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Isolation and Identification of Cardenolide Compounds of *Gomphocarpus sinaicus* and Their Fungicidal Activity Against Soil Borne and Post Harvest Fungi

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Abstract: This study was undertaken to explore new antifungal compounds from the methanolic extract of *G. sinaicus*. Two cardenolide compounds were isolated and identified by GC-MS as cardenolide glycoside, 15-hydroxy-3,4,5,6-dehydrocalotropin and cardenolide genin, 3,4,5,6-dehydrocalotropagenin. The antifungal activity of these compounds was assessed. Results revealed that both compounds showed pronounced fungicidal activity against both soil borne fungi, *R. solani*, *F. oxysporium*, and postharvest fungi, *R. stolonifer*, *P. digtatum*, compared to the standard fungicides, flutolanil and copper oxychloride, respectively. The EC_{50} values of the cardenolide genin were 0.703, 13.63 and 4.22, 8.403 µg/mL for *R. solani*, *F. oxysporium* and *R. stolonifer*, *P. digtatum* respectively. On the other hand, the EC_{50} values of the standard fungicide, flutolanil, were 9.49 and 61.22 µg/mL against *R. solani* and *F. oxysporium*. While the EC_{50} values of copper oxychloride were 279.94 and 187.13 µg/mL against *R. stolonifer* and *P. digtatum*, respectively. The results showed that cellulase, PME, PPO of the tested fungi was more sensitive than to cardenolide genin. The strong antifungal activity of cardenolide genin reported in this study indicated that has a potential to be used as fungicides.

Key words: *Gomphocarpus sinaicus*, cardenolide glucosides, fungicidal activity, fungitoxicity, enzymes.

1. Introduction

Gomphocarpus sinaicus (Boiss.) is atoxic plant, due to its high content of cardiac glycosides. *G. sinaicus* (Boiss.) plant which grows wild in the sandy mountains regions in South Sinai Governorate, Egypt under the common name "Hargel". It is rich in a number of cardenolides, in particular cardenolide glucosides with unusual doubly linked sugars El-Askary et al. [1, 2]. Therefore, the present work was designed to isolate and identify the major active cardenolides from the methanol extract of *G. sinaicus* and to evaluate their fungicidal activity against some of the soil borne and post harvest fungi. Also, investigation of the effects of these compounds on certain fungi enzymes in an attempt to throw some light on their mode of action.

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2. Materials and Methods

2.1 Plant Material

Aerial parts of *Gomphocarpus sinaicus* (Boiss.) were collected form Sinai in may, 2005 at the flowering and fruiting stage. The plants were identified according to the taxonomic characters of Tackhlom [3]. The samples were air-dried at room temperature and then finely powdered.

2.2 Instruments

IR spectra were performed with Perkin Elmer 1430 Ratio Recording Infrared Spectrometer in KBr disks. ^{1}H and ^{13}C NMR spectra were recorded at 500MHz and 125 MHz respectively, on a JEOL JNM ECD 500 Spectrophotometer in $CDCl_3$.

2.3 Test Organisms

Four fungal pathogens were used in this study. Two

soil born fungi, *Rhizoctonia solani* (Kuhn.) and *Fusarium oxysporium* (Schiech.), and two post harvest fungi (*Rhizopus stolonifer* (Ehren) and *Penicillium digtatum* (Pres.) sacc, were used. The fungi were maintained during the course of the experiments on Czapek-Dox Agar (CDA) at 25 ºC.

2.4 Isolation and Identification of Cardenolide Compounds from G. sinaicus Extracts

Batches of air dried aerial parts of *G. sinaicus* each of (1 kg) were percolated with MeOH and the extracts were concentrated, decanted from an insoluble wax, diluted with H_2O and extracted with $CHCl_3$. The wax was triturated with petroleum ether and the residue was added to the $CHCl_3$ extract, which was then filtered under reduced pressure through powdered charcoal. The charcoal was washed with $CHCl_3$ and the combined filtrates were concentrated under reduced pressure to give a dark brown gum. The gum in $CHCl_3$ was fractionated by chromatography column (CC) of silica gel and eluted with MeOH-$CHCl_3$ 10% to give 10 eluates (each of 100 mL), controlled by TLC, and grouped as follows: the solvent of the first six eluates was evaporated under reduced pressure to yield fraction I and the second four eluates yield fraction II.

