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Research

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Ultrasound-Assisted Extraction for Simultaneous Quantitation of Potential Sweetening Compounds from *Derris reticulata* Aqueous Extracts: A Response Surface Methodology Approach

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Abstracts

Background: *Derris reticulata* or Oi Sam Saun is a highly sweet Thai plant, rich in bioactive compounds, and widely used for its medicinal properties. In this study, sweet aqueous extracts from the stems of Oi Sam Saun were extracted using ultrasound-assisted extraction (UAE). Phenolic, flavonoid, and sugar compound extraction was optimized using the Box-Behnken design (BBD) and response surface methodology (RSM).

Methods: Three independent variables—extraction temperature (40–80°C), sonication time (20–60 min), and extraction ratio (1:10–1:30 g/mL)—were investigated, and the optimal condition was used to determined phenolics, flavonoid and 18β-glycyrrhetinic acid by High performance liquid chromatography-diode array detector (HPLC-DAD). Sensory evaluation was also performed.

Results: The values of 84°C, 64 min, and ratio 1:8 g/mL were found to be optimal. Under these conditions, experimental values were well correlated with predicted values, and phenolic, flavonoid, and sugar contents were determined as 0.4725 mgGAE/gDW, 0.1489 ± 0.033 mgCE/gDW, and 4.802 ± 0.651 mg/gDW, respectively. Gallic acid, *p*-coumaric acid, quercetin, and kaempferol was also found in optimal condition. Moreover, the extract contained 18β-glycyrrhetinic acid (0.529 ± 0.002 mg/100 mg) and was 166 times sweeter than sucrose.

Conclusion: High level of phenolics, flavonoids and sugars was detected in optimal condition of the extract. Therefore, this Thai medicinal plant, which has several pharmacological benefits, is newly potentially and applicable as a sweetening agent or sugar substitute in foods.

Keywords: ultrasound-assisted extraction, phenolics, flavonoids, 18β-glycyrrhetinic acid, sweet Thai plant, response surface methodology, Box-Behnken design

Introduction

Sweetness is one of five basic tastes and plays an important role in human diet. Most sweet tastes originate from sugars. However, excessive sugar intake can cause diseases, such as dental caries, hypertension, obesity, and diabetes (Malik et al. 2006). Currently, non-nutritive sweeteners and non-sugar sweetening agents are being consumed to avoid health problems associated with high sugar intake. Most non-nutritive sweeteners that are currently available globally are artificial sweeteners, such as aspartame, sucralose, saccharin, and acesulfame-K, some which have been reported to be harmful to life (Whitehouse et al. 2008). Many research attempts have been made to discover and develop natural non-nutritive sweetening compounds from plants. These attempts have been largely successful, but only a few of these compounds have been commercialized as sweeteners, including stevioside, rebaudioside, glycyrrhizin, mogroside, brazzein, and thaumatin. However, sweetening agents of plant origin, including phenolics, flavonoids, terpenoids, and sugars have both aglycone and glycone structures (Kim and Kinghorn et al. 2002). Some flavonoid or phenolic compounds are sweet, such as glycyphyllin, naringin dihydrochalcone, and dihydroquercetin 3-*O*-acetate (Kim and Kinghorn et al. 2002). A well-known low-calorie sweetener is glycyrrhizin, a glycosylated pentacyclic triterpenoid, containing one molecule of 18β-glycyrrhetinic acid, and two molecules of glucuronic acid. Additionally, the compound, 18β-glycyrrhetinic acid, is found in *Glycyrrhiza glabra* L. or licorice (local name in Thai "Cha-em"). It is a widely used herbal medicine, native to southern Europe and parts of Asia, including Thailand (Khattak and Simpson, 2010). Thailand has a biodiversity of interesting plants such as "Oi Sam Saun" or "Cha em Nua" (*Derris reticulata* Craib.), found in semi-shaded areas of dry evergreen forests, the edge of evergreen mixed (dipterocarp) forests, bamboo forests, or along streams (50–450 m). The stems of this plant give a sweet taste similar to licorice; it is used as a sweetener in local medicine and as a laxative (Sirichamorn et al. 2012). Although some flavonoids, such as lupinifolin,

74 dereticulatin, and pyranoflavonone, were reported in Oi Sam Saun (Yusook et al. 2017;
75 Mahidol et al. 1997; Mahidol et al. 2002), these compounds did not show sweet tastes.

76 Generally, sweetening compounds were extracted via conventional methods, such as
77 maceration, soxhlet extraction, and supercritical fluid extraction. Xia et al. (2008) extracted
78 sweetening components from *Siraitia grosvenorii* or monk fruit by soxhlet extraction method.
79 Further, Koh et al. (2009) extracted sweetening compounds from Chinese sweet tea plant
80 (*Rubus suavissimus* S. Lee) by soaking in water and precipitating with alcohol. Choi et al.
81 (2002) extracted stevioside from stevia leaves by supercritical fluid extraction. Extraction is a
82 crucial step for the isolation of bioactive sweetening compounds from plant materials.
83 However, conventional methods have certain limitations, such as low yield, too much solvents,
84 and bitter tastes caused by the materials used (Armenta et al. 2008). Therefore, modern
85 techniques, such as ultrasound-assisted extraction (UAE), microwave assisted extraction, and
86 pressurized liquid extraction, are modified approaches that have significant advantages over
87 conventional methods (2006). These techniques have been applied in extracting commercial
88 natural sweeteners, such as stevioside, glycyrrhizin, and mogroside (Pan et al. 2008; Rao et al.
89 2012; Charpe and Rathod, 2012).

90 UAE has received considerable attention as a promising alternative to conventional
91 methods (Maran and Priya, 2016). It has been applied in several research and development
92 fields, including phytochemical product extraction and the food industry (Esclapez et al. 2011).
93 It is a simple, low cost, and highly effective technique that exhibits a high efficiency yield in a
94 short time (Al-Dhabi et al. 2017). According to some reports, UAE has been applied for the
95 extraction of natural sweeteners; Charpe and Rathod (2012) extracted glycyrrhizic acid from
96 licorice root using ultrasound, and Liu et al. (2010) extracted total carbohydrate and
97 rebuadioside A from stevia leaves by UAE.

