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Fatimae Ituralde Mariano
Department of Pharmacy, Universidad de Zamboanga, Zamboanga City, Philippines

Corresponding Author:
Fatimae Ituralde Mariano
Department of Pharmacy, Universidad de Zamboanga, Zamboanga City, Philippines

Antioxidant and anti-arthritic activity of peels from citrus fruits

Fatimae Ituralde Mariano

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Abstract
Citrus fruits were the most consumed fruits during the COVID-19 pandemic because of its health benefits. The peels from citrus fruits are rich in bioactive compounds than the commonly consumed juice or pulp. This study aims to utilize the discarded peels, converting them to value added products. The main objective of this study is to determine the peels' antioxidant and anti-arthritic potential. With current arthritis treatment causing different side effects, natural products are promising alternatives. The antioxidant activity was determined using 2, 2-diphenyl-1-picrylhydrazyl (DPPH), and nitric oxide radical scavenging assay. Anti-arthritic activity used protein denaturation and proteinase inhibition assay. Phytochemical study revealed that citrus peels still contain high amounts of flavonoids, a bioactive compound contributing to antioxidant and anti-arthritic activities. In the DPPH assay, the 500ug/mL exhibited higher antioxidant activity while in the nitric oxide scavenging assay, the lowest concentration (5ug/mL) exhibited the highest activity. This means that the lowest concentration already has significant antioxidant activity. In the anti-arthritic activity assays, lower concentration (5ug/mL) of the extracts was also found to have good anti-arthritic activities both in the protein denaturation and proteinase inhibition assay. Hence, the citrus peel wastes have significant antioxidant and anti-arthritic activity in vitro. The correlation between flavonoid content antioxidant and anti-arthritic activities proved that flavonoids may be responsible for the two activities.

Keywords: Philippines, citrus, arthritis, antioxidant, *in vitro*, zamboanga city

Introduction
Arthritis, a chronic systemic inflammatory disease affecting 0.5-1% of the worldwide population, leads to joint deformities and even functional disabilities because of damage to cartilage and bone destruction (MacFarlane *et al*., 2017). In the Philippines, according to the Philippine Rheumatology Association, arthritis, specifically gout, affects 1.6 million Filipinos. Furthermore, one of every six adult Filipinos suffer from arthritis and rheumatism which affect people of all ages, contrary to common perception that these conditions go with old age [1].
In a study conducted by Penserga *et al*. (2015), two hundred and sixty (260) cases of Filipinos with rheumatoid arthritis were successfully included in the Rheumatoid Arthritis Database and Registry (RADAR) of the Philippine General Hospital alone which showed a high female to male ratio, long delay in diagnosis and high attrition rate [2].
Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used for the treatment for rheumatism, such as rheumatoid arthritis and pain. In spite of their extensive usage, NSAIDs are associated with many adverse effects. Chronic usage of Aspirin, a NSAID leads to gastric ulceration and liver damage. Diclofenac has also been a widely used conventional treatment of rheumatic pains, but abdominal pain, constipation and feeling of indigestion have often limited its clinical utilization [3].
Citrus fruits and juices are an important source of bioactive compounds including antioxidants such as ascorbic acid, flavonoids, phenolic compounds and pectins that are important to human nutrition. As far as the peel is concerned, extracts from this part of the fruit were found to have a good total radical anti-oxidative potential [4].
Utilization of peel in several possible ways helps in reducing solid-waste handling along with adding value to these peel waste. In a study conducted by Williams (2013) regarding the potential of orange peels as dietary supplement, no adverse effect or any side effect from the peel has been reported. Also, according to Lang (2019), the peels from lemon has no reported side effects and is recognized by the Food and Drug Administration as safe for human consumption [5].

Utilization of peels into value-added products addresses Sustainable Development Goal (SDG) 12-Responsible Production and Consumption - where wastes are kept in use and regenerated. Also, this study is aligned to Sustainable Development Goal (SDG) 3 which aims to ensure good health and well-being, providing access to safe, affordable, and effective medicines for all.

Furthermore, no study thus far has been conducted to evaluate if the citrus peel "waste" contains anti-oxidant and anti-arthritic activities. The studies found in the literature only evaluated on fresh peels or fruits purchased in the market then peeled but not on waste utilization - the peels discarded by companies or stalls selling fruit juices and the like. Furthermore, studies only explored on the potential of the peels as antioxidants but not on its anti-arthritic activity using the methods employed in this proposal.

Hence, this study is conducted to explore on the potential of the citrus peels for future drug product development (capsules, tablets, or candies) as alternative or in synergistic combination with synthetic drugs already available in the market.

