SCREENING OF Anogeissus latifolia FOR PHYTOCHEMICAL AND ANTIMICROBIAL PROPERTIES AGAINST HUMAN PATHOGENS

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1. INTRODUCTION

The use of plants for treating diseases is as old as the human species. Popular observations on the use and efficacy of medicinal plants significantly contribute to the disclosure of their therapeutic properties, so that they are frequently prescribed, even if their chemical constituents are not always completely known (Maciel *et al*., 2002). From 250 to 500 thousand plant species are estimated to exist on the planet, and only between 1 and 10% are used as food by humans and other animals (Cowan, 1999). The use of medicinal plants as a source for relief from illness can be traced back over five millenniums to written documents of the early civilization in China, India and the north east, but it is thoughtless as art as old as mankind (Mahesh and Satish, 2008). The potential of higher plants as a source for new drugs is still largely unexplored. Among the estimated 2,50,000-500,000 plant species, only a small percentage have been investigated phytochemically and the fraction submitted to biological or pharmacological screening. Compound of natural or synthetic origin has been the source of innumerable therapeutic agents (Gerhartz *et al.*, 1985). Medicinal plants are rich sources of antimicrobial agents. Plants are used medicinally in different countries and are the source of potential and powerful drugs. A wide range of medicinal parts are used to get different rasayanas which possess different medicinal properties against different microbes. Evidently, there are not many scientific studies that confirm the antimicrobial properties for most of the plants collected from our country. The phytochemical research based on ethnopharmacological informations is generally considered an effective approach in the discovery of new anti-infective agents from higher plants. There are a few reports on the use of plants in traditional healing by either tribal people or indigenous communities of Tamil Nadu. The development of drug resistance in human pathogens against commonly used antibiotics has necessitated a search for new antimicrobial substances from other sources including plants. Screening of medicinal plants for antimicrobial activities and phytochemicals is important for finding potential new compounds for therapeutic use (Kroschwitz *et al.*, 1992).

Infection by bacteria happens when harmful bacteria start reproducing at a fast rate in the body causing mild to severe infections, the latter of which is exemplified by tuberculosis, cholera and plague. Bacterial infections like ear infection and sour throat occur commonly in children. Some infections are specific to the organ they invade; for example surgical wounds may get infected with *Staphylococcus aureus*. Treatment with appropriate antibiotics, which act through either by killing the bacteria or prevent their reproduction, can cure diseases caused by bacterial infections. Penicillin was the first antibiotics discovered, and the drug is still in use. Other drugs such as tetracycline, erythromycin, bacitracin, fluroquinolones, and cephalosporins, are in the market and have helped humanity control infection. The biggest threat however is the emergence of some

bacterial strains that have developed resistance to one or more types of antibiotics. The clinical incidence of drug-resistant microbes has increased dramatically in recent decades, leading to hundreds of thousands of hospitalizations and tens of thousands of deaths annually in the United States alone (Kamatenesi-Mugisha *et al.*, 2008).

Long before mankind discovered the existence of microbes, the idea that certain plants had healing potential, indeed, that they contained what we would currently characterize as antimicrobial principles, was well accepted. Since antiquity, man has used plants to treat common infectious diseases and some of these traditional medicines are still included as part of the habitual treatment of various maladies. For example, the use of bearberry (*Arctostaphylos uva-ursi*) and cranberry juice (*Vaccinium macrocarpon*) to treat urinary tract infections is reported in different manuals of phytotherapy, while species such as lemon balm (*Melissa officinalis*), Garlic (*Allium sativum*) and tee tree (*Melaleuca alternifolia*) are described as broad-spectrum antimicrobial agents (Hemraj and Anil, 2012).

