Antioxidant, Antifungal and Insecticidal Activity of Triterpenoids Spinasterol, 22,23-Dihydrospinasterol Isolated from Colocynthis (*Citrullus colocynthis* L.) Leaves.

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Research article

Keywords: Antioxidant activity, Pesticidal activity, Spinasterol, 22, 23-dihydrospinasterol; LC50; EC50; Citrullus colocynthis

Posted Date: October 28th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-96914/v1

Abstract

Terpenoids from natural plants resources are valuable for diverse biological activities which exhibited important part in medical and agrochemicals industry. This study aimed to assess the antioxidant, antifungal and insecticidal activity of a mixture of Spinasterol, 22,23-dihydrospinasterol isolated from *Citrullus colocynthis* leaves. 1, 1-diphenyl-2-picrylhydrazyl (DPPH) was used to assess the antioxidant activity whereas, antifungal activity was tested by mycelium growth inhibition assay on three pathogenic fungi *Magnaporthe grisea*, *Rhizoctonia solani* and *Phytophthora infestans.* Insecticidal activity against *Brevicoryne brassicae* was also determined by using *In-vitro* and *In-vivo* assays. The outcome of the study exposed that Spinasterol, 22, 23-dihydrospinasterol afforded prominent antioxidant activity even at lower concentrations i.e. 19.98, 31.52, 36.61 and 49.76% at 0.78, 3.0, 12.5 and 50μgmL^{-1} respectively. However, moderate fungicidal activity of Spinasterol, 22; 23-dihydrospinasterol was recorded as being EC_{50} values 129.5 and 206.1μgmL^{-1} against *R. solani* and *M. grisea* respectively. On the other hand, Boscalid and Carbendazim being a positive control proved highly effective against all fungi except for *M. grisea* and *P. infestans* with EC_{50} values 868 and 272109μgmL^{-1} respectively. The pronounced insecticidal activity of Spinasterol, 22,23-dihydrospinasterol was afforded *via* residual as well as greenhouse assay being LC_{50} values as 42.46, 54.86, 180.9 μgmL^{-1} and 32.71, 42.46 and 173.8μgmL^{-1} at 72, 48 and 24 h respectively. The study concluded that isolated compound Spinasterol, 22,23-dihydrospinasterol possess promising antioxidant and aphicidal activity with moderate fungicidal activity which could be a suitable candidate as an alternative to synthetic pesticidal agents.

1. Introduction

Natural plants are venerated source of phytochemical compounds responsible for biological activities and are employed in pharmacological as well as agrochemical industry. Although, synthetic chemicals are easily available source which are widely used as antioxidant, antimicrobial, antifungal and pesticidal purposes, however, their rigorous and continuous use has caused resistance development in pest and also poses harmful effects on human's health and the environmental concerns [1].

*Citrullus colocynthi*s belongs to order Cucurbitales and family Cucurbitaceae is an imperative plant from the medicinal as well as pesticidal point of view. *Citrullus colocynthis* exhibited carcinogenic, antibacterial, antifungal, antidiabetic, antioxidant properties and also possess insecticidal potential against various harmful insects [2–5]. Several biologically active compounds have been described from *C. colocynthis*, including cucurbitacins E, I, J, K and L [6], cucurbitacins glycosides [7, 8], the cucurbitacins glucosides I and L [8], flavonoids and flavone glycosides [8, 9]. *Citrullus colocynthis* has also been evaluated against numerous insect pests for its insecticidal activity [10]. In a recent study, a biological compounds i.e. spinasterol, 22,23-dihydrospinasterol was characterized from the leaves of *C. colocynthis* and was evaluated against *Brevicoryne brassicae* (Hemiptera: Aphididae) showed their significant insecticidal properties [11]. Previously, pronounced antioxidant activities were also reported from the leaves and roots of *C. colocynthis* extracts [12].

