



Extraction of Bioactive Component from Herbal *Anoectochilus formosanus* Hayata by Microwave, Ultrasound and Lactic Fermentation

Le Thi Kim Ngan^{1,a}, Nguyen Thi Ly^{1,b}, Nguyen Thi Tham^{1,c}, Dang Thi Kim Thuy^{2,d},
Do Dang Giap^{2,e}, Lieu My Dong^{1,f,*}

¹Faculty of Food Technology, Ho Chi Minh city University of Food Industry, Viet Nam

²Institute of Tropical Biology, Ho Chi Minh city, Viet Nam

*Corresponding author

ARTICLE INFO	ABSTRACT
<p>Research Article</p> <p>Received : 03/10/2018 Accepted : 01/03/2019</p> <p>Keywords: <i>Anoectochilus formosanus</i> Hayata Bioactive compounds Microwave-assisted extraction Ultrasound-assisted extraction Fermentation Lactic acid</p>	<p><i>Anoectochilus formosanus</i> Hayata was demonstrated to have a benefit healthy due to containing active pharmaceutical ingredients. However, <i>A. formosanus</i> is usually processed to produce tea bags which would destroy the bioactive compounds because of the processing procedure. The aim of this study was to evaluate the influence of extracted methods including microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), and fermentation by <i>Lactobacillus acidophilus</i> ATCC-4356 to extract the active pharmaceutical ingredients from <i>A. formosanus</i>. The extracted liquid was analyzed total phenolics, total polysaccharide, and antioxidant activity. The results showed that three methods have a positive effect on the extraction of bioactive compounds of <i>A. formosanus</i> in which the fermentation showed the best result. The total phenolic content, total polysaccharide content and antioxidant capacity that extracted by the fermentation method were 11.762 mg GAE/g; 48.914 mg GE/g, and 1.582 mgVit C/g compare to MAE and UAE which were 7.818 mg and 8.128 GAE/g samples; 41.22 and 37.91mg GE/g samples; 1.032 and 1.163 mgVit C/g respectively. The <i>A. formosanus</i> fermentation method by <i>L. acidophilus</i> promotes bioactive compounds of high biological value. This study would suggest a novel use of lactic fermenting <i>A. formosanus</i> in the production of functional foods.</p>

^a lieudong289@gmail.com

^b <https://orcid.org/0000-0002-6519-0488>

^c lyngan06dhtp@gmail.com

^d <https://orcid.org/0000-0001-6010-4096>

^e suonghatham@gmail.com

^f <https://orcid.org/0000-0003-1948-6269>

^e dtkthuy@gmail.com

^f <https://orcid.org/0000-0002-4212-6942>

^e dodanggiap@gmail.com

^f <https://orcid.org/0000-0001-6618-7670>

^f lieudong289@gmail.com

^f <https://orcid.org/0000-0003-2836-5290>



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Introduction

Anoectochilus formosanus Hayata and different pharmaceutical ingredients extract from *A. formosanus* was demonstrated that they are antioxidant (Tsenga et al., 2006), and useful for prevention and treatment of cirrhosis (Shiha et al., 2005), reducing respiratory distress syndrome of the fetus (Mayumi et al., 2004), improving the immune system, anti-tumor (Tseng et al., 2006), prevention of osteoporosis after menopause (Shih et al., 2004), treatment of neurological diseases, cardiovascular and hypertension. However, *A. formosanus* is usually processed to produce tea bags which would destroy the bioactive compounds because of the processing procedure. Thus, the extraction of bioactive compounds from medicinal plants is necessary to bring health benefits to consumers. Currently, methanol extraction is often used to improve the extraction efficiency. However, in food application, the extraction of bioactive compounds by methanol should be considered for use because methanol could be toxic at low concentration (200 ppm) (Clary., 2013). Therefore,

researching the alternative extraction methods is necessary to obtain bioactive substances. Ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) are one of the potential technologies to extract compounds in herbal drug due to decreasing times and temperature of extraction and enhancing the bioactivity compounds (Vinatoru et al., 2017; Diego et al., 2016). Besides, the process of fermentation is known as a method to extract bioactive compounds effectively (Chang-Chai et al., 2011). Fermentation processing breaks down or converts the composite substrates into functional components under the action of microbial enzymes, which modified the characteristics of the product or changed the content of bioactive compounds (Ahtesham et al., 2016). Fermentation techniques also showed the increased the obtaining ability of bioactive compounds from *A. formosanus* (Chang-Chai et al., 2011). However, though these extraction methods were very promised, the study of comparison of these extracted methods including

ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE) and fermentation was reported poorly. Therefore, the present study was conducted to evaluate the extraction efficiency of the bioactive compounds from *A. formosanus* of these methods by evaluating criteria of total phenolics, total polysaccharide, and antioxidant activity.

Materials and Methods

Anoectochilus formosanus Hayata and Microbial Strain

Biomass of *A. formosanus* in vitro was provided by plant cell Technology lab, Institute of Tropical Biology HCM city and preserved at 4°C.

L. acidophilus ATCC 4356 was obtained from the strain collection of the Faculty of food technology of the Ho Chi Minh University of food industry. *L. acidophilus* was multiplied on Man Rogosa Sharpe (MRS) at 37°C after 22h culture, biomass collection and use for the next process.

Method of Extract

Preparation of *A. formosanus*: *A. formosanus* Hayata removed roots and rotten leaves, washed and dried. 100 ml of sterile water was added into 250 ml the Becher containing 20 ± 0.01 g of the milled sample.

Microwave treatment: MAE experiments were performed on a microwave machine (Electrolux 1250 W) with microwave power 600 W, 800 W, 1000 W; microwave time 30s, 45s, 75s, 105s, 150s, 180s, 210s. Then, the mixture was centrifuged at 5000 rpm for 10 min and collected the supernatant fluid.

Ultrasonic treatment: UAE experiments were performed on equipment Sonics with maximum power 750 W, frequency 20 kHz at power 20%, 25%, 30%, 35%, 40% with ultrasonic times 5 min, 10 min, 15 min with a pulse ON time of 10 sec followed by a pulse OFF time of 10 sec. Then, the mixture was centrifuged at 5000 rpm for 10 min and collected the supernatant layer.

Fermentation processing with *L. acidophilus*: The biomass of *L. acidophilus* was calibrated by spectrophotometer to reach the concentration of *L. acidophilus* of 10^7 CFU/ml, 10^8 CFU/ml, 10^9 CFU/ml and fermented at 24h, 48h, 72h, 96h. After completed fermentation process, the mixture was centrifuged at 5000 rpm for 10 min and collected the supernatant fluid.

The control samples: 100 ml of sterile water added into the Becher (250 ml) that contains $20 \pm 0,01$ g of the milled sample then kept in 30 min. After that, the mixture was centrifuged at 5000 rpm for 10 min and collected the supernatant fluid.

Analytical Methods

Total phenols were determined according to the method of Li Ru-Lai et al. (2013), with slight modified. Brief, 0.1 ml of *A. formosanus* extract added 0.9 ml two times distilled water and 5 ml Folin-Ciocalteu reagent, after 3 min, added 4 ml Na_2CO_3 7.5% (w/v). The mixture stored in dark condition to allow reaction in 30 min. The reaction mixtures were measured at 765 nm absorbance. The results were calculated by mg as gallic acid equivalent per gram (GAE/g) that based on the calibration curve of Gallic acid (10-80 ppm).

The total polysaccharide was determined based on the method described by Dong et al. (2016), with a slight modified. Brief, 0.1 ml of *A. formosanus* extract added 1.9 ml two times distilled water, 8 ml 0.2% of anthrone reagent in concentrated sulfuric acid solution. The mixtures kept under room temperature. The reaction mixtures were measured at 630 nm absorbance. The result was calculated by mg D-glucose equivalent per gram (GE/g) that based on the calibration curve of D-glucose (4-50 ppm).

