3

EXPERIMENTAL PROCEDURE

The aim of the study was to investigate the antilithiatic and antioxidant potential of the selected plants. The study was conducted using *in vitro* and *in vivo* model systems.

The study focused on analyzing the antilithiatic potential of selected medicinal plants. The plants selected for the study were chosen based on their traditional usage for lithiasis. *Tribulus terrestris* (leaf), *Aerva lanata* (flower), *Scoparia dulcis* (leaf), and *Tridax procumbens* (leaf), were collected from Kalapatti, Coimbatore. The leaf and flower samples were identified and certified by the Botanical Survey of India, Coimbatore (BSI/SRC/5/23/2014-15/Tech/19). The leaves and flowers were washed thoroughly in running tap water in order to remove any dirt or soil particles adhered and blotted gently between folds of tissue paper to remove any water droplets. The samples were shade dried and powdered to fine particles in a blender (Multipurpose domestic mixer grinder). The powered sample was sieved using 0.2mm sieve. The extraction was carried out using solvents of increasing polarity by hot percolation method. The residue was dried and used for further analysis.

The study was performed in three distinct phases. In the first phase, the i*n vitro* antilithiatic potential of selected plant extracts was analyzed and the plant with maximum activity was selected. The second phase was formulated to study the *in vivo* protective effects of the selected plant extracts against ethylene glycol induced crystal formation in the kidneys. *In vitro* cell based Assays were performed to study the potency of the extract against crystallization. The third phase, focused on an attempt to identify the major active components in the leaves using various spectral techniques.

The experimental procedure pertaining to the present study "***In vitro* and *In vivo* Investigation of Antilithiatic and Antioxidant Activity of Aqueous Extract of *Aerva lanata*"** are elaborated with the details of the experimental conditions and the steps of the procedures employed to study the various parameter which are presented in this chapter.

PHASE I

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3.2. Assessment of *in vitro* antilithiatic potential of selected plant extracts

3.2.1. *In vitro* calcium oxalate assays

3.2.1.1. Nucleation assay

3.2.1.2. Growth assay

3.2.1.3. Aggregation assay

PHASE II

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3.6.5. FT- IR spectrum

3.6.6. GC-MS

3.6.7. TLC

3.6.8. ^{1}H NMR spectrum

3.7. Statistical analysis

Chemicals

All the chemicals used in the present study were of analytical grade.

Plate 1

Tribulus terrestris

Plate 2

Aerva lanata

Plate 3

Scoparia Dulcis

Plate 4

Tridax procumbens

PHASE I

This phase involves the collection of samples and screening of the plant extracts for its response against the three critical stages of stone formation-nucleation, growth and aggregation.

3.1. Solvent extraction

In order to understand the bioactive principle present in the plant, the powdered samples were sieved (0.2mm), packed in a thimble and subjected to individual extraction using Soxhlet apparatus. Solvents of increasing polarity namely chloroform, methanol and water were used.

The samples were extracted using hot percolation method. The extraction was repeated until the plant material becomes colourless. The extract was evaporated and residue was stored in airtight containers and refrigerated at 4°C.

Preparation of aqueous extract

Aqueous extract of the plant sample also prepared as follows. To 1g of the powdered sample, added 100mL of distilled water and kept in a water bath at 60°C for 2 h. Filtered using Whatman filter paper and centrifuged thrice at 5,000 rpm for 5mins, and the supernatant was collected, evaporated in a flash evaporator and stored in an air tight container in the refrigerator at 4°C.

3.2. Assessment of *in vitro* antilithiatic potential of selected plant extracts

3.2.1. *In vitro* calcium oxalate assay

The solvent extracts of selected medicinal plants were tested for its antilithiatic potential in respect of nucleation, growth and aggregation assays.

3.2.1.1. Nucleation assay

The method used was as described by Hennequin *et al.* (1993) with some minor modifications.

Principle

The nucleation assay is based on the crystal dissolution per cent, absorbance increases with increase in the crystal dissolution which is measured at 620nm spectrophotometrically (Genesys 10-S, USA)

Reagents

1. Tris buffer (0.05M) containing sodium chloride (0.15M), pH 6.5
2. Calcium chloride (3mM)
3. Sodium oxalate (0.5mM)

Procedure

Solutions of calcium chloride and sodium oxalate were filtered thrice through 0.22µm filter, from which 950µL of calcium chloride was mixed with 100µL of extract at different concentrations (50µg - 3200µg/mL). Crystallization was initiated by adding 950µL of sodium oxalate solution. The final solution was magnetically stirred at 800 rpm using a PTFE-coated stirring bar. The temperature was maintained at 37ºC. The optical density of the solution was monitored at 620nm. The rate of nucleation was estimated by comparing the induction time (the delay before the appearance of crystals that have reached a critical size and thus become optically detectable) in the presence of the extract with that of the control in which corm extract was not added.

3.2.1.2. Growth assay

The extent of calcium oxalate crystal dissolution was assessed by the protocol described by Chaudhary *et al.* (2009).

Principle

The rate of crystal growth was determined by the addition of COM crystals to calcium chloride and sodium oxalate solutions in the presence and absence of the extracts. The turbidity is measured at 214nm.

Reagents

1. Tris buffer (10mM) containing sodium chloride (10mM), pH 7.2
2. Calcium chloride (4mM)
3. Sodium oxalate (4mM)
4. COM seed preparation (Pak *et al.*, 1975): COM seed crystals were prepared by mixing equal volumes of 0.01 M calcium chloride and 0.01 M sodium oxalate by drop wise addition of sodium oxalate solution to calcium chloride solution, with constant stirring for 72h at 4ºC. The solution was centrifuged at 2000xg (RCF) for 10min at room temperature. The crystal pellet was washed with distilled water followed by methanol and then air dried and was used for further studies.
5. COM slurry preparation: The crystal slurry was prepared by equilibrating seed crystals in 50mM sodium acetate and adjusts pH at 5.7 by adding glacial acetic acid. To this COM crystals (1.5mg/mL) were added and used for growth assay.

Procedure

4mM calcium chloride and 4mM sodium oxalate of 1mL each were added to a 1.5mL solution containing sodium chloride (10mM) buffered with Tris base (10mM) at pH 7.2. To this 30µl of COM crystal slurry (1.5mg/mL of 50mM sodium acetate buffer of pH 5.7) was added. Consumption of oxalate begins immediately after addition of COM crystal slurry and was monitored for 600 sec for the disappearance of absorbance at 214nm. When the corm extract was added to this solution, depletion of free oxalate ions would decrease if the extract inhibited calcium oxalate crystal growth. Rate of reduction of free oxalate was calculated using the baseline value and the value after 30 sec incubation with or without the extract. The relative inhibitory (Ir) activity was calculated as

$$Ir = \frac{Turbidity_{sample}}{Turbidity_{control}} \times 100$$

3.2.1.3. Aggregation assay

The crystals in solution stick together to form large particles called aggregates and the inhibitory effect in the plant extracts was determined by the protocol described by Hess *et al.* (1989).

Principle

The aggregation assay is based on the crystal dissolution per cent as turbidity increases with increase in the crystal dissolution and measured at 620nm.

Reagents

1. Calcium chloride (50mM)
2. Sodium oxalate (50mM)
3. COM crystal seed preparation: 50mM of calcium chloride and sodium oxalate were mixed and equilibrated to 60°C in a water bath for one hour, cooled and left overnight at 37°C. The crystals were harvested by centrifugation and then completely dried at 37°C.
4. Tris buffer (0.05M) containing sodium chloride (0.15M), pH 6.5

Procedure

COM crystals were used at a final concentration of 0.8mg/mL buffered with 0.05M Tris containing sodium chloride (0.15M) at pH 6.5. Experiments were conducted at 37°C in the presence and absence of the corm extract after the arrest of stirring. The rate of aggregation was estimated as below by comparing the slope of the turbidity in the presence of the extract and with that obtained in the control.

$$Ir = \frac{Turbidity_{sample}}{Turbidity_{control}} \times 100$$

PHASE II

To further substantiate the results of the *in vitro* assays the *in vivo* analyses were performed using male Wistar rats. *In vitro* cell based assays to represent essential aspects of *in vivo* pharmacology and toxicology was performed using NRK 52E cell lines.

3.3. Assessment of antilithiatic potential of *Aerva lanata*

Animal selection

In urolithiatic study, male rats were selected as a model system to induce renal stones as the urinary system of male rats resembles that of humans (Khan, 1997) and males are more prone to kidney stone formation compared to females. Healthy male Wistar albino rats were obtained from Small Animal Breeding Station, Mannuthy, Thirssur, Kerala, India. Animals of 8 weeks old weighing 150-200g were chosen for the study. The animals were acclimatized for two weeks in polypropylene cages and maintained at 27±2ºC, under 12h light/dark cycles, provided with rat chow and drinking water *ad libitum*.

Induction of lithiasis using ethylene glycol

Ethylene glycol is a metabolic precursor of oxalate. The oxalate formation starts after 24-72h of administration. Treatment groups were fed with ethylene glycol (0.75%) in drinking water for induction of kidney stones except the control group of animals.

