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Isolation and characterization of novel protein with anti-fungal and anti-inflammatory properties from *Aloe vera* leaf gel

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ABSTRACT

The Aloe protein of 14 kDa from the *Aloe vera* leaf gel was isolated by an ion exchange chromatography using DEAE-cellulose and CM-cellulose column. The purified Aloe protein exhibited a potent anti-fungal activity against *Candida paraprilosis*, *Candida krusei* and *Candida albicans*. In addition, the purified Aloe protein also showed an anti-inflammatory property against pure lipoxygenase and cyclooxygenase-2 with 84% and 73% inhibition, respectively, and was verified by binding with these proteins by real time method by the phenomenon of surface plasmon resonance. This Aloe protein is a novel protein possessing antifungal and anti-inflammatory properties and thus sets a platform to be used as a medicinal plant product.

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1. Introduction

Since 1986 *Aloe vera* has been used as a traditional medicine and as an ingredient in many cosmetic products. It has gained high importance for its diverse therapeutic properties. The plant, being succulent, contains 99.5 per cent water and the remaining solid material contains over 75 different ingredients including vitamins, minerals, enzymes, sugars, anthraquinones or phenolic compounds, lignin, tannic acids, polysaccharide, glycoproteins, saponins, sterols, amino acids and salicylic acid [1]. *A. vera* provides nutrition, shows anti-inflammatory action and has a wide range of antimicrobial activity [1]. In-vitro experiments have been carried out on numerous organisms and have regularly shown that, in normal strength, *A. vera* is either bactericidal or bacteriostatic against a number of common wound pathogens like *Candida* infections.

Scientific research shows strong immunomodulatory and antitumor properties [2] of *A. vera* polysaccharides that help to boosts the immune system function [3] while destroying cancer tumors [1,4]. It inhibits pain-producing substances like thromboxane & bradykinin [1,5–8]. It protects the body from oxidative stress, which showed the anti-oxidant effect. It alkalizes the body, helping to balance overly acidic dietary habits. It boosts the oxygenation of blood, thereby enhancing the quality of the blood and has a significant impact on reducing heart attacks and strokes. Eating *A. vera* is like adding an all-natural non-stick additive to the blood flow [9–11]. It stabilizes blood sugar and reduces triglycerides in diabetics [1]. This study reports the isolation and characterization of a novel protein with antifungal and anti-inflammatory properties of molecular weight 14 kDa from *A. vera* leaf gel.

2. Materials and methods

A. vera was provided from the nearby garden. DEAE-cellulose was purchased from GE Healthcare and CM-cellulose was purchased from Sigma. All chemicals were of research grade purchased from Sigma and Merck and the protein molecular mass marker was from Fermentas. The sensor chip CM5, surfactant P20, the amine coupling kit containing NHS, EDC and ethanolamine hydrochloride was purchased from Pharmacia Biosensor AB, Uppsala, Sweden.

2.1. Purification of the Aloe protein from the leaf-gel of A. vera

The *A. vera* leaves were rinsed thoroughly in distilled water. The mucilaginous leaf gel was scrapped out from the leaf. The gel was thoroughly homogenized and filtered with cheesecloth.

Abbreviations: SPR, surface plasmon resonance; NHS, N-hydroxysuccinimide; EDC, N-ethyl-N′-3 diethylaminopropyl carbodiimide; NTA, nitrilotriacetic acid; PBS, phosphate buffer saline.

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The filtered gel was precipitated with 30% ammonium sulphate. The precipitated gel was centrifuged at 10,000 rpm for 30 min. The supernatant was dialyzed in 10 mM Tris–HCl (pH 8.5) at room temperature.

The purification steps were followed by ion exchange chromatography using DEAE-cellulose and CM-cellulose column. The leaf extract was loaded to a column of DEAE-cellulose, which had previously been equilibrated with 10 mM Tris–HCl buffer (pH 8.5). After removal of the unadsorbed fraction, the adsorbed peaks were eluted with 100 mM NaCl to 1 M NaCl in the same buffer gradually. The anti-fungal activity was checked in unadsorbed and in eluted fractions and was observed only in unadsorbed fraction. Hence, the unadsorbed fraction was loaded onto CM-cellulose column equilibrated with 10 mM MES buffer (pH 4.5). The elution was carried out using a linear gradient formed with 10 mM MES buffer containing 100 mM NaCl up to 1 M NaCl (pH 4.5).

