

## ANTIFUNGAL AND CYTOTOXIC ACTIVITIES OF *NANNORRHOPS RITCHIANA* ROOTS EXTRACT

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**Abstract:** This study was designed to evaluate the antifungal and cytotoxic activities of the *Nannorrhops ritchiana* (Mazari Palm) 80% methanol extract (NR-M) and its four crude extracts i.e., petroleum ether (NR-A), dichloromethane (NR-B), ethyl acetate (NR-C) and butanol (NR-D). The antifungal activity was determined by agar tube dilution method against nine fungal strains; *Aspergillus flavus*, *Trichophyton longifusus*, *Trichophyton mentagrophytes*, *Aspergillus flavus* and *Microsporum canis* were susceptible to the extracts with percentage inhibition of (70–80%). Extracts exhibited significant and good antifungal activity against various fungal strains. The results were deduced by comparing with those for miconazole, amphotericin B and ketoconazole as standard drugs. The fractions of methanolic extract were assayed for their brine shrimp cytotoxic activity. They exhibited low toxicity with LC<sub>50</sub> values ranging from 285.7 to 4350.75 µg/mL at the concentration of obtained results warrant follow up through bioassay guided isolation of the active principles, future anti-infectious research.

**Keywords:** *Nannorrhops ritchiana*, antifungal activity, brine shrimp, cytotoxic activity

Natural products obtained from various sources like, plants, animals, and microorganisms are considered to be strong candidates of pharmaceutical drugs since long period of time and they constitute a vast majority of chemotherapeutic agents currently in use for various types of infectious diseases provoked by pathogenic bacteria and fungi, cardiovascular diseases, cancers and many others. The interest in using medicinal plant's extracts and their isolated components has increased not only in developing countries but also getting more popularity in developed countries. According to World Health Organization, medicinal plants would be the best source for variety of drugs (1) having less toxicity and being more economical. Therefore, medicinally important plants should be investigated for better understanding of the chemistry and chemical composition of their bioactive constituents, their efficacy and safety (2).

Pakistan occupies a unique position among developing countries due to widespread medicinal plants on account of its topology. It is noticeable that more than 50% of the population in Pakistan is still

being treated with the herbal medicines by the traditional practitioners and over 350 herbal drugs have been reported. Pakistan flora is rich in variety of rare plants and offers a great chance to discover a lead molecule, that is a step forward towards the pharmaceutical drugs (3).

The compounds resulting from natural products are the most important biochemical tools that demonstrate the specific mode of action in various fungal pathogenic diseases, but there is still a great urgency of developing new antifungal agents that are more efficient, less toxic, having minimum side effects (4). Fungal pathogens exist in various forms and some of them cause severe infections of skin, hairs, nails, lungs, ears and joints in humans and animals (5) whereas others cause severe infections of central nervous system (CNS) in humans. At present, about 300 metabolites have been reported to be toxic to man and animals (6) and the main toxic effects are carcinogenicity, genotoxicity, teratogenicity, hepatotoxicity and immune suppression (7, 8). The human fungal diseases also include aspergillosis, actinomycosis, histoplasmosis and candid-

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iomycosis. The discovery of antifungal drugs is the need of this era due to their versatility and the second most important factor is that they showed an enhanced degree of resistance to the existing antifungal drugs.

*Nannorrhops ritchiana* (Arecaceae) is the sole species of this genus (9). It is native to Southwestern Asia, from Southeast of the Arabian Peninsula to east through Iran and Afghanistan to Pakistan. It is widely distributed in the various areas of Balochistan province of Pakistan particularly in Harnai, Khuzdar and Barkhan at an altitude of 1600 m. It is one of the most versatile palms that can survive both in normal and under drastic weather conditions (10). The young leaves of plant with sweet astringent taste have been used as a purgative in livestock (11). The fruit is edible (12) and used by local communities for the treatment of diarrhea and dysentery (13). In Balochistan (14) it is used against various infectious diseases like gastrointestinal disorder (15). The constituents of plant ash have been quantitatively estimated in relation to biogeochemistry of the plant, the presence of minerals like calcium, magnesium, iron and nickel in soil and plant ash opened new avenue for the researchers (16). Therefore, research work is initiated on the roots of *N. ritchiana* to investigate antifungal and cytotoxic activities of various crude extracts.

In search of effective nontoxic oral drugs and topical applications, there is dire need of natural sources to be explored and exploited for relief.

## EXPERIMENTAL

### General

All reagents and solvents used were obtained from Sigma Aldrich (Shalimar Chemicals supplier, Pakistan). Commercial methanol used for extraction was purchased locally.

### Plant material

The *Nannorrhops ritchiana* species was collected from Barkhan and Harnai areas in Balochistan, Pakistan. A voucher specimen No. UoB/230 has been deposited in the Herbarium of the Department of Botany, University of Balochistan, Quetta, Pakistan.

