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Mining the Indigenous Fruit Trees of Mindanao for Essentials Oils with Antibacterial Activity

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ABSTRACT

Indigenous fruit trees are underutilized local resources but of great interest as potential sources of new chemical entities for drug discovery. In this work, the antimicrobial potential of essential oils (EOs) extracted from indigenous fruit trees of Mindanao was evaluated. Thirty-four (34) fruit trees were screened for the presence of EOs using the Clevenger apparatus for hydrodistillation. EOs were subjected to disk diffusion assay for antibacterial evaluation. The result showed that *Lansium domesticum* (lanzones) leaves, *L. domesticum* (lanzones) pericarp, *Psidium guajava* (bayabas) leaves, and *Citrus maxima* (pomelo) pericarp yielded a significant amount of EOs. The said EOs were shown to inhibit the growth of *B. subtilis* and *E. coli*. However, only EOs from *P. guajava* leaves inhibited the growth of three other bacteria namely *S. aureus*, *S. enteretica* and *P. pseudomonas*. The EOs from these plants may further be investigated as potential drug candidates against microbial drug resistance. Moreover, these EOs could be combined for possible synergistic action to maximize their antimicrobial potential.

Keywords: indigenous fruit trees, essential oil, antibacterial, hydrodistillation

INTRODUCTION

The Philippines, which represents 5% of the world's flora, is home to 152 indigenous fruit species that have various ecological and economic importance for food and nutrition, and are a potential source of new chemical entities for drug discovery (Magcale-Macandog et al., 2005). Several of these indigenous fruit trees are found in Mindanao such as nangka (*Artocarpus heterophyllus*), santol (*Sandoricum koetjape*), durian (*Durio zibethinus*), and marang (*Litsea perrottetti*), all of which are indigenous and endemic, with some that are locally found but are introduced from other tropical countries (Miranda et al., 2018; Alipon et al al., 2022).

Aside from various ecological and potential economic importance, these plants are sources of phytochemicals that have profound positive health benefits. They are potential sources of bioactive compounds in fighting several diseases such as cancer, diabetes and microbial disease-causing infections which are one of the prominent causes of health problems, physical disabilities, and mortalities worldwide (Adebayo et al., 2016). These phytochemicals may be obtained in concentrated amounts by extracting the essential oils (EOs) from fruit trees. Such effort not only promotes the utilization of local resources but also encourages the conservation and reproduction of these fruit trees before becoming endangered.

EOs, volatile or ethereal oils, are aromatic oily liquids obtained from plant material like flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots (Guenther, 1948). They are the highly concentrated version of the natural oils in plants and can be obtained by expression, fermentation, enfleurage, extraction, and most commonly by distillation through steam or water (Van de Braak and Leijten, 1999). Technically they are not oils because they contain no fatty acids. But they contain highly concentrated plant compounds which make plants more resistant to disease and insect invasion. This protection in plants also has a wide range of uses for humans. Essential oils have been long recognized for their bactericidal, virucidal, fungicidal, antiparasitic, insecticidal, medicinal and cosmetic applications (Bassole and Juliani, 2012). There are approximately 3000 EOs known, about 300 of which are commercially important (Van de Braak and Leijten, 1999) finding their way into the pharmaceutical, sanitary, cosmetic, agricultural and food industries. The benefits of EOs and the recent enhancement of green consumerism have renewed the scientific community's interest in providing a novel insight into the perspective use of essential oil volatile constituents to combat microbial infections and expands on the current state of knowledge on the potential efficacy of essential oils. Thus, this paper was carried out on the extraction of EOs from leaves and pericarps of indigenous fruit trees in Mindanao for screening on its antibacterial efficacy against six health and food-borne pathogens.

