Published in final edited form as:

J Ethnopharmacol. 2015 August 22; 172: 195–201. doi:10.1016/j.jep.2015.06.019.

Developing an Activity and Absorption-based Quality Control Platform for Chinese Traditional Medicine: Application to Zeng-Sheng-Ping

Taijun Yin¹, Guanyi Yang², Yong Ma¹, Beibei Xu¹, Ming Hu^{1,2}, Ming You³, and Song Gao^{1,2,*}

¹ Department of Pharmacological and Pharmaceutical Sciences, College of Pharmacy, The University of Houston, 1441 Moursund Street, Houston, TX, 77030, USA

² Hubei University of Medicine, 30 South Renmin Road, Shiyan, Hubei, China

³ Medical College of Wisconsin Cancer Center and Department of Pharmacology and Toxicology, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226.

Abstract

Ethnopharmacological relevance—Zeng-Sheng-Ping (ZSP) is a marketed Chinese traditional medicine used for cancer prevention.

Aim of the study—Currently, for the quality control of Chinese traditional medicines, marker compounds are not selected based on bioactivities and pharmaceutical behaviors in most of the cases. Therefore, even if the “quality” of the medicine is controlled, the pharmacological effect could still be inconsistent. The aim of this study is to establish an activity and absorption-based platform to select marker compound(s) for the quality control of Chinese traditional medicines.

Materials and methods—We used ZSP as a reference Chinese traditional medicine to establish the platform. Activity guided fractionation approach was used to purify the major components from ZSP. NMR and MS spectra were used to elucidate the structure of the isolated compounds. MTT assay against oral carcinoma cell line (SCC2095) was performed to evaluate the activities. UPLC-MS/MS was used to quantify the pure compounds in ZSP and the active fraction. The permeabilities of the identified compounds were evaluated in the Caco-2 cell culture model. The intracellular accumulation of the isolated compounds was evaluated in the SCC2095 cells.

Results—The major compounds were identified from ZSP. The contents, anti-proliferation activities, permeabilities, and intracellular accumulations of these compounds were also evaluated. The structure of these purified compounds were identified by comparing the NMR and MS data with those of references as rutaevine (**1**), limonin (**2**), evodol (**3**), obacunone (**4**), fraxinellone (**5**), dictamnine (**6**), maackiain (**7**), trifolirhizin (**8**), and matrine (**9**). The IC₅₀ of compounds **5**, **6**, and **7** against SCC2095 cells were significantly lower than that of ZSP. The uptake permeability of compounds **5**, **6**, and **7** were $2.58 \pm 0.3 \times 10^{-5}$, $4.33 \pm 0.5 \times 10^{-5}$, and $4.27 \pm 0.8 \times 10^{-5}$.

* To whom correspondence should be addressed [Tel: (832) 842-8306; Fax: (832) 842-8305. sgao3@uh.edu].

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respectively in the Caco-2 cell culture model. The intracellular concentrations of these compounds showed that compounds **5**, **6**, and **7** were significantly accumulated inside the cells.

Conclusion—Based on the activity against oral carcinoma cell line as well as the absorption permeability, compound **5**, **6**, and **7** are selected as quality control markers for ZSP. A activity and absorption-based platform was established and successfully used for the quality control of ZSP.

Keywords

Zeng-Sheng-Ping; Quality control; Oral cancer; Anti-proliferation; Absorption

1. Introduction

Chinese traditional medicines (CTM) has been paid increasing attention because of their significant therapeutic efficacy, fewer side effects, relatively low cost, high accessibility, and high acceptability due to a long history of use (Cheng, 2000). However, quality control is the major complaint for CTM. So far, in almost half of the medicines in the Chinese Pharmacopoeia, only one major component is selected as marker compound. It is reported that in the Chinese Pharmacopoeia (2010 version), among 1203 herbs, 529 are regulated by measuring only one marker compound (Song et al., 2013). This is a relatively easy approach to control the quality of a CTM for the manufactures. However, the question is that how to select the marker compound. In some cases, bioactive compound is selected, but, in most of cases, the major component is selected as a marker (Song et al., 2013). As a result, the quality of a CTM may not correlated to its efficacy because the major compound may not active and/or may not be absorbed and reach the target organ. There is a need to develop an activity and absorption-based platform to select reasonable marker compound(s) to control the quality of a CTM.

