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Influence of Solvent Polarity and Conditions on Extraction of Antioxidant, Flavonoids and Phenolic Content from *Averrhoa bilimbi*

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Abstract: This paper presents the influence of solvent polarity and extraction conditions on the extraction of total flavonoid, total phenolic and antioxidants from *Averrhoa bilimbi*. The experiment was performed using a different solvent at different extraction conditions, including extraction time (15-240 min), temperature (30-70 °C) and agitation speed (50-300 rpm). Results showed that yields of extraction varies with solvent polarity. Extraction using 50% aqueous methanol gives the highest antioxidant activity and flavonoid content. The highest total flavonoid content (193.3 μg quercetin equivalent/g dry weight), total phenolic content (717.8 μg gallic acid equivalent/g dry weight) and antioxidant activity (77%) was achieved using 50% methanol, at 70 °C and agitation speed of 300 rpm. This work may be useful for obtaining higher bioactive compounds during the extraction process of *A. bilimbi*.

Key words: Extraction, flavonoid, belimbing buluh, phenolic, antioxidant.

1. Introduction

Averrhoa bilimbi (vernacular name: belimbing buluh) is traditionally used in Malaysia for treatment of cough, cold, itches, boils, rheumatism, syphilis, diabetes, whooping cough and hypertension [1]. Previous scientific studies revealed that extract of *A. bilimbi* contained many useful bioactive compounds such as amino acids, citric acid, cyaniding-3-O-β-D-glucoside, phenolics, potassium ion, sugars and vitamin A [2-4]. It is known to have excellent source in bioactive components with different medically useful functional properties. *A. bilimbi* is known to have high antioxidant content, although, the physicochemical characteristics of *A. bilimbi* depend on its maturity stage [3].

Effectiveness of functional food derived from *A. bilimbi* in preventing diseases depends on the bioavailability of the active ingredients. The first step to recover and purify bioactive compound from plant materials involves an extraction process. The yield of bioactive compound in the extract is dependent on the solvent used, extraction method and condition. Successful extraction of bioactive compounds from plant material is largely dependent on the type of solvent used. During extraction, solvent diffuses into the solid plant material and solubilize compounds with similar polarity [5]. It is known that extracts from the same plant material may vary widely with respect to their antioxidant, phenolic and flavonoids concentrations and activities. The maceration technique was employed by several researchers [2, 3, 6] to extract the polyphenol and antioxidant from *A. bilimbi*. However, none of these studies considered the effect of varying solvent polarity and operating conditions to the yield of polyphenol and antioxidant

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extraction from *A. bilimbi* and hence this is the main objective of this work.

2. Materials and Methods

2.1 Chemicals

Methanol, ethanol, propanol, n-hexane, Follin reagent, sodium hydroxide, sodium nitrate and aluminum chloride were purchased from Merck (Darmstadt, Germany). Gallic acid and quercetin standards were from Acros Organics (NJ, USA). 2,2-diphenyl-1-picrylhydrazyl (DPPH) and sodium carbonate was obtained from Sigma-Aldrich (St. Louis, MO, USA). All chemicals and reagents used in the study were of analytical grade.

2.2 Preparation of Samples

The fruit samples were collected from Jaya Gading, Kuantan. Samples were washed thoroughly using tap water to remove dirt and other foreign materials. Samples were cut into slices and stored at minus 80 °C before lyophilization using freeze dryer (Biotron model Cleanvac 12S, Korea). The lyophilised samples were ground into powder form and kept at minus 20 °C prior to extraction process.

2.3 Extraction Procedures

Extraction of antioxidant and polyphenol from *A. Bilimbi* fruit was performed via maceration technique. Firstly, the ability of different solvent (n-hexane, ethanol, methanol, propanol, 50% propanol, 50% ethanol, 50% methanol) to extract polyphenol and antioxidant was investigated. It was carried out in a conical flask (wrapped with an aluminum foil) by mixing the lypolized fruit powder with solvents at ratio 1:60 (w/v). The experiment was performed at 50 °C, agitation at 200 rpm for 4 h. All extracts were centrifuged (Eppendorf 5810 R) at 10,000 rpm for 10 min and filtered using filter paper (Whatman No. 1) to obtain clear solution. They were kept at minus 20 °C prior to analysis. Analysis of variance (ANOVA) of the triplicate data was performed by using the data analysis tools in Microsoft Office 2010. The solvent which yielded the highest total phenolic content, flavonoid content and antioxidant activities was selected for the remainder of this study to evaluate the effect of extraction time and temperature.

