APPENDIX 1

Determination of pH

The pH was determined by AOAC official method of analysis (2005).

Principle

The pH is the measurement of $H^+$ ion activity and indicates acidity. It may be measured by determining electric potential between glass and reference electrodes, using commercial apparatus standardized against primary standard pH buffers.

Apparatus and reagents

1. pH meter
2. Standard buffer solutions: pH 4.0 buffer and pH 7.0 buffer.
4. Balance: With capacity of $\leq 2$ kg and sensitivity of 0.1 g.

Determination of pH:

Rinse and blot electrodes. Immerse electrodes in sample and read pH, letting meter to stabilize for 1 min. Rinse and blot electrodes and repeated on fresh portion of sample. Determine 2 pH values on each sample. Readings in close agreement indicated that sample was homogenous. Report the values to 2 decimal places.
APPENDIX 2

Determination of ash content

The ash content was determined by AOAC official method of analysis (2005).

Determination of ash content

Weighed accurately 5gm of crude sample and taken in a weighed petridish and heated in a furnace for 30 minutes at 500-550°C, break up the charred mass in petridish, added hot water, filtered through ashless paper and washed thoroughly with water. The contents in paper were returned to dish, dried and heated for 30 minutes at 525°C or until all carbon was burnt off. Cooled in desiccators and weighed. Repeated the process of heating, cooling and weighing at half hour interval till the difference in weight in two consecutive weighing was less than 1mg. Note the lowest weight.

Ash content was calculated by the formula:

\[
\text{Total ash content in } \% = \frac{W_2 - W}{W_1 - W} \times 100
\]

W2 – Weight of dish with ash in gram

W1 – Weight of dish with crude sample taken for test in gram

W – Weight of empty dish in gram
APPENDIX 3

Determination of moisture content

The moisture content was determined by AOAC official method of analysis (2005).

Determination of moisture content

Weighed accurately 5 gm of sample into a dried tarred metal dish (about 7.5 to 8 mm diameter provided with closely fitted lid) contained about 2 gm of finely divided glass fiber filter. Moistened with hot water, mixed thoroughly, evaporated almost to dryness on steam bath and dried for 6 hours in vacuum oven at 70 ± 1°C reduced pressure equal to 100 mm Hg. Admitted a slow current of dried air (dried by passing through H₂SO₄). Replaced the cover, cooled the dish in desiccators and weighed. The process of heating, cooling and weighing at half hour interval was repeated till the difference in weight in two consecutive weighing was less than 1 mg. Note the lowest weight.

Moisture content is calculated by the formula:

\[
\text{Moisture content in \% = } \frac{M1 - M2}{M1 - M} \times 100
\]

M2 – Weight of dish with dried material in gram

M1 – Weight of dish with material before drying in gram

M – Weight of empty dish in gram
APPENDIX 4

Phytochemical screening for secondary metabolites

The preliminary phytochemical screening of secondary metabolites was done by Harborne method (1973).

Alkaloids

1. Dragendorff’s test:

To 0.5 ml of alcoholic extract, added 2 ml of hydrochloric acid and 1 ml of Dragendorff’s reagent. Orange red precipitate or reddish brown color was obtained which showed the presence of alkaloids.

2. Wagner’s test:

To 10 ml of alcoholic extract, added few drops of 1.5% hydrochloric acid and Wagner’s reagent. Brown or yellow precipitate was obtained confirmed the presence of alkaloids.

3. Mayer’s test:

To 1 ml of acidic alcoholic solution of extract added few drops of Mayer’s reagent. Cream or white or pale precipitate formation confirms the presence of alkaloids.

4. Hager’s test:

To the extract, added few drops of Hager’s reagent. Alkaloid was confirmed by formation of yellow color precipitate.
Preparation of reagents for alkaloids

Dragendorff’s reagent: 8 g of bismuth nitrate was dissolved in 20 ml of nitric acid and 2.72 g of KI was dissolved in 50 ml water. The solutions were mixed and allowed to stand till potassium nitrate crystallizes out. The supernatant was diluted to 100 ml with distilled water.

Wagner’s reagent: 1.0 g of iodine and 2.0 g of potassium iodide was dissolved in 5 ml of sulfuric acid and solution was made up to 100 ml.

Mayer’s reagent: 1.36 g of HgCl$_2$ was dissolved in 60 ml distilled water and 5 g of potassium iodide was dissolved in 10 ml water. The above solutions were mixed and made to 100 ml with distilled water.

Hager’s reagent: Saturated picric acid solution.

Flavanoids

1. Shinoda test:

   To 0.5 ml of alcoholic extract added 5 - 10 drops of dilute hydrochloric acid and small piece magnesium or zinc and content was boiled for some minutes. Dirty brown color or reddish pink showed the presence of flavonoids.

2. With sodium hydroxide:

   To the extract added aqueous sodium hydroxide and then passed ammonia vapor. Yellow color was formed which confirmed the presence of flavonoids.
3. Lead acetate test:

To the extract, added 10% lead acetate solution. Formation of yellow precipitate showed the presence of flavonoids.

4. With conc. sulfuric acid:

To small portion of extract added concentrated sulfuric acid. Yellow to orange color formation confirmed the presence of flavones.

Glycosides

1. Legal’s test:

Added 1 ml of pyridine and some drops of sodium nitroprusside solution to methanolic extract and then the solution was made alkaline with NaOH. Blood red color or pink to red color appearance showed the presence of cardiac glycosides.

2. Borntrager’s test:

To the methanolic extract added 10% ferric chloride and 1 ml of concentrated HCl, heated in water bath, cooled and the solution was extracted with benzene. Benzene layer was separated and shaken with ammonia solution. Formation of rose pink to cherry red color in ammonical layer confirmed the presence of anthraquinone glycosides.

3. Extract was dissolved in 1ml water and then added aqueous sodium hydroxide and solution was extracted with benzene. Benzene layer was separated and shaken with ammonia solution. Yellow color formation showed the presence of glycosides.
4. Keller-kiliani test:

To 2 ml of extract added 1 ml of glacial acetic acid, few drops of ferric chloride and concentrated sulfuric acid. Appearance of greenish blue color confirmed the presence of cardiac glycosides.

Terpenoids and steroids:

Steroids:

The extract was dissolved in 5 ml of chloroform and this solution was used for the following tests.

1. Libermann buchard’s test:

To 1 ml of extract added 2 - 3 drops of acetic anhydride and 1 ml of chloroform, boiled and cooled and then added few drops of sulfuric acid along sides of test tubes. Formation of brown ring at junction of two layers showed the presence of steroids.

2. Salkowski test:

To 1 ml of chloroform solution added few drops concentrated sulfuric acid. Brown color was formed which indicated the presence of phytosterols.

Terpenoids:

1. Hirshorn test:

To 1 ml of the extract added 2 ml of trichloroacetic acid and heated and gives yellow color which changes to red showed the presence of terpenoids.
2. Lieberman starch morasky test:

To 1 ml of the extract, 3 ml of warm acetic acid was added with few drops of concentrated sulfuric acid. Color changes from red to blue indicated the presence of terpenoids.

Saponins, resins and thiols

1. Foam’s test:

The extract was diluted with 20 ml of water and it was agitated for 15 minutes in graduated cylinder and formation of 1 cm foam layer confirmed the presence of saponins.

2. To 5ml of extract few drops of sodium bicarbonate was added, shake vigorously and kept for 3 minutes. Formation of honey comb like froth indicated the presence of saponin.

3. To 2 ml of extract added 5 - 10 ml acetic anhydride, gentle heating, cooled and then added 0.5 ml of concentrated sulfuric acid leads to the formation of bright purple color showed the presence of resins.

4. To 0.5 ml of the extract added enough ammonium sulfate to saturate and then added 2-4 drops of 5% sodium nitro prusside and 1- 4 drops concentrated nitric acid. Magenta color formation occurs confirmed the presence of thiols.

Tannins and phenols

1. Lead acetate test:

To 1.5 ml of extract, added few drops of 10% lead acetate solution. White precipitate was obtained which confirmed the presence of tannins.
2. Gelatin test:

    To the extract added 1% Gelatin in 10% sodium chloride and white precipitate was obtained indicated the presence of tannins.

3. Ferric chloride test:

    To extract added 2 to 3 ml of 10% alcoholic ferric chloride. Dark blue or greenish gray or bluish black was obtained showed the presence of tannins.

4. The methanol extract was spotted on a filter paper and then added a drop of phosphomolybdic acid reagent and was exposed to ammonia vapor. Blue coloration on the spot confirmed the phenols.

Fixed oils and fats: (petroleum ether extract)

1. Spot test:

    Small amount of extract was separately pressed between two filter papers. Oil stain on paper appearance showed the presence of fixed oils.

2. Saponification test:

    Added few drops of 0.5N alcoholic KOH to small amount of extract and then added a drop of phenolphthalein. The solution was heated on water bath for 1 to 2 hours. Alkali neutralization occurs partially or soap formation indicated the presence of fixed oils and fats.
Carbohydrates:

A small amount of various solvent extracts were dissolved in 4 ml distilled water separately and then filtered. The filtrate was used for following tests for detection of presence of carbohydrate.

1. Molisch’s test:

   To the filtrate added 2 to 3 drops 1% alcoholic α-napthol and 2 ml of concentrated sulfuric acid was added along sides of test tubes. Red-violet ring appeared at junctions of two liquids confirmed the presence of carbohydrate.

2. Fehlings test:

   To the filtrate added 1 ml of Fehling’s solution A and B and the solution was heated on water bath and formation of red color precipitate indicated the presence of reducing sugar.

3. Benedict’s test:

   To the filtrate added 5 ml of Benedict’s reagent and heated for 5 minutes. Brick red color precipitate obtained showed the presence of reducing sugar.

4. Anthrone test:

   To the filtrate added 2 ml of anthrone reagent and green or blue color was obtained which confirmed the presence of carbohydrates.
APPENDIX 5

Assay of Alkaline phosphatase

Alkaline phosphatase was estimated by King and Armstrong method (1965).

Principle

Alkaline phosphatase hydrolyses a variety of phosphomonoester at alkaline pH (10.0) to liberate phenol and inorganic phosphate. The amount of phenol liberate is ionized to phenolate ion, under alkaline condition reacts with folins phenol reagent to give a blue-green complex which is measured at 660 nm.

Reagents

1. Carbonate – bicarbonate buffer 0.1 M, pH 10.0
2. Substrate: Phenyl phosphate disodium salt solution, 0.01 M
3. Folin Ciocalteau reagent
4. Sodium carbonate 10% (w/v)
5. Standard: 100 μg of phenol per 1 ml of distilled water.

Procedure

5 ml of buffered substrate was incubated at 37°C for 10 minutes. 0.2 ml of serum was added and incubated for 15 minutes. The reaction was arrested by adding 1.8 ml of Folin-Ciocalteau reagent and centrifuged for 15 minutes. After centrifugation, 2.0 ml of 10% sodium carbonate was added to the supernatant. The tubes were kept for incubation at 37°C for 10 minutes. A set of standards were also treated in the same manner. The color developed was read at 640 nm.

The enzyme activity was expressed as IU/L in serum.
APPENDIX 6

Assay of Aspartate transaminase

Aspartate transaminase was assayed by the method of Reitman and Frankel (1957).

Principle

The enzyme catalyses the following reaction:

L-Aspartate + α-Oxoglutarate → Oxaloacetate + L-Glutamate

The oxaloacetate is measured by the reaction with 2,4-dinitrophenylhydrazine giving a brown colored hydrazone after the addition of sodium hydroxide. The color developed is read at 520 nm using a spectrophotometer.

Reagents

1. Phosphate buffer: 0.1 M pH 7.4
2. Dinitrophenyl hydrazine (DNPH) color reagent
3. Sodium hydroxide 0.4 N
4. SGOT substrate: 2.66 g of Aspartic acid and 29.2 mg of α-keto glutarate was dissolved in 100 ml of phosphate buffer. The pH was adjusted to 7.5 with 1 N sodium hydroxide.
5. Standard pyruvate: 11 mg of sodium pyruvate was dissolved and made up to 100 ml with distilled water.
Procedure

0.5 ml of buffered substrate was incubated at 37°C for 5 minutes. To this 0.1 ml of serum was added, mixed well and incubated at 37°C for 60 minutes. To this 0.5 ml DNPH color reagent was added, mixed well and allowed to stand at room temperature for 20 minutes. Then added 5.0 ml of 0.4 N sodium hydroxide mixed well and allowed to stand at room temperature for 10 minutes. A set of standard pyruvate were also treated in a same manner. Absorbance was read at 520 nm against water blank.

Activity of AST was expressed as IU/L in serum.

APPENDIX 7
Assay of Alanine transaminase

Alanine transaminase was assayed by the method of Reitman and Frankel (1957).

