

Attractiveness of essential oils of three *Cymbopogon* species to *Tribolium castaneum* (Herbst) adults

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SUMMARY

Behavior bioassays were conducted in the laboratory ($23 \pm 1^\circ\text{C}$ and $50 \pm 5\%$ r.h.) using the olfactometer to determine the effects of essential oils of three plant species in the *Cymbopogon* genus (Lemongrass), *Cymbopogon nervatus*, *C. proximus* and *C. schoenanthus*, on adults of *Tribolium castaneum*. The effect of essential oils was compared to a commercial biopesticide based on azadirachtin at three concentrations (0.0001, 0.001 and 0.01%).

The results showed that all essential oils and the azadirachtin-based biopesticide had significant ($p < 0.05$) repellent effects on *T. castaneum* adults at all tested concentrations, except *C. proximus* essential oil which showed a neutral effect at the lowest concentration. The highest concentrations of the essential oils of *C. nervatus* and *C. proximus* had significantly stronger repellent effects ($p < 0.05$) than the lowest concentration. At the concentrations of 0.0001 and 0.001% all tested essential oils and azadirachtin showed a similar repellent effect without statistically significant difference, whereas the oil of *C. nervatus* had the highest repellent effect on adults of *T. castaneum* at the 0.01% concentration.

Considering all tested variations, the essential oils of plants of the genus *Cymbopogon* showed similar or stronger repellent effects on *T. castaneum* adults than the biopesticide based on azadirachtin.

Keywords: Essential oils; *Cymbopogon*; Red flour beetle; Behaviour; Repellency

INTRODUCTION

Red flour beetle, *Tribolium castaneum* (Herbst) is one of the most important species of storage insects in economic terms, especially in mills and other facilities for

processing and storage of plant products, where it causes direct losses. Besides direct damage, their presence and excrements contaminate food which affects product trade name reputation (Rees, 2004; Mahroof & Hagstrum, 2012). Due to the specificity of food processing facilities

and processed commodities only a small number of synthetic insecticides (fumigants and contact insecticides) can be used to control this and other stored-product insect pests (Fields & White 2002; Arthur, 2008; Arthur & Subramanyam, 2012). However, the use of synthetic insecticides is limited due to the resistance of certain populations of *T. castaneum* and other storage insects to insecticides (Kljajić & Perić, 2005; Boyer et al., 2012; Andrić et al., 2010) and increasingly restrictive standards relating to food and environment safety (Arthur, 1996; Arthur & Subramanyam, 2012). As a result, alternative methods of protection of stored products are being tested and introduced (Kljajić, 2008; Phillips & Throne, 2010).

One of potential alternative solutions is to use plant extracts and essential oils (EOs). Although a large number of plant species have shown insecticidal activity (Phillips & Throne, 2010; Pugazhvendan et al., 2012; Regnault-Roger et al., 2012; Sung-Wong et al., 2013; Nenaah, 2014), only a few are currently used on a commercial scale. Pyrethrum, a commercial mixture of compounds derived from *Chrysanthemum cinerariifolium* and various extracts from the neem tree, *Azadirachta indica*, with azadirachtin as the active compound, are the most common options (Phillips & Throne, 2010). Products based on azadirachtin have been proved to have lethal effects on insects by inhibiting their growth and development, and interfering with their mating, feeding and oviposition (Gahukar, 2012; Surajwo et al., 2016). However, as EOs are assumed to usually require high doses for achieving lethal effects on storage insects, the influence of EOs on insect behavior has been further examined in recent years. The focus has been on whether EOs attract or repel insects at certain concentrations, and whether their attractant or repellent potentials can be used for improving the existing pest management programs for storage insects (Koul, 2004; Adarkwash et al., 2010; Caballero-Gallardo et al., 2012; Laznik et al., 2012; Regnault-Roger et al., 2012; Licciardello et al., 2013).

Various plant species and their products are traditionally used in many countries as stored-product protection agents, such as essential oils from the genus *Cymbopogon*. These are perennial plants belonging to the grass family (Poaceae), distributed throughout the warm and tropical regions of the Old World and Oceania (Bertea & Maffei, 2010). Earlier research has shown that *Cymbopogon* essential oils can be effective against different insect pests important in agriculture (Rajendran & Sriranjini, 2008; Nerio et al., 2010; Hernandez-Lambrano et al., 2015; Wang et al., 2016), but significant differences have also been revealed regarding the strength of effect, depending on plant species and applied concentration (Bossou et al., 2015).

