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The extraction of estrogen-like compounds from *Pueraria mirifica*, the properties characterization and the preparation of nucleated nanoparticles

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ABSTRACT

Pueraria mirifica (PM), also known as Kwao Krua Khaw, has been used as an herbal plant for many decades in the Northern part of Thailand. The root of PM contains estrogen-like compounds that are less soluble in water resulted in low bioavailability in an aquatic animal application. To increase solubility of the compounds, six different polarity solvents such as hexane, benzene ethyl acetate, acetone, ethanol, and deionized distilled water were employed for extraction. It found that deionized distilled water exhibited the highest extraction percentage yield. However, a high amount of phenolic compounds and flavonoids were obtained in ethyl acetate fraction (EAF). In addition, the results of scavenging radical assay using DPPH IC_{50} showed the highest activity of EAF due to the high content of total phenolic content and flavonoids content. Moreover, EAF, significantly exhibited the highest proliferation activity against MCF-7. Therefore, EAF was selected for developing polymeric micelles. The HPβCD, Pluronic F-68, and Pluronic F-127 were employed with Tween80 to prepare suitable nanoparticles. The proper ratio of EAF-loaded Pluronic F-127 (EAF-F127) product in nanosize range elicited zeta potential value of nearly zero mV and exhibited the transparent aqueous dispersions in DI water. The results promised further *in vivo* studies.

Keywords: *Pueraria mirifica*, antioxidant, MCF-7, polymeric micelles, nanoparticles

INTRODUCTION

Pueraria mirifica (PM) is locally called Kwao Krua Kao in Thai. It is an estrogenic plant that has been used as a traditional herb and a folkloric medicine in Thailand (Intharuksa et al., 2020; Suthon et al., 2016), Myanmar, Laos, Vietnam (Intharuksa et al., 2020; Peerakam et al., 2018) and other southeast Asian countries for over a hundred years (Malaivijitnond, 2012). The roots of PM have been used to improve menopausal stage symptoms or absent periods such as vaginal dryness, hot flashes, irritability, night sweats, depression (Intharuksa et al., 2020). They have been used as a health promoter such as anti-aging, anti-wrinkle agent, increasing hair growth agents (Sirisa-ard, et al., 2018), and bone loss prevention agent. In orchidectomized and ovariectomized rats, the roots are used as an estrogen supplement. (Urasopon et al., 2007; Urasopon et al., 2008). They possess an estrogenic effect on other hormone-sensitive cells (Lin et al., 2017). Unfortunate, most bioactive compounds in PM roots were flavonoids and coumarins (Lee et al., 2017; Jeon, et al., 2005), which were less soluble in water, resulted in low bioavailability in the aquatic animal application. In general, 95% of ethanol was used for the extraction of bioactive compounds from this plant (Peerakam et al., 2018) from the viewpoint of medicinal purposes and the environment. However, to have insoluble compounds with maximum purity, the solvents with different polarities were employed, as indicated by Yodthod et al. (2020).

In this study, we tried to obtain estrogen-like compound fractions using different solvents with different polarities. Moreover, the antioxidant activity, the total phenolic content, the flavonoids content, and MCF-7 proliferative activity were investigated. The selected fraction would be prepared in nano-sized particles by using polymeric micelles formulation technology in order to enhance the solubility. The nanoparticles were developed by using HPβCD ((2-Hydroxypropyl)-β-cyclodextrin), Pluronic F-68, and Pluronic F-127. Particle size and size distribution were determined to characterize the particles. The Zeta potential of surface charge of

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particles was also performed for predicting their stability.