Spinasterol, 22, 23-dihydrospinasterol is a triterpenoids which also exhibited by other natural plants. Study on phytochemical analysis from the leaves of *Bryonies callus* Rattler revealed that it possess ß-sit sterol, triterpens, spinasterol, 22, 23-dihydrospinasterol, glycosides and phenolic contents. Meanwhile, the extract from *B. callus* was found effective for the control of *Aides aegypti* larvae and this mortality may attributed to the existence of phenolic contents and spinasterol, 22,23-dihydrospinasterol. Moreover, larvicidal activity of the extract from *Heliotropium indicum* and *Melothria maderaspatana* was also reported [13]. The extract from the leaves of *Mukia maderaspatana* also possess potential antioxidant properties because of the presence of spinasterol, 22, 23-dihydrospinasterol, flavonoid and phenolic contents [14]. It can also scavenge ABTS and DPPH radical molecules which also possess reducing power [15]. The pharmacological study of *Bougainvillea spectabillis* stems have shown that it has been used against hepatitis disease. It possess caffeic acid and Spinasterol, 22, 23-dihydrospinasterol which was used in herbal medicines against cancer hepatitis causing agents [16]. The leaves of *Vitex negundo* L. exhibited salicylic acid and 22,23-dihydro-α-spinasterol-β-d-glucoside showed repellency as well as toxicity properties against different strains of *Tribolium castaneum* [17].

Two Cucurbitane-type triterpenoid saponins were identified from the solvent extract of *C. colocynthis* fruit, but were not assessed as antioxidant, antifungal and insecticidal activities [18]. Similarly, a blend of spinasterol, 22,23-dihydrospinasterol was isolated and characterized from *Bermeuxia thibetica* (Lamiales: Lamiaceae) roots but was not evaluated as antimicrobial or insecticidal agent [19]. However, some biological activities of a triterpenoid spinasterol, 22,23-dihydrospinasterol contained by *Melothria maderaspatana* (Cucurbitales: Cucurbitaceae) was described by [20].

Although, a little research has been made on separation and characterization of several biological compounds from natural plants resources including spinasterol, 22, 23-dihydrospinasterol but its isolation and identification from *C. colocynthis* and their consumption as antioxidant and antifungal activity was not appraised so far. Keeping in view the detailed literature reviewed significant biological activities of the compound, the current innovative work was assessed for the first time for the evaluation of this biochemical compounds as antioxidant activities, antifungal activities against (*Magnaporthe grisea*, *Rhizoctonia solani* and *Phytophthora infestans*) and as insecticidal agent against *Brevicoryne brassicae.*

2. Results

2.1. Antioxidant Activity

1,1-diphenyl-2-picrylhydrazyl (DPPH) is a steady free radicle molecule with properties of dark-colored crystalline powder commonly used in the laboratory research for antioxidant assay. It dissolved readily in methanol and recognized by absorption of color on spectrophotometer at wavelength of 517 nm. Antioxidant molecules trap (scavenger) for other radical by the involvement of hydrogen particles, as it has violet color in the solution, and become colorless or pale yellow when neutralized and, thus, resulted in reduction of absorbance. Data obtained by DPPH inhibition (%) by scavenging action of free radical is

revealed in (Table 1). Results revealed that at 50μgmL^{-1} concentration maximum inhibition (%) afforded by a mixture of Spinasterol, 22,23-dihydrospinasterol was 49.46 followed by 36.61, 31.52 and 19.98 at 12.5, 3.0 and 0.78μgmL^{-1} respectively. Results also demonstrated that inhibition % decreased significantly on decreasing concentration of the compound.

Table 1
Antioxidant activity of Spinasterol, 22,23-dihydrospinasterol

Concentration (μgmL^{-1})	DPPH inhibition %
0.78	19.98 ± 1.66^{a}
3	31.52 ± 0.94^{b}
12.5	36.61 ± 0.79^{c}
50	49.76 ± 0.12^{d}
Statistics summary	2270.36
S.S	756.79
M.S	3
D.F	11140.69
F	0.000
P	

Values are denoted as mean of the five replicates ± standard error. Different letters given as superscript in the column are not significantly unlike according to (DMRT) at *P = 0.05* level.