However, the UAE method needs to be optimized depending on several factors that can influence the phytochemical extraction yield, including the extraction temperature, ultrasonic time, solvent composition, particle diameter, liquid-solid ratio, and electrical acoustic intensity (Sheng et al. 2017). Currently, response surface methodology (RSM) is a mathematical tool widely used in the research and food industry. Its advantages include decreasing the number of experimental runs, evaluating the effect of several variables, and optimizing conditions. Among many classes of RSM designs, Box-Behnken designs (BBDs) are a class of rotatable or nearly rotatable second-order designs that are based on three-level incomplete factorial designs. BBDs are slightly more efficient with fewer experimental runs than the central composite designs (CCDs) (Ferreira et al. 2007).

To the best of our knowledge, there is a lack of information on sweetening compounds and their related compounds from *Derris reticulata* Craib., and there are only few reports on suitable extraction methods for these compounds. Therefore, this study was aimed to extract such compounds (phenolics, flavonoids, and sugars) from Oi Sam Saun using UAE, optimizing them using RSM, and investigating their sweetness potent.

Materials and Methods

Chemicals

Folin-Ciocalteu reagent, sodium carbonate, methanol, acetonitrile, and formic acid were purchased from Sigma (Singapore). All solvents and standard compounds were of HPLC grade and all chemicals were of analytical grade.

Plant materials

Derris reticulata Craib. (Leguminosae) or Oi Sam Saun stems were collected in July, 2015 from central regions of Thailand. Botanical identification was graciously performed by Assoc.

Prof. Saranya Vajrodaya of the Faculty of Botany, Kasetsart University, Bangkok, Thailand. Voucher specimen (BK no. 069447) was then deposited at the Forest Herbarium-BKF, Bangkok, Thailand. Stems were dried at 50°C until they attained constant weight (12% moisture content), ground to powder, sieved through a 40-mesh sieve, and kept at room temperature prior to the experiments.

Extraction of Oi Sam Saun by Ultrasound-assisted extraction (UAE)

UAE was carried out using an ultrasonic bath (Elmasonic E70H) set to 120 W and 37 Hz. Plant powder was extracted using distilled water at different extraction temperatures (40, 60, and 80ºC), times (20, 40, and 60 min), and ratios (1:10, 1:20 and 1:30 w/v). At designated extraction intervals, the mixture was taken and filtered with Whatman No. 1 filter paper; the Oi Sam Saun UAE extracts were collected to determine the yield, colour, and sweetening compounds.

Determination of extraction yield, colour, and sweetening compounds in Oi Sam Saun extract

Extraction yield

Oi Sam Saun UAE extracts were collected, the water was removed under vacuum using a rotary evaporator (Buchi, Germany) at 40ºC, and they were freeze-dried. The percentage yield of the dried crude extracts was determined and calculated according to Equation 1:

$$\% \text{ yield} = (\text{weight of crude extract}/\text{weight of dried sample}) \times 100 \qquad \text{Equation 1}$$

Colour measurement

Colours of the Oi Sam Saun UAE extracts were measured using a HunterLab colorimeter (Miniscan EZ; Hunter Associates Laboratory Inc., Reston, USA). Each sample was measured in triplicates and analysed using the CIE $L^* a^* b^*$ system. Here, L^* represents the lightness of the

148 colours from 0 (dark) to 100 (light), a^* represents the greenness/redness parameter (negative a^*
149 is green and positive a^* is red), and b* represents the grade of blueness/yellowness (negative b^*
150 is blue and positive b^* is yellow). The angular coordinates of the hue angle ($h°$) were calculated
151 according to Equation 2:

152 $h° = \tan{-1}\ (b^*/a^*)$ when $a^* > 0$ and $b^* > 0$ Equation 2a

153 $h° = 180° + \tan{-1}\ (b^*/a^*)$ when $a^* < 0$ Equation 2b

154 $h° = 360° + \tan{-1}\ (b^*/a^*)$ when $a^* > b^* \times$ and $b^* < 0$ Equation 2c

155

156 For the browning index (BI) of Oi Sam Saun UAE extracts, BI was calculated using
157 Equations 3a and 3b:

158 $$BI = \frac{100(x-0.31)}{0.172}$$ Equation 3a

159 Where:

160 $$X = \frac{a^* + 1.75L^*}{5.645L^* + a^* - 3.012b^*}$$ Equation 3b

161 The browning index (BI) represents the purity of the brown colour or brown pigment
162 concentration (Guerrero et al. 1996; Palou et al. 1999).

163

164 ***Total phenolic content***

165 Phenolic content was determined using the Folin-Ciocalteu method described by Gonçalves et
166 al. (2013). Folin reagent (2.5 mL, diluted 10×) was added to 0.5 mL of the extract, to which
167 2 mL sodium carbonate (5 g/L) was then added. The mixture was then placed in the dark for
168 1 hr before the absorbance was read at 760 nm. Gallic acid was used as a reference standard
169 and the total phenolic content was expressed as gallic acid equivalents (GAE, mg/g extract).

170

171 ***Total flavonoid content***

Total flavonoids were determined using a spectrophotometric assay developed by Dini (2011). About 0.5 mL of the extract and catechin standard (20–100 mg/L) were mixed in a test tube with 2 mL of distilled water, and 5% sodium nitrite (0.15 mL) was added to the test tube. After 5 min, 10% aluminium chloride (0.15 mL) was added to the mixture. At 6 min, 1 M sodium hydroxide (1 mL) and distilled water (1.2 mL) were added, and the mixture was thoroughly mixed. The absorbance of the mixture was measured against a blank at 510 nm. The total flavonoids of the extracts were expressed as mg catechin equivalent (CE)/g dry weight of the plant.

Total sugar content

Total sugar content was determined based on a colorimetric method (Dubois et al. 1956). The extract (1 mL) was mixed with 5% phenol (1 mL), and 5 mL of concentrated sulfuric acid was then added. The reaction mixture was incubated at room temperature for 20 minutes, and the absorbance was measured at 490 nm with glucose as standard. The total sugar content was expressed as mg glucose/g dry weight of plant (mg/g DW).

Experimental design

Box-Behnken experimental design (BBD) with three levels and three factors was selected to investigate the influence of process factors on RSM. The effects of three extraction factors (ratio, temperature, and sonication time) on three responses (phenolic, flavonoid, and sugar contents) were determined. **Table 1** lists the original and code values of the extraction factors and their levels in the extraction process. The experimental design involves 17 experimental runs, including five replicates at centre points, used to allow for the estimation of a pure error sum of squares, and the total number of experimental runs were evaluated from the following equation (Maran and Priya, 2016).

