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Methanolic extract of *Smilax perfoliata* Lour. ameliorates L-arginine-induced acute pancreatitis through modulation of oxidative stress and inflammatory mediators

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Acute pancreatitis (AP) is a severe, potentially life threatening inflammation of the pancreas with no clinically approved therapeutic intervention. Oxidative stress and inflammation are key drivers of the disease, contributing to pancreatic damage and worsening its progression. This study was designed to evaluate both the *in vitro* and *in vivo* effects of the methanolic extract of the rhizome of *Smilax perfoliata* (*MESPR*), a plant known for its traditional medicinal properties, on LPS-induced oxidative stress and inflammation in RAW 264.7 macrophages and L-arginine-induced AP mice model. In the *in vitro* model, *MESPR* exhibited significant antioxidant activity in RAW 264.7 macrophages by suppressing LPS-induced nitrosative-oxidative stress. MESPR treatment in mice reduced L-argininеinduced pancreatic enzyme changes, oxidative stress, and inflammation. It suppressed NF-κB p65, nitrotyrosine, and iNOS expression while increasing Nrf-2 and SOD1, thus mitigating oxidative stress and inflammation in acute pancreatitis. These results indicate that *MESPR* alleviated L-arginine-induced acute pancreatitis by reducing oxidative-nitrosative stress and inflammation.

Keywords: Proteolytic zymogen, Acinar cell, Macrophage, Myeloperoxidase, Inflammatory cytokine, Reactive oxygen species

Acute pancreatitis (AP) is one of the common but poorly understood pancreatic ailments that can be associated with extensive morbidity with mortality rate up to 20%[1]. AP is an inflammatory process that mainly results from the premature activation of proteolytic zymogens within the exocrine pancreas. The activated zymogens outflow into the interstitium of the pancreas, which lead to auto digestion of the gland[2]. The trigger for zymogen activation within pancreatic acinar cells remains unknown, but once activated, these enzymes cause cell membrane disruption, oedema, haemorrhage, necrosis, and inflammation, leading to pancreatic damage[3]. AP is characterized elevated α-amylase and lipase, acinar cell vacuolization, leukocyte infiltration, and pancreatic oedema[4]. Despite of extensive preclinical and clinical studies, no specific treatment exists for AP, and treatment relies on supportive measures like pain management, IV fluids, and fasting[5]. Since there is no specific medical treatment available for AP, there is increasing interest in ethnobotanical agents as potential sources for new, safer therapies[6]. Recently, evidences suggests that the up-regulation of pro-inflammatory mediators such as interleukin IL-1β, IL-6, tumour necrosis factor-α (TNF-α) and monocyte chemotactic protein 1 (MCP-1) may initiate the local response to pancreatic acinar cells and cause damage and further, the intra-acinar proenzymes activation trigger the development of AP[5].

The genus *Smilax* L. (Family: Smilacaceae) have 262 species distributed across tropical and subtropical regions of the world (http://www.plantsoftheworldonline.org). *Smilax perfoliata* Lour. is one such climber distributed in warm tropical region of the Indian sub-continent, North East India (Sikkim, Darjeeling Hills of West Bengal, Assam and Arunachal Pradesh), Nepal,

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Bhutan, Indo-China, Southern China and Taiwan[7]. Traditionally, the *Smilax* genus including the species *Smilax perfoliata* is known as "*Bari Chobchini"* among the Ayurvedic healers in India, "*Hazina"* among the folk healers of the Garo tribal community of Northeast India, and the decoction of the rhizome is mainly used for treatment of painful inflammation during urination, gouty arthritis, syphilis and dermatitis[8]. The *Smilax glabra,* which is closely related to *Smilax perfoliata* is also mainly used for curing jaundice, dispel bodily toxicity, clear heat and dampness from the skin tissues[9]. Presently, there are also growing scientific evidences reporting the therapeutic potential of the *Smilax* species for the treatment of inflammation, rheumatoid arthritis, liver injury, hyperinsulinemia and cancer[10,11].

The *Smilax* extracts have been reported to possess several active ingredients. Rhizomes of *Smilax* extracts reportedly attenuated oxidative stress by lowering oxidized glutathione (GSSG) and reactive oxygen species (ROS) levels while increasing catalase (CAT) and superoxide dismutase (SOD) activity[12,13]. The aim of the present study is to evaluate the protective effects of *Smilax perfoliata* rhizome extracts against LPS-induced oxidative stress and inflammation in RAW 264.7 macrophages and L-arginine-induced AP model.

Materials and Methods

Chemicals

L-arginine, ascorbic acid, bovine serum albumin (BSA), Griess reagent, 2-thiobabituric acid (TBA), sodium dodecyl sulphate (SDS), sodium chloride, reduced glutathione, sodium nitrite, xylene, DPX mounting agent, glycine, 5,5′dithio-bis (2-nitrobenzene acid) (DTNB), eosin, hematoxylin, glacial acetic acid and myeloperoxidase kits were procured from Sigma Aldrich USA. Biochemical kit for amylase, lipase, serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT) levels were purchased from Accurex Biomedical Private Limited. Enzyme linked immunosorbent assay (ELISA) kits for IL-1β, IL-6, and TNF-α were procured from eBioscience, USA. Primary antibodies against β-actin, nitrotyrosine, iNOS, SOD1, Nrf-2 and the HRP conjugated anti-mouse and anti-rabbit antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, USA, whereas total nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB p65) and phosphorylated NF-κB p65 were procured from Cell Signaling Technologies, MA, USA.

Plant material

Rhizomes of *Smilax perfoliata* (SP) were collected from the Botanical Garden of Rajiv Gandhi University, Rono Hills, Doimukh, Arunachal Pradesh, India. The voucher Specimen No. HAU/HT/1621/2017 were authenticated at Herbarium of Botanical Survey of India, Arunachal Pradesh Regional Centre, Itanagar (ARUN) and deposited in the Herbarium of Arunachal University (HAU), Department of Botany, Rajiv Gandhi University, Rono Hills, Doimukh, Arunachal Pradesh, India for future reference. The accepted name and distribution ranges were verified at http://www.plantsoftheworldonline.org (POWO). The rhizomes were sliced, shade-dried, and pulverized. The powdered material underwent cold maceration with 80% aqueous methanol for 6-8 h. The extract was filtered, concentrated using a Rotary Evaporator (IKA RV10) at 40°C, and dried in a hot air oven until constant weight of the extract was obtained with yield value of 34.5% (w/w).

Gas chromatography–Mass spectrometry (GC-MS) analysis of methanol extract of *Smilax perfoliata* rhizome (*MESPR*)

GC-MS analysis of methanol extract of *Smilax perfoliata* rhizome *(MESPR)* was carried out on a GC Clarus 500 Perlin Elmer system comprising an AOC 20i autosampler and gas chromatograph interfaced to a Gas chromatography–Mass spectrometry (GCMS) instrument. 1 µL sample volume was injected with a splitless mode into a GC-MS-QP2010 Ultra system (Shimadzu) consisting of TSQ Quantum XLS GC-MS/MS (Thermo scientific co.). TG-5MS GC column was used for analysis with an inner diameter of 0.25 mm, 30 m length, and 0.25 µm film thickness. The carrier gas used was helium gas at a flow rate of 1 mL/min. The external sample using the M/C technique was analysed injecting at 250^{O}C followed by 2 AC/min to 250 AC and finally with 24 min isothermal at 280^{O}C. Mass spectra were acquired using full scan monitoring mode with a mass scan range of 40-650 m/z. The chromatograms and the mass spectra were evaluated by using the Xcalibur^{TM} software embedded in the GC-MS/MS system. Phytocompounds were identified by their molecular mass, structure, and calculated fragments, with confirmation from Wiley and NIST libraries.

