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# Application of Aqueous Micellar Two-Phase System for Extraction of Bioactive Compounds

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**Abstract.** In this study, the AMTPSs which composed of different types and concentrations of a single nonionic surfactant (i.e. Pluronic L-121, L-81, L-61, and L31) were formed and their respective cloud point temperature was determined. The clouding phenomenon was highly dependent on the molecular weight of the hydrophobic polypropylene oxide of the Pluronic surfactants. The extraction of bioactive compounds from the *Carica papaya* leaves using the temperature-induced aqueous micellar two-phase system (AMTPS) was demonstrated. The phenolic compounds exhibited an overall preference to the micelle-rich bottom phase for all the AMTPS investigated. A total of 60.1% total phenolic content with a DPPH free radical scavenging activity of 81.9% was recovered in the micelle-rich phase of the 10% (w/w) Pluronic L-61 AMTPS with a log partition coefficient, $K_{TPC}$ of 1.27. The AMTPS can serve as an alternative extraction technique for the recovery of various bioactive compounds from other plants.

## INTRODUCTION

*Carica papaya*, also known as *paw paw* [1], can grow up to 45 cm long and 30 cm in diameter. The fruit of *C. papaya* is very nutritious and has a sweet taste and soft texture when it is ripe [2]. In the early of the 21st century, the global papaya fruit production has grown tremendously up to 13.05 million tonnes in 2016 due to the increase in production in India and the demand by United States [3]. Recently, the *C. papaya* leaves extracts have been uncovered to be linked with various health benefits as it contains many bioactive compounds, such as phenol, tannin, flavonoids, carotenoid that exhibit antioxidants activity [4]. Several studies reported that the consumption of *C. papaya* leaves extract could enhance the recovery of wound [5], reduce the risk of cardiovascular disease [6], alleviates allergic disorders [7], serve as an immune-adjuvant for vaccine therapy [8] as well as treat gout [9].

Several conventional methods such as the Soxhlet extraction, maceration extraction, and hydrodistillation have been applied to extract bioactive compounds. Although these methods are relatively lower in cost, but they are not feasible in large-scale production in view of its poor recovery yield, long processing time, and harsh extraction conditions [10]. In order to overcome these limitations, alternatives extraction methods, such as the ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), pressurized liquid extraction (PLE) are introduced. Nevertheless, these methods are not widely applied at large scale to date due to their high operating and equipment costs [11].

The Aqueous Micellar Two-Phase System (AMTPS), which is also known as the cloud point extraction or surfactant-mediated phase separation, have been adopted as an alternative to the conventional solid-liquid or liquid-liquid extraction methods for the recovery of biomolecules. The AMTPS offers several process advantages, such as the high enrichment factor, high recovery, less complexity and low cost [12]. At a relatively low concentration of surfactant, two immiscible phases (i.e. a micelle-rich phase and a micelle-poor phase) are formed when the surfactant solution was heated beyond its cloud-point temperature [13]. Upon increasing the temperature above the cloud point temperature, the solubility of the surfactant molecules decreases and undergo macroscopic phase

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separation due to the self-aggregation of the surfactant molecules and the dehydration of its polyethylene oxide units. The reusability of surfactant after extraction is another remarkable factor which brings the advantage of reducing the overall cost of the extraction process [14].

There are various types of surfactants that are available which include the ionic and non-ionic surfactants. Among these two types of surfactants, the non-ionic surfactants were selected as the phase-forming component for the AMTPS for the extraction of bioactive compounds from the *C. papaya* leaves. An example of non-ionic surfactant is the Pluronic surfactant, which is also known as the Poloxamer, that has been widely applied in the pharmaceutical industry for the drug delivery [15]. Hence, it is anticipated that this Pluronic non-ionic surfactant, which is relatively inexpensive and less toxic compared to the ionic surfactant, could serve as a desirable solvent with high extractability while preserving the bioactivity of the bioactive compounds.

In this study, the AMTPS that composed of Pluronic non-ionic surfactants will be applied to extract the bioactive compounds from the *C. papaya* leaves. Firstly, the phase separation behavior (i.e. cloud point temperature) of different surfactants Pluronic L-121, L-81, L-61, and L-31 will be investigated. The effect of different types of Pluronic surfactant on the partitioning efficiency, the extraction efficiency and the antioxidant activity of the extracted total phenolic content will be evaluated to study for their extractability of TPC from the *C. papaya* leaves.

