



In Vitro Investigation of Anti-inflammatory and Antioxidant Activities of *Curcuma Longa* Rhizome Methanol Extract

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Abstract

Dietary turmeric is well known for its health-beneficial potencies. The current study was done to assess the anti-inflammatory activity of turmeric (*Curcuma longa*) rhizome methanol extract by employing *in vitro* methods. We investigated the protective effect of *Curcuma longa* (*C. longa*) using *in vitro* heat-induced and hyposaline-induced human red blood cell (HRBC) membrane stabilization to explore the anti-inflammatory potential of rhizome. Inhibition of protein denaturation and ability to stabilize the human RBC membrane were studied to establish the mechanism of the anti-arthritis effect of the rhizome. A significant antioxidant nature of turmeric rhizome methanol extract (600 µg/ml) was found as revealed by DPPH assay (67.38%), and hydrogen peroxide reducing assay (63.77%). Further, the extract showed strong heat-induced protein denaturation inhibition activity (67.32%), anti-proteinase activity (71.16%), membrane stabilizing property against heat (68.87%), and hyposaline (63.73%) at a concentration of 600 µg/ml. This study demonstrates the significant anti-inflammatory and anti-arthritis properties of *C. longa*. The results of the investigations justify the folklore use of *C. longa* rhizome in the treatment of inflammation during arthritis, and this plant is suggested to undergo further chemical and pharmacological investigations in future studies.

Keywords: Natural products, Turmeric, Human red blood cell membrane, Inflammation, Arthritis.

Introduction

Inflammation is a multifaceted biological response of vascular tissues to potentially harmful stimuli. When stimulated, immune cells become activated and release inflammatory mediators such as vasoactive amines and eicosanoids, which remodel the local vasculature (Ansar and Ghosh, 2016). Inflammation is a complicated process that is triggered by a variety of factors, including molecules ranging from bacteria to chemicals, and results in cellular trauma or death (Furman et al., 2019). The complex biological reaction of vascular tissues to harmful stimuli includes inflammation. It is a defence mechanism used by body cells in response to harm, infections, allergic reactions, or chemical irritants (Chen et al., 2017).. Due to the dilated blood vessels that cause an increase in blood flow in that area, which causes immune cells like neutrophils and macrophages to migrate toward the inflamed areas along with the fluids that cause edema, this reaction is characterized by certain inflammatory features such as redness, pain, swelling, heat, and loss of function. (Spite et al., 2010).

Oxidative stress, which is a factor in the emergence of diseases like diabetes, cancer, cardiovascular disease, Parkinson's disease, Alzheimer's disease, and ageing, is brought on by an excessive production of free radicals/ROS (Liguori et al., 2018). When intracellular reactive oxidative species come into contact with proteins, they can fragment, aggregate, and negatively interact with ion channels, cell receptors, and oxidative phosphorylation. Intracellular ROS also alter intracellular proteins through oxidation and prevent the uptake of glucose (Anwar et al., 2020a). The damage caused by oxidative stress is exacerbated if the antioxidant enzymes, including superoxide dismutase (SOD), themselves are inactivated (Younus and Anwar, 2017). Inflammation has been linked to discomfort, altered membranes, increased vascular permeability, and protein denaturation (Sakat et al., 2010). Persistent inflammation, on the other hand, has been linked to the development of tumours. Increased ROS generation at the site of inflammation causes tissue damage and endothelial dysfunction in inflammatory diseases (Rahmani et al., 2022; Mittal et al. 2014).

Inflammatory cells organize the microenvironment of tumour cells, which is an essential part of the neoplastic strategy that promotes proliferation, survival, and migration (Baghban et al., 2020). The majority of innate immune signalling molecules, such as chemokines, selectins, and their particular invasion receptors, migration, and metastases, have been taken over by tumour cells (Coussens and Werb, 2002; Zhao et al., 2021). Chronic inflammation has been linked to the development and spread of tumours. The NF-B pathway might be a link between cancer and inflammation. The sustained production of pro-inflammatory cytokines that result from its activation in tumor-associated cells promotes the survival of the tumour (Singh et al., 2019).

The accumulation of various reactive oxygen species (ROS) characterises oxidative stress (Younus and Anwar, 2016). ROS are highly active oxidant molecules that contain an extra electron (Anwar et al., 2022). Superoxide dismutase is one of the most important antioxidant molecule (Anwar et al., 2014). As a result of the tissue injury caused by this trauma, reactive oxygen species (ROS), and cytokines are released. Immune system

disorders have been linked to increased expression of pro-inflammatory mediators such as cytokines, NADPH oxidase, NF kappa B, myeloperoxidase, and Inos (Mittal et al., 2014). Hemolysis can occur, however, when erythrocytes are irreversibly damaged by oxidative stress and high glucose levels (Marar, 2011).

The majority of carboxylic acid-containing NSAIDs (non-steroidal anti-inflammatory drugs) are aspirin, indomethacin, propionic acid-based drugs (ibuprofen, ketoprofen, and flurbiprofen), phenyl acetic acid derivatives, and salicylates (diclofenac). These organic acid-containing drugs inhibit the cyclooxygenase pathway by preventing arachidonic acid (AA) from accessing the enzyme's active site. NSAIDs' excellent anti-inflammatory properties, however, have limited their therapeutic use due to their serious side effects such as gastro-intestinal (GI) ulceration, perforation, obstruction, and bleeding. The majority of NSAIDs' acidity and the inhibition of the mucosal protective prostaglandin (PG) production cause mucosal irritation, which leads to gastric erosion (Qandil, 2012; Shaikh et al., 2015). In the meantime, due to the rising popularity of herbal remedies, extensive research into the anti-inflammatory properties of natural substances is accelerating at a faster rate.

Natural products especially those derived from plants represent the safest, effective, and alternative source for chemical drugs (Thomford et al., 2018). It has been already well established that people can use their dietary choices to lower their risk of illness and treat existing illnesses. In addition, a proper nutritional balance could help to fight against a variety of health problems (Yahia and Anwar, 2020). *Curcuma longa* (*C. longa*), also known as turmeric, is a Zingiberaceae family perennial herb grown in Southeast Asia. The rhizome of turmeric is used in traditional medicine, food, and carminative and diuretic treatments for tumours, infections, and inflammation (Kumar and Sakhya, 2013). Curcumin is a major component in *C. longa* L., being responsible for its biological action such as anti-parasitic, antispasmodic, anti-inflammatory and gastrointestinal effects; and also inhibition of carcinogenesis and cancer growth (Sharifi-Rad et al., 2020; Chaturvedi, 2009). Turmeric extract has been shown to have anti-inflammatory, antioxidant, anti-carcinogenic, anti-mutagenic, anti-coagulant, anti-fertility, anti-diabetic, anti-bacterial, anti-fungal, antiviral, antivenom, antiulcer, hypotensive, and hypocholesteremic properties (Bhutia and Sharangi, 2017; Jabborova et al., 2021). In this study, we looked into the curcuma longa methanol extract's *in vitro* anti-inflammatory and antioxidant activity.