Liquid chromatography-mass spectrometry (LC-MS) analysis of *MESPR*

The liquid chromatography-mass spectrometry (LC-MS) analysis of the *MESPR* was performed using

an LC-MS (Agilent Technologies) equipped with a quaternary pump (QuatPump G1311B), auto sampler (ALS G1329G), column oven (TCC G1316A), and variable wavelength detector (VWD G1314B). 20 µL samples were injected for analysis. The draw speed and eject speed were 200 µL/min. The UV detector spectral was recorded between 190 to 400 nm. Flow rate was 400 µL/min, Solvent A: HPLC grade MeOH and solvent B: HPLC grade H_2O (98:2 ratio). Mass spectra were obtained in an ESI ion mode with 4000V (positive mode) and a temperature of 300^{O}C, using spray ion source. Spectra were recorded between m/z 100 -1000 with scan duration 2 s/scan. The total running time of the LC-MS/MS was 60 min.

Cell culture and cell viability assay

RAW 264.7 macrophages were obtained from the National Centre for Cell Science (NCCS), Pune, India. Cells were grown in DMEM medium with high glucose supplemented with 10% fetal bovine serum and 100 IU/mL penicillin and streptomycin. Cell viability was evaluated by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay following the method suggested previously[14]. The cell viability of untreated cells was considered as 100%. The effect of different concentrations of *MESPR* on cell viability of RAW 264.7 cells was evaluated.

Measurement of nitric oxide (NO) levels

To assess NO levels in RAW 264.7 macrophages, cells were treated with 2 µg/mL LPS and *MESPR* (3.12, 6.25, and 12.50 µg/mL) for 24 h. Then an equal volume cell culture supernatant was mixed with Griess reagents (Sulfanilamide and N-(1-naphthyl) ethylenediamine), incubated for 10 min in the dark, and absorbance was measured at 540 nm following the method recommended previously[15].

2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

The DPPH assay was carried out to evaluate the antioxidant activity of *MESPR* as per the earlier described method with slight modifications[16]. 100 mM solution of DPPH was prepared in methanol and added to the *MESPR* extract at different concentrations (1-100 µg) at 1:1 ratio and absorbance was taken at 570 nm after 30 min of incubation. All the tests were performed in triplicate and percentage inhibition was calculated using the method previously described[17].

Dichlorofluorescin diacetate (DCFDA) assay

DCFDA assay for cellular ROS was done by pre-treating the cells with *MESPR* (3.125, 6.25 and 12.5 µg/mL) for 24 h. Then, cells were treated for 30 min with lipopolysaccharides (LPS - 2 µg/mL) for the induction of oxidative stress. Later, these cells were further incubated with 10 µM DCFDA for 15 min and by using multimode plate reader, fluorescence intensity was measured using the method described previously[18].

Animals

Male Swiss albino mice (6-7 weeks old, 25-30 g) were procured and acclimatized for 1 week under a 12 h light/dark cycle at 22 ± 2°C, with food and water *ad libitum*. Experiments followed standard operating procedure (SOP) approved by the Institutional Animal Ethical Committee (IAEC) of NIPER-Hyderabad (Protocol No. NIP/01/2018/RT/273) and complied with of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines 2018.

Experimental design

Animals were randomly divided into six groups, each group comprising of six mice were included in the study (Fig. 1). Three doses of *MESPR* (30, 100 and 300 mg/kg) were selected. AP was induced by high dose of L-arginine (4 g/kg)[19,20]. A sterile solution of L-arginine was prepared in normal saline and pH was adjusted to 7.0. The sterile L-arginine solution was administered intraperitoneally (i.p.) to the fasted animals at the dose of 4 g/kg, after first administration of L-arginine, animals were returned to the cages and allowed free access to water and food. After 1 h, animals were administered with the second dose of L-arginine 4 g/kg i.p. Doses of the *MESPR* was selected on the basis of previous literatures[21,22]. Group I: Normal control (NC); Group II: L-arginine control group (DC, received 2 doses of L-arginine 4 g/kg at 1 h interval for the induction of AP); Group III: *MESPR* control group, *MESPR* alone was administered orally at a dose of 300 mg/kg; Group IV: *MESPR* 30 mg/kg, orally (LD) + disease induction with L-arginine 4 g/kg (i.p.) 2 doses at 1 h interval; Group V: *MESPR* 100 mg/kg orally (MD) + disease induction with L-arginine 4 g/kg (i.p.) 2 doses at 1 h interval; Group VI: *MESPR* 300 mg/kg orally (HD) + disease induction with L-arginine 4 g/kg (i.p.) 2 doses at 1 h interval. Body weights of all the animals were taken on 1st, 4th and 7th day and after

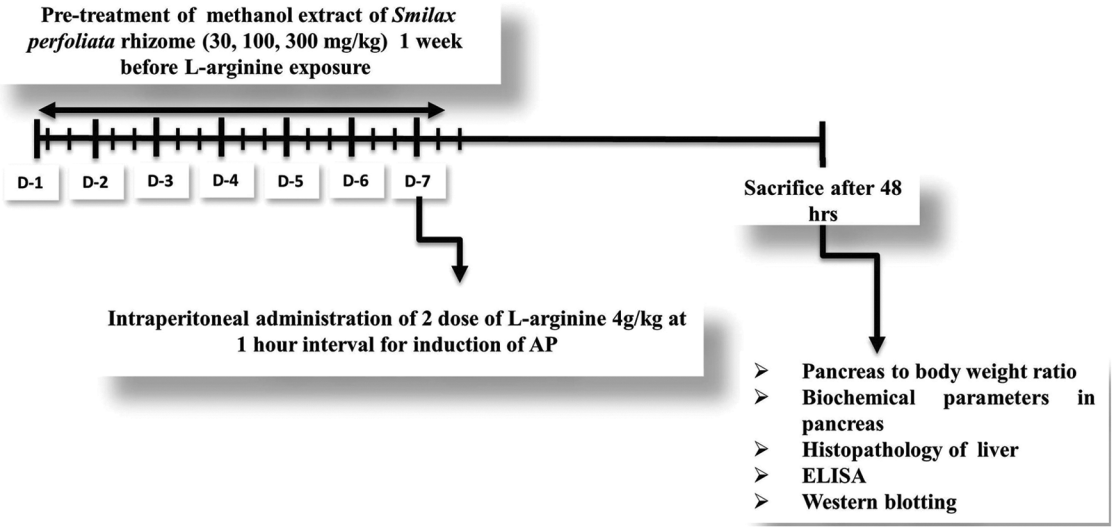

Fig. 1 — Experimental design of L-arginine-induced AP and intervention of *MESPR.*

48 h of the last injection of L-arginine, all animals were sacrificed and pancreatic weights were measured after properly drying and removal of accessory adjacent tissue. Blood was collected for the analysis of various enzymes. Plasma was separated from blood by centrifugation at 10,000 rpm for 10 min at 4°C. A small portion of the pancreas and liver was stored in neutral buffer formalin (10% v/v) for histological examination. For further biochemical analysis, western blot and ELISA, plasma and tissues were stored at -80°C.

