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Phytochemical profiles, total phenolics contents, and antioxidant activities of juices from three lemon (*Citrus limon*) varieties available in Cagayan de Oro, Philippines

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Abstract

Lemon (*Citrus limon*) is a small fruit-bearing tree from the Rutaceae family. This study compared the phytochemical composition, the total phenolics (an antioxidant group of substances), and the antioxidant activity measured as free radical scavenging activity of the juices of three lemon varieties from Cagayan de Oro City, Philippines. The phytochemical screening was conducted by applying qualitative tests for carbohydrates, reducing sugars, glycosides, steroids, saponins, phenols and tannins, flavonoids, anthraquinones, alkaloids, and proteins. Then, the Folin-Ciocalteu test assessed the total phenolics content of the samples. Moreover, the percent scavenging effect via the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity determined its antioxidant activity. Finally, the Pearson correlation test examined the statistical correlation between total phenolics content and antioxidant activity. The lemon juice samples contain many bioactive compounds, including reducing sugars, glycosides, steroids, saponins, phenols and tannins, flavonoids, alkaloids, and proteins. All three juices exhibited high and significantly different total phenolics contents ranging from 460.57 – 686.18 mg/L. The juices also showed high antioxidant activities against free radicals which varied significantly from 89.84 – 92.80 % scavenging effect. The correlation between these two sets of data—total phenolics and free radical scavenging—indicates the very big contribution of the phenolic compounds to the antioxidant activity.

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Introduction

The element oxygen is essential for life. The cells of living organisms use oxygen to produce energy in the form of ATP (adenosine triphosphate). However, one of the by-products of this metabolic process is the formation of free radicals. These free radicals are often reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS and RNS can also come from exogenous sources, such as cooking (smoked meat, used oil, fat), industrial solvents, water and air pollution, tobacco smoke, alcohol, heavy or transition metals (Cd, Hg, Pb, Fe, As), specific medications (cyclosporine, tacrolimus, gentamycin, bleomycin), and radiation. ROS and RNS are double-edged compounds that can provide favorable and unfavorable results. These compounds favor immune function and cellular responses at low to moderate concentrations. They generate oxidative stress at high concentrations, a harmful process that can damage all cell structures. Oxidative stress causes chronic and degenerative diseases such as cancer, arthritis, aging, autoimmune disorders, and cardiovascular and neurodegenerative diseases. Protective mechanisms in the human body combat oxidative stress by generating antioxidants. Nevertheless, exogenous intake of antioxidants is vital in helping the endogenous antioxidants in the body scavenge free radicals effectively (Pham-Huy *et al.,* 2008). Research studies have shown that increased dietary intake of natural phenolics is associated with reduced coronary heart disease, cancer mortality, and longer life expectancy.

These polyphenolic compounds are known for their potent antioxidant, anticancer, antiviral, and anti-inflammatory activities. Furthermore, interest in naturally occurring antioxidants found in plants as secondary metabolites has grown due to issues regarding the safety of widely used synthetic antioxidants like butylated hydroxyanisole (BHA) and tertiary butylhydroquinone (TBHQ) (Ghafar *et al.,* 2010). Beyond antioxidants, many other phytochemicals are associated with a number of favorable health effects.

One source of natural antioxidants is lemons. Its antioxidant activity primarily comes from phenolic compounds (mainly flavonoids) and Vitamin C (Rizaldy *et al.,* 2023). It is also rich in folate, potassium, and limonins. Nutrients in lemon juice play a role in immunity, promoting the improvement of damaged livers, preventing stroke, and reducing cardiovascular disease and cancer risks. The citric acid in lemons benefits those suffering from kidney stones. For pregnant women, folate is crucial to preventing neural tube abnormalities. Lemon juice is also known for its role in weight loss and antimicrobial properties. This study determined and compared the presence of phytochemicals and the antioxidant activity of the juice of locally grown lemon and selected imported lemon varieties available in Cagayan de Oro City.

