

# Inhibition of *Rhizoctonia solani* Growth and Its Extracellular Hydrolytic Enzymes by Different Extracts of Cinnamon (*Cinnamomum cassia*) and Black Cumin Seeds (*Nigella sativa*)

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Docking

**Abstract:** The present study evaluated the ability of different extracts of cinnamon and black cumin seeds to inhibit the growth of the phytopathogenic fungus *Rhizoctonia solani* and its extracellular cell wall degrading enzymes. Concentrations of 300 and 450 ppm of methylene chloride and hexane extracts of cinnamon completely inhibited the growth of *R. solani* after 72 hours of incubation at  $25 \pm 1^\circ\text{C}$  *in vitro*. Methylene chloride or hexane extracts of black cumin seeds at 4000 ppm after 72 hours inhibited the growth of *R. solani* by 37 and 39% respectively. Moreover, black cumin seed hexane extract at 2000 ppm inhibited 55% and 38% of the activity of pectin lyase (PL) and polygalacturonase (PG) respectively. In addition, the methanolic extract of black cumin seeds at 2000 ppm exhibited a significant reduction of exo-protease activity (74.8%). GC-MS analysis results showed that linoleic acid is the main component of the fixed oil fraction of black cumin seed hexane extract while (E)-cinnamaldehyde is the main component in both hexane and methylene chloride extracts of cinnamon. HPLC-MS analysis of black cumin seeds methanolic extract showed that amentoflavone, was the main component. Docking was used to identify the major component interaction with pectin lyase A and exo-protease.

## 1 Introduction

*Rhizoctonia solani* Kühn is a widespread soil borne pathogen, responsible for serious damage to numerous monetarily significant crops worldwide (Elwakil et al 2009). *R. solani* is known to cause rice sheath blight, black scurf on potatoes or damping off, root rot and stem rot diseases of a wide range of vegetable and crops (Ogoshi 1996). Control of *R. solani* is extremely difficult because it can form a sclerotia-dormant that permits the

fungus to endure unfavorable conditions and lives in the soil as a highly saprophytic competitive with a wide range of the soil microbial community (Zachow et al 2011). Examination of plant tissues infected by *R. solani* revealed the presence of pectic enzymes and cellulases (Gawade et al 2017). Cell wall degrading enzymes such as pectic enzymes, cellulases, hemicellulases and proteases, are considered the responsible for damage that accrues to plant cell walls during pathogenesis (El-Abyad et al 1992, Gvozdeva et al 2006). Various strategies have been utilized to

control *R. solani* such as cultural practices, resistant cultivars and chemical control (Ajayi-Oyetunde and Bradley 2018). Despite the increasing efforts to develop resistant cultivars, chemical control is still the main method to control many phytopathogenic fungi including is *R. solni* (Barilli et al 2017). The widespread use of synthetic fungicides has raised ecological issues because of their significant expense, unfavorable impact on environment and potential resistance inducing in the pathogens (Rathmell 1984). With these facts in mind efforts have been made to control *R. solani* through natural products and bioactive compounds. Previous studies demonstrated that cinnamon (*Cinnamomum cassia*) and black seeds (*Nigella sativa*) have antifungal properties against phytopathogenic fungi (Lee et al 2020, Matloob et al 2021).

The current study aims to evaluate the ability of different extracts of cinnamon (*Cinnamomum cassia*), black cumin seeds (*Nigella sativa*) to inhibit *Rhizoctonia solani* growth and its extracellular hydrolytic enzymes, for the purpose of developing eco-friendly fungicides to control *R. solani*.

## 2 Materials and Methods

### 2.1 Preparation of plant extracts

Cinnamon bark (*Cinnamomum cassia*) and of black cumin seeds (*Nigella sativa*) were obtained from the local market at Cairo, Egypt and grounded into a fine powder. The obtained powder was extracted by hexane followed by methylene chloride and then methanol 70% (v/v) at room temperature (28°C). Extraction was carried out by macerating 50g of each plant in 500 ml of hexane in a 1-liter flask for overnight and then mixture was filtered by Whatman No.1 (11cm) filter paper and air dried of hexane before macerating it in 500 ml of methylene chloride for overnight and then filtered and air dried from methylene chloride. The last step was to macerate the plant material resulted from the previous two steps in methanol 70% (v/v) for overnight and filter it. Every extraction step is repeated 3 times. The obtained extracts were air dried at room temperature (28°C) to dry weight and stored in freezer for further use (Abubakar and Haque 2020).

### 2.2 Fungal isolate

*Rhizoctonia solani* AG4-HGI was previously isolated from faba bean roots naturally infected with root rot disease. The pathogenicity of the fungus was tested and identified by (Helmy et al 2015). The fungus was used for test all plant material extracts in this study.

### 2.3 Antifungal assay of plant extracts

Antifungal assay was done using the agar dilution method (Menon et al 2001). Potato dextrose agar medium (PDA) was prepared and sterilized by autoclaving at 121 °C for 20min. The required amount of each extract was dissolved in 1 ml pure dimethyl sulfoxide (DMSO) and was added to the melted medium by a sterilized syringe filter (0.22µm Millipore) and thoroughly mixed to provide 200, 250, 300, 350, 400, 450, 500, 1000, 2000 and 4000 ppm of cinnamon hexane as well as methylene chloride extracts. Hexane or methylene chloride extracts of black cumin seeds and methanolic extract of both plants were prepared at 1000, 2000 and 4000 ppm. Control medium received only DMSO without any extracts, and the final concentration of DMSO in all media was 1%. Approximately 20 ml of treated or untreated medium at about 45°C was poured into a Petri plate (9cm diameter). After solidification of media, a 1cm disc from seven days old fungal culture was placed in the center of each petri plate. There were five replicates of each treatment. Petri plates were incubated at 25±1°C and the linear growth was recorded when the mycelium growth reached the edges of control plates (after 72h). The lowest concentration completely inhibited mycelium growth was considered to be the minimum inhibitory concentration (MIC). The concentration that inhibit 50% of mycelium growth (LC<sub>50</sub>) values were calculated by plotting the regression line using Microsoft Office Excel software. The percentage of mycelium growth inhibition calculated according to the following formula:

$$\text{Growth reduction \%} = (C - P / C) * 100$$

Where C = diameter of colony growth in control, and P = diameter of colony growth in tested extract.

### 2.4 Production of *R. solani* extracellular enzymes in liquid medium

Production of pectin lyase (PL), polygalacturonase (PG) and carboxymethyle cellulase (cmcase) was assayed using the medium described by (Macmillan

and Vaughn 1964), the medium consists of 10g glucose, 5g peptone, 5g yeast extract and 5g  $K_2HPO_4$  in 1-liter distilled water, pH 7.2 and glucose was replaced with citrus pectin, polygalacturonic acid or carboxymethyl cellulose respectively. The production of protease enzyme was assayed using the medium described by (Perombelon and Hadley 1965). The medium consists of 10g glucose, 31.2g skimmed milk powder (32.12% casein), 0.5g yeast extract, 1g  $KH_2PO_4$  and 0.5g  $MgSO_4 \cdot 7H_2O$  in 1-liter distilled water, pH 5.5. Media was prepared and distributed in 5 flasks and then sterilized by autoclaving at 121°C for 20min. Each flask was then inoculated with 1cm disc of seven days old fungal culture, maintained in the laboratory and incubated at 25±1°C for 7 days. The supernatant was obtained by filtration on Whatman no 1 (11cm) filter paper and centrifugation at 5000 rpm for 15min. The supernatant used as crude enzymes extracts.

## 2.5 *Rhizoctonia solani* extracellular enzymes assay

Plant extracts were prepared by dissolving 200 mg of each extract in 1ml pure dimethyl sulfoxide (DMSO). PL activity was determined spectrophotometrically by measuring the increase in  $A_{235}$  as described by (Alaña et al 1989). The reaction mixture (2 ml) contained 1% citrus pectin (w/v) dissolved in 0.05 M citrate buffer (pH 5) as a substrate, 980µl of crude enzyme that pre-adjusted to pH 7.5 with concentrated NaOH and 20µl plant extract as inhibitor. The reaction mixture was pre-incubated without the substrate at 40°C for 15min. The reaction was started with the addition of the substrate and then the mixture was incubated again for 20 min at 40°C. The reaction was stopped using 3.5 ml 0.5 M  $H_2SO_4$ . For the blank tube, acid was added to the mixture first and then the substrate. One unit of activity is the amount of enzyme which produces an increase of 1 unit of  $A_{235}$  per min.

