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Ultrasonic approach in *Clitoria ternatea* (butterfly pea) extraction in water and extract sterilization by ultrafiltration for eye drop active ingredient

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Abstract

The aim of this study is to evaluate butterfly pea ultrasound-assisted extraction (UAE) in water and sterilize the extract using ultrafiltration (UF) membrane for use as an active ingredient in phytopharmaceutical eye drops. The effect of petal-to-leaf (PTL) ratio and extraction time on anthocyanin concentration and antibacterial activity has been studied. The result showed that the optimum configuration was PTL ratio of 1:0 and extraction time of 30 minutes, which significantly yielded anthocyanin concentration of 35.41 ± 0.62 mg/l and exhibited the highest antibacterial activity in terms of inhibition zone against *S.aureus* of 14.75 ± 1.06 mm. Subsequently, feasibility of using UF membrane to sterilize the butterfly pea extract was also studied. Number of bacteria in the extract was varied to check whether UF was able to sterilize the extract regardless of the degree of bacterial contamination. The results showed no presence of bacteria in the permeate during 21 days of observation and an increase in anthocyanin concentration as well as antibacterial activity of the extract. In conclusion, UF was very effective to sterilize butterfly pea extract without the application of heat, thus, preserve the heat-sensitive compounds such as anthocyanins.

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Keywords: butterfly pea; ultrasound-assisted extraction (UAE); sterilization; ultrafiltration (UF) membrane; anthocyanins.

1. Introduction

As estimated by WHO, 80% of the world's population relies on herbal medicines for their healthcare needs [1]. Therefore, development of phytopharmaceutical drugs from natural resources is an interesting subject to be

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explored. *Clitoria ternatea* L., commonly known as the butterfly pea, is a medicinal plant native to Asian tropical equatorial countries such as Indonesia. Butterfly pea is believed to possess beneficial effects towards health such as memory enhancement and a potent cure for several diseases such as insomnia, mouth ulcers, and eye conjunctivitis. Butterfly pea contains anthocyanins which have promising potential for development as an antibacterial agent [2]. According to [3], aqueous butterfly pea extract is made up of the following compounds:

Table 1. Constituents in butterfly pea petal

Composition	**Concentration (nmol/mg petal)**
Flavonoids	20.07 ± 0.55
Flavonol glycoside	14.66 ± 0.33
Kaempferol glycoside	12.71 ± 0.46
Anthocyanins	5.40 ± 0.23
Quercetin glycoside	1.92 ± 0.12
Myricetin glycoside	0.04 ± 0.01

Furthermore, a previous study on eye drop development from butterfly peas showed that anthocyanins contained in butterfly pea petal extract is heat-sensitive; the amount of anthocyanins decreased after heat sterilization namely using autoclave and pasteurization [4]. Based on this finding, elimination of intense heat during extraction and sterilization processes is promising.

UAE has been applied widely because of its potency to increase yield and shorten extraction time. UAE provides the opportunity to enhance extraction of heat-sensitive material at lower processing temperatures [5]. UAE is a therefore promising approach to extract the heat-sensitive compounds in butterfly pea. In UAE, pressure waves are propagated, which result in the cavitation phenomena, which aids the mass transfer of extractable compounds.

In addition, pathogenic microorganisms that enter the eyes may cause diseases resulting from several factors such as poor personal hygiene, lack of nutrition, as well as the toxic nature of the microorganism itself. Consequently, another concern of this study also lies in the importance of sterility for eye drop with anthocyanins derived from butterfly pea as its active ingredient. Eye drops have to be sterile in order to prevent any further infections of the eyes caused by pathogenic microorganisms, therefore, sterilization has to be done to kill or remove all bacteria present in the butterfly pea extract.

In this study, the use of UF membrane to sterilize the butterfly extract will be evaluated. UF is a type of membrane filtration that employs physical separation based on the size of the constituents in a mixture. UF membrane pore ranges from 0.1 to 0.01 micron. Several studies reported that membrane filtration is the best method, in terms of feasibility and economical value, to sterilize materials that are heat-sensitive or undergo degradation after irradiation because it is able to remove the microorganism yet preserve the desired material due to the elimination of heat, chemicals, or radiation [6],[7].

