Malaysian Journal of Chemistry, 2024, Vol. 26(1), 88-95
(Special Issue: *International Conference on Natural Products, ICNP 2023*)

Quality Control Assessment on *Baeckea frutescens* (Myrtaceae) Raw Material Optimal Drying Temperature and Harvesting Time through HPLC and Xanthine Oxidase Inhibition Analyses

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Known for its therapeutic properties, *Baeckea frutescens* (Myrtaceae) or locally known as *cucur atap* is commonly used in traditional medicine for its antibacterial, antidysentery, antipyretic and diuretic properties. With increased acceptance of nature-derived products, the quality, efficacy and safety become a crucial issue that need to be ensured. Plant sample quality is determined by raw material and the specifications throughout the development and manufacturing process. In this study, we aimed to investigate the harvesting time and optimal drying temperature for *B. frutescens* by employing HPLC analysis to quantify the biomarker, 6-methyl quercetin and the major compound myricitrin. Additionally, we evaluated the activity of *B. frutescens* on xanthine oxidase inhibitory assay to ensure the quality of the raw material. The concentrations of specific biomarker, 6-methyl quercetin and also myricitrin were quantified in the samples processed at different drying temperatures and harvesting time. The results revealed significant variations in the concentrations of the biomarker in relation to the harvesting time and drying temperature. The highest percentage of 6-methyl quercetin (0.14%) was observed at the optimum drying temperature of 60°C. For optimal harvesting time, the results suggest that the optimal harvest period for obtaining higher levels of 6-methyl quercetin is at 6 months with 0.09%, however considering the biomass of the raw material, the appropriate time to harvest is suggested between 12 to 18 months. The results of this study provide valuable information on the harvesting and drying practices of *B. frutescens*, enabling the production of high-quality raw materials with consistent levels of 6-methyl quercetin and myricitrin.

Keywords: *Baeckea frutescens*; Harvesting time; Drying temperature; HPLC analysis

Received: September 2023; Accepted: November 2023

Baeckea frutescens L. belongs to the family Myrtaceae and subfamily Myrtoideae and is a medicinal plant known for its traditional use in Southeast Asia. It is a small tree found in the mountainous regions of Southern China, Hong Kong, Southeast Asia, and Australia. Known as cucur atap in Malay, this plant has needle-like leaves that are small and narrow, ranging in length from 6 to 15 mm in length. When crushed, the leaves give off a resinous, aromatic fragrance [1]. The plant is valued for its various therapeutic properties, including anti-inflammatory, antimicrobial, and antioxidant activities. It has long been employed as a folk remedy to treat various inflammatory ailments, such as rheumatism, dermatitis, and colds. Previous phytochemical studies have focused primarily on the leaves and roots of *B. frutescens* - which led to the discovery of phloroglucinols, sesquiterpenoids, chromones and chromanones, flavanones and biflavonoids [2].

Research by Fadzureena et al. in 2011 showed that the methanol extract of *B. frutescens* inhibited the enzymatic activity of xanthine oxidase. Further investigation led to the isolation of a naturally occurring flavonoid compound, 6-methyl quercetin, which exhibited potent xanthine oxidase inhibition with an IC_{50} value of 3.584 µg/mL. Cytotoxicity tests against two normal cells showed that 6-methyl quercetin had an IC_{50} value of ≥ 20 µg/mL, indicating low toxicity to normal cells [3]. Myricitrin, along with 6-methyl quercetin, is also believed to be another main component of *B. frutescens*, particularly in the leaves, which are known for their bioactivity and potential health benefits. These biomarkers have been implicated in the anti-inflammatory, antioxidant, and other pharmacological activities of *B. frutescens* [4]. Therefore, the biomarkers can be used for quality control monitoring in the production of *B. frutescens*-based products to ensure their effectiveness and safety in various applications.

