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Investigation of Possible Mechanism Behind *In-Vitro* Anti-Inflammatory of *Benincasa* Seed Extracts

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ABSTRACT

Inflammation cascades can lead to the development of diseases such as chronic asthma, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, and psoriasis. The currently available drugs have several adverse effects and are expensive to be used. Reported phytochemical work on *Benincasa hispida* seeds indicated the presence of steroid, flavonoid, and terpenoid. In the present work extracts of *Benincasa hispida* seeds were assessed for their *in-vitro* anti-inflammatory activity at different concentrations by various models. *In-vitro* anti-inflammatory activity was estimated using proteinase inhibitory activity, albumin denaturation assay, membrane stabilization, and antilipoxygenase activity. Aspirin was employed as a standard drug. The ethanolic extracts of the seeds exhibited notable anti-inflammatory activity at a concentration of 50 µg/ml as compared with the petroleum ether extract. Hence it can be proposed that the anti-inflammatory efficacy of *B. hispida*can be a good prospect for inflammatory drug development.

INTRODUCTION:

Inflammation is a normal protective response to tissue injury that is caused by physical trauma, noxious chemicals, or microbiological agents. It is characterized by redness, swollen joints, joint pain, stiffness, and loss of joint function. Inflammation cascades can lead to the development of diseases such as chronic asthma, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, and psoriasis. Many of these diseases are debilitating and are becoming increasingly common in our aging society. Rheumatoid arthritis and osteoarthritis are the major inflammatory diseases affecting people worldwide. Several classes of drugs, such as corticosteroids, NSAIDs, and biologics, are used to treat inflammatory disorders. All these drugs possess quite a lot of adverse effects and are expensive to be used[1-2].

The mechanism of inflammation injury is attributed, in part, to the release of reactive oxygen species from activated neutrophils and macrophages. This overproduction leads to tissue injury by damaging the macromolecule and lipid peroxidation of membranes. In addition, ROS propagate inflammation by stimulating the release of the cytokines such as interleukine-I, tumor necrosis factor-α, and interferon-γ, which stimulate the recruitment of additional neutrophils and macrophages. Thus free radicals are important mediators that provoke or sustain inflammatory processes and consequently, their neutralization by antioxidants and radical scavengers can attenuate inflammation[3-5].

Various methods were employed to screen and study drugs, chemicals, herbal preparations that exhibit anti-inflammatory properties or potentials. Drugs from natural origin have limited side effects are desirable to substitute chemical therapeutics[6]. Qualitative analysis of whole plant extract of *Benincasa hispida* revealed the presence of alkaloid, saponins, steroid, flavonoid, glycoside, phenol, tannin, and terpenoid. Hence, in the present work extracts of *Benincasa hispida* seeds were assessed for their *in-vitro* anti-inflammatory activity at different concentrations by various models.

MATERIALS AND METHODS

Collection of plant materials

Plant specimen for the present study was collected from a medicinal plant vendor. Care was taken to select healthy plant materials. The fruit was peeled off and seeds were removed. Seeds were separated, raised using tap water, and dried in an oven at 38^0C for 24 h. The

prepared seeds were stored in a dark place at ambient temperature. The seed powder was obtained using a grinder mill.

Extraction

The 100 g of powdered material was extracted with petroleum ether and ethanol using the Soxhlet apparatus. The extract was stored in a glass bottle in refrigerated condition throughout the experiment.

Chemicals and Instruments

Drugs used in the present study include potassium chloride, bovine serum albumin (BSA), sodium hydroxide, dextrose, dimethylformamide, ethanol, potassium dihydrogen phosphate, sodium chloride, hydrochloric acid, disodium hydrogen phosphate, and sodium citrate. The instruments used were a digital photoactometer, pH meter, and ultraviolet (UV) spectrophotometer.

