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EFFECT OF SOLVENT IN COMBINATION WITH ULTRASONIC WAVE ASSISTANCE ON TRITERPENOID EXTRACTION FROM RED LINGZHI MUSHROOM (*Ganodermalucidum)*

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ABSTRACT

Triterpenoid is one of the compounds that contribute tored lingzhi mushroom's major significant therapeutic activities (*G. lucidum)*. Triterpenoids have anti-allergic, anti-cancer, anti-tumor, and anti-aging effects, enhance oxygen utilization, free radical scavenging, lowerblood pressure, inhibit cholesterol biosynthesis, and enhance liver function and blood pressure, inhibit cholesterol biosynthesis, and improve liver functionetc. The beneficial effects of triterpenoids in *Ganoderma lucidum* on human health have generated interest in developing methods for extracting them from the fruit bodies of *G. lucidum*. This article presents the effect of solvent in combination with ultrasonic wave assistance in triterpenoid extraction ability from red lingzhi mushroom. Before carrying out triterpenoid extraction; Red lingzhi mushroom was ground 2mm in size. Four parameters of the extraction process, including the ethanol concentration, ratio (v:w) between ethanol solvent and mushroom, extraction time, , extraction temperature, ultrasonic time and ultrasonic intensity were determined. The extraction without ultrasonic waves for 180 minutes was used as a control. The total triterpenoid content was collected during the extraction process. The results showed that red lingzhi mushroom was extracted by ethanol solvent at a concentration of 85%, the ratio between ethanol solvent and mushroom was 3:1 (v:w), ultrasound intensity was 58 W/cm^2, frequency was 20 kHz), extracting temperature was 55°C, removing time was 5 min had total triterpenoid content 3.06 times higher than the control.

Keywords: Redlingzhi mushroom, triterpenoid, ultrasound, extraction.

INTRODUCTION

Recently, the red lingzhi mushroom (*Ganodermalucidum*) has been successfully cultivated in Vietnam, with a total yield of about 10 tons annually, bringing high economic efficiency (Duong *et al*., 2020). Several studies have shown that the red lingzhi mushroom (RLM) provides many biologically active substances. RLM contains more than 400 compounds including triterpenoids, polysaccharides, nucleotides, sterols, steroids, fatty acids, proteins, peptides, and trace elements, with biological activities such as anti-atherosclerotic, anti-inflammatory, immune-boosting, anti-cancer, anti-bacteria and viruses, protect the liver, anti-aging, etc. (Sanodiya *et al*., 2009, Russell *et al.,* 2006). More than 150 triterpenoid compounds have been extracted from RLM. In which, there are 50 triterpenoids found only on RLM. Therefore, RLM is identified and confir-med as one of the plants that has rich triterpenoid compounds (Chang and Miles, 2004). Triterpenoids from RLM are considered important substances because they can fight cancer cells, enhance oxygen use, redox free radicals in the body, anti-aging, lower blood pressure, inhibit cholesterol biosynthesis, and to improve liver function (Sakai and Chihara, *1995*). Extracting triterpenoids from dried RLM is not easy eventhough we also use traditional extraction methods such as decoction in water, extraction with methanol, alcohol or concentrated form according to the standard ratio. However, tradetional methods usually only obtain triterpenoids in mycelium and fruit bodies, but it is difficult to separate the shell of fungal spores (Chen *et al.,* 2011). The extraction of biologically active subs-tances in RLM depends on many factors such as solvent type, extraction temperature, extraction time, etc. There are many methods to assist the extraction of triterpenoids in plant materials such as enzymes, microwaves, supercritical CO_2, etc. The basis of using ultrasound energy in the extraction process increases the mass transfer rate of the extract, making it easier to diffuse the section from the inside of the material to the outside (Liu *et al.,* 2010). Therefore, in this study, we evaluated the effect of some parameters (concentration of ethanol solvent, material/solvent ratio, extracting temperature, ultrasound intensity, ultrasound time) on the total content of triterpenoids from RLM during processing with the assistance of ultrasonic wave. This is the premise for indepth studies on the extraction of triterpenoids from RLM.

MATERIALS AND METHODS

Materials: Fresh red lingzhi mushrooms were harvested ona farm in Ba Vi district, Hanoi, Viet-nam, packaged in 20 kg plastic baskets, lined with paper and transported to the laboratory within 1 h. Then mushrooms were then selected, cleaned, and ground 2 mm in size, before used in this experiment.

