Identification, Characterization and Isolation of Bioactive compounds from Plant sources

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Abstract
Bioactive compounds from plants provide us the alternative sources for drugs or medicines with minimum side effects. Due to the demand of the plant-based drugs, more research has been attempted for the extraction of valued compounds from plants. The phytochemical analysis and biological activities like anti-oxidant, anti-inflammatory have also been studied.

Keywords: Bioactive compounds, Anti-oxidants, DPPH, Phytochemical constituents, Anti-inflammatory assays.

Introduction

Plant extracts either as powdered form or liquids form are used to prepare new varieties of drugs. Humans mostly depend on plants and plant products for health remedies. India is the place where diverse varieties of medicinal plants are available to cure different diseases. *Ficus, Ixora, Solanum lycopersicum, Acacia, Polyalthia longifolia, Tabernaemontana divaricate, Centella, Costus, Syzygium cumini, Polyalthia longifolia, Solanum lycopersicum* are few species that exhibit anti-microbial activity and possess secondary metabolites that play an important role in defense mechanism. It is reported that the plant extracts possess many useful important compounds for human health like anti-oxidants, anti-inflammatory, anti-proliferative properties etc. which play a beneficial role in human life.

The process of extracting medicinal compounds from plants is done through many years. At present, characterization of the bioactive compound is quantified through biomolecule assay, phytochemical assays, anti-oxidant assays, anti-microbial assays etc. on selected plant sources.

Material and Methods

Collection of plant samples: The tropical areas of Visakhapatnam are where the plant leaves are gathered. At first, leaves are washed thoroughly and incubated for 24 hours in phosphate buffer solution. The next day, leaves are ground in motor and pestle to fine paste. The paste is then filtered and centrifuged for 10 minutes at 4000 rpm. Eppendorf tubes are used to collect and keep supernatant.

These collected plant extracts are subjected for biomolecule assay for the estimation of protein by Lowry's method and used to study anti-microbial activity and to find out secondary metabolites with the help of phytochemical assays and to access their anti oxidative capacity, anti-inflammatory assay and anti proliferative assays.

Phytochemical analysis: Various phytochemical analyses were done to find the amount of activity exhibited by the collected plant samples. Assays on phytochemicals and assays of biomolecules were performed. The most important of these plant phytochemicals are flavonoids, alkaloids, tannins, terpenoids, glycosides, quinones, saponins, coumarins, anthocyanins and phenolic compounds.

These compounds are responsible for biological actions including antioxidant, anti-inflammatory, anti-microbial, immune modulatory and anti-proliferative activities. The immune system can be stimulated or suppressed by chemicals known as immunomodulators. Immunosuppressants and immunological stimulators are substances that either suppress or activate the immune system. Assay of biomolecules was conducted on both proteins and carbohydrates. Estimation of proteins on different plant extracts was done by Lowry's method and estimation of carbohydrates was done by Anthrone method.

Assays on phytochemicals: The lives of living things depend heavily on plants. The majority of plants have therapeutic qualities. Both primary and secondary metabolites are present in plants. Flavonoids, saponins, glycosides, tannins, alkaloids, coumarins, glycosides, terpenoids and anthocyanins are examples of secondary metabolites with therapeutic significance that are used to treat a variety of illnesses[5].

Few of the most important plants that grow in our surroundings that possess medicinal properties are discussed here namely *Ficus, Ixora, Solanum lycopersicum, Tecoma, Chrysanthemum, Tagetus erecta, Solanum melongena, Acacia, Polyalthia longifolia, Tabernaemontana divaricata, Centella, Costus, Syzygium cumini.*

At first these plant leaves were collected, washed and soaked in PBS for 24 hours and ground with the help of motor and pestle to make a fine paste and filtered with the help of Whatmann filter paper. Centrifuge those plants extracts at 4000 rpm for 10 minutes after filtration is complete. Phytochemicals are detected using the collected supernatant[19].

a) Test for Phenols - Ferric chloride test: 0.5ml of a 5% $FeCl_3$ solution is added to 3ml of the test solution. Phenols are present when bluish black or brownish green colour is formed[13].

b) Test for Saponins - Foam test: 3ml of 1% distilled water should be added to 1ml of plant extract. The presence of saponins is shown by the formation of stable foam[1].

c) Test for Tannins - Braymer's test: To 1ml of filtrate, add 3ml of distilled water and 3 drops of 10% $FeCl_3$ solution. The presence of tannins is indicated by the bluish green colour.

d) Test for Flavonoids: Add 2ml of 10% lead acetate solution to 1ml of plant sample. Precipitate with a yellow colour is formed when flavonoids are present[2].

