Available online at http://www.journalijdr.com

ISSN: 2230-9926

International Journal of Development Research
Vol. 5, Issue, 10, pp. 5661-5667, October, 2015

International Journal of DEVELOPMENT RESEARCH

Full Length Research Article

FUMIGANT TOXICITY AND CYTOTOXICITY EVALUATION OF MONOTERPENES AGAINST FOUR STORED PRODUCTS PESTS

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ARTICLE INFO

Article History:
Received 30th July, 2015
Received in revised form
19th August, 2015
Accepted 20th September, 2015
Published online 31st October, 2015

Key words:
Fumigant Toxicity,
Insect Pests,
Monoterpenes,
Cytotoxicity.

ABSTRACT

In the present study, eight pure monoterpenes: citronellol, geraniol, linalool, eugenol, thymol, cinnamaldehyde, p-cymene and α-pinene were tested for their fumigant toxicity against four stored product pests, *Callosobruchus analis, Sitophilus oryzae, Stegobium paniceum* and *Tribolium castaneum*. Monoterpenes tested showed varying degrees of fumigant toxicity against different species of stored product pests but were highly dependent upon dosage and exposure duration. Cinnamaldehyde, p-cymene and α-pinene at a concentration of 5 -10 µl/mL air caused 90 - 100% mortality among all the four pests after 24 hrs of the treatment. α- pinene was highly toxic with LC_{50} values of 0.03, 0.12, 1.21 and 1.43 µl/mL air after 12 hrs of treatment for *C. analis, S. paniceum, S. oryzae* and *T. castaneum* respectively. Citronellol was least toxic having LC_{50} of 1.0, 1.6, 2.3 and 2.8 µl/mL air after 12 hrs of treatment for the same sequence of the four insects, respectively. Eugenol and thymol also showed potent fumigant toxicity causing high mortality for all the insect species. LC_{50} values of all these monoterpenoids tended to decrease at longer exposure times. In general, monoterpene hydrocarbons exhibited high toxicity as compared to oxygenated monoterpenes. Among the four insect species tested, *C. analis* and *S. paniceum* were the most susceptible, having highest mortality of the species tested for each compound, while *T. castaneum* and *S. oryzae* were the most tolerant of the four insect species tested. These monoterpenes have also been examined for cytotoxicity towards BV2 (microglia) cell line by employing MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and found that monoterpenes exhibit low cytotoxicity.

INTRODUCTION

The environmental problems due to overuse of synthetic pesticide have been the matter of concern for everyone. Synthetic insecticides such as methyl bromide or phosphine have many hazards effects causing severe environmental problems like ozone depletion, environmental pollution, toxicity to non−target organisms, pesticide residues and non-biodegradable properties (Lee *et al*., 2004; Isman, 2006). Therefore to reduce the negative impacts on human health and environment natural products are the best alternative to synthetic pesticides. Pesticides derived from plant essential oils or their constituents have established great efficiency against the broad range of stored products insect pests, responsible for post-harvest losses. The natural products based on plants are relatively less harmful to environment and mammalian health as compared to synthetic chemical pesticides.

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It has been proved that monoterpenes are main components of plant essential oils and are biodegradable and non-persistent in soil and water (Misra and Pavlostathis, 1997). In recent times essential oils of botanical origin and their important constituents, often various monoterpenoids, due to their insecticidal, repellent and antifeedant properties have attracted attention as potential pest control agents (Amos *et al*., 1974; Grundy and Still, 1985; Stamopoulos, 1991). Essential oils have potential fumigant activity against a large number of stored product insects (Huang *et al*., 1997; Liu and Ho, 1999), and their monoterpenoid components are highly volatile, strongly toxic to insect pests but exhibits very low toxicity to warm-blooded animals (Lee *et al*., 2001a; Lee *et al*., 2001b). Monoterpenoid compounds are among the best known pest control agents due to their acute toxicity, antifeedent properties and wide range of repellency against insect pests (Watanabe *et al*., 1993; Hough-Goldstein, 1990), and also possess biological activity as ovicides, antifeedents, fumigants, and contact toxicants against various insect pests (Karr and Coats, 1988; Rice and Coats, 1994; Tsao *et al*., 1995).