Investigation of *in-vitro* anti-inflammatory activity

1. Inhibition of albumin denaturation

The anti-inflammatory activity of Benincasa hispida seed extract was performed by using the inhibition of albumin denaturation method according to Sakat*et al.*[7]slight modifications. The reaction mixture consisted of test extracts and a 1% aqueous solution of bovine albumin fraction. The pH of the reaction mixture was adjusted using 1N HCl. The sample extracts were kept at 37^0C for 20 min and then it is heated to 50^0C for 20 min. After cooling the samples the turbidity was recorded at 660nm using UV Visible Spectrophotometer (Model 371, Elico India Ltd). The experiment was performed in triplicate.

The percentage inhibition of protein denaturation was calculated by the following formula:

Percentage inhibition = (Abs Control –Abs Sample) X 100/ Abs control

2. Anti-proteinase assay

The assay was performed according to the modified method described by Oyedepo and Femurewa,[8]and Sakat*et al.*[7] The reaction mixture (2 ml) contained 1 ml 20 mM Tris HCl buffer (pH 7.4), 0.06 mg trypsin, and 1 ml test sample of different concentrations (10 -50 µg/ml). The mixture was kept warm at 37^0C for 5 min. To this 1 ml of 0.8% (w/v) casein was

added. The mixture was kept warm for an extra 20 min. 2 ml of 70% perchloric acid was added to it to arrest the reaction. Then the cloudy suspension was centrifuged and the absorbance of the supernatant was recorded at 210 nm against buffer as blank. The experiment was repeated thrice. The percentage inhibition of proteinase inhibitory activity was calculated by the following formula:

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

3. Membrane Stabilization Method

Preparation of Red Blood cells (RBCs) suspension[7,9]

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

Heat-induced haemolysis[7,10]

The reaction mixture (2ml) consisted of a 1 ml test sample of various concentrations (10 -50 µg/ml) and 1 ml of 10% RBCs suspension. Aspirin was used as a standard drug. The centrifuge tubes containing the reaction mixture were incubated on a water bath at 56^0C for 30min. At the end of the incubation period, the tubes were cooled under running tap water. The absorbance of the supernatants was recorded at 560 nm. The experiment was repeated thrice. The percentage inhibition of Haemolysis activity was calculated by the following formula:

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

Hypotonicity-induced haemolysis[11]

Different reference samples, different concentrations of extract (10-50µg/ml), and control were individually mixed with 2ml of hyposaline, 1ml of phosphate buffer, and 0.5ml of HRBC suspension. Diclofenac sodium (100µg/ml) was used as a standard drug. All the assay mixtures were kept warm at 37^0C for 30 minutes and centrifuged at 3000rpm. The supernatant liquid was poured and the hemoglobin content was recorded by a

spectrophotometer at 560nm. The percentage of hemolysis was calculated approximately by assuming the hemolysis produced in the control as 100%.

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

4. Anti-lipoxygenase activity[10]

Anti-Lipoxygenase activity was performed by using linoleic acid as substrate and lipoxidase as the enzyme. Test samples were dissolved in 0.25ml of 2M borate buffer pH 9.0 and additional 0.25ml of lipoxidase enzyme solution (20,000U/ml) is added and kept warm for 5 min at 25^0C. After which, 1ml of linoleic acid solution (0.6mM) was added, mixed well and absorbance was measured at 234nm. A dose-response curve was plotted to establish the IC50 values.

The percent inhibition was calculated by using the following formula,

% inhibition= [{Abs control-Abs sample}/Abs control] x 100

Statistical analysis

Results are represented as Mean ± SD. The difference between samples was compared by One Way Analysis Of Variance (ANOVA). This is followed by the Dunnet Multiple comparison tests (control Vs test) using Graph Pad Instat.

RESULTS AND DISCUSSION

It is supposed that currently available drugs such as opoids and non –steroidal anti-inflammatory drugs (NSAIDs) are having limited use in inflammatory disorders, owing to their side effects and potency[12]. Herbal drugs have a wide variety of phytochemicals from which new anti-inflammatory agents can be exposed. During the past two centuries Research on pharmacological actions of plants has yielded compounds for the development of modern medicines[13].

