Extraction and determination of flavonoid compounds in citrus fruit waste

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Abstract. Citrus is a globally cultivated fruit crop that has been heavily industrialised to manufacture food products. It is commonly sought out for its nutritional benefits. However, parallel to rapid industrialisation, parts of the crop's physical composition (inner and outer peel, seeds etc.) are often discarded to the environment as waste. Recent advancement in technology has led researchers to look for alternatives to recover potential therapeutic compounds from citrus fruit waste, directly extending the life of the waste and indirectly solving waste management concerns. Citrus fruit peels are especially rich in flavonoid compounds, a subclass of the many phytochemicals largely present in the body of the fruit. Flavonoid compounds have the capacity to be act as antioxidants, leaving room for the potential of the flavonoids present in fruit waste to be commercialized as a natural bioresource. This study aims to extract and recover the flavonoid compounds present in the peels of citrus fruits calamansi (*Citrus microcarpa*), kaffir lime (*Citrus hystrix*), and key lime (*Citrus aurantiifolia*) via ethanolic extraction and test the presence of recovered flavonoid compounds via alkaline reagent test. In addition, this study also aims to measure the antioxidant activities of all three citrus fruit peel samples via the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. This study hopes to shed light on the therapeutic potential of citrus fruit peel waste as a bioresource which could benefit communities in the future.

1. Introduction

Citrus fruits are heavily commercialised globally and unsurprisingly, 18% of worldwide citrus crops are present in industrial processes [1]. Some parts of the crops consisting of peels, seeds and pulps often end up in landfills as they are deemed as waste at the end of the production line in citrus processing industries [2]. A rising concern of the commercialization of citrus fruit is the mismanagement of its waste. Bioactive compounds present in citrus fruit peels could alleviate pollution problems if they seep into water bodies as they raise the water's Biochemical Oxygen Demand, (BOD) thus subsequently harming aquatic life. Traditional methods including incineration and landfilling to eliminate discarded citrus waste proves to be inadequate as they fail to be energy efficient, environmentally friendly, and economically viable [1].

Polyphenols are the major target compounds present in the by-products of citrus fruit waste. Most scientific literature which exists mainly discusses the biological activities of fruit by-products on food applications for the health and well-being of the community. Depending on the technology used to process the crops, citrus peel waste makes up around 50% to 70% of the w/w of processed fruits [2]. Biological activities in fruits mainly occur in the peel rather than the more heavily consumed fruit pulp [3]. The peels of citrus fruits are rich in flavonoids, phytochemicals which exhibit antioxidant activity and are described as a dietary bioactive [4]. Recent developments in technology have opened new doors for us to properly treat citrus waste via appropriate extraction and purification techniques to recover valuable therapeutic compounds present in parts of the waste. These recovered compounds have the potential to serve as raw material for the benefit of many industries.

2. Methodology

A general three-stage methodology was utilized in the isolation, extraction, and quantification of antioxidant capacity of the flavonoid compounds extracted from peels of citrus fruits calamansi (*Citrus macrocarpa*), kaffir lime (*Citrus hystrix*), and key lime (*Citrus aurantiifolia*) obtained from discarced waste from local markets/food centres in Wangsa Maju, Kuala Lumpur. Prior to the extraction process of a flavonoids in the samples, the samples first went through a series of pre-treatment steps which consisted of drying citrus fruit peels in an oven at 40°C for 3 days in an oven prior to undergoing extraction. This drying step is crucial as it inhibits bacterial growth and increase yield. Next, the dried samples were grounded into finer particles before being dissolved in 60% ethanol solution with a sample to solvent ratio of 1:10 for 72 hours in a shaking incubator. The flavonoid contents of the samples were extracted via ethanolic extraction using 60% ethanol. Samples in the form of crude extracts were then filtered via vacuum filtration and the filtrate was further concentrated using a rotary evaporator. At the final stage of pre-treatment, the percentage yield of extraction Y (%) was calculated using the formula below:

$$Y\ (\%) = \frac{\text{mass of dried sample after extraction process}}{\text{dry mass of the fresh sample prior extraction}} \text{ x } 100 \qquad (1)$$

The presence of flavonoids was then screened via a simple alkaline reagent test where the colour changes of each sample were observed [5]. Ascorbic acid was chosen as the positive control for this experiment. Next, serial dilution was performed on each sample to obtain sample solutions of each fruit peels species in different concentrations of 0 μg/mL – 60 μg/mL in increments of 15 μg/mL. Next, the samples were tested via the DPPH assay where the colour change of each sample was observed to test each sample's capability to scavenge free radicals, suggesting their antioxidant capabilities [5]. The ability of the samples to reduce the purple-coloured DPPH radical solution was used to determine anti-radical activity. The scavenging effect was estimated as in Equation 2 where $A_{control}$ represents the absorbance of control and Asample represents the absorbance in the presence of the citrus fruit peel extract samples.

