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ORIGINAL PAPER

Toxicity and repellence of Taiwanese indigenous djulis, *Chenopodium formosaneum*, against *Aedes albopictus* (Diptera: Culicidae) and *Forcipomyia taiwana* (Diptera: Ceratopogonidae)

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Abstract The biological activity of djulis (*Chenopodium formosaneum*) extracts was evaluated against mosquitoes and biting midges. Djulis extracts were relatively nontoxic to *Aedes albopictus* larvae. However, they showed interesting repellence against adult mosquitoes as estimated by the median effective dosages (ED_{50}). ED_{50} values for djulis extracts against mosquito adults in descending order were: seed extracted with methanol (0.83 %), seed extracted with dichloromethane (0.66 %), leaf extracted with methanol (0.50 %), and leaf extracted with dichloromethane (0.40 %). Field tests also suggested that djulis methanol extracts were effective at about a 1 % level against biting midges (*Forcipomyia taiwana*). A total of 15 and 20 compounds accounting for 88.8 and 79.9 % in the seed and leaf extract, respectively were identified by gas chromatography coupled to mass spectrometry (GC–MS). Among these, 9, 12-octadecadienoyl chloride, (Z, Z) was found in both as well as being the major constituent in the leaf extract (35.7 %). Further studies on the repellent property of the extracts against mosquitoes and biting midges are warranted.

Keywords Repellency · *Chenopodium formosaneum* · *Aedes albopictus* · *Forcipomyia taiwana*

Eddie Hang Chio and En Cheng Yang contributed equally to this study.

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Introduction

Many mosquitoes and biting midges are notorious blood-sucking insects. The bites of these insects are not only bothersome to humans, but they also transmit pathogens. Several species of mosquitoes in tropical and sub-tropical regions, such as *Aedes aegypti* and *Aedes albopictus,* are vectors of yellow and dengue fever (Amakua et al. 2011; Gubler 1998), and *Anopheles gambiae* is a major vector of malaria which is responsible for numerous fatalities, the majority of whom are young children in sub-Saharan Africa (Murray et al. 2012).

Biting midges are also serious pests in some parts of the world. Though biting midges are not a vector of known pathogens, their bites usually cause discomfort to residents and disrupt work-related activities *Forcipomyia taiwana* is a small (ca. 1.4 mm), slender biting midge that is present island-wide in urban and suburban habitats in Taiwan (Chuang et al. 2000). Since this biting midge only feeds on human blood in the day time (Yeh and Chuang 1996), it is one of the most annoying blood-sucking pests in scenic sites and public parks in central Taiwan. Their presence has already resulted in adverse effects on recreational activities and land development, especially tourism.

Several million dollars are invested in chemical eradication of arthropod vectors annually by insecticide

application, however little success has been reported in most parts of the world (Silva et al. 2008). Moreover, the usage of insecticides not only pollutes the environment but is also harmful to non-target organisms, including humans. Therefore, one alternative for management of blood-sucking insects is the prevention of breeding through the use of natural repellents.

For the sake of environmental safety and human health, alternative control strategies are needed, especially focused on natural product alternatives for pest control in developing countries (Regnault-Roger et al. 2012). A number of chemical repellents with different formulations and trade names are available commercially. Among them, DEET (*N*,*N*-diethyl-m-toluamide) is the main active constituent in most of the preparations, in concentrations ranging from 10 to 90 % (Stuart et al. 2000). Though DEET is effective and safe with regular or casual use (Antwi et al. 2008), nervous and immune toxicity resulting from long-term applications have been reported (Corbel et al. 2009), especially for children experiencing seizures (Lipscomb et al. 1992) and dermatitis (Wantke et al. 1996). Moreover, a recent study showed that DEET suppresses humoral immunological functions in B6C3F1 mice (Keil et al. 2009). The demand for natural, non-persistent, and non-toxic insecticides/repellents is gradually increasing (Katz et al. 2008).

