

Anti-Inflammatory Effect and Chemical Composition of *Bupleurum chinense* and *Bupleurum kaoi*

Szu Hsiu Liu*, Hui Ping Tsai, Jiun Lin Guo, Lain Tze Lee, Yi-Hung Wen, Cheng Li Fang, Cheng Yu Lee and Shu Jiau Chiou

Biomedical Technology and Device Research Laboratories, Industrial Technology Research Institute, Taiwan

Abstract

Bupleurum species represent one of the most successful and widely used herbal drugs for treatment of many diseases over the past 2000 years. *Bupleurum chinense* (*B. chinense*) is officially listed in China and *Bupleurum kaoi* (*B. kaoi*) is the only indigenous *Bupleurum* plant in Taiwan. Therefore, the aim of our study is to investigate the chemical components, anti-inflammatory, and antioxidants of *B. kaoi* and *B. chinense*. We report highly sensitive and specific assay for anti-inflammatory with a TNF- α luciferase plasmid vector. In preliminary experiments, we found that *B. kaoi* have stronger anti-inflammatory effects than *B. chinense*. However, it was no significant difference in the free radical scavenging activities of *B. kaoi* and *B. chinense*. We also demonstrated that saikosaponins (SS) a, SS_c and SS_d are the major compounds of *B. kaoi* and *B. chinense* in thin layer chromatography (TLC) analysis. The SS_a, SS_c and SS_d content in *B. kaoi* has been reported to be higher than that in *B. chinense*. We combined TLC chromatograms with hierarchical clustering analysis (HCA) to classify into *B. chinense* and *B. kaoi*. The major impact feature were R_f 0.58 (SS_d) and R_f 0.44 (SS_c). These studies showed that TLC chromatograms with HCA could be as tool in to differentiation and identify different species of herbal medicine in the future.

Keywords: *Bupleurum chinense*; *Bupleurum kaoi*; Anti-inflammatory; Thin layer chromatography (TLC); Hierarchical clustering analysis (HCA); Saikosaponin

Abbreviations

PMA: Phorbol-12-myristate-13-acetate; LPS: Lipopolysaccharide; IC₅₀: Half Maximal Inhibitory Concentration; MTT: 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide; PBS: Phosphate-buffered Saline; TNF- α : Tumor Necrosis Factor; ANOVA: Analysis of Variance; SS: Saikosaponin; SS_a: Saikosaponin a; SS_c: Saikosaponin c; SS_d: Saikosaponin d; RSD: Relative Standard Deviation; TLC: Thin Layer Chromatography; HCA: Hierarchical Clustering Analysis; R_f: Retention Factors

Introduction

In traditional Chinese medicine, *Bupleuri Radix* possesses many biological effects, such as anti-inflammatory, antipyretic, and anti-hepatitis activity [1,2]. A number of factors affect the saikosaponin content of *Bupleuri Radix* including: local growing conditions, harvest time, root portion, plant condition, individual plant characteristics, fertilization, and cultivation methods. The major bioactive components of *Bupleuri Radix* are the saikosaponins (SSs), which belong to the oleanane-type triterpene saponins. Although more than 75 monomer SSs have been isolated from *Radix bupleuri*, only SS_a, SS_b, SS_c, and SS_d have been pharmacologically examined [3-7]. Different monomer SSs have been reported to exhibit different predominant pharmacological effects. Previous studies showed that the SS_a and SS_d are reported to have anti-cancer and anti-inflammatory effects. In addition, SS_a has been shown to increase corticosterone secretion and decrease blood cholesterol, while SS_d has immunoregulatory, anti-bacterial, and liver protection effects [4]. However, herbal medicines come from different species within the same genera or different locations within the same species, the quality and efficacy are somewhat different according to their origins, growing conditions, and so on.

Due to its medicinal importance, the demand for *Bupleuri Radix* has increased steadily in recent years. However, *Bupleurum chinense* (*B. chinense*) is used worldwide for medicinal purposes, but is especially common in China, Japan, and South Korea [8]. *B. chinense* is one of the standard medicinal plants in Chinese Pharmacopoeia 2005. *Bupleurum kaoi* (*B. kaoi*) is the species native to Taiwan, which

contains more proportion of saikosaponins than other *Bupleurum Radix* [9]. Therefore, the aim of our study is to investigate the SSs content and biological activity of *B. kaoi* and *B. chinense*.