†Paper presented at the International Conference on Natural Products 2023 (ICNP 2023)

The efficacy and safety of herbal products derived from medicinal plants are highly dependent on the quality of the raw materials used [5]. Therefore, it is crucial to establish effective quality control measures to ensure consistent chemical composition and levels of bioactive compounds. High-Performance Liquid Chromatography (HPLC) is a widely used analytical technique to separate, identify and quantify different compounds in complex mixtures [6]. HPLC is a powerful technique for rapid analysis of bioactive constituents because it enables systematic profiling of the complex plant samples and specifically focuses on their identification and consistent evaluation of the identified compounds [7].

A crucial aspect of quality control of herbal raw materials is the determination of the optimal drying temperature and harvest time. The drying process plays a critical role in preserving bioactive compounds and preventing degradation or loss during storage [8]. The time of harvest, on the other hand, influences the physiological state of the plant and can influence the chemical profile of the raw material [9]. Therefore, studying the effects of drying temperature and harvest time on the chemical composition and bioactivity of *B. frutescens* is essential to optimize the production of high-quality raw materials. By understanding how drying temperature affect the chemical composition and harvest time affect the chemical composition and bioactivity of *B. frutescens*, this research aims to contribute to the standardization and quality control of raw materials, thereby ensuring the production of herbal products with consistent quality and potency. Moreover, it provides an opportunity to explore the potential therapeutic applications of *B. frutescens* and its bioactive compounds in various health conditions.

EXPERIMENTAL

Chemicals and Materials

Raw Material Preparation for Harvest Time Experiment

The experimental plot of *B. frutescens* was established at the FRIM Research Station in Maran. A total of 88 plantlets, from the FRIM Research Station in Setiu, were prepared for the study. The planting activities were carried out on May 20, 2021. Plant materials were placed into the designated holes and rows, with 1 x 2 m spacing between plants. Observations at the plot made weekly to confirmed the absence of pests, ensuring the ongoing health and well-being of the *B. frutescens* plants. Routine cleaning maintenance at the plot functioned as preventative measures against pest infestations. Additionally, each plant received a 100 g application of organic fertiliser every three months. The plant material was confirmed by FRIM botanist and voucher specimens (SBID043/18) were deposited at FRIM Herbarium. Subsequently, 10 individuals were selected at an interval of 6, 12, and 18 months of plant age. The leaves and stems of each individual were carefully separated, dried, ground, sieved with a 500 µm analytical sieve. Samples were packed in plastic bags, securely sealed, appropriately labelled, and stored in a desiccator. All samples collected are subjected to qualitative and quantitative analysis by HPLC. Six-month-old plants were identified as a basis for quality control of their chemical composition to allow observation of compositional variations over the following months.

Drying Experiment

Fresh plants were harvested from the *B. frutescens* experimental plot. The plant was sorted manually for use in the experiment. Drying experiments were performed with a laboratory-scale convection oven (UFE 500 type, Memmert, Germany) at specific temperatures of 40°C, 50°C, 60°C and 70°C. A control drying temperature of 25°C was used. The mass of the sample was measured periodically until equilibrium weight was reached. The final moisture content, determined with a Halogen moisture analyser below 10% (wet basis), indicated the completion of drying. The dried samples were then ground and packed in plastic bags, securely sealed, appropriately labelled, and stored in a desiccator prior to HPLC analysis.

Qualitative and Quantitative HPLC Analysis

Instrumentation

HPLC chromatograms were generated using the Waters HPLC system consisting of a quaternary pump (Waters 600E), an autosampler (Waters 717), a PDA detector (Waters 2996 PDA) scanning from 190 nm to 400 nm and using Waters XBridge™ C18 column (4.6 i.d. x 250 nm, 5 µm). The chromatograph is processed with Empower 3 software.

Sample Preparation for HPLC Analysis

The dried and ground sample (0.5 g) was extracted in 15 mL of HPLC grade methanol by sonication in a closed vial for 15 min. The solution is filtered with a 0.45 µm filter before being subjected to HPLC analysis. An aliquot of 10 µL was injected for analysis.

Preparation of Standard Solutions for HPLC Analysis

A stock solution of the reference compounds 6-methyl quercetin and myricitrin with a concentration of 1,000 µg/mL each is prepared in methanol. A series of dilutions of the reference compound solution ranging from 1 µg/mL to 1,000 µg/mL are prepared for the determination of the calibration curve.