Djulis (*Chenopodium formosaneum*) seed is a traditional food source used by aboriginal people, especially those who live in the "Peace Village" in Taiwan. Djulis has colorful leaves, but its bright red seeds are mostly likely why it is called "Hung Li" in Chinese. Djulis seed is also one of the ingredients used to make local wine (aka small-grain wine) preferred by the aboriginals (Tsai et al. 2010). This plant has been established in Taiwan for centuries, but its identity was unknown until recently. One species of djulis commonly cultivated by the aboriginals has recently been identified as *C. formosaneum* (Koidz) (Tsai et al. 2010). Taiwanese aboriginals believe that djulis is an excellent insecticide and insect repellent for biting midges (*F. taiwana*) and apple snails (*Pomacea canaliculata*). (Personal communication with Dr.Yi-Yuan Chuang at Kaohsiung District Agricultural Research and Extension Station, Taiwan). Under laboratory conditions, methanol extracts of djulis show some interesting repellence against Asian tiger mosquito (*A. albopictus*) adults with median effective dosage (ED_{50}) ranging from 0.53 to 0.93 % (Chio and Yang 2008). Follow-up studies were therefore conducted to further investigate the potential of djulis extracts as insect repellents. We report here the toxicity and repellency of djulis crude extracts using two solvents against mosquitoes under laboratory conditions. We will also report some preliminary field trial data of djulis methanol extract against biting midges.

Materials and methods

Preparation of djulis extract

Djulis foliage from plants in the reproductive stage were collected from Ping-Tung county of Taiwan with assistance from the aboriginals at August 2007. This plant was later identified as *C. formosaneum* (Koidz) by Prof. Yang YP in the Department of Biological Sciences, National Sun Yat-sen University, Taiwan. Leaves were first oven-dried then ground with a pestle and mortar. Djulis seeds were provided by the National Plant Genetic Resources Center of the Taiwan Agricultural Research Institute. Seeds were ground up directly without oven-drying, with a pestle and mortar. Powders of leaf or seed were mixed with methanol or dichloromethane at 1.5 g powder per 100 mL solvent ratio. After continuously stirring the powders with solvents for about 8 h, the un-dissolved powders were removed by filtration through filter paper. Methanol or dichloromethane extracts were then placed in a lyophilizer overnight or until solvents were completely evaporated. Lyophilized extracts were collected and kept at 4 °C until used. Before the experiments, extracts were serially diluted with the appropriate solvent. Djulis leaf (L) extracted with methanol (M) was labeled as LM, seed (S) extracted with dichloromethane (D) was labeled as SD, and so on. Similar sample preparation procedures had been used successfully for extracting active compounds from green algae (Chou et al. 2008).

Insect preparation

The laboratory colony of the Asian tiger mosquito (*A. albopictus*) was used for the toxicity (third instar larvae) and repellenc bioassays (5–14 days old adult), respectively. Both larvae and adults were raised and maintained in our Department for over 10 years at 27 °C and 80 % relative humidity under a 12:12 h light:dark cycle (Gerber et al. 1994). Adults were provided with a 10 % honey solution ad libitum. Live mice (random stock ICR strains purchased from the Laboratory Animal Center of National Taiwan University (NTU)) were used to provide blood meals for mosquitoes. Larvae were raised at densities of 100 larvae/L distilled water and fed with ground fish food.

Toxicity assays

The toxicity assays were performed in a 96-well microtiter plate with third instar larva of *A. albopictus* according to the procedure described by Chio (2007). In brief, consecutive double dilution of djulis extracts was made in methanol (i.e., from 10,000 to 4.9 ppm) and 100 µl of each dilution were transferred to a column of 96-well plate (i.e.,

8 wells/dilution). After evaporation to complete dryness by a heat block, 100 µl distilled water with five-third instar larvae were pipetted into each well (i.e., 40 larvae/dilution). Four replicates for each treatment were used and larvae mobility and mortality at 24 h post treatment were recorded for probit calculation. Methanol and permethrin (0.5 %) were used in parallel as negative and positive controls, respectively. Permethrin is well-known for mosquito control and induced 100 % larvae mortality for *A. albopictus* in the current assays.

Mosquito repellent assay

The mosquito repellence test was performed between 10:00 and 14:00 with adult mosquitoes that were 5–14 days old according to the procedure described by Chio and Yang (2008). Serial dilution of 1 mL djulis extract in methanol was needed when applied to a small fiberglass window screen (5 × 12 cm) with mesh size of 2.5 × 2.5 mm. Methanol and OFF@ (15 % DEET or *N*,*N*-diethyl-m-toluamide) were applied as negative and positive controls, respectively. These treated screens were later used to make feeding cages hosting a live mouse. The number of mosquitoes that landed on the feeding cage at the end of 2 min was recorded. This test procedure was similar to that described by Chio and Yang (2008).

