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Accelerated stability study of *Orthosiphon stamineus* standardised ethanolic extract and its solid dispersion

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Abstract. The objective of the present study is to develop accelerated stability of *Orthosiphon stamineus* standardised ethanolic extract (SEE) and its solid dispersion (ESD). The stability study of SEE and ESD has been performed using high-performance liquid chromatography (HPLC) and attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) analyses. The spectroscopic datasets of ESD were applied to the principal component analysis (PCA) to extract the maximum information of the ATR-FTIR spectra. SEE and ESD were stored at three different temperatures with two different humidity conditions (30 °C/75% RH, 40 °C/75% RH and 60 °C/85% RH) for six months. Overall, the degradation of marker compounds; rosmarinic acid (RA), 3'-hydroxy-5, 6, 7, 4'-tetramethoxyflavone (TMF), sinensetin (SIN) and eupatorin (EUP) at high temperature (60 °C/85% RH) was higher compared to low temperature (30 °C/75% RH) for both samples. Moreover, the degradation of RA, TMF, SIN and EUP in ESD was slower compared to SEE. The deterioration of marker compounds for both samples followed the first-order reaction kinetics. The shelf life of SEE and ESD is based on the estimated shelf life RA, TMF, SIN, and EUP present in the samples. The shelf life of RA, TMF, SIN, and EUP in ESD were significantly enhanced ($p < 0.001$) compared to the same markers in SEE with EUP was showing the highest shelf life (15 months), while RA showed the lowest shelf life (7 months) when stored at the temperature below 30 °C. The shelf life of all marker compounds in SEE was less than two months when stored at the same temperature (below 30 °C). Based on ATR-FTIR fingerprinting datasets analysed with PCA, ESD kept at 30 °C/75% RH were still preserved of its chemical properties, which indicates that low temperature is better to keep the formulation.

1. Introduction

3'-hydroxy-5, 6, 7, 4'-tetramethoxyflavone (TMF), sinensetin (SIN), eupatorin (EUP), and caffeic acid derivatives including rosmarinic acid (RA) are among the main component isolated from *Orthosiphon stamineus* Benth. (Cat's Whiskers). It was reported to have a potential therapeutic properties, as they are shown to exert antioxidant properties [1-2], diuretic, and uricosuric actions in rats [3] and anticancer properties [4]. However, due to the low aqueous solubility of TMF, SIN, and EUP in *O. stamineus* extract, it is suffering from poor oral bioavailability and incomplete absorption. Hence, it limited the therapeutic properties of *O. stamineus* [5].

In order to overcome the issues, many formulation strategies, including nanoparticles, liposomes, complex with phospholipids, cyclodextrins, and solid dispersions, which appear to provide more prolonged circulation, better permeability, and resistance to metabolic processes [6-8]. Among these approaches, solid dispersion is the most promising method due to the ease of preparation, optimisation, and reproducibility of the manufacturing method [9]. Solid dispersion has shown many advantages for solubility enhancement including reducing the particle size to molecular level, reduce the agglomeration of drug particles in the formulation, enhancing wettability and porosity, as well as changing drug crystalline state to amorphous which leads to faster dissolution for *in vivo* study [10].

Another problem with *O. stamineus* extracts, which limits its therapeutic properties, is the stability of extract and its products concerning the markers (RA, TMF, SIN, and EUP). The ethanolic extract is highly hygroscopic. Therefore, the physical appearance, texture, and colour, get easily oxidised, and the chemical components, particularly the bioactive markers such as RA and EUP degraded overtime. Thus, using solid dispersion, it helps to encapsulate standardised ethanolic extract (SEE) with selected co-polymers (PVP/P407) as a carrier to enhance the stability and solubility of the extract. Therefore, the present study provides the analysis of accelerated stability of SEE and its solid dispersion (ESD) to compare the quantity, quality and shelf life of RA, TMF, SIN, and EUP in both samples under variable conditions (elevated conditions). The stability was carried out following the study protocol of the International Conference on Harmonization (ICH), as suggested by the Working Party of Herbal Medicinal Products (WPHMP) of the European Agency for the Evaluation of Medicinal Products [11-12]. For chemical quality assessment, ATR-FTIR analysis was performed qualitatively with a combination of principal component analysis (PCA) for ESD to extract the maximum information of the ATR-FTIR spectra.

