Ethnobotany is the study of traditional human uses of plants, and is recognized as an effective way to discover future medicines. Medicinal plants have been identified and used throughout human history, and play a crucial role in modern era (Elumalai and Eswariah, 2012) Plants have the ability to synthesize a wide variety of chemical compounds that are used to perform important biological functions, and to defend against attack from predators such as insects, fungi and herbivorous mammals. The use of herbs to treat disease is almost universal among non-industrialized societies, and is often more affordable than expensive modern pharmaceuticals. Herbs, now-a-days, are in great demand for its scent, flavor, or therapeutic properties.

In the present study two plant species viz., *Boerhavia diffusa* and *Tecomella undulata* have been selected.

1. **Boerhavia diffusa** Linn.

*B.diffusa* belongs to the Family: Nyctaginaceae, found in tropical and subtropical parts of the world.

**Habitat:** *B. diffusa* is a perennial herb, Grows as common weed, growing upto 0.1 m to 0.5 m. It flowers from June to September. The flowers are hermaphrodite, it can grow in light (sandy) and medium (loamy) soils and prefers well-drained soil, acid, neutral and basic (alkaline) soils all are suitable. It cannot grow in the shade. It prefers dry or moist soil and can tolerate drought. In India, it is found in the warmer parts and up to an altitude of 2000m. It is found growing in waste lands, road sides, road dividers, near railway tracks, on ruins of old buildings, on rubles, and near old earthen ponds.

**Common name:** commonly known as Punarnava (meaning that which rejuvenates or renews the body), Raktapunarnava, Shothaghni, Kathillaka, Kshudra, Varshabhu, Raktapushpa, Varshaketu and Shilatika, red spiderling,

**Distribution:** _Boerhavia diffusa_ is found in the tropical, subtropical and temperate regions of the world. It is distributed in China, India, Australia, Pakistan, Egypt, Sudan, Srilanka, U.S.A. and South Africa. It is also found in a number of countries of the Middle East. This plant is indigenous to India and U.S.A. it is found in Indian regional flora such as Madras, Tamil Nadu, Goa, Diu, Daman, Nagarhaveli, Cannanore, Eastern Karnataka, Gujrat, Rajasthan. In Rajasthan, it is found in Ajmer, Alwar, Jhalawar, Jodhpur, Jaipur, Kota, Nagaur, Siriska, Samod and Udaipur. About 40 species are distributed in tropical, subtropical and temperate regions. Among these, 6 species, _B. erecta, B. diandra, B. repens, B. procumbens, B. rubicunda, B. saha_ are reported in India and _Boerhavia diffusa (B. diffusa)_ is indigenous.

**Morphological or Macroscopic description:** _B. diffusa_ is a terrestrial, prostrate, diffusely branched annual or perennial herb, 1-1.5 m long.

**Roots:** Well developed, fairly long, slight tortuous, cylindrical, 0.2-1.5 cm in diameter, yellowish brown to brown coloured, surface soft to touch but rough due to minute longitudinal striations and root scars, fracture, short.

**Stems:** Prostrate, decumbent or ascending, 4-10 cm long, rather slender, divaricately branched, Greenish purple, stiff, slender, cylindrical, swollen at nodes, minutely pubescent or nearly glabrous, branches from common stalk.

**Leaves:** opposite, unequal, ex-stipulate; petiole distinctly sulcate above and convex beneath, lamina ovate-orbicular, triangular. Inflorescence axillary or terminal branched cymose; flowers small, 3.8-4.0 x 3-3.2 mm, 2-14 together.
Medico-Ethnobotanical Background

Flowers: Very small, pink coloured, nearly sessile or shortly stalked, 10-25 cm, in small umbells, arranged on slender long stalks, 4-10 corymb, In pendunculate, glomerulate clusters arranged in slender, long stalked, axillary or terminal corymbs. Perianth-short, tubular about 3mm long and deeply constricted in the middle. The lower tubular part is greenish, pesrsisent and is covered with glandular hairs. The upper limb is light rode, funnel shaped and 5 lobed. The stamens are 2 or 3, Filaments united in the ovary at the base and not exerted outside of the perianth. The anthers are small and 2 celled.

Fruits: One seeded nut, 6 mm long clavate, Ovoid or sub-ellipsoid, rounded above, slightly cuneate, below broadly and bluntly 5 ribbed, viscidly glandular.

Phytochemistry: phytochemical characterization revealed, sterols, beta-sitosterols, alkaloids, sugars, ursolic acid (Singh and Udupa, 1972; Srivastava et al., 1972; Desai et al., 1973; Garg et al., 1980; Shukla, 1982; Kokate et al., 2005). Flavonoids, alkaloids, steroids, triterpenoids, lipids, lignins, carbohydrates, proteins and glycoproteins (Jain and Khanna, 1989; Kadota et al., 1989; Lami et al., 1990; Mahesh et al., 2012; Pathak et al., 2012). Rotenoids and alkaloids terpenes, phenylpropanoids, indol compounds, norisoprenoids, and oxalic, ketoglutaric, pyruvic, quinic and fumaric acids were identified. (Pereira et al., 2009), phenolic compounds- 2,6-dimethoxybenzoquinone, catechin,isorhamnetin 3-O-β-D-glucopyranoside and rutin (Lien et al., 2011).

Antimicrobial study: B. diffusa have been shown to exhibit potent antibacterial activity against various Gram-negative and Gram-positive. inhibitory effect on Gram-positive bacteria like Staphylococcus aureus, Bacillus subtilis, Streptococcus faecalis, Micrococcus luteus and all Gram-negative bacteria (Olukoya et al., 1993; Abo and Ashidi, 1999; Awasht and Verma, 2006; Goyal et al., 2010; Banjare et al., 2012; Ramachandra et al.,
Antifungal activity (Inhibition in sporulation) against dermatophytic fungi Microsporum gypseum, M. fulvum and M. canis (Agrawal et al., 2003)

**Pharmacology:** Punarnava, The flowers and seeds are used as contraceptive (Chopra et al., 1956), anti-inflammatory (Bhalla et al., 1968), renal disorders, kidney stones, cystitis, and nephritis (Singh and Udupa, 1972). Astringent to bowels, useful in biliousness, blood purifier as a diuretic (Gaitonde et al., 1974), gallbladder pain and stones, urinary tract disorders (Mudgal, 1975; Cruz, 1995), anthelmintic febrifuge, antileprosy, anti-asthmatic, antiscabies, and anti-urethritis (Nadkarni, 1976), anticonvulsant (Adesa, 1979), anti-nematodal activity (Vijayalakshmi et al., 1979). It works as antistress agent, antihepatotoxic (Mishra, 1980; Chandan et al., 1991; Rawat et al., 1997), anti fibrinolytic (Jain and Khanna, 1989), antibacterial (Olukoya et al., 1993). The leaves are useful in dyspepsia, tumours, spleen enlargement, abdominal pains, appetizer, alexiteric, useful in opthalmia, in joint pains, expectorant, carminative, useful in lumbago, scabies, for all types of liver disorders (including jaundice and hepatitis) (Rawat et al., 1997), Thrombolytic activity, cytotoxic activity (Apu et al., 2012), antimicrobial activity (Baskaran et al., 2012).

