Study on Nutrient Elements, Essential Oil and Antimicrobial Activity of *Citrus hystrix* DC. Peel

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Abstract

Citrus hystrix DC. (Shout-nu) used in household remedies for medicinal purposes was chosen for the present study. The aim of the study is to analyse elements besides essential oil extracted from *Citrus hystrix* peel and to investigate antimicrobial activity of the selected plant. As a nutrient element, the peel sample had relatively the highest content of potassium according to Energy dispersive X-ray fluorescence (EDXRF). Essential oil (0.03 g, 0.06 %) was extracted from the peel 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). Twenty-two compounds of terpenes and their derivatives were detected. By solvent extraction method, four crude extracts of *Citrus hystrix* peel were prepared with various solvents: petroleum ether, ethyl acetate, 96 % ethanol and water. The antimicrobial activity of four crude extracts was investigated against eight microorganisms by agar well diffusion method. It was found that all tested peel extracts exhibited eight tested microorganisms with inhibition zone diameter range between 12~40 mm. From the results, it could be suggested that *Citrus hystrix* peel should be applied as a local health remedy for the local indigenous communities of our country.

Keywords*:* *Citrus hystrix* peel, elements, essential oil, antimicrobial activity

Introduction

Citrus hystrix DC. derived from the family Rutaceae is famous for the treatment of some diseases such as gastrointestinal (GI) problems and paralyses. The plant is called Shout-nu in Myanmar, wild lime in English and Nann-non is its 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 Figure 1(a). The fruit shown in Figure 1(b) is rough and green, and ripens to yellow with the size of 4 cm width (Mabberley, 1997). The tree is native to tropical Southeast Asia and widely distributed in Myanmar (Kress, *et al.*, 2003). *C. hystrix* peel contains high amount of citronellal, β-pinene, limonene, terpinene-4-ol, α-pinene, α-terpinene, γ-terpinene and α-terpineol (Borusiewicz, *et al.*, 2017). In addition, essential oil mainly contains in the peel and leaf of the plant and it is often used for aromatherapy and folk medicine. The essential oil is "essential" in the sense that it contains the "essence" of the plant's fragrance. In traditional Indian medicine, *C. hystrix* peel is used for insecticide, antioxidant, antidandruff, bactericide, fungicide, larvacide, edema, gout and rheumatism (Zaibunnisa and Chutima, 2012). Other uses are shampoos, soap, toothpastes, hair oils, body lotion, perfume, fingernail polishes, cleanser for clothing and hair. In addition, it is also applied in food industry such as biscuit, juice, cake and candy. The peel and leaf are 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 tends to examine elements in extracted essential oil and investigate antimicrobial activity of *C. hystrix* peel sample. In the study, the sample collection, investigation of nutrient elements, extraction and

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identification of essential oil, preparation of various crude extracts and screening of antimicrobial activity from the peel sample have been carried out.

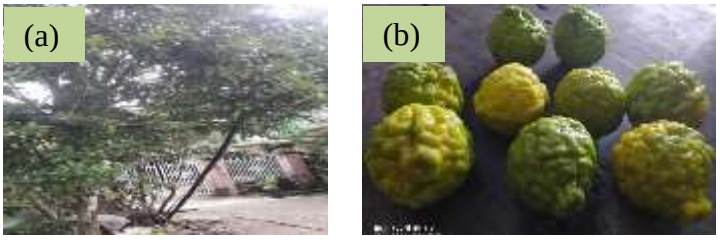


Figure (1) (a) *C. hystrix* plant (b) *C. hystrix* fruit

Materials and Methods

Plant Materials

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

Elemental Analysis of *C. hystrix* Peel by EDXRF Spectrometry

Elemental analysis of *C. hystrix* Peel was analysed by Energy dispersive X-ray fluorescence spectrometer (Shimadzu's EDX-7000/8000) at Monywa University. The dried powder sample was fabricated into pellets by using pellet making machine. It can analyze the elements from sodium to uranium. The individual elements comprising in the sample re-emit their own characteristic X-rays. The X-rays are detected by using semiconductor detector [Si (Li)] which permits multi-element, simultaneous analysis.

Extraction of Essential Oil from *C. hystrix* Peel by Hydro-distillation Method

The fresh *C. hystrix* peel (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 filteration. The yield percentage of the essential oil was calculated.

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

The functional group of essential oil extracted from *C. hystrix* peel 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* Peel by Solvent Extraction Method

The dried powdered sample (50 g) was extracted with petroleum ether (PE), ethyl acetate (EtOAc), 96 % ethanol (EtOH) 500 mL in separated conical flask for three weeks at the room temperature and filtered. Water extract of the peel sample was prepared by boiling 50 g of the 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 was determined and then stored in the refrigerator for the screening of antimicrobial activity.

