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IN VITRO ANTIOXIDANT AND ANTI-INFLAMMATORY STUDIES ON LEAF EXTRACT OF GREWIA HETEROTRICHA MAST AND GREWIA SERRULATA DC.

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ABSTRACT

Methanol leaf extract of *Grewia heterotricha* Mast. and *Grewia serrulata* DC. (Family: Malvaceae) were assessed for their antioxidant and anti-inflammatory activity by invitro methods. Quantitative analysis of phytocomponents such as total phenolics, flavonoids and tannins were estimated by standard spectrophotometric methods. The results obtained showed that the methanol leaf extract of *G.heterotricha* (MEGH) and *G.serrulata* (MEGS) have significant DPPH radical scavenging and H_2O_2 scavenging activity with IC_{50} values 98.95µg/ml, 110.1µg/ml (MEGH) and 102.1µg/ml, 143.3µg/ml (MEGS) respectively. Extracts also exhibited good reducing ability of 131.8 ±11.67 mg ascorbic acid/g dry extract (MEGH) and 79.92 ±5.92 mg ascorbic acid/g dry extract (MEGS). Methanol extracts of both the plants showed promising anti-inflammatory activity by inhibiting protein denaturation with IC_{50} values 1440µg/ml (MEGH) and 1685µg/ml (MEGS). HRBC membrane stabilizing assay of MEGH and MEGS showed significant inhibition values of 91.33±0.25% and 88.11±0.24% respectively. Proteinase inhibitory activity of MEGH and MEGS was found to be 67.91±0.08% and 52.81±0.12% respectively. The results obtained in the present study suggest that the phytocomponents like phenolics, flavonoids and tannins present in the extracts may be responsible for antioxidant and anti-inflammatory activity and hence can be a potent source for the development of therapeutic drugs.

KEYWORDS: Anti-inflammatory, protein denaruration, proteinase inhibition, HRBC.

INTRODUCTION

Inflammation is a complex protective response to tissue injury, which is frequently associated with pain and involves occurrences such as: the increase of vascular permeability, increase of protein denaturation and membrane alteration.[1] It is a defensive response that is characterized by redness, pain, heat and swelling and loss of function in the injured area.[2] Harmful stimuli including pathogens, irritants or damaged cells initiate response of vascular tissue as inflammation. Therefore it is a protective response by the organism to remove injurious stimuli and to initiate the healing process for the tissue.[3] If inflammation is not treated, it leads to the onset of many diseases such as atherosclerosis, rheumatoid arthritis and cancer.

Excessive activation of phagocytes during inflammatory disorders result in the production of oxygen,. OH radicals as well as non-free radical species (H2O2).[4] These free radicals then initiate a chain reaction which leads to the formation of various other free radicals leading to oxidative stress which in turn results in the productivity of reactive oxygen species and reactive nitrogen species causing lipid peroxidation (LPO) and tissue damage.[5] Tissue damage then provokes inflammatory response by production of mediators and chemotactic factors.[6] Hence, the agents that can scavenge these reactive oxygen species can be beneficial in the treatment of inflammatory disorders.

Antioxidants are chemical compounds that play an important role to protect the human body against damage by reactive oxygen species. They can scavenge free radicals that are formed in the body due to normal physiological process.[5] The study of plants that have been used traditionally for curing inflammation is still fruitful and logical research strategy in the source of new anti-inflammatory drugs.[7]

Many bioactive compounds from medicinal plants showed anti-inflammatory and antioxidant activities. It is evident that several plants have been used in traditional ayurvedic medicine for treatment and management of distinct inflammatory disorders and wound healing activities.[8] Some *Grewia* species have free radical scavenging activities which may be responsible for the therapeutic action against tissue damage.[9] In the present study attention will be focused on *Grewia heterotricha* Mast. and *Grewia serrulata* DC. belongs to the family Malvaceae. It is reported that various parts of these plants are used in traditional medicine in wound

healing, fever, bronchitis, to cure upset stomachs, some skin and intestinal infections and seem to have anti-inflammatory and analgesic properties.

