

IN VITRO PROTECTIVE EFFECT OF PROTEINS OF *TERMINALIA CHEBULA* FRUITS ACTION ON INHIBITION OF HEMOLYSIS, PROTEINASE, AND PROTEIN DENATURATION PROCESSOMKAR NG¹, VEDAMURTHY JOSHI², DINESHA RAMADAS^{3*}

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ABSTRACT**Objective:** The study helps to evaluate the anti-inflammatory activity of *Terminalia chebula* fruit protein.**Methods:** The crude protein extracted from *T. chebula* fruits was subjected to analysis for its hemolysis inhibitory, proteinase inhibitory, and protein denaturation inhibitory activities.**Results:** In the antioxidant property model, the crude protein exhibited an inhibitory activity of 52% compared to 58% and 61% for ascorbic acid and alpha-tocopherol, respectively. In the hemolysis inhibition studies, the crude proteins demonstrated an inhibition of 42.1%. In the protein denaturation inhibitory study, the crude protein showed a maximum inhibition of 28%, while in the proteinase inhibitory studies, it exhibited a maximum inhibition of 43.1%.**Conclusion:** The results of the study revealed that the crude proteins possess antioxidant and anti-inflammatory properties in various model systems.**Keywords:** *Terminalia chebula* fruits, Crude proteins, Anti-inflammatory activity, Proteinase inhibition, Haemolysis inhibition.© 2023 The Authors. Published by Innovare Academic Sciences Pvt Ltd. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>) DOI: <http://dx.doi.org/10.22159/ajpcr.2023v16i11.47985>. Journal homepage: <https://innovareacademics.in/journals/index.php/ajpcr>**INTRODUCTION**

Inflammation is a response that occurs when living tissues are damaged, aiming to eliminate irritants and aid in tissue repair. It is considered a defense mechanism to protect the body from infections and injuries [1]. The purpose of inflammation is to localize and remove the harmful agent and eliminate damaged tissue components [2]. This inflammatory response can be observed through changes in blood flow, blood vessel permeability, and the movement of fluid, proteins, and leukocytes from the site of tissue damage. The release of cytokines by leukocytes relocating from the venous system to the site of damage plays a significant role in the inflammatory response [3]. Cytokines help increase blood flow to the injured site, promoting vasodilation and improved permeability of the capillaries. Anti-inflammatory drugs are used to manage these inflammatory conditions; however, they often have adverse side effects such as gastric irritation and can induce oxidative stress and liver injury [4]. In recent years, extensive research has been conducted to explore natural anti-inflammatory agents.

Numerous research studies have demonstrated that natural dietary constituents, including proteins, alkaloids, curcuminoids, carbohydrates, polyphenols, flavonoids, and more, possess a wide range of biological activities [5]. Incorporating these phytochemicals into our diet or using them as herbal remedies can aid in the prevention of various chronic diseases [6]. *Terminalia chebula*, also known as black or chebulicmyrobalan, is a species of *Terminalia* with a multitude of medicinal properties. The fruit is commonly used as a laxative, stomachic, tonic, alterative, antispasmodic, analgesic, wound healer, and anti-inflammatory agent. It has been found to be effective in the treatment of conditions such as ophthalmia, hemorrhoids, dental caries, bleeding gums, and oral ulcers. While previous studies have extensively explored the phytochemicals present in *T. chebula*, the protein component of the fruit has not been widely reported. Therefore, the authors aim to investigate and report the medicinal properties of the proteins present in *T. chebula* fruits.

METHODS

T. chebula fruits, obtained from a reliable source, were identified by Mr. Maheshwar KV, a botanist (Herbarium ID AM109). The fruits were thoroughly cleaned, and the seeds were separated before being powdered and stored in a glass container. The necessary chemicals and reagents were procured from Hi-Media and Sigma Aldrich, while all other chemicals used were of analytical grade.