Materials and Methods

Materials

Trichloroacetic acid, ascorbic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric chloride, Folin-Ciocalteu reagent, potassium ferricyanide, gallic acid, quercetin, and trypsin were purchased from Sigma Co. St. Louis, Missouri, USA. Hydrochloric acid, aluminum chloride, mono sodium dihydrogen phosphate, DMSO, ethanol, methanol, disodium hydrogen phosphate, sodium hydroxide, sodium carbonate, and hydrogen peroxide were purchased from Merck, Darmstadt, Germany. All the reagents and chemicals were of analytical grade. The solvents used were of HPLC grade.

Collection of plant material:

Preparation of extracts

Our standardized procedure yielded a methanol extract of *C. longa* rhizome (Anwar et al., 2020a). The rhizomes were cleaned with distilled water, cut into smaller pieces, and allowed to air dry for three days at room temperature. The air-dried rhizomes weighed 100 g. Using an electronic mixer grinder, the dry ingredients were finely ground to create the powder. The methanol samples were obtained by soaking approximately 100 g of powdered plant material in 1000 ml of 97% methanol in a magnetic shaker for three hours at 37° C in a magnetic shaker. To obtain crude active component, the extracts were purified by filtration before being condensed using rotary evaporators at reduced pressure and 40°C. The extracts were kept at 4°C for future use. The extract yield was calculated using the equation below (Rahmani et al., 2022a).

Yield (%) = [Weight of sample extract / Initial weight of sample] × 100.

Phytochemical screening

A previously published paper was used to conduct phytochemical screening of carbohydrates, phenolics, alkaloids, saponins, flavonoids, tannins, steroids, as well as phenolic compounds (Anwar et al., 2021). Carbohydrate detection was accomplished by mixing 1 ml iodine solution with 3 ml methanol extract separately. Carbohydrates might be confirmed if the color turns to purple. The existence of saponins was determined by mixing 5 ml distil water with 5 ml extracts in a test tube and aggressively shaking the mixture before warming the test tube. The presence of saponins is indicated by the development of stable froth. Meanwhile, 2 ml of extract were placed in a test tube and 2 ml of distil water was added. The existence of condensed tannins is shown by the formation of green precipitate after adding a few drops of FeCl₃. The availability of flavonoids in the extract has been verified by that of the development of a strong yellow color when 2 ml of extracts were mixed with a 20% sodium hydroxide solution. However, after the addition of dilute HCl, the yellowish color becomes colorless. When a few drops of FeCl₃ were combined with the extracts, the development of such a blue black color reported the existence of phenolic chemicals. Two ml of acetic anhydride was added to 0.5 g ethanolic extract of each sample with 2 ml H₂SO₄. The colour changed from violet to blue or green in some samples indicating the presence of steroids. Plant extract treated with 2 ml glacial acetic acid containing a drop of FeCl₃. A brown colour ring indicates the presence of positive test.

Determination of total polyphenols

Within this experiment, methanol extract (500 µl, 1 mg/ml) were applied separately to various test tubes containing Folin-Ciocalteu reagent (2500 µl, 10%). Sodium carbonate (2 ml, 7.5 %) was eventually added to each tube. After thorough mixing, the tubes were maintained at 37° C in the absence of light for 30 minutes.

After incubation, the absorbance of solutions was measured with a spectrophotometer at a constant wavelength of 760 nm. A standard calibration plot was created using various amounts of gallic acid (50-250 µg/ml). This calibration curve was being used to calculate the total phenolic content of the ethanol extracts. The total phenolic content was measured in milligrams of gallic acid equivalents (GAE). All tests were conducted in duplicate. The findings were shown as mg gallic acid equivalents per gm sample extract. Following formula was used to calculate total phenolic content. (Anwar et al., 2020a).

$$\text{Total phenolic content} = Z \times V / m$$

Where, Z is concentration of gallic acid in mg/ml; V is volume (ml) of sample used in the extraction; m is the weight of pure dried sample used (g).

Total flavonoid content estimation

Estimation of total flavonoid content in the extract was carried out using aluminum chloride (AlCl₃) colorimetric method (Anwar et al., 2020a). A standard calibration curve was prepared by using quercetin (20-250 µg/ml). In brief, 500 microliter of extract (50 µg/ml) or standard quercetin solution was added to 500 µl of AlCl₃ (2%). The reaction mixture was shaken intermittently. After 1 hour at room temperature, the absorbance was measured at 420 nm using a spectrophotometer against methanol as blank. The total flavonoid content was calculated as quercetin equivalent (mg/g) (mg QUE/g) by following equation to estimate the total flavonoid content (TPC) (Rahmani et al., 2022a).

$$\text{TPC} = Z \times V / M$$

Where, Z is concentration of quercetin (mg/ml); V is volume of plant extract (ml); m is the weight of pure dried sample used (g).

Scavenging of hydrogen peroxide (H₂O₂)

A slightly modified method of Ruch et al. (1989) was used to investigate antioxidant activity (Ruch et al., 1989; AlSahli et al., 2021). A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Various concentrations (25, 50, 75, 100, 150, 200, 300, 400, 500, and 600 µg/ml) of the extract or ascorbic acid (100 and 200 µg/ml) as the control were added to a hydrogen peroxide solution (1 ml, 40 mM). The absorbance of hydrogen peroxide at 230 nm was recorded after 10 min against a blank solution containing phosphate buffer only. Triplicates of each sample were tested. The hydrogen peroxide percentage scavenging activity was then calculated using the following equation. The percentage of scavenged H₂O₂ was calculated using the following equation (Anwar et al., 2020a).

$$\text{H}_2\text{O}_2 \text{ scavenging ability (\%)} = [(O_c - O_s) / O_c] \times 100$$

Where, O_c is the absorbance of H₂O₂ solution; and O_s is the absorbance of solution having both extract and H₂O₂ solution.

DPPH assay

Antioxidant potential was investigated on the basis of scavenging 1,1 diphenyl-2-picryl-hydrazyl (DPPH) radical by method described in our previously published article (AlSahli et al., 2021; Almatroodi et al., 2020a; Anwar et al., 2020a). Briefly, 2.5 ml of increasing concentration of methanol extract (25, 50, 75, 100, 150, 200, 300, 400, 500, and 600 µg/ml) were mixed with 1 ml of DPPH (0.3 mM; prepared in analytical grade methanol). After 30 minutes, the absorbance was measured at 517 nm against methanol like a blank. The DPPH solution in methanol, on the other hand, was used as a control. The percentage radical scavenging activity was calculated as $[(O_c - O_s) / O_c] \times 100$ (Anwar et al., 2020a).

