ACTIVITY OF APIGENIN AND QUERCETIN ON RAT HEPATIC MITOCHONDRIAL PERMEABILITY TRANSITION PORE

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Abstract

Background: Alteration of mitochondrial functions such as permeability transition has been found to play a vital role in the apoptotic process induced by certain anti-cancer agents. Phenolics such as quercetin and apigenin commonly found in many fruits and vegetables, is known for its potential anti-tumor properties. Objective: This study was carried out to investigate the effect of quercetin and apigenin *in vitro* on mitochondrial membrane permeability transition pore (MMPT). Methodology: Opening of MMPT pore was assessed as mitochondrial swelling and was monitored spectrophotometrically as changes in absorbance at 540 nm under succinate-energized condition. Results: The results obtained showed that calcium ion induced the opening of MMPT pore significantly ($P<0.05$) in rat hepatic mitochondria while spermine inhibited calcium-induced opening of MMPT pore. In contrary to the effect of quercetin in the absence of exogenous Ca^{2+}, apigenin significantly ($P<0.05$) induced pore opening in a concentration dependent manner. The inductive effect of apigenin is higher than that caused by Ca^{2+} only. The percentage induction of apigenin at 10μg/ml, 20μg/ml, 40μg/ml and 80μg/ml are 88.50%, 91.72%, 91.93% and 92.02% respectively while that of Ca^{2+} is 88.28%. Also, in the presence of Ca^{2+}, apigenin further induced the opening of the pore significantly ($P<0.05$) in concentration dependent manner when compared with the calcium only. Apigenin at all concentration tested enhanced the mitochondrial F_1F_o ATPase activity. Our results demonstrate that apigenin induces cell death through mitochondria-mediated pathway of apoptosis. These findings suggest that apigenin could be useful in situations of dysregulated apoptosis such as cancer.

Keywords: *Apoptosis, Mitochondrial permeability transition pore, Apigenin, Quercetin.*

Introduction

The mitochondrial respiratory chain is considered the power house of cells, generating more than 90% of energy in the form of ATP [1]. When compromised, it results to increased reactive oxygen species production and metabolic disorder. The F_1F_0 ATP synthase is a molecular machine that uses proton motive force to pump protons downhill the gradient. The chemiosmotic coupling hypothesis explains that the 'electron transport chain and oxidative phosphorylation are coupled by a proton gradient across the inner mitochondrial membrane'. Intact mitochondrion synthesizes ATP but if not intact hydrolyzes ATP resulting in the release of inorganic phosphate and ADP.

The process of mitochondria uncoupling or proton leak occur under certain situations where protons re-enters the matrix of mitochondrial without making any impact to ATP synthesis. The process is as a result of facilitated diffusion of protons into the matrix which leads to unharnessed potential energy of the proton electrochemical gradient liberated as heat [2].

Permeability transition (PT) is a phenomenon caused by the opening of a voltage-dependent, high conductance channel known as the permeability transition pore located in the inner mitochondrial membrane. It is a sudden increase of inner mitochondrial permeability to solutes with molecular mass up to 1.5KDa. PT is implicated in apoptosis or necrosis as an important event in the control of cell death or survival [3, 4].

Apoptosis is an essential process in all eukaryotic cells that ensures normal tissue turnover and prevents the accumulation of genetic mutations [5, 6]. It occurs under physiological circumstances such as development and homeostasis of multicellular organisms as well as aging. It also occurs under pathological circumstances like disease states or during exposure to noxious agents. It is distinct from cell death by generalized cellular dissolution, termed as necrosis. Apoptosis is important in cancer biology because programmed cell death is a critical process through which undesirable cells are removed. Several studies have shown that deregulation or mutation of apoptosis is linked to the development of a variety of pathological conditions, including neurodegenerative diseases, autoimmunity, and cancer [7, 8].

The two major routes of apoptosis are the intrinsic or mitochondrial pathway and the extrinsic or death receptor pathway [6]. The extrinsic pathway is activated by the binding of death receptors (Fas and other receptors, like TNF receptor 1 and its relatives) with their cognate ligands. Binding of ligand causes the death receptors to induce formation of death-inducing signaling complex by recruitment of caspase-8 which lead to the activation of caspase cascade. The activated caspase-8 can either directly activate effector caspases such as caspase-3 or indirectly initiate activation of the intrinsic pathway of apoptosis [9].

