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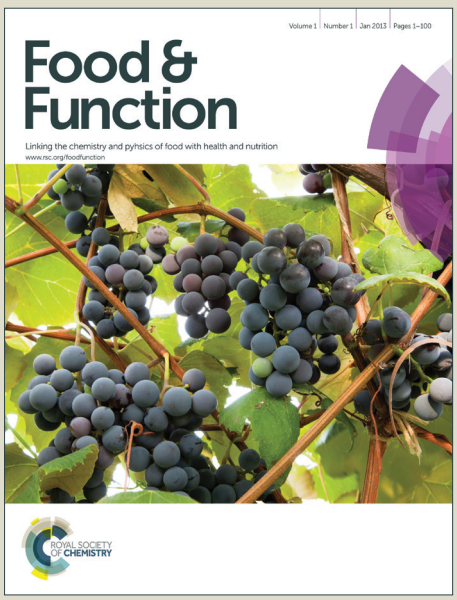

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Investigation on the antidepressant effect of sea buckthorn seed oil through the GC-MS-based metabolomics approach coupled with multivariate analysis

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Abstract: Depression is one of prevalent and serious mental disorders and the number of depressed patients has been on the rise globally during the recent decades. Sea buckthorn seed oil from traditional Chinese medicine (TCM) is edible and has been widely used for treatment of different diseases for a long time. However, there are few published reports on antidepressant effect of sea buckthorn seed oil. With the objective of finding potential biomarkers of the therapeutic response of sea buckthorn seed oil in chronic unpredictable mild stress (CUMS) rats, urine metabolomics based on gas chromatography-mass spectrometry (GC-MS) coupled with multivariate analysis was applied. In this study, we discovered a higher level of pimelic acid as well as palmictic acid and a lower level of suberic acid, citrate, phthalic acid, cinnamic acid and sumiki's acid in urine of rats exposed to CUMS procedures after sea buckthorn seed oil administered. These changes of metabolites are involved in energy metabolism, fatty acid metabolism as well as synthesis of neurotransmitter and other metabolic pathways and it is helpful to facilitate the efficacy evaluation and mechanism elucidating of sea buckthorn seed oil for depression management.

Keywords: sea buckthorn seed oil; metabolomics; antidepressant; gas chromatography-mass spectrometry; chronic unpredictable mild stress.

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Introduction

Depression, a widespread neuropsychiatric disorder, affects 350 million diagnosed patients and their families worldwide[1]. And this number has increased rapidly for decades. The World Health Organization (WHO) calculated that depression will be the first leading cause of global disability by 2030. Depression is characterized by depressed mood, anxiety, anhedonia, disturbed sleep and cognitive impairment even suicidal ideation[2], and increases our vulnerability to other common complex diseases such as type II diabetes[3], cardiovascular disease[4] and dementia[5]. In recent years, many synthetic antidepressants were introduced to treat depression, such as tricyclic antidepressants, selective serotonin reuptake inhibitors, and so on. However, their therapeutic effects are not satisfying with a variety of side effect such as psychomotor impairment and dependence liability[6]. Under this situation, effective and safe therapies for depression are in great demand. Recently, traditional Chinese medicines (TCM), with a long historical clinical practice and less side-effects, are being attracted great interest all over the world. And rather than conventional medicines, traditional Chinese medicine (TCM) emphasize on holistic point of view and devote to repair the organism with consideration of overall health with no or low toxicity. So, an ideal antidepressant from TCM is desperately needed.

Sea buckthorn (*Hippophae* L.), a thorny nitrogen-fixing deciduous shrub, has been used extensively for treatment of different diseases in system of medicine for 1000 years in China[7]. This plant is considered to be of high medicinal and nutritional properties. For example, various preliminary studies verified that sea buckthorn had significant antimicrobial and antioxidative[8], cytoprotective[9], hepatoprotective[10], immunomodulatory properties[11]. The sea buckthorn seed oil, supercritical CO_2-extracted from sea buckthorn seed, is rich in bioactive substances like fatty acids, carotenoids, tocopherols and phytosterols. And it is reported to be effective in cardiovascular diseases[12], atopic dermatitis[13] and gastric ulcers[14]. At present, sea buckthorn seed oil is becoming more and more popular as a special ingredient and food supplement all over the world[15]. However, few studies on anti-depression function of sea buckthorn seed oil were reported. For this reason, edible sea buckthorn seed oil as diet therapy would be a promising alternative for depression management.

Chronic unpredictable mild stress (CUMS), a classical model of depression, has a greatly predictive and constructs validity. This was established largely to induce changes in depression-like behaviors of rats, and most of these changes are reversed by anti-depressant treatments[16, 17]. Usually, the central contribution is indispensable to allocate a statistical relationship with peripheral changes and the central and peripheral pools interrelate by systemic homeostatic mechanisms. So, peripheral changes of NTs and their metabolites in biofluids such as plasma or serum as well as urine could reflect changes that occur in the brain. In the other word, the plasma or serum and urine metabolomics may facilitate the diagnosis of depression and therapeutic evaluation of antidepressants.

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Metabolomics, a powerful top-down systems biological approach, is capable of simultaneously measuring global metabolic compositions in any biosystem chosen for study[18]. Metabolomics profiling coupled with multivariate statistical analysis can capture considerable subtle systemic response to pathophysiological stimuli or stress by identification of potential biomarkers using some advanced data acquiring methods such as nuclear magnetic resonance (NMR) spectroscopy[19], gas chromatography -mass spectrometry (GC-MS)[20], and liquid chromatography-mass spectrometry (LC-MS)[21], which providing insights into the underlying mechanisms and treatments of disease[22, 23]. GC-MS, has been extensively used in metabolomic studies because of its existence of extensive databases on the basis of fragmentation patterns, which help the identification of metabolites[24]. Recently, the application of the metabolomic based on GC-MS has shown value in the evaluation of the therapeutic effects and elucidation of the therapeutic mechanisms of drugs[25].

