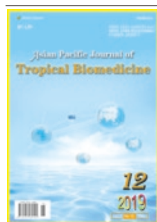

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Aloe barbadensis Miller peptide/polypeptide fraction alleviates inflammation through inhibition of proinflammatory cytokines and mediators *in vitro* and in rats with Freund's adjuvant–induced hind paw edema

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ABSTRACT

Objective: To evaluate the anti-inflammatory potential of peptide/polypeptide fraction of *Aloe vera* through *in vitro* and *in vivo* studies.

Methods: The peptide/polypeptide fraction from *Aloe vera* was obtained through trichloroacetic acid precipitation. The anti-inflammatory property of the peptide/polypeptide fraction was tested by protein denaturation, membrane stabilization assays. The effect of the fraction on RAW 264.7 cell viability was examined by MTT assays. The nitric oxide level was determined through Griess reagent. TNF-α and IL-6 levels were estimated using ELISA kits. *In vivo* studies were carried out in male Wistar rats through injection of Freund's adjuvant in the hind paw. Paw edema was measured through the Vernier scale and levels of alanine aminotransferase, aspartate transaminase, TNF-α, IL-6, and secretory phospholipase A2 were estimated through their respective kits after fourteen days of treatment. GraphPad Prism6 was used for analyzing the results.

Results: The peptide/polypeptide extract inhibited protein denaturation with an IC_{50} value of (218.9±15.6) μg/mL and stabilized the membrane of red blood cells with an IC_{50} value of (275.9±19.1) μg/mL. The extract showed no changes in cell morphology or cytotoxicity up to the concentration of 20 μg/mL in MTT assays. The peptide/polypeptide fraction markedly reduced the levels of proinflammatory markers and mediators in both *in vitro* and *in vivo* studies.

Conclusions: The results indicate that the peptide/polypeptide fraction of *Aloe vera* has anti-inflammatory property through inhibition of inflammatory markers and mediators responsible for NF-κB and mitogen-activated protein kinase pathways.

1. Introduction

Inflammation is an important defense mechanism protecting the body from pathogens and infections[1]. Nevertheless, excessive inflammation in the body leads to diseases such as arthritis, asthma, inflammatory bowel disorder, diabetes, *etc*. Cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin 6 (IL-6), nitric oxide (NO), chemokines, cyclooxygenase 2 (COX2) are all involved in the inflammatory response[2]. The expression of these factors is further activated through nuclear factor (NF-κB) and mitogen-activated protein kinase (MAPK) pathways. Various drugs available in the market target these inflammatory mediators to decrease the inflammation level. However, these drugs have side effects like lesions, cardiovascular disorders, bleeding and also affect the gastrointestinal tract and immune system[3,4]. Hence, it is needful to search alternative remedies from natural sources with therapeutic

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potential and minimal side effects.

Medicinal plants offer a plethora of bioactive constituents with many beneficial properties. Plant-based remedies are preferred due to their availability, better efficacy, and efficiency with lesser side effect[5,6]. One such plant is *Aloe barbadensis* Miller, which is commonly referred to as *Aloe vera*. It is considered as a miracle plant having medicinal properties such as anti-inflammatory, anti-diabetic, anti-cancer, anti-oxidant, immunomodulation and wound healing properties[7]. Previous studies have reported the anti-inflammatory potential of *Aloe vera* gel, where the gel exerts its anti-inflammatory property through inhibition of COX2, prostaglandins, bradykinins[8] and through the inhibition of NF-κB, JNK and extracellular signal-regulated kinase pathway[9]. It is necessary to identify the bioactive component from the gel with better therapeutic potential. Peptides or polypeptides are one of the bioactive constituents present in *Aloe vera*, and are considered to have anti-inflammatory property. There are few reports available on the anti-inflammatory potential of peptides. Lunasin derived from soybean exerts its anti-inflammatory activity through inhibition of NF-κB[10] while cyclic peptides from *Annona squamosa* seeds could reduce proinflammatory cytokines *in vitro*[11]. Peptides from myofibril protein of salmon have shown anti-inflammatory potential through the reduction in the levels of TNF-α, IL-6, inducible nitric oxide synthase, and COX2[12]. It is reported that A 14 KD glycoprotein from *Aloe vera* could exert its anti-inflammatory action through inhibition of COX2 and lipoxygenase[13]. The available literature on anti-inflammatory property of peptides/polypeptides from *Aloe vera* is insufficient, and the study was aimed to explore the anti-inflammatory property of peptide/polypeptide fractions from *Aloe vera* gel and the possible mechanism of action through both *in vitro* and *in vivo* studies.

