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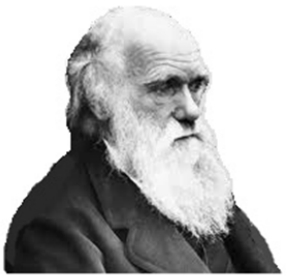

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Assessment of phytochemical constituents and antifungal activities of four selected green leafy vegetables from Owerri, South-East Nigeria

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ABSTRACT

Medicinal plants contain numerous phytochemicals like tannin, alkaloids, steroid, saponin and flavonoid. Plants are being used as valuable source of food and medicine for prevention of illness and maintenance of human health. The following medicinal plants were used: Scent leaves (*Ocimum gratissimum*), Bitter leaves (*Vemonia amygdalina*). Uziza leaves (*Piper guineense*) and Utazi leaves (*Gongronema latifolium*). Soxhlex apparatus was used to extract active ingredients from plants. Methanol and hot water solvents were used for extraction. Methanol and hot water extracts of the plants were used against identified isolated. Standard microbiological and molecular methods were used in the isolation and identification of moulds from stored rice, maize, wheat and groundnut. Sabouraud dextrose agar and potato dextrose agar were used for cultural isolation. Moulds species were identified using 18S rRNA gene sequencing method. Fungal susceptibility testing was performed to determine the minimum inhibition concentration. The following moulds *Aspergillus flavus*, *Aspergillus tamarii*, *Aspergillus niger*, *Aspergillus brunneoviolaceus* and *Penicillium chrysogenum* were isolated and identified from stored grains and legumes. The medicinal plants contain the following phytochemicals tannin, alkaloids, steroid, saponin and flavonoids. Percentage (%) growth inhibition of moulds by methanol extract was greater than hot water extract of medicinal plants. Percentage growth inhibition of moulds by methanol and hot water extract of scent leaf gave the highest inhibition followed by bitter leaf, utazi while uziza gave the least growth inhibition. Minimum inhibitory concentration of plant extracts was greatest at 100 mg/ml and least at 12.5mg/ml. Minimum growth inhibition increases with increase in concentration of medicinal plants. Medicinal plants gave varying levels of growth inhibition against varying isolates and should be used both at home and clinical settings. Therefore, the importance of medicinal plants to our society cannot be overemphasized.

***Keywords*:** Antifungal susceptibility, *Aspergillus* species, Medicinal plants, Stored grains and legumes

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1. INTRODUCTION

Medicinal plants may also be known as medicinal herbs. It has been discovered and used since prehistoric ages. These plants have are able to synthesize many chemical compounds for defense against fungal diseases and insect infestation [1]. Many phytochemical constituents in plants have been discovered. Rigorous scientific research need to be done to ascertain the efficacy and safety nature of the use of the phytochemicals and pharmacological actions, if any, harnessed from these medicinal plants.

Medicinal plants have been known and used in the practice of traditional medicine. Medicinal herbs in Nigeria are becoming popular and needs to be screened for antifungal properties of their extracts against known organisms. The medicinal value of plants lies in the bioactive phytocomponents present in the plants [2].

In Nigeria, a lot of plants have both nutritional value as well as medicinal values. Plants can be used as food and medicine for the prevention and treatment of various diseases. Plants can also be used to maintain good health. There is a global shift to produce drugs from plants and as such attention has been given to medicinal plants to ensure safety to health and better economy of a nation [3]. Medicinal plants contain different phytochemical constituents like alkaloids. Alkaloids have profound physiological effect on animals and are seen in most valuable drugs.

They also have a great physiological effect on animals [4] and have a lot of pharmaceutical activity. Tannin has astringent qualities and is very useful in medicine. Both alkaloids and tannin have anti-herbivore defense function in plants. The presence of alkaloids and tannin in medicinal plants can be a deterrent to grazers. Saponin is glycosides produced in a lot of plants. They prevent parasitic fungal invasion in plants [5]. It is also used to an extent as an emulsifying agent and as an expectorant. Flavonoids are very common phenolic constituents having 15- compound generally distributed throughout the kingdom of plants. Some flavonoids have antibacterial functions among gram-positive species which are more sensitive to isoflavanones than their gram-negative species. Phenols and phenolic compounds are known to have both antifungal and anti-microbial effects [6, 21-26].

2. MATERIALS AND METHODS

Sample collection and preparation

A total of one hundred and twenty (120) Whole grains /fine powder of rice, maize, wheat and groundnut randomly obtained from the 2 markets were stored in four different storage materials (sack, polyethene, plastic containers and metal containers) for three months at ambient temperature in a dry environment. Thirty grams (30 g) each of the stored samples were labeled and transported immediately to laboratory and kept in cool place prior to mycological analysis [7].