Induction of lithiasis using ethylene glycol

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Treatment Groups

(5 rats in each group- As instructed by the Institute Animal Ethics Committee)

1. Control (Untreated) - Regular diet and potable water for 28 days
2. Lithiatic control - Ethylene glycol (0.75%) in drinking water for 28 days
3. Extract control - Extract of selected plant (1600µg/kg body weight) administration by gavage for 28 days
4. Preventive regimen - Ethylene glycol (0.75%) in drinking water and selected plant extract administration by gavage for 28 days
5. Curative regimen - Ethylene glycol (0.75%) water for 1-14 days, followed by selected plant extract administration from 15th to 28th day by gavage
6. Standard drug - (Cystone) Ethylene glycol (0.75%) water for 1-14 days, followed by Cystone administration from 15th to 28th day by gavage

This experimental protocol was approved by the Institute Animal Ethics Committee (Approval No. AUW.IAEC.2013-14.BT:05). Urine, serum, liver and kidney homogenates of the control and treatment rats were utilized for the following biochemical assays.

3.3.1. *In vivo* analysis in experimental animals

All animals were kept in individual metabolic cages and the urine was collected on 0, 7th, 14th, 21st and 28th day of the study period. Animals had free access only to drinking water during urine collection period. The urine was analyzed for volume, pH, calcium, oxalate, inorganic phosphorus, uric acid and creatinine.

At the end of the study all the rats were subjected to mild anesthesia (diethylether) and blood was collected by cardiac puncture. Then the rats were killed by cervical dislocation and liver and kidneys were dissected out. This was used for biochemical and histopathological studies.

3.3.1.1. Volume of urine and pH

The volume of urine collected on 0, 7th, 14th, 21st and 28th day was recorded. The pH of the urine was noted using Systronics digital pH meter.

3.3.1.2. Estimation of calcium

Calcium was estimated in the urine and serum by the method proposed by Clark and Collip (1985).

Principle

Calcium is precipitated directly from urine and serum as oxalate. The precipitate was dissolved in acid and titrated against 0.01N Potassium permanganate.

Reagents

1. 4% Ammonium oxalate
2. 2% Ammonia
3. 0.1N Sulphuric acid
4. 0.01N Potassium permanganate

Procedure

To 2.0mL of the urine, 2.0mL of water and 1.0mL of 4% ammonium oxalate was added and allowed to stand overnight. The precipitated calcium oxalate was centrifuged. The supernatant was removed without disturbing the precipitate and 3.0mL of 2% ammonia was added down of the tube, mixed with the precipitate and centrifuged. This was repeated till the supernatant gave no precipitate with calcium chloride. This was done to remove excess of ammonium oxalate. Finally 2.0mL of 0.1N Sulphuric acid was added and mixed well until the precipitate was dissolved. This was warmed by placing in a beaker containing boiling water to complete the dissolution of oxalate. Then it was titrated against 0.01N potassium permanganate while keeping the mixture at 70-75ºC to a faint pink colour which persisted for about one min. A blank was titrated with 2.0mL of 0.1N sulphuric acid to same end point and the difference between the two titre values gave the volume of 0.01N Potassium permanganate required to titrate calcium oxalate precipitate (1mL of 0.01N $KMnO_4$ is equivalent to 0.2mg of calcium).

$$\text{Amount of calcium present in 100ml of urine} = \left(\frac{\text{Titre value}_{sample} - \text{Titre value}_{blank}}{2}\right) \times 0.2 \times 100$$

3.3.1.3. Estimation of oxalate

Oxalate was estimated in the urine by the method proposed by Hodgkinson and Williams (1972).

Reagents

1. Electrolyte Zinc: Electrolyte zinc wire (3mm dia) was cut into the short length measuring approximately 5mm and approximately 250mg was weighed. Immediately before use, the zinc was cleaned by immersing briefly in a freshly prepared 10N HNO_3 (two volumes of concentrated HNO_3 to one volume of water) and washed thoroughly in distilled water.
2. Chromotropic acid solution: 1g of 4, 5, dihydroxynapthalene 2, 7-disulphonic acid and disodium salt “for formaldehyde determination” was dissolved in 100mL of distilled water and stored at 4°C.
3. Oxalic acid standard: 1.023g of potassium oxalate monohydrate was dissolved in 100mL of distilled water and stored at 4°C. This solution contained 5mg of anhydrous oxalic acid per mL.

Procedure

Urine sample was acidified by the addition of Concentrated HCl (1mL per 100mL of the urine) to ensure any crystals of calcium oxalate which may be present were dissolved in the solution. 0.5mL of the urine was transferred into 25mL of graduated stoppered centrifuge tube followed by 1.5mL of water and a drop of 0.04% bromo-thymol blue indicator solution. pH was adjusted to 7 by the addition of 0.1N diluted sodium hydroxide or diluted acetic acid solution.

Then 2mL of the saturated aqueous calcium sulphate solution was added followed by 14mL of ethanol and the contents were mixed gently and allowed to stand at room temperature overnight.

This solution was centrifuged at 2000rpm for 3min. The supernatant fluid was carefully poured off and the tube was allowed to drain for a few min on a filter paper. Mouth of the tube was wiped with clean tissue and the precipitate was dissolved in 5ml 2N H_2SO_4 solution. A piece of the freshly cleaned zinc was added and heated in a boiling water bath for 30 min (the tubes were left without stoppered to allow evaporation to final volume less than 0.5mL)

The zinc was moved to the mouth of the tube with a bent glass rod and washed with 0.5mL of 1% chromotropic. This operation was most conveniently carried out by fixing the tube almost horizontally in a report clamp to allow washing the piece of zinc.

Concentrated H_2SO_4 was added slowly while mixing and heating in a boiling water bath for 30 min. (The tubes need not be stoppered). Then the tubes were cooled and diluted to 20mL with 10N H_2SO_4 and the optical density was measured at 540nm. The developed colour was stable for several hours.

Stock standard oxalic acid solution was diluted 100 times (50µg of oxalic acid per mL). Six tubes containing 0, 0.2, 0.4, 0.6, 0.8, and 1mL of diluted standard oxalic acid solution (corresponding to 0, 10, 20, 30, 40, and 50µg of anhydrous oxalic acid respectively) were prepared along with a blank. Water was added to make the final volume of the reaction mixture to 1mL, followed by 1mL of 4N H_2SO_4 and a piece of freshly cleaned zinc and then proceeded as described in procedure.

The concentration of oxalic acid in the original sample of urine was given by the equation:

$$mg\ of\ anhydrous\ oxalic\ acid/100ml = \frac{Reading\ from\ calibration\ curve\ (\mu g) \times 100}{0.5} \times 1000$$

3.3.1.4. Estimation of phosphate

The amount of inorganic phosphate present in urine and serum was determined by the Fiske and Subbarow method (1925).

Principle

Phosphorus reacts with molybdic acid to form phosphomolybdic acid and the digested solution is treated with ammonium molybdate and 1,2,4 amino naphthol sulphonic acid. It is selectively reduced to produce a deep blue colour (molybdenum blue) which is probably a mixture of lower oxides of molybdenum. The blue colour developed is then compared with the standard treated in the similar manner in a colorimeter at 660nm.

Reagents

1. 10N Sulphuric acid
2. Ammonium molybdate I: 25g of analytical grade ammonium molybdate was dissolved in 200mL of distilled water and transferred to one litre volumetric flask containing 500mL of 10N sulphuric acid and then the volume was made up to the mark with water and mixed well.
3. Ammonium molybdate II: Dissolved 25g of ammonium molybdate in 200mL of distilled water and transferred to one litre volumetric flask containing 300mL of 10N sulphuric acid and then made up to the mark with water and mixed.
4. 1,2,4 amino napthol sulphonic acid (ANSA): Into a glass stoppered cylinder 195mL of 15% sodium bisulphite solution, 0.5g of 1,2,4 amino napthol sulphonic acid and 5mL of 20% sodium sulphate were added and shaken well until the powder was dissolved. The solution was transferred to a bottle and stored under refrigerated condition.
5. Stock standard phosphate solution: To 35.1mg of potassium dihydrogen phosphate in water, 1.0mL of 10N sulphuric acid was added and diluted to 100mL with water and mixed well.

6. Working standard solution: 10mL of stock standard phosphate was diluted to 100mL. One mL of the solution contains 8µg of phosphorus.

Procedure

Into a series of test tubes, 8, 16, 24, 32 and 40µg concentration of standard phosphorous solution was pipetted out. To this one mL of molybdate I solution and 0.4mL of ANSA reagent were added and the volume was made up to 10mL with water. The urine was also treated in a similar way except for the addition of molybdate II, 25µl of urine mixed with 975µl of water and treated as above. The colour developed after 20 min was read in a colorimeter using red filter against a reagent blank at 660nm. Concentration of phosphorus present in the sample solution was calculated by plotting the concentration of phosphorus on X-axis and the colorimeter reading on Y-axis. From the standard graph, the amount of phosphorus present in the urine was calculated.

3.3.1.5. Estimation of uric acid

The amount of uric acid present in the given sample was estimated by the method of Caraway (1955).

Principle

Uric acid reduces sodium phosphotungstate in alkaline medium to give a blue colour which is measured colorimetrically at 640nm.

