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Proteins and lunasin from *Glycine soja* inhibited inflammation by activating the Hippo pathway through phosphorylation of YAP1 kinase

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Abstract

The Hippo pathway has attracted scientific interest as a target for anti-inflammation and anti-cancer therapy. Our objective was to elucidate and compare the potential anti-inflammatory mechanism of digested whole flour (DWF), total protein extract (TPE), lunasin-free total protein extract (LFP), and enriched lunasin protein extract (ELPE) from wild-type soybean (*Glycine soja*) on the Hippo pathway, using a human monocytic cell (THP-1) as a model. ELPE (56% to 73% purity) showed increased lunasin concentrations (52 – 87 mg/g of defatted flour, DF) compared to TPE (16 – 33 mg/g, DF). TPE significantly decreased IL-6, MCP-1, and TNF-α production (96%, 76%, and 52%). *G. soja* ~~effectively~~ inhibited IL-6 production (74% – 98%) more effectively compared to MCP-1 (6% – 99%). ELPE and TPE significantly ($p \leq 0.05$) decreased the expression of dephosphorylated YAP1 and increased phosphorylated YAP1 ($p \leq 0.05$). ELPE significantly increased ($p \leq 0.05$) cytoplasmic YAP1 retention. *G. soja* proteins and peptides inhibited inflammation by decreasing pro-inflammatory cytokines IL-6, IL-1β, and MCP-1, phosphorylating YAP1 and LATS1/2, and increasing YAP1 cytoplasmic retention, thus activating the Hippo pathway. The results suggest that soybean proteins and peptides inhibited inflammation through the Hippo pathway, offering novel developments of functional food ingredients or supplements for a healthier diet.

Keywords: cytoplasmic retention, inflammation, kinase phosphorylation, plant phytochemicals, proteins, soybean

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1. Introduction

The Hippo pathway is important in cell proliferation and differentiation, organ growth and development, embryogenesis, tissue regeneration and numerous other biological processes [1, 2]. As this pathway is involved in both inflammation and cancer progression, there has been increasing research interest on how to modulate this pathway to treat and prevent chronic diseases[3, 4]. In mammals, key regulators of these pathways are the macrophage stimulating 1 (MST1/2), large tumor suppressor kinase (LATS) 1/2, salvador-homolog 1 (SAV1), MPS one binder kinase activator 1 (MOB1), and Yes-associated protein (YAP)/transcriptional coactivator with PDZ-binding motif (TAZ) [5 - 7].

During the activation of this pathway, MST1/2 complexed with SAV1 to phosphorylate the LATS1/2 and MOB1 complex, thus activating them and phosphorylating the YAP/TAZ, leading to YAP/TAZ retention in the cytoplasm and proteosomal degradation[8 - 10]. Nuclear translocation of YAP/TAZ is inhibited in the active Hippo pathway, and in inactive Hippo pathway, YAP/TAZ accumulates in the nucleus, leading to gene transcription which could lead to cancer growth and proinflammatory cytokine secretion[11 - 13]. Overexpression of YAP1 was associated with promotion of M1 pro-inflammatory macrophage polarization and increased IL-6 level[14 - 16]. YAP1 expression was found to be upregulated during monocyte to macrophage differentiation, but not TAZ[3]. MST1/2 have also been found to aggravate inflammation after stimulation by lipopolysaccharide (LPS)[17].

Dysregulation of this pathway will result in a variety of diseases such as cancer, cardiac and pulmonary diseases[16, 18]. Core kinases of Hippo (MST1/2, LATS1/2 and YAP1) have been targeted for drug development efforts[19]. Verteporfin is a drug that targets YAP1 and was found to be able to inhibit inflammation as well as decreasing the expression of

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MST1/2 and LATS1/2 [18, 20 - 22]. VT02956 and XMU-MP-1 are other drugs developed to target LATS1/2 and MST1/2 kinases respectively[23 - 25]. Phytochemicals such as ursolic acid from herbal plants, cucurbitacin B from the Cruciferae and Cucurbitaceae, curcumin from turmeric, and naringin from tomatoes and grapefruits have been studied for their effects on Hippo pathway[26 - 28]. However, not much research has been found on the effects of plant-based peptides on Hippo pathways as most inhibitors were synthetic peptides.

Plant-derived peptides and proteins have been receiving more attention for their potential to prevent inflammation and inflammation-related diseases. Soybean-derived peptides and proteins have been shown to have anti-cancer, antioxidant and anti-inflammatory potentials [29, 30]. Soybean-derived lunasin has been receiving much attention for its anti-inflammation and chemopreventive potential[31]. However, the bulk of studies were done on lunasin from commercial soybean *Glycine max* (*G. max),* while studies on lunasin from wild-type soybean *Glycine soja* (*G. soja)* were scarcer. Extensive studies on the anti-inflammatory potential of soybean proteins and peptides on the three classical signaling pathways (NF-kB, JAK-STAT, MAPK) have also been done, but not so much on the Hippo pathway. *G. soja* is the ancestor of the commercial *G. max*, thus studies on the bioactivity of its peptides could inform the selection and breeding process for future health applications.

Our objective was to elucidate and compare the potential anti-inflammatory mechanisms of digested whole flour (DWF), total protein extract (TPE), lunasin-free total protein extract (LFP), and enriched lunasin protein extract (ELPE) from wild-type *G. soja* soybean on the Hippo pathway, using THP-1 human cells as the inflammation model. We hypothesized that ELPE will be most effective at inhibiting inflammation compared to

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DWF, TPE and LFP *G. soja* treatments, through phosphorylation of core kinases, thus activating the Hippo pathway and decreasing pro-inflammatory cytokine production.

2. Materials and methods

2.1 Materials and reagents

Wild-type (*G. soja*) soybeans were cultivated and harvested at the Central Crop Research Station of North Carolina State University, Clayton, NC, USA. The *G. soja* accessions used in this research were selected from 88 different accessions based on their high protein and lunasin concentrations and with no apparent differences in their protein profiles[32]. Detergent compatible (DC) protein assay kit, 2× Laemmli sample buffer, tricine sample buffer, 10× tris/glycine/SDS buffer, 10x tris/tricine/SDS buffer, mini-PROTEAN® TGX™ gels (4%–20%, 10 well-comb, 30 µL), and Precision Plus Protein™ Dual Xtra standard were purchased from Bio-Rad (Hercules, CA, USA). Simply Blue Safe Stain was purchased from Invitrogen (Carlsbad, CA, USA); 50% acetonitrile (ACN, LC/MS grade, Fisher) and 5% formic acid (FA, LC/MS grade, Fisher). Purified lunasin control (85% purity) was previously purified in our laboratory by Cavazos et al. (2012) (Urbana, Illinois, USA). Verteprofin (>94% purity, HPLC) used as positive control was purchased from Millipore Sigma (Burlington, MA). Primary antibodies GAPDH (Cat# A21994, RRID:AB_2535905), YAP1 (Cat# PA1-46189, RRID:AB_2219137), p-YAP1 (Cat# MA5-33207, RRID:AB_2812021, Ser127), LATS1/2 (Cat# PA5-115498, RRID:AB_2900134), p-LATS1/2 (Cat# PA5-64591, RRID:AB_2664907, Ser909, Ser872), MST1/2 (Cat# PA5-36100, RRID:AB_2553367), p-MST1/2 (Cat# PA5-104616, RRID:AB_2816091, Thr183, Thr180), MOB1 (Cat# PA5-14268, RRID:AB_2145382), SAV1 (Cat# PA5-98927, RRID:AB_2813540) were purchased from Thermo Fisher Scientific (Waltham, MA).

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ELISA kits for human IL-1β (ELH-IL1b-1), IL-6 (ELH-IL6), TNF-α (ELH-TNFa) and MCP-1 (ELH-MCP1) were purchased from RayBiotech (Peachtree Corners, GA). All other reagents were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise stated.

2.2 Protein extraction from defatted soybean flour and protein quantification

Protein extraction from defatted soybean flour was done using the method described peviously[32]. Extracted protein was then freeze-dried and stored at –20 °C for 3 months maximum or until further use. Soluble protein quantification was done using DC protein assay kit according to the manufacturer's protocol (Bio-Rad).

2.3 Purification of lunasin from selected glycine soja accessions

Purification of lunasin was done according to the protocol described in previous publication with some modifications[33]. Briefly, lyophilized soy protein extract was solubilized in Tris-HCl buffer (pH 7.5) then loaded in pre-equilibrated diethylaminoethyl column (DEAE XK 50/30, 30 cm length and 50 mm inside diameter, Cytiva, NJ, USA) with a flow rate of 3 mL/min. Unbound proteins were eluted using Tris-HCl buffer (pH 7.5) with flow rate 3 mL/min, and bound proteins were eluted using NaCl (0.4 M) in Tris-HCl buffer with the same flow rate. The fractions containing lunasin were pooled and concentrated using 3 kDa molecular weight cut-off (MWCO) centrifugal filter (7,500x*g*, 4 °C, 15 min), then loaded again into the column. Fraction containing lunasin was saved and high molecular weight proteins were removed using 50 kDa ultracentrifugation membrane (7,500x*g*, 4 °C, 15 min). Filtrate was passed through 3 kDa ultracentrifugation membrane (7,500x*g*, 4 °C, 15 min) to further remove salts and water. Retentate was saved, freeze-dried and stored at – 20°C for 3 months.

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2.4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Samples were mixed at 1:1 with buffer (2% β-mercaptoethanol and 98% Tricine buffer) and loaded into each well of Tris/Tricine gel. Gel electrophoresis was run with Tris/Tricine/SDS running buffer at 100 V for 300 min. Gel was stained for 1 h with SimplyBlue Safe Stain and washed with distilled water. ImageQuant 800 was used to capture the gel image and ImageJ software was used for analysis. The estimated percentage of lunasin was calculated as the intensity of lunasin band at 5 and 10 kDa (in comparison to purified lunasin) over the sum of the total intensity of all the protein bands.

2.5 Proteomics sample preparation and LC-MS analysis of lunasin

Lunasin bands in the sample were identified using purified lunasin control (85%), and the bands were then cut from the rest of the gel. The bands cut from SDS-PAGE gel were destained with 50% acetonitrile until the dye was removed. The samples were then digested with 1.5 µg of trypsin (Thermo Scientific) in 50 mM triethylammonium bicarbonate buffer (Sigma) overnight at 37°C. The digested peptides were extracted with 5% formic acid in 50% ACN. The combined samples were vacuum-dried. The samples were suspended in 0.1% trifluoroacetic acid (Applied Biosystems) and desalted with StageTips. After drying, the samples were suspended in 20 µL of 5% ACN with 0.1% FA and 1 or 3 µL of each sample was injected into a Ultimate3000 RSLCnano system coupled to a Fusion Orbitrap Tribrid Mass Spectrometer (Thermo Scientific). The peptides were separated with a 50 cm µPAC C18 analytical column (PharmaFluidics) maintained at 30°C over the course of the run. Mobile phases of 0.1% FA (A) and 0.1% FA in 80% ACN (B) were used for the separation. The gradient was 5% to 12.5% B over 10 min, 12.5% to 45% B over 45 min, 45 to 62.5 over 5 min, and then 62.5% to 94% B over 4 min,

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followed by column washing and equilibration. The mass spectrometer was operated in positive polarity in the top speed mode with MS1 scans from 300-2000 m/z at 120 K resolution (100 ms max IT; 2e5AGC) followed by CID fragmentation (35%) of the most abundant ions. The MS2 scans were collected in an ion trap with an isolation window of 1.2 m/z, a maximum IT of 60 ms, and an AGC target of 1e4. Unassigned charged ions were excluded from selection for MS2, and the dynamic exclusion window was 60 s. The raw LC-MS data was processed with Mascot v2.8.2 (Matrix Science Inc, Boston, MA, USA) to identify the proteins. The peptide mass tolerance was set to 10 ppm, and the fragment mass tolerance was set to 0.3 Da. A tryptic digest with a maximum of 2 missed cleavages was specified along with variable modifications of methionine oxidation and protein N-terminal acetylation. A reverse decoy database strategy was used to calculate the false discovery rate (FDR) of the analysis. Searches were done against the Uniprot *G. soja* (75060 entries; downloaded July 2024) reference proteome.

