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IN VITRO AND IN VIVO EFFECTS OF ALPHA STONE, A POLYHERBAL FORMULA ON MITOCHONDRIAL PERMEABILITY TRANSITION PORE IN RAT LIVER

Biochemistry

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ABSTRACT

Mitochondrial Permeability Transition (mPT) pore opening is critical to cell survival. Alpha stone is used in the treatment of fibroid but the possible mechanism has not been explored. Twenty (20) Swiss albino mice (15-18 g) were orally treated with 100, 200 and 400 mg/kg body weight (bw) dose of alpha stone daily for seven days. Mitochondria were isolated and mPT, F_1F_0 ATPase, lipid peroxidation and cytochrome c release were assessed spectrophotometrically. Similar assays were determined *in vitro*. The mPT pore opened maximally with induction fold of 14 at 640 µg/ml (*in vitro*) and, 26 at 400 mg/kg bw (*in vivo*) in the absence of calcium. This was reversed *in vitro* in the presence of calcium. Lipid peroxidation occurred in both cases while cytochrome c release was significantly high (*in vitro*). Alpha stone caused mPT pore opening both *in vitro* and *in vivo* and this may cause mitochondrial-induced apoptosis which is highly beneficial in diseases where apoptosis is down-regulated such as cancer and some fibroids

KEYWORDS

Phytomedicine, ATPase, lipid peroxidation, cytochrome c

Introduction:

It is estimated that a greater percentage of the world population depends on herbal medicine either for curative effects or preventive measures. The use of herbal preparations as remedies for major diseases is on the increase and there is also a growing concern about their efficacy, safety and control. The guidelines for the use of these herbal preparations are not comparable with those applicable for drugs in general [1]. This is because in most places, herbal preparations are mostly taken without specific regimen. These effective alternatives to the orthodox medicine had been identified and they provide acceptable, affordable and sustainable primary healthcare [2]. The cultivation and processing of medicinal plants is necessary so as to enhance the production of herbal medicines [3]. In most cases, these phytomedical preparations come in mixtures; a situation that paves way for each constituent to express its therapeutic effects. Although, the mechanism of actions of these herbal preparation may not have been studied, it is strongly believed that it is either they modulate specific pathways in the organism or play a significant role in the normal functioning of organelle such as mitochondrion. Mitochondrial functions are central to the existence and well being of cell in particular and the organism as a whole. Because of the important roles played the mitochondrion, this has attracted the attention of scientists for decades, The study of this organelle has grown tremendously.

Consequently, beyond their function in energy generation, mitochondria are also critical in cell signaling [4], generation of reactive oxygen species [5], regulation of apoptosis and cellular ageing [6]. The content and functions of these organelles increase with physical training [7], decrease with ageing and physical inactivity, and are altered in diseases such as type 2 diabetes [8-9], and malaria [10].

The mitochondrial Permeability Transition (mPT) is an abrupt increase in the inner mitochondrial membrane (IMM) permeability to solutes that are greater in size than 1500 Da.

Persistent mPT pore opening is associated with mitochondrial dysfunction because its occurrence leads to mitochondrial depolarization, cessation of ATP synthesis and its consequent hydrolysis, Ca^{2+} release, pyridine nucleotide depletion, inhibition of respiration, *in vitro* matrix swelling, concomitant release of cytochrome c, the rupture of outer mitochondrial membrane (OMM) and eventual release of proapoptotic proteins such as cytochrome c, endonuclease G and Apoptosis Inducing Factor (AIF) [11-12]. Inadequate cell death, especially in cells that undergo aberrant proliferation, is the hallmark of disease conditions such as tumour, fibroid and cancer; therefore it seems likely that drugs or herbal formulation used in the treatment of these diseases enhanced cell death by different mechanisms.

Alpha stone, formulated by a combination of leaves fruits, seeds and formulated with honey is a polyherbal decoction common in the Middle East and is used to treat different diseases in a short period of time. Some of these diseases are: tumours, pile, infections, ulcers, fibroids, malaria, high blood pressure, asthma, rheumatism and most pains.

Alpha stone is formed from the boiling and heating of different Middle East fruit, leaves, herbs, shoots, barks, roots and honey. These plants are leaves and acorns of Palestine oak, leaves and shoots of terebinth (*Pistacia palestine*),Golden chamomile, Carob (*Ceratonia siliqua*), leaves of Dominican sage (*Salvia dominica*), leaves of field marigolds (*Calendula arvensic*),Tulips (*Tulipa systola*), shoots of the white broom (*Retama raetan*), the fruit of the bitter almond tree (*Amygdalius arabica*), seeds of fennel (*Foeniculum vulgare*) and shoots of the leafless ephedra (*Ephedra Foeminea*) which is very rich in phenolics and flavonoids [13]. These are blended together and formulated with honey.