2. Research method

The butterfly pea UAE was conducted in an ultrasonic bath (Sonorex Super 10P, Bandelin) with variations in PTL ratio (i.e. 1:0, 1:1, 1:2, and 2:1) and extraction time (i.e. 15, 30, and 45 minutes). Other variables such as temperature and power were kept constant to minimize variability of the results. The extraction was carried out at a constant temperature of 30˚C with a power of 3%. Power of 3% was chosen to minimize temperature increase during extraction that might affect the heat-sensitive compounds in the extract such as anthocyanins. The extracting solvent used was distilled water as it offered the suitable polarity for the target compounds such as anthocyanins.

Prior to UAE, the blue and white part of the butterfly pea petals were separated. Next, the blue part of the butterfly pea petals as well as the butterfly pea leaves to be extracted were cut into small pieces and weighed using a digital balance (Pioneer PA214, Ohaus). Distilled water was added with a ratio of 1:4 between the cut butterfly pea and the distilled water respectively. Extract was collected by passing the mixture through a filter paper. For control, extraction of butterfly pea was also done without using ultrasonic bath.

Anthocyanin concentration of the extracts was analysed using pH differential method which relies on the structural transformations of anthocyanins as a function of pH [8]. The extracts were diluted in KCl pH 1.0 buffer as well as citric acid pH 4.5 buffer with appropriate dilution factor. The absorbances of these dilutions were measured

using a spectrophotometer (Genesys 10 UV, Thermo Electron Corp.) at 547 nm and 700 nm for haze correction.

Antibacterial activity in terms of inhibition zone of the extracts against *S.aureus* was measured using agar well diffusion method. *S.aureus* concentration was adjusted to 0.5 M McFarland solution prior to inoculation on Mueller-Hinton agar in a sterile petri dish. Wells were made on the inoculated agar using sterile tips. Each well was filled with 70 µl of extracts. The petri dish was stored in a referigerator for approximately 30 minutes to allow diffusion of the extract into the agar. Subsequently, the petri dish was incubated for 24 hours at 35˚C. Inhibition zone was obtained by measuring the clear zone around the agar well using a ruler.

A configuration of PTL ratio and extraction time that resulted in the highest anthocyanin concentration and largest inhibition zone against *S.aureus* was selected for the sterilization stage by means of UF membrane. Bacteria concentration was varied to be original and modified. Modified extract was obtained by putting additional bacteria into the original extract to simulate as if the extract was highly contaminated with bacteria.

Hollow fibre UF membrane made of polysulfone material with a molecular weight cut-off (MWCO) of 20 kDa was used. The UF cartridge was connected to a pressure indicator and an ultrafiltration pump operating at a pressure of 1 bar. The ultrafiltration process was run for 10 minutes in a cleanroom to minimize re-contamination of the permeate (i.e. sterilized extract). The schematic diagram of the UF process is shown in Figure 1 as follows:

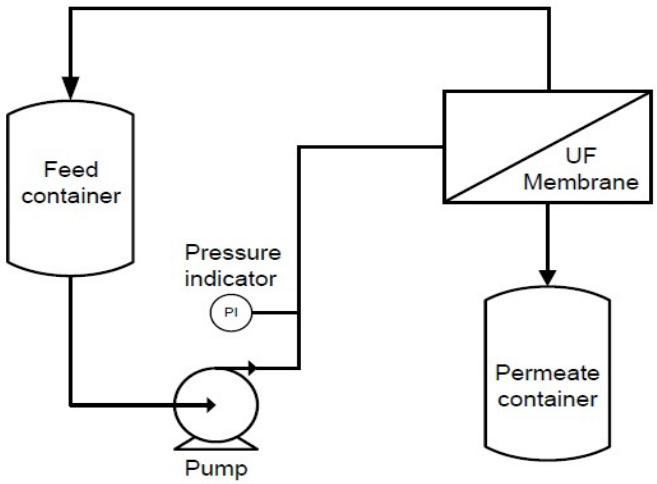

Figure 1. Schematic drawing of the ultrafiltration process.

