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Development of Nanoliposome of *Labisia pumila* Standardized Extract

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ABSTRACT

Background: *Labisia pumila* has been used traditionally for the treatment of several ailments such as gonorrhoea, dysmenorrhoea and as tonics for females.

Aims: To investigate the standardization procedure of *Labisia pumila* extract (LPE) and evaluation of its nanoliposomes.

Methods: *Labisia pumila* extract was standardized by high performance liquid chromatography (HPLC), and gallic acid, caffeic acid, rutin and 2,4-Di-*tert*-butylphenol were quantified in the extract. Then, the standardized extract was prepared as nanoliposome using soybean phospholipid by the film method and after that was characterized by zetasizer, zeta potential, UV-Vis spectrophotometer and FTIR techniques.

Results: For the standardization, the mean percentage recovery values of the concentration studied were 98.49±1.43, 97.01±2.04, 97.70±1.55 and 99.43±3.04 % for gallic acid, caffeic acid, rutin and 2,4-Di-*tert*-butylphenol, respectively. The accuracy values were between 95.06 and 104.86% for the marker compounds, while the corresponding precision values were between 0.09 and 5.18% for within-day and between-day analysis, respectively. The average particle size for LLP was 174.20±4.58 nm with zeta potential of particles surface charge from −43.40 to − 44.40 mV. The polydispersity index was 0.19±0.02 and the morphology and presence of liposomes were further confirmed by transmission electron microscopy which revealed the presence of spherical liposomes of < 200 nm.

Conclusion: HPLC method for the simultaneous determination of selected marker compounds has been developed; the method was reliable, repeatable and reproducible. The method was successfully applied in standardization of LPE. LPE was successfully prepared as nanoliposome using soybean phospholipid.

Keywords: *Labisia pumila*, standardization, nano liposome, gallic acid, caffeic acid, rutin, high performance liquid chromatography, phospholipid, soybean, zetasizer, particle size, zeta potential

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1. Introduction

Labisia pumila Benth, (Myrsinaceae) is known as Kacip Fatimah in Malaysia. It has been used traditionally for the treatment of gonorrhoea, dysmenorrhoea and as tonics for females [1]. Recent research reveal that *L. pumila* has antioxidant, antiaging,antiinflammatory,phytoestrogenic and anticancer effects [2,3,4]. Standardization of plant extracts refers to assuring a consistent, measurable concentration of an identified active constituent throughout the shelf life of the product. Furthermore, the natural inconsistencies in herbal products due to of seasonal, geographical, postharvest handling, and horticultural factors results in significant variations in the quality and potency of the final herbal products [5]. Herbs such as Milk Thistle and St. John's Wort are commonly standardized to guaranteed marker compound concentrations to supply the consumer a therapeutically safe and effective dose. A single or more concentric lipid bilayers separated by water compartments generate artificial vesicles called liposomes [6]. High potential exists for liposomes to transport active compounds in effective delivery systems [7]. Liposome formulations have various features such as improving efficacy, solubility, improved bioavailability decreased toxicity, and enhanced product stability. This will consequently lead to increased patient compliance to therapy. Phospholipids from soybean lecithin are mostly used in liposome formulations due to their availability, safety and low cost [6]. For instance, numerous pharmaceuticals (such as, anti-inflammatory drugs, antifungals, antibiotics, etc.) and plant extracts (such as *Dracocephalum moldavica* and *Callendula officinalis*) have been encapsulated in liposomal systems to date [8]. Therefore, this research was aimed to standardize of *Labisia pumila* extract (LPE), as well as preparation and characterization of nanoliposome of *Labisia pumila* extract (LLP).

2. Material and Methods

2.1. Chemical and reagents

Gallic acid, caffeic acid, rutin, 2,4-Di-*tert*-butylphenol (HPLC grade) and uranyl acetate were purchased from Sigma Aldrich, Switzerland. Soybean lecithin was procured from Hong Aunkimia Sdn Bhd, Malaysia. Acetone, chloroform and ethanol were obtained from Quality Reagent Chemical, Malaysia.

