

Butanol extract of *Manihot esculenta* leaf modulated cigarette butt leachate-mediated liver mitochondrial membrane permeabilization and its functional capacity

*Ajayi E.I.O., Iyoha A.E., Abdulazeez F.O.

Abstract

Objective: The experiment was designed to investigate the modulatory effects of the butanol extract of *Manihot esculenta* leaf, *in vivo*, and against the effects of cigarette butt leachate, *in vitro* on liver mitochondrial membrane permeability transition (MMPT), and its possible hepatoprotection in female Wistar rats.

Methods: The powdery form of fresh, air-dried and pulverized *M. esculenta* leaves were extracted in 95% butanol using Soxhlet apparatus and concentrated to a sticky mass using rotary evaporator. Ethical approval for animal use was obtained from Ladoko Akintola University of Technology, Osogbo, Nigeria (LTH/EC/2014/10/237). Twelve Wistar rats were randomly assigned into 2 groups of 6 rats each, and a separate group of 4 rats were used for the *in vitro* study. The first set of rats was orally treated daily with the extract for 21 days. Following an overnight fast, the rats were sacrificed by cervical dislocation and the liver mitochondria isolated by standard methods. Mitochondrial swelling and liver function assays were done spectrophotometrically.

Results: *In vivo*, the extract significantly induced low amplitude pore opening in the absence of exogenous Ca^{2+} ; whereas when it was present, the extract slightly inhibited same. Two concentrations of cigarette butt leachate caused appreciable low amplitude swelling in control and treated animals in the presence of Ca^{2+} ; in the absence of which highly significant inhibitory effects were observed at the same concentrations, *in vitro*. Significant increases were also recorded for AST, ALT, bilirubin, total protein and ALP, *in vivo*.

Conclusion: The extract showed promise for hepato-protection against cigarette butt leachate toxicity in the liver of normal rats in the presence of exogenous Ca^{2+} , *in vitro*.

Keywords: *Manihot esculenta* leaf extract, cigarette butt leachate, mitochondrial membrane permeability transition pore, liver function

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Extrait de butanol de *Manihot esculenta* feuilleté de mégot de cigarette à médiation par lixiviat mitochondrial et sa capacité fonctionnelle

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Resume

Objectif: L'expérience a été conçue pour étudier les effets modulateurs de l'extrait de butanol de *Manihot esculenta* *in vivo* et contre les effets du lixiviat des mégots de cigarettes *in vitro* sur la transition de la perméabilité membranaire mitochondriale du foie (MMPT) et son éventuelle hépatoprotection chez la femelle Rats Wistar.

Méthodes: La forme pulvérulente de feuilles de *M. esculenta* fraîches, séchées à l'air et pulvérisées a été extraite dans du butanol à 95% en utilisant un appareil Soxhlet et concentrée en une masse collante à l'aide d'un évaporateur rotatif. L'approbation éthique pour l'utilisation des animaux a été obtenue auprès de l'Université de Technologie Ladoko Akintola, Osogbo, Nigeria (LTH / EC / 2014/10/237). Douze rats Wistar ont été répartis au hasard en 2 groupes de 6 rats chacun, et un groupe séparé de 4 rats a été utilisé pour l'étude *in vitro*. Le premier groupe de rats a été traité par voie orale tous les jours avec l'extrait pendant 21 jours. Après un jeûne d'une nuit, les rats ont été sacrifiés par dislocation cervicale et les mitochondries du foie ont été isolées par des méthodes standard. Gonflement mitochondrial et les tests de la fonction hépatique ont été réalisés par spectrophotométrie.

Résultats: *In vivo*, l'extrait induit significativement une ouverture de pores de faible amplitude en l'absence de Ca^{2+} exogène; alors que lorsqu'il était présent, l'extrait inhibait légèrement le même. Deux concentrations de lixiviat de mégots de cigarettes ont provoqué un gonflement de faible amplitude appréciable chez les animaux témoins et traités en présence de Ca^{2+} ; en l'absence de quoi des effets inhibiteurs hautement significatifs ont été observés aux mêmes concentrations, *in vitro*. Des augmentations significatives ont également été enregistrées pour l'AST, l'ALT, la bilirubine, la protéine totale et l'ALP, *in vivo*.

