ORIGINAL ARTICLE

Extraction of *Morinda Citrifolia* by Subcritical Water: Effect of Temperature on Antioxidant and Total Phenolic Activity

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ABSTRACT

Introduction: Oxidative stress occurs when there is a lack of balance between the generation of free radicals and their elimination from the body, which leads to various harmful effects including cell damage and other metabolic disorder. There is a rise in demand of natural drugs being used in medicine and giving better results compared to other conventional drugs. It is possible to encapsulate bioactive compounds from natural medicinal plants into nanoparticles which acts as drug delivery carriers, to get enhanced therapeutic effects. **Materials and methods:** This research focuses on the effect of temperature on the antioxidant and phenolic content of *Morinda citrifolia*, which was extracted using subcritical water extraction (SWE) by applying three different extraction temperature: 100, 120 and 140°C. The extracts were subjected to High Performance Liquid Chromatography (HPLC) to analyse the bioactive compounds. Total Phenolic Content (TPC) and antioxidant assay was carried out to find out the phenolic content and antioxidant activity respectively. **Results:** The results showed that the highest extraction yield for SWE was found with extraction temperature 120°C and it was seen that increased temperature had a significant effect as it increased its phenolic content and antioxidant free radical scavenging activity. The HPLC results also showed higher yield of scopoletin and rutin from sample extracted with 140°C. **Conclusion:** Thus, this study showed that sample of M.citrifolia which was extracted at 140°C, had the highest scopoletin and rutin content and highest phenolic content compared to the other samples.
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Keywords: Scopoletin, *Morinda citrifolia*, Subcritical water extraction, rutin, antioxidant activity.

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INTRODUCTION

The human bodies naturally produce harmful substance known as reactive oxygen species (ROS), and it has a system in place to control and eliminate these substances [1]. When there is an uncontrollable increase of ROS in the body, it causes "oxidative stress", which is a major reason for many metabolic diseases affecting various organs like the heart and brain [1]. Medicinal plants have traditionally been used in folk medicine as a natural healing remedies. They can either be consumed directly, or boiled and drunk as beverages or by made into a paste to treat various ailments as they consist of a vast reservoir of medicinal compounds [2].

Plants like *Cinnamomum zeylanicum*, *Eucalyptus globulus* and *Olea europia* are known to have antidiabetic and higher insulin production effects [2]. *Cistus salviifolius* and *Pistacia lentiscus* are known to have very high antioxidant activity [3]. Natural bioactive compounds that possess beneficial effects such as antibacterial, antiviral, anti-inflammatory, antiallergic, antithrombotic, and vasodilatory actions to treat certain disease such as diabetes, cardiovascular-related illness and cancer have been reported to exhibit excellent antioxidant properties. Antioxidants are collections of substances that counteract and neutralize harmful free radicals and ROS within the cellular environment and converts them into harmless products [4]. It protects against ROS by preventing or stopping the generation of free radicals and also by donating an electron to free radicals rendering them unreactive, thus preventive free-radical induced diseases [5].

However, there is limited bioavailability of both natural and synthetic antioxidants, due to low absorption, poor solubility, penetration of cell membranes, and degradation during the delivery process, ultimately hampering their effectiveness [6]. By encapsulating the bioactive compounds into nanoparticle structure, it presents numerous benefits when compared to conventional methods. They are biodegradable and nontoxic, and the advantages encompasses safeguarding bioactive components in the cellular environment, enhancing their absorption within the body, increasing stability, precision in delivering antioxidants to specific targets, and the ability to regulate their release at the intended site of action [6]. Recently, the use of engineered nanostructures particles has come into focus, as an innovative way which helps to enhance the properties of antioxidants. These nanoparticles, whether modified with natural antioxidants or antioxidant enzymes, serve as effective carriers or delivery vehicles. They also improve antioxidant activity and facilitates their targeted delivery of those that struggle to permeate cell membranes and be taken up by cells [7].

