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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 presence of steroid, flavonoid and terpenoid. In the present work extracts of *Benincasa hispida* seeds were assessed for its *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 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 inflammatory drug development.

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INTRODUCTION:

Collection of plant materials

Plant specimen for the present study was collected from 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 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 grinder mill.

Extraction

The 100 g of powdered material was extracted with petroleum ether and ethanol using soxhlet apparatus. The extract was stored in a glass bottle in refrigerated condition throughout the period of 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. Instruments used were 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 inhibition of albumin denaturation method according to Sakat *et al*.[7] slight modifications. The reaction mixture consisted of test extracts and 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 660 nm using UV Visible Spectrophotometer (Model 371, Elico India Ltd). The experiment was performed in triplicate.

The Percentage inhibition of protein denaturation was calculated by 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 in order 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 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 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. At 3000 rpm for 10min the tubes were centrifuged and were washed three times with equal volume of normal saline. The volume of blood was determined and re constituted as 10% v/v suspension with normal saline.

Heat induced haemolysis[7, 10]

The reaction mixture (2 ml) consisted of 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 reaction mixture were incubated on water bath at 56^0C for 30 min. 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 following formula:

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

Hypotonicity-induced haemolysis[11]

Different reference sample, different concentration of extract (10-50 µg/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 3000 rpm. The supernatant liquid was poured and the haemoglobin content was recorded by a spectrophotometer at 560 nm. The percentage haemolysis was calculated approximately by assuming the haemolysis 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 enzyme. Test samples were dissolved in 0.25 ml of 2M borate buffer pH 9.0 and additional 0.25ml of lipoxidase enzyme solution (20,000 U/ml) is added and kept warm for 5 min at 25^0C. After which, 1 ml of linoleic acid solution (0.6 mM) 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 Dunnet Multiple comparison test (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 the 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 the prostaglandins and leukotriene inhibition leads 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 recommended to achieve maximum anti-inflammatory activity with gastric safety. The results of study suggested that *B. hispida* seed extract may be used as 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]. In order to investigate the mechanism of the anti-inflammation activity, the ability of 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). Asipirin showed the maximum inhibition, 84.58% at the concentration of 50 µg/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.

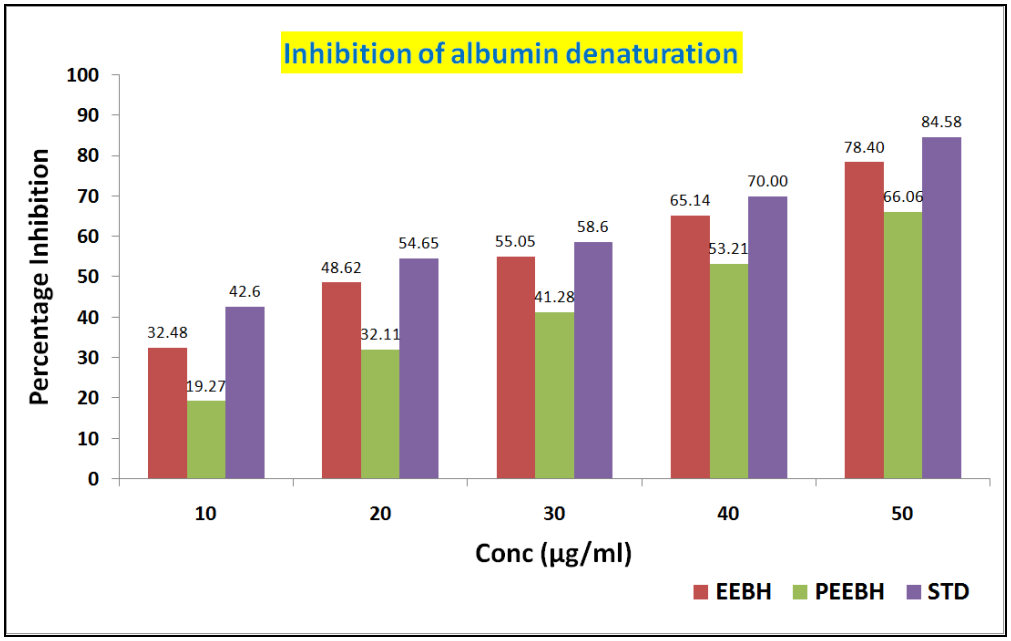

Fig. No. 1: Effect of different extracts of *Benincasa hispida* seeds on heat induced protein denaturation

Anti-Proteinase assay

Neutrophils are known to be a rich source of proteinase. It was previously reported that leukocytes proteinease play an important role in the development of tissue damage during inflammatory reactions and 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 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.

