Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env.Pharmacol. Life Sci., Vol 4 [3] February 2015: 43-55
©2014 Academy for Environment and Life Sciences, India
Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD
Global Impact Factor 0.533
Universal Impact Factor 0.9804

ORIGINAL ARTICLE

Mitochondrial Protection against Arsenic Toxicity by a Novel Gamma Tocopherol Analogue in Rat

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ABSTRACT

Gamma-tocopherol along with the other forms of vitamin E plays a vital role in maintaining tissue homeostasis and protecting against oxidative stress and lipid peroxidation. Recent evidences suggest that gamma-tocopherol has characteristics that are not shared by alpha-tocopherol. In present study, based on the significant role of gamma-tocopherol as a particular antioxidant and its substantial effect on the mitochondria, a new derivative of gamma-tocopherol for improved penetration in mitochondrial matrix and also enhanced anti oxidative activity was synthesized and its protective effects were evaluated. Our results showed that this derivative protected mitochondria more efficiently than gamma tocopherol against oxidative damage caused by arsenic. As mitochondria are presumably the involved organelle in the pathogenesis of many chronic diseases including diabetes and cancer, our results may perhaps open a new view for treatment or prevention of those disorders.
Keywords: gamma-tocopherol; mitochondria; reactive oxygen species; arsenic; glutathione

Received 23.11.2014 Revised 30.12.2014 Accepted 11.01.2015

INTRODUCTION

Oxidative damage considered to be a major predisposing factor in causing chronic diseases including cancer, cardiovascular and neurodegenerative disorders [1,2]. Oxygen free radicals can damage cellular components that normally have important biological functions for maintaining homeostasis [1,3]. For this reason, various natural antioxidants and their derivatives were studied or are in clinical use for treatment or prevention of several disorders [4,5]. Studies showed that natural products such as tocopherol derivatives beside their overall antioxidant properties, affected other organelles of the cell. Interestingly, the effect of tocopherol on the mitochondrial electron transport chain is considered to be a promising therapeutic target in the treatment of cancer [6]. For instance, a vitamin E derivative, alpha tocopheryl succinate, exhibited a potential anti-cancer activity [7].

Vitamin E is a fat-soluble antioxidant found in the cell membrane and other lipophilic components of mammalian cells and plays a vital role in maintaining tissue homeostasis, protecting the organism from free radicals, oxidative stress and lipid peroxidation [8-10]. Generally, Vitamin E family includes four tocopherols and four tocotrienols and based on the number and position of methyl groups on the aromatic ring labeled as α, β, γ and δ isomers [11]. As antioxidant, a hydrogen atom through the phenolic hydroxyl group is easily transfers to lipid peroxyl radical to stop the progress of the lipid peroxidation chain[12].

The major types of the vitamin E family in diet are α and γ-tocopherol (GT) partly due to their higher bioavailability and vitamin characteristics. While bioavailability, bioactivity and plasma concentrations of γ-tocopherol are lower than α-tocopherol, it has distinctive properties that are very important for human health[1,13]. It is a major part of tocopherols in the North American diet and the second most common

tocopherol in human serum (10-20%) [14]. Furthermore, epidemiological studies suggested that γ-tocopherol deficiency compare to α-tocopherol may provide a risk factor for certain types of cancer and myocardial infarction. These findings have encouraged further research on vitamin E and consequently, many in vivo studies have been conducted on tocopherol derivatives [15]. Recent evidence illustrated that gamma-tocopherol has characteristics that are not shared by alpha-tocopherol. Chemical reactivity and interference with the cell metabolism are among those unique biological activities [16].

One interesting role of gamma-tocopherol supposed to be achieved by mitochondrial exploitation. Mitochondria are organelles found in eukaryotic cells and generate a large number of ATP required for their life [17]. Mitochondria are also a major source of intracellular reactive oxygen species (ROS) and are potentially vulnerable to oxidative stress. When ROS production exceeds the cellular capacity for detoxification and repair, oxidative damage to proteins, DNA, and phospholipids could ensue. Impairment of mitochondrial oxidative phosphorylation, potentially leads to cell dysfunction and death. Beside their pathological role, ROS can also act as signal for cell redox capacity [18,19]. Studies have shown that in many chronic states including type 2 diabetes, neurodegenerative disorders, cancer, and also aging, mitochondrial dysfunction may be presented [20]. Interestingly, a number of mitochondrial dysfunction induced toxicants including arsenic (As) were linked to aforementioned diseases. Conversely, there are many reports that connected arsenic exposure to diabetes and cancer [21-24]. Because of distinctive role of mitochondria for intracellular ROS production and their vulnerability to deleterious ROS action, several vitamin E derived antioxidants have rigorously been tested to protect mitochondrion[25-30]. In the present study a new **g**amma-**t**ocopherol**d**erivative (GTD) was synthesized and its protective effects on mitochondria were evaluated.Firstly, we focused on gamma tocopherol (GT), the recently rediscovered form of vitamin E because of its amazing effects on the regulation of cell signaling involved in proliferation and/or degeneration. We chemically modified its structure for improved penetration and also enhanced anti oxidative activity in the mitochondrial matrix. Rationally, improved penetration would be achieved by shortening the hydrocarbon residue compared to parent molecule and more efficient anti oxidant property would be conferred by adding an unsaturated bond to the modified derivative.

MATERIALS AND METHODS

Materials:

As_2O_3, 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES), D-mannitol, 3-(4,5-dimethylthiazol-2-yl)- 2,5diphenyltetrazolium bromide (MTT), dithiobis-2-nitrobenzoic acid (DTNB), 2/,7/-dichlorofluoresceindiacetate (DCFH-DA), sucrose, rhodamine 123 (Rh 123), Coomassie blue, ethylene glycol-bis(2-aminoethylether)-N,N,N/,N/-tetra acetic acid (EGTA), bovine serum albumin (BSA), bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), n-hexan, ethyl acetate, buthyl acetate, propylene carbonate,γ-Butyrolactone, isobutylmethyl ketone, diethyl ketone, toluene, formic acid, p-Toluenesulfonic acid, $FeCl_2$, $AlCl_3$, P_2O_5, BF_3, $TiCl_4$, $SnBr_2$, Silica Sulfuric acid (SiO_2-OSO_3H), acetic acid, were obtained from Merck, Fluka, Sigma-Aldrich (Darmstadt, Germany). The progress of the reaction was followed with TLC using silica-gel SILG/UV 254 plate. Merck Silica gel (100-200 mesh) was used for column chromatography. The IR spectra were recorded on PerkinElmer BX II FT IR spectrophotometer with KBr pellets. ^{1}H NMR spectra were recorded by a Bruker AC 300 MHz spectrometer. ^{13}C NMR spectra were recorded by a Bruker DPX 400 MHz spectrometer. Mass spectra were determined by a FINNIGAN-MAT 8430 mass spectrometer operating at an ionization potential of 70 eV.