The release of mitochondrial intermembrane space proteins into the cytosol triggers the intrinsic pathway. In both cases, when a cell is stimulated by either extracellular or intracellular signals, the outer mitochondrial membrane becomes permeable to internal cytochrome c, which is then released into the cytosol. The released cytochrome c activates caspases by forming apoptosome, a protein complex composed of cytochrome c and apoptotic peptidase activating factor 1, leading to caspase-9 and subsequently caspase-3 activation and culminating at the conclusion of the extrinsic pathway of apoptosis [10].

The key factor responsible for mitochondrial permeability transition pore opening is mitochondrial calcium overload especially when accompanied or coupled with free radicals and oxidative stress, adenine nucleotide depletion, elevated phosphate concentrations, mitochondrial depolarization, release of intra-mitochondrial solutes, large amplitude swelling and outer membrane rupture [11].

Flavonoids are a large group of polyphenolic compounds that comprise an essential class of secondary metabolites in plants. Epidemiological studies suggest that flavonoids play an important role in the prevention of many diseases [12, 13]. The most important groups of flavonoids are anthocyanins, flavonols, flavones, catechins, and flavanones.

Quercetin is a flavonoids commonly found in fruits and vegetables, especially in onions, citrus, apples, green tea and red wine. Quercetin and its derivatives have been reported in various studies as

potent antioxidants. It has been shown to act as free radical scavengers by exerting a protective effect in reperfusion of ischemic tissue damage [14]. It has also been reported to possess a number of health promoting benefits and pharmacological activities including antimicrobial, antiviral and anticancer [15].

Apigenin belongs to the flavone group of flavonoids [16] and it is a polyphenolic bioflavone (4,5,7-trihydroxyflavone) with a nonmutagenic chemopreventive agent. It is reported as a natural flavonoid present in the leaves and stems of vascular plants, including fruits and vegetables [17].

Foods rich in apigenin include apples, endive, beans, broccoli, celery, cherries, cloves, grapes, leeks, onions, barley, parsley and tomatoes, chamomile, seasonings, while plant-derived beverages containing apigenin include tea and wine [18, 19].

Apigenin has gained attention due to its health benefits and ability to inhibit cancer development; it is a potent inhibitor of skin, prostate, colon, breast, and thyroid cancer cell proliferation [20, 21]. Apigenin has been reported to act via several mechanisms, including promotion of cell cycle arrest and apoptosis [22, 23, 24], inhibition of cell transformation, inhibition of mutagenesis, and suppression of signal transduction, gap junction function, and angiogenesis [25, 26]. One of the most recognized mechanisms of apigenin is the capability to promote cell cycle arrest and induction of apoptosis through the p53-related pathway [27]. However, the effect of apigenin on mitochondrial membrane permeability transition remains unknown. Therefore, this study aims at investigating the effect of apigenin on MMPT pore.

Methods

Chemicals and Reagents

Adenosine triphosphate (ATP), 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), ethylene glycol-bis (2-aminoethylether)-tetraacetic acid (EGTA), Trichloro acetic acid (TCA), Tris-HCl, Bovine serum albumin (BSA), Sodium Dodecyl Sulfate (SDS), Folin-Cioclateau, Spermine, Mannitol, Sucrose, Sodium succinate, Rotenone, Apigenin were obtained from Sigma-Aldrich, USA. $CaCl_2$, Na^+K^+ tartarate, Na_2PO_4, Na_2CO_3, NaOH, $CuSO_4.5H_2O$, NH_4Molybdate, L-ascorbate, KCl, were obtained from BDH Chemicals Ltd, Poole, England.