Principle

The enzyme catalyses the following reaction:

L-Alanine + α-Oxoglutarate → Pyruvate + L-Glutamate

The pyruvate is measured by the reaction with 2, 4-dinitro phenyl hydrazine giving a brown colored hydrazone after the addition of sodium hydroxide. The color developed is read at 520 nm using a spectrophotometer.

Reagents

1. Phosphate buffer 0.1 M pH 7.4
2. Dinitrophenyl hydrazine color reagent

3. Sodium hydroxide - 0.4 N

4. SGPT Substrate:

    1.78 g of alanine and 29.2 mg of α-ketoglutarate was dissolved in 100 ml of phosphate buffer. The pH was adjusted to 7.5 with 1 N sodium hydroxide.

5. Standard pyruvate:

    11 mg of sodium pyruvate was dissolved and made up to 100 ml with distilled water.

Procedure

0.5 ml of buffered substrate was incubated at 37°C for 5 minutes. To this 0.1 ml of plasma was added, mixed well and incubated at 37°C for 60 minutes. To this 0.5 ml DNPH color reagent was added, mixed well and allowed to stand at room temperature for 20 minutes. Then added 5.0 ml of 0.4 N sodium hydroxide, mixed well and allowed to stand at room temperature for 10 minutes. A set of standard pyruvate were also treated in a same manner. Absorbance was read at 520 nm against water blank.

Activity of ALT was expressed as IU/L in serum.
APPENDIX 8

Estimation of Protein

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

Principle

Protein reacts with Folin’s phenol reagent to give blue colored complex. The color formed is due to the reaction of alkaline copper with the protein and reduction of phosphomolydate by tyrosine and tryptophan present the protein. The intensity of the color is proportional to the amount of these aromatic aminoacid which is read at 640 nm.

Reagents

1. TCA 10%
2. Sodium hydroxide 0.1 N
3. Alkaline copper reagent
   Solution A: 2% sodium carbonate was dissolved in 0.1 N sodium hydroxide.
   Solution B: 0.5% CuSo4 was dissolved in 1% sodium potassium tartarate.
   50ml of solution A and 50ml of solution B were mixed prior to use.
4. Folin’s phenol reagent
   Commercial reagent was diluted 1:2 with distilled water
5. Stock standard
   100 mg of bovine serum albumin was dissolved in 100 ml of water in a standard flask.
6. Working standard

10 ml of the stock standard was diluted to 100 ml to get a working standard containing 0.1mg/100ml.

Procedure

0.5 ml of tissue homogenate or serum was mixed with 0.5 ml of 10% TCA and centrifuged for 10 minutes. Supernatant was discarded and the precipitate was dissolved in 1.0 ml of 0.1 N NaOH. From this an aliquot was taken and made up to 1 ml with water. To this 4.5 ml of alkaline copper reagent was added and allowed to stand at room temperature for 10 minutes. 0.5 ml of Folin’s phenol reagent was added and the blue color developed was read after 20 minutes at 640 nm. A series of standards in the range of 20-100 μg were treated in a similar manner.

The concentration of protein was expressed as g/100 ml in serum and mg/g in tissues.

APPENDIX 9

Estimation of Bilirubin

Bilirubin was estimated by Malloy and Evelyn method (1973).

Principle

Vanden Bergh developed a color reaction for detecting a bilirubin. The undergoing principle is the conversion of bilirubin to purple colored azobilirubin when coupled with diazotized sulphanilic acid.
Reagents

1. Diazo reagent

   Solution A: Dissolved 100 mg of sulphanilic acid in 1.5 ml of concentrated hydrochloric acid and made up to 100 ml with distilled water.

   Solution B: Dissolved 500 mg of sodium nitrite in 100 ml of water.

   10 ml of solution A was mixed with 0.3 ml of solution B just prior to use.

2. 15% hydrochloric acid

3. Standard bilirubin (100 µg/ml)

   10 mg of bilirubin was dissolved in 100 ml of chloroform.

Procedure

0.2 ml of serum and 1.8 ml of distilled water was taken in two test tubes marked as test and blank and add 2.5 ml of methanol into test tubes. Then 0.5 ml of diazo reagent to test and 0.5 ml of hydrochloric acid to blank was added. 0.1 to 0.4 ml of bilirubin was taken as standards and made up to 2 ml of distilled water. Then 2.5 ml of methanol and 0.5 ml of diazo reagent was added. All the tubes were allowed to stand for 30 minutes at room temperature and read at 540 nm against water as blank.

   The amount of bilirubin was expressed as mg/dl.
APPENDIX 10

Assay of Lipid peroxidation

TBARS were estimated by thiobarbituric acid assay method of Ohkawa et al (1979).

Reagents

1. Tris-HCl buffer (pH 7.5; 0.025 M)
2. TBA-TCA-HCl reagent (1:1:1 v/v)
3. Trichloroacetic acid: 15% w/v
4. 0.375% W/V thiobarbituric acid in 0.25 N HCl
5. Standard (1,1',3,3'-Tetramethoxy propane)

0.16 ml of 3 M solution of standard tetramethoxy propane was made up to 100 ml with double distilled water. 1.0 ml of this was taken and made up to 100 ml with double distilled water, which served as working standard.

Procedure

The tissue homogenate was prepared in Tris-HCl buffer (pH 7.5; 0.025 M). To 1.0 ml of the tissue homogenate, 2.0 ml of TBA–TCA–HCl reagent was added and mixed thoroughly and kept in boiling water bath for 15 minutes. The flocculent precipitate was removed by centrifugation at 1000 g for 10 minutes. A series of standard solution in the concentration of 2-10 nmoles were treated in a similar manner. The absorbance of the sample was read at 535 nm against a reagent blank.

TBARS were expressed as nmoles of MDA formed/mg protein in tissue.
APPENDIX 11

Assay of Catalase

The catalase activity was assayed by the method of Sinha (1972).

Principle

Dichromate in acetic acid is converted to perchromic acid and then to chromic acetate, when treated with hydrogen peroxide. The catalase preparation was allowed to split hydrogen peroxide for different period of time. The reaction was stopped at different time intervals by the addition of dichromate/acetic acid mixture and the remaining hydrogen peroxide as chromic acetate is determined colorimetrically at 620 nm.

Reagents

1. Phosphate buffer (0.01 M; pH 7.0)
2. Hydrogen peroxide - 2 M
3. Potassium dichromate - 5% (w/v)
4. Dichromate - acetic acid reagent

5% potassium dichromate and glacial acetic acid were mixed in the ratio 1:3 (v/v).

5. Standard H₂O₂ - 2 μmoles/ ml

Procedure

To 1.0 ml of phosphate buffer, 0.1 ml enzyme preparation and 0.4 ml hydrogen peroxide were added. The reaction was stopped at 30 seconds by the addition of 2.0 ml dichromate: acetic acid reagent. The tubes were kept in boiling
water bath for 10 minutes, cooled and read at 620 nm. A system devoid of enzyme served as control. A series of standards ranging from 20-100 μmoles of H₂O₂ were also processed as above along with blank containing distilled water.

Activity of catalase was expressed as nmoles of hydrogen peroxide liberated/min/mg protein.

APPENDIX 12
Assay of Superoxide dismutase

Superoxide dismutase (SOD) activity was assayed by the method of Kakkar et al (1984).

Principle

The assay of SOD based on the inhibition of formation of NADH phenazine methosulfate nitroblue tetrazonium formazon. The reaction is initiated by the addition of glacial acetic acid. The color formed at the end of the reaction is extracted into the butanol layer and measured at 560 nm.

Reagents

1. Sodium pyrophosphate buffer (0.025 M; pH8.3)
2. Phenazine methosulphate - 186 μM
3. Nitroblue tetrazolium - 300 μM
4. NADH - 780 μM
5. Glacial acetic acid
6. n-butanol
7. Chloroform

8. Ethanol

Procedure

0.5 ml of tissue homogenate was diluted to 1 ml with water. Then 2.5 ml of ethanol and 1.5 ml chloroform (all reagents chilled) were added. This mixture was shaken for 1 minute at 4°C and then centrifuged. The enzyme activity in the supernatant was assayed.

The assay mixture contained 1.2 ml of sodium pyrophosphate buffer (0.025 M, pH 8.3), 0.1 ml of 186 μM phenazine methosulphate, 0.3 ml of 300 μM nitroblue tetrazolium, 0.3 ml of 780 μM NADH, enzyme preparation and water in a total volume of 3.0 ml. Reaction was started by the addition of NADH. After incubation at 30°C for 90 seconds the reaction was stopped by the addition of 1.0 ml glacial acetic acid. The reaction mixtures were stirred vigorously and shake with 4.0 ml of n-butanol. The intensity of the chromogen in the butanol was measured at 560 nm against butanol blank. A system devoid of enzyme served as control.

The activity of SOD was expressed as 50% inhibition of NBT reduction/minute/mg protein.
APPENDIX 13
Assay of Glutathione peroxidase

Glutathione peroxidase was assayed by the method of Rotruck et al (1973).

Principle

A known amount of enzyme preparation is allowed to react with hydrogen peroxide in the presence of GSH for a time period and then the remaining GSH content is measured at 412 nm.

Reagents

1. Phosphate buffer [0.4 M; pH 7.0]
2. Sodium azide solution - 10 mM
3. Trichloroacetic acid (10%) (w/v)
4. EDTA solution - 0.4 mM
5. Hydrogen peroxide solution - 0.2 mM
6. Glutathione solution - 2 mM
7. Ellman’s reagent

19.8 mg of 5,5′-dithiobisnitrobenzoic acid (DTNB) in 100 ml of 0.1% sodium citrate

Procedure

To 0.2 ml of buffer, 0.2 ml EDTA, 0.1 ml sodium azide, 0.2 ml tissue homogenate (homogenized in 0.4 M phosphate buffer, pH 7.0) were added. To this mixture, 0.2 ml glutathione, followed by 0.1 ml hydrogen peroxide were
added. The contents were mixed well and incubated at 37°C for 10 minutes along with a control tube containing all reagents but no enzyme. After 10 minutes, the reaction was arrested by the addition of 0.4 ml 10% TCA. The tubes were centrifuged and the supernatant was assayed for glutathione content by using Ellman’s reagent.

The activity of glutathione peroxidase was expressed as µmole of GSH utilized/min/mg protein.

**APPENDIX 14**

**Estimation of Reduced glutathione**

Reduced glutathione was determined by the method of Ellman (1959).

**Principle**

Glutathione reacts with DTNB which gives yellow color and measured at 412 nm.

**Reagents**

1. Phosphate buffer (0.2 M; pH 8.0)
2. Trichloroacetic acid 5% (w/v)
3. Ellman’s reagent
   
   19.8 mg of DTNB was dissolved in 100 ml of 0.1% sodium citrate.
4. Standard glutathione
   
   10 mg of GSH was dissolved in 100 ml of distilled water.
Procedure

A known weight of tissue was homogenized in phosphate buffer (pH 8.0; 0.2 M). 0.5 ml of tissue homogenate or plasma was pipetted out and precipitated with 2.0 ml of 5% TCA. 1.0 ml of the supernatant was taken after centrifugation and 0.5 ml of Ellman’s reagent and 3.0 ml of phosphate buffer were added to it. The yellow color developed was read at 412 nm. A series of standards were treated in a similar manner along with a blank containing 3.5 ml of buffer.

The amount of glutathione was expressed µg/mg protein in tissue.

APPENDIX 15

Estimation of Vitamin C

Vitamin C was estimated by the method of Omaye et al (1979).

Principle

Ascorbic acid is oxidized by copper to form dehydroascorbic acid and diketoglutaric acid. These products are treated with 2,4 dinitrophenyl hydrazine to form the derivative of bis 2,4 dinitrophenyl hydrazine. This compound in strong sulfuric acid undergoes a rearrangement to form a product with an absorption band that is measured at 520 nm. The reaction is run in the presence of thiourea to provide a mildly reducing medium which helps to prevent interference from non-ascorbic acid chromogens.

Reagents

1. 5% TCA
2. 65% Sulfuric acid
3. DTCS reagent: 3 g of 2,4 dinitrophenyl hydrazine, 0.4 g thiourea and 0.05 g copper sulfate were dissolved in 9 N sulfuric acid and made up to 100 ml with the same.

4. Standard solution:

Ascorbic acid was prepared in the range of 4-20 µg/ml in 5% oxalic acid.

Procedure

1.0 ml of 10% homogenate was precipitated with 5% ice cold TCA and centrifuged for 20 minutes at 3500 rpm. 1.0 ml of supernatant was mixed with 0.2ml of DTCS reagent and incubated for 3 hours at 37°C. Then 1.5 ml of ice cold 65% sulfuric acid was added, mixed well and allowed to stand at room temperature for 30 minutes. A set of standards containing 20-100 µg of ascorbic acid were taken and processed similarly, along with a reagent blank. The color developed was read at 540 nm.

Values were expressed as mg/dl in serum.

APPENDIX 16

Estimation of Glucose

Glucose was estimated by the method of Trinder (1969).