Isolation of fungi

Two mycological media (Sabouraud Dextrose Agar and Potato Dextrose Agar) were prepared according to standard methods. An antibacterial agent (50 mg/l, chloramphenicol) and 0.1ml of lactic acid were incorporated to inhibited the bacterial and yeasts growth respectively [8]. Standard dilution and streaking technique method was adopted. The samples were serially

diluted up to dilution factor of 10^{-3} and 10^{-5}. One-tenth milliliter (0.1 ml) of suspension was inoculated onto the freshly prepared surface dried media and incubated at 25 ± 2 °C for 7 days for mould growth. Moulds grown on media were subculture on various media for further characterization and identification [9]

Morphological and microscopic identification

The isolated moulds were identified based on colonial morphology and microscopic examination. The moulds were mounted on a clean grease slide, flooded with lactophenol-cotton blue stain to determine mould structures. Microscopically, moulds were identified on the basis of spore characteristics, pigmentation and septation [10, 11].

Molecular characterization of moulds

The DNA of mould isolates were extracted using deoxyribonucleic acid extraction kit protocol. The extracted DNA was amplified using polymerase chain reaction (PCR) amplification protocol described by [12].

Sequencing protocol

PCR products were cleaned using Exosap Protocol, sequenced using the Nimagen Brilliant dye Terminator cycle sequencing kit [12]. The sequenced data were subjected to Basic Local Alignment Search Tool Nucleotide (BLASTn) to identify corresponding organisms from National center for bioinformatics information (NCBI) as described by [13].

The following medicinal plants were used:

1. Scent leaves (*Ocimum gratissimum*)
2. Bitter leaves (*Vemonia amygdalina*)
3. Uziza leaves (*Piper guineense*)
4. Utazi leaves (*Gongronema latifolium*)

Antifungal susceptibility tests using medicinal plants were done according to [14].

Collection of leaves samples and preparation

Samples of the 4 fresh leaves were collected from Relief market in Owerri. The plants were identified at the Department of Plant Science and Biotechnology, Imo State University owerri. The leaves were washed and air dried at room temperature for 14 days. The dried leaves were blended using a blender. The powdered samples were screened through 1mm sieve. The samples were used for extraction.

Extraction

The organic solvents used were Methanol and Hot water.

Methanol Extraction

Fifty grams (50g) of samples of each leaves were used. Using a soxhlex apparatus, the active ingredients of the ground particles were extracted. For extraction, 250 ml of methanol was used. The extract was filtered using sterile filter paper. The filtrate was evaporated to

dryness using the rotary vacuum evaporator at the boiling temperature range of the solvent (79 °C). The solvent was recovered in the recovery flask while the extracts remain in the sample holder, which was collected and stored in the refrigerator at 4 °C. The extract was sterilized with UV light before use.

Hot water Extraction

Fifty grams (50g) of samples of each leaves were used. Samples were soaked in hot water for 24 hours; the active ingredients of the ground particles were filtered.

Fungal susceptibility testing

Extract-Sabouraud dextrose Agar (SDA) mixtures were prepared by mixing extract with molten SDA before the agar solidified (62 g SDA/ liter sterile water). The extract was sterilized before preparing mixtures by exposing to UV light. Discs of the fungus grown on SDA (3 mm in diameter) were cut with cork borer and placed in the Centre of Petri plates containing different concentrations of the extracts. Controls were Petri plates prepared with SDA but without the addition of extracts. Replicates of each treatment were incubated at 28 °C for 4 days. Radial growth was measured with a metric rule in extract-treated and controls. Radial growth was monitored for both extract treated and control to quantify the effect of the plant extract on fungal development. Reduction in radial fungal growth as compared with control was expressed as percentage [14].

Minimum inhibitory concentration (MIC)

Aliquot of the extract (200 mg) was dissolved in 2 ml of sterile distilled water to obtain concentration of 100 mg/ml. This 100mg/ml concentration was double diluted in sterile distilled water to obtain 50 mg/ml, 25 mg/ml, and 12.5 mg/ml. One milliliter (1 ml) of each concentration of extract was transferred to a sterile Petri plate and 10 ml of cooled Sabouraud dextrose agar were poured into the plates, swirled to mix and allowed to solidify. This was incubated at 28 °C for 4 days and observed for fungal growth. The lowest concentration of the extract that inhibited the growth of fungi was recorded as the minimum inhibitory concentration. Plates without the test extracts were used as control [14]. Determination of Photochemical constituents was done according to [15].