2. **Tecomella undulata** Seem.

Tecomella undulata belongs to **Family:** Bignoniaceae.

**Common names:** Roheda, honey tree, desert teak, marwar teak (English), roheda, rohida (Hindi), rakhtroha, raktarohida (Marathi), chalacchada, dadimapspaka (Sanskrit). Rayana, Pittaraj, Roda (Bengali), Rohido (Gujrati), Ruhedu (Pjabi), Yaradumala, Mutale (Karkataka), Mulumoduga cettha (Telegu). Baluchistan: Rori; Bolan: Parpuk; Bombay: Lohera, Lohuri, Rakhtreora, Rugtrora, Roira; Marara: Rohira, Roira; Punjab: Lahura, Luar, Rohira, Roir; Pushtu: Raidawan, Rebdan, Rebdun; Sind: Khen, Lahero, Lohuri.
Synonyms: *Tecoma undulata* G. Don, *Bignonia undulata* Sm.

**Habitat:** *Tecomella undulata* is a deciduous or nearly evergreen tree of arid and semi-arid regions. It occurs on flat and undulating areas including gentle hill slopes and sometimes also in ravines. It is well adapted to drain loamy to sandy loam soil having pH 6.5-8.0. The species thrives very well on stabilized sand dunes, which experience extreme low and high temperatures. It grows in areas of scanty rainfall and high temperature. It can withstand low and high temperature. The tree demands strong light. It is drought, frost, fire and wind hardy.

**Distribution:** Distribution of is restricted to the drier parts of the Arabia, southern Pakistan and northwest India up to an elevation of 1200 metres. In Pakistan in Attock kala chita mountain. It is found in Baluchistan and Sindh. In India, it occurs naturally in Maharashtra, Gujarat, Rajasthan, Punjab and Haryana. The species is mainly found to occur in western parts of Rajasthan. In other states its population is scanty and very rare. In Rajasthan, found in Ajmer, Alwar, Barmer, Jaisalmer, Jodhpur, Pali, Ajmer, Nagaur, Bikaner, Churu and Sikar districts.

**Morphological or Macroscopic description:** It is a medium sized tree with height ranging from 8m to 15m with drooping branches. Stellalety grey-tomentose innovations, otherwise glabrous.

**Roots:** Brown colored, deep and developed lateral root system.

**Stems:** Bark is curved in shape, outer surface dull brown grey or grey in colour, inner surface dark brownish.

**Leaves:** are simple, 1 to 3.2 cm long, narrowly oblong, obtuse, entire with undulate margins.
**Flowers:** are inodorous in corymbose few flowered racemes, terminating short lateral branches, pedicles are 6-13 mm long, Calyx 9.5 - 11 mm long, campanulate. Lobes are 3mm long, broadly ovate, obtuse, mucronate. Corolla is 3.8-6.3 cm long, orange yellow, campanulate, veined. Lobes are 5 subequal rounded. Stamens are exserted and filaments are glabrous. Stigma are 2 lamellate, lobes are spathulate-oblong, rounded.

**Fruits:** Fruit is capsule, slightly curved, 15-20 cm long pods, 8 mm broad, thin, flattened and slightly crooked and seeds are winged, 2cm long and 8 mm broad.

**Phytochemistry:** Different chemical constituents such as alkaloids, saponins (Hungund and Pathak, 1971), flavonoids (Taneja et al., 1975), iridoid glucosides (Joshi et al., 1975; Verma et al., 1986), 2-methyl-5,7-dihydroxycromone 7-O-β-d-glucopyranoside (Gujral et al., 1979), glycosides, alkaloids, tannins (Thanawala and Jolly, 1993), Rader-machol, Undulatin, Lapachol, Tecomelloside, Stigmasterol, β-amyrin, β-sitosterol, β-sitosteryl acetate, compesterol, stigmasterol (Nagpal et al., 2010), Radermachol, lapachol, tecomaquinone-I, α-lapachone, β-lapachone, stigmasterol, β-sitosterol, oleanolic acid, ursolic acid and betulinic acid. (Dhir and Shekhawat, 2012), Tecomin (Nagpal, 2013), α-lapachone, tectol isolated from heartwood, bark and leaf (Rohilla and Garg, 2014).

**Antimicrobial study:** antiviral activities reported by Rastogi and Dhawan, 1990, antibacterial activity with methanolic and aqueous extracts of Tecomella undulata, were more active against Gram-positive bacteria than against Gram-negative bacteria. Leaves show good activity against salmonella typhi (Gehlot and Bohra, 2000), The most susceptible bacteria were B. subtilis, followed by S. epidermidis, (Jain et al., 2012) reports show activity of *Tecomella* extracts against the fungal strains *Candida albicans* and
Curvularia lunata, A. flavus, P. notatum, A. niger, C. carrionii and Mucor spp. (Danya et al., 2012).

**Pharmacology:** It possesses light and dry attributes, tumors, blood disorders, flatulence, abdominal pain and cough. The bark has traditionally been used in treatment of syphilis, painful swellings and cancer. Also antibacterial activity has been reported in stem extract as well (Kritikar et al., 1993; Nadkarni, 2000). Anti inflammatory, analgesic potential and also contains anti-HIV agents (Azam, 1999). Analgesic potential, antibacterial, antioxidant (Parekh et al., 2005; Laghari et al., 2013). It is also used in curing urinary disorder, enlargement of spleen, gonorrhea, leucoderma, liver diseases and remedy for syphilis (Gautam et al., 2007; Chal et al., 2011). In indigenous system of medicine, it is used against spleen, liver and abdominal complaints (Parekh and Chanda, 2007). The plant has been extensively screened for wide range of pharmacological activities. Hepatoprotective activity (Khatri *et al* 2009), It is used in the diseases like ascites, obesity (Alvala *et al*., 2013).
The present study was focused on two economically important plants, viz., *Boerhavia diffusa* and *Tecomella undulata* belonging to the family Nyctaginaceae and Bignoniaceae respectively. The *B. diffusa* was collected from the University of Rajasthan, Jaipur, Campus. A specimen was submitted to the Herbarium, Department of Botany, University of Rajasthan, Jaipur and the voucher specimen no. RUBL211299 was given. Likewise, the *T. undulata* was collected from University of Rajasthan, Jaipur, Campus, and the voucher specimen no. RUBL211300 was allotted by Herbarium, Department of Botany, University of Rajasthan, Jaipur. The plant material was shade dried and different plant parts were collected separately, powdered and used as the experimental plant material for further experimentation.

**Biochemical Analysis**

**Powder Analysis**

Shade-dried and coarsely milled aerial parts of *B. diffusa* and *T. undulata* were treated individually with various chemical reagents (Table 1) to assess the response and behavior of the selected plants or the crude drugs.

