II. Materials and Methods

A. Materials

1. Collection and processing of the plant material

Different bark samples (apical rind, inner bark and mature outer inner bark) of *A. latifolia*, *C. religiosa*, *P. marsupium* and *T. arjuna* were collected from the hilly regions (Radhanagari, Kagal and Vadgoan) of Kolhapur district. In the winter season the bark was collected in the month of January and summer collection was followed in the month of May. The bark samples were cut into pieces, sun-dried then oven dried at 60°C. Dried bark samples were ground into powder and stored in an air tight plastic container.

2. Microorganisms

Five different strains of bacteria *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Salmonella typhi* were used for testing antibacterial activity of bark extracts. The test organisms used in this study were obtained from the department of Microbiology, Shivaji University, Kolhapur, Maharashtra, India. The bacterial strains were cultured on nutrient agar slants. The cultures were
maintained by subculturing periodically and preserved at 4ºC until further use.

B. Methods

1. Preparation of the extract

Oven dried 10g of powdered bark material was weighed accurately and placed in soxhlet extraction chamber which was suspended above the flask containing 100mL of 80% methanol and below a condenser. The flask was heated and the methanol evaporated and moved into the condenser where it was converted into a liquid that trickled into the extraction chamber containing the plant material. The extraction chamber was designed so that when the solvent surrounding the sample exceeded at certain level it overflowed and trickled back down into the boiling flask. At the end of the extraction process, the flask containing the methanol extract was removed and methanol was evaporated by using rotary evaporator. The weight of the residual extract was measured and percent yield was calculated. The residue of the extract was dissolved in 25ml of pure methanol and stored in air tight glass vials at 4ºC until further use (Harborne, 1973).
Extract yield % = \( \frac{W1}{W2} \times 100 \)

Where

\( W1 = \) Net wt of powder in grams after extraction

\( W2 = \) total wt of wood powder in grams taken for extraction.

2. Antibacterial Activities

a. Preparation of the media

Accurately weighed 28g of nutrient agar (Himedia) was dissolved in the 1000ml of distilled water. The medium was sterilized under 15Lb pressure for 15 minutes in an autoclave. 30ml of this sterilized semisolid nutrient agar medium was poured in pre-sterilized 90mm glass petriplates under aseptic conditions in laminar flow. The plates were allowed to cool at room temperature to solidify the medium.

b. Determination of antibacterial activity by agar well diffusion method

Agar well diffusion method described by Perz et al. (1990) was employed to determine antibacterial activity. Well of 10mm diameter was prepared with sterilized cork-borer. Standard
antibiotics, Streptomycin and Chloramphenicol at 50µg/ml were served as positive control and Methanol as negative control. The plates inoculated with different bacterial species were incubated at 37°C in incubator for 24 h and the zone of inhibition was measured (Diameter in mm).

3. Biotonic studies

Certified seeds of mung bean (variety Gold-9) and Wheat (variety Hd-2189) were procured from the college of Agriculture Kolahapur.

a. Preparation of the aqueous extracts

The oven dried bark of A. latifolia, C. religiosa, P. marsupium and T. arjuna were ground separately on mechanical grinder. 0.25g, 0.5g, 0.75g, 1g, 2g and 5g of bark powder of each species was added to 100mL distilled water and kept for 24 hours at room temperature (28-30°C). The resultant extracts were filtered through Whatman No.1 filter paper and stored in conical flask.
b. Germination studies

The effect of resultant bark extracts on seed germination was studied by placing 20 seeds of each test crop in triplicate in petridishes containing three layers of Whatman No. 1 filter paper. Aqueous extract (15mL) of each plant bark was added in petridishes. Control plates were prepared by using distilled water in the place of plant extract. The effect of bark extract on seed germination, plumule length and radical length was recorded after 120 hours of germination.

