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Bioactivity and efficacy of essential oils extracted from *Artemisia annua* against *Tribolium casteneum* (Herbst. 1797) (Coleoptera: Tenebrionidae): An eco-friendly approach

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ABSTRACT

Tribolium casteneum is a major stored grains pest causing huge loss by secreting toxic quinones' which make the grains unfit for human consumption. Increasing concern about the fast-growing resistance in *T. casteneum* against fumigants has evoked more intense research worldwide. Therefore, finding an eco-friendly alternative for the management of the pest is of great importance. In this study, the insecticidal activity of the essential oils (EOs) of *Artemisia annua* is evaluated. Chemical composition of the EOs eluted with methanol and petroleum ether was analysed through Gas chromatography-mass spectrometry (GC-MS). The result has reported a total of 13 & 16 compounds in the methanol and petroleum ether EOs respectively. In contact toxicity studies, adults were found more susceptible to the petroleum ether EOs (LD_{50} = 0.43 mg adult^{-1}) than the methanolic EOs (LD_{50} = 1.87 mg adult^{-1}). Petroleum ether EOs was also superior in fumigant assays against both the adults (0.81 mg L air^{-1}) and larvae (0.65 mg L air^{-1}). Moreover, the same was also recorded as a strong repellent. The bio-molecular studies conducted to gain an insight into the extent of metabolic disturbances inflicted in the treatment sets has shown a significant increase in Lipid peroxidase and decrease ($p<0.01$) in protein, Acetylcholinesterase, Glutathione S Transferees, Reduced Glutathione level. This indicates the major signs of oxidative stress in the treatment sets. The Results ascertain the knowledge to develop natural insecticides from *Artemisia annua* using a potential solvent to be used in the future as an efficient management tool against *T. casteneum.*

1. Introduction

Cereal grains are the primary source of calories and proteins for humans and livestock. Hence, its maintenance is of utmost importance. However, grains are prone to insect infestation while stored in warehouses. *Tribolium casteneum* is one of the highly resistant pests and known to damage a wide range of stored grains (Hagstrum, 2017). Resistance development in them is aided by the qualities like sexual selection by the females for fitness of the progeny (Fedina and Lewis, 2008), easy adaptation to a new environment (Bergerson and Wool, 1988), and rapid dispersal to colonise new food patches (Arnaud et al., 2005). Hence, it continues to cause economic damage, which brings down the market price and nutritional efficiency of the grains (Shafique et al., 2006). It is estimated that every year, approximately 40% of weight loss of wheat flour is caused due to *T. casteneum* infestation (Ajayi and Rahman, 2006). Apart from financial loss, the beetle secretes toxic quinones' which is marked by the characteristic colour change of the flour (Ladisch et al., 1967). Quinones' are carcinogenic thus possess serious health risks to human consumption (El-Mofty et al., 1989).

Quality maintenance of grains in storage is a major concern of all nations. Nevertheless, grain deterioration in warehouses is an unfortunate reality. Control measures exclusively rely on fumigation with methyl bromide and phosphine for managing a wide range of stored grains pests (Bell, 2000). However, the use of methyl bromide was banned worldwide due to its direct association in ozone layer depletion (Carter et al., 2005; Anbar et al., 1996). Moreover, phosphine was found to be least effective in controlling different stored grains pests, including *T. castenuem,* due to the fast-growing resistance of the beetle (Benhalima et al., 2004). Hence, the development of natural, eco-friendly management tools are of great health and economic importance (Okwute, 2012; Chaudhary et al., 2017).

Numerous studies were designed and conducted across the globe to

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find a better alternative to these fumigants (Kedia et al., 2015). Amongst all, plant-based research has drawn global attention for possessing a wide range of secondary metabolites including EOs (Sasidharan et al., 2011). EOs are also reported to be effective against different groups of insects including stored grains pests (Isman, 2000; Caballero-Gallardo et al., 2011). Moreover, plant-based components do not accumulate into the environment and are least toxic to non-target organisms, including humans (Benelli, 2015; Govindarajan et al., 2016).

Artemisia annua, a species of Asteraceae family, is well known for its medicinal properties and used extensively in Asian countries as a combined therapy for Malaria (Klayman, 1985; Das, 2012). Apart from medicinal benefits, EOs from the *Artemisia scoparia* Waldst and Kit were reported to be an efficient fumigant in controlling *T. casteneum* (Negahban et al., 2004). Another species of the plant viz. *A. sieberi* Besser has shown insecticidal properties against three stored grain pests (Negahban et al., 2007). Moreover, EOs from *Artemisia princeps* was described to be an effective repellent and insecticidal candidate against two major stored grain pests (Liu et al., 2006). Based on the previous reports, the insecticidal properties of the genus could be recognised.

Bioactive elements present in EOs act by triggering different modes of action and may reduce the chance of resistance development in *Tribolium* (Upadhyay and Jaiswal, 2007; Beltagy & Omar, 2016). A recent report on the efficacy of EOs which acted by interfering with the metabolic functions in insects was also demonstrated (Maia and Moore, 2011). However, studies to decipher a better solvent for the extraction of EOs from *A. Annua* have not been recorded yet. Hence, the authors analysed and compared the potential of EOs of *A. annua* extracted with both polar (methanol) and non-polar (petroleum ether) solvents against *T. casteneum*. In the present study, we explored the contact and fumigant toxicity of *A. annua* EOs against the pest. EOs obtained by hydro-distillation was evaluated through Gas chromatography-mass spectrometry to identify the major chemical constituents. Additionally, metabolic interference inflicted in the treatment sets of *T. casteneum* was assessed.

2. Materials and methods

2.1. Insect

Tribolium casteneum was collected from the laboratory cultures maintained in the division of Entomology of the Department of Zoology, The Maharaja Sayajirao University of Baroda, for the last three years. Unsexed adults were reared in the defined culture media of wheat flour, wheat grains, and yeast in the ratio of 6:3:1. Insects were maintained in the humidity chamber at a temperature of 27 ± 2 °C and humidity of 70 ± 5 RH. Newly emerged adults (1–10 days old) and final larval stages (14 days old) were used for the toxicity assays. All experiments were conducted in the dark at 27 ± 2 °C and 70 ± 5 RH.

2.2. Plant material and extraction of essential oils

Dried, finely grounded leaves of *A. annua* were procured from the Department of Botany, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India. Plant powder was stored in reclosable sterile plastic zip bags (Procured from Fisherbrand™ Sterile Polyethylene Sampling Bags of 5 kg capacity; free from impurities) at 4 °C until it is used for the extraction of EOs.

Plant powder (25 g) was subjected to hydro-distillation using a modified Clevenger-type apparatus for the extraction of EOs (Clevenger, 1928). The onset of distillation was marked by the boiling of the solvent (300 ml) in the round bottom flask. The hydro-distillation continued for 16 h with methanol and for 1 h with petroleum ether (40–60 °C). The oil layer was separated from the aqueous phase using a separating funnel. EOs were then collected and dried over anhydrous sodium sulphate to remove water. Crude extracts were further processed in the rotary evaporator to remove excess solvents at their boiling range. Oil yield was calculated on a dry weight basis employing yield (%) formula.

$$Yield\ (\%) = \frac{W_{EO}}{W_I} \times 100$$

where W_{EO} is the weight of dry EOs, and W_I is the weight of fresh plant powder taken for extraction. Extracts were then stored in an airtight dark container at 4 °C until needed.

2.3. Gas chromatography-mass spectroscopy (GC- MS)

Gas chromatographic analysis was carried out using Agilent 7890N instrument equipped with a flame ionization detector and HP-5MS (30 m × 0.25 mm × 0.25 μm) capillary column, while the EOs components were identified on an Agilent Technologies Jeol mass spectrometer. The GC settings were as follows: The initial oven temperature was held at 60 °C for 1 min and ramped at 10 °C min^{-1} to 180 °C for 1 min, then ramped at 20 °C min^{-1} to 280 °C for 15 min. The injector temperature was maintained at 270 °C. The samples (1 μl) were injected neat, with a split ratio of 1:10. The carrier gas was helium at a flow rate of 1.0 ml min^{-1}. Spectra were scanned from 20 to 550 m/z at 2 scans s^{-1}. Most EOs constituents were identified by comparing their retention indices with those of the literature and previous studies. The retention indices were determined with respect to the homologous series of N-alkanes (C8–C24) under the same operating conditions. Further identification was made by comparing mass spectra on both columns with those stored in the NIST library or with mass spectra from literature. Component relative percentages were calculated based on GC peak areas without using correction factors.

2.4. Repellency by filter paper arena test

Repellency in insects was evaluated according to Cosimi et al. (2009), where pests were exposed to different concentrations of methanol and petroleum ether derived EOs. EOs were dissolved in acetone to make desired concentrations. Filter papers measuring 7 cm in diameter were cut into two halves. One half filter paper was treated with the desired concentration of EOs and another half with acetone. After drying for 2 min, both halves of filter paper were attached to the underside of the Petri plate using cellotape. Ten unsexed adults were released into the centre of the plate. Five duplicates were maintained for each concentration. Readings were taken at the interval of 1, 2, 3, 4, 5, 6, 12, and 24 h. Insects were transferred to plastic vials (25 ml) containing culture media and checked regularly for three days if any mortality is recorded. Raw data were converted to calculate percentage repellency using the following formula:

$$PR = 2(C - 50)$$

Where C is the percentage of insects recorded on the untreated half of the disc. Positive values expressed repellency and negative values denote attractancy. The result of PR was analysed using ANOVA and Tukey's pairwise comparison test.

2.5. Contact toxicity

To evaluate contact toxicity, following the method of Huang and Ho (1998), adults and larvae were treated with the desired concentrations of EOs. 10 unsexed adults or larvae were taken in a plastic vial (25 ml) and kept in the freezer for 1 min. This reduces the mobility of the pest and hence, their handling became easy. An aliquot of 5 μl of EOs was then topically applied to the mesothoracic region. After 2 min, they were transferred to the plastic vials containing culture media. The mortality was recorded for three days.

