

SCREENING OF SOLVENT EXTRACTS OF *CYCAS REVOLUTA* FOR ISOLATION OF ANTIMICROBIAL COMPOUND

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ABSTRACT

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 studies report the isolation and characterization of a novel molecule, 2, 3-dihydro-4'-O-methyl-amentoflavone from the chloroformic extracts of leaves and female cones of the plant. The molecule was 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. The results hereby concluded that the extracts or the molecule can be utilized in formulating new antimicrobials which can be utilized in treating several infections caused by drug resistant pathogens. However further studies are however needed to study and evaluate its broad spectrum profile and to screen other different pharmacological activities.

Key words: Antimicrobial activity, *Cycas revoluta*, pathogenic and drug resistant strains, flavones.

INTRODUCTION

Plants have served as a source of new pharmaceutical products and inexpensive starting materials for the synthesis of many known drugs. Plant-based anti-microbial represents a vast untapped source of medicines with enormous therapeutic potential (Cowan, 1999). Reports are available on the use of several plant by-products, which possess antimicrobial properties, on several pathogenic bacteria and fungi (Bylka *et al.*, 2004; Shimpi and Bendre, 2005; Kilani, 2006). Some of the important and common plants based drugs employed in medicine are in use today.

The number of plant species in India is estimated to be over 45,000 representing about 7% of the world's flora. Medicinal plants, as a group, comprise approximately 8000 species and account for about 50% of all the higher flowering plant species of India (Gaur, 1999a, 2004b; Rawat *et al.*, 2009a, 2010b). The antimicrobial, antioxidant and anti-inflammatory activities of different plants were investigated (Mathur *et al.*, 2011 a & b). Antimicrobial and antioxidant activities in leaves of *Cycas revoluta* were previously reported (Mathur *et al.*, 2011c). In the present investigation, chloroformic and hydro-alcoholic extracts of the leaves and female cones of *Cycas revoluta* were investigated for

antimicrobial activity and isolation of the antimicrobial compound.

MATERIALS AND METHODS

All the chemicals and reagents used in the experiments were procured from C.D.H and Ranchem. Glass wares used were of Borosil. The media and broth used for microbial culture was procured from Hi-Media Ltd., Mumbai.

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 ITLS, 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) isolated from clinical specimens viz. pus and blood of infected patients were procured with authentication for the study. The standard bacterial and fungal cultures used for the study were procured from Roorkee Research & Analytical Labs Pvt. Ltd., Roorkee (U.K), India and MRSA strains were procured from Shooloni University, H.P., India.

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 extract:

The portion of the dry extracts was subjected to the Phytochemical screening using the method adopted by Trease and Evans (1983) 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. 1ml 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, 1983).

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 a 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. H₂ SO₄ 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 2ml of glacial acetic acid containing 1 drop of 1% FeCl₃. This was under laid with conc. H₂ SO₄. 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:

1ml 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:

5ml 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.

Isolation of the compound via chromatographic techniques:***Conventional preparative TLC***

Silica gel G used for thin layer chromatography (TLC) was activated in hot air oven at 110°C for one hour.

Preparation of thin layer plates and loading of sample:

A quantity of the finely divided absorbing agent silica gel G was prepared by the absorbent with twice the weight of distilled water and the mixture was made homogeneous by vigorous shaking for 5 minutes, then it was applied to the glass plate in a thin and uniform layer by using a Stahl-type applicator or by means of a spreading device. The thickness of the applied layer was maintained at 2 mm to 4 mm for leaves fraction and the plates were activated by being dried in a hot air oven, usually for 24 hours at 60°C. Preparative TLC was used to purify limited quantities of (<50 mg) semi pure fractions of the plants with 2 or 3 compounds on preparative TLC. Preparative TLC is one of the cheapest methods available for the isolation of a component or compounds from the mixture, only small amounts can be obtained from each fractionation procedure. Fractions of the potent plant extracts chromatographed within the column were applied in the form of band on TLC plate. The plates used in this method were 0.5-1 mm thick (analytically TLC uses plates of 0.25 mm thickness). This allowed a greater amount of sample to be loaded on the plate. The plates were developed in the solvent, toluene: diethyl ether: 1.75 M acetic acid (1:1:1) to separate the polar compounds. For separation of non polar/basic compounds, Chloroform: Benzene in a ratio of 50:05 was used as solvent system. A non destructing method was used to detect the compounds. The Iodine chamber was used as a detection system for detection of compounds on the chromatogram.