197 $$N = 2K (K-1) + C_0$$ Equation 4

198 Where K is the number of experimental factors and C_0 is the number of central points.

199 A second-order polynomial equation was fitted to the data by linear regression. The
200 model equation is:

201 $$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i}^{k} \sum_{i}^{k} \beta_{ji} X_i X_j + \varepsilon$$ Equation 5

202 Where Y is the dependent variable (flavonoid and phenolic), β_0 is the model constant, β_i, β_{ii},
203 β_{ij} are the model coefficients, X is the independent variable, k is the number of independent
204 factors, and ε is the error. The parameters, β_i, β_{ii}, β_{ij} represent the linear, quadratic, and
205 interaction effects of the variables, respectively.

206

207 **Optimization of sweetening compound extraction conditions**

208 BBD experimental data were used to determine the optimal conditions for the model. All
209 response variables (Total phenolic, flavonoid, and sugar contents) were kept at maximum, and
210 the independent variables (X_1, X_2, and X_3) were kept within the desired range (between lower
211 and higher level). Statistica 9.0 software (StatSoft Inc., Tulsa, OK) generated the optimal
212 conditions based on BBD data. Further, the optimal condition gave the model a predicted value
213 for each response, for comparison with the experimental value. Hence, the experiments were
214 performed at the suggested optimal condition, and the response values obtained were compared
215 with the model's predicted values.

216

217 **Determination of phenolic and flavonoid compounds**

218 Under optimal conditions, Oi Sam Saun UAE extract (~10 mg) was hydrolysed using 10 mL
219 of 1 M trifluoroacetic acid (TFA) at 90°C for 60 min. Next, the solution was mixed with 40
220 mL methanol and taken to the rotary evaporator at 40°C, until the remaining TFA was removed.

The hydrolysed sample was mixed with 20 mL deionised water and filtered with a 0.45 μm syringe filter used for phenolic and flavonoid determination.

Phenolic and flavonoid compounds were determined under optimal Oi Sam Saun UAE conditions. The extracts were analysed using an HPLC-DAD (diode array detector; 1200 Series, Agilent Technologies, USA), using an Eclipse XDB-C18 column (4.6 mm ID x 250 mm, 5 μm) and a linear gradient with water (pH 2.5) containing TFA (A) and acetonitrile (B) for 65 min at a flow rate of 1.0 mL/min. The gradient was set as follows: 0–20 min, 95–90% A; 20–50 min, 95–70% A; 50–55 min, 70–50% A; 55–60 min, 50–95% A; 60–65, 95% A. The samples (5 μL) were loaded into the HPLC-DAD. The DAD detector was set at 280 and 350 nm for phenolic and flavonoid detection, respectively.

Sugar analysis

Sugar type and content were identified by high performance liquid chromatography-evaporating light scattering detector (HPLC-ELSD; Alltech, Buchi, Switzerland). Oi Sam Saun UAE extracts, under optimal conditions, were dissolved in distilled water at a concentration of 1,000 ppm and filtered through 0.45 μm (Millipore) filters. Separation was achieved using a Rezex RPM Monosaccharide column (300 mm × 7.8 mm ID, 8 μm particle size). The mobile phase was water in isocratic elution with a flow rate of 0.6 mL/min for 30 min. The detection of analytes was carried out using an evaporative light scattering detection (Alltech, Buchi, Switzerland) technique which detects organic molecules by mass; hence, it is useful in the quantitative determination of non-UV-sensitive compounds. The drift tubes for ELSD were set at 105°C and the flow rate of nebulizing gas (N_2) was 2.6 standard litre per minute (SLM). The sugar standard chromatograms were for glucose, fructose, and sucrose.

Determination of 18β-glycyrrhetinic acid

The 18β-glycyrrhetinic acid content of the Oi Sam Saun UAE extract, under optimal conditions, was identified by the modified method of Esmaeili et al. (2010), using 1200 series Agilent HPLC system with a 20 μL sample loop attached to a DAD. HPLC analysis was completed using a reversed phase XDB-C18 column (250 × 4.6 mm, 5 mm) and 18β-glycyrrhetinic acid was determined using an acetonitrile/phosphoric acid (3/1, v/v; pH=2.5) mobile phase at flow rates of 1.0 mL/min (0–8 min) and 0.6 mL/min (8–20 min) at a detector wavelength of 230 nm.

Sweetness potency by sensory evaluation

Sensory evaluation of Oi Sam Saun UAE aqueous extracts

Sensory evaluation of Oi Sam Saun UAE extracts, under optimal conditions, was conducted by 10 semi-trained panellists (3 males and 7 females) aged 24–30 years old. Sensory profiling was performed by selecting extracts from the best condition. The intensity of each attribute, including sweet, sour, and bitter tastes, was scored on a scale of 1 to 9. Sucrose, citric acid, and caffeine were used as standards for sweet, sour, and bitter tastes, respectively. The individual panellists who took part in the study were trained before the tasting and ranking test. The sweet solution (10 mL), at a concentration of 1, 2, 5, and 10% sucrose, was selected for the first session, and 0.5, 1, 2, and 3% sucrose were used for training in the second session. UAE aqueous extracts (1 mL) were served in opaque disposable plastic cups at room temperature (Meilgaard et al. 2006).

Sweetness intensity estimation

The sweetness potency of each sample, relative to that of sucrose, was calculated using the following formula:

$$\text{Sweetness potency} = B/A \times 100 \qquad \text{Equation 6}$$

Where A is the concentration (%w/v) of sample solution at 10%, and B is the concentration (%w/v) of sucrose with the same sweetness as sample. B was calculated from the linear regression formula of the sweetness score (1–9 point) against sucrose concentration (0–15% w/v) (Yoshikawa et al. 2002; Darise et al. 1984).

Statistical analysis

All tests were performed in triplicate. Statistical analysis was performed by the analysis of variance (ANOVA) and least significant difference (LSD) test, using SAS statistical software, version 9 (SAS Institute Inc., Cary, NC, USA), at 95% confidential interval or probability at ≤ 0.05. The three-dimensional (3D) response surface plots of the experimental model were generated using the Statistica 9.0 program (StatSoft Inc., Tulsa, OK).

