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FULL PAPER

An attempt to improve the solubility and stability of *Ocimum tenuiflorum* aqueous leaves extract

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Abstract

Medicinal plants have gained attention in recent years due to the presence of various bioactive compounds which promote distinct health benefits and is less toxic. However, the plants face common and serious problems due to their active compounds which are the solubility and stability. *Ocimum tenuiflorum*, one of the ancient medicinal plants were its medicinal values have been widely studies. Yet, study to improve its quality still lacking. Thus, an effort was taken to study and improve the solubility and stability of the plant. Solid dispersions were prepared with extract and different types of surfactants at different ratio have used to improve the stability. Lecithin (1:1 ratio) have improved the solubility significantly ($p<0.05$) when compared to extract. Enhancement in solubility, hence improved the stability when stored at 4°C of the solid dispersion of extract. Overall, the study has successfully improved the quality of the extract with lecithin complex whereby the solubility and increases stability.

1. Introduction

Herbal medicines have been used worldwide for several decades now on account of the large amounts of active phytoconstituents they contain (Jahan *et al.,* 2016). Medicinal plants are generally characterized with remarkable *in vitro* properties, but their efficiency *in vivo* is quite limited because of their low aqueous solubility, lipophilic properties and larger molecular size. These phenomena result in reduced absorption and bioavailability as the active compounds are unable to pass through the cell membrane (Gunasekaran *et al*., 2014). The term "solubility" should not be confused with the dissolving ability of a substance. According to the Biopharmaceutics Classification System (BCS), the solubility of a drug or compound could be classified as follows: class I - drugs with high solubility and permeability, class II - drugs with low solubility and high permeability, class III - drugs with low solubility and high permeability and class IV - drugs with low solubility and low permeability (Savjani *et al*., 2012).

Stability issues in natural products pose great concern to researchers. The active compounds of many medicinal plants are prone to degradation, particularly during storage, which also causes a loss of efficacy (Thakur *et al.,* 2011). Scientists have discovered that solubility and stability problems could be solved by inclusion or encapsulation of the target compounds into polymer-like cyclodextrins (CDs). CDs can improve the solubility and stability of various drugs and compounds (Ansari and Parveen, 2016). Besides CDs, phospholipids complexity (PC) can also improve the solubility, stability and permeation of compounds in the form of pharmacosomes (Semalty, 2014).

The genus *Ocimum* is a member of the Lamiaceae family. Plants from this genus are broadly distributed in most regions worldwide, including tropical, subtropical, and warm regions (Upadhyay *et al.*, 2015). *Ocimum tenuiflorum* belongs to the genus *Ocimum* and is commonly known as holy basil in some countries, such as India and Thailand. This aromatic medicinal plant is one of the most promising plants used in Ayurvedic treatments given its natural therapeutic properties (Upadhyay, Nachiappan, and Mishra, 2015). *O. tenuiflorum* contains various biologically potent phytoconstituents, including volatile oils, fatty acids, polysaccharides, and phenolic compounds (Rastogi *et al.*, 2015).

Our recent work on *O. tenuiflorum* (Rohini *et al*., 2019) revealed that leaf extracts of this plant have potential pharmacological properties and other researchers for past five years (Parasuraman *et al*., 2015; Mousavi *et al*., 2016; Francis *et al.*, 2017), suggest that leaves extract have potential pharmacological properties that could be beneficial for the medical field. Given the

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scarcity of research on the topic, the present study was carried out to improve the solubility and stability of *O. tenuiflorum* leaf extracts, which are known to have various pharmacological properties (Asha *et al.*, 2011; Suanarunsawat *et al.,* 2011; Jeba *et al.*, 2011; Ramteke *et al*., 2012; Basu *et al*., 2013) and synergistic effect (Soonthornsit *et al.,* 2017).