e) Test for Quinones - Sulfuric acid test: To 1ml of concentrated liquid for every 1ml of extract, add H_2SO_4. Quinones are indicated by the red colour[14].

f) Test for Alkaloids - Mayer's test: 2 drops of potassium mercuric iodide should be added to 1 millilitre of extract to detect the presence of alkaloids by their pale cream colour[10].

g) Test for Terpenoids - Salkowski's test: Add 2ml of chloroform and 1ml of concentrated H_2SO_4 to 5ml of the sample. When a reddish brown precipitate is formed at the contact, terpenoids are present[6].

h) Test for Glycosides - Legal test: Add 1ml of the 0.3% sodium nitroprusside reagent and 2 drops of 10% sodium hydroxide to 5ml of the extract. Glycosides are indicated by a pink to red colour[4].

i) Test for Coumarins - NaOH test: Add a few drops of chloroform and 1ml of 10% NaOH to 1ml of sample. The presence of coumarins is indicated by the yellow colour[12].

j) Test for Anthocyanins: To 2ml of sample extract, add 2ml of 2N HCl and mix them well. Pinkish red colour indicates the presence of anthocyanins[16].

Quantitative Assay of Biomolecules: Various assays on biomolecules were conducted on selected plant extracts. Biomolecules like proteins and carbohydrates are estimated by using Lowry method for protein estimation and Anthrone method for carbohydrate estimation.

Estimation of proteins: Biomolecule assay by the estimation of protein was done on *Ficus, Ixora, Solanum lycopersicum, Tecoma, Chrysanthemum, Tagetus erecta, Solanum melongena, Acacia, Polyalthia longifolia, Tabernaemontana divaricata, Centella, Costus, Syzygium cumini*. The reduction of copper ions in an alkaline environment, which results in the formation of a complex containing peptide links, is the basic idea of Lowry's procedure. The reduction of Folin's reagent by the copper-peptide bond complex also results in a colour shift of the solution to blue, with a calorimeter reading of 650–750 nms for the absorption[21].

Lowry's method: It is the simplest, accurate method to determine the protein concentration. At first, solution A containing NaOH and Na_2CO_3 are prepared. Then solution B with sodium potassium tartrate and copper sulphate solution is prepared. Solution C is prepared by taking solution A and solution B in 4:1 concentration[21]. Take a test tube for blank and six test tubes for samples.

As a reference protein, bovine serum albumin is added to all test tubes with the exception of the blank. With distilled water, each test tube's volume is made up to 1 ml. Folin's reagent and solution C are also added to each test tube in an identical amount. The tubes were incubated with Folin's reagent for 30 minutes, after which the absorbance at 660 nms was measured using a calorimeter. Protein concentration is computed and a typical graph is drawn with samples on the X-axis and protein concentration on the Y-axis.

Assay of Biological activities: The biological activity of selected plant samples was estimated by different assays like anti-microbial assay, antioxidant assay, anti proliferative assay and anti-inflammatory assay.

Antioxidant Assay: Anti oxidative properties of the selected plant extracts were calculated by the following techniques: Anti-oxidant assays are of two categories which includes enzymes and which do not include enzymes i.e. Enzymatic parameters and non-enzymatic parameters. Enzymatic parameters include catalase assay and ascorbic acid oxidase assay and non enzymatic parameters include DPPH, FRAP, TAC, ascorbic acid assay.

i) Enzymatic parameters

(a) Catalase Assay: At first sample extracts were prepared. To 0.5ml of sample extract, add 2.5ml of 0.9% hydrogen peroxide. Incubate at 28℃ for 3mins[7]. After incubation, add 5ml of conc. sulfuric acid to arrest the reaction. Now the above solution is titrated against potassium permanganate sol. Titrate until the colour changes to pink. 8ml of 0.1M phosphate buffer with a pH of 7.5 are used to maintain a blank. A graph is plotted taking % inhibition at X axis and concentration of sample at Y axis.

(b) Ascorbic acid oxidase Assay: It is simple and accurate method to analyze percentage antioxidant nature of the given compound. To 2 ml of sample extract, add 8ml of reaction mixture.100 µl of ascorbic acid (0.5 mM), 600 µl of potassium phosphate buffer (50 mM) and 100 µl of H_2O_2 made up the reaction mixture (0.1 mM). For five minutes, the optical density was measured at 290 nm every 30 seconds against a blank screen. The protein unit[9] used to express the enzyme activity was mg^{-1}.

ii) Non enzymatic parameters

(a) Ascorbic acid Assay: A blank is made by mixing together 1 ml of acetic acid and 5 ml of distilled water in a test tube. A standard is made by mixing together 1 ml of acetic acid and 5 ml of standard ascorbic acid. Add 1 ml of acetic acid to the test samples and thoroughly combine them. Blank, standard and test samples are titrated against dye until the colour changes to pink. Dye is prepared by taking 20mg

of 2,6-dichloro phenol indophenol[20] and 16mg sodium bicarbonate in 500 ml of distilled water. % inhibition is calculated and a standard graph is plotted.

