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Effect of lactic fermentation, microwave, and ultrasonic extraction on the bioactive compounds from *Anoectochilus formosanus* Hayata

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Abstract

Anoectochilus formosanus (family Orchidaceae) is a perennial herb that contains many bioactive valuable. In this study, the extract efficiency of bioactive compounds from *Anoectochilus formosanus* Hayata by microwave-assisted extraction (MAE), ultrasonic-assisted extraction (UAE) treatments, and lactic fermentation in individual and combined impacts were evaluated through the total phenolic content, total polysaccharide content, and antioxidant activity. The results showed that MAE and UAE treatments and lactic fermentation enhanced the effective extraction of bioactive compounds compared to the control samples. The bioactive compound contents from *A. formosanus* fluid trend differences in individual treatment factors. However, there was no significant difference between these treatment factors in which the fermentation process requires more time to reach the expected extraction efficiency. The ultrasonic pretreatment combined lactic fermentation process would bring many benefits; first, no need for more the MAE treatment; second, reduce fermentation time; third, bring a probiotic source as well as valuable metabolic products from the lactic fermentation process.

1. Introduction

Anoectochilus formosanus (family Orchidaceae) is a perennial herb that contains many bioactive valuable. In the world, there are about 35 species of Anoectochilus distributed in India, the Himalayas, Southeast Asia, Indonesia, New Caledonia, and Hawaii (Tseng *et al.,* 2006). In recent decades, many bioactive compounds from *A. formosanus* were isolated and demonstrated that capable of preventing and against diabetes, antioxidants, and reducing cholesterol (Tang *et al.,* 2018). The result of many studies proved that *A. formosanus* is a plentiful source material of medicinal compounds extremely important. Du *et al.* (2008) isolated many powerful antioxidants, their derivatives which were determined as glycoside, kinsenoside and acid butanoic. The diarylheptanoid and three flavonoid glucosides are four of the main phenolic compounds in component BuOH of *A. formosanus*, which is determined with strong antioxidant characteristics (Wang *et al.,* 2002). Therefore, studies on the extraction of bioactivity components from *A. formosanus* is a potential approach.

Many different extraction methods were used in which the fermentation method was applied in many fields as food, pharmaceuticals, and cosmetics. The fermentation process could improve the bioavailability of plants, vegetables, and herbs (Ng *et al*., 2011; Lieu *et al*., 2020). More specific, this process cause disruption or biotransformation from substrate heterogeneous which are herbal ingredients that become corresponding components under the influence of enzyme leading to creating product characteristics or changing the quantity and quality of biological compounds as well as the formation of new more valuable biological compounds (Hussain *et al.,* 2016). The *Angelica dahurica* root extracted fluid fermented by *Lactobacillus acidophilus* showed the highest efficiency with a dosage of 1.5 mg/ mL that could completely inhibit the melanin generation (Wang *et al.,* 2017). However, though the fermentation process could improve the bioactive compound extraction capability, it was not extracted completely. Therefore, the microwave pretreatment or/and the ultrasonic pretreatment that combines with the fermentation process could enhance extraction efficiency.

The microwave-assisted extraction (MAE) enhanced

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extraction yield in a short time at the same temperature, saved solvent, increased phenolic compounds, and significantly increased oxidation, which was friendly with the environment (Proestos *et al.,* 2008). Besides, ultrasonic-assisted extraction (UAE) also achieved the same results. The ultrasonic-assisted extraction method increased polyphenol content and antioxidant activity extracting from defatted hemp, flax, and canola seed cakes that were two times higher compared to the control (Teh *et al.,* 2014). Similarly, the study of spinach extraction by the ultrasonic method increases antioxidant content in a short time, and low cost because of reducing the amount of solvent (Altemimi *et al.,* 2015). These suggest that the MAE and UAE treatments increased the extraction efficiency of the bioactive substances. In the previous study, ultrasonic, microwave, and lactic fermentation enhanced the effective extraction of bioactive compounds from *Anoectochilus formosanus* (Ly *et al.,* 2019). Though the study of bioactive compounds extraction from the herbal plan by MAE and UAE treatments was well-reported, the evaluation of extracted efficiency from *A. formosanus* by lactic fermentation with MAE and UAE pretreatment was poorly reported. In this study, the extract efficiency of bioactive compounds from *Anoectochilus formosanus* by MAE, UAE treatment, and lactic fermentation in individual and combined impacts were evaluated through the total phenolic content, total polysaccharide content, and DPPH antioxidant activity.

