Isolation of Antioxidant Compound by TLC-based Approach from Limau Kasturi (Citrus macrocarpa) Peels Extract

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ABSTRACT

This work reports a study on isolation of phenolic compound extraction from the peel of limau kasturi by screening using different solvent extracts for antioxidant activity. The availability of phenolic compounds in limau kasturi peels as a source of antioxidant is important for the protection of DNA from cancer-causing damage. In this research, the crude extract of limau kasturi was separated using analytical thin-layer chromatography (TLC). The solvents applied TLC developing systems were also tested. The extracted compounds were tested for their potential antioxidant activity using TLC-bioautography method. The mobile phase used in this experiment was a combination of polar and non-polar solvents such as ethyl acetate, diethyl ether, chloroform, methanol, acetone, hexane and water at different ratios. While for the selection of the best solvent extract, methanol, ethanol, acetone, hexane and water extract were tested. The active compound was identified by comparing its colour and Rf (retention factor) value with rutin standard. The results concluded that ethyl acetate, methanol and hexane were the best mobile phase system in this assay with ratio of 5:1:2. In fact, for the best solvent extract, methanol was showed high Rf value, antioxidant activity, and TPC with value of 0.669, 82.18 %, and 69.41 mg GAE/g DW, respectively. Thus, methanol was chosen as the favorable solvent for the antioxidant compound extraction.

Keywords: Limau Kasturi; Phenolic Compounds; Thin-layer Chromatography; Bioautography; Antioxidants

INTRODUCTION

In Malaysia, limau kasturi is one source of economic income. In 2013, a plan was made by the local people in Selangor to increase income residents through the production and sale of agricultural limau kasturi for the next 10 to 15 years. This due to the potential usage of limau kasturi to be used as limau kasturi cordial, limau kasturi juice, cosmetic products based limau kasturi extract. However, due to extensively used in this application, the peels are often removed as waste and their disposal will cause environment problems [2]. In fact, in limau kasturi peels are contained of phenolic compounds which have the capability of reducing the risk of degenerative diseases by reduction of oxidative stress (the main mechanism responsible for cardiovascular diseases) and inhibition of macromolecule oxidation apart reducing bad cholesterol in the blood circulatory system [1,9]. In research, the phenolic...
compounds can be separated using thin layer chromatography (TLC). In 1946, it was the first time that antibiotic was detected using paper chromatography and bioautographic [3]. This technique is widely used because it is ease of use, wide application to a great number of different samples, high sensitivity, speed of separation, and relatively low-cost [8]. This method is also to avoid the time-consuming isolation of inactive compounds. It is involved combination of chromatographic separation and in situ activity determination facilitating the localization and target-directed isolation of active constituents in a mixture [7]. In addition, the antioxidant compounds can be detected using bioautography method which is quick, low-cost, and allows a better bioassay directed fractionation of bioactive compounds [4]. The potential usage of limau kasturi peels have come to our interest to investigate the feasibility of extracting bioactive phenolic compounds from these waste materials. As far as concerned, the research that focused on the recovery of limau kasturi peels as a source of phenolic for antioxidants is still in its infancy.

Materials and Methods

2.1. Sample preparation:
Limau kasturi were purchased from local market in Kangar, Perlis. The fruits were peeled manually and heated at 45°C for 24 h in a convection oven and the dried peels were sieved to fine powder for one hour. The ground powders were then packaged using a tight plastic sealer.

2.2. Chemicals:
Methanol, ethanol, acetone, water, hexane, ethyl acetate, chloroform, diethyl ether, hydroxytoluene (BHT), DPPH (1,1-diphenyl-2-pycrilhydrazol), rutin.

2.3. Soxhlet extraction procedure:
About five grams of sample were packed in a nylon fabric before be placed in an extraction chamber, which is suspended above a flask that containing of solvent and below a condenser. The flask then was heated using heating mantle until the solvent is evaporates and move up into the condenser. The solvent vapor then is converted into a liquid form that trickles into the extraction chamber containing the sample. The time taken for the solvent to evaporate will be regulated. At the end of the process, the round bottom flask was taken to undergone separation process using rotary evaporator.

2.4. Application of aluminum thin-layer chromatography (TLC) assay:
2.4.1. TLC bioautography for antioxidant activity:
Bioautography screening was analyzed in order to check the antioxidant activity of separated compounds on TLC plate. An aliquot of crude extract was spotted onto the silica gel plate and allowed to dry for two minutes. For the evaluation of antioxidant capacity, the developed air dried plate was sprayed with methanolic solution of 2.54 mM DPPH antioxidant reagent. Then, the plates were air-dried again. Bands with the antioxidant capacity were detected as yellow bands on purple background [6].

