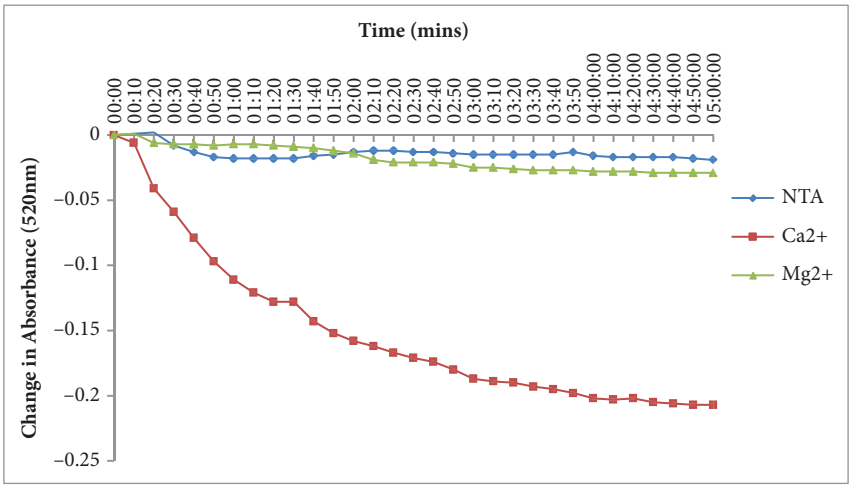
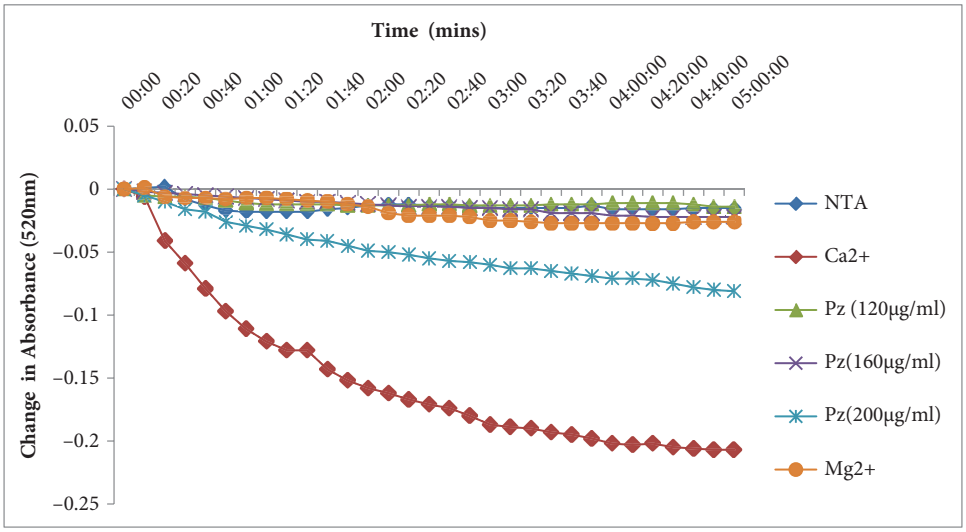


Table 3B: Percentage MPTP induction of methanolic extract of *Plumbago zeylanica* in protein-energy malnourished rats.

Group	Mean ± SEM	Induction (%)
NTA	−0.013 ± 0.001	
120 μg/ml	−0.011 ± 0.001	0
160 μg/ml	−0.013 ± 0.001	18
200 μg/ml	−0.046 ± 0.001	72

Values are expressed as means of thirty-one (31) replicates ± SEM.

Figure 2B: Dose- (μg/ml) and time-dependent course of MPT induction of methanolic extract of *Plumbago zeylanica* (Pz), TA (Ca^{2+}), inhibition (Mg^{2+}), and NTA (−Ca^{2+}) in isolated rat liver mitochondria of protein-energy malnourished rats.



in the PEM state. Ca^{2+} increased MPTP induction in the PEM state of the animals when compared to the presence of both Ca^{2+} and Mg^{2+} in isolated liver mitochondria. However, inhibition by Mg^{2+} is pronounced in this case as shown in their respective percentage induction and inhibition in Table 3A. The significant difference in MPTP induction and inhibition of Ca^{2+}, Mg^{2+}, and NTA (−Ca^{2+}) is graphically illustrated in Figure 2A.

MPTP inductive effect of methanolic extract of Pz was tested at different doses in comparison to MPTP induction of Ca^{2+} in protein-energy malnourished animals in their respective isolated liver mitochondria, as shown in Table 3B. The results showed abrupt decrease in the MPTP induction of the extract at 120 and 160 μg/ml when compared to Ca^{2+}. However, at 200 μg/ml, there was an increase in the MPTP inductive effects of the extract. However, at this dose (200 μg/ml), the effects of the extract on MPTP induction were inefficient to keep pace with the corresponding MPTP induction of Ca^{2+} as shown in Figure 2B.

4. DISCUSSION

The research results reveal that the methanolic extract of Pz induces MPTP at different degrees for both control and experimental (protein energy malnourished) Wistar rats in their isolated liver mitochondria, respectively. The increased Ca^{2+} MPTP induction of experimental animals when compared to the control could be attributed to induced malnutrition. Malnutrition may possibly induce generation of RORs beyond which the endogenous antioxidant enzymes could readily scavenge; such free radicals could act synergistically with Ca^{2+}, thereby stimulating increase in MPTP induction [15]. This suggests that PEM may have a synergistic effect on MPTP induction of Ca^{2+} through ROR generation. These results partly conform to the work reported by Nina *et al*. that Ca^{2+} induced mitochondrial permeability transitions. Similarly, methanolic extract of Pz significantly induces MPTP at different doses and timings that could plausibly be attributed to their bioactive compounds. However, malnutrition induced in experimental animals steadily reduces the activity of the methanolic extract of Pz when compared to the Wistar rats in the control group. The significant alteration in MPTP inductive effect of the extract in experimental group when compared to control could be attributed to ROR produced via PEM. This suggests that the free radicals generated by PEM may have altered the potential

activity of the bioactive compounds in the extract in inducing MPTP as observed in the result. In addition, our results further showed that Mg^{2+} inhibits MPTP induction of Ca^{2+}. This is consistent with the work reported by Bernardi *et al.* that Mg^{2+} inhibits MPTP induction via direct competition with Ca^{2+} for binding at the matrix surface.

5. CONCLUSION

PEM increased the MPTP induction of Ca^{2+} and decreased the MPTP induction of methanolic extract of Pz in dose- and time-dependent pattern. Taken together, PEM increases the MPTP induction of Ca^{2+} and may mitigate the prophylactic/therapeutic effect of the extract in other diseases related to mitochondria targeting.

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Author Contributions
All authors contributed equally.

Conflict of Interest
All authors have no conflict of interest to report.

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