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Identification of Active Compounds of Ethanol Extract of *Citrus amblycarpa* leaves by Analysis of Thin-layer Chromatography and Gas Chromatography-Mass Spectrometry as Bioinsecticide Candidates for Mosquitoes

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Abstract

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Keywords: Thin-layer chromatography; Gas chromatography-mass spectrometry; Bioinsecticide; *Citrus amblycarpa*; *Aedes aegypti*

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BACKGROUND: The use of active compounds from plants becomes an alternative to control mosquitoes nowadays and in the future because they are environmentally-friendly and do not cause health problems. *Citrus amblycarpa* is a local orange of South Kalimantan potential as bioinsecticidal, which commonly used for controlling mosquitoes. Therefore, research needs to be done to find out the benefits of *C. amblycarpa* leaves as bioinsecticidal.

AIM: The research aimed to identify active compounds contained in the extract ethanol of *C. amblycarpa* leaves as bioinsecticidal against mosquitoes.

RESULTS: Based on thin-layer chromatography test, there were some secondary metabolite compounds found such as terpenoids/steroids, flavonoids, polyphenols, and saponins. Gas chromatography-mass spectrometry (GC-MS) test revealed that there were ten primary components of the fraction. The components were Maragenin I (18.82%), 1,3-benzenedicarboxamide (12.28%), 2,3,8-trioxocephalotaxane (10.39%), aristolone, 2H-cyclopropa[a] naphthalene-2-one, noruns-12-ene (7.46%), palmitic acid, n-hexadecanoic acid (7.21%), stigmasterol, demecolcine (7.03%), alpha-tocopherol (5.88%), 2,4,5-trimethylphenol, pseudocumol (4.21%), germacrene-D (3.45%), and 9-octadecenoic acid (3.36%).

CONCLUSION: These active compounds possess biological activity as bioinsecticidal. It was expected that those active compounds in *C. amblycarpa* leaves could be applied for controlling mosquitoes by replacing the use of resistant temephos.

Introduction

Dengue fever, both in tropical and sub-tropical, is a disease [1], [2] transmitted through the bites of *Aedes aegypti* or *Aedes albopictus* and caused by Dengue virus [3], [4]. South Kalimantan is a province which belongs to dengue fever endemic where 13 cities/regencies have been affected by the diseases [5]. A report from Health Agency of South Kalimantan Province showed that there are 1079 cases of dengue fever with 33 people died over 2013. In 2014, there are 363 cases of dengue fever with eight people died (incidence rate/1000 people is 1103), while in 2015 there is a significant increase in the incidence of dengue fever reaching 1.216 cases with 19 people died. The highest case occurs in Banjarmasin, Banjarbaru, and Banjar Regency [6]. The fluctuated condition of

dengue fever incidence encourages a need to control *A. aegypti*. One of the methods to break the cycles and kill mosquitos' larvae is using insecticides [7].

The constant use of synthetic insecticides (temephos/abate, malathion, cypermethrin, lambda sihalothrin, and deltamethrin) on mosquitoes vector causes resistance, bioactive characteristics which are harmful for the environment, toxic substances, in the insecticides will have adverse impact on human health. Plant-based insecticides become an alternative to control mosquitoes using more environmentally friendly plants to suppress the use of synthetic insecticides and anticipate negative impacts on health [8].

In Indonesia, there are around 2.400 species of plants potential for bioinsecticides [9]. One of the local plants and abundant in South Kalimantan and contains active compounds to be used for bioinsecticides

against *A. aegypti* is *C. amblycarpa*. The plants contain several active secondary metabolites such as flavonoids, tannins, saponins, and alkaloids [10]. The extracted fresh peel of *C. amblycarpa* is proven to be lethal for *A. aegypti* third larval instars within 7 h in all concentrations [11]. The aim of the study was to identify and analyze chemical components of *C. amblycarpa* and its potential as bioinsecticides against *A. aegypti*.

