ANTIFUNGAL AND PHYTOCHEMICAL CONSTITUENTS OF AQUEOUS LEAVES EXTRACT OF *Hyptis spicigera* LAM. ON *Aspergillus* AND *Fusarium* SPECIES

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Abstract: The use of most synthetic fungicides have been restricted because of high acute toxicity, long degradation period, pathogen resistance, bad effect on human, plants, animals and the environment at large. Due to high usage of chemical fungicides on farm land near water bodies, there is an increase release of toxin in water bodies which will invariable entered into the food chain. The aim of the research was to determine the phytochemical and antifungal activities of the aqueous leaf extract of *Hyptis spicigera* on *Aspergillus* and *Fusarium* species. The phytochemical screening was done using standard methods. The antifungal activities were carried out to determine the zones of inhibitions, minimum inhibitory concentration and minimum fungicidal concentrations using standard methods of Clinical and Laboratory Standard Institutes. The qualitative phytochemical constituents reveal the presence of anthraquinones, unsaturated sterols and triterpenes, cardiac glycosides, flavonoids, tannins, saponins and alkaloids. The quantitative phytochemical screening revealed high concentration of saponin to be 490 mg/g, phenolic was 280 mg/g, flavonoids was 220 mg/g, alkaloids was 120 mg/g and tannins had the least concentration of 50 mg/g. The sensitivity of the extract on the test organism revealed highest diameter of inhibition on *Aspergillus parasiticus* with mean diameter of inhibition zone of 21.33±0.33 mm and the least inhibition zone of the extract was observed on *Aspergillus niger* with mean diameter of inhibition zone of 16.00±0.58 mm. The MIC of the fungi sensitive to the extract ranges between 3.13 to 12.5 mg/ml of the extract and their MFC ranges between 6.25 to 25 mg/ml of the extract. The result of the analyses of the diameter of inhibition revealed that there was a significant difference between the extract and the control fungicides (Mancozeb). The result obtained from this research revealed that the leaves extract could be used as bio-fungicidal product for the control of fungal diseases of some species of *Aspergillus* and *Fusarium*.

Keywords: Phytochemicals, antifungal *Hyptis spicigera*, methanol, flavonoid fraction, *Aspergillus*, *Fusarium*

Introduction

Biologically active compounds present in the medicinal plants have always been of great interest to scientists working in this field. The use of most synthetic fungicides have been restricted because of high acute toxicity, long degradation period, pathogen resistance, bad effect on human, plants, animals and the environment at large (Wuyep *et al*., 2017). Due to high usage of chemical fungicides on farm land near water bodies, there is an increase release of toxin in water bodies which will invariable entered into the food chain.

Plant diseases are mostly controlled by chemical pesticides, bactericide, fungicide and in some cases by cultural practices. No doubt the use of chemicals has been found to be effective in controlling these diseases, but some major problems threaten to limit the continued use of these chemical fungicides. One problem is the tendency of fungi to develop resistance to chemicals, necessitating a higher dose or the development of new chemicals to replace those to which fungi are resistant which will virtually cause increased released of toxic substances into the environment (Shukla *et al*., 2010; Bhagwat and Datar, 2014).

Fungal contamination of agricultural production is a chronic problem in developing countries and results in a decline in quality of agricultural crops. According to an investigation, nearly 20% decrease in the yield of major food and crops are due to fungal (Agrios, 2005). Crops are naturally contaminated with fungi in the field, during drying, processing, transportation and subsequent storage and it may be difficult to completely prevent mycotoxins formation in contaminated commodities, particularly those that are produced in tropical and subtropical climates, in countries where high temperature and humidity promote the growth and proliferation of fungi (Kumar *et al*., 2008). Thus, they are often colonized by fungi, including species from the genus *Aspergillus*, *Penicillium* and *Fusarium*, which cause significant reductions in crop yield, quality and safety due to their ability to produce mycotoxins (Alkenz *et al*., 2015).

Aspergillus mold fungus is a large genus consisted of over 200 species to which humans are constantly exposed. Only few of these species are pathogenic among which more than 95% of the infections are caused by three species of Aspergillus including *A. fumigatus*, *A. flavus*, *A. niger* (Anaissie *et al*., 2009) Fusarium diseases that affect most crops are caused by several individual *Fusarium* or more commonly, co-occurring species. *Fusarium* spp. can cause indirect losses resulting from seedling blight or reduced seed germination, or direct losses such as seedling foot and stalk rots; however, the most important diseases in cereals due to severe reduction in yield and quality are head blight of small cereals such as wheat, barley, oat and ear rot of maize (Nganje *et al*., 2000; Munkvold, 2003).

