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Phytochemical composition and antifungal activity of *Allium sativum* (garlic) extracts on fungi isolated from groundnut seeds in Makurdi, Benue State, Nigeria

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Abstract

Background and Objective: Groundnut seeds are susceptible to pathogenic fungal attacks due to their high nutrition content. Synthetic chemical fungicide treatment is the common strategy for controlling the seed-borne fungi. These fungicides are not eco-friendly, and their fungi resistance is of great concern. There is a need to find new substances with efficient antifungal potential. This study aimed to determine the phytochemical composition and antifungal activity of *Allium sativum* (garlic) extract on fungi associated with groundnut seeds.

Materials and Methods: Groundnut seeds were purchased from the Wurukum, Wadata, High level, and Modern markets. The garlic used in this study was purchased from the Wurukum market in Makurdi Local Government area, Benue State, Nigeria. Three fungi of *Aspergillus niger*, *Aspergillus flavus*, and *Rhizopus stolonifer* were identified on groundnut seeds. Fungal isolation was done according to the Standard Blotter Method. Saburoud Dextrose Agar (SDA) medium was used to culture the fungal isolates. Aqueous garlic extracts were prepared using the maceration technique. The antifungal activity of the extract was tested using the pour-plating method. Phytochemical constituents were determined according to standard laboratory methods.

Results and Conclusion: The occurrence of *Aspergillus niger* ranged from 0.33-2.67, *Aspergillus flavus* 1.33-2.67, and *Rhizopus stolonifer* 0.67-1.33 across all the markets samples. The study identified *Aspergillus flavus* as the most dominant fungi on groundnut seeds. The aqueous extract of *Allium sativum* inhibited the mycelia radial growth of the tested fungi. The higher extract concentration (100% w/v) showed the higher inhibition than the lower concentration (50% w/v). This study revealed that garlic extract contains several bioactive compounds with antifungal potency (i.e., alkaloids, tannins, anthraquinones, saponins, terpenoids, cardiac glycosides, flavonoids, phenolic compounds, and steroids), which can be used to control the fungi associated with groundnut seeds.

Keywords: Antifungal, *Arachis hypogaea*, Mycelia growth, Pour plating, Standard Blotter Method

1. Introduction

Groundnut (*Arachis hypogaea* L.) is a member of the family Fabaceae. It is also called by other names such as peanut, African nut, monkey nut, Chinese nut, manila nut, goobers pea, earthnut, and ground bean. Groundnut originated from South America and was introduced to Africa by the Portu

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guese [1]. Groundnut is an essential cash crop and a significant component of the diet of many households in developing countries including Nigeria. It is consumed widely in raw, roasted, boiled, or processed forms such as cookies, flakes, and candies [2]. About 80% of the world's population consumes groundnut and groundnut products. By virtue of its high protein content, groundnuts are promoted by nutritionists and agriculturists as a suitable supplement for human and animal protein [2]. Groundnut is rich in protein (26-39%), fat (47-59%), carbohydrates (11%) as well as vitamins E, K and B complex [3]. Apart from direct consumption, it is also helpful for various industrial purposes such as extraction of oils for home and industrial uses [2]. Groundnut seeds are susceptible to pathogenic fungi attack due to their high nutrition content [1]. Fungi in the genus Aspergillus, Penicillium, and Rhizopus are reported to be associated with groundnut seeds, and cause several diseases such as seed rot, necrosis, leaf spot, rust, crown rot, damping off, and wilt of groundnut [4]. The fungi also produce toxic substances called mycotoxin [5]. Aflatoxins are the most toxic and carcinogenic compounds among the known mycotoxins, and are mainly produced by *Aspergillus flavus* and *A. parasiticus* [6,7]. Several synthetic chemicals (fungicides) such as mancozeb, thiram, carbendizim, thiabendazole, maneb fludioxonil, shavit, prothioconazole, picoxstrobin, and fluoxastrobin have been developed and extensively used for treatment of the seeds to control seed-born fungi [8]. However, using these fungicides has raised concerns about potential risks to human health, environmental contamination, and developing fungicide resistance of the pathogens. In light of these concerns, a control strategy based on antagonistic microorganisms or bio-control has emerged as a promising alternative [9]. Among the recent alternative strategies used for plant disease management, significant success has been achieved using plant-derived products. The activities of plant extracts have been shown to be environmentally friendly and effective against plant pathogens [10]. *Allium sativum* (garlic) is an herbaceous, annual, bulbous plant in the family of Amaryllidaceae. It is one of the most essential vegetables worldwide, with a total harvested area of 1.437.690 hectares, and annual production of 24.255.303 tonnes of dry bulbs [11]. The importance of garlic is due to its use for therapeutic and medicinal purposes in both traditional and modern medicine [12]. Though several researchers have worked on the antimicrobial activity of garlic extract, there is a need to more studies about the antifungal activity of this herb especially against the growth and survival of seed-borne fungi of groundnut. In this study, we aimed to evaluate the phytochemicals and also antifungal activity of garlic extract in suppression of seed-borne fungi of groundnut.