METHODOLOGY

Collection and preparation of Plant samples

Indigenous fruit trees were collected around and nearby places in Bukidnon based on accessibility,

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availability, and rarity. These are: *Annona muricata* L. (guyabano), *Annona squamosa* Linn. (atis), *Artocarpus odoratissimus* Blanco (marang), *Averrhoa bilimbi* L. (kamias), *Averrhoa carambola* Linn. (balimbing), *Carica papaya* L. (papaya), *Chrysophyllum caimito* L. (Caimito), *Anacardium occidentale* L. (Kasoi), *Citrus maxima* Merr. (Pomelo), *Citrus sinensis* L. (Dalandan), *Lansium domesticum* Correa (Lanzones), *Manilkara zapota* (L.) P. Royen (Chico), *Muntingia calabura* L. (Aratiles), *Nephelium lappaceum* L. (Rambutan), *Psidium guajava* L. (Bayabas), *Sandoricum koetjape* (Burm.f.) Merr. (Santol), *Syzygium cumini* (L.) Skeels. (Duhat), *Syzygium samarangense* (Blume) Merr.& L. M. Perry (Makopa), *Tamarindus indica* Linn. (Sampalok), *Persia americana* Mill. (Avocado), *Garcinia mangostana* L. (Mangosteen), *Syzygium aqueum* (Burm.f.) Alston (Tambis), *Artocarpus heterophyllus* Lam. (Nangka), *Passiflora edulis* Sims (Passion fruit), *Theobroma cacao* L. (Cacao) and *Durio zibethinus* L. (Durian). The pericarp of the fruit and the leaves were manually chopped, air-dried at room temperature, and ground.

Extraction of Essential Oils

Dried pericarp and leaves were subjected to oil extraction using the Clevenger-type steam distillation apparatus. A varying weight of both the pericarp and leaves per species were placed in the still. The boiling flask was filled with two (2) liters of distilled water and allowed to boil. Boiling was continued for six (6) hours to allow the formation of steam that heated up the plant samples inside the still. This caused the release of essential oil from the plant tissues. The EOs were evaporated and made to travel through a tube into the condensation chamber. Here, the essential oil vapors condensed with the steam and were collected in the receiving funnel forming a filmy layer. The oil produced was measured following the formula below:

$$\text{Yield of extraction} = \frac{\text{The volume of oil extract}}{\text{Weight of fresh material}}$$

For each type of essential oil, five concentrations were prepared: pure, 1:1 (50 µl essential oil + 50 µl diluent), 1:5 (20 µl essential oil + 80 µl diluent), 1:10 (10 µl essential oil + 90 µl diluent), and 1:20 (5 µl essential oil + 95 µl diluent). The diluent was prepared by adding 100 µl of 10% aqueous DMSO and 100 µl of 10% aqueous Polysorbate 20 to 800 µl distilled water.

Test Organisms

Broth culture of *Bacillus subtilis*, *Escherichi coli, Staphylococcus aureus, Salmonella enterica, Pseudomonas aeruginosa*, and *Xanthomonas campestris* were used. To ensure purity, these test organisms were re-streaked several times in NA plates. Before assay, each inoculum was standardized following these steps. An isolated colony from a 24–48-hour culture of the test organisms was inoculated in 10ml NB tubes and incubated at room temperature for 8-10 hours with continuous shaking. After incubation, the optical density of the cultured organisms was checked and adjusted to 0.08 to 0.1 at 625 nm in the microplate reader. This is equivalent to 0.5 McFarland standard. This was done by placing 100µl of the adjusted pathogens in 3 wells and 100µl of media (NB) in another 3 wells in a 96-well plate. The optical density in wells with pathogen, media and blank was read at 625 nm in a microplate reader. Blank subtraction was not done if the optical density of the empty wells was nearly close to the media wells. If the reading is beyond 0.1, the pathogen is diluted further using the formula:

$$\text{Volume of pathogen} = \frac{\text{(Desired reading) (Desired volume)}}{\text{Actual reading}}$$

