Microchemical Journal 142 (2018) 394–402

Contents lists available at ScienceDirect

Microchemical Journal

journal homepage: www.elsevier.com/locate/microc

# Bioactive assay and hyphenated chromatography detection for complex supercritical $CO_2$ extract from *Chaihu Shugan San* using an experimental design approach

Min He[a,*], Liang Hong[a], Zhi-Yu Yang[a,b], Tian-Biao Yang[a], Jun Zeng[a]

[a] Department of Pharmaceutical Engineering, School of Chemical Engineering, Xiangtan University, Xiangtan 411105, People's Republic of China
[b] Medical Metabolic International Collaborative Center, Xiangya Hospital, Central South University, Changsha 410008, People's Republic of China

ARTICLE INFO

*Keywords:*
*Chaihu Shugan san*
Supercritical carbon dioxide extraction
ACGWO
LC-MS-IT-ToF
Anti-inflammatory
Antioxidant

ABSTRACT

In this study, Super Critical $CO_2$ (SCC) was applied to extract the active ingredients from Multi-herbal formula *Chaihu Shugan San* (CSS). Response Surface Methodology (RSM) coupled with an improved chaotic Gray Wolf Optimization (ACGWO) was employed to the experimental design. After gas chromatography–mass spectrometer (GC–CI/EI-MS) detection, liquid chromatography–mass spectrometry- ion trap-time of flight (LC–MS-IT-ToF) further indicated that several phthalides are mainly responsible for the SCC extract; the next is α-Cyperone in *Cyperus rotundus*; and Meranzin hydrate, Isomeranzin in *Citrus reticulata Blanco* again. Further pharmacological tests were also performed for the SCC extracts. THP-1 macrophages were used to observe the anti-inflammatory effects. The expression of IL-6 was obviously suppressed by the SCC extract from CSS, through a positive correlation with the dose. Then, ROS content in a cell model was used to detect the antioxidant capacities. The results showed that the SCC extract from CSS, exert dose-dependent inhibitory effects on the oxidative stress of the cells.

## 1. Introduction

The different combination of herbs can be found in traditional Chinese medicine (TCM) or functional food. *Chaihu Shugan San* (CSS), one of the typical representatives, is mainly used for the diseases like mental disorders, liver diseases [1, 2]. CSS formula is consisting of *Pericarpium citri reticulatae*, *Radix bupleuri*, *Ligusticum chuanxiong hort*, *Rhizoma cyperi*, *Fructus aurantii*, *Paeonia lactiflora* and *Glycyrrhiza uralensis fisch*, in which the semi-volatile Phthalides and Terpenoids are two of the most important ingredients [3]. Nevertheless, the traditional decocting method was mainly accounts for the extracts of seven herbs, which easily cause the degradation and isomerization of those compounds.

At present, many methods are applied to separate the bio-active constituents from herbs, including hydro-distillation [4], microwave-assisted extraction [5], continuous subcritical water extraction [6], ultrasonic extraction [7], etc. These methods are time-consuming and environmental unfriendly. In contrast, supercritical $CO_2$ (SCC) extraction has been widely used in the separation of effective substances in natural products [8], due to its environment friendly, low temperature operation, and so on. The Extraction Yield (EY) is usually influenced by multiple factors in SCC extraction. The experimental design [9] is aims to explain the factors under conditions that are hypothesized to reflect the variation. There are many chemometric methods utilized to analyze the interaction between these factors and results, including Response Surface Methodology (RSM) [10], Excel [11], Taguchi Design Method [12]. Among them, RSM based on Small Face Central Composite Design (SFCCD) has been widely used to optimize various operating parameters of the extraction process, as well as the EY modeling. The ideal RSM results indicated that the experimental data can be applied to a mathematical equation, which is an effective statistical model [13]. In the past years, some good statistical approaches were used, such as Gray Wolf Optimization (GWO) [14], Particle Swarm Optimization (PSO) [15], genetic algorithm [16], and hybrid particle swarm-ant colony algorithm [17], etc. Here, GWO algorithm enjoys simple principle, few adaptable parameters, fast convergence speed and good global search capability, which can be applied in the supercritical process [18]. However, GWO also has many disadvantages, such as, an over-reliance on the initial population, premature convergence, prone to local optimum and the unstable convergence process.