2.2. Molecular mass determination

The gel was run in 12.5% resolving and 5% stacking gel concentration by the method of Laemmli and Favre [12]. After electrophoresis, the gel was stained in Coomassie brilliant blue R-250. The molecular mass of the protein was determined by comparison of its electrophoretic mobility with those of the molecular mass protein marker. The gel was also stained with silver stain to ascertain the purity of the protein.

2.3. N-terminal sequence analysis

The N-terminal sequence analysis of the Aloe protein was done by Edman degradation on a Procise Protein Sequencer (Applied Biosystems). The database was searched for other antifungal proteins with similar sequences using BLAST (http://www.ncbi.nlm.nih.gov/BLAST).

2.4. Assay of antifungal activity

The chromatographic fractions at various stages of purification were tested for antifungal activity after lyophilization. The assay for antifungal activity toward *Candida parapsilosis* (ATCC 22019), *Candida albicans* (ATCC SC 5314), and *Candida krusei* (ATCC 6258) was carried out in 90 mm × 10 mm Petri plates containing 20 ml of potato dextrose agar. These fungal species have been shown to be sensitive to a variety of antifungal proteins. After the mycelial colony had developed, wells were punctured at three corners of the plates. An aliquot (75 μl of 0.5 mg/ml) of the protein solution was added to the wells. The positive and negative controls were 25 μg flucanazole and assay buffer without protein, respectively. The plates were incubated at 37 °C for 72 h until mycelial growth had enveloped the wells containing the control and zones of inhibition form around wells containing Aloe protein [13–15].

To determine the IC_{50} value of the Aloe protein, three doses of it were added separately to three aliquots each containing 4 ml of potato dextrose agar at 45 °C, mixed rapidly and poured into three separate small Petri dishes. After the agar had cooled down, a small amount of mycelia of each fungal colony was added. Buffer without Aloe protein served as a control. After incubation at 37 °C for 72 h, the IC_{50} was determined by the following formula [16]:

$$\text{The percentage inhibition of fungal growth} = \frac{\begin{array}{c}\text{area of mycelial colony in the absence of antifungal protein}\\ -\text{area of mycelial colony in the presence of antifungal protein}\end{array}}{\text{area of mycelial colony in the absence of antifungal protein}} \times 100\%$$

2.5. Assay of anti-inflammatory activity

2.5.1. Analysis by spectrophotometer: inhibition studies of the Aloe protein against LOX and COX-2 by biochemical assay

For inhibition studies, 1:1 molar ratios of purified LOX and the Aloe protein were incubated for 45 min which was then added to the reaction mixture containing 50 mM potassium phosphate buffer, 0.15 mM linoleic acid (substrate) and 0.2% Tween-20. The change in absorbance was monitored at 234 nm and the percentage inhibition was calculated [17]. A control reaction was always carried out without any Aloe protein to check the activity of protein under experimental conditions.

In case of COX-2 activity, the peroxidase assay was performed to estimate the formation of prostaglandin at 610 nm. For the inhibition assay, the purified COX-2 was incubated with Aloe protein in 1:1 molar ratio in the assay buffer for 45 min at 25 °C [18]. The decline in activity was determined and the percentage inhibition of Aloe protein was then calculated [19].

