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Traditional Chinese medicine extraction method by ethanol delivers drug-like molecules

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[ABSTRACT] Traditional Chinese Medicine (TCM) is an important reservoir for bioactive natural products. TCM extraction methods by water decoction and wine tincture are an integral part of TCM and essential for their widely acknowledged efficacy. In this study, we selected 6 common TCMs that are rich in chemistry to investigate whether the TCM extraction methods deliver molecules with drug-like physical chemical properties. Six TCM herbal materials were extracted by water, 95% ethanol, and sequential hexane, dichloromethane and methanol. The extracts were analyzed by HPLC and ^{1}H NMR. Isolation on one of the extracts yielded 32 compounds, their physical chemical properties were analyzed by Instant JChem. Our results showed that ethanol extraction, which mimics TCM wine tincture, delivered compounds with physical chemical properties compliant to Lipinski's rule of 5.

[KEY WORDS] Extraction; Traditional Chinese medicine (TCM); Natural products; Lipinski's Ro5Physicochemical properties

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Introduction

Traditional Chinese medicine (TCM) has been a mainstay of the Chinese healthcare system for thousands of years. Enormous knowledge has been accumulated through the observation of clinical efficacy and safety of TCM targeting a wide variety of diseases [1]. Extraction methods are an integral part of TCM and essential for their widely acknowledged efficacy. In the long history of evolution of TCM, various forms of herbal preparation had been developed. Among them, decoction in water and wine tincture are the two dominant ways to administrate herbal medicine due to their simple application, easy preparation, low cost and proven medicinal efficacy [2]. Water decoction involves boiling herbal formula in hot water, while wine tincture includes soaking herbal material in wine at different temperature. Traditionally, yellow wine (15%–20% of alcohol) is used with heating to process herbs, while white wine (50%–60% of alcohol) is used by soaking herbs at room temperature.

Lipinski's rule of 5 (Ro5) has long been used to predict the drug like properties of small molecules in modern drug discovery [3, 4]. By analysing the physicochemical properties of thousands of orally administrated medicines, Lipinski concluded that small molecules are more likely to be orally active if they have no more than one violation against the Ro5: molecular weight (MW) ≤500 Da, octanol-water partition coefficient (expressed as log P) ≤5, hydrogen bond donors (HBD) ≤5 (expressed as the sum of OHs and NHs), and hydrogen bond acceptors (expressed as the sum of Ns and Os) ≤10. Compound classes that are substrates for biological transporters are exceptions to the rule [4]. Natural products generally obey Lipinski's Ro5. An analysis of 126, 140 unique natural products sourced from *Dictionary of Natural Products* revealed that 60% of the natural products had no violation of Ro5 and 80% had nil or one violation and are thereby Lipinski compliant [5]. Our previous study also found that many TCM compounds possess favorable physical chemical properties required by Ro5, and large proportion of the compounds fall into physicochemical space occupied by small molecular drugs [6]. In the case of more complex natural products, other factors, such as the chameleon property of compounds, such as cyclosporin, could provide cell permeability, however, these chameleon properties of natural products are found in only a few natural products [7]. When Ro5 is modified to exclude these few categories, only a very few exceptions can be found [7]. Two decades have passed since

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Ro5 ushered in the concept of drug-like properties, it continues to play a significant role in decision making by industry leaders and scientists in seeking new medicines [8].

Studies have since been carried out to optimize extraction protocols that can front-load natural products with drug like properties [9]. One well established method used hexane to de-fat the plant material followed by the extraction with dichloromethane (DCM) and methanol, cross-linked N-vinylpyrrolidone-divinylbenzene (NVP-DVB) copolymer was then used to retain highly lipophilic components in the extract followed by C_{18} HPLC fractionation [9, 10]. The HPLC fractionation step provided a second LogP filter allowing high LogP components to be excluded. The LogP value of the compounds, a key indicator for drug like property, can be predicted by their retention time from the HPLC fractionation [9, 11].

