EXTRACTION OF ESSENTIAL OIL, SCREENING OF ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES OF *CITRUS MEDICA VAR. ACIDA* BRANDIS (SHAUK THAKWA) LEAF

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Abstract

Citrus medica var. *acida* Brandis (Shauk thakwa), belonging to the Rutaceae family, has been chosen to investigate phytochemical constituents and some bioactivities. Firstly, preliminary phytochemical tests on *C. medica* leaves revealed the presence of alkaloids, carbohydrates, flavonoids, glycosides, phenolic compounds, reducing sugars, saponins, starch, steroids, tannins, and terpenes. The EDXRF elemental analysis showed that the leaves contained Ca, Al, K, and S as major elements. In the GC-MS analysis of the essential oil extracted by steam distillation method, the thirteen organic compounds detected (2.30 %) were 4-hydroxy-4-methyl-2-pentanone,-3-acetoxy-4-(1-hydroxy-1-methyl ethyl)-1-methyl-cyclohexene, (*R*)-(+)-citronellic acid, 2-(2-hydroxy-2-propyl)-5-methyl cyclohexan-1-ol, (*E*)-3-octadecence, octacosanol, methyl eugenol, benzyl benzoate, *cis*-9, 12-methyl linoleate, methyl (9E)-9-octadecenoate,di-isobutyl phthalate, heptadecan-1-ol and methyl stearate. The antimicrobial activity of leaves extracts in various solvents (pet-ether, ethyl acetate, 95 % ethanol, and water) was tested against six species of microorganisms: *Bacillus subtilis, Bacillus pumilus, Salmonella typhi, Pseudomonas aureginosa, Escherichia coli,* and *Candida albicans,* by the agar well diffusion method. It was found that the ethyl acetate extract (Inhibition zone diameter, 10–-14 mm) and watery extract (10 – 13 mm) exhibited antimicrobial activity against four tested microorganisms except *C. albicans and E. coli*. The 95 % ethanol extract exhibited the more potent antimicrobial activity (10 - 18 mm) against all tested microorganisms. In the screening of antioxidant activity, the watery extract (IC_{50} = 148.58 μg/mL) showed more antioxidant activity than 95 % ethanol extract (IC_{50} = 636.27 μg/mL).

Keywords: *Citrus medica var. acida* Brandis, phytochemical tests, antimicrobial activity, antioxidant activity, essential oil

Introduction

Citrus medica var. *acida* Brandis (Shauk thakwa) is cultivated in many countries all over the world and grows in hot subtropical or tropical regions such as Southern Florida, India, Mexico, Egypt, and West Indies (Onyilofe *et al.*, 2015; Taiwo, 2005). The decoction of pounded leaves is drunk for stomach aches, used as an eyewash, and used to bathe feverish patients. A poultice of leaves is applied to ulcer wounds, and skin disease, and is also applied to the abdomen after childbirth. Crushed leaves are applied to the forehead to treat headaches, and to treat nausea and resuscitate fainting individuals (Khan, 2010). Infusions of *C. medica* leaves have been given to treat fever with jaundice, sore throat, and oral thrush (Kunow, 2003). A decoction of the flower helps induce sleep for those with insomnia. In southwest Nigeria, the roots, bark, stem twigs, leaves, and fruits are used in the treatment of malaria (Khare, 2007).

Scientific Classification of *Citrus medica* var. *acida* Brandis (Shauk thakwa)

Family : Rutaceae
Botanical name: *Citrus medica var. acida* Brandis
Myanmar name: Shauk thakwa
Common name: Citron
Synonym name: *Citrus aurantifolia* (Christm)

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Figure 1. Photographs of *Citrus medica* var. *acida* Brandis

C. medica juice is used to increase stamina, treat dysfunctional uterine bleeding, be used as a facial wash to rejuvenate the skin and remove stains, be drunk to control epistaxis, and be given in pure form as a remedy for arthralgia, diabetes, and atherosclerosis (Onyilofe *et al.*, 2015). *C. medica* juice is sometimes mixed with oil, used as a vermifuge, and is also incorporated into a weight management diet (Akhtar, 2013). The main aim of the research work is to investigate some phytoconstituents, extract essential oils, and screen the antimicrobial and antioxidant activities of Shauk thakwa leaves (Figure 1).

