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Laboratory evaluation of repellency of traditional Czech homemade repellents against *Aedes aegypti*

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ABSTRACT

Objectives: In the Czech Republic, autochtonous transmission of mosquito borne diseases is not common; however, the need for personal protection should not be underestimated. Many people still rely on homemade repellents utilizing recipes based on local folk wisdom that are published annually in local Czech media. Despite minimal disease risk, nuisance biting and potential allergic responses make it essential to evaluate the chemical composition, effect, and duration of four homemade repellents often used and determine the necessity for public health education on application and use of alternative repellent products.
Methods: A review of local web-based media was conducted to identify the most commonly advertised homemade repellent products. The top four products were rosemary (*Rosmarinus officinalis*), sagebrush (*Artemisia absinthium*), walnut-tree (*Juglans regia*) leaves and clove (*Syzygium aromaticum*). These repellents were then prepared following the published recipes to evaluate their repellency effects, and reveal potential allergen presence. A bioassay against *Aedes aegypti* was conducted on ten volunteers for each repellent and the chemical composition was detected using gas chromatography.
Results: Significant initial repellency effect was found in mixtures of the clove (73.1%) and walnut leaves (49.0%) with ALPATM herbal embrocation after 10 minutes. The efficacy decreased to 46.5% and 34.3 % after 30 minutes, respectively; and, 30.3 and 18.2%, 60 minutes after the application. The remaining two samples, *Rosmarinus officinalis* and *Artemisia absinthium* solutions, exhibited no significant effects against *Ae. aegypti*. The evidence of allergens including cinnamic aldehyde, eugenol and coumarin were detected indicating potential concerns for product safety.
Conclusion: The homemade repellents reviewed were either ineffective or had unstable repellency effect within one hour. The low efficacy of these products may be appropriate to decrease nuisance biting, but should not be considered for primary prevention against mosquito borne diseases in areas with active disease transmission. Additionally, more research is needed to assess rates of allergic responses to homemade repellent products.

KEYWORDS
personal protection – insect repellent – efficacy test – allergen content

SOUHRN

Kulma M., Bubová T., Koleška D., Ševčík V., Koya Allen, Galková Z.: Laboratorní hodnocení účinnosti tradičních českých domácích repelentů proti *Aedes aegypti*
Cíl práce: Autochtonní přenos nemocí, kde komáři figurují v roli vektorů, není v České republice častým jevem, a komáři jsou zde proto považováni především za obtížný hmyz, jenž při kalamitách významně snižuje kvalitu života v postižených oblastech. Osobní ochrana by proto neměla být podceňována. V současné době jsou zde stále s oblibou využívány repelenty domácí výroby. Cílem tohoto článku bylo přinést nové poznatky o jejich účinku a chemické složení, včetně detekce potenciálních alergenů, těchto repelentů.
Metodika: Na základě on-line dostupných receptur byly vybrány a následně připraveny čtyři varianty domácích repelentů, s obsahem listů z rozmarýnu (*Rosmarinus officinalis*), pelyňku (*Artemisia absinthium*), ořešáku (*Juglans regia*) a celých hřebíčků (*Syzygium aromaticum*). Byliny použité k přípravě testovaných repelentů byly získány z běžně dostupných komerčních zdrojů bez bližší specifikace. Účinnost takto připravených repelentů byla otestována na komárech *Aedes aegypti* na 10 dobrovolnících pro každý přípravek. Dále byla provedena plynová chromatografie za účelem detekce potenciální přítomnosti alergenů.
Výsledky: Po 10 minutách od aplikace repelentu vykázal vyšší repelentní účinek pouze hřebíčkový extrakt (73,1 %) a výluh ořešákových listů (49 %), oba v kombinaci s přípravkem ALPA™. V průběhu testování se účinnost postupně snižovala na 46,5 %, respektive 34,3 % po 30 minutách a 30,3 %, respektive 18,2 % po 60 minutách, kdy byl test ukončen. Výluhy z rozmarýnu a pelyňku neměly žádný repelentní efekt. Screeningovým měřením byly v testovaných vzorcích detekovány některé potenciální alergeny včetně cinnamaldehydu, eugenolu či kumarinu.
Závěr: Repelence testovaných přípravků byla během 60minutového testu nevýznamná nebo nepříliš stabilní. Repelenty vyrobené z hřebíčku a ořešáku v kombinaci s alkoholovým přípravkem mohou být považovány za alternativu ke snížení obtížnosti bodavého hmyzu, nicméně je nelze považovat za ochranu spolehlivou a vhodnou pro oblasti s častým výskytem komáry přenosných onemocnění. Vzhledem k prokázané přítomnosti potenciálních alergenů je třeba zvážit nejen rizika využití repelentů domácí výroby z hlediska přenosu patogenů, ale i možných alergických reakcí u citlivějších jedinců.

