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Aqueous Enzymatic Extraction Conditions of Bioactive Compounds from Ultrasound Pretreated Noni (*Morinda Citrifolia* L.) Extract

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ABSTRACT

Four different factors involved in the aqueous enzymatic extraction of the bioactive compounds, including total polyphenols, total flavonoids, total saponins, and vitamin C, from ultrasound pretreated noni (*Morinda citrifolia* L.) powder were examined. The study showed that the most suitable enzymatic extraction conditions for the content of the bioactive compounds were obtained at the concentration of enzyme (mixture of pectinase and cellulase, at 1:1, w/w) at 0.5% dry weight (d.w.), the water-to-powder ratio of 16/1 (mL/g), and the incubation temperature at 60°C for 45 min when ultrasonic pretreatment was fixed at 50 ± 5°C for 10 min. Under those conditions, the highest content of total polyphenols, flavonoids, saponins, and vitamin C was found to be 12.53 ± 0.09 mg GAE/g d.w., 6.45 ± 0.61 mg QE/g d.w., 56.02 ± 2.45 mg AE/g d.w., and 1.04 ± 0.09 mg AA/g d.w., respectively. For comparison, the results indicated that the application of ultrasound and enzymes has shown to be effective in improving aqueous extraction. Therefore, it can be concluded that the present extraction method is a novel and green alternative method for extracting bioactive compounds from noni fruit.

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1. Introduction

Morinda citrifolia L. (Noni) is a small tropical tree. Its fruits have been used as a folk medicine for thousands of years for the treatment of many diseases including diabetes, high blood pressure, inflammation, or cancer [1]. Many studies have found that there are more than 200 compounds in noni containing organic acids, alcohols, phenols, saccharides, anthraquinones, carotenoids, triterpenoids, flavonoids, glycosides, lactones, iridoids, ketones, lactones, lignans, nucleosides, triterpenoides, sterols, and aromatic compounds. Specifically, noni contains many compounds including polyphenol, flavonoids, saponin, and vitamin C with various biological activities that are beneficial to human health. Those bioactive compounds are reported to be high antioxidant capacity, which contributes to the prevention of free radicals in the human body [2], anti-bacterial [3], and anti-cancer [4] as well as lowering blood pressure [5]. Since there are health-promoting effects and no acute toxicity, it can be considered as a potential source of bioactive compounds from noni in the future.

The extraction method plays a very important role in order to ensure the quality of the resultant extract. Recently, it has been reported that combined novel extraction techniques over the novel extraction methods can be effectively developed to extract bioactive compounds from plant matrix due to increased extractability, higher extraction yield, lower impurities in the resultant extract, shorter extraction time, lower production cost, lesser solvent usage, and lesser energy consumption. Importantly, the combined techniques result in lesser degraded heat-sensitive compounds and the biological activities of the resultant extract are preserved [6]. Each of the extraction techniques, when applied individually, has limited advantages, including low yield or decreased bioactivity. However, the synergistic effect should be obtained when they are combined in terms of improving extraction yield, bioactivity, and quality of the resultant extract. In general, the combination of enzyme and ultrasound is able to overcome the disadvantages of both individual methods and make the extraction process economical and eco-friendly [7]. This is because enzymatic extraction aids in disrupting cell membrane and penetrating solvent, whereas ultrasound aids in improving extraction yield and shortening extraction time. Before enzymatic extraction, ultrasound pretreatment could be applied to weaken or disrupt the cell walls of the plant material. Afterward, during the enzymatic extraction, enzymes such as pectinase and cellulase can also aid in cell wall disruption [8]. Therefore, the extraction efficiency and extraction duration should be improved. For instance, Wang et al. [9] reported that ultrasonic cellulase extraction was successfully employed to extract polyphenols from passion fruit. The results indicated that the combined method was found to be more effective compared to single extraction and Soxhlet extraction methods due to 1.5-2 times higher content of polyphenols. However, most of the studies focused on the noni roots [10] - [13] or noni fruit [14] using individual advanced extraction techniques such as ultrasonic assisted extraction, hot water extraction, and pulsed electric field assisted extraction. Importantly, due to very limited studies on the combination of ultrasound and enzyme, it is desirable to carry out the enzymatic extraction conditions of bioactive compounds from noni fruit that is pretreated with ultrasound.

In the enzymatic extraction technique, enzymes are applied to hydrolyze the plant wall. Some phytochemicals in the plant matrices are dispersed in the cell cytoplasm, and some compounds are retained in the polysaccharide-lignin network by hydrogen bonding or hydrophobic interactions, which are not accessible with a solvent in a routine extraction process. Enzymatic treatment has been considered a novel and effective way to release bounded compounds and also to increase overall yield. The addition of specific enzymes such as cellulase and pectinase during extraction enhances recovery by breaking the cell wall and hydrolyzing the structural polysaccharides and lipid bodies [15]. There are various factors such as type of enzyme and concentration, the particle size of plant materials, water-to-solid ratio, temperature, and hydrolysis time are well-known as important factors for extraction [16]. Depending on the plant matrix, several critical factors should be considered. As such, the objective of this study was to investigate the enzymatic extraction conditions, including enzyme concentration, water-to-powder ratio, temperature, and time, of ultrasound-pretreated noni fruit extract to achieve the highest content of bioactive compounds in terms of total polyphenol, flavonoids, saponin, and vitamin C. Furthermore, the resultant extract was also compared with the ones extracted using enzymatic extraction without ultrasound pretreatment and ethanolic extraction method in order to determine the effect of the combined extraction method.

2. Materials and Methods

2.1. Chemicals

In this research, commercial enzymes consisting of pectinase (pectinase activity of 108654 PBU/g) and cellulase (cellulase activity of 5436 CMC U/g) were purchased from Hung Thinh Vietnam Co., Ltd., Ha Noi, Vietnam. Folin-Ciocalteu (FC) reagent, sodium carbonate, vanillin, sulphuric acid, sodium nitrite, aluminum chloride, sodium hydroxide, oxalic acid, 2,6-dichlorophenol-indophenol (DCPIP), and ethanol were of analytical grades. All analytical chemicals were obtained from Bach Khoa Co., Ho Chi Minh City, Vietnam. Gallic acid (G7384), quercetin (Q4951), aescin (E1378), and ascorbic acid (A92902) were purchased from Sigma-Aldrich, Singapore, and used as a standard to analyze the total polyphenols, flavonoids, triterpenoid saponins, and ascorbic acid contents, respectively.