## MATERIALS AND METHODS

### Materials

Pluronic L-35, Pluronic L-64, Pluronic L-121, Pluronic F-68, Gallic acid, Fast Blue BB salt, sodium hydroxide, 2,2-diphenylpicrylhydrazyl (DPPH) powder, and ascorbic acid were purchased from Sigma Aldrich (St. Louis, USA). Sodium carbonate and methanol were obtained from Merck (Darmstadt, Germany). The nonionic surfactants were used without further purification.

### *Carica papaya* Leaves Preparation

*C. papaya* leaves were sourced from West Malaysia. The leaves were rinsed with distilled water and cut into small pieces of around 0.5 cm × 0.5 cm size. The leaves were freeze-dried and grinded into powder form. The *C. papaya* leaves powder was sieved using a 500 μm pores size sieve and stored in the fully-sealed bottle at 4 °C [4].

### Coexistence curve of AMTPS

The coexistence curves of the AMTPS, which composed of different types of Pluronic surfactant, were constructed according to the method described by Vicente et al [16]. Surfactant solutions at concentrations ranging from 5% (w/w) to 20% (w/w) were heated in a silicon oil bath until the solutions turned turbid. The solutions were subsequently cooled at a rate of 0.5°C per min. The cloud point temperature at which the solutions turned into a single and transparent phase was recorded.

### AMTPS Extraction Experiments

For the AMTPS extraction of phenolics compounds from the *C. papaya* leaves, 10.0g of AMTPSs were prepared by mixing 10% (w/w) of the different non-ionic surfactants (i.e. Pluronic L-121, Pluronic L-81, Pluronic L-61 & Pluronic L-31), 0.1% (w/w) of *C. papaya* leaves powder, and ultrapure water in a 15 ml centrifuge tube [17]. Then, the mixture was thoroughly mixed using the vortex mixer, equilibrated in a shaking water bath at 40 °C for 10 min and centrifuged at 10000 rpm for 10 min. Both the top and bottom phases' volume were recorded for the determination of the volume ratio, $V_R$. Phase sample solution was collected from the top phase and bottom phase to quantify the amount of total phenolic content and the free radical scavenging activity. Methanol was used to dilute

the viscous phase sample solution if necessary, prior to the sample analysis. All the extraction experiments were performed twice.

## Quantification of Total Phenolic Content via FBBB

In this study, the Fast Blue BB (FBBB) assay [18] was applied to measure the total phenolic content of the phase sample solution. The FBBB assay was adopted instead of the Folin-Ciocalteu (F-C) method due to the formation of fine solids and cloudy resultant mixture were observed in the latter when the phase sample solution was mixed with the F-C reagent [19]. A 0.1% (w/v) of FBBB reagent was prepared by mixing 50 mL of ultrapure water with 50 mg of FBBB salt. 1.5 g of sodium hydroxide was dissolved in 30 mL of ultrapure water to prepare 5% (w/v) sodium hydroxide. For quantification of the total phenolic content, 0.1 mL of 0.1% (w/v) FBBB reagent was added to 1 mL of sample solution. Subsequently, 0.1 mL of 5% (w/v) of sodium hydroxide was added to the mixture, well mixed, and incubated at 25°C for 90 min. The absorbance of each mixture was measured at 420 nm using the microplate reader. Gallic acid is used as the standard for calibration ($y = 4.0921x + 0.053$, $R^2 = 0.99$).

## Quantification of DPPH Free Radical Scavenging Activity

The free radical scavenging activity of the sample solution was determined according to the method described by [20] with some modifications. A mass of 4.0 mg of DPPH powder was added into 50 mL of ethanol to prepare 0.2 mM DPPH reagent. The 0.2 mM DPPH reagent, which was prepared by dissolving 4.0 mg of DPPH powder in 50 mL of ethanol, was mixed with the sample solution in an equal volume of 100 µL. The mixture was left to sit in the dark at room temperature for 30 min. The absorbance of the mixture was measured at 517 nm. All the measurements were quantified in triplicate and the results were reported as ascorbic acid equivalent antioxidant capacity (AEAC) in µg/mL. The DPPH free radical scavenging activity was calculated using Eq. (1):

$$\text{Scavenging Activity (\%)} = \left[\frac{A_c - (A_s - A_b)}{A_c}\right] \times 100\% \tag{1}$$

where $A_c$ is the absorbance value of DPPH reagent without sample at 517 nm, $A_s$ is the absorbance value of DPPH with sample at 517 nm, and $A_b$ is the absorbance of the sample at 517 nm.

