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RESEARCH ARTICLE

ISOLATION OF POLYCRYSTALLINE FERROELECTRIC COMPOUND FROM *CYCAS REVOLUTA*

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ABSTRACT

The present study was performed in order to investigate the nature and antimicrobial property of the compound isolated from leaves and female cones of *Cycas revoluta*. 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 was observed on TLC plate with a retention factor (R_f) value of 0.82 found similar to that of standard compound (having Rf value 0.84). The pure compound was further subjected to HPLC and FT-IR analysis which confirmed the compound as polycrystalline ferroelectric compound (2, 3-dihydro-4′-*O*-methyl-amentoflavone).

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INTRODUCTION

Chemistry plays a major role in defence mechanism and pollination and in the production of wide variety of the so-called secondary metabolites that are involved in such interactions between organisms with most secondary metabolites derived from just a few building blocks (Wallace, 2004). The escalation of plant defences has resulted in increased diversity and the role of constitutive and induced plant defences have come regarded as essential components in our understanding of biodiversity. The enormous chemo-diversity in the plant kingdom producing a wide variety of secondary metabolites in response to or in interaction with the environments, and which have evolved during the course of evolution, provided potential for the discovery of new bioactive compounds. The inherent greater structural diversity coupled with the chemical novelty of low molecular weight drug like properties of plant molecules provide them with an advantage in human disease therapy mainly due to more favorable compliance and bioavailability properties against antibiotic resistant strains (Verpoorte, 1998; Panchal, 2009; Rodriguez *et al*., 2009). Antibiotics are major tools for fighting with the infectious diseases.

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The first therapeutically used antibiotic, penicillin was discovered by Alexander Flemming in 1928. Some antibiotics are broad spectrum (which act against diverse group of bacteria) and some are narrow spectrum (which act against particular group of bacteria). Microorganisms have the ability to become resistant to the major therapies used against them. Resistance in microorganisms by the continuous use of antibiotics or antimicrobial agents are well defined (Wright, 2003; MacLean *et al*., 2010). Plants represent still a large untapped source of structurally novel compounds that might serve as lead for the development of novel drug. Inflammation is considered as a primary physiologic defense mechanism that helps body to protect itself against infection, burn, toxic chemicals, allergens or other noxious stimuli, an uncontrolled and persistent inflammation may act as an etiologic factor for many of these chronic illnesses (Kumar *et al*., 2004). The present study was focussed in order to isolate and characterize the polycrystalline ferroelectric compound from *Cycas revoluta* (Sago palm).

MATERIALS AND METHODS

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 2-4 mm for leaves fraction and the plates were activated by being dried in a hot air oven, usually for 24 hr 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.

De-replication

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 371-600 nm. 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 using a computerized FTIR spectrometer (Perkin Co., Germany) in the range of 4000-400 cm^{-1} by the KBr pellet technique.

Determination of antimicrobial activity of the isolated compound

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 ^{0}C for 18 h and suspension was checked to provide approximately, 10^5 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.