Germination percentage was calculated by using the formula-

\[
\text{Germination} \% = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds taken for germination}} \times 100
\]

Vigour index was calculated by using the formula proposed by (Abdul-Baki and Anderson, 1973)

\[
\text{VI} = (\text{Root length} + \text{Shoot length}) \times \text{Germination percentage}
\]
Mobilization Efficiency was determined with the help of formula given by Shrivastava and Sareen (1974).

\[
\text{ME} = \frac{\text{Dry Wt. of embryonic axis}}{\text{Dry Wt. of Residual grain}} \times 100
\]

4. Qualitative tests for phytochemicals

The methanolic Soxhlet extracts were screened for the presence of alkaloids, anthroquinones, coumarins, flavones, phenols, saponins, Sterols, tannins, terpenoids, xanthoprotein and sugars following the methods of Trease and Evans (1985); Brindha et al. (1981) and Lala (1993).

a. Phenols

To the bark extract, few drops of 0.1M ferric chloride solution were added. Development of bluish green or red colour indicated the presence of phenols.

b. Flavones (Shindo’s test)

The bark extract was mixed with a few magnesium turnings and a few drops of concentrated hydrochloric acid, boiled for five
minutes, the appearance of orange red colour exhibits the presence of flavones.

c. Flavonoids

200 mg of bark material was extracted in 10 ml ethanol and filtered through whatman filter paper from this 2 ml extract was reacted with 2ml filtrate conc. HCl and magnesium ribbon. The development of pink-tomato red color indicated the presence of flavonoids.

d. Tannins

The bark extract was mixed with basic lead acetate solution; the white precipitate indicated the presence of tannins.

e. Terpenoids (Noller’s Test)

The bark extract was warmed with a piece of tin and a few drops of thionyl chloride, appearance of violet colour indicated the presence of terpenoids.

Qualitative analysis of triterpenoids by TLC

Thin layer chromatographic technique was employed for qualitative analysis of triterpenoids from bark samples of A. latifolia, C. religiosa, P. marsupium and T. arjuna.
Preparation of bark extract

1g of powdered drug was extracted with 10mL methanol on water bath for 15 minutes and centrifuged at 5000rpm. The residue was discarded and the supernatant was used for the thin layer chromatography of triterpenoids. TLC plates were prepared following the method described by Kurt Randerath (1966). Thin uniform layer of slurry of silica gel G (40g of silica gel G containing binder 13% CaSO$_4$, Himedia) mixed with 80 ml distilled water was coated on the glass plates (6 cm x 12 cm and 6 cm x 18 cm). The plates were dried in air and then activated in oven at 100°C for 1h.

Triterpenoids were separated according to the method described by Wagner and Bladt (2003). 35µL extract of each bark sample was loaded on silica plates with the help of micropipette with frequent drying under hot air of hair dryer. The plates were subjected to expose the solvent system Ethyl acetate-glacial acetic acid—formic acid-water (100:11:11:26) in chromatographic glass chamber (8X2.5X20). Then the plates were removed from the TLC chamber and dried in air. The TLC plate were sprayed with 10ml of anisaldehyde-sulphuric acid reagent (0.5mL of anisaldehyde mixed with 10mL glacial acetic acid followed by
85mL of methanol and 5mL of concentrated sulphuric acid. After spraying the reagent, TLC plates were air dried and heated at 100°C for 5 minutes in oven. The developed plates were observed under visible transilluminater. The triterpenoids were visible with blue violet coloured bands.

f. Saponins

The bark extract was shaken with water; copious lather formation indicated the presence of saponins.

g. Alkaloids

The extract (1ml) was mixed with a little amount of dilute (2.5N) hydrochloric acid and Mayer’s reagent (13.6 g MgCl2 in 600 ml D.W. + 8.50 g of KI dissolved in 100 ml D:W mix these two solutions and made the final volume 1 L with distilled water). The developed white precipitate indicates the presence of alkaloids.

