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

Dissertation work submitted to

The Tamilnadu Dr. M.G.R. Medical University, Chennai

In partial fulfillment for the award of degree of

MASTER OF PHARMACY

IN

PHARMACOLOGY

Submitted by

Reg no: 261525752

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CERTIFICATE

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DECLARATION

I Hereby I declare that this thesis work **"A PHARMACOLOGICAL EVALUATION FOR THE ETHANOLIC EXTRACT OF *ALPINIA CALCARATA* RHIZOME FOR IT'S ANTI – ASTHMATIC, ANTIOXIDANT AND ANTI – INFLAMMATORY ACTIVITIES",** has been originally carried out by myself under the guidance and supervision of **Prof. Dr. R. Anandan, M.Pharm. Ph.D.,** Professor, Department of Pharmacology, Padmavathi College of Pharmacy and Research Institute, Periyanahalli, Dharmapuri, Tamilnadu – 635 205.This work has not been submitted for any degree at any university.

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EVALUATION CERTIFICATE

This is to certify that dissertation entitled **"A PHARMACOLOGICAL EVALUATION FOR THE ETHANOLIC EXTRACT OF *ALPINIA CALCARATA* RHIZOME FOR IT'S ANTI – ASTHMATIC, ANTIOXIDANT AND ANTI – INFLAMMATORY ACTIVITIES",** constitutes the original work carried out by **Mr.Akhil.S.B Reg.No: 261525752**, under the guidance and supervision of **Prof. Dr. R. Anandan, M.Pharm. Ph.D.,** Professor, Department of Pharmacology, Padmavathi College of Pharmacy and Research Institute, Periyanahalli, Dharmapuri, Tamilnadu – 635 205, has been evaluated on_____

Evaluators:

1. 2.

DEDICATED TO MY BELOVED
FAMILY, TEACHERS AND FRIENDS

INTRODUCTION ABOUT HERBAL DRUGS

The term "**medicinal plant**" include various types of plants used in herbalism ("herbology" or "herbal medicine"). It is the use of plants for medicinal purposes, and the study of such uses. The word "**herb**" has been derived from the Latin word, *"herba"* and an old French word *"herbe".* Now days, herb refers to any part of the plant like fruit, seed, stem, bark, flower, leaf, stigma or a root, as well as a non-woody plant. Earlier, the term "herb" was only applied to non-woody plants, including those that come from trees and shrubs. These medicinal plants are also used as food, flavonoid, medicine or perfume and also in certain spiritual activities.

Plants have been used for medicinal purposes long before prehistoric period. Ancient Unani manuscripts Egyptian papyrus and Chinese writings described the use of herbs. Evidence exist that Unani Hakims, Indian Vaids and European and Mediterranean cultures were using herbs for over 4000 years as medicine. Indigenous cultures such as Rome, Egypt, Iran, Africa and America used herbs in their healing rituals, while other developed traditional medical systems such as Unani, Ayurveda and Chinese Medicine in which herbal therapies were used systematically.

Traditional systems of medicine continue to be widely practiced on many accounts. Population rise, inadequate supply of drugs, prohibitive cost of treatments, side effects of several synthetic drugs and development of resistance to currently used drugs for infectious diseases have led to increased emphasis on the use of plant materials as a source of medicines for a wide variety of human ailments.

Among ancient civilizations, India has been known to be rich repository of medicinal plants. The forest in India is the principal repository of large number of medicinal and aromatic plants, which are largely collected as raw materials for manufacture of drugs and perfumery products. About 8,000 herbal remedies have been codified in AYUSH systems in INDIA. Ayurveda, Unani, Siddha and Folk (tribal) medicines are the major systems of indigenous medicines. Among these systems, Ayurveda and Unani Medicine are most developed and widely practiced in India.

Recently, WHO (World Health Organization) estimated that 80 percent of people worldwide rely on herbal medicines for some aspect of their primary health care

needs. According to WHO, around 21,000 plant species have the potential for being used as medicinal plants.

As per data available over three-quarters of the world population relies mainly on plants and plant extracts for their health care needs. More than 30% of the entire plant species, at one time or other were used for medicinal purposes. It has been estimated, that in developed countries such as United States, plant drugs constitute as much as 25% of the total drugs, while in fast developing countries such as India and China, the contribution is as much as 80%. Thus, the economic importance of medicinal plants is much more to countries such as India than to rest of the world. These countries provide two third of the plants used in modern system of medicine and the health care system of rural population depend on indigenous systems of medicine.

Treatment with medicinal plants is considered very safe as there is no or minimal side effects. These remedies are in sync with nature, which is the biggest advantage. The golden fact is that, use of herbal treatments is independent of any age groups and the sexes.

The ancient scholars only believed that herbs are only solutions to cure a number of health related problems and diseases. They conducted thorough study about the same, experimented to arrive at accurate conclusions about the efficacy of different herbs that have medicinal value. Most of the drugs, thus formulated, are free of side effects or reactions. This is the reason why herbal treatment is growing in popularity across the globe. These herbs that have medicinal quality provide rational means for the treatment of many internal diseases, which are otherwise considered difficult to cure.

Medicinal plants such as *Aloe, Tulsi, Neem, Turmeric* and *Ginger* cure several common ailments. These are considered as home remedies in many parts of the country. It is known fact that lots of consumers are using Basil (*Tulsi*) for making medicines, black tea, in *Pooja* and other activities in their day to day life.

In several parts of the world many herbs are used to honour their kings showing it as a symbol of luck. Now, after finding the role of herbs in medicine, lots of consumers started the plantation of tulsi and other medicinal plants in their home gardens.