In Vitro Study on the Antimicrobial Activity of *C. hystrix* Peel by Agar Well Diffusion Method

Agar well diffusion method was used for the detection of antimicrobial activity of four crude extracts from *C. hystrix* peel. 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 eight cultural microbial strains: *Escherichia coli* AHU5436, *Bacillus subtilis* IFO90571, *Bacillus pumilus* IFO90571, *Candida albicans* NITE09542, *Pseudomonas fluorescens* IFO94307, *Staphylococcus aureus* AHU8465, *Agrobacterium tumefaciens* NITE09678 and *Malassezia furfur* UY. 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 agar well was made with 8 mm sterile cork borer from each agar. Then, the wells were filled with test sample (extracts) to be tested. The assay medium in absence of microorganisms was utilized as negative control and culture with microorganisms was positive control, and antibiotics chloramphenicol was also used as standard for the study. After overnight incubation at 27 °C, the zones of inhibition diameter including 8 mm well were measured.

Results and Discussion

EDXRF Analysis of *C. hystrix* Peel

Under vacuum condition, EDXRF spectra of *C. hystrix* peel sample was shown in Figure 2 and Table 1. In *C. hystrix* peel, potassium (K) peak was also the most prominent and so it showed potassium was the highest content of the peel sample. Calcium (Ca), sulphur (S), phosphorus (P), iron (Fe), copper (Cu), manganese (Mn) and zinc (Zn) were minor component in the sample. From the result, it could be deduced that *C. hystrix* peel may support for human health. From this study, physicochemical properties of the peel sample was studied by the investigation of elemental content and it retards the growth of bacteria.

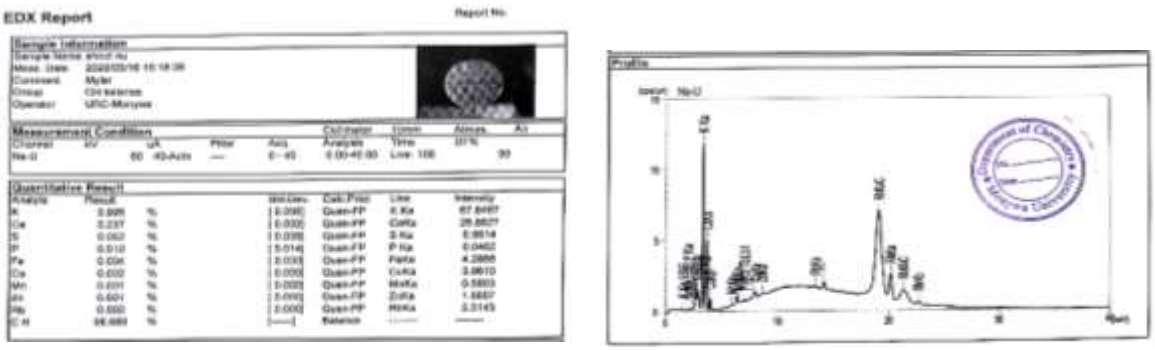


Figure (2) EDXRF spectrum of *C. hystrix* peel

Table (1) Elemental Analysis of *C. hystrix* Peel by EDXRF

No.	Element	Relative Abundance (%)
1.	K	0.995
2.	Ca	0.237
3.	S	0.062
4.	P	0.010
5.	Fe	0.004
6.	Cu	0.002
7.	Mn	0.001
8.	Zn	0.001
9.	C/H	98.689

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

The fresh peel sample of *C. hystrix* was extracted by hydro-distillation method with deionized water in Clevenger apparatus and yielded colourless essential oil (0.03 g, 0.06 %). 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 bending modes are not vibrational modes because they couple with the vibrations of adjacent groups. The absorption bands at 2960 cm^{-1}, 2924 cm^{-1}, 2857 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 ketone was exhibited at 1723cm^{-1}. Bending vibration of C=C alkene group showed at 1482 cm^{-1}. The absorption of 1377 cm^{-1} showed asymmetric bending of methyl group. Branching on the carbon atoms adjacent to the oxygen usually lead to splitting of the C-O-C band. Isopropyl ether shows a triplet structure in the 1121cm^{-1} region (Sliverstein, 1991). In addition, the GC-MS analysis of essential oil could be deduced as containing twenty-two compounds with these respective retention time (RT). These compounds could be assigned as α, 4-dimethyl-3-cyclohexene-1-acetaldehyde (RT: 4.515 min), 3,7-dimethyl-1,6-octadien-3-ol (linalool) (RT: 4.860 min), p-menth-1(7)-en-9-ol (RT: 5.243 min), dl-isopulegol (RT: 5.569 min), terpinen-4-ol (RT: 6.060 min), L-α-terpineol (RT: 6.260 min), geraniol (RT: 6.873 min), 3,7-dimethyl-6-octenoic acid (citronellic acid) (RT: 7.859 min), (1α-3β-4β)- p-menthane-3,8-diol (RT: 8.181 min), α- copaene (RT: 8.561 min), γ- muurolene (RT: 8.710 min), caryophyllene (RT: 9.163 min), 1,5,9,9-tetramethyl-1,4,7-cycloundecatriene (RT: 9.612 min), β-copaene (RT: 9.919 min), 1,2,4A,5,6,8A-hexahydro-4,7-dimethyl-1-(1-methylethyl)-naphthalene (α-muurolene) (RT: 10.340 min), 4-ethenyl-α,α-4-trimethyl-3-(1-methylethenyl)-[1R-(1α,3α,4β)]- cyclohexane-methanol (RT: 10.735 min), nerolidol 2 (RT: 10.805 min), γ-eudesmol (RT: 11.740 min), 1,2,3,4,4A,5,6,8A-octahydro-α,α-4a,8-tetramethyl-(2R-cis)-2-naphthalenemethanol (RT: 12.028 min), tetradecanoic acid (RT: 16.074 min), phytol (RT: 18.583 min) and Bis-(2-ethylhexyl) pathalate (RT: 26.641min) in figures 4 (a) to (v) and table (2). From the results of FT IR and GC-MS spectral data, it was concluded that extracted essential oil from the peel sample includes terpenes and their derivatives are used for the treatment of some diseases such as cancer, inflammatory and neuro disorder. Therefore, it would be suggested that *C. hystrix* peel may be used as alternative medicine.

