Influence of extraction methods on bioactive compounds from Ngoc Linh ginseng callus

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Abstract

Panax vietnamensis Ha et Grushv or Vietnamese ginseng is a valuable medicinal herb with high economic value in the world. In this study, three target compounds were surveyed as polyphenols, polysaccharides, and saponins to evaluate the effects of factors including microwave, ultrasound, enzyme amylase, and cellulase for the first time. The results of the study showed that with different extraction conditions, the recovery efficiency of polyphenol, polysaccharide, and saponin was also different. With the addition of 1.2% of the enzyme amylase (v/v) in 8 hrs of incubation, the highest total content of polyphenol and polysaccharide was obtained, equivalent to 6.48 mg GAE/g sample and 312.48 mg Glu/g sample. While the total saponin content reached the highest value of 4.60 mg/g sample at 4 hrs of incubation using 0.8% of cellulase enzyme (v/v). The effect of microwave and ultrasound also showed a significant recovery efficiency for the three analyzed compounds. But compared to the use of two enzymes (amylase, cellulase), the efficiency was lower. The use of these extraction techniques was based on the advantages of environmental friendliness, simple operation, lower investment costs, and power saving. At the same time, it can improve the extraction and recovery efficiency as well as the activity of bioactive compounds.

1. Introduction

Ngoc Linh ginseng (*Panax vietnamensis* Ha et Grushv.) is a herb with high economic and medicinal value. Like other species in the ginseng genus (such as *P. ginseng*, *P. notoginseng*, *P. quontheofolium*, and *P. japonicus*), the root or rhizome of Vietnam ginseng used to strengthen and recover health and support for treatment of liver diseases (Tran *et al.*, 2001), anti-tress, antidepressants (dela Peña *et al.*, 2017), anti-cancer cells (Van Le *et al.*, 2015), treatment of neurological and cardiovascular diseases (Duc *et al.*, 1994). The main compounds in Ngoc Linh ginseng are saponins triterpene, which have a hydrophobic steroid framework associated with hydrophilic sugar groups or hydroxyl groups (Nag *et al.*, 2012). Ngoc Linh ginseng contains more ginsenoside than other ginseng varieties including more than 52 types with majonoside - R2, which the main ingredient of saponins with more than 5% dry weight, founded in any other ginseng (Tran *et al.*, 2001). Besides that, Ngoc Linh ginseng contains 14 fatty acids, 16 amino acids with 8 irreplaceable amino acids, and 18

macro and trace elements (Tran *et al.*, 2001). In recent years, many other studies had also confirmed the presence of other bioactive substances in Vietnam ginseng, such as polyphenols, flavonoids. In addition to that, Ngoc Linh ginseng contains a high content of triterpene glycosides, especially ocotillol-type derivatives and many other compounds (Chirikova *et al.*, 2019). This showed that the bioactive compounds from Ngoc Linh ginseng is important. But their natural distribution was dramatically decreasing due to overexploitation, search for materials can be proactive while still ensuring medicinal activity is necessary. In recent years, many studies had demonstrated that Ngoc Linh ginseng callus cells cultured *in vitro* contains ginsenoside (Kevers *et al.*, 2004), phenolic compounds such as 5- O - caffeoylquinic acid, 3 - O - caffeoylquinic acid, 5 - O - feruloylquinic acid (Olennikov *et al.*, 2012). This proved that the *in vitro* callus cells can accumulate bioactive compounds akin to mature plants and can become an alternative material in studies. However, previous studies on Ngoc Linh ginseng mostly mentioned the qualitative and pharmacological effects of

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the compounds.