In this study, GC-MS based urine metabolomics coupled with multivariate statistical analysis was used to identify biomarkers for intervention of the sea buckthorn seed oil, where the changes in endogenous metabolites concentration may contribute to efficacy evaluation and underlying mechanism of sea buckthorn seed oil.

Materials and methods

Chemicals

All chemical reagents were of analytical grade. Pyridine, chloroform, anhydrous ethanol, n-hexane, sodium hydroxide, and ethyl chloroformate (ECF) were commercially obtained from China National Pharmaceutical Group Corporation (Beijing, China). L-2-chlorophenylalanine (Shanghai Intechem Tech. Co. Ltd., China) was used as an internal quality standard. Venlafaxine hydrochloride capsules (Chengdu KangHong Pharmaceutical, Lot No. 090705) were purchased from the First Hospital of Shanxi Medical University (Taiyuan, China).

Animals

Male S.D. rats weighed 180-220 g were commercially obtained from Vital River Laboratories SCXK (Jing) 2012-0001 and were acclimated to the research facility for 1 week. All animals were housed in a temperature-controlled environment (22 ± 1℃) and were maintained on a 12:12 h light/dark cycle (lights on at 8:00 a.m.) with free access to water and food, except when the CUMS procedure was being conducted.

CUMS procedure and drug administration

In this study, all protocols were performed in accordance with the National Institutes of Health regulations for the care and use of laboratory animals in research. After a habituation for two weeks, all rats were randomly divided into 6 groups (n=10 in each group): healthy control group (HC group, no stress and no treatment), CUMS-models group (CUMS group, stress plus pure 1 mL/kg of normal soya bean salad oil), high dose group of sea buckthorn seed oil group (SH group, stress plus sea

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buckthorn seed oil at the dose of 1.40 g/kg body weight), middle dose group of sea buckthorn seed oil group (SM group, stress plus sea buckthorn seed oil at the dose of 0.7 g/kg body weight), low dose group of sea buckthorn seed oil group (SL group, stress plus sea buckthorn seed oil at the dose of 0.35g/kg body weight) and venlafaxine hydrochloride group (VH group, stress plus venlafaxine hydrochloride 50 mg/kg body weight). All rats were housed individually except the control group, where the CUMS procedure was conducted and the drugs were administered for 4 weeks on all animals except controls. During the CUMS procedure, all of rats except controls were subjected to various mild stressors for 4 weeks including 5-min of forced swimming in cold water (4℃), 24 h of food deprivation, 24 h of water deprivation, 5 min of exposure to a hot environment (45℃), a 2-min tail pinch (1 cm from the proximal end of the tail), and a foot shock (36 mV with a duration of 10 s for each shock). Additionally, each of additional restraint stress and ultrasonic (40 MHz) electromagnetic radiation stress was applied for 3 h. All of these stressors were scheduled in a semi-random order to make the animals unpredictable. On average, each animal exposed to only one stressor at a time daily. Venlafaxine hydrochloridewas dissolved with saline to the required concentration and sea buckthorn seed oil was dissolved with soya bean salad oil to the required concentration. All drugs were administered at a volume of 1 mL/kg to the CUMS-treated rats via gastric intubation for four weeks. The NS and MS groups were given 1 mL/kg of normal soya bean salad oil.

Behavior test

Open-field test

The open-field test was conducted between 8:00 am to 12:00 am in a quiet room (≤60 dB) as described previously[26]. The open-field apparatus (square area, 100 cm×100 cm; high, 40 cm) was marked with a grid dividing it into 25 equal-size squares. Each animal was placed in the central square and observed for 5 min. Scores for each animals were calculated by the number of rearing (defined as standing upright on its hind legs) and the number of crossing (grid lines it crossed with at least three paws).

Sucrose preference test

After water and food deprivation for 24 h, each animal was placed in an individual metabolic cage, where two bottles containing 1% sucrose solution and water were placed, respectively. The ratio of the amount of sucrose solution to that of total solution ingested within 1 h was defined as the parameter of hedonic behavior.

Statistics analysis

Quantitative data were recorded as mean ± SD. The significance of variation of data between groups in behavior changes was determined via paired-sample *t*-test using SPSS 17.0. Here, the $p < 0.05$ level with 95% confidence intervals was considered statistically significant.

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Sample collection and preparation

After four weeks of the experimental procedure, urine samples from all rats were collected for 12 h overnight fast in metabolism cages, where sodiumazide was added to the collection tubes as an antibacterial agent. After centrifugation at 4 ℃ at 13,000 r/min for 15 min, urine samples were immediately divided into equal aliquots and stored at −80 ℃ until GC-MS analysis.

The preparation of samples was conducted as previous literature[27]. In brief, each 600 μL of urine was added to a screw-top glass vessel. Another 100 μL of L-2-chlorophenylalanine (internal standard, 0.1 mg/ml), 400 μL of anhydrous ethanol, 100 μL of pyridine and 50 μL of ECF were successively added for derivatization. After a vortex-mixing for 2 min, 500 μL of n-hexane was immediately added and a pH ≈ 9~10 for the mixture was subsequently made by sodium hydroxide solution (7mol/L). Additional 50 μL of ECF was added for another derivatization. 300 μL of supernatant was obtained after a centrifugation at 4 ℃ at 13,000 r/min for 10 min. The procedure was repeated with another 500 μL of n-hexane into the residues and 300 μL of supernatant was transferred. Finally, the 700 μL of filtrate was dried under a stream of nitrogen gas and isolated mixed with 200 μL of chloroform following GC-MS analysis.