Preparation of 2RS,7,8-Trimethyl-2-(4-methylpent-3-en-1-yl)-6-hydroxy-3-Chromane (GTD):

The experimental procedure involved charging the reactor with 0.69 gr (0.005mole) of 2,3-dimethyl hydroquinone, 0.272 gr (0.002 mole) of $ZnCl_2$ and 50 ml of butyl acetate. Reaction mixture was stirred for 2 hours, flushed with nitrogen and heated up to about 130 °C, so that the azeotropic mixture of butyl acetate and water was distilled off. Next 0.77g (0.005 mole) of linalool alcohol was added drop wise. After completion of the reaction, as indicated by TLC (5 hours) and cooling down the reaction mixture, the catalyst (zinc dichloride) was removed. The mixture then extracted (3 times) by aqueous NaOH (8 % wt.), followed by aqueous HCl (5 % wt.) and finally by water. Subsequent to the last washing, organic layer was carefully separated, dried over anhydrous $MgSO_4$, filtered and the solvent was removed under low pressure. The product was purified by column chromatography using eluent mixture of hexane: ethyl acetate (5:1 v: v). Finally, pure product (component 3 (GTD), figure 2 and 3) as a yellowish-brown oil (70% yield) was obtained; FTIR (KBr, cm^{-1}): 3392, 3026, 2933, 2869, 1618, 1493, 1454, 1223; ^{13}CNMR (400 MHz, $CDCl_3$): δ 147.38, 146.75, 130.61, 123.17, 120.97, 116.81, 114.37, 113.42, 74.68, 38.13, 29.86, 24.65, 23.12, 22.40, 21.24, 19.57, 18.72, 16.56.

Animals:

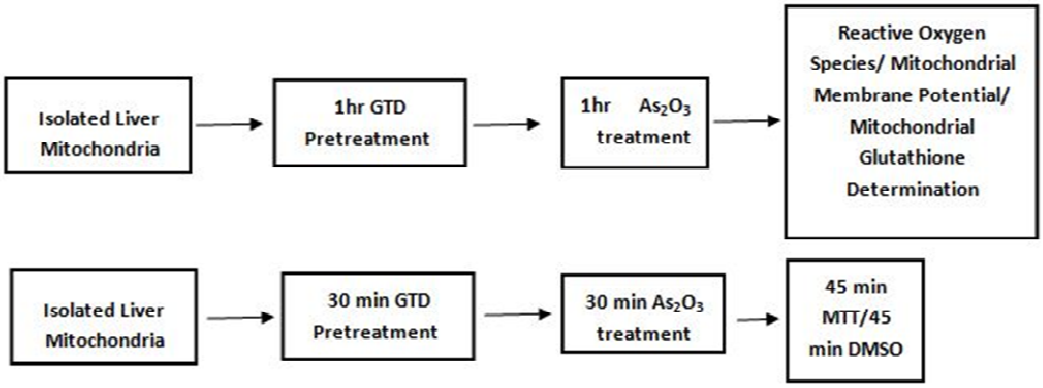
Male Wistar rats (200–250 g) were kept in polypropylene cages and were fed with standard chow and drinking water ad libitum. The animals were maintained at a controlled condition of temperature (25±2

◦C) with a 12 h light: 12 h dark cycle. All experiments carried according to the standards outlined by the local University's Ethical Committee.

Mitochondrial isolation:

Mitochondria were prepared from Wistar rat's liver using differential centrifugation. The liver was removed and minced with a small scissor in a cold mannitol solution containing 200 mM D-mannitol, 70 mM sucrose, 1 mM EGTA, 0.1% (w/v) BSA, 10mM HEPES-KOH, pH 7.4 prepare freshly. The minced liver was gently homogenized in a glass homogenizer. Nuclei and cell debris were sedimented through centrifugation (600×g, 10 min, 4ºC) and the supernatants were centrifuged at10,000×g for 15 min. Supernatant was carefully discarded and the pellet (mitochondria) washed gently by suspending in the isolation medium and centrifuged again for 15 min at 10,000×g. Finally, pellet was suspended in the mannitol solution. For each test, mitochondria were prepared freshly and used within 2 h of isolation. Protein concentrations were determined through the Coomassie blue protein-binding method as explained by Bradford, 1976 [31]. Mitochondrial samples (500 µg protein/ml) were incubated with various concentrations of GTD (1, 5, and 10 µM) (as illustrated below, figure 1) for 30 min at 37 °C followed by As_2O_3 (20, 40 and 100µM) exposure for 30 min. Aliquots of mitochondrial suspension were used to determine the protective effects of GTD against arsenic toxicity. All experiments were performed and repeated at least three times.

Figure 1. Schematic design for toxicological assays



ROS determination:

The mitochondrial ROS measurement was performed using the fluorescent probe DCFH-DA. Briefly, isolated mitochondria suspensions (0.5 mg protein/ml) were incubated with various concentrations of GTD, and then incubated with different concentrations of Arsenic. 10 µM of DCFH-DA was added to the mitochondrial solution. The fluorescence was measured using PerkinElmer LS-50B Luminescence fluorescence spectrophotometer at the excitation and emission wavelengths of 490 and 535 nm, respectively [32].

Mitochondrial damage determination:

The mitochondrial uptake of the cationic fluorescent dye, rhodamine123, has been used for the determination of mitochondrial membrane potential. The mitochondrial suspensions (0.5 mg protein/ml) were incubated with various concentrations of GTD, and then incubated with different concentrations of Arsenic. 10 µM of rhodamine123 was added to the mitochondrial solution. The fluorescence was measured using PerkinElmer LS-50B Luminescence spectrofluorometer at the excitation and emission wavelengths of 490 and 535 nm, respectively [33].

