

Original Research Article

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Study of the Antimicrobial Profile and Phytochemical Composition of Solvent Extracts of Leaves and Female Cones of *Cycas revoluta*

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ABSTRACT

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In the ethno-pharmacological approach, local knowledge about the potential uses of the plants is very useful as compared to the random approach where indigenous knowledge is not taken into consideration. Phytochemicals produced in plants are secondary compounds responsible for metabolic activities and defense in purpose. Phytochemicals are produced by specific biochemical pathways, which occur inside the plant cells. The gymnosperm, *Cycas revoluta* is a natural companion for palms, working well as under planting or in the foreground. In the present investigation, the chloroformic and hydro-alcoholic extracts of leaves and female cones of *Cycas revoluta* were screened for its antimicrobial potential and in urge for isolation and characterization of the potent molecule responsible for antimicrobial potential against pathogenic and drug resistant strains. The results showed that both the leaves and female cones of *Cycas revoluta* are potent antimicrobial agents. It was found that chloroformic extracts of the cones was having potent antimicrobial activity in comparison to the chloroformic extracts of leaves. The extracts were found to be promising antimicrobial agent against methicillin resistant *Staphylococcus aureus* (MRSA), *E. coli*, *Salmonella abony*, *Aspergillus niger*, *Candida albicans* and other pathogens reported in the study.

Introduction

Plants have served as a source of new pharmaceutical products and inexpensive starting materials for the synthesis of some known drugs. Components with medicinal properties from plants play an important role in conventional Western medicine. In 1984, at least 25% of the Western medicine issued in the US and Canada were derived from or modelled after plant natural products and 119 secondary metabolites were used globally as drugs (Farnsworth, 1994). It has been estimated that 14-28% of higher plant species

are used medically. Only 15% of all angiosperms have been investigated chemically and 74% of pharmaceutically active plant derived components were discovered after following up on ethno medical use of the plant (Farnsworth, 1991). The value of ethno-medicine and traditional pharmacology is nowadays gaining increasing recognition in modern medicine because the search for new potential medicinal plants is frequently based on an ethno-medicinal basis (Hasan *et al.*, 2009; Prajapati, 2002; Jaradat *et*

al., 2004). Different pharmacological properties from plants of North west Himalaya Garhwal region were determined (Mathur *et al.*, 2010a,b; 2011a,b). In the ethno-pharmacological approach, local knowledge about the potential uses of the plants is very useful as compared to the random approach where indigenous knowledge is not taken into consideration. Different polycrystalline ferroelectric materials were investigated for antimicrobial and pharmacological properties (Heravi *et al.*, 2016). The current study was performed in order to assess the antimicrobial properties of solvent extracts of leaves and female cones of *Cycas revoluta*, a common gymnosperm in lieu of isolation of antimicrobial polycrystalline ferroelectric materials.

Materials and Methods

Plant materials

The leaves and female cones of the plant, *Cycas revoluta* (Sago-Palm) were collected from the local gardens of Forest Research Institute (FRI), Dehradun. The materials of the plant were taxonomically identified by Botanists/Taxonomists. Records of the specimens were deposited in FRI, Dehradun for future reference.

Chemicals

Analytical reagent (AR) grade ethanol and chloroform were purchased from Ranchem Pvt. Ltd., India. Nutrient agar/broth and Sabouraud's Dextrose agar/broth were purchased from Hi Media Pvt. Ltd., Mumbai, India. Positive controls, Erythromycin and Fucanazole were obtained as the gift samples from Ranbaxy Pvt. Ltd., India.

Preparation of plant extracts

The leaves and female cones of the plant were separated, washed with distilled water, dried

under shade and pulverized. The method of Alade and Irobi (1993) was adopted for preparation of plant extracts with little modifications. Briefly 20 g portions of the powdered plant material were soaked separately in different solvents *viz.* hydro-alcohol (50% v/v) and chloroform on the basis of decreasing polarity for 72 h. Each mixture was stirred every 24 h using a sterile glass rod. At the end of extraction, each solvent was passed through Whatmann filter paper No. 1 (Whatmann, England). The filtrates obtained were later concentrated in vacuo using water bath at 30 °C.

Determination of antimicrobial activity

Culture media

For antibacterial and antifungal activities, Nutrient agar/broth and Sabouraud's dextrose agar/broth respectively was procured from Hi Media Pvt. Bombay, India.