Materials and Methods

Chemicals and reagents

Lipopolysaccharide (LPS, from *Escherichia coli*), DMEM medium, phosphate buffer saline (PBS), antibiotics, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), L-glutamine, and trypsin-EDTA were purchased from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Hyclone Laboratories Inc. (USA). Saikosaponin a, Saikosaponin c, Saikosaponin d were purchased from CHENGDU MUST BIO-TECHNOLOGY CO., LTD (China). HPTLC Silica gel 60 F₂₅₄ was purchased from Merck. Other chemicals were purchased from Echo Chemical (Taiwan) and Sigma-Aldrich (Sigma-Aldrich, Missouri, USA).

Plant materials

Ten batches of raw material samples of *Bupleurum kaoi* and *Bupleurum chinense* were collected from Taiwan and China and listed in Table 1.

Sample preparation

Two grams of *Bupleuri Radix* powder were accurately weighed and extracted with 40 ml Ammonia solution/ Methanol (volume ratio 1:19) by refluxing for 1 h. After filtering, the filtrate was transferred into a

*Corresponding author: Szu Hsiu Liu, Biomedical Technology and Device Research Laboratories, Industrial Technology Research Institute, Rm 201 Bldg 13, 321 Sec 2, Kuang Fu Rd, Hsinchu - 30011, Taiwan, Tel: +88635743949; Fax: +88635732359; E-mail: SzuHsiuLiu@itri.org.tw

Received June 06, 2014; Accepted June 22, 2014; Published June 25, 2014

Citation: Liu SH, Tsai HP, Guo JL, Lee LT, Wen YH, et al. (2014) Anti-Inflammatory Effect and Chemical Composition of *Bupleurum chinense* and *Bupleurum kaoi*. Nat Prod Chem Res 2:139. doi:10.4172/2329-6836.1000139

Copyright: © 2014 Liu SH, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Sample Name	Scientific Name	Sources	Description
Bk-01	<i>Bupleurum kaoi</i>	Taiwan, Chuang Song Zong Chinese herbal medicine pharmacy	Dried
Bk-02	<i>Bupleurum kaoi</i>	Taiwan, Chuang Song Zong Chinese herbal medicine pharmacy	Dried
Bk-03	<i>Bupleurum kaoi</i>	Taiwan, Chuang Song Zong Chinese herbal medicine pharmacy	Dried
Bk-04	<i>Bupleurum kaoi</i>	Taiwan, Chuang Song Zong Chinese herbal medicine pharmacy	Dried
Bk-05	<i>Bupleurum kaoi</i>	Taiwan, Chuang Song Zong Chinese herbal medicine pharmacy	Dried
Bc-01	<i>Bupleurum chinense</i>	Chain, Hubei, song Lin Chinese herbal medicine pharmacy	Dried
Bc-02	<i>Bupleurum chinense</i>	Chain, Kim Kee Baoan Chinese herbal medicine pharmacy	Dried
Bc-03	<i>Bupleurum chinense</i>	Chain, Hebei, An Guk Chinese herbal medicine pharmacy	Dried
Bc-04	<i>Bupleurum chinense</i>	Chain, Beijing, Beijing University of Chinese Medicine	Dried
Bc-05	<i>Bupleurum chinense</i>	Chain, Xonjiang, Altayskiy Kray	Dried

Table 1: Raw herbs used in this study.

flask and was then evaporated to dryness. The residue was dissolved with 10 ml methanol.

Cell culture and TNF- α plasmid preparation

The macrophage cell line RAW 264.7 was purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan). The cells were cultured in DMEM (GIBCO BRL Life Technologies) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 100 U/ml penicillin-streptomycin. They were maintained at 37°C in a humidified atmosphere containing 5% CO₂. The reporter plasmid contains a *Homo sapiens* TNF- α promoter fragment (Panomics, Inc.) linked to a luciferase reporter gene. The colony was expanded in 300 ml of LB medium at 37°C overnight. The cells were collected, and the DNA was extracted and purified using the Qiagen Miniprep kit (Qiagen, Chatsworth, CA). The pCDNA3 plasmid containing the Geneticin (G418) resistant gene was prepared similarly. This plasmid was used in co-transfection studies for the preparation of a TNF- α reporter stable cell line.