## Partitioning Coefficient, Volume Ratio and Yield

The partitioning coefficient ($K$) for the TPC was calculated using Eq. (2):

$$K_{TPC} = \frac{Total\ phenolic\ compounds\ in\ bottom\ phase\ of\ AMTPS}{Total\ phenolic\ compounds\ in\ top\ phase\ of\ AMTPS} \tag{2}$$

The volume ratio, $V_R$ was calculated using Eq. (3):

$$V_R = \frac{V_T}{V_B} \tag{3}$$

where the $V_T$ and $V_B$ are the top phase volume and bottom phase volume of AMTPS, respectively.
The extraction yield of the TPC in the bottom phase, $Y_{TPC}$, of the AMTPS was determined using Eq. (4).

$$Y_{\text{TPC}}(\%) = \frac{100}{1 + \frac{V_R}{K_{\text{TPC}}}} \tag{4}$$

## RESULTS AND DISCUSSION

### Coexistence Curves of Pluronic Series Surfactants

The coexistence curves for several Pluronic non-ionic surfactants (L31, L61, L81, and L121), in a range of 5-20% (w/w), were determined and shown in Fig. 1. The region above each curve corresponds to the formation of the two-phase region at the given clod-point temperature and surfactant's concentration [14]. All the investigated non-ionic surfactants exhibited a cloud point temperature which was lower than 35°C, suggesting that these surfactants would be biocompatible for the separation for thermo-sensitive bioactive compounds. The Pluronic non-ionic surfactants applied in this study are tri-block copolymers which made up of the hydrophilic polyethylene oxide (PEO) monomers and the hydrophobic polypropylene oxide (PPO) monomers in the sequence of PEO*n*-PPO*m*-PEO*n*, where *n* and *m* correspond to the number of EO and PO blocks, respectively [21]. When the temperature of the Pluronic non-ionic surfactant was increased beyond its cloud point temperature, the tri-block copolymer will self-aggregate, forming a hydrophilic micelle core which is surrounded by the hydrated PEO units and a hydrophobic interior which is composed of PPO units.