h. Cardiac glycosides

Cardiac Glycosides were determined by Keller-kiliiani test (2 ml filterate + 1 ml glacial acetic acid + FeCl₃ + conc. H₂SO₄). The developed greenblue colour indicates the presence of cardiac glycosides.
5. Quantitative estimation of phytochemicals

a. Total polyphenols

The total polyphenol contents were determined according to the method of Folin and Denis (1915). Dry powdered bark (500mg) was homogenized in 15 ml acetone (80 %) and filtered through Buchner’s funnel. The residue was washed several times with 80 % acetone and the final volume was adjusted to 50 ml with 80% acetone. The reaction mixture in Nessler’s tubes consisted 1ml of plant extract, 10ml of 20% Na$_2$CO$_3$ and 2ml of Folin-Denis reagent (prepared by mixing 100g of sodium tungstate with 20g phosphomolybdic acid in about 800 ml distilled water and 200 ml 25% Phosphoric acid and the mixture was refluxed for 2-3 hours to room temperature and final volume was adjusted to 1000 ml with distilled water). The reaction mixture was adjusted to final volume 50ml with distilled water. The absorbance of the blue colour developed after 20 min was measured at 660nm on double beam UV-visible spectrophotometer. Total polyphenols were calculated with the help of std. curve of 0.1mg/mL tannic acid and expressed as g.100g$^{-1}$ dry weight.
b. Total flavanoids

Total flavonoids were estimated by the method of Luximon-Ramma et al. (2002). 500mg powdered bark was extracted in 10 ml, acetone (80 %) using mortar and pestle. The homogenate was filtered through Buchner’s funnel using Whatman No. 1 filter paper. The volume of filtrate was adjusted to 50ml with 80 % acetone. The reaction mixture contained 1.5 ml of the plant extract and 1.5ml, 2% Methanolic Aluminum Chloride (2g Aluminium chloride dissolved in 100 ml pure methanol). Blank was prepared with distilled water in place of sample. The absorbance of the reaction mixture was measured at 367.5nm on a UV-visible double beam spectrophotometer (Shimadzu-190). Total flavonoid contents were calculated with help of standard curve of rutin (0.3 mg/ml) and values were expressed as g/100g of dry weight.

c. Water soluble tannins

Method of Schanderl (1970) was employed for determination of water soluble tannins. 500mg of the powdered bark sample along with 75ml distilled water were transferred to a 250 ml capacity conical flask. The flasks were gently heated on
hot plate and material boiled for 30 minutes and centrifuged at 2000rpm for 20 minutes. The residue was discarded and the volume of supernatant was adjusted to 100 ml with volumetric flask. This extract was used for the estimation of the tannin in the bark sample. 1mL of the reaction mixture was transferred to 100mL capacity volumetric flask containing 75mL distilled water. 5ml of Folin-Denis reagent was added followed by 10ml of sodium carbonate solution and diluted to 100 ml with water. Contents in the flasks were thoroughly mixed and after 30 minutes absorbance was measured at 700nm on double beam UV-visible spectrophotometer (Shimadzu-190). A blank was prepared with water instead of the sample. Water soluble tannins were estimated and calculated with the help of standard curve of tannic acid (0.1mg/mL) and expressed as g/100g of dry weight.

d. Total alkaloids

The total alkaloid contents in the bark samples of A. latifolia, C. religiosa, P. marsupium and T. arjuna was measured using 1, 10-phenanthroline method described by Singh et al. (2004) with slight modifications. 100mg bark powder was extracted in 10 ml 80% ethanol. This was filtered through muslin cloth and centrifuged at 5000rpm for 10 min. Supernatant
obtained was used for the further estimation total alkaloids. The reaction mixture contained 1ml plant extract, 1 ml of 0.025M FeCl$_3$ in 0.5M HCl and 1ml of 0.05M of 1, 10- phenanthroline in ethanol. The mixture was incubated for 30 minutes in hot water bath with maintained temperature of 70 ± 2°C. The absorbance of red coloured complex was measured at 510nm against reagent blank. Alkaloid contents were estimated and it was calculated with the help of standard curve of colchicines (0.1mg/mL, 10mg dissolved in 10ml ethanol and diluted to 100mL with distilled water). The values were expressed as g.100 g$^{-1}$of dry weight.