Conclusion: L'extrait s'est avéré prometteur pour la protection hépatique contre la toxicité des lixiviats de mégots de cigarettes dans le foie de rats normaux en présence de Ca^{2+} exogène, *in vitro*.

Mots clés: Extrait de feuille de *Manihot esculenta*, lixiviat de mégots de cigarette, pore de transition de perméabilité membranaire mitochondriale, fonction hépatique

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INTRODUCTION

Cells under stress activate survival and death signaling pathways. Cell death signaling frequently converges on mitochondria, a process that is controlled by the activities of pro- and anti-apoptotic BCL-2 proteins (1). Studies have shown that oxidative stress induces apoptosis (2, 3). Inhibition of the mitochondrial apoptosis pathway through pro-apoptotic BCL-2 proteins could prevent eventual release of cytochrome c from the mitochondrial intermembrane space to the cytosol (4). BCL-2, BCL-XL and MCL-1 modulation of Ca^{2+} signalling during oxidative stress may also play a vital role in nephroprotection. Hence manipulating the pro-survival BCL-2 family members may be beneficial in developing future therapies for neurodegenerative disorders (1). Calcium is a universal messenger regulating many physiological functions, including the ability of the cell to undergo apoptosis (5). In physiopathological conditions including diabetes, apoptotic processes become dysregulated, leading to tissue wastage due to excessive apoptosis. Compounds that inhibit pore opening, including lycopene, zeaxanthin, ginsenosides Rg3, ginkgolides A, B and C; withanolide, icarin, epimedeside and breviflavone could alter calcium levels (6).

The MMPT involves a sudden, and initially reversible increase in permeability of the inner mitochondrial membrane to solutes up to 1.5 kDa due to the pore formed across the inner and outer membranes of mitochondria (7). The pore opening causes depolarization of the mitochondrial membrane potential, loss of the H^+ gradient, uncoupling of oxidative phosphorylation, ATP depletion, and mitochondrial swelling (8, 9). It is commonly defined by its inhibition by potent inhibitors including spermine, cyclosporin A or their derivatives that bind to mitochondrial cyclophilin (peptidyl-prolyl-cis-trans-isomerase) such as N-methyl-Val-4-cyclosporin A. Spermine- and cyclosporin A-mediated inhibition of the permeability transition is transient (lasting 60 min) (10). A diverse range of stimuli can control the MMPT in both isolated mitochondria and intact cells. Full-blown permeability transition causes uncoupling of the respiratory chain with collapse of the electrochemical proton gradient $\Delta\Psi_m$ and cessation of ATP synthesis, matrix Ca^{2+} outflow, depletion of reduced glutathione, depletion of NADPH, hypergeneration of superoxide anion, and mitochondrial release of

intermembrane proteins (11). Several of the consequences of permeability transition themselves favour opening of the permeability transition pore, implying that permeability transition is a self-amplifying process (12). Periodic reversible opening of the permeability transition pore allows for the release of Ca^{2+} from the mitochondrial matrix, thereby participating in Ca^{2+} homeostasis and/or the generation of Ca^{2+} waves (Ca^{2+} -induced Ca^{2+} -release) (13). The intrinsic (mitochondrial) pathway of apoptosis leads to the release of cytochrome c following two crucial events including massive colloid-osmotic swelling of the mitochondrial matrix and rupture or bursting of the outer membrane. Indeed, this seemed to be the case in a number of experiments performed using isolated mitochondria *in vitro* (14, 15, 16). The aim of this experiment therefore was to assess the effect of butanol extract of *Manihot esculenta* leaf on liver mitochondrial swelling in otherwise normal rats, *in vivo*; the modulatory effects of the extract pretreatment against cigarette butt leachate mutagenic perturbations of the mitochondrial membrane permeability transition pore opening, *in vitro* and the effects of oral administration of the extract on liver function.

Cassava leaves, particularly the first matured leaves and the tender petioles and stem are a major food in Africa (17). It has become a staple vegetable and forage in African countries such as Congo, Sierra Leone, Madagascar and Nigeria due to its richness in protein and beta-carotenoids (18), the latter which are potent antioxidants (19).