Biting midge repellence test

Since the midges accept only human blood (Yeh and Chuang 1996) and are not easy to rear under laboratory conditions, we used our legs as bait for the repellent bioassay for both LM and SM extracts. All human subjects provided written informed consent before participating in studies. The study was carried out in the Tai-Hang area, which is notorious for its biting midge infestation in Taiwan (Chuang et al. 2000). Experiments were performed in June to August from 10:00 to 14:00, which is the most heavily infested season and time in Taiwan (Chuang et al. 2000). The bioassay was performed in an approximately 50 cm^2 area (radius 4 cm circle) on our lower legs. On one leg, the area was treated with 200 µl of various concentrations of LM or SM extracts, and on the other leg, the area was treated with the same volume of methanol alone (negative control). A commercial repellant (Earth Chem. Co., Ltd, Japan; containing 5.85 % DEET) was used as a positive control in a parallel assay. Both legs were simultaneously exposed in the field to attract the midges. The numbers of bites located on the 50 cm^2 area, with different treatments were recorded at the end of 3 min. These studies were repeated every 30 min up to 180 min. Therefore, the residual effects of the LM and SM could be accessed in a time-course manner.

Gas chromatography-mass spectroscopy (GC–MS)

Compositions of djulis extract were determined using a Focus GC chromatography, coupled with a Polaris Q mass instrument (Thermo) and equipped with a DB-5MS fused capillary silica column (30 m × 0.25 mm; film thickness 0.25 µm), under the following conditions: helium as carrier gas at 1.0 mL/min; injector split at 250 °C (split ratio 1/30); transfer line temperature 300 °C, ion source temperature 200 °C, column temperature program 40 °C during 3 min, with 5 °C increase per min. to 220 °C, ending with a 3 min. isothermal at 220 °C. The mass spectra were taken at 70 eV with scanning speed of 0.58 scan/s from 50 to 650 *m*/*z*.

Components of djulis extract were identified on the basis of comparison of their retention indices and computerized matching of the acquired mass spectra with those stored in both Wiley and NIST 98 mass spectral libraries of the GC/MS data system containing over 330,000 spectra. Peak identities were further confirmed using a standard index (SI) value determined from a direct match of the unknown spectra with the library spectra. Moreover, a reverse standard index (RSI) value was also evaluated which ignores any mass peaks in the unknown that are not in the library spectrum (Warren et al. 2007). A perfect match would result in a value of 1,000 for either matching factor. For evaluation purposes, any value over 700 is considered to be a good match. Percentage composition was calculated using a peak normalization method.

Data analyses and statistics

The percent of repellence from each concentration was rounded off to the nearest integer and determined by the formula described by Weaving and Sylvester (1967).

$$R = (1 - T/C) \times 100$$

where R = Percentage repellency, T = number of mosquitoes land on treated screens, and C = number of mosquitoes landing on methanol control screen. For the biting midge field study, repellence was determined by the same formula by comparing biting rates on the treated and the negative-control legs. Four replicates for each treatment were used and the median effective dosage (ED_{50}) was then calculated by Probit-log concentration analysis (Finney 1971).

Results

Toxicity of djulis extract

LC50 values for djulis extracts for *A. albopictus* larvae were several thousand ppm, significantly higher than that of permethrin (Table 1). According to Ponlawat et al. (2005), the

Table 1 Toxicity of djulis extracts against *A. albopictus* larvae

	LC_{50} ppm (95 % CI)	Slope
A. albopictus		
LM[a]	9900 (6140–42620)	2.001
SM	8250 (6560–11570)	5.322
LD	6530 (5150–8490)	5.055
SD	4030 (3200–5120)	5.024
Permethrin	2–23 ppb (resistant strain)	(Ponlawat et al. 2005)

[a] *LM* djulis leaf extract with methanol, *LD* djulis leaf extract with dichloromethane, *SM* djulis seed extract with methanol, *SD* djulis seed extract with dichloromethane

LC50 of permethrin ranged from 7–30 to 2–23 ppb for resistant strains of *A. aegypti* and *A. albopictus*, respectively, in Thailand. These results suggest that the djulis extracts were relatively non-toxic to mosquito larvae.

