Phenanthrene-Enriched Extract from *Eulophia Macrobulbon* Using Subcritical Dimethyl Ether for Phosphodiesterase-5A1 Inhibition

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Research

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Abstract

Eulophia macrobulbon (E.C.Parish & Rchb.f.) Hook.f. contains a natural PDE5A1 inhibitor, the phenanthrene, 1-(4'-hydroxybenzyl)-4,8- dimethoxyphenanthrene-2,7-diol (HDP) a potential treatment for erectile dysfunction. This investigation aimed to improve extraction efficiency of HDP from *E. macrobulbon* by using greener extraction methodology, subcritical fluid dimethyl ether extraction (sDME) rather than classical solvent extraction (CSE) and ultrasound-assisted extraction (UAE). The efficiency and quality of obtained extracts were evaluated by: %process yield; solvent amount; extraction period; temperature; %HDP content by LC-MS assay, bioactivity as inhibition of phosphodiesterase-5A1 (PDE5A1) by radio-enzymatic assay; and chemical profiles by LC-QTOF-MS analysis. sDME yielded the highest content of HDP in the extract at 4.47%, much higher than using ethanol (0.4-0.5%), ethyl acetate (1.2-1.7%), or dichloromethane (0.7-1.4%). Process yield for sDME (1.5-2.7%) was similar or less than that observed with other solvents (0.9-17%), but providing that process yield is not prohibitively low, concentration is a more important metric for clinical application. Optimal sDME extraction conditions were: extraction period, 40 mins; 200% water as a cosolvent; sample-to-solvent ratio of 1:8; temperature, 35°C. Phenanthrene aglycone and glycoside derivatives were major constituents in sDME extracts and lesser amounts of phenolic compounds and sugars. Inhibition of PDE5A1 by sDME (IC_{50} 0.67±0.22 µg/mL) was 10-fold more potent than the ethanolic extract and other extraction methods, suggesting a high likelihood of clinical efficacy. Thus, sDME was more efficient, faster, solvent-sparing, greener extraction methodology and more selective for phenanthrene when extracted from *E. macrobulbon*.

Introduction

Erectile dysfunction (ED) or impotence is the inability to achieve penile erection and seriously impinges on the quality of life of patients and their partners (McCabe et al. 2016, Hatzimouratidis et al. 2010). Erection occurs following a cascading reaction triggered by nitric oxide released from neural cells, which leads to increased 3',5'-cyclic guanosine monophosphate (cGMP), a pleotropic cell signaling molecule, and ultimately vascular smooth muscle relaxation leading to increased penile blood flow. The cGMP action is curtailed by a large family of phosphodiesterases (PDEs), of which PDE5A1 predominates in penile erection (Corbin 2004). PDE5A1 inhibition causes cGMP accumulation and sustained penile erection. Sildenafil, commonly sold under the brand name Viagra, is a PDE5A1 inhibitor but causes side effects including visual disturbances, priapism (Boyce and Umland 2001) nausea, headache, and cutaneous flushing (Hatzimouratidis and Hatzichristou 2007). These side effects are caused through Sildenafil's actions on other PDEs and the ATP-binding cassette transporter C5 (Subbotina et al. 2017). Thus, there is demand for more selective PDE5A1.

There has been increasing interest in drugs derived from plant-based extraction processes (Kassing et al. 2010). Several herbal remedies claim efficacy for ED including *Panax ginseng* C.A.Mey., *Lepidium meyenii* Walp., *Ferula hermonis* Boiss., and *Ginkgo biloba* L., (Zhang et al. 2019, Choi et al. 2013, Dell'Agli et al. 2006, Kim et al. 2011, Dell'Agli et al. 2008). The orchid *Eulophia macrobulbon* (E.C. Parish & Rchb.f.) Hook.f. also displays PDE5A1 inhibition embodied in its phenanthrenes, particularly 1-(4'-hydroxybenzyl)-4,8-dimethoxyphenanthrene-2,7-diol (HDP)(Temkitthawon et al. 2017). *E. macrobulbon* relaxes human corpus cavernosal muscle *in vitro* (Preedapirom et al. 2018, Jansakul et al. 2019), relaxes rat pulmonary arteries ex vivo and reduces experimental pulmonary hypertension in rats (Wisutthathum et al. 2018a, Wisutthathum et al. 2018b). Traditionally, *E. macrobulbon* is an aphrodisiac. Indeed, it promoted erection in aged male rats at dose 15mg/kg for 21 days (Preedapirom et al. 2018). Moreover, anti-inflammatory and antioxidation effects of *E. macrobulbon* extract have also been reported (Schuster et al. 2017). Taken together, these studies suggest that extraction of HDP from *E. macrobulbon* is likely to lead to promising clinical applications. All extant studies of HDP/*E. macrobulbon* utilized moderately low doses or extract concentrations and suggest clinical application of *E. macrobulbon*.

Nevertheless, the extraction process needs to be easy and selectively targeted for the therapeutically active compound(s) while minimizing unpalatable and toxic constituents thereby improving efficacy, safety and cost. Many extraction processes for plant-based compounds are tedious, resource-intensive and time-consuming which limits use of natural products (Zhang et al. 2018). Nowadays, the extraction methodology with green and sustainability has been considerably gained attention from researchers.

Furthermore, there is increasing pressure to limit the use of non-polar solvents, such as hexane and dichloromethane to extract active components of herbal feedstock, thereby reducing environmental degradation (Chemat et al. 2019). Supercritical fluid CO_2 extraction has been applied for extraction of several plants (Baldino et al. 2017, Salinas et al. 2020, Yousefi et al. 2019), but the high operating pressure needed imposes prohibitive energy needs (Li and Makino 2014, Subratti et al. 2019). As an alternative, liquid dimethyl ether (DME) has several favorable properties for extracting non-polar/semi-polar compounds (Li and Makino 2014, Subratti et al. 2019), (i) easy to liquefy and store in light-weight canisters, (ii) relatively inert including towards ozone and relatively resistant to auto-oxidation, unlike other alkyl ethers (Naito et al. 2005), (iii) appears to have low toxicity, (iv) synthesizes from biomass on an industrial scale, (v) absorbs 1.5% water thus avoiding pre-drying of the fresh plant (Li and Makino 2014, Azizi et al. 2014, Holldorff and Knapp 1988). Accordingly, it is approved for the food and cosmetic/pharmaceutical industries by the European Food Safety Authority (EFS 2015) and has been used for extraction of some plant materials (Subratti et al. 2019, Boonnoun et al. 2019, Goto et al. 2015).

At ambient pressure and temperature, DME is a gas (boiling point -24°C), the saturated vapor pressure at 20°C is 0.51 MPa, thus readily removed by a depressurized step leaving the final product free of solvent (Azizi et al. 2014). Thus, liquefied DME offers many advantages over a wide range of commonly used solvents.

Application of liquefied (subcritical) DME for extractions has not been applied to *E. macrobulbon* roots. Therefore, this study aims to compare enrichment of bioactive constituents from *E. macrobulbon* by liquefied DME with classical solvent maceration and with/(without) ultrasound-assisted extraction. The chemical identity of bioactive contents (HDP content) of extracts, the inhibition of PDE5A1 activity and chemical constituent profiles were also characterized.