2.6 Lunasin quantification by enzyme-linked immunosorbent assay (ELISA)

Lunasin quantification by ELISA was done according to previously optimized protocol[32, 33]. Briefly, 100 µL of sample (diluted 1:10,000) was loaded per well and left to incubate overnight at 4°C to bind to the plate. The well was washed three times with phosphate buffered saline (PBS, 0.01 M, pH 7.4) before being blocked by 5% bovine serum albumin (BSA, 300 µL per well) for 1 h at 4°C. The washing process was repeated before incubation with the primary lunasin antibody (rabbit polyclonal) diluted 1:200 in 3% BSA (50 µL per well) overnight at 4°C. The wells were washed again followed by incubation by secondary anti-rabbit IgG conjugated to alkaline phosphatase (1:2000 dilution, 50 µL per well). The washing process was repeated and 100 µL of p-nitrophenyl

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phosphate (pNPP) was added to each well. The plate was read at 410 nm after 20 min of incubation at room temperature (24°C), before followed by addition of 100 µL of NaOH (3 M) to each well to stop the reaction. Reading was taken again at 410 nm after 5 min. Previously purified lunasin (85% purity) was used to build the standard curve (0 – 1000 ng/mL).

2.7 Simulated gastrointestinal digestion

Soybean whole flour (defatted) was digested using the harmonized INFOGEST static in vitro simulation of gastrointestinal food digestion protocol[32,34]. Briefly, defatted flour was combined with simulated digestive fluids (salivary, gastric, intestinal) at 1:1 ratio and appropriate proteases during each digestive phase (pepsin for gastric phase; pancreatin for intestinal phase). Digested samples were freeze-dried and stored at – 20°C for 3 months for further testing.

2.8 Cell culture and cell viability assay

Human monocyte THP-1 cell line was cultured in Roswell Park Memorial Institute (RPMI) 1640 growth medium supplemented with 10% FBS, 1% penicillin/streptomycin, and 0.05 mM β-mercaptoethanol at 37°C in 5% CO_2/95% air. THP-1 monocytes were differentiated into macrophages by incubation with 150 nM of phorbol-12-myristate-13-acetate (PMA), then followed by 24 h incubation in PMA-free RPMI medium[35]. Macrophages were seeded at confluency of 5 x 10^4 cells/well in 96-well plate, then incubated with samples (DWF, TPE, LFP and ELPE from PI407207, PI407159, PI407018, PI424088; concentrations 0.1 – 2.0 mg/mL) for 30 min before stimulated with LPS (1 µg/mL) for 24 h. Verteporfin (0.5 µM for Western blot and 2 µM for ELISA) was used as

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the positive control[36]. Cell viability was measured by a colorimetric MTS assay and calculated using the following equation:

$$\%CellViability = \frac{AbsorbanceofTreatment}{AbsorbanceofControl} \times 100$$

2.9 ELISA pro-inflammatory cytokine interleukin in cell culture supernatant

THP-1 cells were seeded and differentiated with 150 nM PMA at confluency of 1 x 10^6 cells/mL in 6-well plates before being treated with different concentrations of DWF, TPE, LFP and ELPE (0, 0.1, 0.5, 1.0 and 2.0 mg/mL) and stimulated with LPS (1 µg/mL). Based on Table 2, IC_{50} values of IL1β were not statistically different for most samples and accessions, except DWF, which was significantly ($p \leq 0.05$) higher.

Supernatant from cell culture treatment was collected to use in ELISA. Protein concentration in the supernatants was quantified and used to adjust and normalize the expression of pro-inflammatory markers in ELISA (IL-1β, IL-6, TNF-α, MCP-1) according to the manufacturer's protocol (Raybiotech).

2.10 Western blot for expression of Hippo pathway kinases

THP-1 macrophages (1 x 10^6 cells/mL) were seeded and differentiated in each well of a 6-well plate and treated with IL-1β IC_{50} values previously determined for DWF (0.6 – 1.9 mg/mL), TPE (0.012 – 0.070 mg/mL), LFP (0.021 – 0.144 mg/mL) and ELPE (0.017 – 0.038 mg/mL). Cell lysates were collected to be used in Western blot[32]. Briefly, 20 µg of sample was loaded into each well and gel electrophoresis was run at 200 V for 35 min, before transferred to membrane. Membrane was blocked with blocking buffer (5% milk in TBST), then with primary antibodies (GAPDH, 1:1000; YAP1, 1:1000; LATS1/2, 1:1000; p-YAP1 (Ser127), 1:500; p-LATS1/2 (Ser909, Ser872), 1:1000; MST1/2, 1:500; p-

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MST1/2 (Thr183, Thr18), 1:500; MOB1, 1:1000; SAV1, 1:1000) overnight at 4°C and secondary IgG anti-rabbit and anti-mouse antibody (1:1000) for 2 h at room temperature (24°C).

2.11 Confocal microscopy and immunofluorescent staining

Immunofluorescent staining and confocal microscopy imaging was done according to previous protocol[37]. Briefly, 3 x 10^5 cells/mL was seeded in Ibidi μ-dish 35 mm and differentiated using 150 nM of PMA. Cells were then treated with samples and stimulated with LPS (1 μg/mL) for 24 h, before they were washed three times (5 min each) with PBS and fixed with 4% formaldehyde aqueous solution (Electron Microscopy Sciences, Hatfield, PA) for 30 min at 25°C. Cells were washed PBS, and permeabilized with 0.1% Triton X 100 in PBS for 15 min at 25°C. Cells were washed once with PBS and incubated with ultracold HPLC-grade methanol for 15 min at −20°C before incubated with PBS at 25°C for 30 min. Cells were blocked with Image-iT FX Signal Enhancer (Life Technologies, Carlsbad, CA) for 30 min at 25°C, washed once with PBS, and incubated with YAP1 (1:200) antibody 3 h at 25°C. Cells were washed three times (5 min each) with PBS and incubated with Alexa Fluor 568 Goat Anti-Rabbit IgG (Life Technologies) secondary antibody (1:200) for 90 min at 25°C in the dark. Cells were washed three times (5 min each) with PBS and cured with ProLong Gold antifade reagent with DAPI (4',6-diamidino-2-phenylindole) (Life Technologies) for 24 h at 25°C in the dark. The microscopy chamber plate was stored at 4°C in the dark until further analysis. The cells were visualized using Carl Zeiss LSM 880 Laser Scanning Microscope (Carl Zeiss AG, Germany) with 63×oil immersion objective. The laser was set at 561 nm wavelength excitation and 645 nm wavelength emission with 1.0 AU pinhole to visualize YAP1. For

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DAPI, the laser was set a 405 nm wavelength excitation and 475 nm wavelength emission with 0.99 AU pinhole. Five different fields were imaged per treatment and the integrated density of YAP1 expression was analyzed from each field using the FIJI software[38] with the average of the integrated density results normalized per cell. Images were scanned in high-resolution format (512×512 pixels; 0.07 UM X 0.07 UM, 0.26 μM x 0.26 μM scaling per pixel). Fluorescence integrated density of total, cytoplasmic and nuclear YAP1 expressions were taken. Single cells were measured by free-hand drawing tool in FIJI software. Cytoplasmic YAP1 fluorescence integrated density was calculated as the fluorescence integrated density of nuclear YAP1 expression subtracted from the fluorescence integrated density of total YAP1 expression in cell. Results were presented as the average fluorescence integrated density of five different fields, and as the nuclear:cytoplasmic YAP1 expression ratio.

2.12 Statistical analysis

Data was expressed as the means of two independent replicates. The results were analyzed using one-way and two-way ANOVA followed by Tukey's multiple comparison test. Differences were considered statistically significant at $p \leq 0.05$. GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA) was used to perform all the statistical analyses.

3. Results

3.1 ELPE contained 50% more lunasin than TPE

Previous study found that out of 88 *G. soja* accessions, four accessions were found to have the highest lunasin concentration and these were selected to purify lunasin and

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studied their anti-inflammatory potentials[32]. Lunasin polyclonal antibody identified bands at 5 and 10 kDa from fractions eluted at 8 and 9 min from the ion exchange chromatogram (**Figure 1A – B**). Proteomics analysis of *G. soja* accession PI424088 found a fragment of lunasin peptide sequence HIMEK in protein band at 5 kDa at elution time 8 min, and HIMEKIQRGDDDDDDDDD in protein band at 5 kDa at elution time 9 min (**Figure 1C**), confirming the presence of lunasin peptide. Lunasin antibody was able to bind to protein band at 10 kDa, indicating that lunasin was present as both monomer at 5 kDa and dimer at 10 kDa (**Supplementary Figure 1**). Quantification of lunasin concentration by ELISA revealed that ion-exchange chromatography increased concentration of lunasin significantly ($p \leq 0.05$) in ELPE of accessions PI407207, PI407159, PI407018, PI424088 (51.6 – 86.9 mg/g DF) in comparison to lunasin concentration in TPE (16.2 – 32.9 mg/g DF) (**Figure 1D**). Analysis of SDS-PAGE results indicated that lunasin purity ranged from 55.5% – 72.5% (**Figure 1E**). These results demonstrated that lunasin was present in ELPE as dimer and monomer with purity up to 73%, and its concentration was significantly increased compared to TPE.

3.2 Digested whole flour, total protein extract, lunasin-free protein and enriched lunasin protein extract from *G. soja* accessions significantly lowered the levels of pro-inflammatory cytokines IL-1β, IL-6, and MCP-1

Treatments of THP-1 macrophages with different concentrations (0.1, 0.5, 1.0 and 2.0 mg/mL) of DWF, TPE, LFP, ELPE from *G. soja* did not significantly decrease cell viability compared to untreated macrophages ($p \leq 0.05$) (**Supplementary Figure 2**). Treatments with 0.5 μM and 2 μM verteporfin (positive control) also did not significantly decrease viability of macrophages compared to untreated cells ($p \leq 0.05$). These results indicated

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that *G. soja* treatments did not have cytotoxic effect.

Production of pro-inflammatory cytokine IL-1β was inhibited significantly after treatment with different concentrations (0.1, 0.5, 1.0, 2.0 mg/mL) of *G. soja* DWF, TPE, LFP and ELPE in LPS-stimulated macrophages. **Table 1** presents the results of IL-1β IC_{50} (mg/mL) for the different samples tested. IC_{50} is defined as the amount (mg/mL) of sample needed to inhibit 50% of the activity, in this case the production of IL-1β. Lower IC_{50} value indicated higher effectiveness of sample to inhibit 50% of IL-1β production. IL-1β IC_{50} values of DWF (0.6 – 1.9 mg/mL), TPE (0.012 – 0.070 mg/mL), LFP (0.021 – 0.144 mg/mL) and ELPE (0.017 – 0.038 mg/mL) were used to test other biomarkers. DWF of all *G. soja* accessions (PI407207, PI407159, PI407018, PI424088) had significantly higher ($p \leq 0.05$) IC_{50} values compared to TPE, LFP and ELPE, indicating that the latter were more effective than DWF in inhibiting IL-1β production. **Table 1** also shows that IL-1β production for TPE, LFP and ELPE was not statistically different for all accessions. IC_{50} values of *G. soja* ELPE were significantly higher ($p \leq 0.05$) than *G. max* ELPE (more potent) for all accessions. IC_{50} values of *G. soja* LFP were significantly lower ($p \leq 0.05$) than *G. max* ELPE for all accessions except for PI424088. These results also indicate that different accessions of DWF, TPE, LFP and ELPE have different efficacy to inhibit inflammation.

After treatment with IC_{50} values, obtained from assessing the IL-1β inhibition of all samples, the production of pro-inflammatory cytokine IL-6 was significantly decreased ($p \leq 0.05$) by treatments of *G. soja* DWF, TPE, LFP and ELPE in LPS-stimulated macrophages (**Figure 2A – D**). Comparing among different sample types within the same accession (**Figure 2E**), treatments with TPE and LFP in some accessions (PI407207 and

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PI424088) significantly ($p < 0.05$) lowered IL-6 production compared to DWF and ELPE. These results demonstrated that production of IL-6 was significantly decreased ($p \leq 0.05$) in comparison to the control LPS by all sample types and all accessions; however, some samples in different accessions showed different inhibitory potential. *G. soja* LFP of several accessions seemed the most effective at inhibiting IL-6 production as it demonstrated the highest inhibition (98%).

