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Phytochemical composition and efficacy of ethanolic leaf extracts of some *Vernonia* species against two phytopathogenic fungi

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ABSTRACT

Phytochemicals in the form of ehanolic extracts of three *Vernonia* species *viz., V. ambigua, V. amygdalina* and *V. cinerea* were evaluated *in vitro* for their efficacy against *Cercosporell apersica* and *Curvularia lunatus* isolates of groundnut leafspot disease. The extracts at the concentrations of 100, 200, 300, 400 and 500 mg/mL were tested on Potato Dextrose Agar medium (PDAM) for their effect on radial mycalial growth. Maximum growth inhibition of *C. persica* was observed at 200 mg/mL for *V. ambigua* and 300 mg/mL *for V. amygdalina* and *V. cinerea* while maximum inhibition of *C. lunatus* was at 300 mg/ml for all the extracts. The extracts had superior inhibition effect over Dithane M_{45} in fungitoxicity against the pathogens. The phytochemical screening of the extract showed the presence of flavonoids, alkaloids, terpenes, saponins, tannins among others at varied concentrations while GC-MS analysis of the extract revealed a mixture of compounds which may be responsible for the observed biological activity. The exploitation of these extracts will greatly contribute to the available ecofriendly biofungicides in the control of plant diseases due to these fungi.

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INTRODUCTION

Many fungi have been identified as causal organism of plant diseases. Fungal diseases often reduce crop yield and lower crop quality by producing toxins which are hazardous to human health. In Nigeria, about 308 phytopathogenic fungi have been recorded (Onifade, 2008). Among the economically important ones are *Cercosporella* and *Curvularia* species which have been reported by many researchers as leafspot pathogens. For example Ilondu *et al.* (2010) implicated *Cercosporella* as the causal pathogen of leafspot disease of sweet potato; Machado *et al.* (2012) reported that leafspot of Brazillian ginseng (*Pfaffiaglomerata)* was caused by *Cercosporellafaffiae*. Similarly, *Curvularialunatus* has been implicated as leafspot pathogen of so many crops such as *Telfairiaoccidentalis* (Maduewesi, 1977) *Dioscorearotundata* (Green, 1995), wheat (Enikuomehin*et al.,* 1999) oil palm seedlings (Odigie, 2000) and sweet potato (Ilondu, 2012b). The increased recognition and importance of phytopathogenic fungi, the difficulties encountered in their control and the increase in resistance to antifungal, have stimulated the search for natural alternatives (Barrera-Necha*et al.,* 2009) with a view to countering obvious pollution problems in the environment and avoiding the toxic effects on non-target organism associated with use of synthetic chemicals among others. Hence, it is obligatory to find out some alternate methods to produce the usage of chemical fungicides. The present day global interest in control of plant pathogens by biocontrol agents has direct impact on economic assistance to farmers. Antifungal activity of higher plants has long been speculated as an important factor to disease resistance and control against a wide range of fungi that infect crops (Oluma and Elaigwu, 2006). In recent times, applications of plant metabolites for plant disease management have become important viable component of integrated pest management as they are eco-friendly where botanicals play an important role (Satish*et al.,* 2010). Natural products either as pure compounds or as standardized plant extracts provide unlimited opportunities for the development of antimicrobials because of the great diversity in their chemical structure and novel mechanism of action and this has led to the screening of several

medicinal plants for potential antimicrobial activity (Okoli*et al.,* 2009).

Vernonia ambigua Kotschy and Peyr, commonly called iron weed is an annual herb of about 65cm high and a weed with no economic significance in Nigeria. It is used as remedies for cough and fever with great antimicrobial activities (Kunle and Egharevba, 2009; Kunle*et al.*, 2010; Abubakar*et al.,* 2011). *Vernonia cinerea* (Linn) is called little iron weed, an annual herb of about 120cm high and a common weed of cultivated farmlands, roadside and waste area (Akobundu and Agyakwa, 1998). The leaf is an ingredient of 'agbo' infusion for treatment of malaria, snake bite and a remedy for pile (Latha*et al.,* 2011). *Vernonia amygdalina* known as bitter leaf is Ewuro (Yoruba) Shiwaakaa (Hausa)) the most common and most readily available species with a lot of medicinal values, for example in the treatment ofdermatophitic diseases (Ilondu and Okeogwale, 2002), antibacterial activities (Bankole*et al.,* 2003), hepato protective and antioxidant activities (Iwalokun*et al.,* 2006) and control of sapolegnisis disease of fish (Ilondu*et al.,* 2009).

