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PHYSICO-CHEMICAL CHARACTERIZATION OF ESSENTIAL OIL FROM THE PEEL AND LEAF OF DALANGHITA (*Citrus nobilis*)

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Abstract: **Citrus ranks as the fourth most important type of fruit produced in the Philippines after banana, mango, and pineapple. *Citrus nobilis*, which is grown primarily in Guimaras and the Panay islands, is sold mainly for its juice and pulp while the peels and the leaves are left to waste. In order to maximize the value of the plant, essential oils will be extracted from both the peels and leaves of *C. nobilis* via steam distillation. Physicochemical analysis of the dalanghita peel (PEO) and leaf (LEO) essential oils will determine their potential application in the industry. Investigation of their physical properties showed that the density, color, odor, congealing point, and refractive index of both essential oils were nearly identical. GC-MS analysis identified seven compounds in the PEO, and twelve compounds in the LEO. D-limonene, gamma-Terpinene, beta-Linalool, and diethyl phthalate were identified to be present in both essential oils. Antioxidant property of the oils were tested using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay with the LEO showing an IC_{50} = 150ppm while the PEO has an IC_{50} = 493 ppm. Antibacterial properties of the oils were tested using the Kirby Bauer Disc Diffusion method where both oils showed activity against *E. coli* and *S. aureus*.**

Keywords: **citrus leaves, citrus nobilis, citrus oils, dalanghita, dalanghita essential oil, dalaghita oil composition, essential oil, GC-MS analysis of citrus oils, phyisicochemical properties of oil, antioxidant property of oils, antibacterial property of essential oil.**

I. INTRODUCTION

Essential oils are widely used in several consumer goods. They are usually included in the list of ingredients for soaps and detergents, pharmaceuticals, perfumes, confectionary food products, soft drinks, distilled alcoholic beverages, and insecticides. Technically, they are steam volatile, aromatic oils that are primarily composed of terpenes, and their oxygenated derivatives. In plants, they serve as protective compounds and can be extracted by rupturing one or more parts such as bark, flowers, fruits, leaves, rhizomes, roots, seeds, stems, & woods (Baser, *et al*., 2010).

Extraction of essential oils can be done by pressing, or the application of heat. Most plant oils may be extracted using a solvent that would enter into the plant cells and dissolve the oil, causing it to come out. Thus, the oils may be produced via distillation or solvent extraction.

Citrus species have found wide applications, especially in the perfume industry because of their refreshing and hormone-balancing effects (Natsheh *et al*, 2013). The *C. nobilis*, which is widely cultivated in Guimaras and the Panay Islands is usually sold for their pulp and juice but the rinds are put to waste and leaves allowed to wither without any significant use. This study will extract and compare the essential oils from the peel and leaves of *C. nobilis*, as well as analyze some of its physical and chemical properties in order to determine its potential use.

II. BODY OF ARTICLE

Essential oils can be extracted from one or more plant parts such as flowers, peels or rinds, leaves, stems, woods, and seeds. Citrus oils have attracted a lot of researches because of the increase consumption in several industries, especially in perfumery.

Dalanghita (*Citrus nobilis*) is a widely cultivated plant in the Philippines. It is a small tree with leaves that are smooth and oblong, and flowers that are white in color and mostly solitary. Its fruits, which are good sources of Vitamin C, are green in color, which may turn to yellow, greenish yellow, or orange over time. The rinds are known to contain volatile oil that is used in flavouring (Philippine Medicinal Plants, 2013). There is a wide distribution of dalanghita fruits in the Philippines, but the leaves are only left to wither, and the rinds are mostly considered wastes.

Fig 1: Dalanghita fruit

Most of the modern methods employed in the analysis of essential oils rely greatly on chromatographic procedures, which enable both separation and identification of the oils. The main objective in any chromatographic separation is the complete resolution of the samples in a very short period of time. This can be achieved using the most appropriate analytical column and enough chromatographic parameters to limit the enlargement of peaks.

III. METHODOLOGY

Preparation of samples:

Dalanghita leaves and fruit samples were taken from Capiz, Iloilo. The samples were washed, stored at room temperature, and steam-distilled within five (5) days.

Extraction of Essential Oils:

The essential oils from the leaves and peels of *C. nobilis* were extracted via steam distillation. Steam distillation was carried out by passing a steam into a round-bottomed flask containing about 300 grams of the sample for about 120 minutes. The condensate (water and oil) was allowed to separate directly and the oil was collected. The percentage of the oil was calculated and the oil was then stored for future analysis. Figure 5 shows the distillation setup employed in the experiment.