Phytochemical tests

Tannin

Tannin was determined according to [15]. Two grams of the samples were boiled in 20 ml of 45% ethanol for 5 minutes. The mixture was cooled and the filterate was used for analysis.

a) Ferric chloride test: One ml of filtrate was diluted with 2.0 ml of distilled water and 2 drops of ferric chloride solution was added and observed for transient greenish to black colour.
b) Lead acetate test: One milliliter (1 ml) of filtrate was added to 3 drops of 5% lead acetate solution and observed for gelatinous precipitate.
c) Bromine water test: one ml of filtrate was added to 0.5 ml of bromine water and observed for pale brown precipitate.

Alkaloid

A quantity of 0.1g of the ground samples was boiled with 5ml of 2% hydrochloric acid on a steam bath. This was filtered and 1 ml portion of the filtrate reacted with 2 drops of the following reagents.

a) Dragendroff's reagent (Bismuth potassium iodide solution) and was observed for orange precipitate.
b) Wagner's reagent (Iodine in potassium iodide solution) and shall be observed for reddish brown precipitate.
c) Meyer's reagent (Potassium mercuric iodide solution), and was observed for creamy coloured precipitate.
d) Picric acid solution (1%) and was observed for yellow precipitate.

Steroid

The test for steroid was done by the Liberman acid test. A portion of the organic extract was treated with three drops of acetic anhydride. Then concentrated H_2SO_4 was carefully added by the side of the test tube. The presence of a brown colour at the boundary of the mixture was taken as positive result.

Saponin

A quantity of 0.1g of the powdered samples were boiled with 5 ml of distilled water for 5 minutes and decanted while still hot. The filtrate was used for frothing and emulsion tests.

Frothing test

One milliliter (1 ml) of the filtrate was diluted with 4ml of distilled water and the mixture was shaken vigorously and observed on standing for suitable froth.

Emulsion test

This was performed by adding 2 drops of olive oil to the frothing solution and shaken vigorously.

Flavonoid estimation

Two gram (2g) of sample was heated with 10 ml of 5% ethyl acetate in a boiling water bath for 3 minutes. The mixture was filtered and 4.0 ml filtrate was shaken with 1 ml of 1% aluminium chloride and 1 ml of 1% dilute ammonia solution. Yellow colouration of ammonia layer gave positive result.

3. RESULTS AND DISCUSSION

Table 1 showed the cultural and microscopic characteristic of identified moulds. The Table 2, shows the Molecular sequence 18S rRNA identity of various moulds. The result of the qualitative phytochemical screening of different solvent extracts of the four selected green leafy vegetables showed the presence of phytochemicals in the different leaves (Table 3).

Table 1. Cultural and microscopic characteristic of identified moulds.

Cultural	Microscopic	Probable moulds
Green Colonies	Hyphae are septate and hyaline.	*Aspergillus* spp.
Dark brown colonies	Conidia head with long chain of conida,	*Aspergillus* spp.
Black colonies	Septated hyphae, long smooth and colourless	*Aspergillus* spp.
Brown to dark brown colonies	Hyaline or pigmented longer stipes	*Aspergillus* spp.
Blue greenish colonies	Septate hyphae branched	*Penicillium* spp.

Table 2. Molecular sequence 18S rRNA identity of various moulds.

S/N	SEQQUENCE ID	PERCENTAGE (%)	NCBI MATCH	ISOLATES
1	AY373852.1	91	*Aspergillus niger* AY373852	*Aspergillus niger*
2	NR138279.1	97	*Aspergillus brunneoviolaceus* NR138279	*Aspergillus brunneoviolaceus*
3	NR111041.1	99	*Aspergillus flavus* NR135325	*Aspergillus flavus*
4	AF004929.1	100	*Aspergillus tamarii* MN339986	*Aspergillus tamarii*
5	NR138306.1	99	*Penicillium chrysogenum* MH793845	*Penicillium chrysogenum*

The plant phytochemicals are shown in Table 3. Tables 4, 5, 6 and 7 showed the percentage growth inhibition of moulds by methanol and hot water. Percentage (%) growth inhibition of moulds by methanol and hot water extract of scent leaf gave the highest inhibition followed by bitter leaf, utazi and uziza gave the least growth inhibition. For scent leaf, *Aspergillus brunneoviolaceus* (19%) gave the highest growth inhibition at 100mg/ml, followed by *Aspergillus tamari* (15%), *Aspergillus flavus* (14%), *Aspergillus niger* (7%) then *Penicillium chrysogenum* (3%). For Bitter leaf extract, *Aspergillus tamari* (15%) gave the highest growth inhibition at 100mg/ml followed by *Aspergillus brunneoviolaceus* (12%), *Aspergillus flavus* (11%), *Aspergillus niger* (5%) then *Penicillium chrysogenum* (3%). For utazi leaf, *Aspergillus brunneoviolaceus* (10%) gave the highest growth inhibition at 100mg/ml followed by

