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In Vitro Antifungal Activity of Moringa Oleifera Lam Leaf on Some Selected Clinical Fungal Strains

KEYWORDS: Antifungal, clinical, extracts, strains, susceptibility.

ISITUA, C. C.	IBEH, I. N	OLAYINKA, J. N.
Department of Biological Sciences, College of Sciences, Afe Babalola University, Ado Ekiti, P.M.B. 5454, Ekiti State, Nigeria.	Department of Medical Laboratory Sciences, School of Basic Medical Sciences, College of Medicine, University of Benin, P.M.B. 1154, Benin City, Edo State, Nigeria.	Department of Pharmacology and Therapeutics, College of Medicine and Health Sciences, Afe Babalola University, Ado Ekiti, P.M.B. 5454, Ekiti State, Nigeria.

ABSTRACT *Human beings all over the world face health problems from cradle to the grave. Some of these health problems such as microbial infections account for high percentage of morbidity among populations in different parts of the world and so far no known cures have been documented for some of these ailments. Literatures suggest that some of the drugs currently used to manage these ailments tend to precipitate other health complications thus creating the need for newer drugs and therapeutic agents. The antifungal properties of Moringa oleifera leaf was investigated in this study. The susceptibility of some clinical fungal strains to M. oleifera leaf was determined using agar diffusion and microbroth dilution methods with treatments arranged in a completely randomized design and replicated four times. The minimum inhibitory concentration (MIC) of the leaf extract against the fungi were determined. The susceptibility test carried out on Candida albicans, Aspergillus niger, Aspergillus flavus, Aspergillus fumigatus and Cryptococcus neoformans showed susceptibility of these isolates to different concentrations of the plant extract. The ethanol extract showed a higher potency. Among the fungal isolates tested, A. flavus showed the highest susceptibility while A. niger showed the least susceptibility and the MIC for aqueous extract was 128µg/ml and 32µg/ml for the ethanol extract. The extracts showed comparable zones of inhibition to the conventional antibiotic (Nystatin) used. M. oleifera leaf extracts showed an appreciable inhibitory activity against the clinical fungal isolates tested which implies its effectiveness in infection therapy particularly those caused by the organisms under study.*

INTRODUCTION

The pressure to find remedies for old, emerging and re-emerging disease entities has propelled man to great reliance on the rich flora and fauna of the world as sources of raw materials. It is well documented that the Cinchona trees provided good antidotes to malaria that threatened to halt the spread of Western influence to certain parts of the world in the pre-millenium era (Ibeh and Onwuchekwa, 2010), while the fungus *Penicillium,* provided the antibiotic-penicillin that was successfully used to prosecute the Second World War (Ibeh, 2009).

Today, public health problems include hypertension (Ebeigbe, 2002), diabetes (Moussa, 2007) and a plethora of other diseases caused by microbial infections (Ibeh and Onwuchekwa, 2010). Infections have increased to a great extent and resistance against antibiotics becomes an ever increasing therapeutic problem (Austin *et al.*, 1999). Many research groups are now engaged in medicinal plants research because natural products of higher plants may give a new source of antimicrobial agents (Samy *et al.*, 1998). Any plant, which either in part or whole is able to provide ingredients employed in healthcare delivery, is described as a medicinal plant (Faleyimu and Osiyemi, 2009). In the absence of modern medicinal remedies people relied on herbal remedies derived from herbs and spices. There are many medicinal herbs and spices, which find place in day-to-day uses, many of these are used as herbal remedies.

Several plant parts either used singly or in combination with others have been utilized effectively for the treatment of virulent ailments and the knowledge of the medicinal properties of plants is passed down usually orally from one generation to the other. Sometimes treatment of particular ailment or disease such as epilepsy and mental disorder is the exclusive right of a particular family in parts of Africa. Such family secrets are usually jealously guarded from outsiders (Jimoh, 2005).

