EFFECTS OF EXTRACTS OF THE ANTI-ASTHMATIC PLANT THONNINGIA SANGUINEA ON ANAPHYLAXIS IN THE GUINEA PIG

By

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D E D I C A T I O N

Dedicated to:

Mr. Kwaw Nseboah (Late father)

Mr. G.F. Djomoah (Late brother)

Madam Janet Yeboah (mother)

and the entire family.

D E C L A R A T I O N

The experimental work described in this thesis was carried out by me at the Department of Biochemistry, University of Ghana, Legon, under the supervision of Dr. Marian E. Addy.

I certify that this work has not been previously accepted for any degree and is not being submitted in candidature for any other degree.

Signed:

..... Candidate
(Alex K. Nyarko)

..... Supervisor
(Dr. Marian E. Addy)

Date: 21st October 1983.

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A B S T R A C T

Thonningia sanguinea is one of the herbal preparations used prophylactically against asthma at the Centre for Scientific Research into Plant Medicine. Preparations of this plant are used with *Desmodium adscendens* which has been shown to be anti-anaphylactic. The anaphylactic reaction is a basic underlying phenomenon in asthma. In this work, the effects of *T. sanguinea* preparations were evaluated on some aspects of the disease.

The method adopted was experimental anaphylaxis in the guinea pig. Both *in vivo* and *in vitro* assay systems were used to evaluate extracts prepared from the plant material.

The results showed that the plant material when administered orally

(i) inhibited anaphylactic contractions in ileal pieces ($p < 0.05$),

(ii) acted to reduce the sensitivity of ileal pieces to histamine ($p < 0.05$),

(iii) inhibited the anaphylactic release of histamine from lung tissues ($p < 0.05$),

(iv) reduced the amount (or effect) of mepyramine-resistant spasmogens released anaphylactically from lung tissues ($p<0.05$), and

(v) reduced the total histamine content of lung tissues ($p<0.05$).

The crude extract, and some of its fractions, when assayed *in vitro*, inhibited the anaphylactic contractions in the ileal pieces in much the same way as when the aqueous extract was administered orally ($p<0.05$). However, the *in vitro* effects on histamine-induced contractions differed from the effect observed when the crude extract was administered orally. Since *Thonningia* preparations are administered orally, the *in vitro* assay system involving histamine-induced contractions, was considered to be of little or no use in evaluating the anti-asthmatic effect of the plant.

These results are discussed with respect to the possible ways by which the plant could interfere with anaphylaxis and thereby prevent the incidence of asthmatic attacks. The results suggested a similar mechanism of action for both *Thonningia* and *Desmodium*.

Qualitative analysis indicated the presence of enolic and/or phenolic steroids. The densitometer scan as well as R_f values and colours of resolved components could be used to control and standardize the quality of *Thonningia* preparations for future use.

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A B R R E V I A T I O N S

Ab	-	antibody
Ag	-	antigen
c AMP	-	cyclic adenosine monophosphate
CSRPM	-	Centre for Scientific Research into Plant Medicine
ED_{50}	-	median effective dose
Ig E	-	immunoglobulin E
Km	-	Michaelis constant
MC	-	mast cell
PC	-	paper chromatography
PGE	-	prostaglandin E
PGF	-	prostaglandin F
S.E.	-	standard error
SRS-A	-	slow reacting substances of anaphylaxis
TLC	-	thin layer chromatography
V_{max}	-	maximum velocity

GLOSSARY OF TERMS

Allergen - an antigen that produces an allergic response.

Anaphylaxis - a hypersensitivity in which the first administration of an antigen to an animal is harmless, but the second administration leads to an intense secondary immune response accompanied by pathological reactions.

Antibody - a protein of the globulin type that is formed in an animal organism in response to the administration of an antigen and that is capable of combining specifically with the antigen.

Antigen - a substance, frequently a protein, that can stimulate an organism to produce antibodies and that can bind specifically with the antibodies thus produced.

Atopy - an immediate-type hypersensitivity, such as asthma or hay fever, that is due to the production of reagins and that tends to occur as an inherited tendency.

Autocoid - any internal secretion of the body that acts on a restricted area within the body e.g. hormones and kinins.

Cytophilic antibody - an antibody that can adhere specifically to macrophages.

Cytotropic antibody - an antibody that binds to target cells, particularly mast cells, thereby sensitizing the animal for anaphylaxis.

Decoction	-	extraction of an essence or part of a substance by boiling in water.
Eczema	-	an inflammation of the skin causing an itchy, flaky or ulcerated surface.
Eosinophilia	-	abnormal increase in number of eosinophils in the blood characteristic of allergic states.
Etiology	-	the study of causes of diseases.
Homocytotropic antibody	-	cytotropic antibody that binds to, and sensitizes target cells in the same species in which it was produced.
Hypersensitivity	-	the altered immunological state that is produced in man and animals by their previous exposure to an antigen and that is characterised by the occurence of different and pathological reactions upon their subsequent exposure to either the same antigen or structurally related substance .
Reagin	-	a homocytotropic antibody of the Ig E class that is formed in response to an allergen and that, upon combination with allergen, causes the release of histamine and other vasoactive agents of immediate-type hypersensitivity.
Receptor	-	a target site at the molecular level to which a substance becomes bound as a result of specific interaction. The site may be on the cell membrane or an enzyme and substance bound may be an antigen, hormone or drug.

Rhinitis	-	inflammation of the mucuous membrane of the nose.
Sympathomimetic	-	stimulating sympathetic nervous action in physiological effect: adrenergic.
Tachycardia	-	an abnormally rapid rate of otherwise normal heart beats - whether physiological or pathological.
Vermifuge	-	a substance taken to purge the intestines of worms.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1. GENERAL INTRODUCTION:

The use of herbs in the treatment of diseases has been, and still is, a tradition in a large number of societies. In Ghana the tradition of using herbs for the treatment of diseases plays an important role in the health care delivery system of the country. This type of health care caters for a large percentage of the population, especially the rural folk, whom modern medicine does not adequately reach. In this practice of health care, various herbal preparations serve as a source of medicaments and some of them are even claimed to be effective in the treatment of some prevalent tropical diseases against which orthodox medicine is ineffective, e.g. the use of Combretum mucronatun and Hileria latifolia in cases of guinea worm infestation (Ampofo, 1977).

The government of Ghana, realising the important role played by herbal medicine with regards to the health care needs of the people, established the Centre for Scientific Research into Plant Medicine (CSRPM) in 1973. This Centre, situated at Mampong, Akwapim, has been investigating local medicinal plants used for the treatment of certain diseases including hypertension, diabetes and asthma, in an attempt to develop substitutes for imported drugs.

The Centre has compiled a list of many herbs claimed by herbalists to be effective in the treatment of many diseases but only a few,

have actually undergone clinical trials (Ampofo, 1977). T. sanguinea is claimed by herbalists to have anti-asthmatic properties. This claim has been substantiated at the Centre, where the plant preparation has been shown to be a prophylactic agent against bronchial asthma, especially in children (Ampofo, 1977).

Asthma is a disease that affects the lung tissues resulting in difficulty in breathing. The disease is widely distributed and attacks are frequent in both children and adults. The state of some patients often gets worse with time and they ultimately become disabled. Asthma virtually has no cure. Apart from disodium cromoglycate (DSCG) which has been reported to be an effective prophylaxis against the disease (Bochner et al, 1978(a)), most of the popular agents used in the management of asthma, such as anti-histamines (Douglas, 1975), SRS-A antagonist (Adams and Lichtenstein, 1977; Drazen et al, 1979), bronchodilaters like Salbutamol (Ventolin), Orciprenaline (Alupent), corticosteroids - prednisone and sympathomimetic amines like epinephrine, all appear to offer only symptomatic relief. Thus any herbal preparation appearing to be an effective prophylaxis against asthma is worth investigating with a view to developing it into a plant preparation that could supplement if not replace anti-asthmatic drugs currently on the market. This thesis, therefore was aimed at investigating the scientific basis for the therapeutic action or the observed clinical effects of T. sanguinea. In these studies the model of experimental anaphylaxis in the guinea pig was adopted.

Information gathered from this work may help to explain some of the observed clinical effects of T. sanguinea. Such information may also contribute to the development of our folk medicine and pharmacopia. It is a fact that many valuable drugs indispensable to modern medicine have come into use through the study of folk medicine, and natural products, especially those of plant origin, have served as prototypes for synthetic drugs. Similarly, investigations into our anti-asthmatic herbal preparations may lead to the identification of a chemical substance which may be used in the effective ultimately management of bronchial asthma.

1.2 BRONCHIAL ASTHMA:

Asthma is quite often referred to as an atopic allergic disease in humans. The disease however may sometimes be non-allergic. In each case it is characterised by episodic or recurrent difficulty in breathing as a result of an increased bronchial irritability. Factors that may provoke the disease include exposure to allergens, cold air and certain chemicals (Reed, 1968).

Asthma is a manifestation of hypersensitivity. Hypersensitivity refers to a state of increased reactivity to a foreign agent. It is acquired by a prior exposure to an allergen, or a chemically related substance, usually a protein. The hypersensitive state may be of an immediate type or delayed type. In the former, response to a shocking dose of allergen may appear

within a few minutes after its administration. This type of response is antibody mediated. The latter type is however cell mediated and response to the administration of allergen is much delayed.

Immediate type hypersensitivity is specific. It depends on, and is transferable by cytotropic antibodies (Gray, 1970). Clinically, the hypersensitive state has several independent components which may occur in various combinations and in varying severity in different individuals. Some of the other major manifestations of hypersensitivity besides bronchial asthma are (i) allergic rhinitis (ii) eosinophilia and (iii) eczema (Reed, 1968).

The origin of asthma is explained by the "Allergic" and "β-adrenergic blockade" theories. The allergic theory considers the disease to be the consequence of antibody-antigen reactions. This theory inadequately explains non-allergic or "intrinsic" asthma but well explains the allergic type or "extrinsic" asthma. The β-adrenergic theory asserts that asthma is due to a defect in the homeostatic mechanism controlling the balance between the antagonistic adrenergic receptors (Szentivanyi, 1968). The theory provides a unifying basis for the pathogenesis of both allergic and non-allergic asthma, especially for some of the puzzling non-immunological features of the disease that cannot be explained by the allergic theory. The β-adrenergic blockade theory, for instance, accounts for the epinephrine reversal in asthmatics as well as the therapeutic response shown to corticosteroids (Coffey and Middleton, 1977).

The two theories acknowledge an allergic component in the etiology of asthma, and reaginic antibodies of the immunoglobulin E (Ig E) class play an important role. Predisposition towards allergic asthma is heredity-determined and hereditary factors influence the capacity to produce reaginic antibodies (Levy and Osler, 1967). Thus most of the symptoms observed during an attack of the disease are accounted for by the release of chemical mediators of the immediate-type allergic reaction. These chemical mediators released cause the contraction of smooth muscles. The sequence of reactions that ensue culminates in the narrowing of the smaller airways. These may be chronically constricted resulting finally in difficulty in breathing.

1.3 ANAPHYLAXIS:

Anaphylaxis is principally an experimental phenomenon demonstrated in laboratory animals; it, however, occasionally occurs in humans. The phenomenon is a manisfestation of the immediate-type hypersensitive state and it is characterised by an acute systemic response of hypersensitive animals following parenteral administration of a soluble antigen or materials to which animals have become sensitive after a previous exposure to it.

Most animals are reported to be capable of demonstrating anaphylaxis (Barret, 1975). A number of chemical mediators are released during

anaphylactic shock and these cause various pathological states. Symptoms of the pathological states however depend on the animal species and the shock organ.

In addition to systemic anaphylaxis, the phenomenon is also demonstrable in vitro using isolated tissues and organs from actively or passively sensitized animals (Austen and Humphrey, 1963).

Studies of the anaphylactic reaction show that mast cells are damaged in allergic reactions. This has been demonstrated in whole guinea pigs (Mota and Vugman, 1956), isolated guinea pig preparations (Mota, 1959) and in patients of bronchial asthma (Ishimova, 1977). These emphasise the role of mast cells in the anaphylactic type reactions.

1.4 ASTHMA-ANAPHYLAXIS RELATIONSHIP:

Asthma and anaphylaxis are both manifestations of the hypersensitive state. The immunological basis of hypersensitivity is the production of reaginic or reagin-like antibodies and the ability to produce these antibodies is genetically controlled (Levine and Vaz, 1970; Levey and Osler, 1967). In both asthma and anaphylaxis the specific union of homologous antigen or allergen with cell fixed antibodies leads to the release of pharmacologically active substances.

The release of histamine has been shown to be common to anaphylaxis in guinea pig tissues (Schild, 1939) and human bronchial and lung tissues (Schild et al, 1951). Similarly, the release of another spasmogenic substance - Slow Reacting Substances of Anaphylaxis (SRS-A) has been demonstrated during anaphylactic shock in the guinea pig (Brocklehurst, 1960) and from passively sensitized human lungs (Sheard et al, 1967). Similarities also exist in the biochemical pathway leading to the release of chemical mediators during anaphylaxis in the guinea pig (Schild, 1937) and from sensitized human lung tissues (Kaliner and Austen, 1973). The release of chemical mediators in each of these cases has been shown to be dependent on divalent cations, an intact glycolytic pathway and a modulation by intracellular concentration of 3'5' c-AMP. Finally, shock tissues in various mammalian species during anaphylactic shock and in asthma include smooth muscles or prominent areas of smooth muscles (Barret, 1975).

The above information provides evidence for a direct relationship between the anaphylactic phenomenon and bronchial asthma, and also strengthens the earlier assertion that the two conditions are manifestations of the same type of primary lesion leading to the hypersensitive state.

1.5 CHEMICAL MEDIATORS OF ANAPHYLAXIS:

Following passive sensitization of target tissues by homocytotropic antibodies, a specific union of the target tissue - antibody complex with a homologous antigen lead to the release of a battery of chemical mediators. These mediators include histamine, SRS-A, serotonin bradykinin and the prostaglandins. The main properties of these substances in relation to anaphylaxis are reviewed in the following sections.

1.5.1 Histamine:

Histamine, a β-imidazolylethylamine, is formed *in vivo* through the decarboxylation of the amino acid L-histidine by the histamine - forming specific enzyme L-histidine decarboxylase. The chemical is widely distributed in mammalian tissues, however, its concentrations appear to be particularly high in the skin, intestinal mucosa and the lungs. These are tissues that come into direct contact with the environment outside the living system. Generally, tissue mast cells (MC) and basophils of blood serve as storage sites. These cells synthesise histamine and store it as a complex with heparin.

The anaphylactic release of histamine is initiated by antigen-antibody interactions and the liberated chemical exhibits varied pharmacological effects. Histamine causes contractions of many smooth muscles such as human and guinea pig bronchi and the gut of the guinea pig. The same mediator, however, powerfully causes relaxation of other smooth muscles like fine blood vessels.

All the pharmacological effects of histamine are initiated through the stimulation of histamine H_1 and H_2 receptors. Anti-histamines and other H_1 antagonists e.g. mepyramine offer a striking protection against anaphylactic bronchospasm in various species but not in man where other bronchoconstrictor autocoids, e.g. SRS-A, are more important in eliciting contractions (Douglas, 1975). Although both H_1 and H_2 blockers are more effective in inhibiting the bronchoconstrictor effect of histamine, none of them inhibits the release of this mediator. They are effective only when the substance has been released.

1.5.2 Slow Reacting Substances of Anaphylaxis:

A group of substances collectively referred to as Slow Reacting Substances of Anaphylaxis (SRS-A) produces slow but prolonged contraction of the isolated guinea pig ileum. Unlike histamine SRS-A does not exist preformed in tissues, they are formed de novo (Vogt, 1969). Like histamine however, antigen-antibody reaction induces a sequence of enzymic process which lead to their release.

The chemical nature and properties of SRS-A appear to be both complicated and controversial. In any case SRS-A is known to be resistant to inactivation by proteolytic enzymes e.g. chymotrypsin (Vogt, 1969), they are lipid-like, and acidic (Vogt, 1969).

Several authors consider SRS-A to be responsible for the powerful contractions and bronchospasms observed in asthmatics (Adams and Lichtenstein, 1977; Carpenter, 1975; Kabat, 1976; Vogt, 1969) and therefore a major contributor to the pathophysiology of asthma and anaphylaxis. In fact, SRS-A have been shown to be a selective contractile agonist for peripheral airway tissues (Drazen et al, 1979).