During our ongoing research in bioactive natural products from TCM [6, 12-13], we have now investigated whether TCM extraction methods can afford compounds with drug-like properties. We selected six herbal materials that are known to contain many metabolites [14, 15]; *Polygonum multiflorum* Thunb, *Pueraria lobata* (Willd) Ohwi, *Acorus tatarinowii* Schott, *Uncariae ramulus* Cum Uncis, *Gastrodia elata* BI and *Ligusticum chuanxiong* Hort. Five extraction methods were compared by using boiled water, 95% ethanol, and sequential organic solvents, namely hexane, dichloromethane and methanol. The chemical composition and their LogP of the extracts were analysed by HPLC chromatography using the same conditions as that in the literature [9, 11], as well as by ^{1}H NMR spectroscopy. Further isolation was carried out on *Gastrodia elata* BI ethanol extract, resulting in 32 compounds. The physical chemical properties of the compounds were evaluated against Ro5. We herein report the comparison of the extraction methods, as well as the evaluation of the physical chemical properties of the isolated compounds.

Material and methods

Plant materials

Gastrodia elata BI and *Ligusticum chuanxiong* Hort herbal materials were purchased from Luosi Wan TCM wholesale Market in Kunming, Yunnan, China. These two TCM were authenticated by Prof. Jingui Shen from Shanghai Institute of Materia and Medica, Chinese Academy of Science. The herbal materials were stored at 4 °C until extraction. Other TCM materials, *Polygonum multiflorum* Thunb, *Pueraria lobata* (Willd) Ohwi, *Acorus tatarinowii* Schott, and *Uncariae ramulus* Cum Uncis, were obtained from Nature Bank, the biota repository at Griffith Institute for Drug Discovery.

General experimental procedures

Waters HPLC system with Phenomenex C_{18} monolithic HPLC column (100 mm × 4.6 mm) was used for the fractionation of the extracts. Waters and Thermo Fisher HPLC system with Phenomenex C_{18} Luna column (250 mm × 21.2 mm and 250 mm × 10 mm, 5 μm) were used for isolation of compounds from the extract. Bruker Ascend™ 800 MHz spectrometer was used to acquire ^{1}H-NMR spectra with DMSO-d_6 as solvent. Thermo Fisher LC-MS system with Thermo Scientific Accucore C_{18} column (150 mm × 2.1 mm, 2.5 μm) was used for MS analysis. All solvents used for extraction, chromatography and MS were HPLC grade or otherwise indicated.

Extraction

TCM material was extracted to afford 5 extracts for each sample.

Hot water extraction Dried and ground TCM material (600 mg) was placed in a glass tube (20 mL), then boiled water (15 mL) was added and the solution was kept boiling on heating mantle for three hours. The water supernatant was filtered and dried using rotary evaporator under vacuum followed by freeze-drying to afford the water extract.

Ethanol extraction 95% ethanol (15 mL) was added to TCM material (600 mg) in the glass tube, and the mixture was sonicated for 1 hr (15 min intervals, four times). The supernatant was decanted before the TCM material was extracted twice more using the same extraction method. The combined ethanol extracts were dried by rotary evaporator under vacuum followed by freeze-drying to afford the EtOH extract.

Sequential organic solvent extraction the dried and ground TCM material (600 mg) was extracted by hexane (15 mL × 3) under the sonication for 1 h (15 min intervals, four times). The hexane supernatant was decanted and dried under N_2 to give the hexane extract. The plant residue was then extracted by DCM (15 mL × 3) in the same way as that of hexane. The combined DCM extracts were dried under N_2 to afford the DCM extract. Using the same methods as that of hexane and DCM, the plant residue was further extracted by MeOH (15 mL × 3) to give the MeOH extract.

HPLC analysis

The TCM extract (2 mg) was dissolved in DMSO (100 μL). The extract mixture was then injected onto a Phenomenex C_{18} monolithic HPLC column (4.6 × 100 mm) with a gradient elution from 10% MeOH/90% H_2O (0.1% TFA) to 100% MeOH (0.1% TFA) over 7 min, then kept at 100% MeOH until 11 min, at a flow rate of 4 mL·min^{-1}.

