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Development of *Orthosiphon stamineus* ethanolic solid dispersions for solubility improvements of lipophilic flavones

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Abstract. This study was conducted to develop an ethanolic solid dispersion of *Orthosiphon stamineus* (ESD) using polymers as carriers, namely polyvinylpyrrolidone (PVP), poloxamer 188 (P188), and poloxamer 407 (P407) *via* the solvent evaporation method. The purpose of preparing the formulation is to improve the solubility of the lipophilic flavones, namely sinensetin (SIN), eupatorine (EUP), and 3′-hydroxy-5, 6, 7, 4′-tetramethoxyflavone (TMF) and caffeic acid derivatives, namely rosmarinic acid (RA). The optimized ESD was characterized using High-Performance Liquid Chromatography (HPLC), Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) fingerprints, and physicochemical methods (particle size, zeta potential, TEM, and SEM). The effect of pH on stability and solubility in buffer and water, *in-vitro* release, and antioxidant properties (DPPH assay) indicated that the nano-formulation ESD using polymers (PVP/P407) with a ratio of extract to polymers (1.0:1.1:0.3 w/w) enhanced the lipophilic flavones (TMF=3.56 ± 0.01% w/w, SIN=2.46 ± 0.01% w/w and EUP=7.87 ± 0.01% w/w) and RA (20.66 ± 0.01% w/w) compared to the same compounds in ethanolic extract ($P< 0.0001$) with particles size less than 200 nm. In conclusion, the successful development of ESD using water-soluble copolymers (PVP/P407) has enhanced the solubility of lipophilic flavones and other compounds (RA), thereby further improving its pharmacological properties.

1. Introduction

Lipophilic flavones (SIN, TMF, and EUP) and rosmarinic acid (caffeic acid derivatives) are major compounds isolated from *Orthosiphon stamineus* or also known as 'Misai Kucing' (Malaysia), Java tea (Indonesia), and Cat's Whiskers (European countries). These compounds possess potential therapeutic properties, including diuretic and uricosuric effect [1], antioxidants [2, 3], anticancer [4], and anti-inflammatory properties [5]. Previous studies have reported that EUP has had an antiproliferative effect against selected cell lines (cancer cells). However, it showed no cytostatic impact on a cell line (human) [6, 7, 8].

There is a growing interest in the pharmacological properties of SIN, TMF, and EUP isolated from *O. stamineus* extract. However, the knowledge of the solubility, rate of dissolution, bioavailability, and stability of these compounds is still lacking. A previous study stated that SIN, TMF, and EUP were very low in oral bioavailability and had incomplete absorption, thus limiting the therapeutic

properties of *O. stamineus* [9]. Solid dispersion is the most promising strategy to overcome these problems because of the simplicity of preparation, ease of optimization, and reproducibility of the production process [10]. Solid dispersion also increased the lipophilic drug's solubility, reduced particle size, decreased agglomeration of drug particles, improved wettability and porosity, and changed its crystalline state amorphous, resulting in the faster dissolution of the *in vivo* sample [11].

This research focused on the development of *O. stamineus* ethanolic solid dispersion (ESD) using water-soluble polymers as carriers, namely polyvinylpyrrolidone (PVP), poloxamer 188 (P188), and poloxamer 407 (P407), to improve the solubility of lipophilic compounds through the solvent evaporation process. The optimized formulation was further analyzed for its solubility using HPLC analysis, particle size, zeta potential, TEM, SEM, stability under various pH conditions, FTIR profiles, *in-vitro* release, and the DPPH assay.

2. Materials and Methods

2.1. Preparation of ethanol solid dispersion (ESD) of O. stamineus

O. stamineus ethanol solid dispersions (ESDs) were prepared using the solvent evaporation method following the previous procedure [12] with slight modification. Different ratios of ethanol extract, PVP, P407, and P188 were dissolved at 40°C in ethanol (10 mL) (Table 1). A magnetic stirrer was used to homogenize the extract and sonicate it at 700 rpm (30 min). The ethanol mixture was added into 50 mL deionized water into the medium and mixed at 1000 rpm. The evaporator was used at 50°C to extract the ethanol solvent, then dried again in an oven (5 h) at 50°C before further use.

