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Anti-allergic effects of the ethanol extract of *Syzygium formosum* (Wall.) Masam leaves and its immunoregulatory mechanisms.

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Abstract*Ethnopharmacological relevance*

As documented in a Vietnamese traditional medical encyclopedia, *Syzygium formosum* (Wall.) Masam leaves have been routinely used among indigenous Vietnamese people for treatment of various allergy-like symptoms including dermatitis and rhinitis.

Aim of the study

Anti-allergic activity of *S. formosum* leaves was examined with a mouse model of chicken ovalbumin (cOVA)-induced food allergy, and mechanisms underlying the anti-allergic effect were explored.

Material and methods:

BALB/c mice were administered i.p. cOVA (20 µg) plus alum (2 mg) twice on day 0 and 14 for sensitization (immunization). Two weeks after the second immunization, the mice were administered cOVA (50 mg) p.o. 5 times every 3 days to induce food allergy symptoms (i.e., anaphylaxis, diarrhea, and drop in the body temperature). Ethanol extract of dried leaves of *S. formosum* (80 mg/ kg or 200 mg/ kg body weight) was administered p.o. daily during the induction (challenge) period.

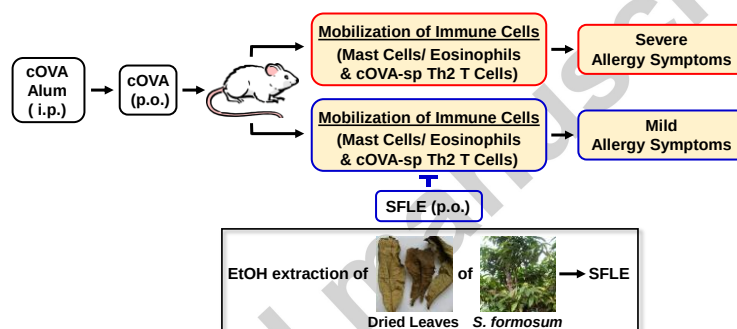
Results:

Treatment with the *S. formosum* leaves ethanol extract ameliorated the allergic symptoms to a significant extent and in a dose-dependent manner. The treatment also resulted in a significant improvement in the inflammatory lesion in the small intestine and reduction in the numbers of mast cells and eosinophils recruited to the lesion. The treatment also brought about a significant reduction in the levels of Th2 cytokines produced by the mesenteric lymph node cells cultured ex vivo with cOVA. The passive anaphylaxis experiment also showed that the extract treatment impaired the mast cell function.

Conclusion:

Our study provides a scientific basis for the traditional (indigenous) use of the *S. formosum* leaves extract for the treatment of various allergy symptoms in Vietnam. In addition, the results show that the extract has activities to suppress antigen-specific Th2 T cell immune responses and the mast cell function, which are directly related with its anti-allergic effect.

Graphical Abstract



1. INTRODUCTION

Food allergy, IgE-mediated hypersensitivity reaction against innocuous food antigens, affects approximately 5-8% of population in developed countries (Gupta et al., 2011; Prescott et al., 2013). The severity of allergic reaction is not predictable, and can range from a mild itching and vomiting to life-threatening systemic anaphylaxis which results in thousands deaths each year (Boyce et al., 2010). Last decades the number of people suffering from food allergy has increased significantly, and, accordingly, efforts to better understand the mechanisms responsible for the induction of food allergy as well as demands for more effective preventive therapies have been also escalating (Oyoshi et al., 2014). Generation of Th2 CD4⁺ T cells during the primary immune response against a food allergen is known to be critical for induction of food allergy. Th2 cytokines (e.g., IL-4, IL-5, IL-10) play a critical role in antibody class switching toward IgE (Yu et al., 2016).

Mast cells, basophils and eosinophils also play a key role in the initiation and propagation of immediate hypersensitivity reactions (Stone et al., 2010). Mast cells and basophils are decorated by IgE via interaction with the high affinity receptor for IgE (FcεRI) expressed on the surface of these cells (Finkelman, 2007). Cross-linking of the IgE-FcεRI complexes by food allergen triggers mast cell activation resulting in release of the granules with histamine, lipid mediators, chemokines, serine proteases such as mast cell protease-1 (MCPT-1), and cytokines (e.g., TNF-α, IL-4, IL-5, IL-6, IL-10, IL-13) (Wang and Sampson, 2011).

Hen's egg allergy is one of the most common IgE-mediated food allergy, and chicken ovalbumin (OVA) was identified as the major allergen to induce Th2-mediated immune responses (Omata et al., 2005). In light of this knowledge, Brandt et al. have established a mouse model of food allergy to use cOVA for the initial systemic sensitization and the oral

challenge afterward (Brandt et al., 2003; Osterfeld et al., 2010). Allergic mice in this model show high levels of cOVA-specific IgE antibodies, severe diarrhea, mastocytosis, intestinal edema, anaphylaxis and elevated production of Th2 cytokines consistent with those described in human subjects suffering from food allergy (Johnston et al., 2014).

Syzygium formosum (Wall.) Masam, a flowering plant that belongs to the myrtle family, *Myrtaceae*, is widely found in India, Malaysia, and Vietnam (Soh and Parnell, 2015). Previously it was shown that *S. formosum* leaves extract (SFLE) has a very high level of antioxidant activity demonstrating the remarkable free radical scavenging activity and the strong inhibition of lipid peroxidation (Phuong et al., 2006). In the Vietnamese folk medicine, SFLE has been traditionally used for treatment of skin allergic and skin inflammation treatment (Do HB, 2004). In addition, hot-water extracts of *S. formosum* leaves-containing mixed herbal formula have been also used as indigenous herbal recipes for treatment of boils, scabies, skin eruptions, skin allergy and diarrhea (Do HB, 2004). Despite the fact, the scientific evidences to prove the anti-allergic effects of SFLE are not established yet. The aims of this study are to determine whether SFLE can inhibit or attenuate allergic responses in a mouse model of OVA-induced food allergy and to gain new insights into the immunological mechanism underlying the anti-allergic effects of SFLE.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

RPMI 1640 medium, fetal bovine serum (FBS), penicillin-streptomycin-glutamine solution, DNP-BSA were purchased from Thermo Fisher Scientific (Carlsbad, USA). cOVA (grade VI), Alum [$\text{KAl}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$], Eosin, Hematoxylin and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, USA). Toluidine Blue was purchased from Samchun Chemical (Pyungtack, Korea). IL-4 and IFN- γ ELISA kits were purchased from Biolegend (San Diego, USA). IL-5 and IL-10 ELISA kits were purchased from BD Pharmingen (San Diego, USA). Biotinylated anti-mouse total IgG and IgG2a were purchased from Jackson ImmunoResearch (West Grove, USA). Biotinylated anti-mouse IgG1 and anti-mouse IgE were purchased from Biolegend and BD Biosciences, respectively. Horseradish peroxidase (HRP)-conjugated streptavidin was purchased from Biolegend. Mouse MCPT-1 ELISA kit was purchased from eBioscience (San Diego, USA). Anti-DNP IgE was purchased from Sigma-Aldrich.

