Agr. Nat. Resour. 56 (2022) 805–814

AGRICULTURE AND NATURAL RESOURCES

Journal homepage: http://anres.kasetsart.org

Research article

Gas chromatography-mass spectrometry, feature-based molecular networking and biological activities against *Solenopsis invicta* of *Citrus* peel essential oils from Vietnam

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Article Info

Article history:
Received 5 January 2022
Revised 22 May 2022
Accepted 24 May 2022
Available online 26 August 2022

Keywords:
Citrus species,
C. reticulata,
Molecular docking,
Molecular networking,
Solenopsis invicta

Abstract

Importance of the work: Reducing the consumption of synthetic insecticides and increasing the use of natural and safe products have become important new strategies.

Objectives: To evaluate molecular networking-based chemical components and biological activities of *Citrus* essential oils against *Solenopsis invicta*.

Materials & Methods: Using the new molecular networking strategy based on the Global Natural Products Social web platform, fumigant toxicity was tested against worker ants and the efficacy of ant-repelling activity. Molecular docking simulation of a fire ant chymotrypsin protein (PDB: 1EQ9) was undertaken with 40 metabolites.

Results: Various chemical classes of three essential oils were identified, namely monoterpenes, oxygenated monoterpenes, ester derivatives, sesquiterpenes, aromatic compounds and volatile amines. The *C. reticulata* essential oil with a mean (± SD) lethal concentration 50 (LC_{50}) value of 47.74 ± 13.99 mg/mL exhibited stronger fumigant toxicity against worker ants than the oils from *C. limonia* and *C. sinensis*. Furthermore, *C. reticulata* essential oil had a significant repellent effect on *S. invicta* during outdoor environmental tests. The molecular docking results indicated that monoterpene-based ester derivatives and several volatile amines with binding energies in the range 5.1–6.4 kcal/mol could be promising fire ant chymotrypsin inhibitors that could be applied to control fire ants by reducing the digestive enzymes in the larval stage.

Main finding: This was an important study on a molecular networking strategy based on gas chromatography-mass spectrometry reported in 2020 and on the biological activities of three *Citrus* essential oils against *S. invicta*. These results could prompt further research on natural, safe and non-polluting insecticides for low-income populations in the tropics.

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https://doi.org/10.34044/j.anres.2022.56.4.15

Introduction

The red imported fire ant (RIFA) *Solenopsis invicta* is one of the most serious agricultural insect pests in the USA, New Zealand, Australia and Asian countries (Allen et al., 2004; Wylie et al., 2020). This ant invades houses, gardens, lawns and schoolyards and breeds and spreads rapidly. RIFA inflicts a fiery sting, causing severe pain and life-threatening allergic reactions in some individuals (Solley et al., 2002). The USA has spent almost USD 7 billion annually on fire ant management (Lard et al., 2006). Currently, combating invasive RIFA is performed using baits containing synthetic toxins that have substantially heightened the risks to human health and environmental pollution. Thus, reducing the consumption of synthetic pesticides and increasing the use of natural, safe and non-polluting insecticides have become the new strategies for the 21st century. Accordingly, major effort has been focused on bioactive compounds derived from natural sources.

Citrus spp. fruits, commonly known as mandarins, lemons and oranges, belong to the family Rutaceae (Rafiq et al., 2018). These fruits are economically important horticultural products worldwide (Liu et al., 2012). *Citrus* species are mainly consumed as fresh and canned products and are also utilized in pharmaceutical products (He et al., 2011; Kelebek and Selli, 2011). During the processing of *Citrus* spp. fruit products, substantial amounts of waste and by-products are dumped in public landfill. However, these by-products are particularly rich in bioactive components, such as antioxidants and essential oils (Hardin et al., 2010). The chemical constituents of essential oils from these *Citrus* peels contain approximately 97% monoterpenes and 1.8–2.2% alcohols, aldehydes and esters (Ballistreri et al., 2019). In addition, the major monoterpenes of these essential oils were identified as limonene, γ-terpinene, myrcene, α-pinene, α-thujene and terpinolene. The biological activities of these essential oils exhibited anti-inflammatory, antibacterial, antiparasitic, antidiabetic, anti-obesity properties, antifungal, larvicidal and antileishmanial activities (Jing et al., 2015; Ademosun et al., 2018; Dias et al., 2020; Klimek-Szczykutowicz et al., 2020; Rezende et al., 2020; Oliveira et al., 2021; Shorbagi et al., 2022;) Nevertheless, there are relatively few reports on molecular networking-based chemical profiling and the biological activities of essential oils from the peel of *Citrus* species against *S. invicta* in the Mekong Delta of Vietnam. Therefore, the current study investigated the chemical compositions of three *Citrus* peel essential oils (*Citrus reticulata* Blanco, *Citrus limonia* L. Osbeck and *Citrus sinensis* L. Osbeck) using a molecular networking strategy and also studied the biological activities of these oils against the fire ant *S. invicta*.