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Fig 2: Steam Distillation Setup employed in the experiment

Physical Properties:

Odor:

The odor of the essential oils was determined by placing a small amount of the oil in a paper towel, and gently waving the paper towel under the nose. The odor was described according to the following common terms used to describe the scent of essential oils (floral, woody, citrus, herbal, spicy, minty, camphor, pine, vanilla-like), and sub categories of each (soft, strong, mellow, sharp, light, tangy, smooth, harsh, bitter, sweet, sour, full, and flat) (Birch Hill Happenings Aromatherapy, 2015).

Color:

The color of the oils was noted based on their physical appearance. A sample of essential oil was placed in a clear bottle and the color was described.

Density:

The density of the essential oils was measured using a 2mL pycnometer available in the Chemistry Department of UPV. The weight of the empty pycnometer was recorded. The pycnometer was filled with the essential oil and weighed again. The experiment was carried in triplicates. The density of the essential oils at 23 ˚C was calculated using the formula

$$\boldsymbol{Density} = \frac{\textbf{mass}}{\textbf{volume}}$$

Congealing point:

About 1 mL of oil was placed in a dry test tube. It was cooled in water at a temperature that is about 4℃ lower than the supposed congealing point of water. For congelation to start, the inner walls of the tube was rubbed up and down with a thermometer until it began to solidify. The temperature was read in 5-minute interval and the congealing point was recorded as the highest temperature observed after it has solidified.

Refractive Index:

The refractive index of the essential oils at 30˚C was measured using a Bausch & Lomb ABBE-3L refractometer available in the Department of Chemistry, UPV.

Chemical Properties:

Fourier Transform Infrared Spectroscopy

The IR spectra of the essential oils were collected using a Thermo Nicolet Avatar 330 FT-IR spectrometer equipped with DTGS-KBr detector. Measurements were obtained at $4cm^{-1}$ between 4000-600 cm^{-1}.

Oil sample was placed on a ZnSe single bounce horizontal attenuated total reflectance (HATR) accessory. The spectra were accumulated by scanning.

Gas Chromatography-Mass Spectrometry

Samples were sent to the Chemistry laboratory of Ateneo de Manila University for GC-MS analysis. It was performed using a Shimadzu GCMS QP2010 Ultra, equipped with a DB-5 column. Helium gas was used as the carrier gas. One microliter (1 µL) of the sample was injected using an autosampler in the split less mode. The column temperature was held at 60˚C for 5 min before ramping to 230˚C at a rate of 10˚C/min. MS analysis was carried out by electron impact ionization scanning from m/z 50 to 450 using a single quadruple mass analyzer.

Antibacterial Property:

The antibacterial activity of the extracted essential oils against *S. aureus and E. coli* was determined via disc diffusion method.

Agar plates were freshly prepared and were seeded with the test inoculums to obtain a lawn culture. A sterile Whatmann filter paper discs (6mm in diameter) with different oil concentrations (20%, 50%, 100% v/v) in DMSO, were placed on the inoculated plates along with the positive and negative controls. Post incubation was the done at 24 hours and plates were read for zone of inhibition around the disc.

Antioxidant Property:

The antioxidant properties of PEO and LEO were determined using DPPH (2,2-diphenyl-picrylhydrazyl) radical scavenging assay.

The assay was carried out by mixing 0.75 methanolic solution of each of the oil with 1.50 mL of 20 ppm methanolic DPPH solution at five final concentrations (100, 250, 350, 500 and 1000 ppm). The mixtures were incubated in the dark for 30 minutes at 25˚C and the absorbance at 517 nm was recorded as (A_{sample}), using the Spectrophotometer available in the Department of Chemistry, UPV. A blank experiment was also carried using the same procedure to a solution without the test material. The absorbance was recorded as (A_{blank}). The free radical scavenging activity of each oil was calculated as percent inhibition following the equation:

$$\%\ Inhibition = (100)\frac{Absorbance\ of\ Blank - Absorbance\ of\ Sample}{Absorbance\ of\ Blank}$$

IV. RESULTS AND DISCUSSION

Steam distillation has been recognized as the least expensive, and the safest way to produce essential oils (Wong, *et al*., 2014). It is considered safe since it only requires water as solvent. And because oils do not mix readily with water, the two can be separated easily. This process was carried out to produce essential oils from the peel and leaves of dalanghita. The essential oils produced were then analyzed and characterized via GCMS.