There were not many studies about the effects of these methods in the above bioactive compound extraction. Extraction is an important step to recover bioactive compounds for plants in general. There are many extraction methods used to improve the efficiency of obtaining bioactive substances from medicinal plants including microwaves, ultrasound, and enzymes. Pan *et al.* (2002) showed that the microwave process improved the extraction efficiency of compounds from *Codonopsis javanica* than other popular methods such as heat reflux extraction, soxhlet extraction, as well as shorter extraction time (Pan *et al.*, 2002). Furthermore, the use of enzyme amylase to extract polysaccharide from Korean ginseng (Song *et al.*, 2018) or the recovery of carotenoids and phenolics using cellulase and pectinase enzymes (Neagu *et al.*, 2014) both led to better extractions rather than traditional methods. The impacts such as microwave or enzyme had positive effects on the efficiency of the extraction of bioactive compounds from medicinal plants. However, the evaluation and comparison of the effect of microwaves, ultrasound, and enzymes on the recovery of biological compounds from Ngoc Linh ginseng has not been thoroughly discussed. Comparing the extraction efficiency of bioactive compounds by different impacts were performed on the same object. In this study, the efficiency of obtaining bioactive compounds from Ngoc Linh ginseng include phenolics, polysaccharides, and saponins triterpene by microwave, ultrasound, and enzymes. The evaluation process was to determine the role of single impacts as well as comparing the extraction efficiency between them to the extraction process of bioactive compounds from Ngoc Linh ginseng.

2. Materials and methods

2.1 Material

Ngoc Linh ginseng was derived from Ngoc Linh Mountain, which is located at 15°04'00"N 107°59'00"E in the Central Highlands region of Vietnam. Ngoc Linh ginseng embryos was cultured in the SH medium (Schenk and Hildebrandt) supplemented with 2 g/L casein hydrolysate.

A weight of 2 g of Ngoc Linh ginseng callus (Figure 1) was added to 70 mL of distilled water, then treated with microwave, ultrasound, and enzymes.

Figure 1. Ngoc Linh ginseng callus

2.2 Microwave-assisted extraction (MAE)

The samples were extracted by microwave (EMM2308X, Electrolux, Sweden) with the following conditions: power (450 W, 650 W, and 800 W), time (45, 60, 75, 90, and 105 s). Then, the extract was filtered and examined for biological activity.

2.3 Ultrasound-assisted extraction (UAE)

The samples were analysed using ultrasonic equipment (VC 750, Sonics, USA). The extraction times at various ultrasound powers were set for 5, 10, and 15 mins respectively (10 s on the pulse and 10 s off pulse) at 20, 25, 30, 35, and 40% wattage, respectively. The extract was filtered and examined for biological activity.

2.4 Enzyme-assisted extraction (EAE)

The samples were individually supplemented with enzymes amylase (100,000 UI/mL), cellulase (700 EGU/g, Novozyme, Bagsvared, Denmark) at different rates (0.4, 0.8 and 1.2% (v/v)). Samples were incubated at 55° C for 2, 4, 8, 12, and 24 hrs. The extract was then filtered and examined for biological activity.

2.5 Analytical method

2.5.1 Total phenolics

The total polyphenol content was determined according to the Folin-Ciocalteu method (Leamsomrong *et al.*, 2009). The test sample solution of 1 mL was mixed with 5 mL of 0.2 M Folin-Ciocalteu reagent and 4 mL of 7.5% sodium carbonate solution. The solution was left in the dark at room temperature for 30 mins before being analysed at 765 nm. Total phenolic content was expressed as mg of gallic acid equivalents/g sample).

2.5.2 Total polysaccharide

The total polysaccharide content was determined according to the Anthrone-sulfuric acid method (Brummer and Cui, 2005). The sample solution of 1 mL was added to 5 mL of ethanol, incubated for 24 hrs at 4° C. Then, the mixture was centrifuged to obtain the precipitate and dissolved again with hot water at 70°C. The solution of 2 mL was then to 8 mL of anthrone 0.2% reagent in concentrated H₂SO₄ acid. The absorbance of the samples was read at 630 nm.

2.5.3 Total saponin

The total saponin content was determined according to the vanillin-perchloric acid method (Yang *et al.*, 2004). Approximately, 0.2 mL of the sample solution was added to 0.2 mL of vanillin/5% acetic acid, 1.2 mL of 70% perchloric acid, and incubated at 70°C for 20 mins. After the end of the incubation process, samples

were rapidly cooled for 2-3 mins. Then, the mixture was added to 5 mL ethyl acetate. The absorbance of samples was read at 550 nm.