Honey is the natural sweet, viscous substance produced by honeybees from the nectar of blossoms or from the secretion of living parts of plants or excretions of plant sucking insects on the living parts of plants, which honeybees collect, transform and combine with specific substances of their own, store and leave in the honey comb to mature. Honey is one of nature's wonders. Honey has been a common sweetener for foods and a powerful medicinal tool for centuries. It is the simplest and often the best way to soothe a sore throat and it can be taken at any time [14]. Honey is often eaten as an energy food. It has simple sugars that are absorbed directly into bloodstream without digestion. Honey mixes well as a sweetener in hot and cold drinks. It goes with nearly all foods. The moisture absorbing quality of honey helps breads, cakes, cookies and candies stay fresh longer. Natural medicinal products have been used for millennia in the treatment of multiple ailments. Although many have been superseded by conventional pharmaceutical approaches, there is currently, resurgence in interest in the use of honey and honey products by the general public.

Honey applied to wounds, burns and ulcers promotes faster healing by

clearing infections and rendering sterility, through promotion of tissue growth and regeneration, and preventing dehydration of the infected site. Honey has been used as medicine in many cultures for a long time. However, it has limited use in medicine due to lack of scientific report. In recent days, honey is becoming acceptable as a reputable therapeutic agent. Its beneficial role has been endorsed due to its antimicrobial, anti-inflammatory and anti-oxidant activities as well as boosting of the immune system [15]. There is paucity of information on the mechanism by which alpha stone exerts its biochemical effects on fibroids and tumours as claimed in folkloric medicine.

This research was therefore carried out to assess the *in vivo* and *in vitro* effects of alpha stone and its overall effects on mitochondrial-mediated cell death. via the opening of the mPT pore.

Materials and Methods:

Chemicals

Mannitol, sucrose, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), (ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), Bovine Serum Albumin (BSA), Rotenone, spermine, sodium succinate dibasic hexahydrate, 2,4-dinitrophenol, Folin-Ciocalteu's phenol reagent, 2N calcium chloride dehydrate, Tris base, Tris hydrochloride, Sodium Dodecyl Sulphate (SDS), Adenosine triphosphate (ATP), thiobarbituric acid (TBA), acetic acid, butan-1-ol and cytochrome c were obtained from Sigma-Aldrich Chemicals, 2033 Westport Center Dr, St. Louis, MO 63146, USA, potassium hydroxide was obtained from Qualichem Fine Chem Pvt. Ltd, India, trichloroacetic acid was obtained from Kermel, China, sodiumtrioxocarbonate (IV), sodium hydroxide, Na-K^+-tartrate, copper (II) tetraoxosulphate (VI) pentahydrate, potassium chloride, ammonium molybdate, and iron (II) tetraoxosulphate (VI) were obtained from The British Drug House Ltd, BDH Laboratory Chemicals Group, Poole, England.

Collection and preparation of alpha stone:

Alpha stone, a dark brown substance, was obtained from a local distributor in Ibadan, Oyo state, Nigeria. Ten percent stock was prepared with distilled water.

Animal source, grouping and treatment:

All procedures that were used in this study, involving animals, conformed to the guidelines of the care and use of animals in research and teaching of the National Institute of Health (NIH) and according to the 1996 revised version.

Forty-five male Wistar Swiss albino mice were obtained from the Animal House Section of the Institute of Advanced Medical Research and Training (IAMRAT), University of Ibadan, twenty of which were orally treated once daily with 100, 200 and 400 mg/kg bw dose of alpha stone for seven days. The control group received the vehicle only. After seven days, mitochondria were isolated from these groups by differential centrifugation. The remaining animals were used for the *in vitro* assay.

