

***Salix tetrasperma* Roxb. extract alleviates neuropathic pain in rats via modulation of NF- κ B/TNF- α /NOX/iNOS pathway**

Materials and methods

HPLC-MS analysis

A ThermoFinnigan LCQ-Duo ion trap mass spectrometer (ThermoElectron Corporation, Waltham, Ma, USA) with an Electrospray ionization (ESI) source (ThermoQuest Corporation, Austin, Tx, USA) was used. A Discovery HS F5 column (15 cm x 4.6 mm ID, 5 μ m particles, Sigma-Aldrich Co Steinheim, Germany) was utilized with the ThermoFinnigan HPLC system. The mobile phase was water and acetonitrile (ACN) (Sigma-Aldrich GmbH, Steinheim, Germany) (0.1 % formic acid each). At 0 min, ACN was 5%, then increased to 30% over 60 min, and finally to 90% within the last 30 min. The flow rate was kept at 1 mL/min with a 1:1 split before the ESI source. The MS operated in the negative mode as previously reported (Ghareeb et al. 2018).

In vitro antioxidant activities

The total antioxidant capacity (TAC) assay was investigated using the commercially available TAC ELISA kit (MBS726896, my BioSource, Inc., San Diego, CA, USA) following the manufacturer's instructions as Cyclooxygenase (COX) and lipoxygenase (LOX) inhibition assays

The potential inhibition of ovine COX-1 and COX-2 by the extract was evaluated with an enzyme immuno assay (EIA) kit (Cayman Chemical, AnnArbor, MI, USA) as per manufacturer's instructions and previous reports (Abdelall, Lamie et al. 2016). To study the inhibition of lipoxygenase we used the screening assay kit (Cayman Chemical, AnnArbor, MI, USA) as per manufacturer's instructions and previous reports (Abdelall, Lamie et al. 2016).

Carrageenan-induced hind-paw edema

Freshly prepared carrageenan solution (1% in 0.9% NaCl, 0.1 mL) was injected into the sub plantar tissues of the right hind paw of rats to induce the edema. One hour earlier, the extract (200, 400 and 600 mg/kg, p.o.), diclofenac (10 mg/kg), or vehicle (10 mL/kg) were orally applied to the rats. A caliper ruler was used to measure the paw thickness (mm) in the dorsal-plantar axis before and after carrageenan injection as previously described (Sobeh, Mahmoud et al. 2018).

Recruitment of leukocyte to peritoneal cavity in mice

Swiss albino mice (n=5-8/ group) were treated with the extract (200, 400 and 600 mg/kg, p.o.) or the vehicle (1 mL/100 g, p.o.) 30 min before either 0.1 mL carrageenan solution (500 μ g/mice) or 0.1 mL sterile saline were injected (i.p.). The experiments were carried out as previously described (Sobeh, Mahmoud et al. 2018).

Acetic acid-induced vascular permeability

The acetic acid-induced vascular permeability was performed as previously described (Lee, Choo et al. 2009). Briefly, mice treated with the extract (200 and 400 and 600 mg/kg, p.o.), diclofenac (20 mg/kg) or vehicle. 1 h. later 0.2 mL Evans blue (0.25% solution in normal saline) was injected in the tail vein. After 30 min, acetic acid (0.6% in normal saline, 1 mL/100g) was injected in the peritoneal cavity of mice. Another group of mice was injected with normal saline only and served as normal control. After another 30 min, the mice were sacrificed by cervical dislocation. The abdominal cavities were washed with 3 mL saline and the washings were then centrifuged at 3000 rpm for 10 min. The vascular permeability is proportional to Evans blue dye content of the supernatant that was detected at 610 nm using a plate reader (BioTeK, Vt, USA).

Anti-nociceptive activity

Acetic acid-induced abdominal writhing and the hot plate test were carried out as described in our previous work (Sobeh, Mahmoud et al. 2018).

Induction of fever in mice using Brewer's yeast

To study a potential antipyretic effect in mice we employed the Brewer's yeast model of pyrexia as previously described (Sengar, Joshi et al. 2015; Liu, Su et al. 2017) with some modifications. After recording the rectal temperature of mice with a lubricated digital thermometer, a 30% Brewer's yeast suspension in normal saline was injected subcutaneously (1 mL/100 g). After 18 h, rectal temperatures were measured (T_0) again. Only animals with at least 0.5°C higher temperature were included in the test. These mice were treated with 200 and 400 mg extract/kg, paracetamol (150 mg/kg) or vehicle. Again, the rectal temperature was measured at 30 min, 1, 2, 3 and 24 h after treatment.

Results

The secondary metabolites were identified based on their molecular weights, fragmentation pattern, retention times, available literature and authentic compounds (**Table S1**). A photo of the flowers is shown in **Figure S1**. The fragmentation pattern of some selected compounds in the flower extract is documented below (**Figures S2–S18**).

Table S1. Secondary metabolites from *Salix tetrasperma* flower extract

No.	Tentatively identified compounds	Previous identification	Reference
1	Protocatechuic acid- <i>O</i> -hexoside*	<i>S. tetrasperma</i>	El-Wakil et al., 2015
2	Chlorogenic acid**	<i>S. alba</i> <i>S. amplexicaulis</i> <i>S. babylonica</i> <i>S. eleagnos</i> <i>S. fragilis</i> <i>S. purpurea</i> <i>S. triandra</i>	Gligorić et al., 2019
3	Caffeoylmalic acid***	-	Pobłocka-Olech et al., 2010
4	Salicin malate***		
5	Salicin*	<i>S. tetrasperma</i> <i>S. alba</i> <i>S. Americana</i> <i>S. cv. Aquatica</i> <i>S. arbusculoides</i> <i>S. aurita</i> <i>S. babylonica</i> <i>S. callicarpa</i> <i>S. alba</i> <i>S. amplexicaulis</i> <i>S. babylonica</i> <i>S. eleagnos</i> <i>S. fragilis</i> <i>S. purpurea</i> <i>S. triandra</i>	El-Shazly et al., 2012 Boeckler et al. 2011 Gligorić et al., 2019
6	Coumaroylquinic acid*	<i>S. tetrasperma</i>	El-Wakil et al., 2015
7	Coumaroyl malic acid	-	Csernatoni et al., 2014
8	Phloretic acid***	-	Tanagornmeatar et al., 2014
9	Sinapic acid 3- <i>O</i> - glucoside***	-	Nićiforović and Abramović, 2014
10	Quercetin dihexoside	<i>S. myrsinifolia</i> Salisb.	Nybakken and Julkunen-Tiitto, 2013
11	2'- <i>O</i> -Acetyl-salicin	<i>S. lasiandra</i> <i>S. glandulosa</i>	Boeckler et al. 2011; Kim et al., 2015
12	Coumaroylgalloyl glucose***		
13	Quercetin pentosyl-rutinoside***		Barros et al., 2012

14	Salicortin	<i>S. alba</i> <i>S. Americana</i> <i>S. cv. Aquatica</i> <i>S. arbusculoides</i> <i>S. aurita</i> <i>S. babylonica</i> <i>S. callicarp</i>	Boeckler et al. 2011
15	Quercetin pentosyl-hexoside***		Bakour et al., 2019
16	Isorhamentin pentosyl-rutinoside***		
17	Rutin	<i>S. chaenomeloides</i> <i>S. alba</i> <i>S. amplexicaulis</i> <i>S. babylonica</i> <i>S. eleagnos</i> <i>S. fragilis</i> <i>S. purpurea</i> <i>S. triandra</i>	Mizuno et al. 1991 Gligorić et al., 2019
18	Quercetin 3-O-glucoside	<i>S. chaenomeloides</i> <i>S. raddeana</i>	Mizuno et al. 1991 Xu et al., 2007
19	Isorhamentin pentosyl hexoside***	-	Wang et al., 2012
20	Kaempferol 3-O-glucoside	<i>S. raddeana</i>	Xu et al., 2007
21	Kaempferol 3-O-galactoside***	-	Löffler et al., 1997
22	Isorhamentin 3-O-glucoside	<i>S. chaenomeloides</i>	Mizuno et al. 1991
23	Trichocarposide	<i>S. martiana</i>	Boeckler et al. 2011
24	Kaempferide 3-O-hexoside***	-	
25	Isorhamentin 3-O-rhamnoside***	-	Sobral et al., 2017
26	Dihydrocinnamoyl salicin***	-	
27	Cinnamoyl salicin	-	Kumari et al., 2016 Keefover-Ring et al., 2014
28	Tremulacin*	<i>S. tetrasperma</i> <i>S. petiolaris</i> <i>S. triandra</i>	El-Shazly et al., 2012 Boeckler et al. 2011
29	Apigenin coumaroyl-glucoside (Terniflorin)	<i>S. alba</i>	Ahmad et al., 1984
30	Apigenin	<i>S. lindeyana</i>	Thapliyal and Bahuguna et al., 1993
31	Kaempferide***		Przybyłek and Karpiński et al., 2019
32	Coumaroyl dihydrobenzoyl salicin***		
33	Coumaroyl dihydrobenzoyl salicin isomer***		
34	Hydroxy-octadecadienoic acid***		
35	Hydroxy-octadecatrienoic acid***		Chechetkin et al., 2019
36	Oleanolic acid derivative***		
37	Sitosterol glucoside linoleic acid***		
38	Acutifoliside glucoside linoleic acid***		

