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Evaluation of Antitussive and Mast cell stabilizing Activity of *Piper longum fruits extracts.* A therapeutic approach for treatment of Asthma.

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ABSTRACT

The mast cell and cough has long been associated with asthma, since it releases a variety of preformed and newly synthesized mediators that account for several features of asthma. Among the mediators, histamine is a well characterized and the most potent vaso-active mediator in acute broncho-constriction. The present study was carried out to evaluate In vitro and In vivo mast cell stabilizing and anti-tussive activity of ethyl acetate and methanolic extract of fruit of *Piper lignum*

Keywords: Asthama, Mast cell stabilizing, Antitussive, Piper longum

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INTRODUCTION

Bronchial asthma is an inflammatory disorder of the airways characterized by airway obstruction, inflammation and bronchial hyper-responsiveness and is a global health problem that results from a complex interplay between genetic and environmental factors. Among several respiratory diseases affecting man, bronchial asthma is the most common disabling syndrome. Nearly 7–10% of the world population suffers from bronchial asthma [1.]. Bronchial Asthma according to the GINA guidelines final update November 2006 is clearly defined as: "A chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation is associated with airway hyper responsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness and coughing, particularly at night or in the early morning." These episodes are associated with airflow obstruction within the lung that is often reversible either spontaneously or with treatment [1]. Asthma is reported in 1.2% to 6.3% adults in most of countries. The overall burden of asthma in India is estimated at more than 15 million patients. Asthma in adults is generally reported as 2.7 to 4.0% in most European countries, 12% in England, 7.1% in United States and higher 9.5 to 17.9% in Australia. The morbidity and mortality of asthma have increased over the past two decades, particularly in Western countries [2]. In most cases, mild-to moderate asthma is controlled by inhalational steroid. However, long-term steroid therapy is often associated with adverse effects [3]. Many side effects of steroids including adrenal suppression and reduction in growth velocity have been reported [4,5]. There is a need for development of additional effective treatments with fewer side effects.

Mast cells are constituents of virtually all organs and tissues and are important mediators of inflammatory responses such as allergy and anaphylaxis [6]. In which histamine remains the best characterized and most potent vasoactive mediator implicated in the acute phase of immediate hypersensitivity upon release [7]. Mast cells and basophils play a central role in inflammatory and immediate allergic reactions. On stimulation, they are able to release potent inflammatory mediators, such as histamine, proteases, chemotactic factors, cytokines and metabolites of arachidonic acid that act on the vasculature, smooth muscle, connective tissue, mucous glands and inflammatory cells. Mast cells settle in connective tissues and usually do not circulate in the blood stream. Basophils are the smallest circulating granulocytes with relatively the least known function. They arise in the bone marrow, and following maturation and differentiation, are released into the blood circulation. Adequately stimulated basophils may settle in the tissues. There are two categories of inflammatory (anaphylactic) mediators in mast cells and basophils.

Preformed mediators, stored in secretory granules and secreted upon cell activation, include a biogenic amine, typically histamine, proteoglycans, either heparin, over-sulphate chondroitin sulphates or both, and a spectrum of neutral proteases. Released histamine acts at H1, H2 and H3 receptors on cells and tissues, and is rapidly metabolized extracellularly. The proteoglycan imparts the metachromatic staining characteristic of mast cells when exposed to certain basic dyes such as toluidine blue. It has two functions, (i) may package histamine and basic proteins into secretory granules, and in mast cells and (ii) appears to regulate the stability of the protease called tryptase. Neutral proteases, which account for the vast majority of the granule protein, serve as markers of mast cells found in serosal, mucosal and brain region. Newly generated mediators, often absent in the resting mast cells, are typically produced during IgE-mediated activation, and consist of arachidonic acid metabolites, principally leukotriene C4 (LTC4) and prostaglandin D2 (PGD2) and cytokines. Of particular interest in humans is the production of tumour necrosis factor, IL-4, IL-5 and IL-6. In the cytoplasm of both mastocytes and macrophages are special lipid bodies, where metabolism of arachidonic acid occur and their products, including leukotrienes, may be stored [8, 9].