GC-MS spectroscopy acquisition

The procedure was conducted as preliminary study[27] with minor adjustment. Each 1.0 μL of these derivative was injected into a gas chromatograph coupled with a PolarisQ Ion Trap mass spectrometer (Thermo Fisher Scientific Inc.,Waltham, MA, USA) in a split mode (10:1) and with electron impact ionization (70 eV). A DB-5MS capillary column (30 m×250 μm i.d., 0.25 μm film thickness; 5% diphenyl cross-linked 95% dimethylpolysiloxane; Agilent J&W Scientific, Folsom, CA) was used to make a separation with helium carrier gas with 1 mL/min of a flow rate. Either the interface temperature or the injection temperature was adjusted to 260 ℃; and the ion source temperature was set at 200 ℃. Initial GC oven temperature was 80 ℃ and rose to 140 ℃ with ℃/min for 2 min after injection, then to 240 ℃ with 4 ℃/min, to 260 ℃ at a rate of 10 ℃/min again. Data acquisition was conducted in the full scan mode (m/z 50-500).

Identification of the endogenous metabolites

Low molecular weight metabolites in collected urine samples were analyzed and represented as the chromatographic peaks in the GC total ion current (TIC) chromatograms. Peaks with intensity exceeding 10-fold of the signal-to-noise (S/N) ratio were integrated and recorded. The AMDIS (version 2.1, DTRA/NIST, USA) software was used to interpret EI-MS spectra of these peaks. Identification of metabolites was based on the existing database NIST library 2005 and most of them were further validated using the commercial available standards via comparing their MS fragmentation patterns and retention times. In addition, the Human Metabolome Database (HMDB) (http://www.Hmdb.ca) and published references are also used as the reference.

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Data analysis

According to preliminary study[27], all of the GC-MS metabolite profiles were converted into a NetCdf file format by Xcalibur, and then processed via the XCMS toolbox (http:// metlin.Scripps.edu/download/) using the settings including: xcmsSet (full width at half-maximum: fwhm = 4; S/N cutoff value: snthresh = 10, max = 20), group (bw= 20). The table obtained was exported into a Microsoft Excel, where normalization was performed prior to multivariate analysis. The resulting three-dimensional matrix involving sample names (observations), peak index (RT–m/z pair) and normalized peak area percent were introduced into SIMCA-P 13.0 software package (Umetrics AB, Umea, Sweden), which utilizes principal component analysis (PCA) for a natural separation among the six groups in this study by visual inspection of score plots, a supervised pattern recognition approach orthogonal projection to latent structures discriminant analysis (OPLS-DA) to maximize difference between groups by incorporating the known classification and VIP statistics to extract novel potential biomarker ions in the OPLS-DA model. The novel potential biomarker ions was extracted based on the variable importance in the project(VIP) of the established OPLS-DA model analysis (VIP $\geq$ 1.00) and a independent-samples t-test (p<0.05) using SPSS 16.0. Then the correlations among metabolites were obtained by deriving Spearman's correlation coefficient between each pair of potential biomarkers via MetaboAnalyst software (http://www. metaboanalyst.ca/). Correlation matrixes were used to visualize the correlation between metabolites. The analysis of potential biomarkers as well as metabolic pathways affected by sea buckthorn seed oil administration were carried out using HMDB and KEGG database on internet.

Results

Behavioral tests in CUMS-treated rats

The results of body weight and open-field test as well as sucrose preference test during the experimental period were summarized in **Table 1**. After four-week CUMS exposure administrated, rats in CUMS group showed a significant decrease in body weight, while rats in control group and those groups with drugs treated increased significantly (p<0.05), indicating that CUMS procedure could bring a decrease in body weight of rat and sea buckthorn seed oil played an important role in the reversion. During the sucrose preference test, the percentages of sucrose consumption of rats in CUMS group showed a significant decrease compared with health controls and rats with a CUMS exposure and sea buckthorn seed oil or venlafaxine treated on the 28th day (p<0.01), suggesting that an decreased interest was induced by CUMS procedure and sea buckthorn seed oil had an obvious improvement of this symptom. In the pen-field test, a significantly fewer number of crossings and rearings were performed by rats in CUMS group compared to those in HC group (p<0.01) on the 28th day, while this number increased after the treatment of sea buckthorn seed oil or venlafaxine (p<0.01).This results indicated that CUMS procedure caused a less activity occurred frequently in depressed patients but sea buckthorn seed oil had an good reversion for it. The

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ability of the animals to maintain body weight, the sucrose preference test and the open field test are all indications of the success of the duplication of depression that is produced by this animal model. The behavioral results of the present study show that the CUMS procedure was very effective in duplicating a depressive state and sea buckthorn seed oil had an obvious antidepressant effect.

Table 1 Effect of sea buckthorn seed oil and venlafaxine hydrochloride on changes of behavior in rats exposed to CUMS on the 28th day.

Groups	HC	CUMS	SH	SM	SL	YH
Crossing	46 ±4.1[a]	3 ±1.6	42 ±5.2 [a]	36 ±4.0 [a]	51 ±5.7 [a]	25 ±2.6 [a]
Rearing	15 ±2.8 [a]	1 ±0.7	14 ±3.0 [a]	8 ±2.1[a]	12 ±2.9 [a]	5 ±0.7 [a]
Body weight change	351.0 ±14.9 [a]	263.2 ±15.2	338.2 ±18.6 [a]	316.3 ±10.9[a]	330.2 ±11.3 [a]	315.5 ±18.2 [a]
Glucose consumption	72.4 ±7.3 [a]	56.7 ±7.4	74.6 ±8.4[a]	73.9 ±2.8 [a]	77.8 ±5.1 [a]	69.5 ±6.0 [a]

Data are expressed as mean ± SD. n=10. HC, control group; CUMS, CUMS-model group, YH, venlafaxine hydrochloride group, SH, high dose group of sea buckthorn seed oil; SM, middle dose group of sea buckthorn seed oil; SL, low dose group of sea buckthorn seed oil.

[a] Means a statistically significant difference at $p < 0.01$ when compared with CUMS-model group.