It has been reported that prostaglandins and leukotriene inhibition lead to anti-inflammatory reaction[14]. The inhibition of COX may cause gastric side effects due to the possible mucosal damage; thus inhibition of COX along with 5-LOX is recommended to achieve maximum anti-inflammatory activity with gastric safety. The results of the study suggested that *B. hispida*seed extract may be used as an anti-inflammatory agent with additional gastric safety.

Inhibition of albumin denaturation

Denaturation of proteins is a well-documented cause of inflammation. Anti-inflammatory drugs such as Phenylbutazone, salicylic acid, etc, have shown dose-dependent ability to thermally induced protein-protein denaturation[15]. To investigate the mechanism of the anti-inflammation activity, the ability of the extract to inhibit protein denaturation was evaluated. It was effective in inhibiting heat-induced albumin denaturation at different concentrations as shown in (Table 1). Maximum inhibition of 78.40% was observed at the concentration of 50 µg/ml of ethanol extract (Fig 1). Aspirin showed the maximum inhibition, 84.58% at the concentration of 50µg/ml.

Table No 1: Effect of different extracts of *Benincasa hispida* seeds on heat-induced protein denaturation

Conc (µg/ml)	Percentage Inhibition		
	Ethanol Extract (EEBH)	Pet ether extract (PEEBH)	Std (Aspirin)
10	32.48	19.27	42.6
20	48.62	32.11	54.65
30	55.05	41.28	58.6
40	65.14	53.21	70.00
50	78.40	66.06	84.58
IC50 Values	**29.04**	**36.66**	**26.70**

Each value represents the mean ± SD. N=3, Experimental group were compared with control.

**p<0.01, considered extremely significant.

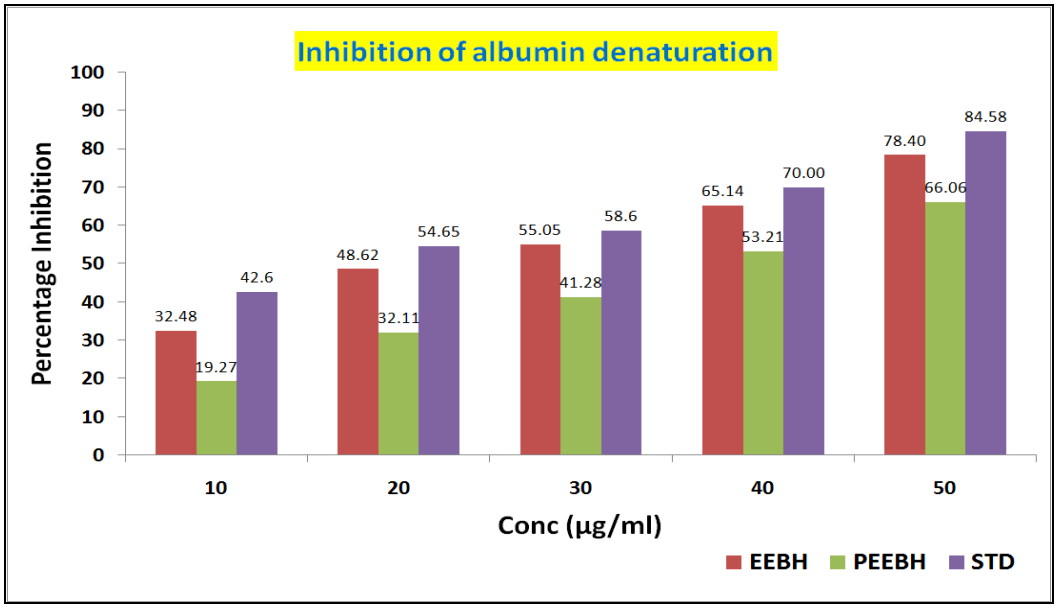

Figure No 1. Effect of different extracts of *Benincasa hispida* seeds on heat-induced protein denaturation

Anti-Proteinase assay

Neutrophils are known to be a rich source of proteinase. It was previously reported that leukocytes proteinase play an important role in the development of tissue damage during inflammatory reactions and a significant level of proteinase was provided by proteinase inhibitors[16-17].*B. hispida* ethanolic extract exhibited significant antiproteinase activity at different concentrations(Fig 2). Ethanol extract showed maximum inhibition of 70.80% at 50µg/ml. Aspirin showed the maximum inhibition of 74.67 % at 50µg/ml (Table 2).