Stable transfection

The macrophage cell line RAW 264.7 (1 \times 10⁵ cells/well) were plated on a 24-well plate and allowed to grow for 24 hours before the transfection. The cells were washed twice with DMEM and incubated for 6 hours in 1 ml of transfection medium containing TNF- α luciferase plasmid (1.5 μ g/ml), pCDNA3 plasmid (0.5 μ g/ml), and LipofectAMINE (GIBCO Life Technologies) (24 μ g/ml). After transfection, the cells were washed with culture medium containing 10% FBS. Twenty-four hours later, the medium was replaced with a selection medium containing 0.5 μ g/ml of G418. The medium was changed every 2 days, and the culture was allowed to continue for 2 weeks.

Luciferase activity of stably transfected reporter cells

The macrophage RAW 264.7 cells were co-transfected with the TNF- α luciferase plasmid and the pCDNA3 plasmid that provides

a selectable marker. Luciferase activity was measured by enzyme-dependent light production using a luciferase assay kit. In each well of 96-well plate, stably transfected reporter cells (1.6 \times 10⁵ cells/well) were treated with 0.1 ng/ml Lipopolysaccharide (LPS) for 6 h at 37°C, a known stimulator of TNF- α . After each experiment, cells were washed and incubated at room temperature for 10 minutes in 30 μ l of lysis buffer (Promega). Mix 20 μ l of cell lysate with 100 μ l of Luciferase Assay Reagent and measure the light produced. Output is quantitated as relative light units (RLU) per μ g protein of the sample.

Cytotoxicity assay

Cytotoxicity assays were performed by the MTT method. Briefly, cells were incubated with 100 μ l of 1 mg/ml MTT for 1 h at 37°C under 5% CO₂. DMSO (100 μ l) was added to dissolve the crystals and the OD₅₆₀ was measured with an ELISA reader (Spectrafluor Plus, Tecan, Switzerland). The results were expressed as cell viability percentages among LPS-stimulated cells.

Thin layer chromatography analysis

Thin layer chromatography (TLC): HPTLC Silica gel 60 F₂₅₄ (glass, 10 \times 10 cm) was used. Developed with developing solvent system (EA : CH₃OH : H₂O= 8:2:1) a path of 8 cm followed by drying. Stained the plate by spraying it with 10% H₂SO₄ spray reagent and heated to 105° for 2 min.

Hierarchical Clustering Analysis (HCA)

TLC fingerprint data: After inspecting the TLC fingerprint of 10 batches of *Bupleurum* samples, the spots shown on the TLC plate was transformed to the optical density after Scion Image software treatment. It was used as the data for analysis. 9 spots were chosen for cluster analysis. Cluster analysis: heat map [10,11] was applied to evaluate the overall features of TLC fingerprint among the 10 batches of *Bupleurum* samples, and heat map-based feature selection was then used to reduce the number of features and to find impact factor of them. The greater of variance in the same group of the heat map cluster result was chosen and repeated this steps until the cluster result was changed. The general idea of this method was to choose the features that can be most distinguished between classes.

Statistics

All the experiments were repeated at least three times. Data were expressed as means \pm SD and analyzed using one-way analysis of variance (ANOVA) followed by a Tukey's test to determine any significant differences. A value of P<0.05 was considered statistically significant.

Results and Discussion

Luciferase activity of stably transfected reporter cells

The macrophage RAW 264.7 cells were co-transfected with the TNF- α luciferase plasmid and the pCDNA3 plasmid that provides a G418 selectable marker for 2 weeks. Luciferase activity was determined by the luciferase assay. In the presence of LPS stimulation, all of the clones exhibited strong and inducible luciferase activities. The maximum TNF- α luciferase concentration was observed 6 hours after LPS stimulation (Data not shown).