2. Materials and methods

2.1. Chemicals and reagents

HPLC grade of RA, TMF, SIN, and EUP were purchased from Indofine Chemicals (New Jersey, USA). Acetonitrile and formic acid (HPLC grade) were purchased from Merck (Petaling Jaya, Selangor, Malaysia). Deionised water used in HPLC analysis was prepared using the Ultra-pure water purifier system (Thermo Scientific, Barnstead). Polyvinylpyrrolidone (PVP-K29/32) and Poloxamer 407 (P407) were purchased from International Specialty Products, USA.

2.2. Plant materials and extraction

Orthosiphon stamineus leaves were purchased from Universiti Malaysia Perlis, Perlis, Malaysia. Taxonomic authentication was performed with the voucher specimen no. 11009 and deposited at the Herbarium, School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia [13]. The leaves were dried in the oven at a temperature of 40-50 ℃. The grounded leaves (500 g) were extracted with ethanol (5 L) using Soxhlet extraction (8 hours) and repeated in triplicate. The extract was filtered using Whatman filter paper No. 1 (Whatman, England) before further concentrated with a rotary evaporator (Buchi, Germany) under vacuum at 60 ℃. Finally, the concentrated extract was freeze-dried (Scanvac coolsafe, Denmark). The dried extract was kept in an air-tight container until further use.

2.3. Preparation of ethanolic solid dispersion

ESD was prepared using the solvent evaporation technique [14]. Briefly, the extract (1 g) was mixed with PVP and P407 (1.1:0.3 w/w) and co-dissolved in ethanol (10 mL) at 40 ℃ sonicated and the mixture was then homogenised using a magnetic stirrer (700 rpm) for 30 min. Next, each ethanolic mixture was added separately dropwise to deionised water (50 mL) by means of a syringe attached to a 27G needle inserted directly into the medium while mixing at 1000 rpm. The ethanol was evaporated at 50 ℃, frozen at -20 ℃ overnight and vacuum freeze-dried (Labconco FreeZone; Labconco, Kansas City, Missouri) at -90 ℃ for 48 h. ESD was dried in the oven for 5 h at 50 ℃ before used.

2.4. High-performance liquid chromatography (HPLC) analysis

The HPLC analysis of SEE and ESD was performed based on the validated published method [15] using Dionex-Ultimate® 3000 Rapid Separation LC system (Dionex, Germany), which was equipped with an autosampler, a column oven, a quaternary pump, a degasser, and a DAD detector.

2.5 Preparation of the marker compounds and samples

The stock solutions of RA, TMF, SIN, and EUP were prepared by weighing 5 mg of each standard and dissolving them in 5 mL of methanol: water (1:1) (HPLC-grade). Each solution was filtered through a 0.45 µm Whatman filter paper. A series of standard working solutions (0.195 to 100 µg/mL) were prepared by diluting the above solution with the same solvent (methanol: water). These standard solutions were stored at 4 ℃ prior to being used. For the preparation of SEE and ESD, the weight of ESD, which is equivalent to 1000 µg/mL SEE was dissolved in methanol. The solution was sonicated for 10 min and transfer to the HPLC vial (1.5 mL) before starting the analysis. All the samples and markers (RA, TMF, SIN, and EUP) were analysed in triplicate (n=3).

2.6. Stability study protocol

The stability study of SEE and ESD was conducted following the standard protocol established by ICH guideline as suggested by the Working Party of Herbal Medicinal Products (WPHMP) of the European Agency for the Evaluation of Medicinal Products [11-12]. The samples were kept in screw-capped transparent glass bottles and exposed to 30 °C/75% RH, 40 °C/75% RH and 60 °C/85% RH. The percentage of markers and release profiles were monitored periodically for six months (0, 1, 2, 3, 4, 5, and 6) using HPLC analysis. The chemicals kinetic parameters, including the order of reaction, rate constant (K), Activation Energy (Ea), Pre-exponential Factor (A), and shelf life reaction, were calculated for stability assessment of both samples. All samples were analysed in triplicate (n=3).