MATERIALS AND METHODS

Plant samples

The mature roots of PM were collected on October 15, 2019, from Tambon Thapa, (18.173540, 99.378623), Muang Lampang District, Lampang, Thailand. The roots were authenticated by herbal experts at Rajamangala University of Technology Lanna (RMUTL). Upon arrival, fresh PM roots were washed with tap water, followed by distilled water consequently. The skin of roots was peeled off by using the paring knife. The thin-sliced samples were dried in a hot air oven at 50 °C until a constant weight was obtained. The dried sample was ground by using an electric blender, and the achieved fine powder was collected by using 80-mesh sieves before wrapping in a vacuum plastic bag and keeping in a deep freezer at -20 °C.

Extraction

Extraction was performed according to the sequential extraction method (Yodthong et al., 2020) using six different solvents, including hexane, benzene, ethyl acetate, acetone, ethanol, and deionized distilled water. Each solvent extraction was performed in triplicate, as described by Yodthong et al. (2020). Ten grams of PM powder were immersed in 300 mL of solvent at ambient temperature and was agitated at 100 rpm assisted by an orbital shaker for 180 min, three times a day, and then filtered using filter paper. The remaining residue was subjected to re-extraction. The filtrates of each cycle were concentrated by using a rotary evaporator, while deionized water filtrate was dried by using a freeze dryer. The fractions were stored at -20 °C in a vacuum bag for further analysis.

DPPH assay

The 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) radical scavenging activity was examined using the modified method described by Sassa-deepaenget al. (2019). DPPH solution (oxidized form) was prepared in absolute ethanol to get the final absorbance of 1.0 ± 0.2 at 517 nm. One hundred µL of various concentrations of samples were mixed with 900 µL of DPPH radical solution and then incubated at the ambient temperature for 30 min in the dark. The solution was measured against the blank at 517 nm using V-1200 spectrophotometer with UV-Professional analysis software. The percentage of inhibition of antioxidant was calculated using the equation:

$$\% \text{ Inhibition} = [(OD_{max} - OD_{sample})/OD_{max}] \times 100$$

The linear curves were generated by plotting the percentage of inhibition against the concentration in µg/mL ($R^2 = 0.99$). After getting the trend line with the regression equation in Microsoft Excel 2019 software, put y=50, and calculated the x value, the IC_{50} of DPPH.

Total phenolic content (TPC)

The content of phenolic compounds was determined by using the Folin-Ciocalteu (FC) colorimetric method using a modified method described by Yodthong et al. (2020). Twenty µL of various concentrations of the extract was mixed with 100 µL of FC reagent in 1,980 µL of DI water; consequently, incubated for 5 min at the ambient temperature. After adding 300 µL of 7% of Na_2CO_3 and incubated for 60 min at the ambient temperature in the dark. The absorbance of the solution was measured at 765 nm using V-1200 spectrophotometer with UV-Professional analysis software. The TPC quantitation was performed in triplicate. The gallic acid at different concentrations was used to generate the standard calibration curve ($R^2 = 0.99$). The TPC was expressed as microgram gallic acid equivalent (GAE) per milligram dry weight (Sassa-deepaeng et al., 2019).

Flavonoid content

The flavonoid content (FC) was examined using the aluminum trichloride ($AlCl_3$) colorimetric method with some modification by Sassa-deepaeng et al. (2019). Briefly, 100 µL of various concentrations of the extract was added to 300 µL of DI water followed 100µL of 5 % $NaNO_2$ and incubated for 5 min at the ambient temperature. Afterward, 100 µL of 10% $AlCl_3$ was added and then incubated for 6 min at the ambient temperature. Finally, 400 µL of 1M NaOH was mixed and then incubated for 30 min in the dark at the absorbance temperature. The absorbance was measured at the wavelength of 415 nm using V-1200 spectrophotometer with UV- Professional analysis software. The flavonoid content was carried out in triplicate and expressed as microgram quercetin equivalent (QE) per milligram dry weight.