DPPH (1,1-diphenyl-2-picrylhydrazyl) antioxidant activity: Measurement of DPPH scavenging activity was determined according to Chang et al (2011) with slight modified. Briefly, 1 ml of *A. formosanus* extract added to 1 ml two times distilled water, 10 ml of 0.8 μM DPPH methanolic solution were mixed and stored in dark condition to allow reaction at 60 min. The reaction mixtures were measured at 517 nm absorbance. The result was calculated by mg Vitamin C/g that based on the calibration curve of L-ascorbic acid (0-50 ppm).

Statistical Analysis

The treatments were repeated three times to calculate the average value, standard deviation. The experimental results were recorded as mean \pm standard deviation. One-way analysis of variance was performed by ANOVA procedures LSD to verify LSD with $\alpha=0.05$ on Statgraphics XV, calculate and show graphs at sigmaplot 11.

Results and Discussion

Effects of Microwave-Assisted Extraction on Bioactive Components of *A. formosanus* Extract

The effect of the MAE method on total polyphenol content and total polysaccharide content was shown in Fig 1. These results showed that the contents of total polyphenol and total polysaccharide of the microwave-assisted sample at the optimal conditions were higher than those of the control sample ($P<0.05$). With microwave power 600W, 800W, 1000W, contents of polyphenolic and polysaccharide were highest in 180s (7.025 mg GAE/g sample, 40.114 mg GA/g sample), 75s (7.818 mg GAE/g sample, 41.220 mg GE/g sample) and 75s (7.338 mg GAE/g sample, 37.220 mg GE/g sample) respectively.

These results indicated that the polyphenolic and polysaccharide contents also increased similarly in the same point in time in the experiment. Time and microwave power of the MAE method effected significantly on the recovery of the phytochemical components (Fig 1). The high of electric-field energy at the high power have formed the rotation of dipole, leading to the friction among molecules, and the high pressure which breaks the biological matrix and release the biological components (Ali et al., 2007). At the low microwave power leading to the microwave energy was low, so the prolonging of microwave time was necessary to reach the boiling point of water. The disruption of friction into cell walls and the increase of microwave time have enhanced the penetration of extracting solvent through the matrix facilitating the obtainment of the target compounds (Vinatoru et al., 2017). On the other hand, in the same of microwave power, if the extraction time were much longer, the obtained component contents increased more rapidly. Nevertheless, the high of microwave power and the long-time of extraction

decreased the compound contents rapidly. For 210s of irradiation time, the content of polyphenol and polysaccharide reduced 7.2%, 48.67%, 58.86% and 10.67%, 22.45%, 34.02% in 600W, 800W, 1000W respectively. The high of microwave power, as well as the long-time extraction, has increased the extracted temperature leading to decreasing the obtained compounds.

DPPH radical scavenging activity of MAE sample increased higher than that of the control sample ($P < 0.05$) and the highest antioxidant activity was achieved at the time of 105, 75, 45 seconds corresponding to the microwave power of 600W, 800W, 1000W (Fig. 2). These results corresponded with the results of the content of total phenolics and polysaccharide (Fig 1), this is because they are the compounds with radical-scavenging activity (WonWoo et al., 2013). The DPPH radical scavenging activity was decreased strongly at the different microwave power when prolonging the extraction time up to 210s. The antioxidant activities of the heat-sensitive compounds decreased with the prolonging of microwave time. These results showed that the optimal conditions to obtain the highest recovery were 75s at 800W (Fig 1, 2).

Effects of Ultrasonic Treatment on Bioactive Components of A. formosanus

The ultrasonic-assisted sample obtained the higher polyphenol and polysaccharide contents than that of the control samples ($P < 0.05$) (Fig.3). Ultrasonic time affected significantly the content of polyphenol and polysaccharide. In the same ultrasonic time, the content of total polyphenol and polysaccharide increased with the ultrasonic power from 20% to 25%, the highest content at 25% power but decreased at high ultrasonic power 30%, 35%, 40%. In the 25% of ultrasonic power, the content of polyphenol and polysaccharide increased with the extension of the radiation time from 5 to 10 minutes, which demonstrated that the extension of ultrasonic time led to the increase of the recovery rate. Ultrasonic method is widely used to extract various substances from a plant by creating microscopic bubbles that believed in to create high-shear gradients by causing micro-streaming which disrupts the cell walls leading to accelerates the penetration of solvent into cells and the release of components from cells into the solvent, and simultaneously significantly enhances the mass transfer rate (Yuting et al., 2013).