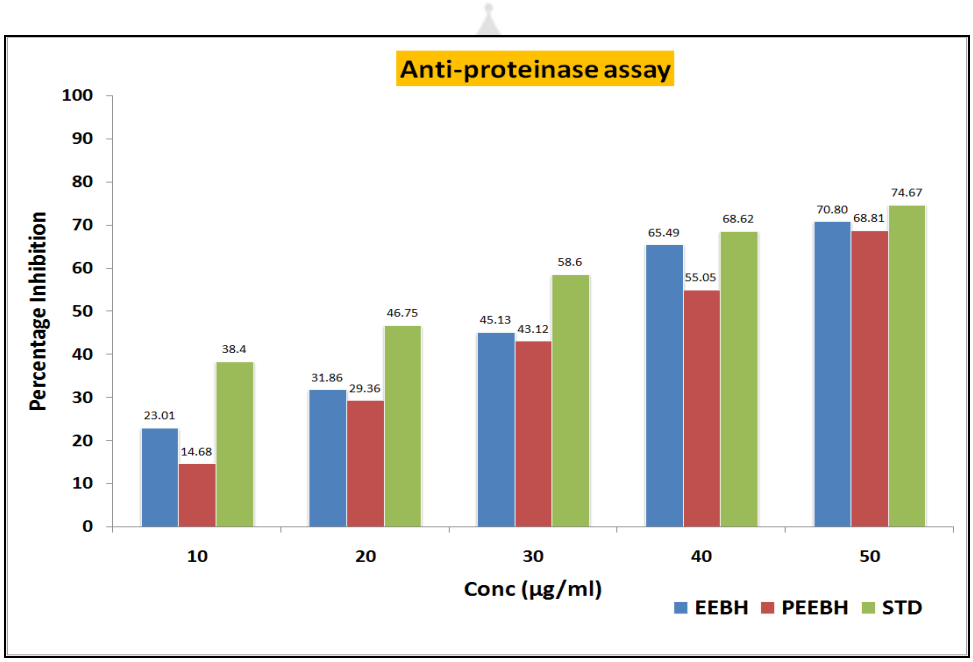

Fig. No. 2: Effect of different extracts of *Benincasa hispida* seeds on Anti-Proteinase assay

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 the 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 surface area/volume ratio of the cells, which could be brought about by an expansion of membrane or the shrinkage of the cells and an interaction with membrane proteins[18].

Heat induced haemolysis

The HRBC membrane is similar to lysosomal membrane the study was performed to verify the stability of HRBC membrane by ethanol extract of *B. hispida*. The extract was effective in inhibitory the heat induced haemolysis at different concentrations. The results showed that ethanol extract of *B. hispida* seeds at concentration 50 µg/ml protect significantly ($p < 0.01$) the erythrocyte membrane against lysis induced by heat (Table 3). Aspirin showed significant ($p < 0.01$) protection against damaging effect of heat solution.

Hypotonicity induced haemolysis

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 anti- inflammatory activity of *B. hispida* extract. The haemolytic effect results in the rupturing of its membrane leading to free radical induced lipid peroxidation[19]. Membrane stabilization leads to the prevention of 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 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 haemolysis 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.

