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SEASONAL DYNAMICS IN THE NUTRITIONAL AND ANTINUTRITIONAL STATUS OF STEM BARK OF *ANOGEISSUS LATIFOLIA*.

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ABSTRACT: *Anogeissus latifolia* (Roxb.) is an important medicinal plant used as herbal drug in the cardiac diseases management. In the present investigation seasonal variations in the stem bark samples (Apical bark, Middle bark and Mature inner bark) with respect to total reducing sugars, total sugars, amylose, amylopectin, starch, crude fibers, crude protein, total ash value, total polyphenols, water soluble tannins, total flavonoids, total alkaloids, nitrates, and total oxalate. The concentration of constituents except oxalate and total ash was found higher in the apical stem bark than the middle and mature inner bark. The oxalate and total ash were higher in the mature inner bark than the apical stem bark and middle bark samples.

Key Words: *Anogeissus latifolia,* total polyphenols, tannins, total flavonoids, total alkaloids.

INTRODUCTION

Anogeissus latifolia (Roxb.) is medium sized deciduous tree belonging to the family combretaceae and it is commonly known as "Ghatti". It is distributed throughout India (Warrier, 1994). The plant is common in dry deciduous forest, except E. Bengal and Assam. It is found in Sub-Himalayan tract, from the Ravi to Nepal, Bhihar, Chota Nagpur and ascends to south India (Chopra *et al*., 1956). The bark is effective in anemic conditions and urinary discharges, piles (Kirtikar and Basu, 1975). According to Jain (1991) stem bark is useful in diarrhea, dysuria, cough, colic, liver complaints, snakebite and skin diseases. Bark is remedy for chronic cough called 'Dangya Khokala' (Patil, 2006). In the present study an attempt has been made to standardize the drug pharmacognosticaly.

MATERIAL AND METHODS.

Materials

Different bark samples (apical rind, middle bark and mature inner bark) of *Anogeissus latifolia* were collected from the hilly regions Radhanagari 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 60^0C. Dried bark samples were ground into powder and stored in an air tight plastic container.

Methods

Qualitative analysis

Priliminary phytochemical analysis was done according to the methods given br Brindha *et al*., (1981) and Lala (1993).

Reducing sugars

The reducing sugars were estimated by employing arsenomolybdate 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 powdered bark tissue with 80% neutral ethanol. The extract was filtered through Buckner'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 $CuSO_4$, $5H_2O$, 24g anhydrous Na_2CO_3, 16g Na-K-tartarate and 180g anhydrous Na_2SO_4 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, 3g sodium arsenate dissolved in 25ml 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 spectophometer (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.

Total sugars

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 sealed with cork and the suspension was incubated in hot water bath and maintained at 60^0C 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 0.2ml plant extract in a test tube, 1ml 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 (0.1mg ml^{-1}) and expressed in g.100 g^{-1} dry tissue.

Starch

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 Buckner'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 (0.1mg.ml-1) and was expressed in g.100g^{-1} dry tissue.

Amylose

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 amylose 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 $g.100g^{-1}$ of dry tissue.

Amylopectin

Amount of Amylopectin was calculated by subtracting the amylose content from the starch content and expressed as $g.100g^{-1}$ of dry weight.

Crude fibers

Crude fiber contents in the bark samples 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_2SO_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_2SO_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 ± 2^0C for 2h. The ashing dish was cooled and weighed (W2g). The residue was ignited at 600 ± 15^0C. Ashing dish was cooled and weighed (W3g). The Crude fiber content in the bark samples was calculate by using following formula and expressed $g.100g^{-1}$ of dry weight.

Loss in weight on ignition (W2-W1) - (W3-W1) X 100 / Weight of the sample

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 Buckner'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_2CO_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 minutes 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.

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 250ml 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^{-1} of dry weight.