2.2. Pretreatment used prior to aqueous enzymatic extraction

Morinda citrifolia L. fruit was purchased at maturity of pale yellow or white, fairly hard, 8-14 cm long from Tien Giang province, Vietnam. The fruits were put inside an insulated hard plastic container to avoid light and temperature exposure during transport and used on the same day. The noni fruits were then kept in the laboratory freezer at -10ºC before starting the experiment. The frozen Noni fruits were sliced into 4.5 mm thickness and air-dried at 60ºC for 7 h until the final moisture content of approximately 9.2% was obtained. The dried fruit was then finely powdered (≤ 500 µm), vacuum packed, and kept at ambient temperature prior to further extraction experiments.

An ultrasonic water bath (Model S180, Elma, Germany) was used to perform the ultrasonic pretreatment. The ultrasound conditions were fixed, for all experiments, at a frequency of 37 kHz, a temperature of 50 ± 5°C and a time of 10 min which were chosen based on our preliminary trials. These parameters were also fixed throughout the study. About 5 g of noni powder was dissolved in distilled water (a ratio of water to powder ranging from 8/1 to 20/1 mL/g) in a 150-mL conical flask and ultrasound pretreatment conditions were chosen based on our preliminary trials.

2.3. Effects of enzymatic extraction conditions on bioactive compounds

After ultrasound pretreatment, the investigated amount of enzyme (0, 0.1, 0.5, and 1%, w/w) was added to the mixture. Next, the mixture was incubated in an incubation bath at desired temperatures (40, 50, and 60°C) and times (30, 45, and 60 min). Finally, the sample was coarsely filtered with cheese cloth and filtered again with filter paper (pore size 15-20 µm) before analysis.

The one-factor experiments, including enzyme concentration, a ratio of water to noni powder, temperature, and time, were randomly designed to investigate the effects of aqueous enzymatic extraction conditions on the content of total polyphenols, flavonoids, triterpenoid saponin, and vitamin C of the resultant noni extract. The most suitable enzymatic extraction conditions were selected for the next experiment. All the extraction runs were carried out in triplicate.

2.4. Comparison

The content of the bioactive compounds in the resultant extract using the enzymatic extraction method was compared with the enzymatic extraction without ultrasound pretreatment and the ethanolic method. For comparison, the conditions of enzymatic extraction without ultrasound pretreatment were carried out similar to the most suitable conditions obtained from Section 2.4 without ultrasound pretreatment. The optimal ethanolic extraction conditions were performed according to the procedure reported by Thoo et al. [17] with slight modifications. Briefly, the noni powder, which was prepared as described in Section 2.2, was extracted with 75% ethanol (a ratio of solvent to noni powder of 10/1) using a heating magnetic stirrer at 57˚C for 40 min and 5000 rpm. Subsequently, the sample was treated similarly to enzymatic extraction as described above before analysis.

2.6. Analytical methods

2.6.1. Total phenolic content

The total phenolic content (TPC) was measured according to Singleton et al. [18] with slight modifications. First, 0.5 mL of sample was mixed with 2.5 mL of Folin-Ciocalteu 10%. After 5 min, 2 mL of 7.5% sodium carbonate solution were added. The mixture was allowed to stand at room temperature in the dark environment for 60 min. The absorption was read at 765 nm using a spectrophotometer (Model 8453, Agilent, US). Gallic acid was used as a standard and the results were expressed as gallic acid equivalents (GAE) per g dry weight (d.w.) of the sample (mg GAE/ g, d.w.).

2.6.2. Total flavonoid content

The total flavonoid content (TFC) was determined according to Marinova et al. [19] with slight modifications. About 1 mL of sample was added to a 10-mL volumetric flask containing 4 mL distilled water. The flask was added with 0.3 mL of 5% $NaNO_2$. After 5 min, 0.3 ml of 10% $AlCl_3$ were added. At the 6th minute, 2 mL of NaOH (1 M) was added, and total volume was made up to 10 ml with distilled water. The solution was mixed well in a vortex and the absorbance was read against at 510 nm. Quercetin was used as a standard and the results were expressed as quercetin equivalents (QE) per g dry weight (d.w.) of the sample (mg QE/ g, d.w.).

2.6.3. Total saponin content

The total saponin content (TSC) was calculated according to Tan et al. [20] with slight modifications. First, 0.3 mL of sample was mixed with 0.3 mL of 8% (w/v) vanillin solution and 3 mL of 72% (v/v) sulfuric acid. The mixture was incubated at 60°C for 15 min and then cooled on ice for 10 min. The absorption was recorded at 560 nm using a spectrophotometer. Aecsin was used as a standard and the results were expressed as aecsin equivalents (AE) per g dry weight (d.w.) of the sample (mg AE/ g d.w.).

2.6.4. Ascorbic acid content

Ascorbic acid content (AA) was determined by 2, 6-dichlorophenol indophenol (DCPIP) titration method [21]. About 1 mL of working standard (1 mg/mL) of ascorbic acid and 2 mL of 4% oxalic acid were pipetted out into a 150-mL conical flask. The contents in the flask were titrated against the dye solution (V1) until the appearance of a pale pink colour persisted for a few minutes. Approximately 1 mL of the test sample was similarly titrated against the dye solution (V2). Ascorbic acid content was presented in mg AA/ g d.w.