*Previously isolated from *S. tetrasperma* leaves

**Compound confirmed using a reference compound

***New compounds in genus *Salix* (Tentatively identified)



Figure S1. A photo of *S. tetrapserma* flowers

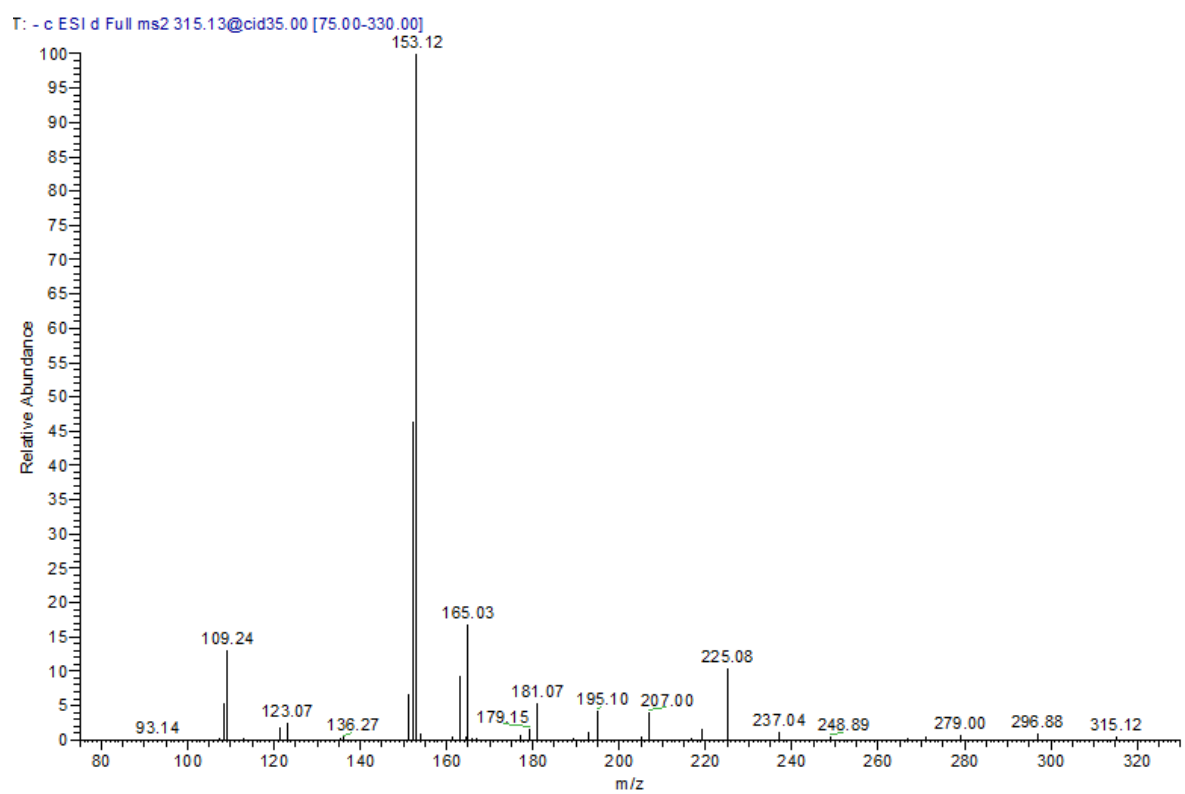


Figure S2. MS/MS profile of protocathechuic acid 3-*O*-hexoside

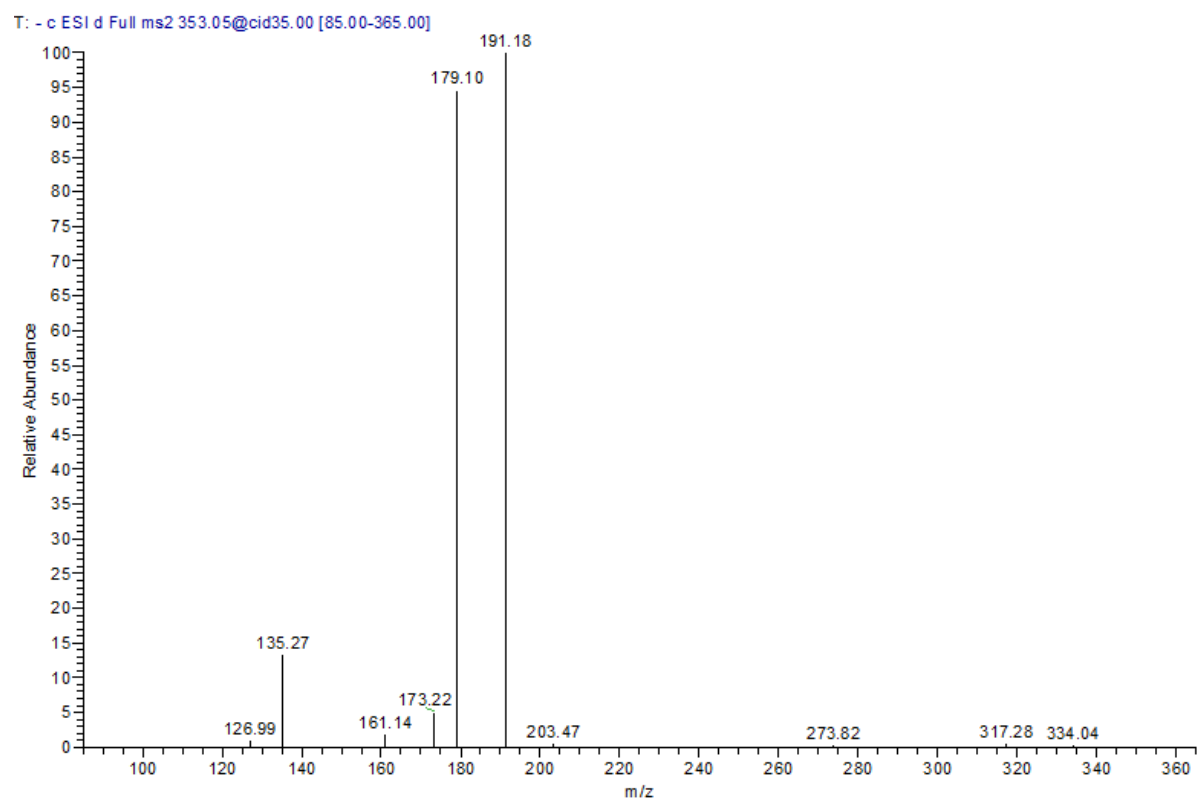


Figure S3. MS/MS profile of chlorogenic acid

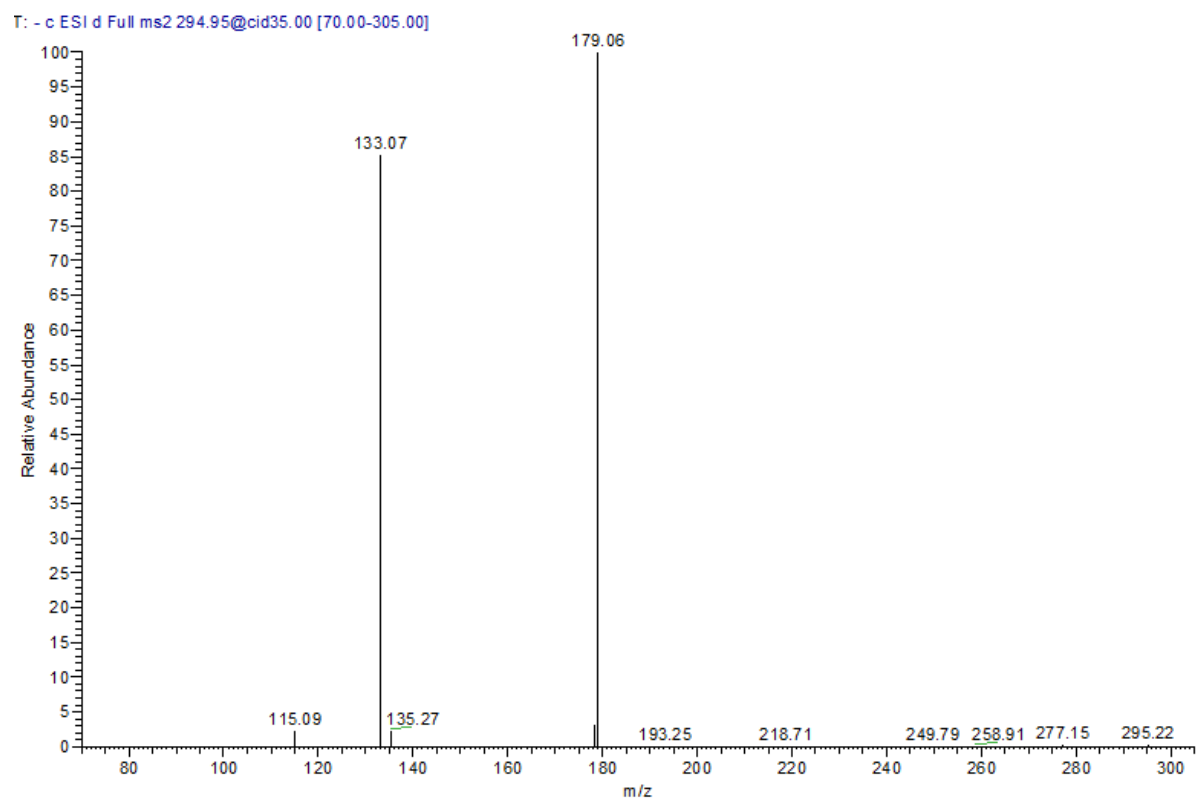


Figure S4. MS/MS profile of caffeoylmalic acid

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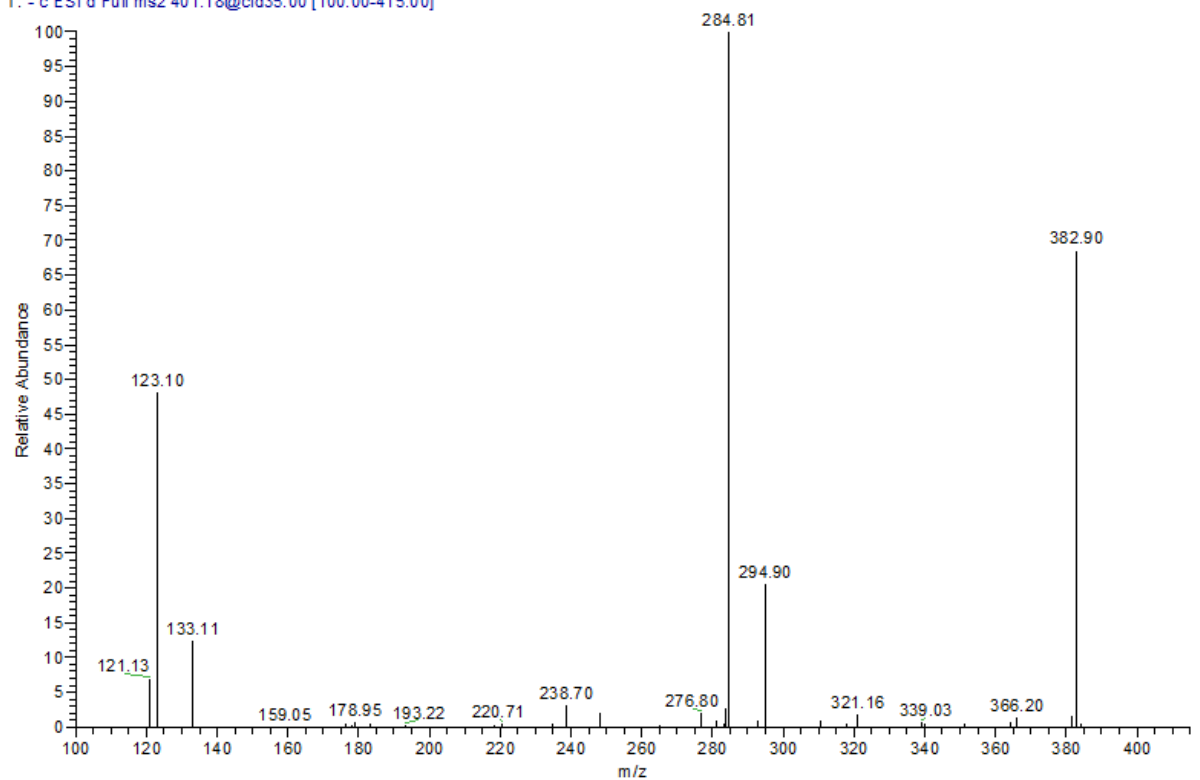


