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Antibacterial Activity of *Cycas rumphii* Miq. Leaves Extracts against Some Tropical Human Pathogenic Bacteria

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ABSTRACT

Cycas rumphii is traditionally used to cure wide variety of ailments including malignant ulcers, wounds healing, sore throats and tuberculosis showing its great potential as an antimicrobial agent. Hence, aim of the present study was to explore antibacterial potential of the leaves of *C. rumphii* Miq. Dried leaves were separately macerated with methanol and double distilled water at room temperature to obtain methanol and aqueous (AQ) extracts. The methanol extract was further successively extracted with light petroleum ether (LPE), benzene (BZ), ethyl acetate (EtOAc) and methanol (MeOH) on hot water bath to get respective extracts. These extracts were evaluated for their antibacterial efficacy at six different concentrations (500 μg, 1, 2, 5, 10 and 15 mg mL^{-1}) against seven G^{+ve} and eleven G^{-ve} hospital isolated bacterial strains causing several tropical diseases using disc diffusion method. EtOAc and MeOH extracts showed maximum antibacterial activity against most of the bacteria taken into account. The most susceptible Gram positive bacteria was *S. albus* with inhibition zone of 22 mm while most susceptible Gram negative bacteria was *S. boydii* with 21 mm inhibition zone with EtOAc extract while the most resistant bacteria was gram negative *S. typhi*. Present findings are suggestive of antibacterial agents in the leaves of *C. rumphii* which can be used in future for formulation of broad spectrum herbal antibacterial products. Results of present study also support various traditional uses associated with this plant which were suggestive of possible antibacterial potential of this plant. Hence, EtOAc and MeOH extracts of the leaves of *C. rumphii* deserve further investigations.

Key words: *Cycas rumphii,* crude extracts, successive extraction, antibacterial activity, gram-positive bacteria, gram-negative bacteria

INTRODUCTION

Since antiquity, plants have traditionally been used to cure a broad continuum of human diseases (Abu-Rabia, 2005). Ayurveda, Unani and Siddha are important Indian medical practices which have played a significant role to congregate information on traditional medicinal plants in the course of many centuries (Muthu *et al*., 2006). According to survey conducted by ethnobotanists in India, it was found out that about 2500 different plant species are used to cure various disorders by folklore practitioners in India (Shengji, 2001). During the past few decades, various scientific

attempts have been made to study medicinal plants and their traditional uses in different parts of the world (Lev, 2006; Karim *et al*., 2011). Ethnomedical studies on traditional medicinal plants have significantly helped pharmaceutical industries around the world in finding out new therapeutic agents for the various fields of tropical biomedicine (Cordell, 2000). Recently in the past twenty years, many of the plant species have been documented pharmacologically which are endowed in phytochemicals with marked activity on human pathogenic bacterial strains (Cox, 1994; Khan *et al*., 2010, 2011).

Inability of existing antibiotics to cure clinical infections efficaciously due to resistant bacteria has urgently demanded the need for new antimicrobial agents to overcome chronic bacterial infections (Sule *et al*., 2011a). Alleviating the problem may require the use of new antimicrobials to mimic the body's natural defense against infection and plants are considered potent candidate for this aim. Employment of antibiotic resistance inhibitors of plant origin is one of the ways to reduce antibiotic resistance and adverse effects on host associated with pathogenic microbes (Nostro *et al*., 2000; Houghton, 2001).

Cycas rumphii Miq. (*C. rumphii*) (family: Cycadaceae; common name: Queen Sago) is a native species of Indonesia that inhabits closed wood-lands or forest near shorelines in S. Borneo, N.E. Java, Maluku Papua and Sulawesi (Barceloux, 2008). *C. rumphii* grows up to 10 m in height with the trunk diameter reaches up to 400 mm. The grey bark is characteristically fissured into diamond and rectangular-shaped (Lindstrom and Hill, 2007). Different parts (leaves, seeds and bracts) of *C. rumphii* are traditionally used in India, Bangladesh and Indonesia to cure wide variety of ailments including malignant ulcers, wounds healing, boils, itchy skin lesions, nephritic pains, edematous swellings, dizziness, headaches, sore throats, flatulence, vomiting of blood, gynecological disorders and tuberculosis (Jain, 1991; Barceloux, 2008). In India, male bracts are also used as a narcotic, stimulant and aphrodisiac agents (Jain, 1991).