Materials And Methods

General materials

Dimethyl ether or DME (Spray-work air can 420D) was used for extraction and purchased from Siam Tamiya Co., Ltd., Thailand (Commercial grade). The cGMP, crude snake venom (Crotalus atrox), histone from calf thymus, bovine serum albumin (BSA), ethylene glycol tetra-acetic acid (EGTA), imidazole, Tris ((trishydroxymethyl)aminomethane), magnesium chloride ($MgCl_2$), DEAE-Sephadex, phenylmethylsulfonyl fluoride (PMSF) were bought from Sigma-Aldrich (St Louis, MO, USA) [3H]cGMP and scintillation cocktail Ultima Gold^{TM} was purchased from Perkin Elmer (Waltham, MA, USA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin (Pen-Strep), and Geniticin (G418) were purchased from Gibco by Life Technologies (Paisley, Scotland). Lipofectamin® 2000 (Invitrogen) was purchased from ThermoFischer Scientific (Waltham, MA, USA). A Hipure plasmid Maxiprep kit was bought from ThermoFischer Scientific. Human embryonic kidney (HEK)293 cell lines were purchased ATCC (Virginia,USA). Genistein (A) (purity >98%) was purchased from Apex Biotechnology (Boston, USA). Sildenafil citrate (purity >98%) was purchased from the European Directorate for Quality of Medicines and Health care (EDQM), Council of Europe (Strasbourg, France). ACN, water and MeOH (LC-MS grade) were purchased from RCI Labscan, (Bangkok, Thailand). Formic acid (AR grade) was obtained from Merck (Darmstadt, Germany). The organic solvents (analytical grade) were purchased from Burdick & Jackson (B&J) (UK). TLC aluminium sheets and silica gel 60 F254 were purchased from Merck (Darmstadt, Germany).

Plant material

E. macrobulbon was collected from Prachinburi mountain (in July 2018), Thailand and identified by Asst. Prof. Dr. Anupan Kongbangkerd, Faculty of Sciences, Naresuan University. The herbarium specimen (No. 002716) is kept in the Biology Department, Faculty of Science, Naresuan University, Thailand. The fresh roots were chopped and air-dried at 55°C for 3 days. The dried plant was milled into fine powder (4kg) and sieved (150-170 µm) and stored in a desiccator at ambient until use.

Isolation of the main bioactive compound from E. macrobulbon

Isolation of HDP followed previous reports with some modifications (Temkitthawon et al. 2017). In brief, dried powders of *E. macrobulbon* (4kg) were macerated with 95% EtOH 2 times (28L), then filtered, and the solvent was removed under reduced pressure to provide 450g of crude extract (11.2%yield). The extract (384.4g) was dissolved in 100% MeOH and partitioned with hexane two times. The hexane part was discarded and MeOH part diluted with DI water to make 20% MeOH and partitioned twice with DCM. The DCM part was dried under reduced pressure to yield 19.9 g of crude extract. The DCM residue was mixed with silica and placed on a silica gel chromatography column (i.d. 103 x 40 mm). The mobile phase for gradient elution was 100%DCM to 0.5- 4% MeOH in DCM. Eighteen fractions were collected (EMD-1-18). The target compound was monitoring to reference standard of HDP by TLC with DCM:MeOH (9.5:0.5 %v/v) as the mobile phase (Rf value was around 0.3). The fraction of EMD-14 was obtained 0.49 g and chosen for further isolation. EMD-14 (0.24 g) was dissolved in methanol and subjected in a sephadex LH-20 column (i.d. 1.5 x 200 cm) eluting with 100% MeOH to yield 19 fractions. Three fractions (EMDLH14-10 to EMDLH14-12) were pooled, and evaporated and recrystallized with MeOH/DCM to obtain 0.19 grams of crystalline bioactive compound (HDP). The spectroscopic data of ^{1}H-NMR and MS were in agreement to literature (Temkitthawon et al. 2017). The isolated HDP was used as reference standard to quantitatively control a quality of the extracts using LC-MS.

Methods of classical solvent and ultrasound-assisted extraction

Classical solvent extraction: Fine powder of *E. macrobulbon* root (10g) was macerated in different solvents, (i) 95% EtOH, (ii) EtOAc, or DCM. Sample to solvent ratios (w/w) were varied from low to high (1:6.25, 1:10, and 1:20), each maceration period for either 24, 48, or 72 hr.

Ultrasound-assisted extraction; the fine powder (10g) was macerated with various organic solvents, EtOH, EtOAc and DCM in fixed sample to solvent ratio of 1:10 at 40^{o}C for 40 mins. The ultrasound frequency was set with low to high intensity (100kHz-1MHz) (Transonic, Themo Fisher Scientific, Göteborg - Sweden). Whenever extraction process reached the time course, the extraction samples were filtered (Whatman paper 2 µm) then dried under reduced pressure to provide the crude extract, then dried over desiccant for 48 hr, and the extract was weighed.

Subcritical fluid dimethyl ether extraction

Dried powder (5g) was mixed with the required volume of water or co-solvent, the mixture loaded into cellulose thimbles (30x100 mm) along with a magnetic bar of 15.9 x 8 mm (length x diameter). The DME extractor was applied for this work and apparatus was schematically presented in reference of (Boonnoun et al. 2017). The thimble was then placed into an extractor (stainless-steel total volume 100-ml with closed system). Liquefied DME was filled into the extractor at required solvent to solid weight ratio. The extraction was conducted at a controlled temperature at a stirring rate of 500 rpm required time (see below). After extraction, then DME and liquid sample were passed through a stainless-steel filter (5 µm pore diameter, Swagelok). The chamber was inverted over a 75-ml Erlenmeyer flask. The remaining liquid sample was then dried over desiccant for 48 hr, the amount weighed and yield determined.

Optimization of dimethyl ether extraction

Extraction conditions were optimized by comparing;

1. *amount of solvent* as ratio of dried sample to DME solvent (w:w) was varied form 1:2, 1:4, 1:6, 1:8, 1:10, 1:12 and 1:13.5. The optimal ratio was selected for the extraction period.
2. *extraction period* was varied (20, 30, 40, 60, 90 and 120 mins). The minimum time necessary to achieve asymptotic HDP content was selected.
3. *extraction temperatures* were set at 30±1, 35±1, 40±1, or 50±1 °C and
4. *the amount of the co-solvents*, water from 0%, 10%, 20%, 40%, 60%, 100% and 200%, or EtOAc from 0%, 10%, 40%, 100%, 200% and 500% of the powder weight.

All samples were determined and assessed the %HDP content and chemical profile using LC-MS/MS. For all extraction methods, the efficacy of extraction was reported as %process yield, %bioactive (HDP) content, solvent volume used, the duration of the extraction process, and PDE5A1 inhibitory bioactivity. These efficacy measurements were evaluated.

Quantitative determination of HDP content in E. macrobulbon extracts using LC-MS

A method for determining HDP in *E. macrobulbon* samples by LC-MS was developed and validated. This used an Agilent 1260 infinity Series HPLC coupled to an Agilent-6540 Q-TOF-MS spectrometer. Chemical constituents were separated on an EC-C18 (50x3 mm, 2.7 cm) column. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in ACN (B). The following gradient system began from 0-5 min, 40% and 5-8 min, 20% B with a post run 2 min. Injection volume was 5 µl, flow rate was 0.3 ml/min and the column was maintained at 35ºC. The optimized MS conditions were: drying gas flow 10 L/min, drying gas temperature 350°C, nebulizer 30 psig, capillary voltage 3500V, skimmer 65 V, and octapole RFV 750 V. The ESI negative ionization in the Scan and SIM mode was used. The validation data was analyzed by Agilent MassHunter Quantitative Analysis Software Version B.05.02/Build 5.2.365.0. The analysis method was validated, and standard curve HDP was established. The stock solution of HDP standard was freshly prepared by dissolving in 100% MeOH to obtain stock concentration of 100 µg/mL. This solution was further diluted with MeOH to make standard concentrations for the creation of calibration curves (0.25, 0.5, 1.0, 2.5, 5.0 and 10.0 µg/mL). Samples were dissolved in 100% MeOH giving solutions of 5 mg/mL then diluted to 50 µg/mL. All samples and standards were filtered through nylon syringe filters (0.45 µm pore) before injection. All analyses were performed in triplicate.

