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Effect of Vernodalol, a Sesquiterpene Lactone, on Economically Important Agricultural Fungi

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Abstract:- The status of food insecurity in the world is devastating and fungal based diseases are a major cause that result in approximately 40-60% crop yield loss. Although synthetic chemical fungicides have been the preferred immediate method for management of fungal diseases, they have undesirable effects on human and animal health; causes environmental hazards, and are expensive. This study investigated whether the natural products from *Vernonia glabra* (Asteraceae) could be used as alternative biofungicide against disease causing agricultural fungi. Fractions were separated from *V. glabra* methanolic flower crude extract, using bioassay-guided fractionation. The single pure fraction yielded was selected for bioassay tests, and was isolated and characterized using chromatographic and spectrometric methods. *In vitro* bioassay test was performed against spore germination of *Fusarium graminearum*, *F. oxysporum*, *F. verticillioides*, *Zymoseptoria tritici*, *Ustilago maydis*, and *Pyricularia oryzae*. A known sesquiterpene lactone, Vernodalol was identified using high resolution mass spectrometry. *In vitro* bioassay results revealed that spores of *Z. tritici* were the most susceptible to Vernodalol, (42% spore inhibition) at 33 µg/100 µL compared to Azoxystrobin, a standard fungicide, (100% inhibition) against the fungus at 0.005 µg/100 µL. Vernodalol sprayed on 7 days old wheat seedlings that were inoculated with spores of *Zymosepteria tritici*, *Puccinia triticina*, and *Helminthosporium sativum*, at 50 and 200 ppm did not exhibit any disease control under greenhouse conditions. Azoxystrobin exhibited 90-100% disease control. *V. glabra* extracts have sesquiterpene lactones which are antifungal, however Vernodalol needs further research on its structural modification to increase efficacy against fungal pathogens.

Keywords:- *Plant Fungal Diseases; Alternative Biofungicides; Sesquiterpene Lactones; Food Security.*

I. INTRODUCTION

Globally, more than 800 million people lack sufficient food and about 1.4 billion live on less than $ 1.25 a day, with the vast majority of these people living in developing countries [15]. The current projection to global food production losses due to plant diseases is between 10 to 16% [49], whereby in developing countries it may be much higher [12; 44]. Fungal pathogens are known to cause 40-60 % yield loss from total plant pathogenic losses worldwide [56]. Majority of the fungal pathogens belong to the Ascomycetes and Basidiomycetes divisions [53].

According to Ranum [47], cereal grains have provided humankind with more nourishment than any other food class. The three most important cereal crops in the world are maize, wheat, and rice [37], accounting for 94% of all cereal consumption in the world [47]. Developing countries depend more on cereal grains for their nutritional needs than the developed world with close to 60% of calories derived directly from cereals in developed countries and 80% calories in the poorest countries [37].

According to Gitonga [29], cereal grain consumption surpasses production in Kenya, creating a deficit that is offset by imports. In recent years, especially from 2008 to-date, Kenya has been facing severe food insecurity, as over 10 million people in the country are food insecure, with majority of them living on food relief [31]. Among the major challenges in cereal production in Kenya are pests and diseases that affect staple food crops, and contamination of the grains with mycotoxins, which negatively affect food quality [8], hence threaten food security and safety [14].

Although synthetic chemical fungicides have been the preferred immediate method for management of agricultural fungal diseases, they have undesirable effects on human and animal health, prompt environmental hazards, lead to outbreak of new fungal species and diseases resistant to the available fungicides and are expensive for the farmers [2]. Therefore, there is a need for application of alternative methods such as natural products from pesticidal plants that are safe, environmentally friendly and cheaper.

Plants produce a wide variety of secondary metabolites, which help them withstand attacks from fungi, bacteria, viruses, and UV radiation [36]. These compounds take the form of terpenoids, phenolics and alkaloids [36]. Sesquiterpene lactones in terpenoid class of compounds are commonly produced from most genera in Asteraceae family, and they are mostly found in leaves and flowering heads of plants, available in a range of cell types [18]. They are usually produced in large amounts, or in some species they may be held in storage organs such as trichomes. Such compounds act as phytoalexins; molecules produced *de novo* in reaction to microbial attack, antifeedant to deter herbivores, and as well as attractants of predators [4; 25], hormones [40], allelochemicals [6; 13] and UV protection [17]. Therefore, sesquiterpene lactone production is one of the main mechanisms of plant defense; a major biological benefit to plants. This study investigated *in vitro* effect of a known sesquiterpene lactone, Vernodalol isolated from *V. glabra*, on the economically important agricultural fungal pathogens, *Fusarium graminearum*, *F. oxysporum*, *F. verticillioides*, *Zymoseptoria tritici*, *Ustilago maydis* and *Pyricularia oryzae*, for altenative biofungicide properties. Thereafter, Vernodalol was screened under greenhouse conditions against *Zymoseptoria tritici*, *Puccinia triticina* and *Helminthosporium sativum* infected on 7 days old wheat seedlings, for assessment of protectant and curative properties.

