Chapter 5

Materials and Methods
Materials and methods

Materials

Powder of bark of *Terminalia arjuna* (TA) was purchased from Herby House, Kolkata, India. Benzoic acid (purity ≥ 99.5%), cupric chloride, ascorbic acid and all the other chemicals used including the solvents were of analytical grade obtained from Sisco Research Laboratories (SRL), Mumbai, India, Qualigens (India/Germany), SD fine chemicals (India), Merck Limited, Delhi, India.

Preparation of the aqueous bark extract of TA

Five (5) gm of TA bark powder was dissolved in 25ml of double distilled water. After proper mixing, it was kept overnight (approximately 16 hours) with cotton plugging. Then it was centrifuged twice at 1300g for 10 minutes and the supernatant was collected and lyophilized. The yield of the aqueous bark extract of TA was 10%.

Collection of human placenta

Human placenta was collected from Vision Care Hospital. For all patients, informed consent was obtained, and the study protocol was approved by the Institutional Human Ethics Committee of Dept. of Physiology, University of Calcutta (IHEC/P02/08 dated 26.03.2008). The experiments were carried out with mitochondria obtained from 10 different placenta collected from different human subjects (i.e., immediately after mother(s) has delivered their baby) under the supervision of Doctors at Vision Care Hospital, Kolkata.

Preparation of human placental mitochondria

Human placental mitochondria were isolated according to the procedure of Hare et al. (1980). After collection of placenta, it was brought into laboratory in sterile plastic container kept in ice. Then, the tissue was cleaned and cut into pieces. Five gm of tissue was placed in 10mL of sucrose buffer [0.25(M) sucrose, 0.001(M) EDTA, 0.05(M) Tris-H$_2$SO$_4$ (pH 7.8)] at 25°C. Then the tissue was blended for 1 minute at low speed by using a Potter Elvenjem glass homogenizer (Belco Glass Inc., Vineland, NJ, USA), after which it
was centrifuged at 1500rpm for 10 minutes. The supernatant was poured through several layers of cheesecloth and kept in ice. Then it was centrifuged at 4000rpm for 5 minutes. The supernatant obtained was further centrifuged at 14000rpm for 20 minutes. The supernatant obtained was discarded and the pellet was resuspended in sucrose buffer and was stored at -20°C for further use.

**Determination of mitochondrial intactness by using Janus green B stain**

The freshly prepared mitochondria were spread on a slide. After that a few drops of Janus green stain were put on the slide and was left for 5 min for staining in moist chamber. The mitochondria were rinsed once with distilled water, so that a diluted stain remained. Then, the mitochondria were mounted in a drop of distilled water with a cover slip and imaged with a confocal system (BD Pathway 855, USA). The digitized images were then analyzed using image analysis system (ImageJ, NIH Software, Bethesda, MI) and the intactness of mitochondria of each image was measured and expressed as the ‘percent area’. Besides, 100µl of mitochondrial suspension, after incubation, were mixed with 20µl of Janus Green B solution and incubated at room temperature, in dark. The mitochondria, stained with Janus Green B were analyzed by using flow cytometry (BDFACS Versa, USA) (Cooperstein et al., 1960).

**Incubation of placental mitochondria with copper (Cu^{2+}) and ascorbic acid in presence and/or absence of aqueous bark extract of TA and/or benzoic acid**

The incubation mixture containing mitochondrial protein (6 mg/ml), 50 mM potassium phosphate buffer (pH 7.4), 0.2 mM Cu^{2+} and 1 mM ascorbic acid in a final volume of 1.0 ml was incubated at 37°C in an incubator for 1 hour. The reaction was terminated by the addition of 40µl of 35mM EDTA.

The mitochondria were co-incubated with copper-ascorbate and three different concentrations of the aqueous bark extract of TA (5, 10, 20mg/ml) or benzoic acid (0.058, 0.115, 0.23μg/ml). The reaction was terminated as described above. Following incubation,
the intactness of mitochondria, the nitric oxide (NO) concentration, the levels of biomarkers of oxidative stress like lipid peroxidation (LPO), reduced glutathione (GSH) and the protein carbonyl (PCO) content, the activities of mitochondrial antioxidant enzymes, some of the Kreb’s cycle enzymes, respiratory chain enzymes, mitochondrial membrane cardiolipin content, mitochondrial swelling, di-tyrosine level, NADH autofluorescence, mitochondrial DNA damage were measured as described below.

