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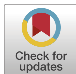

# Evaluation of the anti-inflammatory and wound healing potential of Ayapana protein extracted from fresh leaves of *Ayapana triplinervis* Vahl - *In vitro*

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ABSTRACT

Peptide-based therapeutics represent promising candidates in modern drug discovery due to their pleiotropic and multipotent activities. Notably, plant-derived proteins and peptides provide an untapped source of novel pharmacological agents, with plant peptides constituting a rich and underexplored reservoir of bioactive molecules. In this study, we delineate the wound-healing and chronic wound healing mediated anti-inflammatory efficacy of the Ayapana protein, isolated from the fresh leaves of *Ayapana triplinervis* Vahl. Ayapana protein inhibited heat-induced erythrocyte lysis by 70 % at 2.5 μg/ml in a membrane stabilisation assay and suppressed albumin denaturation with an IC50 value of 298.42 μg/ml. Moreover, it scavenged nitric oxide radicals generated by sodium nitroprusside. In RAW 264.7 macrophages, the proteins significantly down regulated proinflammatory mediators COX-2, IL-6, TNF-α, and Leukotriene B4. In a scratch wound healing assay, Ayapana protein promoted 99 % fibroblast (L929) cell migration within 48 h, without inducing cytotoxic effects. Together, these results highlight the dual activity (anti-inflammatory and wound healing) of Ayapana protein and underscore its promise as a novel therapeutic candidate for tissue repair and inflammation-mediated chronic wound repair.

## 1. Introduction

Wounds are defined as disruptions in the integrity of the skin or underlying tissue, and they are a significant global health challenge. Wounds could be due to various causes like trauma, burns, surgery, or infection. Wounds are broadly classified into two types: acute and chronic [1]. Acute wounds could heal by the normal healing process, whereas chronic wounds are due to underlying medical conditions such as Cardiac and respiratory failure, Regional Ischemia, Local Ischemia, neovascularisation, Angiogenesis, Endocrine disorders like diabetes and other haematological conditions [2]. Diabetic foot ulcers, pressure ulcers, and venous leg ulcers are some of the Chronic wounds. It fails to heal within an expected time, and they persist due to infection, inflammation and poor perfusion [3].

Inflammation is the first sign of injury, which helps in preventing infection, but also initiates the tissue repair process. Prolonged inflammation may lead to disruption in the wound healing mechanism due to high levels of cytokines at the wound site. Neutrophils and Macrophages are prominent in producing proinflammatory cytokines, such as Interleukins (IL) and Tumour Necrosis Factor alpha (TNF-α) [4]. Increased levels of proinflammatory cytokines increase Matrix Metalloproteinase (MMPs) but also reduce the Tissue Inhibitor of Metalloproteinase (TIMP). This leads to an increase in the extracellular matrix (ECM) degradation, disruption in cellular migration, and reduction in fibroblast proliferation and collagen synthesis, which converts an acute wound into a chronic wound [5].

Treating chronic wounds is the greatest challenge for the wound care industry due to their heterogeneous pathophysiology. Homeostasis, Inflammation, Proliferation, Remodelling and Maturation are the major sequence of events in wound healing. These wounds not only impair quality of life but also place a substantial economic strain on healthcare systems due to prolonged treatment and frequent hospitalisations. Primary wound treatment includes debridement, control of infection using antibiotics, use of wound dressings, application of growth factors and skin substitutes [6].

Recently, bioengineered skin grafts, negative pressure wound therapy, the use of stem cells, and nanotechnology-based wound treatments have been under research. Plant-based therapeutics have renewed interest among people due to their promiscuous properties. Many plants have potent antibacterial, anti-inflammatory, antioxidant, and analgesic

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activities, which have been scientifically validated [7]. The phytoconstituents like flavonoids, tannins and alkaloids are key components explored to promote cellular growth and regeneration [8]. Proteins and peptides also play an important role in wound healing. Defensins and cathelicidins (LL 37, Thymosin β4) are naturally occurring antimicrobial peptides which elicit antimicrobial activity and modulate the inflammatory response [9]. Some growth factors, like Epidermal growth factor (EGF), Transforming growth factor β (TGFβ), are essential for cell proliferation and tissue remodelling [10]. Synthetic and recombinant peptides are also being developed as bioactive agents to support various stages of the healing process. LL 37, along with the plant-derived polyphenols, are capable of promoting antibacterial, anti-inflammatory and Type H vascularized bone regeneration [11]. Another, Peptide RL-QN15 (*Rana limnocharis* frog) accelerates skin wound regeneration by regulating the functions of epidermal stem cells [12]. The study aims to isolate natural plant-based therapeutic proteins/peptides from the traditional plant *Ayapana triplinervis* Vahl and to explore their antimicrobial, anti-inflammatory, and wound-healing properties using *in vitro* models.

## 2. Materials and methods

The cell lines RAW 264.7 (Mouse macrophage) and L929 (Mouse Embryo Fibroblast) were procured from National centre for cell Sciences (NCCS, Pune), Leukotriene B4 (ELISA kit) from Cayman Chemicals, Cell culture medium: DMEM- High Glucose, Fetal Bovine Serum, D-PBS; Adjustable multichannel pipettes and a pipettor (Benchtop, USA), Lipopolysaccharide (LPS), FITC Mouse Anti-Human TNF-Alpha (BD Biosciences), COX-2 Monoclonal FIC antibody (Cayman Chemical), PE Rat-Anti-Mouse IL-6 (BD Biosciences), MTT Reagent (HiMedia), Allantoin-50ug/ml (Sigma).

### *2.1. Plant collection, propagation and extraction*

The *Ayapana triplinervis* Vahl plant saplings were collected from the nursery, Institute of Forest Genetics and Tree Breeding (IFGTB), Coimbatore, Tamil Nadu, India, and the same were authenticated by the Botanical Survey of India, TNAU, Coimbatore, Tamil Nadu, India, as *Ayapana triplinervis Vahl* (*Ayapana triplinervis*) belonging to the Asteraceae family. The extraction of bioactive plant protein/peptides from the leaves of *Ayapana triplinervis Vahl* by the EB-TA method was carried out as reported in our earlier work [13]

### *2.2. Anti-inflammatory activity of the Ayapana protein by biochemical assays*

#### *2.2.1. RBC membrane stabilization assay – heat-induced hemolysis*

Bovine erythrocytes (2 % v/v) were prepared as reported in previous studies [14]. The test contains 2 % (v/v) bovine erythrocyte suspension, with Diclofenac sodium serving as the standard drug [15]. The assay mixture consists of 2 ml of hyposaline (0.25 % w/v) sodium chloride, 1.0 ml of 0.15 M sodium phosphate buffer (pH 7.4), 0.5 ml of 2 % (v/v) bovine erythrocyte suspension, and 0.5 to 2.5μg/ml of drugs (Ayapana protein); the final reaction mixtures were adjusted to 4.5 ml with isosaline. Drugs were omitted in the blood control, while the drug control did not include the erythrocyte suspension. The reaction mixtures were incubated at 56 °C for 30 min in a water bath, followed by centrifugation at 5000 rpm for 10 min at room temperature. The absorbance of the released haemoglobin content into the supernatant was measured at 560 nm in a UV–Visible spectrophotometer (Shimadzu).