Analysis of GC-MS profiles

Typical GC-MS chromatograms of urine samples from the six groups were shown in **Fig. 1**. According to the existing database NIST library 2005 and HMDB as well as published references, the typical urine spectra were dominated by amines, various organic acids including lactate, citrate and succinate and a range of lipids (in **Table S1)**.

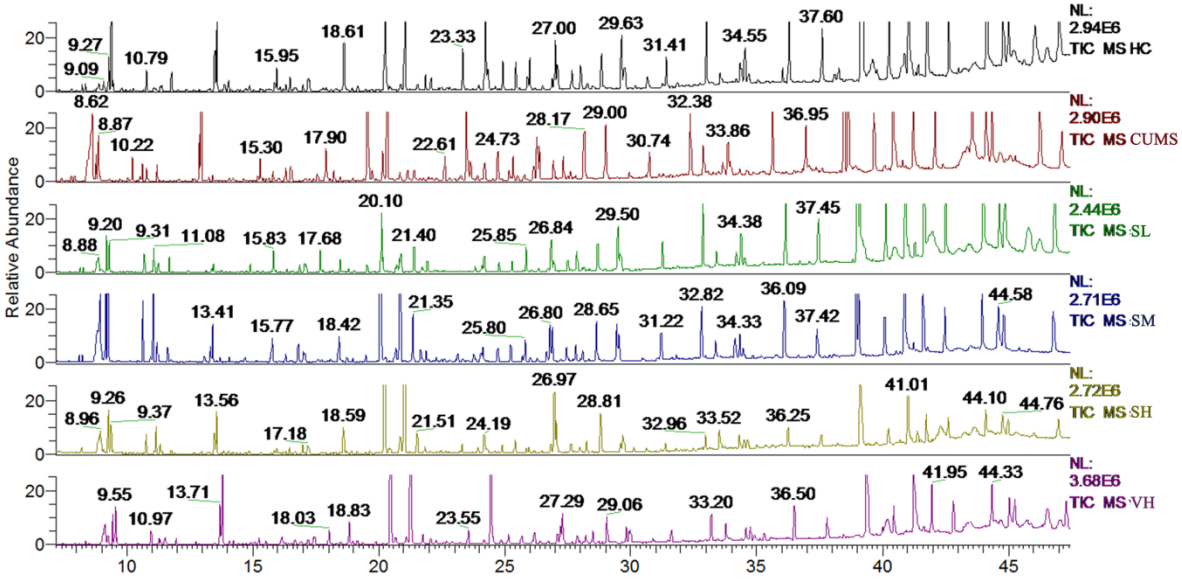

Fig.1 The GC–MS total ion chromatogram of NS group, CUMS group,SL group, SM group, SH group, and VH group.Keys:1,Linolenic acid; 2.Formic acid;3,Malonic acid; 4,L-arginine; 5,Linoleic acid; 6,Oxalic acid (allyl octyester); 7, Orthophosphoric acid; 8,Succinic acid; 9,Benzoic acid; 10,Maleic acid; 11,Glycine; 12,Glutaric acid; 13,Alphatoluic acid; 14,Malic acid; 15,Alanine; 16,Hexanedioic acid; 17,L-valine; 18,,L-leucine; 19,L-isoleucine; 20,Pimelic acid; 21,L-proline;

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22,Cl-Phenylalanine; 23, 4-Methoxyphenylacetic acid; 24,Suberic acid; 25,sumiki's acid;26,Citrate; 27,Cinnamic acid; 28, Isonipecotic acid; 29,Benzoyl-glycine; 30,Phenylalanine; 31,Phthalic; 32, Aminohippuric acid; 33,Indolepyruavte; 34,Acetyl citrate; 35,Stearic acid; 36,Palmitic acid; 37,Glycerol 1-hexadecanoate; 38,Glycerol 1-octadecanoate. HC, healthy control group; CUMS,CUMS-models group; SL, low dose group of sea buckthorn seed oil; SM, middle dose group of sea buckthorn seed oil; SH, high dose group of sea buckthorn seed oil; VH, venlafaxine hydrochloride group.

Multivariate statistics

Visual inspection of these spectra presented obvious difference among these groups but the complexity of GC-MS spectra impeded further comparison the six groups. So, a 3D matrix encompassing arbitrarily was formed by the output data after a pre-treatment via XCMS and Microsoft Excel software. All of resulting data sets were subsequently analyzed to examine the clustering of each group by multivariate statistics OPLS-DA, which can reveal any possible variables contributing to CUMS procedure or sea buckthorn seed oil treatment. A clear separation between HC group and CUMS group was initially observed in Fig.2a, indicating a biochemical perturbation occurred in model group. Using the strategy mentioned before, all of groups with drugs treated exhibited a good separation from CUMS group, suggesting that the sea buckthorn seed oil had an good antidepression effect (in **Fig. 2**). Both the corresponding S-plot and *t*-test suggested that the rats in groups with drugs administrated had lower urinary levels of suberic acid, sumiki's acid, phthalic acid, cinnamic acid as well as citrate and urinary higher levels of pimelic acid and palmitic acid compared CUMS group, which was shown in **Table 2**.