In the present study indicated that the antioxidant activity in 5 min, 10 min, 15 min was achieved the highest at the ultrasonic power of 35%; 25% and 25% respectively and when increasing the ultrasonic power up to 35%, 40%, the free-radical scavenging activity decreased gradually. The increases of the antioxidant activity were due to the content of polyphenol and polysaccharide was high achieved which contributed to the antioxidant activity (WonWoo et al., 2013). At a power sufficiently high, water molecules can be broken generating highly reactive free radicals ($H_2O \rightarrow H^+ + OH^-$) that may react with and modify other molecules (antioxidant) (Ana Cristina et al., 2010). In the present study showed that the ultrasonic treatment with the power of 25% in 15 min, the effects of the recovery of bioactive substances was the highest (Fig 3, 4).

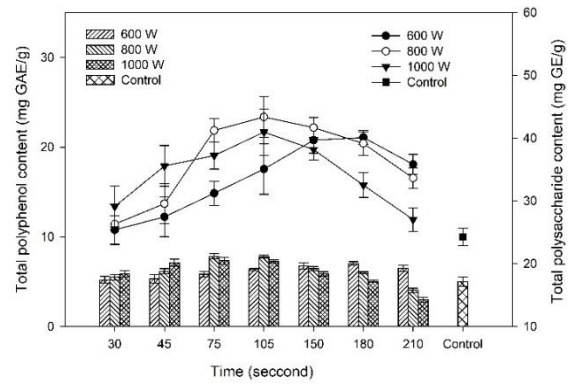


Fig 1 Effects of microwave treatment on content of total phenolics, total polysaccharide

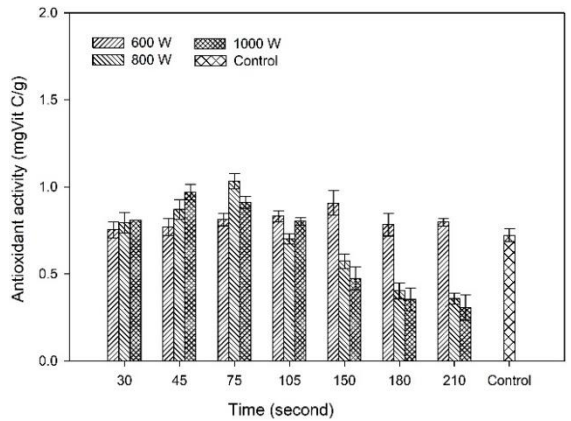


Fig 2 Effects of microwave treatment on antioxidant activity

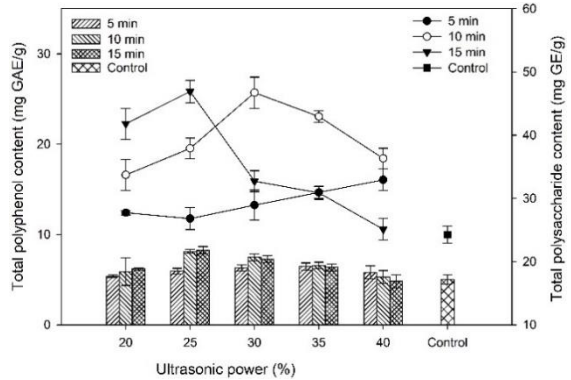


Fig 3 Effects of ultrasonic-treatment on total phenolics, total polysaccharide

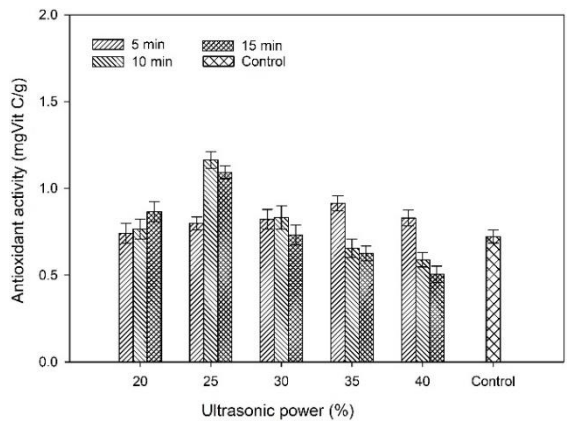


Fig 4 Effects of ultrasonic treatment on content of antioxidant activity

Effects of Fermentation on Bioactive Substance of A. formosanus.

