Enhancing the oil extraction process and exploring phytochemical composition and bioactivities of bitter melon seeds (*Momordica charantia* L.)

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ARTICLE INFO

Research Paper

Received: August 03, 2024

Revised: October 28, 2024

Accepted: October 29, 2024

Keywords

Bitter melon seeds

Lipid

Quality requirements

Secondary compounds

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ABSTRACT

This study was conducted to determine the phytochemical composition of bitter melon seeds (*Momordica charantia* L.) grown in Long An province (Vietnam), to investigate optimal conditions for lipid extraction, and to evaluate the extracted lipid's quality. The seeds had a moisture content of 5.27%, total ash of 1.85%, total flavonoid content of 91.10 mg/100 g, and total polyphenol content of 478.95 mg/100 g. The seeds were also free of highly toxic metals such as lead and cadmium. Using the Soxhlet method, optimal lipid extraction was achieved with a material-to-solvent ratio of 1:80 (w/v) over 4 hours, resulting in a lipid extraction efficiency of 13.74%. The acid, saponification, ester, and peroxide values were 1.01 mg KOH/g, 355.60 mg KOH/g, 354.59 mg KOH/g, and 3.82 meq O_2/kg, respectively, in compliance with the quality requirements of Vietnam and Codex standards. The extracted lipids had antioxidant activity at an IC_{50} value of 119 mg/mL and inhibited the growth of two microbial strains *Staphylococcus aureus* and *Bacillus subtilis* subsp. *spizizenii*. These findings suggest that bitter melon oil has potential applications in the food, pharmaceutical, and cosmetic industries

Cited as: Nguyen, A. T. V., Pham, T. T. A., Dang, H. T., Pham, L. T., & Phung, H. V. C. (2024). Enhancing the oil extraction process and exploring phytochemical composition and bioactivities of bitter melon seeds (*Momordica charantia* L.). *The Journal of Agriculture and Development* 23 (Special issue 1), 170-187.

1. Introduction

Momordica charantia Linn (*M. charantia* L.), commonly known as bitter melon, is an annual climbing vine from the Cucurbitaceae family. Besides its widespread use as a food in many countries, bitter melon has long been used in traditional medicine throughout Asia, Africa, and Latin America due to its rich content of over 60 bioactive phytochemicals, including glycosides, saponins, alkaloids, fixed oils, triterpenes, proteins, and steroids (Behera et al., 2020). Notably, bitter melon seed oil has been applied in treating various diseases such as diabetes, inflammation, and cancer, and is also utilized in cosmetics, poultry, aquaculture feed, and biodiesel production (Horax et al., 2010; Ajuru et al., 2017; Singh et al., 2019). The oil is composed of monounsaturated fatty acids (MUFA), saturated fatty acids (SFA), and polyunsaturated fatty acids (PUFA), with conjugated linoleic acid (CLnA) as the predominant component (Liu et al., 2010; Yoshime et al., 2016).

Traditional extraction techniques for plant-derived bioactive compounds, such as pressing, maceration, and shaking water bath extraction, are widely used for their simplicity and cost-effectiveness. However, these methods often require long extraction times and involve the use of harmful solvents (Pitipanapong et al., 2007; Zaini et al., 2018; Sasongko et al., 2019). On the other hand, advanced techniques like Soxhlet extraction, ultrasonics, supercritical CO_2 (SC-CO_2) extraction, and enzyme-assisted methods offer greater efficiency, reduced extraction time, lower solvent consumption, and better selectivity (Nyam et al., 2009; Xu et al., 2014; Naik et al., 2021), though they come with higher costs and more complex procedures.

The choice of extraction method depends on various factors such as the chemical structure and physicochemical properties of the sample, as well as the research objectives. Thus, the objective of the study aimed to develop a simplified extraction method for bitter melon seeds to produce oil that complies with safety standards, retains the seeds' bioactive components, extends shelf life, and is suitable for multiple applications (Figure 1).

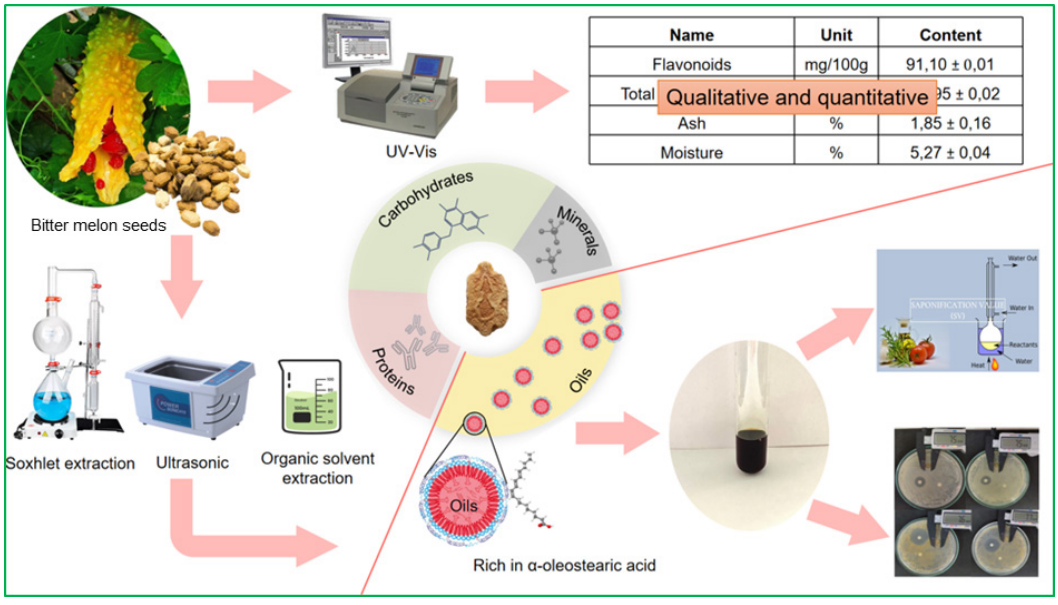

Figure 1. Graphic abstract.