Tobacco is the single greatest cause of preventable death in developed countries and an important cause of premature death worldwide. As many as half of people who use tobacco die from the results of this use. Each year, tobacco causes about 6 million deaths (about 10% of all deaths) with 0.6 million of these occurring in non-smokers due to second hand smoke (20). Environmental tobacco smoke or secondhand smoke has been shown to cause adverse health effects in people of all ages (21). The prevalence of cigarette smoking among youths and adults in Nigeria is high and alarming (22). In restaurants and bars, spent tobacco in the form of ashes and butts are handled and disposed indiscriminately leading to environmental pollution. Cigarette butts are the most usual form of litter in the world, as approximately 5.6 trillion cigarettes are smoked every year worldwide (23). Owing to the ubiquitous nature and magnitude of cigarette

butts discharged into the environment; studies are needed to determine whether cigarette butt waste can exert 'mitotoxic' effects in the course of exposure to the leachate. A plethora of highly toxic chemicals are introduced to the environment via cigarette particulate matter (tar) and butt-ashes leachate. These may include carbon monoxide, hydrogen cyanide, nitrogen oxides, polycyclic aromatic hydrocarbons, ammonia, acetaldehyde, formaldehyde, benzene, phenol, argon, pyridines and acetone, over 50 of which are known to be carcinogenic to humans (24).

Smoked cigarette tobacco, (un)smoked cigarette filters litter waste bins, dumpsites and roadsides through which arsenic, nicotine, polycyclic aromatic hydrocarbons and heavy metals are released into the environment (25, 26, 27). The risks of poisoning from these toxicants resulting from long-term exposure may manifest in many forms of cancer including kidney cancer (28), cancer of the larynx and head and neck (29, 30), bladder cancer (31), cancer of the esophagus (32), cancer of the pancreas (33) and stomach cancer (34).

MATERIALS AND METHODS

Plant and Cigarette Materials: Fresh *M. esculenta* leaves were collected from a farm in Osun State University, Osogbo. They were identified and authenticated (Voucher Number: UIH002/1094) by Mr. S. B. Adeyemi at the Plant Biology Department, University of Ilorin, Kwara State, and were deposited at the herbarium. The leaves were air-dried for three (3) weeks, and extracted in 95% ethanol using Soxhlet apparatus. The extract was concentrated at a lower temperature under reduced pressure in a rotary evaporator (LIDA XMT-J7000/RE52-3 iDNA). The cigarette butt leachate was prepared by soaking 40 mg cigarette ashes from the same cigarette brand in 2 ml distilled water for 24 hours. This was spun in table centrifuge at 3000 X g for 10 minutes and the supernatant was used for this study.

Animals and Treatment Regimen: Ethical approval for animal use was obtained from Ladoko Akintola University of Technology, Osogbo, Nigeria (LTH/EC/2014/10/237). The rats weighing about 80 – 120 g were kept in metabolic cages, acclimatized for 2 weeks, fed normal rat chow and allowed access to water, *ad libitum*. After the acclimatization, the butanol extract was suspended in olive oil at 750 mg/kg

and administered by oral gavage for 21 days. The separate group of 4 rats was used for the *in vitro* study over 200 – 1400 µg/ml extract concentration range.

Liver Mitochondrial Membrane Permeability

Transition Pore Opening: At the end of the administration of butanol extract of *M. esculenta* leaf, liver mitochondria were prepared by the method of Sanz *et al.* (35) with slight modifications. Briefly, the rats were sacrificed by cervical dislocation and the livers dissected and trimmed to remove excess tissue, rinsed in Buffer C, patted dry and weighed. The liver was then minced with a pair of scissors and poured into the homogenizing flask adding 10% MSTHE buffer (210 mM mannitol, 70 mM sucrose, 5mM TRIS and 5 mM HEPES-KOH, pH 7.4 and 1 mM EGTA) was added and then homogenized with Potter-Elvehjem homogenizer. The whole process was carried out on ice to preserve the integrity of the mitochondria. The homogenized tissue was then implored into a high-speed cold centrifuge, where the nuclear fraction, unbroken cells and cell debris were sedimented by centrifugation at 2500 × g for 10 minutes. The supernatant was poured into a new centrifuge tube and the pellet was discarded. The supernatant was then centrifuging again at the speed of 9000 × g for 20minutes to sediment the mitochondria pellets. The brown pellets were washed with MSTHE buffer containing 5% BSA at 6000 × g for 10 minutes, after which the pellet was made homogenous following aspiration and suspension in MSTHE buffer lacking EDTA in aliquots kept on ice. The assessment of liver MMPT was done within 4 hours after mitochondria isolation following the protocol of Lapidus and Sokolove (7), which works on the principle that mitochondria that have accumulated calcium can be induced to undergo a permeability transition. The inner membrane becomes non-selectively permeable to small (1500 Daltons) solutes and show colloid-osmotic i.e. large amplitude swelling which results in a decreased photometric absorption at 540 nm as their refractive index changes so that less light is passed across the cuvette which results in a decrease in the light absorbance measured with a spectrophotometer. To avoid any complications that changes in the redox state of the respiratory chain components might cause, the wavelength of the incident light should be at the isobestic point from the cytochromes (520 nm or 540 nm) as used in several studies on isolated