All chemicals used in this study met the quality standards of laboratory-grade analytical chemicals. Ultrasound equipment TJS-3000 intelligent Ult-rasonic Generator V6.0 (Hangzhou Success Ultr-asonic Equipment Co.,Ltd), capacity 20 l, freq-uency 20 kHz.

Methods

Ethanol solvent concentration: 02 kg of ground mushrooms were extracted by ethanol solvent at concentrations of 70, 75, 80, 85, 90 and 96%, the ratio between ethanol and mushroom was 3:1 (v:w),theintensity of ultrasonic wave was 58 W/ cm^2 at 20 kHz frequency, extracting temperature was 55°C and extracting time was 5 min. After filtering, the extract was measured the triterpenoid content.

The ratio between solvent and red lingzhi mush-rooms: 2 kg of ground mushrooms were extracted by ethanol solvent at concentrations of 85% with the balance between ethanol and mushroom was 1:1, 2:1, 3:1, 4:1, 5:1, 6:1 (v:w), the intensity of ultrasonic wave was 58 W/cm^2 at 20 kHz fre-quency, extracting temperature was 55°C and extracting time was 5 min. After filtering, the ext-ract was measured the triterpenoid content.

Extraction temperature: 2 kg of ground mush-rooms were extracted at temperatures of 35, 45, 55, 65, 75 and 85 ± 2°C by using ethanol solvent at concentrations of 85%, the ratio between ethanol and mushroom was 3:1 (v:w), the int-ensity of ultrasonic wave was 58 W/cm^2 at 20 kHz frequency and extracting time was 5 min. After filtering, the extract was measured the triterpenoid content

Duration and intensity of ultrasonic wave: 2 kg of ground mushrooms were extracted by an ultrasonic wave at a power of 25, 35, 45, 55 and 58 W/cm^2 for 0, 2, 3, 4, 5, 6 and 7 min, 20 kHz frequency, the ratio between ethanol 85% and mushroom was 3:1 (v:w)and extracting time was 5 min. After filtering, the extract was measured the triterpenoid content

Determination of total Triterpenoid: The sample after extraction was filtered and diluted 10 times; aspirate 0.2 mL of the diluted sample into a test tube, add 0.2 mL of vanniline-acetate (10%), 1.2 mL of $HClO_4$ respectively, and then heat in a water bath and incubate at 70°C for 15 min. After 15 min, the test tubes were cooled for 2 min; ethyl acetate was added so that the total volume was 5 mL. Total triterpenoid was analyzed based on colorimetric spectroscopy, measuring absorbance at 548 nm using spectrometers (Ultrospec 2000, Pharmacia Biotech)(Lu *et al., 2012*).

Data analysis: The data were processed by analysis of variance (ANOVA) to determine the difference between the mean values, significant with con-fidence $p < 0.05$. Using Statgraphics Plus software, version 5.1.

RESULTS AND DISCUSSION

The effect of the concentration of ethanol solvent on triterpenoid extraction: The impact of the concentration of ethanol solvent on the ability to extract triterpenoid in the red lingzhi mushrooms by ultrasonic wave assistance is shown in Figure-1. In general, different ethanol concentration gaved if ferenttriterpenoid ability extraction.

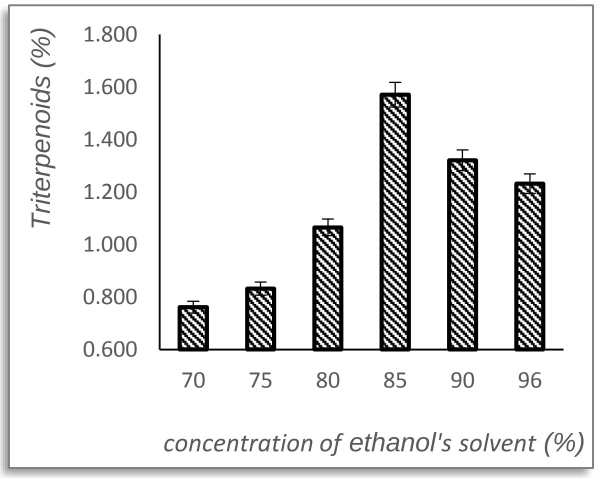

Figure 1: Effect of the ethanol solvent's concentration on triterpenoid content of the *G.lucidum* extract