2.6. Fumigant toxicity

Fumigant toxicity was assessed as per the method suggested by López et al. (2008). One half of the filter paper (What man No.1; 7 cm in diameter) was treated with different concentrations of methanol and petroleum ether EOs, which ranged from 0.24 to 2.37 mg L^{-1} air and 0.14–1.42 mg L^{-1} air respectively. The other half of the paper was treated with acetone and used as control. Papers were air-dried for 2 min to evaporate the solvent. Impregnated papers were then sealed on the screw cap of the plastic vials (25 ml). Ten insects were released in the vial. Five sets were maintained for each concentration. Mortality was determined regularly for 3 day at an interval of 12 h. Loss of antennal and leg movement was taken as an indication for mortality.

Data obtained at the end of the third day was considered as final and processed further for statistical analysis. Probit analysis using Medcalc software was employed for analysing dose-mortality response in the acute toxicity assays. Percentage mortality would be calculated using Abbott's formula (Abbott, 1925) to correct natural mortality, if any, in the control group.

2.7. Biochemical analysis

Quantitative analyses of biochemical constituents in viable (LD_{50}, LD_{90}) and control sets were assessed. Protein profiling by Biuret method (Reckon Diagnostics Pvt. Ltd.) and enzymatic activities of Acetylcholinesterase (AChE), Glutathione S Transferase (GST), Reduced Glutathione (GSH) and Lipid Peroxidase (LPO) were performed following the methods of Ellman et al. (1961), Habig et al. (1974), Jollow et al. (1973) and Buege and Aust (1978) respectively. Analysis of Variance (ANOVA) and Tukey's Pairwise Comparison Test was employed using PAST statistical software package to compare means.

3. Results

3.1. Chemical composition of the essential oils

The oil yield of EOs of *A. annua* was found to be 27.16% and 1.36% w/w for methanol and petroleum ether, respectively. The gas chromatogram result has recorded a total of 13 compounds from the methanolic EOs, accounting for 99.98% of the total oil (Table 1). Major constituents identified in the oil were 1-Docosene (29.57%), I-Valine, N-Heptafluorobutyryl-, nonylester (22.99%), 3-Methylcyclopentadecylcarbamic acid, T- Butyl ester (12.12%) and 5α-Pregn-16-en-20-one, 3β,12α-dihydroxy-, diacetate (5.33%). The relative content of the remaining nine minor components was ranged from 1.69% to 4.67%.

Petroleum ether derived EOs was reported with 16 different compounds which account for 98.15% of the total oil (Table 2). Among the major chemical constituents, 3,4-Hexadienal, 2-butyl-2-ethyl-5-methyl- (22.06%), Deoxyqinghaosu (10.84%), Acetic acid,(1,2,3,4,5,6,7,8-octahydro-3,8,8-trimethylnaphth-2-yl)methylester (9.68), 2-Isopropyl-4,4,7a-trimethyl-2,4,5,6,7,7a-hexahydro-benzofuran-6-ol (7.84%), 1-Eicosanol (7.68%) and 2H-1-Benzopyran-2-one (7.03%) were enlisted. It is important to mention that Isoaromadendrene epoxide and Spiro (4,5)decan-7-one, 1,8-dimethyl-8,9-epoxy-4-isopropyl- existed in their stereoisomeric form and hence reported twice with two different retention time in the EOs.

3.2. Repellency test

Methanolic and petroleum ether EOs have demonstrated 91.63% and 95% repellency, respectively, at the highest concentration of 0.90 mg cm^{-2} (Table 3). Repellency was time and dose-dependent in both the treatment sets. However, an insignificant increase ($p < 0.01$) in repellency with the increase in concentration was seen.

3.3. Contact toxicity

No mortality was recorded in the control sets. Based on LD_{50} and LD_{90} values, *T. castaneum* adults and larvae were found more susceptible to petroleum ether EOs (Table 4). A comparison of two stages showed that the immatures were more vulnerable to the methanolic extract than the adults as no overlap in 95% confidence interval is seen. However, no significant ($p^{>}0.01$) difference between adults and larvae was found with the petroleum ether EOs (Overlap in 95% Confidence interval).

Table 1
Chemical composition of the essential oils of *A. annua* extracted with methanol.

Peak No.	Compounds	RI[a]	RI[b]	ID[c]	Relative content (%)[d]
1	Spiro(2,7)dec-4-ene,1,1,5,6,6,9,9-heptamethyl-10-methylene	1656	–	MS	1.76
2	Tricyclo(3.3.1.1.$^{<}$3,7$^{>}$)decane, tricyclo(3.3.1.1.$^{<}$3,7$^{>}$)decylidene	1825	–	MS	3.63
3	Deoxyqinghaosu	1794	–	MS	1.69
4	3,4-Hexadienal, 2-butyl-2-ethyl-5-methyl	–	–	MS	3.38
5	Phytol	2045	2104	MS, RI	3.05
6	I-Valine, n-heptafluorobutyryl-, nonylester	1807	1786	MS, RI	22.99
7	5β,7βH,10α-Eudesm-11-en-1α-ol	1651	–	MS	4.58
8	Nonadecane	1910	1900	MS, RI	3.72
9	3-Methylcyclopentadecylcarbamic acid, t- butyl ester	2600	–	MS	12.12
10	1-Docosene	2198	2188	MS, RI	29.57
11	Vitamin E (α tocopherol)	–	–	MS	3.49
12	5α-Pregn-16-en-20-one,3β,12α-dihydroxy-,diacetate	2732	–	MS	5.33
13	Squalene	2914	2828	MS, RI	4.67
Total					**99.98%**
	Grouped components (%)				
	Oxygenated Sesquiterpene				5.07
	Saturated Hydrocarbons				39.72
	Alcohols				7.63
	Vitamin				3.49
	Other Metabolites				44.07

[a] Retention indices were calculated using a homologous series of n-alkanes (C8–C24).
[b] Retention indices reported in previous studies.
[c] Identification of volatile components was carried out by comparing Mass spectrum (MS) and Retention indices (RI) of components with those of the authentic standards in the NIST library and previous study.
[d] Results obtained by peak-area normalization.

Table 2
Chemical composition of the essential oils of *Artemisia annua* extracted with Petroleum ether.

Peak No.	Compounds	RI[a]	RI[b]	ID[c]	Relative content (%)[d]
1	Oxirane, tetradecyl-	1702	–	MS	1.46
2	2H-1-Benzopyran-2-one	1374	1414	MS, RI	7.03
3	Caryophyllene oxide	1507	1576	MS, RI	2.95
4	Isoaromadendrene epoxide	1281	1590	MS, RI	5.32
5	Vitamin E (α tocopherol)	–	–	MS	3.36
6	Spiro(4,5)decan-7-one,1,8-dimethyl-8,9-epoxy-4-isopropyl-	1626	–	MS	4.73
7	Isoaromadendrene epoxide	1281	1590	MS,RI	4.35
8	Acetic acid,(1,2,3,4,5,6,7,8-octahydro-3,8,8-trimethylnaphth-2-yl)methylester	1763	–	MS	9.68
9	Spiro (4.5)decan-7-one,1,8-dimethyl-8,9-epoxy-4-isopropyl-	1626	–	MS	2.77
10	4-(1-Hydroperoxy-2,2-dimethyl-6-methylene-cyclohexyl)-pent-3-en-2-one	1835	1477	MS,RI	3.82
11	Deoxyqinghaosu	1794	–	MS	10.84
12	3,4-Hexadienal,2- butyl-2-ethyl-5-methyl-	–	–	MS	22.06
13	2-Isopropyl-4,4,7a-trimethyl-2,4,5,6,7,7a-hexahydro-benzofuran-6-ol	1604	–	MS	7.84
14	Heptacosane	2705	2700	MS, RI	1.15
15	Squalene	2914	2818	MS, RI	3.11
16	1-Eicosanol	2252	2276	MS, RI	7.68
	Total				**98.15%**
	Grouped components (%)				
	Oxygenated Sesquiterpene				42.57
	Saturated Hydrocarbons				4.26
	Alcohols				5.84
	Ethers				4.41
	Esters				16.71
	Vitamin				3.36
	Other Metabolites				19.16

[a] Retention indices were calculated using a homologous series of n-alkanes (C8–C24).
[b] Retention indices reported in previous studies.
[c] Identification of volatile components was carried out by comparing Mass spectrum (MS) and Retention indices (RI) of components with those of the authentic standards in the NIST library and previous study.
[d] Results obtained by peak-area normalization.

3.4. Fumigant toxicity

T. castaneum adults were unaffected to the fumigants of both the EOs compared to their larval counterpart (Overlap in 95% Confidence interval). When LD_{90} values were compared, test insects were found more vulnerable to the fumigants of petroleum ether EOs (Table 5). Control mortality was zero.

3.5. Biochemical analysis

3.5.1. Biomolecular profiling of contact toxicity (Table 6)

The protein concentration of the whole body homogenate of adults and larvae decreased significantly ($p<0.01$) from control to lethal sets. A significant difference was observed between LD_{50} and LD_{90} sets as well. AChE activity significantly ($p<0.01$) decreased from control sets to lethal doses. However, both the EOs depicted a slight and statistically insignificant ($p>0.01$) reduction in AChE level of adults between LD_{50} and LD_{90}. AChE level in the larval stage showed a significant ($p<0.01$) relationship between the control and LD_{90} group. GST and GSH level has reduced significantly ($p<0.01$) in the LD_{90} set compared to LD_{50} of both the life stages. However, an insignificant ($p>0.01$) reduction in the GST level was recorded in the adults between LD_{50} and LD_{90} sets. The level of LPO was found to increase significantly ($p<0.01$) from control to lethal sets.

3.5.2. Bio-molecular profiling of fumigant toxicity (Table 7)

The protein level was significantly ($p<0.01$) decreased in the lethal (LD_{50} and LD_{90}) sets. AChE activity inhibited significantly in the lethal groups when compared with the control. However, no significant reduction was seen between LD_{50} and LD_{90} sets. GST level in the larvae and adults treated with petroleum ether EOs has reported significant ($p<0.01$) decrease between control and treatment sets, whereas the

Table 3
Repellency of solvent derived essential oils of *A. annua* against *T. castaneum* adults using filter paper arena test.

