Journal of Ethnopharmacology 231 (2019) 386–393

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Bioassay-guided isolation of active compounds from *Adenosma buchneroides* essential oil as mosquito repellent against *Aedes albopictus*

Yongpeng Ma[a,b], Mingxiang Li[a,b], Hongxia Zhang[a], Hongzheng Sun[c], Honghai Su[c], Yuhua Wang[a], Zhizhi Du[a,*]

[a] Key Laboratory of Economic Plants and Biotechnology, Yunnan Key Laboratory for Wild Plant Resources, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, China
[b] University of Chinese Academy of Sciences, Beijing 100049, China
[c] Kunming City Center for Disease Control and Prevention, Kunming 650034, China

ARTICLE INFO

Keywords:
Adenosma buchneroides
Ethnobotanical survey
Mosquito repellent
Carvacrol
Structure-activity relationship

ABSTRACT

Ethnopharmacological relevance: A folk herb *Adenosma buchneroides* found in the previous ethnobotanical investigation plays an important role as an insect repellent among the Aini people in southwest of China, but the active compounds responsible for repellent activity of the plant have not yet been investigated.
Aim of the study: The main purpose of the study is to identify the active components of the essential oil which responsible for its repellent activity against *Aedes albopictus* to support the usage of the plant as mosquito repellent by Aini people. In addition, to supply a class of potential alternatives characterized carvacrol analogues to develop natural repellent products.
Material and methods: The essential oil from aerial part of *Ad. buchneroides* was extracted by hydrodistillation. A systematic bioassay-guided isolation of repellent compounds from the essential oil was conducted through chromatographic fractionation combined with in-cage mosquito repellent bioassay. The identification of the essential oil components was accomplished by GC-MS and GC-FID techniques. The structural elucidation of compounds was performed on the basis of IR, HR-ESI-MS and NMR. Larvicidal activity and cytotoxicity of all repellent compounds also tested by larval bioassays and MTS assays, respectively. Structure-activity relationship (SAR) of carvacrol analogues was investigated by in-cage mosquito repellent bioassay.
Results: The essential oil of the plant showed strong mosquito repellent activity with minimum effective dosage (MED) of 0.019 ± 0.007 mg/cm^2, compared to reference standard *N,N*-diethyl-3-methylbenzamide (DEET) (0.031 ± 0.014 mg/cm^2). 26 compounds representing 97.8% of the essential oil were identified. Carvacrol, carvacrol methyl ether and a new fragrant compound, adenosmin A (**1**) were found to be repellent compounds by systematic bioassay-guided isolation, with MEDs in the range of 0.011–0.125 mg/cm^2. An investigation on SAR of carvacrol analogues led to the discover of three analogues with further lower MEDs (0.002–0.009 mg/cm^2) than that of DEET, and other three compounds with similar MEDs (0.029–0.039 mg/cm^2) to that of DEET. Carvacrol (LD_{50} of 24.8 ppm) was the best larvicide among tested repellent compounds. The essential oil and repellent compounds against seven mammalian cell lines revealed low or no cytotoxicity.
Conclusions: Scientific evidences reported here validate the plant's traditional use as insect repellent and imply promising application of the essential oil and carvacrol analogues as natural mosquito repellents.

1. Introduction

Mosquitoes can spread a variety of mosquito-borne diseases, such as malaria, yellow fever, dengue fever, Chikungunya, Zika virus disease, west Nile fever, epidemic encephalitis B and filariasis, etc. Until now, more than 40 kinds of virus reported result in mosquito-borne diseases (Brown and Hebert, 1997). Every year more than 700 million people are infected with mosquito-borne diseases worldwide, and most of them infected with malaria by Anopheles biting (Taubes, 2000). The World Health Organization reported that the number of malaria infections in

Abbreviations: MED, minimum effective dosage; DEET, *N,N*-diethyl-3-methylbenzamide; IC_{50}, the concentration of 50% of inhibition; LD_{50}, 50% lethal dose; LD_{90}, 90% lethal dose; CI, confidence interval; DF, degree of freedom; AR, analytical reagent; AChE, acetyl cholinesterase; CAT, catalase; CarE, carboxylesterase

* Corresponding author.
E-mail address: duzhizhi@mail.kib.ac.cn (Z. Du).

https://doi.org/10.1016/j.jep.2018.11.031
Received 14 September 2018; Received in revised form 20 November 2018; Accepted 20 November 2018
Available online 22 November 2018
0378-8741/

the world had dropped from 262 million to 214 million from 2010 to 2015, and the number of malaria deaths declined from 840,000 to 440,000 annually. This remarkable result was attributed mainly to application of pesticides and artemisinin-based combination therapy. However, the development of mosquito resistance to insecticides and plasmodium resistance to artemisinin were becoming a serious threat to achieving global malaria control, similar as dengue fever, epidemic encephalitis B, Zika virus disease and other mosquito-borne diseases (World Health Organization, 2015).

Indeed, the use of insecticides and repellents are major strategies of preventing mosquito-borne diseases (Hoel et al., 2010) and accelerating the process of new drug research and development is the most important measure to control mosquito resistance. However, few new insecticides have been developed to fight mosquito resistance (Meepagala et al., 2013). DEET (*N*,*N*-diethyl-3-methylbenzamide) used most often as repellent on the market has caused neurotoxicity, dermatitis, and allergic reactions (Corbel et al., 2009; Tabanca et al., 2013a). The development of mosquito resistance to DEET has also been reported (Brown and Hebert, 1997). Hence, an important research effort is necessary to continue to identify new mosquito repellents as alternatives to DEET, especially botanical repellents to meet the public demand for more natural products. Prior to the arrival of synthetic chemicals, utilization of plant-derived materials to repel or kill medically important arthropods including mosquitoes has occurred for centuries. Recently, plant essential oils have got greater concern as main sources for new natural repellents, due to their broad-spectrum activities against target insects, biodegrading to nontoxic products, and low side effects of non-target organisms and environment (Nerio et al., 2010; Isman, 2006; Ali et al., 2015). The genus *Adenosma* (Scrophulariaceae) contains about 15 species worldwide distributes in tropical eastern Asia and tropical Oceania, with essential oils from most of the species and traditionally used for herbal medicine. There are 4 species in China, among them 3 species are in Yunnan province of southwestern China (Editorial Committee of Flora of China, 1998). The activities of essential oils from the genus are rarely documented, only Huixing et al. (1998) reported positive insecticidal activity of the essential oil from *Adenosma buchneroides* against *Callosobruchus maculatus*.

In the 1980s, Prof. Pei found a herb named fleagrass with pleasant smell in ethnobotanical field survey of Xishuangbanna in southwestern Yunnan province. This fleagrass was then identified taxonomically as *Adenosma buchneroides* Bonati and called as “Lao-wo-shou-du” by local Aini people (one branch of the Hani minority group). It was also cultivated in swidden fields together with upland rice as an inter-cropping system and used as insecticide and folk medicine for treatment of cold and cephalalgia. Especially, it was not only hung in the rooms and spreaded on the beds to repel insects such as fleas and mosquitoes, but also boiled into potions to smear or wash the bump bitten by mosquitoes for relief of swelling and pain (Shen et al., 1988). Duo to the potential of fleagrass for making insecticide and herbal medicine, its introduced cultivation had been made by Xu et al. (2008) and is becoming a new productive economically industrial crop.

