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ARTICLE

Insecticidal Potential of α-Pinene and β-Caryophyllene against *Myzus persicae* and Their Impacts on Gene Expression

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ABSTRACT

Myzus persicae (*M. persicae*) is now considered a threat to agricultural crops due to economic losses. Numerous synthetic insecticides applied every year against *M. persicae*, are reported to be unsafe for environment, humans, and beneficial insects. Furthermore, several species of *Myzus* have been found to develop resistance due to over application of these insecticides. Therefore, it is required to find some novel insecticide that would be safe for the environment as well as for humans. In the current study, two major pure constituents α-pinene and β-caryophyllene were evaluated for their insecticidal potential against *M. persicae* using a fumigant toxicity assay. Furthermore, impact of α-pinene and β-caryophyllene on expression of five different genes, e.g., HSP 60, FPPS I, OSD, TOL and ANT responsible for reproduction, dispersion, and growth of *M. persicae* has also been investigated. To perform fumigant toxicity assay, five different concentrations (3.5, 4, 4.5, 5 and 6 μL L^{-1}) of α-pinene and β-caryophyllene were prepared. Lethal concentration (LC) was calculated, and gene expression studies were executed through qRT PCR at LC_{30} of α-pinene and β-caryophyllene. Both constituents demonstrated excellent fumigant toxicity effects against *M. persicae* at all five concentrations. However, α-pinene shows significantly better results (98%) as compared to β-caryophyllene (80%) after 72 h at 6 μL L^{-1} of dose. The highest upregulation in expression was demonstrated at LC_{30} dose of α-pinene in five in three out of five genes understudy (TOL, ANT, and FPPS I). Conversely, two genes HSP 60 and OSD demonstrated downregulation at LC_{30} dose of β-caryophyllene. Conclusively, our results highlighted the promising insecticidal potential of both compounds α-pinene and β-caryophylleneby interfering with the reproduction and development related processes in *M. persicae*, allowing us to recommend the phytoconstituents under investigation as an ecofriendly alternative to synthetic insecticides.

KEYWORDS

Fumigation; *M. persicae*; gene expression; real time PCR; α-pinene; β-caryophyllene

1 Introduction

Pests cause nearly 20%–50% of damage in agricultural crop production [1,2]. Among them, the aphid (*Myzus persicae*) is the most disastrous pest, categorized into more than 4300 species [3,4]. *M. persicae* usually causes harm in three ways; directly feeding on a plant, persisting as a vector by transmitting viral pathogen in the plant, or secreting honeydews, which helps several secondary pathogens to spread on the surface of the plant [5]. Due to different ways of damaging ability, *M. persicae* has become a significant crop hazard, triggering millions of dollars in losses yearly in the agricultural sector [6]. Several insecticides belonging to different classes are being utilized to control *M. persicae* through their multiple application on crops [7]. However extensive usage of these insecticides causes ecological contamination, pesticide remnants in food, and adverse effects on beneficial insects, organisms, and humans. Moreover, *M. persicae* can develop resistance against insecticides due to its high reproduction rate [8,9]. Owing to the current situation, there is a dire need to adopt eco-friendly practices to control *M. persicae* effectively.

Plant essential oils (EOs), a combination of bioactive volatile constituents, are regarded as one of the most important aspects of botanical insecticides due to their safety against the environment, non-target organisms, and low-level resistance [10,11]. Nowadays, essential oils and their active components are emerging as a substitute for insect control with no harmful effects on mammals and the environment [12,13]. Active constituents from the essential oil, such as *Asteraceae* and *Foeniculum vulgare* have demonstrated promising insecticidal activity against *M. persicae* [14,15]. Similarly, numerous studies have shown that essential oil-based secondary plant metabolites may be a worthy substitute for synthetic insecticides in controlling *M. persicae* [16–18].

