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

Maqsood Ahmed  
Shenyang Agricultural University

Ji Mingshan  
Shenyang Agricultural University

Peiwen Qin (qinpeiwen@syau.edu.cn)  
Shenyang Agricultural University

Research article

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

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

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
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 EC₅₀ 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 EC₅₀ 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 LC₅₀ 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 colocynthis 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-sitosterol, 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* (Lamiaceae: 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 50μg/mL 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 0.78μg/mL respectively. Results also demonstrated that inhibition % decreased significantly on decreasing concentration of the compound.

| Concentration (μg/mL) | DPPH inhibition % |
|-----------------------|-------------------|
| 0.78                  | 19.98 ± 1.66<sup>a</sup> |
| 3                     | 31.52 ± 0.94<sup>b</sup> |
| 12.5                  | 36.61 ± 0.79<sup>c</sup> |
| 50                    | 49.76 ± 0.12<sup>d</sup> |

Statistics summary

- **S.S**: 2270.36
- **M.S**: 756.79
- **D.F**: 3
- **F**: 11140.69
- **P**: 0.000

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<sub>50</sub> value shown by Spinasterol, 22,23-dihydrospinasterol was 129.56 μg/mL against *R. solani* showed its activity against this fungus. However, the activity of this compound against *M. grisea* was moderate with EC<sub>50</sub> value as 206.09 μg/mL whereas, negligible control was recorded against *P. Infestans* being EC<sub>50</sub> as 1093 μg/mL. On the other hand Boscalid found highly effective against *R. solani* and *P. infestans* with EC<sub>50</sub> 1.64 and 1.62 μg/mL except for *M. grisea* where the EC<sub>50</sub> values increased to 868 μg/mL. Whereas, Carbendazim showed excellent results against *M. grisea* and *R. solani* with EC<sub>50</sub> values as <0.78 μg/mL except for *P. infestans* where it found in-effective with huge EC<sub>50</sub> 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$_{50}$ (µg mL$^{-1}$) | 95% F.L | Slope ± SE | $\chi^2$ |
|-----------|----------|--------------------------|---------|------------|--------|
|           |          |                          | 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. $\chi^2$; 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. (µg mL⁻¹) | Residual Mean mortality (%) with time (h) | Greenhouse Mean mortality (%) with time (h) |
|----------------|------------------------------------------|-------------------------------------------|
|                | 24                                       | 48                                        | 72                                        |
| 0.78           | 0.00 ± 0.00b                             | 0.00 ± 0.00b                              | 0.00 ± 0.00c                              |
| 3.12           | 3.33 ± 5.77b                             | 6.67 ± 5.77b                              | 10.0 ± 10.0bc                             |
| 12.5           | 6.67 ± 5.77b                             | 20.0 ± 17.3b                              | 16.7 ± 5.77b                              |
| 50             | 26.7 ± 5.77a                             | 50.0 ± 10.0a                              | 56.7 ± 5.77a                              |
| CK             | 0.00 ± 0.00b                             | 0.00 ± 0.00b                              | 0.00 ± 0.00c                              |

Static summary:

|        | S.S   | df   | M.S   | F      |
|--------|-------|------|-------|--------|
| Residual |       |      |       |        |
|        | 1493  | 4    | 373   | 18.6***|
|        | 1826  | 4    | 456   | 17.1***|
|        | 5306  | 4    | 1326  | 15.3***|
|        | 6600  | 4    | 1650  | 49.5***|
|        | 6293  | 4    | 1573  | 78.6***|
|        | 7826  | 4    | 1956  | 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 IC₅₀ values as 6 µg mL⁻¹ whereas, fruit aqueous extract presented IC₅₀ values as 241.25 µg mL⁻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, *a-cadinol* and *T-muurolool* 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*, *isocarassane safrrole* 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.
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 *Blattella 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 spectabilis* 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-glucopyranosylcurcurbitacin 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 were 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) (1H-NMR and 13C-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) (C18H13N5O6) 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.

\[
\text{Inhibition} (\%) = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100
\]  

Where: \( A_{\text{blank}} \) = (absorbance of control treatment); \( A_{\text{sample}} \) = (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). Commercial synthetic fungicides, Boscaild 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 µg mL⁻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 treatments 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 LC₅₀ values by using 1.5 version EPA Probit analysis program. Whereas, Inhibition ratio and EC₅₀ 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

**References**

1. Mvumi C, Marais D, Ngadze E, du Toit ES, Tsindi A: *Effect of moringa extract on the leaf anatomy and yield potential of tomato infected by Alternaria solani*. *South African Journal of Plant and Soil* 2018, **35**(5):389-392.

