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A PHARMACOLOGICAL EVALUATION FOR THE ETHANOLIC EXTRACT OF KAEMPFERIA ROTUNDA RHIZOME FOR IT'S ANTI - ASTHMATIC, ANTIOXIDANT AND ANTI - INFLAMMATORY ACTIVITIES

Mrs. Rubeeya Lodhi*, Mr. Mohd Mohiuddin, Sana Begum, Summaiya Afreen, Fasiha Siddiqui, Gulam Mohammed Khan

Deccan school of pharmacy,Dar-us-salam,Aghapura, Hyderabad.Telangana.

Abstract:

The study includes the phytochemical and pharmacological investigation of the ethanolic extract of Kaempferia rotunda rhizomes. The powdered rhizomes were extracted by means of soxhlet using the solvent ethanol. Preliminary phytochemical screening revealed the presence of carbohydrate, cardiac glycoside, protein, alkaloids, steroids, flavonoids, tannins and phenolic compounds. These constituents may be represented the presence for biological activities of the plant. The acute toxicity study revealed that there is no mortality with the ethanolic extract of Kaempferia rotunda rhizomes up to the dose level of 2000 mg/kg. Histamine contracts the tracheal-bronchial muscle of guinea pig, goat, horse, dog and man. Guinea pig trachea is used for the screening of anti-asthmatic activity. The H_1 receptor after stimulation produces well-ordered dose related contraction of isolated guinea pig trachea. In current study Kaempferia rotunda significantly inhibited histamine induced contraction of isolated guinea pig trachea preparation indicating itsH_1 receptor antagonist activity and supports anti asthmatic property of the plant. The antioxidant property was studied by hydrogen peroxide scavenging assay and reducing power assay. Hydrogen peroxide scavenging ability of ethanolic extract of Kaempferia rotunda rhizomes revealed that the extract scavenge the hydrogen peroxide. However, the hydrogen peroxide scavenging ability was low comparing to standard (ascorbic acid). Reducing power of ethanolic extract of Kaempferia rotunda rhizomes significantly increased with increasing concentration. The in vitro anti-inflammatory potential of ethanolic extract of Kaempferia rotunda rhizomes had shown concentration dependent inhibition of protein denaturation, hypotonicity induced haemolysis of rabbit red blood cell membrane stabilization.

Key words: *Ethanolic Extract, Kaempferia Rotunda Rhizome, Anti - Asthmatic, Antioxidant, Anti - Inflammatory*

Corresponding author:

Mrs Rubeeya Lodhi,
Department of Pharmacology
Deccan school of pharmacy,
Dar-us-salam,Aghapura, Hyderabad.
Telangana.
Email. Id: lodhirubeeya@gmail.com

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INTRODUCTION:

Asthma is a common inflammatory disease that affects the lungs and people worldwide are affected in millions due to this obstructive lung disease [1-3]. Important advancements have been made in the drugs used for therapy on the care, and its prevalence has not decreased much in recent decades. Although the mortality rates are considerably low, it can be avoided in many cases due to routine care [4]. But it has been projected to be the leading cause of death worldwide due to the incidence of industrialization and rapid change in the weather conditions of the earth that the pollutants and asthma causing agents can travel to far off places. It would cause impairment in the quality of life physiologically.

The major symptoms of asthma include bronchial hyper-responsiveness, increased mucus production and narrowing of airways and its remodeling which are due to the infiltration of the immune cells into the lungs and the subsequent consequences causing lung inflammation. Due to this, the patients may exhibit narrowing of the airways and accumulation of the mucus causing shortness of breath, chest discomfort, wheezing, and cough [4-6].

Inflammation is essential for human life, leading to the elimination of noxious stimuli and restoration of tissue homeostasis [7]. However, if the inflammatory response is prolonged, continuous recruitment of inflammatory cells and overproduction of reactive oxygen and nitrogen species (ROS/RNS) are observed, inducing tissue damage [8,9].

High amounts of ROS/RNS (e.g., superoxide radical anion -O2•−- and nitric oxide -•NO) are produced by activated immune cells (e.g., neutrophils and macrophages) to protect the organism against, for instance, bacteria or intracellular parasites [10,11]. ROS/RNS can also activate several essential inflammatory pathways, which will result in the release of several proinflammatory cytokines (e.g., interleukin -IL-6, tumor necrosis factor -TNF-α) and chemokines (e.g., IL-8) with the ability to recruit more immune cells to the site of inflammation [12,13]. IL-6 is a particularly important mediator of the acute phase response, inducing fever, stimulating the production of neutrophils in the bone marrow, and supporting the growth and differentiation of B cells [14]. Another cytokine with an important role in the pathogenesis of inflammatory conditions is TNF-α to regulate cell growth and proliferation, the release of adhesion molecules, and the expression of inflammatory mediators [15].

In recent year there has been tremendous increase in demand for herbal drugs because of its safety, efficacy and better therapeutic results. Due to its economic pricing as compared to synthetic or allopathic drugs, which have several therapeutic compilations. *Kaempferia rotunda* is also considered to be therapeutically important in traditional system of medicine. Aim of the study to evaluate the anti-asthmatic, antioxidant and anti-inflammatory activities of ethanolic extract of *Kaempferia rotunda* rhizome.

MATERIALS:

Plant selected

In the present study, *Kaempferia rotunda* was selected because of its traditional uses. The part used was rhizomes.

Chemicals and reagents used

- Carboxy methyl cellulose (Spectrum reagents and chemicals pvt. Ltd.)
- Ascorbic acid (Spectrum reagents and chemicals pvt. Ltd.)
- Histamine (NICE chemicals pvt.Ltd.)
- Hydrogen peroxide (Spectrum reagents and chemicals pvt. Ltd.)
- Glacial acetic acid (Ozone international, Mumbai)
- Trichloro acetic acid (NICE chemicals pvt. Ltd.)
- Diclofenac sodium (Rajesh chemicals, Mumbai)

Drugs used

- Chlorpheniramine maleate (Abbott Laboratories pvt. Ltd.)
- Dexamethasone (Zydusbiogem, cadila health care Ltd.)

Animals

Swiss albino mice (25-40 gm) and Guinea pig (400-600 gm) were used to carry out the activities. The animals had free access to standard commercial diet and water. Animals were housed in cages under standard conditions i.e., 12:12 hour light or dark cycle at 25 ± 2^0 C. The experiments were carried out as per the guideline of CPCSEA, New Delhi, India.

METHODS

Collection and authentication of *Kaempferia rotunda*

The dried rhizomes of the *Kaempferia rotunda* were collected locally from Chittoor District, AP, India and authenticated by Prof. Madhav Shetty, Dept. of

botany, Taxonomist, SV University, Tirupati. A voucher was kept in the Department of Pharmacognosy for reference. The rhizomes were cleaned and shade dried and milled into coarse powder by a mechanical grinder. Preparation of plant extract. The powdered rhizomes were extracted using ethanol by soxhlet extractor. In this process the powdered drug is placed into the extractor with ethanol as solvent. After extraction the extract was concentrated by evaporation then it was kept in a refrigerator for further use.

Preliminary phytochemical screening

The ethanolic extract of *Kaempferia rotunda* rhizomes were subjected for the following chemical tests for the identification of various active constituents.

ACUTE TOXICITY STUDIES

Acute toxicity of *Kaempferia rotunda* was done as per OECD guidelines 423. The substance was administered in a single dose by gavage using specially designed mice oral tube. Animals were fasted prior to dosing with food but not water withheld overnight. Following the period of fasting, the animals were weighed and the test substance was orally at a dose of 5, 50, 300 and 2000 mg/kg. The animals are observed continuously for first three hours, four any toxic manifestations like increased motor activity, salivation, acute convulsion, coma and death. Changes in the animal behavior should be noted before and after administration for 24hours. Treated animals are to be further observed for 14 days. If the extract does not produce mortality at the highest dose, then the $1/10^{th}$ or $1/20^{th}$ of the dose was selected for experiment.

