**Research Article**

**Extracts of Adenopus breviflorus induce opening of rat liver mitochondrial membrane permeability transition pore**

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**ABSTRACT:** The induction of programmed cell death (apoptosis) through the modulation of Mitochondrial Membrane Permeability Transition (MMPT) pore signalling pathway by tropical medicinal plant extracts is useful in the treatment of tumours resistant to chemotherapy. Fortunately, nature has provided potential phytochemicals which are capable of inducing opening of the MMPT pore in cells where little or no apoptosis takes place. The effect of aqueous and methanol extracts of *Adenopus breviflorus* (AB) used locally against the outbreak of measles and chicken pox was assessed on rat liver MMPT pore. Fifty male albino rats weighing between 120 – 150g were used in this study. For *in vivo* studies, 50 rats of ten animals per group were assigned to five groups and treated with distilled water (control) and varying doses of the aqueous extract of *Adenopus breviflorus* (AEAB), (50, 100, 200 and 400 mg/kg respectively). Rat liver mitochondria were isolated by differential centrifugation and MMPT assayed spectrophotometrically. The results obtained from *in vitro* studies revealed that calcium ion induced MMPT pore opening by 10.0 folds, however, spermine significantly reversed calcium-induced MMPT pore opening in rat liver mitochondrial by 85%. The result further revealed that the AEAB induced MMPT pore opening in a concentration dependent manner. The extract at 50, 150, 250 and 350µg/ml significantly induced pore opening by 1.0, 10.0, 13.0 and 18.0 folds respectively. In the presence of calcium, the AEAB further opened MMPT pore by 16.0, 17.0, 16.0 and 15.6 folds respectively. Similarly, the methanol extract of *Adenopus breviflorus* (MEAB), significantly induced MMPT pore opening by 0.4, 1.1 and 8.0 folds at 150, 250 and 350µg/ml respectively and further opened the MMPT pore in the presence of calcium by 10.8, 12.0, 10.4, and 10.3 folds at 50, 150, 250 and 350µg/ml respectively. The results obtained from the *in vivo* studies, revealed that, the oral administration of the AEAB for 7 days, significantly induced MMPT pore opening by 6.3 folds only at the dose of 400 mg/kg. Similarly, MMPT pore opening was significantly induced in animals orally exposed to the AEAB for 14 days. Specifically, MMPT pore was induced by 8.0, 13.0, 8.0 and 25.0 folds at 50, 100, 200 and 400mg/kg. These findings, suggest that potential phytochemicals that are capable of inducing apoptosis are present in the aqueous and methanol extracts of *Adenopus breviflorus* and these bioactive agents may serve as potential drug in the treatment of tumours.

**KEYWORDS:** Adenopus breviflorus, Apoptosis, Cytochrome c, Mitochondrial Membrane Permeability Transition Pore
INTRODUCTION

Mitochondria are important organelles in the cells, responsible for the generation of energy (Parsons and Green, 2010), and a host of other processes, however, these organelles have been found to play essential roles in the survival of the eukaryotic cells. Recent studies have implicated the mitochondria in several forms of cell death (Vakifahmetoglu-Norberg et al., 2017). Vital signals that decides whether the cell lives or dies culminate on the mitochondria, making the mitochondria a crucial regulator of the intrinsic / mitochondrial-mediated pathway of apoptosis (Fulda et al., 2010; Rasola and Bernardi, 2014; Bernardi and Lisa, 2015)).

Several signals are known to converge outside and inside the mitochondria, inducing the release of proteins which are responsible for the induction of apoptosis and / programmed cell death, high matrix calcium concentration, reactive oxygen species, are sequestered and produced from the mitochondria inducing the mitochondrial mediated-pathway of apoptosis through the opening of the mitochondrial membrane permeability transition (MMPT) pore (Vakifahmetoglu-Norberg et al., 2017). The MMPT pore structure has not been fully identified, however, the opening of the pore ensures that a cell dies in a programmed fashion with loss of membrane potential, ATP depletion, swelling of the mitochondrial matrix and the release of pro-apoptotic proteins such as cytochrome c (Liu et al., 1996), AIF (Susin et al., 1996), Smac/Diablo (Li et al., 2001), from the intermembrane space of the mitochondria to the cytosol where released cytochrome c activates pro-caspase 9, leading to the formation of an apoptosome and consequently activation of downstream caspases 3, 6 and 7. These cascade lead to cleavage of structural proteins, membrane blebbing and other morphological features characteristic of apoptotic cells which are phagocytosed by macrophages (Savill and Fadok, 2000). Although, programmed cell death or apoptosis is a highly regulated process, however, deregulation in the apoptotic pathway may lead to cancer, autoimmune and degenerative diseases (Parsons and Green, 2010).