2.2. Antifungal Activity

The data on fungicidal activity offered by Spinasterol, 22,23-dihydrospinasterol, Boscalid and Carbendazim is presented in the (Table 2). The results revealed that EC_{50} value shown by Spinasterol, 22,23-dihydrospinasterol was 129.56 μgmL^{-1} against *R. solani* showed its activity against this fungus. However, the activity of this compound against *M. grisea* was moderate with EC_{50} value as 206.09 μgmL^{-1} whereas, negligible control was recorded against *P. Infestans* being EC_{50} as 1093 μgmL^{-1}. On the other hand Boscalid found highly effective against *R. solani* and *P. infestans* with EC_{50} 1.64 and 1.62 μgmL^{-1} except for *M. grisea* where the EC_{50} values increased to 868 μgmL^{-1}. Whereas, Carbendazim showed excellent results against *M. grisea* and *R. solani* with EC_{50} values as <0.78 μgmL^{-1} except for *P. infestans* where it found in-effective with huge EC_{50} value as 872109E.

Table 2
Antifungal activity of Spinasterol, 22,23-dihydrospinasterol against *Magnaporthe grisea*, *Rhizoctonia solani* and *Phytophthora infestans*.

Name of the product	Conc. μgmL^{-1}	Inhibition ratio %			EC_{50}		
		M. grisea	*R. solani*	*P. infestans*	*M. grisea*	*R. solani*	*P. infestans*
Spinasterol, 22,23-dihydrospinasterol	0.78	0.088	0.016	0.158	206.09	129.56	1093.1
	3.12	0.115	0.073	0.195			
	12.5	0.218	0.208	0.247			
	50	0.373	0.336	0.341			
Boscalid	0.78	0.055	0.153	0.333	868.02	1.64	1.62
	3.12	0.139	0.913	0.616			
	12.5	0.212	0.964	0.966			
	50	0.255	0.964	0.994			
Carbendazim	0.78	0.955	0.653	0.118	<0.78	<0.75	872109E + 24S
	3.12	0.997	0.879	0.187			
	12.5	1.000	1.000	0.141			
	50	1.000	1.000	0.170			
Whereas, EC_{50}; (Half maximal effective concentration)							

2.3. Insecticidal Activity

The data presented in (Table 3) revealed the insecticidal activity of Spinasterol, 22,23-dihydrospinasterol against cabbage aphid *B. brassicae*. Highest mortality was observed at 72 h of exposure with LC_{50} 42.46 $mgmL^{-1}$ followed by 54.86 and 180.9 $mgmL^{-1}$ at 48 and 24 h exposure respectively *via* residual assay. Likewise, Maximum mortality *via* greenhouse assay was recorded after 72 h with LC_{50} 32.71 $mgmL^{-1}$ followed by 42.46 and 173.8 $mgmL^{-1}$ at 48 and 24 h exposure respectively. In comparison, greenhouse assay afforded higher mortality than residual assay.

Table 3
Probit analysis of Spinasterol, 22,23-dihydrospinasterol against *Brevicoryne brassicae*

Bioassay	Time (h)	LC_{50} (μgmL^{-1})	95% F.L		Slope ± SE	χ2
			Lower	Upper		
Greenhouse	24	173.8	59.77	6796	1.01 ± 0.31	1.04
	48	42.46	25.11	107.2	1.38 ± 0.29	1.99
	72	32.71	19.40	73.57	1.47 ± 0.38	2.18
Residual	24	180.9	65.58	9889	1.17 ± 0.38	0.56
	48	54.86	31.38	166.2	1.42 ± 0.32	1.28
	72	42.46	25.11	107.1	1.38 ± 0.29	1.99

F.L; Fiducial limits. χ2; Chi-square. LC_{50}; Lethal concentration.

