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Author	**EZIKE, Adaobi Chioma**
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PHARMACOLOGICAL CHARACTERIZATION OF THE ANTIASTHMATIC CONSTITUENT OF THE LEAVES OF *Asystasia gangetica* (L.) T. Anderson (ACANTHACEAE)

BY

EZIKE, ADAOBI CHIOMA
PG/Ph.D./02/33341

DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY,

FACULTY OF PHARMACEUTICAL SCIENCES

UNIVERSITY OF NIGERIA, NSUKKA

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A THESIS SUBMITTED TO THE DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY, FACULTY OF PHARMACEUTICAL SCIENCES, UNIVERSITY OF NIGERIA NSUKKA, FOR THE AWARD OF DOCTOR OF PHILOSOPHY (Ph.D.) DEGREE IN PHARMACOLOGY

PROF. P. A. AKAH (Ph.D)
(SUPERVISOR)

DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY
FACULTY OF PHARMACEUTICAL SCIENCES,
UNIVERSITY OF NIGERIA, NSUKKA.

CERTIFICATION

Ezike, Adaobi Chioma, a postgraduate student of the Department of Pharmacology and Toxicology, with the Registration No., PG/Ph.D./02/33341, has satisfactorily completed the requirements of research work for the award of the degree of Doctor of Philosophy in Pharmacology. The work embodied in this thesis is original and has not been submitted, in part or full, for any degree of this or any other university.

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Ezike, A. C.
Student

.....

PROF. P. A. Akah (Ph.D.)
Supervisor

.....

Dr. C. O.Okoli
Ag. Head of Department

.....

External Examiner

DEDICATION

To God be the glory; Great things He has done for me.

To my parents Chief (Hon) and Barr.(Mrs) Cornel Umeh , thank you.

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University of Nigeria, Nsukka. 2007

ABSTRACT

Asthma is currently a worldwide problem, with increasing prevalence in both children and adults; a prevalence rate of 5 – 10% has been reported for Nigeria. The pathophysiological features of the disease are bronchoconstriction, airway hyperresponsiveness and chronic inflammation. Drugs used in the management include bronchodilators which are short-term relievers and anti-inflammatory drugs which are long-term controllers. Despite the availability of oral and inhaled medications, the prevalence of asthma is on the rise. The challenge of developing new effective, safe and long lasting antiasthmatic drugs from natural products appears inevitable. The leaves of *Asystasia gangetica* L. (T). Anderson sub species *micrantha* (Nees) Ensermu (Acanthaceae), a traditional anti-asthma remedy, offer great potential for the development of a novel anti-asthmatic agent. The leaves have been shown to possess antihistaminic, bronchospasmolytic and anti-inflammatory properties. The aim of this research was to isolate and pharmacologically characterize the anti-asthmatic constituent of the leaves of this plant using bioactivity-guided fractionation of the leaf extract.

The leaves of *A. gangetica* were sun dried in open air for 48 h and pulverized to coarse powder. The leaf powder was extracted by cold maceration in 100% methanol for 48 h. The extract obtained was concentrated in a rotary evaporator under reduced pressure to afford the methanol extract (ME). The median lethal dose (LD_{50}) of ME was determined in mice using the intraperitoneal route. The ME was fractionated in a silica gel (70 – 230 mesh) column successively eluted

with hexane: ethylacetate (7:3) and methanol (100%) to obtain fractions A, B and C. Screening of the three fractions for bronchospasmolytic activity using inhibition of histamine induced contraction of the guinea pig trachea and relaxation of pre-contracted trachea (pathological tissue) as bioactivity-guides showed that fraction B exhibited the greatest effect in both tests. Based on the results, fraction B was further separated using gradient solvent mixtures of hexane: ethyl acetate (9.5:0.5, 9:1, 8:2, 7:3, 6:4, 5:5, 0:1) to obtain eight fractions (Fr I, Fr II, Fr III, Fr IV, Fr V, Fr VI, Fr VII, Fr VIII). Activity-guided tests on these fractions revealed greatest potency in Fr VIII, which on chromatographic separation successively eluted with petroleum ether: ethyl acetate (7:3) and ethylacetate (100%) yielded two fractions (Fr 1 and Fr 2). Fr 2 yielded a brown amorphous powder AG-1 which at 0.4 mg/ml completely inhibited the contractions of the guinea pig trachea induced by histamine. The methanol extract (ME), fraction B, Fr VIII and Fr 2 were subjected to phytochemical analysis for identification of constituents. The effect of the extract and fractions (200 and 400 mg/kg) on systemic acute inflammation was studied in rats using the paw edema induced by carrageenan. Also, the effect of the extract and fractions (400 mg/kg) on carrageenan-induced leukocyte infiltration into the pleural cavity as well as pleural exudate formation was evaluated in rats. The data obtained was analysed using One Way Analysis of Variance in SPSS Version 11.

Phytochemical tests showed that the ME tested positive to alkaloids, glycosides, saponins, reducing sugars, terpenes and carbohydrate. Fraction B gave positive

reactions for flavonoids and terpenes while Fr VIII tested positive to flavonoids, terpenes and steroids. Fr 2 gave positive reactions for terpenes and steroids. The intraperitoneal median lethal dose (LD_{50}) of ME in mice was estimated to be greater than 5,000 mg/kg. In the isolated tissue tests, the ME, Fr B, Fr VIII and AG-1 exhibited varying degrees of dose-dependent inhibition of contractions of the guinea pig trachea induced by histamine. The ME and the fractions also relaxed the guinea pig trachea pre-contracted with histamine. In the whole animal experiments, ME, Fr B and Fr VIII significantly ($P<0.05$) suppressed the development of acute edema in the rat paw with peak effect at 1 h post induction of inflammation. In the rat pleurisy study, ME, Fr B and Fr VIII reduced the volume of pleural exudate with the effect of ME (400 mg/kg) and Fr VIII (400 mg/kg) being significant ($P<0.05$). The ME, Fr B and Fr VIII reduced the total leukocyte count (TLC) with ME (400 mg/kg) and Fr VIII (400 mg/kg) causing a significant ($P<0.05$) reduction in TLC.

The results established the anti-asthmatic potency of the leaves of *A. gangetica* and suggest that AG-1 may be the constituent of the leaves responsible for the anti-asthmatic action.

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Akah, P. A., Ezike, A. C., Nwafor, S. V., Okoli, C. O., Enwerem, N. M. (2003) Evaluation of the anti-asthmatic property of *Asystasia gangetica* leaf extracts. *J. Ethnopharmacol.* 89: 25 – 36.

CHAPTER ONE

INTRODUCTION

1.0 DEFINITION OF ASTHMA

Asthma is as a chronic inflammatory airway disorder characterized by obstruction of the airways. Narrowing of the airway occurs because of inflammation and mucus hypersecretion, and is exacerbated as the smooth musculature in the bronchiolar walls becomes hyperresponsive (to nonspecific stimuli), leading to intermittent airway constriction. Intermittent airway constriction gives rise to the asthmatic symptoms of wheeze, cough, chest tightness and shortness of breath. Over time, the bronchioles may become fibrosed or scared and the airflow limitation may become permanent (Cookson, 1999).

The airway inflammation in asthma is due to an immune-mediated process in which inflammatory cells and inflammatory mediators enter airway tissues to cause disordered lung function; it is evident that in asthma, many inflammatory cells play a role, particularly mast cells, eosinophils and T lymphocytes (NHLBI/WHO, 1995). In susceptible individuals, the already inflamed airways respond to an asthma trigger through bronchoconstriction causing recurrent episodes of wheezing (a high-pitched noise due to turbulent airflow through a narrowed airway), dyspnea, and cough particularly at night and/or early morning. These symptoms are usually associated with widespread but variable airflow limitation that is at least partly reversible either spontaneously or with treatment. The inflammation also causes an associated increase in airway responsiveness to a variety of stimuli (NHLBI/WHO, 1995). Therefore, the pathophysiological

components that define asthma include inflammation, bronchoconstriction, airflow limitation and airway hyperresponsiveness.

Common triggers of an asthmatic attack include common allergens, irritants, drugs e.g. non-steroidal anti-inflammatory drugs (NSAIDs) and upper respiratory tract infections; oftentimes there is no identifiable trigger. The exact etiology of asthma is unknown, though there is often a family history of atopy in affected individuals inferring a genetic component.

1.1 CLASSIFICATION OF ASTHMA

Asthma may be classified on the basis of etiology, severity, and pattern of airflow limitation, (NHLBI/WHO, 1995).

1.1.1 Classification based on etiology

The exact etiology of asthma is unknown. However, there is often a family history of atopy in affected individuals inferring a genetic component to the disease. Asthma is classified into two types based on etiology, though much overlap occurs (NHLBI/WHO, 1995).

(i) ***Extrinsic asthma***

Also called atopic or allergic or reagin mediated asthma. Extrinsic asthma is a Type1 hypersensitivity reaction that involves immunoglobulin E (lgE) binding to mast cells. It is a childhood condition that most commonly occurs in patients with a history of allergy and is usually mild (Greene and Harris, 2000). Also in this class are occupational asthma and allergic bronchopulmonary aspergillosis (Kobzik and Schoen, 1994). However, in addition to lgE mechanisms, sensitizing

occupational agents may produce asthma through other cellular and immunological mechanisms that do not depend on the immediate hypersensitivity response.

(ii) ***Intrinsic asthma***

Also called non-atopic or cryptogenic asthma (Greene and Harris, 2000); it is induced by non-specific, diverse, non-immune mechanisms e.g. cold, viruses, respiratory infections, pollutants e.g. sulphur dioxide, stress, exercise and drugs (e.g. NSAIDs). In other words, it is not associated with a history of allergy and it may be accompanied by chronic bronchitis. It starts in adulthood and is more persistent (Kobzik and Schoen, 1994; Greene and Harris, 2000).

A major drawback of this classification is that there are some patients in whom no environmental cause can be identified. Identification of a specific environmental cause for asthma in a patient should be part of the clinical assessment as it enables the use of avoidance strategies in asthma management (NHLBI/WHO, 1995).

The recent association found between serum IgE levels and indices of asthma in all age groups, including non-atopic individuals, raises the possibility that all forms of asthma relate to a mucosal inflammatory response initiated by environmental or other antigens (Burrows *et al.*, 1989).

1.1.2 Classification based on severity

This classification is based on a combination of history of asthma symptoms, and measurements of lung function before the initiation of therapy (MerckMedicus, 2007). Asthma is sub-divided by severity into four classes (Table

1); mild intermittent, mild persistent, moderate persistent and severe persistent (NHLBI/WHO, 1995; NAEPP, 1997; GINA, 1998; AAAAI, 1999). History of asthma symptoms includes the frequency of daytime and night-time symptoms and the effect of the exacerbations on normal daily activities; while measurements of lung function include predicted values for peak expiratory flow rate (PEFR), forced expiratory volume in one second (FEV_1) and the morning to evening variability of PEFR. The presence of one feature of a more severe class suffices to place the patient in that class of severity, and it is best to classify asthma severity before the commencement of therapy (NHLBI/WHO, 1995). This classification is useful because asthma therapy has a stepwise approach in which the level of therapy is increased as the severity of the asthma increases.

An individual should be assigned to the most severe grade in which any feature occurs. The classification characteristics may overlap over time because asthma is highly variable, also an individual's classification may change over time (NHLBI/WHO, 1995).

Although airway inflammation increases with asthma severity, patients at any level of severity can have mild, moderate or severe exacerbations. Some patients with intermittent asthma can experience a severe and life-threatening exacerbation. Physicians may have difficulty assessing the severity of airway obstruction because symptoms of asthma and the use of asthma guidelines vary. This could lead to under-classification of the severity of a patient's asthma with subsequent poor asthma control (Shim and Williams, 1980; Osborne *et al.*, 1999). Consequently, under-classification and under-treatment may contribute to

increased morbidity and mortality rates for asthma over the past two decades (Bousquet *et al.*, 1996).

Table 1: Classification of Asthma Severity

Classification	Symptoms	Lung Function
Step 1: Mild-Intermittent	• Daytime ≤ 2 times per week • Nighttime ≤ 2 times per month • Brief exacerbations • Asymptomatic between exacerbations • Intensity of exacerbations varies	• PEF Variability < 20% • $FEV_1 \geq 80\%$ • PEF ≥ 80%
Step 2: Mild-Persistent	• Daytime > 2 times per week but < 1 time per day • Nighttime > 2 times per month • Exacerbations may affect activity	• PEF Variability range of 20% to 30% • $FEV_1 \geq 80\%$ • PEF ≥ 80%
Step 3: Moderate-Persistent	• Daytime symptoms daily • Nighttime symptoms > 1 time per week • Exacerbations affect activity • Exacerbations ≥ 2 times per week • Exacerbations last for days • Daily use of inhaled short-acting $ß_2$-agonists	• PEF Variability > 30% • FEV_1 > 60% but < 80% • PEF > 60% but < 80%
Step 4: Severe-Persistent	• Daytime symptoms continual • Nighttime symptoms frequent • Symptoms limit physical activity • Exacerbations frequent	• PEF variability > 30% • $FEV_1 \leq 60\%$ • PEF ≤ 60%

PEF = peak expiratory flow, FEV_1 = forced expiratory volume in 1 second
Adapted from National Asthma Education and Prevention Program. *NIH.* 1997;97-4051:iii-86. Kelly HW, et al. *Pharmacology: a Pathophysiologic Approach* 5th ed. New York: McGraw-Hill; 2002. p. 475-510.

1.1.3 Classification based on pattern of airflow limitation

Asthma may also be classified according to time trend patterns of airflow limitation monitored by peak expiratory flow (PEF) measurements (Turner-Warwick, 1977).

1.2 EPIDEMIOLOGY

Asthma is currently a worldwide problem, with increasing prevalence in both children and adults. Total prevalence is estimated to be 7.2% of the world's population (6% in adults, 10% in children) (MerckMedicus, 2007). It occurs in all countries irrespective of the level of development, but seems to be more common in affluent populations (NHLBI/WHO, 1995). Asthma has become an epidemic affecting 155 million individuals worldwide (Cookson, 1999). A prevalence rate of 7.5 – 10% has been reported for Nigeria (Chukwu *et al.*, 2000).

Despite the availability of a good number of oral and inhaled medications, the incidence, prevalence of asthma and its associated morbidity and mortality continues to rise in both children and young adults (Burr, 1987; Teo *et al.*, 1988; Aberg, 1989; Burney *et al.*, 1990; Barnes, 1993a; Rona *et al.*, 1995; Cookson, 1999). The rise in prevalence has been attributed to; (a) changes in the environment (indoor and outdoor) and involve aeroallergens e.g. domestic mites (Carrasco, 1987; Charpin *et al.*, 1988; Peat and Woolcock, 1991), (b) Climate; damp and warm climate is conducive for mite and mould growth (NHLBI/WHO, 1995), (c) Pollution of cities due to urbanization (von Mutius *et al.*, 1992; WHO, 1995), and (d) Improvement in diagnosis and increased reporting of symptoms (Rona *et al.*, 1995). Boys are affected twice as often as girls up to the age of ten, but thereafter, the incidence in both sexes is the same (Flenly, 1990). However in adults (47 – 74 years), the gender ratio reverses, with asthma being more prevalent in women. Prior to the 1960s asthma was generally not considered a

fatal disease, but the situation is different now as asthma mortality and morbidity appears to be on the rise (Sears, 1991).

1.3 RISK FACTORS IN ASTHMA PATHOGENESIS

1.3.1 Risk factors involved in the development of asthma

The risk factors for asthma can be grouped into three:

Predisposing factors

These make an individual susceptible to the disease, and include:

(i) Atopy

Atopy is the propensity to produce abnormal amounts of lgE on exposure to environmental allergens and it is apparently the strongest identifiable risk factor (NHLBI/WHO, 1995). Population studies have revealed that many asthmatic patients are atopic (NHLBI/WHO, 1995) and also that the prevalence of asthma increases with increasing levels of lgE and that those with low serum lgE levels have low prevalence of asthma (Burrows *et al.*, 1989; Sears *et al.*, 1991).

(ii) Gender

Asthma is more prevalent in the male child than the female child (NHLBI/WHO, 1995). However, the increased risk for males in childhood appears to be due to narrower airways (Lesoeuf, 1993) and increased airway tone (Smith *et al.*, 1971; Landau *et al.*, 1993) in boys that predispose them to increased airflow limitation in response to many triggers. The difference in prevalence disappears after ten years of age when the airway diameter/length ratio is the same in both sexes, probably because of changes in thoracic size that occur in males during puberty but not in females (NHLBI/WHO, 1995).

(iii) Race

Greater morbidity and mortality is observed in African-American asthmatics compared with Caucasian asthmatics probably due to socioeconomic and environmental factors (NHLBI/WHO, 1995).

Causal factors

Causal risk factors sensitise the airways and cause the onset of asthma. The most important causal factors of asthma in terms of number of people exposed are probably inhaled allergens (from, for example, domestic mites, furred animals, pollens, fungi) (NHLBI/WHO, 1995). Allergens sensitize atopic subjects by stimulating the development of specific T lymphocyte clones and the production of specific lgE antibodies (NHLBI/WHO, 1995). Once a subject is sensitized (i.e. he has developed memory T lymphocytes and specific lgE), he is predisposed to develop allergic inflammation and asthma exacerbations upon re-exposure to the same allergens.

There are indoor and outdoor allergens:

1. Indoor allergens include house-dust (domestic) mites, animal allergens, cockroach allergen and fungi. The presence of carpets and rugs in homes has increased indoor allergens, as they are ideal habitats not only for domestic mites, cockroaches, and other insects, but also moulds and bacteria.

(i) Domestic mites: Domestic (house-dust) mites are the most common potential indoor allergen and a major cause of asthma worldwide (Platts-Mills, 1992; Sporik *et al.*, 1992). House dust is composed of several organic and inorganic compounds, including fibers, mould spores, pollen grains, insects and

insect faeces, mammalian danders, mites and mite's faeces. Domestic mite allergens are present in mite bodies, secreta and excreta and constitute the main source of dust derived allergens. The principal domestic mite species are the pyroglyphid mites *Dermatophagoides pteronyssinus, Dermatophagoides farinae, Dermatophagoides microceras, Euroglyphus mainei* and *Blomia tropicalis*, which is commonly found in houses in tropical and subtropical regions (NHLBI/WHO, 1995). In addition to the pyroglyphid mites, there are storage mites, which inhabit stored food products and survive under high humidity. The allergens of domestic mites have been identified as cysteine proteases, serine proteases, and amylase; the most important ones have proteolytic activity and thus may have an easier access to the immunocompetent cells (NHLBI/WHO, 1995).

(ii) Animal allergens

Household warm-blooded animals release allergens in secretions (saliva), excretions (urine, faeces) and danders. Cats are potent sensitizers; the principal allergen, Fel d l, is found in cat pelt. Other sources of cat allergens are the sebaceous secretions, saliva and voided urine from male cats. Allergic sensitivity to dogs is not as common as to other mammals. Sources of dog allergens are dog hair and dander, and dog saliva. Allergens are also present in urine proteins of rodents such as mice and rat, cockroach, and fungi (e.g. *Penicillium, Aspergillus, Candida, Alternaria*).

2. Outdoor allergens

The most common outdoor allergens that cause asthma in susceptible people are pollens (from trees, grasses, weeds) and fungi (*Alternaria* and *Cladosporium*).

3. Drugs

Drugs that are causal risk factors for asthma include aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) (NHLBI/WHO, 1995). They may also cause asthma exacerbations i.e. they are triggers. It is yet to be established whether aspirin and other related drugs may indeed cause the development of asthma. Nevertheless, once aspirin or non-steroidal anti-inflammatory drug intolerance develops, it is present for life (Szczeklik, 1990; Hunt and Rosenow, 1993). In about 4 to 28% (depending on the methodology used) of adults with asthma (particularly in those with nasal polyps and sinusitis), but rarely in children with asthma, aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) are causal risk factors for asthma.

4. Occupational sensitizers

These are probably the only firmly documented cause of asthma in adults. Subjects develop asthma only after exposure, and in a few cases, asthma is caused and maintained only by the exposure to the sensitizing occupational agent (Table 2) (NHLBI/WHO, 1995).

Table 2: Some agents which cause asthma in selected occupations

Occupation / Field	Agent
Poultry farmers	Poultry mites, droppings and feather
Nurses	Psyllium
Bakers	Flour, amylase
Hospital workers	Disinfectants (formaldehyde, chloramines)
Sawmill workers, carpenters	Wood dust
Granary workers	Storage mites, *Aspergillus*, grass pollen
Automobile painting	Dimethyl ethanolamine diisocyanates
Anesthesiology	Enflurane
Laboratory animal handlers	Dander and urine proteins

Adapted from NHLBI/WHO, 1995.

Contributing factors

Contributing factors increase the likelihood of asthma developing upon exposure to a causal factor; they may even increase susceptibility to asthma (NHLBI/WHO, 1995). These contributing factors include;

1. Tobacco/cigarette smoke, active and passive smoking.
2. Air pollution
   (i) Outdoor pollutants e.g. nitrogen oxides, ozone, acidic aerosol, particulate matter, sulfur dioxide, carbon monoxide.

(ii) Indoor pollutants include nitric oxide, nitrogen oxides, carbon monoxide, carbon dioxide, sulfur dioxide, formaldehyde, biologicals such as endotoxin (Samet *et al.*, 1987).

Sources of these indoor pollutants include;

- Cooking on wood, kerosene or coal; burning stoves which produce carbon monoxide, nitrogen oxides and sulfur dioxide as well as respirable particles.
- Cooking with natural gas or liquid propane, which produces carbon monoxide, carbon dioxide, nitric oxide and nitrogen oxides.
- Heating with gas, wood, coal and kerosene units and fire places, which produce carbon monoxide, carbon dioxide, nitric oxide, nitrogen oxides, respirable particles and particulate soot.
- Building and furnishing with foam installations, glues, fireboard, pressed board, plywood, particle board, carpet backing and fabrics that contain the volatile organic compound formaldehyde, and using paints or other materials that release isocyanates.

Among problems related to indoor pollution are nose irritation, respiratory infections, bronchitis and lung cancer as a result of respirable particles; nose irritation, impaired lung function and increased infections in children as a result of nitrogen oxides; difficulty in breathing and asthma symptoms as a result of formaldehyde.

3. Viral respiratory infection

There is a temporal association between viral respiratory infections and the development of asthma in childhood (NHLBI/WHO, 1995).

4. Small size at birth

Disproportionate fetal growth (large head and small trunk), which is often associated with a birth weight of less than 2.5 kg may carry an increased risk of developing asthma during childhood or adolescence (Schwartz *et al.*, 1990). The mechanism though unclear may involve reduced airway size and caliber, increased susceptibility to allergen sensitization, increased susceptibility to viral infections, and consequent viral-induced enhanced airway hyperresponsiveness.

5. Diet

The influence of diet on asthma is unclear, but there are some reports of the protective roles of breast milk, abstinence from egg and inclusion of fish in the diet (Chandra *et al.*, 1985; NHLBI/WHO, 1995).

1.3.2 Risk factors that exacerbate asthma (triggers)

Triggers are risk factors that cause asthma exacerbations by inducing inflammation or provoking acute bronchoconstriction or both. Generally, they cannot cause asthma to develop initially, but can exacerbate asthma once it is present. Amongst triggers are further exposures to causal factors that have already sensitized the airways of the person with asthma (NHLBI/WHO, 1995). Triggers may vary from person to person and from time to time, and include allergens, occupational sensitizers, air pollutants, irritants (e.g. wood smoke, kerosene stove smoke, tobacco smoke, household sprays, polishes, cooking oils), viral respiratory

infections (Busse *et al.*, 1993) exercise and hyperventilation (Blackie *et al.*, 1990; Bar-Yishay and Godfrey, 1993), adverse weather conditions (such as freezing temperatures, high humidity, and thunderstorms), sulphur dioxide, food and food additives e.g. monosodium glutamate, some food preservatives and colouring agents (Bousquet *et al.*, 1992). Preservatives in many beverages (including wine and beer) and in some foods contain metabisulphite, which may release sufficient sulphur dioxide to provoke bronchoconstriction (Bousquet *et al.*, 1992). Others are drugs (NSAIDs, β-blockers), extreme emotional expression, rhinitis, sinusitis, gastroesophageal reflux, menstruation (Chien and Mintz, 1993), and pregnancy (Barron and Leff, 1993).

1.4 PATHOGENESIS OF ASTHMA

An understanding of the pathogenesis of asthma is essential to achieving a rational pharmacotherapy (Rang *et al.*, 1998; Boushey, 1998). Chronic airway inflammation involving many cell types and inflammatory mediators underlies the bronchial hyperresponsiveness of asthma (Kobzik and Schoen, 1994; Greene and Harris, 2000). Inflammation is present even when patients are asymptomatic (Greene and Harris, 2000).

The current concept of asthma pathogenesis is that a characteristic chronic inflammatory process involving the airway wall causes the development of airflow limitation and increased airway hyperresponsiveness, thereby predisposing the airways to narrow in response to a variety of stimuli. Increase in the inflammatory response is associated with the recurrent episodes of symptoms and reversible airflow limitation that characterize disordered lung function

(NHLBI/WHO, 1995). Mechanisms of airway inflammation in asthma involve a cascade of events – the release of immunological mediators in both lgE dependent, T-lymphocyte dependent mechanisms and lgE independent, T-lymphocyte dependent mechanisms. The net result is the recruitment of inflammatory cells from the circulation which involves upregulation of endothelial adhesion molecules and their reciprocal ligand expanded on leukocytes. No single pathogenic mechanism accounts for the features of clinical asthma. At least five have been proposed which can co-exist in the same patient (Flenley, 1990). These are:

Mast cell activation

This is the acute phase. The pivotal step is the activation of T-lymphocytes by antigens presented by the antigen presenting cell (APC) (e.g. dendritic cells, macrophages and B-lymphocytes) and involves the major histocompatibility complex, antigen-MHC complex binds to the surface receptors of T-lymphocytes, thereby activating cellular immune defense mechanisms.

Type 2 helper T-lymphocytes (Th 2) produce inter leukin-4 (IL-4) which signals B-lymphocyte cells to synthesize lgE (Coffman *et al.*, 1988). IgE binds to its high-affinity receptor (FC∈RI) on mast cells and other effector cells (Cookson, 1999), hence the individual is sensitized. When a sensitized individual is exposed to allergen/stimuli, there is cross-linking of lgE/FC∈RI complexes leading to mast-cell degranulation and the initiation of inflammation.

Activation of cholinergic vagal fibers

This also contributes to the immediate reaction (Kobzik and Schoen, 1994). Activation of the efferent cholinergic vagal fibres leads to bronchoconstriction secondary to the stimulation of the sub epithelial afferents by inflammatory mediators e.g. histamine. The fact that anticholinergics alone are not particularly effective in asthma means that this mechanism does not play a major role (Flenley, 1990).

Contraction of the airway vascular smooth muscle

The vascular supply to the sub-mucosa originates from the vessels on the external surface of the airways, which then supply the sub mucosa by vessels running between the airway smooth muscle fibers. This anatomical arrangement means that the contraction of these smooth muscles could occlude venous drainage from the sub mucosal space thereby increasing vascular engorgement and mucosal edema and ultimately enhancing airway narrowing.

Airway Inflammation

This accounts for the late-phase reaction. The current concept of asthma pathogenesis is that a characteristic chronic inflammatory process involving the airway wall causes the development of airflow limitation and airway hyperresponsiveness, thereby predisposing the airways to narrow in response to various non-specific stimuli (NHLBI/WHO, 1995).

The mechanisms of airway inflammation in asthma involve a cascade of events; ultimately resulting in the recruitment of inflammatory cells from the circulation to asthmatic airways via the endothelial adhesion molecules or their

activation *in situ.* These cells include mast cells, macrophages, eosinophils, lymphocytes, dendritic cells, basophils, neutrophils and platelets (NHLBI/WHO, 1995; Barnes *et al.*, 1998).The immunologic cascade and the subsequent inflammatory reaction result from an interaction of inflammatory mediators released by the resident and infiltrating inflammatory cells (Kay 1991; Gauldie *et al.*, 1993; Holgate, 1993), and involves infiltration of cytokine-releasing T-cells and activation of inflammatory cells particularly eosinophils, with the resultant release of mediators e.g. leukotriene C_4 (LTC_4), leukotriene D_4 (LTD_4),platelet activating factor (PAF), and eosinophilic granule proteins, namely eosinophilic major basic protein (EMBP) and eosinophilic cationic protein (Chung, 1993).

The released mediators, directly or indirectly (via axon reflex release of excitatory neuropeptides), cause the inflammatory features of vasodilation, edema, mucus secretion and bronchospasm, ultimately resulting in bronchial hyperresponsiveness (Chung, 1993; Rang *et al.*, 1998). Also, the eosinophilic products are cytotoxic to various cells particularly to the airway epithelium. Damage to the airway epithelium leads to shedding of the mucosa and exposure of airway nerves, which may be directly stimulated by other mediators like bradykinin (Barnes, 1986; Chung, 1993). The resultant effect is bronchial hyperresponsiveness, bronchoconstriction, mucus production and airway microvascular leakage (Chung, 1993; Rang *et al.*, 1998). Proteins such as major basic protein (MBP) may directly induce airway hyperresponsiveness (Gundel *et al.*, 1991). Eosinophil is probably the link between inflammation and airway hyperresponsiveness.

Other cells like mast cells, airway epithelial cells also contribute to the sustenance of chronic inflammation through the release of inflammatory mediators.

1.4.1 Mechanisms in allergic (atopic) asthma pathogenesis

Atopy is defined as the propensity to produce lgE mediated responses on exposure to non-specific stimuli. Allergy originally refers to 'deviation from the original state' when vaccination and injection with proteins and sera led to a variety of harmful immune reactions. Allergy is now used to describe type 2 helper (Th2) -associated immune reactions to common environmental proteins, (allergens); presently, allergy is virtually synonymous with atopy (Barnes, 1999). Allergy is acknowledged as a major risk factor for asthma, and inhalation challenge of atopic (allergic) asthmatics with specific allergen evokes a biphasic response comprising discrete acute – and late – phase reactions separated in time by several hours (Galli and Costa, 1995; O'Byrne, 1997; Barnes *et al.*, 1998b; Holt *et al.*, 1999). Allergens cause disease because of the intensity of the immune reaction which they provoke (Cookson, 1999). Typical allergen sources include house dust mite, grass pollens, and animal danders.

In atopic or allergic asthma, genetically disposed individuals are sensitized on exposure to allergens and on subsequent re-exposure, asthma attack results (Rang *et al.*, 1998). This sensitization involves the production of lgE-antibodies which bind to lgE receptors on mast cells on the airway mucosa. The processed allergen is presented to naive Th-precursor (ThP) cells by antigen presenting cells (APCs) within a cytokine milieu that favours the selective expansion of Th2-

polarized memory cells (Th2M), resulting eventually in production of specific lgE by B lymphocytes. Re-exposure to allergen elicits an acute phase response that is triggered through cross linking of antibody and loaded high-affinity lgE receptors on mast cells/basophils (Holt *et al.*, 1999). This is followed after several hours by a late-phase response involving inflammatory mediator production by Th2 cells and eosinophils with additional contributions from mast cells/basophils, $CD8^+$ T-cells, neutrophils, platelets and probably macrophage (Holt *et al.*, 1999). On re-exposure, the asthmatic attack consists of two phases;

Immediate / Acute phase:

This occurs within minutes after re-exposure and is mainly due to bronchospasm (Rang *et al.*, 1998) associated with the immediate fall in forced expiratory volume in one second (FEV_1) (Boushey, 1998), mucus secretion and edema.

The acute phase of allergic asthma reaction represents classical immediate-type hypersensitivity reaction, triggered through allergen-induced cross linking of specific lgE anti-body bound to mast cells through high-affinity receptors (Holt *et al.*, 1999) IgE binds to its high affinity receptor FC∈RI on mast cells and other effector cells (Corry and Kheradmand, 1999). Exposure to allergen in a sensitized individual leads to cross linking of lgE / FC∈RI complexes, which causes mast-cell degranulation and the initiation of inflammation. The mast cells on degranulation release a range of granule associated pre-formed mediators which are responsible for the immediate symptoms of the acute allergic response (in this case, bronchoconstriction), and which contribute directly to some aspects (e.g.

edema) of the late phase reaction (Holt *et al.*, 1999). Mast cells also release a variety of chemokines and cytokines, which contribute to recruitment and activation of secondary effectors particularly eosinophils (O'Byrne, 1997; Barnes *et al.*, 1998a), which are the hall marks of the late phase of allergic reaction.

In the case of airborne antigens, the reaction occurs first on sensitized mast cells on the mucosal surface; the resultant mediator release opens the mucosal intercellular tight junctions and enhances penetration of antigen to the more numerous sub mucosal mast cells and eosinophils; and subsequent activation (Kobzik and Schoen, 1994). The net result is the release of additional mediators and release of histamine and serotonin from their granules. In addition, direct stimulation of subepithelial vagal (parasympathetic) receptors provokes bronchoconstriction through both central and local reflexes (including those mediated by unmyelinated sensory C-fibers).

At the cellular epicenter of this allergic response are $CD4^+$ T-helper memory cells (Corry and Kheradmand, 1999). These produce an array of cytokines that directly or indirectly programme the leucocytes that are responsible ultimately for acute and chronic allergic inflammation in the airways (Holt *et al.*, 1999). The principal type 2 helper (Th 2) cytokines implicated in this process include interleukin (IL)-4, which is required to drive production of allergen-specific immunoglobulin E (lgE) (Coffman *et al.*, 1988), IL-3 which controls mast-cell and basophil development (Hogan *et al.*, 1998) and IL-5, which in conjunction with IL-3 and granulocyte-macrophage colony stimulating factor

(GM-CSF), regulates the eosinophil component of allergy (Kay, 1997; Hogan *et al.*, 1998).

The mediators of lgE triggered reactions include primary and secondary mediators (Kobzik and Schoen, 1994).

Primary mediators include: (a) Histamine which causes bronchoconstriction, increased vascular permeability and increased bronchial secretions and (b) eosinophilic and neutrophilic chemotactic factors which attract eosinophils and neutrophils e.g. leukotrienes.

Secondary mediators include: (a) Leukotrienes (LTC_4, LTD_4 and LTE_4) which cause prolonged bronchoconstriction, and increase vascular permeability and mucus secretion (Kobzik and Schoen, 1994), (b) Platelet activating factor (PAF) which causes aggregation of platelets, (c) Cytokines such as IL-l, Tumor necrosis factor (TNF), and IL-6, some of which have been found to exist in a preformed state within the mast cell granules, and (d) Prostaglandin D_2 which elicits bronchoconstriction and vasodilatation.

Late-phase:

This starts 4 – 8 hours later and may last for 12 – 24 hours (Kobzik and Schoen, 1994). The mediators released in the acute phase recruit additional mediator-releasing cells from the blood (e.g. eosinophils) signaling the initiation of the late phase (Kobzik and Schoen, 1994). This phase involves production of inflammatory mediators principally by Th2 cells and eosinophils. Mast cells/basophils, macrophages, $CD8^+$ T-cells, neutrophils and platelets may contribute to the production of inflammatory mediators. The late phase response is

characterized by airway perivascular edema, mucus plugging and the presence of activated Th2 cells which are the principal source of the cytokines responsible for the sustained recruitment and activation of eosinophils (Holt *et al.*, 1999).

This phase is essentially a progressing inflammatory reaction initiated during the acute phase. The inflammation in asthma is unique in that there is infiltration not only by the usual inflammatory cells, but also and more specifically, by activated cytokine-releasing Th2 cells and eosinophils, whose products cause damage and loss of epithelium with consequent repercussions. The epithelial loss means that irritant receptors and C fibres are more accessible to irritant stimuli; this is thought to be the basis of the hyperreactivity (Corrigan and Kay, 1992).

1.4.2 Non-atopic asthma

The pathogenetic details of non - atopic asthma have not been well studied, but it is most frequently triggered by respiratory tract infection mainly due to viruses (Busse *et al.*, 1992). It is postulated that virus-induced inflammation of the respiratory mucosa lowers the threshold of the subepithelial vagal receptors to irritants (Boushey, 1998).

Not all the features of asthma can be explained by the atopic allergic reaction. Some asthmatic adults have no evidence of immediate hypersensitivity to allergens and most severe exacerbations of asthma appear to be provoked by viral respiratory infection rather than by recent exposure to an unusual quantity of allergen. The severity of asthma symptoms correlates poorly with the quantity of allergen in the atmosphere. Also bronchospasm can be provoked by non-antigenic

stimuli such as distilled water, exercise, cold air, sulphur dioxide and rapid respiratory maneuvers (Boushey, 1998).

1.5 INFLAMMATORY CELLS AND MEDIATORS OF ASTHMA

1.5.1 Inflammatory cells

These are the various cell types implicated in the inflammatory process in asthmatic airways. Inflammatory cells involved in asthma include mast cells, granulocytes and lymphocytes, and airway structural cells (e.g. airway epithelial cells). Granulocytes include mast cells, eosinophils, basophils, and macrophages, and are known to contain many granules. Lymphocytes lack granules and manufacture other kinds of proteins that are involved in the inflammatory process.

Mast cells: Mast cells are increased in numbers in asthmatic tissue, and are usually in a degranulated state. They not only release histamine, but also contribute to chronic inflammatory mechanisms, as they produce cytokines such as TNF-α under IgE-dependent activation (Chung, 1993).

Basophils: The basophils in the circulating blood are similar to the large mast cells located immediately outside many of the capillaries in the body (pericapillary). IgE also attaches to basophils, which rupture to release histamine and other mediators.

Eosinophils: The eosinophil is the inflammatory cell most closely associated with asthma (Murray and Nadel, 2000). Unlike mast cells that are fixed in various tissues throughout the body, eosinophils are very mobile and exhibit chemotaxis. In association with asthma, elevated numbers of eosinophils have been identified

in various tissue compartments, including (Wardlaw *et al.*, 1988; Murray and Nadel, 2000):

a) Biopsies of lung tissue; particularly in the bronchial wall of patients with asthma.

b) Peripheral blood circulation; the increase in peripheral blood eosinophils in asthma is probably due to inflammatory cells or mediators coming from the lungs to cause increased production of eosinophils by the bone marrow.

c) Fluid specimens obtained from the lung using a bronchoscope; with this method, fluid that "washes out" the bronchi and alveoli, termed bronchoalveolar lavage (BAL) fluid, is obtained by inserting a fiberoptic scope down the air passages and into the lungs.

d) In secretions or sputum of patients with asthma; a sputum specimen is basically a coughed-up sample of the mucus that is coating the airway lining.

The mobility of eosinophils indicates that they can be stimulated to leave the blood stream and enter the tissues. Mast cells and basophils release an eosinophils chemotactic factor that causes eosinophils to migrate towards the area of inflammation. In asthma, eosinophils move from the blood into the bronchi (as documented in bronchial biopsies and in BAL fluid) and onto the surface of the airway lining (as documented in sputum). The levels of eosinophils in each of these compartments demonstrate a rough correlation with the disease state of asthma and even with the clinical severity of asthma. When activated, eosinophils release several pre-formed mediators from within their granules. These granules contain several proteins, among which are eosinophilic cationic protein, major

basic protein (MBP), eosinophils-derived neurotoxin (EDN), and eosinophils peroxidase (Chung, 1993).

Eosinophils appear to play a role in virtually all types and severities of asthma. Although mast cells play a central role in allergic asthma, the activity of eosinophils is evident in both allergic and non-allergic asthma. Also, there is clear documentation of increased eosinophil numbers (eosinophilia) and increased eosinophil activation in the blood, lungs, and sputum of asthmatics (Chung, 1993; MerckMedicus, 2007).

