In vitro effects of 2-methyl-3-propylbutane-1,4-diol purified from *Alstonia boonei* on erythrocyte membrane stabilisation and mitochondrial membrane permeabilisation

John Oludele Olanlokun[1*], Olubukola Titilope Oyebode[1], David Popoola[1], Olusola Bodede[2,], Thomas Oyebode Idowu[3], Roshila Moodley[2] and Olufunso Olabode Olorunsogo[1]

1. Laboratories for Biomembrane Research and Biotechnology, Department of Biochemistry, College of Medicine, University of Ibadan, Nigeria
2. Natural Products Research Laboratory, School of Chemistry and Physics, University of Kwazulu-Natal, Westville, Durban, South Africa.
3. Department of Pharmaceutical Chemistry, Obafemi Awolowo University, Ile-Ife, Nigeria.

Abstract

A recent review on the ethnomedicinal, chemical, pharmacological, and toxicological properties of *Alstonia boonei* revealed the plant's potential in the treatment and management of a range of diseases. However, most of these pharmacological effects are only traceable to the crude form of the plant extract and not specific natural products. Phytochemical investigation of the methanol fraction of the methanol extract of the stem-bark *Alstonia boonei* led to the isolation and identification of 2-methyl-3-propylbutane-1,4-diol. The structures were elucidated by the application of 1D-, and 2D-NMR spectroscopic analyses and by comparison with literature data. In this study, the membrane stabilizing activity, mitochondrial membrane permeability transition pore opening, cytochrome c release, mitochondrial ATPase activity and prevention of mitochondrial lipid peroxidation activity of 2-methyl-3-propylbutane-1,4-diol (MPBD) isolated from *A. boonei* were determined. The results showed that MPBD significantly (P<0.05) prevented peroxidation of mitochondrial membrane lipids and hemolysis using both the heat-induced and hypotonic solution-induced membrane stabilization

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assays. On the other hand, the compound caused large amplitude swelling of rat liver mitochondria in the absence of calcium, significant (P<0.05) cytochrome c release and enhancement of mitochondrial ATPase activity *in vitro*. Our findings suggest that MPBD showed characteristic biological properties useful in modulating cell death.

Keywords: *Alstonia boonei,* Cytochrome c, Lipid peroxidation, Mitochondria, , Mitochondrial adenosine triphosphatase.

✉ Oludele John Olanlokun
Jodel72000@yahoo.com
ORCID- 0000-0001-8882-1601

1. Laboratories for Biomembrane Research and Biotechnology, Department of Biochemistry, College of Medicine, University of Ibadan, Nigeria
2. Natural Products Research Laboratory, School of Chemistry and Physics, University of KwaZulu-Natal, Westville, Durban, 4000, South Africa.
3. Department of Pharmaceutical Chemistry, Obafemi Awolowo University, Ile-Ife, Nigeria.

Introduction

Alstonia boonei is well known as a perennial tree with pharmacological properties. Several bioactive compounds with their pharmacological effects have been purified from the plant. Alkaloids such as echitamine and related compounds have been purified from this plant[1]. Recently, we purified tetrahydro-4-(7-hydroxy-10-methoxy-6, 14-dimethyl-15-m-tolylpentadec-13-enyl) pyran-2-one and isobutyryl acetate from the plant and reported their biological effects on some mitochondrial proteins[2]. The mitochondrial permeability transition (mPT) pore is a high conductance channel in the mitochondria whose short opening serves a physiological role in mitochondria. However, long-term opening results in mitochondrial depolarization, low mitochondrial membrane potential, reversal of mitochondrial ATP synthase function to phosphatase, oxidative stress, mitochondrial swelling and release of pro-apoptotic proteins, e.g., cytochrome c into the cytosol[3-4]. We have previously reported the influence of plant extract from *Alstonia boonei*[5] and a medicinal plant cocktail called 'alpha stone' plants on mitochondrial permeability transition pore, the physiological, pathological and pharmacological effects[6]. The mitochondrial permeability transition pore has evolved as a pharmacological target in a variety of diseases[7]. An extensive list of drugs has been reported to delay or prevent permeability transition pore opening, which invariably can be used to prevent tissue degeneration[8]. There is also a quest to find drugs that can cause large amplitude swelling of the mitochondria via induction of the permeability transition pore opening[9]. This is helpful in situations where apoptosis is down regulated.

Although, the exact molecular components of the pore remain elusive, there are different plausible proposals that certain proteins such as the Voltage Dependent Anion Channel (VDAC) of the outer mitochondrial membrane, the Adenine Nucleotide Translocase (ANT)

of the inner mitochondrial membrane and cyclophilin D are components of the pore[9-11]. However, recent evidence has shown the involvement of ATP synthase, a key player in ATP synthesis, in permeability transition pore opening[12]. Biological agents that inhibit mitochondrial respiration, induce mitochondrial swelling or loss of the mitochondrial membrane potential can result in cellular energetic failure via ATP hydrolysis. The mechanism by which this occurs include increase in the cytosolic concentration of inorganic phosphate, Ca^{2+} overloading, loss of osmotic regulation and generation of reactive oxygen species[13].

An increase in reactive oxygen species has been shown to elicit membrane damage via peroxidation of membrane lipids[14]. Cytochrome c release and increase in the cytosolic concentration of inorganic phosphate, among other factors, are important predicators of apoptosis; a crucial biological event in eukaryotic cells, necessary to maintain tissue homeostasis[15]. Crude extracts of medicinal plants and their secondary metabolites have been shown to induce cell death[16-17]. *A. boonei* is a medicinal plant that is commonly used for the treatment of several ailments including, rheumatic and inflammatory disorders[18].

In this study, we present a new compound purified from the methanol fraction of *A. boonei* and some of its biological properties including its interaction with erythrocyte membranes. Erythrocytes have organized membrane structures, which interact and react to drugs and drug candidates[19]. Natural products such as saponins have been shown to have hemolytic properties via the release of hemoglobin from the red blood cells into the surrounding fluids[20]. While several studies have established a strong link between structure-activity relationships on bioactive compounds, it has been shown that the role played by functional groups of these compounds may be critical to their biological functions. Although, several studies have

established the presence of some natural products in *Alstonia boonei* and their pharmacological response has been well studied[1]. It is difficult to purify and identify all phytochemicals present in a medicinal plant in a single study, therefore, a careful repeat of purification procedures of a once studied plant may lead to the discovery of novel and important natural product(s). Moreover, most pharmacological studies of medicinal plants terminate at the extract and fraction level hence, it is better to further purify extracts of medicinal plants to determine the biological effects of new compounds isolated. Furthermore, this will help to ascribe the biological effects of these medicinal plant to specific phytochemicals and possibly optimize their pharmacological use. It is in this regard that we investigated the erythrocyte membrane stabilizing and mitochondrial pore opening potential of this new phytochemical from the methanol fraction of *A. boonei* stem bark extract.

