

## Identification of a fat-soluble fraction of *Sparganium stoloniferum* that inhibits cervical cancer HeLa cells

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### ABSTRACT

**Context:** The rizoma of *Sparganium stoloniferum* has been used as a traditional Chinese medicinal herb for thousands of years. *Sparganium stoloniferum* is a stasis-breaking drug to treat a wide range of diseases including cancer, however, its activity of extract on cervical cancer HeLa cells and the mechanisms remains unknown.

**Objective:** This study aimed to screen *Sparganium stoloniferum* extract for its inhibitory effects on cervical cancer HeLa cells.

**Materials and methods:** *Sparganium stoloniferum* was extracted with 95% ethanol under reflux, and the extracts were preliminary separated by silica gel column chromatography. MTT assay and flow cytometry were used to determine the inhibitory effects of three fractions on HeLa cells. In addition, GC-MS was performed to analyze the chemical composition of the active fraction.

**Results:** Sample II showed a dose-dependent inhibition of HeLa cell growth, with an inhibition rate of more than 30%, whereas the inhibition rates of Samples I and III were less than 30%. Interestingly, Samples I and III had no effect on apoptosis, in contrast to Sample II, which significantly promoted HeLa cell apoptosis, in a dose-dependent manner. GC-MS was performed to analyze the chemical components of Sample II: steroids were found to be the major components with a relative content of 73.905%, while six known compounds were obtained for the first time.

**Discussion and conclusion:** This study provided a novel insight for further research of active fractions of *Sparganium stoloniferum*, in accordance with the basic principle and theory of traditional Chinese medicine (TCM), and will promote the shift of effective substance study, from monomeric chemical compounds to active fractions.

**Keywords:** *Sparganium stoloniferum*; fat-soluble active fraction; cytotoxicity; HeLa cells;

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## INTRODUCTION

*Sparganium stoloniferum* Buch.-Ham, as a Chinese herbal medicine, is often used for blood-activation and mass-elimination in clinical traditional Chinese medicine (TCM); its efficacy for the treatment of blood stasis, pelvic mass, lump, amenorrhea and indigestion pain has been confirmed. Pelvic mass and lump in TCM refer to ovarian cyst, hysteromyoma and tumor in modern medicine [Chinese Pharmacopoeia Commission, 2010; Wang, 2012; Zhao et al., 2011; Han et al., 2012]. Though various compounds from *Sparganium stoloniferum* have been extracted, isolated and analyzed, these molecules all show low concentration and poor water-solubility [Zhang et al., 1996; Shirota et al., 1997; Zhu et al., 2010; Hua et al., 2007; Dong et al., 2008; An et al., 2009; Kong et al., 2011; Osamu et al., 1996; Ying et al., 2009; Hao et al., 2012]. Therefore, the active ingredients of *Sparganium stoloniferum* remain unknown, and its pharmacological mechanism is yet to be clarified.

Since TCM emphasizes an overall concept, the chemical compositions and pharmacological mechanisms are rather complex both in single and formulated herb drugs [Zhang, 2008; Tu et al., 2012; Xiao et al., 2012]. For these reasons, research progress in characterizing the therapeutic basis of TCM is slow with little success. Some studies have confirmed that the therapeutic effect of chemical constituents of a given Chinese herb does not represent its overall properties. Interestingly, the extracts of *Sparganium stoloniferum* were reported to inhibit the proliferation activity of human breast cancer MCF-7 cells [Cho et al., 2006], and the tumor cell vaccine supplemented with *Sparganium stoloniferum* and *Curcuma zedoaria* extracts significantly improved B16 cell inhibition [Xu et al., 2001]. Based on these results, we aimed to isolate fat-soluble compounds from *Sparganium stoloniferum* using column chromatography, screen for effective components by testing the bioactivity of chromatography fractions against cervical cancer HeLa cells, and determine the chemical composition using gas chromatography-mass spectrometry (GC-MS).