Methodology

This study is mainly a preliminary study focused on determining the antioxidant and anti-arthritic activity of Citrus Limon and Citrus sinensis, two of the common citrus fruits sold in the market, whose juices are sold in food establishments and the peels are just discarded as wastes. This study takes advantage of the discarded peels and for potential product development in the future.

Collection and Preparation of Samples

The lemon (Citrus Limon) and orange (Citrus sinensis) peels were collected from stalls selling fruit juices in selected areas in Zamboanga City. This study did not utilize "fresh" citrus peels. The peels came from the waste of the stalls selling fruit (lemon and orange) juices. No approximate time after peeling was considered in the study since fresh peels were not used. The peels collected (not less than 5 kilograms) were cleaned and washed with distilled water to ensure removal of dirt and other extraneous materials.

Preparation of Extracts

The collected peels of C. Limon and C. Sinensis were oven-dried at 60°C. It was then be pulverized using a pharmaceutical blender until the appearance of a powdery consistency. See Figure 1 and 2. An accurately weighed number of powdered peels were macerated with 95% ethanol, enough to submerge the plant material in 1000 ml Florence flask. The flask was sealed tightly and was kept for 72 hours. The supernatant was filtered using Whatman filter paper No.1 and evaporated using a rotary evaporator. The extraction yield was further dried in an evaporating dish under an electric stove at medium heat to evaporate minute amounts of the maceration solvent. The percentage yield of the extract was calculated and determined.

Calculation of percentage (%) yield extract

$$\text{Extraction yield} = \frac{(\text{Wt. of crude dried extract} \times 100)}{\text{Wt. of original sample}}$$

The prepared extracts were sent to University of Immaculate Conception, Davao City for characterization which included the Field Emission Scanning Electron Microscope (FESEM) and Fourier Transform Infrared Spectroscopy (FTIR) in order to compare its chemical properties with the commercially available anti-oxidant, Ascorbic Acid.

Fig 1: Collected lemon and orange peels

Fig 2: Powdered lemon and orange peels for extraction

Preparation of Test Solutions

A stock solution of C. Limon and C. sinensis crude dried extract as prepared with a concentration of 1mg/ml. From the stock solution, serial dilutions for three concentrations were made for the anti-arthritic and antioxidant assays, labeled as TSCL1, TSCL2, and TSCL3 for C. Limon while TSCS1, TSCS2, and TSCS3 for C. Sinensis. The test solutions of C. Limon (TSCL) and C. Sinensis (TSCS) extracts were prepared for three trials with triplicates. For the positive control of the anti-arthritic and antioxidant assays, a stock solution of 1 mg/ml was also prepared which was serially diluted into three concentrations. Three concentrations was prepared for the positive control for the purpose of establishing a direct relationship with the TSCL and TSCS in terms of (%) inhibitory activities.

I. Anti-arthritic Assays

The diluent that was used in the preparation of test solutions for the anti-arthritic assays was distilled water, for the reason that protein denaturation method and proteinase enzyme inhibition particularly used bovine serum albumin. This chemical denatures in ethanol and other organic solvents which could affect the results of the study [6].

The formula C1V1 = C2V2 was used to calculate the serial dilution of test solutions wherein, C1 is the concentration of the stock solution, V1 is the volume removed or aliquoted from the stock solution, C2 is the final concentration of the diluted concentration, and V2 is the final volume of the diluted solution or the total solution volume.

II. Antioxidant Assays

The diluent that was used in the preparation of test solutions for the antioxidant assays was ethanol. Ethanol does not affect the antioxidant activity of sample solutions and this solvent was based on the model paper used for the antioxidant methods. The same formula (C1V1 = C2V2) from the anti-arthritic assays was used for the calculation of the serial dilution of test solutions for both the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and nitric oxide radical scavenging assays.

Phytochemical Screening: The obtained extract was subjected to preliminary phytochemical screening to test for the presence of secondary metabolites such as phenolic compounds, flavonoids, alkaloids, and tannins. Phytochemical test for phenolic compounds was the Lead acetate test. For flavonoids, the Shinoda and Lead acetate test were conducted. For the presence of alkaloids, Dragendorff's reaction and Mayer's test were performed. Lastly, for the presence of tannins, the Ferric chloride solution test was conducted.

Determination of Total Flavonoids: An aliquot (0.5 ml) of the extracts (TSCL 1000 µg/ml and TSCL 1000 µg/ml) or the standard solution (5-500 µg/ml) was transferred in a test tube containing 2 ml distilled water and 0.15 ml of 5% $NaNO_2$. After 5 minutes, 0.15 ml of 10% $AlCl_3$ solutions was added, and the mixture was allowed to stand for another 5 minutes, after which 1 ml of 1 M NaOH was added. The reaction mixture was mixed and allowed to stand for 15 minutes. Sample blank for all extracts and all the dilution of standard rutin was prepared in a similar manner by replacing aluminum chloride solution with distilled water. The absorbance was measured at 510 nm against the blank. From a calibration curve of rutin, the total flavonoid content was calculated and the result was expressed in mg rutin equivalent per gram dry weight extract.