Repellence of djulis extract

In terms of repellence against the mosquitoes, the djulis extracts showed interesting results. Most of the

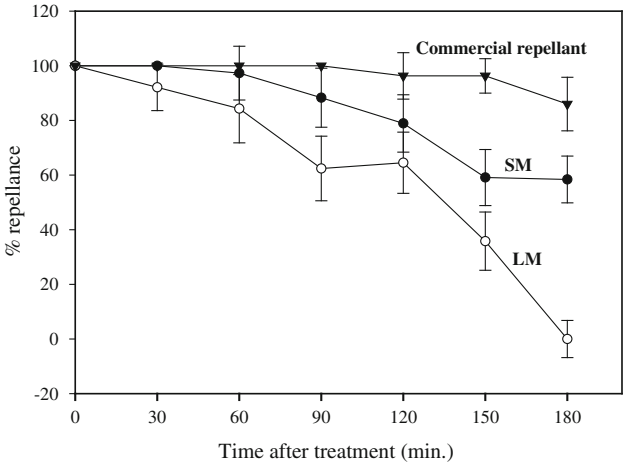

Fig. 1 Time-course repellent effect of djulis extracts against *F. taiwana*. The test area (50 cm^2) was treated with 200 µl of 1 % methanol extract of djulis or commercial repellant with 5.85 % DEET (positive control), and the same-size area on the other leg was treated with the same volume of methanol alone (negative control). Both legs were simultaneously exposed in the field to attract the midges at an interval of 30 min. The numbers of biting midges appeared on the test area were recorded during 3 min exposure at each time point. The results are the means of four independent measurements

Table 2 Percentage repellency of djulis extracts against *A. albopictus* and *F. taiwana*

Conc.(%)	LM	SM	LD	SD	DEET
A. albopictus					
5	95.96 ± 4.57	96.43 ± 2.54	100.0 ± 0.00	100.0 ± 0.00	NA
2.5	90.91 ± 3.48	90.48 ± 3.04	100.0 ± 0.00	90.00 ± 3.65	NA
1.25	77.78 ± 5.47	67.86 ± 2.56	81.82 ± 3.98	77.50 ± 2.61	NA
0.63	64.65 ± 4.64	40.48 ± 4.83	63.64 ± 2.41	50.00 ± 1.63	NA
0.31	29.29 ± 2.16	10.71 ± 3.62	40.91 ± 3.07	17.50 ± 2.38	NA
15	NA	NA	NA	NA	100
p value	<0.0001	<0.0001	<0.0001	<0.0001	
F. taiwana					
1	100.0 ± 0.00	100.0 ± 0.00	NA	NA	NA
0.2	92.70 ± 4.40	98.70 ± 3.03	NA	NA	NA
0.04	58.20 ± 4.59	83.00 ± 2.98	NA	NA	NA
0.008	61.30 ± 2.22	45.50 ± 2.70	NA	NA	NA
0.0016	0.00 ± 0.00	9.60 ± 2.29	NA	NA	NA
p value	<0.0001	<0.0001			

NA not applicable

p value was derived from the statistic comparison among different concentrations of essential oil by linear regression

Table 3 ED_{50} of djulis extracts against *A. albopictus* and *F. taiwana*

Djulis extract	ED_{50} (%)	Slope	Upper limit at 95 %	Lower limit at 95 %
A. albopictus				
LM	0.500	1.925 ± 0.182	0.684	0.312
SM	0.829	2.570 ± 0.197	0.936	0.729
LD	0.408	1.888 ± 0.318	0.495	0.309
SD	0.664	2.612 ± 0.213	0.821	0.516
F. taiwana				
LM	0.013	1.178 ± 0.110	0.039	0.001
SM	0.010	1.633 ± 0.174	0.011	0.008

mosquitoes tended to stay away from the 2.5 % or higher concentrations of djulis-treated screens and this behavior seemed to be dosage related (Table 2). For objective comparison, their median effective dosages (ED_{50}) were calculated by Probit-log concentration analysis. The ED_{50} data suggested that the djulis leaf contained more active components than its seed counterparts. The most repellent extract was that from LD, followed by LM, SD, and SM (Table 3).

The lowest effective concentrations of LM and SM extracts are approximately ~0.008–0.0016 %, respectively (Table 2), and the ED_{50} values for LM and SM are 0.013 and 0.010 %, respectively (Table 3). The time-course experiments showed the repellent effect of the 1 % djulis extracts decreased over time and lasted up to 2.5 h for LM extract and 3 h for SM extract. The commercial repellant was the most repellent and still worked well (86 %) over 3 h under the field conditions (Fig. 1).

