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STABILITY AND TOXICITY PROFILE OF SOLUTION ENHANCED DISPERSION BY SUPERCRITICAL FLUIDS (SEDS) FORMULATED *Andrographis paniculata* EXTRACT

S. Y. Lee[1], L. Chuah Abdullah[2], R. A. Rahman[1,3],
F. Abas[4] and G. H. Chong[1,3*]

[1] University Putra Malaysia, Faculty of Food Science and Technology, Department of Food Technology, Selangor Darul Ehsan, Malaysia.
ORCID: 0000-0003-1542-3594; ORCID: 0000-0001-7358-0390; E-mail: gunhean@upm.edu.my - ORCID: 0000-0002-6521-1832
[2] University Putra Malaysia, Faculty of Engineering, Department of Chemical and Environmental Engineering,
Selangor Darul Ehsan, Malaysia. ORCID: 0000-0003-4785-9296
[3] University Putra Malaysia, Faculty of Food Science and Technology, Supercritical Fluid Centre, Selangor Darul Ehsan, Malaysia.
[4] University Putra Malaysia, Faculty of Food Science and Technology, Department of Food Science, Selangor Darul Ehsan, Malaysia.
ORCID: 0000-0002-8110-9424

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Abstract - The main objective was to evaluate the stability and toxicity of a Solution Enhanced Dispersion by Supercritical Fluids (SEDS) formulated *A. paniculata* extract that was intended for food applications. The best formulated SEDS *A. paniculata* extract with improved dissolution of andrographolide (data not shown) was obtained using 25 mg/mL *A. paniculata* extract (maceration in acetone) and 6 mg/mL Eudragit L100-55 with acetone as feed solvent at the following SEDS co-precipitation conditions: 150 bar, 40 ℃, 15 L/min CO_2 flow rate (1 bar, 25 ℃), 0.5 mL/min liquid feed flow. Sticky *A. paniculata* extract with 16% w/w andrographolide was transformed into SEDS co-precipitates concentrated with 20.4% w/w andrographolide. Only 20-30% of andrographolide was degraded after two-month storage under 5, 30, 45 ℃ at 75% relative humidity (RH). About 30-60% of andrographolide was degraded after addition into drinking water, orange drink, soybean milk for a day. This would imply its unstable nature after being dispersed and wetted in liquid food. SEDS co-precipitate was found to be practically acetone-free (< 0.1 ppm) by Headspace Gas Chromatography-Mass Spectrometry (GC-MS). This showed the capability of the SEDS process in stripping off acetone. Higher cytotoxic potential of SEDS co-precipitates (LC_{50} = 46.46 μg/mL) than extract (LC_{50} = 150.45 μg/mL) was observed.
Keywords: Stability; Toxicity; SEDS; *Andrographis paniculata*; Co-precipitates.

INTRODUCTION

Herbs are processed into forms of essential oils, ointments, salves, and rubs for topical application or into forms of whole herbs, teas, syrups, capsules, and tablets for oral consumption (Wachtel-Galor and Benzie, 2011). However, liquid forms of herbal products are usually found to have lower chemical, physicochemical and microbiological stability that often hinder their applications. Therefore, formulation of liquid herbal product into solid counterparts has been developed to improve their quality and to ensure safe applications (Ameri and Maa, 2006; Bakowska-Barczak and Kolodziejczyk, 2011; Bhandari and Howes, 1999; Bott et al., 2010; Jiménez-Aguilar et al., 2011; Rocha et al., 2012; Yatsu et al., 2011). Transformation of those liquid herbal extracts into dry extracts has resulted in higher concentration of active compounds, ease of

* Corresponding author: Gun H. Chong - E-mail: gunhean@upm.edu.my

standardization, ease of transportation, less storage space required, as well as ease of future translation into different solid pharmaceutical forms for the dry extract (Oliveira et al., 2010).

The common transformation methods used include spray drying, spouted bed drying as well as freeze drying (de Oliveira et al., 2012). While spray drying is the most common method used in herbal processing industries, supercritical fluid (SCFs)-based particulate technology has been found to be feasible for producing other herbal products such as powdered green tea extract, paprika extract, ginger and thyme extract (King, 2014). Application of SCFs is a greener technique that eliminates the most common drawbacks associated with the conventional methods such as thermal degradation, chemical degradation and high residual solvent content due to the larger diffusivities of SCFs than those of liquids, enabling high mass transfer rates during the transformation of liquid herbal extract into dry extract under moderate pressure-temperature conditions.