Experimental

Extraction of the stem bark of *Alstonia boonei*

The stem bark of *A. boonei* were peeled from the plant growing in an uncultivated farmland in Ibadan. Samples were authenticated by Mr. Omotayo F.L., Ekiti State University Herbarium and a specimen (UHAE 013) was deposited in the herbarium. The stem bark (100 g) was air-dried at room temperature for five weeks, crushed and soaked in methanol (MeOH) for 72 h with constant shaking. The extract was sieved and concentrated using a rotary evaporator at 40 ºC under reduced pressure.

Purification of the bioactive compound

The MeOH fraction (2 g) was fractionated successively using ethyl acetate (EtOAc, 100%), EtOAc: MeOH (1:1) and MeOH (100%) on vacuum liquid chromatography. Based on biological activity, using the mitochondrial permeability transition pore opening, the EtOAc-MeOH fraction was further purified on column chromatography starting with 100% chloroform that was sandwiched and stepped

by 10% to 100% EtOAc. - Subsequently, the polarity of the eluting solvent system was increased by adding MeOH at 5% intervals until 20% MeOH.. Certain volume of eluting solvent system (200 mL or 300 mL) was added to obtain eight fractions of each eluting solvent system. These collected fractions were analysed by TLC in (write the solvent system used, e.g. CH_2Cl_2 - MeOH (9 : 1.5) and fractions having the same TLC patterns were bulked together, concentrated *in vacuo* to dryness and weighed; resulting in five fractions coded A (1-9), B(10-27), C(28-42), D(43-72) and E(73-104). Fraction C was further purified using preparative TLC to yield 2-methyl-3-propylbutane-1,4-diol. The purified compound was subjected to 1D (^1H and ^{13}C) and 2D (COSY, HMBC and HSQC) nuclear magnetic resonance (NMR) spectroscopic analyses for full characterization and identification.

Isolation of mitochondria from rat liver

Mitochondria were isolated from Wistar rat liver according to a previously described method by Johnson and Lardy[21]. The rats were sacrificed by cervical dislocation, dissected, the liver was removed, rinsed in isolation buffer (210 mM mannitol, 70 mM sucrose, 5 mM HEPES-KOH (pH 7.4) and 1 mM EGTA), and a 10% minced suspension of the liver in isolation buffer was homogenized on ice-cold medium. The homogenate was loaded into a cold centrifuge (Sigma 3-30K, Germany) and spun twice at 590 g for 5 min each to remove cell and nuclear debris and unbroken cells. The supernatant was spun at 19000 g for 10 min to sediment the mitochondria. Mitochondria pellets were washed twice with washing buffer (210 mM mannitol, 70 mM sucrose, 5 mM HEPES-KOH (pH7.4) and 0.5% BSA) at 16000 g for 10 min each time after which, mitochondria were dispensed into aliquots in Eppendorf tubes with suspension buffer (210 mM mannitol, 70 mM sucrose and 5 mM HEPES-KOH (pH7.4)). Mitochondria used for ATPase activity were isolated similarly except sucrose buffer (25 M) was used throughout.

Mitochondrial Protein determination

Mitochondrial protein was determined as previously described using bovine serum albumin (BSA) as the standard[22]. Mitochondria (10 µL) were pipetted into 990 µL of distilled water and 3 mL of a 100:1:1 solution of 2% Na_2CO_3, 2% Na-K tartrate and 2% $CuSO_4.5H_2O$ was added. The mixture was incubated at room temperature for 10 min, after which, a five-fold diluted Folin-Ciocalteau (0.3 mL) was added. This was incubated at room temperature for 30 min, vortexed and the absorbance was read at 750 nm using a spectrophotometer (752N UV-Visible spectrophotometer, China). Mitochondrial protein was quantified from the BSA standard curve.

Mitochondria permeability transition pore opening

The opening of the mitochondrial pore by MPBD was assessed according to the method described by Lapidus and Sokolove[23]. This method was first used to determine the quality and suitability of the isolated mitochondria for mPT assay as follows: Mitochondria protein (0.4 mg/mL) was incubated in suspension buffer in the presence of rotenone (0.8 µM) for 3.5 min after which, succinate (5 mM sodium succinate) was added and the absorbance was monitored at 540 nm for 12 min at 30 sec intervals. Furthermore, the pore opening effect of calcium; a standard pore opening inducer was monitored using the same mitochondria protein incubated in suspension buffer in the presence of rotenone for 3 min after which, calcium (3 µM $CaCl_2$) was added. Thirty seconds later, succinate was added to energize the medium and the absorbance was read. Spermine reversal of calcium-induced opening was done by incubating the same mitochondria protein quantity in suspension buffer in the presence of rotenone and 5 mM spermine for 3 min. Thereafter, calcium was added. The reaction medium was energized with succinate 30 sec later and the absorbance was monitored for 12

min at 30 sec intervals. Mitochondria whose calcium-induced pore opening is effectively reversed by spermine to the tune of ≥80% were adjudged to be intact, retain their integrity, uncoupled and therefore, suitable for the assay.

To assess the effect of MPBD in the absence of calcium on mPT, mitochondria were incubated in the presence of graded concentrations (10-80μg/mL) of this compound in suspension buffer and rotenone and further energized with succinate. Effect of MPBD on mPT in the presence of calcium follows similar process only that calcium is appropriately added.