Inoculum

The bacteria were inoculated into Nutrient broth and incubated at 37°C for 18 h and suspension was checked to provide approximately, 10⁵ Cfu/ml. The same procedure was done for fungal strains and there strains were inoculated into Sabouraud's dextrose broth but the fungal broth cultures were incubated at 48–72 h.

Microorganisms used

Pure cultures of various pathogenic bacterial and fungal strains, *E. coli* NCIM 2065, *Lactobacillus plantarum* NCIM 2083, *Micrococcus luteus* ATCC 9341, *Salmonella abony* NCIM 2257, *Candida albicans* NCIM 3471, *Aspergillus niger* NCIM 1196 and Methicillin resistant strains of *Staphylococcus aureus* (MRSA) 101 *Staphylococcus aureus* (MRSA) 102 isolated from clinical specimens

viz. pus and blood respectively of infected patients were procured with authentication for the study.

Determination of diameter of zone of inhibition by well diffusion method

The agar well diffusion method (Perez *et al.*, 1993) was modified. Nutrient agar medium (NAM) was used for bacterial cultures while Sabouraud's dextrose agar/broth was used for the growth of fungal cultures. The culture medium was inoculated with the bacteria separately suspended in nutrient broth while the culture medium was inoculated with the fungus separately suspended in Sabouraud's dextrose broth.

A total of 8 mm diameter wells were punched into the agar and filled separately with leaves and female cones extract (1g/ml) and solvent blanks. Solvents, chloroform and hydro-alcohol were used as negative controls. Standard antibiotic (Erythromycin, 1 mg/ml) was simultaneously used as the positive control. The plates were then incubated at 37°C for 18 h.

The antibacterial activity was evaluated by measuring the diameter of zone of inhibition observed. For assaying, antifungal activity of plant extracts, Sabouraud's dextrose agar/broth medium plates were used. The same procedure as that for determination of antibacterial property was adopted and then after, the diameter of zone of inhibition was observed after 48–72 h. Fucanazole (1 mg/ml) was used as standard for determination of antifungal activity. The procedure for assaying antibacterial and antifungal activity was performed in triplicates to confirm the readings of diameter of zone of inhibition observed for each of the test organism. The pure compound isolated was further assayed for antimicrobial activity by the above method.

Determination of Minimum Inhibitory Concentration (MIC)

MIC value of potent plant extracts was determined by the method adopted by Vollekova *et al.*, (2001) and Usman *et al.*, (2007), with some modifications. Plant extract was prepared in highest concentration (1g/ml) in sterile distilled water and was serially diluted with N-saline (0.85% NaCl) and similar quantity of bacterial/fungal suspension was added to different test tubes and incubated for 48 h. The inhibition of turbidity appeared in the minimum dose at which total growth of bacteria gets killed is known as minimum lethal concentration (MLC) while little turbidity appeared in the minimum amount of dose of plant extract which inhibits the growth of bacteria is known as Minimum Inhibitory Concentration (MIC).

Phytochemical screening of the extracts

The portion of the dry extracts was subjected to the Phytochemical screening using the method adopted by Trease and Evans (1989) and Harbourne (1983). Phytochemical screening was performed to test for alkaloids, saponin, tannins, flavanoids, steroids, sugars, cardiac glycosides and anthraquinones (Sofowora, 1993).

Test for alkaloids

The 0.5 g of the plant extract was dissolved in 5 ml of 1% HCl and was kept in water bath for about 2 minutes. 1 ml of the filtrate was treated with Wagner's reagent. Reddish orange colored turbidity or precipitation was taken as indicator for the presence of alkaloids.

Test for tannins

About 0.5 g of the sample was dissolved in 10 ml of boiling water and was filtered. Few ml

of 6% FeCl₃ was added to the filtrate. Deep green color appeared confirmed the presence of Tannins (Trease and Evans, 1989).

Test for flavanoids

About 0.2 gm of the extract was dissolved in methanol and heated for some time. A chip of mg metal was introduced followed by the addition of few drops of conc. HCl. Appearance of red or orange color was Indicator of the flavanoids.

Test for saponin

About 0.5 g of the plant extract was stirred with water in the test tube. Frothing persists on warming was taken as an evidence for the presence of saponin.

Test for steroids

Salkowski's method was adopted for the detection of steroids. About 0.5 g of extract was dissolved in 3 ml of chloroform and filtered. To the filtrate, conc. H₂SO₄ was added to form a lower layer. Reddish brown color was taken as positive for the presence of steroids ring (Agarwal *et al.*, 2011).