Aspergillus tamarii (9%), *Aspergillus flavus* (7%), *Aspergillus niger* (3%) then *Penicillium chrysogenum* (1%). Uziza, leaf, *Aspergillus tamarii* (7%) gave the highest growth inhibition at 100mg/ml followed by *Aspergillus brunneoviolaceus* (5%), *Aspergillus flavus* (4%), *Aspergillus niger* (3%) then *Penicillium chrysogenum* (1%). While the control gave no growth inhibition. Tables 8 and 9 showed the Minimum inhibitory concentration of plant extracts at 50 mg/ml and 100 mg/ml

Table 3. Qualitative analysis of phytochemical constituents.
Phytochemical screening of plant extracts.

Plant extracts	Tannin	Phytochemicals Alkaloid	Steroid	Saponin	Flavonoid
Scent leaf	+	+	+	+	+
Bitter leaf	+	+	+	+	+
Utazi leaf	+	+	+	+	+
Uziza leaf	+	+	+	+	+

Key: + - Presence of Phytochemical.

Table 4. Percentage growth inhibition of moulds by methanol and hot water extract of scent leaf.

Isolates	Concentration of extracts			
	12.5 mg/ml	25 mg/ml	50 mg/ml	100 mg/ml
Control	0.00	0.00	0.00	0.00
Methanol				
Aspergillus flavus	0.00	0.00	8.00	14.00
Aspergillus tamarii	0.00	0.00	10.00	15.00
Aspergillus niger	0.00	0.00	4.00	7.00
Aspergillus brunneoviolaceus	0.00	0.00	14.00	19.00
Penicillium chrysogenum	0.00	0.00	2.00	3.00
Hot water				
Aspergillus flavus	0.00	0.00	4.00	7.00
Aspergillus tamarii	0.00	0.00	6.00	10.00

Aspergillus niger	0.00	0.00	2.00	3.00
Aspergillus brunneoviolaceus	0.00	0.00	10.00	12.00
Penicillium chrysogenum	0.00	0.00	1.00	3.00

Table 5. Percentage growth inhibition of moulds by methanol and hot water extract of bitter leaf.

Isolates	Concentration of extracts			
	12.5 mg/ml	25 mg/ml	50 mg/ml	100 mg/ml
Control	0.00	0.00	0.00	0.00
Methanol				
Aspergillus flavus	0.00	0.00	6.00	11.00
Aspergillus tamarii	0.00	0.00	8.00	15.00
Aspergillus niger	0.00	0.00	2.00	5.00
Aspergillus brunneoviolaceus	0.00	0.00	10.00	12.00
Penicillium chrysogenum	0.00	0.00	2.00	3.00
Hot water				
Aspergillus flavus	0.00	0.00	3.00	4.00
Aspergillus tamarii	0.00	0.00	4.00	7.00
Aspergillus niger	0.00	0.00	1.00	2.00
Aspergillus brunneoviolaceus	0.00	0.00	4.00	6.00
Penicillium chrysogenum	0.00	0.00	0.00	2.00

Table 6. Percentage growth inhibition of moulds by methanol and hot water extract of utazi leaf.

Isolates	Concentration of extracts			
	12.5 mg/ml	25 mg/ml	50 mg/ml	100 mg/ml
Control	0.00	0.00	0.00	0.00
Methanol				
Aspergillus flavus	0.00	0.00	4.00	7.00

Aspergillus tamarii	0.00	0.00	5.00	9.00
Aspergillus niger	0.00	0.00	0.00	3.00
Aspergillus brunneoviolaceus	0.00	0.00	5.00	10.00
Penicillium chrysogenum	0.00	0.00	0.00	1.00
Hot water				
Aspergillus flavus	0.00	0.00	0.00	2.00
Aspergillus tamarii	0.00	0.00	0.00	3.00
Aspergillus niger	0.00	0.00	0.00	0.00
Aspergillus brunneoviolaceus	0.00	0.00	0.00	3.00
Penicillium chrysogenum	0.00	0.00	0.00	0.00

Table 7. Percentage growth inhibition of moulds by methanol and hot water extract of uziza leaf.

