Chromatographic methods for isolation and characterization of bioactive molecules from medicinal plant *Mesua ferrea* Linn

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**ABSTRACT**

Partially purified fraction of methanol extract of leaf of *Mesua ferrea* Linn was assessed for its phytochemical screening. Thin layer chromatographic studies, antimicrobial activity, anti-inflammatory activity, antidiabetic activity and antioxidant activity experiments were conducted. Phytochemical screening reflects the presence of alkaloids, flavonoids, coumarins, terpenoids, steroids, emodins and quinone derivatives. Thin layer chromatographic study was carried out using solvent system of various polarities of *n*-butanol, acetic acid and acetone. TLC profiling shows pure band of peptide at 254 and 366 nm. Protein content was determined using Nanodrop technique. Antimicrobial efficiency was determined using well diffusion method. Partially purified fractions of *Mesua ferrea* Linn was most sensitive against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Protease mirabilis* with maximum zone of inhibition in diameter 16, 21 and 17 mm respectively at a concentration of 20 µg. *In vitro* anti-inflammatory activity was evaluated using albumin denaturation (84.67%) membrane stabilization assay (73.6%) and proteinase inhibitory activity (90.62%). Aspirin was used as standard drug for the study of anti-inflammatory activity. *In vitro* antidiabetic activity was determined by α-amylase inhibition assay. Antioxidant activity was performed by reducing power assay. Fraction #3 has higher absorbance 0.94 at 500 µg/ml followed by fraction #2 absorbance 0.89 at 500 µg/ml.

**Keywords:** *Mesua ferrea* Linn, TLC, antimicrobial, antioxidant, anti-inflammatory, antidiabetic activity, phytochemical screening.

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**INTRODUCTION**

The increase in prevalence of multiple drug resistance has led to the development of new synthetic antimicrobial, anti-oxidative, anti-diabetic and anti-inflammatory drug (Ibrahim et al., 2015) moreover a novel approach is necessary to search for new antimicrobial, antioxidative, anti-diabetic and anti-inflammatory sources from alternative sources.

*Mesua ferrea* Linn is a medium to large evergreen tree from the family Clusiaceae; widely distributed in countries like India, Burma, Thailand and New Guinea (Sharma and Yelne, 2000; Chahar et al., 2013). In India, it occurs in the lower Himalayas from Nepal eastwards to Bengal (Gupta and Tandon, 2005) extending to Andhra Pradesh and Andaman and Nicobar Islands while also ascending up to a height of 1500 m. The plant is extensively used in traditional medicine for various pharmacological actions. *M. ferrea* seeds, leaves and stem bark have been studied for the array of medicinal properties such as antioxidant and antimicrobial activity, analgesic, antispasmodic and anti-venom activities, immunomodulatory and anti-arthritic potentials (Sharma and Yelne, 2000). The dried flowers of the plant are aromatic and used for the treatment of bleeding piles, dysentery, cough, irritability of the stomach, excessive perspiration and also used as carminative (Suresh et al., 2014). *M. ferrea* flowers have also been studied for their anti-convulsant, anti-neoplastic, hepatoprotective and immunomodulatory activities *in-vivo* and *in-vitro* antioxidant activity of
methanol and ethanol extracts of \( \textit{M. ferrea} \) flowers have also been reported (Khanapur et al., 2014). Leaves are used in the form of poultice which is applied to head in severe colds. Leaves and flowers are astringent and stomachic and are also used for snake bite and scorpion stings (Chahar et al., 2013).

**MATERIALS AND METHODS**

**Sample collection**

Leaves samples of \( \textit{Mesua ferrea} \) Linn were collected from Nakshatra Garden and kept for drying at room temperature.