Isolation of mitochondria:

Mitochondria were isolated by the method of Johnson and Lardy [16]. The animals were sacrificed by cervical dislocation, opened and the livers were excised, washed in isolation buffer (210 mM mannitol, 70 mM sucrose, 5 mM HEPES-KOH (pH 7.4 and 1 mM EGTA), weighed, minced and homogenized in a porter Elvedgem homogenizer. The ten percent homogenate was loaded into a cold centrifuge (Sigma 3-30K, Germany) and the unbroken cells and cell debris were sedimented twice at 2, 300 rpm (4°C) for five minutes each. The supernatant was decanted and centrifuged at 13, 000 rpm for ten minutes to pellet the mitochondria. Mitochondria were washed twice with washing buffer (210 mM mannitol, 70 mM sucrose, 5 mM HEPES-KOH (pH7.4) and 0.5% BSA) for ten minutes each time. The pelleted mitochondria were suspended in assay buffer (210 mM mannitol, 70 mM sucrose and 5 mM HEPES-KOH (pH7.4)) and kept on ice until used.

Protein determination:

Protein concentration was determined by the method of Lowry *et al*., [17] using BSA as standard. Briefly, 10 µL of the mitochondria was diluted to 1 mL and added to 3000 µL of Reagent D (a 100:1:1 mixture of 2% Na_2CO_3 in 0.1M NaOH, 2% Na-K-tartrate and 1% $CuSO_4.5H_2O$ respectively) and allowed to stand for 10 minutes. Thereafter, 300 µL of a five-fold dilution of Folin-Ciocalteu reagent was added to all the test tubes in duplicates and allowed to stand for 30 minutes, vortexed and absorbance was read at 750 nm wavelength on a 752N UV-visible spectrophotometer (BOSCH, Brand, China) against a reagent blank.

Assessment of mitochondrial membrane permeability transition in mouse liver mitochondria:

Permeability transition was detected in isolated mitochondria by the changes in the absorption of light (measured at 540 nm) as a result of matrix swelling based on the procedure of Lapidus and Sokolove [18]. Mitochondria were pre-incubated in a glass cuvette in the presence of 0.8 µM rotenone in suspending buffer for about 3 minutes at room temperature prior to the addition of 3 µM $CaCl_2$. Thirty seconds later, 5 mM sodium succinate was added to energize the reaction and mitochondrial permeability was quantified as decrease in absorbance at 540 nm. This was done to assess the intactness of the isolated mitochondria and their suitability for the experiment. Intact mitochondria with negligible change in absorbance at 540 nm were later assessed for their susceptibility to Ca^{2+}-induced mPT pore opening by adding 3 µM $CaCl_2$ 30 seconds before the addition of succinate. Reversal of calcium-induced pore opening was assessed by the incubation of mitochondrial in spermine for 3 minutes before the addition of calcium.

In each case, absorbance readings were monitored continuously for 12 minutes at 30 seconds interval and swelling rate was quantified as ΔA_{540}/min. For the *in vivo* mPT pore assessment, varying volumes of mitochondria isolated from the treated groups containing similar mitochondrial protein concentration (0.4 mg/mL), corresponding to that of mitochondria isolated from a normal mouse, which was intact at that volume was used to assess the effect of the drugs administered on mPT.

Assay of mitochondrial ATPase activity:

Lardy and Wellman [19] method was used for the *in vitro* study and also adopted for the *in vivo* experiment. Mitochondria isolated for this assay followed the procedure described by Johnson and Lardy 1967 except that 0.25M sucrose was used as buffer for the isolation. To each test tube in duplicates, 0.25 M sucrose, 5 mM KCl and 0.1 M Tris-HCl were added. Thereafter, 200, 400 800 and 1600 µg/mL concentrations of alpha stone were added to the designated tubes respectively and the solutions were made up to 2000 µL accordingly. To the uncoupler test tube, 2, 4 dinitrophenol was added followed by 0.01 M ATP. One milliliter Sodium Dodecyl Sulphate (SDS) (10%) was added to the zero time tube before the addition of mitochondria. The SDS was added to zero time tube to stop the reaction after adding ATP. After adding ATP, mitochondria were added to other test tubes every 30 seconds and the set up was incubated at 27°C for 30 minutes in a shaking water bath. After incubation, 1mL of (10%) SDS was added to each test tube (except zero time) every 30 seconds to stop the reaction. Thereafter, 1mL of 1.25% ammonium molybdate was added to each test tube followed by 1mL of 9% ascorbate. The solutions were allowed to stand for 20 minutes and absorbance was read at 660 nm.

Evaluation of lipid peroxidation (*in vitro*):

A modified TBARS assay (Ruberto *et al.,* [20] was used to measure the lipid peroxide formed using mitochondria as lipid rich media. Mitochondria (1 mg/mL) were incubated with 50 µL of 0.07 M $FeSO_4$ to induce lipid peroxidation, the mixture was incubated for 30 minutes at 37°C. Then, 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°C for 60 minutes. After cooling, 3.0 mL of butan-1-ol was added to each tube and centrifuged at 3,000 rpm for 10 minutes. The absorbance of the organic upper layer was measured at 532 nm and inhibition of lipid peroxidation was calculated accordingly.