Anti-arthritic Activity

A. Protein Denaturation Method: In this procedure, 0.5% Bovine Serum Albumin (BSA) was the protein subjected for denaturation and phosphate buffer saline (PBS) was used to maintain a constant pH. In preparing 0.5% BSA, exactly 0.5 g of BSA was dissolved in 100 ml distilled water. For the preparation of PBS pH 6.3, eight (8) g of sodium chloride (NaCl), 0.2 g of potassium chloride (KCl), 1.44 g of disodium hydrogen phosphate (Na_2HPO_4), 0.24 g of potassium dihydrogen phosphate (KH_2PO_4) was dissolved in 800 ml distilled water. The pH was adjusted to 6.3 using 1 N HCl and the volume was made up to 1000 ml with distilled water.

Procedure: This procedure was adapted from Vaijayanthimala (2019), Anoop and Bindu (2015), and Shivhare (2011) with minor modifications. Approximately 0.05 ml of TSCL and TSCS of various concentrations (50, 250, 500 µg/ml) and positive control, diclofenac sodium (50, 250, 500 µg/ml) were taken respectively and 0.45 ml (0.5% w/v BSA) was mixed. The prepared solutions were incubated at 37°C for 20 minutes and the temperature was increased to keep them at 57°C for 3 minutes. After cooling, 2.5 ml of phosphate buffer was added to the above solutions. The absorbance was measured using UV-Visible spectrophotometer at 660 nm. For the control, 0.05 ml distilled water was instead of the extracts while the product control lacks 0.5% BSA. The results was compared with the positive control, Diclofenac sodium. The percentage inhibition of protein denaturation was calculated as:

$$\text{Percentage (\%) Inhibition} = 100\% \quad \frac{(\text{Abs. of test solution} - \text{Abs of product control}}{\text{Abs. of Control}} \times 100)$$

B. Proteinase Inhibition Assay: In this procedure, 0.1% Bovine Serum Albumin (BSA) was used as the protein substrate for the enzyme proteinase. It was prepared by dissolving 0.1 g BSA in 100 ml of distilled water. Twenty (20) mM of tris-HCl buffer pH 7.4 was used to make a solution with trypsin (serine proteinase). It was prepared by dissolving 3.94 g Tris powder in 800 ml of deionized water and the pH was adjusted to 7.4 using 1 M hydrochloric acid (HCl) and made up to 1000 ml with deionized water.

Procedure: This assay was evaluated by the method of Reshma *et al.* (2014) with slight modifications. The samples consisted of 1 ml of 0.1% BSA and 1 ml of TSCL and TSCS of various concentrations (5, 10, 20 µg/ml). This was incubated at room temperature for 5 minutes. The reaction was inhibited by the addition of 2 ml of 80 µg/ml trypsin followed by centrifugation at 3000 rpm for 10 minutes. The drug Diclofenac sodium was used as the positive control. The absorbance of the supernatant was measured at 280 nm keeping buffer as the control test while product control lacks 0.5% bovine serum albumin. The percentage inhibition of proteinase was then calculated.

$$\text{Percentage (\%) Inhibition} = 100\% \quad \frac{(\text{Abs. of test solution} - \text{Abs of product control}}{\text{Abs. of Control}} \times 100)$$

Antioxidant Activity

A. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Assay

DPPH was stored in the freezer, protected from light, and the time out of the freezer was minimized. For the DPPH solution, approximately 0.004 g of DPPH was added to 100 ml of ethanol (0.1 mM) and was measured with a graduated cylinder into a 100 ml volumetric flask wrapped in foil to protect the solution from light. DPPH solution should always be prepared fresh every experimentation. Exactly 2 ml was obtained from TSCL and TSCS of different concentrations (50, 250, 500 µg/ml) and was added with 2 ml of DPPH solution (0.1 mM) and shaken well. For the positive control, 2

ml was taken from the diluted concentrations (50, 250, 500 µg/ml) of Ascorbic acid in ethanol stock solution (1 mg/ml) and 2 ml of DPPH solution (0.1 mM) was added and shaken well. Lastly for the blank, 2 ml of distilled water was added with 2 ml of DPPH solution and mixed in a test tube. All prepared solutions were stored for 30 minutes in the dark and the absorbance was determined against the blank at 517 nm.
After 30 minutes of incubation, the absorbance was read at 517 nm for each sample on the UV/Vis Spectrophotometer. All data was converted into percentage of radical scavenged.