Generally, essential oils are the amalgam of versatile chemical constituents such as terpenoids, terpenes, oxygenated terpenes, sesquiterpenes, phenylpropanoids, alcohols, esters, ketones, etc. [19]. Terpenes are plant-derivative chemicals with potent insect antifeedant and toxic properties to herbivorous insects [20]. α-pinene, a bicyclic terpene abundantly occurring in the *Rosemainus officinalis*, *Piper nigrum* or *Juniperus* species, and *Cupressus sempervirens* essential oils, have been reported to own numerous biological activities such as revolting and antifeedant activities against the Mosquito *Culexpipiens*, *Spodoptera litura*, *Sitophiluszeamais*, silverfish and stored grain insects [21–28]. β-caryophylleneis another naturally occurring bicyclic terpene of *Psidium guajava*, *Cephalotaxus sinensis* essential oils [29] with remarkable insecticidal potential against *M. japonica*, *P. xylostella*, and *Aedes aegypti* [30,31]. Considering the appreciable deterrent and insecticidal potential of both phytoconstituents α-pinene and β-caryophyllene against various insect species, the present study was designed to assess the fumigant toxicity of α-pinene and β-caryophyllene against *M. persicae*. Furthermore, a comparison of the effects of α-pinene and β-caryophyllene on the expression of five genes in *M. persicae* (HSP 60, FPPS I, OSD, TOL, and ANT) was made. The current study provides a detailed understanding of the molecular actions underlying *M. persicae*'s reproductive, developmental, and trauma responses to phytoconstituents under investigation. The conclusions of this study could lay the groundwork for developing original eco-friendly and decomposable insecticides derived from eatable plant essential oils.

2 Materials and Methods

2.1 Source of Constituents

To examine the insecticidal potential of phytochemicals under investigation against *M. persicae*, active constituents α-pinene and β-caryophyllene with 97% and 98% purity respectively were purchased from Innochem Science and Technology Co., Ltd. (Beijing, China).

2.2 Myzus Persicae Culture

Stock culture of *M. persicae* was sustained under an innocuous and insect-free environment at the insectarium of Anhui Agriculture University (China) for over four years. Cabbage plants were used to nurture the insects and then placed in an incubator at ± 26°C and 78% relative humidity in a photoperiod of 16:8 h (L:D) [32].

2.3 Fumigant Assay

To assess the fumigant toxicity of compounds α-pinene and β-caryophyllene caryophyllene extracted from Rosemary (*Rosemainus officinalis*) and *Piper cubeba* essential oil. For the fumigation bioassay, a 500 ml glass container was used. In each treatment, three replication and 30 adult *M. persicae* were placed. Five different doses were chosen (3.5, 4.0, 4.5, 5.0, and 6.0 μL L^{-1}). Using a microinjector, drops of each dose of α-pinene and β-caryophyllene were functional to a part of filter paper (3 × 3 cm) attached to the bottom side of the jar lid. Cabbage leaf was used to provide a suitable environment for *M. persicae*. Then glass containers were preserved in the culturing environments stated overhead. Those insects which were deprived of the constituent's handling were taken as a control. All dealings and panels were executed thrice autonomously. Insect death was noted at 24, 48, and 72 h post-treatment. Abbott's formula was applied to calculate the mortality of *M. persicae* [33]. Furthermore, the lethal concentrations (LC_{30} and LC_{50}) were calculated using probit analysis.

2.4 Real-Time Quantitative PCR (qRT-PCR)

qRT-PCR was carried out to probe the countenance levels of five selected genes under LC_{30} strength of both compounds α-pinene and β-caryophyllene. After 24 h of post-treatment, at least 50 *M. persicae* were composed and immediately deposited in liquid nitrogen, where they were preserved at −80°C until further analysis. The constructor's commands extracted the entire RNA from each treatment using TRIZOL reagent (Invitrogen, Carlsbad, MX, USA). The Biophotometer Plus (Eppendorf, Hamburg, Germany) was used to assess and quantify RNA integrity. To improve RNA stability, cDNA was synthesized using the Prime Script™ reagent kit (Takara, Dalian, China) according to the manufacturer's instructions. For the quantification of OSD and gene, qRT-PCR analysis was performed using *β*-actin as internal control, whereas the ACE gene was used as an inner switch for quantification of ANT, HSP 60, and FPPS I. All primers for qRT-PCR analysis are enlisted in Table 1. Bio-Rad iCycler iQ Real-Time Detection System (Bio-Rad, Hercules, CA, USA) which contained 2x UltraSYBR Mixture (Progema Corporation, Beijing, China) 7.5 μL, cDNA 2 μL, each primer (10 μM) 1 μL, and of RNase-free water 3.5 μL in a final volume of 15 μL, was used for the execution of qRT-PCR. Every treatment was repeated three times for all qRT-PCR. The $2^{-\Delta\Delta CT}$ technique was used to quantify the gene expression (mean ± SD) as a relative fold change [34]. The comparative quantifiable fold appearance change was assessed using one-way ANOVA [35].

