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MANAGEMENT OF OCHRATOXIGENIC FUNGI BY PHYTOCHEMICALS OF *AMARANTHUS VIRIDIS* L.

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ABSTRACT

Ochratoxigenic fungal species are a major cause of various infections in plants and posed serious threat to their consumers, including humans and animals. The objective of this study was to examine the *in vitro* efficacy of different concentrations of leaves methanolic extract of *Amaranthus viridis* L. against target pathogenic ochratoxin producing fungal species (*Trichoderma viride* Pers., *Trichoderma harzianum* Rifai. and *Cladosporium cladosporioides* (Fresen.) G.A. de Vries). For this purpose, different concentrations viz. 2%, 4%, 6%, 8% and 10% of leaf methanolic extract was prepared and tested for their antifungal potential in a completely randomized design (CRD). Results revealed that a 10% concentration of *A. viridis* significantly suppressed the growth of all the tested fungi. The phytochemical analysis of *A. viridis* depicts the presence of glycosides, alkaloids, saponins, tannins and coumarins. Bioassay-guided fractionation was executed to check the antifungal activity of various fractions *in vitro* against the test ochratoxin producing fungi. The ethyl acetate fraction showed the highest antifungal activity among all other fractions. GC-MS (Gas chromatography-mass spectroscopy) analysis of ethyl acetate fraction revealed the presence of twelve compounds viz. 1,2,3-propanetriol monoacetate; *n*-decanoic acid; 1-hexadecene; dodecanoic acid; 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-[R]; tetradecanoic acid, 2-pentadecanone; 6,10,14-trimethyl-; phenol,2- methyl-5-[1-methylethyl]-; *n*-hxadecanoic acid; 2[4H]-benzofuranone; phytol; 9,12- octadecadienoic acid and heptacosane. The presence of these compounds might be responsible for the antifungal potential of *A. viridis* and it can be concluded that the methanolic leaf extract of the tested plant proved to be beneficial for inhibiting the growth of test ochratoxigenic fungi.

Keywords: Bioassay, GC-MS, ochratoxin, phytochemicals.

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INTRODUCTION

Mycotoxicoses is a disease caused due to the production of secondary metabolites known as mycotoxins in plants and animals. The consumption of myctoxins affects the quality of foodstuff, which leads to the development of various diseases in consumers (Jeswal and Kumar 2015). Mycotoxins are odourless, tasteless and colourless compounds that occur sporadically in nature (Fapohunda 2014). According to data, up to 25% of the crops worldwide are contaminated by mycotoxins, and around 4.5-5.0 billion individualsare at the threat of chronic mycotoxins exposure (Tiffany 2013). Mycotoxin producing fungi are ubiquitous and commonly associated with cereal crops. Mycotoxins also caused contamination of eggs, meat, coffee, milk and wines (Okello *et al.,* 2010). The production of mycotoxins depends upon agronomic practices, topographical zones and the dissemination of fungal species (Jonathan and Esho 2010). The significant mycotoxins are aflatoxins and ochratoxin produced by Penicillium, Aspergillus, Fusarium, Alternaria, Trichoderma, Trichotheium species (Shephard 2008). Cladosporium species also causes pre and post-harvest ochratoxin infections (Tasic and Tasic 2007). Cladosporium species produced iso-cladosporium, ergot alkaloids, cladosporin and emodin mycotoxins. On the other hand, despite the biocontrol effect of Trichoderma genera, these fungi are also responsible for producing mycotoxin like trichothecene, trichodermin and harziandione (Shentu *et al.,* 2014).

Plant-based natural products have been used for centuries in disease prevention caused by microorganisms. Pre and post-harvest strategies are needed to lessen the mycotoxin contamination threat in food, including biocontrol, suitable cultural applications, use of resistant varieties and detoxification of mycotoxins (Richard 2003; Dorner 2004; Bhatnagar 2010). The chemicals used to detoxify the agronomic foodstuffs contaminated with mycotoxins create environmental pollution (Kohl *et al.,* 2011). Hazardous effects of these chemicals can be reduced by using plant-based natural products (Bashir *et al.,* 2019; Naeem *et al.,* 2020). Plants contain a vast range of secondary metabolites, and these chemicals serve to safeguard plants against pathogen attack. Plant extracts comprised of harmless antifungal products which can control phytopathogens (Khan *et al.,* 2018).