Materials and Methods

The study was an experimental laboratory conducted in the Laboratory of Entomology and Protozoology, Department of Parasitology, Faculty of Veterinary, Airlangga University. Extraction, isolation, and analysis of chemical compounds were carried out in the Laboratory of Faculty of veterinary, Airlangga University. Around 2.5 kg fresh samples of Limau Kuit leaves were collected from Kaliukan Village, Astambul, Banjar Regency, and South Kalimantan. Several materials used for extraction, isolation, and identification were ethyl-alcohol p.a. (E. Merck), technical ethanol, and aquadest. A set of maceration, rotary evaporator, pipette, test tube, evaporating dish, analytical balance, vial bottle, micropipette, falcon tube, Erlenmeyer glass, capillary pipe, drop plate porcelain, UV lighting ($\lambda = 245$ nm), chromatography chamber, thin-layer chromatography (TLC), chromatography column, test pipette heater, electrical stove, 20 W fluorescent bulb, and a set of gas chromatography-mass spectrometry (GC-MS).

The samples of *C. amblycarpa* leaves were sorted and cleaned by washing the leaves using clean water, drained and distributed on the paper to reduce the water content. After that, 2.5 kg of samples were dried weight, aired for 7 days by putting the samples in the shade places. The samples were then mashed to generate powder. 1 kg of simplisia was macerated using ethyl alcohol solvent for 3 days. Filtration was conducted every day and the filtrates were collected and steamed using rotary evaporator to generate 52 g extracted dry leaves of *C. amblycarpa*. Phytochemical screening of extract ethyl alcohol and the most active fractions include alkaloids, flavonoids, saponins, phenolics, triterpenoids, quinones, and terpenoids and steroids. TLC analysis on the collected extract was run with mobile phase, a combination of ethyl alcohol p.a with various comparisons, and silent phase used silica gel of 60 GF25. The composition of the best TLC

was then employed as a mobile phase in collected extract of chromatography column. Silent phase in the chromatography column was 60 G silica gel. Extract of Limau Kuit leaves and fractions of chromatography column were tested for their activities using BSLT method. The solution making for activity tests was carried out with 3 times replications.

Fraction LC₅₀ was used because it was the most active fraction. The fraction was then analyzed its components using Agilent 6980N Network GC system, detector Agilent 5973 inert MSD. Around 1 μ L sample was injected to GC-MS operated using glass column for 30 m, diameter of 0.25 mm and thickness of 0.25 μ m. Oven temperature was 50°C (5 min), 10°C/min, and 280°C (15 min). Flow in the column was 1 ml/min (constant), Wiley Reference of version 7.0. The method was employed to identify a compound, either one or mixed components [12]. Precise spectrometry mass was employed to determine fragmentation and molecules and also to identify components contained in small amounts [13].

Results

Screening of phytochemical of *C. amblycarpa* results of the study showed that there were some compounds of secondary metabolites such as free terpenoid/steroid, flavonoid, polyphenol, and saponin. The identification result of chemical compounds of *C. amblycarpa* leaves is presented in Table 1 and Figure 1.

Table 1 shows positive test result in free terpenoids/steroids, flavonoids, polyphenols, and saponins but shows negative test in alkaloids.

Analysis of GC-MS of *C. amblycarpa* leaves extracted using ethanol

The samples were analyzed using GC-MS Agilent 6980 N Network GC System, detector Agilent 5973 inert MSD. Chromatogram of *C. amblycarpa* leaves is presented in Figure 2, while the chemical components are shown in Table 2.

Table 2 shows that there are ten main components of fraction obtained from GC-MS analysis. The components are Maragenin (18.82%), 1,3-benzenedicarboxamide (12.28%), 2,3,8-trioxocephalotaxane (10.39%), aristolone, 2H-cyclopropa[a] naphthalen-2-one, noruns-12-ene (7.46%), palmitic acid, n-hexadecanoic acid (7.21%),

Table 1: Screening of phytochemical of *Citrus amblycarpa* leaves extracted using ethanol

Phytochemical test	Reagent	Staining appearance	Result
Alkaloids	Dragendorff	Orange	-
Free Terpenoids/Steroids	Sulfate acid anisaldehyde	Red purple or purple	+
Flavonoids	Ammonia evaporation	Intensive yellow	+
Polyphenols	2% FeCl ₃	Brown to black	+
Saponins	a drop of 2N HCl	Stable foam for more than 30 min	+