In spite of the capability of in minimizing undesirable changes during the transformation process, the stability of bioactive compounds in the final dry extract is equally important to preserve good quality of the dry extract. Studies related to the stability of andrographolide from different formulations such as those in powdered *A. paniculata, aqueous A. paniculata* extract, isolated andrographolide from *A. paniculata* extract, solid dispersion in polyvinylpyrrolidone (PVP-K30) as well as solid dispersion in silicon dioxide (SiO_2) have been conducted and reported (Ibrahim and Chong, 2008; Lomlim et al., 2003; Pholphana et al., 2004; Plubrukarn et al., 2006; Wongkittipong et al., 2004; Zhang et al., 2016). Thus far, first and second-order kinetics of degradation of andrographolide have been reported (Lomlim et al., 2003; Plubrukarn et al., 2006; Wongkittipong et al., 2004). High storage temperature such as 45 to 70℃ and 86 to 97℃ resulted in degradation of andrographolide and the degree of degradation increased with temperature (Lomlim et al., 2003; Plubrukarn et al., 2006; Wongkittipong et al., 2004), while lower storage temperatures such as 5 to 30 ℃ caused no significant reduction of andrographolide along the study period (Ibrahim and Chong, 2008).

Beside stability, toxicity of the formulated extract is undeniably another important factor to be considered for both formulators and consumers. The solvent residue in the final treated product is always the concern, especially if solvent is involved in the process. Throughout the studies reported on SCFs-treated compounds, it was found that the residual amount of solvent, particularly Class III solvents such as dichloromethane (DCM), acetone, and dimethyl sulfoxide (DMSO) was much lower than the International Conference on Harmonization (ICH) requirements (5000 ppm) (Miao et al., 2018; Zhao et al., 2010). While solvent residue content of product could indirectly tell its toxicity potential, a general bioassay proposed by Meyer et al. (1982) could be used to identify the *in vivo* toxicity of co-precipitates towards newly hatch nauplii. In a brine shrimp study (Mamatha, 2014), crude *A. paniculata* extract was found to have a LC_{50} in the range of 100 to 300 µg/mL.

In this study, stability and toxicity of a Solution Enhanced Dispersion by Supercritical Fluids (SEDS) formulated *A. paniculata* extract which was intended for food applications were evaluated. Degradation kinetics of andrographolide from SEDS co-precipitates was investigated under different storage conditions (5, 30, and 45℃ at 75% relative humidity (RH)) for a two-month storage period. The half-life ($t_{1/2}$) and shelf life ($t_{90\%}$) predicted were used to identify the most effective storage condition for the SEDS co-precipitates. On the other hand, the stability of andrographolide from SEDS co-precipitates after their respective addition into three different Ready-To-Drink (RTD) beverages of different pH, namely drinking water, orange drink, and soybean milk was determined to evaluate their application stability for three days. The toxicity profile of the SEDS co-precipitates was elucidated through a solvent residue test by Headspace Gas Chromatography-Mass Spectrometry (GC-MS) analysis and brine shrimp bioassay.

MATERIALS AND METHODS

Materials

Fresh flowering *A. paniculata* were collected from a cultivated herb nursery named "Pusat Pembangunan Komoditi Sendayan" located in Negeri Sembilan, Malaysia. The aerial parts were authenticated by Dr. Shamsul Khamis, a botanist from the Biodiversity Unit of Institute of Bioscience, Universiti Putra Malaysia (UPM). A voucher specimen (Voucher No. SK 2767/15) was prepared and deposited in the herbarium. The collected *A. paniculata* were first cleaned with tap water, drained, then cut into 5-6 cm long pieces and dried under shade for three weeks. It was then milled with a 3 horsepower (hp) tea bag cutting mill (RT-CR30S, Rong Tsong Precision Technology Co., Taiwan) into coarse *A. paniculata* powder, followed by storage in a freezer until further use. High Performance Liquid Chromatography (HPLC) grade methanol with purity of 99.99% was used in HPLC analysis and in brine shrimp bioassay as both control and solvent while acetone of Gas Chromatography (GC) Resolv grade with purity of 99.5% was used in GC-MS analysis as standard (Fisher Scientfic, UK). Analytical grade acetone with purity of 99.98% was used for other analyses (Fisher Scientific, UK). Andrographolide standard with a purity of 98% was used (Sigma-Aldrich, USA). Three RTD

beverages of different pH to simulate three different pH media such as SeaMaster Drinking Water (pH 7.03) (RO Water Sdn. Bhd., Malaysia), MARIGOLD orange drink enriched with Vitamin C (pH 3.89) (Malaysia Milk Sdn. Bhd., Malaysia), and Yeo's Soy Bean Milk Less Sugar (pH 6.66) (Yeo Hiap Seng (Malaysia) Bhd, Malaysia) were used. Brine shrimp eggs in sea salt premix (47 g of sea salt and 3 g of Artemia Cysts from the Great Salt Lake) (Ocean Nutrition, Belgium) and sodium chloride of ACS grade (Fisher Scientific, UK) were used for the brine shrimp bioassay. All chemicals were used as received.

Extraction of *A. paniculata*

Extraction of *A. paniculata* was conducted as described by Sule et al. (2011). Powdered *A. paniculata* (1.5 kg) was extracted by macerating in acetone (6 L) at room temperature for 24 h, followed by filtration using filter paper (Whatman no. 1). The filtrate was concentrated under vacuum at 40 ℃. The extraction process was repeated twice. The dark blackish green *A. paniculata* extract obtained was stored in a freezer until further use.