Estrogen sensitive cell lines testing

The MCF-7 human breast cancer cells from the American Type Culture Collection were cultured in a 75 mL sterile culture flask containing DMEM supplemented with 10 % FBS and 10% of antibiotic-antimycotic in a humidified atmosphere of 5 % CO_2

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at 37°C until cells reach 90% of confluent. The proliferative activity assay was conducted by using a modified method described by Sassa-deepaeng et al. (2017). Cells were harvested with trypsin prior to seeding (5×10^5 cells/well) in 96-well plates. Various concentrations (12.5, 25.0, 50.0, and 100.0 μg/mL) of the fractionated PM were added and then incubated at the same temperature for 20 h, while DMSO was also used as the control. Afterward, the medium was removed by using multichannel pipettes for 12-well plates. The 100μL of (5 mg/mL) 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) reagent was added to each well and further incubated at the same temperature for 4 h. Finally, the formazan product in cells was dissolved by adding dimethyl sulfoxide (DMSO), resulting in a purple solution. The absorbance was measured at the wavelength of 570 nm with a microplate reader, and absorbance at 630 nm was used to correct for nonspecific background values. The percentage of proliferative activity was calculated based on comparative growth with control in quadruplicate.

Polymeric micelle preparation

The HPβCD, Pluronic F-68, and Pluronic F-127 were employed for polymeric micelle formation according to the method of Sassa-deepaeng et al. (2016).The selected fraction was prepared by dissolving it in ethanol. The fraction solution was added dropwise with stirring a solution of HPβCD, Pluronic F-68, or Pluronic F-127 solution to obtain the mixture of fraction-polymer at the weight ratios of 1:1,1:2, 1:3, 1:4, and 1:5. Afterward, 100 µL of Tween80 was added prior to adding deionized water to the volume. The mixture was frozen at -20°C prior to lyophilization under vacuum for 24 h. Finally, the obtained dry fraction-loaded HPβCD, Pluronic F-68, and Pluronic F-127 micelles were re-suspended in deionized water to the desired drug concentration for further studies.

Particle size, Size distribution, and Zeta potential

Particle size and size distribution (PDI) of polymeric micelles were determined using Zeta-sizer Nano Zs working on the principle of photon correlation spectroscopy (PCS) as described by Sassa-deepaeng et al. (2019). One milliliter of each suspended product in water was transferred into a quartz cuvette prior to exposure to laser light diffraction. The intensity of the peak with the highest population was recorded. The Zeta potential was also measured using the same instrument in a folded capillary zeta potential cell. The average data were automatically calculated by the instrument software.

Statistical analysis

The data were analyzed using a tool pack of the Microsoft Excel 2016 software. One-way analysis of variance, and the least significant difference (LSD) were used to examine significant differences, and the differences at $P< 0.05$ were considered significant.

RESULTS AND DISCUSSION

Sequential extraction procedures and different polarity solvents are the critical steps responsible for dissolving the endogenous active compounds from PM root. To achieve the highest yield of active compound, the solvents including hexane, benzene, ethyl acetate, acetone, ethanol, and DI water with the polarity of 0.0, 0.1, 0.2, 0.4, 0.6, and 1.0 were used. Based on the solubility in the different polarity of the solvent, active compounds in samples could be extracted in different fraction by using appropriate solvents. The yield obtained from the extraction shows in Table 1.

The maximum yield was obtained in DI water fraction followed by ethanol, hexane, acetone, ethylacetate, and benzene fractions, respectively. It can be suggested that the major phytochemicals in PM root have mostly high polarity, as indicated by Peerakam et al. (2018). Interestingly, the color of hexane extract was creamy white with an oily odor, while the others were yellowish without an oily smell. The possible description for this inspection was the presence of lipid or lipophilic compounds in hexane fractions, such as lipids or sterols, as indicated by Jeon et al. (2005). However, the results cannot be indicated in which fractions possess the active ingredients which exhibited antioxidant property. Therefore, the DPPH assay was firstly conducted, and the result is indicated in Figure 1.