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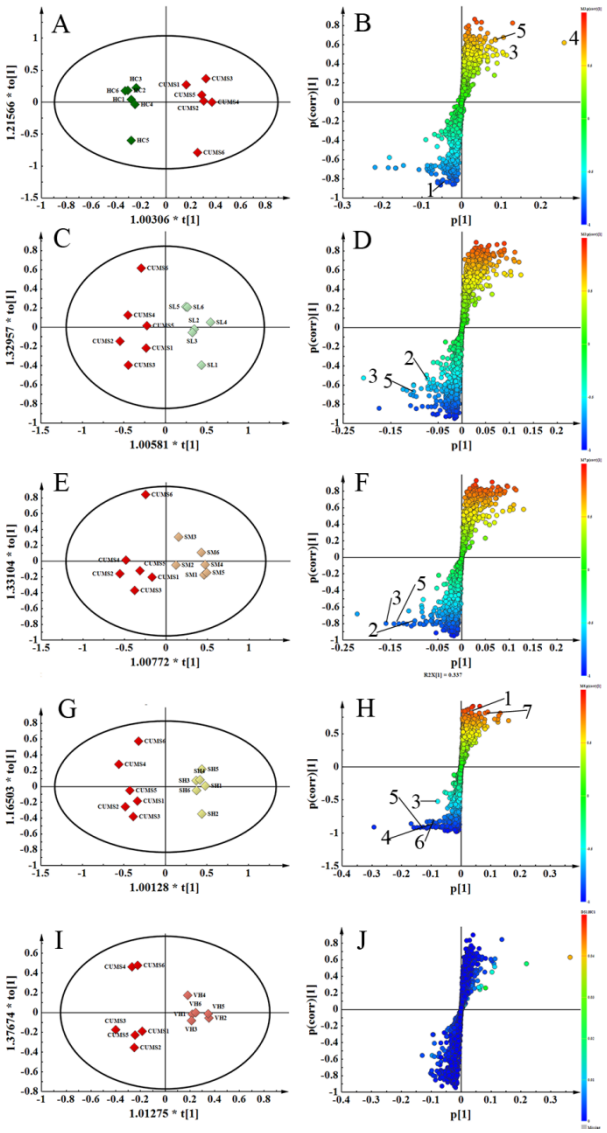

Fig.2 OPLS score plots and S-plots of the HC and CUMS group in (A and B, R2X = 0.59, R2Y = 0.969, Q2 (cum) = 0.753); OPLS score plots and S-plots of the CUMS and SL group in (C and D , R2X = 0.543, R2Y = 0.969, Q2 (cum) = 0.901);OPLS score plots and S-plots of the CUMS and SM group in (E and F , R2X = 0.546, R2Y = 0.858, Q2 (cum) = 0.701); OPLS score plots and S-plots of the CUMS and SH group in (G and H , R2X = 0.657, R2Y = 0.988, Q2 (cum) = 0.901);OPLS score plots and S-plots of the CUMS and VH group in (I and J, R2X = 0.501, R2Y = 0.996, Q2 (cum) = 0.514). HC, healthy control group; CUMS, CUMS-models group; SL, low dose group of sea buckthorn seed oil; SM, middle dose group of sea buckthorn seed oil; SH, high dose group of sea buckthorn seed oil; VH, venlafaxine hydrochloride group.

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Table 2 The relative peaks signal intensity of potential biomarkers for the anti-depression effect of sea buckthorn seed oil in the urine of CUMS rats (Data* 10^{-3})

NO.	Metabolites	HC group	CUMS group	SL group	SM group	SH group	VH group
1	Pimelic acid	0.9 ±0.2 [a]	0.4 ±0.1	0.8 ±0.3 [b]	0.8 ±0.3 [b]	0.7 ±0.2 [a]	0.8 ±0.4 [b]
2	Suberic acid	4.9 ±0. 9 [b]	7.4 ±1.0	3.4 ±2.8 [b]	4.4 ±2.5	4.2 ±1.9 [b]	7.5 ±4.2
3	Sumiki's acid	8.7 ±1.6 [b]	11.3 ±2.2	6.4 ±3.1 [b]	7.6 ±2.3 [b]	6.3 ±3.1 [b]	10.0 ±2.8
4	Citrate	39.5 ±8.9 [b]	63.0 ±18.0	28.9 ±23.0 [b]	23.0 ±7.0 [a]	37.9 ±14.8 [b]	63.8 ±38.6
5	Cinnamic acid	7.0 ±1.3 [b]	9.2 ±1.6	5.5 ±2.6 [b]	5.9 ±2.7 [b]	6.6 ±2.7 [b]	7.8 ±1.8
6	Phthalic acid	1.6 ±0.9 [b]	3.6 ±1.5	6.9 ±2.3	2.0 ±1.4 [b]	1.1 ±0. 3 [a]	2.0 ±0. 4 [b]
7	Palmitic acid	1.1 ±0.3 [a]	0.5 ±1.4	1.5 ±0.8 [b]	0.8 ±2.4 [b]	0.6 ±0.4	1.0 ±0.5 [b]

Data are expressed as mean ± SD. HC group , healthy control group; CUMS group, CUMS-models group; SL group, low dose group of sea buckthorn seed oil group; SM group ,middle dose group of sea buckthorn seed oil; SH group, high dose group of sea buckthorn seed oil; VH group ,venlafaxine hydrochloride group.

[a] Means a statistically significant difference at $p < 0.01$ when compared with CUMS-model group.

[b] Means a statistically significant difference at $p < 0.05$ when compared with CUMS-model group.

The correlations among these potential biomarkers were shown in **Fig. 3**, where the color in each cell indicated the strength of the relationship, from green (negative relationship) to red (positive relationship), and the Spearman's correlation coefficient between each pair of metabolites was also presented. There were changes in levels of these metabolites significantly correlated with each other in samples of rats with sea buckthorn seed oil treatment. Change of citrate level was positively correlated with changes in levels of suberic acid, sumiki's acid and cinnamic acid while negatively correlated with changes in levels of pimelic acid, palmitic and phthalic acid. And these increases in fatty acids including pimelic acid and palmitic acid were correlated with reduction in suberic acid, sumiki's acid,cinnamic acid, and were highly correlated with each other.

