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Assessment of various pretreatment and extraction methods for the extraction of bioactive compounds from *Orthosiphon stamineus* leaf via microstructures analysis

by

Chung-Hung Chan

Please email to rykenz87@yahoo.com for any inquiries

ABSTRACT

The impacts of various methods such as mechanical grinding, ultrasonic-assisted extraction (UAE), microwave-assisted extraction (MAE), and also sample pretreatments using acid and alkali on the microstructure of plant sample were studied for the extraction of bioactive compounds from *Orthosiphon stamineus* leaf. From scanning electron microscopy (SEM) analysis of the extracted sample, UAE and MAE induced significant disruption on glandular trichomes structure, which is the main site for biosynthesis of plant secondary metabolites. This improves the diffusion of bioactive compound and resulted in approximately 86-95% of the total extraction yield quantified by conventional Soxhlet extraction. Chemical pretreatments generally imparted weaker microstructures disruption thus slight improvement on the extraction yields was observed. In this case, acid reagent is more suitable for the pretreatment as the presence of alkali decomposes the bioactive compounds. In a nutshell, the performance of an extraction strongly depends on its degree of disruption on the plant sample microstructure.

Keywords: bioactive compounds, microstructure, extraction systems, ultrasonic-assisted extraction, microwave-assisted extraction

INTRODUCTION

Orthosiphon stamineus or Java tea is a traditional herbal plant that can be found in Asia Pacific mainly Malaysia and Indonesia [1]. It contains bioactive compounds such as sinensetin, 3'-hydroxy-5,6,7,4'-tetramethoxyflavone, rosmarinic acid and eupatorin and has been traditionally used to treat kidney problems, gout, and diabetes [2]. Natural products of this plant are available in the form of herbal tea product and concentrated extract in the market [2, 3]. They are conventionally manufactured through series of processes involving drying, grinding and extraction. The fresh leaves from plantation are usually sun-dried to remove the moisture, followed by grinding to break the plant cells for the ease of elution of bioactive compounds in herbal tea processing. Other method such as extraction is relevant in the production of concentrated extract as it extracts the bioactive compounds from the intact cells after grinding process. The conventional extraction process often subjected to a long heating process which causes the degradation of bio-active compounds and energy wastage. Thus, it is important to look into other methods to enhance the extraction yield of natural product effectively.

The methods to be considered are those can impart structural disruption to biomass sample in order to facilitate efficient mass transfer of bioactive anti-oxidant compounds during extraction process. For instance, grinding of plant sample creates more broken cells, which facilitates the elution of bioactive compounds to extraction solvent. The limitation is that breaking the plant particle to very fine sizes requires huge energy consumption and complicated machinery. Other advanced methods such as ultrasonic-assisted extraction (UAE) and microwave-assisted extraction (MAE) can also improve the extraction efficiency. Ultrasonic treatment creates micro-pores on the structure of plant sample through cavitation [4, 5]. It has been applied in the extraction of bioactive compounds from tobacco seeds [6], pomegranate peel [7] and tomatoes [8]. On the other hand, microwave treatment provides localised heating which increases the internal pressure of the cells and subsequently ruptures them [9]. Its employment can be seen in the extraction of bioactive compounds from cocoa leave [10], green tea [11], and *Morinda citrifolia* [12]. Sample pretreatments with acid and alkali are another method of improving the extraction yield of biomass. This method is able to break down crystalline cellulose of biomass as observed in the cases of rice husk [13], sugar cane [14], and switch grasses [15], which may improve solvent penetration into plant sample during the extraction process. Despite the methods above demonstrated promising

extraction performance, their performances relative to one another and their mechanisms are not well understood. Therefore, this work assessed the impacts of the pretreatment and the extraction methods on plant microstructure and their resulted effects on the extraction yields. The extraction and pretreatment methods in this work can be incorporated into the existing herbal tea processing to enhance the release of therapeutic bioactive compounds in the product.

In this study, the methods, i.e. grinding for particle size reduction, UAE, MAE and chemical pretreatments with acid and alkali were applied to extract bioactive compounds from *Orthosiphon stamineus* leaves. To assess the methods, the improvements on the extraction yield contributed by UAE, MAE and chemical pretreatments were determined and compared with conventional Soxhlet extraction, after excluding the contribution due to the particle size of the plant sample. The impacts on plant microstructure by these methods were observed and their acting mechanisms were discussed.

