



Research Paper

Phenolic composition and effects on allergic contact dermatitis of phenolic extracts *Sapium sebiferum* (L.) Roxb. leaves

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ABSTRACT

Ethnopharmacological relevance: The leaves of *Sapium sebiferum* (L.) Roxb. have long been used in Traditional Chinese Medicine (TCM) for the treatment of eczema, shingles, edema, swelling, ascites, scabs, and snakebites, among other maladies. The present study was an outreach research behind our previous study and aimed to analyze the chemical composition of phenolic extracts of *Sapium sebiferum* leaves and evaluate their effects on allergic contact dermatitis (ACD).

Materials and methods: The main compounds of *Sapium sebiferum* leaves were identified using UPLC-PDA method by comparing retention times and UV–vis spectra with those of reference standards. Their effects on ACD were examined using a dinitrofluorobenzene (DNFB) induced mice ACD model. Chemical parameters including reactive oxygen species (ROS), MDA and GSH/T-GSH ratio of ear tissue were also determined.

Results: Seven compounds including gallic acid, ellagic acid, hyperin, isoquercitrin, astragalin, quercetin and kaempferol were identified from *Sapium sebiferum* leaves, and their contents were also determined; ellagic acid, isoquercitrin and astragalin were in the majority. Phenolic extracts of *Sapium sebiferum* leaves exhibited dose-dependent inhibitory effects on edema induced by ACD at doses of 0.03, 0.1 and 0.3 mg/ear. The application of extracts also decreased ROS and MDA levels and increased GSH/T-GSH ratio of ear tissue.

Conclusion: The present study demonstrated that the bioactivity of *Sapium sebiferum* leaves may be due to the existence of the identified phenolic components, and several high polarity compounds were also active. The beneficial effect of *Sapium sebiferum* leaves on skin diseases is based on its antioxidant activity or effects on antioxidant defense system.

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1. Introduction

Allergic contact dermatitis (ACD) is a highly prevalent skin inflammatory disease, which is also known as contact hypersensitivity mediated by hapten-specific T-cells caused by repeated exposure of skin to the hapten, such as 2,4-dinitrofluorobenzene (DNFB) (Peiser et al., 2012). ACD consists of two distinct phases, the sensitization phase and the elicitation phase. In the latter phase, an inflammatory response is elicited to the skin, and proinflammatory substances including cytokines and chemokines were released (Grabbe and Schwarz, 1998; Shi et al., 2012). Animal models have been widely used in the study of ACD. DNFB is the most frequently used hapten, which can induce the reactive oxygen species (ROS) production (Kim et al., 2009). ROS has been proved to be involved in skin inflammation diseases (Esser et al., 2012). Oxidative stress can

emphasize the inflammatory reaction, and then form a vicious cycle. Another research suggested that GSH/GSSG ratio (the ratio of the reduced (GSH) versus the oxidized (GSSG) form of glutathione) imbalance plays a crucial role in ACD (Mizuashi et al., 2005). These backgrounds implied that antioxidant might be beneficial for the treatment of ACD. Most current topical therapies for ACD include glucocorticosteroids, non-steroid anti-inflammatory drugs, and immunomodulators. However, these agents are often related with severe adverse effects. Therefore, there is a great need for the discovery of new and effective drugs for ACD. Natural products have been found to be an excellent source of new active substances (Vuorela et al., 2004; Kato, 2010).

Sapium sebiferum (L.) Roxb. (Euphorbiaceae), English name is Chinese Tallow Tree, which originated from China (referred to “Wu Jiu” in Chinese), now grows primarily in the subtropical regions of the world and is cultivated as an ornamental plant and has been regarded as an invasive species in the U.S. *Sapium sebiferum* is not only an energy plant but also a medicine. The leaves of *Sapium sebiferum* have been long used as anti-inflammatory medication