Mitochondrial dehydrogenase activity (MTT assay):

The activity of mitochondrial complex II (succinate dehydrogenase) was assayed through the measurement of MTT reduction. Briefly, 1mL of mitochondrial suspensions (0.5 mg protein/ml) was incubated with different concentrations of GTD, and then incubated with different concentrations of Arsenic. suspension was centrifuged at 10,621×g for 1 min. pellet was suspended in 970 µL of isolation medium and 500 µL of 0.5 µM MTT and incubated at 37 C for 45 min. The purple formazan crystals which formed were dissolved in 800 µL DMSO, and the absorbance measured at 570 nm spectrophotometrically (UV-1650 PC, Shimadzu, Japan) [34].

Table1. Effect of various catalysts for synthesis of Compound (3)[a]

No	Catalyst	Time (h)	Condition	Yield[b] %
1	HCOOH	7	reflux	20
2	MeCOOH	7	100˚C	25
3	PTSOH	7	80˚C	15
4	$FeCl_2$	7	reflux	40
5	BF_3/ HOAC	7	r.t	17
6	P_2O_5	7	r.t	30
7	P_2O_5/ SiO_2	7	r.t	35
8	$ZnCl_2$	7	r.t	66
9	SiO_2-OSO_3H	7	r.t	8
10	$AlCl_3$	7	r.t	18
11	$TiCl_4$	10	r.t	Trace
12	$SnBr_2$	10	r.t	Trace

Mitochondrial glutathione content:

GSH content was determined using DTNB reagent by spectrophotometric method in isolated mitochondria. The developed yellowish color was read at 412 nm using a spectrophotometer (UV-1650 PC, Shimadzu, Japan). GSH content was expressed as µg/mg protein[35].

Statistical Analysis:

Results were presented as mean ± SD. All assays performed triplicate, and the mean was used for the statistical analysis. Statistical significance was established using the one-way ANOVA test, accompanied by the post hoc Tukey´s test. Statistical significance has been set at $P<0.05$.

RESULTS

2RS,7,8-Trimethyl-2-(4-methylpent-3-en-1-yl)-6 hydroxy-3-Chromane (GTD) preparation:

Treatment of methyl hydroquinone with isoprenoid alcohol in the presence of Lewis acid catalysts appears to be appropriate route for chromane structure preparation. Thus in order to investigate the possibility and limitations of this route, the reaction of 2,3-dimethyl hydroquinone with linalool alcohol in the presence of variety catalysts (Lewis and Brϕnsted acids) and solvents were tested. The results obtained are summarized in Table 1 and Table 2.

Among the various catalysts and solvents used, two were particularly helpful to form compound 3, entries 8 and 4 (Table 1,2). Then a reaction using different quantities of $ZnCl_2$ was performed. With lower amount of $ZnCl_2$ at molar ratio of 2,3-dimethyl hydroquinone : linalool : $ZnCl_2$ = 1 :1 : 0.2, the yield of product decreased to 55% and with higher amount of $ZnCl_2$ at molar ratio of 2,3-dimethyl hydroquinone: linalool: $ZnCl_2$ = 1: 1: 0.6, the yield of product increased to 72%.

[a]Reaction condition: 2,3-dimethyl hydroquinone=1 mole, linalool alcohol= 1 mole, catalyst= 0.4mole [i]solated yield

As shown in figure 2, four products were recognized by their spectroscopic data in these reactions: 3, 4, 5 and 6, of which only compound 3 which was produced in all reactions in different yields was the goal. This compound separated and purified by column chromatography in reported yields (Tables 1 and 2) at 130°C in the presence of $ZnCl_2$ for 7 hours. Compound 3 was isolated from the crude reaction mixture by column chromatography and identified by NMR, MS, and IR spectroscopy. Compound 4, 5 and 6 are all derivatives of the major product 3 and are formed in different ratios depending on the reaction conditions. They were tentatively identified by spectroscopic analysis of crude reaction mixtures. No attempts were made for isolating these compounds.

Structure of (3) was established by its spectroscopy data: mass, IR, ^{1}HNMR and ^{13}CNMR.The fragmentation observed in the mass spectrum of compound 3 followed familiar courses reported for tocopherols [36]. This spectrum provided useful structural information for compound 3 (figure 2).^{1}HNMR spectrum (300MHz, $CDCl_3$), exhibits resonance signals at δ 6.57 – 6.69 (2H, H_5, H_3/), 4.63 (1H, H_6), 2.50 – 2.74 (2H, H_4), 1.47 – 2.11(13H, H_7, H_8, H_3, H_2/, H_3/), 0.91 – 1.38 (8H, H_6/, H_1/, H_5/) ppm.^{1}HNMR spectrum of compound 3 shows a remarkable resemblance to that of γ-tocopherol. In summary, we found that the best condition for preparation of compound 3 is through the use of $ZnCl_2$ as catalyst and butyl acetate as solvent at 130°C for 7 hours as shown in table 1 and 2.

Table2. Condensation of (1) and (2) for the synthesis of Compound (3) in different solvents using $ZnCl_2$

No	Solvent[a]	Condition	Yield[b] %
1	Toluene	reflux	15
2	Diethyl ketone	reflux	8
3	Isobutylmethyl ketone	reflux	25
4	Buthyl acetate	reflux	70
5	γ-Butyrolactone	reflux	16
6	Propylene carbonate	reflux	20