Solvent used	Concentration (mg cm^{-2})	Duration of exposure (in) hour								Percent Repellency over 24 h
		1	2	3	4	5	6	12	24	
		Mean Repellency (% ± SD)								
Methanol	0.54	53 ± 12*	60 ± 20*	57 ± 29*	60*	60 ± 35*	53 ± 42*	80 ± 20*	73 ± 31*	**62.00**
	0.63	53 ± 12*	53 ± 31*	73 ± 23*	87 ± 12*	87 ± 23*	100*	100*	87 ± 12*	**80.00**
	0.72	53 ± 24*	73 ± 23*	80 ± 20*	83 ± 21*	100*	100*	100*	100*	**86.13**
	0.81	80 ± 20*	73 ± 31*	87 ± 12*	93 ± 12*	93 ± 11*	100*	100*	100*	**90.75**
	0.90	80 ± 20*	80 ± 20*	93 ± 12*	87 ± 12*	93 ± 11*	100*	100*	100*	**91.63**
Petroleum ether	0.54	80 ± 20*	80 ± 20*	93 ± 12*	93 ± 12*	87 ± 23*	87 ± 23*	93 ± 12*	93 ± 12*	**88.25**
	0.63	60 ± 20*	80*	67 ± 12*	80*	80*	80*	80*	80*	**82.125**
	0.72	87 ± 12*	87 ± 12*	87 ± 12*	93 ± 12*	93 ± 12*	93 ± 12*	93 ± 12*	93 ± 12*	**90.75**
	0.81	93 ± 12*	100*	100	93 ± 12*	93 ± 12*	100*	100*	100*	**97.375**
	0.90	100*	93 ± 12*	93 ± 12*	87 ± 23*	87 ± 23*	100*	100*	100*	**95**

Mean (± SEM) followed by * indicate no significant difference ($p < 0.01$) according to the ANOVA.

Table 4
Contact toxicity of essential oils of *A. annua* applied topically to *T. castaneum* at 30 °C and 70 ± 80% RH.

Plant Extract	Life stage	LD_{50} (mg adult^{-1}	95% Confidence interval	LD_{90} (mg adult^{-1}	95% Confidence interval	Slope ± SE	χ2 (DF)
Methanol	Adults	1.87	1.60–2.13	2.94	2.59–3.54	1.18 ± 0.2	51.735 (1)*
	14 days old Larvae	1.24	0.94–1.48	2.22	1.91–2.75	1.31 ± 0.24	50.562 (1)*
Petroleum ether	Adults	0.43	0.22–0.58	0.98	0.82–1.29	2.33 ± 0.49	37.148 (1)*
	14 days old Larvae	0.60	0.42–0.74	1.20	1.02–1.51	2.16 ± 0.40	40.821 (1)*

LD_{50} = Lethal dose that kills 50% of the test organisms.
LD_{90} = Lethal dose that kills 90% of the test organisms.
χ2 = chi square.
DF = degrees of freedom.
* = Significant (P ˂0.01).
Lethal values are expressed as mean of five replicates.

Table 5
Fumigant toxicity of essential oils of *A. annua* to *T. castaneum* exposed for 24 h at 30 °C and 70 ± 80% RH.

Extract	Life stage	LD_{50} (mg L^{-1} air)	95% Confidence interval	LD_{90} (mg L^{-1} air)	95% Confidence interval	Slope ± SE	χ2 (DF)
Methanol	Adult	1.64	1.44–1.89	2.51	2.19–3.10	1.49 ± 0.27	41.486 (1)*
	14 days old Larvae	1.35	1.16–1.55	2.14	1.88–2.58	1.63 ± 0.27	49.890 (1)*
Petroleum ether	Adult	0.81	0.69–0.93	1.28	1.13–1.55	2.72 ± 0.46	49.968 (1)*
	14 days old Larvae	0.65	0.53–0.77	1.13	0.99–1.39	2.68 ± 0.46	47.869 (1)*

LD_{50} = Lethal dose that kills 50% of the test organisms.
LD_{90} = Lethal dose that kills 90% of the test organisms.
χ2 = chi square.
DF = degrees of freedom.
* = Significant (P ˂0.01).
Lethal values are expressed as mean of five replicates.

Table 6
Effect of methanol and petroleum ether derived essential oils of *A. annua* on protein and enzymatic profile of *T. casteneum* subjected to contact toxicity bioassays.

Extracts	Life stages	Treatment	Protein (μg ml^{-1})	AChE (μmoles/min/ml of enzyme)	GST μmoles/min/ml of enzyme	GSH (μmoles/mg protein)	LPO (nmole of MDA/gm of tissue)
Methanol	Adult	Control	983 ± 4.58a	0.227 ± 0.015a	0.293 ± 0.051a	238.03 ± 0.252a	19.01 ± 0.662a
		LD_{50}	890 ± 3b	0.036 ± 0.002b	0.163 ± 0.025b	200.75 ± 0.511b	35.24 ± 0.655b
		LD_{90}	842 ± 5.57c	0.023 ± 0.003b	0.102 ± 0.016b	186.56 ± 0.225c	39.42 ± 0.659c
	Larva	Control	320 ± 4.51a	0.12 ± 0.021a	0.185 ± 0.005a	216.57 ± 0.461a	15.42 ± 0.445a
		LD_{50}	290 ± 4.58b	0.093 ± 0.004 ab	0.089 ± 0.005b	193.83 ± 0.207b	29.01 ± 0.355b
		LD_{90}	256 ± 6.51c	0.076 ± 0.004b	0.073 ± 0.005c	180.43 ± 0.184c	32.69 ± 0.752c
Petroleum ether	Adult	Control	956 ± 8.737a	0.23 ± 0.021a	0.30 ± 0.050a	236.32 ± 1.506a	19.01 ± 0.662a
		LD_{50}	875 ± 6.11b	0.031 ± 0.002b	0.102 ± 0.079b	198.19 ± 0.422b	36.583 ± 1.44b
		LD_{90}	835 ± 9.292c	0.022 ± 0.002b	0.093 ± 0.004b	184.59 ± 0.525c	41.273 ± 1.217c
	Larva	Control	320 ± 4.509a	0.123 ± 0.021a	0.185 ± 0.005a	216.28 ± 0.815a	14.653 ± 0.717a
		LD_{50}	282 ± 4.163b	0.085 ± 0.003 ab	0.089 ± 0.006b	190.55 ± 0.478b	30.68 ± 0.493b
		LD_{90}	246 ± 7.55c	0.058 ± 0.033b	0.073 ± 0.006c	177.933 ± 0.98c	32.833 ± 0.588c

Mean (± SEM) followed by the same letters indicate no significant difference ($p < 0.01$) between different treated groups (Control, LD_{50} & LD_{90}) according to the Tukey's test.

Table 7
Effect of methanol and petroleum ether derived essential oils of *A. annua* on protein and enzymatic profile of *T. casteneum* subjected to fumigant toxicity bioassay.

Extracts	Life stages	Treatment	Protein (μg ml^{-1})	AChE (μmoles/min/ml of enzyme)	GST μmoles/min/ml of enzyme	GSH (μmoles/mg protein)	LPO (nmole of MDA/gm of tissue)
Methanol	Adult	Control	975 ± 1a	0.253 ± 0.031a	0.257 ± 0.031a	235.73 ± 0.161a	18.31 ± 0.067a
		LD_{50}	724 ± 4.58b	0.029 ± 0.006b	0.128 ± 0.009b	195.6 ± 0.07b	36.09 ± 0.035b
		LD_{90}	671 ± 2.65c	0.017 ± 0.003b	0.078 ± 0.008c	178.81 ± 0.165c	39.92 ± 0.145c
	Larva	Control	302 ± 4.04a	0.153 ± 0.025a	0.18 ± 0.01a	210.21 ± 0.275a	14.65 ± 0.04a
		LD_{50}	236 ± 2.65b	0.085 ± 0.004b	0.09 ± 0.008b	201.5 ± 0.475b	30.82 ± 0.275b
		LD_{90}	202 ± 3.512c	0.073 ± 0.006b	0.07 ± 0.007b	187.11 ± 0.24c	35.83 ± 0.151c
Petroleum ether	Adult	Control	976 ± 0.864a	0.253 ± 0.031a	0.262 ± 0.03a	235.81 ± 0.227a	17.75 ± 0.694a
		LD_{50}	718 ± 1.035b	0.018 ± 0.002b	0.099 ± 0.01b	193 ± 0.21b	38.167 ± 0.666b
		LD_{90}	664 ± 1.002c	0.017 ± 0.002b	0.076 ± 0.005b	175.33 ± 0.534c	43.513 ± 0.520c
	Larva	Control	300 ± 2.081a	0.157 ± 0.031a	0.179 ± 0.01a	209.53 ± 1.038a	13.543 ± 1.06a
		LD_{50}	279 ± 1.012b	0.073 ± 0.003b	0.082 ± 0.008b	197.993 ± 0.99b	31.233 ± 0.586b
		LD_{90}	196 ± 0.946c	0.06 ± 0.003b	0.077 ± 0.004b	186.59 ± 0.525c	35.667 ± 0.586c

Mean (± SEM) followed by the same letters indicate no significant difference ($p < 0.01$) between different treated groups (Control, LD_{50} & LD_{90}) according to the Tukey's test.

reduction is insignificant between LD_{50} and LD_{90} groups. However, GSH showed significant ($p^{<}0.01$) reduction between all the three groups of adults treated with methanolic EOs. LPO level has significantly ($p^{<}0.01$) increased in LD_{90} from LD_{50}.