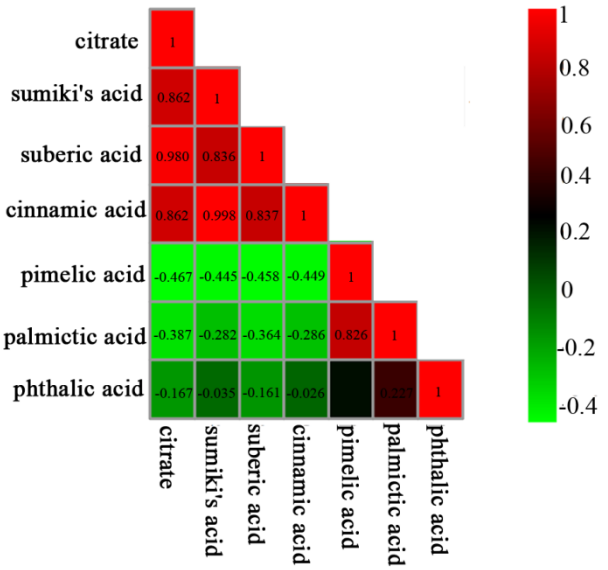

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Fig.3 the correlation heat map displaying correlation coefficients (Spearman) between biomarkers for treatment response of sea buckthorn seed oil. The color-coded scale of correlation is at right, where a red colour indicates a positive correlation, while a green color indicates a negative correlation.

Discussion

Chronic unpredictable mild stress (CUMS) is considered to be the most promising and valuable animal model of depression and has been widely used for investigating the pathophysiology of depression as well as the efficacy of associated therapeutic interventions. Recently, metabolomics combined with high throughput techniques has successfully applied to analyze various biological samples and extract meaningful biological information from the resultant complex and huge data sets via multivariate statistics[28, 29]. Urine has been extensively used in metabolomics studies because it is readily available and minimally invasive[30]. GC-MS is a powerful approach to detect metabolic signatures from complex biofluid samples. In this study, a GC-MS metabolomics approach was applied to analyze metabolites in the urine of rats exposed to CUMS procedure as well as the treatment of sea buckthorn seed oil. We discovered that a set of metabolites including phenylalanine, citrate, lipids (palmitic acid, suberic acid, and pimelic acid) other metabolism molecules (cinnamic acid and phthalic acid). These metabolites were related to energy metabolism pathways, fatty acid metabolism and synthesis of neurotransmitter as well as a disturbance gut microbiota. And these perturbations could be reversed by sea buckthorn seed oil intervention. This gives rise to the characteristic metabolomic profiles of depression and the intervention of sea buckthorn seed oil (**Fig.4**), which will be discussed in further detail below.

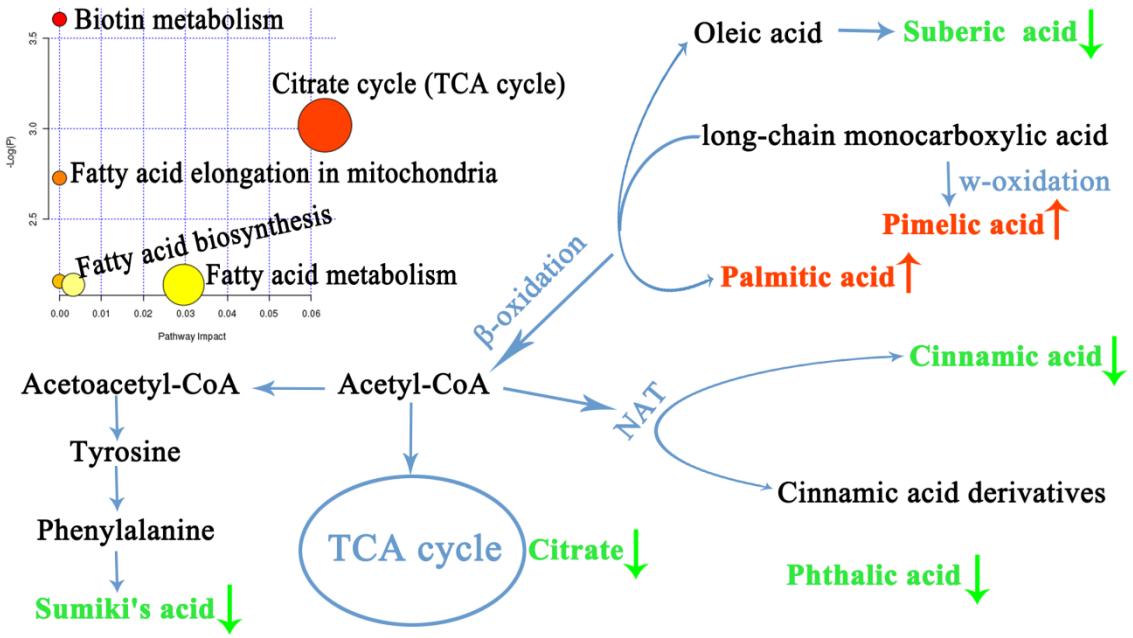

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Fig.4 the overview of metabolic pathways related to sea buckthorn seed oil treatment. The notations are as follows: ↓, higher than CUMS group and ↓, lower than CUMS group. Pimelic acid , palmictic acid ,suberic acid, citrate, phthalic acid, cinnamic acid and sumiki's acid were considered as biomarkers for antidepressive response of sea buckthorn seed oil. It indicates sea buckthorn seed oil may play an important role in antidepression by regulating energy metabolism (citrate), fatty acid metabolism (pimelic acid, palmictic acid and suberic acid), Synthesis of neurotransmitter (sumiki's acid) and other metabolism (phthalic acid, cinnamic acid).