The mitochondrial pathway of apoptosis is mainly regulated by proteins from the Bcl-2 family, which are responsible for the control of the release of pro-apoptotic factors from the intermembrane space of the mitochondria (Khan et al., 2010). Pander et al., in 2011, identified the mitochondrion as a novel target for chemotherapy through the induction of apoptosis. The MMPT pore is a large conductance pathway (Halestrap, 1999; Crompton et al., 2003) formed between the inner and outer mitochondrial membranes, and has been well implicated in the etiology of several diseases. Inhibition and induction of the MMPT pore is well implicated in diseases associated with uncontrolled cell growth, i.e. cancers (Fulda, 2010), and in diseases where too many apoptosis takes place such e.g. neurodegenerative conditions. High matrix calcium ion concentration, elevated levels of reactive oxygen species, (ROS), ceramide (Halestrap and Brenner, 2003), lots medicinal plants extracts and dietary agents possess potent bioactive compounds that can induce both the extrinsic and intrinsic pathways of apoptosis.

Natural compounds from fruits, vegetables and some medicinal plants have been proven to induce the mitochondrial-mediated pathway of apoptosis (Khan et al., 2010), examples include betulinic acid, curcumin (Anto et al., 2002), Fisetin (Khan et al., 2008) which are isolated from fruits, vegetables and medicinal plants. Dietary components in foods e.g, quercetin in onions (Martin, 2006) and natural products of plants such as polyphenols in fruits, have been found to induce apoptosis in some forms of cancers cell lines (Tan et al., 2011). Adenopus breviflorus is a tropical medicinal plant. The fruit is used in herbal medicine against the outbreak of measles and chicken pox by placing the fruit at the corners of the room. The decoction from the fruit of Adenopus breviflorus is used as an abortifacient. It is also used in treating stomach disorder in women and gonorrhoea in men (Elujoba et al., 1985). Traditionally, Adenopus breviflorus is also used in the treatment of prostate cancer with no scientific proof. The aim of this study therefore, was to identify the phytochemicals present in the whole fruit of Adenopus breviflorus and to assess the effect of the extracts of the fruit of Adenopus breviflorus on mitochondrial-mediated cell in isolated rat liver mitochondria.

MATERIALS AND METHODS

Materials

Mannitol, sucrose, N-2-hydroxy-ethyl-pipe-razine-N-2-ethanesulfonic acid (HEPES), rotenone, spermine, Bovine serum albumin (BSA) were purchased from Sigma chemical Co. (St. Louis, MO, USA). Calcium chloride, sodium hydroxide, sodium carbonate, sodium-tartrate, copper sulphate, EGTA, disodium salt of ATP and other reagents used were of analytical grade and were purchased from Sigma, USA and British Drug Houses, Poole, England.

Fresh fruit of Adenopus breviflorus were purchased from Bode market at Ibadan, Nigeria, and authenticated (UIH22500) at the Department of Botany, University of Ibadan, Nigeria. Two kilogram each of the fruit of Adenopus breviflorus were washed with distilled water and chopped in...
to small cubes, after which, they were soaked separately in distilled water and methanol for 48 hours after which the filtrate collected were concentrated to dryness in a rotary evaporator at 40°C. The aqueous and methanol extracts (AEAB and MEAB) were kept at 4°C for experimental use.

Phytochemical screening

The method of Harborne, 1998 was used to determine the presence of alkaloid, anthraquinones, cardenolides, tannins, saponins, polyphenols, and flavonoids.

Experimental animals

Male Wistar strain albino rats weighing between 150.42g ± 10.1 used for this study were purchased from the Preclinical Animal House of the College of Medicine, University of Ibadan, Ibadan, Nigeria. The rats were kept in ventilated cages and fed rat pellets and water ad libitum. They were allowed to acclimatize for two weeks before the start of the experiment. For in vivo studies, 50 rats of ten animals per group were assigned to five groups and treated with AEAB (50, 100, 200 and 400 mg/kg respectively) for seven (7) and fourteen (14) days, respectively. Group 1 received distilled water (control), group 2 received 50mg/kg AEAB, group 3 received 100mg/kg AEAB, group 4 received 200mg/kg AEAB and group 5 received 400mg/kg AEAB, respectively.

