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Antibacterial and Antioxidant Activities of Three Citrus Leaves Extracts

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ABSTRACT

This paper highlighted the antibacterial and antioxidant properties of the leaves extracts of three citrus plants namely: Citrus microcarpa (calamansi), Citrus aurantium (dalandan) and Citrus maxima (pomelo). All citrus leaves extract exhibited antibacterial activity against S. aureus but not in E. coli. C. microcarpa ethanol extract significantly recorded the highest diameter zone of inhibition of 13.53 mm while C. maxima ethanol extract had the lowest diameter (11.26 mm). Among crude extracts, C. maxima significantly showed the widest diameter zone of inhibition of 10.49 mm. In DPPH scavenging assay, C. microcarpa significantly recorded the highest scavenging activity of 48.67%, followed by C. maxima having 43.51%. C. aurantium had the lowest activity. Interestingly, C. microcarpa contained the highest total phenolic content of 309.38 mg AAE/g sample whereas C. aurantium had the lowest phenolic content of 228.21 mg AAE/g sample. Therefore, leaves of citrus plants contain valuable compounds with medicinal and pharmacological importance.

Keywords: Citrus plants, antibacterial, antioxidant, phenolic content.

INTRODUCTION

Nature is a depository of useful and medicinal plants. Medicinal plants have been a valuable resource of natural products and utilized as natural remedy for several diseases. For a long period of time, plants are used in medical treatment and pharmaceutical development, and has significant role in maintaining healthy lifestyle. The active secondary metabolites of plants are effective antimicrobial and antioxidant agents that can be of great significance in pharmaceutical industry.

Citrus plants, belonging to family Rutaceae, are one of the most important commercial fruit crops grown in the Philippines. They are small trees with smooth, oblong to broadly lanceolate, and narrowly winged short petioled leaves. The fruit has loose skin and leathery pericarp, with a sweet sour juicy pulp which are mainly process as juice drinks. These plants are also considered important medicinal plants. For instance, the flavonoids from *Citrus* exhibit in vitro and in vivo anti-inflammatory, anticancer, antioxidant, and cardiovascular protective activities [1, 2].

With an increasing demand on cheap and effective natural compounds for the treatment of many diseases, this study investigated the antibacterial and antioxidant activities of *Citrus microcarpa* (calamansi), *Citrus aurantium* (dalandan) and *Citrus maxima* (pomelo) leaves extracts. The antibacterial property was tested in two human pathogenic bacteria using disc diffusion method whereas the antioxidant activity was determined using DPPH radical scavenging activity and total phenolics.

MATERIALS AND METHODS

2.1 Source of Plant Samples:

Leaves of the three citrus plants were collected from Barangay Bambanaba, Cuyapo, Nueva Ecija, Philippines, and separately placed in a plastic bag with proper label. Samples were washed three times and air-dried in a shaded condition for 7 days. These were pulverized and processed for extraction.

2.2 Crude and Ethanol Extraction:

Five hundred grams of the fresh sample leaves of each citrus plant were milled using a blender and squeezed to obtain the crude extract. On the other hand, 20 g of the air-dried milled leaves of each citrus plant were soaked in 500 ml of 95% ethanol for 48 hours. After which, these were filtered using Whatman No. 2 filter paper to separate the plant material. Each filtrate was evaporated in a rotary evaporator to remove the solvent used. Extracts were labeled and prepared for the antibacterial assay.

2.3 Antibacterial Screening:

The antibacterial activities of the crude and ethanol extracts of citrus plant leaves were determined following the paper disc diffusion method of Bauer et al [3] Gram positive *Staphylococcus aureus* and Gram negative *Escherichia coli* were cultured in 9 ml of nutrient broth (NB) medium and incubated at 37 °C. After 24 hours, the turbidity of each bacterial culture was adjusted to equal that of 0.5 McFarland standard, which approximated 1.5×10^8 ml^{-1}. The bacterial suspension was spread using a sterile cotton swab on nutrient agar plate. Six millimetre diameter paper discs impregnated with crude extract (20 µL) and ethanol extract (20 µL), and streptomycin as standard were placed equidistantly on the medium. Plates were incubated at 37 °C, and the zones of inhibition were measured using vernier calliper after 24 hours. Each test was done in triplicate.

2.4 DPPH radical scavenging activity assay:

The stable 2,2'-diphenyl-1-1picrylhydrazyl (DPPH) radical was used to estimate the free radical scavenging activity of the samples, following the standard method of Shimada et al. [4]. A 100 µl of test sample in ethanol was added with 5 µl DPPH solution (5 mg DPPH powder in 2 ml of ethanol) in 96-well microtitter plates. The mixture was shaken vigorously and left to stand for 30 min in the dark, and the absorbance was then measured at 517 nm. The inhibition of DPPH free radicals was calculated.

2.5 Estimation of total phenolic content:

The total phenolic content was estimated using Folin-Ciocalteu method of Slinkard and Singleton [5] with modifications. Sample solution (50 µl) was mixed 500 µl of 10% Folin-Ciocalteu reagent (Folin:Methanol, 1:1, v/v). After 2 min, 50 µl of 7.5% saturated was added and kept in the dark for 1h before absorbance was taken at 765 nm. A calibration curve was obtained using various concentrations of ascorbic acid. The total phenolic content of the sample was expressed as mg of ascorbic acid equivalents (AAEs) per gram of sample.

2.6 Statistical Analysis:

Data were analyzed using Analysis of Variance (ANOVA). Means were compared using Duncan Multiple Range Test (DMRT) at 5% level of significance.