Zeng Sheng Ping (ZSP), also known as Antitumor B, is a Chinese traditional medicine used for the prevention of alimentary tract cancer. It was reported that in a clinical trial of 1729 subjects who were identified as marked esophageal dysplasia, the incidence of esophageal cancer in the ZSP group (3.9%) was reduced by 53% as compared to that of the placebo group (8.3%) after three years of treatment (Lin et al., 1988). Another clinical trial with 2531 cases of marked esophageal dysplasia showed that the cancer rate drop by 52.2% and 47.3% with 3 and 5 years ZSP treated respectively as compared to control (Lin et al., 1990). Other than the prevention of esophageal cancer, ZSP was also reported to be active in the prevention of oral, lung, and bladder cancer in clinical trial or animal model (Fan, 1993, Zhang et al., 2004, Wang et al., 2009, Wang et al., 2011, Sun et al., 2010). For example, Sun *et al* reported that in a clinical trial on 112 patients with oral leukoplakia, ZSP (4 tablets, 3 times per day for 8-12 months) reduced the size of oral lesion in 67.8% (40/59) patients, whereas the placebo was effective in 17% (9/53) patients ($P < 0.01$) (Sun et al., 2010).

The mechanism studies showed that ZSP down-regulated the expression of epidermal growth factor receptor (EGFR) and phosphorylated EGFR (Wang et al., 2011). In addition, ZSP prevents carcinogenesis at gene level. For example, it was reported that in the Benzo(a)pyrene induced lung tumor model in A/J mice, 114 out of the 284 tumor related genes changed their expression toward the normal levels in tumors when the animal was

treated with ZSP (Zhang et al., 2004). Moreover, ZSP exhibited an enhanced inhibitory effect in animals harboring genetic alterations (*Kras2*, *p53*, and *Ink4a/Arf*), which are often seen in human lung adenocarcinomas.

Due to the promising efficacies against multiple type of cancers, ZSP is worthy of being further investigated in cancer prevention. However, the quality control of ZSP is a major concern. ZSP tablet, which is approved by State Food and Drug Administration of China (SFDA), is made from six herbals comprising *S. tonkinensis* (18-24%, w/w), *P. bistorta* (17-21%, w/w), *P. vulgaris* (18-25%, w/w), *S. brachyotus* (17-23%, w/w), *D. bulbifera* (3-6%, w/w), and *D. dasycarpus* (8-12%, w/w) (Peizhong, 1990). According to the patent, the tablet is made by multiple steps including 1) boiling the first five herbals in water ; 2) removing the solvent under vacuum to form a sticky residue; and 3) mixing the residue with the powder of the last one in the above recipe (Peizhong, 1990). In the Chinese Pharmacopoeia, the quality of ZSP tablet is controlled by the content of matrine, an alkaloid reported from *S. flavescens* (Yang et al., 2010). The reason using matrine as marker compound for the quality control of ZSP is not provided.

Other than above concern, ZSP is made by mixing water extracts of five herbs with the crude powder of the sixth herbs, which does not meet the requirement of a model drugs. Traditional medicines are formulated into tablets, pill, and oral liquid using modern techniques in order to improve their accessibility and controllability. However, it is unusual to use crude plant material to make the tablet directly because plant material could increase the variability during formulation. Moreover, the active components are difficult to be absorbed directly from plant material because only soluble substrates can be absorbed in the intestine (Gao et al., 2012). The aim of this study is to extract ZSP tablets to afford a more potential fraction and establish a reasonable quality control based on the activity and the absorption of major components.