1.5.3 Bradykinin:

The kinins are basic peptides. Bradykinin, made up of nine amino acids, is an example of the kinins. The formation of bradykinin is dependent on the presence of plasma, and kallikrein. The formation of kallikrein is catalysed by certain antigen-antibody aggregates (Kaplan and Austen, 1972).

Pharmacologically, bradykinin resembles SRS-A; it slowly but powerfully produces contractions of certain smooth muscles. This effect is neither blocked by ganglionic blocking agents nor atropine (Douglas, 1975).

1.5.4 Serotonin:

Serotonin, a 5-hydroxytryptamine (5-HT), is synthesised through decarboxylation of dietary L-tryptophan in mammals. This chemical is also released as a result of antigen-antibody interactions.

The effects of serotonin include rapid contractions of certain smooth muscles thus causing bronchoconstriction and increased vascular permeability,

although its bronchoconstrictor effect is uncommon in man except in asthmatic patients (Robard and Kira, 1972). Both lysergic acid diethylamide and reserpine inhibit the effects of serotonin (Davis et al, 1970).

1.5.5 Prostaglandins:

The prostaglandins are a series of compounds that are lipid soluble. The most abundant of these are prostagladin E and F series denoted PGE and PGF respectively. These substances are synthesised in most mammalian cells in vivo from essential fatty acids.

Generally, the prostaglandins show numerous and diverse pharmacological effects. PGF series are known to cause contraction of bronchial and tracheal muscles and asthmatics have been reported to be particularly sensitive to $PGF_{2\alpha}$ which causes intense bronchospasm (Douglas, 1975). On the other hand PGE series are known to relax certain smooth muscles.

1.6 SOME MODEL SYSTEMS FOR ASTHMA-ANAPHYLAXIS:

Although anaphylaxis can be demonstrated in almost all mammals, the guinea pig is used extensively as a model for its study, because this animal is known to react uniformly and intensely upon exposure to antigen (Davis, et al, 1970) producing homocytotropic antibodies of gamma-one type which are similar to human Ig E. In addition to this the shock organ in the guinea pig is the lung-bronchioles, as found in human allergic asthma (Austen and Humphrey, 1963).

In vitro anaphylaxis is best studied by suspending an isolated sensitized guinea pig ileum in an aerated Tyrode's physiological solution. The addition of a homologous antigen causes violent contractions as a result of the release of chemical mediators. This phenomenon was first observed by Schultz (1910) and was later confirmed by Dale (1912) using the guinea pig uterus in place of the ileum. It is therefore commonly referred to as the Schultz-Dale reaction and it is used in most pharmacological assays to study anaphylaxis. In this original model, each ileal piece is used to assay only one antigen concentration.

Okpako's modification of the Schultz-Dale reaction (1970) serves as a good model, easily adaptable for studies of anti-anaphylactic and potential anti-asthmatic agents. In this model, increasing doses of antigen are applied to the same piece of tissue and the contractions obtained are used to construct the antigen dose-response curve. This model has the advantage of minimising distortions due to desensitization in the tissues, and the dose-response curve is easy to construct. In addition, the number of tissues required for the construction of the dose-response curve is reduced and the inherent error due to variation in the sensitivity of the pieces of tissues, from sections of the same intestine of the same tissue, is reduced (Okpako, 1970).

Some direct effects of antigen on smooth muscle from sensitized animals has also been observed. It has been pointed out that there are

anaphylactic responses that are independent of histamine and other autocoids (Mongar and Schild, 1962). Evidence shows that antigen can directly act on the membranes of sensitized cells to cause depolarization and contraction (Alonso De-Florida *et al*, 1968).

Autocoid release from, and contraction of airway tissues are also good *in vitro* models. Perfused guinea pig lung or chopped guinea pig lung, human lung passively sensitized (Assem and Mongar, 1970, Hammond *et al*, 1982) and pieces of monkey lung actively or passively sensitized have been used (Assem and Mongar, 1970). Others include the use of guinea pig tracheal rings and human circumferential bronchial strips (Adams and Lichtenstein, 1977). The anaesthetised guinea pig (Mahajarni and Kulkarni, 1977) or the unanaesthetised guinea pig (Drazen and Austen, 1974) have also been used as models to study both pulmonary compliance and respiratory resistance.

Autocoids released in *in vitro* anaphylaxis are either assayed pharmacologically using the isolated guinea pig ileum or fluorometrically in the case of histamine and serotonin (Shore, 1971; von Redlich and Glick, 1968).

1.7 INHIBITORS OF THE ANAPHYLACTIC REACTION:

Investigations have been going on over the years with a view to identifying chemicals or potential drugs that may either be used to inhibit the anaphylactic reaction and hence allergic asthma or eliminate them altogether. In one of such studies Mongar and Schild (1957) identified several substances which were however of no clinical value. In vitro anaphylactic reactions have been reported to be blocked by anti-histamines like mepyramine and diphenhydramine (Adams and Lichtenstein, 1977). Anti-histamines, however, have been shown to have no effect on the contraction of human bronchi strips in the presence of antigen challenge (Sheard and Blair, 1970). Since antigenic challenge is known to be followed by the release of a battery of chemical substances the need to investigate inhibitors to specific mediators arose. Several specific inhibitors have thus been identified. Included here is an SRS-A antagonist coded FPL 55712 (Adams and Lichtenstein, 1977).

Aspirin and related analgesics are known to offer symptomatic relief to bradykinin-induced pain, and other salicylates as well as glucocorticoids have been reported to inhibit kallikrein activation. Also fernamate phenylbutazone (Collier, 1971 (a) and (b)) as well as certain prostaglandin analogues like 7-oxa-13,14-prostynoic acid (Mashiter and Field, 1974) antagonise some in vitro effects of prostaglandin on muscle. Both methylsergide and cyproheptadine antagonise serotonin.

The specificity of the various antagonists to the chemical mediators suggests that a combination of specific antagonists or a drug which will be capable of inhibiting the release of multiple mediators will be needed in order to control the anaphylactic reaction. The β-blockade theory however allows for the study of sympathomimetic and β-adrenergic agents. Assem and Schild (1969) have reported that sympathomimetic amines and β-adrenergic agents as well as methylxanthine (Lichtenstein and Margolis, 1968) inhibit autocoid release. According to Hammond et al (1982) betamethasone sodium sulphate suppresses the release of SRS-A without affecting histamine release in passively sensitized human lung. Generally, agents capable of increasing intracellular levels of c-AMP inhibit autocoid release (Ishizaka et al, 1970) whereas those that lower intracellular levels of 3'5' c-AMP ehnance such release (Orange et al, 1971).

1.8 DRUGS CURRENTLY BEING USED TO TREAT ASTHMA:

Drugs currently in use may be broadly divided into bronchodilators, steroids and cromoglycate. The basic aim of treatment is to enable the asthmatic patient to lead as normal a life as possible with the lowest effective dose of any drug being used. The choice of drug depends on the patient's pattern of disease. For instance, occasional attacks provoked by colds or exercise require only an aerosol bronchodlilator, to be used

at the earliest sign of a wheeze, whereas continuous or frequent attacks call for regular treatment with steroids or cromoglycate supplemented by bronchodilators (Seaton, 1978).

Bronchodilators may be in the form of tablets or aerosol, examples of these are Salbutamol as Ventolin tablets or Ventolin inhaler, and Orciprenaline as Alupent tablets or Alupent spray. Both Salbutamol and Orciprenaline are highly selective adrenergic stimulants. Their cellular action appears to be initiated by stimulation of adenyl cyclase leading to increase in the production of 3'5' c-AMP (Kennedy and Simpson, 1969; Koivikko, 1974).

Steroid preparations are also available as either tablets or aerosol. For instance betamethasone is packed as Betnesol (aerosol) or Betnelan tablets. Another important anti-inflammatory agent is prednisone. The steroids may also be in the form of depots for intramuscular administration. Steroids generally suppress harmful inflammatory or immunological as well as lymphoid activity (Grodsky, 1977).

Other drugs like aminophylline or theophylline and epinephrine are administered during emergencies. Epinephrine, a β-adrenergic stimulant, and theophylline, a phosphodiesterase inhibitor, effect muscle relaxation as a result of increases in 3'5' c-AMP.

The drugs discussed above only offer symptomatic relief. They are therefore largely administered during periods of attack. Since the effect of these drugs are not long lasting, patients who experience frequent attacks usually have to be on them constantly. The result is that the patients experience their adverse effects. For instance, prolonged use of steroids may affect adrenal function, and patients who use bronchodilators are known to be prone to tachycardia palpitations and generally show signs of excessive sympathetic activity (Bochner et al, 1978(b)).

Presently disodium cromoglycate coded FPL-670, which is used prophylactically, has been shown to suppress the release of chemical mediators thereby inhibiting the anaphylactic reaction (Assem and Mongar, 1970; Cox, 1967; Sheard and Blair, 1970). Now it appears cromoglycate does not only prevent the anaphylactic release of spasmogenic substances but also reduces the lung content of histamine (Mahajarni and Kulkarni, 1977). In a preliminary study by the same authors, Picrorhiza kurroa root powder exhibited similar effects. Aqueous extracts of Desmodium adscendens have

also been demonstrated to be capable of reducing the release of histamine from sensitized guinea pig lung, and lowering histamine content of such tissues (Awumey, 1981). It has also been shown to be anti-anaphylactic when administered orally (Awumey, 1981) and in *in vitro* anaphylactic experiments (Gbewonyo, 1980).

1.9 SOME PLANTS USED IN THE THERAPY OF ASTHMA:

In different societies, different plants are used in the treatment of the same disease. It is therefore not uncommon that in folkloric treatment of asthma numerous plant preparations are found in use. A few of these plants which show anti-asthmatic properties will be discussed in this section.

In the Mediterranean region, the plant *Ammi visnaga* from which cromolyn was extracted has had a long history of use in the treatment of asthma(Lewis and Elvin-Lewis, 1977). Also the root bark of *Picrorhiza kurroa* (Mahajarni and Kulkarni, 1977) as well as the leaves of *Tylophora indica* (Shipvuri *et al*, 1969) are used as anti-asthmatic agents in India.

In Ghana, several herbal preparations are known to be used by traditional healers to treat asthma. Some of these anti-asthmatic plants include *Sterculia foetida*. A preparation of this plant in the form of powder is marketed under the trade name of "Asthmapot" (Noamesi, Personal Communication, 1983). *Anthocleista nobilis*, currently undergoing pharmacological investigations (Lutterodt, Personal Communication, 1983) is also used effectively in the treatment of asthma. In addition to these

are Desmodium adscendens, Deinbollia pinnata and Thonningia sanguinea. Preparations of these three last plants are administered to asthmatic patients attending clinic at CSRPM, Mampong.

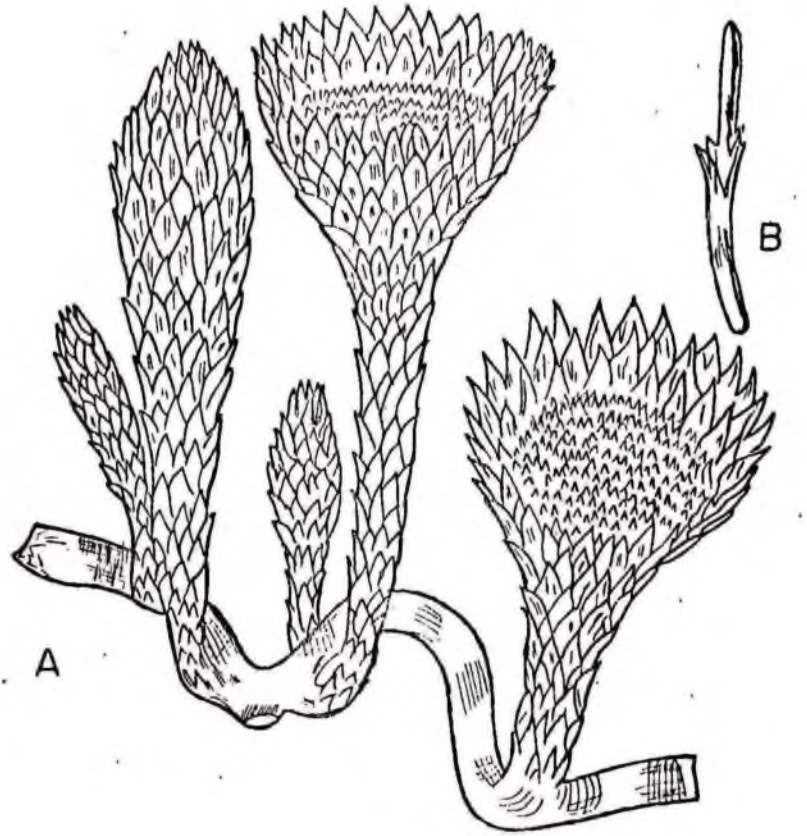
1.10 THONNINGIA SANGUINEA:

Thonningia sanguinea is a forest plant belonging to the family BALANOPHORACEAE. Some of its local names include KWABEDWAA or ANANSE ABEDWAA in Akan or Twi, and KPADEO-ABLETO-TA in Ewe (Ayensu, 1978).

The herb is fleshy, parasitic on roots of indigenous forest trees and shrubs. It is destitute of chlorophyll and may be annual or perennial. The rhizomes of T. sanguinea are subterranean and tuberous at the point of attachment (Fig. 1). The plant is also branched and pubescent with variable peduncles (Holland, 1922; Hutchinson and Dalziel, 1954).

T. sanguinea is used to treat asthma. It has been shown at CSRPM that this plant preparation can be taken prophylactically against bronchial asthma. Ampofo, (1977), reported that this herbal preparation when taken over a period is capable of deferring or stopping completely attacks of asthma. The two other plants used at the Centre, D. adscendens and D. pinnata, have each been shown to be potent against bronchial asthma

Fig. 1 DIAGRAM OF THONNINGIA SANGUINEA SHOWING (A) FEMALE FLOWER HEADS AND (B) MALE FLOWER. (Adapted from Hutchinson and Dalziel, 1954)



especially in children. However, Ampofo (1977) has reported that the best clinical results at the Centre was obtained when T. sanguinea was administered in combination with D. adscendens.

All Thonningia preparations are administered orally at the Centre. Roots of the herb are dried and pulverized. Two dessertspoonfuls of the powder are mixed thoroughly in approximately 250 g of honey and administered in doses of one teaspoonful to one tablespoonful three times daily according to age. Alternatively, the preparation may be made into an alcoholic extract (Ampofo, 1977).

In addition to its use as an anti-asthmatic agent, Thonningia has other uses. The flower head or stem and twig (rhizome) of the plant with other medicines are used as vermifuge (Dalziel, 1948). The decoction of the flower head is also used to treat sore throat as well as dysentery (Holland, 1922) while the entire plant is used in ointment against skin diseases (Ayensu, 1978).

Inspite of the available information on the traditional uses of T. sanguinea preparations, there has been no pharmacological or phytochemical work reported in the literature.

An anti-asthmatic herbal preparation which works prophylactically could do so by being anti-anaphylactic. T. sanguinea preparation when administered orally works prophylactically. This herbal preparation is used together with D. adscendens which has been shown to be anti-anaphylactic when administered orally. In view of these, the present work was undertaken to find out if T. sanguinea was also anti-anaphylactic and whether the scientific basis for its therapeutic action differed from that of D. adscendens.

Experimental anaphylaxis in the guinea pig was adopted in this work. Both in vivo and in vitro assays of the plant preparation were carried out. In the in vivo studies the plant preparation was administered orally to sensitized guinea pigs. This was necessary because all Thonningia preparations for the treatment of asthma are administered orally.

In the in vitro work, the plant preparations were added to the tissue in the organ bath. This assay method was necessary because it was less cumbersome and much faster to assay the other fractions of the plant extract whose quantities were also too small to be administered orally.

CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS:

Guinea pigs were bred at the Department's animal house and at Korle-Bu Teaching Hospital. These were supplemented with occasional purchases from other sources. Animal feed was purchased from Tema Food Complex.

Supplies of powdered roots of T. sanguinea were obtained from CSRPM, Mampong.

Histamine diphosphate, histamine free base, o-phthalaldehyde (OPT) and atropine sulphate were purchased from Sigma. Crystalline egg albumin, acetic acid, hydrochloric acid and Amberlite IR45-OH were purchased from British Drug House (BDH). Sodium chloride, potassium ferricyanide, magnesium chloride, sodium bicarbonate, glucose, perchloric acid, sodium dihydrogen phosphate, ethylene glycol and chloroform were of Analar grade. Ferric chloride, potassium chloride and mepyramine maleate were purchased from May and Baker (M & B). Methanol, ethanol, n-butanol, heptane, sodium hydroxide and calcium chloride were purchased from Fluka. Chromatography paper (Whatman No.1) and silica gel G (Searle Brand) were also used.