Inhibition of LPS-induced TNF- α production

LPS significantly induced the production of TNF- α in stably transfected cells. The inhibitory effects of *B. kaoi* and *B. chinense* on inflammatory cytokines were evaluated by measuring the amounts of

secreted TNF- α in LPS-stimulated stable transfect cells. In Table 2, the 95% ethanol extract of *B. kaoi* and *B. chinense* exhibited potent anti-inflammatory effect against LPS-induced TNF- α release with an IC₅₀ value of 59.8 \pm 3.2 and 68.2 \pm 2.4 μ g/ml, respectively. We also compared with the anti-inflammatory drug SB203580 (p38 inhibitor) served as positive controls. The anti-inflammatory effects of *B. kaoi* and *B. chinense* could be attributed to the inhibition of inflammatory cytokine production or a reduction in the number of stably transfected cells through cytotoxicity. The latter possibility was excluded by comparing the numbers of cells cultured with the different concentrations of *B. kaoi* and *B. chinense*, wherein no significant decreases in cell viability were observed when the concentration was 1000 μ g/ml. The results showed *B. kaoi* have stronger anti-inflammatory effects than *B. chinense*.

DPPH radical scavenging activity

To evaluated as antioxidants according to a DPPH radical scavenging activity assay. The IC₅₀ values of *B. kaoi* and *B. chinense* extracts have been furnished in the Table 2. All samples of *B. kaoi* and *B. chinense* in serial diluted concentrations corresponding to DPPH radical scavenging activities and their values of IC₅₀ are 48.0 \pm 7.1 are 48.7 \pm 3.4 μ g/ml, respectively (Table 2). It show that no significant

Scientific Name	Inhibition of Luciferase Activity IC ₅₀ (μ g/ml)	Inhibition of DPPH radical scavenging activity IC ₅₀ (μ g/ml)
<i>Bupleurum kaoi</i>	59.8 \pm 3.2*	48.0 \pm 7.1
<i>Bupleurum chinense</i>	68.2 \pm 2.4*	48.7 \pm 3.4

TNF- α production in cells treated with SB203580 at 30 μ M was reduced to 62.1 \pm 0.5 % of that in untreated cells. All the experiments were repeated at least three times. Data were expressed as means \pm SD and analysed using one-way analysis of variance (ANOVA) followed by a Tukey's test to determine any significant differences. A value of P<0.05 was considered statistically significant

Table 2: Anti-inflammatory and DPPH radical scavenging activity of *Bupleurum chinense* and *Bupleurum kaoi*.

difference (p<0.05) in the free radical scavenging activities of *B. kaoi* and *B. chinense*.

TLC Analysis of *Bupleurum kaoi* and *Bupleurum chinense*

To give the most chemical information and best separation in the *B. kaoi* and *B. chinense*, the TLC analysis was investigated in this study. The main bioactive components of *Bupleuri Radix* are minor and triterpenoid saponins, such as saikosaponins a (SSa), SSb2, SSb, and SSd (Figure 1). Figure 2 was showed the TLC conditions were developed and optimized using three SSs standards(SSa, SSb, and SSd). In our results showed that SSa, and SSd are the major components of *B. kaoi* and *B. chinense* (Figure 3). In Figure 3, the TLC profiles indicate the highest difference in saikosaponins roots content was between *B. kaoi* and *B. chinense*. *B. kaoi* has greater SSa and SSd than *B. chinense*.

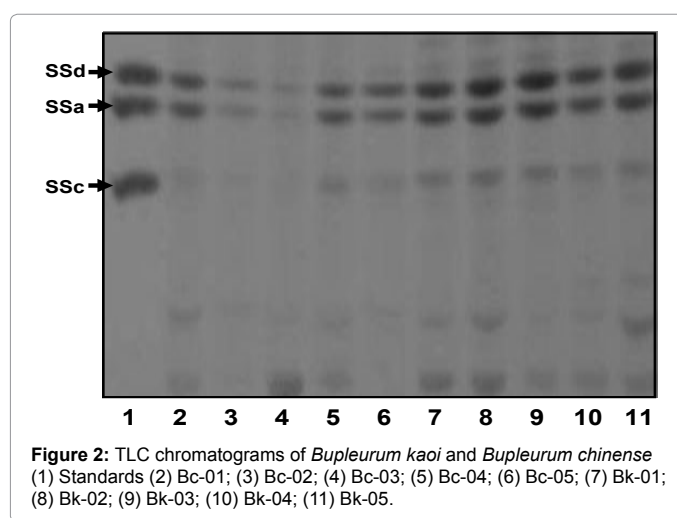


Figure 2: TLC chromatograms of *Bupleurum kaoi* and *Bupleurum chinense* (1) Standards (2) Bc-01; (3) Bc-02; (4) Bc-03; (5) Bc-04; (6) Bc-05; (7) Bk-01; (8) Bk-02; (9) Bk-03; (10) Bk-04; (11) Bk-05.