Indigenous knowledge and practices by local people demonstrated the extensive information available associated with native plants with potential usages to repel or kill medically important arthropods such as mosquitoes and is well documented (Tisgratog et al., 2016). Few of these traditional knowledge and usages have been investigated systematically to determine the efficacy and the chemical constituents responsible for the activity. Although *Ad. buchneroides* plays an important role in Aini people's daily life for its traditional usages on variety of purposes, especially as insect repellent, there is no report about its related research. Only a preliminary study showed γ-terpinene (40.26%), cavacrol(34.98%), *p*-cymene (6.60%), *α*-terpinene (4.05%), carvacrol methyl ether (3.42%) were identified as main components in the essential oil of fresh stem and leaves (Xu et al., 2008). A preliminary bioassay experiment at our lab revealed that essential oil of *Ad. buchneroides* has potent repellent activity against *Aedes albopictus*.

One of the objectives of our current study was to identify the components of the essential oil of *Ad. buchneroides* responsible for mosquito (*Ae. albopictus*) repellent activity by bioassay-guided fractionation of the oil. To discover and develop more and stronger mosquito repellents, structure-activity relationship (SAR), larvicidal activity and cytotoxicity of active compound were also investigated.

2. Materials and methods

2.1. Chemicals

The following materials were used: AR grade *n*-hexane (Damao, Tianjin, China), AR grade acetone (Rionlon, Tianjin, China), AR grade methanol (Huada, Guangzhou, China), AR grade dimethyl sulfoxide (DMSO, Fengchuan, Tianjin, China), petroleum ether (bp. 60–90 °C, Damao, Tianjin, China), chloroform (Rionlon, Tianjin, China), ethyl acetate (Jige, Tianjin, China), ethanol absolute (Damao, Tianjin, China), sulfuric acid (Xilong Chemical Co. Ltd., Guangdong, China), vanillin (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China), water (Wahaha Group Co. Ltd, Hangzhou, China). Carvacrol (CAS# 499-75-2), carvacrol methyl ether (CAS# 6379–73-3), *p*-cymene (CAS# 99-87-6), and thymol methyl ether (CAS# 1076-56-8) were purchased from Sigma-Aldrich (Shanghai, China). Thymol (CAS# 89-83-8) and 3-methyl-4-isopropylphenol (CAS# 3228-02-2) were purchased from YuanYe Bio-technology Co., Ltd. (Shanghai, China). *p*-Menthan-2-ol (CAS# 60320-28-7) was purchased from FCH Group Reagents for Synthesis (Riga, Latvia). 4-Methyl-2-isopropylphenol (CAS# 4427-56-9) and 2-methyl-4-isopropylphenol (CAS# 1740-97-2) were purchased from J & K Scientific Ltd. (Guangzhou, China). 4-Methyl-3-isopropylphenol (CAS# 4371-46-4) was purchased from Atomaxchem Co., Ltd. (Shenzhen, China). DEET (CAS# 134-62-3) was purchased from Usolf Chemical Technology Co., Ltd. (Qingdao, China).

2.2. General experimental procedures

Optical rotations were determined with a Horiba Sepa-300 polarimeter (Horiba, Tokyo, Japan). UV spectra were measured using a Shimadzu UV-2401A spectrophotometer (Shimadzu, Tokyo, Japan). CD spectra were recorded by a Chirascan circular dichroism spectrometer (Chirascan, Surrey, UK). A Tensor 27 spectrophotometer (Bruker, Bremen, Germany) was used to obtain infrared (IR) spectroscopy using KBr pellets. Nuclear magnetic resonance (NMR) spectra were acquired on a Bruker AVANCE III 500 spectrometer (Bruker, Bremen, Germany) at 500 MHz for ^{1}H NMR and at 125 MHz for ^{13}C NMR (chemical shift values (δ) are in ppm with reference to the solvent signals). Electrospray ionization mass spectrometry ESI-MS data were obtained on a Bruker/HCT Esquire spectrometer (Bruker, Bremen, Germany). High resolution electrospray ionization mass spectrometry (HR-ESI-MS) data were recorded on an Agilent G6230 TOF MS spectrometer (Agilent Technologies, Santa Clara, CA).

Column chromatography (CC) was performed using silica gel (200–300 mesh, Qingdao Marine Chemical Co., Ltd., China), silica gel H (10–40 μm, Qingdao Puke Parting Materials Co., Ltd., China), and Sephadex gel LH-20 (GE Healthcare Bio-Sciences AB, Sweden). Thin layer chromatography (TLC) was conducted on glass-backed plates precoated with silica gel GF_{254} (50 × 100 mm, 10–40 μm, Qingdao Marine Chemical Co., Ltd., China), and visualization was made by heating silica gel plates sprayed with 1% vanillin and 10% H_2SO_4 in ethanol.

2.3. Plant material

Aerial part of *Ad. buchneroides* Bonati was collected from Mengla county of Yunnan Province, China, and identified by Professor Shenji Pei at Kunming Institute of Botany, Chinese Academy of Sciences. A voucher specimen (KUN 1341141) was deposited in Herbarium of

Kunming Institute of Botany, Chinese Academy of Sciences.

2.4. Extraction of the essential oil

Aerial part of *Ad. buchneroides* was subjected to hydrodistillation for 3 h using the standard apparatus described in the Chinese Pharmacopeia (State Pharmacopeia Commission, 2015). The essential oil was separated from the aqueous phase produced during distillation and dried by Na_2SO_4. Bright yellowish oil (70.2 g) was obtained from the air dried *Ad. buchneroides* (6.0 kg) with 1.17% extraction yield.

2.5. Gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) analyses

The GC-MS analyses of essential oil from *Ad. buchneroides* and its subfractions were carried out using an Agilent 5975 C chromatographer with a mass spectrometer detector (Agilent Technologies, Santa Clara, CA, USA). An apolar HP-5 capillary column (Agilent 19091J-115, 5% diphenyl polysiloxane, 50 m × 0. 2 mm i.d., 0.33 μm film thickness) was used. The oven temperature was programmed rising from 50 °C to 250 °C at a rate of 5 °C/min, and then kept at 250 °C for 10 min, and finally programmed rising to 300 °C at a rate of 10 °C/min (total run time of 55 min) with helium as the carrier gas (2.1 mL/min). The injector temperature was set at 250 °C and all injections were performed in split mode adjusted at 20:1. The mass spectrometer conditions were as follow: ionization potential 70 eV; ion source temperature 150 °C; electron ionization mass spectra were recorded over the mass range 50–550 *m/z*.

The GC analyses were performed using an Agilent 7890A GC chromatographer (Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionization detector (FID) whose temperature was set to 250 °C, and the same operational conditions described for the GC-MS analyses were used.

Relative percentages of the separated compounds were calculated from integration of the peak areas in the GC-FID chromatograms. Components of the essential oil were identified by comparison with reference standards (including *p*-cymene, carvacrol, thymol and carvacrol methyl ether) or by comparison with their relative retention index reported in the literature (RRI_{lit}) by Adams (2007), determined relative to the retention times of a series of *n*-alkanes (C_8–C_{30}) (Curvers et al., 1985), and by comparison with their fragmentation patterns in the mass spectra with those listed in commercial mass spectral libraries (NIST14).

2.6. Bioassay-guided fractionation and purification of active compounds

The essential oil from *Ad. buchneroides* was evaluated for its mosquito repellent activity against *Ae. albopictus*. A systematic bioassay-guided fractionation was carried out using in-cage mosquito repellent bioassay to isolate and identify compounds with mosquito repellent activity.

Ad. buchneroides essential oil (60.00 g) was fractionated by column chromatography (φ 9.6 cm × 53 cm) on silica gel with petroleum ether-EtOAc solvent system as eluent. After gradient elution with petroleum ether (7.2 L), petroleum ether-EtOAc (98:2, 10.8 L; 95:5, 8.1 L; 90:10, 7.2 L; 80:20, 4.5 L; 50:50, 9.9 L; 25:75, 6.3 L), five fractions were obtained on the basis of TLC analysis.