Figure S5. MS/MS profile of salicin malate

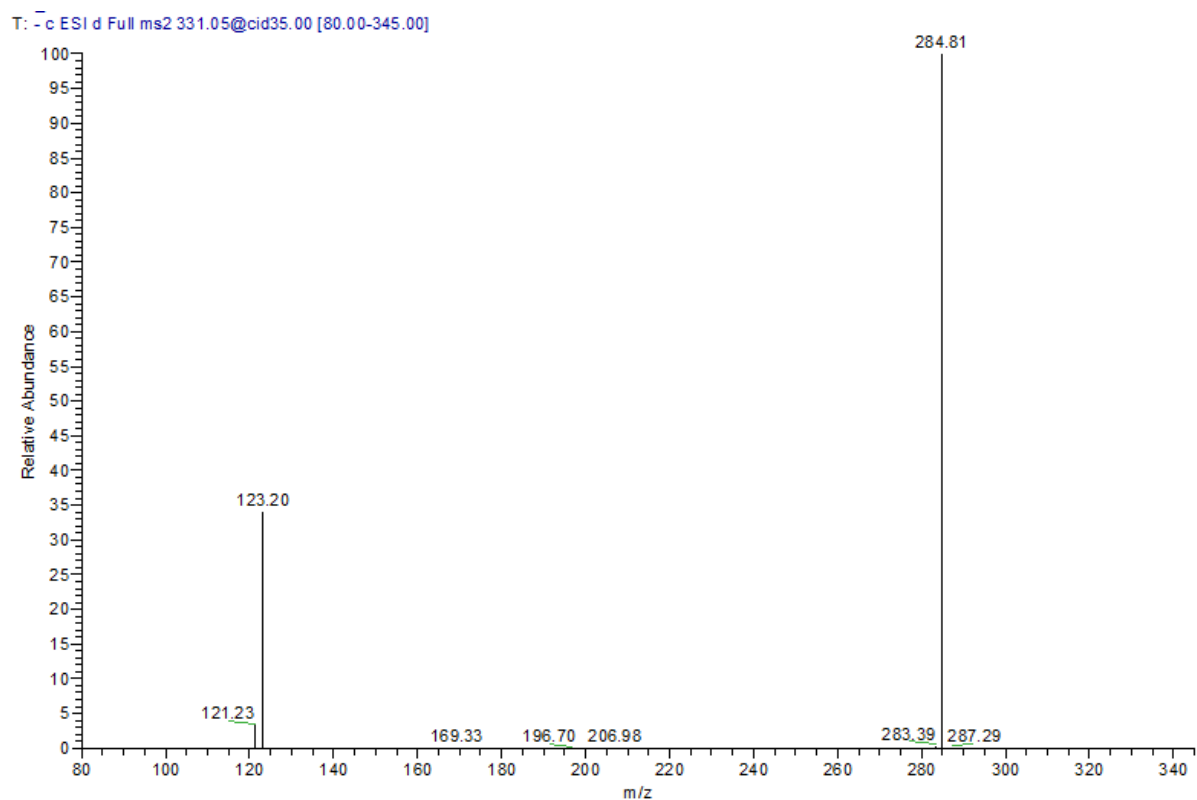


Figure S6. MS/MS profile of salicin [M-H + 46, formic acid adduct]

