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Phytochemical Screening and Antifungal Evaluation of Leaf Extracts of *Nephrolepis undulata* Afzel. Ex Sw. (Nephrolepidaceae) against selected Fungi

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ABSTRACT: *Nephrolepis undulata* (sword fern) is a medicinal plant used traditionally for treatment of various ailments including stomach ache, infections and cough. Therefore, the objective of this study is to undertake a phytochemical screening and antifungal activity evaluation of leaf extracts of *Nephrolepis undulata* Afzel. Ex Sw. (Nephrolepidaceae) against selected fungi *(Aspergilus fumigatus*, *Rhodotorula mucilagenosa*, *Trichophyton rubrum*, *Mucor indicus*, *Candida parapsilosis* and *Cryptococcus neoformans*) using appropriate methods. Phytochemical screening was done using standard methods of analyses while antifungal evaluation of the extracts was done using the agar diffusion method. Phytochemical screening of the extracts revealed the presence of alkaloids, anthracene derivative, carbohydrate, flavonoid and saponin in the methanol, chloroform and petroleum ether extracts. Tannins was present in the methanol extract but absent in chloroform and petroleum ether extracts. The antifungal sensitivity test revealed that the extracts had concentration dependent inhibitory activities against the clinical isolates used in the study; with the methanol extract having the best and broadest spectrum of activity. It had the lowest Minimum Inhibitory Concentration (MIC) of 0.3 mg/mL against *A. fumigatus*. However, its activity was only next to Ketoconazole, the reference drug. The extracts contained active phytochemicals which may be responsible for the varied antifungal actions of the extracts. These positive finding validates the ethnomedicinal application of the plant in the management of various ailments and further demonstrates the applicability of the plant as a possible raw material, in the pharmaceutical industry.

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Plants as sources of medicinal compounds continue to play dominant role in maintenance of human health and the interest in phytomedicine as well as plant derived bioactive compounds, has risen over the years. The increase in interest may be attributed to increasing microbial resistance to conventional antibiotics, problems of adverse side effects and the ready availability of natural herbs (Okigbo and Mmeka, 2006). *Nephrolepis undulata* is a fern native to tropical Africa and Madagascar. It is however distributed worldwide especially in Northern Australia, Southern America and Asia. It belongs to the family Nephrolepidiaceae. The tufts have finely toothed sword-shaped fronds which rise from short, erect, hairy leaf stems. It grows to 2 - 3 feet tall in its terrestrial habitat and sometimes, found as epiphytes on oil palm trees (Patil and Dongare, 2014). It has many common names including annual sword fern, helecho and ladder fern. In India, young leaves are cooked as vegetables. It is also used to treat cough and skin diseases. Decoctions of fresh fronds are used to treat fever. The rhizomes are used for rheumatism, chest congestion and anorexia. The leaves are used in the treatment of jaundice, stomach ache, wounds and

as pregnancy booster (Kalembe *et al*., 2014). In some parts of Nigeria (especially in the Niger-Delta region), there are claims that herbal preparations of *N. undulata* are effective in the treatment of menstrual disorders, infections and ulcers. Due to the limited information available in literature with regards to the effectiveness of *N. undulata* on various fungi, this study was designed.

MATERIALS AND METHODS

Materials: Materials used include Sohxlet apparatus, table-sized autoclave, mettler weighing balance and glass wares of pyrex, England. All solvents were of the Analytical grade, obtained from JHD, Guandgua Chemical Ltd., China. Microbiological media were obtained from Biotech., India and include; Chromogenic agar, Sabouraud Dextrose agar and Sabouraud Dextrose broth.

Collection and Identification of Plant: The plant was collected from Ovia North East Local Government Area of Edo State and authenticated at the Forestry Research Institute of Nigeria (FRIN), Ibadan, Oyo state; where a voucher specimen with assigned herbarium number: FHI110381, was deposited.

Preparation of the plant extracts: The leaves were collected and rinsed in distilled water. It was then air dried under sun for five days before mechanical milling into fine powder. Two hundred grams (200 g) each of the pulverized powder was extracted separately with 1.5 L each of methanol, chloroform and petroleum ether; using the Soxhlet apparatus. The extracts were then concentrated to dryness at 60^{o}C, using a thermostatically controlled water bath. The yields were weighed and percentage yields calculated to be 13.11%, 9.07% and 7.13%, respectively. The extracts were introduced into sterilized sample bottles and stored in the refrigerator at 4^{o}C for subsequent analysis.