Since CSS extract is multi-components mixtures, the chemical identification is thus of great importance, not only for the clarification

* Corresponding author.
*E-mail addresses:* hemin1976@xtu.edu.cn, dahai8214813@gmail.com (M. He).

https://doi.org/10.1016/j.microc.2018.07.016
Received 7 June 2018; Received in revised form 14 July 2018; Accepted 15 July 2018
Available online 18 July 2018

of pharmacodynamic basis, but also for providing scientific evidence for the quality control. Because of efficient separation and abundant information, Mass Spectrometry (MS) coupled with Liquid Chromatography (LC) or Gas Chromatography (GC) has been used to identify the composition in herb or food [19]. Due to the herbal complexity, the chemometric resolution and some *in-silico* approaches have been introduced in the data analysis of GC–MS and LC–MS [20]. These methods provide an accurate, fast, cheap quantitative analysis in the presence of heavy interference by other analytes.

Further pharmacological tests should also be performed for the TCM formula or their extracts. Kuai et al. found that Sheng-ji Hua-yu formula can promote the diabetic wound healing of re-epithelization [21]. Sui et al provided the first direct evidence demonstrating Zhi-Zhen-Fang can attenuate multidrug resistance by repressing Hedgehog signaling [22]. The study by Zhou et al. indicated that Huolingshengji Formula possess neuroprotective effects in SOD1G93A mouse model [23]. Many herbs have been proved to possess anti-inflammatory [24, 25] and antioxidant activity [26, 27]. However, the TCM Formula is too complex, their pharmacological activities can't be explained clearly. Therefore, the multi-angle, multi-level study of different extracts should be adopted in the bioactivity researches of TCM Formula.

The paper discussed the experimental procedure of the SCC extraction from the formula CSS. In this procedure, the EY model was optimized by an improved chaotic Gray Wolf Optimization (ACGWO) algorithm. After the morphological examination of herbal particles, the chemical composition was analyzed by GC–MS and LC–MS apparatus. Finally, the anti-inflammatory and antioxidant researches were performed in the SCC extracts from CSS formula.

## 2. Material and methods

### 2.1. Materials

The herbs, such as *Pericarpium citri reticulatae*, *Radix bupleuri*, *Ligusticum chuanxiong hort*, *Rhizoma cyperi*, *Fructus aurantii* and *Paeonia lactiflora*, were purchased from Hunan Academy of Chinese Medicine. The crude herbs were air-dried indoors before smashing into several grades. $CO_2$ (99.9%) was provided by Chang Gang gas Co. Ltd. (Changsha, China). Alkane standard solutions of $C_8$-$C_{20}$ (no. 04070) were purchased from Fluka Chemika (Buchs, Switzerland). Ligustilide and α-Cyperone were purchased from the Chinese National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). A human monocytic leukemia cell line (THP-1) and HepG2 cells were purchased from American Type Culture Collection (ATCC, USA). Lipopolysaccharide (LPS), RPMI 1640 medium were purchased from Sigma-Aldrich and Thermo Fisher companies, respectively. Human IL-6 ELISA kit and human TNF- α kit were purchased from Enzo life sciences, Inc. $H_2O_2$ was purchased from Kun Feng Chemical Co., Ltd. (Jinan, China). DCFH-DA fluorescent probe (Invitrogen A/S Taastrup, Denmark). DMSO and Celecoxib was purchased from Macklin Biochemical company (Shanghai, China) and Pfizer pharmaceuticals Ltd. (New York, America). Penicillin and streptomycin were purchased in XiangTan central hospital. The solvents, such as methanol and n-Hexane, were of HPLC grade.