During the analysis, the LC-MS system stability was checked by using QC1 (concentration at 1.5 µg/mL) before starting each experimental batch and it was also randomized to inject at beginning, middle, and the end of the experiment to assess the LC-MS system and the stability of HDP throughout analysis of the sample batch.

Qualitative analysis of E. macrobulbon extracts by LC-ESI-QTOF-MS

The conditions for LC-MS for measuring secondary metabolites in *E. macrobulbon* samples were determined using a ZORBAX Eclipse Plus C18 (4.6x100 mm, 3.5µm) column and gradient elution with 0.1% formic acid in water (A) and 0.1% formic acid in ACN (B). The elution program ran for 0 min, 5%B; 0-6 min, 35%B; 6-10 min, 50%B; and 10-18 min, 20% B with a 2 min post-run. The flow rate was 0.6ml/min, the injection volume 10µl, and column temperature maintained at 35°C.

The MS condition was: ESI negative ionization in Scan and SIM mode; the drying gas flow 10 L/min at 350°C; nebulizer 30 psig; capillary voltage 3500V; skimmer 65V; octapole RFV 750V; and the fragmentor in negative mode used 250V. The mass range was set at 100-1200 m/z and collision energy of target MS/MS operated at 10, 20, and 40V. The data from LC-MS/MS were collected by Agilent LC-MS-QTOF MassHunter Data Acquisition Software version of B.05.01 and Agilent MassHunter Qualitative Analysis Software B 06.0 for structural elucidation. For the structural elucidation, compounds were compared to previous literatures with ion molecular mass and fragmentation pattern or with the MassHunter Metlin Metabolite PCD/PCDL database (Agilent Technologies), from Scifinder (https://scifinder.cas.org), Chemspider (http://www. Chemspider.com) and/or Massbank (http://www.massbank.jp).

The samples from suitable extraction method were prepared with 5 mg/ml in 100% MeOH and diluted to be 50 µg/ml then filtered through nylon syringe filters with a 0.45 µm pore size before injection in the LC system.

Preparation of phosphodiesterase-5 (PDE5-A1)

HEK293 cells were grown in DMEM supplemented with 10% FBS, in 75 mm flasks at 37°C in a humidified 5%CO_2. A human PDE5A1 plasmid, a gift from Professor Dr Joseph A. Beavo, University of Washington, Seattle, WA, USA, were sub-cloned into a pcDNA3 vector containing an ampicillin resistant gene. The human PDE5-A1 plasmid was scaled up and purified using Hipure plasmid Maxiprep kit (Invitrogen-PureLink™). HEK293 cells were transfected with human PDE5A1 plasmid using Lipofectamine® 2000 reagent following the company protocol. After 2 days of transfection, PDE5-A1 expression was induced by a selective antibiotic (Geneticin (G418, Gibco), 1 mg/ml) for 7 days. The surviving cells were sub-cultured in DMEM, supplemented with 10% FBS in 175 mm flasks at 37°C in a humidified 5% CO_2 atmosphere, and the cells further cultured until they reached 90–100% confluence. The cells were then harvested using a scraper and lysed by sonication in 1 ml of Tris buffer [50 mM Tris pH 7.5, 2 mM EDTA, 1mM dithiothreitol (DTT) and 1:100 of 100 mM PMSF]. The homogenate was centrifuged at 4°C for 20 mins and the supernatant was used as a source of PDE5A1. A PDE5 inhibitor, sildenafil, was used to confirm the presence of PDE5A1 enzymatic activity.

Measurement of PDE5-A1 enzyme activity

To assess PDE5A1 inhibition, a reaction mixture comprising 20 µl of reagent A (100 mM TrisHCl (pH 7.5), 100 mM imidazole, 15 mM MgCl2, 1.0 mg/ml BSA and 2.5 mg/ml snake venom), 20 µl of 10 mM EGTA, 20 µl of PDE5A1 solution, and either 20 µl of test sample or solvent (5% DMSO) only as a control. The reaction was started by adding substrate 20 µl of 5 µM [^{3}H]cGMP (~50,000 cpm) and performed at 30ºC for 40 min. Then, 100 µl of 50% DEAE resin was added to the reaction. After shaking for 10 min, the resin was allowed to settle (20 min), the supernatant was treated with a second cycle of 50% DEAE resin. This supernatant (100 µl) was shaken with 200 µL of Microscint® 20 and tritium counted on a TopCount NXT scintillation counter (PerkinElmer, USA) for 2 h. The PDE5A1- hydrolyzed <25% of the substrate. Each was performed in duplicate in 96-well plates. [27,28].

In preliminary screening, samples of plant extract and pure compound were tested at final conc. 50 µg/ml and 10 µM respectively. All samples were dissolved in DMSO and diluted with water. DMSO was limited to 1% in the final assay medium. When PDE5A1 inhibition was >80%, samples were further diluted and re-analyzed. IC_{50}s were calculated using Prism software (Graph Pad Inc., San Diego, CA). Sildenafil was used as the positive control.

Data analysis

The %PDE5A1 inhibition was calculated and plotted against log10 [sample] and thereafter, half maximum inhibitory concentrations (IC_{50}) were interpolated by Graph-Pad Prism v. 8 (San Diego, USA). Data were processed by analysis of variance (ANOVA) or Tukey's multiple comparison tests. Results were considered significant where $P<0.01$. Means and SDs were all calculated from at least 3 determinations of each sample.

Results And Discussion

Extraction of E. macrobulbon root

The main bio-active compound in *E. macrobulbon* is a phenanthrene, 1-(4'-hydroxybenzyl)-4,8- dimethoxyphenanthrene-2,7-diol (HDP), and has been reported to be a potent PDE5 inhibitor. HDP is around 50 times more potent than the next strongest PDE5 inhibitor among compounds isolated from *E. macrobulbon*, so it was used as the main biomarker for this study.

Classical solvent and ultrasound-assisted extractions

Commonly, the yield of extract as the percentage of process yield (Y) from the starting material is used to gauge extraction efficiency, since the solvent amount used (v) and extraction period (t) are important determinants of the extractable amounts, efficiency is defined as Y/v or Y/t. However, process yield does not measure the purity or concentration of the resultant extract. For medicinal purposes, it is often important to assess the concentration of the biologically active compound within the final extract. Here, we measured the %HDP in the resulting extract (B). Low %HDP values would necessitate further purification or could cause unpredictable therapeutic efficacy. Thus, the yield of the target bioactive compound depends on B/v and B/t. Moreover, extraction recovery of HDP, the extractable amount of HDP from dried plant was calculated and compared in mg/kg unit.