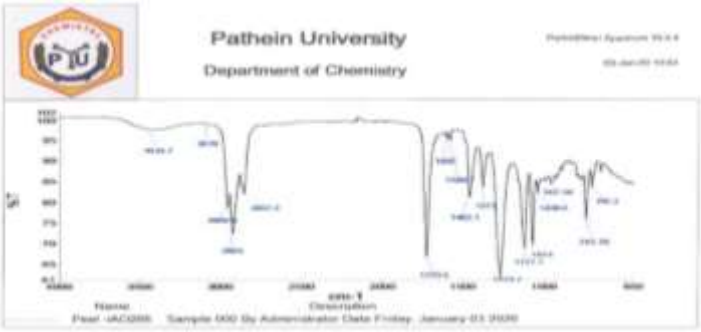


Figure (3) FT IR spectrum of essential oil from *C. hystrix* peel

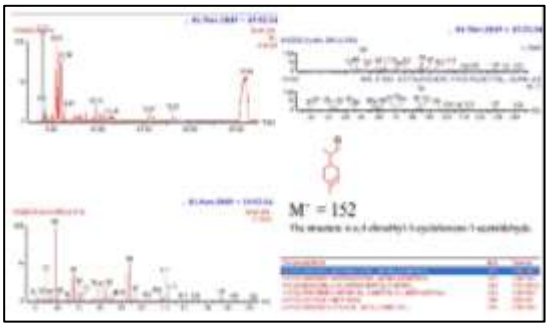


Figure (4.a) GC-MS spectrum of compound 1 from essential oil at RT 4.515 min

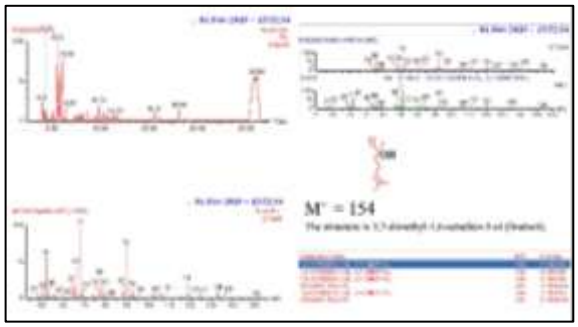


Figure (4.b) GC-MS spectrum of compound 2 from essential oil at RT 4.860 min

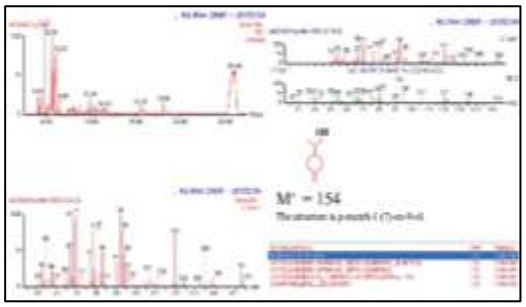


Figure (4.c) GC-MS spectrum of compound 3 from essential oil at RT 5.243 min

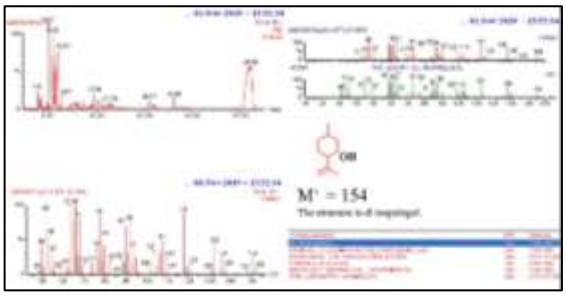


Figure (4.d) GC-MS spectrum of compound 4 from essential oil at RT 5.569 min

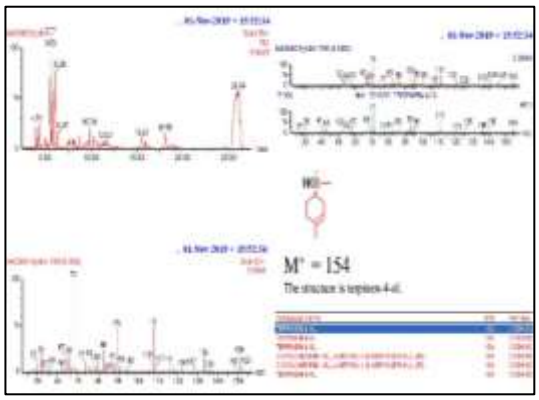


Figure (4.e) GC-MS spectrum of compound 5 from essential oil at RT 6.060 min

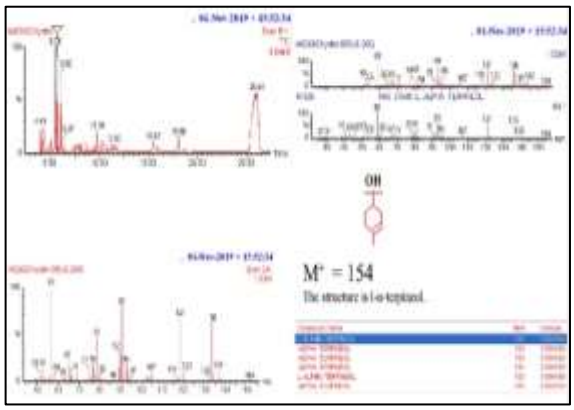


Figure (4.f) GC-MS spectrum of compound 6 from essential oil at RT 6.260 min

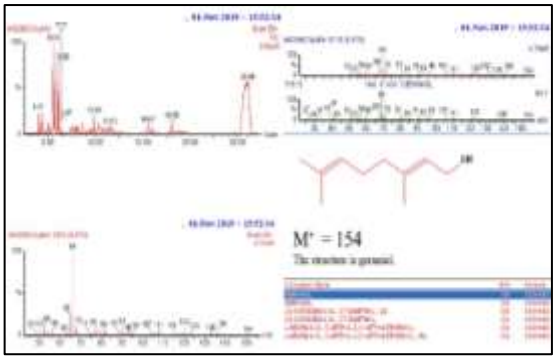


Figure (4.g) GC-MS spectrum of compound 7 from essential oil at RT 6.873 min