Fumigant toxicity of some naturally occurring monoterpenoids have been proved against *Sitophilus oryzae,* the red flour beetle, *Tribolium castaneum* and the sawtoothed grain beetle, *Oryzaephilus surinamensis* (Lee *et al*., 2003). Therefore the present study was carried out to investigate the fumigation toxicity of eight monoterpenoid compounds against four major stored-product insect pests, the red flour beetle, *Tribolium castaneum,* (Herbst), the rice weevil, *Sitophilus oryzae,* (L.), the drugstore beetle, *Stegobium paniceum* (L.) and the pulse beetle, *Callosobruchus analis* (F.) For this purpose, the monoterpenoid compounds were tested in vapour form against adult insect pests and cytotoxicity towards living cell line was examined for their comprehensive safety evaluation.

MATERIALS AND METHODS

The following compounds were tested: citronellol, geraniol, linalool, eugenol, thymol, cinnamaldehyde, α-pinene, p-cymene were provided by Sigma Aldrich, India. The choice of chemical was made in order to provide a large variety of characteristic structures of monoterpenes: aliphatic, cyclic, hydroxylated, unsubstituted, phenolic, methoxyl and aldehyde (Fig. 1). Most of them were identified as major components of essential oils which showed a strong insecticidal effect (Regnault-Roger *et al*., 1993).

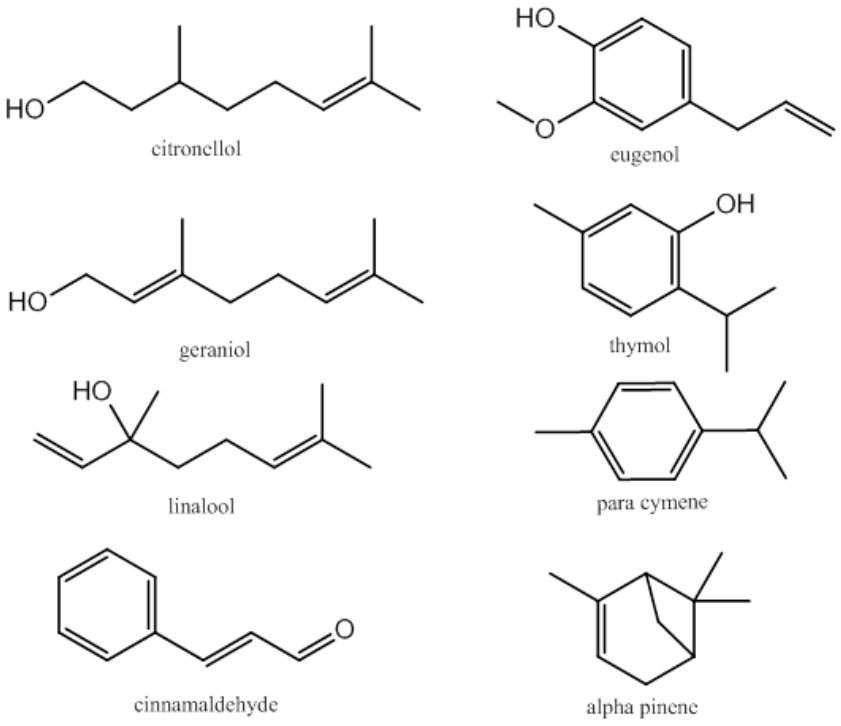

Fig.1. Chemical and structural formulae of different monoterpenes used in the experiment.