Amaranthus viridis "chowlai" belongs to the family Amaranthaceae is well known for producing

phytochemicals such as rutin, quercetin and flavonoids. *A. viridis* also possess antioxidant, antimicrobial and anticancer potential due to the vast wide existence of flavonoids. The plant leaves also proved to be beneficial in the healing of various inflammations, acne problems and skin laxatives as well (Bagepalli *et al.,* 2009). Its is widely occurring in Asia, specifically in rural areas of Pakistan, where it used as a leafy vegetable. Despite that, very little data is available related to the pharmacognostic, phytochemical and nutritional importance of this plant (Khan and Khan 2012). So, the present work was planned to search for eco-friendly antifungal phytochemicals from *A. viridis* against target ochratoxigenic fungal species.

MATERIALS AND METHODS

Leaves of *A. viridis* (100 g) were surface sterilized using 10% sodium hypochlorite followed by sun-drying and grinding. The test ochratoxin producing fungal strains *Trichoderma viride* (FCBP-671)*, Trichoderma harzianum* (FCBP-1277) and *Cladosporium cladosporioides* (FCBP-976) were obtained from Fungal Culture Bank, University of the Punjab, Lahore and the cultures were preserved on 2% MEA (Malt Extract Agar).

Antifungal activity of *A. viridis* was evaluated against the test ochratoxigenic fungi. An investigation was conducted in Plant Physiology and Fungal Biotechnology Laboratory, Lahore College for Women University, Lahore, in Sept-Oct 2019 using the protocol of (Sherazi *et al.,* 2016). For this purpose, 250 mL of methanol was used to soak 100 g test plant material. It was then subjected to filtration using an autoclaved muslin cloth to obtain the crude mass of *A. viridis*.

Five concentrations of test plant material viz. 2%, 4%, 6%, 8% were made by adding and thoroughly mixing 3, 6, 9, 12 and 15 mL of stock solutions in 27, 24, 21, 18, 15 mL of Malt Extract broth medium to make total volume upto 30 mL. The *in vitro* experiment was conducted in a completely randomized design. Three replicates were made for each treatment, while plant extract was not included in the control treatment. All respective flasks were then allowed to incubate for a week at 25 °C after being endowed with 50 mg chloromycetin. Media in incubated flasks was then filtered using pre-weighed Whatman no. 1 filter papers after a week, and their dry weight was recorded after oven drying for 1-2 hours for each flask. The inhibition potency of each concentration towards each fungal strain was calculated using the formula mentioned below:

$$\text{Growth inhibition (\%)} = \frac{G\ \ ii\ c\ \ -G\ \ ii\ ti}{G\ \ ii\ c} \times 100$$

For further studies, methanol extract was segregated using solvents chloroform, *n*-hexane, *n*-butanol and ethyl acetate according to their polarity. The extract was evaporated at room temperature, and crude gluey masses of each fraction were attained. Using agar serial dilution method, in vitro antifungal activity of all the organic fractions by making two concentrations viz. 0.01% and 0.1% using the methodology of Jabeen and Javaid (2008) was conducted with three replicates for each concentration.

The methanol extract was tested to identify the existence of biochemical entities/phytochemicals that accounts for its antifungal potential (Parekh and Chanda, 2007).

After confirming the presence of secondary metabolites, further chemical analysis for the best fraction, i.e. ethyl acetate, was carried out via Gas Chromatography-Mass Spectroscopy. For GC-MS examination sample was run on a chromatogram (GC-MS-QP 2010) separated by capillary column DB-5MS (0.25 µm, 0.25 mm, 30 m). The helium gas was used as carrier gas. Temperature adjustment for the analysis was as follows: 150-220 °C at 10 °C min^{-1}, 220 °C for 2 min, 70-120 °C at 3°C min^{-1}, 70 °C for 2 min, 40-70 °C at 2 °C min^{-1} and 40 °C for 5 min. While the temperature of the injector was 200 °C and the detector temperature was 250 °C. Mass detector settings were source temperature 230 ºC, m/z 29-540 mass scanning range and 70 eV ionization voltages. The volatile compounds percentage proportion was calculated from GC peak regions. The qualitative examination was constructed on mass spectra, indices and retention time comparison was madmade with the matching records from the NIST Library 2010 (word software).

A one way analysis of variance (ANOVA) followed by least significant difference (LSD) using Statistix 8.1 software was used to scrutinize data statistically at a significance level of $P \leq 0.05$.