[a]50 ml solvent; [b]Isolated yield

Figure 2. Hydroquinone and linalool reaction

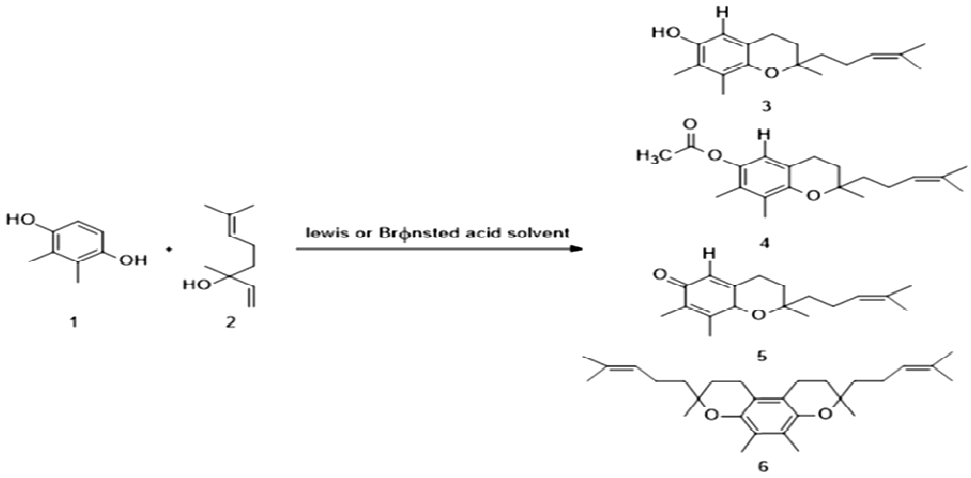
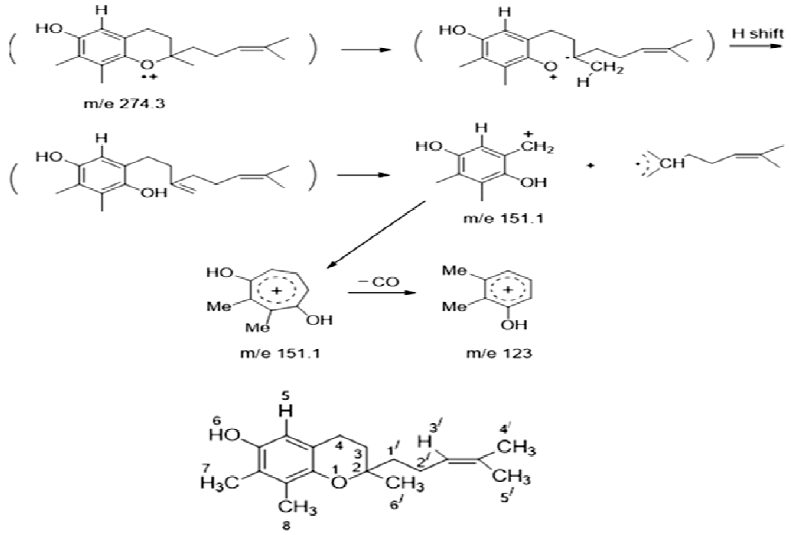


Figure 3. Formation and structure of the compound 3 (GTD)



In order to determine the biological activity of compound (3) (GTD), we conducted the following experiments:

GTD role in the decrease of As-induced ROS generation:

Fig.4a shows the increased level of oxygen radicals upon arsenic exposure in which the amounts of ROS were increased by higher concentration of arsenic. When gamma tocopherol (GT) pretreatment applied, significant lower levels of oxygen free radical were detected. The results indicated that GT′s highest influence on ROS formation attenuation has been achieved by 5 and 10 µM at concentrations of 20 and 40µM arsenic respectively. ROS reduction by the use of different concentrations of gamma-tocopherol was not as far as the negative control; But in the absence of As, when gamma tocopherol applied, significant reduction of ROS were obtained ($P < 0.05$).

As shown in fig.4b, gamma tocopherol derivative (GTD) has also been able to reduce ROS formation in the presence of arsenic. Unlike the GT, GTD even at concentration as low as 1µM, reduced ROS formation

significantly. In the presence of arsenic, GTD reduced the level of ROSdose-dependently and the highest effect was accomplished at a concentration of 10μM of GTD. In contrast to GT, GTD at 1 μM significantly reduced ROS levels formed under 40 μM of arsenic compared to the control (P<0.05).

As shown in fig.5a, b and c at 40 μM of arsenic, GTD in comparison with GT, reduced the level of ROS drastically (P <0.05). Decrease in ROS achieved by 10 μM gamma-tocopherol was comparable to that yielded by GTD at the same concentration (P <0.05). Fig.5b shows significant differences for ROS amounts with 1, 5 and 10 μM of GTD compared to GT. At the highest concentration of arsenic (100 μM), again, GT could never be the same as GTD in inhibiting the formation of free radicals (P <0.05).

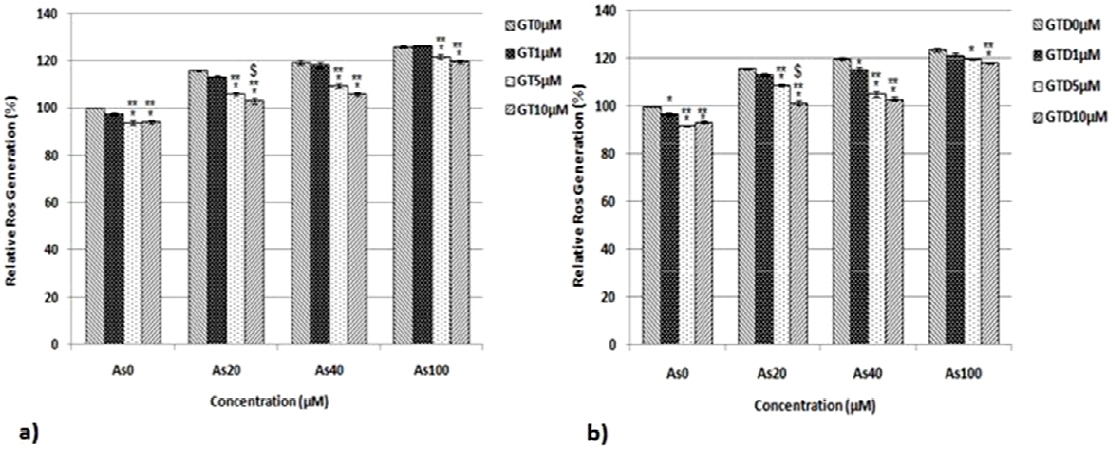


Figure 4. Gamma tocopherol (GT) and its derivative (GTD) reduced mitochondrial ROS generation under different concentrations of As_2O_3.

Reactive oxygen species (ROS) determined using H_2DCF-DA oxidation. Relative DCF fluorescence intensity reflects different ROS amount in different groups. Rat liver mitochondria were isolated, purified (0.5mg/ml protein) and incubated in buffer contains mannitol, sucrose, HEPES and EGTA (pH 7.4) followed by 1 hour exposure to As_2O_3. Fluorimetric measurements were made at $\lambda_{excitation}$=500, $\lambda_{emission}$=520 nm.

