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Quantitative HPLC method and alternative green solvents for extraction of [6]-gingerol from ginger

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Abstract: A simple and validated HPLC method for quantitative analysis of [6]-gingerol in ginger extracts was established. The optimum HPLC conditions involved a TSK-gel ODS-80 Ts column (5 µm, 4.6 × 250 mm) eluted with acetonitrile and 1% aqueous acetic acid (48:52, v/v), at 30ºC, with a flow rate of 1 mL/min and a quantitative UV detection at 280 nm. A good selectivity, linearity (R^2 of 1.0000), accuracy (Recovery of 96.7-103.2%), intraday- and interday-precision (Relative standard deviation (RSD) of less than 2% and 5%, respectively) and sensitivity (LOD and LOD of 0.48 and 0.80 µg/mL, respectively) were obtained. A microwave-assisted extraction was used for preparation of [6]-gingerol enriched ginger extracts. Based on HPLC analysis, the concentrations of [6]-gingerol of the extracts obtained from 20% v/v glycerin in ethanol (0.45 mg/mL) as well as 20% v/v natural deep eutectic solvents (NADES) of sucrose & citric acid in ethanol (0.43 mg/mL) were not significantly different from ethanol (0.42 mg/mL). However, glycerin (20% v/v) or NADES of sucrose & citric acid (20% v/v) might be used as the alternative green solvents for preparation of [6]-gingerol extract that could be directly used for formulation of anti-nausea and vomiting pastilles.

Keywords: Ginger, [6]-gingerol, green solvent, HPLC, microwave.

INTRODUCTION

Ginger (*Zingiber officinale* Roscoe, family Zingiberaceae) has been used in traditional Chinese, Ayurvedic and Unani-Tibb medicines for treatment of various diseases, including gastrointestinal diseases, such as nausea and vomiting, and respiratory diseases (Ali *et al*., 2008). The rhizomes contain volatile oil and oleoresin. The oleoresin is composed of a homologous series of phenolic compounds, i.e., [6]-shogaol, [4]-gingerol, [6]-gingerol, [8]-gingerol, and [10]-gingerol. [6]-Gingerol is a major pharmacologically active compound, which possesses anti-nausea, anti-emetic, anti-inflammatory, antioxidant, anticancer and antitumor activities (Wang *et al*., 2014; Wang *et al*., 2018). In addition, clinical studies and meta-analysis have supported the use of ginger powder for suppressing the sensation of nausea and vomiting. However, neither of these clinical studies used a standardized ginger powder (Viljoen *et al*., 2014; Giacosa *et al*., 2015; Nikkhah Bodagh *et al*., 2019). Therefore, consistency of its efficacy from batch to batch may be in doubt.

The green extraction method is a strategy applied in herbal product development that has gained a lot of attention in recent times (Lateh *et al*., 2019; Sae-Lim *et al*., 2019). Most conventional extraction approaches require high energy, lengthy time, and large volume of organic solvents. There are various reports on ginger extractions using volatile and hazardous organic solvents that are currently prohibited in phytopharmaceutical products, including benzene, chloroform, hexane, acetone, and methanol (Usman *et al*., 2013). Furthermore, supranational entities and regulatory agencies have also expressed an urgent need to reduce the footprint of hazardous chemicals in both the environment and consumer products. The EU in its framework of environmental policy and legislation for 2010-2050 indicated that reducing the use of noxious solvents is a key priority (Bubalo *et al*., 2018). The 'green extraction' approach with natural deep eutectic solvents can enable the attainment of these worthwhile objectives.

Some pharmaceutical excipients, such as isopropyl myristate, mineral oil, propylene glycol, glycerol and natural deep eutectic solvents are already being used as alternative green solvents for herbal extraction. The natural deep eutectic solvents (NADESs) are a mixture of natural eutectic components, i.e., sugar, sugar alcohol, polyalcohol, organic acid, amino acid, choline, and urea (Vanda *et al*., 2018). The use of NADESs as alternative green solvents stems from their biodegradability, affordability, and low toxicity (Kudłak *et al*., 2015). NADESs have been used for extraction of phytochemicals, including curcuminoids (Liu *et al*., 2019), anthocyanins (Panić *et al*., 2019), rutin (Huang *et al*., 2017) and isoflavones (Bajkacz and Adamek, 2017). Quite recently, the NADES of betaine, lactic acid and water has been used as alternative green solvent for extraction of phenolic compounds from ginger using an ultrasound assisted extraction (Tzani *et al*., 2021). On the

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**Corresponding author:* e-mail: pharkphoom.p@psu.ac.th other hand, a microwave extraction reportedly increased the yield of [6]-gingerol as well as reduced the time for extraction (Liu *et al*., 2014; Guo *et al*., 2017; Peng *et al*., 2017). Therefore, combining this extraction method with the use of alternative green solvents may increase the quality of herbal extracts and reduce the cost of production due to reduced energy and time.