2.4 Analysis

2.4.1 Total Phenolic Content

The total phenolic content (TPC) were analysed using Folin-Ciocalteau assay [7]. 40 µL of sample was mixed with 1.8 mL of 10-fold diluted Folin-Ciocalteau reagent. After 5 min, 1.2 mL of 7.5% sodium bicarbonate was added. The solution was homogenized and allowed to stand at room temperature for 60 min. Then, the absorbance was measured using a calibrated ultraviolet-visible spectroscopy (Hitachi U-1800, Japan) at λ = 725 nm. The result was expressed as mg gallic acid equivalent/g dried sample through the calibration curve with gallic acid.

2.4.2 Total Flavonoid Content

The total flavonoids content (TFC) were analysed using aluminum chloride colorimetric assay [8]. 1 mL of sample was diluted with 4 mL distilled water and added with 0.3 mL 5% (w/v) $NaNO_2$. After 5 min, 0.3 mL of (10% w/v) $AlCl_3$ was added. After 6 min, 2 mL of 1 M NaOH was added and the volume was made up to 10 mL immediately by the addition of 2.4 mL distilled water. The solution was mixed vigorously, and the absorbance of the solution was measured a calibrated ultraviolet-visible spectrocopy (Hitachi U-1800, Japan) at λ = 510 nm. The result was expressed in µg quercetin equivalent/g dried sample by comparison with the quercetin standard curve, which was made under the same condition.

2.4.3 Antioxidant

The free-radical scavenging (antioxidant) activity of the extract was measured by means of DPPH assay [9]. 0.1 mL of 10-fold diluted sample or methanol (as blank) was added to 3.9 mL of a 6×10^{-5} mol/L DPPH solution in methanol. The mixture was then shaken vigorously and incubated for 30 min at room temperature in a dark

room. The absorbance was determined using a calibrated ultraviolet-visible (Hitachi U-1800, Japan) at λ = 515 nm. Triplicate measurements were carried out and the percentage of DPPH scavenging activity was calculated according to Eq. (1):

$$\% \text{ DPPH scavenging activity} = \left(\frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}}\right) \times 100 \quad (1)$$

2.5 Statistical Analysis

ANOVA of the triplicate data was performed by using the data analysis tools in Microsoft Excel 2010, and a least significant difference (LSD) test was used to compare the means with a confidence interval of 95%.

3. Results and Discussion

3.1 Effect of Solvents

Effect of solvent type on the extraction yield was studied by fixing the temperature at 50 °C and agitation speed of 200 rpm for 4 h. Results in Table 1 show that aqueous solvent is preferable for phenolic, flavonoid and antioxidant extraction from *A. bilimbi* as opposed to pure solvent with lower polarity such as n-hexane and propanol. Pure solvent such as methanol and ethanol, which has the polarity index of around 5.1 and 5.2 yielded a much higher yield of TFC, TPC and antioxidant content. Addition of water, which has the polarity index of 9.0 enhanced the solubility of both the methoxylated and hydroxylated compounds and hence improved the overall extraction yield. The difference at $P > 0.05$ for TFC and antioxidant obtained using 50% propanol, 50% ethanol and 50% methanol are not significant and hence is not sufficient to reject the null hypothesis. Nevertheless, the results in Table 1 showed that 50% methanol has the highest combined yield of mean TPC (105.2 mg gallic acid equivalent/g dry weight), TFC (341.2 μg quercetin equivalent/g dry weight) and antioxidant (80.0%), and hence was employed for the remainder of this work.

Greater recovery of antioxidant compounds with methanol is consistent with previous studies [8, 10]. Polarity of solvents played a vital role in the extraction process since it would increase the solubility of antioxidant compounds [11]. Most of the pure solvents had a weaker extraction power in this study. Hexane was found to be the least effective to extract the bioactive compounds from *A. bilimbi* which may be attributed by its lowest relative polarity index. Aqueous solvent has a better extraction power than the pure solvent because mixing of non-polar solvent and water may increase the polarity index of solvents and hence may further enhance it extraction efficiency [12]. The result in this study showed that 50% aqueous solvent gave a better extraction of bioactive compounds compared to those of pure solvent. This is due to the difference in polarities of extracting solvents, which affects the solubility of chemical constituents in the sample and its extraction yield [13]. For instance, Cacace and Mazza [14] reported a notable increase of anthocyanins extraction from blackcurrants using aqueous ethanol until the water composition reached a maximum of 60% but decreased afterwards. Maceration extraction using 50% aqueous methanol gives the highest yield of phenolic

Table 1 Influence of solvent on TPC, TFC and antioxidant extraction from *A. bilimbi*.