HPLC-grade acetonitrile, methanol and ethylacetate were purchased from Burdick & Jackson (Morris Plains, USA) and formic acid was purchased from Sigma-Aldrich. Purified water was prepared by using Milli-Q purification system (Millipore, USA). All standard compounds, such as gallic acid, isoquercitrin, quercetin, maslinic acid, corosolic acid, betulinic acid, oleanolic acid and ursolic acid were provided by Natural Products Laboratory at College of Pharmacy in Chungnam National University (CNU).

2.2. Animals

Five weeks old female BALB/C mice were purchased from SAMTAKO Inc. (Osan, South Korea). Mice were housed in the Core Animal Facility in CNU under SPF condition. Animal experiments were carried out in accordance with the protocol (CNU-00570) approved by IACUC of CNU.

2.3. Preparation of SFLE

2.3.1 Preparation of the ethanol extract of *S. formosum* (Wall.) Masam leaves

S. formosum (Wall.) Masam leaves were harvested in the area of Quoc Oai, Hanoi, in April 2015 and air-dried under the sunlight; the identity of the plant was confirmed by Dr. Bui Van Thanh at Institute of Ecology and Biological Resources in Vietnam Academy of Science and Technology (VAST). A voucher specimen (IEBR-0417) was deposited at the herbariums of Institute of Ecology and Biological Resources (VAST) and College of Pharmacy (CNU).

The leaves of *S. formosum* were dried further in dry oven (70°C overnight) and grounded to the powder. The dried powder was put in sealed bottle and kept at 4 °C before use. The leaf powder (150.0 g) was submerged in ethanol (1.0 L) for 4 hours at room temperature with gentle shaking for extraction. The leaf powder was recovered and extracted with ethanol (1.0 L) two more times. After extraction, ethanol (3.0 L) was evaporated with a rotary vacuum evaporator at 45°C to obtain the greenish-brown slurry (23 g).

2.3.2. Preparation of SFLE and standard samples for HPLC analysis

For chemical profiling of SFLE, the SFLE slurry (100.0 mg) was suspended in water (10.0 mL) and partitioned with ethylacetate (10.0 mL) 3 times to yield ethylacetate fractions. The ethylacetate fractions were combined together, and ethylacetate was evaporated using a rotary vacuum evaporator. The dried material was dissolved in methanol (1.0 mL) and filtrated with

0.22 μ m PVDF membrane filter (CTK Co., Korea). The filtered SFLE solution was then subjected to HPLC analysis. Each standard compound (1.0 mg) was dissolved in 1.0 mL of methanol and kept at 4 °C before use.

2.3.3. Preparation of SFLE samples for oral gavage.

For in vivo experiment, the SFLE slurry described 2.3.1 was pulverized into powder, and the SFLE powder was mixed with Tween 20-Saline solution to the final concentration 1 mg/mL and 2.5 mg/mL, respectively (Hadziabdic et al., 2015).

2.4. HPLC-DAD analysis and HPLC-ESI-MS analysis

The samples prepared as described in 2.3.2 were analyzed with a LC-10AD series system (Shimadzu, Kyoto, Japan) consisting of two pumps, a column oven, and diode array detector (DAD) over 150 min period. LabSolutions software was used for instrument control and data acquisition during the analysis. The separation was performed using a Hecator C₁₈ column (250 x 4.6 mm, 5 μ m; RStech, Korea). The mobile phase was 0.05 % formic acid in water (A) and 0.05 % formic acid in acetonitrile (B). The separation was achieved by a linear gradient of 5-100% B over 120 min, followed by isocratic elution of 100% B for 30 min. The flow rate was 1.0 mL/min and the column temperature was maintained at 30 °C. The UV-Vis spectra was recorded from 190 to 300 nm with selective wavelength monitoring at 205 nm.

HPLC-ESI-MS experiment was performed using a high performance liquid chromatography mass spectrometry (LC-ESI-MS) (8040 Series; Shimadzu) apparatus in both positive and negative modes. Electrospray ionization (ESI) was operated under optimized conditions, with an interface voltage of 4.5 kV for positive mode and -3.5 kV for negative mode. The desolvation line temperature was set at 250 °C, with a heat block temperature of 400 °C, and a

nebulizing gas flow rate of 3 L/min. Separation was performed using a Phenomenex C₁₈ column (150 x 2.0 mm, 5 µm, Phenomenex, USA) at a flow rate of 0.3 mL/min. The analytical conditions were the same as for HPLC-DAD analysis. Eight compounds SFLE were identified by comparing retention times, absorption spectra and mass spectra to those of authentic standards (including gallic acid, isoquercitrin, quercetin, maslinic acid, corosolic acid, betulinic acid, oleanolic acid and ursolic acid). Asiatic acid was identified by comparing the chemical properties of the peak with those of asiatic acid reported in the literature.

2.5. A mouse model of cOVA-induced food allergy.

Mice were immunized i.p. with cOVA (20 µg) plus alum (2 mg) twice with an interval of two weeks. OVA-alum mixture was prepared as described previously (Uebel et al., 2012). One week after the second immunization the blood was drawn from the mice, and levels of cOVA-specific IgG in the serums were determined with ELISA; 100% of the immunized mice were confirmed to produce the IgG to a varying extent. The mice producing cOVA-specific IgG at a level in the range of mean $\pm$ SD (OD_{600nm} 0.58 $\pm$ 0.11) were chosen to minimize the variation in the final results due to the variation in the extents of immunization (sensitization) and randomly divided into 5 groups (6 mice per group). Two weeks after the second immunization, the mice were challenged p.o. with cOVA (50 mg) five times once every three days. During the challenge period, mice were either left untreated or treated daily with SFLE formulated as described in 2.3.3 or Dexamethasone (p.o.). Immediately after the fifth challenge, the mice were examined for allergic symptoms (i.e., change in the rectal temperature, diarrhea, and anaphylaxis) and the severities of allergic symptoms were rated and scored as reported previously (Brandt et al., 2003). The rectal temperature was measured

using a rectal probe thermometer (Physitemp, Clifton, USA). When anesthesia is needed, isofluran was used as an anesthetizer; 1.5 or 3% isofluran in 1% O₂.