Phytochemical investigations of *C. rumphii* have revealed the presence of different class of phytoconstituents including cycasin; β-glycosidase; amentoflavone; podocarpusflavone A; 2, 3-dihydroamentoflavone; 2, 3-dihydrohinokiflavone; isoginkacin and bilobetin (Rastogi and Mehrotra, 1991, 1993; Rastogi, 1995; Uddin *et al*., 2004).

Literature has revealed many medicinal uses associated with this plant species particularly in the management of malignant ulcers, wounds healing, sore throats and tuberculosis showing its great potential as an antimicrobial agent. However, its antibacterial potential has never been evaluated scientifically. Hence, present study was an attempt to evaluate the possible antibacterial potential of the crude leaves extracts of *C. rumphii* Miq. against some tropical human pathogenic bacterial strains.

MATERIALS AND METHODS

Collection of plant material: Fresh leaves (5 kg) of *C. rumphii* were collected during winters (December 2009) from different localities of Aligarh district, U.P., India. Plant samples (Voucher specimen number: AV023, AV2080) were deposited in the Herbarium of Department of Botany, Faculty of Life Sciences, Aligarh Muslim University, Aligarh-202002, U.P., India.

Preparation of non-polar and polar extracts: Shade dried leaves of *C. rumphii* (1.75 kg) were pulverized in an electric grinder and powder was stored in a desiccator. The grinded material was used for the extraction process. Five hundred grams plant powder was macerated with 95% methanol in a round bottom flask at room temperature for about 24 h. Mother liquor was filtered out and residual plant material was again macerated with 95% methanol for another 24 h. The

entire process was repeated four times to ascertain the maximum yield of methanol soluble compounds. The extract was evaporated to dryness at 35°C under reduced pressure using rotary evaporator to get concentrated methanol extract. The extract was freeze-dried to give a final yield of 55 g (11.0%) methanol extract and kept in the chiller at -18°C till further use. Freeze-dried methanol extract was reflexed with light petroleum ether (60-80°C) for 5 h on the hot water bath. Light petroleum ether soluble portion upon filtration was evaporated under reduced pressure and freeze-dried to obtain light petroleum ether extract (LPE) (10.0 g, 2.0%). Light petroleum ether insoluble fraction of methanol extract obtained in above process was further reflexed with benzene for 5 h, filtered and evaporated under reduced pressure and freeze-dried to obtain benzene extract (BZ) (12.5 g, 2.5%). Benzene insoluble fraction was reflexed with ethyl acetate for 5 h, filtered and evaporated under reduced pressure and freeze-dried to obtain ethyl acetate extract (EtOAc) (8.0 g, 1.6%). Ethyl acetate insoluble fraction was further reflexed with methanol for 5 h, filtered and evaporated under reduced pressure and freeze-dried to obtain methanol extract (MeOH) (13.0 g, 2.6%) while methanol insoluble residue was discarded. During the entire successive extraction, each step was repeated five times to ensure maximum yield of every desirable extract.

Five hundred gram plant powder macerated with double distilled water and left for 72 h at room temperature under dark. The flask was then reflexed over hot water bath for 10 h and the mother liquor was filtered. The distilled water was again added reflexed and filtered and the entire process was repeated for four times. The filtrate, thus obtained, was evaporated to complete dryness under reduced pressure and freeze-dried to obtain aqueous extract (AQ) (40.0 g, 8.0%) (Harborne, 1998).

Microorganisms: Leaves extracts were tested for possible antibacterial activity through disc diffusion method against eighteen (18) human pathogenic bacteria viz., *Staphylococcus aureus*, *Staphylococcus aureus* ATCC 25953, *Staphylococcus albus*, *Streptococcus haemolyticus* Group A, Streptococcus haemolyticus Group B, *Streptococcus faecalis*, *Bacillus subtilis*, *Escherichia coli*, *Edwardsiella tarda*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Shigella boydii*, *Shigella dysenteriae*, *Shigella flexneri* and *Plesiomonas shigelloides*. All bacterial strains taken into consideration were obtained from the bacterial stock, Department of Microbiology, Jawaharlal Nehru Medical College, Aligarh, U.P., India. The bacterial cultures were maintained at 4°C on nutrient agar.