2.2. Preparation of LPE

Labisia pumila var. *alata* was obtained from Herbagus Sdn Bhd, Penang, Malaysia. Herbarium specimen has been deposited and voucher specimen number was assigned (No. 11607) at the Herbarium Department, Universiti Sains Malaysia, and authenticated by Dr. Rahmad Zakaria of the School of Biological Sciences, University Sains Malaysia, Penang, Malaysia. *Labisia pumila* var. *alata* dried whole plant including roots (500 g) was extracted with 5 L of ethanol (50%) for 5 days using Soxhlet apparatus. The crude extract was filtered, concentrated under reduced pressure using a rotary evaporator, and further dried in oven (Memmert, Germany) at 45^{o}C for 12 hours. The dried extract was then kept in the fridge at -20^{o}C prior to experiment.

2.3. Standardization of crude extract by HPLC method

2.3.1. Development and validation of LPE by HPLC method

The stock solutions (100 µg/mL) of the marker compounds gallic acid, caffeic acid, rutin and 2,4-Di-*tert*-butylphenol were prepared in methanol. A series of working standard solutions were prepared by diluting the stock solution of gallicacid, caffeic acid, rutin, and 2,4-Di-*tert*-butylphenol (0.17-51, 0.35-44.80, 1.56-25 and 0.60-21.60 µg/mL), respectively with the mobile phase (aqueous acetic acid : acetonitrile).

2.3.2. Chromatographic conditions

The chromatographic separation was carried out using HPLC system (1100 series, Agilent Technologies, USA, on a reversed phase-C18 Eclipse column (250 × 4.6 mm, 5 µ particle size, Agilent, USA). The samples (10 µL) were eluted at a flow rate of 1 ml/min, at λ_{max} 270 nm, with a gradient mobile phase comprising of 0.2% aqueous acetic acid (A), acetonitrile (B), initial run conditions were 80% A: 20% B for 5 min, after that between 5.1 to 20 min there was linear increase of solvent B up to 90%. Between 20.1 to 30 min there was a linear decrease to the starting run conditions (80% A: 20% B).

2.3.3. Linearity

Linearity for gallic acid, caffeic acid, rutin, and 2,4-Di-*tert*-butylphenol was observed at (0.17-51,0.35-44.80, 1.56-25 and 0.60-21.60 µg/mL), respectively. The linearity for each marker compound was calculated by plotting peak area (mAU*S) versus concentration (µg/mL). The limit of detection (LOD) values of the marker compounds were determined by analysing successively in 2 fold dilutions with the mobile phase of the standard solutions. The concentration with the smallest detectable peak, at a noise to signal ratio of 3 was taken as the limit of detection (LOD) value, whereas the lower linearity range limit at a noise to signal ratio of 10 was taken as the limit of quantification (LOQ)[9,10].

2.3.4. Accuracy and precision

Each working standard solution was injected (n=5) to determine precision of the method and values were expressed as coefficient of variation (% CV). Within-day accuracy and precision analysis were carried out by injecting the working standard concentrations (n=5/day) while the between-day accuracy and precision were evaluated by injecting the standard working concentrations one time/day for 5 consecutive days [9,10].

2.3.5. Recovery

Three working standard concentrations of each gallic acid, caffeic acid, rutin and 2,4-Di-*tert*-butylphenol were used to determine recovery (%). Although, 100% analyte recovery is ideal, reproducibility in recovery is of great importance in an analytical validation methodology [15]. The % recovery of each analyte was calculated according to following equation:

Recovery (%) = (Amount detected – Amount original) * 100/ Amount spiked.....[10].

2.3.6. Quantification of the selected marker compounds in LPE

Gallic acid, caffeic acid, rutin, and 2,4-Di-*tert*-butylphenol were quantified in LPE. LPE (1 mg/mL) was prepared in deionised H_2O for HPLC analysis. All samples were filtered by 0.45 µm PTFE syringe filter. The selected marker compounds in LPE were quantified in triplicates using linear regression equations of the calibration curves [11].