2. Materials and methods

2.1 Chemicals

Formic acid (LC-MS grade) was purchased from sigma (St. Louis, MO). Acetonitrile, methanol, and water were HPLC grade bought from EMD (Gibbstown NJ). Other chemicals (typically analytical grade) were used as received. ZSP was obtained from the manufacture, Tianjin Central Pharm Inc. (Tianjin, China). Limonin (> 90%), formononetin (>99%), and matrine (> 90%) were bought from sigma (St. Louis, MO). Obacunone (> 95%), fraxinellone (> 95%), rutaevine (> 95%), evodol (> 95%), and dictamnine (> 95%) were bought from Yuanye Ltd (Shanghai, China), Maackiain (> 95%) was bought from Ruicong Ltd (Shanghai, China).

2.2 HPLC for semi-preparation

The HPLC conditions were as follows: an Agilent 1050 running ChemStation software with a 759 A absorbance detector (Applied Biosystem, Foster city, CA); column, Phenomenex luna 5 μ m, 250 $\times$ 10 mm(Phenomenex, CA); mobile phase A (MPA), water, mobile phase B, acetonitrile; detection wave length, 210 nm.

2.3 General instruments for structure identification

The NMR spectra were recorded in CDCl₃ with a Bruker DRX instrument at 500 or 800 MHz for ¹H NMR and 125 or 200 MHz for ¹³C NMR using residual CHCl₃ as the internal standard. Chemical shift values (δ) are given in parts per million (ppm), and the coupling constants are in Hz. MS spectra were recorded on an API 3200 Qtrap mass spectrometer.

2.4 UPLC for components quantification

The ultra performance liquid chromatography (UPLC) conditions were: system, Waters Acquity™ with diode array detector (DAD); column, Acquity UPLC BEH C₁₈ column (50 × 2.1 mm I.D., 1.7 μm, Waters, Milford, MA, USA); mobile phase A (MPA), 0.1% formic acid; mobile phase B (MPB), 100% acetonitrile; gradient for negative scan, 0- 0.5 min, 5.0 % MPB, 0.5-1.5 min, 5.0-50 % MPB, 1.5 -4.0 min, 50-95 % MPB, 4.0-4.5 min, 95 % MPB, 4.5-4.6 min, 95-5.0 % MPB; 4.6-5.0 min, 5.0 % MPB; gradient for positive scan: 0- 0.5 min, 5.0 % MPB, 0.5-1.5 min, 5.0-50 % MPB, 1.5 -2.5 min, 50-95 % MPB, 2.5-3.0 min, 95 % MPB, 3.0-3.5 min, 95-5.0 % MPB; 3.5-4.0 min, 5.0 % MPB; flow rate, 0.5 ml/min; column temperature, 60 °C; injection volume 10 μL.

2.5 Mass spectrometer for components quantification

The quantification was performed in API 3200 Qtrap triple quadrupole mass spectrometer (Applied Biosystem/MDS SCIEX, Foster City, CA, USA) equipped with a TurboIonspray source by using multiple reactions monitoring (MRM) method with ion pair transition to monitor each analyte. Unit mass resolution was set in both mass-resolving quadrupole Q1 and Q3, negative or positive mode. In the negative mode, the ionspray voltage, -4.5 kV; ion source temperature, 650°C; nebulizer gas (gas 1), nitrogen, 30 psi; turbo gas (gas 2), nitrogen 20 psi; curtain gas, nitrogen 10. In the positive mode, ionspray voltage, 5.5 kV; ion source temperature, 650°C; nebulizer gas (gas 1), nitrogen, 30 psi; turbo gas (gas 2), nitrogen 20 psi; curtain gas, nitrogen 10. The Compounds dependent parameters are in Table 1. For example, the MS/MS method for matrine was: positive mode, ionspray voltage, 5.5 kV, ion source temperature, 650 °C; nebulizer gas (gas 1), nitrogen, 30 psi; turbo gas (gas 2), nitrogen 20 psi; curtain gas, nitrogen 10 psi; declustering potential (DP), 96 V; Collision cell entrance potential (CEP), 23 V; collision energy (CE), 49 V; collision cell exit potential (CXP) 2 V.