A quantitative HPLC method for [6]-gingerol has been described by Cafino *et al*. (2016) using an Inertsil ODS-3 column, with an isocratic elution of methanol and water (90:10, v/v). However, the method produced poor resolution of [6]-gingerol in the sample, consuming high amounts of methanol for sample preparation. Moreover, a full validation of the analytical procedures is not yet established. Although a green RP-HPTLC-densitometric method for determination of [6]-shogaol and [6]-gingerol has been recently described (Foudah *et al*., 2020), the method required many steps that are time consuming and also high amounts of methanol needed for sample preparation. The present study, therefore, established a simple and validated quantitative HPLC method for [6]-gingerol as well as a green microwave extraction approach for preparing [6]-gingerol enriched ginger extracts based on alternative green solvents from pharmaceutical excipients currently used in pastilles, such as NADESs and glycerin.

MATERIALS AND METHODS

Reagent and chemicals

Standard 6-gingerol was obtained from Sigma-Aldrich (St. Louis, MO, USA). Methanol and acetonitrile (HPLC grade), ethanol and acetic acid (analytical grade) were purchased from Labscan Limited (Songkhla, Thailand). Ultrapure water was obtained using a Milli-Q system (Millipore, Bedford, MA, USA). Glycerin was purchased from S. Tong Chemicals Co., Ltd. (Nonthaburi, Thailand). Citric acid, sucrose and glucose were purchased from High Science Ltd. Partnership (Hat-Yai, Thailand).

Plant material

Ginger rhizomes were obtained locally in Hat-Yai, Southern Thailand. A voucher specimen (SKP 117 06 06 01) was domiciled at the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand. Briefly, fine ginger rhizome powder was obtained in four steps in sequence, viz; cutting the fresh rhizomes into smaller pieces, drying them in a hot air oven (55°C for 48 h), pulverizing the dried sample and sieving it through a no. 45 sieve.

HPLC analysis

The HPLC analysis was performed using a Shimadzu LC-20A Series High Performance Liquid Chromatography coupled with an SPD-M20A photodiode array (PDA) detector and SIL-20AHT autosampler. A good resolution of [6]-gingerol was achieved at 30°C using TSK-gel ODS-80 Ts column (5 µm, 4.6 × 250 mm) as the stationary phase, acetonitrile and 1% v/v aqueous acetic acid (48:52, v/v) as the mobile phase, eluted at 1.0 mL/min with sample/standard injection volume at 20 µL and detection at 280 nm.

Standard [6]-gingerol solutions

A stock solution of the standard [6]-gingerol was prepared by accurately weighing 2 mg of [6]-gingerol, dissolve in methanol and the volume adjusted to 10 mL. Five concentrations (12.5-200 µg/mL) of the standard solution were prepared from the stock solution by a serial 2-fold dilution. The solutions were filtered through a 0.45 µm membrane filter and subsequently used to construct the calibration curve.

Sample preparation

Dried ginger powder (4.0 g) was extracted with ethanol (100 mL) using a microwave extraction (2,450 MHz and 450 W), at 68±2°C, with three irradiation cycles (1 cycle was 60 sec power-on, and 30 sec power-off). The extract was then filtered and evaporated under reduced pressure at 40°C. The dried extract (20 mg) was reconstituted and adjusted to 10 mL with methanol. All samples were filtered through a 0.45µm membrane filter prior to quantitative HPLC analysis.

Method validation

The method was validated according to the International Conference on Harmonization of Technical Requirement for the Registration of Pharmaceuticals for Human Use (Guideline, 2005). The parameters of specificity, linearity, accuracy, precision, limit of determination (LOD) and limit of quantitation (LOQ) were evaluated to validate the HPLC method. The experiments were performed in triplicate.