+: Contain chemical compound, -: No chemical compound

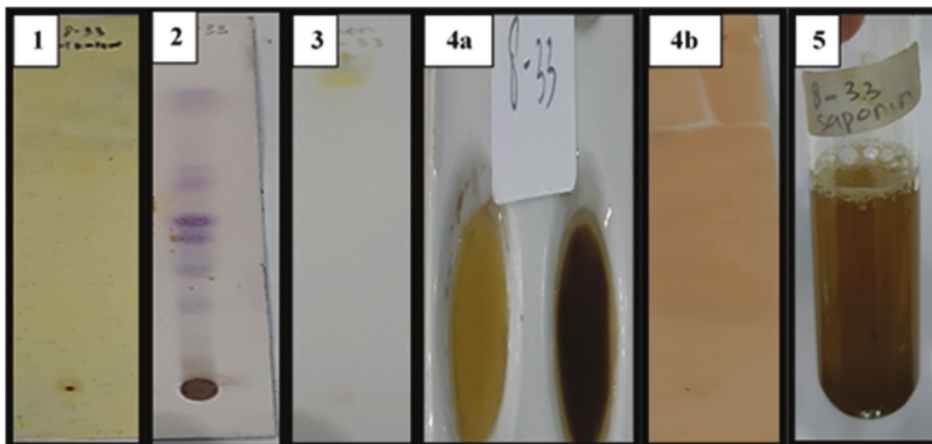


Figure 1: (1) Samples do not show orange spot (contain negative [-] alkaloids), (2) purple spot (contain positive (+) terpenoids/free steroids), (3) intensive yellow spot (contain positive (+) flavonoids), (4a) based on Ferric chloride test, samples display black-blue green spot (contain positive (+) polyphenols), (4b) based on KLT test, samples show blackish spot (contain positive (+) polyphenols), and (5) based on foam test, the foam can last for 30 min (contain positive (+) saponins)

stigmasterol, demecolcine (7.03%), alpha-tocopherol (5.88%), 2,4,5-trimethylphenol, pseudocumol (4.21%), germacrene-D (3.45%), and 9-octadecenoic acid (3.36%).

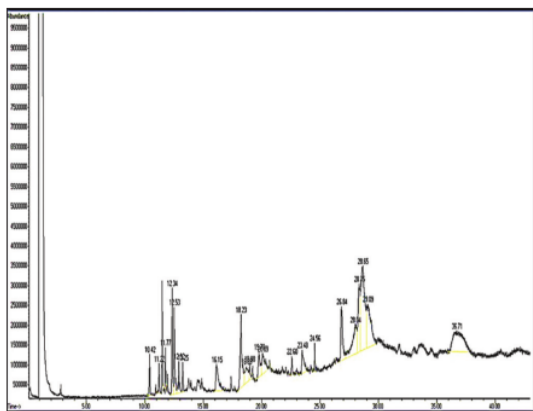


Figure 2: Chromatogram of Limau Kuit (*Citrus amblycarpa*) leaves extracted using ethanol, analyzed using gas chromatography-mass spectrometry

Discussion

Indonesia possesses a wide variety of local plants potential for biopesticidal [14]. In the present study, we are interested in *C. amblycarpa* because the plant is a local orange and abundant from South Kalimantan. Moreover, it could also be plant-based insecticides. The plant contains secondary metabolite compounds such as alkaloids, saponins, tannins, and flavonoids [10]. Principally, plant cells contain primary and secondary

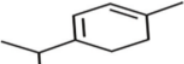

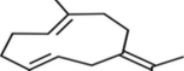
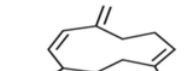


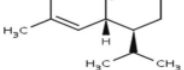
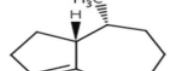
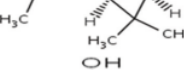
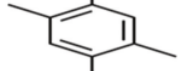




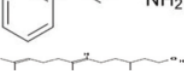
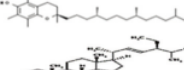



metabolites. Primary metabolites are carbohydrate, amino acids, lipids, and vitamins, while secondary metabolites are a source for pharmaceuticals, food additives, perfume ingredients, or pesticides [15]. The secondary metabolite compounds are a relatively safe insecticidal to environment and human health because it possesses insufficient risks [16].

