Materials and Methods
3. MATERIALS AND METHODS

The objective of the work is to isolate phytochemicals from the dried leaves of Mentha spicata L. that have antioxidant activity by solvent fractionation and evaluate their antigenotoxic properties. The herbaceous plant is widely used throughout India in making “chutneys” in combination with other spices and to add flavour in food preparations. It is also used in ayurveda, siddha, unani and folk medicines. Its essential oil is of economic value for industries making toothpaste, chewing gum, perfume, soap, confectioneries and pharmaceuticals. Part-I of the work consists of solvent fractionation of ethanol extract of dried leaves of the plant, estimation of total antioxidant activity (TAA), estimation of relative antioxidant activity (RAA) relative with quercetin, beta-carotene, ascorbic acid and glutathione; and quantitation of polyphenolics (phenolic and flavonoids) and pigments (chlorophyll and carotenoids). Part-II of the work consists of evaluation of antigenotoxic property of ethanol extract and the solvent fractions against a known genotoxin, 4-nitroquinoline-1-oxide (4-NQO). Part-III of the work involves experiments that aim to find the ability of ethanol extract and solvent fractions to reduce the genotoxin induced lipid peroxidation (LPO) and their ability to modulate enzymatic and non-enzymatic antioxidants.
PART – I

3.1.1 Ethanol extraction and solvent fractionation

Ethanol extraction and solvent fractionation was carried out according to Villasenor et al. (2002).

Reagents

Hexane, chloroform and ethyl acetate (SRL chemicals, India). All are of analytical grade.

Procedure

Eight hundred grams of shade dried leaf powder immersed in a volume of 4L of 95 percent ethanol was left with constant stirring for 24h and filtered. Again, 2L of ethanol was added, left with constant stirring for 24h and filtered. This was repeated for the third day also. Over 72h, a total of 3.5L filtrate was collected. The filtrate was concentrated under reduced pressure at 40°C, using vacuum rotary evaporator. The yield of ethanol extract (EE) was 42g (5.25%).

Ethanol extract was partitioned between 780ml of hexane and 130ml of water (5:1 v/v) using separating funnel. This mixture was thoroughly mixed for 15 minutes and after 6h the hexane fraction (HF) was collected. The aqueous layer (90ml) was further fractionated with 540ml of chloroform (CF) and then with 540ml of ethyl acetate (EAF). All fractions were concentrated with vacuum rotary evaporator. The yields of these fractions were 18g, 12g and 6.7g respectively constituted about 43, 29 and 16 percents of the ethanol extract.
The aqueous fraction (90mL; AF) was lyophilized (Flexi-Dru μp at 50MT and -85°C) and this fraction weighed 3.5g or about 8 percent of the ethanol extract.

3.1.2 Estimation of total antioxidant activity (TAA) and relative antioxidant activity (RAA).

Total antioxidant activity was estimated by following the method of Pellegrini et al. (1999).

Principle

The blue / green ABTS radical is generated through the reaction between ABTS and potassium persulfate. The radical shows maximum absorbance at 410, 671, 752 and 845nm (Figure 9). However, in this study absorbance was measured at 734nm, as there would be less interference from the colored compounds of the sample and at this wavelength, the antioxidant activity to be measured would be maximum than would be at other absorbance wavelengths maxima (Arnao, 2000). Antioxidants transfer a hydrogen atom to the radical and cause discoloration of the solution. The percentage discoloration is proportional to the concentration of the antioxidants. The antioxidant activity can be expressed, relative to the antioxidant activity of standard compound such as quercetin, β-carotene, L-ascorbic acid and glutathione, as relative antioxidant activity (RAA).
Reagents

7 mM 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)

140 mM potassium persulfate (K₂S₂O₈)

Standards: 1 mM β-carotene in acetone, 1 mM quercetin in ethanol, 1 mM L-ascorbic acid and glutathione in double distilled water (sterilized).

