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ORIGINAL PAPER

Insecticidal properties of phenols on *Culex quinquefasciatus* Say and *Musca domestica* L

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Abstract Thirteen simple phenols and 8 phenolic acids were tested for toxicity to *Culex quinquefasciatus* larvae and *Musca domestica* adults. It was found that while the phenolic acids (except salicylic acid) showed little or no effect on acute toxicity, all the tested simple phenols caused mortality within 24 h after application. Lethal doses for acute toxicity of *C. quinquefasciatus* were successfully estimated for 12 substances using probit analysis. Thymol, carvacrol, 2-ethylphenol, and salicylaldehyde showed significantly the highest efficiency, for which the lethal doses LD_{50} were estimated as 30, 36, 38, and 43 μg/ml, respectively. Lethal doses for acute toxicity of *M. domestica* adults were successfully estimated for ten substances. Thymol, carvacrol, and 2,6-dimethoxyphenol showed significantly the highest efficiency, for which the lethal doses LD_{50} were estimated 53, 69, and 87 μg/fly, respectively.

Introduction

Flies and mosquitoes are the most important groups of insect vectors of human and veterinary diseases, responsible for the transmission of a wide variety of pathogens such as viruses, bacteria, fungi, protozoan, and metazoan parasites in humans and animals.

The housefly (*Musca domestica* L.) is a worldwide distributed pest organism and the dominant synanthropic fly species in animal production, homes, and restaurants. Houseflies cause annoyance to humans and animals and vector many medical and veterinary pathogenic organisms. *M. domestica* has been reported to be involved in the dissemination of enteric pathogens such as *Escherichia coli*, *Shigella* spp., *Salmonella* spp., *Helicobacter* spp., *V. cholerae* non-O1. Furthermore, the passage of microorganisms through the alimentary tract of this fly species may provide an opportunity for the multiplication of enteric pathogens (Ketonok and Chicherin 1977; Grubel et al. 1997; Kobayashi et al. 1999; Banjo et al. 2005).

Mosquitoes are responsible for spreading serious vector-borne diseases like malaria, filariasis, Japanese encephalitis, yellow fever, dengue, etc. Especially, lymphatic filariasis caused by *Wuchereria bancrofti* and transmitted by the mosquito *Culex quinquefasciatus* Say is found to be more endemic in the Indian subcontinent. All over the world, more than 50% of persons with filariasis receive their infections from the bites of mosquitoes, very particularly *C. quinquefasciatus* (Lowrie et al. 1989; Rajasekariah et al. 1991; Muturi et al. 2008).

The only efficacious approach to minimizing the incidence of these diseases is to eradicate and control fly and mosquito vectors, mainly by applying insecticides to larval or adult habitants, and to educate the public (Corbel et al. 2004). Chemical control is an effective strategy used extensively in daily life. Today, synthetic insecticides are at the forefront of fly and mosquito-controlling agents. Nevertheless, controlling these insects has become complicated because of their resistance to synthetic insecticides, as well as the toxicity of insecticides to fish and other non-target organisms (Chandre et al. 1998; Rohani et al. 2001). There is an urgent need to develop new materials for controlling flies and mosquitoes in an environmentally safe way, using biodegradable and target-specific insecticides against them (Isman 2006; Pavela 2007a; Rattan 2010).

Plant essential oils have been suggested as alternative sources for insect control, because some are selective,

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biodegrade to nontoxic products, and have few effects on non-target organisms and the environment (Singh and Upadhyay 1993; Isman 2006; Pavela 2007a). Essential oils and their volatile constituents are widely used in the prevention and treatment of human illnesses. Various essential oils have also been documented to exhibit acute toxic effects against the insect. Several experiments have been conducted to investigate insecticidal properties of essential oils against various mosquitoes (Shalaby et al. 1998; Park et al. 2005; Zaridah et al. 2006; Pavela 2009) or houseflies (Shalaby et al. 1998; Pavela 2005, 2007b, 2008a, b). However, after comparing insecticidal efficiencies of essences with respect to their majority substances, we noticed that essential oils containing a majority share of phenols are very often highly efficient. Their biological efficiency often significantly exceeds other terpenoids (Lee et al. 1997; Manou et al. 1998; Amer and Mehlhorn 2006; Kokošková and Pavela 2007; Pavela 2009).

Phenolic compounds are characterized by the presence of a hydroxy (−OH) group, attached to a benzene ring or other complex aromatic ring structures, e.g., pyrogallol, catechol, or resorcinol. They range from simple phenol to polyphenols, such as flavonoids and tannins. Plant phenolics have been regarded as one of the important defenses against insects in various studies (Bruneton 1999).