Table No 2: Effect of different extracts of *Benincasa hispida* seeds on Anti-Proteinase assay

Conc (µg/ml)	Percentage Inhibition		
	Ethanol Extract (EEBH)	Pet ether extract (PEEBH)	Std
10	23.01	14.68	38.4
20	31.86	29.36	46.75
30	45.13	43.12	58.6
40	65.49	55.05	68.62
50	70.80	68.81	74.67
IC50 Values	**32.83**	**35.87**	**28.79**

Each value represents the mean ± SD. N=3, Experimental group were compared with control.

**p<0.01, considered extremely significant.

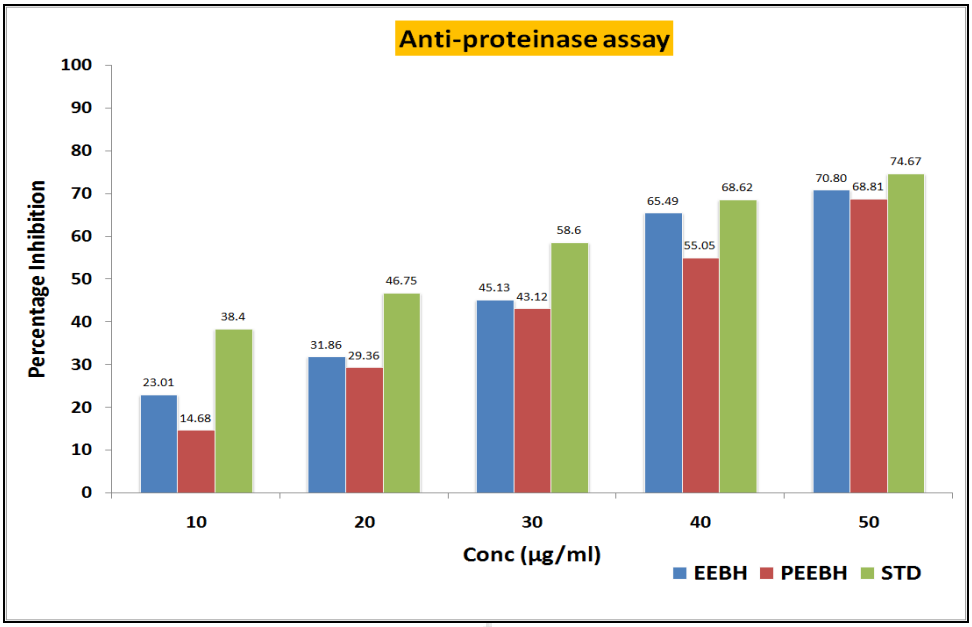

Figure No 2. Effect of different extracts of *Benincasa hispida* seeds on Anti-Proteinase assay

Membrane stabilization test

Stabilization of the RBCs membrane was studied to further establish the mechanism of anti-inflammatory action of *B. hispida*. The extract was effective in inhibiting heat-induced hemolysis at different concentrations. These provide evidence for membrane stabilization as an additional mechanism of their anti-inflammatory effect. Although the precise mechanism of this membrane stabilization is needed to be elucidated, it is possible that the *B. hispida* produced this effect due to a surface area/volume ratio of the cells, which could be brought about by an expansion of membrane or the shrinkage of the cells and interaction with membrane proteins[18].

Heat-induced hemolysis

The HRBC membrane is similar to the lysosomal membrane the study was performed to verify the stability of the HRBC membrane by ethanol extract of *B. hispida*. The extract was effective in inhibiting heat-induced hemolysis at different concentrations. The results showed that ethanol extract of *B. hispida* seeds at concentration 50 µg/ml protects significantly (p

<0.01) the erythrocyte membrane against lysis induced by heat (Table 3). Aspirin showed significant (p <0.01) protection against the damaging effect of heat solution.

