IMMUNOGENIC AND SOME PHARMACOLOGICAL ACTIVITIES OF CRUDE EXTRACTS OF CASSIA TORA LINN (FAMILY - CAESALPINIACEAE)

BY

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DECLARATION

This is to certify that I carried out the work reported in this thesis in the Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaira, under the joint supervision of Dr. (Mrs) H. O. Kwanashie, Prof. K. S. Gamaniel and Prof. J. O. Adekeye.

The works of other investigators are acknowledged and referred to accordingly.

I solemnly declare that no part of this thesis has been submitted elsewhere for a degree.



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THESIS APPROVAL

This project report/thesis/dissertation entitled Immunogenic and some Pharmacological Activities of Crude Extracts of *Cassia tora* Linn. [Family - Caesalpiniaceae] by Chidume, Florence Chimezie meets the regulations governing the award of the degree of Master of Science in Pharmacology of Ahmadu Bello University, and is approved for its contribution to knowledge and literacy presentation.

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EXTERNAL EXAMINER

DEDICATION

To

Heavenly Father

and

All my Dear ones

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My profound appreciation and gratitude I give to my very able supervisors, Dr. (Mrs) H. O. Kwanashie, Prof. K. S. Gamaniel and Prof. J. O. Adekeye for their wonderful guidance, understanding, patience and encouragement.

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This appreciation will not be complete without my acknowledging Him for whom I am. I therefore give all the glory, all the honour, all the adoration to God, our heavenly Father.

ABSTRACT

Cassia tora Linn (family - Caesalpiniaceae) is a shrub widely used ethnomedically in Africa for impetigos, ulcers, helminthiasis and as a purgative.

In the present study, C. tora leaves were soxhlet extracted with methanol and the yield was 34% w/w. C. tora seeds were extracted with normal saline and the supernatant was tested on human, cow and goat blood for agglutination. The methanolic leaf extract was studied for its effect on immune response, acute toxicity, anti-inflammatory and analgesic activities in mice and rats. The effect of the methanolic extract was also tested on smooth muscles of guinea pig ileum and rabbit jejunum. The antimicrobial activity of the methanolic extract against some bacteria was also tested.

Methanolic extract of C. tora leaf in low doses (100 - 200 mg/kg i.p.) showed a significant ($P < 0.05$) increase in mice total leucocyte count. At the same dose levels, lymphocytes were significantly ($P < 0.05$) increased while neutrophils were significantly ($P < 0.05$) reduced. At high dose of 400 mg/kg i.p., no significant difference was seen in the mice total and differential leucocyte counts.

The methanolic extract (25 - 200 mg/kg i.p.) caused a significant increase ($P<0.05$) in the induration induced by 5% (v/v) suspension of sheep red blood cell in mice foot pads. The highest effect was seen with the highest dose used (200 mg/kg).

C. tora leaf extract (50 - 200 mg/kg i.p.) did not cause any significant difference on the antibody litre of 10 mg/ml egg albumin (1 ml x 3 s/c) -

sensitised rats. However, egg albumin (0.025 ml) was precipitated by an equal volume of *C. tora* extract in the absence of antisera.

Seed extract of *C. tora* (0.5 ml) non - specifically agglutinated all the human ABO blood (0.5 ml) at different temperatures (4, 25 and 37°C). However, in goat and cow, it agglutinated some blood and did not agglutinate others (0.5 ml each).

No obvious pharmacological effect such as dizziness, excitement, nervousness or even death was seen in mice treated with *C. tora* leaf extract (10 - 2000 mg/kg i.p. and p.o.). No changes in weight were observed in mice treated for 8 days with 25 - 200 mg/kg of the extract, i.p. and p.o.. The extract (100 - 200 mg/kg i.p.) showed a significant ($P < 0.05$) anti-inflammatory activity on egg albumin - induced oedema in rats, as did aspirin (150 mg/kg i.p.).

C. tora extract (200 - 400 mg/kg i.p.) significantly ($P < 0.05$) reduced the number of writhes due to glacial acetic acid - induced pain (chemical pain) in mice and was comparable to aspirin (150 mg/kg i.p.). The extract (50, 100 mg/kg i.p.) also significantly ($P < 0.05$) increased the ability of mice to bear force-induced pain (mechanical pain) confirming its analgesic activity which was dose-dependent.

C. tora leaf extract (0.04 - 25.6 mg/ml) exhibited contraction of smooth muscles of guinea pig ileum in a concentration-dependent manner. Atropine (0.04 µg/ml) reversibly blocked the contractile effect of the extract (0.8 mg/ml). Also, in a concentration - dependent manner, mepyramine (0.8 - 3.2 u,g/ml) remarkably reduced the contractile amplitude due to *C. tora* extract (1.6 JLig/ml).

The methanolic extract (0.04 - 2.56 mg/ml) caused a transient relaxation followed by an obvious concentration - dependent contraction of the smooth muscle of rabbit jejunum. Atropine (0.04 µg/ml) also reversibly blocked the contractile effect of 0.32 mg/inl of the methanolic extract on rabbit jejunum. Similarly, mepyramine (1.6 - 16.4 µg/ml) in a concentration-dependent manner blocked the contraction due to the extract (1.28 mg/ml).

The methanolic extract of *C. tora* leaves (10 - 250 mg/ml did not show antimicrobial activity against *Staphylococcus aureus, Salmonella typhi, Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa.*

These results generally suggest that *C. tora* extract has a potential of being a good immunogenic, purgative, anti-inflammatory and analgesic agent and may explain why it is widely acceptable in ethnomedical practice.

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GLOSSARY OF ABBREVIATIONS

i.p.	-	intraperitoneal
p.o.	-	per os
p.s.i.	-	pound per square inch
M.I.C.	-	minimum inhibitory concentration
M.B.C.	-	minimum bactericidal concentration
N.C.T.C.	-	national centre for type cultures
A.T.C.C.	-	American type collection center
W.B.C.	-	white blood cell
R.B.C.	-	Red Blood Cell
S.R.B.C.	-	sheep red blood cell
S.E.M.	-	standard error of mean
P.H.A	-	Phytohaemagglutinin
DNA	-	Deoxyribonucleic Acid
GMP	-	Good Manufacturing Practice
CT-B	-	Cholera Toxin – B
LT-B	-	Heat-labile enterotoxin B submit
DTH	-	Delayed Type Hypersensitivity
AIDS	-	Acquired Immunodeficiency Syndrome
SRS-A	-	Slow Reacting Substances of Anaphylaxis

CHAPTER ONE

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1.0 INTRODUCTION

1.1 The Body immune system

1.1.1 Immunity

Immunity is the state of a living organism whereby it resists or is non-susceptible to infection (Zink, 1980). Reactions that take place between infecting micro-organism and the host are quite complex, and the degree and speed with which the invaders may do harm depend entirely on the resistance or lack of resistance of the host. It is not possible to differentiate sharply between states of immunity and of susceptibility since these terms are relative.

The virulence of invading bacteria, viruses or fungi, and the level of resistance offered by body tissues and cells are of utmost importance in determining the outcome of an infection. With the development of an adequate level of immunity, the host adapts itself to the presence of foreign proteins, displays marked resistance to the invasive powers of infecting micro-organisms, and is capable of protecting itself against the deleterious action of their toxic products (Zink, 1980; Ivan, 1988).

It is worth noting that in some individuals, an infection may be so mild that the disease never reaches the clinical stage (subclinical infection). Antibodies however, may be produced, and in sufficient amount so that the individual will become immune to possible future infection by the same species of micro-organisms.

In routine immunological studies, reports have been made of presence of protective antibodies to a variety of infections in individuals who have never

had a clinically recognised case of the disease in question. This is the case particularly, with many of the viral diseases including HIV/AIDS. The degree of immunity possessed is the determining factor in successful resistance against future exposures. It has been shown that a level of immunity can be entirely effective against a moderate contact with a given disease, yet become ineffective in the event of exposure to an overwhelming dose of infective organisms. Other factors, relating to the general level of health contribute, along with specific immune substances, to the resistance offered against invading pathogenic organisms.

The immune mechanism involves the presence of an antigen (immunogen) which provokes the production of antibody molecules or sensitise lymphocytes depending on the nature of the stimulating antigen. This results to 'humoral' and 'cellular' immunity respectively. The criteria for what constitutes an antigen are dependent not only upon the individual source or chemical configuration but the species or biologic relationship of the animal being stimulated (Karush, 1962).

1.1.2 Antibody and Humoral Immunity

Antibodies can be defined as specific serum proteins produced as a result of stimulation by an antigen. The production of antibodies is the function of the humoral or plasmacytoid division of the immune mechanism. Usually, antibodies are directed against antigens that gain access to the blood such as transfused blood cells, and some kinds of bacteria and viruses. They are capable of chemical union both *in-vivo* and *in-vitro* with the particular antigen responsible for their production.

Antibody molecules are produced in special free circulating lymphocytes upon stimulation by specific antigenic structure called the 'determinant'. These antibody - producing cells referred to as 'immunocytes' are derived from bone marrow stem cells. The cells produced by bone marrow that are destined for antibody production are called the 'B' cells. Antibodies are involved in 'Immediate Hypersensitivity Reactions' (Stewart, 1980).

1.1.3 Lymphocytes and Cellular Immunity

This involves the system responsible for rejection of organ transplants or skin graft as well as the defence mechanisms against many types of parasites and endogenous neoplastic (tumour) growths. It also relies on antigen stimulation for its activation. Small lymphocytes are the cells responsible for cellular immunity and also originate from the bone marrow stem cells and are called 'T-cells'. They have been incriminated in 'Delayed Type Hypersensivity' reactions as in Allergic Contact Dermatitis (Stewart, 1980; Ivan, 1988).

Following initial contact with a chemical compound which is capable of producing cell-mediated hypersensitivity, a process of sensitisation occurs in some individuals and in the course of a minimum of six days, lymphocytes located in lymph glands become sensitised and are released into the blood stream. Following subsequent exposure to the specific sensitiser, circulating lymphocytes accumulate at the site and dermatitis becomes manifest one to two days later at the skin site of re-exposure.

Such delay in the appearance of dermatitis at the site of the re-exposure is indicated by an alternative term for the reaction, namely 'Delayed Hypersensitivity' (Mitchel, 1975). Delayed hypersentivity may be detected by means of a 'patch test', a chemical compound is applied to the skin, by

necessity, in a non-irritant dose, and the skin site is examined 48 h later, a positive patch test reaction indicates a state of delayed hypersensitivity (Mitchel, 1975; Shough, 1980).

1.1.4 Agglutination Reactions

Agglutination involves the cross-linking of cells or large particles by antibody directed against surface antigens. This is quite unlike precipitation reaction that involves cross-linking of multivalent protein antigens by antibody. For an obvious agglutination to occur, a reasonable number of antibody links between two cells is required.

Agglutination reactions are used to identify bacteria. They have been observed with leucocytes and platelets and even with spermatozoa in certain cases of male infertility due to sperm agglutinins. They are also used to type red blood cells (Ivan, 1988).

The initial demonstration of immune haemagglutination was performed in 1900 by Ehrlich and Morgenroth (Grubb, 1951). They immunised goats with red blood cells of other goats and the resultant antibodies were capable of distinguishing the difference between the red blood cells of individual goats. These antigens were later referred to as 'Iso-antigens' or 'Allo-antigens' the type that confers individual specificity and allows one to distinguish individuals of the same species (Milgrom, 1966).

There are two agglutinable antigens A or B on the surface of the red blood cells of some individuals. This confers in these individuals natural antibodies in the serum directed against the antigen that is absent from their own cells (Landsteiner, 1901). However, some other group of individuals possess both A

and B antigens on their red blood cells and therefore have neither A nor B antibodies in their sera (Decastello, and Sturli, 1902). Also, individuals in group O were considered amorphs because they possess neither A nor B antigen. This is correct only in a relative term because their cells do possess a large amount of an antigen called 'H' antigen (Morgan and Watkins, 1948). The 'H' antigen is an important antigen as it appears to be the basic ground substance from which the A and B antigens were derived. This is why the ABO system is sometimes referred to as the ABO (H) system.

1.2 Drugs and the Immune System

Immunopharmacology can be defined as the study of the effect of drugs on the immune system of living organisms. Modification of immune function has been of great use in therapeutics. Different pharmacological agents have been known to cause either immune stimulation or suppression both of which could be useful depending on the therapeutic requirement. The usefulness of such immune related drugs cannot be overemphasized. This is because most of the side effects seen in drug therapy have been related to the pharmacological activity of the drug while about 15% of all side-effects of drugs are thought to be immune mediated (Schlumberger *et al*, 1993).

According to Stewart, (1980), an immunosuppressive drug is one that can attenuate the expression of at least one type of immune response. Some classical cytotoxic immunosuppressants include methotrexate, azathioprine and cyclophosphamide, all of which act through different mechanisms aimed at inhibiting nucleic acid synthesis thereby thwarting the stimuli for proliferation of the lymphocytes involved in immune response. This also explains why many of the immunosuppressive drugs now employed were first used in cancer chemotherapy because of their toxicity to rapidly dividing cells (Ivan, 1988;

Gilman *et al*, 1990). Great care is however needed in the use of immunosuppressants because patients on immunosuppressive agents are usually immunocompromised and they tend to be susceptible to infections (Stewart, 1980; Ivan, 1988).

Immunosuppression will be desired in conditions as allotransplantation or allografting, which is the transplanting or grafting of an organ or tissue from one person to another who does not share the same transplantation antigens. The most common allografting procedure is probably blood transfusion where unfortunate consequences of mismatching occur. Other procedures may involve solid grafts such as skin (Ivan, 1988). Other conditions where immunosuppression will be desired include auto-immune diseases which are usually associated with the development of an immune response to normal body or self tissues as in rheumatoid arthritis, nephrosis, thyroiditis, early stages of insulin - dependent diabetes mellitus etc. (Handschumacher, 1990).

Immunostimulants on the other hand describe drugs capable of increasing the resistance of an organism against stressors of variable origin. They achieve this enhancement primarily by non-specific mechanisms of action. Immunostimulants generally stimulate, in a non-antigen dependent manner, the function and efficiency of the non-specific immune system in order to counteract microbial infections or immunosuppressive states (Wagner, 1996). With respect to the mechanisms of action, immunostimulants influence primarily the humoral and cellular immune system. It has been shown that immunostimulants are effective prophylactically as well as therapeutically and must be applied at relatively low doses to achieve optimal effects. They are therefore, suitable for regulative or modulating medication i.e. for restoring body homeostasis (Wagner, 1996).

Immunostimulation has formed one of the basis for the development of prophylactic and in some cases therapeutic vaccines for conditions such as cancer (Drew, 1998), chicken pox, measles and tetanus. According to Drew (1998), cancer vaccination is therapeutic, involving attempts to activate immune responses against antigens in the tumor to which the immune system has already been exposed.

Immunostimulation has also been found to be of value for body defence against infections in individuals with defective immune responses such as premature infants, children with primary immuno-deficiency or protein malnutrition or patients on steroid treatment. Acquired Immunodeficiency Syndrome (AIDS) which is a fatal disease condition caused by a Human Immunodeficiency Virus (HIV) is also another of such immunodefective conditions (Ivan, 1988).

1.3. Plants and Medicine

Plants constitute one of the greatest resources of nature. Throughout the ages, plants have provided man with substances, which are essential for living. They include food, medicine and raw materials for the manufacture of clothing, shelter, etc. Plants have been the source of medicinal agents since the earliest times of human existence. Today, plants are still playing a significant role in the health care of a large proportion of the population in developing countries like Nigeria.

It has been estimated that about 50% of all therapeutic agents (e.g. anti-diabetic, antibiotics, hormones and alkaloids) are derived directly or indirectly from natural sources (Iwu, 1996). In 1980, the total world market value of plant - based medicines was estimated at 8 billion dollars (Hussain, 1992). In May, 1987, the fourth World Health Assembly urged member states to:

i. Initiate a comprehensive programme for the identification, evaluation, preparation, cultivation and conservation of medicinal plants used in traditional medicine; and
ii. Ensure good quality of drugs derived from medicinal plants by using modern techniques and Good Manufacturing Practices (GMP).

Furthermore, in 1989, the World Health Assembly adopted resolution WHA 42:43 in support of national traditional medicine programme and drew attention to herbal medicine as being of great importance to the health of individuals and communities. The World Health Organization, in recognition of the immense value of herbal medicine to Primary Health Care, has advocated for the proper identification, sensible exploitation, scientific development and appropriate utilisation of herbal medicines which provides safe and effective remedies.