*Significant difference as compared to GTD0μM (P<0.05)

**Significant difference as compared to GTD1μM (P< 0.05)

$ Significant difference as compared to GTD5μM (P< 0.05)

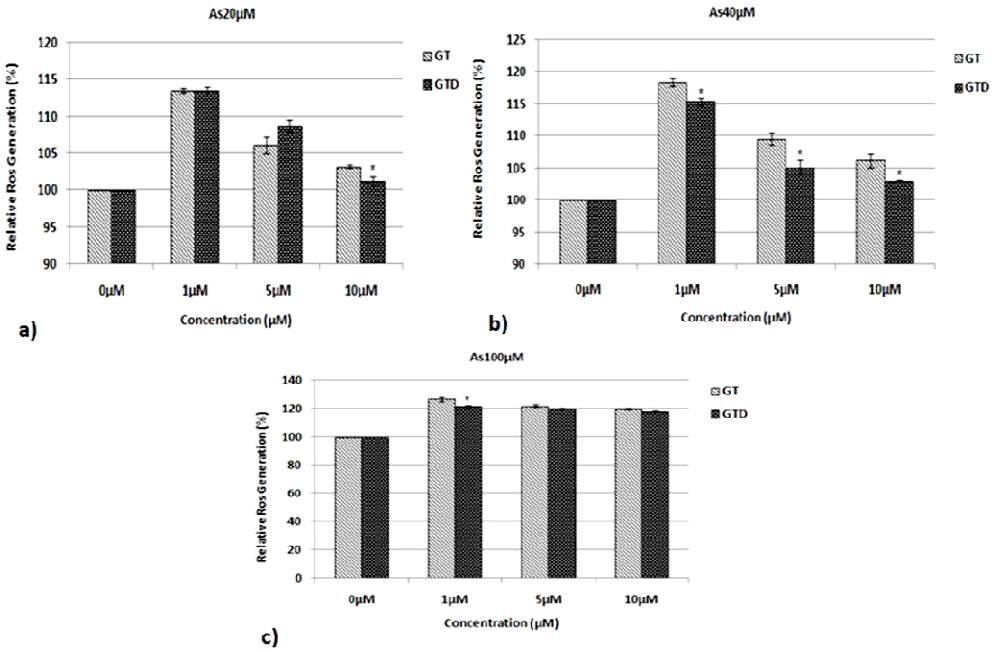


Figure 5. Gamma tocopherol (GT) and its derivative (GTD) reduced mitochondrial ROS generation comparatively at different As_2O_3 concentrations.

Reactive oxygen species (ROS) determined using H_2DCF-DA oxidation. Relative DCF fluorescence intensity reflects different ROS amount in different groups. Rat liver mitochondria were isolated, purified (0.5mg/ml protein) and incubated in buffer contains mannitol, sucrose, HEPES and EGTA (pH 7.4) followed by 1 hour exposure to As_2O_3. Fluorimetric measurements were made at $\lambda_{excitation}$=500, $\lambda_{emission}$=520 nm.

*Significant difference as compared to corresponding GT concentration (P<0.05)

GTD role in preventing oxidation of glutathione:

Glutathione levels were measured in isolated mitochondria by spectrophotometric assay under the different concentrations of arsenic (40, 20 and 100) using DTNB as an indicator. Fig.6 demonstrates a significant depletion of glutathione for all three applied arsenic concentrations (P <0.05) in a concentration dependent manner. The results showed that mitochondrial GSH levels increased with the use of GTD in which 5 and 10 µM of GTD in the presence of arsenic contributed the higher levels.

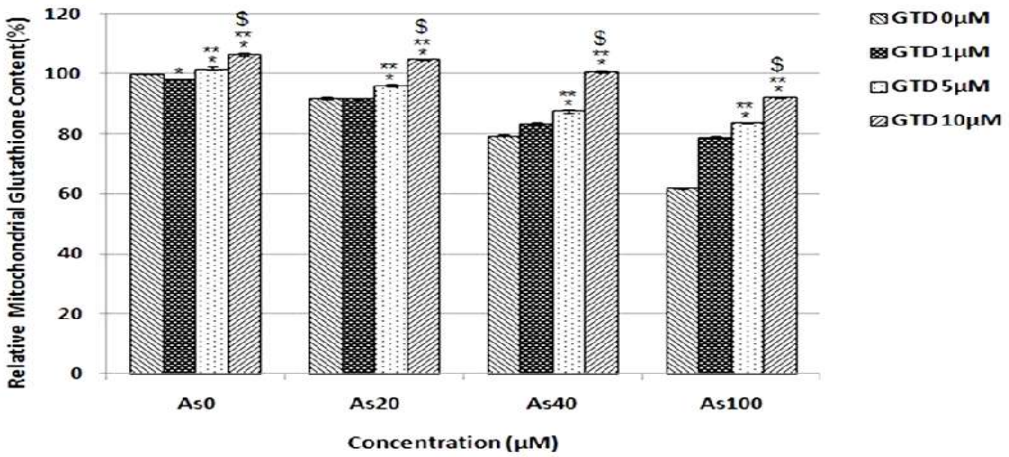


Figure 6. Gamma tocopherol derivative (GTD) modified mitochondrial glutathione contents affected by different concentrations of As_2O_3.

Glutathione contents determined using DTNB. Rat liver mitochondria were isolated, purified (0.5mg/ml protein) and incubated in buffer contains mannitol, sucrose, HEPES and EGTA (pH 7.4) followed by 1 hour exposure to As_2O_3. The yellowish color developed was read at 412 nm spectrophotometrically.

*Significant difference as compared to GTD0µM (P<0.05)

**Significant difference as compared to GTD1µM (P< 0.05)

$ Significant difference as compared to GTD5µM (P< 0.05)

GTD role in preventing mitochondrial membrane potential collapse:

As seen in Fig.7a mitochondrial membrane collapse was concentration-dependently increased following the arsenic concentration elevated. GT protected the mitochondrial membrane when exposed to arsenic. However, except for 10 µM GT at 20 and 100 µM of arsenic, results showed no significant differences (P <0.05). In the absence of arsenic, gamma-tocopherol derivative (GTD) in comparison with GT, at high concentration affected the membrane potential (Fig.7b). Using the GTD pretreatment, the damage was diminished and the GTD highest impact in reducing mitochondrial membrane damage achieved by 5 and 10 µM at 40 µM of arsenic.

As shown in Fig.8a, b and c, GTD more effectively reduced vulnerability of mitochondrial membrane to arsenic than the GT particularly at arsenic concentrations of 40 and 100 µM (P <0.05). At 20 µM of arsenic, results did not show any significant differences between GT and GTD (P <0.05). In this context, Fig.8b shows significant difference at the concentration of 5 µM (P <0.05). Same results were obtained for 1 µM of GTD at 100 µM arsenic concentration. Collectively, the results showed that at the same values used, GTD protected mitochondrial membrane more effectively than GT.