Extraction and isolation of compounds from Gastrodia elata BI

The ground plant material of *Gastrodia elata* BI (2 kg) was extracted with 95% ethanol (2 L × 3) for 1 hr, and the combined ethanol extracts were dried under the vacuum followed by freeze-drying to afford the crude extract. The ethanol extract (30.0 g) was dispersed in minimal amount of methanol (about 120 mL) and mixed thoroughly with 50 g of C_{18} bonded silica 150A 35–70 U (Alletch), then dried and loaded on top of a glass column (ϕ 110 cm) packed with C_{18} silica gel. The column was then eluted sequentially with 5%, 10%, 20%, 30%, 40%, 50%, 70% and 90% methanol in water (2 L each) to afford 8 fractions. ^{1}H-NMR spectrum of 5% ethanol fraction indicated the presence of sugars, while other fractions contained compounds that warranted further purification.

The samples (200 mg) were pre-absorbed onto cotton and

then packed into a stainless-steel cartridge (10 mm × 30 mm) which was connected to the semi preparative column (Phenomenex C_{18} Luna column 250 mm × 21.2 mm). A gradient methanol in water (0.1% TFA) was run over 60 mins at a flow rate of 9 mL·min^{-1}. Sixty fractions (60 × 1 min) were collected and analysed by ^{1}H-NMR. Further purification of 10% MeOH fraction with several injections on C_{18} semi-preparative HPLC eluting with gradient MeOH/H_2O led to the isolation of compounds **1** (31.6 mg, 0.002%), **2** (56.7 mg, 0.003%), **19** (2.0 mg, 0.0002%), **20** (1.8 mg, 0.0002%), and **26** (1.8 mg, 0.0001%); purification of 20% and 30% using Phenomenex C_{18} Luna column HPLC eluting with gradient MeOH/H_2O yielded compounds **3** (2.9 mg, 0.0002%), **4** (21.1 mg, 0.001%), **5** (3.7 mg, 0.0004%), **6** (18.7 mg, 0.001%), **7** (20.3 mg, 0.001%), and **8** (2.5 mg, 0.0002%); purification of 40% fraction using similar conditions gave compounds **15** (17.7 mg, 0.001%), **18** (3.0 mg, 0.0002%), **27** (2.0 mg, 0.0001%), and **28** (1.5 mg, 0.0001%); purification of 50% on Phenomenex C_{18} Luna column HPLC eluting with gradient MeOH/H_2O provided compounds **9** (8.0mg, 0.0004%), **10** (1.4mg, 0.0001%), **11** (3.1mg, 0.0002%), **12** (9.9 mg, 0.0005%), **14** (1.4 mg, 0.0001%), **21** (6.6 mg, 0.0003%), **23** (1.3 mg, 0.0001%), **24** (2.2 mg, 0.0001%), **25** (1.5 mg, 0.0001%), and **30** (2.0 mg, 0.0001%); finally purification of fraction 70% and 90% on C_{18} semi-preparative column eluting with gradient MeOH–H_2O led to the isolation of compounds **13** (7.5 mg, 0.0004%), **16** (3.1 mg, 0.0002%), **17** (1.5 mg, 0.0001%), **22** (1.0 mg, 0.0001%), **29** (8.5 mg, 0.0006%), **31** (1.5 mg, 0.0001%), and **32** (2.5 mg, 0.0002%). The yield was calculated based on the dried weight of TCM material. Thirty-two pure compounds (**1**–**32**) were isolated, their chemical structures were determined by NMR spectroscopic data, and by comparison with the NMR data in the literature.

Calculation of physicochemical properties

The chemical structures were converted to SMILE code, and then uploaded to Instant *Jchem* software (version 18.5.0) (ChemAxon) (http: //www.chemaxon.com) to calculate their physicochemical properties [16].

Results

Six TCM herbal materials, namely *Polygonum multiflorum* Thunb, *Pueraria lobata* (Willd) Ohwi, *Acorus tatarinowii* Schott, *Uncariae ramulus* Cum Uncis, *Gastrodia elata* BI and *Ligusticum chuanxiong* Hort, were selected. These herbal materials are known to be rich in metabolites [14, 15]. They produced a diverse class of compounds, such as stilbene glucoside, anthraquinones and phospholipids from *Polygonum multiflorum* Thunb [17-18], hydroxybenzyl derivatives from *Gastrodia elata* BI [19-21], volatile oils and phenolic acids from *Ligusticum chuanxiong* Hort [22], isoflavones and triterpenoids from *Pueraria lobata* (Willd) Ohwi [23], and indole alkaloids from *Uncariae ramulus* Cum Uncis [24].

