

Antiviral, antifungal, and insecticidal activities of *Eucalyptus* bark extract: HPLC analysis of polyphenolic compounds

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ABSTRACT

In this study, the antiviral, antifungal, and insecticidal and HPLC analysis of polyphenolic compounds of *Eucalyptus camaldulensis* Dehnh. bark extract (ECBE) were evaluated. Three fungi, namely *Fusarium culmorum* MN398395, *Rhizoctonia solani* MN398397, and *Botrytis cinerea* MN398399 were used to colonize wood blocks of chinaberry that was previously treated with different concentrations of ECBE at 1%, 2%, and 3%. Antiviral evaluations (protective, curative, and inactivating activities) of the extract at 100 µg/mL were assayed against *Tobacco mosaic virus* (TMV) MG264131 using the half-leaf method to determine the inhibitory percentage towards the number of local lesions. The protective treatment of *Nicotiana glutinosa* leaves exhibited excellent activity (72.22%) with a 91.1-fold reduction in TMV-CP accumulation in infected tissues. Furthermore, Real-time quantitative PCR revealed that the expression level of PAL and PR-1 (salicylic acid marker) genes were significantly up regulated at four days-post inoculation (dpi) for all treatments compared to untreated leaves. The insecticidal effect was screened by the contact and fumigant methods against *Tribolium castaneum* (Herbst) and *Sitophilus oryzae* L. *in vitro*. In contact assay, all concentrations 1, 5, 10, 20 and 30 ppm caused 100% toxicity to the two tested pests within 24 h, whereas the fumigant assay, gave the highest mortality against *T. castaneum* and *S. oryzae* by 20 ppm (61.66%) and 30 ppm (57.77%), respectively after 24 h. The HPLC analysis of ECBE revealed that benzoic acid, quinol, salicylic acid, myricetin, and rutin were the most abundant polyphenolic compounds found in the extract. In conclusion, when the extract concentration increases, the growth of fungal mycelia was decreased compared with the control, especially against *F. culmorum*. According to the hypotheses of the results, the ECBE recommended to prevent the wood from discoloration, fungal molds by acting as bio-preservative, also trigger the resistance of plants against viral infection and high toxicity against stored-product insects.

1. Introduction

Plant extracts and essential oils had many applications in a lot of industries, have a great impact on inhibiting the fungal and bacterial growth, and could be embedded in possible or minimal processing strategies in a way that could be considered as a solution for preservation [1–4].

Strawberry is one of the most important crops in Egypt for exportation due to the good characteristics and early appearance in the market. Several fungi have been reported to attack strawberry roots and

shoots, i.e., *Rhizoctonia solani*, *Fusarium culmorum*, *F. solani*, *F. oxysporum* and *Macrophomina phaseolina* causing root rot diseases. The *Botrytis cinerea* fungal pathogen develops the grey mold on the strawberry fruit. These diseases lead to economic losses in strawberries and other crops [1,5–9].

The worst destructive crop diseases worldwide caused by plant viruses [9]. *Tobacco mosaic virus* (TMV) causes severe damages and economic losses of crop production and quality worldwide [10–12]. One of the most critical plant viruses inside the poll of the plant virology community [13].

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Table 1
Gene-specific primers used in qRT-PCR analysis in this study.

Primer name	Direction	Sequence (5'-3')	Reference
PAL	Forward	GTTATGCTCTTAGAACGTCGCC	[40]
	Reverse	CCGTGTAATGCCTTGTCTTGA	
Actin	Forward	GGGTTTGTGGAGATGATGCT	[41]
	Reverse	GCTTCGCACCAACATATGCAT	
PR-1	Forward	GTCCATACTAATTGAAACGACC	[41]
	Reverse	CCACTTCAGAGGATTACATATA	
TMV-CP	Forward	ATTAGACCCGCTAGTCACAGCAC	[42]
	Reverse	GTGGGGT TCGCCTGATTTT	

Table 2
Fungal isolates accession number used in this study.

Isolate	Accession number
<i>Fusarium culmorum</i>	MN398395
<i>Rhizoctonia solani</i>	MN398397
<i>Botrytis cinerea</i>	MN398399

Tribolium castaneum (Herbst) (Coleoptera: Tenebrionidae) and *Sitophilus oryzae* (Coleoptera: Curculionidae) are the most widespread and destructive insect pests of stored grains as well as stored products [14–16]. Botanical insecticides might be successful in giving novel methods of activity against the stored product beetles that can decrease the danger of cross-resistance as well as leads the designing of target-specific molecules [16–19]. Li et al. [20] reported that isolated fraction from leaf of *Eupatorium adenophorum* Spreng. showed insecticidal activity against *S. oryzae* (LD₅₀-15.5 mg/L). Saljoqi et al. [21] observed promising results on repellency and toxicity against *S. oryzae* using ethanol extract of bakain fruits at 10% concentration. The work of Othira et al. [22] mentioned fumigant, repellent and feeding deterrence activity from hexane extract of *Hyptis spicigera* Lam. against *S. zeamais* and *T. castaneum* in stored maize. The contact application of methanolic root extracts of *Decalepis hamiltonii* Wight at 250 mg/kg seed showed significant toxicity against *S. oryzae*, *R. dominica* and *T. castaneum* [23].

Eucalyptus species are known to be a rich source of bioactive compounds, including phenolic, flavonoid, terpenoids, tannins,

phloroglucinol, and cardiac glycosides, which had potential antimicrobial activities [24–27]. In Egypt, the *Eucalyptus camaldulensis* leaf methanolic extract analysis reported having possible antibacterial activity due to the phenolic compounds found in, ellagic acid, quercetin 3-O-rhamnoside, quercetin 3-O-b-D-glucuronide, caffeic acid, chlorogenic acid, ferulic acid and p-coumaric acid [27]. Phytochemical extracts from leaves and bark of *E. camaldulensis* are rich in bioactive compounds such as flavonoids, saponins, tannins, and glycosides [28–30]. Ellagic acid, protocatechuic acid, flavonol, ellagitannin, quercetin, and gallic acid were identified in the bark extract of *E. camaldulensis* [31].

Table 3
Fungal mycelia inhibition percentages of *E. camaldulensis* bark extract.

Concentration (%)	Inhibition Percentage of Fungal mycelial Growth (%)		
	<i>F. culmorum</i>	<i>B. cinerea</i>	<i>R. solani</i>
	Mean ± SD	Mean ± SD	Mean ± SD
0	0.00d	0.00d	0.00
1	15.66 ± 1.15c	33.33 ± 2.51c	0.00
2	36.33 ± 1.52b	37 ± 1.00b	0.00
3	49.66 ± 1.52a	43.33 ± 1.52a	0.00
p-value	<0.0001	<0.0001	–
LSD0.05	2.306	2.927	–

Means with the same letter within the same column are not significantly different according to LSD0.05.

Table 4
Protective, curative and inactivation efficacies of ECBE against TMV as antiviral activities.