(b) DPPH (2,2-diphenylpicrylhydrazyl) Assay: The main reason to follow this method is simple and is highly sensitive[15]. To 0.07 mg DPPH, add 100ml of methanol and wait for half an hour. After incubation add 3ml of DPPH in 1ml sample extract and keep at room temperature for 40mins[17]. Colour changes can be observed from purple to light yellow. An accurate absorbance reading at 517nms is taken. % Inhibition was calculated and standard graph is plotted. Here as the absorbance increases, antioxidant activity decreases.

(c) FRAP (Ferric reducing ability of plasma) Assay: This is one of the reliable techniques used. 2.5ml of 0.2M phosphate buffer should be added to 1ml of sample extracts and then maintained at pH 6.6 and mix them well with 2.5ml of 1% potassium ferrocyanide[3]. Incubate in water bath at 50℃ for 20mins. After incubation, add 2.5ml of 10% Trichloroacetic acid. Centrifuge the whole solution at 3000rpm for 5 minutes. Collect 2.5ml of top most supernatant and transfer into another test tube. 2.5 ml of distilled water and 0.5 ml of 0.1% ferric chloride should be added to it. Blue colour is observed. Absorbance readings taken at 670nm. A standard graph is plotted.

(d) TAC (Total Antioxidant Capacity) Assay: The phosphomolybdenum test was used to evaluate total antioxidant activity. To 0.1ml of sample extract, add 1ml of reaction mixture and incubate for 90mins at 95℃ in boiling water bath[8]. These tubes underwent a 20–30-minute normalization period at room temperature following incubation.

Molybdate Reagent Solution Preparation: In 20 ml of distilled water, 1 ml each of 0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate were added. The volume was then increased to 50 ml by adding more distilled water[18]. An absorbance reading at 675nms has to be taken. The positive reference standard utilized was ascorbic acid. For each extract, the average values from three different samples were calculated. A milligram of gallic acid equivalent per gram of dry weight is used as the unit of measurement.

Anti-microbial assay: At first, nutrient agar is prepared and poured in Petri plates. Allow for solidification. After agar gets solidified, bacteria were streaked on agar in their respective plates. On the agar plates, wells were subsequently perforated. In accordance, plant extracts and antibiotics were added to the wells. At 37°C, plates were incubated for 24 hours. Zone of inhibition is seen after 24 hours of incubation. It measures this zone of inhibition. Maximum is the zone of inhibition; maximum is the antimicrobial activity[11].

Lowry's method: The total protein concentrations in different plant extracts was estimated.

Table 1
Phytochemical analysis

TEST	Ficus	Ixora	S. Lycopersicum	Tecoma	Chrysanthemum	T. Erecta	S. Melongena	Acacia	P. Longifoloia	T. Divaricata	Centella	Costus	Syzygium	Syzygium
Phenol	_	+	+	+	+	+	_	+	_	+	+	+	+	
Saponins	_	+	+	+	+	+	+	_	+	+	+	+	+	
Tannins	+	+	+	_	+	+	+	+	+	_	+	_	+	
Flavanoids	+	+	+	+	+	+	_	_	+	+	+	_	+	
Terpenoids	_	+	+	_	_	+	+	_	+	_	+	+	+	
Alkaloids	+	+	_	+	+	+	+	_	_	+	_	_	+	
Quinones	_	+	_	+	_	_	+	_	+	+	_	_	+	
Glycosides	_	+	_	+	+	+	+	+	+	_	_	_	+	
Coumarins	+	+	_	+	+	+	+	+	_	+	+	+	+	
Antocyanin	_	+	+	_	_	_	+	_	_	_	_	_	+	

Here + indicates positive response for that particular activity. -- indicates negative response for that particular activity.