Isolation of Liver Mitochondria

Low ionic strength mitochondria were isolated according to the method described by Johnson and Lardy [28]. The animals were sacrificed by cervical dislocation, dissected and the liver was immediately excised and trimmed to remove excess tissue. The liver was washed several times in isolation buffer (210mM Mannitol, 70mM Sucrose, 5mM Hepes and 1mM EGTA at pH 7.4), until a clear wash was obtained, then weighed and minced with a pair of scissors. A 10% suspension was prepared by homogenizing the liver in a Teflon-glass cup homogenizer. The whole process was carried out on ice to preserve the integrity of the mitochondria. The suspended tissue (liver) in isolation buffer was implored into a refrigerated Sigma (3-30K, Germany) centrifuge, where the nuclear fraction and cell debris were sedimented by low speed centrifugation twice at 2,300rpm for 5 minutes. The supernatant thus obtained was centrifuged at 13,000rpm for 10 minute to pellet the mitochondria. The brown mitochondria pellet obtained after the supernatant was discarded was washed by re-suspending in washing buffer (210mM Mannitol, 70mM Sucrose, 5mM HEPES and 50% BSA at pH 7.4) and centrifuged at 12,000rpm for 10 minutes. This washing stage was done twice. The mitochondria were immediately suspended in a solution of ice-cold MSH Buffer (Mannitol, Sucrose, HEPES-KOH, pH 7.4), then dispensed in Eppendorf tubes in aliquot and placed on ice for immediate use.

Mitochondria Protein Determination

Mitochondria protein content was estimated by the procedure of Lowry *et al.* [29] using Bovine Serum Albumin (BSA) as standard. 3ml of Reagent D was added to protein samples mixed and allowed to stand at room temperature for 10 minutes. 0.3ml of Reagent E was added after 10 minutes. Quickly, the mixture was shaken vigorously and left for another 30 minutes after which the absorbance was read at 750nm wavelength on Camspec M105 spectrophotometer. The results obtained were used to plot a standard protein curve.

Mitochondria Swelling Assay

Mitochondria swelling were assessed according to the method of Lapidus and Sokolove [30]. Mitochondria (0.4mg/ml) were pre-incubated in the

presence of 200mM Rotenone for 3.5 minutes, prior to the addition of 5mM Sodium succinate. Assay for mitochondrial swelling in the absence of a triggering agent (Ca^{2+}) involved pre-incubation of the mitochondria in the presence of MSH buffer (swelling buffer), 0.8µM rotenone for $3^1/_2$minutes after which 5mM succinate was added to energize the reaction. Assay for mitochondrial swelling in the presence of a triggering agent (Ca^{2+}) involved pre-incubation of the mitochondria in the presence of MSH buffer (swelling buffer), 0.8mM rotenone for 3 minutes after which Ca^{2+} was added. Thirty (30) seconds later Succinate was added to energize the reaction. Assay for mitochondrial swelling inhibition in the presence of Spermine involved pre-incubation of mitochondria in the presence of MSH buffer, 0.8mM rotenone and 1mM Spermine for 3minutes after which Ca^{2+}, the triggering agent was added then 30 seconds later, 5mM Succinate was added to energize the reaction. Absorbance was taken at a wavelength of 540nm in a Camspec M105 spectrophotometer every 30 seconds for 12 minutes. Swelling was measured as decrease in absorbance within the time space of 12 minutes. The temperature was maintained at 37°C and swelling rate quantified as an A540/min/mg.

Isolation of Mitochondrial for ATPase Activity

The animals were sacrificed by cervical dislocation, dissected and the liver was immediately excised and trimmed to remove excess tissue. The liver was washed several times in sucrose, until a clear wash was obtained, then weighed and minced with a pair of scissors. A 10% suspension was prepared by homogenizing the liver in a Teflon-glass cup homogenizer. The whole process was carried out on ice to preserve the integrity of the mitochondria. The suspended tissue (liver) in sucrose was implored into a refrigerated MSE centrifuge, where the nuclear fraction and cell debris were sedimented by low speed centrifugation at 2300rpm for 5 minutes. The supernatant was re-centrifuged at the same speed and time to remove unbroken cells. The supernatant thus obtained was centrifuged at 13000rpm (10,000g) for 10 minutes to sediment the mitochondria. The brown mitochondria pellet obtained after the supernatant was discarded, was washed and re-suspended in sucrose and re-centrifuged at 12000rpm (9000g) for 10 minutes. This washing stage was done twice. The mitochondria were immediately suspended in a solution of ice-cold of very little sucrose and then dispensed in Eppendorf tubes in aliquot and placed on ice for immediate use. Protein determination was carried out by Lowry et al (1951).

Determination of Inorganic Phosphate

This was performed according to the method described by Ronner *et al.* [31]. Different concentrations of 1mM Na$_2$PO$_4$ were used for the standard curve as below in the protocol. To the 5ml of de-proteinizised supernatant in a test tube, 1000µl of 1.25% Ammonium Molybdate and 1000µl of 2% freshly prepared solution of Ascorbic acid were added. After through by mixing with gentle shaking, the tube was allowed to stand for 30 minutes. The absorbance was read at 660nm wavelength on Camspec M105 Spectrophotometer against distilled water as blank. The graph of standard PO$_4$ was obtained by plotting absorbance against phosphate concentration.