**Preparation of extract**

Freshly collected plant leaf samples were air dried at room temperature for 5 to 6 days. Dried leaf sample was grinded to powder using mechanical grinder. Dried leaf powder was homogenised in a gradient of 10 ml (1:1) methanol: water and was extracted on a rotatory shaker in a centrifuge tube at 350 rpm overnight (Daayf et al., 1997). Crude extract was filtered through Whatman No.1 filter paper and evaporated in a steam bath at 30°C for 30 min. Extract was stored at -20°C for further study (Vandana and Upadhayaya, 2012).

**Phytochemical screening**

Phytochemical studies were carried out for methanol extracts of \( \textit{M. ferrea} \) leaves and for partially purified fraction to detect the presence of different phytochemical constituents like steroids, terpenoids, tannins, flavonoids, saponins, glycosides, amino acids etc. by using standard procedures:

**Test for alkaloids:** Added 2 ml of extract to 2N HCl and decanted the aqueous layer formed and added a few drops of Mayer’s reagent. A creamy precipitate was observed indicating the presence of alkaloid.

**Test for phenolic compounds:** Added 3 to 5 drops of 5% FeCl₃ solution to 2 ml of the extract. Formation of deep blue colour indicated the presence of phenolic compounds.

**Test for flavonoids:** To 2 ml of extract was added 2 to 5 drops of 1N NaOH. Formation of yellow orange colour indicated the presence of flavonoids.

**Test for saponins:** To 2 ml of extract was added 6ml of water in a test tube. Observation of a persistent foam indicated the presence of saponins.

**Test for tannins:** To 2 ml of aqueous extract in 2 ml of distilled water was added a few drops of FeCl₃. Formation of green precipitate indicated the presence of tannins.

**Test for leucoanthocyanins:** Added 5 ml of aqueous extract to 5 ml of isooamy alcohol. Appearance of a red colour in the upper layer indicated the presence of Leucoanthocyanins.

**Test for quinines:** To 2 ml of extract was added concentrated HCl. Formation of yellow precipitate indicated the presence of quinines.

**Test for coumarins:** 3 ml of 10% NaOH was added to 2 ml of aqueous extract. Formation of yellow colour indicated the presence of coumarins.

**Test for steroids:** Dissolved 1 ml of the extract in 10 ml chloroform and added equal volume of concentrated H₂SO₄. While the upper layer turned red theH₂SO₄ layer showed yellow green fluorescence indicating the presence of steroids.

**Test for emodins:** To 2 ml of extract was added concentrated HCl. Formation of yellow precipitate indicated the presence of emodins.

**Test for Phlobatanins:** Added 2 ml of aqueous extract to 2 ml of 1% HCl and the mixture was boiled. Deposition of a red precipitate indicated the presence of Phlobatanins.

**Test for anthocyanin:** Added 2 ml of aqueous extract to 2 ml of 2N HCl and Ammonia. The colour of the solution turned blue-violet from pink-red indicating the presence of anthocyanins.

**Column chromatography and TLC**

**Column chromatography**

The solvent system that exhibited the best separation of was chosen for column chromatography. The methanol extract of \( \textit{Mesua ferrea} \) Linn was adsorbed onto silica gel. The column (2 cm × 25 cm) was packed with a solution of silica gel in water using the wet slurry method, which involved preparing a solution of silica gel in water and subsequently adding into the column till it is about three-fourth filled.

The solution was stirred and quickly added to the column after which the gel settles. This method was used to prevent the trapping of air bubbles. A ball of wool (glass wool) was pushed into the column to settle atop the packed silica gel.

The solvent system of n-butanol: acetic acid: water (4:1:1) was poured continuously into the column and allowed to drained and about 8 fractions of 5 to 6 ml was collected in sterile centrifuge tube.

The fraction eluted on column was tested with same solvent system by TLC for the presence of active compounds.

**Thin layer chromatography (TLC)**

Fraction eluted from silica gel column was subjected to TLC as per conventional one dimensional ascending method using silica gel (60F₂₅₄ MERCK) pre-coated plate. Plates were cut with ordinary household scissors and markings were made with a soft pencil. For TLC spotting, sample volume 1 µl was applies using a glass capillary and the solvent system included n-butanol: acetic acid: water. TLC plate after loading with sample was run using mobile phase approximate 20 min needed for development of band. After the run the plates were dried and were observed under wavelengths 254 and 366 nm for band detection.