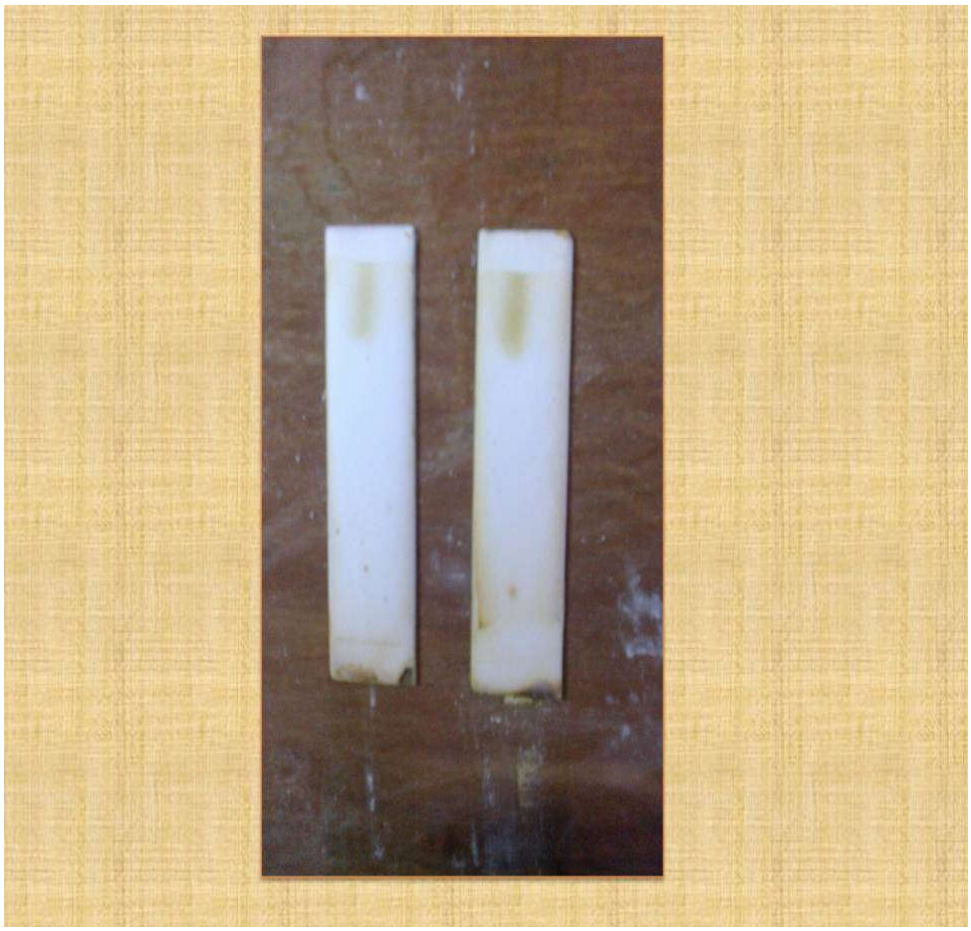

Figure 1. Polycrystalline ferroelectric compound separation in chloroformic leaves and female cones by TLC