Where, the absorbance of a solution without extract is O_c , whereas the absorbance of a solution containing extract is O_s .

Albumin denaturation inhibition activity

Evaluation of anti-inflammatory potential of extract was evaluated by inhibition of albumin denaturation as described earlier (Sakat et al., 2010; Anwar et al., 2020a, Almatroodi et al., 2020b) with slight modifications. Ibuprofen was used as reference drug for this experiment. The reaction mixture consisted of 500 µl of bovine serum albumin (BSA) (1% aqueous solution) and 100 µl of varying concentration of extract (25, 50, 75, 100, 150, 200, 300, 400, 500, 600 µg/ml) or standard ibuprofen (100 & 200 µg/ml) in separate tubes. Then, the samples were incubated at 37 °C for 20 min in an incubator. The tubes were heated in a water bath at 71°C for 10 min to induce the denaturation of BSA. After cooling, the turbidity of samples was spectrophotometrically recorded at 660 nm, and distil water was used as blank. The experiment was performed in triplicate. The percentage inhibition of protein denaturation was calculated as follows: (Anwar et al., 2020a).

$$\text{Percent inhibition} = [(O_c - O_s) / O_c] \times 100$$

Where, O_c represents absorbance of control; O_s denotes to absorbance of sample having extract/ibuprofen.

Inhibition of proteinase action

The inhibitory activity on trypsin was performed according to Sakat et al (2010) and Almatroodi et al. (2020b). The testing solutions (2000 µl) had 1000 µl of tris HCl buffer (20 mM; pH 7.4), trypsin (60 µg), and 1000 µl of varying concentration of extract (25, 50, 75, 100, 150, 200, 300, 400, 500, and 600 µg/ml) or 0.10 & 0.20 mg/ml of diclofenac sodium. After 5 min at room temperature, 1000 µl of casein (0.8%) was included to the tubes. The tubes were kept at room temperature for 5 min, and 1 ml of casein (w/v) was added. To cease the reaction, 2000 µl of 70% perchloric acid was included after 20 min that resulted in the formation of a cloudy

solution. The centrifugation was carried out at 2500 rpm for 5 min. At 210 nm, the supernatant's absorbance was tested against a blank containing just buffer. Triplicates of each sample were taken.

$$\text{Percentage inhibition of proteinase action (\%)} = [(Z_c - Z_s) / Z_c] \times 100$$

In which, Z_c signifies the absorbance of a control sample, and Z_s denotes the absorbance of sample having either the extract or diclofenac.

Inhibition of denaturation of egg albumin

Phosphate buffer saline (2800 μ l, pH 6.4), raw egg albumin of hen (200 μ l), and 2000 μ l of different concentrations (25, 50, 75, 100, 150, 200, 300, 400, 500, and 600 μ g/ml) of extract were taken in the reaction solution (5 ml) (Almatroodi et al., 2020a). Diclofenac sodium was used as a standard diclofenac drug (100 & 200 μ g/ml). In a BOD incubator, various tubes containing these solutions were kept for 15 min at $37 \pm 2^\circ\text{C}$. The reaction mixtures were then heated for 5 minutes at 70°C . After cooling, their absorbance were taken at 660 nm spectrophotometrically using buffer as a blank. The below equation was used to determine the percentage inhibition of denaturation of egg albumin: $\text{Percentage Inhibition} = [(Z_c - Z_s) / Z_c] \times 100$

In which, Z_c signifies the absorbance of a control sample, and Z_s denotes the absorbance of sample having either the extract or diclofenac.

Membrane stabilization ability

a. Red blood cell (RBC) suspension preparation

Fresh blood was obtained from healthy volunteers. Before collecting blood, it was made sure that administration of any anti-inflammatory and anti-contraceptive drug was not taken place at least for a week before donating blood. The collected blood was transferred to the tubes having sterilized Alsever's solution in an equal amount. The tubes containing resultant blood solution were centrifuged for 10 min at 3000 rpm. The supernatant was discarded and erythrocytes were washed three times. Normal saline in equal volume was added to wash erythrocyte sediments. The contents were reconstituted with isotonic phosphate buffer (Anwar et al., 2020a; Chanda and Juvekar, 2019).

b. Hemolysis by heat induction

The 2 ml reaction mixture was prepared by taking 1 ml of extract of differential concentrations (25, 50, 75, 100, 150, 200, 300, 400, 500, and 600 μ g/ml) or aspirin (100 & 200 μ g/ml), and 1 ml human RBC suspension (10% v/v). In control solution, normal saline replaced extract or aspirin. Aspirin was taken as reference drug. The tubes were gently inverted to mix the contents, and were incubated at 56°C for half an hour. After cooling the reaction mixtures were centrifuged at 2500 rpm for 5 min at room temperature. The supernatants were collected, and its absorbance was recorded at 560 nm. The blank had only phosphate buffer (Sakat et al., 2010;

Anwar et al., 2022a). The percentage membrane stabilization activity or percentage protection from heat induced denaturation of RBC membrane was calculated by the formula mentioned below.

$$\text{Percentage protection} = [(Bc - Bs) / Bc] \times 100$$

Where Bc denotes the absorbance of control, and Bs denotes the absorbance of sample in the presence of extract/aspirin.

c. Hypotonicity induced hemolysis

In this assay, the inhibitory action of turmeric extract on hemolysis induced by hypotonicity was performed according to the modified method of Chanda & Juvekar (Chanda & Juvekar 2019; Anwar et al., 2022a). HRBC suspension (0.5 ml, 10 % v/v), 0.1 M phosphate buffer pH 7.4, hyposaline 2 ml, and 1 ml of extract with concentrations from 25, 50, 75, 100, 150, 200, 300, 400, 500, and 600 µg/ml taken in various tubes. The reference drug, diclofenac (100 and 200 µg/ml) was used dissolved in distilled water. The control was made from distilled water. All the reaction mixtures were incubated at 37°C for 30 min and centrifuged for 10 min at 3000 rpm to separate the supernatant. The supernatant absorbance was checked at 540 nm. The percent hyposalinity-induced hemolysis was calculated by supposing the 100% hemolysis in control.

$$\% \text{ protection} = 100 - [(Ds/Dc) \times 100]$$

Where, Dc denotes the absorbance of control, and Ds represents the absorbance in presence of sample/diclofenac sodium

Results

The color, odor, texture, and the percentage yield of methanol extract of *C. longa* rhizome are provided in table 1. Further, total phenolic compounds in methanol extract were found to be 564.14 ± 0.31 mg gallic acid equivalent/100 g dry weight of extract. The phenolic contents indicate the product of defense against pathogens or stress in environment and is not related with the growth functions and development of plant tissues (Anwar et al., 2020a). Reducing sugars, particularly fructose, ascorbic acid, and protein, may have an effect on the quantity of total polyphenols. Polyphenols contribute to health-promoting and sensorial properties of fruits and vegetables. Further, polyphenols are responsible for varying structure-dependent stability during processing and shelf-life (Hanuka Katz et al., 2020).