Measurement of mitochondrial lipid peroxidation (LPO) level and reduced glutathione (GSH), total glutathione (TSH) and protein carbonyl (PCO) content

The level of lipid peroxidation in placental mitochondria were measured in terms of thiobarbituric acid reactive substances (TBARS) using the method of Buege and Aust (1978). Two ml of TBA-TCA-HCl reagent (15% TCA, 0.375%TBA and 0.25(N) HCl) and suitable amount of mitochondrial sample was added to the incubation mixture and heated for 20 minutes at 80⁰C. The absorbance of the sample was determined spectrophotometrically at 532nm. The level of TBARS was calculated using an extinction coefficient of $1.56 \times 10^5 \text{M}^{-1}\text{cm}^{-1}$.

The GSH content (as acid soluble sulphydryl) was estimated by its reaction with DTNB (Ellman’s reagent) following the method of Sedlak et al. (1968) with some modifications. Incubated mitochondria were mixed with Tris–HCl buffer, pH 9.0, followed by DTNB for color development. The absorbance was measured at 412 nm using a UV–VIS spectrophotometer to determine the GSH content. The values were expressed as nmole GSH/ mg of protein. Total sulphydryl content in mitochondria was measured following the method as described by Sedlak and Lindsay (1968). The values were expressed as nmoles GSH/mg of protein.

Protein carbonyl content was estimated by the method of Levine et al. (1994). 0.25 ml of incubated mitochondrial suspension was taken in each tube and 0.5 ml DNPH in 2.0 M HCl was added to the tubes. The contents of the tubes were vortexed every 10 min in the
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Dark for 1 h. Proteins were then precipitated with 30% TCA and centrifuged at 4000g for 10 min. The pellet was washed three times with 1.0 ml of ethanol: ethyl acetate (1:1, v/v). The final pellet was dissolved in 1.0 ml of 6.0 M guanidine HCl in 20 mM potassium dihydrogen phosphate (pH 2.3). The absorbance was determined spectrophotometrically at 370 nm. The protein carbonyl content was calculated using a molar absorption coefficient of $2.2 \times 10^{-4} \text{ M}^{-1} \text{cm}^{-1}$. The values were expressed as nmoles/mg of protein.

Determination of the activities of antioxidant enzymes

Manganese superoxide dismutase (Mn-SOD) activity was measured by pyrogallol autooxidation method (Marklund et al., 1974). To 50 μl of the mitochondrial suspension (as the source of enzyme), 430 μl of 50 mM of Tris–HCl buffer (pH 8.2) and 20 μl of 2 mM pyragallol were added. An increase in absorbance was recorded at 420 nm for 3 min in a UV/VIS spectrophotometer. One unit of enzyme activity is 50% inhibition of the rate of autooxidation of pyragallol as determined by change in absorbance/min at 420 nm. The enzyme activity was expressed as units/mg of protein.

The glutathione peroxidase (GPx) activity was measured according to the method of Paglia et al. (1967) with some modifications. The assay system contained, in a final volume of one ml, 0.05 M phosphate buffer with 2 mM EDTA, pH 7.0, 0.025 mM sodium azide, 0.15 mM glutathione, 0.25 mM NADPH and suitable amount of mitochondrial suspension as the source of enzyme. The reaction was started by the addition of 0.36 mM $H_2O_2$. The linear decrease of absorbance at 340 nm was recorded using a UV/VIS spectrophotometer. The specific activity was expressed as Units/mg of protein.

The glutathione reductase (GR) activity was measured according to the method of Krohne-Ehrich et al. (1977). The assay mixture in a final volume of 3 ml contained 50 mM phosphate buffer (pH 7.0), 200 mM KCl, 1 mM EDTA and water. The blank was set with this mixture. Then, 0.1 mM NADPH was added together with suitable amount of incubated mitochondrial suspension as the source of enzyme into the cuvette. The
reaction was initiated with 1 mM oxidized glutathione (GSSG). The decrease in NADPH absorption was monitored spectrophotometrically at 340 nm. The specific activity of the enzyme was expressed as units/mg of protein.

The glutathione-S-transferase (GST) activity of the incubated mitochondria was measured according to Habig et al. (1974). The enzymatic reaction was measured by observing the conjugation of 1-chloro, 2, 4-dinitrobenzene (CDNB) with reduced glutathione (GSH). One unit of enzyme will conjugate 10 nmol of CDNB with reduced glutathione per minute at 25°C. The rate where the reaction is linear is noted spectrophotometrically at 340 nm. The molar extinction of CDNB is 0.0096 μM⁻¹cm¹. The enzyme activity was expressed as units/mg of protein.