The literature survey indicated no report has been made to evaluate the anti-inflammatory potential of *G.heterotricha* Mast. and *G.serrulata* DC. by *in-vitro* method. Hence, the present study was undertaken to evaluate anti-inflammatory and antioxidant activity of *G.heterotricha* Mast. and *G.serrulata* DC. by *in-vitro* methods.

MATERIALS AND METHODS

Preparation of extract

The powdered leaves (75g) of both plants were extracted with 350ml of methanol using Soxhlet extractor for 24hr. The extracts were concentrated by evaporation using rotary vacuum evaporator to obtain dark viscous semi-solid. The extracts were stored in refrigerator and were used for further study.

Phytochemical Screening

The methanol leaf extracts were tested for the presence of flavonoids, terpenoids, alkaloids, glycosides, tannins, phenolics, saponins carbohydrates and proteins by using standard methods.[10,12] The total phenolic content, total flavonoid and tannin content were determined.[13,15]

In-vitro antioxidant activity

DPPH radical scavenging activity

DPPH radical scavenging activity was measured according to the method of Chu et al. (2000)[16] and Barku *et al.*[17] An aliquot of 2ml of 0.004% DPPH solution in methanol and 1ml of plant extract in methanol at various concentrations (100, 200, 300, 400 and 500 µg) were mixed and incubated at 25°C for 30 min. and absorbance of the test mixture was read at 517nm using a spectrophotometer against a DPPH control containing only 1 ml of methanol in place of the extract. Ascorbic acid was used as a standard. Percent inhibition was calculated using the following expression:

$\% \text{ Inhibition} = (A_0 - A_1) / A_0) \times 100$

Where A_0 and A_1 stand for absorbance of the blank and absorbance of tested extract solution respectively.

Hydrogen peroxide scavenging activity

The ability of leaf extracts to scavenge hydrogen peroxide was determined by the method of Avani Patel *et al.*[14] and Gupta et. al[18] with minor modifications. 1.0 ml of extract (100-500µg/ml) was added to 2.0 ml of 40 mmol hydrogen peroxide solution prepared in 40 mmol phosphate buffer (pH 7.4). The absorbance was read at 230 nm after 10 min. Ascorbic acid was taken as standard. The percentage inhibition was calculated.

% Inhibition = $(A_0–A_1)/A_0) \times 100$

Where A_0 and A_1 stand for absorbance of the blank and absorbance of tested extract solution respectively.

Reducing power assay

The reducing power assay of leaf extracts were determined by the method described by Oyaizu (1986).[19] 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of $K_3Fe(CN)_6$ (1% w/v) were added to 1.0 ml of extract(500µg/ml). The resulting mixture was incubated at 50°C for 20 min, followed by the addition of 2.5 ml of Trichloro acetic acid (10% w/v). The mixture was centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml)was collected and mixed with distilled water (2.5 ml) and 0.5 ml of $FeCl_3$ (0.1%, w/v). The absorbance was then measured at 700 nm against blank sample. Ascorbic acid was used as standard. Increased absorbance of reaction mixture indicates increase in reducing power.

In-vitro anti-inflammatory activity

Inhibition of albumin denaturation

Method of Mizushima et al (1968) and Sakat et al (2010)[20,21] was followed with minor modifications. The reaction mixture was consisting of test extract at different concentrations and 1% aqueous solution of bovine albumin fraction. pH of the reaction mixture was adjusted using small amount of 1N HCl. The samples were incubated at 37°C for 20 min and then heated at 57°C for 20 min. After cooling the samples, the turbidity was measured spectrophotometrically at 660 nm. Percent inhibition of protein denaturation was calculated as follows:

Percentage inhibition = (Abs control – Abs sample) X 100/ Abscontrol

Membrane stabilization

Preparation of Red Blood cells (RBCs) suspension: RBC suspension was prepared by following the method of Sakat et al (2010) and Oyedepo et al (1995).[21,22] The Blood was

collected from healthy human volunteer who has not taken any NSAIDs (Non Steroidal Anti-Inflammatory Drugs) for 2 weeks prior to the experiment and transferred to the centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10min and were washed three times with equal volume of normal saline. The volume of blood was measured and re constituted as 10% v/v suspension with normal saline.