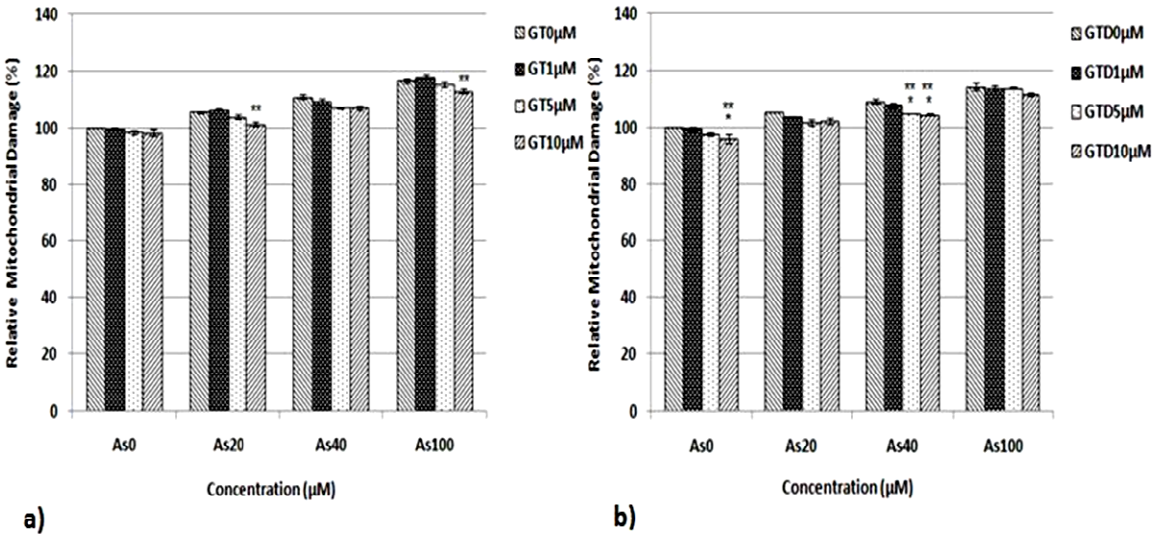


Figure 7. Gamma tocopherol and its derivative (GTD) reduced mitochondrial damage under different concentrations of As_2O_3.

Mitochondrial membrane potential (MMP) (ΔΨm) determined using Rhodamine 123. Relative fluorescence intensity reflects different (MMP) amount in different groups. Rat liver mitochondrial were isolated, purified (0.5mg/ml protein) and incubated in buffer contains mannitol, sucrose, HEPPES and EGTA (pH 7.4) for 2 hr. Fluorimetric measurements were made at $\lambda_{excitation}$=490, $\lambda_{emission}$=535 nm.

*significant difference as compared to GTD0μM (P<0.05)

**Significant difference as compared to GTD1μM (P< 0.05)

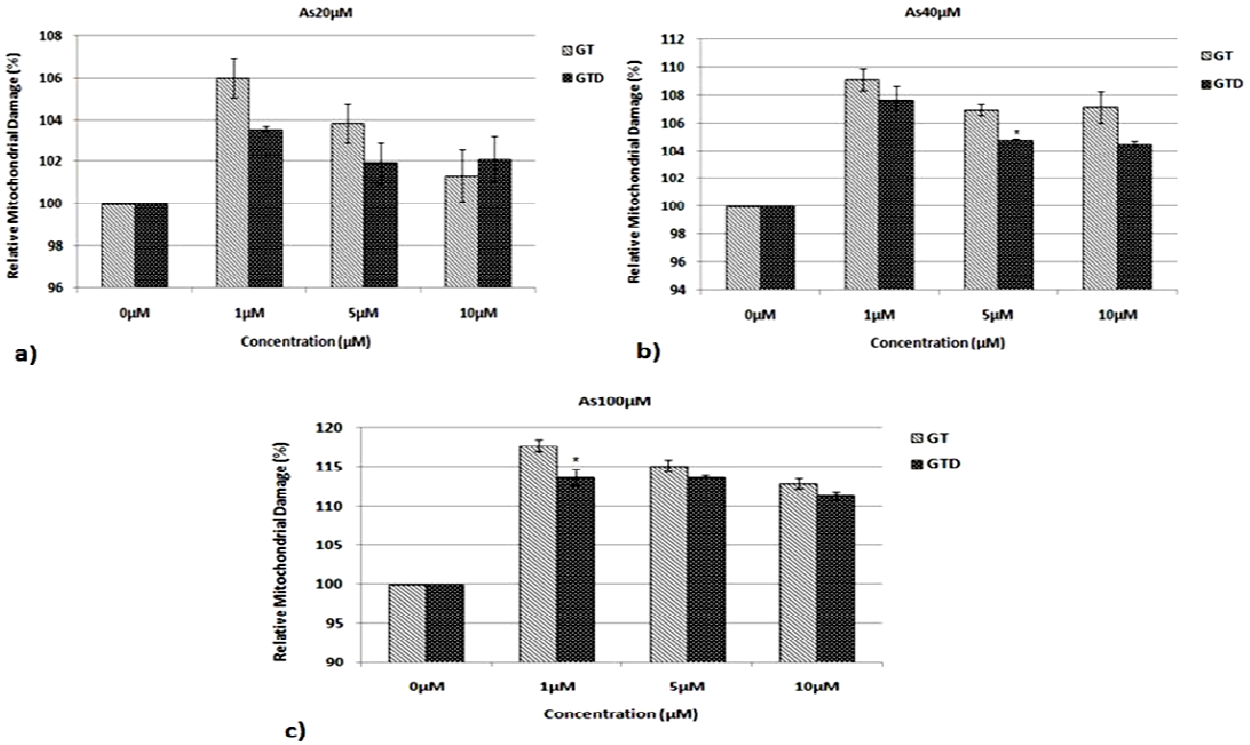


Figure 8. Gamma tocopherol and its derivative (GTD) reduced mitochondrial damage comparatively under different concentrations of As_2O_3.

Mitochondrial membrane potential (MMP) (ΔΨm) determined using Rhodamine 123. Relative fluorescence intensity reflects different (MMP) amount in different groups. Rat liver mitochondrial were isolated, purified (0.5mg/ml protein) and incubated in buffer contains mannitol, sucrose, HEPPES and EGTA (pH 7.4) for 2 hr. Fluorimetric measurements were made at $\lambda_{excitation}$=490, $\lambda_{emission}$=535 nm.