2.6. Histology

Histology experiments were performed as describes by others (Meyerholz et al., 2009; Sridharan and Shankar, 2012). The small intestines were collected 1 day after the last challenge, washed with PBS, fixed in 10% formalin, processed and embedded into paraffin blocks. Five µm thick tissue sections were made with microtome (Leica Microsystem, Germany) and stained with hematoxylin-eosin (H&E) for general analysis or with Toluidine Blue for mast cell evaluation. Stained sections were observed under the autofocus microscope (Motic, China) and numbers of eosinophils and mast cells in high power field (HPF) were counted as described (Sethi et al., 2015; Stucke et al., 2015)

2.7. Measurement of immunoglobulin (IgE/ IgG/ IgG2a/ IgG1) and MCPT-1 levels in the serum

To measure cOVA-specific immunoglobulins and MCPT-1, the blood was collected from each group of mice 1 day after the last challenge. OVA-specific IgE/IgG/IgG2a/IgG1 antibody levels in the serum samples were determined using ELISA as described previously (Shin et al., 2015). Briefly, 96-well plates (Nunc, Denmark) were coated overnight at 4°C with 100 µl of 2 µg/ml cOVA in the coating buffer (pH 9.5). Next day plates were washed 3 times with the washing buffer and blocked with 1% (w/v) bovine serum albumin in PBS (200µl/well) for 1 hour followed by 3 washes with the washing buffer. The serum samples were diluted 50-8.000 folds (100 µl/ well) and incubated for 2 hours. After incubation plates were thoroughly washed, and diluted biotin-conjugated anti-mouse IgE, IgG2a, IgG or IgG1

were added (100µl/well), respectively. After 1 hour of incubation plates were thoroughly washed, and streptavidin-HRP was added and incubated 30 minutes. Plates were thoroughly washed 5 times, 100 µl of tetramethylenbenzidine was added. Reaction was stopped by adding 100 µl 2N H₂SO₄. The optical density (OD) was read using an automated microplate reader (Emax, Molecular Devices) at 450 nm.

Mouse mast cell protease-1 (MCPT-1) levels in the serum (1:100) were quantified using MCPT-1 ELISA kit in accordance with the manufacturer's instructions.

2.8. Ex vivo cytokine production in mesenteric lymph node (mLN) cell culture

mLNs were harvested from each group of mice 1 day after the last challenge and homogenized to prepare single cell suspensions. Isolated mLN cells were resuspended in the complete RPMI complete medium, and 1 X 10⁶ cells were cultured in the presence or absence of 100 µg/ml cOVA in 96-well plates in humidified incubator 37°C with 5% CO₂. Three days later culture supernatants were taken after centrifugation at 1100rpm for 5 min, and the levels of the cytokines in the culture supernatants (IFN-γ, IL-5, IL-4, IL-10) were quantified using respective ELISA kits in accordance with the manufacturer's instructions.

2.9. Passive anaphylaxis

BALB/C mice were injected i.v. with 3 µg of anti-DNP mAb (IgE isotype) one day before injection (i.v.) of 80 µg DNP-BSA for induction of allergic anaphylaxis reaction (Wex et al., 2014). Thirty minutes after injection of DNP-BSA, the rectal temperature of the mice were measured using a rectal probe thermometer. One hour after the injection, the blood was drawn from each mouse and the serum was prepared for analysis of the level of MCPT1.

Mice were treated (p.o.) with SFLE or Dexamethasone daily for 5 days beginning 3 days prior to the injection of the mAb.

2.10. Immunotoxicity assessment

One day after the last challenge, mice were sacrificed by cervical dislocation, and the sizes of the spleens were estimated with the sliding calipers by measuring three dimensions – maximum length (L), maximum width (W), maximum thickness (T); the spleen volume was calculated using the standard clinical ellipsoid equation of $L \times W \times T \times 0.523$ (Faustino-Rocha et al., 2013). Single cell suspensions were prepared from the spleen and the mLN, and the numbers of the cells in those organs were counted using a hemocytometer.

2.11. Data analysis and statistics

Data are presented as the mean $\pm$ standard deviation. Statistical evaluation of the results was carried out using PRISM 7 software (GraphPad Software, La Jolla, CA). Significance of a difference between the groups was calculated with either nonparametric one-way ANOVA (Kruskal-Wallis test) or Student's two-tailed t-test. $P < 0.05$ was considered statistically significant.

3. RESULTS

3.1. Anti-allergic effect of SFLE in a mouse model of cOVA-induced food allergy.

For examining an effect of SFLE on the experimental food allergy symptoms (i.e., diarrhea, decrease in the body temperature, anaphylaxis), BALB/c mice were divided into 5 groups (**Fig. 1B**) and immunized and challenged with cOVA and treated with SFLE as described in **Fig. 1A**. The allergy symptoms were evaluated immediately after the fifth challenge, and the severities were scored according to the guidelines shown in **Fig. 1C** (Arias et al., 2009; Jonsson et al., 2012). Severe allergic symptoms were readily observed in the sham group: average diarrhea score of 2, average anaphylaxis score of 2 as well as a significant decrease in the rectal temperature compared to that of the naïve group (**Fig. 1D, E and F**). Meanwhile, administration of SFLE to the sensitized mice during cOVA oral challenge significantly improved the food allergy symptoms. In contrast to the mice in the sham group, almost all mice in SFLE treated group showed no diarrhea and no rectal temperature decrease; the average anaphylaxis score was also decreased to 0.7 points (**Fig. 1D, E and F**).

Expectedly, treatment of the mice with Dexamethasone (Dexa) (25 mg/kg) used as the control agent also improved the allergic symptoms to a significant extent; the effect of Dexa appeared modestly better than that of SFLE (200 mg/kg) (**Fig. 1D, E and F**). Nevertheless, conforming to its celebrated side effects, the Dexa treatment resulted in a significant weight loss and a marked reduction in the numbers of cells in the spleen and the mLN (**Supp Fig. 1**). In contrast, the mice treated with SFLE at 200 mg/kg dose showed neither significant weight loss nor decrease in the cellularity of the spleen and the mLN compared with those of the naïve mice. Here, it is worth noting that the sizes of the spleen and the mLN of the sham

mice were considerably larger than those of either naïve or the SFLE-treated mice (**Supp Fig. 1**), indicating that a high level of active immune responses were ongoing in the sham mice.

3.2. Amelioration of the allergic inflammatory lesion and inhibition of mast cells and eosinophils infiltration to the gut by SFLE.

Changes in the intestinal mucosa were evaluated on the next day after the fifth cOVA oral challenge. The histological features of the small intestines of the naïve mice were typical of a normal structure, whereas the pathological changes revealing ongoing allergic inflammation were found in the sham group; i.e., thickening of the intestinal wall particularly in the area of serosa and muscularis external (**Fig. 2A Left**). These changes might represent mucosal edema in response to the allergen challenge. Of note, the SFLE treatment ameliorated the inflammatory lesion to a significant extent; thus, the thickening of the intestinal wall in the SFLE-treated mice was much less evident compared to that of the sham mice.