The number of bacteria present in the extract was investigated using total plate count (TPC) method. Serial dilutions of the extract in sterile 0.85 M saline solution were made. 1 ml of each dilution was mixed with TPC agar and incubated for 72 hours. For accuracy and convenience, dilution which resulted in number of bacteria colonies of 30-300 was chosen to be counted.

Number of bacteria and anthocyanin concentration of the original and modified extracts before and after sterilization was measured. In addition, permeate flux and bacteria rejection rate of the UF membrane was also calculated. All data obtained in this study were analysed statistically using ANOVA followed by post hoc analysis using Tukey's HSD prior to drawing conclusion.

3. Results and discussions

3.1 UAE of butterfly pea with varying PTL ratio and extraction time

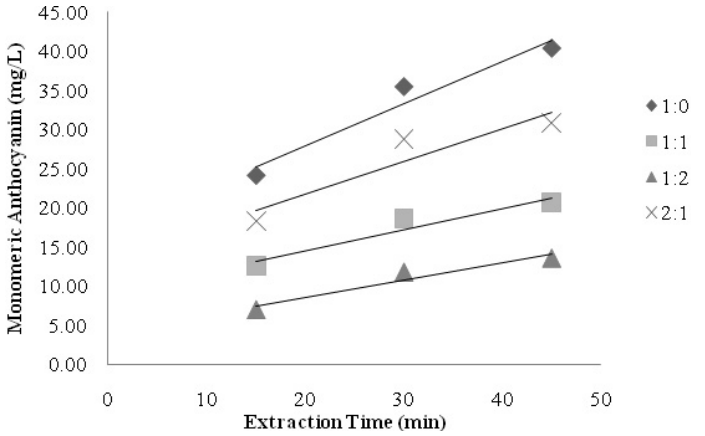

Figure 2. The effects of PTL ratio and extraction time on monomeric anthocyanin content of the extracts.

As deduced from Figure 2, the extracts containing no butterfly pea leaves (i.e. PTL ratio equals 1:0) yielded the highest monomeric anthocyanin at all extraction time followed by extract 2:1, 1:1, and 1:2. The reason was possibly butterfly pea leaves contained no anthocyanins. It can also be seen in Figure 2 that monomeric anthocyanin content increased as extraction time increased. ANOVA followed by Tukey's HSD analysis showed that all levels in both PTL ratio and extraction time had significnat impact in anthocyanin content of the extracts. Therefore, extract with PTL ratio of 1:0 extracted for 45 minutes yielded the highest anthocyanin concentration of 42.02 ± 0.23 mg/l than any other extracts.

In UAE, micro-sized bubbles were formed, grew, and collapsed violently due to fluctuations in pressure which is called the cavitation phenomenon. The burst of these bubbles might rupture cellular material, promoting interpenetration of water (solvent) into the butterfly pea petal cells, and therefore making the compounds such as anthocyanins more accessible to be dissolved by water. This might also lead to the increase of mass diffusivities and thus the increase of monomeric anthocyanin content in the extract. This result is in accordance with a study on UAE of polyphenols and anthocyanins from jambul fruit peels [9]. Longer extraction time possibly allowed bubbles to grow to a size sufficient to cause violent disruption. Longer extraction time might also increase contact time between water and the extractable components like anthocyanins, resulting in higher anthocyanin content in the extract.