In recent years, the gas chromatography-mass spectrometry (GC-MS)-based molecular networking strategy has been increasingly applied in natural product analysis. GC-MS data enable the creation of networks in the Global Natural Products Social (GNPS) networking platform for data sharing and accumulation of public knowledge. The online application of this platform can be assessed at https://gnps.ucsd.edu under the head "GC-MS EI Data Analysis" (Aksenov et al., 2021). This emerging computer-based approach allows rapid identification of the mass fragmentation spectra of natural product mixtures, based on their structural similarity with public spectral reference libraries. Hence, the current research project aimed to apply molecular networking and mass fragmentation spectra to investigate the phytochemical compounds of essential oils from the peels of three *Citrus* species.

Materials and Methods

Plant material

The fruits of mandarin (*C. reticulata* Blanco), lemon (*C. limonia* L. Osbeck) and orange (*C. sinensis* L. Osbeck) were purchased from Xuan Khanh market, Ninh Kieu district, Can Tho city, Vietnam. The samples were botanically authenticated by Dr Pham Ha Thanh Tung, from Department of Botany, Hanoi University of Pharmacy, Vietnam. The fruit peels were collected and processed in a grinder to obtain the raw material powders. Samples were stored at 5 °C for less than 1 wk before being used.

Steam distillation and gas chromatography-mass spectrometry procedure

The powders (100 g) were put into a Clevenger apparatus device for 30–180 min with a solid-to-liquid ratio of 1:3–1:5 (weight per weight, w/w). The steam distillation method to isolate essential oils was designed as follows: heating mantle for a 500 mL round-bottomed flask with three necks (one neck connected with thermometer adapter, another neck attached with a separatory funnel and a final neck connected with a condenser), with the condenser attached to a Clevenger extractor. Then, the essential oils were separated from the oil-water mixtures using petroleum ether 60-90

(Xilong Co.; China) and dehydrated with anhydrous Na_2SO_4 (Xilong Co.; China). The steam distillation process for each condition was repeated thrice. The yields were calculated by dividing the mass of the extracted oil (in grams) by the initial plant biomass (in grams). The essential oils were stored at -5 °C for further analysis. The constituents of the essential oils were analyzed using the GC-MS spectrophotometer (Thermo Scientific system; USA) equipped with a TB-SQC column (15 m × 0.25 mm × 0.25 µm), as shown in Table S1. The essential oils (1.0 µL) were automatically injected using the split mode. Helium was used as the carrier gas.

Molecular networking

Molecular networking was generated using the online workflow at the GNPS web platform (https://gnps.ucsd.edu; Aksenov et al., 2021). The initial MS data was converted from the '.raw' format to the '.mzXML' format using the MS Convert software (Microsoft Corp.; Redmond, WA, USA), which was subsequently imported into the Mzmine 2.53 software. Next, the noise level of mass detection was set at $2.0\times E^3$, while the parameters of the ADAP chromatogram builder were according to Myers et al. (2017). After that, spectral deconvolution was built according to the hierarchical clustering algorithm. The parameters of ADAP alignment were a score threshold and score weight of 0.75 and 0.1, respectively. A missing peaks search was based on the peak finder (multithreaded) module under the following parameters: intensity tolerance, *m/z* tolerance and retention time tolerance were 0.05%, 0.45 *m/z* and 0.05 min, respectively. Then, the data were exported in the MZmine format and subsequently imported to the GC-MS spectral library search and molecular networking on the GNPS web platform under the parameters: fragment ion mass tolerance of *m/z* 0.5 Da; and minimum number of common fragment ions and minimum cosine score of 4 and 0.7, respectively. In addition, other advanced search options were selected, including: library class applying bronze or gold methods with the top hits per spectrum being 10; the maximum of neighbor nodes for one single node and the maximum number of nodes for a sub-network were set at 10 and 100, respectively; and Kovat's calculation was turned on. After the GNPS jobs had been completed, the essential oil phytochemicals of the *Citrus* species were predicted via various parameters (cosine score, shared peaks, total ion chromatogram and retention time queries, Kovats index and used libraries). Among them, cosine score and shared peaks were important values that reflected the high quality of spectrum matches. After the prediction step, the Cytoscape 3.6.0 software was applied to visualize the molecular network results. In Cytoscape, the colors of each node represented the total abundance of the compound (gradient from yellow to purple).

Toxicity assay against Solenopsis invicta

Bioassay was performed as described by Souto et al. (2012), with minor modifications. Briefly, RIFAs were collected at the garden of the College of Natural Sciences, Can Tho University and kept under room temperature at the animal laboratory. Filter papers (4 cm × 5 cm) were impregnated with 1.0 mL of oil solution in acetone solvent at various concentrations (0, 50, 100, 200 mg/mL) or in limonene (purity ≥ 94.0%; Merk; Germany) and placed within beakers (50 mL) containing 20–30 ants. The beakers were covered to prevent the ants from escaping. These experiments were repeated independently thrice. Ant survival was evaluated after 1 h, 2 h, 4 h and 6 h, using Equation 1:

$$\text{Ant survival (\%)} = \frac{\text{Number of surviving ants at the assessed time}}{\text{Number of surviving ants at the initial time}} \times 100 \quad (1)$$