There is a dearth of information on the use of the extract of these *Vernonia* species in the control of phytopathogenic fungi. However, attempts have been made with the use of *V. ambigua*and *V cinerea*in the *in-vitro* control of *Sclerotiumrolfsii* (Ilondu, 2012a). Similarly, the prophylactic effects of the extract of *V. amygdalina*on fungal foliar disease of groundnut (Ogulumba, 2007) and control of leafspot disease of sweet potato (*Ipomoea batatas*) (Ilondu, 2012b) have been reported. The present study was therefore undertaken to evaluate the efficacy of ethanolic leaf extract of *Venonia ambigua* Del., *V. amygdalina* and *V. cinerea* (Family Asteraceae) on the *in-vitro* control of two phytopathogenic fungi: *Cercosporellapersica* and *Curvularialunatus*and to identify their chemical constituents to justify their use as antifungal agents. This will contribute to the available eco-friendly biofungicides in the control of plant diseases due to these fungi.

MATERIALS AND METHODS

Plant Collection and extraction Procedures

Fresh leaves of Bitter leaf, *V. amygdalina, V. ambigua* and *V. cinerea* were collected from within and around Abraka in Delta State Nigeria (Latitude $05^0$47N and Longitude $06^0$6E) the taxonomic identification of the plant species was done using Akobundu and Agyakwa (1998), Odugbemi and Akinsulire (2006) and were placed in their respective polythene bags, properly labelled and taken to Department of Botany laboratory (Herberium section), Delta State University Abraka for further processing. The leaves were washed thoroughly in tap water and air-dried on laboratory bench for 2 weeks. Dried leaves were separately ground into powder with electric blender (Philip Comfort HR 1727) before extraction (Ilondu, 2012b).

The method of extraction was adopted from Ilondu (2012a). One hundred gram of each pulverized sample was put into Sohhlet extractor and three hundred millilitre of absolute ethanol (HPLC grade was added and extracted for 8hrs for each batch of sample. The extracts were evaporated on a rotary evaporator at 40 ^{0}C to remove excess alcohol. The extract yield of each plant sample was weighed and calculated.

Phytochemical tests

One gram of powdered sample was subjected to phytochemical test for alkaloid (Myers reagent), Flavonoids were determined by magnesium rebbon test, Sapoins by chloroform and H_2SO_4 tests, Inulin by Molischs reagent, Tannins, by Ferric salt test, Sterol by Chloroform-acetic anhydride, Terpenes and phenols by adopting the procedures described by Oyewale and Audu (2007).

Extract analysis

GC-MS analysis was done at National Research Institute for Chemical Technology (NARICT) Zaria, Kaduna state, Nigeria. A SHIMADZU GCMS-QP 2010 Plus system was used.The GC-MS was operated under the following conditions: Column oven temperature: 70^{o}C; Injection temperature: 250^{o}C; Injection mode: split; Pressure: 104.1kPa; Total flow: 6.2ml/min; Column flow: 1.59ml/min; Linear velocity: 46.3cm/sec; Purge flow: 3.0

mL/min; and Split ratio: 1.0. The generated chromatogram was recorded. The identification of the components was carried out using the peak enrichment technique of reference compounds and computer matching with those of NIST.05 library mass spectrum.

Source of test fungi

The isolates of *Cercospor ellapersica* and *Curvularia lunatus* were previously obtained from leafspot disease of ground nut (*Arachis hypogaea*) grown in the Department of Agricultural Education Teaching and Research Farm, Delta State University Abraka. They were maintained on PDA slants at 4^0c in the laboratory until needed. The isolates were revived twice on PDA before use.