Results and discussion

Fitting the response surface model

The preparation process to prepare Oi Sam Saun UAE extracts and overall experiment is illustrated in **Fig. 1**. In this study, UAE involved three important parameters—temperature, sonication time, and extraction ratio—which can strongly influence the amount of yield, colour, and phenolic, flavonoid, and sugar contents of Oi Sam Saun extracts. Experiments were applied to determine the optimum temperature, time, and extraction ratio for yield, colour, and phenolic, flavonoid, and sugar compounds in the extract, based on BBD. However, the extraction yield, colour (L* a*, b*, and h°), and browning index (BI) for all crude Oi Sam Saun extracts showed no significant difference ($p > 0.05$; data not shown). Additionally, there was no correlation between yield, colour, browning index, and phenolic, flavonoid, and sugar contents because statistical analysis of the results revealed no significant difference. Moreover, some phenolic and flavonoid compounds were colourless (Kelebek et al. 2010), which meant that the colour and

296 browning index of Oi Sam Saun UAE extracts were not different under every extraction
297 condition. Therefore, phenolic, flavonoid and sugar contents were selected to determine the
298 optimal condition for the Oi Sam Saun UAE process.

299 The experimental results for the 17 experimental points included five central points from
300 the BBD, which were calculated from Equation 5, and are shown in **Table 1**. The quadratic
301 model was applied to show the influence of variables over phenolic, flavonoid, and sugar
302 contents in the extract. The quadratic model regression analysis equations, which were calculated
303 from Equation 6 of Oi Sam Saun, were obtained. The results showed that the experimental data
304 fit a quadratic model in the phenolic, flavonoid, and sugar contents of Oi Sam Saun based on
305 ANOVA (**Table 2**), with significant R^2 values (> 0.90) for the effect of extraction temperature,
306 sonication time, and extraction ratio on phenolic, flavonoid, and sugar contents. The second-
307 order equations for these variables are shown as Equation 7 to 9, respectively.

308

309 $$\text{Phenolic (mg/g)} = 0.233 + 0.028X_1 + 0.027X_2 - 0.147X_3 + 0.039X_1X_2 + 0.056X_2^2 +$$
310 $$0.089X_3^2 \qquad \text{Equation 7}$$

311

312 $$\text{Flavonoid (mg/g)} = 0.037 + 0.003X_1 + 0.002X_2 - 0.058X_3 + 0.004X_1^2 + 0.002X_1X_2 -$$
313 $$0.002X_1X_3 - 0.001X_2X_3 + 0.003X_2^2 + 0.027X_3^2 \qquad \text{Equation 8}$$

314

315 $$\text{Sugar (mg/g)} = 2.010 + 0.140X_1 - 1.415X_3 - 0.210X_2X_3 + 0.741X_3^2 \qquad \text{Equation 9}$$

316

317 where X_1: temperature (°C), X_2: sonication time (min), and X_3: solid/liquid ratio (mL/g).

318 ANOVA was used to evaluate the significance of the quadratic polynomial models. The
319 linear terms of temperature (X_1), time (X_2), and ratio (X_3) showed a significant effect ($p \leq 0.05$)
320 on phenolic content. The quadratic terms of time (X_2^2) and ratio (X_3^2) on phenolic content also

exhibited a significant effect ($p \leq 0.05$), whereas the effect of temperature was insignificant ($p > 0.05$). The combined effect on phenolic contents was significantly ($p \leq 0.05$) influenced by temperature and time.

The linear and quadratic effects of all three parameters on flavonoid content were significant ($p \leq 0.05$). ANOVA showed that, in combination, flavonoid contents were significantly influenced by temperature (X_1) and time (X_2) ($p \leq 0.05$). Moreover, in combination, flavonoid content was also significantly influenced by extraction temperature (X_1) and ratio (X_2), and time (X_1) and ratio (X_3) ($p \leq 0.05$).

The linear effects of temperature (X_1) and ratio (X_3), and the quadratic effect of ratio (X_3^2) on sugar content in the Oi Sam Saun UAE extract was significantly different ($p \leq 0.05$). In combination, only the interaction between time and ratio had a significant ($p \leq 0.05$) effect on sugar content.

Moreover, the highest values of estimated regression coefficients for extraction ratio ($\beta_3 = -0.147, -0.058$, and -1.415) indicated that it was the most important linear variable influencing phenolic, flavonoid, and sugar contents. The negative value implied that phenolic, flavonoid, and sugar contents increased with decreasing extraction ratio. In addition, the model fitness was investigated using the lack-of-fit test ($p \leq 0.05$), which indicated the suitability of models for accurate prediction of the variation (Yolmeh et al. 2014).

Optimization of the extraction process

The 3D response surface plots and two-dimensional (2D) contour plots were constructed from the regression equations to visualize and study the relationship between the response (temperature, sonication time, and extraction ratio) and sweetening compound (phenolic, flavonoid, and sugar) extraction variables shown in **Fig. 2**.

For phenolic compounds in the Oi Sam Saun extracts, the 3D and 2D RSM plots are shown as a function of temperature and sonication time in **Fig. 2a**. ANOVA results indicated that X_1 (temperature) and X_2 (sonication time) had positive interaction effects on phenolic content ($p \leq 0.05$; **Table 2**). Phenolic content increased with increasing extraction temperature and time, probably due to enhanced mass transfer rate and diffusibility at higher temperatures of solvent into the plant cell matrix. Further, the solubility of phenolic compounds also increased at higher temperatures. These phenomena create bubble cavitation in liquid medium and cause bubble collapse, which damages the plant cell matrix (Charpe and Rathod, 2012; Esclapez et al. 2011). It can be concluded that the maximum phenolic content of Oi Sam Saun UAE extract was attained when extraction temperature and sonication time were ~84°C and 64 min, respectively.

The 3D and 2D RSM plots of Oi Sam Saun flavonoid contents are shown in **Fig. 2b–2d**. Based on ANOVA, the combined effect of temperature and sonication time, temperature and ratio, and sonication time and ratio was statistically significant ($p \leq 0.05$). The flavonoid content increased with increasing temperature and sonication time (**Fig. 2b**). Increasing the sonication time influenced flavonoid content due to swelling, and hydration of plant material could be accelerated by the cavitation effect of ultrasound waves during the initial extraction period. The asymmetric collapse of micro-bubbles near surfaces was also associated with micro-jets that could cause the disruption and penetration of water into the matrix through diffusion, improving the washing out of flavonoid content from plant material to surrounding water and enhancing extraction (Maran and Priya, 2016). **Fig.2c** and **Fig.2d** show the effect of temperature and extraction ratio and sonication time and extraction on flavonoid content, respectively. As seen in **Fig. 2c**, flavonoid content increased with increasing temperature and decreasing extraction ratio. **Fig. 2d** shows that increasing sonication time and decreasing extraction ratio increased flavonoid content. This phenomenon was also reported in the UAE

of polyphenols from *Sparganium stoloniferum* (Wang et al. 2013). Taken together, it can be concluded that flavonoid content was the highest when the extraction ratio, temperature, and sonication time were 1:8 g/mL, 84°C, and 64 min, respectively.