2. Materials and methods

2.1 Anoectochilus formosanus Hayata and Microbial strain

Biomass of *Anoectochilus formosanus* Hayata in vitro was provided by Tropical Biology Institute (Figure 1) and preserved at 4°C. The fresh *A. formosanus* biomass was washed by saline water 0.9% (w/v). Then, 100 mL of sterile water was added into 250 mL of the Becher containing 10±0.01 g of the milled sample. The milled *A. formosanus* samples were used for the next process or used as the control samples.

Figure 1. *Anoectochilus formosanus* Hayata

Bifidobacterium bifidum AS 1.1886 was obtained from the strain collection of the Faculty of Food Science and Technology, Ho Chi Minh City University of Food Industry. The strain was incubated on Man Rogosa Sharpe (MRS) medium at 37°C in 24 hrs. Biomass was harvested and used for the experiments

2.2 The individual and combined influence of microwave, ultrasonic, and fermentation by Bifidobacterium bifidum on bioactive components of Anoectochilus formosanus extract

2.2.1 Fermentation processing by Bifidobacterium bifidum

The milled *A. formosanus* samples were added *B. bifidum* biomass (to reach the concentration of 8 Log CFU/mL) and fermented at 37°C in 48 hrs. Then, the extract was filtered and examined for biological activity.

2.2.2 Microwave-assisted extraction

The milled *A. formosanus* samples were performed on microwave equipment (Electrolux 1250 W) at 600 W in 3 mins with a pulse on time of 10 s followed by a pulse off time of 10 s. The mixture after pretreatment was analyzed (Microwave-assisted extraction (MAE) treatment samples) and used as fermentation material (MAE-F treatment samples).

2.2.3 Ultrasonic-assisted extraction

The milled *A. formosanus* samples were performed on a Sonics equipment (750 W), frequency 20 kHz at power 25% in 10 mins with a pulse ON time of 10 sec followed by a pulse of time of 10 s. The mixture after pretreatment was analyzed (Ultrasonic-assisted extraction (UAE) treatment) and used as fermentation material (UAE-F treatment samples).

2.2.4 Treatment by microwave combined ultrasonic

The mixture after pretreatment by microwave treatment was continued ultrasonic process. The mixture after pretreatment was analyzed (Microwave combined ultrasonic (UCM) treatment samples) and used as fermentation material (UCM-F treatment samples).

2.2.5 Fermentation with microwave-assisted extraction and ultrasonic-assisted extraction

The milled *A. formosanus* samples after pretreatment processing were added *B. bifidum* biomass and fermented at 37°C in 60 hrs. The extract was filtered and examined for biological activity in fermentation processing every 24 hrs.

2.3 Analytical methods

2.3.1 DPPH antioxidant activity

The determination of DPPH antioxidant activity was following the method by Ng *et al.* (2011) with

modification as followed, samples after the processing of microwave, ultrasonic were centrifuged at 5000 rpm for 10 mins and collected the extract. An aliquot of *A. formosanus* extract was diluted with 1 mL distilled water and added 10 mL of 0.1 µM DPPH methanolic. All the solutions were mixed and stored in dark, at room temperature for 30 mins. The absorbance was measured thrice at 517 nm wavelength by spectrophotometer. The result was calculated by mg Vitamin C/g sample based on the calibration curve of L-ascorbic acid.

2.3.2 Determination of total polyphenol content

The measurement of total polyphenol content has followed the method of Ly *et al.* (2019) with modifies as follows: samples after the processing of microwave, ultrasonic was centrifuged at 5000 rpm for 10 mins and collected the extract. The samples ferment both leaves and stems which were not centrifuged only collected fermentation extract. 1 mL *A. formosanus* extract was added to 0.9 mL distilled water and 5 mL Folin-Ciocalteu reagent. After 3-5 mins, added 4 mL Na_2CO_3 7.5%. The reaction mixtures were measured absorbance at 765 nm wavelength by spectrophotometer. The result was calculated by mg GAE/g sample based on the calibration curve of Gallic acid.