In many countries, especially in Asia, Herbal medicine has become an integral part of the health care delivery system on the same basis as orthodox medicine (Sofowora, 1982). However, in most countries in Africa, this is not yet the case. Even in the later situation, herbal medicine is recognised as an important component of the health care system especially among the rural dwellers who constitute about 70% of the population (Sofowora, 1982). Furthermore, in both rural and urban communities, many people depend partly on herbal remedies for their primary health care needs. Even in the developed countries, the popularity of crude herbal products is on the increase (Blumenthal, 1998). In these technologically advanced societies, consumers' preference is shifting from purely synthetic to natural based drugs and this is dictating the basis for the resurgence in the utilisation of such products.

It is obvious from these realities that due to the inherent value of herbal medicine and the immense socio-economic demands for adequate pharmaceutical supplies in the rural areas, transportation difficulties, the needed expertise for the rational use of drugs, the availability and cost of these products, herbal medicine remains the most viable way to bridge the gap in medical practice.

It must however, be emphasized that important as it is, to aim at bridging the gap between the availability and the need for orthodox medicine, what has a lasting influence on our people and others in the world is self-reliance vis-a-vis development of herbal medicines, which are safe and effective.

1.4. Plant Constituents as Immunogenic Substances

1.4.1. Prospects

Man has over a long time used herbs or plant products as medicine (for developing immunity or resistance) against cold, coryza, joint pains and fever. A good number of these herbal remedies e.g. *Echinacea purpurea, E. pallida, Viscum album* and *Baptisia tinctoria,* have stood the test of time. However, there is not yet enough scientific data regarding the identity and effectiveness of most of these herbs (Gupta, 1994; Wagner, 1996). More so, most medicinal plants investigated and documented neither reach stage 1 of clinical trial nor gain confidence for clinical use by the practitioners of modern medicine (Gupta, 1994). However, as difficult as it may seem, further detailed investigations on medicinal plants are still going on and this has helped to reveal their involvement in enzymatic, endocrinal, immunomodulating functions. These have helped to widen their profile of activity and opened new vistas of therapeutic applications (Gupta, 1994).

Among plant constituents documented to modulate immune responses are saponins from *Albizzia lebbeck* through synthesis of reaginic antibodies (Tripathi *et al*, 1979), alcoholic extract of *Tylophora indica* (asthaticus) which prevents egg albumin induced anaphylaxis in guinea pigs (Harnath and Shamia, 1975) and horse serum induced broncho-constriction in sensitised rat lung (Gupta, 1975; Nanyampalli and Shet, 1979). Chewing of leaves of *T. indica* for 6 days gives protection to 71% cases of bronchial asthma on antigen challenge (Shrypuri and Agrawal, 1971). The plant saponins from *Clerodendron serratum* (Gupta and Tripathi, 1973a), *A. lebbeck* (Tripathi *et al,* 1978) as well as the alkaloidal fraction of *Solanum xanthocarpum* (Chitravanshi *et al*, 1990) and *T. indica* (Geetha *et al*, 1981) have been shown to protect sensitised mast cells from degranulation on antigen shock thus confirming the immuno-suppressive and membrane stabilising effects as seen with sodium chromoglycate. *T. indica* (Gupta and Tripathi, 1973b) as well as saponins of *A. Lebbeck* (Tripathi *et al*, 1977) and *Gardenia latifolia* have also been found to potentiate beta-adrenergic activity. This effect brings about broncho-dilatation and is considered to be helpful for relieving bronchospasm in asthmatic patients.

T. bellerica and *Ocimum sanctum* have been shown to give protection against histamine as well as pollen (*Acacia arabica*) induced bronchospasm in guinea pigs and the latter has also inhibited antigen-induced histamine release from sensitised mast cells and gave relief in allergic bronchial asthma (Palit *et al*, 1983). The anti-allergic action of *O. sanctum* has been found to be associated with significant production of IgE antibodies (Sen, 1993).

Among the several plants also investigated for anti-asthmatic effects are saponins isolated from *C. serratum* (Gupta *et al*, 1968), *Gardenia turgida* (Gupta, 1971a), *A. lebbeck* (Tripathi *et al*, 1977) and *Solanum xanthocarpum* (Gupta, 1971b). They accorded protection to sensitised guinea pig against

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histamine as well as antigen (egg albumin micro-acrosols). The protective effect of *C. serratum* saponin was found to be associated with the augmentation of anti-allergic activity in the tissues as the lung extracts from the treated animal inhibited histamine and SRS - A responses on guinea pig ileum to a greater extent and for longer period as compared to the extracts from the untreated control animals (Gupta *et al,* 1968). Some of these plant medicines therefore seem to be promising sources for development of anti - asthmatic and anti - allergic drugs.

A good number of plants also have anti-inflammatory activity. Such plants include *Tinospora cordifolia* - bitter principle (Gupta and Chrivastava, 1966), *Balsamodendron mukul* - olioresin - and *Curcuma longa* - volatile oil - (Chandra and Gupta, 1972).

Development of body resistance or immunity against infection has been reported after treatment with *Abutilon indicum* and *Sida cardifolia* as evidenced by enhanced production of anti - *Salmonella typhi* 'O' antibody and protection against tissue damage (Dixit *et al,* 1978). Thus, some of these plant products seem to activate body defence mechanism to fight against the disease.

Helleborous purpurascens (Ranunculaceae) extract was reported to have increased leucocytes, neutrophils and phagocytosis in animals transcutaneously inplanted with it (Bogdan *et al*, 1990). Septilin is shown to increase the total count of leucocytes and percentage of polymorphs in the peripheral blood and is extensively used in the treatment of several acute/chronic infections. It was also shown to protect mice from cyclophosphamide - induced myelosuppression and subsequent leucopenia (Praveenkumar *et al*, 1997). There are some plant-derived immuno - stimulatory drugs available on the drug market and these include extracts of *Echinacea* species (*E. purpurea, E. pallida*) used

therapeutically and prophylactically against chronic and recurrent infections of respiratory organs and urinogenital organs, retarded wound healing and infected wounds, malignant diseases (in combination with chemotherapy or irradiation) - (Bauer and Wagner, 1991). The treatment of leucopenia cancer patients undergoing radiotherapy with a phytopreparation containing *Echinacea* extract in combination with *Baptisia* species extract resulted in a significant increase of the number of leucocytes (Bendel *et al*, 1989).

Plants have also been sources for development of anti-cancer compounds or anti-viral interferons (Dhawan, 1987). The interferon stimulator (SNMC) derived from *Glycerrhiza glabra* has been reported to give protection to patients of subacute hepatic failure known to be fatal in majority of cases (Acharya *et al*, 1993). Successful treatment of subacute hepatic failure with interferon stimulators bring into focus the trial for many other indigenous drugs for the treatment of viral hepatitis and many other similar diseases including cancers. The possibility of development of a herbal drug for treatment of auto-immune diseases like multiple sclerosis and the most dreaded disease AIDS cannot be ruled out.

In addition to the inherent properties of some plants that have made them to modulate immune system, there have also been advances in recombinant DNA technology whereby a variety of foreign genes encoding therapeutic proteins are transferred into plant species such as potato and tobacco (Arakawa and Langridge, 1998). Plant biotechnology techniques have been used to create plants which contain a gene derived from a human pathogen, the resultant plant tissues will accumulate an antigenic protein encoded by the foreign DNA (Mason *et al*, 1992; Thanavala, 1995; Haq *et al*, 1995; Mason, 1996; Arakawa and Langridge, 1998).

In pre-clinical trials, it was found that antigenic proteins produced in transgenic plants retained immunogenic properties when purified; if injected into mice, the antigen caused production of protein - specific antibodies (Thanavala, 1995). In some experiments, where the plant tissues were simply fed to mice, a mucosal immune response occurred (Haq *et al*, 1995; Mason, 1996; Arakawa and Langridge, 1998).

Plants have been selected as bioreactors because their eukaryotic nature often permits appropriate post-translational modification of newly synthesized foreign proteins, which thus retain their biologic activity (Arakawa and Langride, 1998). Also, a number of studies have proven that plants are efficient large - scale cost effective bioreactors for production and delivery of bacterial and viral antigens, which upon oral or parental administration, induce significant immune responses in the recipient. Potato and tobacco plants have been shown to synthesize and assemble Norwalk virus capsid protein (Mason, 1996), hepatitis B virus surface antigen (Mason *et al*, 1992; Thanavala, 1995) and bacterial enterotoxin such as the pentameric B subunits from enterotoxigenic *Escherichia coli* (LT-B) and *Vibrio cholerae* (CT-B). Transgenic potatoes producing CT-B and LT-B evoke both systemic and intestinal immune responses in mice that eat the raw plant tissue and protect the animals from toxin challenge (Haq *et al*, 1995; Arakawa *et al*, 1997; Arakawa and Langridge, 1998).

Most importantly, plant - based production of biologically active recombinant proteins such as pathogen - specific antibodies (Ma, 1995; Ma *et al*, 1998) and vaccine antigens (Haq *et al*, 1995; Tacket *et al*, 1998; Arakawa and Langrdige, 1998) for preventive immunotherapy, enables convenient oral delivery through consumption of edible plant tissues. According to Tacket et al (1998), oral vaccines when compared to vaccine delivery by injection offer the hope of more convenient immunisation strategies. Oral vaccines act by stimulating the

immune system at effector sites (lymphoid tissue) located in the gut and use of edible plants has made this possible.

1.4.2. Phytohaemagglutination

It has been shown that plants contain substances capable of agglutinating erythrocytes. The active substances being 'Phytohaemagglutinin' (PHA) or simply plant 'lectin' (protein). The highest concentration of these phytohaemagglutinin (PHAs) are present in seeds from which they are readily extractable with saline (0.85% - 1.00% w/v) – (Tom and Western, 1971).

Stillmark (1888) first described the agglutination of animal erythrocytes by ricin, a protein isolated from the seed of *Ricinus communis* (Euphorbiaceae). Also, Hellin, (1891) discovered abrin in *Abrus precatorius*. The antigenicity of these PHAs (ricin and abrin) made possible the classic studies through which certain fundamental immunological principles were established.

Ehrlich (1891) found that after gradually increasing doses of abrin or ricin to mice over a period of time, a subcutaneous dose two hundred times greater than the initial lethal dose could be tolerated. Also, the first local immunisation studies showed that repeated small doses of abrin applied to one conjunctiva of a rabbit led to the development of immunity to toxic doses only in the treated eye (Romer, 1901).

Danysz (1902) demonstrated that a single large dose of anti-ricin neutralised more ricin than a series of aliquots of equivalent total volume added over a period of 24 h, a phenomenon now recognised as/and generally applied to antigen - antibody reactions. One of the first indications of the reversibility of antigen - antibody reactions was the observation by Landsteiner (1902) that if

erythrocytes agglutinated by abrin were washed with saline at 56°C, the abrin was eluted in active form.

PHAs have been of value in identification of certain human blood groups. Usually, blood typing is done so as to appropriately match the blood group of the recipient and the group of the donor blood. In order to achieve this, 'typing sera' from humans and animals are usually used. However, studies have shown that some of these PHAs were capable of agglutinating these red blood cells specifically (in which case they only agglutinate a particular blood group/red blood cell surface antigen). This gave the idea that plants might afford alternatives to isohaemagglutinins for the purpose of blood group diagnosis. On the other hand, other PHAs agglutinate the red blood cells non-specifically (i.e. they agglutinate the red blood cells irrespective of their blood group/red cell surface antigen).

The common lima bean, *Phaseolus limensis* and *Vicia crasa* extract for instance provide a highly specific lectin for blood group 'A' - specific antigen. Few anti – B lectins are available and non has been adopted for routine clinical use. According to Tom and Western (1971), some PHAs of many species agglutinate the red blood cells of all human blood groups giving approximately the same titre in each case (i.e. non-specific). *Canavalia ensiformis* (Martin and Bomchill, 1966), *Phaseolus coccineus* (Beckman *et al*, 1962) all of leguminosae family showed non-specific phytohaemagglutination. *Ulex europaeus, Lotus* species and *Laburnum* species show specificity for the H - substance.

PHAs are also of value in the serological distinction of various animals species. Several of them react with erythrocytes of some but not all animal species. Studies on inhibition of PHAs by simple sugars has contributed towards an understanding of the structures of erythrocyte receptors. The agglutination of

erythrocytes by lectins has formed one of the sources of information concerning the chemistry of A, B, H antigen and has also been useful in the taxonomy of the leguminosae. Some PHAs serve as important adjuvants while some can distinguish between T - and B - cells (Tom and Western, 1971).

It has been shown that plants belonging to the leguminose family contain specific proteins (lectins) capable of binding to or agglutinating certain cells such as red blood cells (where they are called phytohaemagglutinin), malignant tumour cells etc, by binding to certain specific carbohydrate groups on cell surfaces (Lehninger, 1990).

In leguminosae, 55.9% of the 198 genera tested and 40.3% of the 1597 species and varieties tested have proved to be PHA positive. In the sub-family, caesalpiniaceae (of the family leguminosae), 50.0% of genera tested proved positive and 25.4% of species tested proved positive (Tom and Western, 1971). *Cassia tora,* a specie in the sub-family, caesalpinaiaceae has been chosen to be tested for some of its pharmacological effects on the body. It is also to be tested for its phytohaemagglutination capacity; knowledge of which could be useful in serology especially in area of blood grouping.

1.5. *Cassia Tora* Linn (Family - Caesalpiniaceae)

The plant is of the main family leguminosae, which is further subdivided into three sub-families - Caesalpiniaceae, mimosaceae and papilionaceae.

C. tora belongs to the sub-family caesalpiniaceae. Some authors have referred to it as *Foetid cassia*. It is a shrub of up to 10 ft. high. It has pinnate leaves with 4 - 6 leaflets. The flowers appear singly or in pairs and are bright yellow coloured. The flowers appear from January to December. The plant has pods, which are usually paired and are up to 8 inches long. The pods are almost cylindrical, slightly curved and very slender when young. The pod contains many seeds in one series arranged longitudinally and is inflorescence. *C. tora* has a widespread distribution in Tropical Africa and in the Tropics generally. Its habitat include cultured lands and flat lands inundated in the rainy season (Hutchinson and Dalziel, 1958; Irvine, 1961).

C. tora is widely known in different parts as of Africa and elsewhere. In northern Nigeria, it is referred to by the Hausas as 'tafasa' or 'Zark amo' while the fulanis call it 'ubulo'. In Southern part of Nigeria, the Yorubas call it 'ako rere'; Ibos call it 'nlesimwo' or 'oke-osiedi' while the people from Benin call it 'Ihemenbika'. It is popularly known in Ghana by the 'Twi' people as 'mmofra brode'; by 'me' people of Sierra Leone as 'nje-pai'; by 'Wol' people of Gambia as 'mbundur' or 'n dour'. The Arabs call it 'kawal' (Dalziel, 1955).



Plate 1: *Cassia tora* plant in its natural habitat.

The plant has been widely used for varying purposes. The young leaves are sometimes eaten as vegetable in soup in Sudan, Nigeria etc. The pounded and fermented leaves and stems are powdered and added to food as a condiment. The seeds are eaten by the Moors in times of scarcity. The plant is used as food for ostriches (Dalziel, 1955). The whole plant, but especially the roots, are used as purgative and anthelmintics. The roots or fresh leaves or pulverised seeds, with lime juice and usually with senna plants are applied to the skin for ringworm or itch. The powdered leaves are applied to ulcers and to parasitic skin conditions, a fresh leaf decoction may be used as lotion for the same purpose.

In Nigeria, the plant decoctions are often mixed with other remedies for fever and cough. The seeds are often an ingredient in charms used in cutting weapons (Dalziel, 1955; Irvine, 1961). The leaves are used for impetigo, ulcers, ringworms. In India, the seeds are used in dyeing along with Indigo. The seeds are also used as suspending and emulsifying agents (Joshi and Varma, 1964)

Phytochemical examination has shown that the seeds which like those of *Cassia occidentalis* are roasted, ground and used as a coffee substitute contain an active principle - emodin - which resembles chrysophanic acid which is used for eruptive skin complaints (Staner, 1937). Also from phytochemical studies, it was found to have anthraquinone principle as the main constituent like other species of *Cassia* plant. These anthraquinones and their derivatives are heterocyclic compounds and are important as far as exertion of cathartic activity is concerned, hence responsible for some of its pharmacological actions. The

anthraquinones having purgative effects form a pharmacologically distinct group and from the chemical structure all anthraquinone laxatives contain a substituted anthracene ring structure (Twari and Behari, 1972).