Physical Properties of PEO and LEO:

As shown in Table I, the essential oils isolated via steam distillation from the peel and leaves of dalanghita were found to be transparent and yellowish in color and yields 4.16mL oil/kg sample, and 3.17 mL oil/kg sample, respectively. These results are comparable to the results of Thavanapong, which showed density of ***C. maxima*** (pomelo) peel to be 0.8433, and refractive index of 1.4685 (Thavanapong, 2006).

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Table I: Physical Properties of PEO and LEO

SAMPLE	COLOR	ODOR CATEGORY	SUB-CATEGORY	DENSITY g/mL	CONGEALING POINT ˚C	REFRACTIVE INDEX
PEO	Transparent	Citrus	Light/fresh	0.90	3.0	1.48
LEO	Yellowish	Citrus	Strong	0.87	3.0	1.48

The values for density, congealing point, and refractive Index of the oils are very close to each other. This is because the components of the oils are very similar, as shown in the GCMS results (Tables V and VI).

Antioxidant Property:

The DPPH-radical scavenging activities of PEO, and LEO are shown in Table I and Fig. 3. DPPH is a stable free radical, which decolorizes in the presence of antioxidants. The decolorization of DPPH when accepting an electron donated by antioxidants can be measured quantitatively from the changes in the absorbance (Saha, *et al*., 2008). IC_{50} was calculated based on the equation of the line for each sample. Lower IC_{50} value indicates higher antioxidant activity.

Table II: DPPH-radical scavenging activity of PEO and LEO

Concentration ppm	Absorbance		Percent Inhibition		IC_{50} (ppm)	
	PEO	LEO	PEO	LEO	PEO	LEO
100	1.20	0.79	15.79	44.56		
250	1.00	0.58	29.82	59.30	492.08	149.89
350	0.86	0.52	39.65	63.51		
500	0.72	0.43	49.47	69.82		
1000	0.38	0.21	73.33	85.26		

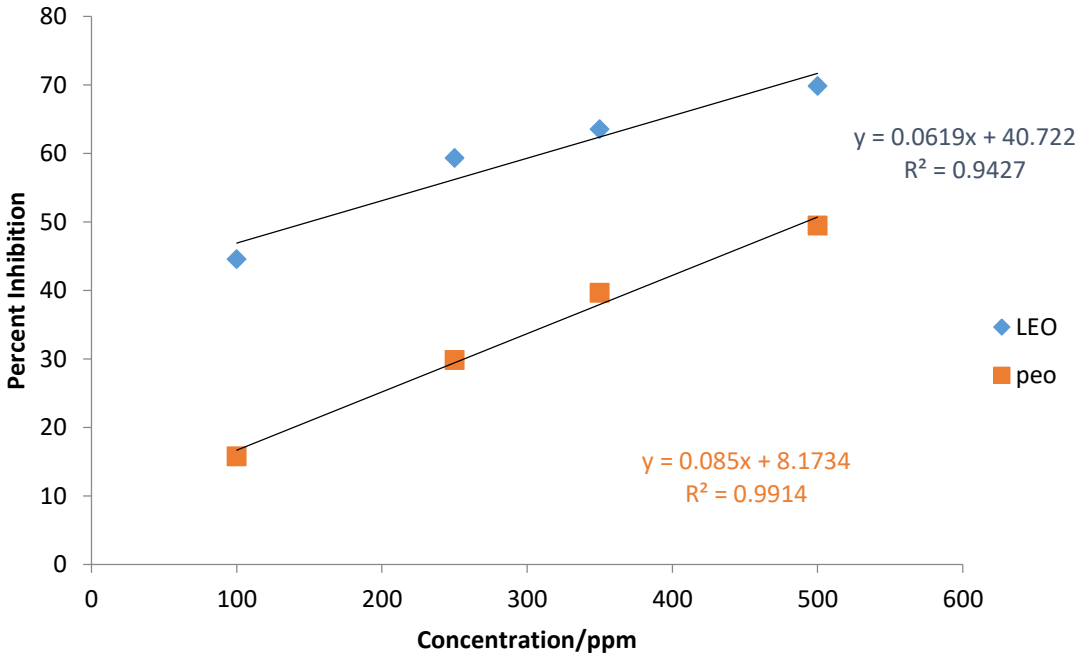

Fig 3: DPPH-radical scavenging activity of PEO and LEO

Based on the graph (Fig. 3), PEO would need around 492 ppm in order to cause 50% inhibition, while LEO would only need around 150 ppm. This means that LEO exhibits higher antioxidant activity than PEO. This can be accounted to the fact that LEO contains more components, with antioxidant properties, than PEO. A well-known antioxidant, ascorbic acid has an IC_{50} value of 55.89 ppm (Saha, *et al*., 2008). This is more or less three (3) times more potent than LEO.