Cough is the most common symptom of asthma. It is an essential protective and defensive act whose action secures the removal of mucus, noxious substances, and infections from the larynx, trachea, and larger bronchi. On the other hand, a number of patients have nonproductive cough, which is not associated with mucus clearance and may have a different stimulation. It may be the first overt sign of disease of airways or lungs and may significantly contribute to the spread of airborne infections and, in some instances, may result in severe functional and structural damage to the organism. The primary action of currently available cough suppressants (opiates, dextromethorphan, etc.) is on the central cough pathway. The significant side effects of these agents such as constipation, respiratory depression, dependence, drowsiness, and death from this action limit their use in human [10].

Piper longum L. (Piperaceae), popularly known in India as Pippali, is used as traditional medicine in Asia, especially in Indian medicine and in Pacific islands. Various Piper species, widely distributed in the tropical and subtropical regions of the world, have been used as a spice and also as a folk medicine [11-14]. *P. longum* L. has been used in traditional remedies as well as in the Ayurvedic system of medicine against various disorders[15,16].

MATERIAL AND METHOD

Collection of plant*:*

Fruits of *P.longum* were collected from botanical garden, Indore (M.P) and identified and authenticated at Department of Botany, Holkar Science College, Indore. A voucher specimen has been kept in the herbarium of our department for future references.

Drugs and chemicals:

Fruits of Piper longum, Petroleum Ether, Chloroform, Ethyl Acetate, Ethanol, H_2SO_4, Aq Sodium Hydrogen Sulphite, Ammonium hydroxide, Codine Phosphate, Dextromethaphan, horse serum, triple antigen containing bordetella pertussis organism, ringer-locke.

Preparation of extract :

Fruits were purchased from local market of indore and identified and authenticated at Department of Botany, Govt. Agriculture College, Indore. A voucher specimen has been kept in the herbarium of our department for future references. shade dried and and powdered Fruits were subjected to successive solvent extraction using Petroleum ether, Chloroform, Ethyl acetate, Methanol as a solvent. All the four extract obtained were filtered, concentrated on water bath, dried in vacuum and stored in refrigerator for further experiment. Since main phytoconstituents flavonoids and alkaloids were found in ethyl acetate as well as in methanolic extract thus these two extract were taken for the further studies.

Experimental animals

For mast cell stabilizing activity (In vitro and In vivo study):

Animals-Male Wister rats (200-250g) were obtained from the experimental animal house The animals were housed in polypropylene cages under standard conditions (12 h light; 12 h dark cycle; 25± 5oC; 35-60% humidity). They were fed with standard pellet diet (Pranav Agro Ltd, Dehradun) and water *ad libitum*. The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC/PCP/2014/49).

For both the model of antitussive activity Swiss albino mice of either sex (20-30g) were used in the study. The animals were housed in polypropylene cages under standard conditions (12 h light; 12 h dark cycle; 25± 5oC; 35-60% humidity). They were fed with standard pellet diet (Pranav Agro Ltd, Dehradun) and water *ad libitum*. The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC/PCP/2014/49).

Pharmacological study:

Mast cell stabilizing activity:

In-vitro mast cell stabilizing activity

Degranulation studies

Sensitized mast cell were obtained from animals sensitized with egg albumin. The doses being given on the 1st, 3rd and 5th day. The sensitized mast cells were degranulated using egg albumin (1mg/ml) on the 10th day of sensitization. The normal mast cell were degranulated using compound 48/80 (100mcg/ml). The cell suspension of mast cells was treated as follows. To 0.1 ml of the peritoneal mast cell suspension, 0.1ml of the test agent in the saline was added and incubated in a constant temperature water bath (37oc) for 15 minutes. Then 0.1 ml of degranulaing agent (Egg albumin 1 mg/ml or compound 48/80 100mcg/ml) was added and further incubated for a period of 10 minutes. The cell were then stained with 0.1% toluidine blue for 5-10 min and the tissue was then washed in acetone and then xylene (2 changes each) for 5 min each wash. The stained cells were viewed through a digital light microscope at 100x magnification and 100 mast cells were counted. The number of intact and fragmented or disrupted mast cells was noted. A mast cell was considered disrupted if four or five granules were found around the mast cells. The number of fragmented or disrupted mast cells as well as of the intact mast cells were counted [17].