2. Materials and Methods

2.1. Materials

The fruit of *M. charantia* L. was purchased from a garden in Long An province, Vietnam. After removing impurities and separating the seeds from the flesh, they were dried at 50 ± 5°C until their moisture content was below 13%. The dried seeds were then crushed and sieved through a 1 mm diameter sieve. Raw powder was stored in zip-lock bags at room temperature until further use.

The equipment used in this study included a reflux extraction system (Isolab, Germany), a Soxhlet extraction system, an ultrasonic cleaner (WUC-32, Jiayuanda, China), an evaporator (Heidolph, Germany), a drying oven (Memmert, Germany), and an ultraviolet-visible spectrophotometer (Model 752N, Jenway, England).

Chemicals used in the study included Dragendorff (Cas#39775-75-2) and Wagner reagents (Cas#39775-75-2), NaOH (Cas#1310-73-2, Xilong), HCl (Cas#D1128, Duksan), $FeCl_3$ (Cas#7705-08-0, Xilong), hexane (Cas#110-54-3, Xilong), Folin-Ciocalteu reagent (Cas#1090010100, Merck), Na_2CO_3 (Cas#1063920500, Merck), phenolphthalein (Cas#77-09-8), KOH (Cas#1310-58-3, Xilong), $Na_2S_2O_3.5H_2O$ (Cas#10102-17-7), and KI (Cas#7681-11-0, Xilong).

2.2. Methods

2.2.1. Phytochemical analysis of bitter melon seeds

Determination of moisture content

The moisture content was determined according to TCVN 7975:2008 (VS, 2008). Initially, the raw material was accurately weighed to 5 ± 0.0001 g into the moisture dish (which was dried at 105°C, cooled, and recorded its mass). The dishes containing the samples were placed in a drying oven and dried at 105 ± 2°C for 4 - 6 h. Subsequently, they were transferred to a desiccator until reaching room temperature, and their mass was re-recorded. This process was repeated multiple times until the difference in weight between two consecutive weighings did not exceed 5 mg. The moisture content of the raw material was calculated using the equation (1):

$$W = \frac{m_1 - m_2}{m_1 - m_o} \times 100 \qquad (1)$$

where m_o, m_1, and m_2 was the mass of the moisture dish, the mass of the moisture dish and the sample before drying, and the mass of the moisture dish and the sample after drying (g), respectively.

Qualitative analysis

Seeds extract preparation: Two types of solvents (70% ethanol and distilled water) were used for extraction. Five g of seed powder were soaked in 30 mL of each solvent. Then, coupled with 20 mL of the same solvents, the residues were extracted by ultrasound-assisted extraction for 15 min. The extract was filtered through a Whatman No.1 filter paper and stored at 4°C for further use.

The biochemical compositions of the seed extracts, including alkaloids, flavonoids, phenolic acids, saponins, and tannins, were qualitatively determined as previously described by Pham et al. (1998) (Table 1).

Table 1. Phytochemical screening methods of seed extracts

Compounds	Reagents	Observations (Indicating Positive Test)
Alkaloids	Wagner	Formation of brown to reddish brown precipitate
	Dragendroff	Formation of red-orange to red precipitate
Flavonoids	NaOH 10%/HCl 10%	The color changed to red-orange or orange and became lighter or discolored when HCl was added.
	Metal magnesium/HCl conc.	The solution was pink to red.
Phenolic acids	$FeCl_3$ 5%	The solution was moss-green to bluish-black
	Iodine	The solution was red
Saponins	Foam test	Persistent foam for 15 min
Tannins	$FeCl_3$ 5%	The solution was moss-green to bluish-black
	Lead acetate 10%	Formation of white precipitate

Quantitative analysis

Based on the qualitative results, the main biological components of the seed extracts were quantified, as presented in Table 2.

Table 2. Quantitative analysis methods of seed extracts

Compounds	Methods
Flavonoids	Flavonoids were measured using the aluminum chloride colorimetric assay. Flavonoids in the sample were extracted using ethanol and mixed with aluminum chloride ($AlCl_3$) and potassium acetate (CH_3COOK), leading to the formation of a yellow-colored complex. The reaction mixture was incubated for 30 minutes, after which the absorbance was measured using a UV-Vis spectrophotometer at 415 - 430 nm. The total flavonoid content was quantified using a standard curve generated with quercetin as the reference compound (Chang et al., 2002).
Polyphenols	The total polyphenol content was quantified by the Folin-Ciocalteu (FC) method (Waterman & Mole, 1994). The FC reagent was used to quantify total polyphenol content through a redox reaction, where phenolic compounds were oxidized by donating electrons to molybdenum (Mo) and tungsten (W) complexes in the reagent, reducing them from Mo(VI) and W(VI) to Mo(V) and W(V). This reduction produced a blue color, with the intensity proportional to the polyphenol concentration. The reaction required an alkaline medium, typically provided by sodium carbonate, to enhance phenolic reactivity by deprotonating them into phenoxide ions. The blue complex's absorbance was measured at 765 nm, and the results were expressed as gallic acid equivalents (GAE). Gallic acid was used as a control.