Five extraction methods were used for each plant material. Hot water and ethanol extraction mimicked TCM's water decoction and wine tincture and was compared with sequential organic solvent extraction by hexane, DCM and methanol. Five extracts were obtained for each TCM material, namely water, ethanol, hexane, DCM and methanol extracts. The chemical composition of each extract was analysed by a reversed phase C_{18} HPLC with a gradient elution from 10% MeOH to 100% MeOH (Fig. 1). The extracts were also analysed by ^{1}H NMR spectroscopy (Figs. 2 and 3).

Using the same HPLC condition as those in the literature [11], HPLC chromatograms (Fig. 1) exhibited a general pattern. For all six TCM materials, hexane and DCM delivered relatively nonpolar compounds with retention times ≥ 5 min, while methanol and water yielded more polar compounds which eluted from the HPLC before 6 min and 4 min, respectively. In comparison, ethanol afforded molecules across the HPLC chromatogram, with most compounds having a retention time between 0–7 mins. Compounds that elute within 7 min in this HPLC method normally have Log P ≤ 5 [11], hence are likely to be orally active. Ethanol extracts contained compounds with retention time ≤ 5 min. Further examination on the number of peaks in the chromatograms suggested that ethanol extracted almost all the compounds that were present in water, methanol, DCM, and to some extent, hexane extracts. In the case of *Acorus tatarinowii* Schott (Fig. 1), ethanol also yielded compounds between 6–7 mins, these were not present in either DCM or hexane extracts.

The ^{1}H NMR spectra were also examined to further compare the chemical compositions of the extracts (Figs. 2 and 3) [25], confirming the rich chemical constituents by ethanol extraction. For both *Gastrodia elata* BI and *Ligusticum chuanxiong* Hort, ^{1}H NMR signals that were present in hexane, DCM, methanol and H_2O extracts can mostly be accounted for in ethanol extracts (Figs. 2 and 3).

Further isolation work was carried out on *Gastrodia elata* BI ethanol extract. Major metabolites were targeted, and their physicochemical properties were evaluated against Ro5. The dried and ground *Gastrodia elata* BI herbal material was extracted exhaustively by 95% ethanol. The extract was fractionated on C_{18} flash column eluting with gradient methanol/water. The fractions from flash chromatography were further purified by Phenomnex C_{18} Luna column HPLC eluting with gradient methanol/water (0.1% TFA) to afford 32 pure compounds (Fig. 4), namely 4-hydroxybenzyl alcohol (**1**) [26], gastrodin (**2**) [27], parishin A (**3**) [28], parishin B (**4**) [28], parishin C (**5**) [28], parishin E (**6**) [29], parishin H (**7**) [30], parishin K (**8**) [31], bis(4-hydroxybenzyl) sulphide (**9**) [32], bis(4-hydroxybenzyl) sulfide (**10**) [33], bis(4-hydroxybenzyl) sulfide (**11**) [34], 4, 4′-dihydroxydiphenylmethane (**12**) [35], 2, 4-bis(4-hydroxybenzyl)phenol (**13**) [36], 4, 4′-dihydroxydiphenylmethane-*O*-*β*-D-glucopyranoside (**14**) [35], plumieride (**15**) [37], bis(2-ethylhexyl) phthalate (**16**) [38], dibutyl phthalte (**17**) [38], *S*-(4-Hydroxybenzyl) glutathione (**18**) [39], adenosine (**19**) [40], 6-*N*-(4-Hydroxybenzyl)-2-oxoadenosine (**20**) [41], 4-hydroxyl benzaldehyde (**21**) [42], 3-(4-hydroxyphenyl)-

2-propenoic acid (**22**) [43], ferulic acid (**23**) [43], aspartic acid (**24**) [44], 3-hydroxy-4-methoxy benzaldehyde (**25**) [26], citric acid (**26**) [28], 4-methoxyphenyl glucopyranoside (**27**) [45], 4-(hexopyranosyloxy) benzaldehyde (**28**) [27], 3-phenoyl-2-propenoic acid (**29**) [46], 4-hydroxyacetophenon (**30**) [47], cymbinodin A (**31**) [48], and 4, 5, 6-trimethoxyfuro[2, 3-b]quinolone (**32**) [49]. The structures of the pure compounds were determined by LCMS, 1D-, and 2D-NMR, and confirmed by the comparison of the NMR data with those reported in the literature. The raw data files are included in the Supporting Information.