In-vivo mast cell stabilizing activity

All groups rats were sensitized by injecting subcutaneously 0.5 ml of horse serum along with 0.5 ml tripple antigen containing 20,000 million Bordetella pertussis organism . The sensitized rats will be divided in to 4 groups of 6 animals each . Rats of group 1 received water (vehicle) and served as control Rats of group 3 and 4 were administered herbal extracts p.o respectively, once a day for 14 days . Group 2 rats received 10 mg/kg of prednisolone (reference) orally for same duration. On day 14,the rats were sacrificed two hours after treatment and the intestinal mesentery was taken for the study on mast cells. Mesenteries of sacrificed rats along intestinal pieces were kept in Ringer –Locke solution .The mesenteric pieces were challenged with horse serum for 10 minutes after which the mast cell were stained and examined microscopically for the number of intact and degranulated mast cell. Pieces of intestinal mesentry will be mounted on slide which will be air dried and then stained with 1% toludine blue, at room temperature for 5 min. Mast cell will be readily identified by their metachromatic cytoplasmic granules under light microscope [18].

Antitussive activity:

Swiss albino mice were divided into four groups, each group containing six mice. The control group was treated with distilled water orally, and the positive control was treated with Codiene Phosphate. The remaining groups were treated with the ethyl acetate and methanol extract at doses of 500 mg/Kg body weight respectively.

Sulfur dioxide gas induced cough reflex in mice

The experimental model is shown in Figure 1 where A is a 500 ml three-necked flask which contains aqueous saturated sodium hydrogen sulphite solution. By opening the stop-cock of a burette (B), the concentrated sulphuric acid was introduced to generate sulphur dioxide gas.

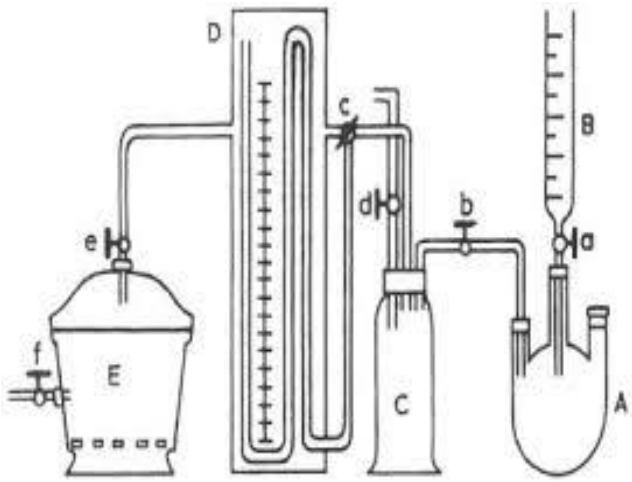

Figure 1: Apparatus used in sulfur dioxide gas induced cough model

A: Saturated NaHSO3 solution in 500ml flask, **B**: Conc. H2SO4 in burette, **C**: Gas Cylinder, **D**: Water manometer, **E:** Dessicator and a, b, c, d, e, f are stop cocks.

The chemical reaction which occurred in flask A is as follows:

2 NaHSO3 + H2SO4 → 2 SO2 + Na2SO4+ 2 H2O

Flask A and gas cylinder C were filled with sulphur dioxide (SO2) gas. Cocks c and b were opened to elevate pressure in gas cylinder C, which was recorded by water manometer D. Stop-cock b was then closed and stop-cock d was opened slightly until pressure in D (11 mm, i.d.) reached 75 mm H2O, when stop-cock d was closed. The procedures were conducted in a draught. Cough response of all the groups are observed (0 minute) by placing the animals in desiccators E. The cocks c, f and e are opened in order and when the pressure in D became 0 mm of H2O, all the cocks are closed immediately. A certain amount, 5ml sulfur dioxide gas is induced into the desiccator and this way. After a minute of introducing the gas, the animal is taken out of the dedicator and frequency of cough is observed for five minutes in an un-ended filter funnel with a stethoscope at the tip in which mice is confined. In the same fashion the frequency of cough are observed for all the animal groups after every 30 minutes for 2 hrs [19].