Phytochemical screening: Qualitative screening of the phytochemical components in the plant extracts was carried out using the method outlined by Harborne (1998) to detect the presence of alkaloids, anthracene derivatives, carbohydrates, cyanogenic glycosides, flavonoids, saponins and tannins.

Sources of test organisms: The test organisms were sourced from previously isolated, characterized and authenticated stock cultures of fungi obtained from Medical Microbiology Laboratory of University of Benin Teaching Hospital (UBTH). The test fungi include *Aspergilus fumigatus, Rhodotorula mucilagenosa, Trichophyton rubrum, Mucor indicus, Candida parapsilosis* and *Cryptococcus neoformans.*

Antifungal assay of the extracts: Antifungal assay of the extracts of *N. undulata* against the test fungi was carried out by the method described by Firas *et al*. (2008). Broth cultures of 48 hours (exponentially growing) of the selected fungi *(Aspergilus fumigatus, Rhodotorula mucilagenosa, Trichophyton rubrum, Mucor indicus, Candida parapsilosis* and *Cryptococcus neoformans*) were adjusted to 0.5 McFarland turbidity standard and further diluted (1:100 using normal saline solution) to yield microbial suspension of approximately 10^{6}cfu/mL which were uniformly streaked on the agar surface using sterile swab sticks. Wells of 7 mm in diameter were made into the uniformly streaked Sabouraud dextrose agar plates. Each well was filled with 0.1 mL of the extract at varying concentrations. The same quantity of Tween-80 (10%) solution served as negative control and 0.01 mg/mL of Ketoconazole served as positive control, for the fungi. All plates were incubated at room temperature (25^{o}C) for 72 hours. The absence or presence of growth was observed on the plates and the diameter of clear zone was measured in mm and recorded. The experiments were done in triplicates and the mean zones of inhibitions calculated.

Determination of Minimum Inhibitory Concentration's (MIC's) of the extracts: The MIC's of the organisms susceptible to the inhibitory effect of the extracts recorded as Inhibition Zone Diameters (IZDs) were determined by the agar dilution method (NCCLS, 2003; Lalitha *et al*., 2004). From the extract stock concentration of 100 mg/mL, lower concentrations were prepared by incorporation into the molten sabouraud dextrose agar at different volumes to obtain a range of concentrations of between 0.1 - 16 mg/mL. Then a loop-full volume of one in hundred dilution (1:100) of 0.5MacFarland turbidity standard of microbial suspensions obtained from the 72 hours broth were spotted on the surface of the agar plates at marked segment of the various plate concentrations of the test extracts. Plates were incubated at room temperature (25 ± 2^{o}C) for 72 hours after which the lowest concentration at which there was no observable fungal growth was recorded as the MIC.

Statistical Analysis: Statistical analysis was done using SPSS software version 16 (SPSS Inc. Chicago) and results presented in tables as mean ± standard error of mean. Paired t-Test was used to compare data for level of significance.

RESULTS AND DISCUSSION

The qualitative phytochemical analysis of the extracts detected the presence of alkaloids, anthracene derivatives, carbohydrates, flavonoids, saponins and tannins (Table 1).

Table 1: Qualitative phytochemical constituents in *N. undulata* extracts

Constituents	Methanol	Chloroform	Petroleum ether
Alkaloids	+++	+++	+++
Anthracene derivatives	+++	+	++
Carbohydrates	+++	++	++
Flavonoids	++	+	+
Saponins	+++	+	+
Tannins	++	-	-

Key: - (absent), + (scantily present), ++ (moderately present), +++ (highly present)

