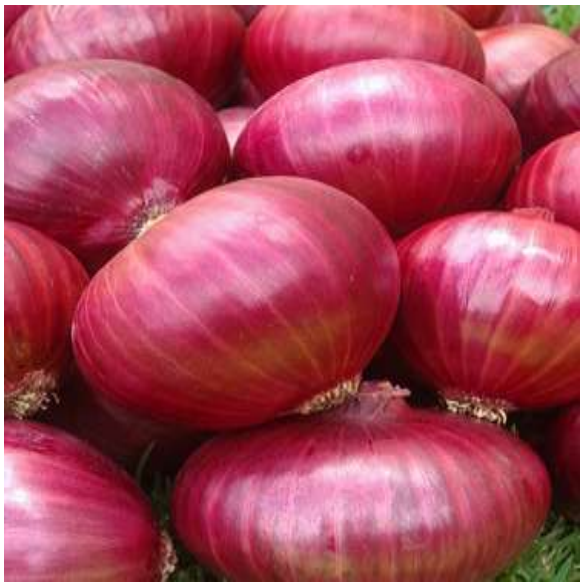
Materials & Methods

Plate 1

***Allium cepa*- Red**



***Allium cepa*- White**

***Allium cepa*- Small**

2. MATERIALS AND METHODS

All chemicals and reagents used in the present study were of molecular and analytical grade and they were purchased from Sigma Chemicals Company, St. Louis, MO, USA; and Sisco Research Laboratories (SRL), Mumbai, India. Quercetin standard was purchased from Sigma Chemicals Company for the assessment of TLC and HPLC. The standard drugs Ciproflaxacin, for Gram positive, Gram negative bacteria and Ketoconazole for fungi were procured from High media Company.

2.1 Materials

2.1.1 Collection of plant material

Fresh onions [Big (Red, White) and Small] [Plate 1] were procured from the local market in Chennai, Tamilnadu, India. The voucher specimens of the plants were authenticated by Prof. A. Manoharan, Taxonomist, Department of Plant Biology and Plant Biotechnology, Presidency College, Chennai, Tamil Nadu, India

2.1.2 Preparation of onion extracts

2.1.2.1 Preparation individual extracts

i) Preparation of individual aqueous extract of *A.cepa* varieties [Big (Red and White) and Small]

Fresh onions were peeled off their outer layer and 1 kg of onion was washed thoroughly with distilled water and then the bulb was cut into pieces and was made into a coar paste. This paste was soaked in 1 litre of distilled water for 24 hours at 4°C it was then filtered thrice using Whatman filter paper. The filtrate was poured

into a beaker and concentrated on a water bath at 100°C to obtain semi-solid residue. The residue was weighed and stored in a refrigerator for further analysis.

ii) Preparation of individual ethanolic extract of *A.cepa* varieties [Big (Red and White) and Small]

After cleaning 1kg of onion as described earlier they were made into a coar paste which was soaked in 500ml of ethanol for 15 days at room temperature then it was filtered using Whatman filter paper and the filtrate was poured into a beaker and concentrated on a water bath at 70-80°C to obtain semi-solid residue. The weight of the yield was noted and then stored in a refrigerator for further analysis.

iii) Preparation of individual chloroform extract of *A.cepa* varieties [Big (Red and White) and Small]

After making a coar paste of 1kg onion of different variety as described earlier they were separately soaked in 300 ml of chloroform for a week at room temperature. It was then filtered using Whatman filter paper and the filtrate was concentrated in a beaker at 60-62°C to obtain semi-solid residue. This was weighed and stored in a refrigerator for further analysis.

iv) Preparation of individual petroleum ether extract of *A.cepa* varieties [Big (Red and White) and Small]

Following the earlier procedure, onion bulb of different varieties were prepared and soaked in 200 ml of petroleum ether for 15 days at room temperature. Then it

was filtered and the filtrate was concentrated at 40-60°C. The extract was weighed and later stored in the refrigerator for further use.

2.1.2.2 Preparation of successive extracts

i) Preparation of successive chloroform extract of *A.cepa* varieties [Big (Red and White) and Small]

Crude content of the petroleum ether extraction was allowed to dry for a day and its weight was noted. To this crude content, 200ml of chloroform was added and kept for a week. After a week, it was filtered and the filtrate was concentrated in a water bath at 60-62°C to obtain the residue. The weight of the extract was recorded and stored in the refrigerator for further analysis.

ii) Preparation of successive ethanolic extract of *A.cepa* varieties [Big (Red and White) and Small]

The crude content of petroleum ether followed by chloroform extraction was allowed to dry for a day and its weight was noted. To this crude content, 200ml of ethanol was added and allowed to stand for a week and then it was filtered. The filtrate was concentrated in a water bath at 70-80°C to obtain the residue. It was weighed and stored in a refrigerator for further studies.