2. Materials and methods

2.1. Chemicals

All standard chemicals, TNF-α, and IL-6 ELISA kits, lipopolysaccharide (LPS), complete-Freund's adjuvant were procured from Sigma Aldrich (USA), alanine aminotransferase (ALT) and aspartate transaminase (AST) from Arkray Healthcare Pvt Ltd (India) and secretory phospholipase A2 (sPLA2) kit from Cayman (USA). RAW264.7 cell line was purchased from National Centre for Cell Sciences, Pune. Diclofenac and aspirin drug was purchased from Cipla. Other chemicals of analytical grade were purchased from Sisco Research Laboratories, Mumbai, India.

2.2. Plant collection, extract preparation and enrichment of peptide/polypeptide fraction

Aloe vera plant was collected from Southern India. It was authenticated through Botanical Survey of India, Southern Regional Center, Coimbatore, India (BSI/SRC/5/23/2018/Tech/728). The *Aloe vera* extract was prepared according to Noor *et al*[14]. The peptide/polypeptide fractions from the extract were enriched through 30% trichloroacetic acid (TCA) precipitation[15]. In brief, the *Aloe vera* extract was dissolved in water and kept for shaking for 24 h. TCA solution was added to the extract and centrifuged at 5 000 rpm. The pellet was added with acetone and washed three times under the same condition. The resulting peptide/polypeptide fraction (PPF) obtained was stored at -80 ℃ for further use. Bradford assay was carried out to estimate the amount of protein[16] and 15% SDS-PAGE was performed.

2.3. Anti-inflammatory potential of PPF of Aloe vera

2.3.1. Albumin denaturation inhibition assay

Different concentrations of PPF (50, 100, 150, 200 and 250 μg/mL) were mixed with 1% bull serum albumin and phosphate-buffered saline and the assay was carried out according to Osman *et al*[17] with aspirin as standard drug. The percentage of inhibition was calculated as follows:

Percentage of inhibition =[(Ac-As)/Ac]×100

Where Ac = absorbance of the control reaction (all reagents except the test sample), As = the absorbance of the test sample.

2.3.2. Membrane stabilization assay

The rat blood was mixed with Alsever solution and centrifuged at 3 000 rpm. Cells obtained were washed with saline, 10% red blood cell (RBC) suspension was made and the assay was carried out according to Chowdhury *et al*[18] with diclofenac as standard drug. The percentage of protection was calculated as follows:

Percentage of protection = 100 – (As/Ac)×100

Where Ac = absorbance of the control reaction (all reagents except the test sample), As = the absorbance of the test sample.

2.4. In vitro cell culture studies

2.4.1. Cell culture

RAW 264.7 cells were cultured and passaged in Dulbecco's Modified Eagle Medium supplemented with 10% FBS with 0.4% penicillin and streptomycin and maintained in a CO_2 incubator with 5% CO_2 at 37 ℃.

2.4.2. Cell viability assay

RAW 264.7 cells were seeded at a density of 5×10^3 cells/well in 96 well plates. After 12 hours of incubation, the cells were treated with various concentrations of PPF (5, 10, 15, 20, 25, 30 μg/mL) and further incubated for 24 h. Viability of cells were assessed through 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT)[19].

2.4.3. Estimation of NO, TNF-α and IL-6 levels

RAW 264.7 cells were seeded at a density of 1×10^4 in a 96 well plate with 10 and 20 μg/mL of PPF for 2 h. LPS (1 μg/mL) was added and incubated for 24 h. The supernatant was collected and mixed with an equal amount of Griess reagent and NO levels were studied[20]. The remaining supernatant was used to estimate the levels of TNF-α[21] and IL-6[22] through respective ELISA kits (Sigma Aldrich). Diclofenac was used as a standard drug[23].

