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Antifungal Activity of *Hyptis spicigera* Methanol Leaf Extract and Flavonoid Fraction

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ABSTRACT: Control of plant fungal diseases using synthetic fungicides continue to cause major problems to human health and the entire ecosystem. The aim of this research was to investigate the phytochemical and antifungal properties of *Hyptis spicigera* methanol leaf extract and flavonoid fraction on *Aspergillus* and *Fusarium* species, with a view to uncovering effective bio-fungicides for development as substitutes to chemical fungicides. Phytochemical screening revealed the presence of sterols and triterpenes, cardiac glycosides, flavonoids, tannins and alkaloids. The quantitative analysis showed that saponin (690 mg/g GAE), phenolics (220 mg/g GAE) and flavonoids (140 mg/g GAE) were found to be in high concentration. The antifungal effects of *H. spicigera* methanol extract on *F. graminearum* (21 mm) was significantly ($p<0.05$) higher than the control fungicide (Mancozeb). Similarly, the flavonoid fraction was more effective on *A. parasitic* (18 mm) than the control fungicide used. The extract and fraction exhibited MIC with range 3.13-12.5 mg/mL and MFC 6.25-12.5 mg/mL, indicating promising antifungal efficacies against *A. flavus* and *F. graminearum*. Our findings have revealed that *H. spicigera* flavonoid rich fraction has potential for development as effective bio-fungicide to control plant fungal diseases of the *Aspergillus* and *Fusarium* species.

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Plant fungal diseases are controlled usually by synthetic chemical fungicides and sometimes by cultural practices. This is because chemical fungicides are found as effective method against fungal diseases. However, they constitute major health problems to the entire ecosystem due to acute toxicity and long degradation period. Moreover, the indiscriminate use of chemical fungicides has resulted to pathogen resistance due to selective pressure (Shukla, 2013; Bhagwat; Datar, 2014). Hence, the need for safe, effective and environmentally sustainable approach to controlling plant fungal diseases especially with the 25-50% loss in agricultural crops due to fungal infections (Chuang *et al*., 2007; Zaker, 2014). Fungal genus such as the *Aspergillus* consists of over 200 species with 95% of its infections caused by *A. fumigatus, A. flavus, A. niger* (Anaissie *et al.*, 2009). *Fusarium* is also an economically important genera of plant fungi that infects agricultural produce according to crops, geography and environmental conditions (Longrieco *et al*., 2002; Vander Lee *et al*., 2015). Thus, the search and development of effective biopesticides from plants is necessary to overcome the loss in agricultural crops due to fungal infections. Previous report showed that numerous plant extracts have exhibited potent antifungal activities (Masih *et al.*, 2014; Wuyep *et al*., 2017). *Hyptis spicigera* (Lamiaceae family) is a flowering plant, erect and annual herb with about 300 to 400 species. The plant is frequently grown as a food crop for its seeds in tropical West Africa. It is aromatic and commonly known as bush mint found in tropical North and South America. *Hyptis spicigera* is used in Nigerian traditional medicine for gastrointestinal disturbances, wounds, skin infections and insect bites (Patricia *et al*., 2014). Several species of *Hyptis* genus have similar ethnopharmacology with related phytochemical profile (McNeil *et al.*, 2011). Previous phytochemistry of the *Hyptis* genus showed that *β*-sitosterol, oleanolic acid and urs-12-en-3*β*-ol-27-oic acid have been isolated from the roots of *Hyptis suaveolens* (Misra*et al.*, 1981). The report by Moreira *et al*. (2010) also showed that *H. suaveolens* essential oil from Brazil contain largely eucaliptol (48%) with antifungal activity on *Aspergillus* species. However, *H. spicigera* essential oil from Nigeria was found to contain *β*-caryophyllene (68%) as major component (Onayade *et al*., 1990). In this study, we report the quantitative analysis of *H. spicigera* methanol extract and subsequent isolation of flavonoid fraction with evaluation of antifungal activity on *Aspergillus* and *Fusarium* species. This is with a view to uncovering

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biopesticides for effective control of plant fungal diseases of the *Aspergillus* and *Fusarium* species.

MATERIALS AND METHODS

Plant material: Hyptis spicigera Lam. fresh leaf was collected within Zaria environs located between Latitude 11° 55″ N and Longitude 7° 99″ E. The leaf was identified at the herbarium unit of the Department of Botany, Ahmadu Bello University Zaria, where a Voucher number (ABU2050) was deposited.