Morinda citrifolia (*M.citrifolia*), also known as Noni fruit, a plant with roots in traditional Polynesian medicine, is commonly ingested for its potential to address a range of health issues such as hypertension, immune system enhancement, and the treatment of infections caused by bacteria, viruses, and fungi [8, 9]. Studies have also proven that some phytochemicals in *M.citrifolia* have shown to contribute to its antioxidant properties as observed in both in vivo and human trials [10]. Its efficacy in managing various ailments has prompted numerous scientific inquiries aimed at exploring the phytochemicals found in various parts of the *M.citrifolia* plant and their associated therapeutic properties [11]. Approximately, 200 phytochemicals have been found in *M.citrifolia*, with the primary functional components falling into three major groups: phenolics, alkaloids, and organic acids. The most significant compounds among these include scopoletin (a phenolic coumarin), alizarin, rutin, damnacanthal, morindone, morindin, aucubin, asperulose, rubiadin, and glycosides of anthraquinone [9, 12].

Subcritical water extraction (SWE) is an eco-friendly method which has the ability to extract particular phenolic compounds from plants by using water as the solvent, at higher concentrations compared to conventional extraction methods like maceration technique [13, 14]. It is also very flexible due to their parameters being easily modifiable, for example, by changing the dielectric constant, surface tension, and viscosity of water it is possible to control the characteristics of the extraction [15, 16]. SWE is also gaining more interest because of its safety, effectiveness and environment friendliness; also, a subsequent number of bioactive compounds like polysaccharides, antioxidants, and polyphenols could be successfully extracted using this technique [16]. Some plants that can be extracted successfully using subcritical water extraction includes medicinal herbs like Ginseng, vegetables like garlic, along with other fruits, oilseed crops, tea leaves, shrubs and legumes [17]. By carrying out the extraction process using SWE, it is possible to encapsulate the extract within nanoparticles for drug delivery, meeting therapeutic requirements [7].

Among the numerous compounds identified in *M.citrifolia*, rutin and scopoletin are two of the main flavonoids. Scopoletin, also known as 6-methoxy-7 hydroxycoumarin, is one of the naturally occurring coumarin that is frequently found in many edible plants, possesses significant impact on human health [18]. It has proven to have several pharmacological effects, such as antidiabetic, anti-inflammatory and antioxidant abilities [19]. Furthermore, this substance substantially elevated the levels of peroxisome proliferator-activated receptor γ2(PPARγ2), and the findings suggested that the component scopoletin has the potential to improve insulin resistance in an in vitro model, offering promising treatment of metabolic disorders [18, 19]. While rutin is known for its antioxidative, anticancer, anti-inflammatory, cardioprotective properties [20]. There is a lack of comprehensive research on *M.citrifolia* extracts obtained by the subcritical water extraction method that relates with antioxidant research and how temperature can affect the extract. Therefore, the effect of antioxidant and total phenolic content of *Morinda citrifolia* due to different extraction temperatures via subcritical water extraction is analysed.

METHODS AND MATERIALS

Sample preparation

Morinda citrifolia, was collected from local farmers in Kedah, Malaysia. A cleansing procedure using tap water was carried out to remove any contaminants. Following that, they were sliced thinly and stored in -80°C until they were frozen and then transferred to the freeze dryer for two days until they reached a uniform weight. The freeze-drying process helps to remove water from a sample by the process of sublimation though gradual heating process under vacuum. This helps to preserve the sample's bioactive compound and increases its shelf life. Once dried, the specimens were crushed using a Commercial blender (Waring®,United States) and sifted to achieve an approximate particle size of 0.50 mm - (Fig 1(a)). The processed samples were then stored at -20 °C.

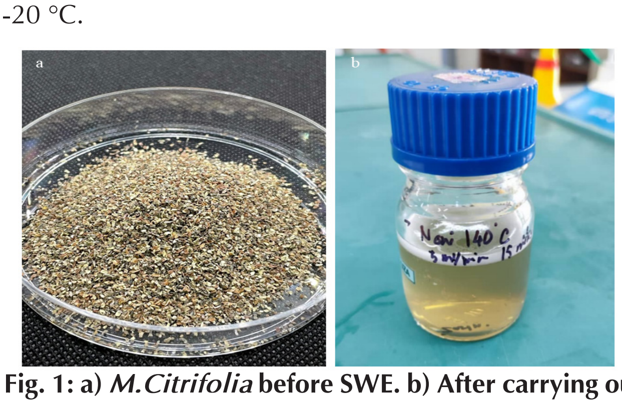

Fig. 1: a) *M.Citrifolia* before SWE. b) After carrying out SWE.

