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Original Article

Antifungal activity and Nystatin Interaction with crude aqueous and Methanolic extracts of *Azadirachta indica* (Neem) on some fungal isolates

A.A. Adegoke and N.U. Asamudo

Department of Microbiology, University of Uyo, P.M.B**. 1017** Uyo, Akwa Ibom State, Nigeria.

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ABSTRACT

Antifungal activities of crude aqueous and methanolic extracts of *Azadirachta indica* on some fungal isolates were determined. Standard microbiological methods and methods in phamacognosy were used to determine phytochemistry, for antibiotic susceptibility testing (AST), and antibiotic-extract **interaction**. The results obtained from this research showed higher quantity of saponins in methanolic extracts while terpenes were more in aqueous extracts. The antifungal effect showed by the extracts increased with increasing concentrations (12.5 mg/mL, 25.0 mg/mL, 50 mg/mL, 100 mg/mL, 200 mg/mL) on *Aspergillus niger* (inhibition zone range: 10.2 ± 0.2 mm-19.5 ± 0.1 mm) *Penicillium* sp. (inhibition zone range: 6.0 ± 0.2 mm-16.3 ± 0.5 mm) and *Candida albicans* (inhibition zone range: 8.3 ±2.0 mm- 18.0 ± 1.2 mm) for both methanolic and aqueous extracts. Aqueous extracts showed higher potency than methanolic extracts ($p < 0.01$). **A**ctivity index as high as 1.8 was observed. MIC as low as 125 µg/mL and 250 µg/mL were also observed. Combined extract and nystatin at MIC and ½ MIC showed synergy against *Aspergillus niger* and *Candida albicans*, but showed indeterminate effect on *Penicillium* sp. The traditional use of Neem plant for treating diverse infectious diseases is hereby justified, while its combination with synthetic antifungal drugs when necessary would be effective.

Keywords: methanolic extracts, aqueous extracts, synergy, antagonistic, infectious disease

INTRODUCTION

There is an increasing demand for medicinal plants and plants products as alternative to orthodox medicines especially in developing countries [1-3]. Reports of proximate analysis show that plants like neem consists of essential molecules, including ascorbic acid (n-hexacosanol) and amino acid (7-desacetyl-7-benzoylazadiradione, 7-desacetyl-7-benzoylgedunin, 17-hydroxyazadiradione) [4]. This justifies the use of such medicinal plants by traditional healers with positive therapeutic results [5]. Different parts used in folklore medicine include: root, stem flower, fruit, twigs, exudates and modified plant organs. Some of these raw drugs of medicinal plants are collected in smaller quantities by the local communities and folk healers for local uses. Many other raw drugs are collected in larger quantities and traded in the market for many herbal industries [6], supporting the need to study the potentials as antimicrobial agent. *Azadirachta indica* (neem) is an evergreen tree that has been used as a traditional medicine for centuries in the Indian culture and many tropical countries [5]. It is a fast growing tree that can reach a height of 15-20 meters (49-66ft) [7]. Neem plants'parts have been used locally to boost immune system, treat malaria, wounds and swellings; and has been rationalized by some formal reports [8, 9].

Meanwhile, the need for potent antifungal drugs continues as ever [10]. Fungal infections are estimated to occur in over a billion people each year and recent evidence suggested the rate is increasing. [11, 12]. Fungi can infect any part of the body including skin, nails, respiratory tract, or can be systemic [10]. If fungal infections enter systemic circulation, consequences can be deadly [13]. There are limited therapeutic options for invasive fungal infections [10]. Three classes of antifungal molecules are currently used in clinical practice and in the last three decades, only one new class of antifungal drugs have been developed [10]. It is therefore worthwhile to further study neem's antifungal activity and the effects of its combine**d** administration with an existing antifungal drugs, while also considering its phytochemistry, for which the study aimed.

***Corresponding Author Address:** Dr. Anthony A. Adegoke Department of Microbiology, University of Uyo, P.M.B. 1017 Uyo, Akwa Ibom State, Nigeria; aayodegoke@gmail.com; anthonyadegoke@yahoo.co.uk*

EXPERIMENTAL

Plants and Pathogens: The leaves of *A. indica* were collected from the Pharmacy Garden of the University of Uyo, Uyo, Akwa Ibom State Nigeria. It was identified by Mr Etefia of the Department of Pharmacology and Natural medicine, Faculty of Pharmacy University of Uyo and some were deposited for reference in the departmental herbarium. The fresh leaves were allowed dry and ground to powder using wooden pestle and mortal. Exactly 250 g of the powdery form of the leaf was placed in a container and was defatted using petroleum ether and macerated using 300 mL of 95%v/v methanol in order to obtain the methanolic extracts of the plant. The mixture was stirred and kept for 24 hours. Clinical isolates of fungi used (*Aspergillus niger*, *Penicillium* sp., and *Candida albicans*), were obtained from the University of Uyo Teaching Hospital and were re-characterized to ascertain identification. All fungal isolates used were cultured on sabouraud dextrose agar (SDA) (Oxoid, UK).