Antimicrobial Property:

The antimicrobial activities of both essential oils are assessed based on the rating system described by Gutierrez *et al*.,2013: (1)<(10) mm zone of inhibition is expressed as inactive; (10) to (13) mm zone of inhibition is expressed as partially active; (14) to (19) mm zone of inhibtiion is active; and >19 mm zone of inhibition is very active (Gutierrez *et al*.,2013). Results from the disc-diffusion assay are summarized in Table III. Both oils showed inhibiting activity against the tested bacteria (E.coli and S. aureus). In particular, both oils showed effectiveness inhibiting ***E.coli*** and ***S.aureus*** at the middle concentration (50 %v/v). Pure oils may be too viscous to scatter around the area that is why their most effective concentration is at 50% v/v.

Table III: Antimicrobial activity of PEO and LEO against *E. coli* and *S. aureus*

Negative Control: DMSO

Positive Control for ***E.coli***: Cetriaxone

Positive Control for ***S.aureus***: Tetracycline

Disk diameter: 6mm

Sample	Concentration %v/v	Zone of Inhibition for *E. coli* (mm)	Zone of Inhibition for *S. aureus* (mm)
PEO	20	1.0	0
	50	1.7	15.7
	100	0	9.0
	N	0	0
	P	20.3	16.3
LEO	20	3.5	0
	50	6.7	23
	100	3.3	20
	N	0	0
	P	17.7	16.6

These results are comparable to the antimicrobial activity of Korean Citrus unshiu peel against ***S. aureus***, in which, 8.2mm was observed for 4 µL/disc of oil (Kang *et al*, 2013). The middle concentration of the PEO showed comparable effectiveness to the positive control (PEO: 15.7mm, positive control: 16.3mm) while the LEO exhibits higher antimicrobial activity than the positive control (LEO: 23mm, positive control: 16.6mm). The PEO is classified as active, while LEO is classified as very active

GCMS and FTIR Analyses:

The components of the oils were determined and verified via GCMS and FTIR. Results from these analyses are shown in Table IV and Figures 4 and 5.

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Fourier Transfor-Infrared:

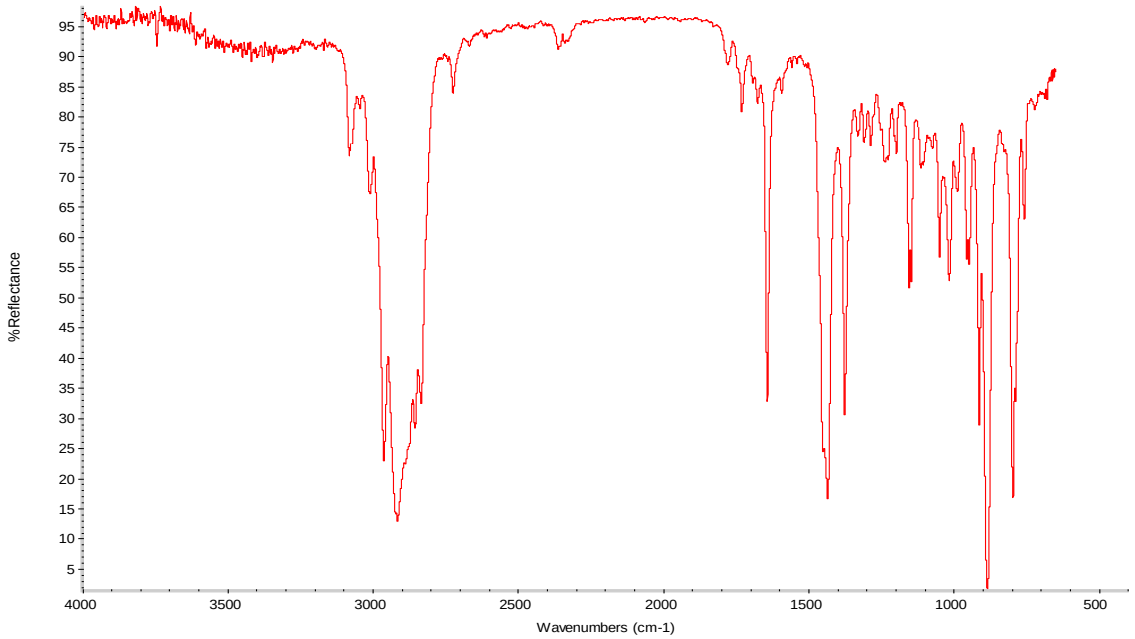

Fig 4: FT-IR Spectra of PEO

The spectra of PEO (Fig. 4) is very similar to the spectra of D-limonene. The signals presented at 2910 cm^{-1} (stretching vibration of C-H bond), 1650 cm^{-1} (stretching vibration of C=C, alkene), 1380 cm^{-1} (bending CH_2, CH_3), and 798 cm^{-1} (oop, tri substituted alkene), are the important peaks that may verify the presence of D-limonene. It is also possible that D-limonene is the major component of this oil, as the FTIR spectra of PEO is very similar to the FT-IR spectra of pure D-limonene.