Method of HPLC Analysis

The samples were analysed using an HPLC system consisting of a quaternary gradient pump, an autosampler and a PDA detector. The HPLC column used was a Waters XBridge™ C18 (5 µm, 250 mm x 4.6 mm) with a gradient system consisting of two solvents: solvent A (0.1% analytical grade formic acid in water) and solvent B (acetonitrile). The gradient program for the analysis was as follows: 10-30% B (0-10 min), 30-60% B (10-25 min), 60-100% (25-30 min) and 15 min at hold with 100% B to the initial conditions. The flow rate was set to 1 mL/min with a sample volume of 10 µL. Retention time data and UV spectra for clear and distinct peaks were analysed and recorded.

All chromatographic tests were performed at room temperature and the separated compounds were monitored at 254 nm. The Empower 3 software was used to control the analytical system, data acquisition and processing.

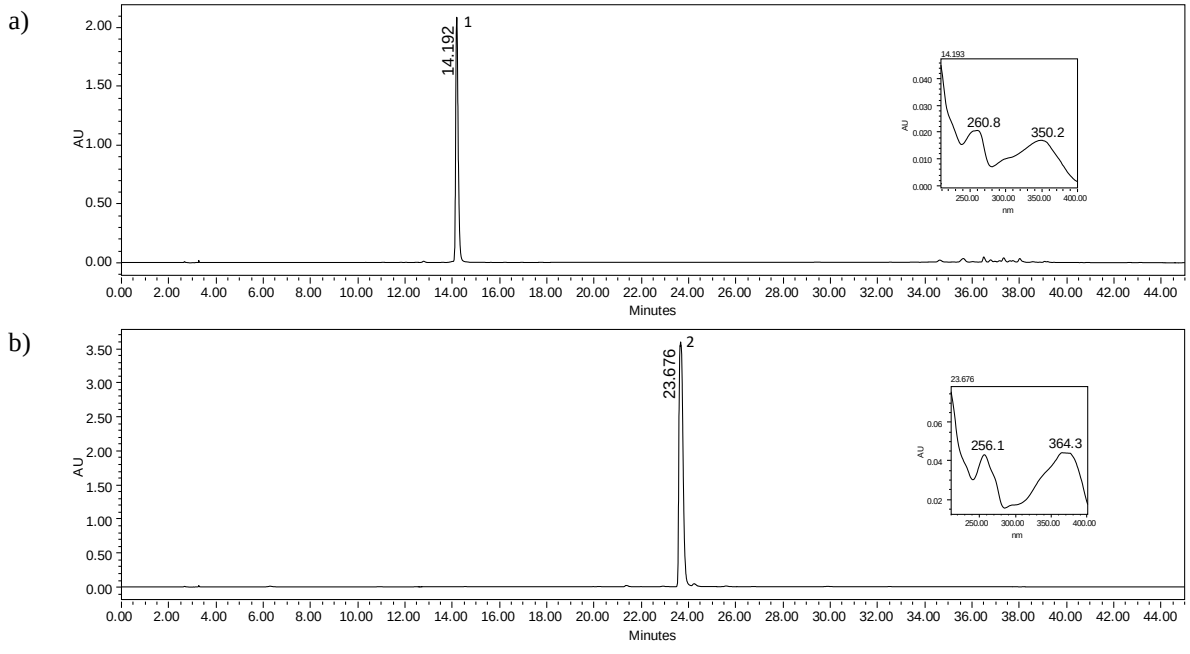
Xanthine Oxidase Inhibitory Assay

The inhibitory activities of the enzyme xanthine oxidase (XO) using xanthine as a substrate were determined using spectral techniques according to [8]. The test sample extract was dissolved with 100% Dimethyl sulfoxide (DMSO) at a concentration of 20 mg/mL. The test solution consisted of potassium phosphate buffer (KH_2PO_4) (0.05M, pH 7.5). A total of 130 µL of KH_2PO_4 solution, 10 µL of sample and 10 µL of xanthine oxidase (XO) enzyme was pipetted into a 96-well microtiter plate and then incubated at 25°C for 15 minutes. The enzymatic reaction was started by adding 100 µL of substrate solution and pre-incubated for 10 minutes. Subsequently, uric acid production was measured with a spectrophotometer at a wavelength of 295 nm. Percent inhibition is represented by the mean of triplicate observations. Allopurinol, a potent xanthine oxidase inhibitor, was used as a positive control. The percent inhibition of XO in the test combination technique described above is as follows.

