CHAPTER II
MATERIALS AND METHODS

2.1 COLLECTION OF TODDY SAMPLES

Toddy was collected directly from the tapper and also from the licensed toddy shops from Kottayam, Ernakulam, Trichur and Palakkad districts of the state of Kerala.

Eighty samples of each randomly collected, twenty each from these four districts were analyzed in the study.

2.2 EXPERIMENTAL ANIMALS

Male albino rats of Wistar strain, two months of age weighing 130 - 150 g were used for the experimental purpose. The animals were purchased from small animals breeding centre of Kerala Agriculture University, Mannuthy, Thrissur and were housed in polypropylene cages in the animal house of School of Biosciences, Mahatma Gandhi University, Kottayam and given standard pellet diet (M/S Hindustan and Lever Ltd Bombay) and drinking water ad libitum. The animals were maintained at a controlled condition of temperature of (26°C - 28°C) with a 12h light: 12h dark cycle. They were given a week’s time to get acclimatized to the laboratory conditions. Initial body weight of each animal was recorded. Animal studies were conducted according to the Institutional animal ethics committee regulations approved by the committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA) Reg No. 2442009/2 and conducted humanely.

2.3 CELL LINE

Chang liver cell line was procured from National Centre for Cell Science (NCCS), Pune, India and grown as a monolayer in DMEM containing HEPES and sodium bicarbonate supplemented with 10% FBS and 1% antibiotic-antimycotics. Cells were maintained in a tissue culture flask and kept in a humidified incubator (5% CO₂ in air at 37 °C).
2.4 CHEMICALS AND DIAGNOSTIC KITS

Diazepam, phenobarbitone and chloral hydrate were purchased with the permission of CPCSEA.

a. Diazepam - 7-Chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one
   Trade name-Valium - Ranbaxy, India

b. Phenobarbitone - 5-Ethyl-5-phenylbarbituric acid
   Trade name: Luminal, Gardenal- Nicholas Piramal India Ltd, Biodeal Labs, Mumbai
c. Chloral hydrate – 2,2,2-Trichloroethane-1,1-diol

\[
\begin{align*}
\text{OH} & \\
\text{HO} & \\
\text{CCl}_3 &
\end{align*}
\]

Trade name - Aquachloral, Novo-Chlorhydrate, Sigma-Aldrich

**Chemicals purchased from Merck, Mumbai, India**

- Absolute alcohol
- Acetaldehyde
- Acetic acid
- Acetone
- Acetylcholine chloride
- Ammonia
- Ammonium molybdate
- Anhydrous Sodium Sulphate
- Antimony potassium tartrate
- Ascorbic acid AR
- Bismuth sub nitrate
- Bismuth subnitrate
- Calcium
- Chloroform
- Dimethylsulphoxide (DMSO)
- Di-sodium hydrogen phosphate
- Glacial acetic acid
- Gluteraldehyde
- Glycine
- Hydrochloric acid
- Hydroxylamine
Isoamyl acetate
Mercurous nitrate
Methanol
Potassium
Potassium dihydrogen phosphate
Potassium iodate
Potassium iodide
Potassium iodide
Potassium permanganate
Pyrazole
Rotenone
Silica gel G
Sodium
Sodium acetate
Sodium carbonate
Sodium chloride
Sodium dihydrogen phosphate
Sodium hydroxide
Sodium lactate
Sodium lactate
Sodium phosphate
Solvent Ether
Starch
Sulphuric acid
Tri sodium citrate
Tween-80
Vitamin C
Chemicals purchased from Central Drug House (CDH), New Delhi, India

Thiobarbituric acid (TBA)

Ferric chloride

Chemicals purchased from Nice chemicals, Kochi

Formaldehyde solution

Hematoxylin and Eosin

Hydrogen peroxide ($\text{H}_2\text{O}_2$)

Chemicals purchased from Sisco Research Laboratories (SRL)

Reduced glutathione (GSH)

Oxidised glutathione (GSSG)

$5,5'$-dithiobis-(2-nitrobenzoic acid) (DTNB)

1-chloro-2,4-dinitrobenzene (CDNB)

Trichloroacetic acid (TCA)

Ethylene diamine tetra acetate (EDTA)

Tris-HCl

NADPH

Iodine

Sodium azide

Nitroblue tetrazolium (NBT)

Bovine serum albumin

Acetyl thiocholine iodide

Diagnostic kits

MTT assay kit : HiMedia, India

JC-1 staining kit : Invitrogen, USA
2.5 INSTRUMENTS

Rotary evaporator : Superfit DB3135H, India
Spectrophotometer : Hitachi U-2800, Japan
Weighing balance : Sartorius, Germany
Inverted microscope : Motic AE 21, Germany
pH meter : pH 700, Eutech Instruments, Singapore
Microscope : Magnus, India
Microplate reader : BioRad, USA
Semi autoanalyser : RMS BCA201, India
Rotary microtome : Biotech master precision ultra thin rotary microtome

Atomic absorption spectrometer-iCE 3000
Ultra violet-visible spectrophotometer-Evolution 201
Gas chromatograph(GC)-Agilent 7890 A
Transmission electron microscope- Hitachi H-7650
Scanning electron microscope-Hitachi 2400
Nephelometric Turbidity Unit
Muffle Furnace
Desiccator

2.6 GLASSWARE AND PLASTIC WARES

Glassware and plastic wares were obtained from Borosil, Mumbai and Tarsons, Kolkata, respectively.
2.7 EXPERIMENTAL DESIGN

2.7.1 Analysis of selected parameters of natural toddy and adulterated toddy

A comparative study based on physical properties, chemical properties and percentage composition of the elements phosphorous, sodium, potassium and calcium were conducted. Analyzed the shop toddy for the presence of diazepam, phenobarbitone, chloral hydrate, sodium lauryl sulphate, silicates and saccharin, which are the common adulterants. Estimated the ascorbic acid, total acidity and ash content. Determined the turbidity, percentage transmission of supernatant liquid in both toddy samples. More than 80 samples from various districts in the state of Kerala were analyzed in the present study.