Materials and Methods

Study area

The study was carried out in the Department of Pharmacognosy and Drugs Development, Ahmadu Bello University Zaria Located in Kaduna State between Latitude 11º 09º N and Longitude 7º 39º E at an altitude of 672 meters above sea level and Department of Botany, Ahmadu Bello University Zaria located between Latitude 11º 15º N and Longitude 7º 65º E and an altitude of 652 meters above sea level using Global Positioning System (GPS) Android Version 9.10.11

Source of plant materials

Fresh leaves samples of *Hyptis spicigera* Lam. was collected within Zaria Environs located between Latitude 11º 55º N and Longitude 7º 99º E, the leaves were taken to the herbarium unit of Department of Botany, Ahmadu Bello University Zaria for proper identification and documentation and the Voucher number of the plant was documented as ABU2050

Preparation of plant materials

The fresh leaves were dried at room temperature (25 – 30^{0}C) for 21 days, the dried leaves were grinded into fine powder

using the leaves grinding machine; the powdered leaves were weighed and kept for further Analyses.

Extraction of plant materials

The powdered materials were extracted using the method described by (Kokate *et al.,* 2002) with some modifications, 250 g of the powdered plant material was extracted with distilled water at room temperature (25 – 36ºC) for 24 h. The filtrate was further concentrated to dryness on a water bath set at 60^0 C, the powdered extract was kept in the desiccator for further analyses

Qualitative phytochemical screening

The leaves extract was tested for Carbohydrate, anthraquinone, saponins, cardiac glycoside, flavonoids, tannins, unsaturated sterols and triterpenes and alkaloids using standard methods described by Trease and Evans (2009).

Quantitative phytochemical screening

The leaves extract was tested for various quantities of the basic secondary metabolites;

Total phenolic content was measured spectroscopically by Folin Ciocalteu Colometric method, using Gallic acid as standard and expressing the result as Gallic acid equivalent (GAE) per gram of sample (Alhakmani *et al.,* 2013)

Total flavonoids contents determined by Aluminium chloride colometric assay (Zhishen *et al.,* 1999). The TFC was determined as mg/g per quercetin equivalent with the help of calibration curve of quercetin.

Total alkaloids content was determined by spectroscopic method, this method was based on reaction between alkaloid and bromocresol green (BCG), the concentration of total alkaloids contents was expressed in mg/g/atropine equivalent (Shamsa *et al.,* 2008; Sharief *et al.,* 2014).

Total tannin content was determined spectroscopically by Folin Ciocalteu method. The tannin content was expressed in terms of mg/g/gallic acid equivalent (Marinova *et al.,* 2005; Rajeev *et al.,* 2012; AfifyAel-M *et al.,* 2012)

Total saponin content was determined according to the method described by Makkar *et al.* (2007). The standard calibration curve was obtained from suitable aliquots of Diosgenin (0.5 mg/ml). The total saponin concentration was expressed as mg/g/ Diosgenin equivalent.

Source of fungal isolates

Four different characterized species each of *Aspergillus* and *Fusarium* known to affect crops and had been identified by Innovative Medicine Initiative (IMI) was used for the study. Already cultured isolates were collected from the Department of Crop Protection Ahmadu Bello University, Zaria. The four species of *Aspergillus* used are: *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus parasiticus* and *Aspergillus fumigatus*. The four species of *Fusarium* used are: *Fusarium verticilloides*, *Fusarium graminerum*, *Fusarium oxysporum* and *Fusarium proliferatum*

Media preparation

Potatoe Dextrose Agar (CRITERION™) was prepared according to the manufacturer's specifications, autoclaved at 121°C for 15 min. The prepared medium was dispensed in universal bottle with caps covered and kept in the refrigerator prior to usage.