6. Antioxidant Potential

a. DPPH radical scavenging activity

The free radical scavenging effect of different bark samples was assayed in vitro by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) according to the method described by Wang et al. (1998) with slight modifications. Equal volumes of methanolic solutions of DPPH (100µM) and crude extract containing (20-200µg/mL) were mixed together. The reaction mixture was shaken well and allowed to stand at room temperature for 30 minutes. The absorbance of the coloured complex was measured at 516nm on double beam UV- spectrophotometer against methanol as blank.
The L-ascorbic acid (100µg/ml) was used as standard and the percent scavenging effect was calculated by using the formula

\[
\% \text{Inhibition} = \left( \frac{A_c - A_{E}/A_s}{A_c} \right) \times 100
\]

Where,

- \(A_c\) is the Absorbance of Control (DPPH),
- \(A_E\) is the Absorbance of DPPH + plant extract,
- \(A_s\) is the Absorbance of standard

b. Total antioxidant activity by phosphomolybdenum method

The total antioxidant activity (TAC) of the crude methanolic extract was determined according to the method of Prieto et al. (1999). The plant extract 0.3ml (50-100µg/ml) was mixed with 2.5ml of phosphomolybdenum reagent (0.6M sulphuric acid, 28mM sodium sulphate and 4mM ammonium molybdate were mixed together in 250mL distilled water). The absorbance of the reaction mixture was measured after 15 minutes at 695nm on UV-double beam spectrophotometer. The L-ascorbic acid (500µg/ml) was used as reference standard. The antioxidant activity was expressed as µg equivalent of ascorbic by using the standard graph of ascorbic acid.
C. Reducing power

The reducing power of the methanolic extract was determined according to the method given Oyaizu, (1986). 1 ml of the bark extract containing (10-100µ/ml) in 1ml of the deionised water mixed with 2.5ml of phosphate buffer (0.2M, pH 6.6) and 2.5ml potassium ferrocyanide (1%). The mixture was incubated at 500C for 20 minutes. 2.5ml of TCA (10%) and centrifuged at 3000rpm. The upper layer of the solution was mixed with 2.5ml distilled water and FeCl₃ (0.5ml, 0.1%). The absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicated the higher reducing power. The absorbance compared with the standard ascorbic acid (100µg/ml).

d. Nitric oxide scavenging activity

Nitric oxide effect of different bark samples was assayed according to the method given by Singh et al. (2009) with slight modifications. Sodium nitroprusside (5µM) was dissolved in standard phosphate buffer (0.025M, pH 7.4). 2ml of sodium nitroprusside was added to 1ml plant extract (50µg/ml) and the reaction mixture was incubated at 25°C for 5 hours. 0.5mL of the incubation solution was removed and diluted with Griess reagent
(Diluted equal volume of 1% sulphanilamide in 2% phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride in water). The absorbance of the chromphore formed was measured at 546nm on UV-visible double beam spectrophotometer. Control experiment was also carried out in the similar manner and distilled water was used in place of extract. The ascorbic acid (50µg/ml) was used as reference standard and the percent scavenging effect was calculated by using the formula

\[
\% \text{ scavenging activity} = \left( \frac{A_c - A_E}{A_s} \right) \times 100
\]

Where,

- \( A_c \) is the Absorbance of Control reaction.
- \( A_E \) is the Absorbance of plant extract.
- \( A_s \) is the Absorbance of standard.

e. Hydroxyl radical scavenging activity

The scavenging of the hydroxyl radical was measured according to the method described by Halliwell et al. (1987). The
assay mixture contained 0.1ml of 1mM EDTA, 0.01ml of 10mM feCl3, 0.1ml of 10mM H2O2, 0.36ml of 10mM dexoy ribose and 1ml of extract (100µg/ml) dissolved in distilled water, 0.33ml of phosphate buffer (50 mM, pH 7.4) and 0.1mL ascorbic acid. The mixture was incubated at 37°C for 1 hr. 1ml of incubated mixture mixed with 1ml of 10% TCA and 1ml of 0.5% TBA (in 0.025M NaOH containing 0.025% BHA). The intensity of the pink coloured complex developed measured at 532 nm. The ascorbic (100µg/ml)) acid was used as standard. Scavenging % of the hydroxyl radical was calculated by using the formula

\[
\% \text{ scavenging activity} = \left( \frac{\text{Ac} - \text{A}_E}{\text{A}_S} \right) \times 100
\]

Where,

\( \text{Ac} \) is the Absorbance of Control reaction.