2.7.1.1 Physical parameters

2.7.1.1.a Odour

Toddy has a strong smell of fermented sap. The odour of Natural Toddy is very characteristic and distinguishable from smell of other fermented liquids. It can be identified by smelling the sample of toddy. The smell is noted in various dilutions up to 48 hr fermentation.

2.7.1.1.b Turbidity

Natural Toddy is highly turbid liquid after a few hours of collection in the pot. The turbidity can be measured using a turbidity meter in NTU (Nephelometric Turbidity Unit). The turbidity of the sample shall be measured in two conditions. One after shaking and other after centrifuging at 5000 rpm for five minutes in all stages of a sample.

2.7.1.1.c Percentage transmission of light through supernatant liquid.

(Willard et al., 1986)

Principle

The transmittance T of a sample is the ratio of the light that has passed through the sample (I) to the intensity of the light when it entered the sample (Io).

\[ T = \frac{I}{I_0} \]
Light was passed through the supernatant liquid placed in a cuvette. The percentage transmission of light was observed using ultraviolet spectrophotometer.

2.7.1.2 Chemical Parameters

2.7.1.2.a Estimation of Ethyl Alcohol

Estimation of ethyl alcohol is made using (i) Specific gravity method prescribed in the Indian Standard method of tests for alcoholic drinks-IS-3752-1967 and also by (ii) gas chromatographic method.

(The Bureau of Indian Standards (BIS) is the national Standards Body of India working under the aegis of Ministry of Consumer Affairs, Food & Public Distribution, Government of India. It is established by the Bureau of Indian Standards Act, 1986 which came into effect on 23 December 1986)

Estimated the alcohol percentage from the time of collection at an interval of 6 hrs upto 48 hrs. Fermentation was arrested by the addition of a fixed quantity of benzoic acid in the same interval.

i Specific gravity method

Principle

The specific gravity = \( \frac{\text{weight of sample}}{\text{weight of water}} \)

Take 100ml of toddy in a 100ml standard flask and note the temperature of the sample. Transfer the whole toddy quantitatively into a distillation flask and slowly distill and collect about 90 ml of distillate in 20-30 minutes in a 100ml standard flask placed in an ice bath. Bring the distillate into the temperature at which the sample was initially measured by placing the distillate in a water bath and make up the distillate to 100ml using distilled water of the same temperature. The specific gravity of the distillate at room temperature is found out and read out the percentage of alcohol at 15.56°C from the standard conversion table in Ref. Tables, Appendix C of official methods of Analysis of AOAC.
ii. Gas chromatographic method (GC)

**Principle**

Gas chromatography is based on the principle of partition (differential distribution) of a volatile compound (gas) in two phases - a liquid phase covering the adsorbent surface and a gaseous phase of the eluting gas. With a fixed set of parameters (length and diameter of column, temperature and flow rate of the eluting gas etc.) the time taken between injection of the sample and its detection in the elute (Retentions time) is constant for a particular compound and can be used for identifying the compound.

Carbowax-60, a metallic packed column was used for the GC analysis. Carrier gas is nitrogen (30 ml/min) and a mixture of hydrogen and air (300 ml/min) is used to produce flame in the flame ionization detector (FID).

Sample injection volume - 1 micro litre

Sample – supernatant liquid of toddy

Calibration curve was plotted using different concentrations of ethyl alcohol (external standard). Using the calibration curve concentration of the sample was calculated.

2.7.1.2.b pH measurement

The basic principle of the pH meter is to measure the concentration of hydrogen ions. Acids dissolve in water forming positively charged hydrogen ions (H+) pH of the sample was measured using a pH meter.

2.2.1.2.c Total acidity

Total acidity can be estimated as per the method No.7 prescribed in the Indian Standards Methods of tests for alcoholic drinks, which is the authority to prescribe the norms, working under the aegis of Ministry of Consumer Affairs, Food & Public Distribution, Government of India. It is established by the Bureau of Indian Standards Act, 1986 which came into effect on 23rd December 1986 - IS-3752-1967.
10 ml of toddy sample is titrated against standard 0.1 N NaOH solution using phenolphthalein as indicator and the acidity is calculated in terms of tartaric acid (gm/L).

2.7.1.2. *d* Total ash content


*Procedure*

Evaporate 100 ml of toddy in a dried dish on a water bath. Heat the content until thoroughly charred. Place the dish in a muffle furnace maintained at 450 to 500 °C for about an hour. Cool the dish in desiccators and weigh.

Calculation

\[
\text{Ash content, percent (m/v)} = \frac{100(m_2 - m_1)}{V}
\]

Where

- \(m_2\) = mass of the dish with ash
- \(m_1\) = mass of empty dish
- \(V\) = volume in ml of toddy taken for ash determination.

2.7.1.2.e Water insoluble ash


*Procedure*

Transfer the ash to about 25ml of distilled water. Cover with a watch glass and boil for 5 minutes. Filter through an ash less filter paper wash the filter paper with hot water and filter through the same filter paper. Heat the content until thoroughly charred. Place the dish in a muffle furnace maintained at 450 to 500 °C for about an hour. Cool the dish in desiccators and weigh.
2.7.1.2. **Water soluble ash**


Water soluble ash = Total ash - water insoluble ash

2.7.1.2.g **Alkalinity of water soluble ash**

Estimated as per IS 3077(1972).

Procedure

Transfer the ash to about 25ml of distilled water. Cover with a watch glass and boil for 5 minutes. Filter through an ash less filter paper. Collect the filtrate. Wash the filter paper with hot water until the filtrate no longer turns red litmus blue and collect the washings in the same beaker. Reserve the entire filtrate for the determination of alkalinity of soluble ash.

2.7.1.2.h **Estimation of Phosphorous**


**Principle**

The technique used is the ascorbic acid method. Ammonium molybdate and antimony potassium tartrate react in an acid medium with dilute solutions of orthophosphate-phosphorus to form an intensely coloured antimony-phospho-molybdate complex. This complex is reduced to an intensely blue-coloured complex by ascorbic acid. The colour is proportional to the phosphorus concentration.