#### *2.2.2. Albumin denaturation assay*

The Elevation in Albumin denaturation at damaged tissue triggers an inflammatory response, which leads to delayed wound healing. The potential to stabilise protein denaturation by Ayapana protein was assessed by the Albumin denaturation assay [16–18] reaction mixture contains 0.05 mL of extract at variable concentrations of 100, 200, 300, 400, and 500 μg/mL in 10 % v/v polyethene glycol and 0.45 mL of bovine serum albumin (5 % w/v aqueous solution). The pH was adjusted to 6.3 by adding 0.1 N HCl, and the samples were incubated at 37 °C for 20 min, then heated at 57 °C for 3 min. After cooling, 2.5 ml of phosphate-buffered saline (pH 6.3) was added to each tube. The resulting turbidity was measured spectrophotometrically at 660 nm. For control tests, 0.05 ml of distilled water was used instead of the extract, while product control tests did not include bovine serum albumin. The inhibition of Albumin denaturation was calculated, with the control representing 100 % protein denaturation. The results were compared with Diclofenac sodium, which was used as a positive control in the present investigation.

#### *2.2.3. Griess assay*

The impact of the Ayapana protein on Nitric oxide radical scavenging was investigated using the Griess Illosvoy reaction [19,20]. Spontaneous decomposition of Sodium nitroprusside releases nitric oxide, which reacts with oxygen to form the nitrite ion. Quantifying the amount of Nitrite ion in the reaction mixture using the Griess Illosvogy reagent will elicit the scavenging potential of Ayapana protein. In this study, Griess Illosvovy reagent was modified by substituting naphthyl ethylene diamine dihydrochloride (0.1 % w/v) for 1-naphthylamine (5 %). The 3 ml reaction mixture contained 10 mM sodium nitroprusside (2 ml), phosphate buffer saline (0.5 ml), the extract (10–50 μg/ml), along with 0.5 ml of standard solution (ascorbic acid), and it was incubated at 25 °C for 150 min. An equivalent amount of methanol was also included in a control sample. After incubation, 0.5 ml of the reaction mixture was drawn and mixed with 1 ml of sulfanilic acid reagent (0.33 % in 20 % glacial acetic acid) and allowed to stand for 5 min to complete the diazotisation reaction. Further, 1 ml of naphthyl ethylene diamine dihydrochloride was added, mixed, and allowed to stand for 30 min at 25 °C. The nitrite concentration was measured at 540 nm, and the concentration was estimated using the absorbance value against standard nitrite solutions. Ascorbic acid was used as the standard. The Nitric oxide scavenging activity of Ayapana protein was calculated, and the graph was plotted against standard Ascorbic acid.

### *2.3. Anti-inflammatory activity of Ayapana protein - in vitro*

The Mouse Macrophage cell line (RAW 264.7) was purchased from NCCS, Pune, India. Both the cells were maintained in DMEM High Glucose media supplemented with 10 % FBS along with the 1 % antibiotic-antimycotic solution and 1 % l-Glutamine (200 mM) in an atmosphere of 5 % $CO_2$, 18–20 % $O_2$ at 37 °C temperature maintained in the $CO_2$ incubator (Healforce, China) and subcultured at 2-day intervals.

#### *2.3.1. Cell viability assay (MTT ASSAY)*

The MTT assay was performed to assess the cytotoxicity of the Ayapana protein on RAW 264.7 [21]. 200 μl (20,000 cells/well) of RAW 264.7 were seeded onto a separate 96-well titer plate and were grown for 24hours. 1 μg/ μl of Lipopolysaccharide (LPS), which served as a standard for RAW264.7. The medium without any culture was used as a blank, and the culture without any drug was used as untreated. The Ayapana protein of 25 to 400 μg/ μl was treated with the RAW 264.7 and L929 cell lines. The plate was incubated for 24hours at 37 °C in a 5 % $CO_2$ atmosphere. The 0.5 mg/ ml of MTT reagent was added to the spent medium and incubated for 3 h at 37 °C, followed by the addition of 100 μl of DMSO and the changes in absorbance were read at 570 nm using an ELISA Plate reader (ELX-800, BioTek, USA)

#### *2.3.2. Effect of ayapana protein on lipopolysaccharide (LPS) stimulated macrophage cell line (RAW 264.7) and expression of proinflammatory cytokines*

The anti-inflammatory activity of the Ayapana protein was assessed by studying the expression of proinflammatory cytokines (IL-6, TNFα,

COX-2) in the LPS-stimulated (RAW 264.7) mouse macrophage cell line [22]. The cell density of $3 \times 10^5$ cells/2 ml cell culture was seeded onto a 6-well plate and incubated in a $CO_2$ incubator overnight at 37 °C for 24 h. Three hours before drug treatment (Excluding the untreated well), 1μg/ml of LPS was used for stimulation, and the cells were harvested. Primarily, cells were washed with PBS, and 0.5 mL of Cytofix solution was added. Again, the cells were washed with 0.5 % BSA in 1X PBS and 0.1 % Sodium azide. 20 μL FITC-Mouse Anti Human TNF Alpha antibody, COX-2 Monoclonal FITC antibody and PE Rat-Anti-Mouse IL-6 were added separately to the corresponding wells and incubated at 20 °C- 25 °C in the dark for 30 min. The cells treated with LPS were used as a negative control for anti-inflammatory marker expression, and cells without LPS treatment were used as a control. LPS-stimulated cells were treated with 200 μg/ml Ayapana protein. Proinflammatory cytokine expression was studied by BD FACS Calibur flow cytometry, Cell Quest Pro software (Version 6.0).

#### 2.3.3. *Effect of Ayapana protein on lipopolysaccharide (LPS) stimulated macrophage cell line (RAW 264.7) and expression of leukotriene B4 (LTB4) marker*

LTB4 is identified as a potent mediator of inflammation. The cell density of $3 \times 10^5$ Cells was seeded at a density of 2 × 10ˆ6 cells/mL into a 6-well plate and incubated overnight in a $CO_2$ incubator at 37 °C for 24hours. The cells were pre-treated with the Ayapana protein with a concentration range of 12.5 to 200 μg/ml for 1 hour (except the untreated wells). 1 μg/ ml LPS was used to stimulate the cells (Except the untreated well). The cell-free supernatant was quantified for LTB4expression using the Leukotriene B4 ELISA kit (CAYMAN Co., Ann Arbour, USA). The cells without treatment were used as a negative control, and the cells alone (without LPS stimulation) were used as a blank.

### 2.4. *Wound healing activity of Ayapana protein in vitro*

The Mouse Embryo Fibroblast Cell line (L929) was purchased from NCCS, Pune, India. The cells were maintained in DMEM High Glucose media supplemented with 10 % FBS along with the 1 % antibiotic-antimycotic solution and 1 % l-Glutamine (200 mM) in an atmosphere of 5 % $CO_2$, 18 to 20 % $O_2$ at 37 °C temperature maintained in the $CO_2$ incubator (Healforce, China) and subcultured at 2-day intervals.