2.6 Extraction and isolation

The extraction was guided by anti-proliferation assay in SCC2095 cell line. ZSP powder (0.1 kg) was extracted with water (5 L× 3) by shaking at room temperature for 4 hours to generate extract 1 (33.2g), 2(10.2g), and 3 (6.0g), the residual was extracted with 95% ethanol (4 L× 3) to obtain extract 4 (3.8g). The extracts 3 and 4 were combined for further liquid-liquid partition. The crude combined extract was suspended in water (1.0 L) which was extracted by ethyl acetate (1L× 3) and n-butanol (1L× 3) consequentially to obtain fraction A (ethyl acetate, 2.3g), B (n-butanol, 2.9g), and C (water). The active fractions (Fr. A and B, re-named as GS409) were combined for further isolation study.

The fraction GS409 was subjected to column chromatography over silica gel made up in dichloromethane (DCM), and was eluted with DCM:MeOH =10:0; 9:1; 4:1; 1:1, and 0:10 to

afford 5 sub-fractions (Fr 1-5). The active sub-fraction 2 was further isolated over a silica gel column which was eluted by DCM:MeOH=40:1 to 0:1 to afford 12 fractions (Fr. 2a-2l). The active Fr. 2j was passed through a Sephadex LH-20 column eluted by MeOH to obtain 6 sub-fractions (Fr. 2j₁-2j₆), among which the active fraction (Fr. 2j₃) was the most active one. The Fr. 2j₃ was further purified through semi-preparative HPLC using ACN/H₂O as mobile phases to afford compounds **1** (13.0 mg), **2** (15.2 mg), **3** (3.5 mg), **4** (37.2 mg), and **6** (21.7 mg). The active Fr. 2i was submitted to Sephadex LH-20 column eluted by MeOH to afford compound **7** (2.1 mg). The active Fr. 3 was submitted to Sephadex LH-20 column eluted by MeOH to obtain compound **8** (16.3 mg). Compound **5** (26.3 mg) was crystalized from Fr. 2a in MeOH and compound **9** (37.3 mg) was crystalized from fraction 2b.

2.7 Sample preparation for quantification

The standard curve for UPLC-MS analysis was prepared in 50% MeOH from the stock solution (10 μ M, in DMSO/EtOH=1:4) of each of the individual standard compounds. Predetermined amounts of the stock solutions were mixed in 50% MeOH, which was further diluted by the same solvent to afford the standard curve samples. The ZSP and GS409 samples were prepared also in 50% of MeOH by diluting the stock solution. Formononetin (0.2 μ M in MeOH) was used as internal standard (I.S.). Ten microliters of I.S. was added to 200 μ L of samples. The injection volume was 10 μ L.

2.8 Anti-proliferation assay

The SCC2095 (oral squamous cell carcinoma cell line) cell line was used in the anti-proliferation assay according to the previously described procedure (Guo et al., 2012). The cells were cultured in MDMEM medium (Hyclone, USA), supplemented with 10% of fetal bovine serum in 5% CO₂ at 37 °C. The assay were performed followed that MTT method in 96-well plate. Briefly, cells were seeded into each well and allowed to adhere for 24 hours before treatment. Then the medium was removed and the drug in the medium at suitable concentration was added to each well, which was further incubated for 48 hours for activity evaluation. The cell viability was measured and a cell growth curve was plotted. IC₅₀ values were calculated by Reed and Muench's method.

2.9 Cellular uptake

The cellular uptake studies were carried out as described previously (Ha et al., 2010). Briefly, SCC2095 cells were seeded in 6-well plates at a density of 5×10^5 cells per well in complete growth medium and allowed to grow for 6 days, with the medium changed once every other day. Before the uptake experiment, medium was aspired followed by incubating the cells with 3 ml of blank HBSS (pH, 7.4) at 37°C for 1 hour. The blank HBSS was removed and 3.0 mL of GS409 (200 μ g/ml in HBSS) was added, which was incubated at 37 °C for 2 or 4 hours. At the end of the experiment, SG409 was removed and the wells were washed by ice-cold blank HBSS for 3 times. Cells were collected and sonicated in 0.5 mL HBSS at low temperature (iced water bath) to break up the cells. The mixture was centrifuged at 15,000 rpm for 15 min. The supernatant (450 μ L) was dried under N₂ and the residue was re-constituted in 50% of acetonitrile (100 μ L) for LC-MS analysis.