**Reagents**

1. **Reagent A**

   Dissolve 12 g of Ammonium molybdate and 0.291g of Antimony potassium tartarate in 250 ml and 100 ml water respectively.
Add both the solutions to 1000ml of 5N sulphuric acid taken in a 2000ml standard flask. Mix thoroughly and make up to 2L with distilled water

2. **Reagent B**

Dissolve 1.056g of Ascorbic acid AR in 200ml Reagent and mix well. This reagent shall be prepared fresh.

3. Phosphorous stock solution(100ppm):- Dissolve 0.445g sodium dihydrogen phosphate monohydrate or 0.439g Potassium dihydrogen phosphate in 500ml distilled water and add 25ml of 7N sulphuric acid to the solution. Make up this solution to 1000ml in a standard flask.

4. Working standard:- Dilute 5ml, 10ml, 15ml, 20ml, 25ml and 30ml of the stock solution to 100ml with distilled water so that working standard solutions of 5ppm, 10ppm, 15ppm, 20ppm, 25ppm and 30ppm are obtained.

**Procedure**

1. Preparation of calibration curve

   Pipette out 1ml each of all working standard solution of phosphorous to separate 25ml standard flask. Add 5ml Reagent B to all flasks and make up to 25ml with distilled water. A blank solution is also prepared using 1ml water instead of standard solution. Absorbance of solutions at 660nm is measured using reagent blank in a spectrophotometer and standard curve is prepared by plotting concentration in X axis and absorbance in Y axis.

2. Estimation of Phosphorous in Toddy

   Centrifuge a small quantity of toddy taken in a centrifuging tube at 5000rpm. Pipette out 0.5ml of clear supernatant liquid into 25ml standard flask and add 5ml Reagent B and dilute to 25ml using distilled water.

   After 30 minutes, the absorbance of the blue coloured solution is measured at 660nm. Concentration of Phosphorous is read out from the standard curve. Multiply the value by 2 and express in ppm.
2.7.1.2.i Estimation of Potassium and sodium

(Chatwal and Anand, 1995)

Using Atomic absorption Spectrometer

Principle

Atomic absorption spectroscopy is a spectroanalytical process used to determine the quantitative of chemical elements using the optical radiation absorption by free atoms within gaseous state. Atomic absorption utilizes the principle that each atom absorbs light at a specific wavelength. Therefore, at a specific wavelength the quantity of the absorbing element can be measured and is proportional to its concentration. It requires standards with known analyte content to establish the relation between the measured absorbance and the analyte concentration and relies therefore on the Beer-Lambert Law which is the linear relationship between absorbance and concentration of an absorbing species.

Beer Lamberts Law

\[ A = \varepsilon \times b \times c \]

A= absorbance (-)
\( \varepsilon \)= molar absorbtivity with units of \( \text{L mol}^{-1}\text{cm}^{-1} \)

b= path length of the sample (cuvette)

c= Concentration of the compound in solution, expressed in \( \text{mol L}^{-1} \)

Procedure

Centrifuge about 10 ml of toddy at 5000rpm for five minutes and make up 500 µl of supernatant clear liquid to 1000ml in standard flask with distilled water. Multipoint calibration curves for Potassium are prepared using appropriate concentration of standard solutions of each element in an Atomic Absorption Spectrometer. From the calibration curves, concentration of elements in the sample is calculated applying dilution factor and the value is expressed in gm/L.
2.7.1.2. Ascorbic acid

Ascorbic acid content in toddy was estimated as per British Pharmacopoeia 2003 page 163-164 after certain modifications.

**Principle**

The assay is based on oxidation-reduction titration. Iodine is a strong oxidizing agent. It oxidizes the ascorbic acid to dehydroascorbic acid and itself reduces to hydrogen iodide.

**Ascorbic Acid Oxidation**

![Chemical Reaction Diagram]

The reaction:

\[
I_2 + C_6H_8O_6 \rightarrow C_6H_6O_6 + 2H^+ + 2I^-
\]

Iodine  Ascorbic acid (vit C)  dehydroascorbic acid  hydrogen ion  iodide ion

**a. Reagents**

1. Iodine solution

0.5gm of Potassium iodide and 0.268 Potassium Iodate are dissolved in 200ml of distilled water and add 30ml of 3M sulphuric acid. Dilute to 500ml with distilled water.

2. Vitamin C solution

Dissolve 250mg vitamin C in 100ml distilled water and make up to 250ml with distilled water.

3. Starch solution

Dissolve 0.5gm starch in 100ml water, boil and cool.
c. **Standardization of vitamin C (Ascorbic acid)**

Pipette out 25 ml of vitamin C solution into a 250 ml conical flask and add 5 ml starch solution. Titrate this against the iodine solution taken in a burette and end point is the appearance of blue colour persisting after 20 seconds. Record the titer values and repeat the titration for concordant values (Vs).

d. **Estimation of Ascorbic acid in Toddy**

Pipette out 25 ml of toddy into a conical flask, add 5 ml of starch solution and titrate against the same iodine solution used in standardization of ascorbic acid. Record the titer values and repeat the titration for concordant values (V₁).

e. **Calculation**

1. Titre value for standardization - Vs
2. Quantity of ascorbic acid corresponds to 1 ml iodine - 25/Vs
3. Titer value for test sample - Vt
4. Ascorbic acid in 25 ml of sample (Wt) - 25/Vs x Vt
5. Ascorbic acid in 100 ml of sample - Wt x 4

2.7.1.2.k. **Detection for basic and acidic compounds**

Isolation and identification of Drugs by E.G.C Clarke

Acid, alkali and neutral extracts of 25 ml toddy is analyzed using TLC for the detection of basic and acidic compounds. 25 ml of toddy is made acidic with dil. H₂SO₄ and extract with 20 ml of solvent ether and collect ether extract after drying with anhydrous Sodium Sulphate. The aqueous solution is then made alkaline with ammonia and extract with 20 ml chloroform. The chloroform layer is separated and dried over anhydrous Sodium Sulphate. Dry both the ether and chloroform extracts and the residues are used for TLC analysis.
Adsorbant: silica gel G

2. Methanol: Ammonia (100:1.5)

Spray reagent 1. Dragendorff’s reagent for detection

Dragendorff’s reagent

a. Mix together 2g of bismuth subnitrate, 25ml of acetic acid and 100ml H₂O.
b. Dissolve 40g of KI in 100ml H₂O.