2.5.2. Analysis by BIAcore 2000: binding with LOX and COX-2

The binding property of Aloe protein was investigated by SPR. All SPR measurements were performed at 25 °C using the BIAcore-2000 (Pharmacia Biosensor AB, Uppsala, Sweden), which is a biosensor-based system for the real-time specific interaction analysis [20]. The LOX was immobilized on the sensor chip CM5 at a flow rate of 10 μl/min at 25 °C using amine-coupling kit [17]. The dextran on the chip was equilibrated with running buffer 10 mM HBS-EP (pH 7.4) and the carbomethylated matrix was activated with an EDC/NHS mixture. 210 μl of LOX (50 μg/ml) in 10 mM sodium acetate (pH 4.8) was injected and the unreacted groups were blocked by passing ethanolamine (pH 8.5). The SPR signal for LOX was found to be 4015 RUs. The three different concentrations of Aloe protein (71×10^{-9} M, 213×10^{-9} M and 355×10^{-9} M) were passed at a flow rate of 10 μl/min over the immobilized LOX. The rate constants K_A and K_D were obtained by analyzing sensogram data using the BIA evaluation 3.0 software.

The interaction of COX-2 with Aloe protein was performed by immobilizing 60 μl of recombinant His-tag COX-2 (50 μg/ml) on Ni^{2+}-(NTA) sensor chip. The SPR signal for COX-2 was found to be 900 RUs [18]. The three different concentrations of Aloe protein (71×10^{-9} M, 213×10^{-9} M and 355×10^{-9} M) were passed over the immobilized COX-2 at a flow rate of 10 μl/min for 4 min and the change in RU was observed.

2.6. Assay for protease activity

For protease activity casein solution was freshly prepared by dissolving 0.5 g of casein in 2.5 ml distilled water and 2.5 ml of 0.2 M NaOH [21]. 50 ml each of purified *A. vera* protein (test sample) and trypsin (positive control) were incubated for 25 min at room temperature with 350 μl of casein solution followed by addition of 1 ml of 4% (w/v) trichloroacetic acid was added. The supernatant was obtained by centrifugation at 8000 rpm for 15 min. The absorbance of the casein fragments produced in the supernatant by the proteolytic action was observed at 280 nm against water as a blank.

2.7. Assay for protease inhibitory activity

For the protease inhibition assay, Aloe protein was incubated with trypsin in 50 mM Tris–HCl buffer (pH 8) at 25 °C for 30 min

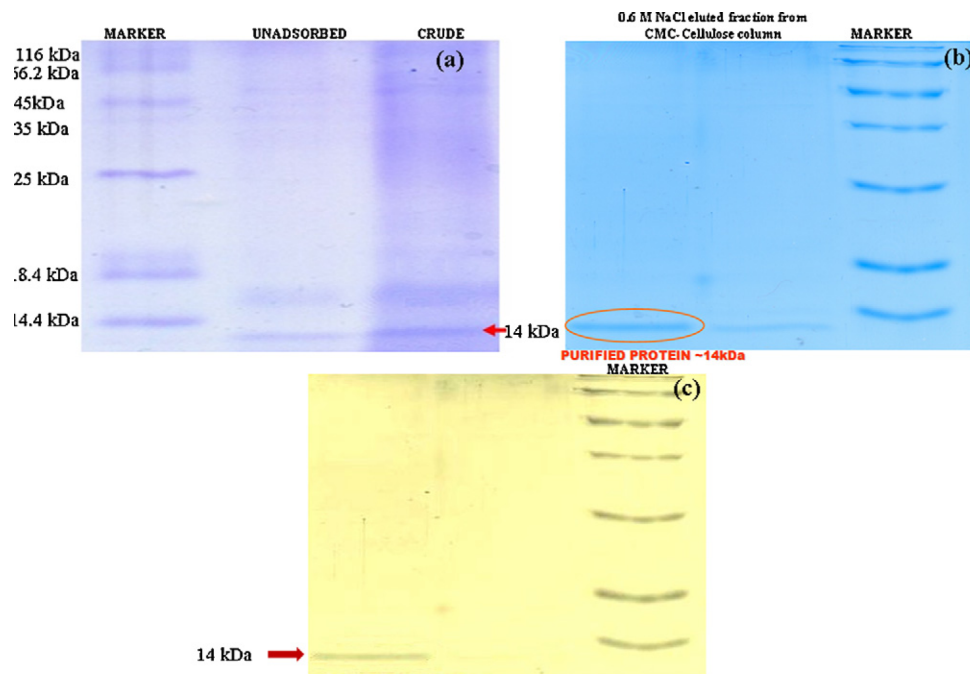

Fig. 1. Purification of Aloe protein showing (a) unbound fraction from DEAE-cellulose column, (b) single band in 0.6 M NaCl eluted fraction from CM-cellulose column stained in Coomassie brilliant blue and (c) silver stain of the CM cellulose fraction.

and then assay carried out using a similar protocol as for the protease activity for the enzyme. The absorbance of supernatant was recorded at 280 nm [21].