Medicinal plants are considered as a rich resources of ingredients which can be used in drug development either pharmacopoeial, non- pharmacopoeial or synthetic drugs. A part from that, these plants play a critical role in the development of human cultures around the whole world. Moreover, some plants are considered as important source of nutrition and as a result of that they are recommended for their therapeutic values. Some of these plants include ginger, green tea, walnuts, aloe, pepper and turmeric etc. Some plants and their derivatives are considered as important source for active ingredients which are used in aspirin and toothpaste etc.

Apart from the medicinal uses, herbs are also used in natural dye, pest control, food, perfume, tea and so on. In many countries different kinds of medicinal plants/ herbs are used to keep ants, flies, mice and flee away from homes and offices. Now a day's medicinal herbs are important sources for pharmaceutical manufacturing.

Recipes for the treatment of common ailments such as diarrhoea, constipation, hypertension, low sperm count, dysentery and weak penile erection, piles, coated tongue, menstrual disorders, bronchial asthma, leucorrhoea and fevers are given by the traditional medicine practitioners very effectively.

Over the past two decades, there has been a tremendous increase in the use of herbal medicine; however, there is still a significant lack of research data in this field. Therefore, since 1999, WHO has published three volumes of the WHO monographs on selected medicinal plants.

Importance of some herbs with their medicinal values

- Herbs such as black pepper, cinnamon, myrrh, aloe, sandalwood, ginseng, red clover, burdock, bayberry, and safflower are used to heal wounds, sores and boils.
- Basil, Fennel, Chives, Cilantro, Apple Mint, Thyme, Golden Oregano, Variegated Lemon Balm, Rosemary, Variegated Sage are some important medicinal herbs and can be planted in kitchen garden. These herbs are easy to grow, look good, taste and smell amazing and many of them are magnets for bees and butterflies.
- Many herbs are used as blood purifiers to alter or change a long-standing condition by eliminating the metabolic toxins. These are also known as 'blood

cleansers'. Certain herbs improve the immunity of the person, thereby reducing conditions such as fever.

- Some herbs are also having antibiotic properties. Turmeric is useful in inhibiting the growth of germs, harmful microbes and bacteria. Turmeric is widely used as a home remedy to heal cut and wounds.
- To reduce fever and the production of heat caused by the condition, certain antipyretic herbs such as *Chirayta*, black pepper, sandal wood and safflower are recommended by traditional Indian medicine practitioners.
- Sandalwood and Cinnamon are great astringents apart from being aromatic. Sandalwood is especially used in arresting the discharge of blood, mucus etc.
- Some herbs are used to neutralize the acid produced by the stomach. Herbs such as marshmallow root and leaf. They serve as antacids. The healthy gastric acid needed for proper digestion is retained by such herbs.
- Indian sages were known to have remedies from plants which act against poisons from animals and snake bites.
- Herbs like Cardamom and Coriander are renowned for their appetizing qualities. Other aromatic herbs such as peppermint, cloves and turmeric add a pleasant aroma to the food, thereby increasing the taste of the meal.
- Some herbs like aloe, sandalwood, turmeric, sheet raj hindi and khare khasak are commonly used as antiseptic and are very high in their medicinal values.
- Ginger and cloves are used in certain cough syrups. They are known for their expectorant property, which promotes the thinning and ejection of mucus from the lungs, trachea and bronchi. Eucalyptus, Cardamom, Wild cherry and cloves are also expectorants.
- Herbs such as Chamomile, Calamus, Ajwain, Basil, Cardamom, Chrysanthemum, Coriander, Fennel, Peppermint and Spearmint, Cinnamon, Ginger and Turmeric are helpful in promoting good blood circulation. Therefore, they are used as cardiac stimulants.
- Certain medicinal herbs have disinfectant property, which destroys disease causing germs. They also inhibit the growth of pathogenic microbes that cause communicable diseases.
- Herbal medicine practitioners recommend calmative herbs, which provide a soothing effect to the body. They are often used as sedatives.

- Certain aromatic plants such as Aloe, Golden seal, Barberry and Chirayata are used as mild tonics. The bitter taste of such plants reduces toxins in blood. They are helpful in destroying infection as well.
- Certain herbs are used as stimulants to increase the activity of a system or an organ, for example herbs like Cayenne (Lal Mirch, Myrrh, Camphor and Guggul).
- A wide variety of herbs including Giloe, Golden seal, Aloe and Barberry are used as tonics. They can also be nutritive and rejuvenate a healthy as well as diseased individual.
- Honey, turmeric, marshmallow and liquorice can effectively treat a fresh cut and wound. They are termed as vulnerary herbs.

As our lifestyle is now getting techno-savvy, we are moving away from nature. While we cannot escape from nature because we are part of nature. As herbs are natural products they are free from side effects, they are comparatively safe, eco-friendly and locally available. Traditionally there are lot of herbs used for the ailments related to different seasons. There is a need to promote them to save the human lives.

These herbal products are today being the symbol of safety in contrast to the synthetic drugs, that are regarded as unsafe to human being and environment. Although herbs had been priced for their medicinal, flavouring and aromatic qualities for centuries, the synthetic products of the modern age surpassed their importance, for a while. However, the blind dependence on synthetics is over and people are returning to the naturals with hope of safety and security. It's time to promote them globally.[64]

1.1. ASTHMA

Asthma is a chronic inflammatory lung disease that can cause repeated episodes of cough, wheezing and breathing difficulty.

During an acute asthma episode, the airway lining in the lungs becomes inflamed and swollen. In addition, mucus production occurs in the airway and muscles surrounding the airway spasm. Combined, these cause a reduction in air flow. [1]

1.1.1. Asthma is characterized by:

- Airway inflammation: The airway lining becomes red, swollen, and narrow.
- Airway obstruction: The muscles encircling the airway tighten causing the airway to narrow making it difficult to get air in and out of the lungs.
- Airway hyper-responsiveness: The muscles encircling the airway respond more quickly and vigorously to small amounts of allergens and irritants.