Specificity

Specificity of the method was performed using a comparison of retention times as well as UV absorption spectra produced by a PDA detector of the standard [6]-gingerol and sample peaks. UV absorption spectra were taken at three different points of the peaks to determine peak homogeneity.

Linearity

Calibration curves were prepared using five concentrations (12.5-200 µg/mL) of [6]-gingerol and plotting peak areas against corresponding concentrations. Linearity of the standard curve was determined by means of linear regression. The coefficient of determination (R^2) must be no less than 0.9990.

Accuracy

Accuracy of the method was determined using a standard addition method. Three different concentrations of the standard [6]-gingerol (100, 50 and 25 µg/mL) were spiked into the sample solution at ratio of 1:1 (v/v). The concentration of [6]-gingerol in the sample was determined prior to the standard spiking for calculation of actual recoveries. A good accuracy is achieved when the percentage recoveries are in the ranges of 90-110%.

Precision

Precision of the method was evaluated based on repeatability (intra-day precision) and reproducibility (inter-day precision). A sample solution was analyzed in six replicates within a day to evaluate repeatability, while reproducibility evaluation was performed by sample extraction and HPLC analysis on three different days. The results were used to calculate a percentage relative standard deviation (%RSD) for evaluation of repeatability (RSD of less than 2%) and reproducibility (RSD of less than 5%).

LOD and LOQ

Serial dilutions of the sample solutions were performed in methanol and analyzed by the HPLC method to determine LOD and LOQ, which were expressed by means of the minimum concentrations that produce the signal-to-noise ratios of 3:1 and 10:1, respectively.

Preparation of NADESs

NADESs of sucrose and citric acid, sorbitol and citric acid, as well as glucose and citric acid were prepared by mixing both components at 1:1 molar ratio in 30% water and stirred at 80ºC until a clear solution was obtained. The NADESs were stored tight-lid containers at 26°C and protected from light.

Determination of NADESs for extraction

NADESs were dissolved in ethanol to produce 50% v/v NADESs. Dried ginger powder (1.0 g) was extracted with 50% v/v NADESs in ethanol and absolute ethanol (25 mL) using a microwave extraction (2,450 MHz and 900 W), at 68 ± 2°C, for 1 min. The extracts were then filtered and the volume adjusted to10 mL with methanol. The diluted extract was filtered via a 0.45 µm membrane filter and then analyzed. The experiments were performed in triplicate.

Determination of sucrose & citric acid NADES and glycerin for extraction

Four concentrations of the NADES (sucrose and citric acid) in ethanol as well as glycerin in ethanol (10, 20, 30, and 50% v/v) were evaluated for their ability to increase the extraction of [6]-gingerol using the method described above. All extracts (1 mL) were diluted and adjusted to 10 mL with methanol, then filtered through a 0.45 µm membrane filter and subjected to the quantitative HPLC analysis. The experiments were performed in triplicate.

Greenness assessment

The AGREE metric technique was used to evaluate the greenness of the HPLC analysis. The software documentation for the analytical greenness calculator version 0.5 was used to calculate the scale (0.0-1.0) of the HPLC analysis. Twelve principles of green analytical chemistry were determined as the assessment criteria, including direct analytical techniques should be applied to avoid sample treatment, minimal sample size and minimal number of sample are goals, *in situ* measurement should be performed, integration of analytical process and operations save energy and reduces the use of reagents, automated and miniaturized methods should be selected, derivatization should be avoided, generation of large volume of analytical waste should be avoided and proper management of analytical waste should be provided, multianalyte or multiparameter methods are preferred versus methods using one analyte at a time, the use of energy should be minimized, reagents obtained from renewable source should be preferred, toxic reagents should be eliminated or replaced, and the safety of the operator should be increased (Pena-Pereira *et al.*, 2020).

STATISTICAL ANALYSIS

Results were presented as mean ± SD. The statistical analysis was calculated using one-way analysis of variance (ANOVA) and Tukey's test by SPSS version 16.0. Statistical significance was considered as $P<0.05$.

RESULTS

HPLC analysis and method validation

The appropriate HPLC conditions for quantitative HPLC analysis of [6]-gingerol in ginger extracts were successfully obtained using a reverse phase HPLC system consisting of a TSK-gel ODS-80 Ts column and an isocratic elution with acetonitrile and 1% aqueous acetic acid (48:52 v/v). The peak of [6]-gingerol was observed at 12 min with a good resolution (fig. 1).