Fr. 1–5 subfractions were analyzed by GC-MS and all fractions were evaluated for mosquito repellent activity against *Ae. albopictus*. Subfractions (Fr. 1–5) were as follows: Fr. 1, 28.968 g (39.7% γ-terpinen, 39.2% *p*-cymene, 15.4% *β*-bisabolene, 5.7% various minor components); Fr. 2, 7.458 g (90.8% carvacrol methyl ether, 9.2% various minor components); Fr. 3, 12.306 g (92.1% carvacrol, 7.9% various minor components); Fr. 4, 186 mg (87.7% adenosmin A, 12.3% various minor components); Fr. 5, 5.628 g (various minor components).

A portion of Fr. 1 (3.467 g) was chromatographed on a Sephadex gel LH-20 column (φ 2.3 cm × 116 cm) with acetone as eluent to yield *β*-bisabolene (**2**) (124 mg). Fr. 2 (7.458 g) was purified by silica gel column (φ 3.6 cm × 38 cm) and eluted with petroleum ether-$CHCl_3$ as eluent in a gradient mode (99:1, 4.5 L; 98:2, 3.6 L; 95:5, 3 L) to afford carvacrol methyl ether (**3**) (5.970 g). Fr. 3 (12.306 g) was purified by silica gel column (φ 4.1 cm × 60 cm) and eluted with petroleum ether-$CHCl_3$ as eluent in a gradient mode (12:1, 6 L; 6:1, 4.8 L; 3:1, 2 L) to afford carvacrol (**4**) (10.637 g). Fr. 4 (186 mg) was purified by silica gel H reduced pressure column (φ 3.0 cm × 11 cm) and eluted with petroleum ether-$CHCl_3$ as eluent in a gradient mode (1:0, 400 mL; 7:1, 300 mL; 3:1, 120 mL) to afford adenosmin A (**1**) (151 mg).

2.7. Compound characterization

*Adenosmin A (**1**):* Colorless oil; $[\alpha]_D^{21}$–5.97 (*c* 0.25, MeOH) ; UV (MeOH) λ_{max} (nm) (log *ε*): 203 (4.86), 219 (4.37), 277 (3.67) ; CD (MeOH) λ_{max} (Δ*ε*) 202 (-0.56) nm; IR (KBr) ν_{max} cm^{-1}: 3437, 2962, 2930, 2872, 1724, 1631, 1507, 1460, 1384, 1251, 1127, 1024 ; ^1H and ^{13}C NMR data, see Table 2. ESI-MS *m/z* 325 $[M+Na]^+$, *m/z* 341 $[M+K]^+$, *m/z* 627 $[2M+Na]^+$; HR-ESI-MS *m/z* 325.2139 $[M+Na]^+$ (calcd for $C_{20}H_{30}O_2Na$, 325.2138).

2.8. In-cage mosquito repellent bioassay

The pupae of *Ae. albopictus* from the Kunming City Center for Disease g and Prevention (Kunming, China), were reared in the culture room at 26 ± 1 °C and 60 ± 10% relative humidity (RH) under a cycle of light and dark (12 h:12 h). Female adults aged 5–10 days were preselected as experimental mosquitoes using a hand-draw box (Posey and Schreck, 1981). Repellency was defined as the minimum threshold surface concentration (MED) that resulted in no more than a given number of bites through the treated surface (Schreck and Smith, 1977). Approximately 500 (± 10%) mosquitoes were selected and put into a stock cage (45 cm × 35 cm × 37.5 cm) for 25 (± 2.5) min before testing (Tabanca et al., 2013b).

An initial mass of test sample was weighed in 5-mL vials containing 2 mL of ethanol as solvent, then 1 mL of solution was removed and uniformly absorbed on 50 cm^2 cotton cloth (5 cm × 10 cm) by 1-mL pipette (Dragonmed, United-Bio Co., Ltd., ShangHai, China), which would make an initial concentration of 1.5 mg/cm^2 on the cloth. Serial dilutions were then performed to produce corresponding concentrations (i.e. 1.5, 0.75, 0.375, 0.187, 0.094, 0.047, 0.023, 0.011, 0.005, and 0.002 mg/cm^2) on the cloth using the remaining 1 mL solution. The treated cloth was allowed to dry for 3–5 min before starting the bioassay.

Two layers of PE plastic film (Cleanwrap, Cleanwrap Plastic Co., Ltd., ShangHai, China) covered hand and arm of a volunteer to avoid bites. An opening (4 cm × 8 cm) was then cut on the half way between the wrist and elbow in order to allow odors from the skin surface to escape out and attract mosquitoes to the opening. The 32 cm^2 opening cut was covered with sample-treated cotton cloth during testing.

Zero to four bites through the treated cloth denoted pass of a concentration of sample during 1 min test period, then lower treatment concentrations were assessed until it failed. If a concentration of 1.5 mg/cm^2 on cloth resulted in failure, the MED was then recorded as ineffective at this highest concentration. Due to decreasing behavioral activity of mosquitoes under repeated exposure to samples and attrahent odors from the arm, the tested mosquitoes were permitted a 15 min recovery time after 10 successive tests. There were four male volunteers in the assay, and all volunteers signed written informed consent. The protocol was approved by the Kunming Center for Disease Control and Prevention. In the study, the oil, subfractions and pure compounds (*β*-bisabolene, carvacrol methyl ether, carvacrol and adenosmin A) from *Ad. buchneroides* and eight carvacrol analogues (*p*-cymene, *p*-menthan-2-ol, carvacrol methyl ether, thymol, 3-methyl-4-isopropylphenol, 2-methyl-4-isopropylphenol, 4-methyl-2-

isopropylphenol, and 4-methyl-3-isopropylphenol) were used as test samples. DEET was used as a positive control. Ethanol was used as the solvent control.

2.9. Larval bioassays and cytotoxicity assay

Larvicidal activity of essential oil and repellent compounds from *Ad. buchneroides* against *Ae. albopictus* was determined by the high-throughput screening larval assays described by Pridgeon et al. (2009). In short, eggs were hatched in a culture room (at a temperature of 26 ± 1 °C and a cycle of 12:12 (L:D) h) by loading a piece of filter paper with eggs in a beaker full of 100 mL of deionized water comprising a small amount of larval diet [2% slurry of rat feeds (Specialty Feeds, Specialty Feeds Pty Ltd., Perth, Australia)]. Larvae were then maintained overnight in a beaker at a temperature-controlled culture room (at a temperature of 26 ± 1 °C and 60 ± 10% RH at a cycle of 12:12 (L:D) h). Five 1-day-old first instar larvae of *Ae. albopictus* were sucked into each well of 96-well plates by a 8-cm Pasteur pipet with a droplet of water. 40 µL of larval diet and 950 µL deionized water were added to each well using a Dragonmed pipette. All samples to be evaluated were diluted in dimethyl sulfoxide (DMSO). Diluted test sample (10 µL) was added to the labeled wells containing a total volume of 1 mL of larvae testing liquid. As solvent control, 10 µL of DMSO alone was added to each well. Larval mortality was noted 24 h after treatment. Larvae without movement in the well after manual disturbance of water by a pipet tip denoted dead. A series of dosages (four to six concentrations) providing a range of mortality between 0 and 100 were used in each larval assay. Treatments were replicated 5–10 times at each concentration.

The detailed experimental procedure of cytotoxicity assay of the essential oil and isolated repellent compounds was described in the Supplementary material, Text S1.