The activity of PG and cmcase was determined by measuring the increase in reducing groups using dinitrosalicylic acid (DNS) reagent as described by (Bailey et al 1992). The reaction mixture (2ml) contains 1% (w/v) polygalacturonic acid as a substrate, 250µl crude enzyme that had been previously adjust to pH 5.5, 1.0 ml Na-acetate buffer (0.05 M, pH 4.5), 230µl distilled water and 20µl plant extract as inhibitor in the

case of PG and contained 1% (w/v) carboxymethyl cellulose dissolved in 0.05 M citrate buffer (pH 5) as a substrate, 500µl crude enzyme, 480µl distilled water, 20µl plant extract as inhibitor in the case of CMCase. After 20min both reactions were terminated by adding 3 ml DNS and boiling for 10min. Pre-boiled enzyme used in blank tube. The color was measured at 546nm using a spectrophotometer. Glucose at various concentrations was used to establish a standard curve of reducing sugars. One unit of activity is the amount of enzyme which produces 1µg of reducing sugars per min.

Exo-proteases activity was determined by measuring the increase in free amino acids using ninhydrine reagent as described by (Zhang et al 2013). The reaction mixture (2 ml) contained 0.5% (w/v) skimmed milk powder (32.12% casein) as a substrate, 500µl of crude enzyme, 1.230 ml distilled water, 20µl of plant extract as inhibitor. After 20min the reaction was terminated by adding 750µl of trichloroacetic acid 16% and centrifuging at 3,000 rpm for 10min. In the case of blank tube, trichloroacetic acid was added to the mixture first then the substrate. 1.5 ml of the supernatant was mixed with 1 ml ninhydrine and 2.5 ml distilled water and incubated in water bath at 100°C for 15min. The reaction was stopped by adding 5 ml ethanol 50% and the color measured at 470nm using a spectrophotometer. Glycine at various concentrations was used to create a standard curve of free amino acids. One unit of activity is the amount of enzyme which produces 1µg of free amino acids per min.

The control consisted of all the components of the reaction mixture except for the plant extract, it was replaced with 20µl DMSO. The final concentrations of DMSO and plant extract in the reaction mixtures were 1% (w/v) and 2000 ppm respectively. The percentage of enzymes inhibition is calculated according to the following formula:

$$\text{Enzyme inhibition \%} = (C - P / C) * 100$$

Where C = activity of the enzyme in control, and P = activity of the enzyme in presence of tested extract.

## 2.6 Chemical composition

Hexane and methylene chloride extracts of cinnamon and fatty acids extracted from black cumin seeds hexane extract were analyzed using GC- MS while black cumin seeds methanol extract was analyzed using HPLC- MS.

### 2.6.1 GC-MS analysis

Black cumin seeds hexane extract preparation: 1g of the extract was mixed with 10 ml of 2 N alcoholic KOH in a screw-capped tube and heated in a water bath at 70°C for 2h. The saponified fatty acids were separated from the un-saponified matter and then recovered to fatty acids by concentrated HCl. Fatty acid methyl esters were obtained by mixing fatty acids with 10 ml of 30% methanol sulphonic (prepared by mixing 15 ml of H<sub>2</sub>SO<sub>4</sub> with 35 ml absolute methanol) and heating in an oven at 70°C for 45min (Kramer et al 1997).

Hexane extract of cinnamon was injected into a GC1310-ISQ mass spectrometer (Thermo Scientific, Austin, TX, USA) with a direct capillary column TG-5MS (30 m x 0.25mm x 0.25 µm film thickness). The oven temperature was initially held at 60°C and then increased by 7°C /min to 200 °C hold for 2min and then increased to a final temperature of 300°C by 10°C /min and hold for 2min. The injector and MS transfer line temperatures were kept at 270, 260°C respectively, and; Helium was used as a carrier gas at a constant flow rate of 1 ml/min. The solvent delay was 3 min and a 1µl diluted sample was automatically injected using autosampler AS1300 coupled with GC in the split mode. EI mass spectra were collected at 70 ev ionization voltages over the range of 50–650 m/z in full scan mode. The ion source temperature was set at 250 °C. Methylene chloride extract of cinnamon and fatty acid methyl esters obtained from hexane extract of black cumin extract were injected into GC Agilent, 5977 mass spectrometer with capillary column Agilent HP-5ms (30 m x 0.25 mm x 0.25 µm film thickness). The oven temperature was initially held at 50°C for 3 min and then increased by 20 °C/min to 300 °C and hold for 5 min. The injector and MS transfer line temperatures were kept at 270, 260°C respectively; Helium was used as a carrier gas at a constant flow rate of 1 ml/min. The solvent delay was 5 min and a 1 µl diluted sample was manually injected in the splitless mode. EI mass spectra were collected at 70 ev ionization voltages over the range of 40–550 m/z in full scan mode. The ion source temperature was set at 230 °C. The components were identified by comparison of their retention times and mass spectra with those of WILEY 09 and NIST 11 mass spectral database.

### 2.6.2 HPLC-MS analysis

Black cumin seeds methanol extract was analyzed using Shimadzu LC-2040 instrument equipped with LC-2040 Pump, LC-2040 autosampler and LC-2030/2040 PDA detector (254 nm). The compounds were separated with Shimadzu uplcms 8045 C-18 column (1.7 mm x 2.1 mm x 50 mm). A gradient of two solvents, A (water) and B (acetonitrile) were used, ranging from 5% acetonitrile to 95% at flow rate 0.2 ml/min. The injected volume was 2 µl. Interface heater and voltage were 300°C and 4.00 kv respectively; the drying gas (nitrogen) flow was set to 10.00 L/min and corona needle voltage was 4.5 kv. The compounds were detected using Q3 Scan negative ESI mood while the collision-induced dissociation gas (CID) was off (all the ions passed through Q1 and Q2 and were scanned by Q3). The mass spectra were recorded in the range of 100–1200 m/z with 3000 u/sec scan speed. The major components were identified by comparison of their retention times and mass spectra with those of WILEY 09 and NIST 11 mass spectral databases and literature.

### 2.7 Molecular docking

Molecular docking simulation of pectin lyase and exo-protease were performed by autodock 4 software (Morris et al 2009). The software was downloaded from the scripps research institute website. The PDB files of pectin lyase A (1IDK, ID: 1) and protease (1GDN, ID: 1) were downloaded from protein data bank (RCSB PDB) and ligands structure were drawn by PubChem draw structure tool (<https://pubchem.ncbi.nlm.nih.gov/#draw=true>) and converted to PDBQT format. Proteins were prepared by deleting water molecules, adding polar hydrogen atoms, calculating Kollman charges and saving the structure as PDBQT format by autodock Tools 1.5.6. Ligands were optimized by adding polar hydrogen atoms, calculating Kollman and Gasteiger charges, minimizing its energy through local energy minimization, detecting torsion root and saving the structure as PDBQT format by autodock Tools 1.5.6 (Sanner 1999). A grid box of size 58 × 50 × 60 was created with 0.329 × 48.064 × -6.436 grid point spacing of 0.375 ° A incase of PL and 46 × 40 × 40 with 3.767 × -1.355 × -6.377 grid point spacing of 0.375 ° A incase of exo-protease to determine the searching space by autogrid4. Docking was performed using the Lamarckian genetic algorithm (LGA) (Morris et al 1998). The outputs were analyzed and visualized by PyMOL software.

## 2.8 Statistical analysis

Statistical analysis was done using the software IBM SPSS Statistics. The replicates were subjected to a one way ANOVA test and the mean values were compared using Duncan and LSD test at a significance level of  $P \leq 0.05$  (Snedecor and Cochran 1980).