2.2 METHODS

2.2.1 Determination of water content in different *A.cepa* varieties [Big (Red and White) and Small]

Water content of *A.cepa* varieties (Red, white and small onion) was determined using the method of the Nwinuka *et al.* (2005). Thermal drying method was used in the determination of water content of the samples. 10g bulbs of different *A. cepa* varieties were weighed in triplicate and placed in washed, dried and weighed crucible. This was placed in an oven and dried at 105°C (Hot air oven, AUSCO Company, Chennai) for three hours. The samples were allowed to cool in a desiccator and then reweighed. The percentage of water content was calculated by expressing the loss in weight on drying as a fraction of the initial weight of sample used and multiplied by 100.

$$\mathbf{WC\ (\%)= \frac{W_o}{W_i}\ X\ 100}$$

Where, **Wo = loss in weight (g) on drying and Wi = initial weight of sample (g).**

2.2.2 Determination of organic content in different *A.cepa* varieties

From the total weight of *A.cepa* varieties used (10gms), the amount of water, and ash content were subtracted to find out the organic content.

Organic content (%) = Total weight of *A.cepa* used-Water content + Ash content.

Inorganic content (%) = Organic content-Total ash content

2.2.3 Determination of Total ash content in different *A.cepa* varieties

A known weight of *A.cepa* varieties dry bulb which ignited to ash and the weight of the ash thereby obtained was expressed in terms of percentage.

In a clean clay crucible, three varieties of *A.cepa* dry bulb were taken and weighed. Weighed dish was placed over a tripod stand carefully. The crucible were opened partially and directed to the tip of the flame for gradual heating in 500°C. The onion samples were heated gently to avoid catching fire. When the smoke subsides the burner was placed underneath the dish. It was gradually ashed continuously, till it becomes a white ash. Then the dish was cooled to room temperature and weighed with its contents. Again the sample was heated to effect for any possible ashing and weighed. The process was repeated till two consecutive weighing and complete combustion was taken. The total ash was then determined and recorded. The percentage of ash content was calculated using the formula:

$$\text{Ash}\,(\%) = \frac{M_a}{M_s} \times 100$$

Where, **M_a = Mass of ash (g) and M_s = Mass of sample used (g).**

2.2.4 Determination of pH in different *A.cepa* varieties [Big (Red and White) and Small]

pH of different *A.cepa* varieties were determined as per the method of Park and Chin (2010) and the pH values of *A.cepa* were measured using a digital pH-meter (Elico, LII20, digital pH-meter, Taiwan). A 10g bulbs of different *A. cepa*

varieties were homogenized with 90 ml of double distilled water, after which the pH values were measured three times for its concordency and expressed in average values.

2.2.5 Preliminary phytochemical screening of *A.cepa* varieties [Big (Red and White) and Small]

Qualitative phytochemical screening of different extracts (aqueous. ethanol, chloroform, petroleum ether, successive chloroform and successive ethanol) of *A.cepa* varieties [Big (Red, White) and Small] were analyzed in the present investigation by the methods of Harbone and Baxter (1993).

2.2.5.1 Phytochemical qualitative organic analysis of *A.cepa* varieties [Big (Red and White) and Small]

i). Test for phytosterols

Liebermann burchard test:

Extracts of about 10mg were dissolved in 3ml of acetic anhydride. To this solution, 2 drops of concentrated sulphuric acid was added along the sides of the test tube. Appearance of bluish green colour indicated the presence of phytosterols.

ii). Test for triterponoids

To 1ml of the extract, 2 drops of thionyl chloride was added. Formation of pink colour recorded the presence of triterponoids.

iii). Test for flavonoids

To test tube containing 1ml of different extracts, magnesium turnings and 2 drops of concentrated hydrochloric acid were added and heated gently. Formation of red colour indicated the presence of flavonoids.

iv). Test for phenols

To 1ml of the different extracts, 2 drops of alcoholic ferric chloride solution was added. Formation of any colour, confirmed the presence of phenols.

v). Test for tannin

To 1ml of different extracts of *A.cepa,* 0.5ml of 10% lead acetate was added. Appearance of white precipitate indicated presence of tannins.

vi). Test for alkaloids

Hager's test:

Hager's reagent: 1 g of picric acid was dissolved in 100 ml of distilled water.