Disk Diffusion Microbial Assay

Mueller Hinton Agar (MHA) was prepared according to the manufacturer's instruction. MHA plates were inoculated with the standardized test organism. Sterile filter paper disks (6 mm) were impregnated with 10 ul of the corresponding treatments as follows: five concentrations of the EOs, 2% DMSO (diluent control), water (negative control) and 15 µg Cefazolin (Phizolin) (positive control). The disks were placed on the surface of the inoculated MHA plates. All disks were distributed evenly so that they were not closer than 24 mm to the center or too close to the edge of the plate. One disk was impregnated with 10 µl 2% DMSO (negative control), another with 10 ul water (negative control) and another with 15 µg Cefazolin (Phizolin) (positive control). For the three (3) remaining disks, 10µl of each concentration (15 mg/ml, 30 mg/ml, and 50 mg/ml) was pipetted. After impregnating all the disks, the plates were upright for 5 minutes. The plates were then incubated at 37°C for 18-24 hours. This assay was done in at least (3) replicates. After incubation, the diameters of the zone of complete inhibition (ZOI) including the disk were measured in millimeters using a ruler. The ZOI was considered if the unaided eye sees no obvious visible growth surrounding the disk. The faint growth of tiny colonies at the edge of the zone of inhibition detected by the magnifying lens was ignored. The test was repeated when the zone of inhibition was shaped irregularly and only circled ZOI was considered. The test was repeated if colonies are present in the zone of inhibition.

Statistical Analysis

The results obtained were statistically analyzed using a One-way Analysis of Variance (ANOVA) at 5% level of probability ($P<0.05$). Significant differences between means were then analyzed using Tukey's post hoc test. Data were computed as mean ± SD of the three replicates.

RESULTS AND DISCUSSION

Description and Yield of Crude Essential Oil

There are thirty-four (34) fruit trees screened for the presence of essential oils. Of these, only the leaves of *L. domesticum* (lanzones) and *P. guajava* (bayabas) and

pericarp of *L. domesticum* (lanzones) and *Citrus maxima* (pomelo) yielded a significant amount of EOs.

Psidium guajava obtained the highest yield with 0.66 µl per gram of dried leaves followed by *L. domesticum* (0.62 ul) per gram of dried leaves and 2.52 ul for dried pericarp while least in *Citrus maxima* (pomelo) with 0.40 µl per gram of dried pericarp. The oils of *P. guajava* and *L. domesticum* are light yellow and produce a strong yet non-irritating aroma. The oil observed in C. maxima was cloudy yellow and produced a strong, non-irritating aroma. In *P. guajava* and *L. domesticum*, the first drop of oil was observed at 25 minutes after boiling. In contrast, in *C. maxima*, the first drop of oil was observed after 21 minutes.

This study employed the hydrodistillation method of extracting essential oil using the Clevenger apparatus. The plant material was soaked for some time in the water, after which the mixture was heated, and volatile materials were carried away in the steam, condensed, and separated. The Clevenger apparatus is the standard method for extracting EOs for quality control. However, the extraction yield using steam distillation is relatively more minor since a part of the essential oil becomes dissolved in the distillation water, making it an inefficient method for the complete extraction of essential oil from aromatic crops (Rao, et. al., 2004). Microwave and microwave-ultrasonic extractions methods were much more efficient than ultrasonic methods as well as conventional steam distillation extraction methods in terms of both the extraction time and obtained yields (Jaradat et.al., 2016). Moreover, the extraction yield of essential oil depends on different factors, such as the regulation of biosynthetic genes, climatic variation, and expression of metabolites (Bora et al., 2020). Tran et.al. (2019), using hydrodistillation to extract the essential oil from Vietnamese powdered mandarin (C. reticulata Blanco), reported that the size of the fruit peel samples, the water-to-peel ratio, the temperature extraction, and the time of extraction could affect the yield of essential oils extracted through hydrodistillation. Also, essential oil yield from natural sources depend on climate, geography, source, degree of freshness, period of harvest, and extraction method, among other factors (Lawrence,1986).