Total Flavonoids

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 Buckner's funnel using Whatman No. 1 filter paper. The volume of filtrate was adjusted to 50ml with 80 % acetone. The reaction mixture contained 1.5ml of the plant extract and 1.5ml, 2% Methanolic Aluminum Chloride (2g Aluminium chloride dissolved in 100ml 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 flavonoids contents were calculated with help of standard curve of rutin (0.3 mg/ml) and values were expressed as g.100g^{-1} of dry weight.

Total alkaloids

The total alkaloid contents in the bark samples were measured using 1,10-phenanthroline method described by Singh *et al*. (2004) with slight modifications. 100mg bark powder was extracted in 10ml 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, 1ml 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^{0}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.100g^{-1}of dry weight.

Crude protein content: Crude protein contents were calculated by multiplying the total nitrogen content by factor 6.25.

Nitrate content

The nitrate contents in bark powder were determined using rapid colorimetric method given by Cataldo *et al*. (1975). In 50mL test tube, 100mg of dry bark powder was suspended in 10ml of de-ionized water. The suspension was incubated at 45^{0}C for one hour. After incubation, to sediment the residue the sample was centrifuged at 5000rpm for 15 minutes. The residue was discarded and the supernatant was taken for nitrate estimation. In 50ml test tubes, 0.2ml of the extract mixed thoroughly with 0.8ml of 5% (w/v) salicylic acid (prepared in concentrated H_2SO_4). After 20 minutes at room temperature, to raise the pH above 12, 19ml of 2N NaOH were added slowly. Samples were cooled to room temperature and absorbance at 410nm was determined on a double beam spectrophotometer (Shimdtzu UV-190). The amount of nitrate (µg of NO_3.g^{-1} dry tissue) was calculated with the help of a standard curve obtained by taking different concentrations of KNO_3 and following similar procedure as employed for the extract.

Oxalic acid content

The oxalic acid contents were estimated according to the method given by 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^{0}C, cooled and filtered to remove interfering ferrous ions containing precipitate.

The filtrate thus obtained was heated to 90-100^{0}C on water bath and to this 10ml 5% $CaCl_2$ 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 Ca^{++} or not, few drops of 5% sodium oxalate were added to 3ml 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_2SO_4 and this was diluted to 125ml with double distilled water and transferred to 250ml conical flask. The content of the conical flask was heated to 90 – 100^{0}C and titrated against 0.05N $KMnO_4$. The percentage of oxalate was calculated by using following formula,

$$ml KMnO_4 \text{ used} \times 0.05 \times 45.02 \times 100 / 1000 \times \text{dry weight} \times 50/100$$

Total ash content

Total ash content was determined as described in Indian Pharmacopeias (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^{0}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.

RESULTS AND DISCUSSION

Preliminary phytochemical analysis of methanolic extract of apical, middle and mature inner bark is shown in the Table No. 1. The intensity of different phytoconstituents was higher in the apical bark and lower in the mature inner bark while moderate in the middle bark of *Anogeissus latifolia*. Seasonal alterations in the different phytochemicals are shown in the table No. 2. The concentrations of reducing sugars, total sugars, crude fibers, crude protein and total ash was higher during summer while that of amylose, Amylopectin was starch was higher during winter. Maximum 5.93% reducing sugars were noticed in apical bark during summer and lower 2.89% was observed in mature inner bark during winter. These are in the range of reducing sugar content in Black locust bark (0.9% to 3.2%, (Siminovitch *et al*., 1953).Total sugars varied from minimum 3.66% in mature inner bark during winter to maximum 10.63% in apical bark during summer while sugar content in the middle bark ranged from 8.26-9.95%. These values are lower than the *Saraca asoca* (15%), *Polylthia liongifolia* (33%) and *Saraca declinata* (11- 12%) reported by Khatoon *et al.* (2009) and *Eucalyptus globulus* bark (62.47%) estimated by Vazquez *et al*. (2008). Concentration of Amylopectin, one of the components of starch was maximum in apical bark (18.77%) during winter and lowest in mature inner bark (12.66%) during summer while that of amylose was recorded maximum in apical bark (1.07%) and lowest in mature inner bark (0.62%) during winter and summer respectively. Amylose content in middle bark ranged from 0.76-0.93%.