#### 2.4.1. *Cell viability assay (MTT ASSAY)*

The MTT assay was performed to assess the cytotoxicity of the Ayapana protein on L929 cell lines [21]. 200 μl (20,000 cells/well) of L929 cells were seeded onto a separate 96-well titer plate and were grown for 24 h. 12.5 μM concentration of Doxorubicin, which served as a standard for the L929 cell line. The medium without any culture was used as a blank, and the culture without any drug was used as untreated. The Ayapana protein of 25 to 400 μg/μl was treated with the L929 cell lines. The plate was incubated for 24hours at 37 °C in a 5 % $CO_2$ atmosphere. The 0.5 mg/ml of MTT reagent was added to the spent medium and incubated for 3 h at 37 °C, followed by the addition of 100 μl of DMSO and the changes in absorbance were read at 630 nm using an ELISA Plate reader (ELX-800, BioTek, USA).

#### 2.4.2. *Wound healing activity of Ayapana protein by scratch wound healing assay*

The Wound healing activity of the Ayapana protein was assessed as described by Martinotti and Ranzato [23,24]. Mouse fibroblast cell line (L929–2.5 × $10^5$ cells per well) was seeded into 12-well tissue culture plates containing DMEM with high glucose media supplemented with 10 % FBS. The cells were grown until they reached 80–100 % confluency as a monolayer for an incubation period of 24 h. Gently and slowly scratch the monolayer with a new 200 μL pipette tip across the center of the well. After scratching, gently wash the well twice with medium to remove the detached cells. Replenish the wells with fresh medium and grow the cells. The Ayapana protein (400 μg/ml) was added to the test wells, and 50 μg/ml of Allantoin was used as a standard drug in a separate well. The cells without any treatment served as a control. The cell images were captured at different time intervals (0, 24 and 48 hr), and the gap distance was measured using Image J software. The percentage of wound healing was calculated, and the graph was plotted

$$\textit{Wound healing}\ (\%) = \frac{(\text{Initial Area} - \text{Final area})}{100}$$

### 2.5. *Statistical analysis*

The results were expressed as mean ± SD using GraphPad Prism (version 7). We used a student's paired test, an unpaired *t*-test, a post hoc test and a one-way analysis of variance (ANOVA) where appropriate. The statistical method applied in each study was described in each figure. The Results were considered to be significant when p-values were ($p < 0.05$) *, ($p < 0.01$) * * ($p < 0.001$) ***, ($p < 0.0001$) ****.

## 3. Results

### 3.1. *Anti-inflammatory activity of the Ayapana protein by biochemical assays*

#### 3.1.1. *RBC membrane stabilization assay – heat-induced hemolysis*

Inflammatory mediators are released from the lysosomes of cells under stress, which could be a cause of inflammation. The lysosomal membrane stabilization was a key step in the inflammatory process. The membrane of human red blood cells (HRBC) mimics the membrane of lysosomes. In this assay, the erythrocytes are subjected to heat stress and assessed for the efficiency of ayapana protein to stabilize the HRBC membrane. The Ayapana protein extract showed inhibition of HRBC membrane degradation in a range of 13 % (for 0.5 μg/ml) to 60 % (for 0.5 μg/ml). In contrast, the standard drug Diclofenac sodium exhibited an inhibition range of 4 % to 55 % (Fig. 1A). The Ayapana protein had shown inhibition of erythrocyte membrane degradation in a dose-dependent manner, which was compared with the standard drug Diclofenac Sodium. The statistical two tailed *t*-test data reveal that Ayapana protein significantly inhibited HRBC membrane degradation ($p < 0.05$) compared with standard Diclofenac sodium (Fig. 1B).

#### 3.1.2. *Albumin denaturation assay*

The albumin denaturation assay was a common technique for analyzing the anti-inflammatory activity of the Ayapana protein. The protein denaturation leads to an inflammatory response. The inhibition of protein denaturation was a crucial activity during inflammation. The Ayapana protein showed dose-dependent inhibition of albumin denaturation. 95 % and 72 % of albumin denaturation was inhibited by the standard drug (Diclofenac sodium) and ayapana protein, respectively (Fig. 2A). The IC50value was calculated. The extract exhibited 50 % inhibition at a concentration of 298.42 (μg/mL), which was less compared with the standard drug Diclofenac sodium (Table 1). The statistical two tailed *t*-test p-value of 0.0004 ($p < 0.01$) shows that the Ayapana protein extract was highly significant on comparing with the standard drug (Fig. 2B).

#### 3.1.3. *Griess assay*

Nitric oxide (NO) free radicals are released as a result of oxidative stress and inflammation. Nitric oxide combines with oxygen, forming nitrite ion. The quantification of Nitrite ion colorimetrically by the Griess Illosvory reaction was a method to investigate the scavenging action of the Ayapana protein [25]. Scavenging these free radicals in the biological system is important to reduce inflammation. The study on Ayapana protein had shown that the scavenging of NO radical was dose-dependent. The inhibitory range of 14 – 67 % was observed on the Ayapana protein, and 26–71 % inhibition in standard ascorbic acid (Fig. 3A). This indicates that the extract showed potent NO scavenging

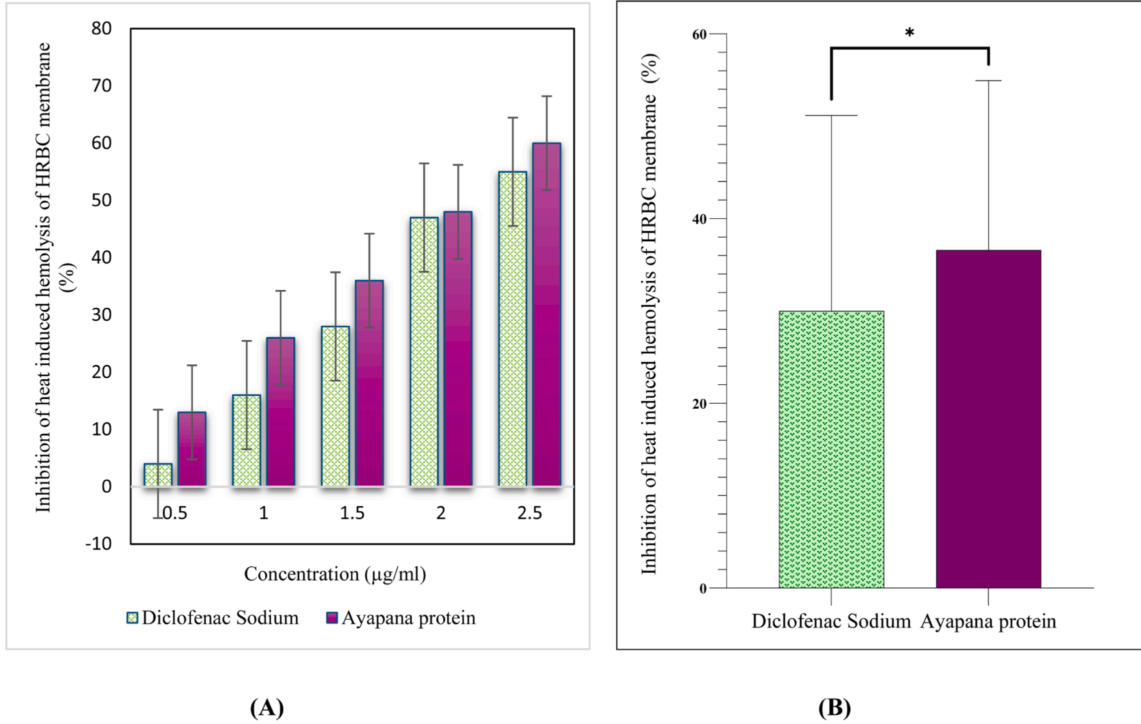



**Fig. 1.** (A) Inhibition of heat-induced hemolysis of HRBC by Ayapana protein. (B)The significance of Ayapana protein with standard diclofenac sodium was statistically determined by a two-tailed t-test as 0.0155 (which was $p < 0.05$) *.