EVALUATION OF ANTI ASTHMATIC ACTIVITY

***In vivo* anti-asthmatic activity**

Histamine aerosol induced bronchoconstriction in guinea pigs

Histamine was dissolved in distilled water to prepare 0.2% w/v solution. Experimentally bronchial asthma was induced in guinea pigs by exposing histamine aerosol by a nebulizer in an aerosol chamber. The required time for appearance of preconvulsive dyspnoea produced by the histamine was noted for each animal. Each animal was placed in the histamine chamber and exposed to 0.2% histamine aerosol.The preconvulsion time (PCT), i.e. the time of aerosol exposure to the start of dyspnoea leading to the appearance of convulsion, was noted. As quickly as the preconvulsion dyspnoea (PCD) was recorded, the animals were removed from the chamber and positioned in fresh air for recover. This time for preconvulsive dyspnoea was recorded as basal value. Guinea pigs were then allowed to recover from dyspnoea for 2 days. After that, the animals were allotted to four different groups of 4-5 animals per group. Animals in group 1 served as control and received carboxy methyl cellulose. The animals of group 2 and 3 were given, by oral intubation, 100 and 200 mg/kg of the plant extract, respectively, while group 4 received the standard drug - Chlorpheniramine maleate, intraperitoneally. After receiving the drugs, all the animals were again exposed to histamine aerosol in the chamber, one hour, four hours and 24 hours, to determine pre convulsive time (PCT).

Milk induced leukocytosis and eosinophilia

Mice were divided into 4 groups with six in each group. Blood samples were collected from retro-orbital plexus. Group 1 served as control and received carboxy methyl cellulose solution, groups 2-3 received plant extract at (100-200 mg/kg) group 4 received dexamethasone at 50 mg/kg i.p. All the groups injected boiled and cooled milk (4 ml/kg, s.c.) 30 min after treatments. Total leukocyte and eosinophile count was done in each group before administration of test compound and 24 hours after milk injection. Difference in total leukocytes and eosinophile count before and after 24-hour drug administration was calculated.

***Ex vivo* anti-asthmatic activity**

Isolated guinea pig tracheal preparation

Isolated guinea pig tracheal tissue was obtained by, Animals were sacrificed by cervical dislocation and carotid bleeding. The trachea was dissected out and transferred into a dish containing Krebs solution and cut crosswise between the section of the cartilage of the trachea and continuously ventilated and maintained at 37 + 0. 5°C.The adjourned trachea was allowed to make steady for at least 15 minutes. On equilibrium, the bath was supplied with Krebs solution for every 15 minutes Dose response curve of histamine (10 µg/ml) in plane Krebs solution and in 1 mg/ml of plant extract act in Krebs solution was taken. Percentage of maximum contractile response on ordinate and concentration of histamine on abscissa was plotted to record dose response curve of histamine, in absence and presence of plantextract.

***IN VITRO* ANTIOXIDANT ACTIVITY**

Hydrogen peroxide scavenging

Hydrogen peroxide solution (20 Mm) was prepared with standard phosphate buffer (pH 7.4). Extract samples (25, 50, 100, 200 and 400 µg/ml) in distilled water were added to hydrogen peroxide solution (0.6 ml). Absorbance of hydrogen peroxide at 230 nm was determined after 10 minutes against a blank solution containing phosphate buffer without hydrogen

peroxide. Ascorbic acid was used as the reference standard.

Reducing power assay

The reducing power of the extract was determined by the method. 1 ml of the extract solution (25, 50, 100, 200 and 400 µg/ml) was mixed with 2.5 ml phosphate buffer (0.2 M, Ph 6.6) and 2.5 ml of potassium ferricyanide ($[K_2 Fe (CN)_6]$ (10g/l)), then the mixture was incubated at 50^0 C for 20 minutes. A portion (2.5ml) of trichloroacetic acid (TCA) (15%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 minutes. Finally, 2.5 ml of the supernatant solution was mixed with 2.5 ml of distilled water and 0.5ml ferric chloride ($FeCl_3$. 0.1%) and absorbance was measured at 700 nm in UV- visible spectrophotometer. The experiments were performed in triplicate. Increased absorbance of reaction mixtureindicates stronger reducing power.

***IN VITRO* ANTI-INFLAMMATORY ACTIVITY**

Protein denaturation

A solution of 0.2% of bovine serum albumin (BSA) was prepared in tris buffer saline and pH was adjusted to 6.8 using glacial acetic acid. Test drug of different concentration (25, 50, 100, 200 and 400 µg/ml) was prepared using ethanol assolvent. 50 µl of each test drug was transformed to test tubes using micropipette.5 ml of 0.2% w/v of BSA was added to the test tubes. The control consists of 5 ml of 0.2% w/v of BSA solution and 5µl alcohol. The test tubes were heated at 72^0 C for5 min and then cooled for 10 min. The absorbance of these solution was determined using UV–visible spectrophotometer at 660nm. Diclofenac sodium was used as standard and treated similarly for determination of absorbance. The rabbit red blood cell membrane stabilization method

Preparation of red blood cell suspension (RBCs suspension)

The fresh whole rabbit blood (5 ml) was collected from marginal ear vein to syringes containing sodium citrate to prevent clotting. The tubes were centrifuged at 3000 rpm for 10 minutes and were washed 3 times with equal volume of normal saline. The volume of the blood was measured and reconstituted as 10% v/v suspension with normal saline.

Membrane stabilization test by hypotonicity induced haemolysis

The reaction mixture consists of 1 ml of test sample of different concentration (25, 50, 100, 200 and 400 µg/ml) in normal saline and 0.5 ml of 10% RBC suspension, 1 ml of 0.2 M phosphate buffer, 1 ml hypo saline were incubated at 37^0C for 30 minutes and centrifuged at 3000 rpm for 20 minutes and the haemoglobin content of the supernatant solution was estimated spectrophotometrically at 560 nm. Diclofenac sodium was used as standard and a control was prepared without extract.

STATISTICAL ANALYSIS

The statistical analysis was carried out by using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. The results are expressed as Mean±S.E.M., n=6

COLLECTION & AUTHENTIFICATION OF *KAEMPFERIA ROTUNDA* RHIZOME

The rhizomes of *Kaempferia rotunda* were collected and authentified.

EXTRACTION OF PLANT MATERIAL

Kaempferia rotunda rhizomes were collected, washed and shade dried. Dried rhizomes were crinkled in to powdered form, weighed out. Extraction of coarse powder was done by soxhlet extraction with ethanol. The percentage yield of the product was found to be 17 % w/w.

PRELIMINARY PHYTOCHEMICAL SCREENING OF ETHANOLIC EXTRACT OF *KAEMPFERIA ROTUNDA* RHIZOMES (EEAC)

The phytochemical screening of the ethanolic extract of the *Kaempferia rotunda* rhizomes indicate the presence of carbohydrate, cardiac glycoside, protein, alkaloids, steroids, flavonoids, tannins and phenolic compounds.

Table No.1: Preliminary phytochemical analysis

Sl. No:	Constituents	Presence/absence
1	Carbohydrate	+
2	Proteins	+
3	Amino acids	_
4	Fats and oils	_
5	Steroids	+
6	Cardiac glycosides	+
7	Anthraquinone glycoside	_
8	Saponin glycosides	_
9	Cyanogenic glycosides	_
10	Coumarin glycosides	_
11	Flavonoids	+
12	Alkaloids	_
13	Tannins	+
14	Phenol	+

(+: presence, -: absence)

ACUTE TOXICITY STUDIES

Acute toxicity studies of ethanolic extract of *Kaempferia rotunda* rhizomes was performed according to OECD guidelines 423 using *swiss albino mice*. At the dose 2000 mg/kg, the ethanolic extract were neither produced mortality nor the sign of morbidity. Hence the dose 100 mg/kg (1/20th of 2000 mg/kg) and 200mg/kg (1/10th dose of 2000 mg/kg).