At least 90 % of the plate was covered only the exposed part was sprayed with the detection system. The active fractions/ pure compounds was scraped from the Silica gel plate and eluted from the silica gel with ethanol. The active compounds were filtered through Millipore filters (0.45 µm and 0.22 µm) to remove the silica gel and this yielded more of compound(s) fraction.

Combination of fractions:

From TLC results, fractions were combined according to the similarity of their chemical profile. Combined fractions were placed under air current at a slowly blowing fan to facilitate drying and crystallization. Once dried the fractions were weighed to calculate the total mass fractionated and the crystallized fractions were washed with the combination of solvents to obtain pure compounds. Active fractions were further chromatographed through Silica gel TLC in order to obtain the pure compounds.

Dereplication:

A system was established to identify isolated compounds from the crude extract. The dereplication method relies on the R_f value. The pure compound isolated and chromatographed on Silica gel TLC plate was detected by using the Iodine chamber. These parameters of the pure compounds were compared with that of crude extract to confirm the identity of the isolated compounds.

Structure elucidation of isolated compounds by combination of different techniques:

Identification of compounds was done by using a combination of different techniques including HPLC, FT-IR and NMR. Besides these characterization techniques, R_f values and melting point of the active compounds were also determined.

High-performance liquid chromatography (HPLC):

HPLC analysis was performed in Roorkee Research and Analytical Laboratory Pvt. Ltd., Roorkee(Uttarakhand), India using a Shimadzo LC- 2010 HPLC system (Kyoto, Japan), equipped with a Shimadzo LC 2010 UV-VIS

detector with a thermostated flow cell and a selectable two wavelengths of 190 - 370 nm or 371–600nm. The detector signal was recorded on a Shimadzo LC2010 integrator. The column used was a C-18 block heating-type Shim-pack VP-ODS (4.6 mm interior diameter × 150 mm long) with a particle size of 5 µm. Mobile phase was designed as per the nature of the compound, containing 50 % acetonitrile along with 50 % Phosphate buffer was used at a flow rate of 3.0 ml/min, column temperature 25°C. Injection volume was 40 µl and detection was carried out at specific wavelength having maximum absorbance as calculated by UV absorption spectra at maximum wavelength.

Fourier Transform Infrared (FTIR) studies:

The IR spectrum of isolated compound was recorded in Roorkee Research and Analytical Laboratory Pvt. Ltd., Roorkee (Uttarakhand), India using a computerized FTIR spectrometer (Perkin Co., Germany) in the range of 4000–400 cm⁻¹ by the KBr pellet technique.

RESULTS AND DISCUSSION

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 and *Salmonella abony* NCIM 2257 while there was no antibacterial potential found against *E. coli* NCIM 2065 and MRSA strains. 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. 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*