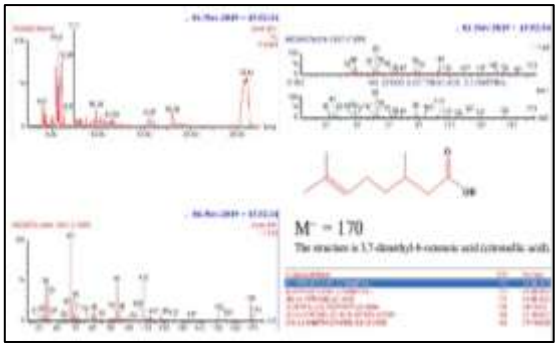


Figure (4.h) GC-MS spectrum of compound 8 from essential oil at RT 7.859 min

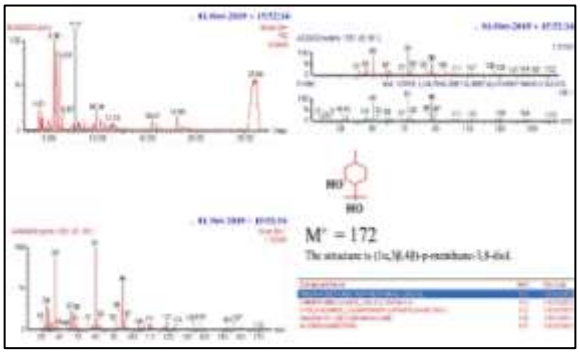


Figure (4.i) GC-MS spectrum of compound 9 from essential oil at RT 8.181 min

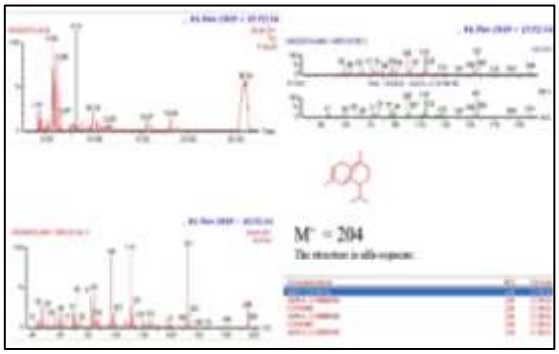


Figure (4.j) GC-MS spectrum of compound 10 from essential oil at RT 8.561min

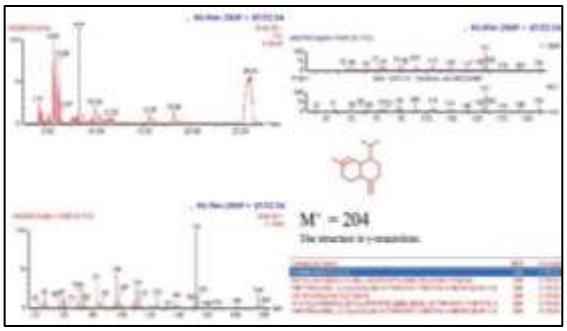


Figure (4.k) GC-MS spectrum of compound 11 from essential oil at RT 8.710 min

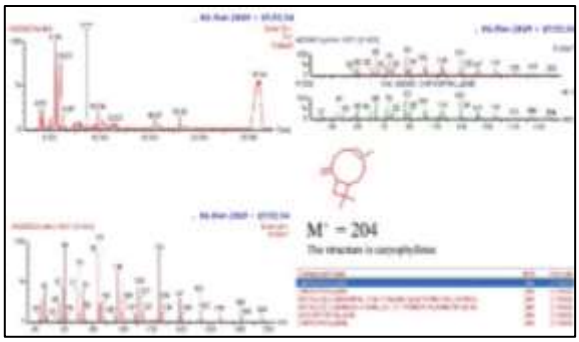


Figure (4.l) GC-MS spectrum of compound12 from essential oil at RT 9.163 min

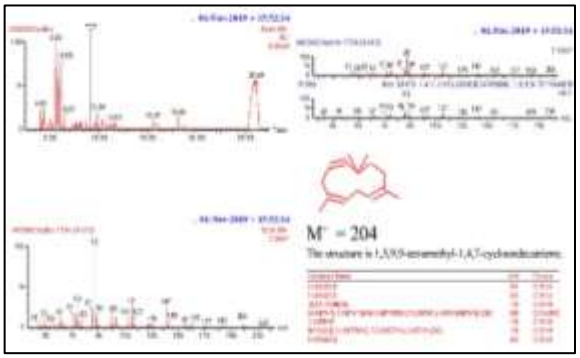


Figure (4.m) GC-MS spectrum of compound 13 from essential oil at RT 9.612 min

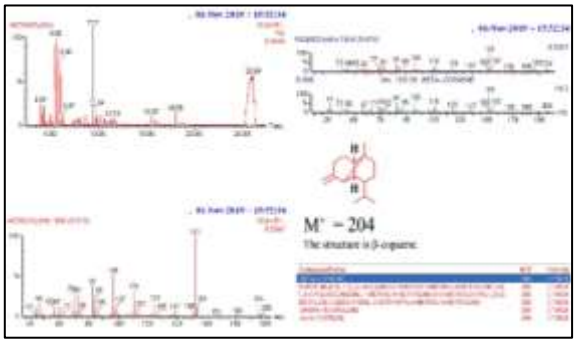


Figure (4.n) GC-MS spectrum of compound 14 from essential oil at RT 9.919 min

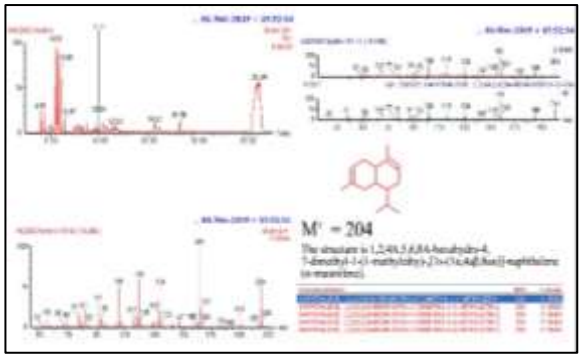
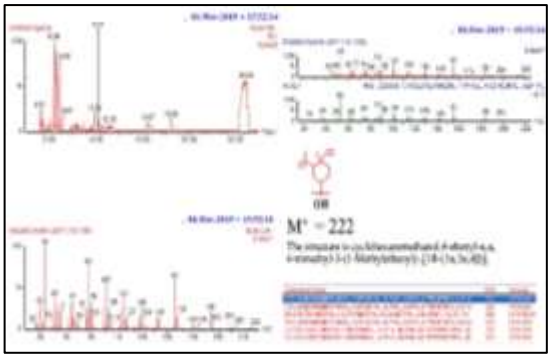