Table 1. Extraction yields of using the different polarity solvent extraction (%)

Solvent	Hexane	Benzene	Ethyl acetate	Acetone	Ethanol	DI water
Yield (%)	2.03±0.46	1.37±0.31	1.44±0.23	1.47±0.21	3.63±0.5	11.87±1.5

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The free radical scavenging activity was determined in terms of IC_{50} value (μg/mL). The smaller IC_{50} value means a higher antioxidant activity. It was found that there was a significant variation in the IC_{50} of the different extracts (2.5±0.91 to 2125.95±18.76). The ethyl acetate fraction showed the highest DPPH radical scavenging activity comparing to other fractions, while the water faction exhibited the lowest IC_{50}. The value of scavenging activity increased from acetone, benzene, and ethanol to hexane fraction, respectively. It was indicated that mainly antioxidant compounds were deposited in moderate polar solvent extracts contributed to reducing power, as documented by Peerakam et al. (2018). To investigate the source of scavenging activity, the TPC and flavonoid contents were measured, and the result is shown in Figure 2.

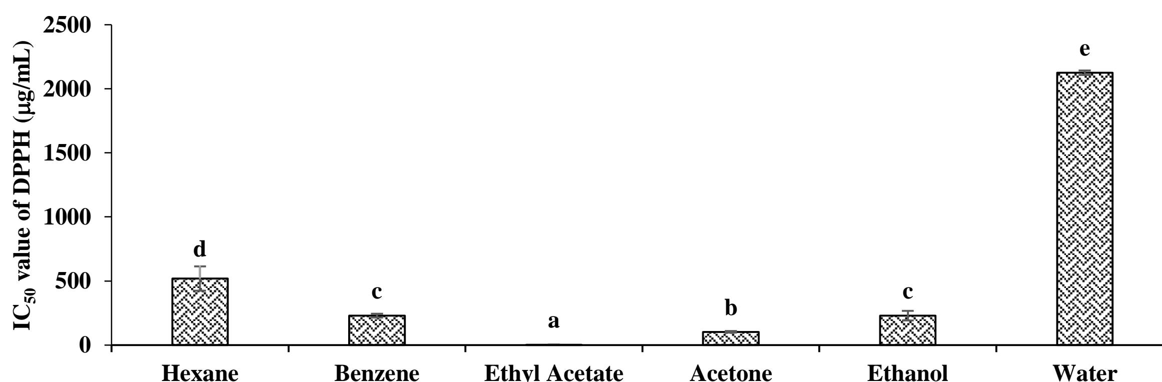

Figure 1. Antioxidant activity of different polarity extracts measured by the DPPH assay.

Different letters indicate statistically significant values (P< 0.05).

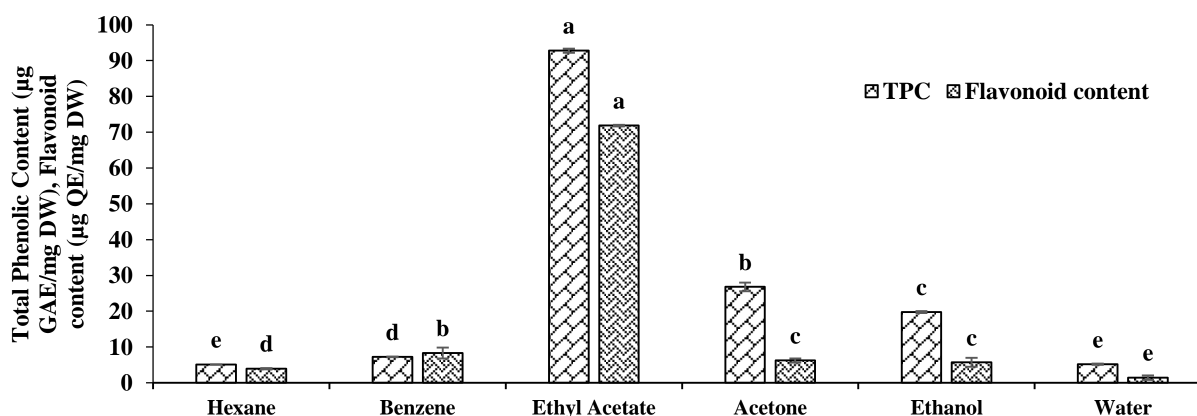

Figure 2. Total phenolic content and flavonoid content of different polarity fractions.