2.6 Statistical analysis

The experiments repeated three times, results presented as mean $\pm$ standard deviation. Data were processed using Excel 2016 and SPSS 20 software with the Duncan test used to compare the differences between groups, significance level 5.

3. Results and discussion

3.1 Effects of microwave-assisted extraction on bioactive components from Ngoc Linh ginseng

The efficiency of the effect of microwave power and time for the extraction of Ngoc Linh ginseng callus was presented in Figure 2. The result from the graph showed that the total content of polyphenol, polysaccharide and saponin of the microwave sample was higher than ($p < 0.05$) the control. At the same time, when the energy level was increased to 650 W, the concentration of the compounds obtained was higher than when 450 W was used. The total polyphenol content at 75 s was 4.27 mg GAE/g sample (650 W) and 4.06 mg GAE/g sample (450 W). As the microwave power was continually increased to 800 W, compounds were quickly extracted in a shorter period, the total polysaccharide content reached 223.56 mg Glu/g sample in just the first 60 s. While at 450 W, the maximum value was 217.35 mg of Glu/g sample, and the 650 W was 221.5 mg of Glu/g sample for longer than 75 s (Figure 2). However, the results found that there were no significant differences between the two microwave energy levels 650 W and 800 W, within the same period ($p > 0.05$).

Figure 2. Effect of microwave treatment on the total content of polyphenols, polysaccharides, and saponins from Ngoc Linh ginseng callus.

The microwave treatment could influence the

efficiency of extraction of substances from plant cells based on the electrical conductivity and dielectric polarization caused by the microwave radiation and accumulating internal pressure (Li *et al.*, 2010). Increasing the microwave power and time significantly increased the total content of polyphenols, polysaccharides, and saponins (Figure 2). At low microwave power (450 W), the recovery time of compounds prolonged led to a slower diffusion speed (Del Nobile *et al.*, 2012). Research conducted by Chandrasekar *et al.* (2015) shows that the total phenolics content extracted from apple pomace, increased gradually when the microwave power was increased from 100 – 900 W, and the highest phenolic content obtained was 15.8 mg GAE/g sample at 149 s with an energy level of 735 W (Chandrasekar *et al.*, 2015). The results from the study indicated that more bioactive compounds were obtained when higher microwave power was used in a shorter time (Figure 2). A higher microwave power causes the temperature to rise rapidly in a short time, which vaporizes the solvent molecules, causing a sudden increase in pressure, breaks the cell structure and creates favourable conditions for dissolved compounds that are then released into the solvent (Pan *et al.*, 2003). Considering the same power level, when the microwave time was prolonged, it resulted in the decrease of target compounds, with 800 W microwave power, the total polyphenols content for 75 s was 4.91 mg GAE/g sample and reduced to 4.66 mg GAE/g sample at 90 s (Figure 2). Similar results were reported by Li *et al.* (2010), the yield of triterpene saponin extract from the defatted residue of yellow horn (*Xanthoceras sorbifolia* Bunge.) kernel increased from 2.75 to 3.31% when microwave time was increased from 3 to 7 mins, but with times longer than 7 mins, it showed signs of reduction as longer extraction times causes excessive power consumption and leads to the decrease of bioactive compounds (Li *et al.*, 2010). At high temperatures, solvents infiltrates and dissolves more substances while surface tension and viscosity of the solvent decreased with increasing temperature, which improved cell wall permeability and long extraction time made the extraction process inefficient because the increased temperature led to the degradation of less heat-stable compounds such as polyphenols (Cacace *et al.*, 2003). The results showed that the microwave condition was 650 W for 75 s. The total content of the obtained bioactive compounds reached the highest value as seen in Figure 2.

3.2 Effects of ultrasound-assisted extraction on bioactive components from Ngoc Linh ginseng

Figure 3 shows the influence of ultrasound on bioactive compounds from Ngoc Linh ginseng through

two factors, ultrasonic power, and time. The ultrasonic process treated samples showed higher efficiency in recovering analytical compounds compared with the control samples ($p < 0.05$).

Figure 3. Effect of ultrasonic treatment on the total content of polyphenols, polysaccharides, and saponins from Ngoc Linh ginseng callus.