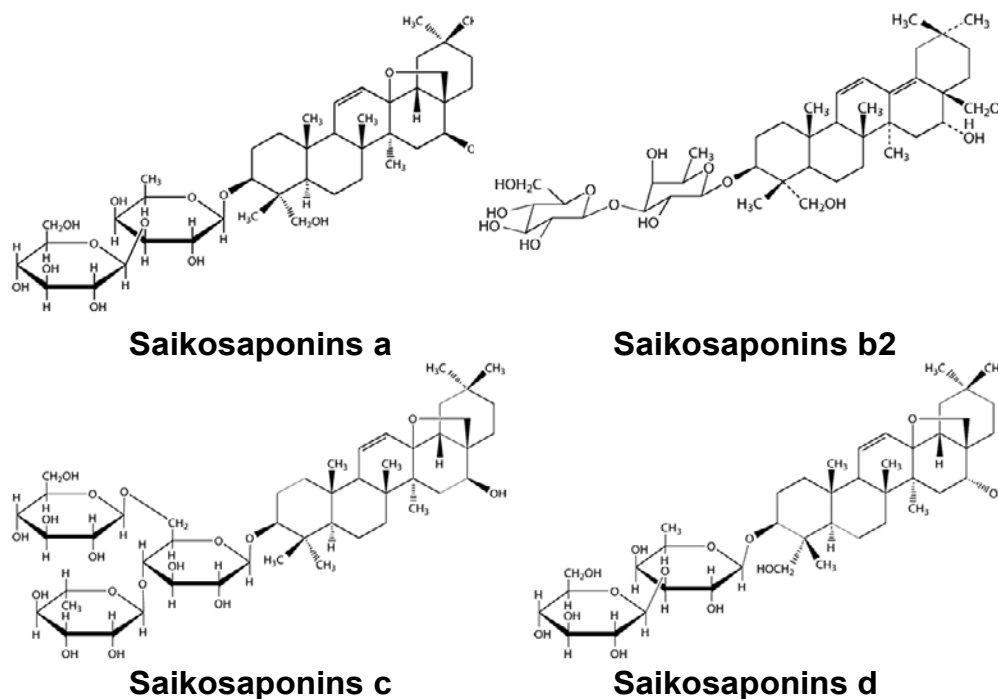


Figure 1: Chemical structures of the major saikosaponins.

Bk-03 (*B. kaoi*) has highest Σ area (SSa+d) in 10 batches of raw material samples. All the percentage of SSa, c and d were estimated from the spot area produced by TLC of each saikosaponin (Table 3). The vertical analysis showed significantly differences in SSa, SSc and SSd content in 10 batches. The SSa, SSc and SSd content in *B. kaoi* has been reported to be higher than that in *B. chinense* (2.0 to 7.5-fold). The horizontal species analysis showed that the percentage ratios' SSa+d/SSa+c+d roots content were 0.84 in *B. kaoi* and 0.83 in *B. chinense*. The percentage ratios' SSc/SSa+c+d roots content were 0.16 in *B. kaoi* and 0.17 in *B. chinense*. It was found that *B. kaoi* roots SSa, SSc, and SSd was higher than *B. chinense*. Interestingly, the percentage ratios' SSa+d/SSa+c+d and SSc/SSa+c+d were similar with *B. chinense*. Similar results were also observed by HPLC analysis (unpublished data).

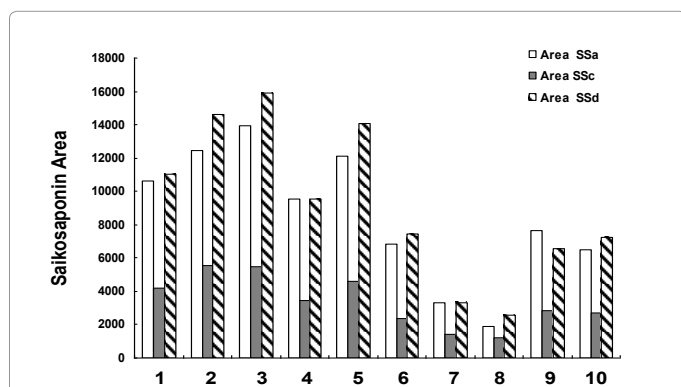


Figure 3: Saikosaponins a, c, and d average area on *Bupleurum kaoi* and *Bupleurum chinense*. (1)Bk-01; (2) Bk-02; (3) Bk-03; (4) Bk-04; (5) Bk-05; (6) Bc-01; (7) Bc-02; (8) Bc-03; (9) Bc-04; (10) Bc-05.