Heat induced haemolysis

This test was done according to the method of Sakat et al., (2010) and Shinde et al. (1999).[21,23] The reaction mixture (2ml) consisted of 1 ml test sample of different concentrations (100 - 500 µg/ml) and 1 ml of 10% RBCs suspension, instead of test sample only saline was added to the control test tube. Aspirin was used as a standard drug. All the centrifuge tubes containing reaction mixture were incubated in water bath at 56 ºC for 30min. At the end of the incubation the tubes were cooled under running tap water. The reaction mixture was centrifuged at 2500 rpm for 5 min and the absorbance of the supernatants was taken at 560 nm. The experiment was performed in triplicates for all the test samples. The Percentage inhibition of Haemolysis was calculated as follows:

Percentage inhibition = (Abs control –Abs sample) X 100/ Abs control

Hypotonicity-induced haemolysis

This activity was carried out by the method of Azeem et al., (2010).[24] Different concentration of extract (100-500µg/ml), reference sample and control were separately mixed with 1ml of phosphate buffer, 2ml of hyposaline and 0.5ml of HRBC suspension. Diclofenac sodium (100µg/ml) was used as a standard drug. All the assay mixtures were incubated at 37^0c for 30minutes and centrifuged at 3000rpm. The supernatant liquid was decanted and the haemoglobin content was estimated by a spectrophotometer at 560nm. The percentage hemolysis was estimated by assuming the haemolysis produced in the control as 100%.

Percentage protection = 100- (OD sample/OD control) x 100

Proteinase Inhibitory Activity

The test was performed according to the modified method of Sakat et al (2010) and Oyedepo et al (1995).[21,22] The reaction mixture (2 ml) was containing 0.06 mg trypsin, 1 ml 20 mM Tris HCl buffer (pH 7.4) and 1 ml test sample of different concentrations (100 - 500 µg/ml). The mixture was incubated at 37oC for 5 min and then 1 ml of 0.8% (w/v) casein was added. The mixture was incubated for an additional 20 min. 2 ml of 70% perchloric acid was added to arrest the reaction. Cloudy suspension was centrifuged and the absorbance of the

supernatant was read at 210 nm against buffer as blank. The experiment was performed in triplicate. The percentage inhibition of proteinase inhibitory activity was calculated.

Percentage inhibition = (Abs control –Abs sample) X 100/ Abs control

RESULTS AND DISCUSSION

Phytochemical Screening

The phytochemical analysis of methanol leaf extracts of *G.heterotricha* Mast.(MEGH) and *G.serrulata* DC.(MEGS) showed the presence of tannin, flavonoids, di-terpenoids, triterpenoids, phenols, steroids, coumarins and saponins. It was reported that phenolic compounds and flavonoids were associated with antioxidant activity. The total phenolic content of MEGH and MEGS were 87.58±2.52 mg CE/g and 61.16 ±1.77 mg CE/g respectively and the total flavonoid content of were 217.0±18.32 mg QE/g and 142.5± 7.94 mg QE/g respectively. The MEGH and MEGS showed the tannin content values of 130.7±8.05 mg TAE/g and 103.1±5.69mg TAE/g respectively, which support the possible bioactivity of these plant extracts. (Table 1-3).

Table 1: Total phenolic content present in the methanol leaf extracts of *Grewia heterotricha* Mast. and *Grewia serrulata* DC.