Carbohydrates and reducing sugar were present in the extract. Several studies has detected same in the leaves of other plants especially as the seat of photosynthetic activities, though some such as the novel mono-substituted carbohydrate fatty acid (CFA) esters are known to be fortified with side chains of bioactive compounds (Okwu, 2004; Nobmann *et al*., 2009; Josephs and Dowe, 2016). Alkaloid was also confirmed to be highly present in the extracts. It has been previously investigated for many pharmacological properties including antibacterial, antifungal, antiprotozoal, cytotoxic, antidiabetic and anti-inflammatory properties (Edeoga *et al*., 2005; Oduak and Lawrence, 2008). Anthracene derivatives was also present in the extracts. They are compounds such as anthracene-9-carboxylic acid, anthracene-9-carboxaldehyde, 9-(hydroxymethyl)-anthracene and anthrone with polycyclic aromatic hydrocarbon (PAH) of formula $C_{14}H_{10}$, consisting of three fused benzene rings. They have been reported to show good activity against a variety of microorganisms including fungi (Wuthi-udomlert *et al*., 2010). The presence of flavonoids was confirmed in the extracts. Flavonoids are known to be synthesized by plants in response to microbial infection and have been found *in-vitro* to be effective against a wide array of microorganisms (Manikandan *et al*., 2006; Habbu, *et al.,* 2010). Tannins was confirmed to be present moderately in the methanol extract. They have an astringent property with many physiologic activities and a wide range of anti-infective actions (Okwu, 2004; Manikandan *et al*., 2006). Saponin has bitter taste, foaming property and serve as mild detergent that solubilises cell permeability barriers and consequent lysing of bacterial and fungal cells (Okwu, 2004). In a related study, Habamu *et al*. (2010) reported tannins, alkaloids, anthracene, flavonoids and saponins as phytochemicals with antimicrobial as well as antifungal properties. Members of the genus *Nephrolepis* has gained wide application to herbal medicine practitioners owing to their negligible toxicity and the presence of a wide array of bioactive phytochemicals (Savoia, 2012). Studies revels that other species of *Nephrolepis* such as *N. cordifolia* and *N. biserrata* are highly nutritious containing ascorbic acid, carbohydrate, protein and some mineral elements; with very low level of oxalate and cyanide (Oloyede *et al*., 2014). Oloyede *et al*. (2014) reported the presence of cardiac glycosides in *N. abrupt*, *N. brownie* and *N. davalliae.* This is in addition to flavonoids, alkaloids, tannins, saponins and phenols. The presence of saponins in these ferns contribute immensely to their antimicrobial activities. Saponins are secondary plant metabolites with potent antifungal, antibacterial, anti-inflamatory and phytoprotectant properties. This means that the primary function of phytochemicals and secondary metabolites are to protect the plants against microbial attack and to aid their survival or adaptation (Savoia, 2012). However, when these plants are consumed by herbivores or for health reasons, their supposedly phytoprotectant compounds and nutrients are transferred and utilized for similar purposes in the consumer (Okwu, 2004; Dowe *et al*., 2016). The results of inhibition zone diameters (Tables 2, 3 and 4) show a concentration dependent activity of the extracts against the test fungi. The positive control (Ketoconazole) was also active against the test fungi as shown by their IZDs, when compared with the negative control (10% Tween-80 solution) which showed no activity. The IZDs on seeded agar plates are measured as an index of destruction or inhibitory action of the extracts against the test microorganisms.

Table 2: Antifungal activities of the methanol extract at different concentrations

Organisms	Zones of Inhibition (mean ± S.E.M mm)					
	Concentrations (mg/mL)				KET	Tween-80
	10	20	30	40	0.01mg/mL	(10%)
A. fumigatus	16.5±1.5	20.0±1.0	24.3±1.7	27.8±0.2	32.5±0.5	−
C. parapsilosis	12.7±0.3	17.7±0.3	22.5±0.5	24.5±1.5	33.3±0.7	−
R. mucilagenosa	14.0±0.0	18.5±0.5	23.0±1.0	26.5±0.5	31.5±1.5	−
T. rubrum	13.2±0.8	17.2±0.8	22.0±1.0	27.5±1.5	30.8±0.3	−
M. indicus	13.8±0.2	18.3±1.7	24.0±0.0	26.8±1.2	31.5±1.5	−
C. neoformans	10.0±1.0	14.0±0.0	18.0±1.0	23.5±0.5	30.5±0.5	−

Key: S.E.M = Standard Error of Mean, − = No activity, KET = Ketoconazole

Extracts are considered active at inhibition zone diameters (IZDs) of >7 mm; a benchmark established due to the well/cork borer size used. In a similar vein, > 9 mm IZDs was established as benchmark of activity by Ndukwe *et al*. (2005) and Usman *et al*. (2005) upon the use of 9 mm cork borer. A stronger antimicrobial agent will create a larger IZD because a low concentration of the agent is enough to diffuse into the agar to inhibit the growth of the microorganism (s). In Table 2, the methanol extract was most effective against *A. fumigatus* based on the IZDs produced and least effective against *C. neoformans*. It however has a broad spectrum of activity. The IZDs ranged from 10.0±1.0 to 27.8±0.2 mm. The least IZD (10.0±1.0 mm) was recorded at 10 mg/mL against *C. neoformans* while the highest (27.8±0.2) was recorded at 40 mg/mL against *A. fumigatus*. The zones of inhibitions varied with the different fungal species and the concentration of the extract. However, even at a higher concentration of 40 mg/mL, the positive control (Ketoconazole) was observed to show higher inhibition zone diameters. This may be attributed to the crude nature of the extract as opposed to ketoconazole, which already is in its purified and compounded form..

