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A Comprehensive Pharmacological Evaluation of the Effectiveness of Plant Extract *Antidesma Montanum Blume*

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ABSTRACT

The study is aimed at phytochemical investigation and antimicrobial activity of the plant belonging to the family Phyllanthaceae which is widely used in Udupi, Dakshina kannada districts of Karnataka for their medicinal property. The aim of the survey is to determine the zone of inhibition of the extract on some bacterial and fungal strains. The antimicrobial activity was determined using agar well diffusion method. The antibacterial and antifungal activity of the extract of the plant Antidesma montanum blume was tested against one gram positive Bacillus spp and one negative Pseudomonas spp. And two fungal strains Penicillium and Aspergillus. The zone of inhibition of the extract determines antibacterial and antifungal activities. Aqueous plant extract was highly effective against the bacterial and fungal strain. The phytochemical analysis of the plant was carried out. The antimicrobial activity of the plant extract of Antidesma montanum blume was due to the presence of various secondary metabolites such as total phenol, tannin, flavonoid and antioxidants were determined. Hence the plant can be used in the development of new pharmaceutical research activity. Biofilm is a complex microbial community. Biofilms show high resistance to antimicrobials. Effective Inhibition of the anti-biofilm activity in the present study was observed at 2.5µl concentration. Antibiofilm test used to determine the antibiofilm property of an organism focuses on the therapeutic properties of the medicinal plant and their effect.

Keywords: *Antimicrobial activity, Antibiofilm activity, phytochemical analysis, antioxidant* assay, *Antidesma montanum blume*

INTRODUCTION

Antidesma montaum blume is a wild variety plant commonly named as mountain current tree. *Antidesma montanum blume* is used as a common household remedy from ancient times. Plant is used by the traditional medicinal users for the swollen mammary gland of the cow and also for the swollen joints in humans.

Antibiotics are important for inhibiting bacterial growth and have benefited human and animal life. Investigation of new drugs is a necessity for the world.

Drugs discovered from natural sources play an important role in treatment of animal and human diseases. Plants are widely used in traditional medicine and their curative potentials are well documented. Plants are rich in secondary metabolites such as alkaloids, tannins, phenolic contents which are found in vitro to have antimicrobial properties [1]. Medicinal plants having antimicrobial properties are investigated from different parts of the world. The microorganisms which are harmful can be controlled with the medicinal drugs and alarming clinical situations are created in the treatment of infection [2].

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Antidesma montanum blume executed significant antimicrobial and antibiofilm activity possessing properties that supported folkloric use in treatment of diseases [3]. Thus in the current investigation, screening of aqueous extracts of *Antidesma montanum blume* leaves against pathogenic bacteria and fungi is done in order to detect new sources of antimicrobial agents. Biofilm is a community of bacteria which can grow in living and nonliving surfaces and it can cause various bacterial and fungal infections. Inhibition of biofilm is the major drug target for the treatment of infection. The plant extract was tested against the biofilm [4].

METHODS

Collection of Plant Material

The leaves of *Antidesma montanum blume* were collected from Udupi district of Karnataka state, India in the shallow forest collected in the month of November.

Preparation of Plant Extract

The extract of *Antidesma montanum blume* leaves was carried out using known standard procedure. Plants were weighed separately and washed with water to remove the contaminants. The leaves were shade dried and powdered in a mechanical grinder. The powder initially mixed with deionized water and boiled.

The aqueous extracts were filtered using cellulose filter paper. More yields of the extracts were collected by this method of extraction. The extracts were then dried and kept aseptically under refrigerated condition until further use. The dry weight of the plant extracts was 100mg/ml obtained. The extracts were preserved at -2 to -4°c [5].

PHYTOCHEMICAL ANALYSIS

Qualitative Analysis

Fehling's test

1ml of the extract was mixed with equal volume of Fehling's A and Fehling's b reagents and incubated in a boiling water bath for a few minutes. Green color solution turns reddish brown with precipitate is the change that needs to be noted. Change in color was observed and recorded.

Benedict's test

1ml of Benedict's reagent was added to 2ml of extract and heated in a boiling water bath for a few minutes. Change in color that is orange red precipitate is observed and recorded.

Tests for proteins and amino acids:

Ninhydrin test

1ml of extract was heated with 1ml of ninhydrin reagent and the change in the color was noted.

Biuret test

1ml of biuret reagent was added to 1ml of extract and observation was made for change in color.