Determination of pancreatic weight index

Pancreatic oedema was measured by calculating the ratio between the pancreatic wet weights to the body weights of animals and represented as percent organ weight index[23].

Organ weight index (%) = [wet weight of pancreas (g) / body weight (g)] × 100.

Determination of plasma biochemical parameters for AP

All the plasma amylase, lipase, ALT and AST were assessed by the standard protocol provided in the commercial kits.

Determination of oxidative stress parameters and myeloperoxidase (MPO) activity

The extent of lipid peroxidation was measured by estimating MDA levels by reaction with thiobarbituric acid as previously described[24]. Quantification of pancreatic total protein content was done by the standard Lowry method[25]. Nitrite content in the tissue homogenate was done by the spectroscopic method by using Griess reagent. Pancreatic glutathione (GSH) levels were determined according to previously described method[26]. L-arginine induced AP is accompanied by the infiltration of neutrophils in the pancreas. The sequestration of neutrophils was estimated by measuring the MPO activity in pancreatic tissues[27]. MPO activity in pancreatic tissues were measured as per the earlier described protocol[28].

Histological examination

After sacrifice of the experimental mice, a portion of pancreatic and liver tissues were fixed in 10% neutral buffer formalin for 48 h and embedded in a paraffin wax. 5 μm thickness pancreatic and liver sections were prepared by using microtome (Leica, Germany) and stained with hematoxylin and eosin (H & E) and toluidine blue staining as per the standard protocols[26]. After staining, sections were examined by light microscopy under an upright microscope. The severity of AP was blindly graded by a semi-quantitative assessment of inflammatory cell infiltration, oedema, and acinar cell necrosis and each change was scored on a scale from 0-3 (normal appearance - 0; minimal change - 1; medium change - 2; and severe change - 3).

Assessment of inflammatory cytokines by ELISA

ELISA was carried out to evaluate the effect of *MESPR* on IL-1β, IL-6, and TNF-α in L-arginine induced AP. IL-1β, IL-6, and TNF-α ELISA kits were purchased from eBioscience, USA[29]. Manufacturer instructions were strictly followed while carrying out the assays.

Western blotting assay

The pancreatic tissues were lysed with radioimmunoprecipitation assay (RIPA) lysis buffer containing protease inhibitor cocktail. The whole tissue lysates were then collected and probe sonicated followed by centrifugation. The supernatant was collected and protein concentration was estimated using Bradford′s method. The proteins were separated according to the molecular weight by polyacrylamide gel electrophoresis (PAGE), and the separated proteins were then transferred onto nitrocellulose membrane. The protein expression was assessed by western blotting method as per the earlier described protocol[14,30]. The blots were detected using chemiluminescence detector FusionFx (Vilber Lourmat, France) after exposure to enhanced chemiluminescence (ECL) reagent. Densitometric analysis of protein bands was performed by using ImageJ software.

Immunohistochemistry

Immunohistochemistry (IHC) was performed as per the laboratory established protocol[14,31]. Briefly, pancreatic sections were deparaffinized in xylene and rehydrated in series of gradient alcohol. Antigen retrieval was performed by heating sections for 30 min in citrate buffer, followed by removal of endogenous peroxidase by using 3% H_2O_2. In the next step, sections were blocked with 3% BSA to avoid non-specific background and incubated with the primary antibodies including rabbit anti-NF-kB p65 overnight at 4°C. HRP-linked polymer detection system was used to develop brown colour reaction, and sections were counterstained with hematoxylin, mounted with DPX mounting medium and slides were observed under the microscope. The percent immunopositive area was calculated by using ImageJ software (NIH, USA).

Statistical analysis

All the data were expressed as mean ± standard error of the mean (SEM) and were analyzed by using GraphPad Prism version 6.0. Statistical analysis was done by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. The value of $P<0.05$ indicated statistically significant.

Results

Characterization of *MESPR* using GC-MS & LC-MS

The GC-MS analysis of *MESPR* have revealed 29 phytocompounds (Fig. 2A) belonging to different

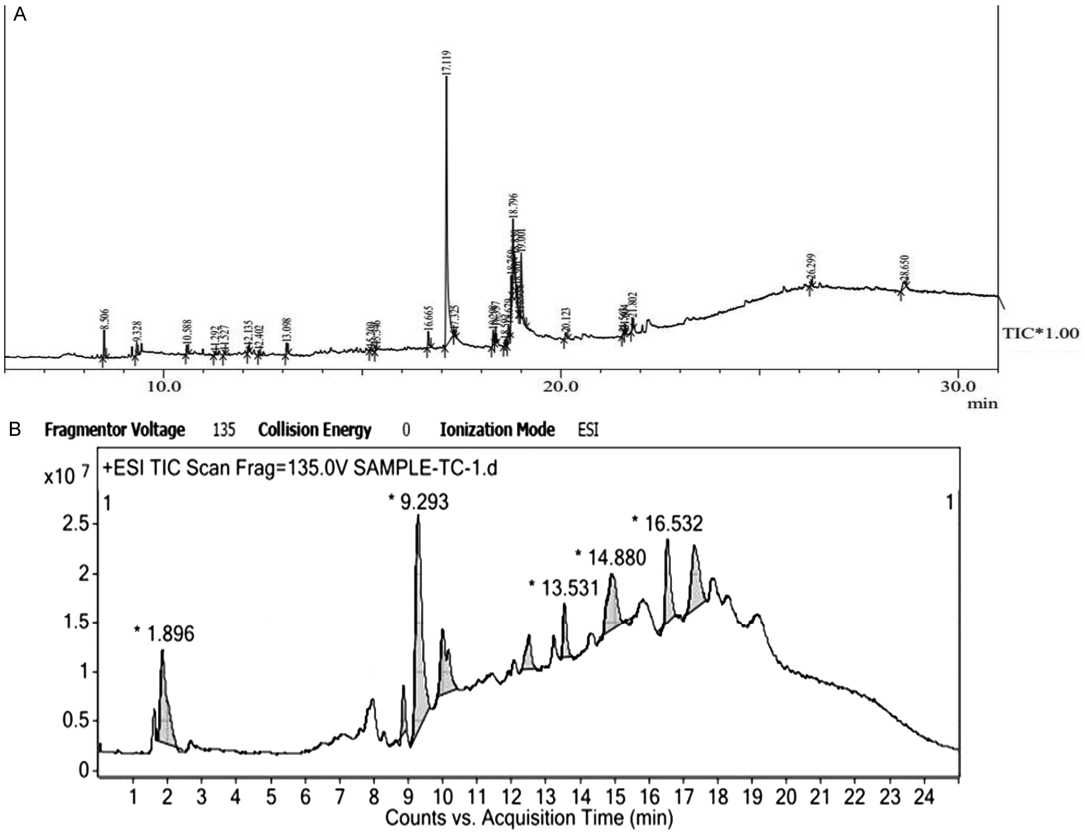

Fig. 2 — GC-MS chromatograms of MESPR (A), LC-MS chromatograms of MESPR (B).