Principle

Glucose is oxidized by glucose oxidase to give D-Gluconic acid and hydrogen peroxide. In the presence of peroxidase, hydrogen peroxide oxidatively couples with 4-amino antipyrene and phenol to produce red quinoneimine dye. The
intensity of color is directly proportional to the glucose concentration in the sample.

\[
\text{D-Glucose + H}_2\text{O + O}_2 \xrightarrow{\text{GOD}} \text{D-Gluconic acid + H}_2\text{O}_2
\]

\[
\text{H}_2\text{O}_2 + 4\text{-aminoantipyrene + phenol} \xrightarrow{\text{POD}} \text{Red Quinonemine dye + H}_2\text{O}
\]

Reagents

1. Stock standard: Dissolved 10 g of dextrose in enough distilled water that was saturated with benzoic acid (3 g/liter) to make 1 litre of solution.

2. Working standard: Prepared dilutions of stock glucose standard with saturated aqueous benzoic acid solution to give standards containing 250, 500, 750, 1000 mg of glucose/ litre.

3. Working solution:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer Ph 7.0</td>
<td>170 mM</td>
</tr>
<tr>
<td>Glucose oxidase</td>
<td>15000 IU/L</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>1500 IU/L</td>
</tr>
<tr>
<td>4-aminoantipyrene</td>
<td>0.28 mM</td>
</tr>
<tr>
<td>Phenol</td>
<td>16 mM</td>
</tr>
</tbody>
</table>

The required amount of working solution was pre-warmed at room temperature before use.
Procedure

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working enzyme reagent</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10.0 µl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glucose standard</td>
<td>-</td>
<td>10.0 µl</td>
<td>-</td>
</tr>
<tr>
<td>Serum</td>
<td>-</td>
<td>-</td>
<td>10.0 µl</td>
</tr>
</tbody>
</table>

The assay mixture was mixed well and incubated at 37°C for 15 minutes and absorbance was measured at 505 nm.

Calculation

\[
\text{Glucose in mg %} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 100
\]

Blood glucose values were expressed as mg/dl.

APPENDIX 17

Estimation of Cholesterol

The cholesterol was estimated by the method of Zak’s et al (1953).

Principle

Cholesterol oxidation is done by addition of acid solution of ferric chloride to precipitate the proteins and subsequent addition of sulfuric acid forms a reddish purple colored complex. The intensity of color is proportional to the concentration of cholesterol and read at 560 nm.
Reagents

1. Stock Cholesterol standard: 1 mg cholesterol per 1 ml glacial acetic acid.
2. Stock ferric chloride reagent: 640 mg of FeCl₃ in 100 ml of glacial acetic acid.
3. Ferric chloride precipitating reagent: 10 ml of stock FeCl₃ was diluted to 100 ml of glacial acetic acid.
4. Ferric chloride diluting reagent: 85 ml of stock FeCl₃ was diluted to 100 ml of glacial acetic acid.
5. Working Cholesterol: 5 ml of stock cholesterol was made up to 100 ml with ferric chloride diluting reagent.

Procedure

0.1 ml of serum was added to 4.9 ml of ferric chloride precipitating reagent. Mixed well using a glass rod and centrifuged for 15 minutes. To 2.5 ml of supernatant added 2.5 ml of ferric chloride diluting reagent and then added 4 ml of concentrated sulfuric acid and mixed thoroughly. Various concentration of working standard of cholesterol ranging from 0.5 to 2.5 ml were taken and treated in similar manner as test. The color developed was read at 560 nm.

The amount of cholesterol was expressed as mg/dl.
APPENDIX 18

Estimation of Triglycerides

The triglyceride was estimated by the method of Foster and Dunn (1973).

Principle

The triglyceride is extracted by isopropanol which upon saponification with potassium hydroxide yield glycerol and soap. The glycerol liberated is treated with metaperiodate which releases formaldehyde, formic acid and iodide. The formaldehyde released reacts with acetyl acetone and ammonia forming a yellow colored compound which is read at 405 nm spectrophotometrically.

Reagents

1. Isopropanol

2. Alumina, activity grade 1 for chromatography was washed with water and dried in an oven overweight.

3. Saponifying agent: 50 g of potassium hydroxide was dissolved in 600 ml water and 400ml isopropanol.

4. Sodium metaperiodate reagent: 77 g anhydrous ammonium acetate was dissolved in 700 ml water, 60 ml glacial acetic acid and then 650 mg sodium metaperiodate was added and made up to a litre with water.

5. Acetyl acetone reagent: 7.5 ml acetyl acetone was added to 200 ml isopropanol, mixed and then added 800 ml water.

6. Standard triolein: 1 g triolein was dissolved in 100 ml isopropanol.

7. Working standard: 1 ml of stock standard was diluted to 100 ml isopropanol.
Procedure

To an aliquot of serum added 4 ml of isopropanol, mixed well and then added 400 mg washed alumina. This was placed in a mechanical rotor for 15 minutes and then centrifuged. To 2 ml of supernatant, 0.6 ml potassium hydroxide was added, stoppered and incubated at 60-70°C for 15 minutes. Cooled and added 1 ml of metaperiodate solution and 0.5 ml acetyl acetone reagent. Mixed, stoppered and incubated at 50°C for 30 minute. A series of standards at concentration 8 to 40 µg triolein were treated similarly along with blank containing only the reagents. Cooled and read at 405 nm against a reagent blank.

The triglyceride content was expressed as mg/dl in serum.

APPENDIX 19
Estimation of Glycogen

The glycogen content was estimated by using anthrone reagent by Carrol et al (1956).

Principle

Glycogen is treated with 45% alcohol to remove glucose. Glucose is dehydrated by sulfuric acid to furfural derivative which then complex with anthrone to give green colored complex which is read at 620 nm.

Reagents

1. Extraction of glycogen:

100 mg of liver tissue was minced thoroughly in a beaker kept at 0°C. The minced liver was then homogenized with 5% TCA (2-3 ml/g tissue). The
homogenate was centrifuged at 3000 rpm for 10 minutes in cold. The supernatant was collected and re-homogenized the sediment with half the volume of TCA and centrifuged in cold. To the supernatant added twice the volume of 45% ethanol and kept overnight for glycogen precipitation. The precipitate was collected by centrifugation and dissolved in minimal volume of water and re-precipitated as before by adding twice the volume of ethanol. The precipitate obtained was again washed with ethanol and dissolved in 5 ml of water.

2. Anthrone reagent:

    200 mg of anthrone was dissolved in 100 ml of concentrated sulfuric acid.

3. Stock standard:

    100 mg of glucose was dissolved in 100 ml of distilled water.

4. Working standard:

    10 ml of stock standard was diluted to 100 ml with distilled water.

Procedure

To 0.5 ml of sample added 0.5 ml of distilled water. Glucose standard was taken ranging from 0.2 to 1.0 ml and made up to 1 ml with distilled water. Then added 4.0 ml of anthrone reagent to all the tubes and heated in a boiling water bath for 8 minutes, cooled and read at 620 nm.

The amount of glycogen was expressed as mg/g tissue.
APPENDIX 20

Short term in vitro anticancer activity

Short term in vitro anticancer activity was investigated by using Dalton’s Lymphoma Ascites (DLA) cells by method of Gupta (2002).

This test relies on the breakdown in membrane integrity determined by the uptake of a dye such as (tryphan blue, erythrorisine and nigrosin) to which the cell is normally impermeable.

Requirements

1. Mice
2. Dalton’s Lymphoma Ascites (DLA) cells
3. Drug dilutions
4. Haemocytometer
5. Centrifuge
6. Tryphan blue dye
7. Hank’s Balanced Salt Solution (HBSS)

Procedure

1. DLA cells were cultured in peritoneal cavity of mice by injecting intraperitoneally a suspension of DLA cells (1.0x 10^6 cells/ml).
2. The DLA cells were withdrawn from the peritoneal cavity of the mice between 15-20 days with the help of sterile syringe.
3. The cells were washed with HBSS and centrifuged for 10-15 minutes at 10,000 rpm. The procedure was repeated thrice.

4. The cells were suspended in known quantity of HBSS and the cell count was adjusted to 2x10^6 cells/ml.

5. The diluted cell suspension was distributed into Eppendorff tubes (0.1 ml containing 2x10^6 cells)

6. The cells were exposed to Decalepis hamiltonii and incubated at 37°C for 3 hours.

7. After 3 hours, dye exclusion test i.e. equal quantity of the drug treated cells and tryphan blue (0.4%) were mixed and left for a minute. It was then located in a hemocytometer and viable and non-viable count was recorded within two minutes. If kept longer, live cells also generated and take up color. Viable cells do not take up color, whereas dead cells take up color.

The % growth inhibition was calculated by using the following formula:

$$\text{% Growth inhibition} = 100 - \left( \frac{\text{Total Cells} - \text{Dead Cells}}{\text{Dead cells}} \right) \times 100$$
APPENDIX 21

In vitro Cytotoxicity activity

In vitro cytotoxicity activity was determined by MTT assay by Mossman method (1993).

Principle

The principle of this assay is tetrazolium salt 3-(4,5 dimethyl thiazole-2yl)-2,5-diphenyltetrazolium bromide (MTT) is cleaved to blue colored formazan catalyzed by succinate dehydrogenase, a mitochondrial enzyme. Number of cell is found to be proportional to degree of formazan production used by the cells.

Requirements

1) Confluent mono layer cell cultures
2) F-12 Conn’s medium with antibiotics
3) New born calf serum
4) Micro titer plate (96 wells)
5) MTT (prepared in HBBS without phenol red, 2mg/ml)
6) Propanol

Procedure

1. TPVG was used to trypsinize the monolayer cell culture and cell count was adjusted to $1.0 \times 10^5$ cells per ml using 10% of serum of new born calf as medium.
2. 0.1 ml of diluted cell suspension (roughly 10,000 cells) was added to each well of micro titer plates which contain 96 wells.

3. After the formation of partial monolayer i.e., after 24 hours supernatant was removed and monolayer was washed once and then added 100 µl of methanolic extract of (1000 to 15.6 µg/ml) Decalepis hamiltonii to cells in micro titer plates. Then the plates were incubated in 5% CO₂ atmosphere for 3 days at 37°C and microscopic examination was carried out and observations were recorded for every 24 hours.

4. Drug solution in wells were removed after 72 hours and then added 50 µl of MTT to each well (MTT was prepared in Hank’s balanced salt solution without phenol red) [(HBSS-PR), 2 mg per ml, Sigma Chemicals].

5. Plates were gently shaken and then incubated in CO₂ atmosphere at 37°C for 3 hours.

6. The supernatant was discarded and added 50 µl of propanol and then the plates were shaken gently for solubilization of formed formazan.

7. Absorbance was read at 540 nm by using micro titer reader (ELISA Reader, Biored).

Percentage growth inhibition was calculated using the following formula

\[
\text{% Growth inhibition} = 100 - \left( \frac{\text{Test OD} - \text{Blank OD}}{\text{Control OD} - \text{Blank OD}} \right) \times 100
\]
APPENDIX 22

Estimation of Hemoglobin

The hemoglobin content was estimated by Sahli’s method of D’Armour et al (1965).

Principle

The Sahli method is based on converting hemoglobin to acid hematin and then visually matching its color against a solid glass standard. Dilute hydrochloric acid is added to a graduated cylinder containing a blood sample until the color of the diluted blood sample matches that of the glass standard. The quantity of dilute acid added will be determined by the hemoglobin level of the blood sample.

Requirements

1. Sahli hemoglobinometer (solid glass standard and a calibrated graduated cylinder)

2. Sahli blood pipette (calibrated to 0.02 ml)

3. Small glass rod for stirring or wooden applicator sticks if glass rods are not available

4. Dropper for adding the hydrochloric acid

5. Dilute 0.1 M hydrochloric acid

6. Detergent
Procedure

1. Fill the Sahli graduated cylinder to the 2 g mark with dilute 0.1 M hydrochloric acid (approximately 0.15 ml).

2. Clean the fingertip with cotton wool soaked with 70% alcohol. The alcohol was allowed to dry. A drop of blood was obtained by puncturing the fingertip with a sterile lancet. The first drop of blood was wiped away.

3. Draw the blood to the 0.02 ml mark using the Sahli blood pipette. Don’t pipetted out by mouth.

4. Wiped any residual blood from the exterior of the pipette. Recheck that the blood still reaches the 0.02 ml mark.

5. Added blood to dilute acid and mixed the blood and acid thoroughly by flushing the pipette several times. The acid/blood mixture was allowed to stand for ten minutes.

6. Diluted the solution by adding few drops of distilled water at a time carefully until color of reaction mixture matches with glass plate in the comparator.

7. The level of fluid was noted at its lower meniscus and the reading corresponding to this level on the scale was recorded in gram/dl.
APPENDIX 23

Enumeration of Red blood cells

The red blood cells were enumerated by hemocytometry method of D’Armour et al (1965).