2.7. ATR-FTIR and principle component analysis (PCA)

ESD was further analysed by the ATR-FTIR spectra device (Nicolet iS10, Thermo Scientific, USA) to compare the fingerprint profiles in different storage conditions. Briefly, the ATR-FTIR analysis was recorded in the region of 4000 to 600 cm^{-1} with 16 scans for each spectrum. The fingerprint spectra from FTIR (1800 – 800 cm^{-1}) were further analysed using principal component analysis (PCA) from The Unscrambler X, CAMO [16]. The baselines of spectra from FTIR data were corrected using Omnic software (Thermo Scientific, USA). Samples were then analysed in triplicate (n=3).

2.8. Statistical analysis

All samples were analysed in triplicate, and the results were presented as mean ± standard deviation. For stability data, an independent t-test was used to compare the significant value between ESD and SEE.

3. Results and discussion

3.1. The ethanolic solid dispersion (ESD) was successfully prepared using selected polymers (PVP/P407) as a carrier to encapsulate the ethanolic extract for solubility enhancement. The optimum extract-to-polymers ratio was selected based on the solubility enhancement of selected lipophilic flavonoid compounds (TMF, SIN, and EUP) as well as RA analysed using HPLC analysis. From the HPLC analysis, the optimized ESD has shown remarkable increment of RA (539.95 ± 0.24 mAU), TMF (33.26 ± 0.04 mAU), SIN (73.20 ± 0.67 mAU), and EUP (153.65 ± 0.38 mAU) with p <0.0001 compared to markers in ethanolic extract (SEE) with the value of 75.21 ± 0.24 mAU, 2.55 ± 0.06 mAU, 12.08 ± 0.06 mAU, and 23.94 ± 0.28 mAU for RA, TMF, SIN, and EUP, respectively.

3.2. Stability study using HPLC analysis and chemical kinetic parameters

The percentage loss of the markers in SEE and ESD stored at 30 °C/75% RH, 40 °C/75% RH and 60 °C/85% RH is indicated in Table 1. It shows that the decomposition of compounds in SEE had a faster

degradation compared to the same markers in ESD stored under the same condition. The result also indicates that increasing temperature could cause an increasing decomposition rate of marker compounds, which is in line with the previous study [17]. In this study, it is suggested that solid dispersion with PVP/P407 was stable during storage compared to SEE, and the encapsulation of the extract with co-polymers can preserve the marker compounds from degradation over time and temperature.

The order of degradation rate was calculated using the graphic method by plotting the graph of zero (time *vs* percentage remaining concentration), first (time *vs* natural logarithm of remaining concentration), and second-order (time *vs* 1/C) graphs for each temperature. The regression coefficient (R^2) was calculated, and the best linearity describes the order of reaction for each marker. Based on the linearity plot of different curves of zero and first-order, the best linearity for all markers at three different storage conditions is the first-order reaction. Thus, the degradation of marker compounds in both samples have followed the first-order reaction kinetics. In other words, the percentage of remaining concentration was reduced with increasing storage temperature.

Table 1. Percentage loss of marker compounds in standardised ethanolic extract and ethanolic solid dispersion

Samples	Storage conditions	Percentage loss of markers (%)			
		RA	TMF	SIN	EUP
Standardised Ethanolic Extract (SEE)	30 °C/75 ± 5% RH	28	67	40	67
	40 °C/75 ± 5% RH	27	70	42	71
	60 °C/85 ± 5% RH	45	80	66	74
Ethanolic Solid Dispersion (ESD)	30 °C/75 ± 5% RH	19	22	19	16
	40 °C/75 ± 5% RH	24	31	20	18
	60 °C/85 ± 5% RH	62	64	56	26