2.5 Statistical Analysis

The Log-Probit model analysis was applied to mortality rate of *M. persicae* to determine the 30% and 50% lethal concentrations (LC_{30} and LC_{50}). The data for mortality rate of *M. persicae* was statistically analyzed by three-way analysis of variance (ANOVA) using the Statistical Package for the Social

Sciences (SPSS) version 16.0. The Tukey HSD analysis was applied at 5% probability of Type I error (α) to separate the means of the obtained data using ANOVA. The mean values were compared through alphabetical letters using Statistix 8.1 and standard error were calculated through software MS Excel version 16.0.

Table 1: List of selected gene forward or reverse primers that were used for the gene expression in *Myzus persicae* under LC_{30} of both constituents (α-pinene and β-caryophyllene)

Name of gene	Accession number	Forward primers	Reverse primers	Gene size	Reference
ANT	DQ407505	GCCGGTAATTTAGCATCAGG	CCTTGGACAAACAGTCTCCA	151	[36]
OSD	AJ634652	TCCCGAAGGAGCTGAACTTA	GCTTAGGGTCCCATTTGTCA	164	[36]
TOL	EB714328	AGCGCTTTCTGACGGAAATA	AGCATTCGAAGAAGCGATTG	177	[36]
HSP 60	EU334430	AGCATTGACCATGCCATGTA	AAACATCGGTCATTGCATCA	122	[36]
FPPS1	AJ250348	CGAACAGGCCATTTACCAGT	GACCCATCGCAGTTTTCATT	107	[36]
β-actin	[37]	GGTGTCTCACACACAGTGCC	CGGCGGTGGTGGTGAAGCTG	90–120	[37]
ACE	[37]	TAACGTAGTAGTGCCAAAGC	CACTGTAGAGCCATTAGCTG	90–120	[37]

3 Results and Discussion

Two major phytoconstituents' (α-pinene and β-caryophyllene) have demonstrated tremendous promise in pest management and may have an ecologically acceptable alternative to conventional insecticides. In the present study, the pure phytoconstituents (α-pinene and β-caryophyllene) in five ascending concentrations (3.5 to 6.0 μL L^{-1} of air) were used to investigate the insecticidal potential against *M. persicae*.

3.1 Fumigant Toxicities of α-Pinene and β-Caryophyllene

We performed a fumigant bioassay to investigate the toxicity of α-pinene and β-caryophyllene against the horticultural pest *M. persicae*. Five different doses (3.5, 4, 4.5, 5, and 6 μL L^{-1}) were finalized for both constituents. The LC_{30} and LC_{50} values of mortality rate after 24, 48, and 72 h of α-pinene and β-caryophyllene application were calculated through probit analysis (Table 2). After 24 h LC_{30} and LC_{50} α-pinene and β-caryophyllene were 4.2, 5.2, and 5.5, 6.8 μL L^{-1} of air. The constituent's fumigant control proved effective against *M. persicae*. Both constituents performed well; however, the response of α-pinene was superior to that of β-caryophyllene. The highest mortality rates of 98% and 74.80% after 72 h were observed by exposing to the highest concentration of α-pinene and β-caryophyllene (6 μL L^{-1} of air). At the same time, at the lowest concentration of constituents α-pinene and β-caryophyllene (3.5 μL L^{-1} of air) mortality rate was also low up to 15.6% and 4.3% after 24 h of exposure, respectively (Fig. 1 and Table S1).

Table 2: The LC_{30} and LC_{50} of α-pinene and β-caryophyllene were exposed to *M. persicae* at different times

Major constituents	Lethal concentration	24 h	48 h	72 h
α-pinene	LC_{30}	4.2	3.6	3.0
	LC_{50}	5.2	4.7	2.9
β-caryophyllene	LC_{30}	5.5	5.1	4.2
	LC_{50}	6.8	5.7	5.0