Mast cells are tissue-based inflammatory cells primarily located in association with blood vessels and at epithelial surfaces (Stone et al., 2010). Their activation through FcεR1, which leads to rapid release of mediators of allergic inflammation (e.g., histamine, leukotriene, serine proteases), is central to the pathogenesis of IgE-mediated food allergy. Expectedly, an average number of mast cells in the small intestines of the mice in the sham group was significantly increased compared to that in the naïve group (**Fig. 2A center** and **2B**). Meanwhile, administration of Dexamethasone or SFLE during the oral challenge significantly reduced the numbers of mast cells residing in the small intestine, indicating that the mast cell differentiation and/or infiltration to the small intestine were discouraged by the treatment.

Eosinophils also play a critical role in the allergic inflammation through the release of inflammatory mediators and cytokines (Jacobsen et al., 2012; Wills-Karp and Karp, 2004). In accordance with the allergic symptoms described above, a marked infiltration of eosinophils was found in the sham group: an average 20 cells/HPF compared to 5 cells/HPF in the naïve group. Treatment with SFLE resulted in significant reduction in the average number of eosinophils in the small intestine (12 cells/HPF) compared to the sham group (**Fig. 2A right and 2B**).

3.3. Lack of change in the serum levels of cOVA-specific antibodies in the serum after SFLE treatment.

We next investigated the effect of SFLE on the serum levels of allergen-specific immunoglobulins (Ig). Sera were collected one day after the fifth challenge. Expectedly, high levels of cOVA-specific antibodies were detected in the sensitized mice, and were totally absent in the naïve mice (**Fig. 3**). In particular, Th2-related antibodies such as IgE and IgG1 which are critical for the induction of food allergy existed at high levels in the sera of the sham mice (**Fig. 3A and B**).

Despite the strong anti-allergic activities demonstrated by the experimental results described above (Figure 1 and 2), the treatment with either SFLE or Dexa appeared to have little effect on the levels of cOVA-specific IgE and IgG1 in the serum (**Fig. 3A and B**). Thus, comparable levels of cOVA-specific IgE and IgG1 were detected in the serums of the sham mice and the SFLE-treated (or Dexa-treated) mice. We also measured the levels of cOVA-specific total IgG and IgG2a, and found that there were little differences among the mice from the sham and SFLE-treated groups (**Fig. 3C and D**). Different from its effect on cOVA-

specific IgE and IgG1, however, Dexamethasone treatment appeared to have a significant effect on the level of cOVA-specific total IgG.

3.4. Suppression by SFLE of Th2-type immune responses mounted in the course of cOVA challenge.

It is well known that allergen-specific Th2 CD4⁺ T cells play a central role in the initiation and progression of the allergic immune response (Akdis and Akdis, 2011; Maggi, 2010). For examining how SFLE had effect on cOVA-specific T cell immune responses mounted in the course of the allergen challenge, the cells isolated from the draining lymph nodes (i.e., mLN) were cultured *ex vivo* with cOVA for 3 days, and the levels and types of cytokines production by T cells were examined by measuring the concentrations of cytokines in the culture supernatants by ELISA.

As shown in **Fig. 4**, all of the Th2-related cytokines (i.e., IL-4, IL-5, IL-10) were readily detected in the culture supernatants obtained from the cells from the sham mice while they were hardly detected in the culture supernatants obtained from the cells from the naïve mice. Of note, levels of the Th2 cytokines production by the cells obtained from the SFLE-treated mice were significantly lower than those from the sham mice, indicating that the SFLE treatment had effect on the T cell immune responses mounted in the course of the cOVA challenge to compromise the *ex vivo* recall responses (**Fig. 4A, B, and C**). In contrast to the Th2 cytokines, a Th1 cytokine, i.e. IFN- γ , was detected to a measurable level even in the culture supernatants from cells from the naïve mice. The level of IFN- γ production by the cells from the sham mice appeared higher than that by the cells from the naïve mice, but the difference turned out not to be statistically significant ($p > 0.05$). The level of IFN- γ

production by the cells from the SFLE-treated mice was also not significantly different from those by the cells from either the naïve or the sham mice (**Fig. 4D**).

3.5. Direct inhibition of the mast cell function by SFLE

MCPT-1, a serine protease, is secreted to the extracellular space when mast cells are activated by an allergen to cross-link IgE-FcεRI complexes on the surface of the cells (Vaali et al., 2005). Indeed, it was found that MCPT-1 levels in the serums of the sham mice were markedly elevated compared to those of the naïve mice. Of note, the serum levels of mMCPT-1 in the SFLE-treated mice were significantly lower than those in the sham mice, indicating that SFLE inhibited the mast cell activation or degranulation (**Fig. 5A**).

In order to know whether mast cells were directly affected by SFLE, we carried out passive anaphylaxis experiment. For the experiment, mice were injected with IgE specific for dinitrophenol (DNP) followed by administration of BSA conjugated with the hapten (DNP-BSA) (**Fig. 5B**). Expectedly, when DNP-BSA was injected into the mice carrying anti-DNP-IgE, a significant decrease in the body temperature was observed within 30 min after the allergen injection, indicating that the systemic allergic reaction was induced by the allergen. Accordingly, a high level of MCPT-1 was also detected in the serums of those mice within 60 min after DNP-BSA injection (**Fig. 5C and D**). Treatment of the mice with SFLE significantly reduced the extent of the temperature change in a dose dependent manner as well as the level of MCPT-1 in the serums. Of note, the effects of SFLE at 320 mg/kg dose was almost same as those of Dexa at 10 mg/kg dose, the highest dose of Dexa not to cause a severe immunotoxicity. These results strongly suggested that SFLE had direct effect on mast cells to compromise their function.

3.6. Chemical profiling of SFLE by HPLC-DAD and HPLC-ESI-MS/MS

To have an idea about what kinds of molecules were responsible for the anti-allergic effects of SFLE, chemical profiling of the extract was carried out using HPLC-DAD. Molecules in the extract were analyzed at the range of wavelength between 190 and 300 nm. As shown in **Fig. 6**, the three-dimensional HPLC profile revealed that various polyphenol, flavonoids and triterpenoids were present in the extract. Note that relative amounts of the molecules in the extract were determined at 205 nm as the extract contained high levels of triterpenoids. The identities of a few molecules in the extract were also confirmed with HPLC-ESI-MS. The names and the contents of nine compounds confirmed to be present in the extract were tabulated in **Table 1**.