### Preparation of extracts

The air-dried roots (15 kg) of *Nannorrhops ritchiana* were extracted with 80% aq. MeOH at room temperature for three times. The combined MeOH extracts were evaporated under vacuum to

get crude extract (NR-M) 1.5 kg. Methanol crude extract was dissolved in cold distilled H<sub>2</sub>O (10 L) and partitioned with petroleum ether (25 L) to afford after concentration the fraction NR-A. The aqueous layer was then extracted with CH<sub>2</sub>Cl<sub>2</sub> (10 L) to obtain fraction NR-B, ethyl acetate (10 L) – fraction NR-C and butanol (10 L) – fraction NR-D, respectively. The fractionated extracts were tested for the antifungal activity and based on that planned for further phytochemistry studies.

## Antifungal assay *in vitro*

### Test organisms

Clinical isolates of human pathogens *Microsporum canis*, *Candida albicans*, *Candida glaberata* and *Aspergillus flavus*; animal pathogens, *Tricheophyton mentagrophytes*, *Fusarium moniliformum* and plant pathogens *Trichophyton longifusus*, *Fusarium solani lycopersici* and *Fusarium oxysporum lycopersici solanivar* were used in this study.

### Agar tube dilution method

Agar tube dilution method (17) was used for the evaluation of antifungal activity. Sabouraud dextrose agar (SDA) was used for the growth of fungus and stock solution was prepared by dissolving 24 mg of each extract in 1 mL of sterile DMSO. Acidic medium (pH 5.5–5.6), containing high concentration of glucose or maltose prepared by mixing 32.5 g/500 mL of distilled water was put in the screw capped tubes and autoclaved at 121°C for 15 min. Tubes were then allowed to cool to 50°C and non-solidified SDA was loaded with 66.6 µL of extract, pipetted from the stock solution and allowed to solidify at room temperature. Then, tubes were inoculated with 4 mm piece of inoculums and incubated at 27–29°C for 7–10 days and relative humidity of incubation room was maintained at 40–50%. After this period, percentage growth inhibition was calculated with reference to the negative control by the formula:

$$\% \text{ inhibition} = \frac{\text{linear growth in test (mm)}}{\text{linear growth in control (mm)}} \times 100$$

Miconazole and amphotericin B were used as standard drugs, while miconazole, amphotericin B and DMSO were used as positive and negative controls (18–22).

### Brine shrimp lethality assay

The brine shrimp lethality test (BST) was performed to predict the cytotoxic activity in the different crude extracts of *N. ritchiana*. The eggs were hatched in artificial sea water as per conventional method (23). Shrimp were transferred by pipette to each tube and sea water was added to make total

volume of 5 mL. The test tubes were kept under illumination so that the nauplii can easily be counted macroscopically in capillary. The survivors were counted with the help of magnifying glass (×3), the brine shrimp (LC<sub>50</sub>) was determined.

## RESULTS AND DISCUSSION

During this study, different fractions obtained from 80% methanol extract (NR-M) of roots of *Nannorrhops ritchiana* plant, petroleum ether (NR-A), dichloromethane (NR-B), ethyl acetate (NR-C), and butanol (NR-D) extracts were screened for antifungal activity (Fig. 1). *N. ritchiana* extracts showed the maximum inhibition of fungal growth and thus indicate the presence of interesting antifungal agents in the roots extract of this plant.

The antifungal activity resulting from crude methanol extract fraction (NR-M) exhibited good activity against *Tricheophyton mentagrophytes*, – 77.3 MIC µg/mL. Fraction NR-A showed significant

antifungal activity against *Microsporium canis* 80%, *Trichophyton longifusus* 67%, good activity against *Tricheophyton mentagrophytes* 70%; extract NR-B showed good activity against *Trichophyton longifusus* 70% but moderate activity against *Microsporium canis* 60%, while extracts NR-C and NR-D exhibited medium activity against *Trichophyton longifusus* 60% and good against *Aspergillus flavus* 70%, respectively.

The brine shrimp lethality assay results presented in Table 2, show that the root extracts were found virtually non-toxic. They show negligible toxicity with LC<sub>50</sub> > 100 µg/mL.

The young leaves of *Nannorrhops ritchiana* are used as a purgative in livestock (11) and fruit (12) is used in traditional medicine by local communities for the treatment of diarrhea, dysentery (13, 14) and against various infectious diseases like gastrointestinal disorder (15). These claims have been supported on the bases of the current bioassay findings, that showed the activity against fungal pathogens.

Table 1. Antifungal activities (MIC µg/mL) of crude fractions of *N. ritchiana* roots extract.

Fungal species*	% inhibition of standard drug <sup>ii</sup>	% inhibition of different fractions				
		A	B	C	D	M
<i>M. canis</i>	110.8 <sup>c</sup>	80	60	– <sup>i</sup>	– <sup>i</sup>	– <sup>i</sup>
<i>T. mentagrophytes</i>	100 <sup>e</sup>	67	– <sup>i</sup>	– <sup>i</sup>	– <sup>i</sup>	77.3
<i>A. flavus</i>	20 <sup>d</sup>	– <sup>i</sup>	– <sup>i</sup>	– <sup>i</sup>	70	– <sup>i</sup>
<i>T. longifusus</i>	70 <sup>b</sup>	70	70	62	– <sup>i</sup>	– <sup>i</sup>

<sup>i</sup> – not active. Reference standard drug: <sup>b</sup>miconazole (MIC µg/mL), <sup>c</sup>miconazole (MIC µg/mL), <sup>d</sup>amphotericin B (MIC µg/mL), <sup>e</sup>ketocazole (µg/mL). Fractions: A: petroleum ether, B: dichloromethane, C: ethyl acetate, D: butanol. M: methanol crude extract. (\*only those fungi, which were significantly inhibited by respective sample have been included).

