STUDY ON ESSENTIAL OIL AND SOME BIOACTIVITIES OF *Citrus hystrix* DC. LEAF

Ei Ei Khaing[1] and Ohn Mar Tin[2]

Abstract

Citrus hystrix DC. (Shout-nu) used in household remedy for medicinal purposes was chosen for present study. Gastrointestinal tract problems and some diseases caused by mosquito become challenges in Myanmar people that many researchers have revealed the finding results of therapeutic properties from natural sources. The aim of this study is to analyze essential oil extracted from *Citrus hystrix* leaf Essential oil (0.02 g, 0.04 %) was extracted from leaf sample by hydro distillation method and was analysed by Fourier transform infrared (FT IR) spectroscopy. The chemical composition with molecular mass in essential oil was then determined by gas chromatography-mass spectrometry (GC-MS). Eight compounds of terpenes: alpha-pinene, D-limonene, beta-ocimene, gamma-terpinene, isopulegol, citronellol, caryophyllene and beta-bisabolene were detected. By solvent extraction method, four crude extracts of *Citrus hystrix* leaf were prepared with various solvents: petroleum ether, ethyl acetate, 96 % ethanol and water. Antimicrobial activity of four crude extracts was investigated against ten microorganisms by paper disc diffusion method. It was found that all tested leaf extracts exhibited ten tested microorganisms with the inhibition zone diameters range between 9~27 mm. Nevertheless, acute toxicity of 96 % EtOH and water extracts from *Citrus hystrix* leaf was evaluated by the method of (OECD) guidelines 423 that all tested *Citrus hystrix* leaf sample showed no toxicity. In addition, larvicidal activity of PE, EtOAc, water extracts and essential oil was studied by WHO standard method. According to larvicidal activity test, PE extract and essential oil have the highest larvicidal activity *in vivo* test larvae model, 3rd and 4th instar *Aede*s larvae. This research can support to the formulation of antimicrobial drugs and can provide on alternative source of mosquito control agents.

Keywords: *Citrus hystrix* leaf, essential oil, terpenes, antimicrobial activity, acute toxicity, larvicidal activity

Introduction

Citrus hystrix DC. derived from the family Rutaceae is famous for household remedy and its edible fruit in Myanmar. It is called Shout-nu in Myanmar, wild lime in English and Nann-non in local name. The fruit and leaf of the plant are well-known in tropical Southeast Asia as their medicinal uses (Okuda, 2005). It is a thorny bush tree, 2 to 11 meters tall with aromatic and distinctively shaped "double leaves" shown in Figures 1(a) and (b). The fruit shown in Figure 1 (c) is rough and green, and ripens to yellow with the size 4 cm wide (Mabberley, 1997). The tree is native to tropical Southeast Asia and widely distributed in Myanmar (Kress, *et al.*, 2003). *C. hystrix* leaf contains high amounts of citronellal, ascorbic acid, limonene, sabinene, comphene, linalool, nerolidal, citronellic acid and terpinolene. In traditional Indian medicine, *C. hystrix* leaf is used for digestion, inflammation, detoxifying the blood, oral health, improving skin, reducing stress and strengthening immune system (Zaibunnisa and Chutima, 2012). The other uses are shampoos, soap, toothpastes, hair oils, body lotion, lipstick, facial makeup, perfume and fingernail polishes. In addition, it is also applied in food industry such as biscuit, juice, cake and candy. The leaf is very popular for ingredients in noodle soup in Thailand (Vermal, *et al.*, 2014). In developing countries, plants are the main source of medicine (Siripongvutikorn, *et al.*, 2014). Today, Myanmar government encourages indigenous forms of medicine. Therefore, this study intends to analyse the extracted essential oil and investigate some bioactivities of *C. hystrix* leaf sample. In the study, the sample collection, extraction and identification of essential oil, preparation of various crude

[1] Dr, Associate Professor, Department of Chemistry, Hinthada University
[2] Dr, Professor (Head), Department of Chemistry, Hinthada University

extracts, screening of antimicrobial activity, determination of acute toxicity and assessment of larvicidal activity from the leaf sample have been carried out.

Figure 1 (a) *C. hystrix* plant (b) *C. hystrix* leaf (c) *C. hystrix* fruit

Materials and Methods

Plant Materials

C. hystrix leaf was collected from Myanaung Township, Ayeyarwady Region. The plant was identified at Department of Botany, Yangon University. The sample was washed, cleaned and dried at room temperature for three weeks. Then the dried sample was powdered and stored in air-tight container.

Extraction of Essential Oil from *C. hystrix* Leaf by Hydro Distillation Method

The fresh *C. hystrix* leaf (50 g) was distilled with deionized water (400 mL) in Clevenger apparatus for 48 h. After the hydro distillation, the essential oil was collected as hydrosol form and then separated two layers of immiscible liquids: water and oil were partitioned with petroleum ether in a separating funnel. The petroleum ether soluble portion was taken and dried with anhydrous sodium sulphate followed by filtration. The yield percent of essential oil was calculated.

Characterization of Essential Oil Extracted from *C. hystrix* Leaf

The functional group of compounds in the essential oil extracted from *C. hystrix* leaf was analyzed by Fourier transform infrared (FT IR) spectroscopy and the chemical composition with molecular mass was determined by gas chromatography mass spectrometry (GC-MS).