The rate constant (K) of the marker compounds at 30 °C/75% RH, 40 °C/75% RH and 60 °C/85% RH in both samples was obtained from the slope of the curves of the first-order reaction. Arrhenius equation was obtained by plotting (ln K) against the inverse of temperature (1/T Kelvin^{-1}). Data derived from linear regression was used to calculate degradation rate constant (K) for the markers at 25 °C by plotting the Arrhenius equation. The Arrhenius plots of the marker compounds are given in Figure 1. The rate constant of chemical degradation of TMF in SEE stored at 25 °C/60% RH, 30 °C/75% RH, 40 °C/75% RH, and 60 °C/85% RH was found to be the highest followed by EUP at the same storage conditions. In ESD stored at 25 °C/60% RH, 30 °C/75% RH, and 40 °C/75% RH, the rate constant of chemical degradation of RA was found to be the highest compared to other markers followed by EUP which was stored at 60 °C/85% RH. It also indicates that the rate of chemical degradation of RA, TMF, SIN, and EUP was found to be increasing with the rise in storage temperature. The result is in line with an earlier study reported by [18] whereby the rate of a chemical reaction increases by a factor of between two to three-fold for every 10 °C rise in temperature.

The Ea of markers in SEE and ESD was calculated from the slope of the straight line of the Arrhenius plot (Figure 1). The calculated Ea and Pre-exponential Factor (A) of markers in both samples are shown in Tables 2. The results illustrate that in SEE, the Ea of EUP and SIN are lower compared to RA and TMF. This happens due to the solubility of these compounds is less than RA and TMF. The stability trend of markers in SEE based on their Ea is found to be TMF > RA > SIN > EUP. However, in ESD, the Ea of all markers is significantly enhanced ($p < 0.001$) compared to SEE. The stability trend of markers in ESD based on their Ea is found to be EUP > TMF > SIN > RA. The higher value of Ea indicates a greater temperature dependence of the reaction rate and a more stable condition throughout the storage condition.

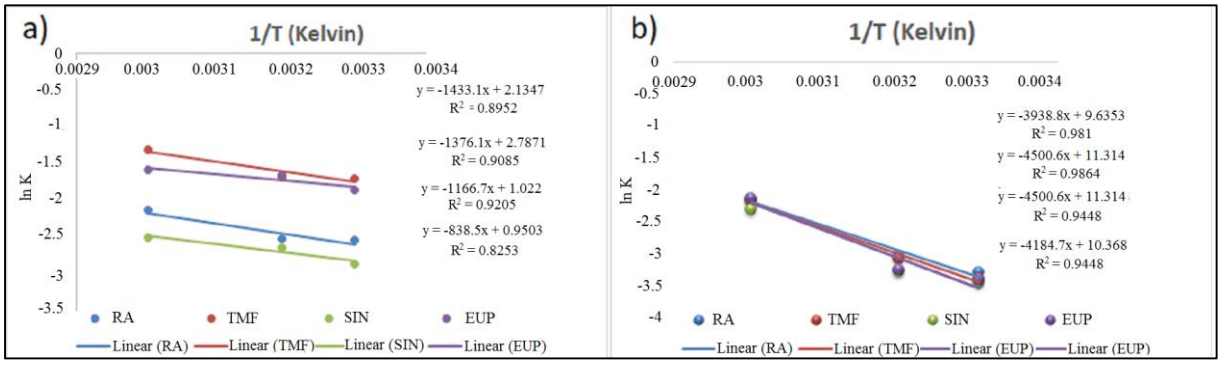

Figure 1. Arrhenius plot of RA, TMF, SIN and EUP in (a) standardised ethanolic extract and (b) ethanolic solid dispersion

Table 2. Activation energy (Ea) and pre-exponential factor (A) of RA, TMF, SIN, and EUP in standardised ethanolic extract (SEE) and ethanolic solid dispersion (ESD)

Markers/ Samples	Ea (KJ/mol) at 25 °C		A (S^{-1})	
	SEE	ESD	SEE	ESD
RA	11.91	32.75	3.65×10^{3}	4.30×10^{11}
TMF	12.03	34.79	1.11×10^{4}	2.45×10^{12}
SIN	9.70	33.63	4.59×10^{2}	8.10×10^{11}
EUP	6.97	37.42	9.33×10^{1}	1.58×10^{13}