Determination of mitochondria ATPase activity

Adenosine triphosphatase activity was determined by a modification of the method of Lardy and Wellman [32]. Each reaction vessel contained 65mM Tris-HCl buffer at pH7.4, 0.5mM KCl, 1mM ATP and 0.25mM Sucrose in a total reaction volume of 2mls. Distilled H$_2$O or test compound was added accordingly. The reaction which was started by the addition of the mitochondrial fraction was allowed to proceed for 30 minutes with constant shaking at 37°C. Mitochondria were put in all the tubes at 30 seconds interval except the tube containing ATP only. 1ml of sodium dodecyl sulfate (SDS) was put in the zero time test tube after the addition of the mitochondria and the reaction in the other reaction tubes was stopped by the addition of 1ml of sodium dodecyl sulfate (SDS) after which 1000µl ammonium molybdate was put into all the test tubes. L-ascorbate was then added to the test tubes at 30 seconds interval for 20 minutes, after the 20th minute the absorbance reading was taken at 660nm.

Statistical analysis

Results were analyzed using student's t test and one-way ANOVA followed by Dunnett's post-hoc test for comparison between control and test groups. All data were expressed as mean ± standard error of the mean. '*p*' values < 0.05 were considered

significant. GraphPad Prism 5 and Microsoft Excel 2003 were used for the analyses.

Results

Figure 1 depicts the result obtained from the assessment of calcium-induced permeability transition in normal rat liver. The result shows a drastic reduction in absorbance at 540nm in the presence of exogenous Ca^{2+}. Ca^{2+} significantly induced the opening of mitochondria pore (0.639 ± 0.024) when compared with the control (mitochondria in the absence of calcium: 0.058 ± 0.002). The percentage inhibition of mitochondrial swelling by spermine was 52.46%.

Figure 2 and 3 shows the effect of the quercetin on MMPT in the presence and absence of exogenous Ca^{2+}. Quercetin inhibited the opening of the pore in a concentration dependent manner in the absence of Ca^{2+}. In the presence of exogenous Ca^{2+}, quercetin also induced the opening of mitochondrial membrane permeability transition pore in a concentration dependent mammer with the lowest concentration 5µg/mL having the highest inductive effect.

Figure 4 and 5 shows the effect of the apigenin on mitochondrial membrane permeability transition in the absence and presence of exogenous ca^{2+}. In the absence of exogenous ca^{2+}, apigenin significantly ($P<0.05$) induced the opening of mitochondrial membrane permeability transition pore in a concentration dependent manner. The inductive effect of apigenin is higher than that caused by ca^{2+} only. The percentage induction of apigenin at 10µg/ml, 20µg/ml, 40µg/ml and 80µg/ml are 88.50%, 91.72%, 91.93% and 92.02% respectively while that of ca^{2+} is 88.28%. Also, in the presence of ca^{2+}, apigenin further induced the opening of the pore significantly ($P<0.05$) in concentration dependent manner when compared with the calcium only.

Figure 6 shows the effect of apigenin on mitochondrial ATPase. The ATPase activity of the extract increased significantly ($P<0.05$) in concentration dependent manner. The uncoupler (2,4-dinitrophenol) significantly ($P<0.05$) uncouples the mitochondria when compared with the control and the apigenin.

Discussion

Quercetin is found in abundance in onions, broccoli, apples and berries. Quercetin and its derivatives have a number of health benefits and are reported in various studies as potent antioxidants against free radicals. It has also been reported to possess a range of biological and pharmacological activities which includes antimicrobial, antiviral and anticancer [15]. Apigenin is a flavonoid commonly found in many fruits and vegetables such as parsley, chamomile, celery, and kumquats. Apigenin have been identified as major cancer-preventive components of human diets due in part to their anti-oxidative, free radical scavenging, and anti-inflammatory activities [33]. Recently, recognition of apigenin as a cancer chemopreventive agent has increased. Significant progress has been made in studying the chemopreventive aspects of apigenin both *in vitro* and *in vivo*. Several studies have demonstrated that the anticarcinogenic properties of apigenin occur through regulation of cellular response to oxidative stress and DNA damage, suppression of inflammation and angiogenesis, retardation of cell proliferation, and induction of autophagy and apoptosis.

