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RESEARCH ARTICLE

In Vitro Activity of Leaf Extracts of *Eupatorium Odoratum* against Dematiaceous Fungi Isolated From Streams in Awka, Anambra State, Nigeria

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ABSTRACT

The antifungal activity of crude extracts of *Eupatorium odoratum leaf was* investigated using agar-well diffusion method. The plant was selected based on its ethno medical uses. The minimum inhibitory and minimum fungicidal concentrations of the leaf extracts were determined using two fold serial dilution method at concentration of 400mg/ml to 50mg/ml. The activity index was determined using the ratio of the tested extracts and the standard antifungal agent (Ketconazole). The result showed variable pattern of susceptibility, the extracts were active against most of the tested organisms. The activities of the ethanol extracts were higher than that of the aqueous extracts. The ethanol extracts of *Eupatorium odoratum* had the highest inhibition zone diameter on *Xylohypha bantiana* ((23±0.81) and *Alternaria alternata* (23±0.81) respectively. *Wangiella dermatitidis* was generally less susceptible to the plant extract than other isolates. The inhibiting effects differed significantly among the extracts and the control, with the exception of susceptibility of *Alternaria alternata* and *Xylohypha bantiana* to ethanol extract of *Eupatorium odoratum leaf.*

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INTRODUCTION

During the last two decades, the development of drugs as well as the appearance of undesirable side effect of certain antibiotics has lead to the search of new antibiotics of new antimicrobial agents mainly among plant extracts with the goal to discover new chemical structure, which overcome the above disadvantages (Okemo *et al.,* 2003; Bouamama *et al*., 2006). *Eupatorium odoratum* is a shrub of the sunflower family (Asteraceae) that is native to Central and South America and found distributed all over tropical Asia, Western Africa and in parts of Australia, including subtropical areas of the world (Akinmoladun *et al*., 2007). The plant is a perennial shrub that forms dense tangled brushes 1.5-2.0 m in height. It occasionally reaches to its maximum height of 6cm (as a climber on the plants) and its stem branch freely with lateral branches developing in pairs from the axillary buds. The older stems are brown and woody near the base; tips and young shoots are green and succulent. The root system is fibrous and does not penetrate beyond 20-30cm in most soils (Akinmoladun *et al*., 2007). It is an aggressive competitor that occupies different types of soil where it forms dense mass that prevent the establishment of other flora. It causes menace in plantations and other ecosystems (Akinmoladun *et al.,* 2007).

Traditionally, the plant has been associated/ used for wound healing and diuretic activity. A decoction of the leaf is used as cough remedy and as ingredient with common grass and guava leaves for the treatment of malaria. The juice pressed out of the crushed leaves is applied to cuts to stop bleeding (Phan *et al*., 2001). Dematiaceous fungi include large group of organisms that are darkly pigmented. In most cases, the pigment is melanin and specifically dihydroxynaphthalene melanin (Dixon and Polak-wyss, 1991). The term "dematiaceuos" refers to the characteristic dark appearance of this group of fungi as it grows on agar. Colonies are dark grey, brown, or black and importantly, have a black reverse when the bottom of the agar plate is examined. This distinguishes the dematiaceous fungi from fungi with black conidia but an otherwise pale mycelium, such as *Apergillus niger* (William, 2003). They are widely distributed in the environment, and occasionally cause infections in humans, animals and plants (Silveria and Nucci, 2002). These fungi can be isolated from soil, air,

rotten wood, plants and plants roots, birds net, straw, water, cereal, decaying food etc. in countries with tropical and subtropical climate (Jiang *et al.,* 2001).

The present study is therefore carried out to investigate the pharmaceutical components of *Eupatorium odoratum* and antifungal activity of the aqueous and ethanol leaf extracts against dematiaceous fungi that are agents of subcutaneous mycoses.

MATERIALS AND METHODS

Collection of Plant Materials

The fresh leaves of *Eupatorium odoratum,* were collected from Awka, Anambra State.The selection was based on the ethno medical uses in folk medicine.