Insecticidal efficiency of these phenols probably depends on their ability to block GABA or the octopaminergic system (Priestley et al. 2003; Bakkali et al. 2008). Nevertheless, phenols cause not only acute toxicity, but they also provide antifeedant, anti-ovipositional, and growth inhibitory effects (Koul 2005; Park et al. 2005; Pavela 2007b; Bakkali et al. 2008). In spite of this fact, there are very few papers comparing the insecticidal efficiency of phenols depending on their chemical structure, although such studies allow for explaining the way of plant defenses against phytophagous pests and provide important information for the development of botanical insecticides.

Application of contact insecticides by their spraying has been still viewed as the most efficient way of fighting against flies; on the contrary, in fighting against mosquitoes, a more efficient way consists in prevention of emergence of adults by destroying their larvae using larvicides. Although in our previous work, we found thymol to be very perspective for the development of botanical pesticides suitable for fighting against mosquitoes (Pavela 2009) as well as against houseflies (Pavela 2008a, b), it is not known whether structurally similar phenols and their acids also exhibit similar efficiencies. The aim of our work was to determine whether even more efficient compounds than thymol can be chosen. In our paper, we have therefore studied 21 simple phenols different in structure and their acids in relation to acute toxicity of *C. quinquegasciatus* Say larvae and *Musca domestica* L. adults.

Material and methods

Chemicals

The compounds (see Table 1) were purchased from Sigma-Aldrich, Chemical Co., Czech Republic. All chemicals were used without further purification. Stock solutions (50% *w/v*) of each compound were prepared in dimethyl sulfoxide/acetone (ratio 1:1) on the day of experiments and were used immediately.

Test organisms

The test organism, namely *C. quinquefasciatus* Say, was reared in the laboratory. The larvae were fed on dog biscuits and yeast powder in the ratio 3:1. Adults were provided with a 10% sucrose solution and a 1-week-old chick for blood feeding. Early fourth-instar larvae were used in the study.

Adult houseflies, *Musca domestica* L., (female, 3–6 days after emergence) were obtained from an established laboratory colony. Housefly larvae were reared in the mixture of sterilized bran with milk powder and water. Housefly adults were given free access to water and to a thick paste of condensed milk and to milk powder.

All the tested insects were treated and maintained at the temperature of 25°C±1°C, in the humidity of 50–70% RH, and in a 16:8 photoperiod (L/D). All experiments were performed under the same conditions.

Bioassays

Larvicidal toxicity Mosquito larvicidal trials were carried out according to WHO standard procedures (1996), with slight modifications. The compounds were diluted in dimethyl sulfoxide/acetone (ratio 1:1) in order to prepare a serial dilution of the test dosage. For experimental treatment, 1 ml of serial dilution was added to 224 ml of distilled water in a 500-ml glass bowl and shaken gently to produce a homogeneous test solution.

The larvae were transferred in distilled water into a bowl of the prepared test solution, with the final surface area of 125 cm^2 (25 larvae/beaker). Four duplicate trials were carried out for every sample concentration, and for each trial, a negative control was included using distilled water containing the same amount of dimethyl sulfoxide/acetone as the test sample. A different series of concentrations (resulting from the previous screening) was used for each extract in order to obtain mortality ranging between 10% and 90%. At least five concentrations were selected for the calculation of lethal doses. A dose of 300 μg/ml was determined to be the uppermost limit for ascertaining larvicidal activity of the compounds. Mortality was deter-

Table 1 The compounds used in study and their functional groups

Compounds	Functional group					
	R1	R2	R3	R4	R5	R6
Phenols						
Thymol	OH	$CH(CH_3)_2$	H	H	CH_3	H
Carvacrol	OH	CH_3	H	H	$CH(CH_3)_2$	H
Eugenol	OH	OCH_3	H	$CH_2CH{=}CH_2$	H	H
4-Ethylphenol	OH	H	H	CH_2 CH_3	H	H
2- Ethylphenol	OH	CH_2 CH_3	H	H	H	H
Salicylaldehyde	OH	COH	H	H	H	H
2-Methoxy-4-methylphenol	OH	O CH_3	H	O CH_3	H	H
4-Ethylguaiacol	OH	O CH_3	H	CH_2 CH_3	H	H
Isoeugenol	OH	O CH_3	H	CH=CH- CH_3	H	H
Guaiacol	OH	O CH_3	H	H	H	H
2,6-Dimethoxyphenol	OH	O CH_3	H	H	H	O CH_3
Vanillin	OH	O CH_3	H	COH	H	H
Phloroglucinol	OH	H	OH	H	OH	H
Phenolic acids						
Gallic acid	OH	OH	OH	H	COOH	H
p-Coumarid acid	OH	H	H	CH=CH-COOH	H	H
Syringic acid	OH	O CH_3	H	COOH	H	O CH_3
Vanillic acid	OH	O CH_3	H	COOH	H	H
Salicylic acid	OH	COOH	H	H	H	H
Sinapic acid	OH	O CH_3	H	CH=CH-COOH	H	O CH_3
Gallic acid	OH	OH	OH	H	COOH	H
Ferulic acid	OH	OCH_3	H	CH=CH-COOH	H	H

mined after 24 h of exposure, during which no food was offered to the larvae.