Compounds	Methods
Tannins	Tannins was quantified according to AOAC 955.35. The extract was prepared with hot distilled water, and then impurities were removed via filter paper. Five milliliters of the extracted solution were taken into a 250 mL Erlenmeyer flask, followed by the addition of 150 mL of distilled water and 5 mL of 0.25% Indigo carmine, and the mixture was shaken well. The mixture was then titrated with 0.1 N $KMnO_4$ solution until it turned yellow.
Saponins	Saponins was quantified according to TCCS 231:2017/TTKNII with some adjustments for suitability (DAH, 2017). The powder material was extracted using 80% methanol. The methanol was removed, and the residue was dissolved in hot water. This solution was then shaken sequentially with diethyl ether and saturated n-butanol. The n-butanol layer was separated from the mixture and concentrated using rotary evaporation. The resulting residue was dried at 80°C until the mass was constant and weighed to determine the saponin content in the material.

2.2.2. Investigating the oil extraction process of bitter melon seeds

Maceration

One g of the sample was measured and placed in a 100 mL beaker (Schott-Duran, Germany). Hexane solvent was then added, and the sample was allowed to macerate for different durations (12, 24, and 36 h) at 70°C under various material-to-solvent ratios (1:60, 1:80, 1:100 w/v), corresponding to each experimental condition. After extraction, the sample was filtered through Newstar 101 filter paper, and the solvent was evaporated using a rotary vacuum evaporator (Heidolph, Hei-VAP Core ML/G3 XL, Germany). The oil was dried at 70°C for 6 - 8 h and cooled in a desiccator and re-weighed the flask. Repeated the experiment three times and calculated the oil yield using the equation (2):

$$W(\%)= \frac{m_1-m_0}{m \times(1-h)} \times 100 \qquad (2)$$

where W was the oil yield (%), m was the mass of the sample used (g), m_0 was the mass of the rotary flask (g), m_1 was the total mass of the rotary flask and the oil after drying (g), and h was the moisture of the sample (%).

Soxhlet extraction

One gram of the sample was placed into a Soxhlet extraction thimble (Isolab, Germany). Reflux extraction was performed for 4, 6, and 8 h using material-to-solvent ratios of 1:60, 1:80, and 1:100 (w/v). Hexane remained the solvent used in this method. After extraction, the solvent was removed by a rotary evaporator and the results were calculated according to formula (2).

Ultrasonic extraction

One gram of the sample was weighed into a 100 mL beaker (Schott-Duran, Germany), and hexane solvent was added at material-to-solvent ratios of 1:60, 1:80, and 1:100 (w/v). The sample was then extracted using an ultrasonic bath (Hwashin, South Korea, 500 W power) for 5, 10, and 15 min at 30°C. After the extraction process, the sample was filtered through Newstar 101 filter paper, and the results were calculated based on equation (2).

2.2.3. Evaluating the quality of oil extracted from bitter melon seeds

Minerals

The mineral element content of oil was quantified using atomic absorption spectrometry according to TCVN 6496:2009 (VS, 2009) with minor modifications. Approximately 1 g of the sample was accurately weighed and placed into a reaction tube. Initially, 10 mL of a 1:1 mixture of HNO_3 and H_2O was added, mixed well, and heated at 95 ± 5°C for 15 min, then cooled to room temperature. Subsequently, 5 mL of 65% HNO_3 was added and heated at 95 ± 5°C for 30 min; this step was repeated if brown-red fumes were observed until they ceased. The sample was then heated for an additional 2 h, ensuring it did not dry out, and cooled again. Next, 2 mL of deionized water and 3 mL of 30% H_2O_2 were added, heated until bubbling decreased, and cooled. Additional 1 mL increments of 30% H_2O_2 were added, heating until bubbling stopped, not exceeding 10 mL in total, followed by another 2-hour heating period. After cooling, 10 mL of 37% HCl was added and the sample was heated for 15 min and cooled. The extract and remaining solid were transferred to a 50 mL volumetric flask, filled to the mark with deionized water, filtered through Whatman No.1 filter paper, and analyzed using flame atomic absorption spectrometry. The mineral content in the sample was calculated using the following formula (3).

$$W=(C \times f \times V) / m \quad (3)$$

where W was the minerals content in the sample (mg/kg); C was concentration of minerals in the test sample according to the standard curve (mg/L); f was dilution factor of the test sample; V was volume of the test sample (mL) and m was the mass of the sample (g).

Acid value (AV)

The sample was weighed into a 250 mL Erlenmeyer flask and dissolved in a neutralized solvent (a 1:1 ratio of ethanol and diethyl ether) by gently heating. After adding the phenolphthalein indicator, the solution was titrated with standardized 0.1 mol/L KOH while continuously shaking. The titration was considered complete when adding a drop of 0.1 mol/L KOH produced a faint but stable color change lasting for at least 15 sec. The AV was determined according to TCVN 6127:2010 (VS, 2010a):

$$W_{AV} = (A \times f \times 5,6)/m \quad (4)$$

where W_{AV} was the acid value, A was the volume of the 0.1 mol/L KOH used (mL), f was the concentration of KOH used (mol/L), and m was the mass of the sample (g).