Bacterial susceptibility test: Disc diffusion method (Bauer *et al*., 1966; Colle and Marr, 1989; Vaghasiya *et al*., 2007) was used to determine susceptibility of plant extracts. Standardized inoculum (1-2×10^7 CFU mL^{-1} 0.5 McFarland standard) was introduced on the surface of the plates containing Mueller Hinton agar (NCCLS, 2004) which was spread evenly with a glass spreader. Paper disc (6 mm) were impregnated with 500 μg mL^{-1}, 1, 2, 5, 10 and 15 mg mL^{-1} plant extracts. The discs were dried in an oven at 37°C and placed on the agar surface with the help of a sterile forceps, aseptically. Finally the discs were pressed with forceps to make complete contact with the surface of the medium. Inoculated petridishes were incubated at 37°C overnight and the inhibition zones were recorded. The experiments were repeated thrice and the mean of the triplicate of the results is summarized in Table 1. Standard sensitivity discs of chloramphenicol (10 μg/disc) (Span Diagnostics Limited, Surat, India) were used to test the sensitivity profile against the reference bacteria. Each experiment was done in triplicate and the mean values are presented. The diameters of the inhibition zones were measured in mm with respect to evaluate antimicrobial activity of all extracts *in vitro*.

Table 1: Yields of non-polar and polar freeze dried extracts of the leaves of *C. rumphii*

Plant extracts	Yield (g)	Yield (%)
Aqueous (AQ) extract	40.0	8.0
Methanol (MeOH) extract	55.0	11.0
Light Petroleum Ether (LPE) fraction	10.0	2.0
Benzene (BZ) fraction	12.0	2.5
Ethyl Acetate (EtOAc) fraction	8.0	1.6
Methanol (MeOH) fraction	13.0	2.6

RESULTS

Five hundred gram pulverized leaves of *C. rumphii* produced = 40.0 g (8.0%) freeze-dried AQ extract. Five hundred gram pulverized leaves of *C. rumphii* yielded = 55.0 g (11.0%) freeze-dried methanol extract. Methanol extract upon successive extraction with non-polar and polar organic solvents at room temperature subsequently yielded LPE = 10.0 g (2.0%), BZ = 12.0 g (2.5%), EtOAc = 8.0 g (1.6%) and MeOH =13.0 g (2.6%) freeze-dried extracts (Table 1).

In the present study, the investigation of antibacterial activity of the aforementioned extracts of the leaves of *C. rumphii* against seven Gram-positive and eleven Gram-negative bacteria was studied using disc diffusion method. The data pertaining to the antimicrobial potential of non-polar (LPE and BZ) and polar (EtOAc, MeOH and AQ) extracts of the leaves of *C. rumphii* are presented in Table 2. The results revealed variability in the inhibitory concentrations of each extract for given bacteria. All extracts presented antimicrobial activity to at least four of the tested microorganisms. None of the extracts except EtOAc extract of the leaves of *C. rumphii* were active against *S. typhi* while all the extracts were active against *S. aureus*, *S. haemolyticus* (group B) and *K. pneumoniae*; the LPE extract was inactive against *S. albus*, *S. faecalis*, *B. subtilis*, *E. coli*, *S. typhi* and *S. boydii*. BZ extract was found inactive against *S. aureus* (ATCC 25953), *S. haemolyticus* (group A), *P. vulgaris*, *P. aeruginosa*, *S. typhi*, *S. dysenteriae* and *S. flexneri*, however, EtOAc extract did not show any antibacterial activity against *E. tarda*, *P. vulgaris* and *S. typhi*. AQ extract showed no inhibitory activity against *E. coli*, *P. mirabilis*, *P. aeruginosa*, *S. typhi* and *S. dysenteriae*. The most susceptible Gram-positive bacteria was *S. albus*, inhibition zone = 22 mm (EtOAc extract) and Gram-negative bacteria was *S. boydii*, inhibition zone = 21 mm (EtOAc extract), respectively. The most resistant bacteria was Gram negative *S. typhi* strain with almost no antibacterial activity against all extracts except EtOAc extract which displayed 11 mm inhibition zone at 15 mg mL^{-1} (Table 2).