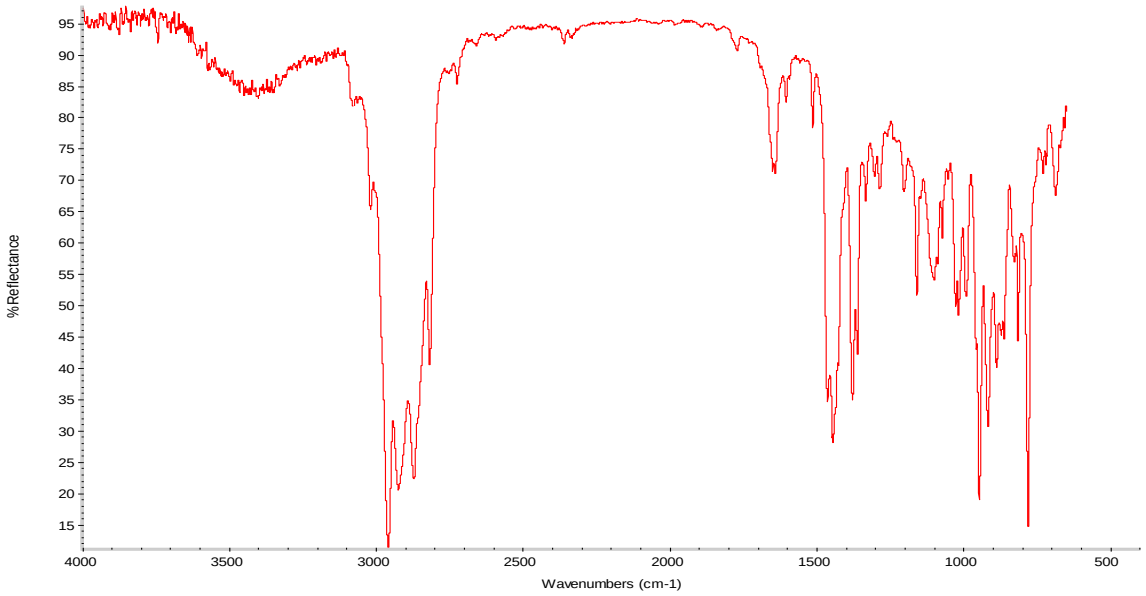

Fig 5: FT-IR Spectra of LEO

In LEO, the signal presented at 2960 cm^{-1} is assigned to the stretching vibration of O-H in carboxylic group, while the characteristic band at 3680 cm^{-1}- 3270 cm^{-1} is assigned to the stretching vibration of O-H in alcohols (Figure 8). Although GCMS results showed that PEO also contained an alcohol terpene, it is only in LEO spectra that the characteristic band is more pronounced. Probably, the relative abundance of the terpene alcohol present in LEO is greater compared to that in PEO.

The FT-IR spectra of PEO and LEO illustrate absorption bands with characteristic frequency, which can be accounted to the different functional groups present in the samples. In both oils, characteristic bands are presented at 3000-2810 cm^{-1} (OH stretch; carboxylic acids), 1640 cm^{-1} (-C=C- stretch; alkenes), and 1450 cm^{-1} (C-H bend; alkanes), 1370 cm^{-1} (C-H rock; alkanes), 1150 cm^{-1} (C-O stretch; alcohols, phenols, esters, and ethers), and 798 cm^{-1} (oop, tri substituted alkene; aromatics).