2.8. Assay for hemagglutinating activity

The hemolytic activity of the Aloe protein was determined by using human RBCs [22]. The human RBC was centrifuged for 15 min to remove the buffy coat and washed 3 times with phosphate buffer saline (PBS: 35 mM phosphate buffer, pH 7/ 150 mM NaCl). 100 μl of 4% (v/v) RBC was suspended in PBS was plated into sterilized 96-well plate and then 100 μl of the Aloe protein solution was added to each well. The plate was incubated for 1 h at 37 °C and centrifuged at 1000 × g for 5 min. The hemoglobin release was monitored by using ELISA plate reader by measuring the absorbance at 414 nm. Gentamycin was used as positive control.

3. Results

3.1. Purification of the Aloe protein from the leaf-gel of A. vera

The chromatographic fractions were assayed for antifungal activity. The unadsorbed fraction of DEAE-cellulose column gave positive antifungal test and this unadsorbed fraction was in turn applied on the CM-cellulose column, it yielded an inactive unbound fraction and active adsorbed fraction when eluted with 0.6 M NaCl in MES buffer (pH 4.5). Fig. 1 showed the molecular mass of crude sample, unadsorbed fraction from DEAE-cellulose and 0.6 M NaCl fraction CM-cellulose column.

The homogenized crude sample of *A. vera* showed a clear ~14 kDa band on SDS PAGE along with many other bands of different molecular masses ranging from 110 kDa to 14 kDa. This same band of ~14 kDa was also observed in the unbound fraction of DEAE-cellulose (Fig. 1a). The unadsorbed fraction from the DEAE-cellulose column was reloaded in the pre-equilibrated CM-cellulose column. SDS-PAGE was run with the unadsorbed and all the bound fractions were eluted from the CM-cellulose column. A single band of ~14 kDa was observed in the 0.6 M NaCl eluent from the CM-cellulose column, after staining with Coomassie brilliant blue (Fig. 1b) and silver staining (Fig. 1c) which indicates that the presently purified Aloe protein is without any carbohydrate residues and is not a glycoprotein of 14 kDa which was reported earlier [23]. The single band that appeared in the SDS-PAGE was designated as Aloe protein of interest. The protein concentration obtained in the final fraction from CM-cellulose column was 0.2 mg/ml which was quantified by Bradford method. The yield was not very good. The purification process was repeated several times to get good amount of protein to perform all the experiments.

3.2. N-terminal sequence analysis

The purified protein exhibited the N-terminal sequence (using Shimadzu PPSQ-20): A S Q L N A G Q T L G T G Q S, which is different from any of the pathogenesis-related protein. The database for other antifungal proteins with similar sequences using BLAST (http://www.ncbi.nlm.nih.gov/BLAST) showed the homology with three proteins with 100% coverage given in Table 1. But it is different from the N-terminal sequence of the any other anti fungal protein. Thus, the 14 kDa protein isolated was a novel finding from the leaf gel of *A. vera*.

3.3. Assay of antifungal activity

All the fractions from the DEAE-cellulose and CM-cellulose column were tested for antifungal activity. The unbound fraction from the DEAE-cellulose exhibited antifungal activity against *C. parapsilosis*, *C. albicans* and *C. krusei*.

The mycelium growth had advanced till it reached the peripheral discs containing the test sample and both the control. The zone of inhibition around the test sample containing *A. vera* protein and the positive control with fluconazole were observed (Fig. 2), confirming the antifungal activity of *A. vera* protein. The IC_{50} values of the antifungal activity against , *C. parapsilosis*, *C. albicans* and *C. krusei* fungal species were 252 μM, 75.61 μM and 50.41 μM, respectively.