2. Memon U, Brohi A, Ahmed SW, Azhar I, Bano H: *Antibacterial screening of Citrullus colocynthis*. *Pakistan journal of pharmaceutical sciences* 2003, **16**(1):1-6.

3. Mohammad D, Al-Khateeb M, Riyadh E, Al-Hashem F, Nabil B, Mohammad K: *In vivo, acute, normohypoglycemic, antihyperglycemic, insulinotropic actions of orally administered ethanol extract of Citrullus colocynthis (L.) Schr pulp*. *American Journal of Biochemistry and Biotechnology* 2009, **5**(3):119-126.

4. Kumar S, Kumar D, Jusha M, Saroha K, Singh N, Vashishta B: *Antioxidant and free radical scavenging potential of Citrullus colocynthis (L.) Schrad. methanolic fruit extract*. *Acta Pharmaceutica* 2008, **58**(2):215-220.

5. Dehghani F, Panjeh SM, Talaei KT, Mesbah AS, Azizi M: *Toxic effects of hydroalcoholic extract of Citrullus colocynthis on pregnant mice*. 2008.

6. Sturm S, Schneider P, Seger C, Stuppner H: *Analysis of Citrullus colocynthis cucurbitacin derivatives with HPLC-SPE-NMR*. *Scientia pharmaceutica* 2009, **77**(7):254.

7. Seger C, Sturm S, Mair ME, Ellmerer EP, Stuppner H: *1H and 13C NMR signal assignment of cucurbitacin derivatives from Citrullus colocynthis (L.) Schrader and Ecballium elaterium L. (Cucurbitaceae)*. *Magnetic Resonance in Chemistry* 2005, **43**(6):489-491.

8. Delazar A, Gibbons S, Kosari AR, Nazemiye H, Modarresi M, Nahar L, Sarker Sd: *Flavone C-glycosides and cucurbitacin glycosides from Citrullus colocynthis*. *DARU Journal of Pharmaceutical Sciences* 2006, **14**(3):109-114.

9. Maatooq GT, El-Sharkawy SH, Afifi M, Rosazza JP: *Cp-hydroxybenzoylglycoflavones from Citrullus colocynthis*. *Phytochemistry* 1997, **44**(1):187-190.

10. Soam PS, Singh T, Vijayvergia R: *Citrullus colocynthis(LINN.) and luffa acutangula(L.) roxb, schrad. source of bioinsecticides and their contribution in managing climate change*. *IJABPT* 2013, **4**(4):7-9.
11. Ahmed M, Qin P, Ji M, An R, Guo H, Shafi J: Spinasterol, 22, 23-Dihydrospinasterol and Fernenol from Citrullus colocynthis L. with Aphicidal Activity against Cabbage Aphid Brevicoryne Brassicae L. *Molecules* 2020, **25**(9):2184.

12. Ahmed M, Ji M, Peiwen Q, Liu Y, Gu Z, Sikandar M, Iqbal M, Javeed A: Phytochemical screening, total phenolics and flavonoids content and antioxidant activities of Citrullus colocynthis L. and Cannabissativa L. *Appl Ecol Environ Res* 2019, **17**:6961-6979.

13. Ramamurthy V, Krishnaveni S: Larvicidal efficacy of leaf extracts of Heliotropium Indicum and Mukia maderaspatana against the dengue fever mosquito vector Aedes aegypti. *J Entomol Zool Stud* 2014, **2**:40-45.

14. Petrus A, Bhuvaneshwari N, Alain J: Antioxidative constitution of Mukia maderaspatana (Linn.) M. Roem. leaves. 2011.

15. Raja B, Pugalendi KV: Evaluation of antioxidant activity of Melothria maderaspatana in vitro. *Central European Journal of Biology* 2010, **5**(2):224-230.