The results obtained from this study showed that calcium ions induced the opening of MMPT pore significantly ($P<0.05$) in rat liver mitochondrial, while spermine inhibited calcium-induced opening of MMPT pore. Spermine has been reported to protect mitochondria from damage and that this protective effect reflects inhibition of the inner membrane permeability transition [30]. Toninello *et al.*, [34] also reported that spermine blocks the collapse of membrane potential and release of Mg^{2+}, adenine nucleotides and Ca^{2+} observed when mitochondria are exposed to damaging concentrations of inorganic phosphate and Ca^{2+}.

In this study quercetin inhibited the opening of the pore in the absence of Ca^{2+} but significantly induced the opening of MMPT pore in the presence of Ca^{2+} in a concentration dependent manner with the lowest concentration also having the highest inductive effect. Contrary to the effect of quercetin in the absence of calcium, apigenin significantly induced the opening of MMPT pore in a concentration dependent manner in the absence of exogenous Ca^{2+}. It was observed that the inductive effect of apigenin was significantly higher than that caused by Ca^{2+} only. It was reported by earlier study that apigenin induced a dose-dependent reduction in viability of human promyelocytic leukemia HL-60

cells through a rapid induction of caspase-3 activity, proteolytic cleavage of poly-(ADP-ribose) polymerase (PARP), loss of mitochondrial transmembrane potential, elevation of reactive oxygen species (ROS) production, release of mitochondrial cytochrome *c* into the cytosol, and subsequent induction of procaspase-9 processing [35]. Observation from this study showed that apigenin further induced the opening of the pore significantly in the presence of Ca^{2+} in a concentration dependent manner when compared to the Ca^{2+} only. The effects of apigenin on apoptosis have been studied extensively in cell populations of many different cancers. Apigenin has been shown either to directly induce apoptosis or to sensitize cells to other pro-apoptotic stimuli in cancer cell lines of oral [36], esophageal [37], colorectal [38] hepatic [39, 40] and pancreatic [41] cancers. Recent work in pancreatic carcinoma has indicated the possibility that apigenin was capable to promote cell cycle arrest and induction of apoptosis through p53-related pathways, even where cancers harbor mutations of the p53 tumor suppressor [42]. Several studies have reported that apigenin-induced apoptosis involves the activation of both the intrinsic and extrinsic apoptotic pathways [24]. The induction of apoptosis has been emphasized in anticancer strategies [43, 44]. Apigenin at all concentration tested enhanced the mitochondrial F_1F_0 ATPase activity with increased concentration of phosphate observed when compared with the control using 2,4-dinitrophenol as standard uncoupler. The result is consistent with the opening of MMPT pore in the absence of calcium which eventually leads to the uncoupling of the mitochondrial electron transport chain, and the phosphate released confirms the concentration dependent pore opening.

Conclusion

The influence of apigenin on mitochondrial mediated pathway of apoptosis confirms its anticancer property. The inductive effects of apigenin on mitochondrial membrane permeability transition pore suggest that it may prove useful in situations where increased rate of apoptosis is required for chemotherapy such as cancer and tissue wastage.

Conflict of interests

The author(s) did not declare any conflict of interest.

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Table 1: Change in absorbance at 540nm of the effect of apigenin in the absence of exogenous calcium.

Groups	Change in Absorbance	Induction fold	% Induction
No triggering agent (-Ca^{2+})	0.013 ± 0.005	1.0	-
Triggering agent (+Ca^{2+})	0.110 ± 0.002	8.5	88.28
10µg/ml Apigenin	0.113 ± 0.004	8.7	88.50
20µg/ml Apigenin	0.157 ± 0.003	12.1	91.72
40µg/ml Apigenin	0.161 ± 0.002	12.4	91.93
80µg/ml Apigenin	0.163 ± 0.005	12.5	92.02

Results are expressed as mean of three determinations ± standard error of mean (SEM).