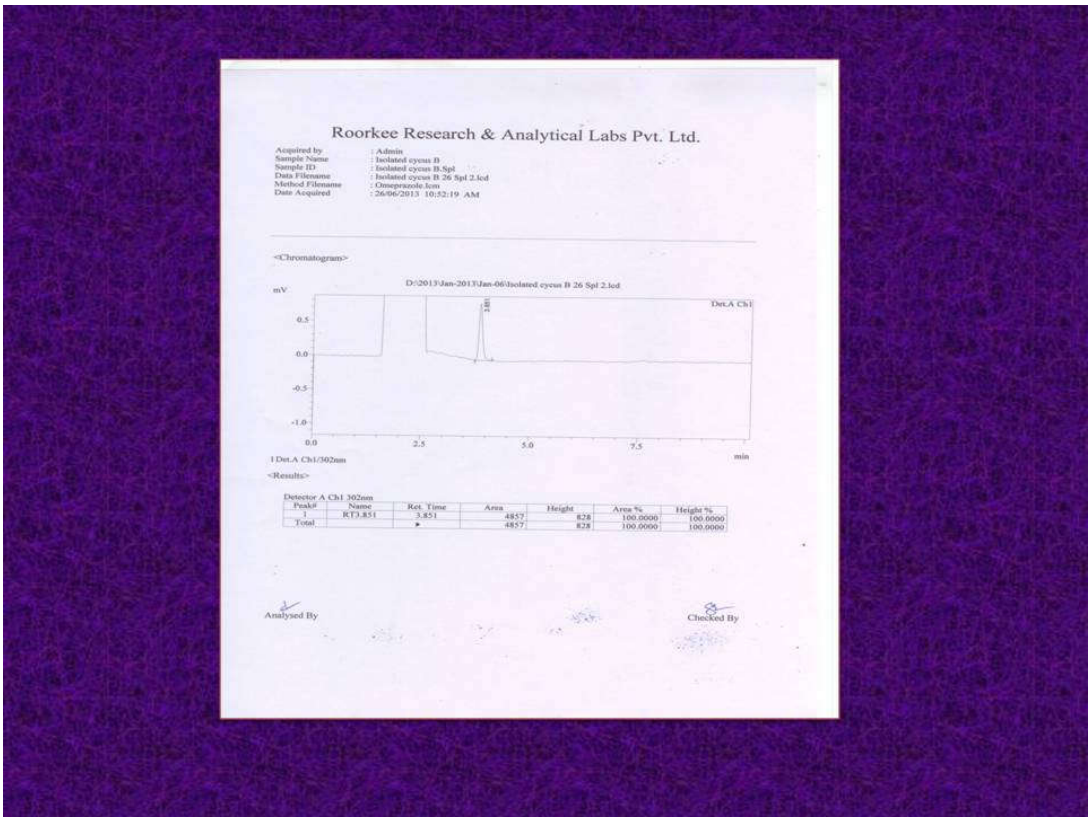

Figure 2. HPLC chromatogram of the standard, Polycrystalline ferroelectric compound