Test insects

Laboratory cultures of *S. oryzae*, *S. paniceum*, *T. castaneum* (5-10 days each) and *C. analis* (0–5 days) was maintained at 30±2 ^{0}C and 68±2% relative humidity. Test insects of *S. oryzae*, were reared on rice kernels, *C. analis* on chickpea grains and wholemeal wheat flour plus brewer's yeast (19:1) was used to rear *S. paniceum* and *T. castaneum* respectively.

Fumigant toxicity bioassay

To test the fumigant toxicity of the monoterpenoids on different insect pests plastic jars with 250 ml capacity with screwed metallic caps were used as exposure chambers. The selected monoterpenes with different doses of 1µl, 5µl and 10µl were diluted with methanol to the appropriate concentrations.

The appropriate dilution of monoterpenoid was administered in 1 ml portions on Whatman No. 1 filter paper (3 cm). After allowing the solvent to evaporate for 10-15 minutes, the filter paper was attached to the inner surface of the screw lid of the jar using adhesive tape. At the bottom of each jar, 10 individuals along with their food source were placed and exposed to the various concenterations of monoterpenes. The insects had no contact with the diffuser and stayed at the bottom of the chamber throughout the experiment. Insect mortalities were determined and calculated after 6, 12, and 24 hrs from exposure, according to the formula of Abott (1925). Three replicates were set up for each dose and control.

Cytotoxicity assay

The MTT (3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) based cytotoxicity test was used to evaluate all of monoterpenes and the tests were carried out on BV2 (microglial cells). Cells were seeded in 96-well flat-bottomed microplates at a density of 5 × 10^4 per mL, 100 µL per well and were allowed to grow for 24 hrs The compounds dissolved in dimethyl sulfoxide (DMSO) were sterilized using a Millipore filter (pore size 0.22 µm) and were added to the culture media over a concentration range of 1-2000 µg/mL.

The cytotoxicity of the monoterpenes was assessed after 24 hrs of exposure. The absorbance was read at 550 nm using a Muliskan PLUS plate reader (Labsystem, Finland).
The statistical analysis was performed using Sigma Stat 3.5.1 and Sigma Plot 11.

Statistical analysis

Data obtained from each dose-response bioassay were subjected to probit analysis in which probit-transformed mortality was regressed against log10-transformed dose, LC_{50s} and LC_{95s} values and 95% fiducial limits were generated (Finney, 1971).

RESULTS

Fumigant toxicity tests

The toxicities of pure commercial eight monoterpenes, including alcohols, phenols, aldehydes and monoterpene hydrocarbons were determined against four stored product pests viz., *C. analis, S. oryzae*, *S. paniceum* and *T. castaneum*. Analysis of the mortality data showed that all the monoterpenoid vapours exhibit a strong toxic action against the adults of four insect pests **(Table1).**

Three monoterpenes cinnamaldehyde, p-cymene and α-pinene at a concentration of 5 -10 µl/mL air caused 100% mortality to *C. analis* and 90% to other three pests after 24 hrs treatment. In alcohol group containing monoterpenes, geraniol showed 100% mortality to *C. analis* and *S. paniceum* followed by linalool. Citronellol showed a weaker activity with the lowest mortality of 75 and 80% against *T. castaneum* and *S. oryzae* while phenolic group bearing monoterpenes, thymol was the most effective against all species tested **(**Fig 2 and 3**)**. No mortality was observed in case of control treatments. Of the eight monoterpenoids screened, the monoterpene hydrocarbons such as α-pinene and p-cymene exhibited the highest activity followed by cinnamaldehyde, phenols and alcohols (Table1).

Among the four insect species tested, the pulse beetle and drugstore beetle were the most susceptible, having the highest mortality for each compound, while the red flour beetle was the most tolerant having the lowest mortality values for any compound used. *S. oryzae* and *T. castaneum* were the most tolerant of the four insect species tested. The concentration of monoterpenes and exposure duration significantly influenced the percentage adult mortality of all the insects tested. In general, the mortality increased with increasing doses of the compounds and exposure times.