4. Discussion

The primary focus of the research was to decipher the influence of solvents in drawing potent chemical groups from the plant species. In this study, both the EOs of *A. annua* has demonstrated acute toxicity against *T. casteneum* adults and larvae. However, the degree of lethality varied with time, concentration, and solvent used to elute EOs. The contact toxicity studies have shown that the larvae were more susceptible than the adults to the methanolic EOs. However, adults were comparatively more susceptible to the petroleum ether EOs. While comparing the two EOs, adults, as well as larvae, were comparatively more susceptible to the petroleum ether EOs. In a similar study, the toxicity of n-hexane derived EOs of two species of *Aloysia* viz. *A. citriodora* and *A. polystachya* against *Tribolium casteneum* and *Tribolium confusum* was evaluated (Benzi et al., 2014). The study has shown the efficacy of non-polar solvent in eluting potent insecticidal components which could effectively control the pests. However, *T. casteneum* was found more tolerant of the EOs of *Aloysia* sp. While comparing the lethal values, it is found that a higher concentration of non-polar solvent derived EOs was required to pose a lethal effect in our study. The outcome can be justified by the fact of resistance development in the pest due to its continuous exposure to the synthetic fumigants. The study is supported by the outcome of Cao et al. (2018) where high LC_{50} values of β-Caryophyllene (41.7 μg cm^{-2}) point towards a resistant strain.

Testing of EOs as a fumigant against the beetle produced positive Results. Comparing the response of the adult beetle revealed that the EOs derived with petroleum ether had a profound effect as depicted in LD_{50} values. The same was true for the larval stage as well. Our result differs from the work of Negahban et al. (2007) where *Artemisia sieberi* has shown a lethal effect (LD_{90}) at a very low dose of 57.32 μL/l air. The work of Boyer et al. (2012) and Jagadeesan et al. (2012) have demonstrated fast-growing resistance in the pest, which could be a probable reason for the high dose which was required to display lethal effects.

Results of the present study have verified petroleum ether derived EOs as a better insecticidal candidate for the control of *T. casteneum* and have added new dimensions to the previous findings (Tripathi et al., 2000). The success might be due to the presence of novel compounds like 3,4-Hexadienal, 2-butyl-2-ethyl-5-methyl- (22.06%), Deoxyqinghaosu (10.84%) in considerably higher percentage. Moreover, a lesser amount of compounds like Caryophyllene oxide and squalene, well known for their antimicrobial and anticancer properties, were also detected in both the extracts (Bui et al., 2014; Falowo et al., 2019). However, further research is needed to claim a possible synergistic effect of different constituents present in petroleum ether EOs (Tak et al., 2016).

Studies on *A. Annua* EOs have deciphered the presence of volatile groups like sesquiterpene, coumarins, phenolic compounds, and flavones (Bora and Sharma, 2011). Among all, 1, 8- Cineole has gained considerable attention for being the core component of EOs which was responsible for insecticidal properties (Durden et al., 2011). The presence of oxygenated sesquiterpene, hydrocarbons, alcohols, vitamins, and diverse chemical groups in both the EOs was deciphered. Deoxyqinghaosu, 3, 4-Hexadienal, 2-butyl-2-ethyl-5-methyl, and Squalene were reported in both the EOs with different percentage composition, and hence, we suggest their possible action in insecticidal activities (Chauhan et al., 2015). Moreover, additional components like esters (16.71%) and ethers (4.41%) in the petroleum ether EOs was a significant finding of our work. Their presence indicated the possibility of a synergistic effect for the higher insecticidal potential of the EOs. The Results are supported by various scientists who portrayed EOs as the best management tools against stored grains pests (Sarwar and Salman, 2015; Bett et al., 2016).

Further, we examined the effect of both the EOs on the bio-molecular profile of *T. casteneum* adults and larvae to gain an insight into the extent of metabolic disturbances inflicted in the treatment sets. Our experimental evidence has indicated the action of EOs on the nervous system of *T. casteneum.* They worked by disturbing the normal course of action of different detoxifying enzymes like AChE, GST, and LPO in the treated sets. While biomolecular profiling of LD_{50} and LD_{90} strains was conducted, the level of AChE, GST, and GSH were found to decrease, whereas the LPO level has increased. Scientists working in the same trail showed similar Results where Coumaran has inhibited the activity of AChE (Rajashekar et al., 2014). Another set of experiments has depicted contradictory results with increased activity of GST in the multi-resistant strains of *T. casteneum* (Cohen, 1986). Moreover, the protein level has demonstrated a significant decrease in the lethal sets as compared to the control group. The results are supported by the work of Koodalingam et al. (2011). LPO level in the study increased significantly in the treatment sets thereby increasing the oxidative stress in the pest. Our findings are consistent with the result of Hasspieler et al. (1990) who emphasised on choosing a non-resistant strain for the study. The result dictates that the *Tribolium* strain used for toxicity assays was not resistant towards the *A. annua* EOs.

In conclusion, the present study has validated the insecticidal potential of petroleum ether derived EOs of *A. annua.* Moreover, the identified allelochemicals of EOs are potent in vivo suppressor of life-supporting biomolecules in the acute toxicity assays. Hence, there is potential for these compounds to be used in synergy to interfere with the enzyme-mediated detoxification process in the target insects. Additionally, by probing the modes of action of fumigants through experiments, future research will evaluate that if the enzyme inhibitors can act as synergists by employing potent non-polar solvents to elute EOs from *A. annua* to increase toxicity against other stored grain pests.

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Declaration of competing interest

Authors declare no conflict of interest.

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Chemical Composition and Bioactivity of the Essential Oils Derived from *Artemisia annua* against The Red Flour Beetle

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Triboliumcasteneum **is the mostresistant stored grainspest causingmore than 40% grains loss per year.Replacement of the conventional fumigants with an eco-friendly alternative seemed an intelligent move to control the pest which has inclined global research towards the efficacy of pesticidal plants. In the race of finding a better insecticidal candidate, we focused on to the chemical compositionof the essential oils (EO) derived with polar and non-polar solvents from *Artemisia annua*and their possible bioactivity against the pest species. GC-MS analyses of Chloroform and n-Hexane derived EOs showed the dominance of Oxygenated Sesquiterpenein the extract. Adults were found more vulnerable to n-Hexane EO (LD_{50}= 0.71 mg adultÉ^{1}) than to chloroform derived EO (LD_{50}= 0.97mg adultÉ^{1}) in contact toxicity assays. In the fumigant bioassayboth the adults and larvae were found susceptible towardsn-Hexane EO with LD_{50} 0.66 & 0.53 mg L airÉ^{1} respectively.Evaluation of the biomolecular profile of adults and larvae at their lethal doses to understand the molecular mechanism underlying oxidative stress** has shown significant downfall (pÂ0.01) in the activities of protein, AChE, GST, GSH whereasup regulation of LPOwas distinctly marked. The basic knowledge of employing potential solvent in eluting EOs of ***A.annua*** **would prove to be an efficient environmental friendly management tool against *T.casteneum*.**

Keywords: *Artemisia annua*, Biomolecules, Contact toxicity, Essential oils, Fumigant toxicity,*Triboliumcasteneum*.

Global demand for food is increasing continuously due to overshooting population. Thisposes great challenge to the sustainable utilization of stored grains which accounts for about more than 70% of their total yield. Unfortunately, stored conditions are a major attraction to different types of infestation mainly by insects due to infinite food resources and favourable abiotic factors (Howe, 1943).*Triboliumcasteneum* (Herbst, 1797) is the most resistant species among the huge list of stored grains pests and known to exploit a wide range of stored products (Hagstrum, 2017). They aretestified as the primary pest of wheat flour & other milled products and secondary pest of wheat grains (Good, 1933). Studies reported that more than 40% quantitative and qualitativedevastation of wheat ûour is caused by the beetle (Ajayi&Rahman, 2006). The flour beetle is also known to secrete toxic quinones which turns the flour greyish and hence reduces its aesthetic and nutritive values (Ladisch et al., 1967). Moreover, carcinogenic attribute of the quinones areaffirmed thus poses

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serious risk to human health (El-Mofty et al., 1989). Hence controlling the pest is of utmost importance.

Fumigation through methyl bromide and phosphine is the most dominant measurepracticed in most of the countries to manage stored product pest (Bell, 2000). However, methyl bromide is banned worldwide due to its association in ozone layer depletion (Anbar et al., 1996). Use of phosphine has already triggered much negative impactdue to their fast growing resistance (Benhalimaet al., 2004). Replacement of these conventional fumigants with an eco-friendly alternative seemed an intelligent move to control the pest which has inclined global research towards the efficacy of pesticidal plants. In the race of finding a better insecticidal candidate, different variants of plants are tested andfortunately emerged as a potent solution towards the challenge (Okwute, 2012; Chaudhary et al., 2017).Plants are known to contain a wide range of essential oils (Sasidharan et al., 2011). The Essential Oils (EO) contain plethora of organic compounds which are relatively non-toxic for the environment and can be used as a potent alternative for the synthetic pesticides (Isman, 2000; Caballero-Gallardo et al., 2011).The views were also supported by previous studies whereEOsof *Ricinuscommunis* seeds and *Daturastramonium* extracts were found effectivein controlling the red flour beetle(Abbasipour et al., 2011; Babarinde et al., 2011).

Genus *Artemisia* is the most widely distributed genera of Asteraceae family (Chu et al., 2012) and is extensively used for its medicinal properties in Asian countries (Das, 2012). *Artemisia* and its EOsare the subject of research interest since decades which is reflected in the wide range of studies conducted across the globe (Bora & Sharma, 2011).The genus was reported to pose toxic effect against pathogens and can be used in human diets and animal fodder (Janssen et al., 1987). It was also testified to possess insecticidal and antifeedant activities (Liu et al., 2006; Gonzalez-Coloma et al., 2012). EOs from the *Artemisia sieberi*Besser has shown insecticidal properties against three stored grain pest (Negahban et al., 2007). Moreover, EOs from *Artemisia princeps*are described to be an effective repellent and insecticidal candidate against two major stored grain pests (Liu et al., 2006).