Saponification value (SV)

The saponification value was determined according to TCVN 6126:2015 (VS, 2015). Specifically, 2 g of the sample was accurately weighed and added to 25 mL of 0.5 mol/L KOH solution in ethanol. It was refluxed for 2 h for oils with high melting points and difficult saponification. About 0.5 - 1 mL of phenolphthalein solution was added to the hot mixture and titrated with standardized 0.5 mol/L HCl solution until the pink color of the indicator disappears. The blank sample was prepared using 25 mL of KOH solution in ethanol, but without the test sample. The SV value was determined using the equation (5):

$$W_{sv} = \frac{(V_0 - V_1) \times 28}{m} \quad (5)$$

where W_{SV} was the saponification value, V_0 was the volume of the standardized HCl for blank (mL), V_1 was the volume of the standardized HCl for sample (mL), and m was the mass of the sample (g).

Peroxide value (PV)

The peroxide value was determined according to TCVN 6121:2010 (VS, 2010b). Two grams of the oil were placed into a 250 mL Erlenmeyer flask, followed by the addition of 20 mL of a mixture of CH_3COOH in a 2:1 (v/v) ratio and 5 mL of saturated KI solution. The mixture was shaken well, sealed, and kept in the dark for 10 min. Afterward, 30 mL of distilled water and a few drops of 5% starch solution were added, and the liberated iodine was titrated with 0.002 N $Na_2S_2O_3$ solution until the blue color disappeared. The blank was processed using the same steps as the test sample, but without the addition of the test sample. The PV was determined using the equation (6):

$$W_{PV} = \frac{(V - V_0) \times C_{thio} \times 0.0002538 \times 1000}{m} \quad (6)$$

where W_{PV} was the peroxide value, V was the volume of the $Na_2S_2O_3$ for sample (mL), V_0 was the volume of the $Na_2S_2O_3$ for blank (mL), C_{thio} was the concentration of the $Na_2S_2O_3$ (mol/L), m was the mass of the sample (g), and 0.0002538 g iodine corresponded to 1 mL 0.002 N $Na_2S_2O_3$ solution.

Antibacterial activity

Antibacterial activity was determined by the Kirby-Bauer method (Bauer et al., 1959). The oil was tested for antibacterial activity on *Staphylococcus aureus* (*S. aureus*) (ATCC® 6538™) and *Salmonella* sp. (ATCC®700623™). A volume of 0.1 mL of the bacterial suspension was spread across the surface of LB agar plates. Sterile filter paper discs, soaked in oil, were arranged in a triangular pattern on the agar surface. The plates were then incubated at room temperature for observation. Inhibition zones were measured after 24 h for *Salmonella* and 48 - 72 h for *S. aureus*. Ampicillin 50 ppm was used as positive control and all experiments were conducted in triplicate.

Antioxidant activity

The antioxidant activity of the oil was estimated by DPPH assay (Chanda et al., 2009). The oil was filtered and diluted to different concentrations. Each 0.5 mL of sample was added to the test tube content of 3 mL 96% ethanol, and 1 mL of 0.5 mM DPPH. The mixture was incubated in darkness at room temperature for 30 min. The absorbance was measured at 517 nm. A control experiment was conducted using 70% ethanol instead of the sample. Ascorbic acid was used as a reference at 10 - 50 mg/L. The antioxidant activity was determined according to the equation (7):

$$\%AA = \frac{OD_c - OD_s}{OD_c} \times 100 \quad (7)$$

where %AA was the antioxidant activity (%), OD_c was the absorbance of the control, and OD_s was the absorbance of the sample.

A correlation was established between sample concentration and antioxidant capacity based on the linear regression equation y = ax + b, from which the IC_{50} value (the concentration at which 50% of free radicals were captured) was determined to be a basis for comparing the antioxidant capacity between experiments. The lower the IC_{50} value, the higher the antioxidant activity.

2.2.4. Data analysis

The data was expressed as mean ± standard deviation (SD) of triplicate measurements. Two-way ANOVA followed by Tukey's test was conducted using GraphPad Prism version 9 to assess significant differences among treatments ($P < 0.05$).

3. Results and Discussion

3.1. Chemical composition of bitter melon seeds

3.1.1. Phytochemical analysis of bitter melon seeds

Qualitative analyses were performed to determine the presence of biologically active compound groups within bitter melon seed extract to explain the metabolic mechanism when used as a product. The results were shown in Table 3.

Table 3. Qualitative results of some secondary compounds in bitter melon seed powder

Compounds	Reagents	Result
Alkaloids	Wagner	No red-orange precipitate was observed (-)
	Dragendroff	No brown to dark brown precipitate was observed (-)
Flavonoids	NaOH 10%/HCl 10%	Suspicious
	Metal magnesium/HCl conc.	Suspicious
Phenolic acids	$FeCl_3$ 5%	No white fluffy precipitate was observed (-)
	Iodine	No blue-black precipitate was observed (-)
Saponins	Foam test	Foam column persists for 30 min (+)
Tannins	$FeCl_3$ 5%	No blue-black precipitate appeared.
	Lead acetate 10%	No red-orange precipitate was observed (-)

+: Presence; –: Absence.