1.1.2. Common signs and symptoms of an acute asthma episode include:

- Coughing
- Wheezing
- Breathlessness
- Respiratory rate increased
- Chest tightness
- Chest or abdominal pain
- Fatigue, feeling out of breath
- Agitation
- Increased pulse rate

1.1.3. Causes

- Allergens from nature, typically inhaled, which include waste from common household pests.
- Indoor air pollution from volatile organic compounds.
- Medications, aspirin,βadrenergic antagonists (beta blockers), and penicillin.
- Food allergies such as milk, peanuts, and eggs.

1.1.4. Pathophysiology and pathogenesis of asthma

Airflow limitation in asthma is recurrent and caused by a variety of changes in the airway. These include:

- **Bronchoconstriction**

In asthma, the dominant physiological event leading to clinical symptoms is airway narrowing and a subsequent interference with airflow. In acute exacerbations of asthma, bronchial smooth muscle contraction (bronchoconstriction) occurs quickly to narrow the airways in response to exposure to a variety of stimuli including allergens or irritants. Allergen-induced acute bronchoconstriction results from an IgE-dependent release of mediators from mast cells that includes histamine, tryptase, leukotrienes and prostaglandins that directly contract airway smooth muscle.

- **Airway edema**

As the disease becomes more persistent and inflammation more progressive, other factors further limit airflow. These include edema, inflammation, mucus hypersecretion and the formation of inspissated mucus plugs, as well as structural changes including hypertrophy and hyperplasia of the airway smooth muscle. These latter changes may not respond to usual treatment.

- **Airway hyper responsiveness**

This is an exaggerated bronchoconstrictor response to a wide variety of stimuli is a major, but not necessarily unique, feature of asthma.

- **Airway remodeling**

In some persons who have asthma, airflow limitation may be only partially reversible. Permanent structural changes can occur in the airway; these are associated with a progressive loss of lung function that is not prevented by or fully reversible by current therapy. Airway remodeling involves an activation of many of the structural cells, with consequent permanent changes in the airway that increase airflow obstruction and airway responsiveness and render the patient less responsive to therapy.[2]

1.1.5. Pathophysiologic mechanism in the development of air way inflammation

Inflammation has a central role in the pathophysiology of asthma. As noted in the definition of asthma, airway inflammation involves an interaction of many cell types and multiple mediators with the airways that eventually results in the characteristics pathophysiologic features of the disease, bronchial inflammation and airflow limitation that result in current episodes of cough, wheeze, and shortness of breath. The process by which these interactive events occur and leads to asthma are still under investigation. The pattern of airway inflammation in asthma, however does not necessarily vary depending upon the disease severity, persistence and duration of disease. The cellular profile and the response of the structural cells in asthma are quite consistent.[3]

1.1.6. Inflammatory Cells

- Lymphocytes

  An increased understanding of the development and regulation of airway inflammation in asthma followed the discovery and description of subpopulations of lymphocytes, T helper1 cells and T helper2 cells with distinct inflammatory mediator profiles and asthma as a Th2 disease, recognizing the importance of number of families of cytokines and chemokines has advanced our understanding of the development of airway inflammation effects on airway function. After the discovery of these distinct lymphocyte subpopulations in animal models of allergic inflammation, evidence emerged that, in human asthma, a shift, or predilection, toward the Th2-cytokine profile resulted in the eosinophilic inflammation characteristic of asthma. In addition, generation of Th2 cytokines (e.g., interleukin-4 (IL-4), IL-5 and IL-3) could also explain the overproduction of IgE, presence of eosinophils, and development of airway hyperresponsiveness. There also may be a reduction in a subgroup of lymphocytes, regulatory T cells, which normally inhibit Th2 cells, as well as an increase in natural killer (NK) cells that release large amounts of Th1 and Th2 cytokines. T lymphocytes, along with other airway resident cells, also can determine the development and degree of airway remodeling.

- Mast cells

  Activation of mucosal mast cells releases bronchoconstrictor mediators (histamine, cysteinyl-leukotrienes, prostaglandin D_2). Although allergen activation occurs through high affinity IgE receptors and is likely the most relevant reaction, sensitized mast cells also may be activated by osmotic stimuli to account for exercise induced bronchospasm (EIB). Increased number of mast cells in airway smooth muscle may be linked to airway hyperresponsiveness. Mast cells also can release a large number of cytokines to change the airway environment and promote inflammation even though exposure to allergens is limited.

- Eosinophils

  Increased numbers of eosinophils exist in the airways of most, but not all, persons who have asthma. These cells contain inflammatory enzymes, generate leukotrienes, and express a wide variety of pro-inflammatory cytokines. Increases in eosinophils often correlate with greater asthma severity. In addition, numerous studies show that treating asthma with corticosteroids reduces circulating and airway eosinophils in parallel with clinical improvement. However, the role and contribution of eosinophils to asthma is undergoing a reevaluation based on studies with an anti-IL-5 treatment that has significantly reduced eosinophils but did not affect asthma control. Therefore, although the eosinophil may not be the only primary effector cell in asthma, it likely has a distinct role in different phases of the disease.

- Neutrophils

  Neutrophils are increased in the airways and sputum of persons who have severe asthma, during acute exacerbations, and in the presence of smoking. Their pathophysiological role remains uncertain; they may be a determinant of a lack of response to corticosteroid treatment. The regulation of neutrophil recruitment, activation, and alteration in lung function is still under study, but leukotriene B_4 may contribute to these processes.