Different lower case letters within the bar indicate significant differences (P< 0.05) analyzed by using the ANOVA calculation.

It was found that TPC values in different polarity fractions ranged from 5.11±0.05 to 92.78±0.58 μg GAE / mg dried weight (DW). The highest TPC value was obtained in ethyl acetate fraction (EAF), and the lowest TPC value was found in DI water fraction. Interestingly, the result was positively related to flavonoid content. The highest amount of flavonoid content was also found in EAF (71.86±0.21μg QE/mg DW), while the water fraction exhibited the lowest flavonoid content. The result confirmed that phenolic compounds, as well as flavonoids, were the most responsible for the antioxidant property, as documented by Kaurinovic and Vastag (2019). However, some researchers recommended that the appropriate ratio of ethanol was also effective and economically used for single-step extraction (Peerakam et al., 2018). To confirm the bioactivity correlations of flavonoids and total phenolic contents with their estrogenic activity, the proliferative activities of fractions on the estrogen-sensitive cell line, MCF-7,were investigated by MTT method. The percentage of cell growth curve was generated and presented in Figure 3.

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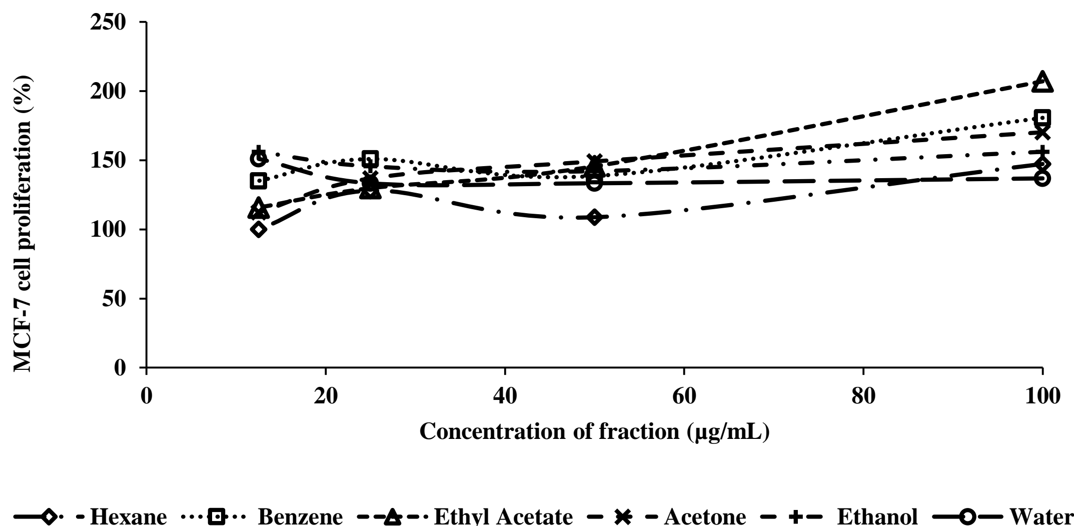

Figure 3. Proliferation curves of MCF-7 cell lines

The MCF-7 proliferation assay is an accepted method for evaluation of estrogenic effect challenged by phytochemicals or extract based on estrogen receptor alpha (ERα) mediates proliferation (Wanda et al., 2006; Innocenti et al., 2007). The mean EAF at 100 µg/mL exhibited a significantly higher proliferation against MCF-7. The result was similar to the report of Cherdshewasart et al. (2008), who summarized that a high dose of *P. mirifica* extract revealed proliferation in MCF-7. Therefore, the EAF was selected for nanoparticle nucleation through polymeric micelles formulation.