Concentration	Average inhibition rate (%)		
	Protective effect	Curative effect	Inactivation effect
<i>E. camaldulensis</i> bark extract (100 µg/mL)	72.22 ± 3.7 ^a	50.43 ± 1.9	33.33 ± 2.1

^a Values are presented as mean ± SD.

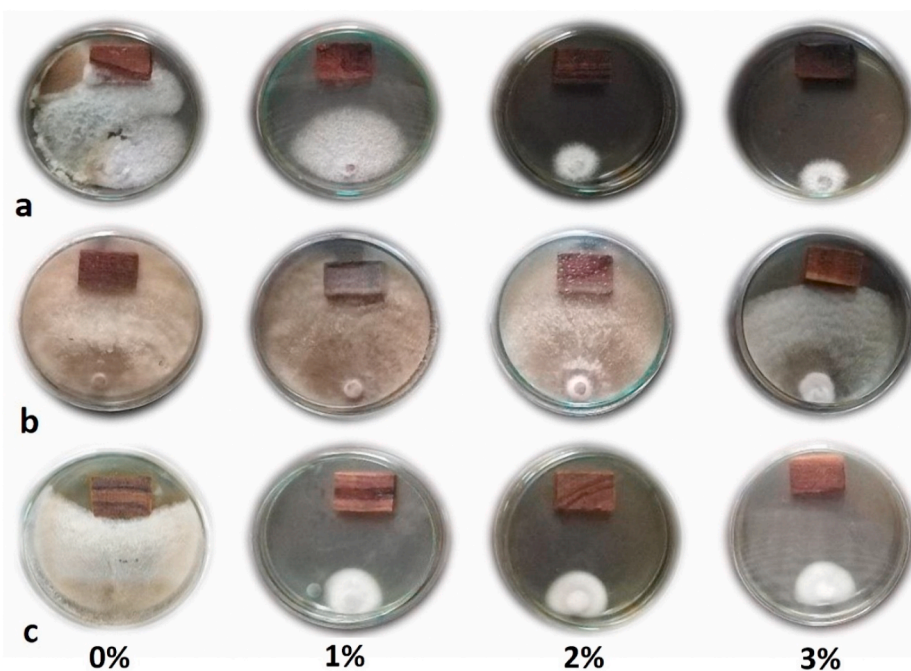


Fig. 1. *E. camaldulensis* bark extract (ECBE) treated-chinaberry wood blocks against the growth of three fungi: (a) *Fusarium culmorum*; (b); *Rhizoctonia solani* and (c) *Botrytis cinerea*.

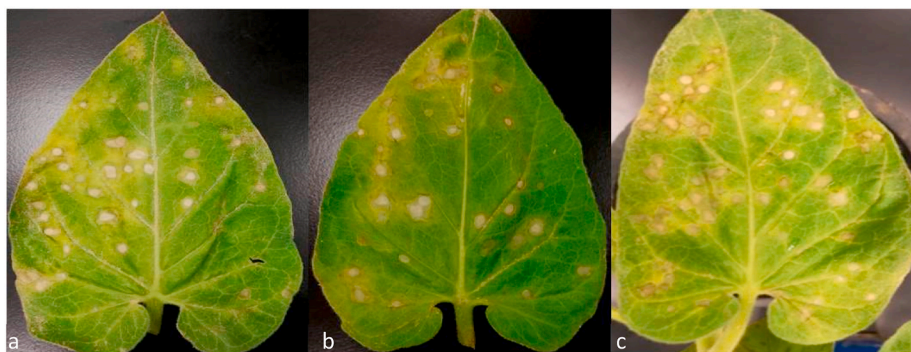


Fig. 2. Comparison of a; protective, b; curative, and c; inactivating activities of ECBE (100 µg/mL) against TMV in *Nicotiana glutinosa* leaves. All the half-left side of the leaves were inoculated with TMV without any treatments and the half-right side with the antiviral agent.

Regarding phytopathogenic microorganisms, few studies have revealed the antiviral activity of essential oils or extracts. This research area is a particular interest and still insufficiently explored and requires the further gathering of information that would enable a complete understanding of the ways and mechanisms of antiviral actions of these natural compounds. Our study aims to analyze the *E. camaldulensis* bark extract (ECBE) by HPLC, to study the antifungal activities of ECBE against three fungi, *Fusarium culmorum*, *Rhizoctonia solani*, and *Botrytis cinerea*, to evaluate the insecticidal activities of ECBE against two pests of stored products, *T. castaneum* and *S. oryzae* and, to assay ECBE antiviral activity against Tobacco mosaic virus.

2. Materials and methods

2.1. Preparation of the extract and HPLC analysis of phenolic compounds

Stem bark sample of *Eucalyptus camaldulensis* collected from a tree growing in Alexandria, Egypt in April 2018, was air-dried at room temperature for two weeks then ground to a fine powder using a small laboratory mill. About 50 g of powdered *E. camaldulensis* bark was extracted by the soaking method [6] with 150 mL of acetone solvent for three days. The extract was filtered with Whatman filter paper no.1, and then concentrated with evaporating the solvent using a rotary evaporator to afford the *E. camaldulensis* bark extract (ECBE). The extract was dissolved in 10% dimethyl sulfoxide (DMSO), and the following concentrations 1%, 2%, and 3% were prepared.

An Agilent 1260 Infinity HPLC Series (Agilent, Santa Clara, CA, USA), equipped with a Quaternary pump and a Zorbax Eclipse plus C18 column (100 mm × 4.6 mm i.d.) (Agilent Technologies, Santa Clara, CA, USA) was operated at 30 °C. The separation achieved using a ternary linear elution gradient with (A) HPLC grade water 0.2% H₃PO₄ (v/v), (B) methanol, and (C) acetonitrile. The injected volume was 20 µL. A VWD detector was set at 284 nm to identify the phenolic compounds [1,7,32,33] in the ECBE. The standard phenolic and flavonoid compounds used were pyrogallol, quinol, gallic acid, catechol, *p*-hydroxy benzoic acid, chlorogenic acid, vanillic acid, caffeic acid, syringic acid, vanillin, *p*-coumaric acid, ferulic acid, ellagic acid, benzoic acid, *o*-coumaric acid, salicylic acid, cinnamic acid, rutin, myricetin, quercetin, naringenin, rosmarinic acid and kaempferol.

2.2. Fungal isolation, and ITS sequencing

Infected strawberry plants showing root rots, cankers, and grey mold symptoms were collected from the fields of El-Behira governorate, Egypt. The fungal isolation, the morphological characteristics of the isolated fungi (hyphal shape, production, and arrangement of spores), and ITS molecular characterization, including the DNA extraction, purification, and PCR reactions were carried out according to previous work [7]. ITS region amplicons were degenerated and sequenced by

Macrogen, Inc., Seoul, Korea. Sequences were deposited in GenBank portal with illustrating their accession numbers.