Acute toxicity Acute topical toxicity was examined with *M. domestica* adults (female, 3–6 days after emergence). A micro-electric applicator was used to deliver 1 μl doses of different dosages, mixed with acetone, to the pronota of anesthetized (CO_2) flies. Initial studies were conducted to determine appropriate ranges for the testing concentrations. Certified acetone was used as the control treatment. A dose of 500 μg/adults was determined to be the uppermost limit for ascertaining the activity of phenolic compounds on acute toxicity. A minimum of four concentrations were replicated at least for three times (50 flies per single replication) in the final bioassays. Mortality was assessed 24 h after the treatment was performed. Flies that did not respond were considered dead.

Statistical analysis

Experimental tests demonstrated that more than 20% of the controlled mortality was discharged and repeated. When the controlled mortality reached 1–20%, the observed mortality was corrected by the Abbott's formula (Abbott 1925) and an LC_{50}, LC_{90} regression equation, and a 95% confidence limit was calculated using probit analysis (Finney 1971).

Results

Larvicidal toxicity Efficiency of 21 phenols on larvicidal toxicity in the fourth instar of *C. quinquefasciatus* larvae is provided in Table 2. After application of the highest dose (300 μg/ml), 12 substances (thymol, carvacrol, eugenol, 4-ethylphenol, 2-ethylphenol, salicylaldehyde, 2-methoxy-4-methylphenol, 4-ethylguaiacol, isoeugenol, guaiacol, 2, 6-dimethoxyphenol, and salicylic acid) caused 100% mortality. Other substances exhibited little or no toxicity.

Lethal doses (Table 2) were successfully estimated for 12 substances using probit analysis. Thymol, carvacrol, 2-ethylphenol, and salicylaldehyde showed significantly the highest efficiency, for which the lethal doses LD_{50} were estimated as 30, 36, 38, and 43 μg/ml, respectively, and LD_{90} doses were estimated as 44, 60, 51, and 62 μg/ml, respectively.

Somewhat less effective were the substances eugenol, 4-ethylphenol, isoeugenol, and 4-ethylguaiacol whose lethal

Table 2 Acute toxicity of compounds against larvae of *Culex quinquefasciatus* and adults of *Musca domestica*

	Culex quinquefasciatus				*Musca domestica*			
	300 μg/ml[a]	LD_{50} (CI_{95})	LD_{90} (CI_{95})	Chi[b]	500 μg/fly[a]	LD_{50} (CI_{95})	LD_{90} (CI_{95})	Chi[b]
Phenols								
Thymol	100.0	30 (28–35)	44 (40–49)	4.464	100.0	53 (48–65)	91 (82–137)	0.679
Carvacrol	100.0	36 (31–39)	60 (52–65)	0.663	100.0	69 (57–78)	131 (108–155)	2.245
Eugenol	100.0	61 (58–64)	82 (76–92)	0.474	100.0	124 (115–134)	218 (195–259)	0.225
4-Ethylphenol	100.0	58 (55–60)	71 (69–79)	3.403	72	391 (296–411)	896 (834–967)	0.434
2- Ethylphenol	100.0	38 (35–42)	51 (46–60)	0.035	84	294 (245–321)	683 (628–711)	0.876
Salicylaldehyde	100.0	43 (39–46)	62 (56–68)	5.177	100.0	101 (88–116)	157 (120–183)	1.592
2-Methoxy-4-methylphenol	100.0	112 (105–119)	150 (139–170)	0.471	29.3	>500	>500	
4-Ethylguaiacol	100.0	95 (88–103)	171 (153–198)	0.333	100.0	176 (144–196)	483 (435–511)	0.570
Isoeugenol	100.0	60 (57–63)	80 (74–91)	5.503	100.0	336 (278–405)	617 (475–823)	0.011
Guaiacol	100.0	118 (113–122)	139 (134–147)	3.454	100.0	181 (144–198)	461 (385–512)	0.477
2,6-Dimethoxyphenol	100.0	146 (138–153)	174 (165–182)	2.058	100.0	87 (72–96)	142 (130–195)	4.770
Vanillin	18.7	>300	>300		0.0	>500	>500	
Phloroglucinol	10.6	>300	>300		0.0	>500	>500	
Phenolic acids								
Salicylic acid	100.0	139 (136–143)	160 (153–172)	0.370	45.8	>500	>500	
Caffeic acid	15.6	>300	>300		0.0	>500	>500	
Vanillic acid	11.5	>300	>300		0.0	>500	>500	
Ferulic acid	10.2	>300	>300		0.0	>500	>500	
Syringic acid	5.0	>300	>300		0.0	>500	>500	
Gallic acid	0.6	>300	>300		0.0	>500	>500	
Sinapic acid	0.5	>300	>300		0.0	>500	>500	
p-Coumarid acid	0.0	>300	>300		0.0	>500	>500	