MATERIALS AND METHODS

Materials and reagents

The standards of the investigated bioactive compounds namely eupatorin (EUP), 3'-hydroxy-5,6,7,4'-tetramethoxyflavone (TMF) and sinensetin (SEN) were purchased from Sigma-Aldrich (USA). The standards were dissolved in chromatography grade absolute ethanol (Merck, Germany) and diluted to desired concentration prior to use in high performance liquid chromatography (HPLC) analysis. The pretreatment reagents, 0.1 M hydrochloric acid and sodium hydroxide solutions, were prepared from concentrated hydrochloric acid (Merck, Germany) and sodium hydroxide pellet (Merck, Germany), respectively. The denatured ethanol used in the extraction was obtained from R&M chemicals (Malaysia).

Pretreatment procedure

Fresh leaf of *Orthosiphon stamineus* was collected from local plantation in Negeri Sembilan, Malaysia. The leaves were washed and dried in a forced convection oven at 45 °C overnight until moisture content achieved below 15 %. After that, the dried sample was grinded and sieved to

various particle sizes: size A (>500 μm), size B (250-500 μm), size C (180-250 μm) and size D (<180 μm). The grounded sample was then stored in an air-tight container at 4°C until further use.

Extraction procedure

To determine the extraction yields due to grinding of plant sample, plant sample with particle size A was rinsed with ethanol at solvent-sample ratio of 100 ml/g. The ethanol extract was then filtered through 0.2 mm RC (Regenerated Cellulose) syringe filter before HPLC analysis. The experiment was repeated with plant sample with particle size B, C and D to investigate the effect of particle size reduction on the extraction yields.

The effects of various methods namely UAE, MAE, sample pretreatments with acid and alkali and Soxhlet extraction were investigated based on plant sample sized B. These methods are conducted at their typical and suboptimal operating conditions. Note that their treatment time and solvent-sample ratios have been optimized in the previous work to prevent mass transfer limitation. Their extraction procedures are elucidated as follows: UAE was performed using sonicator (Qsonica Q500, 20 kHz). The sample of 2 g was mixed with 70% (v/v) aqueous ethanol at solvent-sample ratio of 30 ml/g in a container. The mixture was sonicated at 300 W for 30 min under intermittency ratio, α of 4/5 in which α is defined as the fraction of a cycle when sonication was on, i.e. $\alpha = \tau_{on} / (\tau_{on} + \tau_{off})$ where τ_{on} and τ_{off} are the times in seconds when the sonication was turned on and off, respectively. This intermittency ratio is to prevent overheating of the sonotrode. Upon irradiation, the extract was filtered using fine cloth and RC syringe filter before HPLC analysis.

MAE was performed using domestic microwave oven (Samsung MW718). The sample at 2 g was mixed with 70% (v/v) aqueous ethanol at solvent-sample ratio of 50 ml/g in a closed Duran bottle. The mixture was irradiated at microwave power of 150 W for 20 min. The subsequent analysis procedure was similar to the UAE as previously described.

Sample pretreatments using acid and alkali were performed using reagents, i.e. 0.1M hydrochloric acid (HCl, pH 1) and 0.01M sodium hydroxide (NaOH, pH 12), respectively. The sample was soaked with the selected reagent in a closed Duran bottle for not more than 24 hours. After the pretreatment, the sample was mixed with pure ethanol at solvent-sample ratio of 100 ml/g and the

mixture was stirred for about a minute. The mixture was subjected to HPLC analysis using the similar procedure as in UAE.

Soxhlet extraction was conducted using 2 g of sample and 200 ml of ethanol for 6 hours. The subsequent procedure for HPLC analysis is similar as described in UAE.

HPLC-MS analysis

The bioactive compounds were quantified using Agilent 1200 Series HPLC system (USA) equipped with Agilent ZORBAX Eclipse Plus C18 column, 5 μ m (4.6 mm x 250 mm). There are several methods available for separating the bioactive compound from *Orthosiphon stamineus* [16-18]. However, the following general HPLC method [19] was employed as it does not involve the use of buffer solution and it can separate multi flavonoid compounds from a raw extract. The mobile phase was linear gradient of acetonitrile in water: 5-20% (0-15 min), 20-30% (15-20 min), 30-50% (20-30 min), 50-100% (30-35 min), 100% (35-40 min), and 100-5% (40-50 min) at flow rate of 1.0 ml/min. The injection volume of sample was 10 μ L and the separation is detected by UV-DD at wavelength 340 nm using Agilent 1260 Infinity Diode Array Detector (USA). The retention times of EUP, TMF and SEN were 29.28 and 32.28 and 33.30 minutes, respectively. The extraction yields of the compounds were reported as the mass of extracted bioactive compounds per unit mass of plant sample (mg/g).