Preparation of Leaves Extracts

The leaves were washed with distilled water, dried under shade at room temperature for 14days and pulverized using electronic grinder. A 40g portion of the leaves powder was each extracted by maceration in 400ml each of ethanol and water respectively for 72 hours. The extracts were filtered, evaporated to dryness at 30^{o}C in a steady air current (Nwobu *et al*., 2010, Grillo and Lawal, 2010).

Determination of Extractive Value

The concentrations of the extracts were determined by evaporating 0.1ml of each extract in an evaporating dish of known weight in an oven to dryness. The dish containing the residue was allowed to cool and weighed. The weight of the residue was obtained by subtracting the weight of the empty dish from the weight of the dish and residue. The above process was repeated in triplicate in each case (Vedpyiya *et al*., 2010).

Preliminary Phytochemical Screening of Extracts

This was done according to the method described by Poornima (2011).

Preparation of Test Samples

Test samples of plant extract were prepared in water and ethanol (400mg/ml). This was done by dissolving four gram (4g) of extracts in 10ml of each of the extracting solvents. Serial two-fold dilutions were made (Grillo and Lawal, 2010).

Inoculum Preparation

The fungal isolates used for the screening were *Alternaria alternata*, *Xylohypha bantiana, Curvularia lunata*, *Wangiella dermatitidis*, *Drechslera bisepate, Phialophora verrucosa, Exophiala jeanselmei, Exophiala werneckii* and *Cladosporium carrionii*. The organisms were isolated from streams in Awka and its environs. They were stored in deionized water prior to use. A loopful of colonies of each isolate was inoculated into 4ml peptone water and incubated at 28^{0}C for 5days. Peptone water suspension of the actively growing fungi was made and the turbidity was adjusted to match the turbidity standard of 0.5 McFarland units. This was prepared by mixing 0.05ml of 1% ($^{w}/_{v}$) barium chloride dehydrates with 9.95ml 1% ($^{v}/_{v}$) tetraoxosulphate (vi) acid. The turbidity was equivalent to approximately 1-2x10^{8} colony forming units per millilitre (CFU/ml) (Vedpriya *et al*., 2010).

Antifungal Biossay

Cup-plate agar diffusion using Sabouraud dextrose agar was employed. From the stock of 400mg/ml extract, serial 2-fold dilutions were made to 200, 100, and 50mg/ml (Grillo and Lawal, 2010). Each labeled SDA plate was uniformly incubated with a test organism by using pour plate. A sterile cork borer of 5mm diameter was used to make wells on the medium. About O.1ml of various extract concentration were dropped into each, appropriate labeled well (Atata, 2003; Shahidi, 2004). Ethanol used for the extraction was tested neat for each organism. Ketoconazole 0.05 % was used as positive control. The plates were incubated for 48 to 72 h at room temperature. Antifungal activity was determined by measuring the diameter of zone of inhibition produced after 72 h incubation (NCCLS, 2000).

Determination of Minimum inhibitory Concentrations (MIC)

To measure the MIC values, various concentrations of the stock, 400, 200, 100 and 50mg/ml were made. Each dilution was inoculated with 0.02 ml of the culture of each of the fungal isolates in Sabouraud dextrose broth diluted to 0.5 McFarland standards. Sabouraud dextrose broth inoculated with the isolates without plant extract and SDB with plant extract without microorganisms were set up as controls. The tubes were incubated at 25^{0}C for 72 h. The lowest concentration showing no visible growth was recorded as the minimum inhibitory concentration (MIC) for each organism (Adeniyi *et al*., 2000).

Determination of Minimum Fungicidal Concentration (MIC)

From each negative tube in MIC assay, one millilitre was transferred onto the surface of freshly prepared SDA plates (without antibiotics or extracts) and the plates were incubated at 28^{0}C for 72 hrs. The lowest concentration showing no visible growth on SDA was recorded as minimum fungicidal concentration (MFC) for each organism (Adeniyi *et al*., 2000).