2.10 Transport study by Caco-2 cell culture model

Cell culture was according to previously study in our lab (Yang et al., 2010, Gao et al., 2011). Cells were used at passages 41- 49. The experiment protocol and calculation were described in our previously reports. Briefly, GS409 solution (0.5 mg/ml) was loaded onto the apical or basolateral (donor) side. Five donor samples (500 μ L) and five receiver samples (500 μ L) were taken at 0, 1, 2, 3, and 4 h followed by the addition of 500 μ L of fresh donor solution to the donor side or 500 μ L of fresh buffer to the receiver side. The samples were then analyzed by UPLC-MS/MS. The apparent permeability coefficient (P) was determined by the equation

$$P = (dQ/dt) / (A \times C_o)$$

where dQ/dt is the drug permeation rate (μ mol/s), A is the surface area of the epithelium (cm^2), and C_o is the initial concentration in the donor compartment at time 0 (mM).

2.11 Method Validation for UPLC-MS/MS Quantitative Analysis

The inter-day and intra-day precisions for UPLC-MS/MS analysis were determined by injecting the quality control samples at low (0.0098 μ M), medium (0.316 μ M), and high (5.0 μ M) concentrations using fraxinellone (5), dictamnine (6), and maackiain (7) as indicators in the first day and in each of the following two days. The quality control samples were prepared in HBSS buffer (pH 7.4).

Matrix effect was determined by comparing the peak areas of blank cell extracts spikes with analytes with those of the standard solutions dried and reconstituted with a mobile phase.

3. Results

3.1 An active fraction (GS409) was obtained from ZSP tablets

Guided by MTT assay against oral carcinoma cell line (SCC2095), ZSP powder was extracted by water and alcohol to generated extracts, which were further fractionated by liquid-liquid partition with ethyl acetate, n-butanol, and water to afford a fraction GS409 with 5.2 % in yield (extraction and isolation section). The MTT results showed that the IC_{50} of GS409 (295.7 μ g/ml) was significantly lower than that of ZSP (>500 μ g/ml) (Table 2) indicating that GS409 is more potential against oral carcinoma.

3.2 Nine compounds were purified and identified from the active fraction GS409

By using open columns, and semi-preparative HPLC, nine compounds were successfully purified from GS409 (Figure 1). The chemical structures of these isolated compounds were elucidated by comparing the ^1H NMR, ^{13}C NMR, and MS data with those of references as rutaevine (1) (Zhao et al., 2008), limonin (2) (Brekse et al., 2008), evodol (3) (Murofushi et al., 1988), obacunone (4) (Garcez et al., 2000), fraxinellone (5) (Blaise & Winternitz, 1985), dictamnine (6) (Pusset et al., 1991), maackiain (7) (Kinoshita et al., 1990), trifolirhizin (8) (Zhou et al., 2009), and matrine (9) (Liu et al., 2010, Liu & Shi, 2006). These compounds belong to different classes: compounds 1-4 were diterpene, 5 is a sesquiterpene, 6 and 9 are

alkaloid, 7 and 8 are flavonoids. Among these compounds, **1-6** were reported from *D. dasycarpus*, **7, 8**, and **9** were reported from *S. tonkinensis*.

3.3 An UPLC-MS method was developed to quantify the isolated compounds

An LC-MS/MS method was developed to quantify the above isolated compounds from ZSP and GS409 (Figure 2). The quantification was performed by using a multiple-reaction monitoring (MRM) approach with ion pair transition to monitor each analyte. Unit mass resolution was set in both mass-resolving quadrupoles Q1 and Q3. Compound-dependent parameters are in Table 3. The quantification results showed that the contents of these compounds ranged 0.01 - 34.75 and 35.8 - 785.00 µg/mg in ZSP and GS409 respectively. Obacunone was the most abundant components with 34.75 µg/mg in ZSP and 785.00 µg/mg in GS409. The contents of dictamine, which is the most active compound, in ZSP and GS409 were 23.65 and 650.00 µg/mg. The contents of these isolated compounds in GS409 were significantly higher than those in ZSP suggesting GS409 can be used for further pharmacological study.