Preparation of Crude Extracts of *C. hystrix* Leaf by Direct Extraction Method

The dried powdered sample (50 g) was extracted with petroleum ether (PE), ethyl acetate (EtOAc), 96 % ethanol (EtOH) 500 mL in separate conical flasks for three weeks at room temperature and filtered. Water extract of leaf sample was prepared by boiling 50 g of sample with 500 mL of distilled water for 6 h and filtered. The filtrates were concentrated by rotary evaporator to get crude extracts. The yield % of these extracts were determined and then stored in the refrigerator for the screening of bioactivities such as antimicrobial activity, acute toxicity and larvicidal activity.

In Vitro Study on the Antimicrobial Activity of *C. hystrix* Leaf by Paper Disc Diffusion Method

Paper disc diffusion method was used for the detection of antimicrobial activity of four crude extracts from *C. hystrix* leaf. The test procedure was as follows: the extracts (1 g each) were dissolved in 1 mL of their respective solvents; petroleum ether, ethyl acetate, 96 % ethanol and water, and introduced into sterile petri dishes for testing ten cultural bacterial strains: *Agrobacterium tumefaciens* NITE09678, *Aspergillus parascitius* IFO5123, *Bacillus subtilis*

IFO90571, *Candida albicans* NITE09542, *Micrococcus luteus* NITE83297, *Salmonnella typhi* AHU9743, *Escherichia coli* AHU5436, *Saccharomyces cerevisae* NITE52847, *Pseudomonas fluorescens* IFO94307 and *Staphylococcus aureus* AHU8465. The discs having 8 mm diameter each with 20 µg extract/disc were allowed to dry at 42 ˚C in incubator. The microbial suspension from test broth was streaked evenly into three places on the surface of assay medium agar plates with sterile cotton swab. After inoculation, the assay medium had dried for 5 min, the dried disc impregnated with crude extracts were placed on the agar with flamed forceps and gently pressed down to ensure proper contact. The assay medium in the absence of microorganisms was utilized as negative control, cultured with microorganisms was positive control, and antibiotics chloramphenicol was also used as standard for this study. After overnight incubation at 27 ˚C, the diameters of inhibition zones including 8 mm discs were measured.

In Vivo Study on the Acute Toxicity of *C. hystrix* Leaf on Albino Mice Model

The acute toxicity of 96 % EtOH extract and water extract of *C. hystrix* leaf was done by the method of (OECD) guidelines for Testing of Chemical 423 (OECD, 2001) with albino mice model at Laboratory Animal Services Division, Department of Medical Research (DMR), Yangon. According to the test description, total number of adult female albino mice, weighing (25-30 g) were selected and divided into five groups. Each group contained three animals. They were fasted for 18 h before giving the different doses of *C. hystrix*. Group (1) mice were orally administrated with 96 % EtOH extract of *C. hystrix* 2000 mg/kg dose. Group (2) mice were given orally with 96 % EtOH extract of *C. hystrix* 5000 mg/kg dose. Group (3) mice were also administered with water extract of *C. hystrix* 2000 mg/kg dose. Group (4) mice were orally administrated with water extract of *C. hystrix* 5000 mg/kg dose as shown in Figure 2. Then, Group (5) mice were performed as a control group besides they were treated with clean water and pellet from normal laboratory animal food of Laboratory Animal Services Division, DMR. All groups of mice were kept in the three mice per each cage in the separated room controlling temperature of 26 ± 1º C. After administration of the two extracts of *C. hystrix* on each group of animals were observed first 6 h continuously for mortality and behavior changes. Then, the animals were checked daily for fourteen days of acute oral toxicity experiment. The mortality during this period was noted as nil or percent death.

Figure 2 Acute Oral toxicity test with female albino mice

In Vivo Study on the Larvicidal Activity of *C. hystrix* Leaf on *Aedes aegypti* Mosquito Larvae

Strains of *Aedes aegypti* mosquito larvae and adult *Aedes* mosquitoes emerged from pupae were identified by morphological methods (Rampa and Prachong, 1994). The *Aedes aegypti* mosquitoes were collected from Hlaingthaya Township. Adults were provided with 10 % sucrose solution and 8 weeks old mouse for blood meal. Mosquitoes were held at 26 ± 2 ºC, 65-75 % relative humidity with a photo period of 12 h light and 12 h dark. *Aedes* larvae were used for testing insecticidal properties of *C. hystrix* leaf extracts and essential oil by WHO standard method (WHO, 2005). At first, various concentration of four tested samples: PE extract, EtOAc extract, water extract and essential oil from *C. hystrix* leaf were prepared freshly as 0.1 g, 0.05 g, 0.025 g, 0.0125 g and 0.00625 g in 100 mL each of distilled water in 150 mL plastic cups

respectively. Fifty (50) each 3rd and 4th instars *Aedes aegypti* larvae were put into different concentrations and also negative control (absence of sample) was done simultaneously. Larvae were exposed 24 h for each replication in various concentrations of samples in laboratory at 27-29 ºC and 70 to 80 % relative humidity. Five replicates were carried out and knockdown was checked after 60 min. Then, mortality was recorded after 24 h of exposure period. Knockdown and dead larvae were identified when the larvae failed to move after probing with a needle in the thorax region of the body. Lethal concentration LC_{50} and LC_{90} values for 95 % confidential limits were calculated by the equation of chi-square. Data entry and processing were made by using Microsoft Excel software. The average larval mortality data were subjected to probit analysis for calculating LC_{50} and LC_{90} values and other statistics at 95 % confidence limits of upper confidence limit and lower confidence limit, and chi-square values were calculated using the dose-effect probit analysis (Finney, 1971). Results with probability $p < 0.05$ were considered to be statistically significant.