KLÍČOVÁ SLOVA
osobní ochrana – repelent proti hmyzu – test účinnosti – alergeny

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INTRODUCTION

Globally, mosquitoes are considered the deadliest animals in the world, whose ability to transmit pathogens leads to millions of deaths each year [1]. The geographic distribution of medically important vector species continues to expand due to globalization and anthropogenic factors including international travel, trade, and climate change, leading to increasing numbers of people living in areas at risk for disease introduction and transmission [2, 3].

Emergence of mosquito-borne diseases is of increasing concern across Europe as evidence for introduction and maintenance cycles are established. Totally, 45 mosquito species are currently present in the Czech Republic, including potential vectors belonging to the *Aedes, Culex* and *Anopheles* mosquito species. Moreover, few arbovirus pathogens have recently been detected in the region [4, 5] and emergence of the first human cases or even outbreak is possible [6]. The autochthonous transmission of human *Dirofilaria* recently reported from South Moravia [7] then highlighted the importance of surveillance and public health education for mosquito-borne disease prevention. "Key public health concerns in the Czech Republic remains focused on nuisance-biting of insects; however, the need for personal protection should not be underestimated."

Protection against arthropod bites is usually provided by products containing substances with repelling properties. These products are defined by their ability to force arthropods to move away from a repellent's source, and may be applied directly to skin, clothing or shelter [8]. Historically, people used many plants or smoke to protect themselves; however, these repellents had limited duration. Extensive research began during World War II to find long-lasting repellents, and the breakthrough product, DEET (N, N-diethyl-3-methylbenzamide), is still one of the most widely used repellents [9]. Nevertheless, it has become very modern to replace synthetic products with natural and homemade alternatives in the recent years and this has also been exemplified in the field of personal protection against mosquitoes and other arthropods [10]. This trend is followed by local mass media, which annually publishes articles containing instructions on how to prepare homemade repellents. Even though these instructions are generally based on folk wisdom, they are considered to be natural, cheap, have easy applications, and effective as alternative repellents. The real efficacy of these homemade repellents still remains unknown and has not been scientifically verified. As a result, this article aims to determine the chemical composition, repellency effect and duration of commonly used homemade products. Since the lay public very often considers natural and homemade repellents to be safer than synthetic products, it is also important to acknowledge that some substances commonly present in these repellents may be hazardous [11]. Therefore, this paper also addresses the presence of potential allergens.

METHODS

The study was conducted as a bioassay to determine the efficacy of skin homemade repellents commonly advertised in local Czech Republic media outlets. An adaptation of the guidelines used for similar purposes [12, 13] was used to meet the needs of this study using an individual as his own negative control to determine percentage efficacy and estimate protection time based on the time elapsed between repeated exposures. Gas chromatography was then used to determine the chemical composition of each repellent and identify potential allergens contained within.

Repellent preparations

During January-March 2017, the selection of homemade repellents was determined by a web-based media search and review of local newspapers, magazines and internet sources to identify the most common herbal recipes advertised as an alternative repellent. Four of alternative repellents were chosen and all herbs necessary to prepare the recipes and conduct the study were purchased in dry form from a local commercial source (Bylik, s. r. o., Vrchlabí, Czech Republic).

