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IN VITRO ANTIMICROBIAL ACTIVITY AND PHYTOCHEMICAL SCREENING OF *DRYPETES ROXBURGHII* LEAVES EXTRACTS

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ABSTRACT
Phytochemical screening and antimicrobial activity of *Drypetes roxburghii* leaves aqueous, methanol and hexane extracts was carried out. The antimicrobial activity of different extracts were tested against the gram positive and the gram negative bacterial strains and some fungal strains by observing the zone of inhibition diameter. The gram positive bacteria used in the test were *Staphylococcus aureus, Bacillus subtilis, Enterococcus faecalis, Bacillus cereus* and the gram negative bacteria were *Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris*, fungal strains like *Aspergillus niger*, *Aspergillus flavus* and *Candida albicans* were used. It was observed that aqueous extract of *Drypetes roxburghii* was inactive against all the bacterial strains used in the test, whereas, *Staphylococcus aureus* was the most sensitive strain to the extract with zone of inhibition diameter 21mm and *Bacillus subtilis* was the least sensitive strain to the extract with zone of inhibition diameter 15mm. The plant extracts did not show any inhibitory activity against *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Bacillus cereus*. Aqueous and hexane extracts showed strong antifungal activity against *A. niger* and *Candida albicans* with zone of inhibition diameter 23mm and 21mm and least sensitivity against *Aspergillus flavus*. The results confirmed the antimicrobial activity of *Drypetes roxburghii* leaves extract against various human pathogenic bacteria and fungus.
Keywords: Phytochemicals, crude extract, Antimicrobial activity, *Drypetes roxburghii* (Wall).

INTRODUCTION

The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments. In the last few years, a number of studies have been conducted in different countries to prove such efficiency[1]. Ethno pharmacologists, botanists, microbiologists, and natural-product chemists are searching the earth for phytochemicals which could be developed for the treatment of infectious diseases especially in light of the emergence of drug-resistant microorganisms and the need to produce more effective antimicrobial agents[2]. Their impact is large in developing countries due to relative unavailability of medicines and the emergence of widespread drug resistance. During the last two decades, the development of drug resistance as well as the appearance of undesirable side effects of certain antibiotics has lead to the search of new antimicrobial agents mainly among plant extracts with the goal to discover new chemical structures, which overcome the above disadvantages. Current research on natural molecule and products primarily focuses on plants since they can be sourced more easily and be selected based on their ethno-medicinal uses[3].

The Indian plant *Drypetes roxburghii* Wall, belong to the family Euphorbiaceae. Its synonym is *Putranjiva roxburghii* Wall. It is found wild or cultivated almost in all parts of India, ascending of 750m. It is a dioecious, evergreen tree, attaining a height of upto 18m and a girth upto 2m having grey bark. Leaves long, elliptic oblong to ovate-lanceolate, unequal sides at the base, dark green in colour and shinning in appearance. Phytochemicals present in D. *roxburghii* include saponins, mannitol, arachidic acid, linoleic acid, palmitic acid, glucoputranjivin, putranjivoside, putranoside, β - sitosterol, carboxylic acid, putric acid, putranjivic acid, glucosides, fatty oil, alkaloids and gallo-tannins. Leaves and stones are given in decoction for cold and fever; they are also used in rheumatism, elephantiasis, burning sensation, azoospermia, constipation, sterility. It is also used as an antidiabetic[4-5].

MATERIALS AND METHODS

Plant Material

The leaves of *Drypetes roxburghii* plant were collected in the month of November 2010 from the botanical garden of Forest Research Institute, Dehradun (Uttarakhand), India. The leaves were authenticated by Mr. S.K Srivastava, Scientist D/HOO, Botanical Survey of India. Dehradun (U.K.), India.

Extraction procedure

The collected plant material were dried well under shade and powdered using an electric blender. The 100g/100ml of the powdered sample was transferred to closed containers. The powdered samples were than extracted by means of cold extraction process using the solvents water, methanol and hexane separately for 72 hours by using magnetic stirrer and also by occasional shaking by hand at room temperature, each of the extracts was filtered by using muslin cloth and then by Whatman no.1 filter paper, the filtrate was evaporated to dryness in an evaporating dish on a steam bath at 50 – 60^0c. The extract was then further used in the test.