3.4 The anti-proliferation activities of the isolated compounds were evaluated by MTT assay

To evaluate the activity against oral carcinoma, the isolated compounds were submitted to MTT assay against SCC2095 cell line, which is an oral squamous cell carcinoma cell line (Weng et al., 2010). The results showed that the IC₅₀ of fraxinellone (**5**), dictamnine (**6**), and maackiain (**7**) were less than that of ZSP and GS409 suggesting these compounds are more active than ZSP in anti-proliferation of oral carcinoma (Table 1). Dictamnine (**6**) was the most active one with IC₅₀ value at 22.55 µg/ml, which is similar to that of positive control (5-FU).

3.5 The intracellular accumulation of these isolated compounds was also determined in the SCC2095 cell line

The results showed that dictamnine was the most abundant components accumulated inside the cell (Figure 3). When incubate GS409 with the cell for 2 hours, the intracellular amount of dictamnine was 0.0225 ± 0.004 µg/mg protein. The rank order of the intracellular accumulation was matrine = evodol < limonin = rutaevine < obacunone = fraxinellone < maackiain = trifolirhizin < dictamine. When incubate GS409 with the cell for 4 hours, dictamnine was also the most abundant components in the cell. The rank order was evodol < limonin < rutaevine = Obacunone = fraxinellone < matrine < maackiain < trifolirhizin < dictamine. The intracellular level of these compounds was different when the incubation duration was extended. Further investigation is needed to explain this observation. The intracellular accumulation of active the compounds, fraxinellone (**5**), dictamnine (**6**), and maackiain (**7**), were relatively high among these 9 components at different incubation time.

3.6 The permeabilities of these isolated compounds were determined in the Caco-2 cell

To evaluate the absorption permeability, the GS409 was loaded on the apical side in the Caco-2 transport study. The results showed that the permeabilities of compounds **5**, **6**, and **7** were $2.58 \pm 0.3 \times 10^{-5}$, $4.33 \pm 0.5 \times 10^{-5}$, and $4.27 \pm 0.8 \times 10^{-5}$ respectively (Figure 4).

3.7 The quantification method was validated using fraxinellone (5), dictamnine (6), and maackiain (7) as indicators

The validation results, which were summarized in Table 4, revealed that: 1) the standard curves were linear in the concentration range of 5.0-0.0024 μM fraxinellone and dictamnine, 10.0-0.0024 for maackiain ($R^2 > 0.99$); 2) the precision, and accuracy values for UPLC-MS/MS quantitative analysis were well within the acceptance range (less than 15%); 3) there was no measurable matrix effect observed.

4. Discussion

An active fraction GS409 was obtained from ZSP. Nine compounds were identified (Figure 1) and quantified from the GS409 and ZSP (Table 3). The MTT assay showed that fraxinellone (5), dictamnine (6), and maackiain (7), were the most active compounds (Table 2). The intracellular accumulation of fraxinellone (5), dictamnine (6), and maackiain (7) indicated that, most likely, these three compounds were responsible for the activity (Table 2). The absorption study in the Caco-2 cell culture model showed that the permeabilities of fraxinellone (5), dictamnine (6), and maackiain (7) were above 10-5 cm/sec revealing that the absorption in the intestine of these three compounds is rapid.

An active fraction GS409 was obtained from ZSP, which has high potential to be further investigated in the treatment of oral lesions. ZSP is made by mixing water extracts of five herbs with the crude powder of the sixth herbs. However, using crude plant powder as material to make a formulation is unreasonable because plant material could increase the variability during formulation. In addition, the active components are difficult to be absorbed directly from plant material because only soluble substrates can be absorbed in the intestine (Gao et al., 2012). In this study, an active fraction GS409 was afforded from ZSP by fractionation which was guided by the MTT assay (Section “extraction and isolation”). The MTT results showed that the activity of GS409 is significantly higher than that of ZSP (Table 2). In addition, the contents of the active compounds were increased in GS409 comparing with those in ZSP (Table 3). The content of fraxinellone, dictamnine, and maackiain were 23, 26, and 350-folds higher in GS409 than those in ZSP respectively suggesting the active compounds were concentrated in GS409. Thus, GS409 could be used for future study against oral lesions.