3. Results and discussion

3.1. Chemical analysis of the essential oils

Hydrodistilled essential oil from aerial part of *Ad. buchneroides* was analyzed by GC-FID and GC-MS systems. The identification of chemical constituents of the essential oil was performed through comparison their fragmentation patterns in the mass spectra with those listed in commercial NIST14 mass spectral libraries (MS) and comparison to relative retention index (RRI) reported in the literature (RRI_{lit}). Some of the components including *p*-cymene, carvacrol, thymol and carvacrol methyl ether, of the essential oil were also identified by comparison with reference standards (RS). Active compounds were isolated by bioassay-guided fractionation and identified by NMR and MS. The configuration of a new natural compound, not previously reported, was confirmed by ECD spectrum calculations. The components identified were listed in Table 1 with their relative percentages. Twenty-six compounds representing 97.8% of the oil were identified by using the apolar column. The essential oil of *Ad. buchneroides* was characterized with γ-terpinen (34.86%), carvacrol (22.2%), *p*-cymene (12.1%), carvacrol methyl ether (11.87%) and *β*-bisabolene (7.96%) as major constituents (Fig. 1). Up to now, constituents of essential oil from *Adenosma* genus including *Ad. indianum* (Zeng et al., 2013; Ya et al., 2011; Huang et al., 2011), *Ad. glutinosum* (Dung et al., 1996; Wang et al., 2008), *Ad. bracteosum* (Dai et al., 2015), *Ad. buchneroides* (Xu et al., 2008) and *Ad. indianum* (Dai et al., 2015) had been reported, among which relatively abundant chemical types were composed of monoterpene, sesquiterpene and aliphatic compounds. Likewise, the oil of *Ad. buchneroides* was mainly rich in monoterpene and sesquiterpene in this study.

Table 1
Chemical components of the essential oil of *Ad. buchneroides*.

no.	RRI[a]	RRI_{lit}[b]	compd	relative content (%)[c]	identification[d]
1	935	924	*α*-thujene	0.62	MS, RI
2	946	932	*α*-pinene	0.23	MS, RI
3	982	974	*β*-pinene	0.06	MS, RI
4	994	988	myrcene	0.05	MS, RI
5	1016	1002	*α*-phellandrene	0.05	MS, RI
6	1023	1008	3-carene	1.24	MS, RI
7	1028	1014	*α*-terpinen	1.77	MS, RI
8	1036	1020	*p*-cymene	12.1	MS, RI, RS
9	1041	1024	limonene	1.75	MS, RI
10	1046	1032	(*Z*)-*β*-ocimene	0.07	MS, RI
11	1061	1054	γ-terpinen	34.86	MS, RI
12	1094	1086	terpinolene	0.13	MS, RI
13	1104	1095	linalool	0.06	MS, RI
14	1177	1165	borneol	0.29	MS, RI
15	1185	1174	terpinene-4-ol	0.16	MS, RI
16	1192	1186	*α*-terpineol	0.19	MS, RI
17	1245	1241	carvacrol methyl ether	11.87	MS, RI, RS, NMR
18	1297	1289	thymol	0.55	MS, RI, RS
19	1311	1298	carvacrol	22.2	MS, RI, RS, NMR
20	1427	1417	trans-caryophyllene	0.16	MS, RI
21	1455	1440	(*Z*)-*β*-farnesene	0.06	MS, RI
22	1462	1452	humulene	0.74	MS, RI
23	1521	1505	*β*-bisabolene	7.96	MS, RI, NMR
24	1537	1521	*β*-sesquiphellandrene	0.17	MS, RI
25	1594	1582	caryophyllene oxide	0.1	MS, RI
26	2092	–	adenosmin A	0.33	NMR*, ECD
Total				97.77	

[a] RRI: relative retention indices calculated against a series of *n*-alkanes (C_8-C_{30}) on the HP-5 capillary column.
[b] RRI_{lit}: relative retention indices in literature.
[c] Relative content calculated from FID relative peak area data.
[d] Methods of identification: MS, identification performed on the basis of computer matching of NIST14 mass spectral library; RI, retention indices on the apolar HP-5 column; RS, compounds were identified by comparison to reference standards; NMR, identification conducted by NMR spectroscopy; ECD, identification accomplished by ECD spectroscopy.
* New natural compound, not previously reported.

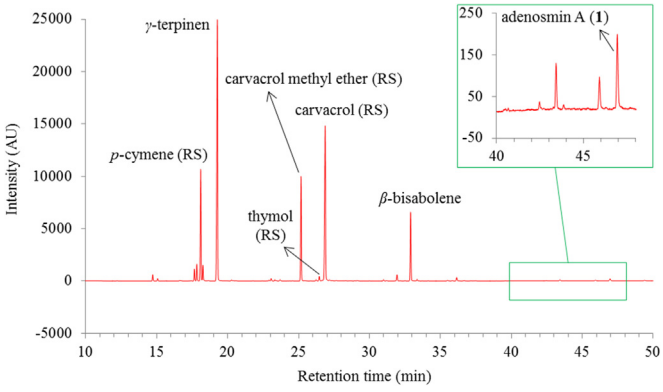

Fig. 1. GC-FID chromatogram of the essential oil from *Ad. buchneroides* on the apolar HP-5 column. RS, compounds were identified by comparison to reference standards.

3.2. The bioassay-guided fractionation of Ad. buchneroides essential oil to identify the active repellent compounds

The essential oil of *Ad. buchneroides* tested by in-cage mosquito repellent bioassay showed strong repellency with a minimum effective dosage (MED) of 0.019 ± 0.007 mg/cm^2 compared to the MED of positive control DEET at 0.031 ± 0.014 mg/cm^2. A systematic bioassay-guided fractionation of *Ad. buchneroides* essential oil was conducted to identify the compounds that contribute to the repellency

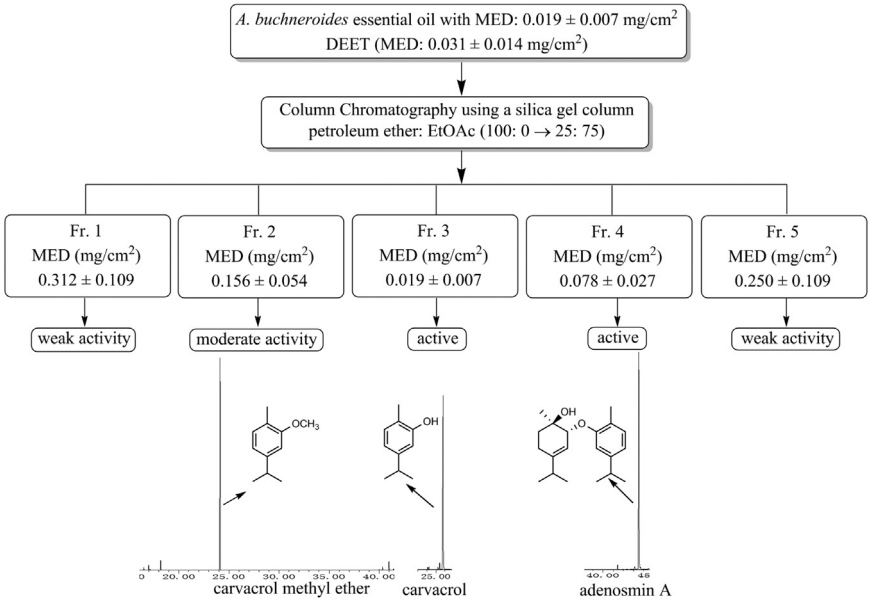

Fig. 2. A flowchart describing procedures of the bioassay-guided isolation of repellent compounds from *Ad. buchneroides* essential oil against *Ae. albopictus*. The minimum effective dosage (MED) was used to evaluate repellency of the oil or fractions or compounds with DEET as a positive control.

of the oil. Five fractions (Fr. 1–5) was obtained by column chromatography, the MEDs for each fraction were then evaluated against *Ae. albopictus* (Fig. 2). Both Fr. 1 (containing 39.7% γ-terpinen, 39.2% *p*-cymene, 15.4% *β*-bisabolene) and Fr. 5 (containing various minor components) were not further isolated due to their weak repellency with a MED of 0.312 ± 0.109 mg/cm^2 and 0.250 ± 0.109 mg/cm^2, respectively. The moderate repellency was displayed for Fr. 2 (90.8% carvacrol methyl ether) with a MED of 0.156 ± 0.054 mg/cm^2. Fr. 3 (rich in 92.1% carvacrol) exhibited the most potent repellency with a MED of 0.019 ± 0.007 mg/cm^2. The adjacent subfraction Fr. 4 (87.7% adenosmin A) was repellent with a MED of 0.078 ± 0.027 mg/cm^2.