Extraction of protein

To prepare the mixture, 10 g of *T. chebula* fruit powder was combined with 200 mL of double-distilled water and vortexed overnight at 10°C. The mixture was then subjected to refrigerated centrifugation at 10,000 rpm, followed by lyophilization. To eliminate unwanted salts, dialysis was performed using a 2 kDa cut-off dialysis membrane against 10 mM Tris buffer. The dialysis process lasted for a period of 72 h, with intervals of 6 h each.

Proximate analysis

The aqueous extract of *T. chebula* fruits contains an abundance of phytochemicals. The protein content was quantified using Bradford's method, while the total sugar content was estimated using the phenol-sulfuric acid method [7,8]. The determination of the total phenolic content was conducted using the Folin-Ciocalteu reagent [9], and the total flavonoid content was assessed using the Quercetin and aluminum chloride methods [10].

DPPH radical scavenging activity

The investigation of DPPH radical scavenging activity was performed with slight modifications [11]. In this investigation, the crude dialyzed protein extracted from *T. chebula* fruit was utilized within the range of 2–10 µg. Subsequently, it was combined with 1 mL of freshly prepared 0.5 mM DPPH ethanolic solution and 2 mL of 0.1 M acetate buffer at pH 5.5. The resulting mixtures were incubated at 37°C for 30 min,

and their absorbance was measured at 517 nm using a Shimadzu UV-Vis Spectrophotometer. As positive controls, ascorbic acid and alpha-tocopherol were employed under the same assay conditions and at the same dosage.

Hemolysis inhibition study

The study was conducted [12] with minor modifications. In brief, a mixture was prepared by combining 0.05 mL of blood cell suspension, 0.05 mL of crude protein, and 2.95 mL of phosphate buffer at pH 7.4. This mixture was then incubated in a shaking water bath at 50°C for 20 min. Following the incubation period, the mixture was centrifuged at 2500 rpm for 5 min, and the absorbance of the resulting supernatant was measured at 540 nm. The extent of hemolysis was calculated using the provided equation. In the formula, the absorption of the control was denoted as A1, while the absorption of the test sample mixture was denoted as A2.

$$\% \text{ inhibition of denaturation} = 100 \times \left[1 - \frac{A2}{A1} \right]$$

Effect on protein denaturation

Protein denaturation studies were conducted with minor modifications, following the procedure outlined in reference [13]. To prepare the reaction mixture, a combination of 0.2 mL of 1% bovine albumin, 4.78 mL of phosphate-buffered saline at pH 6.4, and 0.02 mL of the crude extract was prepared. The resulting mixture was incubated in a water bath at 37°C for 15 min and then subjected to heating at 70°C for 5 min. After cooling, the turbidity of the mixture was measured at 660 nm. The percentage inhibition of protein denaturation was determined using the provided formula, where the absorption of the control was represented as A1, and the absorption of the test sample mixture was denoted as A2.

$$\% \text{ inhibition of denaturation} = 100 \times \left[1 - \frac{A2}{A1} \right]$$

Proteinase inhibitory activity

The proteinase inhibitory activity of *T. chebula* fruit protein was assessed with slight modifications to the method outlined in reference [13]. For the preparation of the reaction solution, a 2 mL mixture was created by combining 0.06 mg of trypsin, 1 mL of 20 mM Tris-HCl buffer at pH 7.4, and 1 mL of the sample (20 µL of sample in 1 mL of methanol). This solution was then incubated at 37°C for 5 min. Subsequently, 1 mL of 0.8% (w/v) casein was added, and the solution was further incubated for 20 min. To stop the reaction, approximately 2 mL of 70% perchloric acid was introduced. The resulting supernatant was analyzed at 210 nm using a UV-vis spectrophotometer, with the buffer serving as the blank and the phosphate buffer solution used as the control. The percentage inhibition of protein denaturation was calculated using the provided formula, where A1 represents the absorption of the control and A2 represents the absorption of the test sample.

$$\% \text{ inhibition of denaturation} = 100 \times \left[1 - \frac{A2}{A1} \right]$$

Statistics

The data are the mean ± standard deviation for the all assays tested in triplicate ($p \leq 0.05$; $n=3$).