Principle

The blood specimen is diluted 1:200 with RBC diluting fluid and cells are counted under high power (40X) objective by a counting chamber. The number of cells in undiluted blood is calculated as number of red cells/ cu mm (µl) of whole blood.

Requirements

1. Neubauer’s chamber with cover slip
2. Red cell pipette.
3. Microscope.
4. Diluting fluid

   A. Trisodium citrate solution:

   Trisodium citrate - 3.8 gm
   Formalin - 1 ml
   Distilled water - 99 ml

   OR

   B. Hayem’s fluid:

   Mercuric chloride - 0.5 gm
Sodium chloride - 1 gm
Sodium sulphate - 5 gm
Distilled water - 200 ml

Procedure

1. Draw blood to 0.5 mark in RBC pipette without letting any bubbles into the pipette by holding the pipette almost horizontally. The pipette must be clean and dry.

2. Wiped the tip clean and drawn the diluting fluid up to the mark 101 (dilution 1 in 200) while filling the bulb, the pipette should be gently rotated to obtain good mixing.

3. The cover slip was placed over the Neubauers chamber so as to cover both ruled platforms evenly.

4. Mixed the contents in pipette and discarded few drops from pipette.

5. By holding the pipette at angle 45° and touching the space between the cover slip and chamber and an appropriate drops of the mixture was allowed to run under the cover slip by capillary action.

6. Allowed the cells to settle for 2 to 3 minutes.

7. The RBC was counted in the four corner squares and a center square which contained totally 80 small squares. Do not count the cells touching the lower and right hand lines but count the cells touching the upper and left hand lines.
Calculation

\[
RBC \text{ count} = \frac{\text{Number of cells counted (N) } \times \text{ Dilution}}{\text{Area counted } \times \text{ depth of fluid}}
\]

\[
RBC \text{ count} = \frac{N \times 200}{1/5 \times 1/10}
\]

\[= 10000 \times \text{No. of cells/cu.mm}\]

**APPENDIX 24**

**Enumeration of White blood cells**

The white blood cells were enumerated by Wintrobe method (1961).

**Principle**

The glacial acetic acid lysis the red cells while gentian violet stains the nuclei of the leucocytes. The blood sample id diluted 1:20 in a WBC pipette with the diluting fluid and the cells are counted using a counting chamber. The number of cells in undiluted blood is reported per cumm (µl) of whole blood.

**Requirements**

1. Neubauer's chamber with cover slip
2. WBC pipette.
3. Microscope.
4. Diluting fluid
   - Glacial acetic acid - 2 ml
1% w/v Gentian violet - 1 ml
Distilled water - 97 ml

Procedure
1. Draw blood to 0.5 mark in WBC pipette without letting any bubbles into the pipette by holding the pipette almost horizontally the pipette must be clean and dry.
2. Wiped the tip clean and drawn the diluting fluid up to the mark 11 (dilution 1 in 20) while filling the bulb, the pipette should be gently rotated to obtain good mixing.
3. The cover slip was placed over the Neubauers chamber so as to cover both ruled platforms evenly.
4. Mixed the contents in pipette and discarded few drops from pipette.
5. By holding the pipette at angle 45° and touching the space between the cover slip and chamber and an appropriate drops of the mixture was allowed to run under the cover slip by capillary action.
6. Allowed the cells to settle for 2 to 3 minutes.
7. The WBC was counted in four ‘W’ marked areas, each having 16 small squares.

Calculation

\[
\text{WBC count} = \frac{\text{Number of white cells counted (N) \times Dilution}}{\text{Area counted \times depth of fluid}}
\]
ANTIBACTERIAL AND PHYTOCHEMICAL STUDIES OF VARIOUS EXTRACTS OF ROOTS OF DECALEPS HAMILTONII WIGHT AND ARN

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ABSTRACT

In the present study, the various root extracts of Decalepis hamiltonii were screened phytochemically for the presence of secondary metabolites and for in vitro antibacterial activity respectively. The in vitro antibacterial activity of the various extracts of Decalepis hamiltonii was studied against Escherichia coli, Klebsiella pneumonia, Salmonella typhi, Proteus mirabilis, Vibrio cholera, Shigella sonnie, Serratia sp. Staphylococcus aureus and Bacillus subtilis by disc diffusion method. Streptomycin and Gentamycin were used as standard reference drugs while DMSO was included as a solubilizing agent as well as a negative control in this study. All the extracts were found to possess different degrees of antibacterial activity except aqueous extract.

Keywords: Decalepis hamiltonii, Phytochemical analysis, Antibacterial activity, Disc diffusion method.

INTRODUCTION

Decalepis hamiltonii Wight and Arn (swallow root) is a monogenic climbing shrub endemic to the Deccan peninsula. Decalepis hamiltonii Wight and Arn commonly called as maredu kummulu or barre sugandhi or marudu gaddalu or makali beru belonging to the family A celep diaceae. Its roots have been used in Ayurveda, the ancient Indian traditional systems of medicine to stimulate appetite, relieve flatulence and as a general tonic. It is also useful as a blood purifier, preservative and as a source of bioinsecticide for stored food gr ains. Its tubers are consumed as pickles and as a juice for its alleged health promoting properties. Earlier studies have shown that roots contain aldehyde, isositols, saponins, amyrins and lupeols as well as volatile compounds such as such as 2-hydroxy-4-methoxybenzaldehyde, vanillin, 2-phenyl ethyl alcohol, benzaldehyde and others. The roots have also been used as a substitute for Hemidesmus indicus in ayurvedic preparations of ancient Indian medicine. It possesses potent antioxidant properties, antulcer, anti inflammatory and antipyretic activities. In addition, 4-hydroxyisophthalic acid, 14-amino tetra decanoic acid, 4-(1-hydroxy-1-methylheptyl)-1-methyl-1,2-cyclohexanediol, 2-(hydroxymethyl)-3-ethoxybenzaldehyde, 2,4,8-trihydroxybicyclo(3.2.1) octan-3-one, bis-2,3,4,6-galloyl-o-β-D-gluco pyranoside, bornorol and ellagic acid have been identified in swallow root. The present study was to analyze the presence of photochemical and to evaluate the antibacterial activity of various extracts of Decalepis hamiltonii against several Gram positive and Gram negative bacterial strains in vitro.

MATERIALS AND METHODS

Plant material

The roots were collected from herbal suppliers in Chennai, India. The root (plant material) was identified and authenticated at Plant Anatomy and Research Center, Chennai, Tamil Nadu, India.

Preparation of plant extract

The roots were air dried under shade and powdered to 40 meshes coarse powder and stored in air tight bottles. 100g of Decalepis hamiltonii root powder was subjected to successive extraction with different solvents in increasing polarity viz. petroleum ether, benzene, chloroform, ethyl acetate, acetone, methanol, ethanol and distilled water by using soxhlet apparatus. The solvents were evaporated under reduced pressure and stored in desiccators at 4°C.

Microorganisms used

The microbial strains were obtained from National Chemical Laboratory (NCL), Pune, India. The organisms were maintained on nutrient agar (Hi Media, India) slope at 4°C and subculture before use. Among 9 microorganisms used, 7 Gram positive bacteria were Escherichia coli, Klebsiella pneumonia, Salmonella typhi, Proteus mirabilis, Vibrio cholera, Shigella sonnie, Serratia sp. Staphylococcus aureus and Bacillus subtilis. Preliminary phytochemical analysis

The qualitative chemical analysis of various extracts were carried out for the presence of alkaldoids, flavonoids, saponins, steroids, glycosides, phenols, thios and resins using the method adopted in similar surveys.

Antibacterial activity

The antibacterial activity screening was performed by disc diffusion method for various extracts. The Mueller Hinton Agar (Hi Media) was used as bacteriological medium. Mueller Hinton Agar plates were prepared by pouring 15ml of molten media into the sterile petriplates. The plates were allowed to solidify for 15 minutes and 0.1% inoculums suspension was swabbed uniformly and inoculums was allowed to dry for 5 minutes. Under aqueous conditions, the plates were exposed to standard drugs (10µg/disc) and incubated at 37°C for 24 hours. The diameter of zone of inhibition produced by the extracts was compared with standard drugs (10µg/disc) and compared to in MHA plates and incubated at 37°C for 24 hours. The diameter of zone of inhibition produced by the extracts was compared with standard drugs (10µg/disc).

RESULTS AND DISCUSSION

Plants are important source of potentially useful structures for the development of new chemotherapeutic agents, the first step towards this goal is in vitro antibacterial activity. The extracts of higher plant can be very good source of antibiotics against various bacterial pathogens. The results of the photochemical screening of the roots of Decalepis hamiltonii are presented in Table 1. The phytochemical analysis revealed the presence of flavonoids, saponins, tannins, steroids, cardiac glycosides. The antibacterial activity of extracts against 9 bacterial strains was presented in Table 2. All the extracts were found to possess different degrees of antibacterial activities except aqueous extract. Petroleum ether extract showed a broad spectrum antibacterial activities may be due to the presence of steroids and glycosides than other extracts. Methanolic extract showed antibacterial activity to all microorganisms except Staphylococcus aureus. Acetone extract showed antibacterial activity.
except to Shigella sonnie. Some of the extracts were ineffective in this study do not possess antibiotic properties or the plant extracts may have antibacterial constituents just not in sufficient concentration so as to be effective.

Various workers have already shown that Gram positive bacteria are more susceptible towards plant extracts as compared to Gram negative bacteria\textsuperscript{17, 18}. These differences may be attributed to fact that the cell wall in Gram positive bacteria is of a single layer whereas the Gram negative cell wall is multilayered structure\textsuperscript{19}. Alternatively, the passage of the active compound through the Gram negative cell wall may be inhibited. Plant based antimicrobials have enormous therapeutic potential as they serve because of lesser side effects. Tannins are well known to possess general antimicrobial properties\textsuperscript{20}. Tannins are quite resistant to microbial attack and are known to inhibit the growth of some microorganisms. It is this antimicrobial effect of tannins that slow down the rate of biodegradation of soil organic matter. Antimicrobial agents can damage pathogens in several ways. The major mode of actions is interference with cell wall synthesis, inhibition of protein synthesis, interference with nucleic acid synthesis, and inhibition of a metabolic pathway\textsuperscript{21}.

The potential for developing antimicrobials from higher plants appears rewarding as it will lead to the development of a phytomedicine to act against microbes. Plant - based antimicrobials have enormous therapeutic potential as they can serve the purpose with lesser side effects that are often associated with synthetic antimicrobials\textsuperscript{22}. However, the present study of in vitro antimicrobial evaluation of Decaleps hamiltonii forms a primary platform for further photochemical and pharmacological studies. The result of present study supports the traditional usage of the studied Decaleps hamiltonii and suggests that some of the plant extracts possess compounds with antimicrobial properties that can be used as antimicrobial agents in new drugs for the therapy of infectious disease caused by pathogens.

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PE – Petroleum ether B – Benzene C – Choroform E – Ethylacetate; A – Acetone M – Methanol E – Ethanol Aq – Aqueous

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Values include cup border diameter (6mm)
Values are mean of three replicates

PE – Petroleum ether B – Benzene C – Choroform E – Ethyl acetate; A – Acetone M – Methanol E – Ethanol Aq – Aqueous; S – Streptomycin G – Gentamycin

Acknowledgement

We thank the department of Microbiology and Biochemistry of Muthayammal College of Arts and Science, Rasipuram, Tamil Nadu, India for their encouragement and support technical support in testing the extracts for activity.

References


HEPATOPROTECTIVE ACTIVITY OF METHANOLIC EXTRACT OF DECALPIS HAMILTONII AGAINST ACETAMINOPHEN-INDUCED HEPATIC INJURY IN RATS

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ABSTRACT

This study was undertaken to investigate the protective effect of methanol extract of Decalpis hamiltonii on acetaminophen-induced hepatotoxicity in rats. Hepatotoxicity was induced by administering an oral dose of acetaminophen (25 mg/kg, 100 mg/kg) and elevated levels of hepatic marker enzymes in serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and bilirubin and a significantly decreased serum levels of total protein were noted, compared to controls. In the liver, significantly elevated levels of lipid peroxidation (LPO), and lowered levels of antioxidant enzymes like catalase (CAT) and superoxide dismutase (SOD) and non-enzymatic antioxidants like GSH and ascorbic acid were observed following acetaminophen administration. When rats with acetaminophen-induced hepatotoxicity were treated with the extract of Decalpis hamiltonii, the serum ALT, AST, ALP, bilirubin and total protein levels reverted to near normal, while the hepatic concentration of CAT, SOD, GSH and ascorbic acid were significantly increased and that of LPO significantly lowered, when compared to acetaminophen-induced rats. Histopathological studies confirmed the hepatoprotective effect conferred by the extract of Decalpis hamiltonii. These results reveal that a methanolic extract of Decalpis hamiltonii is able to significantly alleviate the hepatotoxicity induced by acetaminophen in the rat.