Table 1. Different ratio of *O. stamineus* ethanol solid dispersions (ESDs) using various polymers and ethanol extract.

No.	Formulations	Ratios (w/w)
F0	Extract	-
F1	Extract: PVP	1: 1
F2	Extract: P407	1: 1
F3	Extract: P188	1: 1
F4	Extract:PVP:P407	1:0.5:0.5
F5	Extract:PVP:P188	1:0.5:0.5
F6	Extract:PVP:P407:P188	1:0.3:0.3:0.3
F7	Extract:PVP/P407	1:1.1:0.3

2.2. Aqueous solubility of O. stamineus ethanol extract and ESDs using HPLC analysis

The aqueous solubility of the *O. stamineus* ethanol extract and ESDs were performed using previously validated HPLC analysis based on four compounds (TMF, SIN, EUP, and RA) [13]. In brief, a sample of ESD equal to 10,000 µg/mL of ethanol extract was mixed in deionized water. Each ESD was vortexed (5 min), sonicated (1 min), and centrifuged at 7000 rpm (5 min). Using the HPLC method (Agilent 1260 Infinity, USA), the supernatant was collected and analyzed.

2.3. Characterization of O. stamineus ethanolic solid dispersion (ESD)

2.3.1. ATR-FTIR spectroscopy analysis

FTIR analyzed ethanol extract, polymers (PVP and P407), and ESD in the range of 4000 to 600 cm^{-1}, with 16 scans per spectrum using attenuated total reflection (ATR) equipment (Nicolet iS10, Thermo Scientific, USA) and Omnic software (Thermo Scientific, USA) used to process spectral data [14]. Both analyses have been done in triplicates (n=3).

2.3.2. Measurement of particle size and zeta potential for optimized ESD
The photon correlation spectroscopy (PCS) and Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, Worcestershire, UK) were used to conduct particle size, polydispersity index (PdI), and zeta potential (ζ) following the previous method [15]. In brief, ESD (1000 µg/mL) was dissolved in ultrapure water (18 MΩ) and centrifuged (10 min) at 7000 rpm. The findings are summarized as (n=3) average ± standard deviation (SD).

2.3.3. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) analysis
To confirm the self-assembly of reconstituted solid dispersion into nanoparticles, TEM analysis was conducted. The study applied an earlier approach [15]. The optimized ESD (10,000 µg/mL) was prepared, and a drop of the ESD solution was placed at room temperature on a 400 mesh copper grid coated with an air-dried 5 nm layer of carbon. The prepared samples have been analyzed using Philips CM12 TEM at different magnifications (FEI, Eindhoven, North Brabant, Netherlands). To examine the surface morphology of the freeze-dried sample, SEM was carried out. The same formulation was vacuum freeze-dried (-50°C) and analyzed in the SEM field emission LEO Supra 50 VP (Carl Zeiss SMT, Oberkochen, Germany).

2.3.4. Influence of pH on stability and solubility of ESD in phosphate buffer and water
With some changes, the effect of pH on the stability and solubility of optimized ESD was carried out according to the previous process [15]. Briefly, 50 mg of ESD was reconstituted at pH 1.6, 5.5, and 7.4 in 5 mL of phosphate buffer saline (PBS) to imitate the human body's physiological state in the stomach, digestive system, and blood, respectively. The solutions were vortexed for 1 min (Velp Scientifica, Italy) and incubated for 24 h (37°C) in a water bath (Protech-Electronic, Malaysia). A UV/Vis (PerkinElmer, USA) at 286 nm was used to record samples' absorbance. As a reference, ethanol extract, PVP, and P407 polymer mixtures were used at the same concentration and pH. Simultaneously, the influence of particle size, polydispersity index (PdI), and zeta potential (ζ) was further evaluated for ESD dissolved under various pH conditions (1.6, 5.5, and 7.4). The data are presented as an average ± SD (n=3).

2.3.5. Evaluation of in-vitro release and DPPH effect of ethanol extract and ESD
A dialysis technique was used to conduct an in-vitro release analysis [15] with some modification. In brief, 10 mL of ethanolic extract equivalent (500 µg/mL) ESD was incorporated in PBS and loaded into the dialysis bags. At physiological pH 6.8 (37°C), the experiment was performed with continuous stirring at 100 rpm. The DPPH effect of the released extracts was performed according to the previous method [12].