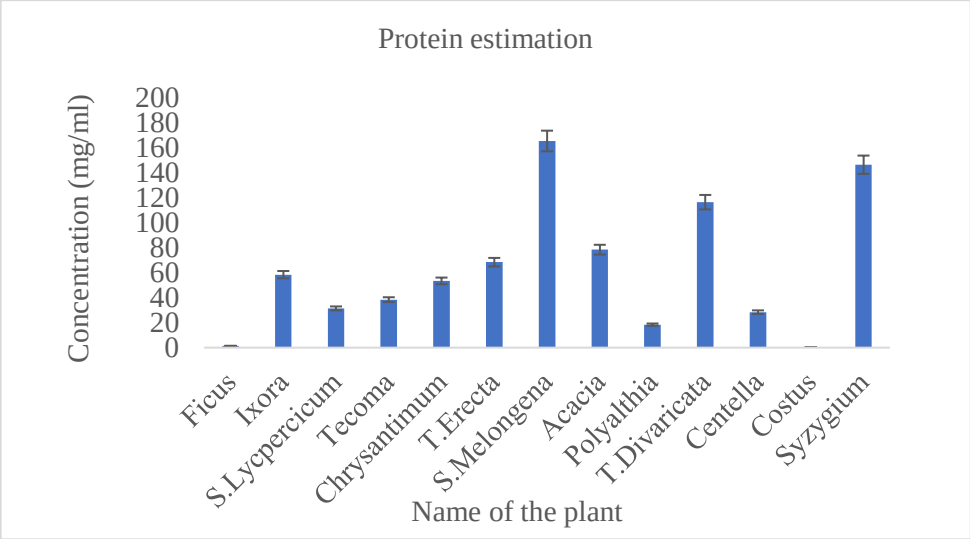

Fig. 1: Estimation of protein by Lowry's method was done. On X-axis, name of the plant is taken and on Y-axis concentration of the protein in mg/ml is taken.

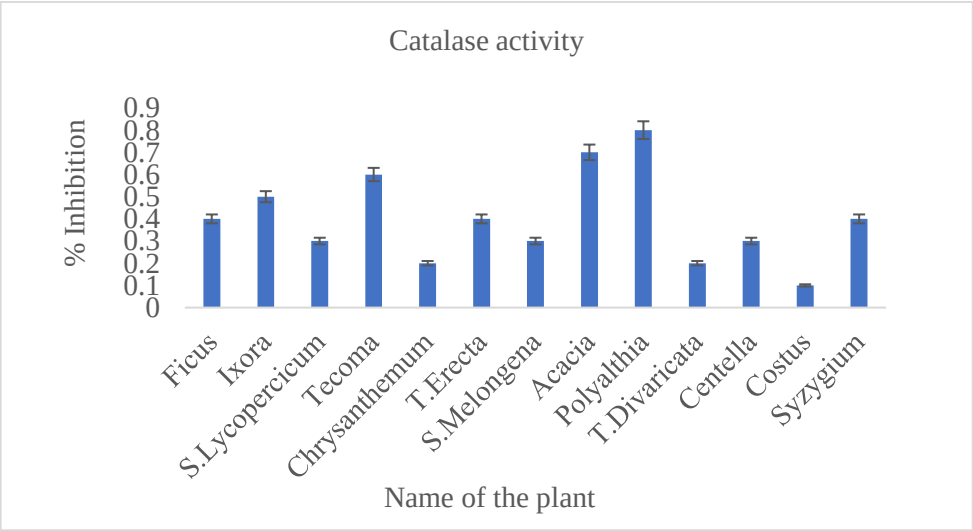

Fig. 2: Percentage inhibition is calculated in catalase activity. Name of the plant is taken on X-axis and % inhibition readings are taken on Y-axis.