Materials and Methods

Sampling of Plant Material and Identification

The leaves of *C. medica* (Shauk thakwa) were collected from Meiktila Township, Mandalay Region, Myanmar, in December 2022. The sample was identified as *Citrus medica var. acida* Brandis by the authorized botanist from the Department of Botany, Meiktila University. The collected fresh leaves were washed, and air dried at room temperature for one week, ground into powder, and then stored in an air-tight container.

Preliminary Phytochemical Investigation of the Leaves of *C. medica*

To classify the types of organic constituents present in the sample, preliminary phytochemical tests were carried out according to the appropriate reported methods (Trease and Evans, 1978; Marini–Bettolo *et al*., 1981; M-Tin Wa, 1972; Robinson, 1983; Finar, 1964; Furniss, 1989).

Determination of Elemental Content in Leaf of *C. medica* by EDXRF Method

Some elements present in leaves of *C. medica* powdered samples were semi-quantitatively determined by using EDX-700, Energy Dispersive X-ray Fluorescence Rigaku spectrometer, at the Department of Physics, Meilktila University.

Extraction of Essential Oil from the Fresh Leaf of *C. medica* by Steam Distillation Method

The fresh leaves (500 g) of *C. medica* were cut into small pieces. The essential oil was extracted from the fresh leaves sample with 6 L of distilled water by using the steam distillation method. The essential oil was extracted with *n*-hexane from the distillate. The *n*-hexane layer was dried by adding a small amount of anhydrous sodium sulphate. The dried *n*-hexane layer was then evaporated at 68 °C, and the essential oil was then weighed until it reached a constant weight. Afterward, the essential oil was stored in an air-tight small bottle and placed in the refrigerator for long-term essential oil storage (Hesham, 2016).

To determine the composition of the essential oil and identify the specific compounds responsible for its aroma and potential therapeutic effects, GC-MS analysis of the essential oil was carried out at the Department of Chemistry, University of Mandalay. The National Institute of Standards and Technology (NIST) database was used to analyse the GC-MS spectrum, comparing the unknown and known components. The name, molecular weight, and structure of each component of the test material were identified (Igwe *et al.*, 2015).

Preparation of Various Crude Extracts

The dried powdered sample (50 g) was extracted with 150 mL of petroleum ether (60-80 °C) for 6 h by using a Soxhlet extractor. The filtrate was concentrated by removing the solvent under reduced pressure to give the respective PE crude extract. Similarly, ethyl acetate, 95 % ethanol, and watery extracts were also prepared according to the above procedure. Each extract was dried at normal pressure in a water bath and stored in a refrigerator for screening the antimicrobial and antioxidant activities.

Screening of Some Biological Activities of the Leaf of *C. medica*

(a) Screening of the antimicrobial activity of various crude extracts by agar well diffusion method

The antimicrobial activity of various crude extracts: 95 % ethanol, ethyl acetate, petroleum ether, and watery extracts was studied by the agar well diffusion method (Collins, 1965) at the Department of Chemistry, Loikaw University The microorganisms used were *Bacillus subtilis*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Bacillus pumilus*, *Candida albicans,* and *Escherichia coli* from the Department of Chemistry, Loikaw University.

(b) Screening of the antioxidant activity by DPPH assay

In this experiment, the antioxidant activity of ethanol and watery extracts was studied by a DPPH free radical scavenging assay (Patil, 2009). DPPH powder (2 mg) was thoroughly dissolved in 100 mL of 95 % ethanol. The stock solution (1.6 mg/mL) of crude extract was prepared by dissolving the respective crude extract in 10 mL of 95 % ethanol. The stock solution was two-fold diluted serially with 95 % ethanol to get the sample solution with the different concentrations of 1600 μg /mL, 800 μg/mL, 400 μg/mL, 200 μg /mL, and 100 μg/mL.