Lipid peroxidation (*in vivo*):

For the *in vivo* experiment, levels of malondialdehyde (MDA) were measured as index of lipid peroxidation in the test samples according to the method of Varshney and Kale [21]. An aliquot of 0.4 mL of test sample (mitochondria) was mixed with 1.6 mL of Tris-KCL buffer to which 0.5 mL of 30 % TCA was added. Thereafter, 0.5 mL of 0.75% TBA was added and the tubes were heated in a water bath for 45 minutes at 80°C. The samples were then cooled and centrifuged at 3,000 rpm for 10 minutes. The clear supernatant was collected and absorbance was measured against a reference blank of distilled water at 532 nm.

The MDA level was calculated using extinction co-efficient of 0.156/µM/cm (Adam – Vizi and Seregi, [22]

$$\text{L.P (nmoleMDA/mg protein)} = \frac{\text{Absorbance x Volume of mixture}}{E_{532nm} \text{ x Volume of Sample x mg protein/mL}}$$

Assay of cytochrome c release:

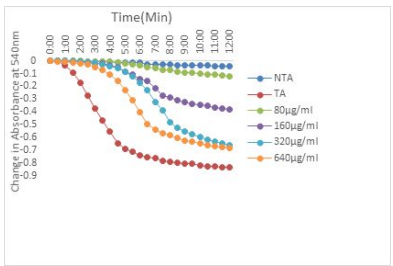
The *in vitro* quantitative determination of cytochrome c released from isolated mitochondria was performed by measuring the Soret (γ) peak for cytochrome c at 414 nm (ε= 100 mM^{-1} cm^{-1}), according to a previously established method by Appaix *et al.,* [23]. Mitochondria were pre-incubated in suspending buffer for 3 minutes at room temperature in the presence of 0.8 µM rotenone prior to the addition of 3 µM $CaCl_2$. Thirty seconds later, 5mM Sodium Succinate was added to energize the reaction and left for 12 minutes. After 30 minutes, the mixtures were centrifuged at 13,000 rpm for 10 minutes, and the supernatant was immediately recovered by aspiration and cytochrome c measured spectrophotometrically at 414 nm. Cytochrome c released was quantified from cytochrome c standard curve.

Statistical analysis:

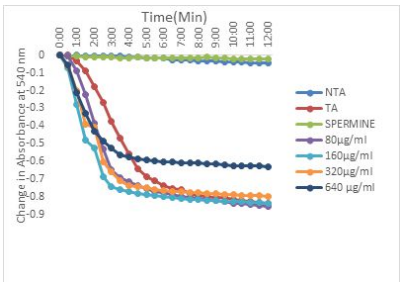
Data were expressed as mean ± SD. Graphpad prism (5. 0 version) was used to analyze data. One-way ANOVA (non-parametric test) was used to test for levels of significance and Tukey's multiple comparison test was used. *P* value less than 0.05 (*P*<0.05) was taken as statistically significant.

Results:

In the absence of calcium, Figure 1a shows that mitochondria respiring on succinate in the absence of calcium did not have significant changes in absorbance. In the presence of calcium, mitochondria swelled and a large amplitude swelling was observed. This swelling was reversed when spermine was added. This shows that the mitochondria were intact *ab initio* and were not uncoupled. They are therefore suitable for further use. Figure 1a further shows that in the absence of calcium, there was a significant decrease in absorbance monitored at 30 seconds interval over a period of 12 minutes when mitochondria were treated with graded concentrations of alpha stone. This indicates that alpha stone caused concentration-dependent opening of the mPT. In Figure 1b, the pore opening effects of calcium was reversed by alpha stone in a concentration-dependent manner. Interestingly, there was a significant decrease in the absorbance at 100, 200 and 400mg/kg bw doses of alpha stone when compared with calcium. This shows that alpha stone at the doses used caused mPT pore opening.