2.4.2. Analytical TLC:
This method was carried out according to Rajauria and Abu-Ghanam [6] with some modification. The analysis was carried out on TLC plates (20 x 20 cm with 0.2 mm thickness, silica gel GF₂₅₄, Merck, Darmstadt, Germany) cut from the commercially available sheets. An aliquot of crude extract was spotted onto the silica gel plate and allowed to dry for two minutes. Then, the plate was developed with less than 10 ml of mobile phase in a beaker at room temperature. After that, the developed plate was dried under normal air and the spots were visualized under visible light. The Rf (retention factor) values of isolated compounds and standard were calculated and compared.

2.4.2.1. Type of mobile phase:
The mobile phase used to developed the separation of compounds was verified into seven combinations (hexane:ethyl acetate:methanol, chloroform:methanol:hexane, chloroform:methanol:water, ethyl acetate:methanol:hexane, diethyl ether:hexane, diethyl ether:hexane:water).

2.4.2.2. Ratio of mobile phase:
The selected mobile phase with high Rf value was verified into seven ratios (5:1:2, 1:5:2, 1:2:5, 2:1:5, 3:3:3, 5:2:1).

2.4.2.3. Type of solvent extractor:
The crude extract with different solvent extractor (methanol, ethanol, acetone, hexane, water) was developed using a selected ratio of mobile phase to separate the compound. The separated compounds were compared with the standard rutin.

2.5. Antioxidant Activity Determination:
Determination of antioxidant activity will be prepared according to FeAs et al. with some modification. DPPH solution will be prepared by dissolving 15.96 mg of DPPH in 675 ml of 80 % methanol to give 6x10⁻⁵ M of concentration. 300 μl of sample with various parameters condition will be mixed with 2.7 ml of DPPH solution and shake. Then, the mixture will be left for 60 min in dark at room temperature (23°C ). Methanol 80 % will be used as blank solution and control solution is only DPPH solution. Reduction of DPPH radical will be
determined by measuring absorption at 517 nm and will be triplicate. Radical scavenging activity will be calculated using this formula:

\[ \text{% RSA} = \left( \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \right) \times 100 \]

IC50 values will be estimated from % RSA against parameters condition plot. BHT will be used as standard.

Results and Discussion

3.1. Thin-layer chromatography bioautography:

In this study, the extraction of phenolic compounds from the peels of limau kasturi was carried out using soxhlet extraction. The parameter conditions were fixed at equal-volume of solvents for 2 hr 30 min of extraction per sample. For the temperature, each sample was tested at boiling point of the solvents. At first, the standard of rutin was tested for the potential biological properties using TLC bioautography. For the screening of antioxidant capacity, the developed plate was sprayed with DPPH reagent. As the result, a single compound of rutin standard has been seen from Figure 1(a) (refer appendixes). For the temperature, each sample was tested at boiling point of the solvents. At first, the standard of rutin was tested for the potential biological properties using TLC bioautography. For the screening of antioxidant capacity, the developed plate was sprayed with DPPH reagent. As the result, a single compound of rutin standard has been seen from Figure 1(a).

3.2. Thin-layer chromatography analysis:

The crude extracts were separated using analytical TLC. In order to get the best separation, the standard of rutin was tested on TLC plate by screening the conditions of parameters (type of mobile phase and ratio of mobile phase). The chromatographic profile of the rutin standard and crude extracts was visualized under ultraviolet (UV) lamp of 254 nm and single compound of rutin was detected and compared. Figure 2(a) (refer appendixes) presents the separation of rutin standard using different combination types of mobile phase. There are seven combinations types of mobile phase (hexane: ethyl acetate: methanol, chloroform: methanol: hexane, chloroform: methanol: water, ethyl acetate: methanol: hexane, diethyl ether: hexane, diethyl ether: hexane; methanol; and acetone: methanol: water). From the result, only two combinations showed inappropriate separations which are chloroform: methanol: water and acetone: methanol: water and the highest Rf value (Rf = 0.965) was measured at TLC plate using ethyl acetate:methanol:hexane (5:1:2).

Figure 2(b) presents the separation of rutin compound using mobile phase ethyl acetate:methanol:hexane at different ratios. There are seven ratios has been varied (5:1:2, 1:5:2, 1:2:5, 2:1:5, 2:5:1, 3:3:3, and 5:2:1). There are three ratios of mobile phase that showed unfavorable separation which are 1:2:5, 2:5:1 and 3:3:3.

The result for the separation of the crude extract on TLC plates was visualized under visible light in Figure 2(c). The chromatographic profile of the crude extract showed the presence of three colour bands on the TLC plate which are brown, green and yellow. The standard of rutin displayed brown colour on TLC plate. So that, this is proved that the phenolic compounds are present in limau kasturi peels extract.