Preparation of fungal inoculum

The spores from the surface of the plates were collected with inoculating needle and suspended in 8 ml of normal saline solution, the mixture was homogenized, 2 ml of 10% tween 20 was added to reduce the clumps of the hyphae to the spore, the mixture was stirred and kept for 6 hours, the spores settle at the bottom, the supernatant was discarded and the spores were gradually decanted into a sterile tube. The suspension was adjusted to 0.5 McFarland standard equivalent to the turbidity of the suspension by a spectrophotometer at a wavelength of 530 nm to obtain a final concentration that will match 0.5 McFarland standard for mould base on the optical density of the solution and the optical density of 1 is taken as 0.5 McFarland which correspond to a spore count between 4×10^5-5×10^6 CFU/ml (EUCAST, 2014).

Sensitivity test

Agar well diffusion method was used to screen the extract against the test organism. 0.1 ml of the test inoculum was smeared across the Petri dish. 0.5 g of the extract was dissolved in 10 ml of 20% DMSO the concentration of the extract obtain was 50 mg/ml, then 0.1 ml of the prepared extract was introduced into the well and incubated at 30°C for 7 days after which the plates was observed for zone of inhibition, the inhibition zone was measured with a meter rule and documented excluding the diameter of the well (CLSI, 2014) A positive control Mancozeb (Fungicide) and a negative control which are Normal Saline and 20% DMSO were set to account for their inhibitory action.

Minimum inhibitory concentration (MIC)

The MIC was determined using the broth dilution method, Potatoe Dextrose Broth was prepared as prescribed by the manufacturer, two-fold serial dilution of the extract was done in the sterile broth to obtain a concentration of 50, 25, 12.5, 6.25, 313 and 1.67 mg/ml. 0.1 ml of the already prepared standard inoculum was introduced into each of the test tube containing varied concentration of the extracts dissolved in the PD broth, incubation was done for 7 days at a temperature of 30°C. The MIC was read as the test tube having the least concentration of the extract with no sign of fungal sporulation; this could easily be seen from the surface of each tube as the spores if present will show visible sign of colored spores depending on the species (CLSI, 2014).

Minimum fungicidal concentration (MFC)

Potatoe Dextrose Agar was prepared; the content of the MIC starting from the test tube which the MIC was recorded and those with increase concentration of the extract in the sterile dilution were sub-cultured onto prepared PDA medium, incubation was done at 30°C for 7 days after whom the plates of the medium was observed for colony growth. The plates with the least concentration of the extract without colony growth is referred to as the minimum fungicidal concentration (CLSI, 2014).

Data analysis

The quantity of the various secondary metabolites and the average mean zone of inhibition of each extract and the controls was subjected to one way Analyses of variance, where significant Duncans Multiple Range Test was used to separate the means using SPSS version 10.9.

Results and Discussion

The qualitative phytochemical screening of aqueous leaves extract of *Hyptis spicigera* indicates the presence of anthraquinone, Unsaturated sterols and triterpenes, Cardiac glycosides, Flavonoids, Tannins, Alkaloids and Saponins (Table 1). The presence of this phytochemicals indicates that the plant could serve as a good potential for several biological activities, the result of the phytochemical screening of this plant is in line with the findings of Sharma and Tripathi (2008); Kelly *et al.* (2015) whom reported similar phytochemicals in other species of *Hyptis.*

Table 1: Qualitative phytochemical screening of aqueous leaf extracts of *Hyptis spicigera* Lam

Phytochemicals	Test	Aqueous extract
Carbohydrates	Molish	+
	Fehlings	+
Anthraquinone	Bontragers	+
Unsaturated	Lieberman-Bucchard	+
Sterols and Triterpenes	Salkwoski	+
Cardiac Glycoside	Kella-killiani	+
	Keddes	+
Saponins	Frothing	+
	Haemolysis	+
Flavonoids	Shinoda	+
	NaOH	+
	Ferric Chloride	+
Tannins	Lead-Acetate	+
	Ferric-Chloride	+
	Bromine-water	+
Alkaloids	Mayers	+
	Dragendoff	+
	Wagners	+

+ = Positive

Table 2: Quantitative phytochemical screening of aqueous leaves extracts of *Hyptis spicigera* Lam

Phytochemicals	Phenolic	Tannins	Flavonoids	Alkaloids	Saponins
Aqueous	280±0.71^{b}	50±0.33^{e}	220±0.00^{c}	120±0.00^{d}	490±0.05^{a}

Mean with the same superscript along each row is not significantly different at P ≥ 0.05