After that, Fr. 2–4 were further purified to afford carvacrol methyl ether (**3**), carvacrol (**4**) and adenosmin A (**1**) as active constituents. The MEDs of the three pure compounds against *Ae. albopictus* were evaluated at 0.125 ± 0.054 mg/cm^2, 0.011 ± 0.000 mg/cm^2 and 0.063 ± 0.027 mg/cm^2, respectively. This study supported that carvacrol methyl ether and carvacrol as repellents against *Ae. aegypti* which had been reported from other plants (Tabanca et al., 2013c). In conclusion, carvacrol and carvacrol methyl ether are major active repellent compounds in essential oil of *Ad. buchneroides*, among which carvacrol plays a key role. In addition, adenosmin A as a minor new compound in the essential oil indicates mosquito repellency for the first time.

3.3. Structural elucidation of compounds

The bioassay-guided fractionation of *Ad. buchneroides* essential oil resulted in the isolation and structural elucidation of a new dimonoterpene ether named adenosmin A (**1**) besides other three known compounds of which NMR data (Fig. S1, Table S1 in Supplementary material) were in agreement with those previously reported. They were identified as *β*-bisabolene (Miyazawa and Kameoka, 1983), carvacrol methyl ether (Alokam et al., 2013) and carvacrol (Bohlmann et al., 1975). The structure of compound **1** was elucidated by spectroscopic analysis including IR, NMR, HR-ESI-MS techniques.

Compound **1** was obtained as colorless oil. The molecular formula of **1** was deduced as $C_{20}H_{30}O_2$ with 6 degrees of unsaturation, on the basis of HR-ESI-MS experiment with a molecular ion peak at *m/z* 325.2139 $[M+Na]^+$ (calcd for $C_{20}H_{30}O_2Na$, 325.2138). The typical absorption peaks at 3437, 1631, and 1251 cm^{-1} in the IR spectrum suggested the existence of a hydroxyl group, phenyl group and ether group, respectively. Analysis of three aromatic proton signals of an ABX-coupling system at δ_H 6.76 (1H, d, 1.5), 6.74 (1 H, dd, 7.5, 1.5), 7.06 (1H, d, 7.5) in the ^{1}H NMR spectrum indicated the typical pattern system of a 1′,2′,5′-trisubstituted benzene ring. Further analysis of the carbon signals in the ^{13}C NMR spectrum revealed that its carbon signals were in good agreement with those of carvacrol (Bohlmann et al., 1975), except that the carbon signals δ_C 120.8 and δ_C 153.6 in carvacrol were shifted downfield, respectively, to δ_C 125.0 (C-2′) and 156.0 (C-1′) in **1** due to the substitution of 1′-OH. In addition to the surplus carbon signals (δ_C 72.0, 80.2, 117.6, 147.1, 25.0, 33.5, 21.9, 34.5, 21.5, 21.5) in the ^{13}C NMR spectrum basically fitted in with those of (1*R*,2*R*)-3-*p*-menthen-l,2-diol (Abraham et al., 1986), except that the carbon signals δ_C 74.8 and δ_C 121.0 in (1*R*,2*R*)-3-*p*-menthen-l,2-diol were shifted respectively to δ_C 80.2 (C-2) and 117.6 (C-3) in **1** due to the substitution of 2-OH. The assignments of all the ^{1}H and ^{13}C NMR signals (Table 2) were accomplished through 1D and ^{2}D NMR techniques, particularly the

Table 2
^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) data of adenosmin A in chloroform-*d* (*δ* in ppm, *J* in Hz).

#	δ_H (multi, *J* values)	δ_C
1		72.0 (C)
2	4.68 (*d*, 2.5, 1H)	80.2 (CH)
3	5.44 (*d*, 2.5, 1H)	117.6 (CH)
4		147.1 (C)
5	2.17 (*m*, 2H,)	25.0 (CH_2)
6	1.86 (*m*, 2H)	33.5 (CH_2)
7	1.35 (*s*, 3H)	21.9 (CH_3)
8	2.23 (*m*, 1H)	34.5 (CH)
9	1.02 (*d*, 6.9, 3H)	21.5 (CH_3)
10	1.00 (*d*, 6.9, 3H)	21.5 (CH_3)
1′		156.0 (C)
2′		125.0 (C)
3′	7.06 (*d*, 7.5, 1H)	130.8 (CH)
4′	6.74 (*dd*, 7.5, 1.5, 1H)	118.3 (CH)
5′		148.0 (C)
6′	6.76 (*d*, 1.5, 1H)	111.2 (CH)
7′	2.20 (*s*, 3H)	16.3 (CH_3)
8′	2.86 (*septet*, 6.9, 1H)	34.2 (CH)
9′	1.24 (*d*, 6.9, 3H)	24.3 (CH_3)
10′	1.24 (*d*, 6.9, 3H)	24.3 (CH_3)

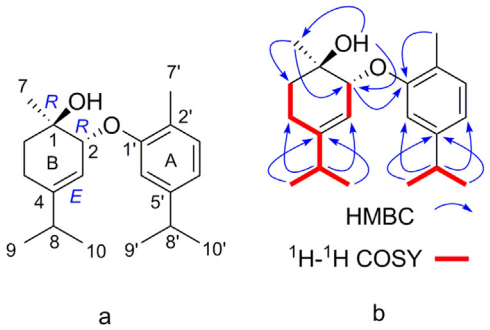

Fig. 3. The structure (a), key ^{1}H-^{1}H COSY and HMBC correlations (b) of **1**.

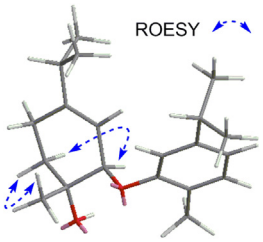

Fig. 4. Key ROESY correlations of **1**.

correlation signal from H-2 to C-1′ by HMBC spectrum (Fig. 3) illuminated that C-2 was linked to C-1′ by an oxygen atom. No correlation between H-2 and H-7 in ROESY spectrum (Fig. 4) demonstrated that the absolute configurations of chiral carbon for C-1 and C-2 were either (1*R*,2*R*)-isomer or (1*S*,2*S*)-isomer. Detailed literatures research manifested that (1*R*,2*R*)-3-*p*-menthen-l,2-diol was isolated from *Alpinia oxyphylla* (Xu et al., 2009), whereas (1*S*,2*S*)-3-*p*-menthen-l,2-diol was all synthesized artificially, not found in natural products. In addition to the optical rotation direction resulting from chiral moiety in B ring of **1** (Fig. 3) was identical with that of (1*R*,2*R*)-(−)-3-*p*-menthen-l,2-diol (Garg and Agarwal, 1988), but that of (1*S*,2*S*)-(+)-3-*p*-menthen-l,2-diol was just the opposite (Suga et al., 1968). Meanwhile, the absolute configuration of **1** was determined by comparison of experimental and theoretical electronic circular dichroism (ECD) spectra. Conformation search using molecular mechanics calculations in Discovery Studio 3.5 Client with MMFF force field gave seven conformers. These predominant conformers were optimized at B3LYP/6-31G (d, p) level. The theoretical calculations of ECD were conducted by time dependent Density Functional Theory (TDDFT) at B3LYP/6-31G (d, p) level in MeOH with PCM model. The ECD spectra of **1** were obtained by weighing the Boltzmann distribution rate of each predominant conformation. The calculated ECD for (1*R*,2*R*)-isomer matched with the experimental curve (Fig. 5) and thus determined the absolute configuration. Above all, compound **1**, was deduced as (1*R*,2*R*)-(−)-4-isopropyl-2-(5′-isopropyl-2′-methylphenoxyl)-1-methyl-3-cyclohexen-1-ol, named adenosmin A.