Reagents

1. 14% Sodium carbonate solution
2. Uric acid reagent: To 50g of sodium tungstate in 400mL of water, added 40mL of 85% phosphoric acid and refluxed gently for 2h. Cooled, transferred to 500mL flask and made up to mark with distilled water. Diluted 1:10 before use.
3. Stock standard uric acid: To 100mg of uric acid with 60mg of lithium carbonate, added 15mL of water. Heated the solution above 60ºC and poured into 100mL standard flask. Made up to the mark with distilled water.

4. Working standard: Diluted 2.0mL of the stock to 100mL with water. This solution contains 20µg of uric acid per mL.

Procedure

To 30µl of urine, added 2.97mL of distilled water. Into different tubes pipetted out 0.5-2.5mL of the working standard, corresponding to 10, 20, 30, 40, and 50µg of uric acid respectively and made up to 3mL, and water served as blank.

In a separate test tube with 8mL of water, added 1mL of serum, 0.6mL of 10% sodium tungstate and 0.5mL of 0.67 N sulphuric acid. Shook well and centrifuged after 15 min. 3mL of the supernatant was taken for the experiment. To all the tubes added 1mL of uric acid reagent followed by 1mL of 14% sodium carbonate and allowed to stand for 25 min for the colour to develop. This was read in a colorimeter at 640nm against a reagent blank.

3.3.1.6. Estimation of creatinine

The amount of creatinine present in urine and serum was estimated by the method of Bones and Taussky (1945).

Principle

The method makes use of the Jaffe's reaction, the production of a mahogany red colour with an alkaline picrate solution. The intensity of the colour developed is compared in a colorimeter against a reagent blank at 540nm.

Reagents

1. 0.04M Picric acid
2. 0.75N Sodium hydroxide
3. Stock solution of creatinine - 100mg of creatinine was dissolved in 0.1N hydrochloric acid and the volume made up to 100mL.
4. Working standard – One mL of stock solution dissolved in 50mL of water. This contains 20µg of creatinine per mL.

Procedure

Tubes containing 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0mL of the working standard solutions corresponding to 10, 20, 30, 40, 50 and 60µg of creatinine were taken. The volume was made up to 3mL with water in all the tubes. 3.0mL of water was taken as blank. One mL of the urine sample was made up to 100mL with distilled water. From this 3mL was taken for the experiment.

For serum creatinine, 3mL of water was added to 2.0mL of serum, 1.0mL of 10% sodium tungstate solution and 2.0mL of 0.67N sulphuric acid and kept for 10 min and centrifuged. 3mL of the supernatant was pipetted out in a test tube. Along with these, a blank was also prepared.

To all the tubes namely the blank, standard, urine and serum tubes, added 1mL of 0.04M picric acid solution and 1mL of 0.75N sodium hydroxide were added and allowed to stand for 20 min for the colour to develop. The tubes were shaken well and the colour so developed was read in a colorimeter at 500nm against the reagent blank.

3.3.1.7. Estimation of magnesium

The amount of magnesium present in urine was estimated by the method of Kolthoff (1927) as described by Sky-Peck (1964).

Principle

The dye, thiazole yellow (methylbenzothiazole- [1,3]- 4,4'-diazoaminobenzol -2,2'- disulfonic acid), combined with magnesium hydroxide in alkaline solution to form a red lake, the intensity of which was proportional to tile magnesium concentration.

Reagents

1. Trichloroacetic acid, 5 and 10 % (w/v)
2. Polyvinyl alcohol, 0.015% (w/v) – Reagent was dissolved by gently warming in water bath and thymol crystals were added as a preservative.

3. Thiazole yellow, 0.0035% (w/v) in 0.015% polyvinyl alcohol. This reagent is light sensitive, and should be stored in brown bottle.
4. Magnesium standard solution (5mg/mL) – In one litre of distilled water 50.67mg of $MgSO_4.7H_2O$ was added.
5. 2N lithium hydroxide – To 83.92g of $LiOH.H_2O$, one litre of distilled water was added and stored in a plastic bottle.

Procedure

One mL aliquot from a well-mixed 24h urine sample was added to 50mL volumetric flask and diluted to the mark with distilled water. To 2mL of aliquot of diluted urine sample, 1mL of 0.0035% thiazole yellow reagent, followed by 1mL of 2N LiOH were added and mixed thoroughly by inversion. The standard was prepared by adding 1mL of 10% TCA to 1mL of the magnesium standard, followed in sequence by 1mL of 0.0035% thiazole yellow reagent and 1mL of 2N LiOH. Blank was prepared by using 1mL of distilled water in place of the standard. All samples were allowed to stand at least for 15 min before reading at 540nm.

The concentration of Mg in urine was calculated using

$$\text{Mg(mg per 24h)} = \frac{\text{O.D. of sample}}{\text{O.D. of standard}} \times \text{Conc.standard}(\mu g) \times 25 \times \text{24h urine volume}$$

3.3.1.8. Estimation of citrate

The amount of citrate present in urine was determined by the method of Millan *et al*. (1987) with a subsequent modification of Levis (1990).

Principle

In alkaline pH, phosphates in the urine were precipitated by magnesium chloride and citrate forms a yellow coloured complex which can be monitored spectrophotometrically at 390 nm.

Reagents

1. 25% Ammonium hydroxide solution.
2. 0.2M Magnesium chloride solution
3. 10M HCl solution
4. Citric acid trisodium salt was used as standard.
5. 18mM Ferric chloride solution was prepared freshly, in deionized water instead of HCl solution.

Procedure

To 0.1mL of ammonium hydroxide (25%), 4mL of sample was added and was mixed well by vortex. To this 0.9mL of magnesium chloride solution was added and mixed on a vortex mixer and the mixture was centrifuged at 4000xg (RCF) for 10 min to obtain phosphate-free urine. The supernatant was then transferred to clear tubes. After adjusting pH of the supernatant to 2 with 0.1mL of 10M HCl, the supernatant was again mixed by vortex. To the mixture 0.25mL of ferric chloride (18mM) was added and mixed on a vortex mixer and absorbance was immediately read against the deionized water at 390nm.

Reagent blank was prepared with the addition of 0.25mL of ferric chloride to 4.75mL HCl solution and read against the deionized water. Test sample was prepared with the addition of 0.75mL of urine sample to 4.25mL of HCl solution and read against the deionized water. Five solutions of citrate (0.312, 0.625, and 1.25, 2.5 and 5.0 mM) were prepared in deionized water and used as standards.

3.3.1.9. Estimation of calcium and oxalate in kidney homogenate

Kidney homogenate were made in 2N HCl by grinding in mortar and pestle. The sample contained equivalent of 100mg of dry weight of kidney in 10mL of hydrochloric acid (Khan *et al*., 2001). The homogenate was heated in water bath at 70ºC for one hour. The solution was later centrifuged at the rate of 2000 rpm for 10 min and calcium was determined in the supernatant as described earlier by the method proposed by Clark and Collip (1985).

Oxalate was precipitated by adding 1M calcium chloride solution and left overnight at 4ºC. The oxalate was then determined by titration with 0.02N KMnO4 while solution was kept at 90ºC. The end point was appearance of pale pink colour which persists for 30 seconds.

3.3.1.10. Estimation of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum, kidney and liver homogenate

Kidney and liver was cut into small pieces on ice and homogenized using mortar and pestle with 10% Tris HCl buffer (0.1M, pH 7.4). The homogenate was centrifuged at 10,000 rpm at 4°C and the supernatant was collected for the estimation of ALT and AST as described by Reitman and Frankel (1957), using Cogent ALT and AST test Kit.

i) Alanine aminotransferase assay

Principle

Alanine aminotransferase catalyzes the transamination of L-Alanine and α-ketoglutarate (α-KG) to form pyruvate and L-glutamate. Pyruvate so formed is coupled with 2,4-dinitrophenyl hydrazine (2,4-DNPH) to form a corresponding hydrazine, a brown coloured complex in alkaline medium and this can be measured colorimetrically (Genesys 10-S, USA).

Reagents

1. Buffered alanine-α-KG, pH 7.4.
2. 2,4-DNPH colour reagent
3. 4N Sodium hydroxide: One mL was diluted to 10mL with distilled water.
4. Working standard: Pyruvate 8mM (150 IU/L).

Procedure

Buffered alanine- α – KG 0.25mL was pipetted out in all the tubes labelled as blank, standard, sample and to control. To the standard tube 0.05mL of standard was pipetted out and to sample tube 0.05mL of kidney or liver homogenate were added, mixed well and incubated at 37ºC for 30 min. To all the tubes added 0.25mL of 2,4-DNPH colour reagent was added. To the blank tube 0.05mL of distilled water was added. They were mixed well and allowed to stand at room temperature (25- 30ºC) for 20 min. At the end of 20 min of incubation 2.5mL of diluted sodium hydroxide was added, mixed well and the O.D. was read against distilled water in a colorimeter using green filter (505nm) within 15 min.

$$ALT\ activity\ in\ IU\ per\ g\ tissue = \frac{A_{sample} - A_{control}}{A_{standard} - A_{blank}} \times Conc.of\ standard$$

ii) Aspartate aminotransferase assay

Principle

Alanine aminotransferase catalyses the transamination of L-Aspartate and (α - KG) to form oxaloacetate and L-Glutamate. Oxaloacetate so formed is coupled with (2,4-DNPH) to form a corresponding hydrazine, a brown coloured complex in alkaline medium and this can be measured colorimetrically (Genesys 10-S, USA).