Preparation and extraction of plant material: Hyptis spicigera fresh leaves were dried at room temperature for 21 days and grinded into fine powder using grinding machine. The powdered sample was weighed and extracted using the method described by Kokate *et al.* (2002) with some modifications. Briefly, 250 g of powdered plant material was extracted with methanol (2L) using cold maceration at room temperature (25°C-36°C) for 24 h. The filtrate was concentrated to dryness at room temperature and kept in the desiccator for further analyses.

Qualitative phytochemical screening: The leaf extract was tested for saponins, cardiac glycoside, flavonoids, tannins, sterols and triterpenes and alkaloids using standard methods (Trease and Evans, 2009).

Quantitative phytochemical screening: The leaf extract was subjected to various quantitative analysis for total phenolics content, total flavonoids contents, total alkaloids, total tannins and total saponins content: The total phenolics content (TPC) and total tannins content (TTC) were measured spectroscopically by Folin-Ciocalteu colometric method, using Gallic acid as standard, expressed as Gallic Acid Equivalent (GAE) per gram of sample (Alhakmani *et al.,* 2013; AfifyAel-M *et al.,* 2012). However, the total flavonoids contents (TFC) was determined by aluminum chloride Colometric assay and expressed as mg/g Quercetin Equivalent (QE) (Zhishen *et al.,* 1999). The total alkaloids content (TAC) was determined by spectroscopic method based on reaction with bromocresol green (BCG) and expressed in mg/g Atropine Equivalent (AE) (Shamsa *et al.,* 2008; Sharief *et al.,* 2014). Then, the total saponins content (TSC) was determined as described by Makkar *et al.* (2007). Diosgenin (0.5 mg/mL) was used for standard calibration curve. The total saponins concentration was expressed as mg/g Diosgenin Equivalent (DE).

Flavonoid isolation and TLC chromatographic profile: Flavonoid was isolated from the methanol extract using standard method as described by Woo *et al.* (1980) (Figure 1).The fraction was spotted on a silica gel coated plate and ran in a TLC tank using butanol-acetic-acid-water BAW (10:1:1) as solvent system. Specific flavonoid chemical test was carried out using aluminum chloride as spray reagent for flavonoid detection on the TLC plates. The plate was sprayed with 1% ethanol solution of aluminum chloride and the appearance of a yellow or red fluorescence in a long wavelength (360 nm) of UV light confirms flavonoids.

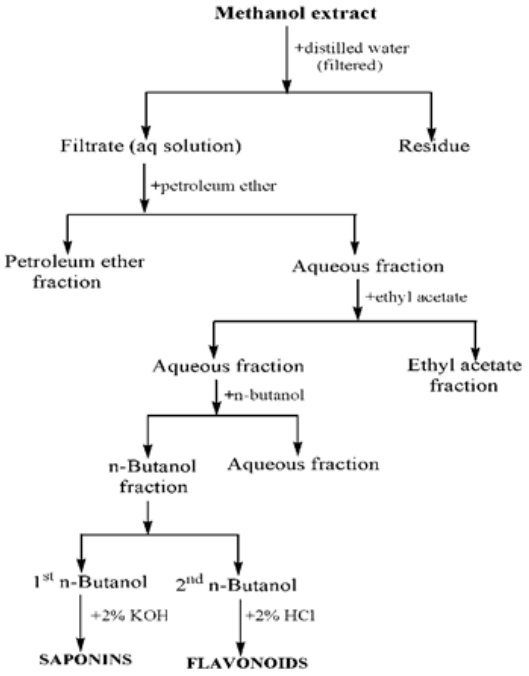

Fig. 1: Fractionation procedure for flavonoid isolation as reported by Woo et al (1990)

Fungal isolates and preparation of inoculum: Four different species each of *Aspergillus* and *Fusarium* 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: *A. flavus, A. niger, A. parasiticus* and *A. fumigatus*. The four species of *Fusarium* used are: *F. verticilloides, F. graminerum, F. oxysporum* and *F. proliferatum.* Potato Dextrose Agar (PDA) was used for the growth of the fungi species and was prepared according to the manufacturer, autoclaved at 121°C for 15 min. The prepared medium (20 mL) each was dispensed in universal bottle with caps covered and kept in the refrigerator prior to usage. The spores from the surface of the plates was collected with inoculating needle and suspended in normal saline solution (10 mL).The mixture was homogenized, 10% Tween-20 (2 mL) was added and heavy particles were allowed to settle down and 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 mold base on the optical density of the solution (0.4-0.5 × 10^6) CFU/mL (EUCAST, 2014).