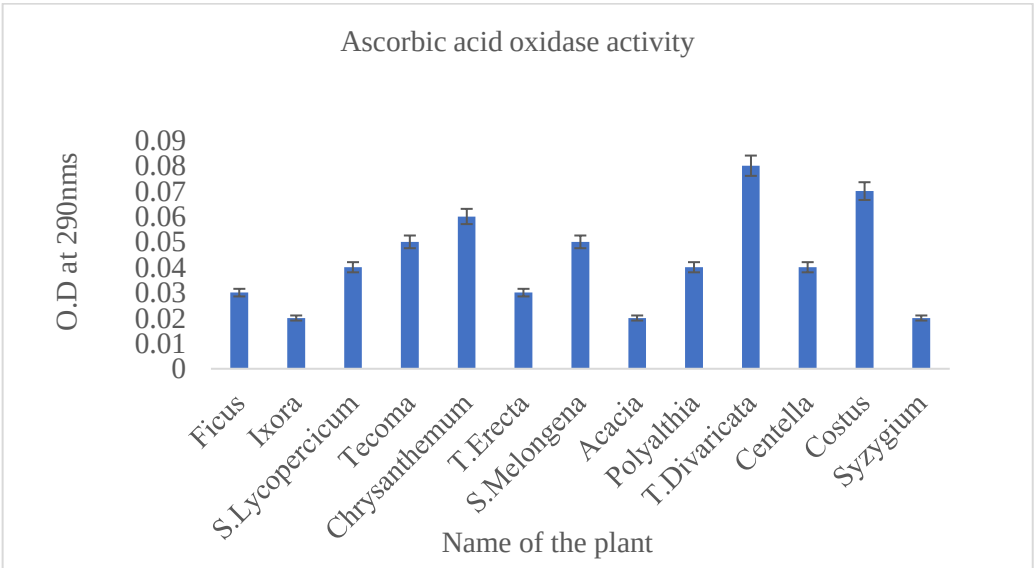

Fig. 3: Name of the plant is taken on X-axis and optical density values are taken on Y-axis.

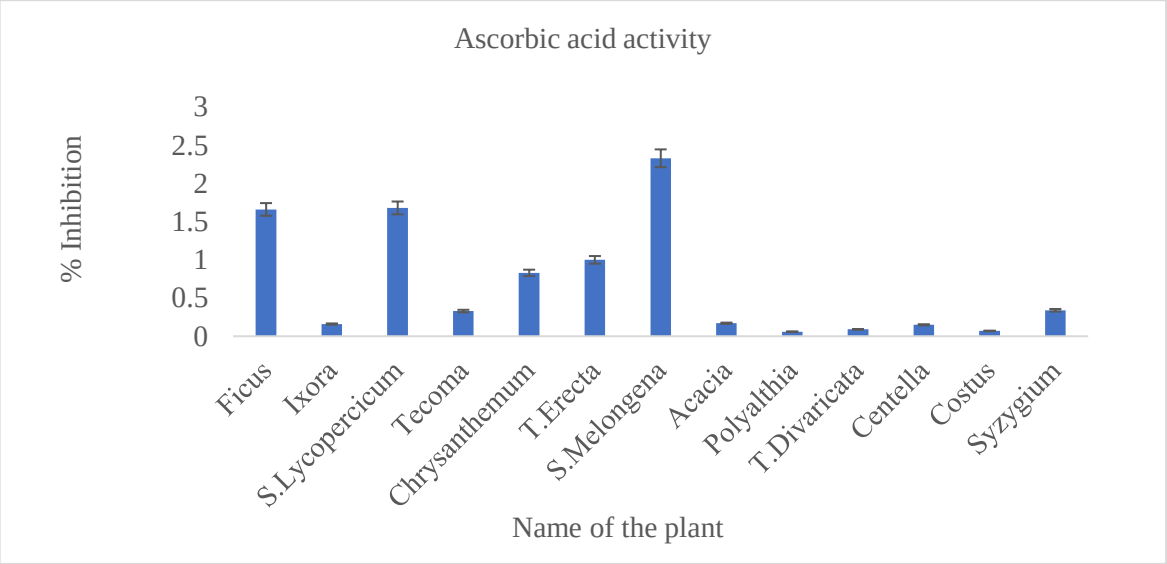

Fig. 4: Name of the plant is taken on X-axis and % inhibition readings are taken on Y-axis.