Reagents

1. Buffered alanine-α-KG, pH 7.4
2. 2,4-DNPH colour reagent
3. 4N Sodium hydroxide: Diluted 1.0mL to 10mL with distilled water
4. Working standard: Pyruvate 6mM (114 IU/L)

Procedure

Pipetted out 0.25mL of buffered alanine- α – KG in all the tubes labelled as blank, standard, sample and to control. To the standard tube pipetted out 0.05mL of standard and to sample tube added 0.05mL of kidney or liver homogenate. Mixed well and incubated at 37ºC for 30 min. To all the tubes added 0.25mL of 2,4-DNPH colour reagent. To the blank tube added 0.05mL of distilled water. Mixed well and allowed to stand at room temperature (25- 30ºC) for 20 min. At the end of 20 min incubation added 2.5mL of diluted sodium hydroxide. Mixed well and read the O.D. against distilled water in colorimeter using green filter (505nm) within 15 min.

$$AST\ activity\ in\ IU\ per\ g\ tissue = \frac{A_{sample} - A_{control}}{A_{standard} - A_{blank}} \times Conc.of\ standard$$

3.3.1.11. Histopathological examination of the kidney architecture

The response of the kidney tissue to lithiatic stress and treatment with the selected plant extract, the tissues were examined for histopathological changes like the necrosis, edema, and changes in nephron, collecting system and peritubular interstitium. The Procedure of Luna (1968) was followed for this study.

Tissue processing

The tissues were placed in 10% formal saline (10% formalin in 0.9% NaCl) for one hour to rectify shrinkage due to higher concentration of formalin. They were then left overnight in running water after securing the mouths of the vessels with cotton gauze. The tissues were dehydrated in ascending grades of isopropanol by immersing in 80% isopropanol overnight followed by 100% isopropanol for one hour. The dehydrated tissues were cleared in two changes of xylene, one hour each. Then the tissues were impregnated with histology grade paraffin wax at 60ºC. The wax impregnated tissues were embedded in paraffin blocks using the same grade wax. The paraffin blocks were mounted and cut with rotary microtome at 3 micron thickness. The sections were flattened on a tissue flatation bath at 40ºC and taken on a glass slide smeared with equal parts of egg albumin and glycerol. The sections were then melted in an incubator at 60ºC and, after 5 min they were allowed to cool.

Tissue staining

The sections were deparaffinised by immersing in xylene for 10 minutes in a staining jar. The deparaffinised sections were washed in 100% isopropanol and stained in Ehrlich’s hematoxylin for 8 minutes. After staining in hematoxylin, the sections were washed in tap water and dipped in acid alcohol (8.3% HCl in 70%alcohol) to remove excess stain. The sections were then placed in running tap water for 10 minutes. The sections were counter-stained in 1% aqueous solution of eosin, for 1 minute. The excess stain was washed in tap water and the sections were allowed to dry. Complete dehydration of the stained sections were ensured by placing the sections in the incubator at 60ºC for 4 minutes. When the sections were cooled, they were mounted in DPX mountant. The cell architecture in the liver was observed under high power objective in a microscope.

In order to minimize the use of animals for research purpose, with a focus to reduce animal sufferings, alternative models were used. The use of alternative experimental systems for studying the antilithiatic property of extracts of selected plant has been standardized, thereby minimizing the use of live animals in future research of lithiasis. This approach is part of a global effort, wherein several alternative systems to replace live experimental animals are being characterized. Towards this purpose the Normal rat kidney cell lines (NRK 52E) were procured from National Centre for Cell Science, Pune, India, and utilized for the study.

3.3.2. *In vitro* analysis using NRK 52E cell lines

The cells were maintained in CO_2 incubator with 5% CO_2 and 95% humidity, supplemented with Dulbecco's Modified Eagles Medium (DMEM) and 10% Fetal Calf Serum (FCS). Penicillin and streptomycin was also added to the medium to 1X final concentration from a 100X stock. Once the cells had attained confluent growth, the cells were trypsinized using Trypsin - EDTA and the number of cells needed for carrying out various assays were seeded into sterile 6-well and 96 well plates. In each well of the 6-well plates, a clean, dry, sterile coverslip was placed before the cells were seeded. Then the plates were incubated in a CO_2 incubator with 5% CO_2 and 95% humidity atmosphere. COM crystals at a concentration of $67 \mu g/cm^2$ (or 0.5mM Oxalate can also be used) was used as lithiatic agent (COM crystals prepared as explained earlier).

The concentration of plant extract used was 1600µg. The cells were treated with the oxalate, both in the presence and the absence of the plant extracts. The exposure of COM crystals were given for 72h at 37ºC. After treatment, the coverslips from the 6-well plates were removed and placed on a glass slide and sealed with vaseline. These slides were used for various staining techniques, whereas in 96-well plates, the medium was removed and replaced with fresh medium. These were used for checking the viability of cells by MTT and SRB assays and cell cytotoxicity by LDH assay as described below.

3.3.1. MTT dye reduction Procedure

The MTT [3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide] reduction assay as described by Igarashi and Miyazawa (2001) was employed to elucidate the cytotoxicity of the sample.

Principle

Living cells convert MTT into its formazon derivative. The number of surviving cells can be determined by the amount of MTT formazon produced, which is measured in a microtitre plate reader after solubilization with a suitable solvent.

Reagents

1. PBS (phosphate buffered saline)
2. MTT – 3mg/mL in PBS
3. Isopropanol in 0.04N HCl (acid-propanol)
4. HCl (0.04N)

Procedure

After the incubation period, the medium was removed. The treated cells (100µl) were incubated with 50µl of MTT at 37°C for 3 h with mild shaking. At the end of the incubation period, 200µl of PBS was added to all the samples and the liquid was carefully aspirated. Acid-propanol (200µl) was added and left overnight in dark. The absorbance was read at 650nm in a microtitre plate reader (Anthos 2020, Austria). The optical density of the oxidant-induced cells were fixed as 100% viabile and the per cent viability of the cells in the other treatment groups were calculated relative to this.

3.3.2. Sulphorhodamine B assay

The sulphorhodamine B (SRB) assay explained by Skehan *et al*. (1990) was employed to determine the cell viability in the presence and the absence of corm extracts in the oxidant-treated cells.

Principle

SRB is a pink coloured aminoxanthane dye with two sulphonic groups. Under mildly acidic conditions, SRB binds to basic amino acids in the proteins in TCA fixed cells to provide a sensitive index of cellular protein content, which is directly proportional to cell viability.

Reagents

1. TCA (40%)
2. TCA (1%)
3. SRB (0.4% in 1% TCA)
4. Acetic acid (1%)
5. Tris (10mM, pH 10.5)
6. PBS

Procedure

After the treatment, the medium was completely removed from each well and washed with 200µl PBS to remove the traces of medium and serum. Ice-cold 40% TCA (350µl) was layered on top of the cells and incubated at 4ºC for one hour, after which the pellet was collected and washed 5 times with cold PBS (200µl). SRB stain (350µl) was added to each well and left in contact with the cells for 30 min at room temperature, after which they were washed 4 times with 350µl of 1% acetic acid to remove any unbound dye. Then, 350µl of 10mM Tris was added to solubilize the protein-bound dye and was shaken gently for 20 min on a gyratory shaker. The Tris layer in each well was transferred to a new 96-well plate and the absorbance was read in a microtitre plate reader (Anthos 2020, Austria) at 496nm. The optical density of the oxidant-induced cells was fixed as 100% viabile and the per cent viability of the cells in the other treatment groups were calculated relative to this.

3.3.3. Lactate dehydrogenase assay

Principle

The target cells are incubated with a cytotoxic agent. During this period, cytoplasmic lactate dehydrogenase (LDH) is released into the medium due to plasma-membrane damage. The LDH activity in the supernatant after pelleting down the cells was measured by a substrate reaction and quantitated with an ELISA plate reader.

LDH was measured using commercial kits according to the manufacturer’s directions (Sigma-Aldrich).

3.3.4. Morphological changes of the cells as observed by Giemsa staining

The morphological changes of the cells were followed in the presence and absence of corm extract and/or oxalate. The treated cells were stained with Giemsa stain and the morphological changes were viewed under Phase Contrast microscope (Nikon, Japan) as explained by Chih *et al*. (2001).

Principle

During cell injury or damage, the cells undergo a series of well-documented morphological changes, which can be observed after staining with Giemsa stain.

Reagents

1. PBS, pH 7.4
2. Liquid Giemsa stain (1:2 dilution in PBS)

Procedure

The diluted Giemsa stain (10µl) was added to the treated cells and the stain was spread by placing a coverslip over it. The cells were observed and photographed under a Phase Contrast microscope (Nikon, Japan) at 400x magnification.

PHASE III

Our next objective was to focus on the enzymic and non-enzymic status of the selected medicinal plant and also it became essential to continue the study to identify the chemical nature of the active component rendering the biochemical activity. Hence, the final phase of the study was formulated to identify the antioxidant status and the active principle(s) rendering the responses evoked by the extract against stone formation.

Natural antioxidants are studied extensively for their capacity to protect organisms and cells from damage induced by oxidative stress (Koksal, 2011). Keeping this in mind, phase III was involved for assessing the antioxidant status of the selected plant sample. Both enzymic and non-enzymic antioxidants were analyzed. The methodology adopted for analyzing these parameters are given below.