## MATERIAL AND METHODS

### Experimental instruments and main materials

The instruments used in this study included a cell incubator (Thermo Scientific 8000, Thermo, USA), a GC-MS device (GC6890-MS5973, Agilent, USA), a microplate reader (Thermo MK3), and a flow cytometer (BD FACSCALIBUR, BD, USA). The chromatography glass column was packed in our laboratory, with silica gel (200-300 mesh) purchased from Qingdao Marine Chemical Corporation (China). Dried tubers of *Sparganium stoloniferum* Buch.-Ham were used and decoction pieces were obtained from Xi'an Shengxing Chinese herb co., LTD (China). It was identification of genuine medicinal materials by professor Wang Jitao from department of pharmacognosy in Shaanxi university of Chinese medicine.

### Column chromatography separation

*Sparganium stoloniferum* decoction pieces (10 kg, before processing) were extracted 3 times under reflux in 5 volumes of 95% ethanol for 2 h, with the first reflux done after over-night soaking. The extraction solution was collected and alcohol recovered by evaporation at 50 °C, leaving 587 g of brown extract. Then, the extract was mixed with silica gel, dried and packed on silica gel column (diameter to height ratio, 1:20) to allow separation. Elution was performed with petroleum ether-ethyl acetate (100:1), petroleum ether-ethyl acetate (100:5) and petroleum ether-ethyl acetate (100:10), with yielded Samples I (1.04 g), II (56.01 g), and III (67.03 g), respectively. An aliquot of each sample was used to test its inhibitory activity against HeLa cells.

### HeLa cell culture

HeLa cells were purchased from ATCC (USA). Cryopreserved HeLa cells were rapidly thawed at 37 °C, centrifuged at 1000 r/min for 5 min, washed with culture medium for 2 times, and then resuspended with basal medium. DMEM (Dulbecco modified eagle medium) complete medium was used for cell culture and replaced daily.

### MTT(3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide) test

Cells seeded at 1000-10000 /well in 96 well plates were incubated at 37 °C in a humid environment containing 5% CO<sub>2</sub>, in presence of samples at different concentrations (0 - 80µg/mL). In general, cells were seeded a day before addition of the drug. After 48h of treatment, 20 µl of a 0.5% MTT solution were added to each well followed by 4h incubation; afterwards, the culture medium was carefully removed, and 150 µl of dimethyl sulfoxide was added to dissolve the formazan product formed. After shaking at low speed for 10 min, OD(optical density) values were read with a microplate reader at 490 nm.

### Detection of cell apoptosis using flow cytometry

HeLa cells at 80% confluency were inoculated in 6-well plates for 48-h in presence of drugs at 0 (containing 1% DMSO, i.e. dimethylsulfoxide), 10, 40, and 80 µg/mL, respectively. After digestion with 0.25% Trypsin, cells were collected by centrifuging method at 2000 rpm for 10 min, resuspended in pre-cooled PBS and re-centrifuged as above. After washes with PBS(Phosphate Buffered Saline), cells were resuspended in 300 µL of 1 × binding buffer, followed by addition of 5 µL of Annexin V – FITC(fluorescein isothiocyanate) and incubation in the dark for 15 min at room temperature. At 5 min before detection, 5 µL of PI dye and 200 µL of 1 × binding buffer were added for staining.

In flow-cytometry dot plots, the upper-left, lower-left, upper-right and lower right quadrants, respectively, corresponded to dead, live, late apoptotic and early apoptotic cells. This study used the sum of early and late apoptotic cells to assess apoptosis rates.

### GC-MS analysis of Sample II

For GC, a DB-5 ms quartz capillary column (30 m × 0.25 mm × 0.25 µm) was used, with helium as carrier gas at a flow rate of 0.8 ml/min; a split ratio of 50 to 1 was used for sample injection and the column head pressure was 50 kPa; an interface temperature of 290°C was employed, with the vaporizing chamber set at 300 °C. The temperature program was as follows: 100 °C for 2 min, increased by 6 °C/min to 290 °C, maintained for 15 min. For MS, EI was used as ion source at 230 °C and ionization was carried at 70 eV; quadrupole mass analyzers were set at 150 °C, and the scanning quality range was 33 – 450 amu.

Samples were dissolved with petroleum ether for GC - MS analysis.