$$\text{DPPH radical scavenging activity } (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \text{ x } 100 \qquad (2)$$

Lastly, the antioxidant ability of each citrus fruit peel extract was measured via spectrometry using the HACH UV-VIS spectrophotometer DR6000. The spectrometer measured the absorbance of each sample. Each sample's absorbance was read at 517 nm and the blank used was absolute ethanol. After the absorbance data was read, a graph to percentage inhibition of DPPH against samples of different concentrations was plotted as shown in Figure 3.

The absorbance data was then used to measure the percentage of inhibition of DPPH and find the half-maximal inhibitory concentration, IC_{50} for each sample of different concentrations. Each sample's absorbance was analysed in triplicates for more accurate results.

3. Results dan Discussion

The percentage yield of each sample after extraction was calculated using Equation 1 and the results are tabulated in Table 1.

Table 1. Percentage yield of extraction (%).

Sample	Yield of extraction (g/g)
Calamansi (*Citrus macrocarpa*)	18
Kaffir lime (*Citrus hystrix*)	23
Key lime (Citrus *aurantiifolia*)	15

The highest yield of extraction as recorded in Table 1 is 23% which is the yield for Kaffir lime (*Citrus hystrix*) followed by 18% and 15% from Calamansi (*Citrus microcarpa*) and Key lime (*Citrus aurantiifolia*), respectively. Overall, the yields of each sample suggest that a lot of mass was lost during the extraction process. Possible reasons of lower extraction yield may include loss of some sample recovered during the whole vacuum filtration and drying process.

The results of the alkaline reagent test were as expected as depicted in Figure 1. In each test tube containing the samples (i) the colour of each sample turned into a vibrant yellow colour (ii) upon the addition of sodium hydroxide solution into the test tubes before subsequently decolorizing after the addition of dilute hydrochloric acid. This indicates that flavonoids are present in each citrus fruit peel sample.

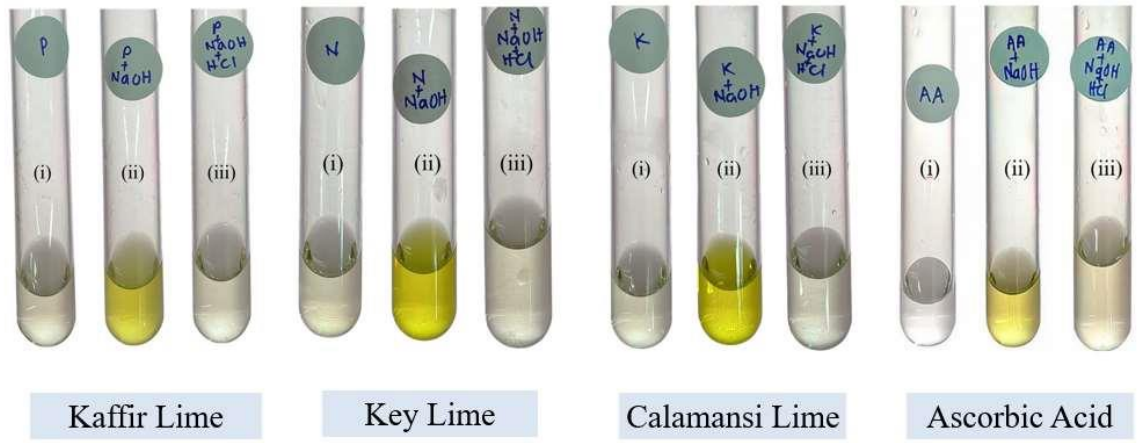

Figure 1. Test tubes showing the colour change of the citrus fruit peel extracts in the alkaline reagent test

The antioxidant activities of flavonoids present in all the samples of different concentrations were assessed via the DPPH assay. The colour change of the violet DPPH solution to a lighter yellow shade shows the radical scavenging activities of the flavonoids in each sample. Figure 2 shows the results of the DPPH radical scavenging assay on kaffir lime extracts of different concentrations.