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with diuretic and parasitocidal effects for the treatment of eczema, shingles, edema, swelling, ascites, scabs, and snakebites, among other maladies, as described in Tradition Chinese medical books (such as Tang Materia Medica and Compendium of Materia Medica) (Peng et al., 2008). Modern pharmacology studies have suggested that the leaves of *Sapium sebiferum* have antioxidant, anti-microbial, anti-inflammatory, anti-hypertensive, and analgesic activities (Hsu et al., 1994; Huang et al., 2004; Hassan et al., 2011; Fu et al., 2013). Some phenolic compounds including gallic acid, ellagic acid, shikimic acid, quercetin, kaempferol, isoquercitrin, hyperin, astragalin, trifolin, and rutin have been identified in *Sapium sebiferum* leaves (Liu and Kong, 2005; Wang et al., 2007). Our previous study indicated that ethyl acetate fractions of ethanol extracts of *Sapium sebiferum* new and fallen leaves (NL and FL) exhibited strong antioxidant activities and efficient therapeutic effects on TPA induced irritant contact dermatitis (Fu et al., 2013). Based on the results of our previous study and related records of *Sapium sebiferum* leaves traditional use, we can hypothesize that *Sapium sebiferum* leaves will be efficient in the treatment of ACD. In the present study, we identified the main compounds and their contents of phenolic extracts of *Sapium sebiferum* new and fallen leaves using UPLC analysis method, and their effects on DNFB induced ACD in murine model were also evaluated. Our results indicated that phenolic extracts of *Sapium sebiferum* leaves exhibited beneficial effects on ACD, which is based on its antioxidant, or effects on antioxidant defense system.

2. Materials and methods

2.1. Standard compounds and other chemicals

Herbal reference standards including gallic acid, ellagic acid, hyperin, isoquercitrin, astragalin, quercetin and kaempferol were obtained from Chengdu Herbpurify Co., Ltd. (Chengdu, China). Methanol, acetonitrile, formic acid and ammonium formate were HPLC grade for UPLC analysis. DCFH-DA and DNFB were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Protein, MDA, T-GSH/GSSG ratio (the ratio of total glutathione (T-GSH) versus the oxidized glutathione (GSSG)) test kits were provided by the Institute of Biological Engineering of Nanjing Jiancheng (Nanjing, China). All other chemicals and reagents used in this study were of analytical grade.

2.2. Preparation of plant extracts

Sapium sebiferum new and fallen leaves were collected from Renshou County in April 2012 and the campus of Sichuan University in November 2011, respectively; both locations were in Sichuan Province, China. A voucher specimen (No. 00721412) was identified by A.P. Jie Bai (School of Life Sciences, Sichuan University) and deposited in the Herbarium of Sichuan University.

The preparation of phenolic extracts of *Sapium sebiferum* new and fallen leaves in the present study was the same as our previous work (Fu et al., 2013). Briefly, the leaves were dried, grounded, and weighed, then extracted with 95% ethanol (v/v) in a Soxhlet apparatus twice. Next, extracts were extracted with petroleum (30–60 °C) (10 times) to remove the pigments and lipids and then extracted with ethyl acetate (8 times). NL and FL were acquired from new and fallen leaves, respectively, and were used for analysis of chemical composition and anti-ACD effects.

2.3. Preparation of standard and sample solutions and UPLC-PDA analysis

Approximately 1.00 mg of each standard was accurately weighed using a Mettler Toledo MS105DU balance and was then brought to a concentration of 2.00 mg/ml with methanol to provide

stock solutions. The stock solutions were mixed and diluted to provide a series of standard solutions at appropriate concentrations for the calibration curves. The extracts of *Sapium sebiferum* leaves were dissolved in methanol at a concentration of 2.00 mg/ml, and diluted to 400 µg/ml for UPLC analysis. All the solutions were stored at –20 °C.

The analysis of phenolic compounds of *Sapium sebiferum* leaves extracts was performed on a Waters Acquity System (Waters Co., Milford, MA, USA) that consisted of a photodiode array (PDA), a binary autosampler, an on-line degasser, and a column oven. Quantitative data processing was carried out using the Empower software (Waters). Sample separation was performed using an Acquity UPLC HSS T3 column (2.1 × 100 mm², 1.8 µm; Waters Co., MA, USA), and the column temperature was set at 40 °C. The mobile phases were (A) acetonitrile and (B) 0.1% formic acid and 10 µM ammonium formate water solution. The gradient was as follows: 17% A for 1 min; 17–20% A for 0.5 min; 20–30% A for 0.5 min; 30–45% A for 1.5 min; 45–50% A for 0.5 min; 50% A for 0.5 min, followed by re-equilibration of the column for 2.5 min with 17% A. The flow rate was 0.5 ml/min and injection volume was 2.0 µl. UV–vis absorption spectra were recorded on-line from 200 to 400 nm during the UPLC analysis.

The PDA detection was conducted at 260 nm for quantitative purpose. The results were expressed as mg of each phenolic compound per 1 g dry extract. Because of the limited commercial standards used in this study, we could not identify and quantify the corresponding compounds of all peaks in the extracts. However, their chemical categories could be identified from their chromatographic behavior and UV spectra. The same categories of phenolics usually have similar chromatographic behaviors and UV spectral characteristics.