2. Materials and methods

2.1 Plant material collection and extraction

O. tenuiflorum leaves were collected at Perak from the botanical garden, identified and authenticated at School of Biological Sciences, University Sains Malaysia (USM) (Herbarium number is 11400). The leaves collected was preweighed and washed with tap water before dried in the oven at 60°C for 24 hrs. The dried leaves again weighed and powdered using heavy-duty blender. The powdered leaves then extracted with distilled water at a ratio of 1:10 according to the method described by Shetty *et al*. (2008) with some modification. Extraction was conducted with slight agitation of the sample using orbital shaker at 160 rpm at 27°C for 24 hrs. The supernatant was collected by centrifuging the mixture at 3500 rpm for 30 mins. Finally, dried crude extract of the leaves was obtained by drying the supernatant using an oven at 60°C.

2.2 Liquid chromatography-mass spectrometry (LC-MS) screening

The crude extract of *O. tenuiflorum* leaves was subjected LCMS screening to identify the bioactive compound present in it. The mobile phase used is 0.1% formic acid in water and acetonitrile (20:80). Injection volume was 2 µL at flow rate of 0.3 mL/min. The total run time was 10 mins. Negative ionization mode was employed with a capillary voltage of 2.5 kV and a source temperature of 200°C.

2.3 Selection of wavelength

Apigenin (API) was chosen as a marker compound based on LCMS screening result in the study. API powder was purchased from MedChemExpress (MCE), USA. About 1 mg accurately weighed and dissolved in 10 mL of dimethyl sulfoxide (DMSO). The solution was filtered through a 0.45 µm nylon filter. Then, about 3 mL of solution was transferred into glass cuvette and scanned between 300 to 400 nm using UV spectroscopy model UV-1600 series. (λ max was 340 nm)

2.4 High performance liquid chromatography (HPLC) setting

The HPLC condition was set up as per Raghu and Agrawal (2016) with slight modifications. The modified method was then validated as per ICH standard guideline (Guideline, 2005) for its linearity, selectivity, the limit of detection (LOD), limit of quantification (LOQ), accuracy, and precision before starting solubility test.

RP Waters, 2487 dual λ absorbance detector with Symmetry® C18 column (5.0 µm × 4.6 mm × 250 mm) was used as the HPLC apparatus. Acetonitrile and 0.1% trifluoroacetic acid in deionized water mixed at a ratio of 40:60 were used as the binary mobile phase. The mobile phase was degassed for 5 min and filtered through a 0.45 µm nylon filter. The chromatographic conditions were as follows: flow rate of 1.0 mL/min flow rate, injection volume of 20 µL, a column temperature of 25°C, and detector wavelength of 340 nm. The analysis was performed through isocratic elution with the total run time of 10 mins.

2.5 Standard preparation

About 1 mg of the standard API powder was accurately weighed and dissolved in 10 mL of DMSO to prepare the stock solution (100 µg/mL). Different concentrations of working solutions (50, 25, 12.5, 6.25, 3.13, 1.56 and 0.78 µg/mL) were prepared through serial dilution from the stock solution. The standard solution was filtered through a 0.45 µm nylon filter into vials. The calibration curve was plotted as the concentration of standard versus peak area response.

2.6 Evaluation of system suitability

System suitability was evaluated by injecting six replicates of the standard solution at the concentration of 50 µg/mL. Chromatograms were obtained. The accepted relative standard deviation (RSD) for the retention time and the peak area of the replicate injection should exceed 2.0%.

2.7 HPLC method validation

2.7.1 Linearity

The linearity of the method was analyzed by injecting the standard solution at eight different concentrations (100 µg/mL to 0.78 µg/mL). The calibration graph was plotted as the standard concentration versus the peak area response. Linearity was calculated through the least-squares regression method.

2.7.2 Selectivity

A placebo solution was prepared per test solution and injected into the HPLC system under the test conditions. The presence of any interfering peak at the retention time of API was determined.

2.7.3 LOD and LOQ

The limit of detection (LOD, signal-to-noise ratio of 3) is the lowest amount of analyte that can be detected. The limit of quantification LOQ, signal-to-noise ratio of 10) is the lowest amount of analyte that can be quantified with acceptable accuracy and precision. LOD and LOQ were evaluated using the following formulas: LOD = 3.3 × SD/S and LOQ = 10 × SD/S, where SD is the standard deviation of the response (peak area), and *S* is the slope of the calibration curve.