Keywords: Acetaminophen; Hepatotoxicity; Decalpis hamiltonii; Free radical; Antioxidants

INTRODUCTION

Liver diseases are mainly caused by toxic chemicals, excess consumption of alcohol, infections and autoimmune disorders. Liver damage is the wide spread pathology which in most cases involves oxidative stress and is characterized by a progressive evolution from steatosis to chronic hepatitis, fibrosis, cirrhosis and hepatocellular carcinoma. Most of the hepatotoxic chemicals damage liver cells mainly by inducing lipid peroxidation and other oxidative damages. Free radical initiated auto oxidation of cellular membrane lipids can lead to cellular necrosis and is now accepted to be important in connection with a variety of pathological conditions. Reactive oxygen species (ROS), from both endogenous and exogenous, are implicated in the etiology of several degenerative diseases, such as coronary artery diseases, stroke, rheumatoid arthritis, diabetes and cancer. It is well known that, free radicals are the reactive species derived from them cause damage through mechanisms of covalent binding and lipid peroxidation with subsequent tissue injury. In order to protect the tissues from damage caused by ROS, organisms possess enzymatic and non-enzymatic antioxidant systems.

Mammalian cells possess elaborate defense mechanisms for radical detoxification. Key metabolic steps are super oxide dismutase (SOD) catalase (CAT) and glutathione peroxidase (GPx), which destroy toxic peroxides. Some non-enzymatic molecules including thioredoxin, thiols and disulphide bonding play important roles in antioxidant defense systems. Some of these compounds are obtained from food such as α-tocopherol, β-carotene and ascorbic acid and such micronutrient elements as zinc and selenium. High consumption of fruits and vegetables is associated with low risk for these diseases, which is attributed to the antioxidant vitamins and other phytochemicals. There is a great deal of interest in edible plants that contain antioxidants and health promoting photochemical, in view of their health implications.

Treating liver diseases with herbal drugs has a long tradition in India, China and Japan. About 170 phytoconstituents isolated from 110 plants belonging to 35 families have been reported to possess liver protective activity. About 600 commercial herbal formulations with claimed hepatoprotective activity are being sold all over the globe. Around 40 patented polyherbal formulations representing a variety of combination of 93 Indian herbs from 44 families are available in Indian market. Some herbal preparations exist as standardized extracts with major known ingredients or even pure compounds. Decalpis hamiltonii Wight and Arn (swallow root) (Family: Asclepiadaceae) commonly called as maredu kummulu or barre sugandhi or maradu gaddalu or makali beru. Decalpis hamiltonii is a monogenic climbing shrub endemic to the Deccan peninsula, which is used as a culinary spice due to its highly aromatic roots. Earlier studies have shown that Decalpis hamiltonii root contain aldehyde, isoalolols, saponins, amyrins and lupeol, as well as volatile compounds such as 2-hydroxy-4-methoxybenzaldehyde, vanillin, 2-phenyl ethyl alcohol, benzaldehyde and others. It has been used as an appetizer, blood purifier, relieves flatulence, preservative and is a source of bioinsecticide for stored food grains; also as a general tonic and as a juice for its alleged health promoting properties. The roots have also been used as a substitute for Hemidesmus indicus in ayurvedic preparations of ancient Indian medicine.

Acetaminophen (Paracetamol) is a widely used antipyretic and analgesic which produces acute liver damage if overdoses are consumed. Acetaminophen is mainly metabolized in liver to excretable glucuronide and sulfate conjugates. However, the hepatotoxicity of acetaminophen has been attributed to the formation of toxic metabolites when a part of acetaminophen is activated by hepatic Cytochrome P-450 to a highly reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI). NAPQI is initially detoxified by conjugation with reduced glutathione (GSH) to form mercapturic acid. However, when the rate of NAPQI formation exceeds the rate of detoxification of GSH, it oxidize tissue macromolecules such as lipid or SH group of protein and alters the homeostasis of calcium after depleting GSH.

In recent years, plant derived natural products such as flavonoids, terpenoids and steroids etc have received considerable attention in recent years due to their diverse pharmacological activity. Decalpis hamiltonii possesses potent antioxidant properties which could be associated with their health benefits and antioxidant activity. Hence, the present study was designed to evaluate the hepatoprotective activity of an extract of Decalpis hamiltonii in an experimental model of acetaminophen-induced hepatotoxicity in Wistar rats. The various biochemical parameters viz., levels of hepatic marker enzymes [serum oxaloacetate transaminases (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP)] bilirubin and total protein, indicators of oxidative stress [lipid peroxidation (LPO)], antioxidant profile were evaluated and the results were correlated with histopathological observations.
MATERIALS AND METHODS

Preparation of the Decalepis hamiltonii extract
The root of Decalepis hamiltonii were collected from local market in Chennai, India and the plant material was identified and authenticated taxonomically at Plant Anatomy and Research Center, Chennai, India. The dried roots were powdered to 40 mesh of powder. The powder was defatted with petroleum ether (60°-80°C) and then extracted with 90% methanol using Soxhlet apparatus. The residue was filtered and concentrated to a dry mass by vacuum distillation; the filtrate thus obtained was used as Decalepis hamiltonii extract.

Experimental design
Healthy adult male albino rats of body weight ranging from 120-150 g were housed in polypropylene cages under controlled conditions of temperature (25±2°C) with a 12-h/12-h day-night cycle, during which time they had free access to food and water ad libitum and fed standard pellet diet (obtained from Sai-Durga feeds and foods, Bangalore, India). Animals were maintained per national guidelines and protocols approved by the Institutional Animal Ethical Committee, proposal number being JSSCP/IAEC/p.cog/06/2010-2011.

The animals were divided into five groups of six rats each:
Group 1: Control rats received only olive oil orally (vehicle) (2g/kg bw)
Group 2: Rats received acetaminophen (2g/kg bw orally after every 72 hours for 10 days).
Group 3: Rats received acetaminophen orally + 100mg of methanolic extract of Decalepis hamiltonii/kg bw orally for 10 days.
Group 4: Rats received acetaminophen orally + 200mg of methanolic extract of Decalepis hamiltonii/kg bw orally for 10 days.
Group 5: Rats received acetaminophen orally + 400mg of methanolic extract of Decalepis hamiltonii/kg bw orally for 10 days.

At the end of the experimental period, all the animals were killed by cervical decapitation. From each animal, blood samples were collected and the hepatic tissue was excised. All the samples were stored at -80°C until analysis.

Preparation of serum and hepatic tissue samples for analysis
From each blood sample, serum was separated by centrifugation at 2500 rpm for 10 minutes for various biochemical estimations. Prior to biochemical analysis, each hepatic tissue (100 mg/ml buffer) was homogenized in 50 mM phosphate buffer (pH 7.0); the homogenate was then centrifuged at 10,000 rpm for 15 min and the supernatant obtained was used for biochemical analysis. All liver parameters were expressed as activity per mg protein. The protein concentration in each fraction was determined by the method of Lowry et al. (1951) using crystalline bovine serum albumin as a standard.

Biochemical analysis
To assess the membrane damage, the activities of liver marker enzymes like ALP (alkaline phosphatase) by king’s method 196522, Alanine transaminase (ALT) and Aspartate transaminase (AST) by Reitman and Frankel method23 were assayed. Serum Proteins by Lowry method24 and serum Bilirubin by Malloy and Evelyn method25 were assayed.

Determination of Lipid peroxidation in hepatic tissue
The extent of lipid peroxidation was assayed by analysis the levels of thiobarbituric reactive substance by Ohkawa et al method26, TRABS in tissues was estimated by the method of Ohkawa et al. To 0.5 ml tissue homogenate, 0.5 ml saline and 1.0 ml of 10% TCA were added, mixed well and centrifuged at 3000 rpm for 20 min. To 1.0 ml of the protein-free supernatant, 0.25 ml of thiobarbituric acid (TBA) reagent was added; the contents were mixed and heated for 1 h at 95°C. The tubes were cooled to room temperature under running water and absorption measured at 532 nm. The levels of lipid peroxides were expressed as nmole of thiobarbituric acid reactive substances (TBARS)/mg protein.

Determination of activities of antioxidant enzymes
The antioxidant enzymes occurring in the liver tissue of the rats were assayed.

Catalase (CAT). CAT activity was determined by the method of Sinha27. In this test, dichromate acetic acid is reduced to chronic acetic acid when heated in the presence of hydrogen peroxide (H2O2) with the formation of perchloric acid as an unstable intermediate. In the test, the green color developed was read at 590nm against blank on a spectrophotometer. The activity of CAT was expressed as units/mg protein (μmol of H2O2 consumed/min/mg protein).

Superoxide dismutase (SOD).The activity of SOD in tissue was assayed by the method of Kakkar et al28. The assay mixture contained 1.2 ml sodium pyrophosphate buffer (pH 8.3, 0.025 mol/L), 0.1 ml phenazine methosulphate (186 mM), 0.3 ml NBT (300 mM), 0.2 ml NADH (780 mM) and approximately diluted enzyme preparation and water in a total volume of 3 ml. After incubation at 30°C for 90 sec, the reaction was terminated by the addition of 1.0 ml glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4.0 ml n-butanol. The color intensity of the chromogen in the butanol layer was measured at 560 nm against n-butanol and concentration of SOD was expressed as units/mg protein. Absorbance values were compared with a standard curve generated from known SOD.

Glutathione peroxidase (Gpx). Gpx activity was measured by the method described by Rotruck et al29. Briefly, reaction mixture contained 0.2ml of 0.4 M Tris-HCl buffer pH 7.0, 0.1 ml of 10 mM sodium azide, 0.2 ml of tissue homogenate (homogenized in 0.4 M, Tris-HCl buffer, pH 7.0), 0.2 ml glutathione, and 0.1 ml of 0.2 mM hydrogen peroxide. The contents were incubated at 37°C for 10 min. The reaction was arrested by 0.4 ml of 10% TCA, and centrifuged. Supernatant was assayed for glutathione content by using Ellman’s reagent (19.8 mg of 5, 5’-dithiobisnitro benzoic acid (DTNB) in 100 ml of 0.1% sodium nitrate).

Determination of levels of non-enzymatic antioxidants
Non-enzymatic antioxidant components of the liver tissue samples of the experimental animals were assayed.

Reduced glutathione (GSH). GSH content was estimated by the method of Ellman30. To measure the reduced glutathione (GSH) level, the tissue homogenate (in 0.1 M phosphate buffer pH 7.4) was taken. The homogenate was added an equal volume of 20% tetrachloroacetic acid (TCA) containing 1 mM EDTA to precipitate the tissue proteins. The mixture was allowed to stand for 5 min prior to centrifugation for 10 min at 200 rpm. The supernatant (200μl) was then transferred to a new set of test tubes and added 1.8 ml of the Ellman’s reagent (5, 5’-dithiobis-2-nitro benzoic acid) (0.1mM) which was prepared (0.3M phosphate buffer with 1% of sodium citrate solution). Then all the test tubes were made up to the volume of 2 ml. After completion of the total reaction, solutions were measured at 412 nm against blank. Absorbance values were compared with a standard curve generated from standard curve from known GSH.

Ascorbate (Vitamin C). Ascorbic acid concentration was measured by Omaye et al. method31. To 0.5 ml of plasma/0.5 ml liver homogenate, 1.5 ml of supernatant, 0.5 ml of DPNH reagent (2% DPNH) and 4% thiourea in 9 N sulphuric acids) was added and incubated for 3 h at room temperature. After incubation 2.5 ml of 8.5% sulphuric acid was added and color developed was read at 530 nm after 30 min.

Histopathological investigation
After sacrificing the rats by cervical decapitation, hepatic tissues were collected, washed in normal saline and fixed in 10% formalin for 24h and dehydrated with alcohol. Hepatic tissues were cleaned and embedded in paraffin, cut in 5-5μm sections, and stained with routine haematoxylin-eosin (H&E) dye and finally observed under
light microscope and morphological changes such as cell necrosis, fatty changes or inflammation of lymphocytes were observed.

**Statistical Analysis**

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Duncan’s multiple range test (DMRT). The values are mean ± SD for six rats in each group. The student's t-test was used to compare the means of specific groups, with p<0.05 considered as significant.

**RESULTS**

The effect of methanolic extract of *Decalepis hamiltonii* on activities of hepatic marker enzymes on serum transaminases and alkaline phosphatase levels in acetalaminophen intoxicated rats are summarized in table 1. A significant (p<0.05) increase in the activities of the serum enzymes AST, ALT and ALP were observed in rats receiving acetalaminophen (group II) when compared to normal (group I) rats administered vehicle alone. However, the activities of these serum enzymes were significantly (p<0.05) lower in rats treated with the *Decalepis hamiltonii* extract (groups III, IV & V) than in group II rats. Interestingly, the mean activities of hepatic marker enzymes were significantly lower in group V rats than those in groups III & IV rats.