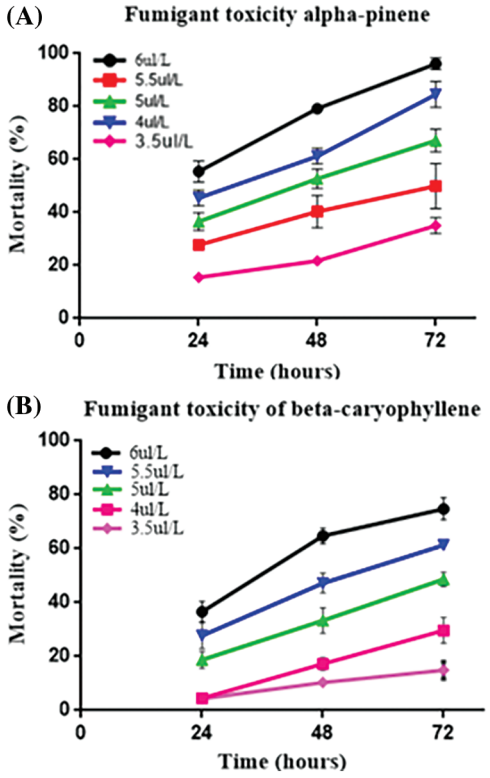

Figure 1: The percentage of fumigant (A) α-pinene and (B) β-caryophyllene toxicity at different times in response to different doses. A percentage represented the mortality rate

Three-way analysis of variance (ANOVA) revealed significant mortality rates individually in treatments, doses, time, and the interactions between treatments × doses and doses × time (Table 3). However, the interactions between treatments × time and treatments × doses × time were non-significant ($p < 0.05$). The interactions between treatments × doses and doses × time were analyzed through two-way ANOVA, and results are given in Tables 4 and 5. The treatment × doses interactions revealed the highest mortality in the sequence of 6.0 μL L^{-1} > 5.5 μL L^{-1} > 5.0 μL L^{-1} > 4.0 μL L^{-1} > 3.5 μL L^{-1}, while application of α-pinene reported more mortality than β-caryophyllene (Table 4). These doses cause a higher mortality rate as time duration increases (Table 5). Applying α-pinene and β-caryophyllene up to 72 h time reported maximum mortality followed by 48 and 24 h of incubation.

Table 3: Three-way analysis of variance (ANOVA) of individual treatments, doses, time, and their interactions

Source	Sum of squares	df	Mean square	F	Sig.
Corrected model	51373.764[a]	29	1771.509	56.302	0.000
Intercept	159895.182	1	159895.182	5.082E3	0.000
Treatments	7578.825	1	7578.825	240.872	0.000
Doses	23340.411	4	5835.103	185.452	0.000
Time	12603.742	2	6301.871	200.287	0.000
Treatments * Doses	6363.189	4	1590.797	50.559	0.000
Treatments * Time	70.747	2	35.374	1.124	0.332

(Continued)

Table 3 (continued)

Source	Sum of squares	df	Mean square	F	Sig.
Doses * Time	1113.465	8	139.183	4.424	0.000
Treatments * Doses * Time	303.385	8	37.923	1.205	0.311
Error	1887.849	60	31.464		
Total	213156.796	90			
Corrected total	53261.613	89			

Note: [a]R Squared = 0.965 (Adjusted R Squared = 0.947).

Table 4: The mortality of *M. persicae* resulted from the interaction between treatments and doses

Doses	α-pinene (% ± SE)	ß-caryophyllene (% ± SE)	Doses total
6.0 µL L^{-1}	77.006 ± 1.892^{a}	58.738 ± 1.452^{bc}	67.872 A
5.5 µL L^{-1}	63.849 ± 2.002^{b}	45.468 ± 0.952^{de}	54.658 B
5.0 µL L^{-1}	52.212 ± 0.274^{cd}	33.604 ± 1.032^{fg}	42.908 C
4.0 µL L^{-1}	39.403 ± 1.074^{ef}	17.189 ± 1.720^{hi}	28.296 D
3.5 µL L^{-1}	24.162 ± 1.362^{gh}	9.868 ± 1.261^{i}	17.015 E
Treatment's total	51.326 A	32.973 B	

Table 5: The mortality of *M. persicae* resulted from the interaction between doses and time

Doses	24 h (% ± SE)	48 h (% ± SE)	72 h (% ± SE)	Doses total
6.0 µL L^{-1}	46.105 ± 1.927^{de}	72.010 ± 2.010^{b}	85.500 ± 1.746^{a}	67.872 A
5.5 µL L^{-1}	36.668 ± 1.263^{efg}	54.287 ± 3.22^{cd}	73.020 ± 1.466^{b}	54.658 B
5.0 µL L^{-1}	27.715 ± 1.327^{gh}	43.063 ± 1.051^{e}	57.947 ± 1.645^{c}	42.908 C
4.0 µL L^{-1}	16.140 ± 0.274^{ij}	28.853 ± 1.261^{fgh}	39.895 ± 1.079^{ef}	28.296 D
3.5 µL L^{-1}	9.945 ± 1.327^{j}	16.083 ± 1.447^{ij}	25.017 ± 1.342^{hi}	17.015 E
Time total	27.315 C	42.859 B	56.276 A	