II. MATERIALS AND METHODS

A. Collection, Drying and Extraction of V. Glabra Flowers

Vernonia glabra was selected for the study based on ethno-medicinal information [7; 53]. The flowers were collected in January, 2013 from one site; Kathiani Sub-County 1º 25’ 00” S and 37º20’ 00” E in Machakos County, Kenya [41]. The flowers were put in five clean khaki bags with mouths left open and transported to an aerated room at the School of Biological Sciences, University of Nairobi. They were allowed to dry for two weeks at room temperature (23 ± 2°C). A voucher specimen of the plant (CK 2013/01) was authenticated using keys, comparison with authentic herbarium materials and expert determination by a Plant Taxonomist at the University of Nairobi. The voucher specimen of the plant in flower was carefully arranged on drying paper, tagged, pressed according to standard herbarium methods [3; 22], and deposited in the University of Nairobi Herbarium.

The dried *V. glabra* flowers were ground into powder using an electric grinder (KM-1500 Cutting mill, mrc laboratory equipment manufacturer, Hagavish 3 Holon, Israel) and extracted using absolute methanol according to standard extraction methods [23]. Two litres of absolute methanol (MeOH) were mixed thoroughly with 40 g of flower powder, and filtered after every 24 hours for three days using cheese cloth. The filtrates were pooled and filtered a second time using a Buchner funnel and filter paper whatman No1. The dry crude extract was obtained by concentrating the filtrate to remove the extraction solvent using a rotary evaporator under vacuum at 40°C. The dry extract was weighed and stored in a vial under low temperatures (4°C) until the next use.

B. Isolation and Identification of Pure Compounds from V. Glabra Methanol Crude Flower Extract

Isolation of the pure compounds was achieved by use of bioassay guided fractionation of the methanolic crude extract from *V. glabra* flower based on [10; 50] methods. Solid-Phase Extraction integrated into automated reversed phase High Performance Liquid Chromatography system, was used for cleaning and separation of 4 g of flower crude extract. The resultant fractions were separated further by Size exclusion column chromatography method based on the size of compounds present and complexity of the fraction [46; 49]. The resultant partially fractionated analytes (fractions) were purified by use of high resolution preparative High performance liquid chromatography (PHPLC) to eliminate most of unwanted aggregates of similar size [49]. During purification, a small amount of 5 mg of the resultant partially purified fractions was weighed using an electronic balance and mixed with 5 mL of absolute methanol (MeOH). The gradient eluent was set on the HPLC system starting with high aqueous conditions, that is, 5% acetonitrile (MeCN) in 95% water mixed with 0.1% formic acid (buffer). About 15-25 µL of the fraction was automatically injected severally into the mobile phase system through the column (Phenomenex Luna C8, 250 x 21.20 mm, 5µm). The organic proportion of the eluent was increased over time to elute all the compounds off the column at 100% acetonitrile (MeCN). Identification of the known pure compounds was done using high resolution electrospray ionization time of flight mass spectrometry (HR-ESI-TOF-LC/MS) [1], incorporated with chemical fingerprint software and a photodiode array detector, to obtain molecular weight and molecular formula of chemical structures, based on the resultant spectral data and comparison of its properties and spectral characteristics with those in literature and online database libraries. Dereplication technique was used to detect compound’s similarities or differences for any match with known structural and biological characteristics using online database libraries [51].

C. Antifungal Assay

The fungal pathogens used for *in vitro* testing were, *Zymoseptoria tritici* ATCC 26518 strain 184, *Ustilago maydis* DAS strain, *Pyricularia oryzae* strain 164, *Fusarium graminearum* strain 2-0369, *F. oxysporum* strain LYCO.R2 0-1080 and *F. verticillioides* NRRL 22172 provided by Dow AgroSciences Plant Pathology and Microbiology laboratories. These pathogens were used to evaluate the antifungal activity of the isolated pure compound and compared to the commercial standard fungicide, Azoxystrobin (with β-Methoxyacrylate as the active ingredient) [11]. The 96-well microtiter plate based inhibition of spore germination assay was performed as described previously by [56], with minor modifications. A 2-fold serial dilution of the isolated pure compound and Azoxystrobin were prepared using stock solutions in absolute Methanol (MeOH), which were dried to powder using a SpeedVac rotary (Savant Explorer Thermo Electron