2.7.4 Precision

The intra- and interday precisions of the system and method were evaluated. A total of six replicates of the standard were injected at the concentration of 50 µg/mL on the same day for the measurement of intraday precision (n = 3). Nine replicates of the standard were injected over 3 consecutive days (n = 9) for the measurement of interday precision. RSD values were calculated.

2.7.5 Accuracy

The accuracy of the method was analyzed by calculating API recovery from spiked samples. Sample solutions were spiked with a known amount of the standard at 25, 50 and 100%. The spiking test was carried out in triplicate, and the recovery amount was expressed in mean percentage.

2.8 Preparation of solid dispersion

The solid dispersions of extract were done using the solvent evaporation method according to Wang *et al.* (2013). Three different types of surfactants, beta cyclodextrin (BCD), hydroxyl-beta cyclodextrin (h-BCD) and soy lecithin (LCT) were used to prepared the solid dispersions of extract. The extract and surfactant at ratio 1:1 and 1:2 were prepared by physical mixture using 30-70% ethanol until complete dissolution. The solutions beakers were closed and allowed to mix until equilibrium was reached for about 3- 4 hrs with agitation at 90 rpm in an orbital shaker.

2.9 Solubility test

About 5-50 mg of extract, solid dispersions 1:1 and 1:2 were weighed and dissolved in 1 mL of distilled water respectively. All the samples were sonicated at 45° C, frequency of 60 kHz for a while to ensure complete dissolution. Then, the samples were filtered into HPLC vials using a 0.45 µm syringe filter. The test was run as per modified and validated HPLC condition mention in section 2.4.

2.10 Storage stability study

Storage stability was studied after improvement of extract solubility has done. One type of solid dispersions was chosen based on the solubility test. The stability study was done with comparison to the extract for 8 weeks considering API as a marker compound. In this study, two forms of the extract and solid dispersions which are the dry and liquid form was evaluated for its stability. All the samples were stored in three different temperatures, room temperature, 3-6°C and 43-47°C with a relative humidity of 72±3%. The content of API in all the samples was quantified using HPLC method (Brinda and Tanuja, 2015) with slight modification.

2.11 Statistical analysis

All the data were analyzed using one-way ANOVA and Tukey multiple range test with SPSS software version 20. Data were plotted as mean ± standard error mean (SEM) and $p<0.05$ is considered statistically significant.

3. Results and discussion

3.1 LCMS screening of aqueous leaves extract

Table 1 shows the list of compounds that were detected in LCMS screening using aqueous leaves extract of *O. tenuiflorum*. The screening proved that the extract contained compounds from sugar, flavonoids, ester, lipid and others.

Table 1. LCMS screening of *Ocimum tenuiflorum* aqueous leaves extract

Compound name	Retention time (RT)	Mass
Glyceraldehyde	0.4036	90.032
Propionylglycine methyl ester	0.4064	145.0736
p-Hydroxymandelic acid	0.4173	168.0426
PS(21:0/18:3(6Z,9Z,12Z))	0.4183	827.5626
epi-4'-hydroxyjasmonic acid	0.4194	226.1207
Apigenin	0.4245	270.0532
Erythrono-1,4-lactone	0.4267	118.0269
7,8-Dihydroxycoumarin	0.4275	178.0272
p-Salicylic acid	0.4302	138.0319
trihydroxy-octadecadienoic acid	0.4311	328.2251
Fisetin	0.4343	286.0483
DL-3-hydroxy caproic acid	0.4373	132.0793
Phenyl acetate	0.4375	136.0527
3-propylmalic acid	0.4473	176.0682
Orthothymotinic Acid	0.4822	194.0942
6-Gingerol	0.4998	294.1821

O. tenuiflorum has been a promising plant since decades ago in treatment of various illness (Suanarunsawat *et al*., 2011) especially the leaves of the

plant where it helps to resolve most of the health issues such as fever, cough, pain, skin diseases and many more (Lahon and Das, 2011). Since the benefits of leaves have been proved traditionally and scientifically, it is important to explore deeper with the leaves. This study was conducted in order to improve the solubility and stability of the plant leaves extract for better improvement of its biological properties.

3.2 Selection of wavelength

Figure 1 shows the UV absorbance spectra of API whereby the maximum absorbance was observed at the wavelength of 340 nm. Thus, this wavelength was selected for further studies using HPLC method.