Anogeissus latifolia is one such plant in ayurvedha used for many ailments such as diarrhea, dysuria, cough, colic, liver complaints, snake bite and skin diseases. Tribals in Udaipur district of Rajasthan, use the bark of this tree in the treatment of fever. Bark is remedy for chronic cough called ‘Dangya Khokala’. Tribal people residing in the forest of Gundlabranhmeswaram wild life sanctuary apply paste of stem bark on scorpion sting. Decoction of bark, two spoons daily is useful as remedy against cough and leaf decoction is effective in epileptic fits. Gum is used as tonic and generally consumed after delivery. Leaf juice is given in purulent discharges from the ear while, fruit is astringent to bowels and cures kapha and biliousness. Skin disease, fever, obesity, anaemia, dropsy, diabetes, urinary calculi, herpes, piles, wound healer. The present study is focused on Screening of *Anogeissus latifolia* for phytochemical and antimicrobial properties against wound pathogens (Patil and Gaikwad, 2011).

TAXONOMIC CLASSIFICATION OF *Anogeissus latifolia*

Kingdom : Plantae

Division : Angiosperms

Order : Myrtales

Family : *Combretaceae*

Genus : *Anogeissus*

Species : *latifolia*

Binominal Name : *Anogeissus latifolia* (Roxb. Ex DC.) Wall. Ex Guill. & Perr (Deeksha *et al.*,2016).

Figure 1: Anogeissus latifolia – Stem, leaves and fruit of A. Latifolia (Roxb.)

Plant Description :

Common name: Axle Wood tree.

Tamil name: Vel naga maram.

2. MATERIALS AND METHODS

2.1 Collection of plant samples

Anogeissus latifolia leaves were collected in the month of January 2018 from Siriya kalvarayan hills Eastern Ghats, Villupuram district. Samples were shade dried and pulverised under the room temperature for 48 hrs.

2.2 Preparation of the extract

The dried powders of leaf of *Anogeissus latifolia* were defatted with methanol (60-80ºC) in a Soxhlet Apparatus by continuous hot- percolation. The solvent was removed by kept into the incubator for 24 hrs. The resultant dried extracts were used for further study.

2.3 Bacteria used for the study

Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Salmonella typhi, and Shigella sp. were used for testing antibacterial activity of leaves extract. The test organisms used in this study were obtained from IMTECH, Chandigarh, India. The bacterial strains were cultured on nutrient agar slants and were maintained by sub culturing periodically and preserved at 4° C until further use.

2.4. Qualitative phytochemical analysis

Qualitative phytochemical screening was done for evaluation of major phytochemical constituents such as tannins, saponins, flavonoids, sterols, terpenoids, carbohydrates, phenols, proteins and amino acids, alkaloids and glycosides using standard procedure of analysis (Brindha *et al.*, 1982)

Table 1: Summary of the procedure followed for phytochemical analysis

S.no	Test	Observation	Inference
1.	To 2ml of the test solution, a few microlitres of chloroform was mixed with 3 to 4 drops of acetic anhydride and 1 drop of conc. H_2SO_4.	Colour change from purple to blue or green	Steroids present
2.	A piece of tin and 2 drops of thionyl chloride was added to 2 ml of the plant extract.	Development of violet/purplish colour	Triterpenoids present.
3.	2ml of test solution was mixed with a very small quantity of anthrone reagent and a few drops of conc. H_2SO_4 and heated.	Green or purple colour developed.	Sugar present.
4.	To 2ml of plant extract, 2ml of Fehling's reagent and 3 ml of water was added.	Formation of reddish-orange colour	Presence of reducing sugar.
5.	To 2 ml of the respective plant extract, 2N HCl was added. The resulting aqueous layer was decanted. To this, a few drops of Mayer's reagent was added.	Turbidity or white precipitate	Alkaloids present.