All were obtained from Sigma chemical Co. (Mo., USA)

50 mM Phosphate buffered saline (pH 7.4)

Test samples: HF in hexane, CF in chloroform, EAF in ethyl acetate and AF in double distilled water (sterilized).
Procedure

ABTS⁺ stock solution was prepared by mixing 5ml of 7mM ABTS and 88μl of 140mM K₂S₂O₈. The mixture was diluted with ethanol or phosphate buffered saline (PBS, pH 7.4) to give an absorbance, 0.75 (± 0.02) at 734nm. The ethanol was used for lipophilic fractions (HF, CF and EAF) and PBS for hydrophilic fraction (AF). The assay was performed in one ml reaction volume. Quercetin and β-carotene were tested in the range of 0 - 7.5μM/ml final concentration. HF, CF and EAF were tested at concentration with range of 0 - 50μg/ml. L-ascorbic acid and glutathione were tested at various concentrations in the range of 0 - 15μM/ml. AF was tested at concentrations in the range of 0 - 30μg/ml. The readings were taken at room temperature. Appropriate blanks were run in each assay and all the experiments were carried out in five replications and the values were averaged.

The TAAs were also expressed as standard equivalents (mM/g of solvent fraction).

\[
\text{Total antioxidant activity (TAA-%)} = \frac{\text{OD ABTS}^+ - \text{OD Sample}}{\text{OD ABTS}^+} \times 100
\]

\[
\text{Relative antioxidant activity (RAA)} = \frac{\% \text{TAA test compound}}{\% \text{TAA Standard compound}}
\]
3.1.3 Estimation of total phenolics

The amount of total phenolics was estimated by following the method of Price and Butlar (1977).

Reagents

0.1M Ferric chloride (FeCl₃; SRL chemical, India)
0.1M Hydrochloric acid (HCl; SRL chemical, India)
0.008M Potassium ferricyanide reagent (K₃ Fe (CN)₆; Loba Chemie, India)

Quercetin was used as standard for phenolic estimation. Solvent fractions were dissolved in their respective solvents.

Procedure

Conical flask containing 25ml of deionised water and 250µl of solvent fractions (HF, CF, EAF and AF) were mixed with 3ml of ferric chloride (0.1M FeCl₃ in 0.1M HCl). After 3 min, 3ml of potassium ferricyanide reagent (0.008M K₃ Fe (CN)₆) was added and allowed to stand for 10 - 15 minutes at room temperature. The OD of blue colour developed was read at 720nm using a Thermospectronic (GENESYS 10-UV) spectrophotometer.

The values were expressed in mg/g solvent fraction.
3.1.4 Estimation of flavonoids

Total flavonoids were estimated according to the method of Lamaison and Carnat (1990).

Reagents

Two percent aluminium chloride in methanol (Central Drug House, India)
Quercetin was used as standard. Solvent fractions were dissolved in their respective solvents.

Procedure

One ml of 2 percent aluminium chloride was mixed with one ml of solvent fraction. The OD of the blue colour was read immediately at 430nm using a Thermospectronic (GENESYS 10-UV) spectrophotometer.

The values are expressed in mg/g of solvent fractions.

3.1.5 Estimation of total chlorophylls

The total chlorophyll was estimated according to the method of Lichtenhaler and Wellburn (1983).

Procedure

Chlorophyll ‘a’ and ‘b’ were spectrophotometrically measured at 645 and 663nm respectively. They were expressed in mg/g solvent fraction using
appropriate equation. The total chlorophyll is the sum of chlorophyll ‘a’ and chlorophyll ‘b’.

\[
\text{Total chlorophyll} = \frac{20.2 \ A_{645} \pm 8.02 \ A_{663}}{a \times 1000 \times W} \times V
\]

Where:
- \(20.2\) = absorption coefficient for chlorophyll ‘a’ at 645nm
- \(8.02\) = absorption coefficient for chlorophyll ‘b’ at 663nm
- \(a\) = length of path light in the cell (usually 1cm)
- \(V\) = volume of the ethanol fraction in ml
- \(W\) = dried weight of the sample in g

3.1.6 Estimation of total carotenoids

Carotenoids were estimated by following the method of Lichtenhaler and Wellburn (1983).