2.4. Preparations of liposome of *L. pumila* extract (LLP)

Liposome of *L. pumila* extract (LLP) was prepared following the method of Aisha *et al* [6]. Crude soybean phospholipids were prepared from soybean lecithin (food grade). The soybean lecithin was dissolved in ethanol (96%) at (0.6:3 W/V) and then refluxed for 30 min at 60°C and left at room temperature to cool. The supernatant was collected and concentrated at 60°C by rotary evaporator. The residue was then washed with acetone (5 L) to produce 63.24 g of soybean phospholipids. LLP was prepared by the film method, LPE was dissolved in ethanol and soybean phospholipids was dissolved in chloroform, the solutions were mixed, and the solvent was evaporated under vacuum using rotary evaporator at 45°C and further dried in oven at 60°C for 1 h.

2.5. Characterization of LLP

2.5.1. Determination of aqueous solubility

Solubility was determined by HPLC method; LPE, and LLP were separately dispersed in deionized water to achieve a concentration of 5 mg/mL. The mixtures were vortexed for 2 min by vortex mixer, Laboratory Medical Supplies, Tokyo, Japan, and then sonicated for 10 min by sonicator bath model UC-10 Lab companion, Korea. After that, they were centrifuged at 10,000 rpm and 25°C for 8 min using CT15RT Benchtop High Speed Refrigerated Centrifuge, (Techcomp limited, China). All samples were analysed in triplicates. Concentration of the 4 marker compounds in LPE and LLP was determined by HPLC as described previously.

2.5.2. Fourier transform infrared spectroscopy (FTIR) and UV-Vis spectrophotometry

The FTIR spectra for LPE, soybean phospholipids and LLP were recorded in the range of 4000 -500 cm^{-1} (n = 6) using fourier transform infrared spectroscopy, Perkin Elmer, USA. For UV-Vis spectrophotometer, LPE, soybean phospholipids and LLP were dissolved in deionized water at concentration of 100 µg/mL, then were sonicated for 5 min and then filtered by 0.45 µm filter and analysed by UV-Vis spectrophotometer, Perkin Elmer, USA, in the range of 500-200 nm against deionized water as blank.

2.5.3. Measurement of particle size, zeta potential and transmission electron microscopy

Photon Correlation Spectroscopy (PCS) using a Zetasizer nano (ZS) (Malvern Instruments Ltd, UK) were used to determined particle size (PS), polydispersity index (PDI) and zeta potential (ZP). The samples were dissolved in ultra-pure water (18 MΩ) at 1 mg/mL and then filtered through 0.45 µm filter. Measurements were carried out in triplicates. To confirm the presence of the liposomal structures, transmission electron microscopy (TEM) was carried out. The samples were studied using CM12 TEM (Philips, Netherlands), 100 µL of LLP (1 mg/mL in water) was deposited on a 400 mesh copper grid coated with 5 nm layer of carbon, air-dried at room temperature for 3 min, and stained with 2% uranyl acetate for 1 min.

3. Results

Standardization of LPE

The calibration curves of marker compounds were linear, all marker compounds showed good linear regressions with high correlation coefficient values ($R^2 \geq 0.999$) between peak area and concentrations of each compound. The limit of detection (LOD) for gallic acid, caffeic acid, rutin and 2,4-Di-*tert*-butylphenol were 0.06, 0.11, 0.52, and 0.20µg/mL, respectively, while the limit of quantification (LOQ) values were 0.17, 0.35, 1.56, and 0.60 µg/mL for gallic acid, caffeic acid, rutin and 2,4-Di-*tert*-butylphenol, respectively. Figure 1 shows HPLC chromatogram of mixed standard compounds, while the HPLC chromatogram of LPE showing the marker compounds are presented in Figure 2.