A



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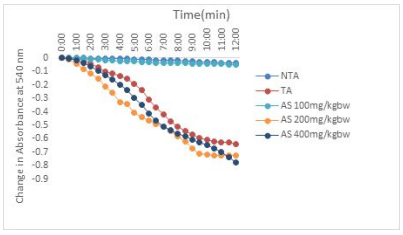
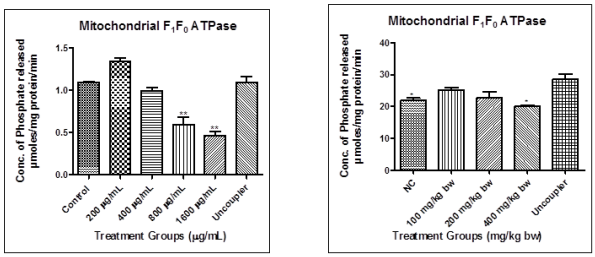


C

Figure 1: Induction of permeability transition in rat liver mitochondria. A, after mitochondria had been preincubated with 5 mM sodium succinate at room temperature, alpha stone (80, 160, 320, 640 µg/ml) as indicated by the key were added without calcium. B, mitochondria were preincubated with 5 mM sodium succinate and 3µM $CaCl_2$ before the addition of calcium. C, mitochondria isolated from animals orally treated with alpha stone (100, 200 and 400 mg/ml) were preincubated in assay buffer before the addition of 5 mM sodium succinate.

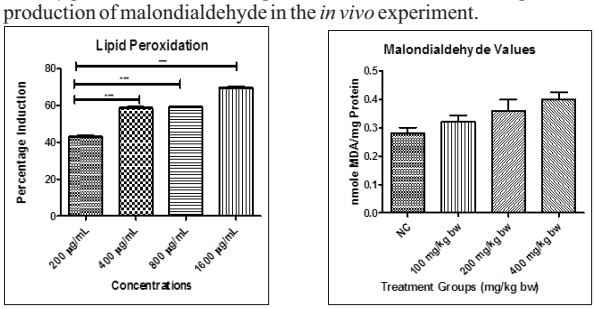
In Figure 2, the *in vitro* and *in vivo* assessment of the effects of alpha stone on F_1F_0 ATPase is presented. Figure 2a shows that F_1F_0 ATPase activity decreased as the concentration of alpha stone increased. The inorganic phosphate released when mitochondria were treated with 800 and 1600 µg/ml significantly (p<0.05) when compared with the uncoupler. Similar results were obtained for the *in vivo* experiment. The inorganic phosphate released at the highest dose of alpha stone was significantly (p<0.05) lower than the value obtained for the uncoupler.



A B

Figure 2: Assessment of the effects of alpha stone on mitochondrial F_1F_0 ATPase activity both *in vitro* (A) and *in vivo* (B). Normal Control (NC). Data are mean ±S.D. ((*n*=6 for *in vitro*); *P<0.0001, **P<0.0001, mean significant when test groups were compared with the uncoupler.

The effects of alpha stone on lipid peroxidation *in vitro,* the generation of malondialdehyde as a byproduct of oxidative damage *in vivo* are shown in Figure 3. Alpha stone enhanced lipid peroxidation maximally at 800µg/mL in the *in vitro* experiment while there was insignificant production of malondialdehyde in the *in vivo* experiment.



A B

Figure 3: Effects of alpha stone on mitochondrial lipid peroxidation both *in vitro* (A) and *in vivo* (B). Normal Control (NC). Data are mean ±S.D. ((*n*=6 for *in vitro*); ***P<0.0001 means comparison of the lower concentrations with the higher concentrations.

The *in vitro* effects of alpha stone on cytochrome c release were presented in Figure 4. Alpha stone enhanced the release of cytochrome c (*in vitro*) at higher concentrations when compared with calcium

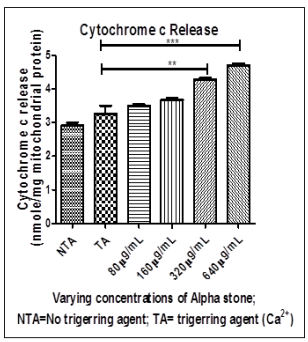


Figure 4: *In vitro* effects of alpha stone on cytochrome c release. No triggering agent (NTA), Triggering agent (TA). Higher concentrations of alpha stone significantly induced cytochrome c release. **P<0.0001, ***P<0.0001 mean comparison of the lower concentrations with the higher concentrations.

Discussion:

Several events are involved in cell death process part of which mitochondrion is actively involved. Previous experiments have shown that mitochondria undergo a series of events that orchestrate cell death or start a cascade of events that may lead to cell death.

In this study, alpha stone induced a large amplitude swelling in the absence of calcium both *in vitro* and *in vivo* while it reversed calcium-induced mPT pore opening *in vitro*. The *in vitro* pore opening effect of alpha stone was corroborated by the *in vivo* experiment. It could be that alpha stone caused the assemblage of the pore components especially at higher concentrations. Therefore the use of alpha stone for the treatment of tumour and fibroid may be justified because these are conditions that require cell death either by extrinsic or intrinsic pathway.