Investigation of *C. tora* by chromatographic methods showed that it contains different types of anthraquinone principles (aglycones). The stem contains rhein, physcion, unknown anthraquinone. The fruit has aloe - emodin, emodin, chrysophanol, physcion, rhein and unknown anthraquinone. Various organs of *C. tora* have been found to contain fatty acids, especially the seeds, cuticle and leaflets. The main constituents of the leaf are palmiate, stearate, oleate, linoleate and arachidate (Akinsanya, 1980).

It has also been shown that *C. tora* seeds yielded water soluble substances coloured brown which had oxytocic actions. This gave reducing sugar on hydrolysis (Yoginder *et al*, 1962).

Antimicrobial studies have shown that the various parts of the plant (leaf, stem and root) contain active constituents responsible for the fungistatic activities of the plant (Elemele, 1983).

Nina (1997) reported that the extract of this plant did not have anti- plasmodial activity.

Some of the traditional uses of the plant, *C. tora* have prompted the investigation of this plant in the light of immunology. The plant is for instance

used as anti-parasitic agent (Dalziel, 1955). Parasites can be ectoparasite or endoparasites and it has been shown that parasitic infections stimulate both humoral and cellular immune responses though the predominant response will be determined by the identity of the parasite. In general however, cell mediated immune responses are more effective against intracellular parasites especially protozoans while antibodies are more effective against extra-cellular parasites in blood and tissue fluids by preventing the re-invasion of the cells particularly in blood borne parasites. T-lymphocytes play a very important role in worm infestation (helminthiasis). T-lymphocytes destroy worms by participating in antibody-dependent cellular cytotoxic (ADCC) reactions. This reaction involves cytotoxic activity of T-lymphocytes on target cells coated with specific IgG antibody (Bellanti and Rocklin, 1978).

The plant is also applied traditionally against itches. Nearly 10% of the population suffer allergies due to extrinsic allergens such as grass pollens, animal danders, the faeces from mites, in house dust etc. (Ivan, 1988). Allergies depend upon an altered immunological status of the individual. Allergic contact dermatitis for instance is mediated by sensitised lymphocytes. Hence, designated 'cell mediated hypersensitivity' and presents clinically as a skin reaction manifestated by redness, vesiculation (small blisters within the epidermis) and itching (Mitchel, 1975). It is therefore possible to use plants that contain active principles that act on the immune system to relieve itches due to allergies.

C. tora has also been traditionally used for fever and cough. Immunologically, fever and cough are basically non-specific types of immune response (though they can also be caused by specific immunologic process). Therefore, any substance that can affect an individual's immune processes can possibly influence cough and the body temperature.

Traditionally also, *C. tora* seed is included as an ingredient in cutting weapons such as arrows (Dalziel, 1955; Irvine, 1961). It is therefore pertinent to study the plant from serological point of view. This is because it is possible that the seeds contain some active principles that have vasculotoxic effect either in terms of lysis or clumping/agglutination of blood cells etc all of which can lead to death. Occurrence of vasculotoxic effect will possibly be a type of mechanism through which weapon poisons act.

All these, in addition to the fact that many plants belonging to the leguminosae family are known to contain specific proteins (lectin) capable of agglutinating certain cells such as red blood cells (RBCs) and malignant tumour cells by binding to certain carbohydrate groups on cells surfaces have prompted the investigation of *C. tora* for its role in immune response.

1.6 Objective of the Study

Since it is known that the immune status of an individual plays an important role in the health of the individual, there is a great need for further investigation of plants to possibly increase the catalogue of agents that can modify immune responses advantage of which can be taken in therapeutics.

The objectives of this study is therefore to evaluate the possible immunogenic and related pharmacological activities of *C. tora* with a view to ascertaining its value in traditional medicine and for future drug development.

Listed below are the specific studies to be carried out:-

(i) Test for the presence of phytohaemagglutinin (PHA) in the seed extract.

(ii) Acute Toxicity Studies on the leaf extract to determine the median lethal dose (LD_{50}) which is the dose of a toxicant that will kill 50% of a group of animals.

(iii) Delayed Type Hypersensitivity Reaction (DTH) to be measured by the degree of induration caused by intradermal injection of sheep red blood cell suspension in foot pads of mice treated with the leaf extract.

(iv) Effect of the leaf extract on the antibody titre of rats sensitised with egg albumin.

(v) Effect of *C. tora* leaf extract on Total Leucocyte Count (TLC) and Differential Leucocyte Count (DLC) in mice.

(vi) Antimicrobial studies to test if *C. tora* leaf extract has some anti-bacterial activities and if it does, to determine its mechanism of action by virtue of its relative potencies in terms of its Minimum Inhibitory Concentrations (M.I.C.) and possibly the Minimum Bactericidal Concentration (M.B.C.).

(vii) Effect of the leaf extract of *C. tora* on inflammatory response using egg albumin – induced oedema in rat hind paw.

(viii) valuation of analgesic activity of *C. tora* leaf extract in mice subjected to chemical and mechanical pains.

(ix) Effect of the leaf extract on smooth muscles of guinea pig ileum and rabbit jejunum as well as the interaction of the extract with antagonists.

CHAPTER TWO

2.0. MATERIALS AND METHODS

2.1. Plant Collection and Identification

The shrub, *Cassia tora* was collected from Zaria, Kaduna State, Nigeria in January, 1999. The plant was authenticated by Mr. A. Musa, a plant taxonomist in the Department of Biological Sciences, Faculty of Science, Ahmadu Bello University, Zaria, Kaduna State, Nigeria and was given a voucher specimen number 1370.

2.1.1. Preparation of the Leaf Extracts

C. tora leaves were air-dried for one week and were then pulverised in a mortar and sieved to fine powder. One hundred and seventy five grams (175 g) of the pulverised leaves were extracted with methanol in a soxhlet extractor for 24 h (Harbone, 1973). The extract was concentrated in a water bath and the product was regarded as the methanolic extract of the pulverised leaves.

2.1.2. Extraction of the Seed Protein - Phytohaemagglutinin (PHA) Preparation

The method of Toms and Western, (1971) was used. The mature dried pods of the plant were opened to release their dried seeds. The seeds were crushed into powder with pestle and mortar and were sieved to fineness. One part of the powdered seeds was extracted with nine parts of saline at room temperature for 16 h. The mixture was further agitated with a mechanical shaker for 2 h. The residue was then removed by centrifuging at 3000 g for 15 mins. The supernatant was retained for PHA studies.

The procedure used by Baker *et al*, (1985) was used to confirm the presence of protein in the supernatant. Turbid precipitation of protein was tested for by adding I ml of 25 % sulphosalicylic acid solution to 3 ml of the clear supernatant.

2.2. Drugs and Chemicals

The drugs and chemicals used in the studies include:
Aspirin (Sigma. USA.), Acetylcholine (Sigma St. Louis, USA), Chicken egg albumin (Sigma Steinheim, Germany), Glacial acetic acid (Searle, Essex, England), Triton x - 100 (Lubley, Newyorkshire, England), Methanol (B.D H. Ltd. Poole, England), Aluminum hydroxide (BDH Ltd. Poole, England) Lab M nutrient agar (Topley House, Bury, England) Nutrient broth No. 2 (Biotech Lab. Ltd., United Kingdom), Histamine (Sigma, USA) Atropine (Sigma, USA) Mepyramine (May and Baker).

2.3. Animals

Wistar rats (160 - 240 g) of both sexes, albino mice (20 - 31 g) of both sexes, male rabbits (2.2 - 2.5 kg), male guinea pigs (350 - 420 g) were used for the investigations and were obtained from the Animal House, Department of Pharmacology and Clinical Pharmacy, Ahmadu Bello University, Zaria, and from the Animal Facility Centre, National Institute for Pharmaceutical Research and Development (NIPRD), Abuja. The animals were fed *ad. libitum* with standard feed and water except when fasting was needed in the course of the study.

Some blood specimens used for the studies were collected from cows and goats (from Zaria abattoir), sheep (from faculty of Veterinary Medicine, Ahmadu

Bello University, Zaria) and human patients (from Ahmadu Bello University Teaching Hospital, Zaria).

2.4. Effect of *C. tora* Leaf Extract on Total Leucocyte Count (TLC) and Differential Leucocyte Count (DLC)

The method adopted for the study was that of Baker *et al*, (1985) and Jain, 1986. Blood was collected from 12 albino mice by applying pressure on their tails (to reduce pain) and then cutting off the tip of each tail. A drop of blood was collected straight on a slide for making a blood film for differential leucocyte count of each mouse. Also, white cell diluting pipette was used to collect blood directly from the tail of each mouse for the total leucocyte count.

The zero reading for the TLC in each of the mice was determined by making 1 in 20 dilution of the respective blood with white cell diluting fluid and then counting with the aid of an improved Neubauer counting chamber under the microscope at X 10 magnification.

The DLC was also determined by preparing thin slide blood film of each mouse and then staining with Leishman's stain. The films were drained and dried at room temperature and were then microscopically examined in an oil immersion at magnification of X 100.

The mice were then grouped into four (4). One male was included in each group. The first group served as the control while the second, third and fourth groups were treated with *C. tora* methanolic extract at doses of 100, 200, and 400 mg/kg i.p. respectively on the first, third, sixth and nineth day. On the tenth day, the TLC and the DLC were repeated using the same procedure as described above.

2.5 Delayed Type Hypersensitivity Reaction (DTH) - An *in-vivo* model for cell mediated immunity

The method of Chong *et al*, (1998) was used for the DTH reaction studies. The method of Dan *et al*, (1973) was adopted for the preparation of 5% (v/v) sheep red blood cell (SRBC) suspension used for this study. About 6 ml of blood was obtained from sheep in the Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria.

- The blood was washed 3 times with about 15 ml normal saline for 7 mins in a centrifuge at 1700 g.

- The cells were then packed for 10 mins at same speed.

- A 5% (v/v) SRBC suspension was made using 1 volume packed SRBC to 19 volumes of normal saline (1:19).

Twenty albino mice of either sex were randomly selected and weighed. The zero readings of the diameter of the mice hind foot pads were taken using a vernier calliper. The mice were then distributed into five groups of four each with one male in each of the groups. Group one served as the control while groups two, three, four and five received *C. tora* leaf extract at doses of 25, 50, 100 and 200 mg/kg i.p. respectively for eight (8) consecutive days. The weights of the mice were determined throughout the treatment period and compared with the weights at onset of treatment.

On the last day of the treatments, the mice were all sensitised with an intradermal injection of 0.02 ml of 5% (v/v) sheep red blood cells (SRBC) suspension into the right front foot pad.

Five days post sensitisation, a challenging dose of 0.02 ml of freshly prepared 5% (v/v) SRBC suspension was similarly injected in the left hind foot pad.

Similarly, a challenging dose of 0.02 ml of the previously prepared 5% (v/v) SRBC suspension (stored in a refrigerator) was injected in the right hind foot pad.

The induration was read 24 h latter using a vernier calliper.

2.6 Antibody Titration - An *in-vitro* model for determination of antibody level in serum

The effect of *C. tora* leaf extract on the antibody level resulting from sensitisation with egg albumin was investigated using 20 wistar rats of either sex which were randomly selected.

The method of Zmijewski and Fletcher, (1972) was used. The rats were distributed into five groups of four rats each. Rats in groups three, four and five were pre-treated with 50, 100 and 200 mg/kg *C. tora* leaf extract i.p. respectively for eight (8) consecutive days. On the 8th, 11th and the 14th days, 10 mg/ml egg albumin (1 ml) + 12 mg/ml aluminium hydroxide (1 ml) were injected subcutaneously into all the groups for sensitisation except the group 1 rats which received only normal saline and served as the control.

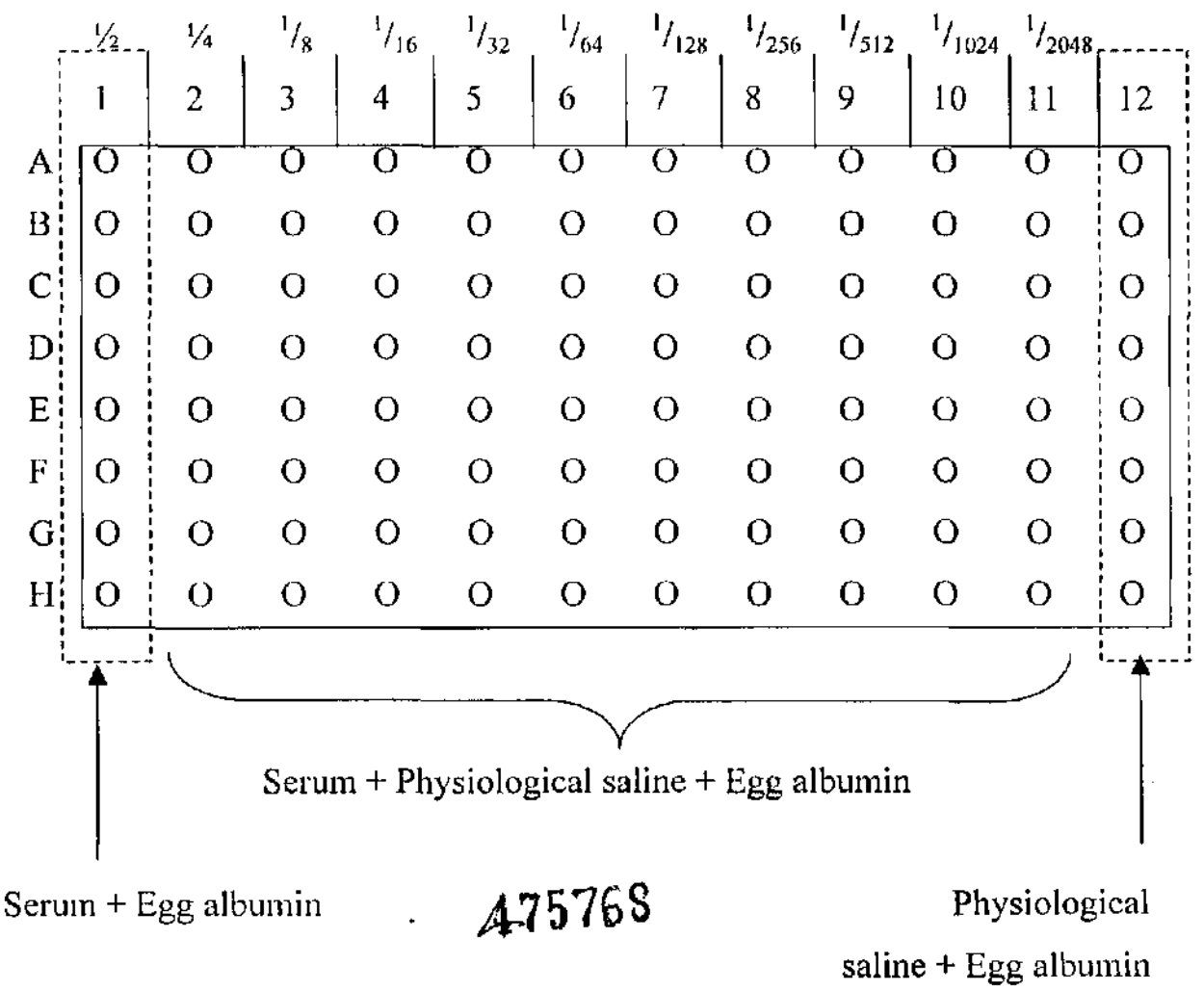
On the 18th day, all the rats were anaesthetized with chloroform and were then opened up for the collection of whole blood straight from the heart. The blood was collected in clean, plain bottles, allowed to stand for 4 h to ensure good clotting. The sera were separated by centrifugation at 2000 g for 10 mins and were then pipetted out into clean bottles.

Each serum sample was titrated to determine the level of antibody produced using the master serial two fold dilution method (Zmijewski and Fletcher, 1972).

The doubling dilution of the sera was done in the microtitre plates with 0.25 ml capacity wells (Tite - Tex - flow laboratories) using 0.025 ml of serum and 0.025 ml of physiological saline. The first well contained only serum while the last well was used as control with only saline present in it.

Microdiluters having the capacity of transferring 0.025 ml of solution were used to mix and transfer the mixture of serum and physiological saline (Serial dilution). An equal volume (0.025 ml) of 10 mg/ml egg albumin solution (antigen) was added to all the wells. The plates were shaken, covered and allowed to stand for 1 h 30 mins. Two preparations were made for each sample.

The pattern of precipitation was observed and the well with the highest dilution with precipitation was recorded as the titre of that antibody.



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Fig. 1: Doubling Dilution in Microtitre Plates.