Isolation of rat liver mitochondria

Low ionic strength mitochondria were prepared from isolated rat liver essentially according to the method of Johnson and Lardy (1967), and as modified by Olorunsogo and Bababunmi (1985) based on differential centrifugation technique. The rats were sacrificed by cervical dislocation, the livers excised, trimmed to remove excess tissue, weighed and washed in ice cold homogenizing buffer.

The liver was minced with a pair of scissors. 10% suspension of tissue in ice-cold homogenizing buffer (210mM Mannitol, 70mM Sucrose, 1mM EGTA, 5mM Hepes-KOH (pH 7.4) was then homogenized. The nuclear fraction and cellular debris were sedimented by centrifuging the homogenate at 2300rpm for 5min in a high speed refrigerated MSE centrifuge at 4°C. The supernant obtained was spun at 13000rpm for 10mins to obtain the mitochondria pellet which was then washed twice with washing buffer (210mM Mannitol, 70mM Sucrose, 0.5% BSA, 5mM Hepes-KOH, pH 7.4) by spinning at 12,000 rpm for 10mins. The mitochondria obtained were immediately re-suspended in appropriate volume of MSH buffer (210mM Mannitol, 70mM Sucrose, 5mM Hepes-KOH, (pH 7.4), dispensed into Eppendorf tubes which were kept at 4°C and used fresh. Mitochondrial protein was determined by the method of Lowry et al., (1951) using BSA as standard.

Protein determination

Mitochondria protein concentration was determined protein according to the method of Lowry et al., (1951), using BSA as standard.

Mitochondrial swelling assay

Mitochondrial membrane permeability transition was monitored by measuring the changes in absorbance of mitochondrial suspension at 540nm in the presence or absence of calcium, the triggering agent in a spectrulab 752s UV/Visible spectrophotometer essentially according to the method of Lapidus and Sokolove (1993). Mitochondria (0.4mg protein/ml) were pre-incubated in the presence of 0.8µm rotenone in a medium containing 210mM Mannitol, 70mM Sucrose, 5mM Hepes-KOH, (pH 7.4), for 3mins at 30°C prior to the addition of 300µm CaCl2 in experiment where calcium was used. Thirty seconds later, 50µm succinate was added and the mitochondrial permeability transition quantified at 540nm for 12mins at 30 secs interval. To test the intactness of the mitochondria, 0.1 mM spermine was added immediately after the addition of rotenone.

Data were analysed using One-way ANOVA on SPSS software version 17. P< 0.05 is considered as significant. All data are expressed as mean ± standard deviation of duplicate. Differences between the control and groups were expressed as induction folds.

<table>
<thead>
<tr>
<th>Phytochemical</th>
<th>Indicators</th>
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<tr>
<td>Alkaloid</td>
<td>+</td>
</tr>
<tr>
<td>Anthraquinones</td>
<td>-</td>
</tr>
<tr>
<td>Cardenolides</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td>-</td>
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<tr>
<td>Saponins</td>
<td>+</td>
</tr>
<tr>
<td>Polyphenols</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>-</td>
</tr>
</tbody>
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Keys: +ve: indicates positive; -ve: indicates negative
RESULTS

Phytochemical screening of the whole fruit of Adenopus Breviflorus

Table 1 shows the result of the phytochemicals present in the whole fruit of AB. Alkaloids, cardenolides, saponins and polyphenols were present in the whole fruit of Adenopus breviflorus.

Effects of calcium ion and spermine on rat liver MMPT pore

Figure 1 shows large amplitude swelling of the mitochondrial when 300µm of Ca\(^{2+}\) (the Triggering Agent, TA) was added to the assay medium, while 0.1mM spermine, a standard inhibitor of the MMPT pore, significantly reversed calcium-induced MMPT pore opening.

Figures 2 and 3, show that AEAB caused large amplitude swelling of the mitochondria by inducing MMPT pore opening by 0.4, 1.1 and 8 folds at 150, 250 and 350µg/ml, respectively. However, in the presence of the triggering agent, calcium induced opening of the MMPT pore by 9.6 folds while, the MEAB potentiated the effect of calcium-induced MMPT pore opening more significantly with inductive folds of 10.8, 12.1, 10.4 and 10.3 at 50, 150, 250, and 350 µg/ml, respectively.