2.4. Effects of NL and FL on ACD

2.4.1. Animals

Male Kunming mice (five weeks old, 18–20 g body weight) were purchased from DOSSY Biotechnology Co., Ltd. (Chengdu, China). Mice were housed under standard conditions with 22 ± 2 °C and a 12 h light/dark cycle, and have free access to standard commercial pellet diet and water. All experiments were approved by the Sichuan University Animal Experimentation Ethics Committee (Permit number: 20140520) and were in complete compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.4.2. DNFB-induced ACD and experimental design

ACD was induced by repeated treatment of DNFB according to a published method with minor modifications (Yuan et al., 2010). Briefly, DNFB was dissolved in AOO (acetone:olive oil=4:1 v/v). For sensitization, DNFB solution (100 µl, 0.5%) was painted on the shaved abdomen of mice. Five days later, the mice were challenged by the application of 10 µl DNFB (0.5%) on inner surfaces of right ears of mice. For the topical application of drugs, groups of mice (n=6) were treated with 10 µl of NL, FL (both at doses of 0.03, 0.1 and 0.3 mg/ear), dexamethasone (Dex, 75 µg/ear) and vehicle (ethanol) on the inner surface of right ears for 1 h, 9 h, 25 h and 33 h respectively after DNFB challenged. The left ears were untreated. Ear thickness was measured before and 48 h after challenged using a digital micrometer (Chengdu Sanhe Measure Tools Co., Ltd.) in a blinded fashion. To minimize variation in technique, a single investigator performed the measurements throughout each experiment. Mice were sacrificed 48 h after challenged, and right ear tissue was collected from each mouse and homogenized for biochemical analysis. The percent inhibition of edema is calculated using the following formula:

Inhibition (%) = $\frac{[1 - \text{Increase in ear thickness (treatment-control)} / \text{Increase in ear thickness (model-control)}] \times 100}{1}$

2.4.3. Determination of the ROS, MDA levels and GSH/T-GSH ratio

ROS level was determined based on the reaction of ROS and DCFH-DA (Ma et al., 2013). Incubation of DCFH-DA with tissue homogenates induced the cleavage of diacetate by esterases and formation of DCFH, which is rapidly oxidized to highly fluorescent DCF by ROS from non-fluorescent statues. Diluted supernatants (100 μ l, 1%, diluted by PBS pH 7.4) were mixed with 100 μ l DCFH-DA solutions (20 μ M, diluted 100-fold by PBS from DMSO stock solution) and incubated at room temperature in darkness for 20 min. The fluorescence was determined using a micro-plate reader (Spectra Max M2, Molecular Device, USA) with an excitation wavelength of 488 nm and emission wavelength of 530 nm. MDA content and GSH/T-GSH ratio were measured using commercial kits according to the manufacturer's protocol.

2.5. Statistical analysis

SPSS (version 19) and GraphPad Prism (GraphPad software Inc., San Diego, CA, USA) were used for statistical analysis of the data. For content analysis, the data are expressed as the mean $\pm$ SD ($n=3$). For animal experiment, the data are expressed as the mean $\pm$ SEM. The statistical evaluations used one-way analysis of variance (ANOVA) with multiple comparisons, followed by Dunnett's *t*-tests. Statistical significance was considered at $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

3. Results and discussion

3.1. Qualitative and quantitative analyses of NL and FL

The typical UPLC chromatograms detected at 260 nm of phenolic compounds of *Sapium sebiferum* leaves compared with standards are shown in Fig. 1. In the present study, we have identified 7 phenolic compounds, including gallic acid (1), ellagic acid (2), hyperin (3), isoquercitrin (4), astragalin (5), quercetin (6) and kaempferol (7) by comparing the retention times and UV-vis spectra (Table S1) with those of reference standards. Meantime, we can speculate that peak 0.964 could be a derivative of gallic acid; peak 2.764 could be another quercetin glycoside; peak 2.915, 3.068 and 3.121 could be three kaempferol glycosides; and peak 2.832 could be a mixture of quercetin glycosides and kaempferol glycosides. As we can see from the chromatograms, ellagic acid, isoquercitrin and astragalin were main components of both NL and

FL, and NL showed high content with respect to these compounds, but less un-retained high polar compounds (about 0.45 min). The UPLC method was subsequently applied to simultaneously determine the above compounds. The quality parameters and contents of compounds are listed in Table 1. The most abundant compounds were ellagic acid, isoquercitrin and astragalin for NL and FL, with the contents of 52.62 ± 0.05 , 74.14 ± 0.01 , 71.21 ± 0.01 mg/g and 33.73 ± 0.33 , 45.76 ± 0.02 , 25.43 ± 0.03 mg/g, respectively. All the compounds had been proved with various bioactivities, including antioxidant and anti-inflammatory (Vattem and Shetty, 2005; Soromou et al., 2012; Choi et al., 2013; Valentova et al., 2014). Ellagic acid also exhibited anti-allergic activity in several models (Xue et al., 2012). Astragalin showed therapeutic effect on atopic dermatitis (Kotani et al., 2000; Matsumoto et al., 2002). Taken together, the identified main compounds of NL and FL should be responsible for their antioxidant and anti-inflammatory activities as we determined in previous study. We can assume that NL and FL could have beneficial effects on ACD due to their traditional application and chemical composition.