Ferric chloride test for tannins and phenolic compounds

1ml of the extract and a few drops of ferric chloride was mixed. Change in color was observed for both the extracts and it was recorded.

Test for flavonoids

1ml of extract mixed with 1ml of sodium hydroxide. The change in the intense yellow color indicates the presence of flavonoids.

Test for saponins

Extract was mixed with 5ml of distilled water in a test tube and it was shaken vigorously. The formation of foam was taken as an indication for the presence of saponins.

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Test for quinones
1ml of extract was treated with little amount of conc. Hydrochloric acid. Observation of yellow precipitate indicates the presence of quinoline.

Test for cardiac glycosides
Keller-killani test: Crude extract was mixed with 2ml of glacialmacetic acid containing a few drops of 2% solution of Ferric chloride. The mixture was then poured into another test tube containing 2ml of concentrated sulphuric acid. A greenish brown ring at the interphase indicated the presence of cardiac glycosides.

Test for terpenoids:

Salkowski's test
Crude extract was mixed with 2ml of chloroform. Then 2ml of concentrated H2SO4 was added drop wise. The appearance of an orange brown ring at the interface was taken as indication for the presence of terpenoids.

Test for steroids:

Liebermann-Burchard's test
1ml of extract was mixed with 2ml of acetic anhydride and conc. sulphuric acid drop wise. Formation of green color indicated the presence of steroids.

Test for alkaloids
1ml of the extract is mixed with 1ml of Hager's reagent. The formation of yellow precipitate indicates the presence of alkaloids. Hager's reagent is a saturated solution of picric acid.

Test for triterpenoids
Conc. Sulphuric acid was added to 1 ml of extract and observed the formation of rings with the lower layer turning yellow. Which henceforth indicates the presence of triterpenoids?

Quantitative Analysis
Determination of total phenolic content
The total phenolic content of the healthy and the infected leaf extracts was determined using the Folin-Ciocalteu method. A stock solution of the extract and water was prepared in the ratio of 1:1 (10mg/10ml). A known volume of this stock solution was made up to 1 ml with distilled water. This was then mixed with 2ml of 20% sodium carbonate solution and this mixture was allowed to stand for 2 minutes at room temperature. Then 0.5ml of 50% Folin Ciocalteau's phenol reagent was added. After incubation for minutes at room temperature in darkness, the absorbance was read at 650 nm using a colorimeter. The total phenolic content was measured by using 0.2ml, 0.4ml, 0.6ml of the solution of Gallic acid (in mg) in water (in ml), which were in the ratio 1:10 respectively.

Determination of the total flavonoid content
Total flavonoid content was determined by the aluminum chloride method. The same stock solution with the same measurements (1:1 ratio) was prepared by mixing extract and water. A known volume of this stock solution was made up to 1 ml with distilled water and 0.3ml of 5% NaNO2 was added and this mixture was allowed to rest for 5 minutes. Then 0.3 ml of 10% AlCl3.H2O solution was added and after 6 min, 2 ml of 1 M NaOH solution was added. The total volume was made up to 10 ml using distilled water. The absorbance against the blank was measured at 650 nm with the help of a colorimeter. Using 0.2ml, 0.4ml, 0.6ml of the solution of quercetin (in mg) in alcohol (in ml), which were in the ratio 1:1 respectively, the total flavonoid content was measured.

Determination of total tannin content

The total tannin content of the healthy and the infected leaf extracts was also determined using the Folin-Ciocalteu method. A stock solution of the extract and water was prepared in the ratio of 1:1 (10mg/10ml). A known volume of this stock solution was made up to 1 ml with distilled water that was then mixed with 0.5 ml of 50% FolinCiocalteau's reagent. Then 1ml of 20% sodium carbonate was added and the absorbance of the mixture was measured at 650 nm using a colorimeter.

Antioxidant ASSAY

DPPH radical scavenging assay: A solution of DPPH(18mg/100ml) in ethanol was prepared and 1 ml of this solution was mixed with 0.6µg/ml concentrations of the stock solution of extract in ethanol (10mg /10ml). The reaction mixture was mixed thoroughly and was left to incubate in the dark at room temperature for 30 min. The absorbance of the mixture was measured at 650 nm using a colorimeter with ascorbic acid as the standard.

Test Microorganism

For antibacterial and antifungal activity testing 2 strains of gram positive and gram negative bacteria were used: *Bacillus spp*. and *pseudomonas spp*. And *Penicillium* and *Aspergillus* are the standard fungal strains used in the agar well diffusion method. Two different Prokaryotic bacteria and two different eukaryotic fungus were used based on their clinical importance. The study was conducted with proper aseptic technique and was followed in the safe handling of bacteria and fungus. Biosafety cabinets were used in conducting the experiment [6].