3. Results and discussion

3.1. Optimization of ESD
In a previous review, poor aqueous solubility and low oral bioavailability of lipophilic compounds (TMF, SIN, and EUP) have been stated to affect their therapeutic applications [15]. These compounds have shown good solubility in organic solvents, including acetonitrile and methanol, but insoluble in water [16]. Due to its high content of lipophilic flavones (TMF, SIN and EUP), rosmarinic acid (RA), antioxidant activity, and low primary metabolite content, including total saponins, polysaccharides, and proteins, macerated ethanol extract of *O. stamineus* was chosen for this work. The optimum extract-to-polymer ratio was selected depending on the improvement of lipophilic flavonoids (TMF, SIN, and EUP) and RA in the aqueous solubility of ESD using HPLC analysis.

Solubility in ethanolic extract (F0) of lipophilic flavones (TMF, SIN and EUP) and RA was found to be 2.55 ± 0.06, 12.08 ± 0.06, 23.94 ± 0.28 and 75.21 ± 0.24 mAU, respectively. F4 (1:0.5:0.5) showed the highest enhancement of TMF (16.06 ± 0.06 mAU), SIN (47.95 ± 0.17 mAU), EUP (99.49 ± 0.59 mAU) and RA (368.78 ± 0.15 mAU), with P< 0.0001, respectively. Hence, F4 was selected for

further optimization to study the PVP polymer ratios from 0.6 to 1.5% and P407 of 0.1 to 0.5%. F7 demonstrated a substantial increase in marker compounds' solubility in an aqueous solution after further formulation, approximately 13-folds of TMF, six-folds of SIN and EUP each, and seven-fold RA compared to F0. The optimized ESD (F7) from the HPLC study showed a remarkable increase ($P< 0.0001$) in TMF, SIN, EUP, and RA with a value of 33.26 ± 0.04, 73.20 ± 0.67, 153.65 ± 0.38, 535.95 ± 0.24 mAU, respectively. Optimized ESD (F7) was found to be a homogeneous, fine, and free-flowing powder, making it ideal for further characterization of this formulation.

3.2. Characterization of O. stamineus ESD

3.2.1. ATR-FTIR analysis

In the region of 4000 to 600 cm^{-1}, FTIR spectra of ethanolic extract, ESD, and polymer mixture (PVP/P407) were recorded. Figure 1(a) shows the ethanolic extract, which displays the characteristic intensities at broad O-H stretch (3550-3200 cm^{-1}). Asymmetric and symmetric stretching (2900-2800 cm^{-1}) refers to the methylene groups (CH_2 and CH_3), methoxy derivatives, and C-H (aldehydes), including cis double bonds [17]. The peaks corresponding to C=O stretching in carbonyl compounds at 1690 cm^{-1} and 1602 cm^{-1} can be characterized by the presence of high terpenoid and flavonoid content in complex extract mixtures [18]. A peak (approximately 1032 cm^{-1}) in the ethanol extract indicated that the extracts were high in flavonoid content [19]. Phenyl and ester carbonyl groups were presented at 1600-1420 cm^{-1} and 1264 cm^{-1}, respectively.

The peak (2879, 1648, and 1100 cm^{-1}) has prominent absorption bands in the polymer mixture (PVP and P407). The band (2879 cm^{-1}) was assigned to aliphatic C-H stretching vibrations, while 1648 and 1100 cm^{-1} were assigned to the carbonyl group of polymers as C=O absorption bands [20]. Owing to the inclusion of ethanolic extract in polymers, remarkable improvements can be seen in the infrared absorption spectrum. It had shown that the broadband at 3412 cm^{-1} moved to 3362 cm^{-1} and the ethanolic extract peak characteristic at 2849 cm^{-1} due to C-H stretching vibration that had disappeared (Figure 1(a)). The band corresponding to C=O stretching in carbonyl compounds (1690 and 1602 cm^{-1}) had shifted, and, following the characteristics of polymers, it became one sharp peak in ESD. The sharp peak in the polymers (1341 cm^{-1}) had disappeared, and in ESD, the peak in the ethanol extract (1360 cm^{-1}) moved to 1371 cm^{-1}. The band (1032 cm^{-1}) corresponding to the flavonoid group may be due to the stretching vibrations of = C-O-C, C-C or bending vibration of C-OH bonds moved to ~ 1100 cm^{-1} due to compounds containing the OH group. The most pronounced spectral changes observed in the fingerprint region between 1800-800 nm (Figure 1(b)) The most pronounced spectral differences observed between 1800-800 nm in the fingerprint region corresponded to the primary and secondary metabolite mixture, which verified the encapsulation of ethanolic extract *via* the solid dispersion phase.