classes of compounds which is presented in Table 1. The major biologically active phytoconstituents found are 1-Tridecanol, Heptadecane, Hexatriacontane, Phenol, 2,4-Bis(1,1-Dimethylethyl), 2-Isopropyl-5-Methyl-1-Heptanol, Heptadecane, N-Hexadecanoic Acid, Octadecanoic Acid, Methyl Ester, 9,12-Octadecadienoic Acid (Z,Z), Octadecanoic Acid, β-sitosterol, γ-sitosterol. LC-MS analyses of *MESPR* have revealed a total of eight phytoconstituents (Fig. 2B) which is presented in Table 2. The interpretation of the target ion was done by comparing its mass: charge (m/z) against the spectral database MassBank. LC-MS analysis of *MESPR* showed the presence of bioactive antioxidants phytoconstituents such as Etoposide, Chrysanthellin B, Cyclogaleginoside, Phosphatidylethanolamine, Silychrystin, Dihydrohesperetin, Dihydrohesperetin-7-O-neohesperidoside, and Procyanidin B2.

Table 1 — List of phytocompounds identified through GC-MS analysis of *MESPR*

Peak	R.Time	Area %	Compound name
1	8.506	3.05	Benzene, 1,3-Bis(1,1-Dimethylethyl)-
2	9.328	1.55	1-Tridecanol
3	10.588	1.23	Heptadecane
4	11.292	0.36	Decane, 2,3,4-Trimethyl-
5	11.527	0.45	Hexatriacontane
6	12.135	0.82	Phenol, 2,4-Bis(1,1-Dimethylethyl)-
7	12.402	0.64	2-Isopropyl-5-Methyl-1-Heptanol
8	13.098	1.52	Heptadecane
9	15.209	0.34	1-Heptanol, 2,4-Diethyl-
10	15.346	0.49	Decane, 3,8-Dimethyl-
11	16.665	2.25	Pentadecanoic Acid, 14-Methyl-, Methyl Ester
12	17.119	50.35	N-Hexadecanoic Acid
13	17.325	0.56	Ethyl Pentadecanoate
14	18.298	1.76	9,12-Octadecadienoic Acid (Z,Z)-, Methyl Est
15	18.357	1.68	9-Octadecenoic Acid (Z)-, Methyl Ester
16	18.592	0.84	Octadecanoic Acid, Methyl Ester
17	18.679	2.57	4-[3,7-Dimethyl-3-Vinyl-1,6-Octadienyl]Phen
18	18.750	3.87	9,12-Octadecadienoic Acid (Z,Z)-
19	18.796	6.52	Heptadecene-(8)-Carbonic Acid-(1)
20	18.839	1.25	Cis-Vaccenic Acid
21	18.901	1.34	(R)-(-)-14-Methyl-8-Hexadecyn-1-Ol
22	18.958	0.37	Ethyl Oleate
23	19.001	9.88	Octadecanoic Acid
24	20.123	1.13	Octadecanoic Acid, 2,3-Dihydroxypropyl Ester
25	21.563	0.22	4-(2-Nitrophenyl)But-3-En-2-One
26	21.604	0.31	2-Hydroxy-3-[(9e)-9-Octadecenoyloxy]Propy
27	21.802	1.37	Methyl Dihydromalvalate
28	26.299	1.00	β-Sitosterol
29	28.650	2.30	γ-Sitosterol

Antioxidant and anti-inflammatory activity of *MESPR* on DPPH scavenging activity, cell viability, cellular ROS and nitric oxide production in RAW 264.7 macrophages

DPPH assay was performed to find the antioxidant potential of *MESPR*, the results revealed the dose-dependent free radical scavenging activity of *MESPR*, where ascorbic acid was used as standard antioxidant as shown in Fig. 3A. The *MESPR* was found to be relatively not cytotoxic upto the concentration of 25 µg/mL, whereas above this concentration produced cell death (Fig. 3B). Further, LPS-induced nitrosative-oxidative stress was evaluated by nitrite and DCFDA assays. LPS stimulation induced oxidative stress in RAW 264.7 macrophages by induction of cellular ROS levels and induced nitric oxide (NO) production, while treatment with *MESPR* at 3.12, 6.25 and 12.5 µg/mL concentrations significantly reduced ROS and NO production as compared to the LPS-treated RAW 264.7 cells (Fig. 3C-E). These results suggest the antioxidant effects of *MESPR* in LPS-induced RAW 264.7 macrophages.

Effect of *MESPR* on L-arginine-induced pancreatitic injury

In L-arginine-induced AP mice, a significant increase in pancreatic oedema was observed due to vacuolization, acinar cell injury, prominent neutrophil infiltration and necrosis in the pancreas without any morphological changes to the Langerhans islet cells. In disease control (DC) group, we found a significant increase in pancreatic weight and body weight ratio compared to the normal control (NC) group. While *MESPR* treatment significantly decreased the pancreas weight to body weight ratio in a dose-dependent manner (Fig. 4A). Moreover, L-arginine elevated the plasma amylase and lipase levels which are important biomarkers for pancreatic acinar cell

Table 2 — List of of phytocompounds identified through LC-MS analysis of *MESPR*

Sl. No.	R.Time	Area	Compound name
1	1.896	132751473	Etoposide
2	8.837	33006296	Chrysanthellin B
3	9.293	287697272	Cyclogaleginoside
4	10.055	104231917	Silychrystin
5	12.456	43324124	Dihydrohesperetin
6	13.531	41172859	Dihydrohesperetin-7-O-neohesperidoside
7	14.88	115728474	Phosphatidylethanolamine
8	16.532	83053115	Procyanidin B2