Y; Percentage of process yield

B; Percentage of HDP content in the extract

t; Extraction period

v; Solvent amount

Extraction by classical methods: From our preliminary experiments with non-polar solvents, hexane and DCM with polarity indices of 0.1 and 3.1 respectively. The extract from hexane presented negligible amount of both HDP content and process yield, only DCM showed acceptable bioactive HDP content but poor process yield (~1%) (Table 1). However, DCM is categorized as carcinogen. The safer, 'greener' solvents, EtOH and EtOAc using classical solvent assisted extractions were compared (Alfonsi et al. 2008) and presented the polarity indices of 5.2 and 4.4 respectively. cEtOH yielded 13-18% of total extract (Y), but HDP content was very low at ~0.5%. Corresponding Y values for EtOAc were 2.0-2.8% (~1.5% HDP content) and for cDCM 0.9-1.7% (~1% HDP content) (Table 1). Thus, the semi-polar solvents cDCM and cEtOAC more selectively extracted HDP from *E. macrobulbon* than EtOH. The clear brown syrup-like appearance for EtOH extracts compared dark brown, almost black solids with EtOAc and DCM for both classic and ultrasonic methods. Extending the extraction period (24-72 hr) tended to increase process yield slightly for cDCM and cEtOH but no consistent yield of bioactive compound or HDP content (Table 1). The process efficiency (Y/t) for all three solvent show little change over the three time points. Increasing all three solvent amounts also increased process yield (Y) at all time points. The percentage of bioactive compound or HDP content (B) in the crude extract was similarly increased. However, both Y and B parameters for cEtOH at 72 hr appeared unaffected by increased solvent. This suggested that under these two conditions (72 hr and 1:20 sample to solvent rato), extraction was near completed. In contrast, 72 hr extraction with cDCM and cEtOAc showed further process yield (Y) and %HDP content. Nevertheless, increased volumes of all three solvents were accompanied by reduced extraction efficiencies Y/v and B/v (Table 1). The HDP extractable amount in mg from kg of dried plant using various solvents were then compared (Table1). The result showed that the overall the HDP extractable amount was greatest for cEtOH (~1000 mg/kg), slightly less for cEtOAc (~400 mg/kg) but miserable for cDCM (~200 mg/kg). However, the extractable mass or crude extract is further used as ingredient in cosmetic, pharmaceutical and food industries so the higher bioactive content in the crude extract is higher therapeutic efficacy. Indeed, the extractable mass from EtOH gave very high %process yield but it was provided negligible %HDP content in the extract (Table1) when compared to other solvents. This is attributed to EtOH was nonspecific phytochemical extraction for *E. macrobulbon* while EtOAc and DCM were better selective HDP extraction.

Ultrasonic assisted extraction produced both process yields (Y) and of HDP content (B) which matched classic extraction using the corresponding solvent and most extreme protocol conditions, but within only 40 min and 1:10 times of sample to solvent ratio. Thus, ultrasonics greatly increases extraction efficiency and HDP extractable amount from dried plant (Table 1).

For both conventional and ultrasonic extraction protocols, DCM and EtOAc were the most selective solvent for extracting the target HDP compound. However, DCM, is toxic and reactive in the atmosphere, a property that misaligns with the idea of herbal medicines being natural and healthier than synthetic drugs.

Subcritical liquid dimethyl ether extraction

Liquid dimethyl ether (DME) is gaining favor for extractions. Here, DME was explored as an alternative solvent to maximize HDP content and bioactivity from *E. macrobulbon*. We started with 35°C and 30 mins as commonly used by others e.g. (Subratti et al. 2019, Boonnoun et al. 2019) and then systematically varied sample-to-solvent ratio, extraction period, extraction temperature, and adding co-solvents, water or EtOAc (Fig. 1). Optimal extraction values were selected for each variable and carried as a fixed value for the next series of determinations.

For every extraction in all protocols with DME, HDP content was consistently higher than classical and ultrasonic extraction methods with DCM, EtOAc, and EtOH with increases of ~9 folds, 5 folds and 4 folds respectively. While the process yield of the extract using DME equated to those with EtOAc and DCM but EtOH extracted a greater bulk (Table1). Eight volumes of DME optimally yielded ~2.8% of HDP after 40 min but larger solvent volumes or prolonged extraction

reduced the apparent bioactive HDP content (Fig. 1a) as seen elsewhere (Andrade et al. 2017). This is due to increasing overall process yield, while also increasing the risks of constituents having adverse reactions.

In the classical extraction protocol, EtOAc was effective solvent to extract HDP and classified as green solvent, thus it was chosen to add as co-solvent in DME extraction protocol. The result showed that supplementing DME with up to ~40% EtOAc increased the %HDP in the extract, but further increase in the EtOAc led to a decrease in %HDP (Fig. 1e). With 500% EtOAc, the solvent yields an extract with similar properties to one with no DME at all.

Water is commonly used as cosolvent in DME because it is partially miscible in DME solvent and has low cost. Initially, 0.5-10 g water was added to 5 g of powder which because a glutinous mass which increased the process yield of extract and increased HDP yield (Fig. 1d). 10% water was likely absorbed by DME at the extraction chamber pressure and temperature (Holldorff and Knapp 1988) and consumed by hydrating the plant root powder constituents (Kanda and Li 2011). The most favorable extraction was seen with 200% water, where most of the water would have form a separate phase in which most of the powder is suspended. Then primarily, most of the extraction would begin in the aqueous phase and non-polar extractants partition into the predominantly DME phase. The low extractant concentration in the aqueous phase then provides a steep diffusion or unbinding gradient between the hydrated particles. In our experiments, the mixture of both phases was collected and dehydrated where the DME forms a temporary storage dump for moderately non-polar compounds. In spite of this mechanistic uncertainly, DME with 200% added water was ~5-fold more efficient for HDP content than the next best extraction protocol, cDCM, cEtOAc or uDCM, uEtOAc (Table 1). Also, the method is quick and requires a fairly economic amount of solvent. Interestingly, the extraction efficiency parameters with B/t and B/v of sDME were 3-fold and 5-fold higher than the best classical and ultrasonic assisted protocol respectively (Table 1). The extractable HDP amount or recovery from dried plant using sDME reached a peak of ~1000mg/kg which was as same as cEtOH (~1000mg/kg) and uEtOH (~1000mg/kg). This suggested that sDME could reach the maximum extracting HDP from dried plant furthermore, the crude extract from sDME was highest HDP content among the classical and ultrasonic assisted extraction using EtOH, DCM and EtOAc.

Other improvements remain to be explored: (i) Further experiments designed to understand the processes that increase the extraction are needed. (ii) Since water improves HDP extraction, using the fresh root blended with an amount water to give required the correct extraction mix could reduce the lengthy drying and pulverizing process. Both proposals rely on separating DME which is aided by differing specific gravities of water and DME (sp. Gravity 6.6 at 5MPa, 20ºC) (Holldorff and Knapp 1988). Using the DME fraction greatly reduces the amount of post-extract drying. Nevertheless, a purpose-designed extraction container is needed for this purpose. All extracts from DME showed brown-black solid appearance with special smell like floral note.

Inhibition of PDE5-1A

The extract using DME/200% water provided the most potent PDE5A1 inhibition (Table 3, Fig S1) compared to DCM and EtOAc (2-fold less) and EtOH (~10 fold less). These differences roughly accord with differences in HDP contents (Table 1). Quantitative variances probably arise from other PDE5A1 inhibitors known to be present in *E. macrobulbon* root (Temkitthawon et al. 2017, Schuster et al. 2017).