% inhibition of enzyme activity = [(Optical density control − Optical density sample)/Optical density sample] × 100%

RESULTS AND DISCUSSION

Preserving bioactive compounds during the drying process is crucial to maintain the quality and potency of herbal raw materials. In the case of *B. frutescens*, the drying temperature plays a significant role in determining levels of marker compounds, 6-methyl quercetin and myricitrin, associated with its therapeutic properties. Figure 1 shows the HPLC profiles of two marker compounds, myricitrin and 6-methyl quercetin, and the raw materials of *B. frutescens* consisting of its stems and leaves.



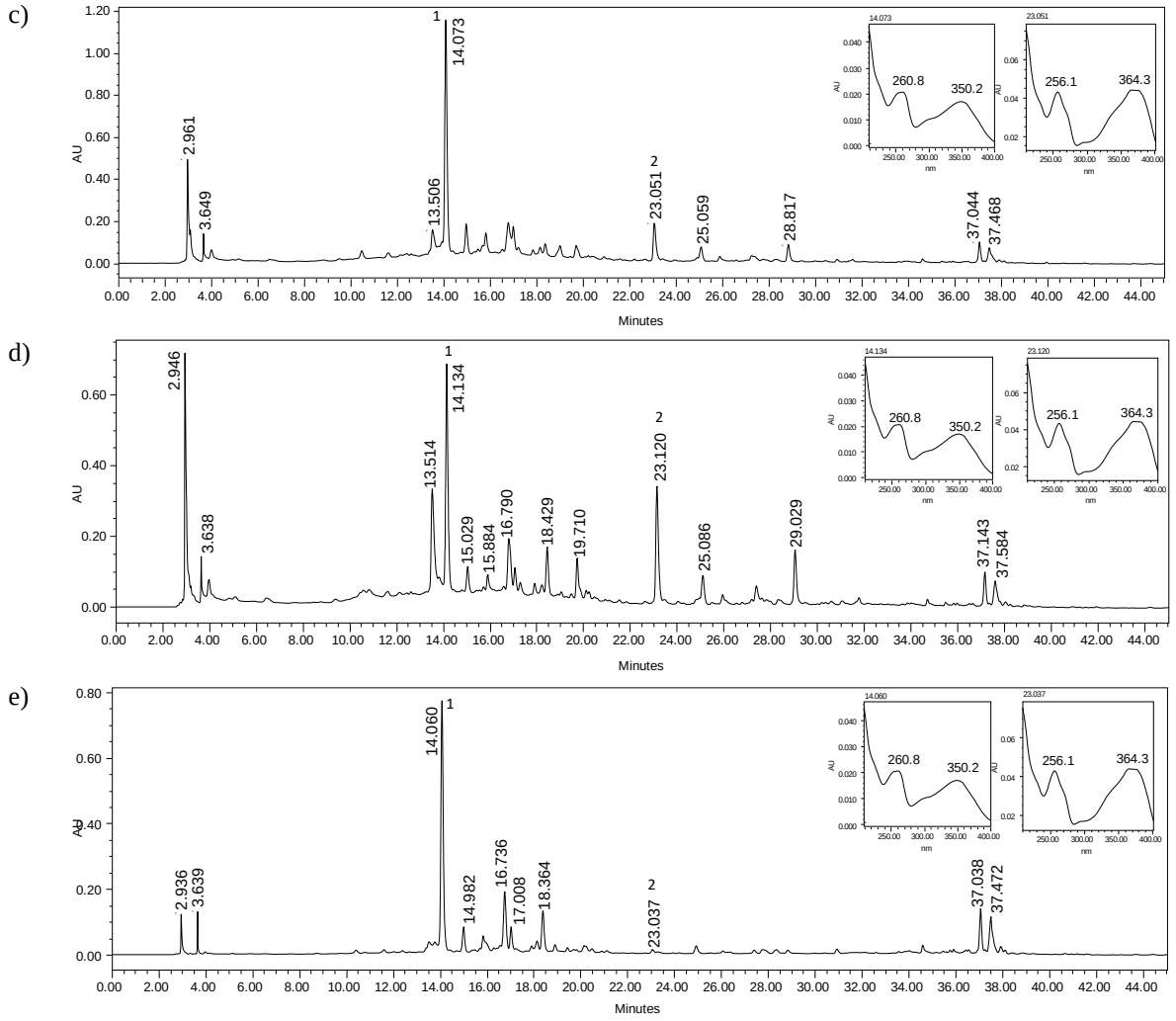


Figure 1. HPLC chromatograms and UV spectra of raw material of *B. frutescens* and marker compounds at UV wavelength of 254 nm, a) myricitrin; b) 6-methyl quercetin; c) mixture of stem and leaves; d) stems; and e) leaves of *B. frutescens*.

HPLC fingerprinting of *B. frutescens* leaves and stems revealed the presence of two distinct peaks, namely 6-methyl quercetin and myricitrin. These compounds were mainly found in the stems, while myricitrin was the compound found most abundantly in the leaves. The identification of these compounds was achieved by comparing their retention time and UV spectra with those of standard compounds.