Energy metabolism

Citrate, which is related to energy metabolism, is a dominant intermediate of the TCA cycle. The entry of new carbon units either from pyruvate (from glycolysis) or from oxidation of fatty acids into this cycle is through acetyl-CoA. The two-carbon acetyl group from acetyl-CoA is transferred to the four-carbon oxaloacetate and yield six-carbon citrate catalyzed by citrate synthase. A dehydration-rehydration rearrangement of citrate yields isocitrate. Two successive decarboxylations produce α-ketoglutarate and then succinyl-CoA, a CoA conjugate of a four-carbon unit. Several steps later, oxaloacetate is regenerated and can combine with another two-carbon unit of acetyl-CoA. Thus, carbon enters the cycle as acetyl-CoA and exits as CO_2. In the process, metabolic energy is captured in the form of ATP, NADH, and enzyme-bound $FADH_2$. The decreasing urine citrate level in depressed patients suggests that depression could bring a suppressed rate of the TCA cycle. This variation was a good agreement with previous studies on depressed patients or animal models[25]. The energy deficiency lead to reduced activity, which is one of the most represented depressive symptoms[31]. And the urinary level of citrate reversed to normal after the intervention of sea buckthorn seed oil suggested that sea buckthorn seed oil may improve depressive symptoms such as reduced activity by regulating the energy metabolism.

Fatty acid metabolism

The change of fatty acid is another significant feature of the metabolites in urine obtained in this study. The levels of palmitic acid were found to be dramatically decreased. Fatty acids are known to represent the major source of energy production (by means of β-oxidation) and energy storage in humans. As mentioned above, the β-oxidation of fatty acids can yield acetylcoenzyme A (CoA) units, which may produce ATP in TCA cycle or be converted into ketone bodies stored in the kidney and liver[32]. Such evident indicated the existence of energy metabolism disturbance in depression and this is in accordance of our earlier findings that CUMS procedure could bring dysfunction of fatty acid metabolism[20]. As discussed above, these findings emphasize depression may block fatty acid transportation and inhibit the TCA cycle, ultimately affect the entire energy metabolism processing. In addition, we found a higher level of suberic acid and a lower level of pimelic acid in urine of rats in CUMS group compared with those in HC group. Suberic acid is metabolic breakdown product derived from oleic acid. Elevated levels of this unstaruated dicarboxylic acid are found in individuals with dicarboxylic acid and

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medium-chain acyl-CoA dehydrogenase deficiency. Pimelic acid, produced from *w*-oxidation of long-chain monocarboxylic acids to long-chain dicarboxylic acids, followed by β-oxidation. Derivatives of pimelic acid are involved in the biosynthesis of lysine, whose deficiency also may result in immunodeficiency. Existing clinical trials has demonstrated that depression increases our vulnerability to other common complex diseases[33]. So, the urinary level of palmitic acid, pimelic acid and suberic acid reversed by sea buckthorn seed oil indicated that the antidepression effects of sea buckthorn seed oil may be based on regulating a dysfunction of fatty acid metabolism.

Synthesis of neurotransmitter

Phenylalanine, an electrically neutral amino acid, is a precursor of the neurotransmitters called catecholamines. It is also a precursor for tyrosine, which can synthesize a monoamine neurotransmitters dopamine. And previous research suggest that phenylalanine was a large neutral amino acid and could affect the 5-HT synthesis[34, 35]. Additionally, it was reported that the excretion of Sumiki's acid was connected with phenylalanine[36]. In this study, the higher urinary level of Sumiki's acid of rats in CUMS group indicated a dysfunction in neuro-transmission as the pathophysiology of depression. And this was reversed after sea buckthorn seed oil treated, suggesting that the antidepressive effect of sea buckthorn seed oil in rats may be associated with changes in neurotransmitter synthesis pathways.

Other metabolism

Phthalic acid, found in tissues or biofluids, is heightened public concern because reports of its potential risk to male reproductive health, being significantly associated with reduced sperm concentration to pesticide concentration in men's urine[37]. Within the reproductive tract, the male is exquisitely vulnerable to the effects of anti-androgens during development due the reliance on the synthesis and action of androgens for the masculinization of the male reproductive tract. The phthalates can suppress androgen synthesis during development and induce testicular dysgenesis together with cryptorchidism. And sexual function decline is always represented in depressed patients. In this study, the increased urinary level of phthalic acid in rats of CUMS group underlined this hypothesis and its decreasing after sea buckthorn seed oil treatment indicating that sea buckthorn seed oil may improve depressive symptoms such as reduced activity sexual function decline by regulating the sexual hormones metabolism. The concentration of cinnamic acid is related to an important enzyme called arylamine N-acetyltransferases (NAT), indicating antidepressant effect of sea buckthorn seed oil in rats may be related to arylamineN-acetyltransferases (NAT).

In summary, these biomarkers were involved in energy metabolism, synthesis of neurotransmitter as well as fatty acid metabolism and other metabolism. The reversed urinary levels of these biomarkers in rats with CUMS procedures after treatment of sea buckthorn seed oil indicate antidepressant effect of sea buckthorn seed oil in rats may be related to regulating their relative metabolism pathways.

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Conclusions

In this study, the biomarkers and biochemical pathways of treatment response of sea buckthorn seed oil were discovered using a GC-MS urine metabolomic profiling technique coupled with multivariate statistical analysis in this study. Application of these biomarkers may provide positive support on optimizing efficacy evaluation as well as illuminating mechanism of sea buckthorn seed oil.

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Figures and tables legend:

Fig.1. The GC–MS total ion chromatogram of NS group, CUMS group, SL group, SM group, SH group, and VH group.Keys:1,Linolenic acid; 2.Formic acid;3,Malonic acid; 4,L-arginine; 5,Linoleic acid; 6,Oxalic acid (allyl octyester); 7, Orthophosphoric acid; 8,Succinic acid; 9,Benzoic acid; 10,Maleic acid; 11,Glycine; 12,Glutaric acid; 13,Alphatoluic acid; 14,Malic acid; 15,Alanine; 16,Hexanedioic acid; 17,L-valine; 18,,L-leucine; 19,L-isoleucine; 20,Pimelic acid; 21,L-proline; 22,Cl-Phenylalanine; 23, 4-Methoxyphenylacetic acid; 24,Suberic acid; 25,sumiki's acid;26,Citrate; 27,Cinnamic acid; 28, Isonipecotic acid; 29,Benzoyl-glycine; 30,Phenylalanine; 31,Phthalic; 32, Aminohippuric acid; 33,Indolepyruavte; 34,Acetyl citrate; 35,Stearic acid; 36,Palmitic acid; 37,Glycerol 1-hexadecanoate; 38,Glycerol 1-octadecanoate.HC, healthy control group; CUMS,CUMS-models group; SL, low dose group of sea buckthorn seed oil.SM, middle dose group of sea buckthorn seed oil; SH, high dose group of sea buckthorn seed oil; VH, venlafaxine hydrochloride group.