An essential oil's unique aroma and other bioactive properties depend on its chemical constituents. The chemical components of plant essential oil differ between species. This chemical difference is directly related to antimicrobial activities against various pathogenic microorganisms (Pichersky, 2006). The leaves of *Psidium ssp*. contains iso-caryophyllene (33.53%), veridiflorene (13.00%), farnesene (11.65%), dl-limonene (9.84%), δ-cadinene (1.75%), α-copaene (2.80%), α-humulene (3.74%), aromadendene (1.70%) and τ-cadinol (0.08%). The volatile oil, "pompelmus" oil, from leaves of *C. maxima* contain d-pinene (0.5-1.5%), d-limonene (90-92%), linalool (1-2%), citrate (3-5%), geraniol (1.2%), linalyl and geranylacetate, citral (25%), free alkaloid (8.61%) and ester (4.38%). The dried peel of *L. domesticum* pericarp produces a dark, semiliquid oleoresin composed of 0.17% volatile oil and 22% resin (Heyne, 1987).

Disk Diffusion Anti-bacterial Assay

Before testing, the EOs were dissolved in DMSO with polysorbate 20. Polysorbate 20 is an emulsifier choice of aroma therapists and cosmetic formulators for mixing EOs in water. At the same time, polysorbate 80 is used to mix heavier oils, such as sunflower oil, olive oil, and argan oil. Without these emulsifiers, the oils would not mix uniformly throughout the product, but would separate or not be homogenous. DMSO with polysorbate 20 acts as a surfactant for the bacterial cells to absorb the oils because of its hydrophobicity and non-polar characteristics. However, DMSO concentration above 10% becomes toxic to cells (Karande et al., 2006).

The antibacterial activity of the essential oils extracted from indigenous fruits revealed that the EO from guava leaves exhibited significant inhibition against *B. subtilis*, *E. coli*, *S. aureus*, *S. enterica*, and *P. aeruginosa*. In *B. subtilis*, inhibition was observed even at 1:5 dilution. For *E. coli*, *S. aureus*, and *P. aeruginosa*, inhibition was observed only when the oil was undiluted (Table 1).

EOs from C. maxima pericarp exhibited inhibition against B. subtilis and E. coli up to 1:5 and 1:10 dilution, respectively (Table 2).

EOs extracted from the leaves and pericarp of lanzones also showed inhibition against *B. subtilis* and *E. coli* at various concentrations but did not inhibit the growth of *S. aureus*, *S. enterica*, *P. aeruginosa* and *X. campestris* (Table 3 and 4). In this study, not all bacteria

Table 1. Mean zone of inhibition (mm) of EO from *P. guajava leaves*.

Treatment	B. subtilis	E.coli	S. aureus	S. enterica	P. aeruginosa	X. campestris
A (Pure)	13.9b	12.5b	12.7b	0.00a	12.7b	0.00a
B (1:1)	12.5b	0.00a	0.00a	12.9b	0.00a	0.00a
C (1:5)	12.7b	0.00a	0.00a	12.9b	0.00a	0.00a
D (1:10)	0.00a	0.00a	0.00a	12.7b	0.00a	0.00a
E (1:20)	0.00a	0.00a	0.00a	0.00a	0.00a	0.00a
F (Water)	0.00a	0.00a	0.00a	0.00a	0.00a	0.00a
G (Diluent)	0.00a	0.00a	0.00a	0.00a	0.00a	0.00a
H (Cefazoline)	33.3c	31.3c	20.6c	18.3c	31.3c	21.6b

Column means of the same letters are not significantly different from each other (P>0.05 ANOVA).

Table 2. Mean zone of inhibition (mm) of EO from *C. maxima pericarp*.