*significant difference as compared to GTD0μM (P<0.05)
**Significant difference as compared to GTD1μM (P< 0.05)

Activity of mitochondrial dehydrogenases:

Dehydrogenases activity examined using the MTT assay at different concentrations of arsenic (20, 40 and 100 μM) (Fig.9). Arsenic alone caused a concentration-dependent inhibition of dehydrogenases activity while GTD in the absence of arsenic significantly increased the activity of complex II. Mitochondrial complex II or dehydrogenase activity improved by GTD and the maximum effect obtained from 1 and 10 μM for arsenic concentrations of 100 or 40 μM respectively.

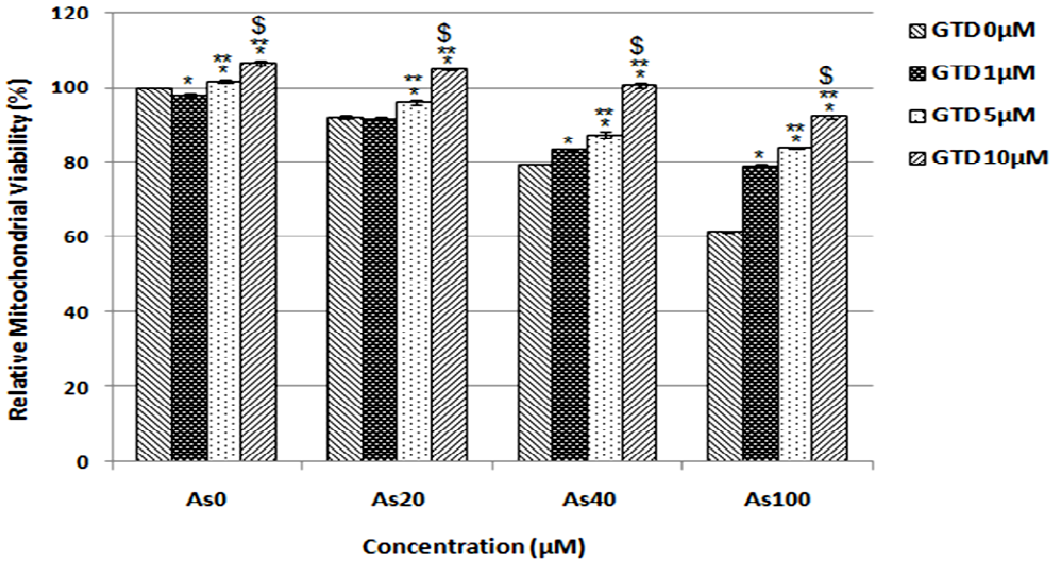


Figure 9. Gamma tocopherol derivative (GTD) potently retained mitochondrial viability affected by different concentrations of As_2O_3.

Mitochondrial viability (total dehydrogenase activity) was assayed by determining the amount of MTT conversion. Rat liver mitochondria were isolated, purified (0.5mg/ml protein) and incubated in buffer contains mannitol, sucrose, HEPES and EGTA (pH 7.4) followed by 1 hour exposure to As_2O_3. The absorbance at 570 nm was measured with a spectrophotometer.

*Significant difference as compared to GTD0μM (P<0.05)
**Significant difference as compared to GTD1μM (P< 0.05)
$ Significant difference as compared to GTD5μM (P< 0.05)

DISCUSSION

Mitochondrial dysfunction has been implicated in pathogenesis of various conditions such as cancer, diabetes, cardiovascular disease and age-related neurodegenerative diseases. Understanding the precise function of mitochondria and its role in cellular toxicity induced by various toxicants provided a number of important targets for treatment and prevention of some related diseases. Besides the nature of mitochondrial genome and its sensitivity to mutations, enzyme activation and also detoxification of different toxicants and xenobiotics in the mitochondrion and its membrane swelling due to the diverse injuries have enormously evaluated [37].

Mitochondrion not only plays a key role in generating energy through the process called Oxidative Phosphorylation (OXPHOS), it also plays a central role in apoptosis, cellular stress responses and genetic diseases [38]. Disruption of OXPHOS leads to changes in the intracellular RedOx states and intracellular organelles, ATP production, the formation of reactive oxygen species and eventually to cell death including apoptosis [39].

Mitochondria are believed to be as target for environmental pollutants including heavy metals. In this context, mitochondrial dysfunction started a noxious process that is believed to be involved in development of many diseases [40]. Since mitochondria play important role in the normal function of cell and its dysfunction were shown to have devastating consequences, in this study, we synthesized a new derivative of gamma-tocopherol and tested its potential protective effects against damage caused by arsenic.

Recent studies have suggested that gamma-tocopherol might be essential for maintaining healthy body mainly by its distinctive features which distinguish it from other members of the tocopherol family [13]. Gamma-tocopherol found to be advantageous in the control of diseases associated with chronic

inflammation, including arthritis, cancer, cardiovascular and neurodegenerative diseases (Alzheimer)[41-45]. As a result, mechanisms beyond the absorption of oxygen free radicals have been proposed for gamma-tocopherol. Nucleophilically, this form is more potent than alpha-tocopherol and can neutralize electrophilic mutagens in the lipophilic parts of the cell[46-48].This neutralizing activity is comparable to inhibiting the electrophilic mutagens by glutathione in the hydrophilic portion of the cell. In this way, one important reaction is the neutralization of peroxynitrite by gamma-tocopherol which inhibits the formation of lipid radicals and protects DNA and proteins. Efficiency of gamma-tocopherol to scavenging oxygen free radicals believed to be more than alpha-tocopherol. This feature may be associated with the structure of gamma-tocopherol since the unsubstituted C-5 position of gamma-tocopherol (Fig. 2), is a nucleophilic center and can potently scavenge oxygen and nitrogen radicals [49].