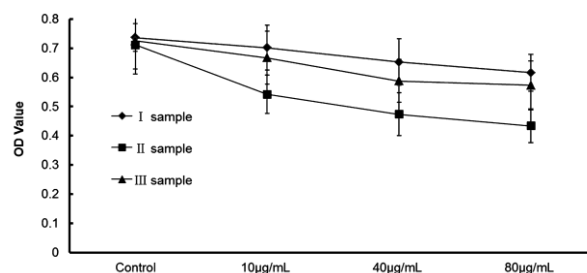
### Statistical analysis

Data are mean ± standard deviation (SD). Intergroup comparison was carried out by *t*-test. *P* < 0.05 was considered statistically significant.

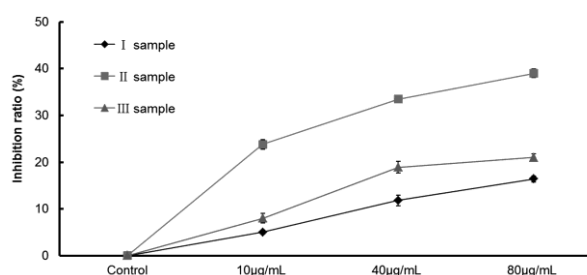
## RESULTS

### Cell growthinhibition

The conversion of MTT into formazan crystals positively correlates with the number of living cells; therefore, the higher the OD value, the lower the growthinhibition rate. The inhibition rate was calculated as 1 – (OD of treatment / OD of control cells). After 48h of treatment, the inhibition rates for the three samples (Samples I-III) increaseddifferentlywithdrugconcentrations. The inhibition rates of Samples I and III were lower than 30%, while that of Sample II exceeded 30% at concentrations greater than 40 µg/ml (Figure1, 2).



**Figure.1** He La cell viability after treatment with Samples I, II, and III



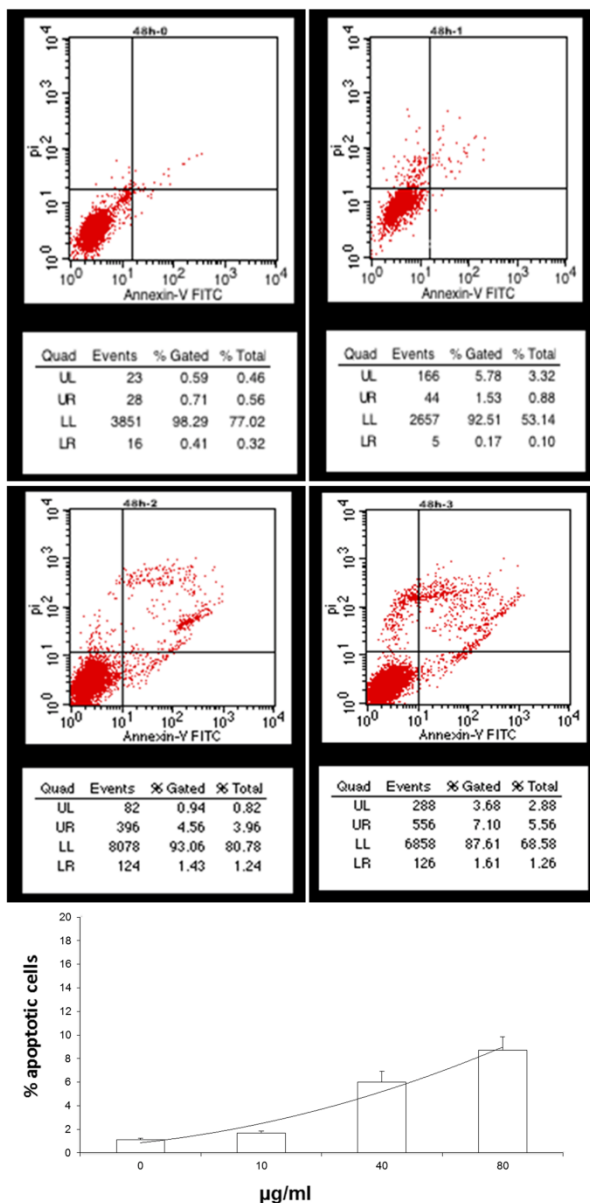
**Figure.2** Inhibition rates of Sample I, II, and III towards He La cells