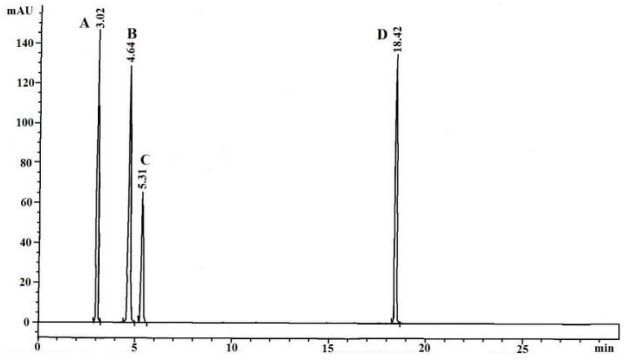

Figure 1: HPLC chromatogram of mixed standard compounds, (A) gallic acid, (B) caffeic acid, (C) rutin and (D) 2,4-Di-*tert*-butylphenol.

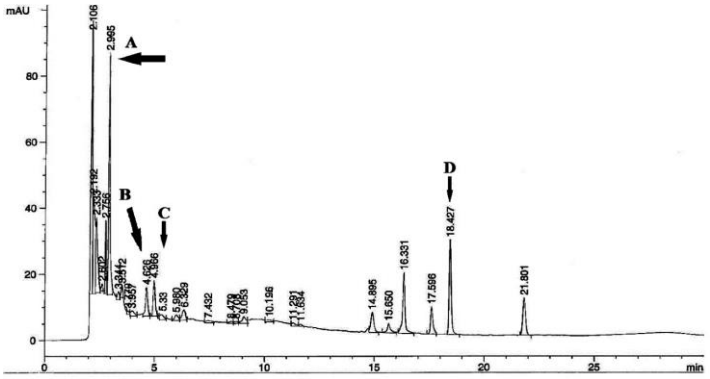

Figure 2: HPLC chromatogram of LPE showing the marker compounds(A) gallic acid, (B) caffeic acid, (C) rutin and (D) 2,4-Di-*tert*-butylphenol.

The % recovery of the marker compounds in LPE is presented in Table 1. The % recovery values of the concentrations studied ranged from 96.93-99.74, 95.19-99.21, 96.15-99.25, 96.61-102.65% for gallic acid, caffeic acid, rutinand 2,4-Di-*tert*-butylphenol, respectively. The % recovery data for the marker compounds was satisfactory thus concluding that the extraction method used did not produce any substantial loss of these compounds. Validation parameters for marker compounds in LPE are shown in Table 1. The accuracy values, expressed as a percentage of true value were between 95.06% and 104.86% for all the marker compounds, while the corresponding precision values expressed as coefficient of variation (% CV) were between 0.09% and 5.18% for within-day and between-day analysis, respectively, this indicates that the method is reliable, repeatable and reproducible.

Table 1: Validation parameters for marker compounds in LPE.

Compounds	Concentration (µg/mL)	Within-day		Between-day		Recovery	
		Accuracy (%)	Precision (CV %)	Accuracy (%)	Precision (CV %)	Mean (%)	Mean (CV %)
Gallic acid	51.00	99.99	0.56	100.40	1.96	98.79	1.24
	12.75	104.86	0.55	99.64	2.57	-	-
	2.55	97.65	0.47	104.51	3.06	99.74	4.65
	0.51	95.20	0.43	100.64	4.12	-	-
	0.17	96.99	0.63	103.15	4.75	96.93	5.96
Caffeic acid	44.80	101.03	2.58	102.37	2.69	96.64	2.31
	11.20	100.81	1.67	103.76	1.48	-	-
	5.60	96.57	2.14	100.83	3.66	99.21	1.20
	1.40	97.10	2.92	97.29	4.71	-	-
	0.35	95.06	3.73	98.05	5.18	95.19	4.78
Rutin	25	99.04	2.53	100.92	3.30	99.25	3.35
	12.5	103.31	0.86	100.21	4.82	-	-
	6.25	95.27	1.49	95.68	3.88	97.70	4.61
	3.12	96.89	0.40	103.49	0.52	-	-
	1.56	99.46	1.42	101.21	3.14	96.15	5.20
2,4-Di-*tert*-butylphenol	21.60	102.15	1.91	98.08	1.62	102.65	1.48
	10.80	97.65	1.34	103.10	0.09	-	-
	7.20	98.37	2.23	97.67	2.70	99.04	2.05
	3.60	100.81	0.48	99.57	1.68	-	-
	0.60	95.61	3.64	95.64	3.36	96.61	3.08