The HPLC analysis of the 32 isolated compounds using Camp's method [9, 11] showed all the compounds were eluted before 7 min (Fig. 4), indicating LogP ≤ 5.

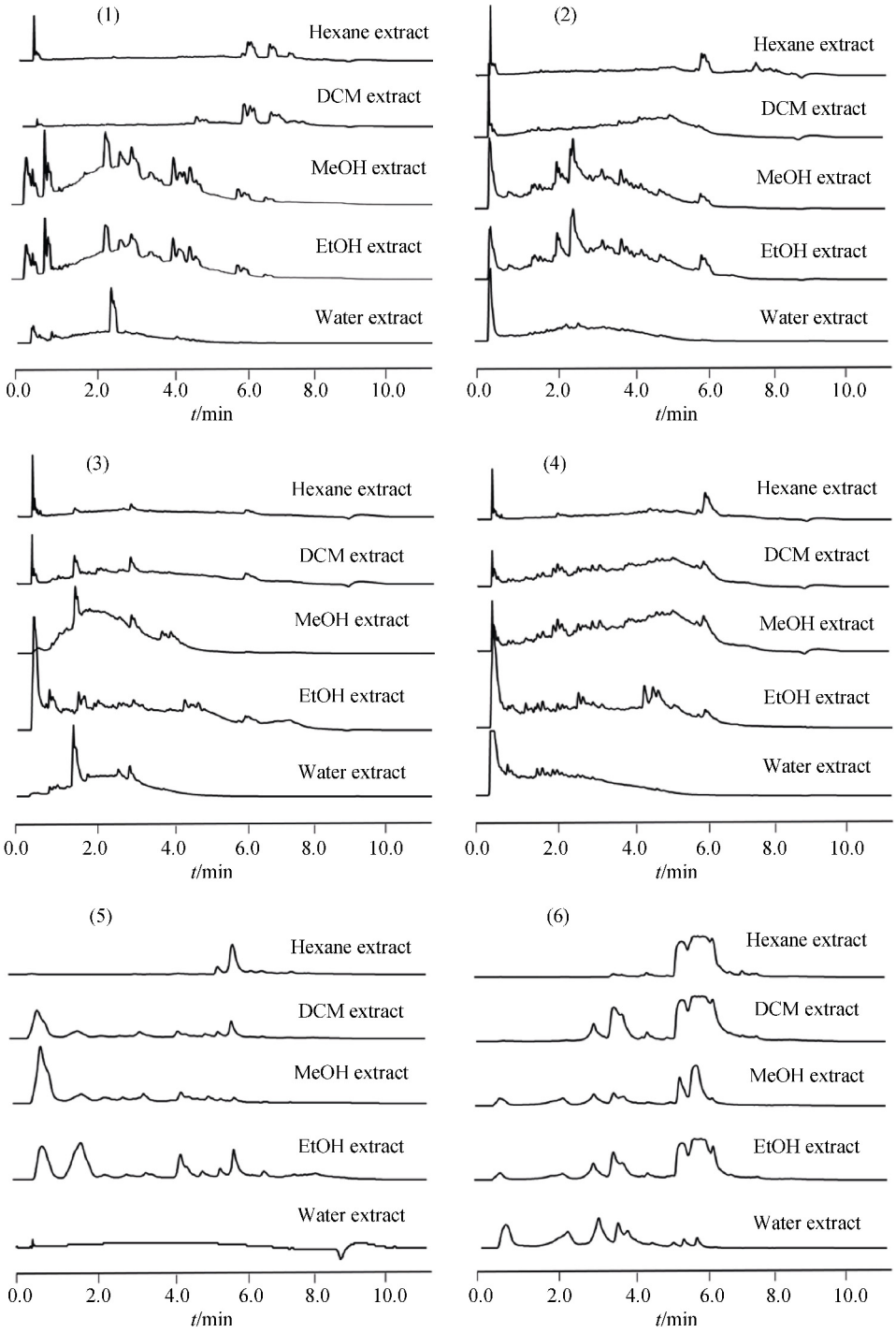

Fig. 1 HPLC chromatograms of extracts from (1) *Polygonum multiflorum* Thunb (2) *Pueraria lobata* (Willd) Ohwi (3) *Acorus tatarinowii* Schott (4) *Uncariae ramulus* Cum Uncis (5) *Gastrodia elata* BI and (6) *Ligusticum chuanxiong* Hort. The chromatograms were at wavelengths of 254 nm