Testing efficacy of ant-repelling activity of essential oils

This assay was performed as described by Aglave and Patel (2017), with some modifications. Cotton balls were prepared in 12-well plates and dampened with 2.0 g of sugar. Each cotton ball was topped with 0.2 mL of essential oils or an industrial ant shield product (ANBIO; Vietnam). These cotton balls were kept the same distance away from an anthill and observed with the naked eye for 3 d (at the same time each day). Experiments were repeated independently thrice at different ant hills. The percentage of repellency at the checked time was calculated based on Equation 2:

$$\text{Ant repellency} = \frac{\text{Attracted ants in negative control - Attracted ants in samples}}{\text{Attracted ants in negative control}} \times 100 \quad (2)$$

Simulation of binding affinity via molecular docking

To predict the binding affinity between ligands and fire ant chymotrypsin *in silico*, a molecular docking study was carried out using the AutoDock tool estimated using the PyRx software (Trott and Olson, 2010). The three-dimensional structure of the fire ant chymotrypsin complex (PDB code: 1EQ9) was downloaded from the Protein Data Bank (http://www.pdb.org) (Botos et al., 2000). The grid of

1EQ9 protein had the following location: center (X: 37.1, Y: 13.3, Z: 36.5); dimension (Angstrom) (X: 18.9, Y: 20.5, Z: 17.8). The results were visualized and analyzed using the Discovery Studio Visualizer software.

Statistical analysis

Values were presented as mean ± SD of three independent experiments. Statistical calculations were performed using the SPSS Statistics 21 software (SPSS, Inc.; USA). Statistically significant values were tested at $p < 0.05$ using Tukey's range test.

Results and Discussion

Chemical profile of Citrus peel essential oils

The essential oils from the peel of three *Citrus* fruits were successfully extracted on a small scale using the steam distillation method. There were several advantages of using this method in the study. First, water from the Clevenger apparatus could be collected and re-added to the distilling flask via a separatory funnel; this step maintained a suitable water level in the distilling flask, while allowing steam distillation to progress. Second, the steam distillation process temperature could be controlled more easily. Finally, several experiments suggested the steam distillation method could efficiently extract the essential oil and minor compounds (Messaoudi et al., 2021).

As shown in Fig. 1A, the solid-to-liquid ratio of 1:4 (w/w) was chosen as the best condition for the next experiments. To evaluate the effect of steam distillation time, the raw materials were extracted with a solid-to-liquid ratio of 1:4 from 30 min to 180 min. The results (Fig. 1B) indicated that a steam distillation time of 120 min obtained a higher yield than 30–90 min. However, when the steam distillation time increased beyond 120 min, the essential oil yields were not different from each other. Overall, the highest yield of essential oil was from *C. sinensis* (max. 2.3%), while *C. reticulata* had a moderate percentage (1.4%) and *C. limonia* had the lowest percentage (0.8%). These oils were analyzed for their chemical constituents, using GC-MS (in the positive-ion mode) and molecular networks were created through the GNPS web platform for the GC-MS raw data (https://gnps.ucsd.edu), with the minimum number of common fragment ions being 4. The final results were visualized using the Cytoscape 3.6.0 software.

In the generated network, individual MS spectra of the *C. reticulata* essential oil were grouped to form two clusters (Fig. 2). The largest cluster could be divided into

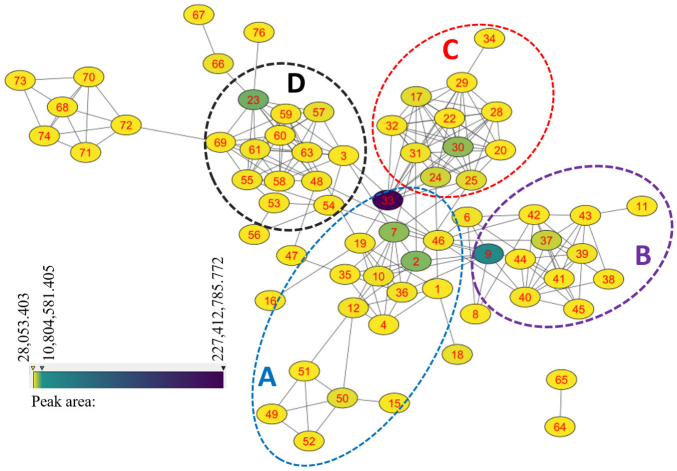

Fig. 2 Molecular networking of essential oil from *C. reticulata* peel generated using GNPS web platform (https://gnps.ucsd.edu)