2. Materials and Methods

2.1. Sample collection

Groundnut seeds were purchased from Wurukum, Wadata, High level, and Modern markets, all within Makurdi Local Government area, Benue State, Nigeria. The Groundnut seeds were packaged in polyethylene bags and taken to Benue State University, Makurdi's botany laboratory for fungal assessment. The garlic bulbs used in this study were purchased from Wurukum market, packaged in polythene envelopes, and taken to the botany laboratory of Benue State University for further studies.

2.2. Isolation of fungi from groundnut seeds

Fungal isolation was done using the Standard Blotter Method as described by the International Seed Testing Association [13]. In practice, 10 seeds were randomly selected from each sample, and their surface was sterilized with 5% sodium hypochlorite for 3-5 min. The seeds were rinsed twice with sterile distilled water, and placed on blotter paper to dry. Then, the seeds were placed on two layers of moistened blotter paper in sterilized petri dishes. The plates were incubated with the seeds at room temperature for five days. Meanwhile, the plates were moistened to avoid drying out. The fungal growth from the points of inoculation was transferred to the petri dishes containing Sabouraud Dextrose Agar (SDA) for subsequent isolation and

identification. The occurrence of fungi was determined by counting each fungus on the plate divided by the total number of fungi grown on all plates.

2.3. Preparation of culture medium

SDA was the medium to isolate the pathogenic fungi (Lord's Mark Indutries Limited, India). It was prepared according to the manufacturer's procedure by dissolving 62 g of the medium in 1000 ml of distilled water, followed by vigorous stirring for homogenization. The flask content was heated on a heating mantle until the solution became clear. After heating, the flask was covered with foil paper and autoclaved at 121 °C for 15 min. The sterile medium was allowed to cool to a temperature at which it could be held with hands, and 2-3 drops of chloramphenicol were added to inhibit bacterial growth, as Benedict et al. described [9].

2.4. Morphological characterization and identification of fungal isolates

The identification of fungi was done macroscopically and microscopically. Colony characteristics such as appearance, change in medium color, and growth rate were observed on the plates for macroscopic identification. Microscopic identification was made by adding a small quantity of the fungus to a glass slide containing a drop of Lactophenol cotton blue, which was then covered with a slip to be observed by a light microscope with ×40 magnification. The observed characteristics of the fungi were compared with a standard pictorial chart for fungal identification by Navi et al. [14].

2.5. Preparation of aqueous garlic extracts

The garlic collected was carefully peeled and washed with sterile distilled water. Then, 100 g and 50 g of the garlic bulbs were weighed, and crushed using a clean mortar and pestle. The ground garlic was then transferred to beaker containing 100 ml of sterile distilled water. The setup was left to stand for 20-40 min, and then sieved using a muslin cloth to obtain the extract (100 and 50 %w/v) [15].

2.6. Antifungal activity

Antifungal activity of the extracts was tested using the pour-plating method. Two ml of each extract (100 and 50% w/v) were separately added to the plates, followed by the addition of 15-20 ml of molten SDA medium. The agar-extract mixture was swirled gently on the bench to ensure the homogeneity of the extract, and left further to solidify. Then, a 4-mm diameter cork borer was used to plug the mycelia from a five-day-old pure culture. A sterile needle was used to obtain the plugged mycelia from the edge. The mycelia were inoculated centrally into the plates containing an agar-extract mixture. These steps were done in three replicates. Controls were the plates containing the organism but no botanical extract. The plates were incubated at room temperature for at least five days. Data were collected for the length and breadth of the fungal growth on days 3, 5, and 7. The effect of the extract on fungal isolates was compared with that of the control. Growth measurement was done using a meter rule. Inhibition of fungal growth was calculated using the formula adopted by Liamngee et al. [16] as shown below.