3.2. Inhibitory effects of NL and FL on ACD

As expected, NL and FL exhibited inhibitory effects on edema after being challenged in dose-dependent manners (Fig. 2). The treatment of vehicles (AOO and ethanol) to the right ear of control group did not affect the thickness. The thickness of ears increased significantly 48 h after being challenged with DNFB, and which was inhibited significantly by the topical application of NL, FL at medium and high doses, or Dex at 75 μ g/ear. The edema was inhibited by 25.55%, 38.83% and 44.47% due to application of NL at 0.03, 0.1 and 0.3 mg/ear, respectively; the treatment of FL (0.03, 0.1 and 0.3 mg/ear) also inhibited increase in ear thickness by 27.71%, 34.20% and 51.48%, respectively. The level of inhibition induced by Dex was 62.87%. High dose (0.3 mg/ear) of NL and FL had effects similar to those of Dex (75 μ g/ear) ($P > 0.05$). NL and FL exhibited almost the same effects on ACD, which was inconsistent with results of content analysis. Even so, these results demonstrated that phenolic extracts of *Sapium sebiferum* leaves have beneficial effects on ACD as recorded traditional use. Because of the hypothesis that anti-inflammatory activity is related to antioxidant activity. Next, we examined the ROS and MDA level and GSH/T-GSH ratio of ear tissue.

The ROS and MDA level and GSH/T-GSH ratio of ear tissue are presented in Table 2. The ROS level of model group was significantly

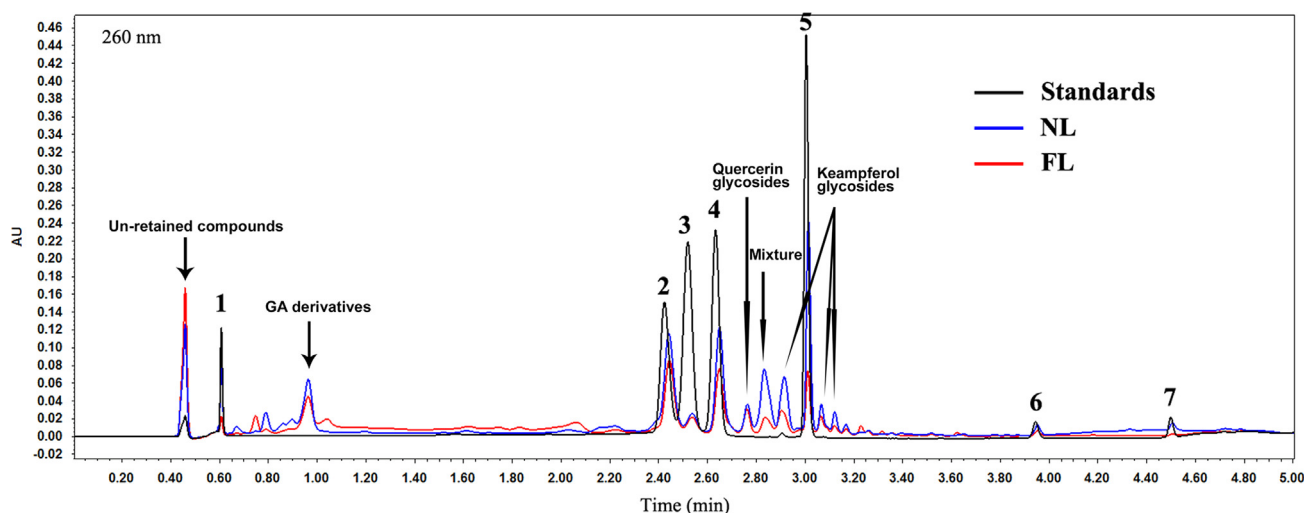


Fig. 1. UPLC chromatograms detected at 260 nm of NL and FL are compared with those of mixed standards. NL, ethyl acetate fraction of the ethanol extract of new leaves; FL, ethyl acetate fraction of the ethanol extract of fallen leaves; gallic acid (1); ellagic acid (2); hyperin (3); isoquercitrin (4); astragalin (5); quercetin (6); and kaempferol (7).