Procedure

Carotenoids were measured at 470nm and expressed in mg/g of solvent fraction using appropriate equation.

\[
\text{Total carotenoids} = \frac{D \times V \times f \times 10}{2500}
\]

Where: \(D\) = absorbance at 470nm in 1.0 cm cell,
- \(V\) = volume of ethanol fraction in ml,
- \(f\) = dilution factor
- 2500 = average extinction coefficient of the pigments.
PART - II

ANTIGENOTOXIC ASSAY

The antigenotoxic property was assayed *in vivo* by estimating the frequency of micronucleated polychromatic erythrocyte cells (MnPCEs) in the bone marrow of mice using 4-nitroquinoline-1-oxide (4-NQO) as reference mutagen. 4-NQO is known to be a strong mutagen and induces DNA strand breaks (Papp-Szabo *et al.*, 2003; Gunji *et al.*, 2006). The 4-NQO induced genetic damage is similar to the UV light induced genetic damage as it also forms DNA adducts at N2 and C8 position of guanine. It is also known to be a carcinogen as it can induce oral, lung, stomach and skin cancers (Kanojia and Vaidya, 2006).

The dose selected, 7.5mg/Kg for the reference mutagen, is based on the published reports. This dose was equal to the 50 percent of LD$_{50}$ dose and induces micronuclei at a significantly higher frequency (Suzuki *et al.*, 1999; Nakajima *et al.*, 1999). 4-NQO was dissolved in a mixture of 50 percent DMSO plus acetic acid in 1:4 ratio (v/v) (Nishikawa *et al.*, 2002).

3.2.1 Dose selection for ethanol extract and solvent fractions

The test doses for ethanol extract (EE) and solvent fractions viz., HF, CF, EAF and AF were determined on the basis of their LD$_{50}$ doses estimated based on preliminary experiments. The doses usually correspond to 50, 25 and 12.5 percents of the LD$_{50}$ doses. The LD$_{50}$ doses and the actual doses employed are given below:
<table>
<thead>
<tr>
<th>Extract / Solvent fraction</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt; dose mg/Kg bwt</th>
<th>Doses used mg/Kg bwt</th>
</tr>
</thead>
<tbody>
<tr>
<td>EE</td>
<td>640</td>
<td>320,160,80</td>
</tr>
<tr>
<td>HF</td>
<td>640</td>
<td>320,160,80</td>
</tr>
<tr>
<td>CF</td>
<td>640</td>
<td>320,160,80</td>
</tr>
<tr>
<td>EAF</td>
<td>320</td>
<td>160,80</td>
</tr>
<tr>
<td>AF</td>
<td>640</td>
<td>320,160,80</td>
</tr>
</tbody>
</table>

Ethanol extract and HF, CF and EAF were dissolved in 50 percent DMSO and AF in double distilled water (DDW).

### 3.2.2 Experimental animals

Healthy Swiss albino mice of either sex, 10 - 12 weeks old weighing 25 - 30g were used for the experiments. All animals were obtained from the King Institute, Chennai and were maintained at the Institute’s animal house under standard environmental conditions (temperature: 25±2°C, humidity: 50±5% and 12h light/dark period). They were housed in polypropylene cages with stainless steel grill top, bedded with rice husk. The animals were provided with standard pelleted diet (M/s. Hindustan Lever Ltd., Mumbai, India.) and water ad libitum. Maintenance of animals was done in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. The Institute’s ethical committee approved all experiments.
2.3 Experimental design

The following experimental protocol has been used to assess the antigenotoxic property. For each group, six mice were used.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 ((X_1))</td>
<td>Control: 50 percent DMSO for EE, HF, CF and EAF and double distilled water for AF. Oral feeding in variable volume corresponding to body weight of mice.</td>
</tr>
<tr>
<td>Group 2 ((X_2))</td>
<td>Feeding EE, HF, CF, EAF and AF by oral gavage for five consecutive days at dose 80mg/Kg bwt.</td>
</tr>
<tr>
<td>Group 3 ((X_3))</td>
<td>Feeding EE, HF, CF, EAF and AF by oral gavage for five consecutive days at dose 160mg/Kg bwt</td>
</tr>
<tr>
<td>Group 4 ((X_4))</td>
<td>Feeding EE, HF, CF and AF by oral gavage for five consecutive days at dose 320mg/Kg bwt</td>
</tr>
<tr>
<td>Group 5 ((X_5))</td>
<td>4-NQO 7.5mg/Kg bwt administered by i.p. injection (50% of LD(_{50}) dose).</td>
</tr>
<tr>
<td>Group 6 ((X_6))</td>
<td>EE, HF, CF, EAF and AF feeding by oral gavage for five days at dose 80mg/Kg bwt + 7.5mg/Kg bwt of 4-NQO by i.p injection on 5th day 2h after feeding test material.</td>
</tr>
<tr>
<td>Group 7 ((X_7))</td>
<td>EE, HF, CF, EAF and AF feeding by oral gavage for five days at dose 160mg/Kg bwt + 7.5mg/Kg bwt of 4-NQO by i.p injection on 5th day 2h after feeding test material.</td>
</tr>
<tr>
<td>Group 8 ((X_8))</td>
<td>EE, HF, CF and AF feeding by oral gavage for five days at dose 320mg/Kg bwt + 7.5mg/Kg bwt of 4-NQO by i.p injection on 5th day 2h after feeding test material.</td>
</tr>
</tbody>
</table>