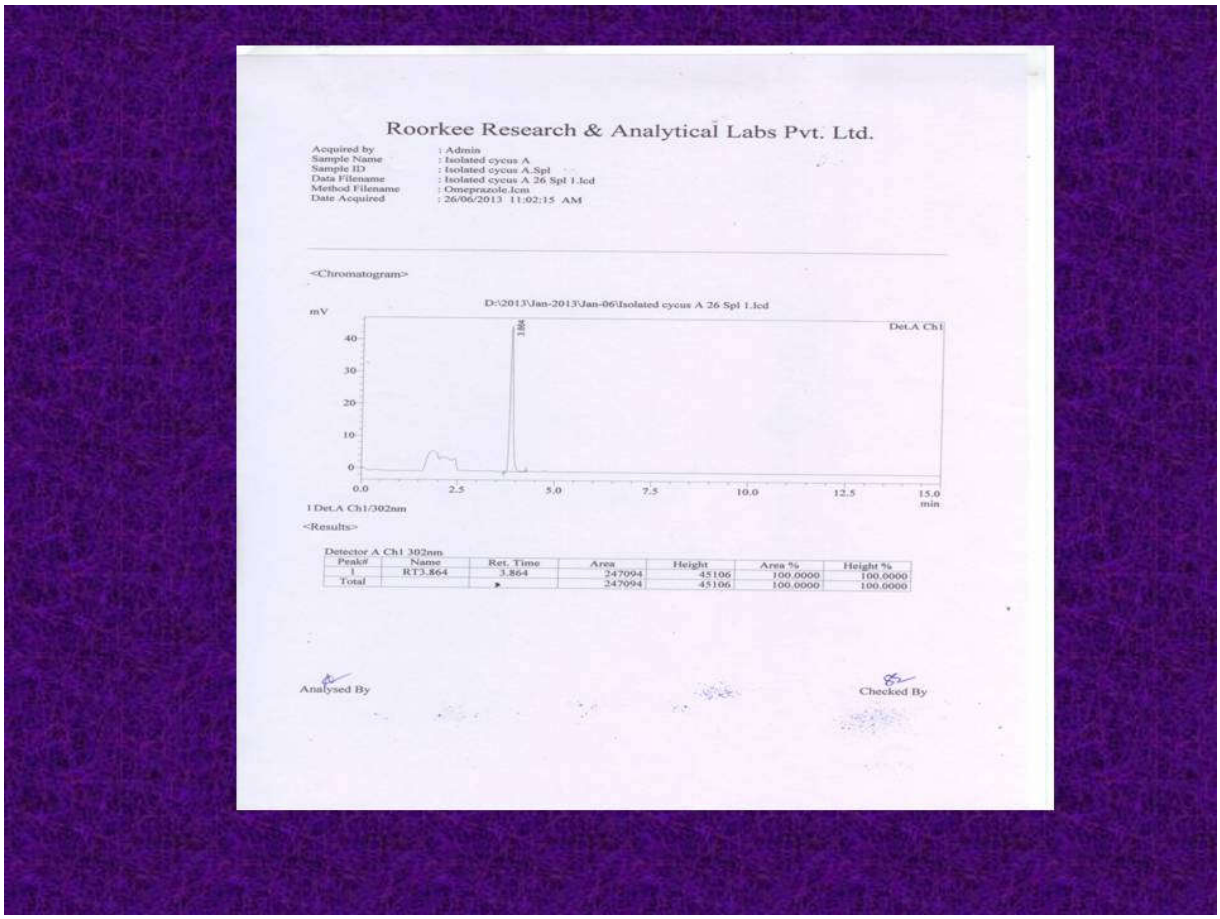

Figure 3. HPLC chromatogram of the isolated ferroelectric polycrystalline compound

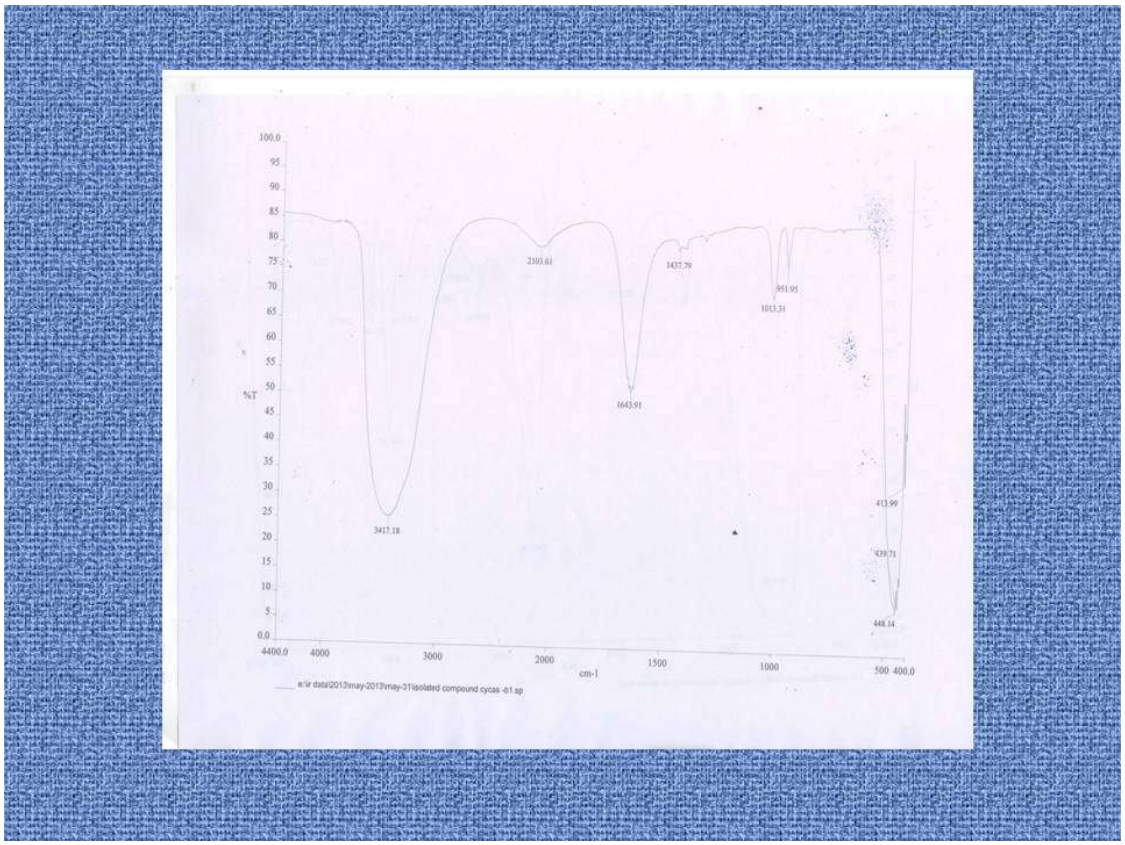

Figure 4. FT-IR spectra of isolated ferroelectric polycrystalline compound

A total of 8 mm diameter wells were punched into the agar and filled with the isolated compound (5 ppm) dissolved in N-saline 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 ^{0}C for 18 h. The antibacterial activity was evaluated by measuring the diameter of zone of inhibition observed.