**Extraction Procedure**

50 gm each of the experimental materials were soxhlet extracted successively with petroleum ether (60°-80°), benzene, acetone, chloroform, ethanol and water for 72hrs. Each of the resultant extract was filtered, dried in vacuo and weighed to calculate the extractive value (%) on dry weight basis. Later, following the established protocols (Paech and Tracey, 1955), each of the test samples were processed further to be used to evaluate the presence of carbohydrates, proteins, tannins, flavonoids, alkaloids, glycosides, phenols, lignins, saponins and sterols. Before doing so, each test sample was reconstituted in the respective solvent and divided into aliquots to perform the following qualitative tests.
Qualitative estimation

Tests for Carbohydrates

1. **Fehling's Test**- To 2 ml of the aliquot, equal volume of freshly prepared Fehling's solution (prepared by mixing solution A: 7.0 gm CuSO₄ 7 H₂O in 100 ml distilled water and B: 24.0 gm KOH and 34.6 sodium potassium tartarate in 100 ml distilled water) was added and the mixture was boiled on a water bath. The development of a rusty brown colour or red precipitate indicated the presence of the carbohydrates.

2. **Benedict's Test**- To 2 ml of the aliquot, a few drops of Benedict's solution (prepared by dissolving 17.3 gm of sodium citrate, 10.0 gm of Na₂CO₃ in 75 ml of distilled water, which was filtered and to this 17.3 gm of CuSO₄.7H₂O dissolved in 20 ml of distilled water was added with agitation and the volume was raised to 100 ml with distilled water) was added followed by boiling the mixture on a water bath. A sequential change in the colour (blue-green-orange) indicated the presence of carbohydrates.

Tests for Proteins

1. **Biuret Test**- To 2 ml of the aliquot, 2 ml of 20% KOH solution was added and mixed thoroughly. To this mixture, 1 ml of 0.5 % CuSO₄ solution was slowly added, which resulted in the development of pale purple colour indicating the presence of proteins.

2. **Ninhydrin Test**- To 2 ml of aliquot, 2-3 drops of 0.1 % Ninhydrin reagent (in acetone) was added along with a few drops of pyridine. The mixture was heated and the appearance of a blue colour indicated the presence of proteins.
Tests for Tannins

1. **FeCl₃ Test** – To 2 ml of the aliquot, 5% aqueous FeCl₃ solution was slowly added and the presence of tannins was indicated by the development of a blue colour changing to olive green, as a result of the addition of more FeCl₃ solution.

2. **Lead acetate test:** Few drops of 10% lead acetate solution were added into 2 ml of extract. Formation of yellow or red precipitate indicates the presence of tannins (Treare and Evans, 1985).

Tests for Flavonoids

1. **Shinoda's Test** – To 2 ml of the test solution, a fragment of magnesium metal (mg⁺⁺) ribbon were added into the test tube, followed by dropwise addition of concentrated conc. HCl. The resulting pink/scarlet/crimson of occasionally green/blue colours indicated the presence of flavonoids (Kokate et al., 2001).

2. **NaOH Tests:** To 2-3 ml of extract, few drops of sodium hydroxide (NaOH) solution were added into a test tube. Formation of intense yellow colour that became colourless on addition of few drops of dilute HCl indicates the presence of flavonoids (Khandewal, 2008).

Tests for Alkaloids

**Iodine Test:** Few drops of dilute iodine solution were added into 2 ml test solution. Blue colour appeared; and disappeared on boiling and reappeared on cooling (Khandewal, 2008).

Test for Glycosides

1. **Keller-Killani Test:** 0.5 gm of extract was dissolved in 5 ml water. Two ml of glacial acetic acid containing one drop of 5% ferric chloride solution was added. This was underlayed with 1 ml of concentrated sulphuric acid. A reddish brown ring at the interface indicated the
presence of a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer (Kumar et al., 2012).

2. **Glycosides test**: 1 ml water was added into the small amount of extract and shaken well. Then aqueous solution of NaOH was added. The appearance of yellow colour indicates the presence of glycosides (Treare and Evans, 1985).

3. **Concentrate H\textsubscript{2}SO\textsubscript{4} Test**: 2ml. glacial acetic acid, one drop of 5% FeCl₃ and conc. H₂SO₄ were added into 5ml extract, the appearance of brown ring indicates the presence of glycosides (Khandewal, 2008).

**Test for Phenols**

1. **Ellagic Acid Test**: The test solution was treated with few drops of 5% (w/v) glacial acetic acid and 5% (w/v) NaNO₂ solution. The solution turned muddy or brown precipitate occurred in the extract. It indicates the presence of phenols solution (Gibbs, 1974).

2. **Phenol Tests**: 0.5 ml of 5% FeCl₃ (w/v) solution was added into 2 ml of test solution, formation of an intense colour indicates the presence of phenols (Gibbs, 1974).

**Test for Lignins**

**Labat test**: 2ml of the test solution was mixed with gallic acid; it developed olive green colour indicating the positive reaction for lignins (Gibbs, 1974).

**Test for saponins**

**Foam Test**: The extract was diluted with 20 ml of distilled water and then was shaken in a graduated cylinder for 15 minutes. Formation of around 1 cm. layer of foam, indicates the presence of saponins (Kokate et al., 2001).
Test for Sterols

1. **Liebermann-Burchard Test**: Chloroform was mixed into 2ml. extract. 1-2 ml. acetic anhydride and 2 drops of concentrated H$_2$SO$_4$ were dropped into the test tube. First red, then blue and finally green colour indicates the presence of sterols (Kokate et al., 2001).

2. **Salkowski’s Test**: 2ml chloroform and 2 ml concentrated H$_2$SO$_4$ were added to the 2 ml extract and shook well. The layer of red chloroform and acid shows greenish yellow fluorescence. It indicates the presence of sterols (Kokate et al.; 2001).

**Terpenoids (Salkowski test)**

Two ml chloroform was added to 0.5 gm of the extract. Then 3 ml concentrated H$_2$SO$_4$ was carefully added to form a layer. A reddish brown coloration of the interface indicates the presence of terpenoids (Kumar et al., 2012).

**Tests for Anthraquinone**

0.5 g of the extract was boiled with 10 ml of sulphuric acid and filtered while hot. The filtrate was shaken with 5 ml of chloroform. The chloroform layer was pipetted into another test tube followed by addition of 1 ml of 10% ammonia. The resulting solution was observed for color changes to violet indicating presence of anthraquinones (Kumar et al., 2012).

**Quantitative Estimation**

**CARBOHYDRATES**

**Extraction Procedure**

A. **Total soluble sugars**

Each of the dried and powdered test sample (50 mg) was macerated in a mortar and pestal with 20 ml of 80% ethanol and left overnight. Each of the homogenates was centrifuged (1200 rpm, 15 min), the supernatants were
removed and concentrated on a water bath. Later each resultant concentrate was raised to 50 ml with distilled water (Ext. I) and processed further following the method of Loomis and Shull (1937) for total soluble sugars.