mitochondria (7).

Thus, the rate of change in absorbance was monitored at 540 nm under energized and de-energized conditions using Camspec M106 spectrophotometer (Spectronic Camspec Ltd, UK), viz: liver mitochondria [(1.0 mg/ml BSA equivalent, by protein determination following Lowry's method (36)] in MSTHE buffer were preincubated in the presence of 2 mM rotenone, energized with 5mM sodium succinate to support swelling in the absence or presence of exogenous Calcium (5.68 mM CaCl_2) and 1mM spermine as triggering and inhibiting agents, respectively. For the effects of the extract on the liver MMPT, *in vitro*, other additions were made to the reaction mixture in the cuvette (200, 600, 1000 and 14000 $\mu\text{g/ml}$ as 10, 30, 50 and 70 μl , respectively). The incubation of the concentrations of cigarette butt leachate (CBL) was done with mitochondria in the presence of 2 mM rotenone at 30 °C for 3 minutes prior to the addition of 5.68 mM CaCl_2 , 30 seconds after which the assay was energised by 5 mM sodium succinate.

Liver Function and Total Protein Quantification:

Following sacrifice of the rats, sera were prepared from blood collected into plain tubes that were allowed to stand at room temperature for 5 minutes and spun in a table top centrifuge at $3000 \times g$ for 20 minutes. Sera were stored at -35°C till further use. Biochemical markers of liver function as well as total protein were determined thereafter. Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) activity and the bilirubin and total protein concentration were determined upon sacrifice after 5 days of treatment by standard methods using Randox[®] kits (England) following manufacturer's instructions. Absorbance for the respective parameter was read at the specified wavelengths (nm) in CamSpec[®] 105 UV-Vis spectrophotometer (UK).

Statistical Analyses: Data were analyzed with ANOVA (SPSS vs 20.0) and results were presented as Mean \pm SD ($p < 0.05$).

RESULTS

The results showed that the extract at 750 mg/kg significantly ($p < 0.05$) triggered low amplitude swelling (-0.034 ± 0.020 , 2-fold) compared to the control groups (-0.017 ± 0.006) in the absence of exogenous Ca^{2+} ; whereas in the presence of exogenous Ca^{2+} , the extract slight inhibited spermine-sensitive liver MMPT pore opening compared to control (-0.093 ± 0.024 ,

2.75-fold; -0.109 ± 0.030 , 2.71-fold, respectively). Concentrations of cigarette butt leachate (200 $\mu\text{g/ml}$, CBL₁₀ and 600 $\mu\text{g/ml}$, CBL₃₀) caused appreciable amplitude swelling of the pore in control (-0.089 ± 0.023 , CBL₃₀) and treated (-0.063 ± 0.019 , CBL₁₀) animals in the presence of Ca^{2+} ; while highly significant inhibitory effects were observed for the CBL in control and treated groups at 200 $\mu\text{g/ml}$ (-0.020 ± 0.008 , CBL₁₀) and 600 $\mu\text{g/ml}$ (-0.024 ± 0.020 , CBL₃₀), respectively, *in vitro*. Significant increases were recorded for AST, ALT, bilirubin, total protein and ALP following oral administration of 750 mg/kg *M. esculenta* leaf compared to the control animals. There were significant increases ($p < 0.05$) in the concentrations of AST, ALT, total and direct bilirubin as well as total protein following oral administration of butane extract of *M. esculenta* leaf to otherwise normal rats: AST level was found to be significantly increased (41.08 ± 1.08 U/L vs 22.44 ± 0.57 U/L). ALT level also significantly in the treated animals compared to control group (92.65 ± 1.5 U/L vs 28.17 ± 0.83 U/L). Total bilirubin was less significantly increased following treatment compared to and direct bilirubin (16.95 ± 0.4 $\mu\text{mol/L}$, 81.83 ± 0.2 $\mu\text{mol/L}$; 13.07 ± 0.35 $\mu\text{mol/L}$, 58.43 ± 0.46 $\mu\text{mol/L}$, respectively). Total protein level was also significantly increased by the extract treatment compared to the control group (30.14 ± 0.86 mg/dL vs 20.82 ± 1.32 mg/dL).