16. Chang W, Chang Y, Lu F, Chiang H-C: Inhibitory effects of phenolics on xanthine oxidase. *Anticancer research* 1994, **14**(2A):501-506.

17. Ling T-J, Wan X-C, Ling W-W, Zhang Z-Z, Xia T, Li D-X, Hou R-Y: New Triterpenoids and Other Constituents from a Special Microbial-Fermented Tea—Fuzhuan Brick Tea. *Journal of agricultural and food chemistry* 2010, **58**(8):4945-4950.

18. Song F, Dai B, Zhang H-Y, Xie J-W, Gu C-Z, Zhang J: Two new cucurbitane-type triterpenoid saponins isolated from ethyl acetate extract of Citrullus colocynthis fruit. *Journal of Asian natural products research* 2015, **17**(8):813-818.

19. Ding L, Chen Y, Wu F: Constituents of the root of Bemeuxia thibetica Decne. *Zhongguo Zhong yao za zhi= Zhongguo zhongyao zazhi= China journal of Chinese materia medica* 1991, **16**(5):289-290, 318.

20. Sinha B, Sasmal D, Basu S: Pharmacological studies on Melothria maderaspatana. *Fitoterapia (Milano)* 1997, **68**(1):75-78.

21. Michaelakis A, Papachristos D, Kimbaris A, Koliopoulos G, Giatropoulos A, Polissiou MG: Citrus essential oils and four enantiomeric pinenes against Culex pipiens (Diptera: Culicidae). *Parasitology research* 2009, **105**(3):769.

22. Lardeux F, Depickère S, Duchon S, Chavez T: Insecticide resistance of Triatoma infestans (Hemiptera, Reduviidae) vector of Chagas disease in Bolivia. *Tropical Medicine & International Health* 2010, **15**(9):1037-1048.

23. Chekroun E, Benariba N, Adida H, Bechiri A, Azzi R, Djaziri R: Antioxidant activity and phytochemical screening of two Cucurbitaceae: Citrullus colocynthis fruits and Bryonia dioica roots. *Asian Pacific Journal of Tropical Disease* 2015, **5**(8):632-637.

24. Benariba N, Djaziri R, Bellakhdar W, Belkacem N, Kadiata M, Malaisse WJ, Sener A: Phytochemical screening and free radical scavenging activity of Citrullus colocynthis seeds extracts. *Asian Pacific journal of tropical biomedicine* 2013, **3**(1):35-40.
25. Hussain AI, Rathore HA, Sattar MZ, Chatha SA, ud din Ahmad F, Ahmad A, Johns EJ: Phenolic profile and antioxidant activity of various extracts from Citrullus colocynthis (L.) from the Pakistani flora. *Industrial crops and products* 2013, **45**:416-422.

26. Chawech R, Mhalla D, Trigui M, Mihoubi M, Fabre N, Jarraya R: Chemical composition and antibacterial activity of extracts and compounds isolated from Citrullus colocynthis (L.) Schrad. *Journal of Pharmacognosy and Phytochemistry* 2015, **4**(4):197.

27. Marzouk B, Marzouk Z, Décor R, Mhadhebi L, Fenina N, Aouni M: Antibacterial and antifungal activities of several populations of Tunisian Citrullus colocynthis Schrad. immature fruits and seeds. *Journal de mycologie médicale* 2010, **20**(3):179-184.

28. Daniel CK, Lennox CL, Vries FA: *In-vitro effects of garlic extracts on pathogenic fungi Botrytis cinerea, Penicillium expansum and Neofabraea alba.* *South African Journal of Science* 2015, **111**(7-8):1-8.

29. Martinez JA: Natural fungicides obtained from plants. In: *Fungicides for plant and animal diseases.* IntechOpen; 2012.

30. Sales MDC, Costa HB, Fernandes PMB, Ventura JA, Meira DD: Antifungal activity of plant extracts with potential to control plant pathogens in pineapple. *Asian Pacific Journal of Tropical Biomedicine* 2016, **6**(1):26-31.