Mix together 10ml of (a), 10 ml of (b), 20ml of acetic acid and 100ml of H₂O.

2.7.1.2Detection of chloral hydrate

Detection of Chloral hydrate was done as per Indian Standard-Alcoholic drinks-Toddy-specification (IS 8538:1988)

Reagents

• Pyridine Solution, freshly distilled
• Sodium hydroxide, 40 %

Procedure

To 2 ml of toddy sample add 5 ml of freshly distilled pyridine and 3ml of sodium hydroxide. Mix the contents thoroughly. Keep in boiling water bath for 5 to 6 minutes. The presence of chloral hydrate shall be indicated by pyridine layer turning pink.

2.7.1.2mDetection of silicates(SiO₃²⁻)

AOAC, 1990 Official Methods of Analysis (15th edn)

Toddy sample is mixed with calcium fluoride and conc H₂SO₄. Warm the mixture by covering with a moistened glass plate. The presence of silicates is indicated by the formation of a white gelatinous precipitate on the glass plate.
2.7.1.2. Detection of sodium lauryl sulphate (SLS)

Indian Pharmacopoeia 1996 (P 698)

Sample is acidified with dil. HCl and add 5ml chloroform. Mix thoroughly and add two to three drops of methylene blue solution. Presence of deep blue colour in the chloroform layer indicates the presence of SLS.

2.7.1.2.a Detection and estimation of starch

(Clark’s Isolation and Identification of Drugs)

a. Iodine solution test

Dissolve 2gm of iodine and 3gm of potassium iodide in sufficient water to produce 100ml.

Add a few drops of the reagent to 1 ml of the sample. Development of blue colour indicates the presence of starch.

b. Estimation using colorimetric method.

Standard starch solution = 0.1gm/100ml

Pipet out 1.5 ml of standard starch solution. Add 1.5 ml distilled water and 600 µl of iodine solution and take the absorbance at 620 nm.

Similarly 1.5 ml of sample solution is mixed with 1.5 ml distilled water and 600 µl of iodine solution and take the absorbance at 620 nm. Calculate the concentration of starch from known standard. Blank is set as distilled water.

2.7.1.2.p Detection of Saccharin

Indian Pharmacopoeia 1996 (P 667)

Mix 20 ml of the sample with 20 mg of resorcinol and add 0.5ml of sulphuric acid and heated over a small flame, until a dark green colour is produced. Allow to cool and add 10ml of water and excess of 2 molar NaOH solution—a fluorescent green colour indicate the presence of saccharin. (Toddy sample is extracted with acid-ether and the test is done)
2.7.1.2. Detection of methyl alcohol


Chromotropic acid test

Principle

The method is based on quantification of classical chromotropic acid – formaldehyde violet spots developed on thin layer chromatography.

Reagents

1. Sodium Bisulphite, AR grade
2. Potassium permanganate solution
   - Dissolve 3 gm of potassium permanganate and 15 ml of phosphoric acid in 100ml distilled water.
3. Sodium salt of chromotropic acid solution - 5% aqueous solution.

Procedure

Take 1ml of distillate and dilute to 5ml using distilled water. Add 2ml potassium permanganate solution. Keep the test tube for 30 minutes. Add few crystals of sodium bisulphate till the disappearance of colour of the solution. Add 1ml of chromotropic acid and 15 ml of sulphuric acid. Heat the test tube on a hot water bath at 60 °C for 10 minutes. The development of violet red colour indicates presence of methyl alcohol.

2.7.2. Toxicity study of toddy adulterants.

Toxicity study of the adulterants like diazepam, phenobarbitone and chloral hydrate were evaluated in male Wistar rats. The rats were fed with natural toddy, shop toddy and different concentrations of the adulterants with toddy and adulterants alone. A control group of rats fed with normal diet were also maintained.
2.8 COLLECTION OF SERUM AND TISSUE SAMPLES

At the end of the experimental period (90 days) the animals except group I and II appeared weak. On the next day, the animals were deprived of food overnight, after recording their body weight were sacrificed by decapitation and blood was collected by cutting the jugular vein. The blood was allowed to clot and centrifuged at 3000 rpm for 20 minutes and serum was separated and kept on ice until use. Liver and kidney were excised and transferred to ice cold containers for different biochemical estimations. Brain was excised and kept in 3 % gluteraldehyde for transmission electron microscopic and scanning electron microscopic analysis.

2.9 BIOCHEMICAL TESTS

The sera from different groups of rats were subjected to biochemical estimation of different parameters such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma glutamyl transpeptidase (GGT), lactate dehydrogenase (LDH), acetyl cholinesterase, alcohol dehydrogenase, aldehyde dehydrogenase, total protein and albumin. For estimating the level of antioxidant enzymes, the liver and kidney tissues were homogenized and subjected to biochemical tests for thiobarbituric acid reactive substances (TBARS), conjugated dienes (CD), reduced glutathione (GSH), glutathione-s-transferase and catalase.

2.10 HISTOPATHOLOGY

Dissected livers were cut into small pieces and fixed in 10 % buffered formalin for histopathological analysis. The liver (5-6 mm thickness pieces) fixed in buffered formalin for 12 hours was processed for paraffin embedding using the micro technique procedure (Galigher et al., 1971). The tissue blocks were cut into 5 μm thick sections (Rotary microtome, Yorco, New Delhi). The paraffin embedded liver were stained with hematoxylin and eosin and examined for histopathological changes under the microscope (Motic A.E 21, Germany). The microphotographs were taken using Moticam 1000 digital camera at original magnification 100X.
2.11 TRANSMISSION ELECTRON MICROSCOPY

Dissected brain were washed in phosphate buffer (pH 7.2-7.4)

Glutaraldehyde fixed samples were dehydrated with grades of acetone, infiltrated and embedded in epoxy resin by polymerizing at 60°C for 3 days. The tissue block is further sectioned using an ultra microtome (Leica UCT) to a thickness of 50-70nm. The sections were then caught on Cu grids (EM Sciences), dried and viewed under transmission electron microscope

Procedure

Fixation

The tissue samples for transmission electron microscopy (TEM) grossed into 1 mm$^3$ size were fixed in 3 % gluteraldehyde in Phosphate buffer (Sorensen, pH 7.4)) for more than 24 hours at 4°C. Processing of the samples for TEM were started after washing the samples with phosphate buffer at 4°C, four changes of 10 minutes each. Thereafter samples were post-fixed in 1 % OsO$_4$ for 2 hours at 4°C. OsO$_4$ acts as a fixative cum primary stain. The samples were again washed in phosphate buffer, four changes of 15 minutes each and finally rinsed in distilled water for 5 minutes.