Table 2 shows the changes in the mean levels of protein and bilirubin in hepatic and serum samples of the experimental rats. Acetalaminophen administration in group II rats resulted in significant (p<0.05) decrease in the levels of protein in hepatic and serum samples and a significant increase in level of serum bilirubin when compared to normal rats (group I). Treatment with *Decalepis hamiltonii* extract in groups III, IV & V rats resulted in significantly higher levels of protein and a significantly lower level of bilirubin (p < 0.05) than that in group II rats.

**Table 1: Effect of methanolic extract of *Decalepis hamiltonii* (MEDH) on activities of hepatic marker enzymes in serum samples of rats in acetalaminophen -induced hepatotoxicity.**

<table>
<thead>
<tr>
<th>Groups</th>
<th>AST (IU/L)</th>
<th>ALT (IU/L)</th>
<th>ALP (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>61.32 ± 4.25a</td>
<td>53.85 ± 2.67a</td>
<td>66.23 ± 1.48a</td>
</tr>
<tr>
<td>Acetalaminophen</td>
<td>175.41 ± 7.82a</td>
<td>126.64 ± 1.72a</td>
<td>120.23 ± 2.16a</td>
</tr>
<tr>
<td>Acetalaminophen+100mg MEDH</td>
<td>145.79 ± 8.53c</td>
<td>102.29 ± 1.77c</td>
<td>85.23 ± 2.52c</td>
</tr>
<tr>
<td>Acetalaminophen+200mg MEDH</td>
<td>106.32 ± 6.11c</td>
<td>96.93 ± 2.38c</td>
<td>77.75 ± 1.75c</td>
</tr>
<tr>
<td>Acetalaminophen+400mg MEDH</td>
<td>66.21 ± 5.54c</td>
<td>68.95 ± 1.37c</td>
<td>68.95 ± 1.37c</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD of six rats in each group. ANOVA followed by Duncan’s multiple range tests. Values not sharing a common superscript differ significantly at P ≤ 0.05.

**Table 2: Effect of methanolic extract of *Decalepis hamiltonii* (MEDH) on levels of protein and bilirubin in hepatic and serum samples of rats in acetalaminophen -induced hepatotoxicity.**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Hepatic protein (mg/dl)</th>
<th>Serum protein (mg/dl)</th>
<th>Serum bilirubin (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>10.04 ± 0.76c</td>
<td>4.5 ± 0.34c</td>
<td>0.91±0.52c</td>
</tr>
<tr>
<td>Acetalaminophen</td>
<td>7.5 ± 0.57b</td>
<td>2.5 ± 0.19b</td>
<td>2.41±0.18b</td>
</tr>
<tr>
<td>Acetalaminophen+100mg MEDH</td>
<td>8.5 ± 0.68de</td>
<td>3.25 ± 0.24d</td>
<td>2.02±0.32c</td>
</tr>
<tr>
<td>Acetalaminophen+200mg MEDH</td>
<td>9.0 ± 0.68d</td>
<td>3.0 ± 0.22c</td>
<td>1.80±0.01d</td>
</tr>
<tr>
<td>Acetalaminophen+400mg MEDH</td>
<td>9.6 ± 0.73d</td>
<td>4.0 ± 0.30d</td>
<td>0.90±0.16d</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD of six rats in each group. ANOVA followed by Duncan’s multiple range tests. Values not sharing a common superscript differ significantly at P ≤ 0.05.

The mean levels of LPO and weight in the hepatic tissue of group II (acetalaminophen -induced) rats were significantly higher than that in group I (normal) rats (table 3). Treatment with *Decalepis hamiltonii* extract in groups II, IV and V rats were found to result in a significant (p < 0.05) lowering of the mean levels of LPO and liver weight, presumably by limiting lipid peroxidation in the hepatic tissue. Interestingly, the mean levels of LPO and liver weight were significantly lower in group V rats than those in groups III & IV rats.

A significant decrease in CAT, SOD and GPx activity was observed in the hepatic tissue of acetalaminophen -induced (group II) rats when compared to normal (group I) rats that had received vehicle alone (table 4). Treatment with the extract of *Decalepis hamiltonii* appeared to exert a beneficial effect since the activities of CAT, SOD and GPx were significantly (p < 0.05) higher in hepatic tissue of groups III, IV and V than those in group II rats. The mean activities of antioxidant enzymes were significantly higher in group V rats than those in groups III & IV rats.

**Table 3: Effect of methanolic extract of *Decalepis hamiltonii* (MEDH) on levels of lipid peroxidation and liver weight in Acetaminophen - induced hepatic damage in rats**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Liver weight (wt/100 g tissue)</th>
<th>Lipid peroxidation (nmol of MDA/mg pro tein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>3.2 ± 0.12a</td>
<td>1.43 ± 0.45c</td>
</tr>
<tr>
<td>Acetalaminophen</td>
<td>6.5 ± 0.19b</td>
<td>3.89 ± 0.89b</td>
</tr>
<tr>
<td>Acetalaminophen+100mg MEDH</td>
<td>5.9±0.16c</td>
<td>2.25 ± 0.26c</td>
</tr>
<tr>
<td>Acetalaminophen+200mg MEDH</td>
<td>5.8 ± 0.12c</td>
<td>2.02 ± 0.31c</td>
</tr>
<tr>
<td>Acetalaminophen+400mg MEDH</td>
<td>3.3 ± 0.19c</td>
<td>1.77 ± 0.15c</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD of six rats in each group. ANOVA followed by Duncan’s multiple range tests. Values not sharing a common superscript differ significantly at P ≤ 0.05.
Table 4: Effect of methanolic extract of Decalepis hamiltonii (MEDH) on activities of enzymatic antioxidants in acetaminophen-induced hepatic damage in rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>CAT (U/mg protein)</th>
<th>SOD (U/mg protein)</th>
<th>GPx (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>34.88 ± 0.90a</td>
<td>3.51 ± 0.592a</td>
<td>20.28 ± 0.31a</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>14.21 ± 0.27b</td>
<td>1.16 ± 0.17b</td>
<td>10.08 ± 0.10b</td>
</tr>
<tr>
<td>Acetaminophen+100mg MEDH</td>
<td>23.23 ± 0.31c</td>
<td>2.50 ± 0.569c</td>
<td>12.34 ± 0.35c</td>
</tr>
<tr>
<td>Acetaminophen+200mg MEDH</td>
<td>29.82 ± 0.83d</td>
<td>2.48 ± 0.49d</td>
<td>14.35 ± 0.36d</td>
</tr>
<tr>
<td>Acetaminophen+400mg MEDH</td>
<td>34.74 ± 0.84e</td>
<td>3.17 ± 0.09e</td>
<td>16.48 ± 0.50e</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD of six rats in each group. ANOVA followed by Duncan’s multiple range tests. Values not sharing a common superscript differ significantly at P ≤ 0.05.

Table 5: Effect of methanolic extract of Decalepis hamiltonii (MEDH) on levels of non-enzymatic antioxidants in acetaminophen-induced hepatic damage in rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>GSH (µg/mg protein)</th>
<th>Vitamin C (mg/100ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>5.5 ± 0.3a</td>
<td>8.0 ± 0.60a</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>1.26 ± 0.26b</td>
<td>2.5 ± 0.19b</td>
</tr>
<tr>
<td>Acetaminophen+100mg MEDH</td>
<td>3.67 ± 0.29c</td>
<td>5.0 ± 0.38c</td>
</tr>
<tr>
<td>Acetaminophen+200mg MEDH</td>
<td>4.97 ± 0.25d</td>
<td>6.21 ± 3.07d</td>
</tr>
<tr>
<td>Acetaminophen+400mg MEDH</td>
<td>5.6 ± 0.4e</td>
<td>7.78 ± 1.34e</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD of six rats in each group. ANOVA followed by Duncan’s multiple range tests. Values not sharing a common superscript differ significantly at P ≤ 0.05.

Table 5 lists the levels of non-enzymatic antioxidants in hepatic tissue samples of the experimental animals. A significant (p < 0.05) decrease in the mean levels of GSH and vitamin C was observed in the hepatic tissue of acetaminophen-induced (group II) rats when compared to normal (group I) rats. Treatment with Decalepis hamiltonii extract in groups III, IV & V rats resulted in a significantly higher concentration of GSH and vitamin C than that in group II rats. The mean levels of GSH and vitamin C were significantly higher in group V rats than those in groups III & IV rats.

Histopathological examinations

When compared to the histo-architecture of the hepatic tissue of group I (normal) animals (Fig.1) with hepatic cells of group II rats (acetaminophen-induced) revealed extensive damage, characterized by the disruption of the lattice nature of the hepatocyte, damaged cell membranes, degenerated nuclei, disintegrated central vein and damaged hepatic sinusoids (Fig. 2). Treatment with methanolic extract at the dose 100, 200 and 400mg/kg body weight is shown in Fig.3, 4 and 5 respectively.
DISCUSSION

The present studies were performed to assess the hepatoprotective activity of methanolic extract of *Decealepis hamiltonii* in rats against acetaminophen as hepatotoxin to prove its claims in folklore practice against liver disorders. Acetaminophen, a widely used antipyretic and analgesic drug, produces acute liver damage if accidental overdoses are consumed. The covalent binding of N-acetyl p- benzoylamine, an oxidation product of acetaminophen, to sulphhydryl groups of proteins resulting in cell necrosis and lipid peroxidation induced by decrease in glutathione in the liver as the cause of hepatotoxicity have been reported earlier. Hepatic cells appear to participate in a variety of enzymatic metabolic activities and acetaminophen produced marked liver damage at the given doses as expected.

In the assessment of liver damage by acetaminophen, the determination of enzyme levels such as serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT) is largely used. Drotman et al. reported that the elevated levels of serum enzymes are indicative of cellular leakage and loss of functional integrity of cell membrane in liver. The disturbance in the transport function of the hepatocytes as a result of hepatic injury causes the leakage of enzymes from cells due to altered permeability of membrane. This results in decreased levels of SGOT, SGPT and ALP in the hepatic cells and a raised level in serum. High levels of SGOT indicate liver damage, such as that due to viral hepatitis as well as cardiac infarction and muscle injury. SGPT catalyses the conversion of alanine to pyruvate and glutamate and is released in similar manner. Therefore, SGPT is more specific to the liver and is thus a better parameter for detecting liver injury.

Serum ALP and bilirubin level on other hand are related to the function of hepatic cell. Increase in serum level of ALP is due to increased synthesis, in presence of increasing biliary pressure. The rise in the level of serum bilirubin is most sensitive and confirms the intensity of jaundice. Bilirubin is one of the most useful clinical clue for the severity of necrosis and its accumulation is a measure of binding, conjugation and excretory capacity of hepatocytes. In our study treatment with methanolic extract of *Decealepis hamiltonii* significantly reduced the levels of these enzymes which are an indication of stabilization of plasma membrane as well as repair of hepatic tissue damage can be considered as an expression of the functional improvement of hepatocytes, which may be caused by an accelerated regeneration of parenchyma cells. Effective control of ALP and bilirubin levels points towards an early improvement in the secretory mechanism of the hepatic cell.

Administration of methanolic extract of *Decealepis hamiltonii* significantly increased the decreased level of protein and also preserves the structural integrity of the hepatocellular membrane and liver cell architecture damaged by acetaminophen, which was confirmed by histopathological studies. Previous studies have proved that lipid peroxidation has been postulated as being the destructive process in liver injury due to acetaminophen administration. Lipid peroxide levels were significantly increased in acetaminophen intoxicated rats were revealed in our study. Guillen- Sans et al. reported an increase in MDA levels (in terms of TBARS) suggested an enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms to prevent formation of excessive free radicals. The treatment with methanolic extract of *Decealepis hamiltonii* significantly reversed these changes. Hence it may be possible that the mechanisms of hepatoprotection of methanolic extract of *Decealepis hamiltonii* is due to its antioxidant effect.

Antioxidants and radical scavengers were to study the mechanism of acetaminophen toxicity as well as to protect liver cells from acetaminophen induced damage. In this study, we observed a decrease in catalase activity in liver tissue during chronic administration of acetaminophen. CAT decomposes hydrogen peroxide and protects the tissue from highly reactive hydroxyl radicals. Inhibition of these protective mechanisms results in enhanced sensitivity to free radical induced cellular damage. Gupta et al. reported that the excessive production of free radicals may result in alterations in the biological activity of cellular macromolecules. Therefore, the reduction in the activity of these enzymes may result in a number of deleterious effects due to accumulation of superoxide radicals and hydrogen peroxide. Administration of methanolic extract of *Decealepis hamiltonii* increases the activity of catalase in acetaminophen induced liver damage in rats to prevent the accumulation of excessive free radicals and protects the liver from acetaminophen intoxication.