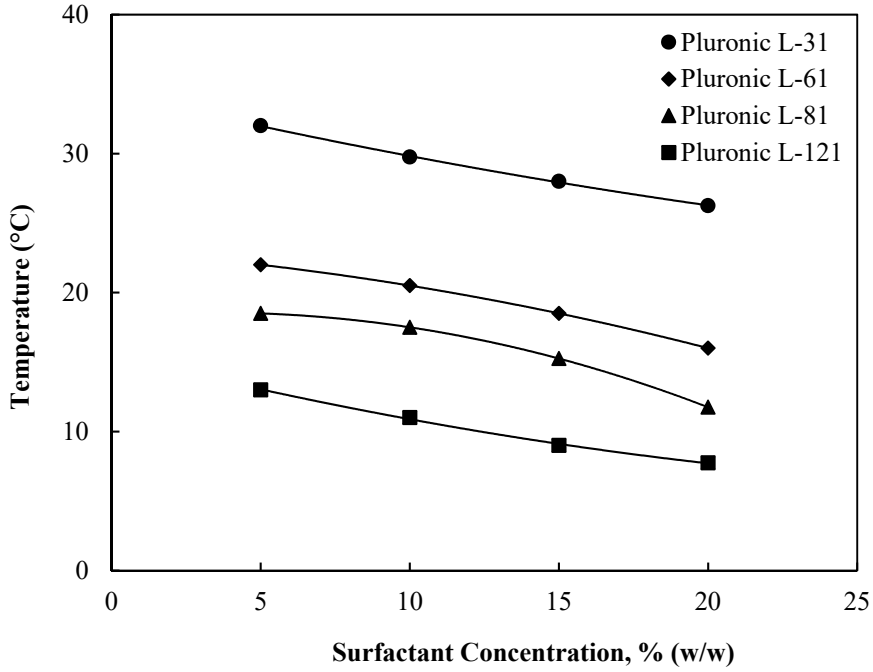


**FIGURE 1**. The cloud point temperature for different concentrations of Pluronic non-ionic surfactants.

Based on Fig. 1, the Pluronic L-121 exhibited the lowest cloud point temperature across the concentration range of 5-20% (w/w) and followed by Pluronic L-81, Pluronic L-61, and Pluronic L-31. It was also observed that the cloud point temperature of these non-ionic surfactants decreased with the increase in the surfactant concentration. The cloud point temperature of the Pluronic surfactants is highly dependent on the alkyl chain length (i.e. molecular weight) of the PPO. Pluronic non-ionic surfactants have similar PPO:PEO composition ratio of 10% EO, but they differ in the molecular weight of the PPO. The molecular weight of the hydrophobic PPO for the Pluronic L-121, L-81, L-61, and L-31 are 4000, 2250, 1750 and 950 g/mol, respectively [22]. As the molecular weight of the PPO units increases, the magnitude of repulsion between the copolymer and water increases and leads to the formation of micelles with larger hydrophobic domain within the copolymer at a lower temperature [23]. Thus, the Pluronic L-121 which possessed a high proportion of PPO units showed the lowest average cloud point temperature 10.2 °C among all the non-ionic surfactants investigated. Although the Pluronic L-121 requires lesser amount of heat energy for the micellization process due to its low cloud point temperature, its feasibility for the extraction process at large

scale could be limited by its high viscosity. Thus, all of the Pluronic non-ionic surfactants were further investigated for their extraction efficiency of TPC from the *C. papaya* leaves. using these

## Effects of AMTPS-forming Surfactant for Extraction of Total Phenolic Compounds

*Partitioning Efficiency of TPC*

The AMTPS formed by 10% (w/w) of different Pluronic non-ionic surfactant were investigated for their extractability of TPC from the *C. papaya* leaves. This specific surfactant concentration was employed for the investigation as it has been reported that a low concentration of these surfactants was sufficient to extract the bioactive compounds from various plant sources [24]. Table 1 shows the partition efficiency of TPC in the AMTPS constituted with different Pluronic surfactants. All the values of the log $K_{TPC}$ were higher than unity. These results indicated that the majority of the extracted phenolic compounds preferentially partitioned to the micelle-rich bottom phase of all the investigated AMTPS due to the inherent hydrophobic nature of the phenolic compounds [25]. The hydrophobic interaction between the micelle core of the Pluronic surfactant and the hydrophobic benzene ring of the phenolic compounds lead to the partition of the extracted TPC towards the micelle-rich bottom phase of the AMTPS. This partitioning behavior was in good agreement with [17] for the recovery of bioactive compounds from the *Garcinia mangostana* peels. The Pluronic L61 showed the highest log $K_{TPC}$ of 1.27, indicating that better partitioning efficiency could be achieved using the AMTPS which composed of Pluronic L61 compared to other types of Pluronic surfactants.

**TABLE 1.** Effect of AMTPS-forming Surfactant on the partitioning efficiency of TPC.

| AMTPS | log $K_{TPC}$ |
|---|---|
| 10% (w/w) P. L-31 | 1.18 |
| 10% (w/w) P. L-61 | 1.27 |
| 10% (w/w) P. L-81 | 1.14 |
| 10% (w/w) P. L-121 | 1.18 |

*Extraction Yield of TPC*

The highest extraction yield of TPC (60.07%) was achieved in the AMTPS which composed of Pluronic L-61 as shown in Fig. 2. A lower yield of TPC was recovered in the micelle-rich phase of the AMTPS which composed of Pluronic L-31 compared to that of Pluronic L-61. This phenomenon could be attributed to the low molecular weight and lower number of PPO units in the Pluronic L-31, which is generally less hydrophobic [17]. Theoretically, the increase in the PPO molecular weight and thus the hydrophobicity of the Pluronic surfactant shall enhance the yield of the TPC in the AMTPS micelle-rich phase. Nevertheless, lower yields of TPC were observed in both Pluronic L-81 and Pluronic L-121 with higher molecular weight. It is likely that these surfactants of higher molecular weight form micelles of more compact conformation, thereby reducing the free volume available in the micelles for separation of the TPC from the *C. papaya* leaves powder [26].