SEDS process for producing co-precipitates

SEDS co-precipitation of *A. paniculata* extract with Eudragit L100-55 was conducted using a SEDS system that was designed and fabricated in-house as reported (Lee et al., 2018). A schematic diagram of the apparatus is shown in Figure 1.

It consisted of two high pressure pumps, one of them (PM-10000C, Delta, Taiwan Supercritical Technology, Taiwan) was used for liquid CO_2 delivery into a preheated water bath that would later lead to the precipitation chamber, while another (260D, Teledyne Isco, USA) was used for liquid feed delivery. A cylindrical vessel of 1 L internal volume (internal diameter = 75 mm) was used as the precipitation chamber. A stainless steel coaxial mixing nozzle (SETOJet 0405R, Kirinoikeuchi, Japan) that could produce a mean droplet diameter down to 50 µm or less was used for spraying of both CO_2 and liquid feed. The nozzle was infixed in the spiral lid of the precipitation chamber. The experiment was started by first cooling CO_2 from a cylinder with the aid of a refrigerated circulated bath with temperature set at 4 ℃ to liquefy CO_2 before being pumped to avoid cavitation. CO_2 was then preheated to 40℃ and pumped into the precipitation chamber through a nozzle. Pressure of the system was measured by a pressure gauge (EN837-1, WIKA, USA) and regulated by a back pressure regulator (26-1700, Tescom, US) located at the exit of the precipitation chamber. Temperature was set by a proportional-integral-derivative (PID) controller connected with an electrically controlled heating jacket. The following parameters: 150 bar, 40 ℃, 15 L/min of CO_2 flow rate

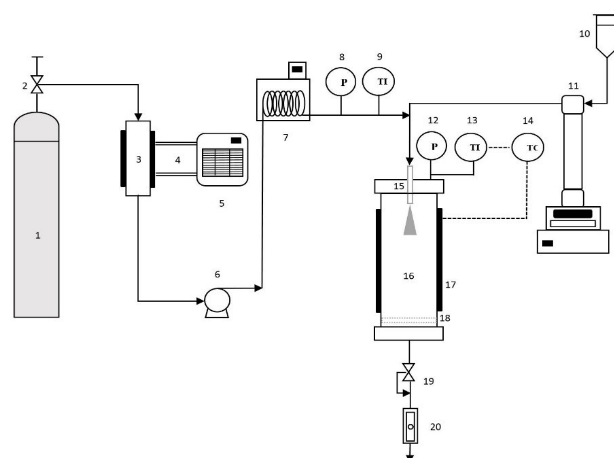

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Figure 1. Schematic diagram of the Solution Enhanced Dispersion by Supercritical Fluids (SEDS) apparatus. (1) CO_2 cylinder; (2) CO_2 cylinder outlet valve; (3) Low pressure heat exchanger; (4) Cooling lines for CO_2; (5) Refrigerated circulating bath; (6) High pressure pump; (7) Water bath for preheating CO_2; (8, 12) Pressure gauge; (9, 13) Temperature indicator; (10) Feed solution; (11) Syringe pump; (14) Temperature controller; (15) Coaxial mixing nozzle; (16) Precipitation chamber; (17) Heating jacket; (18) Filter; (19) Back pressure regulator; (20) Rotameter.

(1 bar, 25 ℃), and 0.5 mL/min of liquid feed flow rate were set constant throughout the experiment as they were the most appropriate conditions that resulted in successful co-precipitation of *A. paniculata* extract with Eudragit L100-55 with improved dissolution of andrographolide (data not shown). After the operating conditions were achieved and remained stable (chosen pressure, temperature, CO_2 flow rate), the liquid feed was delivered by a syringe pump at constant flow rate (0.5 mL/min) into the precipitation chamber. Acetone was used as feed solvent to dissolve both *A. paniculata* extract and Eudragit L100-55, followed by filtration using filter paper (Whatman no. 1) to form 25 mg/mL of *A. paniculata* extract and 6 mg/mL of Eudragit L100-55 in the final liquid feed.

At the end of the liquid feed delivery, sc-CO_2 was further delivered into the precipitation chamber for 45 min as a washing step for removal of any possible residual organic solvent within the chamber that would re-dissolve the formed particles upon depressurization. A filter was placed at bottom of precipitation chamber to collect the produced powder, while at the same time allowing CO_2-solvent to pass through the chamber exit. At the exit of precipitation chamber, the CO_2 flow rate was measured by a rotameter (LZT-08A10M-V,

NBDC, China). At the end of the CO_2 washing step, the CO_2 flow was stopped, the precipitation chamber was slowly depressurized down to atmospheric pressure and the sample powder was collected for further analysis and characterization.

Morphology of SEDS co-precipitates

The morphology of the SEDS co-precipitates was analyzed with scanning electron microscopy (SEM) (JEOL JSM 6400, Japan). Prior to scanning, the sample was mounted on carbon tape and coated with a 450 Å thickness of gold under vacuum for 3 min using a sputter coater (SCD 005, BAL-TEC, Liechtenstein) to avoid charging under the electron beam during viewing.