- Dendritic cells

  These cells function as key antigen-presenting cells that interact with allergens from the airway surface and then migrate to regional lymph nodes to interact with regulatory cells and ultimately to stimulate Th2 cell production from naive T cells.

- Macrophages

  Macrophages are the most numerous cells in the airways and also can be activated by allergens through low-affinity IgE receptors to release inflammatory mediators and cytokines that amplify the inflammatory response.[4]

1.1.7. Inflammatory mediators

- Chemokines are important in recruitment of inflammatory cells into the airways and are mainly expressed in airway epithelial cells. Eotaxin is relatively selective for eosinophils, whereas thymus and activation-regulated chemokines (TARCs) and macrophage-derived chemokines (MDCs) recruit Th2 cells. There is an increasing appreciation for the role this family of mediators has in orchestrating injury, repair, and many aspects of asthma.
- Cytokines direct and modify the inflammatory response in asthma and likely determine its severity. Th2-derived cytokines include IL-5, which is needed for eosinophil differentiation and survival, and IL-4 which is important for Th2 cell differentiation and with IL-13 is important for IgE formation. Key cytokines include IL-1β and tumor necrosis factor-α (TNF-α), which amplify the inflammatory response, and granulocyte-macrophage colony-stimulating factor (GM-CSF), which prolongs eosinophil survival in airways. Recent studies of treatments directed toward single cytokines (e.g., monoclonal antibodies against IL-5 or soluble IL-4 receptor) have not shown benefits in improving asthma outcomes.
- Cysteinyl-leukotrienes are potent bronchoconstrictors derived mainly from mast cells. They are the only mediator whose inhibition has been specifically associated with an improvement in lung function and asthma symptoms. Recent studies have also shown leukotriene B_4 can contribute to the inflammatory process by recruitment of neutrophils.

- Nitric oxide (NO) is produced predominantly from the action of inducible NO synthase in airway epithelial cells; it is a potent vasodilator. Measurements of fractional exhaled NO
  (FeNO) may be useful for monitoring response to asthma treatment because of the purported association between FeNO and the presence of inflammation in asthma).
- Immunoglobulin E (IgE) is the antibody responsible for activation of allergic reactions and is important to the pathogenesis of allergic diseases and the development and persistence of inflammation. IgE attaches to cell surfaces via a specific high-affinity receptor. The mast cell has large numbers of IgE receptors; these, when activated by interaction with antigen, release a wide variety of mediators to initiate acute bronchospasm and also to release pro-inflammatory cytokines to perpetuate underlying airway inflammation.[5]

1.1.8. Treatment

There are number of treatments that can help effectively control or sooth the asthmatic condition. Treatment is based on two important goals, which are (i) specific regimens for the treatment of acute attack by opening swollen airways that are limiting breathing and (ii) prophylactic measures to reduce the inflammation and airway resistance and to maintain airflow. Treatment and prevention involves a combination of medicines, life style advices and identifying and then avoiding potential asthma triggers.

Drugs which are indicated for the treatment of asthma includes the classes of beta2 agonists, corticosteroids, leukotriene inhibitors and xanthenes. They are available in the forms of inhalations, tablets, capsules and injections are used based on medical condition and supervision. Inhalation preparation includes solutions for nebulization, metered dose inhalers and powdered inhalers. Asthma medicines are usually given by inhalers, which are the devices that deliver the drug directly into the airways through your mouth when you breathe.

Drugs for treating asthma are divided into two categories: (1) Quick-relief medications (which are used to relief acute asthma) and (2) Long-term asthma control medications (which are used as prophylactic measures).

1. Quick-relief medications: they are used as needed for rapid, short-term symptom relief during an asthma attack. Types of quick-relief medications are

a) Short-acting beta$_2$ agonists: these inhaled, quick-relief bronchodilators act within minutes to rapidly ease symptoms during an asthma attack. Short-acting beta$_2$ agonists can be taken using a portable, hand-held inhaler or a nebulizer. Examples are salbutamol and terbutaline.
b) Antimuscarinics: these inhaled antimuscarinics act quickly to immediately relax the airways, like other bronchodilators, making it easier to breathe. Examples are ipratropium and tiotropium.
c) Systemic corticosteroids: these systemic corticosteroids (i.e., oral and intravenous routes) relieve airway inflammation caused by severe asthma. However, due to serious side effects when used long term, the systemic routes are used only on a short-term basis to treat severe asthma symptoms. Examples are prednisone and methyl prednisone.
d) Intravenous xanthines: these xanthines relax smooth muscle and to relieve bronchial spasm and are indicated for severe asthma attack. Example is aminophylline.

2. Long-term asthma control medications: they work to reduce the amount of inflammation in the airways and prevent asthma attacks occurring.

a) Inhaled corticosteroids: they are the most effective preventers, however, you may need to use these medications for several days to weeks before they reach their maximum benefit. Examples are fluticasone and budesonide.
b) Long-acting beta$_2$ agonists: these inhaled medications open the airways. Some research shows that they may increase the risk of a severe asthma attack, unless they are used in combination with an inhaled corticosteroid. Examples are salmeterol and formoterol.
c) Leukotriene inhibitors: they act against one of the inflammatory components of asthma and provide protection against bronchoconstriction when taken before

exercise or exposure to allergen or to cold air. Examples of leukotriene inhibitors include montelukast and zafirlukast.

d) Xanthines : apart from the relaxiation of bronchial muscle and bronchial spasm they can stimulant effect on respiration and have anti-inflammatory effects. Example is theophylline.[6]

1.2. INFLAMMATION

Inflammation is defined as the local response of living mammalian tissue to injury due to any agent. It is a body defense reaction in order to eliminate or limit the spread of injurious agent.