2.1.1 Reagents:

The following reagents, sterile physiological saline solution, reagents for preparing Tyrode's physiological solution, citrate buffer 0.1 M pH 4.2 (see appendix) were prepared and stocked in a refrigerator for use. Others include histamine diphosphate 30 mg/cm^3 in citrate buffer, mepyramine 1 mg/cm^3 and atropine 1 mg/cm^3. The following were also prepared and stocked for use: perchloric acid 0.4 M (stored in a dark bottle), HCl 0.1 M and 3 M, NaOH 1 M and 5 M, histamine free base 100 µg/cm^3 in HCl (0.1 M). OPT (1% in reagent grade acetone-free methanol) was always prepared fresh before use. Similarly egg albumen 5 mg/cm^3 and 100 mg/cm^3 in physiological saline solution as well as egg albumen 1% in Tyrode's physiological solution were always prepared fresh before use. Finally, $FeCl_3$ solution 2% in methanol, 1% aqueous solution of $FeCl_3$, 1% aqueous solution of potassium ferricyanide ($K_3Fe(CN)_6$), 20% aqueous solution of phosphoric acid and 25% trichloroacetic acid in chloroform were prepared and stocked for use.

2.2 PREPARATION OF THE CRUDE AQUEOUS EXTRACT:

Known weights of powdered roots of T. sanguinea were extracted continuously with hot water for 48 hours using a soxhlet extractor. The concentration of the aqueous extract was expressed as weight of

sample extracted per litre of solution. For this work a stock solution with a final concentration of 400 g plant material per litre (or 40% solution) was prepared and kept in a refrigerator to prevent it from growing mouldy. Aliquots of this stock were warmed up to room temperature before being administered to the sensitized guinea pigs.

2.2.1 n-Butanol Extract:

A 100 cm^3 sample of the stock aqueous extract corresponding to 40 g of the plant material was used for the n-butanol extraction. Extraction procedure according to Dewidar and El-Munajjed (1970-71) was adopted. Five grammes of NaCl and 50% HCl (to make the solution acid) were added. The resultant solution was then extracted five times, each time with 100 cm^3 of butanol saturated with water.

The aqueous phase left after the butanol extraction was rid of the acid by shaking it with an anion exchanger - Amberlite IR 45-OH and kept for analysis.

The n-butanol fraction obtained above was hydrolysed. This was done by adding 40 cm^3 of ethanol to the butanol fraction followed by HCl to make the resultant solution 4 M with respect to the acid. The mixture was refluxed for 1.5 hours after which 200 cm^3 of water were added and the

mixture allowed to cool. The brown oily residue which settled at the surface was removed and was evaporated to dryness on a water bath.

The aqueous acid phase obtained after hydrolysis was freed of the acid by shaking it with Amberlite IR 45-OH and concentrated (Dewidar and El-Munajjed, 1971).

2.3 QUALITATIVE ANALYSIS:

Some qualitative tests were performed on the solid sample obtained after hydrolysis of the butanol fraction in an attempt to characterise it. As a result, solubility as well as idenfification tests were performed on the sample. The reagents used as solvents for the solubility tests included: water, HCl 1 M, NaOH 1 M, and $NaHCO_3$ 1 M. For the solubility test a small amount of the sample was dissolved in 2 cm^3 of solvent.

Results from the solubility test helped to narrow down on the types of identification tests needed to be performed. Ferric chloride test for phenolic substances was performed on the sample. This was followed by involving the sample in azo-dye formation. This was done by adding a cold solution ($0^{\circ}C$)of the sample in 2 M NaOH to a cold solution ($0^{\circ}C$) of benzene diazonium chloride. This latter test served to confirm the results obtained for the ferric chloride test. In addition to the above,

identifications, tests for amino acids, oximes, ketones and aldehydes were also performed on the sample.

2.4 CHROMATOGRAPHIC ANALYSIS:

The various fractions of the extracted plant material were analysed by paper and thin layer chromatography. In all, five fractions were handled; these were:

(i) the crude aqueous extract

(ii) the n-butanol fraction

(iii) the aqueous phase after the butanol extraction

(iv) the residue after hydrolysis of the butanol fraction; and

(v) the aqueous phase after hydrolysis.

One dimensional paper chromatography (PC) using Whatman No.1 chromatography paper, and thin layer chromatography (TLC) using Silica gel G as the adsorbent and different solvent systems were used in an attempt to resolve the components of the various fractions. Out of the solvent systems tried, only three showed good resolving power. The three were:

Solvent system I: n-butanol-acetic acid-water (40:10:50 v/v)

Solvent system II: Water-methanol-n-butanol (3:3:4 v/v)

Solvent system III: Chloroform-methanol-water (65:35:10 v/v)

About 1 mg of the sample, where solid, was dissolved in 10 cm^3 of butanol. Between 5 µl and 10 µl of the resulting solution were then applied to the paper or thin layer and developed in chromatography tanks which have been equilibrated with the solvent systems for at least an hour at 27°C.

Location of spots after development of the chromatograms were made visually under visible and long wavelength Ultraviolet (UV) lights. In addition to the above the paper chromatograms were treated with the following chemical reagents to aid the location of spots as well as in the identification of some of the chemical substances:

(i) 20% aqueous solution of phosphoric acid

(ii) $FeCl_3$, 2% in methanol; and

(iii) Ferric chloride-potassium ferricyanide system (Neher, 1964).

After the chromatograms had been treated with (iii) above, the papers were rinsed with HCl 0.1 M and then dried at 100°C in an oven.

In order to aid in standardization of the plant extracts, the spots on the TLC plates were also scanned with a densitometer (Schimadzu Dual Wavelengh Chromatogram scanner, model CS-900). The operating parameters were: λs = 505 nm, λr = 660 nm, scan speed = 40 mm/min, sensitivity = X 50. Appropriate slit width and height were chosen.

2.5 SENSITIZATION AND TREATMENT SCHEDULES:

Guinea pigs of mixed sex weighing between 250 g and 350 g were used. Animals purchased from other sources were always quarantined for at least one week before being used for the experiments.

The method of active sensitization was adopted. This was achieved by administering crystalline egg albumen in sterile physiological saline solution as antigen - 100 mg/cm^3 intraperitoneally and 100 mg/cm^3 subcutaneously. Booster doses of the same antigen were administered to the animals seven days after the first administration. These comprised 5 mg/cm^3 of the antigen administered intraperitoneally and another 5 mg/cm^3 given subcutaneously.

In all, three groups of sensitized guinea pigs were used. Animals in the first group were each given, in place of water, 30 cm^3 of 20% crude aqueous extract of T. sanguinea (corresponding to 10 g of plant material extracted) per day. These animals were used to investigate the effects of oral administration of the plant extract on anaphylaxis. The second group of animals were used as controls for the first group and were therefore given water to drink instead of the plant extract. The third group of animals were used to investigate the in vitro effects of the crude extract and the other fractions of the plant material. Each animal here served as its own control and they were also given water to drink.

The above treatment schedules were followed throughout an incubation period which lasted between 21 and 28 days. During this period all the animals were fed ad libitum on elephant grass (Panicum maximum) supplemented with animal feed composed of wheat bran 40%; maize 40% fish meal 17% cod liver oil2%; sodium chloride 1%.

2.6 PREPARATION OF ISOLATED TISSUES:

The animals were killed, between 21 and 28 days after the primary sensitization, either by a sharp blow on the head or by cervical dislocation followed by ex-sanguination. The former method was used when only ileal pieces were required. For isolation of lung tissues, the latter method was adopted. Death was instantaneous with this method, and blood was prevented from being aspirated into the lung (Okpako, 1971).

2.6.1 Ileal Pieces:

After opening the abdomen, a length of ileum was removed and transferred into a dish containing aerated Tyrode's physiological solution. Ileal pieces of about 2 cm long were cut and freed from mesenteric attachments. Sometimes it was necessary to wash out the contents of the gut with warm Tyrode's solution ($37^{\circ}C$). This was done using a pipette slanted at about 30° to the bench. Minimum hydrostatic pressure was achieved this way and thus avoided damaging the tissues. For the same reason, fingers were used to handle the tissues rather than their being gripped with forceps.

2.5.2 Lung Tissues:

The entire lung was dissected out after the thorax had been opened and the aorta tied. The organ was then transferred into a clean vessel containing Tyrode's physiological solution. The lung was then washed free of blood by passing warm (37°C) aerated Tyrode's solution through the pulmonary artery using a Watson-Marlow H.R. Flow inducer. After this known wet weights of the washed lungs were cut and immersed in fresh samples of Tyrode's solution and used for subsequent assays.

2.7 BIOASSAYS:

The pieces of mesenteric membrane-free guinea pig ileum (section 2.5.1) were set up in 10 cm^3 organ baths. In mounting the tissue, threads were attached to each end of the ileal piece, care being taken to avoid closing the lumen of the gut. The shorter thread was then tied to a fixed point in the organ bath while the longer was either tied to the lever of a transducer of an 8" Chart Mover (Havard Apparatus, model 450) or to the lever of a kymograph with a frontal writing point. The tissue was immersed in aerated Tyrode's solution maintained at 37°C. The ileal piece was kept taut or fully relaxed by applying a convenient load (up to 0.5 g) on the lever. The mounted tissue was then allowed about 30 minutes for equilbration. Before all assays the mounted tissue was stimulated 2 to 3 times by the addition of histamine diphosphate in Tyrode solution (200 ng/cm^3).

This resulted in greater constancy of reaction. In the course of the dose-response assays with either antigen or histamine, the mounted tissue was repeatedly washed with fresh warm Tyrode's solution, after each antigen or histamine dose, until the original state of the tissue was obtained once more.

2.7.1 Anaphylactic Contractions of Ileal Pieces:

In order to investigate the effect of oral administration of the crude aqueous extract on anaphylactic contractions, i.e. the *in vivo* effect, ileal pieces, mounted as described earlier, were obtained from animals sensitized and drinking *Thonningia* extract.

A six-fold serial dilution from a 1% solution of egg albumen in Tyrode's solution was made such that when 1 cm^3 of each was added to the organ bath, the final concentration obtained was between 10^{-2} $\mu g/cm^3$ and 10^3 $\mu g/cm^3$. The contractions caused by each of these antigen concentrations were expressed as per cent of the maximum contraction, in the same tissue, caused by a large dose of histamine (200 ng/cm^3).

For the *in vitro* effects of the plant material, a known concentration of the particular fraction was added to the assay bath and incubated for about 10 minutes prior to the addition of antigen. For these effects, tissues were obtained from the group of animals drinking water.

2.7.2 Sensitivity of Ileal Pieces to Histamine:

Tissues obtained from sensitized animals drinking Thonningia extract and mounted as before were used to study the effect of oral administration of the crude extract on the sensitivity of ileal pieces with respect to histamine-induced contractions.

After allowing the tissues to equilibrate, increasing concentrations of histamine were added to the organ bath to give a final bath concentration between 3.4 ng/cm^3 and 24 ng/cm^3. Contractions caused by each of these histamine concentrations were expressed as per cent of the maximum contraction (caused by 200 ng/cm^3 histamine) in the same ileal piece.

To investigate the in vitro effects of the crude extract and the various fractions, tissues were obtained from animals drinking water. Known concentrations of these extracts were individually incubated with the mounted tissues for 10 minutes prior to the application of each histamine dose.

Results from the qualitative analysis (section 2.2.1) showed that both the n-butanol fraction and the hydrolysed sample were insoluble in water. As a result, for the in vitro assays involving these fractions in subsections 2.6.1 and 2.6.2 above, these samples were dissolved in a known volume of ethylene glycol before being diluted with Tyrode's solution to give the desired concentration. In order to eliminate the effect of the ethylene glycol on the tissue, anaphylactic contractions with the various antigen doses and histamine-induced contractions with the various histamine doses.

were observed in the presence of 50 µl ethylene glycol/cm^3 of Tyrode's solution. The effects observed were then substracted from the contractions recorded in the presence of the plant material *in vitro*.

2.7.3 Lung Assays:

The effect of oral administration of *Thonningia* on the anaphylactic release of histamine and autocoids other than histamine from lung tissues were investigated using the non-sensitized guinea pig ileum. The quantity of histamine released anaphylactically as well as the total histamine content in lung tissues were again extracted and estimated fluorometrically according to the method of Shore (1971).

2.7.3.1 Anaphylactic Release of Mediators from Lung tissues:

Approximately 1 g wet weight of lung tissues obtained from sensitized animals drinking the crude extract and those drinking water were challenged with 4 cm^3 of egg albumen, 100 µg/cm^3 in warm aerated Tyrode's solution, for 10 minutes. A 0.5 cm^3 aliquot of each resultant exudate was withdrawn and assayed on the non-sensitized guinea pig ileum in the presence of atropine, 10 µg/l. Prior to this a dose-response curve using between 0 and 20 ng/cm^3 of histamine diphosphate (final bath concentration) was plotted using the same

ileal piece. This served as a calibration from which the amount of autocoids released anaphylactically from the lung tissues were estimated. As was indicated in an earlier section, each application of either exogenous histamine or autocoids released from the lung tissue was followed by thorough washing with fresh Tyrode's solution until the original state of the non-sensitized ileum was obtained once more.

2.7.3.2 Anaphylactic Release of Spasmogens other than Histamine:

Known wet weights of lung tissues from animals drinking the extract and those drinking water were challenged with 4 cm^3 of egg albumen 100 µg/cm^3 in warm Tyrode's solution for 25 minutes. After the 25 minutes incubation period, 3 cm^3 of the resultant exudates were withdrawn and assayed on the non-sensitized guinea pig ileum in the presence of atropine and mepyramine, 10 µg/l each. For the purposes of comparison, the contractions caused by these exudates were expressed as contractions caused by the "spasmogenic substance" per gramme of lung tissue. In the absence of a standard "spasmogen" these contractions were in turn expressed as per cent of the maximum contraction caused in the same ileal piece by 200 ng/cm^3 histamine diphosphate.

2.8 FLUOROMETRIC DETERMINATION OF HISTAMINE:

Histamine was extracted by homogenizing lung tissues obtained from sensitized guinea pigs kept on water or crude extract.

For the extraction of total histamine in the lung, approximately 1 gm wet weight of lung tissue obtained from each of the two groups was homogenized in 9 cm^3 of 0.4 M perchloric acid. For the extraction of histamine released anaphylactically the method described by Shore (1971) was used. Here approximately 1 gm wet weight of the lung tissue was challenged with 5 cm^3 of egg albumin (100 ug/cm^3 in warm Tyrode's solution) for 10 minutes. At the end of the antigenic challenge, 60% perchloric acid was added to the incubation mixture to make the resulting mixture 0.4 M with respect to the acid.

Ten minutes after the addition of the acid each mixture was centrifuged at 1,250 g for 10 minutes. A 4 cm^3 aliquot of the protein-free solution in each tube was transferred into a glass-stoppered shaking tube containing 10 cm^3 n-butanol, 0.5 cm^3 5 M NaOH and 1.5 g of solid NaCl. The tube was shaken mechanically for 5 minutes and then centrifuged. The organic phase was then separated and rid of histidine by shaking with 5 cm^3 of 0.1 M NaOH saturated with NaCl for 1 minute. An aliquot of 8 cm^3 of the butanol phase was transferred into another tube containing 4.5 cm^3 of 0.1 M HCl and 15 cm^3 heptane. The tube was then shaken for 1 minute and 2 cm^3 of the acid phase was transferred into a clean sample bottle for reaction with OPT.

To the 2 cm^3 acid phase, 0.4 cm^3 of 1 M NaOH were added, this was followed by the addition of 0.1 cm^3 1% freshly prepared pure grade OPT. The tube was shaken after each addition. The reaction mixture was allowed to stand for 4 minutes at room temperature after which 0.2 cm^3 of HCl, 3 M, were added to enhance and stabilize fluorescence. The resultant mixture was transferred into a fluorometer cuvette and the fluorescence read at 450 nm from excitation at 360 nm in a spectrophotofluorometer (Turner, model 430).

A calibration curve was prepared using histamine free base over a concentration range (of 0 to 0.5 $\mu g/cm^3$). These concentrations were prepared from a stock solution of histamine free base, 100 $\mu g/cm^3$ in HCl, 0.1 M. The histamine content of the lung tissues and the amount released anaphylactically were then estimated from the calibration curve and expressed as microgrammes histamine per gramme wet weight of lung tissue.