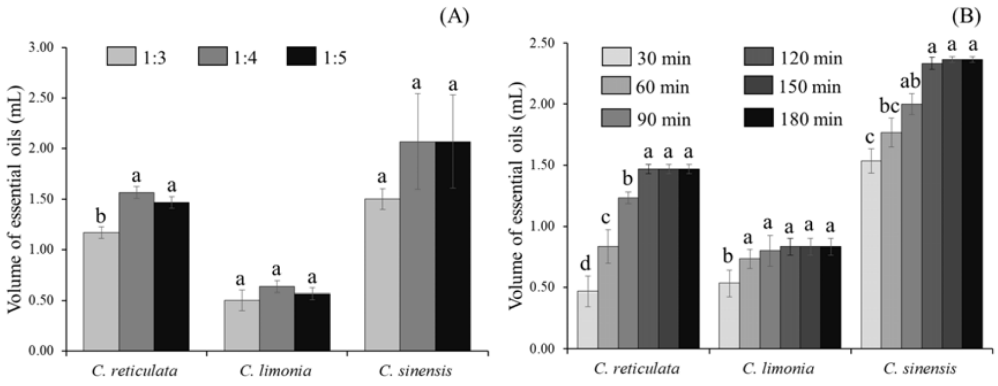

Fig. 1 Essential oils from *C. reticulata*, *C. limonia*, and *C. sinensis* extracted using hydrodistillation method: (A) raw samples extracted for 120 min with solid-to-liquid ratios of 1:3–1:5 (weight per weight); (B) raw samples extracted with solid-to-liquid ratio of 1:4 for 30–180 min, where results expressed as mean ± SD ($n = 3$) and different lowercase letters in same group of each species are significantly different at 5%

four sub-clusters (A–D; Fig. S1). In sub-cluster A, seven out of the 18 metabolites were matched well in the molecular networking, viz., *α*-pinene (2), *β*-pinene (6), *α*-phellandrene (7), limonene (33), *γ*-terpinene (36), *α*-terpineol (49) and *α*-terpinene (50). Further analysis of this sub-cluster identified that other nodes (15, 51 and 52) had the same connection with *α*-terpineol and *α*-terpinene, indicating the underlying structural similarities to terpinene. The remaining nodes in sub-cluster A were suggested as monoterpenes. In sub-cluster B, two compounds were matched from the libraries in the GNPS, with the minimum cosine score of approximately 0.9 and identified as (*E*)-3,7-dimethyl-2,6-octadien-1-ol (9) and linalool (37). From these known ones, the adjacent nodes were putatively identified as oxygenated monoterpenes. In sub-cluster C, five compounds (*E*)-3,7-dimethyl-2,6-octadien-1-yl acetate (17), (*E*)-3,7-dimethyl-2,6-octadien-1-yl propionate (25), dihydrocarvenyl acetate (28), (*E*)-3,7-dimethyl-2,6-octadien-1-yl isobutyrate (29) and terpinyl acetate (32)) were identified, while the remaining nodes were proposed as monoterpene-based ester derivatives. Finally, the identified nodes in sub-cluster D suggested that the *C. reticulata* essential oil also contained minor amounts of volatile amines. In particular, the peak areas of 28 identified compounds obtained about 94.34% of the total chemical composition (Tables 1 and S2).

Table 1 Putative compounds of essential oils from *C. reticulata*, *C. limonia*, and *C. sinensis* peels (peak areas selected from 0.1%) and molecular simulation of 1EQ9 with these compounds

No.	Compound name	Peak area (%)			Fire ant chymotrypsin protein (PDB: 1EQ9)		
		C. reticulata	*C. limonia*	*C. sinensis*	Binding energy (kcal/mol)	K_i (μM)	Hydrogen bond formation
1	*δ*-Cadinene	–	0.48	–	-4.9	254.74	0
2	(+)-3-Carene	–	–	0.11	-4.9	254.74	0
3	(*E*)-3,7-dimethyl-2,6-octadien-1-ol	7.60	12.52	10.23	-4.7	357.10	2
4	(*E*)-3,7-dimethyl-2,6-octadien-1-yl acetate	0.45	2.80	–	-4.7	357.10	2
5	(*E*)-3,7-dimethyl-2,6-octadien-1-yl butyrate	–	–	0.24	-5.1	181.72	2
6	(*E*)-3,7-dimethyl-2,6-octadien-1-yl propionate	0.45	12.66	–	-5.1	181.72	2
7	13-Methyltetradecanoic acid methyl ester	–	0.11	–	-4.2	830.83	1
8	2,6-Diaminopimelic acid	–	–	0.19	-5.4	109.49	**7**
9	3,4-Dimethylbenzyl alcohol	–	–	0.68	-5.1	181.72	1
10	3,5,5-Trimethyl-2-cyclohexen-1-one	0.10	0.25	–	-4.7	357.10	1
11	*α*-Bergamotene	–	0.77	–	-5.3	129.63	0
12	*α*-Bisabolene	–	1.01	–	-5	215.16	0
13	Adamantane	–	0.14	–	-4	1164.67	0
14	Anethole	–	0.89	1.43	-4.3	701.73	0
15	*α*-Phellandrene	1.82	–	1.28	-4.5	500.58	0
16	*α*-Pinene	2.05	3.07	2.63	-5	215.16	0
17	*β*-Pinene	0.20	–	–	-4.7	357.10	0
18	*α*-Terpinene	0.41	0.64	0.28	-4.7	357.10	0
19	*γ*-Terpinene	0.17	12.10	0.19	-4.6	422.80	0
20	*α*-Terpinyl formate	–	1.56	–	-5.2	153.48	2
21	Terpinyl acetate	0.11	–	–	-5.3	129.63	1
22	Camphene	–	–	2.64	-4.7	357.10	0
23	Citronellol	–	0.35	–	-4.8	301.61	2
24	Dihydrocarveol	1.82	0.40	–	-4.9	254.74	2
25	Geranial	0.28	–	–	-4.5	500.58	1
26	Hypoxanthine	0.34	–	–	-5.5	92.48	1
27	Indole-3-carboxylic acid	–	0.18	–	-5.8	55.72	3
28	Isopropylbenzene	–	–	0.25	-4.5	500.58	0
29	Limonene	76.84	28.61	68.83	-4.5	500.58	0
30	Linalool	0.89	0.42	0.19	-4.5	500.58	0
31	Lysinamide	0.13	–	–	-4.6	422.80	4
32	Mecamylamine	–	–	0.19	-4.5	500.58	0
33	Proline	0.13	–	–	-4.5	500.58	2
34	Purine	–	–	0.28	-5.2	153.48	1
35	Pymetrozine	–	0.34	–	-6.4	20.23	2
36	Pyrrole-2-carboxylic acid	–	–	0.11	-4.7	357.10	4
37	Terpinen-4-ol	–	0.69	–	-4.9	254.74	0
38	*β*-Caryophyllene	–	0.24	–	-4.5	500.58	0
39	*β*-Elemene	–	0.13	–	-5	215.16	0
40	Phenylmethanesulfonic acid (PMSF)	–	–	–	-5.1	181.72	3