Hypotonicity induced hemolysis

Human red blood cells (HRBC) membranes are similar to lysosomal membrane competent[20]. Hence the inhibition of hypotonicity red blood cells membrane lysis was taken as a measure of the mechanism of the anti- inflammatory activity of *B. hispida* extract. The hemolytic effect results in the rupturing of its membrane leading to free radical-induced lipid peroxidation[19]. Membrane stabilization leads to the prevention of the release of serum protein and fluids into the tissues during a period of increased permeability caused by inflammatory medication[21]. Ethanol extract *B. hispida* whole plant at a concentration of 10-50 mg/ml protected the human erythrocyte membrane against lysis induced by hypotonic solution (Table 4). Ethanol extract of *B. hispida* possibly stabilized the red blood cell membrane by preventing the release of lytic enzymes and active mediators of inflammation.

Table No 3: Effect of different extracts of *Benincasa hispida* seeds on Heat-induced hemolysis of erythrocyte

Conc (µg/ml)	Percentage Inhibition		
	Ethanol Extract (EEBH)	Pet ether extract (PEEBH)	Std
10	31.86	20.18	40.65
20	40.71	24.77	46.75
30	54.87	45.87	62.34
40	67.26	55.05	70.75
50	75.22	66.97	78.64
IC50 Values	**29.80**	**36.08**	**27.58**

Each value represents the mean ± SD. N=3, Experimental group were compared with control.

**p<0.01, considered extremely significant.

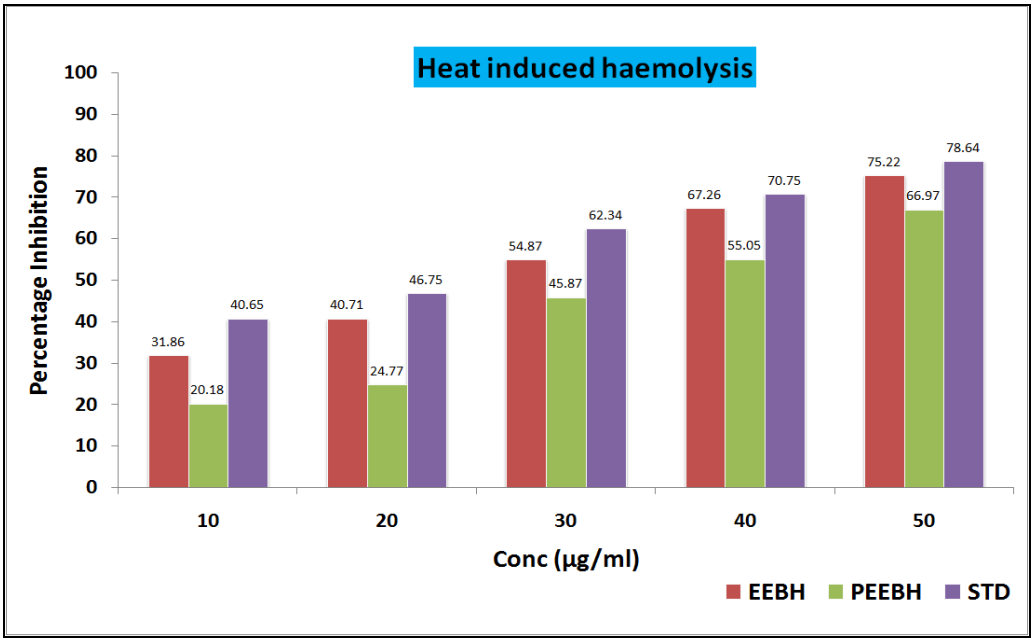

Figure No 3. Effect of different extracts of *Benincasa hispida* seeds on Heat-induced hemolysis of erythrocyte

Table No 4: Effect of different extracts of *Benincasa hispida* seeds on hypotonicity induced hemolysis of erythrocyte

Conc (µg/ml)	Percentage Inhibition		
	Ethanol Extract (EEBH)	Pet ether extract (PEEBH)	Std
10	14.42	10.58	36.48
20	25.96	23.08	41.3
30	43.27	41.35	56.32
40	51.92	50.00	62.34
50	67.31	63.46	70.34
IC50 Values	37.15	39.40	30.94

Each value represents the mean ± SD. N=3, Experimental group were compared with control.