3.4. The structure-activity relationship (SAR) of carvacrol analogues

A variety of terpenes from plant essential oils were regarded as a significant class of potential sources of mosquito-controlling agents (Seo et al., 2015). Carvacrol analogues as monoterpenes are widely distributed in aromatic plants. To provide a broader foundational support for essential oils containing carvacrol analogues in mosquito control and seek efficient repellents, a series of selected carvacrol analogues were purchased and assessed the repellency by in-cage mosquito repellent bioassay (Fig. 6). All carvacrol analogues used as repellent against *Ae. albopictus* for investigation of SAR, including *p*-cymene, *p*-menthan-2-ol, carvacrol methyl ether, thymol, 3-methyl-4-isopropylphenol (Boroomand et al., 2017), 2-methyl-4-isopropylphenol

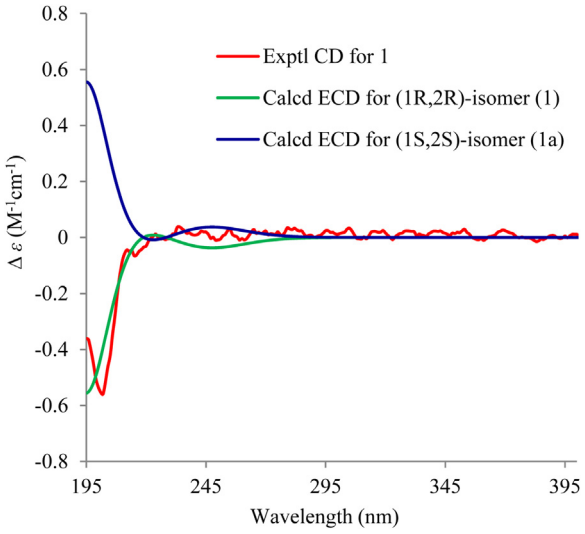

Fig. 5. Experimental CD and calculated ECD spectra for **1** and **1a**.

(Koşar et al., 2008), 4-methyl-2-isopropylphenol (Ezoubeiri et al., 2005), and 4-methyl-3-isopropylphenol (Shao et al., 2008), occurred naturally in various aromatic plants. Compared with carvacrol (MED = 0.011 ± 0.000 mg/cm^2), *p*-menthan-2-ol (MED = 0.009 ± 0.003 mg/cm^2) had little effect on the repellency when phenyl group was saturated totally by hydrogen atom. Tabanca et al. (2016) speculated that the hydroxyl was key factor for repellent activity of carvacrol analogues in a review covering several years of their researches. Further, our research verifies that hydroxyl group affected remarkably the repellency of the analogues. The repellency of *p*-cymene (MED = 1.000 ± 0.433 mg/cm^2) without hydroxyl group on phenyl group disappeared and that of carvacrol methyl ether (MED = 0.133 ± 0.049 mg/cm^2) with a methyl ether group instead of a hydroxyl group was decreased dramatically, which indicates that the hydroxyl group on phenyl group was required for the activity. According to report, odorant molecules were directly bound to odorant receptors (Wicher et al., 2008). Hence, it is speculated that hydroxyl group as a hydrogen bond acceptor may be involved in mosquito's perception of scent. After changing relative positions of three substituents on the benzene ring of carvacrol, these compounds, including 2-methyl-4-isopropylphenol (MED = 0.039 ± 0.013 mg/cm^2), 4-methyl-2-isopropylphenol (MED = 0.035 ± 0.013 mg/cm^2), thymol (MED = 0.035 ± 0.013 mg/cm^2), did not reduce repellency. Excitingly, the MEDs of 3-methyl-4-isopropylphenol (MED = 0.002 ± 0.001 mg/cm^2) and 4-methyl-3-isopropylphenol (0.003 ± 0.002 mg/cm^2) were an order of magnitude lower than that of positive control DEET. These results suggest that repellent activities of carvacrol analogues are significantly increased, when the distance between hydroxyl group and methyl as well as isopropyl group on the benzene ring is furthest. Moreover, it is assumed that both methyl and isopropyl group as hydrophobic group may be bound together to active site of odorant receptor, and the farther away both methyl and isopropyl group are, from the hydroxyl group on the benzene ring, the more beneficial to the combination of odorant molecules with odorant receptors may be.

In conclusion, a research on structure-activity relationship (SAR) of carvacrol analogues indicates that the analogue without hydroxyl group on the benzene ring shows no repellent activity, whereas repellent activities of the analogues are not reduced by the saturation of phenyl group, the methylation of hydroxyl group and the change of relative positions of three substituent groups on the benzene ring of carvacrol analogues.

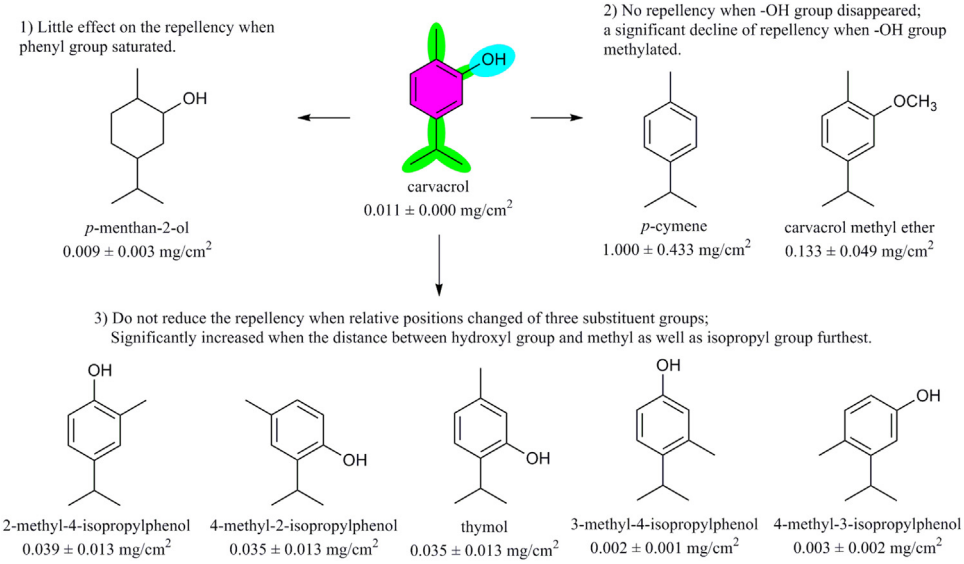

Fig. 6. The summary of the SAR of carvacrol analogues as repellent against *Ae. albopictus*, including the structures and chemical names of carvacrol analogues, and their MEDs.