Concurrently, the total content of polyphenols, polysaccharides, and saponins was proportional with ultrasonic, reached the highest value at ultrasonic power of 25% (187.5W), equivalent to a 5.25 mg GAE/g sample, 241.92 mg Glu/g sample, and 3.67 mg/g sample with an ultrasound time of 10 minutes (Figure 3). The results showed that at the ultrasonic power level of 25%, the recovery efficiency of compounds was higher than the lower power level of 20%, there was no significant difference compared with the higher capacity levels of 30, 35, and 40% (Figure 3). Ye *et al.* (2012) reported that when extracted polyphenols from unripe apples by ultrasonic technique, total polyphenols content increased when increased power from 280 W to 560 W and obtained the maximum value at 490 W (Yue *et al.*, 2012). The ultrasonic process could strengthen efficient extraction because of the propagation of waves (Vilkhu *et al.*, 2008). The compression, expansion, and burst of air bubbles created by the ultrasonic process caused chaotic movement with high speed of microscopic particles in plant materials and created microjets, which impacted the cell surface and led to erosion, surface peeling, and destruction of cell structure (Vilkhu *et al.*, 2008). This increased touch between the cell of material and solvent which increased the ability to extract these substances. This principle was confirmed by samples of mint leaves photographed before and after ultrasonic treatment using an electron microscope to extract menthol. (Shotipruk *et al.*, 2001). At lower ultrasonic power, the mechanical impact created favourable conditions for the mass transfer process, many compounds were extracted (Figure 3). However, with

higher ultrasonic power and longer time, the content of substances reduced. In which the ultrasonic energy level was 40%, the content of three target compounds decreased significantly with the ultrasound time (Figure 3). Research by Zhang *et al.* (2008) shown that a longer ultrasonic time increased the efficiency of oil recovery from flaxseed but prolonging the ultrasonic time for too long caused the recovery efficiency to decreased significantly (Zhang *et al.*, 2008). This showed that the effect of the ultrasonic waves was more effective in the beginning. The ultrasonic waves broke down the plant cell walls, increased the contact area between the solvent and the material, and more target compounds were extracted. This process got weaker and weaker when the ultrasonic time of samples prolonged due to internal hydration and redox (Alonso-Salces *et al.*, 2001). Therefore, ultrasonic energy of 25% for 10 mins of pulse generation was the appropriate parameter when used the ultrasonic action for this study.

3.3 Effects of enzyme amylase and enzyme cellulase on bioactive components from Ngoc Linh ginseng

Survey results on the effects of enzymes amylase and cellulase on the total content of polyphenols, polysaccharides, and saponins from Ngoc Linh ginseng were shown in Figure 4 and Figure 5, respectively.

The results showed that the longer the incubation time, the higher the recovery efficiency of polysaccharide, polyphenols, and saponins. At each additional rate, the values of the three target compounds increased continuously from 2 hrs to 12 hrs of incubation. Polyphenols reached the highest value at 8 hrs incubation with the rate of 1.2% enzyme, equivalent to 6.48 mg GAE/g sample, two times higher than the control, then, there was a sign of gradual decrease but not significantly. Meanwhile, the best recovery time for total polysaccharide content was 12 hrs, equivalent to 312.48 mg Glu/g sample, which increased 66% compared to the control (Figure 4). On the other hand, the highest obtained total saponin content was after 12 hrs of incubation with an enzyme rate of 1.6%, equivalent to 4.18 mg/g of sample, which has increased by 78% compared to the control at the same time. Considering at the same incubation period of 8 hrs, when the enzyme rate was increased to 1.2%, the total content of polysaccharides, polyphenols, and saponins reached the highest value, of 23.5%, 29.6%, 14.1%, respectively, compared to the lowest rate (0.4%). When the enzyme used continued to increase to 1.6%, the total content of polyphenols, polysaccharides, and saponins had no statistically significant difference (Figure 4). Previous studies had demonstrated that the treatment of plant materials with enzymes had a significant effect on the recovery of bioactive compounds. Research by Sunwoo

Figure 4. Effect of enzyme amylase on the total content of polyphenols, polysaccharides, and saponins from Ngoc Linh ginseng callus.