Effect of extracts on fungal growth

Different concentrations (100, 200, 300, 400 and 500 mg/ml) were prepared from each of the extracts. One millilitre of each level of concentration was aseptically incorporated into 20ml of cool molten PDA in sterile test tube. A standard fungicide (Dithane M_{45}) obtained from Delta State Procurement Agency (DAPA) Asaba Delta State was incorporated in equivalent amounts for comparison; each medium was homogenized by gentle agitation before dispensing into sterile 9cm Petri dishes. The control was set up using extract free PDA plates. The plates were allowed to set for 3hrs. The modified method of Chohan *et al.* (2011) was used to determine the effects of the extracts on fungal growth. This was done by inoculating at the centre of 90cm Petri plates with a mycelia disc (4mm) obtained from the colony edge of 7-day old culture of the test fungi. Three replicates of both the control and PDA-extract plates per isolate were incubated at room temperature (28 ± 2^0C) and radial growth was measured with a metric ruler daily for seven days. Colony diameter was taken as the means along two directions on two perpendicular lines drawn on the reverse of the plates. The percentage inhibition was calculated using the formula adopted from Ajayi and Olufolaji (2008).

Data analysis

Data obtained were subjected to Analysis of Variance (ANOVA) using Statistical Package for Social Science (SPSS) version 17.0 and means were separated according to Duncan's Multiple Range Test (DMRT) at 5% probability level.

RESULTS AND DISCUSSION

The yield of the leaf extract was 47.4, 42.00 and 39.00 % for *V. cinerea*, *V. ambigua* and *V. amygdalina* respectively. This study has shown that leaf extracts of the tested plants exhibited broad antifungal activity on the growth of *C. persica* and *C. lunatus in-vitro* (Table 1). Several studies have been carried out and reported on the use of plant extracts in the family of Asteraceae for the control of fungal and other related plant disease (Rai*et al.*, 2004; Okungbowa and Edema, 2007; Ilondu, 2012a). The antifungal activities of *V. amygdalina* (Erasto*et al.*, 2006; Ogwulumba, 2007; Ilondu*et al.*, 2009; Ilondu, 2012b),*V. ambigua* (Kunle, and Egharevba, 2009; Kunle*et al.*, 2010; Abubakar*et al.*, 2011; Ilondu, 2012a) and *V. cinerea* (Latha*et al.*, 2011; Ilondu, 2012a) have been documented.

Latha *et al.*, (2011) had reported that extract of *V. cinerea* completely inhibited the growth of *Candida albicans* and caused alterations in the morphology and complete collapse of yeast cells following 36 hours exposure. Ilondu (2012a) reported that extract of *V. cinerea* was very effective in reducing the mycelia growth of *Sclerotiumrolfsii* and caused 100% inhibition at 80 mg/ml, while *V. ambigua* at 60 mg/ml was more effective in inhibiting the sclerotia germination of the same fungus. The antimicrobial activities of *V. ambigua* have been reported by Kunle*et al.* (2010). On the other hand, Satish *et al.* (2010) reported a significant inhibition of some seed borne fungi of sorghum including *C. lunata*with extract of *Lawsoniainermis.* Increased antifungal activity was observed with a corresponding increase in the concentration of all plant extracts studied. The antifungal study showed that C. *persica* was more sensitive to V. *ambigua* than *V. cinerea* and *V. amygdalina* and the three *Vernonia* species exhibited the same effect on *C. lunatus.* The reduction in mycelia growth of the test fungi in the presence of these extracts is a demonstration that theyare, among other important extracts, capable of checking the spread of many fungal diseases of food crops.

Table 1. Effect of leaf extracts from *Vernonia* species and DM_{45} on radial mycelial growth (cm) and percentage inhibition of two pathogenic fungi after 7 days of inoculation on agar plates.