CHAPTER THREE

RESULTS

3.1 IN VIVO EFFECTS OF THE CRUDE EXTRACT:

3.1.1 Anaphylactic contractions of guinea pig ileum:

Table 1 shows the results obtained when Thonningia extract was administered orally to sensitized guinea pigs. Anaphylactic contractions in ileal pieces obtained from these animals, and from sensitized animals drinking water were measured using the Schultz-Dale reaction (Carpenter, 1975). Figure 2 shows a typical Chart Mover tracing of contractions in the sensitized guinea pig ileum when increasing doses of antigen were applied to the tissues. The contractions were expressed as per cent the maximum contraction produced by a large dose of histamine (200 ng/cm^3) in the same tissue.

FIG. 2 TYPICAL TRACING OF ANAPHYLACTIC CONTRACTIONS IN THE GUINEA PIG ILEUM FOLLOWING ADDITIONS OF INCREASING DOSES OF ANTIGEN. CONTRACTIONS WERE RECORDED WITH AN 8" CHART MOVER (HAVARD APPARATUS MODEL 450). TIME IS SHOWN AS 10 SECONDS INTERVALS AT THE BASE.

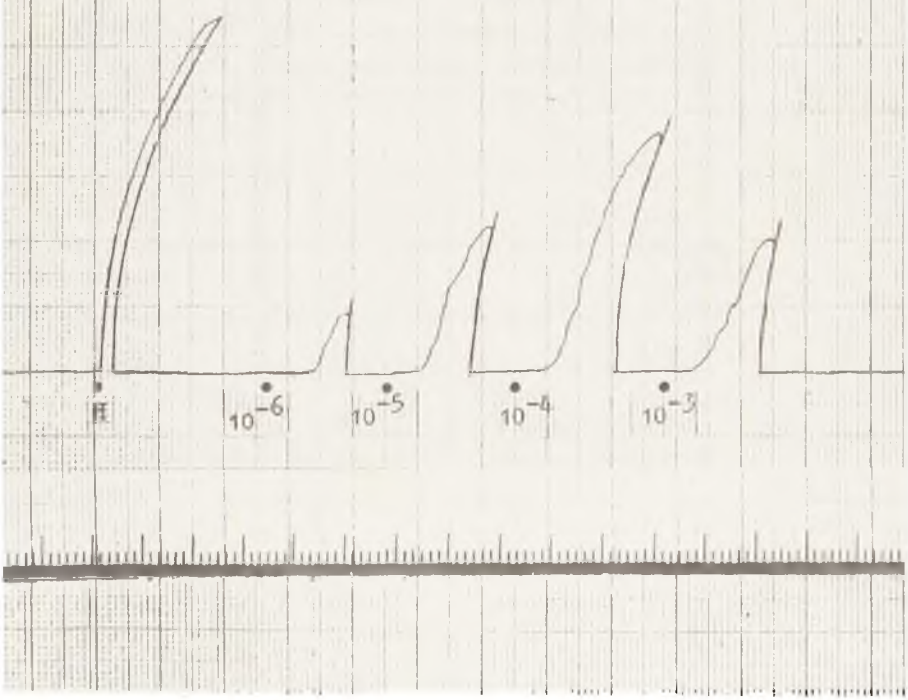


TABLE 1:

Anaphylactic contractions in the guinea pig ileum isolated from sensitized animals drinking water and those drinking the aqueous plant extract. The contractions are in response to increasing doses of antigen. Values are expressed as per cent maximum contractions obtained by the addition of 200 ng/cm^3 histamine.

ANTIGEN DOSE (μg/cm^3)	CONTRACTIONS (% max)	
	Drinking water	Drinking extract
0.1	0	0
1.0	15.0 ± 3.0	9.0 ± 3.0
10.0	57.0 ± 5.0	25.0 ± 4.0
100.0	76.0 ± 6.0	32.0 ± 6.0
1000.0	67.0 ± 5.0	30.0 ± 6.0

Fig. 3 DOSE-RESPONSE CURVES SHOWING THE EFFECT OF ORAL ADMINISTRATION OF AN AQUEOUS EXTRACT OF THONNINGIA SANGUINEA ON ANAPHYLACTIC CONTRACTIONS.
(Each point is a mean of determinations in 5 animals with bars showing standard error).

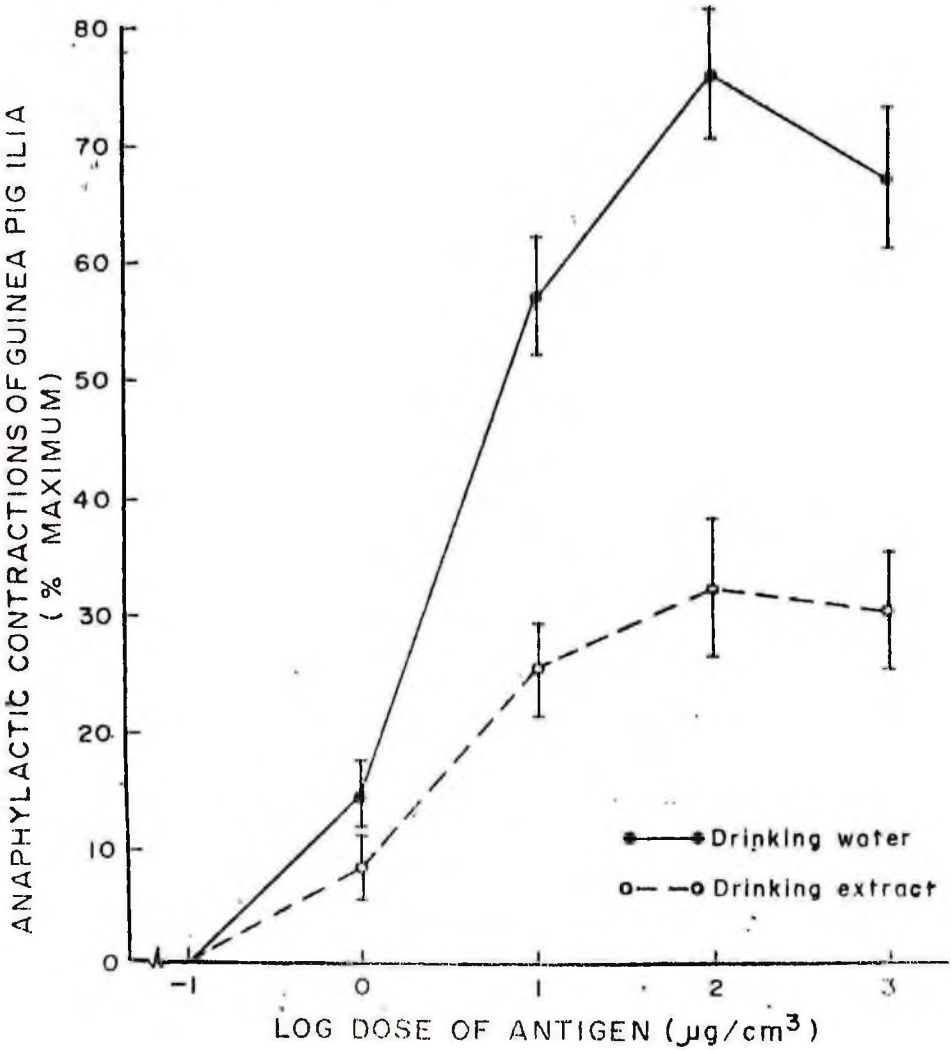


Table 1 shows that 0.1 $\mu g/cm^3$ of egg albumen as antigen caused no contractions in the tissues. Increased contractions were observed as the concentration of the challenging antigen increased to 100 $\mu g/cm^3$. This was followed by a decrease in the observed contractions when 1000 $\mu g/cm^3$ antigen was applied to the tissue. The data show that with the exception of contractions caused by 1.0 $\mu g/cm^3$ antigen, for each antigen concentration, there was a statistically significant difference between contractions recorded in the tissue obtained from animals drinking water (control) and those drinking the extract ($p < 0.05$). The dose-response curves, Figure 3, gives a better graphical representation of this effect. The vertical bars indicate the standard error (S.E) observed at each antigen concentration used.

The results show that when the crude aqueous Thonningia extract was administered orally to sensitized guinea pigs during the period of sensitization, it significantly reduced the anaphylactic contractions in the isolated guinea pig ileum at antigen concentrations known to cause release of mediators (Okpako, 1970).

3.1.2 Histamine-induced Spasms:

Table 2 summarises contractions observed when different concentrations of histamine were applied to sensitized isolated ileal pieces obtained from animals drinking water and those drinking aqueous extract of Thonningia. The results are expressed as per cent the contraction caused by a large dose of histamine (200 ng/cm^3) in the same ileal piece.

The contractions with their standard errors are graphically represented as histamine dose-response curves in Figure 4. The graph shows a parallel shift to the right when the animals were drinking the extract during the sensitization period.

The effective dose of histamine required to cause 50% contraction (ED_{50}) in the ileal pieces were estimated from these curves. The ED_{50} (a measure of the affinity of histamine for its receptors) for tissues obtained from animals drinking the extract was found to be significantly larger than that for tissues from animals drinking water ($p < 0.05$). The figures were 13.7 ± 0.9 ng/cm^3 and 10.5 ± 0.9 ng/cm^3 for tissuses obtained from animals drinking extract and those drinking water respectively.

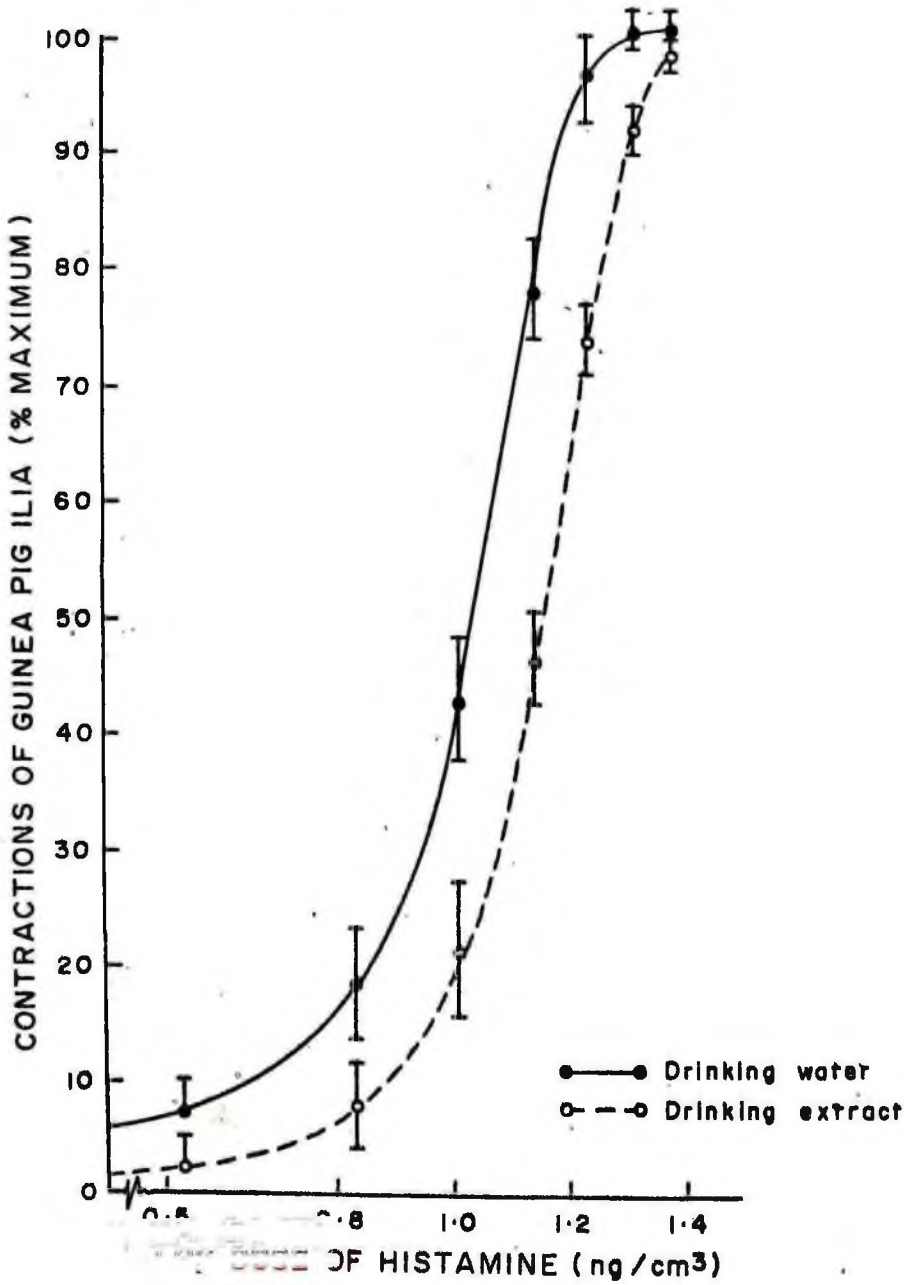
The results show that when guinea pigs were put on Thonningia during the sensitization period, ileal tissues were rendered less sensitive to histamine.

TABLE 2:

Histamine-induced contractions in ileal pieces obtained from sensitized animals drinking water and those drinking extract. The contractions are expressed as per cent maximum caused by 200 ng/cm^3 histamine in the same tissue.

DOSE OF HISTAMINE (ng/cm^3)	CONTRACTIONS (% max)	
	Drinking water (Control)	Drinking extract
3.41	7.0 ± 3.0	3.0 ± 3.0
6.82	18.0 ± 5.0	8.0 ± 4.0
10.23	43.0 ± 5.0	21.0 ± 6.0
13.65	78.0 ± 4.0	46.0 ± 4.0
17.04	96.0 ± 4.0	73.0 ± 3.0
20.45	100.0 ± 2.0	92.0 ± 2.0
23.86	100.0 ± 1.0	98.0 ± 2.0

Fig. 4 DOSE – RESPONSE CURVES SHOWING THE EFFECT OF ORAL ADMINISTRATION OF AN AQUEOUS EXTRACT OF THONNINGIA SANGUINEA ON HISTAMINE INDUCED CONTRACTIONS. (Each point is a mean of determinations in 5 animals with bars showing standard error).



3.1.3 Anaphylactic Release of Mediators from Sensitized Lung Tissues:

Figure 5 shows a typical kymograph tracing of contractions in the atropinised non-sensitized guinea pig ileum.

The contractions were caused by mediators released anaphylactically from lung tissues obtained from sensitized animals that received either extract (T) or water (C) ad libitum. Also shown is a calibration (H) from which the quantity of mediators released was estimated. Mediator(s) release from the weighed tissues was caused by the addition of 100 ug/cm^3 antigen.

Fig. 5 TYPICAL KYMOGRAPH TRACING SHOWING THE EFFECTS OF ORAL ADMINISTRATION OF AN AQUEOUS EXTRACT OF THONNINGIA ON THE ANAPHYLACTIC RELEASE OF SMOOTH MUSCLE STIMULATING SUBSTANCES FROM LUNG TISSUES.

H - contractions in ileal piece due to increasing doses of exogenous histamine

T & C - contractions due to substances released from lung tissue obtained from animals drinking extract and those drinking water respectively.

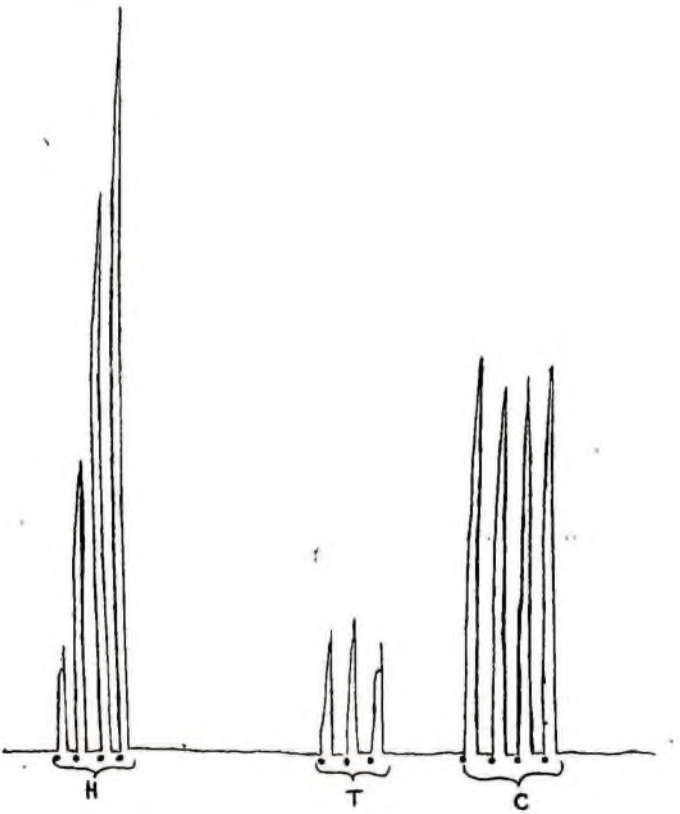


Table 3 summarises the quantity of mediators released anaphylactically from lung tissues obtained from animals drinking extract and those drinking water. The mean values of the mediators released expressed as amount of histamine released anaphylactically from lung tissues with their standard errors were found to be 0.87 ± 0.01 ug/g, and 0.45 ± 0.01 ug/g wet weight of lung tissue for tissues obtained from sensitized animals drinking water and those drinking extract respectively. These values are presented as bar graphs in Figure 6.