To 1ml of different extracts *of A.cepa,* 0.5ml of Hager's reagent was added. The presence of alkaloids was confirmed by the formation of yellow precipitate.

vii). Test for saponin

Foam Test:

The different extracts of *A.cepa* was diluted with 1ml of distilled water separately and shaken well. Stable froth formation indicated the presence of saponin.

viii). Test for Acid

To 1ml of different extracts of *A.cepa,* a few drops of sodium bicarbonate solution was added. The formation of effervescence, confirmed the presence of acid.

ix). Test for proteins

Biuret Test:

In test tube containing 1ml of different extracts of *A.cepa,* 1ml of 5% copper sulphate and 1% sodium hydroxide solution was added. Appearance of deep blue colour indicated the presence of protein.

x). Test for carbohydrates

Molisch´s Test

Reagents: 1. α- Napthol (20%) 20gm of α- Napthol was dissolved in 100ml of alcohol.

2. Sulphuric acid

In a test tube containing 2ml of different extracts of *A.cepa,* 2 drops of freshly prepared 20% alcoholic solution of α- napthol was added. To this solution, 2ml of concentrated sulphuric acid was added so as to form a layer below the mixture. Formation of red violet ring at the junction of the solution, confirmed the presence of carbohydrates.

xi). Test for glycosides

Legal´s Test:

To each 1ml of the hydrolysates of different *A.cepa* extracts, 1ml of pyridine and a few drops of sodium nitroprusside solution were added. Then it was made alkaline with sodium hydroxide solution. Appearance of pink colour indicated the presence of glycosides.

2.2.5.2 Phytochemical qualitative inorganic analysis of *A.cepa* varieties [Big (Red and White) and Small]

A. Test for inorganic analysis

Hundred Grams of *A.cepa* varieties were ashed in a furnace, dissolved in distilled water and used for the analysis of acid and basic radicals.

A_1. Test for acid radicals

Acid radicals like sulphate, sulphide, chloride, phosphate, carbonate, nitrate, nitrite, fluoride and oxalate were tested individually using aqueous ash solution of various *A.cepa*.

i). Test for sulphate

To the solution of aqueous ash, 2ml of dilute hydrochloric acid was added, till the effervescence ceases off. To this, 2ml of barium chloride solution was added with which white precipitate was formed. Concentrated hydrochloric acid was added to this white precipitate, insolubility of the precipitate confirmed the presence of sulphate.

ii). Test for sulphide

To the solution of aqueous ash, lead acetate was added. To this solution, 2ml of concentrated hydrochloric acid was added, and heated. Emission of colourless, rotten egg smell indicated the presence of sulphide.

iii). Test for chloride

Diluted nitric acid was added to the aqueous ash of various *A.cepa*, till the effervescence ceases. To this, 2ml of silver nitrate was added. To the cloudy white

precipitate appeared, ammonium hydroxide solution was added. Complete solubility of this precipitate confirmed the presence of chloride.

iv). Test for Phosphate

To the aqueous ash of *A.cepa* varieties, 2ml of ammonium molybdate solution was added. Addition of concentrated nitric acid to this resulted in the appearance of yellowish precipitate which indicated the presence of phosphate.

v). Test for carbonate

Magnesium sulphate solution was added to the aqueous ash of *A.cepa*. A cloudy white precipitate was formed, which confirmed the presence of carbonate.

vi). Test for Nitrite

To the aqueous ash, 2 drops of acetic acid and benzidine were added. Appearance of yellowish red colour indicated the presence of nitrite.

Vii). Test for Nitrate

To the solution of aqueous ash of various *A.cepa,* eight drops of sulfanilic acid and naphthylamine were added. Appearance of red colour confirmed the presence of nitrate.

viii). Test for Fluoride and oxalate

To the solution of aqueous ash of various *A.cepa,* 2ml of diluted acetic acid and calcium chloride were added. Appearance of cloudy white precipitate confirmed the presence of fluoride and oxalate.

$A_{2.}$ Test for basic radicals

i). Test for lead

To 2ml of aqueous ash, 2ml of potassium iodide solution was added. A yellow precipitate, which was then solubled in hot water, was formed. Reappearance of golden yellow spangles on cooling, confirmed the presence of lead.

ii). Test for aluminium

To the aqueous ash of various *A.cepa*, formation of white precipitate was observed. This white precipitate was solubled in excess sodium hydroxide drops. This indicated the presence of aluminium.

iii). Test for iron

Ash of various *A.cepa* were dissolved in dilute hydrochloric acid directly. To this, 2ml of ammonium thiocyanate was added. Appearance of blood red colour indicated the presence of iron.

iv). Test for zinc, magnesium and mercury

To the aqueous ash, sodium hydroxide solution was added in excess. Formation of white precipitate confirmed the presence of zinc. Then this white precipitate was in soluble with the addition of excess sodium hydroxide, which indicated the presence of magnesium. This was then treated with 2ml of sodium hydroxide solution which resulted in the formation of yellow precipitate confirmed the presence of mercury.

v). Test for calcium

To the aqueous ash, 2ml of 4% ammonium oxalate was added. Appearance of cloudy white precipitate indicated the presence of calcium.

vi). Test for sodium

A.cepa paste was made with its ash in hydrochloric acid. When this was introduced into a blue flame, it glowed into a yellow colour. This confirmed the presence of sodium.

vii). Test for potassium

To the aqueous ash of various *A.cepa*, 2ml of sodium nitrite and 2ml of cobalt in 30% glacial acetic acid were added. Formation of yellow precipitate indicated the presence of potassium.