2.3.3 Determination of total polysaccharide content

The polysaccharide test was referred to Lieu *et al.* (2020) with some modifications. The polysaccharide was recovered after 24 hrs at 4°C from mixture sample: Ethanol (1:5, v/v) by centrifuge 5000 rpm in 10 mins. The polysaccharide was diluted with 10 mL of distilled water (solution B). Then 0.1 mL solution B and 1.9mL twice distilled water was added into 8 mL of Anthrone reagents 2% (w/v). Triplicate measurements of absorbance at 630 nm were carried out. Distilled water was used as blank. The result was displayed as mg GE/g sample based on the calibration curve of glucose.

2.4 Statistical analysis

The data analysis was carried out using Statgraphics 15.1 software. The result was recorded as mean ± standard deviation. The data was analyzed by Microsoft Office Excel 2010 and SPSS statistical software. Significant differences between means were determined by the LSD test of ANOVA procedures ($p < 0.05$). Graphs were described by Microsoft Office Excel 2010.

3. Results and discussion

3.1 Individual influence of microwave, ultrasonic, fermentation, and microwave combined ultrasonic on bioactive components of Anoectochilus formosanus fluid

The extracted efficiency of individual factors showed different effective extraction among these factors (Figure 2). The results showed that MAE and UAE treatments and lactic fermentation enhanced the effective extraction of bioactive compounds from *A. formosanus* compared to the control samples. In the control samples, the total polyphenol, polysaccharide contents, and DPPH values were 4.62 mg GAE/g sample, 26.82 mg GE/g sample, and 0.45 mg VitC/g sample, respectively. In the case of MAE, UAE treatments, and the lactic fermentation process, the total polyphenol, polysaccharide contents, and DPPH values were 6.28; 6.85; and 6.65 mg GAE/g sample, 41.22; 39.45; and 37.95 mg GE/g sample, and 0.65; 0.65; and 0.72 mg VitC/g sample, respectively (Figure 2). The bioactive compound contents from *A. formosanus* fluid trend differences in individual treatment factors. However, there was no significant difference ($p > 0.05$) between these treatment factors. The fermentation process needs more time to reach the expected extraction efficiency (Figure 2).

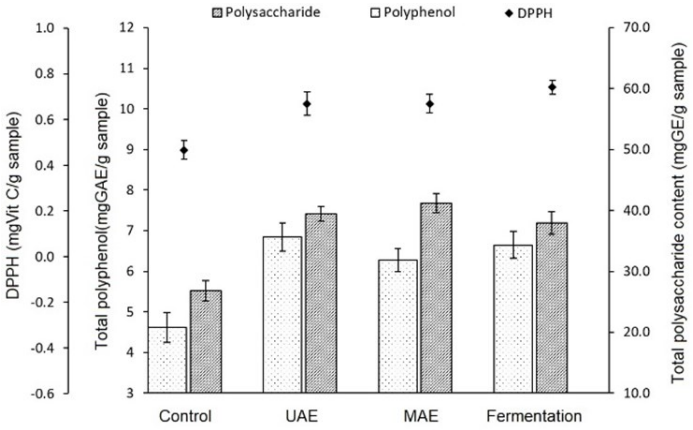

Figure 2. Individual influence of microwave, ultrasonic, fermented on bioactive components of *A. formosanus* fluid. UAE: ultrasonic-assisted extraction, MAE: microwave-assisted extraction.