6.	A drop of neutral ferric chloride (5% solution) was added to 2 ml of the plant extract mixed with a bit of alcohol.	Formation of intense blue-coloured solution.	Phenolic compound(s) present.
7.	To 2 ml of the solution mixed with alcohol, a pinch of magnesium metal and 1-2 drops of concentrated HCl was added and the test tube was heated.	Formation of red to orangish-red colour	Presence of flavonoids.
8.	To 2 ml of alcoholic plant extract, a few drops of Ehrlich's reagent and a few drops of conc. HCl was added.	Development of pink colour.	Presence of catachins
9.	To 2 ml of test solution, a few ml of water was added and the tube was shaken.	Formation of foamy lather upon shaking.	Saponins present.
10.	To 2 ml of test solution, a few ml of water and a few ml of lead acetate solution was added.	Formation of a white precipitate	Tannins present.
11.	To 2 ml of test solution, a few ml of magnesium acetate solution was added.	Pink colour developed.	Anthraquinones present.
12.	To 2 ml of plant extract, a solution of 1% ninhydrin (in alcohol) was added.	Development of blue or violet colour	Presence of aminoacids.

2.5 Determination of antibacterial activity using standard antibiotics by disc diffusion method

Fifteen antibiotics were used for antibiotic susceptibility study against standard and clinically isolated microorganisms. All antibiotic discs were purchased from Hi-Media, Bombay, India. The names and concentration of the antibiotics and antifungal are as follows: Amikacin (10 mcg), Azithromycin (15 mcg), Carbenicillin (100 mcg), Cefaclor (30 mcg), Ceftazidime (30 mcg), Chloramphenicol (30 mcg), Ciprofloxacin (10 mcg), Imipenem (10 mcg), Methicillin (5 mcg), Piperacillin (100 mcg), Tetracycline (10 mcg), Amphotericin-B (100 units), Fluconazole (25 mcg), Ketoconazole (10 mcg), Nystatin (100 units).

2.6 Determination of antibacterial activity by agar well diffusion method

Antimicrobial activity was determined by the well diffusion method according to National Committee for Clinical Laboratory Standards (NCCLS, 2003). Petri plates containing 20 ml of, Nutrient (for bacteria) Agar medium were seeded with 1-3 day cultures of microbial inoculums (standardized inoculums 1-2 X 107 cfu/ml 0.5 Mcfarland standard). Wells (6 mm in diameter) were cut off into agar and 50 µl of plant extracts were tested in a concentration of 100 mg/ml and incubated at 37ºC (bacterial strains) and at 25ºC (fungal strains) for 24-48 h. The assessment of antimicrobial activity was based on measurement of the diameter of the inhibition zone formed around the well.

3. Results and Discussion

The present study was conducted to investigate antibacterial properties of *A. latifolia*. Powdered samples of plant leaves were extracted in four different solvents. The plant material was subjected to soxhlet extraction and the dried paste was weighed and dissolved appropriately in the original solvent used for extraction. This mode of reconstitution of the plant material allows for convenient dissolution of the material,

without precipitation. The yields of extraction from originally used 15-20 g of leaf powder were around 2 g (after soxhlet extraction). Before proceeding with ethanol as the solvent of choice for soxhlation, different solvents such as water, methanol, acetone, chloroform, ethylacetate and hexane (in the order of decreasing polarities) were tried by quickly taking 5g of the plant powder and grinding it with a mortar and pestle, which was then, filtered using a Whatman no.1 filter paper. Then, the extracts were assessed qualitatively for phytochemical composition. Based on the abundance of the phytochemicals present (in terms of qualitative analysis), ethanolic extract was found to possess most of the phytochemical classes.

Figure 2: Photograph of the various solvent extracts of *A. latifolia*

Colour of the extracts were pinkish brown to gold for water (aqueous extract), dark green for both MeOH and EtOH, dark green for acetone, chloroform and EtoAc extracts. A pale green extract was obtained with hexane. Brindha *et al*. method is a compendium of different phytochemical analyses procedures which yield accurate results when assessing plant extracts for their phytochemical/secondary metabolites content. Different extracts were immediately subjected to preliminary phytochemical screening. Among the different extracts used, it was identified that acetone extract tested negative for all the phytochemical classes. Water extract of *A. latifolia* tested positive for all the classes of phytochemicals, except for anthraquinones. Among the several solvents used, both EtOH and MeOH extracts contained two classes of phytochemicals (tannins and catachins). EtOH extract possessed tannins, polyphenols, polyacetylenes, flavonols, terpenoids, sterols and alkaloids (Table 2 and Fig. 2).