B. Nitric Oxide Radical Scavenging Assay
Approximately 1 ml of 10 mM sodium nitroprusside in phosphate buffer pH 7.2 was mixed with 1 ml of TSCL and TSCS of different concentrations (5, 10, 20 µg/ml). The mixture was incubated at 30°C for 150 minutes, followed by mixing with 1 ml of pre-prepared Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid). For the positive control, 1 ml of 10 mM sodium nitroprusside was mixed with 1 ml of Ascorbic acid of different concentrations (5, 10, 20 µg/ml). It was also incubated for 150 minutes, followed by mixing with 1 ml of pre-prepared Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dichloride, and 2% phosphoric acid).
For the blank, 1 ml of ethanol was used instead of the extracts or the ascorbic acid and all chemicals were retained. For all prepared solutions, the absorbance was recorded at 550 nm using a UV/Vis Spectrophotometer.

Data Processing and Analysis
All data were reported as mean ± standard deviation for three trials. The data were analyzed using one-way variance analysis (One Way ANOVA) followed by t-test for pairwise comparison of the (%) inhibition in each sample solution at $p < 0.05$. Correlation between total flavonoid content and the anti-arthritic and antioxidant activities was determined by Pearson product moment correlation.

Results and Discussion
Percentage yield of the two extracts was calculated. For 300 grams of sample, the percentage yield for orange and lemon peels were 20.5% and 7.8%, respectively. This shows that orange peels yield more extracts than the lemon peels.

Chemical Characterization
The citrus peel wastes were analysed in a Fourier transformed infrared spectrophotometer (FTIR) together with the fresh peels for comparison purposes. The FTIR shows the chemical bonds and functional groups present in a particular sample. The figure below shows the spectra for both the fresh lemon and orange peels as compared to the peel waste. There is no difference in the spectra which can prove tat the compounds present in the fresh peels are still present in the waste.