RESULTS AND DISCUSSION

In current studies, methanolic extract of *A. viridis* was used for controlling the *in vitro* growth of ochratoxin producing test fungal species. Different plant concentrations viz. 2%, 4%, 6%, 8% and 10% were made. All the applied concentrations significantly suppressed the test fungi. The 8% and 10% conc. of *A. viridis* inhibited the biomass of *T. viride* and *T. harzianum* upto 64% and 60%, respectively, compared to control. The 10% conc. of *A. viridis* also successfully retarded the growth of *C. cladosporioides* upto 80%. However, minimum reduction in the *in vitro* growth of *T. viride, T. harzianum* and *C. cladosporioides* was observed at 2% concentration, i.e. 48%, 42% and 40%, respectively (Fig. 1-3). Earlier Sadiqa *et al.* (2015) reported that leaves, stem and roots of *A. viridis* caused a significant reduction in the biomass of *Botrytis cinerea*. *A. viridis* is also well known for isolating various

antimicrobial and antioxidant compounds (Sarwar *et al.,* 2016). The test plant *A. viridis* possesses the potential of inhibiting the conidial germination of various pathogenic fungi (Yusoff *et al.,* 2017).

Phytochemical analysis of *A. viridis* revealed saponins, alkaloids, coumarins, glycosides and tannins (Table 1). These phytochemicals probably played a role in the antifungal activity of *A. viridis* against ochratoxigenic fungi. The existence of alkaloids, saponins, flavonoids, tannins and phenolics, having the potential to reduce fungal growth, has been reported (Sivagnanam 2016). The test plant devours the possibility to synthesize a wide range of such antimicrobial compounds (Sadia *et al.,* 2016).

The leaf methanolic extract of *A. viridis* was subjected to fractionation, and different isolated organic fractions were tested against ochratoxin producing fungi. Two concentrations, i.e. 0.1% and 0.10%, of all the isolated fractions (*n*-hexane, *n*-butanol, chloroform, ethyl acetate, and the synthetic fungicide (thiophanate methyl) were assessed. While distilled water which was devoid of any plant extract used as a negative control. Results depicted that at 0.10% concentration of ethyl acetate fraction ascertained to be the best in retarding the biomass of *T. viride* upto 78% *T. harzianum* upto 75% and *C. cladosporioides* 80% (Fig. 4-6). It is probably that diverse natures of compounds affect their solubility in different types of solvents according to their polarity. Earlier the antimicrobial activity of *A. viridis* has been evaluated and found promising results. The ethyl acetate fraction of the seeds of *Citrullus lanatus* markedly showed antimicrobial potential (Maiyo *et al.,* 2010).

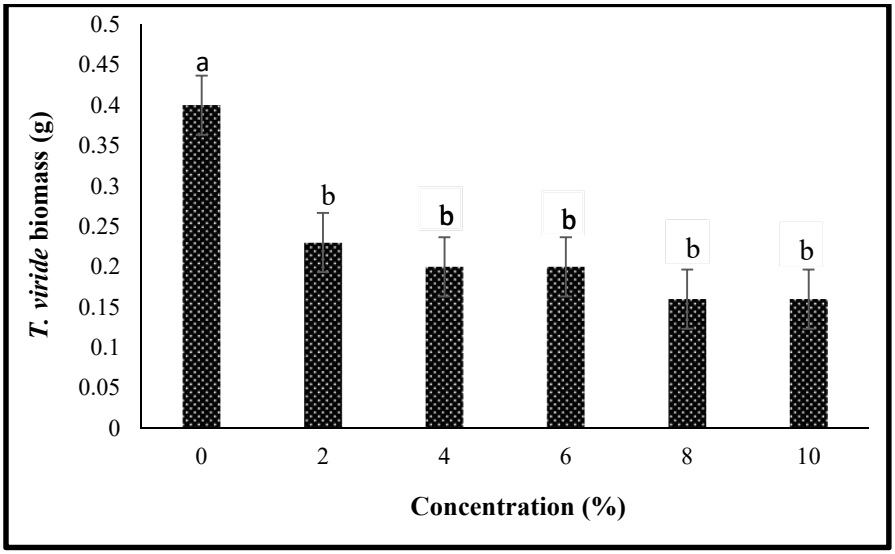

Fig 1: Effect of various concentrations of methanolic extract of *Amaranthus viridis* on *in vitro* growth of *Trichoderma viride*. Values with different letters show significant differences as determined by LSD (P = 0.05). Each value is an average of three replicates.