**p<0.01, considered extremely significant.

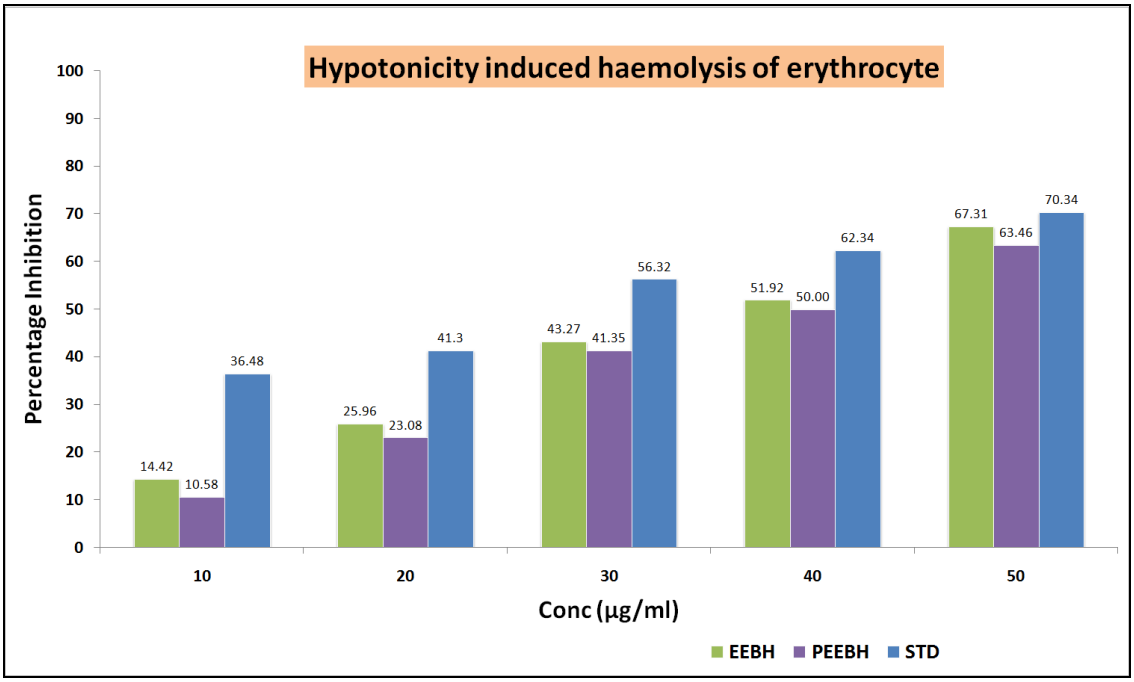

Figure No 4. Effect of different extracts of *Benincasa hispida* seeds on hypotonicity induced hemolysis of erythrocyte

Antilipoxygenase activity

Lipoxygenase (LOXs) are the family of the key enzyme in the biosynthesis of leukotrienes which plays an important role in the pathogenicity of reveal inflammatory diseases. Lipoxygenase is receptive to antioxidants and most of their action may include inhibition of lipid hydroperoxide formation due to scavenging of lipidoxy or lipidperoxy-radicals formed increase of enzyme peroxidation. This can limit the availability of lipid hydroperoxide substrate necessary for the catalytic cycle of LOX[22]. Ethanol extract of *B. hispida seeds* has been checked at 10-50µg/ml. From these results, the strongest inhibition was obtained for ethanol extract at concentration 50 µg/ml. The standard aspirin showed a 78.64% inhibition (Table 5).

Table No 5: Effect of different extracts of *Benincasa hispida* seeds on lipoxygenase inhibitory action

Conc (µg/ml)	Percentage Inhibition		
	Ethanol Extract (EEBH)	Pet ether extract (PEEBH)	Std
10	36.89	20.18	40.65
20	45.08	33.94	46.75
30	54.10	52.29	62.34
40	64.44	59.63	72.84
50	73.77	70.64	78.64
IC50 Values	**30.03**	**32.87**	**27.35**

Each value represents the mean ± SD. N=3, Experimental group were compared with control.