Artemisia annua, the sole producer of Artemisinin, is mainly studied for its efficacy against the malarial parasites, *Plasmodium* (Dhingra et al., 1999). Artemisinin-based combination therapy is emerged as the most efficient antimalarial drug available in the market against MDR strains (Klayman, 1985; Eastman &Fidock, 2009). However, to the best of our knowledge, studies focusing on the candidature of potent solvents for the extraction of EO from *A. annua*are still unknown.Taking into account the serious infestation caused by *Tribolium* which eventually pose negative impact on human health and on country's economy, the very need for the study was sensed. Hence, authors tried to decipher the chemical compositionof the essential oils (EO)eluted with Chloroform (polar) and n-hexane (non-polar) solvents from *Artemisia annua*and testified their possible bioactivity against *Triboliumcasteneum*. In the present study, fumigant and contact toxicity of *A. annua* EOsagainst the pest was evaluated. EO obtained by hydro-distillation was analysed through Gas-chromatography to identify major chemical constituents. Additionally, metabolic interference imposed in the treatment sets of *T. casteneum* (Herbst, 1797) was evaluated.

Experimental methods

Insect

Tribolium.casteneum(Herbst, 1797) were collected from a small culture maintained in thedivision of Entomology of the Department of Zoology, The Maharaja Sayajirao University of BarodaVadodara, Gujarat, India. Unsexed adults werereared in the defined culture media of wheat flour, wheat grain and yeast in the ratio of 6:3:1. Insects were maintained in the humidity chamber at the suitable temperature and humidity ranges of 27±2ÚC, 70±5 RH respectively. Newly emerged adults of 1-10 days old, were used for the toxicity assays. Final larval stages i.e. 14 days old larvae were used in the experiment. All the experiments were conducted in the dark under the same temperature and humidity ranges.

Plant material

The dried, finely grounded leaves of the *A.annua*were procured fromProfessor Neeta Pandya,Department of Botany, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India. Plant powder was then stored in plastic bags at 4ÚC until used for the extraction of EOs.

Extraction of essential oils

Dried plant powder (25 grams) was

subjected to hydro-distillationusing a modified Clevenger-type apparatusfor the extraction of EOs(Clevenger, 1928). Onset of distillation was marked with the boiling of the selected solvents contained in the round bottom flask. 300 mL of both Chloroform and n-Hexane were used separately for 9& 2 hours respectively for the extraction of EOs. Distillation process continued till the solvent becomes transparent in the extraction chamber.The oil layer was then separated from the aqueous phase using a separating funnel. EOs were then collected and dried over anhydrous sodium sulphate to remove extra water. Crude extracts were further processed inrotary evaporator to remove extra solvents at their boiling ranges. Oil yield was calculated on a dry weight basis employing the Yield (%) formula.

$$\text{Yield (\%)} = \frac{W_{EO}}{W_I} \times 100$$

Where, W_{EO} is the weight of dry EO and W_I is the weight of fresh plant powder taken for extraction. Extracts were then stored in the airtight containers in a refrigerator at 4 ÚC until it is used.

Chemical analysis of EO- Gas Chromatography-mass spectroscopy (GC- HRMS)

Gas chromatographic analysis was performed on an Agilent 7890N instrument equipped with a flame ionization detector and HP-5MS (30m × 0.25mm × 0.25ìm) capillary column, while the EO components were identified on an Agilent Technologies Jeol mass spectrometer. The GC settings were as follows: the initial oven temperature was held at 60 °C for 1 min and ramped at 10 °C min”1 to 180 °C for 1 min, and then ramped at 20 °C min”1 to 280 °C for 15 min. The injector temperature was maintained at 270 °C. The samples (1 ìL) were injected neat, with a split ratio of 1:10. The carrier gas was helium at flow rate of 1.0 mL min”1. Spectra were scanned from 20 to 550 m/z at 2 scans s-1. Most constituents were identified using gas chromatography by comparing their retention indices with those of the literature and previous studies. The retention indices were determined in relation to a homologous series of n-alkanes (C8–C24) under the same operating conditions. Further identification was made by comparison of their mass spectra on both columns with those stored in NIST library or with mass spectra from literature. Component relative percentages were calculated based on GC peak areas without using correction factors.

Repellency test

Repellency in insects was evaluated according to Cosimi et al. (2009) where pests were exposed to different concentrations of chloroformand n-hexane derived EOs dissolved in acetone. Filter papers measuring 7 cm in diameter were cut into two equal halves where one half was treated with the desired concentration of EO and other half with acetone. After drying for two minutes, both the halves of filter paper were attached underside with the cellotape and fixed to the petriplate. 10 unsexed adults were released into the centre of the plate. Five replicates were maintained for each concentration. Readings were taken at the interval of 1, 2, 3, 4, 5, 6, 12 and 24 hours. Insects were then transferred to the plastic vials containing media and checked regularly for 3 days if any mortality is recoded. Raw data was converted to calculate percentage repellency using the following formula:

$$PR = 2(C - 50)$$

Where C is the percentage of insects recorded on the untreated half of the disc. Positive values expressed repellency and negative values attractancy. Results of PR were analysed using ANOVA and Tukey’s pairwise comparison test.

Contact toxicity

To evaluate contact toxicity following the method of Huang et al. (1998), adults and larvae were treated with the desired concentrations of chloroform and n-hexane derived EOs. 10 unsexed adults were taken in plastic vials and kept in the freezer for one minute. This made them unconscious and hence their handling became easy. An aliquot of 5µl of EO was then topically applied on the meso-thoracic region. After 2 minutes they were transferred in the plastic vials containing media. The mortality was recoded till 3 days at the interval of 12 hours.

Fumigant toxicity

Fumigant toxicity was assessed according to López et al. (2008). Filter papers (Whatman No.1, 7 cm in diameter) were impregnated with the different concentrations of chloroform and n-hexane derived EOs. Insects were exposed to concentrations ranged from 0.24 to 2.37 mg L airÉ^{1} of polar solvent and 0.14 to 1.42 mg L airÉ^{1}of non-polar solvent. Concentrations were decided after

standardisation of the process. The filter papers were allowed to air dry for 2 minutes post-treatment to evaporate the solvent. Impregnated paper was then sealed on the screw cap of the plastic vials (25mL). 10 unsexed adults and larvae were tested separatelyfor each concentration. Five replicates were maintained for each concentration. Mortality was determined regularly for 3 days at the interval

Table 1. Chemical composition of the essential oils of *A.annua* extracted with chloroform

Sr. No.	Compounds	RI[a]	RI[b]	ID[c]	Relative content (%)[d]
1	2- Pyrrolidinone, 1-methyl-	920	1012	MS, RI	4.89
2	Bicyclo(2.2.1)heptan-2-one,1,7,7, trimethyl-	1121	1136	MS, RI	15.35
3	1- Chloroundecane	1340	1358	MS, RI	1.16
4	2H-1-Benzopyran-2-one, 3,4-dihydro	1392	1350	MS, RI	0.9
5	Bicyclo(7.2.0) undec-4- ene 4,11,11- trimethyl -8- methylene-	1494	1396	MS, RI	3.46
6	2H-1-Benzopyran-2-one	1374	1414	MS, RI	8.2
7	1,6-Cyclodecadiene, 1-methyl-5-methylene-8-(1-methyethyl)-	1515	1480	MS, RI	3.53
8	Phenol,2,4-bis(1,1-dimethyethyl)-	1555	1539	MS, RI	1.42
9	2-Undecanethiol,2-methyl-	1433	1410	MS, RI	3.24
10	Caryophyllene oxide	1507	1576	MS, RI	3.13
11	2-(4a,8-Dimethyl-1,2,3,4,4a,5,6,7-octahydronaphthalen-2-yl)-prop-2-en-1-ol	1745	1732	MS, RI	6.01
12	Oxalic acid, allylhexadecyl ester	2433	-	MS	2.87
13	Pulegone	1212	-	MS	2.51
14	1-Ethyl-3-vinyl-adamantane	1216	-	MS, RI	5.55
15	Oxalic acid, allylhexadecyl ester	2433	1514	MS	2.16
16	7-Hydroxy, 6- methoxy- 2H-1-benzopyran-2-one	1784	1924	MS, RI	2.61
17	Deoxyqinghaosu	1794	-	MS	4.27
18	3,4-Hexadienal,2- butyl-2-ethyl-5-methyl	-	-	MS	10.26
19	Phytol	2045	2104	MS, RI	4.85
20	Oxirane(Tetradecyloxy)methyl-	1877	-	MS	1.45
21	1,4-Methanoazulene-9-methanol,decahydro-4,8,8-trimethyl-	1635	1712	MS, RI	2.11
22	Didodecyldimethylammonium	-	-	MS	1.22
23	1,3-Dimethyl-5-3(2-methoxycarbonyl-2 Acetamidoethyl)-1H-indol-2-yl 1-yl uracil	3566	-	MS	3.02
24	Squalene	2914	2818	MS, RI	2.92
	Total	97.11			
Grouped components (%)					
	Oxygenated Sesquiterpene	20.08			
	Ketones	17.86			
	Esters	14.13			
	Alcohols	12.97			
	Saturated Hydrocarbons	9.91			
	Ethers	4.58			
	Phenol	1.42			
	Other Metabolites	16.14			

[a]Retentionindiceswerecalculatedusingahomologousseriesofn-alkanes(C8–C24).
[b]Identification of volatile components was carried out by comparing Mass spectrum (MS) and Retention indices (RI) of components with those of the authentic standards in NISTlibraryandpreviousstudy.
[c]Resultsobtainedbypeak-areanormalization

of 12 hours. Loss of antennal and leg movement was considered as an indication for mortality.

In the toxicity assays, data obtained at the end of the third day was considered as final and processed further for statistical analysis. Probit analysis (Finney, 1971) using Medcalc software was employed in analysing the dosage- mortality response in both the acute toxicity assays.