In order to study the influence of drying temperature on the composition of 6-methyl quercetin and myricitrin, samples of *B. frutescens* were dried at five different temperatures (25, 40, 50, 60, and 70°C). Quantitative HPLC analysis was performed to analyse the samples. To conform to industry practices for sample drying, a mixture of stems and leaves was used in the experiment because it is impractical in an industrial setting to separate the leaves and stems during the process. The results in Figure 2 and Table 1 show that the drying temperature impacted the levels of 6-methyl quercetin and myricitrin in the samples tested. A control drying condition of 25°C was given. The results show that the highest percentage of 6-methyl quercetin (0.14% in total solution) and myricitrin (2.05% in total solution) was observed at the optimum drying temperature of 60°C. The increase in temperature was favoured by the chemical reactions in the leaves, leading to an increase in the concentration of the two compounds. However, at a certain higher temperature, these compounds decomposed, causing their concentration to decrease. The results are consistent with previous findings [10-12].

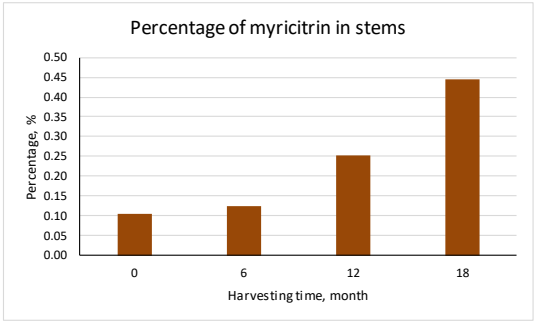
Table 1. Mean percentage of 6-methyl quercetin and myricitrin (% w/w) in mixture of stem and leaves of *B. frutescens*.

Drying Temperature (°C)	Marker Compounds (% w/w)	
	Myricitrin	6-methyl quercetin
25	1.25	0.11
40	1.44	0.12
50	1.34	0.12
60	2.05	0.14
70	1.86	0.11

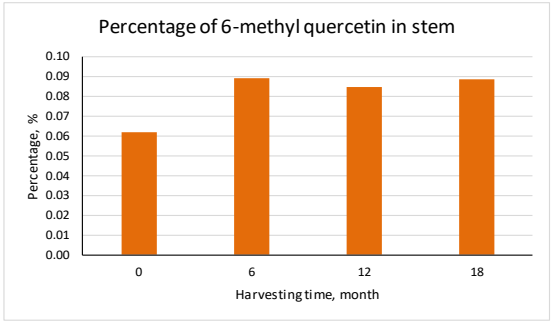
Therefore, the optimum drying temperature as determined in this study was used in the experiment to investigate the influence of harvest temperature on the samples.