Lymphocytes: Asthma is also characterized by airway submucosal infiltration of lymphocytes (Chung, 1993). Lymphocytes lack granules and manufacture other kinds of proteins that are involved in the inflammatory process. B lymphocytes have the important function of manufacturing antibodies, while T lymphocytes play an essential role in events that lead to airway inflammation by orchestrating the entire inflammatory process. T cells release a variety of cytokines that communicate with most other cells in the inflammatory process. Cytokines from T cells can, for instance, activate B cells to make antibodies (even controlling the choice between making lgM, lgG or lgE) or activate eosinophils (Coffman *et al.*, 1988; Walker *et al.*, 1991)

T cells thus initiate and orchestrate a cascade of cytokine-mediated airways inflammation. T cells and their cytokines provide a common pathway for allergic (i.e., lgE-mediated) and non-allergic asthma.

Role of T-lymphocytes in airway inflammation

In addition to the lgE-dependent, T-lymphocyte dependent mechanism described above, T-lymphocytes contribute to the induction and maintenance of allergic reaction through an lgE-independent, T-lymphocyte dependent mechanism. T-lymphocytes are proinflammatory cells in their own right. In addition to being helper cells for promotion of lgE production by B lymphocytes, T lymphocytes through the secretion of cytokines (GM-CSF, IL-3, IL-4, IL-5, IL-9, IL-13) and other protein products capable of stimulating polymorphonuclear leukocytes, attract and activate eosinophils, and other leucocytes, thereby propagating and sustaining airway inflammation (NHLBI/WHO, 1995). Indeed, studies have shown that atopic patients have an increased proportion of Th 2 lymphocytes in the peripheral blood and airways (Del Prete, 1992; Holgate, 1993). T lymphocytes are also capable of modulating eosinophil adherence, locomotion and activation (Holgate, 1993; Montefort *et al.*, 1993) and of stimulating eosinophils, to cause tissue damage (NHLBI/WHO, 1995).

Other components of airway inflammation mechanism are:

Adhesion molecules: These include E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular-cell adhesion molecule-1 (VCAM-1). ICAM-1 may play an important role in the adhesion and subsequent recruitment of eosinophils in asthma (Chung, 1993). IL-4 increases the expression of VCAM-1 on endothelial and airway epithelial cells, and this may be important in eosinophils and lymphocyte trafficking (Schleimer *et al.*, 1992) ; while IL-1 and TNF-α increase the expression of ICAM-1 in both vascular endothelium and airway epithelium

(Tosi *et al.*, 1992) The increase of neutrophils, eosinophils, and lymphocytes in the airway mucosa of worsening asthma is paralled by increased expression of specific adhesion molecules on post capillary venular endothelial cells (Holgate, 1993; Montefort *et al.*, 1993).

The upregulation of these adhesion molecules by mediators and cytokines is the first step in the cascade of events that enables leukocytes to marginally cross the post-capillary venule wall and subsequently migrate to the mucosa. Subsequently, endothelial adhesion molecules engage the activated form of the complementary ligands (either lectins or integrins) on activated leukocytes.

Constitutive/Structural cells: In asthma, normal airway resident cells generate a number of cytokines that may contribute to maintenance of chronic airway inflammation. The epithelium is a source of IL-6, IL-8, GM-CSF, IL-18 and TNF-α. The endothelium can generate IL-8, IL-5 and GM-CSF; and fibroblasts are important source of the mast cell growth factor, stem cell factor, GM-CSF and IL-8 (Gauldie *et al.*, 1993).

1.5.2 Inflammatory mediators of asthma

Inflammatory mediators are secreted cell products which exert functional effects, and produce the typical pathophysiological changes of asthma (Barnes *et al.*, 1998a). Sources of inflammatory mediators are: (a) Inflammatory cells such as mast cells, eosinophils, macrophages, T lymphocytes, dendritic cells, basophils, neutrophils and platelets, and (b) Structural cells such as airway epithelial cells, smooth muscle cells, endothelial cells, and fibroblasts are all capable of

synthesizing and releasing inflammatory mediators (Levine, 1995; John *et al.*, 1997; Saunders *et al.*, 1997; Barnes *et al.*, 1998a).

The activation of these inflammatory and structural cells leads to the release of inflammatory mediators that effect the typical pathophysiological changes of asthma (Barnes, 1996; Barnes *et al.*, 1998a). More than fifty different mediators have been identified in asthma (Barnes *et al.*, 1998a).

Many of the key enzymes involved in the synthesis of inflammatory mediators have been cloned (e.g. 5-lipoxygenase involved in the synthesis of Cysteinyl leukotrienes), leading to the development of specific inhibitors that have useful therapeutic effects e.g. 5-lipoxygenase inhibitors, which inhibit the synthesis of leukotrienes (Barnes *et al.*, 1998a). 5-lipoxygenase inhibitors have been shown to have beneficial effects in the control of clinical asthma and are now available for clinical use (Israel *et al.*, 1996).

Many inflammatory mediator receptors have been cloned e.g. B receptor for platelet-activating factor (PAF) (Honda *et al.*, 1991), receptors for cytokines and growth factors (Kishimoto *et al.*, 1994). Receptor cloning has enabled the elucidation of the signal transduction pathways involved in receptor function. For non-cytokine mediators, inflammatory receptors are often coupled, through G proteins (Gs and Gi), to phosphoinositide hydrolysis and may be other pathways. Cytokine receptors signal through complex pathways, including mitogen activated protein (MAP) kinases and other protein kinases, resulting in the activation of transcription factors. Transcription factors regulate the expression of many genes, including inflammatory genes.

Effects of inflammatory mediators

Inflammatory mediators produce many effects in the airways, including bronchoconstriction, plasma exudation, mucus secretion, neural effects and attraction and activation of inflammatory cells (Table 3); these are acute inflammatory responses (Barnes *et al.*, 1998a). Mediators also effect long lasting (chronic) structural changes in the airways such as fibrosis, hyperplasia and hypertrophy of airway smooth muscle (Knox, 1994), and proliferation of airway vessels (angiogenesis) (Kuwano *et al.*, 1993). There may also be proliferation of mucus secreting cells and changes in the innervation of the airways; these are chronic inflammatory changes and are mediated by the secretion of distinct mediators such as cytokines and growth factors. Some of these changes may be irreversible, leading to fixed narrowing of the airways.

Table 3: Effects of inflammatory mediators implicated in asthma

Mediator	Broncho-constriction	Airway secretion	Plasma exudation	Neural Effects	Chemotaxis	Airway Hyper-responsiveness
Histamine	++	+	+	+	+	-
Serotonin	-	?	_	_	-	-
LTC_4, LTD_4, LT_4	+++	++	++	±	+	±
PAF	++	+	++	+	+++	++
PGD_2 , $PGF_{2\alpha}$	++	+	?	+	?	+
Adenosine	(+)	?	(+)	+	±	-

-, no effect; ±, possible effect; +, small effect; ++, moderate effect; +++, strong effect; uncertain or undetermined effect; parentheses indicate indirect effects.

*Adapted from Barnes *et al.*, 1998a

1.5.2.1 Amine mediators

A. Histamine

Histamine [2-(4-imidazole) ethylamine] was the first mediator implicated in the pathophysiological changes of asthma, when it was found to mimic several features of the disease (Barnes *et al.*, 1998a). Elevated concentrations of histamine have been reported in the bronchoalveolar lavage fluid of asthmatic patients (Wenzel *et al.*, 1988; Liu *et al.*, 1990). Histamine is synthesized and released by mast cells in the airway wall and by circulating and infiltrating basophils (Schroeder and MacGlashan, 1997; Barnes *et al.*, 1998a). Histamine may be released when mast cells and basophils degranulate in response to various immunological (lgE or cytokines) or non-immunological (compound 40/80, calcium ionophore, substance P, opinoids or hypo-osmolar solutions) stimuli (Barnes *et al.*, 1998a).

Four types of histamine receptors have been recognized pharmacologically, namely, H_1, H_2, H_3 (Hill, 1990) and H_4 (Hough 2001). H_1 receptors mediate most of the effects of histamine that are relevant to asthma (Barnes *et al.*, 1998a).

Effect of histamine on the airway

Histamine stimulates phosphoinositol hydrolysis in airway smooth muscle (Grandordy and Barnes, 1987; Hall and Hill, 1988; DayKin *et al.*, 1993), and increases the concentration of inositol-1,4,5-triphosphate (IP_3) in airway smooth muscle, which may result in an increase in intracellular Ca^{2+} (Hardy *et al.*, 1996). Histamine is a bronchoconstrictor, H_1 receptor antagonists e.g. chlorpheniramine and terfenadine are known to cause bronchodilation in asthmatic patients but not

in normal individuals (Eiser *et al.*, 1981; Cookson, 1987). Histamine increases airway mucus secretion via H_2 receptors in humans (Shelhamer *et al.*, 1980) and also directly activates rodent airway goblet cells via H_2 receptors (Tamaoki *et al.*, 1997).

On airway nerves, histamine is known to act on prejunctional H_1 receptors to enhance acetylcholine release (Barnes, 1992). In many species, the bronchoconstricting effect of histamine is partially mediated by a vagal cholinergic reflex.

Histamine also influences the release of cytokines and inflammatory mediators from a variety of inflammatory and immune cells (Falvs and Merety, 1992). The relevance of this is uncertain, because H_1 antagonists do not appear to have significant anti-inflammatory effect (Barnes *et al.*, 1998a). Histamine is a selective chemoattractant for eosinophils (Clark *et al.*, 1975) and activates human eosinophils (Raible *et al.*, 1992) and alveolar macrophages (Cluzel *et al.*, 1990).

B. ***Serotonin***

Though serotonin [5-hydroxytryptamine (5-HT)] causes bronchoconstriction in most animal species, it is not a constrictor of human airway; hence interest in 5-HT is minimal as its relevance in asthma appears doubtful (Barnes *et al.*, 1988; Barnes *et al.*, 1998a). Nevertheless, plasma serotonin levels are elevated in asthma and are significantly related to asthma severity (Lechin *et al.*, 1996).

Serotonin does not constrict human airway smooth muscle *in vitro*, but constricts pulmonary vessels (Raffestin *et al.*, 1985). In animals, serotonin

probably acting via 5-HT_3 receptor increases acetylcholine release from airway nerves (Takahashi *et al.*, 1995).

C. *Adenosine*

Adenosine is a purine nucleoside that plays a key role in nucleic acid energy and protein metabolism. As an extracellular autacoid generated by 5-nucleoside cleavage of adenosine 5-trionophosphate, it is a powerful mediator acting through specific cell surface purinoreceptors. Mast cells release adenosine in response to lgE cross-linking and other stimuli for mast cell activation (Marquardt *et al.*, 1986).

Adenosine elicits little or no contraction of normal airways, but is a potent constrictor of asthmatic airways (Bjorck *et al.*, 1992). Adenosine which accumulates in inflamed mucosa under conditions of cell stress and hypoxia, contributes as a mediator of bronchoconstriction in both acute and chronic asthma (Holgate, 2005). It is likely that the bronchoconstricting effects of adenosine are indirect, resulting from the activation of mast cell degranulation, because adenosine causes histamine release from mast cells (Church *et al.*, 1986; Forsythe *et al.*, 1999). This bronchoconstriction is blocked by histamine and leukotriene antagonists and may be due to the release of mediators from mast cells in asthmatic airways. In vitro studies on isolated human mast cells and basophils revealed that adenosine and A_2 receptor selective analogues augmented inflammatory mediator release from mast cells by stimulating A_2 receptors (Holgate, 2005). Also adenosine acting as a vasodilator can function

synergistically with several inflammatory mediators, leading to increased vascular permeability and airway edema (Barnes *et al.*, 1998a).

1.5.2.2 Lipid derived mediators

A. ***Prostanoids***

Prostanoids include prostaglandins (PGs) and thromboxanes (Tx), which are generated from arachidonic acid, usually by the action of cyclooxygenase (COX). Bronchoalveolar lavage studies have demonstrated increased concentrations of $PGF_{2\alpha}$, PGD_2 and TxB_2 in asthmatics, with PGD_2 having the highest concentration (Liu *et al.*, 1990; Smith *et al.*, 1992; Dworski *et al.*, 1994; Oosterhoff *et al.*, 1995). $PGF_{2\alpha}$, PGD_2 and thromboxanes cause bronchoconstriction of human airways in vitro (Barnes *et al.*, 1998a) and are potent bronchoconstrictors in asthmatic patients (Hardy *et al.*, 1984; Fish *et al.*, 1984; Jones *et al.*, 1992; Saroea *et al.*, 1995), while PGE_2, PGI_2 are bronchodilators (Walter and Davies, 1982; Knight *et al.*, 1995).

B. ***Leukotrienes*** **(LTs)**

Leukotrienes ('leuko' because they are found in white cells and 'trienes' because they contain a conjugated triene system of double bonds), play an important role in the pathophysiological changes of asthma and leukotriene (LT) receptor inhibitors used in the management of asthma have been developed. LTs are potent lipid mediators produced by arachidonic acid metabolism in cell or nuclear membranes (Barnes *et al.*, 1998a). They are derived from arachidonic acid, which is released from membrane phospholipids via the activation of phospholipase A_2. Arachidonic acid is subsequently metabolized by the enzyme

5-lipoxygenase (5-LO) to the unstable leukotriene A_4 (LTA_4). LTA_4 is converted to either LTB_4, or to a series of cysteinyl-leukotrienes (Cys-LTs) starting with LTC_4 which is further metabolized to LTD_4, LTE_4 and LTF_4. LTC_4, LTD_4, and LTE_4, together constitute 'slow-reacting substance of anaphylaxis (SRS-A)', a substance generated in guinea-pig lung during anaphylaxis. LTB_4 is produced mainly by neutrophils and the Cys-LTs mainly by eosinophils, mast cells, basophils and macrophages. LTB_4 is an important mediator in all types of inflammation, while the cysteinyl-leukotrienes (LTC_4, LTD_4 LTE_4 and LTF_4) are of particular importance in asthma (Rang *et al.*, 1999). Cys-LTs act via Cys-LT receptors, Cys-LT_1 and Cys-LT_2, which have been pharmacologically characterized. Cys-LT_1 receptors mediate all the known airway effects of Cys-LTs in human cells (Coleman *et al.*, 1995; IUPHAR, 1995).

Cys-LTs are very potent bronchoconstrictors being approximately 1000-5000 times more potent than histamine as bronchoconstrictors of normal and asthmatic human airways via Cys-LT_1 receptors (Barnes *et al.*, 1984; Drazen, 1988; Krell *et al.*, 1990a). LTB_4 has no direct effect on human airway smooth muscle and does not cause bronchodilation after inhalation in asthmatic patients, even when combined with PGD_2 (Black *et al.*, 1989). Cys-LTs potently elicit increased airway vascular permeability leading to airway edema (Arakawa *et al.*, 1993; Henderson, 1994). Cys-LTs increase mucus secretion, both directly via effects on goblet cells and sub-mucosal gland cells (Goswami *et al.*, 1989; Hoffstein *et al.*, 1990), and indirectly via the activation of airway nerves, leading to reflex secretion from submucosal glands (Maroni *et al.*, 1982). Cys-LTs effect

the release of IL-5 (Underwood *et al.*, 1996), and may increase eosinophilic infiltration (Barnes *et al.*, 1998a). The importance of Cys-LTs in asthma has been highlighted by the clinical usefulness of LT receptor antagonists and 5-LO inhibitors in the management of asthma. Cys-LT production is increased in asthma in response to various challenges that worsen asthma. Cys-LTs are potent mediators of bronchoconstriction, plasma exudation and mucus secretion, and probably increase in eosinophilic inflammation. However, LTs, play a variable role in asthma as some patients respond very well to anti-LTs, whereas others derive little benefit. Anti-LTs are less effective than corticosteroids in asthma treatment, inferring that other inflammatory mediators play important roles in most patients (Barnes *et al.*, 1998a).

C. *Platelet-activating factor (PAF)*

PAF has long been implicated in the pathophysiological mechanisms of asthma, because exogenous PAF closely mimics many of the clinical features of asthma, including airway hyperresponsiveness (Barnes *et al.*, 1998a). PAF is an ether-linked phospholipid synthesized in a wide variety of inflammatory cells, including platelets, neutrophils, basophils, macrophages and eosinophils (Barnes *et al.*, 1989; Chung, 1992). PAF produces acute bronchoconstriction when inhaled by asthmatic patients (Barnes *et al.*, 1989). PAF has little direct effect on human airway smooth muscle contraction *in vitro*, but may elicit constriction through the release of other mediators (Johnson *et al.*, 1992). PAF induced bronchoconstriction is not inhibited by H_1 receptor antagonists (Chung *et al.*, 1988) or thromboxane antagonist (Stenton *et al.*, 1990), but can be inhibited by

LT antagonists (Spencer *et al.*, 1991; Kidney *et al.*, 1993), suggesting the involvement of LTD_4 in this response. PAF is a very potent inducer of vascular engorgement and increased vascular permeability in the airways, leading to plasma exudation of protein rich fluid into the airway lumen (O' Donnell and Barnett, 1987; Evans *et al.*, 1989).

PAF is a potent activator of inflammatory cells; it stimulates chemotaxis and adhesion of eosinophils and neutrophils *in vitro* (Kimani *et al.*, 1988; Kroegel *et al.*, 1988; 1991). PAF enhances LTC_4 release from eosinophils in asthmatic patients (Shindo *et al.*, 1996) and induces eosinophilic infiltration (Henocq and Vargaftig, 1986). However, clinical studies with PAF receptor antagonists have been disappointing.

Other lipid mediators include hydroperoxyeicosatetraenoic acids (HPETEs), mono- and di- HETEs, and lipoxins (Barnes *et al.*, 1998a).

1.5.2.3 *Cytokines*

Cytokines are small protein mediators that play an integral role in the coordination and persistence of inflammation in asthma, although the precise role of each cytokine remains to be determined (Barnes *et al.*, 1998a). With respect to asthma and allergy, cytokines can be classified thus:

(a) *Lymphokines*: These play an important role in immunoregulation e.g. interleukin-2 (IL-2), IL-3, IL-4, IL-5, IL-13, IL-15, IL-16 and IL-17.

(b) *Proinflammatory cytokines*: These are involved in most types of inflammation and appear to amplify and perpetuate the ongoing inflammatory response. They may be important in disease severity and resistance to anti-

inflammatory therapy in asthma (Barnes *et al.*, 1998a). They include IL-1, tumor necrosis factor α (TNF-α), IL-6, IL-11, granulocyte-macrophage colony-stimulating factor (GM-CSF) and stem cell factor (SCF).

(c) *Antiinflammatory cytokines* e.g. IL-10, IL-Ira (Interleukin-1 receptor antagonist), interferon-r (IFN-r), IL-12, and IL-18: Although most cytokines initiate, amplify, or perpetuate inflammation, some cytokines appear to have an inhibitory or anti-inflammatory effect on allergic inflammation, either by blocking the expression or effects of inflammatory cytokines or by shifting the immune response away from the Th 2 pattern of cytokines (Barnes and Lim, 1998).

(d) *Growth factors*: Several growth factors have been implicated in asthma and they include; platelet derived growth factor (PDGF), transforming growth factor-β, (TGF-β), fibroblast growth factor FGF, epidermal growth factor (EGF) and insulin-like growth factor (IGF). Chronic asthma is associated with structural remodeling of the airway; with fibrosis (particularly under the epithelium), increased thickness of the airway smooth muscle layer, increased numbers of mucus-secreting cells, and angiogenesis (Redington and Howarth, 1997). These changes are presumably in response to growth factors secreted from inflammatory and structural cells in the airways (Barnes *et al.*, 1998a).

(e) *Chemokines*: Chemokines are chemotactic cytokines (8 to 10 Daltons) that are involved in attracting leukocytes into tissues. They include IL-8, which is a powerful neutrophil chemoattractant (Kameyoshi *et al.*, 1992; Rot *et al.*, 1992) and RANTES which is a powerful eosinophil chemo- attractant and activator (Barnes *et al.*, 1998a).

Role of cytokines in asthma

1. IL-4 is very important in driving the differentiation of $CD4^+$ Th precursors into Th 2-like cells. Th 2 lymphocytes produce a number of cytokines including IL-3, IL-4, IL-5, IL-9, IL-10, IL-13 and GM-CSF.
2. B lymphocytes under the influence of IL-4 produce specific lgE.
3. The development of mast cells from bone marrow cells represent a process of maturation and expansion involving growth factors and cytokines (e.g. stem cell factor and IL-3) produced by structural cells.
4. The differentiation, migration and pathobiological effects of eosinophils may occur through the effects of GM-CSF, IL-3 and IL-5.
5. Once recruited from the circulation, mature eosinophils in the presence of these cytokines change phenotype into hypodense eosinophils, which show increased survival in bronchial tissue. These eosinophils are primed for ligand-initiated generation of increased amounts of Cys-LTs and for cytotoxicity to other cells, such as those of the airway epithelium. Eosinophils themselves may also generate other cytokines.
6. Cytokines may also play an important role in antigen presentation and may enhance or suppress the ability of macrophages to act as antigen-presenting cells.
7. Growth factors such as platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β) may be released from airway inflammatory cells (such as macrophages and eosinophils) and also from structural cells (such as epithelial cells, endothelial cells and fibroblasts). These growth factors may

stimulate fibrogenesis by recruiting and activating fibroblasts or transforming myofibroblasts.

8. Growth factors may also stimulate the proliferation and growth of airway smooth muscle cells (Hirst *et al.*, 1992; Knox, 1994).
9. Cytokines and growth factors may also play a role in goblet cell hyperplasia and proliferation of mucosal blood vessels.

In summary, cytokines are involved in the development of the atopic state and the chronic inflammatory processes of asthma, ultimately contributing to the release of mediators such as histamine and Cys-LTs, airway remodeling, bronchoconstriction, and bronchial hyper responsiveness (Barnes *et al.*, 1998a).

1.5.2.4 *Proteases*

Proteases secreted in asthma include, mast cell tryptase (Barnes *et al.*, 1998a), mast cell chymase and matrix metalloproteinases (MMP) (Caughey, 1997). Neutrophil elastase, which is a serine protease derived from neutrophils, may also be involved in asthma when neutrophilic inflammation is prominent, such as in severe asthma (Wenzel *et al.*, 1997). Increased levels of tryptase have been reported in bronchoalveolar lavage fluid after allergen challenge (Wenzel *et al.*, 1988) and in induced sputum from asthmatic patients (Louis *et al.*, 1997). Also increased levels of MMP-9 in bronchoalveolar lavage (BAL) fluid from asthmatic patients have been reported (Mautino *et al.*, 1997). Tryptase increases the responsiveness of human airways to histamine *in vitro*, and this effect is more pronounced in sensitized airways (Johnson and Knox, 1997). Tryptase may also increase bronchoconstriction by degrading the bronchodilating neuropeptides,

vasoactive intestinal peptide (VIP) and peptide histidine isoleucine (Tam *et al.*, 1990). Inhaled tryptase causes bronchoconstriction and airway hyperresponsiveness in sheep, effects that are largely mediated by mast cell activation (Molinari *et al.*, 1996). Tryptase is a potent stimulant of airway smooth muscle proliferation (Brown *et al.*, 1995). Tryptase appears to be chemotactic for eosinophils and may interact with other eosinophil chemotactic factors (Walls *et al.*, 1995). It is a potent stimulant of fibroblast proliferation and collagen secretion and appears to act synergistically with other mitogens (Hartmann *et al.*, 1992; Cairns and Walls, 1997). It may therefore play a role in the characteristic sub epithelial fibrosis observed in asthmatic airways. It is also mitogenic for airway epithelial cells and increases the expression of IL-8 and ICAM-1 (Cairns and Walls, 1996).

Other inflammatory mediators of asthma include;

(a) Peptides such as bradykinin, tachykinins, calcitonin gene related peptide (CGRP), endothelins, and complement are involved in asthma (Barnes *et al.*, 1998a).

(b) Small molecules such as reactive oxygen specie (ROS) (Barnes, 1990; Repine *et al.*, 1997), nitric oxide (NO) (Barnes and Belvisi, 1993; Gaston *et al.*, 1994; Barnes, 1995), are involved in asthma.

1.6 PATHOPHYSIOLOGY OF THE AIRWAY IN ASTHMA

The normal airway lumen is relatively free of mucus; the mucus gland provides a protective layer of mucus above the epithelial cells. The bronchial wall is lined and protected by a single layer of ciliated epithelial cells and there are few

eosinophils in the bronchial wall (MerckMedicus, 2007). Generally, airway hyperreactivity /hyperresponsiveness and acute airflow limitation are predominant manifestations of disordered lung function (NHLBI/WHO, 1995).

Asthma is defined by three pathophysiological characteristics: airway inflammation, bronchial hyper-responsiveness and airway obstruction/airflow limitation.

1.6.1 Pathophysiologic features of airway inflammation

Plasma leakage from blood vessels due to increased vascular permeability contributes to bronchial wall edema which results in thickening of the bronchial wall. Eosinophils migrate from the blood stream into the bronchial wall and the airway lumen; eosinophil can release eosinophil cationic protein and leukotrienes. Also, enlarged mucus glands secrete excess mucus that can plug the airway lumen. Moreover, as the airway walls thicken due to these inflammatory reactions, the amount of airway narrowing produced by a given amount of smooth muscle contraction in asthma is much greater than that in normal airway; thus even a small contraction of bronchial smooth muscle can lead to dramatic increases in airway resistance.

1.6.2 Bronchial hyperreactivity/bronchial hyperresponsiveness (BHR).

Bronchial hyperreactivity (BHR) is a hallmark of clinical asthma, and it appears to be caused by airway inflammation. BHR is an exaggerated broncho constrictor response not only to allergens to which the subject is sensitized, but also to a range of non-specific stimuli like cold air (Holt *et al.*, 1999). BHR has been suggested to be as a result of airway inflammatory changes, altered airway

geometry including swelling of airway wall and loss of elastic recoil (James *et al.*, 1989; Hogg 1993; Holt *et al.*, 1999), and imbalance of autonomic control of the airways. Studies have shown that the degree of BHR correlates with the number of inflammatory cells recovered in BAL fluid from the airways of asthmatic patients (Murray and Nadel, 2000).

Clinically, the degree of BHR (measured in research studies by methacholine challenge) has been shown to correlate with general asthma severity, with morning peak expiratory flow rate (PEFR), with the degree of diurnal variation of PEFR, and with the frequency of inhaled beta-agonist use (when taken by patients as needed for symptoms). The degree of BHR appears to decrease when asthma is well controlled with medication. The ultimate result and significance of BHR is the airflow obstruction that occurs when an asthmatic is exposed to a trigger (MerckMedicus, 2007).

1.6.3 Airflow limitation

The recurrent episode of airflow limitation in asthma has four forms each relating to the airway inflammatory process (NHLBI/WHO, 1995). These forms are;

(i). **Acute bronchoconstriction:** This results from (a) lgE dependent release of mediators e.g. histamine, prostaglandins, leukotrienes (which are bronchoconstrictors) (Holgate, 1993) as a result of exposure to aeroallergens; this is the early asthmatic response; (b) airway hyperresponsiveness (NHLBI/WHO, 1995).

(ii). **Swelling of the airway wall**: Airway limitation also results from the edematous swelling of the airway wall (located outside the airway smooth

muscle) with or without bronchoconstriction (NHLBI/WHO, 1995). This component of the asthmatic response is similar to the reduction in airway caliber that characteristically occurs 6 – 24 hours after allergen challenge of the airway i.e. late asthmatic response (Holgate, 1993).There is mucosal thickening and swelling of the airway outside the smooth muscle and loss of elastic recoil pressure secondary to increased airway microvascular permeability and leakage (O' Donnell and Barnett, 1987; Rogers *et al.*, 1988; Evans *et al.*, 1989; Chung, 1993). Airway edema is also accompanied by the presence of exuded plasma in the airway wall interstitium and lumen, probably leading to the shedding of ciliated epithelial cells (Chung, 1993).

(iii). **Chronic mucus plug formation:** This is the more intractable airflow limitation, which takes 6 weeks or longer to resolve following corticosteroid treatment. It is dominated by increased mucus secretion that, with exuded serum proteins and cell debris, forms the mucus plug that characteristically occludes the more peripheral airways in severe asthma (NHLBI/WHO, 1995).

(iv). **Airway wall remodeling**: Chronic inflammation is increasingly being recognized as an important aspect of asthma (Redington and Howarth, 1997), and it results in structural/trophic changes in the airway leading to airway remodeling (Barnes *et al.*, 1998a). Airway remodeling generally refers to the development of specific structural changes in the airway wall in asthma accompanying long-standing and severe airway inflammation (Murray and Nadel, 2000). Some of these changes may be irreversible (even with steroids, bronchodilators or both) leading to fixed narrowing of the airways. A disturbance in the balance of various

growth and inhibitory factors in the airway mucosa secondary to chronic inflammation has been implicated (Holt *et al.*, 1999). Airway remodeling involves the following structural and trophic changes:

(a) Thickening of the airway wall (basement membrane) and fibrosis.

(b) Hypertrophy and hyperplasia of airway smooth muscle (Knox, 1994).

(c) Hyperplasia of goblet cells (Barnes *et al.*, 1998a).

(d) Enlargement of submucus glands.

(e) Angiogenesis (Kuwano *et al.*, 1993).

(f) Changes in the innervation of the airways (Barnes *et al.*, 1998a).

Generally, the lack of uniform ventilation throughout the lung causes ventilation-perfusion ratio (v/q) mismatching resulting in hypoxemia (Wilkins and Dexter, 1993). There is also air trapping and progressive hyperinflation of the lung due to hindered exhalation caused by airway obstruction. Lung hyperinflation plus increased airway resistance raises the work of breathing in asthmatics (Wilkins and Dexter, 1993).

Even in the absence of symptoms and overt airflow limitation, asthma continues to exist in the form of mild airway inflammation and airway hyperresponsiveness (Beasley *et al.*, 1989). Death resulting from asthma is most usually characterized by extensive infiltration of the airway with eosinophils, mast cells and mononuclear cells with extensive involvement of large as well as small airways (Dunnil, 1960). Between these extremes lies the common exacerbation of asthma in which mucosal swelling, excess secretion and increased airway

hyperresponsiveness are features of the inflammatory response (NHLBI/WHO, 1995).

1.7 MANAGEMENT OF ASTHMA

Asthma can be effectively controlled in most patients, although it cannot be cured (NHLBI/WHO, 1995). The goals for successful asthma management are:

(a) To control symptoms, prevent asthma exacerbation, maintain pulmonary function as close to normal levels as possible, minimize anxiety and maintain normal activity levels including exercise.

(b) Minimal (ideally no) need for p.r.n. (as needed) β_2 agonist.

(c) No emergency visits

(d) Near normal PEF and PEF circadian variation of less than 20%.

(e) Minimal or no adverse effects from asthma medications.

(f) Prevention of **irreversible** airflow limitation.

(g) Prevention of asthma mortality (NHLBI/WHO, 1995; Greene and Harris, 2000).

Asthma management could be broadly divided into: non-pharmacological and pharmacological.

1.7.1 Non-Pharmacological Management of Asthma

(a) The identification, avoidance and control of triggers are important steps in asthma management (NHLBI/WHO, 1995). Avoidance of triggers such as indoor allergens (e.g. domestic mites), outdoor allergens (e.g. pollen, fungi), pollutants like smoke (from e.g. kerosene, cigarette), sprays, occupational exposure to chemicals, and medications that exacerbate asthma (e.g. Aspirin, β-blockers) may

in the long term decrease airway inflammation and hyperresponsiveness (Platts-Mills *et al.*, 1982).

(b) Stress reduction.

(c) Prompt control of infections.

(d) Physiotherapy to develop respiratory function e.g. supervised swimming.

(e) Patient counselling and education (Greene and Harris, 2000).

Patients should be educated and encouraged to develop a partnership with their health care providers in asthma management.

1.7.2 Pharmacological management of asthma

Asthma medications which include controllers and relievers are used to prevent and reverse symptoms and airflow limitation. Controllers or long-term control medications are taken daily on a long-term basis for controlling persistent asthma. They include inhaled corticosteroids, cromolyn, nedocromil, leukotriene modifiers and long-acting bronchodilators. Relievers or quick-relief medications relieve bronchospasm and include short-acting $beta_2$ agonists, anti cholinergics and systemic corticosteroids (NHLBI/WHO, 1995; Gross and Ponte, 1998).

1.7.2.1 Bronchodilators

Bronchodilators principally dilate the airways by relaxing airway smooth muscle (Rang *et al.*, 1998), although some have additional effects on mucosal edema or inflammatory cells (Barnes, 1993a). Bronchodilators include $beta_2$ agonists, the xanthines and muscarinic receptor antagonists (anticholinergics) (Rang *et al.*, 1998). Bronchodilators are used for symptom relief in asthma, but generally, have no effect on the underlying inflammation (Barnes, 1999).

Beta$_2$-agonists

Beta$_2$-agonists are the most widely used and effective bronchodilators in clinical practice (Barnes, 1993a). They are classified as short acting and long acting beta$_2$-agonists. This sub classification is useful from a pharmacological point of view, because short acting beta$_2$ agonists are used only for symptomatic relief of asthma, whereas long acting agonists are used prophylactically and as long term medications (Gross and Ponte, 1998; Undem, 2006).

Beta$_2$-agonists act as functional antagonists and reverse airway smooth muscle contraction irrespective of the spasmogen. They are equally effective on large and small airways; and may have effects on cells other than airway smooth muscle, such as mast cells, (to prevent mediator release), on microvascular leakage, on cholinergic neurotransmission and on release of epithelial factors (Barnes, 1993a). Short acting beta$_2$ agonists include salbutamol (albuterol) (most widely used), terbutaline, metaproterenol, pirbuterol, rimiterol, etc. Long acting beta$_2$ agonists which cause bronchodilation and provide protection against bronchoconstriction for over 12 h include salmeterol, bambuterol, formeterol, extended-release albuterol (Lofdahl and Chung, 1991).

Beta$_2$ agonists interact with the membrane bound β_2-receptor to activate adenylcyclase via a G-protein (Gs), thereby increasing cyclic AMP (cAMP) levels in bronchiolar smooth muscle cells.Cyclic AMP activates specific protein kinases, thus reducing both the phosphorylation of myosin light chains and calcium-dependent actin-myosin coupling, resulting in smooth muscle relaxation (Greene and Harris, 2000). The rise in cAMP is also linked to the opening of Ca-activated

K^+- channels (Jones *et al.*, 1990; Miura *et al.*, 1992). $Beta_2$-agonists exert their major effects on the smaller airways (the site of the problem in asthma), where there is the greatest density of $beta_2$ receptors (Green and Harrris, 2000). $Beta_2$-agonists may have the following secondary effects, which contribute to the beneficial prophylactic effects:

(a) Inhibition/modulation of mediator release from mast cells and basophils;

(b) Increase in mucocilliary clearance;

(c) Decrease in vascular permeability and microvascular leakage; and

(d) Reduction of bronchial reactivity to a variety of stimuli i.e. bronchial hyperresponsiveness (Nelson, 1986; Kerrebijn, 1991; Greene and Harris, 2000).

Adverse effects of β_2 agonists include increased heart rate, cardiac arrhythmias, tremor, anxiety, and restlessness. There has been concern about a connection between prolonged use of β_2 agonist and death or near-death from asthma (Suissa *et al.*, 1994). There is some evidence suggesting that regular use of β_2-selective agonists may cause increased bronchial hyperreactivity and deterioration in disease control (Hancox *et al.*, 1999).

Methylxanthines

Methylxanthines used in asthma include theophylline, aminophylline, dyphylline, pentoxifylline and enprofylline (3-propylxanthine) (Barnes, 1993a; Boushey, 1998). They cause a direct relaxation of the bronchial smooth muscle. Methylxanthines have been used for over 100 years, yet their mechanism of

bronchodilation is still unclear. The proposed mechanisms of action of methyl xanthines include:

- Inhibition of phosphodiesterase, leading to an increase in the intracellular concentration of cAMP (Boushey, 1998).
- Inhibition of adenosine receptors (Boushey, 1998; Barnes, 2005).
- Anti inflammatory action partly due to activation of histone deacetylases in the nucleus (Ito *et al.*, 2002). Theoretically, deacetylation of histones could decrease the transcription of several proinflammatory genes and potentiate the effect of corticosteroids (Undem, 2006).

There is increasing evidence that theophylline has anti-inflammatory effects in asthma and chronic obstructive pulmonary disease (COPD) (Barnes, 2003). In patients with COPD, low doses of theophylline reduce the total number and proportion of neutrophils in induced sputum, the concentration of IL-8 and myeloperoxidase and neutrophils chemotactic responses, suggesting that it may have an anti-inflammatory effect (Culpitt *et al.*, 2002)

Adverse effects of methyl xanthines include headache, palpitation, dizziness, hypotension, tachycardia, agitation, emesis, seizures, and anxiety. Theophylline, once the mainstay of asthma treatment, is now considered a third- or fourth-line agent (in patients whose asthma is difficult to control) because of its adverse effect profile and potential interactions with many drugs (Gross and Ponte, 1998; Undem, 2006).

Muscarinic receptor antagonists

Muscarinic receptor antagonists used in the management of asthma include ipratropium bromide, oxitropium bromide and tiotropium. Muscarinic receptor antagonists are competitive inhibitors of acetylcholine at muscarinic receptors (Greene and Harris, 2000). They are bronchodilators that block post-ganglionic efferent vagal pathways (Gross, 1988). They act locally by inhibiting vagally mediated bronchoconstriction and reflex bronchoconstriction caused by inhaled irritants. As non-selective antagonists inhibit prejunctional M_2 receptors and postjunctional M_3 receptors, selective M_3 antagonists should be preferable for use as bronchodilators. Muscarinic receptor antagonists also inhibit the increase in mucus secretion that occurs in response to vagal activity (Boushey, 1998), and may increase mucociliary clearance of bronchial secretions (Rang *et al.*, 1998).

Adverse effects of ipratropium include dry mouth and tachycardia. Anticholinergics are one of the oldest antiasthmatic drugs, but their use declined with the advent of inhaled β_2 agonists. Recently, there has been a renewed interest in anticholinergics due to the availability of ipratropium which is better than prior anticholinergics and the realization that parasympathetic pathways are important in bronchospasm in some asthmatics (Barnes and Hansel, 2004; Undem, 2006). Combined therapy with ipratropium and β_2 adrenergic agonists results in slightly greater and more prolonged bronchodilation than with either agent alone in baseline asthma (Bryant and Rogers, 1992; Undem, 2006).

1.7.2.2. Anti-inflammatory drugs

The understanding of the role of inflammation in asthma has led to the use of anti-inflammatory drugs in the therapy of asthma and the development of new drugs targeted at the inflammatory process. Different classes of anti-inflammatory agents used in the management of asthma include; corticosteroids, cromones and leukotriene modifiers.

Corticosteroids

Corticosteroids are the most effective treatment currently available for long term management of asthma (Rowe *et al.*, 1992; Barnes 1993a; 1999). However, systemic side effects limit the dose that can be given over long periods, and this led to the development of topical steroids given by inhalation. Inhaled corticosteroids have revolutionalized the treatment of asthma and are the first-line treatment for chronic asthma irrespective of age and severity of disease (Barnes *et al.*, 1998b). Inhaled corticosteroids are the most effective long-term control medications for the underlying inflammatory process in the airways (NHLBI/WHO, 1995; Barnes, 2006a).