The green and yellow colors may indicate the presence of chlorophyll and carotenoids pigments in the peels. From the brown bands exhibited on TLC plate for different solvents crude extract, there are no separation detected on hexane extracts, while the highest Rf value was recognized and measured at value of 0.669, followed by ethanol (Rf = 0.656), acetone (Rf = 0.637) and water (Rf = 0.632). This is evinced that methanol is the best solvent types to extract the peels of limau kasturi.

3.3. Antioxidant activity of Citrus macrocarpa peels extract:

The characteristic of antioxidant activity is related to the potential of crude extract which act as a donor for hydrogen atoms or electrons in the transformation of DPPH radical into its reduced form DPPH-H (which is measured spectrophotometrically) [5]. The results in Figure 3 (refer appendixes) showed the antioxidant activity of the methanol, ethanol, acetone, hexane and water extract peels of limau kasturi were measured using DPPH method. The results of DPPH scavenging activity of limau kasturi was compared with butylhydroxytoluene (BHT) as a reference standard which denoted that it has slightly lower antioxidant activity compared to reference standard. The methanol peels extract had showed have the highest antioxidative activity followed by ethanol, acetone, water and hexane. The methanol crude extract of limau kasturi peels was able to reduce the stable radical DPPH to yellow-
colored DPPH-H reaching 82.18% of DPPH scavenging effect at its pure concentration. The percentage antioxidant activity of BHT standard is 83.96% which slightly higher than methanol extracts. While the percentage for ethanol, acetone, water and hexane are 79.52%, 72.33%, 62.61% and 18.73%, respectively. Therefore, methanol was chosen as the favorable solvents for phenolic antioxidant extraction.

3.4. Total phenolic content (TPC) determination:
For phenolic extractions from botanical materials, polar and non-polar solvents such as methanol, ethanol, acetone, water and hexane were used as solvents. Figure 4 (refer appendices) presents the effect of solvent type on total phenolic content from the peels of limau kasturi which is aqueous methanol showed the highest TPC value (69.41 mg GAE/ g DW) compared to ethanol, acetone, water and hexane. Based on the polarity index, the TPC of acetone type is slightly low than methanol although they sharing the same polarity. This showed that phenolic compound from limau kasturi peels present wide coverage of polarity. In addition, methanol is categorized under GRAS (Generally Recognized as Safe) which is applicable in view of the application in food system (Chan et al., 2009). Thus, methanol was chosen as the extraction solvent.

4. Conclusion:
For overall view, phenolic compounds was detected exist in the peels of limau kasturi due to TLC bioautography test. All the solvents crude extract (methanol, ethanol, acetone, water and hexane) showed the presence of phenolic compounds. For the quantitative test, all types of solvent were measured using TLC analysis, antioxidant activity and TPC test. From the result, all test was indicate that methanol is the best solvent extractor (Rf value = 0.669, antioxidant activity = 82.18%, TPC value = 69.41 mg GAE/ g DW). The importance of solvents screening conditions in industry is to produce high yield of production and make the process operates efficiently.

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Author’s contribution:
P.M. Dr. Dachyar Arbain, Madam Nor Helya Iman Kamaludin and Madam Nurul Ain Harmiza developed the idea and had an important role in the result and material section.

References
Appendices:

Fig. 1: TLC bioautography of (a) rutin standard; (b) methanol extract; (c) ethanol extract; (d) acetone extract; (e) hexane extract; and (f) water extract from the peels of limau kasturi.

Fig. 2(a): The separation of rutin standard at different combinations types of mobile phase (1=hexane:ethyl acetate:methanol, 2=chloroform:methanol:hexane, 3=chloroform:methanol:water, 4=ethyl acetate:methanol:hexane, 5=diethyl ether:hexane, 6=diethyl ether:hexane:methanol, and 7=acetone:methanol:water).

Fig. 2(b): The separation of rutin standard at different ratios of ethyl acetate: methanol: hexane (1=5:1:2, 2=1:5:2, 3=1:2:5, 4=2:1:5, 5=2:5:1, 6=3:3:3, and 7=5:2:1).

Fig. 2(c): The separation of crude extracts (1=methanol, 2=ethanol, 3=acetone, 4=hexane, 5=water) using ratio mobile phase of ethyl acetate: methanol: hexane (5:1:2).
Fig. 3: The antioxidant activity of the methanol, ethanol, acetone, hexane and water extract peels of limau kasturi were measured using DPPH method.

Fig. 4: Total phenolic content for type of solvent crude extracts (methanol, ethanol, acetone, water and hexane).