Table1. Percent mortality due to eight monoterpenoids used in experiment of four stored products pests (values are % mortality ± SEM).

% Mortality ± SEM				
Monoterpenes	*C. analis*	*S. paniceum*	*S. oryzae*	*T. castaneum*
Alcohols				
Citronellol	95±3.2	90±6.8	80±9.8	75±8.2
Linalool	95±4.8	92±1.2	85±5.4	80±6.7
Geraniol	100	95±4.2	85±6.8	82±10.2
Phenols				
Eugenol	98±0.8	88±8.5	85±5.7	80±5.9
Thymol	100	90±6.7	88±8.5	85±5.8
Aldehyde				
Cinnamaldehyde	100	95±5.0	90±9.1	88±8.5
Hydrocarbons				
p-cymene	100	95±4.5	92±5.8	90±5.3
α-pinene	100	98±0.8	92±5.7	90±8.5

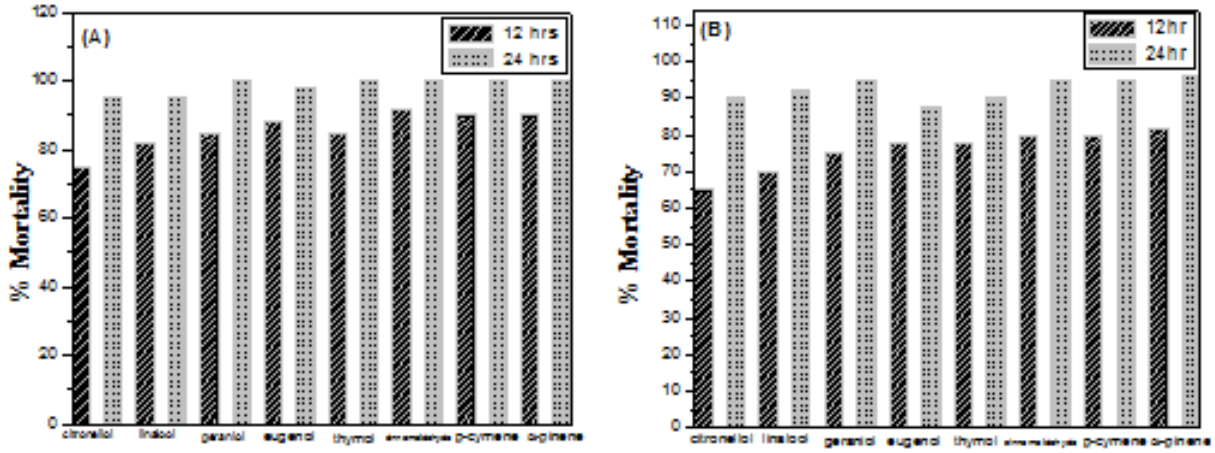

Fig.2 (A) Represent the percent mortality of *C. analis* and (B) represent the percent mortality of *S. paniceum* with different monoterpenes after 12 and 24 hrs of treatment