EVALUATION OF ANTI ASTHMATIC ACTIVITY

***In vivo* anti-asthmatic activity**

Histamine aerosol induced bronchoconstriction in guinea pigs

The present study deals with the screening of anti-asthmatic activity of ethanolic extract of *Kaempferia rotunda* rhizomes by histamine induced bronchoconstriction in guinea pigs. The ethanolic extract of the plant expressively extended the latent period of convulsion followed by exposing to histamine at the dose 200 mg/kg at time 4 hours as compared to standard drug. The % protection was calculated from the latent period of convulsion. The maximum % protection ofethanolic extract of the plant was calculated as 60.79% at 200 mg/kg.

The standard drug used was chlorpheniramine maleate which showed significant % protection at time 1 hour and 4 hours. The plant extract at 100 mg/kg showed 43.2% protection at time 1 hour and the 100 mg/kg plant extract also showed40.2% protection at time 24 hour and also showed 57.2% protection at time 4 hour. The control (Carboxy methyl cellulose) produced 10.9 % protection at time 1 hour and 12.3% protection at time 4 hour and 11.4% protection at time 24 hour. The plant extract at 200 mg/kg showed 48% at time 1 hour and 60.79% at time 4 hour and 44.3% at time 4 hour and 44.3% at 24 hours. The standard drug chlorpheniramine maleate possess 69.76% protection at time 1 hour and 78.3% at time 4 hour and 50.1% at time 24 hour.

Table No.2: Histamine aerosol induced bronchoconstriction in guinea pigs

Group	Latent period of convulsion			
	Before	**1 hour**	**4 hour**	**24 hour**
Control	16.3±2.23	18.36±0.183	18.63±0.186	18.4±0.12
Kaempferia rotunda Ethanolic extract(100 mg/kg)	16.71±1.31	29.65±.28	39.38±0.05*	28.2±0.23
Kaempferia rotunda Ethanolic extract(200 mg/kg)	15.71±0.77	30.5±3.08	40.36±1.04*	28.4±.35
Standard (CPM) (1 mg/kg)	18.46±0.89	60.25±0.03*	68.26±1.01**	36.5±0.55

Values are Mean± S.E.M., where n=6 in each group, P< 0.05 *, P< 0.01 ** (significant) compared with control. Statistical analysis was done by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test.

Figure No.1: Effect of ethanolic extract of *Kaempferia rotunda* rhizomes against histamine induced bronchoconstriction in guinea pigs

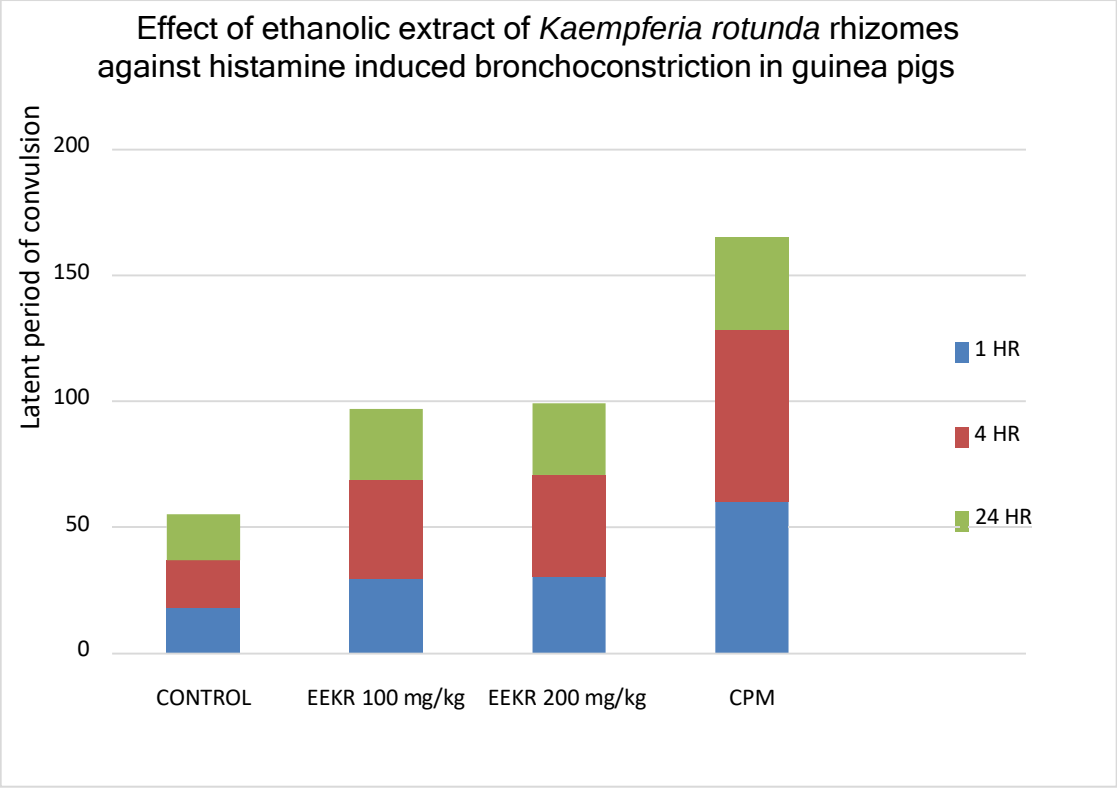
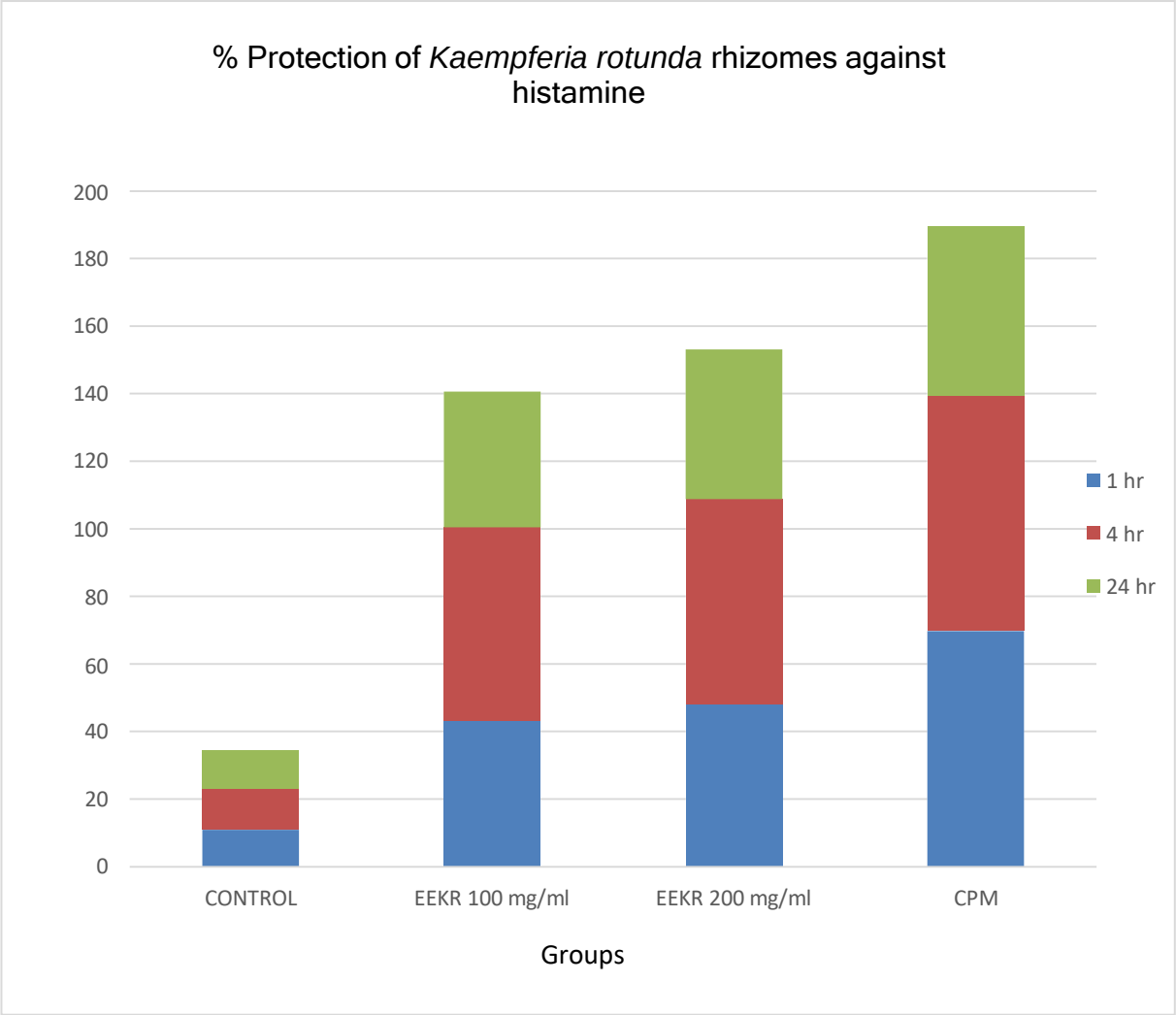