## 3 Results and Discussion

### 3.1 Effect of cinnamon and black cumin seeds extracts on *Rhizoctonia solani* growth

In the current study the growth of *R. solani* was measured in the presence of various concentrations of cinnamon and black cumin seeds extracts after 72h of incubation and the results are presented in **Table 1**.

The results showed that both hexane and methylene chloride extracts of cinnamon and black cumin seeds inhibited *R. solani* growth, whereas no significant effect occurred at all concentrations of methanol extract of both plant species. Cinnamon hexane and methylene chloride extracts caused complete inhibition of *R. solani* growth after 72h at 450 ppm and 300 ppm respectively **Fig 1** while cinnamon methylene chloride extract at 250 ppm inhibited 85% of mycelium growth after 72h. Hexane and methylene chloride extracts of black cumin seeds at 4000 ppm caused inhibition of 38.67 % and 36.67 % of *R. solani* mycelium growth respectively after 72h of incubation **Fig 2**.

### 3.2 Effect of cinnamon and black cumin seeds extracts on extracellular hydrolytic enzymes of *Rhizoctonia solani*

The effect of cinnamon or black cumin seeds extracts at 2000 ppm on *Rhizoctonia solani* extracellular hydrolytic enzymes presented in **Table 2**.

Pectin lyase (PL) was significantly inhibited by treatment with cinnamon extracts (hexane, methylene chloride or methanol) or black cumin seeds extracts (hexane or methylene chloride). In contrast, PL was activated due to treatment with black cumin seeds methanol extract. Hexane and methylene chloride extracts of black cumin seeds reduced PL by more than 50%. Also, hexane and methylene chloride extracts of cinnamon reduced PL activity by 42% and 44% respectively. While methanol extract showed a low inhibitory effect

toward PL (10%) in the case of cinnamon and activated PL by 9.4% in the case of black cumin seeds.

Hexane extract of both plants inhibited polygalacturonase (PG) activity. While cinnamon methanol extract activated PG significantly. Hexane extract of cinnamon and black cumin seeds reduced PG activity by 7.8% and 38% respectively. Methanol extract of cinnamon activated PG by 156.8%. Cinnamon methylene chloride extract and methylene chloride and methanol extracts of black cumin seeds did not show any significant effect on PG activity.

Hexane and methylene chloride extracts of cinnamon or black cumin seeds showed no significant effect on carboxymethyl cellulase (CMCase) activity. Methanol extract inhibited CMCase in the case of black cumin seeds and activated CMCase in the case of cinnamon. Black cumin seeds methanol extract reduced CMCase activity by 12% while methanol extract of cinnamon increased the enzyme activity by 16.5%.

Exo-protease was inhibited by all extracts except for the methanolic extract of cinnamon, which significantly activated Exo-protease. Methanol extract of black cumin seeds showed the best significant effect by the inhibition of 74.8% of the enzyme activity followed by hexane and methylene chloride extracts, which inhibited 49% and 47% of the enzyme activity respectively. Hexane and methylene chloride extracts of cinnamon inhibited 12% and 14% of the enzyme activity respectively while methanolic extract activated exo-protease by 229%.

### 3.3 Chemical composition of cinnamon and black cumin seeds extracts

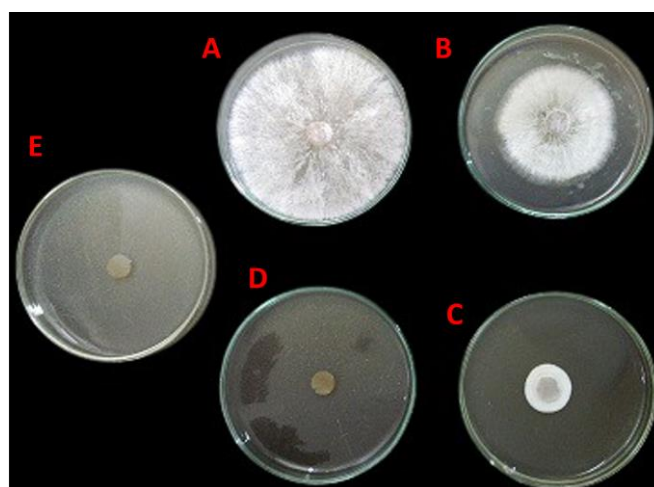
The yield of extracts obtained from the gradient extraction by hexane, methylene chloride or methanol were 12%, 5.54% and 44.5% (w/w) respectively in the case of cinnamon bark and 30.35%, 13.5% and 30% (w/w) respectively in the case of black cumin seeds. Twenty-six compounds were found in cinnamon hexane extract **Fig 3**, the major constituents were (E)-cinnamaldehyde (57.5%), dodecane (7.66%), tetradecane (6.46%) and coumarin (4.89%) **Fig 4**. While the result of GC-MS analysis of cinnamon methylene chloride extract showed only the presence of two constituents (E)-cinnamaldehyde (90.53%) and coumarin (9.46%) **Table 3** and **Fig 5**. The results of GC-MS analysis of black cumin seeds hexane extract fixed oil fraction showed the presence of eight constituents **Table 4** and **Fig 6**, the major constituents were linoleic acid and conjugated linoleic acid 18:2 (75.95%) and palmitic acid (20.85%).

**Table 1.** Effect of black cumin seeds (*N. sativa*) and cinnamon (*C. cassia*) extracts on linear growth of *R. solani* after 72 hours

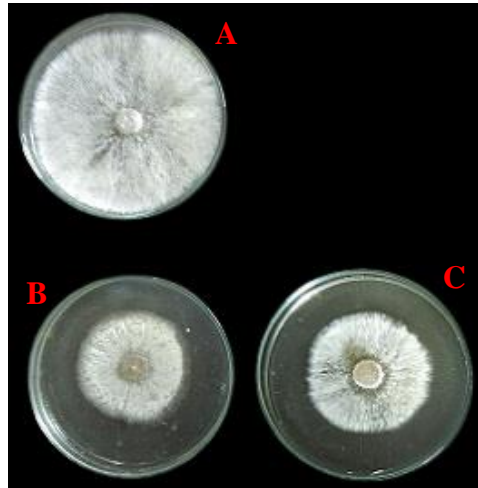
Treatments			% Growth inhibition	MIC
Black cumin seeds	Hexane	1000 ppm	8.22 ±2.00 <sup>j</sup>	>4000 ppm
		2000 ppm	25.33 ±0.41 <sup>h</sup>	
		4000 ppm	38.67 ±2.84 <sup>g</sup>	
	Methylene chloride	1000 ppm	19.33 ±3.25 <sup>i</sup>	>4000 ppm
		2000 ppm	34.22 ±0.41 <sup>g</sup>	
		4000 ppm	36.67 ±1.01 <sup>g</sup>	
	Methanol	1000 ppm	0.00 ± 0.00 <sup>k</sup>	>4000 ppm
		2000 ppm	0.00 ±0.00 <sup>k</sup>	
		4000 ppm	0.28 ±0.27 <sup>k</sup>	
Cinnamon	Hexane	200 ppm	37.11 ±1.14 <sup>g</sup>	450 ppm
		250 ppm	40.88 ±2.92 <sup>f</sup>	
		300 ppm	58.00 ±0.54 <sup>e</sup>	
		350 ppm	67.34 ±0.44 <sup>d</sup>	
		400 ppm	77.56 ±0.41 <sup>c</sup>	
		450 ppm	100.00 ±0.00 <sup>a</sup>	
	Methylene chloride	200 ppm	38.67 ±5.48 <sup>g</sup>	300 ppm
		250 ppm	85.11 ±6.17 <sup>b</sup>	
		300 ppm	100.00 ±0.00 <sup>a</sup>	
	Methanol	1000 ppm	0.22 ±0.22 <sup>k</sup>	>4000 ppm
		2000 ppm	0.67 ±0.44 <sup>k</sup>	
		4000 ppm	0.00 ±0.00 <sup>k</sup>	
L.S.D.			5.0715	

All values present means of five replicates ± SE.