Fraction I (3 × 3 g) was again subjected to CC on silica gel (30 g, 15 mL fractions) (15 × 15 mL) and eluted with $CH_3C(O)CH_3$-$CHCl_3$ (1:4). The eluated fractions were controlled by TLC. Detection of cardenolides by TLC was performed by using kedde's reagent (1:1 mixture of 5% 3,5-dinitrobenzoic acid in MeOH and 2 N KOH) and the solvent systems $CHCl_3$-MeOH 10%, $CHCl_3$-MeOH 15%, $CHCl_3$-MeOH 20%. Evaporation of the solvents of eluted fractions was carried under reduced pressure to yield card. glycoside. Recrystallization of this compound from acetone yielded pure prisms crystals 6.2 g.

Fraction II was purified with MeOH-$CHCl_3$ 20% by CC on silica gel (30 g, 15 mL Fractions.) (15 × 15 mL), MeOH-$CHCl_3$ 30% (15 × 15 mL), MeOH-$CHCl_3$ 50% (15 × 15 mL), MeOH-$CHCl_3$ 70% (15 × 15 mL), MeOH-$CHCl_3$ 90% (15 × 15 mL) controlled by TLC was performed on silica gel plates (PolyGram Sil G/UV 254, Germany) using solvent system $CHCl_3$-MeOH 10%, $CHCl_3$-MeOH 15%, $CHCl_3$-MeOH 20%. Detection was done by kedde's reagent. Evaporation of the solvents of eluted fractions was carried under reduced pressure to yield liquid card. genin (6 mL).

2.5 Antifungal Assay

The antifungal activity of the isolated cardenolide compounds, as well as the standard fungicides, was tested using the radial growth inhibition technique [4, 5]. Appropriate volumes of the stock solutions of the tested compounds in Triton X-100 were added to molten nutrient agar medium (Czapek-Dox Agar; CDA) to obtain a range of concentrations (10, 50, 100, 500, and 1,000 µg/mL before pouring into the Petri dishes (9.0 cm in diameter) at 40-45 °C. Each concentration was tested in triplicate. Parallel controls were maintained with Triton X-100 mixed with CDA medium. The discs of mycelial felt (0.5 cm diameter) of the plant pathogenic fungi, taken from 8-day-old cultures on CDA plates, were transferred aseptically to the centre of Petri dishes. The treatments were incubated at 25 °C in the dark. Colony growth diameter was measured after the fungal growth in the control treatments had completely covered the Petri dishes. Percentage of mycelial growth inhibition was calculated from the formula: Mycelial growth inhibition = [(DC−DT)/DC] × 100 [6], where DC and DT are average diameters of the fungal colonies for control and treatments, respectively. The concentration of the compound inhibiting fungal mycelial growth by 50% (EC_{50}) was calculated using probit analysis.

2.6 Biochemical Measurements

2.6.1 Pectin Methyl Esterase (PME) Activity

Pectin methyl esterase enzyme was secreted from the tested fungi that were grown on Czapeck Dox

medium supplemented with 1% pectin (apple pectin: apipectin 150 SAG), as recommended by El-Gorrani et al. [7].

The activity of PME was measured according to the method described by Smith [8], Talboys and Busch [9] with some modifications.

The reaction mixture was prepared from the following components: pectin (0.5 g), sodium chloride (0.58 g) bromothymol blue solution (0.05%) 2.5 mL, and distilled water up to 1,000 mL chloroform (4 mL) was added to reaction mixture to avoid bacterial contamination. The reaction mixture was immediately adjusted at pH 7.0 with 0.05 N sodium hydroxide solutions.