Sub- Critical Water Extraction (SWE)
In this study, the main components of subcritical water extraction which include drying oven, extraction vessels, the water pump that controls the flow rate and the pressure gauge that controls the pressure, was setup in Centre for Lipid Engineering and Applied Research (CLEAR) UTM. The parameter was referred from the research done by Jamaludin et al., (2021) [13], and it was modified to accommodate the specific condition of this research specially in the category of flow rate, pressure applied and collection time. The machine was flushed at the beginning of every extraction for 10 mins, with a water flow of 10 ml/min and a pressure of 100 bar. This helps to rinse off any residue from the previous extraction process and ensures the accuracy of the extraction contents. 2 grams of *Morinda citrifolia* powder was measured and filled inside a filter bag. The filter bag was then wrapped tightly and loaded inside the extraction vessel of the SWE unit. The parameters are set as Table I.

Table I: The parameters used for each extraction process.

Sample	Temperature (°C)	Particle Size Used (µm)	Pressure (bar)	Flow Rate(ml/min)	Run time (mins)
A	100	500	100	5	15
B	120	500	100	5	15
C	140	500	100	5	15

The subcritical water extraction was carried out at three different temperatures: 100, 120 and 140 °C. This was done to identify and analyse if temperature has any significant effect on the phenolic content, and antioxidant activity. All parameters for extractions were kept constant for accurate comparison, and the extraction process was repeated six times, from which the average extraction yield was calculated to find out the optimum extraction parameters for *Morinda citrifolia*. After the extraction process was completed, the extracts were frozen and then put into freeze drying machine for 48 hours. The retrieved powders' mass was then measured, and percentage extraction yields for each sample were calculated using the Equation 1 as below:

% Extraction Yield=((Dried Extract))/((Raw Extract)) x 100% Eq.1

High Performance Liquid Chromatography (HPLC) Analysis
HPLC is carried out to identify and quantify very precisely the amount of scopoletin and rutin from plant extracts, and it includes the identification of a very wide range of analytes. Thus, it is more suitable for routine analysis procedures. The assessment of phenolic compounds in the extracts was carried out through High-Performance Liquid Chromatography (HPLC, Alliance 2965 system, Waters Corp, USA). This analytical method was previously detailed by Kim and Lim [28]. The phenolic compounds were separated using an XTerra® C18 column with dimensions of 250 Ч 4.6 mm and a film thickness of 5 µm. The mobile phases consisted of two components: 0.5% formic acid (A) and acetonitrile (B). Initially, the composition was set at 80% A and 20% B. Subsequently, the ratio of solvent B was adjusted as follows: changed to 20% over a 15-minute period, increased to 70% over 20 minutes, and then returned to 20% over the course of 30 minutes. The flow rate of the mobile phase was set at 1.0 mL/min. Detection of scopoletin and rutin was accomplished at a wavelength of 350 nm, and quantitative analysis was conducted by comparing the areas of the chromatographic peaks with those of external standards.

Total Phenolic Content (TPC) Analysis
For the TPC, the first process was to make a standardized calibration of Gallic Acid with Folin-Ciocвlteu reagent. Using gallic acid as a calibration standard, the Folin-Ciocвlteu method can provide a reliable and standardized approach to quantify the total phenolic content in various samples, allowing for meaningful comparisons and interpretations of the results. All the samples were prepared with a concentration of 2mg/ml by mixing 20 µL sample with 100 µL of Folin-Ciocвlteu and reagent and 1580 µL of distilled water at room temperature and left for 8 minutes. After that, it was neutralized by adding 300 µL Sodium Carbonate solution and was left in the dark for 2 hours. Gallic acid exhibits a strong absorbance at the wavelength used for measurement in the Folin-Ciocвlteu method, typically around 765 nm. This absorbance response allows for the construction of a calibration curve with good sensitivity and linearity, enabling accurate quantification of the phenolic content in the test samples based on their absorbance values. TPC concentration in all the samples was expressed as milligrams of Gallic Acid equivalents (GAE) per gram of dry *M.citrifolia* extract [21, 22].

Antioxidant assay
This assay was carried out as stated in Miliauskas et al., 2004, with some modifications [23]. DPPH or 2, 2 diphenyl-1-picrylhydrazyl, a purplish, stable free radical, was used to carry out this assay. If there is the presence of antioxidants, then these free radicals are scavenged causing bleaching of the purplish colour.