For sugar compounds, **Fig. 2e** demonstrates the interactive effect of sonication time and extraction ratio on sugar content. ANOVA showed that sugar content was dependent on sonication time and extraction ratio, combined ($p \leq 0.05$). Sugar content increased with increasing sonication time and decreasing extraction ratio. According to **Fig. 2e**, the longer the sonication time, the higher is the sugar content, which is similar to the phenolic and flavonoid contents. Therefore, sugar content was the highest when the extraction ratio and sonication time were around 1:8 g/mL and 64 min, respectively.

Prediction of optimal conditions

The numerical optimization method was used to optimize the UAE conditions. The optimal Oi Sam Saun UAE conditions, which gave the maximum phenolic, flavonoid, and sugar content were 1.2 (temperature), 1.2 (sonication time), and -1.2 (extraction ratio), in coded form. The corresponding actual optimum extraction conditions were 84ºC, 64 min, and 1:8 g/mL, respectively. Under these conditions, the predicted values for phenolic, flavonoid, and sugar contents were 0.4725 mg/g, 0.1688 mg/g, and 4.802 ± 0.651 mg/g, respectively (**Table 3**).

Phenolic and flavonoid profile of optimized Oi Sam Saun UAE extract

Some phenolics and flavonoids have been reported for their sweetening properties (Kim and Kinghorn et al. 2002). Thus, the determination and identification of phenolic and flavonoid compounds in Oi Sam Saun UAE extract was done. Under optimized UAE conditions, the extract of Oi Sam Saun (84ºC, and 64 min, 1:8 g/mL) was investigated in the presence of phenolic and flavonoid compounds. The compounds were identified by comparing their

retention times and UV absorption spectrum with those of standards. Eight compounds were shown in the chromatogram, but only four compounds were identified and quantified—two phenolics (gallic acid, 0.108 ± 0.012 mg/g extract and p-coumaric acid, 0.082 ± 0.007 mg/g extract) and two flavonoids (quercetin, 0.047 ± 0.008 mg/g extract and kaempferol, 0.031 ± 0.002 mg/g extract) (**Fig. 3**; **Table 4**). These four compounds, found in the Oi Sam Saun UAE extract, are not typically present in free form and mostly give a bitter, rather than sweet taste. However, quercetin and kaempferol structures might have similar dihydroflavonol or glycoside forms as other sweet phenolic and flavonoid compounds, such as glycyphyllin, a dihydrochalcone glycoside, or (2*R*, 3*R*)-dihydroquercetin 3-*O*-acetate, a dihydroflavonol (Kim and Kinghorn et al. 2002), thereby giving a sweet taste to Oi Sam Saun extract. Hence, the unknown phenolic and flavonoid compounds may be the key to the sweet taste of this plant extract.

Sugar profile of Oi Sam Saun UAE extract

The sugar composition of the optimized Oi Sam Saun UAE extract was quantified via HPLC-ELSD. Glucose, fructose, sucrose, and maltose were used as sugar standards. The chromatogram results showed that the extract (**Fig. 4**) contained known sugars including sucrose (1.333 ± 0.098 mg/g extract), glucose (0.705 ± 0.051 mg/g extract), and fructose (0.891 ± 0.074 mg/g extract), and three unknown sugars (**Table 4**). The type and concentration of sugars were related to the sweetness intensity of the plant. Glucose in this plant might be in the form of glycoside and impart a sweet taste, like glycyphyllin, which has glucose in its structure. Sucrose also gives a sweet taste to this plant, depending on the concentration. Therefore, analysis of the phenolic, flavonoid, and sugar components suggested that phenolic and flavonoid compounds in Oi Sam Saun UAE extract exist in glycoside forms, which might be responsible for imparting a sweet taste to it (Kim and Kinghorn et al. 2002).

420

421 **The 18β-glycyrrhetinic acid content of optimized Oi Sam Saun UAE extract**

422 The chromatogram in Fig. 4 shows that the 18β-glycyrrhetinic acid content was 0.529 ± 0.002
423 mg/100 mg of optimized Oi San Saun UAE extract (0.529%; **Table 4**). The typical amount of
424 18β-glycyrrhetinic acid found in licorice root was 0.1–1.6%, depending on the region of
425 cultivation and species (Sabbioni et al. 2006). Moreover, 18β-glycyrrhetinic acid gives a sweet
426 taste, but binds to two glucuronic acid molecules. Thus, 18β-glycyrrhetinic acid, found in Oi
427 Sam Saun UAE extract, might be bound to some sugars (such as glucose or fructose), forming
428 some of the unidentifiable compounds from the sugar profile, and possesses a sweet taste. This
429 result is consistent with the sensory profile, which showed that the optimized Oi San Saun UAE
430 extract gave a sweet taste. 18β-glycyrrhetinic acid has been shown to possess several
431 pharmacological benefits, such as an antiulcerative effect, anti-inflammatory activity, direct
432 and indirect antiviral activity, interferon inducibility, an antihepatitis effect, and an
433 antihyperglycemic effect (Kalaiarasi et al. 2009).

434

435 **Sweetness intensity of optimized Oi Sam Saun UAE extracts**

436 The optimized Oi Sam Saun UAE extract (84℃, 64 min, 1:8 g/mL) was selected for sensory
437 test. The sensory scores for each stimulus (sweetness, sourness, and bitterness) on a given
438 adjective scale were detected as 6.94, 1.26, and 0.24, respectively (**Table 3**).

439 The relationship between the sweetness score from the individual panellists who took
440 part in the study and the sucrose concentration was determined, and the equation, $y = 0.6981x$
441 ($R^2 = 0.9656$), was generated, and then used to calculate the B value in **Equation 6**. The Oi
442 Sam Saun extract was 166 times sweeter than sucrose, which could be attributed to the
443 combined effect of several compounds in the plant's crude extracts, such as polyphenols,
444 alkaloids, and other pigments, which contribute to the sweet taste and concentration of

445 sweetening compounds in the crude extracts. Polyphenols are responsible for some important
446 sensory properties associated with foods. This complex and large family of molecules is
447 responsible for the production of taste sensations, ranging from bitter to astringent and pungent,
448 depending on the polyphenol composition of the food (Kaushik et al. 2010). Therefore, the
449 different concentrations of phytochemical compounds, including flavonoids, sugars, and
450 phenolics, elicited similar or better sensory responses from the individual panellists who took
451 part in the study. The presence of phenolic compounds adversely influenced the acceptability
452 of the extracts. In contrast, the presence of flavonoids and sugars enhanced the overall
453 preference for Oi Sam Saun UAE extracts. High concentrations of these compounds may also
454 play a beneficial role when incorporated as part of sweeteners since they contribute to
455 antioxidant activity inherent in UAE extracts.
456