Dehydration

Dehydration of samples was carried out in the ascending grades of acetone in the following order.

- 50% acetone I -10 minutes in cold
- 50% acetone II -10 minutes in cold
- 70% acetone I -10 minutes in cold (stored overnight in Refrigerator)
- 70% acetone II -10 minutes at RT
- 90% acetone I -10 minutes at RT
- 90% acetone II -10 minutes at RT
- 100% acetone I -10 minutes at RT
100% acetone II -10minutes at RT

Dry acetone (acetone mixed with CuSO₄) -10 minutes

After dehydration samples were cleared in Propylene Oxide for 10 minutes.

**Infiltration**

Infiltration of sample with Polybed 812 (Epoxy resin – Polysciences Inc., USA) was carried out as follows.

Propylene Oxide: Resin in 3:1 ratio for 1½ hours

Propylene Oxide: Resin in 1:1 ratio for 1½ hours

Propylene Oxide: Resin in 1:3 ratio kept overnight in vacuum

The next day, samples were infiltrated in pure resin for 1 - 2 hours in vacuum.

**Embedding**

Samples were embedded in moulds containing resin (Poly/bed 812 mixed with dodecenyl succinic anhydride (DDSA – Hardener), Nadic methyl anhydride (NMA -Hardener), Dimethylaminomethyl phenol (DMP - Accelerator) in appropriate ratios as per the kit instructions (Polysciences Inc., USA) and placed in an oven at 60°C for 3 days for polymerization of the resin blocks.

**Ultra thin sectioning**

Once the area of interest was identified from the semi thin sections, the block surface was trimmed for ultra thin sectioning. Ultrathin sections with a thickness of 50 - 70 nm were cut using a diamond knife (Diatome®) and sections were taken on to the shiny side of the copper grid (300 mesh size).

**Staining for TEM**

The ultra thin sections were stained in Uranyl acetate, by immersing the grids with the section side up, for 2 hours. This was followed by washing the sections in methanol – water mixture (100 %, 80 %, 50 %). Thereafter the sections were stained for 10 minutes in Lead Citrate in 0.1 N NaOH (prepared in CO₂ free water). Grids were floated on the drop of the stain with the section side facing
down, placed in a carbon dioxide-free petri dish (by placing sodium hydroxide pellets). Thereafter grids were washed in four changes of distilled water and air dried. Stained ultrathin sections were observed under the electron microscope (Model Hitachi H-7650) at an accelerating voltage of 75 kV and photographs (Kodak Ilford film) were taken.

2.12 SCANNING ELECTRON MICROSCOPY

Dissected brains were washed in phosphate buffer (pH 7.2 - 7.4)

- Fix in 3% gluteraldehyde in phosphate buffer
- Again wash in phosphate buffer
- Dehydration
    - Tissues were put in 30% ethyl alcohol for 15 minutes, followed by
    - 50% ethyl alcohol for 15 minutes, followed by
    - 70% ethyl alcohol for 15 minutes, followed by
    - 90% ethyl alcohol for 15 minutes, followed by
    - 100% ethyl alcohol for 15 minutes.

Finally rinse in isoamyl acetate

- Critical Point Drying

To eliminate the final traces of water, critical point drying was done in a critical point drier using liquid carbon dioxide.

- Gold coating
- Sem observation

The gold coated specimens were observed under Scanning electron microscope.
2.13 STATISTICAL ANALYSIS

The data obtained were analysed for finding the variation between treated and control using one-way-ANOVA followed by tukey's post hoc analysis. The level of significance was set as \( p < 0.05 \).

2.14 PROCEDURES FOR BIOCHEMICAL ANALYSIS

2.14.1 Estimation of Liver Markers

2.14.1.1 Estimation of the activity of aspartate transaminase (AST) (EC 2.6.1.1) and alanine transaminase (ALT) (EC 2.6.1.2)

Activities of AST and ALT in serum were determined by the method of Mohum and Cook, 1957.

Reagents

- Phosphate buffer (100 mM) with \( \alpha \)-oxoglutaric acid (2 mM)
- Substrates (a) Aspartate transaminase. 100 mM L-aspartic acid was added to the above buffer.
  (b) Alanine transaminase. Prepare as in (a) but using 200 mM DL-alanine
- 2,4 Dinitro phenyl hydrazine (1 mM) in 1N HCl
- Sodium hydroxide (400 mM)
- Pyruvate standard (2 mM). Working standard-diluted 1 in 20.

Procedure

1 ml of substrate was pipetted into two tubes and placed in a water bath at 37 °C for a few minutes to reach its temperature. To one (test), 0.2 ml of serum was added and shaken gently to mix. Exactly 1 hr later in the case of AST and after 30 min for ALT, with the test tubes still in the bath, 1.0 ml dinitro phenyl hydrazine was added to both, and 0.2 ml serum to the other (control). Allowed to stand for 20 minutes at room temperature. 10 ml 0.4N NaOH was added to all tubes. Mixed well and absorbance was read at 520 nm after 5 minutes in a colorimeter. For
standard, 1 ml working standard was taken and made up to 1.2 ml with water and proceeded as above. For blank, 1.2 ml water was taken and proceeded as above.

2.14.1.2 Estimation of the activity of Alkaline Phosphatase (ALP) (EC 3.1.3.1).

The method described by Kind and King (1954) was used to assay alkaline phosphatase.

Principle

4-amino-antipyrene reacts with compounds containing phenolic groups in presence of an alkaline oxidizing agent to give a purple colour, which can be measured at 520 nm.