Corticosteroids bind to a cytosolic glucocorticoid receptor, which translocates to the nucleus and binds as a homodimer to DNA to ultimately repress (prevent transcription of) or induce (i.e. initiate transcription of) particular genes (Barnes, 1998). The predominant effect of corticosteroids is to switch off multiple inflammatory genes (encoding cytokines, chemokines, adhesion molecules, inflammatory mediator enzymes and receptors, and proteins) that have been activated during the chronic inflammatory process. They suppress the

multiple inflammatory genes mainly by reversing histone acetylation of activated inflammatory genes through binding of liganded glucocorticoid receptors (GR) to coactivators and recruitment of histone deacetylase – 2 (HDAC2) to the activated transcription complex (Barnes, 2006b) In higher concentrations, they have additional effects on the synthesis of anti-inflammatory proteins and post genomic effects (Barnes, 2006b). Corticosteroids include prednisolone, (for systemic use); new generation, highly potent inhaled corticosteroids for asthma, including budesonide, fluticasone propionate and mometasone furoate, have a high level of anti-inflammatory action with minimal side effects, as the swallowed fraction of drug is largely removed by hepatic metabolism (Barnes *et al.*, 1998b). Other inhaled glucocorticoids are beclomethasone dipropionate, triamcinolone acetonide, flunisolide. Prednisolone is used in short-term systemic corticosteroid therapy to gain initial control of asthma and for treatment of moderate to severe asthma exacerbation (Scarfone *et al.*, 1993).

Corticosteroids are not bronchodilators, but they are highly effective in controlling atopic diseases, and there is increasing evidence that if started early, they may prevent some of the irreversible airway narrowing in asthma (Barnes *et al.*, 1998b). However, they do not cure asthma and allergic inflammation recurs when treatment is stopped.

Common side effects include cough, dysphonia, throat irritation and oropharnygeal candidiasis. Higher doses of inhaled corticosteroids may lead to systemic adverse effects, such as adrenal suppression, osteoporosis and growth

delay in children (Tinkelman *et al.*, 1993; Kamada *et al.*, 1996; Guilbert *et al.*, 2006).

Cromones

They are also called antiallergic drugs or mast-cell stabilizers. The cromones; disodium cromoglycate (cromolyn sodium) and nedocromil sodium are the most specific anti-allergic drugs (Barnes, 1999), and can only be administered by inhalation.

Their molecular mechanism of action remains obscure, but they have been purported to act primarily by preventing both immediate and delayed degranulation of mast cells, thereby preventing the release of mediators of bronchoconstriction (Greene and Harris, 2000). Other effects include reversing increased functional activation in leukocytes obtained from the blood of asthmatic patients (Murphy and Kelly, 1987); suppressing the activating effects of chemotactic peptides on human neutrophils, eosinophils, and monocytes (Kay *et al.*, 1987; Moqbel *et al.*, 1988); inhibiting parasympathetic and cough reflexes (Fuller *et al.*, 1987; Hargreaves and Benson, 1995); and inhibiting leukocyte trafficking in asthmatic airways (Hoshino and Nakamura, 1997).

Cromolyn and nedocromil are very safe agents with mild to moderate anti-inflammatory effect; however, they lack bronchodilating activity. They are good initial long-term control medications in children and pregnant women with mild persistent asthma because of their safety profile (Gross and Ponte, 1998).

Adverse effects include cough, throat irritation and unpleasant taste, wheezing, bronchospasm, laryngeal edema, joint swelling and pain, headache,

rash, nausea, and angioedema (Undem, 2006). The main indication of nedocromil and cromolyn is to prevent asthmatic attacks in individuals with mild to moderate bronchial asthma.

Leukotriene modifiers

Cysteinyl leukotrienes (cys LTs) which are lipooxygenase products of arachidonic acid metabolism are potent inflammatory mediators in asthma and contribute to increased mucus production, bronchoconstriction and eosinophil infiltration. Anti-leukotriene drugs are direct inhibitors of bronchoconstriction and also have anti-inflammatory effect (AAAAI, 1999). Leukotriene modifiers include:

(a). *Leukotriene receptor antagonists*

Zafirlukast, pranlukast and montelukast are leukotriene receptor antagonists; they are selective high-affinity competitive antagonists for the cys-LT1 receptor (Krell *et al.*, 1990b; Jones *et al.*, 1995).

(b). *Leukotriene synthesis inhibitors*

Zileuton is a potent and selective inhibitor of the rate-limiting enzyme 5-lipoxygenase (5-LO) activity, thus inhibiting the formation of all 5-lipoxygenase products (Barnes, 1999).

Although leukotriene modifiers are effective prophylatic treatment for mild asthma, their role in asthma therapy is not clearly defined (Undem, 2006). There is heterogeneity in response to antileukotriene drugs, with patients falling into 'responder' and 'non-responder' groups (Undem, 2006).

Adverse effects include Churg-Strauss syndrome (zafirlukast and montelukast) marked by eosinophilia, vasculitic rash, worsening pulmonary symptoms, cardiac complications, peripheral neuropathy, and elevation in liver enzymes (zileuton).

Anti-lgE Therapy

Omalizumab is a biological drug approved for the treatment of asthma. It is a recombinant humanized monoclonal antibody targeted against lgE. IgE bound to omalizumab cannot bind to lgE receptors (FC∈RI) on mast cells and basophils, thereby preventing the allergic reaction (Undem, 2006). Omalizumab binds tightly to free lgE in the circulation to form omalizumab-lgE complexes that have no affinity for FC∈RI.

At the recommended doses, omalizumab reduces free lgE by more than 95%, thereby limiting the amount of lgE bound to FC∈RI – bearing cells. Omalizumab treatment also decreases the amount of FC∈RI expressed on basophils and mast cells (MacGlashan *et al.*, 1997). It is still under clinical trials for use in asthma even though it is indicated for those older than 12 years with allergies and moderate-to-severe persistent asthma.

1.8 REVIEW OF MEDICINAL PLANTS USED IN ASTHMA THERAPY

Nature is endowed with a variety of herbs with medicinal value. Since the beginning of time, humans have been using plants for healing, and many orthodox drugs evolved from naturally occurring substances through logical drug development.

In Nigeria and some other countries, traditional medicine practitioners are highly esteemed and patronized because they are cheap and easily accessible (Akah and Nwambie, 1994). In line with this, the World Health Organisation (WHO) is encouraging the identification and exploitation of the safe and effective aspects of traditional medicine (Akah *et al.*, 1997).

In different parts of the world, several herbs are used in the treatment of asthma and they are quite popular (NHLBI/WHO, 1995). The mechanisms of action of these anti-asthmatic herbs include bronchodilation, anti-allergic and anti-inflammatory effects (Table 4).' A number of orthodox drugs used in the management of asthma were developed from natural herbs; for example, ephedrine was developed from the *Ephedra* species, atropine from *Atropa belladonna*, and cromolyn sodium was developed from the cromone, khelline found in *Amni visnaga* a West Asian plant.

Leaves of *Datura stramonium* have been used in treating asthma for hundreds of years. The aqueous extract of *Gakani* (vernacular) a poly herbal drug (prepared from six medicinal plants) used as an anti-asthmatic has been shown to inhibit histamine and serotonin induced contractions; it also has anti-inflammatory actions. In practice one teaspoonful is mixed with pap or soaked in a cup of hot water and taken once daily (Akah *et al.*, 1997). Saiboku-to an anti-asthmatic Kampo medicine prepared from ten crude herbs, has been shown to stimulate epithelial nitric oxide (NO) generation (Tamaoki *et al.*, 1995).

Tylophora sylvatica is used in African traditional medicine to treat asthma and many allergic disorders; tylogenin, a steroidal aglycone from *T. sylvatica*

exhibits significant anti-allergic properties by inhibiting antigen-induced mediator release in rabbit basophil-dependent serotonin release and human leukocyte-dependent histamine release models (Gnabre *et al.*, 1994).

Other plants with anti asthmatic actions used in ethnomedicine are; *Garuga pinnata* (Venkatram *et al.*, 1993), *Albizzia lebbeck* (Tripathi and Das, 1977), *Cynanchum komarovii* (Lu *et al.*, 1997), *Phyllanthus niruri* (Singh *et al.*, 1991), and fruits of *Terminalia chebula* (Azeem *et al.*, 1992). In traditional Hawaiian folk medicine, *Bidens campylotheca* (Redl *et al.*, 1993), and in Chinese traditional medicine *Centipeda minima* (Yu *et al.*, 1994) are used as anti-asthmatic. In Papua New Guinea, *Dillenia papuana* is used to treat asthma (Nick *et al.*, 1994), also the trunk bark of *Syzygium brazzavillense* (Myrtaceae) is used in Congo traditional medicine to treat asthma (Ndounga *et al.*, 1991). Others are fruits of *Anethum graveoleus* (Mahran *et al.*, 1992), the bulbs of *Fritillaria imperialis*, *Adhatoda vasica*, and *Picrorhiza kurroa* (Himalayan herb) (Dorsch and Wagner, 1991; NHLBI/WHO, 1995).

Anti asthmatic plants with bronchodilating and antihistamine actions as demonstrated on guinea pig tracheal chains include *Nigella sativa* (Boskabady *et al.*, 2004), and *Bunium persicum* (Boskabady and Moghadas, 2004). *Semen armeniacae* amarum has long been used to control asthma in Korean traditional medicine practice; it has been shown to attenuate airway hyperresponsiveness and airway inflammation possibly as a result of selective inhibition of Th2 response to allergen (DO *et al.*, 2006).

Ding-Chuan-Tang a traditional Chinese medicine has been used in the treatment of bronchial asthma for several centuries. Its anti asthmatic effect is mainly due to bronchodilation and inhibition of eosinophil infiltration in the airway (Kao *et al.*, 2004). *Tylophora asthmatica* has anti asthmatic properties attributed to its action on cell mediated immunity (Haranath and Shyamalakumari, 1974), bronchospasmolytic effect (Dhananjayan *et al.*, 1975; Nayampalli and Sheth, 1979) and anti-inflammatory effects (Gopalakrishnan *et al.*, 1979).

Table 4: Some anti-asthmatic principles isolated from plants

Principle	Metabolite	Plant Part	Plant	Mechanism of anti-asthmatic action	Reference
Tetragalloyl Quinic acid	Quinic acid		*Galphimia Glauca*	Inhibition of bronchial hyper-reactivity and allergic reactions	Neszmelyi *et al.*, 1993
Tylophorine	Alkaloid	Root	*Tylophora Asthmatica*	Immunosuppression Antiinflammatory Bronchodilation	Dhananjayan *et al.*,1975; Nayampalli and Sheth,1979; Gopalakrishnan *et al.*, 1979
Vasicine (parent compound of bromhexine)	Alkaloid	Leaves	*Adhatoda Vasica*	Increased mucociliary clearance Bronchodilation Inhibition of histamine-induced bronchoconstriction	Chopra, 1925; Amin and Mehta, 1959; Atal, 1980
Vasicinone	Alkaloid	Leaves	*Adhatoda Vasica*	Increased mucociliary clearance Bronchodilation Inhibition of histamine-induced bronchoconstriction	Gupta *et al.*, 1977; Cambridge *et al.*, 1962
Forskolin	Diterpene	Root	*Coleus forskohlii*	Activation of adenylate cyclase Bronchodilation	Lichey *et al.*,1984; Tsukawaki *et al.*, 1987
Boswellic acid	Resin	Tree sap	*Boswellia serrata*	Inhibition of 5-lipoxygenase	Wagner,1989; Ammon *et al.*, 1991; Majeed *et al.*,1996; Safayhi *et al.*,1997
Androsin	Phenolic Glycoside	Roots	*Picrorhiza kurroa*	Prevention of bronchoconstriction induced by allergens and PAF	Dorsch *et al.*, 1991
Khelline	Cromone		*Amni visnaga*	Mast cell stabilization Anti-allergic action	
Tylogenin	Steroidal aglycone		*Tylophora sylvatica*	Anti-allergic Inhibition of antigen-induced mediator release	Gnabre *et al.*,1994
Trans-phytol	Triterpenoid		*Phyllantus niruri*		Singh *et al.*, 1991
Euphane triterpenoid	Triterpenoid		*Garuga pinnata*		Venkatram *et al.*, 1993
Vitexicarpin			*Kurroa Vitex trifolia*	Inhibition of histamine induced bronchoconstriction	Alam *et al.*, 2002
Ebeinone	Steroidal alkaloid	Bulb	*Fritillaria imperialis*	Anti-cholinergic	Atta-ur-Rahman *et al.*,1994

1.9 BOTANICAL PROFILE OF *Asystasia gangetica*

1.9.1 Plant Taxonomy

Order	Scrophulariales
Family	Acanthaceae
Genus	Asystasia
Species	gangetica
Subspecies	micrantha (Nees)
Common names	Ugolo oma (Igbo)
	Creeping foxglove (English)
	Ishihobo (Zulu)

1.9.2 Plant Description

"Asystasia" means inconsistency and relates to the fact that the corolla is more or less regular which is unusual in the family Acanthaceae. The word "gangetica" was derived from the Ganges River in India, where it is presumed the species occurs (Jackson, 1990; Elliot, 2004).

Asystasia gangetica an angiosperm dicotyledon is an attractive, fast-growing, spreading, herbaceous groundcover that grows from 300 – 600 mm in height (Saunders, 1958; Elliot, 2004). The plant has bell-shaped, white – cream coloured flowers with tessellated purple markings on the palate (lower petal of the corolla) (Elliot, 2004). About 4 – 6 flowers occur along one side of each unbranched flowering stalk (Saunders, 1958). The fruit is a club-shaped capsule, splitting from tip to base. Fruit is green, but dries to brown after opening. Each

fruit contains four flattened seeds held in place by conspicuous hooks. Seeds are hairless, bone coloured to brownish black. Occuring in opposite pairs, the leaves are green, very slightly saw edged and smooth (Saunders, 1958; CRC, 2003). The leaves are simple, green, oval, sometimes nearly triangular in shape, paler on the underside and may be up to 25 – 165 mm long and 5 – 55 mm wide. The stem roots easily at the nodes, providing a means of spreading and propagation. It forms roots when the nodes make contact with moist soil, ultimately forming a sprawling mass of stems or mats; hence it is regarded as a mat forming creeper (CRC, 2003). The leaves and the stems have scattered hairs (CRC, 2003).

Some literature describe it as a form of Chinese violet (*A. gangetica* ssp. *gangetica*), probably because it is closely related to Chinese violet. The two subspecies are normally confused, but *A. gangetica* ssp. *gangetica* has blue to purple flowers (CRC, 2003).

1.9.3 Geographical Distribution

It is native to Africa, where it is found in countries like Nigeria, Ghana Sierra Leone (West Africa); Kenya (East Africa); Ethiopia (Northeast Africa); Cameroon (West Central Africa); South Africa, Botswana, and Namibia (Southern Africa) (Elliot, 2004; GRIN, 2007). It is also native to India, Sri Lanka and the Malay Peninsula and naturalized in Malaysia, Australia, tropical America, and Oceania (GRIN, 2007). It is found among short grasses and beside paths and grows rampantly in tropical areas, though it thrives in moist conditions or following rainfall; young plants require protection in areas of heavy frost (Elliot 2004). In conducive environment, it has rapid growth rate, early flowering and

high seed production and tolerant to a range of subtropical and tropical climates (CRC, 2003).

1.9.4 Uses of *Asystasia gangetica* In Traditional Medicine

In Nigeria, the leaves are used in the management of asthma; usually, the fresh leaves are macerated in local gin for 24 h, or expressed and the extract drunk (Pers comm, 2000). In the traditional medicine of East Africa (Kenya), it is used as an anthelmintic. Here, the leaves are crushed, boiled in water, and the decoction drunk as a cure for intestinal worms (Kokwaro, 1976).

1.9.5 Other Uses of *Asystasia gangetica*

The plant has been recognized as a potential food source as the leaves have been shown to contain proteins (high amount), amino acids, minerals, sugars, lipids and fibre (Yeoh and Wang, 1993). In South Africa, it is used as a groundcover for large land expanse and as a container plant (hence the name 'African beauty'). Also the leaves have been eaten as spinach by local people (Elliot, 2004). It is a major weed in Malaysia, Indonesia, Papua New Guinea, and other parts of the Pacific Island; where it infests plantations, particularly oil-palm crops, and competes effectively for soil nutrients, reducing productivity and increasing crop management costs. It also grows as a weed in rubber, coffee and other crops (CRC, 2003). In Australia (where it is non-native) specifically, it is on the 'Alert List for Environmental Weeds' ; it is regarded as an environmental weed which smothers other ground plants (groundcovers) and displaces vegetation, thereby reducing the availability of habitat for native plants and animals, ultimately reducing biodiversity.

1.9.6 Pharmacological and Phytochemical Properties

The leaf extracts of *A. gangetica* have been shown to possess anti-histaminic, anti-serotonergic, bronchospasmolytic and anti-inflammatory actions (Akah *et al.*, 2003). Phytochemical tests revealed the presence of carbohydrates, reducing sugars, alkaloids, flavonoids, triterpenes, flavonoids, glycosides (Akah *et al.*, 2003) in the leaf.

1.10 Aim and Scope of Study

In line with the popular use of the leaves of *Asystasia gangetica* in herbal medicine as anti-asthmatic remedy, and the results of preliminary studies, in our laboratory suggesting that the leaves possess anti-asthmatic property (Akah *et al.*, 2003); this research was undertaken to isolate the active principles responsible for the anti-asthmatic effect of the plant and to elucidate the mechanisms of its anti-asthmatic effect. The study employed bioactivity-guided separation technique to isolate the active principle using bronchospasmolytic activity as activity-guide.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Collection, Identification and Preparation of Plant Material

Fresh leaves of *A. gangetica* were collected from August to October, 2004 at Orba, Enugu State, South East Nigeria. The plant material was aunthenticated by Mr. A. Ozioko of Bioresources Development and Conservation Programme (BDCP) centre, Nsukka. The leaves were cleaned, sun-dried in open air for two days to a constant weight and then milled to coarse powder.

2.2 Extraction of Plant Material

About 15 kg of the leaf powder was extracted with 100% methanol by maceration at room temperature (28°C) for 48 h. The mixture was filtered using Whatman filter paper (No.1), the plant material was washed several times with fresh portions of methanol. The extract obtained was concentrated in a rotary evaporator under reduced pressure and completely dried over a water bath at 60°C to obtain 726.93 g of the methanol extract (ME).

2.3 Bioactivity-Guided Fractionation of the Methanol Extract (ME)

The methanol extract (540 g) was fractionated in a silica gel (70 – 230 mesh) column (7.3 cm internal diameter; length of 60 cm) successively eluted with a mixture of n-hexane and ethylacetate (7:3) and methanol (100%). The fractions were collected in aliquots of 10 ml and pooled based on the R_f values of the constituents [visualized on precoated TLC plates developed with hexane: ethylacetate (7:3)] to obtain 3 broad fractions A, B, and C. The three fractions were screened for bronchospasmolytic activity using inhibition of histamine-induced contraction of the guinea pig trachea and relaxation of the trachea precontracted with histamine as bioactivity-guides. Fraction B exhibited the

greatest inhibition of histamine-induced contraction of the guinea pig trachea and relaxation of the precontracted trachea.

2.4 Chromatographic Separation of Fraction B

Based on the results of the bioactivity-guided tests, fraction B was further separated in a silica gel column successively eluted with gradient mixtures of n-hexane and ethyl acetate (9.5:0.5, 9:1, 8:2, 7:3. 6:4, 5:5, 0:1), to obtain eight fractions (I, II, III, IV, V, VI, VII and VIII). Activity-guided tests on these fractions revealed the greatest potency in fraction VIII, which was subsequently subjected to chromatographic separation successively eluted with petroleum ether : ethylacetate (7:3) and ethylacetate (100%) to obtain two fractions; Fr 1 and Fr 2. Fr 2 exhibited greater bronchospasmolytic potency and yielded a light brown coloured amorphous powder AG-1 (30 mg). The powder was washed with ethyl acetate and methanol.

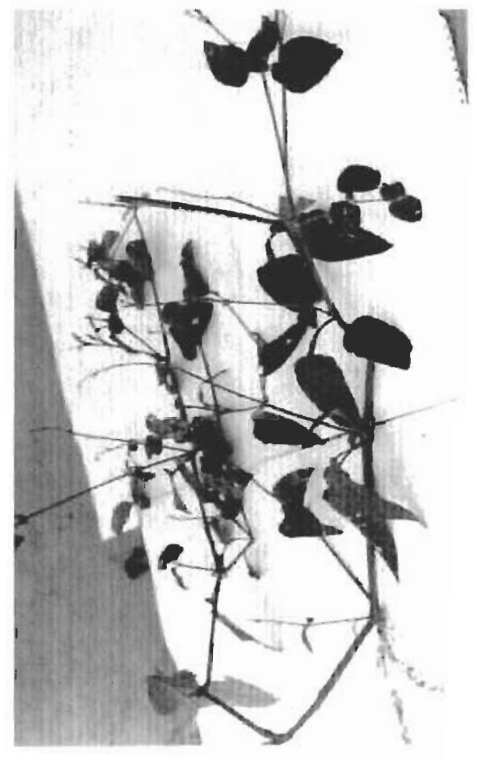

Fig 1: ***Asystasia gangetica*** **(L.) T. Anderson ssp.** ***micrantha*** **(Nees) Ensermu**

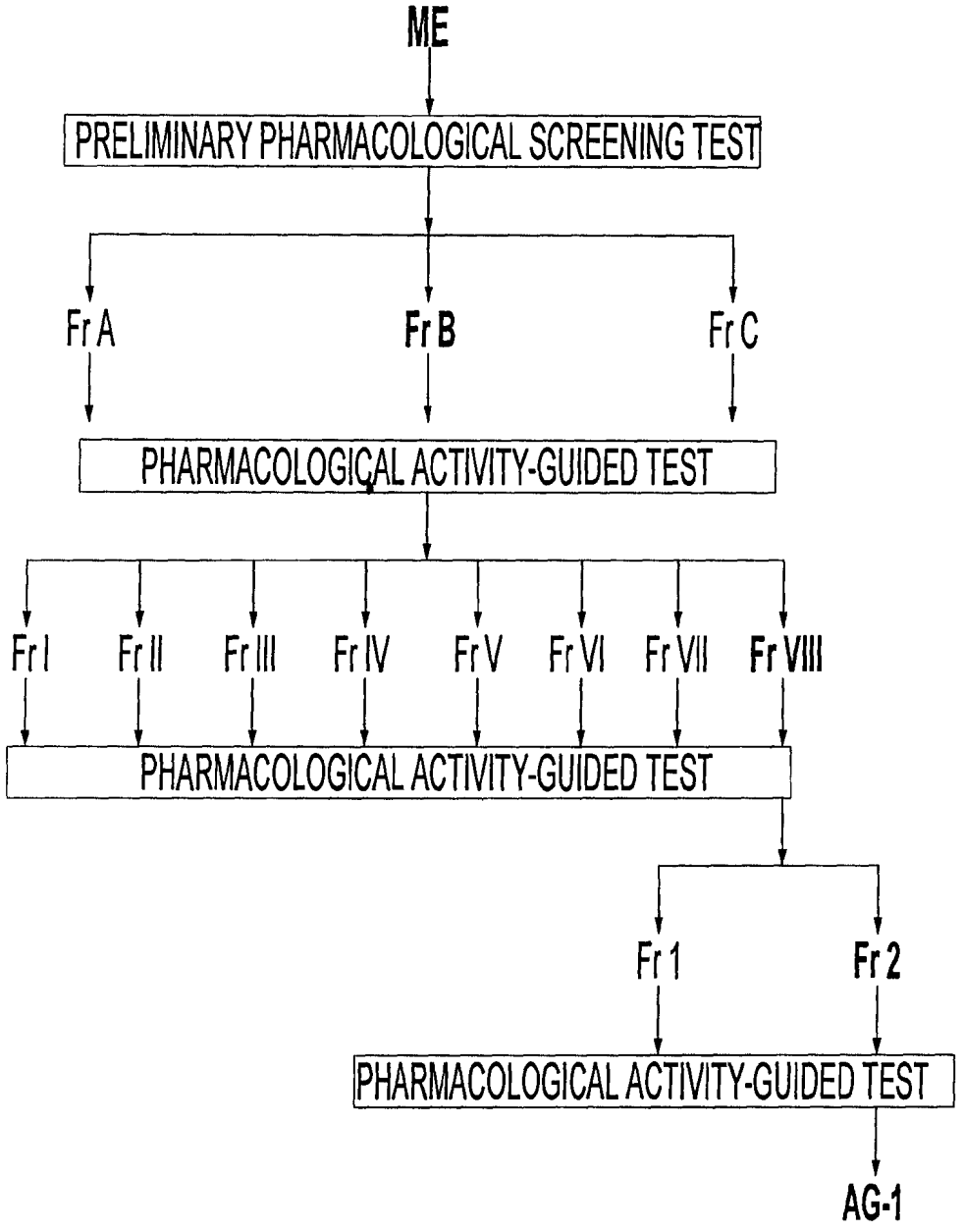

Fig 2: Pharmacological activity-guided fractionation scheme

2.5 Phytochemical Analysis of ME and its Fractions

The ME, Fraction B, Fraction VIII and Fraction Fr 2 were subjected to phytochemical tests using standard procedures (Harborne, 1973; Iwu, 1978; Trease and Evans, 1983).

2.6 Determination of Melting Point of AG-1

The melting point of AG-1 was determined using an analog Electrothermal melting point apparatus.

2.7 Pharmacological Tests

2.7.1 Animals Used

Adult Swiss albino mice (23 – 35 g), guinea pigs (300 – 500 g) and adult Swiss albino rats (100 – 220 g) of either sex, bred in the Laboratory Animal facility of the Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka were used. The rats were housed in stainless steel cages and the mice and guinea pigs were housed in plastic cages within the facility under appropriate conditions. The rats and mice were allowed free access to standard pellets (Bendel Feeds and Flour Mills Ltd., Benin City, Nigeria) and water, while the guinea pigs were fed with local grass, *Penicum maximum* L. The animals were randomly assigned to different control and treatment groups during the experiment.

2.7.2 Acute Toxicity (LD_{50}) Test

The LD_{50} of ME was estimated in adult Swiss albino mice by intraperitoneal route, using the method of Lorke (1983). The test was done in two stages:

***Stage One*:** The animals were divided into three groups of three mice per group. Three different doses (10, 100 and 1000 mg/kg) of ME suspended in 3% v/v Tween 85 were administered to each group respectively. The animals were monitored for 24 h for death.

***Stage Two*:** The doses used in this stage were determined by the result of the first test assessed by the number of deaths per group or per dose. Since no death was recorded in the first stage, three different doses; 1,600, 2,900 and 5,000 mg/kg were administered respectively by the intraperitoneal route to a fresh batch of three animals at one dose per animal. The animals were monitored for 24 h for death.

2.7.3 *In vitro* Pharmacological Studies

Isolated tissue studies were done in triplicates using different animals, to determine the bronchospasmolytic effects of the extract and fractions on the guinea pig trachea.

2.7.3.1 Effect on histamine-induced contractions of the guinea pig trachea

Guinea pigs of either sex starved overnight, but allowed free access to water, were used. The animals were killed by a blow on the head and exsanguinated. The trachea was dissected and cut along its length on the dorsal surface. Incomplete transverse cuts were made between the segments of cartilage to produce a zig-zag strip (Akah *et al.*, 2003).

The isolated trachea was mounted in a 10 ml organ bath containing Tyrode solution maintained at 37 °C and gassed with air (Akah *et al.*, 1997). The tissue was left to equilibrate for 60 min during which the bath solution was replaced

every 10 min (Akah *et al.*, 1997). At the end of the equilibration period, contractions induced by histamine (8 μg/ml) as well as the effect of ME and the subsequent fractions on histamine induced contractions were recorded.

Tissue-histamine contact time was 75 s, since the trachea is a slow contracting tissue. The tissue was bathed in the test substances for 5 min before the addition of histamine. Responses were recorded on an Ugo Basile Microdynamometer Recorder 7004.

2.7.3.2 Effect on the precontracted trachea (pathological tissue)

The guinea pig trachea was prepared and mounted as described above. The trachea was contracted with histamine (8 μg/ml). After 2 min and without washing off the histamine, cumulative doses of ME up to 12.8 mg/ml were added at 2 min interval and the effects recorded. This was done for all the fractions and the negative control (Tween 85).

2.7.4 *In vivo* Pharmacological Studies

2.7.4.1 Studies on acute inflammation

The effect of ME, fraction B and fraction VIII on acute inflammation was studied using carrageenan induced rat hind paw edema as a model of acute inflammation (Winter *et al.*, 1962; 1963). The increase in the linear circumference of the rat hind paw was used as a measure of inflammation.

Adult Swiss albino rats of either sex were used. The animals were fasted for 12 h and deprived of water only during the experiment. Deprivation of water was to ensure uniform hydration and to minimize variability in edematous response (Akah *et al.*, 1993). The animals were divided into 8 groups of five

animals per group, groups 1 and 2 received ME 200 and 400 mg/kg respectively, groups 3 and 4 received fraction B 200 and 400 mg/kg respectively, groups 5 and 6 received fraction VIII 200 and VIII 400 mg/kg respectively, while groups 7 and 8 received piroxicam 50 mg/kg (positive control) and 3% Tween 85 mg/kg (negative control) respectively.

The ME and fractions were suspended in 3% (ME and Fraction B) or 15% Tween 85 (Fraction VIII) and administered orally 1 hour before 0.1 ml 1% carrageenan (w/v in 0.9% saline) was injected into the subplantar surface of the right hand paw of rats. The linear circumference of the right hind paw was measured using a vernier caliper, before and at 0.5, 1, 2, 3 and 4 h after induction of inflammation.

Edema was assessed in terms of the difference between the zero time linear circumference (Co) of the injected paw and its circumference at the different times (Ct) after injection of carrageenan (Hess and Milong, 1972; Akah *et al.*, 1993; Akah *et al.*, 2003). The average edema, level of edema and inhibition of edema were calculated for each group using the relation (Oriowo, 1982; Akah and Njike, 1990; Akah *et al.*, 2003);

$$Average\ edema = \frac{[\sum(C_t - C_o)]}{No.\ of\ animals\ per\ group}$$

$$Percent\ edema(\%) = \left[\frac{E_t}{E_c}\right] \times 100$$

where E_t = average edema of treated group at time t; Ec = average edema of the control group at the same time.

Inhibition of edema (%) = 100 - % edema

2.7.4.2 Studies on carrageenan-induced pleurisy in rats

The effects of methanol extract (ME), fraction B and Fraction VIII on inflammation of the pleural cavity and leucocyte migration as a mechanism of anti-inflammatory action was studied using carrageenan induced pleurisy in rats as described by Cuzzocrea *et al.*, (1999; 2001) with slight modifications. The rats were divided into 5 groups of 5 animals per group. The groups received ME 400 mg/kg, B 400 mg/kg, VIII 400 mg/kg, Dexamethasone 0.3 mg/kg (positive control) and Tween 85 (1 ml/kg) respectively, via the intraperitoneal route 30 min before induction of pleurisy.

Adult Swiss albino rats were anesthetized by ether inhalation and the skin incised at the level of the sixth intercostal space. The underlying muscles were dissected and 0.2 ml of saline (0.9%) containing 1% carrageenan was injected into the pleural cavity. The skin incision was closed with a suture and the animals were allowed to recover. At 4 h after the injection of carrageenan, the animals were sacrificed under ether vapour. The chest was carefully opened and the pleural cavity washed with 2 ml of 10% EDTA in normal saline. The exudates and washing were removed by aspiration and the total volume measured. Exudate contaminated with blood was discarded.

The volume of exudate was calculated by subtracting the volume injected (2 ml) from the total volume recovered. Total leukocyte count (TLC) and differential leukocyte counts were performed in an improved Neubauer chamber.

2.8 Statistical Analyses

Results obtained were analysed using One way Analysis of Variance (ANOVA; SPSS Version 11), subjected to LSD post hoc test and expressed as Mean ± SEM. Differences between means of treated and control groups were regarded significant at $P<0.05$.

CHAPTER THREE

RESULTS

3.1 Extractive yield of ME and fractions

The extraction process afforded 726.93 g (4.86% w/w) of the methanol extract (ME). Concentration of the fractions obtained from the chromatographic process yielded 3.04% w/w of fraction B, 7.41% w/w of fraction VIII, 43.03% w/w of Fr 2 and 34.48% w/w of AG-1. The yield of each fraction was expressed as a percentage of its direct precursor.

3.2 Phytochemical Analysis

Phytochemical analysis showed that the methanol extract tested positive to alkaloids, glycosides, saponins, reducing sugars, terpenes and carbohydrates. Fraction B gave positive reaction for flavonoids, terpenes. Fraction VIII tested positive to flavonoids, terpenes, steroids and resins, while Fr2 gave positive reaction for terpenes and steroids (Table 5).

3.3 Melting Point of AG-1

The melting point of AG-1 was determined to be 134 ± 2^0C

3.4 Acute Toxicity (LD_{50}) Test

Intraperitoneal administration of the ME to mice up to 5,000 mg/kg caused no death. Thus the LD_{50} of ME was greater than 5,000 mg/kg.

Table 5: Phytochemical Constituents of ME and Fractions

Phytochemical Constituent	ME	B	VIII	Fr 2
Carbohydrates	++	-	-	-
Alkaloids	+++	-	-	-
Saponins	+++	-	-	-
Flavonoids	-	++	++	+
Tannins	-	-	-	-
Steroids	-	-	+++	+
Glycosides	+++	Trace	Trace	Trace
Reducing sugars	++	Trace	Trace	Trace
Terpenes	+++	++	++	++
Fats and oil	-	-	-	-

\+ = slightly present

++ = moderately present

+++ = abundantly present

\- = absent

3.5 Effect of ME and fractions on histamine-induced contractions of the guinea pig trachea

The ME (2 – 8 mg), fraction A (2 – 8 mg) and fraction C (2 – 16 mg) potentiated histamine-induced contraction of the guinea pig trachea. The magnitude of potetiation decreased with increasing dose. Higher doses (from 16 mg for ME and fraction A and 32 mg for fraction C) however, caused a dose-related inhibition (Figs 3 and 4, A1 and A2). Fraction B (2 and 4 mg) caused little or no potentiation of histamine-induced contraction while higher doses produced a dose-related inhibition.

Fractions I – VIII, Fr 1 and Fr 2 obtained from subsequent stages of chromatography did not exhibit such initial potentiation of histamine-induced contractions of the guinea pig trachea.

The magnitude of effect caused by the different fractions served as a guide in the chromatographic separation of the methanol extract (ME). The methanol extract (IC_{50} > 12.8 mg/ml) on chromatographic separation yielded three fractions A, B, and C. The order of potency of the fractions was B > A > C based on their IC_{50} values of 1.91, 4.68, and 10.47 mg/ml respectively (Figs 3 – 7, A1and A2).

Fraction B was subjected to further chromatographic separation to yield fractions I – VIII; the order of potency of the fractions was VIII > VII >V > VI > IV = III >I > II as indicated by IC_{50} values of 1.10, 1.12, 1.82, 2.24, 3.35, 3.35, 3.80, and 3.89 mg/ml respectively (Figs 8 – 10, A3 – A9).

Fraction VIII was subjected to further chromatographic separation to yield Fr 2 which precipitated a solid AG-1. AG-1 produced 82% inhibition of the maximal contraction induced by histamine at a concentration of 0.4 mg/ml (Figs 11 and 12). The IC_{50} values are as shown in Table 6.

3.6 Effect of ME and fractions on precontracted guinea pig trachea

On the precontracted guinea pig trachea, ME and the fractions elicited a dose-dependent relaxation of the guinea pig trachea precontracted with histamine (Figs 13 – 16, A10 – A18). They produced varying degrees of relaxation (Table 7 and Fig 17), with fraction VIII producing 77.78% relaxation at a cumulative dose of 16 mg. However, fractions A and C exhibited initial potentiation, relaxing the precontracted trachea from 64 mg.