### Effect of the samples on cell apoptosis

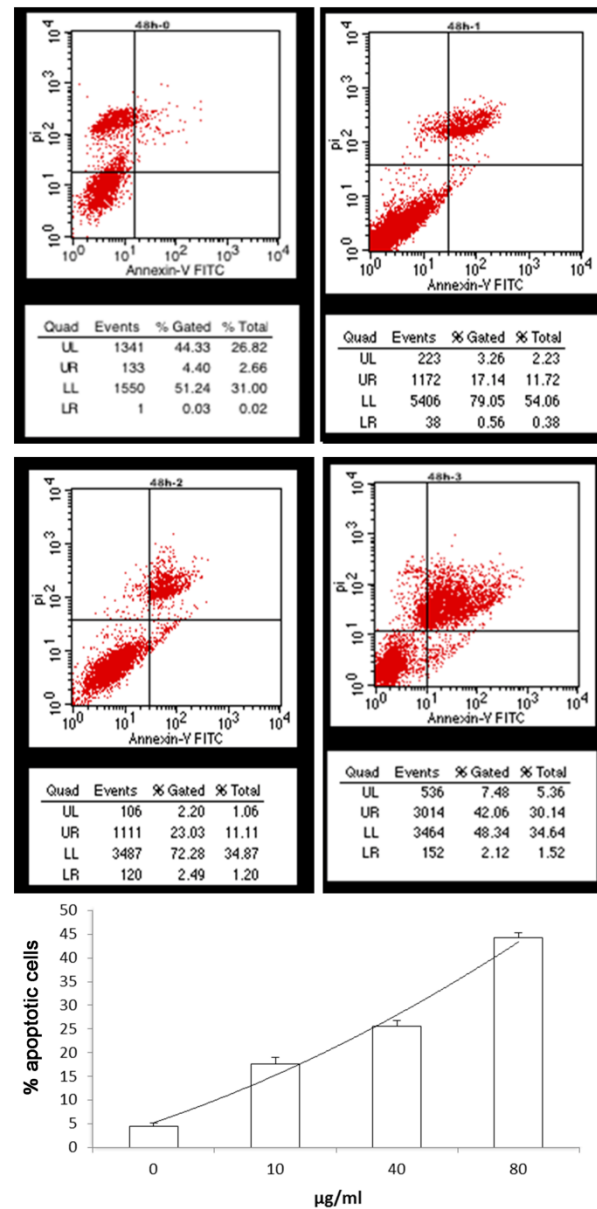
The three samples eluted from chromatography columns were used to treat HeLa cells for 48 h at different concentrations to assess their effects on

cell apoptosis. Annexin V - FITC/PI(Proidium Iodide) double staining showed that sample I barely induced cell apoptosis; in contrast, Sample II induced significantly high apoptosis rates ( $P < 0.05$ ) in HeLa cells compared to the control, in a concentration dependent manner. Sample III at 80  $\mu\text{g/ml}$  slightly induced apoptosis in HeLa cells, whereas the lower concentrations showed no apoptotic effects. These results suggested that sample II promoted HeLa cell apoptosis (Figure 3-5).

Overall, Sample II exhibited high inhibitory activity against HeLa cells, and was considered the active fraction of *Sparganium stoloniferum* extract.