Table 1
Sequence homology of Aloe protein with other antifungal proteins using BLAST (http://www.ncbi.nlm.nih.gov/BLAST).

Accession	Description	Max score	Query coverage	Residue
AAK59994.1	Antifungal protein (*Gastrodia elata*)	46.4	100%	29–43
CAB94298.1	Lectin-like protein (*Gastrodia elata*)	46.4	100%	42–56
AAZ76593.1	Antifungal protein precursor (*Gastrodia elata*)	46.4	100%	29–43
Present study	Antifungal protein (*Aloe vera*)	N.A.	100%	1–15

3.4. Assay of anti-inflammatory activity

3.4.1. Analysis by spectrophotometer

The spectroscopic analysis showed 84.6% loss of activity of inflammatory enzymes, LOX and 73% with COX-2 when incubated with Aloe protein in the presence of the substrate, linoleic acid and arachidonic acid, respectively, revealing that *A. vera* protein exhibits strong anti-inflammatory property by competing with the substrate. Fig. 3a1 and b1 showed the activity profile of LOX and COX-2, respectively, as an increase in absorbance with time in presence of substrate. Fig. 3a2 and b2 showed activity in presence of both Aloe protein and substrate. In case of LOX, the hydroperoxy lipid product containing conjugated diene were monitored at 234 nm. In Fig. 3a1, the optical density (OD) increases with the formation of the product and in Fig. 3a2 the absorbance decline in presence of Aloe protein. Similarly, in case of COX-2 (Fig. 3b1) the TMPD oxidation is monitored by spectrophotometer at 610 nm which forms the product, the OD increases as the oxidation of TMPD increases in presence of substrate arachidonic acid. While in Fig. 3b2, the oxidation process become stable much earlier with the increase of time in presence of Aloe protein. This observation showed Aloe protein inhibit LOX and COX-2 and decreases the rate of formation of the product in presence of substrate.

3.4.2. Analyzed by BIAcore 2000

The anti-inflammatory property was further confirmed by the binding studies using the SPR principle in the BIAcore 2000 real-time machine. The Aloe protein showed very high affinity towards the LOX and COX-2, the K_D values were calculated to be $(2.7 \pm 0.02) \times 10^{-8}$ M and $(4.77 \pm 0.034) \times 10^{-9}$ M, respectively (Fig. 4).

3.5. Assay for protease activity

The Aloe protein did not show any protease activity. The protease, trypsin was used as the control in this experiment. The casein incubated with trypsin was digested to give a high absorbance of 1.2 at 280 nm. In contrast, the Aloe protein did not show any protease activity when incubated with casein fragments and the absorbance obtained was 0.025 at 280 nm, which is highly insignificant in comparison with OD of the control.

3.6. Assay for protease inhibitory activity

The Aloe protein was tested for its ability to inhibit trypsin by incubating it with trypsin and casein in 50 mM Tris–HCl (pH 8) buffer. In one micro centrifuge tube trypsin without protein was

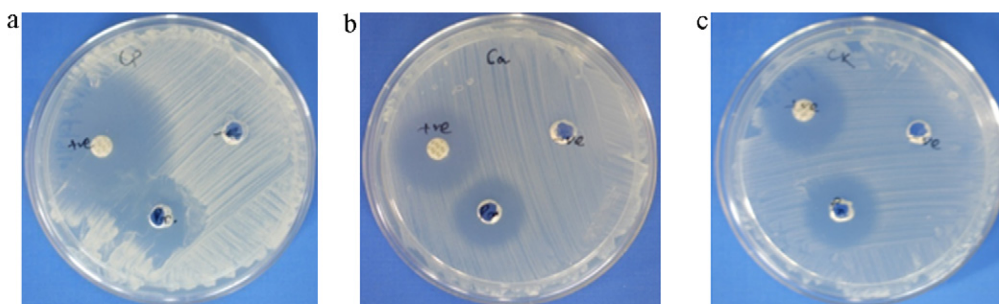

Fig. 2. Antifungal assays of the Aloe protein showing zone of inhibition against (a) *Candida parapsilosis*, (b) *Candida albicans* and (c) *Candida krusei*. Fluconazole discs (25 mg) were taken as positive control while assay buffer with out Aloe protein served as the negative control.

