CHEMICAL COMPOSITION, ANTIBACTERIAL AND ANTIOXIDANT ACTIVITIES OF ACETONE EXTRACT FROM LEAF AND FRUIT PEEL OF *Citrus hystrix*

THAO NGUYEN LUU[1], THI MINH NGOC NGUYEN[1], XUAN SON LY[1], NGOC THUAN NGUYEN[1], TAN VIET PHAM[1], HONG THIEN VAN[1], THANH THO LE[2], QUOC HUNG NGUYEN[2], NGOC NAM TRINH[3*]

[1]*Institute of Biotechnology and Food Technology, Industrial University of Ho Chi Minh City*
[2]*Center of Analytical Services and Experimentation HCMC*
[3]*Office of Science Management and International Affairs, Industrial University of Ho Chi Minh City*
** Corresponding author: trinhngocnam@iuh.edu.vn*

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Abstract. *Citrus hystrix* has been well known for its uses as food and biological activities. In this study, the chemical composition, antibacterial and antioxidant effects of acetone extract isolated from *C. hystrix* leaves and fruit peels were firstly investigated. GC/MS analyses results revealed that there were 56 and 87 chemical components identified from leaf and fruit peel of the studied plant, respectively. The leaf extract was characterized by the predominance of α-pinene, D-limonene, citronellal, caryophyllene oxide and n-hexadecanoic acid while sabinene, β-pinene, D-limonene, citronellol, citronellol acetate, dihydro-α-ionon, δ-cadinene, n-hexadecanoic acid were the major constituents in the fruit peel extract. The two studied extracts showed antibacterial effects against six oral bacteria, including *Bacillus cereus, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella enteritidis* and *Salmonella typhimurium* and displayed a notable antioxidant activity in the ABTS assay with IC_{50} values of 38.59 ± 0.59 µg/mL and 85.89 ± 1.85 µg/mL, respectively.

Keywords: *Citrus hystrix, acetone extracts, chemical components, antibacterial, antioxidant activities.*

1. INTRODUCTION

The Rutaceae family includes over 1600 species belonging to 155 genera. This family is widely distributed in subtropical and tropical areas. A large number of Rutaceae species have been used in medicine, food and essential oil extraction. Rutaceae species also contained many bioactive compounds such as saponins, steroids, cardiac glycosides, alkaloids, and flavonoids [1].

The *Citrus* genus includes 16 species native to China and South-East Asia. *Citrus* species are evergreen aromatic shrubs and small trees [2]. Previous reports demonstrated that the fruits, leaves and flowers of *Citrus* plants were characterized by various bioactive compounds, including flavonoids, vitamin C, potassium, pectin, folic acid, carotenoids, glucarates, coumarins, anthocyanins, limonoids, phenolic acids and essential oils [2], [3]. Notably, the *Citrus* plants have been reported to possess many pharmacological activities such as anti-inflammatory, hypolipidemic, anticancer, antioxidant, antiatherosclerotic, antiallergy antithrombotic, antihypertensive, antiulcer, and antimicrobial activities [2], [3].

Citrus hystrix DC. is commonly known as "kaffir lime" or "makrut lime" in Thai. It is widely cultivated in tropical Asian countries, including Indonesia, Thailand, Malaysia, Laos and Vietnam. The essential oils obtained from different plant parts of this species are widely used in Malaysia as fragrance and flavour agents [4]. The chemical composition and biological activities of essential oils and a large number of extracts from *C. hystrix* have been reported in previous studies [5], [6],

[7], but the acetone extract was limited. In this study, thus, chemical components, antibacterial and antioxidant effects of the acetone extracts from leaves and fruit peels of *C. hystrix* were investigated for the first time.

2. MATERIALS AND METHODS

2.1. Plants

The samples of *Citrus hystrix* were collected from Tri Ton District, An Giang province (Figure 1).