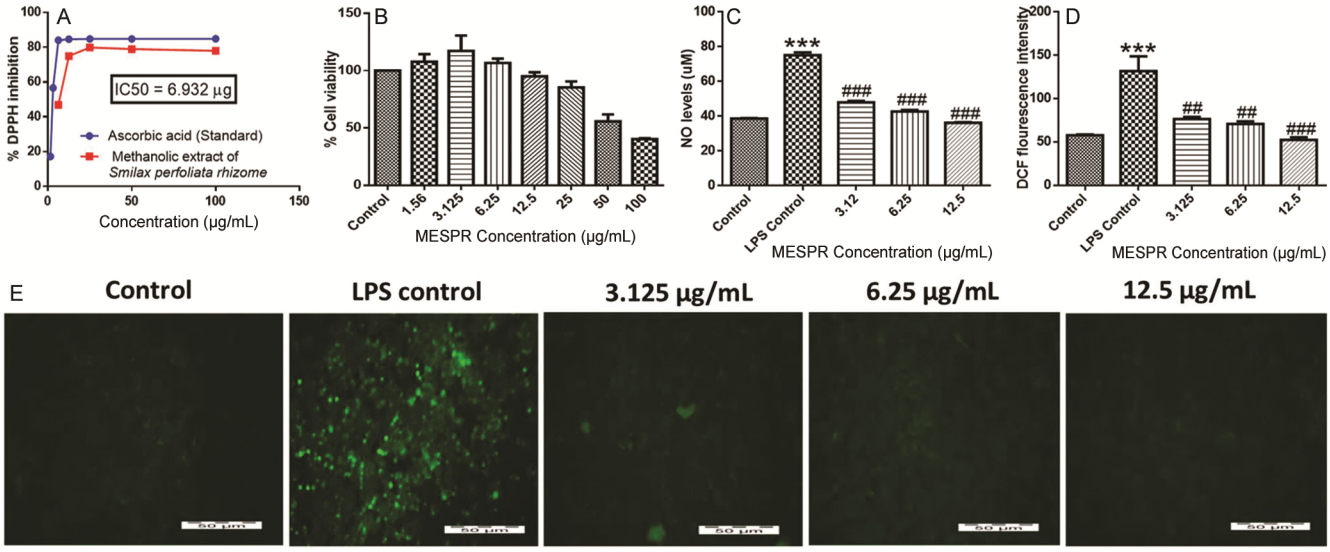

Fig. 3 — Effect of MESPR on DPPH scavenging, antioxidant and anti-nitrosative stress activity. (A) % DPPH inhibition, (B) % cell viability, (C & D) NO levels, and DCF fluorescence intensity, (E) fluorescent microscopic images. *MESPR:* methanolic extract of *Smilax perfoliata* rhizome. [All values are represented as mean ± SEM (n = 3), ***P*<0.01; ****P*<0.001 as compared to control group; ##*P*<0.01; ###*P*<0.001 as compared LPS control group. One-way ANOVA followed by Tukey's multiple comparisons test]

injury. There was a significant increase in plasma amylase and lipase levels in the DC group, whereas treatment with *MESPR* significantly decreased the amylase and lipase levels in a dose-dependent manner (Fig. 6A). Further, we investigated the histopathological architecture of the pancreas after L-arginine exposure. In the NC group, normal architecture of the pancreas devoid of oedema, and inflammation was observed, whereas DC group mice showed significant changes in the tissue architecture including pancreatic oedema due to acinar cell haemorrhage, vacuolization, acinar cell necrosis, and excessive neutrophil infiltration. Treatment with *MESPR* showed the protective effects and restored from the abnormal events resulting from L-arginine exposure. *MESPR* dose-dependently maintained the intact acinar cell morphology and reduced inflammatory cells infiltration compared to the DC group (Fig. 4B-D).

Effect of *MESPR* on L-arginine-induced liver injury

To assess the extrapancreatic effects of L-arginine induced AP model on liver, plasma AST and ALT levels were estimated. In the DC group, there was a significant increase in plasma AST and ALT levels (Fig. 6B). Treatment with *MESPR* significantly decreased the AST and ALT levels in a dose-dependent manner. On the other hand, liver histopathological examination further confirmed the protective effect of *MESPR* as evident from improved cellular morphology and liver architecture as shown in Fig. 4B & E.

Effect of *MESPR* on L-arginine-induced pancreatic inflammation

To measure the neutrophils infiltration into the injured pancreatic tissue, MPO activity was evaluated. Our results showed significant increase in the neutrophils infiltration in L-arginine treated DC mice compared to NC animals via measured MPO activity (Fig. 5A). However, treatment with *MESPR* significantly reduced the MPO activity as compared to the DC group. In addition, *MESPR* control mice showed MPO activity similar to NC group. Inflammatory mediators play a prominent role in the pathogenesis of L-arginine-induced AP. To assess the effect of *MESPR* on L-arginine-induced inflammatory cytokines, we performed ELISA to evaluate the levels of pancreatic TNF-α, IL-6 and IL-1β cytokines. Our results revealed a significant elevation of pro-inflammatory cytokines TNF-α, IL-6 and IL-1β levels in L-arginine-induced DC group, whereas, *MESPR* pretreatment significantly inhibited increased inflammatory cytokine TNF-α, IL-6 and IL-1β levels as compared to the DC animals (Fig. 5B). Previous report showed that NF-κB is a master regulator of inflammatory cytokines expression that plays a critical role in the initiation of pancreatic inflammation.[32] In our study, western blot and IHC results showed that phosphorylated NF-κB p65