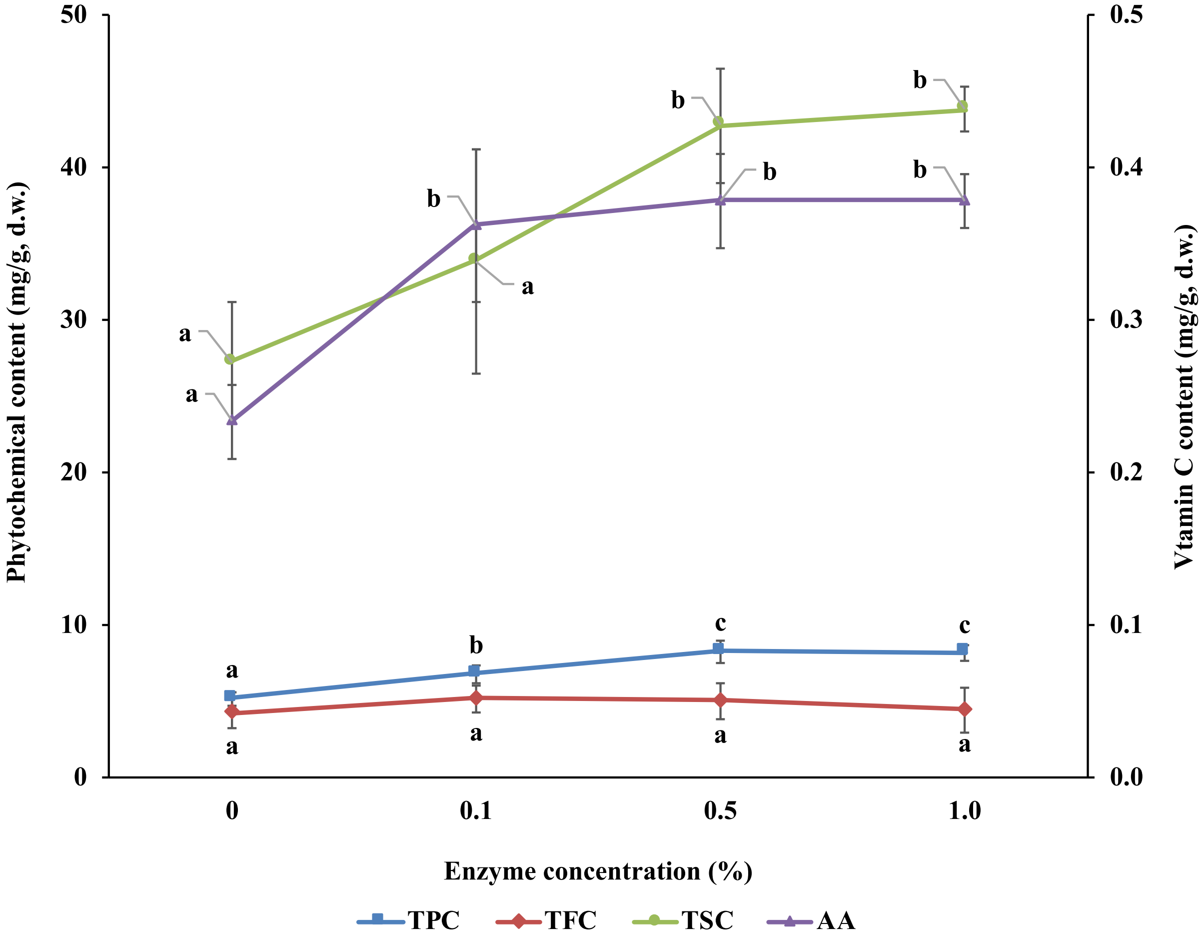
2.7. Statistical analysis

All experiments and analytical measurements were carried out in triplicate. The data were statistically analyzed using SPSS software version 25. The results are presented as mean values and standard deviations (Mean ± SD). The difference between the mean values was analyzed by analysis of variance (ANOVA) and the least significant difference (LSD) at a 5% significance level ($p < 0.05$). The graphs of mean values and error bar were created using Excel version 2016.

3. Results and Discussion

3.1. The effects of enzyme concentration on the extraction of bioactive compounds

The different concentrations of the enzyme were investigated at the fixed factors including a ratio of water-to-noni powder of 8/1, the temperature of 50ºC, and time of 60 min. As shown in **Figure *1***, the effects of the enzyme concentration on the extraction of TPC ($p < 0.001$), TSC ($p < 0.01$) and vitamin C ($p < 0.01$) were found to be statistically significant; however, the TFC was not significantly different ($p > 0.05$). Specifically, the content of polyphenols and saponins tended to



increase with the increase of the enzyme concentration until the concentration of 0.5% was reached. Further increase in the concentration resulted in a non-significant increase in the content of these bioactive compounds in the extract. Under the most suitable enzyme concentration (0.5%), the maximum values of TPC, TFC, TSC, and vitamin C were found to be 8.30 ± 0.75 mg GAE/ g d.w., 5.02 ± 1.20 mg QE/ g d.w., 42.68 ± 1.45 mg AE/ g d.w., 0.38 ± 0.02 mg/g d.w., respectively.

In general, it can be concluded that the ultrasound pretreated before enzyme hydrolysis results in an increase in bioactive compounds in the noni extract. Ultrasonic pretreatment results in random disruption of the cell structure and release of biological compounds, comprising the phenolic through severing its binding to polysaccharides or proteins [22]. At the same time, the collapse of the plant cell wall created favorable conditions for the enzyme to work. Enzymes selectively break down the obtained substrate after sonication, which improves solvent distribution, declines particle size and viscosity, and increases mass transfer rate. Thereby, the recovery of bound phenols and other compounds is enhanced [23]. When there was an excess of substrate, the reaction rate grows as soon as the enzyme is supplemented, but it remained unchanged as the enzyme concentration was increased.