Corporation Speedvac System; Indiana; USA) under vacuum for *in vitro* antifungal assay and Mininimum inhibitory concentration. The dried pure compound and Azoxystrobin were re-dissolved in 2 µL aliquots of dimethyl sulfoxide in sterile distilled water (DMSO: H2O=2:8 v/v=1:4) in the microtiter plate wells. This conveyed a total of 8 concentrations for the isolated pure compound, with a rundown ranging from 33 µg/100 µL to 0.2578125 µg/100 µL, plated from the first to the fourth columns of the microtiter plate. Azoxystrobin too had the same number of 8 concentrations, with a rundown ranging from 0.005 µg/100 µL to 0.000039125 µg/100 µL (5 ppm to 0.015625 ppm), plated from the 5^{th} to 8^{th} columns of the microtiter plate. Dimethyl sulfoxide (DMSO) was used as a negative control, whereby 2 µL were dispensed from the 9^{th} to 12^{th} columns.

The growth medium used for antifungal assay was a synthetic medium consisting of 20 g glucose, 3g Dipotassium-Hydrogen_Phosphate (K_2HPO_4), 3g Potassium-Dihydrogen Phosphate (KH_2PO_4) and 6.7 g of yeast Nitrogen base without amino acids in 1 litre of de-ionized water. Inoculum was prepared by flooding mycelia grown on agar media plates with the above liquid medium to cover the entire surface of the culture and scraped the spores loose with a sterile plastic cell lifter to release the spores and conidia. The inoculum was sieved through a 100 µM nylon mesh filter cell strainer cap (Fisher brand, Thermo Fisher Scientific In., Waltham, Massachusetts, U.S.A) to remove any mycelia debris. Initial inoculum densities were adjusted to 100,000 conidia/or spores/mL for all *Fusarium* pathogens and *Zymoseptoria tritici*, 40,000 spores/mL for *Pyricularia oryzae* and 50,000 spores/mL for *Ustilago maydis*, because these fungal pathogens have different spore sizes and they grow at different rates. Aliquots of 200 µL of the standardized inoculum were dispensed into each microtiter plate well, using a Matrix well-mate multi-dispenser machine (Dow AgroSciences, Indiana; USA). The inoculated microtiter plates were incubated in the dark for 48 hours (*U. maydis*) at 24°C or 72 hours (*P. oryzae* and *Z. tritici*) at 22°C. *Fusarium graminearum*, *F. verticillioides*, and *F. oxysporum* were incubated at 25°C for 72 hours. Initial and final fungal cell/spore density readings were determined using a NepheloStar Nephelometer (BMG Labtech GmbH, D-77799 Ortenberg, Germany). Assays were run in triplicates and percentage inhibition of spore germination was calculated by reference to control wells containing only growth media amended with 2 µL DMSO and inoculum; using the formula by [5], with a few modifications [56].

[i] % inhibition of spore germination= $\mathbf{\frac{[N-(FA_1-IA_1)]}{N}*100}$

Where N= Normal spores density in wells with DMSO only inoculated with test-organism.
FA_1= Final spore density reading in well A_1 inoculated with test-organism and treated with the test compound.
IA_1= Initial spore density reading in well A_1 inoculated with test-organism and treated with the test compound.
Normal spore density (N) = Final spore density reading in wells with DMSO and test-organism after incubation-Initial spore density reading in wells with DMSO and test-organism before incubation.

