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Phytochemical Profile and Antimicrobial Activity of Citrus Medica (Limau Susu) Fruit Peels Extract

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ABSTRACT

Citrus medica, also known as "Limau Susu" in Malaysia, is generally called a citron in English. *Citrus medica* is one of the four original citruses, and many of today's citrus varieties are descended from this variety. Six *Citrus medica* peel extracts were produced from this research using the maceration technique and percentage yield were calculated. Each different extraction procedure inevitably results in variances in biological activity. This study aimed to assess extraction procedures' impact on the yield and microbial activity of *Citrus medica* using the disc-diffusion method. In addition, GC-MS was carried out to identify additional components (qualitative). The phytochemical analysis of each extract was done to distinguish the chemical constituent between the extracts. Results show that both dry and fresh samples of the *Citrus medica* plant give percentage yield of methanol extract. Meanwhile, the hexane extract shows the highest anti-microbial activities due to the presence of 3 major compounds. The high antimicrobial activity is due to the presence of Oxacycloheptadec-8-en-2-one, 2-Cyclohexen-1-ol, and Terpineol as major compounds in the extract

Keywords: *Citrus medica*; Extraction Method; Antimicrobial Activity; GCMS.

1.0 Introduction

Citron (*Citrus medica*) is a real, basic, or main Citrus species. Ripe citron fruits are large and have a yellow peel, the most significant portion of the citron fruit. The flesh of the fruits is pale to greenish-yellow, not particularly juicy, and mildly sour or sweet. Fewer research has been conducted on the chemical makeup of citron's edible portions, such as juice or pulp. Economically significant citrus species, such as lime, lemon, orange, and grapefruit, are hybrids of *Citrus medica, Citrus reticulata,* and *Citrus maxima*. These three ancestors are responsible for the intricate floral anatomy that exists today. Later on, it develops into a complex citrus fruit. Citron is a descendant of lemon and several hybrids known as limes; in fact, it has a comparable flavonoid profile to lemon and limes, with the most common flavanone and flavone components being hesperidin, eriodictyol glycoside, and diosmin, as well as diosmetin and apigenin C-glycosides (Ballistreri et al., 2019).

The phytochemical are essential and non-essential molecules derived from plant secondary metabolites with medicinal potential due to high biological activity. The primary phytochemicals in fruits and vegetables include carotenoids, flavonoids, and phenolics. Like other healthy fruits, Citron has a wide spectrum of phytochemical compounds. The compounds from the class of flavonoids, alkaloids, and carotenoids are found abundantly in fruit. Citron and other citrus fruits have a stronger medicinal efficacy than other significant fruits because of these important phytochemical components (Chhikara et al., 2018). Besides, extraction methods always lead to differences in biological activities due to different phytochemicals content in each extract (chen et al., 2016).

The accumulation of citrus peel waste has been steadily rising, posing a growing environmental concern. This upward trend could exacerbate existing environmental issues. Consequently, there is a pressing need to explore alternative solutions that can not only address the problem of citrus waste but also provide tangible benefits for humanity (Liegeard, & Manning, 2020). In the contemporary world, we are grappling with the formidable challenge of antimicrobial resistance (AMR). This is because when microbial such as bacteria, viruses, fungi, and parasites change over time and no longer respond to the medicine making infection harder to treat and increasing the risk of disease spread and the mortality rate (Hijazi et al., 2019). This work discusses different extraction methods of waste citrus peels and their antimicrobial activity. The profiling of each extract was also been identified.

2.0 Methods and Materials

2.1 Plant Sample

Citrus medica fruits were collected from the market in Kota Semarahan. Healthy fruits were chosen based on their appearance and color consistency. Then, fresh *Citrus medica* fruits were cleaned, and the fruit pulp was separated from the peel. The peels are then cut into smaller pieces.

2.2 Extract preparation

The peels of *Citrus medica* were air dried until they reached constant weight. Then after it dried, the peel was fully soaked in hexane for 72 hours at room temperature. After 72 hours, the hexane will be filtered and concentrated to one-sixth of its initial volume using a rotary evaporator to get the extract. These steps were repeated two more times. Afterward, the total mass of the extract was recorded. After extracted with hexane, then, the peel was further extracted using ethyl acetate, followed by methanol for 72 hours and three-time cycles. The same method was used for fresh peels of *Citrus medica,* where *Citrus medica* peel was directly soaked in hexane followed by Ethyl acetate and methanol (Juradin et al., 2019).