Measurement of Mitochondrial F_0F_1ATPase activity as an index of ATP hydrolysis

Sucrose (0.25 M), KCl (5 mM) and Tris-HCl (0.1 M) were dispensed into test tubes in triplicates per group and the entire volume in each test tube was made up to 1 ml with distilled water. Adenosine triphosphate (0.01 M) was added to all test tubes in the groups except the group labelled 'mitochondria only' tubes. Mitochondria (0.5 mg/mL) were added to all test tubes in the groups except the test tubes in the group labelled 'ATP only'. Uncoupler (25 μM, 2,4-dinitrophenol) was added to the test tubes in its group while 10% sodium dodecylsulphate (SDS) was added to the test tubes in the group labelled 'zero time' immediately after the addition of mitochondria. Varied concentrations of the test compound (10-80 μg/mL) were added to the 'test groups' containing reagents, ATP and mitochondria. The volume in all the test tubes was made up uniformly and the test tubes were transferred to the shaker water bath and they were incubated at 27ºC for 30 min. After incubation, 1 mL of SDS (10%) was added to all the tubes except the test tubes in the group labelled'zero time' tubes to stop the reaction. Thereafter, 1 ml each of the mixture was transferred into separate test tubes and 1 mL each of 1.25% ammonium molybdate in 6.25% H_2SO_4 and 9% ascorbate were added successively and the solution was allowed to stand for 30 min. The absorbance

of the blue color was read at 660 nm. The concentration of inorganic phosphate was estimated from a standard curve prepared using 1 mM solution of potassium dihydrogen phosphate, treated like the deproteinized mixture[24].

Cytochrome c release determination

Cytochrome c release was determined as previously described[25]. Briefly, mitochondrial protein was pre-incubated in suspension buffer, rotenone and graded concentrations (10-80 µg/mL) of the test compound for 3.5 min, thereafter, succinate was added. For the negative control, mitochondria were pre-incubated in the absence of calcium whilst in the positive control mitochondria were pre-incubated in the presence of calcium. The test compound was similarly treated in the absence of mitochondria and the incubation in each case lasted for 30 min after which the mixtures were spun at 16000 g for 10 min. The absorbance of the supernatant was read at 414 nm while the absorbance of the drug control was deducted from that of the test groups. The concentration of cytochrome c release was estimated from the cytochrome c standard curve.

Evaluation of lipid peroxidation

A modified TBARS assay[26] was used to measure the extent of lipid peroxidation using mitochondria as lipid rich media. Mitochondria (1 mg/mL) were incubated with graded concentrations of MPBD (10-80 µg/mL), 50 µL of 0.07 M $FeSO_4$ to induce lipid peroxidation and the mixture was incubated for 30 min at 37 ºC. Thereafter, 1.5 mL of 20% acetic acid (pH 3.5) and 1.5 mL of 0.8% (w/v) thiobarbituric acid in 1.1% SDS were added and the resulting mixture was vortexed and then heated at 95 ºC for 60 min. After cooling, 3.0 mL of butan-1-ol was added to each tube and centrifuged at 1000 g for 10 min. The absorbance of the organic upper layer was measured at 532 nm and inhibition of lipid peroxidation was calculated accordingly.

Membrane stabilizing activity

Heat-induced hemolysis

The procedure for the membrane stabilizing assay was carried out as previously described[27]. The 2 mL assay mixture contained 0.5 mL hyposaline, 1.0 mL of 0.15 M sodium phosphate buffer (pH 7.4), varying volumes of isosaline and 0.5 mL of bovine erythrocyte suspension. The drug control assay contained the above volumes of buffers and reagents without erythrocyte suspension while the blood control contained all the reagents except the drug. The reaction mixtures were incubated at 56 ºC for 30 min in a water bath and cooled to room temperature. Thereafter, mixtures were centrifuged at 1000 g for 5 min and the absorbance of the supernatant was read at 560 nm against the test blank; the buffered sodium chloride solution served as blank. The percentage membrane stability activities were estimated as follows:

$$\frac{\text{100-(drug test value –drug control value)} \times 100}{\text{Control value}}$$

Hypotonic solution-induced hemolysis

Using the hypotonic solution-induced erythrocyte lysis, whole bovine blood was centrifuged and blood cells were washed three times with 154 mM NaCl in 10 mM sodium phosphate buffer (pH 7.4) through centrifugation for 10 min at 1000 g. The sample tubes contained 0.50 mL erythrocyte suspension mixed with 5 mL of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing the compound (2.0 mg/mL) or acetyl salicylic acid(0.1 mg/mL). The control sample consisted of 0.5 mL of erythrocytes mixed with hypotonic buffered saline alone. The mixture was incubated for 10 min at room temperature, centrifuged for 10 min at 1000 g and the absorbance of the supernatant was measured at 540 nm. Acetyl salicylic acid was used as the positive control.

Statistical analysis

Representative profiles were used for the determination of mitochondrial permeability transition while other results were presented as mean ± SD of triplicate determinations. Data were analyzed using descriptive statistics and one way ANOVA with Tukey multiple comparison post-hoc test to compare groups.

Results

In this study, a single compound from the MeOH extract of *A. boonei* was recognized as a mitochondrial pore opening inducer. This compound was therefore isolated, characterized and identified for further study.

Characterisation of the isolated compound

Brown amorphous solid; IR *V*max KBr cm^{-1}: 3407.93, 3378.652, 2906 and 1043.12.^{1}HNMR ($CDCl_3$, 500 MHz): δ_H3.61-3.65 (2H, m, H-1), 1.54-1.56 (1H, m, H-2), 1.18 (3H, H-2a), 1.57-1.60 (1H, m,H-3), 3.57-3.60 (2H, m, H-4), 1.19-1.23 (2H, m, H-1′), 1.34-1.39 (2H, m, H-2′), 0.92 (3H, H-3′). ^{1}HNMR ($CDCl_3$, 500 MHz): δ_C64.71 (C-1), 34.87 (C-2), 14.8 (C-2a), 47.5 (C-3), 62.78 (C-4), 25.90 (C-1′), 18.88 (C-2′), 10.13 (C-3′).

Structure elucidation and Identification of the isolated compound 1, (MPBD)

The ^{1}HNMR spectrum of the isolated compound revealed non-aromatic signals and were analysed together with the aid of ^{1}H/^{1}H COSY and HMBC spectral data, which showed geminal oxygenated methylene proton multiplets at δ_H3.61 – 3.65 (2H, m, H-1) with correlated to the resonance at δc 64.71. A second geminal oxygenated methylene proton multiplet at δ_H3.57 – 3.60 (2H, m, H-4) that correlated to the resonance at δc 62.78 was also observed. The ^{1}HNMR spectrum also revealed a methine resonance at δ_H 1.54-1.56 (1H, m, H-2) that correlated with the resonance at δc 34.87 and coupled to the single methyl resonance at δ_H1.18 (3H, d, *J*=6.8Hz, H-2a) that correlated to δc 14.8.