3.5. Larvicidal activity and cytotoxicity of all isolated repellent compounds

Essential oil of *Ad. buchneroides* showed positive larvicidal activity against 1-day-old *Ae. albopictus* larvae with a LD_{50} value of 68.0 (62.0–75.1) ppm. Carvacrol was the most toxic compound among tested compounds with a LD_{50} value of 24.8 (16.8–26.2) ppm. Carvacrol methyl ether indicated a weak toxicity with a LD_{50} value of 165.1 (150.8–181.3) ppm, whereas adenosmin A demonstrated no larvicidal activity (Table 3). Ali et al. (2015) reported monoterpene hydrocarbons were significantly weak insecticidal activity against insects. These results suggest that carvacrol may be responsible for larvicidal activity of *Ad. buchneroides* essential oil. In a previous study, carvacrol and carvacrol methyl ether were reported to exhibited larvicidal activity against *Ae. aegypti* (Tabanca et al., 2013c). In comparison, in present study carvacrol methyl ether against *Ae. albopictus* larvae exhibited weaker larvicidal activity may result from different species of mosquito. The larvicidal activities of hydrocarbons with benzene, cyclohexadiene and cyclohexene structures were stronger than those of hydrocarbons with bicycloheptane and simple aliphatic structures (Seo et al., 2015). Similar to this report, the presence of bicyclic group of adenosmin A eliminated larvicidal activity. Lu et al. (2009) reported that eucalyptol and α-pinene as the main insecticidal components of *Vitex negundo* essential oil affected the breath of *Sitophilus zeamaise* by distinct inhibitory activities on AChE, CAT and CarE of *Si. zeamaise* in vitro. Ma et al. (2009) pointed out that Na^+, K^+-ATPase was possibly the insecticidal target of terpinen-4-ol as the main insecticidal component in the essential oil of *Sabina vulgaris* Ant. against *Culex pipiens pallens*. Tong, Coats (2010) suggested that insect GABA receptor was molecular target of insecticidal monoterpenes as positive allosteric modulators. These reports denote that the action mechanisms of insecticidal monoterpenes is diverse.

The detailed result and discussion of cytotoxicity of the essential oil and all isolated repellent compounds were described in the Supplementary material, Table S2, Text S2.

Table 3
Toxicity of *Ad. buchneroides* essential oil and isolated repellent compounds from its oil against 1-day-old larvae of *Ae. albopictus* at 24 h post-treatment.

compd/oil	LD_{50} (95% CI) ppm	LD_{90} (95% CI) ppm	χ^2	DF
carvacrol	24.8 (16.8–26.2)	29.6 (27.6–56.7)	7.6	48
carvacrol methyl ether	165.1 (150.8–181.3)	231.6 (211.2–261.7)	12.2	48
adenosmin A	1317.7 (1175.2–1499.9)	2122.128 (1875.254–2491.753)	21.5	58
Ad. buchneroides oil	68.0 (62.0–75.1)	92.5 (84.1–105.0)	14.4	38

4. Conclusion

In this study, a systematic research on *Ad. buchneroides* essential oil was accomplished for its active mosquito repellent compounds according to local folk medical practice of the Aini people. Bioassay-guided fractionation of the essential oil led to the identification of three constituents, carvacrol, carvacrol methyl ether and a minor new compound adenosmin A with significant mosquito repellent activity. The presence of these compounds in essential oil of *Ad. buchneroides* supports the traditional use of this plant as an insect repellent by Aini people in Yunnan of China.

In addition, larvicidal activity, cytotoxicity, and a SAR of carvacrol analogues were investigated. The larvicidal bioassay of the oil against 1-day-old *Ae. albopictus* larvae revealed that larvicidal activity of the oil might be mainly attributed to carvacrol in the oil. Essential oil of *Ad. buchneroides* just showed low cytotoxicity, the new active compound adenosmin A was not individually cytotoxic to any of the tested cell lines. The essential oil and the active carvacrol analogues have good potential for development as plant-based mosquito repellents, especially these two compounds, 3-methyl-4-isopropylphenol and 4-methyl-3-isopropylphenol. These two compound's MEDs are far lower than that of positive control DEET, they are potential constituents to develop new natural repellent products, and contribute to mode-of-action studies on compound-receptor interactions. Further research is needed to investigate the underlying mode of action of the active constituents, and develop formulations for use as mosquito repellents as well as evaluate the safety of the formulations.

Acknowledgments

We thank Professor Shenji Pei for identification of the plant. We would like to thank the efforts of all volunteers participating in the mosquito repellent bioassay.

Declaration of interest

The authors declare no conflict of interest.

Funding

This study was supported by grant from the Plant Germplasm Resources Innovation Project of Chinese Academy of Sciences (ZSZC-012) and National Natural Science Foundation of China (31670337).

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jep.2018.11.031.