3.4. Assessment of antioxidant potential of selected plant extract

The selected plant extract was tested for the activities of enzymic (superoxide dismutase, catalase, peroxidase, glutathione reductase, glutathione-S-transferase and polyphenol oxidase) antioxidants. The procedures used are described below.

3.4.1. Determination of the activities of enzymic antioxidants

3.4.1.1. Assay of superoxide dismutase (SOD)

SOD was assayed according to the method of Kakkar *et al.* (1984).

Principle

The assay of SOD is based on the inhibition of the formation of NADH-phenazine methosulphate-nitroblue tetrazolium formazon. The colour formed at the end of the reaction can be extracted into butanol and measured at 560nm spectrophotometrically.

Reagents

1. Sodium pyrophosphate buffer (0.025M, pH 8.3)
2. Phenazine methosulphate (PMS) (186μM)
3. Nitroblue tetrazolium (NBT) (300μM)
4. NADH (780μM)
5. Glacial acetic acid
6. n-butanol
7. Potassium phosphate buffer (50mM, pH 6.4)

Procedure

Preparation of Enzyme Extract

The fresh plant sample (0.5g) was ground with 3.0mL of potassium phosphate buffer, centrifuged at 2000g for 10 minutes and the supernatants were used for the assay.

Assay

The assay mixture contained 1.2mL of sodium pyrophosphate buffer, 0.1mL of PMS, 0.3mL of NBT, 0.2mL of the enzyme preparation and water in a total volume of 2.8mL. The reaction was initiated by the addition of 0.2mL of NADH. The mixture was incubated at 30°C for 90 seconds and arrested by the addition of 1.0mL of glacial acetic acid. The reaction mixture was then shaken with 4.0mL of n-butanol, allowed to stand for 10 minutes and centrifuged. The intensity of the chromogen in the butanol layer was measured at 560nm in a spectrophotometer (Genesys 10-S, USA).

One unit of enzyme activity is defined as the amount of enzyme that gave 50% inhibition of NBT reduction in one minute.

3.4.1.2. Assay of catalase

The enzyme-catalyzed decomposition of H_2O_2 was measured by the method of Luck (1974) in the selected plant extract.

Principle

The rate of decomposition of H_2O_2 was measured spectrophotometrically from changes in absorbance at 240nm for one min, since H_2O_2 absorbs light at this wavelength.

Reagents

1. Phosphate buffer : 0.067 M (pH 7.0)
2. Hydrogen peroxide (2mM) in phosphate buffer

Procedure

Preparation of enzyme extract

A 20% homogenate of the plant extract was prepared in phosphate buffer at 4ºC. The homogenate was centrifuged and the supernatant was used for the enzyme Assay.

Assay

H_2O_2-phosphate buffer (2.9mL) was pipetted out into a quartz cuvette. The enzyme extract (0.1mL) was rapidly added and mixed thoroughly. The time required for decrease in absorbance by 0.05 units was recorded. The H_2O_2-phosphate buffer served as control.

One enzyme unit was calculated as the amount of enzyme required to decrease the absorbance at 240nm by 0.05 units.

3.4.1.3. Assay of peroxidase

The method proposed by Reddy *et al*. (1995) was adopted for assaying the activity of peroxidase.

Principle

Peroxidase converts H_2O_2 to H_2O and O_2 in the presence of a hydrogen donor pyrogallol. The oxidation of pyrogallol to a coloured product called purpurogalli can be read spectrophotometrically at 430nm. The formation of the product is proportional to the enzyme activity.

Reagents

1. Pyrogallol : 0.05 M in 0.1M phosphate buffer (pH 6.5)
2. H_2O_2 : 1% in 0.1M phosphate buffer, pH 6.5

Procedure

Preparation of enzyme extract

A 20% homogenate of the fresh plant sample was prepared in 0.1M phosphate buffer (pH 6.5), clarified by centrifugation and the supernatant was used for the assay.

Assay

To 3.0mL of pyrogallol solution, 0.1mL of the enzyme extract was added and the spectrophotometer was adjusted to read zero at 430 nm. To the test cuvette, 0.5mL of H_2O_2 was added and mixed. The change in absorbance was recorded every 30 seconds up to 3 minutes in a spectrophotometer (Genesys 10-S, USA). One unit of peroxidase is defined as the change in absorbance/minute at 430nm.

3.4.1.4. Procedure of glutathione reductase

Glutathione reductase (GSH) activity was determined by the method proposed by David and Richard (1983).

Principle

The enzyme glutathione reductase involves in the conversion of oxidized glutathione to its reduced form by using NADPH as a substrate. The amount of NADPH utilized is a direct measure of enzyme activity.

Reagents

1. Phosphate buffer (0.12M, pH 7.2)
2. EDTA (15mM)
3. Sodium azide (10mM)
4. Oxidized glutathione (6.3mM)
5. NADPH (9.6mM)

Procedure

Fresh selected plant extract (0.5g) was crushed and extracted into 2.5mL of phosphate buffer. The debris was removed by centrifugation at 5000g for 10 min and the supernatant was used for the assay.

Assay

The reaction mixture contained in a final volume of 3.0mL, EDTA (0.1mL), sodium azide (0.1mL), oxidized glutathione (0.1mL), enzyme source (0.1mL) and water. The reaction mixture was incubated for 3 min, after which NADPH (0.1mL) was added to the reaction mixture. The absorbance at 340nm was recorded at an interval of 15 sec for 3 min. For each series of measurement, controls were done that contained water instead of oxidized glutathione. One unit of glutathione reductase is expressed as µmole of NADPH oxidized per min.

3.4.1.5. Procedure of glutathione -S-transferase

Glutathione S-transferase was assessed by the method of Habig *et al*. (1974).

Principle

The enzyme is assayed by its ability to conjugate GSH and CDNB, the extent of conjugation causing a proportionate change in the absorbance at 340nm.

Reagents

1. Glutathione (1mM)
2. 1-chloro-2,4-dinitrobenzene (CDNB) (1mM in ethanol)
3. Phosphate buffer (0.1M, pH 6.5)

Procedure

Preparation of Enzyme Extract

The samples (0.5g) were homogenized in a mortar and pestle with 5mL of phosphate buffer. The homogenates were centrifuged at 5000rpm for 10 minutes and the supernatants were used for the assay.

Assay

The activity of the enzyme was determined by observing the change in absorbance at 340nm. The reaction mixture contained 0.1mL of GSH, 0.1mL of CDNB and phosphate buffer in a total volume of 2.9mL. The reaction was initiated by the addition of 0.1mL of the enzyme extract. The readings were recorded every 15 seconds at 340nm against distilled water blank for a minimum of three minutes in a spectrophotometer (Genesys 10-S, USA). The assay mixture without the extract served as the control to monitor non-specific binding of the substrates.

GST activity was calculated using the extinction co-efficient of the product formed ($9.6mM^{-1}cm^{-1}$) and was expressed as nMoles of CDNB conjugated/ minute.

3.4.1.6. Procedure of polyphenol oxidase

Catechol oxidase and laccase activities were estimated simultaneously by the method of Esterbauer *et al*. (1977).

Principle

Phenol oxidases are copper containing proteins that catalyse the aerobic oxidation of phenolic substrates to quinines, which are autooxidized to dark brown pigments known as melanins. These can be estimated spectrophotometrically at 495nm.

Reagents

1. Tris-HCl (50mM, pH 7.2) containing sorbitol (0.4M) and NaCl (10mM)
2. Phosphate buffer (0.1M, pH 6.5)
3. Catechol solution (0.01M)

Procedure

Preparation of Enzyme Extract

The enzyme extract was prepared by homogenizing 0.5g of plant tissue in 2.0mL of the extraction medium containing tris HCl, sorbitol and NaCl. The homogenate was centrifuged at 2000g for 10 minutes and the supernatant was used for the assay.

Assay

Phosphate buffer (2.5mL) and 0.3mL of catechol solution were added in the cuvette and the spectrophotometer was set at 495nm. The enzyme extract (0.2mL) was added and the change in absorbance was recorded for every 30 seconds up to 5 minutes in a spectrophotometer (Genesys 10-S, USA).

One unit of catechol oxidase or laccase is defined as the amount of enzyme that transforms 1µMe of dihydrophenol to 1µMe of quinone per minute.

$$Enzyme\ unit = K \times \frac{\Delta A}{minute}$$

where, K for catechol oxidase = 0.272, K for laccase = 0.242, ∆A = change in absorbance.

3.4.2. Estimation of the levels of non-enzymic antioxidants

The non-enzymic antioxidants analyzed were ascorbic acid, α-tocopherol, total carotenoids, lycopene, reduced glutathione, total phenols, flavonoids and chlorophyll.

3.4.2.1. Estimation of ascorbic acid (Vitamin C)

Ascorbic acid was analysed by the spectrophotometric method described by Roe and Keuther (1943).

Principle

Ascorbate is converted into dehydroascorbate on treatment with activated charcoal, which reacts with 2,4-dinitrophenyl hydrazine to form osazones. These osazones produce an orange coloured solution when dissolved in sulphuric acid, whose absorbance can be measured spectrophotometrically at 540nm.