2.3. Antifungal activity of bark extract

Chinaberry wood blocks with dimensions of 0.5 cm × 1 cm × 2 cm, were air-dried and autoclaved at 121 °C for 20 min, then treated with the prepared concentrations of ECBE (each block have a 100 µl). Fungi *Fusarium culmorum*, *Rhizoctonia solani*, and *Botrytis cinerea* were assayed for linear growths as incubated with the treated woods in the incubator chamber at 20–25 °C with relative humidity 85–92% for seven days, then the percentage of mycelial growth inhibition was measured [6] with the following formula;

$$MGI = [(Ac - At) / Ac] \times 100$$

Where MGI is mycelial growth inhibition, AC and AT are average diameters of the fungal colony of the control and treatment, respectively. Wood samples soaked only with 10% DMSO were used as the control.

2.4. Sources of viruses and inoculum preparation

The Egyptian TMV (Acc# MG264131, strain KH1) was propagated in *Nicotiana tabacum* as TMV-propagation host and purified as described by Gooding and Hebert [34]. The purified inoculum concentration of TMV was diluted to 20 µg/mL with 0.1 M phosphate buffer, pH 7.2 before use.

2.5. Assays of antiviral activity in the greenhouse

The ECBE was dissolved in DMSO (2% (v/v)) and diluted to 100 µg/mL with sterile distilled water. A solution of an equal concentration of DMSO was used as negative control. By using the half-leaf method [35], *Nicotiana glutinosa*, as a local lesion host for TMV, the antiviral activity of the ECBE was tested according to the inhibition percentage towards the number of local lesions. The inhibitory effect was calculated according to the following formula:

$$I = (1 - T / C) \times 100$$

Where I is the inhibition effect, T the number of local lesions on the treated half of the leaves, and C is the number of local lesions on the control half of the leaves. Under greenhouse-controlled conditions, *N. glutinosa* plants at the 5–6 leaf stage were subjected to the following treatments. Each experiment was replicated three times.

Protective treatment: The upper right halves leaves were treated with ECBE extract using a paintbrush. After 24 h, both halves of the leaves were dusted with carborundum and mechanically inoculated with TMV [36,37]. After washing the leaves with water, the local lesion numbers were recorded 4–5 days' post inoculations (dpi).

Curative treatment: The whole leaves were mechanically inoculated

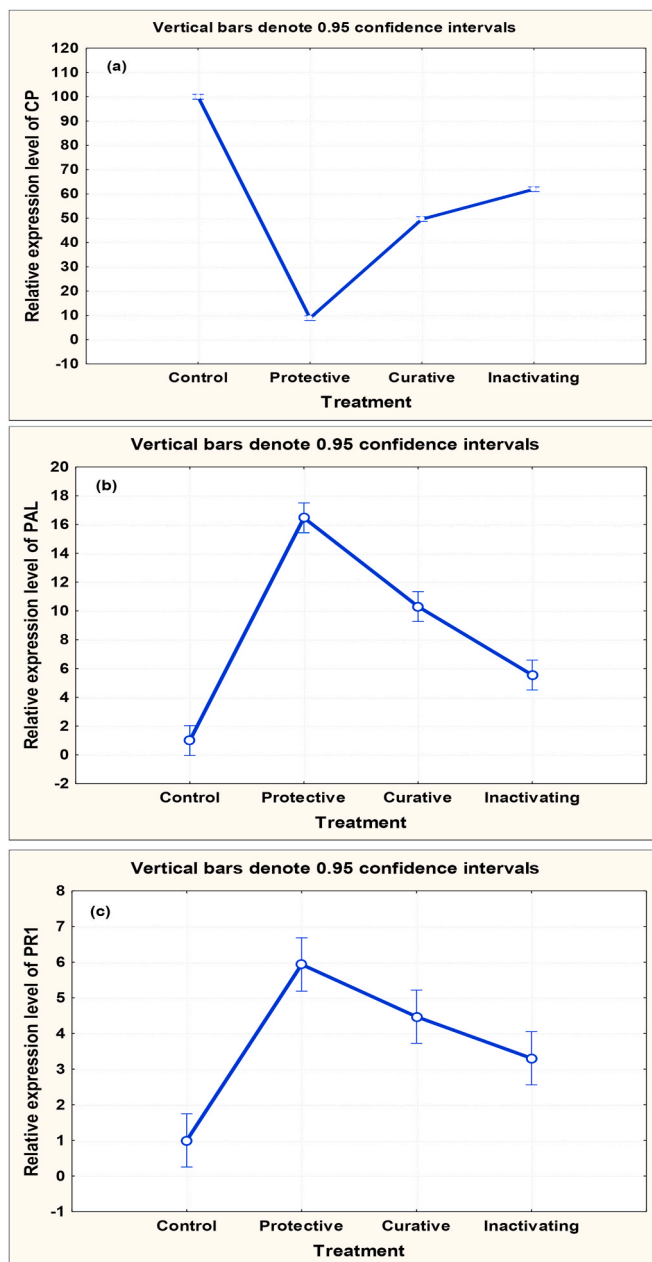


Fig. 3. The relative expression levels of the tested genes upon TMV infection at 4 dpi comparing to control. a: shows comparison between the accumulation levels of TMV-CP gene after protective, curative and inactivating activities of ECBE treatments compared to untreated tobacco leaves. b: shows relative expression level of PAL after protective, curative and inactivating activities of ECBE treatments compared to untreated tobacco leaves. c: shows relative expression level of PR-1 after protective, curative and inactivating activities of ECBE treatments compared to untreated tobacco leaves.

with TMV, as mentioned above, and after 24 h, the upper right halves leaves were treated with ECBE using a paintbrush. Afterthought, the local lesion numbers were counted and recorded (4–5 dpi).

Inactivation treatment: Equal volume of purified TMV was mixed with an equal amount of ECBE and incubated for 1 h; then, the mixture used to inoculate the right side of the leaves, whereas the left side was inoculated with TMV. A 50 μ L volume of TMV suspension was used as inoculum, and the local lesion numbers were recorded 4–5 days after inoculation.

2.6. Molecular investigations

2.6.1. Plant total RNA extraction and cDNA synthesis

Total RNA was extracted from *N. glutinosa* half leaves (0.1 g, fresh weight) and collected at 4 dpi using the RNeasy Mini Kit according to the manufacturer's instructions (QIAGEN). After checking the purity and concentration of extracted RNA, 1 μ g of RNA was used to synthesize cDNA in a reverse transcription reaction (superscript reverse transcriptase enzyme, Invitrogen, USA) according to the manufacturer's instructions [38]. In a thermal cycler (Eppendorf), the reverse transcriptase reaction performed at 42 °C for 1 h and deactivated at 72 °C for 10 min. After holding at 4 °C, the reaction mixture was stored at –20 °C until used.