Solvent type	TPC (mg gallic acid equivalent/g dry weight)	TFC (μg quercetin equivalent/g dry weight)	DPPH (%)
Ethanol	39.0 ± 4.8	336.7 ± 6.76	45.1 ± 1.5%
Ethanol (50%)	120.4 ± 10.8[a]	329.2 ± 40.0[b]	79.5 ± 6.9%[c]
Methanol	71.4 ± 7.5	231.7 ± 21.9	62.0 ± 5.9%
Methanol (50%)	105.2 ± 12.2	341.2 ± 14.3[b]	80.0 ± 3.2%[c]
Propanol	31.6 ± 4.3	235.7 ± 9.6	27.1 ± 1.8%
Propanol (50%)	129.6 ± 8.5[a]	286.7 ± 29.2[b]	79.0 ± 0.5%[c]
n-hexane	ND	43.2 ± 3.0	0.5 ± 0.05%

Means (three replicates) followed by at least one same letter are not significantly different ($P > 0.05$).

compounds and antioxidant from *A. bilimbi* compared to the other solvent studied.

3.2 Effect of Extraction Time and Temperature

Extraction time is an important parameter in the extraction process in order to optimize the recovery of bioactive component from the sample. The effect of extraction time was studied at 15-240 min with 50% aqueous methanol at 50 °C with agitation speed of 200 rpm. Results showed TFC increase from 200-300 µg quercetin equivalent/g dry weight when the extraction time prolonged from 15 min to 60 min (Fig. 1). However, extraction beyond 60 min does not show any improvement due to flavonoids degradation from exposure to heat, light and oxygen [15]. TPC also shows an increasing trend from 62 mg gallic acid equivalent/g dry weight to 110 mg gallic acid equivalent/g dry weight when the extraction time increases from 15 min to 180 min. Phenolic acids are in general less prone to degradation and hence may benefit from prolonged extraction time, nevertheless, no further gain by prolonging extraction beyond 180 min. Optimum extraction time for TPC and TFC differ due to different degrees of phenolic polymerisation, interaction of phenolics with other constituents and solubility of the phenolics which influence the equilibrium time between solid sample and solution [13]. Antioxidant is not affected significantly by extraction time with only about 10% increases by increasing extraction time from 15 min to 240 min. Similar result has been reported by previous researchers [16-17]. In this study, the 60 min extraction showed the highest effect on total flavonoids while for phenolics and antioxidants activity. It marked a significant difference from the highest one. Thus, 60 min extraction was selected for the remainder of this work.

The effect of temperature on *A. bilimbi* extraction was investigated by using 50% aqueous methanol at agitation of 200 rpm for 60 min. The TPC, TFC and antioxidant activity increase significantly by 50%, 45% and 29%, respectively, when the temperature increases from 30 °C to 70 °C. The same phenomenon was also reported by Tagliazucchi et al. [18] in the extraction of phenols from grape skin. Temperature increase improved extraction by enhancing both the solubility of polyphenols and the diffusion coefficient. In the present study, increasing extraction temperatures had an encouraging effect to total phenolics, total flavonoids and antioxidants activity. Similar findings with regards to increased solubility of polyphenol at high temperature are also reported elsewhere [19-22]. In this circumstance, it is believed that the phenolic compounds, flavonoids compounds and antioxidants content from *A. bilimbi* are thermally stable and that the extraction time selected in the second stage is suitable for both moderate to high temperature without leading to unfavourable degradation.