The bioactive compounds in *A. formosanus* have been shown their medicinal values, proving health benefits. The enhancement of extraction efficiency helps to improve the medicinal value of *A. formosanus*. The effect of MAE, UAE pretreatments, and lactic fermentation improved the extracted efficiency of bioactivity substance from *A. formosanus* (Ng *et al.,* 2011; Ly *et al.,* 2019). The enhancement of bioactive compounds extraction capability by the MAE method has been proven in previous studies. The microwave process creates electrical conductivity and dielectric polarization, increasing the internal temperature in the plant cells, leading to enhance extraction efficiency (Li *et al.,* 2010). The bioactive substance increased along with the temperature of the microwave process, the DPPH antioxidant activity in rice bran oil increased 78%, contemporaneous in the process of extracting the α -tocopherol component was not changed (Zigoneanu *et*

al., 2008). When increasing time and microwave power, the extraction yield of mulberry leaves markedly improved (Thirugnanasambandham *et al.,* 2015). Similarly, the UAE treatment impacts plant cells leading to cell disruption due to wave propagation (Ji *et al.,* 2006). When the high amplitude ultrasonic waves pass through a material medium, the formation and bursting of air bubbles can occur near or at the surface of the cell wall causing microscopic cracks (Vinatoru *et al.,* 2017). These cracks would facilitate the release of dissolved substances into the solvent.

Increasing microwave and ultrasonic power would lead to enhancing the bioactivity compounds extraction in a shorter treatment time. A study by Pan *et al*. (2003) showed that at a high-power level, the greater the microwave energy leading to heating rate and pressure also increases dramatically, which break down the cell structure and releases the bioactive compounds into the environment (Pan *et al.,* 2003). However, the increase in power excessively, as well as prolong the treatment time would affect bioactivity compounds (Ly *et al.,* 2019). Quan *et al.* (2006) indicated that at high wave energy (800 W), strong intermolecular fluctuation in solution reaching to boil point rapidly along with a longer extracted time causing the decomposition of bioactive compound from fresh tea shoot (Quan *et al.,* 2006). Similarly, the ultrasonic wave breaks down the cell structure, most of the polysaccharides were released, but when the extraction time was prolonged, leading to the degradation of the polysaccharide (Ying *et al*., 2011). A study by Garcia *et al.* (2018) showed that the DPPH value from *Laminaria digitata* was impacted significantly by amplitude and ultrasonic time (Garcia *et al.,* 2018). This is due to the high temperature that reduced the surface tension, increasing the vapor pressure in the micro-bubbles, which caused an efficient deterioration of the ultrasonic waves (Zhao *et al.,* 2007). The extraction efficiency by MAE or UAE treatment depends on the extracted subject as well as the capacity and time of extraction. The present study showed that the MAE treatment at 600 W of power in 3 min or the UAE treatment at 25% in 10 min had higher extraction efficiency than control samples (Figure 2). Prolong treatment time at high power was caused to overheat, affecting bioactivity compounds (data not shown).

Using the lactic fermentation process to improve bioactive compounds content in plants has been reported in previous studies. A study by Bhat *et al.* (2015) showed that the increase in total phenolic content and antioxidant activity in guava juice was proportional to the fermentation time by *Lactobacillus plantarum* (Bhat *et al.,* 2015). Prolonging the fermentation time would provoke the disruption of the plant cell wall, cause the release or synthesis of different bioactive compounds (Katina *et al.,* 2007). During the fermentation process, microorganisms synthesize enzymes that could break the ester bond and release the binding phenolic acid, improving the nutritional values and increasing their usability (Acosta-Estrada *et al.,* 2014). This increase is due to microbial's enzymes converting hydrolyzed polyphenol complexes into simple polyphenols and other bioactive compounds (Bhat *et al.,* 2015). The present study showed that the fermentation process requires more time than MEA and UEA treatments, but there was no significant difference in polyphenol and polysaccharides contents, and DPPH value between these impacts (Figure 2). However, in the fermentation process, besides these compounds, this process would also synthesize other valuable metabolic compounds that increase natural antimicrobial activity as well as probiotic sources.

3.2 The combined influence of microwave - fermented, ultrasonic – fermented, and microwave – ultrasonic - fermented on bioactive components of Anoectochilus formosanus fluid.