The aim of this study was to investigate the effects of essential oils of three *Cymbopogon* species: *C. nervatus* (Hochst.) Chiov., *C. proximus* Stapf. and *C. schoenanthus* (L.) Spreng, on *T. castaneum* adults. The olfactometer was used in tests with selected concentrations (0.0001, 0.001 and 0.01%) of essential oils and the effects were compared with those of azadirachtin in commercial bioinsecticide.

MATERIAL AND METHODS

Test insects

A laboratory population of *T. castaneum*, reared in the insectary of the Institute of Pesticides and Environmental Protection, Belgrade, Serbia, was used in the tests, and procedures described by Harein and Soderstrom (1966), and Davis and Bry (1985) were applied. *Tribolium castaneum* were reared in 2.5 L glass jars containing wheat flour with 5% yeast. Air temperature in the insectary was $25 \pm 1^\circ\text{C}$, and relative humidity $60 \pm 5\%$. Unsexed 3-5 week old adults were used in all trials. Before using them in the experiment the adults were starved for 24 h (Wakefield et al., 2005).

Essential oils tested

The essential oils were obtained by hydrodistillation from inflorescences of *C. nervatus* originating from West Sudan, leaves of *C. schoenanthus* cultivated in Khartoum (Sudan) and leaves of *C. proximus* purchased from a local store in Khartoum (Sudan). Chemical composition of the oils was analysed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) according to a method described previously (Omar et al., 2016). The essential oil of *C. nervatus* inflorescence was characterized by oxygenated monoterpenes with *p*-menthadiene skeleton, *trans-p*-mentha-1(7),8-dien-2-ol (19.9%), *cis-p*-mentha-1(7),8-dien-2-ol (19.5%), *trans-p*-mentha-2,8-dien-1-ol (16.5%) and *cis-p*-mentha-2,8-dien-1-ol (8.1%) (Omar et al., 2016). The most dominant constituents of the essential oil of *C. schoenanthus* leaves were monoterpenes piperitone (71.0%) and δ -2-carene (12.6%), whereas those of *C. proximus* leaves oil were piperitone (37.9%), sesquiterpenes elemol (16.9%), β -eudesmol (11.4%) and α -eudesmol (9.2%).

The commercial product NeemAzal-T/S (manufactured by Trifolio-M GmbH, Germany) is a formulation of NeemAzal, a high content oil-free extract of *A. indica* seeds containing 34% of azadirachtin-A and around 20% of other isomers. The content of azadirachtin-A in the product is standardized to 1% (10gL^{-1}).

Bioassays

Olfactory responses of *T. castaneum* adults were measured using a two-way airflow olfactometer consisting of two stimulus zones (arms) directly opposite each other, with a central neutral zone separating them (Ninkovic et al., 2013). Air was drawn from the centre of the olfactometer using a vacuum pump, establishing discrete air currents in the side arms.

Insect olfactory response was tested by adding the EOs or biopesticide at a volume of 10 μ L to small pieces of filter paper placed into plastic tubes (4 mm diameter) connected to the holes on the sides of the olfactometer arms. Filter papers with the tested EOs or biopesticide were attached to one side of the olfactometer and control to the opposite side. The experiment was conducted using serial dilutions of EOs and biopesticide in n-Hexane (0.0001, 0.001 and 0.01%), and n-Hexane was used as the control.

Insects were randomly chosen, using a fine paintbrush. A single insect was introduced into the olfactometer through a hole in the top. After an adaptation period of three minutes, insects staying in olfactometer arms were recorded over a 10 minute period.

The accumulated time (in seconds) of stay of a single insect in the arms with different odour sources was regarded as one replicate. Pseudo replication was avoided by using a single insect in each replicate, testing an insect only at a time, and by using a clean olfactometer for each replicate. The number of replications was 20.

The experiment was conducted at $23 \pm 1^\circ\text{C}$ and $50 \pm 5\%$ r.h. in a dark room with a light above the olfactometer.