The interaction between treatment and doses was statistically significant at $p < 0.05$; the values that share different letters in the lower case along the column and row were statistically significant at $p < 0.05$ and vice versa; The treatments and doses values that share different letters in the upper case along last column and last row were statistically significant at $p < 0.05$ and vice versa; SE stands for standard error.

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3.2 Gene Expression

To find out the difference in gene expression patterns, gene expression profiling was performed on *M. persicae* (adult) at sub-lethal concentrations (LC_{30}). Various researchers have examined gene expression

responses to several treatments in relative quantity (RQ) to that in controlled *M. persicae*. A two-fold increase or decrease in expression was considered biologically significant [38]. The variation in the expression of five genes (HSP60, FPPS I, OSD, TOL, and ANT) was studied [39] in adult *M. persicae* exposed to α-pinene and β-caryophyllene for 24 h. OSD, TOL, and ANT (dispersal-related genes) have previously been shown to be overexpressed in *M. persicae* in response to stress. The results of this study indicate all three genes showed varying levels of overexpression, except for OSD genes that showed several times greater upregulation in *M. persicae*, at an LC_{30} dose of α-pinene and β-caryophyllene.

3.3 OSD Gene

In adult *M. persicae*, a 5-fold increase in OSD gene expression was observed after exposure to the lethal dose (LC_{30}) of α-pinene (Fig. 2A). The LC_{30} dose of β-caryophyllene has been shown to have a maximum expression of the OSD gene. At the same lethal dose (LC_{30}) of β-caryophyllene, α-pinene causes a 2-fold less augmentation in the expression of OSD gene in *M. persicae* after the same period. However, essential oil constituents (α-pinene and β-caryophyllene) boosted strongly upregulated gene expression in comparison to the control (Fig. 2A). Initial studies reveal that fecundity can be suppers at enhanced levels of *OSD* in *M. persicae*, which correlates with reduced reproduction [36].