Crystallinity (XRD)

Powder crystallinity of the SEDS co-precipitates was characterized with an X-ray diffractometer (PW3040/60 MPD X'Pert High Pro Panalytical, Philips, Netherlands) with Cu Kα radiation with a K-A2/K-A1 ratio of 0.5, generated under a voltage of 40 kV and a current of 40 mA. Samples were scanned over the most informative range from 10° to 80° with a step size of 0.033° and step time 19.6850 sec.

Andrographolide content

Andrographolide content of the SEDS co-precipitates was quantified by HPLC as described by Panossian et al. (2000). A LC system (1200 series, Agilent Technologies, USA) comprising of a micro vacuum degasser (G1322A, JP73069224), a quaternary pump (G1311A, DE62969377), an auto sampler (G1329A, DE64771769), DAD diode array detector (G1315D, DE64260468), and thermostatted column compartment (G1316A, DE90374060) was used. Chromatographic separation was carried out with a C18 column, 4.6 x 150 mm, 5 µm (Zorbax Eclipse Plus, Agilent Technologies, USA). The mobile phase, composed of 60% methanol and 40% deionized water, was set in isocratic mode with a flow rate of 0.7 mL/min. The detection wavelength was set at 229 nm. The injection volume was 20.0 µL and the total run time was fixed at 10 min. Data acquisition and analysis were performed using Agilent Chemstation Software coupled to the computer. Five mg of SEDS co-precipitates was dissolved in 10 mL methanol, diluted with mobile phase, filtered using a 0.22 µm syringe filter and injected onto the HPLC system for determination. Andrographolide content was quantified by comparison with an andrographolide standard calibration curve. Ten mg of andrographolide standard was dissolved in 10 mL of methanol, then diluted to a series of concentrations (mg/mL) 0.01, 0.05, 0.10, 0.15, 0.20, and 0.25. Andrographolide content of *A. paniculata* extract was determined for comparison. Each determination was conducted in duplicate.

Storage stability study of andrographolide from SEDS co-precipitates

The storage stability study of SEDS co-precipitates was conducted at 5±2, 30±2, and 45±2 ℃ at 75±5% relative humidity (RH) according to Plubrukarn et al. (2006) with slight modifications. Temperature higher than 45 ℃ was not administered due to poor andrographolide stability in those elevated-temperature systems as reported (Lomlim et al., 2003; Plubrukarn et al., 2006; Wongkittipong et al., 2004). Since stability study of andrographolide in dried *A. paniculata* plants at ambient conditions conducted by Pholphana et al. (2004) showed only a decrement of the content of andrographolide during the first three months in a 15-month study period, our study period was adjusted to be less than three months. SEDS co-precipitates were initially filled into separate petri dishes, then placed into closed desiccators containing saturated NaCl solution in order to furnish an atmosphere of 75±5% RH under darkness at different temperatures. The andrographolide content of SEDS co-precipitates was determined before the storage period and consecutively after 21, 49, and 64 days of storage under different conditions as stated. Samples were randomly collected in duplicate during the sampling period for determination of the percentage of andrographolide content (%) using HPLC. The order of the andrographolide degradation was determined by the representation that gave a linear plot over the time course of reaction (Schmitz, 2017). The determination of the order of reaction started from zeroth, followed by first and second order. If a plot of [A] versus t is linear, then it is a zeroth-order reaction. If a plot of ln [A] versus t is linear, then it is a first-order with the slope of plot as the rate constant. If a plot of 1/[A] versus t is linear, then it is a second order reaction. For a second-order reaction, the plot of ln [A] versus t is not linear (Schmitz, 2017). Shelf life ($t_{90\%}$) and half-life ($t_{1/2}$) of SEDS co-precipitates were calculated using equations (1) to (6) as follow:

Zeroth-order: $[A] = [A]_o - kt$

$$t_{90\%} = 0.1[A]_o / k \quad (1)$$

$$t_{1/2} = [A]_o / 2k \quad (2)$$

First-order: $\ln [A] = \ln [A]_o - kt$

$$t_{90\%} = -\ln 0.9 / k \quad (3)$$

$$t_{1/2} = \ln 2 / k \quad (4)$$

Second order: $1/[A] = 1/[A]_o + kt$

$$t_{90\%} = 0.11[A]/k[A]_o \quad (5)$$

$$t_{1/2} = 1/k[A]_o \quad (6)$$

where $[A]_o$ is the initial content of andrographolide, [A] is the content of andrographolide at time t, and k is the rate constant.