S.No.	Test	Ethanol	Aqueous	Methanol	Chloroform	Acetone
1	Anthroquinone	-	-	-	+	-
2	Tannins	+	+	+	-	-
3	Saponins	+	-	-	-	-
4	Flavonoids	+	-	-	-	-
5	Catachins	+	+	+	+	-
6	Sugar	+	-	-	+	-

Table 2: Phytochemical Analysis of *Anogessius latifolia*

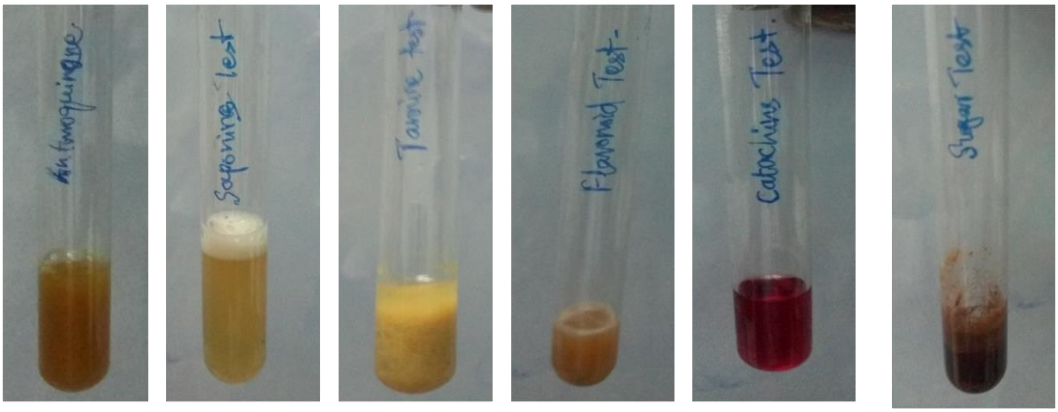

Figure 2: Phytochemical analysis of *A. latifolia* (Roxb.)

The antibacterial assay was carried out within a day after extraction, in order to avoid atmospheric oxygen-mediated oxidation of the plant material. Ethanol extracts of different concentration were tested with positive and negative control against three different pathogens. Both the disc diffusion and the well diffusion assays were carried out; of which better results were obtained from the well diffusion assays, which may be due to comparatively large volumes of plant extracts that wells can hold, whereas, discs are tinier, amenable to contamination (if sterilization is not carried out properly). The wells were punched using autoclaved gel punchers and Mueller Hinton Agar was used as a medium of choice since it allows for luxuriant growth of all three organisms used in the study.

The zones of inhibition were measured by taking five different concentrations of the ethanolic extract – 0.01, 0.05, 0.1, 0.15 and 0.2 mg/ml into wells punched with a sterile, autoclaved gel puncher. Appropriate negative controls (ethanolic disc) as well as positive controls (antibiotic discs) were maintained in order to cross-check the activity of the plant extract against *Klebsiella pneumoniae, E.coli* and *Salmonella typhi* (Fig. 3). The zones of inhibition obtained were tabulated (Table 3).

Bacterial Strains used	Concentration of ethanol extract used					Commercial antibiotics used			
	0.01 mg	0.05 mg	0.1 mg	0.15 mg	0.2 mg	Gen (10 µg)	Amp (10 µg)	Pen (30 µg)	Van (10 µg)
E. coli	0.3	0.5	0.9	1.1	1.5	2.0 cm	-	-	1.8 cm
S. typhi	0.5	0.9	0.7	1.0	1.0	2.9 cm	-	-	None
K. pneumoniae	0.4	0.2	0.7	1.1	1.2	2.5 cm	-	-	None

Table. 2 Zones of inhibition obtained for ethanolic extract of *A. latifolia* against *E.coli, Klebsiella pneumoniae* and *Salmonella typhi.*