Different letters in the same column refer to significant difference (p< 0.05)



**Fig 1.** Effect of different concentrations of cinnamon methylene chloride extract on *R. solani* growth; A: Control, B: 200 ppm, C: 250 ppm, D: 300 ppm, E: 350



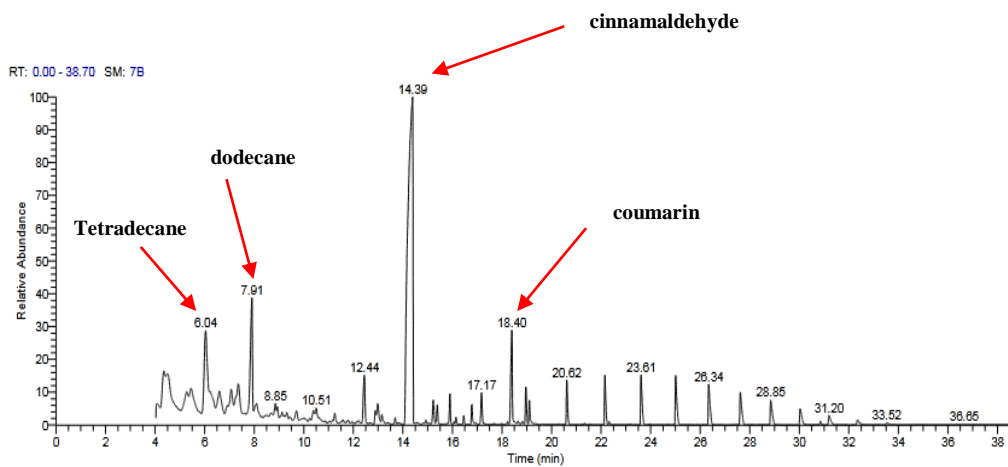
**Fig 2.** Effect of different extracts of black cumin seeds at 4000 ppm on *R. solani* growth; A: Control, B: Hexane ex., C: Methylene chloride ex.

**Table 2.** Effect of cinnamon (*C. cassia*) or black cumin seeds (*N. saliva*) extracts at concentration 2000 ppm on the activity of *R. solani* hydrolytic enzymes

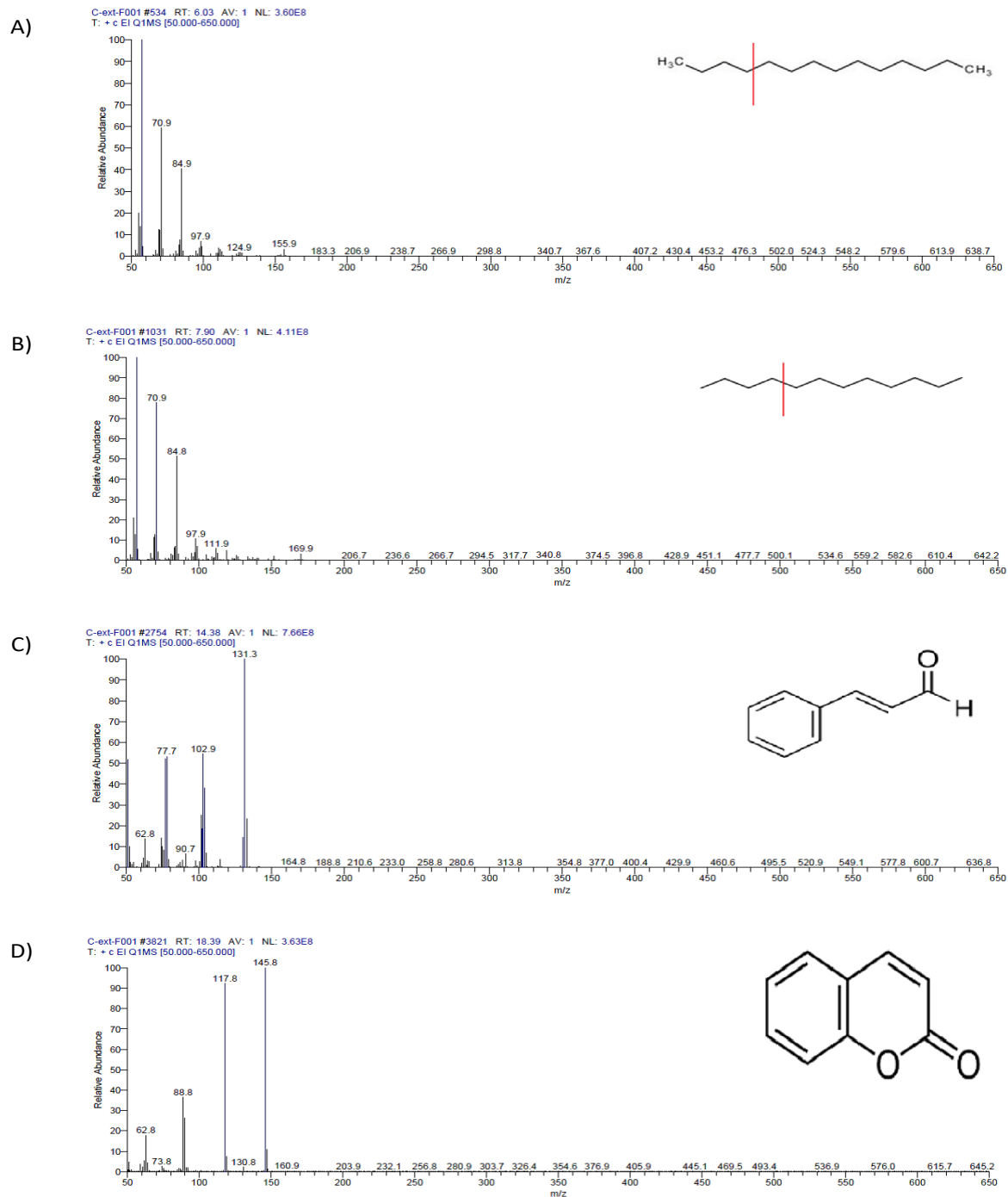
Treatments		Enzymes activities ( IU/ml crude enzyme extract)			
		Pectin layase (PL)	Polyglactourinase (PG)	Carboxymethyl cellulase	Exo-Protease
Control		0.69±0.01 <sup>d</sup>	232.07±0.58 <sup>c</sup>	103.7± 0.53 <sup>b</sup>	14.32±0.05 <sup>f</sup>
Cinnamon	Hexane	0.39±0.01 <sup>b</sup>	213.8±0.92 <sup>b</sup>	104.03± 0.77 <sup>b</sup>	12.55±0.06 <sup>e</sup>
	Methylene Chloride	0.38±0.00 <sup>b</sup>	230.2±0.69 <sup>c</sup>	103.3± 0.83 <sup>b</sup>	12.31±0.03 <sup>d</sup>
	Methanol	0.62±0.00 <sup>c</sup>	596.07±5.34 <sup>d</sup>	120.5± 0.61 <sup>c</sup>	47.18±0.04 <sup>g</sup>
Black cumin seeds	Hexane	0.31±0.00 <sup>a</sup>	143.8±4.01 <sup>a</sup>	104.23± 1.01 <sup>b</sup>	7.27±0.03 <sup>b</sup>
	Methylene Chloride	0.32±0.00 <sup>a</sup>	226.6±2.02 <sup>c</sup>	103.17± 1.11 <sup>b</sup>	7.52±0.03 <sup>c</sup>
	Methanol	0.75±0.00 <sup>e</sup>	230.47±0.35 <sup>c</sup>	91.1± 0.69 <sup>a</sup>	3.61±0.05 <sup>a</sup>
L.S.D.		0.0237	8.1346	1.2383	0.1296

All values present means of three replicates ± SE.