In the present study, the estimated shelf life (t_{90}) of marker compounds in SEE and ESD stored at different conditions are given in Figure 2. The approximate t_{90} of RA, TMF, SIN, and EUP in SEE was less than two months at all storage conditions. By comparing the t_{90} of markers in both samples, it showed that the shelf life of RA, TMF, SIN, and EUP in ESD was significantly enhanced ($p < 0.001$) compared to the markers in SEE. The results also demonstrate that all markers in ESD have a longer shelf life when stored below 30 ℃. This happens due to the stability of the encapsulation process using co-polymers with the extract at low temperatures compared to high temperature.

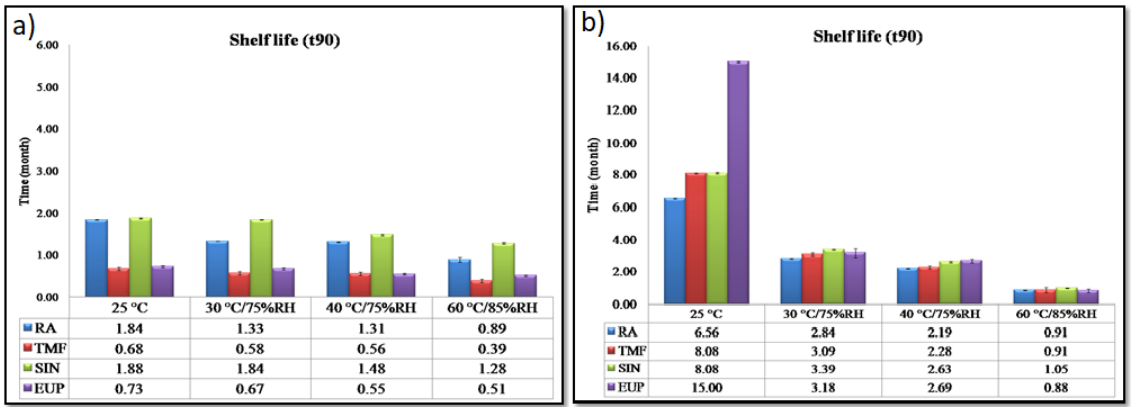

Figure 2. Estimated shelf life of RA, TMF, SIN, and EUP in (a) ethanolic extract (SEE) and (b) ethanolic solid dispersion (ESD).

3.3. Stability study using ATR-FTIR and PCA

The chemical quality assessment of ESD in this study was analysed qualitatively using ATR-FTIR techniques in the fingerprint region (1800-800 cm^{-1}). From Figure 3, the spectra have a similar pattern, and it is difficult to be interpreted on a visual inspection. Thus, powerful chemometric methods needed

to be applied in order to identify the trends. From the absorbance pattern, when ESD was stored at low temperature, no degradation of functional groups was found from zero to six months. However, the functional group started to degrade when ESD was stored at 40 °C/75% RH at month six (Figure 3b). The functional group was further degraded at month three to six at high temperature, 60 °C/85% RH (Figure 3c). To extract maximum information of FTIR spectra, PCA was applied to the spectroscopic datasets. Figure 3d shows a PCA scores scatter plot of ESD stability stored at 30 °C/75% RH, 40 °C/75% RH, and 60 °C/85% RH for six months. Based on the PCA result, the score plot was generated from comparisons of the two PCs; principle component (PC-1) axis and PC-2 axis, which encountered of 95% of total variants. The result demonstrated that two samples stored at 40 °C/75% RH and 60 °C/85% RH were outside the circle, which means that the degradation of markers in these samples was higher compared to the other samples. It is supported by Figure 3b and c, whereby the absorbance pattern of the functional groups for samples stored at 40 °C/75% RH and 60 °C/85% RH were degraded compared to the sample stored at 30 °C/75% RH (Figure 3a). All samples stored at 30 °C/75% RH were still intact of its chemical properties of ESD, which means low temperature is better to keep this solid dispersion.