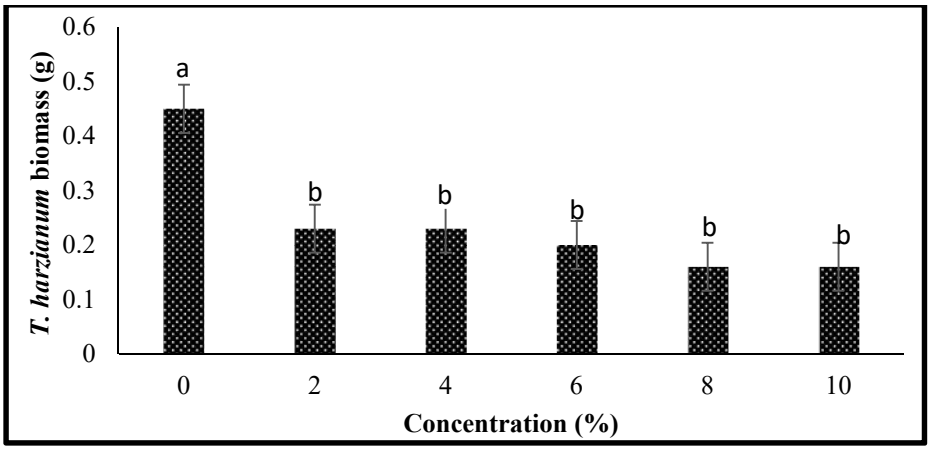

Fig 2: Effect of various concentrations of methanolic extract of *Amaranthus viridis* on *in vitro* growth of *Trichoderma harzianum*. Values with different letters show significant differences as determined by LSD (P = 0.05). Each value is an average of three replicates.

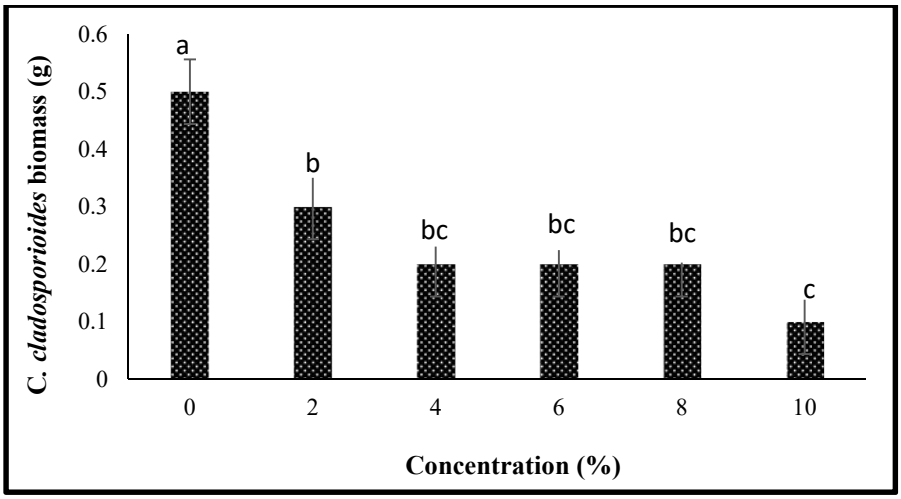

Fig 3: Effect of various concentrations of methanolic extract of *Amaranthus viridis* on *in vitro* growth of *Cladosporium cladosporioides*. Values with different letters show significant differences as determined by LSD (P = 0.05). Each value is an average of three replicates.