Antifungal sensitivity test: Agar well diffusion method was used for the screening on test organism. The test inoculum (0.1 mL) was smeared across the petri dish. The extract (0.5 g) was dissolved in 20% DMSO (10 mL) resulting to concentration of the extract to be 50 mg/mL. The prepared extract (0.1 mL) was introduced into the well and incubated at 30°C for 7 days after which the plates were observed for zone of inhibition, and 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; Potato Dextrose Broth (PDB) was prepared as prescribed by the manufacturer. Two-fold serial dilution of the extract and fraction were carried out in the sterile broth to obtain a concentration of 50 mg/mL, 25 mg/mL, 12.5 mg/mL, 6.25 mg/mL, 313 mg/mL and 1.67 mg/mL. One loop full of the already prepared standard inoculum was introduced into each of the test tube containing varied concentration of the extracts dissolved in the PDB was done and incubation was carried out 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 sporulation of fungi; 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): Potato Dextrose Agar (PDA) was prepared, the content of the MIC starting from the test tube which the MIC were recorded and those with increase concentration of the extract in the sterile dilution were sub-cultured onto prepared medium. The plates were incubated at 30°C for 7 days after which the plates were observed for colony growth. The plate with the least concentration of the extract without colony growth is referred to as the minimum fungicidal concentration (CLSI, 2014).

Data analysis: Quantitative analysis of phytochemicals and the average mean zone of inhibition of each extract and the controls were subjected to One-way Analyses of Variance (ANOVA), where significant Duncan's Multiple Range Test was used to separate the means using SPSS version 20.

RESULTS AND DISCUSSION

Qualitative and quantitative phytochemical analysis: The qualitative screening of phytochemicals from *H. spicigera* leaf methanol extract showed numerous secondary metabolites (Table 1).The presence of these phytochemicals implied that the plant could be a good source of potent therapeutic agents. Previous phytochemical screening shows similar profile with *Hyptis* species as reported by Sharma and Tripathi (2008). The quantitative evaluation revealed that saponins (690 mg/g) had significantly higher concentration than other phytochemicals (Table 1). The saponin concentration of 690 mg/g Diosgenin Equivalent (DE) indicated that the plant is rich in saponin glycosides. The high concentration of saponins is a pointer for broad range antifungal agents capable of controlling plant mycotic infections (Ladan *et al.,* 2014). Previous report attributed the role of saponins from plant extract on lyses of fungal spores and subsequent death of the cells (Masih *et al.*, 2014). The phenolics content (220 mg/g GAE) from *H. spicigera* leaf methanol extract indicated promising antimicrobial agents, because phenolics compounds at very little concentration inhibits the growth and sporulation of several fungi species leading to fungistatic or fungicidal in fungal species (Beatriz *et al.,* 2018).

Table 1: Phytochemical analysis of *H.spicigera*of methanol leaf extract

Phytochemicals	Test	Qualitative	Quantitative (mg/g)
Sterols and triterpenes	*Salkowski*	+	
Cardiac glycoside	*Kella-killiani*	+	
Saponins	*Frothing*	+	690±0.33
	Haemolysis	+	
Flavonoids	*Shinoda*	+	140±0.33
	Ferric Chloride	+	
Tannins	*Lead-Acetate*	+	20±0.17
	Bromine-water	+	
Alkaloids	*Mayer's*	+	90±0.13
	Dragendorf's	+	
	Wagner's	+	
Phenolics			220±0.13

Plant flavonoids possess specific bioactivities against pathogenic microbial agents (Kumar and Pandey, 2013). Thus, *H. spicigera* leaf methanol extract contains flavonoids (140 mg/g GAE) indicating the important role as antifungal therapeutic agents. The effects of flavonoids on fungal pathogens reported previously suggest their structural and chemical diversity as the basis of therapeutic potentials (De Conti Lourenço *et al*., 2013). The alkaloids quantification was low (90 mg/g Atropine Equivalent) nevertheless, alkaloids are associated to intercalate into the microbial cell wall component and cause disruption (Cowan, 1999). *Antifungal activity of H. spicigera extract and flavonoid fraction:* The antifungal effects of methanol extract and flavonoid fraction are presented (Table 2). Five of the eight fungal species were susceptible to both the extract and fraction. The range for zone of growth inhibitions for methanol extract (18-21 mm) and flavonoid fraction (14.33-18.33 mm) as presented. The effect of methanol extract on *F. graminearum* with higher antifungal activity (21 mm) indicates the potency of the crude extract, but no statistically significant ($p \geq 0.05$) difference with flavonoid fraction and the control drug. However, it was observed that the antifungal activity of both extract and fraction was significantly ($p \geq 0.05$) more potent on *A. parasiticus* and *F. oxysporum* than the control drug (Table 2). The resistance of *A. parasiticus* and *F. oxysporum* to the control fungicide (Mancozeb) depicts phytoconstituents from *H. spicigera*as effective bio-fungicides. Previous report showed that *Aspergillus* sp. was susceptible to *Barringtonia racemosa* methanol leaf extract with only 31.3% inhibition (Hussin *et al.*, 2009). Thus, the susceptibility of these fungal species to *H. spicigera* extract and fraction might be due to synergistic interactions of phytoconstituents.