Chemical profiles by LC-MS/MS

Total ion count (TIC) chromatograms from LC-MS/MS are shown in Fig. 2. Extraction of saccharides (retention time, 1-2 min) was prominent for more polar solvents (EtOH and EtOAc) while the DCM only extracted compounds that eluted after ~6 min (Fig. 2 (red line)). In contrast, EtOH extracted material eluting mostly before 10 mins. For DME extraction, 23 compounds were identified. Those compounds with potential pharmacological interest were polyphenols and glycosides (eluting at 3.0-7.5 min), and of current interest, phenanthrenes as glycosides (7.0-9.1 min) and less polar phenanthrene aglycones 9.5-14.5 min) (Table 2).Phenanthrene derivatives were found in the same range with identifiable peak area in percentage for all extracts, cEtOH, cEtOAc, cDCM and sDME were 62.71, 68.26, 64.76 and 62.68% respectively. The more polar phenanthrene glycosides, predominantly existed in cEtOH, cEtOAc and sDME with 48.62, 33.55 and 23.77% respectively. The major compounds of phenanthrene glycoside in those extracts were compounds **5**, **8** and **9**, which possess core aglycone mass of 284 $[M]^+$, which are the same mass as aglycone, compound **14**. Only 1.35% of phenanthrene glycosides was found in DCM. This was due to polarity indices of solvent for extraction. All mass fragmentations of identifiable compounds are in supplementary data (Table S1). Most of phenanthrene aglycones (compound **13**-**21**, see in Fig. 4) were predominantly found in DCM with 63.41% while in the EtOAc and DME phenanthrene aglycones were measured to be 34.71 and 38.9% respectively. Compounds **19** and **21** have been reported to have PDE5A1 inhibitory activity and compound **21** was identified as HDP, the target PDE5 inhibitor in the present study (Temkitthawon et al. 2017). Moreover, the toxicity on human cancerous cell lines of compounds **13**, **14**, **15** and **21** have been reported, the most toxic on human colorectal adenocarcinoma cell line CaCo-2 was compound **15** (Schuster et al. 2017). Worth noting, a peak of natural PDE5A1 inhibitor, HDP (compound **21**) was predominated in sDME with 13.19% of total identifiable peaks, EtOAC was almost as high with 7.80%. Some phenolic compounds in the extracts were found such as 4-hydroxybenzaldehyde and methyl arbutin (Table 2).

Conclusions

This study explored the potential of phenanthrene enrichment extraction using a greener and safer technique: liquefied dimethyl ether extraction method from *E. marcobulbon*. We found that an optimized sDME protocol with an extraction period of 40 minutes, an addition of 200% water relative to sDME (%w/w), sample-to-solvent ratio of 1:8, and a temperature of 35°C yielded 1.55% process yield with a 4.47% concentration of HDP in the resulting extract. The process yield was comparable or in some cases less than the optimal protocols using cDCM, cEtOAc, and cEtOH. However, the concentration of HDP using sDME was dramatically higher than the best non-DME protocol (cEtOAc yielded a maximum HDP concentration of 1.75%), CSE, UAE in all solvents. High concentration of HDP is critical for clinical applications where higher compound purity is likely to yield more predictable and efficacious results. Indeed, we observed that when compared to the uEtOH extract, the extract obtained using our optimized sDME protocol was about 10-fold as effective at inhibition of PDE5A1, suggesting promise for clinical application. In addition to a high concentration of HDP and promising in vitro results, sDME is a greener and safer solvent than the others used here. The chemical-fingerprint profile of the extract was identified using LC-QTOF/MS and could be distinguished into 4 major classes, sugars, phenolic

compounds, phenanthrene glycosides and phenanthrene aglycones. The major component in the extract was phenanthrene derivatives. Thus, use of sDME is a promising technique to selectively enrich phenanthrene extract from *E. macrobulbon*.

Abbreviations

ACN; Acetonitrile, cGMP; 3',5'-cyclic guanosine monophosphate, CSE; classical solvent maceration, DCM; Dichloromethane, DEAE; Diethylaminoethyl cellulose, DME; dimethyl ether. sDME, subcritical dimethyl ether, DMSO; Dimethyl sulfoxide, ED; erectile dysfunction, EtOAc; Ethyl acetate, EtOH; Ethanol, HDP; 1-(4'-hydroxybenzyl)-4,8- dimethoxyphenanthrene-2,7-diol, MeOH; Methanol, PDE5A1; Phosphodiesterase-5A1, UAE; ultrasound-assisted extraction

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All the authors have read and approved the manuscript before submission to Bioresources and Bioprocessing.

Availability of data and materials

All data and materials are available in the main text and supporting information.

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

Protocols were designed by Jukkarin Srivilai, Kornkanok Ingkaninan and Panatpong Boonnoun. Prapapan Temkitthawon, Nattiya Chaichamnong and Jinutda Engsuwan worked on radio-enzymatic assay. Jukkarin Srivilai and Chitaporn Pingyod were responsible for plant extraction experiments. Nitra Nuengchamnong, Tongchai Saesong, and Jukkarin Srivilai designed the chemical analysis assays for all extract samples. Jukkarin Srivilai and C. Norman Scholfield analyzed the data, C. Norman Scofield, Jukkarin Srivilai, and Kornkanok Ingkaninan wrote the paper which was agreed by all authors. All authors have read and approved the manuscript.

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Tables

Table 1 Extraction of *E. macrobulbon* root by classical solvent extraction (cDCM, cEtOAc, and cEtOH), ultrasound-assistance (uDCM, uEtOAc, and uEtOH), and subcritical dimethyl ether (sDME). Time (t) is the duration of extraction, yield (Y mean of 3, ±SD) by weight of extract from dried root powder, and its content of HDP (B) by LC-MS of total extract. All extractions and analyses were done in triplicate.

Sample No.	Extract'n protocol	Time (t) (hr)	Water added w/w%	EtOAc w/w%	Sample/ Solvent ratio	Extraction temp. (°C)	Yield (Y) w/w%	HDP content (B) w/w%	Extraction efficiency parameters				
									Y/t	Y/v	B/t	B/v	B/Y
1	cDCM	24	-	-	1:6.5	Ambient	1.15±0.10	0.88±0.01	0.05	0.18	0.04	0.14	0.77
2		24	-	-	1:10	Ambient	1.20±0.23	0.93±0.03	0.05	0.12	0.04	0.09	0.77
3		24	-	-	1:20	Ambient	1.64±0.15	1.39±0.001	0.07	0.08	0.06	0.07	0.85
4		48	-	-	1:6.5	Ambient	0.97±0.13	0.73±0.07	0.02	0.15	0.02	0.11	0.76
5		48	-	-	1:10	Ambient	1.22±0.07	1.04±0.04	0.03	0.12	0.02	0.10	0.86
6		48	-	-	1:20	Ambient	1.59±0.20	1.45±0.11	0.03	0.08	0.03	0.07	0.91
7		72	-	-	1:6.5	Ambient	1.14±0.06	0.86±0.06	0.02	0.17	0.01	0.13	0.76
8		72	-	-	1:10	Ambient	1.11±0.03	0.92±0.001	0.02	0.11	0.01	0.09	0.83
9		72	-	-	1:20	Ambient	1.71±0.22	1.45±0.11	0.02	0.09	0.02	0.07	0.85
10	cEtOAc	24	-	-	1:6.5	Ambient	2.03±0.03	1.19±0.05	0.08	0.31	0.05	0.18	0.58
11		24	-	-	1:10	Ambient	2.38±0.02	1.44±0.02	0.10	0.24	0.06	0.14	0.61
12		24	-	-	1:20	Ambient	2.81±0.27	1.75±0.03	0.12	0.14	0.07	0.09	0.62
13		48	-	-	1:6.5	Ambient	2.47±0.06	1.17±0.07	0.05	0.38	0.02	0.18	0.48
14		48	-	-	1:10	Ambient	2.48±0.20	1.41±0.06	0.05	0.25	0.03	0.14	0.57
15		48	-	-	1:20	Ambient	2.95±0.22	1.68±0.06	0.06	0.15	0.03	0.08	0.57
16		72	-	-	1:6.5	Ambient	2.17±0.11	1.15±0.02	0.03	0.33	0.02	0.18	0.53
17		72	-	-	1:10	Ambient	2.40±0.10	1.27±0.05	0.03	0.24	0.02	0.13	0.53
18		72	-	-	1:20	Ambient	2.84±0.05	1.51±0.06	0.04	0.14	0.02	0.08	0.53
19	cEtOH	24	-	-	1:6.5	Ambient	14.51±1.37	0.47±0.00	0.60	2.23	0.02	0.07	0.03
20		24	-	-	1:10	Ambient	13.93±0.66	0.54±0.01	0.58	1.39	0.02	0.05	0.03
21		24	-	-	1:20	Ambient	17.39±1.43	0.53±0.02	0.72	0.87	0.02	0.03	0.04
22		48	-	-	1:6.5	Ambient	15.85±0.34	0.50±0.01	0.33	2.44	0.01	0.08	0.03
23		48	-	-	1:10	Ambient	15.96±0.32	0.52±0.03	0.33	1.60	0.01	0.05	0.03
24		48	-	-	1:20	Ambient	17.10±0.69	0.63±0.01	0.36	0.86	0.01	0.03	0.04
25		72	-	-	1:6.5	Ambient	16.46±1.09	0.57±0.02	0.23	2.53	0.01	0.09	0.03
26		72	-	-	1:10	Ambient	17.56±1.11	0.47±0.00	0.24	1.76	0.01	0.06	0.03
27		72	-	-	1:20	Ambient	17.60±10.6	0.54±0.01	0.24	0.88	0.01	0.02	0.04
28	uDCM	40min	-	-	1:10	40	1.87±0.12	1.24±0.01	2.80	0.19	2.29	0.15	0.82
29	uEtOAc	40min	-	-	1:10	40	2.80±0.72	0.95±0.08	4.20	0.28	2.63	0.18	0.63
30	uEtOH	40min	-	-	1:10	40	17.87±0.81	0.53±0.01	26.80	1.79	0.70	0.05	0.03
31	sDME	40min	200	-	1:8	35	1.55±0.08	4.47±0.21	2.33	0.19	6.71	0.56	**4.60**
32	sDME	40min	40	-	1:8	35	1.88±0.08	3.77±0.20	2.82	0.24	5.65	0.47	**2.00**
33	sDME	40min	40	40	1:8	35	2.74±0.03	3.33±0.40	4.11	0.34	4.99	0.42	**1.21**