DISCUSSIONS

S. formosum (Wall.) Masam is an evergreen tree naturally growing in the Southeast Asian countries. The hot water extract of its leaves has been traditionally used to treat various atopy-like symptoms (e.g., skin dermatitis, diarrhea) in Vietnam (Do HB, 2004). Yet, up until now scientific evidences on its anti-allergic effects and explanations for possible mechanisms underlying those effects are not available. The main aim of the present study was to explore anti-allergic effects of the ethanol extract of *S. formosum* leaves and their underlying mechanism. We used a mouse model of cOVA-induced food allergy for those purposes. According to the literature, it was recommended to consume daily about 1 L of hot-water extract (tea) prepared with 100 g dried *S. formosum* leaves. Given that approximately 5 g of dried extract can be obtained from 100 g dried *S. formosum* leaves after hot-water extraction, the traditional dose of *S. formosum* leaves extract can be about 60 – 100 mg/kg. We chose the doses (i.e., 80 and 200 mg/kg) used in the current study based on the estimated traditional dose.

The results that the treatment with SFLE resulted in a significant improvement in the allergic symptoms (i.e., diarrhea, anaphylaxis, drop in the body temperature) induced by orally administered cOVA (**Fig.1**) appeared to confirm the anti-allergic effects of the plant extract indigenously known among Vietnamese for a long time. The results that SFLE also ameliorated the allergic inflammatory lesions in the gut (**Fig. 2**) reassured those effects. In addition, that no significant changes in the body weight and in the cellularities of the lymph nodes and the spleen were observed during the SFLE treatment (**Supp. Fig. 1**) indicates that SFLE cause no unwanted side effects.

The presence of high levels of allergen-specific IgE and IgG1 in the blood is a hallmark of the type 1 (Th2-mediated) food allergy (Hamilton, 2010; Lavine, 2012). It is also conceived that the severities of allergic symptoms of patients are correlated with the levels of cognate allergen-specific IgE in the blood. The results that the treatment with SFLE resulted in little changes in the average levels of either cOVA-specific IgE or IgG1 as well as cOVA-specific total IgG and IgG2a (**Fig. 3**), however, indicate that the anti-allergic activity of SFLE is not attributed to the modulation of antibody production by cOVA-specific B cells and plasma cells.

It is well established that escalation in the production of Th2 cytokines (e.g., IL-4, IL-5, IL-10) by CD4⁺ T cells is closely associated not only with the onset of allergic symptoms but also with the degree of gastrointestinal inflammation in patients with food allergy (Hawrylowicz and O'Garra, 2005; Nakajima-Adachi et al., 2017). While the results presented in **Fig. 4** clearly indicate that SFLE had effect on the T cell immune reaction induced by cOVA, it is yet to be understood how it meddled the immune response. Here, we may speculate two different scenarios. First, it is possible that SFLE may scuew the immune response in favor of Th1 response. Alternatively, it is also possible that the extract may attenuate the T cell activation and proliferation to limit the generation of cOVA-specific effector and/or memory T cells. We assume that the latter is more likely as the treatment with SFSE resulted in no significant change in the levels of IFN- γ production by the cells from different groups of mice (**Fig. 4D**).

The results that the levels of cOVA-specific IgE in the blood were largely unchanged after SFLE treatment while SFLE strongly suppressed cOVA-specific CD4⁺ Th2 responses are

somewhat puzzling. We reason that the development of cOVA-specific IgE producing effector (and memory) B cells and plasma cells are mostly completed in the course of the immunization period and thus cOVA-specific effector CD4⁺ T cells may not have much effect on the production of IgE during the challenge period. It is still likely, however, that cOVA-specific effector T cells may play a role in the maturation and differentiation of mast cell and eosinophil via production of Th2 cytokines such as IL-5, exacerbating the allergic symptoms and the allergic inflammatory lesion.

The passive anaphylaxis experiment indicated that SFLE also had the mast cell stabilizing activity to suppress the release of the granules with allergic effector molecules from mast cells (**Fig. 5**). Taken together, it appears that the anti-allergic effects of SFLE are exerted by compromising multiple immune mechanisms involved in initiation and progression of allergic inflammatory symptoms including antigen-specific T cell immune responses and activation of mast cell function. The analytical study using three-dimensional HPLC and HPLC-MS revealed that high levels of several different triterpenoids were present in SFLE (**Fig. 6 and Table 1**). In light of the anti-allergic effect of triterpenoids, it is of note that the extract contains significant levels of oleanolic acid, ursolic acid and maslinic acid whose anti-allergic activities have been reported by the studies by others (Cordova et al., 2014; Kim et al., 2013; Kishikawa et al., 2017). In particular, a strong anti-allergic effect of oleanolic acid has been demonstrated by a recent study by Cordova *et al.* using a mouse model of allergic conjunctivitis; the study has shown that injection of oleanolic acid (i.p.) significantly relieves the allergic symptoms caused by Ragweed pollen. Given that, it seems likely that the triterpenoids present in the extract made a great contribution to the anti-allergic effects of SFLE shown in the current study.

Although there are a few allergic medications, both popular and effective, in the market, demands for more efficacious and safer allergy medications continue to increase. Our study implies that SFLE or a specific solvent fraction of the extract can be a prospective therapeutics for various allergic disorders and symptoms. Further studies are warranted to more carefully dissect the immunoregulatory mechanisms of SFLE and molecule(s) responsible for the anti-allergic effects of orally administered SFLE.

CONCLUSION

In the current study, for the first time we present scientific evidences for the anti-allergic effects of the leaf extract of *S. formosum* (Wall.) Masam which has been traditionally used as a folk medicine in Vietnam. The results showed that the leaves extract improved the food allergy symptoms and the inflammatory lesion in the gut. The results also showed that the anti-allergic effect of the extract resulted at least in part from inhibition of the allergen-specific T cell immune responses and the mast cell function. The chemical profiling study showed that triterpenoids with various immuno-regulatory activities are contained in the extract in significant levels, suggesting that those triterpenoids should be closely related with the anti-allergic activity of the extract.

Conflict of Interest

Authors declare no conflict of interest associated with this study.

Acknowledgements

We thank Li Linying and Sojin Choi in College of Pharmacy, Chungnam National University for technical supports and discussions. This work was supported by CNU Research Grant (2016-1531-01), a grant for Institute of Drug Research and Development in College of Pharmacy (CNU) (National Research Foundation of Korea), grants from National Research Foundation of Korea (NRF-2015R1D1A1A02062348 and NRF-2014K1A4A3069114) and a grant from Korea Food Research Institute (E0121304-05). Nguyen Thi Minh Nguyet and Maria Lomunova are supported by BK21Plus program by National Research Foundation of Korea.

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Figure Legends

Figure 1. Improvement of the food allergy symptoms by SFLE.