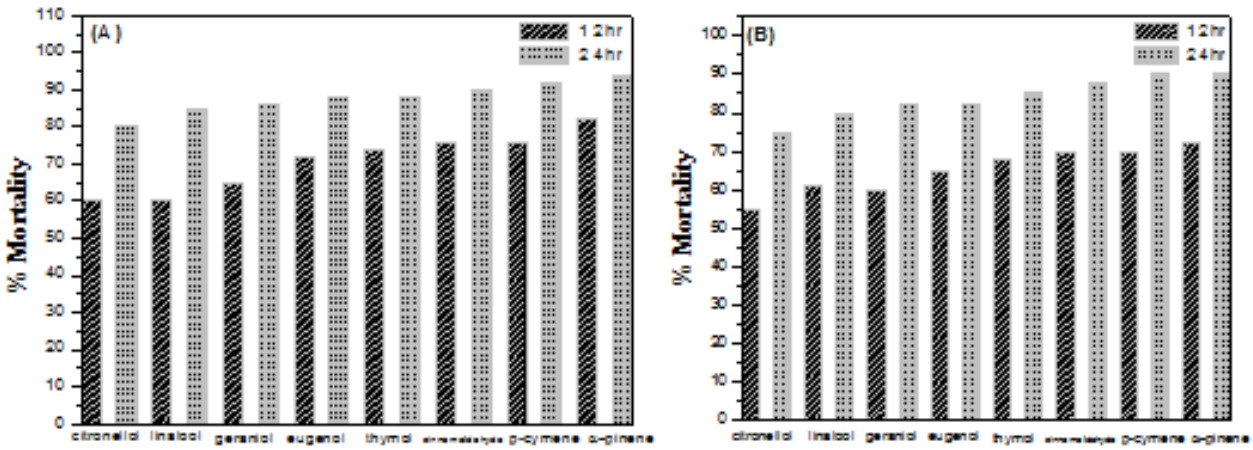

Fig.3 (A) Represent the percent mortality of *S. oryzae* and (B) represent the percent mortality of *T. castaneum* with different monoterpenes after 12 and 24 hrs of treatment.

Table 2. LC_{50} values and 95% Fiducial Limits (FL) of eight monoterpenoids against four stored product insects on different exposure intervals

LC_{50} µl/mL air			
	6 hrs	12 hrs	24 hrs
Citronellol			
C. analis	3.1 (1.3-8.5)	1.0 (0.5-3.1)	0.06 (0.02-0.1)
S. paniceum	3.8 (0.9-5.4)	1.6 (0.6-3.8)	0.08 (0.03-0.1)
S. oryzae	4.5 (3.2-6.4)	2.3 (1.0-3.7)	1.14 (0.5-2.8)
T. castaneum	5.2 (4.8-6.0)	2.8 (1.5-4.5)	1.53 (1.0-3.1)
Linalool			
C. analis	2.1 (0.5-3.4)	0.21 (0 .02-0.5)	0.04 (0.01-0.2)
S. paniceum	2.4 (1.0-4.1)	1.12 (0.2-1.9)	0.05 (0.02-0.7)
S. oryzae	3.0 (2.2-6.3)	1.92 (0.5-3.4)	0.12 (0.02-1.0)
T. castaneum	3.5 (2.5-8.1)	2.0 (0.5-3.5)	0.42 (0.01-1.4)
Geraniol			
C. analis	1.8 (0.1-3.9)	0.14 (0.05-0.2)	0.04 (0.01-0.09)
S. paniceum	2.8 (1.0-5.0)	0.36 (0.1-0.8)	0.05 (0.02-0.09)
S. oryzae	3.0 (1.6-6.8)	1.48 (0.5-2.9)	0.14 (0.1-1.5)
T. castaneum	3.0 (2.0-5.6)	1.87 (1.0-2.4)	0.24 (0.05-0.8)
Eugenol			
C. analis	1.63 (1.1-3.5)	0.02 (0.01-0.1)	0.02 (0.01-0.4)
S. paniceum	1.85 (0.3-4.1)	0.23 (0.04-0.8)	0.02 (0.005-0.1)
S. oryzae	2.1 (1.5-4.1)	0.52 (0.1-0.8)	0.05 (0.02-1.7)
T. castaneum	2.7 (1.8-4.5)	1.42 (0.3-3.7)	0.23 (0.1-1.5)
Thymol			
C. analis	1.38 (0.5-2.3)	0.09 (0.01-2.5)	0.03 (0.01-0.5)
S. paniceum	1.78 (0.5-3.8)	0.15 (0.05-1.5)	0.05 (0.02-1.5)
S. oryzae	2.1 (1.0-4.3)	0.32 (0.08-1.0)	0.05 (0.01-1.8)
T. castaneum	2.4 (1.2-3.9)	0.94 (0.1-1.5)	0.05 (0.02-1.5)
Cinnamaldehyde			
C. analis	1.21 (0.3-2.5)	0.05 (0.01-1.2)	0.02 (0.01-0.2)
S. paniceum	1.56 (0.5-3.2)	0.12 (0.05-0.4)	0.03 (0.01-0.8)
S. oryzae	1.72 (1.0-3.2)	0.24 (0.1-1.9)	0.04 (0.02-0.5)
T. castaneum	1.86 (0.5-3.8)	1.23 (0.5-2.3)	0.10 (0.05-0.6)
p-cymene			
C. analis	1.13 (0.2-1.9)	0.05 (0.01-1.0)	0.02 (0.01-0.2)
S. paniceum	1.41 (0.3-2.7)	0.14 (0.05-0.6)	0.02 (0.01-0.1)
S. oryzae	1.53 (0.5-3.1)	1.20 (0.5-2.0)	0.04 (0.03-0.4)
T. castaneum	1.75 (1.0-3.8)	1.58 (1.0-3.4)	0.05 (0.02-2.5)
α-pinene			
C. analis	1.0 (0.2-2.5)	0.03 (0.005-0.1)	0.01 (0.005-0.1)
S. paniceum	1.39 (0.5-3.1)	0.12 (0.08-0.8)	0.02 (0.01-0.1)
S. oryzae	1.42 (0.5-3.0)	1.21 (0.5-2.3)	0.03 (0.01-0.1)
T. castaneum	1.61 (1.2-4.8)	1.43 (0.5-2.8)	0.04 (0.01-0.2)