2.2.6 Isolation of phenolic rich fraction from aqueous and ethanolic extracts of *A.cepa* varieties [Big (Red and White) and Small]

Following the phytochemical screening the phenolic compounds in the aqueous and ethanolic extracts of *A.cepa* varieties were analysed. Phenols are a group of naturally occurring compounds widely distributed as secondary metabolites in the plant kingdom. They have been recognized for having interesting clinical properties such as anti-inflammatory, antiallergic, antiviral, antibacterial and antitumoral activities. Hence an attempt was made to analyse the phenolic compound of *A.cepa* on thin layer and column chromatography.

2.2.6.1 Thin Layer Chromatography (TLC)

Thin Layer Chromatography was analyzed by the method of Parthasarathy *et al.* (2009).

Principle

TLC is based on the principle of separation. The separation depends on the relative affinity of compounds towards stationary and mobile phase. The compounds under the influence of mobile phase (driven by capillary action) travel over the surface of stationary phase. During this movement the compounds with higher affinity to stationary phase travel slowly while the others travel faster. Thus separation of components in the mixture is achieved.

Procedure

Thin-Layer Chromatography (TLC) is a simple and inexpensive technique that is often used to judge the purity of a synthesized compound or to indicate the extent of progress of a chemical reaction. A commercially available alumina percolated silica gel TLC plate (merk-60 F254 0.25 mm thick was obtained and then plate was made into cut approximately 1.5 cm wide and 4 cm long. The TLC plate was marked using a pencil. Slightly a straight line was drawn parallel to the short dimension of the plate, about 0.5cm from the one end of the plate to placing the substance spots. Then, a drop of solution containing samples of aqueous and ethanolic extracts of *A.cepa* varieties for separation were placed using capillary tube and spotted on an alumina percolated silica gel TLC plate (merk-60 F254 0.25 mm thick). This process is termed ‘spotting’ and the spot is the origin point for the TLC. The TLC plate was then placed in a

chamber containing a small amount of mixture of hexane and ethyl acetate (8:2) solvent used as a mobile phase. The mobile phase ascended the TLC plate (layer of adsorbent) as a result of the capillary action and reached the sample. Then each compound in the mixture travelled a specific distance along the TLC plate leading to a series of spots. When the solvent front was close to the top of the TLC plate, the plate was then removed from the jar and the position of the solvent front is marked with a pencil. To visualize the spots, (that is to identify the number active compounds present in crude mixture of *A.cepa* extracts) the plate was placed in an iodine chamber for few seconds and bands (compounds) were identified. Each component of the deposited mixture was moved a different distance on the plate by the solvent. The components were then appeared as a series of spots at different locations on the plate. After identifying the suitable solvent system for mobile phase and spots (compounds), presence of quercetin compound in the aqueous and ethanolic extract of *Allium cepa* varieties, was confirmed by running quercetin standard and R_f values of each quercetin and other fractions were calculated by the following formula:

$$\textbf{Retardation factor } (\mathbf{R_f}) = \frac{\textbf{Distance that compound has travelled}}{\textbf{Distance that solvent has travelled}}$$

2.2.6.2 High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography was analyzed by the method of Roldan *et al*. (2008).

Principle

In isocratic HPLC the analyte is forced through a column of the stationary phase (usually a tube packed with small round particles with a certain surface chemistry) by pumping a liquid (mobile phase) at high pressure through the column. The sample to be analyzed is introduced in a small volume to the stream of mobile phase and is retarded by specific chemical or physical interactions with the stationary phase as it traverses the length of the column. The amount of retardation depends on the nature of the analyte, stationary phase and mobile phase composition. The time at which a specific analyte elutes (comes out of the end of the column) is called the retention time and is considered a reasonably unique identifying characteristic of a given analyte. The use of pressure increases the linear velocity (speed) giving the components less time to diffuse within the column, leading to improved resolution in the resulting chromatoGram. Common solvents used include any miscible combinations of water or various organic liquids (the most common are methanol and acetonitrile). Water may contain buffers or salts to assist in the separation of the analyte components, or compounds such as Trifluoroacetic acid which acts as an ion pairing agent.