An activity and absorption-based quality control for ZSP was established. Traditional medicine is usually made from multiple herbals, which contains multiple components. In the final product, the reasonable marker compounds for quality control should strictly focus on those components that are strongly correlated to the safety and efficacy. How to select the marker compounds will directly affect the therapeutic effect and the safety. So far, for most traditional medicine, the quality is evaluated by using one compound as a marker in Chinese Pharmacopoeia (2010 version) (Song et al., 2013). We expect that the markers meet the following criteria: 1) active at least in the *in vitro* model; 2) a reasonable concentration in the related organs; 3) belongs to different compound class; 4) high permeability.

For ZSP tablet, matrine is the only compound used for the quality control by the manufacture because it is the major components of one of the composited herbal S.

flavescens (Yang et al., 2010). The result of this experiment showed that matrine was inactive ($IC_{50} > 100 \mu\text{g/ml}$) in the MTT assay against SCC2095, an oral squamous cell carcinoma cell line (Table 2). Therefore, the “chemical quality” of ZSP, which was evaluated based on the content of matrine, could not correlate with the efficacy of ZSP in the treatment of oral lesions even if matrine was detected in the cancer cell line (Figure 3). Based on the results of this study, we suggest to use fraxinellone (5), dictamnine (6), and maackiain (7) as markers to evaluate the quality of ZSP due to the following reasons: 1) the *in vitro* assay showed that these three compounds can significantly inhibit the growth of cancer cells (SCC2095) (Table 2); 2) the intracellular accumulation of these three compounds was detected as high level comparing with the other identified components (Fig. 1); and 3) the permeabilities of these three compounds were above 10^{-5} cm/sec , which may translate to more than 70% absorption in the human intestine following oral administration according to the reference (Figure 4) (Artursson & Karlsson, 1991); and 4) these three compounds belongs to different classes: fraxinellone belongs to terpene, dictamnine is an alkaloid, and maackiain is a flavone (Figure 1). The diversity of these compounds could provide wider chemical information of ZSP comparing with that from a single compound.

5. Conclusion

By using ZSP as a reference medicine, an activity and absorption-based platform is established to select marker compounds for the quality control of Chinese traditional medicines. This platform emphasizes the pharmacological activity and pharmaceutical behaviors, rather than focus on chemical information only. Therefore, the quality of a Chinese traditional medicine should be controlled using the marker compounds selected through this platform.

Acknowledgments

The work was supported by NIH AT003203 to MH at University of Houston.

Abbreviations

UPLC	ultra-performance liquid chromatography
HPLC	high-performance liquid chromatography
I.S.	internal standard
DP	declustering potential
CE	collision energy
CXP	collision cell exit potential
QC	quality control
LLOQ	lower limit of quantification
MPA	mobile phase A
MPB	mobile phase B

ZSP	Zeng-Sheng-Ping
CDCI3	Cholorform-d6
NMR	Nuclear magnetic resonance
DCM	dichloromethane
MeOH	Methanol

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Figure 1.
The chemical structures of the identified compounds (1-9)

Figure 2.
The chromatograms of the isolated compounds in UPLC-MS (A, negative scan mode, B, positive scan mode)

Figure 3.

The intracellular accumulation of the isolated compounds. The experiment was conducted in SCC2095 cell line by incubating GS409 at 37 °C for 2 or 4 hours. After the experiment, the cells were collected and sonicated with 0.4 ml of HBSS for LC-MS analysis. Each data point is the average of three determinations. Bars are standard deviations of the mean.

Figure 4.