The 4 herbs for investigation were rosemary (*Rosmarinus officinalis*), sagebrush (*Artemisia absinthium*), walnut-tree (*Juglans regia*), and clove (*Syzygium aromaticum*). Each were prepared following the recipes identified in the media search. Rosemary, sagebrush and walnut-tree were in leaf form and used to prepare a respective solution for each. For each of these 3 herbs, 100 g of leaves were put into 1 litre of boiling water. After 5 minutes of boiling, the heating source was eliminated and herbs were then left to infuse in the water for 4 hours until the mixture settled at room temperature. For rosemary and sage brush solutions, the solid debris is removed from the liquid solution and 4 ml (approximately one spoon) of apple vinegar was added. The walnut tree leaves solution was then mixed in a 1:3 ratio with commercial ALPA™, alcohol herbal embrocation produced by a local company (Alpa, Velké Meziříčí, Czech Republic), that is commonly used for reflection massages, rheumatic pains of muscles or disinfection. For the last alternative repellent extract, 20 g of whole clove were put into 200 ml of ALPA™ embrocation for 7 days. Afterwards, the solid bits of clove were strained and discarded. Each of the prepared repellents were then stored at 5 °C until the start of bioassay.

Bioassay

Anthropophilic *Aedes aegypti* mosquitoes were selected as the model organism for purpose of the efficacy evaluation. The colony of *Ae. aegypti* used is maintained in the National Reference Laboratory (NRL) for Vector Control at the National Institute of Public Health in Prague (NIPH), Czech Republic. The colony is kept at 27 ± 2 °C and ≥80% relative humidity and larvae are fed on pellets for omnivorous laboratory animals. Before testing, adults were provided with 10% glucose water solution. The mosquitoes used for the test were 7–10 days old, after which, mating occurrence was observed. Laboratory efficacy tests were performed in the laboratory at the aforementioned conditions.

The repellents were applied in 2 ml doses directly on the skin on the forearm (from wrist to elbow) of volunteers. The individual's other forearm remained untreated as a matched negative control. For testing, 10 volunteers (5 men, 5 women) in 2 repetitions for each sample were used. Before the test, both hands of volunteers were

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Table 1. Efficacy of tested homemade natural repellents against *Aedes aegypti* mosquitoes expressed by total number of bites and per cent efficacy

Repellent	Repellent efficacy (%)					
	10 min.		30 min.		60 min.	
	No. of bites (TA/NC)	Efficacy (%)	No. of bites (TA/NC)	Efficacy (%)	No. of bites (TA/NC)	Efficacy (%)
Syzigium + ALPATM	71/263	73.1±18.6	122/240	46.5±24.4	186/268	30.3±17.2
Juglans LL + ALPATM	127/249	49.0±19.2	198/301	34.3±17.0	225/275	18.2±17.4
Rosmarinus LL + AV	232/234	≤ 10	ND	ND	ND	ND
Artemisium LL + AV	184/202	≤ 10	ND	ND	ND	ND

TA: treated arm, NC – negative control, LL – leaf leachate, AV – apple vinegar

washed with fragrance-free soap, rinsed with water and then washed with 70% aqueous solution of ethanol, and dried with a towel. During the efficacy test, both hands were inserted into the cages (size 25 x 25 x 30 cm), which contained cca 50 unfed females *Ae. aegypti* mosquitoes. After 1 minute exposure time, number of mosquito bites was recorded. Efficacy was calculated as the difference between the individual's treated arm (TA) and untreated negative control (NC) arm using the formula in Figure 1. This process was repeated at 10, 30 and 60 minutes post-treatment with the repellent substances. In the case that no significant difference between untreated and treated arm was observed, the test was terminated. Each product was evaluated on a different day and none of volunteers tested more than one repellent per day.

$$\text{Efficacy (\%)} = \frac{\text{Number of bites on NC - Number of bites on TA}}{\text{Number of bites on NC}} \times 100$$

Figure 1. Calculation of percent efficacy of alternative repellents

Gas chromatography

The gas chromatography – mass spectrometry (GC–MS) analyses were performed at the Unit for Chemical Safety of Products at NIPH using a Thermo Trace 1310 GC and Thermo Quantum XLS Ultra (Thermo Scientific, USA), equipped with a Rxi-17MS column (20 m x 0.18 mm i. d., 0.18 µm film thickness; Restek, USA). Helium (99.999%, Linde, Czech Republic) was used as the carrier gas at a constant column flow of 0.6 mL min/1. Split mode injection was used (20:1; 1 µL) and the injector temperature was kept at 250 °C. The oven temperature was held at 50 °C for 0.5 min, ramped at 20 °C min/1 to 145 °C, 1 °C min/1 to 156 °C, then 30 °C min/1 to 290 °C and held for 0.3 min. Transfer line and ion source temperatures were set at 300 and 200 °C. The mass spectrometer was operated in full scan mode from 30 to 400 m/z at 0.1 s per scan.