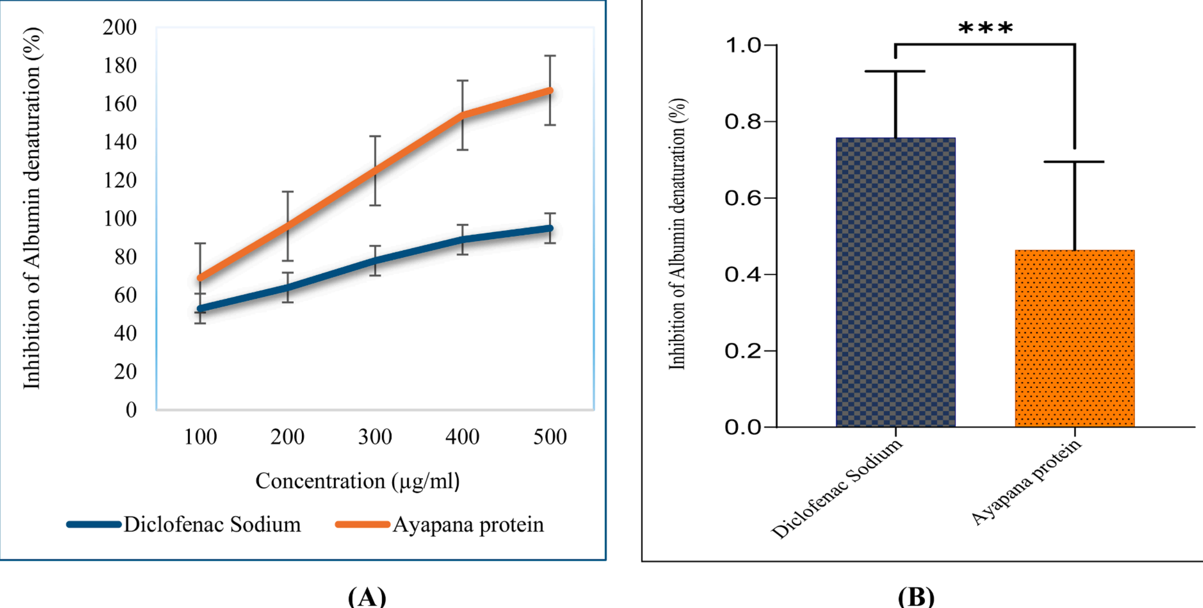



**Fig. 2.** (A) Effect of Ayapana protein on albumin denaturation. (B)The significance of Ayapana protein was statistically determined by paired t-test as 0.0004 (which was $p < 0.01$) ***.

activity with a 50 % inhibitory effect at 69 µg/ml (Table 2). The Ayapana protein was shown to possess Nitric oxide scavenging action with a significance value of 0.00312 ($p < 0.05$) (Fig. 3B).

### *3.2. Anti-inflammatory activity of Ayapana protein - in vitro*

#### *3.2.1. MTT assay – RAW264.7*

The cytotoxicity of Ayapana protein was assessed in the RAW 264.7 cell line by MTT assay. The cell survivability study results revealed that Ayapana protein caused growth inhibition in a dose-dependent manner on the RAW 264.7 (Mouse macrophage cell line). The IC50 value was calculated as 64.61 µg/ml. The LPS-stimulated cells without treatment were observed to show a cell viability percentage of 69.88 % with 1µg/ml, which was very less compared with the cells treated with the Ayapana protein, but the cells without any treatment showed 100 % cell viability (Fig. 4). The statistical one-way Anova test shown that ayapana protein was highly significant with ($p$<0.0001)

**Table 1**
Determination of IC50 value for the Inhibition percentage of heat-induced albumin denaturation by Ayapana protein and Diclofenac Sodium as a standard drug.

| Concentration of samples (μg/mL) | % Inhibition of Albumin Denaturation by | |
|---|---|---|
| | Diclofenac Sodium | Ayapana protein |
| 100 | 53 | 18 |
| 200 | 64 | 32 |
| 300 | 78 | 47 |
| 400 | 89 | 65 |
| 500 | 95 | 72 |
| IC50 value | 327.18 (μg/mL) | 298.42 (μg/mL) |

### *3.2.2. Pro-inflammatory cytokine expression analysis in LPS-stimulated macrophage cell line (RAW 264.7)*

i) COX-2 expression study in the Ayapana protein-treated RAW 264.7 cell line:

Cyclooxygenase-2 (COX-2), also known as prostaglandin G/H synthase 2 (PGHS2), is a 70 kDa microsomal enzyme that belongs to the prostaglandin G/H synthase family. Several cell types, including fibroblasts, vascular smooth muscle cells, endothelium, and monocytes, express it. This catalyses the production of Prostaglandins and Reactive oxygen species, which are responsible for the inflammatory response. The Ayapana protein has been shown to suppress the expression of COX-2 in the LPS-stimulated RAW 264.7 cell line, which was compared with the untreated and only treatment with LPS (Fig. 5) μg/ml of Ayapana protein extract inhibited the COX-2 expression in the LPS-stimulated RAW 264.7 cell line, which was compared with the LPS-stimulated cells without any treatment by measuring the relative fluorescence intensity. The LPS-stimulated cells showed high fluorescence intensity, indicating no suppression in COX-2 expression. However, the cells without LPS stimulation and drug acted as a control, which shows a very minimum relative fluorescence intensity.

ii) IL-6 expression study in the Ayapana protein-treated RAW 264.7 cell line:

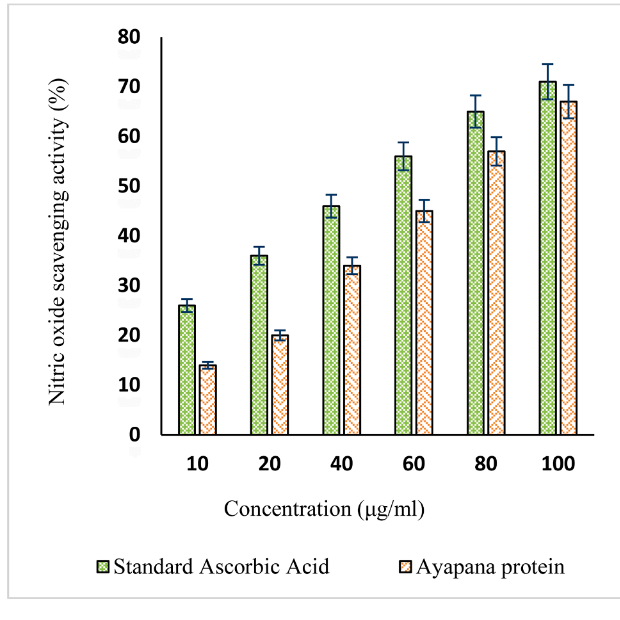
The Ayapana protein was evaluated for anti-inflammatory activity by studying the IL-6 expression in LPS-induced RAW 264.7 cells. Interleukin-6 (IL6) is a phosphoglycoprotein with a molecular weight ranging from 21 to 29 kDa. IL-6 is a multifunctional cytokine that plays a central role in host defense mechanisms, including acute phase reactions, hematopoiesis, and immune responses. Its dysregulation leads to Rheumatoid Arthritis. The suppression of IL-6 by the Ayapana protein suggested that it could be a potent inhibitor of IL-6 expression during the inflammatory response. The relative fluorescence intensity value of Ayapana protein (200 μg/ml) revealed that it shows inhibitory action against the proinflammatory cytokine IL6. The histogram was plotted for the Concentration of the sample vs relative fluorescence intensity (Fig. 5).

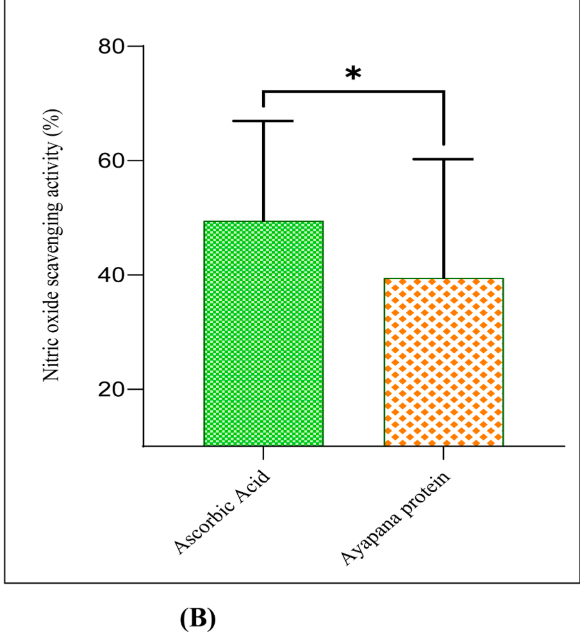
iii) TNF-alpha expression study in the Ayapana protein-treated RAW 264.7 cell line:

The anti-inflammatory activity of Ayapana protein was analysed by studying the expression of TNF-α in LPS-stimulated RAW 264.7 macrophage cell line. TNF-alpha (Tumour Necrosis Factor alpha), designated TNFSF2, is a trimeric glycoprotein active in both membrane-bound and secreted forms. TNF-alpha is produced by several lymphoid cells as well as by astrocytes, endothelial cells, and smooth muscle cells. The histogram was plotted for the concentration of Ayapana protein against the relative fluorescence intensity value (Fig. 5). The Ayapana protein

**Table 2**
IC50 value determination of Ayapana protein for Nitric oxide scavenging activity and Ascorbic acid as standard.

| S.No. | Concentration (μg/ml) | Inhibition (%) | |
|---|---|---|---|
| | | Ascorbic Acid | Ayapana protein |
| 1 | 10 | 26 | 14 |
| 2 | 20 | 36 | 20 |
| 3 | 40 | 46 | 34 |
| 4 | 60 | 56 | 45 |
| 5 | 80 | 65 | 57 |
| 6 | 100 | 71 | 67 |
| IC50 values | | IC50=51.75 μg/ml | IC50=69.36 μg/ml |




(A) (B)

**Fig. 3.** (A) Nitric oxide scavenging activity of Ayapana protein. (B)The significance of Ayapana protein was statistically determined by paired t-test as 0.0312 (which was $p < 0.05$) *.

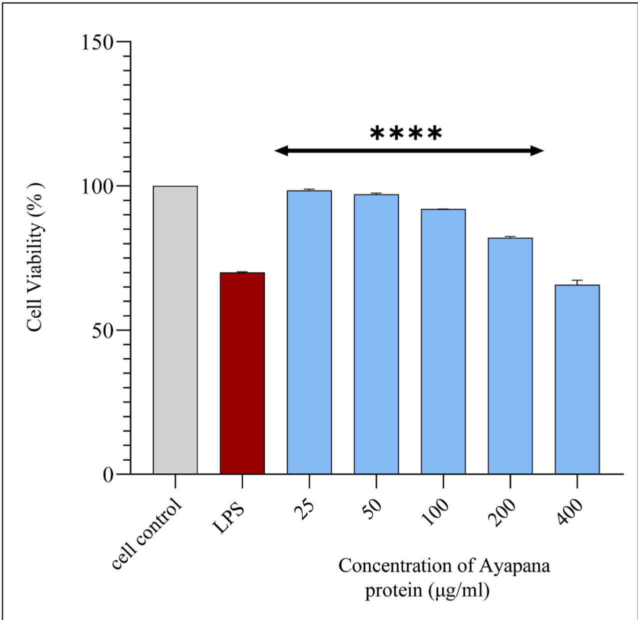



**Fig. 4.** Cytotoxicity analysis of the Ayapana protein of Ayapana triplinervis Vahl on RAW 264.7. The significance of Ayapana protein was statistically determined by one-way ANOVA ($p < 0.0001$) ****.

suppressed the TNF-alpha expression in LPS-stimulated Mouse macrophage cell line (RAW 264.7). Compared with untreated, which show minimum intensity, indicating minimal expression of TNF-alpha since it was not treated with any of the drugs.

The overlay of COX-2 (Fig. 5A), IL-6 (Fig. 5B), and TNF-α (Fig. 5C) expression in Untreated RAW 264.7, LPS-stimulated cells and the LPS-stimulated cells after treatment with Ayapana protein was assessed. Overall, the Ayapana protein at a concentration of 200 μg/ml down-regulates the expression of pro-inflammatory cytokines COX-2, IL-6, and TNF-alpha in LPS-stimulated RAW 264.7 cells (Fig. 6). while upregulation of pro-inflammatory cytokines was observed in the LPS-stimulated RAW 264.7 cell line.

#### *3.2.3. Effect of Ayapana protein on lipopolysaccharide (LPS) stimulated macrophage cell line (RAW 264.7) and expression of leukotriene B4 (LTB4) marker*

The inhibitory percentage of Leukotriene B4 (LTB4) in LPS-stimulated RAW 264.7 macrophage cell line was analyzed for the anti-inflammatory activity of Ayapana protein. LTB4 was a potent mediator of inflammation. It is synthesized from Arachidonic acid with the combined action of 5-lipoxygenase and LTA4 hydrolase. 5 % to 73 % inhibition was observed in Ayapana protein treatment, while the LPS-induced cells showed high levels of LTB4 concentration (Fig. 7). The Ayapana protein blocked the LTB4, mitigating the inflammatory response mediated by LPS in a dose-dependent manner. The IC50 value was calculated as 48.53 μg/ml. The statistical data of Ayapana protein on LTB4 by one-way Anova had shown highly significant p value ($p < 0.0001$) ****

### *3.3. Wound healing activity of Ayapana protein - in vitro*

#### *3.3.1. MTT assay – L929*

The cytotoxic effect of the Ayapana protein was analysed by MTT assay using mouse fibroblast cells (L929). The colourimetric assay reveals that the extract was known to show 100 % cell viability for concentrations >100 μg/ml. The assay shows that the cell was proliferating instead of being inhibited. The results were compared with standard drug doxorubicin, in which 67 % cell viability was observed at 12.5, and the untreated cells showed 100 % cell viability on 24 h of incubation (Fig. 8). The Ayapana protein was non-toxic to the L929 mouse fibroblast cell line. The statistical data of Ayapana protein was determined by one-way ANOVA which shows high significance ($p < 0.0001$) ****.