Composition of djulis extract

Tables 4 and 5 list the constituents identified, percentage composition and biological effect in the order of elution from the DB-5MS capillary column. A total of 15 and 20 compounds accounting for 88.8 and 79.9 % in the seed and leaf extract, respectively were identified. In the seed extract, methyl 6,9-octadecadienoate, methyl oleate, and ethyl linoleate were the major constituents each with percentage compositions greater than 10 %. The major constituents in leaf extract were palmitic acid and 9, 12-octadecadienoyl chloride, (Z, Z)- which accounted for 10.5 and 35.7 %, respectively.

Table 4 Composition from the seeds of djulis characterized by GC–MS

Retention time	Constituents	SI/RSI[a]	Relative composition (%)	Biological effect and references[b]
18.55[c]	(1) Cinnamic acid, *O*-hydroxy-,(E)-	855/859	0.99	Feeding-deterrent (Morimoto et al. 1999; Smith 2011)
	(2) Benzofuran,2,3-dihydro-	841/843		
21.2[c]	Phenol, 4-ethenyl-2-methoxy	876/887	0.88	NA
22.85[c]	(Z)-2-Pentenal	926/959	0.83	NA
26.44	Methyl 3-methoxy-4-hydroxybenzoate	849/953	0.43	NA
34.15	Theobromine	758/890	0.57	NA
35.11	Methyl palmitoleate	741/763	0.56	NA
35.55[c]	Methyl palmitate	805/828	5.17	Repellent pheromones, acaricidal (Posy et al. 1984; Wang et al. 2010; Wang et al. 2009)
36.01	Nitrobenzo(3,4)tricycle [3,2,1,0(2,7)] octene	901/979	4.85	NA
36.20	Palmitic acid	771/783	8.89	Feeding- and oviposition-deterrent (Scheffrahn and Rust 1983; Xu et al. 2006)
36.92	Oleoamide	767/780	4.86	NA
38.74	Methyl 6,9-octadecadienoate	806/806	21.71	NA
38.86	Methyl oleate	806/827	10.61	Anti-oviposition (Bird et al. 1987)
39.43	Ethyl linoleate	792/826	18.67	Anti-oviposition (Bird et al. 1987)
39.54[c]	9,12-Octadecadienoyl chloride, (Z, Z)-	755/792	9.3	NA
40.03[c]	Octadecanoic acid	705/743	0.48	Anti-oviposition (Ganesan et al. 2006)

[a] *SI* indicates standard index, *RSI* indicates reverse standard index

[b] NA indicates not applicable

[a] Common components in between leaf and seed extracts

Discussion

The present study investigated a new botanical-based repellent based on extracts from *C. formosaneum* (the native species of djulis in Taiwan) effective against mosquitoes (*A. albopictus*) and biting midges (*F. taiwana*). In addition, our studies also suggested that the extracts of *C. formosaneum* also exhibit good repellence against *A. aegypti* (supplementary Table 1) and several crop pests (data not shown).

The ED_{50} values of LM (0.500 %) and SM (0.829 %) in this study are in good agreement with a previous investigation reporting repellence of LM at 0.53 % and SM at 0.91 % (Chio and Yang 2008). The repellence of djulis extracts was equivalent to that of neem oil (ED_{50} of 0.58 % against the Asian Tiger mosquito) (Chio and Yang 2008). The commercial OFF^{TM} showed the best repellence under laboratory conditions and no mosquito landed on the OFF^{TM} treated screens. However, it should be noted that the concentration DEET in OFF^{TM} reaches 15 % which is far higher than that of djulis extract. Though another commercial repellent from Earth Chem. Co., Ltd also showed good repellence against *F. taiwana*, it contains 5.85 % of DEET which is also far higher than the concentration of djulis used in these studies. Moreover, the residents in endemic areas have complained that DEET has a poor repellent effect against the biting midge, *F. taiwana*. But the commercial DEET from Earth Chem. Co., Ltd worked well in these studies. This product is based on a micro-encapsulated formulation that slows the release rate and prolongs the repellence. It will be interesting to determine if the methanol extract of djulis, formulated using nano-technology, could also provide longer protection in the field.