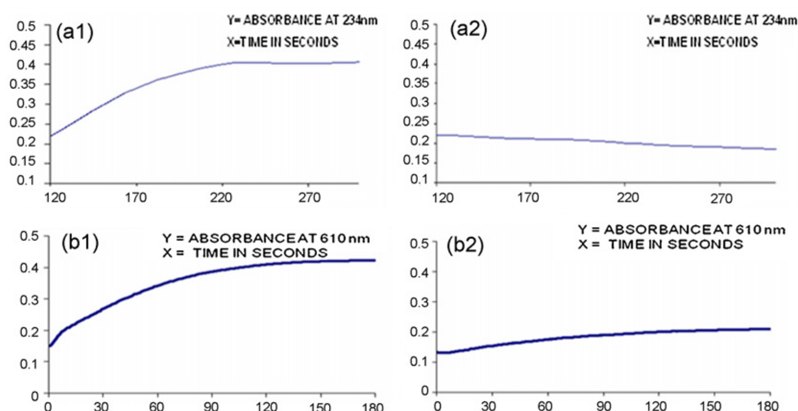

Fig. 3. Anti-inflammatory assay against LOX and COX-2 derived by spectroscopic analysis: (a1) activity profile of LOX in presence of substrate, (a2) inhibition of LOX by Aloe protein, (b1) activity profile of COX-2 presence of substrate, and (b2) inhibition of COX-2 by Aloe protein.

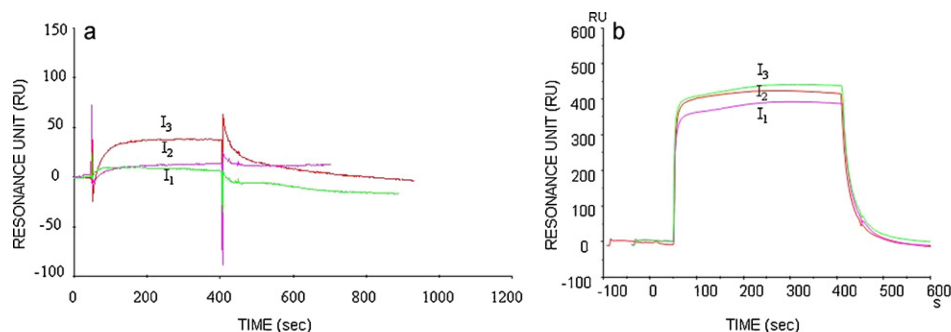

Fig. 4. Binding assay of the Aloe protein using BIAcore. Sensogram showing binding of three different concentrations of Aloe protein ($I_1 = 71 \times 10^{-9}$ M, $I_2 = 213 \times 10^{-9}$ M and $I_3 = 355 \times 10^{-9}$ M) with (a) LOX: on the sensor chip CM5 and (b) COX-2: on the Ni^{2+} NTA sensor chip.

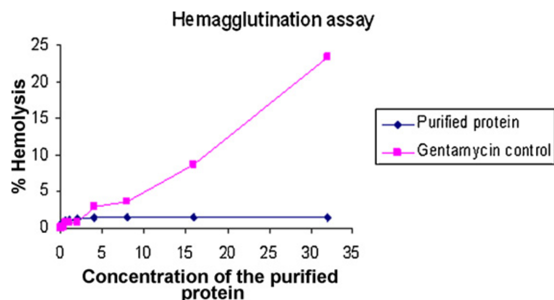

Fig. 5. Hemagglutination assay showing % hemolysis of human RBC by the Aloe protein. Gentamycin was used as control.

taken as control and in the other tube trypsin was incubated with the purified protein, casein being added in both the cases. The absorbance obtained for trypsin without Aloe protein and trypsin incubated with Aloe protein were 1.2 and 0.062, respectively; thus indicating that the Aloe protein has potent protease inhibitory activity.