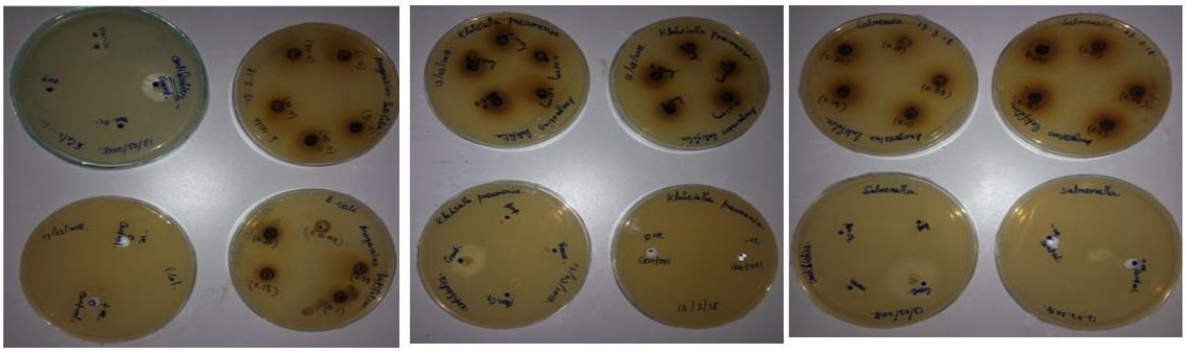

Figure 3. Well diffusion assay of the ethanolic extract of *A. latifolia* against *E.coli, Klebsiella pneumoniae* and *Salmonella typhi.*

Plant extracts often contain secondary metabolites of different types. This reflects the differences in metabolism in the plants themselves, and the mutually beneficial relationships that endophytic organisms may share with the plants, by residing within them. Similar to our immune system of the gut, bacteria residing within the plants may aid in the synthesis and alteration of plant metabolites. Thus, secondary metabolites are akin to the immune system found in animals, and therefore, they would allow only specific organisms to grow (which have association with the plants themselves), and kill the intruding pathogens. This way, secondary metabolites, which are loaded with diverse kinds of chemical compounds, may aid in either bacteriostasis or bactericidal killing of the pathogenic bacteria used as test species in our study (Chattopadhyay *et al*., 2002).

Natural products from plants are of interest for the discovery of antimicrobial compounds. Assays used in the identification of antimicrobial compounds are reviewed in this chapter. The measurement of growth inhibition of microorganisms by diffusion or dilution assays is used for screening antimicrobial compounds and plant extracts. For drug discovery, microbial growth inhibition is not sufficient. Additional studies are required on the mode of action in pathogenic microorganisms such as effects on bacterial cell membranes, fungal cell wall synthesis, DNA replication and repair, ribosome binding, protein synthesis and metabolic enzymes (Verma and Singh, 2008). It is therefore important to study the mode of action of plant antimicrobial compounds after positive screening for microbial growth inhibition. This chapter discusses first the most common mode of action of antibiotics followed by an overview of possible assays which can be used as tools to find antimicrobial compounds and discover novel leads for drug development (Parekh and Chanda, 2008). From the discovery of penicillin in 1928 and during the four decades after World Wall II, many advances were made in antimicrobial therapy. Today, the pace of antimicrobial discovery has slowed. During the 20-year period from 1983 to 2002, the FDA's (Food and Drug Administration) approval of new antibacterial agents decreased by 56%. Between 2004 and 2006, only three new antibacterial agents have been approved. Most antimicrobial agents used for the treatment of bacterial infections may be categorized according to their principle mode of action. The most common modes of action are interference with the cell membrane and cell wall, interference with nucleic acids, and enzyme interactions (Lewis and Ausubel, 2006).

Conclusion

Herbal medicines are an essential and growing part of the international pharmacopeia. Knowledge of their medicinal properties is growing as a result of research and testing, which will make them an increasingly safe alternative or a preferred option to allopathic medicine. The present study reveals the presence of different chemical compounds in *Anogeissus latifolia* with antibacterial properties against human pathogens. Further research in this pursuit, such as separation of individual compounds may land up with new chemical compounds of antibacterial property.

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