World Health Organization defines traditional medicine as the sum total of the knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in prevention, diagnosis, improvement or treatment of physical and mental illness (WHO, 2008). Interest is on the increase by the researcher and users in using plants medically both for traditional uses and as potential new sources of drugs and treatment of human beings and animals.

About seventy-five percent (75%) of the world's population rely on plants or plant extracts as sources of medicines (Miller, 1993). Many plant - synthesized substances are useful for the maintenance of health in humans and other animals. Farmers and local inhabitants possess considerable indigenous knowledge arising from their long utilization of forest products (non-timber), on how plants are used, their distribution, classification and identification in the ecosystem (Eyzagaure, 1995; Ameeruddy, 1994). More than 60 million consumers in the United States take herbal remedies. More doctors are recommending herbal medicines and, some health insurance plans offer coverage for alternative health treatments such as herbal remedies (Idu, 2009).

Green leafy vegetables are food to man and feed to animals, they are also medicine to both man and animals. Food and nutrient play significant roles in the health of a biologic entity. One of such roles is in the maintenance of high level of immunity. Studies have shown that diseases

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and infection always come after lowering of host immunity (Ibeh, 1998; Ibeh, 2005; Isitua and Ibeh, 2012). Therefore, if host immunity could be maintained at peak level by healthy foods and nutrients, it will be possible to reduce or prevent diseases and infections. An instrument that could achieve this could become a useful intervention tool in the cycle of infection and human diseases.

This study was carried out to evaluate *Moringa oleifera* Lam. plant as one of such instruments that can function as an intervention tool in the myriad of health problems that beset mankind. *M. oleifera* locally known in Nigeria as 'ewe-ile' or 'ewe-igbale' (Yoruba), 'zogalla-gandi' (Hausa), 'gawara' (Fulani), 'ikwe oyibo' (Ibo), 'isie'; 'ekie' or 'asie' (Bini) and 'drumstick' tree or 'horse-radish' tree (English) belongs to the family *Moringaceae* and is widely distributed in the Indo-Bangla subcontinent and cultivated throughout the tropical belt (Fahey, 2005). Different parts of this plant are used in the indigenous systems of medicine for the treatment of a variety of human ailments and are also eaten as vegetable (Farjana *et al.*, 2003). The present study therefore aims at determining the *in vitro* antifungal properties of *M. oleifera* leaf with a view to ascertaining its value as a tool to combat diseases caused by fungal infections and establish the basis for its antifungal properties.

MATERIALS AND METHODS

Source and preparation of plant material

The plant materials used for this study are the matured leaves of *Moringa oleifera* Lam. obtained from an orchard within the premises of Abia State University, Uturu, Nigeria. The leaves were removed from the branches, washed properly with sterile distilled water and then air-dried. Upon drying, the leaves were macerated in a sterile grinder into fine powdery form, it was sieved and stored in dry airtight glass jar for extraction and antifungal analyses.

Extraction of plant material

The powdered *M. oleifera* leaf was subjected to aqueous and ethanol extraction using the protocol of Fatope and Hamisu [19]. Briefly, eight (8) batches of fifty grams (50 g) each of the powdered plant materials were weighed and percolated with 500 ml and the ninth batch of twenty grams was weighed and percolated with 200 ml of 95 % ethanol in separate erlenmeyer flasks and allowed to stand for two weeks with intermittent shaking. These were filtered and concentrated using rotavapour machine at 4-10 C. They were combined, air-dried and labelled **EE** (ethanol extract). Fifty grams (50 g) of the plant material was macerated in water, heated to 50 C for eight hours and the extract recovered by percolation. The extract was concentrated *in vacuo* at 40 C to yield the aqueous extract **AE.** All extracts were kept refrigerated before use.

Test organism

The organisms used were clinical fungal isolates of *Candida albicans, Aspergillus niger, Aspergillus flavus, Aspergillus fumigatus* and *Cryptococcus neoformans* obtained from the medical microbiology laboratory of University of Benin Teaching Hospital, Benin City, Nigeria. These isolates were maintained on potato dextrose agar slants in the refrigerator prior to use.