B. Starch

For starch, the residual pellet obtained out of the above process in each case was suspended in 5 ml of 52% perchloric acid and 6.5 ml of distilled water, shaken vigorously (5 min) and then centrifuged (2500 rpm, 20 min; McCready et al., 1950). This step was repeated thrice and the supernatant of each sample were pooled together and the volume was raised to 100 ml with distilled water (Ext. II). Out of this, 1 ml aliquot was measured separately to estimate starch quantitatively.

Quantification

Aliquots (1 ml) of each test sample (Ext. I and II) were used to estimate quantitatively the total levels of carbohydrates following the protocol of Dubois et al., (1951) using phenol-sulphuric acid reagent, which included the preparation of a regression curve for the standard glucose.

A stock solution of glucose (1 mg/ml) was prepared in distilled water, out of which 0.1 to 0.8 ml were separately pipetted into the test tubes and the volume of each was raised to 1 ml with distilled water. Each of these were kept in an ice-chest, 1 ml of 5% aqueous phenol was added and shaken gently. Later, 5 ml of conc. H$_2$SO$_4$ was added rapidly, accompanied with gentle agitation during the addition of the acid. These were allowed to stand in a water bath at 26-30°C for 20 min before taking the optical densities (ODs) of the yellow-orange colour thus developed at 490 nm in a spectrophotometer after setting for 100% transmission against the blank (which was prepared by substituting distilled water for the sugar solution). Three replicates in each were run and their mean values were calculated. A regression curve was computed between its known concentration and the respective OD, which followed the Beer's Law.
Materials and Methods

The concentration values of the total soluble sugar in the test samples were directly worked out from the regression curve of the standard glucose. Five replicates of each experimental sample were taken and their mean values were recorded. The sugar contents in terms of glucose equivalent and the use of conversion factor 0.9 to convert the values of glucose to starch was made in each case.

LIPIDS

Extraction and Quantification

Each of the dried and powdered test samples (1g) was homogenized using a mortar and pestle with 10 ml distilled water (Jayaraman, 1981). The resulted pulp was transferred to a conical flask (250 ml), 30 ml of chloroform-methanol (2:1, v/v) mixture was added and later, mixed thoroughly. The above mixture was kept overnight at room temperature; and then 20 ml each of chloroform and distilled water was added. Transfer the mixture to a separating funnel and mix well, three layers - a clear lower coloured layer of chloroform containing all the lipids, a upper coloured aqueous layer of methanol with all soluble materials and a thick pasty interphase were observed. The methanol layer was discarded and the lower layer was collected. This organic layer in case was taken in pre-weighed beakers and concentrated carefully. On complete evaporation, the weight was determined again which was taken as the weight of total lipids/g of the dried plant material (s).

PROTEINS

Extraction Procedure

Each of the dried test samples (60 mg) was measured in 10 ml of cold 10% TCA solution (30 min), kept at 4°C overnight and centrifuged. The supernatants were discarded in each case and the resultant pellet of each was re-suspended in 10 ml of 5% TCA solution and heated at 80°C in a water bath for 30 min. These samples were cooled, re-centrifuged and the supernatant so
obtained were discarded each time. The pellet was then washed with distilled water and centrifuged. Each of the residues left after the centrifugation was dissolved in 10 ml of 1 N NaOH and left overnight at room temperature (Osborne, 1962).

Quantification

Using 1 ml aliquot of extract, total phenol contents were estimated following the method of Lowry et al., (1951).

A stock solution of Bovine serum albumin (BSA; Sigma chemical Co., St. Louis, USA) was prepared in 1N NaOH (1 mg/ml), out of which 0.1 to 0.8 ml of the solution was separately pipetted in the test tubes and the volume in each case was raised to 1 ml by adding distilled water. To each, 5 ml of the alkaline solution (prepared freshly by mixing 50 ml of 2% Na₂CO₃ in 0.1 N NaOH and 1 ml of 0.5% CuSO₄.5H₂O in sodium potassium tartarate) was added and kept at room temperature (10 ml). Later, to each of these tubes 0.5 ml of Folin-Ciocalteau reagent (CSIR Centre for Biochemicals, Delhi, India; diluted with equal volume of distilled water, just before use) was added rapidly with immediate mixing and after 30 min, the ODs were measured at 750 nm of using a spectrophotometer against the appropriate blank, Five replicates of each concentration were taken and their mean values plotted against their respective concentration to compute a regression curve.

All the test samples were similarly processed as above and the level of total proteins individually was calculated by referring the ODs of the test sample with the standard curve (BSA). Three replicates were examined in each case and the mean values were recorded.
ASCORBIC ACID

Extraction procedure

Each of the fresh experimental materials (400mg) was homogenize thoroughly with 10 ml of acetate buffer (pH 4.8) and centrifuged (1200 rpm, 20 min.). The supernatants were separately collected, out of which 1 ml was measured to other test tube, 4 ml of 4% trichloroacetic acid (TCA) was added, left overnight and later, centrifuged (Roe and Kuenthar, 1943). To the supernatant of each sample, 1 ml of the colour reagent (prepared by mixing 90 ml of 2.2%, 2,4-dinitrophenylhydrazine in 10N H_2SO_4, 5 ml of 5% thiourea and 5 ml of 0.6% CuSO_4 solution), was added and incubated at 57^0C for 45 min. Later, on cooling 7 ml of 65% H_2SO_4 was added to each mixture and cooled again.

Quantification

From the stock solution of ascorbic acid (10 mg/100 ml in 4% TCA), varied concentrations (0.01 to 0.09 mg/ml) were prepared in different test tube. The volume of each was raised to 5 ml by adding 4% TCA solution and left overnight at the room temperature. To these, 1 ml of the colour reagent was added. Later, to each of these, 7 ml of 65% H_2SO_4 was added, brought to the room temperature and the ODs were measured at 540 nm in a spectrophotometer against a blank. A regression curve was computed between the main optical density and the concentration of standard ascorbic acid, which followed Beer’s Law.
FLAVONOIDS

Extraction Procedure

Each of the dried and powdered test samples was Soxhlet for flavonoids using 80% methanol (100 ml/gdw; Subramanian and Nagarajan, 1969) for 24 hours on a water bath. The methanolic extracts were filtered and concentrated in vacuo individually. Each of the residual syrup was fractionated by successive extraction (3 X) with petroleum ether (Fr. I), diethyl ether (Fr. II) and ethyl acetate (Fr. III). In each case, Fr. I (upper layer) was rejected due to its fatty components, whereas Fr. II and Fr. III (upper layer) were analyzed for free and bound- flavonoids respectively. Fraction stored until analysis but, the Fr. III which contained flavonoids glycosides was acid-hydrolysed (7% H₂SO₄; 10 ml/g) for 2 hrs. The hydrolysate was filtered, the filtrate was extracted with ethyl acetate (3X), which were later pooled, washed thoroughly with water till neutrality and concentrated under pressure. Its aqueous fraction was, however, not studied and discarded (lower layer). Both the fraction II and III (the acid-hydrolysed proteins) were constituted in ethanol, before chromatographic analysis and GC-MS analysis.