#### *3.3.2. Scratch wound healing assay*

The wound healing property of Ayapana protein was studied using the L929 cell line by the scratch wound healing assay. The wound area was measured and the graph was plotted for the control, standard Allantoin and the Ayapana protein for different time intervals (0, 24, 48 h) (Fig. 10). The extract had shown 99 % cell migration with 200μg/ml concentration, which was compared with standard Allantoin (50 μg/ml), eliciting 97 % cell migration during 48 h (Fig. 9a). The cell migration on treatment with Ayapana protein shows high healing potential. The cells without treatment showed 22 % cell migration, indicating a delayed wound healing process compared to the standard and the test sample. The wound area measured at every time interval (0, 24, 48 h) showed that Ayapana protein was efficient in stimulating cell migration, and the wound area was decreased faster within 48 h, which was compared with standard allantoin and untreated fibroblast cells (Fig. 9b).

## 4. Discussion

The present study demonstrates that the Ayapana Protein from fresh leaves of *Ayapana triplinervis* Vahl exhibits strong anti-inflammatory and wound healing properties, likely mediated through immunoregulatory mechanisms. The Ayapana protein stabilizes HRBC membranes and inhibits albumin denaturation, suggesting its ability to prevent protein and membrane damage during inflammation. Because the RBC membrane is considered a model for lysosomal instability. Lysosomal disruption in neutrophils triggers the release of proteolytic enzymes (cathepsin), which leads to an inflammatory response. By stabilizing this membrane, the ayapana protein may reduce this cascade, the excessive inflammation can be prevented. Albumin is a major plasma protein that undergoes conformational changes during injury, but the inhibition of its denaturation is a relevant therapeutic property. Ayapana protein demonstrates considerable potential to suppress inflammation, these effects were comparable or superior to several reported research works including *Rhodomonas* crude protein and peptides [26] and *Erioglossum rubiginosum* [27].

The Ayapana protein also displayed significant nitric oxide scavenging activity, aligning with antioxidant findings from other medicinal plants and protein hydrolysates. Importantly, the protein was nontoxic to RAW 264.7 (macrophage) cell line and L929 (fibroblast) cells. Ayapana protein has demonstrated enhanced cell viability and migration rates, than the methanolic extract of *Rhaphidophora korthalsii* [28] and *Plinia. Peruviana* fruit extract [29]. Ayapana protein suppressed the expression of proinflammatory cytokines COX −2, IL-6, TNF-α in LPS stimulated RAW 264.7 cell line. By comparison with research findings of *Sonchus oleraceus* [30] and *Alternanthera sessilis* [31] had low potency in suppressing inflammatory mediators than Ayapana protein. The accumulation of ROS at the wound site triggers the inflammatory mediators causing inflammation. The antioxidant properties of *Ayapana triplinervis* may complement its anti-inflammatory effects which supports fast wound healing.

Taken together, the bioactivity profile of Ayapana protein suggests a dual role in inflammation control and tissue repair. Its immunomodulatory activity coupled with antioxidant protection, may prevent lysosomal destabilization and subsequent release of inflammatory mediators. These findings position the Ayapana protein as a promiscuous candidate for wound healing applications. Ongoing work will aim to identify the specific active component responsible for these effects, assess their incorporation into nanocellulose scaffold for *in vivo* wound healing studies and evaluate their ability to downregulate cathepsin released following lysosomal membrane permeation.