The effects of fermentation process by *Lactobacillus acidophilus* on the content of total phenolics and polysaccharide were shown in Fig. 5.

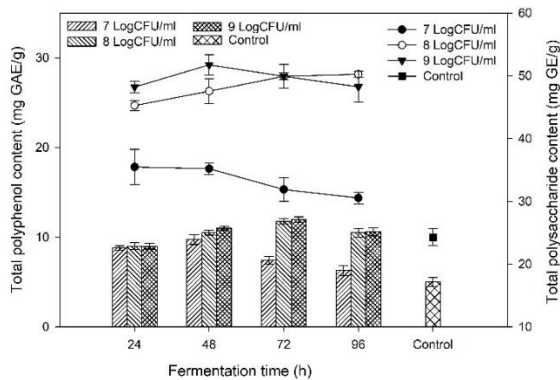


Fig 5 Effects of fermentation on content of total phenolics, total polysaccharide

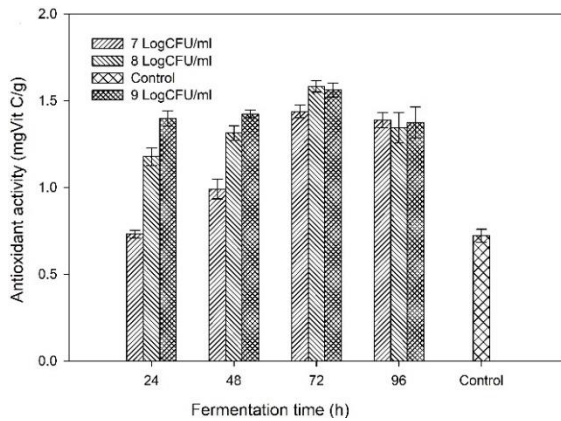


Fig 6 Effects of fermentation on antioxidant activity

These results showed that the obtained total phenolics was higher compared to the unfermented sample ($p < 0.05$). The content of polyphenol achieved from the concentration of 10^7 CFU/ml, 10^8 CFU/ml, 10^9 CFU/ml was higher 1.9 times (9.747 mg GAE/g sample), 2.3 times (11.762 mg GAE/g sample) and 2.4 times (11.962 mg GAE/g sample) compared to the unfermented sample, respectively. In the fermentation processing, *Lactobacillus acidophilus* metabolized or convert the crude matrix into biological

components. The change of the polyphenol-protein, polyphenol-lipid bonds in the fermented process led to release free phenol, reduce the content of the associated phenol (Li-Ru et al., 2013). However, when the time of fermentation kept longer, the polysaccharide content decreased gradually (Fig. 5). This might be explained that lactic bacteria released the active enzyme such as amylase, β -glucosidase which can transfer polysaccharide into monosaccharide and used them to produce ATP for the biosynthesis of lactic acid and the last products (Ratchadaporn et al., 2018). The content of polysaccharide at the concentration of 10^9 CFU/ml (48.207 mg GE/g sample) obtained higher than that of 10^7 CFU/ml (35.466 mg GE/g sample) and 10^8 CFU/ml (45.257 mg GE/g sample) after 24h for fermentation. The total polysaccharide was tended to decrease gradually after 48h, 72h, and 72h fermentation at the concentration of 10^7 CFU/ml, 10^8 CFU/ml, and 10^9 CFU/ml respectively (Fig. 5). This showed that the increase of the concentration of *L. acidophilus* enhanced the catalytic ability to the biochemical reaction which released much more compounds but consumed a lot of energies in their growth process.