Qualitative Phytochemical Analysis of the Plant Extracts: Phytochemical screening of the plant extracts was conducted following the standard procedure as described by Harbone [27], Sofowora [28] and Trease and Evans [29]. For alkaloids', a measure of 0.5 g extract was mixed with 5 ml 1% aqueous hydrochloric acid on a steam bath. A few drops of Dragendorff's reagent were applied on 1 ml of the filtrate. The presence of turbidity or precipitation was reported for the presence of alkaloids. Exact 0.5 g of the extract extract in distilled water in a test tube was warmed. Persistent frothing during warming was recorded as an evidence for the presence of saponins.

Tannin's test was done by dissolving about 0.5 g of the extract in distilled water into which 10 ml of bromine water was added. Bromine discoloration implied tannins' presence. For Fehling's test for Combined Reducing Sugars, CRS, 0.5 g extracts was boiled in 5 ml hydrochloric acid (hydrolyzing) and the resulting solution was neutralised with sodium hydroxide solution. few drops of Fehling's solution was added to the mixture and then heated on a water bath for 2 minutes. Reddish-brown precipitate of cuprous oxide showed the presence of combined reducing sugars. The presence of anthraquinones was determined by Borntrager's test. Exact 0.5 g of the plant extract mixed with benzene layer separated and 10% ammonia solution was applied to 50% portion. A pink, red or violet coloration in the ammoniacal phase showed the detection of anthraquinone. The presence of cardiac glycosides was confirmed by Liberman's test, Salkowski test and Keller-Killani test. Sofowora [28] and Trease and Evans [29].

Antifungal Activity Assay of Plant Extracts: The antifungal activity was determined by both disc diffusion and tube dilution methods. Overnight culture of the isolates was diluted with peptone water to match the McFarland standard turbidity, which was used as the inoculums size to be seeded on the culture plates. One milliliter of the standardized fungal inoculum was transferred into pure agar plates with the aid of a sterile syringe and a sterile spreader was used to spread the inoculum uniform**l**y on the plates. The plant extracts was administered into 5 holes (bored with the aid of an improvised 6 mm sterile cork borer) on each plate with a varying concentrations of 200 mg/mL, 100 mg/mL, 50 mg/mL, 25 mg/mL, and 12.5 mg/mL using a sterile syringe. Likewise, Nystatin (500 000 UI) was titrated to achieve 100 units and sterile distilled water was administered into two additional holes to serve as positive and negative control respectively. For the disc diffusion methods, the plates were allowed to stand for one hour to aid diffusion of extracts into the medium, and then incubated at 25˚C for 24 – 48 hours. The clear zones of inhibition (mm) were measured using a meter rule, while the turbidity or its absence was recorded for the growth and inhibition. The results of both disc diffusion were validated by the observation in broth dilution.

Determination of Activity Index: The activity index (AI) was calculated using the mean inhibition zone of the extract and the mean inhibition zone of a standard antifungal drug (Nystatin).

$$AI = \frac{\text{Mean inhibition zone of sample (leaf extracts)}}{\text{Mean inhibition zone of standard drug}}$$

Determination of Nystatin- *Azadirachta indica* interaction: This was carried out by boring five wells on agar plates seeded with Nystatin only, Methanolic extracts only, Aqueous extracts only, Methanolic extracts + nystatin and aqueous extracts + nystatin. The wells were filled with equal volumes of the respective combination, labelled and incubated at 25˚C for 24 hours (and/or 48 hours for some plates). The zone of inhibition around each well were measured and recorded. This was validated by the checkerboard liquid assay.

Checkerboard liquid MIC assays for *Aspergillus* spp., *Penicillium* spp. and *Candida* spp.
Various lower concentrations of both extracts and nystatin were prepared by titration to achieve MIC and ½ MICs. The test fungal isolates were also standardized. The microtitre plates were incubated

with shaking at 30°C. Optical density readings at 600 nm were obtained after 48 h of incubation. Further reading were taken for the next 5 days. Samples (5 µL) were taken from the wells with no growth and were subcultured on SDA plates to ascertain fungicidal or fungistatic effect.

Statistical Analysis: Triplicate samples were analysed to arrive at the mean from absolute values. The comparison of the antifungal activity of the medicinal extracts with standard antibiotics, and their interactions with same were analysed. Paired sample T-test was employed to analyse the zone of inhibition (mm) and the values were reported as mean zone of inhibition ± standard deviation. One way anova was used to compare the effect of methanolic extract to aqueous extract ($p < 0.01$).