Table No.3: % Protection of the plant *Kaempferia rotunda* rhizomes against histamine induced bronchoconstriction in guinea pig

Group	%protection		
	1 hour	4 hour	24 hour
Control (carboxy methyl cellulose)	10.9	12.3	11.4
Kaempferia rotunda ethanolic extract (100 mg/kg)	43.2	57.2	40.2
Kaempferia rotunda ethanolic extract (200 mg/kg)	48	60.79	44.3
Standard(CPM)	69.76	78.3	50.1

Figure No.2: % Protection of the plant *Kaempferia rotunda* rhizomes against histamineinduced bronchoconstriction in guinea pigs



Milk induced leukocytosis and eosinophilia

Milk induced leukocytosis

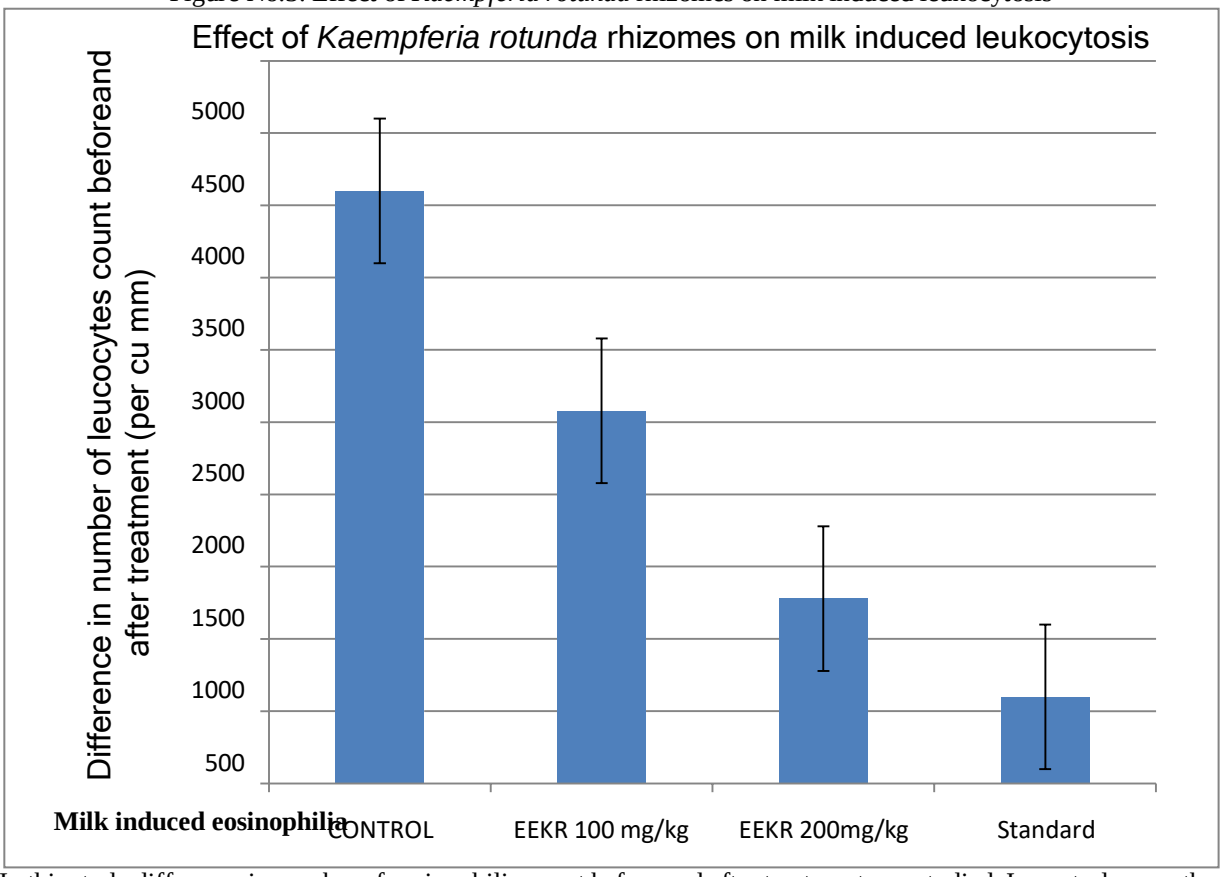
In the milk induced leukocytes the maximum increase in difference of leukocytes count was observed in control group (4100 ± 9) 24 hour after administration of milk. Groups of mice pretreated with ethanolic extract (200 mg/kg) showed significant activity. The ethanolic extract of plant *Alpinia calcarata* (200 mg/kg) showed decrease in number of leukocytes (1280 ± 12) as compared to control. The standard drug possesses significant activity (600±10) and the plantextract (100 mg/kg) showed less significant activity as compared to control.

Table No.4: Effect of ethanolic extract of *Kaempferia rotunda* rhizomes on milk induced leukocytosis

Groups	**Difference in no of leukocytesbefore and after treatment(Cu.mm)**
Control (Carboxy methyl cellulose)	4100±9
Kaempferia rotunda ethanolic extract (100 mg/kg)	2580±8*
Kaempferia rotunda ethanolic extract (200 mg/kg)	1280±12**
Standard (Dexamethasone(50 mg/kg))	600±10**

Values are Mean± S.E.M., where n=6 in each group, P< 0.05 *, P< 0.01 ** (significant) compared with control. Statistical analysis was done by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test.

Figure No.3: Effect of *Kaempferia rotunda* rhizomes on milk induced leukocytosis



Milk induced eosinophilia

In this study difference in number of eosinophilic count before and after treatment was studied. In control group there was maximum increase in difference in number of eosinophilic count (118± 1.414) was observed. The ethanolic

extract of *Kaempferia rotunda* (200 mg/kg) showed significant activity by less difference in number of eosinophilic count (53 ±1.434). The standard drug also exhibited significant activityby less difference in number of eosinophilic count (38 ±1.13). The ethanolic extract of plant (100 mg/kg) showed less significant activity.