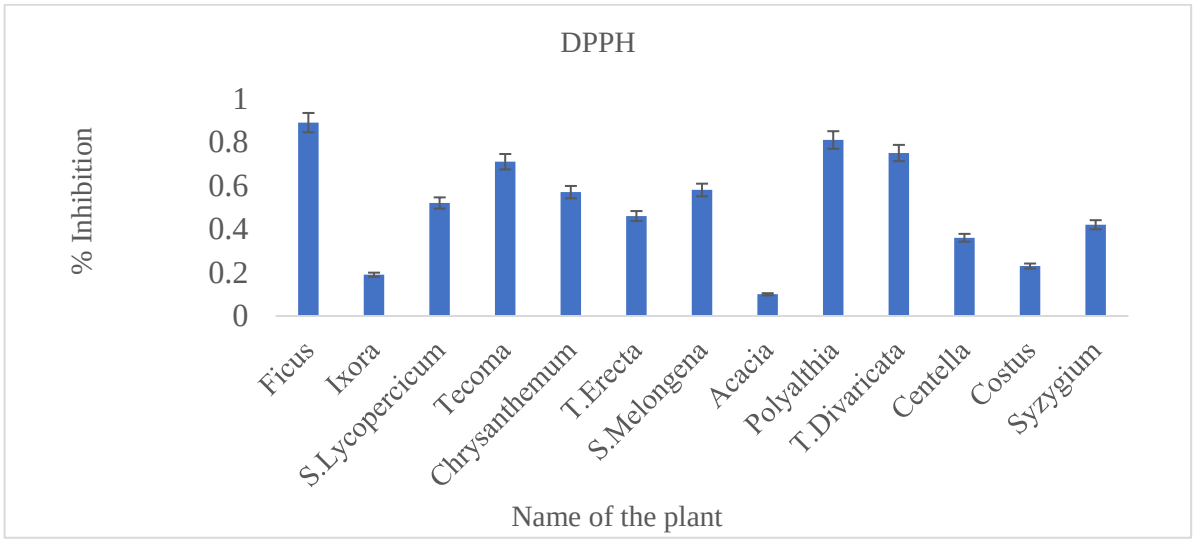

Fig. 5: 2,2-diphenylpicrylhydrazyl activity is observed. Name of the plant is taken on X-axis and % inhibition readings are taken on Y-axis.

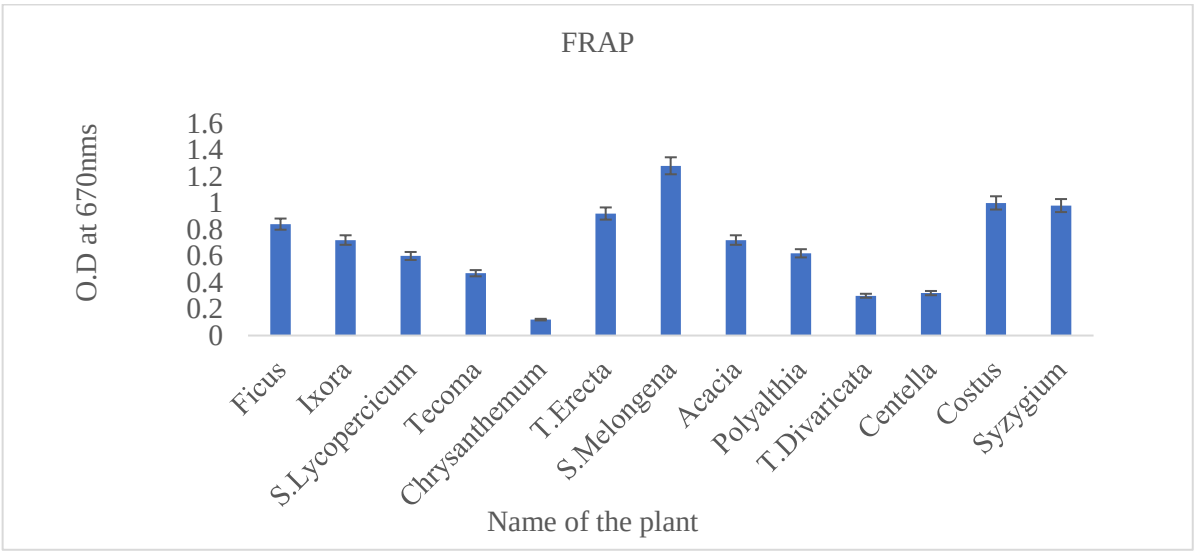

Fig. 6: Ferric reducing ability of plasma was studied on these plant extracts taking name of the plant on X-axis and optical density values on Y-axis.