RESULTS

As described in the materials and methods, the crude proteins from *T. chebula* fruit were subjected to dialysis against water, employing a 2 kDa molecular cut-off membrane. This process aimed to remove free carbohydrates, free polyphenols, and free flavonoids. The results of the proximate analysis revealed that the fruit extract is abundant in proteins while containing only a minimal quantity of bound carbohydrates and polyphenols. Furthermore, it was determined that the extract does not contain any flavonoids.

Antioxidant activity

The antioxidant activity of *T. chebula* fruit protein (TCFP) was assessed by measuring its stable DPPH radical scavenging activity in a dose-dependent manner. Ascorbic acid and alpha-tocopherol were employed as positive controls in the experiment. Fig. 1 illustrates that the crude protein, at a maximum dosage of 10 µg, displayed a 52% inhibition rate. The protein exhibited significant DPPH radical-scavenging activity compared to the group without any inhibitor. At the same dosage of 10 µg, conventional antioxidants such as Ascorbic acid and Vitamin E demonstrated DPPH radical scavenging activities of 58% and 61%, respectively.

Effect of heat induced hemolysis

Fig. 2 illustrates the heat-induced hemolysis inhibition in red blood cells, presenting the percentage inhibition at different concentrations of TCFP ranging from 20–100 µg/mL. The TCFP displayed a concentration-dependent inhibition of hemolysis, with inhibition percentages ranging from 8% to 42%. In comparison, the standard exhibited inhibition percentages ranging from 15% to 45%.

Effect of protein denaturation

In Fig. 3, the dose-dependent inhibitory effect of TCFP on protein denaturation is depicted at various concentrations (20–100 µg/mL). Phenylmethylsulfonyl fluoride (PMSF) was used as the standard in the experiment. The percentage of protein denaturation inhibition by the crude proteins from *T. chebula* fruit ranged from 5.0% to 28.0% within the concentration range of 20–100 µg/mL. In contrast, the standard protease inhibitor PMSF exhibited a range of 11.0–41.0%. Importantly, the crude proteins from *T. chebula* fruit demonstrated a significantly higher level of inhibition ($p < 0.05$) compared to the other standards examined.

Proteinase inhibitory activity

In Fig. 4, the proteinase inhibitory activity of different doses of TCFP is illustrated, demonstrating inhibition levels ranging from 10.0% to 35.0%. Notably, TCFP exhibited significantly higher proteinase inhibition ($p < 0.05$) compared to standard inhibitors like EDTA. The inhibition levels of EDTA ranged from 15.0% to 43.0%.

DISCUSSION

Inflammation, a biological response of the immune system, can be initiated by various factors, including toxic components, pathogens,