2.5. In vivo anti-inflammatory potential of PPF of Aloe vera

2.5.1. Animals

Male Wistar rats weighing around 200 g (3 months old) were obtained from Animal House of the Institute. The rats were housed in an air-conditioned room [(25 ± 1) ℃] with a 12-h light/12-h dark cycle. The animals were acclimatized to the environment before the experimental use, by supplying water *ad libitum* and fed with a standard laboratory diet. The animals were treated according to the guidelines recommended by the Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India. The experiments were approved by the Institutional Animal Ethics Committee (IAEC No: VIT/IAEC/Sep1/05).

2.5.2. Experimental procedure

The animals were randomly allocated to four different groups comprising of six animals each as mentioned below:

Group 1: Normal rats (N); Group 2: Freund's adjuvant-induced rats (IC); Group 3: Freund's adjuvant-induced rats treated with PPF extract[14] (0.45 mg/kg bw) (IC+PPF); Group 4: Freund's adjuvant-induced rats treated with diclofenac[24] (10 mg/kg bw) (IC+STD).

2.5.3. Complete-Freund's adjuvant-induced rat paw edema

Freund's Adjuvant [0.1 mL heat-killed *Mycobacterium tuberculosis* (10 mg/mL) in paraffin oil] was injected intradermally into the footpad of left hind paw[25]. Wherein the PPF and diclofenac were fed to the rats orally after 7 days of adjuvant injection and continued till 14 days. The edema was measured using a Vernier scale. The difference in the volume of paw on day 1 and day 14 was considered as edema. The changes in treated groups were compared to the inflammatory control group and percentage inhibition was calculated as mentioned below:

Percentage inhibition of edema = $(T_c - T_t)/T_c \times 100$

Where T_c = paw thickness in inflammatory control group (IC); T_t = paw thickness in treated groups.

After 14 days, the rats were sacrificed with mild ether anesthesia. The blood was collected through the cardiac puncture and centrifuged. The plasma was collected and stored at -80 ℃. The plasma was used for the estimation of AST and ALT[26], TNF-α, IL-6, and sPLA2[27] levels through respective ELISA kits as per the manufacturer's protocol.

2.6. Statistical analysis

Statistical analysis was performed through GraphPad Prism 6. All results were expressed as mean ± SD. The analysis was performed through One way ANOVA followed by the Bonferroni method. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Anti-inflammatory assays

The protein concentration in *Aloe vera* extract was (1.7±0.3) mg/g. Upon TCA precipitation, the protein concentration was increased to (5.5±0.2) mg/g in the enriched PPF and the same was confirmed through the 15% SDS-PAGE gel (Figure 1). The PPF of *Aloe vera* extract was tested for its anti-inflammatory study through protein denaturation and membrane stabilization assay. In both the studies, the PPF has shown a positive result in a dose-dependent manner (Figure 2). The PPF had the ability to inhibit the protein denaturation with an IC_{50} value of (218.9±15.6) μg/mL and to stabilize the membrane of RBC in a dose-dependent manner with an IC_{50} value of (275.9±19.1) μg/mL.

3.2. In vitro cell culture studies

3.2.1. Cell viability assay

From the cell viability assay, it was observed that there were no changes in cell morphology or cytotoxicity up to the concentration of 20 μg/mL. Two concentrations of PPF were selected (lower 10 μg/mL and higher 20 μg/mL) for the following assays.

3.2.2. Estimation of NO Levels, TNF-α and IL-6 levels

Upon LPS stimulation, the NO levels were significantly increased ($P < 0.001$) by 75.1% compared to normal cells. Treatment of LPS stimulated RAW 264.7 cells with PPF at 10 μg/mL and 20 μg/mL reduced the NO levels by 60.2% and 65.9%, respectively (Figure 3A).

The TNF-α and IL-6 levels were increased significantly ($P < 0.001$) by 76.2% and 68.4%, respectively upon treatment with LPS when compared to normal cells. The TNF-α levels were decreased by 56.8% and 64.5% and IL-6 levels decreased by 52.3% and 65.9% on treatment with 10 μg/mL and 20 μg/mL of PPF, respectively (Figure 3B, 3C).

3.3. In vivo studies

3.3.1. Estimation of AST and ALT levels

The levels of AST and ALT in plasma were measured in adjuvant-induced rats followed by treatment with PPF. In adjuvant-induced rats (IC Group), the levels of AST and ALT were increased significantly by 55.1% and 57.7%, respectively compared to normal rats ($P < 0.001$). Upon administration of PPF, AST and ALT levels were decreased significantly ($P < 0.001$) by around 44.5% and 41.8%, respectively (Table 1).