Table 6: Concentration of ME and fractions that inhibited 50% (IC_{50}) of histamine-induced contractions of the guinea pig trachea

Extract/Fraction	IC_{50} (mg/ml)
ME	>12.8
B	1.91
VIII	1.10
Fr 2	[a]
AG-1	[a]

[a] At ≤ 0.4mg, there was greater than 50% inhibition of histamine induced contraction of guinea pig trachea

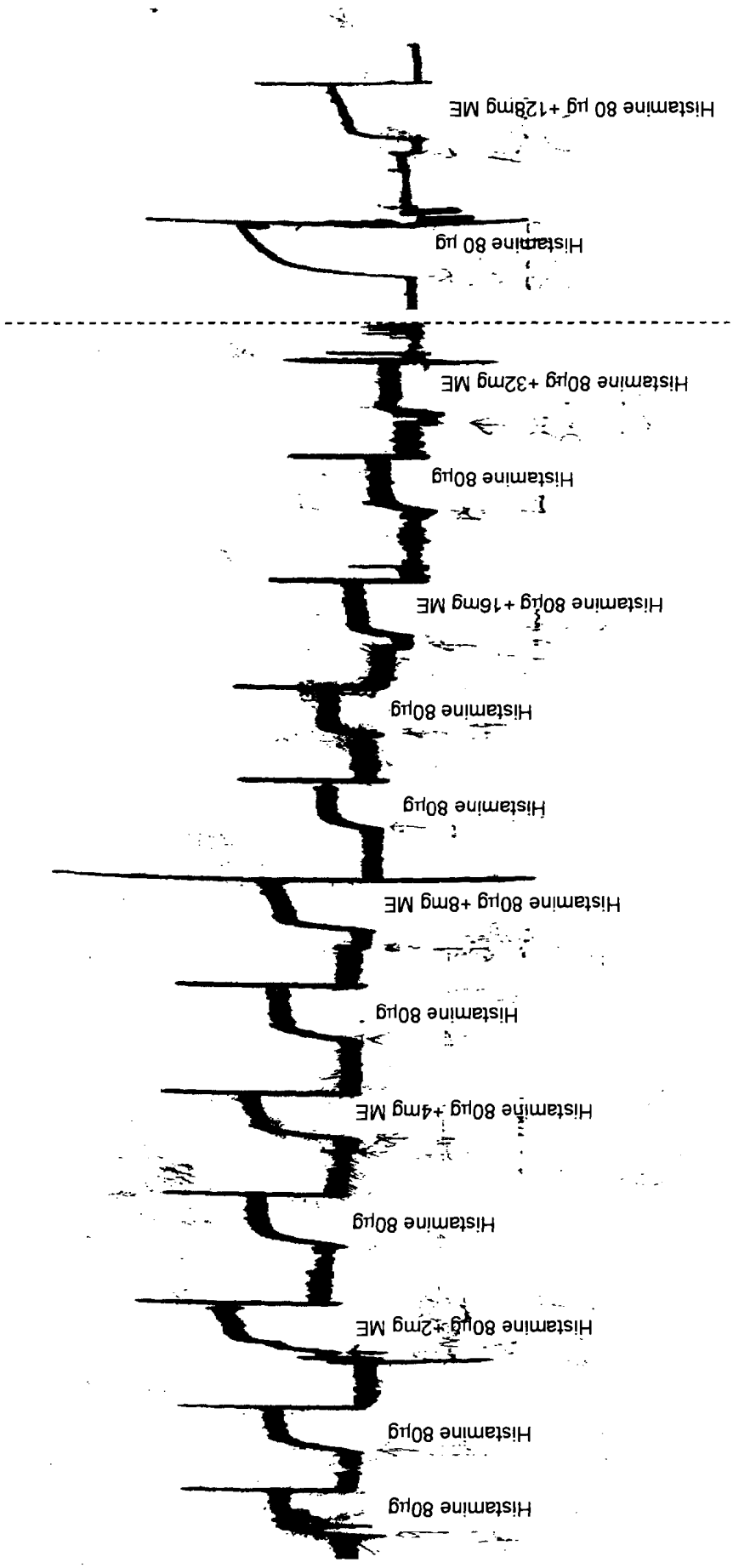

Fig. 3: Effect of ME on histamine-induced contractions of the guinea pig trachea

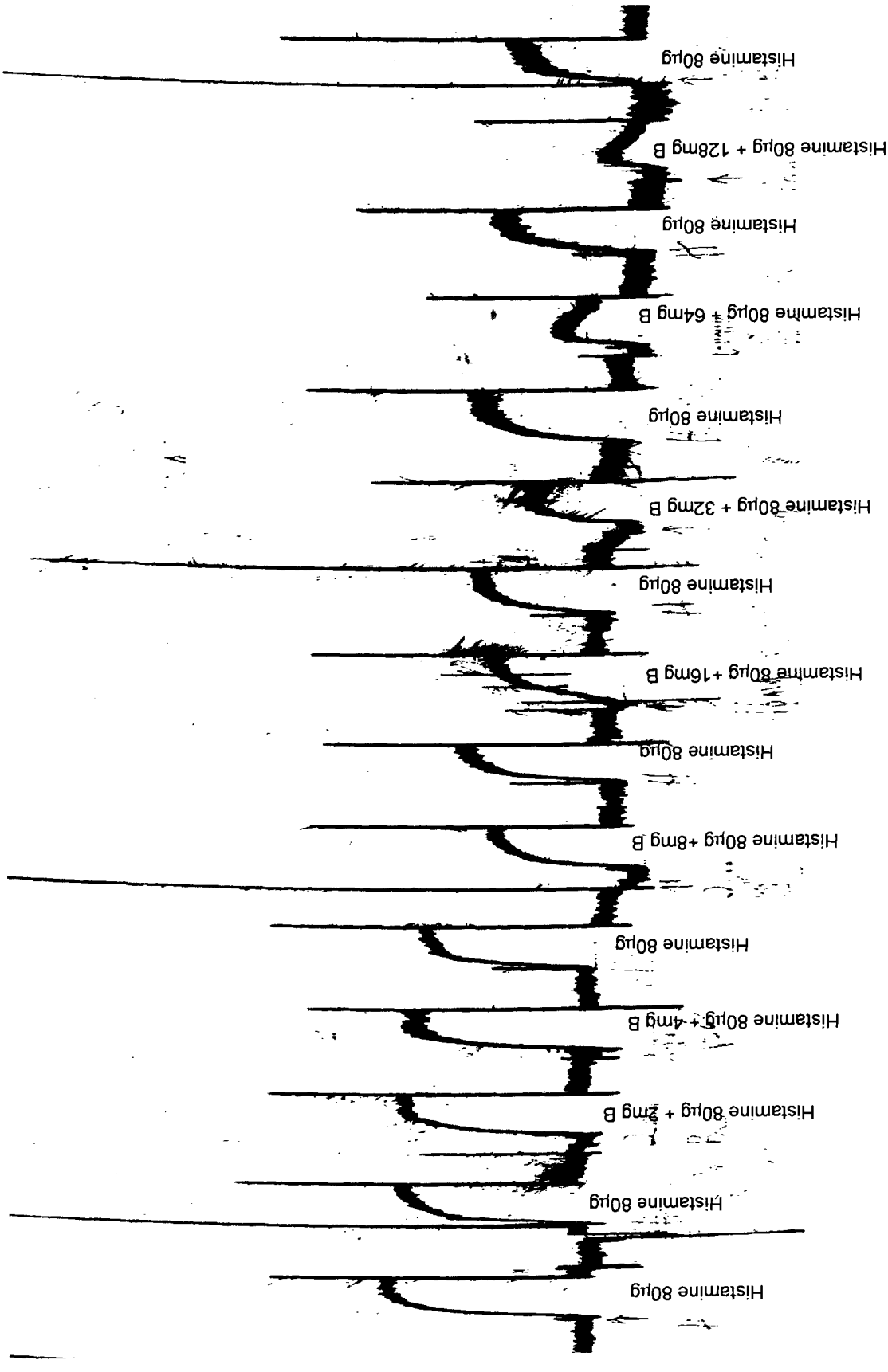

Fig. 4: Effect of fraction B on histamine-induced contractions of the guinea pig trachea

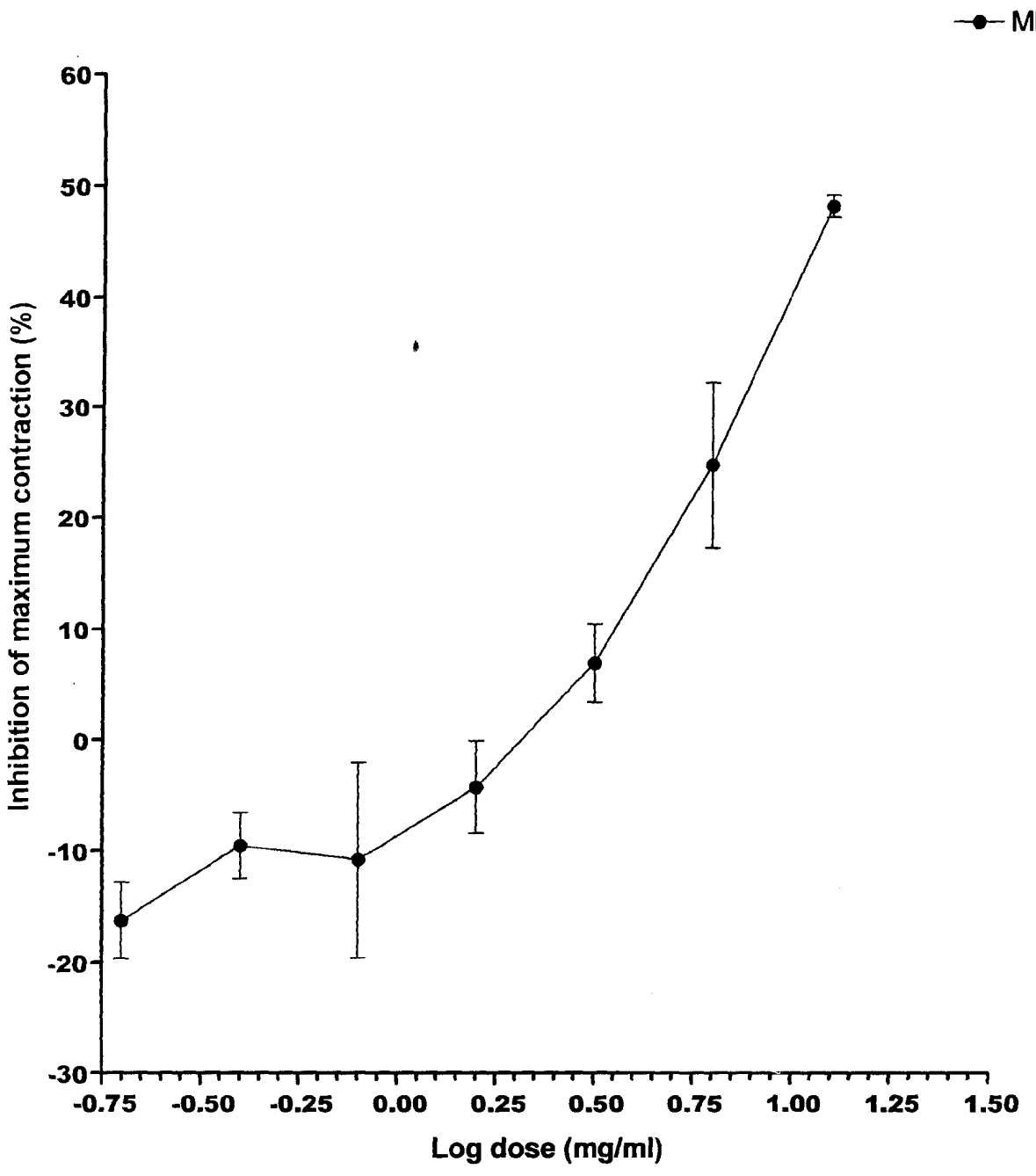

Fig.5: Effect of ME on histamine-induced contractions of the guinea pig trachea

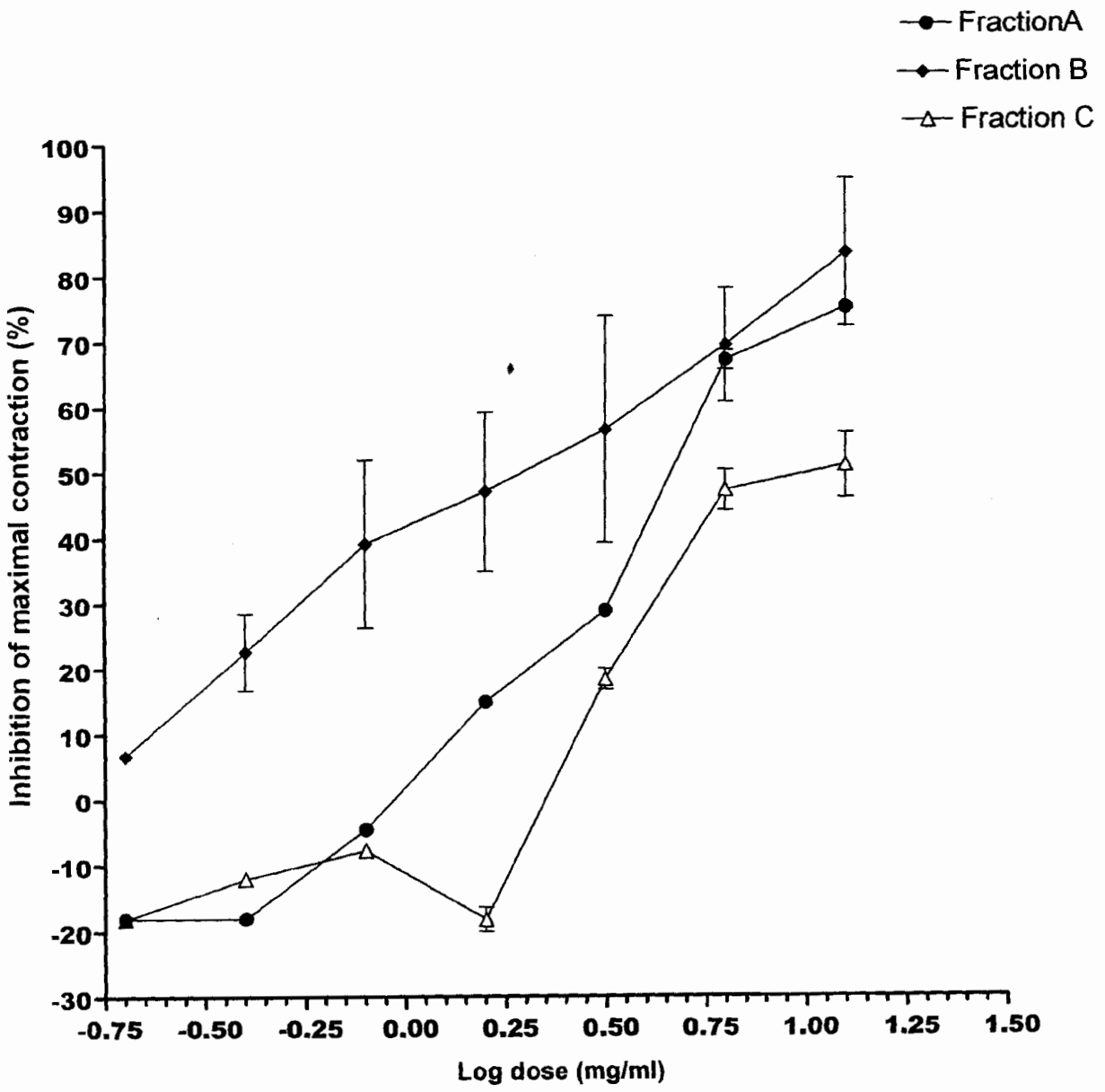

Fig.6: Effect of fractions A, B, and C on histamine-induced contractions of the guinea pig trachea

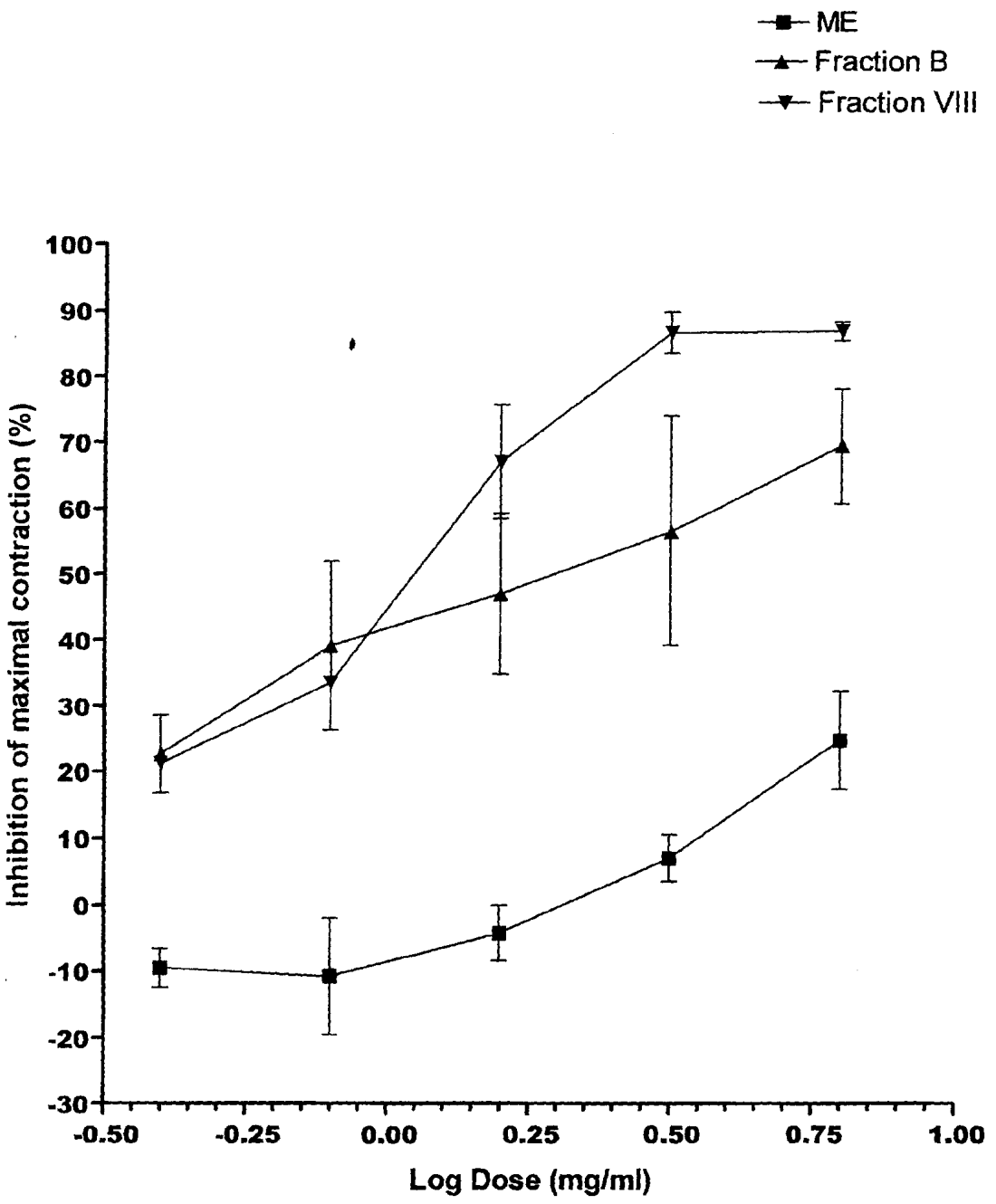

Fig. 7: Effect of ME, fractions B and VIII on histamine-induced contractions of the guinea pig trachea

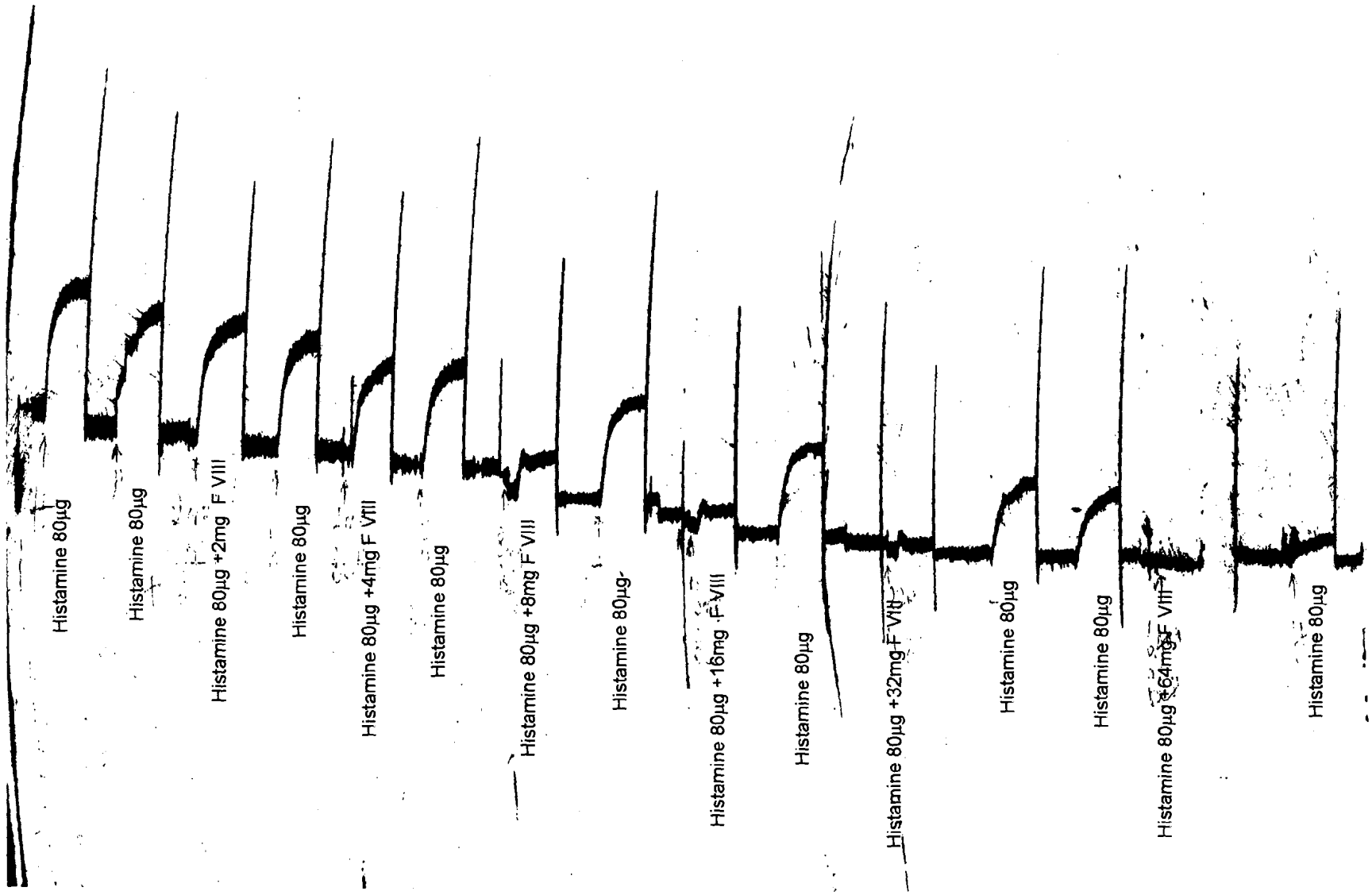

Fig. 8: Effect of fraction VIII on histamine-induced contractions of the guinea pig trachea

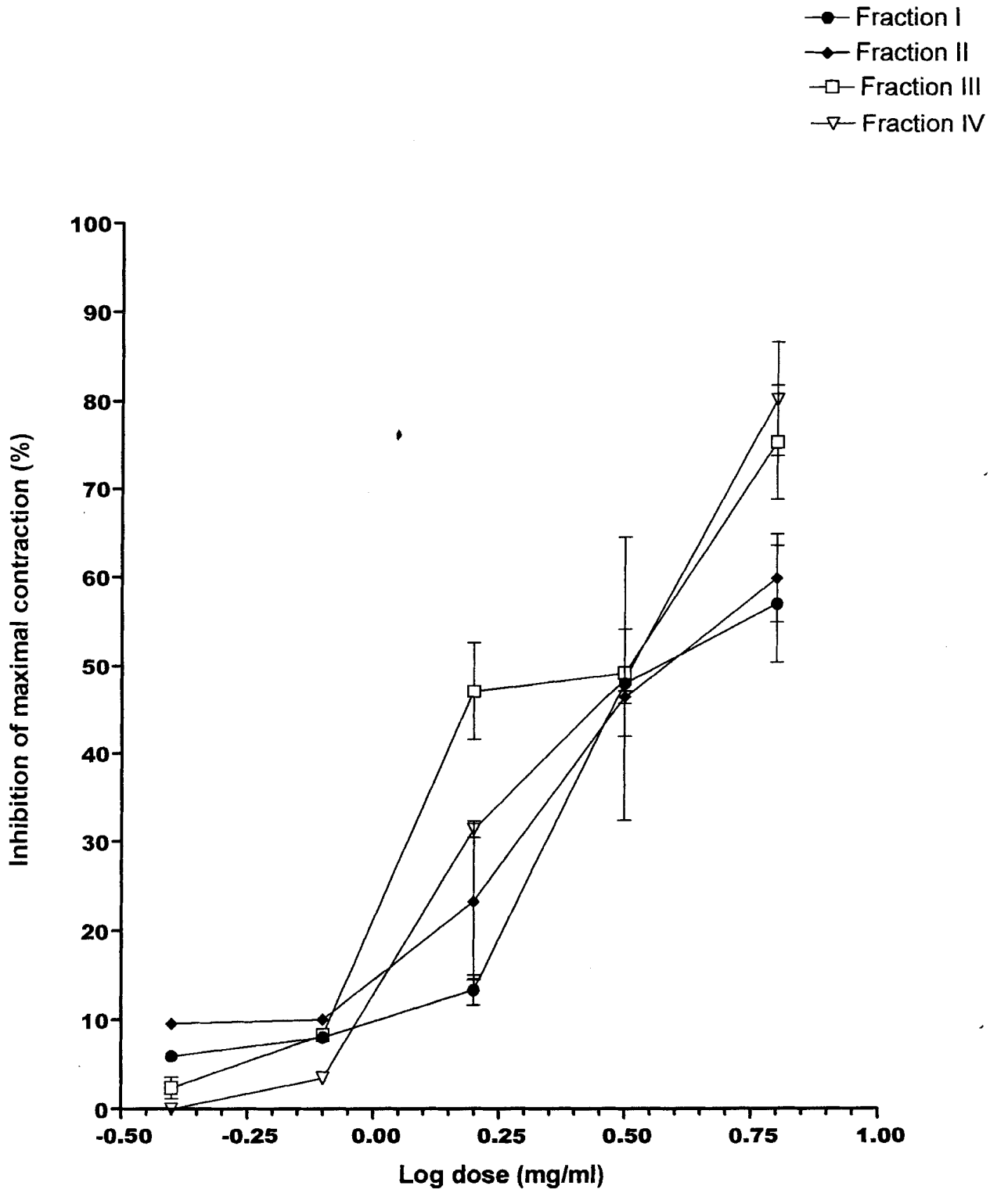

Fig. 9: Effect of fractions I,II,III,and IV on histamine-induced contractions of the guinea pig trachea

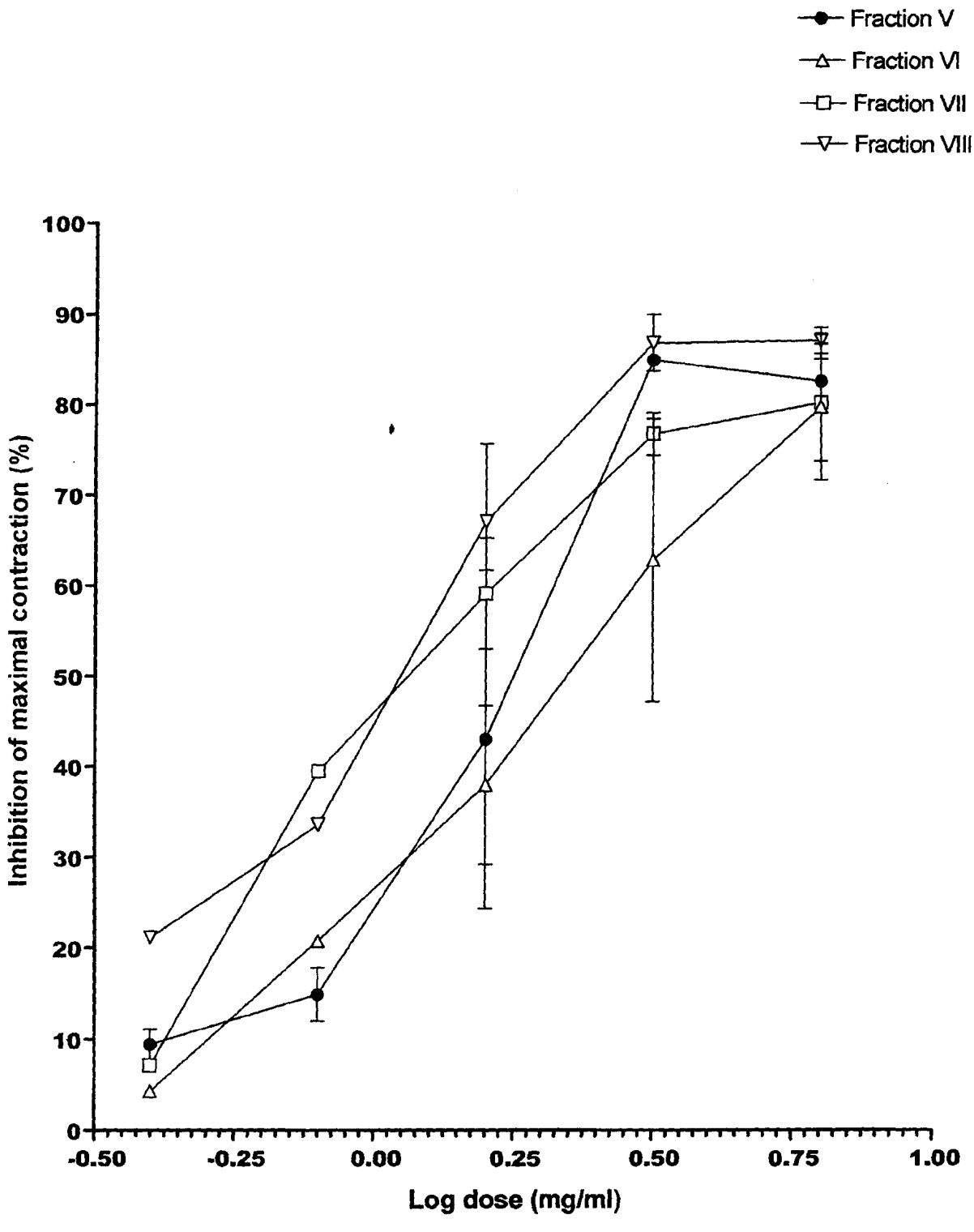

Fig. 10: Effect of fractions V,VI,VII, and VIII on histamine-induced contractions of the guinea pig trachea

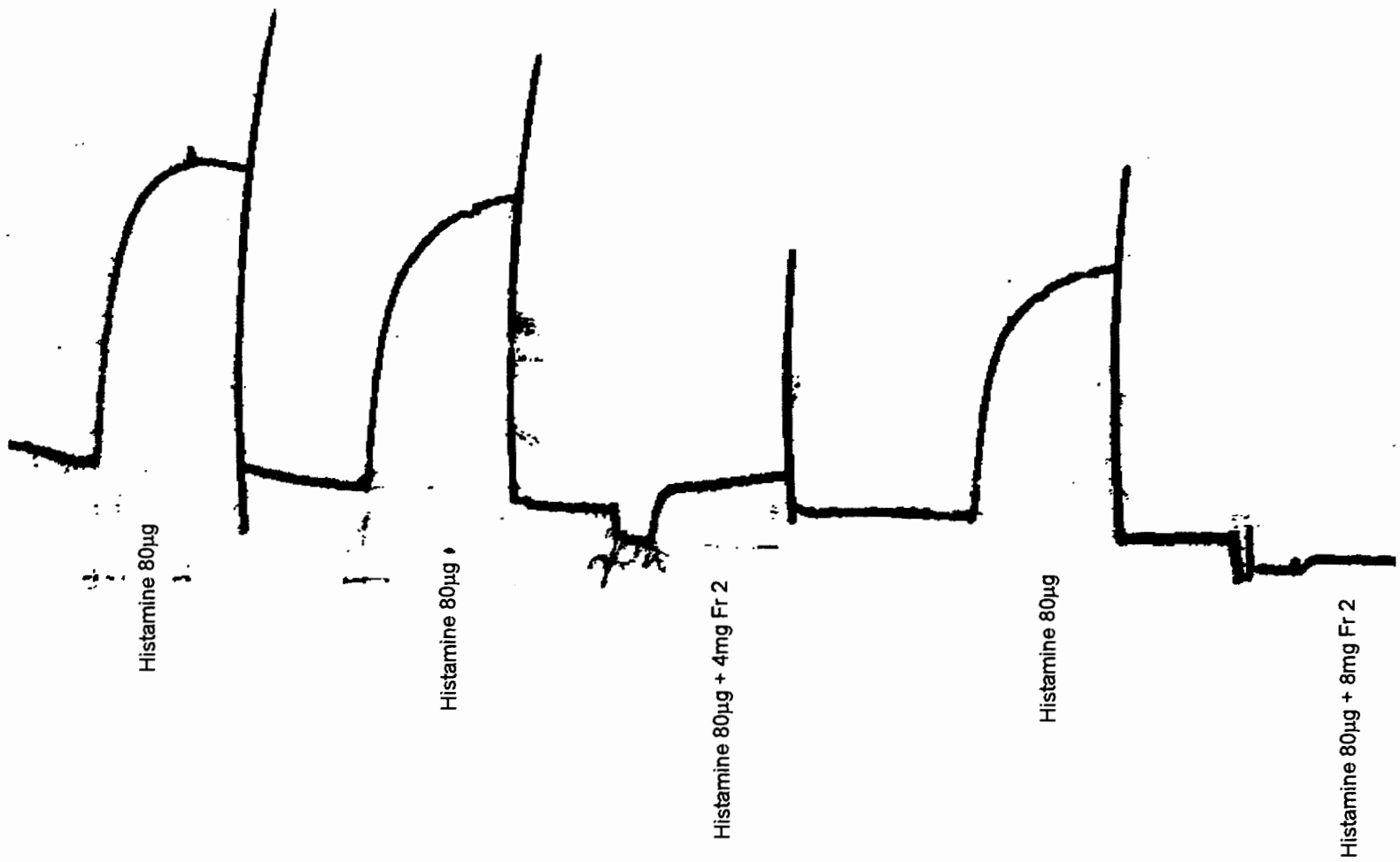

Fig. 11: Effect of fraction Fr 2 on histamine-induced contractions of the guinea pig trachea

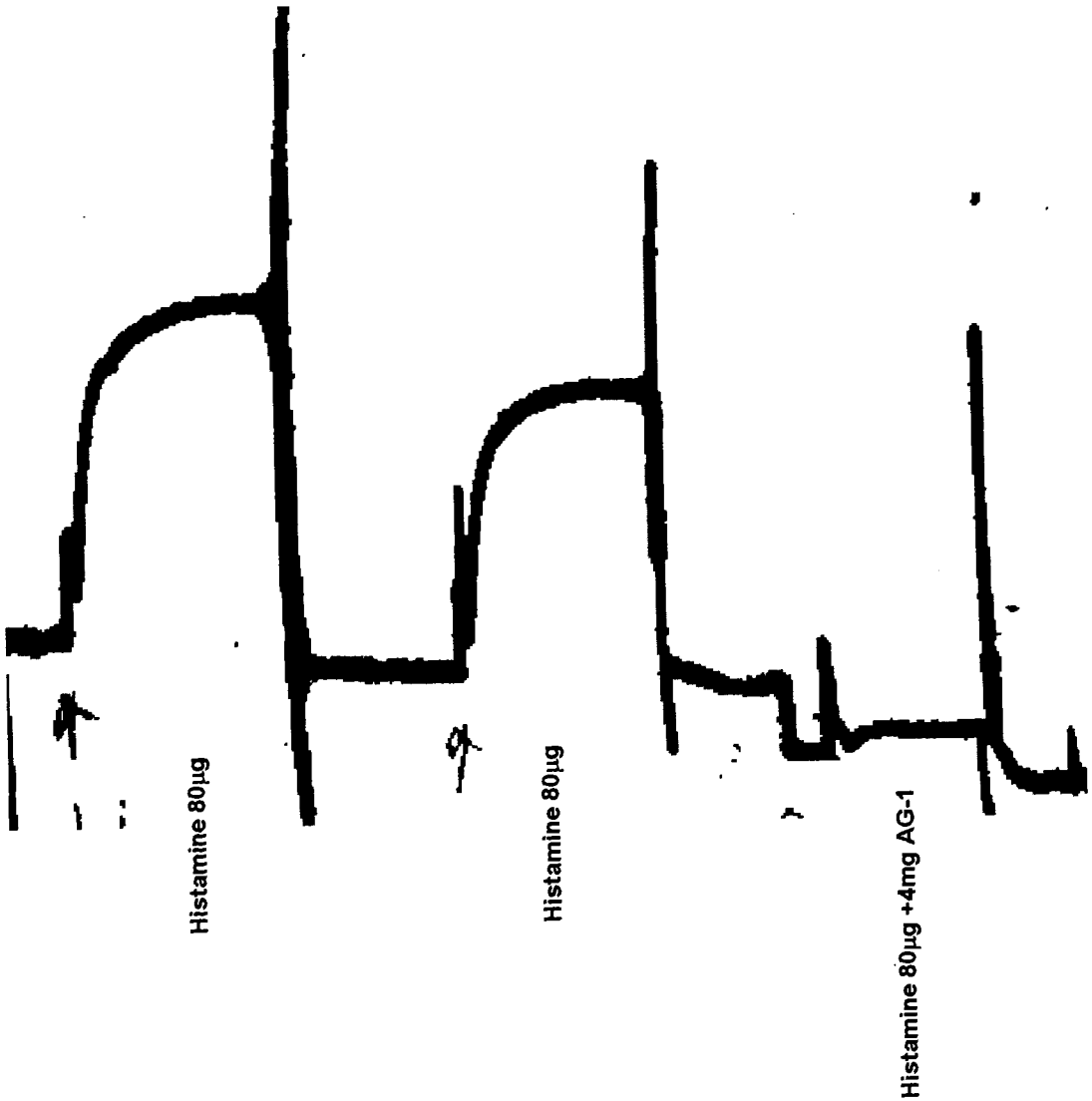

Fig. 12: Effect of 4mg AG-1 on histamine-induced contractions of the guinea pig trachea

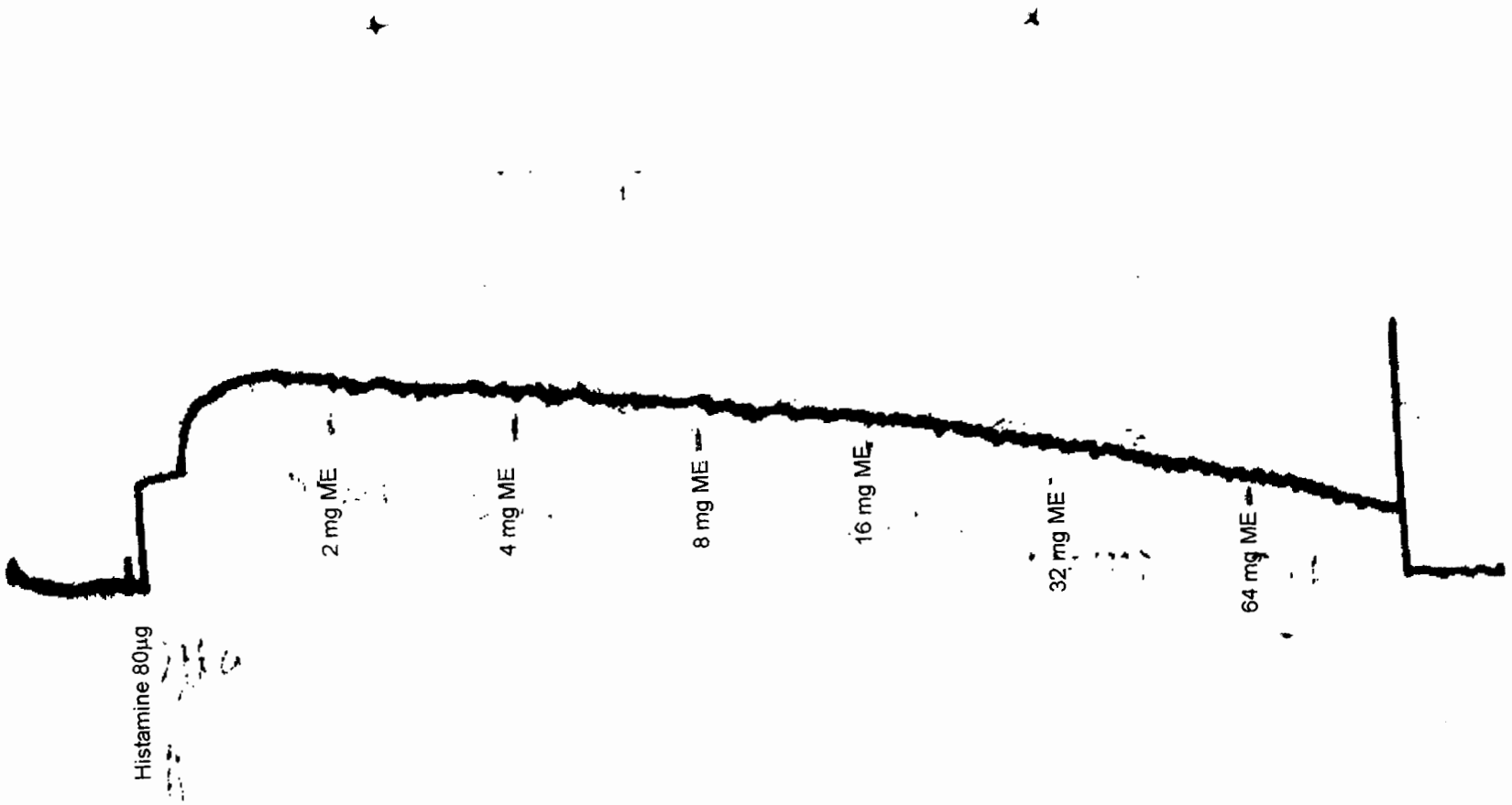

Fig. 13: Effect of cumulative doses of ME on guinea pig trachea pre-contracted with histamine