The protective role of flavonoids has been reported to be against diabetes and its complications, cancer, and cardiovascular diseases. The total flavonoid content of extract was measured with the aluminum chloride (AlCl₃) colorimetric assay using quercetin as standard. The content of total flavonoid in methanol extract of *C. longa* rhizome was 453.87 ± 0.72 quercetin equivalents/100 g dried weight of extract.

Preliminary screening	Rhizome
Weight of dry powder of rhizome	100 g

Yield	19.43 %
Extract	Methanol
Color	Reddish
Odour	Aromatic
Consistency	Solid
Flavonoid (alkaline reagent test)	+
Phenolic compounds (FeCl ₃ test)	+

Table. 1: Preliminary screening of *C. longa* rhizome extract

Phytochemical constituents	Rhizome
Alkaloids	+
Saponins	+
Tannins	+
Flavonoids	+
Glycosides	+
Terpenoids	+
Phenolic compounds	+
Steroids	+

Table. 2: Phytochemical screening of *C. longa* rhizome extract

Hydrogen Peroxide (H₂O₂) reducing activity

Reducing capacity and antioxidant activity are directly correlated to each other. The percentage H₂O₂ scavenging ability of *C. longa* rhizome extract has been presented in figure 1. H₂O₂ reducing activity of *C. longa* rhizome extract was found to be increased in a concentration dependent manner. Moreover, the maximum percentage scavenging capacity is shown by 600 µg/ml of methanol extract. As compared, the reducing capacity of ascorbic acid was highest at a concentration of 200 µg/ml.

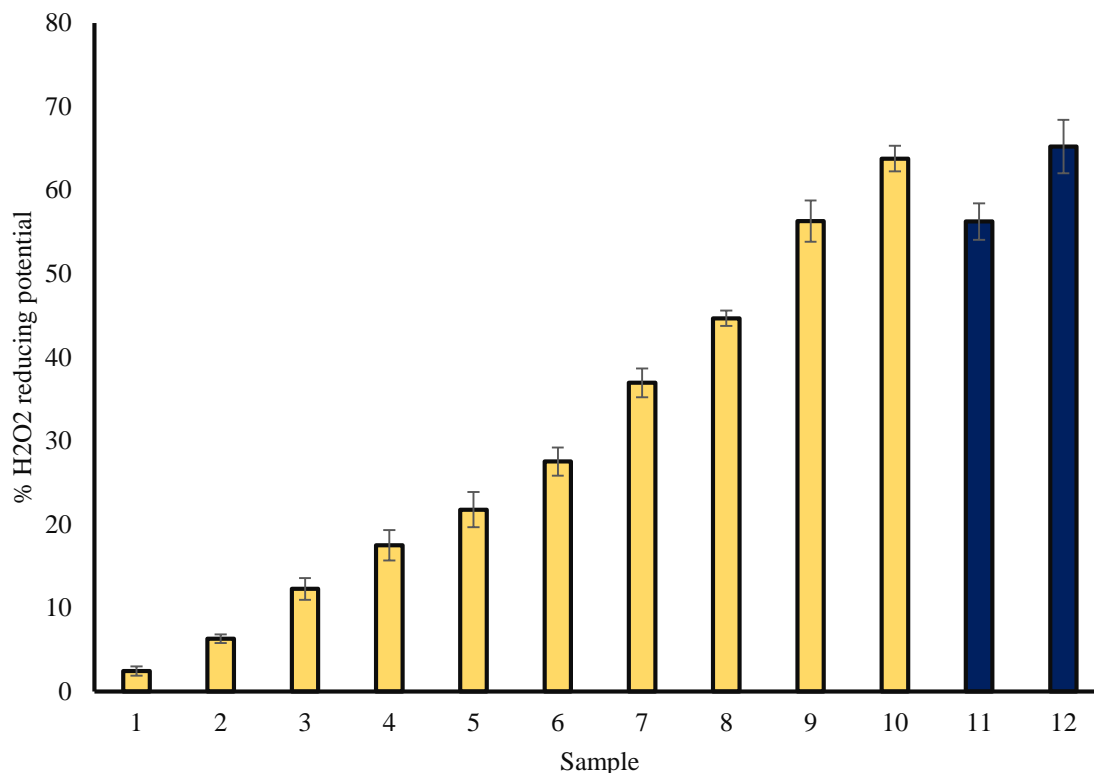


Figure 1- Percentage H₂O₂ scavenging activity of methanol extract of *C. longa* rhizome extract. Sample 1 to 10 corresponds to various concentrations of extract (25, 50, 75, 100, 150, 200, 300, 400, 500 & 600 µg/ml). However, sample 11 & 12 represents 100 & 200 µg/ml of ascorbic acid. The results are presented as means ± SEM (n =3, p <0.05).

DPPH radical scavenging assay

It has been found to be a very high correlation between the concentration of the extract of natural products and percentage of DPPH radical scavenging activity (Anwar et al., 2020a). In our study, methanol extract has showed a concentration dependent DPPH scavenging activity (Figure 2) due to their strong antioxidant nature that was comparable to ascorbic acid.