Table 2: Antifungal activity of *H. Spicigera* methanol extract and flavonoid fraction

	Zone of inhibition (mm)		
Fungal species	**Methanol Extract**	**Flavonoid Fraction**	**Control (Mancozeb)**
A. flavus	18.00 ± 0.33^{b}	16.67 ± 0.33^{c}	20.00 ± 1.16^{a}
A. fumigatus	18.33 ± 0.88^{b}	14.33 ± 0.33^{c}	22.67 ± 0.88^{a}
A. parasiticus	18.33 ± 0.88^{a}	18.33 ± 0.33^{a}	0.00 ± 0.00^{c}
A. niger	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	19.33 ± 0.88^{a}
F. graminearum	21.00 ± 0.00^{a}	17.67 ± 0.33^{c}	18.33 ± 1.45^{b}
F. proliferatum	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	21.00 ± 0.57^{a}
F. oxysporum	18.00 ± 0.58^{a}	17.67 ± 0.33^{b}	0.00 ± 0.00^{c}
F. verticilloides	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	22.00 ± 0.58^{a}

Mean with the same superscript along each row are not significantly different at $p \geq 0.05$

The minimum inhibitory concentration (MIC) represents the low concentration required to inhibit fungal growth. Low MIC translates to potent antifungal activity of the extract or fraction being evaluated. The *H. spicigera* methanol extract demonstrated MIC_m of 12.5 mg/mL on all test organisms. However, flavonoid fraction exhibited MIC_f range of 3.13-12.5 mg/mL indicating most potent antifungal activity (Figure 2). This revealed that the flavonoid rich fraction is effective antifungal agent on *A. flavus* and *F. graminearum* more than the methanol crude extract. Previous report on antifungal efficacy and mechanisms of flavonoids indicated that medicinal plants containing flavonoids are safe antifungal agents that demonstrated enormous therapeutic potentials through disruption of plasma membrane as well as inhibition of cell wall formation, cell division or protein synthesis (Al Aboody and Mickymaray, 2020). The minimum fungicidal concentration (MFC) is the lowest concentration that can completely kill the fungal species being evaluated. Thus, lowest MFC values indicate therapeutic properties of extracts under study. The methanol extract displayed MFC_m (12.5 mg/mL) for all fungal organisms tested. However, the flavonoid fraction demonstrated the lowest MFC_f of 6.25 mg/mL indicating most potent fungicide on *F. graminearum* (Figure 2). This finding has revealed the application of *H. spicigera* flavonoid fractions as effective bio-fungicide to control of plant fungal diseases based on *Aspergillus* and *Fusarium* species.

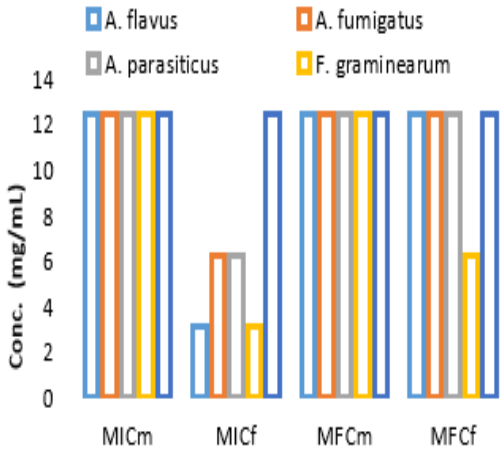

Fig 2: MIC /MFC of *H. specigera* methanol extract and flavonoid fraction

Conclusion: H. spicigera leaf methanol extract was found to contain phytochemicals such as saponins, phenolics and flavonoids in high concentration. The flavonoid rich fraction isolated from methanol extract demonstrated significant antifungal efficacies on *A. flavus* and *F. graminearum*. Our findings have revealed the potential of *H. spicigera* flavonoid fraction for development of bio-fungicides to control plant fungal diseases especially based on *Aspergillus* and *Fusarium* species.

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