D. Greenhouse Evaluation of the Isolated Pure Compound

➢ *Plant Material and Pathogen Inoculation*

The greenhouse efficacy trial was performed according to [56] method. The efficacy of Vernodalol against *Z. tritici* (wheat leaf blotch), *Puccinia triticina* (wheat leaf rust) and *Helminthosporium sativum* (wheat spot blotch of leaf) was tested on wheat (Yuma variety) as the host plant. Seeds (8-12 per pot) were planted into plastic pots with surface area of 27.5 square centimetres (cm^2) containing 50% mineral soil/50% soil-less Metro Mix™ (Scotts, Marrysville, OH), and grown in greenhouse 16 hours in light at 20°C for 7-8 days. The wheat plants were inoculated with aqueous suspension of spores prepared from stock cultures maintained on agar plates (*Z. tritici* and *H. sativum*) and delivered as a fine mist using a Devilbiss sprayer. Spore concentrations were adjusted to 1.0 x 10^7 (*Z. tritici*) and 5.0 x 10^4 (*H. sativum*) spores mL^{-1} prior to addition of Tween 20 at a 0.05% v/v final concentration to facilitate leaf wetting and coverage. Inoculum of *P. triticina* was prepared by suspending harvested urediospores (1 g) in water with the aid of Tween 20, again added at a final concentration of 0.05% v/v. Spore suspensions were filtered through two layers of cheese cloth, and the filtrate was used to inoculate host plants. Plants were inoculated either 3 days prior to (3 day curative timing) or 1 day post (1 day protectant timing) application of Vernodalol. Inoculated wheat plants were kept in a dark dew chamber at 20-22°C and 100% humidity 24 hours (*P. triticina*) or 72 hours (*Z. tritici* and *H. sativum*) to support spore germination and host leaf infection. The infected plants were transferred to greenhouse maintaining temperatures of 20°C (*Z. tritici* and *H. sativum*) or 24°C (*P. triticina*) until disease symptoms were fully expressed on the first leaf of untreated plants. Tests were carried out in triplicate and percentage disease control was calculated using the ratio of disease on treated plants related to untreated plants.

➢ *Test Compound Formulation and Application on Infected Wheat Plants*

For the efficacy testing of *Z. tritici*, *P. triticina* and *H. sativum*, test compound application was made at low spray volume typical of field application practices. For this purpose, an early stage Emulsifiable Concentrate (EC) formulation of the test compound was used along with a standard commercial fungicide (Azoxystrobin) formulation. All formulations were completed using distilled water. The formulated compound and Azoxystrobin were evaluated using rates of 80, 20, 5 and 1.25 g ha^{-1}. The formulated materials were applied to the wheat plants with a spray volume of 200 L ha^{-1}, at a concentration of 50 and 200 ppm using a track sprayer (DeVries Manufacturing, Hollanale, MN, USA) equipped with a Tee Jet 8003E spray nozzle operating at 32 psi. The track speed was 1.8 miles h^{-1}, and the distance between nozzle and bench was 25 in. Treated plants were allowed to dry for 1 hour prior to handling. For tests involving high volume spray application, the test compound and Azoxystrobin were dissolved in acetone,

from which further serial dilutions were made. Final spray solutions were obtained by mixing aliquots of the acetone solutions with 9 volumes of water containing 110 ppm of Triton X-100 to deliver concentrations of 80, 20, 5, 1.25 mg L^{-1}. Applications to seedling plants were made to run off 24 hours prior to inoculation, using an automated turntable sprayer (DeVries Manufacturing, Hollandale USA) fitted with two 6218-1/4 JAUPM spray nozzles (spraying systems Co., Wheaton, IL USA) and operated at 25 psi.

E. Data Analysis

Data analysis was done using R Guide program software version Rx64-bit 3.3.3 for Analysis of Variance (ANOVA) to determine significant factors/or variables that influenced inhibition of spore germination. Tukey's Honest Significant Difference (THSD) test was used for comparison of inhibition of spore germination and determined which factors were significantly different in influencing the inhibition [58].

III. RESULTS

The present research yielded a known compound, Vernodalol (57 mg weight), light green in a glistening oily solid state; UV λmax 254nm, 210 nm; HR-ESI-MS m/z 393.1530, with species ion [M+H]+; Molecular formula C20H24O8; exact mass of 392.146 D; Relative probability score of 93.7% depicting a high chance of being the correct formula; a high double bond equivalent (DBE) of 9 showing presence of unsaturation or double bonds in the compound structure (Table 1 and Figure 1).

Formula	Mass	Species	m/z	Diff (ppm)	Score (MFG)	DBE
C20 H24 O8	392.146	(M+H)+	393.1530	2.93	93.7	9

Table 1:- Molecular formula of Vernodalol of species ion $[M+H]^+$; exact mass 392.146 D, mass-to-charge ratio (*m/z*) 393.1530, relative probability score of 93.7% and a double bond equivalent (DBE) of 9.

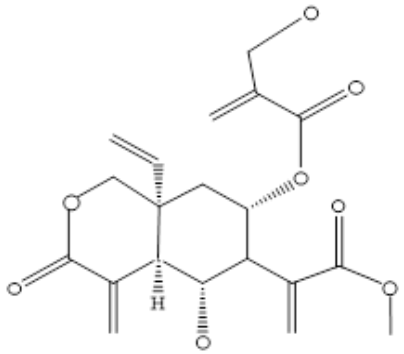

Fig 1:- Chemical Structure of Vernodalol isolated from *Vernonia glabra* flower extract, with molecular formula of $C_{20}H_{24}O_8$ and molecular weight of 392. 146 Da.