(A) The experimental scheme of a mouth model of cOVA-induced food allergy is shown. The treatment schedule is also denoted. (B) Description of the control and experimental groups (6 mice per group) is shown. Thirty, sixty and ninety minutes after the 5th challenge, severities of diarrhea (D) and anaphylaxis (E) and changes in the rectal temperature (F) were examined and scored according to the guidelines shown in the tables (C). Asterisk (*) denotes $p < 0.05$ by nonparametric one-way ANOVA (Kruskal-Wallis Test).

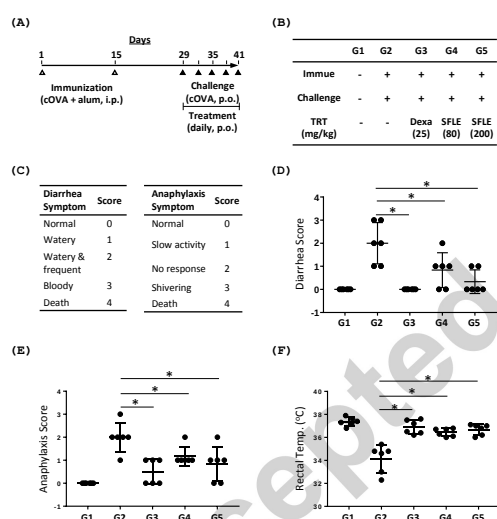


Figure 1

Figure 2. Amelioration of the allergic inflammatory lesion in the gut and obstruction of the infiltration of mast cells and eosinophils to the tissue by SFLE.

The small intestines (jejunum) were taken from the respective group of mice one day after the 5th cOVA challenge and fixed with formalin and sectioned with microtome. The thin tissue sections were stained with hematoxylin plus eosin (H&E) and observed under the microscope for the inflammatory lesions (**A, left**) and for eosinophils (stained dark red and blue) (**A, center**). They were also stained with the toluidine solution and observed for mast cells (stained blue purple) (**A right**). The numbers of mast cells (**B, left**) and eosinophils (**B, right**) in high power fields were counted and the average numbers were plotted. Cell numbers in at least five different HPFs were counted and the means and SDs were calculated. Asterisk (*) denotes $p < 0.05$ by Student's t-test.

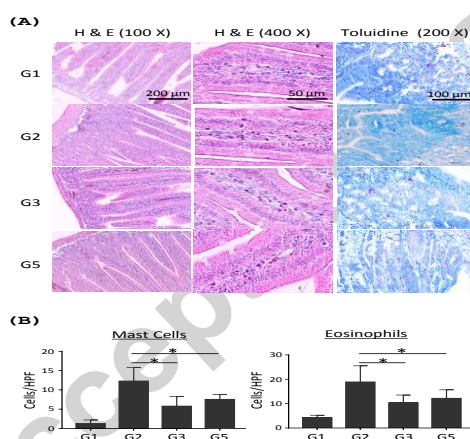


Figure 2

Figure 3. Lack of significant changes in the levels of cOVA-specific antibodies in the serum following the SFLE treatment.

The serums were prepared from the blood drawn from the respective group of mice 15 hrs after the 5th challenge. Levels of cOVA- specific IgE (**A**), cOVA-specific IgG1 (**B**), cOVA-specific total IgG (**C**), and cOVA-specific IgG2a (**D**) in the serums were measured with ELISA. # denotes the dilution factor of the serums. The data represent means $\pm$ SD. Asterisk (*) denotes $p < 0.05$ by Student's t-test.

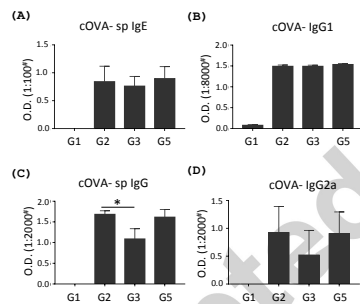


Figure 3

Figure 4. Suppression of Th2-type immune responses by SFLE.

The cells prepared from the mLNs of the respective group of mice 15 hrs after the 5th challenge were cultured for 3 days in the presence (gray bar) or absence (black bar) of cOVA. The concentrations of IL-4 (**A**), IL-5 (**B**), IL-10 (**C**) and IFN- γ (**D**) in the culture supernatants were measured with ELISA. The data represent means $\pm$ SD. Asterisk (*) denotes $p < 0.05$ by Student's t-test.

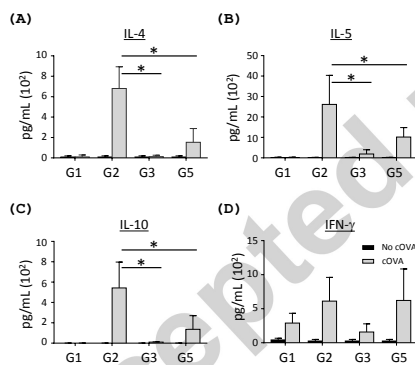


Figure 4

Figure 5. Direct inhibition by SFLE of the mast cell function in vivo.

The concentration of MCPT-1 in the serums prepared as described in Fig. 3 were measured with ELISA (**A**). The passive anaphylaxis experiment was carried out as described in (**B**). The changes in the body temperature caused by the systemic allergic reaction were examined 30 min after injection of DNP-BSA (**C**). One hr after DNP-BSA injection, the blood were drawn from the mice to prepare the serums. The levels of MCPT-1 in the serums were then measured with ELISA (**D**). The experiments were carried out with 3 mice per group. Asterisk (*) denotes $p < 0.05$ by Student's t-test.

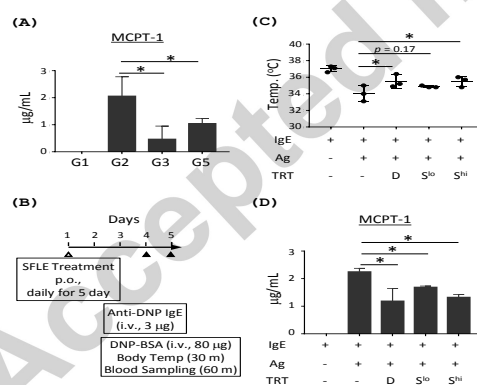


Figure 5

Figure 6. Chemical profiling of SFLE by HPLC-DAD

Three-dimensional high performance liquid chromatography (HPLC) profile of SFLE

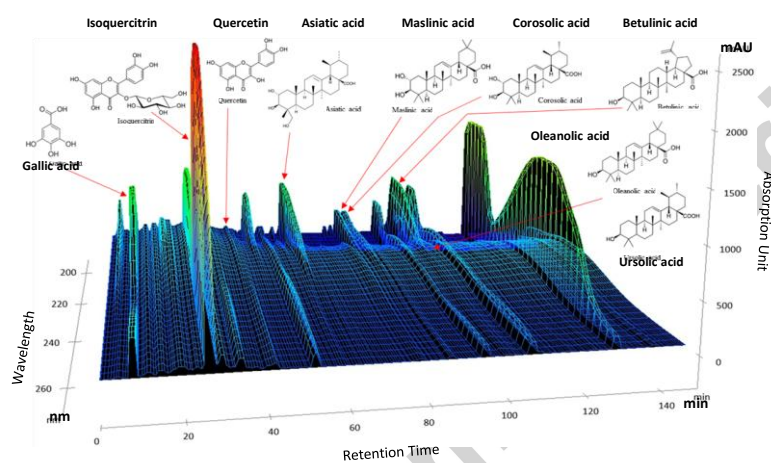


Figure 6

Table 1. Ultraviolet and mass spectral data of representative polyphenols, flavonoids and triterpenoids in SFLE and their contents^{\$}.