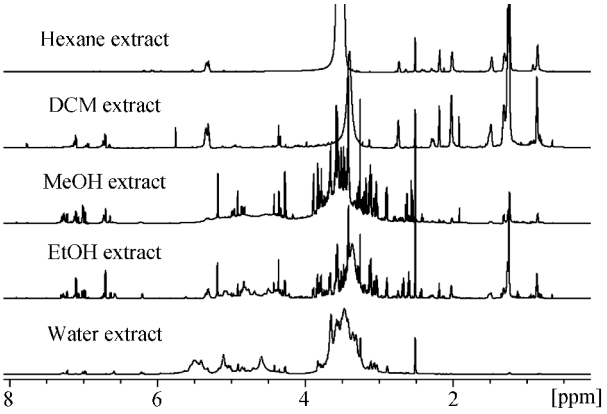

Fig. 2 ^{1}H-NMR spectra of the extracts from *Gastrodia elata* BI. The NMR spectra were acquired on 800 MHz with DMSO-d_6 as solvent

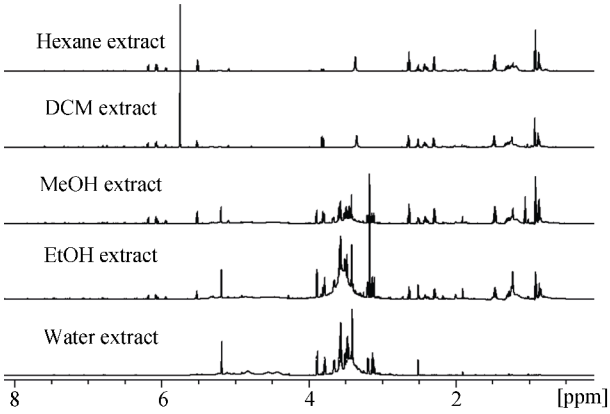

Fig. 3 ^{1}H-NMR spectra of the extracts from *Ligusticum chuanxiong* Hort. The NMR spectra were acquired on 800 MHz with DMSO-d6 as solvent