According to the results from Table 3, bitter melon seeds contain saponin, corroborating the research conducted by Zahan (Zahan et al., 2020). In addition to saponin, which showed positive results, alkaloids and tannins showed negative results, and compounds such as polyphenols and flavonoids yielded inconclusive outcomes. Hence, the project continued to conduct quantification to accurately evaluate the bioactive compounds content of seeds.

3.1.2. Quantitative results

Following the qualitative results, the study continued to conduct accurate surveys of the compound content to establish a database for subsequent experimentation. Quantitative results, such as moisture, total ash (minerals), flavonoids, and total polyphenol, were shown in Table 4.

Table 4. Quantitative results on the phytochemical composition of material

Compound	Unit	Content
Flavonoids	mgQE/100 g	91.10 ± 0.01
Total polyphenols	mgGA/100 g	478.95 ± 0.02
Ash (minerals)	%	1.85 ± 0.16
Moisture	%	5.27 ± 0.04

The results are expressed as the mean ± standard deviation of three replicates and were calculated based on absolute dry samples.

Maintaining an appropriate moisture level during storage was imperative. If the moisture level was too low or too high, it compromised the quality and integrity of medicinal herbs. High moisture fostered an environment for bacteria, mold, and insects to grow, causing spoilage of medicinal materials. The moisture content indicated in Table 4 was 5.27%, which was in accordance with the requirements of the Vietnam Pharmacopoeia (< 13%).

The investigated bitter melon seed powder's measured flavonoids content was 0.91 mgQE/g, and the TPC at 4.79 mgGA/g. These outcomes were equivalent to those of Horax *et al*. (2010), who studied the amount of total polyphenol at about 4.76 mgGA/g.

The research also investigated the quantity of minerals in bitter melon seed extract and recorded the absence of highly toxic metals such as lead (Pb) and cadmium (Cd). In addition, the extract contained some trace elements known for their nutritional and immunity benefits, including iron (Fe), zinc (Zn), manganese (Mn), and small quantities of copper (Cu) and nickel (Ni) (Table 5). These findings contributed to confirming that the quality of bitter melon seed oil conformed with Vietnam Pharmacopoeia V, where heavy metals were not detected exceeding 20 parts per million, equating to 20 mg/kg (MOH, 2011a).

Table 5. Analysis results of some metals in bitter melon seed extract

Element	Unit	Content
Cd	mg/kg	No detection (LOD = 1 mg/kg)
Pb	mg/kg	No detection (LOD = 2 mg/kg)
Cu	mg/kg	11.02 ± 0.05
Zn	mg/kg	48.26 ± 1.26
Fe	mg/kg	87.71 ± 3.20
Mn	mg/kg	66.23 ± 2.58
Ni	mg/kg	4.14 ± 0.06

3.2. The oil extraction process from bitter melon seeds

3.2.1. The oil extraction efficiency of various methods

The oil was extracted as described in section 2.2.2, and the extraction efficiency was shown in Figures 2, 3, and 4. The experiment selected for comparison featured short extraction times and low solvent consumption, yet demonstrated the highest efficiency of each method. The optimal process was then proposed by comparing it with other methods.

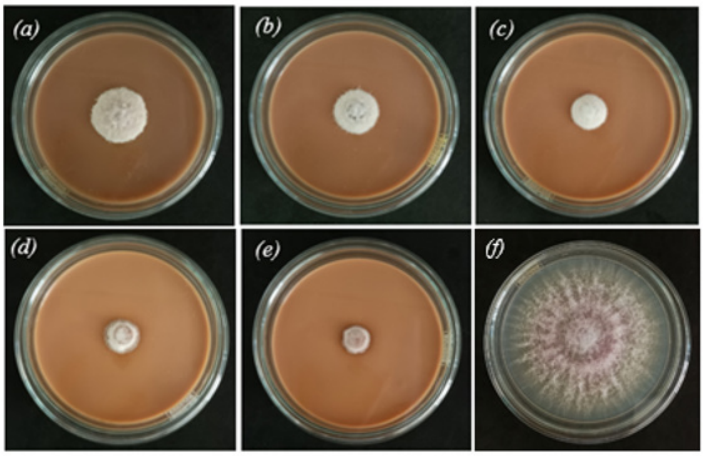

Figure 2. The lipid content was extracted by the maceration method.
ns: non-significant; *: $P < 0.05$; **: $P < 0.01$; and ***: $P < 0.001$.

The maceration method was simple and easy to execute but it was time-consuming. This method involved a static extraction mechanism where the solvent gradually penetrated the seed cells and dissolved the oil externally. As a result, the extraction efficiency was low and significantly dependent on the amount of solvent used and the lipid concentration within each plant. Investigating the impact of the material to solvent ratio and extraction duration on oil content revealed that the 12-h extraction experiment, using a ratio of 1:100, achieved the highest oil extraction efficiency. Therefore, this experiment was chosen as the optimal condition for the organic solvent extraction method.

Soxhlet extraction, using recirculated extraction solvent, was a technique that was easy to perform and required minimal equipment. However, it posed a challenge to apply on an industry scale.