Effects of oral administration of AEAB for 7 and 14 days on rat liver MMPT pore

Table 2 shows the result obtained from the oral administration of the AEAB to rats for 7 and 14 days, respectively. The AEAB induced opening of the MMPT pore significantly only at 200 and 400mg/kg by 0.6 and 6.3 folds. There were no effects at 50 and 100mg/kg, respectively. However, after 14 days oral exposure to AEAB, the extract induced opening of the MMPT pore in isolated rat liver mitochondria by 8.0, 13.0, 8.0 and 25.0 folds at 50, 100, 200 and 400mg/kg, respectively.
Figure 4: Inductive effect of the methanol extract of *Adenopus breviflorus* on MMPT pore opening

Figure 5: Effect of varying concentrations of methanol extract of *Adenopus breviflorus* on calcium-induced MMPT pore.

Table 2: Inductive effects of 7 and 14 days oral administration of aqueous extracts of AB on rat liver mitochondria.

<table>
<thead>
<tr>
<th>Concentration (mg/Kg)</th>
<th>7 Days</th>
<th>14 Days</th>
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<tbody>
<tr>
<td></td>
<td>Mean ± SD Induction Fold</td>
<td>Mean ± SD Induction Fold</td>
</tr>
<tr>
<td>Control</td>
<td>-0.037±0.010 -</td>
<td>-0.024±0.000 -</td>
</tr>
<tr>
<td>50</td>
<td>-0.039±0.000 0.0</td>
<td>-0.215±0.001* 8.0</td>
</tr>
<tr>
<td>100</td>
<td>-0.030±0.004 0.0</td>
<td>-0.334±0.008* 13.0</td>
</tr>
<tr>
<td>200</td>
<td>-0.060±0.005 0.6</td>
<td>-0.223±0.008* 8.0</td>
</tr>
<tr>
<td>400</td>
<td>-0.270±0.009* 6.3</td>
<td>-0.631±0.008* 25.0</td>
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Each value is a mean of 2 determinations ± standard deviation. Significance was estimated by analysis of variance (ANOVA).

* Values are significantly different at p < 0.05 when compared to control

**DISCUSSION**

More attention is given worldwide to the use of herbs in the treatment of a number of ailments. Plants possess many phytochemicals which are useful medicines to man, hence, the use of medicinal plants and their isolated bioactive agents as chemopreventives and chemotherapeutic agents (Martin, 2006; Fulda *et al.*, 2010; Khan *et al.*, 2010). Potential phytochemicals present in medicinal plants elicit their therapeutic effects through a number of mechanisms which ranges from scavenging of reactive oxygen species, induction of mitochondrial-mediated apoptosis e.t.c. Phytochemical screening of the whole fruit of *Adenopus breviflorus* revealed the presence of alkaloids, cardenolides, saponins and polyphenols. However, anthraquinones, tanins and flavonoids were not present in the whole fruit of *Adenopus breviflorus*. Studies have shown that alkaloids (Isah, 2016), cardenolide glycosides (Leu *et al.*, 2014), saponins (Man *et al.*, 2010) and polyphenols (Niedzwiecki, 2016), all have anticancer effects against many cancer cells. Other studies have also shown that compounds isolated belonging to these class of phytochemicals are useful medicines examples include Berberine, a plant derived alkaloid, the cardenolide Ouabain, Honokiol from *Magnolia obovata* and Avicins a triterpenoid sapopnin derived from Acacia Victoriae are potent principles which have all been shown to induce apoptosis through the opening of the MMPT pore. (Zhang *et al.*, 2014; Chen *et al.*, 2014; Fried and Arbiser, 2009, Dallavia *et al.*, 2014).