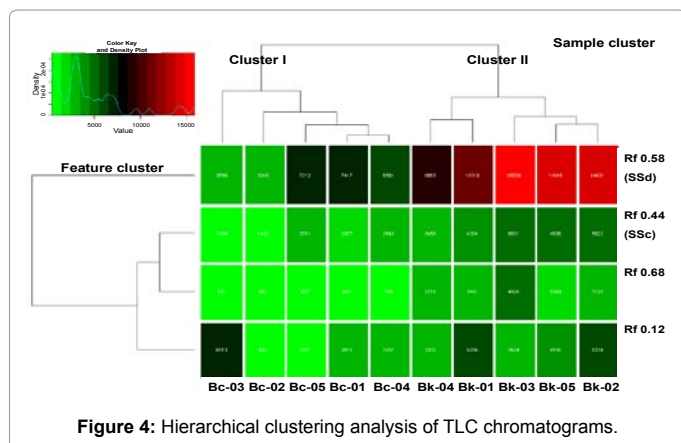


Figure 4: Hierarchical clustering analysis of TLC chromatograms.

Hierarchical clustering analysis (HCA) of TLC fingerprint

Saikosaponins is found to be the major content of *Bupleurum* after TLC fingerprint analysis. SSa and d are the major compounds of *B. kaoi* and *B. chinense*. In order to assess this resemblance and differences of these samples, a hierarchical agglomerative clustering analysis of *B. kaoi* and *B. chinense* samples were performed based on the relative spot areas of TLC. The results of HCA were shown in Figure 4, we found that the samples could be classified into Cluster I (*B. chinense*) and Cluster II (*B. kaoi*). It showed that the major impact feature were retention factors (Rf) 0.58(SSd) and 0.44(SSc), and the next were Rf 0.68 and 0.12.

Conclusion

In traditional chinese medicine, *Bupleuri Radix* possesses many biological effects, such as anti-inflammatory, antipyretic, anti-hepatitis activity [1,2,12]. Anti-inflammatory action of saikosaponins (SSs) isolated from the root of *Bupleurum falcatum L* were examined using female albino rats. Among saikosaponins isolated from *Bupleurum falcatum*, SSa and d, not c, were demonstrated to have anti-inflammatory action [13]. In recently studies demonstrated that the Tumor necrosis factor-alpha (TNF- α) is primarily produced by macrophages, lymphocytes, neutrophils, endothelial cells, keratinocytes, and fibroblasts during acute inflammatory reactions. Therefore, to clarify the anti-inflammatory activity, we establish a highly sensitive and specific assay for TNF- α gene transcription. This assay is based on the use of TNF- α reporter cell line, which was created by stable transfection of a macrophage cell line (RAW264.7) with a TNF- α luciferase plasmid vector. In preliminary experiments, we found that *B. kaoi* have stronger anti-inflammatory effects than *B. chinense*. However, it was no significant difference in the free radical scavenging activities of *B. kaoi* and *B. chinense*.

We demonstrated that SSa, SSc and d are the major compounds of *B. kaoi* and *B. chinense* in TLC analysis. The SSa, SSc and SSd content in *B. kaoi* has been reported to be higher than that in *B. chinense*. We combined TLC chromatograms with hierarchical clustering analysis to differentiate the different species of *Bupleuri Radix*. These studies showed that, HCA could be classified into Cluster I (*B. chinense*) and Cluster II (*B. kaoi*). The major impact feature were Rf 0.58(SSd) and Rf 0.44(SSc). This method could be as tool in to differentiation and identify different species of herbal medicine in the future.

Acknowledgement

The study was financially supported by grants from the Industrial Technology Research Institute. The authors would like to thank Chuang Song Zong chinese herbal medicine pharmacy, Taiwan for providing *Bupleurum kaoi*.