Higher amount of starch was reported in apical stem bark (19.83%) in winter while mature inner bark (13.29%) accumulated lower starch during summer which is higher than the starch content in the bark tissue of six tree species- Ash tree (6.9%), Alder (2.8-2.9%), Oak (2.5-2.7%) , Maple (0.5- 0.6%) and Birch (0.3-0.4%) estimated by Essiamllh and Eschrich (1985) and lower than the *Saraca asoca* (52-55%) and *Saraca declinata* (51-52%) reported by Khatoon *et al*., (2009). Crude fiber content was ranged from minimum 12.45% in mature inner bark during summer to maximum 21.75% in apical stem bark during winter while that of middle bark was moderate (14.05-19.85%).

The crude fiber content estimated in our study are in the range of *Ficus racemosa* (20.5%) as reported by Ahamad *et al*., (2010) and Shri Lankan Cinnamon contained (21.27%) as quantified by Al-Numair *et al*., (2007) and lower than *Diospyros beccarii* (51.56%) estimated by Whitten and Whitten (1987). Amount of Crude protein was higher in apical stem bark (11.04%) in summer while lowest value 4.34% was estimated for mature inner bark during winter and middle bark contained 5.55-8.82% crude protein.

Table No. 1. Qualitative analysis of stem bark samples of A. latifolia.

Sr. No.	Parameter	Samples		
		Apical Bark	Middle Bark	Mature inner Bark
1	Polyphenol	+++	++	+
2	Flavonoid	+++	++	+
3	Tannin	+++	++	+
4	Alkaloid	+++	++	+
5	Flavones	+++	++	+
6	Terpenoids	+++	++	+
7	Saponins	+++	++	+
8	Cardiac glycosides	+++	++	+
9	Sterols	+++	++	+

+++'- High concentration; +'+'- Moderate concentration; '+'- low concentration and '-' absent

Table No. 2. Phytochemical Evaluation

Sr. No.	Parameter	Samples					
		Apical Bark		Middle Bark		Mature inner Bark	
		Summer	Winter	Summer	Winter	Summer	Winter
1	Reducing Sugars	5.93	4.94	5.36	3.79	4.42	2.89
2	Total Sugars	10.63	9.54	9.95	8.26	8.49	3.66
3	Amylopectin	17.17	18.77	13.81	16.90	12.66	16.16
4	Amylose	0.87	1.07	0.76	0.93	0.62	0.79
5	Starch	18.04	19.83	14.57	17.83	13.29	16.95
6	Crude fiber	15.5	21.75	14.05	19.85	12.45	16.3
7	Crude Protein	11.04	9.51	8.82	5.55	7.95	4.34
8	Total ash	13.7	10.1	16.4	13.3	17.8	16.2
9	Total Polyphenols	18.78	14.61	18.54	14.35	14.30	12.66
10	Tannin	6.75	6.35	6.25	6.02	4.43	4.36
11	Flavonoid	4.63	2.96	4.50	2.80	4.34	2.07
12	Total Alkaloid	2.97	2.92	2.37	2.10	1.78	1.44
13	Total Oxalate	12.28	10.87	14.91	14.02	16.97	16.20
14	Nitrate (µg of $NO_3.g^{-1}$ dry tissue)	4863	3087	4208	2459	2077	1148

The Crude protein values in the *Anogeissus latifolia* bark are in the range of *Gmelina arborea* (11.37%) for apical bark, *Careya arborea* (5.37%) for middle bark as reported by Santra *et al*. (2008) and Chinese Cinnamon (4.10±0.09%, Al-Numair *et al*., 2007) for mature inner bark. Values reported in our study are higher than *Picea jezoensis* (1.6±0.14%) and *Abies bomplepis* (1.9±0.05%) respectively (Yokoyama and Shibata 1998).