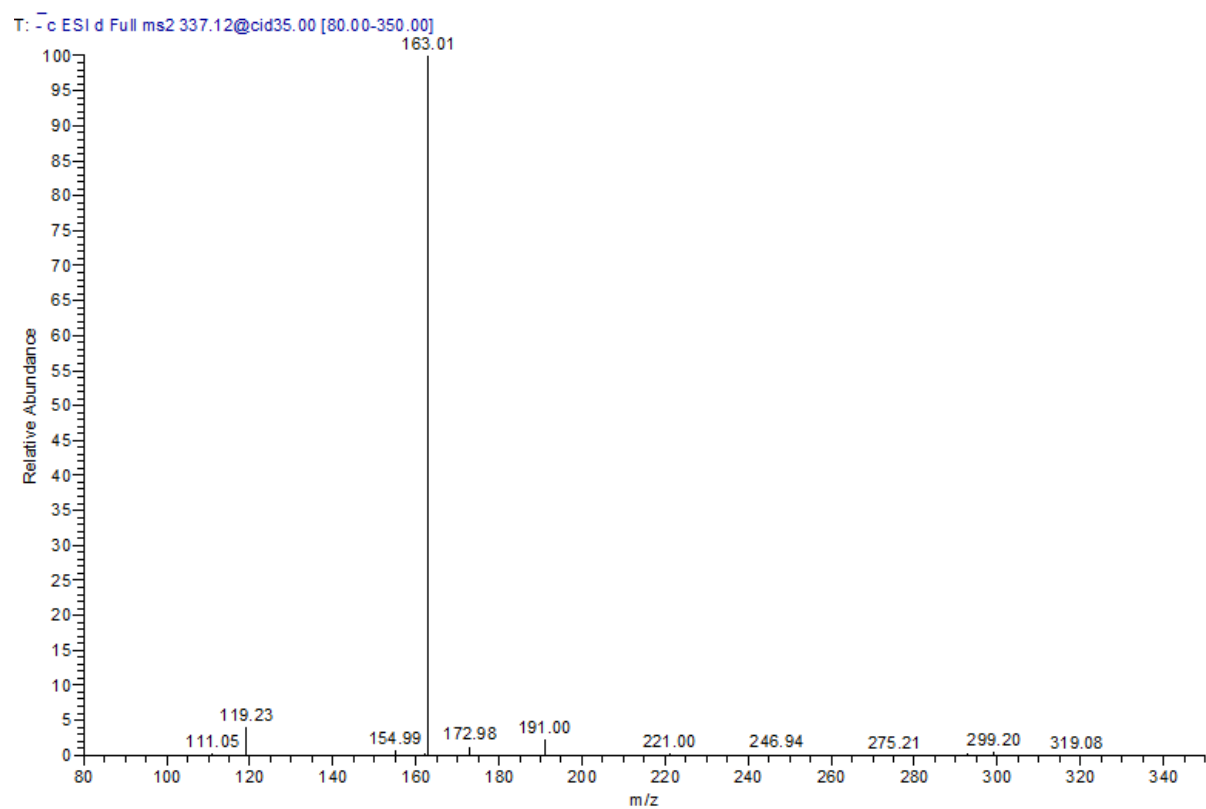


Figure S7. MS/MS profile of coumaroylquinic acid

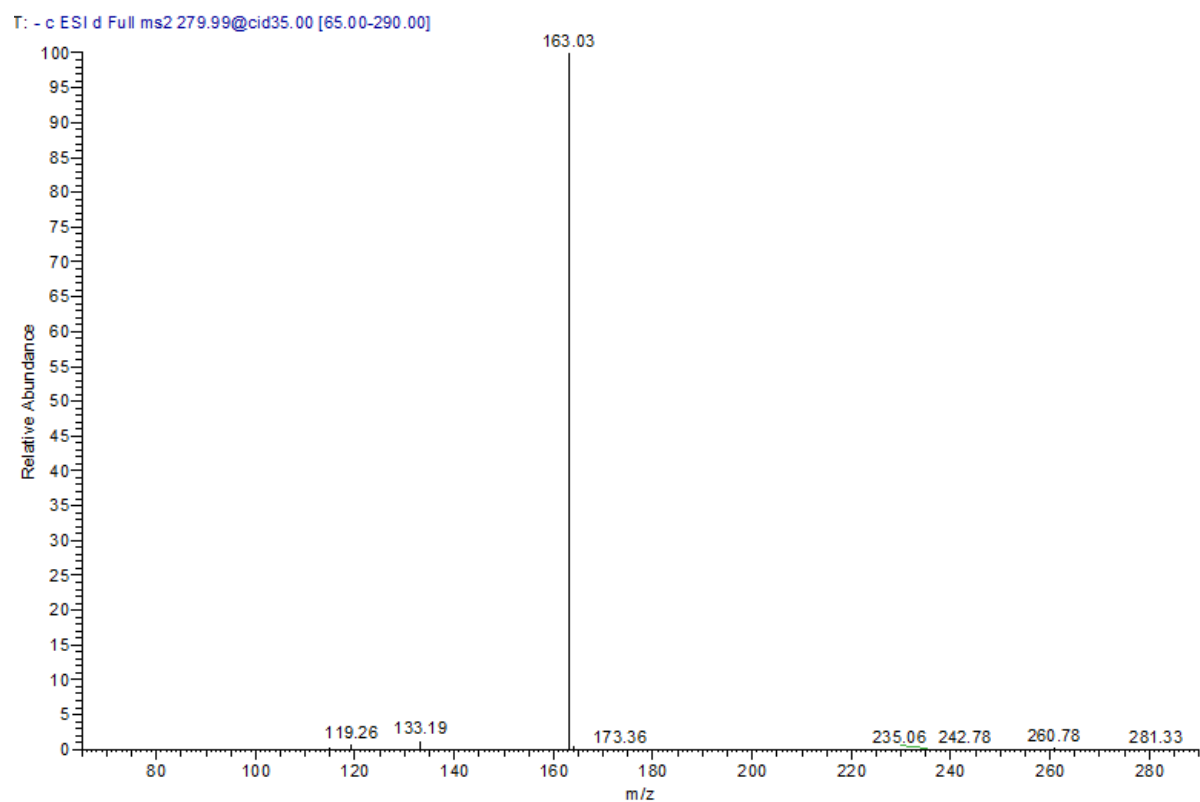


Figure S8. MS/MS profile of coumaroylmalic acid

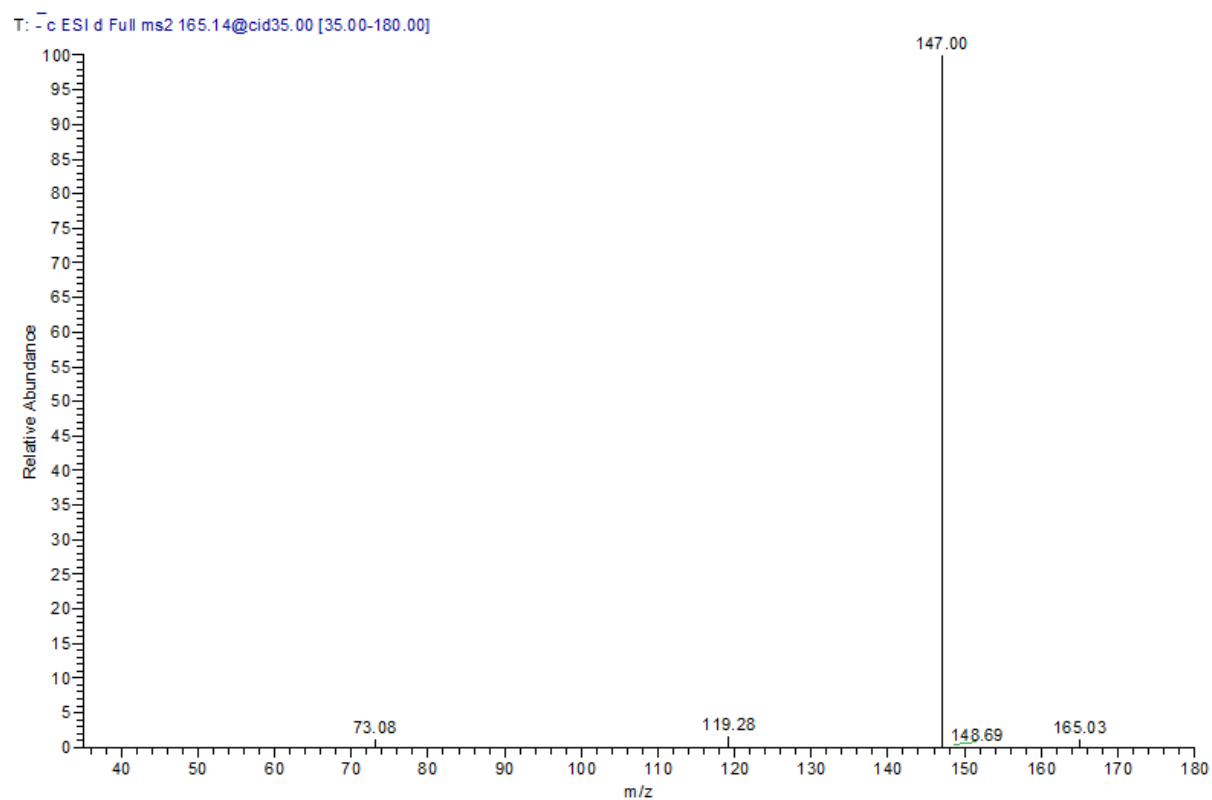


Figure S9. MS/MS profile of phloretic acid

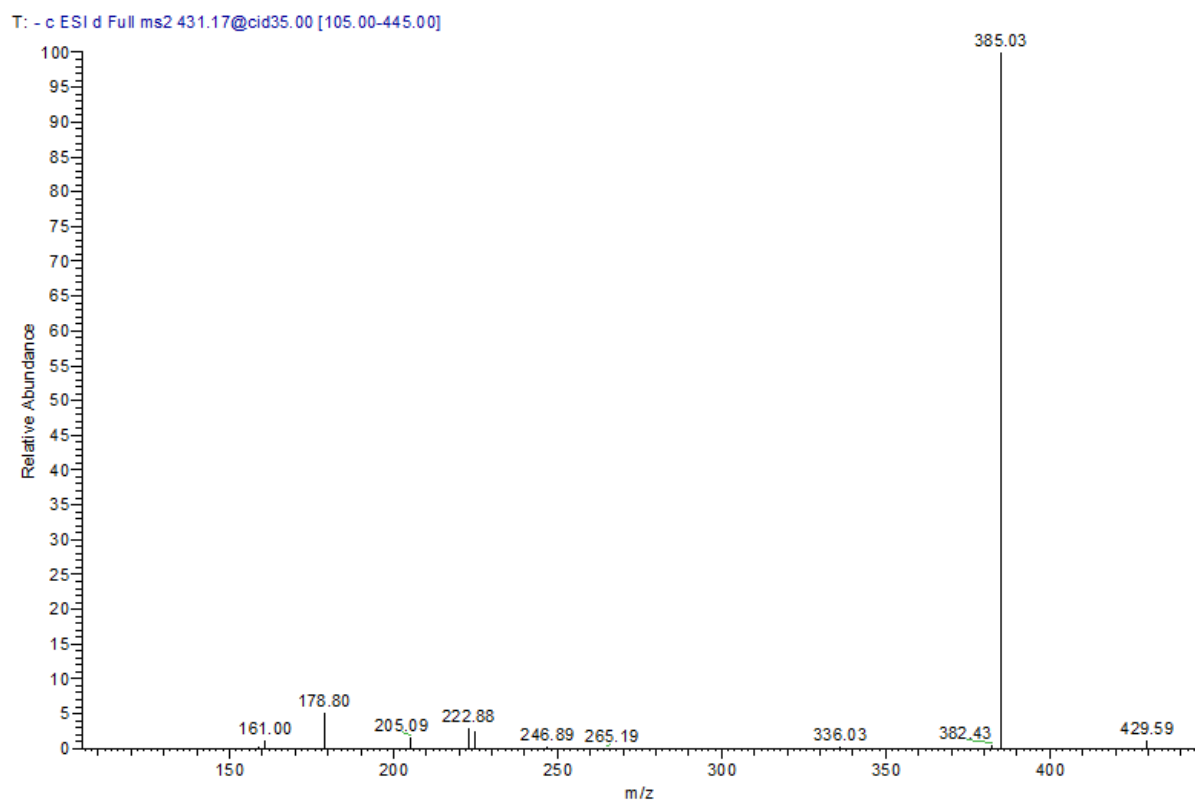


Figure S10. MS/MS profile of sinapic acid 3-O- hexoside [M-H + 46, formic acid adduct]

T: - c ESI d Full ms2 373.01@cid35.00 [90.00-385.00]

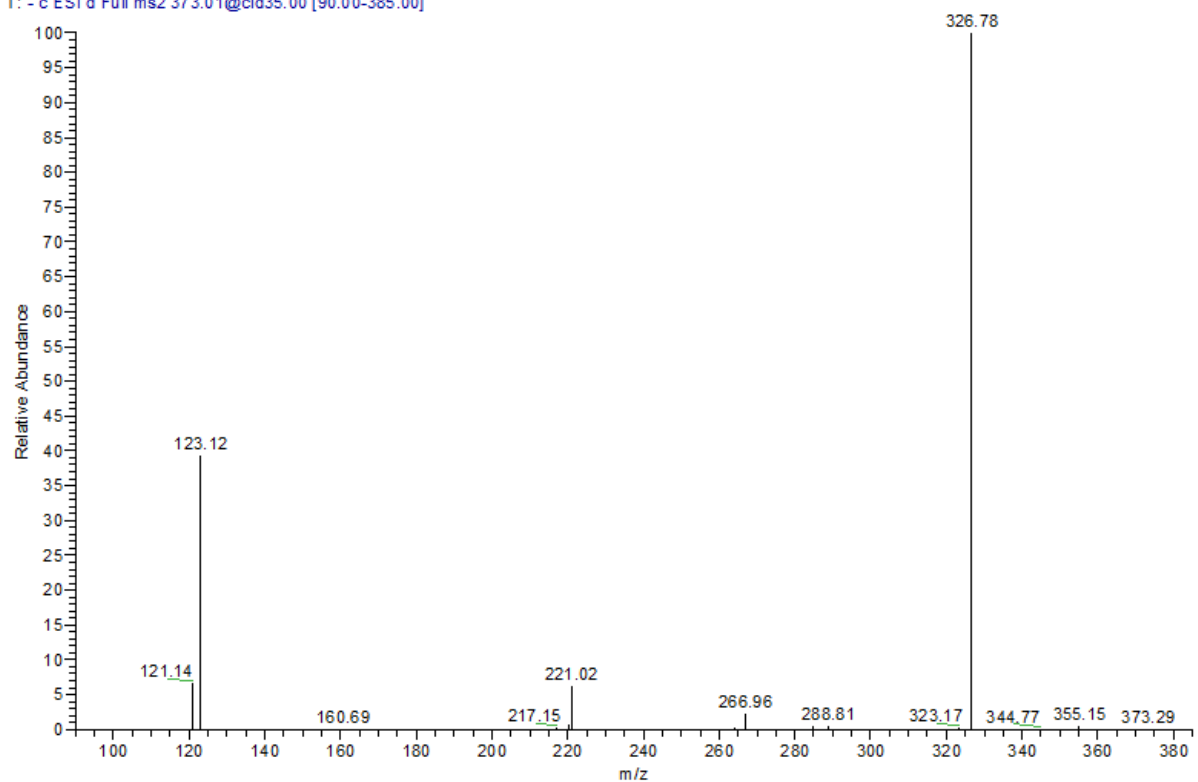


Figure S11. MS/MS profile of 2'-O-acetyl-salicin [M-H + 46, formic acid adduct]

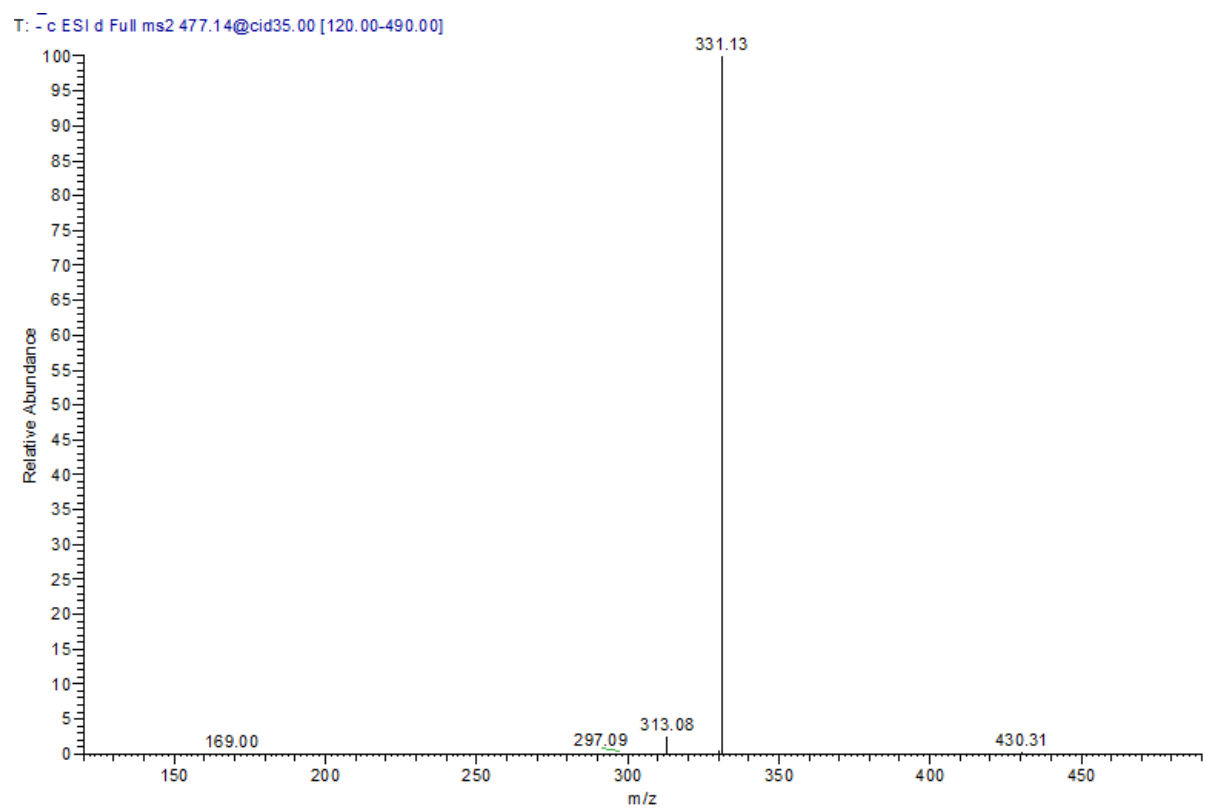


Figure S12. MS/MS profile of coumaroylgalloyl glucose

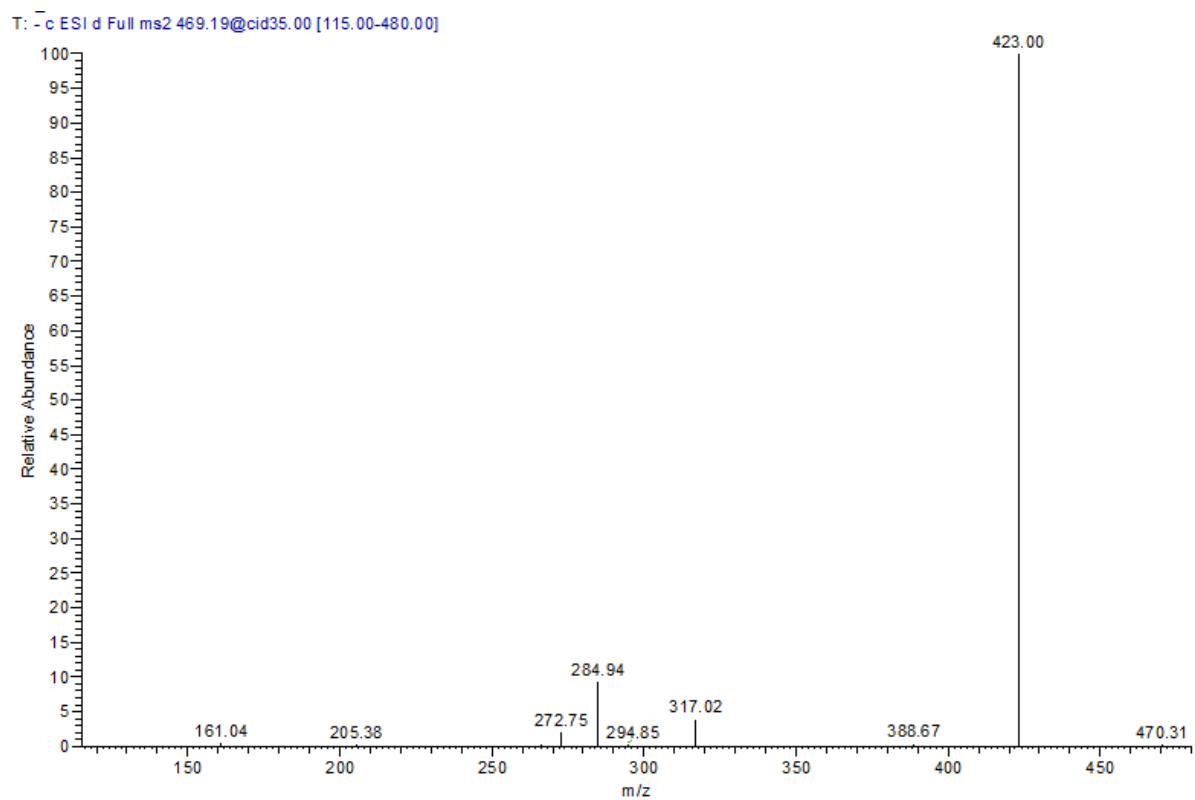


Figure S13. MS/MS profile of salicortin [M-H + 46, formic acid adduct]