2.6.1. External Influence of *C. Tora* leaf extract on the Precipitation Reaction of Egg Albumin and its Antibody

The external interference of the antibody – antigen reactions by the *C. tora* extract was also tested on the sera of the animals not pretreated with the extract. These include sera of the control rats in group 1 which did not receive any treatment except normal saline and those of group 2 which received only antigen treatment (egg albumin).

This also involved the use of 0.025 ml serum and 0.025 ml physiological saline. While the first well contained only serum, the last well contained only the saline

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and served as the control. Following serial dilution of the sera, 0.025 ml of 10 mg/ml *C. tora* leaf extract was added to all the wells and allowed to stand for 10 mins. An equal volume (0.025 ml) of 10 mg/ml egg albumin was then added to all the wells and allowed to stand for 1 h. The pattern of precipitation was also observed.

2.7. Test for the Presence of Phytohaemagglutinins (PHA) in the Seed Extract

The method of Toms and Western, (1971) was adopted. Whole blood was collected in EDTA bottles from twelve (12) people with known A, B and O blood groups. The people were from Ahmadu Bello University Teaching Hospital, Zaria, Nigeria. Four of the blood samples belonged to blood group A, another four to group B and the remaining four samples to group O.

Blood was also collected from six (6) different cows and six (6) different goats from Zaria abattoir.

To 0.5 ml of each of the blood samples, 0.5 ml of the supernatant from seed extraction was added and mixed. For the controls, 0.5 ml of normal saline was added to 0.5 ml of every blood sample. These preparations were made in three (3) batches to be incubated for 2 h. at $4^{O}C$, $25^{O}C$ and $37^{O}C$ respectively.

The presence of agglutination was observed after 2 h.

2.8. Antimicrobial Studies

Test for anti-microbial activity was done using Agar Zone Diffusion method (Bauer *et al*, 1966) and two methods were employed for the determination of anti-microbial activity on the agar. These include;

i. Disc method
ii. Cup plate method.

The organisms used were; *Pseudomonas aeruginosa* (NCTC 27853), *Escherichia coli* (NCTC 10418), *Salmonella typhi* (Lab. Strain), *Staphylococcus aureus* (ATCC 17079), *Bacillus cereus* (ATCC 14579).

In the Agar Zone Diffusion method, nutrient agar was used as the diffusion medium. Nutrient agar solution was prepared according to the manufacturer's instructions. 20 ml of the agar solution was dispensed into 10 bijou bottles and sterilised at 121^{O}C at 15 p.s.i. for 15 mins.

The agar was allowed to cool before pouring into the sterile plates. The agar in the bijou bottles were used as slants or slopes for the different organisms.

The organisms were sub-cultured from their agar slants into nutrient broth and incubated at 37^{O}C for 24 h. The standardisation of the microbial suspension obtained was done in two ways.

The first one involved the use of McFarland scale. 1 ml of the culture (in nutrient broth) was suitably diluted with sterile normal saline such that the turbidity produced matched the turbidity of the standard tube containing a suspension of barium sulphate prepared by adding 0.1 ml of 1% barium chloride

solution to 9.9 ml of 1% sulphuric acid. 1 ml of the diluted broth contained approximately 3 x 10^6 organisms.

The second method involved the use of sterile normal saline to make 1 in 1000 dilution of gram positive organisms and 1 in 5000 dilution for gram negative organisms.

The plates containing the gelled nutrient agar were then flooded with 2 ml of the inoculum (standardised microbial suspension) and the excess decanted. The plates were allowed to dry for 15 mins at room temperature to enable the organisms to be adsorbed onto the agar.

In the disc method, sterile filter paper discs were soaked in different concentrations of the extract, shaken gently and evenly placed on the inoculated plates with the aid of a sterile forceps ensuring that they were not closer than 10 mm to the edge of the plate and were sufficiently separated from each other to avoid over-lapping of the zones of inhibition. A standard disc (mastring – S) comprising of different standard antibiotics with different concentrations was used as standard.

The standard antibiotics were chloramphenicol (25 µg), Erythromycin (5 µg), Penicillin G (1 unit), Streptomycin (10 µg), Tetracyclin (25 µg), Gentamycin (10 µg), Ampicillin (10 µg), Cloxacillin (5 µg).

In the cup plate method, a flamed number 4 cork-borer was used to make holes in the media and the agar around the bored holes were removed with the help of a sterile wire loop. Little quantity of molten agar was placed at the bottom of each of the holes to seal the bottom. Using sterile pipettes, 0.3 ml of the diff ent concentrations of the extract were placed in the holes in each of the

plates made for the different bacterial organisms. The plates were then left at room temperature for 2 h to allow diffusion of the extracts into the agar medium.

In both the disc and cup plate methods, the plates were incubated at 37^{O}C for 24h. The diameter of the clear regions (zones of inhibition) were then measured using a ruler.

The concentration of *C. tora* leaf extract used were 10, 20, 31.25, 40, 62.50, 100, 125 and 250 mg/ml. The study was repeated four times.

2.9. Acute Toxicity Studies (LD_{50})

The acute toxicity (LD_{50}) of the extract was estimated in albino mice by the intraperitoneal (i.p.) and the oral (p.o.) routes as described by Lockes, (1983), which uses few animals.

The method estimates the dose of the extract that will kill 50% of the animal population by the route given. 40 albino mice were starved of feed but allowed access to water 24 h prior to the study. They were then grouped into ten (10) of four (4) mice each. The first five (5) groups and the second five (5) groups were used for intraperitoneal (i.p.) and oral (p.o.) routes of administration respectively. Extract was given at doses of 10, 100, 1000, 1500 and 2000 mg/kg. The mice were then observed for 24 h for pharmacological effects such as dizziness, excitement, nervousness or death.

According to this methodology, (LD_{50}) is estimated by taking the mean of the product of the highest dose that did not cause death and the lowest dose that caused death.

2.10. Studies on Guinea Pig Ileum Preparation

An adult guinea pig was starved for about 17 h with access to water. It was then killed by a blow on the head and the abdomen cut open. The ileum was carefully removed and a small piece of about 2 cm length was suspended in a 25 ml organ bath containing aerated Tyrode solution constituted in millimole (mM) as NaCl 136.8; KCl 2.7; NaH_2PO_4 0.14; D – Glucose 5.5; $NaHCO_3$12.0; $CaCl_2$1.3; $MgCl_2$ 0.5 dissolved in 1 litre of distilled water and maintained at 37^{O}C. The set up was connected to microdynamometer recorder set at sensitivity of 1.5 and speed of 24 mm/min.

At equilibrium, the effect of acetyl-choline, different concentrations of *C. tora* extract were tested. The effects of atropine and mepyramine on the tissue responses to the extract were also tested. The concentration of the extract at which it was half-maximally effective (EC_{50}) was also determined. This involved the conversion of the log organ bath concentration at which the percent of maximal effect was 50 to normal organ bath concentration.

This study was carried out on preparations from four (4) different guinea pigs.

2.11. Studies on Isolated Rabbit Jejunum

An adult rabbit was starved of feed for about 18 h and was killed following a blow on the head. The abdomen was cut open and about 2 cm piece of the excised jejunum was suspended in a 25 ml organ bath containing aerated Tyrode solution maintained at 37^{O}C.

The effects of acetylcholine, the leaf extract, atropine and mepyramine were observed on the piece of jejunum. The responses were recorded on

microdynamometer set at sensitivity of 1.5 and speed of 24 mm/min. The concentration of the extract at which it was half-maximally effective (EC_{50}) was also determined.

The studies were also conducted on four (4) different rabbits.

2.12. Studies on the Effect of *C. tora* Leaf Extract on Inflammatory Response

The technique employed was that of Winter *et al*, (1962) modified by Akah and Nwambie, (1994). The anti-inflammatory activity of the leaf extract was evaluated using egg albumin – induced oedema in rat hind paw. Adult wistar rats of either sex were divided into four groups of four (4) rats each. To two groups, two doses of the methanolic extract (100 and 200 mg/kg) were administered intraperitoneally. Aspirin (150 mg/kg i.p.) was used as a reference standard while the group with normal saline served as the control.

30 mins after drug treatment, oedema was induced by injecting 0.1 ml egg albumin into the sub-planter surface of the right hind paw. The measurement of the paw volume (cm^3) was done using LETICA Digital Plethysmometer (LE 7500) immediately and at 20 mins intervals after the injection of egg albumin for a period of 2 h. The average volume (cm^3) of oedema was calculated for each dose.

2.13. Evaluation of Analgesic Activity of *C. tora* Leaf Extract in Mice

Two models were adopted for this evaluation and they include;

i. Writhing Test model (a test on chemical pain)
ii. Tail Flick model (a test on mechanical pain).

2.13.1. Writhing Test

The Siegmund *et al*, (1957) technique modified by Koster *et al,* (1959) was adopted. The analgesic activity of *C. tora* leaf extract was evaluated on acetic acid – induced writhes in mice.

Two doses of the extract (200 and 400 mg/kg) were administered intraperitoneally to different groups of four mice each. At intervals of 30 mins, 60 mins (1 h), 90 mins, (1 h 30 mins) and 120 mins (2 h) following administration of the extract, 0.75% glacial acetic acid was administered intraperitoneally at the dose of 10 ml/kg.

5 mins after injection of acetic acid, the number of writhes made in 5 mins were counted with the aid of a counter. Similar tests were done using aspirin (150 mg/kg i.p.) as reference standard and normal saline for the control.

2.13.2. Tail Flick Test

This involved the modification of the techniques of Takagi *et al*, (1966); Huong *et al*, (1996). The screening technique involved the use of Ugo Basile Analgesy – meter (Cat. No. 7200) for the evaluation of the force-induced pain. This instrument exerts a force that increases at a constant rate. The force was applied to the animal's tail, which was placed on a small plinth under a cone – shaped pusher with a round tip. The scale was read off at the point when the mouse suddenly withdrew its tail and that marked the force at which the animal felt pain.

Prior to treatment, the nociceptive response for all the mice were taken for the establishment of the baseline. Two doses of the extract (50 and 100 mg/kg i.p.) were administered to two (2) groups of four (4) mice each and aspirin (50

mg/kg i.p.) to another group. The group with normal saline served as the control. The test was then repeated and the nociceptive response was measured every 30 mins over a 120 mins observation period.

2.14. Statistical Analysis

The general results are expressed as mean ± SEM and significance of differences between the controls and treated groups were determined using Student t–test. P-values < 0.05 were taken to be statistically significant. Normal/Gaussian distribution was employed for estimation of 95% confidence intervals in some experiments.

CHAPTER THREE

3.0. RESULTS

3.1. Methanolic Extraction

The percentage yield from the leaves was calculated to be 34% w/w.

3.2. Sulphosalicylic Acid Test

There was an instant reaction between 1 ml sulphosalicylic acid and 3 ml of clear supernatant from seed extraction. The reaction resulted to a very high turbid solution confirming the presence of protein in the extracted supernatant.

This afforded the basis for the use of the supernatant for phytohaemagglutinin studies.

3.3. Effect of *C. tora* Leaf Extract on Total and Differential Leucocyte Counts in Mice

95% confidence intervals estimated from the baseline values for total and differential leucocyte counts are shown in Table 1a. Following the treatment with *C. tora* leaf extract, the low doses of 100 and 200 mg/kg i.p. caused a significant ($p < 0.05$) increase in mice total leucocyte count. Also of importance is the significant ($p < 0.05$) increase in the lymphocyte level and the significant ($p < 0.05$) decrease in the number of neutrophils at these doses. The high dose of 400 mg/kg, on the other hand caused an apparent increase in the total leucocyte and lymphocyte counts. However, the increase was slightly lower than statistical value at $\alpha = 0.05$ (Table 1b).

Table 1a: 95% confidence intervals estimated for the total and differential leucocyte counts of mice before treatment with *C. tora* leaf extract.

Cell Types	**95% Confidence Intervals for the Cell Types**
TLC	3682.00 – 6933.69
Lympho.	28.54 – 69.13
Neutr.	25.00 – 67.59
Mono.	-1.45 – 3.79
Eosin.	-4.58 – 6.58
Baso.	-0.96 – 1.30

n = 12

Table 1b: Effect of methanolic extract of *C. tora* leaves (100 – 400 mg/kg i.p.) on total and differential leucocyte counts in mice.

Treatment Mg/kg	TLC per cubic mm ±SEM	Differential Leucocyte Counts (%)				
		Lympho.	Neutr.	Mono.	Eosin.	Baso.
Control	4220.00 ± 1.53	56.67 ± 1.73	38.00 ± 10.58	1.33 ± 0.67	4.00 ± 1.15	0.00 ± 0.00
C. tora:						
100	8666.67* ± 7.31	96.00* ± 1.15	3.33* ± 0.67	0.67 ± 0.67	0.00 ± 0.00	0.00 ± 0.00
200	8550.00* ± 15.5	100.00* ± 0.00	0.00* ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
400	7000.00 ± 13.11	78.00 5.27	22.00 ± 5.29	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Extract was administered on the first, third, sixth and ninth days.

Key:

TLC	- total leucocyte count;	Lympho.	- lymphocytes
Neutr.	- neutrophils;	Mono.	- monocytes
Eosin.	- eosinophils;	Baso.	- basophils

Values are mean ± SEM (n = 3)

* = $P < 0.05$ (Student t – test).

3.4. Delayed Type Hypersensitivity Reaction (DTH)

95% Confidence interval for thickness (mm) of hind foot pad of mice before treatment with *C. tora* was estimated to be 0.54 – 0.93. Baseline values for the twenty (20) mice were used in the estimation. Following treatment with *C. tora* leaf extract, there was a significant ($p < 0.05$) increase in the thickness of both right and left hind foot pads of mice treated with *C. tora* leaf extract (25 – 200 mg/kg i.p.) for 8 days.

The highest percentage difference was seen at the dose of 200 mg/kg (Table 2).

Table 2: Effect of methanolic extract of *C. tora* leaves (25 – 200 mg/kg i.p.) on the induration induced by 5% (v/v) suspension of SRBC in mice foot pads.

Treatment (mg/kg/day)	Thickness (mm) of Foot Pad Post-Treatment			
	RHFP	% Difference from Control	LHFP	% Difference from Control
Control	14.00 ± 0.7	0.00	9.50 ± 0.3	0.00
C. tora				
25	16.50* ± 0.6	17.86	10.75* ± 0.3	13.16
50	16.50* ± 0.3	17.86	10.75* ± 1.2	13.16
100	16.50* ± 0.9	17.86	12.50* ± 1.0	31.58
200	17.25* ± 0.5	23.21	12.75* ± 0.5	34.21

Key:

RHFP- right hind foot pad treated with previously prepared SRBC

LHFP- left hind foot pad treated with freshly prepared SRBC

SRBC- sheep red blood cell

Values are expressed as the mean ± SEM

n = 4

* = $P < 0.05$ (Student t – test).

3.5. Effect of *C. tora* Leaf Extract on Weight of Mice

At $\alpha = 0.05$, no significant difference was observed between the weight of mice at onset and weight after the 8 days treatment with the leaf extract of *C. tora* (25 – 200 mg/kg i.p.) – (Table 3).

Table 3: Effect of *C. tora* leaf extract (25 – 200 mg/kg x 8 i.p.) on the weight of mice.

C. tora **Dose (mg/kg)**	**Mean Increase in Weight**	**Mean % Increase in Weight**
25	0.80	3.00
50	1.57	7.39
100	1.45	5.63
200	1.76	7.15

Average weight at onset (day 1) = 24.66 ± 1.2

and at the end (day 8) = 26.06 ± 1.1

$n = 4$; $P > 0.05$ for all groups relative to control.

3.6. *Antibody Titration*

There was an apparent increase in the antibody titre of extract-treated groups. However, they were not statistically ($\alpha = 0.05$) different from the group that received only the antigen (egg albumin) – (Table 4).

Table 4: Effect of *C. tora* leaf extract (50 – 200 mg/kg i.p.) on the antibody titre of rats vaccinated with 10 mg/ml egg albumin, after the challenge dose. All the rats received 1 ml of 10 mg/ml egg albumin on 8th, 11th and 14th days except group 1 that received normal saline.

Group	*C. tora* Treatment (mg/kg/day)	Mean Titre ± SEM
1	-	$^1/_{8.73} \pm 0.100$
2*	-	$^1/_{64.00} \pm 0.010$
3	50	$^1/_{93.09} \pm 0.003$
4	100	$^1/_{102.40} \pm 0.002$
5	200	$^1/_{85.33} \pm 0.002$

n = 4; $p > 0.05$ (Student t – test).