Moreover, the EtOAc and MeOH extracts presented the highest activity, i.e., they were able to inhibit 15 (83.33%) types of microorganisms of interest. Furthermore, they also had the highest activity rate against bacteria. On the other hand, AQ extract showed inhibitory activity against 12 (66.66%) types of microorganisms and non-polar extracts (LPE and BZ) revealed antimicrobial activity against 11 (61.11%) microorganisms of interest, respectively. The antibacterial activity was more prominent on the Gram-positive bacteria than the Gram-negative bacteria. Gram-positive bacteria were the most susceptible to growth inhibition by EtOAc and MeOH extracts. The results showed that all the extracts have variable degrees of antibacterial activity and that the inhibition of bacterial growth was dose dependent as inhibitory action of the extracts was found to increase with the increase of concentration against all bacterial strains.

All extracts displayed similar antibacterial activity compared to positive control (Chloramphenicol) though at higher concentration. All extracts up to 1 mg mL^{-1} did not exert any antibacterial activity against all bacterial strains. Highest antibacterial activity was observed at

Table 2: Antibacterial activity of *Cycas rumphii* Miq. leaves extracts against some tropical human pathogenic bacteria

	Gram positive bacteria							Gram negative bacteria										
Extract	1	2	3	4	5	6	7	1	2	3	4	5	6	7	8	9	10	11
LPE																		
500 µg	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1 mg	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2 mg	11	08	-	09	08	-	-	-	08	08	09	07	07	-	-	09	09	-
5 mg	14	11	-	12	11	-	-	-	11	12	12	11	11	-	-	12	13	-
10 mg	16	16	-	15	16	-	-	-	17	17	17	16	16	-	-	18	17	-
15 mg	18	19	-	17	19	-	-	-	20	20	21	19	19	-	-	20	19	-
BZ																		
500 µg	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1 mg	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2 mg	07	-	10	-	08	07	09	08	07	09	09	-	-	-	07	-	-	09
5 mg	12	-	14	-	11	10	12	11	09	13	12	-	-	-	12	-	-	11
10 mg	16	-	15	-	16	12	16	16	12	16	16	-	-	-	16	-	-	16
15 mg	19	-	18	-	19	16	19	19	16	19	18	-	-	-	19	-	-	19
EtOAc																		
500 µg	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1 mg	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2 mg	08	08	07	08	-	07	09	-	08	07	-	07	-	-	09	07	07	07
5 mg	14	14	12	14	07	10	15	-	10	10	-	09	-	07	14	09	09	10
10 mg	17	16	17	19	09	12	19	-	13	13	-	14	-	09	17	11	11	12
15 mg	21	20	22	21	12	18	21	-	17	18	-	19	-	11	21	13	15	17
MeOH																		
500 µg	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1 mg	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2 mg	08	09	08	07	-	07	08	08	-	07	08	-	07	-	-	-	07	07
5 mg	14	15	12	09	07	10	14	14	-	09	11	-	09	-	07	09	09	12
10 mg	17	17	17	13	09	12	17	17	-	12	13	-	12	-	09	11	11	14
15 mg	21	21	20	19	12	16	20	20	-	16	18	-	16	-	11	15	14	16
AQ																		
500 µg	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1 mg	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2 mg	-	07	-	07	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5 mg	10	11	10	10	-	07	08	-	-	8	-	07	-	-	07	-	07	-
10 mg	13	13	13	14	-	10	11	-	08	10	-	09	-	-	08	-	10	-
15 mg	16	17	15	16	08	12	15	-	10	13	-	11	-	-	11	-	12	-
Chloramphenicol																		
10 µg/disc	18	18	16	-	-	-	16	18	16	-	16	18	-	16	17	19	18	20

Gram positive bacteria: 1. *Staphylococcus aureus* 2. *Staphylococcus aureus* ATCC 25953 3. *Staphylococcus albus* 4. *Streptococcus haemolyticus*, Group-A 5 and *Streptococcus haemolyticus*, Group-B 6. *Streptococcus faecalis* 7. *Bacillus subtilis*. Gram negative bacteria: 1. *Escherichia coli* 2. *Edwardsiella tarda* 3. *Klebsiella pneumoniae* 4. *Proteus mirabilis* 5. *Proteus vulgaris* 6. *Pseudomonas aeruginosa* 7. *Salmonella typhi* 8. *Shigella boydii* 9. *Shigella dysenteriae* 10. *Shigella flexneri* 11. *Plesiomonas shigelloides*. Values are the mean of replication of three experiments; -: no inhibition. Zone of inhibition in mm LPE: Light petroleum ether extract, BZ: Benzene extract, EtOAc: Ethyl acetate extract, MeOH: Methanol extract, AQ: Aqueous extract

15 mg mL^{-1} by all extracts against most of the bacterial strains. EtOAc extract exhibited the highest antibacterial activity against most of the bacterial strains in comparison to other extracts of the leaves of *C. rumphii*.