Growth Media

Stock cultures were incubated for 24 hours at 37°c on Muller Hinton Agar and Potato Dextrose Agar respectively, following refrigeration storage at 4°c.The bacterial strains were grown in MHA plates at 37 °c, which were grown in nutrient broth at 37° c and maintained on nutrient agar slant at 4°c.Whereas fungus were grown in the PDA media at Room temperature [7].

Antimicrobial Activities

Antibacterial activity assay

The antibacterial activity of bacteria was analyzed by agar well diffusion assay. Antibacterial activity of Plant extract against 2 pathogenic gram positive and gram negative bacteria. For determination of zones of inhibition bacteria were taken for a comparison of the Results. Extracts were screened for their Antibacterial assay against *Bacillus* and *Pseudomonas spp.*

The 100 mg/ml of aqueous extract were prepared. Muller Hinton agar plates were seeded with indicated bacterial strains and allowed to incubate at 37°c for 24 hours. Control was carried out by the similar condition [8]. The Zone of inhibition was measured after 18 to 24 Hours at 37°c.The sensitivity was analyzed by measuring the sizes of the Inhibitory zone. The uninoculated media were also tested for inhibitory zones and served as control [9].

Antifungal activity assay

Antifungal assay was carried out using the agar well diffusion assay. Fungal Isolates were maintained on potato dextrose agar and the culture were stored at room temperature and sub cultured once a month. The media was dispersed to a sterile Petri plate and allowed to cool down before use [10]. The Petri dish and prepared medium were autoclaved at 121°c for 15 minutes. Prepared mediums were poured into a set of Petri dishes at 5mm under aseptic conditions. After the solidification of the media, The Isolates were allowed to grow before they were used for examination and aqueous extract

of the plant *Antidesma montanum blume* was evenly distributed on the Potato dextrose agar in designated Petri plate. Standard controls were also added. Plates were left overnight for the solution to be absorbed through the media. The tests were carried in triplicate. Results were evaluated by measuring the zone of Inhibition of the bacterial growth [11,12].

Antibiofilm assay

The tubes were filled with 200µl Muller Hinton agar and 10µl test pathogen of optimum density 0.08 at 590nm and 10µl of plant extract were added and mixed well. Standard controls were used. After preparation the tube was incubated at 37°c for 24 hours. After incubation the contents of the tube were discarded and phosphate buffer saline of pH 6.4 used to wash and then it was air dried for 45 minutes. Fix the bacteria with sodium acetate and flooded with crystal violet stain and incubate in the dark for 30 minutes and then wash with distilled water until the dyes are removed. After drying 200 µl ethanol of 95% were added to the tubes and absorbance was measured at 600mm.

RESULT

The aqueous extract of the plant showed that the carbohydrate, protein, amino acid, phenol, flavonoid, tannin, saponins, quinonines, cardiac glycoside, terpenoids, triterpenoids, alkaloid and steroid were present and it also helped in investigating the antioxidant property in the plant (Table 1 & 2) .The leaf extract of Antidesma montanum blume of 10 mg/ml concentration was taken and it was tested against the two pathogenic bacterial strain Bacillus and Pseudomonas spp. And two fungal strains Penicillium and Aspergillus. The test pathogen showed the zone of inhibition against plant extract. The result is given in Table 3.

Statistical Analysis

Table 1: *Results of phytochemical screening of aqueous leaf extracts of Antidesma montanum blume.*

Sl.no	Name of the compounds	Name of the test	Status
1	carbohydrate	Fehling's test	+
2	Reducing sugar	Benedict's test	++
3	Protein and amino acid	Ninhydrin test Biurets test	++ ++
4	Tannin and phenols	Ferric chloride test	+
5	Flavonoids	NaOH	+
6	Saponin	Foam test	+++
7	Cardiac glycoside	Killer killani test	+
8	Terpenoids	Salkowski test	++
9	Steroids	Liebermann-Burchard's test	+
10	alkaloids	Hager's test	+++
11	Triterpenoids	Ring test	+

Table 2: *Antioxidant activity, tannin, phenolic, flavonoid of Antidesma montanum Blume.*

Phytochemical constiuents	Crude extract
Total phenolic(mg/g)content(inGAE*)	1.2 ± 0.360
Total flavonoid (mg/g) content (in QE*)	0.243±0.951
Total tannin (mg/g)content (in TAE*)	1.666±1.035

Results are mean of triplicate determination based on the reference standard ±standard deviation. GAE=Gallic acid equivalent; QE=Quercetin equivalent; TAE=Tannic acid equivalent.