Growth inhibition of fungi = $(R_1-R_2)/R_1 \times 100$

Where,

R_1 = Radial growth of the pathogen in the control plates.

R_2 = Radial growth of the pathogen in the plates containing the extract.

2.7. Phytochemical screening of the aqueous extract

The aqueous extracts of *Allium sativum* were subjected to phytochemical screening to check for the presence of the active ingredients. The phytochemical screening was carried out using the method described by Trease and Evans [17].

For alkaloid determination by Dragendoff's test, two ml of the aqueous garlic extract were mixed with 1% HCl and about six drops of Mayor's reagents. A Creamish or pale-yellow precipitate indicated the presence of alkaloids.

For tannins determination by the Ferric Chloride test, one ml of the extract was treated with a few drops of 0.1% ferric chloride and observed for

brownish green or a blue-black coloration.

For anthraquinones determination by Borntrager's test, one ml of the extract solution was hydrolyzed with diluted H_2SO_4 extracted with benzene. Then, one ml of diluted ammonia was added to the mixture. A rose-pink color showed the presence anthraquinones.

For determination of saponins by Froth test, one ml of the sample was weighed into a conical flask in which 10 ml of sterile distilled water was added and boiled for five min. The mixture was filtered, and 2.5 ml of the filtrate was added to 10 ml of sterile distilled water in a test tube. The test tube was stopped for about 30 sec. It was then allowed to stand for half an hour. Honeycomb froth indicated the presence of saponins.

For determination of terpenoids by Salkowski test, five ml of aqueous *Allium sativum* extract was mixed with two ml of chloroform, and three ml of concentrated H_2SO_4 was carefully added to form a layer. Appearance of a reddish-brown color in the interface showed the presence of terpenoids.

For cardiac glycosides determination by Keller-Killani test, five ml of aqueous extract of *Allium sativum* was treated with two ml of glacial acetic acid containing one drop of ferric chloride solution. It was underlaid with one ml of concentrated H_2SO_4. A brown ring of the interface indicated a deoxy sugar characteristic of cardenolides.

The aqueous extract of *Allium sativum* was treated with a few drops of sodium hydroxide solution for flavonoid determination using the lead acetate test. Formation of intense yellow color, which will become colorless by further addition of diluted acid, indicated the presence of flavonoids.

For determination of phenolic compounds by ferric chloride test, the *Allium sativum* extract was treated with 3-4 drops of ferric chloride solution. Formation of a bluish-black color indicated the presence of phenols.

3. Results and Discussion

Three fungi of *A. niger*, *A. flavus*, and *Rhizopus stolonifer* were isolated and identified from the groundnut seed samples collected in four markets in Makurdi metropolis. The macroscopic and microscopic characteristics of the fungi on SDA are presented in Figures 1-3. *A. niger* on SDA showed clusters of dark brown colonies, and conidiophores were smooth-walled and brownish, with clusters of dark-walled conidia. *A. flavus* had a light green to deep green colony. Conidia were spherical, and conidiophores were hyaline and heavy-walled. *R. stolonifer* on SDA had a whitish-grey cottony mycelium. The sporangiophores were branched, forming a large terminal of globose sporangia.