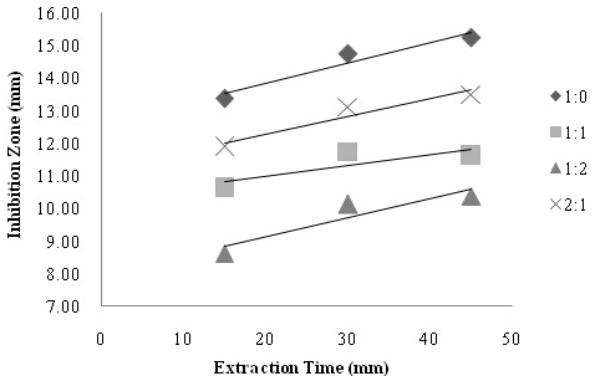

Figure 3. The effects of PTL ratio and extraction time on inhibition zone of the extracts against S.aureus

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Furthermore, Figure 3 shows the inhibition zone of the extracts against *S.aureus.* The extracts with PTL ratio of 1:0 exhibited the largest inhibition zone at all extraction time followed by extract 2:1, 1:1, and 1:2. This was possibly due to less antibacterial agents such as anthocyanins was present in extract 2:1, 1:1, and 1:2. In addition, as extraction time increased, the inhibition zone of the extract also increased as there were more antibacterial agents

extracted by the solvent. This trend was similar with the trend of monomeric anthocyanin content from Figure 2. Statistical analysis showed a positive correlation of 0.95 between monomeric anthocyanin concentration and with inhibition zone of the extract. It suggested that anthocyanins might be active the antibacterial agents that worked against *S.aureus*, but there was also synergy effect caused by the other components in butterfly pea petal and leaves. Statistical analysis using ANOVA followed by Tukey's HSD showed that all levels in PTL ratio resulted in significant impact in inhibition zone, while extraction time of 30 minutes and 45 minutes exhibited not significantly different result. Therefore, the optimum configuration was PTL ratio of 1:0 extracted for 30 minutes with inhibition zone of 14.75 ± 1.06 mm against *S.aureus*.

As reported on the study of antibacterial activity of T.P Wang aqueous extract against *S.aureus* [10] and on the study of antibacterial activity of T. Vulgaris aqueous extract against *S.aureus* [11], the presence of antibacterial agents in the aqueous extracts leads to the lysis of the bacteria by causing a rupture in the bacterial cell membrane. This report suggested a possible mechanism of butterfly pea petal aqueous extract against *S.aureus* in which the extract possibly worked by binding to the bacterial cell wall, which could lead to deformity or formation of pores on the cell wall, causing a leakage of intracellular materials and therefore inhibited the growth of the bacteria and eventually resulted in the death of the bacteria.

In conclusion, from the UAE stage, the extract with PTL ratio of 1:0 extracted for 30 minutes was chosen as the optimum configuration and selected for the sterilization stage as it exhibited the largest inhibition zone against *S.aureus* as desired in antibacterial products such as eye drops.

3.2 Sterilization of extract by UF membrane

Bacteria concentration of the butterfly pea petal extract was varied to be original and modified with amount of bacteria of 3.5 x 10^3 CFU/ml and 9.6 x 10^6 CFU/ml respectively. Figure 4 shows the permeate flux of original and modified extracts during the UF process.

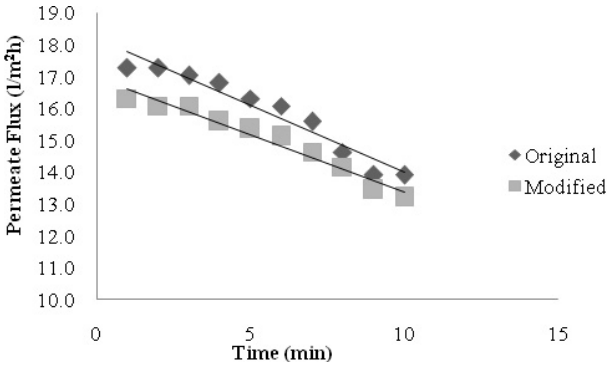

Figure 4. Permeate flux of original and modified extracts.