A. Significant Factors that Determined the Cause of Antifungal Activity

Data analysis showed that the effect of Vernodalol on inhibiting spore germination of *Z. tritici*, *U. maydis, P. oryzae, Fusarium graminearum, F. oxysporum* and *F. verticillioides,* was influenced by the type of chemical class used and the test-organisms screened for the antifungal activity, which exhibited high significance at p≤0.000 (Table 2). The different levels of the chemical concentration obtained after 2-fold serial dilutions, were less significant at p≤0.001 in determining the minimum inhibitory concentrations of Vernodalol compared to Azoxystrobin.

	Df	Sum Sq.	Mean Sq.	F value	Pr (>F)
Chemical Class	2	89477	44739	260.845	<2e-16 ***
Chemical conc.	15	6870	491	2.861	0.000955 **
Test-organism	5	16923	2820	16.444	8.66e-14 ***
Residuals	121	20753	172		

Table 2:- The data in the table depicts that the type of compound class used, compound concentration, and test-organism were the significant factors/or variables that determined the inhibition of spore germination

-----Significant codes: 0.000 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Df=Degrees of freedom;
Sum Sq=Sum of squares;
Mean Sq=Mean sum of squares;
Pr (>F) =P value; DMSO-Dimethyl sulfoxide.

*Footnote

Based on Analysis of Variance (ANOVA), the type of chemical class used, chemical concentration, and test-organism factors (variables) were significant at P≤0.05.

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B. Antifungal Activity of Vernodalol Compared to a Standard Fungicide, Azoxystrobin

Figure 2: shows inhibition of spore germination by Vernodalol, at a concentration of 33 µg/100 µL compared to Azoxystrobin at a concentration of 0.005 µg/100 µL (5 ppm). Vernodalol showed moderate activity of 42% inhibition of spore germination against *Zymoseptoria tritici* and weak activity of 20% against *Fusarium oxysporum*, *Ustilago maydis* (8%), *Pyricularia oryzae* (10%), *Fusarium graminearum* (14%), and *F. verticillioides* (4%). Azoxystrobin had a strong activity against *Zymoseptoria tritici* (100%) at a low concentration of 0.005 µg/100 µL, compared to Vernodalol's which was less active (Table 3). The negative control dimethyl sulfoxide (DMSO) was not active.