Sample preparation

Ethanolic leaf extracts were diluted in acetone and immediately injected to GC. Due to high range of analyte concentrations several dilutions from 1:40 to 1:1000 were used. Oasis Prime HLB SPE cartridge (60 mg, 3 mL, Waters) was first conditioned by 1 mL of methanol followed by 1 mL of deionized water at a flow rate of one drop per second. Then 100 mg of aqueous sample was passed through the column at a rate of 1–3 drops per second on a Supelco SPE vacuum manifold (Sigma Aldrich). After sample loading, the cartridge was washed with 1 mL of deionized water and the cartridge was then dried by a passage of air for 25 minutes (-0.7 bar). The analytes were then manually eluted by 900 µL of acetone into a 2-mL glass vial at a flow rate of one drop per second. Eluate was then injected into the GC.

RESULTS

The repellency effect was found only in mixtures of clove and walnut leaf (Table 1) solutions with ALPA™ herbal embrocation. Initial repellency at 10 minutes after application was found to be 49.0 and 73.1% respectively (see Table 1). The other two samples (*Rosmarinus* and *Artemisia* leaf leachates) exhibited no significant repellency effect. Regarding the chemical compositions, the sample with clove and ALPA™ showed the richest compound content (see Table 2), and no major substances were detected in the mixture of *Artemisia* leachate and apple vinegar.

Table 2. List of chemical compounds detected in tested homemade repellents

Syzigium + ALPA alcohol embrocation	*Rosmarinus* leachate + apple vinegar	*Juglans* leachate + ALPA alcohol embrocation
Eucalyptol	Eucalyptol	Eucalyptol
Isopulegol	Camphol	Isopulegol
Levomenthol	Camphor	Terpinyl acetate
Methyl salicylate	Verbenone	Menthol
Bornyl acetate	Terpineol	–
Copaene	Coniferol	–
Chavicol	–	–
Terpinyl acetate	–	–
Vanillin	–	–
Caryophyllene oxide	–	–
Eugenol acetate	–	–
Humulene	–	–
Caryophyllene	–	–
Menthol	–	–

Allergen composition of the tested repellents is displayed in Table 3. The samples contained 7–8 ingredients that might

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be considered allergens. Limonene, linalool, benzyl alcohol and eugenol were present in all of the tested repellents.

Table 3. The content of hazardous ingredients in tested samples

Analyte	Repellent			
	Syzigium +ALPA	*Juglans* LL +ALPA	*Artemisia* LL	*Rosmarinus* LL
Limonene	*	*	*	*
Linalool	*	*	*	*
Benzyl alcohol	*	*	*	*
Cinnamaldehyd	*	*	–	–
Anisyl alcohol	–	–	*	–
Eugenol	*	*	*	*
Isoeugenol	*	–	*	*
Coumarin	–	–	*	*
Benzyl benzoate	*	*	–	–
Benzyl salicylate	*	*	–	–

Abbreviations: LL – leaf leachate, *-present

DISCUSSION

The laboratory tests were performed to estimate the effect of Czech homemade repellents. A repellency effect was found in only two of the four tested repellents. Laboratory tests serve as an initial effort to gather data on product efficacy. To reveal an accurate efficacy of these repellents in the natural environment of mosquitoes, further research including field tests is essential.

The highest initial repellency was observed at 10 minutes after treatment, in clove alcohol extract, which was more than 70%. Unfortunately, this effect was quite short, with efficacy decreasing to less than 50% after 30 minutes and only 30.3% after 60 minutes. This result could be expected as previous studies by Barnard (1999) [13], Phasomkusolsil et Soonwera (2011) [14], and Sritabutra et al. (2011) [15], all identified clove essential oil as the one of the most efficient plant-based natural repellents. In contrast, effects measured by these authors were much longer and more stable in comparison to this study. These earlier studies found clove-repellents to provide up to 100% protection against *Ae. aegypti* between 56–225 minutes. This may indicate that even though herbal embrocation ALPA™ contains substances whose positive insecticidal effect includes menthol [16] and eucalyptol [17], the manufacture process that is considered traditional in the Czech Republic and which was used for purposes of this study, is probably not the best way to facilitate maximal repellency potential of the *Syzygium* plant.