**Fig.3** Apoptotic rates in HeLa cells exposed to sample I for 48 h



**Fig.4** Apoptotic rates of HeLa cells exposed to sample II for 48 h

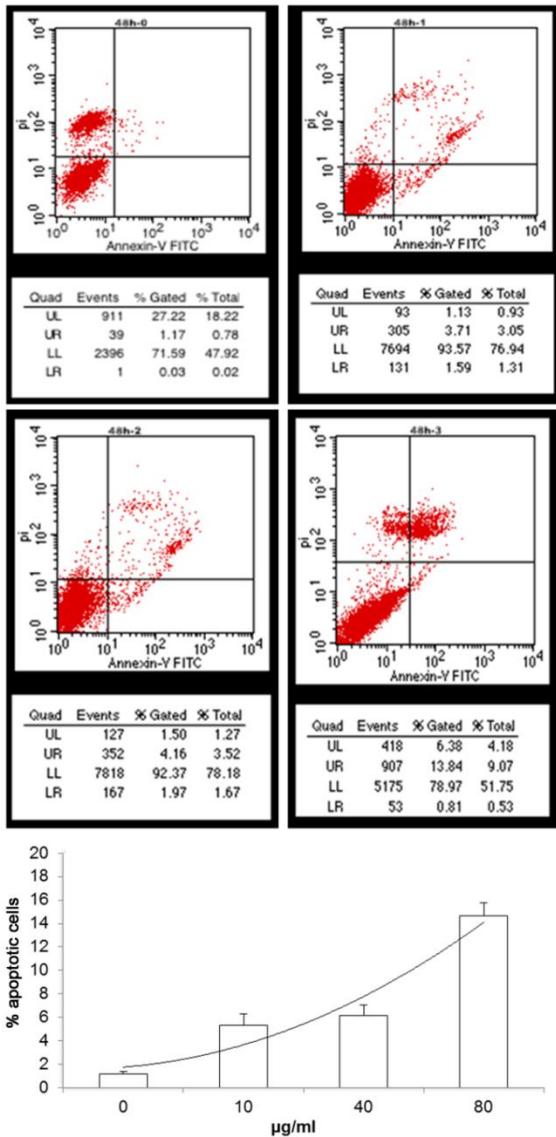


Fig.5 Apoptotic rates of HeLa cells exposed to sample III for 48 h

**GC-MS result**

GC-MS revealed 14 chromatographic peaks from the active fraction (Sample II) of *Sparganium stoloniferum* extract. Their relative contents were calculated by the area normalization method. Each peak was identified mainly by searching the NIST 98 spectrum library and comparing with standard substances. Based on total ion chromatograms of Sample II, 12 peaks were identified as steroids (73.905%), organic acids (6.507%) and other chemicals such as 1, 6-caprolactam (3.695%), 2,2'-(1,4-Phenylene)bis(1H-isoindole-1,3(2H)-dione) (4.602%); the remaining 2 peaks represented unidentified substances (11.291%). The total ion chromatogram is presented below (Figure 6, Table 1).

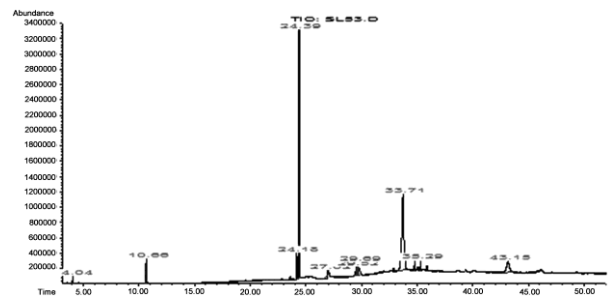
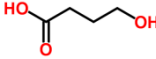
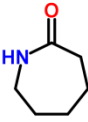



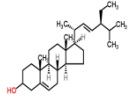
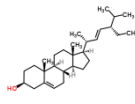
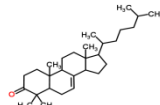
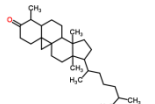
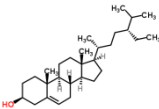
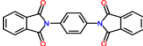
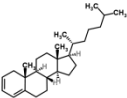