Flasks (100 mL) containing a total definite volume (10 mL) consisted of 7.0 mL reaction mixture (pH 7) + 2.0 mL of crude enzyme + 1.0 mL of a 10, 50, 100, 500 or 1,000 ppm concentration of each tested compound and tested fungicide (flutolanil or copper oxychloride). Each mixture was incubated at 30 °C for 24 h, and then titrated to pH 7.0 with 0.01 N sodium hydroxide. The total sodium hydroxide solution consumed by the blank gave the actual consumption of sodium hydroxide solution due to pectin methyl esterase activity on the substrate. Similar mixtures for each treatment except the crude enzyme (which was substituted by inactivated enzyme kept in a boiling water bath for 10 minutes) was prepared concurrently and considered as a blank treatment. The flasks containing the blank were titrated with 0.01 N sodium hydroxide solutions to the pH 7.0. Each treatment was replicated three times. The inhibition percentage of the activity was calculated by the following equation:

$$I\% = \left[\left(\frac{A-B}{A}\right)\right] \times 100 \quad (1)$$

where *I%* is inhibition percent; *A* is mL of NaOH (0.01 N) in the control treatment; *B* is mL of NaOH (0.01 N) in the treatment.

2.6.2 Polyphenol Oxidase (PPO) Activity

Determination of polyphenol oxidase activity was carried out according to the method that described by Broesch [10]. The czapeck-Dox medium containing the 10, 50, 100, 500 and 1,000 ppm of each tested compound and tested fungicides (flutolanil or copper oxychloride) was prepared in conical flasks. The inoculums discs of the tested fungi were located on the surface of the medium in the flasks. The flasks then incubated until—the hyphal growth in untreated flasks (control) was completed, then the contents of flasks were filtrated under vacuum. The filtrate was used to measure the enzyme activity. The filtrates were centrifuged for 15 minutes at 4,000 rpm. Aliquots of supernatants were assayed for polyphenol oxidase activity using spectrophotometer at 575 nm. The reaction mixture consisted of 2.0 mL of borate buffer (pH 9.0), 1.0 mL of 1% P-aminobenzoic (acid alcoholic solution), 2.0 mL of 1% catechol, 1.0 mL of filtrate. The same reaction mixture was prepared without enzyme source and considered as a blank.

Enzyme activity was measured as optical density (absorbance) after one hour incubation in a shaking water bath at 45 °C.

$$I\% = \left[\left(\frac{A-B}{A}\right)\right] \times 100 \quad (2)$$

where *I %* is inhibition percent; *A* is absorbance of the control sample at 575 nm; *B* is absorbance of the treatment sample at 575 nm.

2.6.3 Cellulase Activity

Cellulase enzyme was secreted from the tested fungi that were grown on Czapeck Dox medium amended with 3% w/v concentration of carboxy methyl cellulose as the sole carbon source and autoclaved (1.5 lb/in2) for 15 min only as recommended by Kavitha et al. [11]. Then cultures were inoculated with the fungus, incubated at 28 °C for 12 days and then filtrated through Whatman No. 1. The filtrate was considered as a source of crude enzyme which was immediately used for assaying the enzyme activity.

The activity of cellulase was monitored by estimation of reducing sugar using the dinitrosalicylic method [12].

The reaction mixture was prepared from the following components (containing 10 g of 3,5-dinitrosalicylic acid, 10 g NaOH, 20 mL of phenol,

0.5 g of sodium sulfate with a final volume of 1,000 mL).

In a test tube, 2 mL of a solution (containing 1 g of CMC, 100 mL of buffer, 0.2 M HAC-NaAC) with pH 4.8 was added to 2 mL of CMC-NaAC buffer. The mixture was kept in a water bath at 50 ºC for 5 min, then 1 mL of crude enzyme was added and the mixture was warmed in a water bath at 50 ºC for 30 min. Then 1 mL of 10, 50, 100, 500 or 1,000 ppm of each tested compound and tested fungicides (flutolanil or copper oxychloride) was added and incubated at 28 ºC for 24 h. Finally 3 mL of the previously prepared reaction mixture solution was added. The absorbance of CMCase was measured at 575 nm with a spectrophotometer after 15 min incubation in a shaking water bath at 50 ºC. Blanks were prepared similarly (3 replicates per treatment) but without the addition of the enzyme sample during incubation.

$$I\% = \left[\left(\frac{A-B}{A}\right)\right] \times 100 \quad (3)$$

where *I%* is inhibition percent; *A* is absorbance of the control sample at 575 nm; *B* is absorbance of the treatment sample at 575 nm.