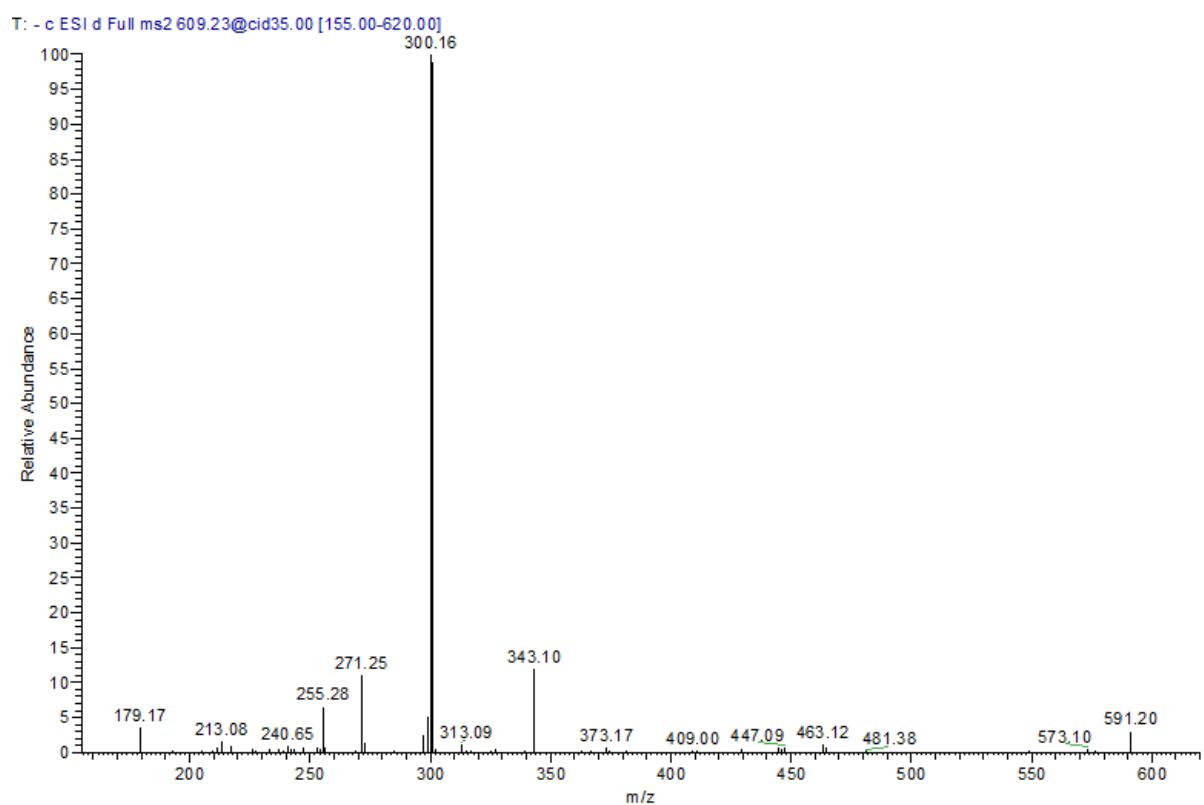


Figure S14. MS/MS profile of rutin

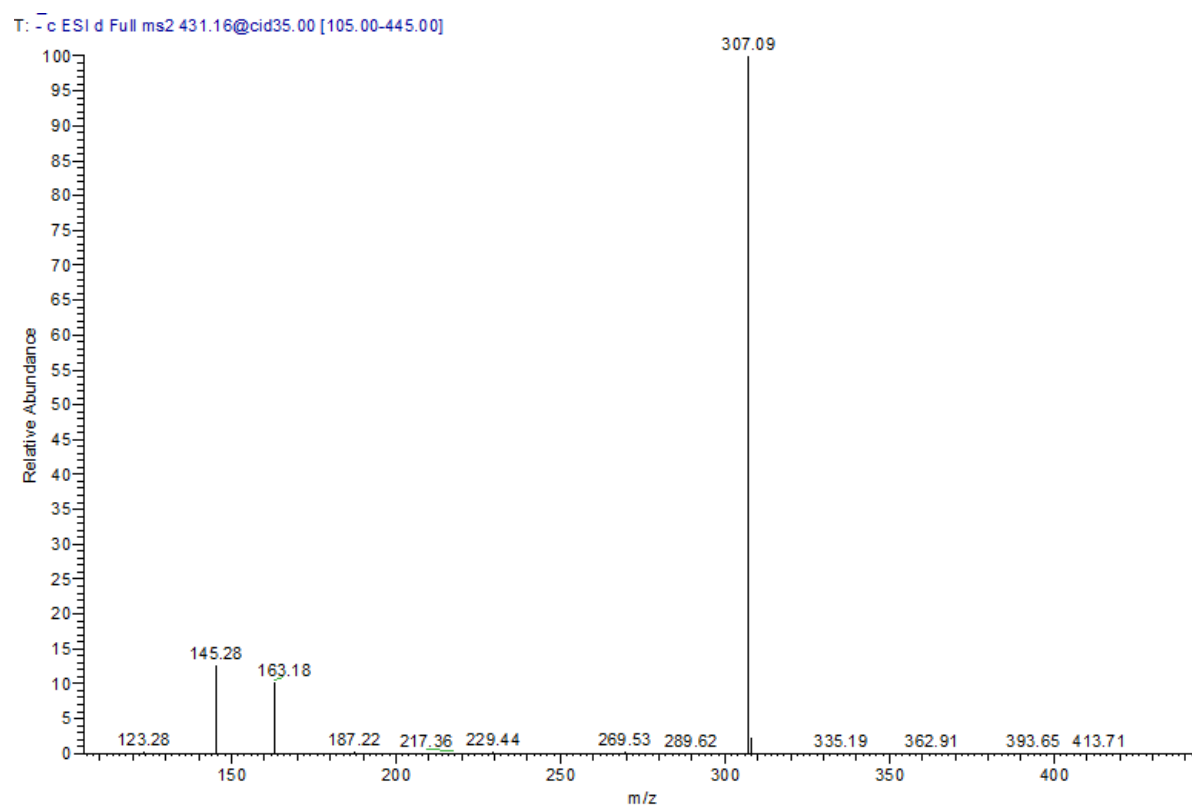


Figure S15. MS/MS profile of trichocarposide

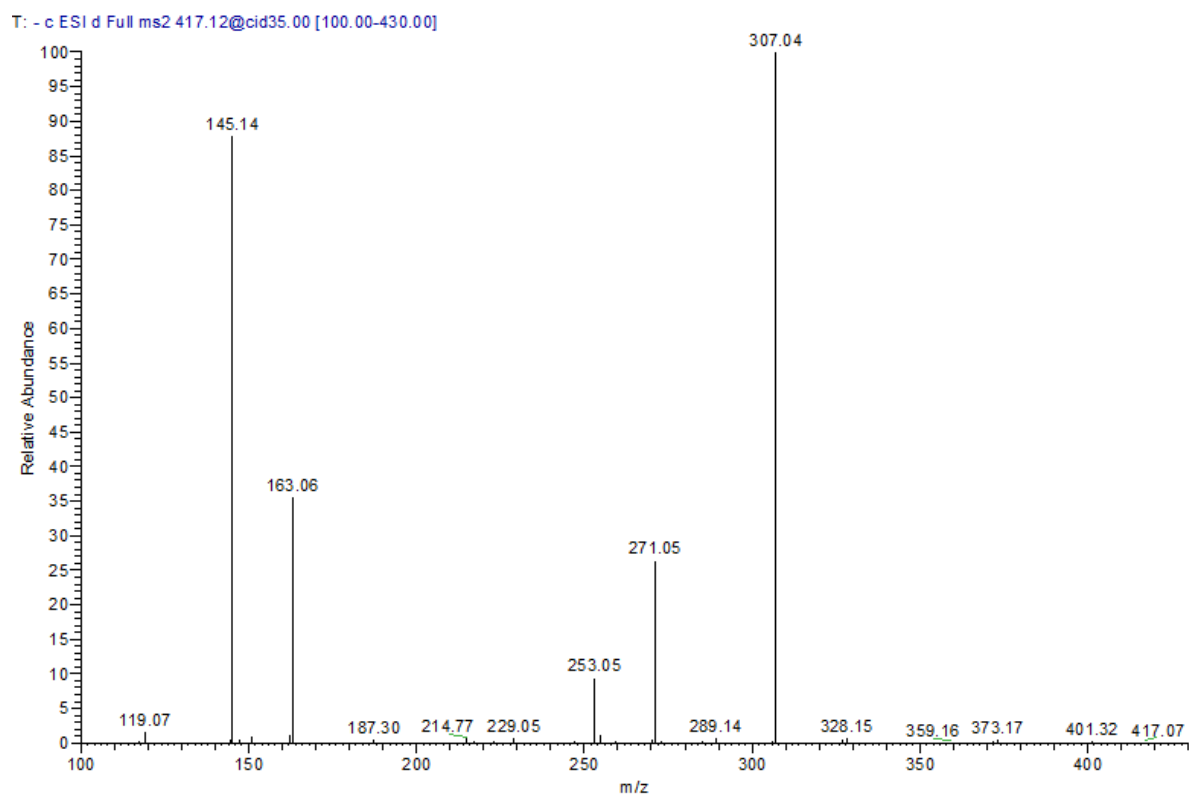


Figure S16. MS/MS profile of dihydrocinnamoyl salicin

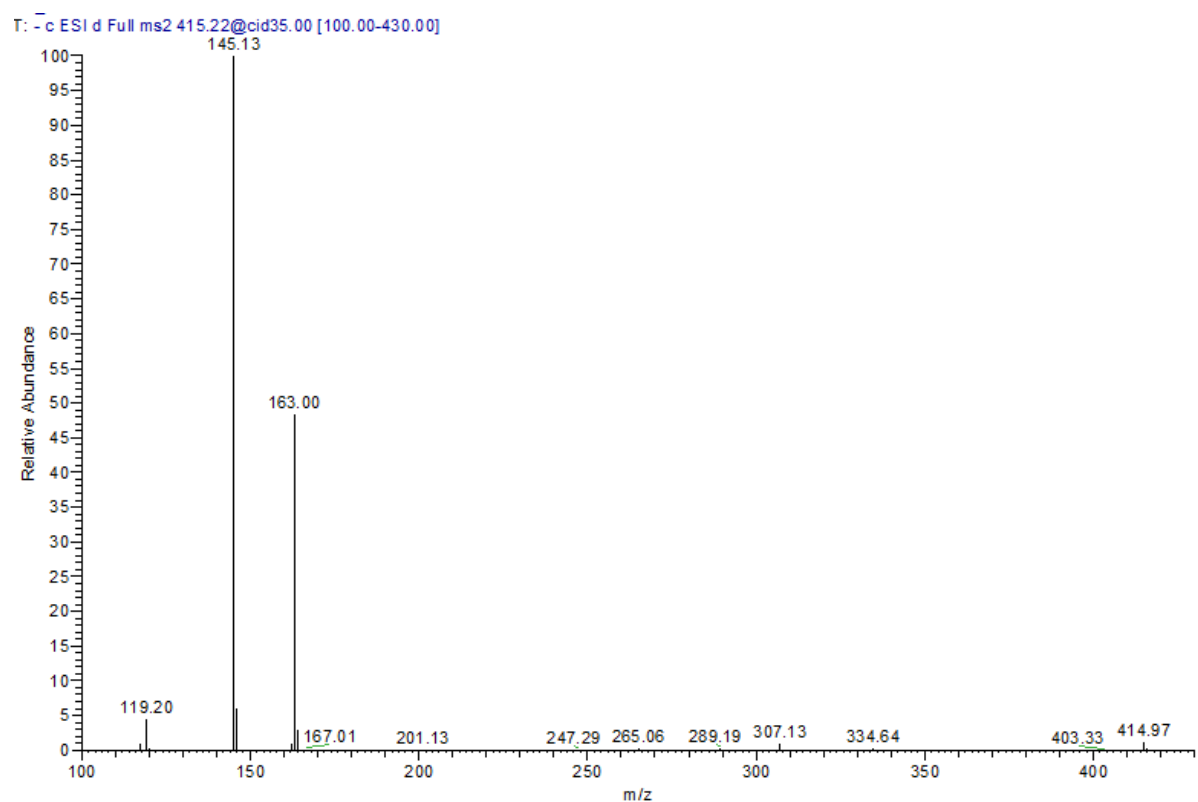


Figure S17. MS/MS profile of cinnamoyl salicin

T: - c ESI d Full ms2 569.11@cid35.00 [145.00-580.00]