Reagents

- Disodium phenyl phosphate (0.01M)
- Sodium carbonate-sodium bicarbonate buffer(0.1M)
- Buffered substrate for use prepared by mixing equal volumes of above two solutions (pH=10)
- Standard phenol solution

Stock solution : 100mg of phenol per 100ml of solution

Working standard: The stock standard was diluted 1 in 10

- Sodium hydroxide 0.5 M
- Sodium bicarbonate 0.5M
- 4-amino anti-pyrine 0.6% in water
- Potassium ferricyanide 2.4g/100ml in water

Procedure

2ml of buffered substrate was measured into each of two test tubes and placed in a water bath at 37 °C for a few minutes. Then to the test 0.1ml of serum was added and incubated for exactly 15 minutes. It was removed from the bath and added 0.8ml of 0.5N NaOH and 1.2ml of 0.5 M sodium bicarbonate to both tubes
and then 0.1ml of sera was added to the second tube (blank). To both tubes, 1ml of amino anti-pyrine reagent and 1ml of potassium ferricyanide were added. For standard, 1.1 ml of buffer and 1ml of phenol standard containing 0.01 ml of buffer and 1ml of water were taken. Then to both tubes NaOH, bicarbonate, amino anti-pyrine and ferricyanide were added as above. Read at 520nm.

2.14.1.3 Estimation of the activity of γ-glutamyl transpeptidase (GGT) (EC 2.3.2.2).

The method of Naftalin et al., (1969) was used for the assay of GGT.

**Principle**

γ-glutamyl transpeptidase catalyzes the transfer of the γ-glutamyl moiety of a γ-glutamyl donor to the accepter L (γ-Glutamyl –p-nitroanilide) + γ-glutamyl-p-nitroanilide + p-nitroaniline. The formation of p-nitroaniline is determined readily from the increase in absorbance at 405 nm. The specific activity was expressed as units of enzyme activity per milligram of protein.

**Reagents**

- Buffer: Prepared by mixing Tris (120mM/L), MgCl₂ (90mM/L) and Glycyl-glycine (pH=7.8).
- Substrate: 1.28g L-γ-glutamyl 4-nitroanilide in 0.15mM/1N HCl and made to 100ml with acid.

**Procedure**

100 µl of serum or liver extract and 1ml of buffer were warmed to 37 °C. The reaction was initiated by adding 0.1ml of substrate and mixing well instantaneously. The reaction was monitored continuously at 405 nm in 1cm cuvette so as to obtain the change in absorbance per minute.

2.14.1.4 Estimation of lactate dehydrogenase(LDH) activity (EC 1.1.1.27)

LDH was estimated using the method of Wroblewski and Due, 1955)
Reagents

- Glycine Reagent: 7.505g glycine and 5.85g NaCl were dissolved in about 900 ml of distilled water and made upto 1 litre.

- Buffered substrate: 125 ml of glycine buffer and 75 ml of 0.1N NaOH were mixed, and then added into it 4g of lithium lactate/ sodium lactate. Mixed well and pH adjusted to 10.

- NAD⁺ Solution: 10 mg of NAD⁺ was dissolved in 2.14 g/dl of Nicotinamide solution.

Procedure

10 ml of buffered substrate, 0.2ml of NAD⁺ solution and 0.2ml of serum were pipetted into cuvette. The mixture was mixed thoroughly and then readings were taken at 340nm after 45 seconds and then at an interval of 1,2,3 minutes. The mean absorbance change per minute was determined.

2.14.1.5 Estimation of serum cholinesterase

The method of Hestrin (1949) was adopted for the assay of serum cholinesterase.

Principle

Cholinesterase are enzymes which hydrolyse esters of choline to give choline and acid. The ester which remains after incubation with the enzyme is converted quantitatively by alkaline hydroxyl amine into a hydroxamic acid and which is measured spectronhotometrically at 540 nm by means of the red-purple complex formed with FeCl₃ in acid medium.

Reagents

- Hydroxylamine hydrochloride 2M (stored in cold)
- Sodium hydroxide (3.5 N)
- Concentrated HCl
d. Ferric chloride reagent. 0.37 M Ferric chloride (FeCl₃·6H₂O) in 0.1 N Hydrochloric acid

e Standard solution. Acetylcholine chloride, 0.004 M in sodium acetate solution (0.001 N of pH 4.5)

f Alkaline hydroxyl amine reagent. Mixed equal volumes of reagent a & b

Procedure

1 ml of reaction mixture was taken to each of two tubes named test and control, and kept in a water bath at 37 °C for some time. Then added 0.2 ml of the enzyme extract/serum to the test and incubated both the tubes at 37 °C for 1 hr. 2 ml of alkaline hydroxyl amine reagent was added to both the tubes. Then added 0.2 ml of enzyme extract to the control. After 1 minute pH of the mixture brought to 1.2 by adding 6 ml of 0.5 N HCl, and mixed well. Centrifuged at 3000 rpm for 10 minutes. The supernatant was read at 540 nm. The unit of activity is expressed as µ moles of choline degraded/h/mg protein.

2.14.1.6 Estimation of total protein (TP)

The protein content was estimated by the method of Lowry et al., (1951).

Principle

Aromatic amino acids like tyrosine and tryptophan reduce the phosphomolybdic acid of Folin’s reagent (Folin Ciocalteau reagent) to give a blue colour. The intensity of the purple blue colour formed depends upon the amount of the amino acids present and is proportional to the amount of protein that was measured in spectrophotometer at 650 nm.

Reagents

- 0.4 M Tris HCl buffer pH 7.0
- 10% w/v Trichloroacetic acid (TCA)
- 0.1 N NaOH (7 gm of NaOH in 1750 ml distilled water)
- Alkaline Copper Reagent:
a) 25 gm Na₂CO₃ in 1250 ml 0.1N NaOH.

b) 125mg CuSO₄·5H₂O in 25 ml distilled water

c) 0.25 gm sodium potassium tartarate(1%) in 25 ml distilled water.