Table 1The retention time, regression equations, correlation coefficients, linearity range and contents in extracts of compounds **1–7** for UPLC-PDA analysis.

No.	Compounds	R_t (min)	Regression equations	R^2	linearity range ($\mu\text{g/ml}$)	NL (mg/g)	FL (mg/g)
1	Gallic acid	0.607	$y = 7876.3x - 5601.4$	0.9977	0.625–20	21.42 ± 0.10	8.42 ± 0.02
2	Ellagic acid	2.441	$y = 16901x - 32452$	0.9978	1.5625–50	52.62 ± 0.05	33.73 ± 0.33
3	Hyperin	2.537	$y = 11524x - 13937$	0.9983	3.125–100	18.85 ± 0.01	11.59 ± 0.03
4	Isoquercitrin	2.649	$y = 10458x - 18540$	0.9985	3.125–100	74.14 ± 0.01	45.76 ± 0.02
5	Astragalin	3.013	$y = 11290x - 25655$	0.9984	3.125–100	71.21 ± 0.01	25.43 ± 0.03
6	Quercetin	3.952	$y = 13041x - 2525.3$	0.9963	0.15625–5	3.90 ± 0.03	2.43 ± 0.01
7	Keampferol	4.506	$y = 12684x - 1321.1$	0.9982	0.15625–5	2.89 ± 0.09	0.73 ± 0.02

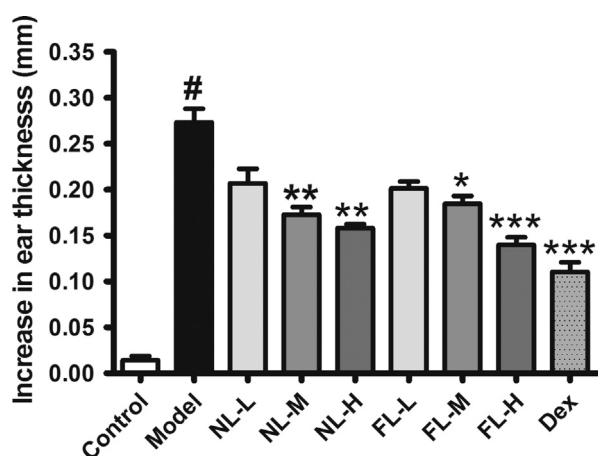
 R_t , retention time; and R^2 , correlation coefficient.

Fig. 2. Inhibitory effects of NL and FL on edema in the DNFB-induced ACD model. The ears of mice were treated with vehicle, NL, FL (L: low dose, 0.03 mg/ear; M: medium dose, 0.1 mg/ear; H: high dose, 0.3 mg/ear), or Dex (75 μg /ear) for 1, 9, 25 and 33 h after being DNFB challenged. Ear edema was measured before and 48 h after being challenged, and expressed as the increase in ear thickness (mm). Each bar represents the mean $\pm$ SEM of the ear thickness ($n=6$). # $P < 0.001$, significantly different from the control; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significantly different from model.

increased due to the occurrence of ACD, which was consistent with the reported results that DNFB can induce the production of ROS (Kim et al., 2009). The application of drugs blocked ROS surge. The effects of medium and high doses of NL and FL, and Dex were significant as compared with those of model group ($P < 0.05$). High dose FL surprisingly decreased the ROS level even lower than control group. The MDA content represents the lipid peroxidation index, which is consequence of oxidative stress and can be ameliorated by GSH. The MDA level of model group was increased but not significant as compared with that of control group ($P > 0.05$). All the treatments rescued MDA levels but only high dose of NL was significant ($P < 0.05$). Interesting results were obtained in the analysis of GSH/T-GSH ratio. The occurrence of ACD did not significantly change the ratio of GSH/T-GSH, but the application of NL and FL exhibited unexpected effects. They increased GSH ratio in dose-dependent manners, and effects of medium and high doses of NL and high dose of FL were significant ($P < 0.05$), especially high dose of NL. Our results indicated that topical application of phenolic extract of *Sapium sebiferum* could suppressed ACD by improving the antioxidant system, which is in line with its strong antioxidant activity.

Taken together, something unusual and interesting should be noted. The content analysis results showed that all the identified main components with anti-inflammatory activities of NL were higher than FL. So NL should exhibit stronger activity than FL, but the fact is not. According to the following facts, we may know why this happened. Firstly, in the biochemical analysis, we found that FL exerts stronger effect in the decreased ROS level, meantime, NL

Table 2

Effects of NL and FL on the antioxidant system indexes of ear tissue.