RESULTS AND DISCUSSION

3.1 Antibacterial Activity of Citrus Plants:

The antibacterial activities of leaves extracts of three citrus plants including *C. microcarpa, C. aurantium* and *C. maxima* were screened in vitro. The diameter zone of inhibitions of the two extracts of the three citrus plant leaves against *S. aureus* is presented in Table 1. Interestingly, all citrus leaves extract exhibited antibacterial activity against *S. aureus* but not in *E. coli* (data not shown). Among the extracts evaluated, ethanol extract of *C. microcarpa* significantly recorded the highest diameter zone of inhibition of 13.53 mm, which statistically comparable with the ethanol extract of *C. aurantium* having 12.93 mm. *C. maxima* had the lowest diameter (11.26 mm) among the ethanol extracts. On the other hand, among crude extracts, *C. maxima* significantly showed the widest diameter zone of inhibition of 10.49 mm. However, no significant difference was noticed on the diameters of *C. microcarpa* and *C. aurantium*. Although inhibition diameters of all citrus extracts are found to be statistically differed with the diameter of streptomycin, these inhibitions against *S. aureus* strongly indicate the great potential for the degree of antibacterial activity of the three citrus plants.

Table 1. Diameter zone of inhibition of the different citrus extracts against *S. aureus in vitro*.

Citrus Plants	Extract	Diameter zone of inhibition (mm)
C. maxima	Crude	10.49[c]
	Ethanol	11.26[c]
C. microcarpa	Crude	9.83[d]
	Ethanol	13.53[b]
C. aurantium	Crude	9.71[d]
	Ethanol	12.93[b]
Streptomycin	-	32.19[a]

In the mean column, means having the same letter of superscripts are not significantly different from each other using DMRT at 5% level of significance.

Citrus plants are known for their antibacterial properties due to their strong bioactive components. For example, the 2-hydroxypropane-1,2,3-tricarboxylic acid, active compound of *C. microcarpa*, exhibited *Citrobacter freundii* (ATCC 8090), *Aeromonas hydrophila* (ATCC 49140), *Pseudomonas aeruginosa* (ATCC 35032), *Streptococcus agalatiae* (ATCC 13813), *Edwardsiella tarda* (ATCC 15947), *Escherichia coli* (ATCC 25922), and *Yersinia enterocolitica* (ATCC 23715) [6]. In addition, mycelial growth of *Aspergillus flavus* decreased in increasing concentration of essential oils of *C. maxima* and completely inhibited the growth at 500 ppm. This oil contained DL-limonene (31.83%), E-citral (17.75%), 1-hexene-4-methyl (15.22%) and Z-citral (13.38%) as the major components [7]. On the other hand, extract of *C. aurantium* contain 8 flavonoids namely, isonaringin, naringin, hesperidin, neohesperidin, naringenin, hesperitin, nobiletin and tangeritin, which are known to exhibit antibacterial property [8].

3.2 Radical Scavenging Activity of Citrus Plants:

Free radicals caused by oxidative stress have been linked to ageing process and pathogenesis of various diseases, such as myocardial and cerebral ischemia, arteriosclerosis, diabetes, rheumatoid arthritis, inflammation and cancer-initiation [9]. Thus, discovering of antioxidants from natural sources to scavenge free radicals is of high interest. In this present study, the radical scavenging activity of the three citrus plants was investigated (Table 2). Among the three citrus plants, *C. microcarpa* significantly recorded the highest radical scavenging activity of 48.67%, followed by *C. maxima* having 43.51%. *C. aurantium* had the lowest scavenging activity. These results clearly dictate that the three citrus plants hold promising antioxidants that protect the human body against various diseases including cancer and cardiovascular diseases.

Table 2. Radical scavenging activity and total phenolics of the three citrus plant leaves

Citrus Plants	Radical Scavenging Activity (%)	Total Phenolics (mg AAE / g sample)
C. maxima	43.51[c]	228.21[b]
C. microcarpa	48.67[b]	309.38[a]
C. aurantium	41.37[c]	246.36[b]
Cathechin	75.38[a]	-

In the mean column, means having the same letter of superscripts are not significantly different from each other using DMRT at 5% level of significance.

3.3 Total Phenolic Content of Citrus Plants:

Phenolic compounds (tocopherols, flavonoids, and phenolic acids), nitrogen compounds (alkaloids, chlorophyll derivatives, amino acids, and amines), carotenoids, and ascorbic acid are natural antioxidants [10, 11] They have broad range of useful antibacterial, antiviral, and pharmaceutical activities, thus, they have tremendous importance to humankind. The phenolic content of the three citrus plants was also determined in this present study and the results are presented in Table 2. Similarly, *C. microcarpa* contained the highest total phenolic content of 309.38 mg AAE/g sample whereas *C. aurantium* had the lowest phenolic content of 228.21 mg AAE/g sample. Although phenolic content of the three citrus plants vary, the presence of this compound indicates their promising medicinal properties.

Duzzioni et al. [12] studied the physicochemical and radical scavenging activities of four citrus fruit varieties cultivated in Brazil and they found that cravo tangerine has the highest content of citric acid, while the pera orange is rich in ascorbic acid. The lima orange has the highest total phenolic contents, and the ponkan is high in total carotenoids. They also added that the radical scavenging activity of these citrus was higher in the aqueous than in

the methanolic or acetone fractions. Moreover, in the study of Prasad and Ashwin Rajkumar [13], the methanolic extract of citrus have the scavenging activity ranged from 0 % (*C. reticulata*) to 80.57% (*C. hystrix*) whereas the *C. aurantifolia* and *C. hystrix* showed better activity ranging from 15% to 76%. The hexane extract have scavenging activity ranged from 7.5% (*C. reticulata*) to 78.50% (*C. aurantifolia*) while *C. hystrix* and *Murraya koenigii* exhibited constant activity.

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