Plantlets of *B. frutescens* originally obtained from FRIM Research Station in Setiu were transplanted and cultivated at the FRIM Research Station in Maran. Plant material was collected at four different time intervals: 0, 6, 12, and 18 months (0 months is count after 6 months acclimatization in the plot). Ten individuals were collected for each harvest period indicated. In this particular experiment, leaves and stems of *B. frutescens* were separated to closely examine the influence of harvest age on the composition of 6-methyl quercetin and myricitrin.

a)



b)



c)

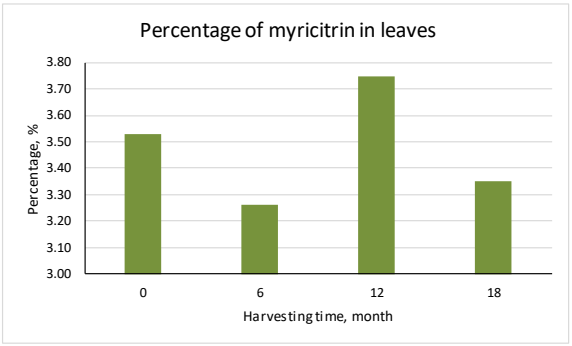


Figure 2. Mean percentage of myricitrin (a) and 6-methyl quercetin (b) in stem and myricitrin (c) in leaves of *B. frutescens*.

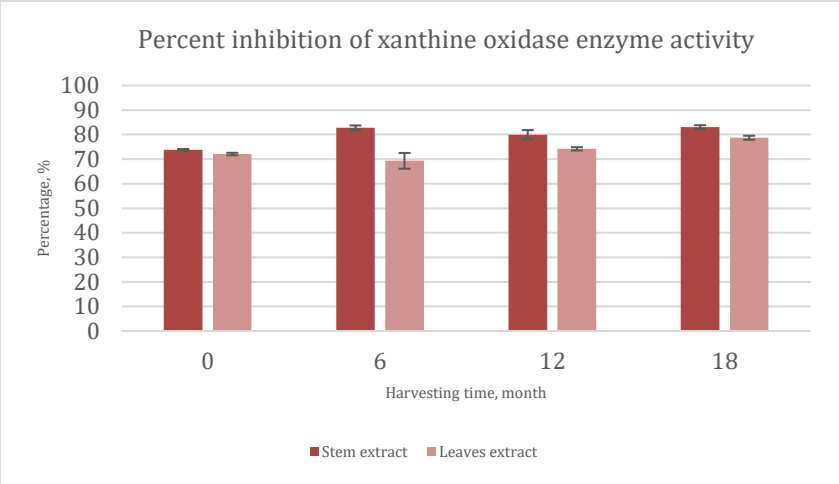


Figure 3. Xanthine oxidase inhibitory activity of *B. frutescens* stem and leaves extract at the age of 0, 6, 12 and 18 months.

Values are presented as mean ± standard error (n = 3).

According to our observation at harvest time, the plants produced flowers. The plants grew well, and their heights were measured regularly to monitor their development. In addition, the plants were fertilised every three months to ensure optimal nutrition for healthy growth.

The results in Figure 3 indicate a clear relationship between harvest time and the composition of myricitrin and 6-methyl quercetin in the stems of *B. frutescens*. Even though myricitrin does not contribute to xanthine oxidase assay, but the percentage is still observed. Over the course of 18 months, the myricitrin content shows a marked increase, with higher concentrations being observed in later harvests. Myricitrin content increases from 0.11% at 0 months to 0.12% at 6 months, further rising to 0.25% at 12 months and peaking at 0.45% at 18 months. In the case of 6-methyl quercetin, despite initially showing a similar trend, increasing from 0.06% at 0 months to 0.09% at 6 months, over the course of 18 months, it drops slightly to 0.08% at 12 months before returning to 0.09% at 18 months. This was also supported by the xanthine oxidase inhibition test result as shown in Figure 3.