Test for cardiac glycosides

About 0.5 g of the extract was dissolved in 2 ml of glacial acetic acid containing 1 drop of 1% FeCl₃. This was under laid with concentrated H₂SO₄. A brown ring obtained at the interphase indicates the presence of deoxy sugar. A violet ring appeared below the ring while in the acetic acid layer a greenish ring appeared just above ring and gradually spread throughout this layer.

Test for reducing sugars

One ml each of Fehling's solutions, I and II was added to 2 ml of the aqueous solution of

the extract. The mixture was heated in a boiling water bath for about 2-5 minutes. The production of a brick red precipitate indicated the presence of reducing sugars.

Test for anthraquinones

5 ml of the extract solution was hydrolyzed with dil/conc. H₂SO₄. 1 ml of dilute ammonia was added to it. Rose pink colour confirmed the presence of anthraquinones.

Results and Discussion

The present study suggests that, the leaves and female cones of predominant gymnosperm, *Cycas revoluta* (Sago Palm) after identification and authentication were collected from high altitudes of North West Himalaya Garhwal region. It is already understood that, angiosperms are the predominant source of medicinal molecules which shows variable pharmacological action but any how less study has been done on gymnosperms (naked seeded/non flowering plants).

The information about general medicinal properties of Sago palm taken for the study was obtained from local/traditional healers of Uttarakhand. These female cones and leaves are used in Ayurveda as medicines but not much work has been emphasized to reveal their socio-economic importance, the active molecules responsible in these parts for treatment of pathogenic diseases and as food supplements in the form of antioxidants. The present brief study was thus initiated to reveal some of the pharmaceutical properties of leaves and female cones of *Cycas revoluta* (Sago Palm) having abundance on high altitudes in Uttarakhand valley. The present investigation leads to the isolation and identification of the compounds from the potent extracts which can be utilized as antimicrobials and antioxidants.

Antimicrobial activity of solvent extracts

In the present investigation, hydro-alcoholic and chloroformic extracts of leaves and chloroformic extracts (only) of cones of *Cycas revoluta* were screened for their antimicrobial potential against pathogenic bacterial and fungal strains along with methicillin resistant strains of *Staphylococcus aureus*. The results were found to be very interesting. It was found that chloroformic extracts of female cones and leaves of the plant were potent antimicrobial agents in comparison to hydro-alcoholic extracts.

It was found that hydro-alcoholic and chloroformic extracts of leaves of the plant showed potent antibacterial activity against bacterial strains studied viz. *Lactobacillus plantarum* NCIM 2083, *Micrococcus luteus* ATCC 9341, *Salmonella abony* NCIM 2257, MRSA 101 and MRSA 102 while there was no antibacterial potential found against *E. coli* NCIM 2065. The extracts also not showed any significant antifungal activity. It was found that *E. coli* NCIM 2065, *Candida albicans* NCIM 3471, *Aspergillus niger* NCIM 1196 and Methicillin resistant strains of *Staphylococcus aureus* (MRSA) were

resistant against these solvent extracts. Although, slight antifungal activity was found of chloroformic extract against *Aspergillus niger* NCIM 1196 (Sharma *et al.*, 2014). The results are shown in table 1 and figure 1.

The chloroformic extracts of cones were much more effective against all the pathogens studied in comparison to chloroformic and hydro-alcoholic extracts of leaves. The results showed that chloroformic extracts of cones were significant antimicrobial agent against all the pathogens studied viz. *E. coli* NCIM 2065, *Lactobacillus plantarum* NCIM 2083, *Micrococcus luteus* ATCC 9341, *Salmonella abony* NCIM 2257, *Candida albicans* NCIM 3471, *Aspergillus niger* NCIM 1196 and Methicillin resistant strains of *Staphylococcus aureus* (MRSA). The results were found to be more surprising when the extracts were found to have significant antibacterial activity against MRSA strains (isolated from pus and blood). Almost similar pattern of antifungal activity of cone chloroformic extracts was found against fungal strains studied viz. *Candida albicans* NCIM 3471, *Aspergillus niger* NCIM 1196. The results are shown in table 2 and figure 2.