2.6.2. qRT-PCR assay and data analysis

To study the TMV accumulation and defense-related genes expression changes after different treatments, qRT-PCR with different sets of primer (Table 1) specific for phenylalanine ammonia-lyase (PAL), pathogenesis-related protein-1 (PR-1) and TMV-CP genes were performed. The housekeeping gene β -actin was used as a reference gene to the normalization of the transcript expression levels. Each sample on all reactions run in triplicate on a Rotor-Gene 6000 (QIAGEN, ABI System, USA) using the SYBR Green PCR Master Mix (Fermentas, USA). The amplification program of thermal cycling included an initial denaturation step at 95 °C for 10 min, followed by 40 cycles consisting of; denaturation at 95 °C for 15s, annealing at 60 °C for 30s and extension at 72 °C for 30s. The relative expression ratio was accurately quantified and calculated [39]. Relative expression levels more than 1 demonstrate an increase of accumulation (up-regulation) and values lower than 1 means a decrease in expression (down-regulation).

2.7. Tested insects

2.7.1. Culture of *Tribolium castaneum*

The adult *Tribolium castaneum* was collected from infested grains purchased from nearby markets and brought to the laboratory. The way of breeding was built up in a plastic container of 25 \times 10 cm and stored at 30 \pm 2 °C and relative humidity of 70–75%. By sieving the culture, the adult insects separated, and they used for subsequent experiments. The culture was continuously maintained in the containers throughout the examination period [43].

2.7.2. Culture of *Sitophilus oryzae*

The beetles of *S. oryzae* were collected from infested wheat grains (*Triticum aestivum* L.). Insect rearing was kept up at the laboratory of Plant Protection Department, Faculty of Agriculture (Saba Basha), Alexandria University, Alexandria, Egypt. The cultures kept up at 28 \pm 2 °C and 65 \pm 5% relative humidity. At first, 50 pairs of 1-2-day-old adults were placed in a jar containing their food grains (1 kg). The jars stayed tied for a maximum period of 7 days to allow mating and oviposition. Parental stocks were removed, the remaining of the content (diet and laid eggs) of each jar was used to infest the fresh seeds, and the diet was covered fastened with pieces of cloth and rubber bands to prevent the insects from escaping. The subsequent progenies of the beetles were used for all tests [44].

2.8. Methods of insecticidal bioassay

2.8.1. Contact toxicity

The contact toxicity of ECBE against *T. castaneum* and *S. oryzae* adults were determined by topical application. The ECBE was dissolved in DMSO to obtain serial dilutions from 1, 5, 10, 20, and 30 ppm. A 0.5 μ L droplet of an ECBE solution was applied onto the pronotum of an adult weevil using a Hamilton's syringe (700 series, Microliter TM Hamilton Company, USA). For each ECBE solution, 15 insects divided into three replicates were used. Insects treated with DMSO alone served

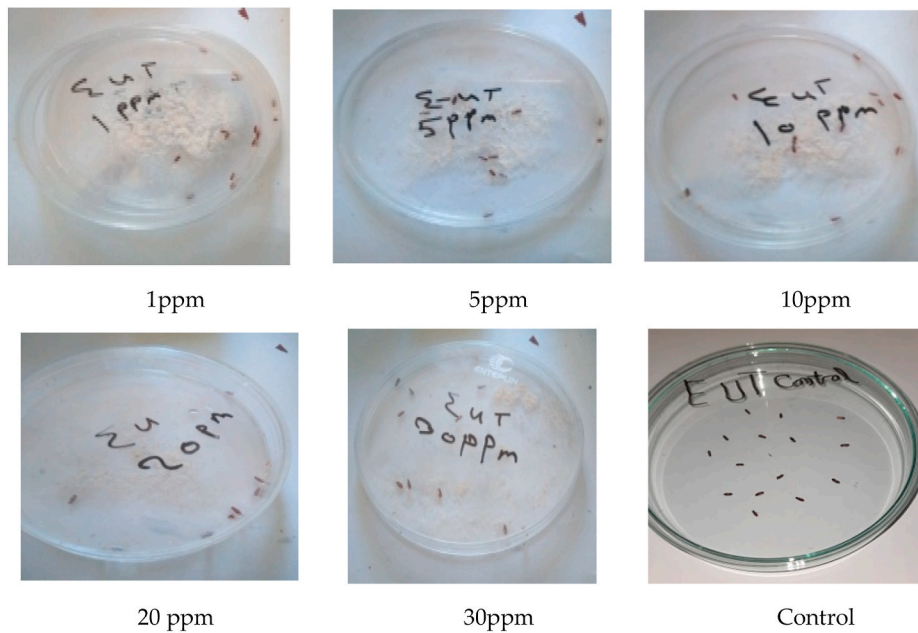


Fig. 4. Contact toxicity of different concentrations of *Eucalyptus camaldulensis* extract against *Tribolium castaneum* adults 24 h after topical application.

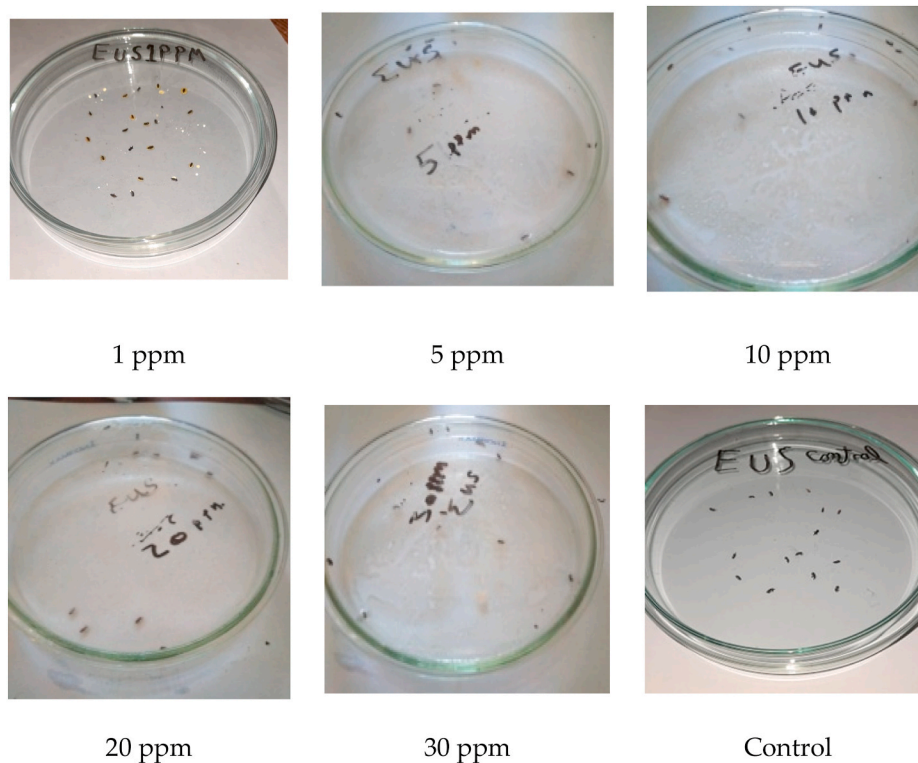


Fig. 5. Contact toxicity of different concentrations of *Eucalyptus camaldulensis* bark extract against *Sitophilus oryzae* adults 24 h after topical application.

as control. After topical application, the insects were confined in Petri dishes within a metal ring (Ø 4.0 × 2.5 cm) covered with metallic net (mesh 1 mm) to prevent insects escape. Every Petri dish provided with five wheat kernels and maintained in the dark at 26 ± 2 °C and 60 ± 5% R.H., The number of dead insects was recorded after 24 h [45].