The percentage frequency of cough reflex was calculated by the formula

% frequency of cough reflex= (1 – T / C) X 100

Where T= Cough reflex in tested drug treated in mice

C= Cough reflex in control group treated mice.

Ammonium hydroxide induced Cough:

Swiss albino mice were divided into four groups, each group containing six mice. The control group was treated with distilled water orally, and the positive control was treated with dextramethorphan. The remaining groups were treated with the ethyl acetate and methanolic extract at doses of 500 mg/Kg body weight respectively. Anti-tussive activity was investigated on a classical mouse cough model induced by ammonia liquor. Each mouse was placed in a 300 ml special glass chamber and exposed to 40µl 25% NH4OH. The cough frequency produced during 2 min exposure period was counted. The cough frequency and latent period of cough were also recorded [20].

The percentage frequency of cough reflex was calculated by the formula

% Frequency of Cough Reflex= (1 – T / C) X 100

Where T= Cough reflex in tested drug treated in mice;

C= Cough reflex in control group treated mice.

RESULTS AND DISCUSSION

All experimental data were expressed as mean ± SEM.(Table 1,2,3,4,5,6 and 7) Statistical analysis was carried out by using one way ANOVA followed by Dunnett's test.

The mast cells have a crucial role in the development of many physiological changes during anaphylactic and allergic responses. Immunoglobulin- E antibodies bind to receptors on the surface of mast cell. Allergen-IgE interaction on mast cell leads to the release of histamine, heparin, proteases and other mediators and the synthesis and secretion of leukotrienes and prostaglandins. These products result in bronchoconstriction, changes in blood vessel tone, increased vascular permeability and myriad other pro-inflammatory effects [21]. The functions of mast cells can be manipulated for therapeutic ends by regulating their function with appropriate drugs. Plant origin constituents may influence differentiation into mast cells, chemical composition and or architecture of mast cell surface membrane. It may influence the synthesis of IgE molecules or binding of IgE on mast cell surface. It is also possible, that the plant drug mayreduce the life span of mast cells [22]. Extract of *Piper longum* markedly protected the sensitized mast cells. However, the effect was less than that observed with the standard drug (Kitotifen) used. The pathological mechanism involved in Type-I allergy has been explained as the degranulation of mast cells and basophils, followed by the release of mediators such as histamine, leukotrienes and prostaglandins from these cells [23]. The degranulation of mast cells occurs in response to the immunological stimuli in which the antigen– antibody interaction on

the cell surface predominates. The present investigation indicates that the extract of *Piper longum* is active against Type-I allergic condition because of their ability to inhibit the release of mediators from mast cells and basophils and thus influences the course of the disease. The preliminary phytochemical tests showed the presence of flavonoids and phenolic compounds in the *Piper longum m*ethanolic and ethyl acetate extract. Mast cells after degranulation shows demonstrated that transgranulation occurs between mast cells and fibroblasts with mast cells apparently transferring their granules to the cytoplasm of fibroblasts or to the mesothelium. It has been reported that mast cell granules are internalized in fibroblasts 1-3 h after C48/80 injection [24]. In the mast cell the extruded granules might be degraded by the extracellular, as the initial compact morphological appearance of the discharged granules is gradually lost, and the granule contents are discharged [25].