3. Results and Discussion

3.1 Isolation and Identification of Cardenolide Compounds of G. sinaicus

Fractionation of the chloroform- soluble fraction of the methanolic extract of the aerial parts of *G. sinaicus* by column chromatography on silica gel afforded two cardenolide compounds, card. glycoside which was isolated in a crystalline form and card. genin, which was isolated in liquid form.

The purity of the two compounds was proved by TLC. The two compounds were visualized on TLC plates using the following techniques; inspection the TL chromatograms under ultraviolet radiation at 254 nm, spraying with α-naphthol-H_2SO_4 chromogenic reagent for sugar moiety and Kedde chromogentic reagent for cardenolides. The two compounds had RF values of 0.83 and 0.51, respectively and showed positive results for both chromogenic spray reagents.

The identifies of the isolated compounds were established by comparing their infrared, IR spectra, mass spectrum, MS, and nuclear magnetic resonance—^{1}H-NMR and ^{13}C-NMR with the literature data by El-Askary et al. [13], Abdel-Azim et al. [14], Abdel-Azim [15] as well as the corresponding data for various molecules given by Weast [16], Robien et al. [17].

Results in Table 1 show that card. glycoside had the molecular formula $C_{29}H_{45}O_{10}$ deduced from its mass spectrum and the presence of 29 carbons observed in the ^{13}C NMR spectrum. The MS spectrum revealed a molecular ion peak at M/Z 553 $\{M\}^+$, 517 $\{M\text{-}2H_2O^+\}$, 414 $\{M\text{-}C_3H_7OH\}^+$, 381 $\{M\text{-}H_2O\text{-}CH_3\}^+$, 272 $\{M\text{-}2H_2O\text{-}CO\}^+$, 213 $\{M\text{-}OCH_3\}^+$ and a base peak at M/Z 159 $\{M\text{-}CH_2{=}CH_2\}^+$.

Table 2 shows the IR spectrum revealed several absorption bands at 4205-4335, 3061-3740, 1219-1536, 907-1190, 1653-1682 and 1682-1939 cm^{-1} which indicated the presence of CH, OH(stretching), OH(bending), CO, C=C and C=O.

Table 3 shows the ^{1}H NMR spectrum, the presence of a signal at δ 2.49 indicated the presence of 3'-hydroxyl group. A signal at δ 0.818 indicated the presence of methyl group and a signal at δ 3.87 indicated the presence of formyl group.

Table 4 shows the ^{13}C NMR spectrum of this compound showed that the tertiary carbon signal for C-18 was shifted from δ 15.6682 to 18.3452, C-6: δ

Table 1 Mass spectrum and relative abundance for of the card. glycoside (crystalline) isolated from *G. sinaicus*.

M/Z	Base peak 100	Genin ion
553	25	M
517	10	$\{M\text{-}2H_2O^+\}$
457	10	$\{M\text{-}C_3H_7OH\}^+$
414	57.5	$\{M\text{-}H_2O\text{-}CH_3\}^+$
381	22.5	
336	27.5	$\{M\text{-}2H_2O\text{-}CO\}^+$
272	42.5	
244	37.5	$\{M\text{-}OCH_3\}^+$
213	57.5	
187	37.5	$\{M\text{-}CH_2{=}CH_2\}^+$
159	65.0	

Table 2 Infrared positions of bonds found in crystalline and liquid compounds isolated from *G. sinaicus*.