Results and Discussion

Extraction and Characterization of Essential Oil from *C. hystrix* Leaf by Modern Techniques

The fresh leaf sample of *C. hystrix* was extracted by hydro distillation method with distilled water in Clevenger apparatus and yielded colourless essential oil (0.02 g, 0.04 %). It was identified by modern methods: FT IR spectroscopy and GC-MS spectrometry. According to FT IR spectrum of essential oil, the following groups could be assigned (Figure 3). The O-H stretching vibration for alcohol performed at 3361 cm^{-1}. The absorption bands at 2926 cm^{-1} and 2854 cm^{-1} showed symmetric and asymmetric C-H stretching of aliphatic hydrocarbon due to methyl and methylene group. The stretching vibration of C=O for ester was exhibited at 1728 cm^{-1}. The bending of methylene and asymmetric bending of methyl groups showed at 1456 cm^{-1}. The absorption of 1377 cm^{-1} showed symmetric bending of methyl group. The C-O stretching vibration of alcohol showed in the 1163 cm^{-1} region (Silverstein, 1991). In addition, the GC-MS analysis of essential oil could be deduced as eight compounds with these respective retention times (RT). These compounds could be assigned as alpha-pinene (RT: 3.46 min), D-limonene (RT: 3.97 min), beta-ocimene (RT: 4.11 min), gamma-terpinene (RT: 4.30 min), isopulegol (RT: 5.50 min), citronellol (RT: 6.45 min), caryophyllene (RT: 9.91 min) and beta-bisabolene (RT: 10.11 min) as shown in Figures 4 (a), (b), (c), (d), (e), (f), (g), (h) and Table 1. From the results of GC-MS and FT IR spectral data, it was concluded that extracted essential oil from leaf sample contained monoterpenes and sesquiterpenes.