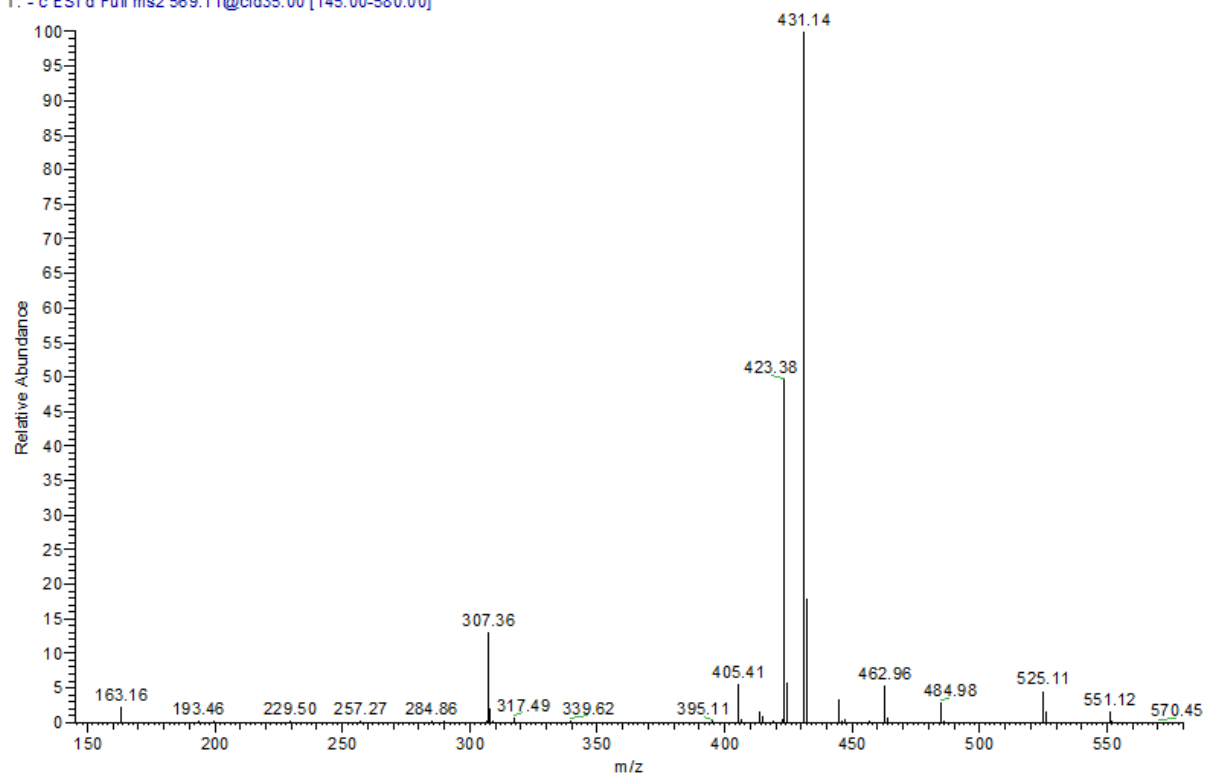


Figure S18. MS/MS profile of coumaroyl dihydrobenzoyl salicin

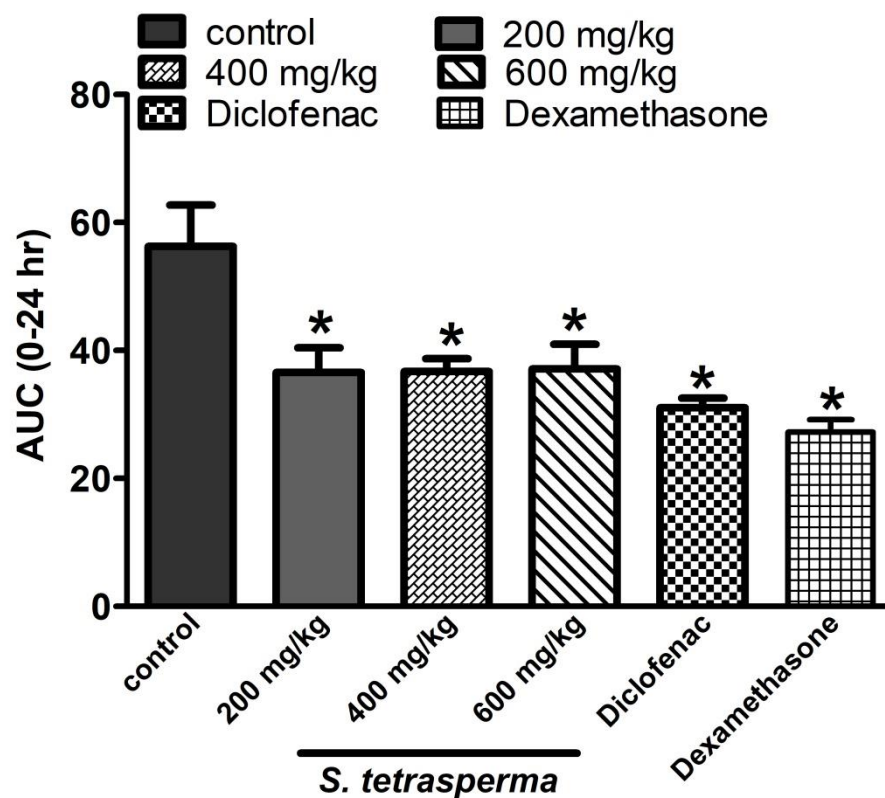


Figure S19. Effect of 1 h prior administration of *S. tetrasperma* (200, 400 and 600 mg/kg, p.o.), diclofenac sodium (20 mg/kg, p.o) or dexamethasone (2 mg/kg, p.o) on carrageenan (1% suspension, 0.1 ml/rat) induced-hind paw edema in rats. Edema thickness (mm) was measured before and then hourly for 5 h and at 24 h after carrageenan injection. Data represents the AUC₀₋₂₄ and is expressed as mean $\pm$ S.E.M (n=5). * p < 0.05 vs. control values.

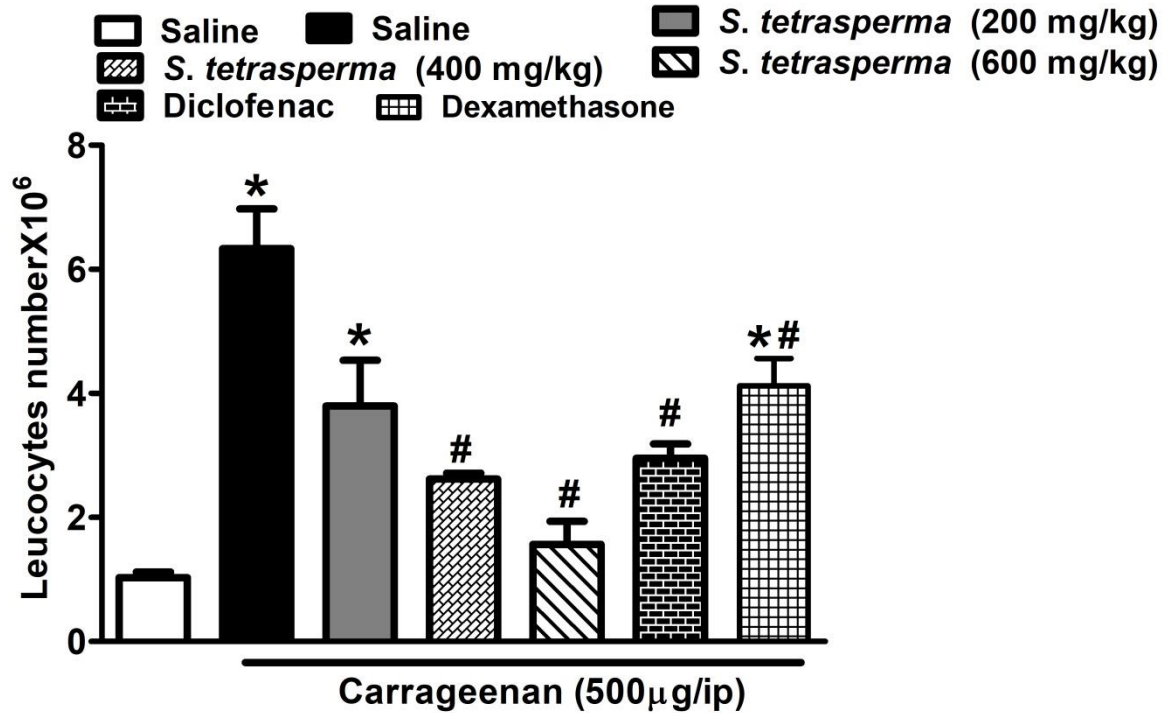


Figure S20. Effect of carrageenan (500 µg, ip) on leukocytes migration into the peritoneal cavity in mice (total number X10⁶) with or without 1h prior treatment with *S. tetrasperma* (200, 400 and 600 mg/kg, p.o.), diclofenac sodium (20 mg/kg, p.o) or dexamethasone (2 mg/kg, p.o). Data is expressed as mean ± S.E.M (n=5-7). **p* < 0.01 vs. vehicle (saline) values, #*p* < 0.01 vs control (carrageenan treated group).