Table 3: Antifungal activities of the chloroform extract at different concentrations

Organisms	Zones of Inhibition (mean ± S.E.M mm)					
	Concentrations (mg/mL)				KET	Tween-80
	10	20	30	40	0.01mg/mL	(10%)
A. fumigatus	11.5±1.5	13.5±0.5	15.0±0.0	18.3±1..7	31.0±1.0	−
C. parapsilosis	-	10.8±0.2	13.8±1.3	17.0±0.0	30.5±1.5	−
R. mucilagenosa	10.0±0.0	11.5±0.5	14.5±0.5	17.5±0.5	33.5±0.5	−
T. rubrum	-	10.5±1.5	12.0±1.0	15.0±1.0	31.3±0.7	−
M. indicus	10.7±0.3	12.0±1.0	14.0±0.0	18.0±0.0	32.5±1.5	−
C. neoformans	-	9.5±1.5	11.0±1.0	14.0±0.5	31.3±1.7	−

Key: *S.E.M = Standard Error of Mean,* **− =** *No activity, KET = Ketoconazole*

Table 4: Antifungal activities of the petroleum ether extract at different concentrations

Organisms	Zones of Inhibition (mean ± S.E.M mm)					
	Concentrations (mg/mL)				KET	Tween-80
	10	20	30	40	0.01mg/mL	(10%)
A. fumigatus	15.0±0.0	18.5±0.5	22.3±0.7	24.8±1.3	33.0±0.0	−
C. parapsilosis	13.5±1.5	16.7±0.3	20.8±1.3	22.0±0.0	30.5±0.5	−
R. mucilagenosa	14.7±1.3	17.5±0.5	21.0±0.0	21.5±0.5	32.5±1.5	−
T. rubrum	13.5±0.5	16.2±0.8	18.0±1.0	19.0±1.0	31.0±0.0	−
M. indicus	14.0±0.0	15.5±1.5	17.8±1.2	21.7±0.3	33.5±1.5	−
C. neoformans	10.7±1.3	14.0±1.0	17.0±0.0	19.0±0.5	31.7±1.3	−

Key: *S.E.M = Standard Error of Mean,* **− =** *No activity, KET = Ketoconazole*

This result is in agreement with those reported by Oshomo and Idu (2012) in which the ethanol extract of *Zanthozylum zanthoxylloides* showed a concentration dependent activity against clinical fungal isolates. In general, larger zones correlate with smaller minimum inhibitory concentration (MIC) of antimicrobial agents.

In Table 3, the chloroform extract was also most effective against *A. fumigatus* based on the IZDs produced and least effective against *C. neoformans*. It was however found to be less effective against the fungi, when compared with the methanol extract.

In Table 4, the petroleum ether extract was most effective against *A. fumigatus* and least effective against *C. neoformans*.

It was however found to be less effective than the methanol extract but more effective than the chloroform extract, against the fungi.

The MIC results (Tables 5, 6 and 7) in this study reflects the pattern of activity shown by the IZDs. In Table 5, the highest MIC of 6 mg/mL was against *C. neoformans* which was the least susceptible of the test fungi. The least MIC of 0.3 mg/mL was against *A. fumigatus*.

In Table 6, the highest MIC of 14 mg/mL was against *C. neoformans* which was the least susceptible of the test fungi. The least MIC of 2 mg/mL was against *A. fumigatus*.

In Table 7, the highest MIC of 6 mg/mL was against *C. neoformans* which was the least susceptible of the test fungi. The least MIC of 0.7 mg/mL was against *A. fumigatus*. The MICs are quantitative indices used to measure the effectiveness of antimicrobial agents against microorganisms and are of importance in fixing benchmark of effective dose concentrations (Bairy *et al*., 2002; Firas *et al*., 2008; Vinothkumar *et al*., 2012).