Data analysis

Prior to statistical data analysis, we calculated the indices obtained by subtracting total time which insects spent in the control arm from total time that insects spent in olfactometer arms with scents of the essential oils or azadirachtin during the same experiment.

Thus we obtained the numbers, indices, which were either positive or negative - positive when the substance acted as an attractant, and negative when it acted as a repellent. The absolute value of the index indicates a potential for attraction or repulsion of that substance for insects.

The indices were further analyzed using the analysis of variance for repeated measurements and the mean values were compared using the t and LSD post hoc tests. The data were run on StatSoft (2005) version 7.1 (StatSoft Inc., Tulsa, Oklahoma).

RESULTS

The essential oils of all three *Cymbopogon* species and azadirachtin at all concentrations showed statistically significant ($p < 0.05$) repellent effects on adults of *T. castaneum*, with the exception of *C. proximus* oil which did show no statistically significant ($p = 0.321$) repellent effect at the lowest concentration on this insect species (Table 1).

Table 1. Attractiveness of essential oils of *Cymbopogon* species and azadirachtin to *T. castaneum* adults

Essential oils	Concentration (%)	Index of attractiveness	SE	Effects	t ^a	df	p
<i>Cymbopogon nervatus</i>	0.0001	-152.75	31.1	repellent	-4.912	19	<0.001
	0.001	-124.00	28.33	repellent	-4.377	19	<0.001
	0.01	-306.00	27.11	repellent	-11.285	19	<0.001
<i>Cymbopogon proximus</i>	0.0001	-49.25	48.36	neutral	-1.018	19	0.321
	0.001	-144.00	32.94	repellent	-4.372	19	<0.001
	0.01	-239.50	33.08	repellent	-7.239	19	<0.001
<i>Cymbopogon schoenanthus</i>	0.0001	-136.75	36.84	repellent	-3.712	19	0.001
	0.001	-213.75	35.18	repellent	-6.075	19	<0.001
	0.01	-141.00	28.18	repellent	-5.004	19	<0.001
Azadirachtin ^b	0.0001	-118.50	34.83	repellent	-3.402	19	0.003
	0.001	-202.25	32.73	repellent	-6.179	19	<0.001
	0.01	-215.25	35.65	repellent	-6.038	19	<0.001

^a t - test for one sample

^bBiopesticide NeemAzal-T/S

Comparing the effects between the tested concentrations of each EO and azadirachtin, no statistically significant difference was found between the concentration of *C. schoenanthus* oil and azadirachtin (Table 2). The highest concentration of *C. nervatus* oil repelled insects significantly more than the lowest concentration. Insects spent 15.8% and 8.4% of total time in the olfactometer arms with concentrations of 0.01% and 0.0001%, respectively (Figure 1). The essential oil of *C. proximus* at the highest concentration (13.9% of total time) had a significantly stronger repellent effect than at the lowest concentration (28.4% of total time), while the mean concentration did not differ from the other two (Table 2, Figure 1)

By comparing the effects of *Cymbopogon* EOs and the commercial product based on azadirachtin at the same concentrations, it was revealed that all tested oils at the concentrations of 0.0001% and 0.001% had the same repellent effect on *T. castaneum* adults as the commercial product (Table 3). At the highest concentration, the oil of *C. nervatus* showed the strongest repellent effect, followed by *C. proximus* oil and azadirachtin with the same level of repellency, while the *C. schoenanthus* oil showed the lowest repellent effect (Table 3).

The differences in index values at the highest concentration are shown in Figure 2.

Table 2. The effect strength of different concentrations of *Cymbopogon* species essential oils and azadirachtin on *T. castaneum* adults

Essential oils	Concentration (%)			Relations ^a	η^2	F ^b
	0.0001 (1)	0.001 (2)	0.01 (3)			
<i>Cymbopogon nervatus</i>	-152.75	-124.00	-306.00	3 < 1, 2	0.378	11.57**
<i>Cymbopogon proximus</i>	-49.25	-144.00	-239.50	3 < 1; 2 = 1, 3	0.242	6.08**
<i>Cymbopogon schoenanthus</i>	-136.75	-213.75	-141.00	1 = 2 = 3	0.092	1.97
Azadirachtin ^c	-118.50	-202.25	-215.25	1 = 2 = 3	0.116	2.49