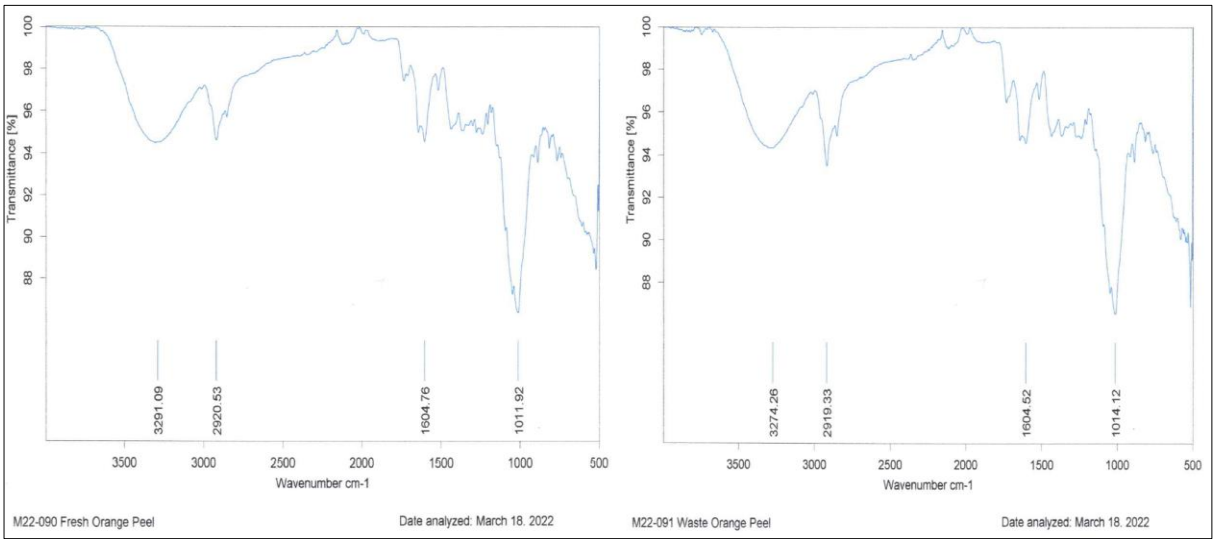

Fig 3: FTIR spectra of fresh orange peel and the orange peel waste

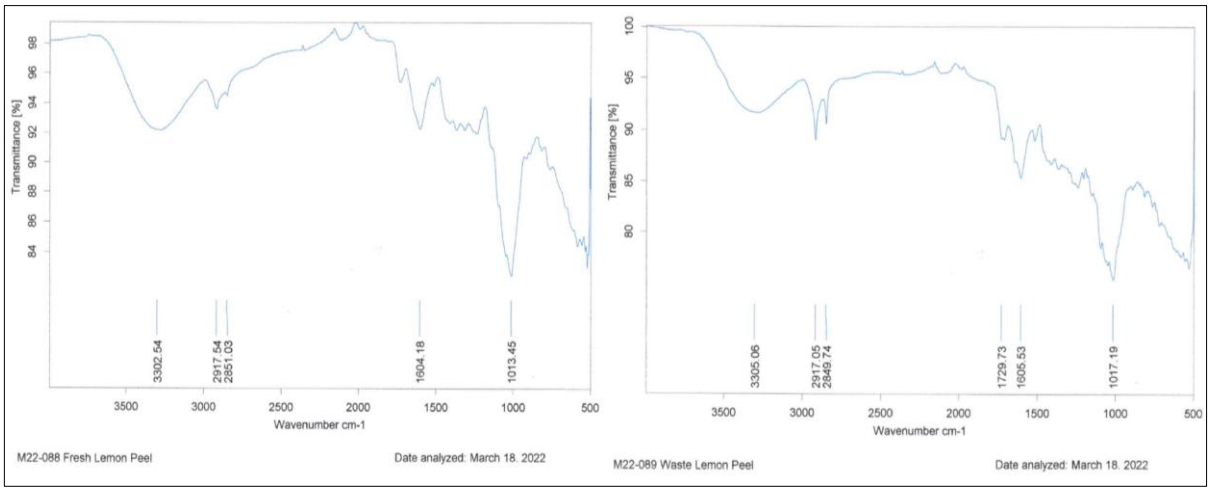

Fig 4: FTIR spectra of fresh lemon peel and the lemon peel waste

Phytochemical Analysis

The phytochemical evaluation of plant extracts was done to demonstrate the existence of secondary metabolites or the bioactive substances which may be responsible for the two investigated activities - antioxidant and anti-arthritic. As seen in Table 1, the phytochemicals present both in lemon and orange peels are alkaloids, tannins, flavonoids and phenols.

Table 1: Phytochemicals present in the peels of lemon and orange

Phytochemical/s	Lemon	Orange
Alkaloids	present	present
Tannins	present	present
Phenols	present	present
Flavonoids	present	present

Determination of flavonoid content

The extracts were also evaluated for flavonoid content. Flavonoid obtained from plants have shown to have high antioxidant activity since they possess a broad spectrum of chemical and biological activities which include the radical scavenging activity [7]. The flavonoid content of lemon and orange was 481.12mg and 445.35mg rutin per gram of dry weight extract. As seen in Table 2, results revealed that there is no significant difference between the two flavonoid concentrations do not vary.

Table 2: Flavonoid Content

Test Sample	Mean Absorbance	T-Value	P-Value	Remarks	Decision on Ho
Lemon	0.069	0.918	0.411	Not-Significant	Do not reject Ho
Orange	0.068				

In a study conducted by Ibrahim and Hamed, results show that the flavonoid content of fresh lemon and orange peels through oven-drying were 241.20 mg and 317.41 mg. This shows that citrus peels have high concentration of flavonoids [7].

Evaluation of Antioxidant Activity

The anti-oxidant activity was evaluated using two different tests: the 2, 2 Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay and nitric oxide radical scavenging assay.

DPPH is a stable organic free radical with an absorption band at 517 nm. It loses the purple color that absorbs at this wavelength when accepting an electron or a free radical species, which results in a yellow color [8].

As seen in Figure 5, Lemon 500 ug/mL has the highest antioxidant activity with 85.6% inhibition compared to the standard, Ascorbic Acid with 84.6%. Orange 500ug/mL also has 83.5% inhibition.