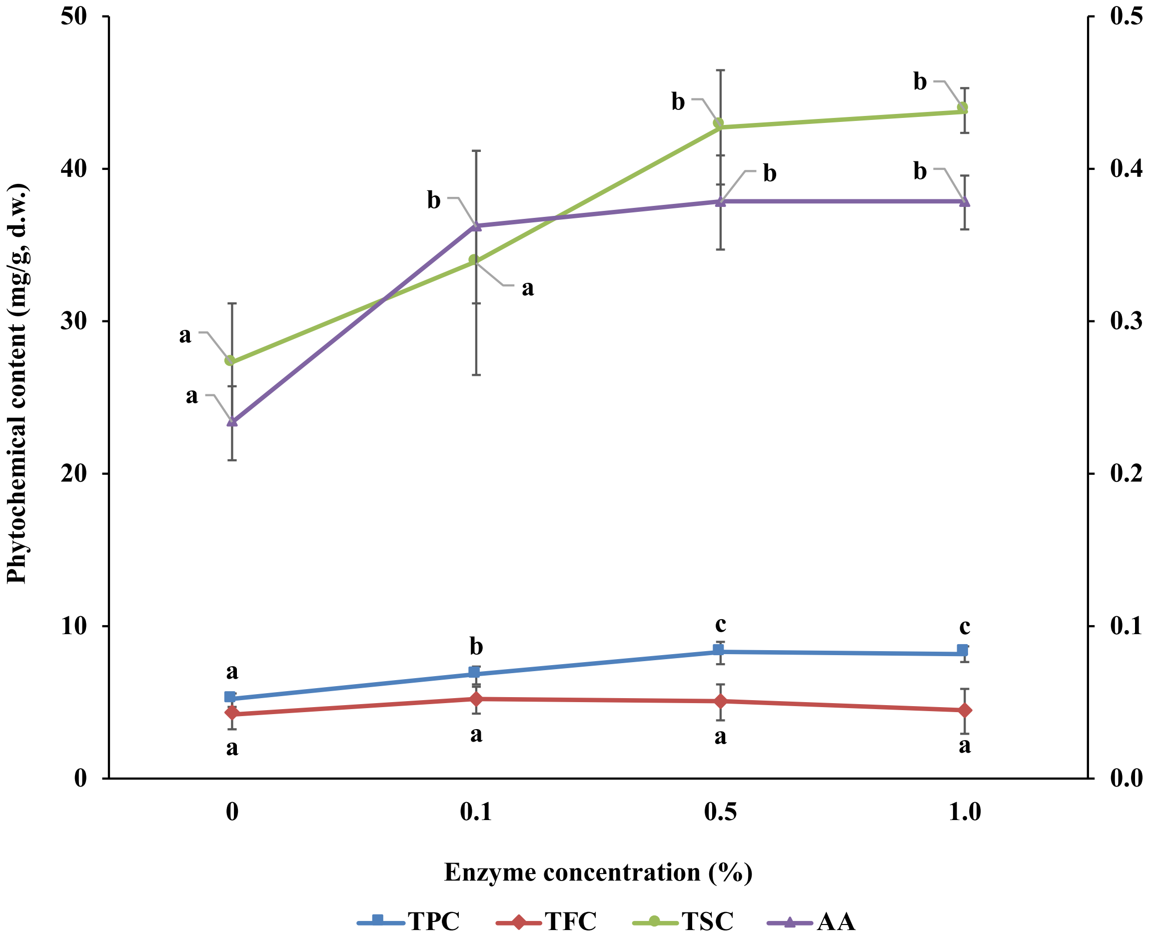


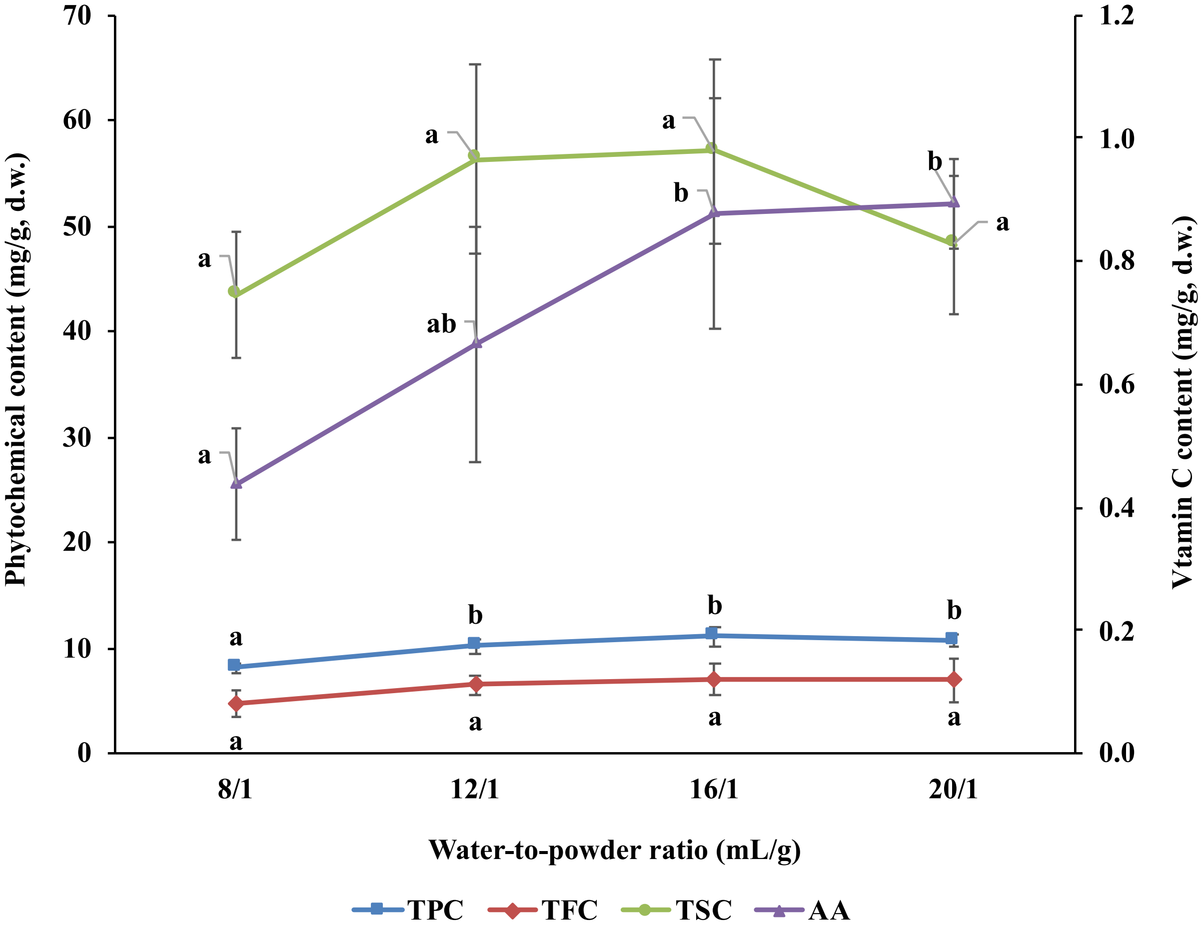
Figure 1. *Effect of enzyme concentration on the content of phytochemicals and vitamin C*

TPC, total phenolic content; TFC, total flavonoid content; TSC, total saponin content; and AA, ascorbic acid content (vitamin C). The values are expressed as mean ± standard deviations.