**Determination of the activities of pyruvate dehydrogenase (PDH) and some of the Kreb’s cycle enzymes**

Pyruvate dehydrogenase (PDH) activity was measured spectrophotometrically according to the method of Chretien et al. (1995) with some modifications by following the reduction of NAD⁺ to NADH at 340 nm using 50 mM phosphate buffer, pH 7.4, 0.5 mM sodium pyruvate as the substrate and 0.5 mM NAD⁺ in addition to the suitable amount of mitochondrial suspension as the source of enzyme. The enzyme activity was expressed as units/mg of protein.

Isocitrate dehydrogenase (ICDH) activity was measured according to the method of Duncan et al. (1979) by measuring the reduction of NAD⁺ to NADH at 340 nm with the help of a UV–VIS spectrophotometer. One ml assay volume contained 50 mM phosphate buffer, pH 7.4, 0.5 mM isocitrate, 0.1 mM MnSO₄, 0.1 mM NAD⁺ and the suitable amount of incubated mitochondrial suspension as the source of enzyme. The enzyme activity was expressed as units/mg of protein.
Alpha-Ketoglutarate dehydrogenase (α-KGDH) activity was measured spectrophotometrically according to the method of Duncan et al. (1979) by measuring the reduction of 0.35 mM NAD⁺ to NADH at 340 nm using 50 mM phosphate buffer, pH 7.4 as the assay buffer, 0.1 mM α-ketoglutarate as the substrate and the suitable amount of incubated mitochondrial suspension as the source of enzyme. The enzyme activity was expressed as units/mg of protein.

Succinate dehydrogenase (SDH) activity was measured spectrophotometrically by following the reduction of potassium ferricyanide [K₃Fe(CN)₆] at 420 nm according to the method of Veeger et al. (1969) with some modifications. One ml of assay mixture contained 50 mM phosphate buffer, pH 7.4, 2% (w/v) BSA, 4 mM succinate, 2.5 mM K₃Fe(CN)₆ and the suitable amount of incubated mitochondrial suspension as the source of enzyme. The enzyme activity was expressed as units/mg of protein.

Immediately before aconitase activity was determined, freshly isolated mitochondria were suspended in 0.5 ml of buffer containing 50 mM Tris-HCl (pH 7.4) and 0.6 mM MnCl₂ and sonicated for 2 s. Aconitase activity was measured spectrophotometrically by monitoring the formation of cis-aconitate from added iso-citrate (20 mM) and suitable amount of mitochondrial suspension as the source of enzyme, at 240 nm and 25ºC following the method of Gardner et al. (1994). One unit was defined as the amount of enzyme necessary to produce 1µmol cis-aconitate per minute (A_{240}=3.6 mM⁻¹cm⁻¹). The enzyme activity was expressed as units/mg of protein.

Fumarase activity was determined spectrophotometrically by measuring the increase in absorbance at 240 nm at 25ºC in the reaction mixture to which 30 mM potassium phosphate (pH 7.4), and 0.1 mM L-malate and suitable amount of mitochondrial suspension as the source of enzyme were added following the method of Racker (1950). One unit (U) was defined as the amount of enzyme necessary to produce 1 µmol fumarate
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per minute ($A_{240} = 3.6 \text{ mM} \cdot \text{cm}^{-1}$). The enzyme activity was expressed as units/mg of protein.

Determination of hydroxyl (•OH) radical scavenging activity
Incubation mixture contained sodium phosphate buffer (0.05mM, pH 7.4) with 100 µl of incubated mitochondrial suspension (0.6mg/ml mitochondrial protein) for 60 minutes in the presence and absence of DMSO (500µM) and different concentrations of the aqueous bark extract of TA or benzoic acid in a volume of 1ml to determine the hydroxyl radical scavenging activity of the aqueous bark extract of TA or benzoic acid in an in vitro system. The reaction was terminated in each case by the addition of 0.1mM EDTA. Methanesulfinic acid (MSA) formed during incubation was measured by the method of Babbs and Steiner (1990) as modified by Bandyopadhyay et al. (2004).