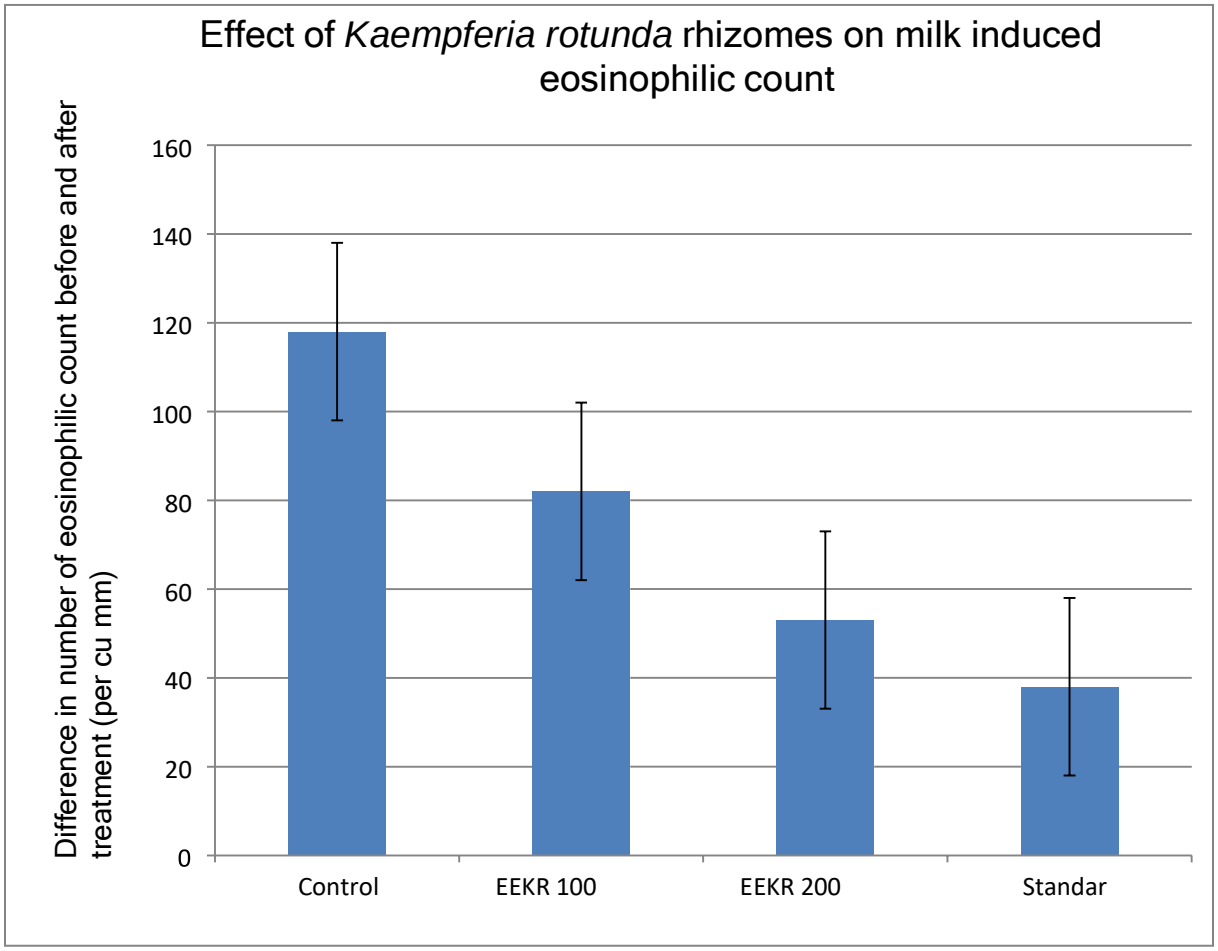
The results were expressed in the table number 7and figure number 6.

Table No.5: Effect of ethanolic extract of *Kaempferia rotunda* rhizomes on milk induced eosinophilia

Groups	Difference in no of eosinophilic countbefore and after treatment(Cu.mm)
Control (Carboxy methyl cellulose)	118±1.414
Kaempferia rotunda ethanolic extract (100 mg/kg)	82±1.2*
Kaempferia rotunda ethanolic extract (200 mg/kg)	53±1.434**
Standard (Dexamethasone (50 mg/kg))	38±1.13**

Values are Mean± SEM, where n=6 in each group, P< 0.05 *, P< 0.01 ** (significant) compared with control. Statistical analysis was done by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test.

Figure No.4: Effect of *Kaempferia rotunda* rhizomes on milk induced eosinophilic count



***Ex vivo* anti-asthmatic study**

Isolated guinea pig tracheal preparation

Histamine (10 µg/ml) produced dose dependent contraction of guinea pigtracheal preparation. Pretreatment with the ethanolic extract of *Kaempferia rotunda*rhizome (1mg/ml) significantly inhibited the contractile effect of histamine.

Concentration Response Curve of guinea pig tracheal preparation before and after administration of plant extract are shown below.

Figure No.5: Concentration Response Curve of Histamine using Guinea pig trachealpreparation (In Absence of Plant Extract).

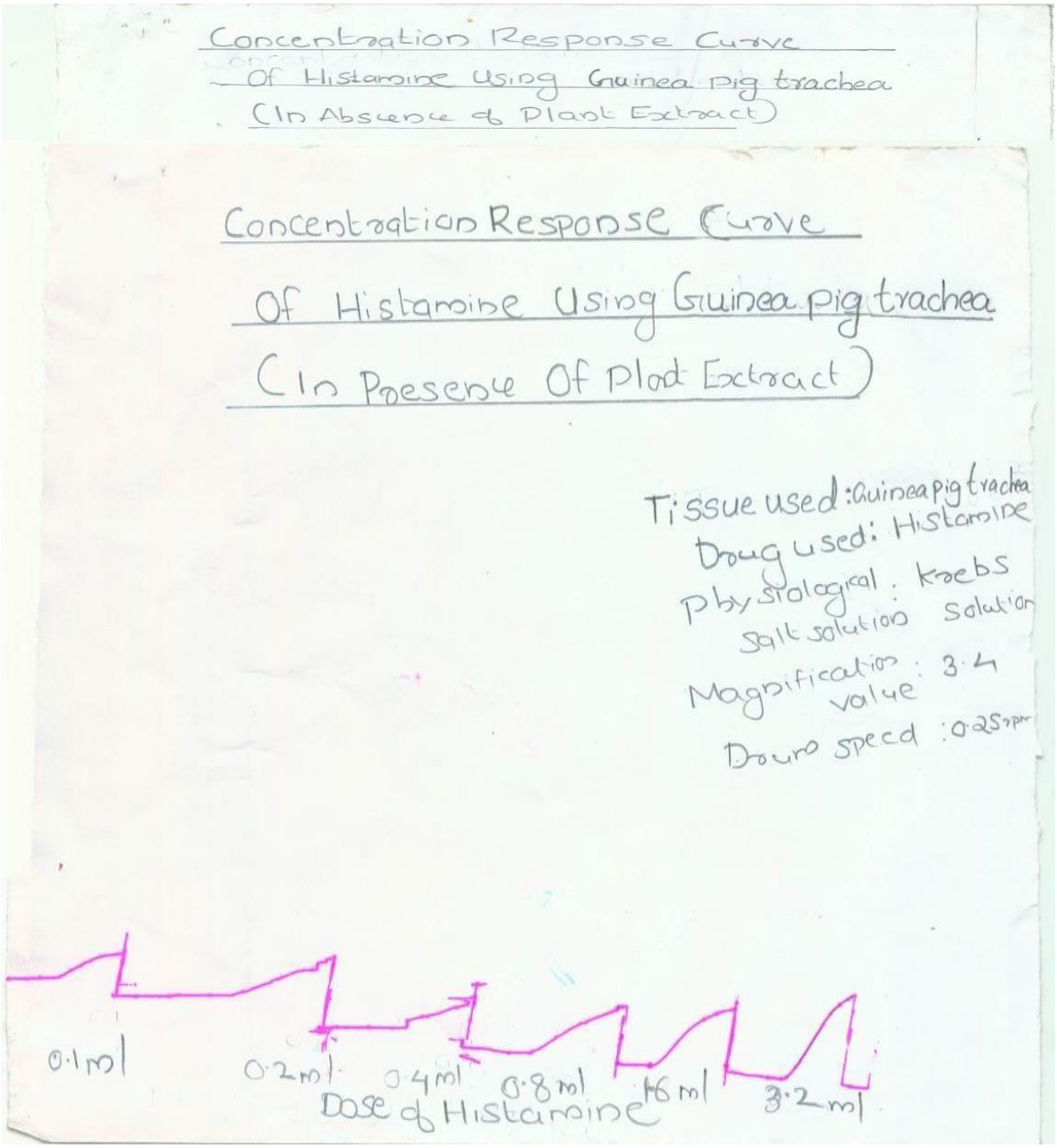


Figure No.6: Concentration Response Curve of Histamine using Guinea pig trachealpreparation (In Presence of Plant Extract).