Fungi	Extraction conc. (mg)	*V. ambigua*		*V. amygdalina*		*V. cinerea*		DM_{45}	
C. persica	0	4.30^{a}	0.00^{c}	4.30^{a}	0.00^{d}	4.30^{a}	0.00^{d}	4.30^{a}	0.00^{f}
	100	1.00^{b}	76.74^{b}	2.20^{b}	48.84^{c}	2.60^{b}	39.53^{c}	2.96^{b}	31.16^{e}
	200	0.00^{c}	100^{a}	1.60^{c}	62.79^{b}	0.86^{c}	80.00^{b}	2.50^{c}	41.86^{d}
	300	0.00^{c}	100^{a}	0.00^{d}	100^{a}	0.00^{d}	100^{a}	2.00^{d}	53.49^{c}
	400	0.00^{c}	100^{a}	0.00^{d}	100^{a}	0.00^{d}	100^{a}	1.40^{e}	67.44^{b}
	500	0.00^{c}	100^{a}	0.00^{d}	100^{a}	0.00^{d}	100^{a}	1.00^{f}	76.74^{a}
C. lunatus	0	4.30^{a}	0.00^{d}	4.30^{a}	0.00^{c}	4.30^{a}	0.00^{d}	4.30^{a}	0.00^{e}
	100	1.50^{b}	65.12^{c}	1.43^{b}	66.74^{b}	3.33^{b}	22.56^{c}	2.30^{b}	46.51^{d}
	200	0.90^{c}	79.07^{b}	1.06^{b}	75.35^{b}	1.30^{c}	69.77^{b}	1.90^{c}	55.81^{c}
	300	0.00^{d}	100^{a}	0.00^{c}	100^{a}	0.00^{d}	100^{a}	1.61^{c}	62.59^{c}
	400	0.00^{o}	100^{a}	0.00^{c}	100^{a}	0.00^{d}	100^{a}	1.10^{d}	74.42^{b}
	500	0.00^{o}	100^{a}	0.00^{c}	100^{a}	0.00^{d}	100^{a}	0.50^{e}	88.37^{a}

Value with the same superscript in the same column are not significantly different at $p>0.05$ by Duncan Multiple Range test.

All the extracts had a superior inhibitory effect over a standard fungicide Dithane M_{45} in fungitoxicity against the pathogens. Satish *et al.,* (2010) reported that extracts of *Lawsoniainermis*gave more inhibitory effect on *C. lunatus*and other fungi than fungicides including Dithane M_{45}. It could be possible that the fungi have developed resistance to the chemical as pointed out by Erasto*et al.,* (2006).The differences in the fungi-toxic effect of these extracts could be attributed to the quality and quantity of their constituents.

The phytochemical screening reveals the presence of various concentrations of secondary metabolites in the extracts. In *V ambigua,* alkaloids are present

in high concentration, saponins in moderate concentration while tannins and phenol are present in low concentration. In *V. amygdalina,* anthraquinoneis present in moderate concentration while alkaloids, saponins and glycoside are present in low concentrations. Similarly, all the Phytochemicals present in *V. cinerea*which included alkaloid, terpenes, saponins and anthraquinoneare present in low concentrations. Phytochemical screening helps to reveal the chemical nature of constituents of the plant extract and the one that predominates over the others. It may also be used to search for bioactive agents that could be used in the synthesis of very useful drugs (Okoli*et al.,* 2009).

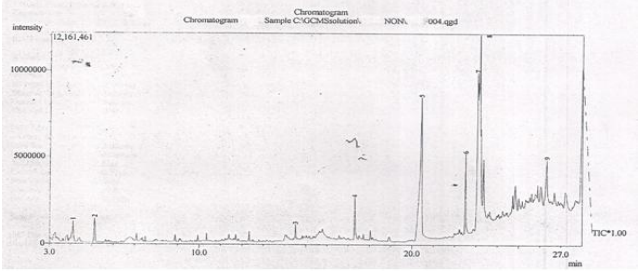

Fig 1. GC-MS Chromatogram for*Vernoniaambigua* leaf extract

Table 2. Major constituents of the ethanolic extract of *Vernonia* spp.