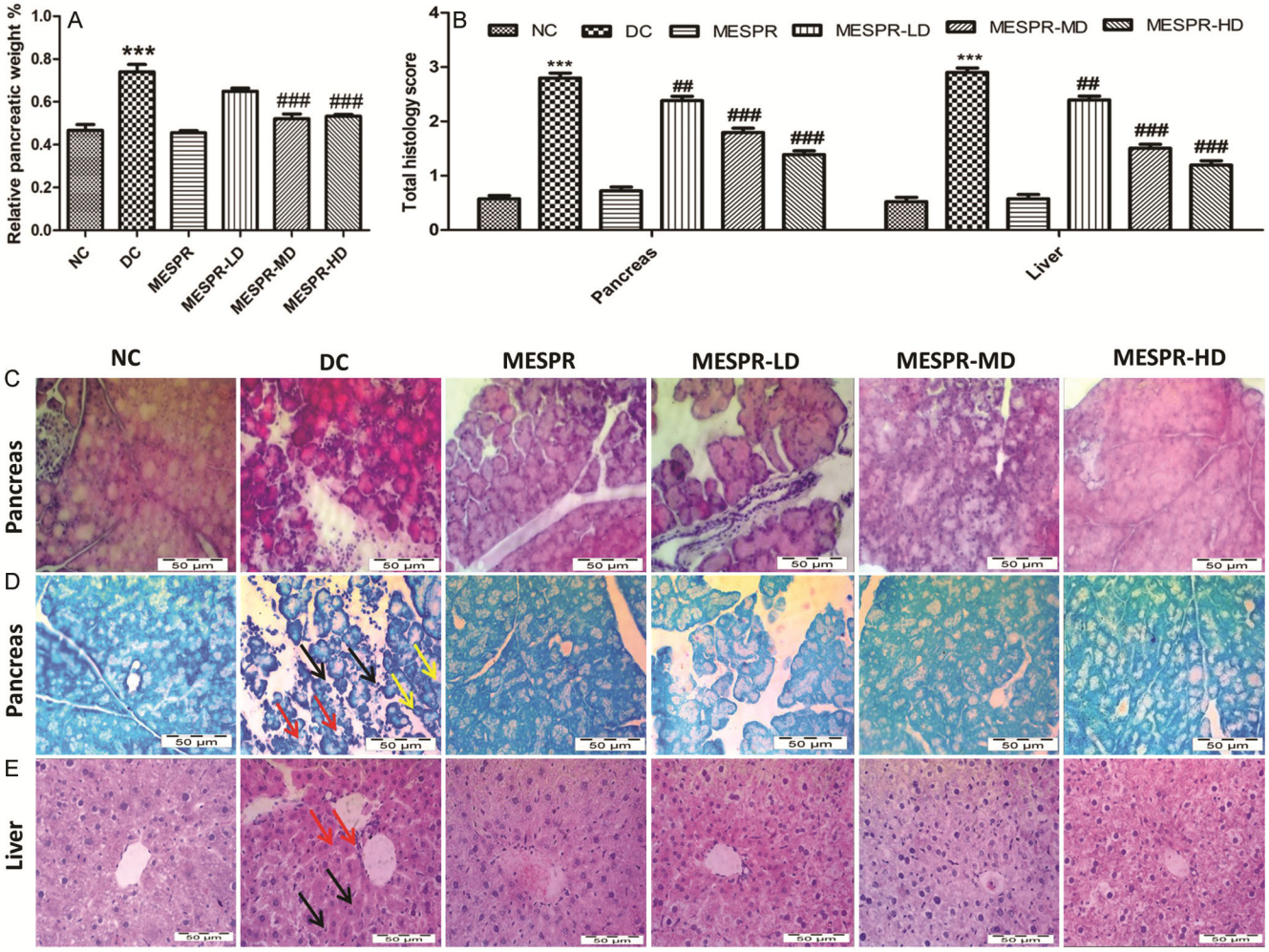

Fig. 4 — Effect of *MESPR* on L-arginine-induced pancreatic and liver injury. (A) Relative pancreatic weight index, (B) Histological scores for pancreatic and liver tissue sections from H & E stained images. (C) Pancreatic tissue sections from H & E stained images observed at a magnification of 40×. (D) Toluidine blue staining images of the pancreatic tissue sections observed at a magnification of 40×. (E) Liver tissue sections from H & E stained images observed at a magnification of 40×. For pancreatic tissue section black arrows indicate the inflammatory cells infiltration, red arrows indicate the acinar cell necrosis and yellow arrows indicate the vacuolization in the exocrine pancreas. For liver tissue section black arrows indicate the Kupffer cells infiltration, red arrows indicate the increased sinusoidal space in the liver. NC: normal control, DC: disease control, *MESPR*: methanolic extract of *Smilax perfoliata* rhizome, *MESPR*-LD: methanolic extract of *Smilax perfoliata* rhizome low dose (30 mg/kg), *MESPR*-MD: methanolic extract of *Smilax perfoliata* rhizome mid dose (100 mg/kg), and *MESPR*-HD: methanolic extract of *Smilax perfoliata* rhizome high dose (300 mg/kg). [All values are represented as mean ± SEM (n = 6). ***P*<0.01; ****P*<0.001 as compared to normal control group; #*P*<0.05; ##*P*<0.01 and ###*P*<0.001 as compared to disease control group. Statistical difference was analyzed one-way ANOVA followed by Tukey's multiple comparisons test]

expression was significantly increased in L-arginine treated DC group, while treatment with *MESPR* significantly suppressed the phosphorylated NF-κB p65 expression compared to the DC group (Fig. 5C-F). However, there were no significant changes in NF-κB p65 expression in *MESPR* alone treated animals. These results suggested that oral administration of *MESPR* produced strong anti-inflammatory effects in L-arginine-induced pancreatic inflammation via inhibiting inflammatory mediators.

Effect of *MESPR* on L-arginine-induced oxidative-nitrosative stress in pancreas

Pancreatic MDA content was estimated to evaluate the severity of pancreatic peroxidation injury. In DC group, MDA levels were significantly increased compared to NC, while treatment with *MESPR* significantly decreased the MDA levels in pancreatic tissue in a dose-dependent manner. Further, pancreatic GSH and nitrite levels were measured to evaluate the extent of oxidative stress. In the DC

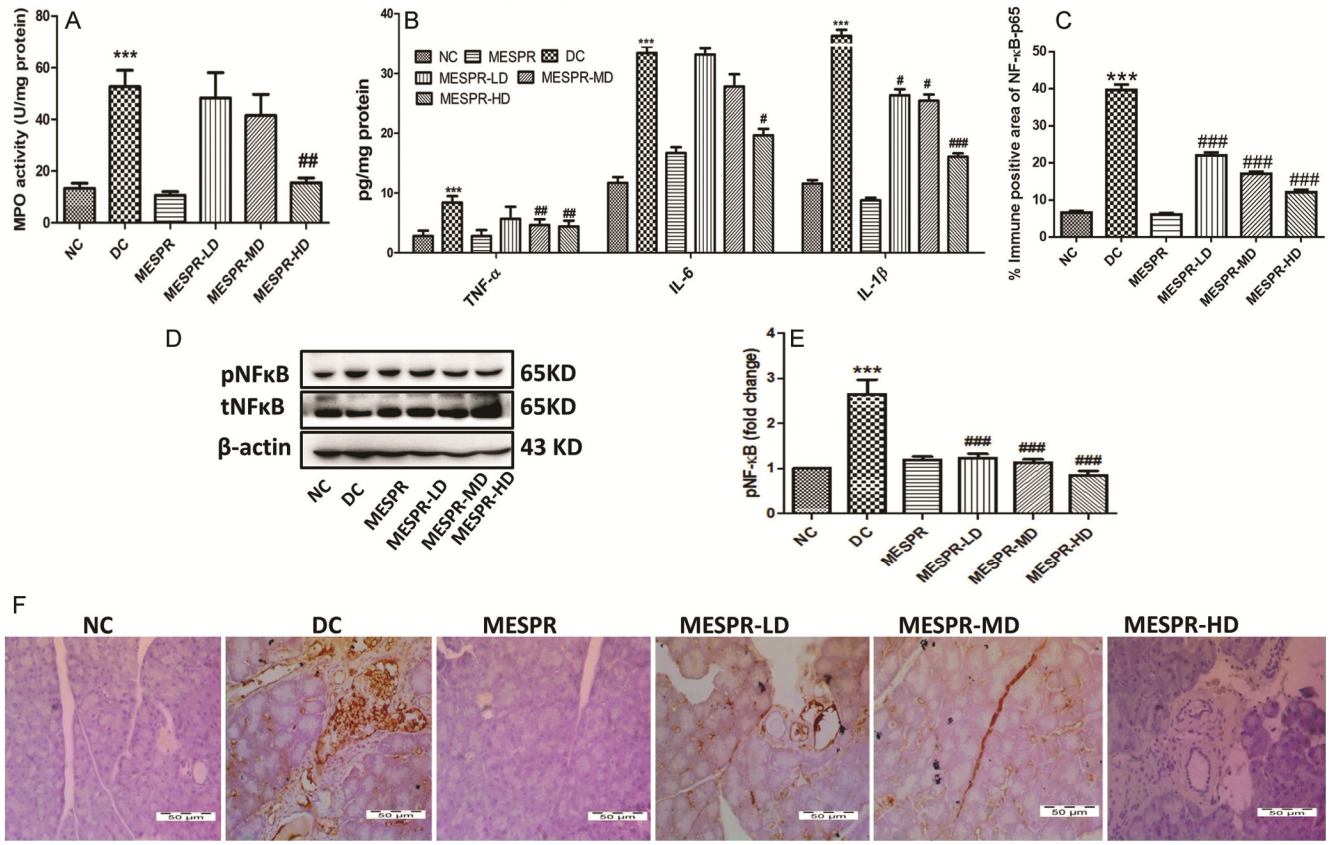

Fig. 5 — Effect of *MESPR* on inflammatory markers in L-arginine induced acute pancreatic tissues. (A) MPO activity, (B) TNF-α, IL-6 and IL-1β levels, (D & E) Immunoblot and quantitative analysis of NF-κB p65, (C & F) Immunohistochemical (IHC) staining images and quantitative analysis of the NF-κB p65 expression by using ImageJ software at 40×. [All values are represented as mean ± SEM (n = 3) ***P<0.001 as compared to control group; #P<0.05; ##P<0.01 and ###P<0.001 as compared to disease control group. Statistical difference was analyzed one-way ANOVA followed by Tukey's multiple comparisons test]