The results presented in (Table 4) revealed that on prolonged exposure period of 72 h and at 50 $mgmL^{-1}$ concentration 63.3% and 56.7% mortality was observed *via* greenhouse and residual assay respectively. However, higher mortality i.e. 56.7% and 50% was also observed at 48 h at the same concentration *via* greenhouse and residual assay respectively. Whereas, 30 and 26.7% mortality was recorded at 24 h exposure *via* greenhouse and residual assay respectively at the same concentration.

Table 4
Insecticidal activity of Spinasterol, 22,23-dihydrospinasterol against *Brevicoryne brassicae*

Conc. (μgmL^{-1})	Mean mortality (%) with time (h)					
	24		48		72	
	Residual	Greenhouse	Residual	Greenhouse	Residual	Greenhouse
0.78	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{d}	0.00 ± 0.00^{e}
3.12	3.33 ± 5.77^{b}	6.67 ± 5.77^{b}	6.67 ± 5.77^{bc}	10.0 ± 10.0^{bc}	10.0 ± 0.00^{bc}	13.3 ± 5.77^{c}
12.5	6.67 ± 5.77^{b}	10.0 ± 0.00^{b}	20.0 ± 17.3^{b}	16.7 ± 5.77^{b}	16.7 ± 5.77^{b}	23.3 ± 5.77^{b}
50	26.7 ± 5.77^{a}	30.0 ± 10.0^{a}	50.0 ± 10.0^{a}	56.7 ± 5.77^{a}	56.7 ± 5.77^{a}	63.3 ± 5.77^{a}
CK	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	3.33 ± 5.77^{cd}	3.33 ± 5.77^{d}
Statics summary						
S.S	1493	1826	5306	6600	6293	7826
df	4	4	4	4	4	4
M.S	373	456	1326	1650	1573	1956
F	18.6***	17.1***	15.3***	49.5***	78.6***	73.4***

Data is designated as mean ± standard deviation with different latters in superscripts is significantly differed according to DMRT" $P > 0.05$). S.S (Sum of square); df (Degree of freedom); M.S (Mean square); F (Significance); CK (Check); *** (Highly significant).

3. Discussion

Natural plants are God gifted treasures for humans which possess a widespread variety of biological compounds involved in pharmaceutical and agricultural industry. These products contain substantial potential as natural antioxidant and also commonly used against various insects [21, 22].

Citrullus colocynthis is a valuable source of antioxidant potential such as butanol extract from *C. colocynthis* fruit showed IC_{50} values as 6 μgmL^{-1} whereas, fruit aqueous extract presented IC_{50} values as 241.25 μgmL^{-1}. Antioxidant properties of *C. colocynthis* leaves and roots extract was also documented as 45.98, 39.81% and 36.65 from leaves as well as 29.12, 35.51 and 33.83% DPPH inhibition was recorded from Hexane, aqueous and ethanol extract respectively [12]. Results documented by Benariba et al. [23] are also in accordance with our findings who reported inhibition of DPPH radical from seed extract

of *C. colocynthis* being IC_{50} values as 500, 580 and 350 μgmL^{-1} via aqueous, hydro-methanolic and ethyl acetate extract respectively. The analysis of *C. colocynthis* extracts documented the existence of various biochemical compounds as tannins, terpenoids, flavonoids and coumarins responsible for the pronounced antioxidant as well as other biological activities of this plant [24]. Initial screening for phytochemical of *C. colocynthis* revealed the existence of plenty of flavonoids and phenols showed the significant antioxidant activity as 88.8% from fruit extract with potential free radical scavenging consequences at a concentration of 2500 μg mL^{-1} [4]. The quantification of phenolic and flavonoids contents from solvent extract of *C. colocynthis* roots, leaves and fruits extracts was evaluated to compare the antioxidant activities. The amounts of total phenolic and flavonoids contents were (3.07–18.6 mgg^{-1} and 0.51–13.9 mgg^{-1} of dry sample respectively, followed by roots and fruits extract. Ethanol extract of leaves possessed the highest antioxidant activity as well as DPPH radical scavenging activities from roots and fruits extract [25].