Table 5: Minimum inhibitory concentrations (MICs) of the methanol extract against the test fungi

Organisms	Concentrations (mg/mL)												
	16	**14**	**12**	**10**	**8**	**6**	**4**	**2**	**0.9**	**0.7**	**0.5**	**0.3**	**0.1**
A. fumigatus	-	-	-	-	-	-	-	-	-	-	-	-	+
C. parapsilosis	-	-	-	-	-	-	-	-	+	+	+	+	+
R. mucilagenosa	-	-	-	-	-	-	-	-	-	-	-	+	+
T. rubrum	-	-	-	-	-	-	-	-	+	+	+	+	+
M. indicus	-	-	-	-	-	-	-	-	-	-	+	+	+
C. neoformans	-	-	-	-	-	-	+	+	+	+	+	+	+

Key: + = Growth, - No growth

Table 6: Minimum inhibitory concentrations (MICs) of the chloroform extract against the test fungi

Organisms	Concentrations (mg/mL)												
	16	**14**	**12**	**10**	**8**	**6**	**4**	**2**	**0.9**	**0.7**	**0.5**	**0.3**	**0.1**
A. fumigatus	-	-	-	-	-	-	-	-	+	+	+	+	+
C. parapsilosis	-	-	-	+	+	+	+	+	+	+	+	+	+
R. mucilagenosa	-	-	-	-	-	+	+	+	+	+	+	+	+
T. rubrum	-	-	-	+	+	+	+	+	+	+	+	+	+
M. indicus	-	-	-	-	-	-	+	+	+	+	+	+	+
C. neoformans	-	-	+	+	+	+	+	+	+	+	+	+	+

Key: + = Growth, - No growth

Table 7: Minimum inhibitory concentrations (MICs) of the petroleum ether extract against the test fungi

Organisms	Concentrations (mg/mL)												
	16	**14**	**12**	**10**	**8**	**6**	**4**	**2**	**0.9**	**0.7**	**0.5**	**0.3**	**0.1**
A. fumigatus	-	-	-	-	-	-	-	-	-	-	+	+	+
C. parapsilosis	-	-	-	-	-	-	-	-	+	+	+	+	+
R. mucilagenosa	-	-	-	-	-	-	-	-	-	+	+	+	+
T. rubrum	-	-	-	-	-	-	-	-	+	+	+	+	+
M. indicus	-	-	-	-	-	-	-	-	-	+	+	+	+
C. neoformans	-	-	-	-	-	-	+	+	+	+	+	+	+

Key: + = Growth, - No growth

The lower the MIC value, the more potent is the antimicrobial agent. Conversely, the higher the MIC value, the less potent is the antimicrobial agent. *C. neoformans* was least susceptible to the inhibitory effect of the plant extracts whereas *A. fumigatus* was most susceptible. In a related study, Krishnan, (2012), reported lower MICs for *R. mucilagenosa* and *A. fumigatus* relative to other fungal isolates used for the study. The higher MIC or resistance showed by *C. neoformans* may be due to the ability of the fungus to produce spores containing multinucleated budding cells and also the cells are usually encapsulated with the ability to produce slimy secretions which is a sort of resistance mechanism encoded in the DNA of the organism (Chessebrough, 2006; Rahman *et al.*, 2011). Microbial cells have evolved various mechanism of resisting the effect of antimicrobial agents ranging from use of barriers such as capsules, membranes, spores, etc to use of efflux pump, production of neutralizing substances, frequent mutations, etc (Rahman *et al.*, 2011; Josephs and Dowe, 2016). However, the destruction of the fungal cells by the plant extracts as indicated by the IZDs and MICs could be due to the presence of bioactive plant compounds, whose effects may either be individualistic or synergistic. Thousands of antimicrobial compounds from plants have been identified and isolated. Studies have shown that some plant extracts are nearly more active than conventional antibiotics. This is because majority of viruses, bacteria and some fungi undergo genetic mutation and also use other mechanisms to confer resistance to toxic effects of drugs (Piddock, 2006; Ojala *et al* 2000). However, plant extracts combine their immunomodulatory and antimicrobial properties especially *in-vivo* to overwhelm infectious agents.

Conclusion: This study has demonstrated a concentration dependent activity of the extracts against the test fungi as opposed to the negative control (10% Tween-80 solution) which showed no activity (unlike ketoconazole which was the reference drug). The study also showed that the phytochemical constituents of the plant may contribute largely to its antifungal properties. Hence, justifying the ethnomedicinal application of the plant as well as its applicability in Pharmaceutical industries.

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