Determination of Activity Index (A.I)

The activity index (A.I) of crude leaves extracts was calculated as described by Vedpriya *et al*., (2010).

Activity index (A.I) = Mean of zone of inhibition of extract / Zone of Inhibition obtained for Standard antibiotic drug

Statistical Analysis

All measurements were replicated three times. The results of the antifungal activity of replicates were expressed as mean ± standard deviation (SD) and student t-test at $p \leq 0.05$ (95% confidence level) was applied to access the different between the means (Chao-Hsun *et al*., 2010).

RESULTS

The preliminary phytochemical analysis of *Eupatorium odoratum* showed the presence of alkaloids, saponins, tannins, flavonoids, phenolics, resins, glycosides and steroids (Table 1).

Table 1: Preliminary phytochemical analysis of leaves extract of *Eupatorium odoratum*

Phytochemical tested	Test used	Leave's Aqueous	extract Ethanol
Alkaloids	Mayer's test	+ +	++
Saponin	Foam test	+++	++
Tannins	Alkaline reagent	++	+++
Flavonoids	Shimoda's test	+	+
Phenolics	Ferric chloride test	++	+++
Resins		+	+
Glycosides	Keller Killani's test	++	++
Steroids		+	++

Keys: +++ Appreciable amount; ++ Moderate amount; + Trace; - Completely absent

Table 2: Antifungal activity of *Eupatorium odoratum* leaf extracts (400mg/ml)

Isolates	Mean zone diameter(mm)±SD(5mmcork borer)		
	AEE	EEE	KET. (0.05%)
A .alternata[b]	20.67±0.94	23±0.81	19±0.81
X .bantiana	9±0.48	11.7±1.25	17±0.48
C . lunata	6.7±0.81	14±0.81	17±0.81
W. dermatitidis	3±0.81	8±0.81	16±0.81
D . biseptate	6±0.81	12.3±0.86	17± 0.81
P. verrucosa	3±0.81	7±0.81	15±0.81
E. werneckii	8±0.81	13±0.81	15.6±0.48
E. jeanselmei	7±0.81	13±0.81	15.3±0.47
C .carrionii	13±0.81	16±0.81	19.3±0.48

Keys: AEE-------Aqueous extract of *Eupatorium odoratum;* EEE--------Ethanol extract of *Eupatorium odoratum*
b---------- P≥0.05

Table 3: The Activity Index of the crude leaves extract

Isolates	Extracts AEE	EEE
A.alternata	1.08	1.21
X.bantiana	0.51	0.66
C.lunata	0.39	0.82
W.dermatitidis	0.18	0.50
D.biseptate	0.35	0.72
P.verrucosa	0.20	0.47
E.werneckii	*0.51*	0.83
E.jeanselmei	0.46	0.85
C.carrionii	0.67	0.83

Keys: AEE---Aqueous extract of *Eupatorium odoratum;* EEE---Ethanol extract of *Eupatorium odoratum*

Table 4: Minimum inhibitory and minimum fungicidal concentrations of *Eupatorium odoratum* extracts against the isolates

Isolates	Minimum inhibitory and minimum fungicidal concentrations (mg/ml)	
	Aqueous extract	Ethanol extract
A. alternata	100(100)	50(100)
X .bantiana	100(200)	100(100)
C. lunata	200(400)	100(200)
W. dermatiitidis	400(-)	200 (400)
D. biseptate	200(400)	100(200)
P. verrucosa	200 (400)	200(400)
E .werneckii	100(200)	100(100)
E .Jeanselmei	200(400)	100(200)
C. carrionii	100(200)	50(100)

Key: Minimum fungicidal concentration in parenthesis

All the isolates showed variable susceptibility to the plant extracts tested (Table 2). The antifungal activity was screened from the zone of inhibition. Ethanol extracts showed higher degree of inhibition than the aqueous extracts. The zones of inhibition of the extracts were compared with those of standard antifungal ketoconazole (0.05%). Ethanol extract of *Eupatorium odoratum* had the highest zone of inhibition diameter on *Alternaria alternata* (23±0.81). *Wangiella dermatitidis* was generally less susceptible to the plant extract than the other isolates. The statistical analysis of the results showed that there is a significant difference between the mean of the test and the mean of the control (P≤0.05) with the exception of susceptibility of *Alternaria alternata* to *Eupatorium odoratum* (P≥0.05). These results are shown in Table 2.