Ethanol at concentration of 70, 75, 80, 85, 90 and 96% obtaining the triterpen-oid content in the extract respectively were0.761; 0.832; 1.065; 1.570; 1.321 and 1.23%. These results demons-trated that the concentration of ethanol solvent affects the ability to extract triterpenoids from red lingzhi mushrooms. This can be explained that at each given solvent concentration in addition to the ability to dissolve triterpenoid, ethanol also can dissolve impurities, reducing the ability to dissolve triterpenoid into the solvent. The highest extract yield was found in the ethanolconcen-tration of 85% (1.570% triterpenoid). Thus, the ethanol concentration of 85% was selected as the solvent for extracting triterpenoidin theredlingzhi mushrooms by ultrasonic wave.Cai *et al*., (2019) found that the total triterpenoid extraction from the medicinal fungus *Sanghuang russanghuang* was optimal at an ethanol concentration of 80% under the conditions of a 1:15 solidliquid ratio, 15 min extraction time 100 W of extraction power and a 60°C extraction temperature. Chew *et al.*, (2011) found that 40% ethanol had a significant ($p<0.05$) effect on phenolic contents (total phenolic content, total flavornoid content and 2,2'-azino-bis (3-ethylbenzo-thiazoline-6-sul-phonic acid) and antioxidant capacities (2,2'-azi-no-bis(3-ethyl –benzothiazoline-6-sulphonicacid and 2,2-diphenyl-1-picrylhydrazyl) of crude extract from *Cente- llaasiatica*.

The effect of the ratio of ethanol solvent and mushroom on the extraction of triterpenoids: Using more solvents for extraction, the greater the ability to diffuse triterpenoids from mushroom to the solvent, but if the amount of solvent is too much, the ability to obtain triterpenoid active ingredients is insignificant, it will not be parac-tical due to costing solvent, time and energy to recover solvent. Therefore, determining the ratio between ethanol solvent and mushroom for triterpenoids extraction to achieve high efficiency is necessary.

Figure 2 showed that using a solvent with different ratios, the extraction capacity of triterpenoids was different, when increasing the amount of the solvent, the triterpenoids concentration in the extract also grew.

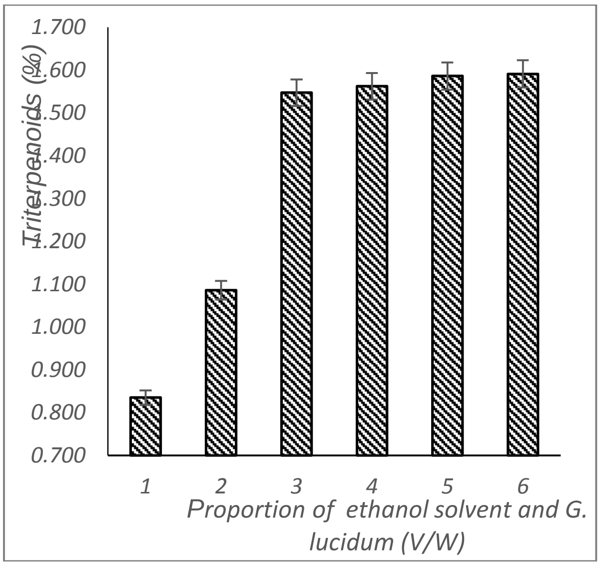

Figure 2: Effect of the proportion between ethanol solvent and *G. lucidumingre*-dient on thetriterpenoid content