DISCUSSION

The extract had a significant induction effect of pore opening (-0.034 ± 0.02 , 2fold) when compared to the status of the pore, *ab initio* in the absence of exogenous Ca^{2+} (-0.017 ± 0.006). This may be a reflection of the ability of the extract to trigger cell death through the mitochondrial pathway. However, the apparently significant low amplitude swelling and induction effect of butanol extract of *M. esculenta* leaf on rat liver MMPT pore opening observed in the presence of Ca^{2+} (2.7 fold) significant mitoprotection in the control (2.71 fold). In the control group, *in vitro* perturbation of the liver MMPT pore opening by the concentrations of cigarette butt leachate (CBL₁₀ and CBL₃₀) slightly induced (2.34 and 1.68 folds) compared to Ca^{2+} (2.71 fold); whereas following treatment, triggering of liver MMPT by CBL₁₀ reduced to 1.60-fold while that of CBL₃₀ increased to 2.78-fold. This may be a case of public health concern as accumulation of arsenic, nicotine, polycyclic aromatic hydrocarbons, heavy metals and a plethora of other toxicants

from smoked cigarette tobacco, (un)smoked cigarette filters may overwhelm the liver. This concern is in consonance with the report of Moriwaki *et al.* (27) that smoked cigarette tobacco and (un)smoked cigarette filters litter waste bins, dumpsites and roadsides through which are released into the environment. Increased AST, ALT, bilirubin and total protein following administration of the extract suggest that though the extract improved protein synthesis by the liver, the rate of break-down of the proteins was also increased leading to elevated levels of bilirubin. The extract may also be toxic to the liver having caused cytolysis of plasma membrane of the hepatocytes as suggested by the increased AST and ALT levels (37).

CONCLUSION

The outcome of this research indicate that the butanol extract of *M. esculenta* possess certain hepatotoxic bioactive compounds that could induce liver MMPT pore opening in normal rats, *in vivo*. Oral administration of the same extract offered mitoprotection against cigarette butt leachate toxicity, *in vitro* in the liver of normal rats in the presence of exogenous Ca^{2+} . Thus it can be concluded that the extract could be useful in the prevention of pore opening that could lead to the activation of the mitochondrial apoptotic pathway caused by exposure to cigarette waste.

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Conflict of Interest: The authors declare that they do not have any conflict of interest or competing/ financial interests.

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Table 1: Effects of butanol extract of *Manihot esculenta* leaf on some serum liver function parameters

Groups/ Biomarkers	AST (U/L)	ALT (U/L)	T-BIL ($\mu\text{mol/L}$)	D-BIL ($\mu\text{mol/L}$)	TP (mg/dl)
Control	22.44±0.57	28.17±0.83	13.07±0.35	58.43±0.46	20.82±1.32
Extract- treated	41.08±1.08*	92.65±1.50*	16.95±0.40*	81.83±0.20*	30.14±0.86*