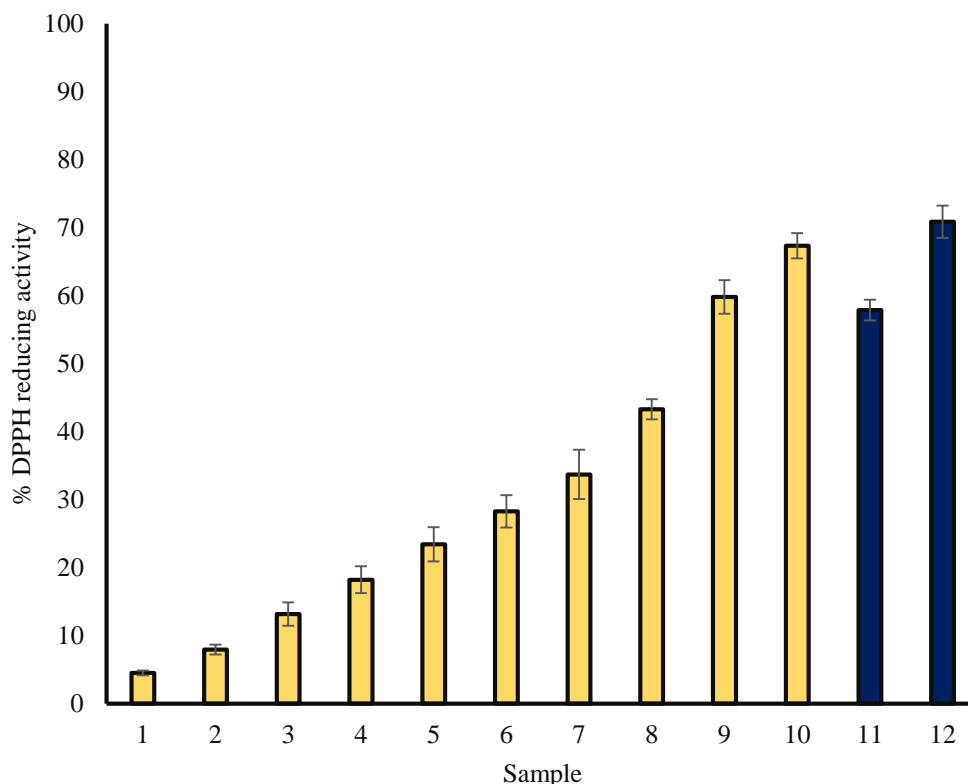


Figure 2. DPPH scavenging activity (%) of *C. longa* rhizome methanol extract. Sample 1 to 10 corresponds to various concentrations of extract (25, 50, 75, 100, 150, 200, 300, 400, 500 & 600 $\mu\text{g/ml}$). However, sample 11 & 12 represents 100 & 200 $\mu\text{g/ml}$ of ascorbic acid. The results are presented as means \pm SEM ($n=3$, $p < 0.05$).

Protein denaturation inhibition: An evaluation of anti-inflammatory activity

During denaturation process, many weak bonds including hydrogen bonds responsible for native structure of protein become broken. Thus, a highly ordered structure of protein become lost due to several factors. Various factors like chemicals or stress induce protein denaturation. Besides, it has been already reported that protein denaturation contributes inflammation significantly. Therefore, the possible anti-inflammatory activity of *C. longa* rhizome was investigated by their potential to protect from protein denaturation. The data suggest that methanol extract has a significant protecting efficacy ($67.31 \pm 3.14\%$) against heat-induced albumin denaturation at a concentration of 600 $\mu\text{g/ml}$ (Figure 3), Ibuprofen, a standard anti-inflammatory drug showed the maximum inhibition ($66.57 \pm 3.27\%$), at the concentration of 200 $\mu\text{g/ml}$.

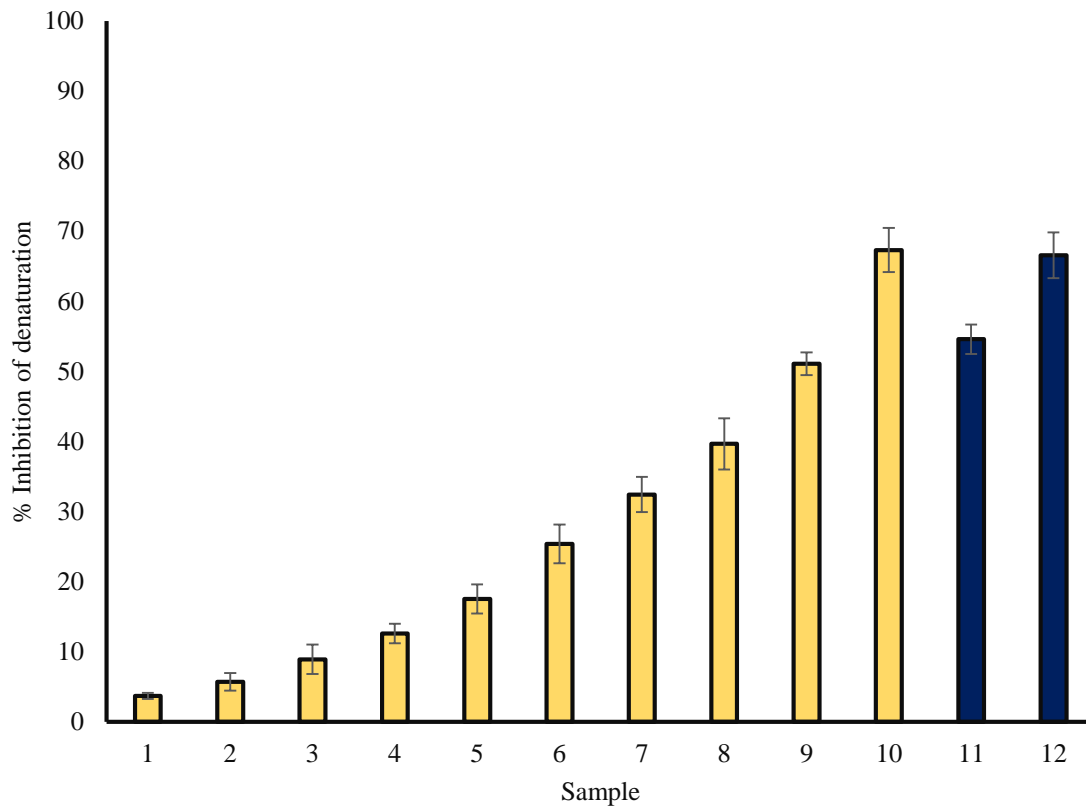


Figure 3. Percentage protection of heat induced protein denaturation. Sample 1 to 10 corresponds to various concentrations of extract (25, 50, 75, 100, 150, 200, 300, 400, 500 & 600 $\mu\text{g/ml}$). However, sample 11 & 12 represents 100 & 200 $\mu\text{g/ml}$ of ibuprofen. The results are presented as means \pm SEM ($n=3$, $p<0.05$).

Anti-proteinase activity

Proteinases have been implicated in arthritic reactions. Proteinase activity was also significantly inhibited by extract. Rhizome methanol extract (600 $\mu\text{g/ml}$) showed the maximum proteinase inhibition activity of 71% (Figure 4). However, diclofenac sodium showed maximum inhibition 68% at 200 $\mu\text{g/ml}$.