Reagents

1. TCA (4%)
2. 2,4-dinitrophenyl hydrazine reagent (2%) in 9N H_2SO_4
3. Thiourea (10%)
4. Sulphuric acid (85%)
5. Standard ascorbic acid solution: 100µg / mL in 4% TCA

Extraction of ascorbic acid

Ascorbate was extracted from 1g of the plant sample using 4% TCA and the volume was made up to 10mL with the same. The supernatant obtained after centrifugation at 2000rpm for 10 minutes was treated with a pinch of activated charcoal, shaken vigorously using a cyclomixer and kept for 5 minutes. The charcoal particles were removed by centrifugation and aliquots were used for the estimation.

Procedure

Standard ascorbate ranging between 0.2-1.0mL and 0.5mL and 1.0mL of the supernatant were taken. The volume was made up to 2.0mL with 4% TCA. DNPH reagent (0.5mL) was added to all the tubes, followed by 2 drops of 10% thiourea solution. The contents were mixed and incubated at 37°C for 3 hours resulting in the formation of osazone crystals. The crystals were dissolved in 2.5mL of 85% sulphuric acid, in cold. To the blank alone, DNPH reagent and thiourea were added after the addition of sulphuric acid. The tubes were cooled in ice and the absorbance was read at 540nm in a spectrophotometer (Genesys 10-S, USA).

A standard graph was constructed using an electronic calculator set to the linear regression mode. The concentration of ascorbate in the samples were calculated and expressed in terms of mg/g of sample.

3.4.2.2. Estimation of tocopherol (Vitamin E)

Tocopherol was estimated in the plant samples by the Emmerie-Engel reaction as reported by Rosenberg (1992).

Principle

The Emmerie-Engel reaction is based on the reduction of ferric to ferrous ions by tocopherols, which, with 2,2'-dipyridyl, forms a red colour. Tocopherols and carotenes are first extracted with xylene and read at 460nm to measure carotenes. A correction is made for these after adding ferric chloride and read at 520nm.

Reagents

1. Absolute alcohol
2. Xylene
3. 2,2'-dipyridyl (1.2g/L in n-propanol)
4. Ferric chloride solution (1.2g/L in ethanol)
5. Standard solution (D,L-α-tocopherol, 10mg/L in absolute alcohol)
6. Sulphuric acid (0.1N)

Extraction of Tocopherol

The plant sample (2.5g) was homogenized using mortar and pestle with 50mL of 0.1N sulphuric acid and allowed to stand overnight. The contents of the flask were shaken vigorously and filtered through Whatman No.1 filter paper. Aliquots of the filtrate were used for the estimation.

Procedure

Into 3 stoppered centrifuge tubes, 1.5mL of plant extract, 1.5mL of the standard and 1.5mL of water were pipetted out separately. To all the tubes, 1.5mL of ethanol and 1.5mL of xylene were added, mixed well and centrifuged. Xylene (1.0mL) layer was transferred into another stoppered tube. To each tube, 1.0mL of dipyridyl reagent was added and mixed well. The mixture (1.5mL) was pipetted out into a cuvette and the extinction was read at 460nm. Ferric chloride solution (0.33mL) was added to all the tubes and mixed well. The red colour developed was read exactly after 15 minutes at 520nm in a spectrophotometer (Genesys 10-S, USA).

$$\text{Tocopherol (µg)} = \frac{A_{520} - A_{450}}{A\ of\ standard_{520}} \times 0.29 \times 15$$

The results are expressed as µg tocopherol/g of sample

3.4.2.3. Estimation of total carotenoids and lycopene

Total carotenoids and lycopene were estimated by the method described by Zakaria *et al*. (1979).

Principle

Total carotenoids and lycopene can be extracted in the sample using petroleum ether and estimated at 450nm and 503nm respectively.

Reagents

1. Petroleum ether (40ºC - 60ºC)
2. Anhydrous sodium sulphate
3. Calcium carbonate
4. Alcoholic potassium hydroxide (12%)

Procedure

The experiment was carried out in the dark to avoid photolysis of carotenoids once the saponification was complete. The sample (0.5g) was homogenized using mortar and pestle and saponified with 2.5mL of 12% alcoholic potassium hydroxide in a water bath at 60°C for 30 minutes. The saponified extract was transferred to a separating funnel containing 10-15mL of petroleum ether and mixed well. The lower aqueous layer was then transferred to another separating funnel and the upper petroleum ether layer containing the carotenoids was collected. The extraction was repeated until the aqueous layer became colourless. A small amount of anhydrous sodium sulphate was added to the petroleum ether extract to remove excess moisture. The final volume of the petroleum ether extract was noted. The absorbance of the yellow colour was read in a spectrophotometer (Genesys 10-S, USA) at 450nm and 503nm using petroleum ether as blank. The amount of total carotenoids and lycopene was calculated using the formulae,

$$Amount\ of\ total\ carotenoids = \frac{A_{450} \times 4 \times \mathrm{V} \times 100}{W} mg$$

where, A=absorbance at 450nm, V=Volume of the sample, W=Weight of the sample

$$Lycopene = \frac{3.12 \times A_{503} \times \text{Vol. of the sample} \times \text{dilution} \times 100}{1 \times Wt.of\ Sample \times 1000}$$

The total carotenoids and lycopene were expressed as mg/g plant extract.

3.4.2.4. Estimation of reduced glutathione (GSH)

Reduced glutathione was determined by the method of Moron *et al.* (1979).

Principle

Reduced glutathione on reaction with DTNB (5,5'-dithiobis nitro benzoic acid) produces a yellow coloured product that absorbs at 412nm.

Reagents

1. TCA (5%)
2. Phosphate buffer (0.2M, pH 8.0)
3. DTNB (0.6mM in 0.2M phosphate buffer)
4. Standard GSH (10nMes/mL of 5% TCA)

Extraction of glutathione

A homogenate was prepared with 0.5g of the plant sample with 2.5mL of 5% TCA. The precipitated protein was centrifuged at 1000rpm for 10 minutes. The supernatant (0.1mL) was used for the estimation of GSH.

Procedure

The supernatant (0.1mL) was made up to 1.0mL with 0.2M sodium phosphate buffer (pH 8.0). Standard GSH corresponding to concentrations ranging between 2 and 10 nmoles were also prepared. Two mL of freshly prepared DTNB solution was added and the intensity of the yellow colour developed was measured in a spectrophotometer (Genesys 10-S, USA) at 412nm after 10 minutes. The values are expressed as nmoles GSH/g sample.

3.4.2.5. Estimation of total phenols

The amount of total phenols in the plant tissues was estimated by the method proposed by Mallick and Singh (1980).

Principle

Phenols react with phosphoMybdic acid in Folin-Ciocalteau reagent to produce a blue-coloured complex in alkaline medium, which can be estimated spectro-photometrically at 650nm.

Reagents

1. Ethanol (80%)
2. Folin-Ciocalteau reagent (1N)
3. Sodium carbonate (20%)
4. Standard catechol solution (100µg/mL in water)

Procedure

The sample (0.5g) was homogenized using mortar and pestle with 10X volume of 80% ethanol. The homogenate was centrifuged at 10,000rpm for 20 minutes. The extraction was repeated with 80% ethanol. The supernatants were pooled and evaporated to dryness. The residue was then dissolved in a known volume of distilled water. Different aliquots were pipetted out and the volume in each tube was made up to 3.0mL with distilled water. Folin-Ciocalteau reagent (0.5mL) was added and the tubes were placed in a boiling water bath for exactly one minute. The tubes were cooled and the absorbance was read at 650nm in a spectrophotometer (Genesys 10-S, USA) against a reagent blank. Standard catechol solutions (0.2-1mL) corresponding to 2.0-10µg concentrations were also treated as above.

The concentration of phenols is expressed as mg/g tissue.

3.4.2.6. Estimation of flavonoids

The method proposed by Cameron *et al.* (1943) was used to extract and estimate flavonoids.

Principle

Flavonoids react with vanillin to produce a coloured product, which can be measured spectrophotometrically.

Reagents

1. Vanillin reagent (1% in 70% sulphuric acid)
2. Catechin standard (110µg/mL)

Extraction of flavonoids

The samples (0.5g) were first extracted with methanol : water mixture (2:1) and secondly with the same mixture in the ratio 1:1. The extracts were shaken well and they were allowed to stand overnight. The supernatants were pooled and the volume was measured. This supernatant was concentrated and then used for the assay.

Procedure

A known volume of the extract was pipetted out and evaporated to dryness. Vanillin reagent (4.0mL) was added and the tubes were heated in a boiling water bath for 15 minutes. Varying concentrations of the standard were also treated in the same manner. The optical density was read in a spectrophotometer (Genesys 10-S, USA) at 340nm. A standard curve was constructed and the concentration of flavonoids in each sample was calculated. The values of flavonoids were expressed as mg/g sample.

3.5. Assessment of radical scavenging potential

The scavenging effects of *Aerva lanata* leaf extracts were evaluated against DPPH, ABTS, hydrogen peroxide, superoxide, nitric oxide and hydroxyl radicals.

3.5.1. DPPH

The ability of the leaf extracts to scavenge the DPPH radical was quantified using a spectrophotometric assay.

Principle

DPPH radical reacts with an antioxidant compound that can donate hydrogen, and gets reduced. DPPH, when acted upon by an antioxidant, is converted into diphenyl-picryl hydrazine. This can be identified by the conversion of purple to light yellow colour.