\( \text{A}_E \) is the Absorbance of plant extract.

\( \text{A}_S \) is the Absorbance of standard.
f. Hydrogen peroxide radical scavenging activity

Ability of the bark extract to scavenge hydrogen peroxide radical was estimated according to the method given by Govindarajan et al. (2003) and Gulcin et al. (2004). 1mL of plant extract containing 200µg/ml residue mixed with 2mL of 10mM phosphate buffered (0.1M, pH 7.4) hydrogen peroxide solution. The reaction mixture was incubated at 37°C and the absorbance of measured at 230nm after 10 minutes against blank containing phosphate buffer solution. The ascorbic acid (200µg/ml) was used as reference standard and the percent scavenging hydrogen peroxide was calculated by using the formula.

\[
\% \text{ scavenging activity} = \left( \frac{A_c - A_{E}/A_s}{A_c} \right) \times 100
\]

Where, Ac is the Absorbance of Control reaction.

\( A_E \) is the Absorbance of plant extract.

As is the Absorbance of standard.

g. Ferrous ion chelating ability

The method proposed by Decker and Welch (1990) was used to determine the metal chelation ability of the plant extract. 2 ml of bark extract (100µg/ml) was mixed with 0.1 ml of 2 mM
FeCl₂ and 0.2 ml of 5 mM ferrozine solutions and allowed to react for 10 minutes at room temperature. The absorbance at 562 nm of the resulting solutions were measured and recorded. The FeCl₂ and ferrozine acted as control solution. The percentage inhibition of the ferrous ion was calculated by comparing the results of the test with that L-ascorbic acid (100μg/ml) of the control using the formula.

\[
\text{% scavenging activity} = \left( \frac{Ac - AE}{AS} \right) \times 100
\]

Where,

\( Ac \) is the Absorbance of Control reaction.

\( AE \) is the Absorbance of plant extract.

\( As \) is the Absorbance of standard.

**Organic constituents**

**a. Starch content**

For estimation of the starch, the insoluble residue along with the filter paper obtained at the beginning after filtering the alcoholic extract of reducing sugar was transferred to a 100ml
capacity conical flask containing 50 ml distilled water and 5ml concentrated HCL. The contents were hydrolyzed at 15lbs pressure for half an hour. After cooling these conical flasks to room temperature, neutralized by addition of anhydrous sodium carbonate and filtered through Buchner’s funnel. Filtrate contains reducing sugars (glucose) formed as a result of hydrolysis of starch. The volume of filtrate was measured and used for the further analysis of starch. These sugars represent the starch content in the residue. Other steps are essentially similar as described for estimation of reducing sugars. The amount of starch was estimated with the help of standard curve obtained by using different concentration of standard glucose solution (0.1 mg.ml⁻¹) and were expressed in g 100 g⁻¹ dry tissue.

b. Amylose content

Amylose content was estimated according to the method described by Sadasivam and Manickam (1992). 250mg of powdered bark sample was taken in 100ml beaker. To this 1ml of distilled ethanol and 10mL of 1N NaOH was added and boiled on water bath for 10 minutes for digestion. The reaction mixture was cooled and transferred to 100mL capacity volumetric flask and diluted to 100mL with distilled water. This extract was used
for the estimation of the amylase. In 50mL test tube 2.5ml plant extract was taken. To 20mL of distilled water and few drops of phenolphthalein indicator (0.1%) were added. 0.1N HCl was added to this test tube until the pink colour disappeared. To this 1mL of iodine reagent (1g of iodine and 10g of KI dissolved in water and diluted to 500mL) was added and volume was made to 50mL. The intensity of the colour measured at 590nm on double beam UV-spectrophotometer. The amylase content was estimated and calculated with the help of standard curve obtained by using different concentrations of standard amylose solution (1mg/mL) and expressed as g/100g of dry tissue.

c. Amylopectin content

Amount of Amylopectin was calculated by subtracting the amylose content from the starch content and expressed as g/100g of dry weight.