- Folin Ciocalteau reagent(1:1 dilution)
- Stock Standard BSA (100mg BSA in 100ml 0.1 N NaOH).
- Working standard BSA (5ml of stock standard was made upto 50ml with 0.1N NaOH.

**Procedure**

To 0.2ml of serum /tissue extract 1.8 ml of 0.1 N NaOH and 5ml of alkaline copper reagent were added and kept for 15 minutes. Then 0.5ml of diluted Folin-phenol reagent was added, mixed, kept for 30 minutes and read at 650 nm. To the blank, 0.2ml of water and to the standard , 0.2ml of working standard were added instead of serum/ tissue extract and treated as above.

**2.14.1.7 Estimation of albumin**

The albumin content was determined by the method of Doumas and Peters(1997).

**Principle**

Albumin binds with Bromocresol green (BCG) at pH 4.2 causing a shift in absorbance of the yellow BCG dye. The blue-green colour formed is proportional to the concentration of albumin present, when measured photometrically between 580-630 nm with maximum absorbance at 625nm.

**Reagents**

- Albumin reagent
  - a. Bromocresol green 0.08 m mol/L
  - b. Succinate buffer (pH 4.2± 0.1 at 25 °C) 50m mol/L
  - c. Sodium azide 1 gm/L

- Albumin standard 3.6 g/dl
**Procedure**

Pippete into tubes labeled test and standard 1ml each of albumin reagent add 10 µl of test sample and standard mix well and take the reading at 625nm after one minute at 37 °C.

\[
\text{Albumin} = \frac{\text{Absorbance of test} \times \text{conc. of standard (g/dl)}}{\text{Absorbance of standard}}
\]

**2.14.1.8 Estimation of aldehyde dehydrogenase (E.C.1.2.1.3) and alcohol dehydrogenase (E.C.1.1.1.1)**

Aldehyde dehydrogenase was assayed by the method of Tottmar et al.,(1973) and Alcohol dehydrogenase was assayed by the method described by Buettner (1965).

**Procedure**

5 g of hepatic tissue was homogenized in 20 ml of sucrose medium(0.25M sucrose, 5mM Tris-HCl, 0.5mM EDTA, pH 7.2) and the homogenate was used as the enzyme medium. Aldehyde dehydrogenase was assayed spectrophotometrically with aldehyde as the substrate by measuring the reduction of NAD$^+$ at 340 nm. By the method of Tottmar et al (1973).

The assay mixture contained 50 mM sodium pyrophosphate pH 8.8, 0.5 mM NAD$^+$ or 2.5 mM NADP$^+$, 0.1 mM pyrazole (to inhibit alcohol dehydrogenase) 0.05-5 mM acetaldehyde and 5 micro mole rotenone in methanol(to inhibit mitochondrial NADH oxidase). The reaction was started by the addition of the substrate. A blank was run simultaneously with the omission of the substrate.

One unit of aldehyde dehydrogenase is micro moles of NADH formed/min/g of protein.

Alcohol dehydrogenase was assayed as described by Buttner(1965). The activity was measured by following the addition of NADH spectrophotometrically with acetaldehyde as the substrate. The reaction mixture contained 50 mM KH$_2$PO$_4$, pH 7.4, 0.15mM NADH and 8Mm acetaldehyde. The reaction was
started by the addition of acetaldehyde. A blank was used with the omission of the substrate.

One unit of alcohol dehydrogenase is micro moles of NAD+ formed/min/g protein.

2.14.2 Estimation of Antioxidant Parameters

2.14.2.1 Estimation of reduced glutathione (GSH)

GSH was estimated by Ellmann’s method of Moron et al., (1979)

Principle

Reduced glutathione (GSH) was estimated by its reaction with dithio-bis-2-nitrobenzoic acid (DTNB) that gives an yellow coloured complex with absorption maximum at 412nm.

Reagents

- Phosphate Buffer 0.2M (pH-8.0)
- 25% w/v Trichloroacetic acid (TCA)
- 5% w/v Trichloroacetic acid (TCA)
- Dithio-bis-2-nitrobenzoic acid (DTNB) 0.6mM
- Standard Glutathione (GSH)- 5mg reduced glutathione was diluted to 50ml with distilled water.

Procedure

To 500 µl of the homogenate, 125 µl of 25% (w/v) TCA was added to precipitate proteins. The tubes were cooled on ice for 5 minutes and the mixture was further diluted with 600 µl of 5% TCA and centrifuged for 10 minutes. 300 µl of the resulting supernatant was taken for GSH estimation. The volume of the aliquot was made upto 1ml with 0.2 M phosphate buffer,pH 8.0 and 2ml freshly prepared DTNB (0.6mM ) was added to the tubes. The intensity of yellow colour formed was measured at 412nm. Values are expressed in mmol/mg tissue.
2.14.2.2 Estimation of Glutathione-S-Transferase (GST) (EC 2.5.1.18)

Glutathione-S-Transferase was analysed by the method of Habig et al.,(1974)

**Principle**

The enzyme is assayed by its ability to conjugate GSH and 1-chloro-2,4-dinitrobenzene(CDNB), the extent of conjugation causing a proportionate change in the absorbance at 340 nm.

\[ \text{GST} \]

\[
\text{Glutathione-SH} + \text{CDNB} \rightarrow \text{Glutathione-S-CDNB}
\]

**Reagent**

- Phosphate buffer : 0.5M, pH 6.5
- 1-Chloro-2,4 dinitrobenzene(CDNB) in 95% ethanol :25mM
- Reduced glutathione : 20mM

**Procedure**

The reaction mixture contained 200 µl phosphate buffer, 20 µl CDNB and 50 µl of tissue homogenate was made up to 1 mL with distilled water. The reaction mixture was pre- incubated at 37°C for 10 minutes. 50 µl of GSH was added and the change in absorbance was read at 340 nm for 3 min at 30 seconds intervals. To the blank, 50 µl of water was added instead of tissue homogenate.

2.14.2.3 Estimation of Catalase (EC 1.11.1.6)

The catalase activity was determined by the method of Machly and Chance, (1954)

**Principle**

\[
\text{CAT} \]

\[ 2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2 \]
The UV absorbance of H$_2$O$_2$ is measured between 230 and 250nm. On decomposition, H$_2$O$_2$ by catalase, the absorption decreases with time. The enzyme activity can be calculated from this decrease.