Groups	ROS fluorescence intensity/mg protein	MDA nmol/mg protein	GSH/T-GSH
Control	0.79 ± 0.08	1.82 ± 0.06	0.44 ± 0.01
Model	1.78 ± 0.12^{aa}	2.89 ± 0.34	0.44 ± 0.01
NL-L	1.18 ± 0.06	1.19 ± 0.09	0.47 ± 0.03
NL-M	$1.01 \pm 0.09^*$	1.28 ± 0.06	$0.49 \pm 0.01^*$
NL-H	$0.97 \pm 0.08^*$	$0.65 \pm 0.05^*$	$0.59 \pm 0.02^*$
FL-L	1.15 ± 0.07	1.17 ± 0.09	0.47 ± 0.01
FL-M	$1.02 \pm 0.07^*$	1.06 ± 0.04	0.49 ± 0.01
FL-H	$0.42 \pm 0.06^{***}$	1.05 ± 0.06	$0.49 \pm 0.01^{**}$
Dex	$0.74 \pm 0.08^{**}$	1.41 ± 0.07	0.39 ± 0.01

NL, ethyl acetate fraction of the ethanol extract of new leaves; FL, ethyl acetate fraction of the ethanol extract of fallen leaves; L: low dose, 0.03 mg/ear; M: medium dose, 0.1 mg/ear; and H: high dose, 0.3 mg/ear.

Each value represents the mean $\pm$ SEM of five animals.^{aa} $P < 0.01$ versus the control group.* $P < 0.05$ versus the model group.** $P < 0.01$ versus the model group.*** $P < 0.001$ versus the model group.

increased the GSH/T-GSH ratio remarkably. Secondly, FL is rich in some extra un-retained compounds than NL. Thirdly, in the previous study, FL has exhibited a little stronger radical scavenging activity than NL. Finally, these identified compounds had been reported to act on single pathways, including NF- κ B (Soromou et al., 2012; Mo et al., 2013; Valentova et al., 2014). The direct radical scavenging activity of some of them seems not to be very strong, such as astragalin. Based on these four facts, we can suppose that these extra un-retained compounds of FL may react with ROS directly, not like the high contents of identified compounds of NL which may act on the signal pathways and influence the expression of genes involved in antioxidant system thereby increasing the GSH ratio. In summary, the possible mechanism of phenolic extracts of *Sapium sebiferum* leaves could be that they exert their bioactivities to directly scavenge ROS or improve the antioxidant defense system.

4. Conclusion

This study is an outreach research of our previous work. The present study demonstrated that the bioactivity of *Sapium sebiferum* leaves may be due to the existence of the identified phenolic components, and several high polarity compounds were also active. Phenolic extracts of *Sapium sebiferum* leaves showed antioxidant, anti-inflammatory and anti-ACD effects. The beneficial effects on the skin were due, at least in part, to their antioxidant activity. Our results provided modern scientific evidence of traditional uses of *Sapium sebiferum* for the treatment of skin inflammation diseases.