Triterpenoid content increased sharply at the ratio of solvent/mushroom was 1:1, 2:1 and 3:1 (l/kg) (0.835, 1.086 and 1.547%, respectively). The results showed that when ataratio of 1:1, the amount of solvent used is small, not enough to penetrate the entire extraction material, the diffusion of solute into the solvent, and a certain amount of solvent will be kept in the raw material is the cause of the reduction in extraction efficiency. Increasing the solvent ratio will promote the dissolution of triterpenoids into the solvent at an early stage. The solutes have good solubility conditions in the solvent because a large amount of solvent will increase the contacting ability of the cell with the solvent, leading to an increase in the concentration difference between the environment inside and outside of the cell. It increases the osmotic pressure difference, and the solutes will diffuse into the solvent, leading to an increase in the triterpenoid content of the extract. Initially, the concentration of Triterpenoids in the material is high, so their diffusion out of the cell is also fast. At high solvent ratios of 4, 5 and 6, the triterpenoid extraction capacity did not increase significantly, respectively 1.562, 1.586 and 1.591 % when compared to a ratio of 3:1 (1.547%). From these results, to save powerful solvent and energy, the ratio between solvent and mushroom of 3:1 (v:w) was the most ideal for extracting triterpenoid in the redlingzhi mushrooms.Cai*et al*., (2019) found that the total triterpenoid yield was removed from the medicinal fungus *Sanghuang-porussanghuang* markedly increased when the solid-liquid ratio ranged from 1:10 to 1:20. In this condition, dissolved medicinal powder increased as the solid liquid ratio increased. When the dissolution of triterpenoid reached an equivalence point, an increase in the solvent volume did not dissolve more triterpenoid; therefore, the extraction yield no longer increased when the solid-liquid ratio reached 1:20.

The effect of ultrasonic temperature on triterpenoid yield: The higher the extracting temperature will increase the porosity of the material (due to the swelling of the material), the viscosity decreases and the active ingredient to dissolve easily into the solvent. Extracting by temperature with ultrasonic waves assistance increased the number of air bubbles formed. However, when the temperature increases, air bubble' intensity breaking will be reduced due to the effect of vapour pressure increasing and playing a role as a buffer layer, preventing the collision of surrounding molecules when the air bubble is broken. Extracting temperature too highly increase, can increase the solubility of some impureties, promoting chemical changes that make the shift in quality of the extract unprofitable. In contrast, the rupture of air bubbles will be difficult when the temperature decreases because the increased environmental viscosity. Therefore, determining the suitable extraction temperature is essential to achieving high productivity and pure extracts.The results in Figure-3 showed that the extraction temperature from 35, 45 to 55°C gave the extraction ability with an enormously increasing triterpenoid content of 0.662, 0.832 and 1.358 % respec-tively.

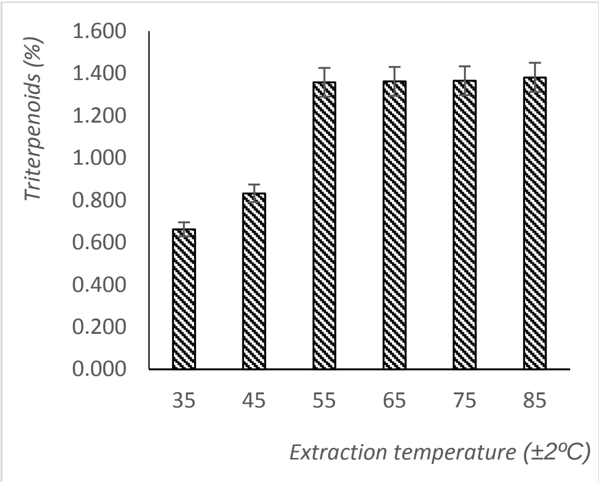

Figure 3: Effect of extraction temperature on triterpenoid content of the extract

The extraction temperature increased from 65, 75 to 85°Cgave triterpenoid content to increase insignificantly by 1.363, 1.365 and 1.381%, resp-ectively. It was found that increasing the ext-raction temperature to 85°C gave a negligible increase in the triterpenoid extraction capacity compared with the temperature of 55°C. From this result can conclude 55°Cis a suitable temp-erature to extract triterpenoid in red lingzhimus-hrooms. An increase in temperature can enhance the permeability of triterpenoid molecules inside the cell. In the range of 30–60°C, the total triterpenoid yield increased as the temperature increased. However, the yield slightly decreased when the temperature exceeded 60°C because high temperatures (greater than 60°C) destroy the triterpenoid molecular structure of the five rings (Cai *et al*., 2019). The extraction temperature affected on the total phenolic content and radical-scavenging properties of the different extracts reported by Onyebuchi and DoğaKavaz (2020). Onyebuchi and DoğaKavaz (2020) also concluded that all extracts' chemical and bioactive properties showed signi-ficant dependence on the extraction temperature and solvent type. The mechanical effect of acoustic cavitation from the ultrasound increases the surface contact between solvents and samples and the permea-bility of cell walls. Physical and chemical proper-ties of the materials subjected to ultrasound are altered and disrupt the plant cell wall, facilitating the release of compounds and enhancing mass transport of the solvents into the plant cells (Dhanani *et al*., 2013). Chew *et al.*, (2011) conclu-ded that extracting temperature at 65°C by 40% ethanol solvent had a significant ($p<0.05$) effect on phenolic contents and antioxidant comp-ounds of crude extract from *Centellaasiatica*.