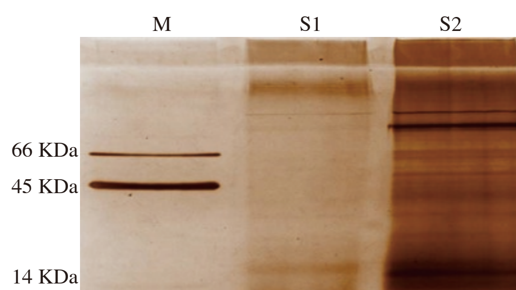

Figure 1. SDS-PAGE (15%) of *Aloe vera* extract and enriched peptide/polypeptide fraction. Lane 1: Marker, Lane 2 (S1): *Aloe vera* extract (5 μg), Lane 3 (S2): peptide/polypeptide fraction (5 μg).

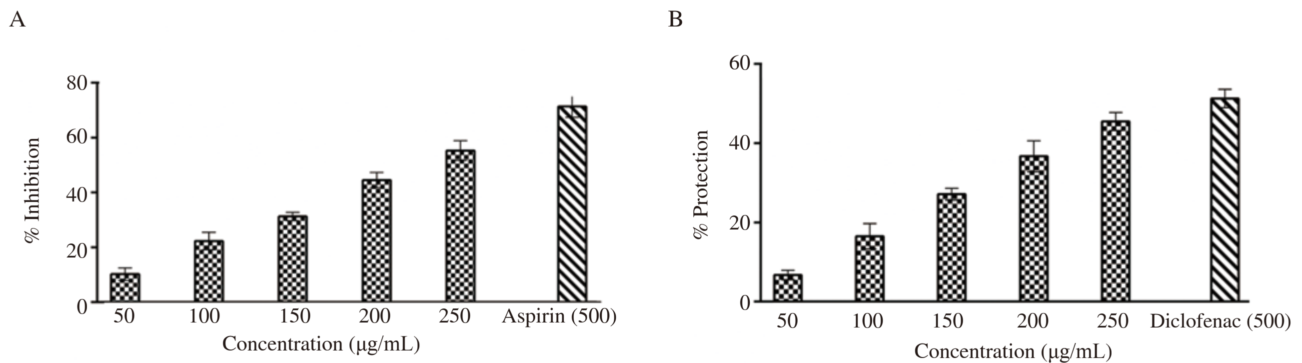

Figure 2. Effect of peptide/polypeptide fraction on protein denaturation (A) and membrane stabilization (B). Values expressed as Mean±SD, n=3.