Values in a line not sharing a letter (a, b) are significantly different from each other ($p < 0.05$).

3.2. The effects of a water-to-powder ratio on the extraction of bioactive compounds

The ratio of water-to-powder in enzymatic extraction has been considered another important factor, which may influence the yield of extracted bioactive compounds. In theory, the higher ratio of water-to-powder, the higher yield of bioactive compounds obtained. As such, the effects of the ratio of water-to-noni powder ranging from 8/1 to 20/1 mL/g were investigated at the fixed enzymatic extraction conditions including the enzyme concentration of 0.5%, the temperature of 50°C, and time of 60 min.



As shown in **Figure** *2*, the content of polyphenols, including TPC and TFC, increased sharply when the water-to-powder ratio was increased from 8/1 to 12/1 mL/g. The content then only gradually increased and reached a plateau as the ratio exceeded 16/1 mL/g. A similar trend was also found for TSC and vitamin C, but the content of TSC and vitamin C decreased when the water-to-powder ratio exceeded 16/1 mL/g. As such, in terms of maximal extraction of TPC, TFC, TSC, and vitamin C, the best extraction yield was achieved with the ratio of water-to-powder of 16/1 mL/g.

This could be explained by the fact that the solvent promotes plant extraction by contributing to the improvement in mass transfer during ultrasonication, increasing the breakdown of cell structure, and releasing biological compounds. Besides, water is the dissolution medium that facilitates the enzyme contact with the substrate, which helps to raise the enzyme affinity, giving rise to boosting the extraction efficiency. Nevertheless, when the solvent was added to a certain ratio (20/1 in this experiment), it resulted in a decrease in the substrate concentration, leading to a limit on the enzyme's ability to act and reducing extraction efficiency.

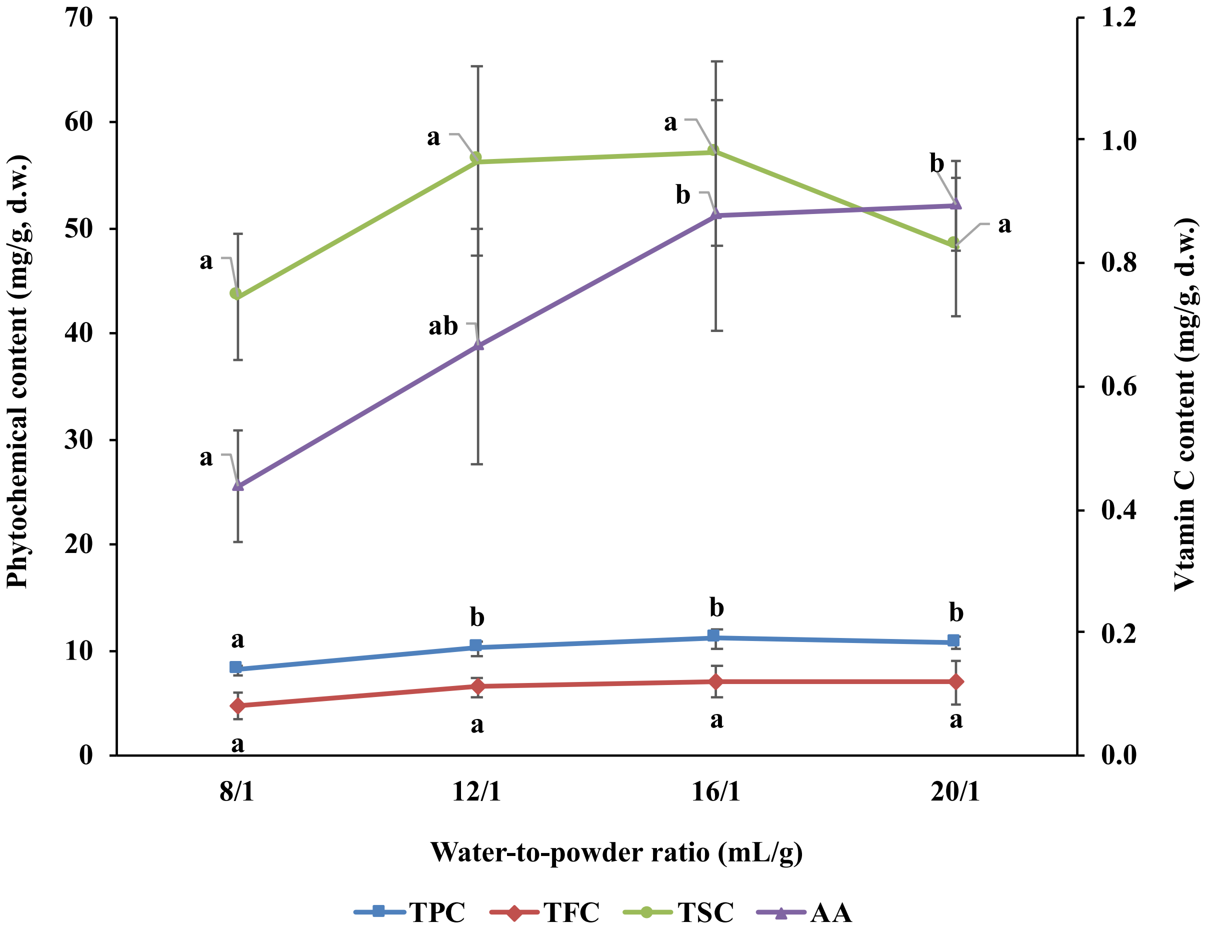


Figure 2. *The effect of a water-to-powder ratio on the content of bioactive compounds*

TPC, total phenolic content; TFC, total flavonoid content; TSC, total saponin content; and AA, ascorbic acid content (vitamin C). The values are expressed as mean ± standard deviations.