Fig.6 Total ion chromatogram of sample II

**Tab.1** Chemical compounds of Sample II detected by GC-MS analysis

No.	Retention time/min	Compound	Formula	Structural formula	Molecular weight	Content/%
1	4.043	gamma.-hydroxybutyric acid	C <sub>4</sub> H <sub>8</sub> O <sub>3</sub>		104	0.658
2	10.655	caprolactam	C <sub>6</sub> H <sub>11</sub> NO		113	3.695
3	24.180	n-hexadecanoic acid	C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>		256	3.753
4	24.392	Non-parsed				
5	26.967	9,12-octadecadienoic acid(Z,Z)-	C <sub>18</sub> H <sub>32</sub> O <sub>2</sub>		280	1.832
6	27.020	9-octadecenoic acid.(E)-	C <sub>18</sub> H <sub>34</sub> O <sub>2</sub>		282	0.264
7	29.517	(22E)-Stigmasta-5,22-dien-3-ol	C <sub>29</sub> H <sub>48</sub> O		412	1.399
8	29.618	(3β,22E)-Stigmasta-5,22-dien-3-ol	C <sub>29</sub> H <sub>48</sub> O		412	5.647
9	29.643	4,4-Dimethylcholest-7-en-3-one	C <sub>29</sub> H <sub>48</sub> O		412	3.281
10	29.686	9,19-cyclocholestan-3-one,4,14-dimethyl-	C <sub>29</sub> H <sub>48</sub> O		412	6.399

11	33.714	(3β)-Stigmast-5-en-3-ol or β-Sitosterol	C <sub>29</sub> H <sub>50</sub> O		414	53.339
12	34.758	2,2'-(1,4-Phenylene)bis(1H-isoindole-1,3(2H)-dione)	C <sub>22</sub> H <sub>12</sub> N <sub>2</sub> O <sub>4</sub>		368	4.602
				#		
13	35.294	cholestadiene	C <sub>27</sub> H <sub>44</sub>		368	3.840
				#		
14	43.148	unidentified				

# known compounds found for the first time

## DISCUSSION

In this study, silica gel column chromatography was used to isolate highly active fat-soluble fractions from the extracts of *Sparganium stoloniferum*, and the MTT test showed that sample II inhibits HeLa cell proliferation in a dose-dependent manner, with inhibition rates of 23.8, 33.5 and 39% when cells were treated at 10, 40, and 80 µg/mL, respectively. In addition, Sample II promoted HeLa cell apoptosis in a dose-dependent manner, with apoptosis rates of 17.74, 25.53 and 44.17%, respectively, when used at 10, 40, and 80 µg/ml. These apoptosis rates were significantly higher compared with control cells ( $P < 0.05$ ).

GC-MS was used to analyze the chemical composition of sample II, which was composed mainly of steroids (73.905%) and organic acids (6.507%). Six known chemicals were found for the first time in this plant: 1, 6 - caprolactam (3.695%), 2,2'-(1,4-Phenylene)bis(1H-isoindole-1,3(2H)-dione) (4.602%), (22E)-Stigmasta-5,22-dien-3-ol (1.399%), cholestadiene (3.840%), 4,4-Dimethylcholest-7-en-3-one (3.281%) and 9,19-cyclocholestan-3-one,4,14-dimethyl- (6.399%). However, 2 peaks were unidentified (11.291%).

Most antitumor studies have focused on activity of monomer compounds, but few on the effective parts of traditional Chinese medicine. In this study, we reported that the antitumor activities of fat-soluble fraction of *Sparganium stoloniferum* and GC-MS was performed to analyze the chemical

composition of the active fraction. GC-MS is a commonly used method to isolate fat-soluble compounds. As the research of the chemical composition of effective parts, the experimental results are reliable. The study has confirmed that the therapeutic effect of chemical constituents of a given Chinese herb does not represent its overall properties. Fat-soluble fractions (sample II) from the extracts of *Sparganium stoloniferum* can inhibit HeLa cell proliferation and promote HeLa cell apoptosis, but steroids and organic acids isolated from *Sparganium stoloniferum* can not.

## CONCLUSIONS

This preliminary research regarding the bioactivity of *Sparganium stoloniferum*'s sample II and its chemical composition emphasizes that the integrity of Chinese herbs is of significance. Indeed, based on the present findings, any of the chemical compounds identified in sample II can hardly inhibit cell proliferation and promote apoptosis independently, as did sample II. Therefore, we would further investigate the molecular mechanisms by which *Sparganium stoloniferum*'s sample II inhibits cervical cancer HeLa cells.