Potential of *G. soja* to inhibit MCP-1 production varied among sample types and accessions. Production of pro-inflammatory cytokine MCP-1 was not significantly decreased ($p > 0.05$) by treatment with DWF in comparison to control LPS, except for macrophages treated with DWF of accession PI407207 (68% inhibition) (**Figure 3A**). Treatments TPE, LFP and ELPE significantly decreased ($p \leq 0.05$) the production of MCP-1 for all accessions (PI407207, PI407159, PI407018, PI424088) compared to control LPS (31% - 99% inhibition) (**Figures 3B – D**). Comparing among different sample types within the same accession, treatments with ELPE of accessions PI407159 (92% inhibition) and PI407018 (97% inhibition) showed significantly lower ($p \leq 0.05$) MCP-1 production compared to treatments with DWF, TPE and LFP of the same accessions. Treatments with DWF, TPE and LFP of accession PI407207 showed significantly lower ($p \leq 0.05$) MCP-1 inhibition, (68%, 64%, 59%, respectively) than ELPE (41% inhibition) of the same accession. These results indicate that different sample type obtained from the same *G. soja* accession could have significantly different ($p \leq 0.05$) inhibitory potential due to the different chemical composition. *G. soja* ELPE of accession PI407018 seemed to be the most promising at inhibiting MCP-1 (97%) (**Figure 3E**).

Production of TNF-α was not significantly decreased ($p > 0.05$) by treatments with

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DWF, TPE, LFP and ELPE compared to control LPS, except for TPE (52% inhibition) of accession PI424088 (**Figures 4A – D**). Verteporfin (2 μM) also did not significantly decrease ($p > 0.05$) production of TNF-α in comparison to control LPS. This marker did not seem to be significantly affected by *G. soja* treatments.

Table 2 provided a summary of the increase and decrease in production of IL-6, IL-1β, TNF-α, and MCP-1 after treatment with samples DWF, TPE, LFP, ELPE from accessions PI407207, PI407159, PI407018, and PI424088 in comparison to LPS. All accessions and sample types were able to significantly inhibit production of IL-6 (74% - 98% inhibition), but not TNF-α. Only TPE of accession PI424088 was able to significantly decrease ($p \leq 0.05$) all four pro-inflammatory cytokines tested (IL-1β, IL-6, TNF-α, MCP-1) in comparison to control LPS. TPE, LFP and ELPE of all accessions were able to significantly decrease ($p \leq 0.05$) production of three pro-inflammatory cytokines (IL-6, IL-1β, MCP-1) in comparison to control LPS. All sample types of accession PI407207 were effective in inhibiting production of IL-6, IL-1β and MCP-1. These results demonstrated that *G. soja* treatments were able to inhibit inflammation *in vitro* through decreased production of pro-inflammatory cytokines.

3.3. Digested whole flour, total protein extract, lunasin-free protein and enriched lunasin protein extract from *G. soja* decreased the expression of dephosphorylated YAP1 and LATS1/2 kinases

Western blot results showed that expression of dephosphorylated YAP1 decreased significantly after treatment by DWF, TPE, LFP and ELPE across all accessions of *G. soja* ($p \leq 0.05$) in comparison to LPS-stimulated macrophages, except for DWF of

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accession PI424088 (**Figure 5A – D**). TPE of accession PI424088 and ELPE of accession PI407207 were the only treatments that significantly ($p < 0.05$) increased the expression of phosphorylated YAP1 (p-YAP1) (**Figures 5A – D**). These results demonstrated that while *G. soja* treatments (DWF, TPE, LFP and ELPE of all accessions) could significantly decrease ($p \leq 0.05$) the expression of YAP1 compared to LPS, only two treatments (ELPE of PI407207 and TPE of PI424088) were able to significantly increase the expression of p-YAP1 compared to the control LPS. These results suggested that *G. soja* TPE and ELPE were able to phosphorylate the Hippo core kinase YAP1.

In addition, *G. max* was tested to compare LFP and ELPE of accessions PI567313 and PI594845 and showed that both treatments were able to significantly decrease ($p \leq 0.05$) the expression of YAP1 compared to control LPS (**Figure 5E – F**). *G. max* LFP of accession PI594845 and *G. max* ELPE of accessions PI567313 and PI594845 were also able to significantly increase p-YAP1 expression compared to control LPS.

G. soja DWF, TPE, LFP and ELPE of all accessions were able to significantly decrease ($p \leq 0.05$) the expression of dephosphorylated LATS1/2 compared to LPS (**Figures 6A – D**). Expression of phosphorylated LATS1/2 (p-LATS1/2) was increased significantly ($p \leq 0.05$) by *G. soja* DWF of accessions PI407207, PI407018 and PI424088 in comparison to control LPS (**Figure 6A**). Expression of p-LATS1 was not increased significantly ($p \leq 0.05$) by *G. soja* TPE, LFP and ELPE of all accessions compared to control LPS (**Figure 6B – D**). *G. soja* treatments of all accessions (PI407207, PI407159, PI407018 and PI424088) and all sample types (DWF, TPE, LFP, ELPE) were able to significantly lower ($p \leq 0.05$) expression of dephosphorylated LATS1/2, however, only *G. soja* DWF of accessions PI407207, PI407018, and PI424088 were able to significantly

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increase ($p \leq 0.05$) expression of p-LATS1/2 compared to control LPS. These results indicate that *G. soja* DWF was able to phosphorylate Hippo pathway central regulator LATS1/2.

G. max LFP of accessions PI567313 and PI594845 were able to significantly decrease ($p \leq 0.05$) LATS1/2 expression and significantly increase ($p \leq 0.05$) p-LATS1/2 expression compared to control LPS (**Figure 6E**); however, *G. max* ELPE of accessions PI567313 and PI594845 were not able to significantly increase ($p \leq 0.05$) p-LATS1/2 expression compared to control LPS (**Figure 6F**).

MST1/2 kinase was not significantly ($p < 0.05$) affected by treatments with *G. soja* (**Figures 7A – D**). Only four treatments (DWF PI407207 and PI407159, LFP PI407207 and ELPE PI407159) were able to significantly decrease ($p < 0.05$) expression of dephosphorylated MST1/2 compared to control LPS. DWF of accession PI407207 was the only treatment that significantly ($p < 0.05$) increased phosphorylated MST1/2 expression compared to control LPS. These results suggested that *G. soja* treatments did not seem to affect MST1/2 kinase.

Treatment with *G. soja* DWF of accession PI424088 significantly decreased ($p \leq 0.05$) expression of MOB1 compared to control LPS (**Figure 8A**), while treatment with *G. soja* TPE of accession PI424088 significantly increased ($p \leq 0.05$) expression of MOB1 compared to control LPS (**Figure 8B**). Treatments with *G. soja* LFP and ELPE did not significantly affect ($p > 0.05$) expression of MOB1 compared to control LPS (**Figures 8C – D**).

Treatments with *G. soja* DWF of accessions PI407159 and PI424088 were able to

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significantly decrease expression of SAV1 compared to control LPS (**Figure 9A**). Treatment with *G. soja* TPE of accession PI407207 was able to significantly decrease expression of SAV1 compared to control LPS (**Figure 9B**). Treatments with *G. soja* LPF and ELPE of all accessions did not significantly affect ($p > 0.05$) expression of SAV1 compared to control LPS (**Figures 9C – D**). MOB1 and SAV1 regulatory proteins did not seem to be significantly affected by *G. soja* treatments.

Table 2 provides a summary of the effect on the expressions of kinases after treatments with different *G. soja* accessions (PI407207, PI407159, PI407018, PI424088) and different sample types (DWF, TPE, LFP, ELPE) in comparison to control LPS. Treatments of macrophages with ELPE of PI407207 and TPE of PI424088 were able to phosphorylate YAP1. Treatments of macrophages with DWF of PI407207, PI407018 and PI424088 were able to phosphorylate LATS1/2. Treatments of macrophages with DWF of PI407159 were able to phosphorylate MST1/2. These results suggested that *G. soja* treatments could modulate the Hippo pathway through phosphorylation of the kinases LATS1/2 and YAP1, which are the central regulator and the core kinase of the pathway, respectively.

3.4 Treatments with *G. soja* ELPE PI407207 and TPE PI424088 increased cytoplasmic YAP1 retention

Cells were treated with ELPE of PI407207 (IC_{50} = 0.017 mg/mL) and TPE of PI424088 (IC_{50} = 0.070 mg/mL) as these treatments had been shown to be able to significantly ($p \leq 0.05$) decrease dephosphorylated YAP1 and increase phosphorylated YAP1 expression compared to control LPS through Western blot. **Figure 10A** shows immunofluorescence staining images of YAP1 expression and DAPI-stained nuclei indicating the location of

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YAP1 in the macrophages. Further analysis showed that cells treated with *G. soja* ELPE PI407207 showed significantly higher ($p \leq 0.05$) cytoplasmic YAP1 expression compared to control LPS (**Figure 10B**), and cells treated with *G. soja* TPE PI424088 showed significantly lower ($p \leq 0.05$) nuclear YAP1 expression compared to control LPS (**Figure 10C**). Calculation of the nuclear:cytoplasmic YAP1 expression ratio showed that cells treated with *G. soja* ELPE PI407207 had a significantly lower ($p \leq 0.05$) ratio compared to control LPS (**Figure 10D**). These results indicated that treatment with ELPE was able to modulate the Hippo pathway by increasing the cytoplasmic retention of its core kinase YAP1.

4. Discussion

Previous lunasin purification studies done using the same method reported lunasin purity that ranged from 80% to 99% [33, 40]. Our results showed higher purity (56% to 73%) compared to lunasin purification done through recombinant protein expression method (46% - 52%) [41, 42]. Previous studies evaluating the anti-inflammatory and other bioactive potentials of lunasin were done using chemically synthesized lunasin with at least 95% purity [43 - 46]. However, there were other studies evaluating the anti-inflammatory potential and other health benefits of lunasin-enriched protein extracts instead of pure lunasin that showed lunasin-enriched protein extracts had antimicrobial and anti-inflammatory potentials[40, 47].

Comparing sample types, DWF of all accessions seemed to be the least effective in mitigating inflammation through reduction of pro-inflammatory cytokines, and DWF of all accessions were shown to have significantly higher ($p \leq 0.05$) IC_{50} values compared to

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TPE, LFP and ELPE. DWF was the only sample type to have gone through simulated gastrointestinal digestion, and previous study had shown that bioaccessibility of protein and lunasin decreased in digested soybean flour[32]. Except for accession PI407207, DWF did not significantly decrease ($p \leq 0.05$) production of MCP-1 compared to control LPS, while TPE, LFP and ELPE of all *G. soja* accessions were able to do so. However, DWF was able to phosphorylate LATS1/2 expression compared to control LPS (**Table 2**). DWF of accession PI407159 were also the only treatment able to phosphorylate MST1/2. These results suggested that *G. soja* DWF could affect the Hippo pathway at the upstream kinases MST1/2 and LATS1/2, thus indirectly regulating YAP1 kinase as studies have shown that MST1/2 and LATS1/2 were involved in the regulation of YAP1 expression and activation [48, 49].

Increased expression of LATS1/2 had been associated with inflammation-related diseases such as inflammatory bowel disease (IBD) and diabetes [50, 51]. The inhibition of LATS1/2 had been shown to suppress growth of cancer cells and inhibit inflammation, therefore, inhibition of LATS1/2 had also been proposed as therapeutic target for cancer and inflammation-related diseases [52 - 55]. A LATS1/2-specific inhibitor such as Src that induce LATS1/2 phosphorylation to modulate the Hippo pathway had also been studied[56], however, much of the research focused on the other kinases and mechanisms that could induce LATS1/2 phosphorylation such as WWC1/2/3 and angiomotins instead of specific compounds[57, 8]. Thus, *G. soja* DWF could be explored as potential LATS1/2-specific inhibitors.

Overexpression of YAP1 was associated with tumorigenesis and reduced patient survival rate in cancer progression [58, 59]. Increased expression of YAP1 was also

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associated with inflammation in diseases such as psoriasis and fibrosis [60, 61, 11]. The inhibition of YAP1 could be a therapeutic target for cancer suppression and inflammation-related diseases [62, 16, 63] and studies had also shown that phosphorylation of YAP1 to be important in anticancer mechanisms [64 - 66]. YAP1 had been considered a therapeutic target for cancer and inflammation, and YAP1-specific inhibitors such as verteporfin and CA3 had been evaluated for their ability to decrease YAP1 protein level [67 - 69]. Several peptides had been shown to be YAP1 inhibitors, however, those were either chemically synthesized or purified through recombinant protein expression method[70]. Treatments with *G. soja* ELPE and TPE were found to be able to phosphorylate YAP1 expression compared to control LPS, thus they could be explored as potential YAP1-specific inhibitors. Phosphorylation of YAP1 had also been shown to inhibit YAP1 nuclear translocation and increase YAP1 cytoplasmic retention[71], and our results had shown that ELPE PI407207 was also able to increase YAP1 cytoplasmic retention.