Different letters in the same column refer to significant difference (p< 0.05)



**Fig 3.** GC/MS chromatogram of cinnamon hexane extract

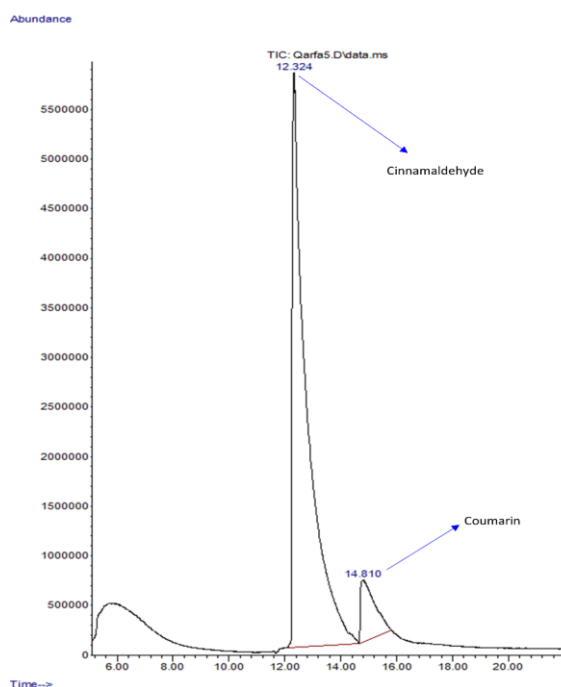


**Fig 4.** Mass spectrum of A) tetradecane; B) dodecane; C) cinnamaldehyde; D) coumarin



**Table 3.** Chemical composition of Cinnamon methylene chloride extract

Compound	RT	MS (m/z)	% Area
Cinnamaldehyde	12.324	131, 103, 77, 51	90.53
Coumarin	14.810	146.1, 118, 89, 63, 43	9.46
<b>Total</b>			<b>99.99</b>

**Fig 5.** GC/MS chromatogram of cinnamon methylene chloride extract

HPLC-MS analysis of methanolic extract of black cumin seeds showed the presence of more than forty compounds **Fig 7**, the major constituents were amentoflavone (36.8%) at a retention time (tR) of 1.57 min, quercetin3-O-sophoroside-7-O-rhamnoside (9.94%) at a retention time (tR) of 11.40 min and procyanidin C2 (16.73%) at a retention time (tR) of 20.29 min. The molecular weight of major compounds were confirmed by comparing retention time and mass spectrums with those of the similar reference compound and literature. Regarding the mass spectrum of amentoflavone **Fig 8A**, it was found that the  $[M-H]^-$  ion was at 537 m/z; the ion at 387 m/z resulted from the fragmentation of C ring at bonds 1 and 3 in flavone I while the ion at 357 m/z resulted from the fragmentation of C ring at bonds 0 and 4 in flavone II plus the loss of water molecule. The base peak resulted from

fragmentation of C ring in both flavones I and II plus the loss of  $CH_2O$  was at 195 m/z **Fig 9A**. The mass spectrum of quercetin3-O-sophoroside-7-O-rhamnoside **Fig 8B** showed only two peaks, the  $[M-H]^-$  ion at 771 m/z (the major peak) **Fig 9B** and the  $[M-H+1]^-$  ion at 772 m/z. The mass spectrum of procyanidin C2 **Fig 8C** showed that the  $[M-H]^-$  ion was at 865 m/z and the base peak resulted from the loss of 2 catechins through quinone methides reaction followed by loss of 2 water molecules was at 251 m/z. The fragmentation pattern of procyanidin C2 presents on **Fig 9C**.

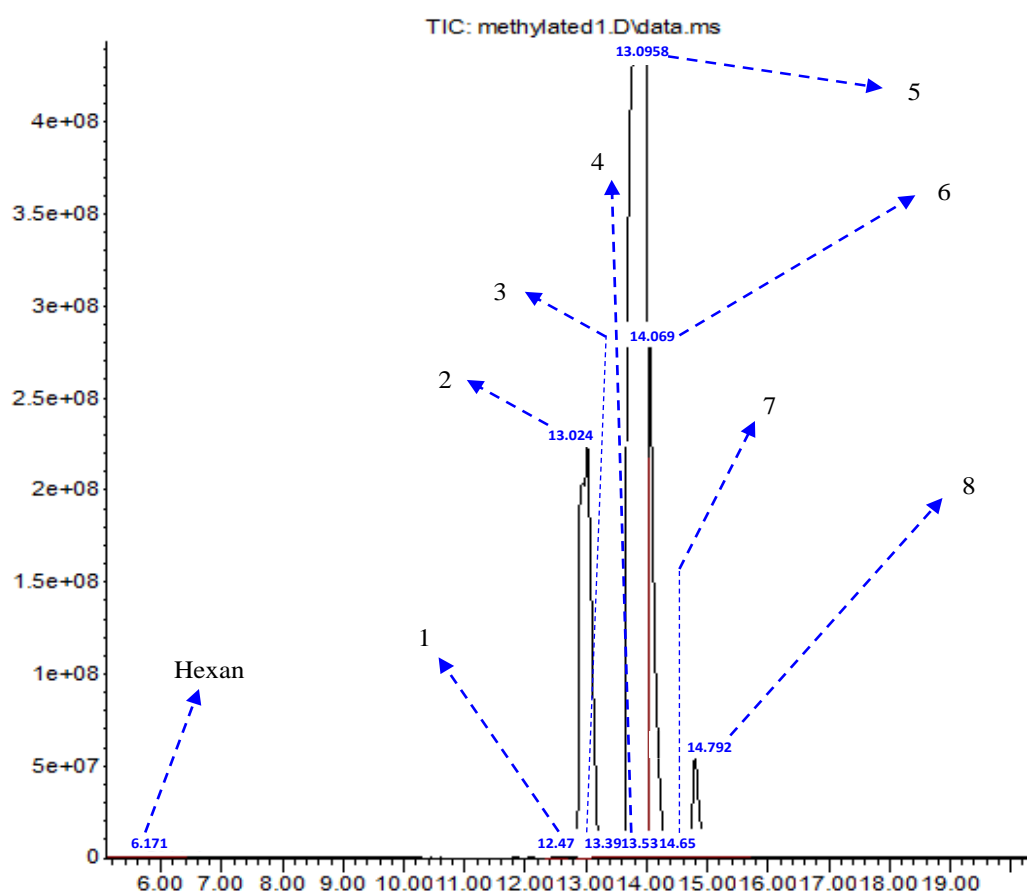
The current study attempted to investigate the efficacy of different extracts of cinnamon and black cumin seeds to inhibit the growth of *R. solani* and its hydrolytic enzymes and to link the results obtained with the major constituents of the extracts which have been identified in this study. The identification of amentoflavone confirmed by comparing its fragmentation pattern with the fragmentation pattern of IC3 – IIC8 linked biflavones reported by (Yao et al 2017) while quercetin3-O-sophoroside-7-O-rhamnoside identification confirmed by comparing its fragmentation pattern with the fragmentation pattern of flavone C,O-hexosides reported by (Tsugawa et al 2019) and procyanidin C2 were confirmed by comparing its fragmentation pattern with the fragmentation pattern of procyanidins reported by (Enomoto et al 2019).

The results showed that methylene chloride and hexane extracts of both plants demonstrated moderate to high antifungal activity against *R. solani* with the lowest minimum inhibitory concentration (MIC) in the case of cinnamon. It has been found that the major constituent of both methylene chloride and hexane extracts of cinnamon is E)-cinnamaldehyde and there is a linear association between the concentration of E)-cinnamaldehyde in the extracts and its antifungal activity which suggests that E)-cinnamaldehyde is the responsible for the antifungal activity of cinnamon extracts. The results of previous studies showed that cinnamon extracts and cinnamon-derived compounds have antifungal activity against several phytopathogenic fungi including *R. solani* (Lee et al 2020). According to the results reported by (Nguyen et al 2009) the direct contact of cinnamon methanol crude extract at 1% to young hyphae of *R. solani* cause complete destroy of the hyphae specially the cell walls which deform and collapse furthermore it has been reported that cinnamaldehyde has good antifungal properties due to the presence of a conjugated double bond and a long CH chain outside