Twenty-four hours after mutagen administration, the mice were sacrificed by cervical dislocation. Femur bone were dissected out and
processed for micronucleus assay. The liver tissue was also collected and stored in 0.9 percent NaCl at −20°C until further use for biochemical assays.

3.2.4 Micronucleus assay

The mouse bone marrow micronucleus test was carried out according to the method of Schmid (1975).

Background

The micronuclei are generated due to chromosomal breakage, spindle dysfunction and non-disjunction of chromosomes. During anaphase, acentric, chromatid and chromosome fragments lag behind when the centric elements move towards the spindle poles. After telophase the undamaged chromosomes, as well as the centric fragments are included in the daughter cells. A proportion of acentric fragments, chromatid fragments and lagged chromosome transform into one or several secondary nuclei called micronuclei, which as a rule are much smaller by about 1/5 – 1/20th the size of the main nucleus. A few hours after completion of the last mitosis, erythroblasts expel their main nucleus but the micronuclei remain in the cytoplasm of the young erythrocyte and can be easily visualized as small spherical bodies. Mature erythrocytes usually stain yellowish brown and are called as normochromatic erythrocytes (NCEs) and young erythrocytes stain bluish and are called as polychromatic erythrocytes (PCEs). The assay is based on estimation of the frequency of micronucleated PCEs (MnPCEs) in the bone marrow of the treated animals in comparison with the untreated animals. This method is highly suitable for routine cytogenetic screening for genetic damage.
Reagents

Fetal bovine serum (Himedia Laboratory, India).

May-Grünwald stain (Sigma)

Two hundred and fifty mg of the May-Grünwald stain powder was dissolved in 100ml of methanol by mixing over a magnetic stirrer for 15 min and filtered through Whatmann No.1 filter paper. The stain was prepared fresh before use.

Giemsa stain (Sigma)

One gram of Giemsa powder was dissolved in 56ml of glycerol by mixing over a magnetic stirrer at 50°C, overnight. After cooling to room temperature, 84ml of methanol was added and kept for mixing for one hour. This was filtered through Whatmann No.1 filter paper and stored in a dark bottle at 4°C.

Slides

The slides were immersed in 10 percent chromic acid, overnight. They were rinsed in tap water and soaked in soap solution for one hour. The slide was then scrubbed well and left in the running tap water for 15 min. They were then rinsed twice in distilled water and blotted using a coarse filter paper. Further they were cleaned with methanol and stored in slide boxes prior to use.
Procedure

At the end of the experimental period, all animals were sacrificed by cervical dislocation. The femur bones were removed and cleaned using a coarse filter paper. The kneecap was gently cut and the marrow of the femur bones was flushed into 2ml FB serum. It was gently aspirated to give a homogenous suspension. The suspension was pelleted by centrifugation for 10 min at 2000 rpm. The pellet was re-suspended in a drop of fresh serum. A drop of the suspension is kept on slide and drawn out as thin smear. The slides were then air-dried for 18h. For each animal three slides were prepared and coded for analysis.

Staining

Initially the air-dried slides were stained in undiluted May-Grünwald stain for 3 min, and later in diluted May-Grünwald stain (1:1 dilution with distilled water) for 2 min and were rinsed in distilled water. Finally, the slides were stained in Giemsa (1:6 dilution with distilled water) for 5-7 min, rinsed in double distilled water and air dried for analysis.