Treatment	B. subtilis	E.coli	S. aureus	S. enterica	P. aeruginosa	X. campestris
A (Pure)	13.7[b]	14.7[c]	0.00a	0.00a	0.00a	0.00a
B (1:1)	13.5[b]	12.8[c]	0.00a	0.00a	0.00a	0.00a
C (1:5)	13.6b	12.8[b]	0.00a	0.00a	0.00a	0.00a
D (1:10)	0.00[a]	12.8[b]	0.00a	0.00a	0.00a	0.00a
E (1:20)	0.00[a]	0.00[a]	0.00a	0.00a	0.00a	0.00a
F (Water)	0.00[a]	0.00[a]	0.00a	0.00a	0.00a	0.00a
G (Diluent)	0.00[a]	0.00[a]	0.00a	0.00a	0.00a	0.00a
H (Cefazoline)	31.3[c]	34.0[d]	33.6b	24.3b	31.3b	23.0b

Column means of the same letters are not significantly different from each other (P>0.05 ANOVA).

Table 3. Mean zone of inhibition (mm) of EO from *L. domesticum leaves*.

Treatment	B. subtilis	E.coli	S. aureus	S. enterica	P. aeruginosa	X. campestris
A (Pure)	13.8b	12.9b	0.00a	0.00a	0.00a	0.00a
B (1:1)	13.7b	12.7b	0.00a	0.00a	0.00a	0.00a
C (1:5)	13.7b	12.8b	0.00a	0.00a	0.00a	0.00a
D (1:10)	12.9b	12.7b	0.00a	0.00a	0.00a	0.00a
E (1:20)	8.2a	0.00a	0.00a	0.00a	0.00a	0.00a
F (Water)	0.00a	0.00a	0.00a	0.00a	0.00a	0.00a
G (Diluent)	0.00a	0.00a	0.00a	0.00a	0.00a	0.00a
H (Cefazoline)	31.3c	34.0c	33.6b	34.3b	31.3b	23.0b

Column means of the same letters are not significantly different from each other (P>0.05 ANOVA).

Table 4. Mean zone of inhibition (mm) of EO from L. domesticum pericarp.

Treatment	B. subtilis	E.coli	S. aureus	S. enterica	P. aeruginosa	X. campestris
A (Pure)	13.9a	14.9c	0.00a	0.00a	12.9b	12.9a
B (1:1)	13.8a	14.7c	0.00a	0.00a	0.00a	12.8b
C (1:5)	13.4a	14.8b	0.00a	0.00a	0.00a	12.8c
D (1:10)	13.5a	12.7b	0.00a	0.00a	0.00a	0.00a
E (1:20)	0.00a	0.00a	0.00a	0.00a	0.00a	0.00a
F (Water)	0.00a	0.00a	0.00a	0.00a	0.00a	0.00a
G (Diluent)	0.00a	0.00a	0.00a	0.00a	0.00a	0.00a
H (Cefazoline)	31.3c	31.3b	34.6b	33.6b	33.0c	30.6b

Column means of the same letters are not significantly different from each other (P>0.05 ANOVA).

tested were susceptible to the four EOs. The EOs extracted from *P. guajava* leaves inhibited only *B. subtilis*, *E. coli*, *S. aureus*. *S. enterica*, and *P. aeruginosa* and not X. campestris. Moreover, the EOs of *C. maxima pericarp* and *L. domesticum* leaves, and pericarp inhibited only *B. subtilis* and *E. coli*. The mode of action of EOs and/or their components is dependent on their chemical composition and may have a single target or multiple targets of their activity (Nazzaro, 2013). *P. guajava*., popularly known as guava, is a small tree belonging to the myrtle family (Myrtaceae). It is one of the plants in folklore medicine used to treat several diseases. In the Philippines, the astringent unripe fruit, the leaves, the cortex of the bark and the roots are used for washing ulcers and wounds, as an astringent, vulnerary, and for diarrhea. P. guajava leaves have been reported to contain fixed oil and volatile oil which is dominated by α-pinene, 1,8-cineole, and *β*-bisabolol (Da Silva et al., 2003) as well as 57 components including 27 terpenes, 14 alcohols and 4-esters (Joseph and Priya, 2011). These compounds may be responsible for the susceptibility of the bacteria except for *X. campestris*. Quercetin is said to be the primary antibacterial compound in guava leaves (Rattanachaikunsopon and Phumkhachorn, 2010). This compound has been shown to inhibit both Gram-positive and Gram-negative bacteria such as *S. aureus*, *S. mutans*, *P. aeruginosa*, *S. enteritidis*, *B. cereus*, *P. vulgaris*, S. *dysenteriae* and *E. coli* (Cowen, 1999). Barret et al. (1994) evaluated a range of antibiotics against Xanthomonas campestris pelargonii and showed that many antibiotics showed reduced or no activity on the latter.