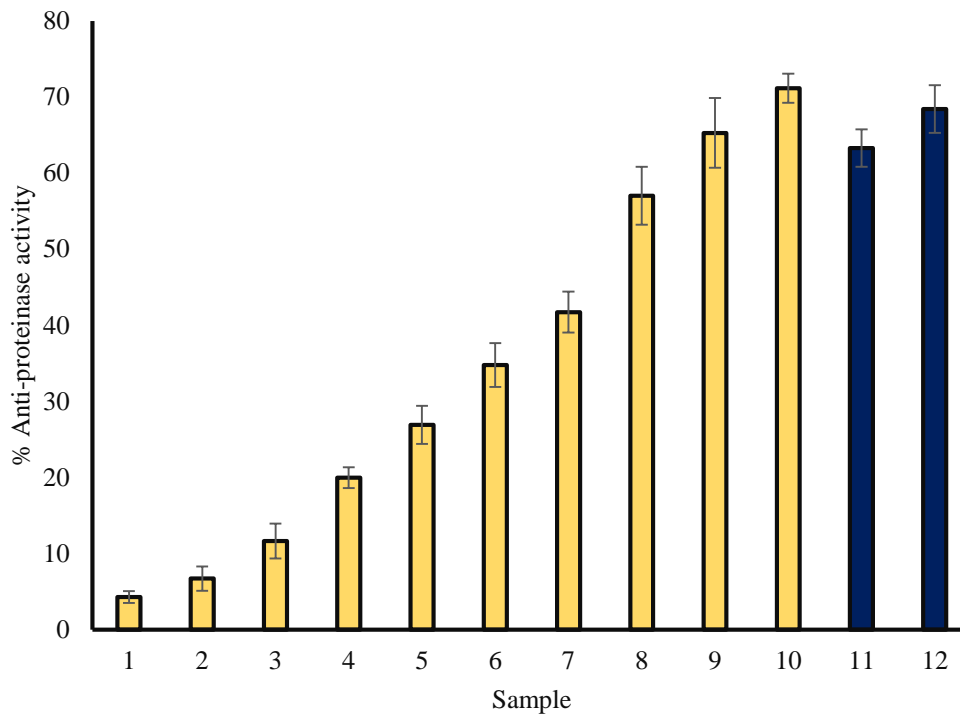


Figure 4. Anti-proteinase activity of methanol extract of *C. longa* rhizome. Sample 1 to 10 corresponds to various concentrations of extract (25, 50, 75, 100, 150, 200, 300, 400, 500 & 600 µg/ml). However, sample 11 & 12 represents 100 & 200 µg/ml of diclofenac sodium. The results are presented as means ± SEM (n =3, p <0.05).

Inhibition of egg albumin denaturation inhibition to evaluate *in vitro* anti-arthritis activity

The anti-arthritis effect of methanol extract was appraised against egg albumin denaturation (Figure 5). *In vitro* anti-arthritis activity by inhibition of egg albumin denaturation method was shown to be increased with an increase in the concentration of rhizome. The significant ability to inhibit the heat induced egg albumin denaturation might be due to the presence of some antioxidant polyphenolic compounds. Diclofenac, a standard drug showed the maximum inhibition 70%, at the concentration of 200 µg/ml.

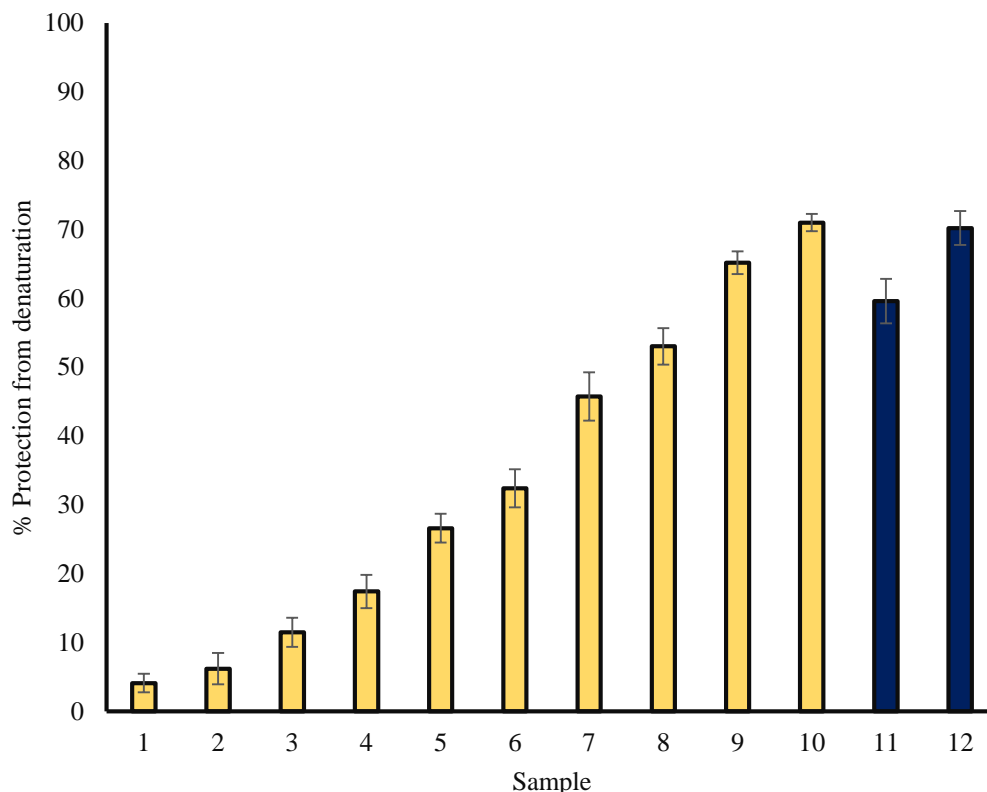


Figure 5. Percentage protection of heat induced denaturation of egg albumin by methanol extract of *C. longa* rhizome. Sample 1 to 10 corresponds to various concentrations of extract (25, 50, 75, 100, 150, 200, 300, 400, 500 & 600 $\mu\text{g/ml}$). However, sample 11 & 12 represents 100 & 200 $\mu\text{g/ml}$ of diclofenac sodium. The results are presented as means \pm SEM ($n=3$, $p<0.05$).

Membrane stabilization test

To establish the mechanism of anti-inflammatory action of *C. longa* rhizome, the stabilization of the RBCs membrane in the presence of extracts was investigated.

a. Heat induced hemolysis

Methanol extract of rhizome were found to have protective effects against heat induced hemolysis at all concentrations. At the site of inflammation, the mechanism behind the protection from heat induced hemolysis most probably involves the inhibition of the lysosomal content release within the neutrophils. The inhibition of heat induced hemolysis was found to be increased significantly with increase in the concentration. The extract showed the maximum inhibition 68.87% at 600 $\mu\text{g/ml}$ (Figure 6). The standard drug aspirin used as standard, and it showed significant protection (67.58%) at concentration 200 $\mu\text{g/ml}$.