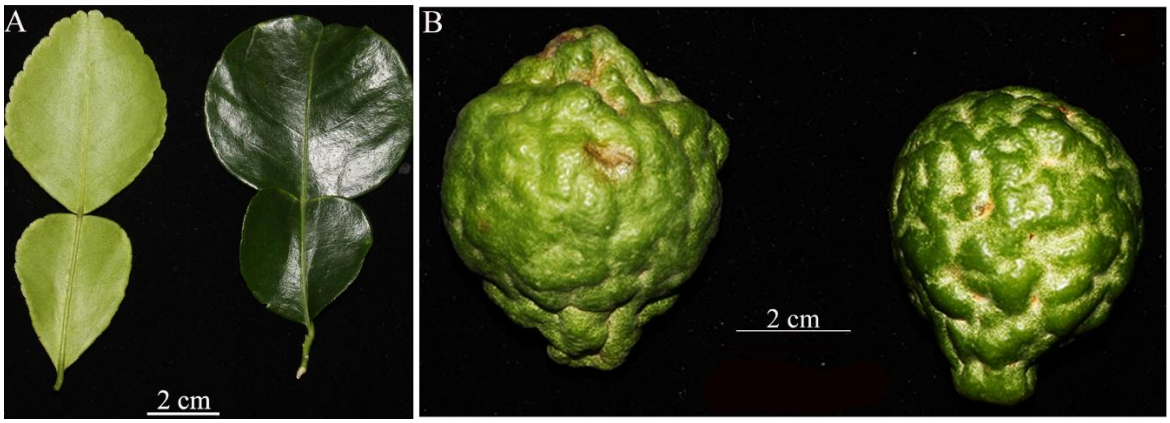

Figure 1: *Citrus hystrix*. A. Leaves. B. Fruits.

2.2. Microorganisms

Six studied bacteria such as *Bacillus cereus* (ATCC 11774), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella enteritidis* (ATCC 13976) and *Salmonella typhimurium* (ATCC 13311) were used to clarify the antibacterial activity of the acetone extracts of the studied species.

2.3. Chemicals

ABTS (2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid) was purchased from Sigma, USA.

2.4. Extraction procedures

The leaves and fruit peels of *C. hystrix* were moderately dried at 50^{o}C until their constant weights. The electric grinder was used to ground the dried samples into powder. 50g of the dried samples were subsequently macerated in 250 ml of 99% acetone solution at room temperature for 72 hours. The extract was then filtered through a Whatman paper. The process was repeated twice. The filtrate was then concentrated under the reduced pressure at 50^{o}C to obtain the brown extract prior to sublimation drying to remove the acetone residue completely [8].

2.5. Gas chromatography-mass spectrometry (GC/MS) analysis

Thermo Scientific TRACE 1310 Gas Chromatograph coupled with Thermo Scientific ISQ 7000 Single Quadrupole was used to analyze the chemical composition of the sample. The GC Column was DB-5MS 30m, 0.25mm, 0.25µm. Carrier gas used was helium with a constant flow rate of 1.2 mL/min. The sample was injected into the GC system at an inlet temperature of 270^{o}C with a split mode ratio of 30:1, split flow rate of 36 mL/min, the rate split line of 30:1 and the splitless time of 1 minute. The temperature column was initiated at 80 ^{0}C and kept constant for 5 minutes. The temperature was then increased by 20^{0}C/min to 280 ^{0}C and kept constant for 10 minutes and then increased to 300^{0}C at a rate of 20^{0}C /min and kept for 3 minutes. The ion source temperature was set at 250^{0}C while the transmission temperature was set at 280^{0}C. The ionization energy was 70

eV. The m/z scan range was between 29-650 m/z and the scan time was 0.2 seconds. The chemical composition of the sample was evaluated based on the comparison between our mass spectrometry and the NIST 2017 library.

2.6. Antibacterial assay

Disc diffusion test was employed to study the antibacterial activity assay using those six bacterial cultures following the CLSI guideline (CLSI, 2010). The bacterial strains were first activated by being cultured in Luria-Bertani Broth until their turbidity was equivalent to 0.5 McFarland. Mueller Hinton agar plate was inoculated with 0.1 mL of the bacterial culture by spread - plate technique before sterile paper discs containing 10ul of the extract solution were placed on its surface. The plate was incubated at 37^0C for 16-18 hours. The positive control used was Gentamycin antibiotic disc (Nam Khoa, Vietnam). The resistance of the extract against the bacterial strains was evaluated by measuring the inhibition zone after 16-18 hours of incubation.