Table 2 Chemical profile of *E. macrobulbon* extracts

Cpd No.		Rt. (min)	Compound	Ionized mass (m/z)	Ref.	Identifiable peak area, %extract				
						cEtOH	cEtOAc	cDCM	sDME	
1	*Polar comps*	1.75	Hexoses	179.0627 $[M-H]^-$	180.0700	Lib	0.30	1.33	0.05	0.71
2		1.92	Sucrose	387.1266 $[M-HCOO]^-$	388.1339	Lib	1.12	0.38	0.01	0.01
3		3.69	Methyl arbutin	331.1142 $[M-HCOO]^-$	286.1053	Lib	15.37	10.02	0.19	5.79
4		5.88	N-Nitroso-3-hydroxypyrolidine	175.0677 $[M-H]^-$	176.0750	Lib	13.34	3.73	1.49	12.57
5	*Glycosides of phenanthrene*	7.15	2-ethyl-6-((4,7,8-trimethoxyphenanthren-2-yl)oxy)tetrahydro-2H-pyran-3,4,5-triol	443.1695 $[M-H]^-$	444.1768	-	29.35	21.59	0.88	16.99
6		7.17	2-((6-ethyl-5-hydroxy-4-((4-hydroxybenzyl)oxy)-2-((4,7,8-trimethoxyphenanthren-2-yl)oxy)tetrahydro-2H-pyran-3-yl)oxy)-6-(hydroxymethyl)tetrahydro-2H-pyran-3,4,5-triol	909.3255 $[M+Cl]^-$	874.3348	-	1.49	1.23	0.01	1.10
7		7.32	4-Hydroxybenzaldehyde	121.0345 $[M-H]^-$	122.0418	-	3.61	6.46	18.91	10.86
8		7.69	2-((6-ethyl-5-hydroxy-4-((4-hydroxybenzyl)oxy)-2-((4,7,8-trimethoxyphenanthren-2-yl)oxy)tetrahydro-2H-pyran-3-yl)oxy)-6-(hydroxymethyl)tetrahydro-2H-pyran-3,4,5-triol	711.2709 $[M-H]^-$	712.2782	-	9.72	6.76	0.39	3.28
9		8.35	2-((6-ethyl-4-((4-hydroxybenzyl)oxy)-5-methoxy-2-((4,7,8-trimethoxyphenanthren-2-yl)oxy)tetrahydro-2H-pyran-3-yl)oxy)-6-(hydroxymethyl)tetrahydro-2H-pyran-3,4,5-triol	761.2647 $[M+Cl]^-$	726.2952	-	3.53	2.45	0.04	0.99
10		8.80	2-ethyl-6-((2-ethyl-6-((2-ethyl-4-((4-hydroxybenzyl)oxy)-5-((3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)-6-((4,7,8-trimethoxyphenanthren-2-yl)oxy)tetrahydro-2H-pyran-3-yl)oxy)-5-hydroxy-4-((4-hydroxybenzyl)oxy)tetrahydro-2H-pyran-3-yl)oxy)tetrahydro-2H-pyran-3,4,5-triol	1173.4110 $[M+Cl]^-$	1138.4621	-	1.11	0.12	0.00	0.02
11		8.91	2-((6-ethyl-5-((3-hydroxybenzyl)oxy)-4-((4-hydroxybenzyl)oxy)-2-((4,7,8-trimethoxyphenanthren-2-yl)oxy)tetrahydro-2H-pyran-3-yl)oxy)-6-(hydroxymethyl)tetrahydro-2H-pyran-3,4,5-triol	853.2932 $[M+Cl]^-$	818.3234	-	1.32	0.94	0.01	0.81
12		9.08	2-((6-ethyl-5-((6-ethyl-3,4-dihydroxy-5-methyltetrahydro-2H-pyran-2-yl)oxy)-4-((4-hydroxybenzyl)oxy)-2-((4,7,8-trimethoxyphenanthren-2-yl)oxy)tetrahydro-2H-pyran-3-yl)oxy)-6-(hydroxymethyl)tetrahydro-2H-pyran-3,4,5-triol	869.3327 $[M-H]^-$	870.3400	-	2.10	0.46	0.02	0.58
13	*Phenanthrene aglycone*	9.56	4-methoxy-9,10-dihydro-2,7-phenanthrenediol	241.0881 $[M-H]^-$	242.0943	(20)	3.66	3.20	18.25	4.27
14		10.73	4,7,8-trimethoxyphenanthren-2-ol	283.0709 $[M-H]^-$	284.1049	-	0.49	5.51	9.94	4.44
15		11.11	4-methoxy-2,7-phenanthrenediol	239.0719 $[M-H]^-$	240.0786	(20)	0.08	1.55	1.91	0.24
16		11.46	8-(4-hydroxybenzyl)-1,5,7-trimethoxy-9,10-dihydrophenanthren-2-ol	427.1085 $[M-H]^-$	392.1387	-	0.28	0.33	0.14	0.71
17		11.53	1,5-dimethoxy-2,7-phenanthrenediol	269.0832 $[M+Cl]^-$	270.0892	(20)	0.62	7.61	12.72	3.13
18		12.04	(E)-6-((4-hydroxycyclohexa-2,4-dien-1-ylidene)methyl)-1,5-dimethoxy-9,10-dihydrophenanthrene-2,7-diol	377.1402 $[M-H]^-$	378.1467	-	0.20	0.57	0.94	2.22