Figure 1: Calcium induced opening of normal rat liver mitochondrial membrane permeability transition pore and the reversal effect of spermine. NTA= No Triggering Agent, TA= Triggering Agent, INH= Inhibitor (Spermine).
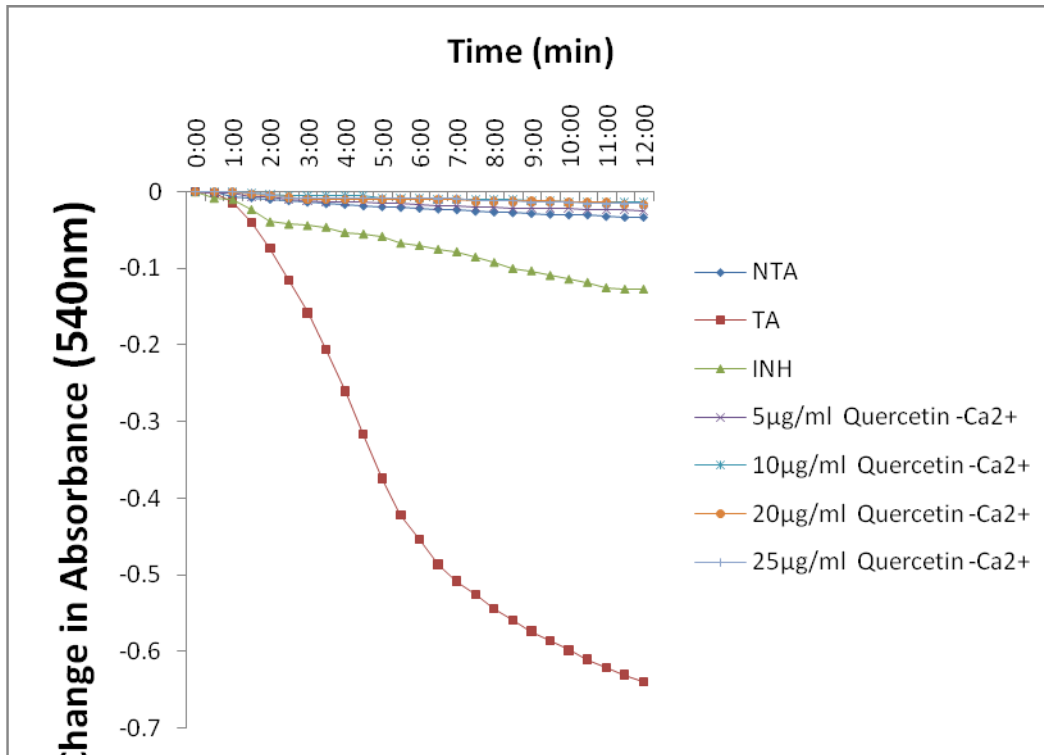
Figure 2: Varying concentrations of quercetin on rat liver mitochondrial membrane permeability transition pore in the absence of exogenous calcium
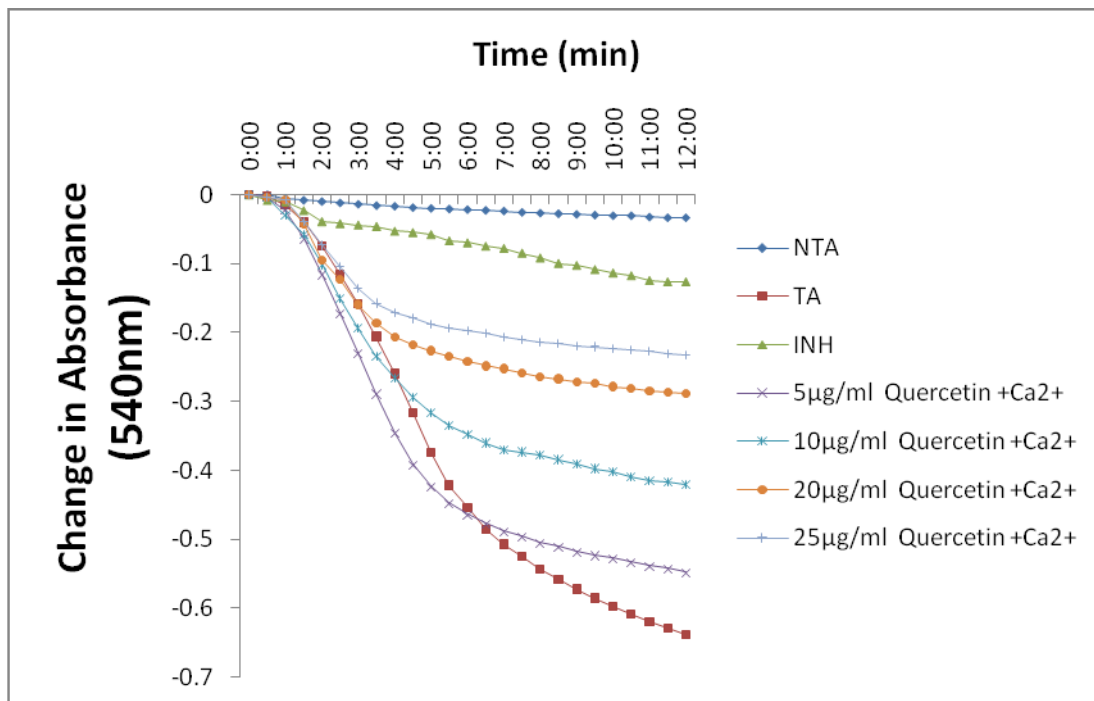