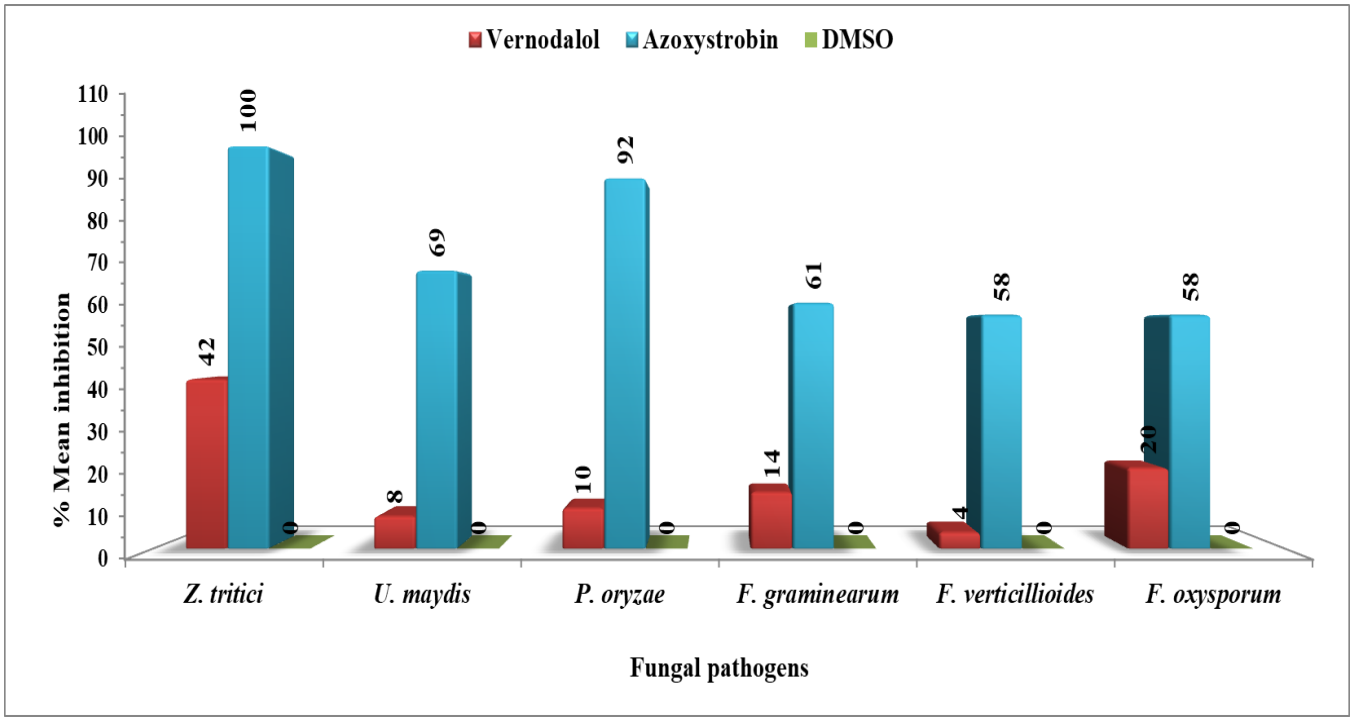

Fig 2:- Percentage inhibition of spore germination by Vernodalol at 33 µg/100 µL concentration against *Z. tritici*, *U. maydis*, *P. oryzae*, *F. graminearum*, *F. verticillioides*, and *F. oxysporum* compared to Azoxystrobin at 0.005 µg/100 µL

C. Minimum Inhibitory Concentration of Vernodalol Compared to Azoxystrobin

Table 3 depicts the effect of chemical concentration as a factor that determined the minimum inhibitory concentration of Vernodalol, which inhibited spore germination against *Z. tritici*, *U. maydis*, *P. oryzae*, *F. graminearum*, *F. oxysporum*, and *F. verticillioides* at 0.26-33 µg/100 µL compared to Azoxystrobin at 0.000039-0.005 µg/100 µL. Vernodalol compound displayed minimum inhibitory concentrations (M.I.Cs) at 8.25 µg/100 µL against *F. verticillioides*, 1.03 µg/100 µL against *U. maydis*, and 0.51 µg/100 µL against *P. oryzae*, *F. graminearum*, and *F. oxysporum*. No M.I.C was determined on Vernodalol against *Z. tritici* at low concentrations, even though its percentage inhibition of spore germination was weak compared to Azoxystrobin. Azoxystrobin's activity was strong even at very low concentrations, and that resulted in a lack of M.I.C, determination against all six tested fungal pathogens.

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Chemical class	Compound Concentration (µg/100 µL) from serial dilutions	Test-organisms /M.I.Cs formed due to % inhibition of spore germination					
		Z. tritici	*U. maydis*	*P. oryzae*	*F. graminearum*	*F. oxysporum*	*F. verticillioides*
Vernodalol	33	42	8	10	14	20	4
	16.5	35	7	10	10	18	3
	8.25	35	4	10	10	16	2
	4.125	30	4	7	9	14	0
	2.0625	29	2	6	9	11	0
	1.03125	26	1	4	4	7	0
	0.515625	18	0	4	3	1	0
	0.2578125	11	0	0	0	0	0
Azoxystrobin	0.005	100	69	92	61	58	58
	0.0025	97	68	90	54	55	51
	0.00125	96	65	90	46	50	45
	0.000625	96	65	89	35	38	32
	0.0003125	96	64	89	26	29	20
	0.0001565	94	61	87	19	22	16
	0.00007825	91	57	81	18	20	13
	0.000039125	79	43	76	8	17	10
DMSO	2	0	0	0	0	0	0
	2	0	0	0	0	0	0
	2	0	0	0	0	0	0
	2	0	0	0	0	0	0
	2	0	0	0	0	0	0
	2	0	0	0	0	0	0
	2	0	0	0	0	0	0
	2	0	0	0	0	0	0

Table 3:- The data presented in the table shows the effect of compound concentration in determining the minimum inhibitory concentration caused by Vernodalol compared to Azoxystrobin against six test fungal pathogens

D. Greenhouse Efficacy of Isolated Vernodalol Compound Compared to Azoxystrobin at 50 and 200 Ppm

The Vernodalol compound tested under greenhouse conditions for 1 day protectant timing (1DP) against *Zymoseptoria tritici*, *Helminthosporium sativum*, and *Puccinia triticina* and 3-day curative timing (3DC) against *Z. tritici* only, did not show any activity or disease control. However, the standard fungicide, Azoxystrobin displayed strong antifungal activity of 100% disease control in 1 Day protectant timing against *Zymoeptoria tritici* and *Puccinia triticina*. At 50 and 200 ppm, Azoxystrobin had 93% and 94% disease control in 1 day protectant timing against *Helminthosporium sativum* respectively. In 3-Day curative timing, Azoxystrobin showed 100% activity against *Z. tritici.*

IV. DISCUSSION

This research yielded a pure compound, known as Vernodalol which is a sesquiterpene lactone. Vernodalol's availability in *V. glabra* from this study was found to be moderate (57 mg). Chadwick [36] reported that sesquiterpene lactones are functional compounds and are liable to change in concentration during plant development according to plant needs. An example is when a plant undergoes floral transition and while in this period, it is likely to produce more defensive compounds to protect its investment in reproductive structures. This means significant changes occur in the plant in a spatial, temporal, and species dependent manner and due to plant use of the produced compounds, the isolated amounts and concentration may turn out to be low [36].