Note: *The antibody titre for *C. tora* treated groups were statistically compared with the titre of group 2 rats (not group 1 rats).

3.6.1. External Influence of *C. tora* Leaf Extract on the Precipitation Reaction of Egg Albumin and its Antibody

Egg albumin (0.025 ml) was precipitated at one in eight ($^1/_8$) dilution by an equal volume of *C. tora* leaf extract in rats not pretreated with egg albumin

(antigen). On the other hand, egg albumin (0.025 ml) was precipitated at one in thirty two ($^1/_{32}$) dilution in sera of rats pretreated with egg albumin, which is the antigen (Table 5).

Table 5: The external influence of 0.025 ml *C. tora* leaf extract (10 mg/ml) on reaction between 0.025 ml egg albumin (10 mg/ml) and antisera.

Group	Sera	$^1/_2$	$^1/_4$	$^1/_8$	$^1/_{16}$	$^1/_{32}$	$^1/_{64}$	$^1/_{128}$	$^1/_{256}$	$^1/_{512}$	$^1/_{1024}$	$^1/_{2048}$	Physiological Saline
		1	2	3	4	5	6	7	8	9	10	11	12
With no antigen pretreatment	Serum 1	-	-	+	+	+	+	+	+	+	+	+	+
	" 2	-	-	+	+	+	+	+	+	+	+	+	+
	" 3	-	-	+	+	+	+	+	+	+	+	+	+
	" 4	-	-	+	+	+	+	+	+	+	+	+	+
With only antigen pretreatment	Serum 1	-	-	-	-	+	+	+	+	+	+	+	+
	" 2	-	-	-	-	+	+	+	+	+	+	+	+
	" 3	-	-	-	-	+	+	+	+	+	+	+	+
	" 4	-	-	-	-	+	+	+	+	+	+	+	+

3.7. Test for Presence of Phytohaemagglutinins in *C. tora* Seed Extract

The supernatant from the seed extraction non-specifically agglutinated the human ABO blood at the different temperatures they were subjected to. On the other hand, it only agglutinated the blood of some cows and goats (Table 6).

Table 6: Effect of *C. tora* seed extract (0.5 ml) on human, cow and goat blood (0.5 ml each) at 4°C, 25°C and 37°C.

Blood Samples	Temperatures (°C)		
	4	25	37
Human			
Group A			
N/S	-	-	-
1	+	+	+
2	+	+	+
3	+	+	+
4	+	+	+
Group B			
N/S	-	-	-
1	+	+	+
2	+	+	+
3	+	+	+
4	+	+	+
Group O			
N/S	-	-	-
1	+	+	+
2	+	+	+
3	+	+	+
4	+	+	+
Cow			
N/S	-	-	-
1	+	+	+
2	-	-	-
3	-	-	-
4	+	+	+
5	-	-	-
6	+	+	+
Goat			
N/S	-	-	-
1	-	-	-
2	-	-	-
3	+	+	+
4	+	+	+
5	-	-	-
6	+	+	+

Keys:N NS = normal saline; ABO = different human blood groups; 1–6 = numbers for individual identification; + = presence of agglutination; - = absence of agglutination.

3.8. Antimicrobial Activity

No zones of inhibition were observed around the methanolic extract of *C. tora* leaves (10 – 250 mg/ml). However, the different standard antibiotics showed zones of inhibition for different organisms.

3.9. Acute Toxicity Studies (LD_{50})

At doses ≤ 2000 mg/kg i.p. and p.o., no pharmacological effect such as dizziness, excitement, nervousness or even death was seen during the observation period of 24 h.

3.10. Guinea Pig Ileum Preparation

3.10.1. *C. tora* leaf extract (0.04 – 25.6 mg/ml) caused a concentration–dependent contraction of smooth muscles of guinea pig ileum as did acetylcholine (≥ 0.04 μg/ml) and histamine (≥ 0.04 μg/ml), (Fig. 2, Appendices I, II and III). The concentration of the extract at which it is half-maximally effective (EC_{50}) on guinea pig ileum is 1.33 mg/ml, an equivalence of log organ bath concentration of 0.125.

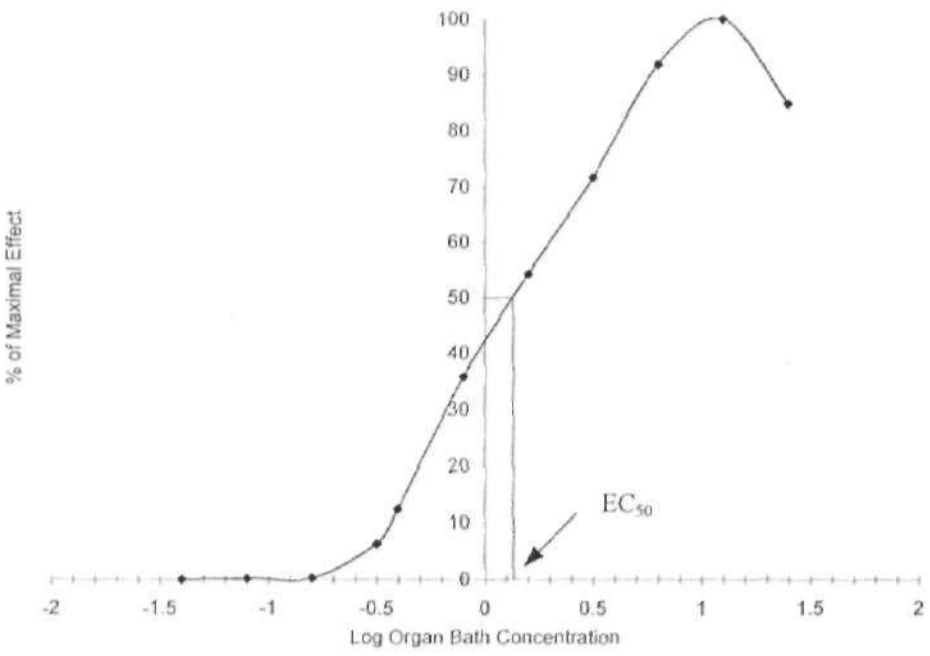


Fig. 2: Log concentration – dependent contraction of guinea pig ileum by methanolic leaf extract of *C. tora* (0.04 – 25.6 mg/ml)
-Data derived from Appendix III-

3.10.2. Antagonism between Mepyramine, Atropine and *C. tora* Leaf Extract on Guinea Pig Ileum

Mepyramine (0.8 – 3.2 μg/ml) reduced the contractile amplitude of histamine (0.16 μg/ml) tested on smooth muscle of guinea pig ileum (Appendix IV). Also, the same concentration of mepyramine reduced the contractile effect of *C. tora* extract (1.6 mg/ml) in a concentration – dependent manner and the effect of *C. tora* reversed after tissue was washed (Appendix V).

Atropine (0.04 μg/ml) completely blocked the contractile effect of *C. tora* extract (0.8 mg/ml) on the smooth muscle of guinea pig ileum (Appendix VI).

3.11. Studies on Isolated Rabbit Jejunum

3.11.1. *C. tora* leaf extract (0.04 – 2.56 mg/ml) caused a transient relaxation followed by a concentration – dependent contraction of the smooth muscles of rabbit jejunum. This is unlike acetylcholine (0.04 μg/ml) and histamine (0.04 – 1.28 μg/ml) which also caused contraction without the transient relaxation (Fig. 3, Appendices VII and VIII). The concentration of the extract at which it was half-maximally effective (EC_{50}) on isolated rabbit jejunum is 0.09 mg/ml, an equivalence of log organ bath concentration of -1.06.

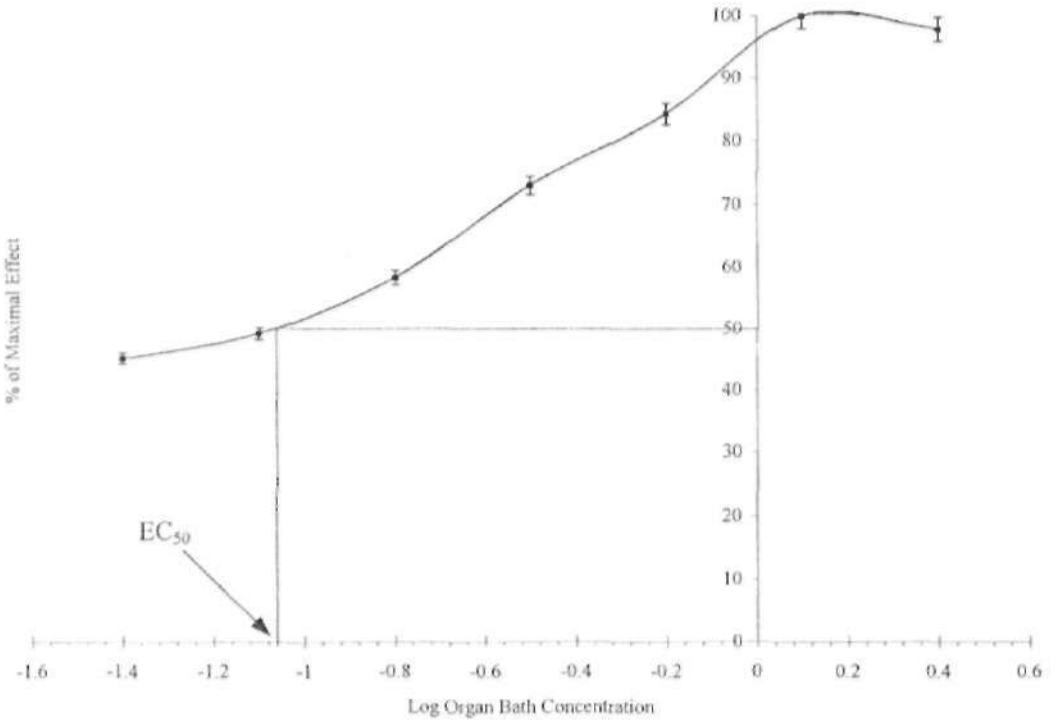


Fig. 3: Log concentration – dependent contraction of rabbit jejunum by methanolic leaf extract of *C. tora* (0.04 – 2.56 mg/ml)
-Data derived from Appendix VIII-

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3.11.2. Antagonism between Atropine, Mepyramine and *C. tora* extract on rabbit Jejunum

Atropine (0.04 µg/ml) blocked the contractile effect of 0.32 mg/ml of the methanolic leaf extract on rabbit jejunum. However, the contractile effect of the extract returned after washing the tissue (Appendix IX).

Mepyramine also reduced the contractile effect of *C. tora* extract in a concentration – dependent manner.

3.12. Anti-inflammatory Activity of *C. tora* Leaf Extract

The methanolic extract of *C. tora* exhibited a significant ($p < 0.05$) anti-inflammatory activity at the tested doses (100 – 200 mg/kg i.p.). The results obtained were comparable to that of aspirin (150 mg/kg i.p.) on egg albumin – induced oedema in rats (Fig. 4, Appendix X).

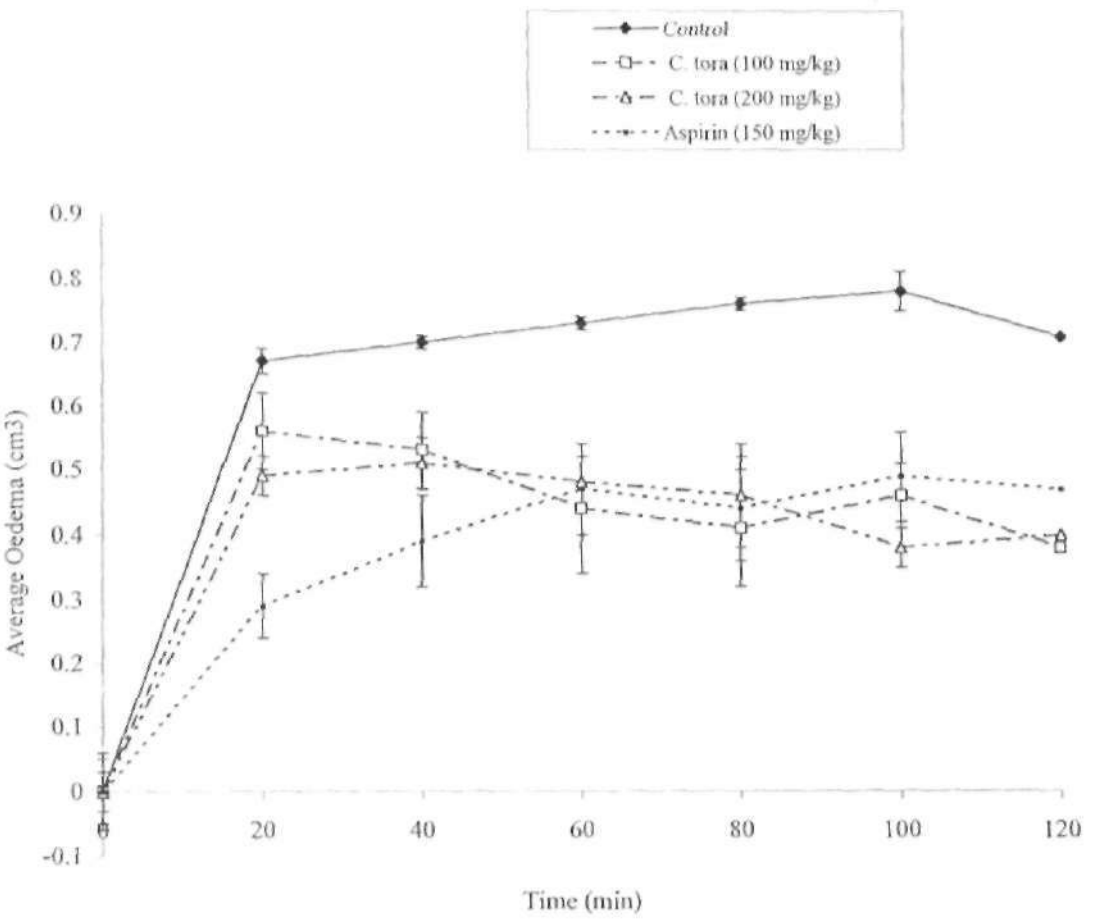


Fig. 4: Effect of *C. tora* leaf extract (100 – 200 mg/kg i.p.) and aspirin (150 mg/kg i.p.) on egg albumin – induced oedema in rats.
A significant difference occurred between the control and treated groups. $P < 0.05$ (Student t-test).

3.13. Analgesic Activity of *C. tora* Leaf Extract

3.13.1. Writhing Test

C. tora leaf extract (200 - 400 mg/kg i.p.) significantly ($p < 0.05$) reduced the number of writhes due to glacial acetic acid - induced pain in mice. The effect was comparable to that of aspirin (150 mg/kg i.p.) which was the reference standard.

The maximal analgesic effects were observed at 60th and 120th minutes for the 200 and 400 mg/kg doses respectively (Fig. 5, Appendix XI).

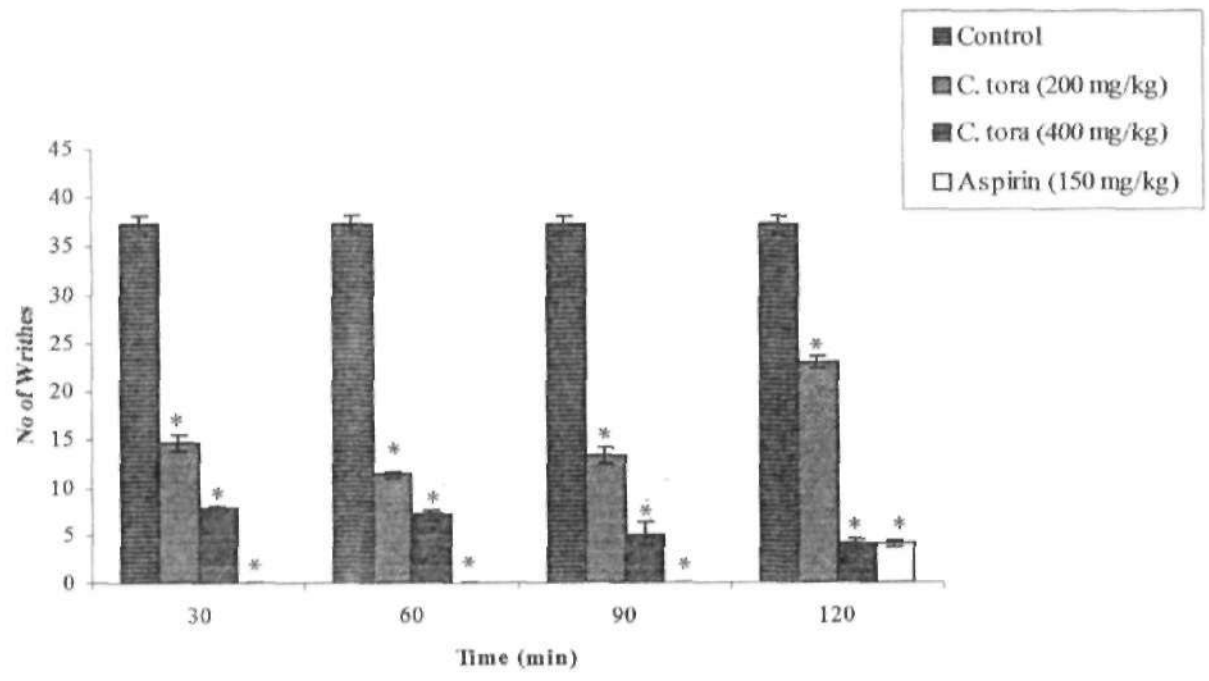


Fig. 5: Effect of *C. tora* leaf extract (200 - 400 mg/kg i.p.) on 0.75% glacial acetic acid (10 ml/kg I.p.) - induced writhing in mice.