**p < 0.01

^a LSD post hoc test

^b Based on repeated measures of ANOVA

^c Biopesticide NeemAzal-T/S

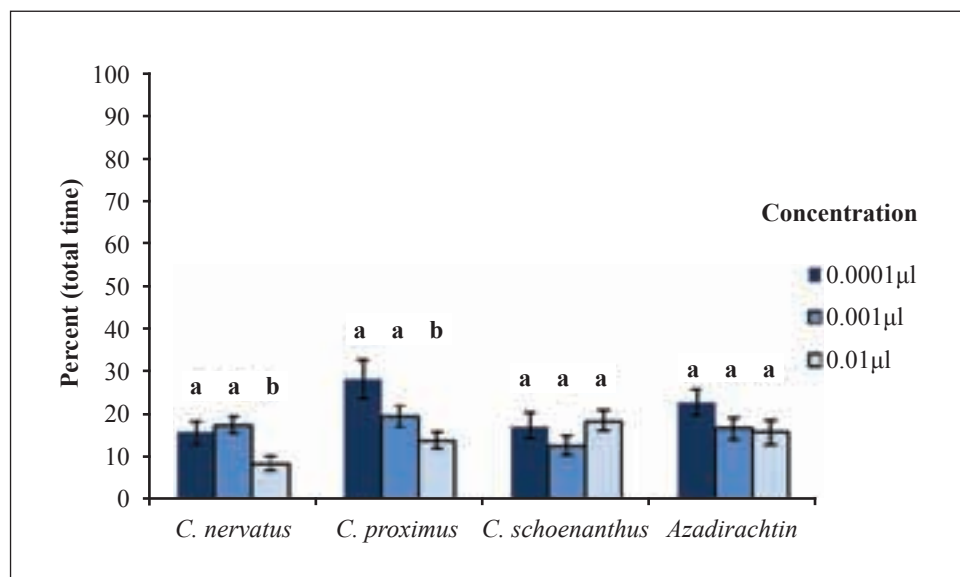


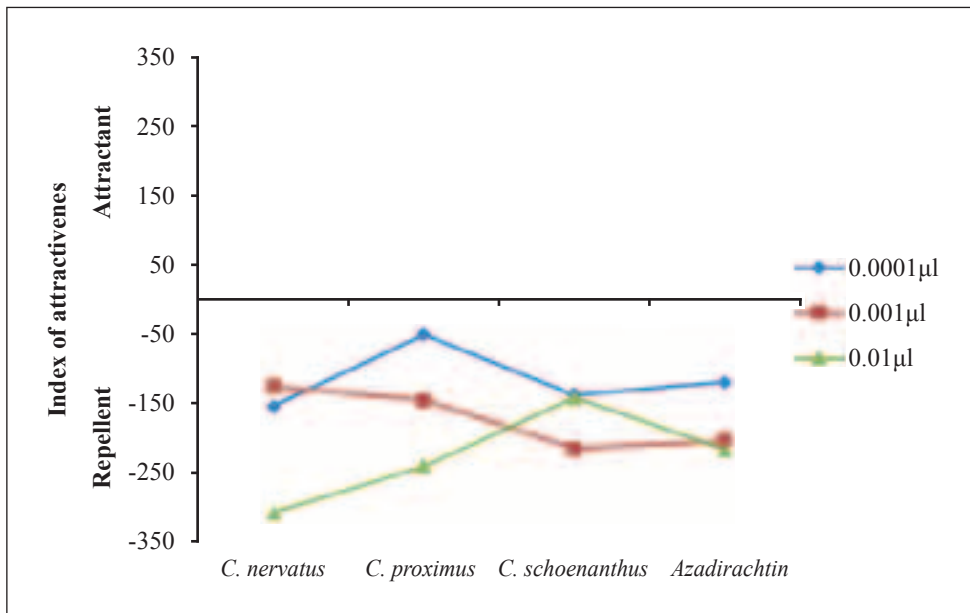
Figure 1. Total time (% \pm SE) that *T. castaneum* adults spent in olfactometer zones with essential oils

* For each essential oil, means marked by the same letter are not significantly different, Fisher's LSD test at $P > 0.05$

Table 3. The effect and relations of the same concentrations of essential oils of *Cymbopogon* species and azadirachtin on *T. castaneum* adults