(i) Extraction, separation, identification and quantification of phenolic rich contents in *A.cepa* extracts

Total phenolic contents in the ethanolic extract of *A.cepa* were analysed by HPLC according to the method of Hertog *et al.* (1992).

Hydrolysis mixture

Extract (10g) plus 25ml methanol and water (80:20, v/v) were mixed with 5ml of 6M HCl solution. Hydrolysis was performed in duplicate. After refluxing at 90°C for 4hours, the extract was allowed to cool, and vacuum filtered, made up to 50ml with methanol. This was sonicated. The extract was filtered through a 0.43µm membranes filter for oraganic solvent prior to injection. Duplicate of 20µl of extract was analyzed by HPLC.

(ii) HPLC analysis of small onion's ethanolic extract

The ethanolic extract of *A.cepa* (Small onion) was analysed in HPLC system from Agilent 1000 series HPLC system, UV- absorbance Diode Array Detector (DAD). The Merk column was C_{18} (4.6 mm X 250 mm, 5 µm at 40°C) and mobile phase constituted of solvent A (0.1% formic acid) and solvent B acetonitrile) with gradient elution, (i.e., solvent B was increased from 7 to 45% within 30 minutes and then dramatically decreased to 7% within 1 minute. The flow rate was 1.4 ml/minutes and detection was made at 275 nm. Identification of total phenolic contents were analysed by HPLC by comparing the retention time of standards.

2.2.7 *In vitro* antioxidant studies

2.2.7.1 DPPH free radical scavenging activity of *A.cepa* varieties [*Big* (Red and White) and Small]

Scavenging of 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical was assessed by the method of Hatano *et al.* (1989).

Principle

The scavenging reaction between DPPH and an antioxidant of the sample (H-A) can be written as:

DPPH + (H-A) ⟶ DPPH-H + (A)

(Purple) (Antioxidant) (Yellow)

Antioxidants of the sample react with DPPH which is a stable free radical and gets reduced to the DPPH-H and as a consequence the absorbance's decreased from the DPPH radical to the DPPH-H form. The degree of discoloration indicates the scavenging potential of the antioxidant compounds of the extracts in terms of hydrogen donating ability.

Reagents

1. **Methanolic solution of DPPH (0.1mM):** DPPH (19.7mg) was dissolved in 500ml of analytical grade methanol.
2. **Ascorbic acid (1%):** Ascorbic acid (1g) was dissolved in 100 ml of methanol.
3. **Extract preparation (Stock):** Each extracts (50mg) were dissolved in 50 ml of analytical grade methanol. The required concentrations of the extracts were diluted accordingly from the stock.
4. **Extract preparation (working)**

   The extract of 0.005ml was made up to 1ml by the addition of 0.995ml of water.

Procedure

DPPH solution (1.0 ml) was added to 1.0 ml of different extracts of *A.cepa* varieties in methanol at different concentrations (5, 10, 15, 20 and 25µg/ml). The mixture was kept at room temperature for 50 minutes and the activity was measured at 517nm. Ascorbic acid at various concentrations (5, 10, 15, 20 and 25µg/ml) were used as standard. The percentage of free radical inhibition was calculated as IC_{50}. It denotes the concentration of the sample required to scavenge 50% of DPPH free radical. The capability to scavenge the DPPH radical was calculated using the following formula,

$$\textbf{DPPH radical scavenged (\%)} = \frac{\textbf{Control OD-Sample OD}}{\textbf{Control OD}} \textbf{ X100}$$

2.2.7.2 Nitric oxide radical scavenging activity of *A.cepa* varieties [Big (Red & White) and Small]

Scavenging of nitric oxide radical was assayed by the method of Garrat, (1964).

Principle

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent scavengers of nitric oxide which compete with oxygen, leading to reduced production of nitrite ions.

Reagents

1. Sodium nitroprusside (10 mM):

Sodium nitroprusside (29.79mg) was dissolved in 100 ml of double distilled water.

2. Phosphate buffer saline (0.1M, pH 7.4):

Sodium chloride (0.8g), 0.2g potassium chloride (KCl), 1.44g sodium orthophosphate ($NaHPO_4$) and 0.024 g of potassium dihydrogen phosphate (KH_2PO_4) were dissolved in 80ml of double distilled water and the pH was adjusted to 7.4 and was made up to 100ml with double distilled water.

3. Sulfanilic acid (0.33% w/v):

Sulfanilic acid (330mg) was dissolved in 100 ml of 20% acetic acid.

4. Naphthyl ethylene diamine dihydrochloride (0.1%, w/v):

Naphthyl ethylene diamine dihydrochloride (100mg) was dissolved in 100ml of double distilled water.