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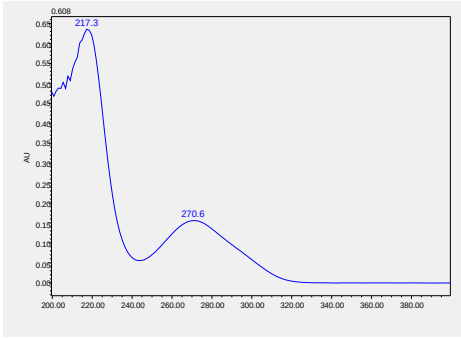
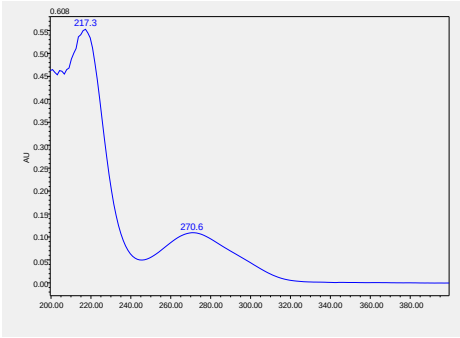
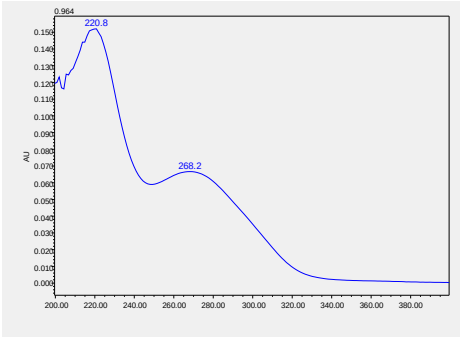
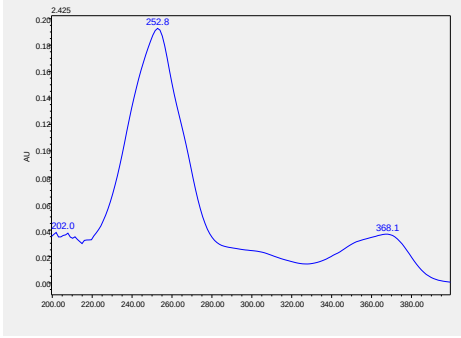
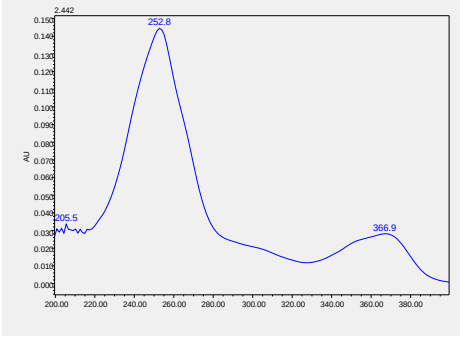
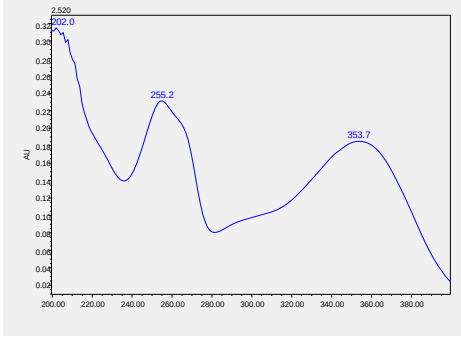
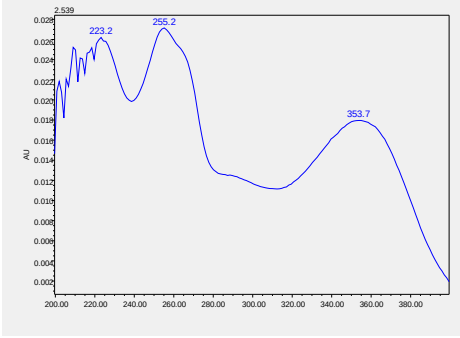
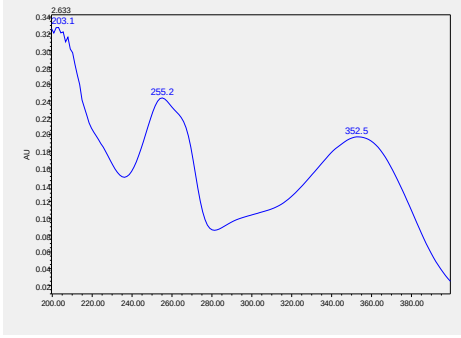
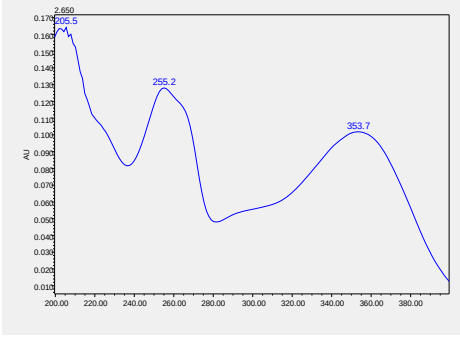
Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.jep.2014.12.072>.

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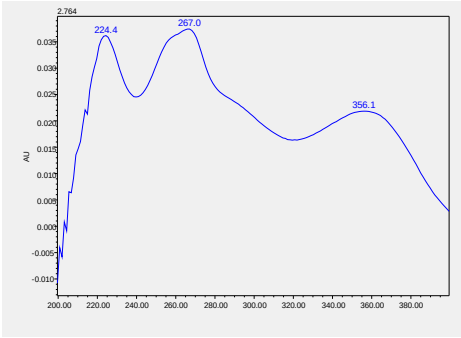
Table S1. UV-vis spectra of standards and main compounds of NL

Compounds	Rt (min)	Standard	NL
Gallic acid (1)	0.607		
Unkonwn Gallic acid derivatives	0.964	no	
Ellagic acid (2)	2.441		
Hyperin (3)	2.537		
Isoquercitrin (4)	2.649		