Colour of the spot and pattern were observed and RF values were calculated using formula:

\[
RF (retention factor) = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by the solvent front}}
\]

**Antimicrobial activity**

**Microorganism used**

The bacterial strains were collected from microbial culture collection laboratory VSBT.

**Well diffusion assay**

The antimicrobial activity was performed by agar well diffusion method (Caron, 2012). In order to extract the solvent 20 ml of media [\( \text{Muller and Hinton (MH)} \) agar] was poured into the petri plates along with the inoculum. A well was prepared in the plate with the help of cork bores (6 mm). 20 µl of the test sample was poured in each well using sterile micropipette. For positive control standard antibiotic tetracycline (30 mcg) was used. The plates were
incubated overnight at 37°C in a BOD incubator. The microbial growth was determined by measuring the diameter of zone of inhibition. The entire process was carried out aseptically in the laminar air flow hood.

Antidiabetic property

Inhibition of α-amylase enzyme

Standard maltose curve: 0.2 to 1 ml of standard maltose (1mg/ml) was taken into different tubes. The volume was made to 1 ml in each case with distilled water. 1 ml of DNSA (Dinitro salicylic acid) reagent was added to each tube and then placed in boiling water bath for 15 min. 8 ml of distilled water was added to each tube and mixed well. Absorbance of the solution was read using a colorimeter at 570 nm against a blank solution (Dhriti et al., 2014).

α-amylase inhibition assay: 100 to 500 μl of extract was taken in different test tubes, and the volume was made to 0.5 ml with phosphate buffer at pH 6.8. Blank was measured using 1 ml of phosphate buffer. The solution taken was treated with 0.5 ml of alpha amylase (0.5 mg/ml). The solution was incubated at 25°C for 10 min. 0.5 ml of 1% starch solution in 0.02 M sodium phosphate buffer at pH 6.9 was added subsequently to all tubes and then incubated at 25°C for 10 min. The reaction was stopped by DNSA and the reaction mixture was kept in boiling water bath for 5 min and then cooled to room temperature.

The solution was mixed with 8 ml distilled water, and the absorbance of the solution was read using a colorimeter at 570 nm against a blank solution. Amount of maltose produced is calculated using a standard maltose curve and the enzyme activity is calculated by using formula (Dhriti et al., 2014).

\[
\text{Enzyme activity} = \frac{\text{Amount of maltose formed} \times 2}{10 \times 342}
\]

Anti-inflammatory activity

Albumin denaturation assay

Method of Mizushima and Kobayashi (1968) and Sakat et al. (2010) was used followed by minor modification. The reaction mixture consisted of the test extract and 1% aqueous solution of bovine albumin fraction. The pH of the reaction mixture was adjusted using small amount of HCl. The sample extract was incubated at 37°C for 20 min and then heated to 51°C for 20 min. After cooling, the sample the turbidity was measured spectrophotometrically at 660 nm. The experiment was performed in triplicate. Percent inhibition of protein denaturation was calculated as follows:

\[
\% \text{inhibition} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100
\]

Reducing power assay

Reducing power assay was evaluated according to the method of Ghadir et al. (2014). Different concentrations of partially purified methanol fraction of M. ferrea Linn (100 to 500 μg/ml) was mixed with 1 ml of 0.2 M phosphate buffer (pH 6.6) and 1 ml of potassium ferricyanide followed by incubation at 50°C for 20 min, then it was cooled rapidly; 1 ml of 10% trichloro acetic acid was added and vortexes. This incubation mixture was centrifuged at 300 rpm for 10 min. The supernatant was taken out and mixed with 2 ml of distilled water and 0.5 ml of 1% ferr chloride. After incubation for 10 min, the absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicates the reducing potential of partial purified fraction. All tests were performed in triplicate and ascorbic acid was used as standard.