Chromatographic Analysis

A. Thin Layer Chromatography (TLC) of Aglycones

1. Qualitative

Thin glass plates coated with silica gel’G’ were air dried at room temperature. The dried plates were activated at 100 C for 15 min in an oven, cooled at room temperature and used to examine free and bound aglycones of each sample obtained. Each of the extracts was applied 1cm above the edge of the chromatographic plates alongwith the reference flavonoids used as marker and developed in an air tight chromatographic chamber which has already been saturated with 200ml of solvent system of benzene- acetic acid – water (125:72:3; Wong and Francis, 1968).
Several other solvent systems, such as n-butanol – acetic acid - water (4:1:5; upper phase), ethyl acetate saturated with water, acetic acid - water (6:4; 85:15), butanol - acetic acid water (TBA; 3:1:1) and Forestal system [acetic acid - conc. HCl - water, (10:3:30)] were also tried but benzene - acetic acid- water (125:72:3, solvent I) gave better separation.

Later, the developed chromatograms of each were exposed to I$_2$ vapors, UV light alone and in the presence of ammonia fumes (100 ml wide mouthered bottle containing conc. ammonia was held in close contact with each spot for 5-10 sec (Table 6 and Plate 3). The extracts resolved in to three spots ($R_f$ 0.56, green yellow; 0.79, green yellow; 0.86, green yellow) were observed in day light, and the spots coincided to the authentic markers, (1) luteolin ($R_f$ 0.56, green yellow) (2) quercetin ($R_f$ 0.79, green yellow) and (3) kaempferol ($R_f$ 0.86, green yellow).

Simultaneously, some of the developed chromatograms were also kept in I$_2$ chamber yellowish-brown spots against white background were observed. After evaporation of I$_2$ by continuous heating, few plates were sprayed with 5% alc. FeCl$_3$ as also with 1% alcoholic AlCl$_3$ separately and heated in an oven at 100°C for 5 min (Mabry et al., 1970). Luteolin ($R_f$ 0.56), Quercetin ($R_f$ 0.79) and Kaempferol ($R_f$ 0.86) and gave positive reactions to the spraying reagents.

2. Preparative

Preparative TLC was performed on silca gel G coated plates, activated, cooled. The extract of *B. diffusa* and *T. undulata* along with authentic markers applied on the preparative TLC plates and developed in the solvent I. Such developed chromatograms coinciding to reference (visualized under UV light) were scrapped and eluted with ethyl acetate separately. Each of the eluates was dried over anhydrous Na$_2$SO$_4$, re-constituted in chloroform and
crystallized using (methanol).

**B. Identification**

Each of the isolated compounds was subject its mp, UV, IR and NMR spectral studies with the authentic samples. Later, on the basis of the colour reactions, TLC behaviour and the spectrophotometric data, the isolated compounds were identified by comparing with that of the standards (Marby et al., 1970; Harbone et al., 1975).

**C. Quantification**

The identified luteolin (L), quercetin (Q) and kaempferol (K) were quantitatively estimated by spectrophotometric methods of Kariyone et al., (1953) and Mabry et al., (1970) respectively, which included the computation, of their regression curves.

Stock solutions of L, Q and K were prepared in methanol (1 mg/ml), out of which varied concentrations (20 µg to 160 µg) were separately spotted on TLC plates, developed above, air-dried and visualized under UV light as also I₂ vapors. The spots marked on the basis or fluorescence was collected along the absorbent in separate test tubes. Later, to each 5 ml of spectroscopic methanol was added shaken vigorously, centrifuged and the supernatants were collected separately. The volume of each was raised to 10 ml by methanol, to which 3 ml of 0.1 M AlCl₃ solution was added by vigorous shaking and kept at room temperature for 20 min. The OD of each of the sample was taken on a spectrophotometer set at 426nm for L, 424 nm for K and 440 nm for Q and the average of five replicates of each was calculated. A regression curve for each of that authentic compound (L, Q, K) was plotted in between the various concentrations and their respective ODs, which followed the Beer's Law.

Likewise, each of the free (F) and bound (B) fractions of were dissolved in 1 ml of methanol, spotted (0.1 ml) on TLC along with the
authentic samples and the fluorescent spots coinciding with those of the authentic markers were marked, scrapped, eluted and processed as mentioned above. The ODs were recorded and the level of flavonoids in each was computed (mg/gdw) from the standard calibration curves. Three such replicates were run and their average value was recorded.

Phytosterols
Extraction Procedure

Each of the dried and powdered test materials were defatted in a soxhlet apparatus in petroleum ether (60-80°C) for 24 hours on a water bath. The residual defatted material was air-dried and refluxed with 30% HCl (v/v) for 4 hours. Each hydrolyzed sample was washed with water till pH 7 and dried. The dried preparation was again extracted with benzene for 24 h. The extract was filtered and dried in vacuo. The crude extract was dissolved in chloroform before chromatographic examination (Kaul & Staba, 1968).

Chromatographic Analysis
A. Thin Layer Chromatography (TLC)
1. Qualitative

For this, silica gel (G) coated plates were used, on which the test extracts reconstituted in benzene were applied along with reference sterols as marked and developed in a solvent system of hexane- acetone (8:2; Heble et al., 1968) as this solvent system gave better separation of the compounds. Other solvent systems such as benzene-ethyl acetate (85:75; Heble et al., 1968) and benzene-ethyl acetate (3:2; Kaul and Staba, 1968) were used as this solvent system gave better separation of the compounds. Such chromatograms were air-dried, visualized under UV light and the fluorescence or the colours were noted. Later, each was sprayed with 50% H₂SO₄ (Bennett and Heftmann, 1962) or anisaldehyde reagent (prepared by mixing minimum 0.5 ml anisaldehyde + ml H₂SO₄ + 50 ml glacial acetic acid; Heftmann, 1965) separately and heated to 100°C for 5-10 min until characteristic colours
developed. The reaction time required for initial appearance of the colour in
day light and after heating for 10 min was recorded (Table 11) and to locate
the spots in unsprayed developed chromatograms on exposure to I₂ vapours
also proved useful.

Four spots (R_f 0.93, 0.86, 0.84, 0.23) were observed uniform in the
extracts and Out of these, four major spots, which were the same in their
position (R_f 0.93, dark brown; D - R_f 0.86, dull red; E – R_f 0.84 pink; F - R_f
0.23, dull brown, sprayed with 50% H₂SO₄ coinciding to, lanosterol, Beta-
sitosterol, stigmasterol, campesterol respectively were observed and
identified. These spots also gave colour reactions comparable to the markers
with anisaldehyde reagent. Since, the remaining spot did not coincide with
any of the reference compounds used and hence, their further identification
was not attempted. Three such replicates in each case were run and their
average R_f values were calculated.