Concentration (%)	Essential oils	Index of attractiveness	Relations ^a	η^2	F ^b
0.0001	<i>Cymbopogon nervatus</i> (1)	-152.75	1 = 2 = 3 = 4	0.087	1.82
	<i>Cymbopogon proximus</i> (2)	-49.25			
	<i>Cymbopogon schoenanthus</i> (3)	-136.75			
	Azadirachtin (4) ^c	-118.50			
0.001	<i>Cymbopogon nervatus</i> (1)	-124.00	1 = 2 = 3 = 4	0.088	1.83
	<i>Cymbopogon proximus</i> (2)	-144.00			
	<i>Cymbopogon schoenanthus</i> (3)	-213.75			
	Azadirachtin (4) ^c	-202.25			
0.01	<i>Cymbopogon nervatus</i> (1)	-306.00	1 < 3 1 = 2, 4 3 = 2, 4	0.177	4.07*
	<i>Cymbopogon proximus</i> (2)	-239.50			
	<i>Cymbopogon schoenanthus</i> (3)	-141.00			
	Azadirachtin (4) ^c	-215.25			

* p < 0.05

^aBased on the LSD post hoc test, only differences are listed^bBased on repeated measures ANOVA^cBiopesticide NeemAzal-T/S**Figure 2.** Differences in attractiveness among the essential oils

DISCUSSION

Overall, our results showed that all three *Cymbopogon* (*C. nervatus*, *C. proximus* and *C. schoenanthus*) EOs had repellent effects on *T. castaneum* adults. This is in agreement with many previous studies which reported

EOs repellency to stored-product insects (Nerio et al., 2010; Ukeh & Umoetok, 2011; Kedia et al, 2015). Earlier studies also showed repellent effects of oils from different species of the genus *Cymbopogon* that we used in our research (Licciardello et al., 2013; Caballero-Gallardo et al., 2012)

Also, the results of these studies showed that azadirachtin from *A. indica* commercial extract of *A. indica* had a strong repellent effect on *T. castaneum* adults. This is consistent with previous research in which it was found that *A. indica* extracts, apart from causing mortality in insect pests, also acted as a repellent (Boeke et al., 2004; Showler et al., 2004; Adarkwash et al., 2010)

In our research, all tested EOs showed a repellent effect at the intermediate concentration (0.001%) and the highest concentration (0.01%). It was found that *T. castaneum* adults spent about 2.5-fold less time in the arm of the olfactometer with the lowest concentration (0.0001%) of *C. schoenanthus* and *C. nervatus* EOs and about 2-fold less time in the arm with azadirachtin, compared to the arm containing n-hexane. The *C. proximus* oil showed no statistically significant effect on *T. castaneum* at the lowest concentration, suggesting a different repellent potential from EOs obtained from various species of the genus *Cymbopogon*.

The experiment conducted by Caballero-Gallardo et al., (2012) revealed greater differences in repellent effect between different *Cymbopogon* species at low concentrations. In those tests it was found that the species *C. flexuosus* and *C. martini* had equally high repellency for *T. castaneum* (> 90%) at the higher EO dose of 0.2 mL cm⁻² filter paper. At a significantly lower dose of 0.000022 mL cm⁻² filter paper, *C. martini* oil showed a significantly greater repellency (46%) than the related species *C. flexuosus* (20%).

Taking into consideration all the EOs and azadirachtin at the highest concentration, *T. castaneum* adults spent 2.5-7.5-fold more time in the olfactometer arm containing n-hexane than in the arm with the tested oils. The highest tested concentrations of *C. nervatus* and *C. proximus* oils caused a double strong repellent effect compared to the lowest concentration tested. A dependence of repellency strength on essential oil concentrations was detected in a research conducted by Licciardello et al. (2013), who found that an EO of *C. nardus* had a statistically significant repellent effect on *T. castaneum* applied at concentrations of 0.005-0.02 mL cm⁻² of filter paper, while a lower concentration of 0.001 mL cm⁻² had no significant repellent effect. In an experiment in which EO was applied to whole oat flour, Olivero-Verbel et al. (2013) reported that increasing concentrations of *C. citratus* oil increased the repellent effect on *T. castaneum*. The highest repellency was recorded at the amount of 5 mL cm⁻² of flour, and minimum effect was observed at the lowest tested dose of 0.0005 mL cm⁻² of flour.