Characterization of LLP

Analysis of the marker compounds by HPLC showed improvement in their concentrations as compared to that obtained in non-formulated extract (Figure 3). The improvement in concentrations for gallic acid and 2,4-Di-*tert*-butylphenol was significant, P = 0.000, P= 0.05, respectively, in LLP compared to that of LPE. The improvement in the concentration of marker compounds could be because of improvement in their solubility.

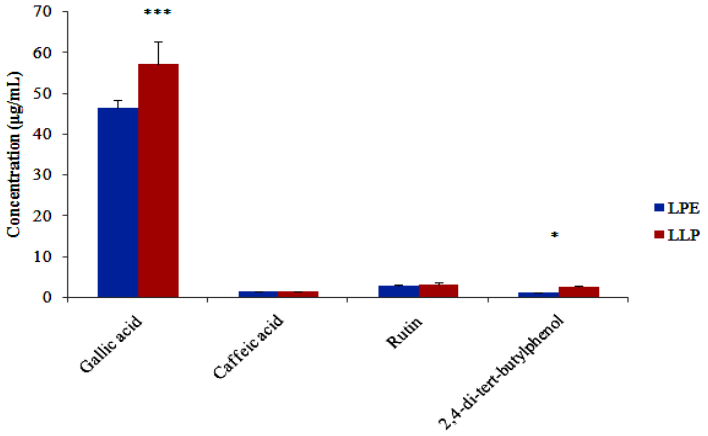

Figure 3: Analysis of the marker compounds in LPE and LLP by HPLC showing improvement in their solubility (*** = P value = 0.000, (** = P value = 0.001, (* = P value = 0.05, Student's t-test).

FTIR spectra of soybean phospholipid, LPE, and LLP were studied in order to get insights into occurrence of interaction between LPE and phospholipids (Figure 4). In soybean phospholipid the broad band centered at 3358 cm^{-1} represents the OH stretching, the principal

bands at 2853 and 2924 cm^{-1} correspond to the symmetric and anti-symmetric stretching in the CH_2 groups of alkyl chains, the strong band centered at 1738 cm^{-1} corresponds to the stretching vibrations of the ester carbonyl groups, the band centered at 1649 cm^{-1} is assigned to C=O stretching, and the scissoring vibrations of the CH_2 groups are represented by the band at 1465 cm^{-1}. The characteristic phosphate group vibrational band assigned to the phosphorus oxide (PO_2) anti-symmetric stretching mode is centered at 1221 cm^{-1} and the PO_2 symmetric stretching mode PO_2 at 1062 cm^{-1}. In *L. pumila* extract, the broad band centered at 3354 cm^{-1} corresponds to OH stretching, vibration at 2930 cm^{-1} correspond to C-H stretching, the bands centered at 1589 cm^{-1} correspond to symmetric and asymmetric of C=C, the vibration at 1393 cm^{-1} correspond to N=O bending, and the vibration at 1044 cm^{-1} corresponds to C=O stretch. In liposome of *L. pumila*, remarkable changes can be seen in the infrared absorption spectra due to incorporation of *L. pumila* extract in soybean phospholipid; the broad band corresponding to OH group is shifted from 3354 cm^{-1} to 3263 cm^{-1}, the vibration at 2930 cm^{-1} shifted to 3009 cm^{-1}, the vibrations at 1589 cm^{-1}, and 1393 cm^{-1} disappeared.