Table IV: Summary of peaks in PEO and LEO

Sample	Frequency (cm^{-1})	bond	Functional Group
PEO			
	3000-2810	O-H stretch	carboxylic acids
	1640	-C=C- stretch	alkenes
	1450	C-H bend	alkanes
	1370	C-H rock	alkanes
	1150	C-O stretch	alcohols, carboxylic acids, esters, ethers
	885	oop C-H	aromatics
	796	oop C-H	aromatics
LEO			
	3600-3240	O-H stretch	alcohols, phenols
	2990-2810	O-H stretch	carboxylic acids
	1640	-C=C- stretch	alkenes
	1450	C-H bend	alkanes
	1370	C-H rock	alkanes
	1150	C-O stretch	alcohols, carboxylic acids, esters, ethers
	1100	C-O stretch	alcohols, carboxylic acids, esters, ethers
	945	O-H bend	carboxylic acids
	798	oop C-H	aromatics

Gas Chromatography-Mass Spectrometry:

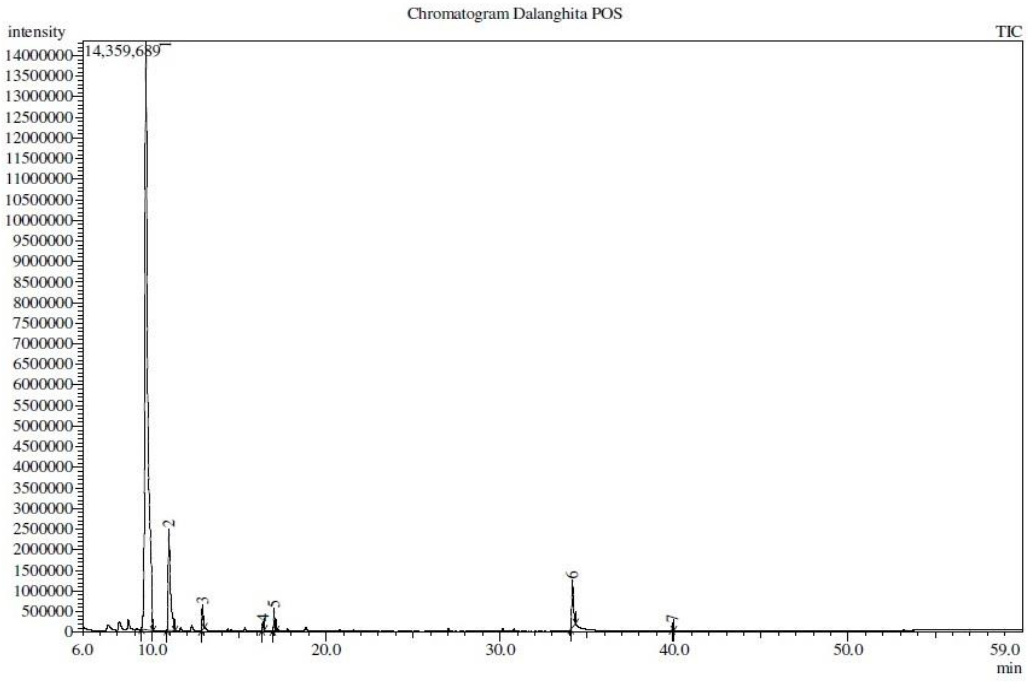

Fig 6: GCMS Chromatogram of PEO

GC-MS results for the qualitative analysis of PEO showed seven (7) peaks, which correspond to seven (7) different compounds. The list of probable compounds in the PEO is summarized in Table V.

Table V: Summary of the identified compounds for PEO

Peak No.	Retention time	Compound
1	9.652	D-Limonene
2	10.971	gamma-Terpinene
3	12.896	beta-Linalool
4	16.367	Terpinen-4-ol
5	17.009	L-alpha-Terpineol
6	34.164	Diethyl Phthalate
7	39.921	α-sinensal

GCMS results of PEO are comparable to the results obtained by Thavanapong, (2006), Chong, *et al*., (2012) and Kang *et al*., (2013), who studied the GCMS components of ***C. maxima*** (pomelo), ***C. microcarpa*** (calamansi) peel, and Korean citrus unshiu peel, respectively. The components that are present among the four citrus species (dalanghita, pomelo, calamansi, and Korean citrus) are listed in Table VI

Table VI: Common compounds found in Dalanghita, Calamansi, Pomelo, and Korean Citrus Peels

	Dalanghita	**Pomelo** (Thavanapong, 2006)	**Calamansi** (Chong *et al*, 2012)	**Korean Citrus** (Kang *et al*., 2013)
D-Limonene	✓	✓	✓	✓
gamma-Terpinene	✓			✓
beta-Linalool	✓	✓	✓	
Terpinen-4-ol	✓			
L-alpha-Terpineol	✓	✓	✓	
Diethyl Phthalate	✓			✓
α-sinensal	✓			