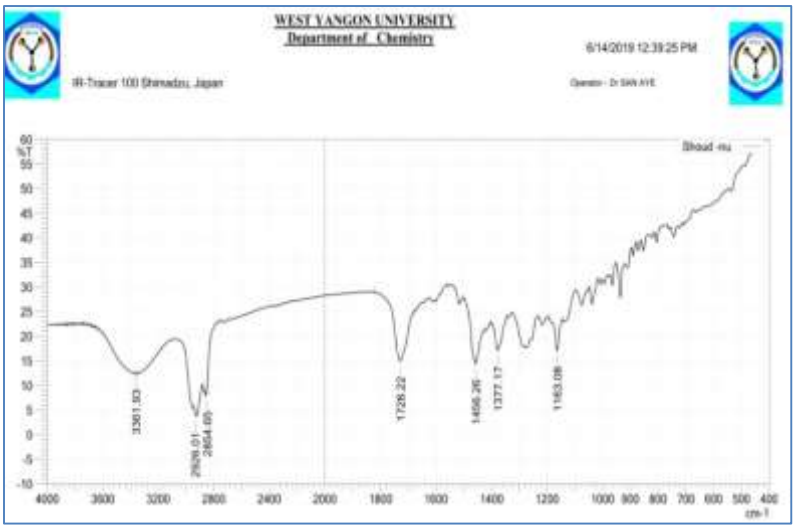


Figure 3 FT IR spectrum of essential oil (leaf oil) from *C. hystrix* leaf

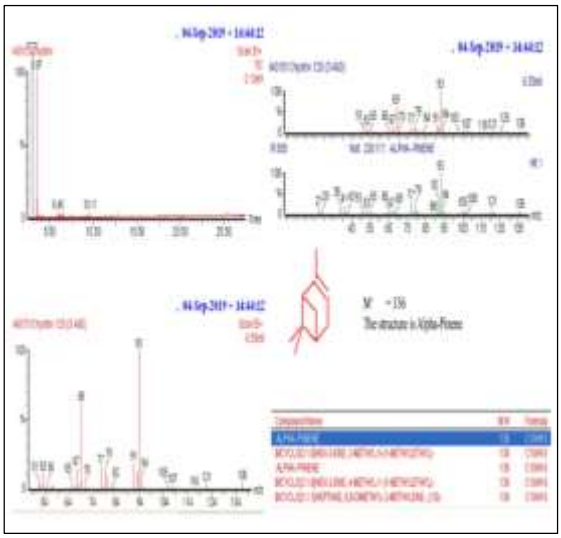


Figure 4 (a) GC- MS spectrum of compound 1from *C. hystrix* leaf at RT 3.46 min

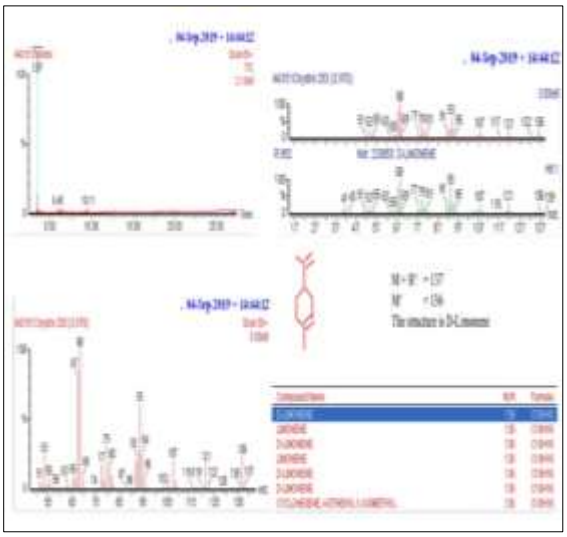


Figure 4 (b) GC- MS spectrum of compound 2 from *C. hystrix* leaf at RT 3.97 min

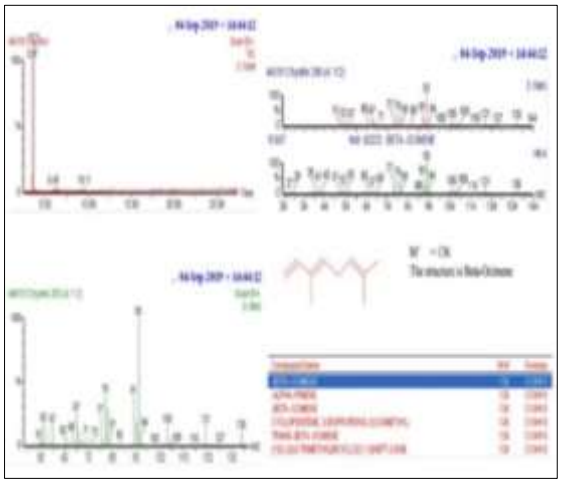


Figure 4 (c) GC- MS spectrum of compound 3 from *C. hystrix* leaf at RT 4.11 min

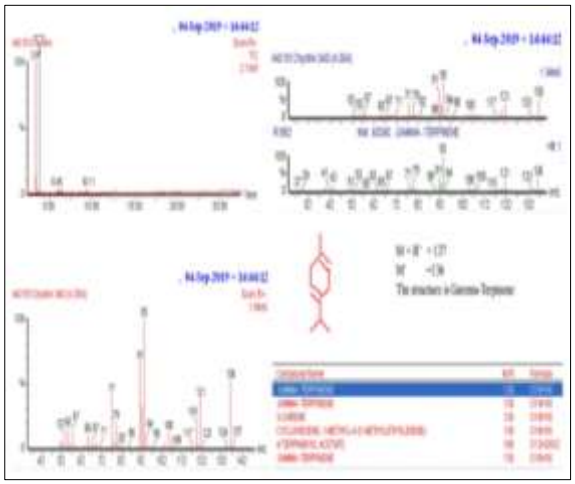


Figure 4 (d) GC- MS spectrum of compound 4 from *C. hystrix* leaf at RT 4.30 min

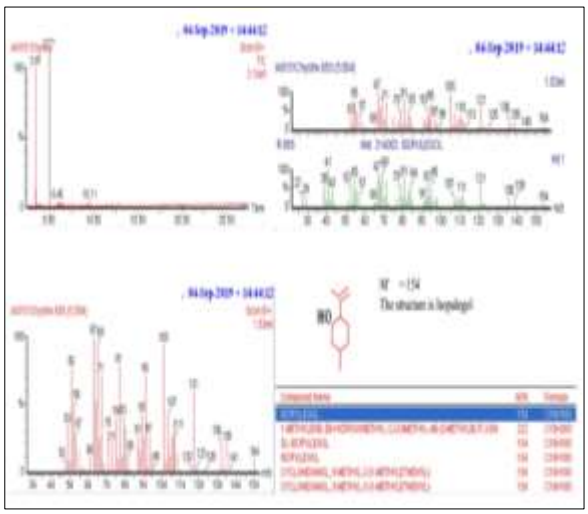


Figure 4 (e) GC- MS spectrum of compound 5 from *C. hystrix* leaf at RT: 5.50 min