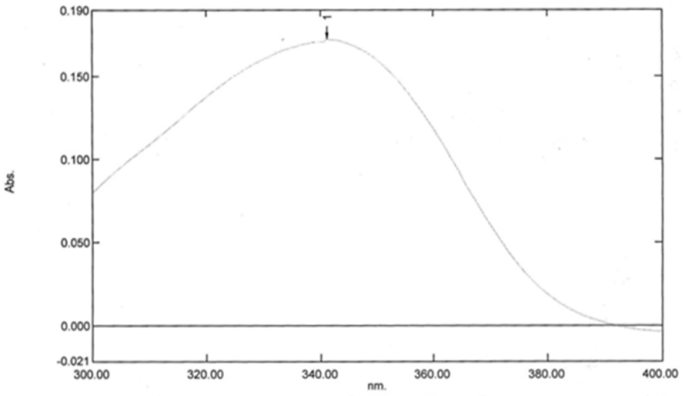

Figure 1. Peak for maximum UV absorbance spectra of API

3.3 System suitability

The HPLC system suitability was evaluated by injecting six replicates of 50 µg/mL of API standard solution. The acceptance criterion should not be more than 2% RSD for the retention time and peak area. The results are shown in Table 2.

Table 2. System suitability study

	Retention Time (min)	Peak Area (mV.s)
Mean (n=6)	8.472	4242.664
SD	0.09	72.12
RSD (%)	1.07	1.70

As preferred by many, oral administration is the most convenient and easy route of administration among other routes (Rabti *et al*., 2014). Solubility is an important factor associated with absorption of compounds that were administered orally. Appropriate solubility in gastrointestinal fluid and permeation through cell membranes is necessary for proper absorption of administered substances (Yousaf *et al*., 2018).

According to Jahan *et al*. (2016), phytochemicals of medicinal plants have relatively low absorption and bioavailability. In this study, the LCMS screening of *O. tenuiflorum* aqueous extract revealed that it contains compounds from varieties of groups like flavonoids, lipids, ester, sugars and others. Thus, it was necessary to improve the solubility of the extract in order to enhance its absorption and bioavailability. Numbers of techniques have been utilized by researchers for the improvement of solubility like inclusion complex (Canbolat *et al*., 2014; Mangolim *et al*., 2014; Qiu *et al*., 2014), micronization (Rasenack and Müller, 2002; Bansal *et al*., 2011; Khadka *et al*., 2014) hydrotrophy (Agrawal *et al*., 2004; Kim *et al*., 2010; Abraham *et al*., 2014), solid dispersion (Patel *et al*., 2008; Sinha *et al*., 2010; Tran *et al*., 2013).

3.4 HPLC method validation

3.4.1 Linearity

The calibration curve plotted for eight concentrations of the standard over peak area response indicate the linearity of the method with correlation coefficient (R^2) equals to 0.9998. The regression analysis is shown in Figure 2.

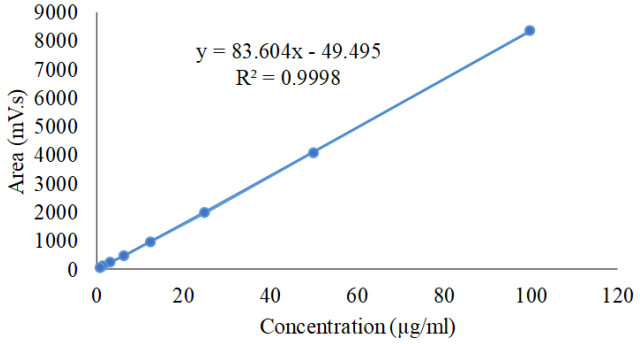

Figure 2. Calibration curve of the standard

3.4.2 Selectivity

Based on Figure 3 and 4, it shows that the system is highly specific and selective as no interfering peak was eluted at the retention time of API.