A methine resonance at δ_H 1.57-1.60 (1H, m, H-3) that correlated to δc 47.50and which coupled to a propyl group at δ_H 1.19-1.23 (2H, m, H-1′) that correlated to δc 25.90 were also observed. A methylene resonance at δ_H 1.34-1.39 (2H, m, H-2′) that correlated to δc 18.88 and which coupled to a methyl resonance at δ_H 0.92 (3H, H-3′) that correlated to δc 10.13 was observed. The $^1H/^1H$ COSY indicated the correlations of H-1 to H-2; H-2 to H-2a and H-3; H-3 to H-4; H-1′ and H-2; H-1′ cross peak to H-3 and H-2′. Based on spectral data and HMBC correlations (Table 1, Figure 1) the isolated compound was identified as 2-methyl-3-propylbutane-1,4-diol (MPBD). The spectral data for MPBD are similar to those reported for 2,3-diethylbutane-1,4-diol[28].

Table 1 ^{13}C NMR, COSY and HMBC data of the isolated compound in $CDCl_3$ at 500 MHz

No	Type of Carbon	δ_C ppm	COSY	HMBC
1	CH_2OH	64.71	H-2, H-3, H-2a	34.87, 14.80, 47.50
2	CH	34.87	H-1, H-3, H-2a	14.80, 47.50, 64.71
2a	CH_3	14.80	H-2, H-2, H-3	34.87, 64.71, 47.50
3	CH	47.50	H-1′, H-4, H-2	25.90, 18.88, 62.78, 34.87
4	CH_2OH	62.78	H-3	47.50, 34.87
1′	CH_2	25.90	H-2′, H-3′, H-3	47.50, 18.88, 10.13
2′	CH_2	18.88	H-1′, H-3′	47.50, 25.90, 10.13
3′	CH_3	10.13	H-2′, H-1′	18.88, 25.90

Effects of 2-methyl-3-propylbutane-1,4-diol (MPBD) on mitochondrial permeability transition

The effects of isolated compound, 2-methyl-3-propylbutane-1,4-diol (MPBD)on mPT, *in vitro*, both in the absence and in the presence of calcium are presented in Figure 2. Initially, the integrity of the isolated mitochondria incubated in rotenone, respiring on succinate in the absence and presence of calcium was established by taking the rate of decrease in absorbance at thirty seconds interval for a period of twelve minutes. It was discovered that in the absence of calcium, there was no significant

change in absorbance for those periods. However, in another experiment, when the same quantity of mitochondria (quantified as 0.4 mg/mL mitochondrial protein) was incubated with rotenone and succinate in the presence of calcium, there was an increase in the change in absorbance denoting a large amplitude swelling of the mitochondria, signifying the opening of the mitochondrial pore. This shows that isolated mitochondria responded to calcium, a standard pore inducer. In another experiment still to confirm that the isolated mitochondria were intact, we incubated the isolated mitochondria in rotenone and succinate in the presence of calcium and a standard pore inhibitor, spermine. It was observed that spermine abrogated the effect of calcium by reversing its (calcium) pore opening effect. Isolated mitochondria with negligible opening of the pore measured as insignificant changes in the absorbance, with significant decrease in the absorbance upon the addition of calcium in which the inductive effect of calcium was favorably reversed by spermine were considered intact and therefore suitable for further use (Figure 2a). These steps were first carried out in each experiment to ascertain the integrity of the isolated mitochondria before the determination of the effects of the purified compound so as not to attribute pore opening or inhibition (whichever the case) as a result of mitochondrial damage to drug or drug candidate's effect(s). Subsequently, the effect of the purified compound on mitochondrial pore in the absence of calcium was investigated. It was discovered that all the concentrations used induced the opening of the pore in a concentration-dependent manner. Again, it was also observed that MPBD, at the lowest concentration used in this study (10 μg/mL) induced mPT pore openning more than the effect of calcium. Although, the pore opening effect of MPBD could not be ascertained whether it was in a specific sequence or series, it was however, noticed that the difference between 10 mg/ml and 20 mg/mL was in many folds compared with the difference between 20 mg/mL and 40 mg/mL or 40 mg/mL and 80 mg/mL (Figure 2a). Whichever the case, all concentrations of MPBD opened the mPT pore in the absence of calcium.

However, in the presence of calcium it was observed that it was only the highest concentration (80 mg/mL) that potentiated the calcium-induced opening of the pore. The pore opening effect of the 40 mg/mL concentration was slightly observed though not as high as that of calcium. Interestingly, the pore opening effects of 10 and 20 mg/mL concentrations that readily opened the pore in the absence of calcium were not observed in the presence of calcium. This implies that MPBD has additive pore opening effect only at the highest concentration as observed in this study (Figure 2b).

MPBD enhances cytochrome c release, F_0F_1 ATPase activity but inhibits lipid peroxidation

In this study, the effects of MPBD on *in vitro* cytochrome c release, activity of mitochondrial F_0F_1 ATPase and lipid peroxidation using the mitochondria as the lipid-rich media were monitored. The results show that in the absence of calcium, only a minimal amount of cytochrome c was released. However, in the presence of MPBD at 10 µg/mL, a significant amount of cytochrome c were released. It was noticed that as the concentration of MPBD increased, there was increase in the concentration of cytochrome c that was released into the reaction medium. It was noticed also that although, concentration-dependent increase in cytochrome c was observed, when isolated mitochondria were treated with MPBD, significant increase (P<0.05) was observed when mitochondria were treated with 40 µg/mL compared with 20 µg/mL concentration of MPBD. It was observed also that although, maximum cytochrome c release by MPBD was observed at 80 µg/mL, there was no significant difference between the effect of exogenous calcium and 80 µg/mL concentration of MPBD (Figure 3a).The effect of MPBD on mitochondrial ATPase at physiological pH (7.4) is represented in Figure 3b. While there was significant difference (P<0.0001) between the effect of various concentrations (10-80 µg/mL) of MPBD and the control, it was also observed that 20, 40 and 80 µg/mL of the isolated compound significantly (P<0.01) enhanced the activity of mitochondrial F_0F_1 ATPase when compared with the least concentration (10 µg/mL) of MPBD used in this study. It is interesting to