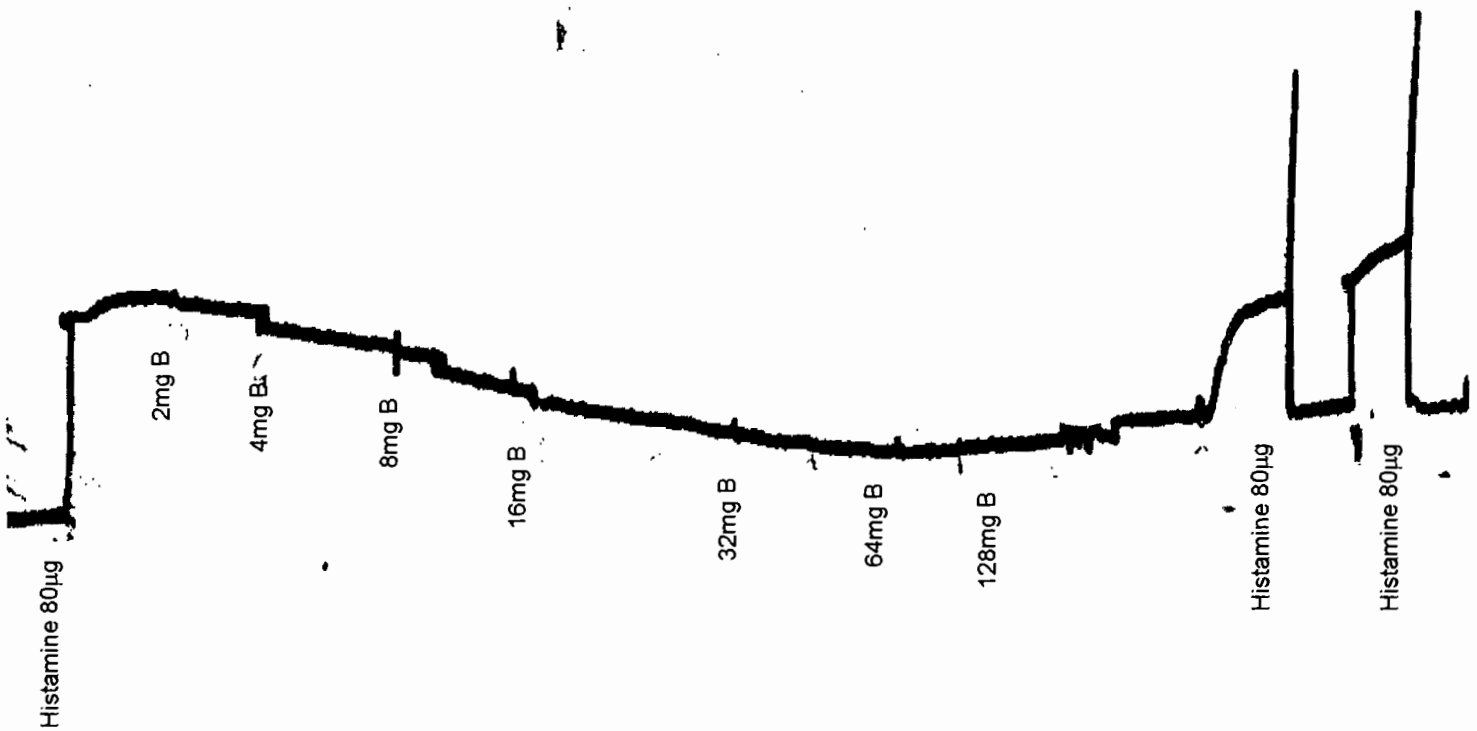

Fig.14: Effect of cumulative doses of fraction B on guinea pig trachea pre-contracted with histamine

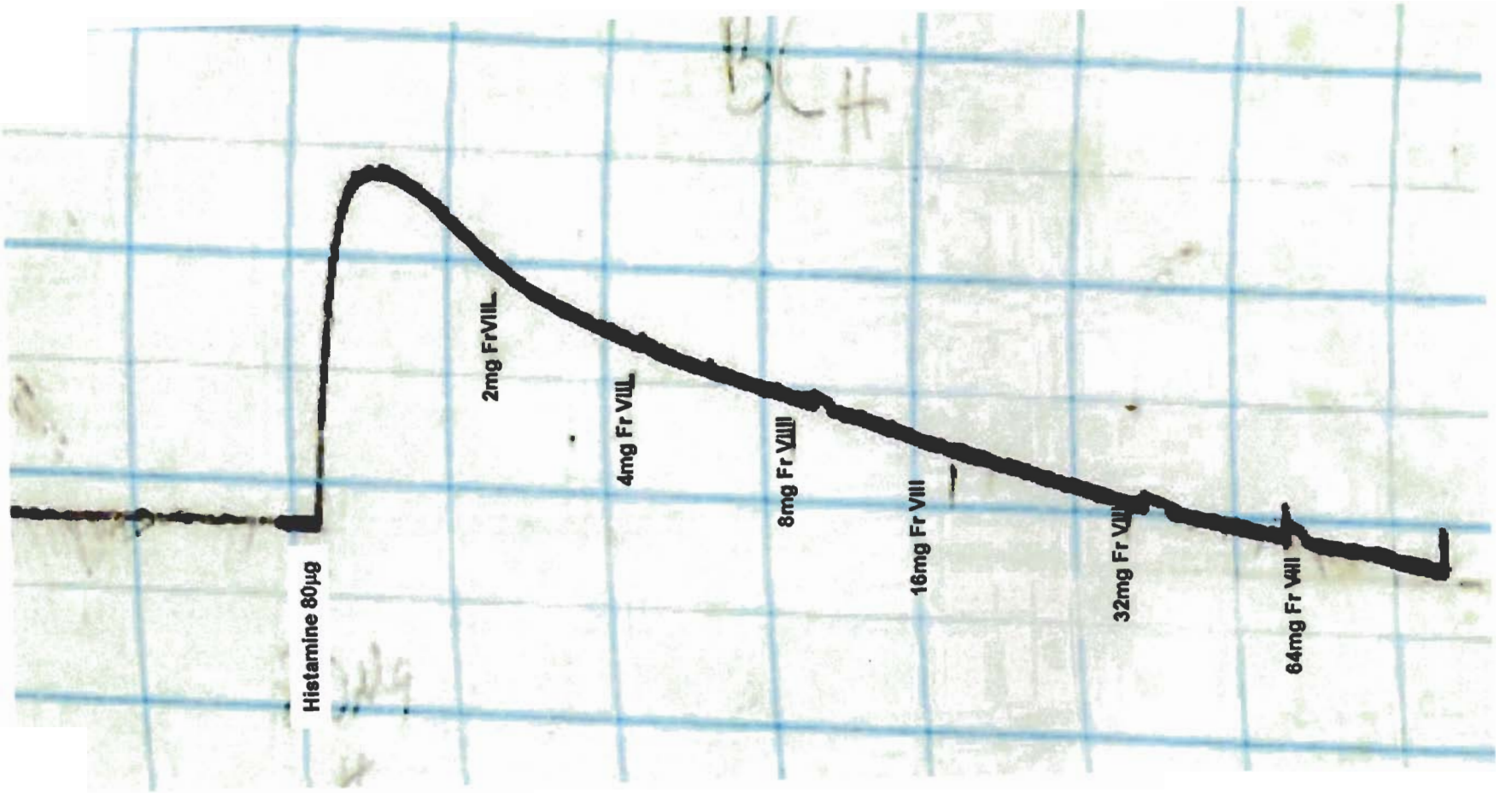

Fig. 15: Effect of cumulative doses of fraction VIII on guinea pig trachea pre-contracted with histamine

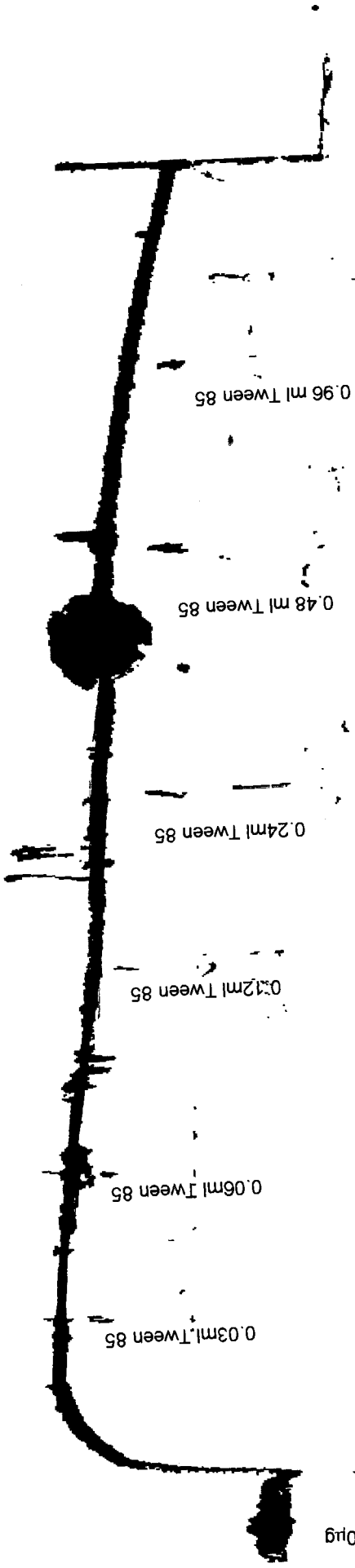

Fig. 16: Effect of control (vehicle) on guinea pig trache re-contracted with histamine

Table 7: Relaxant effects produced by cumulative doses of ME and fractions on guinea pig trachea pre-contracted with histamine

Extract /fraction	Relaxation (%) of Precontracted trachea					
	2 mg	4 mg	8 mg	16 mg	32 mg	64 mg
ME	3.68 ± 0.33*	12.68± 0.18	20.8 ● 0.47*	29.10± 0.67*	42.89 ± 0.29*	55.74 ± 0.41*
B	7.79 ● 0.10*	21.21±7.00*	39.18±10.3*	69.2 ± 6.75*	93.94 ± 3.50*	115.63 ± 0.77*
VIII	25.0 ± 0.00*	41.6 ± 0.00*	61.1 ± 1.39*	77.7 ± 1.39*	86.11 ± 1.39*	100 ± 0.00*
Control	0.00	7.39 ± 0.64	7.22 ± 0.55	12.04 ± 0.46	13.77 ± 1.61	30.03 ± 3.74

* $P<0.05$ compared to negative control (One Way ANOVA; LSD post hoc)

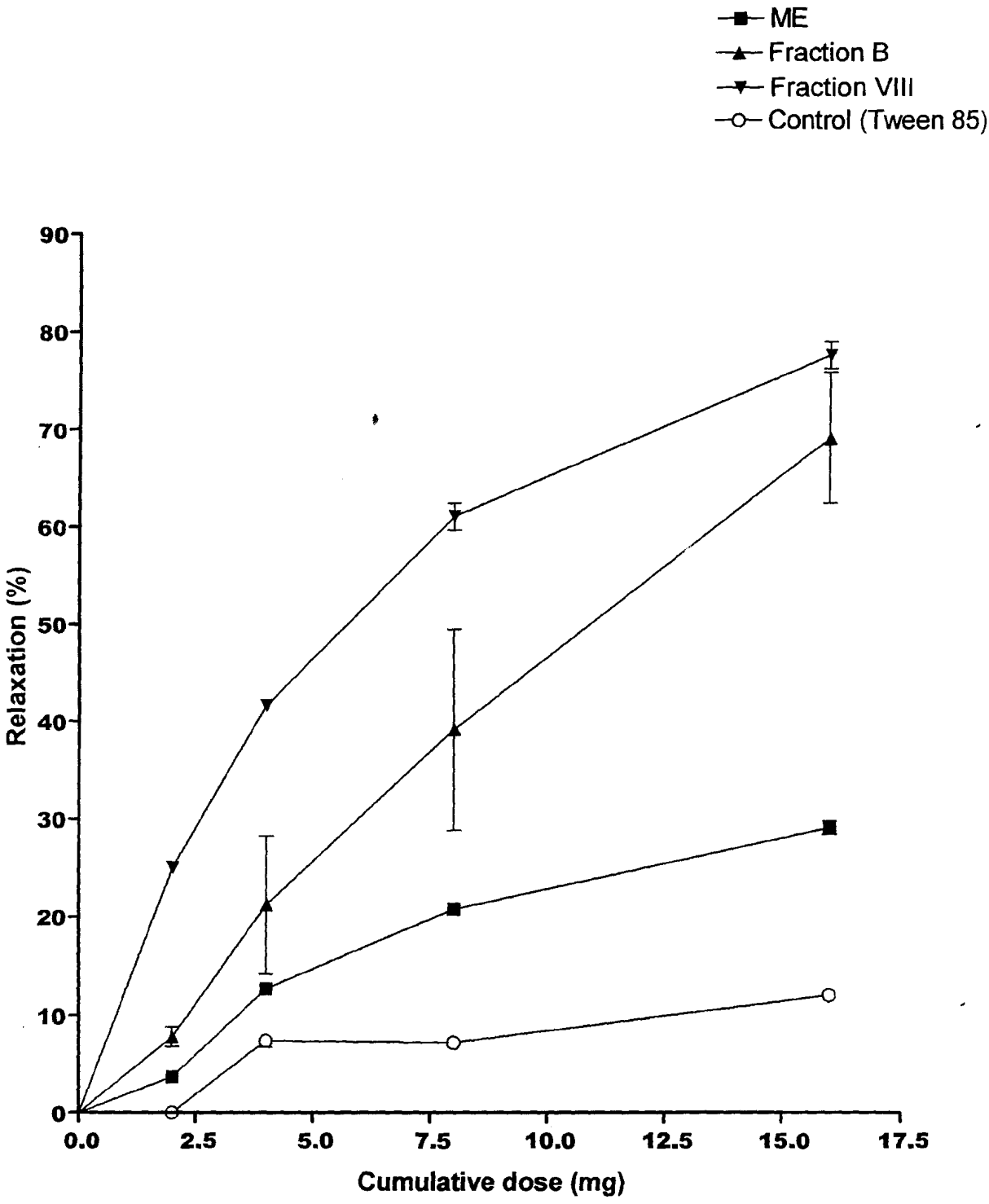

Fig. 17: Relaxant effect of ME, B, and VIII, on guinea pig trachea pre-contracted with histamine

3.7 Effect of ME and fractions on carrageenan induced rat paw edema

In the control rats, carrageenan induced a progressive increase in paw edema for the whole duration of the experiment (4 hours).

The ME produced inhibition of rat paw edema with 400 mg/kg dose causing a greater inhibition of rat paw edema than 200 mg/kg (Fig. 18). Fraction B 200 mg/kg caused a greater inhibition of rat paw edema than 400 mg/kg (Fig. 19). Fraction VIII 200 mg/kg was more potent than 400 mg/kg beyond 30 min, as VIII 400 mg produced the greatest percent inhibition of edema at 30 min (Fig. 20). The percent inhibition of edema produced by VIII 400 mg/kg at 30 min was greater than that produced by piroxicam. Fraction VIII 400 mg/kg also produced a significant ($P < 0.05$) inhibition of paw edema at 30 min and 1hr, after which there was increase in rat paw edema up to 4hr (Fig.20 and Table 8).

The ME and fractions tested produced significant inhibition of rat paw edema at 1 hr. Fraction VIII 200 mg/kg produced significant inhibition of rat paw edema at 1 hr, 2 hr and 3 hr (Table 8).

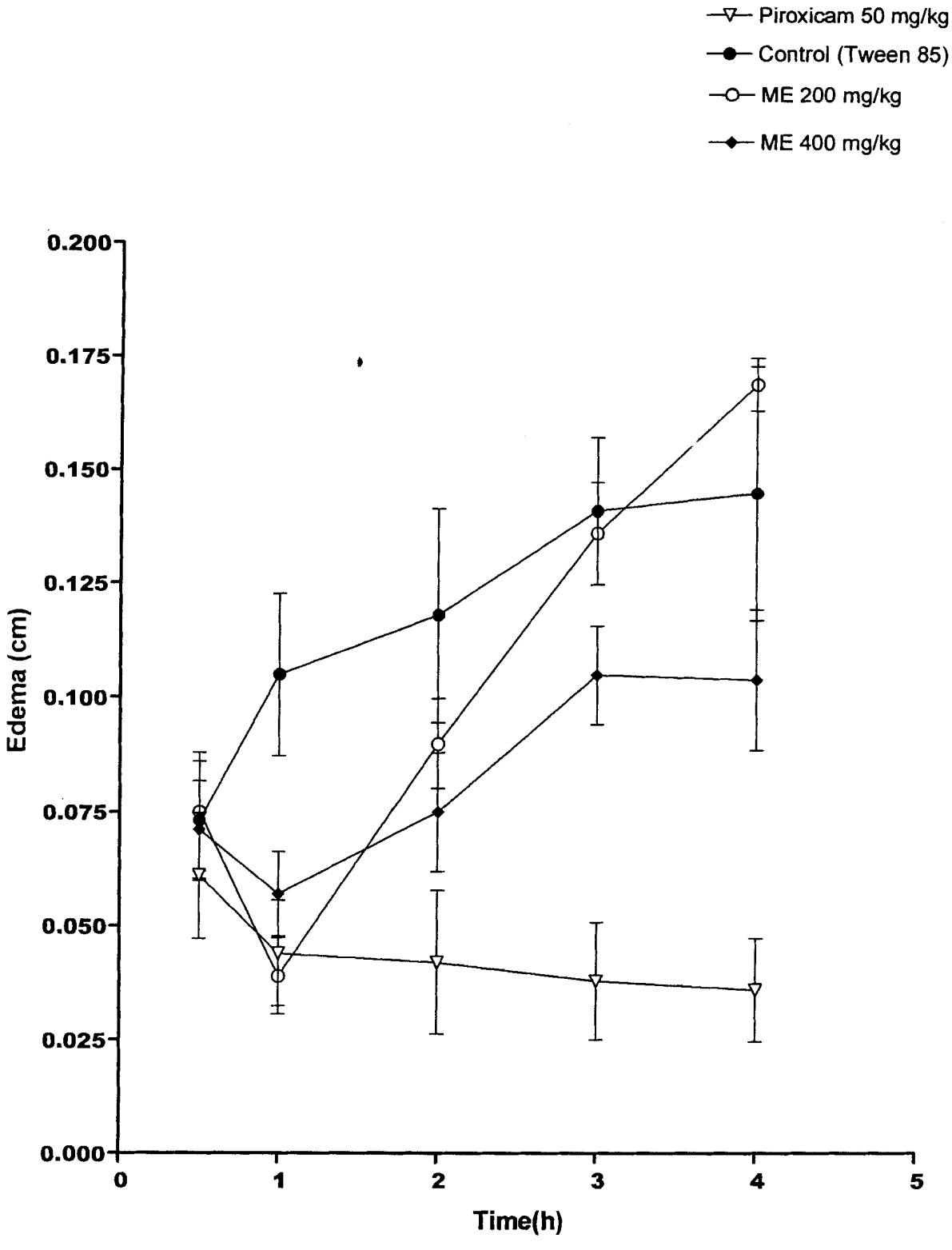

Fig. 18: Effect of ME on carrageenan induced acute edema of the rat paw

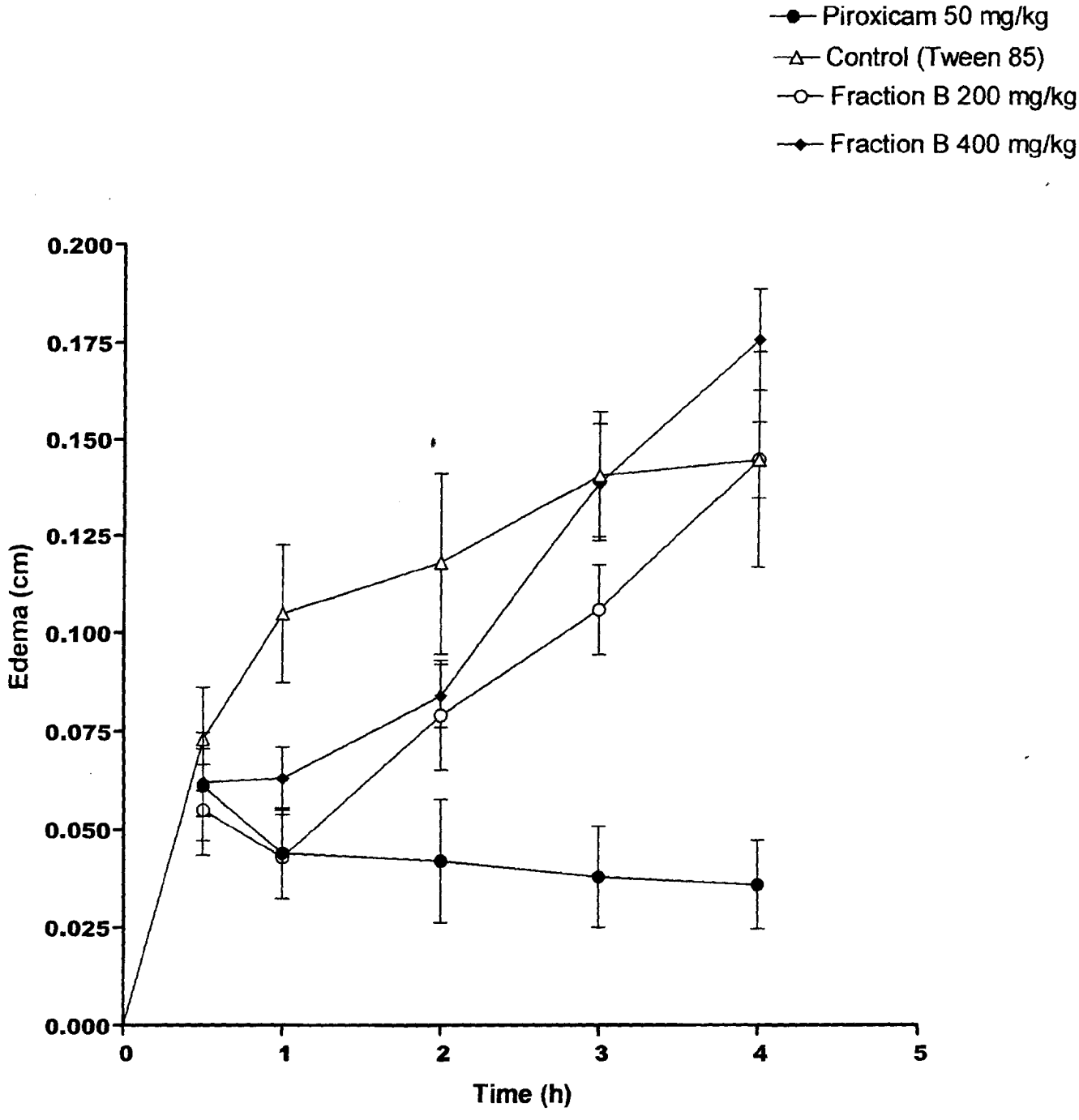

Fig. 19: Effect of fraction B on carrageenan induced acute edema of the rat paw

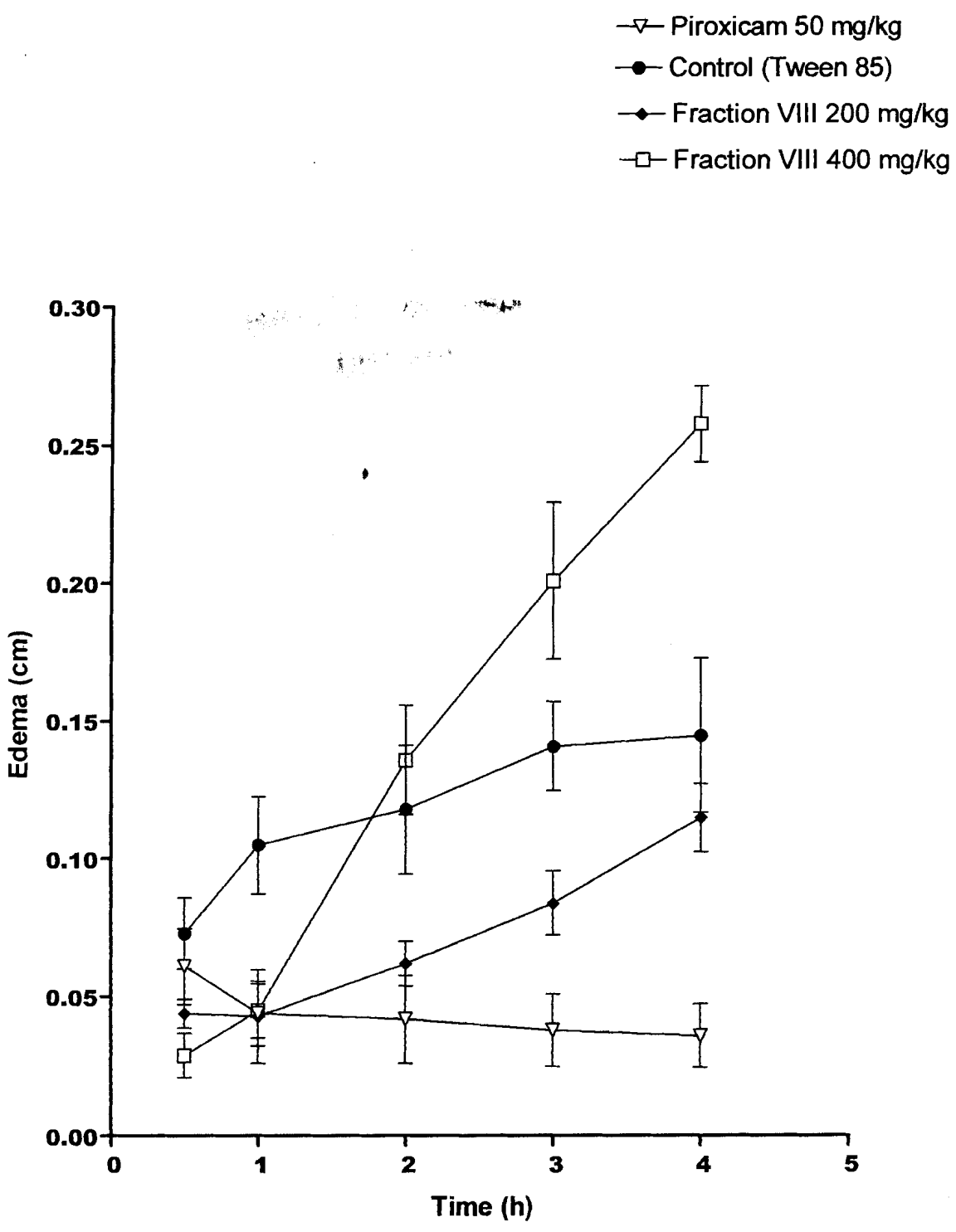

Fig. 20: Effect of fraction VIII on carrageenan induced acute edema of the rat paw

Table 8: Percent inhibition of carrageenan induced rat paw edema

Extract/ Fraction	Dose (mg/kg)	Inhibition of edema (%)				
		0.5 h	1 h	2 h	3 h	4 h
ME	200	-2.74	62.86*	23.73	3.55	-16.55
	400	1.37	45.71*	36.44*	25.53	25.71
B	200	24.66	59.05*	33.05	24.82	0.00
	400	15.07	40.00*	28.81	1.42	-21.38
VIII	200	39.73	59.05*	47.46*	40.43*	20.69
	400	60.27*	57.14*	-15.25	-42.55	-77.93
Piroxicam	50	16.44	58.10*	61.02*	66.67*	63.45*

* $P<0.05$ compared to negative control (One Way ANOVA; LSD post hoc)

3.8 Effects of ME and fractions on carrageenan induced pleurisy in rats

Injection of carrageenan into the pleural cavity leads to pleurisy, infiltration of polymorphonuclear leukocytes, and lung injury.

3.8.1 Effect on volume of pleural exudates

All carrageenan injected rats developed acute pleurisy, with the negative control group producing 0.75± 0.03 ml of turbid exudates. The ME, fraction B, fraction VIII, and dexamethasone all significantly reduced the volume of pleural exudates ($P<0.05$), compared to negative control. The results are as shown in Table 9 and Figure 21.

3.8.2 Effect on leukocyte infiltration into the pleural cavity

The ME, fraction B, fraction VIII, and dexamethasone reduced the total leukocyte count (TLC) (Tables 10 and 11; Fig 22) with ME, fraction VIII, and dexamethasone producing significant reductions of total leukocyte count.

In the differential count, ME, fraction B and fraction VIII increased neutrophil infiltration, while dexamethasone caused a decrease (Tables 10 and 11; Fig 23). The ME, fraction B, and fraction VIII elicited a non-significant reduction in lymphocyte migration, while dexamethasone caused an increase (Tables 10 and 11; Fig 23). The ME and fraction VIII increased eosinophils infiltration, while fraction B, and dexamethasone inhibited eosinophil infiltration. Fraction B and fraction VIII produced no change in monocyte infiltration, while ME and dexamethasone increased eosinophils infiltration (Tables 10 and 11; Fig. 23)

Table 9: Effect of ME and fractions on volume of pleural exudates

Extract/fraction	Dose (mg/kg)	Volume of pleural exudates (ml)	Reduction in volume of pleural exudates (%)
ME	400	0.40 ± 0.00	46.67*
B	400	0.55 ± 0.21	26.67
VIII	400	0.18 ± 0.09	76.00*
Dexamethasone	0.3	0.18 ± 0.09	76.00*
Tween 85	-	0.75 ± 0.03	0.00

* $P<0.05$ compared to negative control (One Way ANOVA; LSD post hoc)

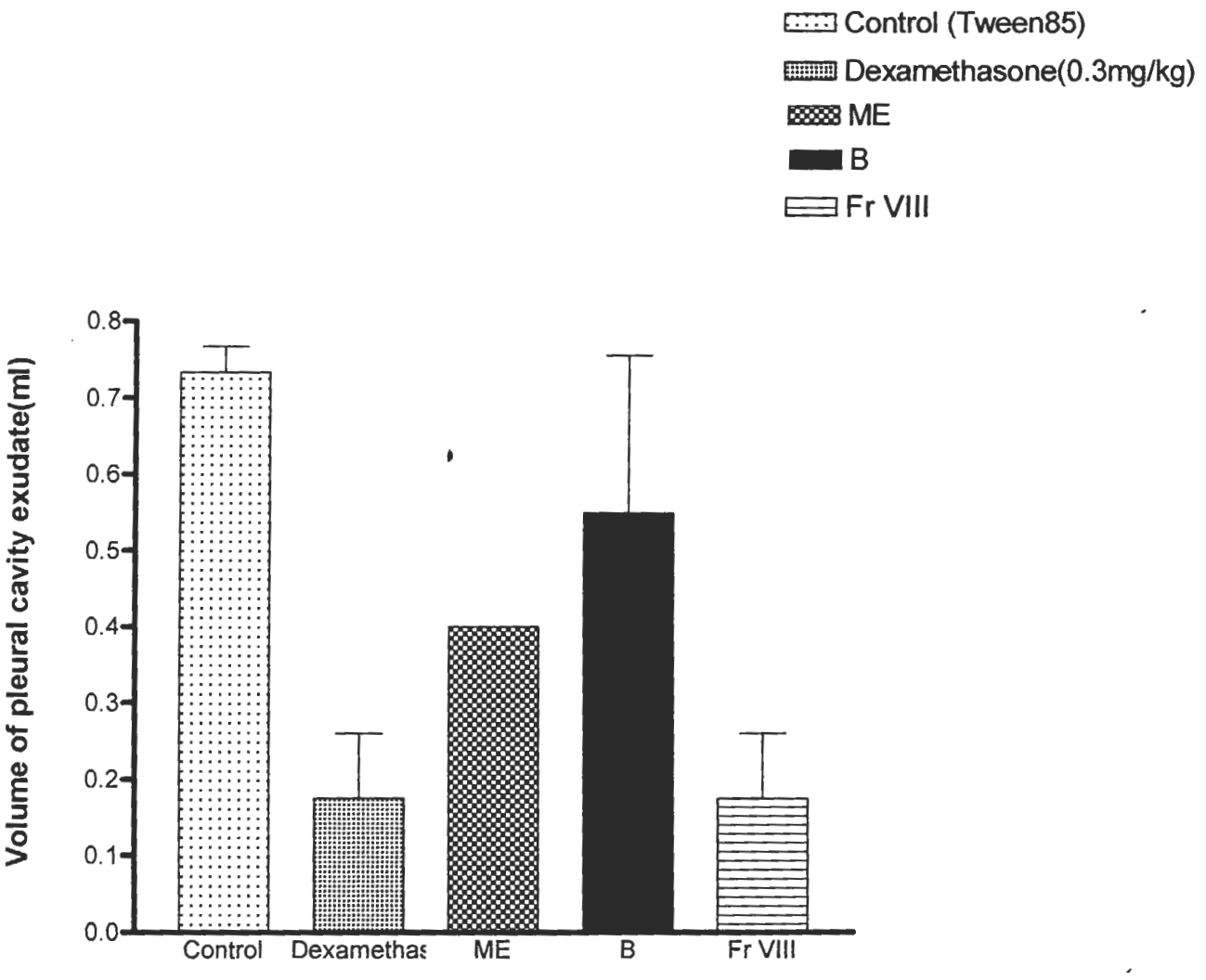

Fig. 21: Effect of ME, B, and VIII on volume of pleural exudate in carrageenan induced pleurisy in rats

Table 10: Effect of ME and fractions on carrageenan induced *in vivo* leukocyte migration into the rat pleural cavity

Extract/ Fraction	Dose (mg/kg)	TLC [Cells(mm^3) $x10^3$]	DLC (%)			
			Eosinophils	Neutrophils	Lymphocytes	Monocytes
ME	400	3.38 ± 0.91	3.50 ± 0.65	71.00 ± 1.68	24.75 ± 1.80	0.75 ±0.48
B	400	10.94 ± 2.79	0.75 ± 0.48	74.00 ± 3.32	25.00 ± 3.44	0.25 ± 0.25
VIII	400	6.59 ± 1.44	2.75 ± 0.63	71.50 ± 3.28	25.50 ± 3.57	0.25 ± 0.25
Dexamethasone	0.3	7.43 ± 1.60	1.25 ± 0.25	65.25 ± 4.59	33.00 ± 4.14	0.50 ± 0.29
Tween 85	-	14.49 ± 1.63	1.75 ± 0.48	65.50 ± 3.30	32.50 ± 2.90	0.25 ± 0.25

Table 11: Percent inhibition of *in vivo* leukocyte migration in rat pleural cavity

Extract/fraction	Dose (mg/kg)	Inhibition (%)				
		TLC	Eosinophils	Neutrophils	Lymphocytes	Monocytes
ME	400	76.67*	-100	-8.40	23.85	-200
B	400	24.50	57.14	-12.98	23.08	0.0
VIII	400	54.52*	-57.14	-9.16	21.53	0.0
Dexamethasone	0.3	48.72*	28.57	0.38	-1.54	-100

* $P<0.05$ compared to negative control (One Way ANOVA; LSD post hoc)

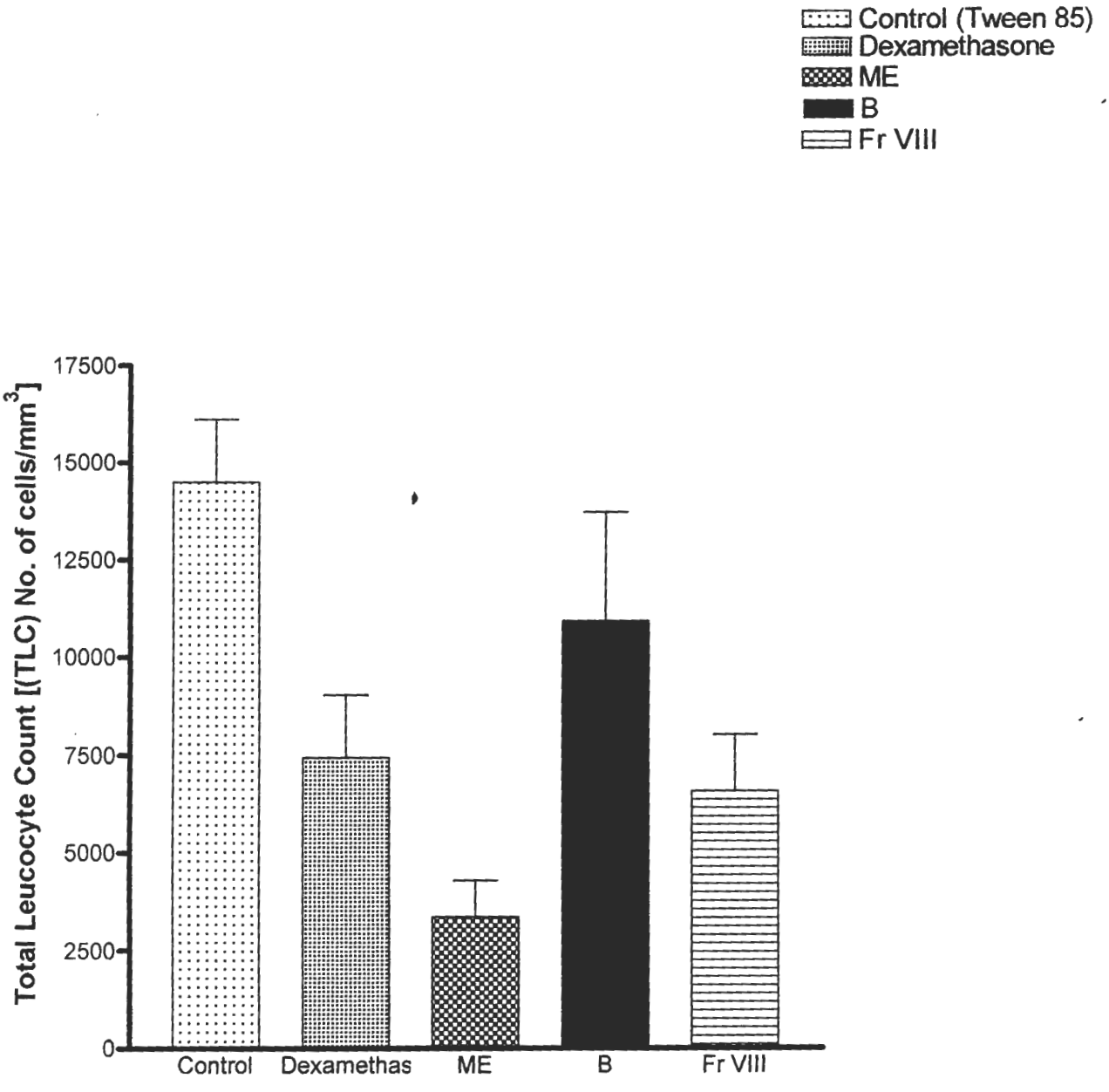

Fig. 22: Effect of ME,B, and VIII on carrageenan induced leukocyte migration in rats *iv vivo*

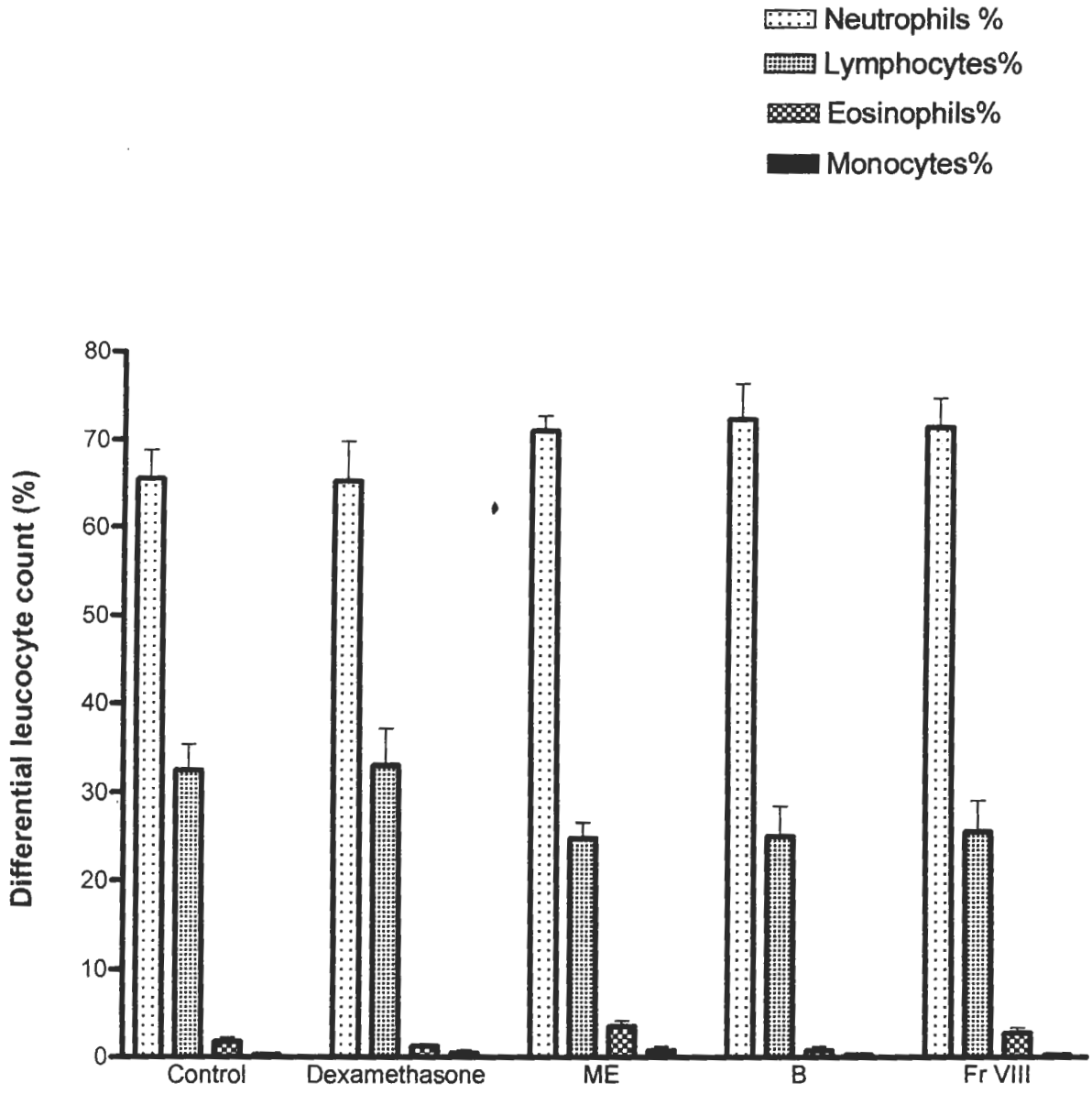

Fig. 23: Effect of ME, B, and VIII on carrageenan induced leukocyte(differential) migration in rats *in vivo*

CHAPTER FOUR

DISCUSSION AND CONCLUSION

The extraction and bioactivity-guided fractionation of the methanol leaf extract of *Asystasia gangetica*, resulted in the isolation of a bronchospasmolytic principle, AG-1.