Transport of the isolated compounds in the Caco-2 cell culture model. GS409 (1.0 mg/ml) was loaded on the apical side of the Caco-2 cell monolayer. The samples were taken from the basolateral side at 0, 60, 120, 180, and 240 min. The buffer used in both donor and receiver sides was HBSS (pH = 7.4). The experiment was performed at 37°C. Each data point is the average of three determinations. Bars are standard deviations of the mean.

Table 1

The Compound-Dependent Parameters in UPLC-MS Analysis

Compounds	Scan mood	Q1(<i>m/z</i>)	Q3 (<i>m/z</i>)	Dwell time (ms)	DP(V)	EP(V)	CEP(V)	CE(V)	CXP(V)
Rutaevine (1)	Negative	485	123	100	-61	-10	-22	-43	-1
Limonin (2)		469	229	100	-62	-10	-23	-35	-1
Evodol (3)		483	421	100	-27	-10	-22	-21	-3
Maackain (7)		283	254	100	-50	-10	-26	-17	-2
Trifolirhizin (8)		445	283	100	-12	-10	-21	-21	-2
Formononetein (I.S.)		267	252	100	-47	-10	-26	-23	-2
Obacunone(4)	Positive	455	161	100	44	10	40	33	4
Fraxinellone (5)		233	215	100	38	10	15	13	2
Dictamine (6)		200	185	100	50	10	15	30	2
Matrine (9)		249	148	100	96	10	23	49	2
Formononetein (I.S.)		269	197	100	30	10	37	28	4

Table 2

Anti-proliferation activity of ZSP, GS409, and the isolated compounds in SCC2095 cell line

Compound	IC ₅₀ (μg/ml)	Compound	IC ₅₀ (μg/ml)
ZSP	>500	Fraxinellone	28.6
GS409	295.7	Dictamnine	22.55
Rutaevine	>100	Maackiain	86.28
Limonin	>100	Trifolirhizin	>100
Evodol	>100	Matrine	>100
Obacunone	>100	5-Fu (positive control)	28.61

Table 3Contents of the isolated compounds in ZSP and GS409^{*}

Compound	Content (µg/mg material)	
	ZSP	GS409
Rutaevine (1)	6.10 ± 0.59	186.50 ± 3.37
Limonin (2)	6.25 ± 1.60	225.50 ± 6.77
Evodol (3)	1.27 ± 0.19	74.00 ± 15.54
Obacunone (4)	34.75 ± 1.04	785.00 ± 23.55
Fraxinellone (5)	19.20 ± 2.30	447.00 ± 26.82
Dictamine (6)	23.65 ± 1.18	620.00 ± 68.70
Maackiain (7)	0.10 ± 0.00	35.80 ± 6.44
Trifolirhizin (8)	9.70 ± 0.87	330.00 ± 34.20
Matrine (9)	23.55 ± 1.98	95.50 ± 13.80

* Data were based on three individual experiments.

Table 4

Intra-day, inter-day precision, accuracy, and matrix effect for fraxinellone, dictamnine, and maackiain

Analyte	Linear range (μM)	Precession and accuracy					Matrix effect [*]
		Intra-day(n=5)			Inter-day(n=5)		
		Concentration (μM)	Accuracy (Bias, %)	Precision (CV, %)	Accuracy (Bias, %)	Precision (CV, %)	
fraxinellone	5.0-0.0024	5.000	104.67	3.12	103.50	15.78	114.10
		0.316	107.00	2.58	97.14	13.64	92.77
		0.0098	102.28	8.91	86.75	13.02	104.96
dictamnine	5.0-0.0024	5.000	106.23	5.43	90.20	7.23	98.76
		0.316	86.65	5.64	91.07	13.06	86.68
		0.0098	96.45	10.22	85.01	15.53	102.13
maackiain	10.0-0.0024	5.000	101.92	4.20	92.7	13.86	107.18
		0.316	107.71	5.18	95.1	9.47	88.27
		0.0098	104.42	6.7	97.6	13.48	90.45

* Matrix effect expressed as the ratio of the mean peak area of an analyte spiked post-extraction to the mean peak area of the same analyte standards multiplied by 100.