References

Abraham, W.R., Stumpf, B., Kieslich, K., 1986. Microbial transformations of terpenoids with 1-*p*-menthene skeleton. Appl. Microbiol. Biot. 24, 24–30.
Adams, R.P., 2007. Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry, 4th ed. Allured Publishing Corporation, Carol Stream, Illinois.
Ali, A., Tabanca, N., Demirci, B., Blythe, E.K., Ali, Z., Baser, K.H., Khan, I.A., 2015. Chemical composition and biological activity of four *Salvia* essential oils and individual compounds against two species of mosquitoes. J. Agric. Food Chem. 63, 447–456.
Alokam, R., Jeankumar, V.U., Sridevi, J.P., Matikonda, S.S., Peddi, S., Alvala, M., Yogeeswari, P., Sriram, D., 2013. Identification and structure–activity relationship study of carvacrol derivatives as *Mycobacterium tuberculosis* chorismate mutase inhibitors. J. Enzym. Inhib. Med. Chem. 29, 547–554.
Bohlmann, F., Zeisberg, R., Klein, E., 1975. ^{13}C NMR-spektren von monoterpenen. Magn. Reson. Chem. 7, 426–432.
Boroomand, N., Sadat-Hosseini, M., Moghbeli, M., Farajpour, M., 2017. Phytochemical components, total phenol and mineral contents and antioxidant activity of six major medicinal plants from Rayen, Iran. Nat. Prod. Res. 32, 564–567.
Brown, M., Hebert, A.A., 1997. Insect repellents: an overview. J. Am. Acad. Dermatol. 36, 243–249.
Corbel, V., Stankiewicz, M., Pennetier, C., Fournier, D., Stojan, J., Girard, E., Dimitrov, M., Molgo, J., Hougard, J.-M., Lapied, B., 2009. Evidence for inhibition of cholinesterases in insect and mammalian nervous systems by the insect repellent DEET. BMC Biol. 7, 1–11.
Curvers, J., Rijks, J., Cramers, C., Knauss, K., Larson, P., 1985. Temperature programmed retention indexes: calculation from isothermal data. Part 1, theory. J. High Resolut. Chromatogr. 8, 607–610.
Dai, D.N., Thang, T.D., Thai, T.H., Ogunwande, I.A., 2015. Chemical constituents of leaf essential oils of four *Scrophulariaceae* species grown in Vietnam. J. Essent. Oil Res. 27, 1–6.
Dung, N.X., Le, V.H., Leclercq, P.A., 1996. A new chemotype of *Adenosma glutinosum* (L.) Druce var. caeruleum (R.Br.) Tsoong from Vietnam. J. Essent. Oil Res. 8, 359–362.
Editorial Committee of Flora of China, 1998. Flora of China. Science Press, Beijing, China.
Ezoubeiri, A., Gadhi, C.A., Fdil, N., Benharref, A., Jana, M., Vanhaelen, M., 2005. Isolation and antimicrobial activity of two phenolic compounds from *Pulicaria odora* L. J. Ethnopharmacol. 99, 287–292.
Garg, S.N., Agarwal, S.K., 1988. New monoterpene diols from essential oil of *Ferula jaeschkeana*. Phytochemistry 27, 936–937.
Hoel, D., Pridgeon, J.W., Bernier, U.R., Chauhan, K., Meepagala, K., Cantrell, C., 2010. Departments of Defense and Agriculture team up to develop new insecticides for mosquito control. Wing Beats 21, 19–34.
Huang, Y., Wu, H.Y., Wei, Z.Y., Xiao, Y.F., Yu, X.L., 2011. Chemical constituents and antibacterial activity of essential oil form *Adenosma indianum*. Chin. J. Exp. Tradit. Med. Formul. 17, 79–82.
Huixing, L., Ruhai, L., Mushan, W., Pinyan, Y., Zhiguo, K., Yusheng, Y., 1998. Effect of 25 plant essential oils against *Callosobruchus maculatus*. In: Proceedings of the 7th International Working Conference on Stored-product Protection, Beijing, China, pp. 849–851.
Isman, M.B., 2006. Botanical insecticides, deterrents, and repellents in modern agriculture and an increasingly regulated world. Annu. Rev. Entomol. 51, 45–66.
Koşar, M., Demirci, B., Demirci, F., Başer, K.H., 2008. Effect of maturation on the composition and biological activity of the essential oil of a commercially important *Satureja* species from Turkey: *Satureja cuneifolia* Ten. (Lamiaceae). J. Agric. Food Chem. 56, 2260–2265.
Lu, C.-B., Xue, M., Liu, Y.-Q., Liu, A.-H., Wang, H.-T., 2009. Insecticidal components and toxicity of *Vitex negundo* (Lamiales: verbenaceae) essential oil to *Sitophilus zeamais* (Coleoptera: curculionidae) and their action mechanisms. Acta Èntomol. Sin. 52, 159–167.
Ma, Z.-Q., Luan, Z.-C., Zhang, X., 2009. Effects of terpinen-4-ol on *Culex pipiens pallens* Na^+, K^+-ATPase. Chin. J. Pestic. Sci. 11, 230–234.
Meepagala, K.M., Bernier, U.R., Burandt, C., Duke, S.O., 2013. Mosquito repellents based on a natural chromene analogue with longer duration of action than *N,N*-diethyl-meta-toluamide (DEET). J. Agric. Food Chem. 61, 9293–9297.
Miyazawa, M., Kameoka, H., 1983. Helianthol A, a sesquiterpene alcohol from *Helianthus tuberosus*. Phytochemistry 22, 1040–1042.
Nerio, L.S., Verbel, J.O., Stashenko, E., 2010. Repellent activity of essential oils: a review. Bioresour. Technol. 101, 372–378.
Posey, K.H., Schreck, C.E., 1981. An airflow apparatus for selecting female mosquitoes for use in repellent and attraction studies. Mosq. News 41, 566–568.
Pridgeon, J.W., Becnel, J.J., Clark, G.G., Linthicum, K.J., 2009. A high-throughput screening method to identify potential pesticides for mosquito control. J. Med. Entomol. 46, 335–341.
Schreck, Posey C.E., Smith K., 1977. Repellent activity of compounds submitted by Walter Reed Army Institute of Research, Part 1. Protection time and minimum effective dosage against *Aedes aegypti* mosquitoes. 〈http://ageconsearch.umn.edu/record/158059〉, (22/03/2018).
Seo, S.-M., Jung, C.S., Kang, J., Lee, H.R., Kim, S.W., Hyun, J., Park, I.K., 2015. Larvicidal and acetylcholinesterase inhibitory activities of *Apiaceae* plant essential oils and their constituents against *Aedes albopictus* and formulation development. J. Agric. Food Chem. 63, 9977–9986.
Shao, Y., Zhao, X.-H., Mei, L.-J., Tao, Y.-D., 2008. GC-MS analyses of the essential oil from var. atropurpurea Pamp. Chin. J. Anal. Lab. 27, 38–41.
Shen, P.Q., Sun, H.D., Pei, S.J., 1988. Ethnobotany of fleagrass (*Adenosma buchneroides* Bonati), a traditional cultivated plant of the Hani people, Xishuangbanna, Yunnan, China. In: Ethnobiology: Implications and Applications: Proceedings of the First International Congress of Ethnobiology, Belém, Brazil, pp. 305–309.
State Pharmacopeia Commission, 2015. Pharmacopeia of the People's Republic of China, 2015 edition. China Medical Science Press, Beijing, China, pp. 203.
Suga, T., Shishibori, T., Matsuura, T.B., 1968. Stereochemical studies of monoterpene compounds. III. stereochemistry and intramolecular hydrogen bonding of 1-hydroxy-*p*-menth-3-en-2-one and its reduction products. Chem. Soc. Jpn. 41, 944–948.
Tabanca, N., Ali, A., Bernier, U.R., Agramonte, U.M., Tsikolia, M., Bloomquist, J.R., 2016. Discovery of repellents from natural products. Curr. Org. Chem. 25, 2690–2702.
Tabanca, N., Ali, A., Bernier, U.R., Khan, I.A., Kocyigit-Kaymakcioglu, B., Oruc-Emre, E.E., Unsalan, S., Rollas, S., 2013b. Biting deterrence and insecticidal activity of hydrazide–hydrazones and their corresponding 3-acetyl-2,5-disubstituted-2,3-dihydro-1,3,4-oxadiazoles against *Aedes aegypti*. Pest Manag. Sci. 69, 703–708.
Tabanca, N., Bernier, U.R., Ali, A., Wang, M., Demirci, C., Blythe, E.K., Khan, S.I., Baser, K.H.C., Khan, I.A., 2013b. Bioassay-guided investigation of two *Monarda* essential oils as repellents of yellow fever mosquito *Aedes aegypti*. J. Agric. Food Chem. 61, 8573–8580.
Tabanca, N., Wang, M., Avonto, C., Chittiboyina, A.G., Parcher, J.F., Carroll, J.F., Kramer, M., Khan, I.K., 2013c. Bioactivity-guided investigation of geranium essential oils as natural tick repellents. J. Agric. Food Chem. 61, 4101–4107.
Taubes, G., 2000. Searching for a parasite's weak spot. Science 290, 434–437.
Tisgratog, R., Sanguanpong, U., Grieco, J., Ngoen-Kluan, R., Chareonviriyaphap, T., 2016. Plants traditionally used as mosquito repellents and the implication for their use in vector control. Acta Trop. 157, 136–144.
Tong, F., Coats, J.R., 2010. Effects of monoterpenoid insecticides on [^3H]-TBOB binding in house fly GABA receptor and $^{36}Cl^-$ uptake in American cockroach ventral nerve cord. Pestic. Biochem. Phys. 98, 317–324.
Wang, C.C., Wei, G., Li, R.M., 2008. GC-MS analysis of volatile oil in *Adenosma glutinosum* (Linn.) Druce. Chin. J. Inform. TCM 15, 36–37.
Wicher, D., Schäfer, R., Bauernfeind, R., Stensmyr, M.C., Heller, R., Heinemann, S.H., Hansson, B.S., 2008. Drosophila odorant receptors are both ligand-gated and cyclic-nucleotide-activated cation channels. Nature 452, 1007–1011.
World Health Organization, 2015. World Malaria Report 2015. (06/03/2018). 〈http://www.who.int/malaria/media/world-malaria-report-2015/en/〉.
Xu, J.-J., Tan, N.-H., Chen, Y.-S., Pan, X.-L., Zeng, G.-Z., Han, H.-J., Ji, C.-J., Zhu, M.-J., 2009. Three unusual new sesquiterpenes from *Alpinia oxyphylla*. Helv. Chim. Acta 92, 1621–1625.
Xu, Y., Cheng, B.Q., Yu, Z., Ding, J.K., 2008. A preliminary study on the new perfume plant *Adenosma buchneroides* Bonati. In: The 7th Proceedings of the Seminar on Fragrance and Flavor China, Hangzhou, China, pp. 26–29.
Ya, Q.K., Lu, W.J., Chen, J.Y., Tan, X., 2011. GC-MS analysis of the chemical constituent of volatile oil from Zhuang drug *Adenosma indianum* (Lour.) Merr. Chin. J. Pharm. Anal. 31, 544–546.
Zeng, Z., Meng, C., Ye, X., Zeng, Z., 2013. Analysis of volatile components of *Adenosma indianum* (Lour.) Merr. by steam distillation and headspace solid-phase microextraction. J. Chem. 2013, 1–7.