* = Significant reduction of writhes at $P < 0.05$ (Student t - test). n = 4.

3.13.2. Tail Flick Test

The leaf extract of *C. tora* (50, 100 mg/kg i.p.) significantly ($p < 0.05$) increased the ability of mice to bear force – induced pain (mechanical pain). The effect was also comparable to that of aspirin (50 mg/kg i.p.). The maximal analgesic effect was observed 30 mins following administration of drug (Fig. 6, Appendix XII).

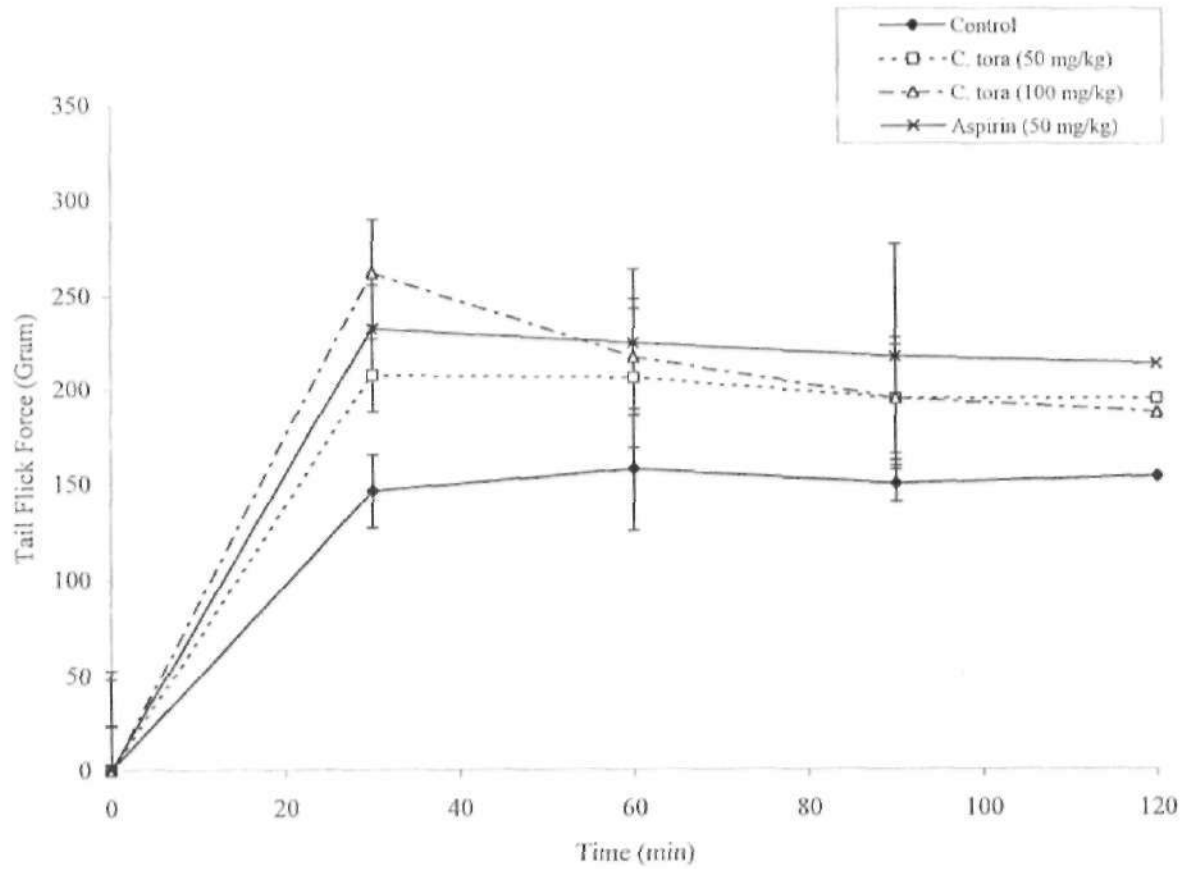


Fig. 6: Effect of *C. tora* extract (50 – 100 mg/kg i.p.) on force – induced pain in mice.

CHAPTER FOUR

4.0. DISCUSSION

4.1. General Discussion

The methanolic leaf extract of *Cassia tora* at low doses of 100 and 200 mg/kg significantly ($p < 0.05$) increased total leucocyte count (an immunostimulatory effect) in mice while the high dose of 400 mg/kg did not statistically cause any significant change in the total and differential leucocyte counts. The increase in the mice total leucocyte count at low doses could be due to an immunostimulatory effect of *C. tora* extract on the mice. The extract could have exerted this effect on bone marrow or caused blast transformation of the white blood cells. On the other hand, the inability of the high dose of 400 mg/kg to cause any significant change could probably be due to an inhibitory effect induced by excessive doses of drug on the site of action as was earlier reported by Goldstein *et al*, (1974). The consequence is that instead of having the 'sigmoid' curves typical of dose – response relationships, 'bell – shaped' curves could be obtained. This observation has been associated with immunopharmacological experiments (Nicola, 1987; Woollard, 1987; Nakai and Hirai, 1989). This is also in line with report of Wagner, (1966) that one characteristic of immunostimulant is that, similar to vaccines, they must be applied at relatively low doses to achieve optimal effects.

Also noteworthy is the significant ($p < 0.05$) increase in the lymphocyte level and decrease in the number of neutrophils. It is known that immunostmulants influence primarily the humoral and cellular immune systems both of which originate from lymphocytes. The cells (lymphocytes) are believed to originate from precursor cells (stem cells) produced in bone marrow (Zink, 1980).

Cassia tora leaf extract therefore behaved like extracts of *Echinacea* species and *Baptisia* species (Bendel *et al,* 1989); *Helleborous purpurascens* (Bogdan *et al,* 1990) and septilin (Praveenkumar *et al*, 1997), which were reported to increase total leucocyte count. The wide ethnomedical use of *C. tora* especially against infection could partially be attributed to its possible immunostimulatory activity.

In the present study, the delayed type hypersensitivity reaction observed as an increase in the induration induced by 5% (v/v) suspension of sheep red blood cell (SRBC) in mice foot pads by the methanolic extract of *C. tora* suggests an enhancement of lymphoproliferative response by the extract. Sheep red blood cell is a T – dependent antigen (Chong *et al*, 1998). Its first dose most likely sensitises the T – lymphocytes to become activated and then produce and release physiologically active substances into the surrounding area. These effectors cause accumulation of macrophages, monocytes, polymorphonuclear cells and probably other non – sensitised leucocytes into the area of antigen contact. The unsensitised cells are transformed into blast cells which mature to produce and release more physiologically active material (Zink, 1980). Thus, the initial effect is amplified until the invading antigen is destroyed. This may be part of the reason why all the groups, including the controls had an increased induration. However, extract – treated groups had higher induration probably because of the extract's ability to particularly activate more T – lymphocytes or stimulated the bone marrow to produce more white blood cells (WBCs).

Because no significant ($p < 0.05$) difference was observed between the antibody titre of the control rats and those treated with *C. tora* leaf extract, it is suggested that probably only a few of the formed lymphocytes from stem cells in the bone marrow were antibody – producing lymphocytes.

The observed pattern of precipitation of egg albumin by *C. tora* extract from serum samples of egg albumin vaccinated and unvaccinated rats shows that the precipitation was better when there was no pre-formed antibody. This was demonstrated by the fact that precipitation of egg albumin by *C. tora* extract occurred at a high dilution of one in thirty two ($^1/_{32}$) in rats earlier vaccinated with egg albumin This probably suggests that there was first an interaction between the egg albumin and its specific antibody, which could not allow the extract to precipitate egg albumin until all antibodies present were used up. Also, in rats not vaccinated earlier with egg albumin, precipitation occurred at a low dilution of one in eight ($^1/_8$). This probably shows that there were no preformed specific antibodies to interact with egg albumin. However, not having an immediate precipitation of egg albumin (at lowest dilution of ½) by the extract in later group of rats suggests that cross reacting antibodies were probably present.

Generally, these results suggest that *C. tora* extract also acted as an adjuvant (a substance incorporated into or injected simultaneously with antigen, which potentiates the immune response). Adjuvants produce 'depot effects' whereby they provide a long – lived reservoir of antigen by preventing free antigen from dispersing rapidly from the local tissue draining the injection site. Such adjuvants include aluminium compounds (phosphates and hydroxide) and Freund's incomplete adjuvant (in which the antigen is incorporated in the aqueous phase of a stabilised water in paraffin oil emulsion). It can therefore be said that *C. tora* extract by virtue of its ability to precipitate egg albumin (the antigen), can produce 'egg albumin depot' which can gradually release the antigen thereby potentiating immune response.

C. tora seed extract did not discriminate between the human blood groups at the different temperatures to which they were subjected (non-specific

agglutination). The agglutination titre was approximately the same in each case. This could mean that the extract has the ability to agglutinate some other very common blood group antigens rather than A, B which in this case could be the 'H' – antigen which is common to all the groups. This is in line with the probable suggestion made by Bird, (1959) that certain non-specific PHAs might be complementary to some other very common blood group antigen. This result therefore showed that *C.tora* seed extract cannot be a good alternative to isohaemagglutinins for human blood group diagnosis.

On the other hand however, the study showed that the seed extract specifically agglutinated cow and goat blood. At this level of study however, it cannot be said which of these animal blood groups were agglutinated.

In addition to these findings, the effect of these seed extracts can be traced back to their traditional uses. Traditionally, these seeds like *Cassia occidentalis* (leguminosae) are roasted, ground and used as a coffee substitute and are often as ingredient in charms used in cutting weapons (Dalziel, 1955; Irvine, 1961). Earlier experiments have however shown that *C. occidentalis* is lethal to calves with the animals showing anorexia, weakness, ataxia, recumbency and death (Dollahite and Henson, 1965; Henson and Dollahite, 1966). It was also shown to similarly affect poultry where it caused diminished egg yield and significant mortality (Simpson *et al*, 1971; Torres *et al*, 1971). *Phaseolus vulgaris* (leguminosae) has been shown to contain a haemagglutinin which is toxic to chicks (Honavar *et al*, 1962) and rats (Jaffe and Vegga, 1968) but they also showed that 'toasting' the beans before feeding the stock detoxifies the haemagglutinin. This habit of roasting the seeds probably explains why these roasted seeds despite their haemagglutinin contents are still used as coffee substitutes.

Different plant toxins have been used as poisons in cutting weapons and they act through different mechanisms. For instance, arrow poison obtained from *Chondodendron* and various species of *Strychnos* are known to contain tubocurarine as their active ingredient (Farnsworth *et al*, 1985; Bisset, 1989). Tubocurarine paralyses skeletal muscle by blocking the nerve impulse at the myoneural junction. It causes release of histamine, fall in blood pressure and death due to paralysis of respiratory muscles (Clarke *et al*, 1975). Also, the use of *Erythrophloeum guineense* ('Sassy bark') as an ingredient of arrow poison is attributable to its cassaine and other alkaloidal contents. In the same way, the use of *C. tora* seed as an ingredient of cutting weapons could be attributable to its phytohaemagglutinin content. As was earlier mentioned, phytohaemagglutinins have lethal effect (except when otherwise 'toasted' for detoxification). It is only logical to reason that since phytohaemagglutinins have the ability of clumping the red blood cells, that the later (RBCs) will be hindered of their principal function which is carriage of oxygen to all the cells, tissues and organs of the body. This will ultimately result to death thereby achieving the aim of using it as ingredient in cutting weapon.

Investigative studies into the antimicrobial activity of *C. tora* was conducted in order to find out if there is a correlation between its traditional use against infections and its scientific properties. The methanolic extract did not produce any zone of inhibition to any of the tested organisms both in the disc and cup plate methods. This probably suggests that the organisms were not susceptible to its activity or that the extract does not have antimicrobial activity at all. However, it is also possible that other micro organisms may be susceptible to it. Also, Elemele (1983) earlier reported that *C. tora* contains active consituents responsible for its fungistatic activities.

The high lethal dose value (LD_{50}) estimated for *C. tora* leaf extract suggests that the extract is relatively safe and possibly justifies its traditional use as vegetable in soups.

The antagonism of methanolic extract of *C. tora* by atropine on both guinea pig ileum and rabbit jejunum suggests that the extract contained some active principles with muscarinic properties. Cholinomimetics are known to stimulate the smooth muscles of gut through the muscarinic receptors and this stimulatory effect is blocked by atropine. The stimulatory effect of the extract can therefore be likened to those of cholinomimetics.

The contractile effect of methanolic extract of *C. tora* leaves on both guinea pig ileum and rabbit jejunum was blocked by mepyramine. This suggests that the extract possibly caused histamine release by the tissue mast cells or contains some active principles that act on H_1 receptors to cause contraction of smooth muscles of the gut. Histamine has been shown to act through three principal types of receptors, H_1 (Ash and Schild, 1966), H_2 and H_3 (Black *et al*, 1972). However, the contraction of the gut is mediated by H_1 receptors (Ash and Schild, 1966), which are readily blocked by mepyramine and such other classical antihistamines described as H_1 – receptor blocking drugs or H_1 blockers.

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The methanolic leaf extract inhibited egg albumin – induced oedema in rats suggesting an anti-inflammatory effect. The results were observed to be similar to that of aspirin. Barnes *et al*, (1990) reported that there are many mechanisms underlying anti-inflammatory action, like the exogenous effect of steroids or endogenous release of glucocorticoids, interaction with prostaglandin biosynthesis, interaction with tachykinin, or other inflammatory mediators. It is however not possible at this stage of the study to say the mechanism through

mechanism for the anti-inflammatory effect. Also, it is important to note that this methanolic leaf extract showing anti-inflammatory effect has earlier been reported to enhance cellular immune response. These are two opposite effects and could mean that the crude extract contains different components that exert these different effects. Fractionation of the plant extract may help in identifying the components responsible for the different activities.

The test for analgesia on chemical pain (writhing test) showed that the methanolic extract of *C. tora* leaves significantly reduced the number of writhes due to glacial acetic acid – induced pain in mice. Also, test for analgesia on mechanical pain (tail flick test) showed that the extract significantly increased the ability of mice to bear force – induced pain. The results were comparable to those of aspirin. These results suggest that the extract contain some active principles with analgesic properties. The maximal analgesic effect was seen at 30th min for the 50 and 100 mg/kg i.p. dose of the extract and at 60th and 120th min for the 200 and 400 mg/kg doses respectively suggesting a dose – dependent analgesic effect.

Analgesics are classified into two – 'Opioid analgesics' which include morphine and related compounds such as codeine and the 'Antipyretic analgesics' (non-opioid analgesics) which includes aspirin, acetanilide and phenacetin.

The opioid analgesics are more consistently effective than antipyretic analgesics in pain associated with trauma or with deep structures such as viscera. The antipyretic analgesics however, are more useful in the treatment of pain associated with headache, connective tissue, arthralgia and pains arising from integumental structures rather than viscera. However, it cannot be said precisely at this stage whether the analgesic effect of *C. tora* extract will be classified as opioid or non- opioid. Further work may be able to inform us about the

mechanism of action of the extract in relation to pain relief. The analgesic property may also be the reason why decoctions from the leaves are applied to ulcers considering the pains associated with ulcers.