2.7. Determination of antioxidant activity of extract

The ABTS radical scavenging activity of the extracts was determined using the method described by Maeng et al. [10] with minor modification. Firstly, 7 mM ABTS was added to 2.45 mM K_2SO_4 of the sample. The mixture was then slightly shaken and placed in the dark for 18 hours at 37ºC (solution A). 0.1 mL extract was added to 3 mL solution A. The mixture was diluted to 5 ml using acetone and then slightly shaken and placed in dark 15 minutes. The absorbance of the solution was later recorded at 734 nm using UV-vis spectrophotometer (UVS 2800, Labome, USA) and UVWin6 Software v6.0.0. Ascorbic acid was used as a reference standard. Ascorbic acid standard curve (0-15 ppm) was constructed with the equation y = -0.0278x + 0.421, R2 = 0.9990, where y is the absorbance at 734 nm and x is the sample concentration (µg/mL). The sample concentration was calculated from the standard curve equation and the results were expressed as µg/mL ascorbic acid.

2.8. Data analysis

Three biological replicates were used for the experiment, and the results were expressed as mean ± standard deviation (SD). The differences between means groups were calculated by Fisher's least significant difference (LSD) procedure using Statgraphics Centurion XV software (Statpoint Technologies Inc, Virginia, USA) with $p < 0.05$.

3. RESULTS AND DICUSSION

3.1. Chemical composition of *C. hystrix* leaf and fruit peel

The chemical constituents of the acetone extract obtained from the *C. hystrix* leaves and fruit peels are shown in Table 1 and 2. A total of 56 and 87 chemical components were detected in the leaf and fruit peel extracts, respectively. Based on the GC chromatogram (Figure 2), the major components in acetone extract isolated from the leaves and fruit peels of studied species were identified. As a result, the leaf extract was characterized by the predominance of sabinene, α-pinene, D-limonene, citronellal, caryophyllene oxide, n-hexadecanoic acid, thunbergol, thunbergol, phytol and cis-vaccenic acid. Meanwhile, sabinene, β-pinene, D-limonene, citronellol, citronellol acetate, dihydro-α-ionone, δ-cadinene, n-hexadecanoic acid, geranyl acetate and oxypeucedanin were the major compounds in the fruit peel extract.