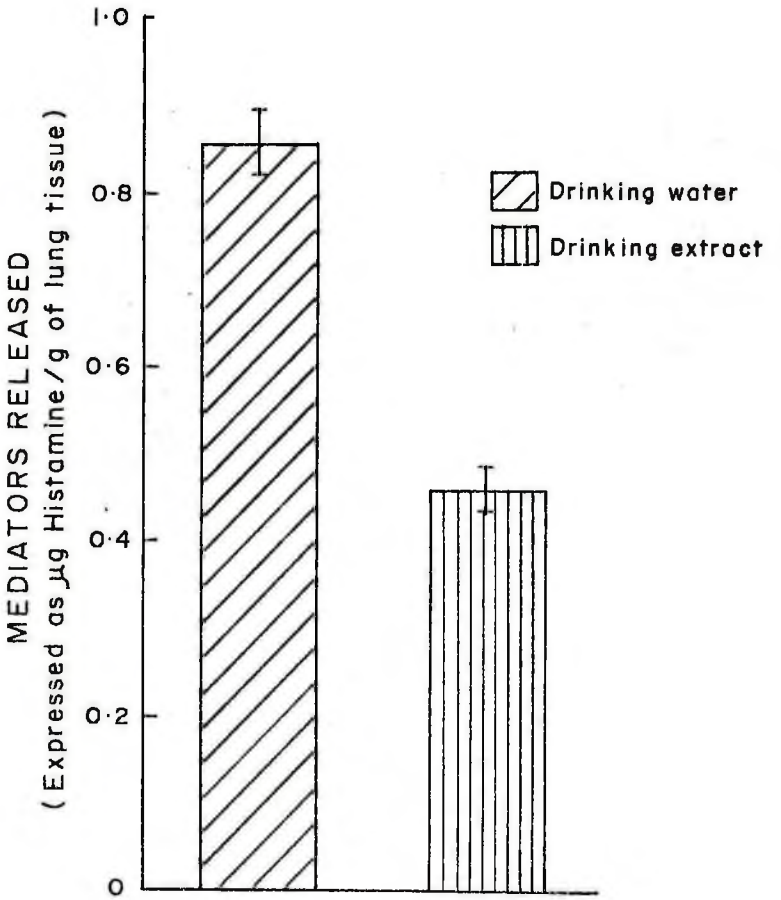
The results show that when Thonningia extract was administered orally to sensitized guinea pigs, it significantly reduced the quantity to spasmogens released anaphylactically from the lung tissues ($p < 0.05$).

TABLE 3:

Quantity of mediators released anaphylactically from lung tissues obtained from eight sensitized animals drinking extract and eight others drinking water. The amount of mediator released was expressed as µg histamine/g lung tissue. The non-sensitized guinea pig ileum was used to estimate the released mediators.

	Drinking water	Drinking extract
µg Histamine released/g lung tissue	0.862	0.434
	0.840	0.475
	0.926	0.448
	0.886	0.502
	0.803	0.432
	0.874	0.432
	0.905	0.407
	0.896	0.510

Fig. 6 BAR GRAPHS SHOWING THE EFFECT OF ORAL ADMINISTRATION OF AN AQUEOUS EXTRACT OF THONNINGIA SANGUINEA ON THE AMOUNT OF MEDIATORS RELEASED ANAPHYLACTICALLY FROM LUNG TISSUES DETERMINED USING THE ATROPINISED NON-SENSITIZED GUINEA PIG ILEUM. (Each bar shows a mean of determinations in 8 animals, standard errors are also shown)



3.1.4 Anaphylactic release of spasmogens other than histamine:

A typical kymograph tracing of contractions caused in the non-sensitized guinea pig ileum in the presence of atropine and mepyramine, 10 µg/l each, is shown in Figure 7. These contractions were caused by spasmogens released anaphylactically from tissues obtained from sensitized animals drinking the plant extract (T), and those drinking water (C). The spasmogens were released by 100 µg/cm^3 antigen. The effect of 200 ng/cm^3 histamine (H) on the tissue in the presence of atropine (10 µg/l) is indicated. Also shown in the figure is the effect of the same dose of histamine on the tissue, this time in the presence of both atropine and mepyramine (H + M). This figure shows that in the presence of mepyramine (10 µg/l) the effect of histamine (200 ng/cm^3) was blocked.

Table 4 summarises the responses in the non-sensitized guinea pig ileum due to spasmogens other than histamine released per gramme of lung tissue. The contractions are expressed as per cent the maximum contraction caused by 200 ng/cm^3 histamine in the same tissue.

The mean/standard errors of the results in Table 4 were found to be /and 74.0 ± 3.0 % and 33.0 ± 2.0 % the contractions caused in the same guinea pig ileum for spasmogens (other than histamine) released from animals drinking water and those drinking extract respectively. These figures are represented as bar graphs in Figure 8.

Fig. 7 TYPICAL KYMOGRAPH TRACING SHOWING CONTRACTIONS IN THE GUINEA PIG ILEUM DUE TO SPASMOGENS OTHER THAN HISTAMINE RELEASED ANAPHYLACTICALLY FROM LUNG TISSUES OBTAINED FROM ANIMALS DRINKING THONNINGIA EXTRACT (T) AND THOSE DRINKING WATER (C).

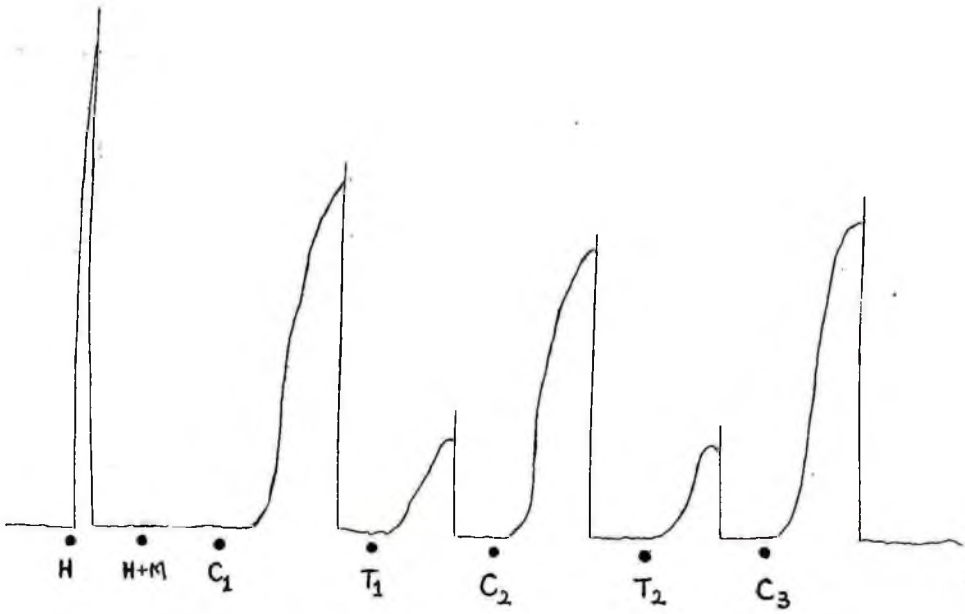
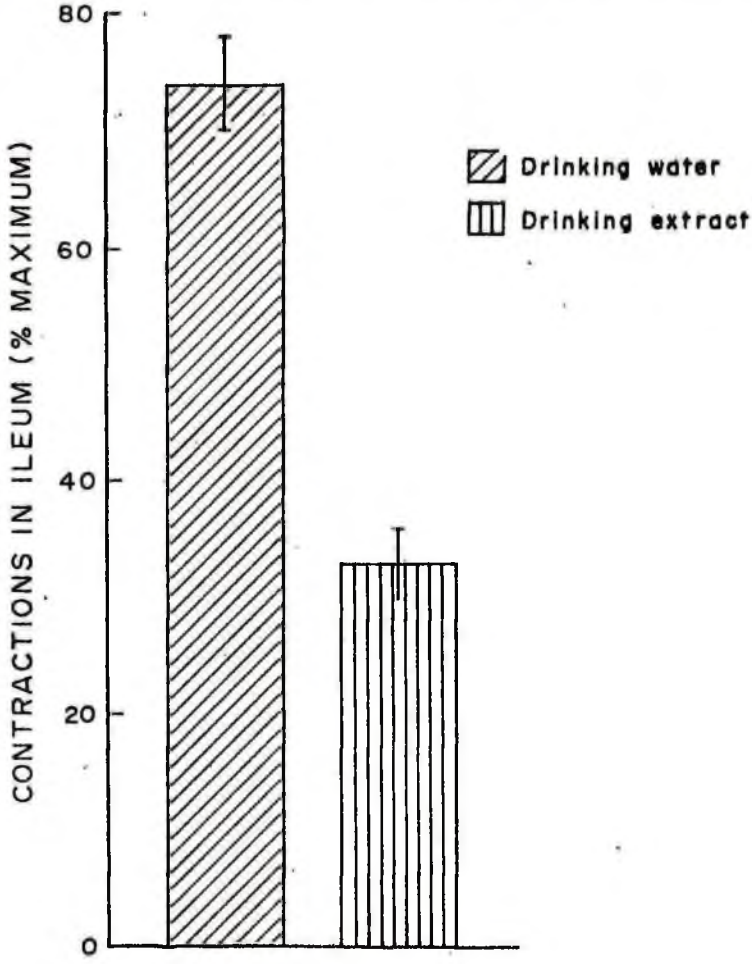


TABLE 4:

Effect of spasmogens other than histamine released from lung tissues on the non-sensitized guinea pig ileum in the presence of atropine and mepyramine, 10 µg/l each. Tissues were obtained from five sensitized animals drinking water and five others drinking extract. Values are expressed as per cent maximum contractions caused by 200 ng/cm^3 histamine.

	Drinking water	Drinking extract
Contractions (% max)	74	28
	64	39
	78	31
	81	35
	74	32

Fig. 8 BAR GRAPHS SHOWING EFFECT OF ORAL ADMINISTRATION OF AQUEOUS EXTRACT OF T. SANGUINEA ON CONTRACTIONS OF ILEAL PIECES CAUSED BY AUTOCOIDS RELEASED FROM LUNG TISSUE FROM SENSITIZED GUINEA PIGS IN PRESENCE OF MEPYRAMINE (10 µg/l) (Each bar is a mean of determinations in 5 animals. Standard errors are indicated)



The results were found to be statisticcally different from each other ($p < 0.05$), suggesting that oral administration of Thonningia extract significantly affected the release of spasmogens other than histamine from sensitized guinea pig lung tissues.

3.1.5: Histamine content of, and release, from lung tissues:

The total histamine in lung tissues from sensitized animals drinking water and those drinking extract, as well as the quantity released anaphylactically were extracted and assayed fluorometrically. The results obtained are presented in Table 5.

The means and standard errors of the data shown in Table 5 for total lung histamine are 4.8 ± 0.2 ug/g, and 2.9 ± 0.2 ug/g wet weight of lung tissues for animals drinking water and those drinking extract respectively. The means and standard errors for histamine released anaphylactically, extracted and assayed the same way (fluorometrically) are 0.80 ± 0.02 ug/g, and 0.3 ± 0.2 ug/g wet weight of lung tissues for animals drinking water and those drinking extract respectively. From these results the percentage histamine released anaphylactically were found to be 16.6 ± 0.7 % and 10.8 ± 0.6 % of the total lung histamine for animals drinking water and those drinking extract respectively. In each of these cases significant differences were observed between animals drinking water and those drinking extract ($p < 0.05$).

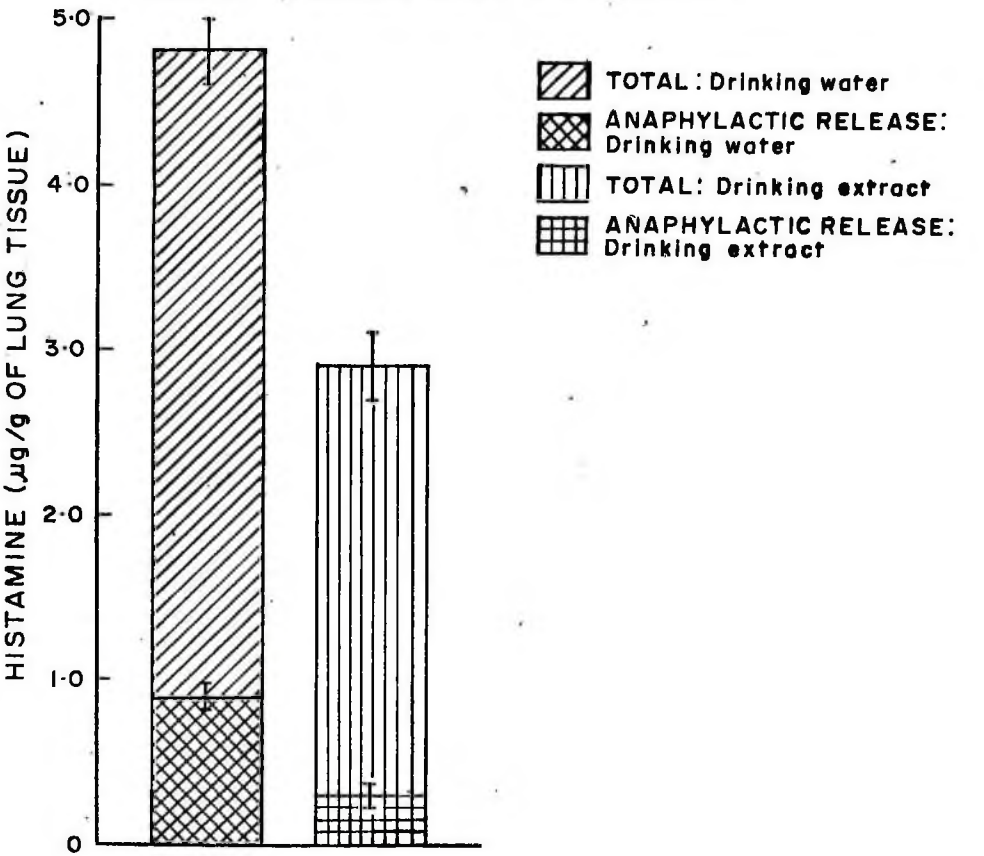
TABLE 5:

Total histamine content of sensitised lung tissues, and the quantity released anaphylactically by 100 µg/cm^3 antigen. Lung tissues were obtained from sensitized guinea pigs drinking water and those drinking extract. Values are expressed as µg histamine/g lung tissue.

Eight animals were used for total histamine, and five animals for histamine released.

	Drinking water		Drinking extract	
	Total Histamine	Histamine released	Total Histamine	Histamine released
µg Histamine/ g lung tissue	5.150	0.746	3.757	0.366
	4.383	0.702	3.129	0.318
	4.167	0.865	3.171	0.279
	4.990	0.783	2.229	0.326
	4.173	0.883	2.443	0.275
	5.221	–	2.830	–
	5.844	–	3.272	–
	4.300	–	2.734	–

Fig. 9 COMPONENT BAR GRAPHS SHOWING EFFECTS OF AQUEOUS EXTRACT OF THONNINGIA SANGUINEA ON THE TOTAL HISTAMINE CONTENT AND THE QUANTITY RELEASED ANAPHYLACTICALLY FROM LUNG TISSUES OBTAINED FROM SENSITIZED GUINEA PIGS. HISTAMINE WAS DETERMINED FLUOROMETRICALLY. (The bars show mean determinations in 8 animals (total histamine) and 5 animals (anaphylactic release). Standard errors are indicated).



The means and standard errors of the total histamine in the lung tissues and the quantity released anaphylactically have been presented as component bar graphs in Figure 9. The results show that when the crude aqueous extract of Thonningia was administered orally to guinea pigs during the sensitization period, it significantly lowered the total lung histamine content; it also significantly inhibited the anaphylactic release of this mediator.