Pomelo is a perennial plant belonging to the

family Rutaceae, scientifically known as *Citrus maxima* (Burm.) Merr. and locally known as lukban or suha (Libunao et al., 2013). This plant has been reported to treat fever, cough, pharyngitis, skin diseases and sore throat (Othman et al., 2016). Li et al. (2019) reported the antibacterial activity of finger citron essential oil (FCEO, *Citrus medica L. var. sarcodactylis*) and its mechanism against food-borne bacteria such as *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis* and *Micrococcus luteus*. They identified 28 components in the oil through gas chromatography-mass spectrometry, in which limonene (45.36%), γ-terpinene (21.23%), and dodecanoic acid (7.52%) were the three main components present in the plant. Limonene has been shown to suppress the development of both Gram-positive and Gram-negative bacteria, but with greater activity on the former than the latter, indicating that it has a broad spectrum of action which includes destroying cell walls and membranes, causing protein and bacterial nucleic acid leakage and inhibiting ATPase activity (Raspo et al., 2020). *Lansium domesticum* Corr. is a fruit tree of the Meliaceae family. It is a popular folk remedy for sore eyes and was reported for treating malaria, dysentery, and even as a mosquito repellant (Orwa et al., 2009). The aqueous extract of Lanzones seed inhibited the growth of *E. coli* and *S. aureus* at high concentrations (Alfonso et al., 2017). Shan et al. (2007) showed that as the concentrations of the fruit extracts increase, the antibacterial activities also increase. This inhibition could be due to components that attack the bacterial cell wall and cell membrane, thereby causing leakage and coagulation of cytoplasmic components. The fresh peel contains 0.2% light-yellow volatile oil, a brown resin and reducing acids while the dried peel has a dark, semi-liquid oleoresin composed of 0.17% volatile oil and 22% resin. The fruit peel also contains three new onoceranoid triterpenes, lansionic acid, 3 β -hydroxyonocera-8,14-dien-21-one, and 21α-hydroxyonocera-8 and 14-dien-3-one. The seed includes five tetranorterpenoid, domesticulide, and 11 known triterpenoids. The seed extract is rich in limonoids which includes andirobin derivates, methyl angolensates, mexicanolides an azadiradione, onoceranoids and dukunolides (Tilaar et al., 2008).

The susceptibility of a particular species to essential oil is hard to predict. Identifying the mode of action of EOs requires much study of the raw material until the singular components are identified, and the mode of action should also be studied in multiple strains and species of microorganisms (Nazzaro et al., 2013).

In this work, the extracted EOs from indigenous fruit trees such as *P. guajava*, *C. maxima* and *L. domesticum* proved to be potential sources of antimicrobials for developing health and cosmetic products. Though the inhibition exhibited by the EOs is lower than that of cefazolin, the antibiotic control. This is not surprising since the antibiotic control is of high purity compared to the crude EOs, which are a mixture of several components.

CONCLUSION

This study extracted EOs from *P. guajava leaves*, *C. maxima pericarp* and *L. domesticum* leaves and pericarp. All four EOs extracted from these plants were shown to inhibit the growth of bacteria particularly *B. subtilis* and *E. coli*, thereby, making these indigenous fruit trees as potential sources of antimicrobials for the development of various health and cosmetic products. It is recommended to develop combined formulations of the extracted EOs to determine their potential synergistic action to maximize their antimicrobial potential.

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