Supercritical Carbon Dioxide Extraction of Flavonoid Compounds from *Hevea* Leaves Clone RRIM 2025

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ABSTRACT

The application of supercritical carbon dioxide in the extraction of flavonoid compounds from *Hevea* leaves clone RRIM 2025 has been investigated. The effects of temperature (40, 50 and 60 °C) and pressure (100, 200 and 300 bar) on total extraction yield and antioxidant activity of *Heve*a leaves extracts were studied using complete randomized design (CRD). The results showed that extraction temperature and pressure had significant effect ($P < 0.05$) on the total extraction yield and antioxidant activity. The effect of temperature is much dominant compared to the effect of pressure. The best extraction yield (25.00 ± 1.67 mg extract/ g sample) was achieved at 50 ˚C and 300 bar. The extracts had higher antioxidant activity from 20.02 ± 2.96 % inhibition to 81.29 ± 3.99 % inhibition compared to butylated hydroxytoluene (BHT) as the reference. The highest antioxidant activity was obtained at 50 °C and 200 bar. These findings suggest that temperature at 50 °C is more convenient to be selected for the extraction of flavonoid compounds from *Hevea* leaves clone RRIM 2025. The phytochemical screening and Liquid Chromatography Mass Spectrometry (LCMS) analysis of extracts also revealed the presence of flavonoids skeleton. It is concluded that supercritical carbon dioxide process is technically feasible for extracting flavonoid compounds with high antioxidant properties from *Hevea* leaves.

INTRODUCTION

Undesirable changes in food quality due to oxidation reactions can be prevented by adding antioxidant compounds into its formulation. The usage of synthetic antioxidant such as butylated hydroxytoluene (BHT) should be replaced by natural antioxidant due to its toxicity [1]. Plant antioxidants can be the natural alternatives to synthetic antioxidants especially in the food industry. Flavonoids most commonly known for their antioxidant activity [2] are polyphenolic compounds that are widely distributed in fruits, leaves and medicinal plants. It has been proven that the supercritical carbon dioxide can extract the active natural compounds from plant matrices [3]. Supercritical fluid have several advantages compared with liquid solvents such as the dissolving power of a supercritical fluid depends on its density, which is highly tunable by changing the pressure or/ and temperature; and the supercritical fluid has a higher diffusivity and lower viscosity and low surface tension than a liquid solvent, leading to a more favorable mass transfer [4,5]. Researchers studying phenolics in *Hevea* leaves have identified them as derivatives of kaempferol or quercetin [6,7]. Flavonoids in plant are known as agents of defense against herbivores and pathogens and they form the basis for allelopathic interactions with other plant species [8]. Flavonoids in plant can occur in free form (aglycones) or linked to sugars (glycosides) [9]. *Hevea* leaves could possibly become a new source of plant antioxidant as they are abundant in source bushes *Hevea* leaves could possibly become a new source of plant antioxidant as they are abundant in source bushes as waste after the pruning process for bud wood collection.

The extraction and identification of these compounds from *Hevea* leaves are important in order to exploit its potential for commercial and medicinal uses. Thus, the aims of this study are to study the effects of temperature and pressure on extraction yield and antioxidant activity of *Hevea* leaves clone RRIM 2025 and to investigate the antioxidant compounds such as flavonoid in *Hevea* leaves.

MATERIALS AND METHODS

The fresh mature leaves of *Hevea* Clone RRIM 2025 were obtained from RISDA's source bush, Sungkai, Perak, Malaysia. Leaves were dried in an oven and then ground in a dry mill blender (Panasonic, Malaysia). Industrial grade liquid carbon dioxide was purchased from Linde Gases Malaysia. Ethanol (99.5%, analytical grade) was obtained from Systerm. Extractions were performed using Supercritical Fluid Extraction system (Thar Instruments, Inc., USA). The supercritical carbon dioxide flow rate was maintained at 30 g/min. The extraction time was fixed to 60 min. Extraction was performed at pressure 100 to 300 bar and at temperature 40 to 60 ˚C. The extracts were weighed for total yield determination and then subjected to phytochemical screening analysis and antioxidant assay. Formation of yellow colour in ammonia layer indicates the presence of flavonoids. Antioxidant activity was determined by detecting the scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical [10-12]. The assay is based on the colour change caused by reduction of DPPH radical which was determined by measuring absorbance at 517 nm (UV-Vis Spectrophotometer, Perkin Elmer, USA). The sample was analysed by a High Performance Liquid Chromatography (HPLC) system (Waters Delta 600) with photodiode array detector. A Phenomenex-Luna C18 (4.6 mm i.d x 250 mm x 5 um) column was used and two solvents; 0.1% aqueous phosphoric acid and acetonitrile were employed. For Liquid Chromatography Mass Spectrometry (LCMS), Q-TOF mass spectrometer with dual ESI source (Agilent 6520) was used and analysed by Agilent Mass Hunter Qualitative analysis. Statistical calculations and analysis were performed using statistical software, SAS Version 9.2.