Analysis

From each animal (experimental/control), 2500 polychromatic erythrocytes (PCEs) (with or without micronuclei) and a corresponding number of NCEs were scored under oil immersion objective (100x CXL plus: LABOMED Microscope) (Figure 10).
Figure 10. Bone marrow smear of Swiss albino mice showing Polychromatic Erythrocytes (PCEs), Normochromatic Erythrocytes (NCEs) and Micronucleated Polychromatic Erythrocytes (MnPCEs)
PART - III

LIPID PEROXIDATION AND BIOCHEMICAL ASSAYS

Lipid peroxidation is a simple inexpensive measure that could be used to reflect the whole body radical activity or overall oxidative stress and also the body’s antioxidant capability. Glutathione peroxidase (GPx), glutathione-S-transferase (GST), superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), Vitamin E and Vitamin C mediate protection against ROS (reactive oxygen species) generated during cellular activity. SOD removes superoxide radical ($O_2^-$) and CAT detoxifies $H_2O_2$. GPx catalyses the reduction of $H_2O_2$ at the expense of GSH. GST detoxifies both exogenous and endogenous substances by conjugating with glutathione. GSH maintains cell membrane sulphhydryl groups and other structural proteins in stable form. Vitamin E is the chain breaking antioxidant and maintains membrane stability. Vitamin C scavenges radicals from extracellular fluids and protects the membranes from oxidative damage; and also regenerates tocopherol.

3.3.1 Preparation of tissue homogenate

Weighed amounts of liver tissues were homogenized in Tris-HCL buffer (0.1M, pH 7.4) with a Teflon-coated homogenizer to give a 10 percent homogenate. The tissue homogenate was used for the estimation of lipid peroxidation (LPO), enzymatic antioxidants (GPx, GST, SOD & CAT) and non-enzymatic antioxidants activity (GSH, Vit E & Vit C).
3.3.2 Estimation of total protein

Total protein was estimated by following the method of Lowry et al. (1951).

Reagents

Alkaline copper reagent:

Solution A: Two percent sodium carbonate in 0.1 N sodium hydroxide. Solution B: 0.5 percent copper sulphate in 1 percent sodium potassium tartrate. 50 ml of solution A was mixed with 1 ml of solution B just before use.

Folin’s phenol reagent (SRL, India).

Standard: 0.02 percent Bovine serum albumin (SRL, India)

Procedure

The 0.1ml of 10 percent homogenate was diluted to 1ml with distilled water. From this diluted sample, 0.1ml was taken and made up to one ml with distilled water. Similarly, standard was made up to one ml with distilled water. Blank contained one ml of distilled water. To all the tubes, 4.5ml of alkaline copper reagent was added and kept at room temperature for 10 minutes. Then, 0.5ml of Folin’s phenol reagent was added. OD of the colour developed was read after 20 minutes at 640nm against reagent blank using Therospectronic (GENESYS 10-UV) spectrophotometer.
Total protein content was expressed in mg/g tissue.

3.3.3 Lipid peroxidation (LPO)

Thiobarbituric acid reactive substances (TBARS) released from endogenous lipid peroxides reflect lipid peroxidation and this was assayed by the method described by Ohkawa et al. (1979).

Reagents

0.15M Tris-HCL buffer, pH 7.4

10mM Potassium dihydrogen phosphate (KH₂PO₄)

One percent thiobarbituric acid (TBA-SRL, India)

Ten percent trichloro acetic acid (TCA- SRL, India)

50 nmoles Malondialdehyde (MDA)/mL as standard (Sigma)

Procedure

The assay mixture consisting 1.6ml Tris-HCl buffer, 0.2ml KH₂PO₄ and 0.2ml 10 percent tissue homogenate was incubated with shaking for 30 minutes at 37°C. The reaction was arrested by the addition of one ml of 10 percent TCA. 1.5ml of one percent TBA was added and heated in a boiling water bath for 20 minutes. The test tubes were cooled and centrifuged at 3000 x g for 10 minutes. The OD of the colour developed in the supernatant was read at 532 nm using Thermospectronic (GENESYS 10-UV) spectrophotometer. Standard was treated similarly and was read against distilled water blank.
The lipid peroxidation was expressed in terms of nmoles of MDA formed/mg protein under incubation conditions.