RESULTS

As observed in this research, plants are important sources of natural macromolecules and life sustaining compounds.

Qualitative Phytochemical Screening of *Azadirachta indica:* It was observed that *Alkaloids* was present in methanolic extracts and absent in aqueous extracts, *Saponins* was present in abundance in the methanolic extracts and moderately present in aqueous extracts. *Flavonoid*s and *Tannin*s were moderately present in both the methanolic and the aqueous extracts. *Terpenes* was moderately present in methanolic extracts but abundantly present in the aqueous extracts. On the other hand, *Cyanogenic glycosides*, combined *Anthraquinones* and free *Anthraquinones* were completely absent in both the methanolic and aqueous inference. *Cardiac glycosides Lieberman* and *Killerkilliani* was present in both methanolic and aqueous extracts, while *Salkowski* was abundantly present in both methanolic and aqueous extracts of *Azadirachta indica* (Table 1).

Antifungal activity and drug interaction of *A. indica* against some fungal isolates

Antifungal activity of *A. indica* on *Aspergillus niger* was observed to be dose-dependent and it increased with increase in concentration from 12.5 to 200 mg/mL. In methanolic extract, the range of the mean zones of inhibition were 10.0 ± 0.2 mm – 13.0 ± 0.3 mm for *Aspergillus* sp., 6.0 ± 0.2 mm – 16.3 ± 0.5 mm for *Penicillium* sp. And 10.0 ± 0.7 mm – 13.1 ± 0.9 mm for *Candida albicans* (Table 2). The result of the mean zone of inhibition for the aqueous extract of *A. indica* as depicted in Table 3 showed a range of 15.5 ± 0.5 mm – 19.5 ± 0.1 mm for *Aspergillus* sp., 9.0 ± 0.3 mm -16.3 ± 0.4 for *Penicillium* sp. and 8.3 ± 0.1 mm -18.0 ± 1.2 mm for *Candida albicans* (Table 3). This zone on the agar diffusion was also consistent with the results of MICs. **MIC as low as** 125 µg/mL was observed against *Aspergillus niger* and *Candida albicans* by aqueous extracts, and against *Penicillium* sp. by methanolic extracts (Table 4). Aqueous extracts **had** higher potency than methanolic extracts ($p < 0.01$).

Interaction between aqueous extracts and standard antifungal agents: The combination between the aqueous extract and the standard antifungal agent at the MIC and at ½ MIC of both the extract and nystatin gave a resultant synergistic activity against *Aspergillus niger* and *Candida albicans*, while it gave indeterminate effect against *Penicillium* sp

Table 1 **Qualitative Phytochemical Constituents of *Azadirachta indica***

Test		Methanolic inference	Aqueous inference
Alkaloids		+	-
Saponins		+++	++
Flavonoids		++	+
Tannins		++	+
Terpenes		++	+++
Cyanogenic glycosides		--	-
Combined Anthraquinone		--	-
Free Anthraquinones		--	-
Cardiac glycosides			
i.	Lebermans	+	+
ii.	Salkowski	+++	+++
iii.	Kellerkilliani	+	+

Key: += Present, ++ = Moderately present, +++ = Present in Abundance, -- = Completely Absent, - = Absent

Table 2. Antifungal activity, drug interaction and activity index of the methanolic extracts of *A. indica* on the fungal isolates

Organism	200 mg/mL		100 mg/mL		50 mg/mL		25.0 mg/mL		12.5 mg/mL		Nystatin	Methanol
	Mean±SD	A.I	Mean±SD	A.I	Mean±SD	A.I	Mean±SD	A.I	Mean±SD	A.I	Mean±SD	
Aspergillus Niger	13.0± 0.3 -	1.1	11.5 ± 0.3	0.9	11.0 ± 0.0	0.9	10.0 ± 0.8	0.8	10.0 ± 0.2	0.7	14.0 ± 1.2	-
Penicillium sp	16.3 ± 0.5	1.8	14.2 ± 0.3	1.5	11.0 ± 0.9	1.2	10.1 ± 0.0	-	6.0 ± 0.2		10.2 ± 0.0	-
Candida Albicans	13.1 ± 0.9	1.4	12.3 ± 1.4	1.1	12.0 ± 0.8	1.1	11.0 ± 0.0	1.3	10.0 ± 0.7	1.3	11.1 ± 1.0	-

Key: A.I = Activity index; SD = Standard deviation

Table 3. Antifungal activity, drug interaction and activity index of the aqueous extracts of *A. indica* on the fungal isolates