Preparative

To isolates the above sterols identified co-chromatographically from
the extracts of the selected species (B. diffusa and T. undulata) PTLC was
performed on Silica gel G coated plates (0.4 - 0.5 mm) along with the
reference markers. These plates were developed in hexane acetone (8:2), air-
dried and exposed to I₂ vapours where upon the spots were marked. Later, I₂
was evaporated by heating in an oven and one column in each plate was
sprayed with 50% H₂SO₄ (Hiai et al., 1976) to relocate the spots
corresponding to the markers. The spots were scrapped separately eluted with
chloroform and the process was repeated until sufficient crystallizable
(methanol-acetone) amount of each of the substance(s) was obtained (Kaul
and Staba, 1968). Each was retested by co-tlc, revealing their homogeneous
nature and subjected for further identification.
B. Identification

Melting points (uncorrected), UV, IR and NMR spectra of each of the isolated compounds were taken and a comparison of the TLC behaviour, colour reaction physical properties and spectral characteristics was made, which was found to be in accordance with those reported for the authentic compounds (Heilbron and Bunbury, 1953).

C. Quantification

In all the plant parts of _B. diffusa_ and _T. undulata_ levels of lanosterol, \(\beta\)-sitosterol, sigmasterol and campesterol were spectro-photometrically estimated following the protocol of Das and Banerjee (1980) which included the computation of standard curves.

Stock solutions (1 mg/ml) lanosterol, \(\beta\)-sitosterol, stigmasterol and campesterol were separately prepared in chloroform, out of which different concentrations (0.01 to 0.09 mg/ml) were separately spotted on TLC (Si gel G; hexane-acetone, 8: 2) and the developed air-dried chromatograms were exposed to I\(_2\) vapours. The positive spots were marked on these, plates, heated (1000°C) of 15 min. to remove any I\(_2\), scrapped, eluted with 5 ml of chloroform, (centrifuged and the supernatants were transferred to separate test tubes, which were later, evaporated to dryness. To each of these, 3 ml of glacial acetic acid was added, shaken for 1 min. at room temperature and the tubes were placed in an ice-chest. To the tubes, 2 ml of freshly prepared chromogenic reagent (0.5 ml of 0.5% anhydrous FeCl\(_3\) in glacial acetic acid +100 ml of 36 N H\(_2\)SO\(_4\); Klyne, 1965) was added dropwise at 0°C and mixed thoroughly. Each or the reaction mixture was incubated at 40°C for 30 min. and their ODs were measured using a spectrophotome set at 540 nm against a blank (3 ml gl. acetic acid + 2 ml 1 of chromogenic reagent). In each case, concentration was plotted against the respective concentrations to compute regression curves, which followed Beer's Law.
Similarly, the extracts of all the plant parts of two plant species were dissolved in benzene were spotted on TLC along with reference markers and process as above. The coinciding spots to the reference compounds in the extracts were marked scrapped, eluted, taken up in 5 ml of chloroform and processed further, the authentic samples. Concentrations of lanosterol β-sitosterol, stigmasterol and campesterol in all selected species were calculated (mg/gdw) by referring the experimental sample with the respective standard regression curve.

**ALKLAOIDS**

**Extraction Procedure** (Family: Nyctaginaceae)

Each of the dried and powdered test sample (10gm) was soaked in 200 mL methanol for several days at room temperature. The mixture was filtered and methanol was removed by rotary evaporator to give the crude methanolic extract. Procedure of extraction and evaporation was repeated three times. The dry methanolic extract was dissolved again in 20 mL methanol in separating funnel and mixed with 200 mL of 0.5 N sulphuric acid. A few drops of ammonia were added to the solution till the whole solution became basic (pH 9-12). 50 ml of chloroform was added in separating funnel and kept at room temperature for 24 hours. Discard the upper layer and taken the lower layer which was further evaporated to give the crude alkaloid mixture (Singh et al., 2000), which was analysed for GC-MS analysis.

**Extraction Procedure** (Family: Bignoniaceae)

Powdered and weighed plant materials were taken in 100mL Erlenmeyer flasks containing distilled water (50mL/g) and 5mL of 0.05 N sulphuric acid was added to it. Mixture was macerated for 3-4 h and boiled gently for 25 minutes. Heavy magnesium oxide (2.5g/g) was added to the mixture and again boiled gently for 20 minutes. It was cooled at room temperature and an equal amount of distilled water was added to make up for
loss of distilled water during boiling. Alcohol was added to remove the mucus. Mixture was filtered through Whatman filter paper (Kogan et al., 1953). Filtrate was evaporated to dryness in vacuo, reconstituted in distill water for further analysis.

**GC-MS analysis of flavonoids, phytosterols and alkaloids**

**Preparation of Extracts**

Extraction of flavonoids, phytosterols and alkaloids was done by established protocols mentioned in extraction procedure. Extracts of all the plant parts of selected plant species were analyzed by GC-MS in Jawahar Lal Nehru University, Advance Instrument Centre, New Delhi.

**GC-MS conditions**

GCMS-QP 2010 Plus was used for identification and quantification of phytoconstituents, using MS libraries previously compiled from purchased standards. For the acquisition of an electron ionization mass spectrum, an ion source temperature of 250°C was used. The GC was equipped with a SE-30 capillary column a split injection piece (270°C) and direct GC-MS coupling (280°C). Helium (1.2 mL/min) was used as the carrier gas with a split ratio of 1:10. The oven temperature program for analyzing the extracts utilized an initial oven temperature of 100°C, maintained for 2 min, followed by a steady climb to 200°C at a rate of 7°C/min allowed to increase to 190°C at a rate of 30°C/min. This oven temperature was again maintained at 190°C for 5 min and then allowed to increase to 300°C at a rate of 7°C/min. This oven temperature was maintained for 2 min and finally ramped to 300°C at a rate of 10°C/min and maintained for a further 22 min. Injection temperature was 270°C and volume 250°C and 1 μL, respectively. The total GC running time was about 43.28 min. The MS operating conditions were as follows, Interference temperature of 260°C, Ion source temperature of 250°C, mass
scan (m/z)-40-450, solvent cut time 7 min, scan speed 2000 amu/s total MS running time-50.28 min and Threshold -1000.

**Identification**

GC-MS is a valuable aid for identifying unknown peak as well as for confirming the identification of identified phytoconstituents. In some cases when no identical spectra were found, the structural type of the corresponding component was suggested only on the basis of its mass spectral fragmentation and retention data. Identification of components was based on direct comparison of the retention times and mass spectral data with those for standard compounds and computer matching with the library (Wiley library, NIST data bank, database NIST 98) as well as by comparison of the retention time.

**Quantification**

Composition was estimated on the basis of calculation of the GC peak areas in percent by setting the total peak areas to 100%.
Sources of test organisms

a. **Fungi**


b. **Bacteria**

The bacterial strains *Escherichia coli* (MTCC 1652), *Staphylococcus aureus* (MTCC 0087) (Gram +ve), *Pseudomonas aeruginosa* (MTCC 4646) (Gram +ve), *Bacillus subtilis* (MTCC 0121), *Klebsiella pneumoniae* (MTCC-0109)(Gram –ve) and *Streptomyces albudenecus*(MTCC 1764), *Enterococcus faecalis* (ATCC- 29212)(gram +ve) were procured from the microbial type culture collection (Institute of Microbial Technology, Chandigarh, India).