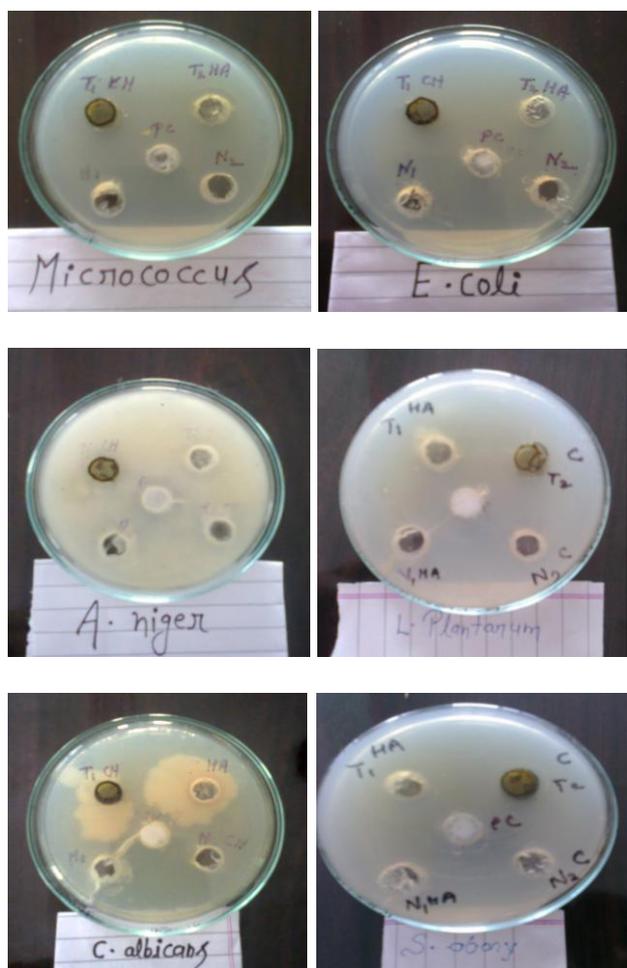
Table 1: Antimicrobial activities of leaves extracts of *Cycas revoluta*

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 35	MRSA 8	<i>B. niger</i>	<i>C. albicans</i>
Hydro-alcoholic extracts	NA	21	17	11	05	NA	NA	NA
Chloroformic extracts	NA	14	22	15	07	NA	05	NA
Erythromycin (1 mg/ml)	10	20	15	18	10	12	NT	NT
Fucanazole (1 mg/ml)	NT	NT	NT	NT	NT	NT	24	20

*NA, No Activity; NT, Not Tested

abony NCIM 2257, *Candida albicans* NCIM 3471, *Aspergillus niger* NCIM 1196 and Methicillin resistant strains of *Staphylococcus aureus* (MRSA).

Figure-1: Antimicrobial activities of leaves extracts of *Cycas revoluta*



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**.

Figure-2: Antimicrobial activities of female cones chloroformic extracts of *Cycas revolute*

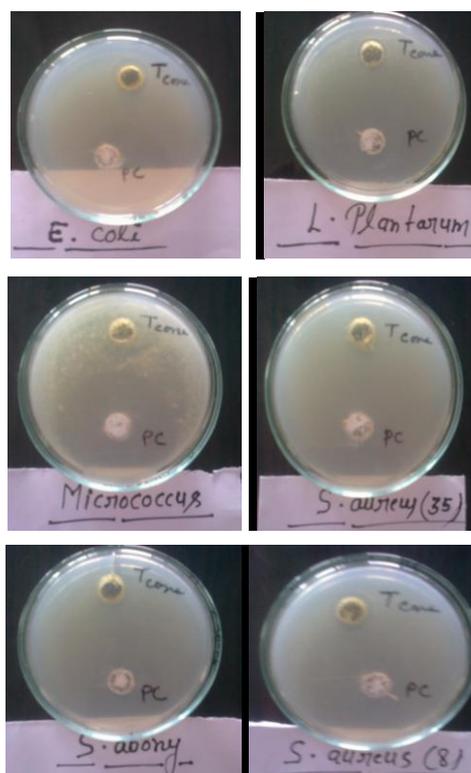


Table 1: Antimicrobial activities of female cones extracts of *Cycas revoluta*

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 35</i>	<i>MRSA 8</i>	<i>A. niger</i>	<i>C. albicans</i>
Hydro-alcoholic extracts	NA	21	17	11	06	NA	NA	NA
Chloroformic extracts	15	17	15	12	15	14	10	10
Erythromycin (1 mg/ml)	15	13	15	11	10	12	NT	NT
Fucanazole (1 mg/ml)	NT	NT	NT	NT	NT	NT	24	20

*NA. No Activity; NT. Not Tested

Minimum Inhibitory Concentration of solvent extracts:

The female cones and leaves chloroformic extracts of *Cycas revoluta* 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.