Unkonwn
Quercetin
glycoside

2.764

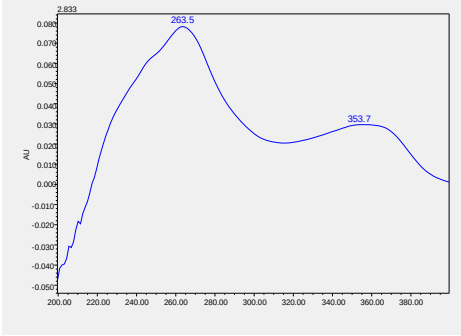
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Unkown
Mixture

2.832

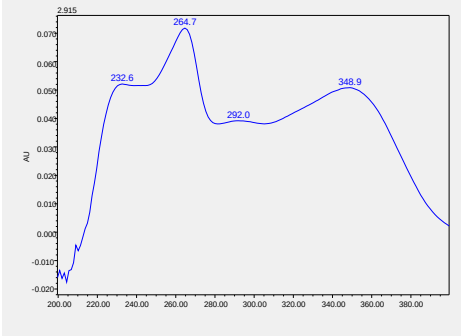
no



Unknown
Kaempferol
glycosides

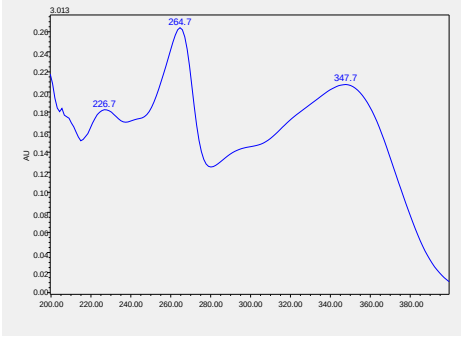
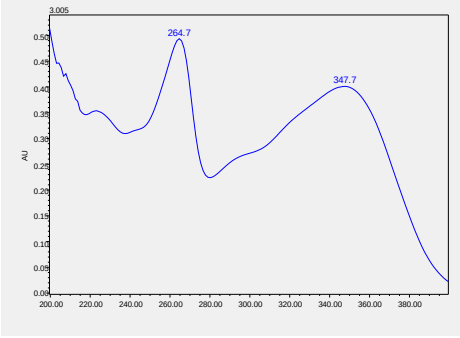
2.915

no



Astragalin (5)

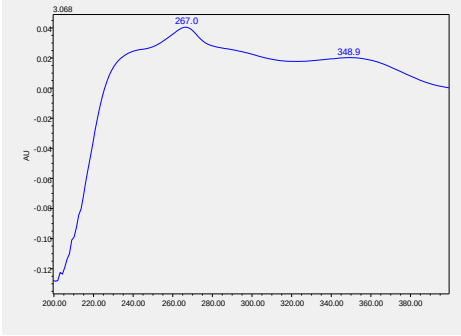
3.013



Unknown
Kaempferol
glycosides

3.068

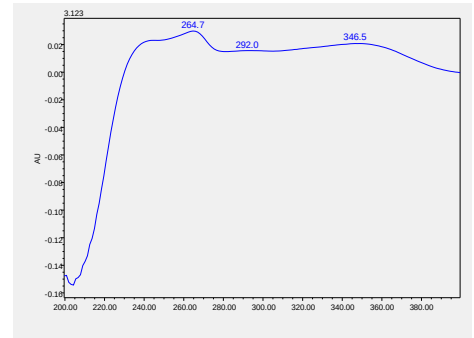
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Unkown
Kaempferol
glycosides

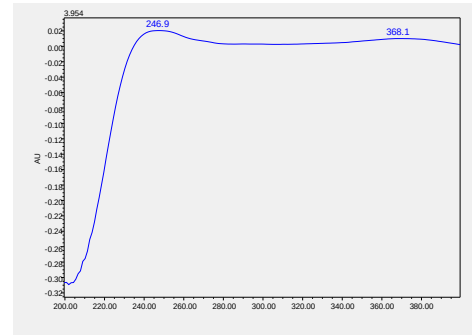
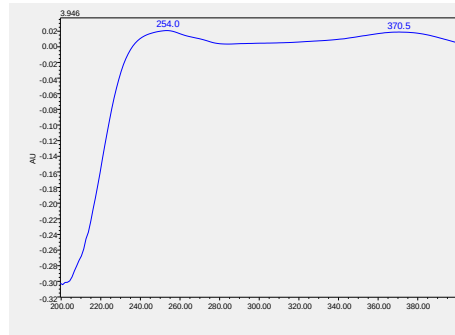
3.121

no



Quercetin (6)

3.952



Kaempferol (7)

4.506