2.3 Phytochemical Analysis of Extract Using GCMS

The phytochemical analysis was done using GCMS according to Zamakshshari et al. (2023). The RTX-5MS GC-MS column was utilized in this project. Fused-silica capillary column (30 m ×0.25 mm i.d.; 0.25 µm film thickness) using helium as the carrier gas and was run at the constant pressure of 100.0 kPa. The injection was done in spitless mode at an injector temperature of 300°C. The oven temperature increased (rate) from 40 to 160 °C (5 min hold) at rate of 4 °C/min, and from 160 °C to 280 °C (15 min hold) at a rate of 5 °C/min. Each extract sample was run for 74 minutes. The temperature of the GC-MS interface was adjusted at 280 °C. MS mode (amu) was used for analytical scanning ranging from 45 to 500 atomic mass units. The temperature of the ion source will be set at 280 degrees Celsius. The peaks will be identified using the National Institute of Standards and Technology Mass Spectral Library (NIST08) and selection criterion of SI 85% and higher.

2.4 Antimicrobial Activity

The bacteria were cultivated in potato dextrose agar. Four bacteria strains were used in this work to evaluate the antimicrobial activities of the extract. There are two are Gram-positive strains (*Bacilius subtilis* and *Staphylococcus aureus*) and two are Gram-negative strains (*Escherichia coli* and *Pseudomonas aeruginosa*). After the plates are incubated at 37 °C for 24 hours, the anti-microbial activity will be assessed by measuring the diameter of the inhibitory zone. 10 mg/ml of the extract should be used for screening, whereas 1 mg/mL will be used for standard compounds (Zamakshshari et al., 2023). Contrast the antimicrobial activity with ampicillin, the positive control.

3.0 Results and Discussion

3.1 Extraction of *Citrus medica*

Based on Table 1, for fresh *Citrus Medica* extraction, methanol extract produces the largest weight of extract and percentage yield (19.78g and 6.598%, respectively), whereas hexane solution produces the lowest weight of extract and percentage yield (2.88g and 0.960%, respectively). As for dry Citrus Medica, the fresh peel of the fruits was dried in oven at 40°C until it reached a constant weight, of 32.058g. The methanol extract produces the highest weight and percentage yields (20.56g and 64.133%). Meanwhile, hexane extract gives the lowest weight of extract and percentage yields (0.783g and 2.442%).

Solvents employed for this extraction were methanol, hexane, and ethyl acetate. The efficacy of each solvent in extracting a certain molecule is determined by various aspects, including the substance's composition, solubility in the solvent, and polarity. Methanol is a polar solvent, which contains a positive and a negative pole, giving it a relatively high dielectric constant. Because of this feature, methanol may dissolve a wide spectrum of polar molecules. Polar molecules such as organic acids, alcohols, aldehydes, and other hydrophilic chemicals can be extracted successfully using methanol (Freakley et al., 2021). Hexane, on the other hand, is a non-polar solvent with a low dielectric constant. Its primary use is the extraction of non-polar substances such as lipids, oils, and hydrocarbons (Bhuiya et al., 2020). Although less polar than methanol, ethyl acetate is more polar than hexane. In terms of polarity, it is intermediate between the two solvents. Ethyl acetate extracts semipolar compounds from the plant (Oladipo & Betiku, 2019).

Table 1 Percentage yield of *Citrus Medica* Extract

Condition	Solution	Mass of sample (g)	Mass of extract (g)	Percentage yield (%)
Fresh	Hexane Ethyl acetate Methanol	300.000	2.880 3.580 19.780	0.960 1.193 6.593
Dry	Hexane Ethyl acetate Methanol	32.058	0.783 1.534 20.560	2.442 4.785 64.133

3.2 Phytochemical Analysis of *Citrus medica* using GCMS

The phytochemical components of all six extracts were analyzed through GCMS, as indicated in Table 2. The dried ethyl acetate extract shows a significant percentage of D-Limonene, which is 89.22%. D-limonene is a naturally occurring chemical that belongs to the cyclic monoterpene family. It is a clear, colorless liquid with a strong citrus fragrance typically in citrus essential oils such as oranges, lemons, limes, and grapefruits (Singh et al., 2021). D-limonene, having the chemical formula $C_{10}H_{16}$, is the most prevalent enantiomer of limonene. It has a bicyclic structure known as the p-methane backbone. Because the chemical is chiral, it possesses two mirror-image forms (enantiomers): D-limonene and L-limonene. D-limonene, on the other hand, is the more prevalent form found in nature and is generally referred to simply as "limonene" (Santiago & Feijoo, 2020).