Table.1 Antimicrobial activities of leaves extracts of *Cycas revoluta*

Solvent extracts of leaves	Diameter of zone of inhibition (mm)							
	Pathogens studied							
	<i>E. coli</i>	<i>S. abony</i>	<i>M. luteus</i>	<i>L. plantarum</i>	MRSA- 101	MRSA-102	<i>A. niger</i>	<i>C. albicans</i>
Hydro-alcoholic extracts (1 mg/ml)	NA	27.5	23.0	28.0	10.0	18.0	NA	NA
Chloroformic extracts (1 mg/ml)	NA	22.2	33.0	25.0	7.0	12.0	7.0	NA
Erythromycin (1 mg/ml)	10	25.0	25.0	22.0	12.0	15.0	NT	NT
Fucanazole (1 mg/ml)	NT	NT	NT	NT	NT	NT	24.0	20.0

*NA, No Activity; NT, Not Tested; MRSA, Methicillin resistant *S. aureus*

Table.2 Antimicrobial activities of female cones extracts of *Cycas revoluta*

Solvent extracts of female cones	Diameter of zone of inhibition (mm)							
	Pathogens studied							
	<i>E. coli</i>	<i>S. abony</i>	<i>M. luteus</i>	<i>L. plantarum</i>	<i>MRSA 101</i>	<i>MRSA 102</i>	<i>niger</i>	<i>C. albicans</i>
Hydro-alcoholic extracts (1 mg/ml)	NA	18.0	22.0	12.0	12.0	NA	NA	NA
Chloroformic extracts (1 mg/ml)	27.0	23.0	22.0	15.0	23.0	27.0	23.0	22.0
Erythromycin (1 mg/ml)	21.0	25.0	25.0	17.0	13.0	26.0	NT	NT
Fucanazole (1 mg/ml)	NT	NT	NT	NT	NT	NT	24	20.0

*NA, No Activity; NT, Not Tested; MRSA, Methicillin resistant *S. aureus*

Table.3 MIC and MLC values of potent extracts of *Cycas revoluta*

Solvent Extracts	Against Pathogen	MIC (mg/ml)	MLC (mg/ml)
Chloroformic leaves extract	<i>Aspergillus niger</i>	0.01	0.1
Chloroformic leaves extract	<i>Micrococcus luteus</i>	0.01	0.1
Chloroformic cones extract	<i>Aspergillus niger</i>	10	12
Chloroformic cones extract	<i>Salmonella abony</i>	10	12

Table.4 Phytochemical screening of the active constituents

Extracts	Phyto-constituents							
	Alkaloids	Tannins	Flavanoids	Saponin	Steroids	Cardiac glycosides	Reducing sugars	Anthraquinones
Cones extracts	-	-	+	+	+	-	+	-
Leaves extracts	+	-	-	+	-	-	+	-