**Culture of test microbes**

For the cultivation of bacteria, Nutrient Broth Medium (NB) was prepared using 8% Nutrient Broth (Difco) in distilled water and agar-agar and sterilized at 121°C for 20 min. A peptone saline solution was prepared (by mixing 3.56gm KH$_2$PO$_4$ + 7.23gm NaH$_2$PO$_4$ + 4.30 gm NaCl + 1gm peptone in 1000 ml of distilled water, followed by autoclaving) and the bacterial cultures were maintained on this medium by regular sub-culturing and test bacteria were incubated at 37°C for 24 hrs. However, for the cultivation of fungi, Potato dextrose agar (PDA) medium was prepared (by mixing 100ml potato infusion + 20gm agar + 2gm glucose, followed by autoclaving) and the fungal cultures were maintained on this medium by regular subculturings and the test fungi were incubated at 27°C for 48hrs.
Preparation of Plates

To prepare the test plates, for both bacteria and fungi, 10 to 15ml of the respective medium was poured into the petri dishes under aseptic conditions. They were then permitted to set at room temperature and were dried so that no drops of moisture remain on the surface. For assessing the bactericidal efficacy, a fresh suspension bacteria was prepared in saline solution from a freshly grown agar slant, while for fungicidal efficacy, a uniform spread of the test fungi was made using sterile swab.

Preparation of test extracts

Powdered different plant parts of (50g) of *B. diffusa* and *T. undulata* were soxhlet extracted with petroleum ether (60-80°C), benzene, acetone, chloroform, ethanol and distilled water successively. Similarly, 10g of different plant parts were homogenized separately with methanol only (Veliky and Latta, 1974) and left overnight at the room temperature. Later, each of the homogenates was filtered and the residue was re-extracted twice for complete exhaustion, the extracts were pooled individually and dried in vacuo. Similarly, various metabolites rich fraction, such as flavonoids, phytosterols and alkaloids were extracted separately. Extraction of flavonoids, phytosterols and alkaloids was done by established protocols mentioned in extraction procedure.

All these fractions were stored at 4°C in a refrigerator until screened, and the sequential extracts were raised to final concentrations of 2mg/ml and 4mg/ml in DMSO, before use.

Fungicidal and Bactericidal Assay

For both, bactericidal and fungicidal assays agar well diffusion method was adopted (Bauer *et al.*, 1996), because of its reproducibility and precision. The nutrient agar (NA) and Potato dextrose agar (PDA) plates were proseeded with different test organism separately using a sterile swab over previously
sterilized culture medium plates and wells were punctured in the culture medium using sterile cork borers. Each of the well was then filled with 40µl of the test extracts, control solvent, streptomycin/gentamycin (1mg/ml) or ketokenazol (1mg/ml) as reference separately. These plates were initially placed at low temperature for 1hr, so as to allow the maximum diffusion of the compounds from the wells into the plate and later, incubated at 37°C for 24hrs in case of bacteria and 48hrs at 27°C for fungi, after which the zones of inhibition could be easily observed and measured around the wells in solidified medium. Three replicates of each test extract were examined and the mean values were then referred.

Determination of MIC
Test for antifungal activity

The micromycetes were maintained on Potato dextrose agar and the cultures stored at 4°C and sub-cultured once a month. In order to investigate the antifungal activity of the extracts, a modified micro dilution technique was used (Hanel and Raether, 1998; Daouk et al., 1995; Espine-Ingroff, 2001). The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% tween 80(v/v). The spore suspension was adjusted with sterile saline to a concentration of approximately 1.0 x10^7 cfu/ml in a final volume of 100µL per well. The inocula were stored at 4°C for further use. Dilutions of the inocula were cultured on solid potato dextrose agar to verify the absence of contamination and to check the validity of the inoculum. Minimum inhibitory concentration (MIC) determinations were performed by a serial dilution technique using 96-well microtiter plates. The microplates were incubated for 72hrs at 28°C, respectively. The lowest concentrations without visible growth (at the binocular microscope) were defined as MICs.
Test for antibacterial activity

The antibacterial assay was carried out by serial microdilution method (Booth, 1971; Daouk et al., 1995) in order to determine the antibacterial activity of compounds tested against the human pathogenic bacteria. The bacterial suspensions were adjusted with sterile saline to a concentration of $1.0 \times 10^7$ cfu/ml. The inocula was prepared daily and stored at $+4^\circ C$ until use. Dilutions of the inocula were cultured on solid medium to verify the absence of contamination and to check the validity of the inoculum. All experiments were repeated thrice.

Microdilution test

The minimum inhibitory concentrations (MICs) were determined by performing a serial dilution technique using 96-well microtitre plates. The bacterial and fungal suspension was adjusted to final concentration of $1.0 \times 10^7$ cfu/ml. The compounds to be investigated were dissolved in respective solvents and added in nutrient broth or potato dextrose broth medium with inoculums. The microplates were incubated for 24hrs at $37^\circ C$ in case of bacteria and 72hrs at $28^\circ C$ in case of fungi. The lowest concentrations without visible growth were defined as concentrations that completely inhibited bacterial growth (MICs). The optical density of each well was measured at a wavelength of 655nm by Microplate reader (Perlong, ENM8602) and compared with a blank and the positive control. Gentamycin/ streptomycin and ketokenazole were used as positive control (1mg/mL DMSO). All experiments were performed thrice.
Methanol extracts of different plant parts of *Boerhavia diffusa* and *Tecomella undulata* were taken for total phenolic content, total flavonoid content and detailed antioxidant potential via different antioxidant systems.

**Total Phenolic and Flavonoid Content**

**Plant Extraction**

2gm each of the dry material was extracted with 25ml of methanol at room temperature for 48 hours, filtered through Whatman paper no 1 filter paper, stored and used for quantification.