Figure (4.o) GC-MS spectrum of compound from essential oil at RT 10.340 min



15 Figure (4.p) GC-MS spectrum of compound 16 from essential oil at RT 10.735min

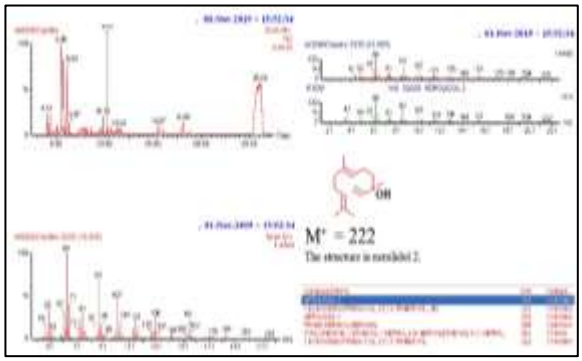


Figure (4.q) GC-MS spectrum of compound17 from essential oil at RT 10.805 min

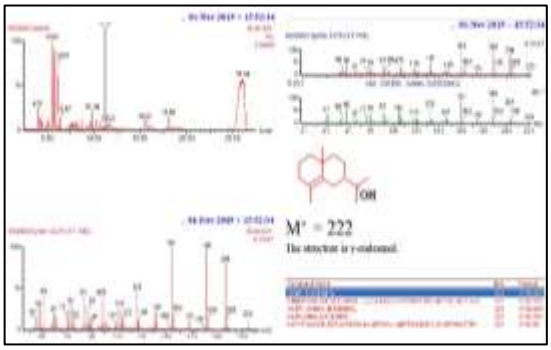


Figure (4.r) GC-MS spectrum of compound 18 from essential oil at RT 11.740 min

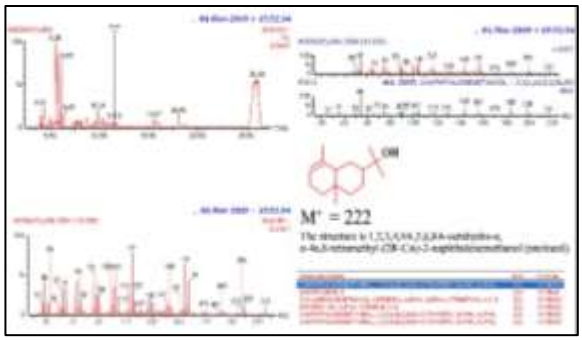


Figure (4.s) GC-MS spectrum of compound19 from essential oil at RT 12.028 min

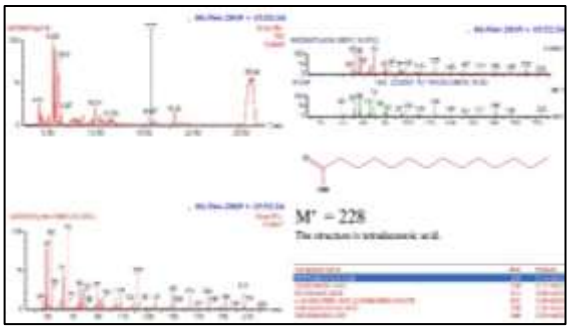


Figure (4.t) GC-MS spectrum of compound 20 from essential oil at RT 16.074 min

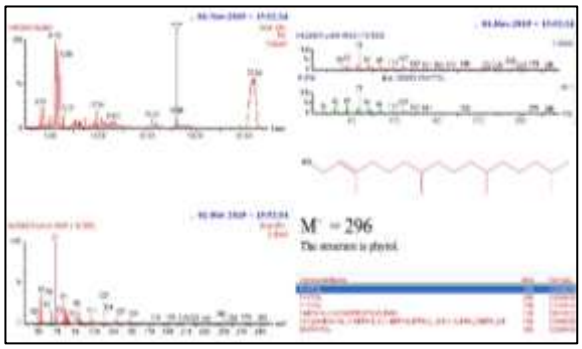


Figure (4.u) GC-MS spectrum of compound 21 from essential oil at RT 18.583 min

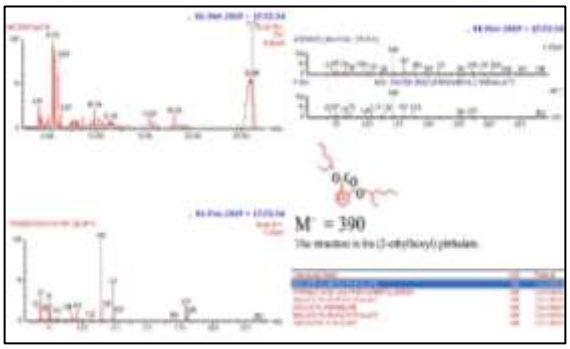
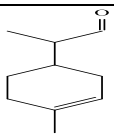
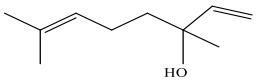
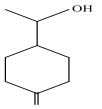
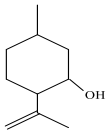
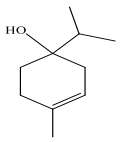
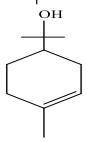
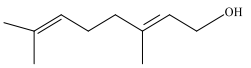
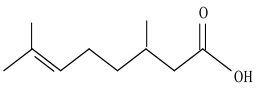
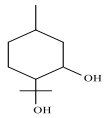
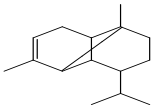
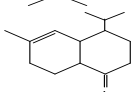
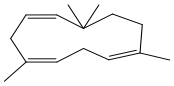
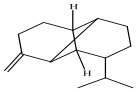
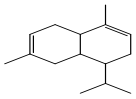