*-, Absent; +, Present

Figure.1 Antimicrobial activities of leaves extracts of *Cycas revolute*

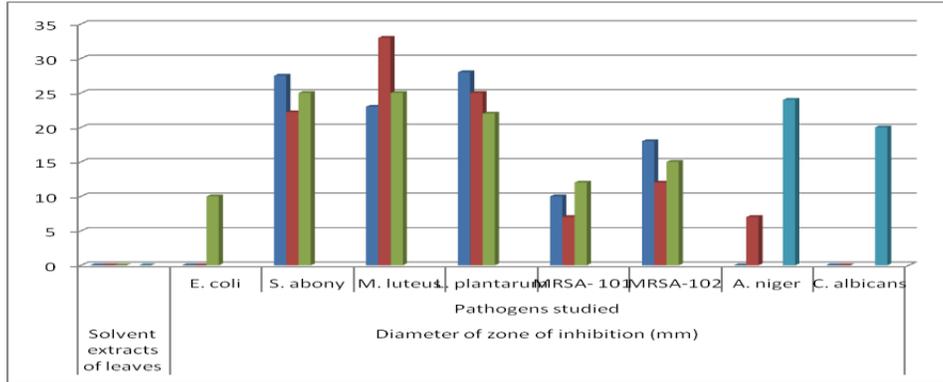


Figure.2 Antimicrobial activities of female cones extracts of *Cycas revolute*

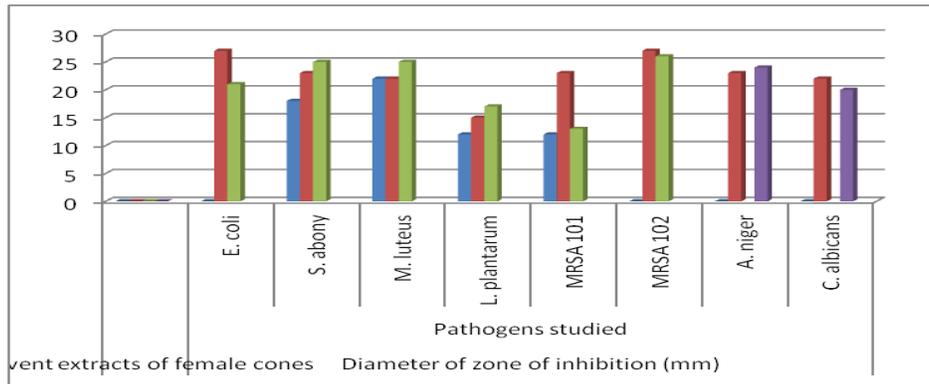
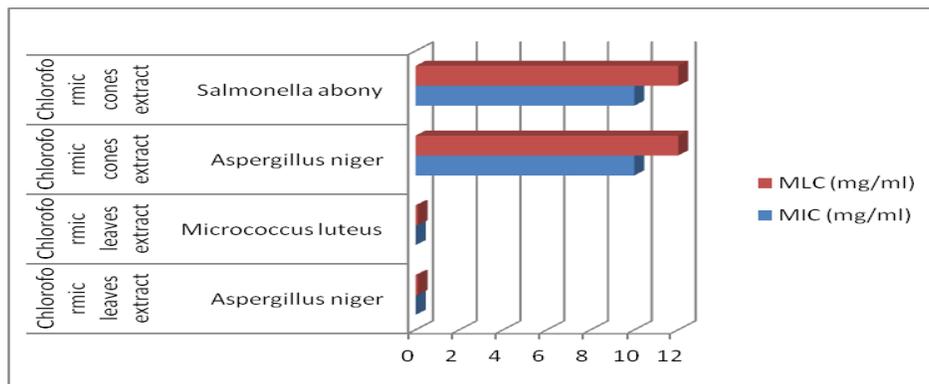


Figure.3 MIC and MLC values of potent extracts of *Cycas revolute*



Minimum Inhibitory Concentration (MIC) of solvent extracts

The female cones and leaves chloroformic extracts of *Cycas revolute* were subjected for

determination of MIC and MLC values. The extracts were serially diluted with N-saline and MIC values were determined along with MLC values against the pathogens which were found to be most sensitive against the same. MIC and

MLC values were determined of chloroformic leaves extract against *Aspergillus niger* NCIM 1196 and *Micrococcus luteus* ATCC 9341 while in case of cones chloroformic extract, values were determined against *Aspergillus niger* NCIM 1196 and *Salmonella abony* NCIM 2257. The results revealed that chloroformic extracts of leaves of the plant showed MIC and MLC values, 0.01 mg/ml and 0.1 mg/ml respectively against *Micrococcus luteus* ATCC 9341 and *Aspergillus niger* NCIM while chloroformic cone extracts of the plant showed MIC and MLC values, 10 mg/ml and 12 mg/ml against *Salmonella abony* NCIM 2257 and *Aspergillus niger* NCIM 1196. The results are shown in table 3 and figure 3.

Phytochemical Screening of the potent extracts

The chloroformic and hydro-alcoholic extracts of the leaves were subjected to phytochemical screening in lieu of determination of active constituents present in the extracts. It was observed that, flavanoids, saponin, steroids and reducing sugars were present in chloroformic extracts of cones while alkaloids, saponin and reducing were present in chloroformic extract of leaves. The results are shown in table 4.

The studies are in correlation with the findings reported by Sharma and Mathur (2014, 2015). In the previous study performed, the potent molecule isolated from *Cycas revoluta* was a flavone.

Statistical analysis

The data recorded from various *in vitro* and laboratory experiments were subjected to statistical analysis. The differences exhibited by treatments in various experiments were tested for their significance at 5 per cent using standard procedure as described (Gomez and Gomez, 1983).

In conclusion the study highlighted the importance of pharmacological activities viz. antimicrobial and antioxidant activities of leaves and female cones of *Cycas revoluta*. The

preparation of hydro-alcoholic and chloroformic extracts of leaves and female cones of *Cycas revoluta* through forward or activity directed approach was emphasized upon and successfully demonstrated in the present study lead to bioactive pharmacological agents which can be utilized as potent antimicrobial molecules. The study makes a strong plea in favor of proper investigation of plant kingdom to generate molecules having variable pharmacological properties. The scope of utilizing plant bioactive(s) in treating infectious diseases, is validated as per the studies concerned.

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