Bond	Mod	Crystalline compound	Liquid compound
C-H	Stretch	*4205.6-4335.7(s)*	*3211.7-4323.3(s)*
C-H	Stretch *C*	*2708.8-3403.1(m)*	*2853.3-3406.4(m)*
O-H	Stretch	*3061.1-3740(s)*	*3052-3747.9(w)*
O-H	Bending	*1219.0-1536.4(s)*	*1216.7-1539.3(w)*
C-C	Stretch	*822.8-1195.9(m)*	*824.8-1165.5(m)*
C-O	Stretch	*907.3-1190.0(s)*	*914.9-1165.5(m)*
C=C	Stretch	*1653.0-1682.5(m)*	*1663.9-1683.9(s)*
C=O	Stretch	*1682.5-1939.5(m)*	*1683.9-1868.7(s)*
C=O (2v)	Stretch (2v)	*34031-3447.5(m)*	*3406.4-3747.9(m)*

S = strong; C = combination frequency; m = medium; 2v = first overtone; W = weak.

Table 3 ^{1}H NMR (500MHZ) Chemical shifts (δ) of card. glycoside (crystalline) and card. genin (liquid) prepared from *G. sinaicus*.

^{1}H	Card. glycoside (δ)	Card. genin (δ)
Hα-3'	2.49	
H-3	3.81	3.78
H-7		6.2885
H-16	2.5988	3.06
H-17	2.784	2.787
H-18	0.818	0.869
H-19	3.87	3.872
H-21	4.8044	4.8395
H-22	5.3485	5.3271

Solvent: $CDCl_3$; ^{1}H = Proton number; (δ) = Chemical shifts.

Table 4 ^{13}C NMR (500MHZ) Chemical shifts (δ) of card. glycoside (crystalline) and card. genin (liquid) prepared from *G. sinaicus*.

Carbon number	Card. Glycoside	Card. Genin
18	15.6682	-
	17.9776	14.2435
	18.3452	-
6	29.2946	22.8031
	29.7656	24.9631
	31.0524	27.3529
	36.6937	29.5014
	-	29.8116
	76.9294	29.9035
	77.1822	32.0405
	77.4349	-
19	-	76.8719
	113.4770	77.1217
	-	77.3775
	207.4254	

Solvent: $CDCl_3$.

29.2946 to 77.4349 and C-19: δ 113.4770 to 207.4254. The ^{1}H and ^{13}C NMR spectra of card. glycoside were very similar to those reported by [12, 14] for cardenolide glycoside, 15β-hydroxy-5,6-dehydrocalotropin, dissolved in the same solvent ($CDCl_3$), except for the presence of two olefinic carbons C-3 and C-4. So, it is suggested that the predicted card. glycoside isolated of *G. sinaicus* may be defined as 15-hydroxy-3,4,5,6-dehydrocalotropin as shown in the following predicted structure showed in Fig. 1.

Card. genin had the molecular formula $C_{23}H_{22}O_3$

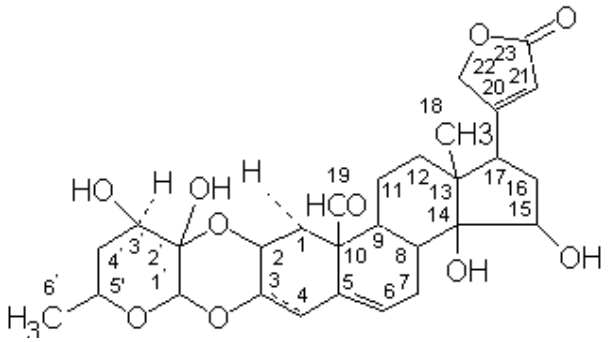

Fig. 1 Predicted structure of the cardenolide glycoside, 15-hydroxyl-3,4,5,6-dehydrocalotropin isolated from *G. sinaicus*.

deduced from its mass spectrum and the presence of 23 carbons observed in the ^{13}C NMR spectrum. The MS spectrum of this compound showed a molecular ion peak at M/Z 346 $\{M\}^+$, 328 $\{M\text{-}H_2O^+\}$, 273 $\{M\text{-}CH_2\text{=}CH\text{-}CH_2\text{-}CH_3\}^+$, 246 $\{M\text{-}CH_2\text{=}CH\}^+$, 201$\{M\text{–}CH_3CHOH\}^+$ and 145$\{M\text{-}CH_2\text{=}CH_2\}^+$ as shown in Table 5.