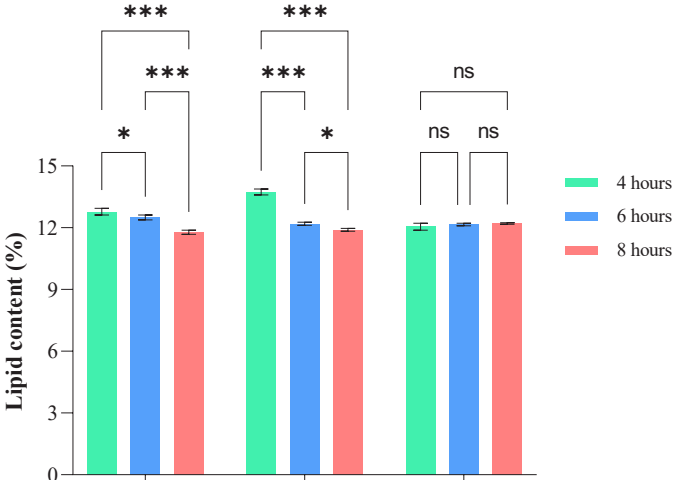

Figure 3. The lipid content was extracted by the Soxhlet method.
ns: non-significant; *: $P < 0.05$; **: $P < 0.01$; and ***: $P < 0.001$.

As shown in Figure 3, the lipid content obtained from the 4-h experiment with the ratio material: solvent of 1:60 and 1:80 was highest at 12.79% and 13.74%, respectively. These results demonstrated that increasing the amount of raw material necessitated more solvent and time extraction. However, once the saturation was reached (as observed in the experiment with a ratio of 1:100), the extraction took too long, proving to be inefficient and wasteful of solvents, which had negative implications for the environment and human health. Therefore, it was necessary to survey and select optimized extraction processes to save time, labor, and financial resources. For this method, the optimal condition was determined to be 4-h extraction time with a material to solvent ratio of 1:80.

The ultrasound-assisted extraction method had been a modern approach that accelerated the process with simple procedures but was expensive due to the need for specialized equipment.

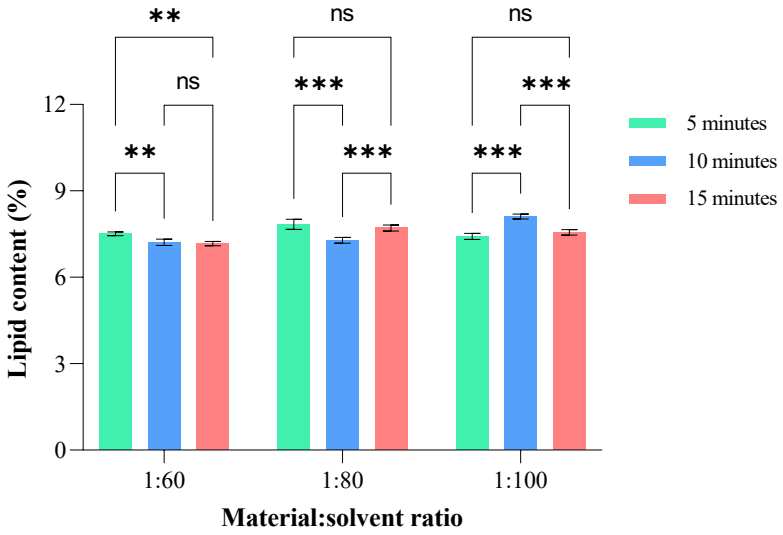

Figure 4. The lipid content was extracted by ultrasound-assisted method.
ns: non-significant; *: $P < 0.05$; **: $P < 0.01$; and ***: $P < 0.001$.

According to the test results, the 10-min extraction with a ratio of 1:100 achieved the highest oil extraction efficiencies (8.10%). Simultaneously, using low temperatures in the ultrasound-assisted method helped preserve the biological activity of secondary compounds, alongside the primary objective of maximizing lipid recovery.

3.2.2. Comparison of oil content obtained between three extraction methods

The experiments that had yielded the highest oil extraction efficiency for each method were subjected to one-way ANOVA analysis using GraphPad Prism version 9. Figure 5 showed that the average efficiency of the Soxhlet method (SL-1:80-4 h, with 13.74%) surpassed that of the maceration method using an organic solvent (M-1:100-12 h, with 11.79%) and the ultrasound-assisted method (UA-1:100-10 min, with 8.10%). These results were lower than the research by Ali et al. (2008), which reported the oil extraction efficiency of bitter melon seeds using the Soxhlet method was 26.00%. However, Ali's experiment utilized petroleum ether, a flammable solvent (boiling temperature 30 - 150°C), which could

irritate the skin, eyes, and mucous membranes and cause severe health issues such as drowsiness, dizziness, lightheadedness, nausea, unconsciousness, and coma when inhaled in high concentrations (Tociu et al., 2021). Thus, this study chose hexane as the solvent because it was not as flammable as petroleum ether (boiling temperature 68.70°C) and lower toxicity, though the extraction efficiency was lower (Herskowitz et al., 1971). Moreover, the differences in crop varieties, geographical conditions, and farming practices also influenced oil extraction efficiency.