Measurement of reactive nitrogen species (RNS) in mitochondria
The concentration of nitric oxide (NO), one of the RNS, in the incubated mitochondria was measured spectrophotometrically at 548nm according to the method of Fiddler (1977) by using Griess reagent (Griess, 1879). The reaction mixture in a spectrophotometer cuvette (1 cm path length) contained 100 µL of Griess Reagent, 700 µL of the sample (i.e., incubated mitochondrial suspension) and 700 µL of distilled water. The NO concentration was expressed as µM/mg of protein.

Determination of activities of respiratory complex enzymes during coupling and uncoupling condition to assess mitochondrial status
NADH-Cytochrome c oxidoreductase activity was measured spectrophotometrically by following the reduction of oxidized cytochrome c at 565 nm according to the method of Goyal et al. (1995). One ml of assay mixture contained in addition to the suitable amount of mitochondrial suspension as the source of enzyme, 50 mM phosphate buffer, 0.1 mg BSA, 20 mM oxidized cytochrome c and 0.5 (M) NADH. The activity of the enzyme was
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expressed as units/ mg of protein. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used as an uncoupler (0.1mM).

Cytochrome c oxidase activity was determined spectrophotometrically by following the oxidation of reduced cytochrome c at 550 nm according to the method of Goyal et al. (1995). One ml of assay mixture contained 50 mM phosphate buffer, pH 7.4, 40 mM reduced cytochrome c and a suitable aliquot of the mitochondrial suspension as the source of enzyme. The enzyme activity was expressed as units/mg of protein. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used as an uncoupler (0.1mM).

ATP synthase activity, measured in the direction of ATP hydrolysis (ATPase activity), was determined by the continuous spectrophotometric assay of Rosing et al. (1975) except that 2 mM EGTA replaced EDTA in the reaction medium. Aliquots of sonicated sample as a source of enzyme (200-400 µg in 20 µl sample volume) were added to the reaction medium containing 60 mM sucrose, 50 mM triethanolamine-HCl, 50 mM KCl, 4 mM MgCl_2, 2 mM ATP, 2 mM EGTA, 1 mM KCN, pH 8.0 (KOH) with 100 µM NADH, 5 units/ml pyruvate kinase and 5 units/ml lactate dehydrogenase. The total volume in the cuvette was 1 ml. The linear reaction was followed for 2 min at 340 nm and 37 °C. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used as an uncoupler (0.1mM).

Measurement of thiolase activity (total and potassium-sensitive)
The mitochondrial acetoacetyl CoA thiolase activity was measured in the presence of 50 mM KCl (total activity) and in its absence (K^+ replaced by an equivalent concentration of Na^+). The latter was taken to represent cytosolic thiolase activity. Mitochondrial acetoacetyl CoA thiolase activity was calculated by subtracting the rate of enzyme activity in the absence of K^+ from that in the presence of K^+. When measuring K^+ activation, care was taken to ensure that neither substrate nor enzyme solution contained K^+, this ion being replaced by Na^+. The rate of the reaction due to formation of acetyl CoA was assessed spectrophotometrically at 303 nm. The reaction mixture contained 100mM Tris-
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HCl (pH 8.1), 50mM CoA, 25mM MgCl₂, 50mM KCl and 10mM acetoacetyl CoA and suitable amount of mitochondrial suspension as the source of enzyme, (Middleton, 1973). The enzyme activity was expressed as mmol/ mg of protein.

Measurement of conjugated dienes, tryptophan and di-tyrosine fluorescence intensity

Conjugated diene formation was determined from the absorbance ratio $A_{233}/A_{215}$ of mitochondria dispensed in concentration 20μg/ml protein with 10 mmol/l phosphate buffer containing 1% Lubrol (Braughler et al., 1986). Fluorescence measurements were performed in solution containing 50μg proteins per ml, 10 mmol/l HEPES, 100 mmol/l KCl (pH 7.0) at 25°C using a spectrofluorimeter.

The fluorescence emission spectra (from 300 to 450 nm, 5 nm slit width) of tryptophan were measured by excitation at 295 nm (2 nm slit width) (Dousset et al., 1994). Emission spectra of di-tyrosine, a product of tyrosine oxidation, were recorded in range 380 to 440 nm (5 nm slit width) at excitation wavelength 325 nm (5 nm slit width) (Giulivi and Davies, 1994). Emission spectra (from 425 to 480 nm, 5 nm slit width) of lysine conjugated with LPO products were recovered at excitation of 365 nm (5 nm slit width). Excitation spectra (from 325 to 380 nm, 5 nm slit width) were measured at 440 nm (5 nm slit width) (Dousset et al., 1994).