Phytochemicals of *Citrus medica var. acida* Brandis (Shauk thakwa) Leaf

The phytochemical tests revealed that alkaloids, α-amino acids, carbohydrates, flavonoids,glycosides, phenolic compounds, reducing sugars, saponins, starch, steroids, tannins, and terpenes were present in the tested sample. Phenolic compounds, flavonoids, and tannins are major contributors to antioxidant activity. Alkaloids, flavonoids, saponins, steroids, tannins, and terpenes play a major role as anti-inflammatory agents. Alkaloids, flavonoids, glycosides, and tannins have antidiabetic activity (Duthie, 2000).

Elemental Analysis of Sample by EDX RF Technique

According to the EDXRF elemental analysis, *C. medica*-leaves contain Ca, Al, S, and K as major elements. Besides Si and P as minor elements, Mn, Fe, Cu, Zn, Sr, and Ba as trace elements were also detected. The advantages of calcium are strong bones and teeth and the regulation of muscle contraction in the human body. Potassium is used in many foods and as a

supplement. Aluminium is also used to treat muscle and joint pain, fatigue and insomnia, symptoms of colds, and flu. The results are reported in Table 1.

Table 1. Relative Abundance of Elemental Content in *C. medica* Leaf by EDXRF

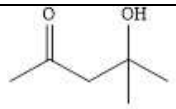
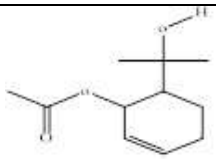
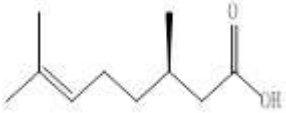
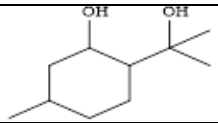
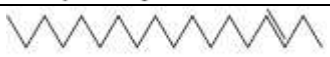
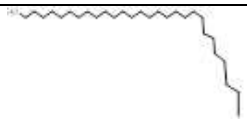
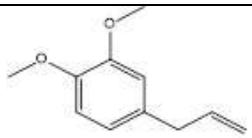
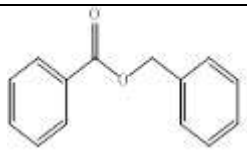
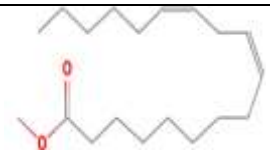
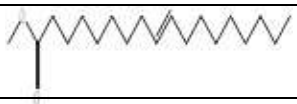
Element	Relative abundance (%)
Ca	38.753
Al	19.813
S	16.953
K	11.392
Si	8.337
P	4.355
Fe	0.102
Cu	0.035
Sr	0.021
Mn	0.019
Zn	0.005
Ba	0.002

Organic Compounds in Essential Oil of the Leaf of *C. medica* by GC-MS

Essential oil from the leaf of *C. medica* was extracted from the fresh leaf samples by steam distillation method (2.30 %). In the GC-MS analysis of the extracted essential oil sample, a total of 13 organic compounds detected are (1) 4-hydroxy-4-methyl-2-pentanone, (2) 3-acetoxy-4-(1-hydroxy-1-methyl ethyl)-1- methyl-cyclohexene, (3) (*R*)-(+)- citronellic acid, (4) 2-(2-hydroxy-2-propyl) -5-methyl cyclohexan-1-ol, (5) (*E*)-3-octadecence, (6) octacosanol, (7) methyl eugenol, (8) benzyl benzoate, (9) *Cis*-9,12-methyl linoleate, (10) methyl (9E)-9-octadecenoate, (11) di-isobutyl phthalate, (12) heptadecane-1-ol and (13) methyl stearate.