Vernodalol which is classified under a subclass sesquiterpene lactone and in terpenoid class is one of the secondary metabolites produced by plants in various chemical classes [57].The chemical class was one of the factors or variables that influenced the inhibition of spore germination against test fungal pathogens *S. tritici*, *U. maydis*, *P. oryzae*, *F. oxysporum*, *F. graminearum*, and *F. verticillioides*.

Structural features of chemical compounds in different chemical classes may influence the mechanism of action of the chemical compounds [34; 16]. According to [33], various reactions of chemical compounds with biological systems, may determine the functional group responsible for evoking a target biological effect in the organism, and it may be interpreted as susceptible, intermediate or resistant.

Sesquiterpene lactones are a subclass of terpenoids and the most distinctive secondary metabolites of the members of plants within the Asteraceae family [35]. However they have been reported from several plant families such as Acanthaceae, Amaranthaceae, Apiaceae, and Magnoliaceae [35]. Guillermo and Soad [21; 35] revealed that most of sesquiterpene lactones display a wide range of activities such as antimicrobial, and act by disrupting the cell wall and cell membrane of fungi and invasive bacteria. They are also antioxidant, insecticidal, and antifeedants to deter herbivores and inhibit growth of competing plants. These activities are mainly related to their characterizing α-methyl-γ-lactone group or α-β-unsaturated carbonyl moiety [35; 21].

Vernodalol isolated from flower extract of *V. glabra* in this study, is a known sesquiterpene lactone [39; 44; 24]. Results of this study exhibited only moderate antifungal activity (42% inhibition of spore germination) against *Z. tritici* and weak antifungal activity to all other five fungal pathogens tested at concentration of 33 µg/100 µL. The inhibition of spore germination of all six fungal pathogens was an indication that Vernodalol possess a broad spectrum antifungal activity.

Erasto *et al*. [45], reported that the activity of Vernodalol is based on the structural features present, that is, the number of functional groups and substituents, whereby Vernodalol has three α-β-unsaturated carbonyl carbons (ketones) and two hydroxyl groups. There is also the presence of an ethenyl (-CH=CH_2) group at C-10, which is free to rotate through the C1-C10 bond so as to attain stable configuration and thus make it more accessible for free reaction, thus leading to antifungal activity. These structural properties may have contributed to the moderate activity of inhibition of spore germination against *Z. tritici*.

Some properties of Vernodalol may have contributed to the weak antifungal activity against all other five fungi tested in this study. Jisaka *et al*. [38] reported that the low activity of Vernodalol could be explained by the loss of hydrophobicity in the acyl moiety.

Wedge *et al*. [9] also observed that the most effective Sesquiterpene lactones are those that contain α-methyl-γ-lactone group (α-M-γ-L), but lack bulky sterically inhibitory groups, which limit access to the α-M-γ-L. The two hydroxyl groups may have contributed to antifungal activity by being able to provide hydrogen bond and increase solubility of the compound for easier interaction with the polar interface of the biological target site.

The concentration of Vernodalol was less significant at P≤0.001 in influencing inhibition of spore germination against the six test fungal pathogens; *S. tritici*, *U. maydis*, *P.oryzae*, *F. graminearum*, *F. oxysporum*, and *F. verticillioides*. Medium to weak antifungal activity of Vernodalol was exhibited despite the use of high concentration of 33 µg/100 µL, compared to the low concentration of Azoxystrobin of 0.005 µg/100 µL which showed strong activity. This observation could be attributed to the presence of low concentration of the active ingredients in the compound or the compound might not be active at all. Nazzaro *et al*. [20] reported that the effect of chemical constituents depends on their amount in pure compounds. According to Charianopoulas *et al*. [42], the presence of other components at very low concentrations may result in synergic, additive, or antagonistic interactions which may lead to strong, medium or no activity.