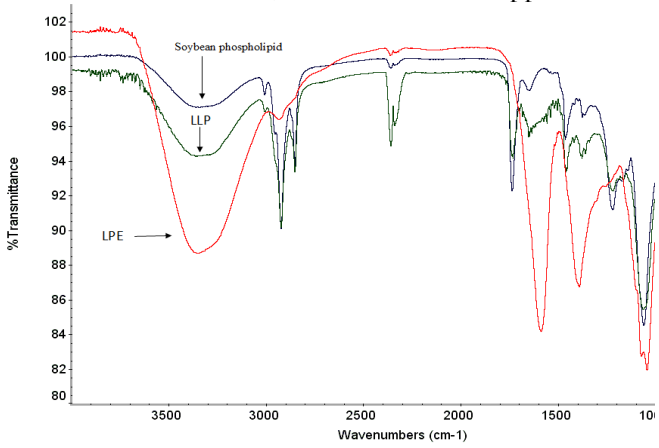

Figure 4: FTIR spectra for LPE, soybean phospholipid and LLP.

UV-Vis spectra showed that in LPE there is a clear peak appeared at 270.96 nm, while in the liposome (LLP) the peak did not appear at 270.96 nm which indicated a change occurred in LPE suggested that the soybean phospholipid encapsulated the extract particles (Figure 5).

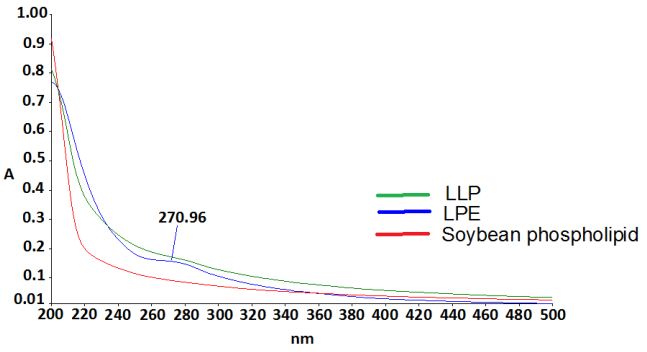

Figure 5: UV spectra for LPE, soybean phospholipid and LLP.

The average particle size for LLP was 174.20±4.58 nm with zeta potential of particles surface charge from −43.40 to −44.40 mV (Figure 6). The polydispersity index was 0.19±0.02. The morphology and presence of liposomes were further assured by TEM which confirms the presence of spherical liposomes of <200 nm. Furthermore, the lipid bilayer of the LLP was revealed obviously at high magnification (Figure 7).

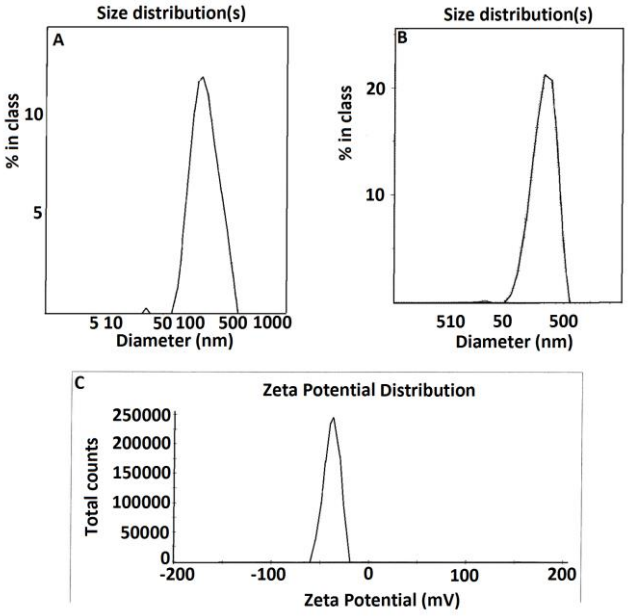

Figure 6: Particle size, zeta potential of LLP sample (A) volume size distribution of LLP particles, (B) intensity size distribution of LLP particles, and (C) zeta potential for LLP particles.