1.2.1. Causes of inflammation

- Infective agents. E.g.: Bacteria, viruses and their toxins.
- Immunological agents. E.g.: Cell mediated and antigen antibody reaction.
- Physical agents. E.g.: Heat, cold, radiation, mechanical trauma.
- Chemical agents. E.g.: Organic and inorganic poisons

1.2.2. Signs of inflammation

The main signs of inflammation are

- Redness (Latin rubor)
- Heat (calor)
- Swelling (tumor)
- Pain (dolor)

1.2.3. Inflammation may be classified as

A) Acute inflammation

B) Chronic inflammation

A. Acute inflammation

Acute inflammation is immediate and early response to tissue. Main features are listed below

a) Vasodilation

b) Vascular leakage and edema

c) Leukocyte emigration

a) Vasodilation

Brief arteriolar vasoconstriction followed by vasodilation

- Accounts for warmth and redness.
- Opens microvascular beds.
- Increased intravascular pressure causes an early transudate into interstitium.

b) Vascular leakage

- Transudate gives way to exudate (protein-rich)
- Increases interstitial osmotic pressure contributing to edema (water and ions)

Five mechanisms known to cause vascular leakiness

1. Histamines, bradykinins, leukotrienes cause an earl, brief (15-30 min) immediate transient response, in the form of endothelial cell contraction that widens intercellular gaps of venules.

2. Cytokines mediators (TNF, IL-1) induce endothelial junction retraction through cytoskeleton reorganization.

3. Severe injuries may cause immediate direct endothelial cell damage (necrosis, detachment) making them leaky until they are repaired (immediate sustained response), or may cause delayed damage as in thermal or UV injury.

4. Marginating and endothelial cell adherent leukocytes may pile-up and damage the endothelium through activation and release of toxic oxygen radicals and proteolytic enzymes.

5. Some bacterial toxins.[7]

c) Leucocyte emigration

Leukocytes leave the vasculature routinely through the following sequence of events:

- Margination and rolling
- Adhesion and transmigration
- Chemotaxis and activation

Chemical mediators

• Plasma-derived:

- Complement, kinins, coagulation factors
- Many in “pro-form” requiring activation (enzymatic cleavage) [8]

• Cell-derived:

- Preformed, sequestered and released (mast cell histamine)
- Synthesized as needed (prostaglandin)[9]

B. Chronic inflammation

Chronic inflammation is defined as prolonged process in which tissue destruction and inflammation occurs at the same time. The characteristic features of chronic inflammation are the presence of chronic inflammatory cells such as lymphocytes, plasma cells and macrophages.[10]

Causes of chronic inflammation

- Chronic inflammation following acute inflammation
- Recurrent attacks of chronic inflammation

Characteristic features

- Necrosis
- Mononuclear cell infiltration
- Proliferative changes

Types of chronic inflammation

a. Non specific

   This type of chronic inflammation is occurred with the formation of granulation tissue E.g: chronic ulcer.

b. Specific

   When the injurious agent causes a characteristic histologic tissue response

   E.g: syphilis[11]

Granulomatous inflammation

Granuloma is defined as the tiny lesion about 1 mm diameter, composed predominantly of collection of modified macrophages called epithelioid cells and rimmed at periphery by lymphoid cells.

Examples of granulomatous Inflammation-Tuberculosis, leprosy, fungal infections.[12]

1.3. ANTIOXIDANTS

The ability to utilize oxygen has provided humans with the benefit of metabolizing fats, proteins, and carbohydrates for energy; however, it does not come without cost. Oxygen is a highly reactive atom that is capable of becoming part of potentially damaging molecules commonly called “free radicals.” Free radicals are capable of attacking the healthy cells of the body, causing them to lose their structure and function.[13]

Cell damage caused by free radicals appears to be a major contributor to aging and to degenerative diseases of aging such as cancer, cardiovascular disease, cataracts, immune system decline, and brain dysfunction. Overall, free radicals have been implicated in the pathogenesis of at least 50 diseases. Fortunately, free radical formation is controlled naturally by various beneficial compounds known as antioxidants. It is when the availability of antioxidants is limited that this damage can become cumulative and debilitating.[14]

Free radicals are electrically charged molecules, i.e., they have an unpaired electron, which causes them to seek out and capture electrons from other

substances in order to neutralize themselves. Although the initial attack causes the free radical to become neutralized, another free radical is formed in the process, causing a chain reaction to occur. And until subsequent free radicals are deactivated, thousands of free radical reactions can occur within seconds of the initial reaction. Antioxidants are capable of stabilizing, or deactivating, free radicals before they attack cells. Antioxidants are absolutely critical for maintaining optimal cellular and systemic health and well-being.[15]

1.3.1. Reactive oxygen species

Reactive oxygen species (ROS) is a term which encompasses all highly reactive, oxygen-containing molecules, including free radicals. Types of ROS include the hydroxyl radical, the superoxide anion radical, hydrogen peroxide, singlet oxygen, nitric oxide radical, hypochlorite radical, and various lipid peroxides. All are capable of reacting with membrane lipids, nucleic acids, proteins and enzymes, and other small molecules, resulting in cellular damage.[16]

ROS are generated by a number of pathways. Most of the oxidants produced by cells occur as:

• A consequence of normal aerobic metabolism: approximately 90% of the oxygen utilized by the cell is consumed by the mitochondrial electron transport system.

• Oxidative burst from phagocytes (white blood cells) as part of the mechanism by which bacteria and viruses are killed, and by which foreign proteins (antigens) are denatured.

• Xenobiotic metabolism, i.e., detoxification of toxic substances.