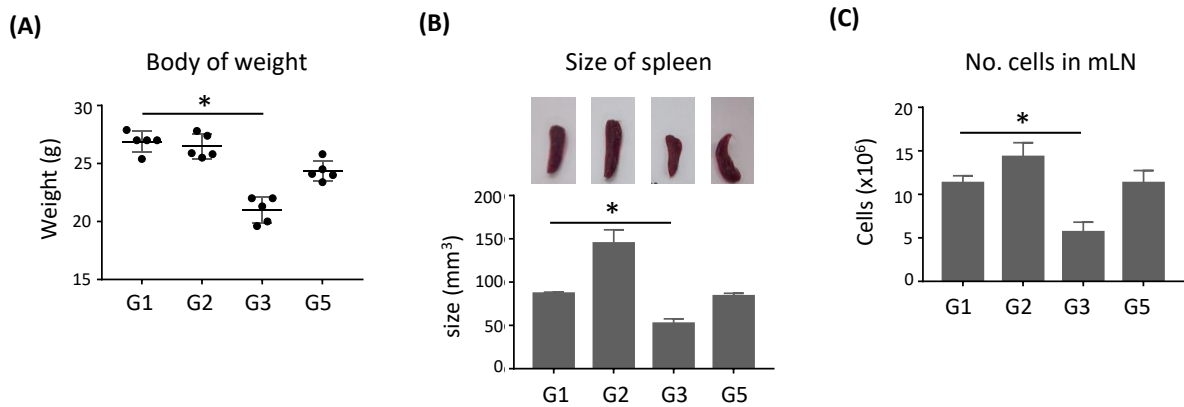
Peak No.	Compound	Retention Time (min)	$\lambda_{\max}$ (nm)	m/z [M-H] ⁻	Content [*] (mg/g)
1	gallic acid	8.18	214, 270	169	0.35
2	isoquercitrin	26.70	248, 366	463	1.48
3	quercetin	40.73	213, 253, 370	301	0.06
4	asiatic acid ^{**}	61.18	205	487	n.d. [#]
5	maslinic acid	83.62	216	471	1.76
6	corosolic acid	87.71	213	471	6.76
7	betulinic acid	102.60	205, 224	455	10.47
8	oleanolic acid	104.74	223	455	0.46
9	ursolic acid	105.10	222	455	0.12

^{\$} LC-ESI-MS spectra of the compounds in this table are shown in **Supp. Fig 2**.

^{*} Content in the dried SFLE slurry.

^{**} Asiatic acid in the sample was identified by comparing UV spectra and mass fragmentation data with those reported in the literature (Nong, 2009; Yang et al., 2011). This peak was eluted at 61.18 min and yielded an [M-H]⁻ at m/z 487 and [M+H]⁺ at m/z 489.

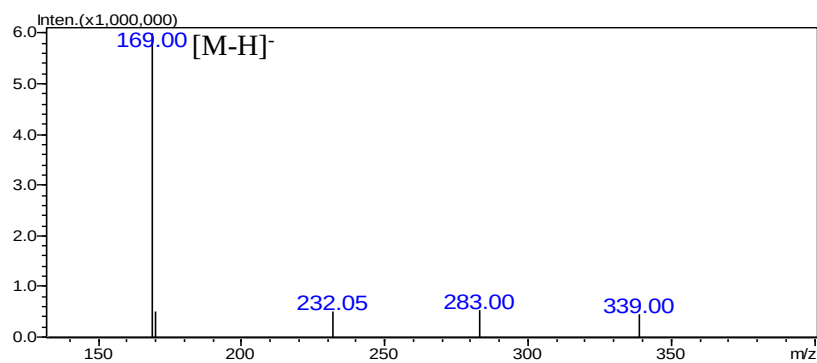
[#] n.d. stands for 'not determined'.



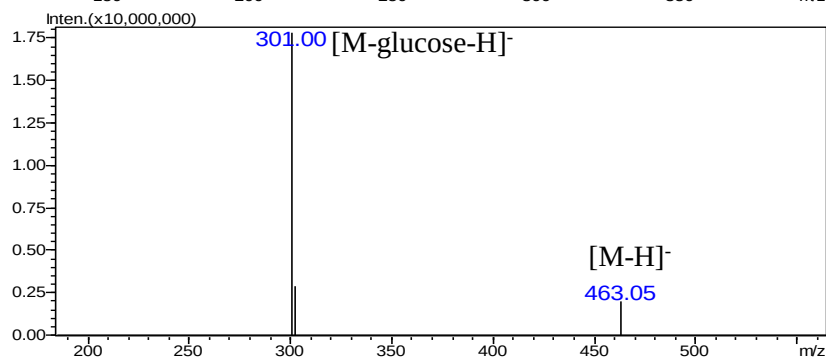
Supp. Figure 1. Lack of SFLE-mediated immunotoxicity; its effects on the body weight and the cellularities of the secondary lymphoid organs.

(A) Body weights of the mice were measured 15 hrs after the 5th cOVA challenge (A). Then, the mice were killed to take the spleens and the mLNs. The volume of the spleen was calculated after measuring the length and the widths of the tissue (B). The pictures show the representative sizes of the spleens of mice in the respective groups (B). The numbers of cells in the single cell suspensions of the mLNs were counted with a hemacytometer (C). The data represent means $\pm$ SD. Asterisk (*) denotes $p < 0.05$ by Student's t-test.