The effect of ultrasound time and ultrasonic intensity on total triterpenoid extraction: Eff-ect of time and intensity of ultrasonic waveon ability of extraction of triterpenoid from the red lingzhi mushrooms is shown in Figure 4, the extraction without ultrasonic wave (control) for 0, 2, 3, 4, 5, 6 and 7 min obtained triterpenoidscontent were 0.238, 0.311, 0.367, 0.378, 0.383, 0.398 and 0.429 %, respectively.

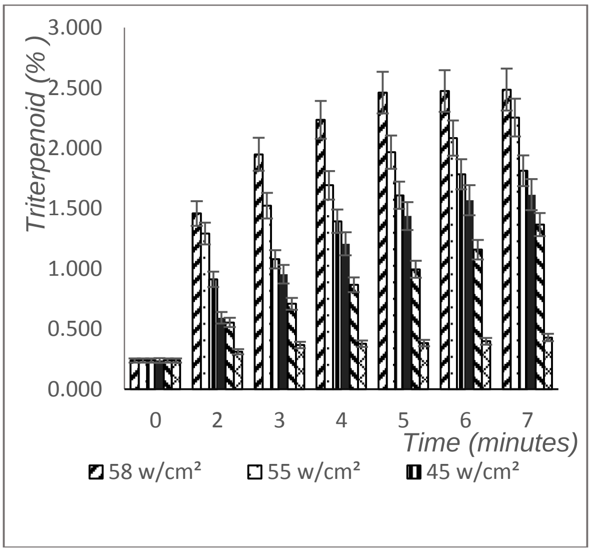

Figure 4: Effect of time and ultrasonicinten-sity on the capacity of triterpenoid extract from *G. lucidum*

The extraction using ultrasonic waves at different intensities from 25 to 58 W/cm^2 for 2, 3, 4, 5, 6 and 7 min obtained triterpenoids content ranging from 0.55 to 2.486%. A significantly diff-erent in extracted triterpenoids content between control and samples extracted by ultrasonic wave at different intensities ($P \leq 0.05$). At a different ext-racting times with different powers also obtained additional triterpenoids content (Figure 4). As seen in Figure 4, the extracted triterpenoids con-tent sharply increased after 2, 3 and 5 min of ext-raction; after that, at 5,6 and 7 min of extraction, the extracted triterpenoids content in the extract did not increase significantly. At extrac-ting cond-itions ultrasonic intensity of 58 W/cm^2 gave higher triterpenoids content when compared to the other extracting conditions with the control. At the ultrasonic intensity of 58 W/ cm² with an ultra-sonic extraction times of 2, 3, 4 and 5 min, triter-penoidcontentin extract increasedby1.458, 1.949, 2.236 and 2.461%, respectively; after 6 and 7 minutes of ultrasonic extraction, the triterpenoid content still increased at 2.474 and 2.486%, res-pectively, at the time level of 5, 6 and 7 min, the triterpenoid content in the extract did not increase significantly. These results justified that at an ultrasonic intensity of 58 W/cm² with an ultra-sonic extraction time of 5 min, the best condition to extract triterpenoid in red lingzhi mushrooms. Chew *et al.*, (2011) found that extracting time for 120min by 40% ethanol had a significant ($p<0.05$) effect on phenolic contents and antioxidant comp-ounds of crude extract from *Centellaasiatica*. The maximum yield of triterpenoids was obtained from medicinal fungus *Sanghuang porussangh-uang* when the ultrasonic time was 20 min. After 20 min, most of the cells were dissolved, and the triter-penoid molecules achieved equivalence between the inside and outside of the cells (Cai *et al*., 2019). The time and intensity of ultrasound were investigated as when the ultrasonic intensity and time increased the ability to extract triterpenoid highly increased and much higher than that of the control (without using ultrasound, for triterpenoid content in the extract is the lowest). The higher the ultrasonic intensity, the higher the effervescence, the high breaking force, which increases the extract's mass transfer rate. The foam breaking also createagitation to help diffuse the extract inside the material to escape more easily (Jian-bing *et al*., 2005).The increase in extraction capa-city is due to the higher the ultrasonic intensity, the higher the effervescent effect of high breaking force, which increases the mass transfer rate of the extract. In addition, the foam bubbling also creates an agitation that allows the diffusion of the extracts inside the material to escape more easily (Jian-bing *et al.*, 2005). The longer the ultrasonic time, the more significant the intensity, the more the particles of the extracted material are destroyed and the more the viscosity and solu-bility increase, respectively. Viscosity increases with increasing ultrasonic intensity and time, the number of short-chain molecules gains, and the thickness also increases.Still, when the intensity reaches a specific value, thethickness decreases. The higher the ultrasonic intensity, which is an agent to contribute to increasing the efficiency of the extraction process, the rapid expansion of bubbles during the negative pressure cycle makes the bubbles no chance of shrinking during the positive pressure cycle. Conversely, when the ultrasonic intensity is smaller, the number of times the bubbles expand and compress will increase, and the extraction time will be longer (Herceg*et al*., 2010). The stronger the ultrasonic wave intensity, the higher the ability to extract the active ingredient in a shorter time. So, to save time and cost, 5 min of ultrasonic extraction at the intensity of 58 W/cm² is the most effective for the extraction of triter-penoidsin red lingzhi mushrooms.