Table 2. Brine shrimp lethality test of *Nannorrhops ritchiana* roots extracts.

No.	Extracts	LC <sub>50</sub> µg/mL	95% CI
1.	Petroleum ether (NR-A)	285.7	189.2–431.4
2.	Dichloromethane (NR-B)	1462.2	685.55–3123.3
3.	Ethyl acetate (NR-C)	4357.77	1274.1–14901.10
4.	Methanol (NR-M)	409.84	253.07–664.10
5.	Cyclophosphamide	16.32	10.60–25.14

The results are presented as LC<sub>50</sub> values (µg/mL) and 95% confidence intervals (CI). Fractions: A: petroleum ether, B: dichloromethane, C: ethyl acetate, D: butanol. M: methanol crude extract. NR = *Nannorrhops ritchiana*

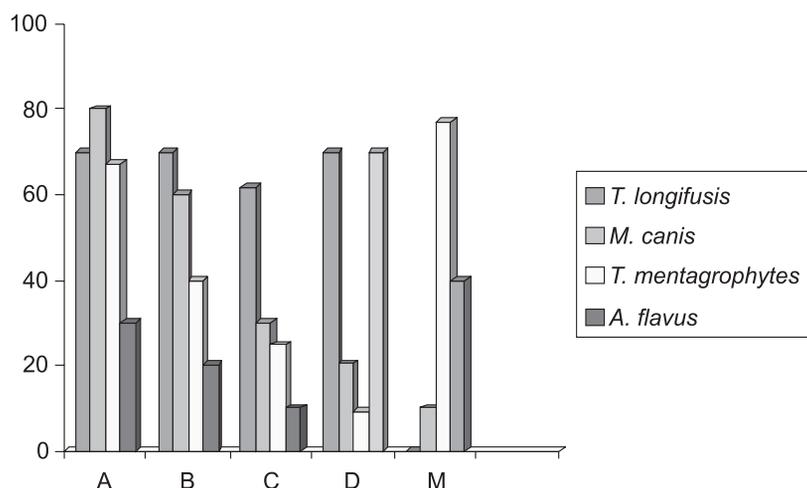


Figure 1. Antifungal activities of fractions of *N. ritichiana*. Fractions: A: petroleum ether, B: dichloromethane, C: ethyl acetate, D: butanol, M: methanol crude extract

The crude methanol roots extract (NR-M) of *Nannorrhops ritichiana* and four fractions i.e., petroleum ether (NR-A), dichloromethane (NR-B), ethyl acetate (NR-C) and butanol (NR-D) extracts exhibited an overall significant antifungal activity (70–80%) against different pathogens but few fungi have low activity against different fractions.

Extracts of the roots exhibited mild cytotoxic activity against brine shrimp larvae with  $LC_{50}$  value ranging from 285.75 to 4357.77  $\mu\text{g/mL}$ , whereas that of the standard anticancer drug was 16.32 (10.50–25.16)  $\mu\text{g/mL}$ . It is worth mentioning that the extract with very low toxicity on brine shrimp in particular are those active against antifungal pathogens.

## CONCLUSION

This study has shown significant fungicidal activity in the roots of *Nannorrhops ritichiana* and it can be exploited as an ideal future drug as an anti dermatophyte. This activity may be attributed to the presence of alkaloids, phenols, polyphenols, saponins, tannins, anthraquinones, sterols and especially alkaloids, found in the crude extract and fractions thereof. These phytochemical classes of natural products are known to display antifungal activities. This also indicates that the root extracts were virtually non toxic on shrimps. They exhibited very low toxicity, giving  $LC_{50}$  values greater than 100  $\mu\text{g/mL}$ . Selectivity of petroleum ether extract with  $LC_{50}$  values 285.77  $\mu\text{g/mL}$  with very low toxicity is not appreciated to be used in anticancer and antitumor activities. It may be speculated here that the extracts would be useful for the treatment of diar-

rhea caused by the gastrointestinal infection. Further research is planned to isolate and identify the constituents present in these fractions. The characterization of the structures of bioactive constituents of these fractions present in this plant should be done in order to determine their full spectrum of efficacy.

## Acknowledgments

The authors are grateful to Prof. Dr. Tareen R.B. Botany Department, University of Balochistan, Quetta for the plant identification and Prof. Dr. M.I. Choudhary, H.E.J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences (ICCBS), University of Karachi, Karachi, Pakistan for research collaboration.

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Received: 4. 11. 2013