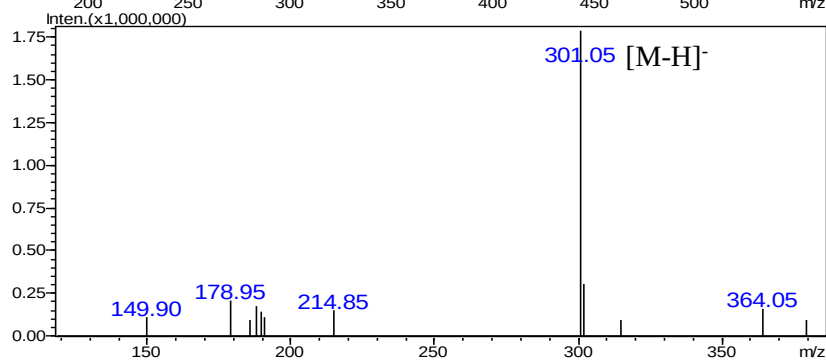
(1)



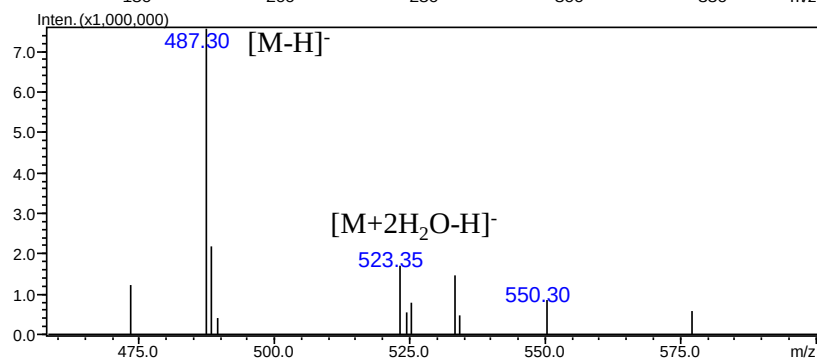
(2)



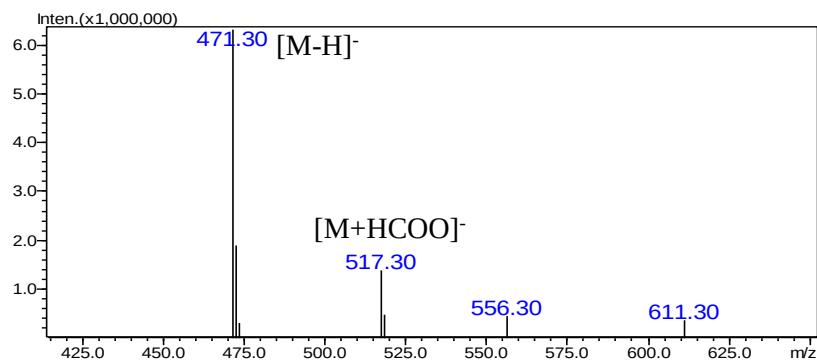
(3)



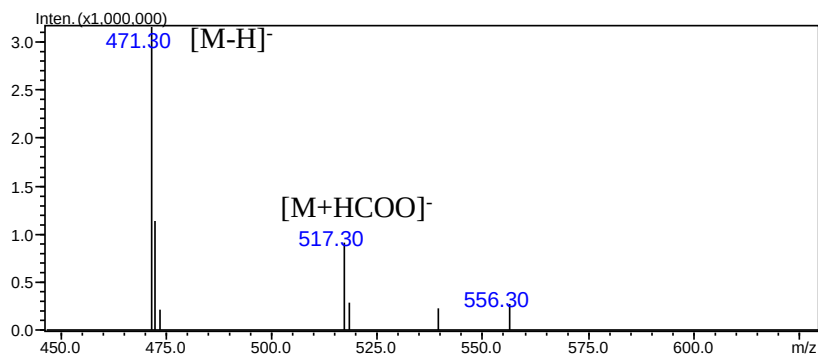
(4)



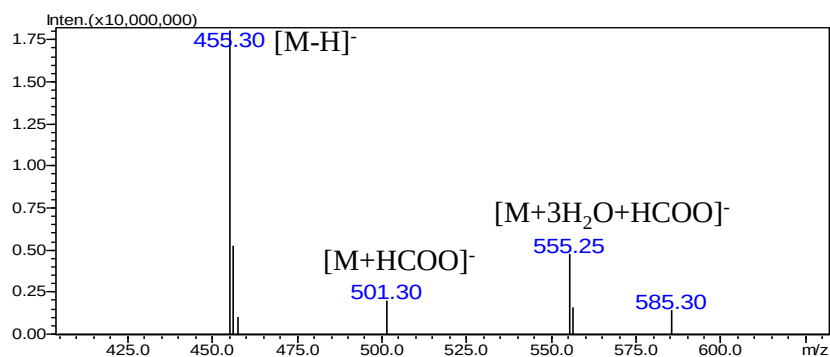
(5)



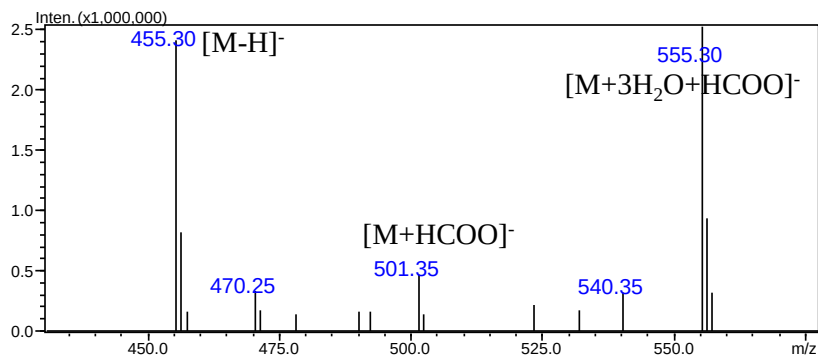
(6)



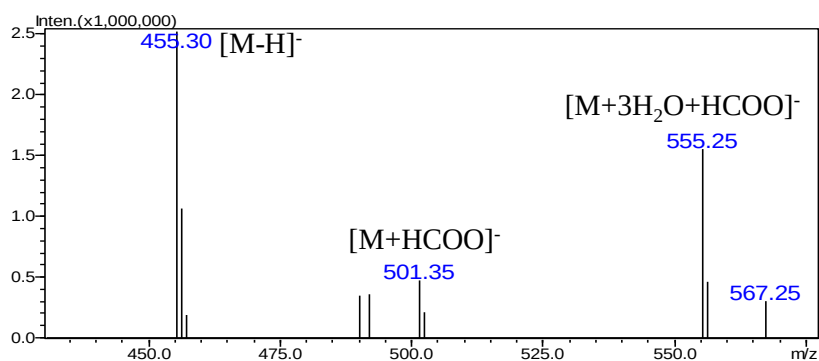
(7)



(8)



(9)



Supp. Figure 2. LC-ESI-MS spectrum of nine compounds in SFLE at negative mode.

(1) Gallic acid, (2) Isoquercitrin, (3) Quercetin, (4) Asiatic acid, (5) Maslinic acid, (6) Corosolic acid, (7) Betulinic acid, (8) Oleanolic acid, (9) Ursolic acid.