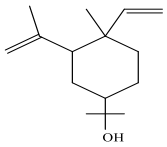
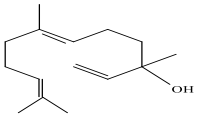
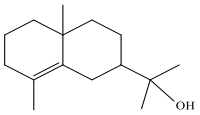
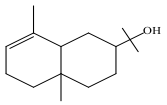
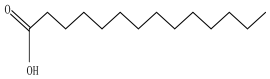
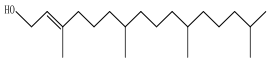
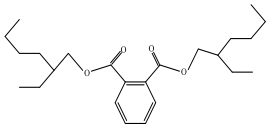


Figure (4.v) GC-MS spectrum of compound 22 from essential oil at RT 26.641 min

Table (2) The Twenty-two Compounds detected by GC-MS in Essential Oil from *C. hystrix* Peel at Different Retention Times (RT)

Compd. (s)	Name	Structure	Molecular weight	Formula	RT (min)
1	α, 4-dimethyl-3-cyclohexene-1-acetaldehyde		152	$C_{10}H_{16}O$	4.515
2	3,7-dimethyl-1,6-octadien-3-ol (linalool)		154	$C_{10}H_{18}O$	4.860
3	p-menth-1(7)-en-9-ol		154	$C_{10}H_{18}O$	5.243
4	dl-isopulegol		154	$C_{10}H_{18}O$	5.569
5	terpinen-4-ol		154	$C_{10}H_{18}O$	6.060
6	L-α-terpineol		154	$C_{10}H_{18}O$	6.260
7	geraniol		154	$C_{10}H_{18}O$	6.873
8	3,7-dimethyl-6-octenoic acid (citronellic acid)		170	$C_{10}H_{18}O_2$	7.859
9	(1α-3β-4β) – p-menthane-3,8-diol		172	$C_{10}H_{20}O_2$	8.181
10	α- copaene		204	$C_{15}H_{24}$	8.561
11	γ- muurolene		204	$C_{15}H_{24}$	8.710
12	caryophyllene		204	$C_{15}H_{24}$	9.163
13	1,5,9,9-tetramethyl-1,4,7-cycloundecatriene		204	$C_{15}H_{24}$	9.612
14	β-copaene		204	$C_{15}H_{24}$	9.919
15	1,2,4A,5,6,8A-hexahydro-4,7-dimethyl-1-(1-methylethyl)-naphthalene (α-muurolene)		204	$C_{15}H_{24}$	10.340