d. Total sugar content

The total sugars were estimated following the Phenol-sulphuric acid method described by Dey (1990). The plant material (0.250mg of oven dried bark powder) was suspended in 20ml of 90% ethanol in 50mL test tube. The test tubes were stoppered with cork and the suspension was incubated in hot
water bath and maintained at 60°C for one hr. The extract was decanted and collected in 25ml capacity volumetric flask and re-extracted with another 10ml volume of 90% ethanol. Both the extracts were collected and final volume was made 25ml with the 90% ethanol. For the estimation, to the 0.2 ml plant extract in a test tube, 1 ml 5% phenol was carefully added and mixed thoroughly. 5ml of concentrated sulphuric acid (analytical grade) was added very carefully to the above test tube. This was mixed thoroughly by vertical agitation with a glass rod with a broadened end. The mixture was cooled at room temperature in air and the absorbance was read at 485 nm against blank containing distilled water. The amount of soluble sugars was estimated with the help of standard glucose (0.1 mg ml\(^{-1}\)) and expressed in g 100 g\(^{-1}\) dry tissue.

e. Reducing sugar content

The reducing sugars were estimated by employing arsenopolybdate reagent introduced by Nelson (1944) for colorimetric determination of the cuprous oxide formed in the oxidation of the sugars by alkaline cooper tartarate reagent. The soluble carbohydrates were extracted from 0.250mg of oven dried powderd bark tissue with 80% neutral ethanol. The extract
was filtered through Buchner’s funnel using Whatman No. 1 filter paper. The filtrate thus obtained was condensed to 5 ml on water bath and to this 2g lead acetate and potassium oxalate (1:1) were added for decolourization, 40 ml distilled water was added and the solution was filtered. The volume of filtrate was measured and it served as an extract for determination of reducing sugars. To the 0.4mL plant extract requisite amount of distilled water was added to make the final volume 1 ml. 1 ml Somogyi’s alkaline copper tartarate reagent (4g CuSO₄, 5H₂O, 24 g anhydrous Na₂CO₃, 16 g Na-K-tartarate and 180g anhydrous Na₂SO₄ dissolved in 1 liter distilled water) was added to each test tube and all these test tubes were transferred to boiling water bath for 10 minutes. Tubes were removed and cooled to room temperature and to this 1 ml Arsenomolybdate reagent (25g Ammonium molybdate dissolved in 450 ml distilled water, 3 g sodium arsenate dissolved in 25 ml distilled water, 21 ml concentrated HCl. These ingredients were mixed well and digested for 48 hours at 37°C) was added to each test tube. The contents were diluted to 10mL with distilled water and after 10 minutes, the absorbance of the reaction mixture was measured at 660nm on UV-visible double beam spectrophotometer (Shimdtzu UV-190). The amount of reducing sugar was calculated with the
help of standard curve obtained by using different concentration of standard glucose solution (0.1 mg ml\(^{-1}\)) and was expressed in g 100 g\(^{-1}\) dry tissue.

**f. Crude fiber content**

Crude fiber contents in the bark samples of *A. latifolia, C. religiosa, P. marsupium* and *T. arjuna* were estimated according to the method described by Maynard (1970). 2g of oven dried bark powder was transferred to 500ml conical flask and 200mL of 0.255N H\(_2\)SO\(_4\) (1.25g of sulphuric acid diluted to 100mL) was added to it. The contents were boiled for 30 minutes with bumping chips on hot plate. The flask were cooled and filtered through muslin cloth. The residue was washed several times with hot distilled water. The residue thus obtained boiled with 200ml, 0.313N NaOH (1.25g of NaOH dissolved in 100mL distilled water). The contents were filtered through the muslin cloth and the residue washed with 25mL 1.25% H\(_2\)SO\(_4\), three 50mL portions of water and 25mL alcohol. The residue was removed and transferred to pre-weighed ashing dish (W1g). The residue was dried at 130 ± 2\(^{\circ}\)C for 2h. The ashing dish was cooled and weighed (W2g). The residue was ignited at 600 ± 15\(^{\circ}\)C. Ashing dish was cooled and weighed (W3g). The Crude fiber content in
the bark samples was calculate by using the formula and expressed g/100g of dry weight.