In the present study, validation of the HPLC method was accomplished by defining the specificity, linearity, accuracy, precision, and sensitivity (LOD and LOQ). The results are shown in table 1 and fig. 2.

Determination of alternative green solvents

The solutions of 50% v/v NADESs in ethanol, including glucose and citric acid, sorbitol and citric acid, as well as sucrose and citric acid, were initially examined for their effectiveness with respect to extraction of [6]-gingerol. We found that the concentrations of [6]-gingerol in the extracts obtained from all solvents were not significantly different ($P<0.05$) (table 2).

The effects of glycerin and NADES concentrations in ethanol (10%, 20%, 30%, and 50%) were further determined for extraction of [6]-gingerol. The results are shown in table 3.

Table 1: Validated data of quantitative HPLC method for [6]-gingerol in ginger extract

Parameters		Validated data
Linearity Equation [a]		Y = 11811X + 4413.6
Coefficient of determination (R^2)		1.0000
Linear range (µg/mL)		12.5 - 200
LOD (µg/mL)		0.48
LOQ (µg/mL)		0.80
Accuracy (%Recovery)	+ 100 µg/mL [6]-gingerol*	103.2 ± 0.9
	+ 50 µg/mL [6]-gingerol*	101.0 ± 2.9
	+ 25 µg/mL [6]-gingerol*	96.7 ± 1.5
Precision (%RSD)	Intraday *(n=6)*	0.4
	Interday *(n=3)*	2.5

[a] Y = peak area; X = concentration (µg/mL), LOD and LOD are limit of detection and limit of quantification, respectively. * Spiked concentration of standard [6]-gingerol

Table 2: Determination of the efficacy of 50% v/v NADESs in ethanol as a green solvent for extraction of [6]-gingerol

Solvents	[6]-Gingerol concentration (mg/mL)
Ethanol	0.42 ± 0.01
50% NADES of glucose & citric acid in ethanol	0.45 ± 0.04
50% NADES of sorbitol & citric acid in ethanol	0.41 ± 0.01
50% NADES of sucrose & citric acid in ethanol	0.43 ± 0.01

Data were expressed as mean ± SD (n = 3).

Table 3: Determination of NADES (sucrose and citric acid) and glycerol in ethanol as a green solvent for extraction of [6]-gingerol

Solvents	[6]-Gingerol concentration (mg/mL)
Ethanol	0.42 ± 0.01^{ab}
10% Glycerin in ethanol	0.40 ± 0.00^{b}
20% Glycerin in ethanol	0.45 ± 0.01^{a}
30% Glycerin in ethanol	0.40 ± 0.01^{b}
50% Glycerin in ethanol	0.40 ± 0.01^{b}
10% NADES in ethanol	0.41 ± 0.02^{ab}
20% NADES in ethanol	0.43 ± 0.02^{ab}
30% NADES in ethanol	0.43 ± 0.04^{ab}
50% NADES in ethanol	0.40 ± 0.02^{ab}

NADES = NADES of sucrose & citric acid, Data were expressed as mean ± SD (n = 3). Statistical significances (*P*<0.05) were shown in different letters in the same column.

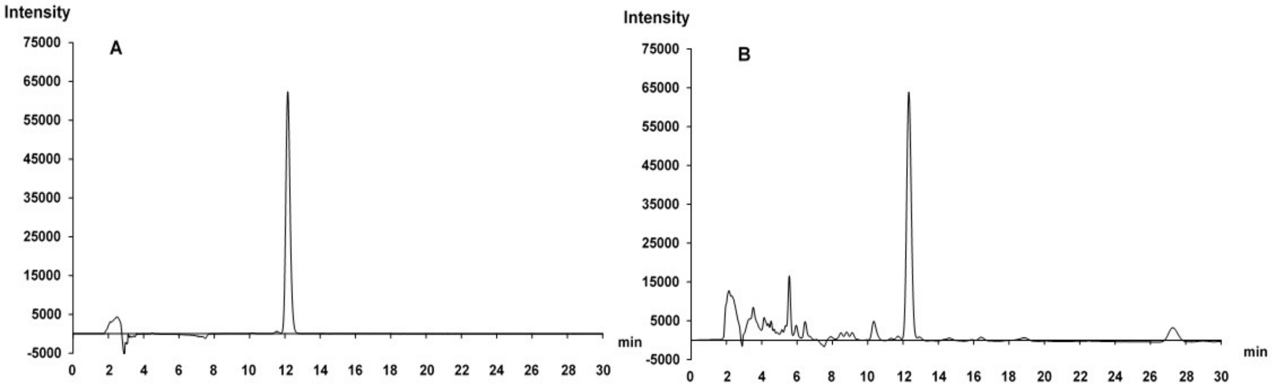

Fig. 1: HPLC chromatograms of [6]-gingerol (A) and ginger extract (B)