TPE of accession PI424088 was also the only treatment that was able to significantly decrease ($p \leq 0.05$) the expression of all pro-inflammatory cytokines tested (IL-1β, IL-6, TNF-α and MCP-1). This treatment was also able to phosphorylate YAP1, ($p \leq 0.05$) expression compared to control LPS. In addition to significantly reducing ($p \leq 0.05$) nuclear YAP1 expression compared to control LPS. Except for TNF-α, *G. soja* of all accessions and sample types were able to significantly decrease ($p \leq 0.05$) production of IL-6 and MCP-1 compared to control LPS. TPE is the total soluble protein extracted from defatted soybean flour, which had been evaluated to include other proteins (lipoxygenase, β-amylase; Kunitz trypsin inhibitor), β-conglycinin, glycinin and Bowman-Birk inhibitor, in addition to lunasin [72 - 74]. **Supplementary Figure 3** shows that LFP contained β-

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conglycinin, as shown by bands appearing between 50 kDa to 75 kDa, but not lunasin. Bowman-Birk inhibitor and β-conglycinin had been shown to exhibit anti-inflammatory potential [75 - 78, 24]. A study also showed that the combination of lunasin, Bowman-Birk inhibitor and Kunitz trypsin inhibitor was able to exert anti-inflammatory potential through decreasing production of nitric oxide, IL-6 and TNF-α[46].

Increased phosphorylation of LATS1/2 and YAP1 had been shown to be able to reduce inflammation[71], therefore the *G. soja* treatments that were able to both decrease the expression of dephosphorylated LATS1/2 and YAP1, as well as increase the expression of p-LATS1/2 and p-YAP1, could be explored as either LATS1/2-specific or YAP1-specific inhibitors. *G. soja* ELPE and TPE could be explored as YAP-1 specific inhibitors, while DWF could be explored as LATS1 specific inhibitors. As YAP1 phosphorylation increased YAP1 cytoplasmic retention and inhibited YAP1 nuclear translocation, we also showed that *G. soja* ELPE and TPE had potential to be YAP1 nuclear translocation inhibitor. TPE showed promising potential as pro-inflammatory cytokine production inhibitor. While there are phytochemicals that had been studied as modulators of Hippo pathway[20], to our knowledge, this is the first report on ancestral soybean proteins and peptides that had been studied for their effect on the Hippo pathway so far. **Figure 11** presents a diagram of the active (left) and inactive (right) Hippo pathway in the presence and absence of the ELPE. This research offered more insight into alternative potential targets for inflammation and inflammation-related diseases.

5. Conclusion

The purification process used was able to significantly increase lunasin concentration,

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which then increased the potential to mitigate inflammation. In general, purified lunasin and total protein extract were most effective in inhibiting inflammation. *G. soja* was able to mitigate inflammation by decreasing pro-inflammatory cytokines IL-6, IL-1B and MCP-1, and activating the Hippo pathway through kinase-specific phosphorylation and YAP1 cytoplasmic retention. These results could be used to develop new functional food ingredients or supplements that offer alternative and novel therapeutic targets for a healthier diet to manage inflammation-related diseases.

Abbreviations

DWF	digested whole flour
ELISA	enzyme-linked immunosorbent assay
ELPE	enriched lunasin protein extract
IL	interleukin
LATS	large tumor suppressant
LFP	lunasin-free protein
LPS	lipopolysaccharide
MCP	monocyte chemoattractant protein
MOB	MPS one binder
MST	macrophage stimulating
SAV	Salvador homolog
TAZ	transcriptional coactivator with PDZ-binding motif
TNF	tumor necrosis factor
TPE	total protein extract
VP	verteporfin
YAP	Yes-associated protein

CRediT authorship contribution statement

Jennifer Kusumah: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Jiazheng Yuan**: Resources, Visualization, Methodology, Investigation, Data curation, Conceptualization. **Elvira Gonzalez de Mejia:** Writing – review & editing, Supervision, Resources, Visualization, Project administration,

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Methodology, Funding acquisition, Conceptualization. All authors read and approved the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at xxx

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Figure legends

Figure 1. (A) Ion-exchange chromatogram. (B) SDS-PAGE of fractions eluted by ion-exchange chromatography. Red box indicate protein band where presence of lunasin was detected. (C) LC-MS results of lunasin peptide sequence found in protein band at 5 kDa from fraction eluted at 9 min; aspartic acid tail and chromatin-binding sequence. (D) Comparison of lunasin concentrations in total protein extract and enriched lunasin protein extract. Data was expressed as means of two independent replicates. Asterisks indicate significant differences according to two-way ANOVA (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). (E) Purity of lunasin (%).

Figure 2. Production of IL-6 expressed as pg per mg of protein in macrophages stimulated with LPS and treated with *G. soja* samples. Data is shown as the mean of two independent replicates and analyzed using one-way ANOVA. (A) Cells treated with digested whole flour. (B) Cells treated with total protein extract. (C) Cells treated with lunasin-free protein. (D) Cells treated with enriched lunasin protein extract. (E) Comparison of IL-6 produced in cells after different treatments. Letters A – C showed significant differences ($p < 0.05$) among different accessions within same sample type. Letters W – X showed significant differences ($p < 0.05$) among different sample type within same accession. LPS= lipopolysaccharide; UNT = untreated cells; VP=Verteporfin used as a positive control.

Figure 3. Production of MCP-1 expressed as pg per mg of protein in macrophages stimulated with LPS and treated with *G. soja* samples. Data is shown as the mean of two independent replicates and analyzed using one-way ANOVA. (A) Cells treated with

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G. soja digested whole flour. (B) Cells treated with *G. soja* total protein extract. (C) Cells treated with *G. soja* lunasin-free protein. (D) Cells treated with *G. soja* enriched lunasin protein extract. (E) Comparison of MCP-1 produced in cells after different treatments. Letters A – D showed significant differences ($p < 0.05$) among different accessions within same sample type. Letters W – X showed significant differences ($p < 0.05$) among different sample type within same accession.

Figure 4. Production of TNF-α expressed as pg per mg of protein in macrophages stimulated with LPS and treated with *G. soja* samples. Data is shown as the mean of two independent replicates and analyzed using one-way ANOVA. (A) Cells treated with *G. soja* digested whole flour. (B) Cells treated with *G. soja* total protein extract. (C) Cells treated with *G. soja* lunasin-free protein. (D) Cells treated with *G. soja* enriched lunasin protein extract. (E) Comparison of TNF-α produced in cells after different treatments. Letters A – D showed significant differences ($p < 0.05$) among different accessions within same sample type. Letters W – X showed significant differences ($p < 0.05$) among different sample type within same accession.

Figure 5. Expression of YAP1 and p-YAP1 represented as fold change compared to LPS. Data was expressed as means of two independent replicates. Different letters showed statistical significance ($p < 0.05$) according to one-way ANOVA. (A) Cells treated with *G. soja* digested whole flour. (B) Cells treated with *G. soja* total protein extract. (C) Cells treated with *G. soja* lunasin-free protein. (D) Cells treated with *G. soja* enriched lunasin protein extract. (E) Cells treated with *G. max* lunasin-free protein. (F) Cells treated with *G. max* enriched lunasin protein extract. Asterisks (*) represent statistically significant difference ($p < 0.05$) in the expression of YAP1 compared to p-YAP1 according to Student's t-test. Different letters A – C represent statistically significant differences for YAP1 expression among different accessions ($p < 0.05$) according to one-way ANOVA. Different letters X – Z represent statistically significant differences for p-YAP1 expression among different accessions ($p < 0.05$) according to one-way ANOVA.

Figure 6. Expression of LATS1/2 and p-LATS1/2 represented as fold change compared to LPS. Data was expressed as means of two independent replicates. Different letters

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showed statistical significance ($p < 0.05$) according to one-way ANOVA. (A) Cells treated with *G. soja* digested whole flour. (B) Cells treated with *G. soja* total protein extract. (C) Cells treated with *G. soja* lunasin-free protein. (D) Cells treated with *G. soja* enriched lunasin protein extract. (E) Cells treated with *G. max* lunasin-free protein. (F) Cells treated with *G. max* enriched lunasin protein extract. Asterisks (*) represent statistically significant difference ($p < 0.05$) in the expression of LATS1/2 compared to p-LATS1/2 according to Student's t-test. Different letters A – B represent statistically significant differences for LATS1 expression among different accessions ($p < 0.05$) according to one-way ANOVA. Different letters X – Z represent statistically significant differences for p-LATS1 expression among different accessions ($p < 0.05$) according to one-way ANOVA.

Figure 7. Expression of MST1/2 and p-MST1/2 represented as fold change compared to LPS. Data was expressed as means of two independent replicates. Different letters showed statistical significance ($p < 0.05$) according to one-way ANOVA. (A) Cells treated with *G. soja* digested whole flour. (B) Cells treated with *G. soja* total protein extract. (C) Cells treated with *G. soja* lunasin-free protein. (D) Cells treated with *G. soja* enriched lunasin protein extract. Asterisks (*) represent statistically significant difference ($p < 0.05$) in the expression of MST1/2 compared to p- MST1/2 according to Student's t-test. Different letters A – C represent statistically significant differences for MST1 expression among different accessions ($p < 0.05$) according to one-way ANOVA. Different letters X – Z represent statistically significant differences for p-MST1 expression among different accessions ($p < 0.05$) according to one-way ANOVA.

Figure 8. Expression of MOB1 represented as fold change compared to LPS. Data was expressed as means of two independent replicates. Different letters showed statistical significance ($p < 0.05$) according to one-way ANOVA. (A) Cells treated with *G. soja* digested whole flour. (B) Cells treated with *G. soja* total protein extract. (C) Cells treated with *G. soja* lunasin-free protein. (D) Cells treated with *G. soja* enriched lunasin protein extract. Different letters A – C represent statistically significant differences ($p < 0.05$) according to one-way ANOVA.

Figure 9. Expression of SAV1 represented as fold change compared to LPS. Data was

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expressed as means of two independent replicates. Different letters showed statistical significance ($p < 0.05$) according to one-way ANOVA. (A) Cells treated with *G. soja* digested whole flour. (B) Cells treated with *G. soja* total protein extract. (C) Cells treated with *G. soja* lunasin-free protein. (D) Cells treated with *G. soja* enriched lunasin protein extract. Different letters A – C represent statistically significant differences ($p < 0.05$) according to one-way ANOVA.

Figure 10. (A) Immunofluorescence staining results as captured by confocal microscopy. (B) Cytoplasmic YAP1 expression in cells treated with LPS, VP, UNT, *G. soja* ELPE PI407207 and *G. soja* PI424088 represented as integrated pixel density (C) Nuclear YAP1 expression in cells treated with LPS, VP, UNT, *G. soja* ELPE PI407207 and *G. soja* PI424088 represented as integrated pixel density. (D) Nuclear:cytoplasmic YAP1 expression ratio in cells treated with LPS, VP, UNT, *G. soja* ELPE PI407207 and *G. soja* PI424088. Different letters showed statistical significance ($p < 0.05$) according to one-way ANOVA. The integrated density has been normalized to the number of cells present in each image (number of DAPI-stained nuclei visible = number of cells present). The bar graph results showed an average of five images per treatment that had been normalized according to the number of cells.

Figure 11. Diagram showing the activated (left) and inactive (right) Hippo pathway. Soybean bioactive compounds mitigated inflammation by increasing the expression of phosphorylated LATS1/2 and YAP1 kinases, leading to decreased production of pro-inflammatory markers IL-6, MCP-1 and IL-1β.

Supplementary Figure 1. SDS-PAGE results of accession PI424088 after filtration with 30 and 50 kDa MWCO filters. Red box indicated lunasin band. Yellow box indicated the bands that were cut from the gel and used for LC-MS/MS analysis to find the lunasin sequence. Previously purified lunasin was used as a control. This figure was adapted from Kusumah et al. (2025).