Citrus species extracts are well-known sources of bio-functional compounds with health-promoting effects. In particular, essential oils are known for their antibacterial activity due to the high content of terpenes. The thirteen compounds identified by GC-MS analysis of the essential oil of *C. media leaves* matched the NIST library data. These compounds with their respective retention time, together with their structures, molecular weights, and molecular formulae are listed in Table 2.

Table 2. Name of the Compounds, and their Structure, Retention Time, Molecular Weight, and Molecular Formula Detected in the Essential Oil of *Citrus medica* Leaf

No.	Name of Compound	Structure	Retention time (min)	Molecular weight	Molecular formula
1	4-hydroxy- 4-methyl-2-pentanone,		2.972	116	$C_6H_{12}O_2$
2	3-acetoxy-4-(1-hydroxy- 1-methyl ethyl)-1-methyl--cyclohexene		15.197	212	$C_{12}H_{20}O_3$
3	(R)-(+)-citronellic acid		16.319	170	$C_{10}H_{18}O_2$
4	2-(2-hydroxy-2-propyl) - 5-methyl cyclohexan-1- ol		16.543	172	$C_{10}H_{20}O_2$
5	(*E*)-3-octadecence		17.410	252	$C_{18}H_{36}$
6	octacosanol		17.567	410	$C_{28}H_{58}O$
7	methyl eugenol		17.881	178	$C_{11}H_{14}O_2$
8	benzyl benzoate		36.840	212	$C_{14}H_{12}O_2$
9	*Cis*-9,12- methyl linoleate		27.580	294	$C_{19}H_{34}O_2$
10	methyl (9E)-9-dctadecenoate		27.790	296	$C_{19}H_{36}O_2$
11	di-isobutyl phthalate		28.426	278	$C_{16}H_{22}O_4$
12	heptadecane-1-ol		28.512	256	$C_{17}H_{36}O$
13	methyl stearate		28.639	298	$C_{19}H_{38}O_2$

Screening of Antimicrobial Activity of Different Crude Extracts of *C. medica* Leaf

The crude extracts of *C. medica* leaves (petroleum ether, ethyl acetate, 95 % ethanol, and watery) were tested on six species of microorganisms such as *Bacillus subtilis, Salmonella typhi, Pseudomonas aeruginosa*, *Bacillus pumilus, Candida albicans*, and *Escherichia coli* by using the agar well diffusion method. From the experimental results (Table 3), all the crude extracts except the PE extract showed antimicrobial activity against four of the six tested microorganisms. It was found that EtOAc extract (ID: 10-14 mm) and H_2O extract (ID: 10-3 mm) exhibited antimicrobial activity against four tested microorganisms except *C. albicans and E. coli* whereas 95 % EtOH extract exhibited antimicrobial activity against all microorganisms tested (ID: 10-18 mm). In general, since the larger the diameter, the greater is the antimicrobial activity, the polar extracts exhibited higher activity than non-polar extracts. *C. medica* also exhibited more potent antimicrobial activity in the polar extract but was inactive in the non-polar extract.

Table 3. Antimicrobial Activity of Crude Extracts of *C. medica* by Agar Well Diffusion Method

No.	Microorganisms	Inhibition zone diameter (mm)			
		H_2O	EtOH	EtOAc	PE
1	*B. subtilis*	11	16	10	–
2	*S. typhi*	10	13	10	–
3	*P. aeruginosa*	13	18	14	–
4	*B. pumilus*	12	16	11	–
5	*C. albicans*	_	10	_	–
6	*E.coli*	–	10	_	–

Agar well diameter- 8mm;
Inhibition zone diameter = 9-14 mm = low activity; 15-20 mm = medium activity
21 mm above = high activity; No zone of inhibition = (-)

Antioxidant Activity of Some Crude Extracts from the Leaf of *C. medica*

The antioxidant activity of 95 % ethanol and watery extracts of the leaves of *Citrus medica* was studied by the DPPH (1,1-diphenyl-2-pricryhydrazyl) free radical scavenging assay method using UV spectrophotometry. This method is based on the reduction of coloured free radical DPPH in ethanolic solution by different concentrations of each sample. The antioxidant activity was expressed as a 50 % oxidative inhibitory concentration (IC_{50}).