Today's advances in research on mitochondria provided a tool to design new drugs for various diseases and conditions related to mitochondrial abnormality. Study on isolated mitochondria has led to better understanding of molecular mechanisms of xenobiotics [32,38,50]. Purified mitochondria have been used for assessing the toxicity and mechanism of injury caused by heavy metals. Such studies revealed that arsenic induced apoptosis and necrosis through release of cytochrome c. Mitochondria are supposed to be the main targets for arsenic toxicity. Arsenic exposure through disruption of mitochondrial electron transfer chain lead to mitochondrial swelling [51]. Arsenic in cancer cell lines induced MPT (mitochondrial permeability transition) and apoptosis [52,53]. Studies show that arsenic increases ROS, lipid peroxidation, and impairment of mitochondrial membrane potential[54,55]. strong evidences have linked arsenic-induced oxidative stress to endothelial inflammation, the major complication in atherosclerosis [52,56]. Although high levels of arsenic inhibited angiogenesis, at lower concentrations in some studies it has been stimulated angiogenesis and it seems that mitochondrial disruption caused by arsenic or oxidative stress plays a key role in the angiogenesis [57,58].

The results of the present study indicated that mitochondrial ROS overproduction was significantly induced by arsenic in a concentration dependent manner that with regard to the mechanisms described above can be caused by impaired electron transport chain and mitochondrial membrane (Fig. 4a). When mitochondria were pretreated with gamma-tocopherol or its derivative (GTD) the amounts of ROS formation were considerably reduced (Fig. 4a-b). Given the role of gamma-tocopherol in scavenging free radicals in the lipophilic phase, these results were expected. Interestingly, gamma-tocopherol derivative, compared to parent tocopherol, inhibited arsenic induced oxygen free radicals production in a more effective manner. This is especially evident for 40 μM arsenic. The presence of unsaturated double bond in the modified derivative may contribute to this higher efficiency. Besides the double bond contribution to antioxidant property, fluidity also plays a significant role. Here the higher fluidity leads to enhanced penetration across the membranes. Our synthesized compound according to its unsaturability has further fluidity and perhaps cross the membrane easier (Fig. 2). In this regard we can take a look at some tocotrienols derivatives which contain three unsaturated double bonds and the antioxidant properties that conferred reported to be higher than gamma-tocopherol [59,60].

As mentioned gamma-tocopherol effects on various diseases such as cancer are not directly related to its antioxidant effects. Studies revealed that tocopherols with similar antioxidant activities have different antiproliferative properties. Lots of evidences suggested that tocopherols alongside their antioxidant properties were also involved in signaling and regulation of gene expression in animals [13,39,61]. To some extent, these effects possibly related to mitochondrial exploitation by gamma-tocopherol. Therefore, in this study, mitochondrial membrane potential in the presence of arsenic, gamma- tocopherol and its derivative have been studied (Fig. 7a-b). Gamma-tocopherol only at high concentrations (10μM) reduced mitochondrial damage while GTD at lesser concentration showed protective effect on mitochondria. In the other words, GTD protected mitochondria more efficiently which may be due to the higher ROS inhibitory effect of this derivative (Fig. 8a-c).

Arsenic-induced damage to the mitochondria thought to be through its stimulation for reactive oxygen species generation. Naturally, 1-2% of oxygen consumed by mitochondria are converted to superoxide radicals [62]. At the presence of arsenic, the formation of superoxide increased and resulted in mitochondrial glutathione oxidation and depletion (Fig. 6). In our study, mitochondrial glutathione depletion in a concentration dependent manner observed for arsenic exposure. GT derivative with respect to its higher antioxidant and penetration rate neutralized the free radicals and prevented the oxidation of mitochondrial glutathione (Fig.6). On the other hand, mitochondrial dehydrogenase activity gradually decreases with increasing arsenic concentrations (Fig.8) and GTD profoundly maintained mitochondrial dehydrogenase activity in such a way that at the highest concentration (10μM) dehydrogenase activity approximately restored to its normal values (control group).

In conclusion, our results showed that the chemically modified derivative of gamma tocopherol protected mitochondria more efficiently against oxidative damage induced by arsenic. As mitochondria are

presumably the affected organelle in the pathogenesis of many chronic diseases including diabetes and cancer, our results may perhaps open a new view for treatment or prevention of those disorders.

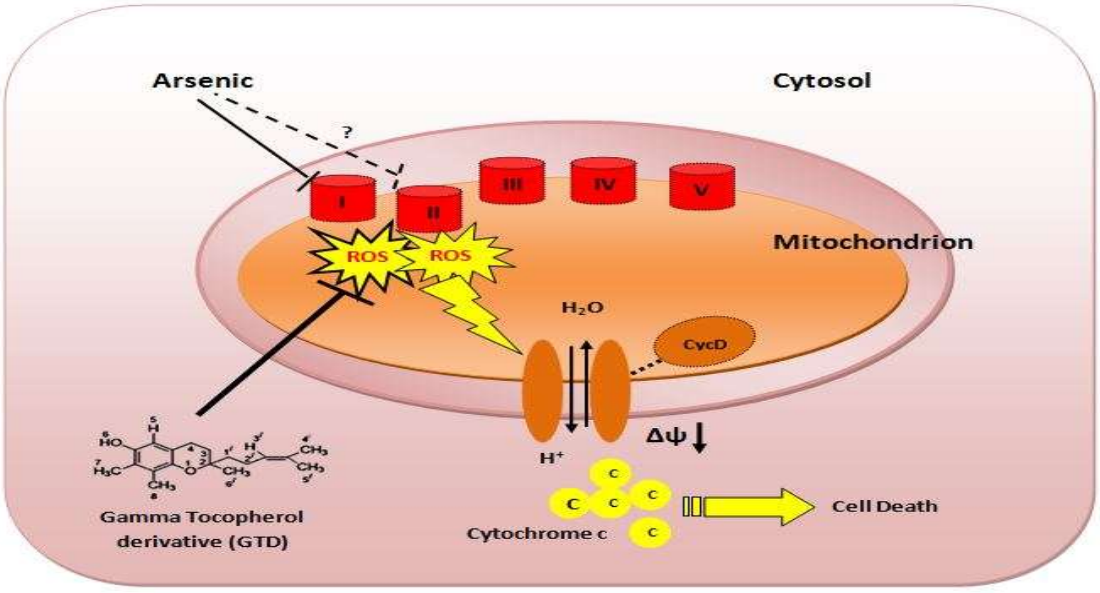


Figure 10. Mitochondrial view of gamma tocopherol derivative (GTD) action

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CITATION OF THIS ARTICLE

Mohammad H, Rashid B, Mohsen R, Mohammad R S, Ali R K. Mitochondrial Protection against Arsenic Toxicity by a Novel Gamma Tocopherol Analogue in Rat. Bull. Env.Pharmacol. Life Sci., Vol 4 [3] February 2015: 43-55