Supplementary Figure 2. Cell viability after treatment with different concentrations of digests, lunasin-free protein, total protein extract and enriched lunasin protein extract from different accessions of *G. soja.* Different letters showed statistical significance ($p < 0.05$) according to one-way ANOVA

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Supplementary Figure 3. SDS-PAGE results of accession PI424088 showing the protein profile of lunasin-free protein (LFP) eluted by Buffer A (Tris-HCl, 1 M, pH 7.5) in comparison to enriched lunasin protein extract (ELPE) eluted by Buffer B (Tris-HCl + NaCl, 1 M, pH 7.5) after first and second purification, and before filtration with 50 kDa MWCO filter. Red box indicated the presence of β-conglycinin and lunasin.

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Table 1. Effect of different sample types from *G. soja* in IL-1β (IC_{50}) in THP-1 macrophages stimulated by LPS.

Accessions	Sample Type	Lunasin (mg/g of defatted flour)	IL-1β IC_{50} (mg/mL)
PI407207	DWF	0.9	$1.50 \pm 0.1^{B, a}$
	TPE	32.6	$0.015 \pm 0.0002^{A, b}$
	LFP	0.000233	$0.021 \pm 0.02^{B, b}$
	ELPE	65.9	$0.017 \pm 0.01^{A, b}$
PI407159	DWF	1.8	$0.68 \pm 0.1^{C, a}$
	TPE	32.9	$0.012 \pm 0.0005^{A, b}$
	LFP	0.000312	$0.024 \pm 0.01^{B, b}$
	ELPE	86.9	$0.036 \pm 0.005^{A, b}$
PI407018	DWF	1.2	0.86 (IC_{30}) $\pm 0.5^{D, a}$
	TPE	16.2	$0.025 \pm 0.008^{A, b}$
	LFP	0.000211	$0.022 \pm 0.01^{B, b}$
	ELPE	65.1	$0.016 \pm 0.01^{A, b}$
PI424088	DWF	0.8	$1.90 \pm 0.6^{A, a}$
	TPE	29.5	$0.070 \pm 0.06^{A, b}$
	LFP	0.00031	$0.144 \pm 0.07^{A, bc}$
	ELPE	51.6	$0.038 \pm 0.004^{A, c}$
PI567313 (*G. max*)	LFP	1.4	0.124 ± 0.08^{A}
	ELPE	19.1	0.0010 ± 0.003^{B}
PI594845 (*G. max*)	LFP	2.4	0.164 ± 0.12^{A}
	ELPE	25.5	0.0029 ± 0.001^{B}

IC_{50} is defined as the amount (mg/mL) of sample needed to inhibit 50% of activity, in this case the production of IL-1β. Lower IC_{50} indicated higher effectiveness of sample to inhibit 50% of IL-1β production. Different letters A – D represent significant differences within same sample type and among different accessions according to one-way ANOVA ($p < 0.05$). Different letters a – d represent significant differences in different sample type and within same accessions according to one-way ANOVA ($p < 0.05$). Digested whole flour (DWF), total protein extract (TPE), lunasin-free total protein extract (LFP), and enriched lunasin protein extract (ELPE).

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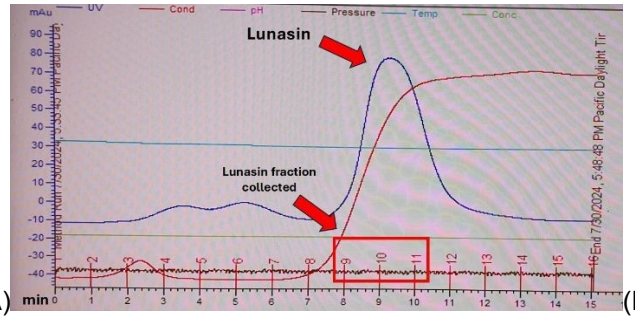
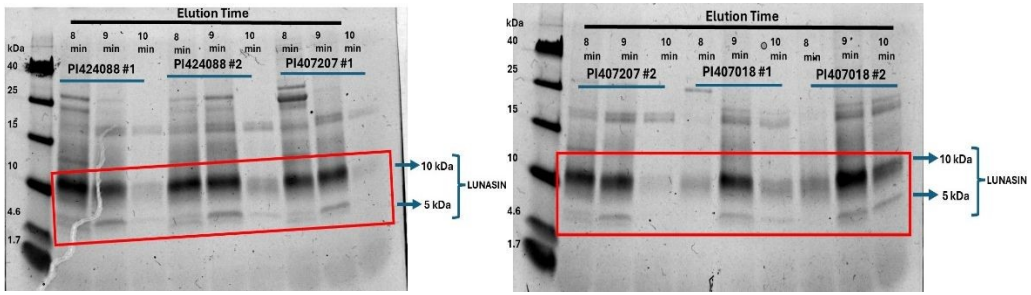
Table 2. Summarized changes in expression of pro-inflammatory markers.

Accessions	Sample Type	Biomarkers											
		IL-6	IL-1β	TNF-α	MCP-1	YAP1		LATS1/2		MST1/2		MOB 1	SAV 1
						P (-)	P (+)	P (-)	P (+)	P (-)	P (+)		
PI407207	DWF	↓*	↓*	NS	↓*	↓*	NS	↓*	↑*	↓*	NS	NS	NS
	TPE	↓*	↓*	↑*	↓*	↓*	NS	↓*	NS	NS	↓*	NS	↓*
	LFP	↓*	↓*	↑*	↓*	↓*	NS	↓*	NS	↓*	NS	NS	NS
	ELPE	↓*	↓*	NS	↓*	↓*	↑*	↓*	NS	↑*	NS	NS	NS
PI407159	DWF	↓*	↓*	↑*	NS	↓*	NS	↓*	NS	↓*	↑*	NS	↓*
	TPE	↓*	↓*	↑*	↓*	↓*	NS	↓*	NS	NS	NS	NS	NS
	LFP	↓*	↓*	↑*	↓*	↓*	NS	↓*	NS	NS	NS	NS	NS
	ELPE	↓*	↓*	NS	↓*	↓*	NS	↓*	NS	↓*	NS	NS	NS
PI407018	DWF	↓*	↓*	NS	NS	↓*	NS	↓*	↑*	NS	NS	NS	NS
	TPE	↓*	↓*	NS	↓*	↓*	NS	↓*	NS	NS	NS	NS	NS
	LFP	↓*	↓*	NS	↓*	↓*	NS	↓*	NS	NS	NS	NS	NS
	ELPE	↓*	↓*	NS	↓*	↓*	NS	↓*	NS	NS	NS	NS	NS
PI424088	DWF	↓*	↓*	NS	NS	NS	NS	↓*	↑*	NS	NS	↓*	↓*
	TPE	↓*	↓*	↓*	↓*	↓*	↑*	↓*	NS	NS	NS	↑*	NS
	LFP	↓*	↓*	NS	↓*	↓*	NS	↓*	NS	NS	NS	NS	NS
	ELPE	↓*	↓*	NS	↓*	↓*	NS	↓*	NS	↑*	NS	NS	NS

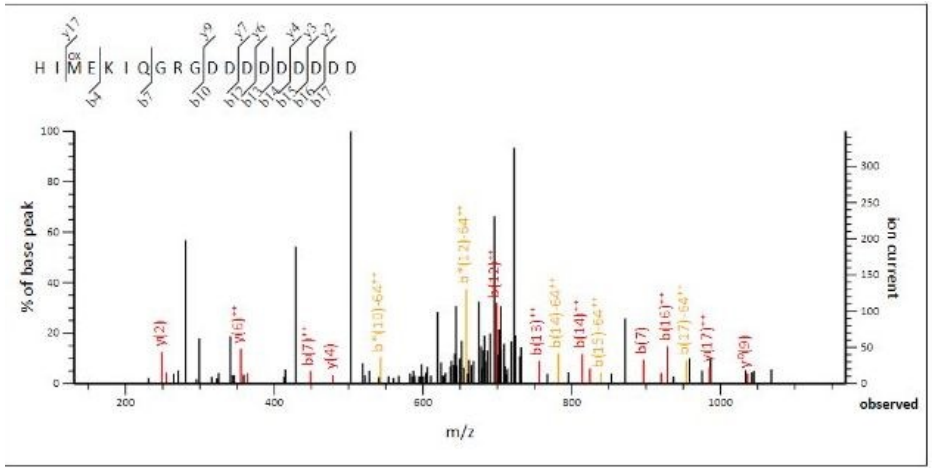
Arrow up (↑) indicates that the expression increased, arrow down (↓) indicates that the expression decreased compared to control, LPS. Asterisk (*) indicated that the decrease or increase is statistically significant ($p \leq 0.05$) compared to control, LPS. * Indicates statistically significant anti-inflammatory potential. P (-) represented dephosphorylated kinase. P (+) represented phosphorylated kinases. Verteprofin: IL-6↓*, 41.7 pg/mg of protein; IL-1β↓*, 5.0 pg/mg of protein; TNF-α, NS, 5.9 pg/mg of protein; MCP-1↓*, 20.9 pg/mg of protein; YAP-1↓*, 0.44 fold change; p-YAP1↑*, 1.69 fold change; LATS1/2↓*, 0.40 fold change; p-LATS1/2↑*, 1.65 fold change; MST1/2↓*, 0.65 fold change; p-MST1/2, NS, 1.08 fold change; MOB1,NS, 0.92 fold change; SAV1, NS, 0.82 fold change. Digested whole flour (DWF), total protein extract (TPE), lunasin-free total protein extract (LFP), and enriched lunasin protein extract (ELPE).



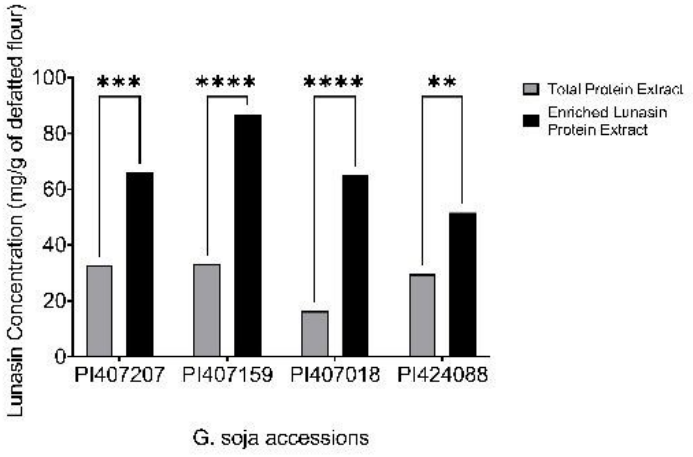
(A)



(B)



(C)

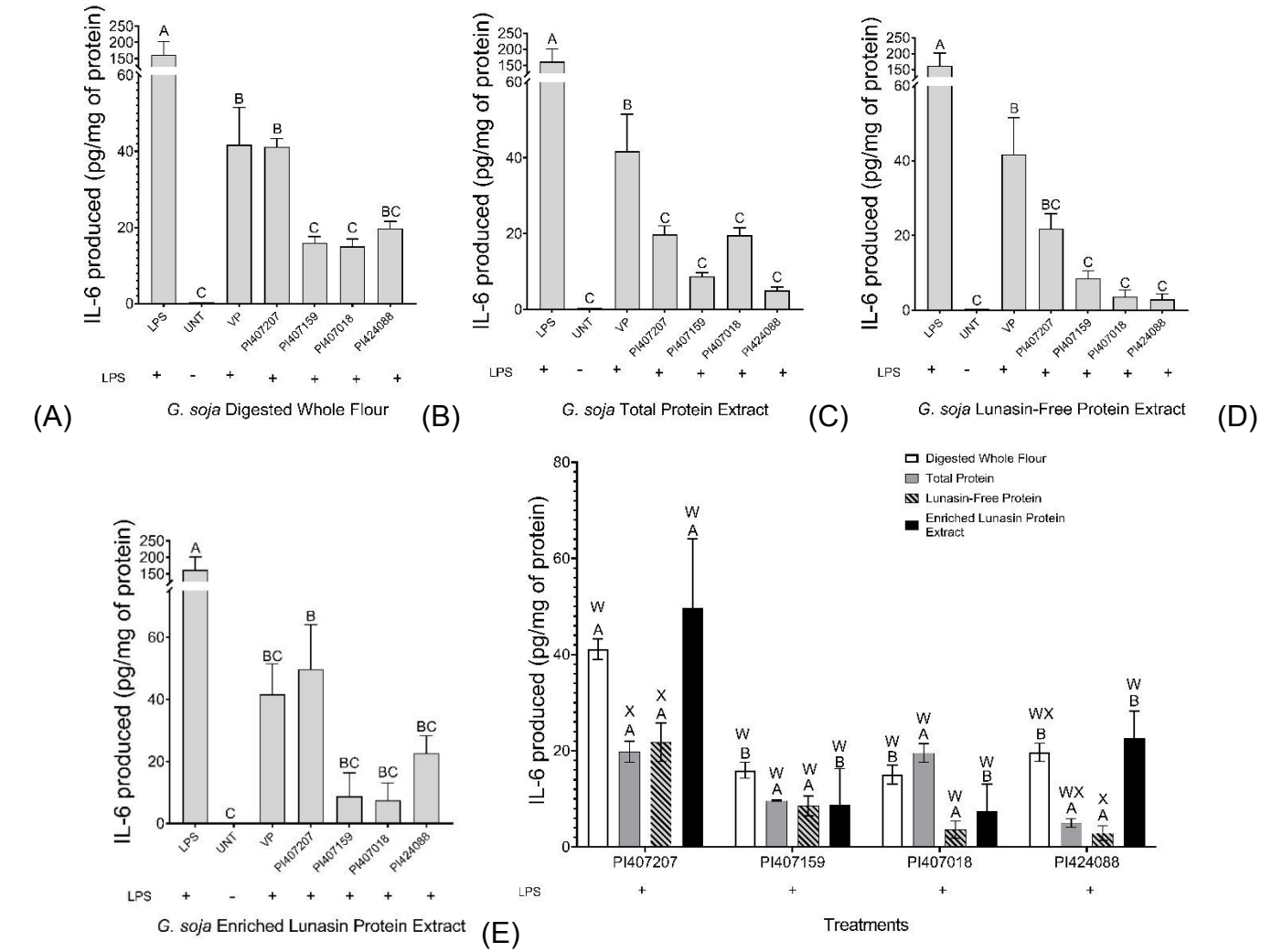


(D)