(Cont'd)

16	4-ethenyl-α,α-4-trimethyl-3-(1-methylethenyl)-[1R-(1α, 3α, 4β)]-cyclohexanemethanol		222	$C_{15}H_{26}O$	10.735
17	nerolidol 2		222	$C_{15}H_{26}O$	10.805
18	γ-eudesmol		222	$C_{15}H_{26}O$	11.740
19	1, 2, 3, 4, 4A, 5, 6, 8A-octahydro-α,α-4a, 8-tetramethyl-(2R-cis)-2-naphthalenemethanol		222	$C_{15}H_{26}O$	12.028
20	tetradecanoic acid		228	$C_{14}H_{28}O_2$	16.074
21	phytol		296	$C_{20}H_{40}O$	18.583
22	is-(2-ethylhexyl) pathalate		390	$C_{24}H_{38}O_4$	26.641

Compd. (s) = Compounds

Screening of Antimicrobial Activity of *C. hystrix* Peel Extracts by Agar Well Diffusion Method

The dried peel powder collected from Myanaung Township, Ayeyarwady Region was extracted with various solvents and the yield % of PE extract (3.00 %), EtOAC extract (5.20 %), 96 % EtOH extract (10.60 %) and water extract (13.20 %) were obtained respectively. These four crude extracts were tested with eight microorganisms such as *Escherichia coli* AHU5436, *Bacillus subtilis* IFO90571, *Bacillus pumilus* IFO90571, *Candida albicans* NITE09542, *Pseudomonas fluorescens* IFO94307, *Staphylococcus aureus* AHU8465, *Agrobacterium tumefaciens* NITE09678 and *Malassezia furfur* UY. The eight tested microorganisms obtained from the source of NITE & Kyowa Hakko Co. Ltd., Japan, Pharmaceutical Research Development (PRD), Ministry of Technology and University of Yangon (UY) were cultured at Biotechnology and Development Center (BDC) of Pathein University and then tested at Department of Chemistry, Hinthada University. The microorganism species used in the test are responsible for typhoid, GI tract infection, diarrhea, septicemia, crown gall disease, food poisoning, dandruff, seborrheic dermatitis and abscess in skin, mouth, nose. The measurable inhibition zone diameter of crude extracts showed the degree of antimicrobial activity (Fig. 5).

From the results given in Table 3, it was observed that all four crude extracts of the *C. hystrix* peel exhibited inhibition zone diameters range between 12~40 mm against eight tested

microorganisms. Thus, it may be effectively used as active remedy for this treatment of their related diseases and fungal infection.

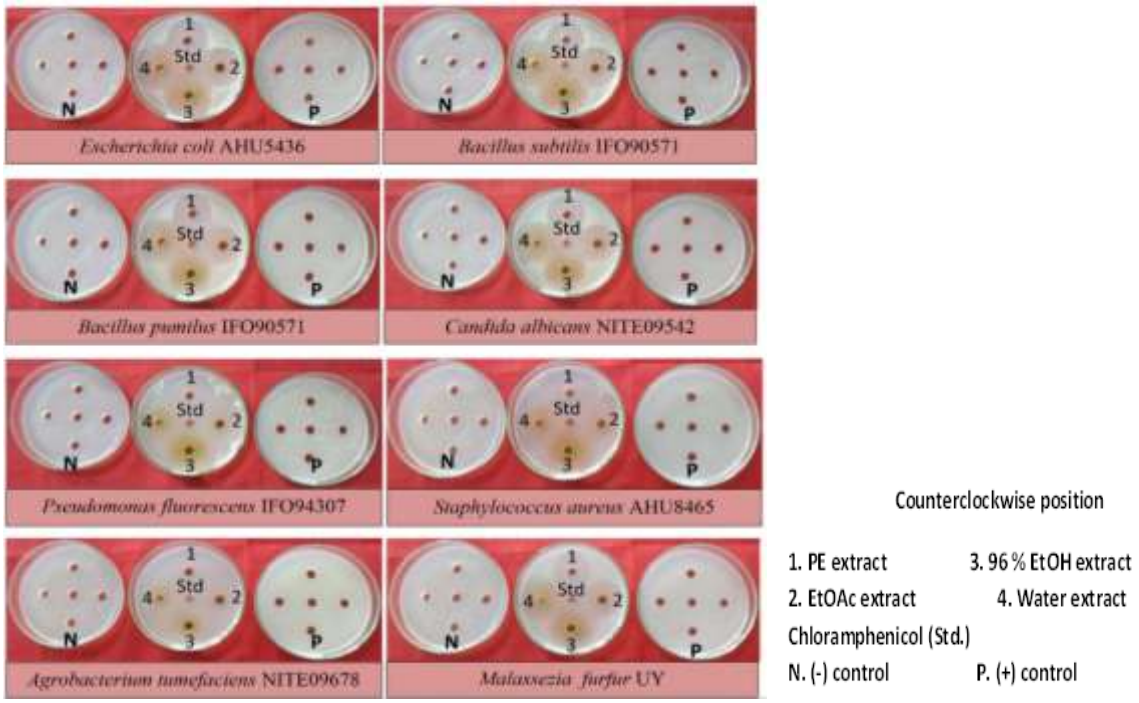


Figure (5) Inhibition zones of four extracts of *C. hystrix* peel against eight microorganisms