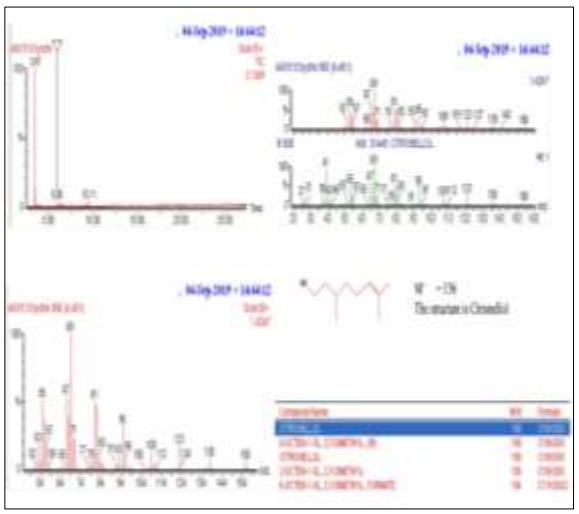


Figure 4 (f) GC- MS spectrum of compound 6 from *C. hystrix* leaf at RT 6.45 min

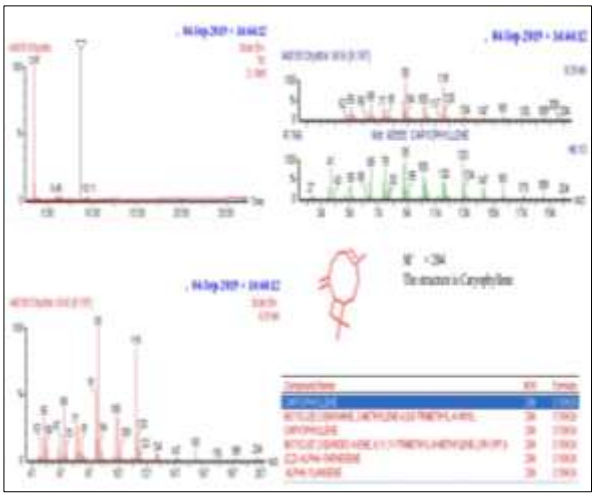


Figure 4 (g) GC- MS spectrum of compound 7 from *C. hystrix* leaf at RT 9.19 min

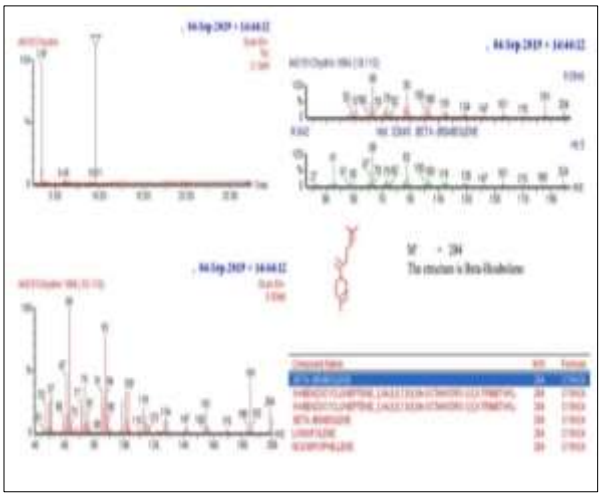
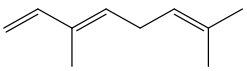
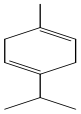
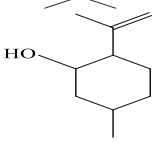
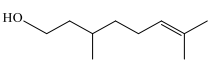
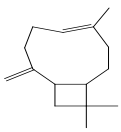
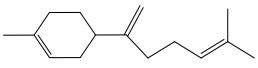


Figure 4 (h) GC- MS spectrum of compound 8 from *C. hystrix* leaf at RT 10.11 min

Table 1 The Nine Compounds detected by GC-MS in Essential Oil from *C. hystrix* Leaf at Different Retention Times (RT)

Compounds	Name	Structure	Molecular weight	Formula	RT (min)
1	alpha-pinene		136	$C_{10}H_{16}$	3.46
2	D-limonene		136	$C_{10}H_{16}$	3.97
3	beta-ocimene		136	$C_{10}H_{16}$	4.11
4	gamma-terpinene		136	$C_{10}H_{16}$	4.30
5	isopulegol		154	$C_{10}H_{18}O$	5.50
6	citronellol		156	$C_{10}H_{20}O$	6.45
7	caryophyllene		204	$C_{15}H_{24}$	9.19
8	beta-bisabolene		204	$C_{15}H_{24}$	10.11

Screening of Antimicrobial Activity of *C. hystrix* Leaf Extracts by Paper Disc Diffusion Method

The dried leaf powder collected from Myanaung Township, Ayeyarwady Region was extracted with various solvents and the yield % of PE extract (3.40 %), EtOAC extract (7.20 %), 96 % EtOH extract (12.60 %) and water extract (18.20 %) were obtained respectively. These four

crude extracts were tested with ten microorganisms such as *Agrobacterium tumefaciens* NITE09678, *Aspergillus parascitius* IFO5123, *Bacillus subtilis* IFO90571, *Candida albicans* NITE09542, *Micrococcus luteus* NITE83297, *Salmonella typhi* AHU9743, *Escherichia coli* AHU5436, *Saccharomyces cerevisae* NITE52847, *Pseudomonas fluorescens* IFO94307 and *Staphylococcus aureus* AHU8465. The ten tested microorganisms obtained from the source of NITE & Kyowa Hakko Co. Ltd., Japan were cultured at Biological Resources and Biotechnology Development Center (BDC) of Pathein University and then screened at Department of Chemistry, Hinthada University. The microorganism species used in the test are responsible for plant diseases, diarrhea food poisoning, GI tract infection and abscess in skin, nose. The measurable inhibition zone diameter of crude extracts showed the degree of antimicrobial activity (Figures 5 and 6).