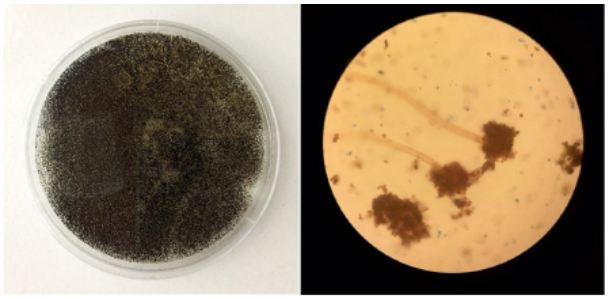

Figure 1- Macroscopic view (left), and microscopic view of *A. niger* (right)

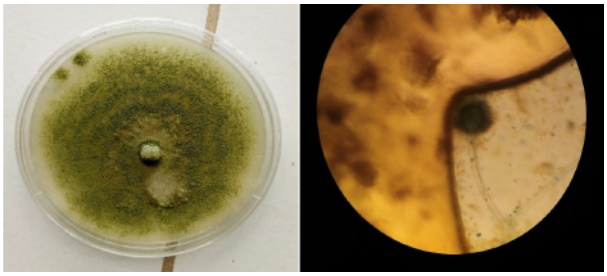

Figure 2- Macroscopic view (left), and microscopic view of *A. flavus* (right)

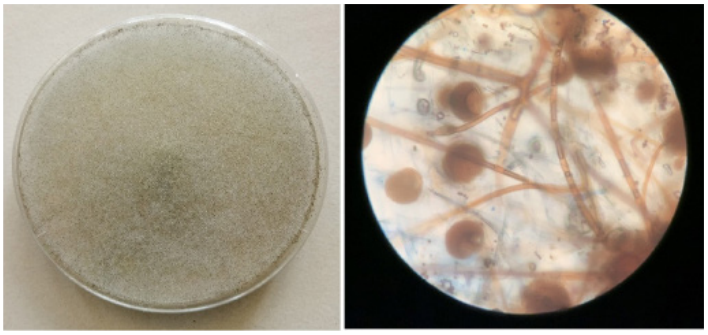

Figure 3- Macroscopic view (left), and microscopic view of *R. stolonifer* (right)

Table 1 shows the occurrence of fungi in the sampling locations. As seen, Wurukum had higher fungal occurrence than High level and Wadata markets, and the Modern market recorded the least occurrence. According to Table 2, the Wurukum market had a higher occurrence of *A. niger* than High level, Modern, and Wadata markets. For *A. flavus*, Wurukum and Wadata markets showed the highest distribution compared to the Modern and the High level markets. For *R. stolonifer*, High level and Modern markets recorded the highest distribution, followed by Wurukum and Wadata markets.

Table 1- Occurrence of fungi on groundnut seeds in different markets of Makurdi

Market	**Fungal occurrence**
High level	4.00
Modern	2.33
Wadata	2.67
Wurukum	5.00

Table 2- Occurrence of fungal species on groundnut seeds in markets of Makurdi

Market	***Aspergillus niger***	***Aspergillus flavus***	***Rhizopus stolonifer***
High level	1.00	1.33	1.33
Modern Market	1.00	2.00	1.33
Wadata	0.33	2.67	0.67
Wurukum	2.67	2.67	1.00

The inhibitory effect of garlic extract on fungal radial growth is presented in Table 3. The results revealed that the mycelial radial growth in the control was higher than the growth in agar-extract treatment for all the tested fungi on all days.

Table 3- Inhibitory effect of garlic extracts on growth of the fungi isolated from groundnut seeds in Makurdi

		Day of incubation		
		3	5	7
Fungus	Concentration (%w/v)		Growth (mm)	
A. niger	0	1.73	2.02	2.75
	50	0.67	0.87	1.65
	100	0.48	0.69	0.95
A. flavus	0	3.07	4.05	4.90
	50	1.10	1.58	1.80
	100	0.56	0.90	1.10
R. Stolonifer	0	2.10	3.10	3.93
	50	1.50	2.00	2.64
	100	0.78	1.10	1.85

Phytochemical screening of the garlic extracts confirmed the presence of alkaloids, tannins, anthraquinones, saponins, terpenoids, cardiac glycosides, flavonoids, phenolic compounds, and steroids.