Stability study of andrographolide from SEDS co-precipitates after addition into model food systems

Three different RTD beverages, namely drinking water, orange drink, and soybean milk, were selected as model food systems for the stability study of andrographolide after SEDS co-precipitates were respectively added into the food model systems. Thirty mg of SEDS co-precipitates were added into 10 mL of RTD beverages, then stirred, sealed, followed by storage in the dark under 5 ℃ for three days only since the intended application of the SEDS co-precipitates is for instant addition into RTD beverages for enrichment purposes before consumption. The andrographolide content was determined before the storage period and consecutively after 1, 2, and 3 days of storage. Samples were randomly collected in duplicate during the sampling period for the determination of the percentage of andrographolide content (%) using HPLC. The pH of RTD beverage mixtures was monitored and recorded before and along the storage period to check on the compatibility of added SEDS co-precipitates with different RTD beverages to help as well in defining the extent of the study period.

Toxicity of SEDS co-precipitates

Solvent residue test

Headspace GC-MS analysis with reference to Jacq et al. (2008) was conducted to detect the acetone residue in SEDS co-precipitates. Prior to GC-MS analysis, 20 mg of SEDS co-precipitates were weighed into a 20 mL headspace vial, followed by addition of 10 mL distilled water. The condition of the Agilent G1888 static headspace sampler was set as follows: vial equilibrium time of 10 min, oven temperature at 80 ℃, loop temperature at 120 ℃, transfer line temperature 120 ℃. Samples were then injected into a DB-624 GC column packed with 94% dimethyl polysiloxane and 6% cyanopropyl phenyl (20 m x 0.18 mm i.d. x 1 µm film thickness, Agilent J & W 121-1324, USA) housed in an Agilent 7890A GC/5975C MSD system comprising a gas chromatograph (GC) coupled with a mass selective detector (MSD). Helium was used as carrier gas at a flow of 1 mL/min. The stepped oven temperature program was set as follows: held at 40 ℃ for 3 min, then from 40 to 120 ℃ at the rate of 30 ℃/min, and from 120 to 220 ℃ at the rate of 40 ℃/min. The injector was set at 250 ℃ and injection mode with split ratio of 50:1 was employed. Solvent delay was 1 min. The total run time was 8 min. The following settings were applied in the operation of MSD: transfer line temperature at 280 ℃, ion source temperature at 230 ℃, quadrupole temperature at 150 ℃, mass scan from 30 to 300 (m/z). The presence of acetone in co-precipitates was identified and confirmed by comparing the retention time with that of acetone standard and the quantitation was conducted using selected ion monitoring (SIM) mode. The acetone standard calibration curve was plotted at the following concentrations: 0.1, 3.14, 6.28, 9.42, and 12.56 ppm.

Brine shrimp bioassay

Sample preparation was conducted as described in Meyer et al. (1982). Fifty mg of extract or sample were dissolved into 5 mL of methanol to form Solution A. Solution B was prepared by diluting 0.5 mL of Solution A to 10 mL with methanol. Amounts of 100 µL Solution B, 50 µL Solution A, and 500 µL Solution A were transferred to a small disc of filter paper to form 10, 100, and 1000 µg/mL samples, respectively, in the final 5 mL of artificial sea water. Control discs were prepared from the lowest amount (50 µL) to the highest amount (500 µL) of methanol without presence of extract or sample. The discs of filter papers were dried in air and placed into 5 mL vials. Ten replicates were prepared for each different dose level. To hatch the brine shrimp, first a clean hatching container was used. An open air tube was installed in the tip to give aeration while a light source was installed at the top of the container. The container was filled with fresh tap water followed by addition of brine shrimp eggs in sea salt premix. Continuous aeration was important to keep cysts in suspension. Hatching was completed after 24 h incubation at room temperature (29 ℃). The aeration and light source were removed from the container to allow the empty cyst shells to float while the live Artemia nauplii settle to the bottom of container. The nauplii were collected, rinsed with artificial sea water (prepared using 35 g NaCl in 1 L of tap water) and transferred into a container of fresh artificial sea water. For the bioassay, ten nauplii were transferred into each vial containing the disc of sample using a disposable pipette, and artificial sea water was added to make 5 mL. The nauplii could be measured macroscopically in the stem of pipette against a lighted background. A drop of yeast suspension (prepared using 3 mg in 5 mL artificial sea water) was added into the vial as food for the nauplii. A light source was provided along the incubation period for 24 h. After 24 h, the number of survivors in each vial was measured.

The percent of death at each dose level and control were determined. In cases where control death

occurred, the data were corrected using Abbott's formula (7):

$$\%\text{death} = \left(\left[\text{test} - \text{control}\right] / \text{control}\right) \times 100\%$$

Lethal concentration (LC_{50}) and 95% confidence interval were determined from the 24 h count through the probit analysis method using Minitab 16 software (Minitab Inc., State College, PA, USA).