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## REFERENCES

- An SY, Qian SH, Jiang JQ. (2009). Chemical constituents of *Sparganium stoloniferum* Buch.-Ham. *Chinese Wild Plant Resources*, 4, 57-59.
- Chinese Pharmacopoeia Commission. (2010). *Pharmacopoeia of China*. Part I: 12.
- Cho SI, Cho SJ, Kim BY, Marshal D. (2006). Anticancer Activities of *Sparganium Stoloniferum* on the Proliferation of MCF-7 Cells. *J Biomed Nanotech*, 2, 125-128.
- Dong X, Wang GR, Yao QQ. (2008). Chemical constituents of *Sparganium stoloniferum*. *Acta Pharmaceutica Sinica*, 1, 63-66.
- Han FJ, Li XP, Liu CF, Wei LH. (2012). Modern mechanism research progress of chinese medicine for cervical cancer and its advantages. *Information on Traditional Chinese Med*, 5, 101-104.
- Hao W, Feng RH, Guan SG, Yu WB, Wei M, Guo JL, Liu X, Yang M, Jiang BH, Wu WY, Zhang LX, Guan SH, Guo D. (2012). Rapid preparative isolation of a new phenylpropanoid glycoside and four minor compounds from *Sparganium stoloniferum* using high-speed counter-current chromatography as a fractionation tool. *J Sep Sci*, 35, 1160-1166.
- Hua HM, Yuan T, Wang YL. (2007). A new aromatic alkaline from the tuber of *Sparganium stoloniferum*. *Fitoterapia*, 78, 274-275.
- Kong LJ, Liang QL, Wu QN. (2011). Chemical constituents of *Sparganium stoloniferum*. *Chinese Traditional and Herbal Drugs*, 3, 440-442.
- Osamu S, Setsuko S, Motoyoshi S, Yan N, Hua WY. (1996). Chemical Constituents of Chinese Folk Medicine "San Leng", *Sparganium stoloniferum*. *J Natural Products*, 3, 242-245.
- Shirota O, Sekita S, Satake M. (1997). Two phenylpropanoid glycosides from *sparganium stoloniferum*. *Phytochemistry*, 4, 695-698.
- Tu PF, Shi SP, Jiang Y. (2012). Strategies and approaches on exploring material basis of Chinese materia medica. *Chinese Traditional and Herbal Drugs*, 2, 209-215.
- Wang H. (2012). Academic thoughts and clinical experience of Zhang Yu Fen and observing Huo Xue Xiao Zheng side treatment of uterine flesh tumour clinical curative effect. *Beijing university of chinese medicine*.
- Xiao XH, Wang JB, Yan D, Zhao YL. (2012). Translational medicine: promoting Chinese materia medica modernization into clinic faster and better. *Chinese Traditional and Herbal Drugs*, 1, 1-8.
- Xu LC, Sun ZH, Chen ZL. (2001). Antitumor effect on murine B16 melanoma by tumor cell vaccine modified with SE (Chinese Traditional Medicine, San-Leng and E-SHU). *Chinese J Cancer*, 12, 1380-1382.
- Ying X, Deng KZ, Guo YQ, Gao WY, Zhang TJ. (2009). New Chemical Constituents from the Rhizomes of *Sparganium stoloniferum*. *Archives of Pharmacol Research*, 5, 717-720.
- Zhang WD. (2008). New Research idea for the modernization of TCM—experience of combing the medicinal chemistry of natural products with biology in natural products research. *China j natural medicine*, 1, 2-5.
- Zhang WD, Wang YH, Qin LP. (1996). Studies on the flavonoids in the Rhizome of *Sparganium stoloniferum* Buch.-Ham. *China J Chinese Materia Medica*, 9, 550-551.
- Zhao H, Liu L, Wang QY, Gu N, Wu XK. (2011). The research progress of traditional Chinese medicine treatment of cervical cancer. *Chinese medicine modern distance education of china*, 19, 165-166.
- Zhu FM, Du B, Li J. (2010). Analysis of the Chemical Composition of essential oil from *Rhizoma sparganii* by GC-MS method. *Natural Product Research Development*, 2, 253-256.

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### Erratum

In Article “Identification of a fat-soluble fraction of *Sparganium stoloniferum* that inhibits cervical cancer HeLa cells”, with the number of DOI: <http://dx.doi.org/10.1590/1678-4324-2016150491>, published in journal Brazilian Archives of Biology and Technology, vol. 59, the 01 page.

that read:

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