(E)

Accessions	Elution Time (Min)	Purity (%)	Accessions	Elution Time (Min)	Purity (%)
PI407207	8	55.5	PI407018	8	72.5
	9	62.5		9	66.0
	10	56.0		10	63.5
PI407159	8	58.0	PI424088	8	63.0
	9	56.5		9	59.5
	10	64.5		10	70.0

Figure 1



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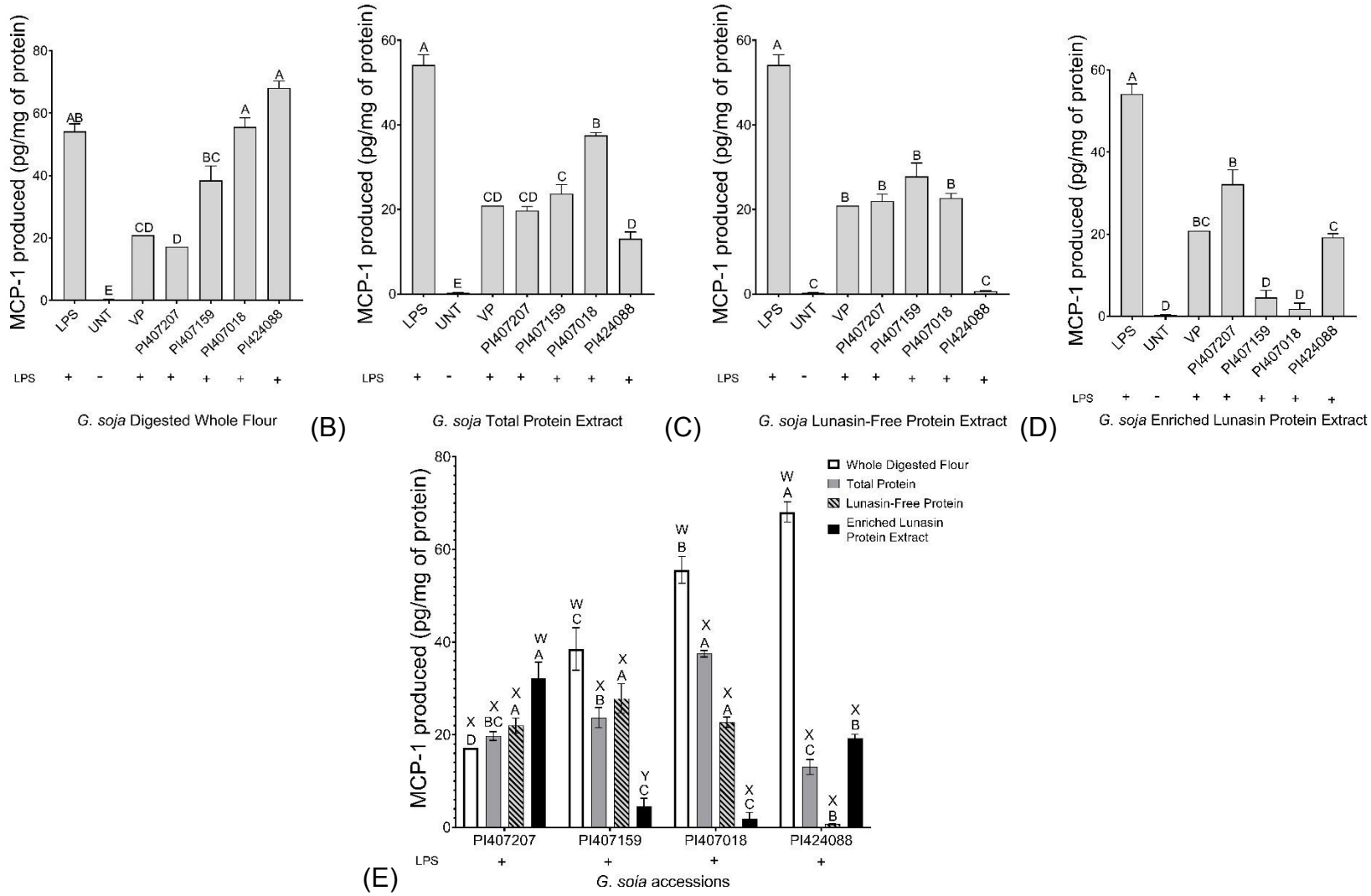

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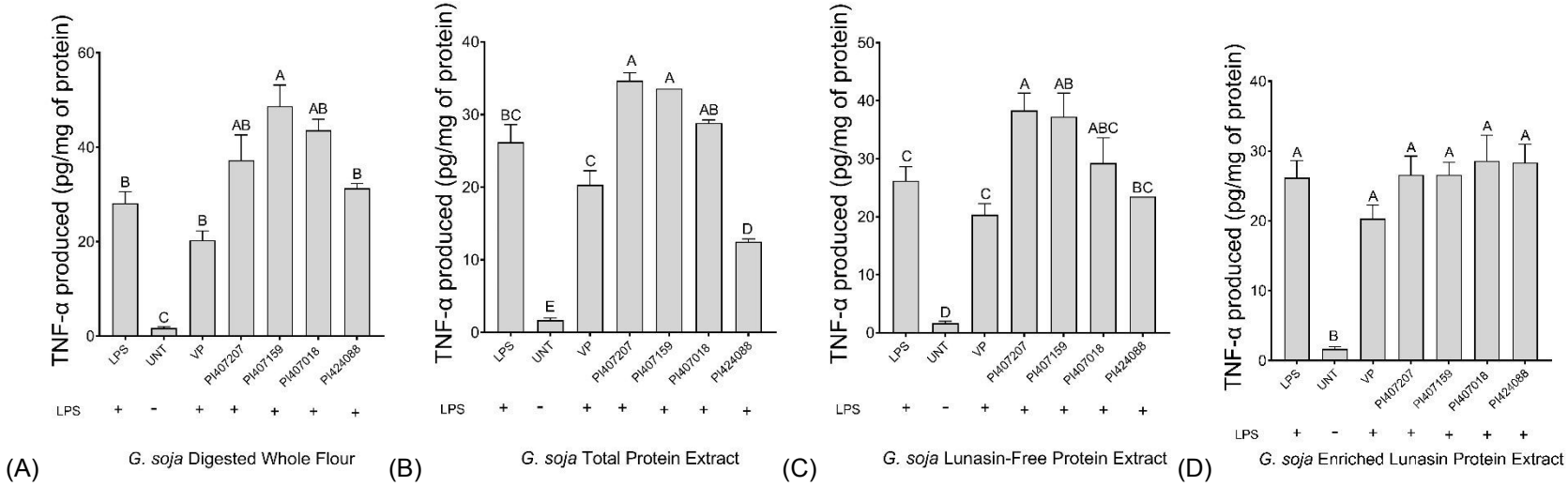

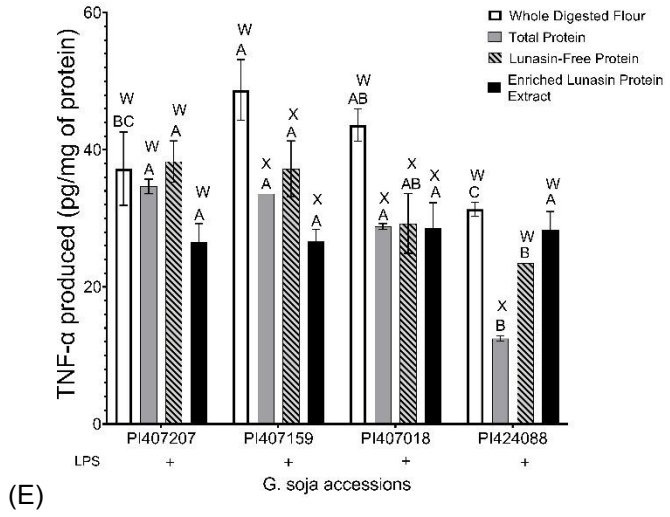

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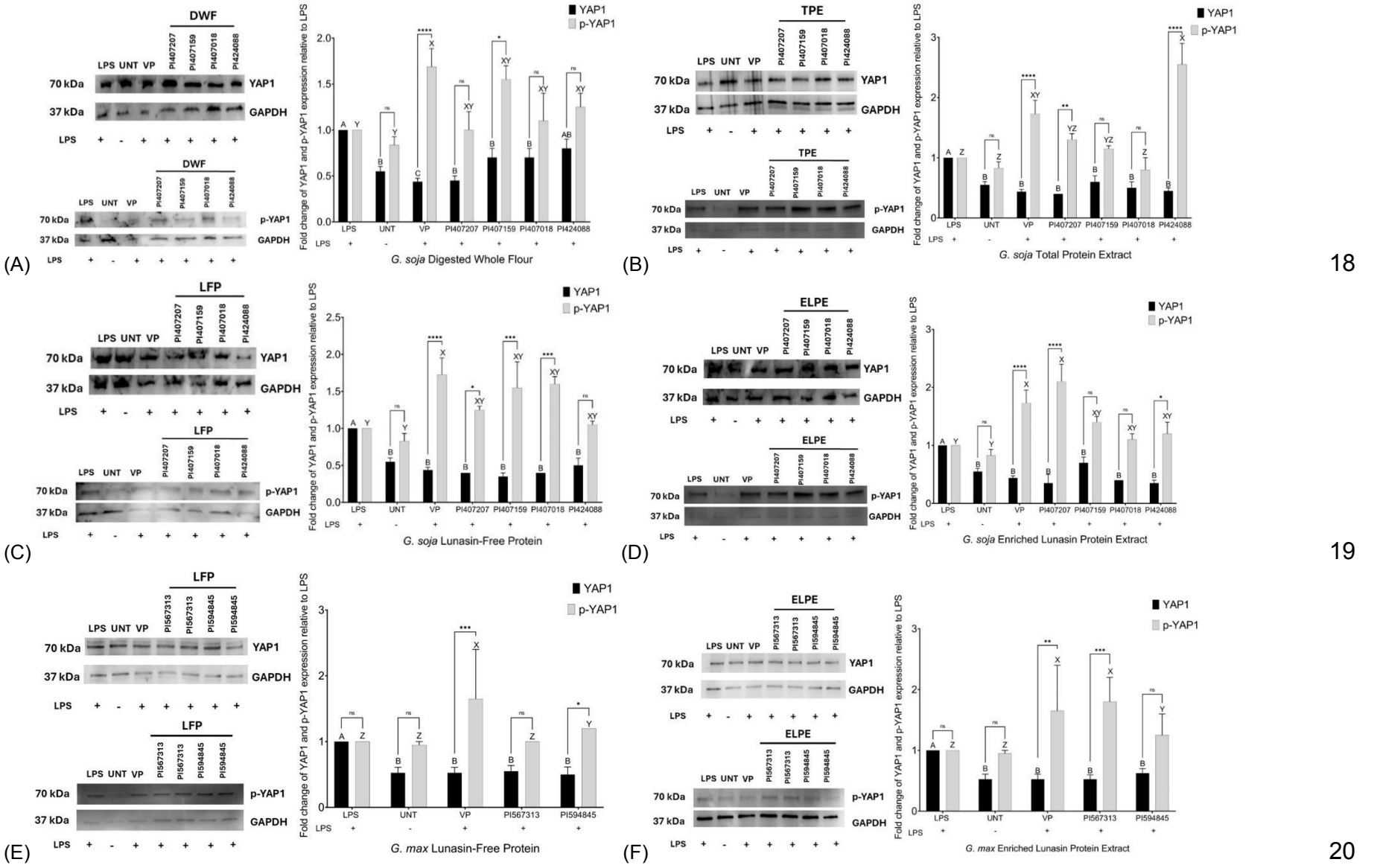