Total polyphenols determined in the present investigation are higher during summer season than the winter. The apical bark (18.78%) during summer accumulated higher amount polyphenols while lowest value was recorded for mature inner bark during winter (12.66%). These values are higher than estimated for estimated total polyphenols in the bark of *Averrhoa carmboloa* (3.01%), *Azadirachta indica* (8.9%) *Casurina equisetifolia* (7.2%) and in *Cinnamon zeylanicum* (4.8%) by Prakash *et al*., (2007). The tannin content of apical and middle bark was estimated in the range of 6% during both season and are lower than tannin content reported in the bark of *Pinus oocarpa* (14.04%) and *Pinus leiophylla* (13.08%) by (Martinez *et al*., 1995).No large difference was noticed in the tannin content of mature inner bark during summer and winter i.e. 4.43 and 4.36% respectively and are in the range of *Labumum vulgare* (4.18%) reported by Alibalic and Murko, 1991). Flavonoid contents increased two fold during summer than winter and ranged from its lowest amount 2.07% in mature inner bark during winter to 4.63% in apical bark during summer. Winter flavonoid are in the range of *Azadirachta indica* (2.52%) by Sultana *et al*. (2005) while summer flavonoid are lower than the *Acanthopanax trifoliatus* bark (9.91%) as estimated by Sithisarm and Jarikasem (2009). Amount of flavonoid determined in the present study is quite higher than estimated for *Stryphonodendron adstringes* (0.03%) by Elizabeth *et al*. (2004). No large differences in the alkaloid content during summer and winter was observed in the three bark samples. Apical bark contained 2.92-2.97%, middle bark accumulated 2.10-2.37% and mature inner bark alkaloid concentration varied from 1.44-1.78% which is lower than the apical and middle bark. Alkaloid level estimated in *Anogeissus latifolia* are higher than *Delphinium occidentale* (1.1%) reported by William (1975) and lower than *Chinchona officinalis* (9.3%) determined by Martin and Gandara (1945).The total ash content in the bark samples was increased from apical to mature inner bark during both seasons and was maximum in mature inner bark (17.8%) during summer and minimum in apical stem bark (10.1%) during winter. This total ash content was maximum than reported for *Stryphnodendron adstringens* (1.6%, Audi *et al*., 2004), Shri Lankan Cinnmon and Chinese cinnamon (3.77% and 2.89%, Al- Numair *et al*. 2007) and lower than *Vatica simalurensis* (26.15%) as investigated by Whitten and Whitten (1987).

Amount of oxalate was also higher in the mature inner bark and decreased to apical stem bark. Higher level of oxalate was recorded during summer in mature inner bark (16.97%) while lowest content was estimated in apical bark (10.87%) during winter. Pandey and Kori (2009) reported that oxalate content in *T. arjuna* bark varied from 7.66 to 20.05%. In the present study the oxalate contents correlates with *T. arjuna* reported by Pandey and Kori, (2009). Nitrate levels in the bark samples increased during summer from mature inner bark to apical bark and were recorded maximum 4863µg/g during summer and minimum 1148µg/g during winter in apical and mature inner bark respectively. These values are lower than the values recorded for white radish (20421µg/g) Celery (10919µg/g) and Red radish (7266µg/g) by Taras (1950).

Conclusion

The bark of *Anogeissus latifolia* contains appreciable amount of secondary metabolite polyphenols, tannins, flavonoids and alkaloids. These phytoconstituents may acts as source of pharmacologically active agents and also natural antioxidants. Low nitrate and oxalate contents points to fearless use of this drug. The present evaluation of various biochemical parameters will be helpful while standardizing the drug for its various pharmacological potentials and to check the adulteration in natural valuable drug at the time of consumption for desire pharmacological effect.

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