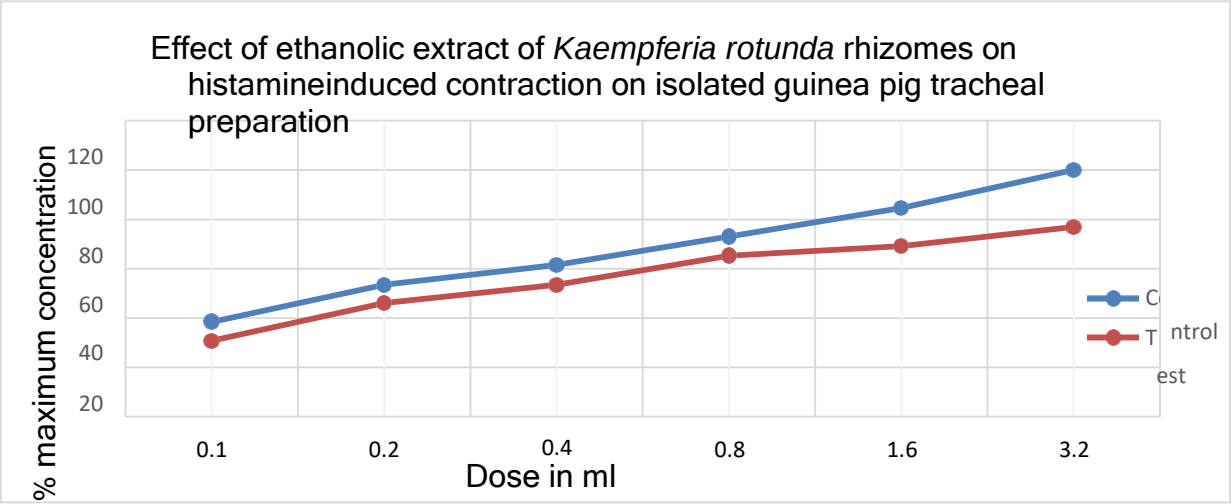
The results were expressed in table number 8 and the effect of *Kaempferia rotunda* rhizomes on histamine induced contraction on isolated guinea pig tracheal is shown in figure number 9

Table No.6: Effect of ethanolic extract of *Kaempferia rotunda* rhizomes on histamine induced contraction on isolated guinea pig tracheal preparation

Sl no	Dose of histamine (10µg/ml) in ml	Control (Histamine 10 µg/ml) % maximum contraction	Test Histamine(10µg/ml)+EEAC(1mg/ml) % maximum contraction
1	0.1	38.46 ± 1.58	30.76 ± 1.32**
2	0.2	53.48 ± 4.23	46.15 ± 2.91**
3	0.4	61.5 ± 3.89	53.48 ± 3.31**
4	0.8	73.07 ± 2.32	65.3 ± 1.76**
5	1.6	84.6 ± 2.13	69.2 ± 1.09**
6	3.2	100 ± 1.07	76.92 ± 2.11*

Values are Mean± S.E.M., where n=6 in each group, P< 0.05 *, P< 0.01 ** (significant) compared with control. Statistical analysis was done by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test.

Figure No.7: Effect of ethanolic extract of *Kaempferia rotunda* rhizomes on histamine induced contraction on isolated guinea pig tracheal preparation



***IN VITRO* ANTIOXIDANT ACTIVITY**

Hydrogen peroxide scavenging

The hydrogen peroxide scavenging activity of ethanolic extract of *Kaempferia rotunda* rhizomes was determined. The percentage hydrogen peroxide scavenging ability of the test extract increased in a dose dependent manner and the reference standard, ascorbic acid (100 µg/ml) exhibited 60.23% hydrogen peroxide scavenging activity. The maximum hydrogen peroxide scavenging activity shown by ethanolic extract of *Kaempferia rotunda* rhizomes was found to be 53.3 % at 400 µg/ml.

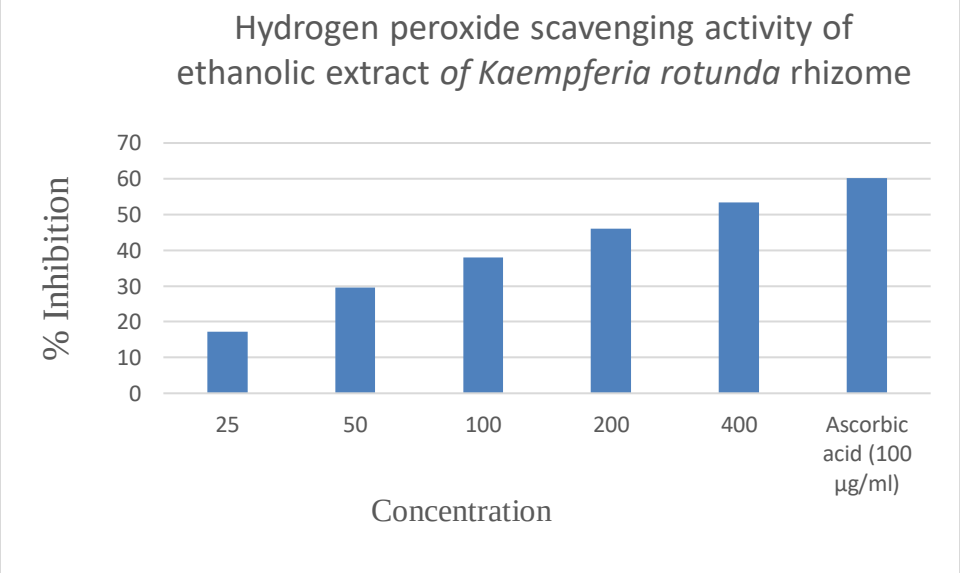
The hydrogen peroxide scavenging effect of ethanolic extract was shown in table number 7.

Table No.7: Hydrogen peroxide scavenging activity of ethanolic extract of *Kaempferia rotunda* rhizomes

Sl no	Concentration(µg/ml)	Absorbance [A]	% inhibition
1	25	0.632±0.0005	17.16
2	50	0.539±0.0052	29.5
3	100	0.474±0.0056	38.04
4	200	0.414±0.0005	46
5	400	0.357±0.0032	53.3
6	Ascorbic acid (100 µg/ml)	0.256±0.056	60.23

(Values are Mean±S.E.M., where n=6) in each group, P< 0.05 *, P< 0.01 **(significant)compared with control. Statistical analysis was done by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test.

Figure No.8: Hydrogen peroxide scavenging activity of ethanolic extract of *Kaempferia rotunda* rhizome.