As seen in Figure 4, permeate flux of modified extract was slightly lower than that of original extract, this was due to the high bacteria amount contained in the modified extract. Feed concentration showed a negative effect on permeate flux, this phenomenon was probably because higher feed concentration induced earlier concentration polarization near the ultrafiltration membrane surface and therefore resulted in lower permeate flux. As also seen in Figure 4, permeate flux of both original and modified extract decreased with time. The permeate flux of original extract started at 17.3 l/m^2h and ended up at 13.9 l/m^2h after 10 minutes, while the permeate flux of modified extract started at 16.3 l/m^2h and ended up at 13.2 l/m^2h after 10 minutes. This happened possibly due to fouling or the deposition of some compounds contained in the extract on the ultrafiltration membrane surface as time increased. Similar result was also found in a study on the effect of UF on black currant juice [12].

To prove whether UF was effective in removing bacteria in the extract, rejection rate of bacteria was calculated. Number of bacteria in original and modified extract before and after ultrafiltration is shown in Table 1 as follows:

Table 1. Number of bacteria in original and modified extracts before and after sterilization by UF membrane.

	Number of Bacteria (CFU/ml)	
	Original Extract	**Modified Extract**
Feed	3.5×10^3	9.6×10^6
Permeate	0	0

No presence of bacteria in the permeate side suggested a bacteria rejection rate of 100%. All the bacteria in the extract frrom the feed was rejected to the retentate side, resulting in the presence of no bacteria in the permeate side, and therefore making the permeate sterile. Bacteria were rejected because they could not pass through the ultrafiltration membrane due to the nature of their big size. Bacteria have a size range between 0.4×10^6 to 8.6×10^6 kDa [13], which is significantly larger than the UF pore size used (i.e. 20 kDa). This size difference separated bacteria from the other smaller particles in the extract (that could pass through the ultrafiltration membrane) such as the anthocyanin pigments.

Other than sterilizing the extracts, UF membrane also concentrated the extracts as shown in Figure 5. Monomeric anthocyanin concentration of the extracts increased significantly after UF. The monomeric anthocyanin concentration of unsterilized original extract was 19.49 ± 0.80 mg/l which increased to 78.83 ± 1.18 mg/l after sterilization. Meanwhile, monomeric anthocyanin concentration of unsterilized modified extract was 12.03 ± 0.45mg/l which increased to 48.17 ± 0.88 mg/l after sterilization.

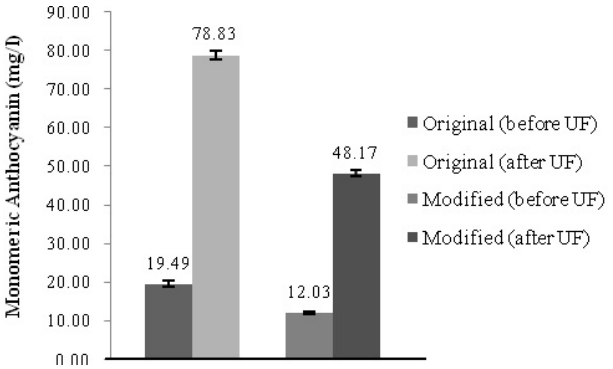

Figure 5. Monomeric anthocyanin of original and modified extract before and after sterilization by ultrafiltration.

The size of anthocyanins in butterfly pea petals range between 470 to 2110 Da, which is significantly smaller than the UF pore size (i.e. 20 kDa). During the UF process, anthocyanins could pass through the membrane while other molecules that had significantly bigger size than the membrane pore could not pass through the membrane. This separated as well as concentrated the extract on the permeate side. This finding was in agreement with a study on concentration of blackcurrant juice and red wine by integrated membrane process [14].

The increase of anthocyanin concentration after UF process lead to the increase of inhibition zone of the extracts because anthocyanins was assumed to be one of the active antibacterial agents in the extract that worked against *S.aureus.* Positive correlation between anthocyanin concentration and inhibition zone was maintained in this sterilization stage. The inhibition zoe of original and modified extract before and after sterilization by UF membrane is shown in Figure 6.