5. Extract preparation (Stock):

Each extracts (100mg) were dissolved in 100ml of analytical grade methanol. The required concentrations of the extracts were diluted accordingly from the stock.

6. Extract preparation (working)

The extract of 0.1ml was made up to 1ml by the addition of 0.9ml of water.

Procedure

The reaction mixture (3ml) containing sodium nitroprusside (10mM, 2 ml), phosphate buffer saline (0.5 ml) and different concentrations (100, 200, 300,400 and

500µg) of extracts of *A.cepa* varieties (0.5ml) were incubated at 25°C for 150 minutes. After incubation, 0.5 ml of the reaction mixture containing nitrite was pipetted out and mixed with 1 ml of sulfanilic acid reagent (0.33% in 20% acetic acid) and allowed to stand for 5 minutes for completing diazotization. Then, 1 ml of naphthyl ethylene diamine dihydrochloride was added, mixed and allowed to stand for 30 minutes at 25°C. A pink colored chromophore is formed in diffused light. Ascorbic acid at various concentrations (100, 200,300, 400 and 500µg) were used as standard. The activity was measured at 550 nm and the results were expressed as % of scavenging using the following formula,

$$\textbf{Nitric oxide radical scavenged (\%)} = \frac{\textbf{Control OD-Sample OD}}{\textbf{Control OD}} \textbf{ X100}$$

2.2.7.3 Super oxide anion scavenging activity of *A.cepa* varieties [Big (Red and White) and Small]

Scavenging of superoxide anion activity was assessed by the method of Liu *et al.* (1997).

Principle

Super oxide anion is generated by Phenazine methosulphate-NADH (PMS-NADH) system by oxidation of NADH and is assessed by the reduction of nitroblue tetrazolium (NBT).

Reagents

1. Tris-Hcl buffer (16µM, pH 8.0):

Tris-HCl (126.08mg) was dissolved in 40 ml of double distilled water. pH was adjusted to 8.0 and then made up to 50 ml with double distilled water.

2. Nitroblue tetrazolium (NBT) (50µM):

Nitroblue tetrazolium (408.82 mg) was dissolved in 10 ml of double distilled water.

3. Phenazine methosulphate (PMS) (10µM):

Phenazine methosulphate (30.63 mg) was dissolved in 10ml of double distilled water.

4. NADH (78µM) for 10 ml:

NADH (517.48 mg) was dissolved in 10ml of double distilled water.

5. Extract preparation (Stock):

Each extracts (100mg) were dissolved in 100ml of analytical grade methanol. The required concentrations of the extracts were diluted accordingly from the stock.

6. Extract preparation (working)

The extract of 0.1ml was made up to 1ml by the addition of 0.9ml of water.

Procedure

Superoxide anions were chemically generated in a mixture of phenazine methosulphate (PMS) and NADH. The reaction was quantified by coupling superoxide generation to the reduction of nitroblue tetrozolium (NBT). In this experiment, the superoxide radicals were generated in 3ml of Tris-Hcl buffer (16mM,

pH 8.0) containing 1ml of NBT (50 µM), 1ml of NADH (78 mM) and 1ml of various concentrations (100, 200, 300, 400, 500 µg/ml) of *A.cepa* varieties extracts. Ascorbic acid at various concentrations (100,200,300,400 and 500µg) were used as standard. The reaction mixture was incubated at 25°C for 5 minutes and the activity was measured at 560nm. Results were expressed as % of scavenging using the following formula,

$$\text{.Super oxide anion scavenged (\%)} = \frac{\text{Control OD-Sample OD}}{\text{Control OD}} \text{X100}$$

2.2.7.4 Protective effect of *A.cepa* varieties [*Big* (Red & White) and Small] varieties on free radical-mediated DNA Sugar Damage

Free radical-mediated DNA sugar damage was assessed by the method of Halliwell and Guteridge (1992).

Reagents

1. Phosphate buffer (0.1M, pH 7.4):

i). NaH_2PO_4 Buffer A:

NaH_2PO_4 0.78g was dissolved in 40 ml of double distilled water, pH was adjusted to 7.4 and made up to 50 ml with double distilled.

ii). Na_2HPO_4 Buffer B:

Na_2HPO_4 (0.88g) was dissolved in 40ml of double distilled water, pH was adjusted to 7.4 and made up to 50ml with double distilled water. 39 ml of buffer A was mixed with 61 ml of buffer B and made up to 100 ml.

2. Ascorbic acid (1mM):

Ascorbic acid (1.76mg) was dissolved in 10 ml of double distilled water.

3. Ferric chloride (100 µM):

Ferric chloride (16.22mg) was dissolved in 10ml of double distilled water.