**p<0.01, considered extremely significant.

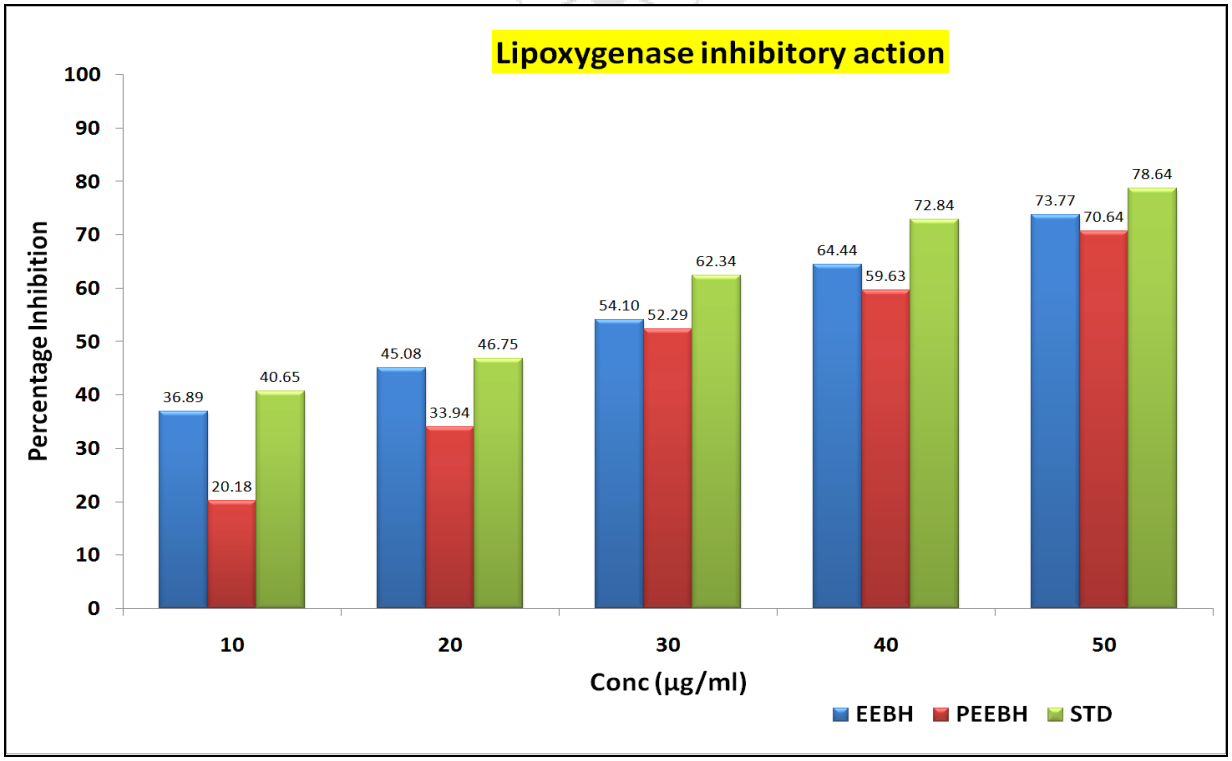

Figure No 5. Effect of different extracts of *Benincasa hispida* seeds on lipoxygenase inhibitory action

CONCLUSION

The results obtained from the present study on an ethanol extract of *B. hispida*seeds possess potential anti-inflammatory activity. The indicated effect might be due to the presence of phenolic compounds, alkaloids, tannins, and steroids. The results indicated that the phytoconstituents from *B. hispida*seeds can be used as a lead compound for designing a potent anti-inflammatory drug. The possible mechanism of action of anti-inflammatory activity of *B. hispida*can is extended from the results of the reported phytochemical screening.

CONFLICT OF INTEREST: Nil

ACKNOWLEDGMENT: Nil

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