From the results given in Table 2, it was observed that all four crude extracts of *C. hystrix* leaf exhibited inhibition zone diameters range between 9-27 mm against ten tested microorganisms. In addition, the inhibition zone diameter (27 mm) of EtOAc extract showed the most potent activity against *Candida albicans* NITE09542. Thus, it may be effectively used as active remedy for this treatment of their related diseases and fungal infection.

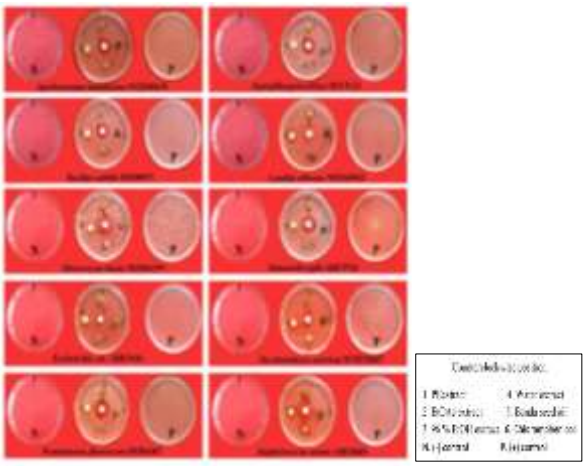


Figure 5 Inhibition zones of various crude extracts from *C. hystrix* leaf against ten species of microorganisms

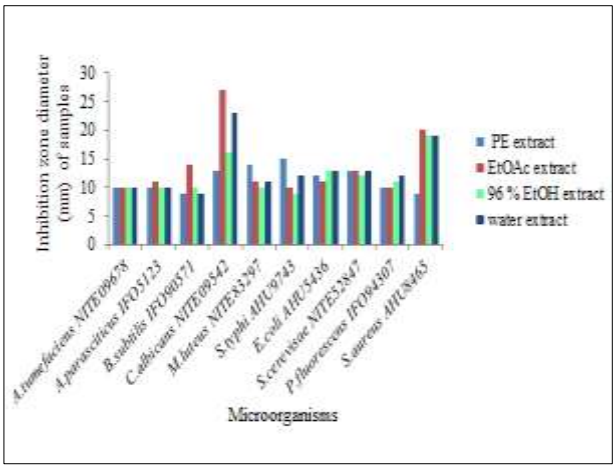


Figure 6 Comparison of inhibition zone diameters for various crude extracts of *C. hystrix* leaf against ten species of microorganisms.

Table 2 Inhibition Zone Diameters of Various Test Samples of *C. hystrix* Leaf Against ten Microorganisms

No	Microorganisms*	Inhibition Zone Diameters (mm) of Test samples				
		PE extract	EtOAc extract	EtOH extract	H_2O extract	Std.
1.	*Agrobacterium tumefaciens* NITE09678	10	10	10	10	26
2.	*Aspergillus parasciticus* IFO5123	10	11	10	10	26
3.	*Bacillus subtilis* IFO90571	9	14	10	9	24
4.	*Candida albicans* NITE09542	13	27	16	23	34
5.	*Micrococcus luteus* NITE83297	14	11	10	11	30
6.	*Salmonella typhi* AHU9743	15	10	9	12	26
7.	*Escherichia coli* AHU5436	12	11	13	13	19
8.	*Saccharomyces cerevisae* NITE52847	15	15	12	13	26
9.	*Pseudomonas fluorescens* IFO94307	10	10	11	12	28
10	*Staphylococcus aureus* AHU8465	9	20	19	19	30

Paper disc diameter– (8mm), 9 mm ~ 14 mm(+), 15 mm ~ 19 mm (+ +), 20 mm ~ above (+ + +)
Tested microorganisms (From the source of NITE & Kyowa Hakko Co. Ltd., Japan *) Std. = Chloramphenico

Study on Acute Toxicity of Ethanol and Water Extracts from *C. hystrix* Leaf

Acute toxicity on 96 % ethanol and water extracts of *C. hystrix* leaf was studied with the dosage of 2000 mg/kg and 5000 mg/kg body weight in albino mice. The condition of mice was recorded after administration for fourteen days. All groups of animals were also observed still alive and did not show any visible symptoms of toxicity like restlessness, respiratory disorders, convulsion, aggressive activities, coma and death. All dosages of *C. hystrix* samples showed no toxicological clinical signs and no mortality of all the groups of mice during fourteen days (Tables 3 and 4).

Table 3 Acute Toxicity Effect of 96 % EtOH and Water Extracts of *C. hystrix* Leaf on Albino Mice Model (after Fourteen Days Administration)

No	Groups of Albino Mice	Administrated Extracts of *C. hystrix* Leaf	Dosages (Single dose) mg/kg	Number of Death	% of Death
1	Group 1	96 % EtOH extract	2000	Nil	0
2	Group 2	96 % EtOH extract	5000	Nil	0
3	Group 3	Water extract	2000	Nil	0
4	Group 4	Water extract	5000	Nil	0
5	Group 5	No administration (Control)	No dosages	Nil	0

Nil = no lethality of the albino mice

Table 4 Observation of Toxic Clinical Signs on 96 % EtOH and Water Extracts *C. hystrix* Leaf in Acute Toxicity Test with Albino Mice