Determination of mitochondrial DNA (mtDNA) damage with agarose gel electrophoresis

The incubated mitochondria with or without copper-ascorbate, copper-ascorbate plus aqueous bark extract of TA, copper-ascorbate plus benzoic acid, aqueous bark extract of TA only and benzoic acid only were lysed with 3 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 0.5% SDS and 0.3 mg/ml of proteinase K overnight at 37°C. Mitochondrial DNA was isolated using extraction with 1 M NaCl for 10 min at room temperature and purified twice with chloroform/isoamyl alcohol, 24:1. Then, the samples
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were precipitated and dissolved in TE buffer, and the DNA, thus obtained, gave an average 260/280 absorbance ratio of 2-2.5. The obtained DNA samples were then mixed with 6X loading dye and resolved in 0.8% agarose gel. The gel was stained with ethidium bromide and DNA bands detected in a Gel-Doc apparatus (Biorad, Hercules CA). The intensity of the DNA bands were then analyzed using image analysis system (ImageJ, NIH Software, Bethesda, MI) and the intensity of each band was measured and expressed as the gray scale of the band.

Measurement of mitochondrial swelling
Mitochondrial swelling was assessed by measuring the changes in absorbance of the suspension at 520 nm (Δ) by spectrophotometry according to Halestrap et al. (1990). The standard incubation medium for the swelling assay contained 250 mmol/L sucrose, 0.3 mmol/L CaCl\(_2\) and 10 mmol/L Tris (pH 7.4). Mitochondria (0.5 mg protein) were suspended in 3.6 mL of phosphate buffer. A quantity of 1.8 mL of this suspension was added to both sample and reference cuvettes and 6 mmol/L succinate was added to the sample cuvette only, and the absorption at 520 nm was recorded continuously at 25°C for 10 min. Swelling of mitochondria was evaluated by the changes in values of absorption at 520 nm.

Determination of mitochondrial reduced nicotinamide adenine dinucleotide (NADH) level
The mitochondrial pyridine nucleotide, reduced nicotinamide adenine dinucleotide (NADH), was monitored by measuring its autofluorescence with excitation and emission wavelengths of 360 nm and 450 nm, respectively according to Minezaki et al. (1994). Mitochondria (2 mg protein) were added to 1.8 mL of phosphate buffer containing 6mmol/L succinate and the autofluorescence of NADH was determined.
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**Determination of mitochondrial membrane potential (ΔΨm)**

Changes of mitochondrial membrane potential was determined by using JC-1(5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide) dye (Sigma-Aldrich Co. LLC, St. Louis, MO, USA). Incubated mitochondrial preparation was stained with help of JC-1 dye. Final concentration of JC-1 staining solution was 0.2µg/ml. The stained mitochondrial sample was analysed and an excitation wavelength of 488 nm and an emission wavelength of band pass filter 586/42 nm were used to measure the mitochondrial membrane potentiality with help of flow Cytometry (BDFACS Versa, USA).

**Determination of mitochondrial membrane cardiolipin content**

Mitochondrial membrane cardiolipin content was determined by using NAO (10-nanoyl acridine orange) dye (Sigma-Aldrich Co. LLC, St. Louis, MO, USA). To 100µl of incubated mitochondria (10mg protein/ml), 10µl of NAO (10µM) was added and the final volume was adjusted with 50mM phosphate buffer. The samples were incubated for 2 min at dark at room temperature, and then centrifuged at 1000g for 5 min. The stained mitochondrial sample was analysed at an excitation wavelength of 488nm and an emission wavelength of band pass filter 586/42nm were used to measure the mitochondrial membrane cardiolipin content with help of flow Cytometry (BDFACS Versa, USA).

**Scanning electron microscopy**

The incubated mitochondrial suspension was centrifuged, and the supernatant was removed. The pellet was fixed overnight with 2.5% glutaraldehyde. After washing three times with PBS, the pellet was dehydrated for 10 min at each concentration of a graded ethanol series (50, 70, 80, 90, 95 and 100%). The pellet was immersed in pure tert-butyl alcohol and was then placed into a 4°C refrigerator until the tert-butyl alcohol solidified. The frozen samples were dried by placing them into a vacuum bottle. Mitochondrial morphology was evaluated by scanning electron microscopy (SEM; Zeiss Evo 18 model EDS 8100).
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**Estimation of protein**
The protein content of the different samples were determined by the method of Lowry et al. (1951).