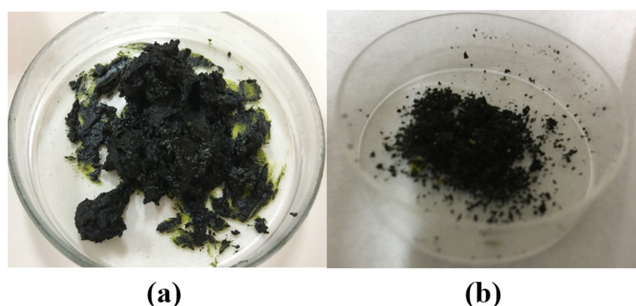
RESULTS AND DISCUSSION

Physical and chemical properties of SEDS co-precipitates

The SEDS process successfully allowed the transformation of the sticky paste of *A. paniculata* extract into SEDS co-precipitates after being formulated with Eudragit L100-55. During the SEDS process, sc-CO_2 acted as anti-solvent to extract out acetone that was used to dissolve both *A. paniculata* extract and Eudragit L100-55, leaving the formation of co-precipitates that were practically solvent-free in the precipitation chamber. As shown in Figure 2, the SEDS co-precipitates produced were visually good in terms of colour retainment and particulate formation when compared to the initial dark blackish green *A. paniculata* paste.

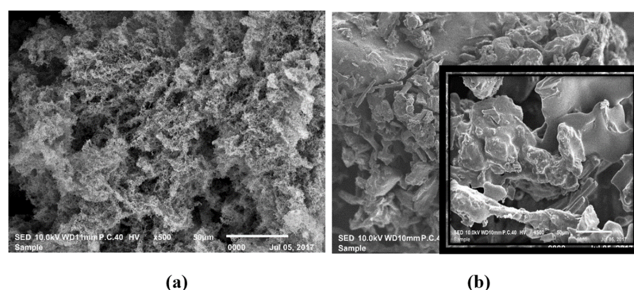
SEM photographs of both SEDS precipitated Eudragit L100-55 powder and SEDS co-precipitates are shown in Figure 3.

From the XRD plot as shown in Figure 4, it could be seen that the SEDS co-precipitates had lower crystallinity as compared to SEDS precipitated *A. paniculata* extract powder. SEDS precipitated *A. paniculata* extract powder was obtained at similar SEDS co-precipitation conditions, but with the absence of Eudragit L100-55 in the formulation. In addition, the SEDS co-precipitation process allowed



(a) **(b)**

Figure 2. Dark blackish green *A. paniculata* paste obtained from maceration using acetone as extraction solvent (a), SEDS co-precipitates 6:25 (Eudragit L100-55:*A. paniculata*) obtained at 150 bar, 40 ℃, 15 L/min of CO_2 flow rate, 0.5 mL/min of liquid feed flow rate, and acetone used as feed solvent (b).



(a) **(b)**

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Figure 3. Scanning Electron Microscopy (SEM) photographs of Solution Enhanced Dispersion by Supercritical Fluids (SEDS) precipitated Eudragit L100-55 (a), SEDS co-precipitates 6:25 (Eudragit L100-55:*A. paniculata*) obtained at 150 bar, 40 ℃, 15 L/min of CO_2 flow rate, 0.5 mL/min of liquid feed flow rate, and acetone used as feed solvent (b).

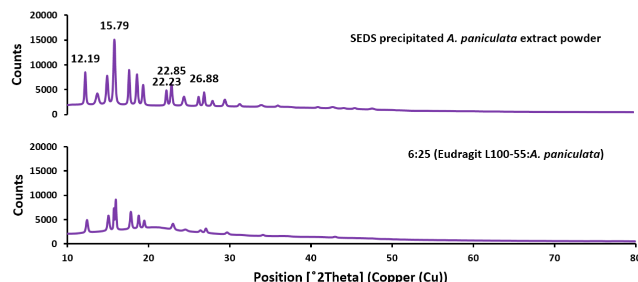

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Figure 4. X-Ray Diffraction (XRD) pattern of SEDS precipitated *A. paniculata* extract powder and SEDS co-precipitates 6:25 (Eudragit L100-55:*A. paniculata*) obtained at 150 bar, 40 ℃, 15 L/min of CO_2 flow rate, 0.5 mL/min of liquid feed flow rate, and acetone as feed solvent.

the co-precipitates formulated to contain 20.4% w/w andrographolide, while the initial andrographolide content of *A. paniculata* extract was 16% w/w. The increase of content of andrographolide in SEDS co-precipitates could be due to extraction of undesired compounds from the crude extract during SEDS co-precipitation, which resulted in an increase of concentration of andrographolide in SEDS co-precipitates compared to the starting material, in this case the crude extract. Similar findings of a concentration effect by the supercritical anti-solvent method, concentrating the active compound from 20% w/w in the crude extract up to 36% w/w in precipitates, has also been reported (Baldino et al., 2018).

Storage stability study of andrographolide from co-precipitates

The degradation of andrographolide from the SEDS co-precipitates during the sampling periods up to 64 days did not fit into zeroth-order kinetics as the plot of percentage of andrographolide versus time t showed curvature rather than being linear. Instead, it fitted first-order kinetics where the linearity was best met when ln [% of andrographolide] was plotted against time t for different storage temperatures as shown in Figure 5.