31. Daferera DJ, Ziogas BN, Polissiou MG: The effectiveness of plant essential oils on the growth of *Botrytis cinerea, Fusarium* sp. and *Clavibacter michiganensis subsp. michiganensis*. *Crop protection* 2003, **22**(1):39-44.

32. Chang H-T, Cheng Y-H, Wu C-L, Chang S-T, Chang T-T, Su Y-C: Antifungal activity of essential oil and its constituents from *Calocedrus macrolepis var. formosana* Florin leaf against plant pathogenic fungi. *Bioresource technology* 2008, **99**(14):6266-6270.

33. Lee H: Fungicidal property of active component derived from *Acorus gramineus* rhizome against phytopathogenic fungi. *Bioresource technology* 2007, **98**(6):1324-1328.

34. Quiroga EN, Sampietro AR, Vattuone MA: Screening antifungal activities of selected medicinal plants. *Journal of ethnopharmacology* 2001, **74**(1):89-96.

35. Abad MJ, Ansuategui M, Bermejo P: Active antifungal substances from natural sources. *Arkivoc* 2007, **7**(11):6-145.

36. Mohamed Amine G, KHELIL AOEH, Bouabdallah G, Noureddine H, Amina Nesrine D, Amel B, Sawsen H, Mokhtar B, Djalal Eddine Houari A: Antimycotoxigenic and antifungal activities of Citrullus colocynthis seeds against Aspergillus flavus and Aspergillus ochraceus contaminating wheat stored. *African journal of biotechnology* 2013, **12**(43):6222-6231.

37. Jung W-C, Jang Y-S, Hieu TT, Lee C-K, Ahn Y-J: Toxicity of *Myristica fragrans* seed compounds against *Blattella germanica* (*Dictyoptera: Blattellidae*). *Journal of medical entomology* 2007, **44**(3):524-529.

38. Yeom H-J, Kang JS, Kim G-H, Park I-K: Insecticidal and acetylcholine esterase inhibition activity of Apiaceae plant essential oils and their constituents against adults of German cockroach (*Blattella germanica*). *Journal of Agricultural and Food Chemistry* 2012, **60**(29):7194-7203.
39. Torkey HM, Abou-Yousef H, AZ AA, Farid H: **Insecticidal effect of cucurbitacin E glycoside isolated from *Citrullus colocynthis* against *Aphis craccivora***. *Aust J Basic Appl Sci* 2009, **3**:4060–4066.

40. Nong X, Fang C-L, Wang J-H, Gu X-B, Yang D-Y, Liu T-F, Fu Y, Zhang R-H, Zheng W-P, Peng X-R: **Acaricidal activity of extract from Eupatorium adenophorum against the Psoroptes cuniculi and Sarcoptes scabiei in vitro**. *Veterinary parasitology* 2012, **187**(1-2):345-349.

41. Ahmed M, Ji M, Sikandar A, Iram A, Qin P, Zhu H, Javeed A, Shafi J, Iqbal Z, Farid Iqbal M: **Phytochemical Analysis, Biochemical and Mineral Composition and GC-MS Profiling of Methanolic Extract of Chinese Arrowhead Sagittaria trifolia L. from Northeast China**. *Molecules* 2019, **24**(17):3025.

42. Ahmed M, Ji M, Qin P, Gu Z, Liu Y, Sikandar A, Iqbal M, Javeed A, Shafi J, Du Y: **Determination of phytochemicals, antioxidant activity and biochemical composition of chinese mugwort (*Artemisia argyi* l.) leaf extract from northeast China**. *Appl Ecol Environ Res* 2019, **17**(6):15349-15362.

43. Hou Z, Yang R, Zhang C, Zhu L-F, Miao F, Yang X-J, Zhou L: **2-(Substituted phenyl)-3, 4-dihydroisoquinolin-2-iiums as novel antifungal lead compounds: Biological evaluation and structure-activity relationships**. *Molecules* 2013, **18**(9):10413-10424.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- [GraphicalAbstratJPG.jpg](GraphicalAbstratJPG.jpg)
- [SupplementryFile.docx](SupplementryFile.docx)