\[
\text{Loss in weight on ignition (W2-W1) - (W3-W1) x 100} \\
\text{Weight of the sample}
\]

g. Total nitrogen content

For the estimation of total nitrogen from the bark the method described by Hawk et al., (1948) was followed. 0.5g of oven dried powdered bark plant material was taken in Kjeldahl’s flask. To this a pinch of microsalt (200 g K\textsubscript{}\text{2}SO\textsubscript{}4 + 5 g CuSO\textsubscript{}4 dehydrated) and 5ml H\textsubscript{}2SO\textsubscript{}4 (1:1) were added. To avoid bumping few glass beads were added to the flasks and the material was digested on low flame. A faint yellow coloured solution was obtained after complete digestion, which after cooling to room temperature, transferred to 100 ml capacity volumetric flask and diluted with distilled water up to the mark. The solution was filtered through Whatman No. 1 filter paper and used further for the estimation of total nitrogen from the bark powder.

2 ml of plant extract of each bark sample was taken in a set of Nessler’s tubes. To each these tubes added one drop of 8 % KHSO\textsubscript{}4 and volume was adjusted up to the mark of 35ml with
distilled water. 15 ml of freshly prepared Nessler’s reagent (Reagent A: 7g KI + 10 g Hgl₂ in 40 ml distilled water, Reagent B: 10 NaOH in 50 ml water. A and B were mixed in proportion of 4:5 only at the time of estimation) was added to each test tube. The reaction between sample and the reagent gives the orange brown coloured product of NH₄Hg₂I₃. The intensity of this colour was measured at 520 nm on a double beam UV-spectrophotometer (Shimdtzu UV-190) after 15 minutes.

Total nitrogen was calculated by using the standard curve obtained by using the different concentrations of standard ammonium sulfate solution (0.236g of oven dried ammonium sulfate dissolved in distilled water and few drops of conc. H₂SO₄ were added. The volume was made 1000 ml with distilled water. This solution contains 0.05mg of nitrogen per ml) and employing the similar procedure as described for the analysis of samples.

**Crude protein content**

Crude protein contents were calculated by multiplying the total nitrogen content by factor 6.25.
8. Estimation of inorganic constituents

a. Total ash content

Total ash content was determined as described in Indian Pharmacopeia (1996). 1g of dry powdered bark was accurately weighed and transferred to the previously ignited and weighed silica crucible. The bark powder was spread at the bottom of the crucible and the crucible incinerated at a high temperature not exceeding 450°C until free from carbon. The crucible was cooled and weighed. The procedure was repeated to get constant weight and the percent of total ash was calculated with reference to the air dried powder.

b. Preparation of Acid digests

Acid digestion method developed by Toth et al., (1948) was followed for the analysis of inorganic constituents. 0.5g of oven dried bark powder was transferred to 100 ml capacity beaker and 20ml concentrated HNO₃ was added to it. The beakers were kept covered with watch glass till the primary reactions completed. These beakers were heated gently on hot plate to dissolve solid particles bark powder. After cooling to room temperature, 60% of 10ml of perchloric acid was added to it and mixed thoroughly. The beakers were again heated strongly
until 2-3 ml a clear and colorless solution was obtained. The beakers were cooled and contents transferred to the 100 ml capacity volumetric flask and diluted up to the mark with distilled water and kept overnight. On next day the extract were filtered through ash less filter paper (Whatman No.44). Filtrates thus obtained were used for estimation of different in inorganic constituents.

The levels of i. calcium, iii. magnesium, iv. iron, v. manganese, vi. copper, vii. zinc, h. chromium, viii. cadmium, and ix. nickel, were estimated using Atomic Absorption Spectrophotometer (Perkins Elmer 3030). In case needed, appropriate dilution of plant extract was made with distilled water.