**Total Phenolic Content**

Total phenolic compound contents were determined by the Folin-Ciocalteau method (Mcdonald *et al.*, 2001; Ebrahimzadeh *et al.*, 2008a,b; Nabavi *et al.*, 2008). The extract samples (0.5 ml; 1; 10 diluted) were mixed with Folin Ciocalteu reagent (5 ml, 1:10 diluted with distilled water) for 5 min and aqueous Na$_2$CO$_3$ (4 ml, 1 M) were then added. The mixture was allowed to stand for 15 min and the total phenols were determined by colorimetric method at 765 nm. The standard curve was prepared using the standard solution of Gallic acid in methanol in the range 20-200µg/ml ($R^2$=0.987). Total phenol values are expressed in terms of Gallic acid equivalent (mg/ g of dry mass), which is a common reference compound. Total phenolic content can be calculated from the formula:

$$T = \frac{CV}{M}$$

Where,

- $T = $ Total Phenolic concentration
- $C = $ Concentration of gallic acid from calibration curve (µg/ml)
- $V = $ Volume of extract (ml)
- $M = $ Wt. of methanol plant extract
Total Flavonoid Content

Total flavonoid content was determined by using aluminium chloride colorimetric method (AlCl₃) according to the known method (Dewanto et al., 2002; Sakanaka et al., 2005) with slight modifications using quercetin as standard. 1ml of test material was added to 10ml volumetric flask containing 4ml of water. To above mixture, 0.3ml of 5% NaNO₂ was added. After 5mins, 0.3ml of 10% AlCl₃ was added. After 6min, 2ml of 1M NaOH was added and the total volume was made up to 10ml with distilled water. Then the solutions were mixed well and absorbance was measured against blank at 510nm. The standard curve was prepared using the standard solution of Quercetin in methanol in the range 0.5- 5.0mg/ml ($R^2=0.991$). Total flavonoid content of the extracts was expressed in milligram of quercetin equivalents/gdw. Total flavonoid content can be calculated from the formula:

$$T = \frac{CV}{M}$$

Where,

$T$ = Total flavonoid concentration

$C$ = Concentration of quercetin from calibration curve (mg/ml)

$V$ = Volume of extract (ml)

$M$ = Wt of methanol plant extract

Determination of antioxidant activity

Plant extraction

10gm each of the plant material was soxhlet extracted with methanol for 24 hours. The extract was filtered with Whatman filter paper no 1 and the crude extract was concentrated to dryness in a rotary flash evaporator under reduced pressure and controlled temperature (40–50°C). The extracts were stored at 4°C in refrigerator for subsequent use in antioxidant assay.
DPPH Assay
The antioxidant activities were determined using 1, 1, diphenyl-2-picrylhydrazyl (DPPH) as a free radical. Experiments were initiated by preparing a 0.25mM solution of DPPH and 1mg/ml solution of different plant parts extracts (stock) in methanol. To the methanol solution of DPPH an equal volume of the extract dissolved in methanol was added at various concentrations. An equal amount of alcohol was added to the control. The setup was left at dark in room temperature and the absorption was monitored after 20 minutes. Ascorbic acid was used as a control. Experiment was performed in triplicate (John, 1984; Sreejayan and Rao, 1996). A control reaction was carried out without the test sample. Absorbance values were corrected for radicals decay using blank solution. The inhibitory effect of DPPH was calculated according to the following formula:

\[
\% \text{ Inhibition} = [1 - \left(\frac{Abs_{\text{SAMPLE}}}{Abs_{\text{CONTROL}}}\right)] \times 100
\]

Linear graph of concentration Vs percentage inhibition was prepared and IC50 values were calculated. The antioxidant activity of each sample was expressed in terms of IC50 (micromolar concentration required to inhibit DPPH radical formation by 50%), calculated from the inhibition curve (Yokozawa et al., 1998; Fejes et al., 2000; HSU, 2006).

Reducing ability (FRAP assay)
The determination of the total antioxidant activity (FRAP assay) in the extract is done by modified method of Benzie and Strain, 1996. The FRAP activity was evaluated in methanol, ethyl acetate and hexane extracts of different plant parts of plants. The stock solutions included 300 mM acetate buffer (0.3 M acetic acid and 0.3 M sodium acetate), pH 3.6, 10 mM TPTZ (2, 4, 6-triprydyl-s-triazine) solution in 40 mM HCl, and 20 mM Ferric chloride(FeCl3·6H2O) solution. The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ, and 2.5 ml FeCl3·6H2O. The
temperature of the solution was raised to 37°C before use. Plant extracts (100 μL each of methanol, ethyl acetate and hexane) were allowed to react with 2900 μl of the FRAP solution for 30 min in the dark condition. Readings of the coloured product (ferrous tripyridyltriazine complex) were taken at 593 nm. The standard curve was linear between 100 and 1000 μM FeSO₄. Results are expressed in mM Fe (II)/g dry mass.

**ABTS radical scavenging assay**

To determine ABTS radical scavenging assay, the method of Re et al., (1999) was adopted. The stock solutions included 0.002M ABTS solution and 0.07M potassium persulphate solution. The working solution was then prepared by mixing the 25 ml of ABTS stock and 0.1 ml of potassium persulphate stock and allowing them to react for 12 hrs at room temperature in the dark. The solution was then diluted by mixing ABTS solution with ethanol to obtain an absorbance of 0.706 ± 0.001 units at 734 nm using the spectrophotometer. Fresh ABTS solution was prepared for each assay. Plant extracts (1 ml) at varying concentration were allowed to react with 3 ml of the ABTS solution and the absorbance was taken at 734 nm after 6 min using the spectrophotometer. The ABTS scavenging capacity of the extract was compared with that of BHT and percentage inhibition calculated as:

\[
\%\text{ Inhibition} = [1 - (\text{Abs}_{\text{SAMPLE}} / \text{Abs}_{\text{CONTROL}})] \times 100
\]

Where, \( \text{Abs}_{\text{control}} \) is the absorbance of ABTS radical + methanol; \( \text{Abs}_{\text{sample}} \) is the absorbance of ABTS radical + sample extract /standard.

**Lipid Peroxidation Assay (LPO)**

LPO activity was calculated using the protocol of Heath and Packer (1968). 0.5gm of dry material was homogenized with 10 ml of 0.1% (w/v) Trichloroacetic acid (TCA). The homogenate was centrifuged for 5 mins (15000g, 4°C). Supernatant was collected and 1 ml of supernatant was mixed
with 4ml of 0.5% (w/v) TBA (Thiobarbituric acid) in 20% (w/v) TCA and then incubated in water bath at 95°C for 30 mins. Reaction was quickly ended by incubating on an ice bath. In case the solution is not clear, centrifuged at 10000g for 10 mins and the absorbance was measured at 532 and 600nm. OD₆₀₀ values were subtracted from MDA-TBA complex values at 532 nm and MDA concentration was calculated using the Lambert-Beer law with an extinction coefficient ε₅₃₂ = 155/ mM/cm. Results were presented as µmols MDA/gm.

**Peroxidase assay**

The method of assay measures the oxidation of pyrogallol to purpurogallin by peroxidase when catalyzed by peroxidase at 420 nm and at 20°C. Plant sample (200mg) was homogenized with 10ml of phosphate buffer and refrigerated centrifuged at 10000 rpm for 20 mins. The clear supernatant was taken as the enzyme extract. The activity was assayed after the method of Chance and Maehly (1955) with some modifications. 2.4 ml of phosphate buffer, 0.3ml pyrogallol (50µM) and 0.2 ml of H₂O₂ (30%) were added. The amount of purpurogallin formed was determined by taking the absorbance at 420 nm immediately after adding 0.1 ml enzyme extract. The extinction coefficient of 2.8/ mM/cm was used in calculating the enzyme activity that was expressed in terms of millimole per minute per gram dry weight.

**Statistical analysis**

Experimental results are expressed as means ± standard deviation (SD). All measurements were replicated three times. IC₅₀ values were also calculated by linear regression analysis.