Preliminary Phytochemical Screening

The different qualitative tests can be performed for establishing profile of the plant extracts for its chemical composition. The freshly prepared extracts were subjected to standard phytochemical analysis to test for the presence of the phytoconstituents like tannins, saponins, alkaloids, carbohydrates, glycosides, phenolics, flavonoids and terpenoids. Chemical tests were carried out on hexane, methanol and aqueous extracts of the powdered specimens using standard procedures for the detection of saponins, alkaloids (Mayer's reagent and Dragendorff's

reagent test), carbohydrates (Molish's and Benedict's test), glycosides (Borntragor's and Legal's tests), phenolics and tannins (Ferric chloride and Lead acetate test), flavonoids (alkaline reagent test), terpenoids (Salkowski test and Trichloro acetic acid test) to identify the phytochemical constituents [7-8].

Test microorganisms used

The microorganisms include gram negative bacteria *Escherichia coli* (MTCC 452), *Pseudomonas aeruginosa* (MTCC 1034), *Proteus vulgaris* (MTCC 1771), gram positive bacteria *Staphylococcus aureus* (MTCC 737), *Bacillus subtilis* (MTCC 441), *Enterococcus faecalis* (MTCC 439), *Bacillus cereus* (MTCC 1306) and fungal strains *Aspergillus niger* (MTCC 1344), *Aspergillus flavus* (MTCC 277), *Candida albicans* (MTCC 227). The test microorganisms were obtained from Microbial Type Culture Collection (MTCC), IMTECH, Chandigarh, India.

Preparation of extracts

The crude extracts were dissolved in 30% dimethyl sulphoxide (DMSO) and further diluted to obtain of each extracted sample of 100 mg/L concentration was used for the determination of antibacterial and antifungal activity.

Preparation of media and inoculum

Nutrient Agar/broth and Potato Dextrose Agar/broth (Himedia, India) were used as the media for culturing of bacterial and fungal strains. A loopful of bacterial pure culture was inoculated in 20ml sterile nutrient broth medium in the test tubes aseptically and this process was repeated for all the bacterial strains. The tubes were incubated at 37^0 C for 24 hr. growth was observed in all the test tubes and this was further used in the experiment.

Standard antibiotic: Ciprofloxacin 0.3% w/v.

Standard antifungal: Itraconazole (25 mcg), Clotrimazole (25 mcg).

Antibacterial activity: Agar well diffusion method

About 1ml of the inoculum was poured in the sterilized nutrient agar media when media attains a temperature of 30-40^{0}c, mixed well, and 20ml of this media was poured in all the petriplates and allowed to solidify. Then four wells of 6mm were made in each petriplate with the help of a sterile cork borer, 50µL of the plant extract was poured in each well using sterilized micropipettes. For each bacterial strain, negative controls were maintained where pure solvents were used instead of the extract. The control zones were subtracted from the test zones, for positive control, standard antibiotic Ciprofloxacin (30 mcg) was used. The plates were incubated overnight at 37°C. Microbial growth was determined by measuring the diameter of zone of inhibition. The entire process was carried out aseptically in the laminar airflow. The experiment was performed in triplicate[9-10].

Antifungal activity: Agar well diffusion method

About 1ml of the inoculum was poured in the sterilized Potato dextrose agar media when media attains a temperature of 30-40^{o}c, mixed well, and 20ml of this media was poured in all the petriplates and allowed to solidify. Then four wells of 6mm were made in each petriplate with the help of a sterile cork borer, 50µL of the plant extract was poured in each well using sterilized micropipettes. For each fungal strain, negative controls were maintained where pure solvents were used instead of the extract. The control zones were subtracted from the test zones, for positive control, standard antifungal Itraconazole (25 mcg) and Clotrimazole (25 mcg) were used. The plates were incubated 28°C for 48–72hrs. Fungal growth was determined by measuring the diameter of the zone of inhibition. The entire process was carried out aseptically in the laminar airflow. The experiment was performed in triplicate[11-12].

Determination of Minimum Inhibitory Concentration (MIC)

MIC is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation.

MIC by "Serial Tube Dilution Method" In this technique, the tubes of broth medium, containing graded doses of compounds are inoculated with the test organism. After suitable incubation, growth will occur in those tubes where the concentration of compound is below the inhibitory level and the culture will become turbid (cloudy). Therefore, growth will not occur above the inhibitory level and the tubes will remain clear.

Preparation of the sample solution

0.64g of the solvent free extract was weighed and dissolved in 100 ml of solvent (DMSO) having concentration 640mcg/ml and further dilutions were made from this sample and likewise.

Preparation of inoculum

The test bacteria grown at 37^0C in nutrient agar medium was diluted in sterile nutrient broth by taking one loopful of test bacterial strain in 20 ml of nutrient broth and the fungal strain grown in potatoes dextrose agar at 28^0C for 72 hrs was diluted in sterile potatoes dextrose broth. This suspension was used as inoculum.