ii. Phosphorus

The phosphorus content was estimated according to the method of Sekine et al., (1965). Phosphorus reacts with molybdate vanadate reagent to give yellow colour complex. The intensity yellow colour complex estimating colorimetrically and by comparing it with the colour intensity of the known standards, phosphorus content was estimated.
2ml of acid digest was taken in the test tubes and to this
equal amount of 2N HNO₃ followed by 1ml of freshly prepared
molybdate vanadate reagent (A - 25 g Ammonium molybdate in
500 ml of distilled water. B - 1.25 g Ammonium vanadate in 500
ml 1 N HNO₃, A and B were mixed equally at the time of using)
were added. Then final volume in each test tube was adjusted to
10 ml with distilled water. The ingredients were mixed well and
allowed to react for 20 minutes. After 20 minutes colour intensity
was measured at 420 nm using a reaction blank containing no
phosphorus. Total phosphorus was calculated with help of
standard curve obtained by using standard phosphorus solution
(0.110 g KH₂PO₄ per litre = 0.025 mg P⁵⁺ ml⁻¹) taking different
concentrations, other steps being essentially similar as described
above. Amount of phosphorus in the plant material was
expressed in g.100 g⁻¹ on dry weight basis.

9. Antinurritional factors

a. Nitrate content

The nitrate contents in bark powder of A. latifolia, C.
religiosa, P. marsupium and T. arjuna were determined using
rapid colorimetric method given by Cataldo et al. (1975). In 50mL
test tube, 100mg of dry bark powder was suspended in 10 ml of
deionized water. The suspension was incubated at 45°C for one hour. After incubation, to sediment the residue the sample was centrifuged at 5000 g for 15 minutes. The residue was discarded and the supernatant was taken for nitrate estimation. In 50ml test tubes, 0.2 ml of the extract mixed thoroughly with 0.8 ml of 5% (w/v) salicylic acid (prepared in concentrated H₂SO₄). After 20 minutes at room temperature, to raise the pH 19 ml of 2N NaOH were added slowly above 12. Samples were cooled to room temperature and absorbance at 410nm was determined on a double beam spectrophotometer (Shimdtzu UV-190). The amount of nitrate (mg NO₃⁻ g⁻¹ dry tissue) was calculated with the help of a standard curve obtained by taking different concentrations of KNO₃ and following similar procedure as employed for the extract.

b. Oxalic acid content

The oxalic acid content in apical rind, inner and mature outer bark of A. latifolia, C. religiosa, P. marsupium and T. arjuna was estimated according to the method given Abaza et al. (1968). For estimation oxalic acid 1g of oven dried powdered bark, 10ml 3N HCl and 65ml of double distilled water were taken in 100ml capacity volumetric flask. The flasks were kept in boiling water bath
for 1hr to digest the plant material. After digestion flasks were removed, cooled and diluted up to the mark of 100ml and filtered through Whatman No. 1 filter paper. Two aliquots of 50ml extract were placed in 150ml beakers and 20ml 6N HCl were added in each beaker to increase acidity and avoid pectin retention. The mixture was evaporated to half of its original volume and filtered through Whatman No. 1 filter paper. The precipitate on the filter paper was washed several times with warm double distilled water. To this filtrate 3-4 drops of methyl red indicator (0.01g methyl red in 100ml alcohol) were added and to this concentrated ammonia solution was added until the solution turned faint yellow. After this the solution was heated carefully on water bath maintained at 90-100°C, cooled and filtered to remove interfering ferrous ions containing precipitate.

The filtrate thus obtained was heated to 90-100°C on water bath and to this 10ml 5% CaCl₂ was immediately added along with 20-25 drops of ammonia solution to restore yellow colour. The solution was kept overnight to settle. On next day, the solution was filtered through ashless filter paper (Whatman Filter Paper No. 44). The precipitate on the filter paper was washed several times with double distilled water to make free from Ca (to check whether the ppt is free from Ca²⁺ or not, few drops of 5% sodium oxalate were
added to 3 ml of washing filtrate in test tube. Formation of turbidity indicated presence of Ca++ and demanded further washing of ppt). The along with residue the filter paper was dissolved in hot 1:5 H$_2$SO$_4$ and this was diluted to 125 ml with double distilled water and transferred to 250 ml conical flask. The content of the conical flask was heated to 90 - 100°C and titrated against 0.05 N KMnO$_4$. The percentage of oxalate was calculated by using following formula,

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\text{% of oxalate} = \frac{\text{ml KMnO}_4 \times 0.05 \times 45.02 \times 100}{1000 \times \text{dry weight} \times 50/100}
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