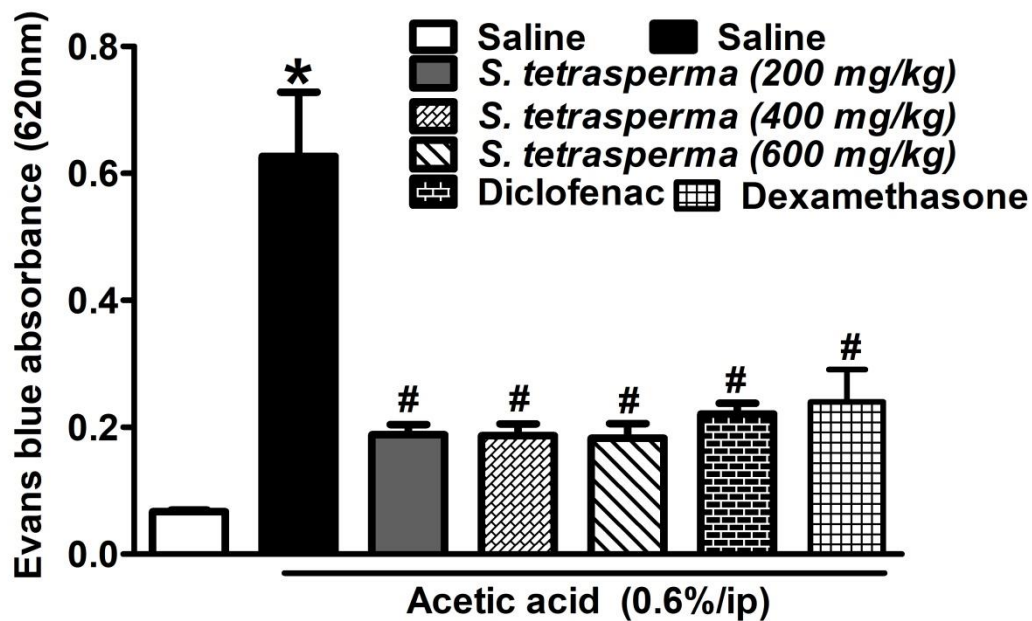


Figure S21. Effect of *S. tetrasperma* extract (200, 400 and 600 mg/kg, p.o.) on acetic acid-induced vascular permeability. The amount of Evans blue dye in the abdominal cavity was measured as an indicator of inflammation degree. The values are expressed as the means $\pm$ SEM (n=5-6). * $p < 0.001$ compared to saline group. # $p < 0.001$ compared to control (acetic acid only treated group).

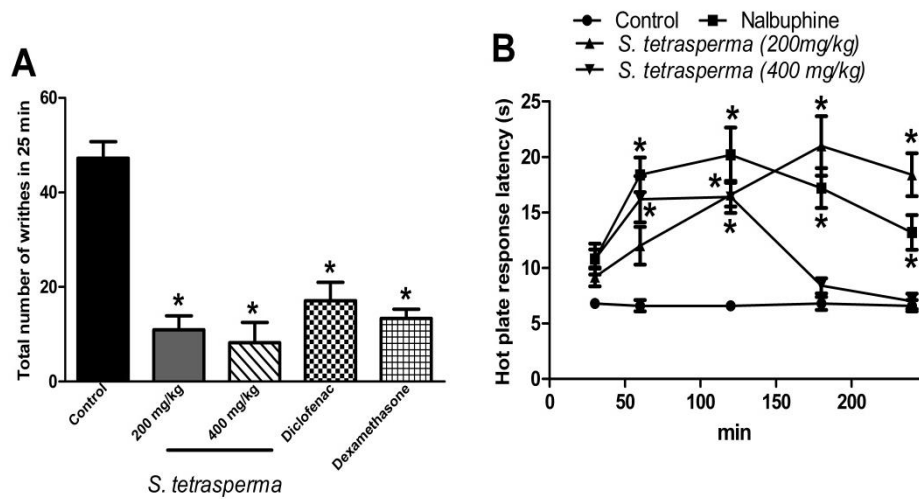


Figure S22. Effect of *S. tetrasperma* (200 and 400 mg/kg, p.o.), diclofenac (20 mg/kg, p.o) or dexamethasone (2 mg/kg, p.o) on acetic acid-induced writhing (0.7%, 1 ml/100 g) in mice (**A**). Hot plate response latency (s) measured 1–4 h after vehicle, the extract (200 and 400 mg/kg, p.o.) or nabuphine (10mg/kg, p.o) administration in mice (**B**). Data is expressed as mean $\pm$ S.E.M (n=5-8). * $p < 0.001$ vs. control values.

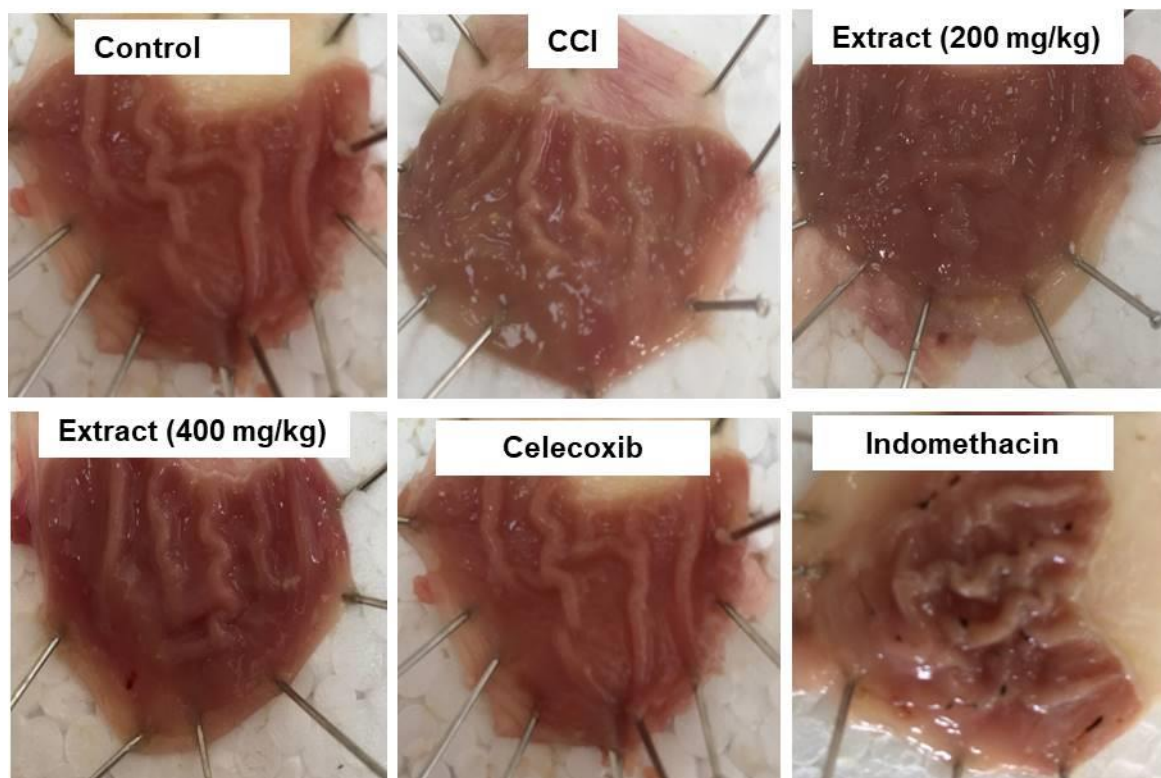
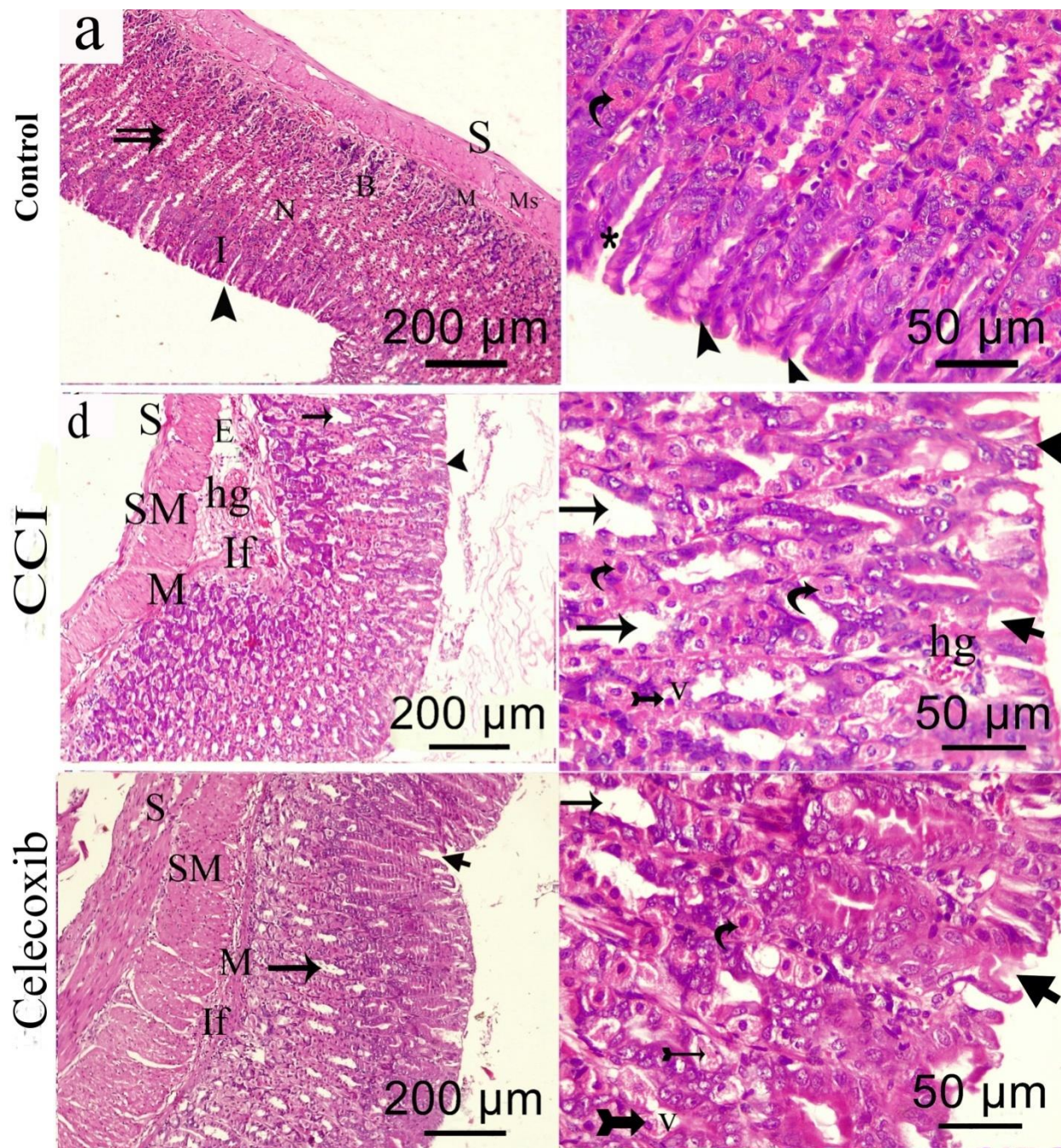


Figure S23. Representative stomach images obtained from CCI rats receiving either the vehicle or different treatments