RESULTS AND DISCUSSION

Column chromatography and TLC

Thin layer chromatographic studies of partially purified methanol fraction of Mesua ferrea Linn was done using silica gel 60 F 254 (MERCK) aluminium plate. The solvent system n-butanol: acetic acid: water (4:1:1) was used for separation of the compounds. Partially purified fraction eluted on column chromatography showed different band patterns at 254 and 366 nm. Spots were characterized by Rf value by viewing them under UV light. For fraction two
spots were detected at 254 nm with an Rf value. For fraction 3, one spot was detected at 254 nm with an Rf value of 0.77 and two spots were detected at 366 nm with an Rf values 0.70 and 0.65. For fraction 4, one spot was visible at 254 nm with an Rf value 0.85 and another spot was visible at 366 nm with an Rf value of 0.78. For fraction 5, a spot was visible at 254 nm with an Rf value of 0.87 and two-spot were visible at 366 nm with Rf values 0.79 and 0.76. For fraction 6, a spot was visible at 254 nm with an Rf value of 0.85 and another spot at 366 nm with an Rf value 0.78. For fraction #7 a spot was visible at 366 nm.

**Phytochemical analysis**

Qualitative physiochemical investigation for the extracts of *Mesua ferrea* leaves and partially purified extract with various specific reagent showed the presence of steroids, flavonoids, coumarins terpenoids, emodins, alkaloids, phenols and quinones (Narendra et al., 2011) reported that methanol extracts of *Mesua ferrea* leaves revealed the presence of steroids, terpenoids, glycosides, tannins, alkaloids, saponins, phenols and carbohydrates. The results of phytochemical screening are shown in Table 1.

<table>
<thead>
<tr>
<th>Name of the phytochemicals</th>
<th>Result</th>
<th>Partially purified fraction # 2, 3 and 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenolic compounds</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Phlobatannins</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Coumarins</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Anthocynins</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Steroids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fatty acid</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Emodins</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Quinones</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**Antimicrobial activity**

Antimicrobial activity of partially purified fraction of leaf extract gave different zones of inhibition on the organism tested. The extract showed antimicrobial activity against Gram +ve positive and Gram negative bacterial strains. Fraction numbers 3, 4 and 6 of partially purified fraction of *M. ferrea* Linn were most sensitive against *Staphylococcus aureus, Pseudomonas aeruginosa* and *Proteus vulgaris* with a maximum zone of inhibition in diameter 16, 21 and 17 mm respectively at a concentration of 20 µg/50 µl. The partially purified fraction did not show any activity against *Bacillus subtilis* (Table 2). *Staphylococcus aureus, Pseudomonas aeruginosa and Proteus vulgaris* are most sensitive N3 and N4 as shown in Figure 1 A, B and D. While *Bacillus subtilis* do not show any sensitivity N7 all against negative control CD used as sterile buffer.

**In vitro anti-inflammatory activity**

**Albumin denaturation assay**

Denaturation of proteins is a well-documented cause of inflammation. As a part of the investigation on the mechanism of the anti-inflammatory activity, the ability of the fractions to inhibit denaturation of proteins was studied. Partially purified fraction were effective in inhibiting albumin denaturation with a maximum inhibition of 84.67% was observed at 500 µg/ml, while aspirin, a standard drug showed a maximum inhibition of 89.23% at conc. 500 µl/ml (Figure 2).

**Membrane stabilization assay**

The HRBC membrane stabilization has been used as a method to study the *in vitro* anti-inflammatory activity because the erythrocyte membrane is analogous to the lysosomal membrane (Kaur and Arora, 2009) and its stabilization implies that the partially purified fraction may well stabilize lysosomal membranes. Stabilization of lysosomal membrane is important in limiting the inflammatory response by preventing the release of lysosome constituents containing activated neutrophil, such as bacterial enzymes and proteases, which causes further tissue inflammation and damage upon extra cellular release. Purified fraction were effective in membrane stabilization at different concentration as
Table 2. Antimicrobial activity of partially purified fraction by well diffusion method.