The toxicities of monoterpenes at different time intervals and their LC_{95} values have been calculated and summarized in the Table 2 and 3 respectively. The most toxic compounds were α-pinene and p-cymene followed by cinnamaldehyde. α- pinene was highly toxic with LC_{50} values of 0.03, 0.12, 1.21 and 1.43 µl/mL air respectively after 12 hrs treatment to *C. analis, S. paniceum, S. oryzae* and *T. castaneum*. Cinnamaldehyde also possessed a similar higher toxicity with LC_{50} values of 0.02, 0.03, 0.04 and 0.10 µl/mL air after 24 hrs of the treatment to the same sequence of insect pests respectively (Table 2).

Table 3. LC_{95} values and 95% Fiducial Limits (FL) of eight monoterpenoids against four stored product insects

	LC_{95} µl/mL air			
	C. analis	*S. paniceum*	*S. oryzae*	*T. castaneum*
Citronellol	6.8 (5.8-7.3)	6.2 (5.5-7.0)	7.1 (6.4-7.5)	8.5 (2.3-12.5)
Linalool	6.0 (5.3-6.5)	5.8 (5.1-6.6)	6.5 (5.8-7.0)	8.0 (1.8- 10.5)
Geraniol	5.4 (4.8-6.1)	5.8 (4.8-6.5)	6.1 (5.0-7.1)	7.6 (6.8-8.1)
Eugenol	5.2 (4.2-6.3)	4.8 (3.9-6.5)	5.0 (3.8-5.8)	6.8 (6.1-7.5)
Thymol	4.8 (3.7-6.1)	5.0 (4.1-6.7)	5.0 (4.1-6.2)	6.5 (5.5-7.5)
Cinnamaldehyde	4.2 (3.1-5.7)	5.5 (4.5-6.5)	5.7 (5.1-6.3)	6.1 (5.6-7.1)
p-cymene	3.8 (2.0-4.8)	4.5 (3.2-5.6)	5.2 (4.0-6.5)	6.0 (5.5-6.8)
α-pinene	3.2 (2.5-4.1)	3.2 (1.9-5.1)	4.5 (3.4-6.1)	5.7 (4.8-6.3)