Table VI showed the similarity of components among the citrus species. It can be noted that among the four (4) citrus species, the most common compound present is limonene, a monocyclic terpene that is present in most citrus oils. This compound, along with gamma-terpinene, adds up to the lemon-like odor of citrus oils (Sun, 2007). It can also be noted that among the three (3) citrus species (Dalanghita, Pomelo, and Calamansi), the two (2) naturally-occurring terpene alcohols (β-linalool, and L-alpha-terpineol) are detected. These terpene alcohols contributed different odor to the overall aroma of the oil. Beta-linalool is known to emit a floral fragrance while L-alpha-terpineol emits an odor that is very similar to lilac (Yao, *et al*.,2005). Another terpene alcohol, Terpinen-4-ol was detected in the PEO. This alcohol is recognized to have good antifungal and antibacterial properties. This compound is also thought to be the reason why the "common juniper" wood resists rotting (Hammer, *et al*., 2012). The peel of Dalanghita also contained an aldehyde group (α-sinensal), which is known to contribute not only in the odor of a citrus fruit but also to its taste (Buchi, *et al*., 1974). Diethyl Phthalate, a compound commonly used as carrier for fragrance materials and cosmetics (Api, 2001), is present in both Dalanghita and Korean Citrus peels.

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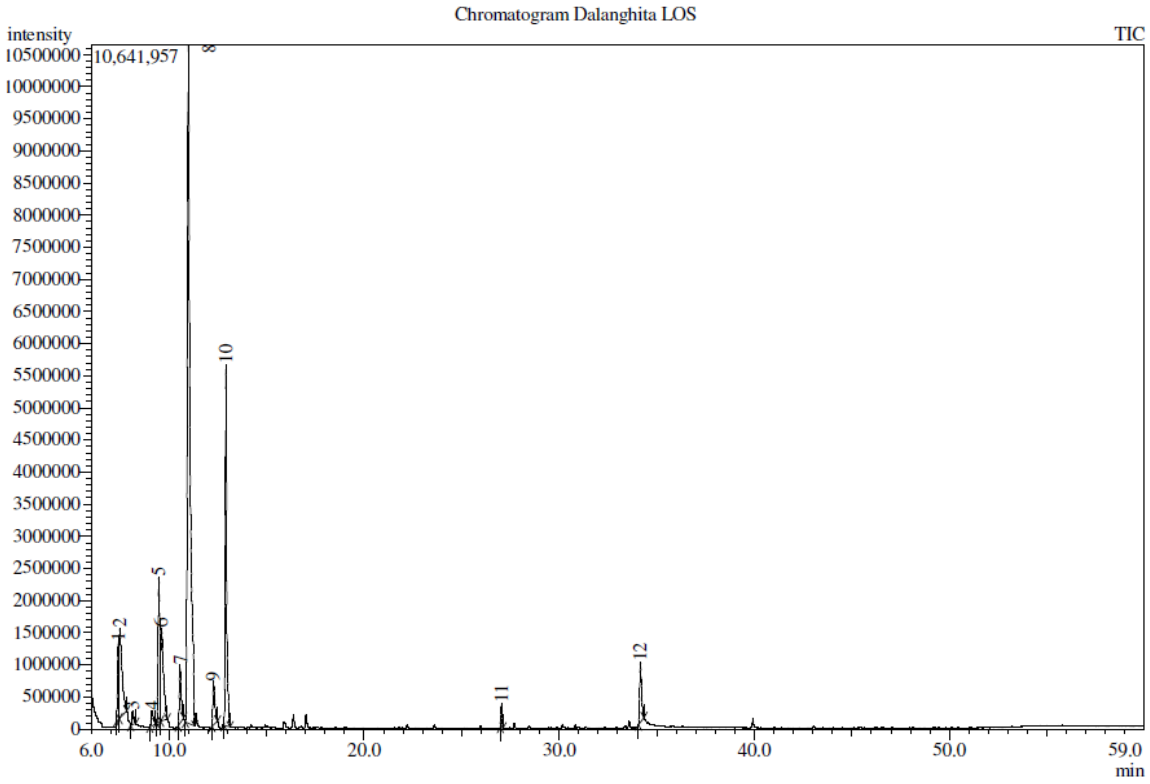

Fig 7: GCMS Chromatogram of LEO

GC-MS results for the qualitative analysis of LEO showed seven (12) peaks, which correspond to seven (12) different compounds. The list of probable compounds in the LEO is summarized in Table VII.