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

Solvent extracts	Phytoconstituents							
	Alkaloids	Tannins	Flavanoids	Saponin	Steroids	Cardiac glycosides	Reducing sugars	Anthraquinones
Chloroformic cones extracts	-	-	+	+	+	-	+	-
Chloroformic leaves extracts	+	-	-	+	-	-	+	-

Phytochemical Screening:

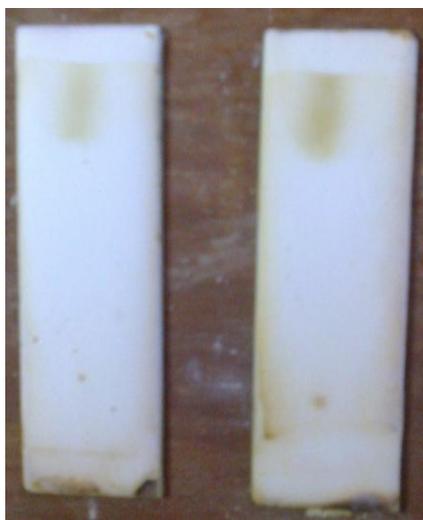
The chloroformic and hydro-alcoholic extracts of the leaves were subjected to phytochemical screening in lieu of determination of active constituents present in the extracts. The results are shown in Table 4.

Isolation and characterization of the compound via chromatographic and spectroscopic techniques:

The compound was isolated by using preparative TLC and further the compound in the form of single spots was scrapped out for characterization via HPLC and FT-IR. The isolated compound was dissolved in appropriate solvent. 5 µl of sample (chloroformic extracts of leaves and cones) were applied to silica gel plates, Merck (Germany) 20 × 20 cm, 0.25 mm in thickness were used. Plates were developed using the solvent system, Benzene: Chloroform (5:50) and the separated zones were visualized using Iodine chamber. A brown colored spot with a retention factor (R_f) value of 0.82 was identified as 2, 3-dihydro-4'-*O*-methyl-amentoflavone. The pure compound was further subjected to HPLC and FT-IR analysis. The results of TLC, HPLC and FT-IR are shown in Figure 3-5. The compound isolated compound was further screened for antimicrobial activity. Results are shown in Table 5 and Figure 6.

The results concluded that both the leaves and female cones of *Cycas revoluta* are potent antimicrobial agents. The studies correlate the findings previously reported by Mathur *et al.*, 2011. The studies report the isolation and characterization of a novel molecule, 2,3-dihydro-4'-*O*-methyl-amentoflavone from the chloroformic extracts of leaves and female cones of the plant.

Figure-3: Separation of compound in Chloroformic leaves and female cones by TLC (Solvent System: Benzene: Chloroform:: 5:50; Detection system: Iodine chamber)



Although this molecule was previously reported in leaflets of *Cycas revoluta* by Das *et al.*, 2005 but this molecule is newly reported in female cones of the plant. The molecule was found to be promising antimicrobial agent against MRSA, *E. coli*, *Salmonella abony*, *Aspergillus niger*, *Candida albicans*, and other pathogens reported in the study.

CONCLUSION

The results hereby concluded that the extracts or the molecule can be utilized in formulating new antimicrobials which can be utilized in treating several infections caused by drug resistant pathogens. However further studies are needed to study and evaluate its broad spectrum profile and to screen other different pharmacological activities.