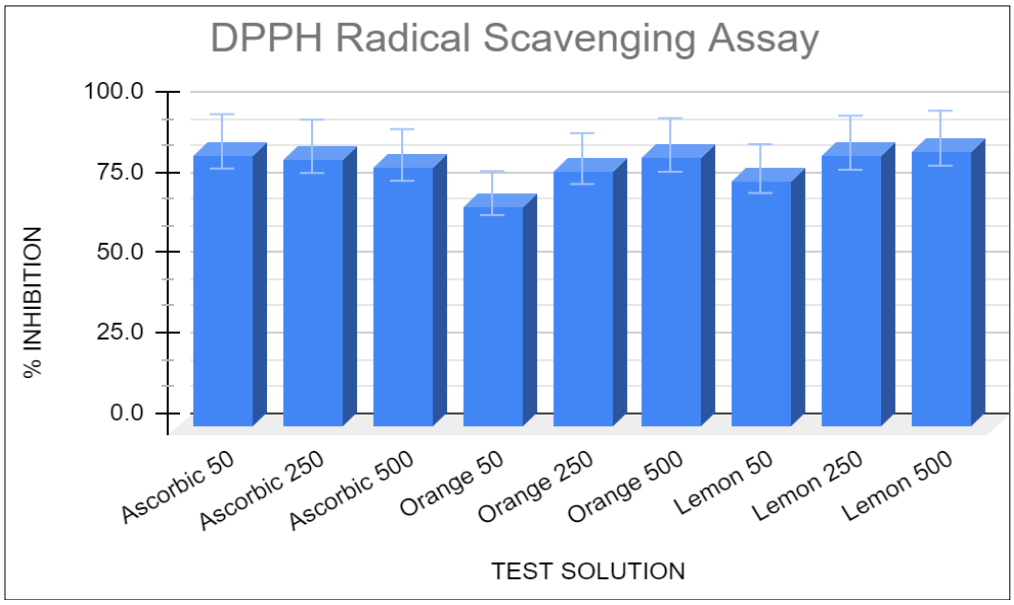

Fig 5: DPPH Radical Scavenging Assay

The One-Way Analysis of Variance reveals that there is a significant difference with the percent inhibition of the different test solution with p value of 0.002. Post hoc analysis using T-test was also conducted to determine which among the test solution is comparable with the commercially available, Ascorbic Acid.

Therefore, citrus peel extracts exhibited good radical scavenging activity, which is comparable to the previous findings reported by Abrosca *et al.* (2007) and Sultana *et al.* (2008) for citrus peels [9, 10].

On the other hand, in vitro quenching of nitric oxide (NO) radical is also one of the methods that can be used to determine antioxidant activity. The procedure is based on the principle that sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions [11]. Absorbance will be measured and the decrease will indicate antioxidant activity.

As seen on Figure 6, the sample with the highest scavenging activity is Ascorbic 5ug/mL with 74.7% followed by Orange 5ug/mL and Lemon 5ug/mL with 70.4 and 71.4% percent inhibition, respectively.

The One-Way Analysis of Variance reveals that there is a significant difference in the concentration of test solutions containing Orange peels with a p value of 0.009.

With the percent inhibition seen in the different test solutions, both lemon and orange exhibit antioxidant activity. Also, with the lowest concentration showing promising antioxidant activities, this shows that even low dose of lemon and orange can already exert significant antioxidant activities. According to Nabavi *et al.* (2009), phenols and polyphenolic compounds, such as flavonoids, have been shown to possess significant antioxidant activities.