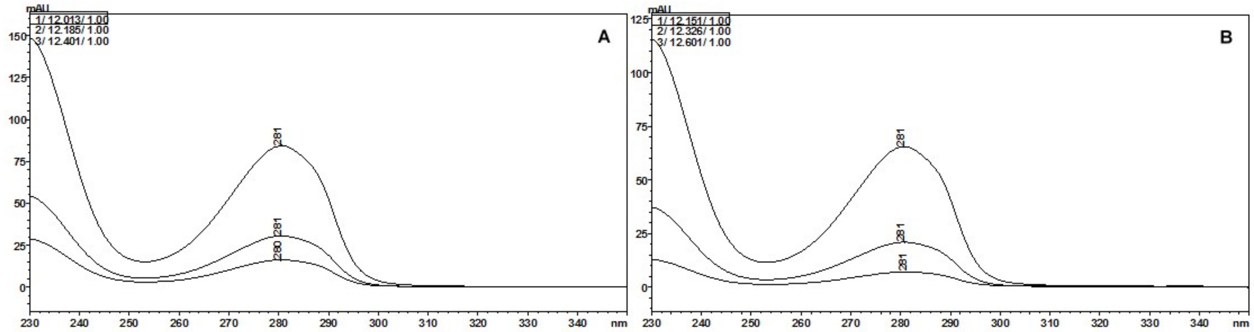

Fig. 2: UV absorption spectra of standard [6]-gingerol (A) and sample peak at 12 min (B)

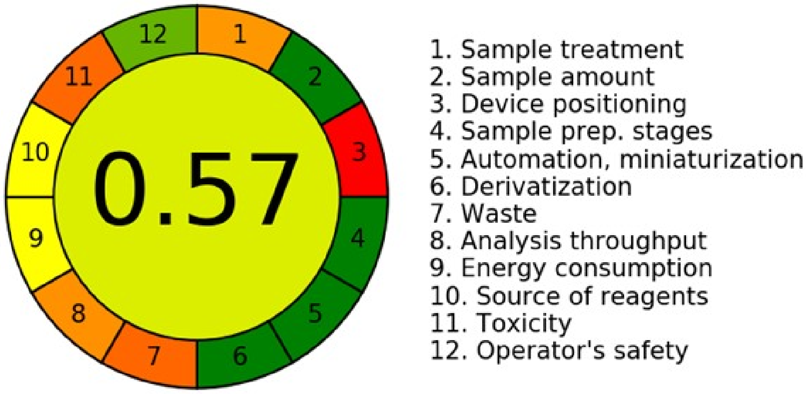

Fig. 3: Analytical GREEnness (AGREE) scale for the HPLC method

DISCUSSION

HPLC analysis and method validation

Various ratios of acetonitrile and acetic acid (1%, v/v) were examined as the mobile phase until a good resolution of [6]-gingerol peak was obtained. The appropriate HPLC conditions for quantitative HPLC analysis of [6]-gingerol were successfully obtained using a reverse phase HPLC system consisting of a TSK-gel ODS-80 Ts column and an isocratic elution with acetonitrile and 1% aqueous acetic acid (48:52 v/v). Although there are some reports currently on HPLC methods for quantitative analysis of [6]-gingerol in *Z. officinale* extracts (Pawar *et al*., 2011; Salmon *et al*., 2012; Schwertner and Rios, 2007; You *et al*., 2019), these methods are accompanied by some notable limitations, including poor resolution of [6]-gingerol peak, time-consuming process of sample preparation and analysis, and the need for a gradient system for mobile phase elution. Moreover, some of these HPLC methods were not validated.

The retention time and UV absorption spectra of the sample peak at 12 min were identical to that of the standard [6]-gingerol (Fig. 2). The results indicated homogeneity of the peak as well as specificity of the method. Linearity was evaluated using an R^2 value calculated from the calibration curve of [6]-gingerol. The calibration curve exhibited good linearity over the concentration ranges of 12.5-200 µg/mL, with an equation of Y = 11811X + 4413.6 and R^2 of 1.0000 (table 1).