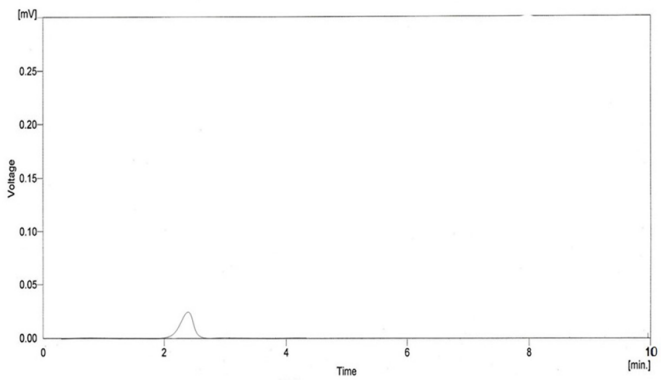

Figure 3. Chromatogram of blank solution

3.4.3 LOD and LOQ

LOD and LOQ values were calculated on the basis of a linear graph equation. The LOD and LOQ values of the triplicate sample injections were 0.034 and 0.102 µg/mL respectively.

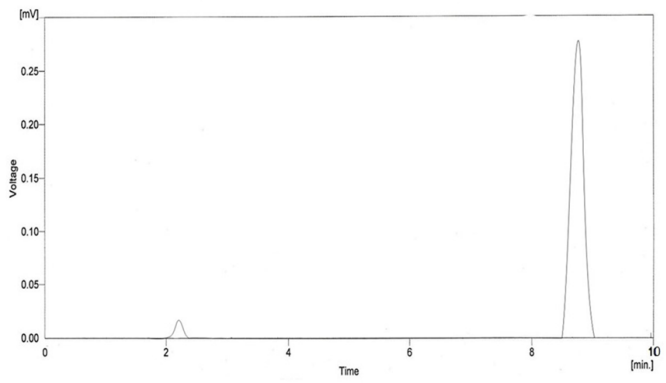

Figure 4. Chromatogram of the standard solution. The acceptance criterion is whereby the RSD % value is below 2%. The percentages of recovery of all three levels were in the range of 101.3 to 101.8%.

3.4.5 Precision

The results of inter- and intraday precision measurements of the developed method are listed in Table 3. The acceptable criterion for the precision of the HPLC method is RSD <2%. The proposed system provided RSD% values of less than <2% for both inter- and intraday measurements. These results suggest that the proposed system could precisely measure API.

Table 3. Peak areas of three replicates injection of standard (50 µg/mL)

	Day 1	Day 2	Day 3
1	4178.803	4312.207	4083.187
2	4196.078	4326.561	4097.964
3	4183.995	4318.475	4088.223
Mean	4186.292	4319.081	4089.791
SD	8.864	7.196	7.512
RSD %	0.212	0.167	0.184

3.4.6 Accuracy

The accuracy of the method was analyzed at three levels which are 25, 50 and 100%. The result was presented in Table 4. The recovery of the analyte found to be below the acceptance criterion whereby the RSD % value is below 2%. The percentages of recovery of all three levels were in the range of 101.3 to 101.8%.

Table 4. Recovery percentage of three replicates injection of standard at three levels

	25%	50%	100%
RSD	0.38	0.21	0.11
Recovery %	101.86	101.33	101.59

3.5 Solubility test

Figure 5 shows the solubility test results of solid dispersions from three different types of surfactants compared with that of the original leaf extract. Extract solubility was significantly ($p<0.05$) improved by using lecithin at 1:1 ratio compared with other types and the ratio of surfactants ($p>0.05$).

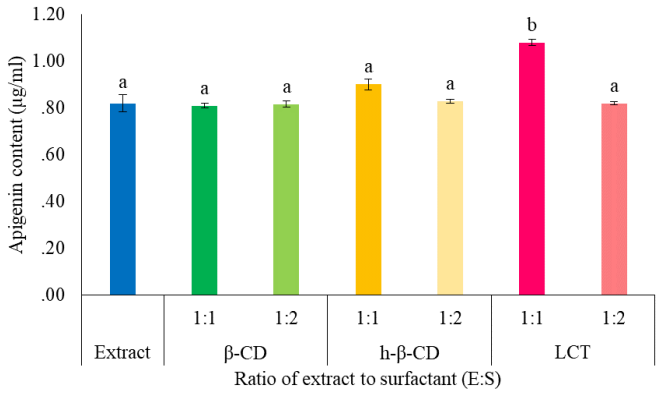

Figure 5. Solubility test of extract and SDs (1:1 and 1:2). Vertical bars represent the standard error mean (n = 3). Bars with different alphabets are statistically significant when ($p<0.05$).