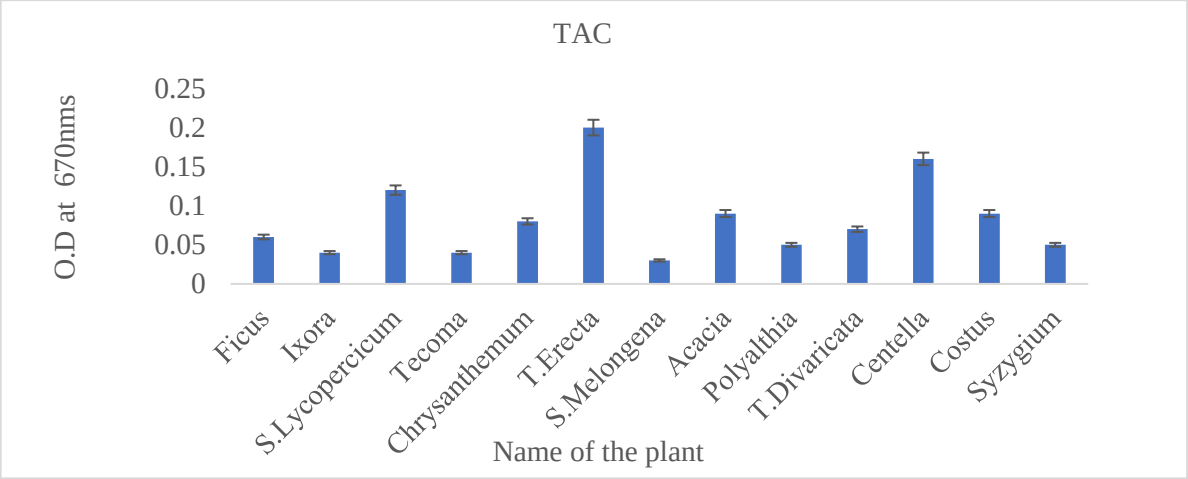

Fig. 7: Total anti-oxidant capacity was measured by taking name of the plant on X-axis and optical density values at Y-axis.

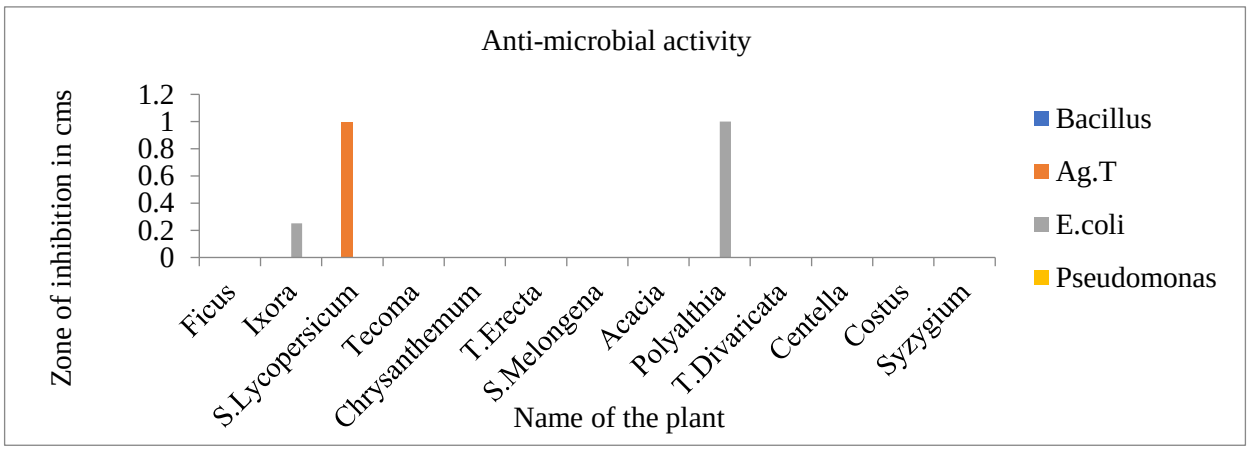

Fig. 8: Zone of inhibition is measured on the following plant extracts. Name of the plant is taken on X-axis and zone of inhibition is taken on Y-axis.

Bacterial strain *E. coli* is coated on the nutrient agar as in fig. 9. Antibiotic refampsin is taken in a respective well. 1 represents *Ficus*, 2 represents *Ixora* and 3 represents *Solanum lycopersicum.* All these three are taken in their respective wells. Zone of inhibition is observed in well no. 2 i.e. *Ixora* and Refampsin.

Fig. 9: Zone of inhibition of *Ixora*. 2 represents *Ixora* and R represents Refampsin and *E* represents *E. coli* strain.

Bacterial strain *Agrobacterium tumefaciens* is coated on the nutrient agar as in fig. 10. Antibiotic refampsin is taken in a respective well. In well no. 3 containing *Solanum lycopersicum* plant extract, maximum zone of inhibition is observed. Zone of inhibition is observed in refampsin containing well.

Fig. 10: Zone of inhibition of *Solanum lycopercicum*. 3 represents *Solanum lycopercicum* and R represents Refampsin and *Ag* represents *Agrobacterium* strain.

Bacterial strain *Eschericia coli* is coated on the nutrient agar as in fig. 11. Antibiotic refampsin is taken in a respective well. The well no. 9 containing *Polyalthia longifolia* showed maximum zone of inhibition. Refampsin also showed zone of inhibition.

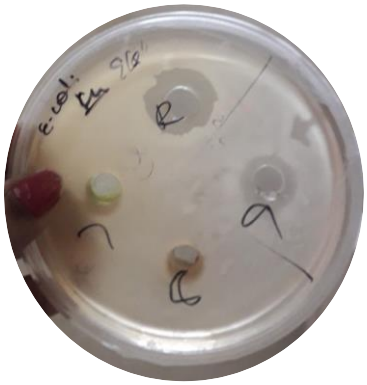

Fig. 11: Zone of inhibition *of Polyalthia longifolia*. 9 represents *Polyalthia longifolia* and R represents Refampsin coated with *E. coli* strain.