The lower oil extraction efficiency of the ultrasound-assisted method was attributed to the short extraction time, limited power (500 W), and the presence of thick cell walls in bitter melon seeds, which led to poor separation efficiency and low dispersion within the cells. In addition, the probe's temperature could increase when the extraction time lasts too long, potentially denaturing biologically active compounds in oil. The maceration method using an organic solvent required an extended duration, ranging from 1 to 3 days, and achieved only 85% efficiency compared to the Soxhlet method. Although the extraction efficiency increased at elevated temperatures, the short circulation time of the solvent resulted in excessively prolonged extraction periods needed to completely extract the oil from the bitter melon seed samples.

Despite these challenges, the Soxhlet method consistently demonstrated the highest extraction efficiency with statistically significant differences among the methods examined. The Soxhlet technique had the potential to be scaled up for industrial use, accommodating volume up to 120 L per extraction process, making it suitable for a wide range of medicinal herbs. Thus, the practical application of this method was feasible.

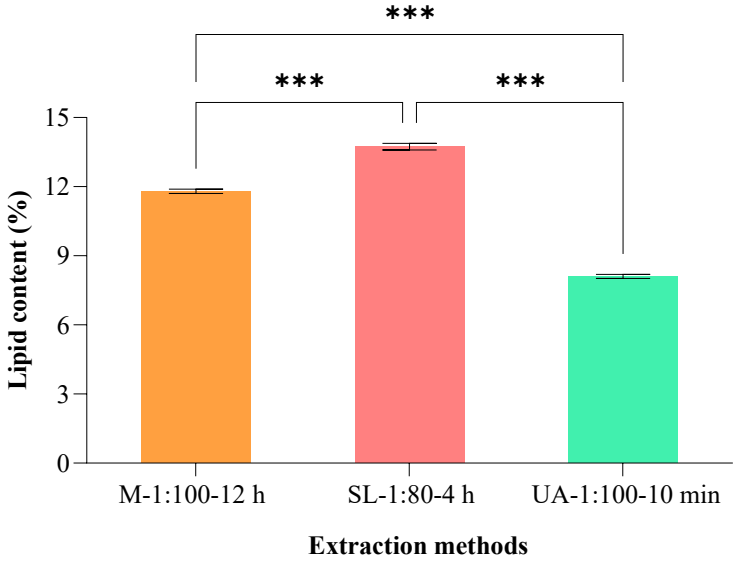

Figure 5. Compare oil extraction efficiency using different methods
ns: non-significant; *: $P < 0.05$; **: $P < 0.01$; and ***: $P < 0.001$.

3.2.3. Evaluating the quality of bitter melon seed oil

Bitter melon seed oil, extracted by the Soxhlet method as selected in the previous section, was evaluated for quality indices such as acid value, saponification value, peroxide value, and ester value (Table 6).

Table 6. Results of quality indices of bitter melon seed oil

Property	Unit	Content
Acid value (AV)	mgKOH/g	1.01 ± 0.01
Saponification value (SV)	mgKOH/g	355.60 ± 1.62
Peroxide value (PV)	meqO_2/kg	3.82 ± 0.02
Ester value	mgKOH/g	354.06 ± 1.32

FFAs, which was hydrolysis products of triglycerides (TGs) found in vegetable oils, primarily occurring during the production and preservation of oils. When fat was damaged, the amount of free fatty acids underwent degradation. Short-chain free fatty acids might be formed from the secondary oxidation of unsaturated aldehydes or other oxidation products originating from the cleavage of lipid hydroperoxides (Skiera et al., 2014; Alexandri et al., 2017). The concentration of FFA in edible oils depends on factors such as quality and variety of raw material, harvesting conditions, processing, storage, and deterioration status (Skiera et al., 2012). The acid value of bitter melon seed oil in this study was 1.01 ± 0.91 mg KOH/g, lower than the results reported by Tran et al. (2021) and Samba et al. (2022), which were 2.93 ± 0.25 mg KOH/g và 3.89 ± 0.37 mg KOH/g, respectively. Additionally, these values were higher than the research of Nkafamiya et al. (2007), which was 0.33 mg KOH/g. The lower AV, the more durable and higher the quality. Acid indices served as a crucial parameter for assessing the edibility of oil, and oil with a low acid number (< 4 mg/g) was suitable for human consumption (Moodley et al., 2007). The bitter melon seed oil had an acid value less than the limit for virgin edible oils (Codex, 1999), so it should be listed as an edible oil.

Determining the PV of edible oil was essential because PV was one of the most commonly used quality parameters to oversee lipid oxidation and control oil quality. The oil was easily oxidized during the processing and storage phases, which negatively affects oil quality and human health. Peroxide value shows the oxidation degree of the unsaturated fatty acids in products. When the peroxide value (PV) surpasses the critical threshold, edible oils could taste rancid and might even cause food poisoning (Gotoh et al., 2006). The peroxide number of bitter melon seed oil was 3.82 ± 0.81 meq O_2/kg, within the allowable standard range (< 10 meq O_2/kg) according to Codex-Stan 210-1999 (Codex, 1999). The peroxide value in this study was much lower than the PV of palm oil (16.08 meq/kg) and sorrel (Hibiscus sabdariffa) (5.00 ± 0.01 meq/kg) (Birnin-Yauri et al., 2011; Betiku et al., 2013).