Scanning electron microscopy (SEM) analysis

The microstructure changes of the plant samples before and after each extraction methods were examined with Quanta™ 200 FESEM scanning electron microscope (FEI, USA) operated at 10 kV accelerating voltage under low vacuum mode. The surface morphology of the extracted plant samples were compared to the raw dry plant sample.

RESULTS AND DISCUSSION

Enhancement of extraction yield by various methods

The effect of particle size reduction on the extraction yields were illustrated in Fig. 1. As expected, more bioactive compounds can be extracted from plant sample with smaller particle sizes due to broken cells. This effect is amplified when the particle size of the plant sample is reduced below the leaf thickness (average value of 240 μm) of the plant sample, as indicated by size D (<180 μm). At this condition, the extraction yield is comparable to exhaustive Soxhlet extraction yield. Nevertheless, excessive grinding of plant particles to achieve powder-like sizes is not feasible as only small portion of the plant samples can be converted into the powder form, not to mention the process requires high energy consumption and long duration.

Fig. 1 Enhancement of the total extraction yield of eupatorin, 3'-hydroxy-5,6,7,4'-tetramethoxyflavone and sinensetin from *Orthosiphon stamineus* plant by various methods. Unshaded region indicates the degree of enhancement. Particle size reduction: rinsing with ethanol, 100 ml/g, (A) >500 μm sample, (B) 250 - 500 μm sample, (C) 180 - 250 μm sample, (D) < 180 μm sample; Ultrasonic: 250 - 500 μm sample, 70% EtOH, 30 ml/g, 300 W; Microwave: 250 - 500 μm sample, 70% EtOH, 50 ml/g, 150 W, 20 min; Acid: soaking with 0.1M HCl, 250 - 500 μm sample, 24 h, 25 $^{\circ}\text{C}$; alkali: soaking with 0.01M NaOH, 250 - 500 μm sample, 1 h, 25 $^{\circ}\text{C}$; Soxhlet: 250 - 500 μm sample, EtOH, 100 ml/g, 6 h

The performances of other extraction methods in Fig. 1 were evaluated relative to the intrinsic yield contributed by the particle size (B). UAE and MAE drastically improve the total extraction yields using shorter treatment time. Their extraction results are about 95% and 86% of the total extraction yields by Soxhlet extraction. The yields of the compounds in this study were about the same with the yield quantified by other conventional technique [1], which is in the range of 0.75-4.72 mg/g of dried mass sample. In view of the sample pretreatment methods, acid reagent is suitable to be used in enhancing the extraction yields compared to alkali, as the latter degraded 5% of the intrinsic yield (after soaking for 1 h). This suggests that the bioactive compounds from the plant might be denatured, degraded or undergone other chemical changes under basic environment. As a whole, chemical pretreatments is less effective compared to UAE and MAE. To further evaluate the methods above, their impacts on microstructure of plant sample is studied and their acting mechanism is discussed in the next section.

Microstructure analysis of plant sample

The SEM micrographs of the raw dry leaf in Fig. 2a and 2b show that the leaf has different upper and lower surfaces. The severe corrugation observed on the leaf surfaces was attributed to heat drying during sample preparation. Throughout the upper surface of the leaf, there are numerous interspersed gland-like structures known as glandular trichome. Glandular trichome consists of a stalk that supports a secretory head, and this is the main site of flavonoids biosynthesis in plant [20]. These gland-like structures are also present in many of the herbal plants, such as thyme, peppermint and basil [20–22]. Under the heat drying condition of sample preparation, the glandular trichomes on the raw dry leaf sample was deflated as shown in Fig. 2(a). Unlike the upper surface, the lower surface of the leaf has scarcer number of glandular trichomes. Besides, it also has numerous stomata openings throughout its lower surface. The orderly arrangement of the upper and lower epidermal surfaces demonstrate that the cell integrity of the raw dry sample remained intact. The intact epidermises ensure minimal loss of bioactive compounds from the leaf sample.