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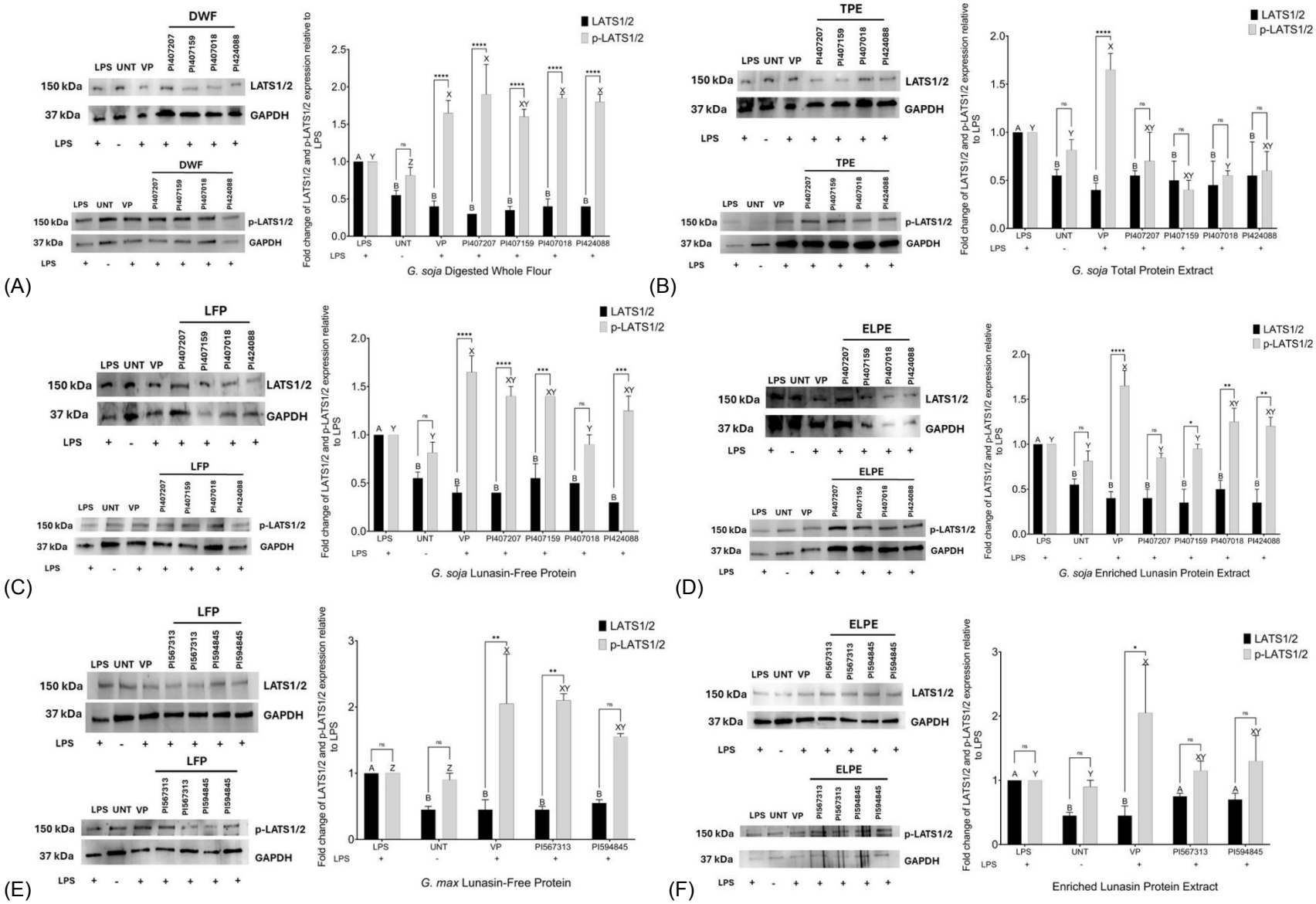

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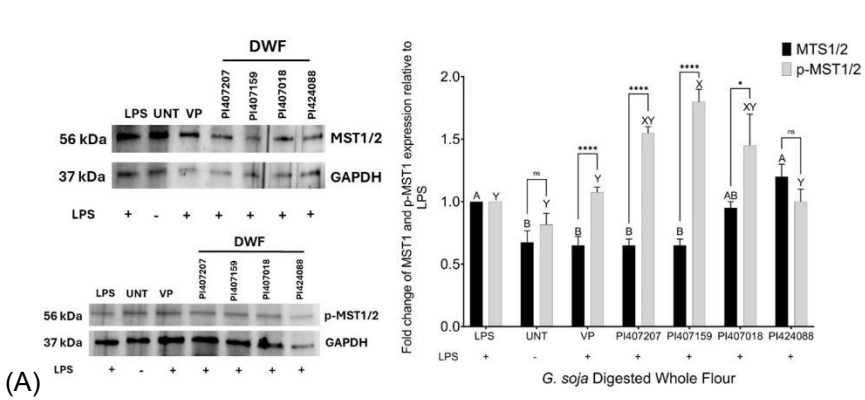
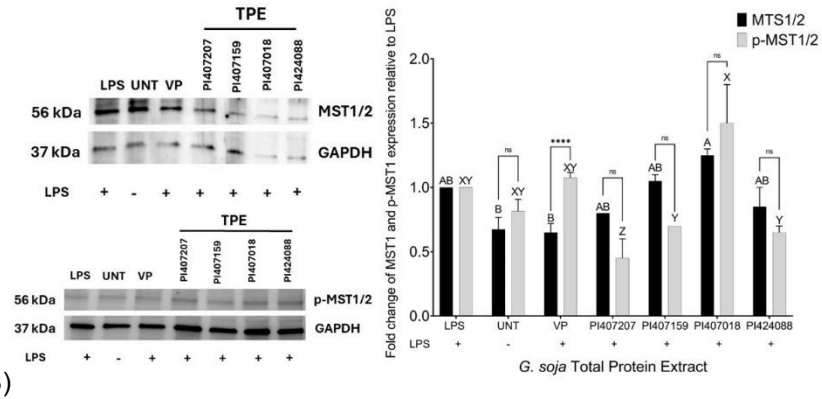


(A)



(B)

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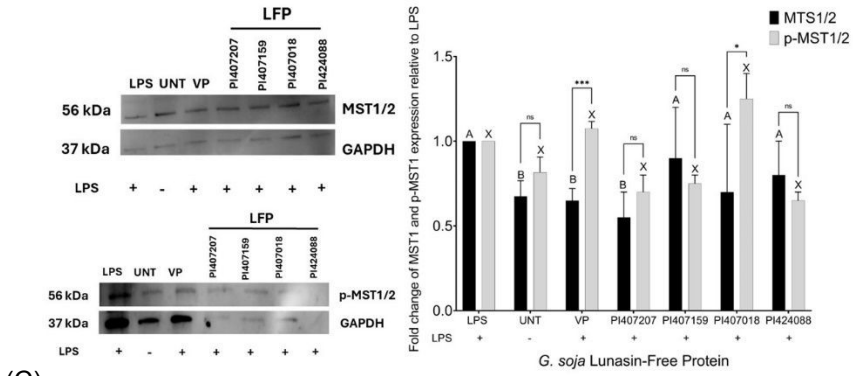

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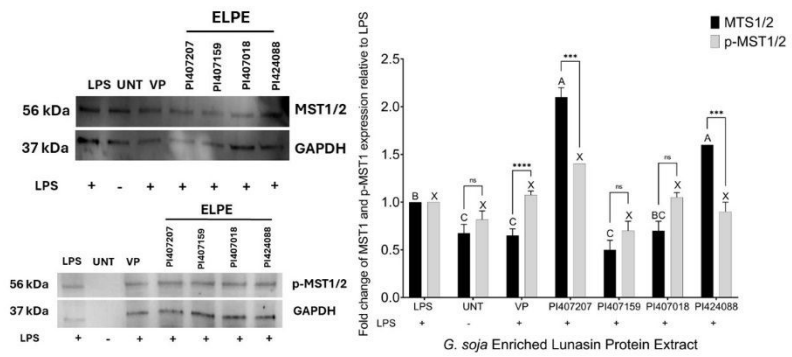

(D)

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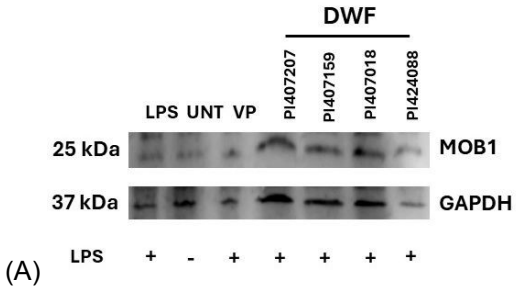
Figure 7.



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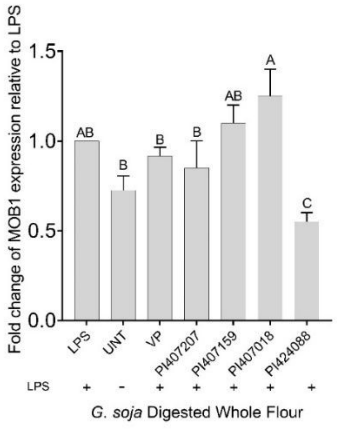

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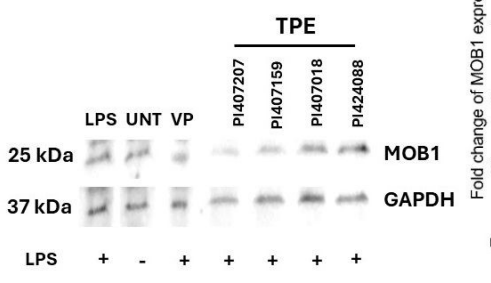

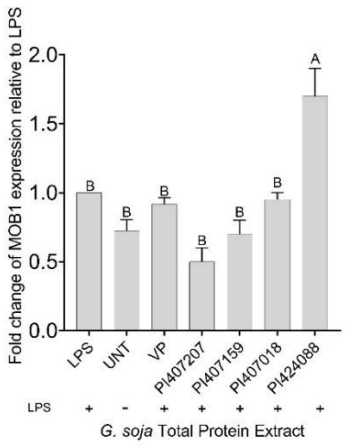

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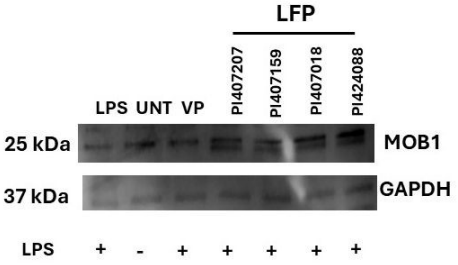

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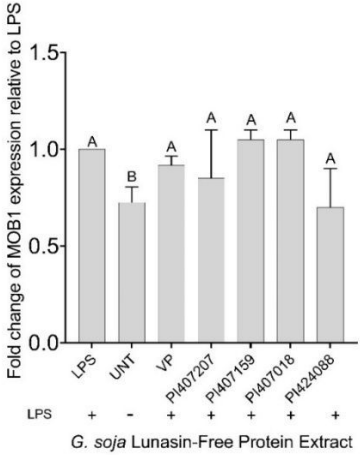

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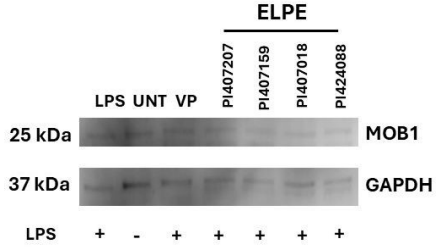