Peak no.	*V. ambigua*			*V. amygdalina*			*V. cinerea*		
	Retention time (min)	Compound name	Abundance (%)	Retention time (min)	Compound name	Abundance (%)	Retention time (min)	Compound name	Abundance (%)
1	4.108	Isoamyl-acetate	6.51	4.125	Isoamyl acetate	5.59	4.150	Isoamyl acetate	5.04
2	5.133	1,1-Diethoxy-3-methylbutane	15.61	5.125	1,1-diethoxy-3-methylbutane	13.42	5.133	1,1-Diethoxy-3-methylbutane	12.09
3	14.517	Caryophy-llene oxide	6.51	17.325	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	8.86	12.342	Caryophyllene	10.92
4	17.308	3,7,11,15-Tetramethyl-2-hexadacen-1-ol	10.31	18.975	Hexadecanoic acid methyl ester	9.63	14.517	Caryophyllene oxide	5.04
5	520.425	Hexadeca-noic acid ethyl ester	13.33	20.367	Hexadecanoic acid	9.50	17.325	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	7.98
6	22.508	Phytol	10.75	20.467	Hexadecanoic acid ethyl ester	11.46	620.358	Hexadecanoic acid	8.56
7	23.067	Linoleic acid ethyl ester	10.15	22.542	Phytol	9.25	22.517	Phytol	8.34
8	23.167	Linolenic acid ethyl ester	11.97	23.075	9,12-Octadecadienoic acid ethyl ester	10.55	23.125	Linolenic acid	7.87
9				23.183	Linolenic acid ethyl ester	10.29	23.325	Octadecanoic acid	8.56
10				25.175	Eicosanoic acid ethyl ester	11.46	27.150	Bata-Amyrin	25.58

Extract analysis using gas chromatography mass spectrometry (GCMS) revealed a complex mixture of compounds. The graph chromatography profiles were shown in Figs 1-3. Eight to nine compounds were identified in the various extracts which are mainly characterized by abundance of terpenoids, fatty acids and their ester (Table 2). As pointed out by Rai (2004), terpenoids constitute a group of compounds majority of which occur in the family Asteraceae. Isoamylacetate, 1,1-diethoxy-3-methyl butane, 3,7,11,15-tetra methyl-2-hexadacen-1-ol and phytoloccur in all the *Vernonia*species but vary in their percentage abundance. Caryophyllene, linolenicacid, octadecanoicacid and Bata-amyrinwere unique to*V. Cinerea* but Beta-amyrin (25.58%) is the most abundant in the extract. Fatty acids including their ethyl and methyl esters occur in varied abundance in all the extracts especially *V. amygdalina.* Its antifungal activities had been reported by Tahany*et al.*, (2010). Fatty acid and their methyl esters have been reported to have antimicrobial and antifungal properties (Agoramoorthy*et al.*, 2007). The growth inhibitory effect of diterpenes including phytol has been reported by Yoshihiro *et al.*, (2005) where phytol damaged cell membranes to allow the leakage of K^+ ions from the cells.

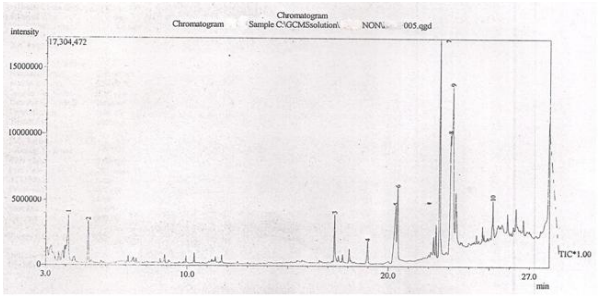

Fig 2. GC-MS Chromatogram for *Vernoniaamygdalina* leaf extract

The GC-MS analysis revealed almost similar constituent in the three extracts while the *in-vitro* screening of the extracts suggest the possibility of their use a biofungicide. Since *V. amigdalina* is used as a vegetable, *V. ambigua* and *V. cinerea* proved to be a good substitute in the control of the pathogens thereby converting weeds to wealth. Further studies should be addressed on the single or blended effects of these constituents of phytopathogenic fungi and attempting the formulation of these extracts for field application.

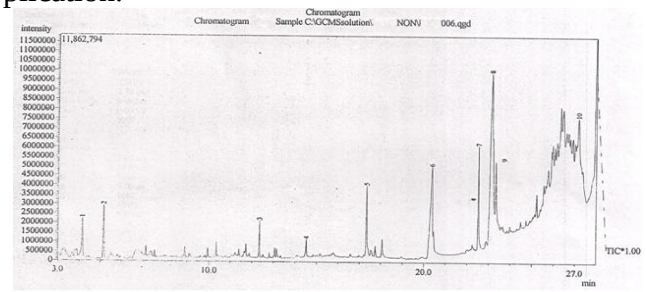

Fig 3. GC-MS Chromatogram for *Vernoniacinerea* leaf extract

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