‘*’ denotes significance at $p < 0.005$

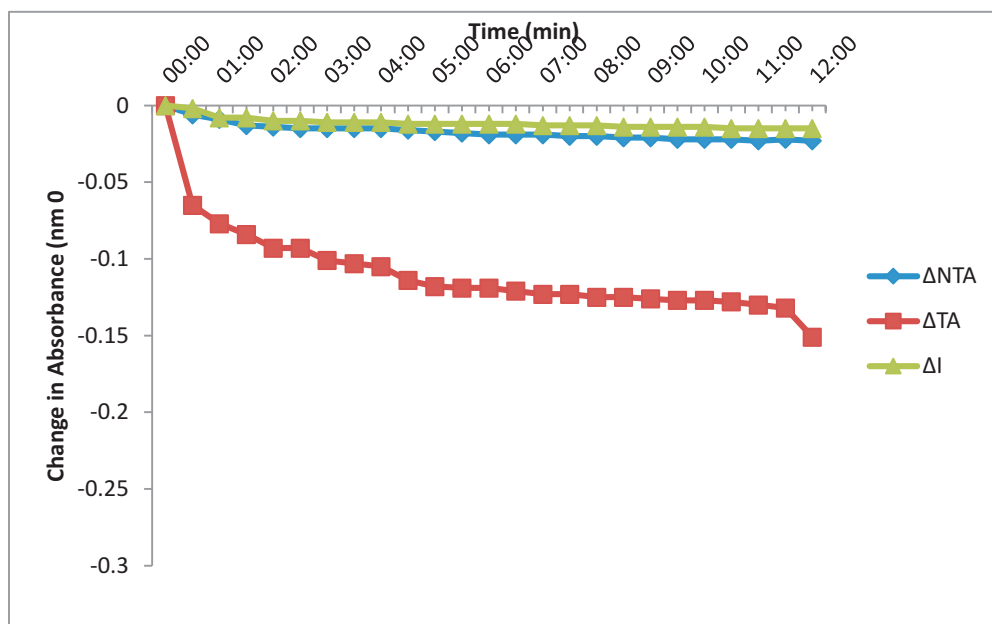


Figure 1: Ca^{2+} -induced opening of liver mitochondrial membrane permeability transition (MMPT) pore and inhibition of the opening by Spermine in normal rats.

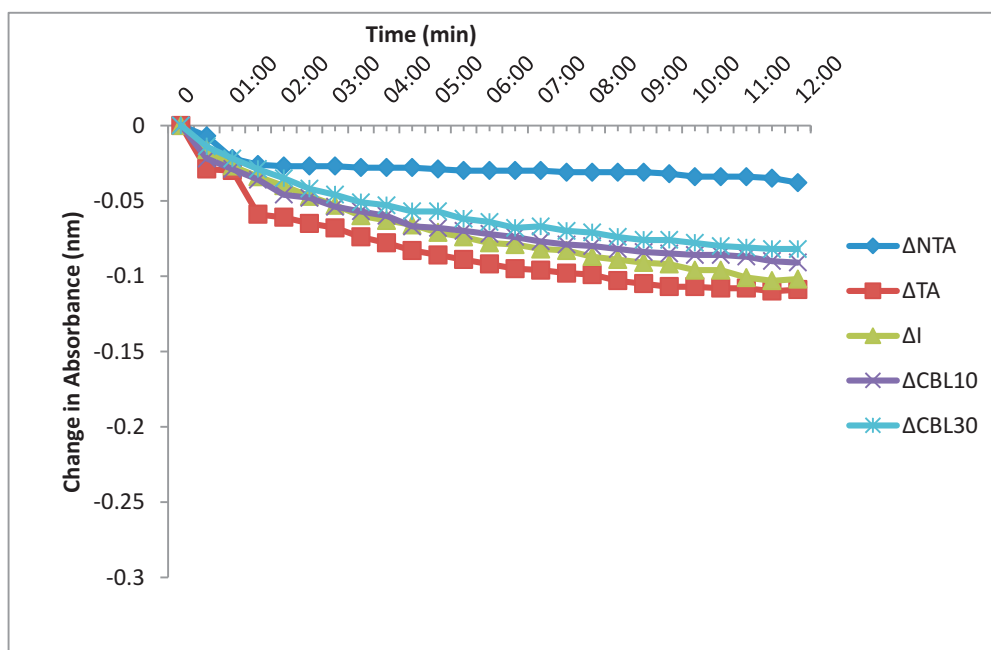


Figure 2 : The effect of varying concentrations of Cigarette Butt Leachate on liver MMPT Pore opening of Normal rat, in vitro. NTA: No Triggering Agent, TA: Triggering agent (5.68 mM CaCl_2), I: Spermine inhibition, CBL₁₀: 200 $\mu\text{g/ml}$, CBL₃₀:600 $\mu\text{g/ml}$.