Extracts	mg Catechol/g dry extract
MEGH	87.58±2.52
MEGS	61.16 ±1.77

Each value represents mean±SD (n = 3).

Table 2: Total flavonoid content present in the methanol leaf extracts of *Grewia heterotricha* Mast. and *Grewia serrulata* DC.

Extracts	mg quercetin/g dry extract
MEGH	217.0 ±18.32
MEGS	142.5± 7.94

Each value represents mean±SD (n = 3).

Table 3: Tannin content present in the methanol leaf extracts of *Grewia heterotricha* Mast. and *Grewia serrulata* DC.

Extracts	mg tannic acid/g dry extract
MEGH	130.7±8.05
MEGS	103.1±5.69

Each value represents mean±SD (n = 3).

In vitro Antioxidant studies

DPPH radical scavenging activity

The antioxidant activity of methanol leaf extracts of *G.heterotricha* Mast. and *G.serrulata* DC. were investigated by DPPH radical scavenging assay using ascorbic acid as a standard. The results were summarized in Table 4 and Fig. 1. The IC_{50} values were found to be 98.95 µg/ml and 102.1 µg/ml respectively which was significantly comparable with free radical scavenging activity of ascorbic acid (IC_{50} 13.44 µg/ml).

Table 4: DPPH radical scavenging activity of methanol leaf extracts of *Grewia heterotricha* Mast. and *Grewia serrulata* DC.

Extracts	Concentration(µg/ml) / % Inhibition					IC_{50}
	100	200	300	400	500	
MEGH	52.60	71.74	85.39	93.80	94.60	98.95
MEGS	49.89	70.48	84.92	88.74	90.44	102.1
Ascorbic acid	82.97	85.95	89.09	93.16	95.72	13.44

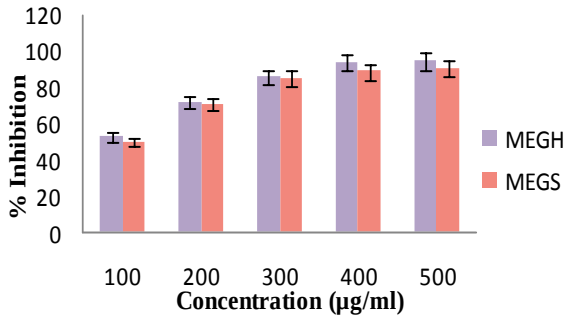

Fig. 1: DPPH scavenging activity of MEGH and MEGS.

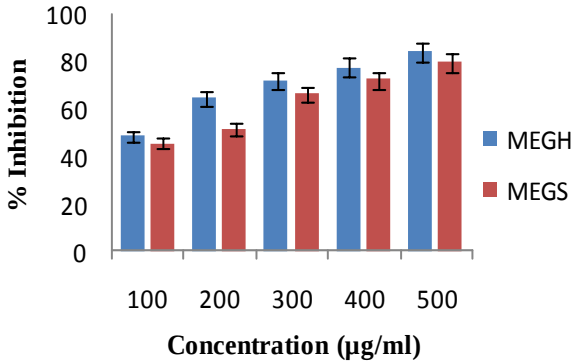

Fig. 2: Hydrogen peroxide scavenging activity of MEGH and MEGS.

Hydrogen peroxide scavenging activity

Scavenging of hydrogen peroxide by methanol leaf extracts were found to be concentration dependent. Maximum inhibition was shown by MEGH with IC_{50} value of 110.1µg/ml than MEGS with IC_{50} value of 143.3µg/ml (Table 5 and Fig. 2).

Table 5: Hydrogen peroxide scavenging activity of methanol leaf extracts of *Grewia heterotricha* Mast. and *Grewia serrulata* DC.