et al. (2013) shown that the use of β - amylase enzyme increased saponin yield in fresh ginseng by 21% during the initial 12 hrs, with no significant change in prolongation incubation time by 24 hrs (Sunwoo *et al.*, 2013). The prolonging incubation time helped the enzyme activity to be maintained, the enzyme had more time to react with the substrate to create the product, increased the recovery efficiency, the longer extraction time composition of the substances contained in the extract were affected (Persson *et al.*, 2006). Research by Hong *et al.* (2013) had also shown that catechin compounds could inhibit enzymes by direct competitive interaction (Hong *et al.*, 2013). On the other hand, recent studies had demonstrated that starchy granules exist in various types of ginsengs (Yu *et al.*, 2011). These starch grains interfered with the extraction of polysaccharides (Sun *et al.*, 2015). For conventional hot extraction methods, there were still quite a few polysaccharides that were not fully extracted. The results obtained from the study indicated that the enzyme amylase was suitable for the extraction of compounds, especially polysaccharides from Ngoc Linh ginseng callus (Figure 4). At the same time, the study results also show that, when the amount of amylase enzyme was increased, compared to the extract, the content of all three target compounds also tended to increase. Therefore, the appropriate condition for using enzyme amylase in this experiment was 1.2% (v/v) for 8 hrs of incubation.

Similar to the amylase-treated samples, the extracted samples were incubated with the cellulase enzyme for increasing the efficiency of the recovery of biological compounds with time and the addition rate (Figure 5). Enzyme-assisted extraction of plant compounds was based on increased mass transfer rate and the penetration of solvents and enzymes into materials to release intracellular products by breaking down the cell wall

Figure 5. Effect of enzyme cellulase on the total content of polyphenols, polysaccharides, and saponins from Ngoc Linh ginseng callus.

(Sunwoo *et al.*, 2013). The results of the study showed that the lowest additional rate was 0.4%, the appropriate time to receive the maximum bioactive compounds was extended to 12 hrs, but still higher than the sample control (Figure 5). Accordingly, the total content of polyphenols, polysaccharides, and saponins reached 4.60 mg GAE/g sample, 227.74 mg Glu/g sample, and 3.75 mg/g sample respectively, increased 54%, 4.6%, and 46.5%, compared to the control sample. However, when the enzyme ratio increased by two times (0.8%), the appropriate time to receive bioactive compounds shortened by three times (4 hrs). Corresponding to the values of polyphenols, polysaccharides, and saponins increased by 23%, 28%, and 23%, respectively, compared to the rate of 0.4%. The incubation time was an important parameter in the processing of materials with enzymes because it directly affected the transformation of the starting material into a different product within a certain limit (Choi *et al.*, 2010). The experimental results showed that the content of the target compounds increased gradually when the incubation time of the enzyme extended. The treatment of oats with the enzyme cellulase (80 mins) showed significantly increased total phenolic content, antioxidant ability compared to the heat treatment when the incubation period lasted from 0 to 80 mins (Chen *et al.*, 2016). Similarly, polysaccharide extracts from pumpkin (*Cucurbita moschata*) were supported by cellulase, and their antibacterial activity showed to increase with continuous increase in incubation time from 20 to 120 mins (Qian, 2014). However, too much increase in enzyme content did not improve the efficiency of the extraction of compounds (Figure 5). The recovery polysaccharides from *Polygonatum odoratum* showed a 5% cellulase concentration was more efficient when used at 6% was reported by Liu *et al.* (2015). Complex