Acute toxicity study on the ME in mice has established an intraperitoneal LD_{50} greater than 5000 mg/kg, thus suggesting that ME is relatively safe (Lorke, 1983) with remote risk of acute intoxication.

Phytochemical analysis has shown that the ME contained copious amounts of carbohydrates, alkaloids, saponins, glycosides, and terpenes. Flavonoids, terpenes, and trace amounts of glycosides and reducing sugars were present in fraction B, while fraction VIII contained steroids, flavonoids, and terpenes. Fr 2, which yielded AG-1, contained terpenes, and trace amounts of steroids and flavonoids. These plant principles are known to have therapeutic activity eliciting a variety of pharmacological actions related to asthma relief, such as bronchodilatation (Amin and Mehta, 1959; Cambridge *et al.*, 1962; Gopalakrishnan *et al.*, 1979; Tsukawaki *et al.*, 1987), anti-inflammatory effect (Dhananjayan *et al.*, 1975; Gopalakrishnani *et al.*, 1979), and inhibition of histamine-induced bronchoconstriction (Gupta *et al.*, 1977). Analysis of AG-1 indicated the presence of terpenoids, suggesting that it may be a terpenoid compound.

Pharmacologically, the management of asthma is hinged on two main principles; the relaxation of the bronchial muscles to relieve airway obstruction and hyperresponsiveness, and the suppression of inflammation via the inhibition

of the release/action of inflammatory mediators of asthma, such as histamine, cytokines, leukotrienes, and others (Undem, 2006).

The ME, fractions A, B and C, produced initial potentiation of histamine-induced contraction of the guinea pig trachea at lower doses which was lost as the doses were increased and on further separation of the fractions. Thus, fractions obtained from subsequent chromatographic separations lacked this activity. The reason for this is not known and seems likely to be due to some constituents of the ME and the earlier fractions. Therefore, the activity of the later fractions may be limited to the inhibition of contractions and exclusive of initial potentiation.

Generally, the extract and fractions exhibited a dose-related inhibition of histamine-induced contraction of the guinea pig trachea to varying extents. The fact that all the fractions were effective in causing this inhibition suggests that the leaves may contain more than one bronchospasmolytic principle. In this study, however, AG-1 exhibited the most potent effect causing 82% inhibition of histamine-induced contraction of the guinea pig trachea at a dose of 0.4 mg/ml; where ME at 12.8 mg/ml produced less than 50% inhibition.

Also, the extract and fractions relaxed the pre-contracted trachea (pathological tissue), with fraction VIII (which yielded AG-1) exhibiting the most potent relaxation of the guinea pig trachea pre-contracted with histamine. The inhibition of histamine-induced contractions and relaxation of trachea pre-contracted with histamine by the ME and fractions suggest they may possess bronchospasmolytic activity. The magnitude of bronchospasmolytic activity increased in the order: ME < fraction B < fraction VIII < Fr 2 < AG-1. Thus, the

anti-asthmatic effect of *A. gangetica* may derive largely in part from bronchospasmolytic activity and AG-1 is an anti-asthmatic principle/constituent of the leaf. The bronchospasmolytic effect may in part be due to inhibition of the action of histamine on smooth muscles of the tracheo-bronchial tree. Since ME, its fractions and AG-1 inhibitied bronchoconstriction in non-pathological and pathological tissues, it then means that the bioactive extractives of the plant may protect the tracheo-bronchial apparatus from acute asthmatic attacks and can as well relieve already established bronchoconstriction through broncho-relaxant effect. Bronchodilators reverse/relax the contraction of airway smooth muscle and are used in the management of asthma. Some may have additional effect on mucosal edema or inflammation (Barnes, 1993a). Histamine H_1 . receptors are found in human and guinea pig bronchial smooth muscles. Although the classical first generation antihistamines (H_1 blockers) are not useful in the management of asthma, some new H_1 blockers e.g. azelastine and cetirizine have anti-inflammatory properties acting on kinin, leukotriene and prostaglandin production and may be the precursors of new anti-asthmatic drugs (Greene and Harris, 2000).

The significance of inflammation in asthma is generally highlighted by the fact that bronchoconstriction/airflow limitation and airway hyperresponsiveness are the direct result of airway wall inflammation (Holt *et al.*, 1999; Prasad *et al.*, 2000). The rediscovery of asthma as a disease of progressive inflammation (Barnes, 1993b; Prasad *et al.*, 2000) necessitated the study of the anti-inflammatory activity of the extract and fractions especially since the leaves have been shown to possess anti-inflammatory activity (Akah *et al.*, 2003). Study of the

anti-iflammatory activity of the extract and fractions revealed some degree of suppression of acute inflammation which peaked at 1 h post induction, thus suggesting a potent inhibition of histamine release. In carrageenan induced rat paw edema, the inflammatory reaction is biphasic in nature. The early phase starts 1 h after the administration of the phlogistic agent and is due to the release of histamine and serotonin, while the late phase starts 3-5 h after induction and is due to the release of prostaglandins, bradykinins and other inflammatory mediators (Vinegar *et al.*, 1969; Di Rosa and Sorrentino, 1969; Flower *et al.*, 1972; Ferreira *et al.*, 1974). This is analogous to the airway inflammatory reaction in asthma. Suppression of inflammation 1 h post injection of phlogistic agent suggests an anti-histamine effect, whereas suppression at 4-6 h suggests an inhibition of the arachidonic acid pathway (Willoughby and Flower, 1993). Some anti-inflammatory drugs act by antagonizing or destroying the mediators of inflammation like histamine, serotonin, leukotrienes, cytokines e.t.c. (Akah *et al.*, 1997). The inhibition of histamine-induced contraction of the trachea may also indicate that the anti-inflammatory activity of the extractives may derive from inhibition of histamine which has been implicated in the pathogenetic mechanism of inflammation in asthma (Kobzik and Schoen, 1994; Barnes *et al.*, 1998a). Histamine is one of the primary mediators released in the early phase of asthma attack, which subsequently leads to the release of other secondary mediators and initiation of the late phase (Kobzik and Schoen 1994; Barnes *et al.*, 1998a). Thus, its inhibition naturally slows down the inflammatory response. In addition to accelerating the destruction or antagonizing the action of these mediators (Vane,

1971; Akah *et al.*, 1993), many anti-inflammatory agents are known to modify the inflammatory response by stabilization of lysosomal membrane (Ignarro, 1971), inhibition of lysosomal enzyme (Anderson, 1968) and mononuclear leukocyte migration (Di Rosa *et al.*, 1971). It is apparent that the anti-inflammatory action of this plant is also partly due to antagonism of inflammatory mediators.

In the acute pleurisy study, the reduction in the volume of pleural exudates caused by the extract and fractions is an indication of inhibition of vascular permeability and plasma exudation and suppression of airway secretion. Histamine is known to increase vascular permeability, plasma exudation, and airway secretion in asthma (Barnes *et al.*, 1998a) and the early phase of carrageenan-induced pleurisy is associated with the production of histamine, leukotrienses, platelet activating factor, and possibly cyclooxygenase products (Cuzzocrea *et al.*, 2001). The exudates, serum proteins and cell debris cause airflow limitation due to airway mucosal edema, swelling of the airway wall, and mucus plug formation. Inhibition of fluid accumulation in the pleural cavity by the extract and fractions suggest they may suppress airway mucosal edema which contributes to airflow limitation in asthma. Polymorphonuclear cells are the predominant content of cellular exudates at 4 h post pleurisy (Meacock and Kitchen, 1979). Although the extract and fractions reduced the total leukocyte count (TLC), they did not reduce the differential leukocyte count for neutrophils, monocytes and eosinophils except for fraction B that elicited a mild reduction in eosinophil count. Lymphocyte count was, however, mildly reduced by ME, B, and VIII. Lymphocytes are among the inflammatory cells implicated in asthma as

chronic inflammation associated with asthma is characterized by airway submucosal infiltration of lymphocytes. T lymphocytes contribute to the induction and maintenance of allergic reactions and are proinflammatory cells (NHLBI/WHO, 1995). It is likely that the mild reduction in lymphocyte infiltration caused by the extract and fractions may be beneficial in asthma as this may slow down the inflammatory process. This may partly account for the use of this plant in asthma.

From the foregoing, it is evident that the anti-asthmatic effect of *A. gangetica* could be largely due to the isolated constituent, AG-1. Although phytochemical tests showed that AG-1 is a terpenoid compound, the precise identity will be fully established when the chemical structure is characterized. Triterpenoids such as trans-phytol (Singh *et al.*, 1991) as well as the diterpene, forskolin (Lichey *et al.*, 1984; Tsukawaki *et al.*, 1987) have been shown to possess bronchospasmolytic activity. Elucidation of the structural identity of AG-1 is ongoing.

In conclusion, the leaf of *Asystasia gangetica* may largely owe its effectiveness in the treatment of asthma to bronchospasmolytic effect in addition to modulation of the acute inflammatory response as well as inhibition of cell migration. AG-1, which is likely a terpenoid compound, may be the active bronchospasmolytic principle.

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APPENDIX

Fig. A1: Effect of fraction A on histamine-induced contractions of the guinea pig trachea

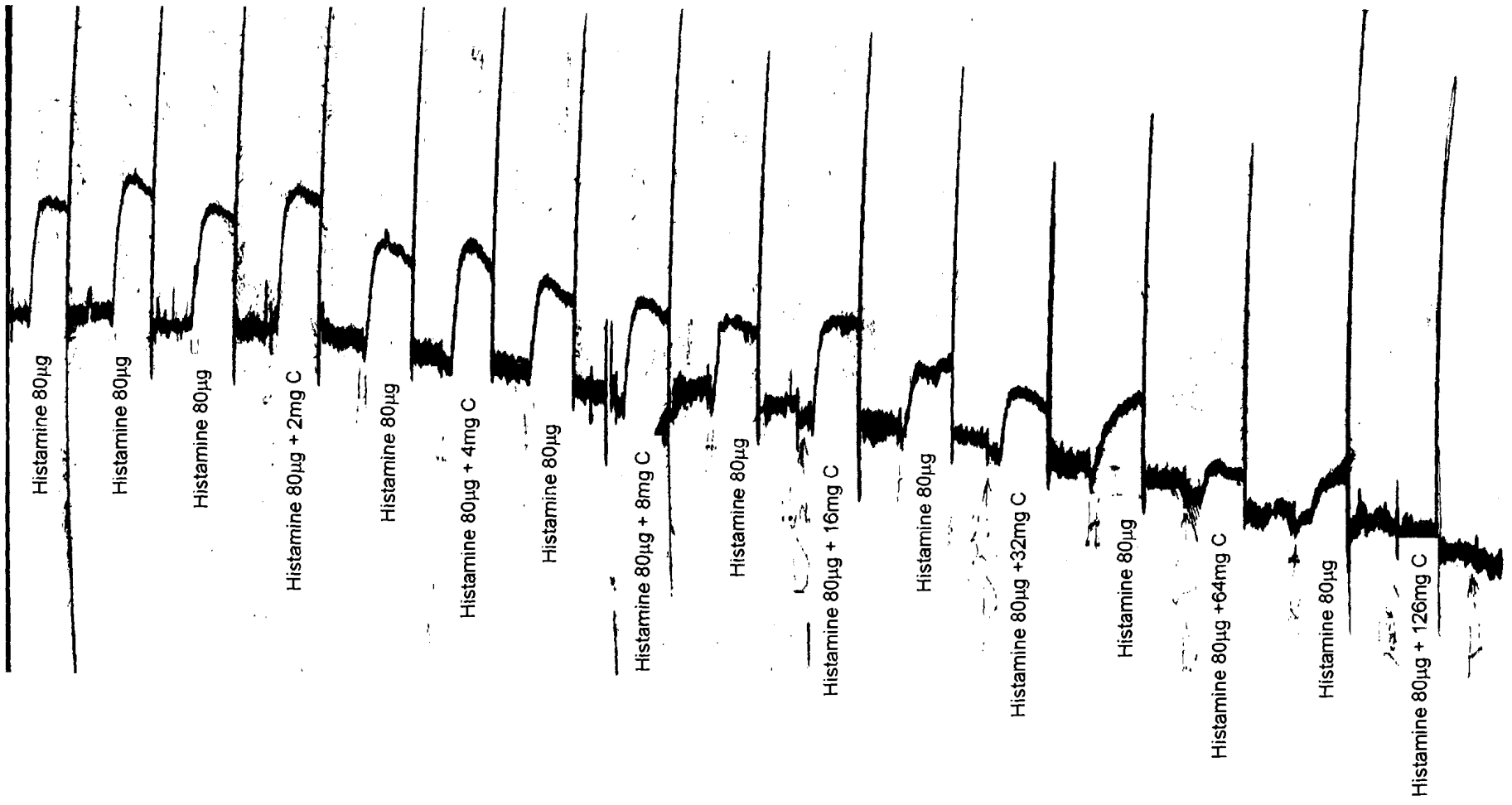

Fig.A2: Effect of fraction C on histamine-induced contractions of the guinea pig trachea

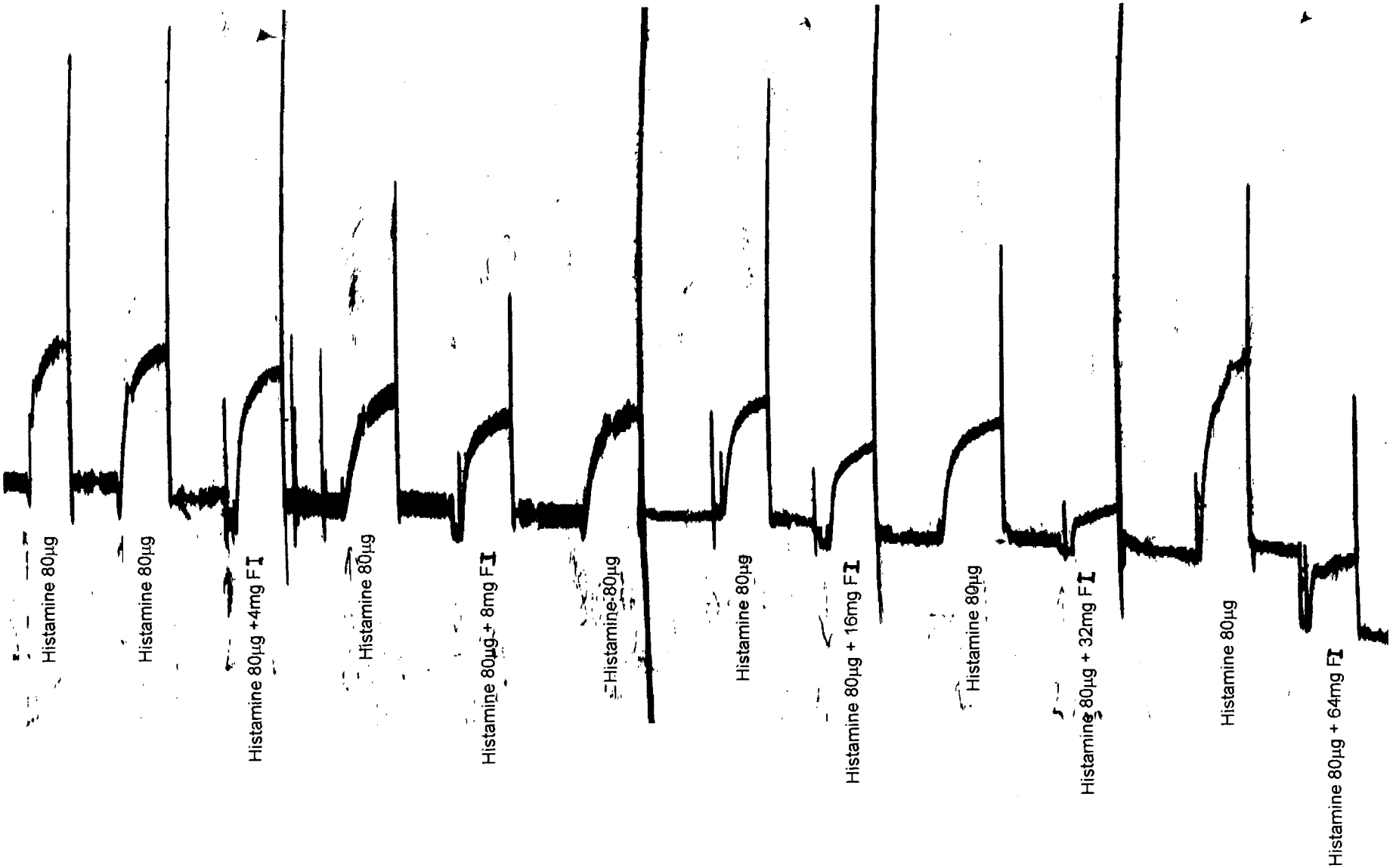

Fig. A3: Effect of fraction I on histamine-induced contractions of the guinea pig trachea

Fig. A4: Effect of fraction II on histamine-induced contractions of the guinea pig trachea

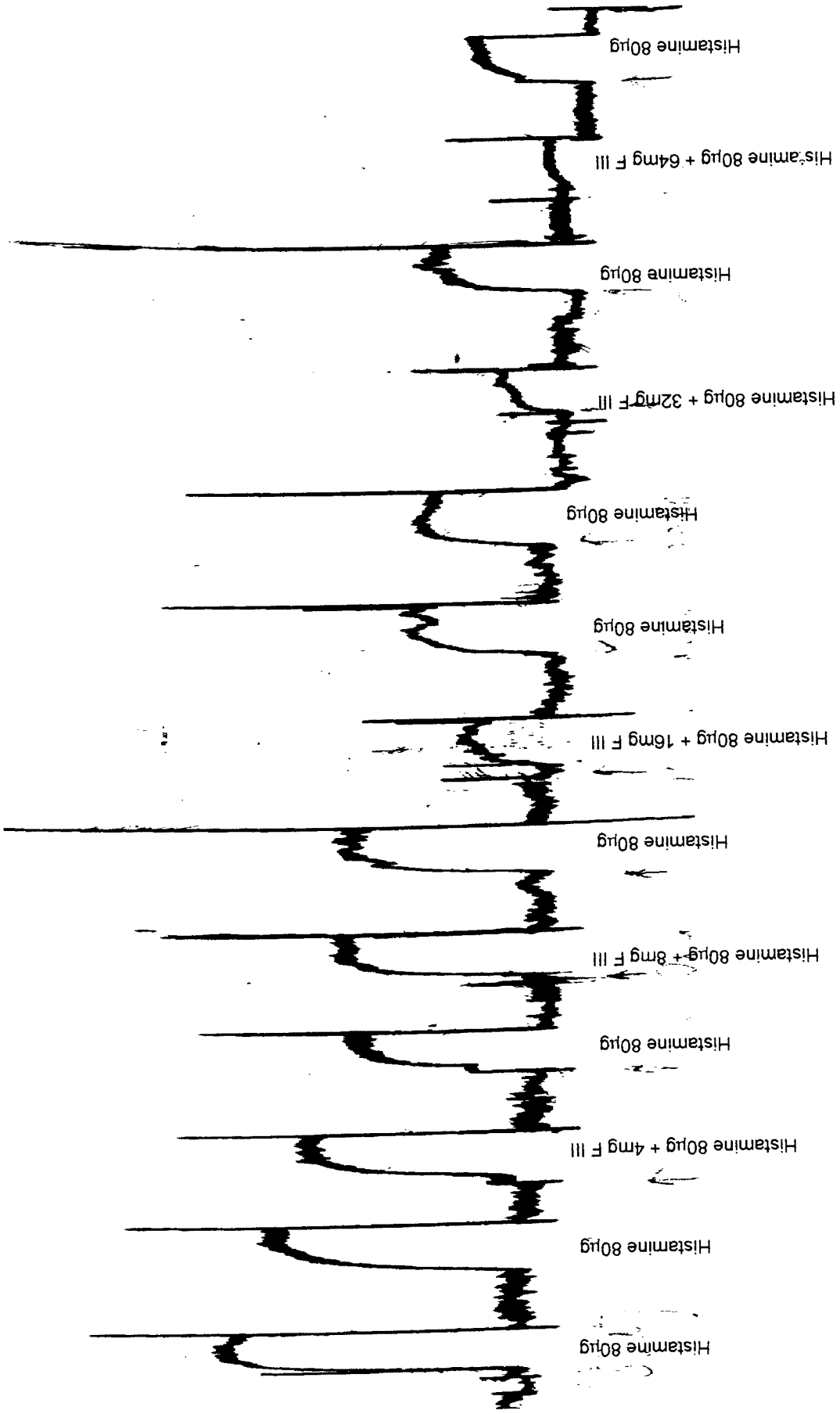

Fig. A5: Effect of fraction III on histamine-induced contractions of the guinea pig trachea

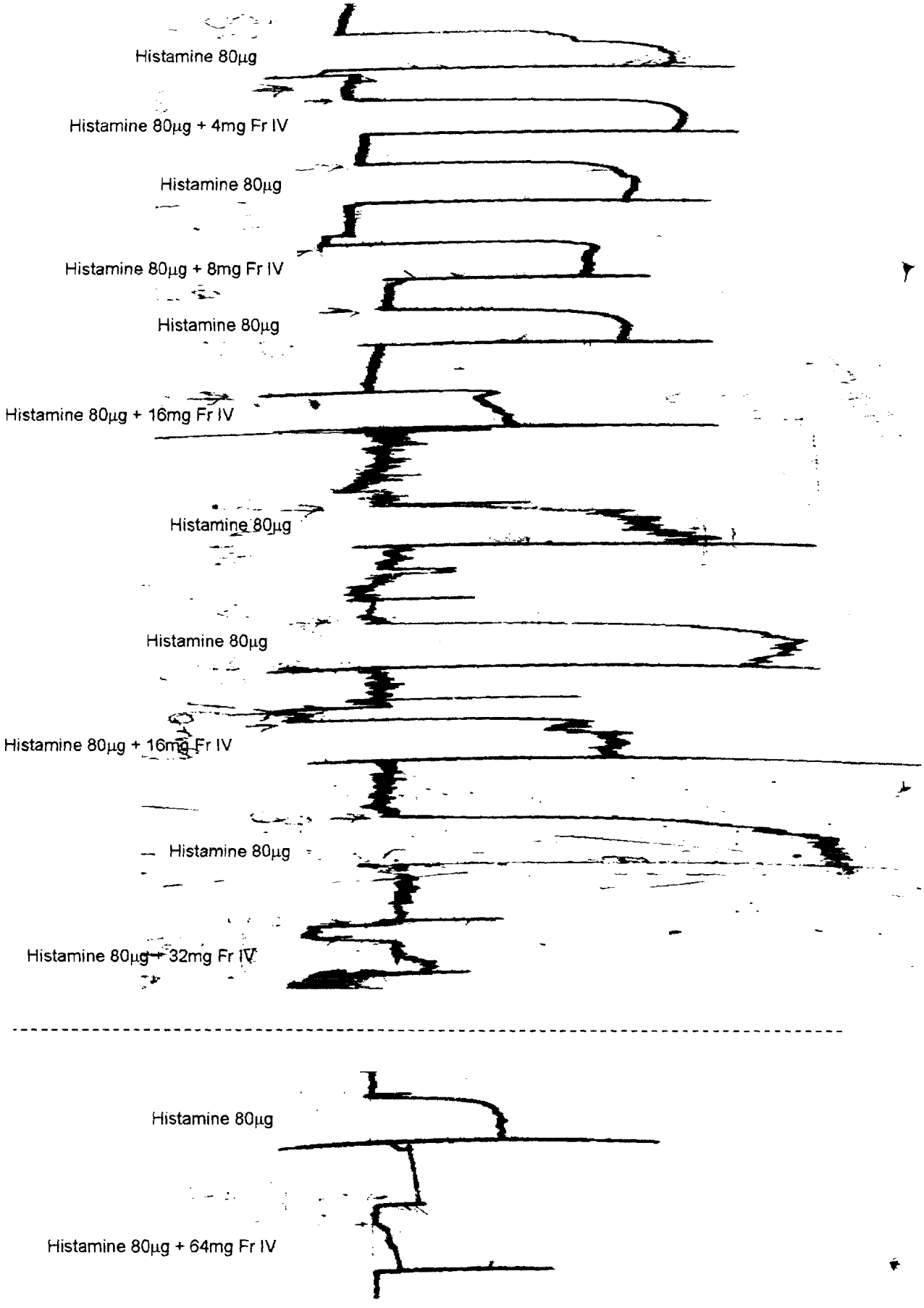

Fig. A6: Effect of fraction IV on histamine-induced contractions of the guinea pig trachea

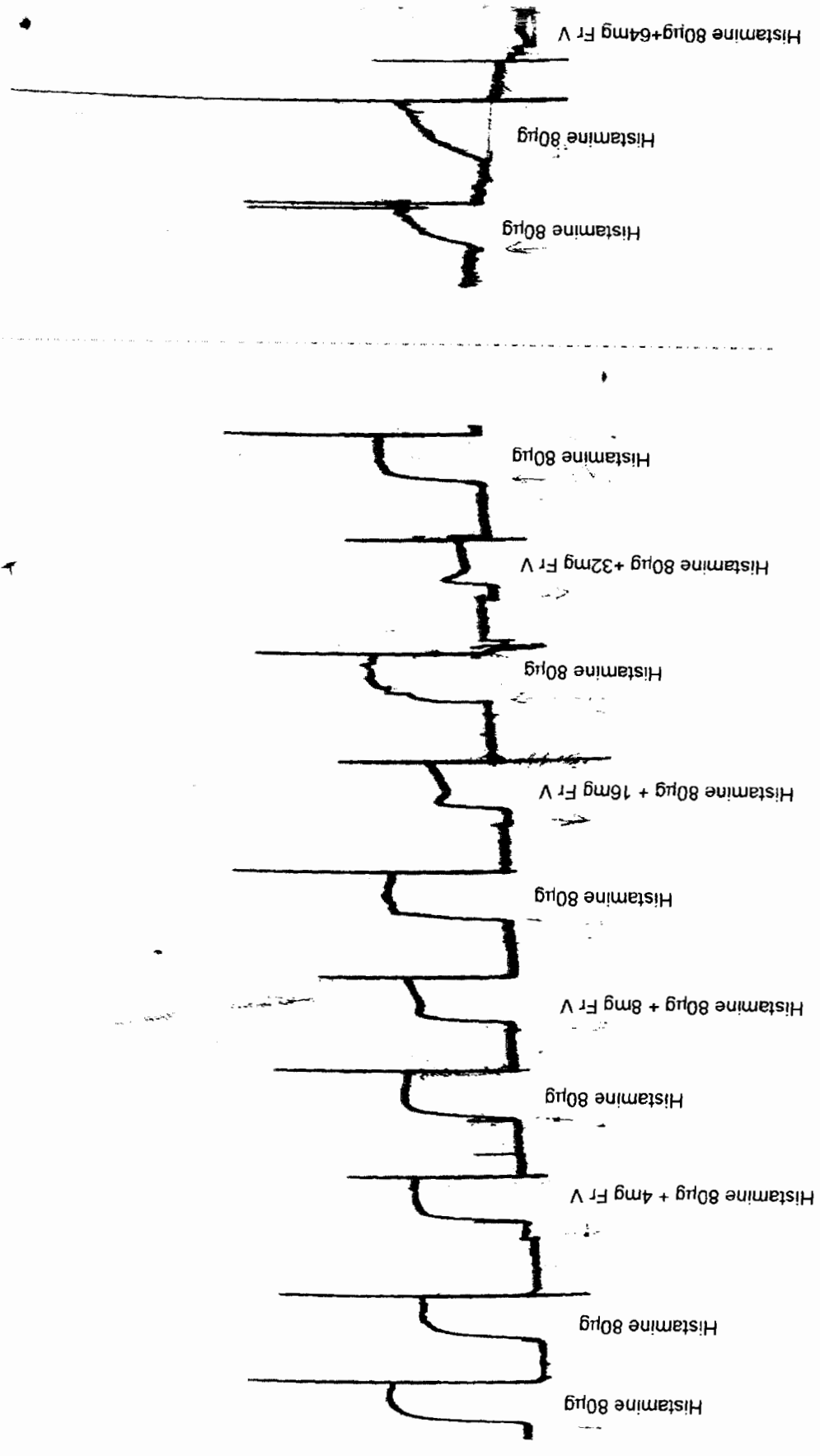

Fig. A7: Effect of fraction V on histamine-induced contractions of the guinea pig trachea

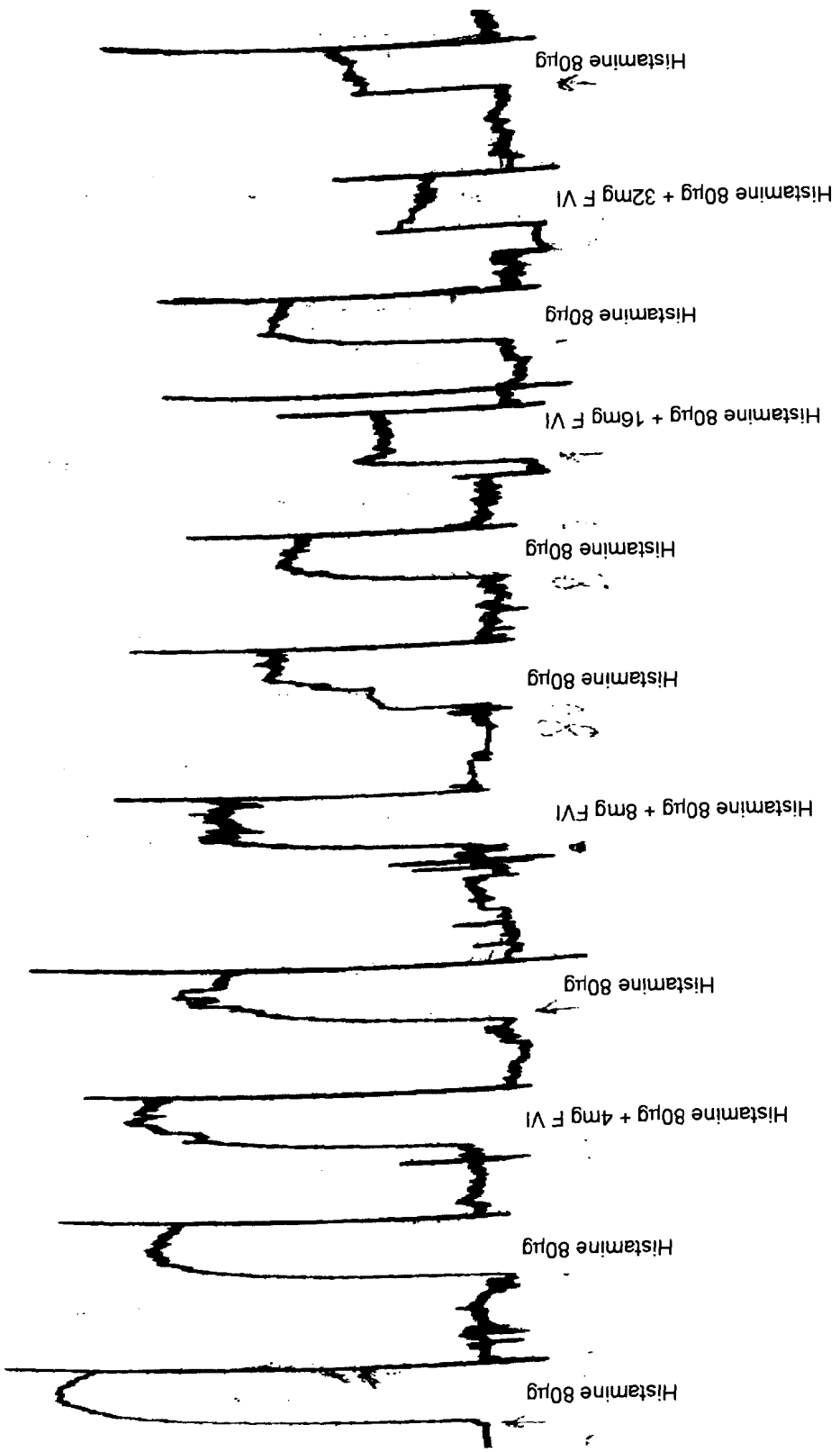

Fig. A8: Effect of fraction VI on histamine-induced contractions of the guinea pig trachea

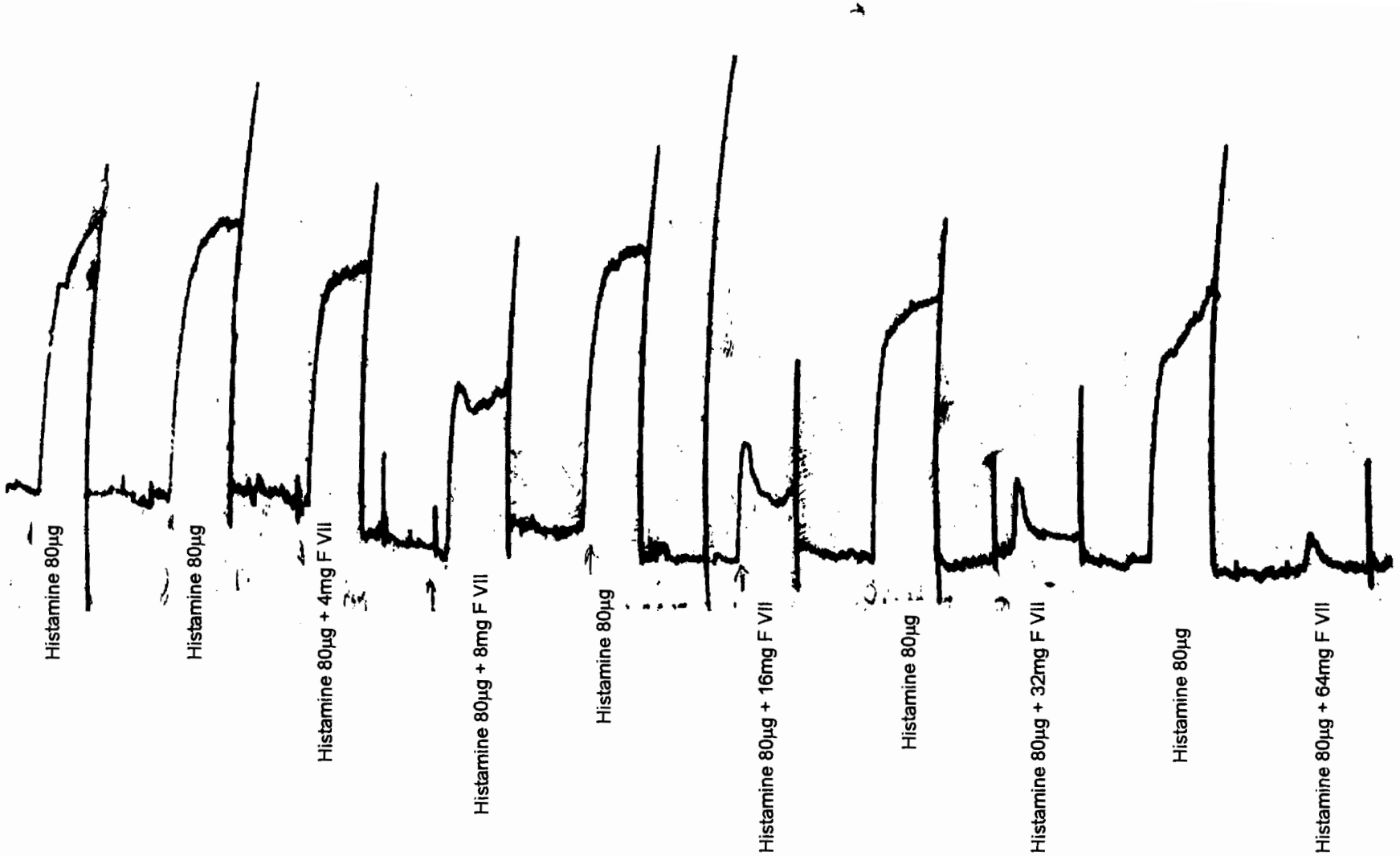

Fig. A9: Effect of fraction VII on histamine-induced contractions of the guinea pig trachea

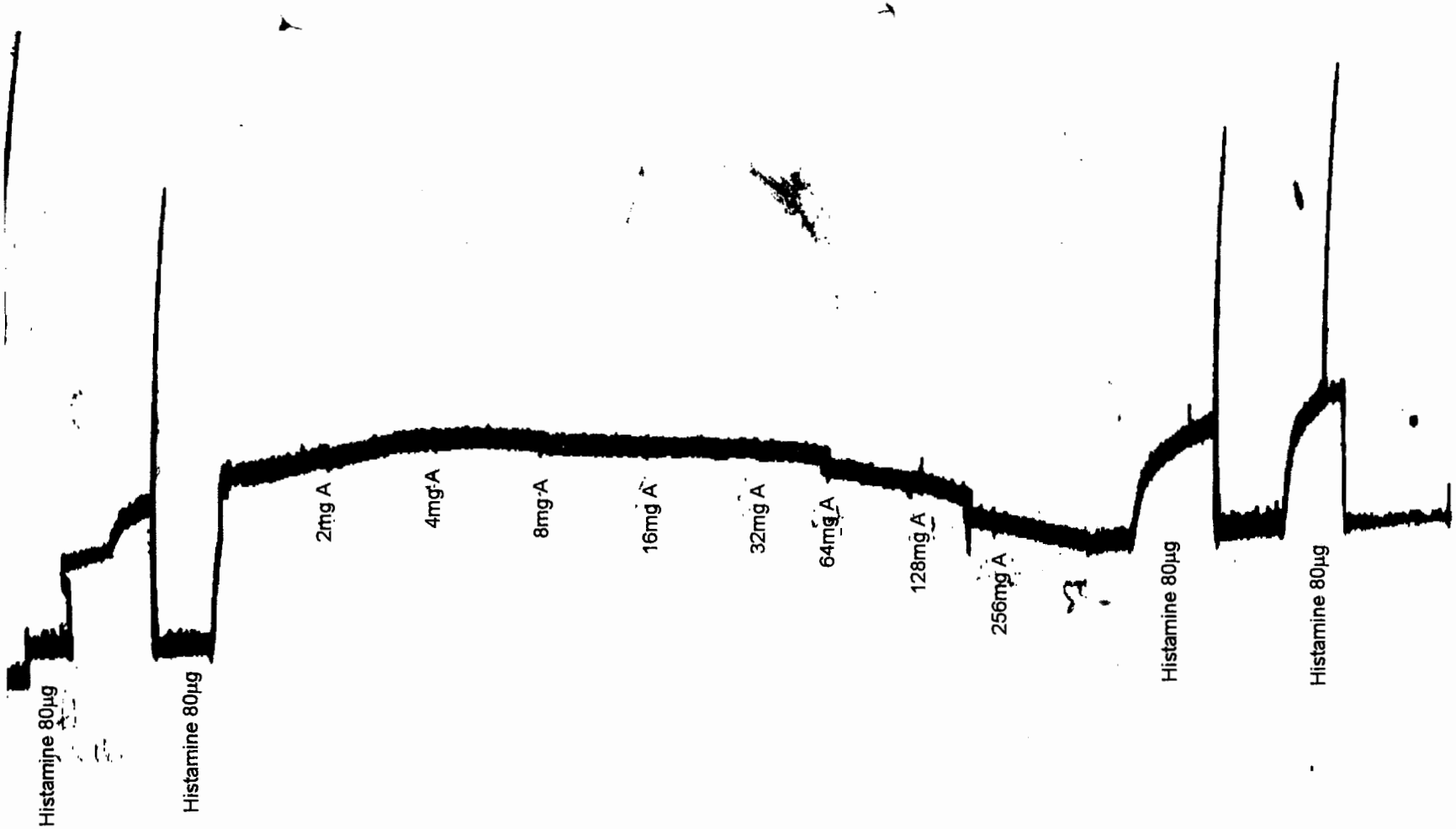

Fig.A10: Effect of cumulative doses of fraction A on guinea pig trachea pre-contracted with histamine