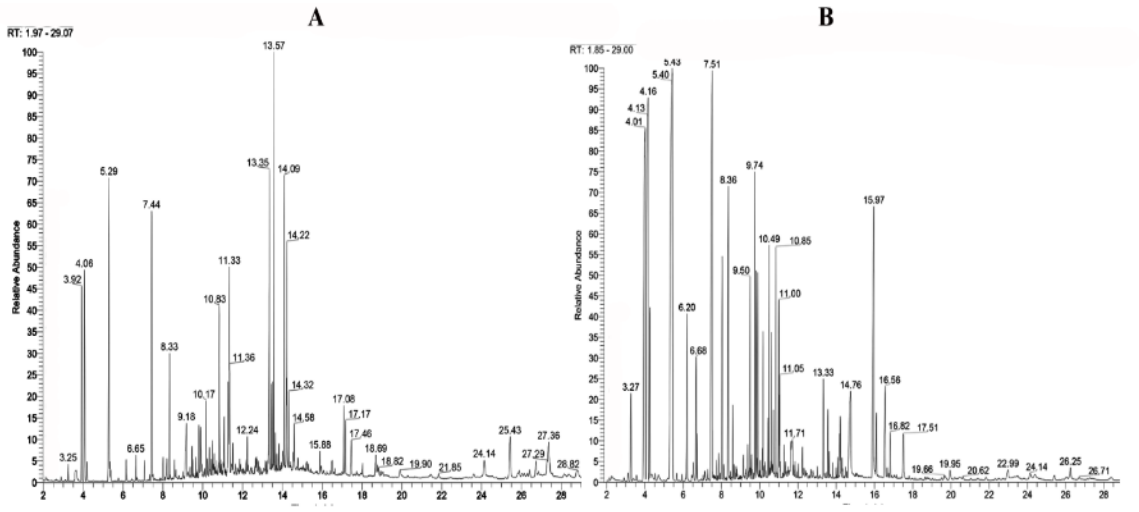

Figure 2: GC chromatogram. A. leaf extract. B. fruit peel extract

Some biological activities have been recorded in several chemical components isolated from the leaf and fruit peel extracts of *C. hystrix*. For instance, D-limonene has been reported to be effective against breast cancer. The effect of D-limonene on the expression of cyclin D1 in tumor tissue was significant with 22 percent reduction, but was hardly found in tissue Ki67, cleaved caspase-3 expression, serum leptin, adiponectin, TGF-b1, insulin-like growth factor binding protein-3 (IGFBP-3), and interleukin-6 (IL-6) levels [11]. Another example was of Terpinen 4-ol. This component was found to have several biological and pharmacological properties, including antiviral, anti-biofilm, anti-cancer, anti-inflammatory activities [12]. In a study by Shapira *et al.* (2016), terpinen-4-ol was proven to be able to inhibit the growth of range of cancer cells, such as colorectal, gastric, pancreatic and prostate cancer cell lines in dose dependent manner [13].

Myrcene has been proven to effect against germinal cells, protoscoleces and murine cyst of *Echinococcus granulosus* and the vector caused Echinococcosis in humans and animals. In a study on chemoprophylactic efficacy, the activity of β-myrcene was equivalent to albendazole, the drug used for echinococcosis treatment in humans [14]. The anti-inflammatory properties and physiological role, metabolism and nutritionnal implications in humans of n-hexadecanoic acid have been recorded [15]. Antidiabetic activity of sitosterol was reported when applied in mice whose diabetes was induced using streptozotocin. Sitosterol helped increase plasma insulin level and anti-hyperlipidemic and anti-hyperglycemic effects. Anti-nociceptive and antioxidant effects was found in phytol [16] while sabinene showed weak inhibition on Balb/3T3 cells [17]. Moreover, α-pinene and β-pinene have been reported to possess larvicidal and insecticidal activities against *Aedes aegypti, Lasioderma serricorne* and *Rhodnius nasutus* [18], [19], [20].

Table 1: Chemical composition from the leaf extracts of *C. hystrix*

No.	RT	Compounds	No.	RT	Compounds
1	3.66	Glycerin	29	11.28	(-)-Spathulenol
2	3.92	Sabinene	30	11.33	Caryophyllene oxide
3	4.06	α-Pinene	31	11.51	Humulene-1,2-epoxide
4	4.18	α-Myrcene	32	12.24	Tetradecanoic acid
5	5.29	D-Limonene	33	13.35	n-Hexadecanoic acid
6	5.33	α-Terpineol	34	13.44	4,8-Decadienal, 5,9-dimethyl-

7	5.38	o-Menthan-8-ol	35	13.57	Thunbergol
8	5.77	Melonal	36	13.64	trans-Geranylgeraniol
9	6.15	cis-β-Terpineol	37	13.72	17-Octadecynoic acid
10	6.64	Linalool	38	13.83	Heptadecanoic acid
11	6.70	β-Terpineol	39	14.09	Phytol
12	7.44	Citronellal	40	14.19	Linoelaidic acid
13	8.01	α-Terpinyl acetate	41	14.22	cis-Vaccenic acid
14	8.33	Citronellol	42	14.32	Octadecanoic acid
15	8.58	Geraniol	43	14.58	Bergaptol
16	9.18	Citronellic acid	44	15.87	Oxypeucedanin
17	9.46	Neomenthoglycol	45	16.49	Pabulenol
18	9.49	Citronellol acetate	46	17.16	Citronellyl palmitate
19	9.65	Menthoglycol	47	17.45	Oxypeucedanin hydrate
20	9.8	Copaene	48	18.01	Squalene
21	10.17	Caryophyllene	49	18.68	Citronellyl linoleate
22	10.24	Shyobunol	50	18.83	17-Pentatriacontene
23	10.33	Epoxy-linalooloxide	51	19.89	Vitamin P
24	10.44	Nerolidyl acetate	52	21.84	Vitamin E
25	10.49	α-Dihydroionone	53	23.6	Campesterol
26	10.71	β-Acorenol	54	24.14	Stigmasterol
27	10.83	Hydroxycitronellal	55	25.43	β-Sitosterol
28	11.07	α-Nerolidol	56	26.73	Lupeol