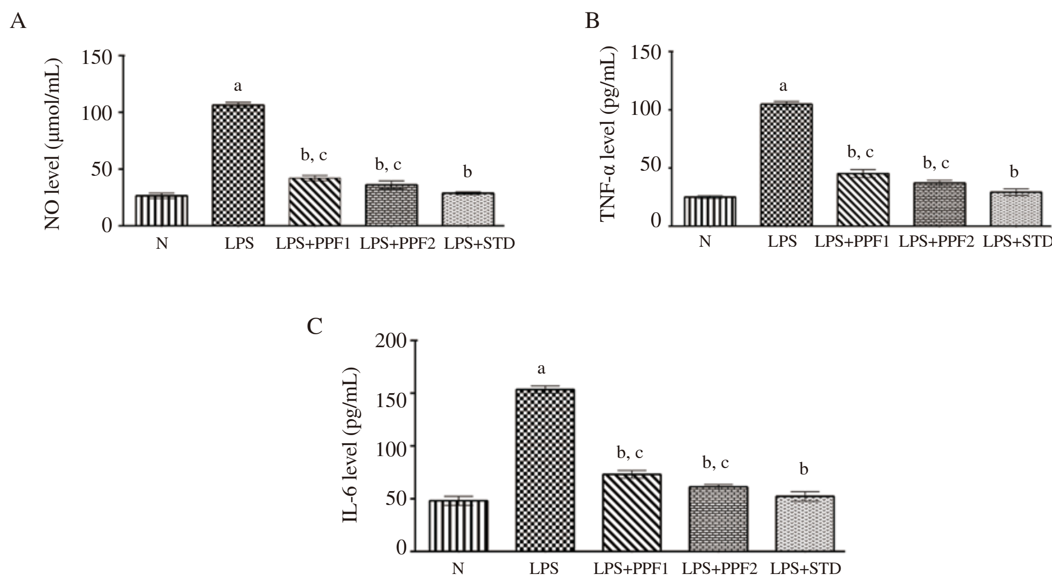

Figure 3. Effect of peptide/polypeptide fraction on NO levels (A), TNF-α levels (B) and IL-6 levels (C) *in vitro*. Values were expressed as Mean±SD, n=6. Data were analysed through One way ANOVA followed by Bonferroni test. N: normal group; LPS: LPS induced group; LPS+PPF: LPS induced+peptide/polypeptide fraction group (PPF1=10 μg/mL, PPF2=20 μg/mL); LPS+ STD: LPS-induced+diclofenac. a: $P < 0.05$, N *vs.* LPS; b: $P < 0.001$, LPS *vs.* LPS+PPF, LPS *vs.* LPS+STD; c: $P < 0.01$, LPS+PPF *vs.* LPS+STD.

3.3.2. Estimation of paw edema, TNF–α, IL–6, and sPLA2 levels

An increase in the paw volume by 74.3% ($P < 0.001$) in inflammatory control rats (Group IC) was observed when compared to the paw of the normal rats (Group N) (Figure 4A). The paw edema was decreased by 60.1% upon treatment with PPF (Group IC+PPF) and 66.6% with the standard drug (Group IC+STD).

The proinflammatory cytokines TNF-α, IL-6, and sPLA2 were increased significantly by 73.4%, 68.35 and 70.2%, respectively in adjuvant-induced rats (Group IC) when compared to the normal group rats ($P < 0.001$). Treatment of PPF reduced the levels of TNF-α, IL-6 and sPLA2 by 60.5%, 56.9% and 62.4%, respectively (Figure 4B, 4C, 4D) when compared to inflammatory control group rats ($P < 0.001$).

Table 1. Levels of alanine aminotransferase and aspartate transaminase upon treatment of peptide/polypeptide fraction in rats.

Groups	Aspartate transaminase level (IU/mL)	Alanine aminotransferase level (IU/mL)
N	59.5±4.5	37.3±3.2
IC	132.4±3.2^{a}	88.2±4.6^{a}
IC+PPF	73.4±2.1^{b,c}	51.3±2.8^{b,c}
IC+STD	63.5±3.2^{b}	40.2±2.5^{b}

Values were expressed as Mean±SD, n=6. Results were analyzed through One-way ANOVA followed by Bonferroni test. N: normal group; IC: Adjuvant-induced group; IC+PPF: Adjuvant-induced+ peptide/polypeptide fraction group; IC+ STD: Adjuvant-induced+diclofenac. $^{a}P < 0.05$, N *vs.* IC; $^{b}P< 0.001$, IC *vs.* IC+PPF, IC *vs.* IC+STD; $^{c}P < 0.01$, IC+PPF *vs.* IC+STD.