From these experimental results (Table 4 and Figure 4), the tested crude extracts were found to have low antioxidant activity Moreover, the watery extract (IC_{50} = 148.58 µg/mL) showed higher antioxidant activity than the 95 % ethanol extract (IC_{50} = 636.27 µg/mL). However, both the watery extract and the ethanol extract were found to have lower antioxidant activity than standard ascorbic acid (IC_{50} = 4.75 µg/mL) because the lower the IC_{50} value, the higher the antioxidant activity of the sample.

Table 4. Percent Inhibition and IC_{50} Values of Crude Extracts from the Leaf of *C. medica*

Extracts	% Inhibition in different concentrations (µg/mL)					IC_{50} µg/mL
	100	200	400	800	1600	
ethanol	24.93	27.13	40.82	57.08	81.35	636.27
watery	36.79	63.98	72.59	78.11	84.18	148.58
Standard	**1**	**2**	**4**	**8**	**16**	**IC50 µg/mL**
Ascorbic Acid	16.63	30.63	46.11	74.60	81.73	4.75

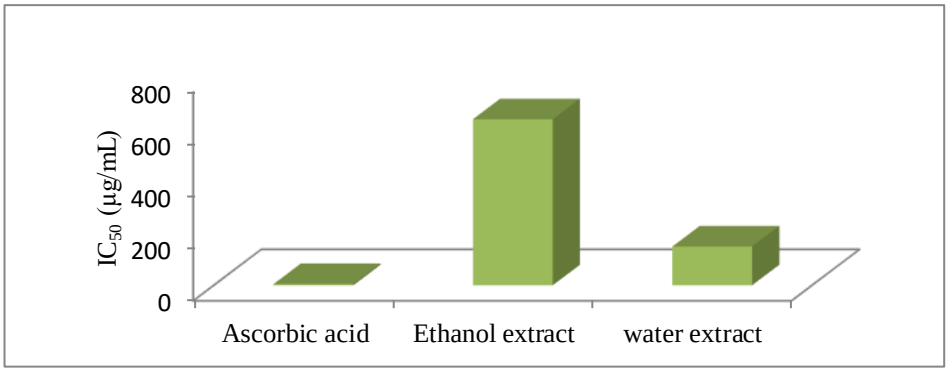


Figure 4. IC_{50} values of watery and ethanol extracts from *C. medica* leaves compared with standard ascorbic acid

Conclusion

From the overall assessment of the present work, the following inferences could be deduced. The phytochemical investigation showed the presence of phytoconstituents which would be beneficial in understanding the pharmacological importance and health risks. From the EDXRF measurement, it can be found that the leaves of *C. medica* contain the main elements: Ca, Al, S, K, and Si. Among them, the content of calcium is the highest (38.753 %). The GC-MS analysis revealed that the essential oil of C. *medica* leaves contained fatty acid esters, monoterpenoids, and unsaturated fatty acids. Among the chemical composition, methyl eugenol is a major constituent in the leaf, and it is a natural constituent of a large number of essential oils of plant origin. Methyl eugenol also has antioxidant activity. According to the antimicrobial activity of the polar and non-polar extracts, it can be deduced that the polar extract of *C. medica* leaves may be effective for the treatment of some diseases. From the screening of the antioxidant activity, the IC_{50} values of ethanol and watery extracts were observed at 636.27 µg/mL and 148.58 µg/mL, respectively. Therefore, the antioxidant capacity of the watery extract was higher than that of the ethanol extract. However, these crude extracts possess lower antioxidant activity than the standard ascorbic acid. Moreover, the *C. medica* leaf exhibited low antimicrobial activity.

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