Extracts	Concentration(µg/ml) / % Inhibition					IC_{50}
	100	200	300	400	500	
MEGH	48.38	64.51	72.04	77.41	83.87	110.1
MEGS	45.36	51.54	65.97	72.16	79.38	143.3
Ascorbic acid	66.21	69.59	79.72	87.83	95.94	57.96

Reducing power assay

The results of reducing power assay are given in the Table 6. The extracts showed potent reducing power ability in a concentration dependent manner. The methanolic extract of *G.heterotricha* Mast. showed highest reducing ability (131.8±11.67) than *G.serrulata* DC. (79.92 ±5.92).

Table 6: Reducing property of methanol leaf extracts of *Grewia heterotricha* Mast. and *Grewia serrulata* DC.

Extracts	mg ascorbic acid/g dry extract
MEGH	131.8 ±11.67
MEGS	79.92 ±5.92

Anti-inflammatory Studies

Inhibition of albumin denaturation

Denaturation of proteins is a well known cause of inflammation. On denaturation most of the proteins in the biological system lose their activity. The cause of denaturation may be heat, external stress or compounds such as, strong acid or base, a concentrated inorganic salt or an organic solvent.[20] In the present investigation, the ability of plant extract to inhibit protein denaturation was studied. The methanol leaf extracts of selected plants showed significant inhibition of egg albumin denaturation in concentration dependent manner. IC_{50} of MEGH and MEGS were observed as 1440µg/ml and 1685µg/ml, respectively. Aspirin was used as a standard anti-inflammatory drug showed IC_{50} value 298µg/ml. Results are presented in Fig. 3 and 4.

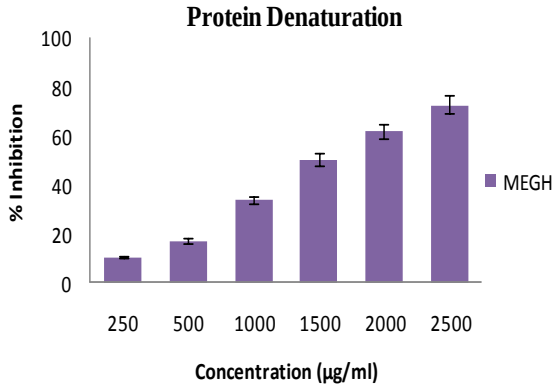

Fig. 3: Inhibition of Protein denaturation activity of MEGH.

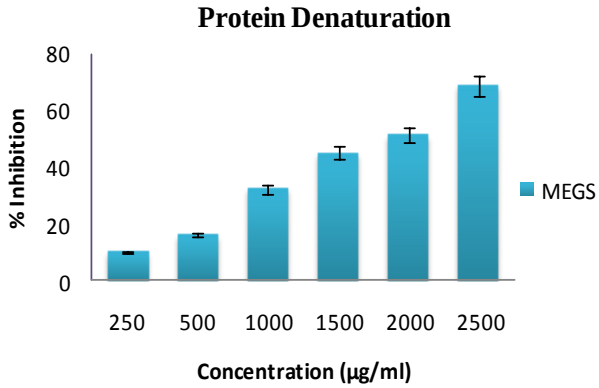

Fig. 4: Inhibition of Protein denaturation activity of MEGH.

Membrane stabilization

At the site of inflammation, lysosomal constituents of activated neutrophils such as proteases and bacterial enzymes are released. These lysosomal contents cause further inflammation and tissue damage. Stabilization of lysosomal membrane can prevent the release of these lysosomal contents and may inhibit tissue inflammation. As erythrocyte membrane is analogous to lysosomal membrane, stabilization of erythrocyte membrane implies to the stabilization of lysosomal membrane.[25,26]

Heat induced haemolysis

The extracts inhibited the heat induced hemolysis of RBCs at different concentrations. MEGH and MEGS showed maximum inhibition of 91.33±0.25% and 88.11±0.24% respectively at 100 µg/ml. Standard Dichlofenac sodium (100 µg/ml) showed maximum inhibition of 91.56±0.25% (Fig 5 & 6).