Accuracy of the method was determined by quantitative analysis of [6]-gingerol in the extracts fortified with three known concentrations of the standard compound and calculated according to the percentage recovery of the spiked standard. Recoveries in the range of 96-103% were obtained for [6]-gingerol (table 1), indicating great accuracy of the method. The % RSD of six measurements in the same day was assessed for evaluation of repeatability or intraday precision as well as for instrumental error, while % RSD value from the determination of three samples, independently prepared in other days, evaluated reproducibility or interday precision. The RSD values of 0.4% and 2.5% for intraday and interday precisions, respectively, indicated good precision of the HPLC method. Based on LOD and LOQ analysis, the method exhibited good sensitivity for [6]-gingerol with LOD and LOQ values of 0.48 and 0.80 µg/mL, respectively. These results indicated that the HPLC method for quantitative analysis of [6]-gingerol in ginger extracts showed accuracy, precision, specificity, and sensitivity. In addition, the method is simple and less time-consuming for both sample preparation and analysis processes, which can be practically used as a routine analytical method for ginger extracts.

Recently, several greenest assessment tools were introduced for selection of the greenest analytical method for reduction of chemical hazards. There are four greenness assessment tools, including National Environmental Methods Index (NEMI), Eco-Scale Assessment (ESA), Green Analytical Procedure Index (GAPI), and Analytical GREEnness metric (AGREE). GAPI and AGREE have been proved to be suitable for greenness assessment (Gamal *et al*., 2021; Płotka-Wasylka and Wojnowski, 2021). AGREE is a comprehensive, flexible, and straightforward assessment (Pena-Pereira *et al*., 2020). Based on the AGREE method, a greener analytical scale of the HPLC method was 0.5 (Fig. 3) indicating that this quantitative HPLC method for [6]-gingerol was quite green by reducing the number of steps, time, energy and toxic solvent.

Determination of alternative green solvents

Although the concentrations of [6]-gingerol in the extracts obtained from all solvents were not significantly different ($P<0.05$) (Table 2), after storing the extract obtained from 50% v/v NADES of glucose and citric acid for 7 days, a precipitate was observed indicating that the extract was unstable. On the other and, pertaining their industrial applications, NADES of sucrose and citric acid is commonly used for formulation of ginger pastille and cheaper than NADES of sorbitol and citric acid. Therefore, NADES of sucrose and citric acid was selected for further study.

It has been reported that a high viscosity of NADESs and glycerol may decrease the extraction efficiency of phytochemicals, due to a slow solute diffusion during extraction (Liu *et al*., 2019; El Kantar *et al*., 2019). But NADESs and glycerol are also usually used as pharmaceutical excipients in pastille formulas. The effects of glycerin and NADES concentrations in ethanol (10%, 20%, 30%, and 50%) were therefore determined for extraction of [6]-gingerol. The results revealed that 20% v/v glycerin in ethanol exhibited a bit higher concentration of [6]-gingerol than other solvents (Table 3). However, the concentration ranges of NADES of sucrose and citric acid in ethanol between 10-50% v/v did not affect the extraction efficiency of [6]-gingerol. In addition, the concentrations of [6]-gingerol obtained from all solvents were not significantly different from ethanol. However, 20% v/v glycerin and 20% NADES of sucrose & citric acid in ethanol were suggested as alternative green solvents for extraction of [6]-gingerol. That was because the boiling points of 20% v/v glycerin and 20% NADES of sucrose and citric acid in ethanol are higher than ethanol alone, as a result, they were easier to handle using a microwave-assisted extraction. Also, the obtained [6]-gingerol extracts could be directly used for formulation of anti-nausea and vomiting pastilles resulting in reduction of ethanol consuming as well as cost of production.

CONCLUSION

A validated HPLC method for quantitative analysis of [6]-gingerol in ginger extracts has been established. The method can be practically applied in routine quantitative analysis of ginger extracts. Furthermore, based on the findings obtained in this study, microwave extraction using 20% v/v glycerin or 20% NADES of sucrose and citric acid in ethanol as an alternative green solvent is recommended for preparation of [6]-gingerol enrich extracts which can be used for formulation of ginger pastilles.

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