Biochemical analysis

Quantitative analyses of biochemical constituents in treated (LC_{50}, LC_{90}) and control sets were assessed. Protein profiling by Biurate kit method (HiMedia Laboratories Pvt. Limited) and enzymatic activities of Acetylcholinesterase (AChE), Glutathione S Transferase (GST), Reduced glutathione (GSH) and Lipid Peroxidases (LPO) were performed following the methods of Ellman et al. (1961), Habig et al. (1974), Jollow et al. (1973) and Buege&Aust (1978) respectively. Analysis of Variance (ANOVA) and Tukey's

Table 2. Chemical composition of the essential oils of *Artemisia annua* extracted with n-hexane

Sr. No.	Compounds	RI[a]	RI[b]	ID[c]	Relative content (%)[d]
1	Bicyclo(2.2.1)heptan-2-one,7,7-trimethyl-	1121	1146	MS, RI	7.2
2	2H -1- Benzopyran- 2- one	1374	1414	MS, RI	7.09
3	2-Isopropenyl-4±,8-dimethyl-1,2,3,4,4a,5,6,7-octahydronaphthalene	1502	1473	MS, RI	3.22
4	Caryophyllene oxide	1507	1576	MS, RI	3.64
5	Isoaromadendrene epoxide	1281	1590	MS, RI	5.59
6	Globulol	1530	1578	MS, RI	3.05
7	3-Cyclohexane-1-carboxaldehyde,1,3,4-trimethyl	1204	1171	MS	3.67
8	4,4-Dimethyladamantan-2-ol	1203	-	MS	7.37
9	2-Propen-1-ol,3-(2,6,6-trimethyl-1-cyclohexane-1-yl)-	1465	-	MS	2.19
10	Cedran-diol, 8S,13-	1786	-	MS	8.29
11	n-Hexadecanoic acid	1968	1942	MS, RI	5.23
12	4,8a-Dimethyl-6-(2-methyl-oxiran-2-yl)-4a, 5,6,7,8,8a-hexahydro-1H-naphthalene-2-one	1742	-	MS	1.49
13	Deoxyqinghaosu	1794	-	MS	6.39
14	3,4- Hexadienal,2- butyl-2-ethyl-5-methyl-2	-	-	MS	20.98
15	Z,Z-5,16-Octadecadien-1-ol acetate	2193	-	MS	1.67
16	11,14,15,16- Tetraoxatetracyclo(10.3.1.0 (4,13). 0(8,13))hexadecane-10-one,1,5,9-trimethyl	1903	-	MS	1.57
17	1,4-Methanoazulene-9-methanol,decahydro-4,8,8-trimethyl-	1635	1712	MS, RI	6.03
18	Squalene	2914	2818	MS, RI	5.34
	Total	100.00%			
Grouped components (%)					
	Oxygenated Sesquiterpene	36.02			
	Alcohols	19.56			
	Esters	8.76			
	Saturated Hydrocarbons	8.56			
	Ketones	7.20			
	Carboxylic acids	5.23			
	Aldehydes	3.67			
	Ethers	3.64			
	Other Metabolite	7.35			

[a]Retention indices were calculated using a homologous series of n-alkanes (C8–C24).
[b]Retention indices reported in previous studies
[c]Identification of volatile components was carried out by comparing MS spectrum and RIs of components with those of the authentic standards in NIST 5 library and previous study.
[d]Results obtained by peak-area normalization.

Table 3. Repellency of solvent derived essential oils of *A. annua* against *T. castaneum* adults using Filter paper arena test

Solvent used	Conc. (mg cmÉ²)	Duration of exposure (in) hour Mean Repellency (% ± SD) 1	2	3	4	5	6	12	24	Percent Repellency over 24 hours
Chloroform	0.54	67±31*	26±31*	33±12*	40±35*	53±23*	80±20*	80±20*	93±12*	59
	0.63	40±35*	80±20*	73±31*	13±46*	53±12*	80±20*	93±11*	93±12*	65.63
	0.72	100*	60±35*	87±23*	67±23*	80±20*	80±20*	93±11*	93±12*	82.5
	0.81	80±20*	100*	93±12*	73±23*	100*	80±20*	100*	100*	90.75
	0.90	93±11*	67±24*	87±23*	73±31*	93±12*	93±12*	100*	100*	88.25
n-hexane	0.54	60*	67±12*	60±20*	87±12*	80±20*	60±20*	80±20*	87±11*	72.625
	0.63	80±20*	87±23*	80±20*	87±12*	87±12*	93±12*	93±12*	80±20*	85.875
	0.72	60±20*	93±12*	87±23*	87±23*	93±12*	87±12*	100*	93±11*	87.5
	0.81	87±12*	93±12*	100*	100*	93±12*	100*	100*	100*	96.625
	0.90	87±23*	87±23*	100*	87±23*	87±23*	100*	100*	100*	93.5

Means (±SEM) followed by * indicate no significant difference ($p < 0.01$) according to the ANOVA.

Table 4. Contact toxicity of essential oils of *Artemisia annua*applied topically to *Triboliumcastaneum* at 30°C and 70±80% r.h

Plant Extract	Life stage	LD_{50} (mg adult-^{1})	95% Confidence interval	LD_{90} (mg adult-^{1})	95% Confidence interval	Slope ± SE	$\chi 2$ (DF)
Chloroform	Adults	0.97	0.48-1.29	2.30	1.93-3.02	0.96±0.2	32.174(1)*
	14 days old Larvae	1.57	1.26-1.86	2.81	2.43-3.48	1.04±0.18	42.870(1)*
n-hexane	Adults	0.71	0.57-0.83	1.19	1.04-1.46	2.60±0.44	54.373(1)*
	14 days old Larvae	0.47	0.25-0.61	1.04	0.87-1.35	2.24±0.46	37.06(1)*

LD_{50}= Lethal dose that kills 50% of the test organisms.
LD_{90}= Lethal dose that kills 90% of the test organisms.
$\chi 2$= chi square.
DF=degrees of freedom.
*= Signiûcant (P <0.01)
Lethal values are expressed as mean of ûve replicates.

Table 5. Fumigant toxicity of essential oils of Artemisia annuato *Triboliumcastaneum* exposed for 24 h at 30?C and 70±80% r.h

Plant Extract	Life stage	LD_{50} (mg adult-^{1})	95% Confidence interval	LD_{90} (mg adult-^{1})	95% Confidence interval	Slope ± SE	$\chi 2$ (DF)
Chloroform	Adult	1.17	0.93-1.38	2.13	1.83-2.68	1.33±0.24	37.944(1)*
	14 days old Larvae	0.98	0.71-1.20	1.94	1. 65-2.46	1.34±0.25	36.638(1)*
n-hexane	Adult	0.66	0.52-0.77	1.17	1.01-1.45	2.48±0.43	43.757(1)*
	14 days old Larvae	0.53	0.37-0.65	1.04	0.89-1.31	2.50±0.47	40.603(1)*

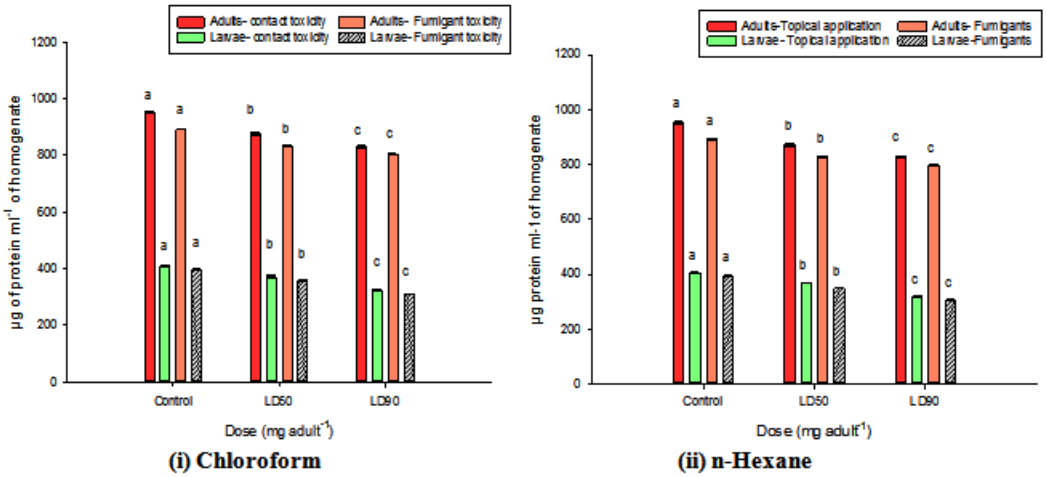
LD50= Lethal dose that kills 50% of the test organisms.
LD90= Lethal dose that kills 90% of the test organisms.
?2= chi square.
DF=degrees of freedom.
*= Signi?cant (P ?0.01)
Lethal values are expressed as mean of ?ve replicates.

Pairwise Comparison Test were employed using Sigma plot 13.0 statistical software package to compare means.

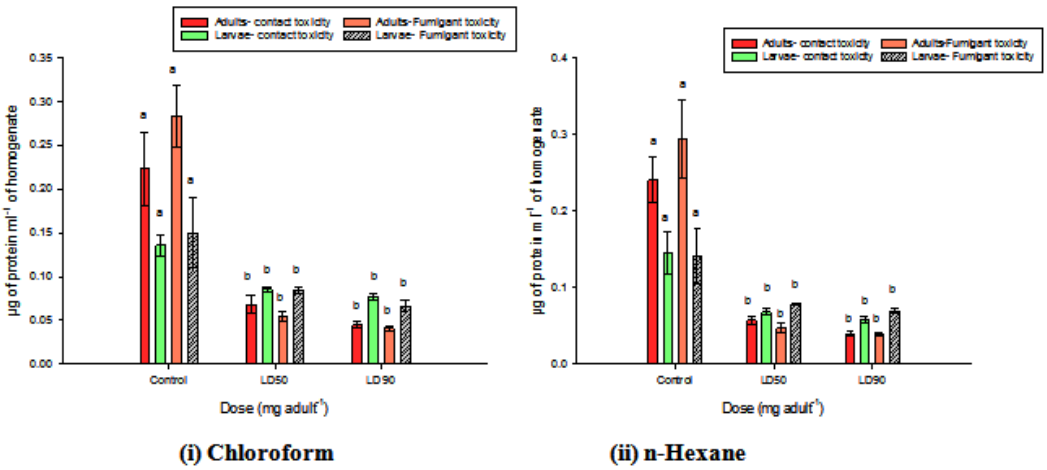
RESULTS

Chemical composition of the essential oils

The average oil yield from the solvent derived EOs of *A.annua* was found to be 19.28% and 3.68% w/w for chloroform and n-Hexane respectively.Results of GC-MS showed the qualitative and quantitative composition of EOs of *A. annua* in Table 1 and 2. Major peaks determined in the solvent extracted EOs of *A. annua*were marked serially along with their retention indices and % relative composition according to the order of their elution. Twenty four major compounds were identified in the chloroform EO accounted for 97.11% of the total oil (Table 1). Among the major chemical constituents, Bicyclo(2.2.1)heptan-