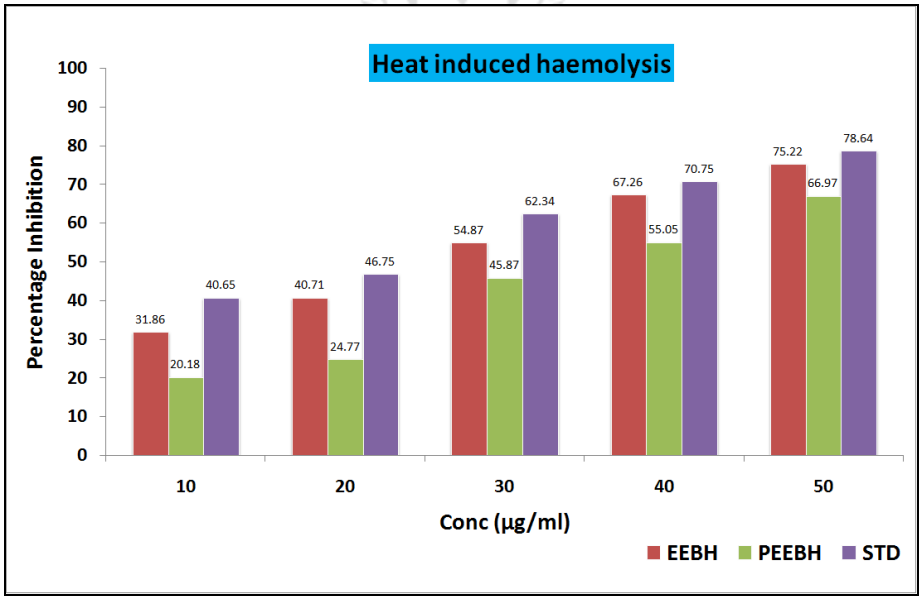

Fig. No. 3: Effect of different extracts of *Benincasa hispida* seeds on Heat induced haemolysis of erythrocyte

Table No. 4: Effect of different extracts of *Benincasa hispida* seeds on hypotonicity induced haemolysis 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.

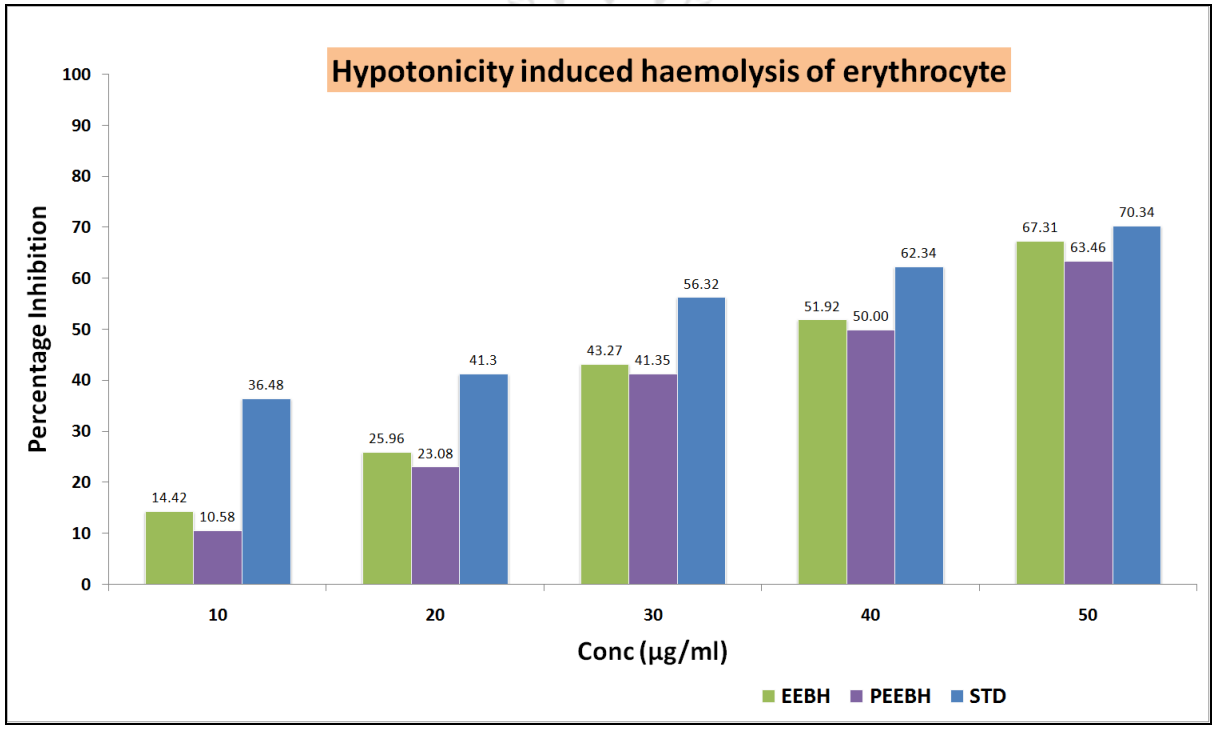

Fig. No. 4: Effect of different extracts of *Benincasa hispida* seeds on hypotonicity induced haemolysis of erythrocyte