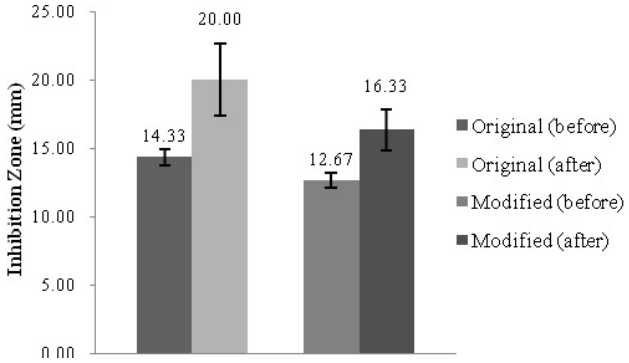

Figure 6. Inhibition Zone of original and modified extract before and after sterilization by UF membrane.

From Figure 6, the inhibition zone of unsterilized original extract was 14.33 ± 0.57 mm which increased to 20.00 ± 2.64 mm after ultrafiltration and the inhibition zone of unsterilized modified extract was 12.67 ± 0.57 mm which increased to 16.33 ± 1.52 mm after UF.

3.3 Sterile stability of the sterilized extracts

Sterile stability refers to how the sterility of the sterilized extract is during storage. After sterilization using UF membrane, sterility of the sterilized extracts was observed continuously for 21 days with a two-day interval. Bacteria could form a resistant structure in response to stress, such as exhaustion of nutrients, as a survival strategy which is called the endospore. Endospores are spherical structures that represent a dormant stage in bacterial growth cycle, commonly formed by genus *Bacillus* and *Clostridium.* Mature spores are usually 0.8 – 1.2 μm in length. Other than dormant, these spores are also highly resistant to heat, pressure, and many chemical disinfectants that is believed to be attributed by the multiple layers of protein that encase the spore. The common spore coats are CotA, CotB, CotC, and CotG that weigh approximately 173 [15].

Being significantly smaller than vegetative bacterial cells, endospores might pass through the UF pores. These bacterial endospores might not be detected by bacteria enumeration conducted right after the sterilization process due to their dormancy. Therefore, observation and measurements conducted in the subsequent 21 days were intended to reveal if there were dormant bacterial endospores present in the extract, which could outgrow into vegetative bacterial cells and re-contaminate the sterilized extracts. Endospores usually take three stages to come out of stasis and begin growing, which are activation, germination, and outgrowth. 21 days were assumed to be enough to allow these stages to happen in the case that there were bacterial endospores in the sterilized extracts. Table 2 shows the number of bacteria in both original and modified sterilized extracts during 21 days of storage. No bacteria was present in both sterilized extracts, therefore, it was concluded that UF membrane was effective to sterilizse the butterfly pea petal extract.

Table 2. Sterile stability of sterilized original extract and sterilized modified extract during 21 days of storage

Day	**Number of Bacteria (CFU/ml)**	
	Original Extract	**Modified Extract**
1	0	0
3	0	0
5	0	0
7	0	0
9	0	0
11	0	0
13	0	0

15	0	0
17	0	0
19	0	0
21	0	0

4. Conclusion

This study suggested that the extract with petal-to-leaf (PTL) ratio of 1:0 (i.e pure butterfly pea petals) significantly yielded the highest concentration of anthocyanin and exhibited the highest antibacterial activity against *S.aureus* at all extraction time compared to the extracts with PTL ratio of 1:1, 1:2, and 2:1. Furthermore, both anthocyanin concentration and antibacterial activity increased with time. The results suggested that the optimum configuration was PTL ratio of 1:0 and extraction time of 30 minutes, which resulted in the highest anthocyanin concentration of 35.41 ± 0.62 mg/l and the highest antibacterial activity in terms of inhibition zone of 14.75 ± 1.06 mm. Furthermore, the use ultrafiltration (UF) membrane was very effective to sterilize butterfly pea extract to be used as active ingredient in phytopharmaceutical eye drops. The UF membrane was able to remove all the bacteria in the extract yet preserved the anthocyanin content and antibacterial activity of the extract itself.

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