note also that the uncoupler (25 μM of 2, 4-dinitrophenol) enhanced mitochondrial F_0F_1 ATPase activity (P<0.01) than the highest concentration of MPBD (80 μg/mL) used in this study. Again, it was noticed that 20, 40 and 80 μg/mL of MPBD enhanced mitochondrial F_0F_1 ATPase activity in concentration-dependent manner albeit insignificantly (Figure 3b). The effect of MPBD on peroxidation of mitochondrial membrane lipids is presented in Figure 3c. It was observed that MPBD significantly (P< 0.05) inhibited the peroxidation of mitochondrial membrane lipids both at 40 and 80 μg/mL when compared with the least concentration (10 μg/mL) used in this study. There was no significant difference between the effect of 20 μg/mL and 10 μg/mL concentrations of MPBD on the inhibition of peroxidation of mitochondrial membrane lipids (Figure 3c).

MPBD stabilizes erythrocyte membrane

Figure 4 shows the effects of MPBD on erythrocyte membrane stability using both heat-induced and hypotonic solution-induced membrane destabilization methods. In Figure 4a, it was observed that MPBD stabilized erythrocyte membrane maximally at the highest concentration although, there was no significant difference between the effects of the lowest and the highest concentrations. In the hypotonic solution-induced method of membrane destabilization however, it was observed that MPBD reversed membrane destabilization maximally at 20 μg/mL when compared with 10 μg/mL (P<0.01) or 40 μg/mL(P<0.05) and highly significant (P<0.05) when compared with acetylsalicylic acid, the control drug (Figure 4b).The results obtained show that while MPBD stabilize the erythrocyte membranes in a concentration dependent manner using the heat-induced method, its stabilizing effect did not linearly correlated with its concentrations when hypotonic solution-induced method of membrane destabilization was used. However, in both methods, MPBD reversed membrane destabilization either by using hypotonic solution or heat.

Discussion

The identification of the isolated compound was achieved on the basis of one-dimensional and two-dimensional NMR spectroscopy and comparison with a similar alkyl di-substituted butane-1,4-diol in literature. There are no reports of isolation of alkyl di-substituted butane-1,4-diol from plants hence, this is reported here for the first time. Simple aliphatic and polar molecules have previously been analyzed and purified in plant and they form part of the complex nature of plant-derived bioactive compounds[29]. However, butane-1,4-diol and its derivatives have been employed in the synthesis of bioactive lactones[27] and other fine chemicals[30].

Mitochondrial permeability transition (mPT) pore opening has evolved as a pharmacological target in which cells can selectively be commuted to death. Once activated, mPT unselectively allows the passage of solutes and water across the inner membrane of mitochondria. The biphasic opening (short-term and long-term) of the pore has both physiological and pathological implications. The latter event may occur as a result of increases in calcium overload, reactive oxygen generation, ATP consumption, mitochondrial membrane depolarization as well as mitochondrial swelling and consequent release of pro-apoptotic proteins are important events that determine the life and death of the cell[31]. Again, the opening of the pore can also be induced during pharmacological intervention for the treatment of some diseases[32]. It is in this regard, that naturally occurring compounds that can influence these processes are considered as pharmacological tools for determining the fate of cells.

Natural compounds that have been classified as mitochondrial permeants are either pro-oxidants or antioxidants[33]. In this study, 2-methyl-3-propylbutane-1,4-diol (MPBD) was isolated from *A. boonei*. The interaction of MPBD with mitochondria and the concomitant release of cytochrome c can likely

be viewed in two ways; this is because of the alkyl and hydroxyl groups present on the compound. The alkyl group of natural products has been found to be a linker connecting another active portion that is considered to be pro-drugs carrying the active principle to mitochondria[33]. On the other hand, the functional group (hydroxyl group) is critical to the pharmacological role of some natural products. For example, the hemolytic effects of some saponins have been linked to the structural differences in the sapogenin (aglycone), sugar residue, and the number of OH, CH_2OH and COOH groups and where the OH group is ionizable to donate free proton, such compound may have antioxidant properties. Interestingly, alkanediols, the group of organic compounds to which MPBD belongs are protic molecules, meaning that they can donate protons[34]. All these features may be responsible for the biological functions of MPBD by interacting with mitochondria to elicit the pore-opening effect. The purified MPBD is a small molecule by virtue of its molecular size. This may be the reason for its extensive localization and interaction with mitochondria. Lipophilic small molecules or those with positive potentials can interact with mitochondria membrane readily than giant molecules. Sometimes, they may have affinity for some receptors or substances that are secreted by mitochondria in order to elicit their biological effects. This way, small molecules may cause mitochondrial damage or activate intrinsic activation of apoptosis[35].

The opening of the mPT is a critical event in mitochondrial-mediated cell death. The large amplitude opening of the mitochondrial pore observed in the presence of MPBD showed that the mPT can be targeted using this compound both for pharmacological and clinical purposes especially in a variety of diseases where apoptosis is deregulated. Furthermore, the opening of the pore that was observed in the presence of calcium at the highest concentration showed that, at this concentration, MPBD potentiate calcium-induced opening of the pore.

One of the major reasons for long-term opening of the mitochondrial pore is the reduction in the mitochondrial membrane potential. To overcome this membrane potential loss, ATP synthase works in reverse mode, hydrolyzing ATP (as an ATPase enzyme). Therefore, a major outcome of mPT opening is the reversal of ATP synthase to function as an ATP hydrolyzing enzyme rather than synthesizing it[29]. In this study, MPBD was found to be a potent biological compound that significantly enhanced mitochondrial F_0F_1ATPase activity. This may be because it is able to cause a decrease in mitochondrial membrane potential or mitochondrial uncoupling. Mitochondrial uncoupling, a pathway that enables proton re-entry into the matrix independent of ATP synthesis, is an energy dissipating cycling that occurs in all eukaryotic cells[36].

Measured in this study as an increase in dephosphorylation efficiency of the mitochondrial ATPase, mitochondrial uncoupling is not entirely harmful because there is an inverse relationship between the proton leak and reactive oxygen species generation in isolated mitochondria. Therefore, therapeutic mitochondrial uncoupling is protective against some disorders such as obesity, diabetes, Parkinson's disease and aging [37-41]. Furthermore, recent findings on the involvement of mitochondrial ATPase in mPT formation may further make this enzyme a therapeutic target in a number of diseases.