3.2 IN VITRO EFFECTS OF FRACTIONS OF THONNINGIA:

3.2.1 Effects of fractions of Thonningia on Anaphylactic Contractions:

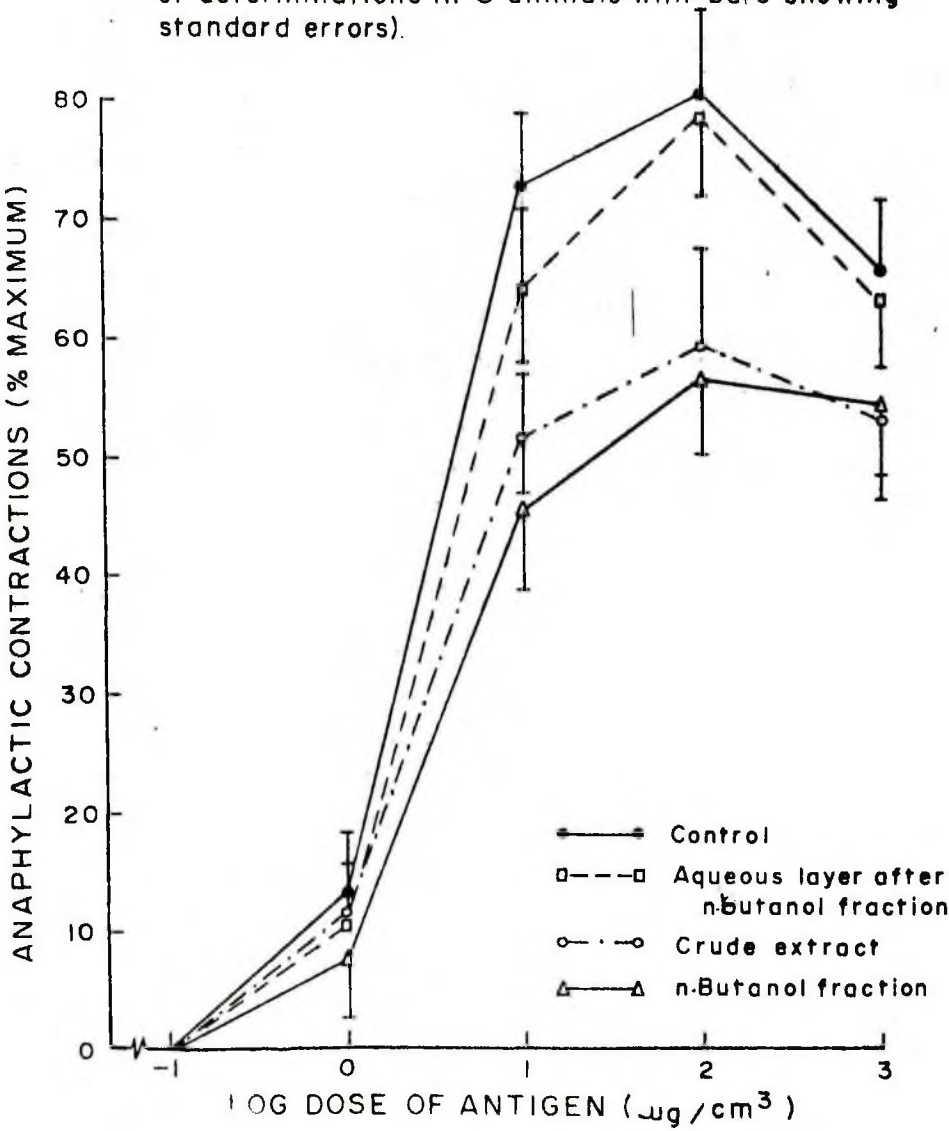
Table 6 is a summary of the anaphylactic contractions recorded as a result of adding increasing doses of antigen to the sensitized isolated guinea pig ileum. The contractions were in the presence of (a) the crude extract containing 20 mg Thonningia extracted/cm^3; (b) the n-butanol fraction, 0.4 mg/cm^3; and (c) 0.5 cm^3 of the aqueous phase left after the n-butanol extraction.

TABLE 6:

Anaphylactic contractions in ileal pieces recorded with or without the addition of Thonningia extracts to the tissue bath. Contractions are expressed as per cent maximum contractions caused in the ileum by 200 ng/cm^3.of histamine.

	CONTRACTIONS (% max)			
ANTIGEN DOSE (ng/cm^3)	Control (no extract)	Crude extract	Butanol fraction	Aqueous phase after butanol extraction
0.1	0	0	0	0
1.0	13.0 ± 5.0	12.0 ± 4.0	8.0 ± 5.0	10.0 ± 3.0
10.0	73.0 ± 6.0	51.0 ± 6.0	46.0 ± 7.0	64.0 ± 7.0
100.0	80.0 ± 7.0	59.0 ± 8.0	57.0 ± 8.0	79.0 ± 7.0
1000.0	65.0 ± 6.0	53.0 ± 7.0	54.0 ± 6.0	63.0 ± 5.0

Fig. 10 DOSE – RESPONSE CURVES SHOWING IN VITRO EFFECTS OF EXTRACTS OF THONNINGIA SANGUINEA ON ANAPHYLACTIC CONTRACTIONS IN THE GUINEA PIG ILEUM. (Each point is a mean of determinations in 5 animals with bars showing standard errors).



As was observed for tissues obtained from animals drinking the plant extract, 0.1 $\mu g/cm^3$ antigen caused no contractions in the sensitized ileal pieces in the absence of, and in the presence of the various fractions of the plant extract. The contractions, however, increased with increasing doses of antigen, reaching a maximum at 100 $\mu g/cm^3$ antigen. This was followed by a decrease in the contraction when an antigen dose of 1000 $\mu g/cm^3$ was applied.

The data in Table 6 are graphically represented as dose-response curves in Figure 10. The results show that the crude extract of Thonningia and the n-butanol fraction assayed in vitro inhibited the anaphylactic contractions caused by 10 $\mu g/cm^3$ and 100 $\mu g/cm^3$ antigen concentrations ($p < 0.05$ for both antigen concentrations). In this assay the aqueous phase left after the n-butanol extraction did not inhibit the anaphylactic contractions.

3.2.2 Histamine-induced Spasms in the Presence of Fractions of Thonningia:

Table 7 shows contractions (in per cent maximum) in ileal pieces due to increasing doses of histamine. These contractions were measured in the presence of (a) the crude extract containing 20 mg Thonningia extracted/cm^3 and (b) butanol fraction 40 $\mu g/cm^3$ and 400 $\mu g/cm^3$ (final bath concentration in each case).

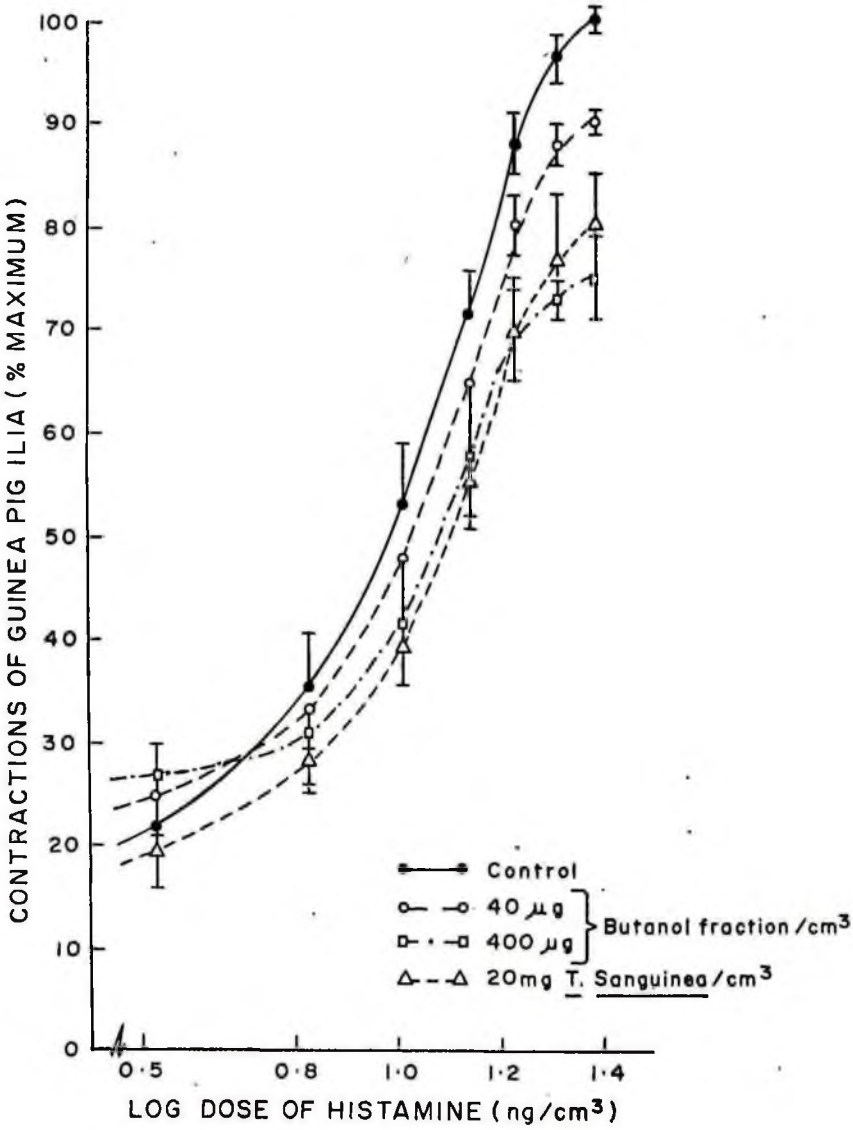
TABLE 7:

Histamine-induced contractions in ileal pieces recorded with or without the addition of (a) the crude extract and (b) the n-butanol fraction to the tissue bath. Contractions are expressed as per cent maximum contractions caused by 200 ng/cm^3 histamine in the same tissue.

Histamine dose (ng/cm^3)	CONTRACTIONS (% max)			
	Control (no extract)	Crude extract	40 µg/cm^3 butanol fraction	400 µg/cm^3 butanol fraction
3.41	22.0 ± 6.0	19.0 ± 3.0	25.0 ± 4.0	27.0 ± 3.0
6.82	36.0 ± 5.0	28.0 ± 3.0	34.0 ± 4.0	31.0 ± 5.0
10.23	53.0 ± 6.0	39.0 ± 4.0	48.0 ± 4.0	41.0 ± 6.0
13.65	72.0 ± 4.0	55.0 ± 4.0	65.0 ± 7.0	58.0 ± 6.0
17.04	88.0 ± 3.0	70.0 ± 4.0	80.0 ± 3.0	70.0 ± 5.0
20.45	97.0 ± 2.0	72.0 ± 6.0	88.0 ± 2.0	73.0 ± 2.0
23.86	100.0 ± 1.0	80.0 ± 5.0	91.0 ± 1.0	75.0 ± 4.0

Fig. II DOSE–RESPONSE CURVES SHOWING THE IN VITRO EFFECTS OF THE CRUDE EXTRACT AND THE n–BUTANOL FRACTION OF THONNINGIA SANGUINEA ON HISTAMINE–INDUCED CONTRACTIONS.

(Each point is a mean of determinations in 5 animals, bars indicate the standard errors)



The data are presented as dose-response curves in Figure 11. Both the crude extract and the n-butanol fraction when assayed *in vitro* reduced the maximum responses of the ileal pieces to histamine. The graph shows a shift to the right when the crude extract and the n-butanol fraction of *Thonningia* were each added to the organ bath. Such a shift is typical of non-competitive type of inhibition. It could also be seen from the graphs that the contractions were greatly suppressed by the plant material at higher histamine concentrations.

The ED_{50} (a measure of the affinity of histamine for its receptors) for each of the curves was estimated. They were found to be 10.0 ± 1.0 ng/cm^3, 12.0 ± 1.0 ng/cm^3, 11.0 ± 1.0 ng/cm^3 and 12.0 ± 1.0 ng/cm^3 histamine, for contractions observed in the absence of plant materials, in the presence of crude extract, and in the presence of 40 $\mu g/cm^3$ and 400 $\mu g/cm^3$ butanol phases respectively.

Though the crude extract and the butanol fraction *in vitro* each rendered the guinea pig ileum less sensitive to histamine, when the values of ED_{50} were considered, these reductions in sensitivity were found to be statistically insignificant. Therefore the extract appears to have no effect on the affinity of histamine for its receptors.

3.2.3 Anaphylactic Contractions in Ileal Pieces in the Presence of Hydrolysed n-Butanol Fraction:

Results obtained when increasing doses of antigen were added to ileal pieces in presence of 40 $\mu g/cm^3$ and 400 $\mu g/cm^3$ of hydrolysed n-butanol the fraction are shown in Table 8.

The data shown in Table 8 are presented as dose-response curves in Figure 12. For these, there were no significant reductions in the anaphylactic contraction by the 400 $\mu g/cm^3$ hydrolysed n-butanol fraction when 1.0 $\mu g/cm^3$ antigen was applied to the tissues. All other contractions elicited in the presence of the same concentration of hydrolysed sample in vitro were significantly reduced ($p < 0.05$). The 40 $\mu g/cm^3$ sample caused no reduction in the anaphylactic contractions.

The results therefore show that a higher concentration of the hydrolysed butanol sample (400 $\mu g/cm^3$) reduced the anaphylactic contractions in the sensitized isolated guinea pig ileum while the less concentrated sample (40 $\mu g/cm^3$) did not.

3.2.4 Histamine-induced Spasms in the Presence of the Hydrolysed Sample:

Table 9 is a summary of the results obtained when two different concentrations of the hydrolysed n-butanol fraction were assayed using the contractions caused in the guinea pig ileum, by increasing doses of histamine.

TABLE 8:

Anaphylactic contractions in ileal pieces obtained *in vitro* in the presence and absence of hydrolysed n-butanol fractions. Values are expressed as per cent maximum contractions caused by 200 ng/cm^3 histamine in the same tissue.

ANTIGEN DOSE ($\mu g/cm^3$)	CONTRACTIONS (% max)		
	Control (no extract)	40 $\mu g/cm^3$ sample	400 $\mu g/cm^3$ sample
0.1	0	0	0
1.0	13.0 ± 3.0	16.0 ± 3.0	5.0 ± 3.0
10.0	72.0 ± 5.0	67.0 ± 5.0	22.0 ± 4.0
100.0	80.0 ± 6.0	76.0 ± 6.0	50.0 ± 6.0
1000.0	64.0 ± 6.0	62.0 ± 5.0	46.0 ± 6.0

Fig. 12 DOSE – RESPONSE CURVES SHOWING IN VITRO EFFECTS OF HYDROLYSED n-BUTANOL FRACTION OF THONNINGIA SANGUINEA ON ANAPHYLACTIC CONTRACTIONS OF GUINEA PIG ILIA.
(Each point is a mean of determinations in 5 animals, bars indicate the standard errors)