The combination of factors (microwave, ultrasonic, fermented) improved the extraction efficiency of bioactive components from *A. formosanus* fluid, and some combination treatments showed a higher effect than individual factors (Figures 2, 3, 4, 5, and 6). In the case of UCM treatment samples, the content of the bioactive compounds was no significant difference compared to individual factors (Figures 2 and 6). The treatments including the fermentation process significantly enhanced bioactive compounds compared to individual factors. In the case of MAE-F treatment samples, the peak point was achieved after 48 hrs with the total polyphenol, polysaccharide contents, and DPPH values were 8.25 mg GAE/g sample, 49.75 mg GE/g sample, and 0.85 mg Vit C/g sample, respectively. The results also indicated that the fermentation time in UAE-F and UCM-F treatments was reduced compared to the MAE-F treatment (Figures 3, 4, and 5). There was no significant difference between UAE-F and UCM-F treatment. The UAE-F treatment reached a peak point after 36 hrs with the total polyphenol, polysaccharide contents, and DPPH values were 8.45 mg GAE/g sample, 51.40 mg GE/g sample, and 0.95 mg VitC/g sample, respectively.

The individual effect of microwave, ultrasonic, and lactic fermentation on bioactive components extracted from *A. formosanus* was reported in the previous study (Ng *et al.,* 2011; Ly *et al.,* 2019). Besides, the combination of these factors showed effectiveness in extracting bioactive compounds from plants. Lin *et al.* (2015) showed that microwave pretreatment could

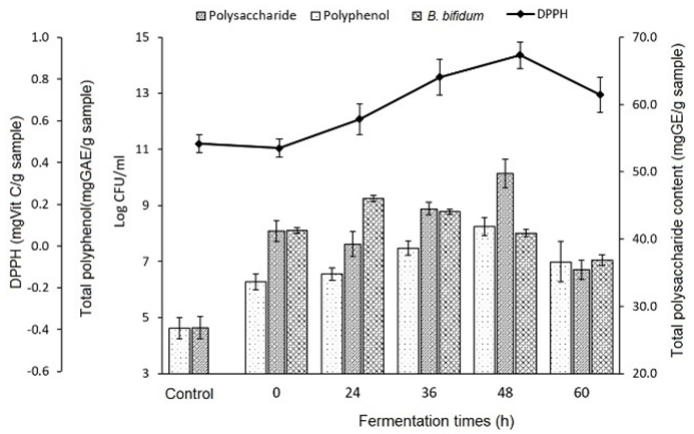

Figure 3. Influence of MAE treatment combined fermentation on bioactive components of *A. formosanus* fluid.

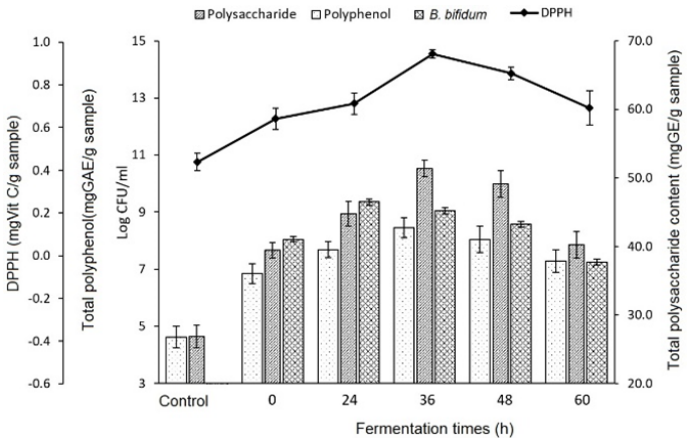

Figure 4. Influence of UAE treatment combined fermentation on bioactive components of *A. formosanus* fluid.