Reducing power assay

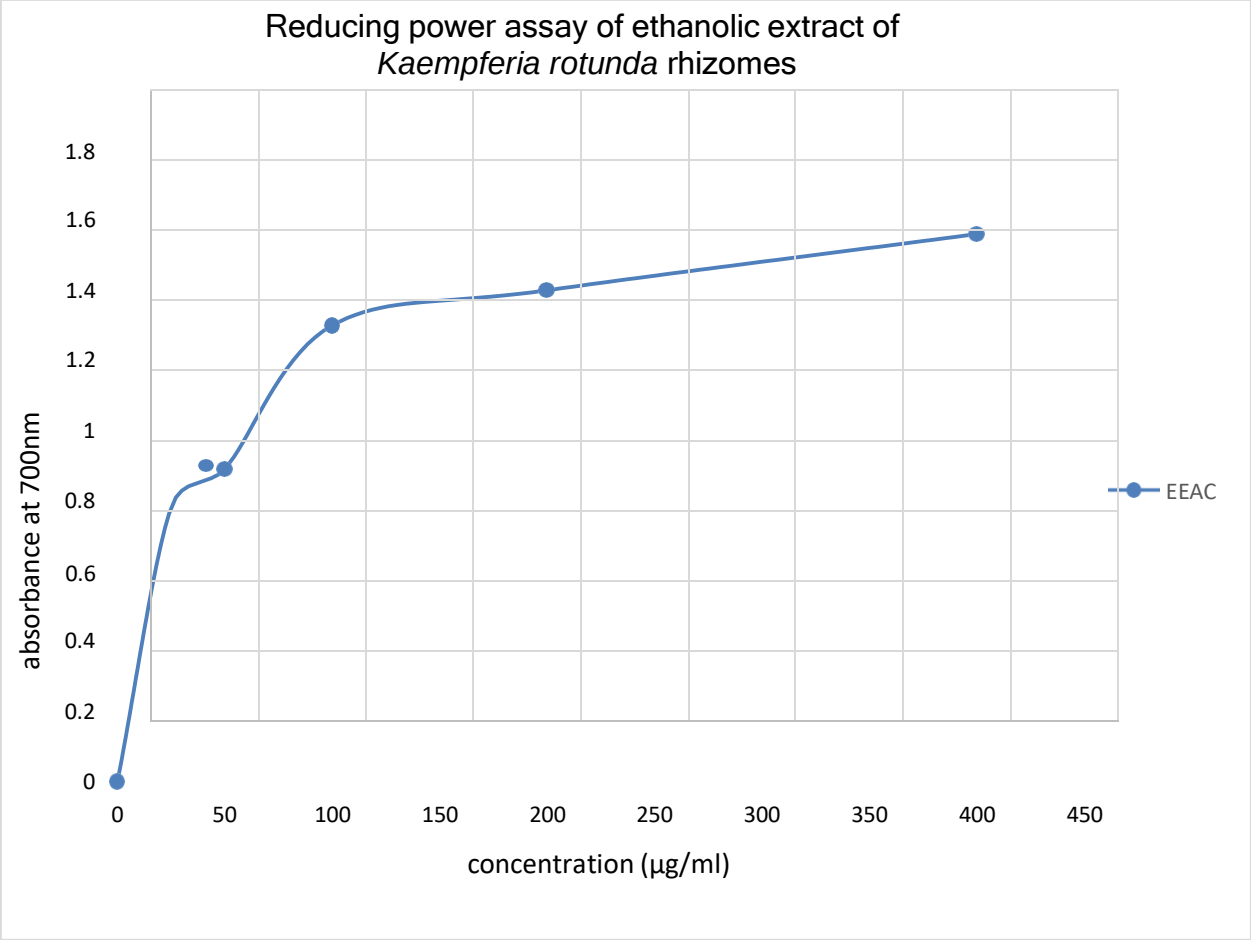
Increase in absorbance of the extract indicates the reducing power of the test sample. Reducing power of ethanolic extract of *Kaempferia rotunda* rhizomes increased with increasing concentration. Results are expressed below

Table No.8: Reducing power activity of ethanolic extract of *Kaempferia rotunda* rhizomes

Sl no	Concentration (µg/ml)	Absorbance [A]
1	25	0.782±0.32
2	50	0.891±0.21
3	100	1.3±0.35
4	200	1.4±0.42
5	400	1.56±0.82

(Values are Mean±S.E.M., where n=6) in each group, P< 0.05 *, P< 0.01 ** (significant) compared with control. Statistical analysis was done by one-way analysisof variance (ANOVA) followed by Dunnett's multiple comparison test.

Figure No.9: Reducing power assay of ethanolic extract of *Kaempferia rotunda* rhizomes



***IN VITRO* ANTI-INFLAMMATORY ACTIVITY**

Protein denaturation

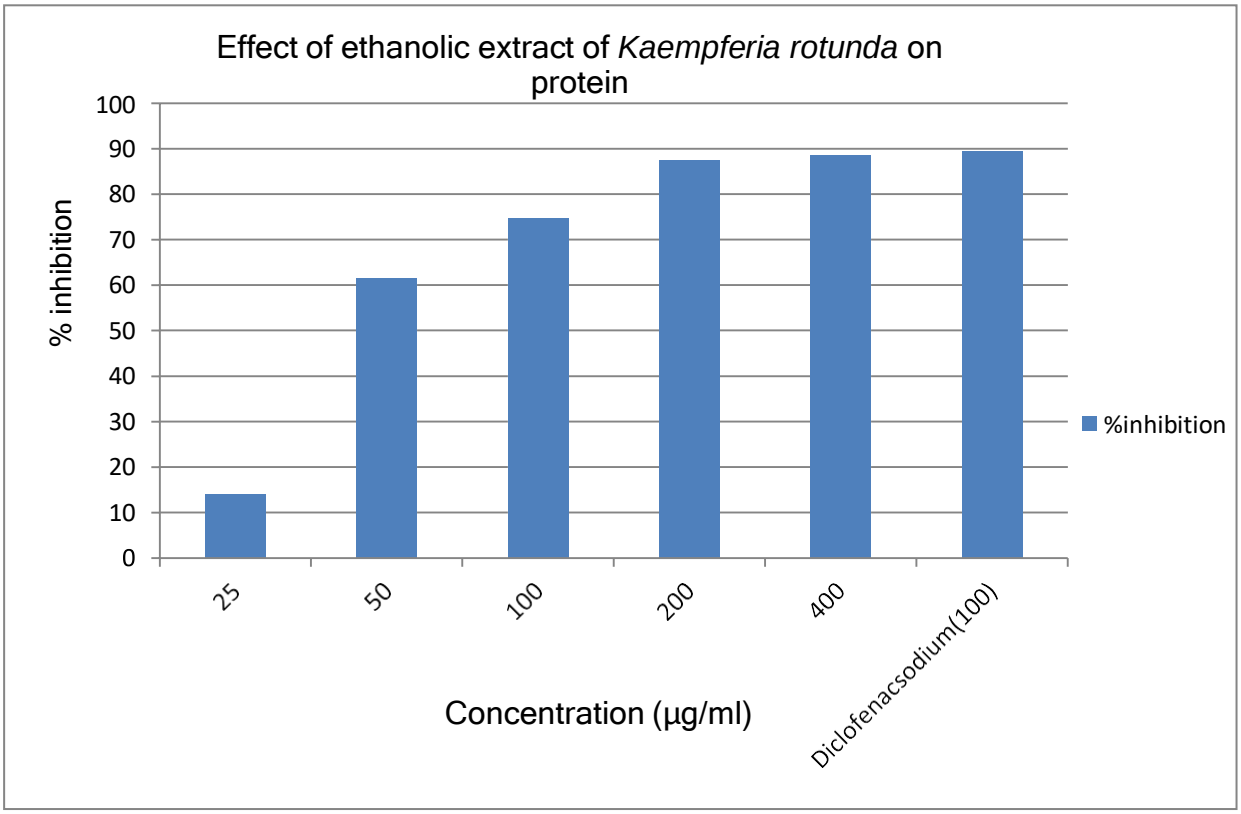
As part of the evaluation of anti-inflammatory activity, ability of plant extract on protein denaturation was studied. It was effective in inhibiting heat induced protein denaturation. Diclofenac sodium a standard anti-inflammatory agent possesses maximum % inhibition. The ethanolic extract of the plant *Kaempferia rotunda* rhizome possess significant % inhibition activity at concentration 200 µg/ml and 400 µg/ml.

Table No.9: Effect of ethanolic extract of *Kaempferia rotunda* on protein denaturation

Sl no	Concentration (µg/ml)	Absorbance [A]	% inhibition
1	25	1.28±0.05	14
2	50	0.578±0.03	61.6
3	100	0.382±0.002	74.63
4	200	0.189±0.01	87.4
5	400	0.172±0.002	88.57
6	Diclofenac sodium (100µg/ml)	0.165±0.005	89.43

(Values are Mean±S.E.M., where n=6) in each group, P< 0.05 *, P< 0.01 ** (significant) compared with control. Statistical analysis was done by one-way analysisof variance (ANOVA) followed by Dunnett's multiple comparison test.

Figure No 10: Effect of ethanolic extract *of Kaempferia rotunda* on protein denaturation.