Results and Discussion

Overall thirteen experimental plants were identified and various assays were conducted on *Ficus, Ixora, Solanum lycopersicum, Tecoma, Chrysanthemum, Tagetuserecta, Solanum melongena, Acacia, Polyalthia longifolia, T.Divaricata, Centella, Costus, Syzygium cumini* to observe their biological activities. At first, phytochemical screening was done on these different plant extracts, out of which *Acacia, Ixora, Polyalthia longifolia, Tecoma and Solanum lycopersicum, Tagetus erecta, Syzygium cumini* have shown more phytochemical activities. Estimation of proteins by Lowry method has also been done in plant extracts in which *Acacia, Tagetus erecta, Solanum melongena, Tabernaemontana divaricate, Syzygium cumini* and *Ixora* have shown high concentration of proteins.

In enzymatic parameters of anti-oxidant assays, *Ixora, Tecoma stans, Acacia, Polyalthia longifolia* and *Syzygium cumini* have exhibited maximum catalase activity. In ascorbic acid oxidase activity, *Ficus, Solanum lycopersicum, Tecoma stans, Chrysanthemum, Solanum melongena, Taberneamontana divaricate, Costus* and *Polyalthia longifolia* have shown maximum activity. In non-enzymatic parameters of anti-oxidant assays, *Ficus, Solanum lycopersicum* and *Solanum melongena* have shown maximum activity. In DPPH assay, *Ficus, Tecoma stans, Chrysanthemum, Solanum melongena, Polyalthia longifolia, Tabernaemontana divaricate* and *Syzygium cumini* exhibited maximum activity.

In FRAP assay, *Ficus, Tagetus erecta, Solanum melongena, Costus, Ixora, Acacia* and *Syzygium cumini* exhibited maximum activity. In total anti-oxidant capacity (TAC) activity, *Solanum lycopersicum, Tagetus erecta, Centella, Costus, Chrysanthemum* and *Acacia* exhibited maximum activity. In anti-microbial activity, *Solanum lycopersicum* has shown high zone of inhibition followed by *Polyalthia longifolia* and *Ixora.*

Conclusion

In the present investigation, among the thirteen plants selected, almost all the plants exhibited positive results for phytochemical analysis. *Ficus* has shown highest activity against catalase activity, ascorbic acid activity, DPPH and FRAP. *Ixora* has shown high activity against catalase activity, FRAP and zone of inhibition is observed while anti-microbial activity is performed. *Solanum lycopersicum* has shown high activity against ascorbic acid oxidase assay, ascorbic acid assay, DPPH, total antioxidant capacity assay and has shown zone of inhibition when anti-microbial activity is performed. *Tecoma* exhibits maximum activity against catalase activity, ascorbic acid oxidase assay and DPPH.

Chrysanthemum has shown maximum activity against ascorbic acid oxidase assay and DPPH. *Tagetus erecta* has exhibited maximum activity against DPPH, FRAP and total anti-oxidant capacity. *Solanum melongena* has exhibited high protein activity against protein estimation by Lowry method and exhibited highest activity against anti-oxidant activity, ascorbic acid oxidase assay, ascorbic acid activity, DPPH and FRAP. *Acacia* exhibited highest activity against catalase activity and FRAP. *Polyalthia longifolia* has exhibited high activity against Catalase activity, DPPH and shown zone of inhibition for anti-microbial activity. *Tabernaemontana divaricate* has recorded high protein content against protein estimation by Lowry method and shown high activity against ascorbic acid oxidase activity and DPPH. *Centella* has showed high activity against ascorbic acid oxidase activity and total anti-oxidant capacity.

Costus has shown maximum activity against ascorbic acid oxidase activity and FRAP. *Syzygium cumini* has exhibited high protein content against protein estimation assay by Lowry method and has shown high activity against anti-oxidant assays, catalase activity, DPPH and FRAP. Hence, it is concluded that *Ficus, Tagetus erecta, Solanum melongena, Polyalthia longifolia, Tabernaemontana divaricate, Costus* and *Syzygium cumini* are rich in phytochemical content, protein content and anti-oxidant properties indicating high medicinal values. Hence, further studies on the characterization of bioactive compounds with these plants as they are showing high potential activities among all the other sources tested can be carried out.

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