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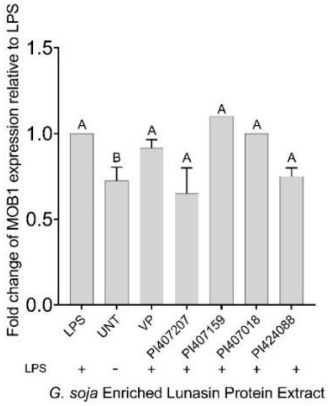

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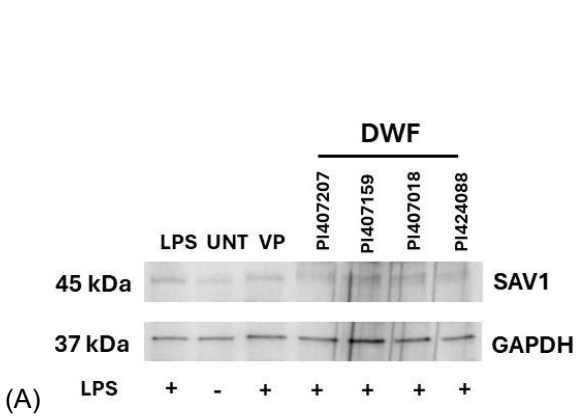

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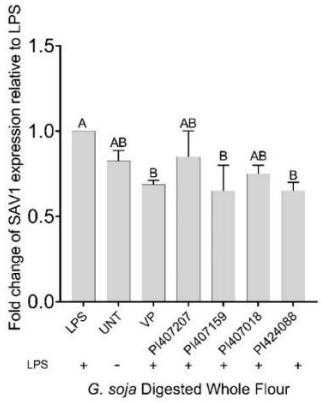

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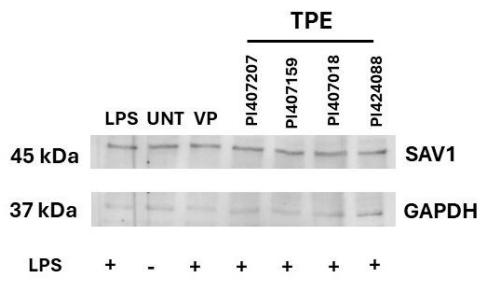

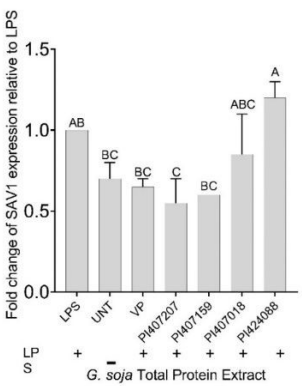

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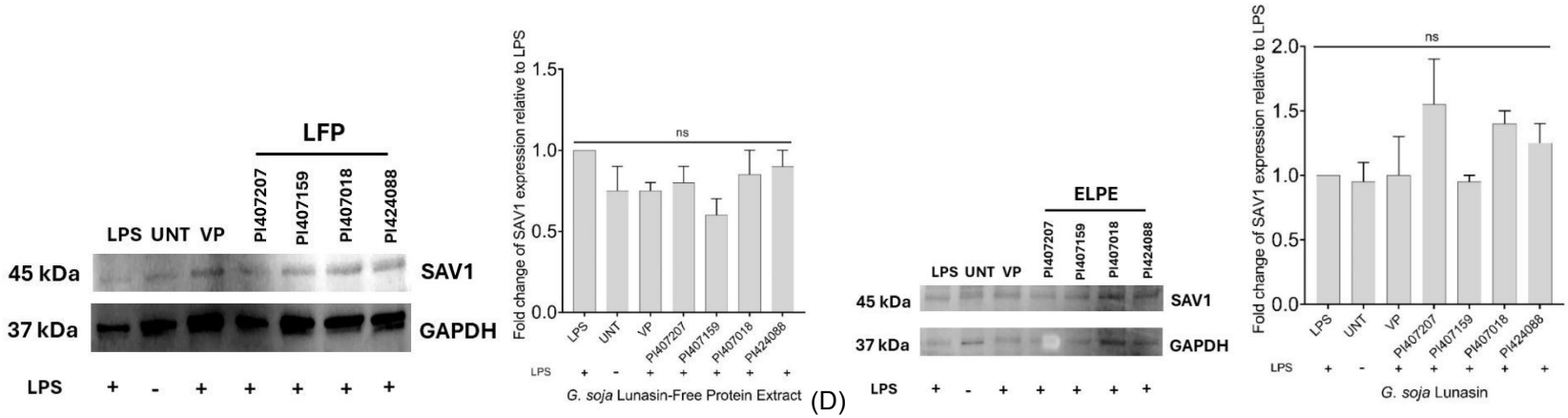

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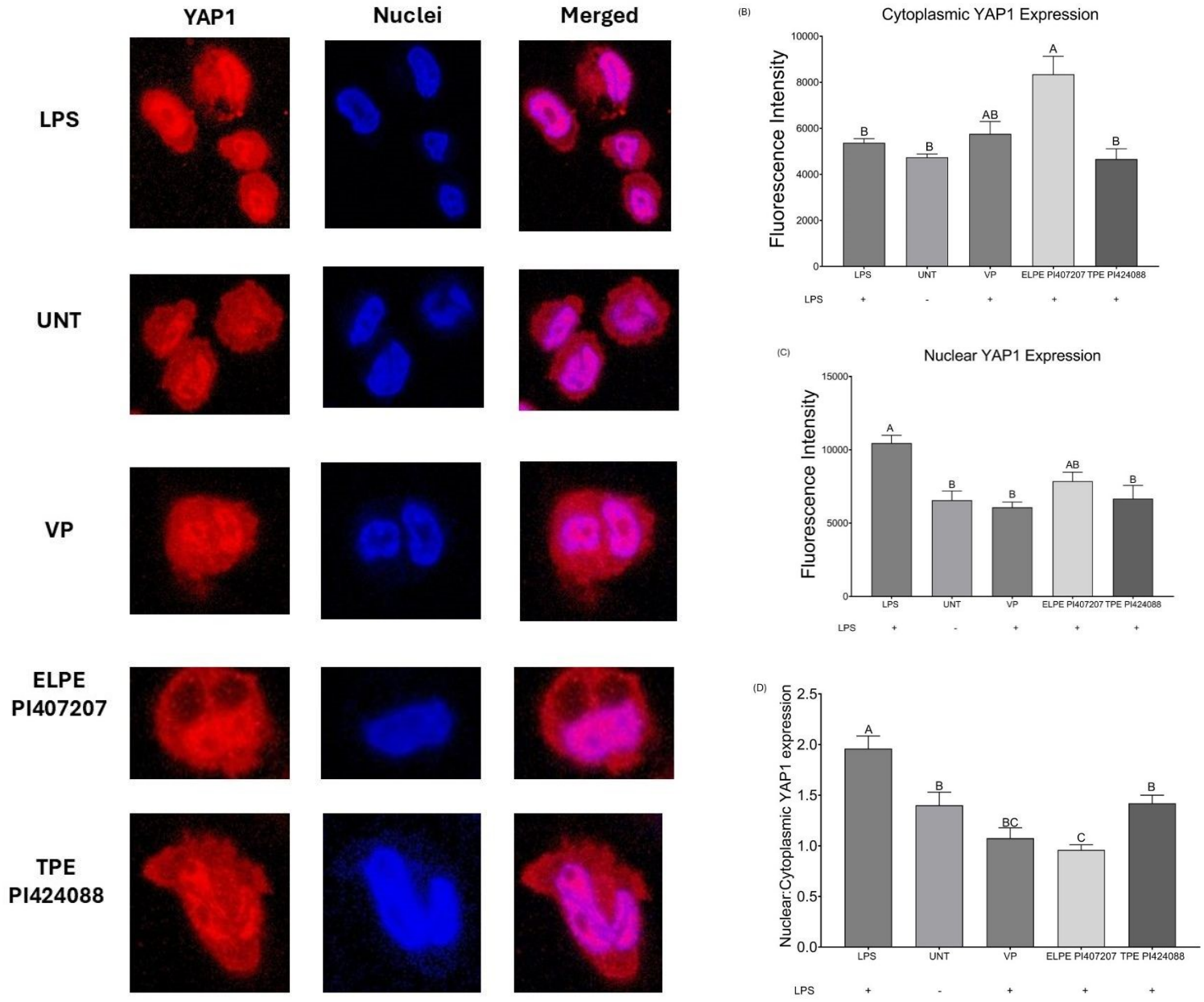

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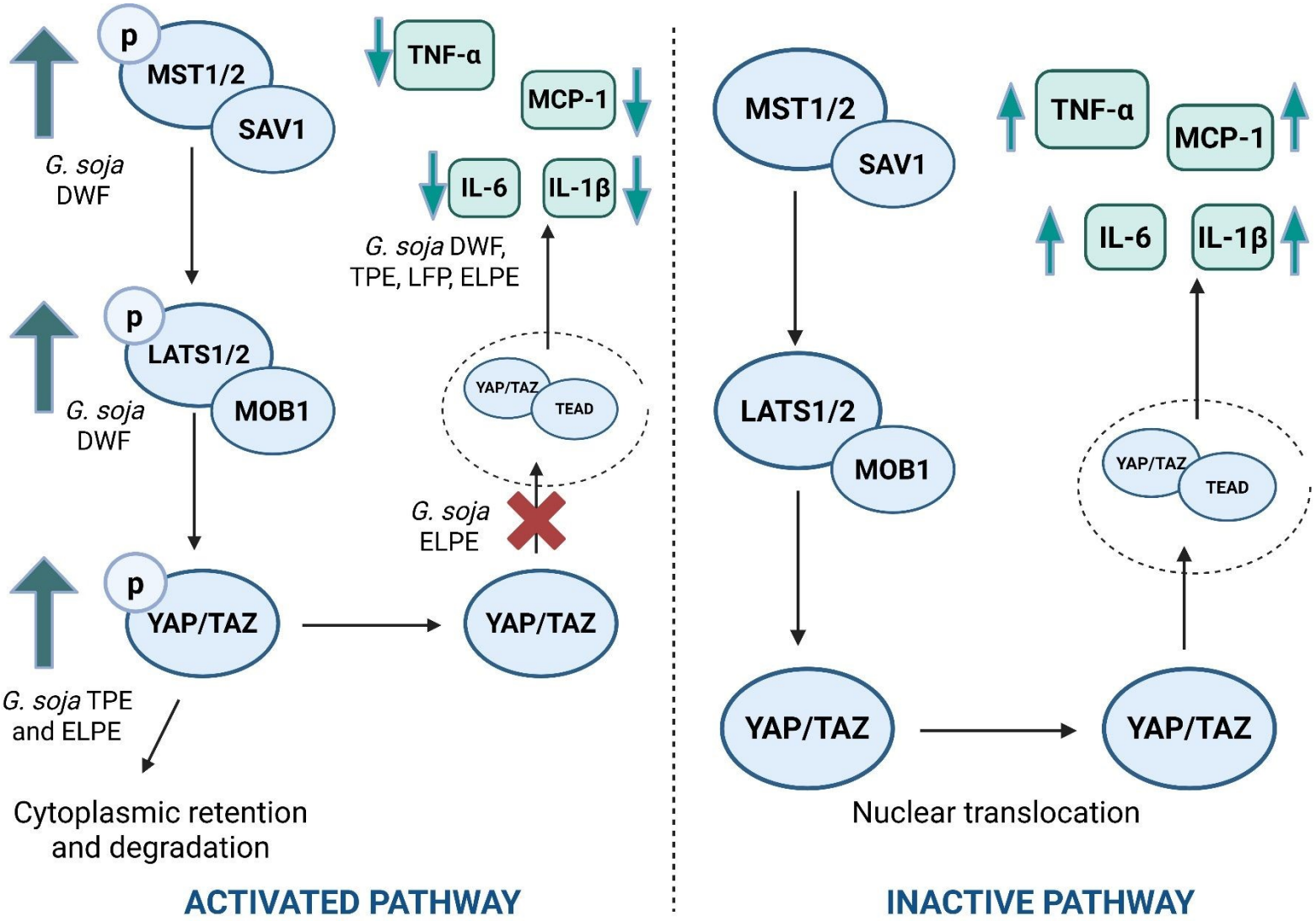

Figure 11.

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Data available within the article or its supplementary materials

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.