457 **Conclusion**

458 For the first time, the phenolic, flavonoid, and sugar compounds from Oi Sam Saun were
459 extracted using UAE. This BBD (three levels, three factors) with RSM optimization study
460 showed that UAE gives the best yield of sweetening compounds. The extraction yield, colour,
461 and browning index of Oi Sam Saun UAE extracts were similar under all extraction conditions.
462 The optimal conditions for Oi Sam Saun extraction were: 84ºC, 64 min and 1:8 g/mL, and the
463 maximum phenolic, flavonoid, and sugar yields were, 0.4725 mgGAE/gDW, 0.1489 ±
464 0.033 mgCE/gDW, and 4.802 ± 0.651 mg/gDW, respectively. The best extraction conditions
465 yielded 0.108 ± 0.012 mg/g gallic acid, 0.082 ± 0.007 mg/g *p*-coumaric acid, 0.047 ±
466 0.008 mg/g quercetin, and 0.031 ± 0.002 mg/g kaempferol. Sucrose, glucose, and fructose
467 concentrations were 1.333 ± 0.098, 0.705 ± 0.051 and 0.891 ± 0.074 mg/g extract, respectively.
468 The amount of 18β-glycyrrhetinic acid was 0.529 ± 0.002 mg/100 mg of extract. Moreover,
469 the Oi Sam Saun UAE extract was 166 times sweeter than sucrose. These results reveal the

potential application of this Thai medicinal plant in foods as a sweetening agent to substitute sugars and provide several pharmacological benefits. Moreover, its application as a sweetening compound may be rationalized for suitable processing to improve taste and sweetness potency. However, the fractionation, purification, and identification processes of individual sweetening compounds need to be investigated. Therefore, further studies should focus on identifying the new sweetening compounds via LC-MS-MS.

Abbreviations

UAE: ultrasound-assisted extraction; BBDs: Box-Behnken designs; RSM: response surface methodology; HPLC-DAD: high performance liquid chromatography-diode array detector; GAE: gallic acid equivalents; CE: catechin equivalent; CCDs: central composite designs; BI: browning index; TFA: trifluoroacetic acid; HPLC-ELSD: high performance liquid chromatography-evaporating light scattering detector; SLM: standard litre per minute; ANOVA: analysis of variance; and LSD: least significant difference; 3D: three-dimensional; 2D: two-dimensional.

Authors' Contributions

KT designed the study and reviewed manuscript concept, structure, topics, performed the research experiments, interpreted the results, acquired the data and wrote the manuscript with original draft, review & editing. NL as supervision designed, reviewed and developed manuscript concept, structure and topics. OK, PV and WM were involved in the analysis. All authors read and approved the final manuscript of publication.

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497 work.

498

499 **Competing interest**

500 The authors declare that they have no competing interests.

501

502 **Availability of data and materials**

503 All data that are relevant to the study are reported within the article.

504

505 **Consent for publication**

506 The authors approved the consent for publishing the manuscript.

507

508 **Ethics approval and consent to participate**

509 All the authors have read and agreed the ethics for publishing the manuscript. The study was
510 conducted in compliance with the ethical principles stated in the ethical guideline in human
511 experiments from King Mongkut's University of Technology Thonburi (2018) and the protocol
512 for this study. The appropriateness of this study was reviewed and approved by the ethics
513 committee of each participating facility. Written informed consent was obtained from all
514 participants in this study.