Organisms	200 mg/mL		100 mg/mL		50 mg/mL		25.0 mg/mL		12.5 mg/mL		Nystatin	
	Mean ±SD	A.I	Mean±SD	A.I	Mean±SD	A.I	Mean±SD	A.I	Mean±SD	A.I	Mean±SD	Water
Aspergillus niger	19.5 ± 0.1	1.3	18.7 ± 0.6	1.3	17.7 ± 0.8	1.3	17.0± 1.0	1.2	15.5 ± 0.5	1.1	14.0 ± 1.2	-
Penicillium sp	16.3 ± 0.4	1.6	15.5 ±1.0	1.5	14.0± 0.6	1.4	13.1 ± 0.0	1.3	9.0 ± 0.3	0.8	10.2 ± 0.0	-
Candida albicans	18.0 ± 1.2	1.6	17.5 ± 0.7	1.6	13.4± 0.0	1.2	10.2 ± 0.5	0.9	8.3 ± 0.1	0.8	11.1 ± 1.0	-

Key: A.I = Activity index; SD = Standard deviation; NZ = No zone

Table 4: Minimum Inhibitory Concentration (MICs) µg/mL of both methanolic and aqueous and methanolic extracts of

Organisms	Methanol (µg/mL)						Aqueous (µg/mL)					
	500	250	125	62.5	MIC	MBC	500	250	125	62.5	MIC	MBC
A. nig er	-	-	+	+	250	ND	-	-	-	+	125	500
Penicillium sp	-	-	-	+	125	250	-	-	+	+	250	ND
Candida albicans	-	-	+	+	250	ND	-	-	-	+	125	500

- = no growth, + =growth, ND = no detected accurately even when repeated severally

DISCUSSION

As observed in this study, presence of phytochemicals must have begat antifungal activity observed. Flavonoids and tannins have been reported to confer protection against microbial infection [14]. Presence of *Terpenes* might make the aqueous extract better as it triggers defense [15]. Flavonoids has also been reported to have greater potential benefit to human health [16]. *Cardiac glycosides Lieberman* and *Killerkilliani* was present in both methanolic and aqueous extracts, while *Salkowski* was abundantly present in both methanolic and aqueous extracts of *Azadirachta indica* (Table 1). This is an agreement with the work of Oseni and Akwetey [16] and Timothy *et al*. [17] which also linked the presence of these phytochemical components to antimicrobial activity of the plant extracts. These constituents have been reported to exhibit antiprotozoal and antibacterial activities [18] and can serve as guide to the source of potent drugs [19, 20].

Aqueous extracts however, showed better antifungal potency than the methanolic extracts with activity index of 1.1 at very low extract concentration (12.5 mg/mL) against *Aspergillus* species. This was in tandem with the reports of Mahmoud *et al*. [21] and Bazie *et al*. [22]. Series of very high activity indices > 1 with respect to nystatin against the three fungal pathogens showed that the extract compared more favourably than the synthetic antibiotic [23]. This is further

exemplified by indices of 1.8 the methanolic extracts at concentration of 200 mg/mL exhibited against *Penicillium* species. Clinically, this plasma concentration of 200 mg/mL may not be achievable but the concentration of the drug's active ingredient in the extract can be achieved. In practice, this active ingredient are obtained by advance purification before clinical trials [23]. Though that level of purification is beyond the scope of this research, the results obtained so far is promising toward addressing the scourge of these fungal pathogens. These fungal pathogens are established human pathogens. *Aspergillus* spp. for instance cause aspergillosis, just as *Candida albicans* is aetiology of some dermatophytosis, candidiasis and a number of emerging diseases. All concentrations of the aqueous extract effectively suppressed the mycelial growth of these fungi and this effect was found to increase with concentration where a maximum. The combination between the aqueous extract and the standard antifungal agent at the MIC and at ½ MIC of both the extract and nystatin gave a resultant synergistic activity against *Aspergillus niger* and *Candida albicans*, while it gave indeterminate effect against *Penicillium* sp. This might be due to unpredictable nature of *Penicillium* sp. due to production of several metabolites that enhance their survival in multiple antibiotic contaminated riverbeds

The World Health Organization, WHO [24] continued to raise concern about the resistance of pathogens to antibiotics. Even though appreciable potency by aqueous extracts of *A. indica* against fungal pathogens, synergy with antibiotics would be more effective therapeutic approach for multidrug resistant fungal infections [25, 26]. This **would** be a way forward for treating infection**s** involving *Aspergillus niger* and *Candida albicans*, though not applicable to *Penicillium* sp as observed in this study. This is in line with approved standards on the interpretation of synergy and indifference [27]. Further research on this is solicited on fractions and their application on laboratory animals (*in vivo).*

Conclusions

A. indica showed antifungal activity and good interaction with nystatin. The aqueous extracts are more promising source of potential antifungal drugs, not only because of its rich phytochemical constituents, but because of its high activity indices compare to the standard antibiotic, nystatin.

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Author Contributions

All authors have equal and complimentary contributions.

Conflicts of Interest

The authors declare no conflict of interest

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