The activity indexes (I.A) of the crude leaves extract are shown in Table 3. The results showed that AEE and EEE have maximum activity against *Alternaria alternata.*

The minimum inhibitory and minimum fungicidal concentrations are shown in Table 4. Generally, the ethanol extract has greater activity against the isolates than the aqueous extract. The ethanol extract of *Eupatorium odoratum* had its lowest MIC of 50mg/ml against *Alternaria alternata* and *Cladosporium carrionii*.

DISCUSSION

The *in vitro* antifungal activity assay of leaf extract of *Eupatorium odoratum* revealed that the ethanol extract had greater activity against the fungal isolates than the aqueous extracts.It is probable that the bioactive compounds in the leaves were more extractable in ethanol than water. This is in line with what was reported by Britto (2001). Ethanol extract of *Eupatorium odoratum* appeared to be more efficacious against *Alternaria alternata*. The inhibition zone diameters obtained was higher than the inhibition zone diameter obtained with the control (ketoconazole).

More so, *Eupatorium odoratum* extracts recorded the lowest MIC value of 50mg/ml against *Alternaria alternata*. This finding compares favourably well with antifungal efficacy reports on other medicinal plants. For example, Sontaya (2007) reported that ethanol extracts of *Eupatorium odoratum* inhibited mycelial growth of *Fusarium oxysporium* and *Collectotrium capsici* by 52.9% and 64.0% respectively. Bark and leaf extracts of Indian spice plant *Cinnamomum zeylanicum* were active against *Alternaria solani* and *Curvularia lunata* at 100 µg/ml and 50 µg/ml respectively (Ajay *et al*., 2009). Paola *et al*. (2011) reported that extracts of *Rosamarinus officinalis* and *Cynara sclarea* showed maximum activity against *Alternaria* species, even at very low concentrations.

Historically pharmacological screening of compounds of natural or synthetic origin has been the source of innumerable therapeutic agents. Random screening as a tool in discovering new biological active molecules has been most productive in the area of antibiotics (Gerhartz *et al*., 1985; Kroschwitz and Howe-Grant, 1992). Even now contrary to common belief, drugs from higher plants continue to occupy an important niche in modern medicine. On a global basis, at least 130 drugs, all single chemical entities extracted from higher plants, or modified further synthetically, are currently in use, though some of them are now being made synthetically for economic reasons (New man *et al*., 2000)

Even though pharmacological industries have produced a number of new antibodies in the last three decades, the resistance to these drugs by micro organisms

has increased (Cohen, 1992). There are many approaches to search for a new biologically active principle in higherplants (Earnsworth and Louis, 1983). One of such approach is systemic screening, which may result in the discovery of novel effective compounds (Janoska *et. al.* 2003). The result obtained from this study showed strong evidence that the crude extract of *Eupatorium odoratum* exerts antifungal activity against the dematiaceous fungi tested. The findings therefore supports local claims since the dematiaceous fungi are agents of subcutaneous mycoses.

The antifungal activity of plants, therefore, tends to establish scientific bases for their application in folk medicine. It is therefore, suggested that these plants be evaluated chromatographically, so as to locate the bioactive ingredient, some of which might prove to be novel chemical compounds. In medicinal plant evaluation, inhibition zone diameter can at best serve as a screening test for antimicrobial potential.

Conclusion

The antifungal efficacy of locally used plant materials, as reported in this study, tends to provide scientific bases for their application in folk medicine. This study, paves the way for further attention and research to identify the active components responsible for the plant antifungal activity. Further studies should be taken to elucidate the exact mechanism of action by which extracts exert their antifungal effect.

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