Graph 1. (i & ii) Protein activity (Mean±SE) in *T. casteneum* exposed to solvent derived EOs of *A. annua* at lethal doses. Columns marked with different letters are significantly different (PÂ0.01; ANOVA and Tukey's honest post-hoc test)

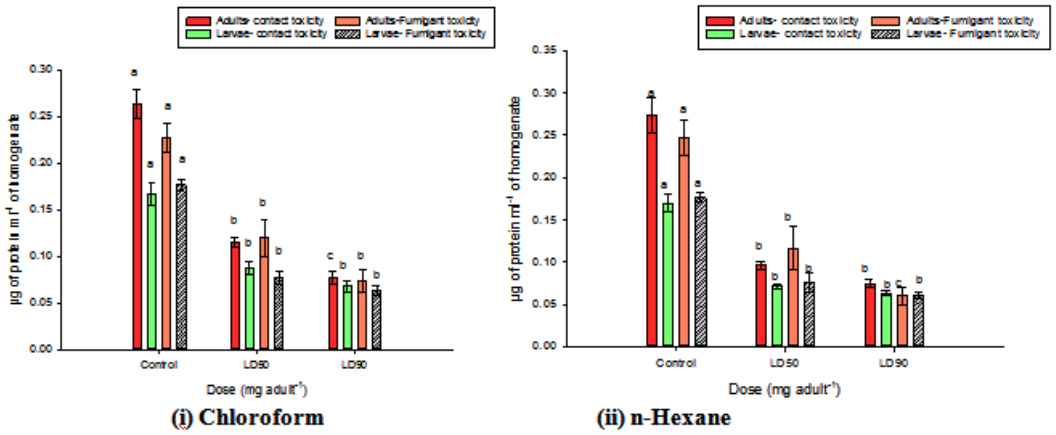


Graph 2. (i & ii) AChE activity (Mean±SE) in *T. casteneum* exposed to solvent derived EOs of *A. annua* at lethal doses. Columns marked with different letters are significantly different (PÂ0.01; ANOVA and Tukey's honest post-hoc test)

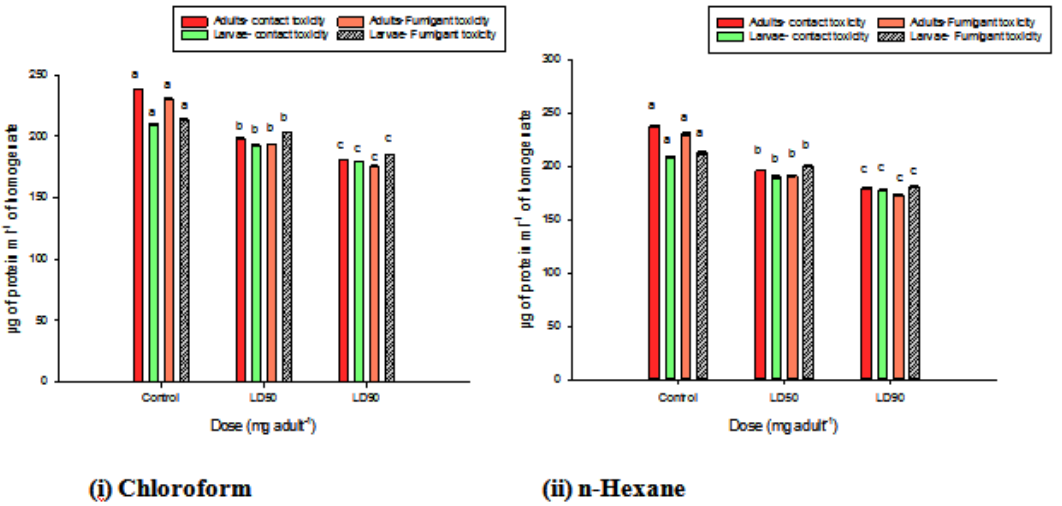
2-one,1,7,7, trimethyl- (15.35%) 3,4-Hexadienal, 2-butyl-2-ethyl-5-methyl- (10.26%), 2H-1-Benzopyran-2-one (8.20%), 2-(4a,8-Dimethyl-1,2,3,4,4a,5,6,7-octahydronaphthalen-2-yl)-prop-2-en-1-ol (6.01%)and1-Ethyl-3-vinyl-adamantane (5.55%) were enlisted. It is important to mention that Oxalic acid,allylhexadecyl ester was existed in their stereoisomeric form hence reported with two different retention time in the analysis.

On the other hand, chemical composition of the n-Hexane eluted EO was recorded to containeighteen different compounds accounting for 100% of the total oil (Table 2). The major constituents as depicted in the Figure 2 were identified as 3,4-Hexadienal,2- butyl-2-ethyl-5-methyl-2 (20.98%), Cedran-diol, 8S,13- (8.29%), 4,4-Dimethyladamantan-2-ol (7.37%), Bicyclo(2.2.1)heptan-2-one,1,7,7-trimethyl- (7.20), 2H -1- Benzopyran- 2- one (7.09), Deoxyqinghaosu (6.39%), 1,4-Methanoazulene-9-methanol,decahydro-4,8,8-trimethyl- (6.03).

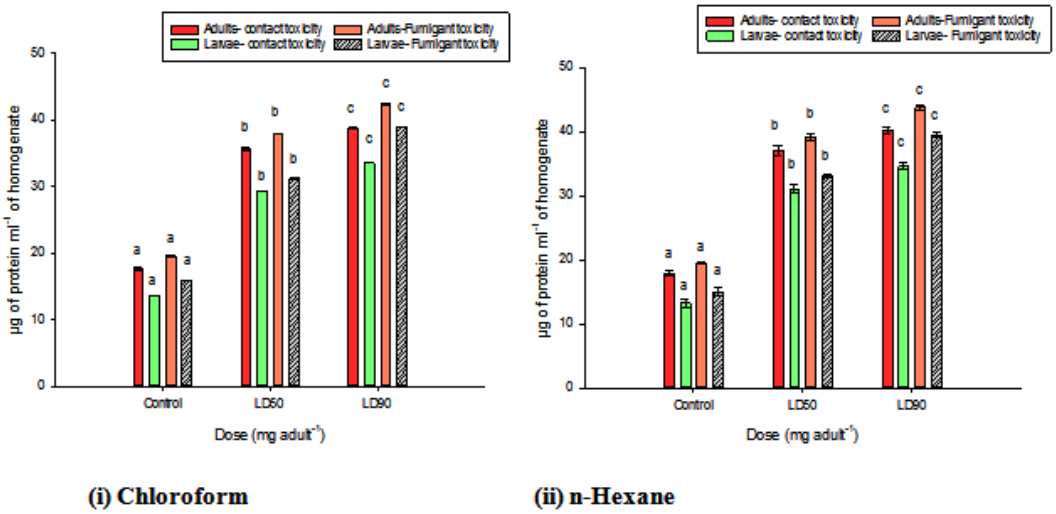
In both the EOs, higher % of Oxygenated Sesquiterpene was recorded with 20.08% and 36.02% in case of chloroform and n-Hexane



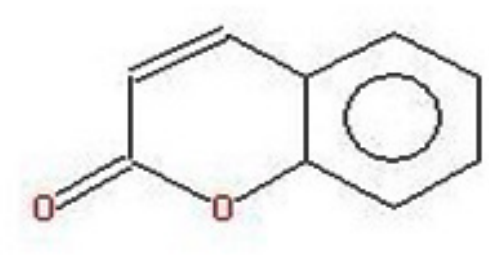
Graph 3. (i & ii) GST activity (Mean±SE) in *T. casteneum* exposed to solvent derived EOs of *A. annua* at lethal doses. Columns marked with different letters are significantly different (PÂ0.01; ANOVA and Tukey's honest post-hoc test)



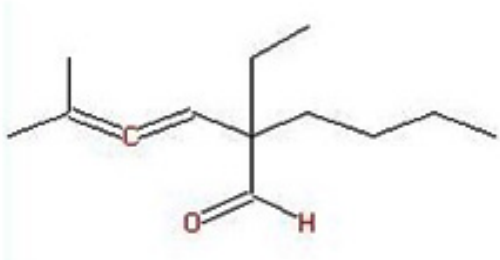
Graph 4. (i & ii) GSH activity (Mean±SE) in *T. casteneum* exposed to solvent derived EOs of *A. annua* at lethal doses. Columns marked with different letters are significantly different (PÂ0.01; ANOVA and Tukey's honest post-hoc test)



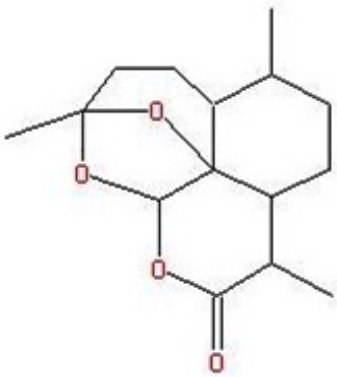
Graph 5. (i & ii) LPO activity (Mean±SE) in *T. casteneum* exposed to solvent derived EO of *A. annua* at lethal doses. Columns marked with different letters are significantly different (PÂ0.01; ANOVA and Tukey's honest post-hoc test)



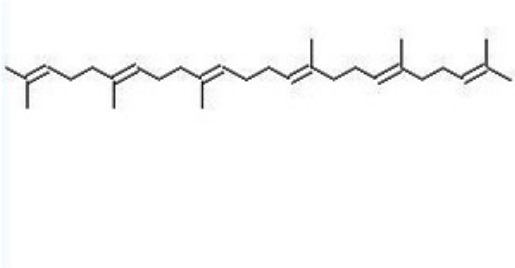
2H-1-Benzopyran-2-one



3,4-Hexadienal,2-butyl-2-ethyl-5-methyl



Deoxyqinghaosu



Squalene

Fig. 1. Chemical structures of the major components of Essential oils of *Artemisia annua*grown in Indian climatic conditions (2H-1-Benzopyran-2-one, 3,4-Hexadienal,2-butyl-2-ethyl-5-methyl, Deoxyqinghaosu and Squalene)

extracts respectively. The second largest group identified was ketones in chloroform EO and alcohol in n-Hexane EO. Chemical groups like aldehydes and carboxylic acids have contributed in the total composition of n- Hexane distillate EO but absent in chloroform distillate (Table 1 & 2).Interestingly, lesser amount of compounds like Caryophyllene oxide and Squalene known for their antimicrobial and anticancer properties were also detected in both the extracts (Falowo et al., 2019; Smith, 2000).