<table>
<thead>
<tr>
<th>Fraction no.</th>
<th>Staphylococcus aureus (mm)</th>
<th>Protease vulgaris (mm)</th>
<th>Bacillus subtilis (mm)</th>
<th>Pseudomonas aeruginosa (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 µg/50 µg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction 3</td>
<td>16</td>
<td>17</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fraction 4</td>
<td>23</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fraction 6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>21</td>
</tr>
</tbody>
</table>

Figure 1. Antimicrobial activity of partially purified fraction of Mesua ferrea Linn against A) Staphylococcus aureus, B) Pseudomonas aeruginosa, C) Bacillus subtilis, D) Proteus vulgaris.

Figure 2. Graphical representation of albumin denaturation, proteinase inhibition and membrane stabilization.
shown (Table 3) maximum inhibition of fraction 3 was 73.6% observed at 500 µg/ml, followed by fraction 4 (68%) and fraction 6 (64%) aspirin a showed a maximum inhibition 79.85% at conc. 500 µg/ml.

**Proteinase inhibitory activity**

Proteinases have been implicated inarthritis reactions. Neutrophils are known to be a rich source of proteinase which carries in their lysosomal granules many serine proteinases (Evangelie et al., 2015). It was previously reported that leukocytes proteinase play an important role in the development of tissue damage during inflammatory reactions and significant level of protection was provided by proteinase inhibitors (Leelaprakash and Dass, 2011). *M. ferrea* Linn partial purified fraction exhibited significant antiproteinase activity at different concentrations as shown in Table 3. Maximum inhibition of fraction 3 was 90.62% observed at 500 µg/ml, followed by fraction 7 (71.25%) and fraction 4 (62.5%) aspirin showed a maximum inhibition 88.48% at a concentration of 500 µg/ml.

**Reducing power assay**

Reducing power activity is often used to evaluate the ability of natural antioxidant to donate electron (Rajeshwar et al., 2005). The reducing power ability of partially purified fraction of *M. ferrea* Linn. Increased constantly with increase in the volume of partially purified fraction from 100 to 500 µg/ml. Fraction 3 has higher absorbance 0.89 at 500 µg/ml followed by fraction 5, which has an absorbance of 0.94 at 500µg/ml. Reducing power of partially purified fraction was compared with ascorbic acid as standard showing higher absorbance 1.02 at 500 µg/ml absorbance listed (Table 4 and Figure 3).

**In vitro antidiabetic activity**

**Alpha amylase inhibition assay**

The intestinal digestive enzyme α-amylase plays a vital role in the carbohydrate digestion. Antidiabetic therapeutic approach reduces the post prandial glucose level in blood by the inhibition of α-amylase enzyme (Dhriti et al., 2014). The in vitro α-amylase inhibitory studies demonstrated that *M. ferrea* Linn has anti diabetic activity. Partially purified fraction showed maximum inhibition of fraction 3 with 84.98% at a concentration of 500 µg/ml and fraction 5 with 70.71% at a concentration of 500 µg/ml as listed (Table 4 and Figure 3).

**CONCLUSION**

Extraction and isolation of bioactive compounds from *M. ferrea* Linn was carried out with potent antimicrobial, antidiabetic, anti-inflammatory and antioxidant in nature. The results of column chromatography and thin layer chromatography purified purification of *M. ferrea* Linn leaves extract. *M. ferrea* Linn partially purified fraction was screened for phytochemical analysis; phytochemical analysis of extract revealed the presence of active phytoconstituents. Partially purified fraction also showed

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**Table 3.** Effect of different fraction of *Mesua ferrea* Linn on albumin denaturation, membrane stabilization and proteinase inhibition.