polysaccharides such as cellulose, hemicellulose, pectin, and lignin created and kept the plant cell walls stable (Gligor *et al.*, 2019). Enzymes had specific properties that broke the bonds of the above substrates, such as a polysaccharide-lignin bond, ether-ester bonding between phenol and polymer (Hong *et al.*, 2013) helped release bioactive compounds inside the cell space. This made the enzyme-assistant extraction process acquire higher amounts of bioactive compounds ($p < 0.05$) than the control (Figures 4 and 5). The results obtained from the study showed that, in the first 2 - 4 hrs, excess substrate, the reaction speed increased when increased the rate of additional enzymes. However, when increased (1.2%), the content of the target compounds did not change significantly (Figure 5). Similar results were observed in the case of incubation with the enzyme amylase when the enzyme content increased to 1.6% (v/v), the extraction efficiency was no longer proportional to the enzyme concentration (Figure 4). This was explained by the phenolics obtained from cellulose hydrolysis by the cellulase enzyme, went back to precipitate, and inhibited irreversible enzyme, the reaction velocity did not change (Qin *et al.*, 2016). The results obtained from this study indicated that the recovery efficiency of the bioactive compounds in this survey was affected by an increased rate of cellulase enzymes (1.2% v/v) or amylase (1.6% v/v) (Figures 4 and 5). An appropriate rate of enzyme and incubation time was necessary to ensure the effective extraction of bioactive compounds from Ngoc Linh ginseng callus.

The effects of different impact factors include microwave, ultrasound, and enzymes (amylase, cellulase) on the total content of compounds extracted from Ngoc Linh ginseng presented in Table 1. The experimental results of the single impact shown that all four impact factors had a certain influence on bioactive compounds from Ngoc Linh ginseng callus. The results study showed that the use of enzyme amylase with the appropriate rate was 1.2% (v/v) in 8 hrs of incubation, obtained the highest total content of polyphenol and polysaccharide. Total saponin content obtained by cellulase enzyme with an additional rate was 0.8% (v/v), incubated for four consecutive hrs. The data table also

showed that, although the microwave process had a positive effect on the content of analytical compounds, compared to the remaining effects in this study, the microwave had the lowest extraction efficiency. Microwaves created the movement of molecules in the material based on ionic, and bipolar interaction led to thermogenesis that decomposed some heat-sensitive compounds (Li *et al.*, 2010). Meanwhile, the use of enzymes directly impacted the material cell walls and broke them down through a specific interaction without causing internal heat generation (Sunwoo *et al.*, 2013), thus protected compounds that are easily degraded by heat, such as polyphenols.

The results study showed that all four impact factors brought positive effects in the extraction process to obtain bioactive substances from Ngoc Linh ginseng. In which the treatment of Ngoc Linh ginseng by enzymes amylase, cellulase was significantly effective while the effect of the microwave with high power levels was less effective. In the upcoming studies, which necessary to investigate the combined effects of factors affecting the ability to acquire bioactive substances from Ngoc Linh callus.

4. Conclusion

The results of the study showed that with different extraction conditions, the recovery efficiency of polyphenol, polysaccharide, and saponin was also different. The enzyme treatment rate was 1.2% (v/v) in the case of enzyme amylase treatment in 8 hrs of incubation, obtained the highest total content of polyphenol and polysaccharide while the total saponin content obtained the highest value with 0.8% (v/v) in the case of cellulase treatment condition in 4 hrs of incubation. Although the extraction process was supported by microwave and ultrasound, which had a positive impact on the recovery efficiency of three analytical compounds from Ngoc Linh ginseng callus, the efficiency was still significantly lower than using enzymes. Concurrently, research results also showed that it was feasible to use callus for extraction. Thus, it increased the initiative and stability of raw materials.

Table 1. Effects of different treatment methods on bioactive compounds from Ngoc Linh ginseng callus.

Methods	Total polyphenol content (mg GAE/g sample)	Total polysaccharide content (mg Glu/g sample)	Total saponin content (mg/g sample)
Control	3.79±0.42 ^e	186.00±11.24 ^e	2.75±0.39 ^c
MAE (650 W/75 s)	4.27±0.11 ^d	221.5±8.11 ^d	2.91±0.15 ^c
UAE (25%/10 min)	5.25±0.22 ^c	241.92±6.44 ^c	3.67±0.12 ^b
Enzyme Amylase 1.2%/8 hrs	6.48±0.09 ^a	312.48±5.33 ^a	3.80±0.12 ^b
Enzyme Cellulase 0.8%/4 hrs	5.67±0.14 ^b	291.43±6.31 ^b	4.60±0.08 ^a

Values are expressed as mean±SD. Values with different superscript within the column are significantly different ($p < 0.05$)

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