Molecular networking of the *C. limonia* essential oil was also obtained in the positive mode and clustered using the Cytoscape software. Then, the putative structures were matchedfrom the libraries in the GNPS. Fig. 3 shows the monoterpenes and their derivatives successfully detected in the largest cluster. In sub-cluster A, four chemical classes were matched well in the molecular networking, including monoterpenes (limonene, α-pinene, γ-terpinene,…), oxygenated monoterpenes [(*E*)-3,7-dimethyl-2,6-octadien-1-ol, dihydrocarveol, linalool], monoterpene-based ester derivatives [(*E*)-3,7-dimethyl-2,6-octadien-1-yl acetate, (*E*)-3,7-dimethyl-2,6-octadien-1-yl propionate, dihydrocarvenyl acetate, (*E*)-3,7-dimethyl-2,6-octadien-1-yl isobutyrate] and sesquiterpenes (α-bergamotene, -cadinene, α-bisabolene, β-elemene, germacrene-B, α-santalene) (Fig. S2). The remaining nodes in this sub-cluster were identified as their derivatives. The major unidentified nodes in sub-cluster B were not matched with any compounds from GNPS libraries, with the minimum number of common fragment ions and the minimum cosine score being 4 and 0.7, respectively. However, several minor nodes were matched with the GNPS reference libraries, including indole-3-carboxylic acid, 1-methyladenosine, 3-methyladenine and pymetrozine. These results indicated that the *C. limonia* essential oil also contained minor amounts of heterocyclic aromatic amines. Interestingly, 4 of those 10 metabolites in cluster C were matched from the libraries in the GNPS, with the minimum cosine score of approximately 0.7–0.8, including 13-methyltetradecanoic acid methyl ester, methyl laurate, 15-methylhexadecanoic acid methyl ester and tetradecanoic acid methyl ester. The results in the current study indicated that the presence of fatty acid methyl esters was detected in *C. limonia* essential oil in small amounts. According to the chemical analysis, the peak areas of 52 identified compounds represented 81.18% of the total chemical composition of the *C. limonia* essential oil (Tables 1 and S3).

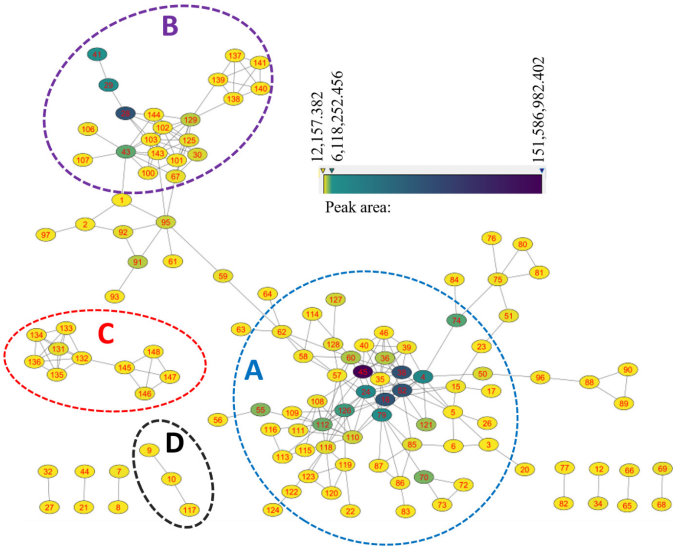

Fig. 3 Molecular networking of essential oil from *C. limonia* peel generated using GNPS web platform (https://gnps.ucsd.edu)