Figure 4 (a): HPLC chromatogram of the standard compound

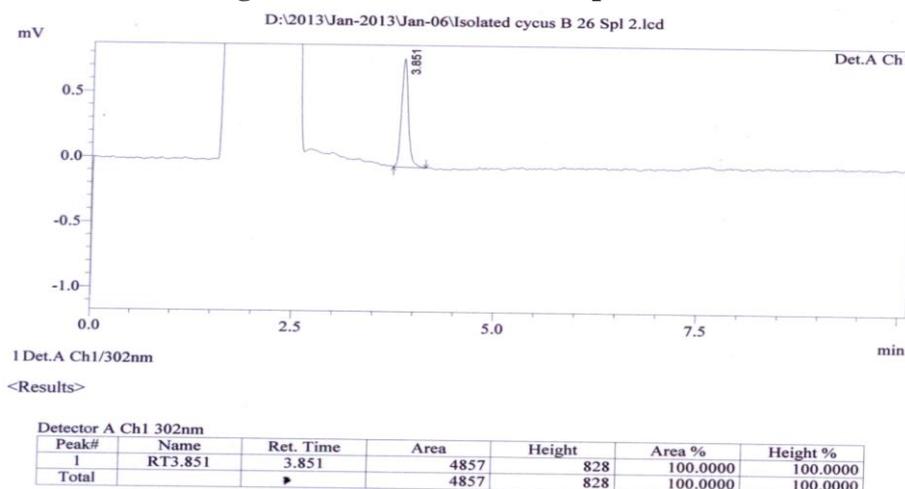


Figure 4 (b): HPLC chromatogram of the isolated compound (flavone)