First-order kinetics of degradation of andrographolide were also reported by Wongkittipong et al. (2004). First-order degradation simply means that the degradation of andrographolide is concentration-dependent, therefore the amount of andrographolide degrading per unit of time is not constant for the co-precipitates. Most drugs tend to degrade by either zeroth- or first-order kinetics (Brooks et al., 2017). However, second-order kinetics of degradation of andrographolide was also reported for herb and solid dispersion in PVP-K30 (Lomlim et al., 2003; Plubrukarn et al., 2006). Second-order degradation means the degradation rate depends on either concentration of two same or two different reactants. Therefore, the difference in terms of degradation kinetics reported could be due to the different matrix or formulation of andrographolide being studied.

The first-order rate equation, rate constant (k), coefficient of determination (R^2) of andrographolide degradation, shelf-life ($t_{90\%}$), and half-life ($t_{1/2}$) of andrographolide of SEDS co-precipitates at different storage temperatures are shown in Table 1. It could be seen that the degradation rate constant of andrographolide from SEDS co-precipitates was found to be the highest at the highest storage temperature. Similar findings have been reported where the increment of temperature resulted in a greater degradation rate constant of andrographolide [rate constant = 0.0085 min^{-1} (86 ℃), 0.0229 min^{-1} (92 ℃), and 0.048 min^{-1} (97 ℃)], at higher temperature (Wongkittipong et al., 2004).

In terms of shelf life and half-life, it could be seen from Table 1 that the increment of storage temperature from 5 to 45 ℃ resulted in a decrement of both shelf life and half-life approximately up to 1.6-fold for SEDS co-precipitates. Because almost the same shelf life and half-life of andrographolide of SEDS co-precipitates are predicted at 5 and 30 ℃, the most probable storage condition suggested would be the ambient condition to be practical and economic in future applications. Similarly, an ambient storage condition for andrographolide from herb has also been suggested (Ibrahim and Chong, 2008).

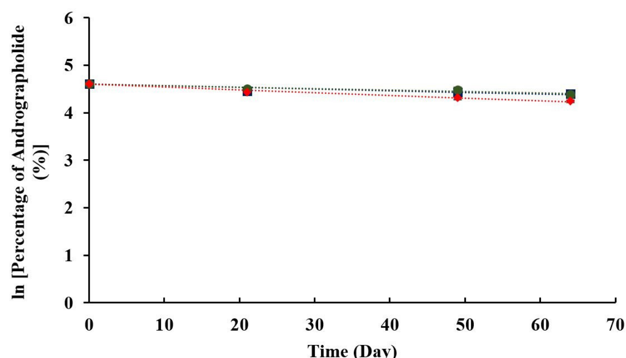

Figure 5. First-order plots of degradation of andrographolide from SEDS co-precipitates at 5 ℃ (■), calculated from the model for 5 ℃ (···), at 30 ℃ (●), calculated from the model for 30 ℃ (···), at 45 ℃ (♦), calculated from the model for 45 ℃ (···).

Stability study of andrographolide from SEDS co-precipitates after addition into model food systems

Along the stability period, the pH of each beverage mixture was found to be constant as shown in Figure 6. This showed compatibility of SEDS co-precipitates with different RTD beverages.

Figure 7 shows the degradation of andrographolide from SEDS co-precipitates after its addition into three different RTD beverages from day 0 to day 3.

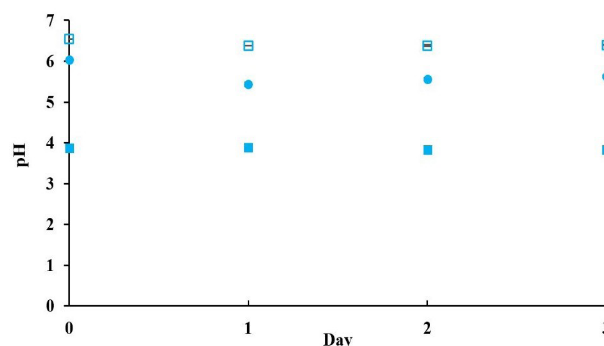

Figure 6. pH of beverage mixtures after addition of SEDS co-precipitates into drinking water (●), soybean milk (□), orange drink (■).

Table 1. First-order rate equation, rate constant (k), coefficient of determination (R^2) of andrographolide degradation, shelf-life ($t_{90\%}$), and half-life ($t_{1/2}$) of andrographolide from SEDS co-precipitates at different storage temperatures.

Temperature (℃)	SEDS co-precipitated *A. paniculata* with Eudragit L100-55			
	Rate equation	k (d^{-1})	$t_{90\%}$ (year)	$t_{1/2}$ (year)
5	ln A= -0.0035t + 4.6052 R^2 = 0.8372	0.0035	0.08	0.54
30	ln A= -0.0032t + 4.6052 R^2 = 0.8998	0.0032	0.09	0.59
45	ln A= -0.0059t + 4.6052 R^2 = 0.9720	0.0059	0.05	0.32

Table 2. Brine shrimp bioassay results.