**Table 4.** Fatty acids profile of black cumin seeds hexane extract

No	Compound		RT	% Area
1	Methyl tetradecanoate	C14:0	12.47	0.0136
2	Hexadecanoic acid, methyl ester	C16:0	12.024	20.8571
3	Hexadecanoic acid, 14-methyl-, methyl ester	Anteiso-C17:0	13.39	0.0149
4	Heptadecanoic acid, methyl ester	C17:0	13.53	0.0108
5	9,11-Octadecadienoic acid, methyl ester, (E,E)-	C18:2 $\Delta^9, \Delta^{11}$	13.0598	64.5672
6	8,11-Octadecadienoic acid, methyl ester	C18:2 $\Delta^8, \Delta^{11}$	14.069	11.3569
7	9,12-Octadecadienoic acid, methyl ester, (E,E)-	C18:2 $\Delta^9, \Delta^{12}$	14.65	0.0265
8	cis-11,14-Eicosadienoic acid, methyl ester	C20:2 $\Delta^{11}, \Delta^{14}$	14.792	2.9683
	<b>Total</b>			99.8153

Abundance



**Fig 6.** GC/MS chromatogram of fatty acid profile of black cumin seeds

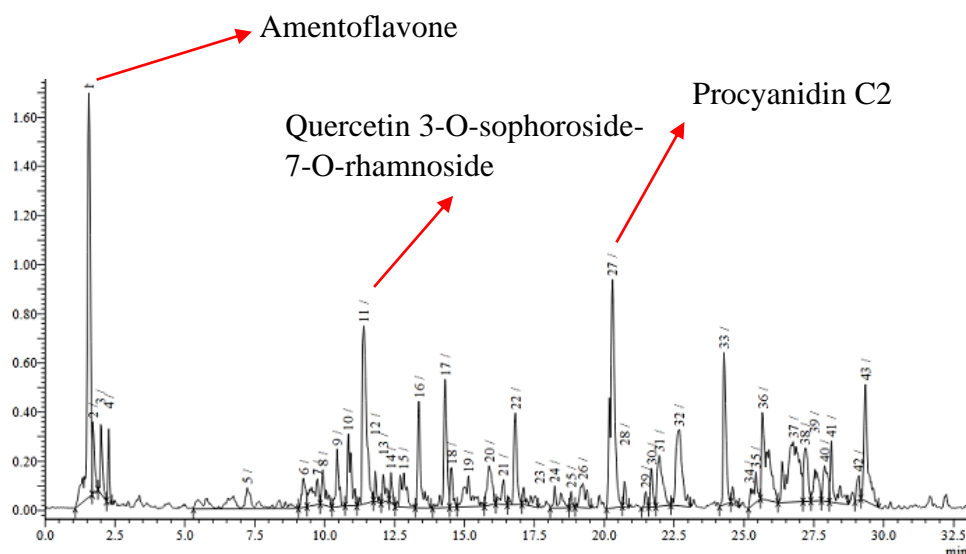


Fig 7. HPLC/MS chromatogram of black cumin seeds methanolic extract

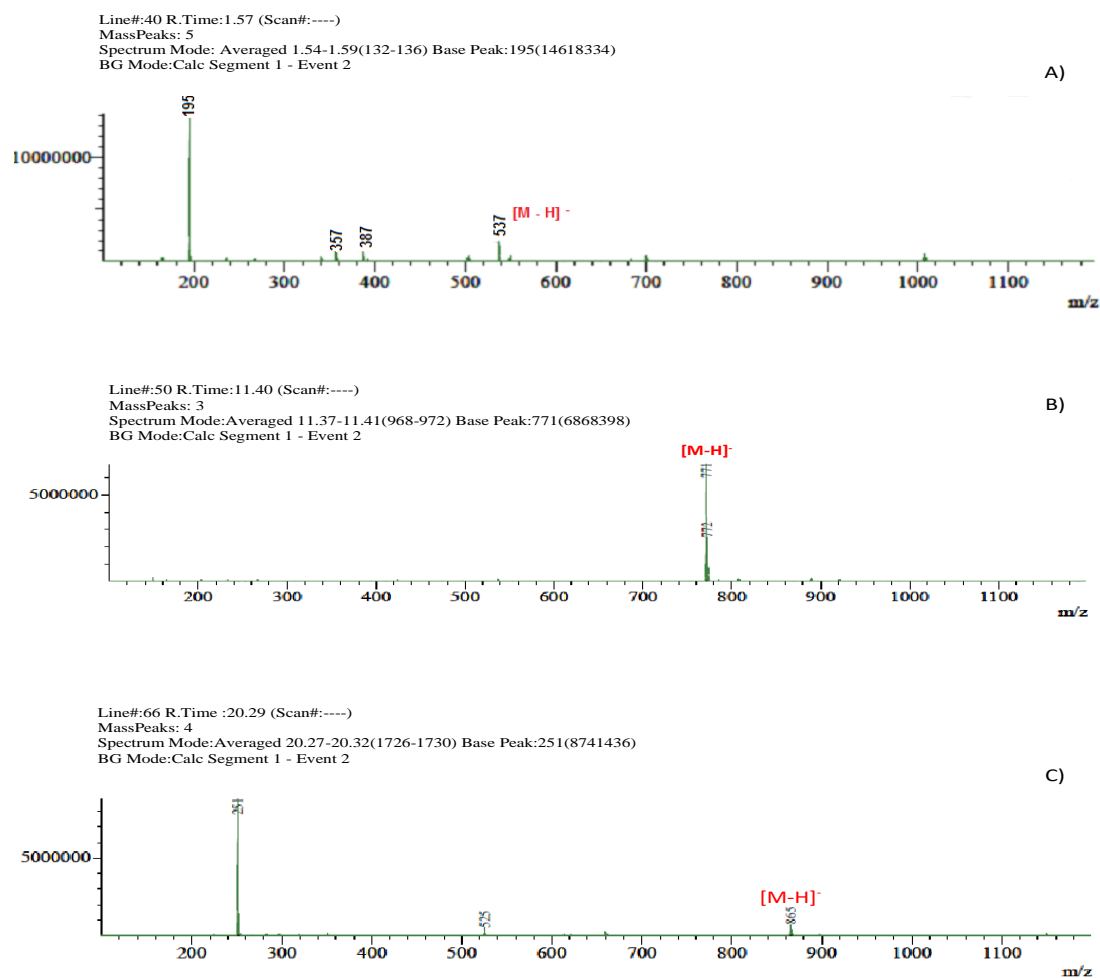
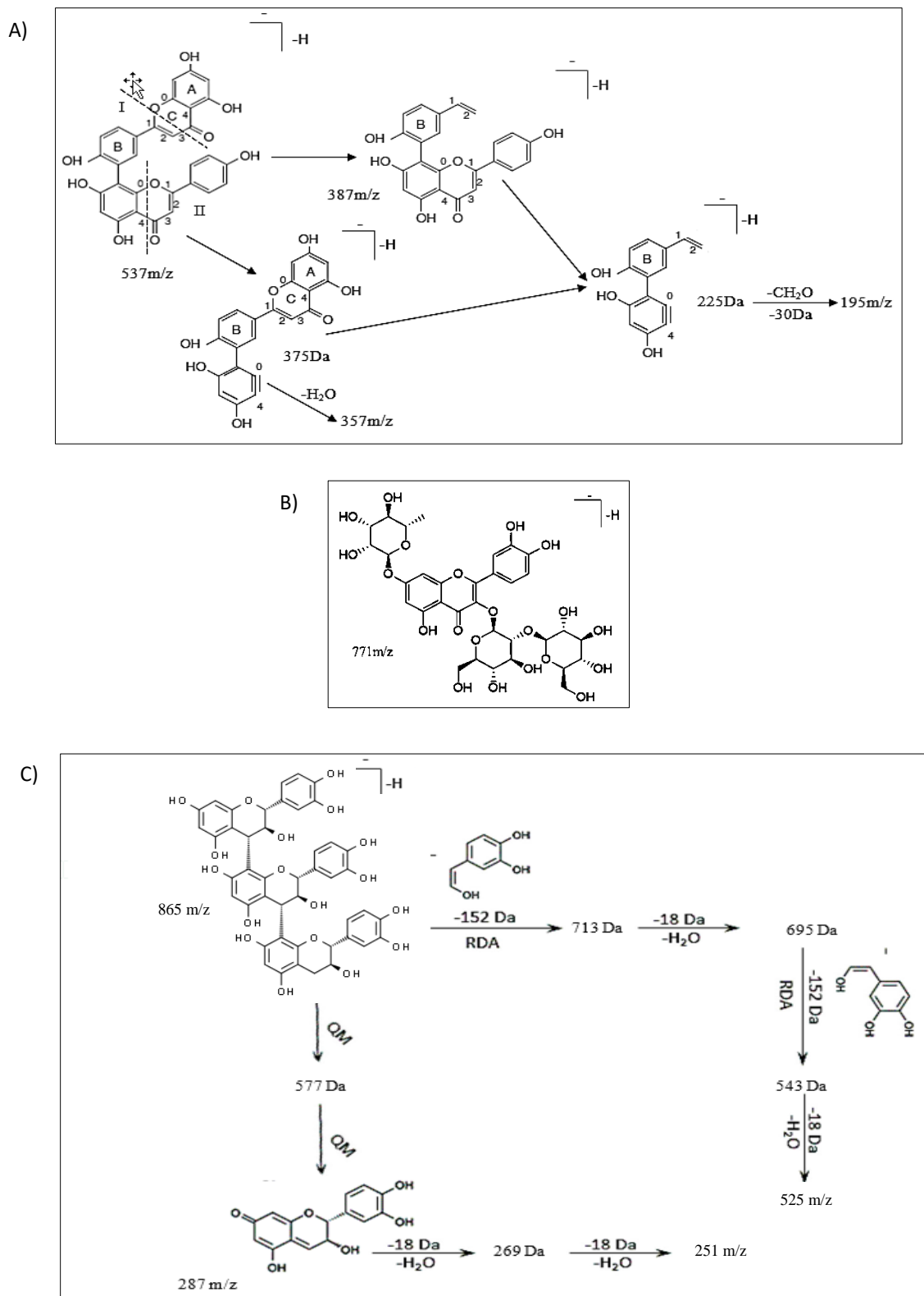