Table 2: Chemical composition from the fruit peel extracts of *C. hystrix*

No.	**RT**	**Compounds**	**No.**	**RT**	**Compounds**
1	3.12	β-Thujene	45	10.31	Nerolidyl acetate
2	3.27	α-Pinene	46	10.44	Humulene
3	3.40	3-p-Menthen-7-al	47	10.49	Dihydro-α-ionone
4	3.56	Camphene	48	10.55	Isogermacrene D
5	4.01	Sabinene	49	10.58	Shyobunol
6	4.16	β-Pinene	50	10.62	β-Copaene
7	4.35	Furan	51	10.71	Bicylogermacrene
8	4.53	Octanal	52	10.85	δ-Cadinene
9	5.43	D-Limonene	53	10.95	Sesquisabinene hydrate
10	5.66	β-Ocimene	54	11.04	Hedycaryol
11	5.93	γ-Terpinene	55	11.07	α-Nerolidol
12	6.20	Sabinene hydrate	56	11.26	Germacrene D-4-ol
13	6.45	p-Mentha-1,4(8)-diene	57	11.34	Globulol
14	6.68	Linalool	58	11.41	Nerolidol
15	6.72	cis-β-Terpineol	59	11.47	Globulol
16	6.93	Ipsenone	60	11.64	D-Mannose
17	7.04	trans-p-Mentha-2,8-dienol	61	11.71	Glucose
18	7.13	cis-Myroxide	62	11.78	Pogostole
19	7.28	Myroxide	63	11..85	Shyobunol
20	7.51	Citronellal	64	12.02	(Z,E)-Farnesol

21	7.75	endo-Borneol	65	12.23	Myristic acid
22	7.85	Terpinen-4-ol	66	13.00	Cholestan-3-ol, 2-methylene-, (3β,5α)-
23	7.93	p-mentha-1(7),8-dien-2-ol	67	13.33	n-Hexadecanoic acid
24	8.04	α-Terpinyl acetate	68	13.56	Thunbergol
25	8.11	Decanal	69	13.64	2-cis-Geranylgeraniol
26	8.36	Citronellol	70	13.82	Heptadecanoic acid
27	8.47	Neral	71	13.99	cis-13-Eicosenoic acid
28	8.59	Geraniol	72	14.08	Phytol
29	8.65	Lilac alcohol B	73	14.21	Linolenic acid
30	8.77	α-Citral	74	14.30	Stearic acid
31	9.29	(-)-Myrtenol	75	14.48	Bergaptol
32	9.44	δ-Elemene	76	14.55	Arborescin
33	9.46	8-Hydroxyneomenthol	77	14.76	Psoralen, 8-hydroxy
34	9.50	Citronellol acetate	78	15.97	Oxypeucedanin
35	9.54	α-Cubebene	79	16.10	Isooxypencedanine
36	9.60	Citronellol epoxide	80	16.56	Pabulenol
37	9.66	8-hydroxymenthol	81	16.82	β-D-Mannofuranoside, farnesyl-
38	9.74	Geranyl acetate	82	17.52	Oxypeucedanin hydrate
39	9.76	Cedrene	83	19.94	Hesperetin
40	9.81	Copaene	84	21.83	Vitamin E
41	9.85	10-Undecen-1-al, 2-methyl	85	24.15	Stigmasterol
42	9.89	cis-β-Copaene	86	25.41	β-Sitosterol
43	10.03	trans-Geranylacetone	87	26.71	Scaposin
44	10.17	Caryophyllene			

3.2. Antibacterial activitiy of *C. hystrix* leaf and fruit peel

The antibacterial activity of the acetone extracts obtained from the leaves and fruit peels of *C. hystrix* was investigated by measuring the diameter of inhibition zones of the extracts on oral bacteria. As a result, both extracts could inhibit the growth of all six studied bacterial strains (Table 3). Accordingly, the leaf extract showed strong antibacterial activities against *B. cereus*, *S. aureus* and *E. coli* with the inhibition zones of 26.8±0.8 mm, 18.3±0.6 mm and 17.2±1.1 mm, respectively. In addition, the leaf extract had moderate antibacterial activities against *P. aeruginosa* (13.3±1.2 mm) and weak effects against *S. enteritidis* (8.2±0.3 mm) and *S. typhimurium* (8.2±0.3 mm). Besides, the fruit peel extract inhibited strongly the growth of *E. coli*, *S. aureus*, *S. enteritidis* and *P. aeruginosa* with the inhibition zone of 25.7±1.5 mm, 23.0±1.7 mm, 19.8±1.1 mm và 16.7±1.2 mm, respectively while 10.8±1.1 mm and 10.7±0.8 mm were the inhibition zones of *B. cereus* and *S. typhimurium* towards the same extract.