RESULTS AND DISCUSSION

Phytochemical screening

The objective of this test is to detect the presence of some important phytochemical constituents that usually exhibit biological activities. The extracts were evaluated for alkaloids, saponins, flavonoinds, tannins, triterpenes and steroids. As shown in Table 1, *Hevea* leaves contain saponins, flavonoids, tannins, triterpenes and steroids. This analysis revealed similar result as a preliminary phytochemical screening performed by Eluyode *et al*. [13]. As for SFE extracts from *Hevea* leaves; flavonoids and steroids were presented in the sample. Therefore, an attempt was then made to study the antioxidant activity and to identify the flavonoid compounds in *Hevea* leaves.

Table 1: Phytochemical screening

	Alkaloids	**Saponins**	**Flavonoids**	**Tannins**	**Triterpenes**	**Steroids**
Hevea leaves	-	+	+	+	+	+
SFE extracts from *Hevea* leaves	-	-	+	-	-	+

+ = Present, - = Absent

Determination of total extraction yield and antioxidant activity

Figure 1 shows that the extraction yield at 300 bar was increased from 40 to 50 ˚C and decreased from 50 to 60 ˚C. The extraction yield keep on decreasing from 40 to 60 ˚C at 100 bar and 200 bar. At 40 ˚C, the mean of yield has not much difference for any setting of pressure. The highest mean of yield can be seen at the setting of 300 bar, while setting at 100 bar gave the lowest mean of yield. Effect of temperature at pressure 200 bar has not much difference for any setting of temperature. At a constant pressure, increasing the temperature reduces the density of supercritical fluid and hence reduces the dissolving power of CO_2. The reduction in CO_2 density will lead to reduction in solubility of analytes and decreases the extraction yield [3,14]. The highest yield was achieved at 50 ˚C and 300 bar which is 25.00 ± 1.67 mg extract/ g sample.

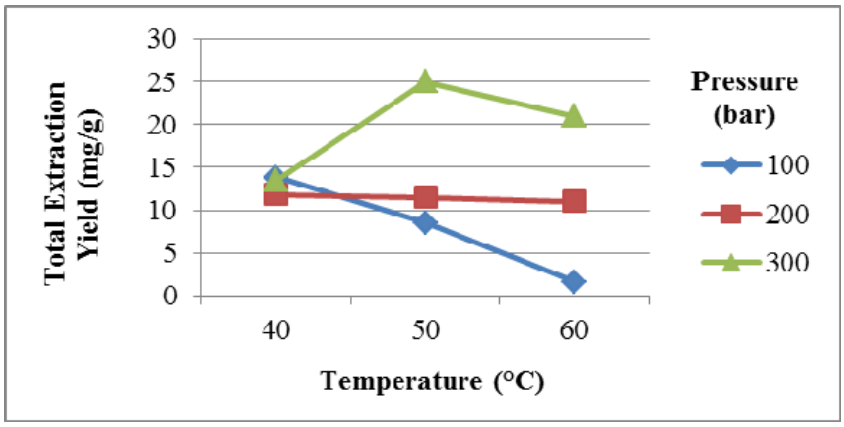

Figure 1: Effect of pressure and temperature on total extraction yield of *Hevea* leaves extracts clone RRIM 2025

Figure 2 presents the effect of pressure and temperature on antioxidant activity of *Hevea* leaves extracts. According to ANOVA analysis, pressure and temperature had significant interaction effect ($P < 0.05$) on the mean of antioxidant activity. The effect of temperature is much dominant compared to the effect of pressure. The extracts had higher antioxidant activity from 20.02 ± 2.96 % inhibition to 81.29 ± 3.99 % inhibition compared to butylated hydroxytoluene (BHT) as a reference. In comparison, BHT scavenged 37.50 % of the initial DPPH free radicals which was lower than the inhibition of extracts. As seen in Figure 7, the antioxidant activity was decreased with increasing pressure from 200 bar to 300 bar. The antioxidants that are extracted at higher pressure having lower antioxidant activity compared to those extracted at lower pressure [15]. The antioxidant activities keep on decreasing from 50 to 60 ˚C for all temperature settings. This might be due to the thermo-sensitivity of antioxidants at high temperature [1]. At 40˚C, the mean of antioxidant activity has not much difference for any setting of pressure. It was clear that the highest antioxidant activity was achieved at 50 °C and 200 bar.