Samples	Percent deaths of brine shrimp after 24 h exposure (μg/mL)			LC_{50} (μg/mL)	95% CI (μg/mL)
	10	100	1000		
Fresh *A. paniculata* extract	11	40	92	150.45±23.01	108.65-200.21
SEDS Co-precipitates	21	67	92	46.46±8.31	32.01-65.70

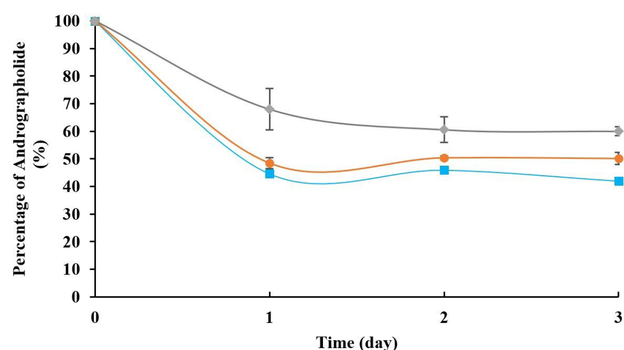

Figure 7. Degradation of andrographolide from SEDS co-precipitates in drinking water (■), orange drink (●), and soybean milk (♦).

Overall, the same trend of andrographolide degradation was observed from SEDS co-precipitates in all three different pH RTD beverages. After addition of SEDS co-precipitates into the RTD beverages for a day at 5 ℃, andrographolide had been degraded by about 40-70% of its initial concentration and the concentration of andrographolide remained in the consecutive days. Andrographolide from SEDS co-precipitates was found to be the least stable in drinking water compared to soybean milk and orange drink. This could be due to higher dissolved oxygen content in drinking water while the presence of different food components in soybean milk and orange drinks might confer a protective effect towards andrographolide. Similar findings on anti-oxidative effect of different food components of skimmed milk and orange drink on astaxanthin compared to water have also been reported (Anarjan and Tan, 2013).

Toxicity study

From the headspace GC-MS analysis, the estimated acetone content in the SEDS co-precipitates was found to be less than 0.1 ppm, which met the requirement set with permitted daily exposure (PDE) of 50 mg/day or 5000 ppm or higher provided that the amount is realistic in relation to manufacturing capacity according to the International Conference for Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Guideline Q3C(R6) on Impurities: Guideline for Residual Solvents (ICH, 2016). Throughout the studies reported on compound treatment by the supercritical anti-solvent process with usage of Class III solvents such as ethanol, acetone, and DMSO, sc-CO_2 acting as anti-solvent was able to extract out the solvent, resulting in an amount of detected solvent residue much lower than the International Conference on Harmonisation (ICH) requirements (Miao et al., 2018; Zhao et al., 2010). This shows the efficiency of the anti-solvent in the SEDS process in removing solvent from the feed during the precipitation and washing process in the precipitation chamber.

From Table 2, it could be seen that all samples tested in the brine shrimp bioassay, including fresh *A. paniculata* extract and SEDS co-precipitates, showed LC_{50} less than 1000 μg/mL. According to Meyer et al. (1982), compounds with LC_{50} less than 1000 μg/mL are considered bioactive in the brine shrimp bioassay.

Overall, the toxicity of both fresh *A. paniculata* extract and SEDS co-precipitates towards brine shrimp were concentration-dependent. The LC_{50} value obtained for fresh *A. paniculata* extract was close to the one reported for whole *A. paniculata* extract, which was in the range of 100 to 300 μg/mL (Mamatha, 2014). For a LC_{50} value in the range of 100 to 500 μg/mL, samples are considered to be non-toxic (Moshi et al., 2010). However, the SEDS co-precipitates with LC_{50} value between 30 and 100 μg/mL could be categorized as mildly toxic. While Eudragit L100-55 had been shown to be a safe drug carrier that displayed insignificant cytotoxicity in a study conducted by Hao et al. (2013), the increased LC_{50} of SEDS co-precipitates could be an indicative of its higher cytotoxic potential after SEDS co-precipitation. SEDS co-precipitation improved the dissolution of andrographolide of our formulated *A. paniculata* extract as determined in our previous study (data not shown) and could be making it more bioactive towards newly hatched brine shrimp.

CONCLUSION

The lower stability of andrographolide from SEDS co-precipitates in liquid food systems could be due to the presence of water as plasticizing agent that increased its molecular mobility and degradation. In view of the lower andrographolide stability in liquid food systems, the SEDS co-precipitates formulated are suggested to be more suitable to be dusted on dry food systems as toppings. For applications of SEDS co-precipitates in liquid food systems such as RTD beverages, instant addition followed by consumption is suggested to be more suitable. From the negligible residual solvent content of SEDS co-precipitates, the SEDS process conditions applied are capable of

removing most of the solvent during co-precipitation. Therefore, the higher cytotoxic potential of the SEDS co-precipitates may not be due to its residual solvent but rather the presence of potent cytotoxic components that warrant further investigation in suitable application dosages before incorporation into food.

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NOMENCLATURE

Ȧ Angstrom as measurement of thin film thickness

LC_{50} Lethal concentration

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