The fate of a cell is largely dependent on a number of signals which meet on the mitochondria. Death signals on the mitochondria lead to the permeabilization of the outer and inner mitochondrial membranes causing the opening of the MMPT pore, loss of mitochondrial membrane potential, inactivation of ATP synthase, mitochondrial matrix swelling and the release of proapoptotic proteins that drive cascades of reactions that ultimately lead to cell death in a programmed fashion (Dallavia *et al.*, 2014). There is a general believe, that the MMPT pore can be opened by any agent which can interact with any of the component of the pore e.g. hexokinase, adenine nucleotide translocase (ANT) (Panda *et al.*, 2011). The effect of varying concentration of the aqueous and methanol extract of *Adenopus breviflorus* on MMPT pore was assessed on rat liver mitochondria in the presence and absence of calcium ion - the triggering agent. The results obtained from the effects of AEAB and MEAB on the opening of the MMPT pore shows that both extracts are capable of inducing opening of the MMPT pore in isolated rat liver mitochondria, even in the absence of the triggering agent. Our findings further revealed, that both AEAB and MEAB induced opening of the MMPT pore significantly in a...
concentration dependent manner.

However, it was also observed that AEAB has more capacity and is more potent in inducing mitochondrial-mediated apoptosis when compared with MEAB as exhibited in the induction fold obtained in the two experiment. Some studies have shown that the potency of any extract depends on the solvents used for extraction, as different classes of phytochemicals are soluble in various solvents (Handa et al., 2008). Furthermore, The results obtained agrees with the findings of Adedosu et al., 2012, where the chloroform extract of Brysocarpus coccineus was more potent in inducing MMPT pore compared with the ethylacetate extract of the same plant. Also in another study by Oyedeji, et al., (2016), the aqueous extract of Citrullus lanatus was more potent in inducing permeabilization of the mitochondrial pore compared with the methanol extract of the same plant. The MMPT pore is believed to be composed of Adenine Nucleotide Translocase, Voltage Dependent Anion Channel, Cyclophilin D, Benzodiazepine receptor, Creatine kinase and Hexokinase II, however, recent studies have indicated that the pore might be formed through dimerization of F-ATP synthase (Fulda et al., 2010; Rasola and Bernardi, 2014 and Bernardi and Lisa, 2015).

Furthermore, the extracts potentiated the effect of the triggering agent by opening the pore further, however, in a non-concentration dependent manner. The effect of AEAB was further assessed on MMPT pore in vivo because of our earlier observation, that AEAB was more potent in inducing mitochondrial-mediated apoptosis by inducing opening of the MMPT pore. Significant p<0.05 induction of opening of the MMPT pore was observed in isolated rat liver of animals orally exposed to AEAB for 7 and 14 days respectively. Maximum induction of the pore was observed at 400mg/kg for animals orally exposed to AEAB for 7 and 14 days, respectively.

These results suggest that the potent bioactive agents are present in both AEAB and MEAB and that these active principles are more potent than calcium in the induction of apoptosis by opening of the MMPT pore. Recent researches have shown that the modulation of the pore may be an important pharmacological target in chemotherapy (Fulda et al., 2010; Panda et al., 2011; Dallavia et al., 2014). It may be that the compound present in the aqueous and methanol extracts of AB are interacting with some components of the pore thereby facilitating induction of the pore. The presence of polyphenols present in the aqueous and methanol extracts of Adenopus breviflorus might be responsible for the modulatory effects of these extracts on MMPT pore, as antioxidants, especially polyphenols have been known to modulate the intrinsic pathway of apoptosis (Martin, 2006).

Adenopus breviflorus is used traditionally as an abortifacient and the fruit has anti-implantation activity (Elujoba et al., 1985). Earlier reports by Oshodi in 1996 have shown that the fruit of Adenopus breviflorus contain linoleic acid as the predominant fatty acid in the dehulled seeds. Studies carried out by Serini et al., (2009) and Bernardi, (2013) have shown that fatty acids such as oleic acid are potent inducers of apoptosis and the MMPT pore. Also certain extracts of medicinal plants and isolated compounds of dietary origin have been scientifically proven to induce the intrinsic pathway of apoptosis through the induction of MMPT pore in isolated mitochondria, e.g. beta-sitosterol (Neuzil, et al., 2007; Fulda, 2010). Betulinic acid, a natural pentacyclic triterpenoid, has been shown to trigger mitochondrial outer membrane permeabilization, cause loss of mitochondrial membrane potential dissipation and cytochrome c release in isolated mitochondria (Fulda et al., 1998).

Conclusion

The results show that the aqueous and methanol extracts of Adenopus breviflorus possess potential phytochemical / phytoneutrients, which can induce apoptosis through the opening of the mitochondrial membrane permeability transition pore and that further studies on the purification, isolation and characterization of the potent bioactive component may be useful in drug development for tumours.

REFERENCES


A horticultural plant can induce opening of rat liver MMPT pore.