SOD dismutates superoxide radicals O₂⁻ into hydrogen peroxide plus O₂, thus participating with other antioxidant enzymes, in the enzymatic defense against oxygen toxicity. In this study, SOD plays an important role in the elimination of ROS derived from the peroxidative process of xenobiotics in liver tissues. Curtis et al. reported that the decrease in serum activity of SOD is a sensitive index in hepatocellular damage and is the most sensitive enzymatic index in liver injury. In the present study, it was observed that methanolic extract of *Decealepis hamiltonii* caused a significantly increased in the hepatic SOD activity of the acetaminophen induced liver damage in rats. This shows methanolic extract of *Decealepis hamiltonii* may be associated with decreased oxidative stress and free radical mediated tissue damage.

Glutathione is a non-enzymatic biological antioxidant present in the liver. Its functions are concerned with the removal of free radical species such as hydrogen peroxide, superoxide radicals, alkoxy radicals and maintenance of membrane protein thiol and as a substrate for GPs and GST. Deficiency of GSH within living organisms can lead to tissue disorder and injury. Example includes liver injury induced by consuming alcohol or by taking drugs like acetaminophen, lung injury by smoking and muscle injury by intense physical activity. All are known to be correlated with low levels of GSH.

Acetaminophen intoxication produces significant depletion of GSH and imbalance of GSH/GSSG ratio. The reduced form of GSH becomes readily oxidized to GSSG on interacting with free radicals. Excessive production of free radicals resulted in the oxidative stress.
which leads to damage to biomolecules e.g., lipids and can induce lipid peroxidation40. In our present study, decreased level of GSH has been associated with an enhanced lipid peroxidation in acetalopinom treated rats. Administration of methanolic extract of Decalepis hamiltonii significantly increased the level of GSH in a dose dependent manner.

Vitamin C acts as an antioxidant in biological systems and scavenge the free radicals thereby increase the antioxidant defense in the body. Vitamin C is an excellent hydrophilic antioxidant; it readily scavenge ROS and peroxo radical41. Also acts as a co-antioxidant by regenerating the vitamin A, E and GSH from radicals42. In our study we have observed a decreased level of vitamin C in serum of acetalopinom induced hepatic damaged rats. Chatterjee43 proved that the decreased level could be the increased utilization of vitamin C in deactivation of the increased levels of ROS or too decreased in the GSH level. Since, the GSH is required for the recycling of vitamin C. Administration of methanolic extract of Decalepis hamiltonii increase the serum level of vitamin C, may be expected to enhance the GSH level or stimulation of the system to recycle the dehydroascorbic acid to ascorbic acid.

Histopathological Study

Histology of liver sections of normal control animals (group I) showed normal hepatic cells with well preserved cytoplasm, prominent nucleus and nucleolus and central vein (Fig 1). Acetalopinom treated animals (Fig 2) shows the liver cells of rats intoxicated with cells have high degree of damage, characterized by necrosis along with various gradation of fatty changes of tiny to large sized vacuoles ( fatty droplets). The normal architecture of liver was completely damaged.

The hepatic cells of rats treated with 100 and 200mg of methanolic extract of Decalepis hamiltonii showed mild fatty change with tiny vacuolation, but is some what similar to normal (Fig 3 and 4). The hepatic cells of rats treated with 400mg of methanolic extract of Decalepis hamiltonii showed almost normal hepatic cells but some damaged cells could also been seen (Fig 5), but as compared to acetalopinom damaged cells, the number of hepatocytes with normal nucleus are much more, and vacuolation in cytoplasm are observed to be low. Methanolic extract of Decalepis hamiltonii treatment exhibited protection against liver damage caused by acetalopinom which is confirmed by the results of biochemical studies.

CONCLUSION

Our photochemical study showed the presence of flavanoids, steroids, tannins and saponins in methanolic extract of Decalepis hamiltonii. It is known that some flavanoids are able to reduce xenoptic induced hepatotoxicity in animals28. The inhibitory activity of flavanoids on free radical production could be related their hepatoprotective effects since exogenous antioxidants may counteract the damaging effects of oxidative stress, co-operating with natural systems like glutathione, tocopherol or protective enzymes33. Our results shows that the hepatoprotective and antioxidant effect of methanolic extract of Decalepis hamiltonii may be due to its antioxidant and free radical scavenging properties. In conclusion, the results of this study demonstrate that methanolic extract of Decalepis hamiltonii has a potent hepatoprotective action upon acetalopinom induced hepatic damage in rats.

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Original Article

Antidiabetic effect of methanolic extract of Decalepis hamiltonii root (wight and Am) in normal and alloxan induced diabetic rats

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Abstract

Diabetes mellitus is a major health growing problem in most countries. Purpose of the study was to evaluate the antidiabetic activity of methanolic extracts of root of Decalepis hamiltonii in normal and alloxan induced diabetic rats. Alloxan monohydrate 120 mg/kg was used to induce diabetes mellitus. The methanolic extract of D. hamiltonii at 200 mg and 400 mg and glibenclamide at 7 mg/kg bwt were administered to normal and alloxan induced diabetic rats which significantly reduced the blood glucose in the normal rats and alloxan induced diabetic rats. Also the administration of extract significantly decreased serum total cholesterol, triglyceride, and AST and ALT levels and at the same time increased liver glycogen content. OGTT was performed by administration of 200 mg and 400 mg of methanolic extract of D. hamiltonii and 7 mg of glibenclamide to different groups respectively which significantly lower at all time points that blood was sampled after oral glucose load. These results suggest that the methanolic extract of root of D. hamiltonii was effective in lowering blood glucose level in diabetic rats.

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1. Introduction

Diabetes mellitus (DM) is a chronic disease caused by inherited or acquired deficiency in insulin secretion and by decreased responsiveness of the organs to secreted insulin.1 Diabetes mellitus is a syndrome, initially characterized by a loss of glucose homeostasis resulting from defects in insulin secretion, insulin action both resulting impaired metabolism of glucose and other energy yielding fuels such as lipids and proteins.2 DM is a leading cause of end stage kidney disease, cardiomyopathy and heart attacks, strokes, retinal degeneration leading to blindness and non-traumatic amputations.3 Dyslipidemia, quite common in diabetic patients, is the main risk factor for cardiovascular and cerebrovascular diseases.

DM is currently one of the most costly and burdensome chronic diseases and is a condition that is increasing in epidemic proportions throughout the world. Diabetes is a serious illness with multiple complications and premature mortality, accounting for at least 10% of total health care expenditure in many countries.4 The prevalence of diabetes of all age groups worldwide is projected to rise from 171 million in

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2000 to 366 million in 2030.\textsuperscript{1} Reason of this rise includes increase in sedentary life style, consumption of energy rich diet, obesity, higher life span, etc.\textsuperscript{2} DM is a major and growing health problem in most countries. It causes considerable amount of disability, premature mortality, and loss of productivity as well as increased demands on health care facilities.

As diabetes aggravates and b-cell function deteriorates, the insulin level begins to fall below the body’s requirements and causes prolonged and more severe hyperglycemia.\textsuperscript{3} Hyperglycemia induces long term complications of diabetes such as cardiovascular complications and microvascular complications such as retinopathy, nephropathy and neuropathy and foot ulcer.\textsuperscript{4}

Several approaches are presently available to reduce the hyperglycemia including insulin therapy which suppresses glucose production and augments glucose utilization and several drawbacks like insulin resistance,\textsuperscript{5} anorexic nervous, brain atrophy and fatty liver\textsuperscript{6} after chronic treatment; treatment by sulfonylurea, which stimulates pancreatic islet cell to secrete insulin; metformin, which acts to reduce hepatic glucose production; α-glucosidase inhibitors, which interfere with glucose absorption. Unfortunately, all of these therapies have limited efficacy and various side effects and thus searching for new classes of compounds is essential to overcome these problems. In spite of the presence of known antidiabetic medicine in the pharmaceutical market, remedies from medicinal plants are used with success to treat this disease.\textsuperscript{7}

Based on the WHO recommendations hypoglycemic agents of plant origin used in traditional medicine are important (WHO, 1980).\textsuperscript{8} The attributed antihyperglycemic effects of these plants is due to their ability to restore the function of pancreatic tissues by causing an increase in insulin output or inhibit the intestinal absorption of glucose or to the facilitation of metabolites in insulin dependent processes. Hence treatment with herbal drugs has an effect on protecting b-cells and smoothing out fluctuation in glucose levels. Most of these plants have been found to contain substances like glycosides, alkaloids, terpenoids, flavanoids etc. that are frequently implicated as having antidiabetic effects.\textsuperscript{9}

Alloxan was one of the most widely used chemical diabetogens during initial research work on experimental diabetes. It is a cyclic urea analog of chemical composition 2,4,5,6-tetraoxo-hexa hydropyrimidine.\textsuperscript{10} Alloxan induces diabetes in animals and impairs glucose induced insulin secretion from b cells of Islets of Langerhans of Pancreas. It has been reported that alloxan rapidly and selectively accumulates in b cells in comparison with non-b cells. Several reports directly or indirectly indicate that alloxan affects the membrane potential and ion channels in b cells.\textsuperscript{11}

In the present investigation, methanolic extract of root of Decalepis hamiltonii was used to evaluate the antidiabetic activity in normal and alloxan induced diabetic rats.

2. Materials and methods

2.1. Plant material

The root of D. hamiltonii used for the investigation was purchased from a plant supplier in Chennai, Tamil Nadu, India. The plant was authenticated taxonomically at Plant Anatomy and Research Center, Chennai, Tamil Nadu, India.

2.2. Preparation of extract

The root of D. hamiltonii were dried in shade, crushed to coarse powder. The powder was defatted with petroleum ether (60\textdegree C) and then extracted with 90% methanol using soxhlet extractor. The solvent was evaporated under reduced pressure and dried in vacuum and the filtrate obtained was used for further studies.

2.3. Animals

Healthy albino wistar rats weighing 150±200 g was used for the present study. They were housed in polypropylene cages under controlled conditions of temperature (25 ± 2 °C) with a 12-h light dark cycles. All the animals were acclimatized for 7 days before the study. They were fed with standard pellet diet obtained from Sai-Durga feeds and foods, Bangalore, India and water ad libitum. All the studies conducted were approved by the Institutional Animal Ethical Committee of JSS College of Pharmacy, Proposal number IAEC/PCog/06/2010-2011.

2.4. Oral glucose tolerance test (OGTT)

The oral glucose tolerance test was performed in overnight fasted (18 h) normal rats. The rats were divided into four groups of six rats each. Group 1 served as normal control received orally 0.3% Carboxy methyl cellulose. Group 2 received orally reference drug Glibenclamide at a dose of 7 mg/kg bwt. Group 3 and 4 received orally 200 mg and 400 mg/ kg of methanolic extract of D. hamiltonii dissolved in 0.3% Carboxy methyl cellulose respectively. After 30 min of treatment, all the groups were orally loaded with 2 g/kg of glucose. Blood samples were collected just prior to glucose administration and at 30, 60, 120 and 150 min after glucose loading. Blood glucose levels were measured using commercial kit.

2.5. Hypoglycemic activity in normal rats

Healthy wistar albino rats weighing 150±200 g were fasted overnight and were divided into four groups of six rats each.

Group 1: Normal control received orally 0.3% Carboxy methyl cellulose.

Group 2: Normal rats received orally reference drug Glibenclamide (7 mg/kg bwt)

Group 3: Normal rats received orally methanolic extract of D. hamiltonii (200 mg/kg bwt) dissolved in 0.3% Carboxy methyl cellulose.

Group 4: Normal rats received orally methanolic extract of D. hamiltonii (400 mg/kg bwt) dissolved in 0.3% Carboxy methyl cellulose.

Blood samples were collected before and 1, 2 and 4 h after treatment and the glucose level were determined by using commercial kit.
2.6. Induction of diabetes

For induction of diabetes in Wistar rats, 150 mg/kg of alloxan monohydrate dissolved in normal saline was administered intraperitoneally in overnight fasted rats. After 1 h, the animals were fed with standard pellet and water ad libitum. After 72 h, the blood glucose levels were estimated and rats having blood glucose level more than 180 mg/dl were selected for the study.

2.7. Hypoglycemic activity in diabetic rats

Healthy wistar albino rats weighing 150±200 g were fasted overnight and were divided into five groups of six rats each.

Group 1: Normal control received orally 0.3% Carboxy methyl cellulose
Group 2: Diabetic rats received orally Alloxan monohydrate (150 mg/kg bwt)
Group 3: Diabetic rats treated orally with reference drug Glibenclamide (7 mg/kg bwt)
Group 4: Diabetic rats treated orally with methanolic extract of D. hamiltonii (200 mg/kg bwt) dissolved in 0.3% Carboxy methyl cellulose.
Group 5: Diabetic rats treated orally with methanolic extract of D. hamiltonii (400 mg/kg bwt) dissolved in 0.3% Carboxy methyl cellulose.

Blood samples were collected before and 1, 2 and 4 days after treatment and the glucose level were determined by using commercial kit.