The results of this study identified three fungi associated with groundnut seeds in Markudi, Benue State, Nigeria. It agrees with the findings of Omugo et al. [4], who isolated *A. niger*, *A. flavus*, and *R. stolonifer* on groundnut seeds in Owerri metropolis, Imo State in Nigeria. Tobin-West et al. [1] also identified these fungi on groundnut seeds in the Port Harcourt metropolis, River State in Nigeria. In a study by Liamngee et al. [18], similar fungi were isolated from common bean (*Phaseolus vulgaris*) seeds in Makurdi. In agreement, Nyirahakizimana et al. [3] also isolated *A. flavus* and *A. niger*, among other pathogenic fungi, on raw and roasted groundnuts from Formal and Informal Markets in Eldoret and Kericho Towns, Kenya. The result further identified *Aspergillus* species as the dominant fungi on groundnut seeds in Makurdi. This finding agrees with the previous study of Raju and Krishnamurthy [19], who identified *Aspergillus* species as the predominant fungi affecting stored groundnut seeds' quality. The dominance of *Aspergillus* spp. could be attributed to their ability to contaminate seeds at various stages, from production to harvest. Aspergillus is a common mold in tropical and sub-tropical countries that causes aflatoxin contamination on stored commodities, such as groundnut, cereal, and cotton seeds [4]. These fungi, especially *A. flavus*, pose a significant health problem to the consumers of groundnuts in Makurdi. It has been reported that *A. flavus* produces a large amount of aflatoxin, especially aflatoxin B1, classified as a class 1 carcinogen [19]. Studies have shown that human consumption of the products contaminated with aflatoxins may lead to liver cancer, stunted growth in children, and immune system disorders through chronic aflatoxicosis [20].

Results of the current study showed that the extract greatly inhibited the radial growth of the fungi. The ability of the garlic extracts to affect the relative abundance of the fungal pathogens is similar to the study of Khan et al. [22]. In agreement, Agi and Azike [23] also reported the antifungal activity of garlic extract on mycelia radial growth of *Aspergillus* spp., *Penicillium* spp., and *Candida albicans*. They reported that various concentrations of garlic extract inhibited the fungal radial growth. In their study, the higher concentration (100% w/v) showed more inhibition than the lower concentration (50% w/v). Clearly, it was due to the high concentration of bioactive compounds [16]. The antifungal activity of garlic extract could be attributed to the sulfur-containing compounds in garlic, particularly allicin [21]. Allicin has potent antimicrobial activity on various pathogenic organisms [12].

The results of the phytochemical screening of the garlic extract revealed the presence of alkaloids, tannins, flavonoids, cardiac glycosides, anthraquinones, terpenoids, saponins, phenolic compounds, and steroids. This finding agrees with previous studies [24,25], who confirmed the presence of these phytochemicals in garlic extract. Phytochemicals are responsible for the color, flavor, and smell, and protect the plants against attack by microorganisms and parasites [26]. The presence of these chemicals is attributed to the medicinal properties of the garlic extracts. It has been shown that saponins are active antifungal agents. Tannins are also known as antimicrobial agents. Tannins have been reported to prevent the development of microorganisms by precipitating the microbial proteins and making the nutritional protein unavailable for the cells [26]. The hydroxyl group of the phenolic compounds has been reported to interrupt the cell membrane of fungi. It reduces the proton-motive force, and thus inhibited the ATP synthesis, causing cell death of the fungi [27]. Flavonoids have been reported to inhibit the cytoplasmic membrane function, thus affecting the growth of microorganisms [28]. Terpenoids and alkaloids are known to affect the cell metabolism of microorganisms by inhibiting their protein synthesis [27]. Cardiac glycosides have been reported to interrupt the cell walls of microbes. Anthraquinones and steroids are also known as antifungal agents that affect fungi

nucleic acid synthesis, inhibiting fungal growth [27,28].

4. Conclusion

This study identified *A. niger*, *A. flavus*, and *R. Stolonifer* as the fungi associated with groundnut seeds in Makurdi. These fungi pose a severe health risk to the consumers of groundnuts because of the production of mycotoxins. The aqueous garlic extracts could effectively reduce the mycelia growth of these fungi at concentrations of 100% and 50% w/v. It is assumed that the antifungal activity of the extracts is due to the presence of phytochemicals, including alkaloids, tannins, flavonoids, cardiac glycosides, anthraquinones, terpenoids, saponins, phenolic compounds, and steroids. Accordingly, garlic extract can be used as an alternative to the synthetic fungicides used in groundnut seed preservation by spraying the extract on the seeds right from the harvest and during storage.

5. Acknowledgment

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6. Conflict of Interest

The authors declare that they have no conflict of interest.

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