Values in a line not sharing a letter (a, b) are significantly different from each other ($p < 0.05$).

3.3. Effect of extraction temperature on the extraction of bioactive compounds

The temperature of enzymatic extraction was considered one of the important factors that could affect the bioactive compounds from noni fruit. In this research, the three different temperatures of 40, 50, and 60˚C were investigated at the fixed factors including enzyme concentration of 0.5%, the ratio of water-to-powder of 16/1 mL/g, and time of 60 min (Figure 3). The results indicated that the content of bioactive compounds was non-significantly different at three temperatures of 40, 50, and 60˚C ($p > 0.05$), except for flavonoids ($p < 0.05$). In particular, when the temperature rose from 40˚C to 60˚C, the flavonoid content increased from 5.90 ± 0.68 and 7.67 ± 0.61 (mg QE/g d.w.), respectively. Statistical results showed that there was a non-significant difference in the flavonoid content between the sample enzymatically treated at the temperature of 40 and 50˚C, and 50 and 60˚C. As such, the temperature of 50˚C was considered the most suitable extraction temperature of bioactive compounds.

In general, high extraction temperatures, which should be in the range of optimal temperature of enzyme activity, can increase the extraction yield of bioactive compounds. The temperature influences enzyme activity and the rheological properties of soluble compounds. The enzyme activity increases proportionally with an increasing temperature, resulting in enhancing the extraction efficiency. In addition, the viscosity of the medium reduces with increasing temperature and the compounds are therefore more soluble. However, high temperature during the extraction causing the coagulation and denaturation of proteins owing to a change in the three-dimensional structure of enzyme are the main barriers to extracting bioactive compounds [23]. In addition, high temperatures may destroy bioactive compounds that are more sensitive to heat, consisting of polyphenols, saponins, and vitamin C. The decrease or instability in the content of those compounds at 60˚C was observed due to the thermal degradation rather than other causes such as oxidation and light. Therefore, the most suitable temperature of 50˚C for extracting the bioactive compounds from noni fruit is highly recommended.