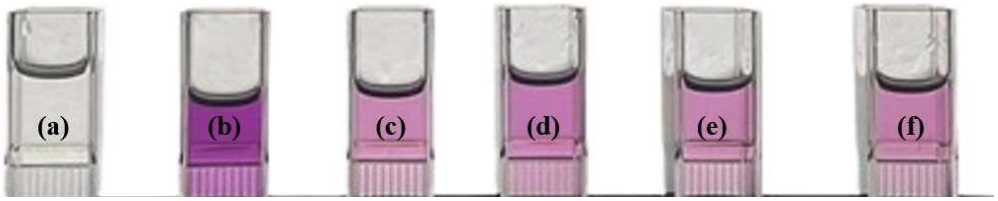

Figure 2. (a) Ethanol solution (blank) (b) Standard DPPH solution (positive control) (c) 15 µg/mL Kaffir lime (*Citrus hystrix*) extract + DPPH solution (d) 30 µg/mL Kaffir lime (*Citrus hystrix*) extract + DPPH solution (e) 45 µg/mL Kaffir lime (*Citrus hystrix*) extract + DPPH solution (f) 60 µg/mL Kaffir lime (*Citrus hystrix*) extract + DPPH solution

As observed in all the three figures below, the violet colour of each sample once added with DPPH solution turned into a lighter shade of violet compared to the standard DPPH solution used as the control. The samples did not turn completely yellow even after inhibition for 30 minutes in a dark room. This lack of colour change could be due to the aliquots of each sample being of too high concentration or the lack of time the DPPH-added samples were left to be incubated in the dark for a proper reaction to occur. However, it can be suggested that the radical scavenging activity of the flavonoids in each sample are still present despite not exhibiting obvious colour change.

A graph of DPPH inhibition against samples of different concentrations was plotted as in Figure 3 after the absorbance of each sample was read.

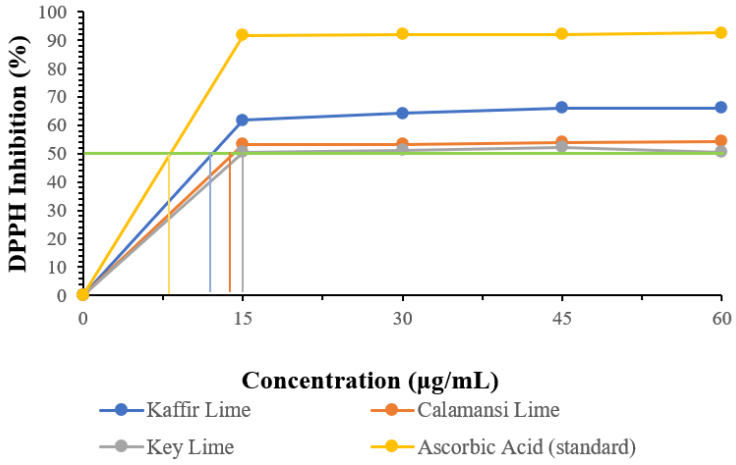

Figure 3. DPPH Radical Scavenging Activity of Citrus Fruit Peels Extracts and IC_{50} values

From the graph in Figure 3, it is demonstrated that the percentage DPPH inhibition (%) of all the four samples differ in a dose-dependent way. Each sample consistently showed the highest percentage DPPH inhibition at the highest concentration of extract, 60 µg/mL. The IC_{50} values were determined graphically, and it is observed that the ascorbic acid solution had the lowest IC_{50} values, followed by the

extracts of kaffir lime, calamansi lime, and key lime, with values of 7.2, 10.5, 14.6, and 15 μg/mL respectively. Ascorbic acid (standard) remains the strongest antioxidant as its IC_{50} value is read to be 7.2 μg/mL. Hence, we can conclude from the graph that ascorbic acid remains the strongest antioxidant among all the samples. However, extracts of kaffir lime, calamansi lime, and key lime also fall under the category of a strong antioxidant as it falls between the range of 10 μg/mL - 50 μg/mL. Furthermore, the radical scavenging activities varied according to the extract of each citrus fruit variety as expected.

4. Conclusion

In conclusion, all three fruit peels of citrus fruits calamansi (*microcarpa*), kaffir lime (*hystrix*), and key lime (*aurantiifolia*) studied in this work were successfully extracted via ethanolic extraction and the presence of flavonoids in the samples were also shown via the alkaline reagent test. Moreover, this study proved that all three citrus fruit peels exhibited great antioxidant activities which have the potential to be replicated and replace the antioxidants which currently exist in the market. However, because they are natural compounds, their compositions and bioactivity may not match synthetic antioxidants. Hence more research should be done on creating stricter standards for effective recovery of bioactive therapeutic compounds from fruit waste. Most studies on extraction of bioactive compounds from citrus fruits are done in the west using more common fruits such as oranges, lemons, and grapefruit. This research hopes to contribute findings on bioactive compound extraction on citrus fruits readily available in South-East Asia.

5. Acknowledgement

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6. References

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