Standardization of inoculum

A loopful of the test isolates were picked using a sterile wire loop and emulsified in 3-4 ml of sterile physiological saline followed by proper shaking. The turbidity of the suspension was matched with that of 0.5 McFarland standards for sensitivity test as described by NCCLS [20]. The McFarland standard was prepared by mixing 0.6 ml of 1 % (w/v) dihydrate barium chloride solution with 99.4 ml of 1 % (v/v) sulphuric acid solution.

Antifungal assay

In vitro antimicrobial screening is generally performed by disc diffusion method [21] for primary selection of the compounds as therapeutic agent. Disc diffusion method is highly effective for rapidly growing microorganisms and the activities of the test drugs are expressed by measuring the diameter of the zone of inhibition.

Sensitivity discs of 6mm diameter were punched out from Whatmann No.1 filter paper, sterilized in Bijou bottles by autoclaving at 121 C for 15 minutes. Sensitivity discs were prepared by weighing the appropriate amount of the extract and serial doubling dilution in Dimethyl-sulfoxide (DMSO) followed by placing the improvised paper discs in the solution such that each disc took up 0.01 ml to make the required disc potency. Disc potencies of 50 µg/disc and 100 µg/disc were prepared for antifungal activity [22].

Standardized inocula of each isolates were swabbed onto the surface of yeast extract agar in separate petri dishes. This was followed by placing the prepared discs of the extracts and standard antibiotic discs (Nystatin 50 µg/disc) which served as control onto the surface of the inoculated media at intervals. The plates were inverted and allowed to stand for 30 minutes for the extract to diffuse into the agar after which the plates were incubated at room temperature (28 ± 2 C) for 72 hours. This was followed by measurement of zones of inhibition formed by the test organisms around each of the extract and standard antibiotic discs [23].

Micro-broth dilution test for minimum inhibitory concentration (MIC)

Minimum inhibitory concentration of the plant extracts were prepared by serial doubling dilutions using sterile distilled water to obtain concentrations of 16 µg/ml, 32 µg/ml, 64 µg/ml and 128 µg/ml. Equal volume of extracts and yeast extract broth (i.e. 2 ml each) were dispensed into sterilized test tubes. Specifically, 0.1 ml of standardized inocula (5.0 x10^4 CFU/ml fungi) was added to each of the test tubes above and the tubes incubated at 28 ± 2 C for 72 hours [26]. Tubes containing broth without plant extracts were inoculated and incubated alongside to serve as positive control. Uninoculated tubes containing broth and plant extracts were incubated alongside to serve as negative control. The tubes were observed after incubation to determine the minimum inhibitory concentration (MIC) as the lowest concentration that showed no evidence of growth (turbidity) or the least concentration without turbidity [23].

Determination of minimum fungicidal concentration (MFC)

Yeast extract agar plates were separately inoculated with sample (1 ml) from each of the test tubes that showed no turbidity and the plates incubated at 37 C until growth was evident (for about 24-48 hours). The minimum fungicidal concentration is the lowest concentration showing no growth or fewer than 3 colonies per plate to obtain approximately 99-99.5 % killing activity [24].

RESULTS AND DISCUSSION

The antifungal activity of aqueous and ethanol extracts of M. oleifera Lam. leaf was investigated using the agar

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disc diffusion method against some selected clinical fungal pathogens Candida albicans, Aspergillus niger, Aspergillus flavus, Aspergillus fumigatus and Cryptococcus neoformans.

The susceptibility of the fungal isolates tested in this study is shown in Table 1. All the fungal isolates showed susceptibility to the ethanol extract at 100 µg concentration while 03 (60%) were susceptible at the lowest concentration (50 µg) of the ethanol extract used. Two (02) (40%) fungal isolates were susceptible at the 50 µg concentration and 04 (80%) fungal isolates were susceptible at the 100 µg concentration of the aqueous extract of *M. oleifera* leaf.

The minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) values of the ethanol and aqueous extracts of *M. oliefera* leaf are shown in Table 2 and 3. *Candida albicans* and *Aspergillus flavus* had MIC of 32 µg / ml, while *Cryptococcus neoformans* had MIC of 64 µg / ml for ethanol extract. *C. neoformans* had MIC of 128 µg / ml for the aqueous extract.

The antibiogram of microorganisms exposed to extracts of *M. oleifera* showed susceptibility of all the fungal strains to both the ethanol and water extracts of the leaf. *Aspergillus flavus* showed highest susceptibility by giving the widest zone of inhibition, while *Aspergillus niger* gave the least susceptibility (Table 1). This finding suggests that the leaf contains biologically active principles which qualify it for medicinal use. The results obtained in this study thus provide a rationale for the use of this leaf in traditional medicine by Asian and African native folks in the treatment of a variety of ailments ranging from all forms of microbial infections to diabetes and malnutrition in West Africa and high blood pressure in India [25]. Thus, if the organisms implicated in these conditions are susceptible to the antimicrobial agents in *M. oleifera*, then its application is justified.

The susceptibility of the fungal strains to the extract *M. oleifera* may be a pointer to its potential as a drug that could be used against these susceptible fungal strains. Also, antimicrobial resistance is an important issue that has created problems in the treatment of infectious diseases and necessitates the search for alternative drugs or natural antimicrobial remedies [26]. The difference in fungal response was possible due to the nature of the fungal strains. It is noted that the ethanol extract of *M. oleifera* leaf exhibited a broad spectrum of antimicrobial effect against all clinical fungal strains studied.

The medium of extraction of *M. oleifera* leaf seem to impact on the potency of the active principles since the ethanol extract produced wider zones of inhibition and higher minimal inhibitory concentrations (Tables 1, 2 and 3). The possible reasons for the differences in the potency of leaf extracts include higher extractability of the active principle in ethanol than in water, which in turn may affect its concentration per unit volume of extract. The other factor is the synergistic effect between ethanol and the active principles of the leaf. Whatever may be the case, this result suggests that ethanol extraction produces better antibacterial effect of the *M. oleifera* extract [27]. Compounds like tannins and polyphenol which are found in *M. oleifera* are soluble in ethanol [28] and have been reported to possess antimicrobial activity [26].

Extracts of *M. oleifera* showed appreciable activity against the fungal isolates when compared with Nystatin (Table 1). This result suggests the need for further studies on *M. oleifera*, although the spectrum of activity of the leaf extracts were narrower than those of the antibiotic Nystatin the use of the plant extract show great potential in terms of cost and health implications associated with the synthetic antifungal drugs. The effectiveness may be improved by testing different extractions methods to increase the concentration of the active biochemical components.

The extract showed strongest antifungal activity against *A. flavus* an organism causing aspergillosis in humans. Based on the leaf extracts activity against the clinical fungal isolates used in this study, *M. oleifera* leaf could be employed in the treatment of aspergillosis, cryptococcosis and candidiasis which usually occur in people whose immune system is weakened, commonly from chemotherapy. And being that this plant is rich in essential nutrients and phytochemicals, its positive impact on hematological parameters based on previous research findings [29] [30] it could therefore double as an immune booster or immunomodulator in the treatment of the aforementioned diseases caused by the clinical fungal strains.

It is vitally important to know about the cell lysis mechanisms of *M. oleifera* extracts on fungal cells so that further development of disease treatment can be conducted accordingly. A study of the morphological change of the cell induced by these extracts would therefore be the preliminary in understanding the lysis mechanism [31]. The result of this study is in agreement with the findings of Chuang *et al.* [32] who worked on the antifungal activity of crude extracts and essential oil of *M. oleifera* Lam. in Taiwan; Patel *et al.* [33] who evaluated the phytochemical and antifungal activity of *M. oleifera* leaf in India and Aisha *et al.* [34] who reported on the phytochemical screening and antifungal activity of *M. oleifera* on some selected fungi in Dutse, Jigawa State, Nigeria.