Table 3: inhibition zone of acetone extracts isolated from *Citrus hystrix* against six bacterial strains

Tested bacteria	Growth inhibition zone (mm)		
	Leaf	fruit peel	Gentamycin
B. cereus	26.8 ± 0.8^{b}	10.8 ± 1.1^{a}	26.5 ± 0.5^{b}
E. coli	17.2 ± 1.1^{a}	25.7 ± 1.5^{b}	16.7 ± 0.6^{a}
P. aeruginosa	13.3 ± 1.2^{a}	16.7 ± 1.2^{b}	15.3 ± 0.3^{b}
S. enteritidis	8.3 ± 0.3^{a}	19.8 ± 1.1^{b}	19.7 ± 1.2^{b}

S. typhimurium	8.2±0.3[a]	10.7±0.8[b]	18.8±1.1[c]
S. aureus	18.3±0.6[a]	23.0±1.7[b]	17.2±0.8[a]

[a,b,c]Different superscript lower-case letters in the same row denote significant difference (p<0.05).

The antibacterial activities of the acetone extract obtained from the leaf and fruit peel of *C. hystrix* could be established by the following chemical components, including α-pinene, β-pinene, linalool, myrcene, D-limonene and sabinene. For instance, α-pinene has been reported to possess antibacterial effects against many bacterial strains, including antimicrobial against *Escherichia coli, Mycobacterium smegmatis, Cylindrocarpon mali, Staphylococcus aureus, S. epidermidis, Streptococcus faecalis, S.* pyogenes, *S. pneumonia, Pseudomonas aeruginosa, Aspergillus niger* and *Stereum purpureum* [21], [22]. Furthermore, β-pinene inhibited the growth of gram-positive bacteria causing potential infectious endocarditis, including *Staphylococcus aureus, S. epidermidis, Streptococcus pyogenes* and *S. pneumonia* [22].

Linalool showed antibacterial activities of three pathogenic bacteria as *P. aeruginosa, E. coli* and *S. aureus* [23]. *Pasteurella multocida* and *Listeria monocytogenes*, other pathogenic bacteria, have also been inhibited by linalool [24] [25]. Also, linalool has been reported to be active against many pathogenic bacteria such as *Fusobacterium nucleatum* subsp. *polymorphum, F. nucleatum* subsp. *nucleatum, F. nucleatum* subsp. *fusiforme, F. nucleatum* subsp. *vincentii, F. nucleatum* subsp. *animalis, Prevotella intermedia, P. intermedia* and *Porphylomonas gingivalis* [26]. In addition, myrcene displayed activity against *S. aureus* [27], *Enterococcus faecalis, Streptococcus salivarius* and *S. sanguinis* [28]. D-limonene had good activity against *B. subtilis, E. coli* and *S. aureus* [29]. Finally, sabinene possessed a strong bacterial activity against *S. typhimurium, E.* coli and *Micrococcus* luteus [30].

To date, the antibacterial effects of the acetone extracts of *Citrus hystrix* are limited. However, the antimicrobial assays of the essential oils and other extracts of this species have been investigated by previous studies. For instance, the essential oil isolated from the fruit of *C. hystrix* was found to be effective against six pathogenic bacteria, including S. aureus, Enterococcus faecalis, E. coli, Proteus vulgaris, Klebsiella pneumoniae and P. aeruginosa [6]. Similarly, the leaf and fruit peel essential oils of *C. hystrix* were found to have an inhibitory effect on S. aureus, Streptococcus pneumoniae and Haemophilus influenza [7]. The ethyl acetate extract of *C. hystrix* was demonstrated to be effective against many bacterial strains such as *S. aureus, B. cereus, Listeria onocytogenes* and *Salmonella* sp. [31].

3.3. Antioxidant activity of *C. hystrix* leaf and fruit peel

Table 4 shows the IC_{50} values of the acetone extracts obtained from the leaf and fruit peel of *C. hystrix*. As a result, the leaf extract showed the stronger antioxidant activity (IC_{50} value of 38.59 ± 0.59 µg/mL) than the fruit peel extract (IC_{50} value of 85.89 ± 1.85 µg/mL). Previous reports on the other extracts from *C. hystrix* have shown their radical scavenging activity. For example, the antioxidant activity of the crude extract and fractions such as n-butanol ethyl acetate and hexane of *C. hystrix* was determined using 1,1-diphenyl-2-picrylhydrazol (DPPH) with IC_{50} values of 0.09, 0.44, 1.54 and 53.84 mg/mL, respectively [5]. Furthermore, the essential oils obtained from twig, leaves-twig, lead and, fruit peel of *C. hystrix* showed DPPH free radical scavenging with IC_{50} values of 8.7, 6.4, 8.4, 6.2 and 10.0 µg/mL [32].