Antilipoxygenase activity

Lipoxygenase (LOXs) are the family of the key enzyme in the biosynthesis of leukotrienes which plays an important role in the pathogenecity of reveal inflammatory diseases. Lipoxygenase are receptive to antioxidants and the 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 10-50 µg/ml. From these results, the strongest inhibition was obtained for ethanol extract at concentration 50 µg/ml. The standard aspirin showed an 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.

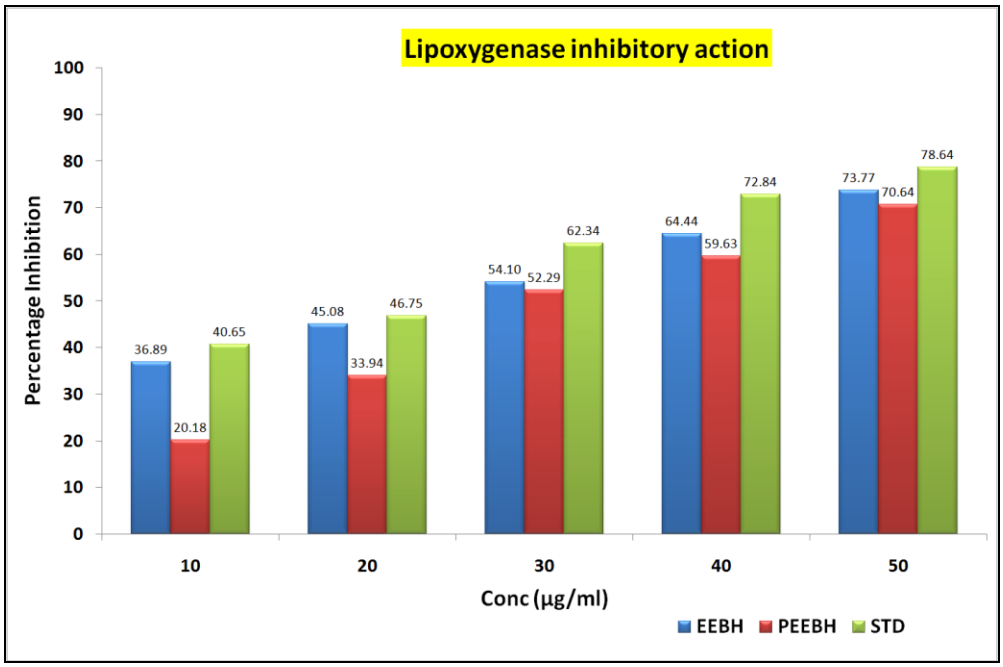

Fig. No. 5: Effect of different extracts of *Benincasa hispida* seeds on lipoxygenase inhibitory action

CONCLUSION

The results obtained from the present study on 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 lead compound for designing a potent anti-inflammatory drug. The possible mechanism of action of anti-inflammatory activity of *B. hispida* can be extended from the results of reported phytochemical screening.

REFERENCES

1. Schimmer B P, Parker K L. Adrenocorticotropic hormone; adrenocortical steroids and their synthetic analogs; Inhibitors of the synthesis and actions of adrenocortical hormones. In: Hardman J G, Limbird L E, Gilman A G, editors. Goodman and Gilman's The pharmacological basis of therapeutics. 10th Edition. USA: McGraw; 2001. pp 1649–1677.
2. Mary J S, Chithra B and Sivajiganesan S. *In-vitro* Anti-Inflammatory activity of the flowers of *Nerium Oleander* (White). Int J Res – Granthaalayah. 2017; 5(6): 123-128.
3. Indian Medicinal Plants, A compendium of 500 species, Vol-1, by Vaidyaratnam P S Varier's, Arya Vaidya Sala, Kottakkal, Coll. No. AVS 1481.
4. Nadkarni K M. Indian Materia Medica, Vol-1, Edited by D R. K. R. Nadkarni, P. NO.114