The results for the effects of the fermentation by *L. acidophilus* on the antioxidant activity displayed in Fig. 6 which showed that the highest antioxidant capacity of samples fermented at the concentration of 10^8 CFU/ml in 72h. These results corresponded with the content of polyphenol and polysaccharide achieved. These results could be explained by the content of bioactive compounds that modified during fermentation through the metabolic activity of microbes. Especially, lactic acid bacteria have an enzyme which can produce the highly antioxidant such as hydroxytyrosol, pyrogallol, 4-vinyl phenol, 4-vinyl guaiacol. The antioxidant activity of the flavonoid aglycone is 10 to 12 times higher compared to flavonoid glucosides (Sheng-Yang et al., 2002). *L. acidophilus* contained β -glucosidase which can cleave flavonoid glucoside into aglycone (Axelle et al., 2017). This enhanced the antioxidant capacity (Fig. 6). According to Chang-Chai et al., (2011), *A. formosanus* is processed to produce tea bags for consumption in Taiwan, however, the processing procedure possibly destroys the functional compounds, which reduces the aroma and flavor of *A. formosanus* herbal tea.

Table 1 Comparison of the different extraction methods

Treatment methods	Total polyphenol content (mg GAE/g)	Total polysaccharide content (mg GE/g)	Antioxidant activity (mg VitC/g)
Control	(5.010 ± 0.5) ^a	(24.266 ± 0.930) ^a	(0,722 ± 0,037) ^a
Microwave	(7.818 ± 0.305) ^b	(41.220 ± 1.86) ^b	(1,032 ± 0,043) ^b
Ultrasonic	(8.128 ± 0.216) ^b	(37.910 ± 1.61) ^c	(1,163 ± 0,047) ^b
Fermentation	(11.762 ± 0.311) ^c	(49.914 ± 3.46) ^d	(1,582 ± 0,032) ^c

^{abc} Representing significant differences (P<0.05) in each experiment

Research on the effect of MAE, UAE, and fermentation methods on the recovery of the bioactive compounds was reported in the previous studies (Ana Álvarez et al., 2017; Vinatoru et al., 2017; Chang-Chai et al., 2011). However, the evaluation of the effects among these methods had not yet been published sufficiently. The results in this study showed that all microwave-assisted, ultrasonic treatment,

fermentation methods effected positively on exploiting the bioactive components from *A. formosanus*. The fermented processing from *A. formosanus* showed that the effects of the recovery rate of biological compounds were higher compared to two other methods (Table 1). Microwave and ultrasonic were the extract process with physical affectations, different from them, the fermentation not only

enhanced extract capacity of the functional components from *A. formosanus* but also create the highly bioactive substances due to the metabolic activity of microbes. In the next study, it suggests evaluating the effects of three combined methods, making a simulation to find the optimal conditions for the highest recovery rate of bioactive components.

Conclusion

The results of this study indicated that all microwave-assisted, ultrasonic-assisted, and fermentation methods enhanced the ability of exploitation of biological substances from *A. formosanus*. The maximum bioactive components recovery from *A. formosanus* including the microwave power was 800W in 75s, ultrasonic treatment at 25% of power in 10min and fermentation with *Lactobacillus acidophilus* at the concentration of 10⁸ CFU/ml in 72h. In which, fermentation achieved the highest bioactive components recovery compared to the other methods, simultaneously, enhanced the biological activity of those compounds. *A. formosanus* fermented by *Lactobacillus acidophilus* at the concentration of 10⁸ CFU/ml in 72h obtained the highest total polyphenol and polysaccharide content which are 11.762 mg GAE/g sample and 48.914 mg GE/g respectively. The antioxidative ability of fermented *A. formosanus* was higher 3 folds than that of the control. This study demonstrated that the fermentation by *L. acidophilus* is the potential technology which can replace other methods to obtain the bioactive compounds from *A. formosanus* and increase pharmacological properties of those compounds

Acknowledgments

The authors gratefully acknowledge Ho Chi Minh City University of Food Industry for the financial support

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