The quantitative phytochemical screening of aqueous leaves extract of *Hyptis spicigera* indicates that among all the phytochemicals saponins had the highest concentration of 490±0.05 mg/g per diosgenin equivalent followed by phenolics with a concentration of 280±0.71 mg/g per gallic acid equivalent, flavonoids had a concentration of 220±0.00 mg/g per quercetin equivalent, alkaloids had a concentration of 120±0.00 mg/g per atropine equivalent and tannins had the least concentration of 50±0.33 mg/g per gallic acid equivalent (Table 2). Saponins had a great potential in resulting to toxicity to broad range of microorganism and also it could serve as chemotheraphy of mycotic infections on plant pathogenic microbes. This is also in line with the findings of Sodipo *et al.* (1991) whom reported that saponins foams in aqueous solution and has a great hemolytic effect and can also bind to cholesterol sites and causes anti-mycotic effects on plants pathogens.

The phenolics content of the extract was 280 mg/g, phenolic acid had been known to bind to adhesion complex on fungal cell wall leading to inactivation of key enzymes that aid in the digestion of nutrient by the fungal spores. Beatriz *et al.* (2018) reports that phenolic compound at a very little concentration inhibits the growth and sporulation of several fungi species leading to fungistatic or fungicidal in fungal species. Similar potential of phenolic was also observed from the juice extract of *Gmelina arborea* on candida species (Adamu *et al.*, 2019). Alkaloids was also detected to be present in the extract at a concentration of 120 mg/g, alkaloids had greatly been associated to intercalate into the cell wall of fungal spores and causes cellular disruption (Cowan, 1999).

The quantity of flavonoids was noticed to be 220 mg/g. In plants, flavonoids play an important role in biological processes. Beside their function as pigments in flowers and fruits to attract pollinators and seed dispersers, flavonoids are involved in UV-scavenging, fertility and disease resistance especially to fungal infection (Schiijlen *et al.*, 2004). Tannins with the least concentration of 50 mg/g. the least concentration of tannins detected could be as a result of the extraction solvent used and possibly the tannin content of the plant is un-hydrolyzed which makes it more soluble in polar solvents.

The sensitivity of the aqueous leaf extract to the test organism showed that five fungal species were sensitive to the extract (Table 3). The inhibitory zone of fungi sensitive to the extract ranges from 16.00±0.58 to 21.33±0.33 mm (Table 3). *A. fumigatus*, *A. parasiticus*, *A. niger*, *F. proliferatum* and *F. oxysporum* were all sensitive to the extract while *A. flavus*, *F. graminearum* and *F. verticilloides* were all resistance to the extract, also the control fungicides used which is mancozeb does not show inhibitory activity on *A. parasiticus* and *F. oxysporum.* The resistance of these fungi species to the fungicide could be as a result of the broad spectrum activity of the fungicide and it's been used for a wide range of fungi species which makes it less specific to certain species of fungi (Abhishek *et al.*, 2014).

Table 3: Zones of inhibitions of the aqueous leaves extract of *Hyptis spicigera* on *Aspergillus* and *Fusarium* species

Fungal species	Aqueous	Control fungicides (mancozeb)
A. flavus	0.00±0.00^{b}	20.00±1.16^{a}
A. fumigatus	17.67±0.33^{b}	22.67±0.88^{a}
A. parasiticus	21.33±0.33^{a}	0.00±0.00^{b}
A. niger	16.00±0.58^{b}	19.33±0.88^{a}
F. graminearum	0.00±0.00^{b}	18.33±1.45^{a}
F. proliferatum	18.33±0.33^{b}	21.00±0.57^{a}
F. oxysporum	18.00±0.58^{a}	0.00±0.00^{b}
F. verticilloides	0.00±0.00^{b}	22.00±0.58^{a}

Mean with the same superscript along each row are not significantly different at p≥0.05

The widest diameter of inhibition was observed on *A. parasiticus* with inhibition zone of 21.33±0.33 mm, *F. proliferatum* showed an inhibition zone of 18.33±0.33 mm, *F. oxysporum* had a mean inhibition zone of 18.00±0.58 mm, *A. fumigates* had a mean zone of inhibition of 17.67±0.33 mm and the least diameter of inhibition was observed on *A. niger* with inhibition zone of 16.00±0.58 mm. The activity observed by the extract on this species could be as a result of the combine reaction effect between the flavonoid contents and the phenolic constituent in the plant which had been known to exert proteolytic and hydrolytic enzyme inhibition in microorganism. Similarly, Ahn *et al.* (2005); Mohanta *et al.* (2007) reported that flavonoids and phenolics exert hydrolytic and enzymatic activity on microorganism by causing substrate deprivation and disruption of cell wall.