It is important to note that some drugs are known to be clinically effective analgesics, even antipyretics but lack significant anti-inflammatory properties e.g. phenacetin, acetaminophen while other drugs are potent anti-inflammatory agents but lack or have only weak analgesic properties e.g. phenylbutazone. Others have both analgesic and anti-inflammatory properties e.g. aspirin. The study of effect of *C. tora* extract on egg albumin – induced eodema in rats showed it to have a significant anti-inflammatory effects while writhing test and tail flick test showed it to have a significant analgesic properties. It is therefore possible that the extract belongs to the same group as aspirin, which has both analgesic and anti-inflammatory properties. These results possibly justify the ethnomedical use of leaf extract of *C. tora* on ulcers and impetigos.

4.2. Conclusion

The project has shown that the leaf extract of *Cassia tora* contain some active principles with potential of being good immunostimulants. The ability of the extract to cause increased amounts of lymphocytes in mice indicates that it may be worthwhile to further investigate this plant for its future development as an immunostimulant.

Also, the ability of the seed extract to agglutinate red blood cells has supported its traditional usage as an ingredient of cutting weapon.

The fact that deleterious effects have not been reported in the traditional usage of *C. tora* leaves as vegetable in soup could be because of its lack of toxicity even at fairly high dosage (> 2000 mg/kg) as found in this work.

The stimulatory/contractile effect of the leaf extract on the smooth muscles of the rabbit jejunum and guinea pig ileum may account for the traditional usage of the plant extract as purgative.

The present study also indicates that the extract has a potential of being an analgesic as was demonstrated by the relief of both chemical and mechanical pains induced in mice. It also has potential as an anti-inflammatory agent because it reduced egg albumin – induced oedema in rats.

In view of all these findings, it will be worthwhile to do more extensive studies on this plant, (especially in the light of immunology, pharmacology and phytochemistry) for future drug development.

4.3. Suggestions for Further Work

These include;

i. Detailed study of the mechanism by which *C. tora* leaf extract preferentially enhanced cell mediated immune response; (such as activation of more T – lymphocytes form stem cell lymphocytes, enhancement of lymphokine production, etc.)

ii. Chronic toxicity studies on *C. tora* leaf extract.

iii. Elucidation of the mechanisms of action for the anti-inflammatory and analgesic effect of *C. tora* leaf extract.

iv. Obtaining the different fractions in *C. tora* leaf so as to identify the fractions with the different immunologic/pharmacologic responses observed.

v. Elucidation of the phytochemical structures of the active ingredients responsible for the different activities.

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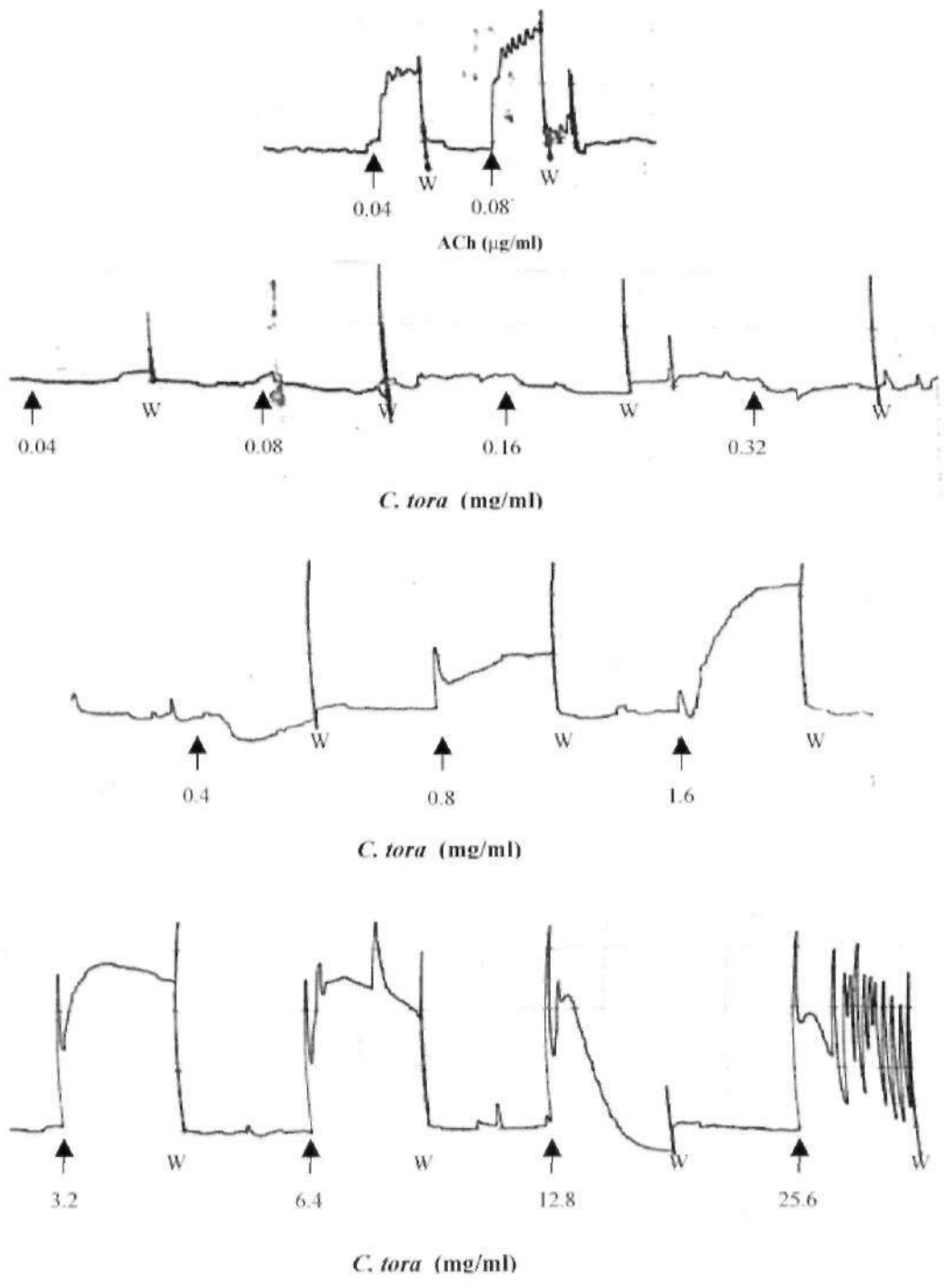
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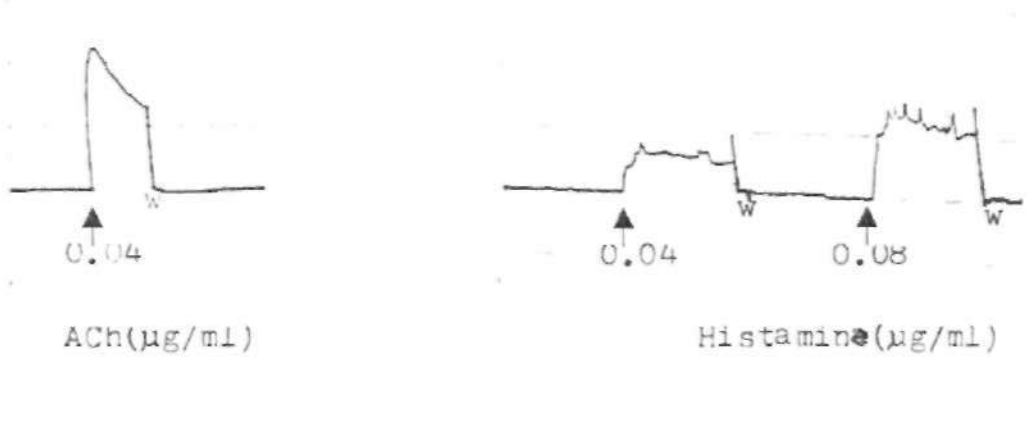
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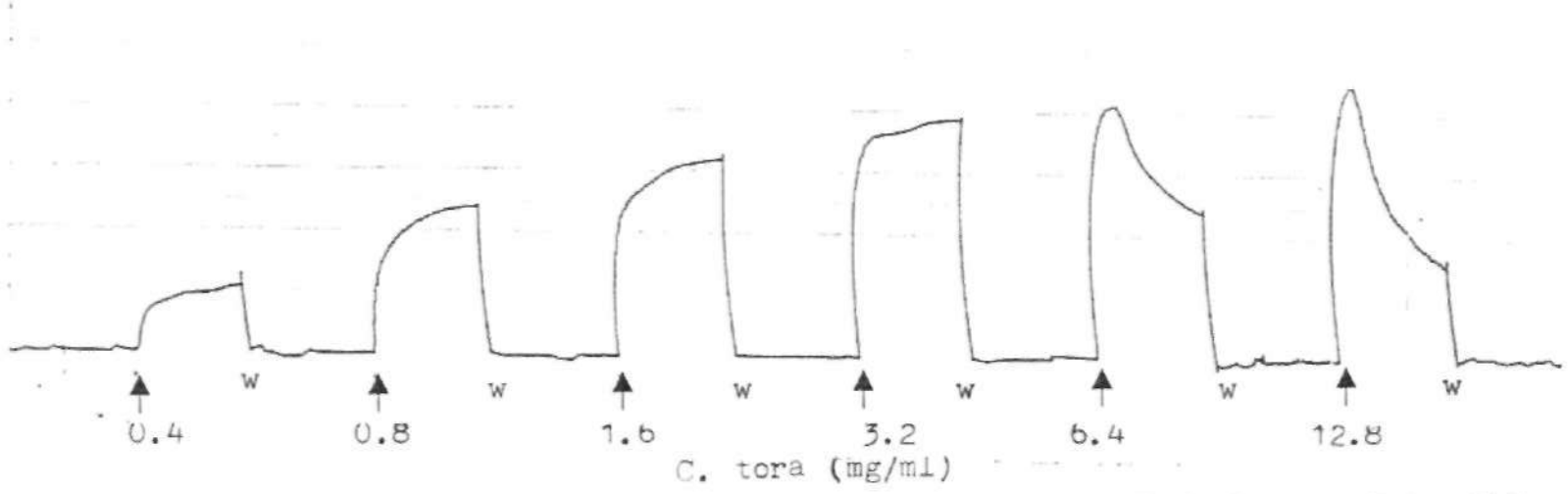


Appendix I: Concentration – dependent effect of *C. tora* methanolic leaf extract (0.04 – 25.6 mg/ml) on the isolated guinea pig ileum.

Sensitivity x 1.5; Speed = 24 mm/min; n = 3

ACh = Acetylcholine



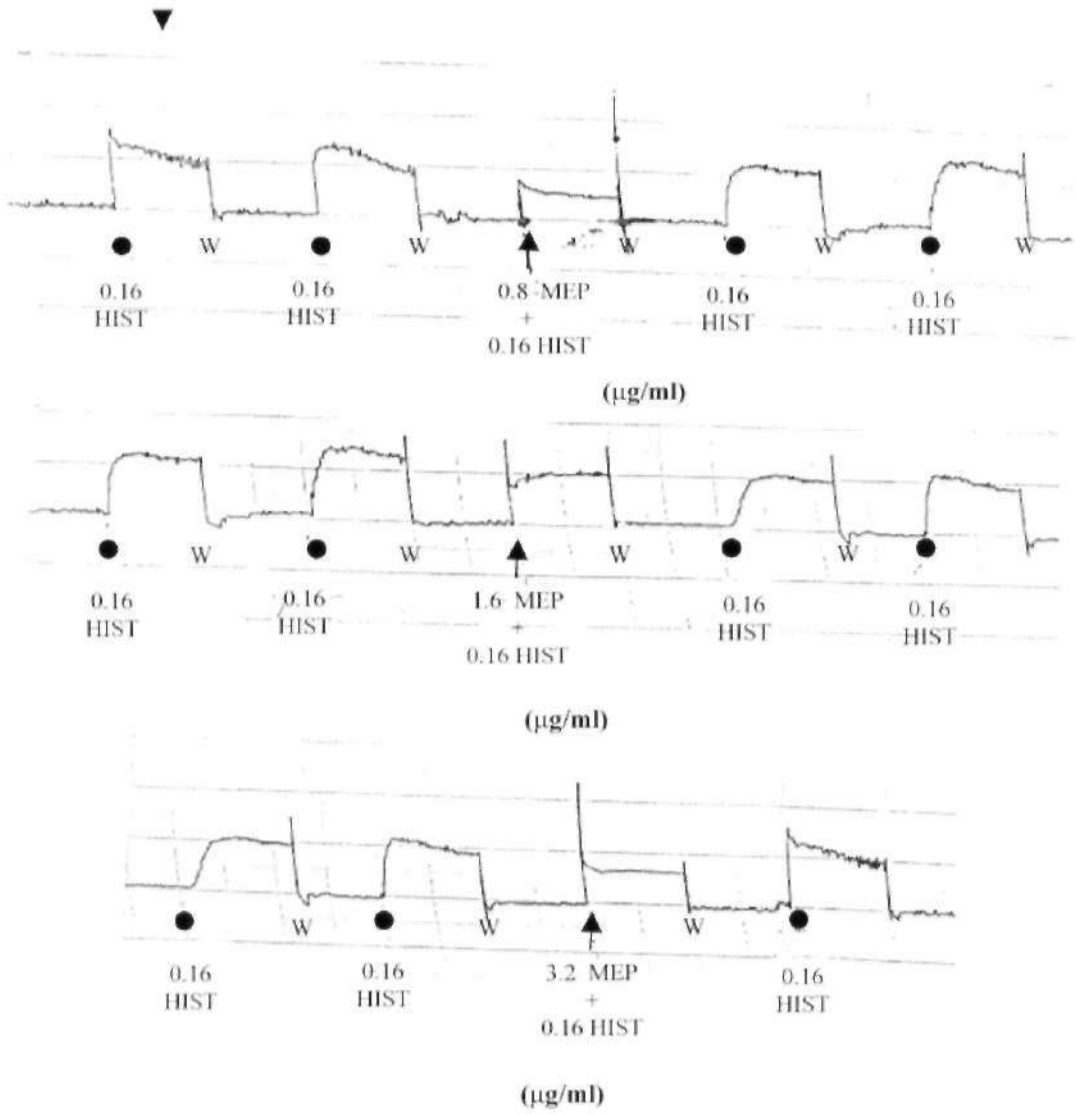


Appendix II:Concentration-dependent effect of *C. tora* methanolic leaf extract (0.4 – 12.8 mg/ml) on the isolated guinea pig ileum.

Sensitivity X 1.5; Speed = 24 mm/min; n = 3; Ach = Acetylcholine

Appendix III: Log – concentration dependent effect of methanolic leaf extract of *C. tora* leaves (0.04 – 25.6 mg/ml) on guinea pig ileum.