Consequently, things like vigorous exercise, which accelerates cellular metabolism; chronic inflammation, infections, and other illnesses; exposure to allergens and the presence of “leaky gut” syndrome; and exposure to drugs or toxins such as cigarette smoke, pollution, pesticides, and insecticides may all contribute to an increase in the body’s oxidant load.[17]

1.3.2. Antioxidant protection

To protect the cells and organ systems of the body against reactive oxygen species, humans have evolved a highly sophisticated and complex antioxidant protection system. It involves a variety of components, both endogenous and exogenous in origin, that function interactively and synergistically to neutralize free radicals. These components include.[18, 19]

• Nutrient derived antioxidants like ascorbic acid (vitamin C), tocopherols and tocotrienols (vitamin E), carotenoids and other low molecular weight compounds such as glutathione and lipoic acid.

• Antioxidant enzymes, e.g., superoxide dismutase, glutathione peroxidase, and glutathione reductase, which catalyze free radical quenching reactions.

• Metal binding proteins, such as ferritin, lactoferrin, albumin, and ceruloplasmin that sequester free iron and copper ions that are capable of catalyzing oxidative reactions.[20]

• Numerous other antioxidant phytonutrients present in a wide variety of plant foods.

Table No.1: Various reactive oxygen species (ROS) and corresponding neutralizing antioxidants

ROS	NEUTRALIZING ANTIOXIDANTS
Hydroxyl radical	Vitamin C, glutathione, lipoic acid
Superoxide radical	Vitamin C, glutathione, flavonoids
Hydrogen peroxide	Glutathione, beta carotene, vitamin E
Lipid peroxides	Beta carotene, vitamin E, ubiquinone

1.3.3. Dietary antioxidants

Vitamin C, vitamin E, and beta carotene are among the most widely studied dietary antioxidants. Vitamin C is considered the most important water-soluble antioxidant in extracellular fluids. It is capable of neutralizing ROS in the aqueous phase before lipid peroxidation is initiated. Vitamin E, a major lipid-soluble antioxidant, is the most effective chain-breaking antioxidant within the cell membrane where it protects membrane fatty acids from lipid peroxidation. Vitamin C has been cited as being capable of regenerating vitamin E.[21,22]

1.3.4. Phytonutrients

A number of other dietary antioxidant substances exist beyond the traditional vitamins discussed above. Many plant-derived substances, collectively termed "phytonutrients," or "phytochemicals," are becoming increasingly known for their antioxidant activity. Phenolic compounds such as flavonoids are ubiquitous within the plant kingdom: approximately 3,000 flavonoid substances have been described. In plants, flavonoids serve as protectors against a wide variety of environmental stresses while, in humans, flavonoids appear to function as "biological response modifiers.[23,24]

1.3.5. Endogenous antioxidants

In addition to dietary antioxidants, the body relies on several endogenous defense mechanisms to help protect against free radical-induced cell damage. The antioxidant enzymes – glutathione peroxidase, catalase, and superoxide dismutase (SOD) – metabolize oxidative toxic intermediates and require micronutrient cofactors such as selenium, iron, copper, zinc, and manganese for optimum catalytic activity.[25,26]

1.3.6. Antioxidant system in our body

The body has developed several endogenous antioxidant systems to deal with the reactive oxygen species.

- Non-enzymatic system

  This include both lipid soluble vitamins and water soluble vitamins

  E.g.: Lipid soluble vitamins-α- tocopherol (vitamin E), carotenes

Water soluble vitamins-ascorbic acid.

- Enzymatic systems

E.g.: Super oxide dismutase, catalase, peroxidase.[27,28]

Herbs are found out to be a source of various phytochemicals which possess antioxidant property.

1.4. ADVANTAGES OF HERBAL MEDICATION

Herbal medicine exhibit fewer side effects and they are very much safe. Plants are gifts of natural to mankind for treating different types of diseases. Herbal medicines are cheaper and easily available. Also for certain diseases like hepatitis, herbs and herbal drugs are the only remedies. The traditional medicine is largely getting popularity over allopathic medicine because of their cost, availability and free from side effects.[29]

The plant *Alpinia calcarata* has been traditionally used for various diseases. In present study rhizomes of the plant *Alpinia calcarata* have been used which traditionally indicated in the treatment of asthma.[30]

1.5. HERBAL DRUGS USED IN ANTI – ASTHMATIC STUDY

Table No.2: List of Herbal Drugs used in Anti – asthmatic study

SL. NO	PLANT	FAMILY	PART	EXTRACT	MODEL	AUTHORS
1	*Aerva lanta*	*Amaranthaceae*	Aerial parts	Ethanol	1) 100 µg/mL in the isolated goat tracheal chain preparation model. 2) 30 and 60 mg/kg doses orally in clonidine-	Kumar D, Prasad DN, Parkash J, Bhatnaga.S. P, Kumar.D.[65]

					induced catalepsy. 3) Mast cell degranulation in mice possesses anti-asthmatic activity	
2	*Ageratum conyzoides*	*Asteraceae*	Leaves	Hydro alcoholic	1) Inhibiting clonidine induced catalepsy in mice	Tote MV, Mahire NB, Jain AP, Bose S, Undale VR, Bhosale [66]
3	*Asystasia gangetica*	*Acanthaceae*	Leaves	Evaluated hexane, ethyl acetate, and methanol extracts	1)Anti-asthmatic activity using guinea pig trachea, 2) Rat stomach strip, 3) Guinea pig ileal preparation, 3) Egg albumin-induced acute inflammation.	Akah PA, Ezike AC, Nwafor SV, Okoli CO, Enwerem. NM[67]