Materials and methods

Sample collection and preparation

The Citrus Hill Plantation (CHP) lemons were from the municipality of Barobo in the province of Surigao del Sur, while the URGN 165 and the Cali Fresh lemons were purchased from supermarkets in Cagayan de Oro City.

The samples were selected through simple random sampling. The Citrus Hill Plantation harvests an average of 350 kilograms of lemons in one setting. This translates into about 3,500 pieces of lemons per harvest. From this population, the sample size was computed using Slovin's formula (shown below) with a 20 % margin of error. The result is a sample size of 25 pieces of lemon per variety. This sample size is in close agreement with the JBT FoodTech Citrus Systems' procedures, which uses 20 fruits to analyze its products.

Slovin's formula: $$n = \frac{N}{1 + Ne^2}$$

Where:

n = sample size

N = population size

e = margin of error

The lemon fruit samples were washed and air-dried at room temperature. The juice was extracted by cutting the fruit in half and squeezing the juice out while straining to separate the seeds and any solid matter. For each lemon variety, the juice of five (5) lemon fruits was combined to constitute one (1) composite sample. There were five (5) replicates of this composite per variety. Three (3) trials were performed for each test from each replicate.

Phytochemical screening

Qualitative tests were employed to determine the phytochemicals present in the juice samples. In each test, 2 mL of juice sample was used. Most of the tests were performed by applying the methods described by Bansode and Chavan (2012). The following standard chemical tests were conducted.

Test for carbohydrates

The juice sample was mixed with 2 mL of iodine solution in the iodine test for carbohydrates. The formation of a dark blue or purple color signaled the detection of the carbohydrate.

Test for reducing sugars

The juice was shaken with 5 mL distilled water and filtered. 1 mL of filtrate was boiled with 2 mL Fehling's solution A&B for 10 minutes. The formation of a red precipitate indicated the presence of reducing sugar.

Test for glycosides

The Salkowski's test was used to detect glycosides. The juice was mixed with 2 mL of chloroform. Next, 2 mL of concentrated sulfuric acid was added cautiously and mixed lightly. The presence of the steroidal ring, or glycone portion of the glycoside, was indicated by a reddish-brown color.

Test for steroid

The Liebermann-Burchard test was used to detect the most common steroid—cholesterol. The juice was mixed with 2 mL of chloroform. Then, 2 mL each of concentrated sulfuric acid and acetic acid were poured into the mixture. The appearance of a greenish coloration indicated the existence of steroids.

Test for saponins

The froth or foam test was done by mixing the juice with 5 mL of distilled water in a test tube and shaking vigorously. The development of stable foam confirmed the presence of saponins.

Test for phenols and tannins

The juice was mixed with 2 mL of 2% Iron (III) chloride. The formation of a blue-green or black coloration confirmed the presence of phenols and tannins.

Test for flavonoids

The alkaline reagent test for flavonoids was conducted by mixing the juice with 2 mL of 2 % sodium hydroxide solution. The formation of an intense yellow color that turned colorless with the addition of a few drops of diluted acid indicated the presence of flavonoids.

Test for anthraquinones

The juice was added with 2 mL of 10 % ammonium hydroxide. A bright pink color signaled the presence of anthraquinones. This was the Borntrager's test.

Test for alkaloids

2 to 3 drops of Wagner's reagent were added to the juice. The formation of a brown or reddish-brown precipitate indicated the presence of alkaloids.

Test for proteins

The test was conducted by boiling the juice with 2 mL of 0.2% ninhydrin solution. The appearance of a violet color suggested the presence of amino acids and proteins.