515

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Figures

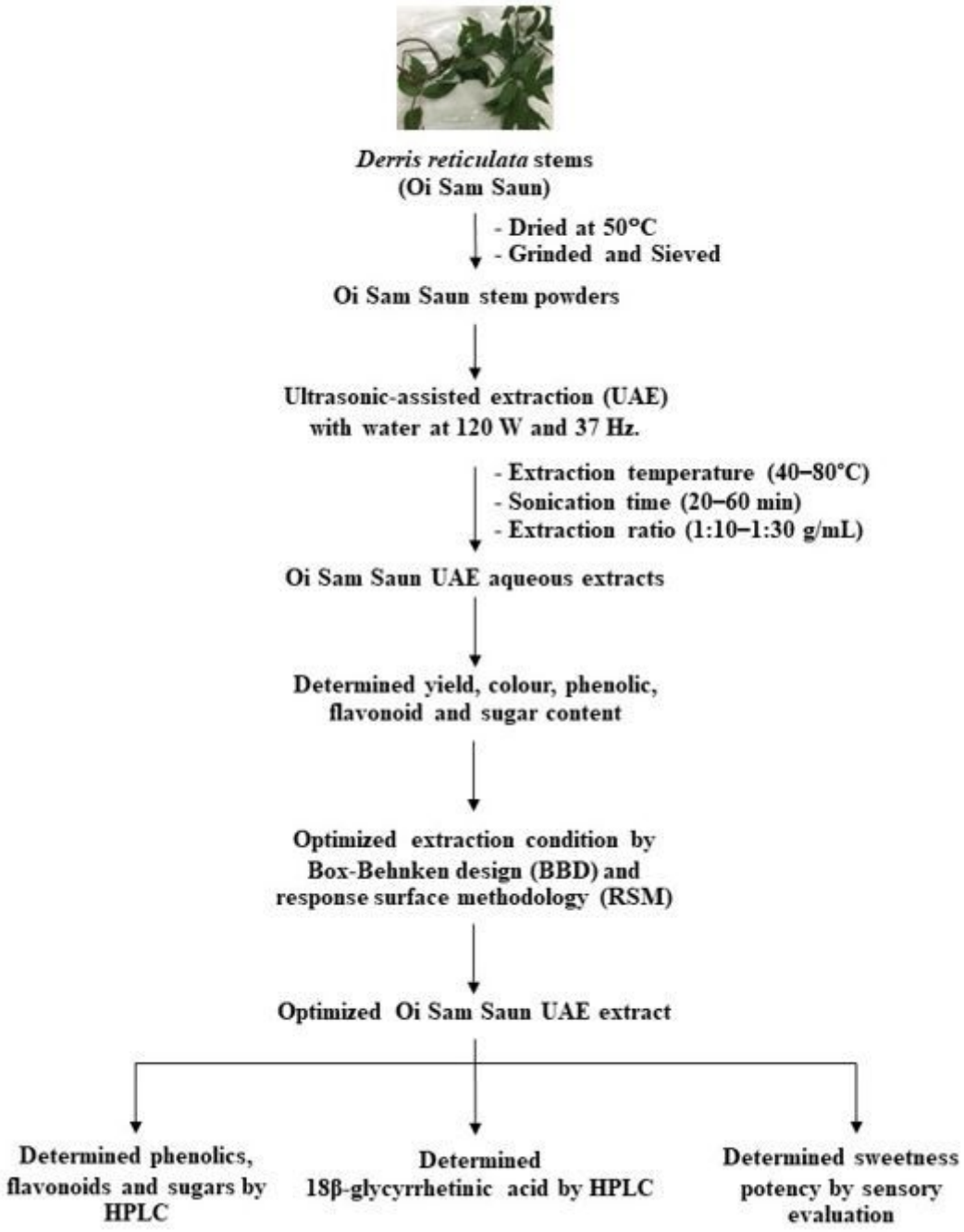

Figure 1

Schematic illustration for the process of Oi Sam Saun UAE extraction and overall experiment.

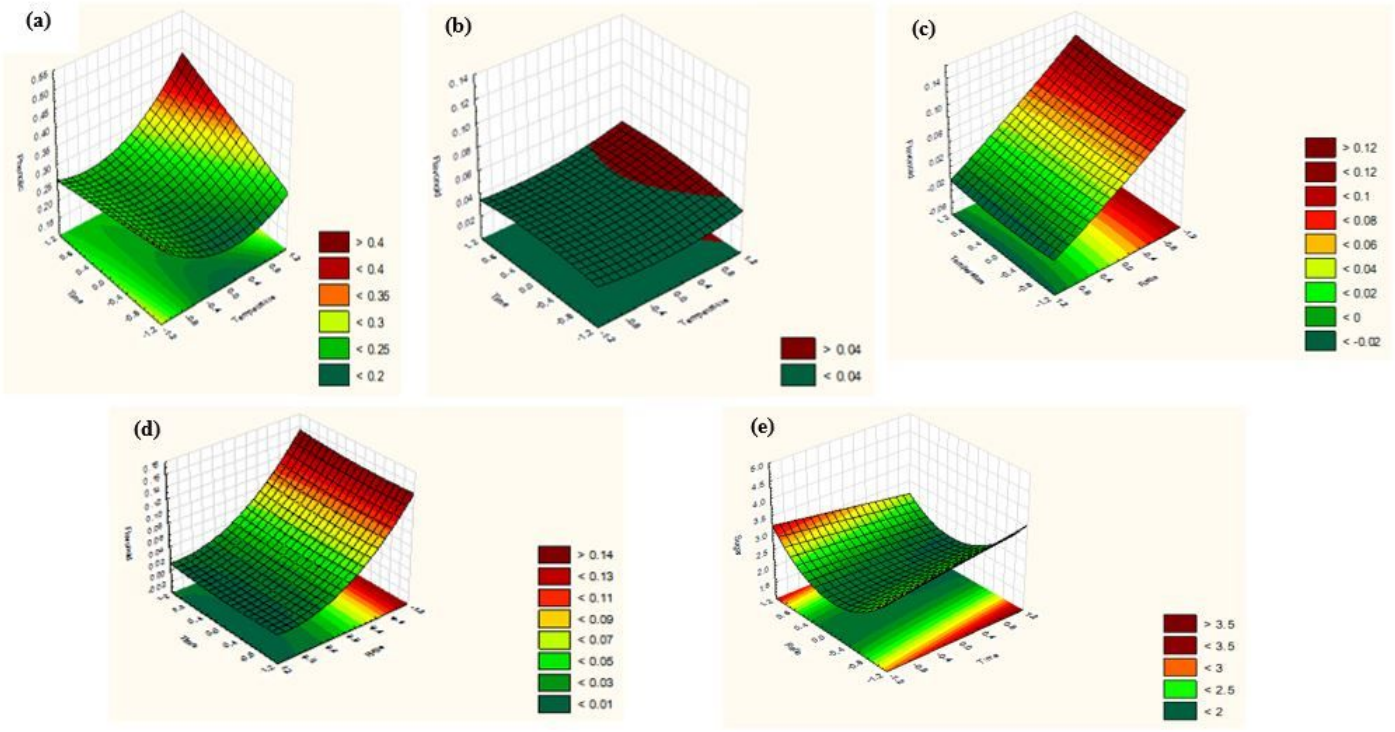

Figure 2

3D response surface graph of Oi Sam Saun of total phenolic content against time and temperature (a); total flavonoid content against time and temperature (b), temperature and ratio (c) and time and ratio (d); total sugar content against time and ratio (e).