Fig.2 OPLS score plots and S-plots of the HC and CUMS group in (A and B, R2X = 0.59, R2Y = 0.969, Q2 (cum) = 0.753); OPLS score plots and S-plots of the CUMS and SL group in (C and D , R2X = 0.543, R2Y = 0.969, Q2 (cum) = 0.901);OPLS score plots and S-plots of the CUMS and SM group in (E and F , R2X = 0.546, R2Y = 0.858, Q2 (cum) = 0.701); OPLS score plots and S-plots of the CUMS and SH group in (G and H , R2X = 0.657, R2Y = 0.988, Q2 (cum) = 0.901);OPLS score plots and S-plots of the CUMS and VH group in (I and J, R2X = 0.501, R2Y = 0.996, Q2 (cum) = 0.514). HC, healthy control group; CUMS, CUMS-models group; SL, low dose group of sea buckthorn seed oil; SM, middle dose group of sea buckthorn seed oil; SH, high dose group of sea buckthorn seed oil; VH, venlafaxine hydrochloride group.

Fig.3 the correlation heat map displaying correlation coefficients (Spearman) between biomarkers for treatment response of sea buckthorn seed oil. The color-coded scale of correlation is at right, where a red colour indicates a positive correlation, while a green color indicates a negative correlation.

Fig.4 the overview of metabolic pathways related to sea buckthorn seed oil treatment. The notations are as follows: ↓, higher than CUMS group and ↓, lower than CUMS group. Pimelic acid , palmictic acid ,suberic acid, citrate, phthalic acid, cinnamic acid and sumiki's acid were considered as biomarkers for antidepressive response of sea buckthorn seed oil. It indicates sea buckthorn seed oil may play an important role in antidepression by regulating energy metabolism (citrate), fatty acid metabolism (pimelic acid, palmictic acid and suberic acid), Synthesis of neurotransmitter (sumiki's acid) and other metabolism (phthalic acid, cinnamic acid).

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Table 1 Effect of sea buckthorn seed oil and venlafaxine hydrochloride on changes of behavior in rats exposed to CUMS on the 28th day.

Data are expressed as mean ± SD. n=10. HC, control group; CUMS, CUMS-model group, YH, venlafaxine hydrochloride group, SH, high dose group of sea buckthorn seed oil; SM, middle dose group of sea buckthorn seed oil; SL, low dose group of sea buckthorn seed oil.

[a] Means a statistically significant difference at $p < 0.01$ when compared with CUMS-model group.

Table 2 The relative peaks signal intensity of potential biomarkers for the anti-depression effect of sea buckthorn seed oil in the urine of CUMS rats (Data* 10^{-3})

Data are expressed as mean ± SD. HC group , healthy control group; CUMS group, CUMS-models group; SL group, low dose group of sea buckthorn seed oil group; SM group ,middle dose group of sea buckthorn seed oil; SH group, high dose group of sea buckthorn seed oil; VH group ,venlafaxine hydrochloride group.

[a] Means a statistically significant difference at $p < 0.01$ when compared with CUMS-model group.

[b] Means a statistically significant difference at $p < 0.05$ when compared with CUMS-model group.

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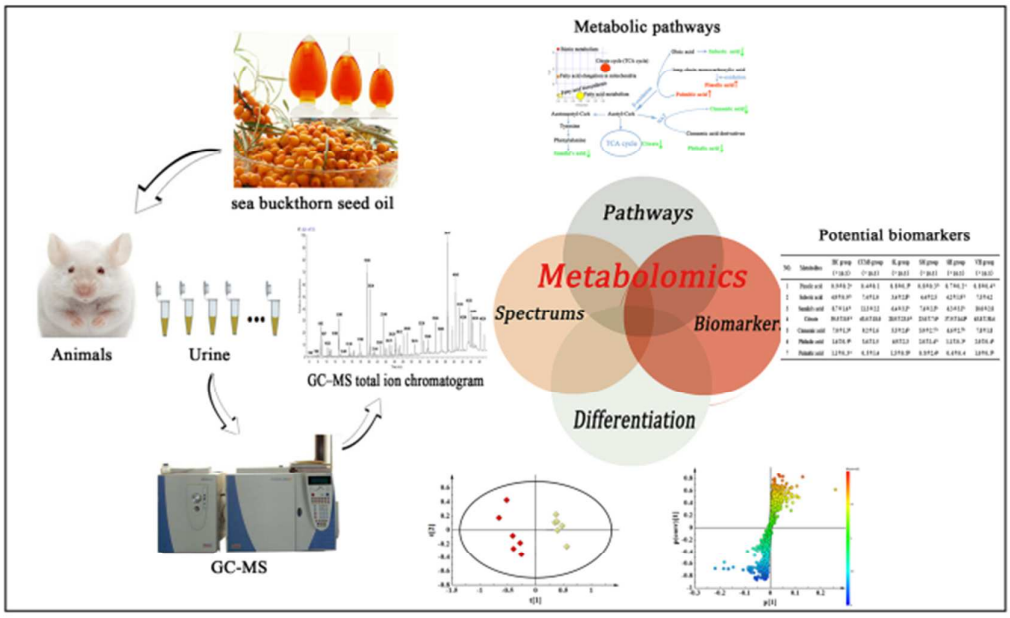
The antidepressant effect of sea buckthorn seed oil was investigated by the GC-MS-based metabolomics approach coupled with multivariate analysis.



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