The present investigation indicates the methanolic and ethyl acetate extract of *Piper longum* is active in the Type-I allergic conditions because of their ability to inhibit the release of mediators from mast cells and thus influence the course of the disease by preventing the harmful effects of the released mediators. The preliminary phytochemical tests showed the presence of flavonoids in the methanolic and ethyl acetate extract. Plant flavonoids are known to inhibit basophil histamine release and neutrophilbetaglucuronidase release, and thereby possess in-vivo antiallergic activity [26]. The flavonoids also inhibited the histamine release induced by 48/80. Plants containing flavonoids have been reported to possess antihistaminic, antiallergic and mast cell degranulation properties [27, 28].

The anti-tussive activity of ethyl acetate and methanol fraction of fruits *Piper longum* in experimental animal model. Anti-tussive agents or cough suppressants are used mainly to suppress dry and painful cough. They act to reduce the urge to cough. The larynx and extrapulmonary airways are richly supplied with non myelinated C- fibres and rapidly adapting receptors having myelinated Aδ- fibres. These are involved in the cough mechanism [29]. Vagal afferent nerve provide inputs to brainstem nuclei, primarily the nucleus of the solitary tract (nTS) that receive inputs from airway cough evoking afferents and generate cough reflex in body . Centrally acting antitussives such as codeine and dextromethorphan act within the central nervous system (CNS) at the level of the brain stem by depolarization or a dulling of the vagus nerve, the nerve leading from the brain stem and serving the chest area. Peripheral antitussive drugs act outside the CNS to inhibit cough by suppressing the responsiveness of one or more vagal sensory receptors that produce cough [30].

Antitussive animal models could be designed by mechanical stimulus, electrical stimulus, and chemical stimulus. In this experiment, chemicals like ammonium liquor and sulfur dioxide were used to induce cough. These models are widely used animal models for evaluating antitussive activity of a traditionally used drug. Cough is a normal physiological response to an irritation of the laryngo-tracheo-bronchial system caused by mechanical or chemical stimulation. It may be painful and require suppression by antitussive drugs [31].

The *in vivo* antitussive activity of the ethyl acetate and methanolic extract of fruit of *P.longum* was investigated for its effect on a cough model induced by sulphur dioxide gas in mice and found to have significant anti-tussive activity when compared with control and the standard drug Codiene phosphate. The ethyl acetate and methanolic extract of *P.longum* plant was orally administered at the dose levels of 500 mg/kg b.w. showed maximum inhibition of cough by 82% and 81% respectively .The standard anti-tussive drug Codiene phosphate (10mg/kg b.w.) showed maximum inhibition of cough by 84%. It was found that both extract of *P.longum* showed anti-tussive activity and obtained percentage inhibition of cough reflex is approximately comparable as standard drug (Table 4 and 5).

Table 1: Effect of extracts on egg albumin induced mast cell degranulation in rats

Treatment N=6	Dose (mg/Kg)	Number of mast cells	Percent inhibition
Control		9 ± 1.3	
Ketotifen	10 mcg / ml	88 ± 1.5*	80.12± 1.02
Ethyl acetae PL	500	62 ± 1.15*	60.11± 1.21
Methanol PL	500	48 ± 1.19*	46.78± 1.18

*: p< 0.05 Vs control n= number of animals.

Table 2: Effect of extracts on compound 48/80 induced mast cell degranulation in rats

Treatment N=6	Dose (mg/Kg)	Number of mast cells	Percent inhibition
Control		6 ± 0.11	
Ketotifen	10 mcg / ml	78 ± 1.26*	75.12 ± 1.08
Ethyl acetae PL	500	57 ± 0.17*	55.58 ± 1.45
Methanol PL	500	42 ± 1.17*	40.28 ± 1.21

*: p< 0.05 Vs. control n= number of animals.

Table3: In-vivo mast cell stabilizing activity of ethyl acetate and methanolic extract of fruit of *P. longum*

Treatment	Doses (mg/kg body weight)	Route of Administration	Granulated mast cell	Non - Granulated mast cell
Control(TWEEN 80,1)#		oral	85.83±1.72	12.33±1.03

Control(TWEEN 80,1)sensitize		oral	23.66±3.32	82.5±3.06
Prednisolone	10	oral	75.66±2.42*	30.16±2.13*
Ethyl acetae PL	500	oral	66.16±1.94*	44.33±2.25*
Methanol PL	500	oral	62.33±1.96*	37.5±1.37*

#not treated with horse serum and triple antigen. values are mean±S.E., n=6, *P<0.001 when compared with control.