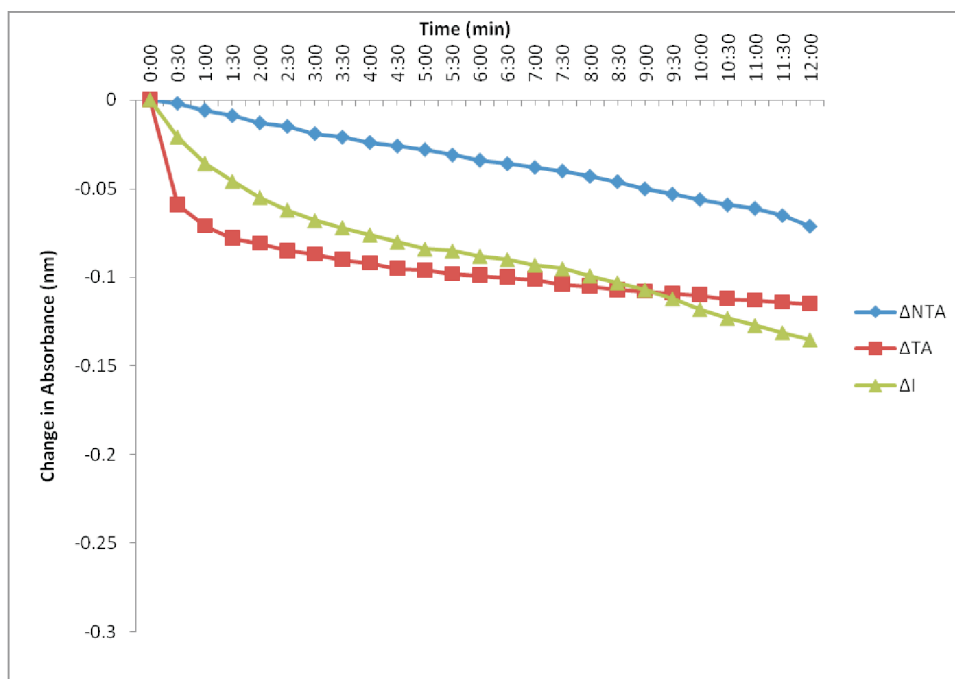


Figure 3: Ca²⁺-induced opening of liver mitochondrial membrane permeability transition (MMPT) pore in rat administered butanol extract of *Manihot esculenta* leaf.

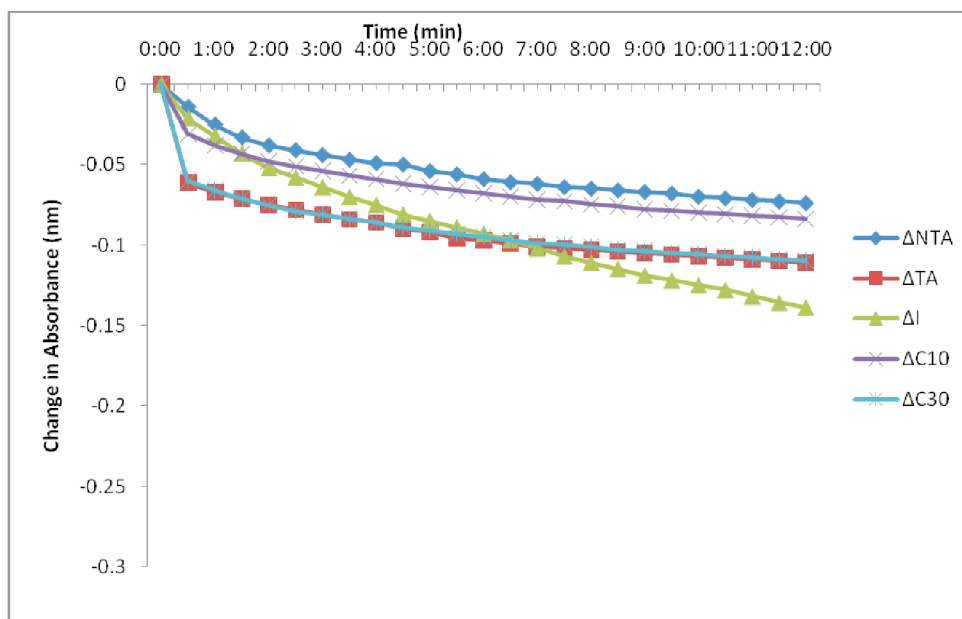


Figure 4 : Effect of *M. esculenta* on perturbation of rat liver MMPT pore opening by Cigarette Butt Leachate, in vitro. NTA: No Triggering Agent, TA: Triggering agent (5.68 mM CaCl₂), I: Spermine inhibition, CBL₁₀: 200 µg/ml, CBL₃₀: 600 µg/ml.