In a study documented by Chawech et al. [26] reported the antibacterial activity of isolated compound Cucurbaticin E and Gluco- Cucurbaticin E from *C. colocynthis* against *Bacillus cereus* and *Enterococcus faecalis*. The minimum inhibitory concentration (MIC) values were 0.625 and 1.25 mgmL^{-1} respectively. Moreover, all of the populations of *C. colocynthis* extract showed antibacterial activity against *Pseudomonas aeruginosa* and *Escherichia coli*, *Enterococcus faecalis* and *Staphylococcus aureus* and antifungal activity against four Candidia species i.e. *Candida krusei*, *Candida glabrata*, *Candida parapsilosis* and *Candida albicans* [27].

Extracts and essential oils from plant origin contain secondary metabolite; phenolic, steroid and terpenoids compounds which are toxic in nature and are stored in the plant cells and bears bio-pesticidal properties against pathogens and insect pests. Moreover, they are easily biodegradable, benefiting their existence without causing severe damage to the environment and humans [28–30]. Literature review showed that there are several examples of plant products used in plant protection measures as a broad spectrum of plant pathogenic fungi, for instance, *thymol* and *carvacrol* have antifungal activity against *Botrytis cinerea* and *Fusarium* spp. Results indicated that these compounds could be employed independently as fungicidal agents against various phytopathogenic fungi [31]. Besides, *α-cadinol* and *T-muurolol* compounds isolated from the *Calocedrus macrolepis* exhibit significant fungicidal activity against *Fusarium oxysporum* and *Rhizoctonia solani* [32]. On the other hand, methanolic extract from the rhizome of *Acorus gramineus* comprises of numerous chemical compounds such as *caryophyllene, a-asarone, methyl isoeugenol, isoasarone safrole* possessed antifungal activities however, *asaronaldehyde* (2,4,5-trimethoxybenzaldehyde) presented complete control of *Phytophthora infestans* in potatoes and tomatoes whereas, it showed 75% control of *R. solani* [33]. Our findings on antifungal activity of triterpenoids (Spinasterol, 22,23-dihydrospinasterol) were supported by Quiroga et al. [34] that lactones, sesquiterpen and triterpenes from *Schinus molle's* fruits and leaves possessed antifungal potential against *Alternaria alternate, Penicillium cyclopium, Aspergillus niger, Aspergillus flavus Microsporum griseum* and *Penicillium italicum*. Similarly, a flavonoid 4'-methoxy-5,7-dihydroxyflavone 6-C-glucoside isolated from the stem and leaves of *Aquilegia vulgaris*, presented its antifungal activity against mold *A.*

niger [35]. The antimycotoxigenic and antifungal activity of alcoholic and distilled water extracts of *C. colocynthis* were evaluated against *Aspergillus flavus* and *Aspergillus ochraceus* and showed an excellent antifungal activity against *A. ochraceus* with good antiochratoxigenic power in the liquid medium which supported findings about antifungal activity and triterpenoids spinasterol, 22,23-dihydrospinasterol [36].

Activity of some of the biological compounds such as camphor, pulegone and verbenone which were isolated from *Myristica fragrans* was assessed against German cockroach *Blattellea germanica* with LC_{50} values as 0.07 $mgcm^{-1}$, 0.06 $mgcm^{-1}$ and 0.07 $mgcm^{-1}$ respectively [37]. Similarly, other isolated compounds like, carvecol, eugenol, p-cymene, isoeugenol and thymol had displayed anti-adulticidal potential at 1 $mgadult^{-1}$ against *B. germanica* [38]. Similarly, isolated compound Spinasterol, 22,23-dihydrospinasterol exhibited medicinal and cytotoxic properties, moreover, the same was characterized from *Bougainvillea spectabillis* exibited sturdy inhibition of enzyme xanthine oxidase with IC_{50} values as 39.21 µM [16]. Our results on toxicity of spinasterol, 22,23-dihydrospinasterol revealed that it exhibited potential insecticidal activity and caused significant mortality of *B. brassicae*. Similar outcomes were described by Torkey et al. [39] who reported activity of the 2-*O*-*β*-D-glucapyranosylcucurbitacin E isolated from *C. colocynthis* against *Aphis craccivora* with momentous mortality of this pest with LC_{50} of 11,003 ppm. Moreover, insecticidal activity of isolated compound from *Eupatorium adenophorum* 9-oxo-10,11-dehydroageraphorone was appraised against *Pseudoregma bambucicola* exhibited mortality of 73.33% at 2 $mgmL^{-1}$ with 6 h exposure. Moreover, 100% control of this pest was recorded at the similar concentration at one month of post exposure in a field experiment [40]