Finally, the molecular network of the *C. sinensis* essential oil was displayed in two clusters. Even though this network was not generated perfectly, the largest cluster could be divided into two sub-clusters (Fig. S3). In sub-cluster A, two structural types were identified, namely the monoterpenes (camphene, limonene, α-terpinene and γ-terpinene) and monoterpene-based ester derivatives [(*E*)-3,7-dimethyl-2,6-octadien-1-yl acetate, (*E*)-3,7-dimethyl-2,6-octadien-1-yl propionate, (*E*)-3,7-dimethyl-2,6-octadien-1-yl butyrate], while other nodes were suggested as monoterpene derivatives. Next, six nodes in sub-cluster B were matched from the libraries in GNPS, with the minimum cosine score of 0.75-0.85 and identified as pyroglutamic acid, pyrrole-2-carboxylic acid, 2,6-diaminopimelic acid, mecamylamine, purine and vigabatrin. Similar to sub-cluster D of the *C. reticulata* essential oil, the *C. sinensis* essential oil also contained minor amounts of volatile amines. The remaining nodes in this largest cluster were putatively identified as monoterpenes and their oxygenated derivatives and aromatic metabolites occurred widely in nature. Taken together, a total of 92 chemical components were suggested, with the presence of 27 identified compounds (90.45%) and 65 unknown structures (less than 9.55%) from this molecular networking (Tables 1 and S4).

Assessment of toxicity effects against Solenopsis invicta of essential oils

To examine the insecticidal action of essential oils, a toxicity assay was performed using RIFA *Solenopsis invicta*. The results in Table 2 show the ant survival of solvent condition was 100% for all the exposure periods (from 1 h to 6 h). Therefore, acetone was determined as the non-toxic solvent of the setup assay. The three essential oils were diluted in the acetone solvent at various concentrations (50 mg/mL, 100 mg/mL, 200 mg/mL) or limonene (10 mg/mL, 25 mg/mL, 50 mg/mL) and exposed to RIFA. The proportion

Table 2 Effects of essential oils from *C. reticulata*, *C. limonia*, *C. sinensis* peels, and limonene on *S. invicta* survival after 1 h to 6 h of exposure

Extract name	Conc. (mg/mL)	Exposure (h)			
		1	2	4	6
Neg. ctl.	-	100±0.00	100±0.00	100±0.00	100±0.00
C. reticulata	50	50.83±3.18^{a}	13.12±4.68^{c}	10.32±0.48^{c}	0±0.00^{d}
	100	35.82±2.58^{b}	2.38±4.12^{d}	0±0.00^{d}	0±0.00^{d}
	200	15.3±1.18^{c}	0±0.00^{d}	0±0.00^{d}	0±0.00^{d}
C. limonia	50	100±0.00^{a}	83.03±5.12^{b}	39.68±9.29^{d}	8.24±4.72^{ef}
	100	100±0.00^{a}	58.33±8.99^{c}	17.03±3.85^{e}	0±0.00^{f}
	200	100±0.00^{a}	33.61±10.42^{d}	9.77±1.54^{ef}	0±0.00^{f}
C. sinensis	50	100±0.00^{a}	79.73±7.19^{b}	21.81±2.03^{d}	7.51±2.3^{ef}
	100	100±0.00^{a}	41.28±6.69^{c}	8.4±0.88^{ef}	0±0.00^{f}
	200	74.33±1.46^{b}	14.4±3.37^{de}	0±0.00^{f}	0±0.00^{f}
Limonene	10	55.34±3.10^{a}	14.02±1.34^{c}	9.59±1.27^{e}	0±0.00^{e}
	25	33.89±1.19^{b}	1.26±1.17^{e}	0±0.00^{e}	0±0.00^{e}
	50	13.82±1.54^{c}	0±0.00^{e}	0±0.00^{e}	0±0.00^{e}

Results expressed as mean ± SD (n = 3)

Different lowercase superscripts in same group of each essential oils or limonene are significantly different at 5%.

of ant survival after treating with *C. reticulata* essential oil significantly exhibited the lowest value for all the exposure periods. In particular, the ant mortality was 100% after 6 h of treatment with this oil at all tested concentrations. However, only moderate activity of ant toxicity was observed for the *C. limonia* and *C. sinensis* essential oils. After 1 h of exposure, only the LC_{50} of *C. reticulata* essential oil could be calculated with the value being 47.74 ± 13.99 mg/mL, while this value could not be determined for other oils. To compare the insecticidal action of the *C. limonia* and *C. sinensis* essential oils, the LC_{50} values at 2 h of exposure period were estimated as 142.87 ± 28.73 and 104.86 ± 14.79 mg/mL, respectively (Table S5). Based on this calculation, it is suggested that the essential oil of *C. sinensis* exhibited better fumigant toxicity against worker ants than *C. limonia*.