The activity of the aqueous extract against microbes investigated in this study is at variance with previous works which showed that aqueous extracts of plants generally exhibited little or no antimicrobial activities [35] [36]. The difference could be attributed to variation in the environment where the plant was collected, the season and the physiological stage of the plant when leaves were harvested [37]. This affects the chemical composition and the amount of compounds in the plant. In general, water extracts are the commonly used and are affordable to resource-limited farmers. The curative advantage is that consumers including animals tend to consume the plant material in large quantities and in high concentrations. This suggests its ability to meet the required physiological levels to inhibit the pathogen growth *in situ*.

In this study, the claim is established that *M. oleifera* ethanol and aqueous extracts had fungistatic and fungicidal properties against all the clinical fungal isolates which are mostly known to be multi-drug resistant [38]. The ability of the ethanol extract to inhibit and kill these organisms is noteworthy even though it was at the highest concentration (100 g) tested. This plant therefore, may well enrich the pool of herbs and shrubs from which modern pharmaceutical industries may rely on for raw materials.

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Table 1: *In vitro* **antifungal activity of ethanol and aqueous extract of** *M. oleifera* **leaf using disc diffusion method**

Fungal strains	Ethanol extract (50µg) mm	Ethanol extract (100µg) mm	Aqueous extract (50µg) mm	Aqueous extract (100µg) mm	Nystatin (50µg) mm
Candida albicans	12	14	00	10	20
Aspergillus niger	00	12	00	00	22
Aspergillus flavus	12	18	11	16	21
Aspergillus fumigatus	11	13	00	08	22
Cryptococcus neoformans	00	15	10	11	25

Mean zone of inhibition (diameter in mm)

Table 2: Minimum inhibitory concentrations (MIC) of *M. oleifera* **extracts based on turbidity**

	Ethanol Extract					Aqueous Extract				
	CA	AN	AF	AFM	CN	CA	AN	AF	AFM	CN
CONC. (µg ml^{-1})										
16	+	++	+	++	++	++	++	+	++	++
32	-	++	-	++	+	++	++	+	++	+
64	-	++	-	+	-	+	++	+	++	+
128	-	+	-	+	-	+	++	+	+	-

Key: CA = *Candida albicans*, AN = *Aspergillus niger*, AF = *Aspergillus flavus*, AFM = *Aspergillus fumigatus*, CN = *Cryptococcus neoformans*, CONC. = Concentration, ++ = More turbid, + = Less turbid, - = No growth.

Table 3: MIC and MFC values of the ethanol and aqueous extracts of *M. oleifera* **leaf against test organisms using microbroth dilution technique**

Extract	*Candida albicans* MIC	*Candida albicans* MFC	*Aspergillus flavus* MIC	*Aspergillus flavus* MFC	*Cryptococcus neoformans* MIC	*Cryptococcus neoformans* MFC
Ethanol extract (µg ml^{-1})	32	**	32	**	64	**
Aqueous extract (µg ml^{-1})	**	**	**	**	128	**

Key: MIC = Minimum inhibitory concentration, MBC = Minimum bactericidal concentration, ** = MIC or MBC value above 128 µg ml^{-1}

CONCLUSION

The results of the antifungal activity of *M. oleifera* leaf of this investigation implicated that the leaf could potentially serve to treat fungal diseases and infection that have become more resistant to most of the common antibiotics used for treatment. The growing incidence of resistance of microorganisms to conventional antimicrobial agents are a source of concern globally. Although the conventional antibiotic (Nystatin) used in this study showed more effect against the pathogens in terms of inhibition zones; the plant extracts, especially the ethanol extract showed comparable zones of inhibition too. These observations confirm the use of the plant in traditional medicine in Africa and Asia for the treatment of infections and diseases caused by microbial pathogens. Therefore, it would be of immense advantage if the active principle in the leaves of *M. oleifera* which is responsible for its antimicrobial activities could be isolated, identified and characterized for proper utilization to better the lots of mankind.

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