LD_{50} and LD_{90} value in micrograms (μg) and CI_{95}–95% confidence intervals, essential oils activity is considered significantly different when the 95% CI fail to overlap

[a] Average mortality in % obtained after application; the uppermost limit for ascertaining the activity of phenolic compounds on acute toxicity

[b] Chi-square value, significant at $P<0.05$ level

doses (LD_{50}) were estimated as 61, 58, 60, and 95 μg/ml, respectively. LD_{50} doses higher than 100 μg/ml were estimated for the other substances.

Acute toxicity Efficiency of 21 phenols on acute toxicity of *M. domestica* adults is provided in Table 2. After application of the highest dose (500 μg/fly), eight substances (thymol, carvacrol, eugenol, salicylaldehyde, 4-ethylguaiacol, isoeugenol, guaiacol, and 2,6-dimethoxyphenol) caused 100% mortality and two (4-ethylguaiacol and 2-ethylphenol) caused mortality >50%. Other substances exhibited little or no toxicity.

Lethal doses (Table 2) were successfully estimated for ten substances using probit analysis. Thymol, carvacrol, and 2,6-dimethoxyphenol showed significantly the highest efficiency, for which the lethal doses LD_{50} were estimated <100 μg/fly (53, 69, and 87 μg/fly, respectively), and LD_{90} doses were estimated <150 μg/fly (91, 131, and 124 μg/fly, respectively).

Somewhat less effective were the substances eugenol, salicylaldehyde, 4-ethylguaiacol, and guaiacol whose lethal doses (LD_{50}) were estimated as 124, 101, 176, and 181 μg/fly, respectively. LD_{50} doses higher than 200 μg/fly were estimated for the other substances.

Discussion

The aim of our work was to compare the efficiency of phenols and their acids on acute toxicity of two vectors important in terms of economy; mosquitoes (*C. quinquefasciatus*) and houseflies (*M. domestica*), and based on the results, to select the most efficient and perspective compounds suitable for the development of botanical pesticides. We therefore selected 13 phenols differing in their structures and 8 phenolic acids that occur most frequently in nature and whose potential production poses no financial demands and/or that occur as majority components in essential oils of aromatic plants.

It was found that while the phenolic acids (except salicylic acid) showed little or no effect on acute toxicity (Table 2), all the tested simple phenols caused mortality within 24 h after application. The differences in efficiency of individual phenols were determined using lethal doses (LD_{50} and LD_{90}).

In our comparison of the lethal dosage values obtained, we found not one but several differences in efficiency. For both tested insect species, thymol and its phenol isomer had the lowest estimated lethal dosages. They can therefore clearly by selected as the most efficient of the tested phenols. These compounds are the only ones of the tested compounds whose isopropyl group is found on R_2 and R_5, and the methyl functional group found on R_5 and R_2, respectively (Table 1). It is thus likely that these functional groups will be responsible for better blockade of the GABA system compared to other phenols. Blockade of the GABA-gated chloride channel reduces neuronal inhibition, which leads to hyper-excitation of the central nervous system, convulsions, and death (Bloomquist 2003). It was suggested that thymol potentiates GABAA receptors through an unidentified binding site (Priestley et al. 2003).

Other phenols showed significantly lower efficiency and/or their efficiency was different depending on the insect species. This difference in efficiency may be caused both by inter-species sensitivity as well as by different developmental stages or different ways of application. For example, eugenol and isoeugenol exhibited equal toxicity for mosquito larvae (LD_{50} 61 and 60 μg/ml, respectively); but after topical application in flies, eugenol showed significantly higher efficiency (LD_{50} 124 μg/fly) compared to isoeugenol (LD_{50} 336 μg/fly). This different effect might be caused by potential gradual evaporation of residues of the active substance in topical application. Similar results were presented also by Huang et al. (2002). The authors observed the efficiency of eugenol, isoeugenol, and methyleugenol on contact toxicity against *Sitophilus zeamais* and *Tribolium castaneum*. The authors estimated significantly different LD_{95} for *S. zeamais* and *T. castaneum*, 47 and 79 μg/mg, respectively, for eugenol, and 102 and 115 μg/mg, respectively, for isoeugenol.