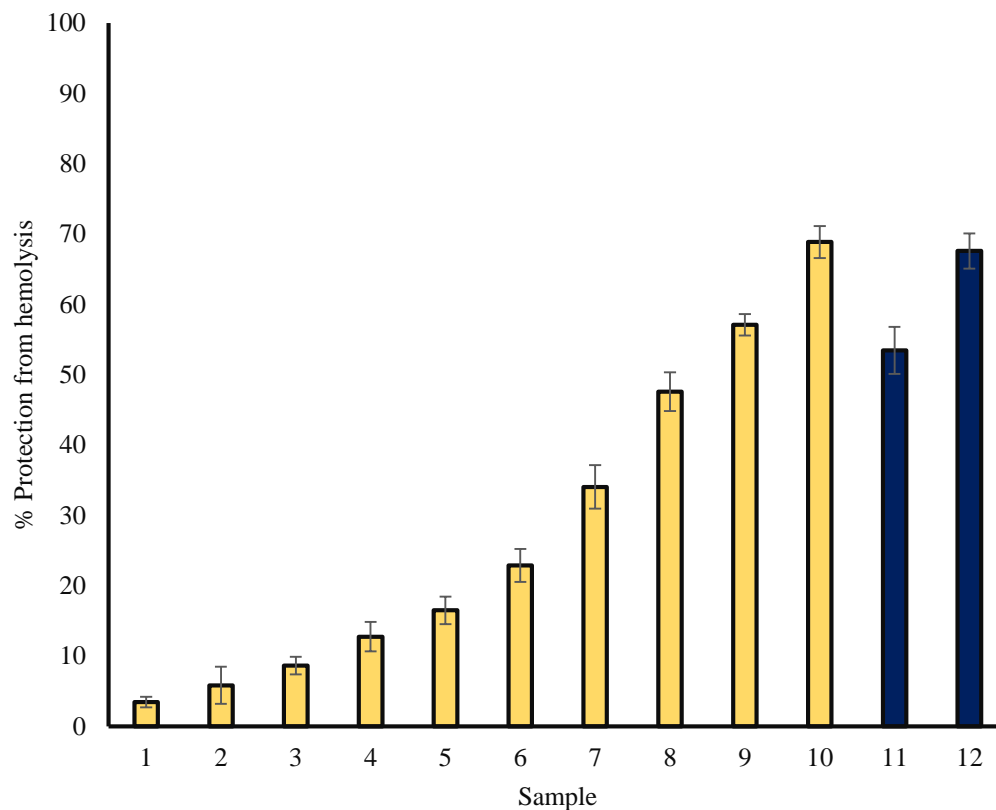


Figure 6. Percentage protection from heat-induced hemolysis. Sample 1 to 10 corresponds to various concentrations of extract (25, 50, 75, 100, 150, 200, 300, 400, 500 & 600 $\mu\text{g/ml}$). However, sample 11 & 12 represents 100 & 200 $\mu\text{g/ml}$ of diclofenac sodium. The results are presented as means \pm SEM (n =3, p <0.05).

a. Protection from hyposaline-induced hemolysis

The methanol extract of *C. longa* rhizome exhibited significant protecting ability against hyposaline induced hemolysis at various concentrations. The osmotic loss has been reported to be caused by hemolysis induced by hypotonicity. Methanol extract inhibited the hemolysis mediated by hypotonicity and protected from concentration variable osmotic loss. The data shows that *C. longa* methanol extract have potential of stabilizing the RBC membrane, and is an effective membrane stabilizer (63.73%) even at higher concentrations (600 $\mu\text{g/ml}$) (Figure 7). Diclofenac sodium showed maximum protection of 66.94% at 200 $\mu\text{g/ml}$.

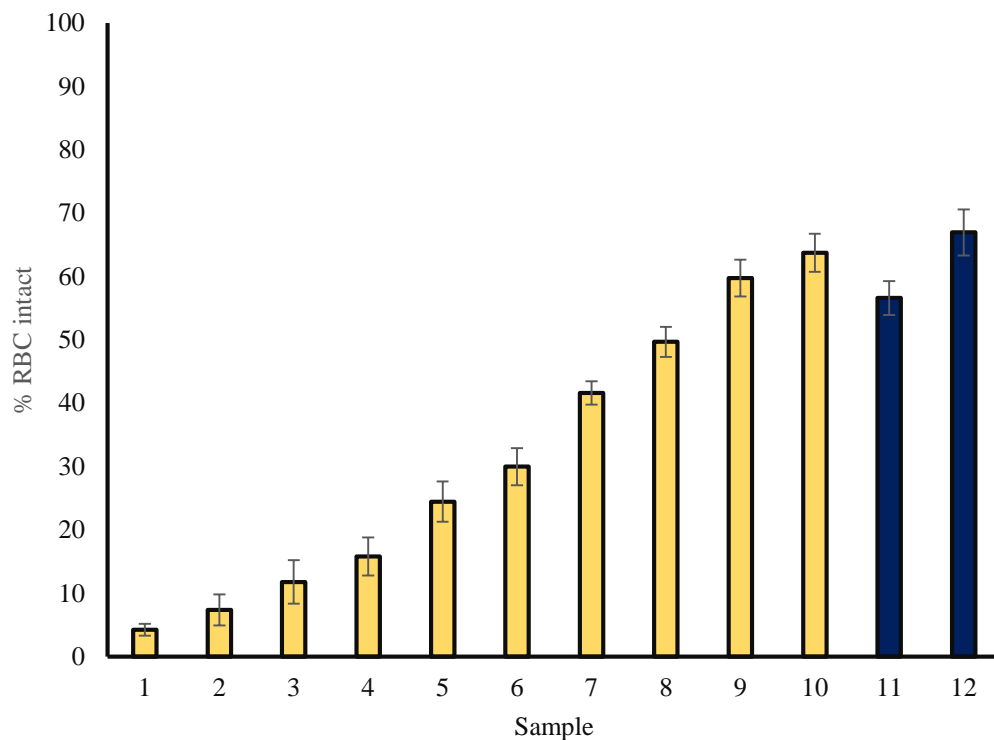


Figure 7. Protection from hyposalinity-induced hemolysis. Sample 1 to 10 corresponds to various concentrations of extract (25, 50, 75, 100, 150, 200, 300, 400, 500 & 600 $\mu\text{g/ml}$). However, sample 11 & 12 represents 100 & 200 $\mu\text{g/ml}$ of diclofenac sodium. The results are presented as means \pm SEM ($n=3$, $p<0.05$).

Discussion

Pharmacologic treatments, such as nonsteroidal anti-inflammatory drugs (NSAIDs) are frequently used to treat osteoarthritis (OA) and musculoskeletal pain. Although NSAIDs are generally effective, they are also linked to safety issues, such as adverse gastrointestinal, hepatic, and cardiovascular events. The majority of OA patients also have other comorbid conditions, such as diabetes, hypertension, and dyslipidemia, which makes it difficult to determine the best course of treatment and can worsen inflammation, accelerating the course of the disease. There is a need for more safe and efficient alternative pain relief options (Sethi et al., 2022). A well-balanced, nutritious diet is thought to be critical for optimal immune response (Anwar et al., 2022b). Inflammation is a reaction to tissue damage and it can last for months to years due to inflammatory cytokines and growth factors. Different cultures have used turmeric for medicinal purposes for a very long time. Turmeric's active ingredient, curcumin, has demonstrated a variety of positive physiological and pharmacological effects (Hewlings and Kalman, 2017). Through interactions with Toll-like receptors (TLRs), which are important for innate immunity, curcumin exhibits anti-inflammatory properties. When it binds, it controls the synthesis of inflammatory mediators like Nuclear Factor Kappa-B (NF- κ B), Activator Protein 1 (AP-1), and Mitogen-Activated Protein Kinases (MAPK). One of the primary targets for the treatment of inflammatory diseases like

rheumatoid arthritis and inflammatory bowel diseases is the Janus kinase/Signal transducer and activator of transcription (JAK/STAT) signalling pathway (Sivani et al., 2022).