group, GSH levels were found significantly decreased compared to the NC group. Treatment with *MESPR* dose-dependently increased the GSH levels while we found the significant elevation of the pancreatic GSH levels at mid and high dose of *MESPR* compared to the DC group. Similarly, in the DC group, nitrite levels were significantly increased compared to NC group indicating the nitrosative stress in pancreas; treatment with *MESPR* at mid and high doses significantly decreased the nitrite activity compared to DC (Fig. 6C).

Nrf-2 is an antioxidant transcription factor that becomes activated and translocate into the nucleus in response to oxidative-nitrosative stress. To evaluate the effect of *MESPR* on the expression of Nrf-2, SOD1, nitrotyrosine, and iNOS in pancreatic tissue of L-arginine-induced AP were analyzed by western blotting. Nrf-2 is a transcription factor helps in the transcription of many antioxidant enzymes. Nrf-2 and antioxidant enzyme SOD1 were significantly decreased in the DC group compared to the NC group, whereas treatment with *MESPR* significantly restored the Nrf-2 and SOD1 protein expression in dose-dependent manner. However, *MESPR* control group did not show any alteration of these protein expressions. Furthermore, we found protein expression of iNOS, and the nitrotyrosine were upregulated in DC group compared to NC group and *MESPR* treatment resulted in significant downregulation of iNOS and nitrotyrosine expression compared to DC group (Fig. 6D & E).

Discussion

Acute pancreatitis (AP) is a life-threatening condition with a high mortality rate and no specific therapy is currently available. It is initiated by the premature activation of proteolytic zymogens within pancreatic acinar cells, leading to cell injury. This injury triggers the release of pro-inflammatory factors such as IL-1β, IL-6, and TNF-α. Subsequently,

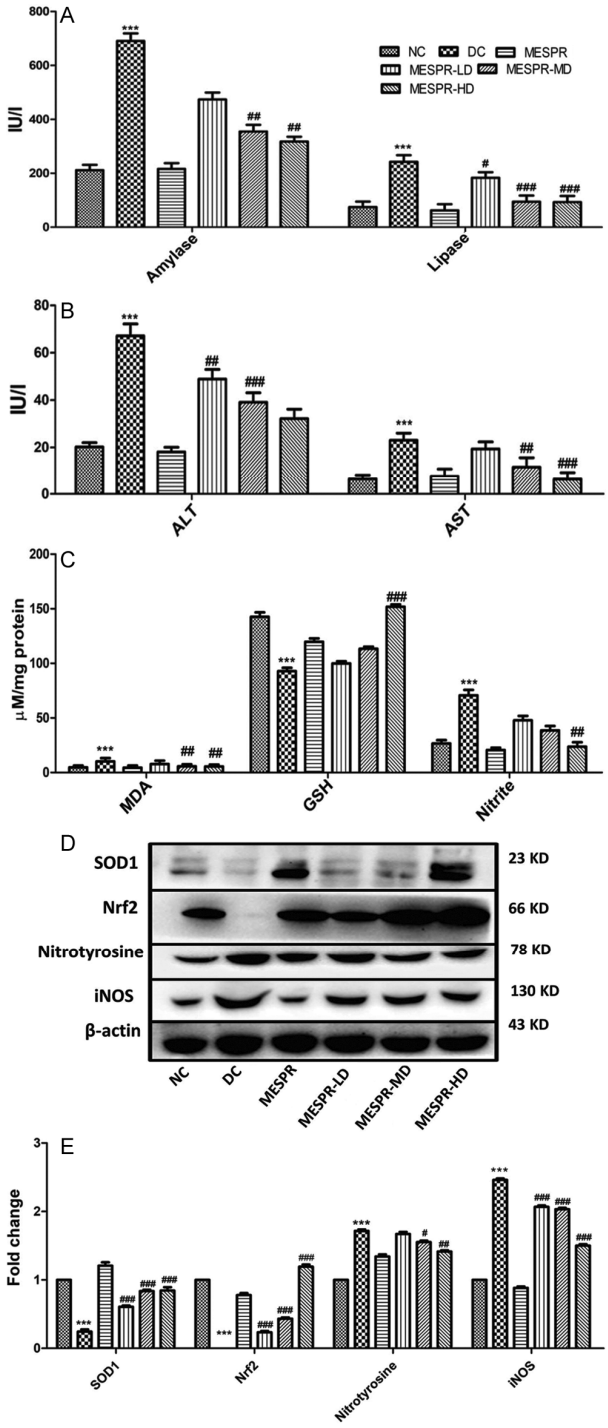

Fig. 6 — Effect of *MESPR* on oxidative-nitrosative stress parameters in pancreatic tissues (A) Amylase and Lipase levels, (B) ALT and AST Levels, (C) MDA, GSH and Nitrite levels. (D & E) Immunoblot and quantitative analysis of Nrf-2, SOD1, nitrotyrosine and iNOS expression. [All values are represented as mean ± SEM (n = 3-6), six mice in each group. ***P<0.001 as compared to control group; #P<0.05, ##P<0.01, and ###P<0.001 as compared to disease control group. Statistical difference was analyzed one-way ANOVA followed by Tukey's multiple comparisons test]

damaged acinar cells release chemokines that attract inflammatory cells, including macrophages and neutrophils, to the pancreas. This accumulation of inflammatory cells exacerbates the local inflammatory response, which can rapidly progress to systemic inflammation[33]. Current treatments for AP are limited to supportive care. Therefore, more effective therapeutic options are needed for better management of this life-threatening condition. Protective effects of natural antioxidants are well known in scavenging ROS for the prevention of oxidative stress induced damage. A variety of antioxidants are known to be present in several medicinal plants, fruits, and vegetables[34]. In this study, antioxidant flavonoid compounds such as Chrysanthellin B, Dihydrohesperetin and Silychrystin were detected in *MESPR* which might be responsible for present antioxidant activities. *MESPR* showed dose-dependent free radical scavenging activity in present study is useful for selecting the concentrations required for its *in-vitro* antioxidant activity in RAW 264.7 macrophages. Our *in vitro* results revealed the promising antioxidant and anti-nitrosative effects of *MESPR* against LPS-induced oxidative and nitrosative stress in RAW 264.7 macrophages. Our results are consistent with the previous reports where methanolic and aqueous extract of *S. glabra* showed antioxidant activity[35]. For the evaluation of *in vivo* activity, L-arginine induced AP mice model was used. Earlier investigations have indicated that L-arginine can be used to establish a model of AP in rodents. L-arginine is a natural amino acid that causes partial inhibition of the L-arginase enzyme which converts L-arginine to L-ornithine and urea. L-arginine is the precursor of nitrate and nitric oxide which induce oxidative stress[36]. It is believed that L-arginine is initiate AP by inducing nitric oxide synthase (NOS), and as a result, interaction of nitric oxide and superoxide radicals leading to the generation of peroxynitrite radicals that are responsible for cellular damage and inflammation[20]. The organ body weight index is an imperative tool for assessing changes in organ size due to the fluid deposition in the organs. In the present study, the DC group showed a significant increase in pancreatic oedema. This effect may be produced due to large doses of L-arginine, which can induce pancreatic oxidative stress and NO production that increased vascular, microcapillary permeability, inflammatory cells infiltration and resulting in pancreatic oedema and acinar cell damage[37]. Oral administration of *MESPR* showed the protective