3.3.4 ENZYMATIC ANTIOXIDANTS

3.3.4.1 Glutathione peroxidase (EC: 1.11.1.9, GPx)

Glutathione peroxidase activity was assayed according to the method of Rotruck et al. (1973).

Reagents

0.32M Sodium phosphate buffer, pH 7.0

0.8mM Ethylene diamine tetra acetate (EDTA)

10mM Sodium azide

4mM Reduced glutathione

2.5mM Hydrogen peroxide

Ten percent trichloro acetic acid (TCA - w/v)

0.3M Disodium hydrogen phosphate

40mg 5,5'-dithiobis (2-nitro benzoic acid) (DTNB) in 0.3M disodium hydrogen phosphate

0.01 Percent reduced glutathione as standard

All chemicals are of analytical grade and were purchased from SRL chemicals, India.
Procedure

Each 0.2ml of EDTA, sodium azide, reduced glutathione, hydrogen peroxide, 0.4ml of sodium phosphate buffer and 0.1ml of 10 percent homogenates were mixed and incubated at 37°C for 10 minutes. The reaction was arrested by the addition of 0.5ml of 10 percent TCA. The samples were centrifuged at 2000 rpm for 10 minutes and the supernatant was collected. To 0.5ml of supernatant, 4ml of disodium hydrogen phosphate and one ml of DTNB were added. The colour developed was read at 420nm immediately using Thermospectronic (GENESYS 10-UV) spectrophotometer. Graded concentrations of the standards were treated similarly.

The activity was expressed as moles of reduced glutathione oxidized/min/mg protein under conditions incubated.

3.3.4.2 Glutathione-S-transferase (EC: 2. 5. 18, GST)

The enzyme activity was assayed by following the method of Habig et al. (1974).

Reagents

0.5M Phosphate buffer, pH 6.5.

30mM 1-chloro 2,4-dinitrobenzene (CDNB) in 95 percent ethanol

30mM Reduced glutathione
Procedure

One ml phosphate buffer, 0.1ml of 10 percent liver homogenate, 1.7ml water and 0.1ml CDNB were mixed and incubated at 37°C for 15 minutes. After incubation, 0.1ml reduced glutathione was added. OD was measured against blank (distilled water) at 340nm using Thermospectronic (GENESYS 10-UV) spectrophotometer.

Enzyme activity was expressed in nmoles of CDNB conjugated/min/mg protein.

3.3.4.3 Superoxide dismutase (EC: 1.15.1.1, SOD)

The enzyme activity was assayed according to the method of Marklund and Marklund (1974).

Reagents

0.1M Tris-HCL buffer pH 8.2

0.05M Tris-HCL buffer pH 7.4

2mM Pyrogallol (SRL) solution in 0.05 M Tris-HCL, pH 7.4

Absolute ethanol (AR)

Chloroform (AR).
Procedure

The homogenate of 0.2ml, 0.25ml ice-cold chloroform, 0.15ml ice-cold ethanol and one ml distilled water were mixed and centrifuged at 3000 x g for 10 minutes. The supernatant was used for enzyme assay. 0.5ml of the supernatant, 2ml of 0.1M Tris buffer and 1.5ml water were mixed. The reaction was initiated by adding 0.5ml pyrogallol. Change in OD (due to autooxidation) was noted at an interval of one minute over a period of three minutes at 480nm. The blank contained 2.0ml distilled water, 2ml of 0.1M Tris buffer and 0.5ml pyrogallol.

The enzyme activity is expressed in units/min/mg protein. One unit corresponds to the amount of enzyme required to inhibit 50 percent autooxidation.

3.3.4.4 Catalase (EC: 1. 11. 1. 6, CAT)

Catalase activity was assayed by following the method of Sinha (1972).