The solubility of complex prepared in water shows in Figure 4.

In the process of polymeric micelle development, Tween80 was added for incorporation into the micelles to solubilized substances. Moreover, it was used in conjunction with nanoparticles to improve specific delivery and was adsorbed on the surface of specific receptors of the blood-brain barrier (Sassa-deepaeng et al., 2016). It was found that the incorporation of Pluronic F127 and Tween80 into EAF resulted in transparent aqueous dispersions were obtained. After the freeze-drying process, the products could be solubilized in water absolutely and still being transparent, as shown in Figure 4(c). The result was in agreement with the previous results that the practically insoluble flavonoid chrysin could be solubilized by polymeric micelles, and transparent aqueous mixtures were obtained (Sassa-deepaeng et al., 2016). To confirm that EAF-F127 was in the nanosize range, size, size distribution (PDI), and zeta potential were determined using Zetasizer Nano Zs. The result is shown in Figure 5.

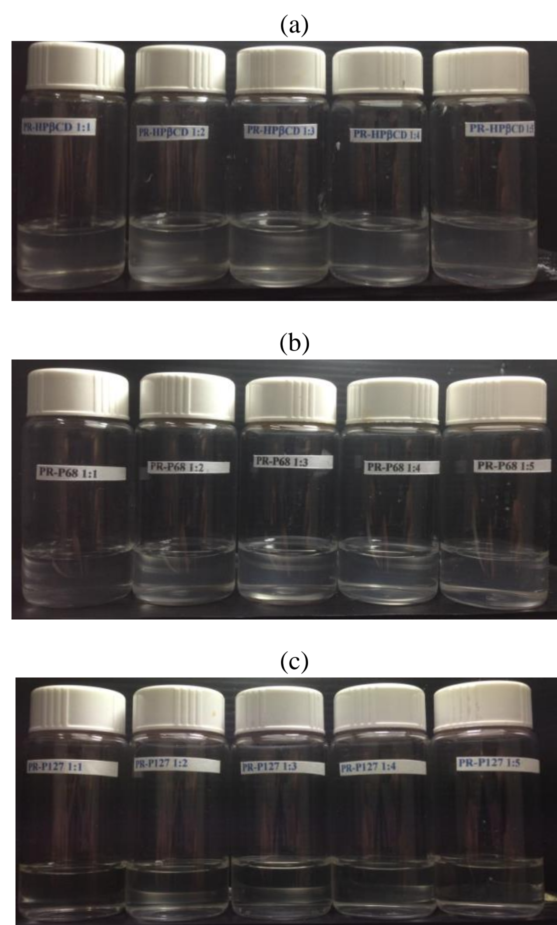

Figure 4. Solubility of (a) EAF-HPβCD, (b) EAF-F68, and (c) EAF-F127 in DI water.

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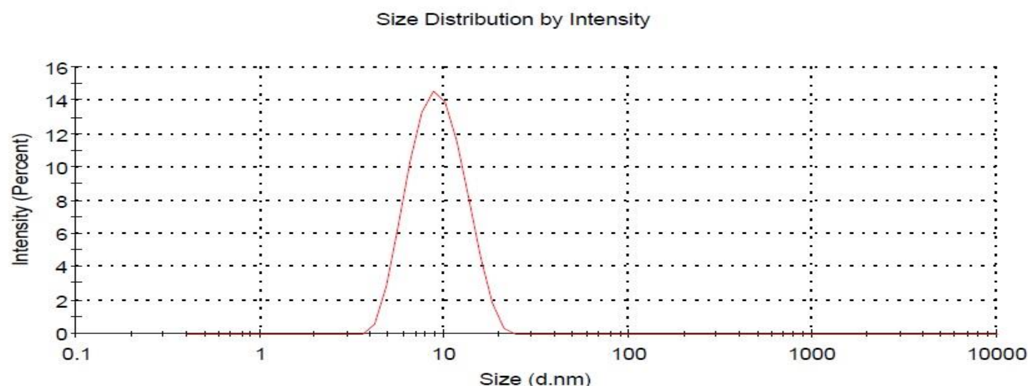

Figure 5. The size distribution by the intensity of EAF-F127.