DISCUSSION

Plants are imperative source of potentially important compounds for the development of new therapeutic agents. Natural products from medicinal plants as secondary metabolites (minor phytoconstituents) have been associated with the conferment of antibacterial activities by many studies (Rios and Recio, 2005; Tamokou *et al*., 2008; Sule *et al*., 2011b). The presence of some of such biological active substances in a substantial amount in the explored part of *C. rumphii* may have bestowed the strong and significant antibacterial activity on the leaves extracts. In this regard, higher concentration of these phytochemicals might have been responsible for a higher degree of inhibition on the bacterial strains.

Formerly, in many studies of similar kinds, polar extracts i.e., acetone, ethanol and methanol have been used as extractants, however this orchestrated strategy could not reveal the furthermost sensitivity in yielding strong antimicrobial compounds that might pave the ways to synthesize new antimicrobial drugs (Cowan, 1999; Ncube *et al*., 2008). For this reason, in the present study, five different solvents based on their different degrees of polarity as determined by the functional group present, i.e., light petroleum ether, benzene, ethyl acetate, methanol and water were used and taken into account to obtain maximum active compounds in the extracts. It is quite noteworthy to observe that a relationship was detected between the extract solubility and antibacterial activity of different fractions. This suggests that in sequential extraction, maximum biological active compounds were solubilized according to their degree of solubility in polar solvents as EtOAc and MeOH extracts (fractions) displayed the highest antibacterial activity followed by AQ extract, BZ and LPE fractions. Parekh *et al*. (2005) and Sule *et al*. (2011a) accounted that methanol is a better solvent for consistent extraction of antimicrobials from medicinal plants. Our results also comply with the same study as polar fractions (EtOAc and MeOH) obtained from methanol extract revealed potent antibacterial activity in comparison to non-polar solvents (LPE and BZ). These results further confirm that significant antibacterial compounds are polar in nature as evidenced by the higher degrees of antibacterial activity of EtOAc and MeOH fractions from the methanol extract of the leaves of *C. rumphii*.

All the extracts exhibited strong antibacterial activity against Gram-positive bacteria in comparison to Gram-negative bacteria. The susceptibility of Gram-positive bacteria towards various plant extracts than those of Gram-negative bacteria has already been reported previously (Bele *et al*., 2009; Vaghasiya and Chanda, 2010). This has been attributed to the fact that the Gram-negative bacterial cell wall outer membrane appears to act as a permeability barrier to many substances including amphipathic (cationic, neutral and anionic) broad spectrum antibiotics (Lewis, 2001). The activity of *C. rumphii* leaves extracts against both Gram-positive and gram-negative bacteria might indicate the presence of broad spectrum antimicrobial compounds. Biological active substances in the form of pure compounds and standardized plant extracts endow infinite prospects for the development of new drugs owing to bountiful unparalleled availability of chemical miscellany (Sati *et al*., 2011; Cos *et al*., 2006; Chanda *et al*., 2011; Sule *et al*., 2011b). These results explicitly suggest that the bioactive compounds present in polar extracts have good potential for development of novel antibacterial herbal products.

CONCLUSION

Results obtained from our study further confirm the traditional use of *C. rumphii* in different ailments associated with pathogenic bacteria. Non-polar and polar extracts revealed varying degrees of antibacterial activity. However, polar extracts i.e., EtOAc and MeOH extracts

demonstrated most pronounced antibacterial activities against bacterial strains taken into consideration. Hence, EtOAc and MeOH extracts of the leaves of *C. rumphii* deserve further investigations to develop new antibiotics that may help in combating several bacterial diseases in tropical countries. This is the first ever report on the antibacterial potential of the leaves of *C. rumphii*.

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