The ester indices represented the amount of milligrams KOH required to saponify the esters contained in 1 g of oil, equal to the difference between the saponification index and the acid index. The ester value of bitter melon seed oil was 355.06 ± 1.77 mg KOH/g. Oil with a high saponification value was important for soap making and the cosmetic industry (Akanni et al.,

2005). The saponification value in this study was higher than argan oil (190.88 mg KOH/g) and olive oil (97.94 mg KOH/g) (Borchani et al., 2010). The solubility of soap in water depends on the quality of fatty acids, denoted by the saponification value. When this value was high, soap made from oil would be more soluble (Nyakudya et al., 2015). For that reason, bitter melon seed oil fulfills the requirement for a high SV and could be exploited as a material for making bath soap, lather shaving cream, and hair shampoo.

3.2.4. Antibacterial activity

The bitter melon seed oil was assessed for its antibacterial activity against two organisms: *S. aureus* and *Salmonella* sp. (Figure 6). These bacteria, commonly found in food and cosmetics, can cause some diseases such as skin infections, intoxication, and diarrhea. The results of the antibacterial activity of the bitter melon seed were presented in Table 7, with tetracycline 0.20% as the positive control and distilled water as the negative control.

Table 7. Results of measuring the diameter of the zones of inhibition

Organism	The diameter of the zones of inhibition (mm)	
	Bitter melon seed oil	Positive control
S. aureus	3.65 ± 0.05	20.50 ± 0.30
Salmonella sp.	3.50 ± 0.01	11.20 ± 0.05

According to Table 7, the oil extracted from bitter melon seeds demonstrated antimicrobial activity against both *Staphylococcus aureus* (3.65 ± 0.65 mm) and *Salmonella* sp. (3.50 ± 0.01 mm). The result was lower than the finding of Anjum et al. (2013), which reported inhibition zones of 22.80 ± 1.20 mm and 24.80 ± 1.30 mm, respectively, for two bitter melon varieties in Pakistan. However, the outcome surpassed the results reported by Tian et al. (2010) regarding to the antibacterial activity against *Salmonella* sp. of *Camellia oleifera* oil (2.50 mm). These differences could attributed to variations in bitter melon variety, solvent extraction, climate, agricultural practices, the growing season of each area, and the concentration of oil used in the experiments.

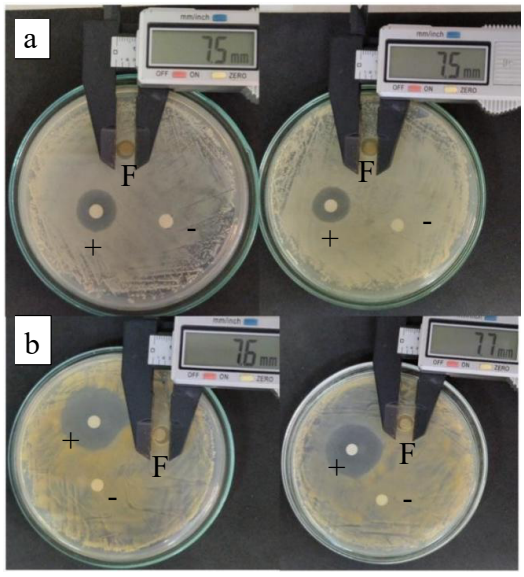

Figure 6. Testing antibacterial activity of bitter melon seed oil.
a) *Salmonella sp*.; b) *Staphylococcus aureus*; (F) Bitter melon seed oil; (+) Positive control: tetracycline 0.20%; (-) Negative control: distilled water.

3.2.5. Antioxidant activity

Based on the experimental results, the linear regression equation showing the correlation between the antioxidant activities and ascorbic acid concentrations was established as y = 1.7072x + 11.8380, with an estimated IC_{50} value of 0.03 mg/mL. Similarly, the linear regression formula showing the correlation between the antioxidant activities and the oil concentrations was established y = 0.2806x - 10.885 with an estimated IC_{50} value of 119.88 mg/mL (Table 8).

Table 8. IC_{50} of bitter melon seed oil

Sample	Linear regression equation	IC_{50} (mg/mL)
Ascorbic acid	y = 1.7072x + 11.8380	0.03
Bitter melon seed oil	y = 0.2806x + 10.885	119.88

The IC_{50} value of the oil was higher than that of ascorbic acid, indicating its weaker antioxidant capacity. However, bitter melon seed oil demonstrated a much better ability to scavenge free radicals compared to the finding on two bitter melon varieties in Pakistan (157.42 mg/mL and 143.59 mg/mL) (Anjum et al., 2013).

4. Conclusions

The qualitative analysis had shown that bitter melon seed contained various compounds, and the metal content in the seed met the standards set by the Ministry of Health in 2011. The best method for extracting oil from bitter melon seed was the Soxhlet method, which involved a 4-h extraction time and a 1:80 ratio of material to solvent, resulting in a 13.74% yield. The quality of the oil, including its acid, saponification, ester, and peroxide values, met the requirements of Vietnam standards for vegetable oils. The bitter melon seed oil also demonstrated antioxidant activity with an IC_{50} value of 119.88 mg/mL. Moreover, the oil showed inhibitory effects on the bacteria *Staphylococcus aureus* and *Salmonella* sp., with inhibition zone diameters of 3.65 mm and 3.50 mm, respectively. These results suggested that the seeds collected from bitter melon by-products were potential sources for oils and their applications in various fields.

Conflict of interest

The authors have declared no conflict of interest.

Acknowledgements

The authors acknowledge support from the Faculty of Biological Sciences and Research Institute for Biotechnology and Environment, Nong Lam University HCMC.

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