A 5mg/ml concentrated solution of each extract was made using a mixture of 90% methanol and 10% water. DPPH solution was made using methanol to a final concentration of 0.1mM. 77µL of extract solution was mixed with 3ml of methanolic DPPH and vortexed until properly mixed. Then the mixture was kept in the dark for 30 minutes at room temperature. The absorbance was measured at 517nm by using a spectrophotometer. The calculations were done as follows:

DPPH(Quenched)%= $(A_{control}- A_{sample})$/A_(control)x100% Eq.2

Here, Ablank is the absorbance of the blank, Acontrol is the absorbance of the control and Asample is the absorbance of the sample.

Statistical Analysis

The statistical analysis was conducted using Microsoft Excel. The data was organized in a structured format and a one-way single factor ANOVA was performed to compare the mean values of the three samples for their extraction yield, TPC and antioxidant analysis.

RESULTS

Extraction Yield Calculation

The results for the extraction yield after carrying out the SWE and freeze-drying process of *M.citrifolia* were shown in the table II.

Table II: Mean±SD of multiple measurements of Extraction Yield after Freeze Drying Process of *M.citrifolia* Fruit

Sample	Temperature of extraction	Particle Size (µm)	Mean Extraction Yield (%)
A	100	500	7.605 ± 0.67
B	120	500	11.218 ± 0.21
C	140	500	9.11± 0.9

The results reveal significant variations in the yields of the three samples. Sample B exhibited the highest yield at 11.218 ± 0.21. In contrast, Sample A and Sample C displayed yields of 7.605 ± 0.67 and 9.11± 0.9, respectively. The ANOVA analysis conducted on the three samples yielded a significant F-Statistic of 7.599 and a p-value of 0.0227. The p-value is seen to be below the commonly accepted level of 0.05. This indicates that the differences in the means of the three samples are statistically significant. This can be attributed to multiple factors, including variations in the plant material's composition and the extraction process itself. Sample B (extracted at 120°C) may have contained a higher concentration of compounds due to its extraction temperature being more suitable compared to the sample C (extracted at 140°C).

Evaluation of HPLC

Table III shows the HPLC chromatographic profiles, with the peak for scopoletin and rutin found in each extract as compared to the standard solution.

Table III: HPLC of scopoletin and rutin found in *M.Citrifolia* Extracts.

Sample	Scopoletin Area (mAU*s)	Rutin Area (mAU*s)
Standard	925.2 (15ppm)	3823.4 (500ppm)
A	59.5	209.1
B	123.2	257.0
C	145.0	276.6

From the results obtained, it is evident that there is an increase in the quantity of scopoletin and rutin as the extraction temperature from SWE was increased. Both scopoletin and rutin content exhibited a significant rise as the temperature increased from 100 °C to 140 °C (Figure 2).

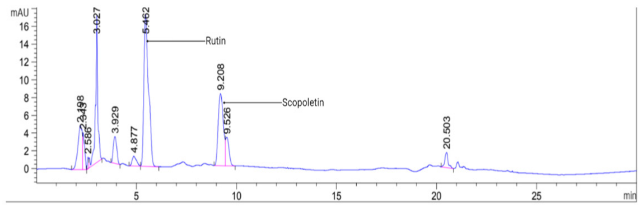

Fig. 2: HPLC results showing peak of Rutin (5.462 mAU) and Scopoletin (9.208 mAU) at 140°C at 350 nm

It could be seen that when the samples were extracted at 100 °C, the scopoletin chromatographic peak had an area of 59.5 mAU*s, which showed a significant increase with increased temperature, 123.2 mAU*s (extracted at 120°C) and 145.0 mAU*s (extracted at 140°C). Similar trend was observed with rutin as well, as its peak area increased from 209.1 mAU*s to 257.0 mAU*s and then finally 276.6 mAU*s. It can be observed from the values that scopoletin showed greater increase compared to rutin.

Evaluation of the total phenolic content

The total phenolic content of *Morinda citrifolia* extract was tested at an absorbance of 765 nm using the Folin Ciocalteu colorimetric method, a linear Calibration curve was constructed and, the R2 value of 0.999 was achieved. Table IV shows the results on the total phenolic content of *M.citrifolia*, which was extracted at 3 different temperatures by SWE. Values are expressed as mean±SD. As observed in our results, Sample A exhibited a TPC value of 0.114± 0.007 mg GAE/G, while Sample B showed a slightly higher TPC which is (0.134 ± 0.02) and Sample C (extracted at 140°C), showed the highest TPC among the three samples, which was 0.144 ± 0.018. For the ANOVA result, the p-value was found to be 0.269 showing no statistically significant difference among the means of the 3 samples. From these results, it can be concluded that *M.citrifolia* extracts tends to get higher phenolic content when extracted in higher temperatures.