Similarly, significantly different efficiency was found also for 2-methoxy-4-methylphenol or salicylic acid, which showed very good efficiency against mosquito larvae; however, they exerted no significant toxic action on housefly adults.

Based on the determined lethal doses, we evaluated the phenols thymol and carvacrol as the most efficient ones in our work. Our results thus confirmed our previous observations where we found out that essential oils containing these compounds were significantly more efficient compared to essential oils (Pavela 2005, 2008a, 2009; Pavela et al. 2009; Žabka et al. 2009). Thymol and its phenol isomer, carvacrol, are the major constituents of essential oils from various aromatic plants such as *Thymus vulgaris* L. (Lamiaceae), *Origanum compactum* (Lamiaceae), *Acalypha phleoides* (Euphorbiaceae), and *Lippia sidoides* (Verbenaceae), *Carum carvi* (Apiaceae) which find their widespread use in folk medicine and aromatherapy (Bruneton 1999). Thymol and carvacrol are commonly used in the food industry and in cosmetics as preservatives and antioxidants (Manou et al. 1998; Ultee et al. 1999). Thymol is also used in mouth rinses for oral hygiene against caries-related pathogens (Bruneton 1999).

Thymol and carvacrol belong to the most active antioxidants found in essential oils (Yanishlieva et al. 1999; Monteiro et al. 2000). They possess multiple biological properties such as anti-inflammatory, antibacterial, antifungal, anticarcinogenic, antiplatelet, and other diverse activities (Bruneton 1999; Ruberto and Baratta 2000; Kokošková and Pavela 2007; Baker 2008).

Moreover, as determined in previous papers, thymol or essential oils with the majority content of thymol, respectively, cause not only acute toxicity of the larvae and adults of our tested vectors, but also its lethal and sublethal doses may cause significant reduction of fertility and life of adults, and vitality of the larvae of the subsequent generation *M. domestica* (Pavela 2007b). Similarly, we found in our research that even relatively short-term exposure of larvae to lethal oil doses can markedly increase their mortality in time, and thus reduce the total number of viable adults of *C. quinquefasciatus*, leading to a possible significant reduction of the total vector population dynamics (Pavela et al. 2009). The significant repellent effect of thyme oil, as well as some of its effective substances, has been known from the work of other authors, as well. For example, Park et al. (2005) determined the repellent efficiency of five monoterpenes (carvacrol, p-cymene, linalool, alpha-terpinene and thymol) derived from the essential oil of *T. vulgaris* against the mosquito *Culex pipiens* pallens. All five monoterpenes repelled mosquitoes effectively based on a human forearm bioassay. Alpha-terpinene and carvacrol showed significantly greater repellency than a commercial formulation, N,N-diethyl-m-methylbenzamide (DEET), whereas thymol showed repellency similar to that of DEET. The duration of repellency after application for all these monoterpenes was equal to or higher than that of DEET. These findings indicate that a spray-type solution containing thymol and carvacrol may serve as an alternative mosquito repellent.

We did not study the repellent effect of oils applied on human skin in our research. Nevertheless, we found that relatively low oil doses exerted a very strong antiovipositional effect on gravid females of *C. quinquefasciatus*

(Pavela et al. 2009). This effect was much higher than that of some other plant extracts. For example, Rajkumar and Jebanesan (2005) determined the oviposition deterrent effects of *Solanum trilobatum* against gravid females of *Anopheles stephensii*. They found an efficiency value of approximately 18% for the solution concentration of 0.01%, whereas in our research, we determined an almost 100% oviposition deterrence for this concentration.

Moreover, as found for example by Al-Bayati (2008), *T. vulgaris* oil may show a very significant synergistic effect with some other essential oils and methanol extracts. Some authors also studied synergistic relationships among individual substances contained purely in essential oils. In particular, some monoterpenes may represent significant synergens (Hummelbrunner and Isman 2001; Pavela 2008b; Yen and Chang 2008).

All these results we obtained, confirmed also by the results of other authors, should lead us to concluding that thymol and carvacrol and/or plant isolates containing these compounds are highly perspective for the development of botanical insecticides suitable for fighting against *C. quinquefasciatus* and *M. domestica*.

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