2.8. Biochemical analysis

At the end of the experiment, the animals were fasted overnight and then rats were sacrificed by cervical decapitation and the blood samples were collected to clot and serum separated by centrifugation at 2500 rpm for 10 min. Serum glucose, total cholesterol, triglycerides, aspartate transaminase (AST), alanine transaminase (ALT) was determined. Serum glucose was estimated by Oxidase method. The activities of serum AST and ALT were assayed by Reitman and Frankel method. Total cholesterol and triglycerides were determined by the respective method. Liver was dissected out and washed in normal saline and stored −80 °C for assay of glycogen content by using Anthrone reagent.

2.9. Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by student’s ‘t’ test. The values are mean T SD for six rats in each group. Statistical significance was considered at p < 0.05.

3. Results

There was a significant elevation of serum glucose, total cholesterol, triglycerides, aspartate transaminase, alanine transaminase while liver glycogen significantly decreased in the diabetic control rats as compared with non-diabetic control group.

3.1. Effect of MEDH in oral glucose tolerance in normal rats

Table 1 showed the blood glucose levels of the control, Glibenclamide (7 mg/kg) and methanolic extract of D. hamiltonii (200 mg and 400 mg/kg) at different time points (0, 30, 60, 120, 150 min) after oral administration of glucose (2 g/kg). There was a peak increase in the blood glucose at 30 min in all the groups. In Glibenclamide and 400 mg of MEDH treated group, there was a decrease in blood glucose level at 150 min when compared to control group.

3.2. Effect of MEDH on blood glucose level in normal fasted rats

Table 2 showed the level of blood glucose in euglycemic rats at 0, 1, 2 and 4 h of administration. The administration of Glibenclamide (7 mg/kg) and methanolic extract of D. hamiltonii (200 mg and 400 mg/kg) on euglycemic rats was not significant at 1 h, while it was significant at 4 h (p < 0.05) as compared to control.

3.3. Effect of MEDH on blood glucose level in alloxan induced diabetic rats

The level of blood glucose in normal and diabetic rats at 0, 1, 2 and 4 h of administration was showed in Table 3. There was a significant elevation of blood glucose level in diabetic group as compared to normal control rats. The administration of Glibenclamide (7 mg/kg) and methanolic extract of

### Table 1: Effect of MEDH in oral glucose tolerance in normal fasted rats.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Group</th>
<th>Blood glucose level mg/dl (Mean T SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fasting</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30 min</td>
</tr>
<tr>
<td>1</td>
<td>Control (0.3% CMC)</td>
<td>72.58 T 0.65</td>
</tr>
<tr>
<td>2</td>
<td>Glibenclamide (7 mg/kg)</td>
<td>76.46 T 1.10</td>
</tr>
<tr>
<td>3</td>
<td>MEDH (200 mg/kg)</td>
<td>77.23 T 0.84</td>
</tr>
<tr>
<td>4</td>
<td>MEDH (400 mg/kg)</td>
<td>70.69 T 0.98</td>
</tr>
</tbody>
</table>
Table 2: Effect of MEDH on blood glucose level in normal rats.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Group</th>
<th>Blood glucose level mg/dl (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fasting</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>Control (0.3% CMC)</td>
<td>72.1 ± 0.58</td>
</tr>
<tr>
<td>2</td>
<td>Glibenclamide (7 mg/kg)</td>
<td>76.33 ± 0.77</td>
</tr>
<tr>
<td>3</td>
<td>MEDH (200 mg/kg)</td>
<td>70.17 ± 1.24</td>
</tr>
<tr>
<td>4</td>
<td>MEDH (400 mg/kg)</td>
<td>75.09 ± 1.27</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD. ANOVA followed by Duncan’s multiple range tests. Values not sharing a common superscript differ significantly at P < 0.05.

D. hamiltonii (200 mg and 400 mg/kg) reduced the blood glucose in diabetic rats as compared to control rats. The 4th day treatment with Glibenclamide and 400 mg of MEDH resulted in significant hypoglycemic effect in diabetic group.

3.4. Effect of MEDH on serum AST and ALT and liver glycogen in alloxan induced diabetic rats

Table 4 showed the level of serum AST and ALT and liver glycogen in normal and experimental rats. There was a significant elevation of serum AST and ALT and decrease in liver glycogen content in diabetic control as compared to non-diabetic control rats. The administration of Glibenclamide (7 mg/kg) and methanolic extract of D. hamiltonii (200 mg and 400 mg/kg) significantly decreased AST and ALT levels and increased glycogen content in diabetic rats as compared to control rats.

3.5. Effect of MEDH on serum cholesterol and triglycerides alloxan induced diabetic rats

There was a significant increase in the cholesterol and triglycerides in diabetic rats as compared to control. The administration of Glibenclamide (7 mg/kg) and methanolic extract of D. hamiltonii (200 mg and 400 mg/kg) brought back the levels of cholesterol and triglycerides to near normal rats (Table 5).

4. Discussion

Diabetes mellitus patients in India are increasing day by day probably due to change in life style change in food pattern i.e. from traditional fiber rich diet to sugary fast food diet and also because of genetic basis. The disorder being chronic in nature needs long term treatment to prevent the complications arising due to persistent high blood glucose level. Pharmacotherapy available for the treatment of diabetes in modern healthcare system includes insulin and oral 16 hypoglycemic drugs. However due to economic constraints, it is not possible for majority of the diabetic patients in developing countries like India to use these drugs on regular basis. Moreover these synthetic antidiabetic drugs are associated with large number of adverse effects. Hence there is increase in the trend to use traditional indigenous plants widely available in India for the treatment of diabetes mellitus. Over 150 plant extract and some of their active principles including flavonoids, tannins, alkaloids etc. are used for the treatment of diabetes.

In the present study, alloxan was used as a diabetogen. It induces diabetes by destroying beta cells of the pancreas partially, through production of relative oxygen species. The present study is the preliminary assessment of antidiabetic activity of methanolic extract of D. hamiltonii in normal and alloxan induced diabetic rats. Alloxan, a beta cytoxixin, induces chemical diabetes by damaging the insulin secreting pancreatic beta cell, resulting in a decrease in endogenous
insulin release, which paves the ways for the decreased utilization of glucose by the tissues.24

In present study, methanolic extract of D. hamiltonii induced a significant decrease in serum glucose level of alloxan induced diabetic rats as compared to diabetic control group. The antidiabetic activity of methanolic extract of D. hamiltonii may be its promote insulin secretion by closure of K⁺-ATP channels, membrane depolarization and stimulation of calcium influx, an initial key step in insulin secretion. In this context, number of other plants has also been reported to have antidiabetic and insulin stimulatory effects.25 Flavanoids, sterols, triterpenoids, alkaloids and phenolics are known to be bioactive antidiabetic principles.26 Flavanoids are known to regenerate the damaged beta cells in the alloxan induced diabetic rats.27 Phenolics are found to be effective antihyperglycemic agents.

Some plants exhibit properties similar to well known sulfonylurea drugs like glibenclamide; they reduce blood glucose in normoglycemic animals. Glibenclamide is reported to enhance the activity of beta cells of pancreas resulting in secretion of larger amounts of insulin, which in turn brings down blood glucose level. Like the plant extract, glibenclamide also produced significant reduction in blood glucose level in alloxan diabetic rats. Since alloxan is known to destroy pancreatic beta cells, the present finding appear to be in consonance with the earlier suggestion of Jackson and Bressler28 that sulfonylurea have extra-pancreatic anti-hyperglycemic mechanism of action secondary to their insulin secreting effect and the attendant glucose uptake into, and utilization by the tissues.

Hyperlipidemia is a metabolic complication of both clinical and experimental diabetes. Previous studies suggested that hyperglycemia and hyperlipidemia are the common characteristics of alloxan induced diabetes mellitus in experimental rats.29 In the present study, total cholesterol and triglycerides were significantly decreased in rats by methanolic extract of D. hamiltonii as compared to diabetic controls. The reduction in cholesterol level may be due to inhibitory effect of methanolic extract of D. hamiltonii on 3-hydroxy-3-methyl-glutaryl Coenzyme A reductase (HMG CoA reductase), the rate-regulatory enzyme of cholesterol biosynthesis30 or by stimulating effect of glucose utilization by peripheral tissues.31 The increased concentration of cholesterol could result in a relative molecular ordering of the residual phospholipids resulting in a decrease in membrane fluidity.32

Accumulation of triglycerides is one of the risk factors in coronary heart disease (CHD). The significant increase in the level of triglyceride of diabetic control rats may be due to the lack of insulin. Since under normal condition, insulin activates the enzyme lipoprotein lipase and hydrolysis triglyceride.33 However, in diabetic state lipoprotein lipase is not activated due to insulin deficiency resulting in hyper-triglyceridemia. Methanolic extract of D. hamiltonii reduces triglycerides in tissues of alloxan-induced diabetic rats and may prevent the progression of CHD.

The abnormally high concentration of serum lipids in diabetes mellitus is mainly due to an increase in the mobilization of free fatty acids from the peripheral fat deposits (adipose tissue) due to the under utilization of the glucose.24 Regarding the mechanism of action of methanolic extract of D. hamiltonii may enhance activity of enzymes involved in bile acid synthesis and its excretion and this may have decreased in serum cholesterol and triglycerides. The lipid lowering effect of the extract might be due to the action of flavanoids and other phenolic compounds, di and triterpenoids, steroids and glycosides. Normalized rate of lipogenesis is due to the insulin-like activity of triterpenoids35 or activating normoglycemia by the insulinitropic effect of flavanoids36 or the lipid lowering property of phenolic compounds.37

Enzymes directly associated with the conversion of aminoacids to ketoacids are AST and ALT. Inflammatory hepatocellular disorders results in extremely elevated transaminase

| Table 4 Effect of MEDH on serum AST and ALT and liver glycogen in alloxan induced diabetic rats. |
|---|---|---|---|
| S. No | Group | AST (IU/L) | ALT (IU/L) | Glycogen (g/100 g) |
| 1 | Control (0.3% CMC) | 49.05 T 2.4⁣a | 55.56 T 3.29⁣a | 3.5 T 0.16⁣a |
| 2 | Diabetic control (Alloxan) | 190.74 T 5.48⁣b | 189.16 T 2.44⁣b | 0.85 T 0.03⁣b |
| 3 | Diabetic Glibenclamide (7 mg/kg) | 79.06 T 2.59⁣c | 87.8 T 2.8⁣c | 0.06 T 0.1⁣c |
| 4 | Diabetic MEDH (200 mg/kg) | 119.78 T 2.83⁣d | 120.6 T 2.8⁣d | 1.8 T 0.13⁣d |
| 5 | Diabetic MEDH (400 mg/kg) | 81.9 T 2.63⁣e | 90.07 T 1.9⁣e | 2.7 T 0.24⁣e |

Values are expressed as mean T SD. ANOVA followed by Duncan’s multiple range tests. Values not sharing a common superscript differ significantly at P < 0.05.

| Table 5 Effect of MEDH on serum cholesterol and triglycerides in alloxan induced diabetic rats. |
|---|---|---|
| S. No | Group | Cholesterol (mg/dl) | Triglycerides (mg/dl) |
levels. The increase in the activities of plasma AST and ALT indicated that diabetes may be induced hepatic dysfunction. Supporting our findings it has been found by Larcan et al. that liver was necrotic in diabetic patients. Chronic mild elevation of aminotransferase is frequently found in type 2 diabetic patients. Therefore, an increase in the activities of AST and ALT in plasma may be mainly due to the leakage of these enzymes from the liver cytosol into the blood stream. On the other hand, treatment of the diabetic rats with methanolic extract of D. hamiltonii caused reduction in the activity of these enzymes in plasma when compared to the diabetic group.

Glucose synthesis in the rat liver and skeletal muscles was impaired during diabetes; hence glycogen content of skeletal muscle and liver markedly decreased in diabetes. Insulin is a stimulator of glycogen synthase system. On the other hand, insulin inhibits glycogenolysis and in lack of insulin, glycogenolysis is not under inhibition of insulin and, therefore, glycogen content of the liver decreases. Since alloxan causes selective destruction of beta cells of islets of pancreas resulting in marked decrease in insulin levels, it is rational that glycogen level in tissues decrease as they depend on insulin for influx of glucose. Treatment with methanolic extract of D. hamiltonii prevented the depletion of glycogen content in liver and skeletal muscle in alloxan-induced diabetic rabbits. This prevention of depletion of glycogen is possibly due to stimulation of insulin release from beta cells.

Further experiments are needed to identify the active components of the root extraction to determine its mechanism of action. Conclusively, it is evident that methanolic extract of D. hamiltonii root contains an anti-hyperglycemic agents capable of lowering blood glucose level and hypolipidemic effect.

Conflicts of interest

All authors have none to declare.

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Authors are thankful to the department of Biochemistry of Muthayammal College of Arts and Science, Raspuram, Tamil Nadu and Dr. B. Duraiswamy, Department of pharmacognosy, ooty, Tamil Nadu for their encouragement and technical support in testing the extracts for activity.

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