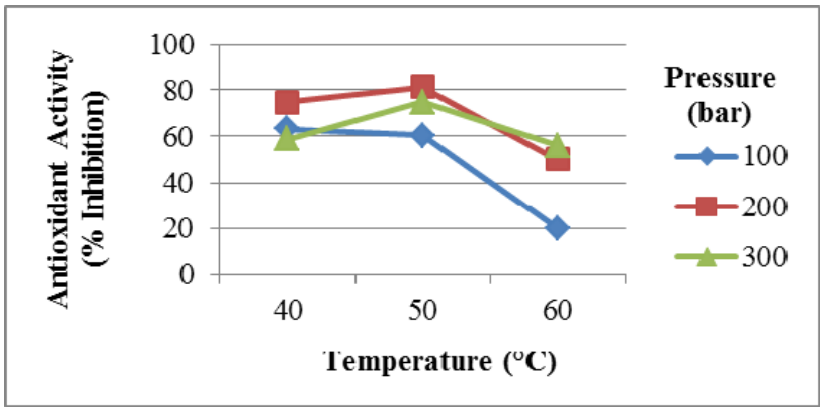

Figure 2: Effect of pressure and temperature on antioxidant activity of *Hevea* leaves extracts clone RRIM 2025

HPLC Profiling Analysis

From the HPLC analysis, it was confirmed that the *Hevea* leaves consist of flavonoids skeleton. From the chromatogram in Figure 3, five peaks of flavonoids were detected at all five ultra violet (UV) wavelengths (200, 254, 290, 300 and 366nm). The flavonoid peaks can be identified by their UV/ DAD spectral pattern with two bands, band I with λ_{max} around 300 – 380 nm and band II with λ_{max} around 240 – 280 nm [9].

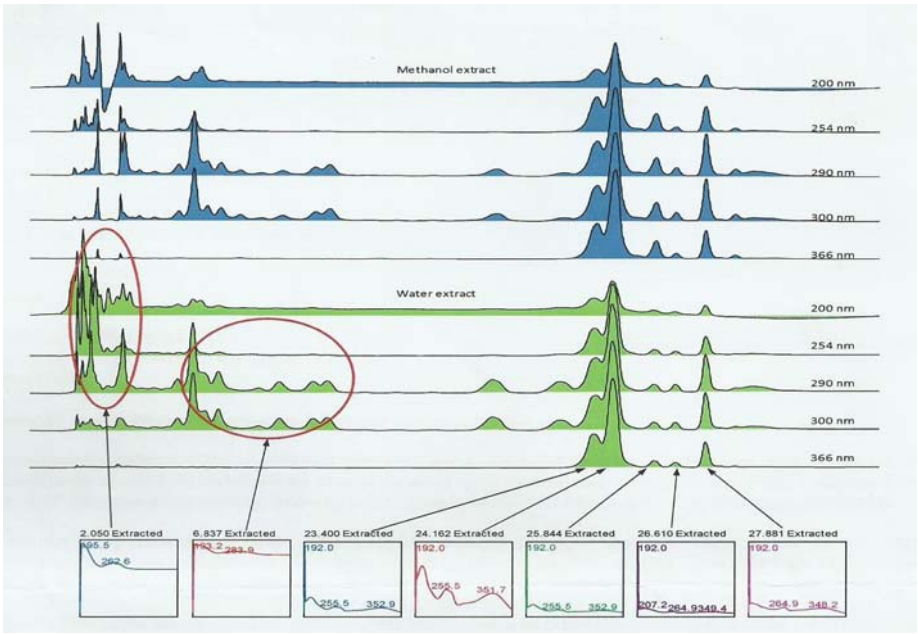

Figure 3: HPLC chromatograms of extracts and UV profiles of flavonoid peak

Liquid Chromatography Mass Spectrometry (LCMS) Analysis

Further investigation would be required for a detailed characterization of the flavonoid compounds by LCMS method. All flavonoid compounds from the extraction were identified by matching the mass or molecular weight and their spectral characteristics against the

compounds in the LC-MS library. The sample preparation has been modified to a 'strong' acid hydrolysis condition which used 6M Hydrocloric acid (HCl) and reflux temperature at 95℃. The possible five flavonoid compounds in the *Hevea* leaves extract were summarized in Table 2. This finding is in agreement with Ghasemzadeh *et. al* [16] and Liza *et. al* [17] who indicated that an abrasive hydrolysis conditions should be applied to perform complete hydrolysis to produce all free aglycone for quantification.

Table 2: The flavonoid compounds

Compound	m/z	Retention Time (min)	Molecular formula
3-tert-butyl-5-methylcatechol	181.1225	9.73	$C_{11}H_{17}O_2$
6-Gingerol	293.1762	11.40	$C_{17}H_{25}O_4$
Sciadopitysin	581.1437	18.98	$C_{33}H_{25}O_{10}$
(±)8-Gingerol	323.2221	19.09	$C_{19}H_{31}O_4$
Licochalcone A	337.1446	19.64	$C_{21}H_{21}O_4$

CONCLUSION

The phytochemical screening and Liquid Chromatography Mass Spectrometry (LCMS) analysis of extracts revealed the presence of flavonoids compounds in *Hevea* leaves extracts of clone RRIM 2025. The results showed that extraction temperature and pressure had significant effect ($P < 0.05$) on the total extraction yield and antioxidant activity. The best extraction yield was achieved at 50 ˚C and 300 bar. The extracts had higher antioxidant activity from 20.02 ± 2.96 % inhibition to 81.29 ± 3.99 % inhibition compared to butylated hydroxytoluene (BHT) as the reference. It is concluded that supercritical carbon dioxide process is technically feasible for extracting flavonoid compounds with high antioxidant properties from *Hevea* leaves.

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