If we compare the repellent effects of the tested EOs, it may be inferred that differences exist only at the highest

concentration, where the oil of *C. nervatus* showed the highest repellent effect, significantly higher than *C. schoenanthus* oil. Our experiment also showed that the highest tested concentration of *C. schoenanthus* oil had a weaker repellent effect on *T. castaneum* adults than the other two tested *Cymbopogon* oils. Azadirachtin in the commercial biopesticide used in our research as a standard did not show a stronger repellent effect than the tested EOs. In an experiment with the species *Aphis gossypii*, Ramakrishna (2008) found high repellency (40-60%) of azadirachtin for that species. In our experiment, azadirachtin at the minimum and maximum concentrations had a slightly lower repellent effect than the oil of *C. nervatus*. Research in which the repellent effect of the commercial synthetic repellent IR3535 was compared to repellent effects of EOs from plants of the genus *Cymbopogon* also revealed that the oils of *C. citratus* (Olivero-Verbel et al., 2013), *C. martinii* and *C. flexuosus* (Caballero-Gallardo et al., 2012) and *C. citratus*, *C. flexuosus*, *C. martinii* (Hernandez-Lambran et al., 2015) had stronger repellency than the commercial repellent.

The results obtained in this study show that the essential oils of *C. nervatus*, *C. proximus* and *C. schoenanthus* demonstrated repellent effects on *T. castaneum* adults. Also, taking into consideration the tested concentrations it can be inferred that the tested essential oils showed a pronounced repellent effect at very low concentrations (0.0001%), which suggests that an analysis of their cost-effective use in practice could be positive. However, a more thorough verification of the potentials of these oils as repellents for *T. castaneum* adults requires detailed studies on treated wheat grain, in the laboratory and/or semi-field conditions.

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Uticaj etarskih ulja iz tri vrste biljaka roda *Cymbopogon* na ponašanje kestenjastog brašnara (*Tribolium castaneum* Herbst)

REZIME

U laboratorijskim uslovima ($24\pm 1^\circ\text{C}$ i $50\pm 5\%$ r.v.v.) je pomoću olfaktometra ispitivan uticaj etarskih ulja iz tri biljne vrste roda limunovih trava (*Cymbopogon nervatus*, *Cymbopogon proximus* i *Cymbopogon schoenanthus*) na ponašanje (atraktivnost/repelentnost) adulta *T. castaneum*. Uticaj etarskih ulja na ponašanje kestenjastog brašnara, poređen je sa uticajem biopesticida na bazi azadirachtina u tri koncentracije: 0.0001, 0.001 i 0.01%.

Rezultati testiranja pokazali su da sva etarska ulja i biopesticid na bazi azadirachtina u svim ispitivanim koncentracijama ispoljavaju statistički značajan odbijajući uticaj - repelentni efekat na adulte kestenjastog brašnara, osim najniže koncentracije ulja iz biljke *C. proximus* koja je ispoljila neutralan efekat na brašnara. Najviša koncentracija ulja iz biljaka *C. nervatus* i *C. proximus* je ispoljila statistički značajno jači repelentni efekat u odnosu na najnižu koncentraciju. Sva ispitivana etarska ulja i azadirachtin su u koncentracijama 0.0001 i 0.001% ispoljila sličan repelentni efekat bez statistički značajne razlike, dok je ulje iz biljke *C. nervatus* u koncentraciji 0.01% prouzrokovalo statistički značajno jači repelentni efekat na adulte kestenjastog brašnara u odnosu na ostale ispitivane supstance.

Na osnovu rezultata istraživanja sa adultima kestenjastog brašnara može se zaključiti da su etarska ulja iz tri vrste biljaka iz roda *Cymbopogon* ispoljila sličan i/ili jači repelentni efekat od biopesticida na bazi azadirachtina. Takođe, dobijeni rezultati pokazuju značajan potencijal upotrebe ulja ovih biljaka kao prirodnog sredstva za zaštitu uskladištenih biljnih proizvoda.

Ključne reči: Etarska ulja; *Cymbopogon*; Kestenjasti brašnar; Ponašanje; Repelentnost