Rabbit red blood cell membrane stabilization method

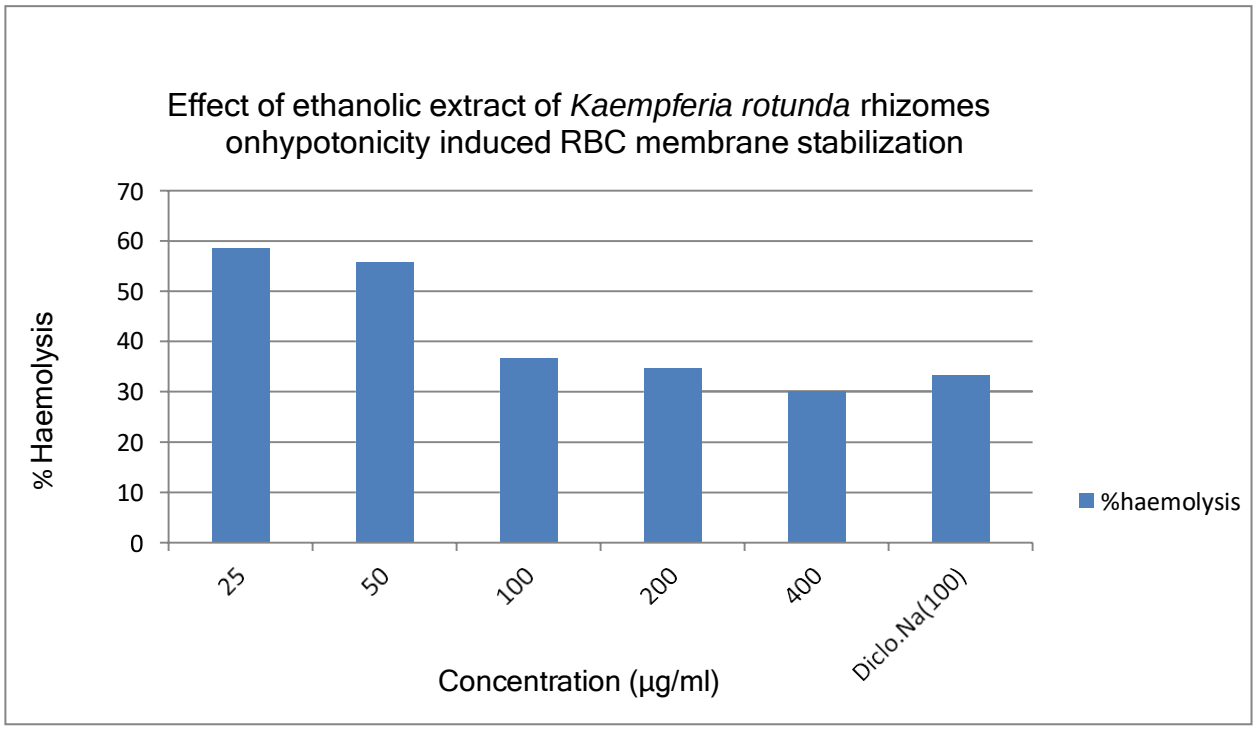
The ethanolic extract of *Kaempferia rotunda* rhizome had shown significant inhibition of haemolysis or the active RBC membrane stabilization comparing todiclofenac sodium the reference standard. The maximum percentage protection shown by test extract was 69.82% at 400 µg/ml and minimum percentage protection was found to be 41.4 at 25 µg/ml. The reference standard diclofenac sodium possesses 66.75% at concentration 100 µg/ml.

Table No.10: Effect of ethanolic extract of *Kaempferia rotunda* rhizomes on hypo tonicity induced RBC membrane stabilization.

Sl no	Concentration (µg/ml)	Absorbance[A]	% Protection	% Haemolysis
1	25	0.61±0.03	41.4	58.6
2	50	0.58±0.002	44.3	55.7
3	100	0.382±0.004	63.3	36.7
4	200	0.36±0.009	65.3	34.7
5	400	0.32±0.007	69.82	30.18
6	Diclofenac sodium (100 µg/ml)	0.34±0.008	66.75	33.25

(Values are Mean±S.E.M., where n=6) in each group, P< 0.05 *, P< 0.01 **(significant)compared with control. Statistical analysis was done by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test.

Figure No.11: Effect of ethanolic extract of *Alpinia calcarata* rhizomes on hypo tonicity induced RBC membrane stabilization



DISCUSSION:

The study includes the phytochemical and pharmacological investigation of theethanolic extract of *Kaempferia rotunda* rhizomes. The powdered rhizomes were extracted by means of soxhlet using the solvent ethanol. Preliminary phytochemical screening revealed the presence of carbohydrate, cardiac glycoside, protein, alkaloids, steroids, flavonoids, tannins and phenolic compounds. These constituents may be represented the presence for biological activities of the plant. The acute toxicity study revealed that there is no mortality with the ethanolic extract of *Kaempferia rotunda* rhizomes up to the dose level of 2000 mg/kg.

Asthma is an allergic disease with the utmost clinical and economic effect isan allergic and inflammatory outward sign of respiratory disorders. Asthma is a respiratory disease. The symptoms of bronchial asthma are characterized by wide blowout narrowing of the bronchial tube due to contraction of smooth muscle inreplay to stimuli subsequently in the release of histamine.

Bronchoconstriction induced by histamine is an immunological model of antigen induced airway obstruction. Histamine when inhaled causes hypoxia and leads to spasm in guinea pigs and causes very strong smooth muscle contraction and capillary dialation in cardiovascular system. Bronchodialators can delay the occurrence of these symptoms. The study revealed the H_1 receptor antagonistic activity and support the plant by anti-asthmatic property.

Herbal formulations used in the treatment of asthma include some anti-stress herbs to enable adoption to stress since excessive stress or nervous debility may aggravate symptoms of asthma. The normalization effect of an adaptogen can be observed in milk induced leukocytosis after administration of milk. Also eosinophilplay a pivotal role in the pathogenesis of allergic disorders. The plant extract showed marked protection against eosinophil cell count, which is a hallmark of allergic asthma as compared to control group. The reduction in eosinophil count means inhibition in eosinophil cell recruitment and inhibition of interleukins such as IL-4, IL-5 and IL-13 which play important role in eosinophil cell count. So the milk induced leukocytosis and eosinophilic study revealed anti asthmatic property of the plant extract.
Histamine contracts the tracheal-bronchial muscle of guinea pig, goat, horse, dog and man. Guinea pig trachea is used for the screening of anti-asthmatic activity. The H_1 receptor after stimulation produces well-ordered dose related contraction of isolated guinea pig trachea. In current study *Kaempferia rotunda* significantly inhibited histamine induced contraction of isolated guinea pig trachea preparation indicating its H_1 receptor antagonist activity and supports anti asthmatic property of the plant.

The antioxidant property was studied by hydrogen peroxide scavenging assay and reducing power assay. Hydrogen peroxide scavenging ability of ethanolic extract of *Kaempferia rotunda* rhizomes revealed that the extract scavenge the hydrogen peroxide. However, the hydrogen peroxide scavenging ability was low comparing to standard (ascorbic acid). Reducing power of ethanolic extract of *Kaempferia rotunda* rhizomes significantly increased with increasing concentration.

The *in vitro* anti-inflammatory potential of ethanolic extract of *Kaempferia rotunda* rhizomes had shown concentration dependent inhibition of protein denaturation, hypotonicity induced haemolysis of rabbit red blood cell membrane stabilization.

CONCLUSION:

The result of the investigation showed that the ethanolic extract of *Kaempferia rotunda* rhizomes possess anti asthmatic activity. The antioxidant and anti- inflammatory property of the plant also supports its anti-asthmatic property. Drugs effective in asthma are mostly steroidal in nature. Phytochemical analysis showed presence of flavonoid and steroids. The anti-asthmatic property showed by the plant may be because of these chemical moieties. The results obtained in the study supports the traditional and also demands further research and to isolate and characterize active principles responsible for anti-asthmatic activity.

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