Comparison of triterpenoid extraction method in red lingzhi mushrooms by using ultrasonic wave and without ultrasonic wave: Table 1 indi-cated that extraction with ultrasonic waves intensity of 58W/cm^{2}at 20 kHz frequency, at 55 ± 2^{0}C, the ratio of ethanol 85% mushroom was 3:1 (v:w), for 5 minutes gave the highesttriterpenoid extraction abilityof 2.47 % and soluble dry matter of 16.30%.

Table 1: Comparison of triterpenoid extraction method in red lingzhi mushrooms by using ultrasonic wave and without ultrasonic wave

Norm	Unit	Triterpenoid extract		
		Ultrasonic intensity 58 W/cm^2, 20 kHz	Without ultrasonic	
Material size	mm	1.8	1.8	30
Proportion of ethanol and *G.lucidum* ingredient	v:w	3	3	6
Extract temperature	°C	55 ± 2.0	55 ± 2.0	98 ± 2.0
Extract duration	min	5	5	180
Triterpenoids	%	2.47±0.02	0.38±0.01	0.81±0.02
Dry matter	%	16.30±0.04	2.12±0.05	16.92±0.04

While the extraction method did not use ultra-sonic waves, extracted at 55 ± 2^{0}C, the ratio of ethanol 85%/mushroomwas 3:1 (v:w),for 5 min gaveless triterpenoidcontent (0.38%) andsoluble dry matter of 2.12 %. Extraction without ultra-sonic wave at 98 ± 2^{0}C (traditional method), a ratio of water/mushroom (d=30mm) was 6:1 (v:w), for 180 min (3 h) gave the possibility of extracting triterpenoid content only of 0.81% and soluble dry matter of 16.92%. Extraction without an ultrasonic wave provides lower triterpenoid content than extraction with an ultrasonic wavea. At ultrasonic wave intensity 58 W/cm^2, 20 kHz frequency for 5 min, triterpenoid content was about 3.06 times higher than non-ultrasound for 3h at 98 ± 2^{0}C. There was a significant reduction in extraction temperature of 98 ± 2^{0}C in the conventional extraction process to 55 ± 2^{0}C during the use of ultrasonic waves. This shows that ultrasonic energy gives the effect of extracting triterpenoid in red lingzhi mushrooms.

CONCLUSIONS

Extracting triterpenoid in red lingzhi mushrooms by 85% ethanol solvent with the ratio between solvent and mushroom was 3:1 combined with ultrasonic wave assistance at ultrasonic intensity 58 W/cm2, frequency 20 kHz, time 5 min at 55 ± 2°C instead of conventional extraction methods provides an attractive technological alternative for obtaining the total triterpenoid content 3.06 times higher than the traditionalextraction meth-ods. This study shows the potential of using ult-rasonic waves is more effective than traditional extraction methods without using ultrasonic waves, which is to shorten the extraction time and more obtain triterpenoids in red lingzhi mush-rooms.

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