To the best of our knowledge, the effect of *Juglans* tree leaf leachate against mosquitoes has not been determined. However, its use as a traditional antiparasitic treatment against lice is known from studies in Italy [18] so a potential repellency effect may be assumed. Considering chemical composition of this solution and subsequent comparison with ALPA™ embrocation composition, repellent compounds presented in the solution were likely provided from the herbal embrocation. The total numbers of active compounds, found by gas chromatography analysis, were even lower than in *Rosmarinus* solution but some repellency effect was observed.

Finally, no effect against *Ae. aegypti* was found for mixtures containing rosemary and sagebrush, even though repellent, acaricidal or insecticidal activity of rosemary [19, 20] and sagebrush [21, 22] formulations have been recently documented. In this study, no difference was found between treated and untreated forearms 10 minutes after application, so the experiment was terminated. However, some repellent substances were found in the rosemary solution, so it is possible that repeated or different application may reveal a repellency effect.

Based on these results, since only the alcohol based extracts worked, the question can be raised of whether it is possible that only the ALPA™ alcohol embrocation itself, used as part of solutions, could have some effect against mosquitoes. While a valid supposition, this study focused solely on the efficacy evaluation of the recipes for homemade repellents as whole mixtures not each component separately. Moreover, no information or record about using only the ALPA™ embrocation for this purpose currently exists.

Obtained results showed, that tested homemade repellents could obviously did not reach as strong an effect as is provided by most common synthetic repellents. For example, formulations containing 10% DEET and IR3535 (ethyl N-acetyl-N-butyl-β-alaninate) are reported to provide 100% protection against *Ae. aegypti* for 65–290 and 64–320 minutes, respectively [23]. Moreover, unpublished data provided by NRL for Vector Control showed that the same effect of products with the same active substances could last up to 4 hours, depending on the concentration.

This study also aims to address the safety of the homemade repellents and potential for adverse effects from allergens, so chromatographic profiles were used to detect potential allergens. Even though it is commonly assumed that natural and homemade repellents must be safer than the synthetic products, this may not be true at all. Plants contain many toxins that can cause significant human or animal health effects [24]. Conversely of synthetic commercial repellents, many plant-based products, with potential natural repellent effect, do not undergo any compulsory safety tests and may contain some substances that can be hazardous to human health, so their concentrations must be taken into account. The list of safe concentrations for some of natural repellents are available by e. g. Strickman et al. (2009) [11], but during the home-made repellent preparation, the line of safe concentration could be easily overcomed. Based on gas chromatography, several allergens were found in the investigated samples. One of them, cinnamic aldehyde presented in *Syzigium* and *Juglans* alcohol mixtures, are considered strong or potent allergens [25], with hypersensitivity of tested persons reaching 1% [26]. Eugenol, which was detected in all of the samples, is considered by Strickman et al. [18] as a sensitive skin irritant, belonging to the group of "rarely found allergens [25]" but caused an allergic reaction in 0.4% of tested persons in a study by Bauman et al. [26]. The same percentage of hypersensitivity was also reported for coumarin, which was detected in *Artemisia* and *Rosmarinus* leaf extracts. The risk of the other detected ingredients, according to Schnuch et al. [25] was too small to consider and their potential to cause a reaction is < 0.1% [26]. Fortunately,

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no allergic reaction was been observed in volunteers during this experiment.

CONCLUSION

Plant-based traditional repellents are cheap and "easy to prepare" alternatives to commercially synthetic products. However, their efficacy was found to be significantly lower if any effect at all. The tested repellents were either ineffective or had a short and unstable repellency effect. Since mosquito density in the Czech Republic, with the exception of irregular flooding, is not very high and generally pathogen-free, some of these repellents could serve as an alternative personal protection and could decrease nuisance biting. On the other hand, it is necessary to consider the potential consequences including allergen contents and no ultimate protection.

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