Fig. 2 SEM micrographs of *Orthosiphon stamineus* leaf showing (a) upper and (b) lower surfaces of freshly dried leaf sample, and (c) upper and (d) lower surfaces of leaf sample subjected to Soxhlet extraction. GT: glandular trichome; S: stomata; RGT: ruptured glandular trichome

The difference in the extraction yields by various extraction methods in Fig. 1 might be due to varying extents of impact of the methods on the microstructure of the sample. In view of the impact by conventional Soxhlet extraction, the surfaces of the extracted leaf was shrunk and covered with cell debris-like particles as shown in Fig. 2c and 2d. Due to the severe corrugation of the leaf surfaces after the extraction, stomata and glandular trichomes were hardly visible at the lower

surface. Despite the fact that the leaf samples after Soxhlet extraction remain almost intact or slightly damaged or ruptured [23], the extraction is able to extract the bioactive compounds from plant sample completely over a duration of time. This is because the Soxhlet apparatus enables repeated event of diffusion of ethanol into leaf cells, which solubilizes the anti-oxidant compounds and transfers of solubilized compounds into the bulk solvent over a duration [24, 25].

UAE is fast and efficient extraction method, where it disrupted the integrity of the leaf surfaces by causing shrinking of epidermis (Fig. 3a and 3b). Furthermore, most of the glandular trichomes were visibly deflated and many were ruptured. The ultrasonic waves propagate into the liquid, results in alternating high-pressure (compression) and low-pressure (rarefaction) cycles. In the rarefaction half-cycle of ultrasonic wave, vast amount of small vacuum bubbles were generated by the high intensity ultrasonic waves [26]. This event was followed by cavitation that is the implosion of small bubbles on the surfaces of the sample during the compression half-cycle of the wave. The imploding of bubbles was accompanied by generation of high local temperature, which was evidenced by the increase in liquid temperature during ultrasonication in this study. Cavitation also results in the formation of high pressure shock wave and generation of powerful liquid jet that is expelled at the leaf surface [27]. The occurrence of these events led to the formation of micropores as observed from the SEM micrograph. Furthermore, micro-streams and micro-turbulent eddies formed during bubble implosion cause micro-stirring in the solvent [28] which enhanced the mass transfer of solvent and release of plant cell materials.

Fig. 3 SEM micrographs of extracted *Orthosiphon stamineus* leaf. (a) Upper and (b) lower surfaces of UAE extracted leaf; (c) upper and (d) lower surfaces of MAE extracted leaf; (e) upper and (f) lower surfaces of acid pretreated leaf; (g) upper and (h) lower surfaces of alkaline pretreated leaf

MAE also imparted obvious disruption to the epidermal surfaces and caused rupture of glandular trichomes (Fig. 3c and 3d). It has been reported and reviewed that microwave irradiation of plant samples leads to intense structural destruction, shrinking of plant part and irregularities in the plant surface [23]. Penetration of microwave heated up the solvent and plant cells by both ionic conduction and dipole rotation mechanisms [29]. The localised heat generated within the cells as well as heat of convection from the surrounding solvent resulted in liquid vaporisation and pressure build up within the plant cells, which in turn ruptured the cells and led to the formation of micropores. The structural disruptions facilitated the permeation of solvent into plant cells and enhanced the extraction of anti-oxidant compounds from cells to bulk solvent [23]. The better efficiency of MAE is attributed to its more efficient heating than Soxhlet extraction.

Compared to the UAE and MAE, chemical pretreatments did not appear to be the preferred pretreatments prior to extraction. The SEM analysis demonstrated that structural disruption imparted by the acid and alkali was not as intense compared to UAE and MAE, where most of the glandular trichomes were deflated with only a few ruptured (Fig. 3e to 3h). In spite of being classified in the same category, hydrochloric acid and sodium hydroxide act on the leaf sample in different ways. The acid indiscriminately hydrolysed amorphous hemicellulose/cellulose in the sample to disrupt the surface structure of the sample [30]. Whereas the alkali mainly remove lignin and fractionally remove hemicellulose/cellulose from the sample [31]. Furthermore, the present of pretreatment reagent alkali in the extraction solvent leads to decomposition of the flavonoids, which was the main cause of the consistently lower extraction yields.

CONCLUSIONS

The performance of extraction method in terms of extraction yields and duration depends on their impacts on the microstructure of plant sample, particularly on the glandular trichome as it is the main site for biosynthesis of plant secondary metabolites. UAE and MAE are able to rupture most of the glandular trichomes of *Orthosiphon stamineus* leaves thus resulted in higher extraction yields and shorter treatment time. Sample pretreatments using chemicals disrupts the epidermal surface of plant sample severely, but gives poor rupture effect on glandular trichome and it may denature the extracted bioactive compounds. In conclusion, there is a proportional relationship

between the extraction performances and the degree of disruption on microstructure of plant sample.

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