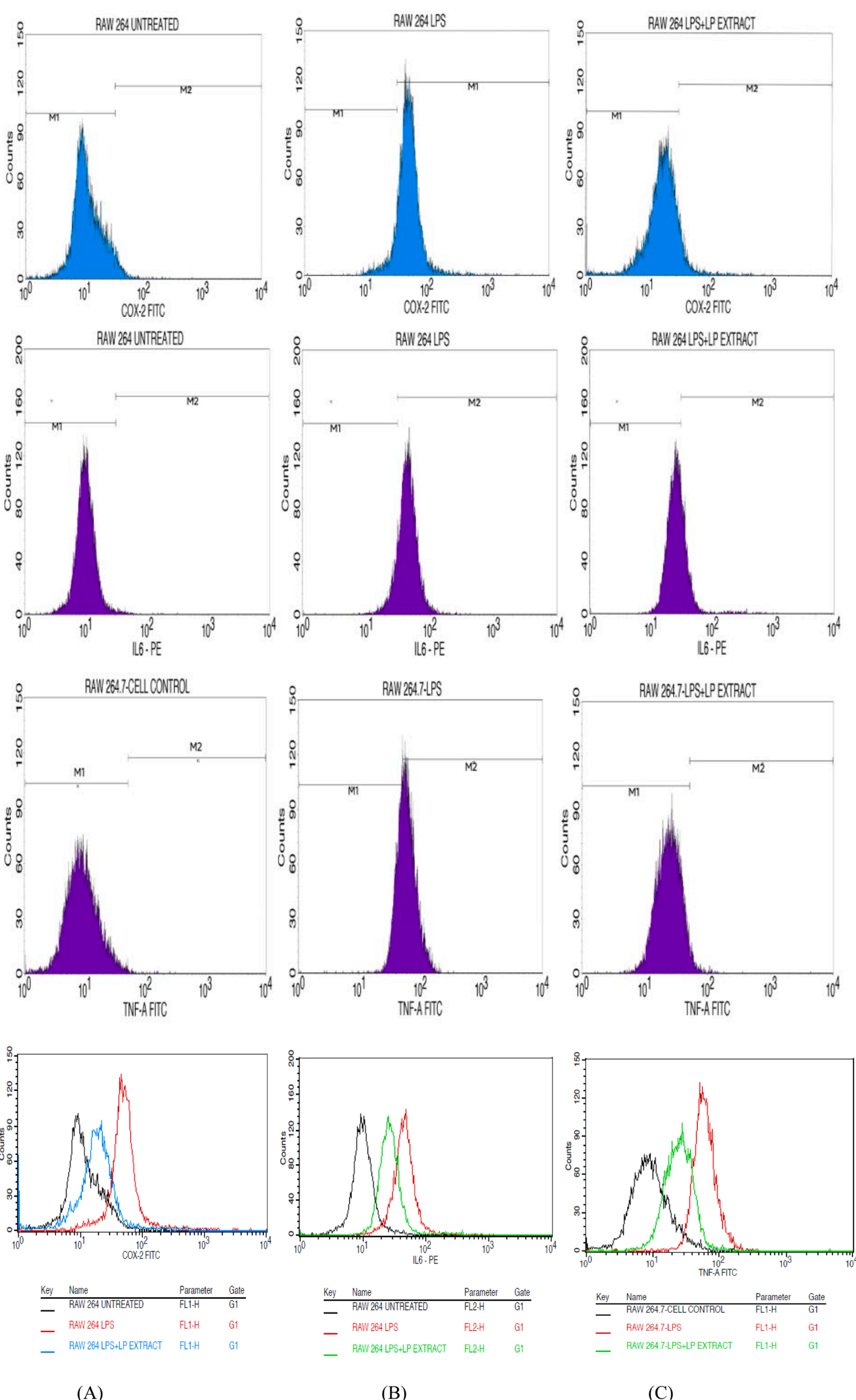



**Fig. 5.** Proinflammatory cytokine (COX2, IL-6 and TNF-alpha) expression study in the Ayapana protein-treated RAW 264.7 cells. Untreated RAW 264.7 mouse macrophage; RAW264.7 + LPS; RAW264.7 + LPS + Ayapana protein (LP extract). Histogram of the gated Raw 264.7 singlets distinguishes cells at the M1 and M2 phases. (M1 refers to the Negative expression/region, and M2 refers to the Positive expression/region). (A) Overlay of COX-2 expression study in Ayapana protein (LP extract). (B) Overlay of IL-6 expression study in Ayapana protein (LP extract). (C) Overlay of TNF-alpha expression study in Ayapana protein (LP extract).

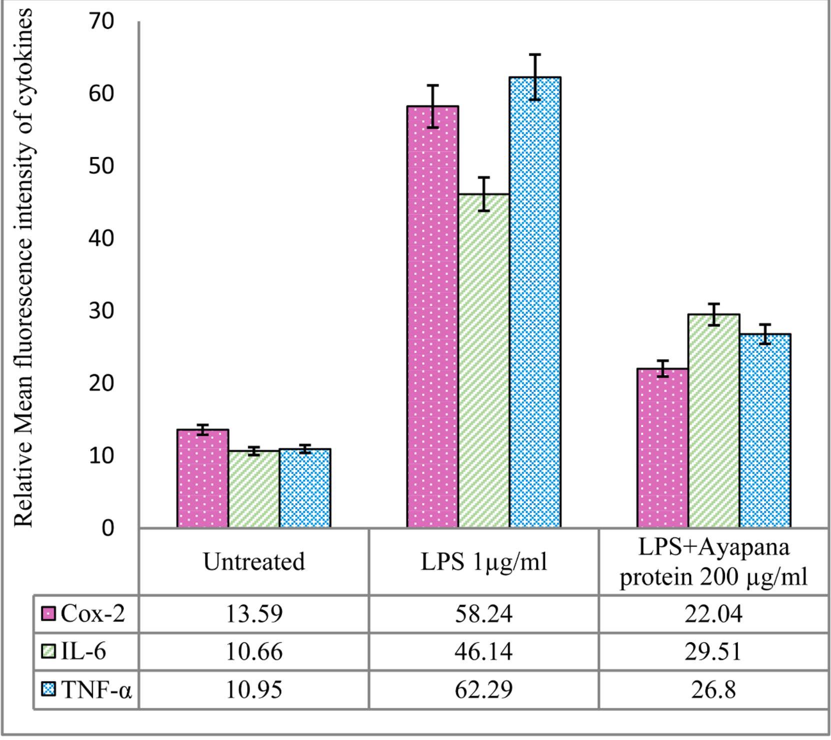



**Fig. 6.** Overall study of the expression of pro-inflammatory cytokines (COX-2, IL-6, TNF-α) in LPS-stimulated RAW 264.7 cells.

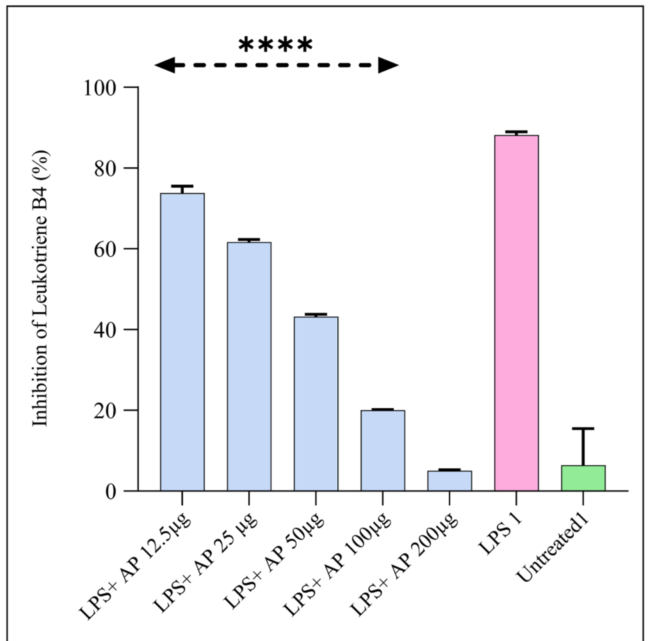



**Fig. 7.** Inhibition of Leukotriene B4 by Ayapana protein of Ayapana triplinervis Vahl. Lipopolysaccharide (LPS) + Ayapana protein (AP) with concentration range of 12.5 – 200µg/ml, Lipopolysaccharide (LPS), untreated. The significance of Ayapana protein was statistically determined by one-way Anova ($p < 0.0001$) ****.

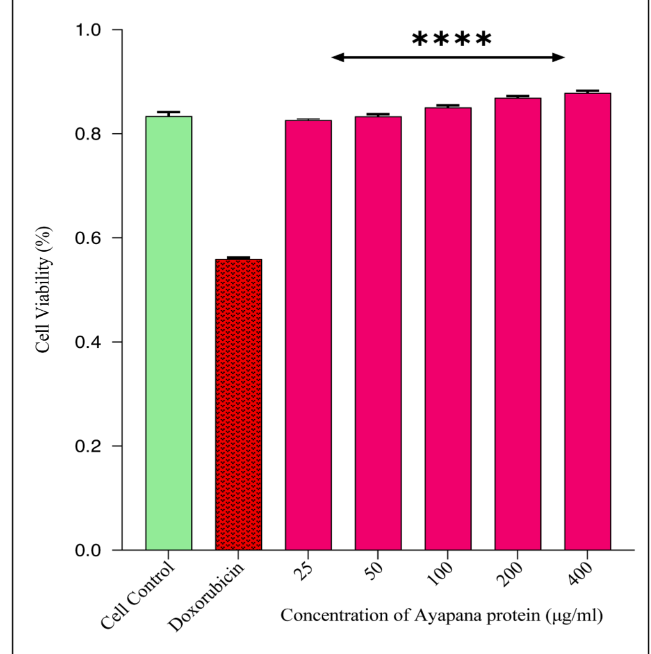



**Fig. 8.** Cytotoxicity study of Ayapana protein extract on L929 mouse fibroblast cell lines by MTT Assay. The significance of Ayapana protein was statistically determined by one-way Anova ($p < 0.0001$) ****.