19	12.10	1-(4-hydroxybenzyl)-4-methoxy-9,10-dihydrophenanthrene-2,7-diol	347.1399 $[M-Cl]^-$	348.1476	(15)	2.46	4.77	8.49	7.11
20	12.32	1-(4-hydroxybenzyl)-9-methoxyphenanthrene-2,7-diol	345.1245 $[M-H]^-$	346.1205	-	1.49	3.38	2.11	3.60
21	12.70	**1-(4-hydroxybenzyl)-4,8-dimethoxy-2,7-phenanthrenediol (HDP)**	375.1361 $[M-H]^-$	376.1438	(15,20)	4.81	7.80	8.92	13.19
22	14.25	4,4'-((8-hydroxy-2,4,7-trimethoxyphenanthrene-1,9-diyl)bis(methylene))dicyclohexanol	507.1606 $[M-H]^-$	508.1679	-	0.95	2.99	4.15	2.28
23	14.26	2,5,7-trimethoxy-8,10-bis((4-methoxycyclohexyl)methyl)phenanthren-1-ol	537.1722 $[M-H]^-$	538.1795	-	2.58	6.84	10.45	5.10
	Total, %					100.00	100.00	100.00	100.00

Note; Lib is Mass Hunter library

Table 3 Inhibition of PDE5-1A by various extracts and %HDP content (the experiment was done in triplicate)

Extraction Method (Sample No.)	%Yield±SD (g/g)	%HDP content (g/g)	IC_{50} (in µg/mL) against PDE5 inhibitory activity
cDCM (No.3)	$1.64\pm0.15^{\#}$	$1.39\pm0.001^{*}$	1.12 ± 0.09^{d}
cEtOAc (No.24)	$2.81\pm0.27^{\dagger}$	$1.75\pm0.03^{**}$	1.30 ± 0.46^{d}
cEtOH (No.21)	$17.39\pm1.43^{\ddagger}$	$0.53\pm0.02^{***}$	4.03 ± 0.16^{c}
uDCM (No.28)	$1.87\pm0.12^{\#}$	$1.24\pm0.01^{****}$	1.24 ± 0.11^{d}
uEtOAc (No.29)	$2.80\pm0.72^{\dagger}$	$0.95\pm0.08^{****}$	1.64 ± 0.17^{c}
uEtOH (No.30)	$17.87\pm0.81^{\ddagger}$	$0.53\pm0.01^{***}$	6.29 ± 0.08^{b}
sDME (No.33)	$1.55\pm0.08^{\#}$	$4.47\pm0.21^{*****}$	0.67 ± 0.22^{a}

The uppercase symbols stand for significant difference ($p<0.05$).

Sildenafil was used as positive control and presented IC_{50} at 0.002±0.0008 µg/mL in triplicate

Figures

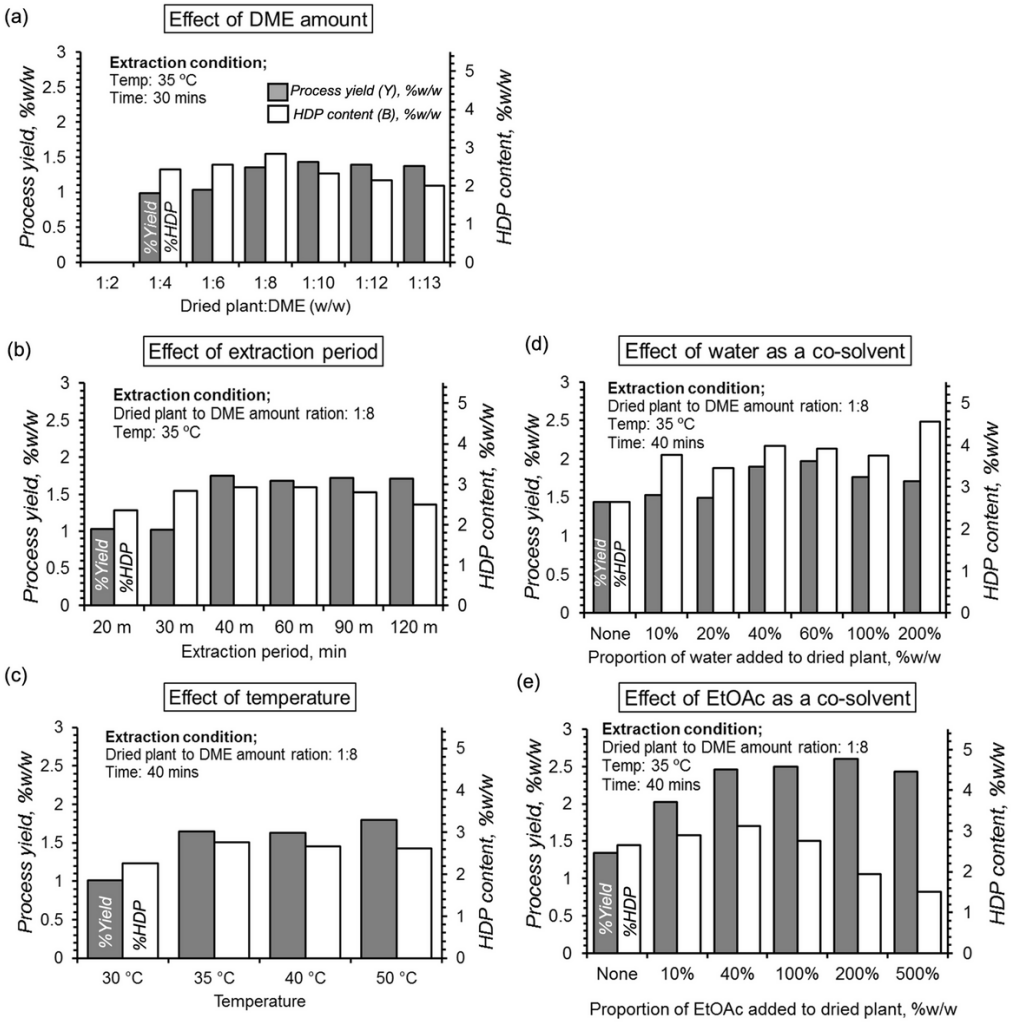

Figure 1

Parameters influencing dimethyl ether (sDME) extraction of E. Macrobulbon root powder as total yield (black bars) and its content of the bioactive ingredient, HDP measured LC/MS (open bars). Values deemed optimal for each parameter were used for the next parameter measures (b-e) which were (a) sDME volume (1:8), (b) (40 min extraction period), and (c) (35oC). Each bar is a single determinations.