The purpose of the study was to identify and analyses the chemical content of *C. amblycarpa* leaves using TLC and GC-MS analysis [20] and also to examine its potency as bioinsecticidal. The active compounds such as alkaloids, terpenoids, flavonoids, and polyphenols in the extract were determined using color reagent, while saponins compounds were tested by foam test. The results showed that extract ethanol of the leaves showed positive test on terpenoids/steroids, flavonoids, polyphenols, and saponins compounds, but showed negative result on alkaloids compounds (Table 1). Ghosh reported that steroids, sitosterols, and stigmasterols compounds are found in maja leaves and possess larvicidal activity for *A. aegypti*, *A. stephensi* and *C. quinquefasciatus* larvae [17]. Steroids are toxic to nerve cells affecting neurotransmission function and inhibiting ion transports making mosquitoes limp and death [18].

Flavonoids contained in the plant affects the respiration of mosquitoes. The compound gets into the nerve cells along with the air through respiratory organs decreasing the amount of oxygen. As a result, the mosquitoes suffer from nervous and spiracle disruptions and then death [19]. Plants containing flavonoids compounds have toxic effect on *Anopheles* and *A. aegypti* larvae, indicated by the loss of chitin layer and abnormal body stretching [20].


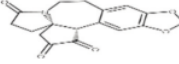
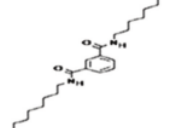
The results of chromatogram and analysis of GC-MS extract ethanol of *C. amblycarpa* leaves showed 22 compounds with ten primary components of

Table 2: Chemical components of *Citrus amblycarpa* leaves extracted using ethanol and analyzed using GC-MS

Peak	Retention time (min)	Area (%)	Chemical formula	Compound name	Biological activities	Chemical structure
1	10.42	1.46	C ₁₀ H ₁₆	Alpha-terpinene	Potential larvicides and mosquito Repellent ¹⁴	
2	11.22	0.99	C ₁₅ H ₂₄	2-Methylene-4,8,8-trimethyl-4-vinyl-Bicyclo [5.2.0] nonane, Beta-Elemene	Potential Insecticide of <i>Aedes aegypti</i> ¹⁵	
3	11.78	0.91	C ₁₅ H ₂₄	Gamma elemene, Germacrene-B	Potential Insecticide of <i>Aedes aegypti</i> ¹⁵	
4	12.34	3.45	C ₁₅ H ₂₄	Germacrene-D	Toxic to <i>Anopheles subpictus</i> , <i>Aedes albopictus</i> and <i>Culex tritaeniorhynchus</i> larvae ¹⁶ , Potential insecticides ¹⁷	
5	12.53	3.13	C ₁₅ H ₂₄	Lepidozene	Mosquito Repellent <i>Aedes aegypti</i> ¹⁸	
6	12.92	1.66	C ₁₅ H ₂₄	Delta-cadinene, beta.-cadinene	Potential insecticides ¹⁹ , Anti feedant ²⁰	
7	13.25	0.91	C ₁₅ H ₂₄	Alpha-Gurjunene., beta.-Neodovlene	Activity of larvicides of <i>Aedes aegypti</i> ²¹ , mosquito repellent ²²	
8	16.16	4.21	C ₉ H ₁₂ O	4-Hydrazinopyrazino [3,2-D] Pyrimidine, -2,4,5 Trimethylphenol, Pseudocumenol	Potential insecticides ²³	
9	18.23	7.21	C ₁₆ H ₃₂ O ₂	Palmitic acid, n-Hexadecanoic acid	Possess biolarvicides effect on <i>Aedes aegypti</i> , <i>Culex</i> sp., and <i>Anopheles sudaicus</i> larvae. ²⁴	
10	18.70	2.95	C ₁₆ H ₃₂ O ₂	Palmitic acid, n-Hexadecanoic acid	Activity of insecticides on <i>Aedes aegypti</i> . ²⁵	
11	18.98	0.81	C ₁₈ H ₃₄ O ₂	9-Octadecenoic acid	Lethal to <i>Aedes aegypti</i> and <i>Culex pipiens</i>	
12	19.78	2.65	C ₁₈ H ₃₄ O ₂	9-Octadecenoic acid	pallens larvae ²⁶	
13	20.09	3.36	C ₁₈ H ₃₄ O ₂	9-Octadecenoic acid		
14	22.60	0.77	C ₃₄ H ₅₈ O ₄	Diocyl ester, 1-2 Benzenedicarboxylic acid	Activity of larvicides vector of <i>Aedes aegypti</i> ²⁷	
15	23.48	2.87	C ₉ H ₉ NO	Cinnamamide	Potential insecticides and antifungal, ²⁸ Repellent ²⁹	
16	24.56	0.80	C ₁₅ H ₂₈ O	Dihydrofarnesol,-dodecatrienol	Antioxidant, antifungal, antibacterial ³⁰	
17	26.84	5.88	C ₂₉ H ₅₀ O ₂	Alpha-tocopherol, Vitamin E	Antioxiidant ³¹	
18	28.04	7.03	C ₂₃ H ₃₆ O	Stigmasterol, Demecolcine	Potential Insecticides ^{32,33}	
19	28.35	7.46	C ₁₅ H ₁₂ O	Aristolone, 2H-Cyclopropa[a] naphthalen-2-one, Noruns-12-ene	Potential Insecticides ³⁴	