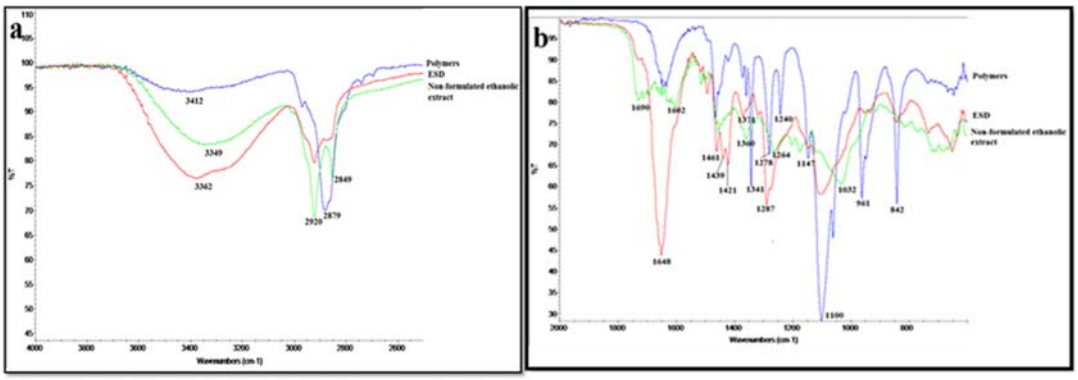

Figure 1. Comparison of FTIR spectra of *O. stamineus* ethanolic extract, polymers (PVP/P407) and ESD; (a) Spectra region from 4000-2500 nm and (b) Fingerprint region from 1800-800 nm.

3.2.2. Particle size, zeta potential, TEM and SEM analysis

A PdI value between 0.1 to 0.25 represents a narrow size distribution based on the results (Table 2), whereas a PdI value of ≥ 0.5 indicates an extensive particle size distribution [21, 22]. These results demonstrate that polymers are strongly correlated with the small mean particle size and uniform distribution of particles observed in ESD (PVP/P407). The zeta potential of ESD was greater than that of polymers in this analysis, indicating that nano-formulated ESD is more robust than polymers. In the ESD morphology, a spherical shape with a particle size of 93.62 nm was seen (TEM analysis) (Figure 2(b)) and was distributed uniformly in the system. These results are consistent with the particle size and PdI measured by the Photon Correlation Spectroscopy (PCS) (Table 2). SEM analysis was performed to confirm that nano-formulated ESD demonstrated the existence of amorphous and highly porous powder (Figure 2(c)).

Table 2. Analysis of particle size and zeta potential of *O. stamineus* ethanolic solid dispersion (ESD).

Items	Particles diameter (nm)	Intensity (%)	Polydispersity index (PdI)	Zeta potential (mV)	Conductivity (mS/cm)
ESD	97.14±0.31	100	0.22±0.01	-22.76±5.99	0.31±0.02
Polymers	141.13±4.20	97.63	0.25±0.02	-12.3±0.62	0.04±0.01