Figure 3: Varying concentrations of quercetin on rat liver mitochondrial membrane permeability transition pore in the presence of exogenous calcium.
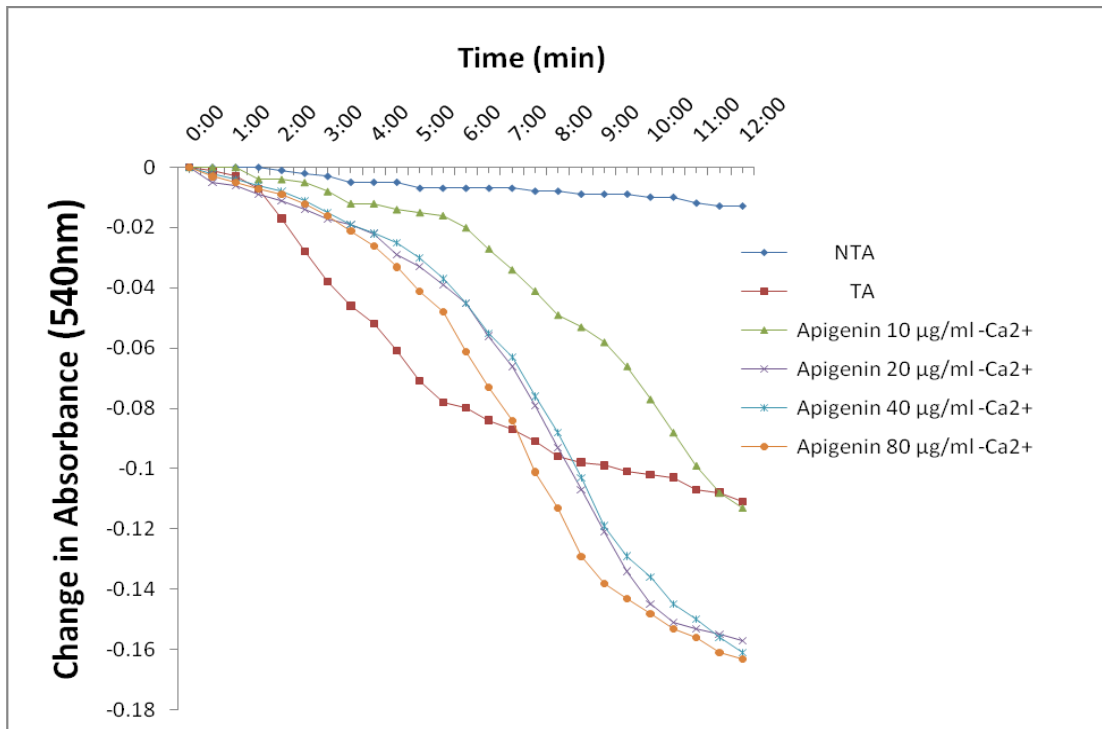
Figure 4: Varying concentrations of apigenin on rat liver mitochondrial membrane permeability transition pore in the absence of exogenous calcium.
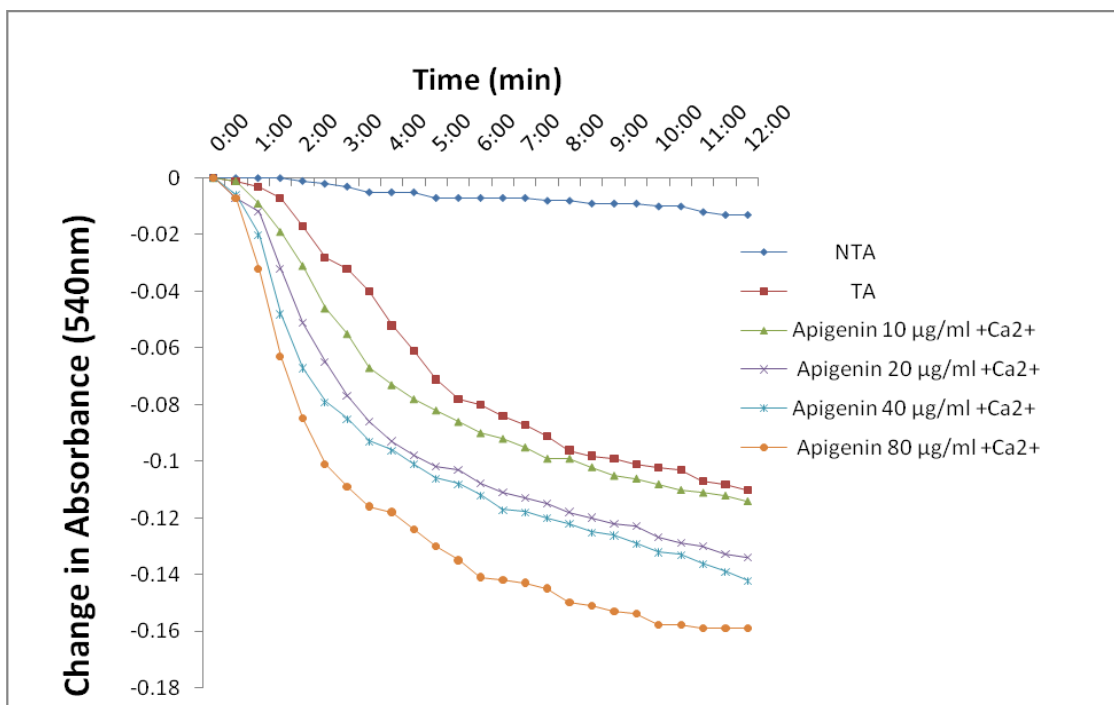