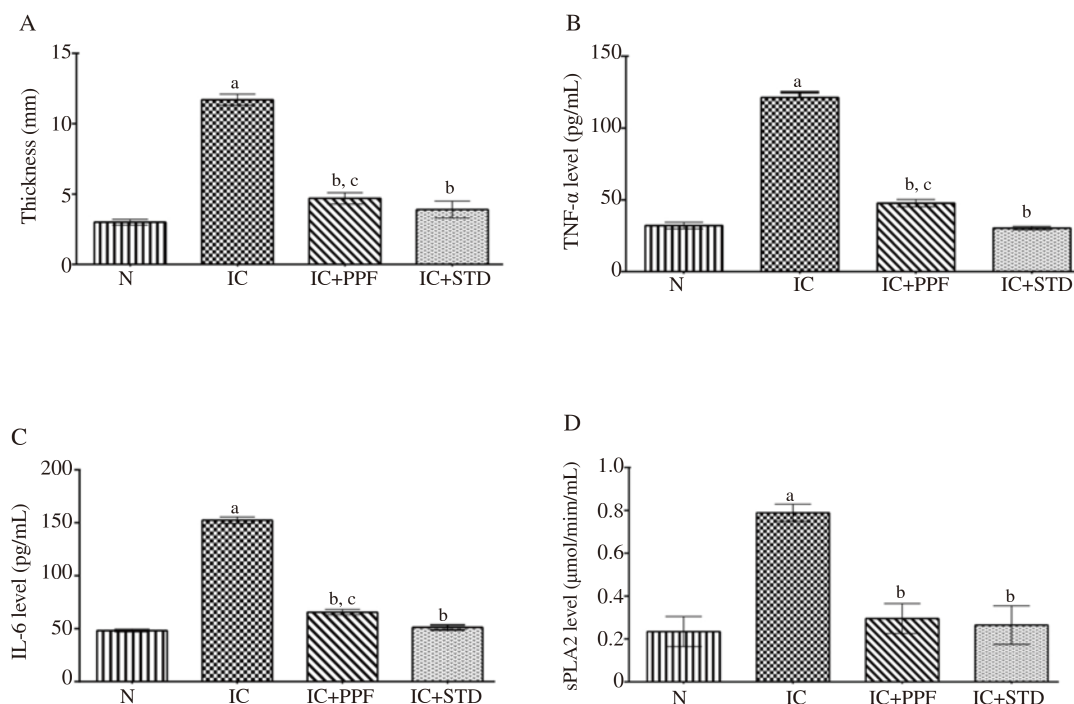

Figure 4. Effect of peptide/polypeptide fraction on paw edema (A), TNF-α levels (B), IL-6 levels (C) and sPLA2 level (D) *in vivo*.Values were expressed as Mean±SD, n=6. Data were analysed through One way ANOVA followed by Bonferroni test. N: normal group; IC: Adjuvant-induced group; IC+PPF: Adjuvant-induced+peptide/polypeptide fraction group; IC+ STD: Adjuvant-induced+diclofenac. a: $P < 0.05$, N *vs.* IC; b: $P < 0.001$, IC *vs.* IC+PPF, IC *vs.* IC+STD; c: $P < 0.01$, IC+PPF *vs.* IC+STD.

4. Discussion

Anti-inflammatory medications available in the market have been reported to cause severe side effects[28] and hence, more studies have focused on herbal medicine and the bioactive constituents present in them[29]. In the present study, the anti-inflammatory potential of peptides/polypeptides from *Aloe vera* was studied through *in vitro* and *in vivo* studies to explore the possible mechanism of action in the alleviation of inflammation.

Inflammation in living tissues is stimulated through injuries, microbial infections, irritations which has been associated with pathogenesis of diseases such as cancer, arthritis, stroke, *etc*[30]. Protein denaturation inhibition assay and membrane stabilization assay are used to determine the anti-inflammatory property of the extracts[17,18]. The PPF of *Aloe vera* extract has the capacity to inhibit the protein denaturation in a dose-dependent manner. Protein denaturation is often correlated to inflammatory stimuli in various inflammatory diseases. Tissue injury can be referred to as protein denaturation in cells and tissues[31]. Hence inhibition of protein denaturation indicates the anti-inflammatory potential of the extracts. In the present study, the PPF was able to protect the RBC membrane in a dose-dependent manner. Infiltration of leucocytes occurs in cells as a defense mechanism in the body against inflammation[32]. Leucocytes release their lysosomal content leading to further damage and injury to the cell membrane. Damaged lysosomal membrane releases phospholipase A2 (PLA2) leading to the production of prostaglandins and leukotrienes which causes further inflammatory responses. The RBC membrane is similar to the lysosomal membrane and hence stabilization of the RBC membrane is one of the assays to determine the anti-inflammatory potential of extracts[33]. As the RBC membrane and lysosomal membrane share similar properties, PPF may stabilize the RBC membrane through inhibition of PLA2 pathway.

Based on the results obtained, the PPF of *Aloe vera* extract was tested for its anti-inflammatory property on RAW264.7 cells. The LPS stimulation leads to the production of various proinflammatory cytokines such as TNF-α, IL-6, IL1, vascular endothelial growth factor and inflammatory mediators like NO, prostaglandins through NF-κB pathway in macrophages[25]. The high levels of these proinflammatory mediators and cytokines have been implicated in autoimmune disorders[34]. Therefore, reduction in the levels of these mediators and cytokines can decrease the inflammatory response. The PPF has the capacity to reduce NO, TNF-α, and IL-6 levels in our *in vitro* studies. It suggested that the PPF has the ability to down-regulate the levels of the above-mentioned cytokines thereby inhibiting the NF-κB pathway.