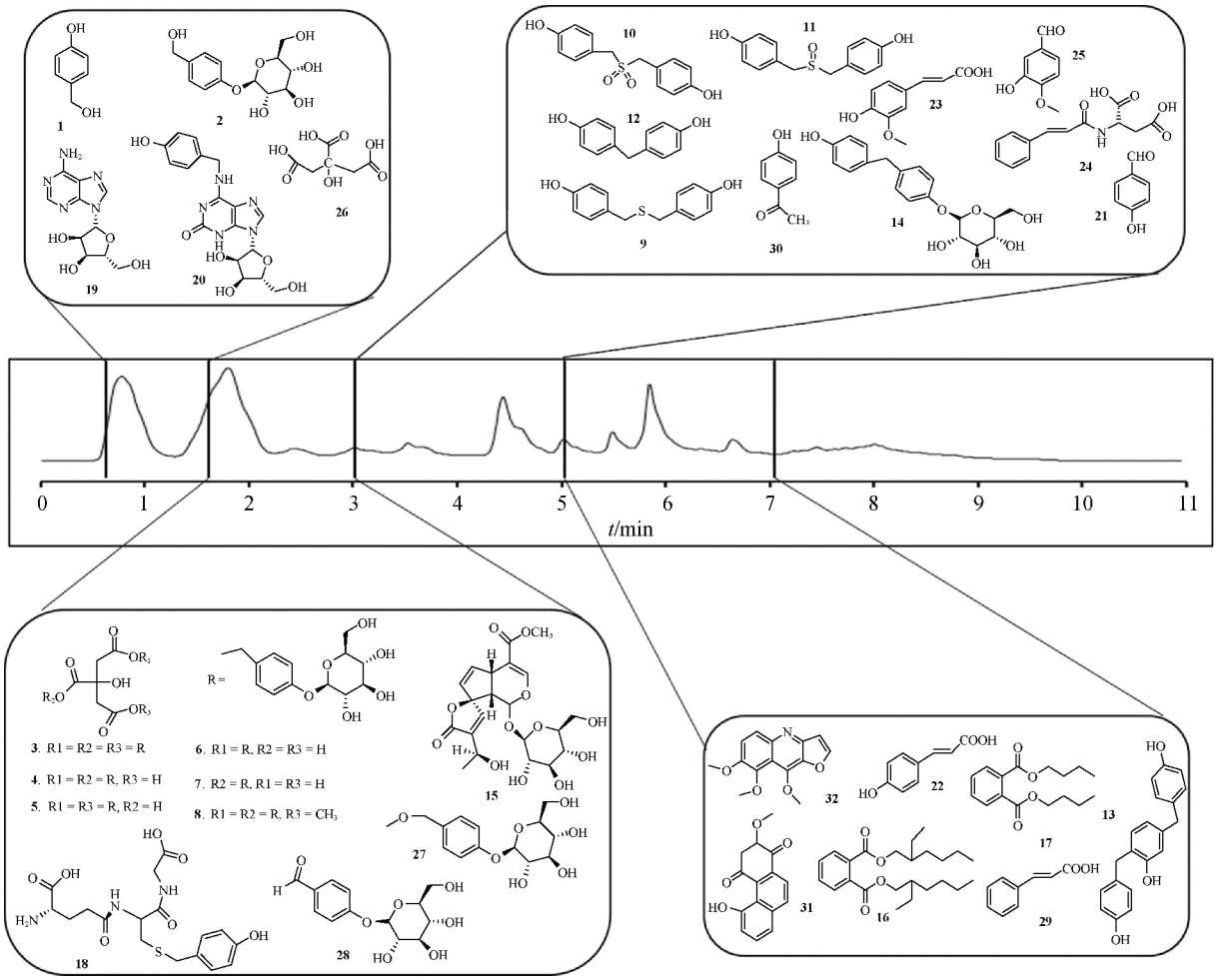

Fig. 4 The chemical structures of the 32 major compounds from *Gastrodia elata* BI ethanol extract

The physicochemical properties of the 32 major compounds were calculated using Instant *J*Chem software (version 18.5.0) (ChemAxon) (http: //www.chemaxon.com). The properties included molecular weight (MW), octanol-water partition coefficient (LogP), hydrogen bond donors (HBD) and hydrogen bond acceptors (HBA), and the results are shown in Fig. 5.

The data showed that most of the compounds isolated from *Gastrodia elata* BI ethanol extract obeyed the Lipinski's rule of 5. Of the 32 compounds, 24 had no violation at

all, while 8 compounds had one or more violations against Ro5. Overall, 87.5% of the compounds had the molecular weight ≤ 500, 96.9% with log P ≤ 5, 81.3% had the HBD ≤5, while 78.1% had the HBA ≤ 10. The outliers mainly included citryl glycosides (parishins), namely parishins A, B, C, E, H and K (**3**–**8**) (Table 1). The sugar groups provided O-functionality and hydroxyl groups, which accounted for the violation of HBD and HBA values, as well as molecular weights. However, the glycosides are likely to be hydrolyzed in the gut, the aglycones obtained by hydrolysis are Ro5 compliant (Table 1) and are likely to be orally bioavailable.