Reagents

0.01M Phosphate buffer, pH 7.0

0.2M Hydrogen peroxide

Dichromate-acetic acid reagent: 5 percent potassium dichromate was mixed with acetic acid in the ratio 1:3 (v/v). The solution was diluted with distilled water, in the ratio, 1:5
Procedure

Ten percent of 0.1ml liver homogenate, one ml of phosphate buffer and 0.5ml of hydrogen peroxide were added in four test tubes and labeled at 0, 30, 60 and 120 seconds. Potassium dichromate: acetic acid reagent was added at their specified times. Standard H₂O₂ in the range of 4 to 20μM were treated similarly. At the end of reaction, the test tubes were heated in boiling water for 15 minutes. After cooling, the green colour developed was read at 570nm using Thermospectronic (GENESYS 10-UV) spectrophotometer.

Catalase activity was expressed in μmoles of H₂O₂ consumed/min/mg protein.

3.3.5 NON-ENZYMATIC ANTIOXIDANTS

3.3.5.1 Reduced glutathione (GSH)

Reduced glutathione was estimated by following the method of Moron et al. (1979).

Reagents

5.33 Percent sodium phosphate

10 Percent trichloro acetic acid (TCA)

0.6mM 5,5'-dithiobis(2-nitro benzoic acid) (DTNB) in 1 percent sodiumcitrate

0.01 Percent reduced glutathione (Standard)
Procedure

One mL of 10 percent liver homogenate was precipitated with one ml of 10 percent TCA and discarded by centrifugation. 0.5mL supernatant, 2.0ml phosphate solution and 0.5ml DTNB reagents were added in a test tube. Standard (0.01% GSH) was treated similarly. The colour developed was read at 420nm using a Thermospectronic (GENESYS 10-UV) spectrophotometer.

Glutathione was expressed in μg/mg protein.

3.3.5.2 Vitamin E

Vitamin E activity was assayed by following the method of Desai (1984).

Reagents

0.2 Percent 4,6-diphenyl-1, 10-phenanthroline in ethanol (SRL, India)

0.001M Ferric chloride in ethanol

0.001M O-phosphoric acid in ethanol

0.01 Percent vitamin E (standard) in ethanol

Absolute ethanol and petroleum ether (SRL, India)
Procedure

One ml of 10 percent liver homogenate and one ml of ethanol mixed in a test tube. Then three ml of petroleum ether was added, shaken rapidly and centrifuged at 2000 rpm for 10 minutes. Two ml of supernatant was taken and evaporated to dryness. To this 0.2ml of bathophenanthroline was added. The assay mixture was protected from light and 0.2ml of ferric chloride was added followed by 0.2ml O-phosphoric acid. The total volume was made up to 3ml with ethanol. The colour developed was read at 530nm using Thermospectronic (GENESYS 10-UV) spectrophotometer.

The enzyme activity was expressed in μg/mg protein.

3.3.5.3 Vitamin C

Vitamin C activity was assayed by following the method of Omaye et al. (1979).

Reagents

5 Percent TCA

65 Percent (v/v) sulphuric acid (H₂SO₄)

0.05 Percent Vitamin C (Standard) in TCA

2,4 dinitrophenyl hydrazine-thiourea copper sulphate reagent (DTC): thiourea 0.4g, copper sulphate 0.5g and dinitrophenylhydrazine 3.0g in 100ml of 9N H₂SO₄.
All chemicals are Analytical grade and sourced from SRL chemicals, India.

Procedure

To 0.5ml of 10 percent liver homogenate, 0.5ml of distilled water and one ml of 5 percent TCA were added, mixed thoroughly and centrifuged for 20 minutes. To one ml of the supernatant, 0.2ml of DTC reagent was added and incubated at 37°C for 3 h. Then 1.5ml of 65 percent of sulphuric acid was added, mixed well. The contents were allowed to stand at room temperature for another 30 minutes. The colour developed was read at 520nm using Thermospectronic (GENESYS 10-UV) spectrophotometer. Graded amount of standard was also treated similarly.

The enzyme activity was expressed in µg/mg protein.

PART - IV

STATISTICAL ANALYSIS

Means were compared by student's t-test. One-way analysis of variance (ANOVA) and Student-Newman-Keuls (SNK) were used for multisampling comparisons. All statistical tests were performed using SPSS package (version-10). P-value of ≤ 0.05 was considered to be significant.