Reagents

- Phosphate Buffer 0.1 M pH 7.0
- H$_2$O$_2$ -30mM in phosphate buffer (340 µl H$_2$O$_2$ made upto 100mL with Phosphate buffer).

Procedure

Tissue was homogenized in 0.025 M TrisHCl buffer of pH 6.5 and centrifuged at 1500rpm for 10 minutes. The supernatant was used for the assay. To about 25 µl tissue extract 1ml of phosphate buffer of 0.1M, pH 7.0 and 250 µl of H$_2$O$_2$ was added. Change in optical density (at zero time, after 30 sec and after 60 sec) was measured at 240 nm. Specific activity is expressed as µmol of H$_2$O$_2$ consumed/min/mg protein.

2.14.2.4 Estimation of Thiobarbituric Acid Reactive Substances (TBARS)

TBARS was estimated by thiobarbituric acid assay method of Nichans and Samuelson, (1968).

Principle

In this method, malondialdehyde and other TBARS were measured by their reactivity with Thiobarbituric acid in the acidic condition to generate a pink coloured complex which was read at 535 nm.

Reagents

- Tris-HCl Buffer 0.25M, pH-7.5
- 15 % w/v Trichloroacetic acid.
- 0.25 N Hydrochloric acid.
- 0.375 % w/v Trichloroacetic acid in 0.25 N Hydrochloric acid.
TCA- TBA-HCl reagent- 125 mL. TBA in HCl is mixed with 125 ml 15% TCA.

Stock- Standard 4.8 mM solution was diluted to get a concentration of 48 mM/ml.

**Procedure**

The tissue homogenate was prepared in 0.1 µl of Tris HCl buffer (pH- 7.5). 1.0 ml of the tissue homogenate was combined with 2.0 ml of TCA-TBA-HCl reagent and mixed thoroughly. The solution was heated for 15 minutes in a boiling water bath. The flocculent precipitate was removed by centrifugation at 2000rpm for 10 minutes. The absorbance of the sample was read at 535 nm against a blank without tissue.

**2.14.2.5. Estimation of Conjugated Dienes (CD).**

Conjugated dienes was estimated according to the method of Beuje and Aust, (1978). This method is based on the arrangement of the double bonds in polyunsaturated fatty acids to form conjugated dienes with an absorbance maximum at 233 nm.

**Reagents**

- Chloroform
- Methanol
- Cyclohexane

**Procedure**

To 1ml of tissue homogenate 5 ml of chloroform-methanol reagent(2:1 v/v) was added, mixed thoroughly and centrifuged for 5 minutes. 3ml of lower layer was then evaporated to dryness. To this 1.5 ml of cyclohexane was added and the absorbance was read at 233nm against a cyclohexane blank.
2.15 IN VITRO CYTOTOXIC AND APOPTOTIC ASSAYS.

2.15.1 MTT assay (Mosmann, 1983)

The cell viability was assessed by MTT assay, which determines themetabolically active mitochondria of intact cells. The assay was carried out using MTT cell assay kit, following the protocols described by the manufacturer’s (HiMedia, India). Briefly, Chang liver cells were seeded in 96-well plates (Greiner, Frickenhausen, Germany) with $5 \times 10^3$ cells/100 µL and incubated for 24 h at 37°C. The cells were then treated with indicated concentrations of diazepam, phenobarbitone and chloral hydrate and DMSO (0.1% v/v) and incubated for another 24 h at 37°C in a 5% CO$_2$ atmosphere. The assay was performed by the addition of premixed MTT reagent, to a final concentration of 10% of total volume, to culture wells containing various concentrations of the test substance and incubated for further 4 h. During 4 h incubation, living cells converted the tetrazolium component of the dye solution into a formazan product. 100 µL of the solubilization solution (provided with the MTT assay kit) was then added to the culture wells to solubilize the formazan product and the absorbance at 570 nm was recorded using a 96-well plate reader (Bio-Rad, Hercules, CA, USA). The experiments were performed in triplicate. Percentage inhibition was calculated using the formula

$$\text{Percentage growth inhibition} = \left( \frac{(\text{Mean absorbance of the control cells}) - (\text{Mean absorbance of treated cells})}{\text{Mean absorbance of control cells}} \right) \times 100$$

2.15.2. JC-1 staining assay (Cossarizza et al.,1993)

Principle

JC-1 (the cationic dye-5,5’,6,6’ tetrachloro-1,1’,3-3’-tetraethylbenzimidazolylcarbocyanine iodide) is a reliable probe for the analysis of mitochondrial transmembrane potential changes occurring very early in apoptosis. It is a mitochondrial lipophilic dye and becomes concentrated in mitochondria in proportion to their membrane potential($\Delta \Psi_m$); more dye becomes accumulated in mitochondria with greater $\Delta \Psi_m$ and ATP generating capacity. Therefore, fluorescence of JC-1 can be considered as an indicator of mitochondrial energy state and the dye exists as a monomer at low concentrations giving green
fluorescence. J-aggregates are multimers of JC-1 formed inside intact mitochondria and their formation and fluorescence responds linearly to increase in mitochondrial membrane potential. Therefore, in JC-1 staining, the apoptotic cells were identified by an increase in green fluorescence and the loss of red fluorescence.

**Procedure**

The apoptotic potential of natural toddy, shop toddy, diazepam, phenobarbitone and chloral hydrate alone and in combination in chang liver cells were carried out by (JC-1) staining. The mitochondrial membrane potential ($\Delta \Psi_m$) was assayed using the cationic dye - JC-1 mitochondrial potential sensor (Invitrogen, USA), according to the manufacturer’s directions. Briefly, chang cells were incubated for 24 h in a 24-well plates (Greiner, Germany) and the cells were treated with indicated concentrations of diazepam, phenobarbitone and chloral hydrate. The treated cells were washed with PBS and incubated for 30 min in 10% RPMI medium without phenol red containing JC-1 at a concentration of 2.5 $\mu$g/mL. The cells were then examined and photographed using fluorescence microscope.