4. Thiobarbituric acid (TBA) (0.67%):

TBA (0.33g) was dissolved in 50 ml of double distilled water.

5. Calf thymus DNA:

Calf thymus (15mg) was dissolved in 15ml of 0.15M of sodium chloride.

6. Extract preparation (Stock):

Each extracts 100mg were dissolved in 100ml of analytical grade methanol. The required concentrations of the extracts were diluted accordingly from the stock.

7**. Extract preparation (working)**

The extract of 0.1ml was made up to 1ml by the addition of 0.9ml double distilled water.

Procedure

The reaction mixture in a total volume of 1.24ml contained 0.5ml of calf thymus DNA, 0.5ml of phosphate buffer, 0.2ml of ascorbic acid (1mM) and 0.0 4ml of ferric chloride. To this reaction mixture various concentrations of *A.cepa* varieties extracts (100, 200, 300, 400, 500 µg/ml) were added. The reaction mixture was incubated for 1 hour at 37°C. After the incubation, 1ml of 0.67% TBA was added to the reaction mixture and then it was kept in boiling water bath for 15 minutes. The

scavenging activity was measured at 535nm and results were expressed as % of inhibition.

$$\textbf{(\%) of inhibition} = \frac{\textbf{Control OD-Sample OD}}{\textbf{Control OD}} \textbf{X100}$$

2.2.8 Screening of antimicrobial activity

The antimicrobial activities of aqueous, ethanol, chloroform and petroleum ether extracts of various *A.cepa* varieties [Big (Red and White) and Small] were studied. Ciprofloxacin and ketaconazole were used as standard drug.

2.2.8.1 Antibacterial activity

Aqueous, ethanol, chloroform and petroleum ether extracts of *A.cepa* [Big (Red and White) and Small] were subjected for antimicrobial activity by disc diffusion method against both Gram positive and Gram negative organism by the method of Gillespie, (2002).

Organisms used were

Gram positive organisms

1. *Staphylococcus aureus ATCC* 6538
2. *Streptococcus pyogenes* ATCC 14289
3. *Micrococcus luteus* ATCC 9341
4. *Staphylococcus epidermidis* ATCC 155
5. *Streptococcus pneumonia* ATCC 33400
6. *Bacillus subtilis* ATCC 6633
7. *Bacillus cereus* ATCC 11778

Gram negative organisms

1. *Streptococcus mutans* ATCC 25175

2. *Escherichia coli* ATCC 8739

3. *Salmonella typhi* ATCC 6539

4. *Klebsiella pneumonia* ATCC 29665

5. *Pseudomonas aeruginosa* ATCC 9027

Reagents

Nutrient agar medium (Indian Pharmacopoeia, 1996).

10gms of Beef extract, 10gms of peptone, 5gms of sodium chloride and 12gms of agar are dissolved in 1litre of distilled water.

(i) Method

In the present study, the paper disc agar diffusion method was used to evaluate the antimicrobial activity of the various *A.cepa* extracts *in vitro.*

Medium preparation and sterilization

All the ingredients were dissolved in distilled water and gently heated. pH of the medium was adjusted to 8.0 to 8.4 with 5M sodium hydroxide and boiled for 10 minutes then filtered. The filtrate was adjusted to pH 7.2 and sterilized by maintaining at 115°C for 30 minutes.

Procedure

The sterilized (autoclaved at 120°C for 30 minutes) medium (40°C-50°C) was inoculated with the suspension of various microorganisms and poured into petridishes to give a depth of 5mm, various concentration of *A.cepa* aqueous (100,

200, 300µg/ml), ethanol (25, 50, 100µg/ml), chloroform (50,100,150µg/ml) and 50,100,150µg of petroleum ether extracts of the various *A.cepa* (Red, White and Small) were prepared separately. Sterile disc (made from Whatman filter paper is sterilized in uv lamp) dipped in specified concentration of the extracts and standard (ciprofloxacin 50µg/ml). The impregnated discs are allowed to dry and dried discs were placed on the surface of agar plates. A disc dipped in solution of different concentration of *A.cepa* extracts, standard and blank were placed on the surface of agar plates. The plates were left for 1hour at room temperature and incubated at 37°C for 24 hour. The diameter of zone of inhibition of extracts and standards were measured.

2.2.8.2 Antifungal activity

(i) Method

Aqueous, ethanol, chloroform and petroleum ether extract of various *A.cepa* were screened for antifungal activity by disc diffusion method against following organisms as described by Gillespie (2002).

Fungus

1. *Aspergillus niger*
2. *Aspergillus fumigates*
3. *Candida albicans*
4. *Aspergillus flavus*

Saubouraud dextrose agar media

Forty Grams of dextrose, 10g of mixture of equal parts of peptide digest of animal tissue and pancreatic digest of casein, 15g of agar and 1 litre of distilled water.