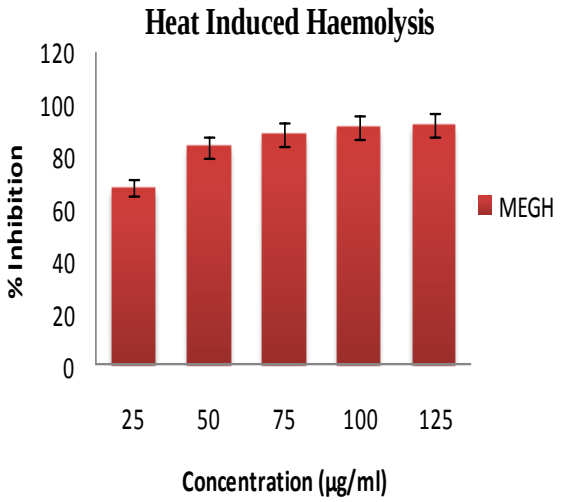

Fig. 5: Inhibition of heat induced haemolytic activity of MEGH.

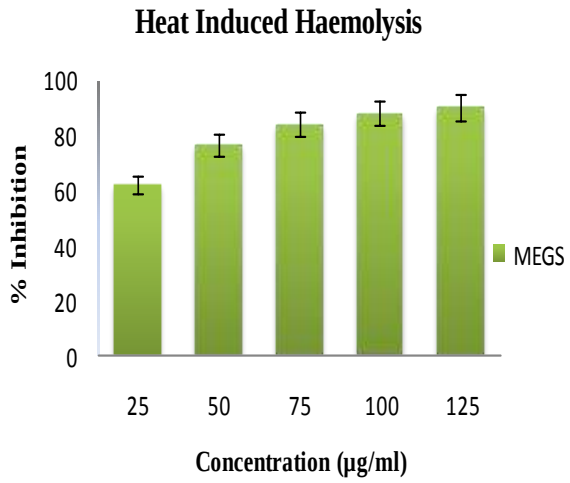

Fig. 6: Inhibition of heat induced haemolytic activity of MEGS.

Hypotonicity-induced haemolysis

The results showed that the methanol extracts of *G.heterotricha* Mast. and *G.serrulata* DC. protect significantly the HRBC membrane against lysis induced by hypotonic solution. At the concentration of 100 µg/ml, the % inhibition of MEGH and MEGS were observed as 75.06±0.26% and 70.64±0.26% respectively. Standard Dichlofenac sodium (100 µg/ml) showed maximum inhibition of 95.58±0.26%(Fig.7&8).

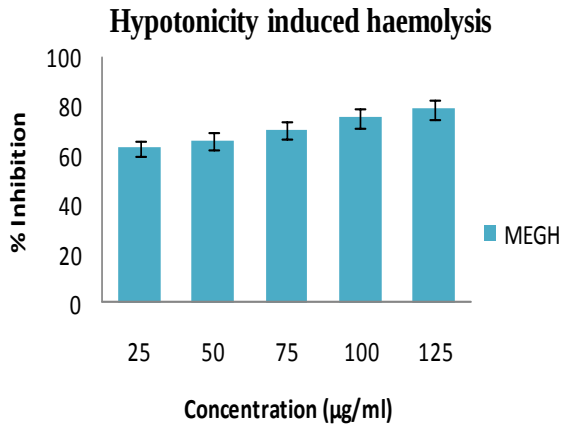

Fig. 7: Inhibition of hypotonicity induced haemolytic activity of MEGH.