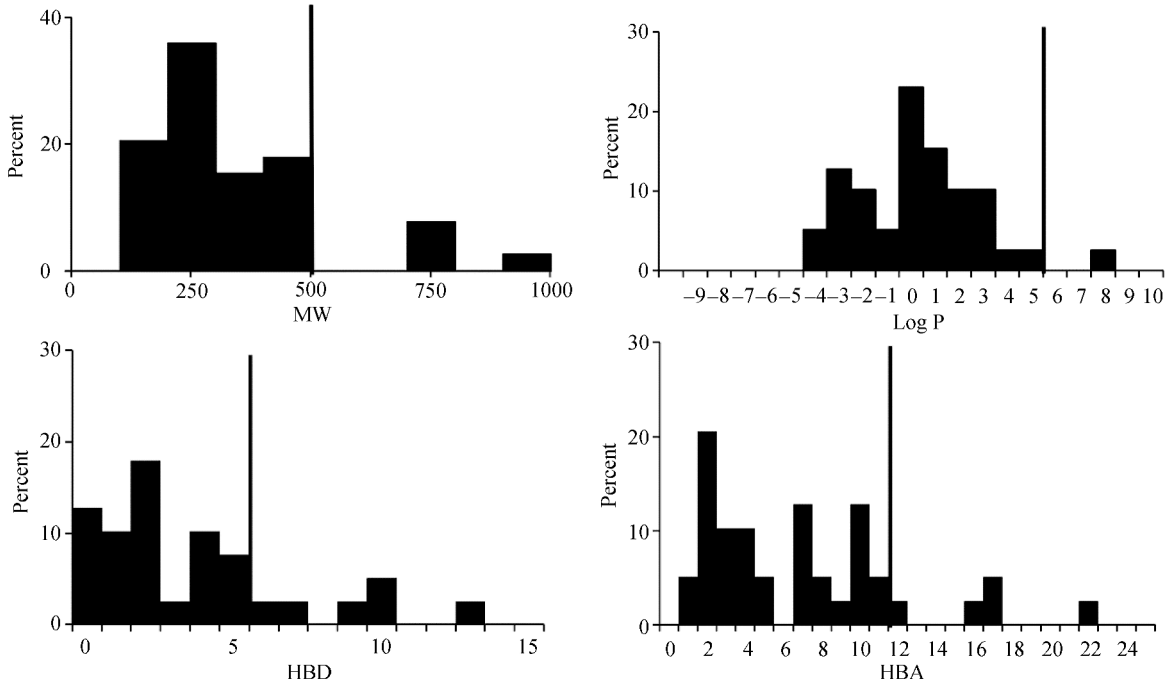

Fig. 5 Physicochemical properties of 32 compounds from *Gastrodia elata* BI ethanol extract, including molecular weight (MW), octanol-water partition coefficient (LogP), hydrogen bond donors (HBD) and hydrogen bond acceptors (HBA)

Table 1 Physicochemical properties of citryl glycosides (3–8) and their respective aglycone

Compounds	MW (Da)	LogP	HBD	HBA
Parishin A (**3**)	996.9	–3.4	13	22
Aglycone of parishin A (**3**)	510.5	3.4	4	7
Parishin B (**4**)	728.7	–2.7	10	17
Aglycone of parishin B (**4**)	404.4	1.8	4	7
Parishin C (**5**)	728.7	–2.7	10	17
Aglycone of parishin C (**5**)	404.7	1.8	4	7
Parishin E (**6**)	460.4	–2.0	6	12
Aglycone of parishin E (**6**)	298.3	0.24	4	7
Parishin H (**7**)	460.4	–2.0	6	12
Aglycone of parishin H (**7**)	298.3	0.24	4	7
Parishin K (**8**)	740.0	–2.6	9	16
Aglycone of parishin K (**8**)	418.4	1.96	3	6

Discussion

Inspired by traditional TCM knowledge, our research mimicked traditional administration method of TCM using 95% ethanol rather than organic solvents, such as hexane and dichloromethane. The experiment led to the isolation of 32 compounds that have physicochemical properties suggesting oral bioavailability. It may be beneficial to use 95% ethanol to isolate the potential orally bioavailable components in TCM.

Even though 21.9% and 18.7% of compounds violated in HBA and HBD requirements, respectively, almost all the compounds (96.9%) had desirable partition coefficient, a key indicator for oral bioavailability. The results confirmed that ethanol extraction, which mimicked the traditional wine tincture

preparation, indeed yielded compounds with favorable physicochemical properties, and hence are likely to be orally available [4].

The design and application of green and sustainable extraction methods have been intensely researched by industries [50]. Alternative solvents, principally water or agro-solvents generated by agriculture process have been recommended given they are less flammable and volatile. Ethanol, produced from sugar or starch, is considered a green, renewable, and non-toxic solvent for extraction compared to other organic solvents such as dichloromethane and methanol. Our research also confirmed that ethanol can deliver molecules that are likely to be orally active.

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