Total phenolics content analysis

Using the method adapted from Ghafar *et al.* (2009), the juice sample (100 µL) was mixed with 0.75 mL of Folin-Ciocalteu reagent (10-fold dilution with distilled water) and allowed to stand at 22°C for 5 min. Afterward, 0.75 mL of sodium carbonate (60 g/L) solution was added to the mixture. Following 90-minute incubation at 22°C, absorbance was measured at 725 nm using a UV-visible spectrophotometer. The total phenolic content was determined using a standard curve of gallic acid at 20 - 100 mg/L concentrations.

Antioxidant activity via DPPH radical scavenging activity

From the method for radical scavenging activity adapted also from Ghafar *et al.* (2009), 1 mL of centrifuged juice and ascorbic acid (positive control) at different concentrations (25, 50, 75, 100, and 150 mg/mL) was mixed with 5.8 mL of 100 μM DPPH (dissolved in 80% ethanol). The mixture was vigorously shaken and left to stand at room temperature for thirty (30) minutes in the dark. The control contained only DPPH solution instead of the sample, while 80% ethanol was used as the blank. Absorbance was read at 515 nm by using a UV-vis spectrophotometer. The scavenging effect was calculated using the equation:

$$\text{Scavenging effect (\%)} = [1 - (\frac{\text{Absorbance sample}}{\text{Absorbance control}})] \times 100\ \%$$

Statistical analysis

Data from total phenolics content and DPPH radical scavenging activity analyses were analyzed using ANOVA followed by Tukey's Honestly Significant Difference (HSD) Test. The correlation between the antioxidant content and the antioxidant activity was analyzed using the Pearson correlation analysis.

Results

Phytochemical screening

Table 1 shows the results of the phytochemical screening of the juice of the three lemon varieties, namely CHP lemon, URGN 165 lemon, and Cali Fresh lemon. Based on the results, all varieties were negative for carbohydrates and anthraquinones. All were positive for reducing sugars, glycosides, steroids, saponins, phenols and tannins, flavonoids, alkaloids, and proteins.

Total phenolics content analysis

Table 2 shows the results for the total phenolics content of the lemons. URGN 165 exhibited the highest result of 686.18 mg/L. This result is not significantly different from that of Cali Fresh, with 623.95 mg/L. Conversely, both values are significantly different from CHP, 460.57 mg/L.

Table 1. Phytochemistry of the juice of the lemon varieties

Lemon	Carbohydrates	Reducing Sugars	Glycosides	Steroids	Saponins	Phenols and Tannins	Flavonoids	Anthraquinones	Alkaloids	Proteins
CHP	-	+	+	+	+	+	+	-	+	+
URGN 165	-	+	+	+	+	+	+	-	+	+
Cali fresh	-	+	+	+	+	+	+	-	+	+

Legend: (+) presence of phytochemical and (-) absence of phytochemical.

Table 2. Total phenolics and antioxidant activity of the lemon varieties

Lemon	Total phenolics (mg/L)	Antioxidant activity (%SE)
CHP	$460.57^{b} \pm 46.41$	$89.84^{b} \pm 0.88$
URGN 165	$686.18^{a} \pm 92.54$	$92.80^{a} \pm 0.26$
Cali Fresh	$623.95^{a} \pm 92.42$	$92.30^{a} \pm 0.99$

Antioxidant activity via DPPH radical scavenging activity

Table 2 also shows the antioxidant activities of the lemon juices accomplished via DPPH assay. URGN 165 lemon had the highest scavenging effect at 92.80 %, followed closely by Cali Fresh lemon with 92.30 %. Both values are significantly different from that of CHP lemon at 89.84 %.

Correlation between total phenolics content and DPPH radical scavenging activity

The correlation between the total phenolics content and the antioxidant activity in terms of DPPH radical scavenging activity was found to be 0.66. This result indicates a significant positive correlation between the two variables.