Cytochrome c is a small soluble electron carrier heme-protein that facilitates cell energy production by transferring electrons from complex III to IV. A consequence of the large amplitude swelling of mitochondria is the release of cytochrome c into the cytosol, which results in cell apoptosis. In this study, MPBD significantly increased cytochrome c release. Previous studies provided both clinical and experimental evidences of cytochrome c release being an indication of cell death through either apoptosis or necrosis[42]. In this study, it was observed that the purified small molecule from *A. boonei*, MPBD, mediated cytochrome c release. This may be as a result of MPBD dislodging the protein from its anchor, thus affecting the membrane-bound cytochrome c pool and consequently affecting

electron transport and energy generation. Cytochrome c release is a critical event in cell death and monitoring its extracellular release may be used as a marker to monitor cell death[43].

Lipid peroxidation damages membrane phospholipids and can also act as a signal for cell death. In this study, MPBD prevented peroxidation of mitochondrial phospholipids, indicating that the cell death-inducing effect of MPBD was not via reactive oxygen species generation and peroxidation of mitochondrial membrane phospholipids. It interesting to note also that cell death effect of MPBD may not be via mitochondrial membrane damage since the integrity of mitochondrial membrane is intact. The results obtained in this study also show that MPBD prevents erythrocyte membrane damage. This indicates that, although the compound could induce mPT pore opening and subsequent cell death via the mitochondrial pathway, it also prevents membrane de-stabilization. This selectivity, coupled with the molecular size have advantages for the biological function and cell permeabilization by MPBD. Molecules that can cross the cell membranes easily are needed in medicine and research[44]. The organization of the cell membranes allows small molecules to permeate and cross the cells easily because of their molecular size. Using Lipinski's "Rule of 5", all parameters favor the predictive passive permeation of cell membrane by MPBD and its bioavailability in the context of small molecule drug development. Its molecular weight is 146 (as against a maximum of 500), there are no more than five hydrogen bond donor (MPBD has 2), there are no more than ten hydrogen bond acceptor (MPBD has 2)[45]. The selective release of cytochrome c and mitochondrial membrane permeabilization by MPBD but inhibited lipid peroxidation show that this molecule can commute some cells to death without causing a gross damage to other cells. The release of cellular contents during necrotic cell death where cell membranes are lysed can cause further damage by chains of reaction. This shows the potential of MPBD as a good drug candidate for the induction of apoptosis in cells but not necrosis. Furthermore, it shows that the outer membranes of the cells being commuted

to death may be intact making treatment with this drug candidate selective thereby preventing further injury.

Conclusion

This study showed that the isolated compound, 2-methyl-3-propylbutane-1,4-diol (MPBD), from *A. boonei* caused mitochondrial-mediated cell death via the induction of mPT, enhancement of mitochondrial F_0F_1 ATPase and cytochrome c release but selectively prevented lipid peroxidation and erythrocyte membrane de-stabilization. Being a small molecule and because of its total number of hydrogen bond donor and acceptor, its bioavailability and membrane permeation is high. Thus, this molecule meets the requirement of the use of small molecule in drug development. Therefore, the compound could find use in applications requiring selective cell death or as inhibitors of lipid peroxidation or as a proton donor.

Competing interest

The authors declare that no competing interest exists.

Authors Contribution

Olanlokun JO conceived and designed this study. Material preparation, data collection and analysis were performed by [Olanlokun JO and Popoola D], cytochrome c release assay and data analysis were performed by [Oyebode OT]. Spectroscopy was performed by [Bodede O and Moodley R] and interpreted by [Idowu OT]. The first draft of the manuscript was written by [Olanlokun JO] corrected by [Olorunsogo OO] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Figure captions

Fig. 1 The structure of 2-methyl-3-propylbutane-1,4-diol showing 1D NMR (^{1}H (A), ^{13}C (B)) and key 2D (Correlation spectroscopy (COSY) (C) and Heteromuclear Multiple Bond Correlation HMBC (D)) analysis.

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Fig. 3 Effects of 2-methyl-3-propylbutane-1,4-diol (MPBD) on cytochrome c release (A), mitochondrial F_0F_1 ATPase (B) and lipid peroxidation (C). No Ca^{2+} indicates in the absence of exogenous calcium; uncoupler used in this study was 2, 4-dinitrophenol. Data were expressed as mean±SD of triplicate determinations. *=P<0.05; **=P<0.01; *** = P<0.001; ****=P<0.0001.

Fig. 4. Inhibitory effect of 2-methyl-3-propylbutane-1,4-diol (MPBD) on erythrocyte membrane destabilization using heat-induced (A) and hypotonic solution-induced (B) methods. Acetylsalicylic acid was used as the standard. Data were expressed as mean±SD of triplicate determinations. *=P<0.05; **=P<0.01.