According to the chemical profile results of the three essential oils and their toxicity effects against *S. invicta*, the major volatile compound, limonene, could contribute significantly to the insecticidal activity. To confirm this suggestion, the insecticidal action of pure limonene was observed after 1 h of exposure period with an LC_{50} value of 12.69 ± 2.68 mg/mL. Therefore, *C. reticulata* essential oil had the highest content of limonene (76.84%) that strongly induced ant toxicity. However, the essential oils of *C. limonia* and *C. sinensis*, with less toxic symptoms, had limonene contents of 28.61% and 68.83%, respectively. Another study revealed that limonene had beneficial biological effects on the prevention and treatment of many diseases and is used in the formulation of cosmetic products, with little risk of side effects (Lopes et al., 2009). Based on the current findings, the *C. reticulata* essential oil as well as limonene could be potential insecticidal agents, without inducing human toxicity. In particular, poor rural populations in developing countries who lack access to safe insecticidal agents could produce these essential oils themselves for application in and around the home.

Efficacy assessment of ant-repelling activity of essential oils

The *C. reticulata* essential oil was chosen for evaluating its repellency against *S. invicta*. In this experiment, a cotton ball soaked with sugar water alone was used as a blank sample, while a ball containing sugar water and an industrial ant repellent was defined as a positive control. As shown in Figs. 4A–4C, after 24 h of outdoor exposure, the negative control attracted a lot of ants, while the positive control repelled the ants completely. Similar to the repellent activity of the industrial ant repellent, the *C. reticulata* essential oil also prevented *S. invicta* from reaching the ball. The same phenomena were recorded after 2 d or 3 d of outdoor exposure (Fig. S4–S6). In addition, when the antennae of the ants contacted with the balls of the positive control or *C. reticulata* essential oil after 2–4 h of outdoor exposure, they immediately retreated (Tables S6 and S7). This study posited the hypothesis that the *C. reticulata* essential oil could interfere with the ants' scent trails, pheromones and communication.

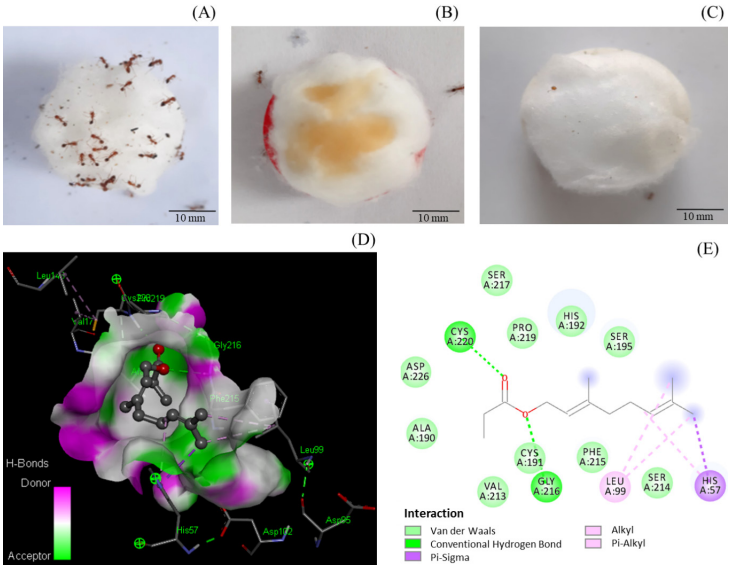

Fig. 4 (A–C) Effects of *C. reticulata* essential oil on repellency against *S. invicta* after 24 h of outdoor exposure: (A) cotton ball with sugar water alone; (B) cotton ball containing sugar water and industrial ant repellent; (C) cotton ball containing mixture of sugar water and *C. reticulata* essential oil; (D, E) 3D molecular docking simulation; (D) and 2D interaction diagram; (E) of the fire ant chymotrypsin protein (PDB: 1EQ9) and (*E*)-3,7-dimethyl-2, 6-octadien-1-yl propionate using BIOVIA Discovery Studio Visualizer

Molecular docking between fire ant chymotrypsin and putative compounds

Fire ant control strategies by reducing digestive enzymes, such as trypsin and chymotrypsin at the larval stage, have emerged in recent decades (Terra and Ferreira, 1994; Lopes et al., 2009; Allen et al., 2018). Until now, there has been no report of *Citrus* essential oils inhibiting the trypsin or chymotrypsin proteins or both and the application of computational docking in this field is still limited due to the lack of reports about the crystal structures of fire ant trypsin and chymotrypsin. Recently, only the structure of *S. invicta* chymotrypsin, an ant proteolytic enzyme, was reported with an atomic resolution of 1.7 Å and the PDB code 1EQ9 (Botos et al., 2000). Therefore, the molecular docking between this protein and the putative compounds of essential oils was investigated as a tool for drug discovery.

Molecular stimulation between the fire ant chymotrypsin protein and ligands was performed using the Autodock software. To evaluate the ligand-protein complex binding affinity, three essential criteria—energy binding (ΔG_{bind}), inhibition constant (K_i) and hydrogen bonds (HBs)—were applied to choose the best complex (Morris et al., 1998). As shown in Table 3,

Table 3 Molecular docking interactions between fire ant chymotrypsin protein (PDB: 1EQ9) with phenylmethanesulfonic acid and putative compounds using BIOVIA Discovery Studio Visualizer