Discussion

Phytochemical screening

A number of phytochemicals have been recognized to improve health beyond essential nutrition and to decrease the risk of major chronic diseases (Liu, 2004). In this study, all lemon juice samples tested positive for reducing sugars, steroids, saponins, phenols/tannins, flavonoids, alkaloids, and proteins. The positive test results for phytochemicals for all three juices can be a favorable sign. Glycosides are known to have pharmacological activities that include analgesic, anti-inflammatory, cardiotonic, antibacterial, antifungal, antiviral, and anticancer effects (Soto-Blanco, 2021). Saponins are linked to lowering a body's cancer risks, cholesterol levels, blood lipids, and blood glucose response (Shi *et al.,* 2004). Phenolic compounds are known to be anticarcinogenic, antimutagenic, anti-inflammatory, and, most importantly, antioxidants, which are significant for cancer prevention and treatment (Roleira *et al.,* 2018; Chung *et al.,* 1998). Alkaloids have many physiological effects, including hypnotic, psychotropic, antibacterial, antimitotic, anti-inflammatory, antitumor, analgesic, and local anesthetic properties (Kurek, 2019).

The results for phytochemical screening have similarities and differences when compared to the results of Bansode and Chavan (2012). The lemon juices in both studies showed positive test results for reducing sugars, glycosides, steroids, phenols and tannins, flavonoids, and proteins. They differ in that the lemon juice of Bansode and Chavan was positive for carbohydrates, negative for saponins and glycosides, and no tests were conducted for anthraquinones and alkaloids. Among the phytochemicals present in the lemon juice samples, the phenolic compounds are the most important groups that determine their biological activity (Klimek-Szczykutowicz *et al.,* 2020).

Total phenolics content analysis

The lemon juices from all three varieties have relatively high total phenolics contents that range from 460.57 – 686.18 mg/L. This result is similar to the result of Rekha *et al.* (2012) and higher than the result of Fejzic and Cavar (2014) with total phenolics contents of 600 mg/L and 322 mg/L, respectively. The total phenolics contents of the lemon samples used in this study varied significantly. The differences in total phenolics contents may be attributed to the differences in lemon variety, cultivar, or hybrid, which are produced to attain desirable organoleptic and industrial fruit qualities (Klimek-Szczykutowicz *et al.,* 2020). The results can also vary due to climate, maturity, cultural practice, fruit quality, fruit handling, processing, packaging, and storage conditions.

Antioxidant activity via DPPH radical scavenging activity

In this study, the lemon juices from all three varieties have relatively high DPPH radical scavenging ability that ranges from 89.84 % to 92.80 %. This result is consistent with that of Rekha *et al.* (2012) which ranges from 86.25 % to 95.0 %. The percent scavenging effect of URGN 165 and Cali Fresh lemon juices is significantly higher than that of CHP. The result of this study suggests that the lemon juice samples effectively counter the DPPH oxidants and is good sources of antioxidants.

Correlation between total phenolics content and DPPH radical scavenging activity

A significant positive correlation exists between the total phenolics content and the DPPH radical scavenging activity. This result is in agreement with that of Ghafar *et al.* (2010) and Rizaldy *et al.* (2023), who have reported strong positive correlations between total phenolics content and DPPH radical scavenging activity.

The result signifies that the total phenolics contents of the lemon juices are directly proportional to their antioxidant activities, which means that the higher the total phenolics content, the greater the antioxidant activity of the lemon juice.

Conclusion

The juice of the three lemon varieties contained a wide assortment of phytochemicals, including reducing sugars, glycosides, steroids, saponins, phenols and tannins, flavonoids, alkaloids, and proteins. The lemon juices differed significantly in their concentrations of the total phenolic substances. The URGN 165 and the Cali Fresh lemons had higher total phenolics contents than the CHP lemon. The lemon juices varied significantly in their antioxidant activities in terms of DPPH Radical Scavenging

Activity. The URGN 165 and the Cali Fresh lemons had higher antioxidant activities than the CHP lemon. There was a positive correlation between the antioxidant content and the antioxidant activity.

Recommendation

Based on the research, all the lemon juices have antioxidative potentials and recommended for advanced research.

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