DPPH spectrophotometric assay

The scavenging ability of the natural antioxidants of the leaves towards the stable free radical DPPH was measured by the method of Mensor *et al*. (2001).

Reagents

1. DPPH – 2,2-diphenyl-2-picryl hydrazyl hydrate (0.3mM in methanol)
2. Methanol

Procedure

The selected plant extract (20μl) were added to 0.5mL of methanolic solution of DPPH and 0.48mL of methanol. The mixture was allowed to react at room temperature for 30 minutes. Methanol served as the blank and DPPH in methanol, without the leaf extracts, served as the positive control. After 30 minutes of incubation, the discolouration of the purple colour was measured at 518nm in a spectrophotometer (Genesys 10-S, USA). The radical scavenging activity was calculated as follows:

$$\text{Scavenging activity (\%)} = 100 - \frac{A_{518}\,[\text{sample}] - A_{518}\,[\text{blank}]}{A_{518}\,[\text{blank}]} \times 100$$

3.5.2. ABTS

The antioxidant effect of plant extract was studied using ABTS radical cation de-colourization assay according to the method proposed by Shirwaikar *et al*. (2006).

Reagents

1. ABTS Solution (7mM with 2.45 mM ammonium persulfate).

Procedure

ABTS radical cations ($ABTS^+$) were produced by reacting ABTS solution (7mM) with 2.45mM ammonium persulphate. The mixture was allowed to stand in the dark at room temperature for 12-16h before use. The extract of plant material (each 0.5mL) was added to 0.3mL of ABTS solution and the final volume made up to 1mL with ethanol. The absorbance was read at 745nm and the per cent inhibition was calculated using the formula,

$$\text{Inhibition (\%)} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

The method is applicable to the study of both water-soluble and lipid-soluble antioxidants, pure compounds, and food extracts.

3.5.3. Hydrogen peroxide

The ability of extract of plant sample to scavenge H_2O_2 was determined by the method proposed by Ruch *et al.* (1989).

Principle

H_2O_2 scavenging activity was measured in terms of a decrease in the absorbance at 230nm spectrophotometrically.

Reagents

1. Phosphate buffer (0.1M, pH 7.4)
2. H_2O_2 in phosphate buffer (40mM)

Procedure

The extract of plant sample was diluted to a concentration of 10mg in 10µl. This extract (10µl corresponding to 10mg) was added to 0.6mL of H_2O_2 solution and the final volume was made up to 3mL with the same buffer. After 10 min, the absorbance values at 230nm of the reaction mixtures were recorded against a blank containing phosphate buffer without H_2O_2 for each sample. The per cent inhibition was calculated using the formula,

$$\text{\% scavenging} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

3.5.4. Hydroxyl radical

The effect of extract of plant sample on oxidant-induced damage to deoxyribose *in vitro* was quantified as the amount of thiobarbituric acid reactive substances (TBARS) formed as explained by Elizabeth and Rao (1990).

Principle

The principle of the assay is the quantification of 2-deoxyribose degradation product, malondialdehyde, by its condensation with thiobarbituric acid.

Reagents

1. Deoxyribose (2.8 mM)
2. Ferric chloride (0.1mM)
3. EDTA (0.1mM)
4. H_2O_2 (1mM)
5. Ascorbate (0.1mM)
6. KH_2PO_4-KOH (20mM, pH 7.4)
7. Thiobarbituric acid (TBA (1%)

Procedure

The reaction mixture contained 0.1mL of deoxyribose, 0.1mL of ferric chloride, 0.1mL of EDTA and 0.1mL of H_2O_2. To 0.1mL of ascorbate, 0.1mL of KH_2PO_4-KOH buffer and 20µL of plant extract in a final volume of 1mL was added. The reaction mixture was incubated at 37°C for 1 h. At the end of the incubation period, 1mL of TBA was added and heated at 95°C for 20 min to develop the colour. After cooling, TBARS formation was measured spectrophotometrically at 532nm against an appropriate blank. The hydroxyl radical scavenging activity was determined by comparing the absorbance of the control with that of the samples. The per cent TBARS production for positive control (H_2O_2) was fixed at 100% and the relative per cent TBARS was calculated for the extract treated groups as given below,

$$\%\ \text{scavenging} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

3.6. Characterization of phytochemical constituents of selected plant extract

The phytochemical analyses were performed with the selected plant extract in order to identify the components responsible for stone dissolution.

3.6.1. Preliminary phytochemical analysis

The selected plant extract was screened for the presence of phytochemicals according to the method of Khandelwal (2002).

3.6.1. Preliminary phytochemical analysis

i) Detection of alkaloids

a) **Mayer's test:** A fraction of the extract was treated with Mayer's reagent (1.36g of mercuric chlorate and 5g of potassium iodide in 100mL distilled water) and noted for a cream coloured precipitate.

b) **Dragendroff's test:** A fraction of the extract was treated with Dragendroff's reagent and observed for the formation of reddish orange precipitate. (Dragendroff's reagent: Mixed together bismuth sub-nitrate 1.7g, 20mL of glacial acetic acid, 80mL of water and 50% solution of potassium iodide in 100mL of water. Store as stock solution. Mix 10mL of stock and 20mL glacial acetic acid and make up volume to 100mL with water which gives the working solution).

c) **Wagner's test:** A fraction of the plant extract was treated with Wagner's reagent (1.27g of iodine and 2g of potassium iodide in 100mL of distilled water) and observed for the formation of reddish brown precipitate.

ii) Detection of flavonoids

a) **Aqueous NaOH test:** To a fraction of the extract, 1N aqueous NaOH was added and observed for the formation of yellow-orange colour.

b) **Concentrated H_2SO_4 test:** To a small fraction of the extract, Concentrated H_2SO_4 was added and the orange colour formed was observed.

c) **Schinodo's test:** To a small fraction of the extract, a piece of magnesium turnings was added, followed by concentrated HCl and then heated slightly and the formation of dark pink colour was observed.

iii) Test for steroids

a) **Liebermann-Buchard test:** To the extract, 2mL of chloroform, followed by 10 drops of acetic anhydride and 2 drops of concentrated sulphuric acid were added. The appearance of rose red colour, which quickly changes from blue to green, indicated the presence of steroids.

b) **Salkowski test:** The extract was dissolved in chloroform and shaken well with an equal volume of Concentrated sulphuric acid. The appearance of red colour, in the chloroform layer and green fluorescence in the acid layer indicated the presence of sterol.

iv) Test for terpenoids

a) **Liebermann-Buchard test:** To the extract, 2mL of chloroform, followed by 10 drops of acetic anhydride and 2 drops of Concentrated sulphuric acid were added and for the formation of dark green colour, indicated the presence of terpenoids.

v) Test for tannin

a) **Braemer's test:** To 10mL of water added 0.5 g extract, boiled and then filtered. A few drops of 10% ferric chloride was added to the filtrate. A dark green, blue or brown colour was observed.

vi) Detection of saponins

a) **Foam test:** A fraction of the extract was vigorously shaken with water and observed for persistent foam.

vii) Detection of phenolic compounds

a) **Ferric chloride test:** A fraction of the extract was treated with 5% $FeCl_3$ solution and observed for the formation of deep blue colour.

b) **Lead acetate test:** A fraction of the extract was treated with 10% lead acetate solution and observed for the formation of white precipitate.

viii) Test for carbohydrates

a) **Misch's test:** Few drops of Misch's reagent (α-naphthol dissolved in ethanol) was added to the extract dissolved in distilled water and this was then followed by addition of 1.0mL of Concentrated H_2SO_4 by the sides of the test tube. The mixture was then allowed to stand for two min and then diluted with 5mL of distilled water. Formation of a red or dull violet colour at the interphase of the two layers was a positive test.

b) **Fehling's test:** 0.5g of extract was dissolved in distilled water and filtered. The filtrate was heated with 5mL of equal volumes of Fehling's solution A and B. Formation of red precipitate of cuprous oxide was an indication of the presence of reducing sugars (Fehling's A: 7% copper sulphate solution; Fehling's B: 25% potassium hydroxide and 35% sodium potassium tartarate).