Table IV: TPC and DPPH analysis.

Samples	SWE extraction temperature (°C)	Total phenolic content (mg GAE/g)	DPPH free radical scavenging activity (%)
A	100	0.114± 0.007	24.7 ± 0.28
B	120	0.134 ± 0.02	25.5 ± 0.52
C	140	0.144 ± 0.018	23.0 ± 2.93

Evaluation of the Antioxidant assay

DPPH free radical scavenging by the *M.citrifolia* extracts at 5 mg/ml was carried out, and the results are shown in Table IV. It was observed that all three of the samples possessed DPPH scavenging activity, thus suggesting that all of them has antioxidant properties. Sample B, extracted at 120 °C, showed the highest scavenging activity of 25.5%, while Sample C (140 °C) showed the least activity among the three of them. The ANOVA results gave the p-value of 0.378, showing no statistically significant difference among the means of the 3 samples. The impact of different temperatures on the DPPH activity did not follow any trend in this situation.

DISCUSSIONS

Studies have shown that higher temperatures help to increase the extraction yield significantly, for example in a study done on Onosma Mutabilis showed a linear increase of yield with increasing temperature, which could be also due to higher vapor pressure allowing greater penetration of water through the sample [24]. Sample C which was extracted at 140 °C giving lower yield, which could be the result of degradation due to high temperature. However, Sample C had the highest content for scopoletin and rutin which is 145.0 mAU*s and 276.6 mAU*s, respectively. In a study conducted about extraction using pressurized hot water it was observed that higher temperature causes improved extraction kinetics due to faster diffusibility, improved absorption of the sample and also reduces electrostatic force which eventually leads to better release of particles [25]. This observation supports that higher extraction temperatures might have contributed to more efficient extraction of different compounds' from *Morinda citrifolia*.

The determination of TPC is a very important as it is essential to identify the potential bioactive compounds of natural extracts to understand the functionalities. In this study, M. citrifolia extracts tends to get higher phenolic content when extracted in higher temperatures which is 140 °C. A proper temperature selection is important in yielding an optimized phenolic content for *M.citrifolia*, but there is also a chance that higher temperature may end up degrading the bioactive compounds of the plant extract [16, 26].

Antioxidants, both natural and dietary supplements, has the capability of reducing the complications that arise due to several diseases [31]. Studies have proven recently that oxidative stress can also contribute to many chronic and degenerative diseases causing factors like diabetes, neurological diseases like Alzheimers, Parkinson's and stroke. The DPPH assay is a widely utilized, simple and cost-effective technique to analyse and measure the antioxidant properties of natural extract. It uses free radicals to test if a substance has the potential to act as a supplier of hydrogen atoms, thus helping in scavenging free radicals. Through this process, the DPPH is reduced, undergoing a reaction resulting in strong absorption of light at 517 nm [22]. In this study, the results for DPPH assay are not following any trend and not influence by temperature.

Another type of extraction using maceration technique was performed to observe the influence of high temperature with degradation of the extracts. *M.citrifolia*, was extracted at 60 °C with methanol as solvent. As a result, DPPH assay shows 15±8.97 (%) which is much lower than SWE. Therefore, the temperature from the SWE technique did not affect the antioxidant properties, and that the decrease in DPPH activity by using maceration technique happened due to lower extraction of bioactive compounds responsible for the antioxidant functionalities. In another research carried out using Onosma mutabolis, it was also proven that SWE showed more efficiency than maceration for bioactive compounds extractions for both roots and stems parts of the plant [25]. It is important to find a proper balance by further investigation to get the optimized temperature value for SWE.

CONCLUSION

This study provides very important insights about the optimization of the Subcritical water extraction process for *Morinda citrifolia*, does have a significant effect on the bioactive potential of the extracts along with higher phenolic content, and thus it can also be concluded that the extract that showed the best result, was Sample C, which was extracted using the highest temperature which is 140 °C. This extract can further be utilized to encapsulate in a nanoparticle and then apply it for drug delivery processes. It is important to find the optimum condition for the extraction process to avoid degradation of heat sensitive phenolic compounds. *Morinda citrifolia* has a great potential to be used as a therapeutic medium as it consists of various medicinal benefits, but further research is required to elucidate the specific compounds that is responsible for these effects.

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