Oxidative stress, systemic hyper-inflammatory reactions, and lysosomal membrane permeabilization are frequently associated with a number of diseases, including diabetes and its complications (Anwar et al., 2022a). Age-related diseases like arthritis, diabetes, cancer, etc., all have oxidative stress as a major contributing factor (Rea et al., 2018). It has been found that methods based on suppressed autoxidation work best for termination-enhancing and chain-breaking antioxidants. However, specific research on preventative antioxidants is needed (Tan et al., 2018). According to our research, *C. longa* has strong antioxidant properties that may contribute to its therapeutic potential for treating oxidative stress, protein denaturation, and membrane stability.

This study suggests that the primary source of the antioxidant and anti-inflammatory activity in the plant *C. longa* is provided by phenolic molecules. A sizable subgroup of the family of naturally occurring polyphenolic compounds known as flavonoids is produced as a byproduct of secondary metabolism in plants (Khan et al., 2022). Flavonoids have been shown to be effective in both the prevention and management of common disease complexes, including cancer. Since flavonoids exhibit a wide range of pharmacological properties, including anti-oxidant activity, anti-inflammation, free radical scavenging, and anti-cancer activity, as well as controlling cellular proliferation, inducing apoptosis, preventing platelet aggregation, and lowering plasma levels of low-density lipoproteins, they have been considered to be of scientific interest (Rahmani et al., 2022b). The result of the chemical analysis showed that the TPC (564.14 ± 0.31 mg gallic acid equivalent/100 g dry weight of extract) and TFC (453.87 ± 0.72 quercetin equivalents/100 g dried weight of extract) was determined in methanol rhizome extract. Regarding antioxidant activity results, a strong DPPH scavenging activity ($67.38 \pm 1.862\%$) was evaluated by 600 $\mu\text{g/ml}$ of methanol extract of the rhizome.

Protein denaturation is a well-known contributor to inflammation, which in turn causes inflammatory illnesses like rheumatoid arthritis (Elisha et al., 2016). Therefore, *C. longa* might be employed as a rheumatoid arthritis treatment. Additionally, studies have been conducted on the potential therapeutic value of compounds that can prevent thermally induced protein denaturation as anti-inflammatory agents. Protein denaturation, which can be brought about *in vitro* by elements like heat, stress, or specific chemical compounds, is a process that occurs when proteins lose their structural integrity and biological function as a result of inflammation. As a result, tissue protein denaturation is recognized as a sign of inflammation (Sherwani et al., 2022). In order to create a new anti-inflammatory drug, it is worthwhile to look for natural substances that can stop protein denaturation. According to the *in vitro* anti-inflammatory activity results (Osman et al., 2016), Curcuma rhizome extract at 600 $\mu\text{g/ml}$ suppressed albumin denaturation by 67.31% and inhibited egg albumin denaturation by 71%.

Lysosomal enzymes are released during inflammation, and a number of common alterations may take place. By stabilising the lysosomal membrane, activated neutrophils are prevented from releasing chemical mediators and lysosomal components, which lessens the inflammatory response (Bonam et al., 2018). The majority of anti-inflammatory drugs work in this way. Due to the similarities between the erythrocyte and lysosome membranes, the stabilisation of the erythrocyte membrane could be extrapolated to the stabilisation of the lysosomal membrane (Gunathilake et al., 2018). We looked at how *C. longa* rhizome extracts in methanol affected the stabilisation of the HRBC membrane. All extracts were discovered to have the capacity to stabilise the RBC membrane in hypotonic solution and to inhibit the hemolysis brought on by heat at various concentrations. The highest percentage of inhibition was seen in rhizome methanol extract at 600 µg/ml, with 68.87% and 63.73%, respectively. These outcomes are on par with the anti-inflammatory effects of common anti-inflammatory drugs.

As a result, it was demonstrated that *C. longa* rhizome could be a reliable source of human erythrocyte stabiliser. Additionally, proteinases have been linked to arthritic reactions. The lysosomal granules of neutrophils contain a large number of serine proteinases, making them a rich source of proteinase. Leukocyte proteinase is thought to be crucial in the occurrence of tissue damage during inflammatory reactions, and proteinase inhibitors offered a significant level of defence. The methanol extract of *C. longa* and diclofenac sodium were also shown in this study to be able to inhibit proteinase activity, suggesting that they may be able to contribute to the healing of tissues during inflammation (Korkmaz et al., 2010). The presence of the polyphenolic compounds (alkaloids, flavonoids, tannins, steroids, and phenols) previously noted may be the cause of these anti-inflammatory properties. The current study raises the possibility of using the *C. longa* rhizome methanol extract to create a potent anti-inflammatory drug for the treatment of a variety of diseases, including cancer, neurological disorders, ageing, and inflammation.

Conclusion

Based on the results of this study, *C. longa* could be suggested as a promising source of natural antioxidants and anti-inflammatory, as evidenced by its strong antiradical scavenging activity of rhizome extract, potent anti-protein denaturation activity of rhizome extract, and high polyphenol content, and it is capable of stabilising HRBC membrane and offering protection from hemolysis. The ability to protect against heat and hypotonic saline-induced erythrocyte lysis is a very good indicator of any agent's anti-inflammatory activity. The structural similarity between the lysosomal membrane and the RBC membrane allows for extrapolation of any substance's effect on stabilising the RBC membrane to stabilising the lysosomal membrane. As a result, our current in-vitro studies showed a notable anti-arthritic activity. This activity may be *C. longa* caused by the presence of active ingredients like flavonoids, glycosides, and tannins. As a result, the rhizome of can be used as a powerful anti-inflammatory and anti-arthritic substance. However, in order to ascertain the mechanisms of the phyto-constituents of the methanol extract of *C. longa* rhizome in this regard, more thorough chemical and pharmacological research is required.

Ethics approval and consent to participate

Not applicable.

Human and animal rights

No Animals/Humans were used for studies that are base of this research.

Consent for publication

Not applicable.

Availability of data and materials

The data supporting the findings of this research are provided within the article.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

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