Figure 5: Varying concentrations of apigenin on rat liver mitochondrial membrane permeability transition pore in the presence of exogenous calcium.
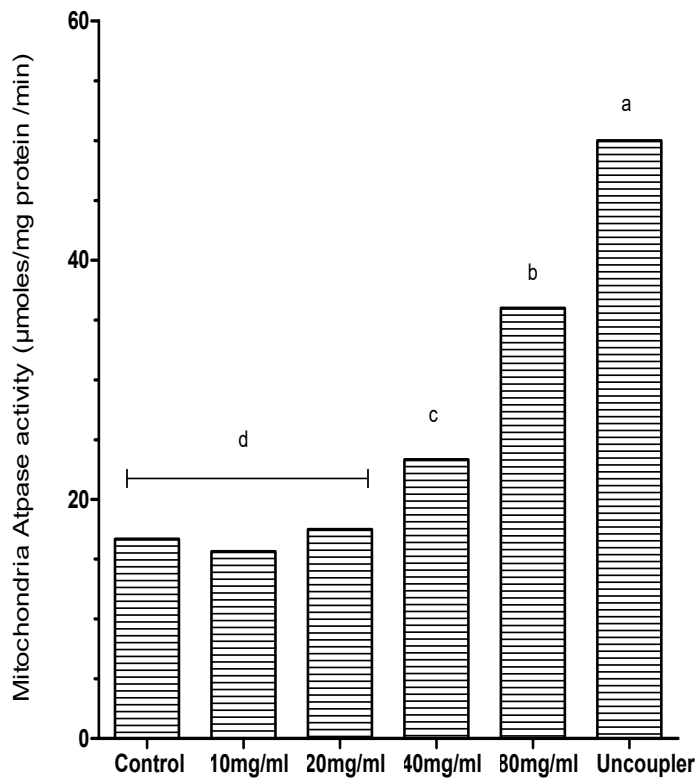
Figure 6: Effect of apigenin on mitochondrial ATPase

Results are expressed as mean of three determinations ±

standard error of mean (SEM).