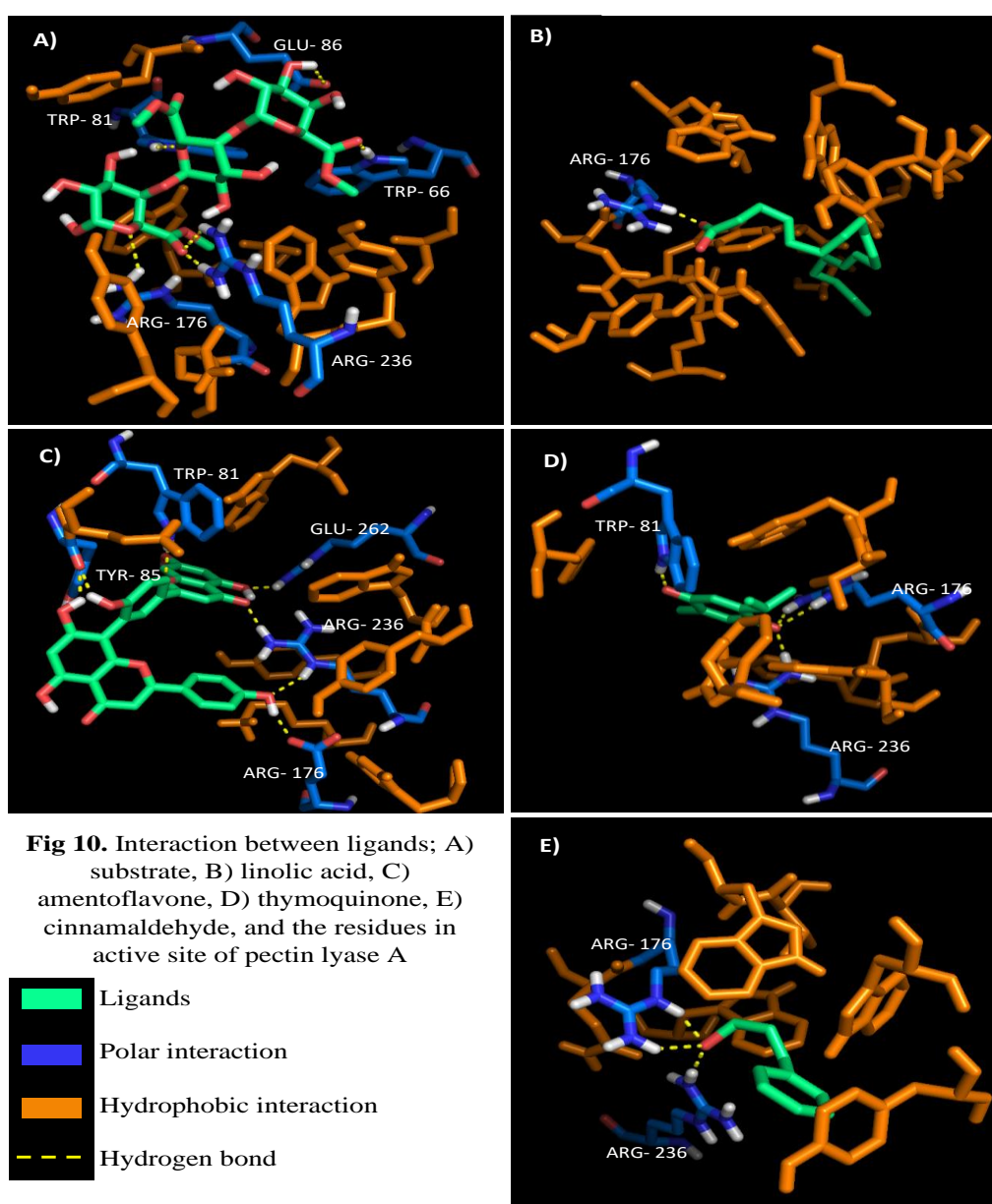
Fig 8. Mass spectrums of A) amentoflavone; B) quercetin3-O-sophoroside-7-O-rhamnoside; C) procyanidin C2



**Fig 9.** Fragmentation pattern of A) amentoflavone (Yao et al 2017); B) quercetin3-O-sophoroside-7-O-rhamnoside (Tsugawa et al 2019); C) procyanidin C2 (Enomoto et al 2019)