Table 4: Effect of ethyl acetate and methanolic extract of fruit of *P. longum* on cough frequency in Sulphur dioxide gas induced cough mice

Treatment	Dose(mg/kg)	COUGH FREQUENCY IN MINUTES				
		0 MIN	30 MIN	60 MIN	90 MIN	120 MIN
CONTROL		77.38±1.58	78.13±1.57	76.88±1.89	75.63±1.44	76.25±1.51
Codiene Phospahte	10	76.38±1.55**	31.75±1.31**	18.25±1.24**	13.88±1.30**	11.5±1.51**
Ethyl acetae PL	500	75.16±1.21**	64.27±1.22**	53.14±1.35**	36.28±1.11**	14.36±1.01**
Methanol PL	500	78.13±1.54**	66.75±1.44**	51.88±1.38**	36.25±1.58**	15.25±1.41**

Values are mean ± SEM, n= No. of animals in each group. ** $p < 0.05$ Significance versus control.

Table 5; Effect of ethyl acetate and methanolic extract of fruit of *P. longum* on % Inhibition in Sulphur dioxide gas induced cough mice

Treatment	% INHIBITION OF COUGH REFLEX			
	30 MIN	60 MIN	90 MIN	120 MIN
Codeine Phosphate	59%	76%	81%	84%
Ethyl acetate PL	17%	31%	52%	81%
Methanol PL	18%	30%	52%	80%

Ammonium hydroxide well-described inducers of bronchoconstriction in individuals and are chemically related and, therefore, may share a common mechanism of action. Acute exposure of ammonium hydroxide causes dryness of nose and throat and a measureable increase in resistance to bronchial air flow.In this model, ethyl acetate and methanolic extract of fruit of *P.longum* orally administered at the dose of 500mg/kg b.w. showed maximum inhibition of cough by 82% and 81% respectively. The standard anti-tussive drug Dextromethorphan (10mg/kg b.w.) showed maximum inhibition of cough by 84% (Table 6 and 7).

Table 6 : Effect of ethyl acetate and methanolic extract of fruit of *P. longum* on cough frequency in Ammonium hydroxide gas induced cough mice

Treatment	Dose(mg/kg)	COUGH FREQUENCY IN MINUTES				
		0 min	30 min	60 min	90 min	120 min
Control		74.16±2.92	76.83 ±2.48	75.5±0.83	74.33±3.07	74.67±3.01
Dextromethapahan	10	76.33± 1.21**	34.83±2.63**	22.33±1.86**	14.5±2.42**	11.5±1.04**

Ethyl acetate ext PL	500	73.33±1.86**	56.16±1.94**	46.33±2.42**	37.16±2.63**	15.33±1.50**
Methanol extract PL	500	74.34±2.92**	56.19±1.37**	48.81±1.20**	38.15±1.92**	15.81±2.08**

Values are mean ± SEM, n= No. of animals in each group. ** $p < 0.05$ Significance versus control.

Table 7: Effect of ethyl acetate and methanolic extract of fruit of *P. longum* on % Inhibition Ammonium Hydroxide gas induced cough mice

Treatment	Dose (mg/kg)	% INHIBITION OF COUGH REFLEX			
		30 min	60 min	90 min	120 min
Dextromethapahan	10	54%	70%	80%	84%
Ethyl acetate ext PL	500	26%	38%	50%	79%
Methanol extract PL	500	26%	35%	48%	79%

CONCLUSION:

To conclude, our study indicated that the ethyl acetate and methanolic extract of fruit of *Piper longum* demonstrated significant mast cell stabilizing and antitussive activity. These effects are the important evidence for the traditional use of fruit of *Piper longum* in the treatment of cough and respiratory disorders.

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