The IR spectrum exhibited characteristic absorption bands for C-H stretching (3211-4323 cm^{-1}), Carbon-Carbon double bond stretching (1663-1683 cm^{-1}), Carbon-Carbon single bond stretching (824-1165 cm^{-1}) and Carbon-Oxygen single bond stretching (1683-1868) in Table 2.

In the ^1H NMR spectrum, the presence of a signal at δ 3.78 indicated the presence of hydroxyl group. A signal at δ 0.869 and 3.872 indicated the presence of methyl group and formyl group, respectively (Table 3).

In the ^{13}C NMR spectrum, the tertiary carbon signal for C-18 was shifted at δ 14.2435, C-6: δ 22.8031 to 32.0405 and C-19: δ 76.8719 to 77.3775 (Table 4). Comparison ^1H and ^{13}C NMR spectrum of card. genin with those reported by [12 , 14] for cardenolide genin dissolved in the same solvent ($CDCl_3$), it is suggested that the predicted card. genin may be a 3,4,5,6-Dehydro-calotropagenin in Fig. 2.

3.2 Antifungal Activity of Isolated Cardenolide Compounds of G. sinaicus

Results of the antifungal activity of isolated compounds against soilborne fungi were recorded in

Table 5 Mass spectrum and relative abundance for of the card. genin (liquid) isolated from *G. sinaicus*.

M/Z	Base peak 100	Genin ion
346	15	M
328	40	$\{M\text{-}H_2O^+\}$
273	15	$\{M\text{-}CH_2\text{=}CH\text{-}CH_2\text{-}CH_3\}^+$
246	10	$\{M\text{-}CH_2\text{=}CH\}^+$
201	22	$\{M\text{-}CH_3CHOH\}^+$
173	30	$\{M\text{-}CH_2\text{=}CH_2\}^+$
145	87.5	

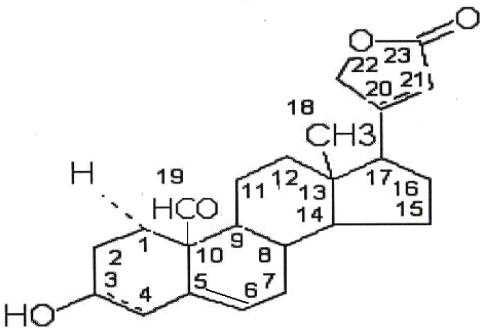

Fig. 2 Predicted structure of the cardenolide genin, 3,4,5,6-dehydrocalotropin isolated from *G. sinaicus*.

Table 6. card. glycoside at 100 µg/mL significantly reduced the mycelial growth of *R. solani* and *F. oxysporium* by 89.81 and 50.0% respectively. Total inhibition of mycelial growth of *R. solani* was obtained at 500 µg/mL. Statistical analysis and the calculated EC_{50} values revealed that *R. solani* was most susceptible to the inhibition by card. glycoside (EC_{50} value = 17.63 µg/mL) than *F. oxysporium* (EC_{50} value = 114.2 µg/mL).

On the other hand, cardenolide genin, at only 50 µg/mL significantly reduced the radial growth of *F. oxysporium* by 80.13%. Total inhibition of *R. solani* and *F. oxysporium* was obtained by 10 and 500 µg/mL, respectively. Again, *R. solani* was more susceptible to inhibition by cardenolide genin than *F. oxysporium* fungus.

Against postharvest fungi, results revealed that in Table 7. cardenolide glycoside at 100 µg/mL significantly reduced the mycelial growth of *R. stolonifer* and *P. digtatum* by 71.48 and 85.19%, respectively. At a concentration of 1,000 µg/mL the corresponding inhibition values were 90.44 and 92.59%

Table 6 Antifungal activity of isolated cardenolide glycoside and cardenolide genin against soilborne fungi.