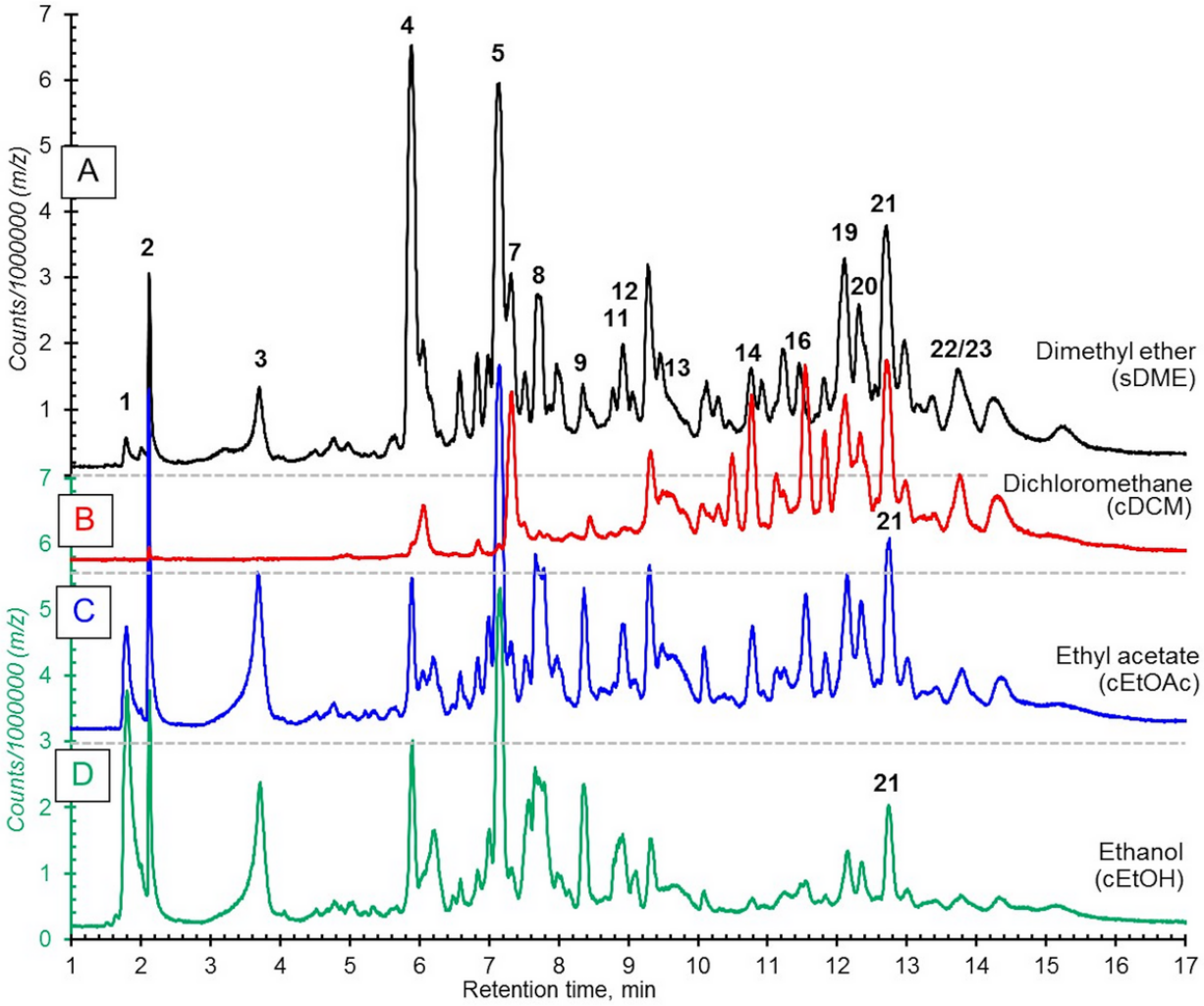

Figure 2

Total ion count LC-MS chromatograms (TIC) from of sample extracts of E. macrobulbon with 50 ug/mL. All chromatograms have the same y-scales but only A and D scales shown. The numbered peaks correspond with compounds identified in table 2. Extraction protocols were: (A) 10g water added 5g powdered E. Macrobulbon root and extracted with 40g DME (method of sample no. 31, table 1); (B) cDCM, method of sample no. 3, (table 1); (C) cEtOAc, method of sample no. 12, (table 1), (D) cEtOH, method of sample no. 12, (table 1)

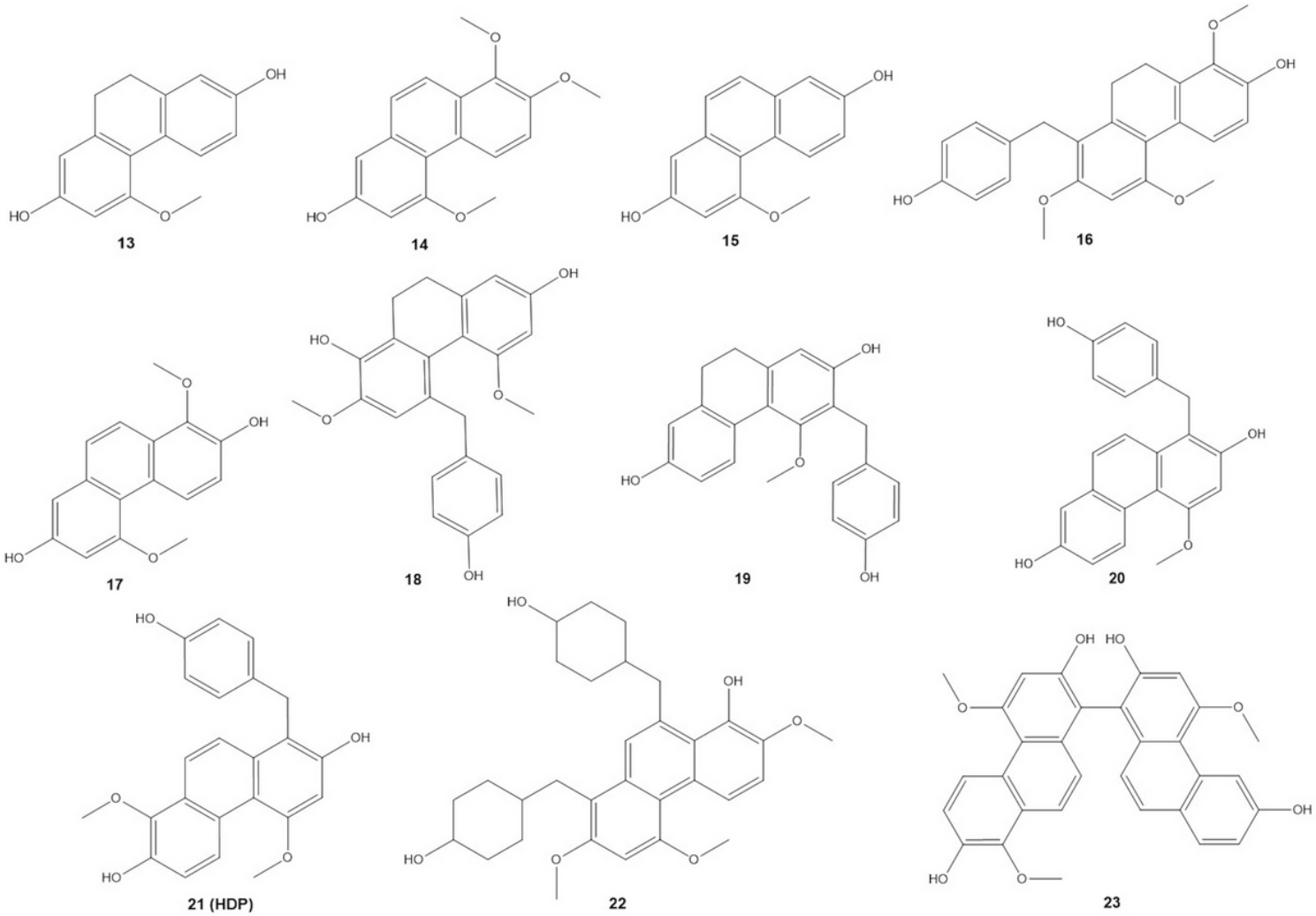

Figure 3

Identifiable compounds of aglycone phrenanthrene structure from E. Macrobulbon root extract using LC-QTOF-MS

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- AdditionalfileSrivilaiJ.docx
- FigureS1Srivilai.png
- GraphicalabstractSrivilai.png