effect *via* reducing the organ and body weight ratio. Next, we found L-arginine treated DC group revealed a significant increase in plasma amylase and lipase levels after 48 h, which are important biomarkers in the diagnosis of AP[38]. Further, AP was evidenced by the histopathological assessment of pancreatic tissue. It was observed an extensive tissue degeneration of pancreatic acinar cells, severe congestion of blood vessels, and perivascular oedema associated with inflammatory cells infiltration[20]. *MESPR* potentially reduced the pancreatic enzyme levels and pathological alterations at three doses and showed anti-pancreatitis potential against L-arginine-induced AP. Release of IL-1β, IL-6, and TNF-α promotes inflammatory cell infiltration including neutrophils and macrophages to accumulate in the pancreatic tissue, which results in exacerbating the local and systemic inflammatory response in the pancreas and other organs[39,40]. In the present study, pancreatic IL-1β, IL-6, and TNF-α levels were increased after 48 h of L-arginine exposure. This effect may be attributed to L-arginine-induced oxidative and nitrosative stress and production of the pro-inflammatory cytokines[41]. Interestingly, oral administration of *MESPR* significantly lowered pancreatic IL-1β, IL-6, and TNF-α levels. This effect may be attributed to the inhibition of reactive species by *MESPR* resulting in inhibition of pro-inflammatory cytokines production. Further, our current results revealed that L-arginine-induced elevated pancreatic MPO activity was significantly suppressed by *MESPR* pretreatment, and these results suggested that *MESPR* exhibits its inhibitory activity on L-arginine-induced AP through down-regulating the inflammatory cytokines and neutrophils accumulation in the pancreas. The signalling pathways responsible for regulating inflammatory response and cytokine production during the progression of AP has been of great interest. One important signalling molecule, NF-*κ*B has been identified as a critical regulator of inflammation-related genes in the pancreas. Activation of NF-*κ*B in acinar cells worsens pancreatitis severity in mice[42,43]. Our results showed that L-arginine-induced DC group significantly increased phosphorylated NF-κB p65 expression, which may result in the upregulation of inflammatory cytokines and neutrophils infiltration in the pancreas. However, our pharmacological intervention with *MESPR* showed anti-inflammatory effects *via* significant downregulation of phosphorylated NF-*κ*B p65 expression and protected pancreatic damage against L-arginine-induced AP mice. These results suggested that inhibition of inflammatory cascades may play a major role in the attenuation of AP[36].

Further, the present study revealed that L-arginine treatment significantly reduced pancreatic GSH concentration. GSH depletion is a hallmark of the initial phase of AP development and assumed to allow the premature activation of digestive enzymes inside acinar cells, which results into the inflammatory response[44]. On the contrary, pretreatment of *MESPR* significantly increased pancreatic GSH concentration compared to DC mice. Further, L-arginine-induced AP significantly increased pancreatic MDA and nitrite concentration in the pancreas. These effects are in accordance with Ren *et al*. (2019) who reported that the high dose of L-arginine can increase the production of lipid peroxide and decreases the antioxidant enzymes such as GSH and SOD in pancreatic tissues[45]. Moreover, treatment with *MESPR* reversed the effects of L-arginine and inhibited L-arginine-induced oxidative-nitrosative stress. The antioxidant effects of *MESPR* may be due to the radical scavenging activity of its components such as glycosides and flavonoids present in the *MESPR*, which could inhibit lipid peroxidation, nitric oxide and prevent reduced glutathione depletion[22,46]. In addition, nuclear regulator of antioxidant protein Nrf-2 expression was significantly suppressed in L-arginine-treated animals, which was similar to what has been reported previously[11,30]. *MESPR* pretreatment to L-arginine challenged mice induced Nrf-2 activation. In addition to Nrf-2 activation, we also observed the increase of SOD1 expression after *MESPR* treatment in the L-arginine treated AP mice. Increased production of oxidative-nitrosative stress also resulted in a significant increase in iNOS and nitrotyrosine expression in the L-arginine exposed mice. This elevated expression of iNOS and nitrotyrosine was effectively inhibited by *MESPR* treatment and resulted in the suppression of L-arginine-induced oxidative-nitrosative stress. The *MESPR* has demonstrated significant medicinal potential, attributed to its rich content of bioactive compounds. Previous studies on *Smilax* extracts have reported key active ingredients, including saponins (Diosgenin and Smilagenin), flavonoids (Rutin and Kaempferol), polyphenolic compound (Vanillic acid), crude tannins, and β-Sitosterol. These compounds have been shown to exhibit anticancer, anti-inflammatory, antioxidant, and immunomodulatory properties[13]. Phytochemical screening of *MESPR* have

revealed presence of 37 phytocompounds, including bioactive compound Vaccenic acid, β-Sitosterol, γ-Sitosterol, Silychrystin, Dihydrohesperetin, Procyanidin B2 of polyphenolic, flavonoid and saponin groups. The potent pancreatic protective effects observed in the present study are likely due to the synergistic action of these active ingredients. Specifically, the saponins, flavonoids, and polyphenolic compounds may have contributed to the suppression of inflammatory processes by downregulating pro-inflammatory mediators. This suggests that *MESPR* holds promise as a therapeutic agent for inflammatory conditions like acute pancreatitis.

Conclusion

Our results strongly indicated that the *MESPR* exhibits promising protective effects against LPS-induced oxidative stress, inflammation in RAW 264.7 macrophages and L-arginine-induced Acute pancreatitis (AP) mice model. *MESPR* reduced severity of L-arginine-induced AP that involved attenuation of oxidative-nitrosative stress and suppression of inflammatory events through the activation of Nrf-2 and suppression of NF-κB pathways. Thus, present study validated the traditional ethnopharmacological use claims associated with the flavonoid, saponin and polyphenol rich *Smilax perfoliata* rhizome extracts for treatment of inflammatory related syndromes. However, our findings also confer scopes for in-depth exploration of the major bioactive principles, namely, flavonoid, saponin and polyphenols in *MESPR* responsible for demonstrated effect in AP models. This would potentially lead to development of novel pancreatic protective phytomedicine for the effective treatment of Acute pancreatitis (AP).

Conflict of interest

The authors have no declare conflict of interest.

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