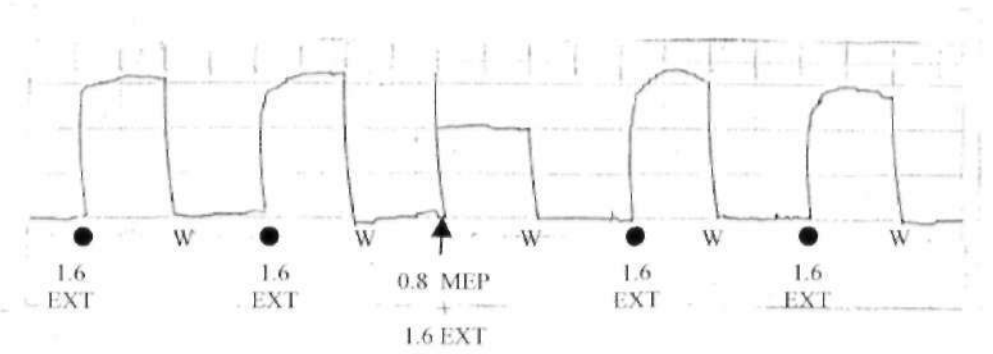
Organ Bath Concentration (mg/ml)	Log Organ Bath Concentration	Maximum Height of Contraction (mm)			Average Height (mm) ± SEM	% of Maximal Effect
		H_1	H_2	H_3		
0.04	-1.4	0	0	-	0.0 ± 0.0	0.00
0.08	-1.1	0	2	-	1.0 ± 1.0	0.25
0.16	-0.8	0	2	-	1.0 ± 1.0	0.25
0.32	-0.5	3	2	-	2.5 ± 0.5	6.25
0.40	-0.4	4	1	10	5.0 ± 2.6	12.50
0.80	-0.1	10	10	23	14.3 ± 4.3	35.75
1.60	0.2	13	21	31	21.7 ± 5.2	54.25
3.20	0.5	20	28	38	28.7 ± 5.2	71.75
6.40	0.8	34	35	41	36.7 ± 2.2	91.75
12.80	1.1	41	34	45	40.0 ± 3.2	100.00
25.60	1.4	35	33	-	34.0 ± 1.0	85.00

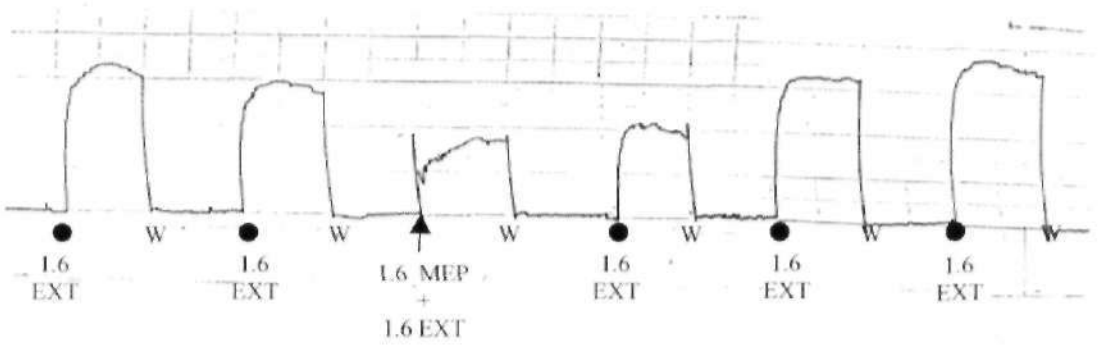


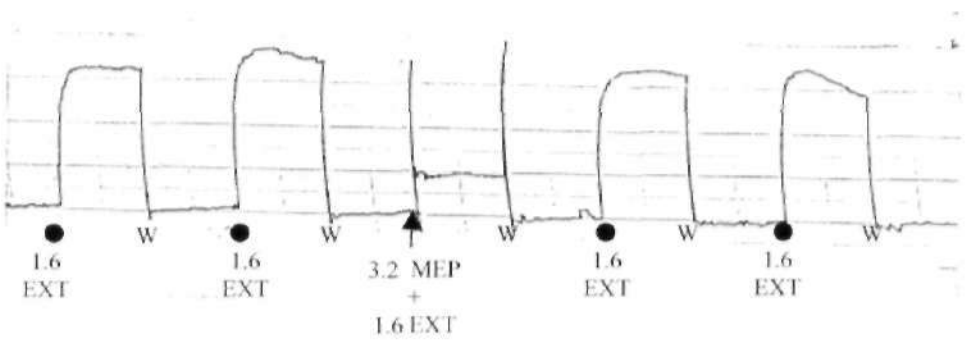
Appendix IV: Antagonism between mepyramine (0.8 – 3.2 μg/ml) and histamine (0.16 μg/ml) on guinea pig ileum.

Sensitivity x 1.5; Speed = 24 mm/min

HIST = Histamine, MEP = Mepyramine



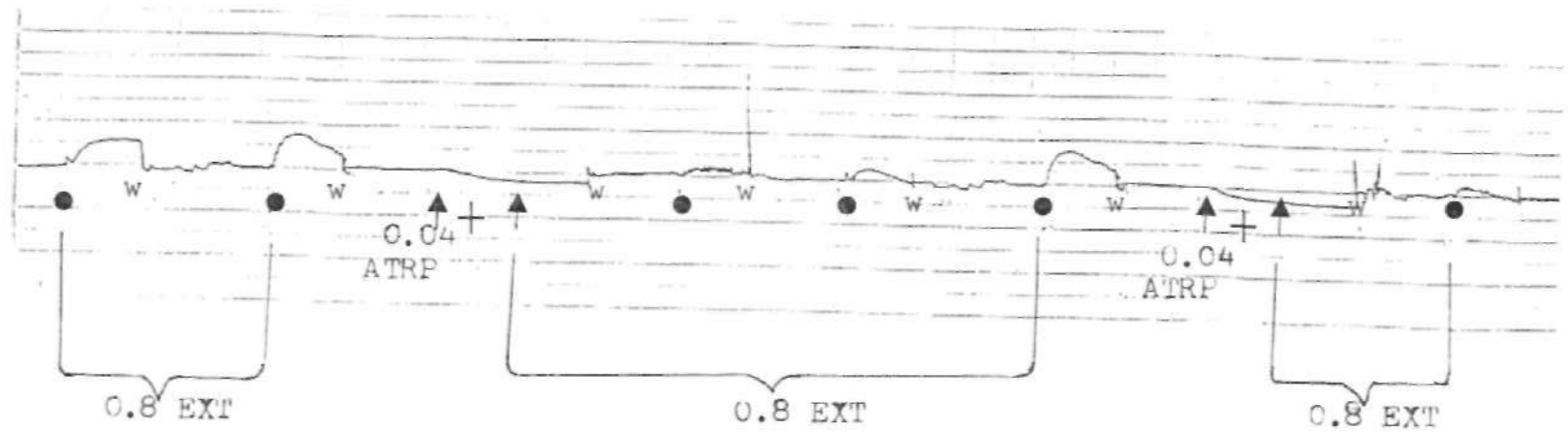




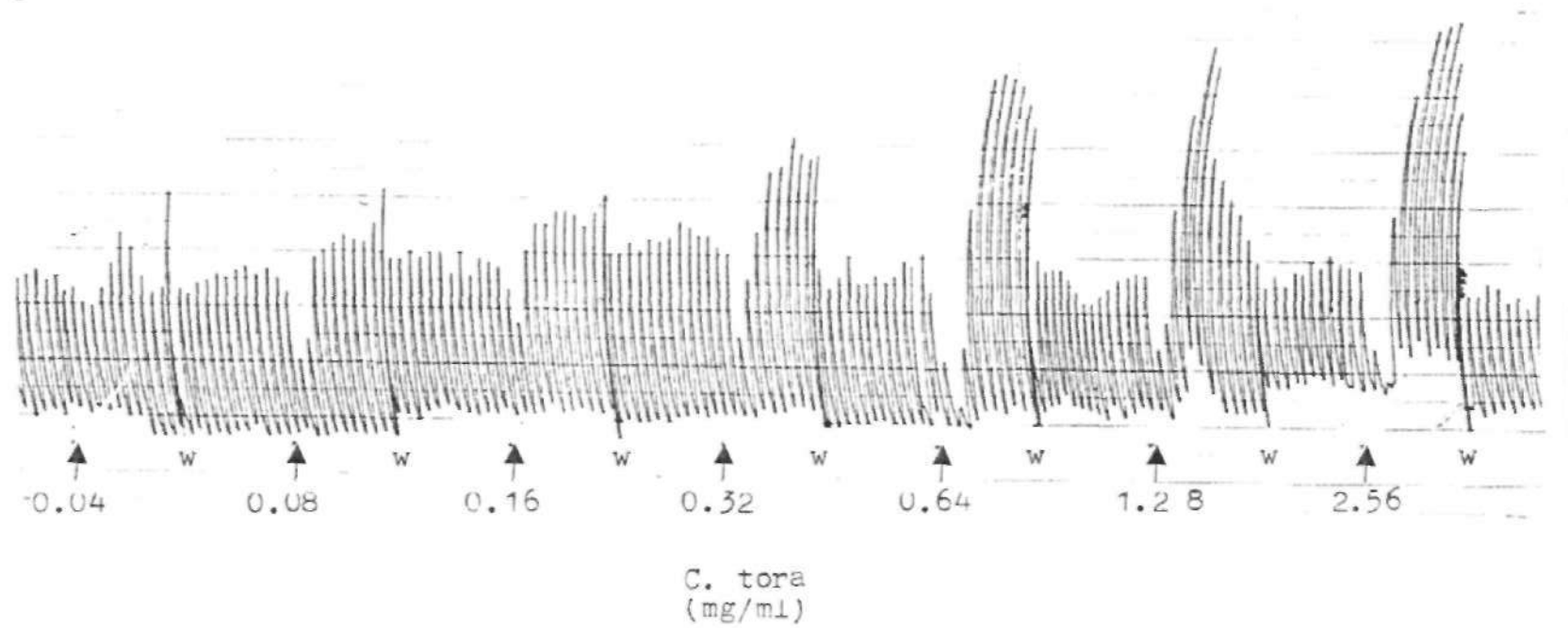
Appendix V: Antagonism between mepyramine (0.8 – 3.2 µg/ml) and *C. tora* methanolic leaf extract (1.6 mg/ml) on guinea pig ileum.

Sensitivity x 1.5; Speed = 24 mm/min

EXT = Extract (mg/ml); MEP = Mepyramine (µg/ml).



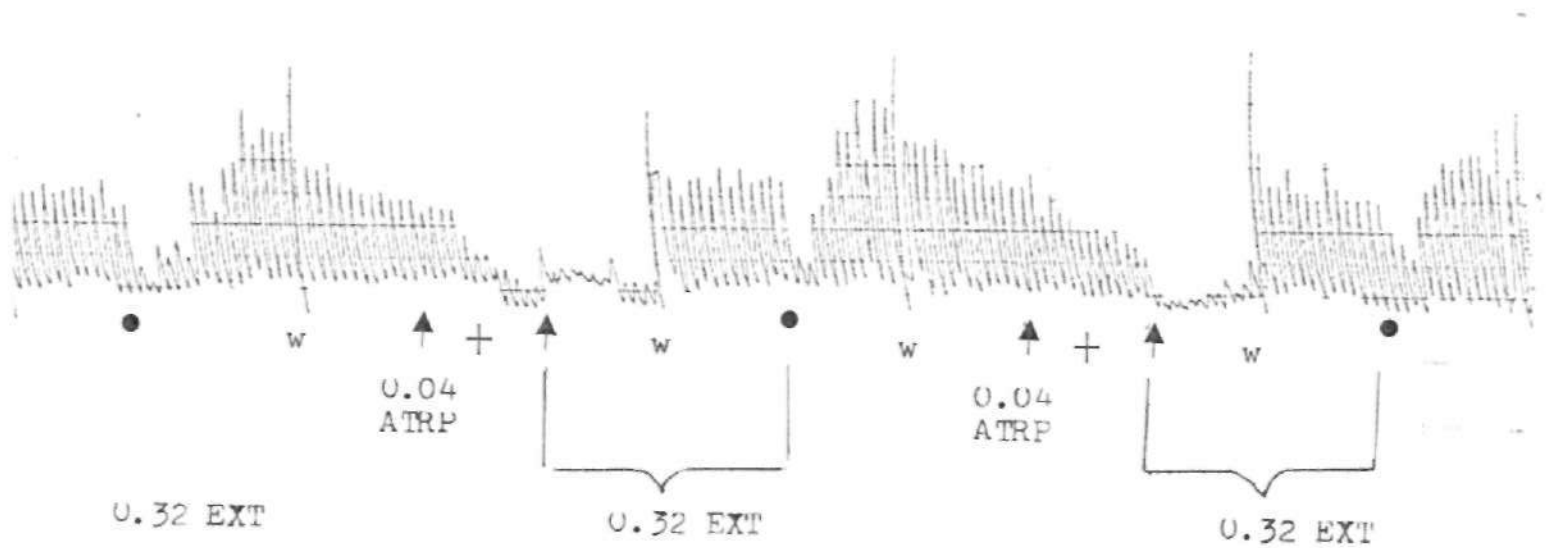
Appendix VI: Antagonism between atropine (0.04 µg/ml) and *C. tora* methanolic leaf extract (0.8 mg/ml) on guinea pig ileum.
Sensitivity X 1.5; Speed = 24 mm/min
EXT = Extract (mg/ml); ATRP = Atropine (µg/ml)



Appendix VII: Concentration-dependent contraction of *C. tora* methanolic leaf extract (0.04 – 2.56 mg/ml) on rabbit jejunum.
Sensitivity X 1.5; Speed = 24 mm/min

Appendix VIII: Log – concentration dependent effect of methanolic leaf extract of *C. tora* leaves (0.04 – 25.6 mg/ml) on the rabbit jejunum.

Organ Bath Concentration (mg/ml)	Log Organ Bath Concentration	Maximum Height of Contraction				Average Height (mm) ± SEM	% of Maximal Effect
		H_1	H_2	H_3	H_4		
0.04	-1.4	33	38	33	27	32.8 ± 2.3	44.93
0.08	-1.1	35	40	36	32	35.8 ± 1.7	49.04
0.16	-0.8	36	56	45	33	42.5 ± 5.2	58.22
0.32	-0.5	51	70	56	36	53.3 ± 7.0	73.01
0.64	-0.2	63	65	68	50	61.5 ± 4.0	84.25
1.28	0.1	69	95	71	57	73.0 ± 8.0	100.00
2.56	0.4	74	70	70	72	71.5 ± 1.0	97.95



Appendix IX: Antagonism between atropine (0.04 µg/ml) and *C. tora* methanolic leaf extract (0.32 mg/ml) on rabbit jejunum.

Sensitivity X 1.5; Speed = 24 mm/min; n = 3

EXT = Extract (mg/ml); ATRP = Atropine (µg/ml)

Appendix X: Effect of *C. tora* leaf extract (100 – 200 mg/kg i.p.) and aspirin (150 mg/kg i.p.) on egg albumin – induced oedema in rats.

Drug	**Time (min)**						
(mg/kg)	**0**	**20**	**40**	**60**	**80**	**100**	**120**
Control	0.89	1.49	1.59	1.62	1.65	1.67	1.60
		[0.67]	[0.70]	[0.73]	[0.76]	[0.78]	[0.71]
C. tora							
100	0.47	1.03	1.00	0.91	0.88	0.87	0.85
		[0.56]	[0.53]	[0.44]	[0.41]	[0.46]	[0.38]
200	0.49	0.98	1.00	0.97	0.95	0.87	0.89
		[0.49]	[0.51]	[0.48]	[0.46]	[0.38]	[0.40]
Aspirin							
150	0.80	1.09	1.19	1.27	1.24	1.29	1.27
		[0.29]	[0.39]	[0.47]	[0.04]	[0.49]	[0.47]

[ ] = Average oedema in cm^3.

Appendix XI: Effect of *C. tora* leaf extract (200 – 400 mg/kg i.p.), aspirin (150 mg/kg i.p.) on 0.75% glacial acetic acid (10 ml/kg i.p.) – induced writhing in mice.

Drug (mg/kg)	**Time (min)**	**Mean Writhe ± SEM**	**% In Relation to Control**
Control	30 – 120	37.25 ± 0.9	$^{37.25}/_{37.25}$ x 100 = 100
C. tora			
200	30	14.67 ± 0.9	$^{14.67}/_{37.25}$ x 100 = 39.4
400	30	7.67 ± 0.3	$^{7.67}/_{37.25}$ x 100 = 20.6
Aspirin			
150	30	0.00 ± 0.0	$^{0.00}/_{37.25}$ x 100 = 0.0
C. tora			
200	60	11.33 ± 0.3	$^{11.33}/_{37.25}$ x 100 = 30.4
400	60	7.00 ± 0.6	$^{7.00}/_{37.25}$ x 100 = 18.8
Aspirin			
150	60	0.00 ± 0.0	$^{0.00}/_{37.25}$ x 100 = 0.0
C. tora			
200	90	13.33 ± 0.9	$^{13.33}/_{37.25}$ x 100 = 35.8
400	90	5.00 ± 1.2	$^{5.00}/_{37.25}$ x 100 = 13.4
Aspirin			
150	90	0.00 ± 0.0	$^{0.00}/_{37.25}$ x 100 = 0.0
C. tora			
200	120	23.00 ± 0.6	$^{23.00}/_{37.25}$ x 100 = 61.7
400	120	4.00 ± 0.6	$^{4.00}/_{37.25}$ x 100 = 10.7
Aspirin			
150	120	4.00 ± 0.4	$^{4.00}/_{37.25}$ x 100 = 10.7

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Appendix XII: Effect of *C. tora* leaf extract (50 – 100 mg/kg i.p.) and aspirin (50 mg/kg i.p.) on force – induced pain in mice.

Drug (mg/kg)	**Tail Flick Force (gram) ± SEM**			
	30 min	**60 min**	**90 min**	**120 min**
Control	146.25 ± 23.22	157.50 ± 18.91	150.00 ± 31.82	153.75 ± 9.44
C. tora				
50	207.75 ± 24.14	206.25 ± 19.72	195.00 ± 37.25	195.00 ± 32.98
100	262.50 ± 52.86	217.50 ± 27.73	195.00 ± 31.22	187.50 ± 29.05
Aspirin				
50	232.50 ± 48.41	225.00 ± 23.72	217.50 ± 39.45	213.75 ± 60.12