4	*Bacopa monnieri*	*Scrophulariaceae*	Leaves	Evaluated petroleum ether, chloroform, methanol and water extracts	1)Mast cell stabilizing activity in rats.	Samiulla DS, Prashanth.D , Amit A [68]
5	*Cassia sophera*	*Caesalpiniaceae*	Leaves	Ethanol	1) Anti-asthmatic activity in carrageenan induced paw edema, 2) Histamine induced bronchoconstriction, 3) Clonidine and haloperidol induced catalepsy, 4) Milk induced leukocytosis, and eosinophilia, 5)Passive paw anaphylaxis animal-models.	Nagore DH, Ghosh VK, Patil MJ.[69]
6	*Casuarina equisetifolia*	*Casuarinaceae*	Wood & Bark	Methanol	1)Antihistami-nic activity by	Aher AN,

					inhibiting the histamine induced contraction of trachea (10-80 mcg/mL), 2) Clonidine induced catalepsy and mast cell degranulation at doses 100 mg/kg.	Pal SC, Pati UK, Yadav SK, Bhattacharya S.[70]
7	*Clerodendrum Serratum*	*Verbenaceae*	Roots	Ethanol	1)Anti-asthmatic activity using isolated goat tracheal chain preparation, 2) Clonidine induced catalepsy, 3) Milk induced leukocytosis and eosinophilia in mice.	Bhujbal SS, Kewatkar SM, Kumar.D, Mudgade.SC , Patil MJ.[71]
8	*Cnidium monnieri*	*Umbelliferae*	Fruits	Ethanol	1) In passive cutaneous anaphylaxis in rats.	Matsuda H, Tomohiro N, Yasuko,

						Kubo M.[72]
9	*Curculigo orchioides*	*Amaryllidaceae*	Rhizome	Alcoholic	1) Induced mast cell degranulation and systemic anaphylaxis, 2) Inhibited histamine-induced contraction in goat trachea, guinea pig ileum, 3)Bronchoconstriction in guinea pigs, 3)Egg albumin induced passive paw anaphylaxis in rats, 4) Milk induced leukocytosis and eosinophilia, 5) Clonidine induced catalepsy in mice.	Venkatesh.P ,Mukherjee. PK, Satheesh Kumar N, Neelesh K Nema, Bandyopadhyay A, Hiroyuki Fukui [73] Pandit P, Singh A, Bafna AR, Kadam PV, Patil MJ [74]

10	*Euphorbia hirta*	*Euphorbiaceae*	Whole aerial part	Ethanol	1) Inhibited the passive cutaneous anaphylaxis and paw anaphylaxis reaction, 2) Protection of mast cell from degranulation.	Youssouf MS, Kaiser.P, Tahir M, Singh GD, Singh S, Sharma VK[75].
11	*Hemidesmus indicus*	*Asclepiadaceae*	Roots	Ethanol	1) Isolated goat tracheal chain preparation, 2) Passive paw anaphylaxis in rat, 3) Clonidine-induced catalepsy in mice.	Bhujbal SS, Kumar D, Deoda RS, Deore TK, Patil MJ.[76]
12	*Ficus bengalensis*	*Moraceae*	Bark	Ethyl acetate, ethanol and aqueous extracts.	1)Antihistami-nic activity by inhibiting clonidine induced catalepsy in mice.	Taur DJ, Nirmal SA, Patil RY[77,78]

1.5. PLANT PROFILE

Figure No.1: *Alpinia calcarata* – PLANT

Figure No.2: Rhizome.

1.5.1. PLANT DESCRIPTION

Selected plant : *Alpinia calcarata*

1.5.1.1. Classification

Kingdom : Plantae

Division : Mangnoliphyta

Class : Liliopsida

Order : Zingiberales

Family : *Zingiberaceae*

Genus : Alpinia

Species : *Alpinia calcarata*

1.5.1.2. Synonyms

Alpinia calcarata Rosk., Alpinia erecta Lodd. and Steud., Alpinia bracheata Rosk., Alpinia cerrnta Sims., Renealmia calcarata Haw., Globba eracta Retx., Languas calcarata Mem.[31]

1.5.1.3. Selected Vernacular Names

Sinhala- Heen aratta, Aratta

English- Galanga, Small galanga

Tamil- Amkolinji

Sanskrit- Rasna[32]

1.5.1.4. Distribution`

It is native to India. Occurs in Southern Malay Peninsula and Sri Lanka. It is common in village gardens in Sri Lanka.[33]

1.5.1.5. Botanical description

Alpinia calcarata is a rhizomatous perennial herb with a non-tuberous rootstock, stems slender, about 75 cm tall; leaves simple, alternate, 25 - 32 cm long and 2.5 - 5 cm broad, lanceolate, acuminate, long-pointed, glabrous on both surfaces and shining on the upper surface, scantily hairy along the margin, petioles sheathing; flowers pinkish white, irregular, bisexual, in pendunculate, terminal, dense flowered panicles 8.5 cm long, two flowers together at each node, one opening earlier than the other, each bearing a pair of bracteoles, the inner one smaller than the outer, bracteoles oblong, papery white, each flower about 4 cm long, pedicels short, hairy; sepals 3, fused into a campanulate tube l cm long, pubescent outside, glabrous inside, apices rounded; petals 3, fused at base but segments free tinged with pink, segments oblong-spathulate, pubescent outside, lateral narrow; staminodes 3, fused at base with the stamen into a tube adnate to corolla, two basal staminodes reduced to minute filaments, the larger one petaloid, 3 cm by 2.3 cm ovate, yellow with vinous red streaks, emarginated, apex frilled and darker, glabrous and shining on both surfaces; stamen, anther tubular, style passing through, filament flat, 1.5 cm long, anther 0.8 cm long, style 3.5 cm long, tinged pink, hairy towards the apex, stigma swollen; ovary inferior, 3 mm long, strongly pubescent, 3-locular with ovules in each loculus on a central axis capsules not seen.[34, 35]