### 2.2. Supercritical $CO_2$ extraction

A supercritical $CO_2$ extraction device (Fig. S I1, Supplemental information I), HA121-50-01, was supplied by HuaAn Supercritical fluid extraction Co. Ltd. (Nantong, China). The extraction system is consisted of one 1 L extraction vessel and two separators. The gas tank with a condenser can offer $CO_2$ fluid, and the output can be increased to the desired pressure by a pump. In this study, 150 g of dried herbs was loaded into a 1 L high pressure vessel, and the liquid $CO_2$ was used to extract the essential oil. Later, the extracts were fed into the separator I and separator II, in which the density of $CO_2$ and the solubility of essential oil could be adjusted by various pressure. Every 60 min in separator II, the liquid oil was released completely from the gaseous $CO_2$, and the latter was flowed into a purifier for recycled use. Finally, the weighed essential oil was stored in −20 °C for further analysis. The extraction yield (EY) can be calculated in accordance with Eq. (1).

$$EY(\%) = \frac{W_{oil}}{W_{powder\ of\ CSS}} \times 100\% \tag{1}$$

$W_{oil}$ refers to the weight of oil, $W_{powder\ of\ CSS}$ refers to the weight of herbs.

### 2.3. Experimental design, RSM prediction and ACGWO optimization

Many parameters may influence the EY of CSS either directly or indirectly. However, traditional single factor design requires many experiments, and often ignore the interaction between the various factors. In our study, three factors (extraction temperature, extraction pressure and particle size) are utilized to investigate their influences on EY. The remaining parameters are specified at the fixed values, such as extraction time at 120 min, $CO_2$ flow rate at 43.67 L/h, and weight of CSS powder was specified as 150 g.

In our study, RSM was applied to analyze the relationships between three input independent variables and the output parameter. After three-factor three-level values in this experiment are set, the experiment is designed by the Small Face Central Composite Design (SFCCD) based on the 15 sets of operating parameters, including four factorial points, six axial points and five center points. When it comes to the experimental results, analysis of variance (AOV) was used to calculate the statistical significance of each coefficient on EY. A nonlinear fitting method was then used to establish the quadratic polynomial regression equation, which was introduced to predict the highest yield.

GWO algorithm is applied to achieve the simulation by building a 4-layer pyramid hierarchical management system, which performs the social hunting behavior. The management system is divided into α (the first layer), β (the second layer), δ (the third layer), ω (the fourth layer). The GWO principle (Supplemental information II) is a novel meta-heuristic algorithm for global optimization, and the position of the prey is the optimal solution after multiple iterations. To maintain the diversity of the population and make the initial population uniformly distributed, the chaotic algorithm was introduced in GWO algorithm (Fig. S II1, Supplemental information II) to accelerate the convergence speed of the algorithm. Chaos randomness and ergodicity can avoid the tendencies of falling into local minimal values during the search process, thus overcoming the shortcomings of GWO algorithm. Furthermore, the coordinated problem between global search and local search were common in the group intelligence algorithms. The strong global search ability can ensure the diversity of the population, and the strong local search capability can guarantee the precision of results. Therefore, it is important to deal with the balance between the global search capability and the local search capability in the GWO algorithm. The linear convergence of the convergence factor *a* can't fully reflect the actual optimization process. To maintain the balance between global search and local search, a nonlinear convergence factor was introduced in the algorithm (Fig. S II2, Supplemental information II).

As observed in Fig. 1, the optimization steps of ACGWO algorithm are explained. Firstly, the algorithm parameters were set. Secondly, the fitness values of individuals in the population are calculated, then the first three values with better fitness are assigned to α, β and δ. Thirdly, the position of ω and the chaotic sequence are updated. Fourthly, the number of iterations and convergence factor *a* were updated, to calculate the value of the improved $\vec{a}$ and update the parameters ($\vec{r_1}$, $\vec{r_2}$, $\vec{a}$, $\overrightarrow{C_{id}}$, $\overrightarrow{A_{id}}$ and $X_n$) etc. Finally, the optimal solution (α) could be obtained.