2.8.2. Fumigant toxicity

The fumigant toxicity of ECBE to *T. castaneum* and *S. oryzae* adults in the presence of wheat grains was assessed using the method described in

literature [46]. A glass container (500 mL) was used as a fumigation chamber. A filter paper (Whatman No. 1) disc (Ø 2.0 cm) was suspended in the center of the chamber by an iron wire attached to the undersurface of its aluminum screw cap. Fifteen adult insects were placed in the chamber, the paper disc treated with an appropriate volume of ECBE, and the glass container tightly closed. In tests with wheat grains, intact kernels (50 g) were placed on the base of the fumigation chamber together with the insects. The tested concentrations of ECBE were 1, 5, 10, 20, and 30 ppm, and the untreated paper disc was used as a control.

Table 5
Fumigant toxicity of the *Eucalyptus camaldulensis* bark extract against the adults of *Tribolium castaneum*

<i>Eucalyptus camaldulensis</i> extract Concentration (ppm)	Adults of <i>Tribolium castaneum</i> Mortality (%)	LC ₅₀ ppm	Lower limit	Upper limit	LC ₉₀ ppm	Lower limit	Upper limit	Slope ±SE
1	13.33	20.73	–	–	443.347	–	–	0.963 ± 0.189
5	13.33							
10	44.44							
20	61.66							
30	44.44							
Control	0.00							

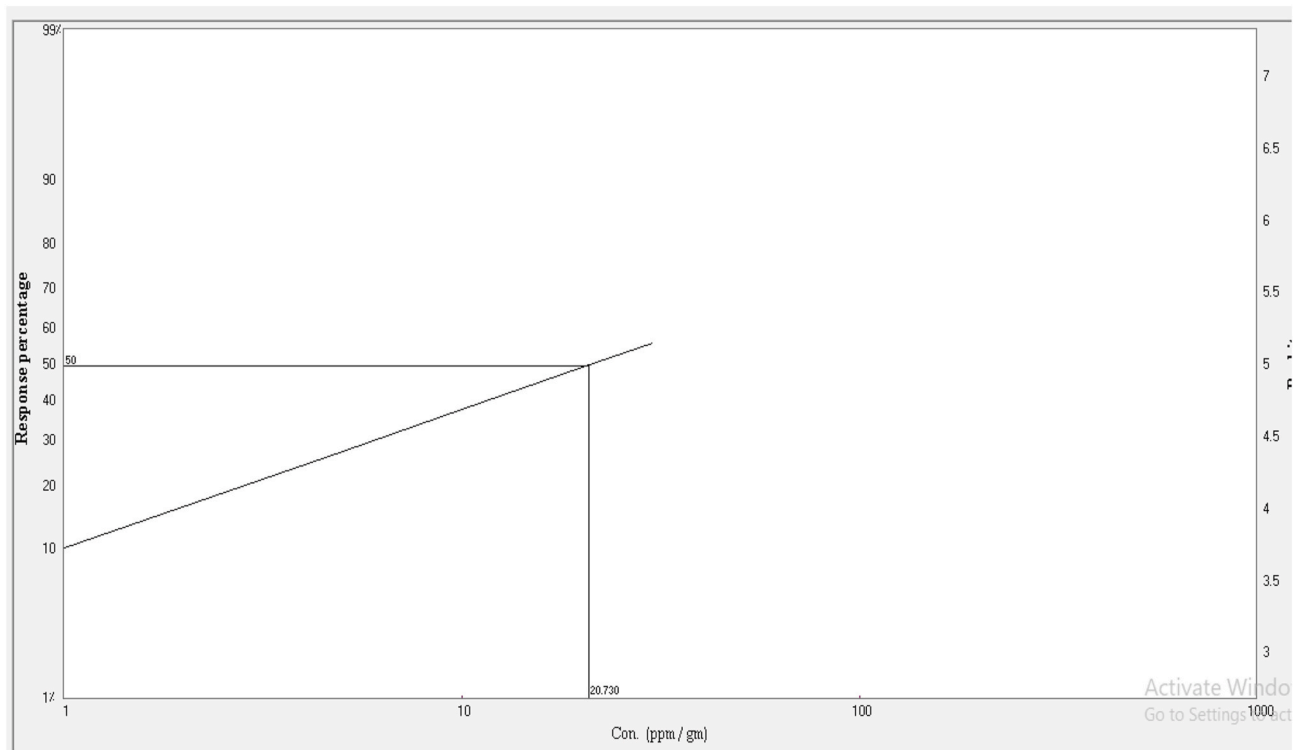


Fig. 6. Toxicity line of *Eucalyptus camaldulensis* bark extract against adults of *Tribolium castaneum*.

Table 6
Fumigant toxicity of the bark extract *Eucalyptus camaldulensis* against the adults of *Sitophilus oryzae*.

<i>Eucalyptus camaldulensis</i> extract Concentration (ppm)	Adults of <i>Sitophilus oryzae</i> Mortality (%)	LC ₅₀ (ppm)	Lower limit	Upper limit	LC ₉₀ (ppm)	Lower limit	Upper limit	Slope ±SE
1	13.33	19.03	12.23	38.39	459.13	143.63	6314.62	0.927 ± 0.188
5	28.88							
10	33.33							
20	55.55							
30	57.77							
Control	0.00							

Three replicates of each concentration and the control were set up. Bioassays were carried out in the dark at 26 ± 2 °C and 60 ± 5% R.H for 24 h. Dead insects were counted after exposure to fresh air in Petri dishes for 12 h. This allowed for recovery of insects immobilized and dead immediately after exposure to the ECBE.

2.9. Statistical analysis

The results of the inhibition percentage of mycelial growth of the three fungi as affected by the tested concentrations (0, 1, 2, and 3%) of the ECBE were statistically analyzed using one-way analysis of variance (ANOVA) with SAS software (SAS Institute, Release 8.02, Cary, North Carolina State University, Raleigh, NC, USA). The means were compared

to the control treatment. Mortality rates were corrected according to Abbott’s formula [47] and plotted against concentrations as log/probit regression lines. LC₅₀, LC₉₀ values, the toxicity index, and the slope of the lines were calculated according to Finney [48] using “LdPLINE®” software (<http://embakr.tripod.com/ldpline/ldpline.htm>).