5. Chatterjee A. The Treatise of Indian Medicinal Plants, National Institute of Science and Communication CSIR 1997, New Delhi. Vol.IV:212-217.
6. Koleva II, Van Beek T A, Linssen J P, de Groot A, Evstapieva L N. Phytochem Anal 2002; 13; 8-17.
7. Sakat S, Juvekar A R, Gambhire M N. *In vitro* antioxidant and anti-inflammatory activity of methanol extract of *Oxalis corniculata* Linn. Int J Pharma and Pharmacol Sci. 2010; 2(1): 146-155.
8. Oyedepo O O, Femurewa A J. Anti-protease and membrane stabilizing activities of extracts of *Fagraz anthoxiloides, Olax subscorpioidesc* and *Tetrapleura tetraptera*. Int J of Pharmacong. 1995; 33: 65-69.
9. Sadique J, Al-Rqobahs WA, Bughaith, EIGindi Ar. The bioactivity of certain medicinal plants on the stabilization of RBS membrane system. Fitoterapia. 1989; 60:525-532.
10. Shinde U A, Kulkarni *et al.* Mast cell stabilizing and lipoxygenase inhibitory activity of *Cedrus deodara* (Roxb.) Loud. Wood Oil. Indian J Exp Biol. 1999; 37(3): 258-261.
11. Azeem A K, Dilip C, Prasanth S S, Junise V, Hanan Shahima. Anti-inflammatory activity of the glandular extracts of *Thunnus alalunga*. Asia Pac J Med. 2010; 3(10): 412-420.
12. Ahmadiani A, Fereidoni M, Semnanian S, Kamalinejad M and Saremi S. Antinociceptive and anti-inflammatory effects of *Sambucus ebulus* rhizome extract in rats. J Ethnopharmacol. 1998; 61(2): 229-232.
13. Arivazhagan S, Balasenthi S and Nagini S. Antioxidant and anti-inflammatory activities of *Mallotus oppostifolium*. J Phytotherapy Re. 2000; 14 (4) 291-293.
14. Sarkar B K, Kumar R, *et al.* Evaluation of *In vitro* Anti-Inflammatory Activity and HPTLC Analysis of Plant *Phyllanthus Fraternus.* Int J Curr Pharm Res. 2017*;* 9 (5), 198-200.
15. Mizushima Y and Kobayashi M. Interaction of anti-inflammatory drugs with serum proteins, especially with some biologically active proteins. J of Pharma Pharmacol. 1968; 20: 169-173.
16. Das S N, Chatterjee S. Long term toxicity study of ART-400. Indian Indg Med. 1995; 16 (2): 117-123.
17. Halliwell B, Whiteman M. Measuring reactive species and oxidative damage *in-vivo* and in cell culture; how should you do it and what do the results mean? Br. J. Pharmacol. 2004: 142: 231-225.
18. Chou C T. The anti-inflammatory effect of *Tripterygium wifordii* Hook F on adjuvant induced paws edema in rats and inflammatory mediators' release. Phytother Res. 1997; 11: 152-154.
19. Das S N and Chatterjee S. Long term toxicity study of ART-400. Indian Indg Med. 1995; 16(2): 117-123.
20. Mounnissamy V M, Kavimani S, Balu V, Drlin Q S. Evaluation of anti-inflammatory and membrane stabilizing properties of ethanol extract of *Canjerarehedi*. Iranian J. Pharmacol Therap. 2008; 6: 235-237
21. Chaitanya RSNAKK, Sandhya S, David B, Vinod KR, Murali S. HRBC Membrane Stabilizing Property of Root, Stem and Leaf of *Glochidion velutinum*. Int. J. Res. Pharmaceu. Biomed. Sci. 2011: 2: 256-259.
22. Kumaraswamy MV, Satish S. Antioxidant and Anti-Lipoxygenase Activity of *Thespesia lampas.* Adv. Biol. Res. 2008: 2: 56-59.