**Measurements of antioxidant activities of the aqueous bark extract of TA in chemically defined system**

(a) **DPPH free radical scavenging activity of the aqueous bark extract of TA**

The DPPH free radical scavenging activity of each sample was determined according to the method described by Joyeux et al. (1995). A solution of 0.1 mM DPPH in methanol was prepared. The initial absorbance of the DPPH in methanol was measured at 515 nm. An aliquot (40 μL) of an extract was added to 3 mL of methanolic DPPH solution. The change in absorbance at 515 nm was measured after 30 min. The antiradical activity (AA) was determined using the following formula:

\[
AA\% = 100 - \left[\frac{(\text{Abs: sample} - \text{Abs: empty sample})}{\text{Abs: control}}\right] \times 100
\]

(b) **Determination of reducing power (Ferric ion reducing antioxidant power, FRAP assay) of the aqueous bark extract of TA**

The reducing power was determined according to the method of Oyaizu (1986). 2.5 ml of extract were mixed with 2.5 ml of 200 mmol/l sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. After 2.5 ml of 10% trichloroacetic acid (w/v) were added, the mixture was centrifuged at 1500g for 10 min. The upper layer (5 ml) was mixed with 5 ml deionised water and 1 ml of 0.1% of ferric chloride, and the absorbance was measured at 700 nm: higher absorbance indicates higher reducing power. Ascorbic acid was used as standard.
Sample preparation for High Performance Liquid Chromatography (HPLC) profiling

10 mg of the lyophilized powder was weighed and solubilised in water-acetonitrile (9:1) mixture, then filtered through 0.22µm filter paper to avoid the minute particles. Standard benzoic acid was also prepared in same way before injecting into the MetaChem PolarisTM Amide C18, 5µm, 4.6 x 250 mm HPLC column.

Sample running through High Performance Liquid Chromatography (HPLC)

HPLC solvents (0.1% orthophosphoric acid and HPLC grade acetonitrile) were also filtered by the same method described above. Thereafter, the HPLC profiling (ZORBAX C18 Extend column) was done using UV detector according to the method as described by Kumar et al. (2008).

Spectral scan of the aqueous bark extract of TA

The spectra of the aqueous bark extract of TA were recorded at wavelengths ranging from 200-800 nm to determine the absorption maxima of the different component(s) using a UV-VIS scanning spectrophotometer (Model: Type 2202).

Analysis of the aqueous bark extract of TA through gas chromatography-mass spectrometry (GC-MS)

The lyophilized powder obtained from the aqueous bark extract of TA was solubilized in water-methanol (0.5:4.5) mixture for identifying its component(s) through GC-MS. GC-MS analysis was carried out using Agilent Technologies 6890 N Network GC system and interfaced to Agilent Technologies 5973 Inert Mass Selective Detector employing the following conditions: column DB-1 ms fused silica capillary column (30X0.25 I.D.X 0.10 Film, composed of 100% Dimethylpolysiloxane) (chosen for improved signal to noise ratio for better sensitivity and mass spectral integrity), operating in electron impact mode; helium (5.0) was used as carrier gas at a constant flow of 1ml/min. The injector, MS
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Source and MS Quadrupole temperature were fixed at 250°C, 230°C and 150°C, respectively, and turbo speed of the pump was 100%.

Oven programming

<table>
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For tuning of the MSD in EI mode Perfluorotributylamine (PFTBA) was used as tuning compound. Mass spectra were taken at 2235 EMVolts and fragments from 69 to 502.

Identification of phyto-compound(s) of the aqueous bark extract of TA

Interpretation of the mass spectrum of GC-MS was conducted using the Wiley/ NBS Registry of Mass spectral data. It consists of a set of mass spectra for over 1, 12,275 compounds with structures for 89, 903. The arrangement is by molecular weight with CAS number and chemical name indexes. The spectrum of the unknown component was compared with the spectrum of the known component inherent in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained.

Statistical evaluation

Each experiment was repeated at least three times. Data are presented as means ± SE. Significance of mean values of different parameters between the incubated mitochondria were analyzed using one way post hoc tests (Tukey’s HSD test) of analysis of variances (ANOVA) after ascertaining the homogeneity of variances between the incubations. Pairwise comparisons were done by calculating the least significance. Statistical tests were performed using Microcal Origin version 7.0 for Windows.