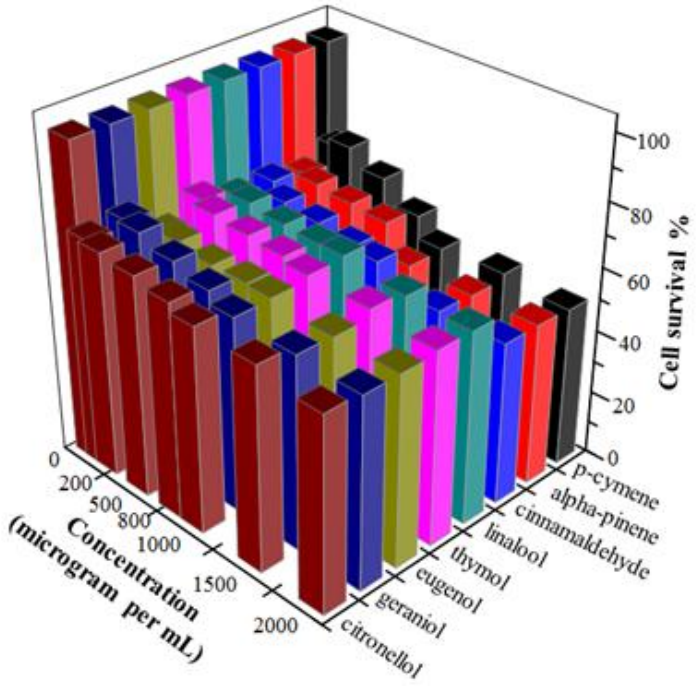

Figure 4. 3D graph represents the cell survival percentage at different concentrations of monoterpenes (citronellol, geraniol, eugenol, thymol, linalool, cinnamaldehyde, alpha-pinene and para-cymene) during MTT assay against BV2 (microglia) cells.

It was found that LC_{50} values decreased with increase in exposure time. Eugenol and thymol showed fumigant toxicity having LC_{50} value of 2.7 µl/mL and 2.4 µl/mL air after 6 hrs treatment respectively, whereas similar compounds exhibit LC_{50} values of 1.42 µl/mL and 0.94 µl/mL air after 12 hrs of treatment respectively against *T. castaneum*. Among the alcohol group containing monoterpenes the linalool and geraniol showed highest toxicity against *C. analis* having LC_{50} value of 2.1 µl/mL and 1.8 µl/mL air respectively. On the other hand, citronellol was less efficacious with higher LC_{50} values of 1.0, 1.6, 2.3, 2.8 µl/mL air for *C. analis, S. paniceum, S. oryzae* and *T. castaneum* respectively after 12 hrs of treatment (Table 2). Above insecticidal study expressed the significant activity of investigated monoterpenes against a panel of stored product pests. It is necessary from application point of view to check the toxicity of botanical insecticides towards mammals. Cytotoxicity of compounds generally indicated by IC_{50} value that signifies the concentration which causes death of 50% of living cells. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was employed to investigate the cytotoxicity of all the investigated monoterpenes towards BV2 (microglia) cell line.

The outcomes of cytotoxicity of monoterpenes indicated that the investigated monoterpenes are low cytotoxic and exhibit toxicity at higher concentration range. Figure 4 displays the order of cytotoxicity at higher concentration range as citronellol < geraniol < linalool < eugenol < thymol < cinnamaldehyde < alpha-pinene < para-cymene that indicate, the hydrocarbon monoterpenes and aldehyde group containing monoterpenes exhibit higher toxicity than alcohol group containing monoterpenes.

These results of cytotoxicity are in corroboration with insecticidal results where hydrocarbon monoterpenes and aldehyde group containing monoterpenes possess significant efficacy against a panel of stored product pests. The cytotoxicity results clearly indicate that para-cymene and alpha-pinene exhibit IC_{50} value of 1000 µg/mL whereas cinnamaldehyde possess IC_{50} value of 1500 µg/mL towards living cell line i.e. BV2. Alcohol group containing monoterpenes such as citronellol, geraniol, linalool and thymol were found to be low cytotoxic by retaining the cell survival up to 60% even at higher concentration range of 2000 µg/mL (Figure 4).