Table 3: *Antimicrobial sensitivity test for the plant extract.*

Test Microorganism	Bacillus spp.	Pseudomonas spp.	Penicillium	Aspergillus
Aqueous extract showing the zone of inhibition	±3cm	±2cm	±3cm	±1.5cm
Standard (kanamycin)	±3.3cm	±1.2cm	±1.8cm	±2.5cm

+ = active; - = inactive

The growth of the bacterial strain and fungal strain ranged from 10mm to 30mm.The antibiofilm activity performed with the Bacillus spp. against the Plant extract at different concentrations. The extract showed 98% Inhibition at 2.5µl. (Table 4.)

Table 4: *Antibiofilm Assay.*

Concentration (µg/mL)	**Biofilm inhibition (%)**
0.5	_
1.0	+
1.5	++
2.0	++
2.5	++

Good (++) antibiofilm activity(>50% inhibition); poor(+)antibiofilm activity (more than 0-50%);No(-)antibiofilm activity(0% or less).

DISCUSSION

Effect of the plant extract is studied at various levels in the world. The study helps in identification of drugs from this plant. Traditional medicine has been effective against both the diseases and infection in human as well animal's. Plant product has therapeutic uses. However, clinical trials are done to check the efficacy and effectiveness of the plant to act as a new drug.

The present investigation showed that plant possess rich phytochemicals which are resistant against bacteria and fungi .Compounds such as alkaloids, saponins, tannins and flavonoids are highly active phytochemicals and responsible for the plant to act as antimicrobial agent. Plant extract successfully inhibited the growth of bacterial and fungal strains. The plant showed antioxidant and antibiofilm properties in it. The result were compared with a previous study done with plant extract of the different antidesma species. Antidesma menasu showed less phytochemical compound and moderate inhibition has been observed by the Staphylococcus aureus at different concentrations. No antimicrobial activity was observed in aqueous extract and both aqueous as well as ethanol extract have not shown any antifungal activity against Candida albicans and Cryptococcus neoformans [13].

Antidesma thwaitesianum fruit extract moderately showed antibacterial effect

against Staphylococcus aureus (zone of inhibition=8mm).and total phenolic content has been detected [14] In the current antimicrobial study with Antidesma thwaitesianum mull. Arg. fruit juice showed that gram positive bacteria were more susceptible then gram negative bacteria and in the seed germination check with the same plant extract retarded the germination of P. digitatum conidia. No mold spore germination was seen in the presence of Antidesma thwaitesianum [15]. Aerial part of Antidesma bunius showed no antibacterial activity against the gram negative bacteria, Pseudomonas aeruginosa and the fungi Candida albicans [16].

In vitro antimicrobial activity of Antidesma bunius extract on oral pathogenic bacteria has been studied. In which green fruit and red fruit extract were found to have exhibited an inhibitory effect on all tested bacteria [17]. However its effect was found to be lower than that of tetracycline (positive control).

The extract from the black fruit and leaves had not shown any inhibitory effect on the tested bacteria [18]. The crude methanol bark extract of antidesma venosum showed promising result for all tested organism had justified medicinal uses of the plant in treating bacterial related infection such as diarrhea and amoebic dysentery [19]. Antibacterial property of methanol extract of Antidesma ghaesembilla tested against brine shrimp and seven pathogenic microorganisms [20]. No zone of inhibition was observed against Shigella sonnei, Vibrio cholera and Bacillus megaterium at concentration 400µg/disc. Phytochemical investigation of the plant Antidesma acidum confirmed the presence of alkaloid,triterpenoid and flavonoids [21]. The leaves of Antidesma acidum used in traditional medicine for the treatment of stomach ache in children, digestion and diabetic issues.

CONCLUSION

The survey of the traditional medicinal plant shows antibiotic sensitivity plays an important role in research and drug developing .The plant Antidesma montanum blume successfully inhibited the growth of the microorganism and acted as antimicrobial and antibiofilm agent. The present study proved that the plant is a natural source of antioxidants and it is helpful in treating the different infections caused in humans and animals. Thus further study with this plant can let us know the wide medicinal importance of the plant and their application.

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