Table 2. Common Phytochemical analysis of Citrus Medica extract using GCMS method

No	Compounds	Percentage (%) of compound in extract (10 mg /mL)					
		Fresh			Dry		
		Hex	EA	MeOH	Hex	EA	MeOH
1	2-Cyclohexen-1-ol	29.18	N. D	N. D	N. D	N. D	N. D
2	Terpineol	29.26	N. D	N. D	N. D	N. D	N. D
3	Decane	N. D	32.67	20.23	N. D	N. D	28.74
4	Benzene	N. D	34.72	N. D	N. D	N. D	23.85
5	Undecane	N. D	13.50	9.82	N. D	N. D	17.24
6	1,3-Dioxepane	N. D	4.97	N. D	N. D	N. D	N. D
7	1,3-Dioxolane	N. D	14.14	N. D	N. D	N. D	N. D
8	1,3,5,7-Cyclooctatetraene	N. D	N. D	13.50	N. D	N. D	N. D
9	1,2,3-trimethyl-Benzene	N. D	N. D	19.40	N. D	N. D	N. D
10	1,2,4-trimethyl-Benzene	N. D	N. D	4.38	N. D	N. D	N. D
11	Trans-3-Caren-2-ol	N. D	N. D	3.86	N. D	N. D	N. D
12	Oxacycloheptadec-8-en-2-one	N. D	N. D	N. D	28.06	N. D	21.47
13	Myrcene	N. D	N. D	N. D	N. D	0.90	N. D

14	D-Limonene	N. D	N. D	N. D	N. D	89.22	N. D
15	Terpinene	N. D	N. D	N. D	N. D	8.68	N. D
16	18-Octadec-9-enolide	N. D	N. D	N. D	N. D	0.89	N. D

* Hex= hexane, EA = Ethyl acetate, MeOH = Methanol, ND= Not Detected.

3.3 Antimicrobial Activity of *Citrus Medica*

The antibacterial properties of all *Citrus Medica* extract tested against Gram-positive and Gram-negative microorganisms. Gram-positive bacteria were more susceptible than Gram-negative bacteria among the microorganisms tested. This might be explained by the differing cell wall architectures of these two types of bacteria. According to Table 3, methanol extract had no antibacterial effect for any strains compared to the other peel essential oils**.** Hexane extract from fresh and dry conditions shows a high antibacterial activity with an inhibition zone of more than 20 mm. This is because of bioactive compounds such as Oxacycloheptadec-8-en-2-one, 2-Cyclohexen-1-ol, and Terpineol in the extract. Previous research shows that, the presence of major compounds of Oxacycloheptadec-8-en-2-one and 2-Cyclohexen-1-ol in the medicinal plant *Senna hirsute* distribute to its antimicrobial activities (Hidayati et al., 2020). Meanwhile, Terpenoids such as terpineol are known to have good antimicrobial activities inhibiting various bacteria strains (Benazzouz et al., 2020).

Table 3. Antimicrobial activity of *Citrus medica* extracts

		Inhibition zone Bacteria strain diameters (mm)			
Condition	Extract	BA	SA	EC	PA
Fresh	Hexane	15.00 ± 0.58	-	21.67±1.15	15.00± 1.00
	Ethyl acetate	14.00 ± 1.00	-	11.67±0.58	10.67 ± 1.15
	Methanol	-	-	-	-
Dry	Hexane	26.55 ± 1.53	-	11.33±0.57	16.00 ± 0.10
	Ethyl acetate	11.33 ± 1.53	-	9.33±1.53	15.00 ± 1.73
	Methanol	-	-	-	-
[redacted]	DMSO	-	-	-	-
	Ampicillin	24.33 ± 0.58	13.67 ± 0.58	28.33 ± 3.21	23.00 ± 1.00

*** BA=*Bacilius subtilis*, SA=*Staphylococcus aureus*, EC=*Escherichia coli*, PA= *Pseudomonas aeruginosa*, ND= Not Detected.**

Conclusion

Citrus Medica peel wastes, an abundant agro-industrial waste in the Malaysia region, were chemically examined, compared, and assessed for phytochemical and antibacterial activity. Differential accumulation of the discovered volatiles (Table 2) was seen among every six extracts. Furthermore, antibacterial activities are found to be related to the type of solvent used to extract the *Citrus medica* peel due to the different chemical constituents found in each extract. The investigations enabled us to identify groups of putative bioactive metabolites that might be involved in the reported antimicrobial activity of the active extract. As a result, both Hexane extract from each condition, dry and fresh, shows good antimicrobial activity due to the present of Oxacycloheptadec-8-en-2-one, 2-Cyclohexen-1-ol, and Terpineol as major compounds. However, further works such as isolation and extraction of these extracts needs to be carried out to determine the active metabolite leads to high biological activities.

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Competing interests. The authors declare no competing interests

Authors' contributions. Sofia Anak Simon: prepared the extract, participated in chemical profiling using gas chromatography-mass spectrometry (GC-MS), and antimicrobial studies, and drafted the manuscript. Nor Hisam Zamakshshari: writing, reviewing, and editing the manuscript.

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