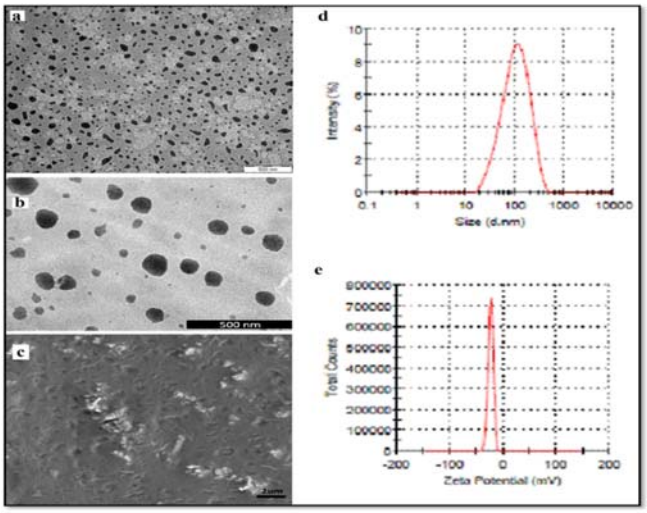

Figure 2. Transmission electron microscopy (TEM) with bar length 500 nm for (a) ethanolic extract (b) nano-formulated ESD (c) Scanning electron microscopy (SEM) with 3000X magnification (d) nano-formulated ESD particle size distribution and (e) zeta potential distribution.

3.2.3. Influence of pH in PBS and water on stability and solubility of ESD

ESD stability and solubility were tested at various pH levels (1.6, 5.5, and 7.4) to predict its stability in the gastrointestinal tract. No precipitation was seen after mixing with PBS at pH 1.6, 5.5, 7.4, and water. The percentage of the soluble fraction (stable), relative to that in water, was 96.91 ± 0.01% (pH 1.6), 96.40 ± 0.05% (pH 5.5), 96.36 ± 0.02% (pH 7.4) and 94.72 ± 0.03% (water). These findings show that ESD is extremely stable at all pH levels, including water. Particle size and zeta potential have been carried out further to validate the stability of ESD at various pH levels. Table 3 indicates that ESD suffered limited degradation of pH 7.4 in PBS saline to mimic the physiological conditions of the body representing blood as 78.19 ± 1.87 nm of particle size obtained. The size obtained is almost identical to aqueous solutions (97.14 ± 0.31 nm). The result also shows that ESD was found to

be stable in both gastric (pH 1.6), intestinal (pH 5.5), and blood (pH 7.4) simulated pH conditions, where the particle size was shown to be between 78.19 ± 1.87 and 172.47 ± 1.55 nm, respectively.

Table 3. pH effects towards the stability of ESD.

pH	Particles diameter (nm)	Zeta potential (mV)	Solubility (%)
pH 1.6	123.83±2.54	-1.98±0.58	96.91±0.01
pH 5.5	172.47±1.55	-6.85±6.17	96.40±0.05
pH 7.4	78.19±1.87	-5.75±2.97	96.36±0.02
water	97.14±0.31	-22.76±5.99	94.72±0.03

3.2.4. In-vitro release and DPPH effect of ethanol extract and ESD

The *in vitro* release study was conducted at pH 6.8 (48 h) to reproduce the intestinal fluids. With a cumulative percentage release (40.24%) at 37°C, ESD could maintain drug release for 48 h. Under the same conditions, the percent release was 90.52% for ethanol extract. This finding provides further insight into the stability of solid dispersion structures (ESD) under test conditions, presumably due to the interaction between ethanolic extract and water-soluble copolymers (PVP and P407). Thus, it allows the active principles to be slowly produced. In both ethanolic extract and ESD, the release extract was further analyzed using DPPH assays. The results indicate a potent antioxidant activity of ESD at IC_{50} of 10.92 ± 0.24 µg/mL compared to ethanolic extract (17.88 ± 0.29 µg/mL), and this result has proven that the encapsulated ethanolic extract with water-soluble polymers (PVP/P407) can improve the antioxidant activity by preserving the active constituents.

3.2.5. Statistical analysis

The data are described as mean ± SD. The data were evaluated for statistical significance using one-way ANOVA (SPSS 20.0), followed by Tukey's posthoc tests. Significant P values of less than 0.05 were considered.

4. Conclusion

To conclude, the ethanolic extract from *O. stamineus* was successfully encapsulated using water-soluble copolymers (PVP/P407) with a ratio of extract to polymers (1.0:1.1:0.3 w/w). The solubility of lipophilic flavones (SIN, TMF, and EUP) and RA (caffeic acid derivatives) was substantially improved by this formulation ($P< 0.0001$), thus further enhancing its pharmacological properties.

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