The activities observed on these fungi species could also be as a result of the high concentration of saponins present in the extract. Saponin is known to cause lysis of the cell and cell wall constituent of microorganism as reported by Sodipo *et al.* (1991), Ladan *et al.* (2014). The sapogenin content could also bind to cholesterol sites there by inactivating key enzymatic reactions between the cell wall and the nutrient medium there by serving as substrate deprivation, also the reaction between the spores of this fungi species with the phenolic compound present in the extract could result in the formation of complex which could inactivate enzyme formation (Cowan, 1999).

The alkaloids present in the extract was detected in moderate quantity despite their rare concentrations in nature, alkaloids had been known to exert a broad spectrum of biological activities. Ladan *et al.* (2011) reported that alkaloids such as cytisine, lupinine, anargynin and sparteinin showed antifungal

activity on *Aspergillus* species from plant pathogenic microbes. This research is similar to the finding of Krishna *et al.* (2007) whom reported that natural occurring alkaloids and their derivatives showed a broad spectrum of antimicrobial activity and tends to serve as poison or toxins to most microorganisms.

Minimum inhibitory concentration (MIC) of Aqueous leaf extract of Hyptis spicigera Lam

The minimum inhibitory concentration of fungi sensitive to the aqueous extract showed that *A. fumigates* and *F. proliferatum* had their MIC at a concentration of 6.25 mg/ml, *A. niger* and *F. oxysporum* showed MIC at 12.5 mg/ml while the MIC for *A. parasiticus* was observed at a concentration of 3.13 mg/ml which implies that at this concentration there was no sign of fungal sporulation in the test tubes (Table 4) the lower MIC values observed on the aqueous extract could be as a result of the higher concentration of alkaloids and flavonoids in the extract, this was in contrast with the findings of Adamu *et al.* (2020) whom reports higher MIC values in the ethyl-acetate leaf extract of this plant.

Table 4: Minimum inhibitory concentration (MIC) of fungi sensitive to aqueous extract

Test organism	Concentration in mg/ml					
	50	25	12.5	6.25	3.13	1.57
A. fumigatus	--	--	--	0¥	+	++
A. parasiticus	--	--	--	--	0¥	+
A. niger	--	--	0¥	+	++	+++
F. oxysporum	--	--	0¥	+	++	+++
F. proliferatum	--	--	--	0¥	+	++

-- = No fungal sporulation, 0¥ = MIC, + = presence of fungal sporulation

Table 5: Minimum fungicidal concentration (MFC) of aqueous extract

Test organism	Concentration in mg/ml				
	50	**25**	**12.5**	**6.25**	**3.13**
A. fumigatus	--	--	0¥	+	++
A. parasiticus	--	--	--	0¥	+
A. niger	--	0¥	+	++	+++
F. proliferatum	--	--	0¥	+	++
F. oxysporum	--	--	0¥	+	++

-- = No colony growth, 0¥ = MFC, + = Slow growth

Minimum fungicidal concentration of Aqueousleaf extract of Hyptis spicigera Lam

The minimum fungicidal concentration of aqueous extract as shown in Table 5 indicated that all the content of the MIC of fungi species sub-cultured did not show any trace or sign of growth at a concentration of 12 mg/ml for *A. fumigatus, F. proliferatum* and *F. oxysporum* while the MFC for *A. parasiticus* and *A. niger* were observed at a concentration of 12.5 and 25 mg/ml.

Conclusion

The finding from this research revealed that the aqueous leaf extract of *Hyptis spicigera* contains unsaturated sterols and triterpenes, cardiac glycosides, flavonoids, tannins, saponins and alkaloids. The aqueous leaf extract showed a broad spectrum of activity against *A. fumigatus, A. parasiticus, A. niger, F. proliferatum*and *F. oxysporum.* The research from this study could be employed in the development of bio-fungicides against these species of fungi.

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Conflict of Interest

Authors declare that there is no conflict of interest reported in this work.

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