(Contd...)

Table 2: (Continued)

Peak	Retention time (min)	Area (%)	Chemical formula	Compound name	Biological activities	Chemical structure
20	28.65	18.82	C ₂₀ H ₃₆ O ₂	Maragenin I	Potential Insecticides ²⁵	
21	29.09	10.39	C ₂₀ H ₃₆	2,3,8-Trioxocephalotaxane	Potential Insecticides ²⁶	
22	36.71	12.28	C ₉ H ₉ N ₃ O ₂	1,3-Benzenedicarboxamide	Potential Insecticides ²⁷	

GC-MS: Gas chromatography-mass spectrometry.

the fraction. *Maragenin I* is a main compound with the highest component found in the leaves. *Maragenin I* is a derivate of triterpenoid [21]. Literature study has been done, *Maragenin I* compound is found to be antiviral [22], anti-microbe, and antioxidant [23]. The compound is able to control the growth of insects and potential as insecticides [24]. It is a derivative of triterpenoid/steroid. Therefore, it is concluded that the compound is potent to be used as biopesticides, and poisonous to *A. aegypti*.

The compound is able to kill *A. aegypti* to 90%. *Stigmasterol* is the main sterol of plasma membrane in the cell of plants [25]. Sterols, in plants known as phytosterol and belongs to the group of alcohol steroids, are natural phytochemical exclusively found in plants. The compound is alcohol soluble. *Stigmasterols* are present in various medical plants and it has been reported that the compounds inhibit the activity of acetyl cholinesterase making them possessing larvicidal effect. Moreover, *stigmasterols* are one of active compounds which contribute to insecticidal [26]. It is potential to prevent insects and to be developed for botanical biopesticides.

D-alpha-tocopherol (Vitamin E) is fat-soluble compound and the main antioxidant for cells. This compound contains highest antioxidant activity of all tocopherols [27]. *Trimethylphenol* compound is found in the extract of *Artemia salina* flowers with cytotoxic that can be used for pesticides. *Germacrene-D* is a compound belonging to sesquiterpenoid hydrocarbon group [28]. This compound has been reported poisonous to *Anopheles subpictus*, *Aedes albopictus*, and *Culex tritaeniorhynchus* larvae. *Germacrene-D* compound causes typical biological activities such as toxic which inhibits food, antiparasitic, and pesticides.

9-Octadecenoic acid, also known as oleic acid, is a compound from fatty acid. Compounds from lipid acids are benefit to prevent pests. The acid can be lethal to *A. aegypti* and *Culex pipiens pallens* larvae. 9-Octadecenoic acid is also an active principle compound obtained from the extract of *Annona glabra*. It is also poisonous that work quickly if applied manually and serves as ingested and contact insecticides. Thus, it affects mortality rate for *Eurema* sp. larvae [29], [30], [31], [32].

Conclusion

C. amblycarpa leaves contain active chemicals such as terpenoids/steroids, flavonoids, polyphenols, and saponins potential as bioinsecticides. The analysis of GC-MS showed that the main components of fraction were *maragenin I*, 1,3-benzenedicarboxamide, 2,3,8-trioxocephalotaxane, aristolone, 2H-cyclopropa[a]naphthalen-2-one, noruns-12-ene, palmitic acid, n-hexadecanoic acid, stigmasterol, demecolcine, alpha-tocopherol, 2,4,5-trimethylphenol, pseudocumenol, and germacrene-D. The active compounds of the leaves could be an alternative to control mosquitoes in the future by replacing the use of resistant *temephos*.

Acknowledgments

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