Conc. (μg/mL)	Inhibition %			
	R. solani		*F. oxysporium*	
	Card. glycoside	Card. genin	Card. glycoside	Card. genin
10	33.33	100	14.81	42.59
50	79.63	100	38.89	80.13
100	89.81	100	50.00	92.59
500	100	100	66.67	100
1,000	100	100	84.22	100
EC_{50} (μg/mL)	17.63	<10	114.2	13.63
U.L	22.01	--	153.47	17.64
L.L	13.45	--	84.53	9.76
Slope	1.795	--	0.935	1.651

U.L = Upper limit; L.L = Low limit.

Table 7 Antifungal activity of isolated cardenolide glycoside and cardenolide genin against postharvest fungi.

Conc. (μg/mL)	Inhibition%			
	R. stolonifer		*P. digtatum*	
	Card. glycoside	Card. genin	Card. glycoside	Card. genin
10	29.63	62.96	35.19	50.67
50	60.11	66.67	62.29	72.30
100	71.48	72.22	85.19	88.19
500	79.89	81.11	84.90	91.41
1,000	90.44	95.03	92.59	95.33
EC_{50} (μg/mL)	31.64	4.22	19.901	8.403
U.L	45.32	--	35.74	14.11
L.L	20.08	--	1.94	3.89
Slope	0.848	0.509	0.888	0.841

U.L = Upper limit; L.L = Low limit.

respectively. On the other hand cardenolide genin at only 10 μg/mL significantly reduced the radial growth of *R. stolonifer* and *P. digtatum* by 62.96 and 50.67%, respectively. At a concentration of 1,000 μg/mL the corresponding inhibition values were 95.03 and 95.33%, respectively. According to the EC_{50} values of cardenolide genin against postharvest fungi, *R. stolonifer* was most susceptible to inhibition by this compound than *P. digtatum*.

Also the antifungal activity of the two isolated compounds was compared with the fungicidal activity of standard fungicides, copper oxychloride for postharvest fungi and flutolnail for soilborne fungi. Against soilborne fungi, cardenolide genin was 13.5 and 4.5 times more toxic to *R. solani* and *F. oxysporium* (EC_{50} values = 0.703 and 13.63 μg/mL, respectively) than flutolnail (corresponding EC_{50} values = 9.49 and 61.22 μg/mL) as shown in Table 8. On the other hand flutolnail was 1.9 and 1.9 times more toxic to the tested fungi than cardenolide glycoside. Against postharvest fungi, again cardenolide genin was 66.3 and 22.3 times more toxic to *R. stolonifer* and *P. digtatum*, respectively than copper oxychloride. Also, cardenolide glycoside was 8.8 and 9.4 times more toxic to the same fungi, respectively, than copper oxychloride.

All of the above results showed that the isolated cardenolide compounds of aerial parts of *G. sinaicus* have significant antifungal activity against the tested fungi since it is widely accepted that plant extractives that are active at EC_{50} values less than 100 μg/mL could be considered to have good potency level by Rios et al. [18].

The highest antifungal activity was obtained from the cardenolide genin. These compounds as well as methanol extract of *G. sinaicus* may be potential

antifungal compounds for use as fungicides. Many plant extracts and plant products have been reported to possess fungicidal activity against soilborne fungi [19-27].

3.3 Biochemical Studies

In an attempt to elucidate the possible mode of the fungicidal action of the two isolated cardenolide compounds, this part of study aims to discover if these compounds have an inhibitory effect on the enzymes, pectin methyl esterase (PME), polyphenol oxidase (PPO) and cellulase, tested of the soilborne and postharvest fungi.

Results in Table 9 show that card. genin was more effective in inhibiting PME, PPO and cellulase enzymes of the soil borne fungi with I_{50} values of 12.01, 11.02, 119.89 µg/mL for *R. solani* and 129.0, 72.25, 109.08 µg/mL for *F. oxysporium* than the card. glycoside.

Table 8 Fungicidal activity of the isolated cardenolide compounds in comparison with standard fungicides.

Tested fungi	EC_{50} values (µg/mL) and their confidence limits		
Soilborne fungi	Card. glycoside	Card. genin	Flutolnail
R. solani	17.63	0.703	9.49 (5.55-16.79)
F. oxysporium	114.2	13.63	61.22 (32.63-144.31)
Postharvest fungi	Card. glycoside	Card. genin	Copper oxychloride
R. stolonifer	31.64	4.22	279.94 (100-1583)
P. digtatum	19.901	8.403	187.13 (81.86-675)

Table 9 Inhibitory effect of the isolated cardenolide compounds and synthetic fungicide on the activity of some enzymes of soil bornfungi.