Sample	Area			Percentage (%)							
	SSa	SSc	SSd	Σ SSa+d	Σ a+c+d SS	SSa	SSc	SSd	Σ SSa+d	Σ SSc	Σ a+c+d SS
Bk-01	10636	4204	11019	21655	25859	0.41	0.16	0.43	0.84	0.16	1.00
Bk-02	12474	5522	14631	27105	32627	0.38	0.17	0.45	0.83	0.17	1.00
Bk-03	13953	5501	15899	29852	35353	0.39	0.16	0.45	0.84	0.16	1.00
Bk-04	9517	3468	9563	29852	22548	0.42	0.15	0.42	0.85	0.15	1.00
Bk-05	12140	4635	14045	26185	30820	0.39	0.15	0.46	0.85	0.15	1.00
Bc-01	6846	2377	7417	14263	16640	0.41	0.14	0.45	0.86	0.14	1.00
Bc-02	3293	1442	3349	6642	8084	0.41	0.18	0.41	0.82	0.18	1.00
Bc-03	1923	1249	2550	4473	5722	0.34	0.22	0.45	0.78	0.22	1.00
Bc-04	7620	2843	6591	14211	17054	0.45	0.17	0.39	0.83	0.17	1.00
Bc-05	6485	2701	7212	13697	16398	0.40	0.16	0.44	0.84	0.16	1.00

Table 3: Saikosaponins a, c, and d average percentage in roots on *Bupleurum kaoi* and *Bupleurum chinense*.

Reference

1. Sakurai MH, Matsumoto T, Kiyohara H, Yamada H (1999) B-cell proliferation activity of pectic polysaccharide from a medicinal herb, the roots of *Bupleurum falcatum* L. and its structural requirement. Immunology 97: 540-547.
2. Yang YY, Tang YZ, Fan CL, Luo HT, Guo PR, et al. (2010) Identification and determination of the saikosaponins in *Radix bupleuri* by accelerated solvent extraction combined with rapid-resolution LC-MS. J Sep Sci 33: 1933-1945.
3. Zhu S, Shimokawa S, Shoyama Y, Tanaka H (2006) A novel analytical ELISA-based methodology for pharmacologically active saikosaponins. Fitoterapia 77: 100-108.
4. Huang HQ, Zhang X, Xu ZX, Su J, Yan SK, et al. (2009) Fast determination of saikosaponins in *Bupleurum* by rapid resolution liquid chromatography with evaporative light scattering detection. J Pharm Biomed Anal 49: 1048-1055.
5. Shyu KG, Tsai SC, Wang BW, Liu YC, Lee CC (2004) Saikosaponin C induces endothelial cells growth, migration and capillary tube formation. Life Sci 76: 813-826.
6. Zong Z, Fujikawa-Yamamoto K, Ota T, Guan X, Murakami M, et al. (1998) Saikosaponin b2 induces differentiation without growth inhibition in cultured B16 melanoma cells. Cell Struct Funct 23: 265-272.
7. Wong VK, Zhou H, Cheung SS, Li T, Liu L (2009) Mechanistic study of saikosaponin-d (Ssd) on suppression of murine T lymphocyte activation. J Cell Biochem 107: 303-315.
8. Sui C, Zhang J, Wei J, Chen S, Li Y, et al. (2011) Transcriptome analysis of *Bupleurum chinense* focusing on genes involved in the biosynthesis of saikosaponins. BMC Genomics 12: 539.
9. Yen MH, Lin CC, Chang CH, Lin SC (1994) Anti-inflammatory and hepatoprotective activity of saikosaponin-f and the root extract of *Bupleurum kaoi*. Fitoterapia 65: 409-417.
10. Ling RF (1973) A computer generated aid for cluster analysis. Communications of the ACM 16: 355-361.
11. Bansal N, Blum A, Chawla S (2004) Correlation Clustering. Machine Learning Journal 56: 86-113.
12. Ashour ML, Wink M (2011) Genus *Bupleurum*: a review of its phytochemistry, pharmacology and modes of action. J Pharm Pharmacol 63: 305-321.
13. Yamamoto M, Kumagai A, Yamamura Y (1975) Structure and actions of saikosaponins isolated from *Bupleurum falcatum* L. I. Anti-inflammatory action of saikosaponins. Arzneimittelforschung 25:1021-1023.