## 5. Conclusion

The protein extract of *Ayapana triplinervis* exhibits significant anti-inflammatory, antioxidant and wound healing properties. By stabilizing cell membranes, suppressing inflammatory responses, and enhancing fibroblast proliferation and migration, the Ayapana protein demonstrates strong therapeutic potential. These findings support its development as a natural biomaterial for wound healing, with future studies directed towards isolating active constituents, exploring molecular mechanisms, and evaluating efficacy *in vivo* models.

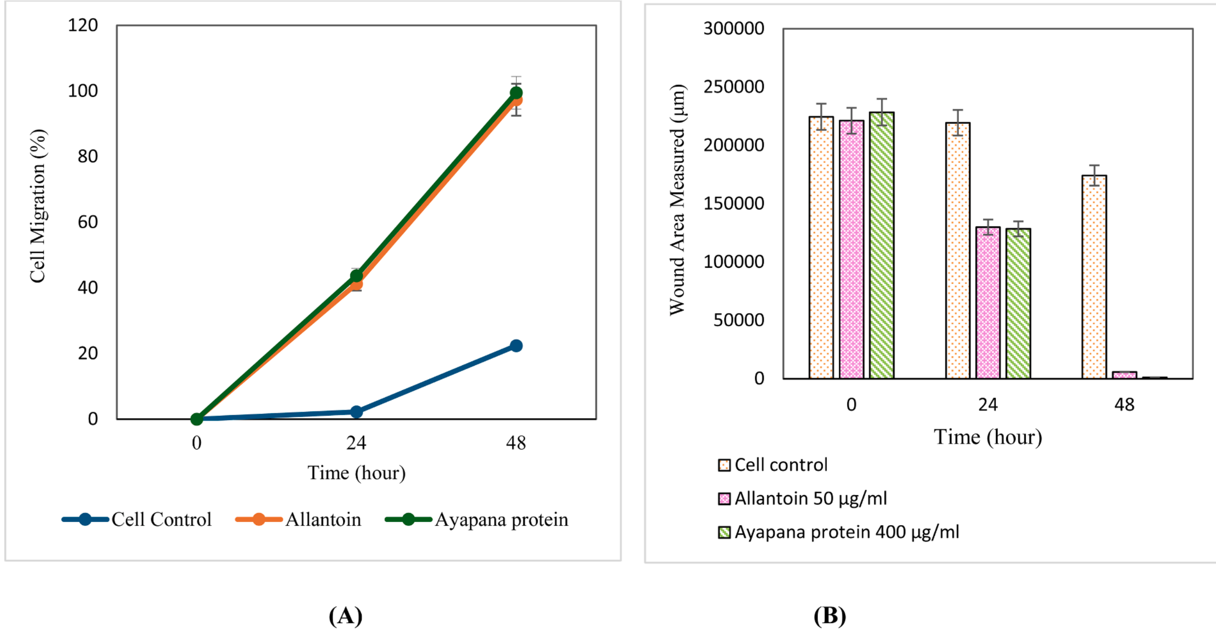



**Fig. 9.** Wound Scratch assay of the Ayapana protein (A) The cell migration was plotted at different time intervals; (B) Wound area measurement.

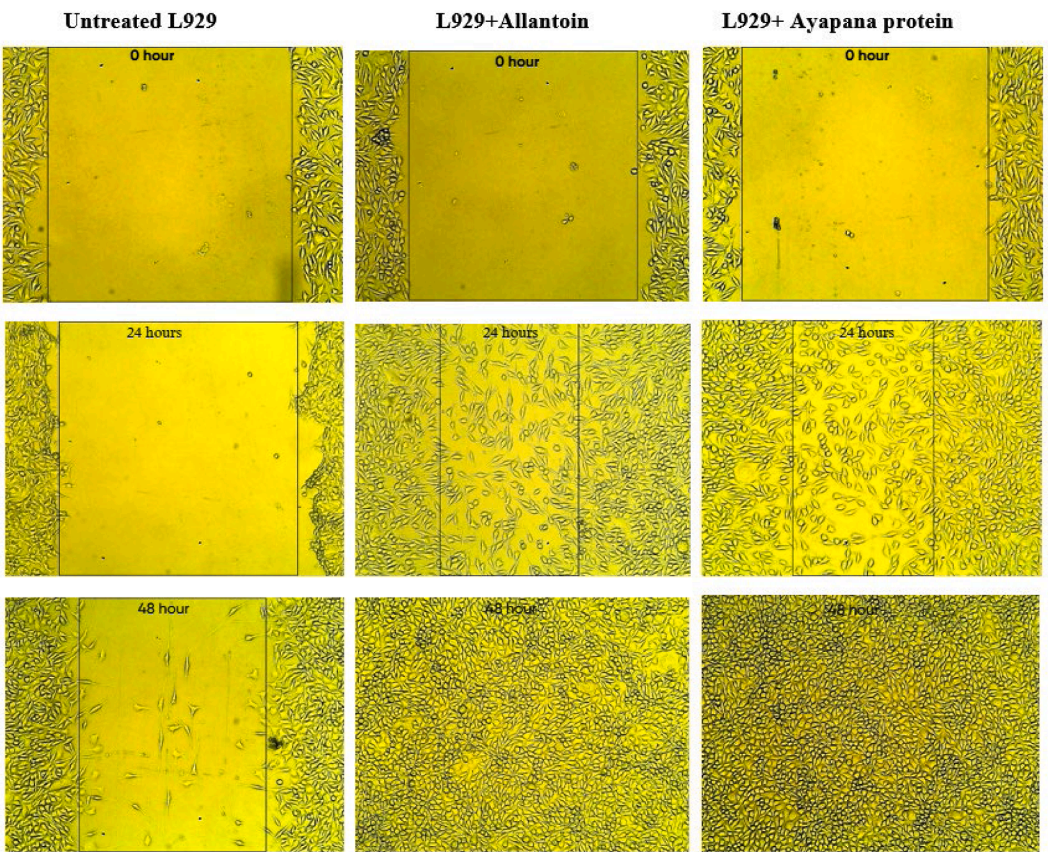



**Fig. 10.** Microscopic view of the wound scratch assay for L929 fibroblast cells at different time intervals.

## 6. Limitations

These findings suggest that the Ayapana protein would be a promising herbal therapeutic agent to manage inflammation and strengthen faster wound healing. However, we acknowledge that further mechanistic investigations are necessary. Future work will focus on identifying the active bioactive components, elucidating the molecular pathways involved (including PANoptosis and lysosomal-dependent cell death), and validating the findings *in vivo* wound-healing models. Additionally, the formulation of the protein into nanocellulose-based scaffolds will be explored to enhance its therapeutic delivery and clinical applicability.

## Ethical approval

Not Applicable.

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## Data availability

The authors confirm that the data supporting the findings of this study are available within the article. Raw data that support the findings of this study are available from the corresponding author, upon reasonable request.

## CRediT authorship contribution statement

**Anitha P:** Writing – original draft, Validation, Methodology, Investigation, Data curation, Conceptualization. **Balasubramanian Murugesan:** Writing – review & editing, Supervision, Conceptualization.

## 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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