Media preparation and sterilization

All the above ingredients were dissolved in distilled water and gently heated. pH of the medium was adjusted at 8.0 to 8.4 with 5M sodium hydroxide and boiled for 10 minutes. This was then filtered. The filtrate was adjusted at 5.6. This is sterilized in an autoclave for 30 minutes.

Procedure

Suspension of microorganisms were added to sterile sabouraud dextrose agar medium at 45°C and the mixture was transferred to sterile petridishes and allowed to solidify. Sterile discs dipped in various concentration of *A.cepa* aqueous extract (100,200,300 µg/ml), ethanolic extract (25, 50, 100 µg/ml) and 50,100,150 µg/ml concentration of chloroform extract and 50,100,150µg/ml concentration of petroleum ether extracts of various *A.cepa* (Red,White and Small) were prepared separately. Sterile discs (made from Whatman filter paper are sterilized in uv lamp) dipped in specified concentration of the extracts and standard (ketaconazole 5 µg/ml). The impregnated discs are allowed to dry. The dried discs were placed on the surface of agar plates. A disc dipped in solution of different concentration of *A.cepa* extracts, standard and blank were placed on the surface of agar plated.

The plates were left for 1 hour at room temperature and incubated at 37°C for 48 hour. The diameters of zone of inhibition of extracts were measured.

2.2.9 Antihelminthic activity

2.2.9.1 Collection and maintenance of parasites *Cotylophoron cotylophorum* (Plate, 2)

C.cotylophorum is a parasite selected for the present study as it is commonly encountered species of paramphistome in the rumen of sheep (*Ovis aries*).

2.2.9.2 Taxonomical position of *C.cotylophorum*

Kingdom : Animalia

Phylum : Platyhelminthes

Class : Trematodes

Sub class : Digenea

Order : Paramphistomida

Super family: Paramphistomidae

Family : Paramphistomatidae

Genus : *Cotylophoron*

Species : *cotylophorum* (Fischoeder, 1901)

2.2.9.3 Taxonomical position of the permanent host of *C.cotylophorum*

Kingdom : Animalia

Phylum : Chordata

Sub phylum : Vertebrata

Class : Mammalia

Order : Artiodactyla

Family : Bovidae

Sub family : Caprinae

Genus : *Ovis*

Species : *aries* (Linnaeous, 1758)

Naturally infected hosts (sheep) were the source of the parasites used in the present study. These parasites were collected immediately after slaughtering the hosts at a local corporation slaughter house at pulianthope, Chennai. *C.cotylophorum* were obtained from the rumen of the Indian sheep *Ovis aries*. The parasites were kept in physiological saline and brought to the lab immediately. The specimens were washed thoroughly several times in saline to eliminate possible contamination from the host and finally in distilled water.

Preparation of medium

Hedon fleig solution:

Nacl	-	7g
KCl	-	0.3g
$CaCl_2$	-	0.1g
$NaHCO_3$	-	1.5g
Mg_2SO_4	-	0.2g
$C_6H_{12}O_6$	-	1g

All the above ingredients were dissolved in 1 litre of distilled water. The pH was maintained at 7.2 for further experimental use.

2.2.9.4 Antihelminthic activity of different extracts of various *A.cepa* varieties [Big (Red and White) and Small onion]

Procedure

After cleaning with normal saline, 10 *Cotylophoron cotylophorum* of uniform size were carefully transferred to 100ml of Hedon fleig (pH 7.2) medium with 1% glucose and maintained. In the present investigation in all survival studies attempted were made to maintain *C. cotylophorum* in aerobic conditions.

C. cotylophorum with uniform size were collected and washed several times in normal saline. 10 *C. cotylophorum* were acclimatized in hedon fleig (pH 7.2) medium with 1% glucose for 6 hours. After the time, the worms were transferred to fresh glucose enriched hedon-fleig (pH 7.2) medium, having different concentration of different extracts of *A.cepa* varieties. Survival of *C.cotylophorum* was noted at an interval of every six hours for 12h, 24h and 48h incubation. Dead parasites were removed to avoid contamination. The lethal concentrations of the extracts were recorded.

2.3 Statistical analysis

All the samples and readings were prepared and measured in triplicate. To evaluate antioxidant activity, the data were subjected to statistical analysis using one-way analysis of variance (ANOVA) and Tukey's multiple range test to assess the significance of individual variations between the groups and results were expressed as mean ± SD using a computer-based software (SPSS 7.5 for windows student version; SPSS, Chicago, IL). In the Tukey's test, the significance was considered at the level of $p<0.05$ (Votto *et al.,* 2011).

Plate 2

Cotylophoron cotylophorum