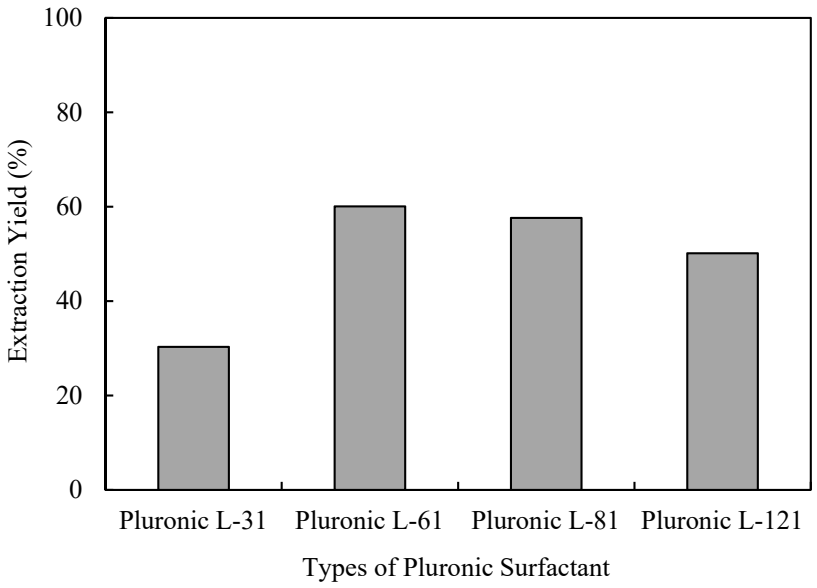


**FIGURE 2**. Effect of AMTPS-forming Surfactant on the extraction yield of TPC.

*DPPH Free Radical Scavenging Activity*

The DPPH free radical scavenging activity of extracted bioactive compounds was depicted in Fig. 3. Pluronic L-61 showed the highest scavenging activity of 81.9% among all the surfactants investigated. The scavenging activity of the extracted sample exhibited a similar trend in accordance with the extraction yield of TPC, whereby the scavenging activity increased steadily at lower molecular weight of Pluronic surfactant but decrease at higher molecular weight of Pluronic surfactant. The scavenging activity is highly dependent on the amount of antioxidant compounds which are usually found in the phenolic form [20]. Hence, the scavenging activity is strongly driven by the presence of high concentration of total phenolic contents.

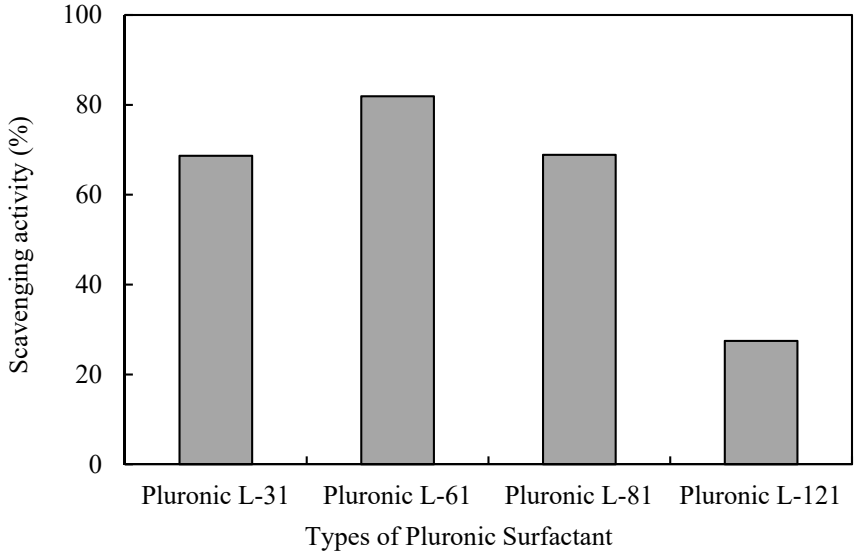


**FIGURE 3**. Effect of AMTPS-forming Surfactant on the scavenging activity of the extracted sample.

## CONCLUSION

In this study, the cloud point temperatures of several types of Pluronic surfactants L-31, L-61, L-81, and L-121 were investigated. The result showed that Pluronic L-121 exhibited the lowest cloud point temperature among all the surfactants investigated, followed by the Pluronic L-81, Pluronic L-61, and Pluronic L-31. A simple AMTPS was performed for the extraction of phenolic compounds from the *C. papaya* leaves. The phenolic compounds were preferentially partitioned to the micelle-rich bottom phase of all the AMTPS investigated. A log $K_{TPC}$ of 1.27 was achieved using the AMTPS that composed of 10% (w/w) Pluronic L-61 with 0.1% (w/w) of sample loading at 40°C, resulting in an extraction yield and DPPH free radical scavenging activity of 60.1% and 81.9%, respectively. Other factors that could enhance the extraction efficiency of the phenolic compounds from the *C. papaya* leaves will be further investigated in the future study.

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