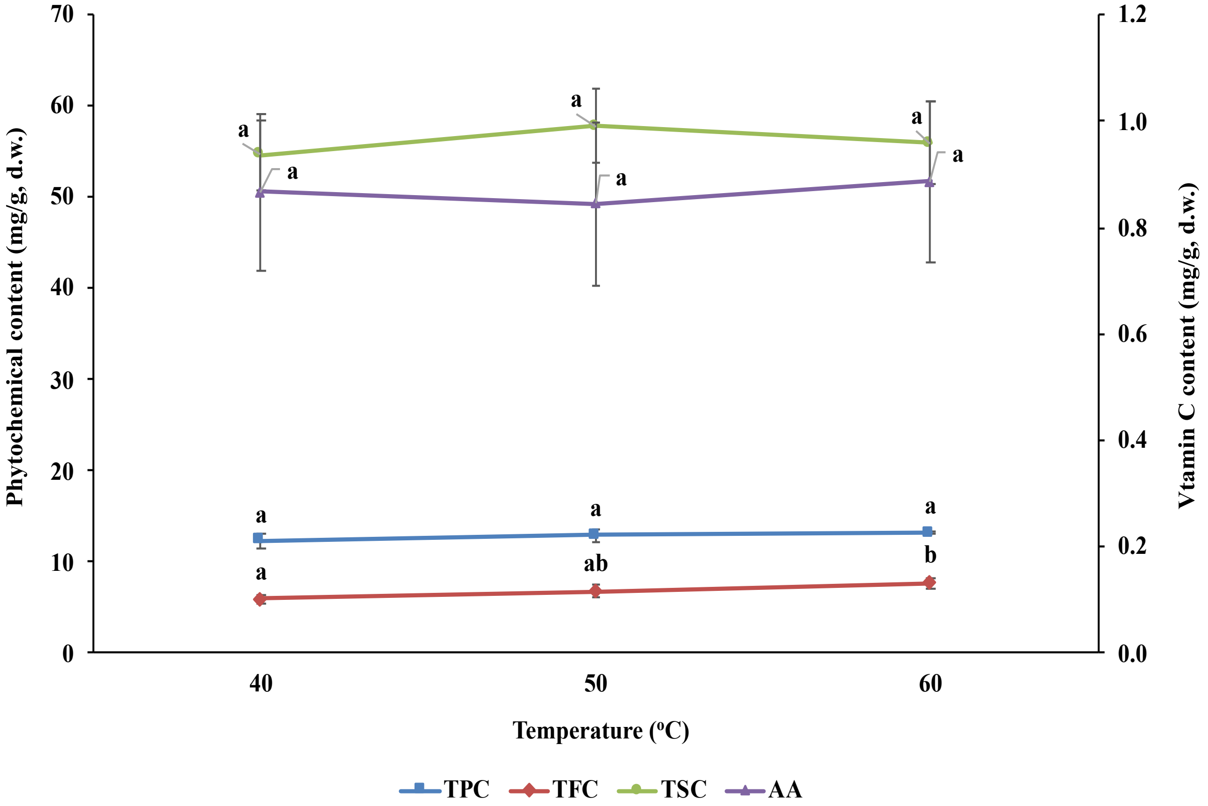


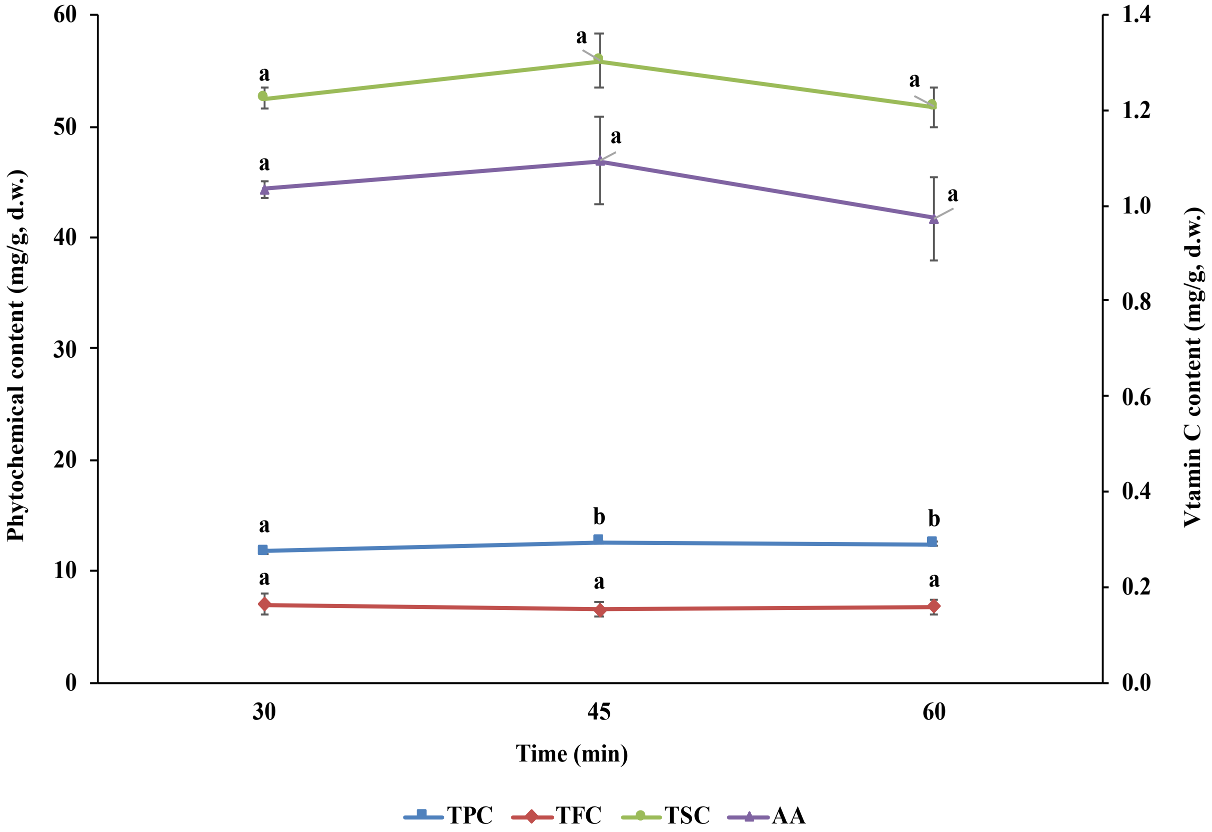
Figure 3. *Effect of incubation temperature on the content of bioactive compounds*

TPC, total phenolic content; TFC, total flavonoid content; TSC, total saponin content; and AA, ascorbic acid content (vitamin C). The values are expressed as mean ± standard deviations.

Values in a line not sharing a letter (a, b) are significantly different from each other ($p < 0.05$).

3.4. Effect of incubation time on the extraction of bioactive compounds

The duration of extraction is also considered an important factor impacting the yield of the bioactive compounds extracted. The longer extraction times can enable more of the bioactive compounds to move into the solution. However, the stability of those compounds may be affected when the incubation time is longer due to oxidation and degradation, particularly under higher temperatures. As such, the effect of incubation time on the content of TPC, TFC, TSC, and vitamin C was investigated under the most suitable conditions obtained from the above experimental results (i.e. enzyme concentration of 0.5%, the ratio of water-to-powder of 16/1 mL/g, and incubation temperature of 50˚C) for three different times of 30, 45, and 60 min.



As shown in Figure *4*, the incubation time had an effect on the content of TPC, TFC, TSC, and vitamin C. In general, the content of those bioactive compounds slightly increased as the incubation time was increased from 30 to 45 min, and then a decrease when the time was further increased to 60 min. Statistical results indicated that the effect of incubation time on TFC, TSC, and vitamin C was found to be non-significant ($p > 0.05$), except for TPC ($p < 0.05$). Solvent permeability and dry matter dissolution were improved when the time increased, resulting in enhanced extraction rate and improved extraction yield; simultaneously this helped enzymes expose to substrate well; thus the content of biologically active compounds in the extract was higher. However, with a further increase in the extraction time, the bioactive compounds may be degraded or oxidized, leading to a reduction of the bioactive compound content in the resultant extract. Therefore, the incubation time of 45 min was desirable.