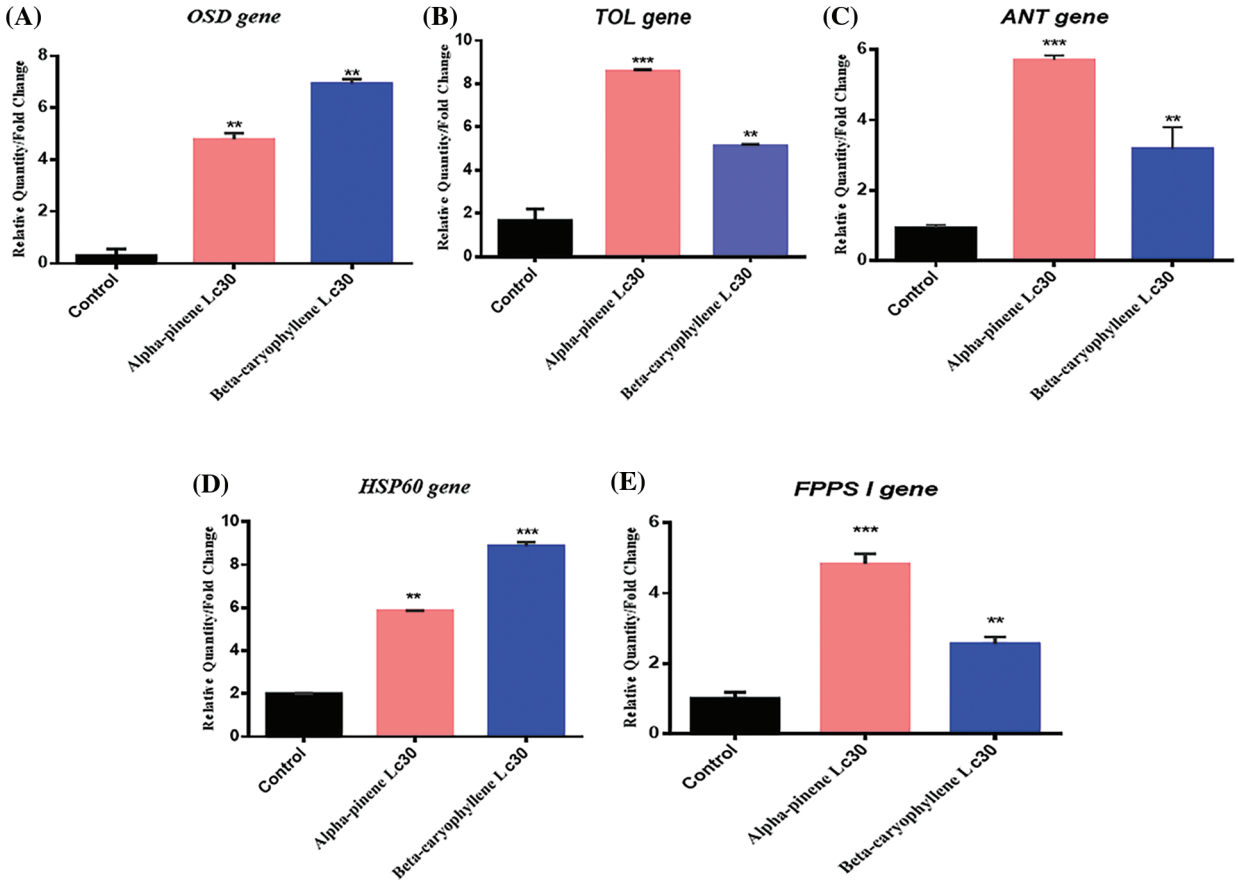

Figure 2: Relative gene expression of five different genes OSD (Olfactory Segment-D), TOL (Take-outlike), ANT (Adenosine nucleotide translocase), HSP 60 (Heat shock protein), and FPPS I (Farnesyl diphosphate synthase) were calculated at a sublethal concentration (LC_{30}) of two constituents of essential oil (α-pinene and β-caryophyllene) in adults *M. persicae* [35]. Mean values and standard deviations (SDs) are indicated by the error bar. ***Significant difference ($p < 0.001$), **significant difference at $p < 0.01$

3.4 TOL Gene

The constituents α-pinene and β-caryophyllene incite markedly increased TOL gene expression at LC_{30} doses. On exposure to *α-pinene* and β-caryophyllene, an unexpectedly different TOL gene expression pattern was recorded compared to the OSD gene. Compared to α-pinene and β-caryophyllene, the highest expression of the TOL gene was recorded at the LC_{30} dose of α-pinene (Fig. 2B). Previous results demonstrate that TOL can be overexpressed in response to starvation [40,41] and fluctuation in JH (Juvenile hormones) titers [42] during courtship and mating [43]. At an LC_{30} dose of α-pinene and β-caryophyllene, significant upregulation of the TOL gene was recorded. It has been previously reported that [41–43] upregulation of; the TOL gene causes starvation due to juvenile hormones binding proteins. It is carried out by fluctuation in JH titers, affecting antennal responses to food.

3.5 ANT Gene

In contrast to OSD gene expression under treatment of α-pinene LC_{30} dose, ANT gene expression is high. However, its expression is lower than TOL gene. A comparison between α-pinene and β-caryophyllene also shows the variation at the same dose. At the LC_{30} dose of α-pinene, ANT gene expression is 2 folds high in *M. persicae* as compared to β-caryophyllene LC_{30} dose (Fig. 2C). ANT gene specifically regulates mitochondrial proteins. These proteins are responsible for vital functions such as carriers of essential metabolites, which facilitate several mitochondrial roles, catalyzing trans-membranous (mitochondrial) transport of ADP to synthesize ATP [44]. With the modest comeback of the ANT gene to an at LC_{30} dosage of β-caryophyllene, ANTgene expresses moderate response, which shows negligible energy expenditures for *M. persicae*.

3.6 HSP 60 Gene

One of the stress response genes is HSP 60, and its expression varies according to each stressor type. Multiple studies have proven the downregulation of the HSP gene family (HSP 60, HSP 70, and HSP 90) associated with a recovery response after prolonged exposure to even mild stress. On the other hand, the upregulation of HSP 60 demonstrates the buildup of impaired proteins after stress or injury to an organism [45]. In addition, HSP accumulation has been reported to decrease fecundity [46]. In this study, after 24 h exposure to topical treatment of adult *M. persicae* with LC_{30} dose of α-pinene and β-caryophyllene, an almost 6 and 9-fold upregulation in the expression of the HSP 60 gene was obtained (Fig. 2D). Meanwhile, three folds reduced expression was noticed in response to LC_{30} dose of α-pinene. Compared to previous studies [46], it can be concluded that α-pinene and β-caryophyllene at higher doses negatively affect *M. persicae* reproduction by reducing fecundity. Results show that α-pinene has a low effect on HSP 60 gene compared to β-caryophyllene.