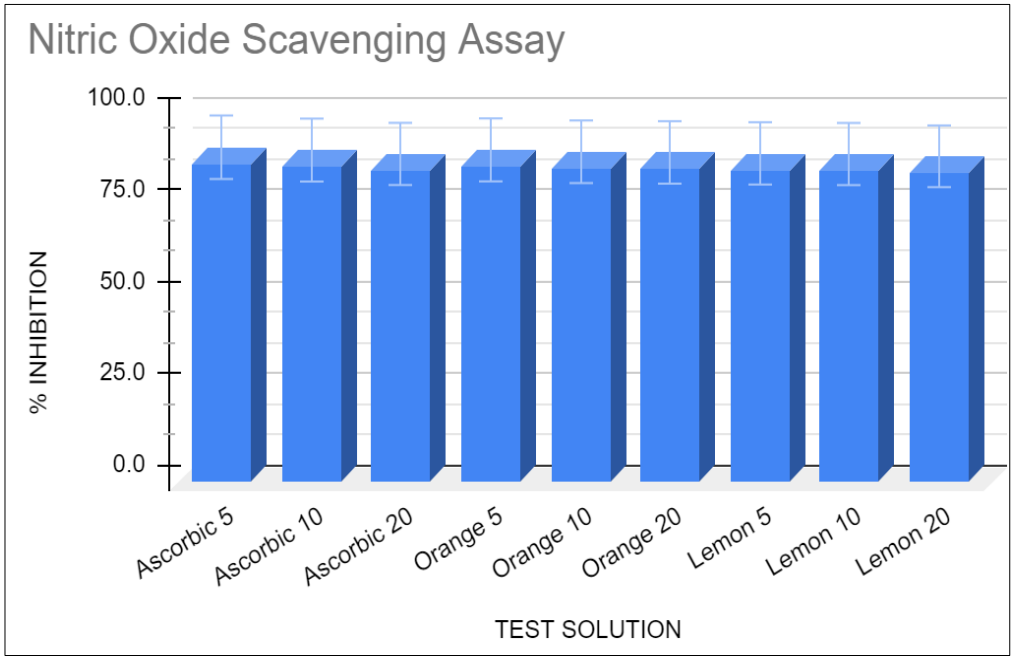

Fig 6: Nitric Oxide (NO) Scavenging Assay

Evaluation of Anti-arthritic Activity

For the in vitro anti-arthritic activity, two different tests were conducted: protein denaturation method and proteinase enzyme inhibition method. Bovine Serum Albumin (BSA), a water-soluble monomeric protein [12] when heated undergoes denaturation and expresses antigens associated to Type III hypersensitive reaction and which are related to diseases such as serum sickness, glomerulonephritis, rheumatoid arthritis and systemic lupus erythematosus [13].

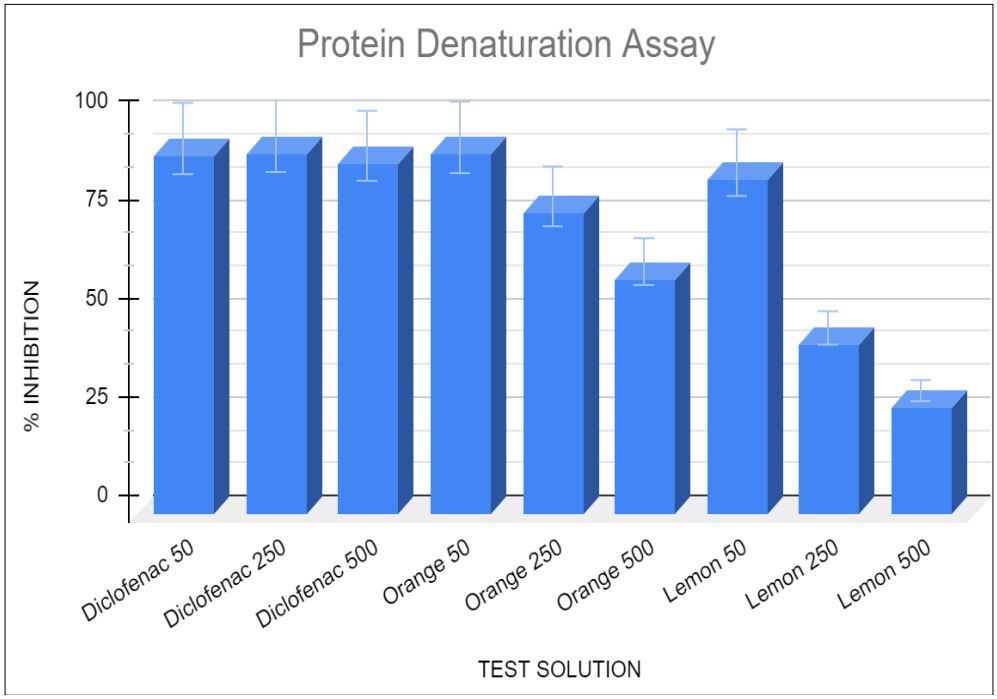

Fig 7: Protein Denaturation Assay

As seen on Figure 7, the test solution with the highest protein denaturation activity is orange 50ug/mL with 90.8% followed by Diclofenac 50ug/kg. Lemon 50ug/kg has low anti-arthritic activity with only 84.4% inhibition when compared with orange and the commercially available, Diclofenac.

Since the lowest concentration exhibited significant anti-arthritic activity, further analysis revealed that the percent inhibition of other concentration is not comparable with lower concentrations. Hence, the lower the concentration of the fruit peel, the higher its anti-arthritic activity.

On the other hand, a protease (also called a peptidase or proteinase) is an enzyme that performs proteolysis; protein catabolism by hydrolysis of peptide bonds. Proteases are responsible for enzymatic cleavage of peptide bonds, which is a basic requirement for completion of diverse biological processes. The proteolytic activity of proteases must be rigorously controlled to avoid inappropriate degradation of proteins. Imbalance in regulation of proteolytic activity can be found in a wide range of diseases, including cancer, rheumatoid arthritis (RA) and osteoarthritis (OA)