Table VII: Summary of the identified compounds for LEO

Peak No.	Retention time	Compound
1	7.400	beta-Phellandrene
2	7.478	beta-Pinene
3	8.128	beta-Myrcene
4	9.126	alpha-terpinene
5	9.479	m-Cymene
6	9.620	D-limonene
7	10.563	beta-ocimene
8	10.989	gamma-Terpinene
9	12.280	(+)-4-Carene
10	12.921	beta-Linalool
11	27.048	Caryophyllene
12	34.166	Diethyl Phthalate

The GCMS results of LEO showed similarity in the results obtained by Thavanapong, (2006), Chong, *et al*., (2012) and Kang *et al*., (2013). In fact, there is greater similarity in results from the components of the leaf of Dalanghita to the peels of Calamansi, Pomelo, and Korean Citrus. This comparison is summarized in Table VIII.

It can also be noted that the reason why LEO exhibit greater antimicrobial properties over PEO is that LEO contains more compounds that are known to have antimicrobial properties (β-pinene, gamma-terpinene, β-phellandrene, β-linalool, caryophyllene, β-myrcene) (da Silve, *et al*., 2012).

Table VIII: Common compounds found in Dalanghita leaf, Calamansi peel, Pomelo peel, and Korean Citrus peel.

	Dalanghita	**Pomelo** (Thavanapong, 2006)	**Calamansi** (Chong *et al*, 2012)	**Korean Citrus** (Kang *et al*., 2013)
beta-Phellandrene	✓		✓	
beta-Pinene	✓	✓	✓	
beta-Myrcene	✓	✓	✓	✓
alpha-terpinene	✓			
m-Cymene	✓		✓	
D-limonene	✓	✓	✓	✓
beta-ocimene	✓	✓	✓	
gamma-Terpinene	✓			✓
(+)-4-Carene	✓		✓	
beta-Linalool	✓	✓	✓	
Caryophyllene	✓	✓	✓	
Diethyl Phthalate	✓			✓

Table VIII showed the similarity of components between LEO and the peels of pomelo, calamansi, and Korean citrus. It can be noted that among the four (4) citrus species, the common compounds present are β-myrcene, β-ocimene, and D-limonene. These compounds are hydrocarbons, which are the dominant compounds present in citrus oils. Other hydrocarbons present include β-phellandrene, β-pinene, m-cymene, carene, and caryophyllene. All of these add to the flavor and scent of the oils but the most unique component among them is caryophyllene. This compound is considered an attractive candidate in biotechnology because of its unique structure that includes a cyclobutane ring. Cyclobutanes have been utilized now in medicine as chemotherapy drugs such as Carboplatin. On top of this, caryophyllene is considered a very unique compound since it is both terpene and a "dietary cannabinoid", a food-stuff which acts as cannabinoid and can bind with the CB2 receptors (The LEAF online, 2015).

In general, the LEO, showed greater resemblance in components to the peel of calamansi. Aside from the hydrocarbon compounds that are present in both samples (dalanghita leaf and calamansi peel), both oils also contain linalool, a naturally-occurring terpene alcohol, which finds wide application in the perfume industry due to its floral scent (Kang *et al*., 2013).

V. CONCLUSION

In this study, the physical properties of oils, its antioxidant activity, antibacterial properties, as well as the probable compounds present in PEO and LEO were identified. GCMS analyses of the PEO and the LEO showed seven compounds in the PEO, and twelve compounds in the LEO. D-limonene, gamma-Terpinene, beta-Linalool, and Diethyl Phthalate were identified in both essential oils. These compounds were branded as common ingredients utilized in perfumery and cosmetics.

Results from antioxidant assay showed that the LEO exhibited more antioxidant activity than the PEO. This is most likely due to the LEO having more compounds that exhibit antioxidant activity. The antibacterial assay of both oils showed good inhibiting capacity against ***E. coli*** and ***S. aureus***.

Analysis of the FT-IR spectra showed that both the PEO and the LEO contain functional groups that are associated with carboxylic acids, (3000-2810 cm^{-1}), alcohols (1150 cm^{-1}), alkenes, (1640 cm^{-1}), and alkanes (1450 cm^{-1}). In the case of the PEO, the spectrum is very similar to the spectrum of D-limonene. This could mean that D-limonene is the major component of the PEO (NIST library). In the case of the LEO, the spectrum showed a more pronounced band for alcohol, which is most likely due to the β-linalool component of the sample.

Based on the results and observations gathered from the experiment, the following recommendations are offered:

a) analyze the components of the samples every day for seven days via GC-MS and FT-IR to determine if the composition of the oils would vary with time; b) use several different concentrations in doing the antioxidant assay to get better and more reliable results; c) use different types of extraction methods such as cold pressing and the use of supercritical CO_2; and d) use other parts of the dalanghita plant as samples such as its roots and seeds.

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