Repellency test

EOsof*A. annua* showed strong repellent activity against the adult beetles. In the present study, repellency was more evident in the treatment sets ofn-hexane derived EO than the chloroform EO. Highest concentration of the EO (0.90mg cmÉ^2) had demonstrated 93.5% and 88.25% of repellency by n-hexane and chloroform derived EOs respectively (Table 3). However, repellency was recorded to increase insignificantly (pÃ0.01) with the increase in concentration.

Contact toxicity

Topical application was employed to evaluate whether the insecticidal activity of the EO of *A.annua*against *T. casteneum*adults and 14 days old larvae was attributable to contact toxicity (Table 4). No mortality was recorded in the control sets. When LD_{50} and LD_{90} values were compared, *T.castaneum* adults were recorded to be more susceptible than its larval stage to chloroform derived EOs (No overlap in 95% confidence interval). Moreover, no significant (pÃ0.01) difference in contact toxicity was seen between larvae and adult beetle when treated with n-HexaneEO as overlap in 95% confidence interval is marked. On the basis of LD_{90} values, adult beetles were found more vulnerable to chloroformderived EO whereas larvae were more responsive towards n-Hexane derived EO.

Fumigant toxicity

Fumigant toxicity was more pronounced in n-hexane eluted EO treated sets than to Chloroform derived EO (Table 8). Zero mortality was found in control set. Results testified the susceptibility of larval stagestowards solvents derived EOs when compared with the adults. While comparing the LD_{90} values, both the stages were found sensitive towards the n-hexane extracted EO treated sets.

Biochemical analysis

Quantitative analysis of biomolecules was performed to assess the changes in their normal range on exposure to the plant EOs. The protein concentrations of the whole body homogenate in the adult and 14-days old larvae were found to be in the ranges of 318- 955 µg mlÉ^1. However, there was a significant (pÂ0.01) downfall in the protein concentration of the treated sets of contact and fumigant bioassays. In chloroform EO treated sets, protein level of adults decreased from 954µg mlÉ^1(control)to 833µg mlÉ^1(LD_{90}) and in larvae 407µg mlÉ^1 in control to 326 µg mlÉ^1 in LD_{90} (Fig. 1 i). Results of n-Hexane EO treated sets were similar (Graph 1 ii). However, reduction in protein concentration was more pronounced in fumigant toxicity assays.

As explained in the Graph2, level of AChE was reduced significantly (pÂ0.01) in the lethal doses when compared with the control. However, insignificant (pÃ0.01) reduction is marked between the LD_{50}& LD_{90}. In contact toxicity bioassays, AChE level in the adults treated with chloroform EO decreased from 0.223 to 0.045µmoles/min/ml of enzyme and 0.136 to 0.077µmoles/min/ml of enzyme in larvae. Similar downfall was documented from the n-HexaneEO treated sets (Graph 2 ii). AChE reduction was more pronounced in fumigant toxicity assays from 0.293 to 0.038 µmoles/min/ml of enzyme in adult beetles and 0.14 to 0.069 µmoles/min/ml of enzyme in larvae.

The GST activity was declined significantly (pÂ0.01) in the treated sets while comparing their normal range recorded in the control group. But, slight and insignificant (pÃ0.01) decrease was noticed in the enzyme activity between the LD_{50}& LD_{90} groups. However, as depicted in the Graph3 i, adults treated topically with the chloroform derived EO has shown significant (pÂ0.01) decrease between the sub-lethal (0.115 µmoles/min/ml of enzyme) and lethal group (0.077 µmoles/min/ml of enzyme). Similar results were documented from n-Hexane eluted EO treated sets of adult beetles (Graph3 ii) where values decline from 0.047 µmoles/min/ml of enzyme in LD_{50} to 0.038 µmoles/min/ml of enzyme in LD_{90} groups.

A significant (pÂ0.01) reduction in GSH activity was well marked in all the treatment sets from their normal range (209- 179µmoles/mg protein in larvae; 237-181µmoles/mg protein in adults). Moreover, the enzymatic activity was

found to be highly downregulated in n-hexane EO treated sets (Graph4 i) than the chloroform EO treatment sets (Fig. 4 ii).

LPO activity was significantly (pÂ0.01) upregulated in the treated sets when compared with the control. Moreover, the enzymatic activity was found to be dose- dependent and increased significantly from the LD_{50} to the LD_{90} groups (Graph 5). Adults exposed to fumigation of both the EOs, has shown a steep increase in the LPO level (17.983 nmole of MDA/gm of tissue in control to 40.25 nmole of MDA/gm of tissue in larvae). Similar downfall is attained in the fumigant assays as well.

DISCUSSION

Our results are quite different from the previous reports. *A.annua* is well established for possessing Artemisininwhich is a potent antimalarial component. However, 1,8-cineole has emerged as a major insecticidal candidate in various studies (Tripathi et al., 2001). Conversely, GC-MS analyses of the present study have clearly depicted the presence of some novel component in the chloroform and n-Hexane derived EOs of *A. annua*in excessively high amount. Oxygenated sesquiterpene, unique to *A. annua*is responsible for pharmacological activity is reported to be the major chemical group present in both the EOs (Martínez et al, 2012; Brown, 2010). The group include compounds like 3,4-Hexadienal,2-butyl-2-ethyl-5-methyl and Deoxyqinghaosu in different proportions (Figure 1) which can be attributable to the insecticidal properties of both the EOs. Insecticidal compounds like2H-1-Benzopyran-2-one (Xiaorong& Taiping, 2008) and Squalene(Chauhan, 2015) were also detected in the EOs which ascertains the insecticidal candidature of plant EOs derived with chloroform and n-hexane.

A.annua showed potent contact, fumigant and repellent activity against *T.casteneum* with chloroform and n-Hexane derived EOs. However, the insecticidal properties of the EOsvaried with solvents and the life stages of the red flour beetle. Presence of Deoxyqinghaosu in the extract was an obvious outcome (Li, 2012; Ni et al., 2012). Correlated to the fact, relatively higherPercent composition of Deoxyqinghaosu in n-hexane EO can be attributable to the better repellent activityand was reported for the first time in the present study from filter paper arena tests.

Results of contact toxicity bioassays have clearly depicted the susceptibility of the adults (LD_{50}= 0.71mg adultÉ^{1}) andlarvae (LD_{50}= 0.47mg insectÉ^{1}) towards the n-hexane EO. Similarly, Fumigant toxicity has represented n-hexane a better eluent of EO for both the life stages. This could be due to the presence of high percentage of 3,4-Hexadienal,2-butyl-2-ethyl-5-methyl in the crude extract. Moreover, higher Percent composition of Squalene whose insecticidal properties (Chauhan et al., 2015) were already described could have surplused the bioactivity of the plants EOs.

Evaluation of the biomolecular profile of *T. casteneum* adults and 14- days' old larvae at their lethal doses were carried out to understand the molecular mechanism underlying oxidative stress following exposure to plant EOs. Decline in protein level with the increase in concentration has been seen in case of both contact and fumigant toxicity bioassays. The results were continuous with a number of earlier investigations where scientists recorded significant downfall in protein level (Smirle et al., 1996; Huang et al., 2004; Macedo et al., 2007). Spectrometric quantification of primary detoxifying enzymes like AChE, GST, GSH and LPO has demonstrated the plants mode of action. Gradual Reduction in the level of AChE, GST and GSH were marked at the lethal doses when compared with the control. In a similar study conducted with the extract of soapnut*Sapindusemarginatus* against *Aedesaegypti* has shown significant reduction in larval AChE concentration (Koodalingam et al., 2011).GST is the key cytosolic enzyme for resistance development in insect's acts by catalysing the conjugation of reduced glutathione to the toxic molecule thus transforms it to less toxic end product (Grant & Matsumura, 1989). Our results on GST level were continuous with a number of previous investigations (Yu, 1982; Vanhaelen et al., 2001). On the contrary, Zibaee&Bandani (2010) has reported a positive correlation between the concentration of plant extract and GST activity after 24 h of exposure. However, reduction in the level of the enzyme was marked with the increase in exposure time. Correlated with GST, GSH level

also experienced a significant downfall in the treated sets (Vontas et al., 2001).Upregulation of LPO levelwas an obvious outcome due to sudden increase in oxidative stress. Similar results were reported by Hasspieler (1990) where an increase in LPO activity was found in mosquito larvae.

CONCLUSION

The present study hasestablishednon-polar solvents as the potent candidate for derivingEOs of *A.annua*with better insecticidal and repellent activity against *T. casteneum*. Moreover, newly identified allelochemicals have emerged as an efficient in vivo suppressor of life supporting biomolecules except LPO in the toxicity assays. Hence, these compounds can be used as a potential synergist in pest management by interfering with enzyme mediated detoxification. Moreover, by analysing the gene expression of bioassay survived individuals, future research can focus on modulation of bioassays to channelize the toxicity of *Artemisia annua*with potent non-polar solvents against other stored grain pests.

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