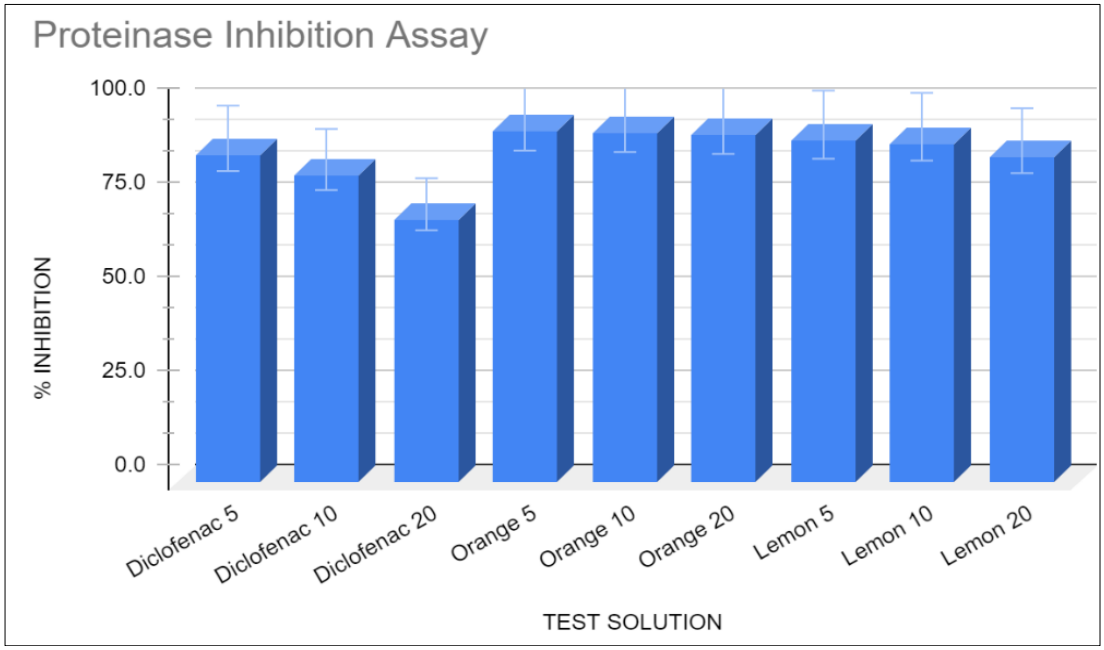

Fig 8: Proteinase Inhibition Assay

Figure 8 shows that the test solution with the highest anti-arthritic activity is seen on all the concentration of orange peels with 929%, 92.5% and 91.9% inhibition for 5ug/mL, 10ug/mL and 20ug/mL, respectively. Lemon 5ug/mL has the highest activity when compared with other lemon concentrations. Diclofenac this time has the lowest anti-arthritic activity.

The One-Way Analysis of Variance reveals that there is no significant difference with the different concentrations of Diclofenac and Lemon. On the other hand, concentrations for orange peel are found to be statistically significant with each other.

Further analysis revealed that the lowest concentration of orange and lemon are found to be statistically significant with the higher concentration which means that their activities are not comparable. Similar to the protein denaturation assay, the lower concentration of lemon and orange are the ones that have exhibited goof anti-arthritic activity.

Lastly, a correlation study was also conducted to determine if the flavonoid content of the two extracts have any relationship with their anti-oxidant and anti-arthritic activities.

Table 11 shows that the flavonoid content and the anti-arthritic and antioxidant activities have perfect correlations which means that the higher flavonoid content seen in the two extracts are responsible for their excellent activities.

Conclusion

The main objective of this study is to determine if the fruit wastes-lemon and orange peels possess still possess antioxidant and anti-arthritic activity. Phytochemical screening was conducted and it was revealed that lemon and fruit peels from waste still possess bioactive compounds namely flavonoids, tannins, phenols, and alkaloids. In the literature, these bioactive compounds are responsible for different pharmacologic activities.

It was also revealed that the orange and citrus fruit peels still contain high amounts of flavonoids, a bioactive compound contributing to antioxidant and anti-arthritic activities.

In the in vitro assay of antioxidant activity, two different methods were used. In the DPPH assay, the 500ug/mL exhibited higher antioxidant activity while in the nitric oxide scavenging assay, the lowest concentration (5ug/mL) exhibited the highest activity. This means that the lowest concentration already has significant antioxidant activity.

IC50 values of lemon and orange for the antioxidant activity ranged from 12.52 mg to 184.85 mg and 8.03mg to 211.61 mg, while the IC50 for the anti-arthritic activity of lemon and orange ranged from 15.71 mg to 207.66 mg and 10.25mg to 261.87 mg, respectively. The lower the IC50 means that the particular fruit peel has better activity.

To determine anti-arthritic activity, two methods were also utilized. Lower concentration of the extracts was found to have good anti-arthritic activities both in the protein denaturation and proteinase inhibition assay.

The flavonoid content also has perfect correlation with the antioxidant and anti-arthritic activities which signifies that this bioactive metabolite present in the peels may have been responsible for the two pharmacologic activities.

Acknowledgment

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Disclosure of Conflict of Interest

The authors declare that they have no conflict of interest.

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