Procedure

Nine test tubes were taken, and marked 1,2,3,4,5,6,7 and the rest two were assigned as TMC (Medium + Compound) and TMI (Medium + Inoculum). 100 ml of nutrient broth (for bacteria) and 100 ml of potatoes dextrose broth (for fungus) was made and sterilized in an autoclave for 15Ibs/sq. inch pressure for 15 min. 9 ml of sterilized nutrient broth was poured in each of the 9 test tubes. 1ml of the sample solution was added to the 1st test tube and mixed well and then 1ml of this content was transferred to the 2nd test tube. The content of the second test tube was mixed well and again 1ml of this mixture was transferred to the 3rd test tube. This process of serial dilution was continued up to the 7 test tubes and mixed well. 10µl of the properly diluted inoculum was added to each of 7 test tubes and mixed well. To the control test tube TMC, 1ml of the sample was added and mixed well and 1ml of this mixed content was discarded to check the clarity of the medium in presence of diluted solution of the compound. 10µl of the inoculum was added to the control test tube TMI, and observed the growth of the organism in the medium used. All the test tubes were incubated at 37^0C for 18-24 hrs for bacterial growth and at 28^0C for 72hrs for fungal growth. The entire experimental process was carried out aseptically in the laminar flow[14,15].

Table 1: Phytochemical screening of *Drypetes roxburghii* leaves extracts

S. No.	TESTS	Hexane extract	Methanol extract	Water extract
1.	Alkaloids Dragendorff's test Mayer's test	 − −	 + +	 − −
2.	Carbohydrates Molisch's test Benedict's test	 − −	 + +	 − −
3.	Glycosides Borntrager's test Legal's test	 − −	 − −	 − −
4.	Saponins	−	−	+
5.	Phenolics & Tannins Ferric chloride test Lead acetate test	 − −	 + +	 − −
6.	Flavonoids	+	+	−
7.	Triterpenoids	+	−	−

Table 2: Determination of antibacterial activity of *Drypetes roxburghii* Leaves
(Including Bore diameter 6mm); Cp – Ciprofloxacin ; (-) no activity.

Bacterial Strains	Zone of Inhibition (mm)			Antibiotic Cp (30 mcg)
	Hexane	Methane	Aqueous	
Staphylococcus aureus	_	21	_	28
Escherichia coli	_	15	_	28
Pseudomonas aeruginosa	_	_	_	26
Proteus vulgaris	_	_	_	20
Bacillus subtilis	_	15	_	18
Enterococcus faecalis	18	_	_	21
Bacillus cereus	_	_	_	21

Table 3: MIC of *Drypetes roxburghii* hexane extract

Marked no. of Test tubes	Nutrient broth added (ml)	*Drypetes* Hexane Extract (µg/ml)	Inoculum added (µl)	Bacterial growth						
				S. aureus	*E. coli*	*P. aeruginosa*	*P. vulgaris*	*B. subtilis*	*E. faecalis*	*B. cereus*
1	1	640	10	_	_	_	_	_	_	_
2	1	320	10	_	_	_	_	_	_	_
3	1	160	10	_	_	_	_	_	_	_
4	1	80	10	_	_	_	_	_	_	_
5	1	40	10	_	_	_	_	_	+	_
6	1	20	10	_	_	_	_	_	+	_
7	1	10	10	_	_	_	_	_	+	_
T_{MC}	1	640	0	_	_	_	_	_	_	_
T_{MI}	1	0	10	+	+	+	+	+	+	+

Table 4: MIC of *Drypetes roxburghii* methanol extract

Marked no. of Test tubes	Nutrient broth added (ml)	*Drypetes* Methanol Extract (µg/ml)	Inoculum added (µl)	Bacterial growth						
				S. aureus	*E. coli*	*P. aeruginosa*	*P. vulgaris*	*B. subtilis*	*E. faecalis*	*B. cereus*
1	1	640	10	_	_	_	_	_	_	_
2	1	320	10	_	_	_	_	_	_	_
3	1	160	10	_	_	_	_	_	_	_
4	1	80	10	_	+	_	_	+	_	_
5	1	40	10	_	+	_	_	+	_	_
6	1	20	10	+	+	_	_	+	_	_
7	1	10	10	+	+	_	_	+	_	_
T_{MC}	1	640	0	_	_	_	_	_	_	_
T_{MI}	1	0	10	+	+	+	+	+	+	+

Table 5: Antifungal activity of Drypetes roxburghii leaves extract
(including 6mm bore diameter)**;** Standard I :Itraconazole; Standard II :Clotrimazole.