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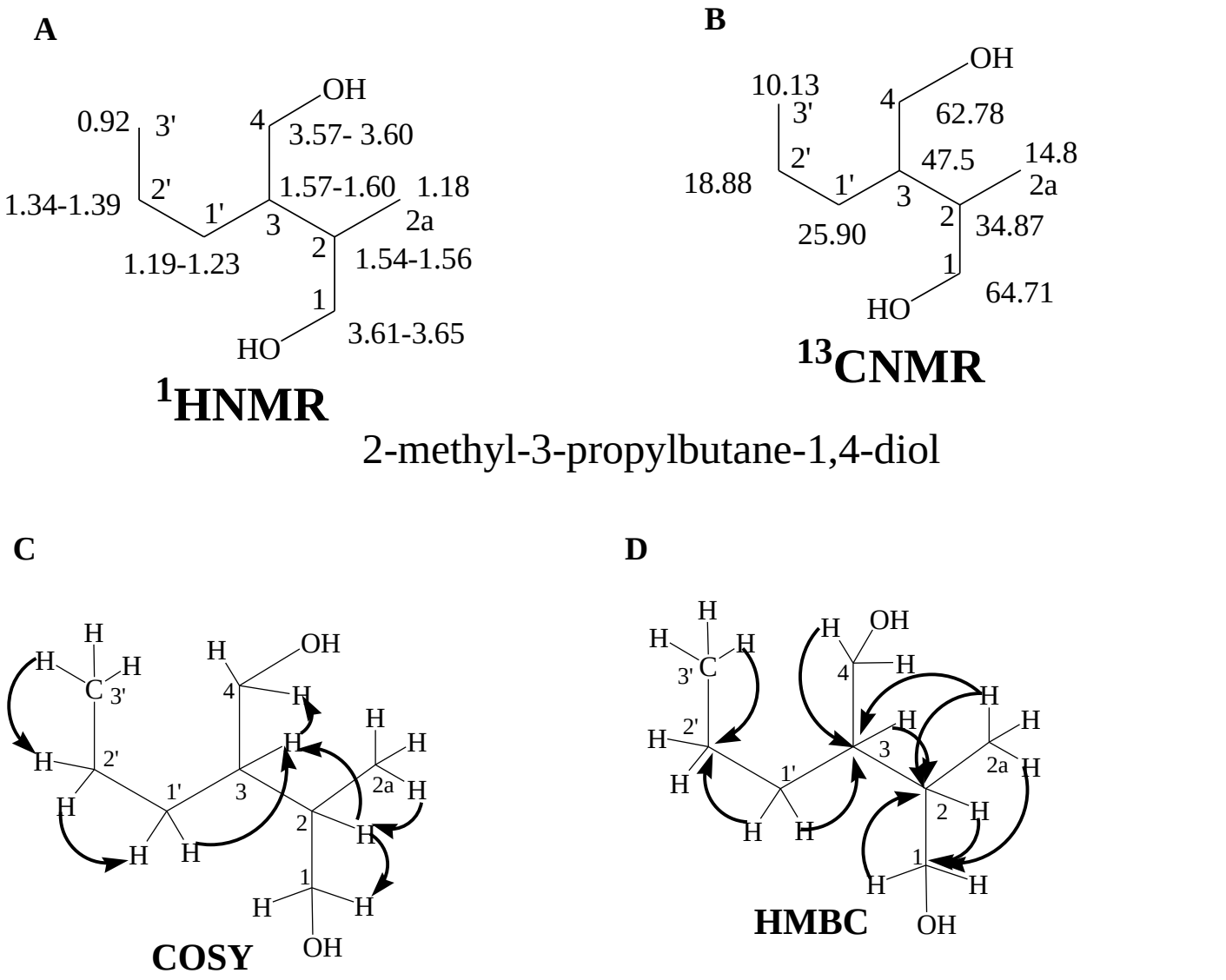

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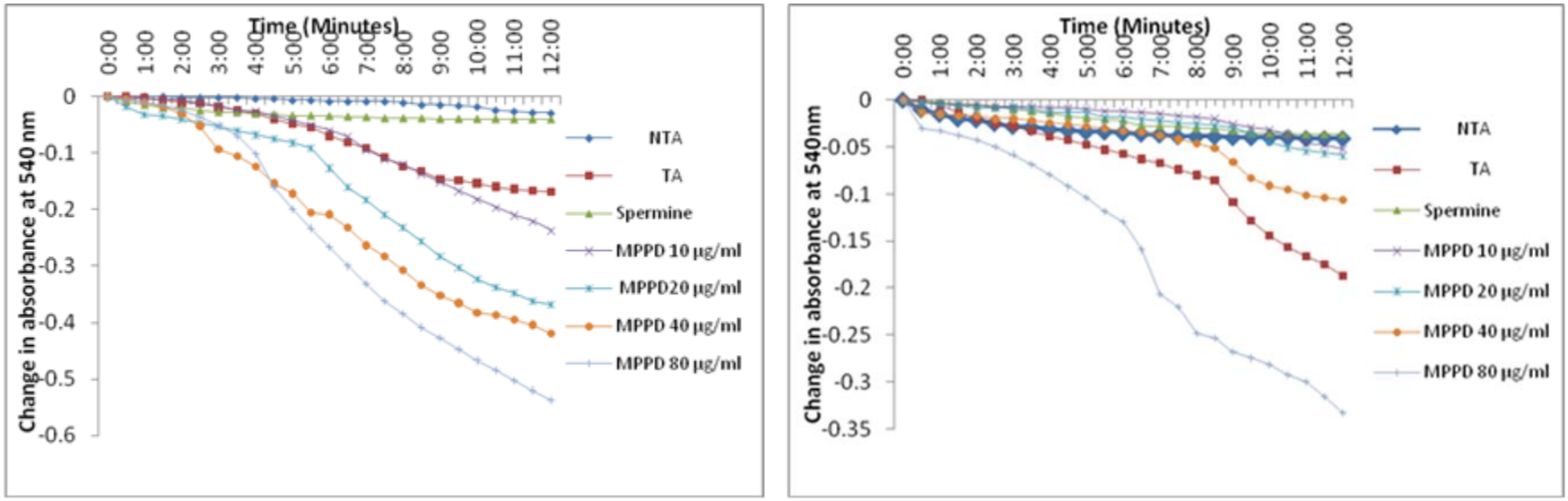