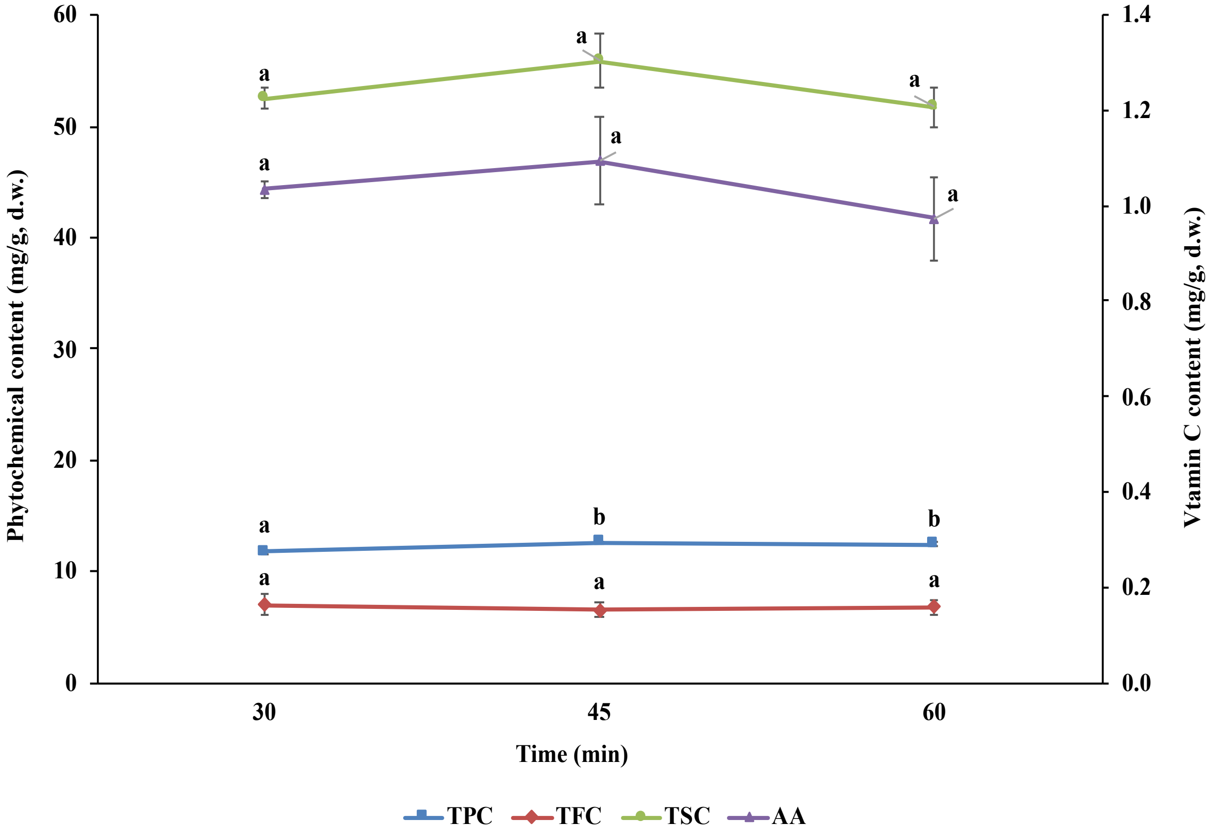


Figure 4. *The effect of incubation time on the content of bioactive compounds*

TPC, total phenolic content; TFC, total flavonoid content; TSC, total saponin content; and AA, ascorbic acid content (vitamin C). The values are expressed as mean ± standard deviations.

Values in a line not sharing a letter (a, b) are significantly different from each other ($p < 0.05$).

3.5. Comparison of aqueous enzymatic extraction and ethanolic extraction methods

In this research, the content of bioactive compounds including TPC, TFC, TSC, and AA extracted by the present method (i.e. aqueous enzymatic extraction after ultrasound pretreated) under the most suitable conditions, aqueous enzymatic extraction without ultrasound pretreated, and the optimal ethanolic extraction conditions (refer to Section 2.5). The results are shown in Table 1.

Table 1. *Comparation of bioactive compounds content between the present, aqueous enzymatic extraction without ultrasound pretreated and ethanolic extraction methods*

	TPC **mg GA/g, d.w.**	**TFC** **mg QE/g, d.w.**	**TSC** **mg AE/g, d.w.**	**AA** **mg/g, d.w.**
Aqueous enzymatic extraction after ultrasound pretreated	12.53 ± 0.09^{a}	6.45 ± 0.61^{a}	56.02 ± 2.45^{a}	1.04 ± 0.09^{a}
Aqueous enzymatic extraction without ultrasound pretreated	9.04 ± 0.04^{b}	4.06 ± 0.14^{b}	30.83 ± 1.00^{b}	0.52 ± 0.09^{b}
Ethanolic extraction	15.80 ± 0.81^{c}	9.33 ± 0.43^{c}	61.85 ± 2.06^{c}	2.09 ± 0.20^{c}

TPC, total phenolic content; TFC, total flavonoid content; TSC, total saponin content; and AA, ascorbic acid content (vitamin C). The values are expressed as mean ± standard deviations.

Values in a column not sharing a superscript letter ($^{a, b, c}$) are significantly different from each other ($p < 0.05$).

As can be seen in Table 1, the content of phytochemicals in the resultant extract using ultrasound pretreated prior to the enzymatic extraction was significantly higher than that obtained without ultrasound and highly comparable with those using the optimal ethanolic extraction method. The low extraction level of AA obtained likely is due to the higher thermal degradation rate of AA compared to the phytochemicals. As a result, there is a need to further investigation on the improvement of the AA retention in the extract. Furthermore, although the use of ethanol as extracting solvent is less harmful and toxic compared with other chemical solvents such as methanol, acetone, hexane among others, the concern of ethanol safety, management, and control of solvent emission during the extraction is important to protect workers and the environment. Particularly, as the resultant extract is applied into foods and/ or pharmaceuticals, consumers are concerned about potential residual solvents in the extract. As such, the use of a green solvent such as only water is more desirable. This research finding show feasibility of combining ultrasound pretreatment and enzymatic extraction for extracting the bioactive compounds from noni powder. In order to improve extraction yield, there is a need to further optimize the extraction conditions using the response surface methodology.

4. Conclusions

This research showed the extracted bioactive compounds including TPC, TFC, TSC, and vitamin C from ultrasonic pretreated using aqueous enzymatic extraction were affected by all four of the factors investigated; the enzyme concentration, the water-to-powder ratio, the temperature, and the time were all important factors which directly affected the content of the bioactive compounds in the resultant extract. The results indicated that the most appropriate enzymatic extraction conditions for ultrasound pretreated noni powder were enzyme concentration of 0.5%, the water-to-powder ratio of 16/1, incubation temperature of 50°C, and time of 45 min. Under those conditions, the content of TPC (12.53 mg GAE/ g d.w.), TFC (6.45 mg QE/ g d.w.), TSC (56.02 mg AE/ g d.w.), and vitamin C (1.04 mg AA/g d.w.) was found to be maximal. Furthermore, the most suitable extraction conditions of the present method were also compared with the enzymatic extraction without ultrasound pretreatment and the optimal ethanolic extraction method to determine the positive effect of the ultrasound pretreated prior to enzymatic extraction. It can be concluded that the present extraction method is a novel and green alternative method for extracting the bioactive compounds, indicated by the high content of bioactive compounds and water used as extracting solvent. Finally, future research on extraction optimization, isolation, and characterization of the extracted bioactive compounds from noni fruit should be carried out for an application in foods and pharmaceuticals.

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