Although, different studies had been conducted on extracts, essential oils and isolated compounds from natural plants as their antioxidant, antimicrobial, antifungal an insecticidal activities but such activities of Spinasterol, 22,23-dihydrospinasterol was not evaluated so for. Thus, this innovative research was performed for the first time to investigate the antioxidant, antifungal and insecticidal properties of the isolated compound Spinasterol, 22,23-dihydrospinasterol .

4. Conclusions

The current investigations specified that Spinasterol, 22,23-dihydrospinasterol isolated from *Citrullus colocynthis* leaves exhibited pronounced antioxidant activities and insecticidal activity against *Brevicoryne brassicae* via residual and greenhouse assay as well as moderate antifungal activities against *Magnaporthe grisea* and *Rhizoctonia solani*. However, in comparison, greenhouse assay showed higher mortality of this pest. Based on the present findings, Spinasterol, 22,23-dihydrospinasterol might be introduced as an antioxidant, antifungal and insecticidal purposes as an alternatives to synthetic chemical agents. However, more research is desirable on the isolation and characterization of other bioactive compounds for their evaluation as antioxidant, antifungal and insecticidal properties.

5. Material And Methods

5.1. Collection of Materials

Samples of the *Citrullus colocynthis* (Cucurbitales: Cucurbitaceae) leaves also locally famous as tumba was collected from desert area of Punjab Province, Pakistan, (29°59'34"N, 73°15'13"E) with latitudinal and longitudinal gradients during the year 2019. The collected samples were identified as *Citrullus colocynthis* at Entomological Research Institute Faisalabad, Pakistan.

Pure colonies of three pathogenic fungi i.e. Rice Blast (*Magnaporthe grisea*), Sheath Blight (*Rhizoctonia solani*) and Phytophthora (*Phytophthora infestans*) were obtained from Department of Pesticides Science, College of Plant Protection, Shenyang Agricultural University, and Shenyang China. The Cabbage aphids were sustained on the cabbage plants which were grown at 20 ± 5 °C and 45 ± 5% R.H in the greenhouse, along with 16:8 (Light: Dark) photoperiod.

5.2. Extraction, Purification and Identification of Biochemical Compound

Extraction, separation, purification and identification of the purified compounds was achieved by solvents/cold extraction, various chromatographic techniques, mass spectrum and nuclear magnetic resonance (NMR) (^{1}H-NMR and ^{13}C-NMR) spectrum respectively as described in a recently published article of the author [11] and presented in the supplementary file.

5.3. Determination of Radical Scavenging Activity of DPPH

In order to assess the antioxidant action of Spinasterol, 22,23-dihydrospinasterol extracted by chromatographic techniques and identified by nuclear magnetic resonance analysis at various concentrations *viz.* 0.78, 3.00, 12.5 and 50 µg/mL accomplished in tween 20 (1% solution in distilled water), stable free radicals molecule 1,1-diphenyl-2-picrylhydrazyl (DPPH) ($C_{18}H_{13}N_5O_6$) a dark colored crystalline powder was employed. In brief, into 3.5 mL freshly prepared DPPH solution (0.002 g 50 mL^{-1} in HPLC grade methanol) and 0.25 mL of various concentration of purified compound prepared in methanol were added, shacked and left for incubation in darkness at 28 °C for half an hour. [12, 41, 42]. Consequently, absorbance was assessed at 517 nm by means of an absorbance micro-plate reader (SpectraMax Model No. 190, made in China and designed at USA) and inhibition percent of prepared 1, 1-diphenyl-2 picrylhydrazyl solution was calculated on reducing of absorbance by using following Eq. (1). Conclusively, a lower absorbance degree validates higher radical scavenging activity.

$$Inhibition\ (\%) = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100 \qquad (1)$$

Where: A_{blank} = (absorbance of control treatment); A_{Sample} = (absorbance of prepared samples).