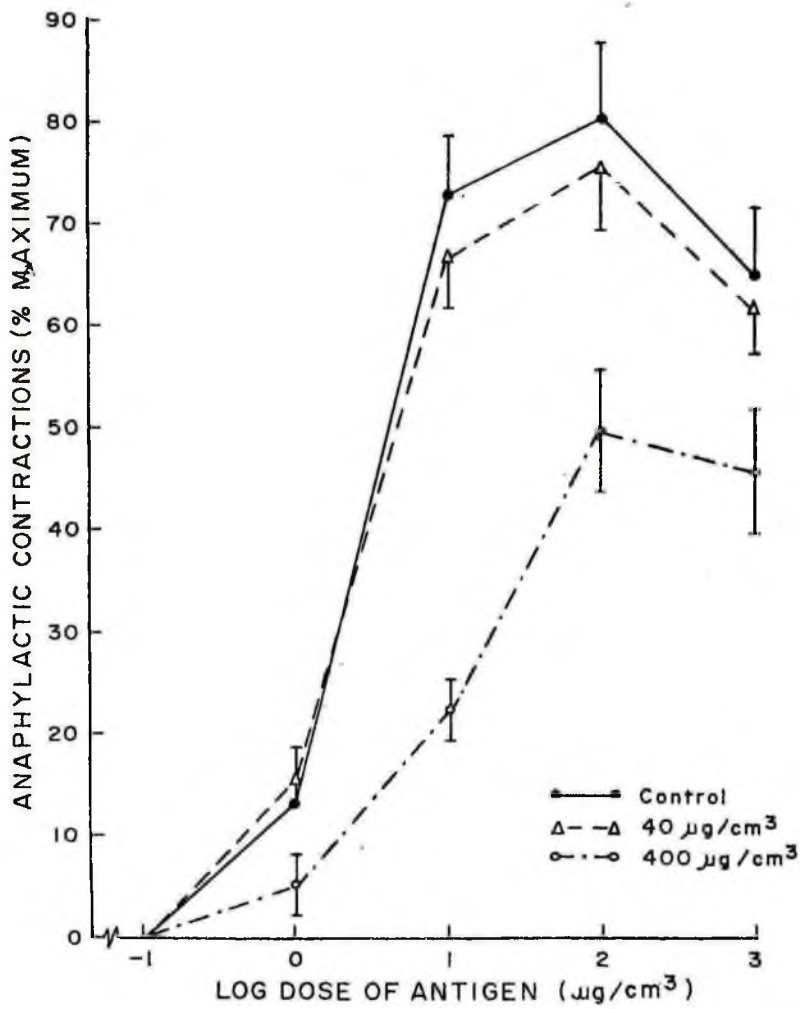
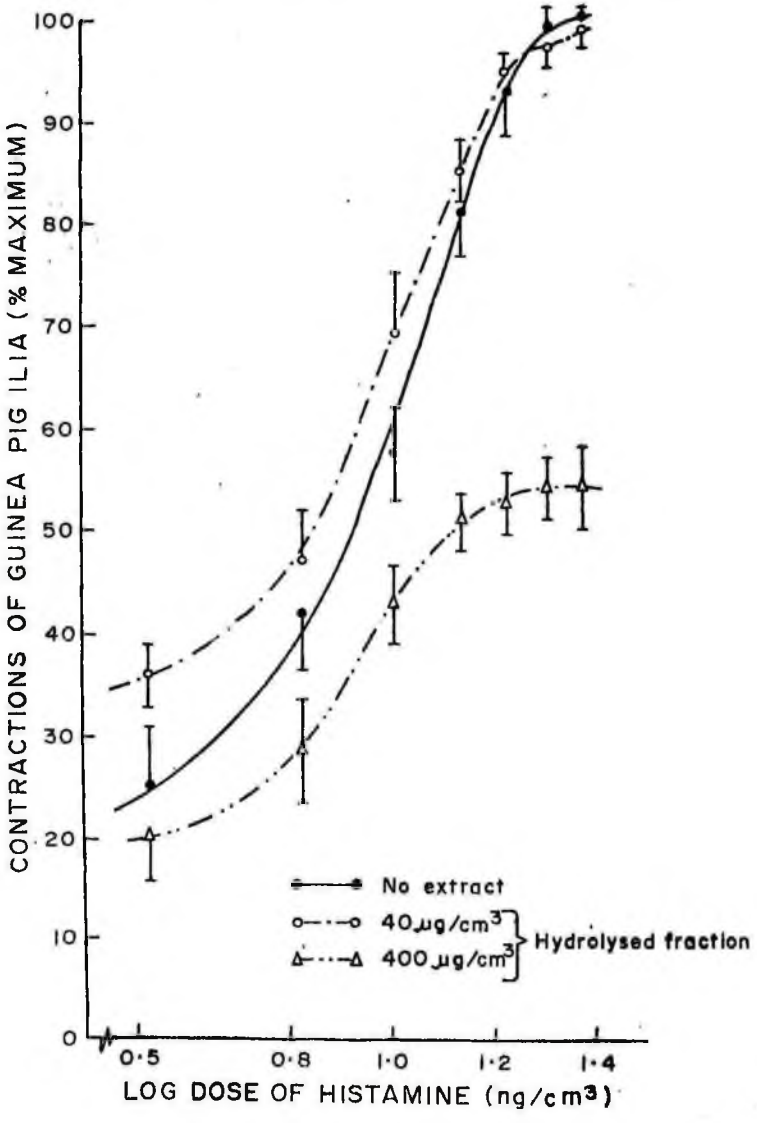


TABLE 9:

Histamine-induced spasms in ileal pieces measured in the presence and absence of the hydrolysed n-butanol fraction. Contractions are expressed as per cent maximum contractions caused by 200 ng/cm^3 histamine.

HISTAMINE DOSE (ng/cm^3)	CONTRACTIONS (% max)		
	Control (no extract)	40 µg/cm^3 sample	400 µg/cm^3 sample
3.41	25.0 ± 6.0	36.0 ± 3.0	20.0 ± 4.0
6.82	41.0 ± 5.0	47.0 ± 5.0	39.0 ± 5.0
10.23	58.0 ± 5.0	69.0 ± 5.0	43.0 ± 4.0
13.65	81.0 ± 4.0	85.0 ± 3.0	51.0 ± 3.0
17.04	93.0 ± 4.0	95.0 ± 2.0	53.0 ± 3.0
20.45	99.0 ± 2.0	97.0 ± 2.0	54.0 ± 3.0
23.86	100.0 ± 2.0	99.0 ± 2.0	54.0 ± 4.0

Fig. 13 DOSE – RESPONSE CURVES SHOWING THE IN VITRO EFFECTS OF HYDROLYSED n-BUTANOL FRACTION OF THONNINGIA SANGUINEA ON HISTAMINE – INDUCED CONTRACTION.
(Each point is a mean of determinations in 5 animals, bars indicate the standard errors)



The data are shown as dose-response curves in Figure 13. The graph shows that when lower histamine doses were applied to the tissues in the presence of 40 $\mu g/cm^3$ of the hydrolysed sample there were potentiation of the contractions caused. This effect of the lower dose of the plant material is quite significant, especially at very low doses of histamine. The converse was true for contractions observed in ileal tissues in the presence of 400 $\mu g/cm^3$ of the hydrolysed sample. Contractions in this case were significantly suppressed by the higher concentration of the hydrolysed material.

The ED_{50} was estimated for each of the three curves in Figure 13. These were found to be (9.0 ± 1.0) ng/cm^3, (7.1 ± 0.8) ng/cm^3 and (13.0 ± 1.0) ng/cm^3 histamine for contractions observed in the absence of plant material, in the presence of 40 $\mu g/cm^3$ and 400 $\mu g/cm^3$ hydrolysed samples respectively. The 400 $\mu g/cm^3$ sample was found to render the ileal pieces less sensitive to histamine ($p < 0.05$).

3.3 QUALITATIVE ORGANIC ANALYSIS:

The aqueous extract of T. sanguinea was dark-brown in colour and foamed readily suggesting the presence of saponins or related substances. Solid material was observed to be deposited after the extract had been left to stand undisturbed for about 2 hours.

The n-butanol fraction was also dark-brown and very viscous. The hydrolysed n-butanol sample contained a brown solid material. The aqueous phases left after the butanol extraction of the crude extract, and after hydrolysis of the butanol fraction were brown and colourless respectively.

Solubility tests performed on the solid material showed that the sample was insoluble in water, an indication that it was a hydrophobic organic substance. This sample was also found to be insoluble in 1 M HCl. The solid material was therefore not basic. It was found to be soluble in 1 M NaOH forming a brown solution. This suggested that the sample was acidic in its nature. The sample, however, did not dissolve in 1 M $NaHCO_3$. This indicated that the sample was a weak acid, suggesting possibly the presence of a phenol, an enol, an oxime or an amino acid.

The identification test with ferric chloride yielded a dirty-green solution. This was an indication of the presence of a phenolic or a poly-hydroxy substance with a ferric reacting group as well as the presence of flavonoid or flavonol substances (Geissman, 1955).

When a sample of the hydrolysed material was used in azo-dye formation, a dark reddish-brown precipitate was formed at the bottom of the test-tube leaving a uniform yellowish-brown suspension. The above results confirmed the presence of a phenol or a polyhydroxy phenolic substance.

Tests for amino acids, oximes and saponins did not indicate their presence. The hydrolysed butanol sample is probably a polyhydroxy phenolic or enolic substance possibly steroidal in nature.

3.4 CHROMATOGRAPHY OF THE EXTRACTS:

Out of the three solvent systems used, viz:

(i) n-butanol-acetic acid-water (40:10:50 v/v)

(ii) water-methanol- n-butanol (3:3:4 v/v)

(iii) chloroform-methanol-water (65:35:10 v/v) ,

the first two were observed to effect good resolution of spots on TLC with Silica gel G as the adsorbent. The third solvent was found to be better for paper chromatography.

After development of the thin layers in the appropriate solvent system, location of resolved spots was done visually under visible light as well as long wavelength ultraviolet light. The results obtained when TLC was used are summarised in Table 10.

The bright green fluorescence under UV light suggests the presence of flavonol compounds, while the brown spots suggest the presence of flavonol-glycosides, anthocyanins or related substances (Geissman, 1955).

TABLE 10:

R_f values and corresponding colours of resolved components of T. sanguinea samples, separated using TLC and viewed under UV light.

Sample	n-butanol-acetic acid-water		water-methanol-n-butanol	
	R_f	Colour	R_f	Colour
Crude aqueous extract	0.10	brown	0.27	brown
	0.12	blue	0.43	brown
	0.17	brown	0.55	yellowish green
	0.21	yellowish green	0.66	yellow
	0.27	brown	0.77	blue
	0.69	blue		
	0.78	light brown		
	0.91	dark brown		
n-Butanol fraction	0.15	brown	0.30	brown
	0.33	yellowish green	0.45	blue
	0.57	yellow	0.53	yellow
	0.70	yellow	0.67	yellow
Hydrolysed n-butanol fraction	0.84	green	0.94	green
	0.97	yellowish green		
Aqueous phase after butanol extraction	0.25	blue	0.31	blue
	0.56	brown	0.46	green
	0.76	yellow	0.78	yellow
	0.92	green		

Figure 14 also shows a typical densitometer scan of the crude aqueous extract of Thonningia on TLC developed in solvent system I.

Table 11 shows the results obtained when paper chromatography was used. The colours of the resolved components after treatment with the indicated detecting agents, which are specific for phenolic and enolic steroids, show that the various fractions of Thonningia extract contain steroidal substances which are either phenolic, enolic or generally ferric reacting in nature.

Treatment of the chromatographs with acid fluorescence reagents such as 20% phosphoric acid and 25% trichloroacetic acid in chloroform, with the view of detecting the presence of sapogenins, did not effect any changes in the observed UV fluorescence.

All but the hydrolysed n-butanol fraction showed dark-brown origins on the paper and thin layer chromatograms after development. These indicated the presence of substances that are immobile in the various mobile phases or substances that strongly adsorbed onto the stationary phase. Likewise, no spots were observed either on paper or on thin layer for the aqueous phase after hydrolysis. The origin of this fraction rather showed intense bright blue fluorescence under UV light.

Fig. 14 A DENSITOMETER SCAN OF THE CRUDE AQUEOUS EXTRACT OF THONNINGIA SANGUINEA SEPARATED ON TLC, USING THE SCHIMADZU DUAL WAVELENGTH CHROMATOGRAM SCANNER.
(Model CS - 900) $\lambda s = 505$ nm; $\lambda r = 660$ nm;
Sensitivity = x 50; Scan speed = 40 mm / min.
Solvent system: n - butanol - Acetic acid - water
(40 : 10 : 50 v/v)

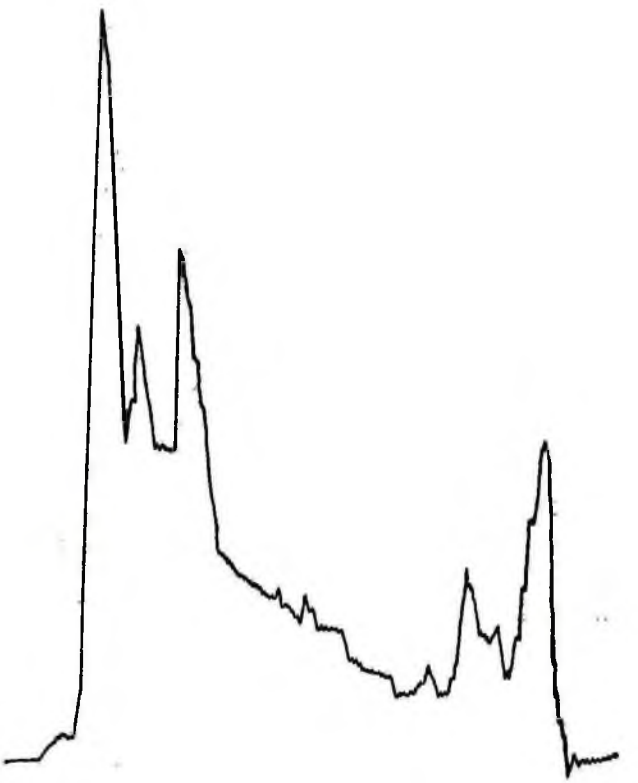


TABLE II:

R_f values and corresponding colours of resolved components of T. sanguinea samples, separated using paper chromatography. Spots were located under UV light and with the aid of detecting agents.

SAMPLE	UV (Long)		$FeCl_3$ in methanol		$FeCl_3$-$K_3Fe(CN)_6$ System	
	R_f	Colour	R_f	Colour	R_f	Colour
Crude aqueous extract	0.23	brown	0.08	light blue	0.13	deep blue
	0.27	yellow	0.33	blue-black	0.27	deep blue
	0.32	yellowish green	0.52	" "	0.53	" "
	0.40	brown	0.83	black	0.56	" "
	0.51	light-blue	0.98	"	0.83	" "
	0.82	blue			0.94	" "
	0.91	bright-blue				
n-Butanol fraction	0.52	light-yellow	0.51	black	0.50	deep blue
	0.57	green	0.59	dark-brown	0.61	" "
	0.94	blue	0.94	blue-black	0.96	" "
	0.98	blue				
Hydrolysed n-butanol fraction	0.96	green	0.99	blue-black	0.99	deep blue
Aqueous phase after butanol extraction	0.36	blue	0.30	brown	0.31	light blue
	0.45	green	0.37	light brown	0.38	" "
	0.68	blue	0.70	blue-black	0.77	deep blue

CHAPTER FOUR

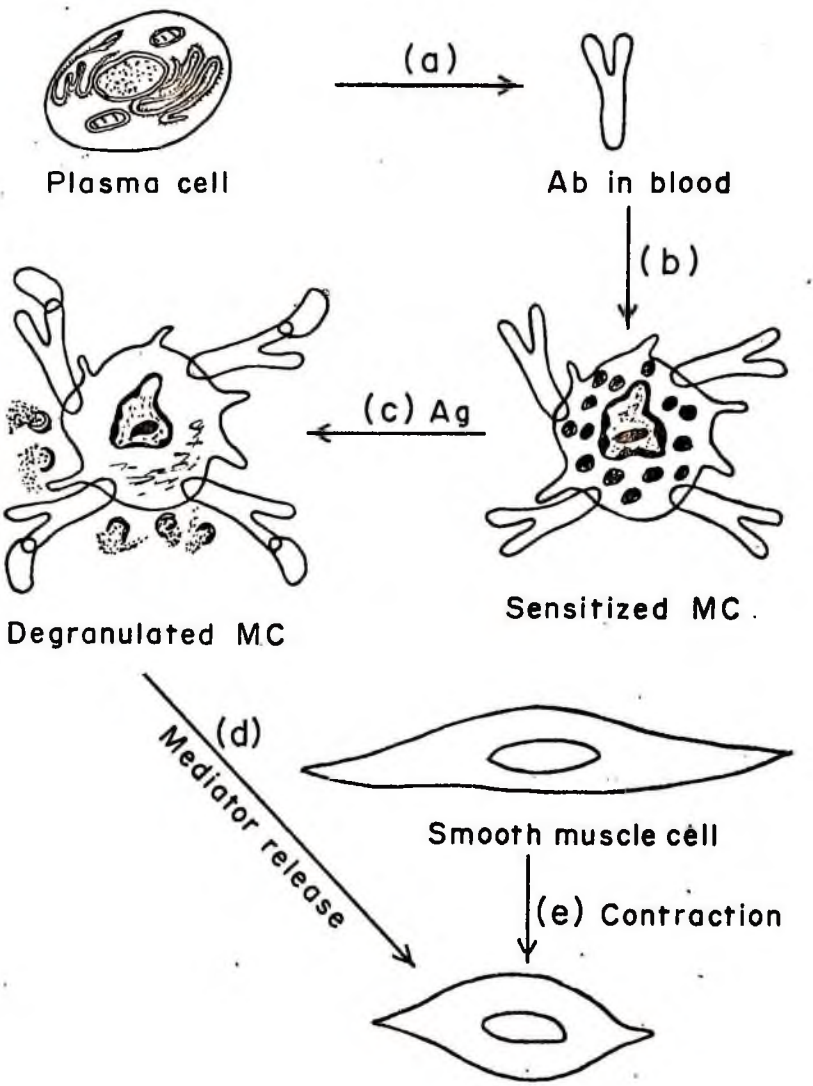
D I S C U S S I O N

The modified Schultz-Dale reaction enabled antigen dose-response curves to be used to evaluate the effect of the crude extract of *T. sanguinea*, a plant used in the treatment of asthma, on anaphylaxis. The results showed a reduction in the anaphylactic contractions recorded in the sensitized isolated guinea pig ileum when the crude extract was administered orally to guinea pigs (Figure 3).