Bioactive agents present in medicinal plants have been adjudged to play modulatory roles in mitochondrial-mediated apoptosis [24]. Locally, the resin obtained from terebinth (*Pistacia Palestine*), an active component of the alpha stone, is antiseptic, antispasmodic, cytostatic, expectorant and vulnerary. It has also been used in the treatment of cancer in folkloric medicine. The pods, seed and stem bark of Carob (*Ceratonia siliqua*) contain high pectin and tannin content which has been used as a herbal remedy for diarrhea. Pectin is a polysaccharide, a water-soluble substance, thought to aid in digestion and bacterial infections. The tannins which are carbohydrates and plant pigments have antioxidant and antibacterial properties. Additionally, the tannins retain water and act as a binding agent resulting in firmer stools. Field marigolds (*Calendula arvensic*) shared similar therapeutic use with *Pistacia Palestine* while flowers of white broom (*Retama raetam*) stops nose bleeding, the seeds are used to treat liver complaints, fevers, bladder and kidney problems. Leaves of field marigolds (*Calendula arvensic*) tulips (*Tulipa systola*), seeds of fennel (*Foeniculum vulgare*) and shoots of ephedra (*Ephedra foeminea*) have antibacterial properties. Taking together, it may be that the synergistic effects of the bioactivities of the natural products found in these medicinal plants are responsible for the effectiveness of alpha stone as seen in this study.

Although alpha stone used in this work opened the mPT pore significantly, our results have shown that this polyherbal formulation did not enhance F_1F_0 ATPase activity, it could be that these therapeutic substances did not induce cell death via this mechanism. To know whether alpha stone induced cell death via oxidative stress leading to the peroxidation of the membrane lipid, we assay for the indices of lipid peroxidation both *in vivo* (levels of malondialdehyde) and *in vitro* (percentage inhibition of lipid peroxidation). Our results showed that there was increase in the levels of malondialdehyde generated *in vivo* albeit insignificant but the *in vitro* experiment showed enhanced peroxidation of membrane lipid maximally at 800µg/mL. This showed that the induction of the mPT pore opening may likely be via peroxidation of membrane lipids. Previous work has shown that mitochondrial energy imbalance and lipid peroxidation are capable of causing cell death [25]. Some phytochemicals such as flavonoids that are known as antioxidants at lower concentrations may show another trend of activity (pro oxidants) at higher concentrations. Apart from the phytochemicals from the medicinal plants that are constituents of alpha stone, the role of honey in the formulation of this decoction cannot be overemphasized. Although honey contain sugars, it also contain a wide range of minor constituents many of which, including polyphenols, have antioxidant properties. Some flavonoids have been found to have antioxidant and antitumoral activities [26-29]. In contrast to these activities, flavonoids, such as quercetin and kaempherol, have been shown to induce nuclear DNA damage and lipid peroxidation in the presence of transition metals [30–33] and interestingly, honey, especially from Argentina and Italy have been found to contain minerals such as iron, manganese, zinc and copper [34].

In the course of mitochondria-mediated cell death, mitochondrial dysfunction shows off as a decrease in the membrane potential, large amplitude swelling, ATP hydrolysis and concomitant release of cytochrome c. High concentrations of alpha stone induced the release of cytochrome c significantly higher than the effects noticed by exogenous calcium. This showed that at high concentrations of alpha stone, mitochondrial-mediated cell death is evidently possible since no cell can survive the release of cytochrome c. Calcium is a major mineral constituent of honey and also of the medicinal plants that are components of alpha stone. Calcium is well known as an inducer of apoptosis but in addition to this, copper and manganese induce apoptosis in yeast cells via different pathways [35].

Taking together, the mechanism of action of the decoction as a phytomedicine in fibroid and cancer treatment may be via the induction of pore opening, in the presence and absence of calcium, induction of lipid peroxidation and release of cytochrome c. it will be of high benefit if the bioactive compound(s) responsible for this biochemical processes can be isolated and characterized.

In conclusion, alpha stone contains phytochemical constituents that are of biomedical importance that have both *in vitro* and *in vivo* inductive effects on mitochondrial pore. Besides, it contains some mineral elements that may work synergistically with the phytochemical in order to cause the opening of mPT pore. This may be useful in pathological conditions that require upregulation of cell death.

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