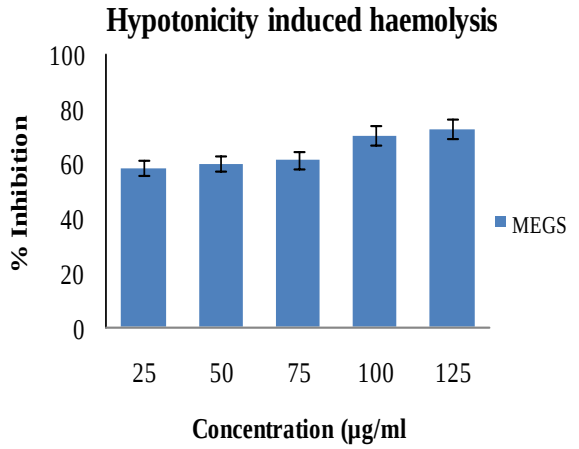

Fig. 8: Inhibition of hypotonicity induced haemolytic activity of MEGS.

Proteinase Inhibitory Activity

Neutrophils are major white blood cells present during inflammatory response. Neutrophils carry lysosomes which are rich in proteinases. These proteinases when released during inflammatory reactions can cause tissue damage. Proteinase inhibitors can provide significant level of protection against proteinases during inflammatory reactions.[27,28] MEGH and MEGS exhibited significant antiproteinase activity 67.91±0.08% and 52.81±0.12% respectively at 1mg/ml. Standard Dichlofenac sodium (100 µg/ml) showed maximum inhibition of 91.27±0.09% (Fig.9&10).

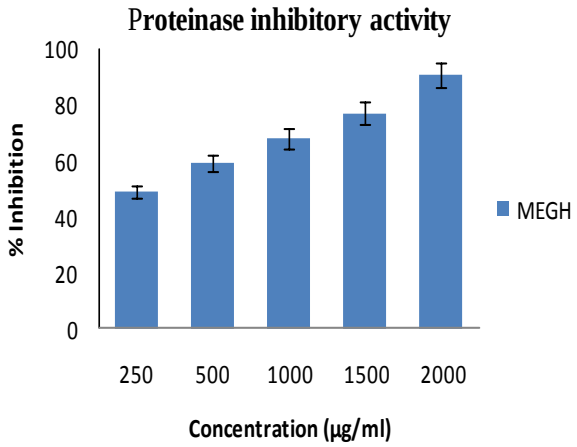

Fig. 9: Proteinase inhibitory activity of MEGH.

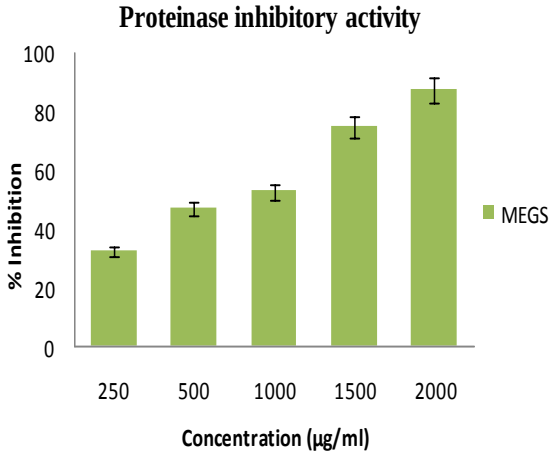

Fig. 10: Proteinase inhibitory activity of MEGS.

CONCLUSION

In the present investigation, the results have shown that the methanol leaf extracts of *G.heterotricha* Mast. and *G.serrulata* DC. have active phytoconstituents and possess significant antioxidant and anti-inflammatory properties. In the present study it was found that the methanol leaf extract of these plants contain significant amount of phenolics, tannins and flavonoids and the presence of these bioactive compounds may be responsible for remarkable antioxidant and anti-inflammatory activity. These components in the extracts serve as free radical inhibitors or scavengers and inhibited the albumin denaturation, proteinase activity and stabilized the membrane of Red Blood Cells. Thus it can be concluded that the methanol leaf extracts of *G.heterotricha* Mast. and *G.serrulata* DC. can be used as potential sources in the development of new antioxidant and anti-inflammatory drugs to treat various diseases such as neurological disorder, inflammation, cancer and aging.

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