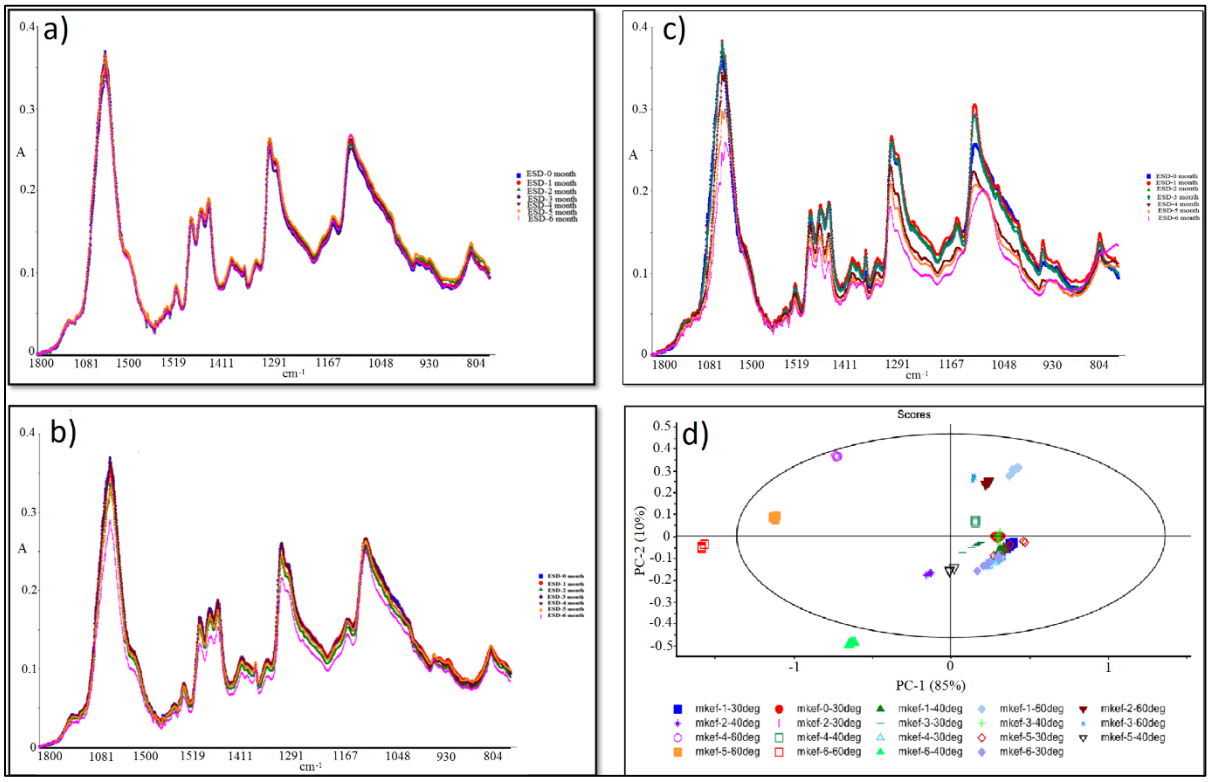

Figure 3. The FTIR spectra of ESD from zero until six months stored at (a) 30 °C/75% RH; (b) 40 °C/75% RH; (c) 60 °C/85% RH (d) Principle component analysis (PCA) of ESD stored at 30 °C/75% RH, 40 °C/75% RH, and 60 °C/85% RH for six months.

4. Conclusion

In conclusion, it is believed that the present study has successfully analysed the accelerated stability of standardised ethanolic extract (SEE) and its solid dispersion (ESD) using HPLC and ATR-FTIR with PCA analysis. The shelf life of RA, TMF, SIN, and EUP in ESD were significantly enhanced ($p < 0.001$) compared to the same markers in SEE. The ESD also was more stable than the extract alone, and the temperature below 30 °C is more suitable to keep ESD to maintain the integrity of the formulated extract for solubility enhancement as well as increasing the therapeutic properties of *Orthosiphon stamineus* extract.

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