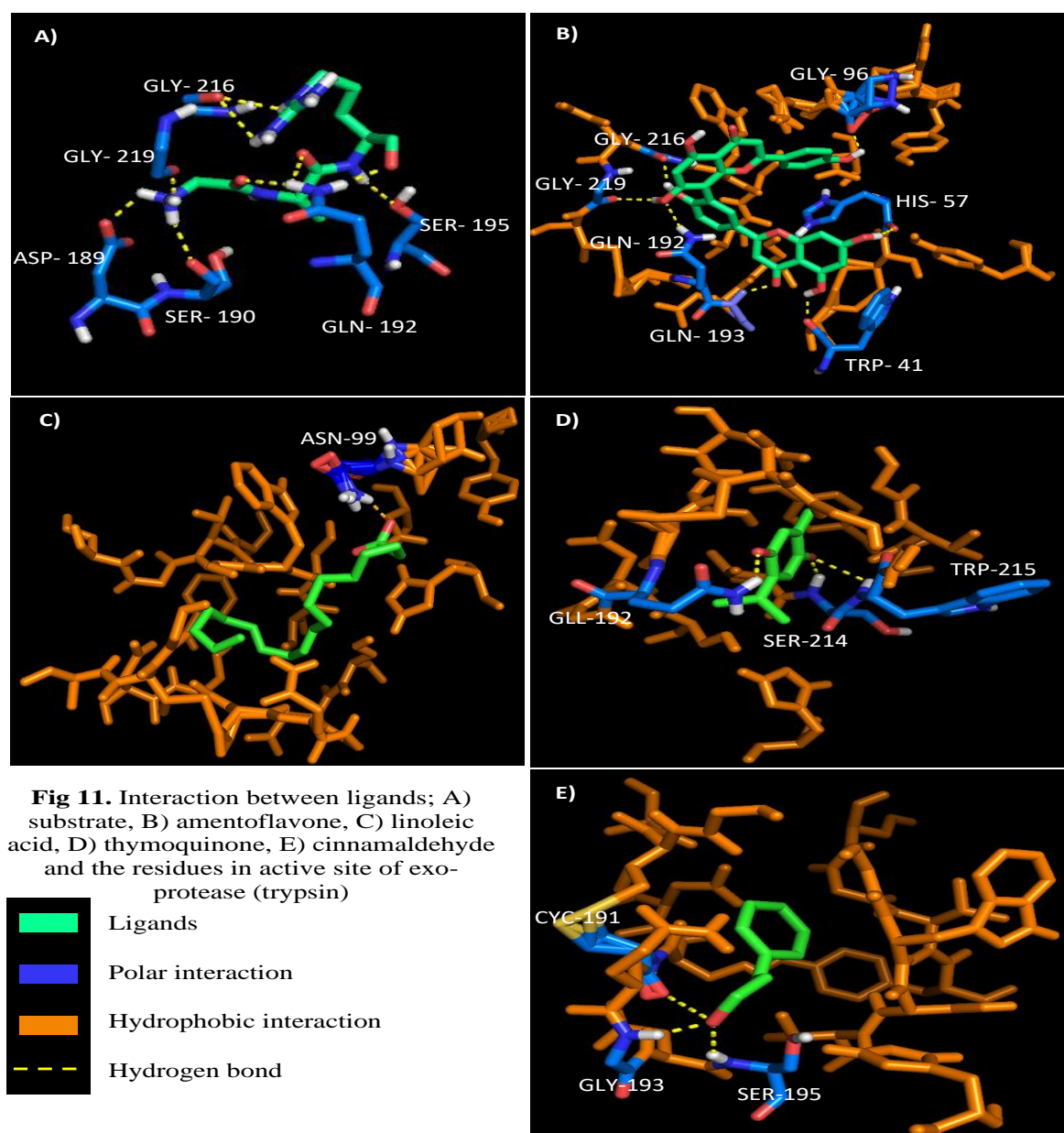
**Table 5.** Interaction between the major components of cinnamon and black cumin seeds extracts and pectin lyase A

Ligand	Type	Binding energy (Kcal/mol)	Ki (mmol)	Hydrogen bonds	Interacted residues
High methoxyl pectin	Substrate	-3.75	–	6	Trp66, Trp81, Glu86 Arg176 and Arg236
Linoleic acid	Inhibitor	-5.04	0.2	1	Arg176
Thymoquinone	Inhibitor	-6.2	0.028	4	Trp81, Arg176 and Arg236
Cinnamaldehyde	Inhibitor	-4.55	0.459	3	Arg176, Arg176 and Arg236
Amentoflavone	Inhibitor	-6.86	0.009	7	Arg176, Arg236, Tyr85, Glu262 and Trp81



**Table 6.** Interaction between the major components of cinnamon and black cumin seeds extracts and exo-protease (trypsin)

Ligand	Type	Binding energy (Kcal/mol)	Ki (mmol)	Hydrogen bonds	Interacted residues
GLY-ALA-ARG	Substrate	-2.41	–	9	Ser195, ser190, asp189, gln192, gly216, gly219
Amentoflavone	Inhibitor	-8.36	0.00074	7	Trp41, his57, gly96, gln192, gly193, gly216, gly219,
Linoleic acid	Inhibitor	-6.62	0.014	1	Asn99
Thymoquinone	Inhibitor	-6.84	0.009	3	Trp215, ser214, gln192
Cinnamaldehyde	Inhibitor	-6.24	0.026	4	Cys191, gly193, ser195





the ring in its structure (Xie et al 2017). In the case of black cumin seeds, the most effective extract against *R. solani* growth is hexane extract which according to (Edris et al 2016) consists of volatile fraction that represent (4.75%) of the extract with thymoquinone as the main component and fixed oil fraction that contains high level of unsaturated fatty acids. In the current study linoleic acid was identified as the main fatty acid of the fixed oil fraction of hexane extract. While the antifungal activity of black cumin seeds hexane extract is not totally interpreted, it could be due to the high level of unsaturated fatty that have the ability to penetrate the lipid membrane and cause metabolic disturbance by breaking down in the more alkaline environment inside the cell (Russell and Diez-Gonzalez 1997) which probably is the case in our study or it could be due to the presence of some bioactive compounds as thymol and carvacrol as reported by (Numpaque et al 2011). Antifungal activity of fatty acids and plant oils against *R. solani* and other phytopathogenic fungi has been detected in previous studies (Liu et al 2008, Hamza et al 2016).

Also, the results showed that the extracts of cinnamon and black cumin seeds inhibited *R. solani* extracellular enzymes, a similar inhibitory effect on the extracellular enzymes of *R. aolani* described by (Marei and Abdelgaleil 2017, Li et al 2020). To further understanding the effect of the extracts on pectin lyase and exo-protease of *R. solani* molecular docking was performed. The docking was performed between the active site of pectin lyase A and six ligands the enzyme substrate (high methoxyl pectin) and the major compounds of each extract. The active site of the enzyme consists of seven aromatic residues Trp66, Trp81, Trp151, Trp212, Tyr85, Tyr211, Tyr215 (binding residues) and three acidic residues Arg176, Arg236, Asp154 (catalytic residues) (Mayans et al 1997). The results presented in **Table 5** shows that all the ligands bind to the active site of the enzyme **Fig 10**. All the major compounds of the extracts (Linoleic acid, thymoquinone, cinnamaldehyde and amentoflavone) demonstrated higher affinity to the enzyme than the substrate indicating that all the previous compounds are highly competitive inhibitors. When comparing the docking output with *in vitro* results in the current study which showed that all extracts have inhibitory effect toward pectin lyase of *R. solani* except for black cumin seed methanol extract that have activation

effect it was found that all compounds docking output were compatible with *in vitro* results expect for amentoflavone. The contrast between the *in vitro* result of black cumin seed methanol extract and the docking output of its major compound, amentoflavone, that has a high affinity towards the active site of the enzyme could be due to that pectin lyase of *R. solani* can breaks down amentoflavone when bound to its active site and lead to the increase of double bonds which in turn cause an increasing in A<sub>235</sub>.

In the case of exo-protease, trypsin (Rypniewski et al 2001) was used for molecular docking because of its similarity with *R. solani* trypsin like protease (Gvozdeva et al 2006). Five ligands were docked in the active site of the enzyme, ASP189, GLY216, GLY219 and SER225 are the residues that form the specificity pocket, ARG35, SER36, ASN37, GLY38, GLY39, PRO40, TRP41 and TYR59c are the residues that give the binding cavity its shape and Ser195, His57 and Asp102 are catalytic residues (Rypniewski et al 1995). The docking output **Table 6** shows that amentoflavone the major compound of black cumin seed methanol extract is the only ligands beside the substrate that binds to the specificity pocket and one of the catalytic residues **Fig 11** with higher affinity than the substrate indicating that amentoflavone is a highly competitive inhibitor and explain the high inhibitory effect of black cumin seed methanol extract toward *R. solani* exo-protease. According to several previous studies bifalvonoids especially amentoflavone have inhibitory effect toward various types of proteases (Pan et al 2005, Shin et al 2006, Wei et al 2019). Cinnamaldehyde binds to one of the catalytic residues SER195 while linoleic acid and thymoquinone don't bind to the active site but bind with high affinity to other residues near the active site.

Despite of the contribution of molecular docking simulation in understanding the effect of the extracts on the enzymes further *in vitro* work needed to confirm its results.

#### 4 Conclusion

Based on the results obtained, it can be concluded that black cumin seeds or cinnamon extracts could be used to control diseases caused by the phytopathogenic fungi *R. solani* due to their inhibitory effect on *R. solani* extracellular enzymes especially hexane and methanol extracts of black cumin seeds that showed significant reduction of pectin lyase and protease enzymes activity and their high inhibitory effect toward *R. solani* growth especially in the case of hexane and methylene chloride extracts of

cinnamon that showed low MIC against *R. solani* mycelium growth. Further studding of black seeds and cinnamon extracts and their main components could lead to development of new eco-friendly fungicides.

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