Solvent artefacts formation could not be overlooked, in that, it may have formed and led to loss of activity of the active ingredients and loss in total yield of active ingredients during isolation and it may be attributed to weak antifungal activity or no activity of the isolated Vernodalol in this study. This observation is in line with Maltese *et al*. [19] who in their studies reported that artefacts formation was associated with weak inhibitory activity or no activity at all.

Sensitivity of test fungi to the isolated Vernodalol used in this study was varied depending on fungal status; such as susceptibility and resistance. *Zymoseptoria tritici* was more susceptible as it was the most affected with higher antifungal activity at high concentration of 33 µg/100 µL. *Fusarium oxysporum*, *F. graminearun*, *Pyricularia oryzae*, *Ustilago maydis*, and *F. verticillioides* were not susceptible when same concentrations were used. All the six tested fungal pathogens were highly susceptible to Azoxystrobin; a standard commercial fungicide at 0.005 µg/100 µL. Chemical concentration was less significant in inhibition of spore germination because very weak activity was reported at high and low concentrations in most fungi.

This difference in sensitivity of the fungal pathogens could be explained by the development of strategies that fungi use to neutralize or overcome detrimental effects of the chemical compounds or there is a possibility that the compound is inactive. According to [30], fungal pathogens develop resistance to antifungals by mutating or overexpressing the genes coding for enzymes that destroy the active site of the chemical compounds and reduce the intercellular accumulation of the toxic compounds, rendering chemical compounds less active or inactive.

The greenhouse *in vivo* efficacy of the isolated Vernodalol did not exhibit any disease control, that is, it didn't display 1-day protectant and 3-day curative properties, although compared to *in vitro* efficacy results, Vernodalol showed medium activity. Therefore it is expected that the efficacy in greenhouse would be low or no activity at all. This observation could have occurred due to tough environmental conditions that natural product antifungals are exposed to in the field. Based on literature [26], there are several factors that could have contributed to poor greenhouse performance, including; environmental conditions, fungal resistance, crop-fungal interactions, chemical compound metabolism, and physico-chemical properties of the applied chemical compound.

Some of the environmental conditions that may have contributed to loss of efficacy of Vernodalol against the whole fungal plant pathogens may have included enhanced fungal and light degradation of the natural chemical compound, Vernodalol; while temperature could have been involved in decomposition of the compound. This argument is in line with Copping *et al*. [32] and Tyson *et al*. [27], who found out that, the dose rate of chemical compounds to be used for efficacy may have been too low due to light degradation and microbial enzyme degradation of the chemical compounds, or fungal mutation which renders the chemical compounds inactive and hence resistance, while temperature could be involved in decomposition of the chemical compounds. The authors also mentioned the crop-fungal interactions and high humidity which may have caused increased fungal growth to high populations or high inoculum (population dynamics) that could have enhanced fungal pathogens' severity and out shadowed chemical compounds' activity respectively, while low humidity may have caused evaporation of the dose rate.

Based on greenhouse results of this study, there could have been poor spreading of chemical compound, Vernodalol on plant canopy, poor permeability of formulated chemical compounds and poor distribution of chemical compounds within the target plant parts. Base on literature [32], these factors could have caused poor surface coverage of plant canopy, poor penetration of the chemical compound due to waxy cuticle of plant leaves, poor translocation of the chemical compound, and metabolism of the chemical compound by plants could have converted the chemical compounds into stored substances or they could have been neutralized into inactive substances.

According to Copping *et al*. [32], the physico-chemical properties of the chemical compound, such as the spray solution behavior in water as a solvent and behavior in spray droplets, may have caused the activity or selectivity of the chemical compound not to be optimized due to varying crystallization (expression) and placement on leaves' surface of the plants.

V. CONCLUSION

The flower extract of *Vernonia glabra* (Asteraceae family) yielded a sesquiterpene lactone, Vernodalol. Vernodalol has different functional groups such as free hydroxyl groups, α-β unsaturated C=C double bonds, conjugated carbonyl groups, methoxy group, and α-Methylene-γ-lactone.

These functional groups have properties such as electrophilic, nucleophilic, hydrophilic, hydrophobic, hydrogen bonding with the surrounding atoms in the aromatic rings and interaction with target biological sites, and toxicity, which are attributed to inhibition of spore germination against *Zymoseptoria tritici*, *Ustilago maydis*, *Pyricularia oryzae*, *Fusarium graminearum*, *F. verticillioides*, and *F. oxysporum*, rendering Vernodalol a broad spectrum antifungal activity.

However, further research on the structural modification of Vernodalol may be required to improve its stability and activity both *in-vitro* and in the field, against recurring aggressive agricultural pathogens and could have potential to be used by farmers in Kenya as alternative, cheap, and environmentally safe biofungicide in crop protection.

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