Ligand	*S. invicta* chymotrypsin – PMSF complex (PDB: 1EQ9)						
	Binding energy (kcal/mol)	K_i (μM)	Hydrogen bond	Van der Waals	π-Cation	Alkyl/ π-alkyl	π-Sulfur/ Amide π-Stacked
Phenylmethanesulfonic acid	-5.1	181.72	His192, Ser195, Ser214	Cys191, Gly193, Val213, Phe215, Gly216, Pro219, Asp226	–	Ala190	His57, Cys220
(*E*)-3,7-dimethyl-2,6-octadien-1-yl butyrate	-5.1	181.72	Cys220, Gly216	Arg41, Ala190, Cys191, Gly193, Ser195, Val213, Ser214, Phe215, Ser217, Pro219, Asp226	–	Cys42, His57, His192	–
(*E*)-3,7-dimethyl-2,6-octadien-1-yl propionate	-5.1	181.72	Cys220, Gly216	Ala190, Cys191, His192, Ser195, Val213, Ser214, Phe215, Ser217, Pro219, Asp226	–	His57, Ser214	–
α-Terpinyl formate	-5.2	153.48	Cys220, Ser217	Cys191, His192, Ser195, Val213, Ser214, Phe215, Gly216, Pro219, Asp226	–	His57	–
2,6-Diaminopimelic acid	-5.4	109.49	Ser195, Ser214, Phe215, Gly216, Cys220, Asp226	His57, Ala190, Cys191, His192, Val213, Ser217, Pro219, Ala221, Val227	–	–	–
Indole-3-carboxylic acid	-5.8	55.72	Ser195, Ser214, Cys220	Ala190, His192, Val213, Phe215, Asp226	–	Pro219	Gly216
Pymetrozine	-6.4	20.23	Ala190, His192	Cys191, Gly193, Ser195, Phe215, Gly216, Asp226, Val227	His192	Ala190, Val213	–

phenylmethanesulfonic acid (PMSF), a co-crystallized ligand of the fire ant chymotrypsin protein, produced values of ΔG_{bind}, K_i and HB formation of -5.1 kcal/mol, 181.72 μM and 03 bonds, respectively. Compared to Botos et al. (2000), PMSF was found to bind similarly to the active site of the 1EQ9 protein through interactions with critical amino acid residues, including Ala190, Cys191, His192, Gly193, Ser195 and Cys220 (Fig. S7). A recent study (Kondapuram et al., 2021) suggested that Autodock software could become an essential tool to predict the docking simulation. As shown in Tables 1 and 3 and Figs. S8–S12, ester derivatives [(*E*)-3,7-dimethyl-2,6-octadien-1-yl butyrate, (*E*)-3,7-dimethyl-2,6-octadien-1-yl propionate and α-terpinyl formate] significantly exhibited ΔG_{bind}, K_i and HBs values similar to PMSF. In particular, several volatile amines [6-diaminopimelic acid, indole-3-carboxylic acid and pymetrozine] were found to bind into the active site, which were similar to the ligand-binding site between PMSF and 1EQ9. The ΔG_{bind}, K_i and HBs values of these volatile amines were lower than for the co-crystallized ligand (Tables 1 and 3). These data contributed additional evidence that essential oils, including monoterpene-based ester derivatives and several volatile amines, could affect the nutrition and reproduction of fire ants by reducing proteolytic digestive enzymes (Chen and Oi, 2020; Zhang et al., 2021).

In summary, based on the molecular networking and GC-MS fragmentation analysis of three essential oils from *Citrus* species, many structural types of these oils were detected and identified, including monoterpenes, oxygenated monoterpenes, ester derivatives, sesquiterpenes, aromatic compounds and volatile amines. The peak areas of identified compounds from essential oils of *C. reticulata*, *C. limonia* and *C. sinensis* represented 94.34%, 81.18% and 90.45%, respectively of their total chemical compositions. During the screening process of these essential oils for their toxicity effect against *S. invicta*, the *C. reticulata* essential oil exhibited the strongest fumigant toxicity against worker ants with an LC_{50} value of 47.74 ± 13.99 mg/mL after 1 h of exposure. This essential oil also had a significant repellent effect on RIFA in outdoor environmental tests. Based on the molecular docking simulation of 40 metabolites of these essential oils, it was suggested that monoterpene-based ester derivatives and several volatile amines could be promising fire ant chymotrypsin inhibitors. Taken together, these results highlight that the *C. reticulata* essential oil could have high potential for observed activity against *S. invicta*, but further research is needed to confirm these observations. Furthermore, basic future investigations by the current authors will focus on a preliminary test of repellency of microencapsulated *Citrus* essential oils against *S. invicta*. In general, these results show applications of the multidisciplinary fields to investigate the utilization of resource waste. The combination of the various experiments involved molecular networking-based chemical profiling, chemical extraction and an animal model for screening the biological activities of essential oils from *Citrus* peel against *S. invicta*. The experiment also provided a new approach for research and utilized agricultural waste as a natural insecticide or insect repellent.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

This work was funded in part by the Can Tho University, Code T2022-27. Some simple experiments were supported by Bachelor in Chemistry students, including Mr. Le-Thien-Vu Huynh, Mr. Huynh-Phuong-Vy Van, Mr. Tan-Dat Nguyen and Mr. Huu-Dat Ma.

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