Fig. A11: Effect of cumulative doses of fraction C on guinea pig trachea pre-contracted with histamine

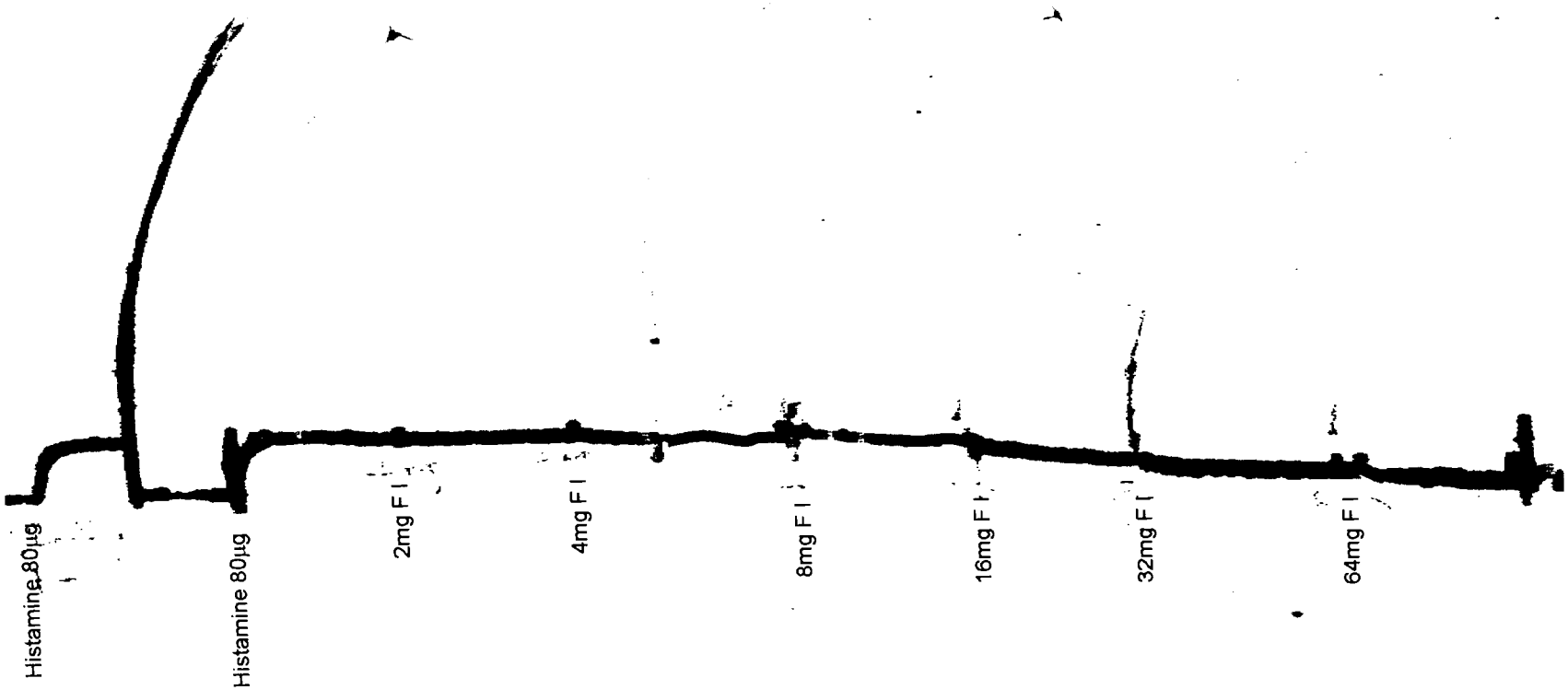

Fig. A12: Effect of cumulative doses of fraction I on guinea pig trachea pre-contracted with histamine

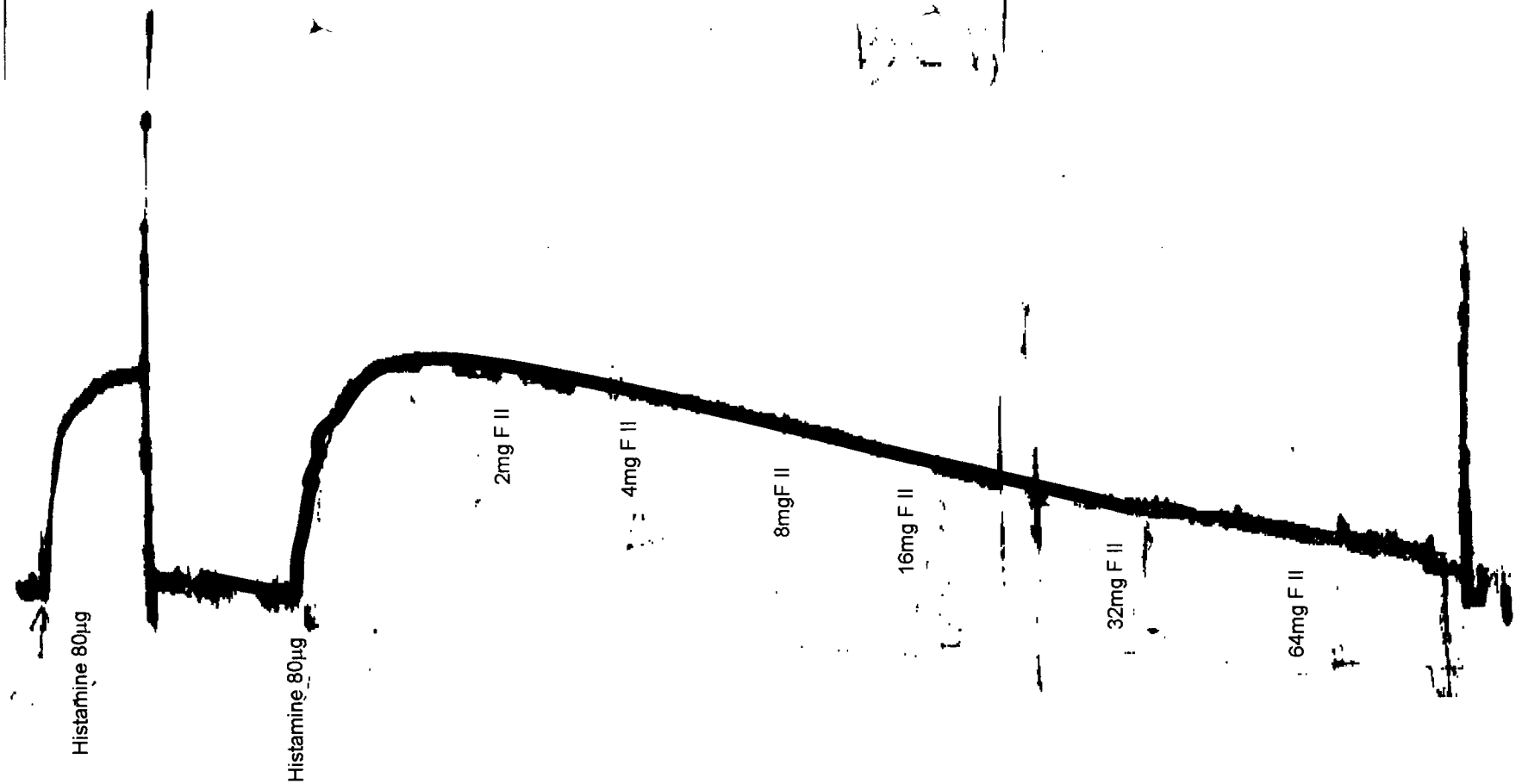

Fig.A13: Effect of cumulative doses of fraction II on guinea pig trachea pre-contracted with histamine

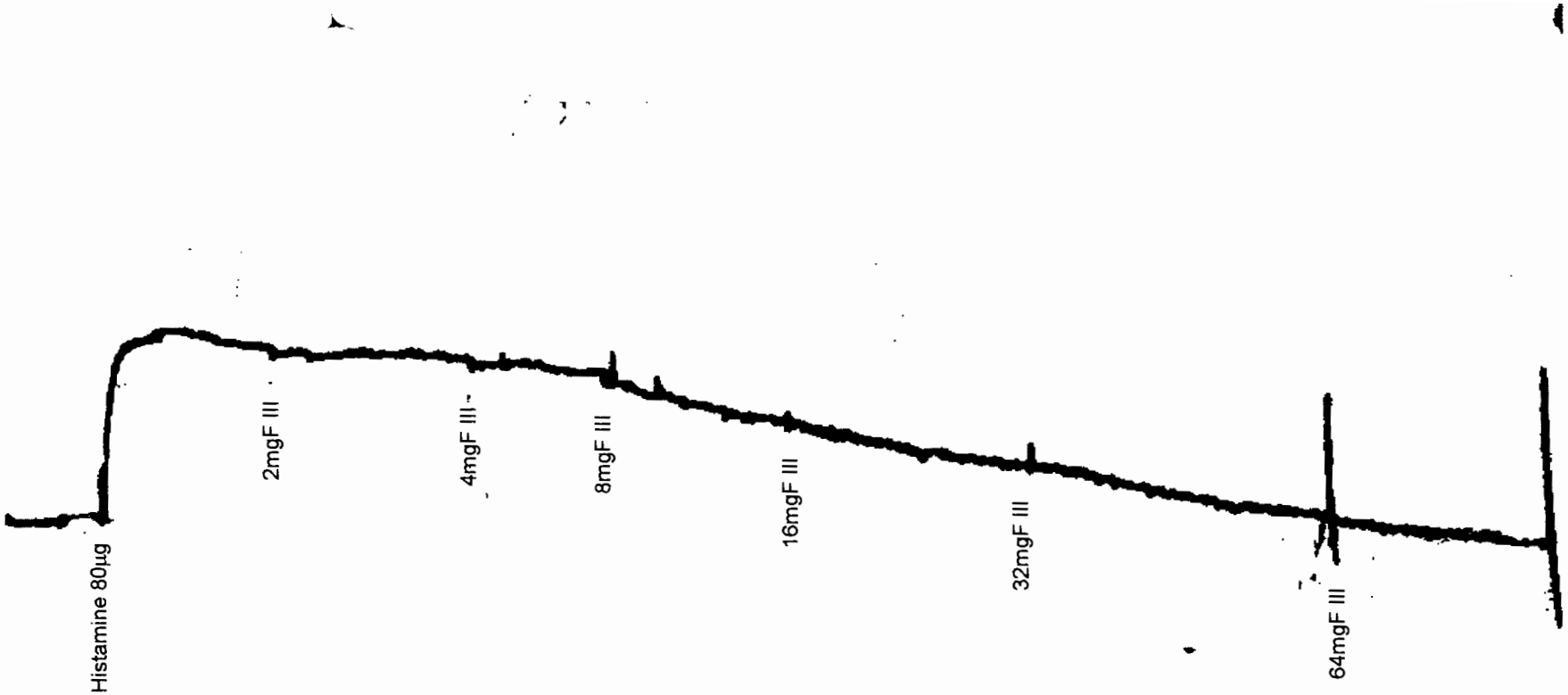

Fig.A14: Effect of cumulative doses of fraction III on guinea pig trachea pre-contracted with histamine

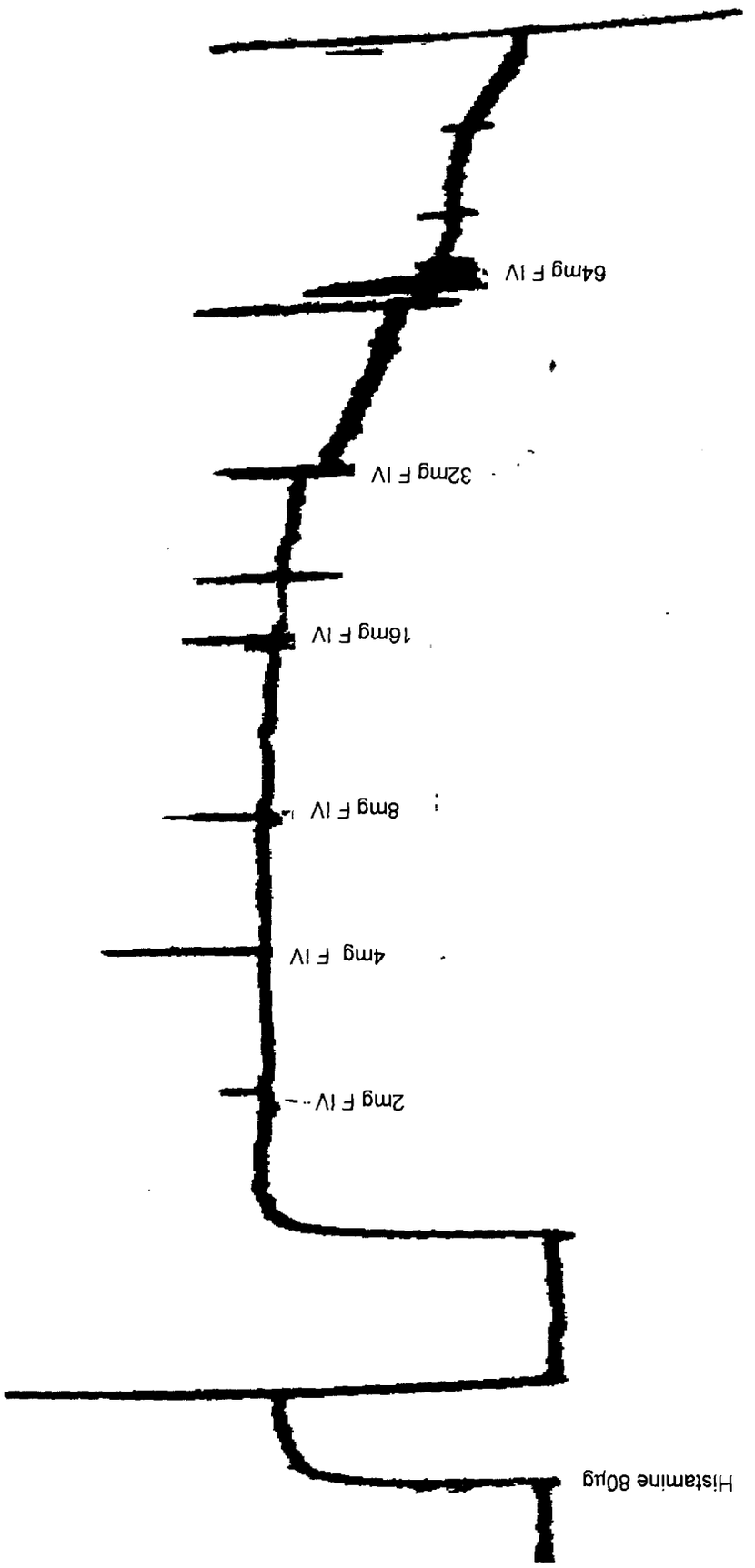

Fig.A15: Effect of cumulative doses of fraction IV on guinea pig trachea pre-contracted with histamine

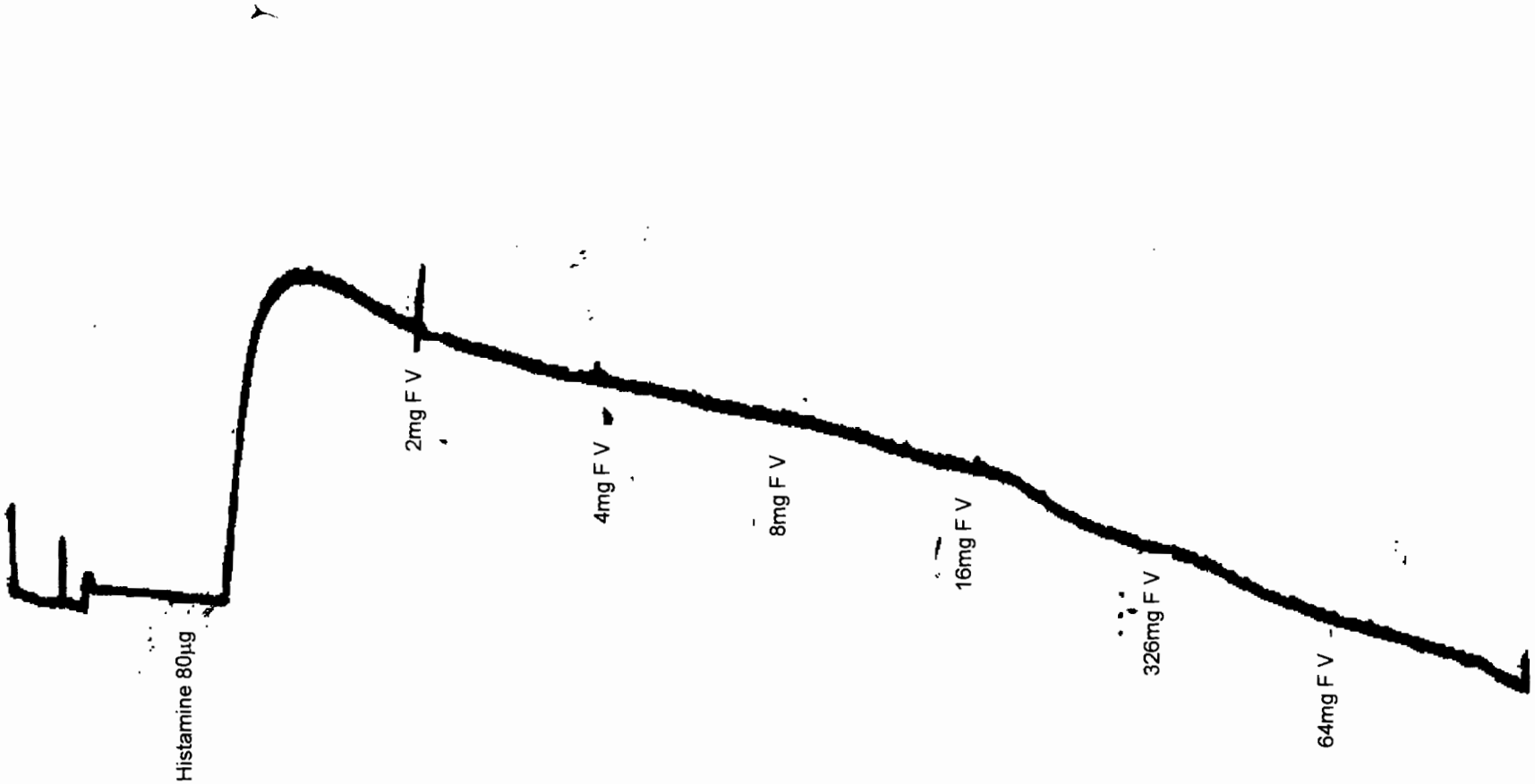

Fig.A16: Effect of cumulative doses of fraction V on guinea pig trachea pre-contracted with histamine

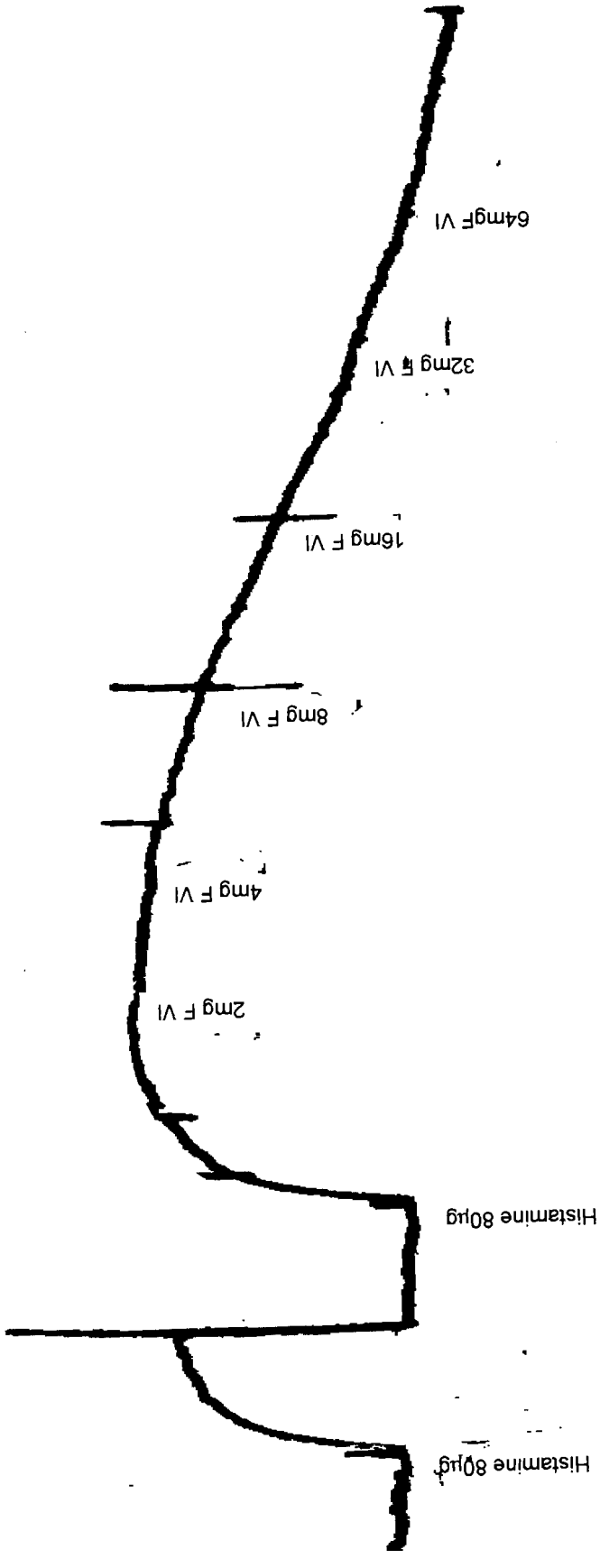

Fig. A17: Effect of cumulative doses of fraction VI on guinea pig trachea pre-contracted with histamine

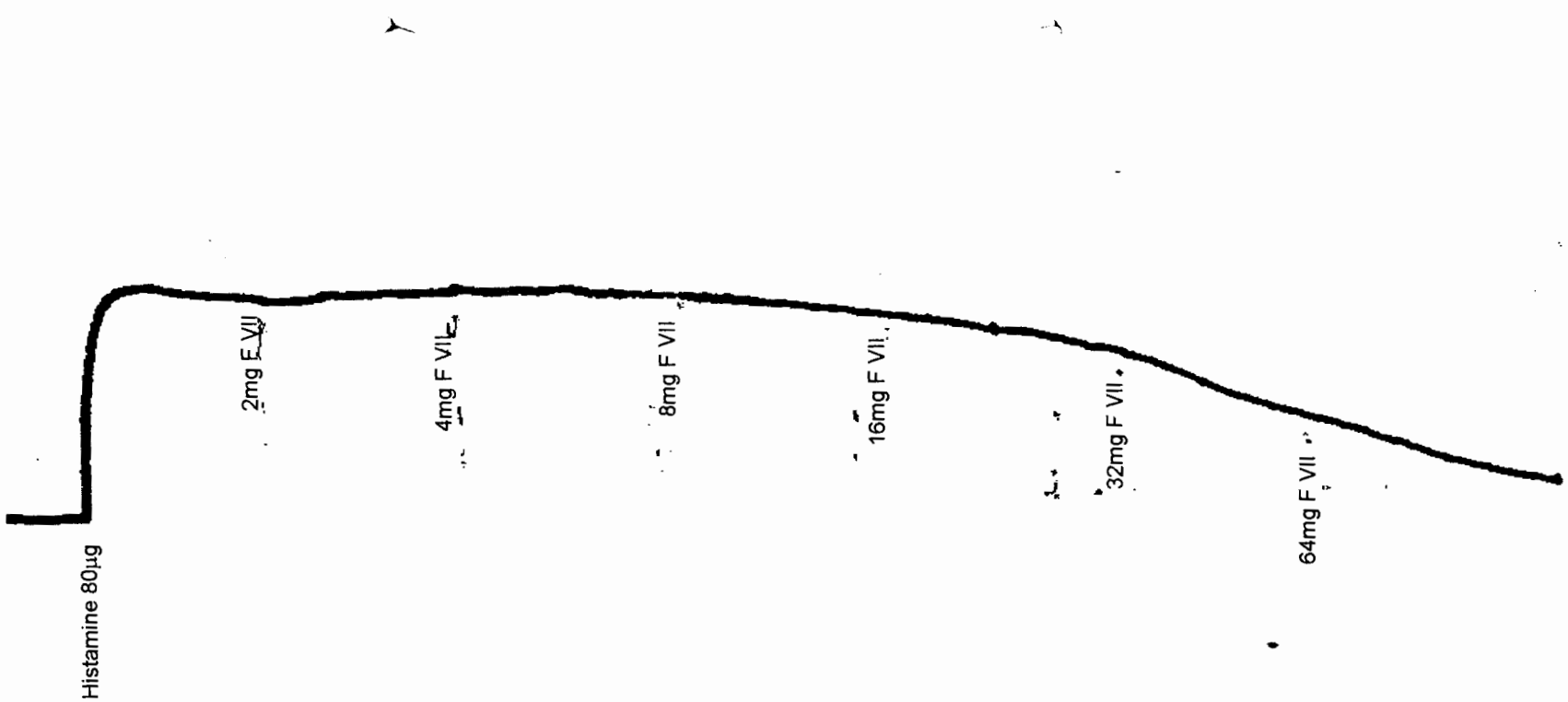

Fig. A18: Effect of cumulative doses of fraction VII on guinea pig trachea pre-contracted with histamine

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Evaluation of the anti-asthmatic property of *Asystasia gangetica* leaf extracts

P.A. Akah [a,*], A.C. Ezike [a], S.V. Nwafor [a], C.O. Okoli [a], N.M. Enwerem [b]

[a] *Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, Enugu State, Nigeria*

[b] *Department of Medicinal Plant Research and Traditional Medicine, National Institute for Pharmaceutical Research and Development (NIPRD), Abuja, Nigeria*

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Abstract

The leaf of *Asystasia gangetica* T. Adams (Acanthaceae) is used in many parts of Nigeria for the management of asthma. This study was aimed at investigating the anti-asthmatic property of hexane, ethylacetate, and methanol extracts of the leaves of *Asystasia gangetica*, obtained by successive sohxlet extraction. The results indicated that the extracts did not exhibit contractile or relaxant activity in isolated tissue preparations; however, they inhibited the contraction evoked by spasmogens; the IC_{50} were calculated, where possible. The extracts relaxed histamine-precontracted tracheal strips in the following degree of potency—ethylacetate extract > hexane extract = methanol extract. The extracts also exhibited anti-inflammatory activity in the order of magnitude—methanol extract > hexane extract > ethylacetate extract. Acute toxicity test estimated an i.p. LD_{50} of 2150 mg/kg in mice for methanol extract while phytochemical screening showed the presence of carbohydrates, proteins, alkaloids, tannins, steroidal aglycones, saponins, flavonoids, reducing sugars, and triterpenoids, with the methanol extract having the highest number of constituents. The study justified the use of the leaf of *Asystasia gangetica* in the management of asthma in Nigerian folk medicine.

Keywords: *Asystasia gangetica*; Anti-asthmatic; Anti-inflammatory; Isolated tissues

1. Introduction

Asthma is a chronic disease of the airways/respiratory system with a worldwide incidence of 155 million (Cokson, 1999), and it is a disease that does not respect the boundaries of race, age, and gender. The availability of effective medications notwithstanding, the prevalence of asthma is increasing (Rona et al., 1995; Cokson, 1999) with 5–10% rate reported for Nigeria (Chukwu et al., 2000). There are incresing demand for the use of traditional medicines in the management of asthma, and *Asystasia gangetica* (L) T. Adams (Family Acanthaceae) is one of such plants with acclaimed potency in asthma.

Asystasia gangetica is a straggling herb usually found among short grasses and along pathways. The leaves are green, oval-shaped with rounded base, very slightly saw-edged, and smooth (Saunders, 1958). The plant is recognized as a potential food source because the leaves have been shown to contain high amounts of proteins, amino acids, minerals, sugars, lipids, and fiber (Yeoh and Wong, 1993).

In the traditional medicine of East Africa (Kenya), *Asystasia gangetica* is used as an anthelmintic. The leaves are crushed, boiled in water, and the decoction drunk as a cure for intestinal worms (Kokwaro, 1976). In Nigeria, the leaves of *Asystasia gangetica* are claimed to be highly effective in the local treatment of asthma (personal communication, 2000). The fresh leaves are macerated in local gin for 24 h or expressed and the extract drunk.

Over 70% of Nigeria's more than 100 million people live in rural areas where traditional medicine practice is well established and patronized. The success of the practice has continued to reveal the potential of plants as therapeutic agents. As part of our continued efforts to screen Nigerian herbal remedies for pharmacological activity (Akah et al., 1997; Okoli and Akah, 2000; Nwafor et al., 2002), and in consideration of the claimed efficacy of *Asystasia gangetica* in native therapy of asthma, we investigated the leaves for anti-asthmatic activity.

* Corresponding author.
E-mail address: peterakah@hotmail.com (P.A. Akah).

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2. Material and methods

2.1. Collection and preparation of plant material

Fresh leaves of *Asystasia gangetica* were collected in June 2000 at Orba, Enugu State, Nigeria. They were authenticated by Mr. A. Ozioko of the Department of Botany, University of Nigeria, Nsukka. A specimen of the plant (no. P02611) was deposited at the University's Herbarium.

The collected leaves were sun-dried in open air for 7 days and reduced to coarse powder using a mortar and pestle.

2.2. Extraction of plant material

About 500 g of the dried powder was successively extracted with *n*-hexane, ethylacetate, and methanol using a sohxlet extractor. The extracts were concentrated in a rotavapor under reduced pressure and completely dried over boiling water to afford dry yield of 1.11, 1.91, and 6.84% for *n*-hexane, ethylacetate, and methanol extracts, respectively.

2.3. Phytochemical analysis

The *n*-hexane, methanol, and ethylacetate extracts are subjected to phytochemical analysis using conventional protocol (Evans et al., 1989).

2.4. Determination of acute toxicity

The median lethal dose (LD_{50}) of the methanolic extract was determined in Swiss albino mice using intraperitoneal (i.p.) route of administration (Lorke, 1983). The experiment was carried out in two phases; the first phase involved three groups of three animals per group that were administered 10, 100, and 1000 mg/kg of the methanol extract, respectively. Since no animal died in all the treated groups in the first phase after a 24-h monitoring period, the second phase comprising of three groups of one animal per group was carried out by administering 1600, 2900, and 5000 mg/kg of the extract, respectively. This is as proposed by Lorke (1983). The animals were again monitored for 24 h. The geographic mean of 1600 mg/kg, which was the least dose that did not kill any of the animal and 2900 mg/kg, which was the least dose that killed one of the animal was taken as the median lethal dose (LD_{50}).

3. Studies on smooth muscles

3.1. Guinea pig trachea

Guinea pigs of either sex (250–500 g), starved overnight but allowed free access to water, were used. The animals were killed by a blow on the head and exsanguinated. The trachea was dissected out and cut along its length on the dorsal surface. Incomplete transverse cuts were made along the segments of the cartilage to produce a zig-zag strip. The isolated trachea was mounted in a 30 ml organ bath containing Tyrode solution, maintained at 37 ± 1 °C and gassed with air. The tissue was equilibrated for 60 min during which the bath solution was replaced every 10 min (Akah et al., 1997). At the end of the equilibration period, histamine (14 μg/ml)-induced contractions as well as the effects of the extracts (up to 2 mg/ml) were recorded. The effects of the extracts on the responses elicited by the spasmogens acetylcholine, serotonin, and histamine were also investigated. A drug–tissue contact time of 1 min was maintained. Responses were recorded on an Ugo Basile Microdynamometer recorder (7004). In addition, the effect of the extracts on histamine-precontracted trachea was evaluated.

3.2. Rat stomach strip

Adult albino rats (200–250 mg) of either sex were killed by a blow on the head and exsanguinated. The abdomen was opened and the stomach removed. The fundal portion of the stomach was cut into a sheet, from where strips of about 2–3 cm long were prepared as described by the Staff of Department of Pharmacology, University of Edinburgh (Anonymous, 1970). The strip was suspended in atropinized (3.5×10^{-7} M) Tyrode solution (Akah et al., 1997) and after 60 min equilibration period, the effects of extract (up to 2 mg/ml) on 5-hydroxytriptamine (5-HT; serotonin)-induced contractions were recorded. Extract–tissue contact time was 1 min before the addition of 5-HT.

3.3. Guinea pig ileal preparation

A segment of the guinea pig ileum (approximately 2 cm long) removed from a freshly killed animal was suspended in a 30 ml organ bath containing Tyrode solution maintained at 37 °C and gassed with air. After an equilibration period of 60 min, contractile responses were established for histamine (2.5 μg/ml) and acetylcholine (2.5 μg/ml). The effect of the extracts on histamine- and acetylcholine-induced contractions were investigated.

3.4. Anti-inflammatory test

The effects of the extracts on egg albumin-induced acute inflammation (Akah and Nwambie, 1994; Okoli and Akah, 2000) were evaluated by the rat right hind paw edema method. Increases in the rat hind paw linear circumference were used as a measure of acute inflammation (Winter et al., 1963; Okoli and Akah, 2000).

Adult albino rats (200–250 g) of either sex were used. Animals were fasted for 12 h and deprived of water only during the experiment. Each group of four animal received either 100 or 200 mg/kg of each extract suspended in 3% (v/v) Tween 85 and administered intraperitoneally. 0.1 ml of fresh undiluted egg albumin was injected into the sub-planter of the right hind paw of the rats 30 min after extract administration. Control animals received an equivalent volume of

3% (v/v) Tween 85 or 100 mg/kg acetylsalicylic acid. The linear circumference of the paw was measured before and at 30-min intervals for 4 h after egg albumin injection (Akah et al., 1997).

Percent inflammation and percent inhibition of edema were calculated (Oriowo, 1982; Akah et al., 1993) using the relation:

$$\text{Percent inflammation} = \frac{\text{average inflammation of treated group at time } t}{\text{average inflammation of control at time } t} \times 100$$

$$\text{Percent inhibition of edema} = 100 - \text{percent inflammation}$$

3.5. Statistical analysis

The results were analyzed using Student's t-test and expressed as mean ± standard error of mean. Differences between means of treated groups and the control were regarded as significant at $P < 0.05$.

4. Results

Results of the toxicological studies established an i.p. LD_{50} of 2150 mg/kg for the methanol extract.

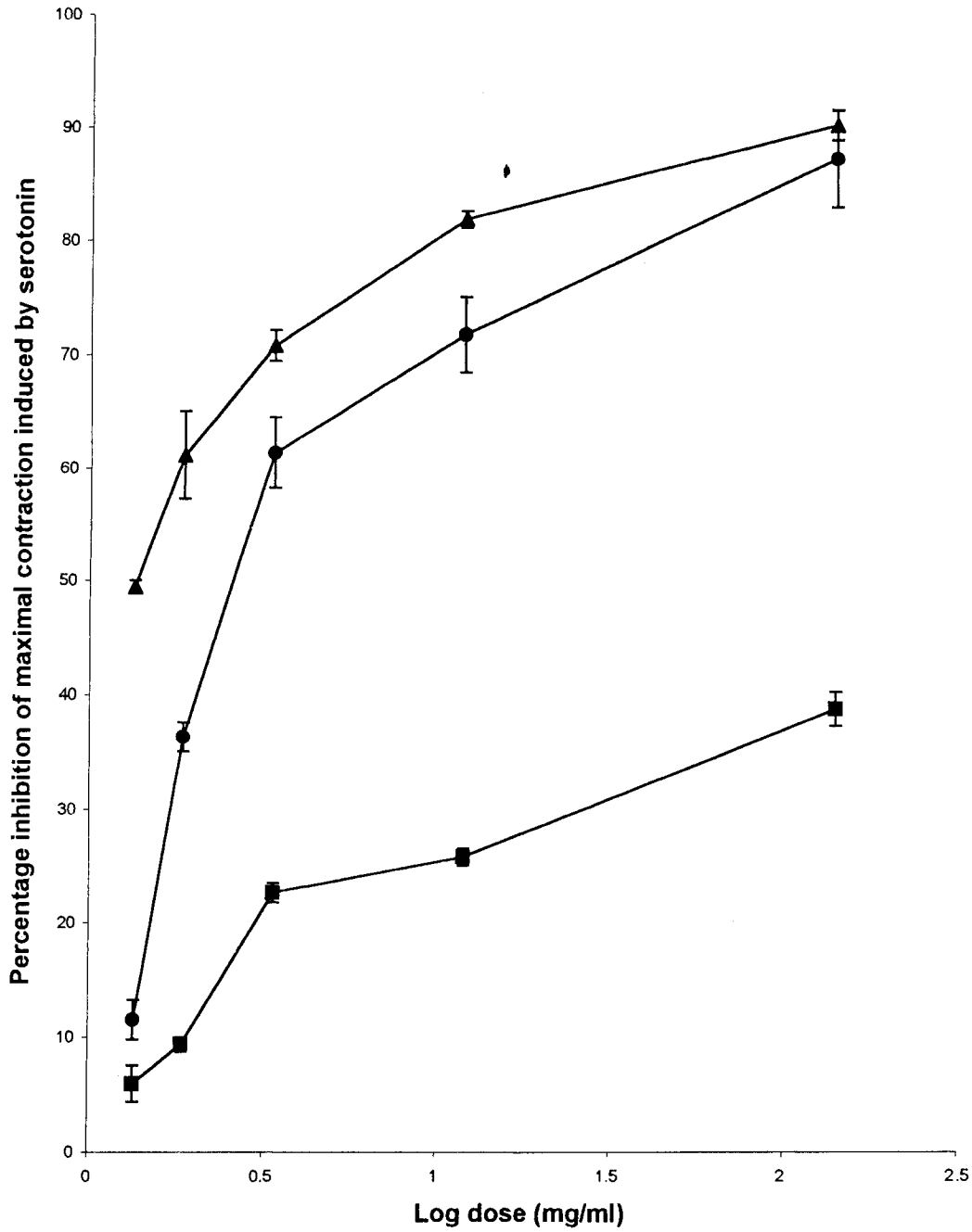

Fig. 1. Effect of the extracts on serotonin-induced contraction of the rat fundus strip. (●) n-Hexane extract; (■) methanol extract; (▲) ethylacetate extract.