3.7 FPPS I Gene

FPPS I gene expression was enhanced post-treatment with LC_{30} of α-pinene and β-caryophyllene (Fig. 2E). At LC_{30} dose of α-pinene, FPPS Igene showed the highest (4 folds) upregulation. β-caryophyllene while 2-fold downregulation in FPPS Igene expression was noted in β-caryophyllene compared to α-pinene (Fig. 2E). Previously, it came to know that JH biosynthesis is affected by [47] *FPPS I* by catalyzing the synthesis of farnesyl diphosphate (FPP). The production of sexual pheromones in *M. persicae* is stimulated by JH. At the same time, increased JH titers in female insects promote the growth of apterous forms via inhibition of wing growth. FPPS I downregulation is related to reduced production of *β-farnesene* (EBF) [47,48], which may raise egg-laying capacity in *M. persicae*.

In short, it was concluded that an enhancement in the expression of *FPPS I* gene could negatively affect the reproduction and fecundity in *M. persicae*. Recent studies show significantly upregulated FPPS I gene expression after 24 h treatment with (LC_{30}) of α-pinene and β-caryophyllene in adult *M. persicae*. Hence,

the phytochemical constituents (α-pinene and β-caryophyllene) were found to be inversely related to the fecundity and reproduction of *M. persicae*.

In this study, α-pinene and β-caryophyllene of Rosemary and *Piper cubeba* oils have demonstrated potent fumigant toxic effects for controlling adults *M. persicae*. A comparative gene expression analysis has further supported that α-pinene upregulates the expression of *TOL*, *ANT*, and *FPPS I* genes more drastically than β-caryophyllene at the same dose (LC_{30}) and time intervals (24 h).

4 Conclusions

In the present study, the major constituents of two essential oils, α-pinene and β-caryophyllene, demonstrate mild to excellent control against *M. persicae* at all concentrations due to their promising anti-insecticidal activity. Gene expression assay identified that α-pinene and β-caryophyllene could affect the reproduction of *M. persicae* even at low concentrations. The findings can offer appreciable strategies for the rational design and discovery of innovative constituents, artificially or from natural products, with improved insecticidal potential and a higher biological safety profile.

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Table S1: The mortality rate of *M. persicae* due to different doses of α-pinene and β-caryophyllene toxicity at different times of incubation

Treatments	Doses	Time	Mortality rate (% ± SE)
α-pinene	6.0 μL L^{-1}	24 h	55.55 ± 2.01
		48 h	79.27 ± 1.91
		72 h	96.19 ± 2.14
	5.5 μL L^{-1}	24 h	27.78 ± 1.11
		48 h	40.38 ± 3.52
		72 h	50.05 ± 2.91
	5.0 μL L^{-1}	24 h	36.65 ± 3.32
		48 h	52.76 ± 3.65
		72 h	67.22 ± 2.34
	4.0 μL L^{-1}	24 h	45.56 ± 2.94
		48 h	61.33 ± 2.92
		72 h	84.66 ± 2.87
	3.5 μL L^{-1}	24 h	15.56 ± 1.11
		48 h	21.82 ± 0.78
		72 h	35.11 ± 1.01
ß-caryophyllene	6.0 μL L^{-1}	24 h	36.66 ± 3.83
		48 h	64.75 ± 2.88
		72 h	74.80 ± 2.04
	5.5 μL L^{-1}	24 h	27.78 ± 2.84
		48 h	47.24 ± 3.65
		72 h	61.38 ± 1.48
	5.0 μL L^{-1}	24 h	18.78 ± 3.04
		48 h	33.37 ± 4.67
		72 h	48.67 ± 2.65
	4.0 μL L^{-1}	24 h	4.50 ± 1.10
		48 h	17.33 ± 2.33
		72 h	29.74 ± 4.81
	3.5 μL L^{-1}	24 h	4.33 ± 0.712
		48 h	10.35 ± 0.21
		72 h	14.92 ± 3.27