No	Signs of Toxicity	Group 1 2000 mg/kg	Group 2 5000 mg/kg	Group 3 2000 mg/kg	Group 4 5000 mg/kg	Group 5 No administration (Control)
1	Difficult breathing	-	-	-	-	-
2	Restlessness	-	-	-	-	-
3	Convulsion	-	-	-	-	-
4	Coma	-	-	-	-	-
5	Death	-	-	-	-	-

- = did not show any visible symptoms of toxicity

Examination of Larvicidal Activity of *C. hystrix* Leaf Extracts (Crudes) and Essential Oil Against 3rd and 4th Instars *Aedes aegypti* Larvae

From the results of current study, it was noted that the lowest knockdown effect occurred at 0.00625 g/mL dilution of *C. hystrix* leaf extracts such as PE, EtOAc, water and essential oil with the percent knockdown 24.0 %, 10.8 %, 6.0 % and 12.8 % respectively. In addition, the highest knockdown effect of *Aedes aegypti* larvae was observed in 96.0 % knockdown at 0.1 g/mL of PE extract followed by 90.0 % knockdown at 0.1 g/mL of essential oil from *C. hystrix* of leaf sample (Table 5). Furthermore, the percent in the lowest mortality effect for *C. hystrix* leaf extracts such as PE, EtOAc, water extracts and essential oil were 28.40 %, 31.6 %, 10.40 % and 32.8 % respectively at 0.00625 g/mL dilution. And then, the highest mortality effect of *Aedes aegypti* larvae was found out 99.20 % mortality at 0.1 g/mL dilution of essential oil followed by 98.40 % mortality at 0.1 g/mL dilution of PE extract of *C. hystrix* leaf (Table 6). The concentration of 50 % mortality (LC_{50}) value of PE, EtOAc, water extracts and essential oil against 3rd and 4th instars

Aedes aegypti larvae were found in 0.0133 g/mL, 0.0123 g/mL, 0.027 g/mL, 0.0114 g/mL and also 90 % mortality (LC_{90}) value of leaf extracts were found to be 0.0528 g/mL, 0.0697 g/mL, 0.122 g/mL, 0.0452 g/mL respectively as shown in Table 7. In addition, the lowest amount of 0.0114 g/mL of essential oil was needed for 50 % mortality (LC_{50}) and 0.0452 g was needed for 90 % mortality (LC_{90}) of *Aedes* larvae. Out of these tested leaf samples, essential oil was found to be the most active against 3rd and 4th instars *Aedes aegypti* larvae.

Table 5 Knockdown Effect (Within 60 min) on Various Dilutions of *C. hystrix* Leaf Extracts and Essential Oil Against 3rd and 4th Instars *Aedes aegypti* Larvae

Concentrations (g / 100 mL)	Number of Knockdown and % Knockdown of *C. hystrix* Leaf Extracts (Crudes) and Essential oil			
	PE extract	EtOAc extract	Water extract	Essential oil
0.1	240 (96.0)	169 (67.6)	157 (62.8)	225 (90.0)
0.05	201(80.4)	114 (45.6)	57 (22.8)	174 (69.6)
0.025	157 (62.8)	80 (32.0)	41(16.4)	90 (26.0)
0.0125	106 (42.4)	41(16.4)	26 (10.4)	65 (26.0)
0.00625	60 (24.0)	27 (10.8)	15(6.0)	32 (12.8)
Control	0	0	0	0

Total larvae = 250

Table 6 Mortality Effect (Within 24 h) on Various Dilutions of *C. hystrix* Leaf Extracts (Crudes) and Esssential oil against 3rd and 4th instars *Aedes aegypti* larvae

Concentrations (g / 100 mL)	Number of Mortality and % Mortality of *C. hystrix* Leaf Extracts (Crudes) and Essential oil			
	PE extract	EtOAc extract	Water extract	Essential oil
0.1	246 (98.4)	238 (95.2)	221 (88.4)	248 (99.2)
0.05	211 (84.4)	209 (83.6)	162 (64.8)	209 (83.6)
0.025	160 (64.0)	161 (64.4)	121 (48.4)	170 (68.0)
0.0125	124 (49.6)	117 (46.8)	69 (27.6)	149 (59.6)
0.00625	71 (28.4)	79 (31.6)	26 (10.4)	82 (32.8)
Control	0	0	0	0

Total larvae = 250

Table 7 Lethal Concentration (LC) Values of *C. hystrix* Leaf Extracts (Crudes) and Essential oil Against 3rd and 4th Instars *Aedes Aegypti* Larvae

Lethal Concentration (LC)	*C. hystrix* Leaf Extracts (Crudes) and Essential oil			
	PE extract	EtOAc extract	Water extract	Essential oil
LC_{50}	0.0133	0.0123	0.027	0.0114
LC_{90}	0.0528	0.0697	0.122	0.0452
Chi square χ^2	0.0742	0.0191	0.0177	0.1561
D_f	4	4	4	4
P value	0.05	0.05	0.05	0.05

LC_{50} = Lethal Concentration dose 50, LC_{90} = Lethal Concentration dose 90, d_f = *degree of freedom*

Conclusion

Colourless essential oil (0.02 g, 0.04 %) was obtained from the plant, *C. hystrix* leaf by hydro distillation method. In addition, GC-MS analysis of essential oil could be deduced as alpha-pinene (RT: 3.46 min), D-limonene (RT: 3.97 min), beta-ocimene (RT: 4.11 min), gamma-terpinene (RT: 4.30 min), isopulegol (RT: 5.50 min), citronellol (RT: 6.45 min), caryophyllene (RT: 9.91 min) and beta-bisabolene (RT: 10.11 min). Crude extracts were prepared from *C. hystrix* leaf using PE, EtOAc, 96 % EtOH and water as their solvent polarity. These extracts were used to test antimicrobial activity, acute toxicity and larvicidal activity.