Dynamic Light Scattering (DLS) analysis was occupied to determine the sized distribution and the polydispersity of colloidal particles of EAF-F127. The major size distribution peak was in the nanosize range of 9.564±3.17 nm with an excellent polydispersity index (PDI) of ~0.2. The dimeter of EAF-F127 was in the size range of polymeric micelles of other phytochemicals (Sassa-deepaeng et al., 2016). To predict the stability of EAF-F127, the zeta potential measurement was performed and revealed in Figure 6.

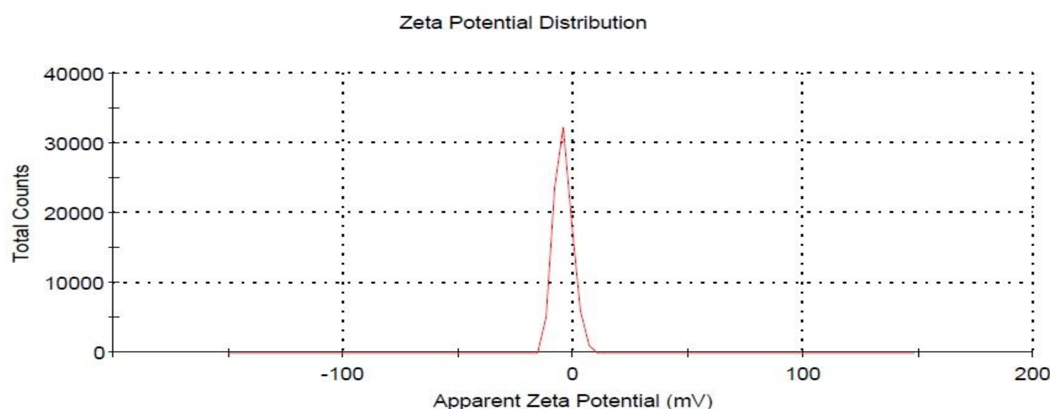

Figure 6. Zeta potential graph of EAF-F127.

The zeta (ζ) potential value is resulted by the net electrical charge contained within the region bounded by the clipping plane of nanoparticles elicited the appearance of a maximum peak at -4.32±3.394 mV. In general, the ζ potential is a parameter commonly used to diagnose cellular interaction with surface charged nanoparticles (Mikolajczyk et al., 2015). Particles that possess a large positive or negative ζ potential are repulsed each other resulted are relatively stable (Sassa-deepaeng et al., 2019). Unfortunately, the ζ potential value of EAF-F127 elicited the appearance of a major peak nearly zero, indicating that the developed EAF-F127 might be less stable and the possibility of aggregation formation. However, the strong interaction between the hydrophilic moiety of Tween80 and the water molecules would support the self-assembly of micelles resulted in the re-solubilization of particles.

CONCLUSIONS

The results demonstrated that the extraction method concerning the solvent selected affects the activity of the obtained PM root. It was observed that ethyl acetate extraction could provide high phenolic content and flavonoid content, which are positively related to proliferative activity against estrogen-sensitive cell line MCF-7. To overcome the less soluble fraction in water, Pluronic F-127 and Tween 80 were employed in the proper ratio. Finally, the obtained EAF-F127 was in the nanosize range with low PDI. Although the ζ potential value was

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nearly zero, the particles were still stable due to the existence of Tween 80 in the system and resulted in the re-assembly of micelles in water to prevent aggregation of nanoparticles. The results indicated enhanced water solubility of EAF- F127 product was promising for further use *in vivo* studies.

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