3.7. Assay for hemagglutinating activity

The hemolytic activity of the Aloe protein was determined to check the toxicity to erythrocytes using human RBCs. The concentration upto 32 mg/ml of the Aloe protein lysed only 0–2% of 4% (v/v) human erythrocytes (Fig. 5). The Aloe protein is less toxic to human erythrocytes as compared to gentamycin.

4. Discussion

A. vera has been traditionally used worldwide as a folk medicine for various diseases because of its multiple biological activities. The species is frequently cited as being used in many herbal medicines. There is some preliminary evidence that *A. vera* extracts may be useful in the treatment of wound [24] and burn healing [1], diabetes [25,26] and elevated blood lipids [27] in humans. These positive effects are thought to be due to the presence of compounds such as polysaccharides, mannans, anthraquinones and lectins [1]. This plant has drawn immense attention in the recent past due to its numerous properties attributed by its unique compositional features [28]. It is a great challenge to relate the functional properties of the gel with the composition present in *A. vera* leaf gel. The present study showed the anti fungal and anti-inflammatory property of new Aloe protein of molecular weight of 14 kDa.

The effect of ethanolic extract of *A. vera* gel on in vitro antibacterial and antifungal activities against several pathogenic microbial isolates of clinical importance were reported [29]. The Aloe protein of this study showed strong antifungal activity and was found to be potent against different *Candida* species.

Inflammatory responses mediated through induced arachadonic acid metabolism can occur by the lipoxygenase (LOX) pathway, which is responsible for leukotriene (LT) synthesis, or the cyclooxygenase (COX) pathway, which results in the production of various prostaglandins (PG) [30]. Aloe protein exhibited both LOX and COX inhibitory activity which suggests this protein may be clinically useful to reduce LT and PG synthesis in the inflammatory processes.

The Aloe protein showed inhibition against inflammatory enzymes like COX-2 and LOX and its percentage inhibition and dissociation constant are comparable with the known COX-2 inhibitor like non-steroidal anti-inflammatory drug (diclofenac; $K_D = 1.45 \times 10^{-7}$ M; nimusulide; $K_D = 2.22 \times 10^{-9}$ M)

The anti-inflammatory properties of *A. vera* gel has been reported in previously published literatures but low molecular weight proteins, other than glycoprotein; exhibiting anti-inflammatory activity is a novel finding of this present work. The ammonium sulphate precipitated fraction of the *A. vera* gel also reported for in-vitro anti-bradykinin activity [31] and a 14 kDa radical scavenging glycoprotein had been reported for inhibiting cyclooxygenase-2 and thromboxane A2 synthase from *A. vera* gel [23]. Another study reported a carboxy-peptidase activity of the *A. vera* leaf gel, inactivates bradykinins and produces an anti-inflammatory effect [32,33]. It has been reported earlier that aqueous extract from *A. vera* gel showed anti-inflammatory activity by inhibiting the action of arachidonic acid pathway via cyclooxygenase [34].

Some antifungal protein isolated previously did not show protease and protease inhibitory activities [21]. The real mechanism of protease inhibition is not known. It can be hypothecated that the addition and incubation of the purified protein to trypsin has blocked the binding site of the protease and thus have reduced the affinity towards casein. As a result, the casein incubated with the Aloe protein and trypsin together was not digested. This Aloe protein is not a protease. It was inhibiting trypsin showing protease inhibition property.

Toxicity to erythrocyte was investigated by using human RBC by hemagglutination experiment. This property of the molecule is very vital for its application as a therapeutic agent. Further an ideal antimicrobial molecule should also have an anti inflammatory property as the infections are associated with inflammation [35,36]. Thus it can be summarized that the Aloe protein is the potent therapeutic agent.

5. Conclusion

The present study reveals the isolation of novel protein of 14 kDa, which showed different functional aspects like anti-fungal and anti-inflammatory. This Aloe protein did not exhibit protease activity, however it inhibited trypsin showing protease inhibitory

function. Though the N-terminal sequence showed it to be a lectin like protein but the absence of hemagglutinating activity confirmed it to be an antifungal protein other than lectin in *A. vera*. This study will further explore different proteins with diverse therapeutic functions present in *A. vera*.

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