Screening of antimicrobial activity of various crude extracts such as PE, EtOAc, EtOH and H_2O extracts from *C. hystrix* leaf sample was also investigated by employing paper disc diffusion method against ten tested microorganisms responsible for plant diseases, diarrhea, typhoid, food poisoning, GI tract infection and abscess in skin, nose. It was observed that all extracts of *C. hystrix* leaf exhibited inhibition zone diameters between 9~27 mm against ten tested microorganisms. Out of these extracts, EtOAc extract of leaf sample was found to be the most potent activity and especially against *Candida albicans* NITE09542 responsible for abscess caused by fungus. By OECD guidelines 423 with albino mice, it was found that there was no acute toxicity in the selected sample. The larvicidal activity of three crudes (PE, EtOAc and water extracts); leaf extracts and essential oil obtained from *C. hystrix* leaf were investigated in the range of 0.00625 to 0.1 g/mL by *Aedes* larvae method at DMR. From the observation, the highest knockdown of *Aedes* larvae was found at the concentration of 0.1 g/mL of PE extract. The highest mortality effect (99.20 %) of *Aedes* larvae was found in the concentration of 0.1 g/mL of essential oil. The lowest mortality effect (10.4 %) of water extract was observed at the concentration of 0.00625 g/mL. Among them, the essential oil showed the highest lethal concentration activity (LC_{50} = 0.0114 g/mL and LC_{90}= 0.0452 g/mL). From larvicidal activity test, the extract provide could an alternative sources of mosquito control agents. In addition, to herbal formulation for maintaining human health.

Acknowledgements

We would like to express our heartfelt gratitude to Dr Nilar Myint, Rector and Dr Mar Lar, Pro-Rector, Hinthada University, for their giving permission to report this research paper. We would also like to extend our gratitude to Dr. Thi Thi Aye, Professor, Department of Chemistry, Hinthada University, for her enthusiastic reading as well as supporting in this research and faculty members of our Department. We deeply thanks to responsible and honorable persons of Myanmar Academy of Arts and Science for giving opportunity to publish this paper.

References

Finney, R. (1971). *Probit Analysis*. Cambridge: 3rd Ed., Cambridge University Press, pp. 9968-9972

Kress, W.J., Defilipps, R.A., Farr, E. and Yin Yin Kyi. (2003). *A Checklist of the Trees, Shrubs, Herbs and Climbers of Myanmar*. Washington, DC, USA: 1st Ed., Department of Systematic Biology- Botany, National Museum of Natural History, p. 87

Mabberley, D.J. (1997). "A Classification for Edible *Citrus* (Rutaceae)". *Telopea*, vol. 7, (2), pp. 167-172

OECD (Organization for Economic Co-operation and Development). (2001). "Guidance Document on Accute Oral Toxicity Testing". OECD Series on Testing and Assessment No. 24, *ENV/JM/MONO*, vol. 4, pp. 8-22

Okuda, T. (2005). "Systematics and Health Effects of Chemically Distinct Tannin in Medicinal Plants". *Phytochemistry*, vol. 66, pp. 2012 – 2031

Rampa, R. and Prachong, P. (1994). "Illustrated Keys to the Medically Important Mosquitos of Thailand". *Southeast Asian Journal of Tropical Medicine and Public Health,* vol .25 (1), pp.1-66

Silverstein, R.M., Bassler, G.C. and Terence, C.M. (1991). *Spectrometric Identification of Organic Compounds*, Singapore: 5th Ed., John Wiley and Sons, Inc., pp. 289-315

Siripongvutikorn, S., Sriket, C. and Sriket, P. (2014). "Phytochemical of Thai Local Edible Herbs". *Inetrnational Food Research Journal,* vol. 21, pp.1009-1011

Vermal, S.C., Vashishth, E., Singh, R., Pant, P. and Padhi, M.M. (2014). "A Review on Phytochemistry and Pharmacological Activity of Parts of *C. hystrix* Used as an Ayurvedic Medicine". *World Journal of Pharmaceutical Research*, vol. 3(5), pp.138-158

WHO (2005). "World Health Organization Guidelines for Laboratory and Field Testing of Mosquito Larvicides". Geneva, *WHO/CDS/WHOPES/GCDPP*

Zaibunnisa, A.H. and Chutima, W. (2012). "Extraction of Volatile Oil from Kaffir Lime Leaves (*Citrus hystrix*) Using Pressurized Liquid Extraction". *International Journal of Food Nutrition and Public Health*, vol. 5, p. 201