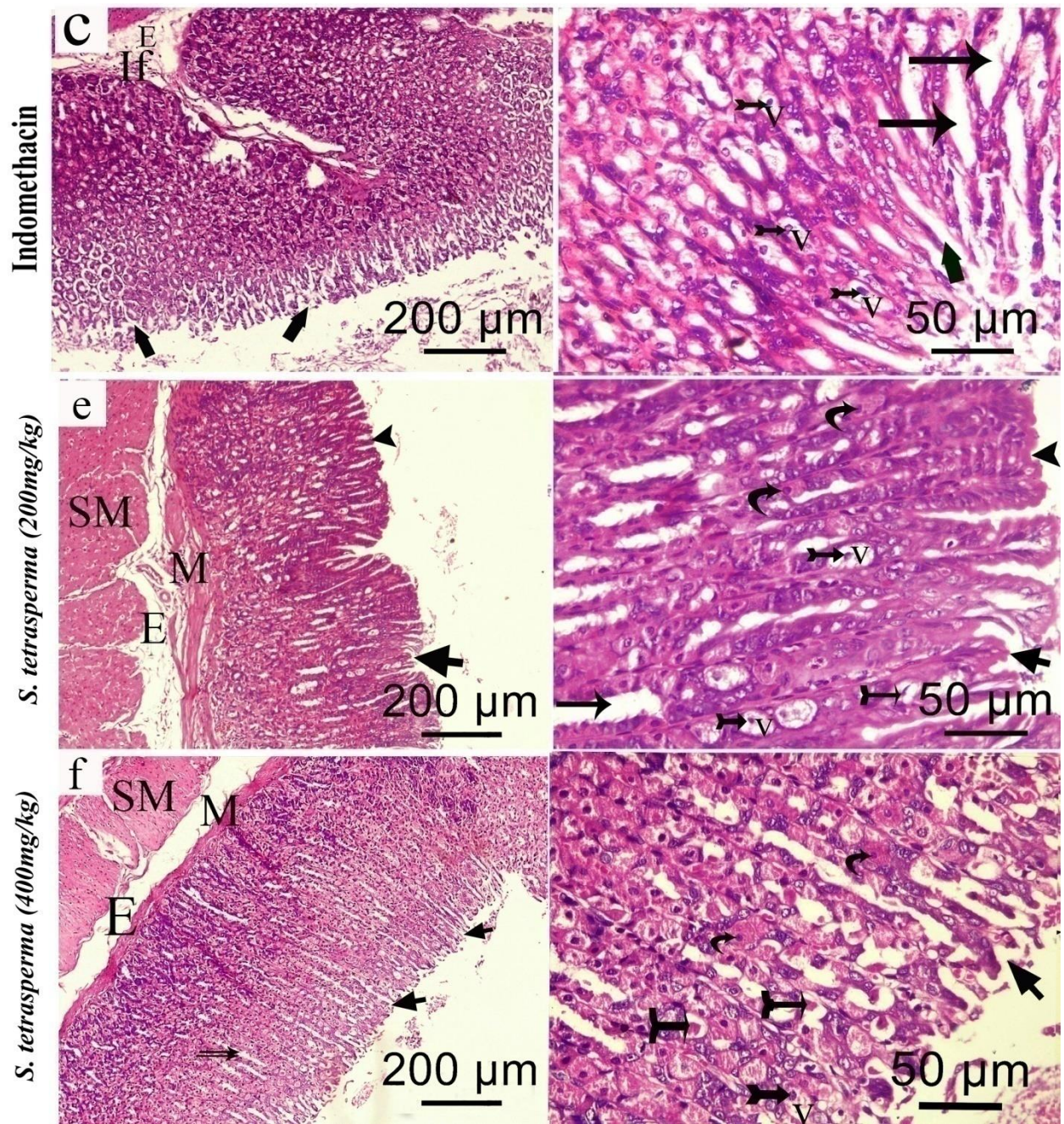


Figure S24. Representative photomicrograph of stomach sections (fundic mucosa) from different groups, (a) control; (b) CCI; (c) celecoxib; (d) indomethacin; (e) *S. tetrasperma* (200 mg/kg) and (f) *S. tetrasperma* (400 mg/kg). The arrow heads, double arrow, arrow, curved arrows, thin bifid arrow, thick bifid arrows and twisted arrows, short arrow, thick arrow and astrexic illustrate normal fundic mucosa, normal fundic glands, wide fundic glands, normal parietal cell, remnant of cytoplasm of parital cells, dark stained nuclei of parital cell, glandular

cells with karyolytic nuclei, slight shedding fundic mucosa, erosion of fundic mucosa and gastric pit respectively. Isthmus (I), N (neck), basic region (B), mucosa (M), muscularis mucosa (Ms), serosa (S), edema (E), infiltration (If), hemorrhage (hg), vacuoles (v) and blood vessel (bv)

Scale bar, 200 μm $\times$ 100, 50 μm $\times$ 400.

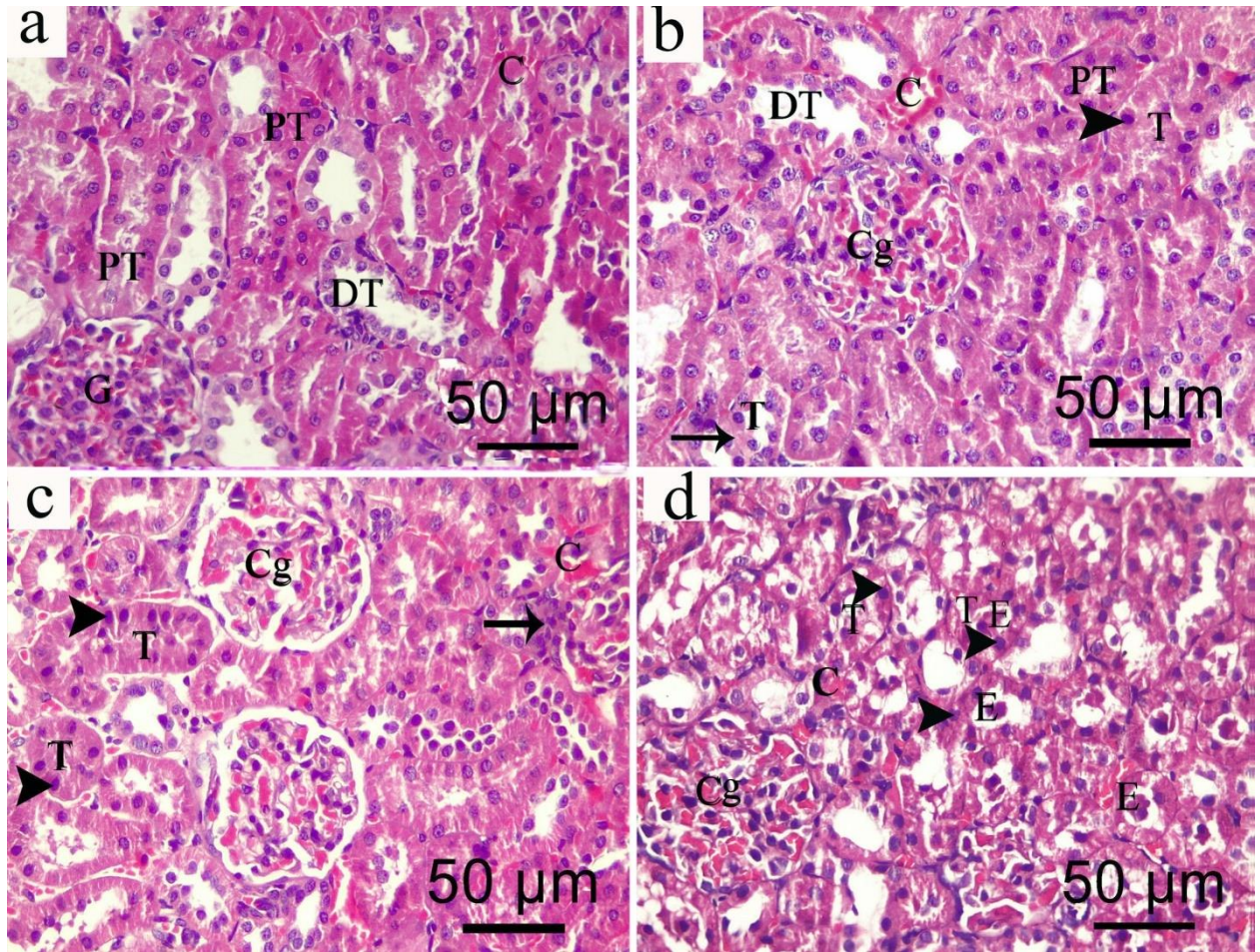


Figure S25. Representative photograph of renal cortex sections of the different groups. (a) control; (b) CCI; (c) *S. tetrasperma* (200 mg/kg); (d) *S. tetrasperma* (400 mg/kg). Arrow and arrow head illustrate the dark stained nuclei and infiltrating cells respectively. G, glomerulus; Cg, congested glomeruli; PT, proximal convoluted tubule; DT, distal convoluted tubule; T, affected tubule; E, exfoliated cells; C, peritubular capillaries.

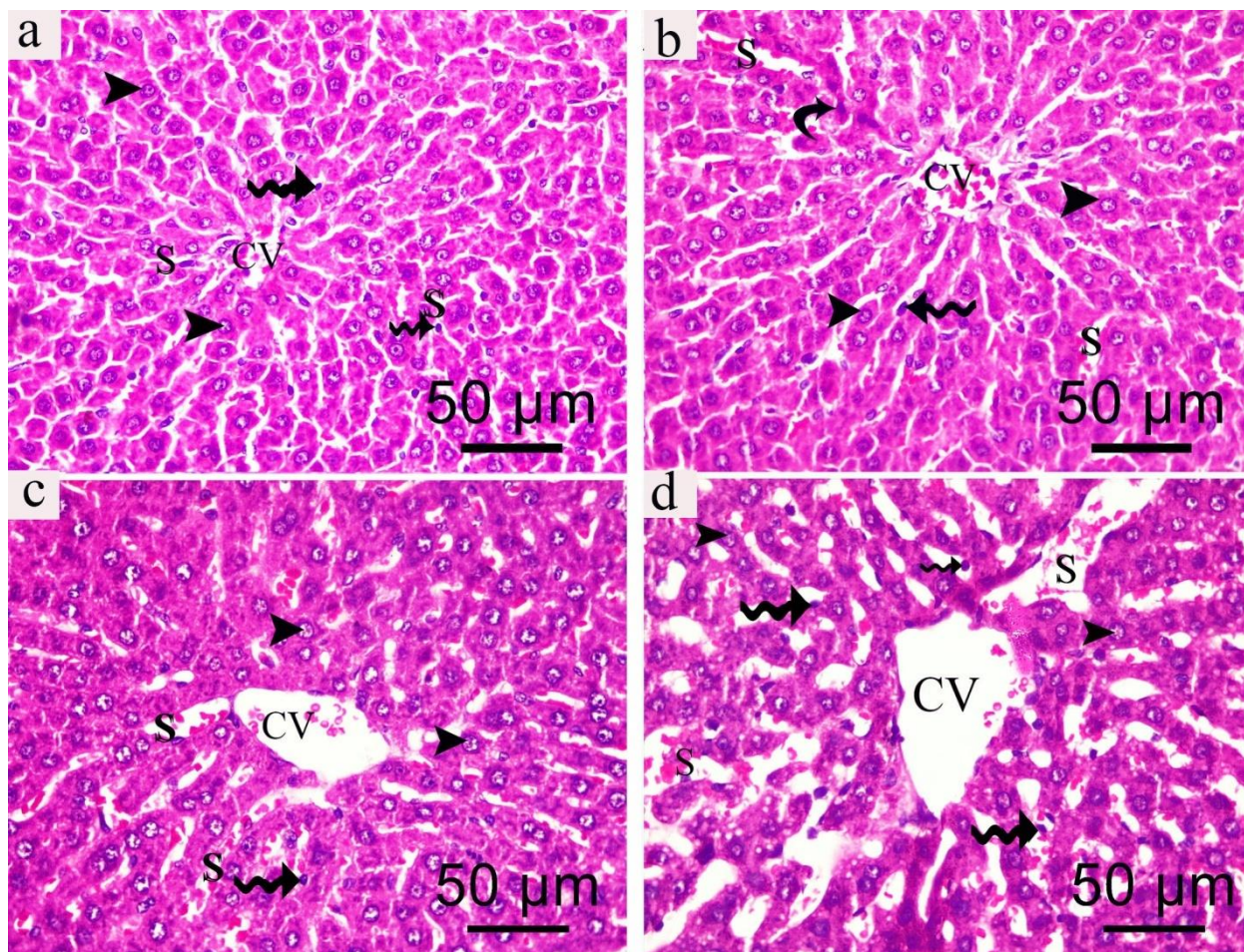


Figure S26. Reprehensive photograph in sections of liver from different groups, (a) control; (b) CCI; (c) *S. tetrasperma* (200 mg/kg); (d) *S. tetrasperma* (400 mg/kg). Arrow head, curved arrow and twisted arrow illustrate normal hepatocytes, Kupffer cells, hepatocytes with dark stained nuclei infiltrations respectively. S, the blood sinusoids; Cv, the central vein, Scale bar, 50 μm $\times 400$.

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