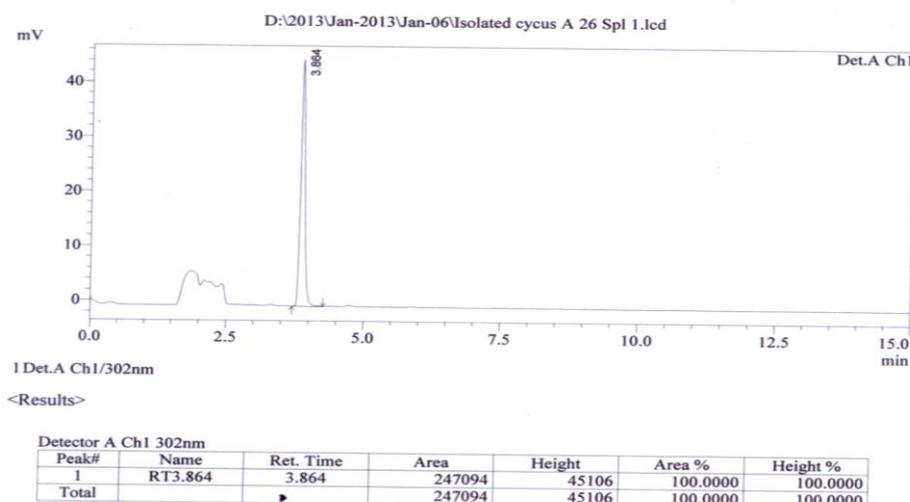
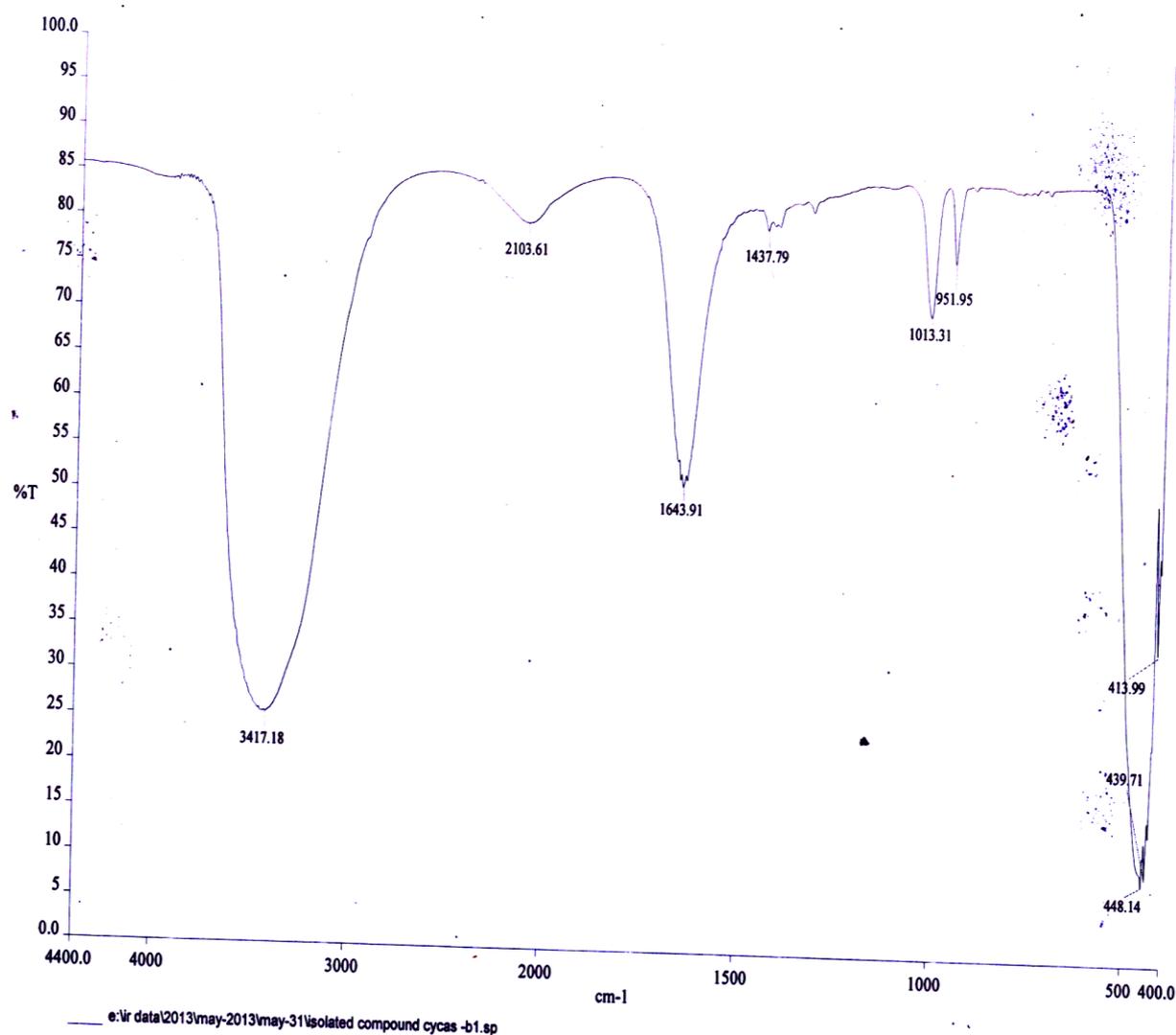


Figure 5: FT-IR spectra of isolated compound (flavone)**Table 5: Antimicrobial activities of isolated compound (from chloroformic extracts of leaves and cones) of *Cycas revolute***

Compound/ Antibiotic	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 35</i>	<i>MRSA 8</i>	<i>A. niger</i>	<i>C. albicans</i>
Isolated Compound	15	17	13	11	08	12	14	10
Erythromycin (1 mg/ml)	15	13	15	11	10	12	NT	NT
Fucanazole (1 mg/ml)	NT	NT	NT	NT	NT	NT	24	20

*NA, No Activity; NT, Not Tested

Figur-6: Antimicrobial activities of isolated compound (from chloroformic extracts of leaves and cones) of *Cycas revolute*



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