3. Results

3.1. Fungal isolates

ITS sequence confirmed the fungal isolates as *Fusarium culmorum*, *Rhizoctonia solani*, and *Botrytis cinerea*, with the accession numbers illustrated in Table 2.

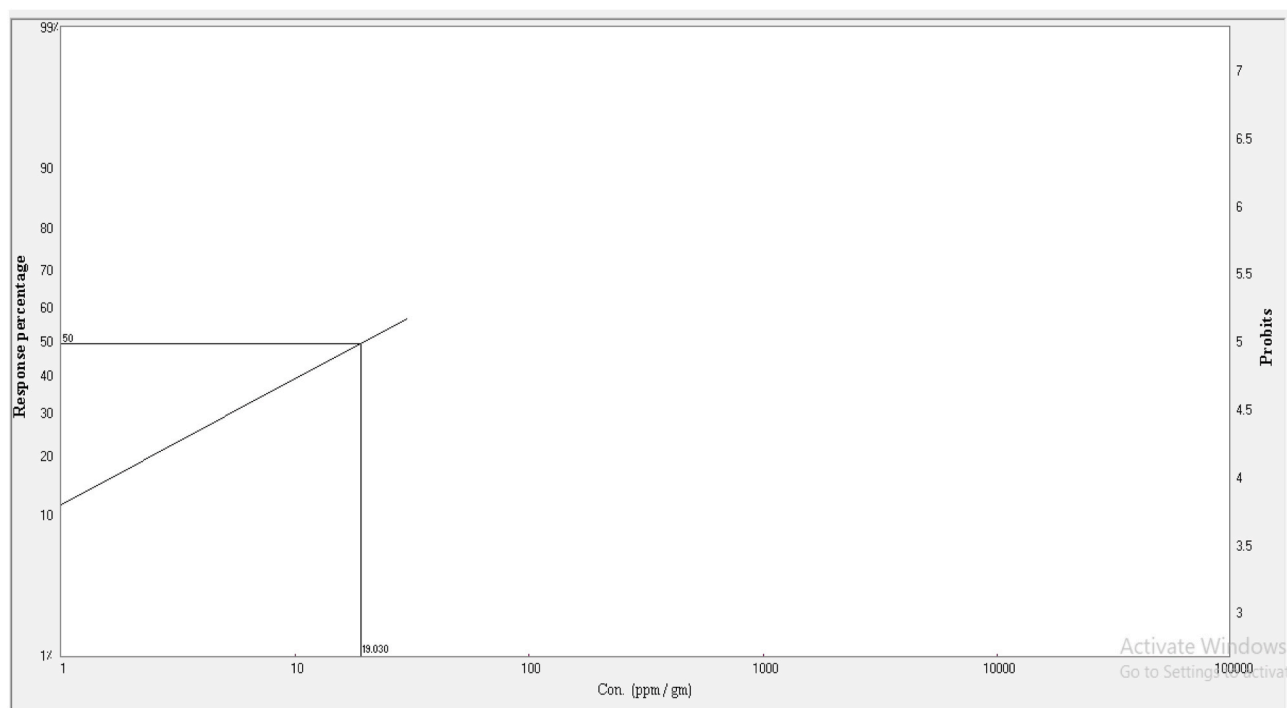


Fig. 7. Toxicity line of *Eucalyptus camaldulensis* bark extract against adults of *Sitophilus oryzae*.

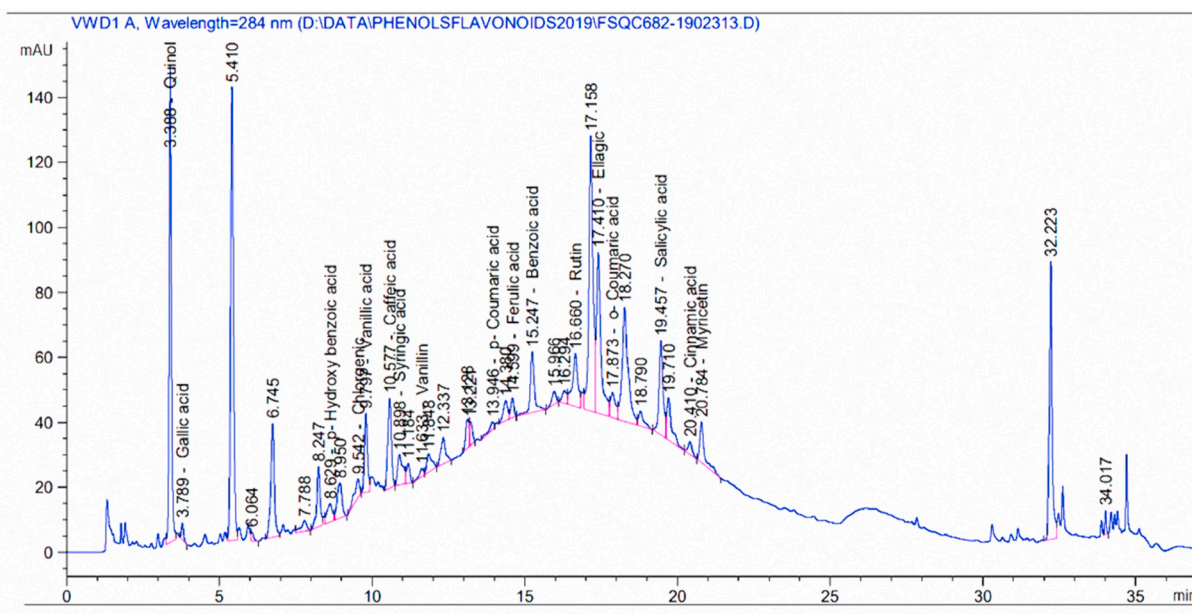


Fig. 8. HPLC chromatogram of phenolic and flavonoid compounds identified in ECBE.

3.2. Antifungal activity of the bark extract

Fig. 1 shows that wood blocks of the chinaberry treated with different concentrations (1, 2, and 3%) of *Eucalyptus camaldulensis* bark extract (ECBE) had inhibition degrees of fungal growth compared to the untreated wood blocks (control treatment with 10% DMSO). The fungal mycelia growth of *F. culmorum*, and *B. cinerea*, were highly inhibited at the concentration 3% with values of 49.66, and 43.33%, respectively, compared to the control. In Table 3, there is no significant effect of the bark extract concentrations (1, 2, and 3%) against *R. solani* isolate compared to the control, where no fungal mycelia inhibition was found.

Further, by increasing the concentration, the percentage of fungal inhibition was significantly increased against *F. culmorum* and *B. cinerea*.

3.3. Anti-TMV activity in the greenhouse

To find anti-TMV activities of ECBE, protective, curative, and inactivating activities were tested, as shown in Table 4 and Fig. 2. ECBE activity was tested by counting lesions on inoculated leaves at four days post-inoculation (dpi). As Fig. 2 shows, the number of a local lesion on the leaf treated with ECBE was significantly less than that without treatment. The results of protective (24 h before virus infection),

Table 7
Chemical composition analysis of phenolic and flavonoid compounds of ECBE by HPLC.