DISCUSSION

The present study demonstrates the monoterpenes have varying degrees of fumigant toxicity against different species of stored product pests but dependent upon the dosage and duration of treatment. Previous studies also evaluated the varying insecticidal activities of monoterpenes against various insect species and limonene, terpinen-4-ol, 1,8-cineole, menthone, carvacrol, myrcene and α-pinene have been proved more toxic than other (Lee *et al*., 1997; Prates *et al*., 1998; Kim and Ahn, 2001). Among the tested compounds, monoterpene hydrocarbons, α-pinene and p-cymene showed relatively strong toxicity than oxygenated monoterpenes against all the insect species. α -pinene, has shown strong fumigant toxicity against the bean bruchid beetle, *Acanthoscelides obtectus* (Say) (Roger and Hamaroui, 1995). Kordali *et al*. (2007) studied toxicities of nine monoterpene hydrocarbons and twenty one oxygenated monoterpenes, against larvae and adults of Colorado potato beetle and monoterpene hydrocarbons, α-pinene, 3-carene, β-citronellene and terpinene were found to be most toxic than the oxygenated ones. In addition, cinnamaldehyde proved to be a more potent insecticide than anethole and eugenol against the two species of beetles, *T. castaneum* and *S. oryzae* (Ho *et al*., 1997). Similar results were reported in the present study where cinnamaldehyde resulted in higher mortality at low LC_{50} values than the oxygenated monoterpenes (alcohols and phenols) against all insect species.

Fumigant toxic activity and reproductive inhibition induced by monoterpenes with chemical skeleton having a non-substituted phenolic moiety was found more against *A. obtectus* than non-phenolic structure or phenol substituted by a methoxy group. (Roger and Hamraoui, 1995). Coats *et al*. (1991) found that exposure of *S. oryzae* for 24 hrs to linalool and d-limonene gave LC_{50} values of 14 and 19 µl/L air but our results showed different toxicities for linalool LC_{50} = 0.12 µl/mL air against the rice weevil may be due to different strains of rice weevil. Ogendo *et al*. (2008) reported that eugenol, at 1µl/L air, resulted in 79%, 61% and 100% mortality of *R. dominica, O. surinamensis and C. chinensis,* respectively, in 24 hrs after treatment and 90 -100% mortality of *T. castaneum* and *S. oryzae* was achieved after 168 hrs treatment. Similarly in present study eugenol, at concentrations of 5 µl/mL air, was potent enough to achieve about 80% mortality of all the test insects except *T. castaneum* within 24 h after treatment. Generally adults of *Callosobruchus* spp. were more susceptible to essential oils or their components than other insect species (Ahmed and Eapen, 1986; Tripathi *et al*., 2003). Present investigation support the findings revealing most susceptibility of *C. analis* and *S. paniceum* than *S. oryzae* and *T. castaneum*. Further, the investigation of cytotoxicity of investigated monoterpenes towards living cells demonstrated that hydrocarbon monoterpenes and aldehyde group containing monoterpenes were found to be cytotoxic in the concentration ranging from 1000 - 2000 µg/mL whereas rest other monoterpenes (citronellol, geraniol, linalool, thymol and eugenol) exhibit low cytotoxicity at similar concentration range. In conclusion, the development of natural or biological insecticides having low cytotoxicity will help to decrease various environmental problems as compared to synthetic chemicals.

The practical implications of these findings are adequate to control the insect pests through monoterpenoids. Monoterpenoids are typically volatile compounds that penetrate into insects rapidly and interfere with their physiological functions. These natural low cytotoxic monoterpenes could be used as an environmentally friendly fumigants to control stored-grain insect pests.

Acknowledgement

Jyotika Brari is thankful to Himachal Pradesh University for the university fellowship and funding support. Jyotika Brari is also thankful to Dr. D. R. Thakur for his guidance in this research work.

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