In contrast, the leaves of *B. frutescens* contain myricitrin, the content of which is also influenced by the time of harvest. The myricitrin content of the leaves is slightly higher at 0 months (3.53%) than at 6 months (3.26%). However, there is a slight increase in myricitrin content at 12 months (3.75%), followed by a slight decrease at 18 months (3.35%). We also measured the xanthine oxidase inhibitory activity of the leaves extract to be compared with the stems extract. The results showed no significant difference was observed between the two extracts evaluated (Figure 3). This finding will support industry practices where it does not affect efficiency and efficacy to separate the leaves and stems for product development [the industrial practices is not published].

During the 18 months of experimental period, there were fluctuations in the concentrations of the compounds in *B. frutescens*, with the maximum concentration occurring at the 18th month. Variations in plant quality during different harvest times are due to several factors which include plant maturity, environmental conditions, seasonal changes, stages of plant development, and external factors such as agricultural inputs [13–17]. Thus, it becomes clear that variations in plant quality at different harvest times are the result of complex interactions between plant biology, environmental conditions, and agricultural practices [18].

The observed fluctuations in myricitrin and 6-methylquercetin content in *B. frutescens* can be attributed to the growth cycle of the plant during different harvest stages. Each phase of the growth cycle can have unique chemical properties, leading to fluctuations in the levels of these compounds [18]. Based on the results of this study, it is evident that the harvest timing plays a vital role in determining the levels of myricitrin and 6-methylquercetin in the stems and leaves of *B. frutescens*. The results suggest that the optimal harvest period for obtaining higher levels of these chemical compounds is at 6 months but considering the biomass of the raw material, it is still appropriate to harvest the plants between 12 to 18 months. By identifying the ideal time to harvest, it becomes possible to maximize yields of myricitrin and 6-methylquercetin, known for their potential bioactivity and therapeutic properties [2, 3].

Our results underscore the importance of optimizing the drying temperature and selecting the appropriate harvest timing to ensure the production of

high-quality raw materials with the desired chemical composition and levels of bioactive compounds. Quantitative HPLC analysis has proven to be a reliable method for assessing the quality of *B. frutescens*. These results will contribute to the standardization and quality control of herbal products from *B. frutescens* and enable the development of safe and effective formulations. Furthermore, the standardized extract of *B. frutescens* demonstrated statistically significant reductions in serum urate levels in hyperuricemic rats [3]. These findings laid the foundation for establishing effective dosages of BF standardized extract. This discovery led to the granting of a patent (MY-182603-A: An extract of *Baeckea frutescens* and its use in the treatment of gout)."

CONCLUSION

Our study investigated the optimum drying temperature and harvest time for *B. frutescens* and assessed the raw material quality using quantitative HPLC analysis. The results showed that myricitrin and 6-methyl-quercetin levels is optimum on drying temperature of 60°C. For harvesting, levels of myricitrin and 6-methylquercetin increased from 0 months to 18 months, indicating the influence of harvest time on the accumulation of these bioactive compounds. Additionally, it was found that *B. frutescens* extract with 6-methylquercetin content has a strong inhibitory effect on the xanthine oxidase enzyme even at exceptionally low concentration, even at a low concentration of 0.06% of 6-methyl quercetin, the xanthine oxidase activity was significantly inhibited, with a 73.8% inhibition rate observed, suggesting a potential therapeutic application. Thus, these results indicate the potential of *B. frutescens* extract as a xanthine oxidase inhibitor, regardless of the age of the samples. For further studies, LCMS or NMR based metabolomics approach are suggested for more comprehensive and accurate result for optimal drying temperature and harvesting time as well as their correlation with xanthine oxidase properties.

ACKNOWLEDGEMENTS

We would like to express appreciation to Forest Research Institute Malaysia (FRIM), Ministry of Natural Resources, Environment and Climate Change for the RMK 12 Funding, Staff of Natural Products Division, Innovation and Commercialization Division, all individuals and organizations who contributed to the completion of this research project and the preparation of this journal paper.

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