To substantiate our results of *in vitro* studies, the anti-inflammatory potential of PPF was also tested *in vivo* in an adjuvant-induced inflammatory model which is a well-known model to investigate the anti-inflammatory potential of extracts. The present study revealed that the administration of Freund's adjuvant increased the paw edema, and the levels of tissue marker enzymes (AST, ALT, cytokines TNF-α and IL-6 and sPLA2 enzyme). Treatment with PPF was able to reduce the levels of paw edema as well as tissue marker

enzymes significantly. High levels of AST and ALT indicate liver and kidney damage during inflammation. The levels of AST & ALT play an important role in the release of bradykinins in the inflammatory process and are involved in bone erosion and osteopenia[35]. The PPF of *Aloe vera* extract was able to reduce the high levels of AST and ALT due to administration of Freund's adjuvant in rats.

It was also observed that the levels of TNF-α and IL-6 were elevated in the plasma of inflammatory control rats which was reduced by treatment with PPF extract. An important aspect of inflammation is that there is a disturbance in the homeostasis of anti-inflammatory and proinflammatory cytokines[25]. The increased levels of proinflammatory cytokines such as TNF-α, IL-6, monocyte chemoattractant protein 1 propagate the progression of inflammation through the infiltration of neutrophils leading to destruction and damage of the joints and bones[36]. TNF-α is one of the pleiotropic cytokines, which is seen mostly in inflammatory disorders. Cells such as synoviocytes, T-cells produce proinflammatory cytokines upon activation leading to synovial inflammation in the joints[25]. Another pleiotropic cytokine is IL-6 which plays a crucial role in the maturation of B cells. IL-6 is also involved in the production of auto-antibodies and plays a role in arthritic progression through elevated levels of vascular endothelial growth factor[37]. Both TNF-α and IL-6 act synergistically in activating the immune cells in synovium leading to bone loss along with oxidative stress[38]. Another enzyme involved in inflammation is secretory sPLA2. These sPLA2 enzymes are active during inflammation and are one of the major targets for drug development against inflammation[39]. They help in the progression of inflammation through mast cell degranulation and the release of proinflammatory cytokines. The levels of sPLA2 are high in the joints of patients affected with the inflammatory disorder[40,41]. Treatment with PPF extract to the adjuvant-induced rats has shown a significant reduction in the levels of these inflammatory cytokines as well as the levels of sPLA2 enzyme.

The results from this study indicate that the PPF of *Aloe vera* has shown anti-inflammatory potential in both *in vitro* and *in vivo* studies. The PPF is able to suppress the inflammatory mediators like NO, TNF-α, IL-6 in RAW264.7 cells and was able to reduce the paw edema volume, AST, ALT, TNF-α, IL-6 and sPLA2 levels in adjuvant-induced rats. The reduced levels of inflammatory mediators may affect the inflammatory pathways such as NF-κB and MAPK pathways. MAPK plays a role in the regulation of LPS stimulated NF-κB pathway[42,43]. This leads to phosphorylation of IκB-α or P65 which further activates the inflammatory genes like TNF-α and IL-6. PPF of *Aloe vera* extract may be related to either of these factors IκB-α or P65, thereby decreasing the levels of TNF-α and IL-6. Further, NF-κB regulates the proinflammatory cytokines TNF-α and IL-6 in macrophages. These elevated levels of cytokines may activate NF-κB in other immune cells and fibroblast cells leading to the increased inflammatory response[44]. The PPF may also be related to these factors such as p50, RelA thereby inhibiting the NF-κB pathway leading to decreased levels of cytokines and other inflammatory mediators[44]. The peptide/polypeptide fraction has promising potential and it needs to be further purified and characterized in order to identify the molecular mass, the nature of the peptides and/or polypeptides with anti-inflammatory action. These peptides/polypeptides may serve as potential candidates for the treatment of inflammatory diseases.

Conflict of interest statement

Authors declare that there are no competing interests.

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Authors' contributions

SNB was involved in data collection, data analysis and interpretation, as well as drafting the article. AN was involved in conception of the work, data analysis and interpretation, drafting the article, critical revision of the article, and final approval of the version

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