Although oral delivery is the preferred administration route for many herbal medicines, this method often reduces the bioavailability of the administered compounds. According to Gonçalves *et al*. (2016), poor aqueous solubility is amongst the main reasons behind the low bioavailability of active compounds after oral administration. Thus, improvements in solubility may also be expected to improve the bioavailability of target compounds after administration.

Amongst the available approaches for solubility enhancement, solid dispersion is one of the most widely used (Kommavarapu *et al*., 2015). Solid dispersions of poorly soluble compounds could be achieved via melting, kneading, solvent evaporation and lyophilization. Solvent evaporation is an excellent technique to enhance the solubility and dissolution rate of various compounds (Yousaf *et al*., 2018).

The solubility test of solid dispersion *O. tenuiflorum* aqueous leaves extract was performed using HPLC equipment. The HPLC setting has been slightly modified and validated to reduce the time consumption for each run. The method developed and validated by (Raghu and Agrawal, 2016), requires a total run time of 40 mins for API separation through isocratic elution. By contrast, the proposed method required a total run time of 10 mins for API separation through isocratic elution. The modified method was validated and optimized with a linearity of R^2 = 0.9998 for different standard concentrations (100–0.78 µg/mL). The proposed system is highly suitable for API detection given that it provided a RSD% of <2%. The system is highly selective for API because no

interfering peak can be observed at the retention time of the standard solution. The system presented satisfactory LOD and LOQ values of 0.034 and 0.102 µg/mL respectively, with acceptable ranges of accuracy and precision below the RSD of 2%. Hence, this method was followed for solubility and storage stability in this study.

The solubility of *O. tenuiflorum* aqueous leaf extract was successfully enhanced by adopting lecithin at a ratio of 1:1 using the solvent evaporation-based solid dispersion method. Improvements in extract solubility may be attributed to the molecular structure of lecithin, which features amphipathic owning the amphipathic property as stated by Wang *et al*. (2013). Lecithin molecules are arranged in the form of bilayer sheets that allow the attachment of hydrophilic and hydrophobic compounds (Saraf, 2010). Similar to lecithin, CD derivatives, such as BCD and h-BCD, have also have been proven to improve the solubility of poorly soluble compounds (Yuvaraja and Khanam, 2014; Zhao *et al.,* 2016). However, in this study, the solubility of the extracts could not be enhanced by the CD derivatives, likely because of the unique structure of CD. CDs are cyclic polymers composed of a hydrophobic interior and hydrophilic exterior that form a cone-shaped structure. This type of structure limits the loading of a compound either small or larger in size (Miranda *et al*., 2011). Semalty (2014) said that higher CD ratios (e.g., 1:3, 1:4 and so on) are required to improve the solubility of larger compounds, especially those from medicinal plants.

3.6 Stability study

Figures 6, 7 and 8 show the stability of extract and solid dispersions of the extract with lecithin 1:1 ratio (dry and liquid). Figure 6 and 7 show a similar trend of API degradation whereby 0% of API content was available in liquid-solid dispersions, liquid and dry extract samples while 98% API content available in dry solid dispersions during the 2nd weeks of study. After 2 weeks, the API in the dry solid dispersions was rapidly degraded, and 0% API was available in the 4th and 8th weeks of the study. Changes in API content in Figure 8 are similar to those in Figure 6 and 7. Specifically, 0% API was available in the dry and liquid extracts by the 2nd week of the study. When the liquid-solid dispersions were stored at 4°C, however, remarkable changes in API degradation were observed. Specifically, API remained available up to the 4th week of the study. Indeed, approximately 90% of the API content of the liquid-solid dispersions remained available up to the 8th week when stored at 4°C.

The loss of active compounds during storage is an important factor that should be considered in evaluating the quality of natural products (Thakur *et al.*, 2011). Active compounds are also highly susceptible to degradation or breakdown into inactive by-products in the circulation, which reduces their bioavailability (Coimbra *et al*., 2011). Because the LCMS result of the *O. tenuiflorum* aqueous leaf extract confirms that it is made up of various compounds, the stability of this extract must be assessed. Thus, the storage stability and bioavailability (*in vitro* drug release) of the extract were studied after its solubility was improved with lecithin at a 1:1 ratio.