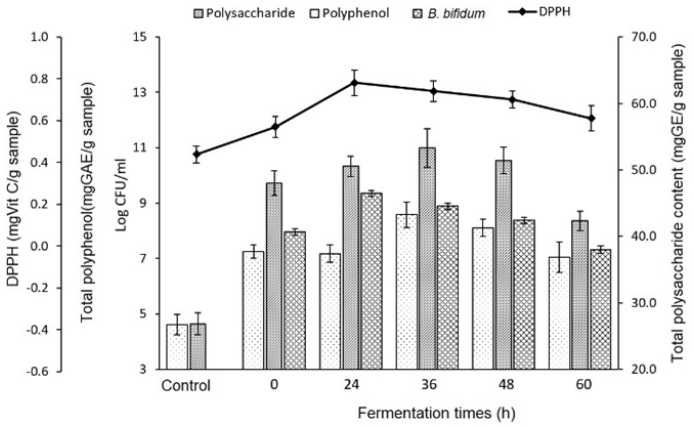

Figure 5. Influence of MAE-UAE treatment combined fermentation on bioactive components of *A. formosanus* fluid.

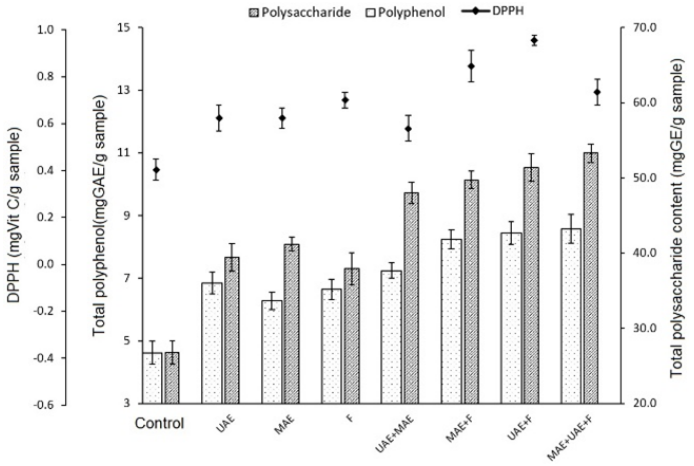

Figure 6. Effects of different treatment methods on bioactive components of *A. formosanus* fluid.

effectively break down the lignocellulose structure of water hyacinth and improve enzymatic digestibility and H_2/CH_4 production. Similarly, the research of Zhou *et al.* (2018) showed that microwave combined ultrasonic treatment increased productivity and quality of extraction of single-cell oil from *Mortierella isabellina* NTG1–121 (MIN) compared to the control samples. Also, the combined pretreatment between microwave and ultrasound makes the extraction efficiency of phenol compounds higher, enhancing the antioxidant resistance from soybean seed (Đurović *et al.,* 2018). However, the study on combining the MAE and UAE pretreatment with the lactic fermentation process on *A. formosanus* fluid was poorly reported. The combining of these factors would improve the content of the bioactive compounds. The results obtained from this study showed that combined impacts had a positive effect on bioactive components extracted from *A. formosanus* compared to individual factors in which the combination using fermentation showed a significant influence (Figures 3, 4, 5, and 6). The pretreatment process facilitated *B. bifidum* to interact with the substance leading to improve bioactive compounds. The results also showed that the UAE-F treatment samples were enhanced bioactive compounds effectively compared to the MAE-F treatment samples (Figures 3 and 4). Besides, UCM-F treatment samples were not enhanced bioactive compounds significantly compare to UAE-F treatment samples (Figures 5 and 6). Similar results were also recorded in UCM treatment samples, which was not significantly different compared to individual factors (Figures 2 and 6). These results would be due to the combination of MAE and UAE in UCM treatment samples that made the bioactive compounds from *A. formosanus* undergo two phases of overheating. These significantly affected the bioactive compounds from *A. formosanus* (Figures 2, 3, 4, 5, and 6).

4. Conclusion

The results showed that the content of polyphenol, polysaccharide and DPPH value in *A. formosanus* extracted fluid were significantly improved by the fermentation process, MAE, and UAE treatments compared to control samples. In case of individual factors showed that there was no significant difference among these factors in which the fermentation process requires more time than other factors. However, the lactic fermentation process would bring a probiotic source as well as valuable products of the metabolic process. The combined factors using fermentation were more effective than individual factors. The results also

indicated that the UAE pretreatment combined fermentation process would bring three benefits: first, no need for more MAE treatment; second, reduce fermentation time; third, bring a probiotic source as well as valuable metabolic products from the fermentation process.

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