Tested fungi and enzyme	I_{50} values (µg/mL)		
	Card. glycoside	Card. genin	Flutolnail
R. soloani			
PME	74.32	12.01	9.10
U.L	108.78	23.94	20.91
L.L	48.27	3.71	1.87
Slope	0.697	0.520	0.499
PPO	23.65	11.02	5.24
U.L	36.46	20.39	9.76
L.L	13.09	4.15	2.03
Slope	0.737	0.616	0.79
Cellulase	440.0	119.89	94.89
U.L	617.88	154.87	119.58
L.L	331.678	92.56	74.57
Slope	1.09	1.11	1.25
F. oxysporum			
PME	410.0	129.0	85.09
U.L	664.87	186.75	127.57
L.L	281.46	89.92	54.38
Slope	0.774	0.748	0.651
PPO	100.34	72.25	52.81
U.L	202.04	97.72	69.76
L.L	46.34	51.8	38.50
Slope	1.13	0.91	1.037
Cellulase	316.55	109.08	85.28
U.L	1,368.76	141.68	169.63
L.L	136.4	83.46	38.38
Slope	1.23	1.07	1.35

U.L= Upper limit; L.L= Low limit.

Table 10 Inhibitory effect of the isolated cardenolide compounds and synthetic fungicide on the activity of some enzymes of postharvest fungi.

Tested fungi and enzyme	I_{50} values (μg/mL)		
	Card. glycoside	Card. genin	Copper oxychloride
R. stolonifer			
PME	373.0	95.44	164.34
U.L	658.01	140.41	231.72
L.L	242.89	63.19	118.79
Slope	0.654	0.689	0.833
PPO	> 1,000	653.25	88.55
U.L	68,358	1095.57	133.64
L.L	1,580.89	444.35	56.42
Slope	0.428	0.855	0.641
Cellulase	500.28	87.78	46.10
U.L	--	126.29	66.34
L.L	--	58.78	29.53
Slope	0.604	0.726	0.771
P. digtatum			
PME	397.0	243.0	225.0
U.L	847.86	359.94	320.79
L.L	234.81	173.04	164.22
Slope	0.526	0.799	0.865
PPO	738.03	602.55	376.1
U.L	1,131.94	844.64	481.54
L.L	539.18	461.12	292.02
Slope	1.13	1.26	1.44
Cellulase	837.88	120.66	113.91
U.L	--	182.63	167.08
L.L	--	79.55	77.06
Slope	0.735	0.654	0.705

U.L = Upper limit; L.L = Low limit.

Flutolnil was more effective in inhibiting the enzymes of soil borne fungi than the card. genin.

Concerning the enzymes, PME, PPO and Cellulase, of post harvest fungi, results in Table 10 revealed that card. genin was more effective in inhibiting the enzymes with I_{50} values 95.44, 653.25 and 87.78 μg/mL for *R. stolonifer* and 243.0, 602.55 and 120.66 μg/mL, respectively, for *P. digtatum* than the card. glycoside.

4. Conclusion

All of the aforementioned results indicate that the isolated cardenolide compounds of *G. sinaicus* have significant fungicidal activity against soil borne and post harvest fungi. According to the available literature, this is the first report on the antifungal activity of the isolated compounds against pathogenic fungi. Also, there was a general correlation between the antifungal activity of the isolated cardenolide compounds and their potency for inhibition of PME, PPO and cellulase of soilborne and postharvest fungi. Thus, the mechanism of fungitoxicity of these compounds may be due in part to the inhibition of cellulase, PME and PPO enzymes as reported by Rosslenbroich and Stuebler [28]. However, the effect of the cardenolide genin on these enzymes is not sufficient to determine that these enzymes are its putative target site. Further investigations on pesticidal and toxicological effects of these cardenolides are highly recommended.

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