Steps involved in the immunological component of asthma and anaphylaxis include: (a) synthesis of reagins or reagin-like antibodies; (b) fixation of reagins to mast cell surfaces; (c) formation of mast cell-bound-reagin-antigen complex; (d) degranulation of mast cell and mediator release; and (e) contraction of smooth muscle cells, (Figure 1). Considering the results represented in Figure 3, one could only state that the plant material is anti-anaphylactic. It seems quite difficult to identify the step at which the plant material effected its observed action.

The anaphylactic responses elicited in the ileal tissues, in the antigen dose-response curves, discussed above, could be attributed to only histamine and serotonin; this is because the contact time of antigen with

Fig. 15 ASTHMA: Immunopharmacology.
(a) Plasma cells synthesise reagins, (b) fixation of reagin to MC, (c) formation of MC-bound Ab-Ag complex, (d) degranulation of MC and mediator release and (e) contraction of smooth muscle cell. (Barret, 1975)



the tissue was less than 4 minutes in which case only histamine and serotonin could have been released (Marquis, 1973).

Histamine dose-response curves are shown in Figure 4. When the law of mass-action is applied to dose-response curves, an observed biologic effect is assumed to be a reflection of the combination of drug molecules with receptors much as the rate of appearance of products in enzyme reactions reflects the degree of combination of substrate molecules with active site of enzymes (Goldstein et al. 1974).

The estimated ED_{50} for each curve could be said to be equivalent to K_m, the Michaelis constant in enzyme kinetic reactions, while the maximum response recorded could be equated to Vmax - maximum velocity in enzyme reactions. From Figure 4, it could be seen that ED_{50} and hence the Km, a measure of the affinity of agonist for the receptor, was found to be significantly larger when the animals were treated with the plant extract compared with those drinking water. The extract when administered orally to the animals therefore affected the Km, which is an intrinsic property of the tissues, such that the effective affinity of the agonist (histamine) for its receptors was significantly lowered. Unlike the Km however, the

Vmax - maximum response of the tissues at "infinite" agonist concentration, remained unchanged for tissues obtained from sensitized animals drinking water and those drinking the crude aqueous extract.

The observed affects of the plant material could also have occurred through an allosteric type of reaction. In this case a potential inhibitor or drug binds to an allosteric site - away from the specificity or binding site for an agonist. The binding then induces a conformational change in the enzyme or tissue to depress its efficiency or effect.

The results discussed above provide evidence to suggest the level at which the plant material effected its action in the results presented in Figure 3. The results in Figure 4 showed that the plant material interferred with contraction of smooth muscle cells. Such interference could occur either at the level of stimulus induction or response in the affector system. The interference is likely to be in the effector system because the crude extract was administered orally to the animals. The significance of the results discussed above is that even if histamine was released in the anaphylactic reaction its effect on smooth muscles would be greatly reduced if the patient was already on the plant preparation.

The pathological state caused by mediators released during anaphylactic shock depends on the shock organ. It was therefore necessary to determine the quantity of such mediators released from the lung tissue, and the effect of this anti-asthmatic plant preparation on them.

The results represented in Figures 6 and 9 showed that the quantity of spasmogens released anaphylactically, expressed as amount of histamine per gramme wet weight of lung tissue obtained from animals drinking Thonningia extract was about one half the quantity released from lungs obtained from animals drinking water. The bulk of these spasmogens could be assumed to be histamine in view of the antigenic challenge time of 10 minutes in the bioassays. Anaphylactic release of histamine reaches a peak within 5 minutes (Marquis, 1973). The time course of histamine release makes serotonin the probable contaminant, however, the contribution of this mediator was greatly reduced or totally excluded especially in the fluorometric determination. In this assay, the operating parameters, excitation and emission wavelengths of the molecules, were specific for histamine.

It could be seen that the results obtained when histamine was determined fluorometrically and that obtained when the non-sensitized guinea pig ileum was used were quite comparable. It could thus be reasoned that the contractions, observed when ileal pieces were used, were due to histamine with little or no contribution from the other mediators.

Plasma levels of histamine in normal and asthmatic children have been reported to be 9 µg/dl and 17 µg/dl respectively (Sokolova, 1977). If these were spill-overs from target tissues then it would be reasonable to suppose

that the quantity of histamine in target tissues of asthmatics is higher than the quantity in normal individuals. Taking this and Figures 6 and 9 into consideration, the results suggested that when Thonningia extract was administered orally, it was capable of preventing histamine release to such an extent that the quantity released was no longer pathological. On the total histamine content of lung tissues, the results suggested that oral administration of the crude extract of Thonningia did not only affect the amount of histamine released anaphylactically, but also the synthesis or breakdown of the amine in such a way that the amount present in the tissues was significantly reduced.

One important spasmogen which significantly contributes to contractions of smooth muscles during asthmatic attacks is SRS-A. It is probable that the contractions elicited in the non-sensitized quinea pig ileum in the presence of mepyramine and atropine (10 µg/l each) were due to SRS-A or an SRS-like material released from the lung tissues. This is because in the bioassays, antigenic challenge of the lung tissues were done for 25 minutes whereas the release of SRS-A reaches its peak in

20 minutes. Similarly, the release of bradykinin reaches a peak in 15 minutes (Marquis, 1973). Despite the challenge time of 25 minutes, the observed contractions would not be attributed to bradykinin. The release of bradykinin unlike the other mediators is dependent on plasma, and in order to either eliminate or significantly reduce its contribution to the observed contractions, the lung tissues were washed free of blood prior to antigenic challenge. The contractions elicited in the ileal pieces were therefore more likely to be due to SRS-A or a related material and possibly serotonin to some extent.

The in vitro assays performed provided a faster and convenient way of evaluating the effect of the crude aqueous extract and fractions from it on anaphylaxis. The fractions isolated from the crude aqueous extract were also too small to be administered orally. The in vitro assay system therefore served as a convenient way of screening those isolated fractions.

It could be seen from the results that the crude extract as well as the n-butanol extract when assayed in vitro interferred with the anaphylactic contractions in the guinea pig ileum whereas the aqueous phase left after the n-butanol extraction did not exhibit such interfernces. A similar

interference was also observed when the hydrolysed n-butanol sample was assayed *in vitro*, (Figure 12). The dose-response curves of these reults shown in Figures 10 and 12 are identical to that in Figure 3, when the animals drank the crude aqueous extract. It is most probable that the anti-anaphylactic effect exhibited by the various samples *in vitro* were achieved through non-specific effects of the chemical substances present in these samples. The non-specific effects possibly included membrane perturbations in the ileal pieces. The results of such perturbations could be an interference with one or a combination of some of the steps immedidately preceding muscle contraction in anaphylaxis.

In the chemical analysis of the hydrolysed n-butanol fraction and the butanal fraction, the presence of substances with hydroxy or poly-hydroxy groups were indicated. The presence of substances which could possibly be steroidal was confirmed by the detecting agents used in the paper chromatography. The azo-dye test also confirmed the presence of these chemical substances. The functional groups of these chemical substances would interact with tissue surfaces non-specifically

to block or modify regions containing receptors for mediators of anaphylaxis or mast cell-antibody aggregates. The result would be a decrease in the anaphylactic response in the tissue either due to an inability to form mast cell-bound antibody-antigen complex leading to low mediator release, or even if the above complex was formed, an inability of the mediator released to interact with its receptors leading to low responses in the tissues. If the plant materials act this way in vitro, then it would be interfering with the anaphylactic contractions at the level of stimulus induction.

The in vitro assay of the crude extract and fractions from it affected the histamine-induced spasms observed in a non-competitive manner. The results represented in Figure 11 showed a reduction in the maximum response obtainable at "infinite" histamine concentration without any significant change in the affinity of the agonist (histamine) for active sites on the tissues. These observations are quite typical of non-competitive type of inhibition. In this case, it could be said that, the inhibitor (plant preparation) was either not directed to the active or specificity site on the tissue, or bound irreversibly at the active site such that there was no competition between it and histamine.

In vitro assay of the hydrolysed butanol sample also demonstrated a non-competitive type of inhibition. In addition the dose-response curve

obtained in the presence of the hydrolysed sample (400 $\mu g/cm^3$) (Figure 13), suggested the presence of "spare or reserve-receptors" for histamine on the tissues (Ariens and Simonis, 1964). This phenomenon was shown as a shift to the right, (by the curve obtained in the presence of 400 $\mu g/cm^3$ of the hydrolysed sample in vitro) which preceded the decline in the observed maximum response (Figure 13). The nature of the curves is also typical of an inhibitor which interferes with induction of stimulus (Ariens, Simonis and van Rossum, 1964).

Potentiation of the histamine-induced spasms observed at lower histamine concentrations in the presence of 40 $\mu g/cm^3$ of the hydrolysed butanol sample could be attributed to a displacement of histamine from "silent-receptors" - sites at which drugs may compete for inert adsorption (Ariens and Simonis, 1964). The result of this displacement could be an increased response in the biologic effect observed in the tissues as a result of an increase in the effective conentration of the exogenous histamine in the organ bath. It would be recalled that prior to the dose-response experiments, the tissues were stimulated with large doses of histamine (200 ng/cm^3). It follows then that a saturation of histamine receptors would result in excess histamine in the assay bath binding to silent receptors without exhibiting any biologic effect.

The in vitro assays, as had been said earlier, were adopted in an attempt to find a faster and convenient method of assaying the crude extract and the smaller quantities of fractions isolated from it. The results obtained,

compared to the ones obtained when the crude extract was orally administered, indicated that the mechanism of action, of the plant material involved in the two assay systems, are different. When histamine-induced spasms were used to evaluate the effect of the plant material, the crude extract caused a significant change in the ED_{50} without any change in the V_{max} when the extract was administered orally. The inhibition of contractions in the ileal pieces in this case appeared to be in the effector system. This was not so in the case of the *in vitro* assay of the plant extracts. The V_{max} observed in each case was significantly altered and the inhibition appeared to be at the level of stimulus induction. Furthermore, the histamine dose-response curves obtained in the two assay systems were not identical. However, when anaphylactic contractions were used to evaluate the effect of the plant extracts, the antigen dose-response curves obtained when the two assay systems were used were identical.

Since all *Thonningia* preparations are administered orally to asthmatic patients, it seems that *in vitro* assays involving histamine-induced spasms would not be useful when evaluating fractions isolated from the plant extract. However, the antigen dose-response curves obtained when the plant extract was administered orally and when assayed *in vitro* appeared to be identical. Therefore, for a quicker way of evaluating fractions, an *in vitro* assay system, making use of anaphylactic contractions in the

guinea pig ileum, could be employed meaningfully, before the oral administration of the potentially effective fraction.

Comparing the results discussed here/those reported on D. adscendens (section 1.8), it could be inferred that the therapeutic effect observed, for the two herbal preparations, is possibly the results of a similar mechanism of action. At CSRPM it has been reported that best clinical results were obtained when T. sanguinea preparation was administered in combination with D. adscendens. The available information does not suggest a synergistic type of interaction between the two herbal preparations.

The presence of saponins or sapogenins could not be indicated, because of the unavailability of reagents which are more specific for their determination. However, their presence could not be excluded. In addition to the chemical substances discussed earlier, the presence of flavonoid, flavonol, anthocyanins and related substances were suggested.

The densitometer scan of the crude extract of Thonningia on TLC, (Figure 14), could be used as a "fingerprint" for an aqueous preparation of T. sanguinea when treated as specified in this report. Furthermore, this "fingerprint", together with the number of resolved spots, their R_f values and corresponding colours, could be used to standardize the quality of the plant material when fresh samples are obtained, in future, from the field.

CONCLUSION

1. The work reported in this thesis indicates that *Thonningia sanguinea* is anti-anaphylactic when administered orally and when fractions of it are assayed *in vitro*.

2. The anti-anaphylactic property which is probably responsible for the therapeutic action or observed clinical effects of *Thonningia sanguinea* is due to the plant's interference with:

   i. the steps leading to the release of pharmacological mediators of anaphylaxis;

   ii. either the synthesis or break-down of histamine in lung tissues thereby causing levels of this mediator to be significantly reduced; and

   iii. contraction of smooth muscle.

3. The results also suggest a similar mechanism of action for both *Thonningia* and *Desmodium adscendens*.

SUGGESTIONS FOR FURTHER WORK

The results of the investigations as reported give some indication about the mechanism leading to the therapeutic action of T. sanguinea. There is still the need for further investigations to be carried out on the plant preparation in order to establish concretely the definite mechanism of action of the plant material. To this end the following suggestions for further work are made:

1. The aqueous extract when administered orally inhibited the anaphylactic reaction. The extract could have affected the quantity of reagins or reagin-like antibodies produced. This would mean a reduction in the effective concentration of mast cell bound-antibody complex formed and therefore a reduction in the quantity of mediators released upon antigenic challenge. There is therefore the need to measure the levels of reagins or reagin-like antibodies in sensitized animals drinking the plant extract and those drinking water.

2. When the crude aqueous extract was administered orally, it reduced the anaphylactic release of autocoids. Since mast cells serve both as synthetic and storage sites for mediators, and play an important role in the anaphylactic reaction, there is the need

to make use of an assay system involving mast cells in order to evaluate the effect of the plant material on autocoid release from these cells.

3. Oral administration of the crude extract has been shown to render ileal pieces less sensitive to histamine. It is therefore necessary to find out whether the plant preparation generally affects all types of smooth muscles e.g. uterus, tracheal and lung parenchymal tissues. The lack of selectivity for the type of smooth muscle may be detrimental to some patients who might be on this preparation constantly.

4. Oral administration of the crude extract was shown to reduce the histamine content in sensitized lung tissues. There is therefore the need for the activities of the enzyme L-histidine decarboxylase as well as histaminases from sensitized animals drinking the plant extract and those drinking water to be studied.

5. Finally, there is the need for a thorough chemical and chromatographic studies to be made on the chemical constituents of Thonningia.

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A P P E N D I X

A.1 Sterile physiological saline solution:

The above sterile solution (0.9%) was prepared by dissolving 9.0 g sodium chloride in a litre of glass-distilled water. The resultant solution was autoclaved under a pressure of 15 lb/sq. in. for 15 minutes. The sterile solution obtained was kept in a refrigerator in a clean, well stoppered bottle for subsequent use.

A.2 Tyrode's physiological solution:

One litre of the above physiological solution was prepared according to the reagents and table below:

Reagent	Concentration	Quantity per litre
NaCl	-	8.0 g
$NaHCO_3$	-	1.0 g
$NaH_2PO_4.2H_2O$	5.0 %	1.3 cm^3
KCl	10.0 %	2.0 cm^3
$MgCl_2$	10.0 %	2.6 cm^3
$CaCl_2$	1 M	1.8 cm^3
Glucose	-	1.0 g

The volume of the above mixture was made up to the litre mark by adding glass-distilled water. Calcium chloride was always added last to avoid precipitating poorly soluble calcium salts. Tyrode's solution was always prepared fresh, from the indicated reagents, before use.

A.3 Citrate buffer:

Citrate buffer 0.1 M, and pH 4.2 was prepared from (i) citric acid 0.1 M and (ii) trisodium citrate 0.1 M. A 100 cm^3 of the buffer was prepared by mixing 54 cm^3 of the citric acid solution with 46 cm^3 of the trisodium citrate solution.

The buffer and reagents used in its preparation were stored in a refrigerator. The pH of the buffer was always checked on a pH meter before use.

A.4 Antigen:

(i) For sensitization purposes, the antigen, 100 mg/cm^3 and 5 mg/cm^3 egg albumen was prepared in physiological saline solution.

(ii) For challenge purposes, the antigen, 1 % egg albumen was prepared in Tyrode's physiological solution.

A. 5 Ferric chloride-ferricyanide reagent:

This reagent was always prepared fresh before use by mixing equal volumes of aqueous solution of ferric chloride (1 %) and aqueous solution of potassium ferricyanide (1 %).