Table 4: Radical scavenging activity of leaf and fruit peel of *C. hystrix*

Samples	**Leaf extract**	**fruit peel extract**	**Vitamin C**
IC_{50}	38.59 ± 0.59	85.89 ± 1.85	1.22 ± 0.08

4. CONCLUSION

The present study identified 56 and 87 chemical constituents of acetone extracts isolated from the *Citrus hystrix* leaves and fruit peels. The chemical constituents in acetone extract of *C. hystrix* leaves mainly contained α-pinene, D-limonene, citronellal, caryophyllene oxide, n-hexadecanoic acid while sabinene, β-pinene, D-limonene, citronellol and citronellol acetate were the major constituents in the fruit peel extract. The fact that the acetone extracts of *C. hystrix* leaf and fruit peel possessed the antibacterial activities against six bacterial strains such as *B. cereus*, *S. aureus*, *E. coli*, *P. aeruginosa*, *S. enteritidis* and *S. typhimurium* will lead to the potential use of those extracts as natural and sustainable alternatives to antibiotics. Finally, the antioxidant capacity of the two extracts partially explained whey they are potent in the treatments of certain diseases.

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THÀNH PHẦN HÓA HỌC, KHẢ NĂNG KHÁNG KHUẨN VÀ KHÁNG OXY HÓA CỦA CAO CHIẾT ACETONE LY TRÍCH TỪ LÁ VÀ VỎ QUẢ CỦA LOÀI *Citrus hystrix*

LƯU THẢO NGUYÊN[1], NGUYỄN THỊ MINH NGỌC[1], LÝ XUÂN SƠN[1], NGUYỄN NGỌC THUẦN[1], PHẠM TẤN VIỆT[1], VĂN HỒNG THIỆN[1], LÊ THÀNH THỌ[2], NGUYỄN QUỐC HÙNG[2], TRỊNH NGỌC NAM[3*]

[1]Viện Công nghệ Sinh học và Thực phẩm, Trường Đại học Công Nghiệp Thành phố Hồ Chí Minh
[2]Trung tâm Dịch vụ Phân tích Thí nghiệm Thành phố Hồ Chí Minh
[3]Phòng Quản lý Khoa học và Hợp tác Quốc tế, Trường Đại học Công Nghiệp Thành phố Hồ Chí Minh
** Tác giả liên hệ: trinhngocnam@iuh.edu.vn*

TÓM TẮT. *Citrus hystrix* được biết đến như là một loài có nhiều hoạt tính sinh học. Nghiên cứu này lần đầu cho thấy thành phần hóa học, khả năng kháng khuẩn và kháng oxy hóa từ cao chiết acetone ly trích từ lá và vỏ quả của loài *C. hystrix*. Bằng phương pháp sắc ký khí ghép khối phổ (GC/MS), nghiên cứu này đã xác định được lần lượt 56 và 87 chất có trong cao chiết lá và vỏ quả. Theo đó, α-pinene, D-limonene, citronellal, caryophyllene oxide và n-hexadecanoic acid là những thành phần chính trong cao chiết lá trong khi sabinene, β-pinene, D-limonene, citronellol, citronellol acetate, dihydro-α-ionon, δ-cadinene và n-hexadecanoic acid là những chất chính có trong cao chiết vỏ quả. Hai loại cao chiết cũng cho thấy có khả năng kháng lại 6 chủng vi khuẩn kiểm định là *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enteritidis* và *Salmonella typhimurium*. Ngoài ra, khả năng kháng lại gốc tự do ABTS của cao chiết lá và vỏ quả với giá IC_{50} tương ứng là 38,59 ± 0,59 µg/mL và 85,89 ± 1,85 ppm.

Từ khóa: *Citrus hystrix, cao chiết acetone, thành phần hóa học, kháng khuẩn, kháng oxy hóa.*

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