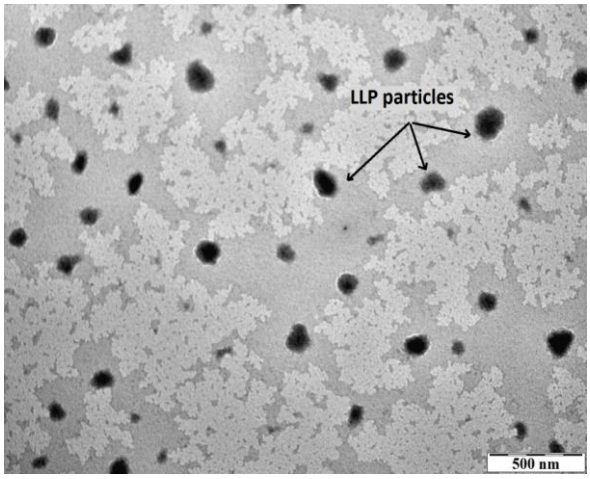

Figure 7: TEM image of LLP.

4. DISCUSSION

High performance liquid chromatography is an accurate and specific method for standardization and quantification of the components of synthetic and natural drugs [12]. Standardization of *L. pumila* extract was successfully carried out using HPLC and the selected marker compounds were quantified, gallic acid concentration was high in the extract compared to the concentrations of caffeic acid, rutin and 2,4-Di-*tert*-butylphenol which explain the claimed effects of the *L. pumila* extract. Gallic acid, caffeic acid, rutin and 2,4-Di-*tert*-butylphenol showed several pharmacological activities such as antioxidant, antiangiogenesis and anticancer effects [13-25]. Liposome contains synthetic phospholipid vesicles, is biocompatible, biologically safe, and protect formulations from external influential factors [20]. Liposomes or nano liposomes are among the earliest target transport systems that have demonstrated excellent results for clinical trials. Currently employed and successful similar technologies abound in the medical field. Nanoliposomes technology can be used for plant extracts and pure compounds [8]. Many plant extracts were prepared as nanoliposomes and showed improved efficacy [26]. The nanoliposome of *L. pumila* extract is successfully prepared and characterized in terms of particle size, zeta potential, polydispersity index, TEM, UV and FTIR analysis. The aqueous solubility of LLP showed enhanced solubility of the marker compounds as compared to LPE which could improve the bioavailability and efficacy of *L. pumila* extract. According to the optical microscopy pictures (Figure 7), the liposomal formulations were produced successfully and exhibited a consistent spherical morphology, and this in agreement with [8]. The liposome has various particle sizes ranging from nanometers to micrometers and seems to be spherical in shape. To preserve stability, regulate release, and increase bioavailability of target compounds, nanoliposome technology is more sophisticated and effective. Most nutrients' solubility and bioavailability can be improved with this technology. Additionally, it is possible to achieve the controlled release at the designated spot. Therefore, liposome technology is used to encapsulate a variety of bioactive compounds and is essential for applications in pharmaceutical, nutraceutical, and functional food products [7].

5. CONCLUSIONS

This study provides valuable data on standardization and formulation of *L. pumila* extract. An HPLC method for the simultaneous determination of the marker compounds has been developed, the method was reliable, repeatable, and reproducible. The method was successfully applied in standardization of LPE. LPE was successfully prepared and characterized as nanoliposome using soybean phospholipid. Furthermore, this study may provide a basic formulation of *Labisia pumila* ethanolic extract for preparation of oral drug delivery system, and possibly topical delivery systems to the skin.

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CONFLICT OF INTEREST

The authors declare that no conflict of interest associated with this work.

CONTRIBUTION OF AUTHORS

Guarantors of integrity of entire study, Mohammed Ali Ahmed Saeed, Zhari Ismail and Abdul Hakeem Memon; study concepts/study design or data acquisition or data analysis/interpretation, all authors;

manuscript drafting or manuscript revision for important intellectual content, all authors; manuscript final version approval, all authors; literature research, Mohammed Ali Ahmed Saeed; experimental studies, Mohammed Ali Ahmed Saeed, Abdul Hakeem Memon, Mohd Shahrul Ridzuan Hamil and Armaghan Shafaei; statistical analysis, Mohammed Ali Ahmed Saeed; and manuscript editing, Zhari Ismail, Amirin Sadikun and Abdul Hakeem Memon.

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