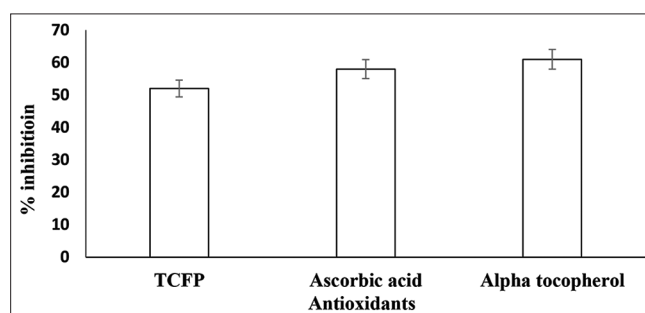


Fig. 1: DPPH radical scavenging activity

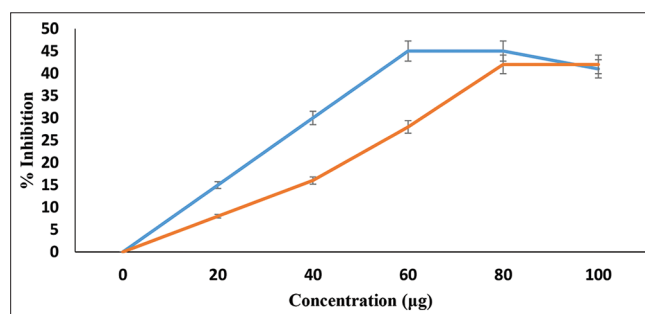


Fig. 2: Hemolysis inhibition studies

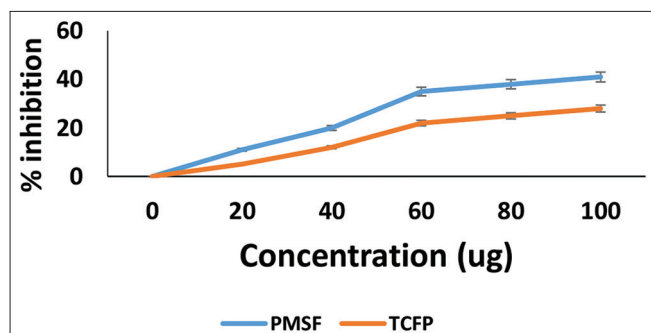


Fig. 3: Protein denaturation inhibitory studies

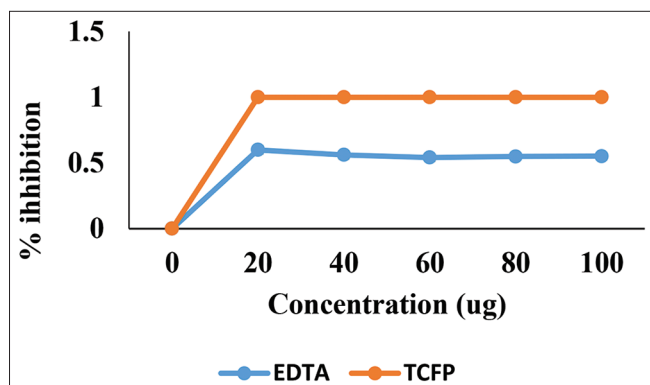


Fig. 4: Proteinase inhibitory activity

and other stimuli. These substances have the potential to trigger inflammatory reactions in different organs, leading to tissue damage or the development of diseases [14]. Such reactions activate signaling pathways that regulate the production of inflammatory mediators in both tissue cells and blood cells [15]. This study specifically focuses on the resemblance between the lysosomal membrane and the red blood cell membrane, highlighting the importance of inhibiting red blood cell hemolysis in understanding the inflammatory process [16]. Preventing cell membrane lysis and cytoplasmic content damage is crucial to minimizing harm and inflammation. Substances that safeguard cell membranes from harmful agents play a significant role in inflammation inhibition. Previous research reported the potential of crude proteins from Pippali seeds (*Piper longum*) in reducing inflammation-related responses [17,18]. Similarly, proteins isolated from *Zingiber officinale* rhizomes have been recognized for their anti-inflammatory properties, exhibiting significant antioxidant and anti-inflammatory effects compared to standard compounds [19]. Neutrophils carry numerous serine proteinases that contribute to arthritic reactions [20]. During inflammation, leukocyte proteinases play a role in causing tissue damage, emphasizing the importance of including proteinase inhibitors for protection against such damage. Recent studies have indicated that plant proteins are responsible for the antioxidant and anti-inflammatory properties observed in numerous plants. Consequently, the bioactive components present in plants and herbs may contribute to their anti-inflammatory activity [21-23].

CONCLUSION

According to the results, the crude proteins extracted from *T. chebula* fruits exhibit antioxidant and anti-inflammatory properties in different model systems, with effectiveness varying across doses.

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AUTHORS CONTRIBUTION

The First author did the *in vitro* studies

The second author designed the studies

The corresponding author involved in designing, and writing the manuscript.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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