Compound	Conc. (mg/100 g)
Phenolic and flavonoid compounds	
Pyrogallol	ND ^a
Quinol (Hydroquinone or p-Hydroquinone)	306.68
Gallic acid	3.32
Catechol	ND
p-Hydroxy benzoic acid	29.80
Chlorogenic acid	10.17
Vanillic acid	41.86
Caffeic acid	18.37
Syringic acid	13.08
Vanillin	2.41
p-Coumaric acid	1.00
Ferulic acid	4.56
Benzoic acid	363.82
Ellagic acid	83.10
o-Coumaric acid	6.34
Salicylic acid	160.30
Cinnamic acid	1.38
Rutin	146.78
Myricetin	175.42
Quercetin	ND
Rosmarinic acid	ND
Naringenin	ND
Kaempferol	ND

^a ND: not detected.

curative (24 h after virus infection) and inactivation efficacies of ECBE at a dose of 100 µg/mL against TMV are given in Table 3. High infection protective of inhibition rate (72.22%) was noticed *in vivo*; infection curative inhibition rate was 50.43% *in vivo*; the inactivation inhibition rate was 33.33%. The results showed that the ECBE compound possessed better protective activity against TMV. The levels of reduction induced by ECBE treatment suggesting that it has strong potential for use against TMV under both greenhouse and field conditions.

Quantification of TMV by qRT-PCR revealed a 91.1-, 50.4- and 38.4-fold reduction in TMV-CP RNA levels in protective, curative, and inactivating-treated plants, respectively, compared with the control (Fig. 3a). To evaluate the mode of action of ECBE-mediated plant protection against TMV, we assessed the expression of two defense-related genes, which was used to represent the elicitation of SAR in *N. glutinosa* due to the inability of extract to directly counteract multiplication of TMV in plant cells.

The expression levels of PAL and PR-1 (salicylic acid marker) genes were differentially up regulated in tobacco after ECBE treatment (Fig. 3 b and c). Compared to the untreated leaves, the protective treatment exhibited the highest expression levels of both PAL and PR-1 followed by curative and inactivating treatments. The expression level of PAL was increased by 16.449-, 10.303- and 5.550-fold, while PR-1 increased by 5.938-, 4.469- and 3.305-fold change higher than untreated leaves (Fig. 3 b and c).

3.4. Contact and fumigant toxicity of the tested *E. camaldulensis* extract

The insecticidal activities of ECBE against *T. castaneum* and *S. oryzae*, were examined by direct contact application method whereas, all concentrations used achieved 100% mortality after 24 h from application (Figs. 4 and 5). While the fumigant application of ECBE against *T. castaneum* and *S. oryzae* adults determined by LC₅₀ and LC₉₀. The toxicity increased by increasing the concentrations, exposure period, and insect species. During the fumigant method, insects moved up towards the lid, and they chose to stay away from the treated discs suggesting the repellent activity of the extract tested (Table 5 and Fig. 5). ECBE was significantly more toxic against *T. castaneum* than *S. oryzae*. The highest mortality in *T. castaneum* was achieved by the applied

concentration of 20 ppm (61.66%). ECBE (LC₅₀ = 20.73 ppm) exhibited more potent fumigant toxicity against *T. castaneum* (Table 5 and Fig. 6). ECBE against *S. oryzae* gave the highest mortality at the applied concentration 30 ppm (57.77%). The LC₅₀ and LC₉₀ were recorded as 19.03 and 459.13 ppm, respectively (Table 6 and Fig. 7).

3.5. Phytochemical constituents of extract

Fig. 8 shows the HPLC chromatograms for the identified polyphenolic compounds of ECBE and presented in Table 7. The most abundant phenolic compounds in mg/100 g were benzoic acid (363.82), quinol (306.68), salicylic acid (160.31), and ellagic acid (83.10); while the identified flavonoid compounds were myricetin (175.42) and rutin (146.78).

4. Discussion

The *E. camaldulensis* bark extract (ECBE) is a useful resource of phytochemicals, which could be the reason for their antimicrobial properties, where, hydroquinone, benzoic acid, ellagic acid, salicylic acid, rutin, and myricetin were found in high amounts. Extracts of leaves and bark are hugely valuable as antibacterial, antiviral, and antifungal agents and their insecticidal activity should not be ignored considering the current cost of therapeutic studies, toxic effects, and insect growth resistance. The certain *E. camaldulensis* plant character such as simple planting, high popularity by cultivation, and fast growth further studies need to exterminate the antimicrobial activity, to increase the market reliability of ECBE drug products. Future studies may start focusing on identifying antimicrobial activity pathways, particularly potential quorum questioning methods besides the improvement of the synergistic effect of other compounds in combination with ECBE.

The methanolic ECBE showed the presence of polyphenolic of phenolic acids, flavonoids, and aldehyde compounds such as gallic, protocatechuic, vanillic, ellagic acids, and protocatechuic aldehyde, eriodictyol, quercetin and naringenin luteolin and kaempferol [31,49]. Water-soluble phenolics from leaf litter of *Eucalyptus* hybrids beneath showed the presence of compounds such as gallic acid, p-hydroxybenzoic, p-hydroxybenzaldehyde, protocatechuic, caffeic acid, gentisic, p-coumaric acid, chlorogenic acid, ferulic acid, hydroquinone, and vanillin [50]. Phenolic compounds of hydroquinone, pyrogallol, hesperitin, resorcinol, protocatechuic acid, chlorogenic acid naringenin, and catechin were isolated from *E. camaldulensis* leaf extract [49]. *E. camaldulensis* leaf extracts proved to present more effective against *F. solani* than stem and bark [51]. Quercetin-7-O-diglucoside isolated from wood and stem bark of *Terminalia brownii*, significantly reduced the mycelial growth of *Aspergillus* and *Fusarium* isolates [52]. However, the derivatives of quercetin were reported having suitable antifungal activities with low MIC values [53–57]. The growth of *Fusarium* spp. has been suppressed by dihydroquercetin isolated from barley [58]. The derivatives of naringenin and naringenin compounds possessed slight antifungal and antimicrobial activity, while in our study, it is not detected at all [59].

Many plant extracts exhibited an inhibitory effect against plant viruses. Antiviral activity of *Boerhaavia diffusa*, *Mirabilis jalapa*, *Boerhaavia diffusa*, *Clerodendrum aculeatum*, *Potentilla arguta*, *Sambucus racemosa*, *Thuja orientalis* extracts could be due to their ability to inducing systemic resistance and inhibiting viral replication [60–64].

Generally, plant extracts, secondary metabolites, are an eco-friendly effective control way against a wide range of plant pathogens [65]. Polyphenolic compounds, phenolic, and flavonoids play essential roles in plant resistance against pathogenic microorganisms, including plant viruses [1,7,8,57,66–70]. In our study, ECBE protection, curative, and inactivating activities against TMV infection as well as expression levels of PAL and PR-1 were evaluated. The results revealed that the application of ECBE (100 µg/mL) were significantly reduced local lesion symptoms of treated tissues when compared to non-treated tissues.