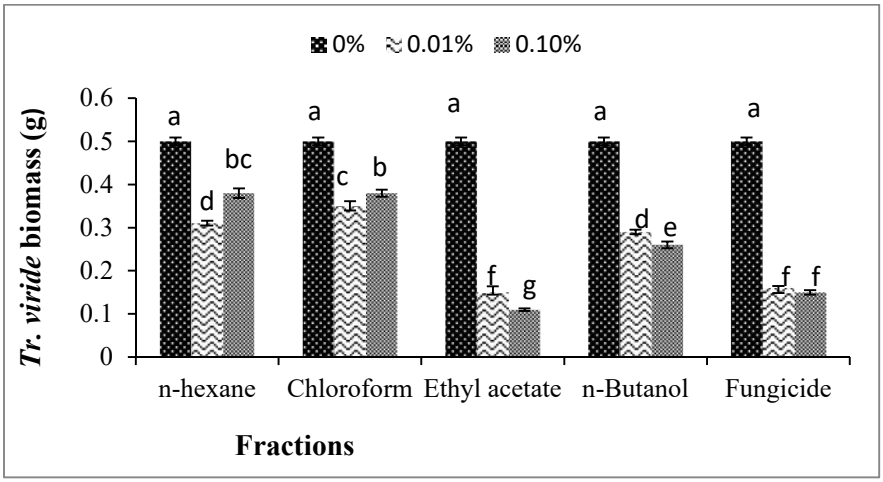

Fig 4: Effect of different fractions of methanolic extract of *Amaranthus viridis* and fungicide on *in vitro* growth of *Trichoderma viride.* Values with different letters show significant differences as determined by LSD (P = 0.05).

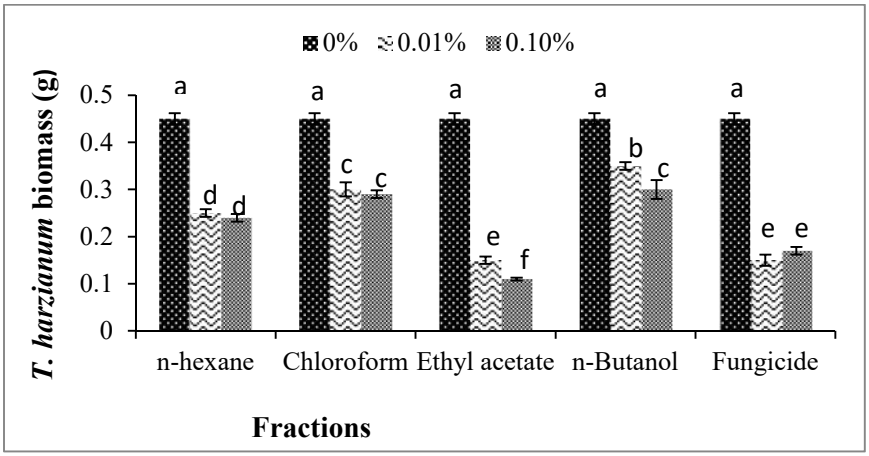

Fig 5: Effect of different fractions of methanolic extract of *Amaranthus viridis* and fungicide on *in vitro* growth of *Trichoderma harzianum.* Values with different letters show significant differences as determined by LSD (P = 0.05).

After, potent phytochemicals were verified in the respective test plant; then, the best-suited fraction,ethyl acetate was selected for compound identification using GC-MS investigation. The results pertaining to GC-MS analysis of ethyl acetate fraction of *A. viridis* lead to identification of 12 bioactive compounds which were 1,2,3-propanetriol monoacetate (alcohol); phenol; 2-methyl-5[1-methylethyl]- ; *n*-decanoic acid (fatty acid); 1-hexadecene (unsaturated hydrocarbon); dodecanoic acid (fatty acids); 2[4H]-benzofuranone (organohetertocyclic compounds); *n*-hexadecanoic acid (saturated fatty acids); tetradecanoic acid (saturated fatty acids); 2-pentadecanone; 6,10,14-trimethyl- (sesquiterpene); phytol (acyclic diterpenoid); 9,12-octadecadienoic acid (polyunsaturated fatty acid) and heptacosane (alkane) respectively (Table 2). 1-hexadecene (0.254%) proved efficient in retarding fungal growth (Babaiwa *et al.,* 2017). 1,2,3-propanetriol monoacetate, *n*-decanoic acid (capric acid), dodecanoic acid/lauric acid and tetradecanoic acid (myristic acid) are all saturated fatty acids identified at peak areas 4.558%, 4.679%, 18.819% and 2.545% are known to possess great antibacterial, antioxidant and antifungal activity. Phenol,2-methyl-5-[1-methylethyl]- identified at peak area 4.132%, also known as carvacrol, is a monoterpenic phenol that occurs in many essential oils of plant and has great significance as antimicrobial, anti-inflammatory, anti-tumour and antioxidant activity (Ezhumalai *et al.,* 2014; Qi *et al.,* 2019). Phytol belongs to diterpene alcohol recognized at the peak area 5.570% having anti-inflammatory and antimicrobial activity (Phatangare *et al.,* 2017). *n*-hexadecanoic acid (26.006%), 2(4h)-benzofuranone (0.755%), and 9,12-octadecadienoic acid (27.890%) reported to possess antifungal, antimicrobial and antimalarial potential (Verma and Bansal 2015). Heptacosane possesses great anti-inflammatory as well as antimicrobial potential identified at the peak area of 2.830%, while 2-pentadecanone, 6,10,14-trimethyl- have antibacterial activity recognized at a peak area of 1.962% (Nurettin *et al.,* 2006).