Extraction of alkaloid, flavonoid, steroid, terpenoid, tannin, saponin and phenols

The extraction procedures followed for alkaloid, phenol, flavonoid and saponin were proposed by Harborne (1973). The methodology of extraction followed for steroids and tannins was outlined by Vitale *et al*. (1995) and Obdoni and Ochuko (2001) respectively. Fresh plant material was used for fraction preparation for UV/visible spectral analysis.

i) Total alkaloid fraction

Fresh plant sample (5g) was extracted with 20mL of ethanol: 28% NH_4OH, (95:5) and kept at room temperature overnight. The extract was filtered and concentrated under reduced pressure to a fumy residue, which was extracted twice with 1N HCl (10mL each) and filtered. Alkaloids were liberated at pH 9.8 by the addition of 0.7M Na_2CO_3. The solution was extracted with methylene chloride (3×5mL). The organic extract was dried over anhydrous sodium sulphate to yield the total alkaloid fraction.

ii) Flavonoid fraction

The phenolic extract was further extracted with petroleum ether (3×5mL), where the flavonoids were present in the aqueous fraction. (See phenol fraction preparation in page 90).

iii) Steroid fraction

Fresh plant sample (2g) was weighed and added to 10mL of methanol. It was kept in a water bath for 15 min. The mixture was filtered, condensed and used.

iv) Terpenoid fraction

10g of fresh plant sample was soaked in alcohol for 24 h. Then filtered, the filtrate was extracted with petroleum ether and this ether extract was treated as terpenoids (Ferguson, 1956).

v) Tannin fraction

Suspended the plant sample in methanol was suspended and allowed it to stand overnight. Refluxed it for 4h. The residue was filtered and washed with methanol and allowed to cool down, observed for any modification and used an aliquot of this to assay tannins.

vi) Saponin fraction

Fresh plant sample (20g) was crushed, transferred to a conical flask and 200mL of 20% aqueous ethanol was added. The sample was heated over a hot water bath for 4 h with continuous stirring at 55ºC. The mixture was filtered and re-extracted with another 200mL of 20% ethanol. The combined extracts were reduced to 40mL over a water bath at 90ºC. The concentrate was transferred into a 250mL separating funnel and 20mL of diethyl ether was added and shaken vigorously. The aqueous layer was recovered and the ether layer was discarded. The extraction was repeated twice with the addition of n-butanol. The combined n-butanol extract was washed twice with 10mL of 5% sodium chloride. The remaining solution was heated in a water bath, evaporated and dried in an oven. The saponin content was calculated as gram percentage.

vii) Total phenol fraction

Fresh plant sample (1g) was crushed using a mortar and pestle and extracted with 20mL of 80% ethanol at 80ºC for 15 min. The extract was clarified by centrifugation and used for the analysis of phenols.

3.6.2. UV/ visible absorption spectral analysis

A preliminary absorption spectral analysis was done by a survey scan of the extract of *Aerva lanata* in a nanospectrophotometer (Optizen, Korea). The instrument was set to scan mode and the absorption spectrum was obtained in the range of 190nm to 900nm.

3.6.3. HPLC

The residue of the selected plant extract was dissolved in an appropriate volume of HPLC grade methanol and 20µL of the sample was injected into the apparatus (Shimadzu, Japan, equipped with a PDA detector and a reverse phase C18 column). The sample analysis was performed at room temperature, in the wavelength range of 210-440nm at 1000 psi and the mobile phase used was acetonitrile and water in the ratio of 50:50 with a run time of 60 min of 1mL/min flow rate.

3.6.4. HPTLC

The selected plant extract (100mg) was dissolved in methanol (1mL) and centrifuged at 3000rpm for 5 minutes. The supernatant was collected and used for HPTLC analysis. The test sample (3µl) was loaded as an 8mm band in the 5 × 10 Silica gel G60 F_{254} plate using a Hamilton syringe and CAMAG INOMAT 5 instrument. After saturation with the solvent vapour, the TLC plate loaded with test and the reference was kept in a TLC twin trough developing chamber with the respective mobile phase (given below) and developed up to 90mm.

The developed plates were dried in hot air oven to evaporate the solvents from the plates. The plates were kept in a photo-documentation chamber (CAMAG REPROSTAR 3) and the images were captured in white light, UV 254nm and UV 366nm. After derivatization with the appropriate

Reagents (as given below), the plates were photo-documented at daylight for alkaloids, phenols, flavonoids, saponins, steroids and tannins. The peak table, peak display and peak densitogram of alkaloids, phenolics, flavonoids, saponins, steroids and tannins were noted.

a. HPTLC analysis of alkaloids

The mobile phase used was ethyl acetate: methanol: water (10:1.4:1). The developed plates were sprayed with Dragendroff's reagent followed by ethanol sulphuric acid. Then the plates were heated at 120ºC for 5 min in a hot air oven. Colchicine was used as the reference standard and the presence of alkaloids was confirmed by the appearance of bright orange coloured zones in the daylight mode. (Dragendroff's reagent: Preparation described in 3.6.1. i) b.).

b. HPTLC analysis of flavonoids

The mobile phase used was chloroform: methanol in the ratio of 96:4. The plate was sprayed with 1% ethanol aluminium chloride reagent and heated at 120ºC for 5 min in a hot air oven. Quercetin was used as the reference standard for flavonoid analysis. The presence of flavonoids was confirmed by the appearance of yellow and yellow-green fluorescence at UV 366nm.

c. HPTLC analysis of steroids

Ethyl acetate-methanol-glacial acetic acid-water (10:2.2:1.1:2.6) was used as the mobile phase. The plate was sprayed with anisaldehyde sulphuric acid reagent (1mL of p-anisaldehyde and 1mL Concentrated sulphuric acid in 20mL of ethanol) and dried at 110ºC for 3 min in a hot air oven. Solasodine was used as the reference standard. The presence of steroids was confirmed by the appearance of blue-violet coloured zones in visible day light.

d. HPTLC analysis of terpenoids

n-Hexane: ethyl acetate (7.2:2.9) were used as the mobile phase. The plate was sprayed with anisaldehyde sulphuric acid reagent (1mL of p-anisaldehyde and 1mL Concentrated Sulphuric acid in 20mL of ethanol) and dried at 100ºC for 3 min in a hot air oven. Artemisinin was used as the reference standard. The presence of terpenoids was confirmed by the appearance of blue-violet coloured zones in visible light.

e. HPTLC analysis of tannins

Isobutanol-acetic acid-water (3:1:1) was used as the mobile phase. The plate was sprayed with 5% Ferric chloride reagent and dried at 100ºC for 3 min in a hot air oven. Tannic acid was used as the reference standard. The presence of tannins was confirmed by the appearance of bluish brown coloured zone in visible light.

f. HPTLC analysis of saponins

The mobile phase used was chloroform: acetic acid: methanol: water in the ratio of 6.4: 3.2: 1.2: 0.8. The plate was sprayed with anisaldehyde sulphuric acid reagent (1mL of p-anisaldehyde and 1mL Concentrated Sulphuric acid in 20mL of ethanol) and dried at 110ºC for 3 min in a hot air oven. Saponin was used as the reference standard. The presence of saponins was confirmed by the appearance of blue or yellowish brown coloured zones in visible light.

g. HPTLC analysis of phenolics

The mobile phase used was toluene: chloroform: acetone (4: 2.5: 3.5). After development, the plate was sprayed with 25% aqueous Folin-Ciocalteau reagent and heated at 120ºC for 5 min in a hot air oven. Eugenol was used as the reference standard for the analysis of phenolics. The presence of phenolics was confirmed by the appearance of blue or blue-grey coloured zones at daylight.

3.6.5. FT- IR spectrum

Infrared light from a suitable source passed through a scanning Shimadzu interferometer and Fourier Transformation gave a plot of intensity versus frequency (Light source is Laser). When a powdered plant sample is placed in the beam, it absorbs particular frequencies, so that their intensities were reduced in the inferogram and the ensuing Fourier transform was the infrared absorption spectrum of the sample.

3.6.6. GC-MS spectral analysis

The extract of *Aerva lanata* was analyzed using a Shimadzu Gas chromatography apparatus (Model QP 5000 GC-MS) using a DB-S capillary column (30m) equipped with QP MS detector (EI, 70ev) with helium as a carrier gas at a flow rate of 1mL/min. The compounds were identified using the WILEY database available in the software provided.

3.6.7. TLC

Separation of the extract of *Aerva lanata* using TLC (Harborne, 1973)

The extract was subjected to thin layer chromatography in order to separate the active compounds present. The plates were prepared using a slurry of silica gel G in distilled water. Silica gel G (20g) was added to 40mL of distilled water and a thick slurry was made. All solid particles were blended well and the uniform silica gel slurry was applied onto the TLC plate at a thickness of 0.25mm. The plate was allowed to dry at room temperature. The dried plate was placed in the oven at $100^{o}C$ for 30 minutes to activate the silica gel. The plate was taken from the oven and kept at room temperature for 15 minutes.

Using a microcapillary tube, a small drop of extract of the flowers was placed on the TLC plate, 3cm above the bottom. This spot was allowed to dry and the TLC plate was placed into the TLC chamber which was saturated with the solvent mixture (Chloroform: methanol, (96:4)) carefully to have uniform solvent level. When the solvent reached 2 cm below the top, the plates were taken out of the chamber.

The major bands observed were scrapped out of the plate. This was dissolved in methanol and centrifuged, the supernatant was decanted. Based on the TLC profile, similar bands were pooled and evaporated to dryness and refrigerated. These bands were used for NMR spectroscopy.

3.6.7. ^{1}H NMR spectrum

NMR spectroscopy has been an important analytical tool for investigating natural compounds for many years. It is an excellent alternative to X-ray diffraction for compounds, NMR analysis gives good quality information (e.g., composition, conformation) about the structures of simple natural compounds obtained from plants. The selected plant extract bands from TLC was also subjected to ^{1}H- NMR (Bruker, 200mHz in $CdCl_3$, internal standard TMS). The chemical shift values were recorded as S values/ppm, relative to the TMS.

3.6.8. Statistical analysis

The parameters recorded in all phases of the study were subjected to statistical analysis using SPSS 21 and SAS statistical softwares. *In vivo* experiments were conducted using two factorial CRD and simple CRD and the treatments were compared with Tukey's 5% level of significance.

The results obtained for various parameters were analyzed in all four phases and the salient findings made during the study were presented in the next chapter.