Phytochemical analysis indicated the presence of several bioactive constituents as shown in Table 1. Solvent removal afforded percentage extractive yield of 1.14, 1.19, and 6.84% for hexane, ethylacetate, and methanol extracts, respectively.

4.1. Effects on smooth muscles

The results of the isolated tissue experiments indicated that the extracts neither contracted nor relaxed any of the isolated tissue preparation. However, the extracts inhibited the contraction evoked by the spasmogens to varying degrees (Figs. 1–6). The ethylacetate fraction was most potent in inhibiting contractions induced by 5-HT on rat fundus strip while the methanol extract was least potent (Fig. 1). On the guinea pig tracheal chain, the methanol extract displayed minimal inhibitory activity against histamine-induced contraction (Fig. 2), had no inhibitory activity against contraction induced by acetylcholine (Fig. 3) and exhibited substantial inhibitory effect

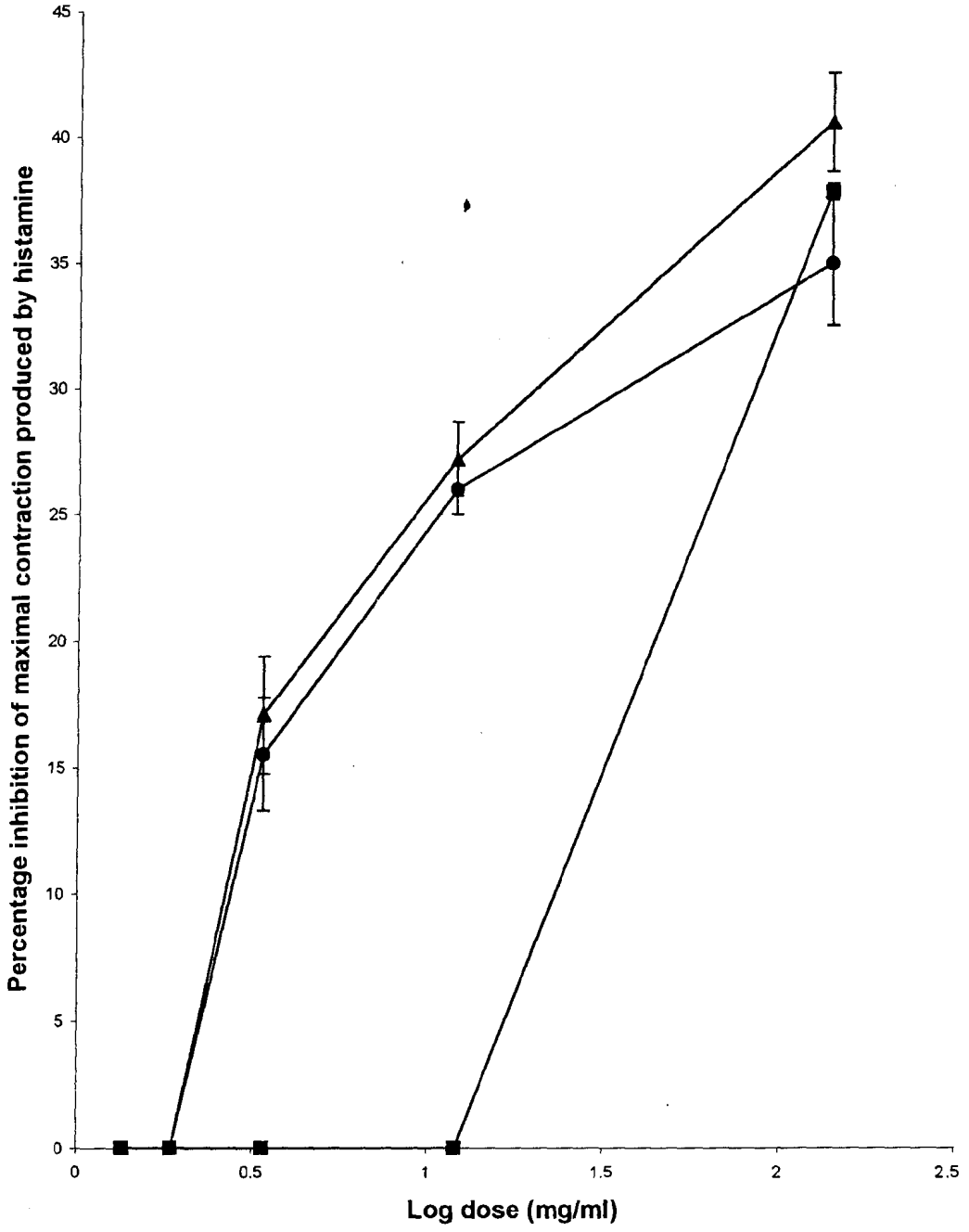

Fig. 2. Effect of the extracts on histamine-induced contraction of the guinea pig tracheal chain. (●) *n*-Hexane extract; (■) methanol extract; (▲) ethylacetate extract.

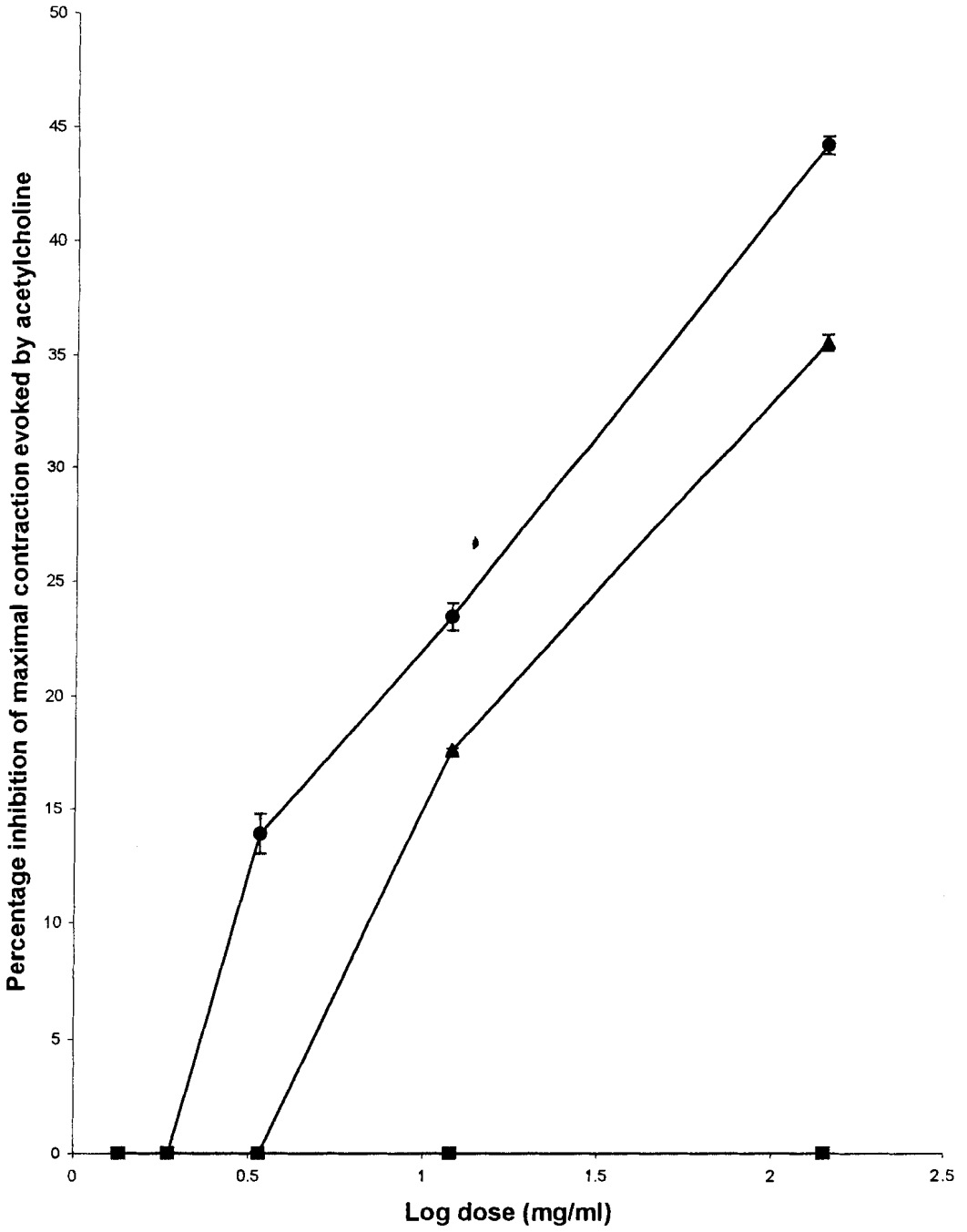

Fig. 3. Effect of the extracts on acetylcholine-induced contractions of the guinea pig tracheal chain. (●) *n*-Hexane extract; (■) methanol extract; (▲) ethylacetate extract.

against 5-HT-evoked contraction (Fig. 4). The extracts showed the lowest inhibitory potential against contraction induced by acetylcholine, where the *n*-hexane extract was most potent (Fig. 5). The *n*-hexane and ethylacetate fractions exhibited about equal degree of efficacy in inhibiting 5-HT-induced contraction in the tracheal chain (Fig. 4) and that evoked by acetylcholine in the ileum (Fig. 5). Not only that the methanol extract appeared to lack any inhibitory activity against contraction induced by acetylcholine in the trachea (Fig. 3), it even potentiated contraction induced by this spasmogen in the ileum, indicating inherent muscarinic receptor agonistic potency (Fig. 5). The contraction evoked by histamine on the guinea pig ileum was most effectively inhibited by *n*-hexane extract while the methanol extract was least effective (Fig. 6). The concentration of the extracts that inhibited 50% of the maximal contraction produced by the various agonists (IC_{50}) were calculated, where possible, and is shown in Table 2. The extracts also relaxed histamine-precontracted tracheal strip (Table 3).

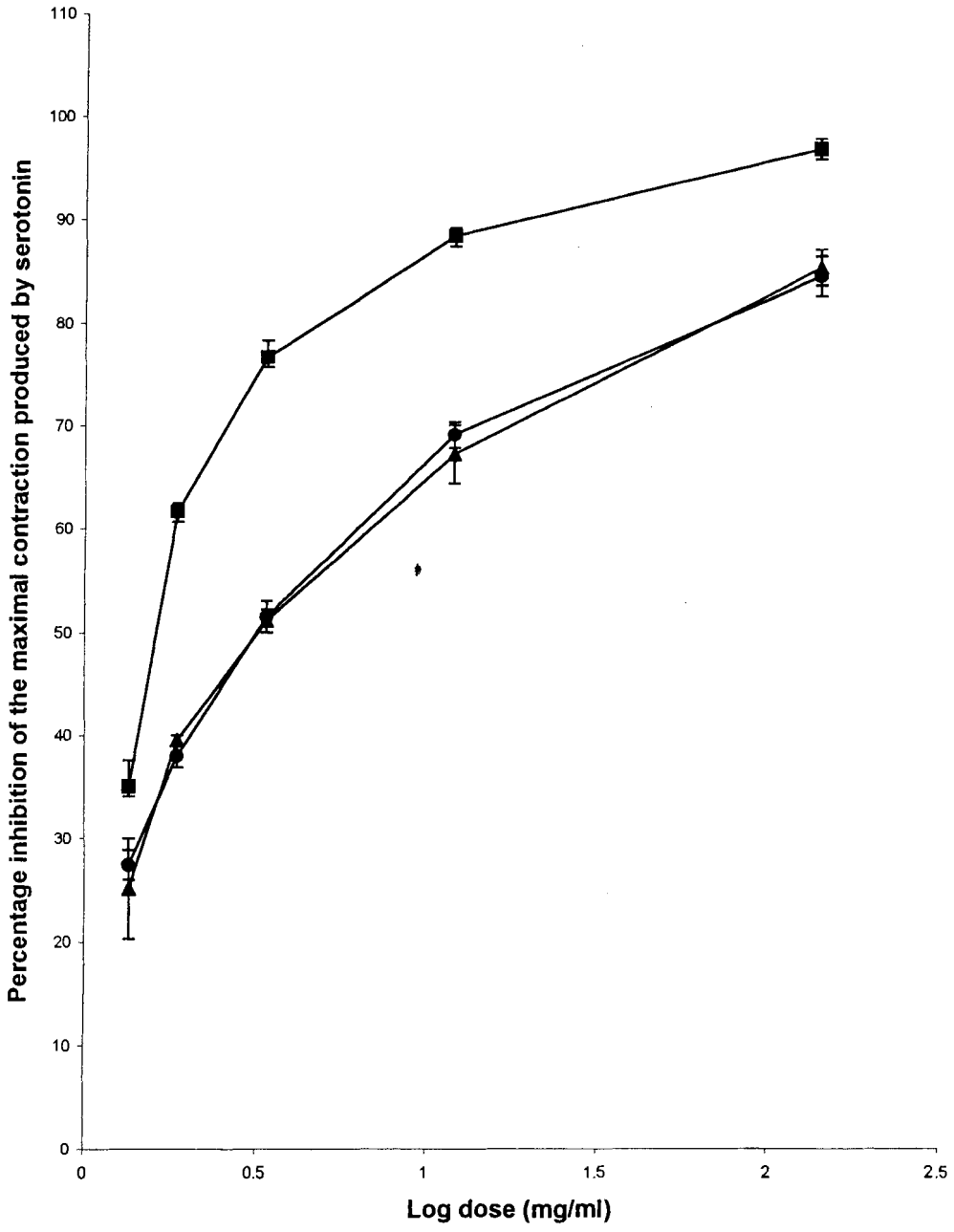

Fig. 4. Effect of the extracts on serotonin-induced contraction of the guinea pig tracheal chain. (●) *n*-Hexane extract; (■) methanol extract; (▲) ethylacetate extract.

Generally, the inhibitory potency of the extracts against contractions induced by the various spasmogens in the different isolated tissue preparations are in the following order: ethylacetate extract > *n*-hexane extract > methanol extract. However, this order of activity is reversed in 5-HT-induced contraction of the guinea pig tracheal chain (Fig. 4).

4.2. Anti-inflammatory activity

Anti-inflammatory activity was assessed in terms of decrease in the linear circumference of the treated rat paw relative to the value at zero time. All the extracts caused remarkable dose-related reduction in paw edema. Peak edema occurred at 30 min in the treated groups. The reduction in paw edema obtained with the extracts was significant ($P < 0.05$) 60 min (90 min for the hexane extract) after edema induction (Figs. 7–9).

5. Discussion

The extracts inhibited trachea contractions induced by histamine, serotonin, and acetylcholine. These agents are implicated in various ways in the pathogenesis of asthma.

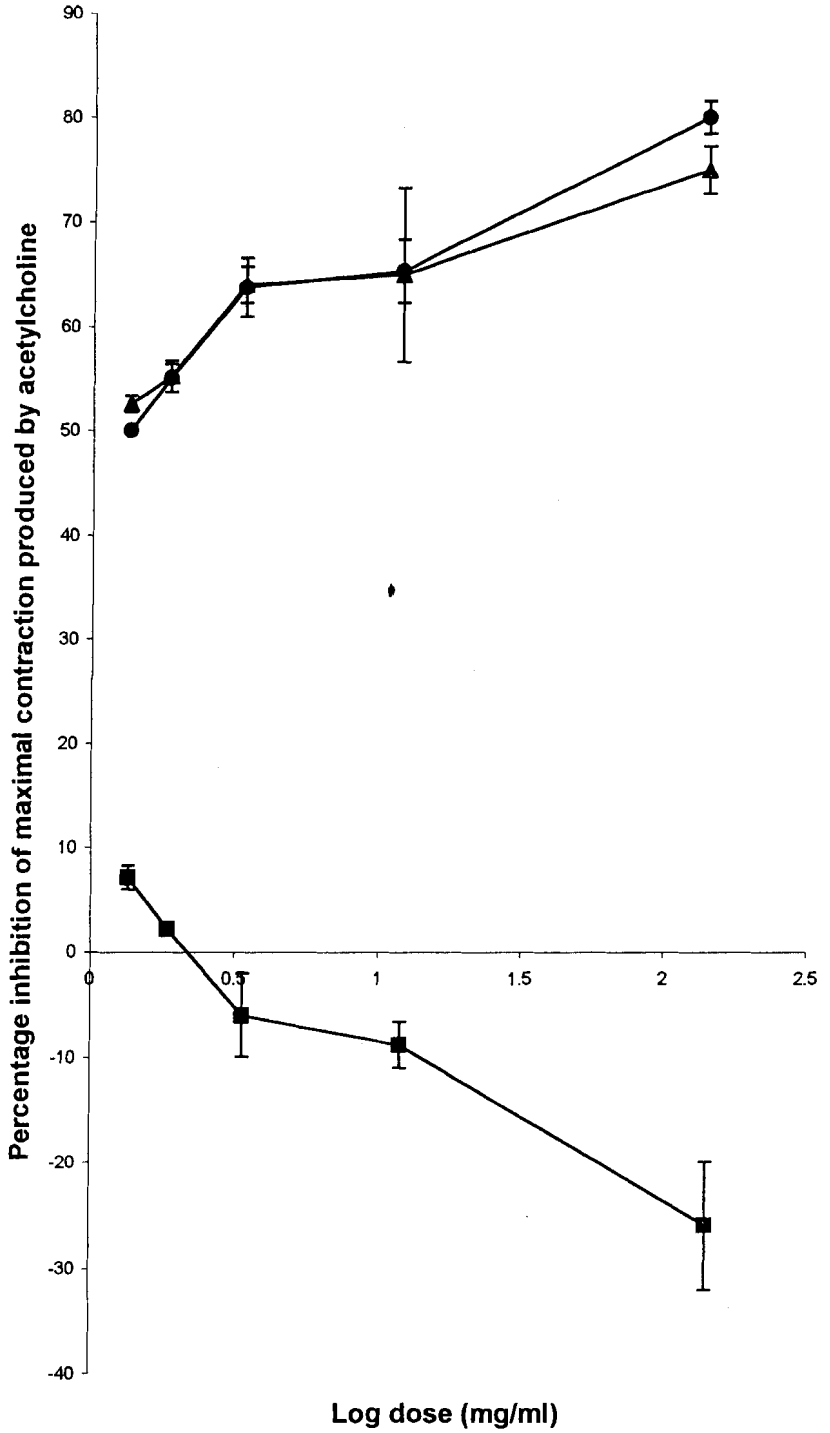

Fig. 5. Effect of the extracts on acetylcholine-induced contraction of the isolated guinea pig ileum. (●) *n*-Hexane extract; (■) methanol extract; (▲) ethylacetate extract.

Histamine is the most implicated mediator in bronchconstriction that accompany asthma (Summers et al., 1981). Although the role of 5-HT in asthma is uncertain, it is a potent bronchoconstrictor (Barnes et al., 1998) and also increases acetylcholine release from airway nerves via 5-HT_3 receptors (Takahashi et al., 1995). Acetylcholine on its own can cause bronchoconstriction by activating efferent cholinergic fibers secondary to the stimulation of the sub-epithelial afferent fibers by inflammatory mediators such as histamine (Flenley, 1990). The methanol extract exhibited the least activity at inhibiting the actions of the spasmogens, however, it was most potent at inhibiting 5-HT-induced contraction of the tracheal. In animals, 5-HT brings about contraction of smooth muscles by stimulating the 5-HT_2 receptors

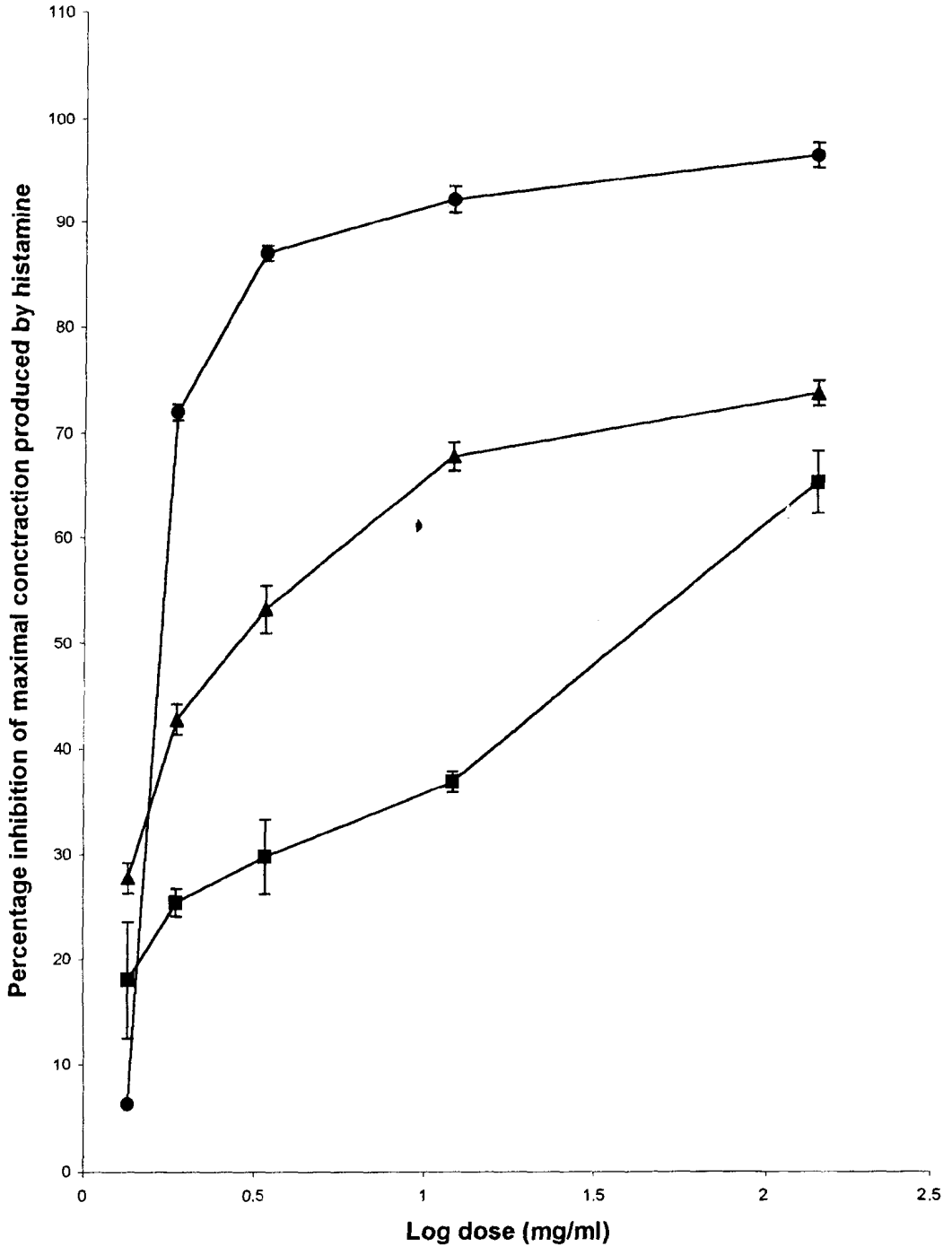

Fig. 6. Effect of the extracts on histamine-induced contraction of the isolated guinea pig ileum. (●) *n*-Hexane extract; (■) methanol extract; (▲) ethylacetate extract.

(Garrison, 1991). The pronounced inhibitory activity of methanol extract against contraction induced by 5-HT in guinea pig tracheal but not that in rat fundus strip indicates that the inhibitory activity may not be entirely mediated through 5-HT_2 receptor antagonism. In guinea pig airways, serotonin inhibits nonadrenergic noncholinergic neurally induced constriction resulting from tachykinin release via a 5-HT_1-like receptor localized to sensory nerve endings (Ward et al., 1994; Dupont et al., 1995). Therefore, it is likely that the methanol extract interacted with 5-HT to bring about enhanced activity at this receptor, leading to physiological antagonism of the contractile effect at the 5-HT_2 receptors.

The ability of the extracts to inhibit the contractions induced by these bronchoconstrictors suggests a possible role in the treatment of asthma. Furthermore, the relaxation of histamine-precontracted trachea by the extracts

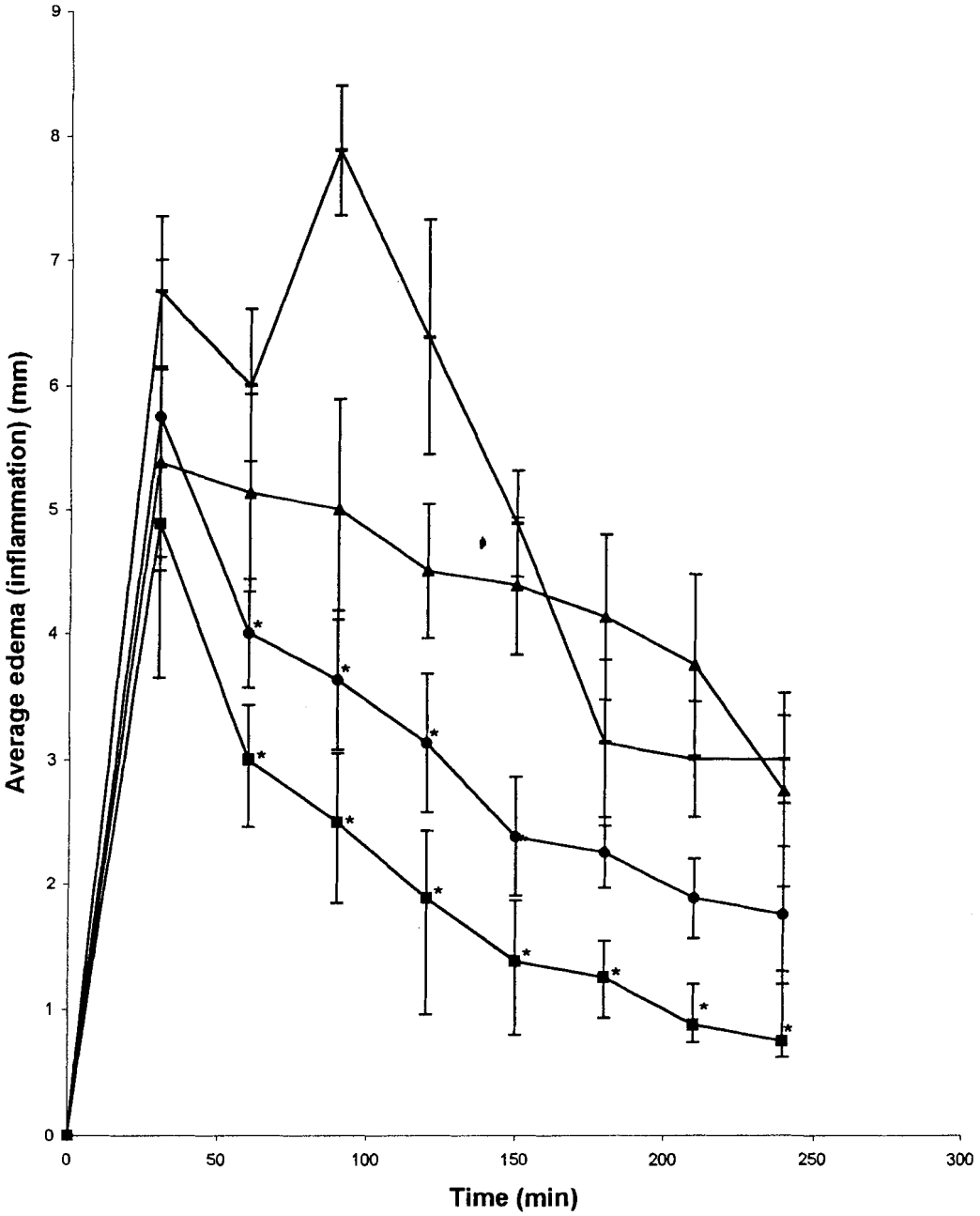

Fig. 7. Effect of the methanol extract on egg albumin-induced paw edema in rats. *$P < 0.05$. (●) *n*-Hexane extract (100 mg/kg); (■) *n*-hexane extract (200 mg/kg); (▲) aspirin (100 mg/kg); (-) 3% Tween 85.

indicates their potency in ameliorating established asthma. Airway hyperresponsiveness in asthma is attributed in part to changes in autonomic regulation particularly increased parasympathetic activity (Rang and Dale, 1988). While other extracts decreased acetylcholine-induced contraction of the guinea pig ileum, the methanol extract caused a decrease, followed by an increase in contraction. It is possible that the methanol extract contains two bioactive constituents acting to oppose the action of each other at the muscarinic receptors, one producing inhibitory activity and the other potently stimulating the receptors, especially at higher doses. It is also likely that one constituent might have acted to produce such opposing effects, eliciting responses characteristic of partial agonists.

Airway obstruction/bronchoconstriction or airway hyperresponsiveness in asthma are believed to be a direct consequence of airway wall inflammation (Holt et al., 1999; Prasad et al., 2000). The three extract of *Asystasia gangetica* exhibited varying degrees of anti-inflammatory activity in the order of magnitude—methanol extract > hexane extract > ethylacetate extract. Mechanisms that possibly underlie this anti-inflammatory activity include inhibition of the actions of inflammatory mediators such as histamine, effect on adrenocorticoid hormone and immunosuppression.

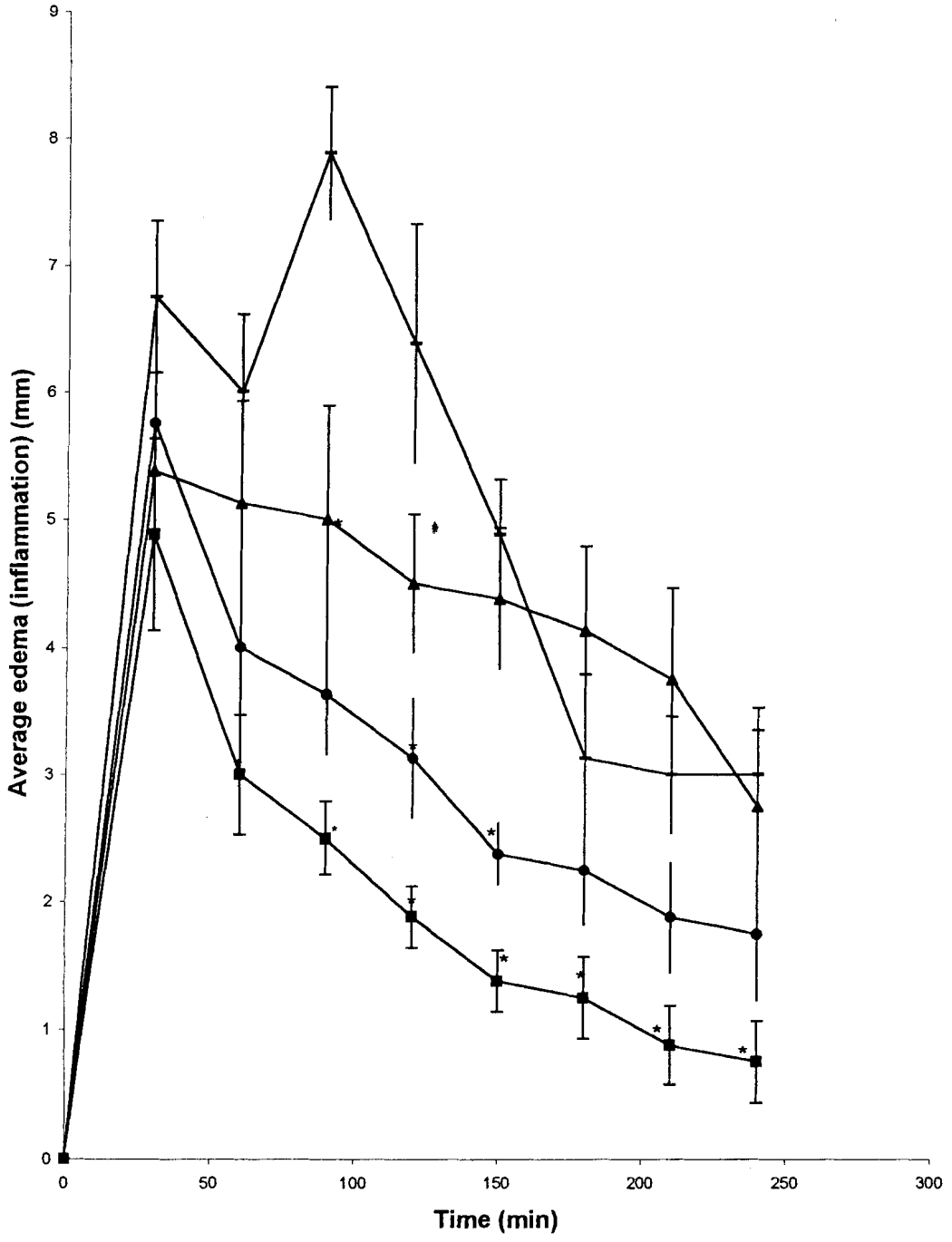

Fig. 8. Effect of *n*-hexane extract on egg albumin-induced paw edema in rats. $^*P < 0.05$. (●) *n*-Hexane extract (100 mg/kg); (■) *n*-hexane extract (200 mg/kg); (▲) aspirin (100 mg/kg); (-) 3% Tween 85.

The orders of potencies (both isolated tissue and anti-inflammatory studies) in this study obviously indicate that there may be more than one bioactive constituent present in *Asystasia gangetica* leaf extract. The component(s) inhibiting the contractions induced by 5-HT and histamine seem to be extracted more by the solvents ethylacetate and *n*-hexane. These solvents generally extract compounds in the middle polar (ethylacetate) to non-polar range (*n*-hexane). Conversely, the constituents(s) exhibiting the most effective anti-inflammatory potency is/are likely to be highly polar in nature since they were extracted by methanol, a very polar solvent.

Our results further showed that the methanol extract was least potent at inhibiting contractions induced by the spasmogens acetylcholine, 5-HT, and histamine. Some of these spasmogens are implicated in the inflammatory processes of asthma (Summers et al., 1981; Takahashi et al., 1995; Barnes et al., 1998). This notwithstanding, the methanol extract displayed the most potent anti-inflammatory activity. Although these spamogens are involved in inflammatory

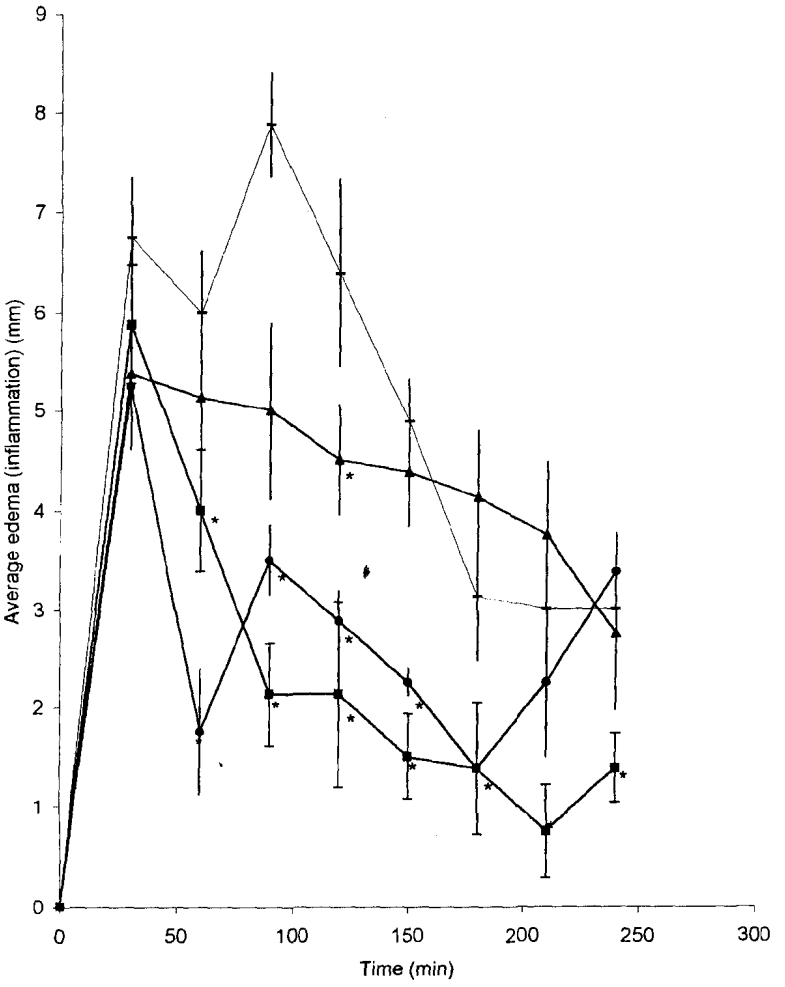

Fig. 9. Effect of ethylacetate extract on egg albumin-induced paw edema in rats. $^{*}P < 0.05$. (●) Ethylacetate extract (100 mg/kg); (■) ethylacetate extract (200 mg/kg); (▲) aspirin (100 mg/kg); (-) 3% Tween 85.

processes, their release is stimulated by multiple inciting stimuli (Bosin, 1978; Schroeder and MacGlashan, 1997). It is possible that the methanol extract bring about more potent anti-inflammatory activity not only by inhibiting the activity of these spasmogens but also by inhibiting the elaboration of the inciting stimuli. Moreover, the inflammatory processes of asthma are of multiple etiologies; prominent among them is immunostimulation by a variety of factors (Barnes, 1996). It is also likely that the methanol extract exhibited more potency at inhibiting these humoral processes than the other extracts, thus accounting for its better anti-inflammatory

Table 1
Phytochemical constituents of the various extracts

Phytochemical constituents	Methanol extract	Ethylacetate extract	Hexane extract
Carbohydrates	+	+	+
Proteins	+	+	−
Alkaloids	+	−	−
Tannins	+	−	−
Steroidal aglycone	+	+	+
Saponins	+	−	+
Flavonoids	+	−	−
Reducing sugars	−	−	−
Triterpenoids	−	+	+

+: present; −: absent.

Table 2
Concentration (mg/ml) of the extracts that inhibited 50% of the contraction produced by the various agonists (IC_{50}) in different smooth muscle preparations

Agonist/extract	*n*-Hexane extract	Methanol extract	Ethylacetate extract
Guinea pig ileum			
Acetylcholine	1.35	–[a]	1.26
Histamine	2.00	39.81	2.82
Guinea pig tracheal chain			
Serotonin	3.16	1.79	3.16
Acetylcholine	–[a]	–[a]	–[a]
Histamine	–[a]	–[a]	–[a]
Rat fundus strip			
Serotonin	2.95	–[a]	1.35

[a] At the concentrations used, the extract did not produce up to 50% inhibition of the maximal contraction evoked by the agonists.

Table 3
Percentage inhibition of the maximal response (relaxation) of the precontracted guinea pig trachea produced by the cumulative administration of the various extracts

Extract	Dose (mg/ml)	Percentage reduction of maximal response after 20 min
Methanol extract	256	25.0 ± 2.1
Ethylacetate extract	64	75.0 ± 3.0
Hexane extract	256	25.0 ± 1.6
Control		12.2 ± 1.2

activity. Inhibition of immunosuppression also incorporates inhibition of the activity of proinflammatory mediators. This proposed mechanism is consistent with previous findings that anti-inflammatory plant principles such as premnazole, an isoxazole alkaloid, and lupeol, a triterpenoid, have been shown to act through control of adrenocorticoid hormone (Barik et al., 1992) and immunosuppression (Singh et al., 1997), respectively.

Inherent anti-inflammatory activity is a desirable property of a putative anti-asthmatic agent, since asthma is a complex chronic inflammatory disease of the airways. The fact that the methanol extract showed the most potent anti-inflammatory activity may justify the local extracting method of macerating the leaves of *Asystasia gangetica* in local gin (which is mainly ethanol) before being drunk.

Sequel to the above discussion, it is apparent that the mechanism of anti-asthmatic property of the leaf extracts of *Asystasia gangetica* is multi-faceted; inhibition of the activity of spasmogens and immunosuppression may be involved. The bioactive component(s) responsible for the observed activities is not precisely known but it may be one or more of the phytochemical constituents established to be present in the leaf extracts.

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