Isolates	Concentration of extracts			
	12.5 mg/ml	25 mg/ml	50 mg/ml	100 mg/ml
Control	0.00	0.00	0.00	0.00
Methanol				
Aspergillus flavus	0.00	0.00	0.00	4.00
Aspergillus tamarii	0.00	0.00	0.00	7.00
Aspergillus niger	0.00	0.00	0.00	3.00
Aspergillus brunneoviolaceus	0.00	0.00	0.00	5.00
Penicillium chrysogenum	0.00	0.00	0.00	1.00
Hot water				
Aspergillus flavus	0.00	0.00	0.00	1.00
Aspergillus tamarii	0.00	0.00	0.00	2.00
Aspergillus niger	0.00	0.00	0.00	0.00
Aspergillus brunneoviolaceus	0.00	0.00	0.00	2.00
Penicillium chrysogenum	0.00	0.00	0.00	0.00

Table 8. Minimum Growth inhibitory concentration of plant extracts at 50 mg/ml.

Isolates	Scent leaf	Bitter leaf	Utazi leaf	Uziza leaf
Control	0.00	0.00	0.00	0.00
Methanol				
Aspergillus flavus	8.00	6.00	4.00	0.00
Aspergillus tamarii	10.00	8.00	5.00	0.00
Aspergillus niger	4.00	2.00	0.00	0.00
Aspergillus brunneoviolaceus	14.00	10.00	5.00	0.00
Penicillium chrysogenum	2.00	2.00	0.00	0.00
Hot water				
Aspergillus flavus	4.00	3.00	0.00	0.00
Aspergillus tamarii	6.00	4.00	0.00	0.00
Aspergillus niger	2.00	1.00	0.00	0.00
Aspergillus brunneoviolaceus	10.00	4.00	0.00	0.00
Penicillium chrysogenum	1.00	0.00	0.00	0.00

Table 9. Minimum Growth inhibitory concentration of plant extracts at 100 mg/ml.

Isolates	Scent leaf	Bitter leaf	Utazi leaf	Uziza leaf
Control	0.00	0.00	0.00	0.00
Methanol				
Aspergillus flavus	14.00	12.00	7.00	4.00
Aspergillus tamarii	15.00	11.00	9.00	7.00
Aspergillus niger	7.00	5.00	3.00	3.00
Aspergillus brunneoviolaceus	19.00	15.00	10.00	5.00
Penicillium chrysogenum	3.00	3.00	1.00	1.00
Hot water				

Aspergillus flavus	7.00	4.00	2.00	1.00
Aspergillus tamarii	10.00	6.00	3.00	2.00
Aspergillus niger	3.00	2.00	0.00	0.00
Aspergillus brunneoviolaceus	12.00	7.00	3.00	2.00
Penicillium chrysogenum	3.00	2.00	0.00	0.00

It was reported that the health benefits of vegetables and other plants lie on their phytochemical compositions [16]. The study of [17] observed that that scent leave (*Ocimum gratissimum*) and other local herbs contain both nutritive (fiber, potassium, phosphorus, ascorbate) and phytochemical (alkaloids, flavonoids, phenol, steroid) composition. These constitutes promotes good health and have antimicrobial activity. According to [18], Uziza leaves (*Piper guineense*) and Utazi leaves (*Gongronema latifolium*) as well as other medicinal plants have some level of antimicrobial activities because of the various phytochemicals they contain. This agreed with this research where there were some levels of susceptibility of moulds to the various medicinal plants. Furthermore, the work of [19] observed that hot water extract of (*Vemonia amygdalina*) and (*Ocimum gratissimum*) showed the presence of different phytochemicals like alkaloid, tannin, flavonoid and steroid. They both contain similar antimicrobial properties though the former contains more quantity, these accounts for their medicinal use. According to the research of [20], four local vegetables leaves which includes bitter leaf (*Vemonia amygdalina*), scent leaves (*Ocimum gratissimum*), utazi leaves (*Gongronema latifolium*) and uziza leaves (*Piper guineense*) on fungal isolates especially *Aspergillus* species showed that the lowest microbial content was found on uziza leaves (*Piper guineense*) followed by utazi leaves (*Gongronema latifolium*), then scent leaves (*Ocimum gratissimum*) and bitter leaves (*Vemonia amygdalina*), having the greatest fungal efficacy.

4. CONCLUSION

Medicinal plants contain phytochemicals and as such gave varying levels of growth inhibition against different moulds and should be used both at home and clinical environment. Therefore the use of medicinal plants cannot be overemphasized.

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