Though some constituents of djulis extract identified by GC–MS showed feeding-deterrent and anti-oviposition

Table 5 Composition from the leaves of djulis characterized by GC–MS

Retention time	Constituents	SI/RSI[a]	Relative composition (%)	Biological effect and references[b]
14.22	4,4-dimethyl-1,3-cyclopentanedione	737/846	0.64	NA
16.33	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	710/754	0.52	NA
18.55[c]	(1) Benzofuran,2,3-dihydro-	842/867	0.96	Feeding-deterrent (Morimoto et al. 1999, Smith 2011)
	(2) Cinnamic acid,O-hydroxy-,(E)-	836/864		
18.74	HMF	881/906	0.99	Feeding-deterrent (Ohmura et al. 1999)
20.45	Vitispirane	829/876	0.74	NA
21.2[c]	Phenol,4-ethenyl-2-methoxy	888/895	7.86	NA
22.85[c]	(Z)-2-Pentenal	769/927	0.83	NA
23.53	1-(3,6,6-trimethyl-1,6,7,7A-tetrahydro-cyclopenta|C|pyran-1-yl)ethanone	753/803	0.59	NA
25.86	Isohomogenol	813/866	0.49	NA
31.50	2-(2-Oxoethyl)-cis-bicyclo[3,3,0]octane-3,7-dione	776/903	1.04	NA
32.21	(-)-Loliolide	790/825	0.88	Ant-repellent (Okunade and Wiemer 1985)
33.75	2-Methyl-octadecyne	768/810	1.54	NA
34.63	2-Methyl-octadecyne	696/789	0.61	NA
35.43	Methyl palmitoleate	745/765	0.52	NA
35.54[c]	Methyl palmitate	782/811	2.51	Repellent pheromones, acaricidal (Posy et al. 1984; Wang et al. 2010; Wang et al. 2009)
36.2	Palmitic acid	796/810	10.5	Feeding-deterrent (Scheffrahn and Rust 1983)
38.82	Ethyl linoleolate	786/807	7.54	NA
39.02	Neophytadiene	773/819	4.39	NA
39.54[c]	9,12-Octadecadienoylchloride, (Z,Z)-	755/844	35.71	NA
40.02[c]	Octadecanoic acid	666/714	0.99	Anti-oviposition (Ganesan et al. 2006)

[a] *SI* indicates standard index, *RSI* indicates reverse standard index

[b] NA indicates not applicable

[c] Common components in between leaf and seed extracts

effect, most of them have never been explored for repellent activity. Interestingly, there are six constituents occurring in both seed and leaf extracts (Table 4, 5) and, in particular, 9,12-octadecadienoyl chloride, (Z, Z) was the prevailing constituent in the leaf extract (35.7 %). This compound also comprised 9.3 % in the seed extract of djulis and has been shown to be the predominant component (44.2 %) of *Cynoglossum zeylanicum* (Boraginaceaceae) and is an emetic (Anitha et al. 2012). However, 9,12-octadecadienoyl chloride, (Z, Z) has never been explored for repellent activity. Methyl palmitate comprised 5.2 and 2.5 % in the extracts of djulis seed and leaf, respectively and has been shown to have repellent and acaricidal activities (Posy et al. 1984; Wang et al. 2010; Wang et al. 2009). Though 4-ethenyl-2-methoxy phenol comprised 7.9 % in leaf extract, it only accounted for 0.9 % of the seed extract and has never been tested for any bioactivity. The other three components (O-hydroxy-,(E)-cinnamic acid (or benzofuran,2,3-dihydro-); (Z)-2-pentenal and octadecanoic acid) all accounted for <1 % in both leaf and seed extracts, although some of them are known feeding-deterrents or have oviposition-deterrent activity (Ganesan et al. 2006; Morimoto et al. 1999; Smith 2011). We are continuing to work on identification of the active principles in djulis extract responsible for the observed repellent property against mosquitoes and biting midges.

The active ingredients of the extracts need to be isolated, purified, and identified before their potential as commercial insect repellents can be fully assessed. For practical purposes, experiments are currently underway to investigate their residual action under field conditions.

Plant natural products represent potential alternative insecticides and/or repellents for the management of blood-sucking insects, since they constitute a rich source of bioactive compounds that are biodegradable, and generally non-toxic to non-target organisms. *C. formosaneum*, a native cereal plant in Taiwan, is easy to cultivate under drought conditions with high yield (46 g seed/plant) and short-harvest times (28 days for leaf and 2.5 months for seed on average). This availability makes it a potential target for implementation in an integrated mosquito and biting midge management program.

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