Significant inhibition of TMV infectivity at all assays, whether applied in a protective, curative, or inactivating manner were reported. Many reports showed that many changes develop in TMV particle morphology after treatment with plant extracts in addition to suppression of disease symptoms and restriction of virus multiplication [71–76]. Consequently, we suggest that the application of ECBE activates the immune defense system besides inhibition of TMV replication. These results are in agreement with those previously reported [77–79].

Overall, there are two main two-pronged mechanisms of antiviral agents: the direct inhibition of virus replication, and the other is indirectly through simultaneous activation of the host innate immune response and inducing SAR against the virus [80,81]. In the current study, the different treatments result revealed that the ECBE possess the two mechanisms of action, but in varying proportions. Besides, it is the first enzyme in the phenylpropanoid pathway linking between primary and secondary metabolism [82], PAL involved in salicylic acid (SA) biosynthesis [83,84]. SA is a vital signal phytohormone molecule of SAR in plants [85], and its role in plant immunity has been known for over two decades. The activation of SA in response to pathogens is associated with the accumulation and expression of PR-1 as SA marker gene [86]. The high transcriptional levels of PAL (16.449-fold) and PR-1 (5.938-fold) genes that associated with TMV inhibition rate 72.22% in protective treatment indicated that ECBE elicited *N. glabrosa* SAR resulted in reduced viral infection and disease severity. The results are in agreement with Jin et al. [87], who showed that the treatment of tobacco plants with *Eupatorium adenophorum* leaf extract induce SAR and inactivate TMV particles.

Moreover, the application of *Clerodendrum inerme*, *Mirabilis jalapa*, *Zingiber officinale*, and *Mentha longifolia* extracts acted as a potential elicitor of SAR against tomato viral infections [88]. Additionally, Venkatesan et al. [89] showed that plant extract associated with induction of PAL, polyphenol oxidase (PPO), peroxidase (POD) upon *Mungbean yellow mosaic* infection. On the other hand, the high expression level of PAL with 5.550-fold and moderate expression level PR-1 with 3.305-fold change that associated with TMV inhibition rate 33.33% with inactivating treatment indicated that the ECBE also significantly inhibited TMV replication. Mixed plant extract composed of *Sophora flavescens*, *Forsythia suspense*, and *Lonicera japonica* induced and up-regulated tobacco PR-1 resulted in the development of systemic resistance against TMV [90]. The inactivating activity of aqueous extract of *Bryophyllum daigremontianum* inhibited systemic and local infection of TMV ranged from 51.45% to 86.08% on different host plants [91]. In comparison with the previous results, our promising data that showed ECBE treatments induce PAL and PR-1, which associated with the accumulation of SA and trigger of SAR, push us to seek the field application of ECBE that may improve the plant disease resistance against viral infection. The present research underlines the importance of *E. camaldulensis* extract, which contains phenolic and flavonoid compounds as a botanical insecticide against *T. castaneum* and *S. oryzae*. Many investigations documented the potential insecticidal activities of *E. camaldulensis* essential oils against major stored-product insects *Sitophilus oryzae* (L.), *Callosobruchus maculatus* (Fab.), and *T. castaneum* (Herbst) [92,93]. A leaf methanol extract of *E. camaldulensis* showed potential larvicidal activity against *Culex quinquefasciatus* (Say) than bark extract [94]. Recent studies also proved the efficacy of plant extracts against storage insects. During the investigations on the toxic properties of combination of *Acorus calamus* L. rhizomes and *Thevetia nerifolia* Juss. seed extract, the lowest LC₅₀ values (43.27 µg/cm) were found against *S. oryzae* at 24 h after treatment [95].

Adeyemi et al. [96] observed 50% feeding deterrent activity of *T. castaneum* with 3,4,5,7, tetrahydroxy flavonol (quercetin-1) at 2 mg/ml isolated from chloroform extract of stem bark of *Bobgunnia madagascariensis* Desv. in stored maize; also, Gressel and Ammann [97] reported that quercetin acts as an allelochemical as well as insecticidal to storage insects. Geng et al. [98] reported that Jolkinolide B and 17 hydroxy Jolkinolide B isolated from ethanolic extracts of roots of

Euphorbia fischeriana Steud. at concentration of 30 ppm possessed vigorous feeding deterrent activity against *S. zeamais* (EC₅₀=342.1 and 543.9 ppm) and *T. castaneum* (EC₅₀=361.4 and 551.5 ppm). The crude leaf extracts and their chromatographic fractions eluted with ethyl acetate of *Cocos nucifera* L. and *Terminalia catappa* L. were found to be insecticidal and fumigants to adults of *S. oryzae* and *T. castaneum* [15]. Ali and Mohammed [99] found that methanol extracts of *Zingiber officinale* Roscoe caused 100% mortality to *S. oryzae*. Jeon et al. [100] studied contact and fumigant toxicity of chloroform fraction of methanolic extract of *Ruta chalepensis* L. and obtained 87.7% mortality to *S. oryzae* at 1.02 mg/cm². Further insecticidal constituents isolated from leaves of *R. chalepensis*, quinoline (0.057 mg/cm²), quinoline-4-carbaldehyde (0.065 mg/cm²) and quinoline-3-carbaldehyde (0.092 mg/cm²) were found to be most toxic to *S. oryzae*. Other studies have shown that *T. castaneum*, *S. oryzae* (up to 50% after 24 h) and *R. dominica* (up to 60% after 24 h) can also be repelled by plant extracts of *Anagallis arvensis* L., *Hibiscus rosa-sinensis* L. and *Lapsana communis* L. [101].

Pure compounds osajin, lupalbigenin, scandinone, sphaerobioside, genistein, and prenylated isoflavones derived from *Derris scandens* Benth. caused 100% toxicity to *T. castaneum* after the 10th day of treatment [102].

5. Conclusions

In the present study, *E. camaldulensis* bark extract at 3% shows high antifungal properties against two mold species tested (*F. culmorum*, and *B. cinerea*). Therefore, it is possible to assert that there are some potential applications of this extract for wood protection. The ECBE showed the highest insecticidal activities and contained a potent inhibitor against TMV infection. Although the isolation of the active antiviral constituents still requires many further studies could be related to the phenolic and flavonoid compounds.

CRedit authorship contribution statement

Ahmed Abdelkhalek: Conceptualization, Data curation, Methodology, Validation, Writing - original draft. **Mohamed Z.M. Salem:** Data curation, Methodology, Writing - original draft. **Ahmed M. Kordy:** Data curation, Methodology, Writing - original draft. **Abdelfattah Z.M. Salem:** Writing - review & editing, Supervision. **Said I. Behiry:** Conceptualization, Writing - original draft, Writing - review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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