The stability of the solid dispersions compounds was stable and remained available about 90% in dry form at 4°C, by referring API as a marker compound. Compared with the original extract, solid dispersion compounds with lecithin demonstrated enhanced stability. Improvement in the stability of the solid dispersions compared with that of the extract may be due to the formation of a coating on the former during lecithin complexation was prepared (Kumar *et al.*, 2014). This coating prevents the leakage of active compounds during storage (Semalty, 2014). However, the stability of solid dispersion compounds stored at room temperature and 45°C could not be maintained by lecithin complexation because lecithin is sensitive to heat (Wang *et al*., 2014).

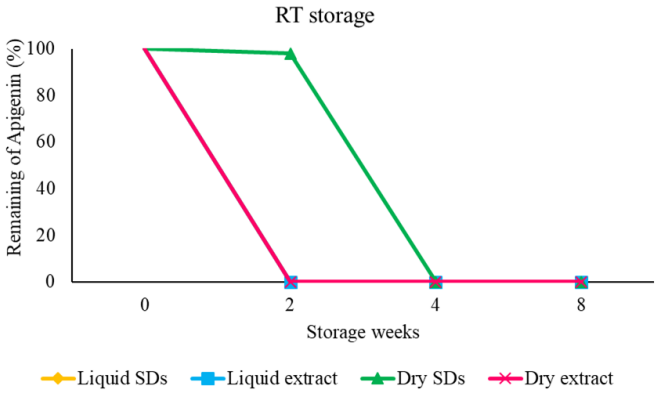

Figure 6. Stability of the extract and SDs samples stored at RT.

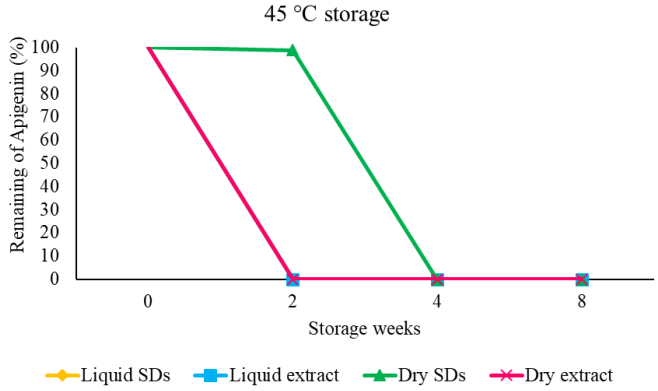

Figure 7. Stability of the extract and SDs samples stored at 45° C

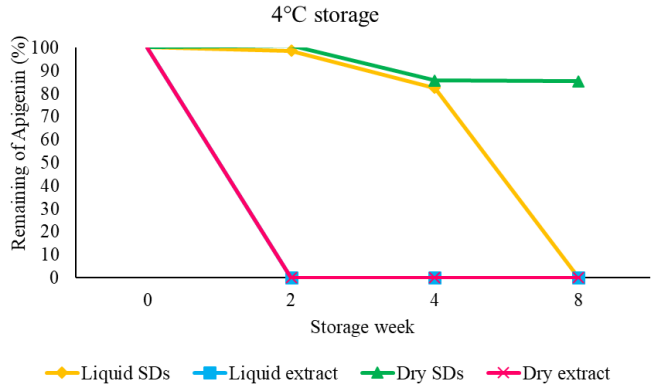

Figure 8. Stability of the extract and SDs samples stored at 4° C

4. Conclusion

This study proved that *O. tenuiflorum* aqueous leaf extracts are poorly soluble because of the various types of compounds they contain. The compounds present in these extracts are not stable in dry or liquid form during storage and have poor dissolution properties. In this work, we improved the solubility of *O. tenuiflorum* aqueous leaf extracts via the solid dispersion method. The solubility of the extracts was successfully enhanced by lecithin complexation, and the stability of the compound in the dry solid dispersion sample was prolonged during storage at 4°C.

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