5.4. *Determination of Antifungal Activity*

Antifungal activity of Spinasterol, 22,23-dihydrospinasterol against *Magnaporthe grisea, Rhizoctonia solani*) and *Phytophthora infestans* was evaluated *in-vitro* using radiated growing test on potatoes dextrose agar (PDA). Comercial synthetic fungicides, Boscalid and Carbendazim were used as positive control. Purified compound was placed in acetone to dissolve and then mixed with PDA to gain various concentrations with standard to lower concentration *viz.* 0.78, 3.00, 12.5 and 50 μgmL^{-1}. Then, the PDA with various concentrations was transferred into petri dishes (90 mm) diameter with 15 mL each in petri dish and then incubated along with 5 mm lumps of *M. grisea, R. solani* and *P. infestans* for each test compound and fungicides. The lumps of fungus were got by pressing at the corner of the mycelia colony from already prepared culture medium of PDA. After an incubation of one week at 25 °C, radius of mycelia growth were calculated at the inhibition percentages comparative to control (CK) with acetone (1%). All the teatmtments were replicated thrice and data was calculated adopting the standard method [43].

5.5. Determination of Insecticidal Activity

The *in-vitro* (residual) and *in-vivo* (greenhouse) insecticidal activity was assessed against cabbage aphid *Brevicoryne brassicae.* For residual assay, freshly cut cabbage leaves were dipped for 10 s in corresponding concentration and on drying, placed in glass petri dishes. Next, 10 wingless adult aphids were transferred on the leaves. Check (CK) was prepared in 1% solution of tween 20 deprived of purified compound and all petri dishes were incubated at room temperature, 60% relative humidity and 16:8 (Light: Dark) photoperiod for 72 h. For greenhouse assay, on clean and healthy plants of 5–7 true leaf stage, 10 wingless adult aphids were released. After one hour of releasing aphids and on complete settling of aphids on plants leaves, were sprayed with corresponding concentrations (2–3 showers; 10 mL) using hand sprayer. For control (CK) plants were sprayed with 1% solution of tween 20 then, treated plants along with check (CK) were placed in greenhouse for 72 h.

Mortality data for both *in-vitro* and *in-vivo* experiments was calculated regularly at 24, 48 and 72 h exposure period by examining the aphids using the binocular microscope. The individual's aphids were considered as dead who offered no response on needle stimulation.

5.6. Statistical Analysis

Analysis of variance (ANOVA) was used to analyze the data. Difference among the treatments was calculated at $P = 0.05$ by Duncan Multiple Range Test (DMRT) with software IBM-SPSS statistics 25.0 version. Probability analysis was accomplished for the calculation of LC_{50} values by using 1.5 version EPA Probit analysis program. Whereas, Inhibition ratio and EC_{50} values were intended by using Log-Probit analysis.

Declarations

Data Availability Statement: All the presented data is available in the manuscript

Competing Interest Statement: The authors have declared no conflict of interest.

Funding: The financial support provided by the National Key Research & Development Program of China (2016YFD0200500) is greatly acknowledged.

Author Contributions: Formal analysis, M.A.; Methodology, M.A.; and P.Q.; Project administration, M.J.; and P.Q.; Software, M.A.; supervision, M.J.; Visualization, P.Q.; Writing—original draft, M.A.; Writing—review & editing, M.J.,

Acknowledgment: Experimental guidelines and supervision provided by Professor Ji Mingshan is greatly acknowledged

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