A **B**

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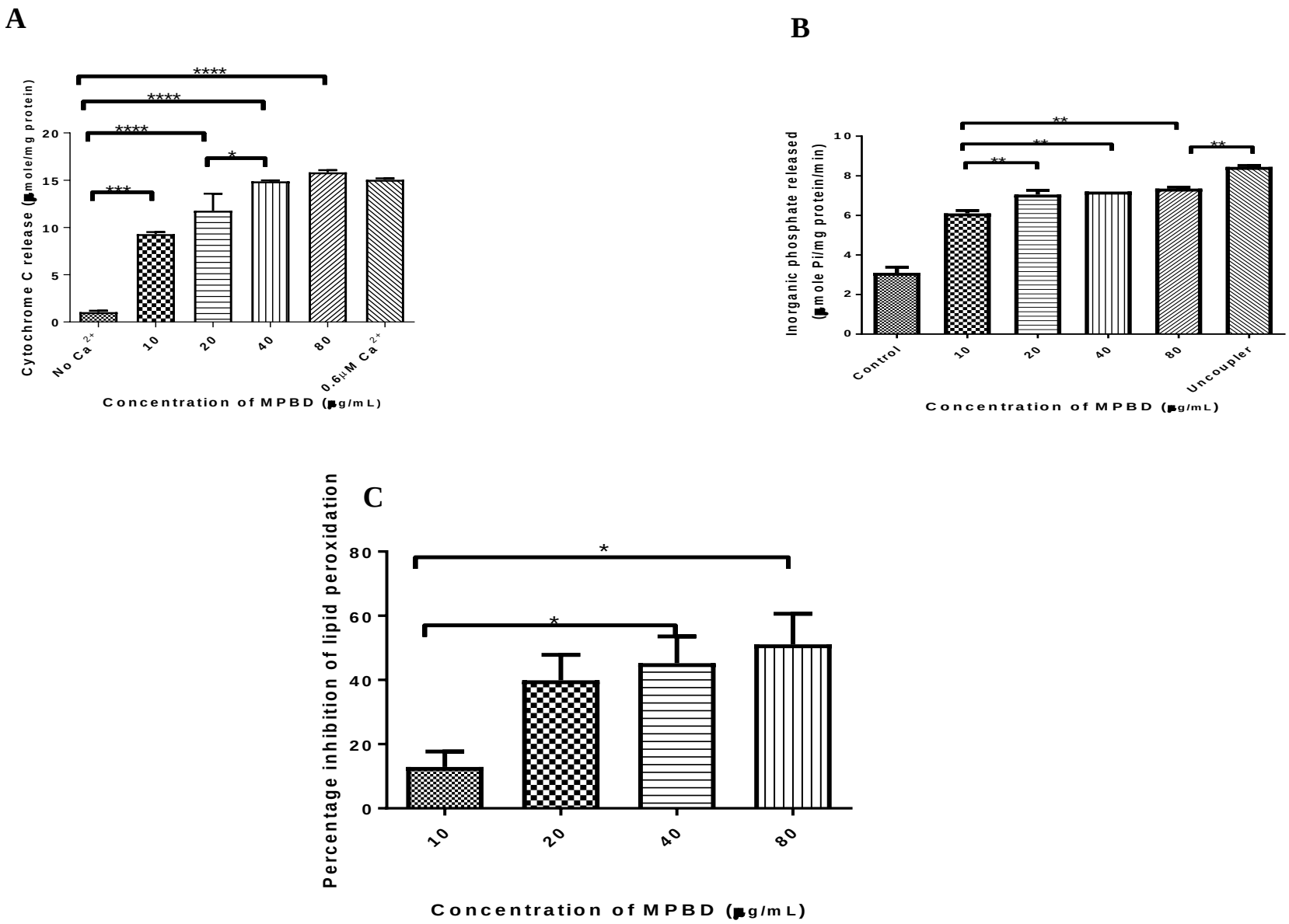

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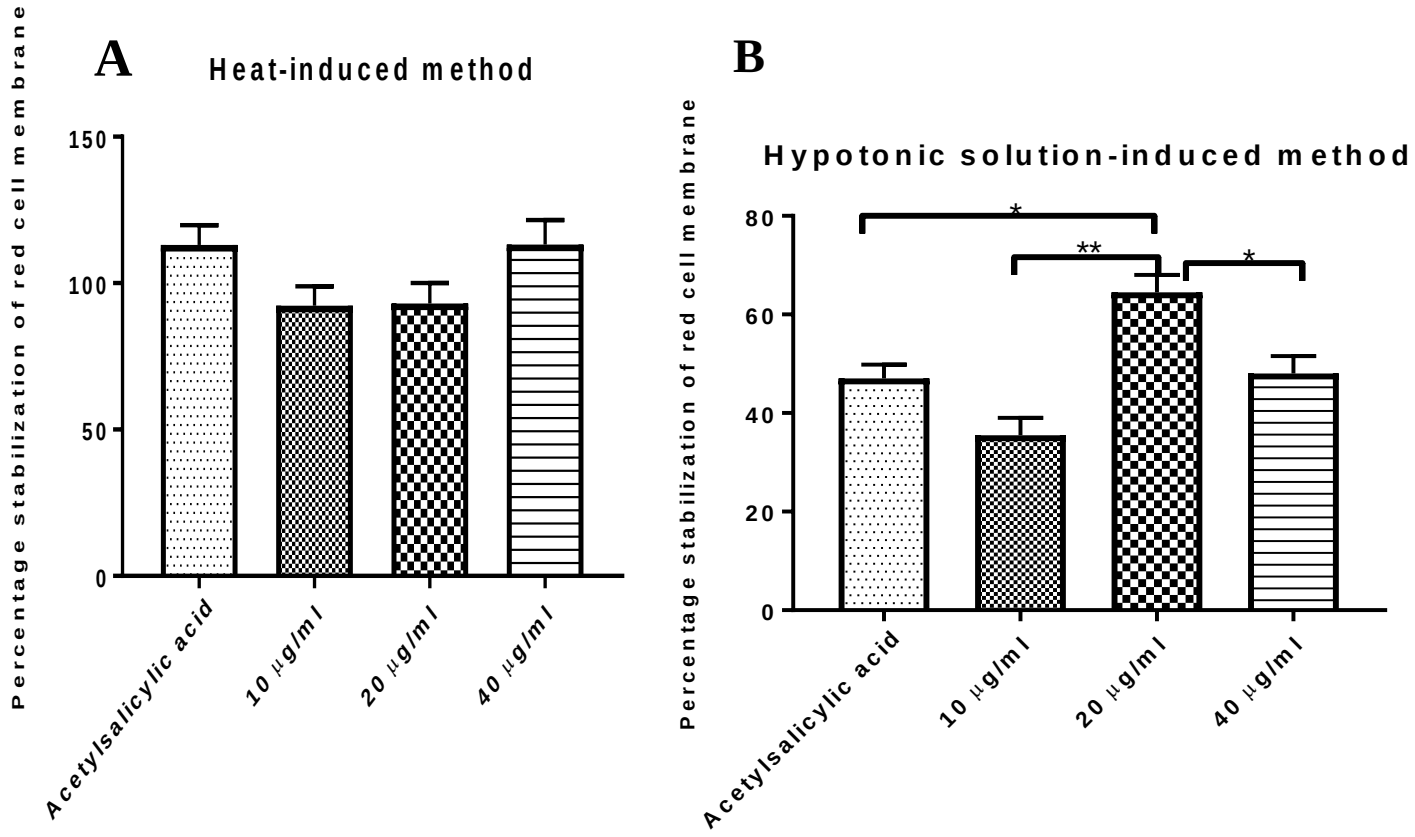

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