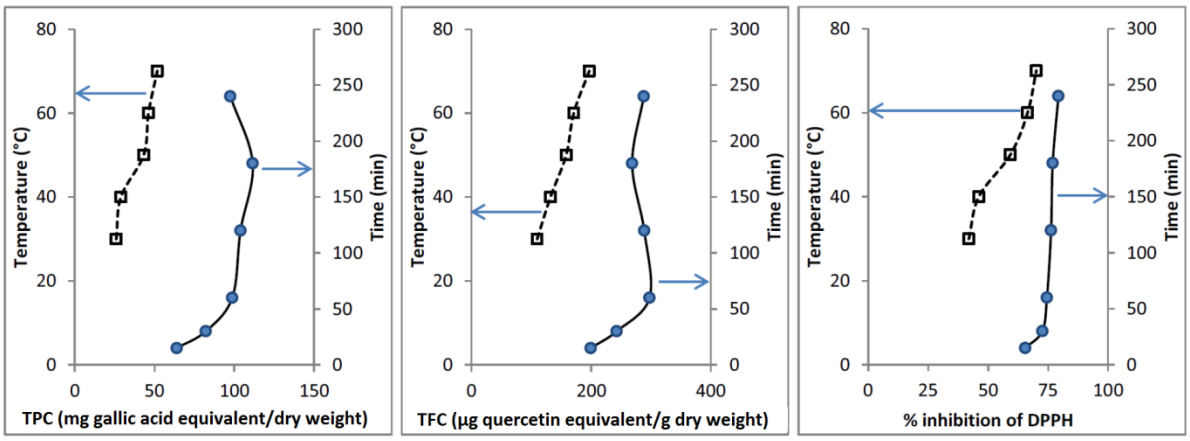

Fig. 1 Effect of time and temperature to TFC, TPC and antioxidant extraction from *A. bilimbi*.

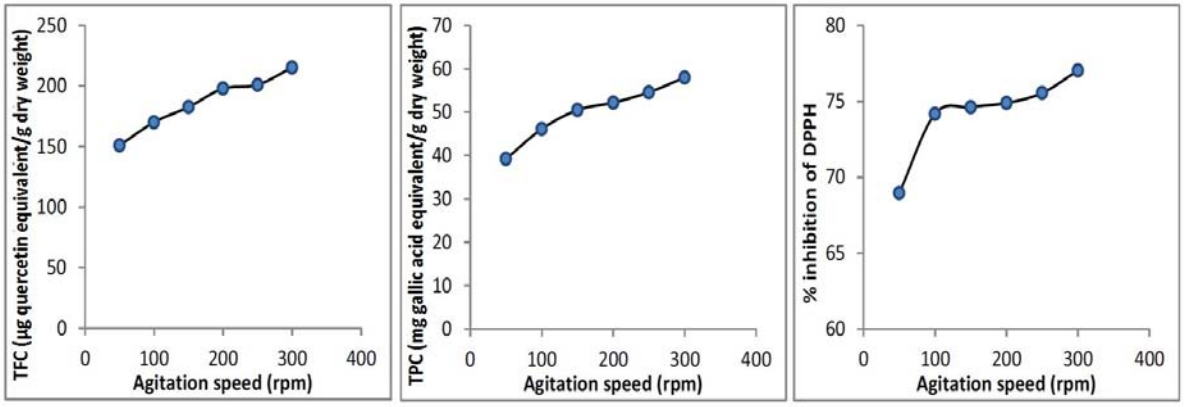

Fig. 2 Effect of agitation speed to TFC, TPC and antioxidant extraction from *A. bilimbi.*

3.3 Effect of Agitation Speed

The effect of agitation speed was studied with 50% aqueous methanol at 70 °C for 60 min. As shown in Fig. 2, the results suggest that TPC, TFC and antioxidant activity increase significantly by 52%, 47% and 10%, respectively, when the agitation speed increases from 50 rpm to 300 rpm. This is consistent with the mass transfer theory. Convective mass transfer occurres at the surface when a fluid is outside the solid [18]. The solute from inside the solid moves to the surface through diffusion and once at the surface, it is limited by the convective mass transfer. Higher agitation rate leads to a higher mass transfer coefficient and improves the convective mass transfer rate leading to increase in extraction yields. Similar findings are also reported by Chan et al. [23], which implied that agitation increased the extraction yield by accelerating the disruption and dissolution of active compounds bound to the sample matrix to the fluid.

4. Conclusions

The present study has demonstrated that solvent polarity and extraction conditions affect the extraction yield of total phenolic, total flavonoid and antioxidants from *A. bilimbi*. Aqueous solvent, i.e., 50% aqueous methanol yielded much higher phenolic content, flavonoid content and antioxidant activities from *A. bilimbi*, as opposed to polar solvent such as hexane and propanol. The highest TFC (193.3 µg quercetin equivalent/g dry weight), TPC (717.8 µg gallic acid equivalent/g dry weight) and antioxidant activity of 77% was achieved using 50% methanol, at 70 °C and agitation speed of 300 rpm. The TFC, TPC and antioxidant activity increased with the agitation speed and temperature. Findings from this work may be helpful to enhance extraction of bioactive compounds from *A. bilimbi* in the future. Further work should include other extraction techniques such as ultrasonic-assisted extraction and microwave-assisted extraction which may provide a faster extraction of antioxidant and polyphenols from *A. bilimbi*.

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