<table>
<thead>
<tr>
<th>Test sample</th>
<th>Albumin denaturation</th>
<th>Membrane stabilization</th>
<th>Proteinase inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction 3</td>
<td>84.67 ± 0.010</td>
<td>73.6 ± 0.010</td>
<td>90.62 ± 0.032</td>
</tr>
<tr>
<td>Fraction 4</td>
<td>69 ± 0.006</td>
<td>68 ± 0.010</td>
<td>62.5 ± 0.008</td>
</tr>
<tr>
<td>Fraction 6</td>
<td>40.89 ± 0.010</td>
<td>56.60 ± 0.010</td>
<td>55 ± 0.008</td>
</tr>
<tr>
<td>Fraction 7</td>
<td>50 ± 0.010</td>
<td>64 ± 0.010</td>
<td>71.25 ± 0.008</td>
</tr>
<tr>
<td>Aspirin</td>
<td>82.23 ± 0.010</td>
<td>79.85 ± 0.006</td>
<td>88.48 ± 0.000</td>
</tr>
</tbody>
</table>

**Table 4.** *In vitro* alpha amylase inhibition method.

<table>
<thead>
<tr>
<th>Conc. µg/ml</th>
<th>Fraction 3</th>
<th>Fraction 5</th>
<th>Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abs.</td>
<td>% inhibition</td>
<td>Abs.</td>
<td>% inhibition</td>
</tr>
<tr>
<td>100</td>
<td>0.361</td>
<td>0.171</td>
<td>0.027</td>
</tr>
<tr>
<td>200</td>
<td>0.794</td>
<td>0.174</td>
<td>0.032</td>
</tr>
<tr>
<td>300</td>
<td>1.114</td>
<td>0.239</td>
<td>0.053</td>
</tr>
<tr>
<td>400</td>
<td>1.415</td>
<td>0.251</td>
<td>0.057</td>
</tr>
<tr>
<td>500</td>
<td>1.323</td>
<td>0.286</td>
<td>0.075</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD; Experimental group were compared with control. **p < 0.01, considered extremely significant.**
Figure 3. Graphical representation of in vitro alpha amylase inhibition assay of partially purified fractions of using maltose as a standard.

**Table 5.** The reducing power ability of partially purified fraction.

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Fraction 3 conc. µg/ml</th>
<th>Absorbance at 700 nm</th>
<th>Fraction 5 conc. µg/ml</th>
<th>Absorbance at 700 nm</th>
<th>Ascorbic acid</th>
<th>Absorbance at 700 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>0.32</td>
<td>100</td>
<td>0.30</td>
<td>100</td>
<td>0.38</td>
</tr>
<tr>
<td>2</td>
<td>200</td>
<td>0.40</td>
<td>200</td>
<td>0.37</td>
<td>200</td>
<td>0.48</td>
</tr>
<tr>
<td>3</td>
<td>300</td>
<td>0.49</td>
<td>300</td>
<td>0.52</td>
<td>300</td>
<td>0.67</td>
</tr>
<tr>
<td>4</td>
<td>400</td>
<td>0.71</td>
<td>400</td>
<td>0.78</td>
<td>400</td>
<td>0.86</td>
</tr>
<tr>
<td>5</td>
<td>500</td>
<td>0.89</td>
<td>500</td>
<td>0.94</td>
<td>500</td>
<td>1.02</td>
</tr>
</tbody>
</table>

Figure 4. Graphical representation of reducing power assay of partially purified fraction of *Mesua ferrea* Linn.
good antimicrobial activity against pathogenic bacteria. Extract fraction showed anti-diabetic activity which is compared with maltose standard. *Mesua ferrea* Linn has good anti-inflammatory activity which is confirmed by inhibiting the heat induced albumen denaturation assay. Proteinase inhibition and stabilized the red blood cells membrane which is compared with aspirin as a standard. This study proves that this medicinal plant could be futuristic alternative for medicine for anti-diabetic and anti-inflammatory properties.

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