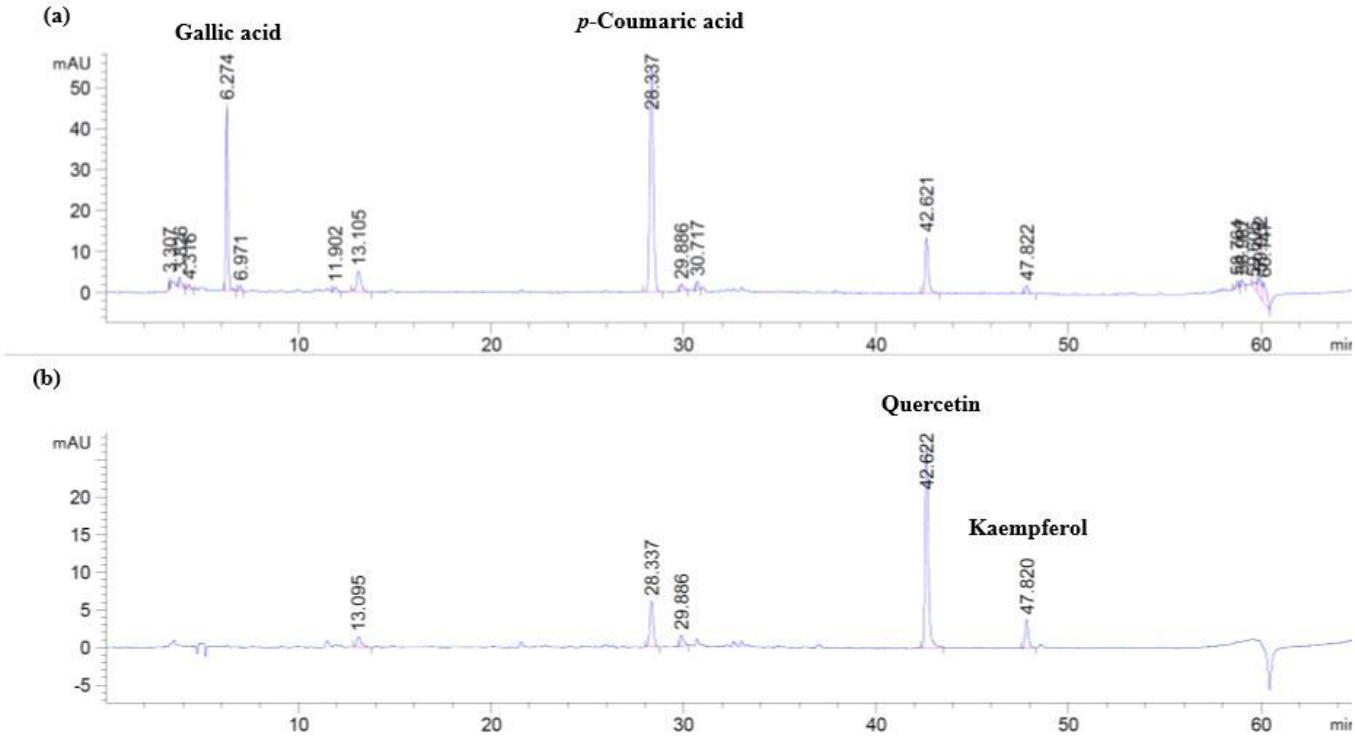

Figure 3

HPLC chromatogram showing the phenolics (a) at 280 nm and flavonoids (b) at 350 nm in the Oi Sam Saun UAE extract.

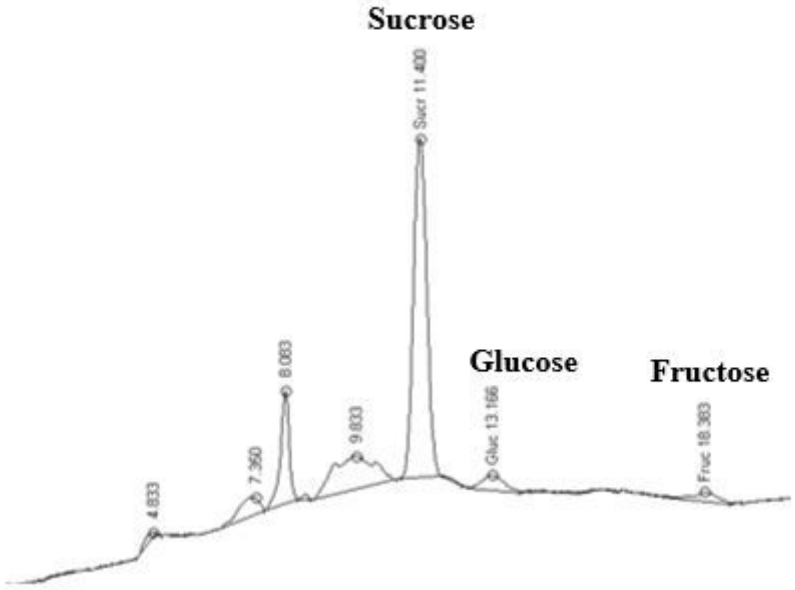

Figure 4

HPLC-ELSD (evaporating light scattering detector) chromatogram showing the sugar profile of the Oi Sam Saun UAE extract.

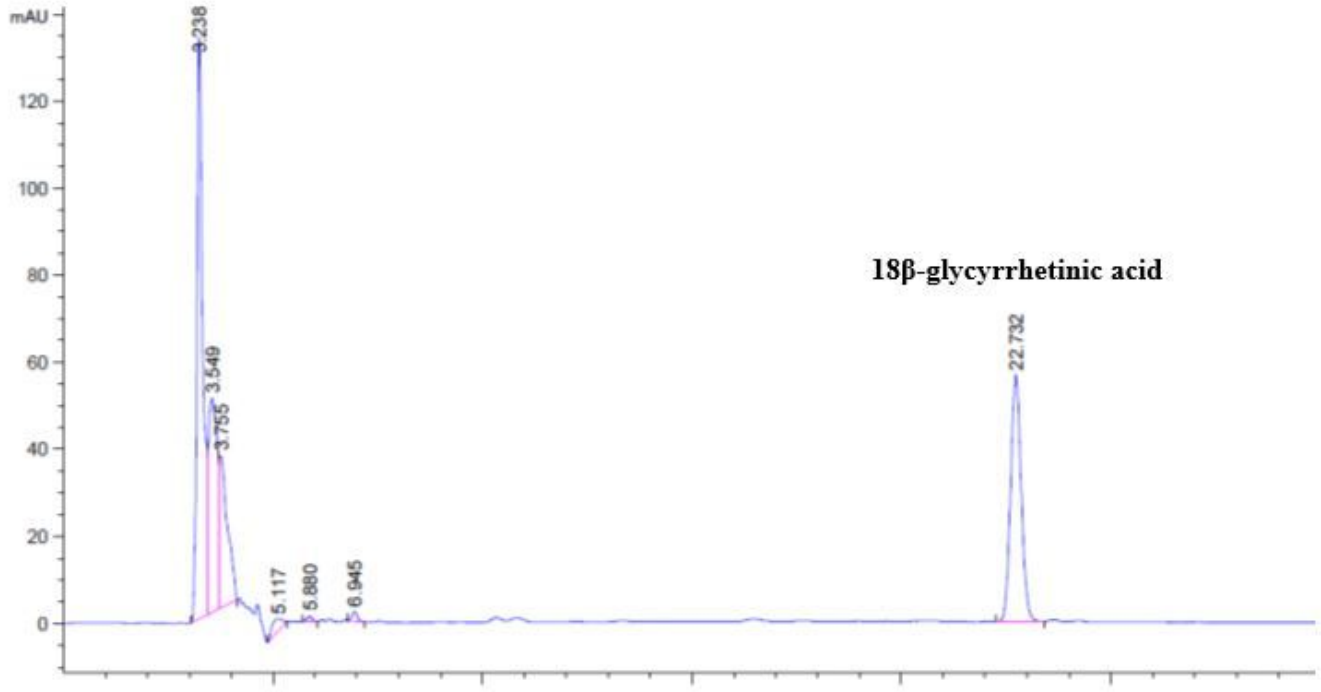

Figure 5

HPLC chromatogram showing 18β-glycyrrhetinic acid in the Oi Sam Saun UAE extract.

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