Table (3) Inhibition Zone Diameters of Four Crude Extracts from *C. hystrix* Peel

Microorganisms*	Inhibition Zone Diameters (mm) of Test Samples				
	PE Extract	EtOAc Extract	96 % EtOH Extract	H_2O Extract	Std.
Escherichia coli AHU5436	32	33	36	39	42
Bacillus subtilis IFO90571	32	35	39	**40**	42
Bacillus pumilus IFO90571	31	30	33	35	40
Candida albicans NITE09542	31	32	34	37	40
Pseudomonas fluorescens IFO94307	32	30	34	22	17
Staphylococcus aureus AHU8465	32	29	30	29	20
Agrobacterium tumefaciens NITE09678	16	**12**	18	19	21
Malassezia furfur UY	30	31	33	33	35

Std. = Chloramphenicol (standard) Agar well– (8 mm)
10 mm-14 mm (+), 15mm-19 mm (++), 20 mm-above (+++)
(+) = low activity, (++) = medium activity, (+++) = high activity

Tested microorganisms – From the source of NITE & Kyowa Hakko Co. Ltd., Japan, Pharmaceutical Research Development (PRD), Ministry of Technology and University of Yangon (UY) *

Conclusion

From EDXRF spectrum, the sample of *C. hystrix* peel had relatively the highest content of potassium whereas minor components of calcium, sulphur, phosphorous, iron, copper, manganese and zinc were observed. Colourless essential oil (0.03 g, 0.06 %) was obtained from *C. hystrix* peel by the hydro-distillation method. The functional groups contained in the essential oil could be determined by FT IR spectroscopy. In addition, GC-MS analysis of essential oil could be deduced as α, 4-dimethyl-3-cyclohexene-1-acetaldehyde (RT: 4.515 min), 3,7-dimethyl-1,6-octadien-3-ol (linalool) (RT: 4.860 min), p-menth-1(7)-en-9-ol (RT: 5.243 min), dl-isopulegol (RT: 5.569 min), terpinen-4-ol (RT: 6.060 min), L-α-terpineol (RT: 6.260 min), geraniol (RT: 6.873 min), 3,7-dimethyl-6-octenoic acid (citronellic acid) (RT: 7.859 min), (1α-3β-4β)- p-menthane-3,8-diol (RT: 8.181 min), α-copaene (RT: 8.561 min), γ- muurolene (RT: 8.710 min), caryophyllene (RT: 9.163 min), 1,5,9,9-tetramethyl-1,4,7-cycloundecatriene (RT: 9.612 min), β-copaene (RT: 9.919 min), 1,2,4A,5,6,8A-hexahydro-4,7-dimethyl-1-(1-methylethyl)-naphthalene (α-muurolene) (RT: 10.340 min), 4-ethenyl-α,α-4-trimethyl-3-(1-methylethenyl)-[1R-(1α,3α,4β)]- cyclohexane-methanol (RT: 10.735 min), nerolidol 2 (RT: 10.805 min), γ-eudesmol (RT: 11.740 min), 1,2,3,4,4A,5,6,8A-octahydro-α,α-4a,8-tetramethyl-(2R-cis)-2-naphthalenemethanol (RT: 12.028 min), tetradecanoic acid (RT: 16.074 min), phytol (RT: 18.583 min) and Bis-(2-ethylhexyl) pathalate (RT: 26.641min). Crude extracts were prepared from *C. hystrix* peel using PE, EtOAc, 96 % EtOH and water as their solvent polarity. These extracts were used to test the antimicrobial activity.

Screening of antimicrobial activity of various crude extracts such as PE, EtOAc, EtOH and H_2O extracts from *C. hystrix* peel sample was also investigated by employing agar well diffusion method against eight tested microorganisms responsible for typhoid, GI tract infection, diarrhea, septicemia, crown gall disease, food poisoning, dandruff, seborrheic dermatitis and abscess in skin, mouth, nose. It was observed that all the extracts of *C. hystrix* peel exhibited inhibition zone diameters between 12~40 mm against eight tested microorganisms.

Based on the findings of present study it may be concluded that *C. hystrix* peel possesses valuable essential oil as well as significant antimicrobial activity. In addition, some nutrient elements were found in the peel sample. In fact, the selected Myanmar medicinal plant, *C. hystrix* peel may be used in herbal formulation for maintaining health and preventing from pathogenic microorganisms. The result of this study is an encouragement for further work that will lead to pharmacological analysis of the essential oils extracted from the selected plant.

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