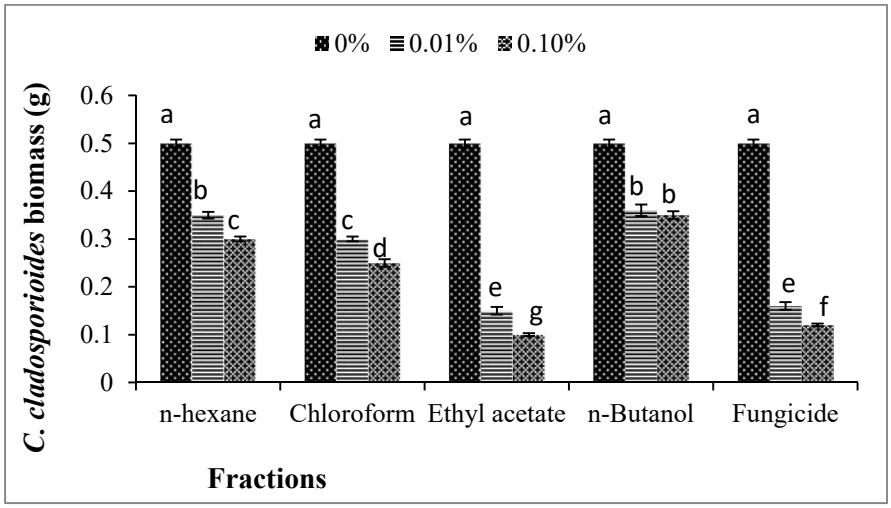

Fig 6: Effect of different fractions of methanolic extract of *Amaranthus viridis* and fungicide on *in vitro* growth of *Cladosporium cladosporioides.* Values with different letters show significant differences as determined by LSD (P = 0.05).

Table 1. Phytochemical investigation of leaf methanolic extract of *A. viridis.*

Phytochemicals	Result
Saponins	++
Terpenoids	-
Alkaloids	++
Phylobatinnins	-
Coumarins	++
Flavanoids	-
Tannins	++
Glycosides	++

Positive sign shows that phytochemicals are present and negative sign shows the absence of phytochemicals.

Table 2. GC-MS exploration of ethyl acetate fraction of *Amaranthus viridis.*

Sr.#	Compound Name	Retention Time	Molecular Weight	Molecular Formula	Peak Area%
1	1,2,3-Propanetriol, monoacetate	9.253	134.13	$C_5H_{10}O_4$	4.558%
2	Phenol,2-methyl-5-[1-methylethyl]-	11.49	294	$C_{19}H_{34}O_2$	4.132%
3	*n*-Decanoic acid	12.000	294	$C_{19}H_{34}O_2$	4.679%
4	1-Hexadecene	13.648	294	$C_{19}H_{34}O_2$	0.254%
5	Dodecanoic acid	14.584	296	$C_{19}H_{36}O_2$	18.819%
6	Tetradecanoic acid	16.617	296	$C_{19}H_{36}O_2$	2.545%
7	2[4H]-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-[R]	15.846	296	$C_{19}H_{36}O_2$	0.755%
8	Phytol	19.924	312	$C_{20}H_{40}O_2$	5.570%
9	*n*-Hexadecanoic acid	18.762	284	$C_{18}H_{36}O_2$	26.006%
10	2-Pentadecanone, 6,10,14-trimethyl-	17.131	284	$C_{18}H_{36}O_2$	1.962%
11	9,12-Octadecadienoic acid	21.002	256	$C_{16}H_{32}O_2$	27.890%
12	Heptacosane	22.053	312	$C_{20}H_{40}O_2$	2.830%

Based on the work conducted, it is suggested that *A. viridis* exhibit a wide range of secondary metabolites which contribute towards its antifungal potential.

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