Fungal strains	Zone of Inhibition (in mm)			Std. Antifungal I, 25mcg/ml	Std. Antifungal II, 25mcg/ml
	Hexane	Methanol	Aqueous		
Aspergillus niger	_	_	23	_	25
Aspergillus flavus	_	12	_	21	23
Candida albicans	21	_	_	17	27

Table 6: MIC of *Drypetes roxburghii* hexane extract

Marked no. of Test tubes	Nutrient broth added (ml)	*Drypetes* Hexane extract (µg/ml)	Inoculum added (µl)	Fungal growth		
				A. niger	*A. flavus*	*C. albicans*
1	1	640	10	_	_	_
2	1	320	10	_	_	_
3	1	160	10	_	_	_
4	1	80	10	_	_	_
5	1	40	10	_	_	_
6	1	20	10	_	_	+
7	1	10	10	_	_	+
T_{MC}	1	640	0	_	_	_
T_{MI}	1	0	10	+	+	+

Table 7: MIC *Drypetes roxburghii* methanol extract

Marked no. of Test tubes	Nutrient broth added (ml)	*Drypetes* Methanol extract (µg/ml)	Inoculum added (µl)	Fungal growth		
				A. niger	*A. flavus*	*C. albicans*
1	1	640	10	_	_	_
2	1	320	10	_	_	_
3	1	160	10	_	_	_
4	1	80	10	_	_	_
5	1	40	10	_	+	_
6	1	20	10	_	+	_
7	1	10	10	_	+	_
T_{MC}	1	640	0	_	_	_
T_{MI}	1	0	10	+	+	+

Table 8: MIC of *Drypetes roxburghii* aqueous extract

Marked no. of Test tubes	Nutrient broth added (ml)	*Drypetes* Aqueous extract (µg/ml)	Inoculum added (µl)	Fungal growth		
				A. niger	*A. flavus*	*C. albicans*
1	1	640	10	_	_	_
2	1	320	10	_	_	_
3	1	160	10	_	_	_
4	1	80	10	_	_	_
5	1	40	10	_	_	_
6	1	20	10	+	_	_
7	1	10	10	+	_	_
T_{MC}	1	640	0	_	_	_
T_{MI}	1	0	10	+	+	+

RESULTS

Aqueous extract of *D. roxburghii* was inactive against all the bacterial strains used, whereas, *S. aureus* was the most sensitive strain to the extract with zone of inhibition diameter 21mm and *B. subtilis* was the least sensitive strain to the extract with zone of inhibition diameter 15mm. The plant extracts did not show any inhibitory activity against *P. aeruginosa*, *P. vulgaris* and *B. cereus*.The standard (Ciprofloxacin 30µg) positive control showed inhibition diameter ranging from 20-28mm against gram-negative bacteria and 18–28mm against gram-positive bacteria. The MIC values of *D. roxburghii* hexane extract against *E. faecalis* is 80 µg/ml. MIC values of *D. roxburghii* methanol extract against *S. aureus, E. coli*, *B. subtilis*, are 40, 160 and 160 µg/ml.

It was seen that aqueous and hexane extract of *D. roxburghii* showed strong antifungal activity against *A. niger* and *C. albicans* with zone of inhibition diameter 23mm & 21mm and showing minimum zone of inhibition diameter of 12mm against *A. flavus*. The MIC values of *D. roxburghii* hexane extract against *C. albicans* was 40 µg/ml, methanol extract against *A. flavus* was 80 µg/ml and aqueous extract against *A. niger* was 40µg/ml. The antifungal agent Clotrimazole demonstrated a higher activity compared to Itraconazole against all the fungi tested, while Itraconazole was only effective against *Aspergillus flavus* and *Candida albicans.*

DISCUSSION

Plant extracts have great potential as antimicrobial compounds against microorganisms. Thus, they can be used in the treatment of infectious diseases caused by resistant microbes. The bioactivity of plant extracts is attributed to phytochemical constituents. The methanolic extracts considered in the study possess maximum phytoconstituents as well as maximum number of compounds as compared to hexane extract. Strong antimicrobial activity is seen in methanol and hexane extract as compared to aqueous extract which draws a conclusion that maximum number of phytoconstituents responsible for the activity are extracted out in hexane and methanol extract. The plant which possess good amount of tannins have antibacterial potential that allows them to react with protein to form stable water soluble compounds thereby killing the bacteria by directly damaging its cell wall[15,16]. The flavonoids, alkaloids, triterpenoids, saponins present in the leaves of *D. roxburghii* may therefore contribute to the antimicrobial activity of the plant.

It may, therefore be concluded from the above investigation that *Drypetes roxburghii* can be used as a drug to treat disease caused by the bacteria and fungi which are sensitive to the above mentioned samples.

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