The pure compound was further subjected to HPLC and FT-IR analysis which confirmed the compound as polycrystalline ferroelectric compound (2, 3-dihydro-4′-*O*-methyl-amentoflavone) (Sharma *et al*., 2014). The results of TLC, HPLC and FT-IR are shown in Figure 1-4. The compound isolated compound was further screened for antimicrobial activity at 5 ppm dose which are shown in Table 1 and Figure 5.

Table 1. Antimicrobial activities of isolated ferroelectric polycrystalline compound

Compound/ Antibiotic	Diameter of zone of inhibition (mm) Pathogens studied							
	E.coli	S. abony	M. luteus	L. plantarum	MRSA 101	MRSA 102	A.niger	C. albicans
Isolated Compound (Dose @ 5 ppm)	22.0	28.0	15.0	12.0	10.0	13.0	24.0	21.0
Erythromycin (1 mg/ml)	18.0	22.0	25.0	23.0	21.0	24.0	NT	NT
Fucanazole (1 mg/ml)	NT	NT	NT	NT	NT	NT	35.0	24.0

*NA, No Activity; NT, Not Tested

For assaying, antifungal activity of the isolated compound, 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.

RESULTS

Isolation and characterization of the compound

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.

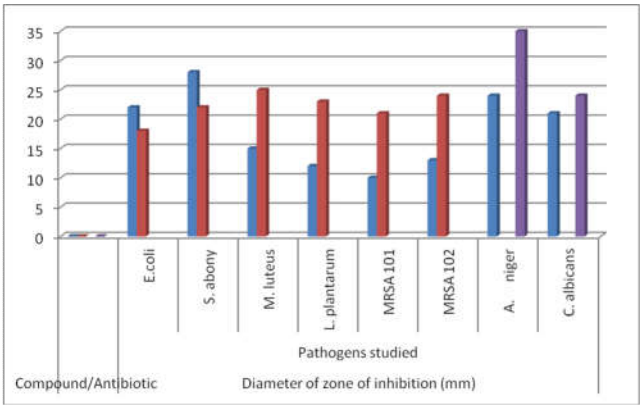

Figure 5. Antimicrobial activities of isolated ferroelectric polycrystalline compound

A brown colored spot was observed on TLC plate with a retention factor (R_f) value of 0.82 found similar to that of standard compound (having Rf value 0.84).

DISCUSSION

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. 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. After fractionation of the chloroformic extract of cones and leaves via column chromatography and series of successive thin layer chromatography, brownish colour compound (showed single spot on TLC plate) was eluted this was further dried. The retention factor (R_f) value of pure compound isolated was found to be 0.82 (showed close resemblance to standard compound having retention factor 0.84). The pure compound was further subjected to HPLC and FT-IR analysis which confirm the compound as 2, 3-dihydro-4′-*O*-methyl-amentoflavone which was found to be a polycrystalline ferroelectric compound. The previous studies performed by Shobha Rani *et al*., 2008; Das *et al*., 2005 and Sharma *et al*., 2015 confirmed the presence of flavones like compounds in *Cycas* leaves extracts. The compound isolated was screened for antimicrobial activity against the above said contaminants at 5 ppm dose.

Conclusion

The isolation and characterization of isolated novel molecule from leaves and female cones of Cycas revoluta viz. ferroelectric polycrystalline compound (2, 3-dihydro-4′-*O*-methyl-amentoflavone) can thus be utilized as a constituent in the formulation of antimicrobial and anti-oxidative agents is validated by the study. However, further studies are needed to optimize preparation of different solvent extracts and molecules isolation (if other) and to correspond its effect on variable pathogens and drug resistant strains.

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