Silvy Mathew et al., (2014)[36] conducted a study on the antimicrobial activity of the plant *Alpinia calcarata* rhizome. The present study was designed to investigate the anti-microbial activity of four solvent extracts (Petroleum ether, Dichloromethane, Acetone and Methanol) of rhizome of *Alpinia calcarata*. The rhizome of this plant is an important antimicrobial agent and a digestive stimulant. The plant extracts showed considerable activity against ten tested strains viz., Pseudomonas aeroginosa, Escherichia coli, Enterobacter aerogens, Bacillus subtilis, Staphylococcus aureus, Streptococcus faecalis, Vibrio cholerae, Salmonella paratyphi, Klebsiella pneumoniae and Proteus vulgaris by using agar disc diffusion assay. This study reveals that *Alpinia calcarata* has antimicrobial activity against gram positive and gram negative bacteria.

Arambewela L et al., (2004)[37] conducted a study on the antinociceptive activities of aqueous and ethanolic extracts of *Alpinia calcarata* rhizomes in rats. Rhizomes of *Alpinia calcarata* (*Zingiberaceae*) possess several bio-activities and are used in traditional medicine of Sri Lanka. However, their antinociceptive activity has not been investigated so far. The aim of this study therefore, was to examine the antinociceptive activity of hot water extract (HWE) and hot ethanol extract (HEE) of *Alpinia calcarata* rhizomes using rats and three models of nociception (tail flick, hot plate and formalin tests). Different concentrations of HWE (100, 250, 500, 750, 1000 mg/kg) and HEE (100, 250, 500, 750, 1000 mg/kg) were made and orally administrated to rats and the reaction times determined. The results showed that the extracts have marked dose-dependent antinociceptive activity, when evaluated in the hot plate and the formalin tests but not in the tail flick test. The antinociceptive effect was slightly higher in HEE than that of HWE. The antinociceptive effect was mediated via opioid mechanisms.

Ramya Rajasekar et al., (2014)[38] proposed a study on the antidiabetic activity of the *Alpinia calcarata* rhizomes. The study was to determine effect of *Alpinia calcarata* on glucose uptake in diabetic rats-an in vitro and in vivo model. Diabetes mellitus is a heterogeneous metabolic disorders characterized by abnormally high levels of blood glucose The main objective of the present work is to study the effect of *Alpinia calcarata* on glucose uptake in streptozotocin (STZ) induced diabetic rats.

The diabetes was induced by single dose of STZ (45 mg/kg) in citrate buffer, while the normal control group was given the vehicle (citrate buffer) only. After induction of diabetes, the diabetic animals were treated with ethanolic extract of *Alpinia calcarata* (200 mg/kg) and glibenclamide (2 mg/kg) for 30 days. Blood glucose estimation was performed every week of the study. At the end of study period, animals were sacrificed for biochemical studies.

Streptozotocin induced diabetic rats shows the altered levels of various biochemical profiles. Those levels were brought back to near normal upon treatment with ethanolic extract of *Alpinia calcarata* and standard drug glibanclamide. No significant changes were observed on treatment with plant.

Lakshmi Arambewela et al., (2011)[39] conducted a study on the safety profile of the plant *Alpinia calcarata* rhizomes. The aim of the present study was to investigate whether *Alpinia calcarata* (Family*: Zingiberaceae*) rhizomes have any toxic effects in rats. *Albino wistar rats* were used as the experimental model and orally administered hot water extract (HWE) and hot ethanolic extract (HEE) of *Alpinia calcarata* rhizomes at a dose of 1500 mg/kg respectively for 42 consecutive days. Administration of the HWE or HEE to rats did not result in any chronic toxic effects as evident from their effects on (a) liver function (b) kidney function (c) hematological parameters such as red blood cell (RBC) count, white blood cell (WBC) count and haemoglobin (Hb) concentration (d) external morphology and wet weights of selected organs. Further, the HWE and the HEE did not appear to mediate any unacceptable effects on food and water intake, % weight gain, consistency of faeces and color of urine. In conclusion, the results of this study have revealed that the HWE and the HEE of *Alpinia calcarata* at the doses tested do not produce any serious toxic side effects in rats.

Mizanur Rahman etal., (2012)[40] conducted a study on the anti-inflammatory, analgesic and gc – ms (gas chromatographic and mass spectrometric) analysis of essential oil of *Alpinia calcarata* rhizome. In the present study, essential oil isolated from *Alpinia calcarata* was analyzed and also assessed for acute toxicity, anti-inflammatory and analgesic activities in animals. The essential oil isolated from *Alpinia calcarata* was analyzed by using GC-MS on a combined GC-MS instrument. For evaluation of the anti-inflammatory property “carrageenan induced paw edema

model" served as acute model. "Acetic acid induced writhing response model" was used to assess analgesic activity in mice. The major components of essential oils isolated from *Alpinia calcarata* were Camphene (3.86%), Betamyrcene (4.39%), Eucalyptol (14.05%), Linalol (2.48%), Pyrazine (1.72%), L-camphor (7.90%) and Berneol (5.67%). Intraperitoneal injection of essential oil isolated from *Alpinia calcarata* significantly suppressedthe paw edema induced by carrageenan in two different dose levels studies namely 400 mg/kg and 380 mg/kg. Intraperitoneal injection of essential oil also significantly attenuated the acetic acid induced writhing response in three different dose levels studies namely 200 mg/kg, 400 mg/kg and 600 mg/kg. These studies suggest that essential oil isolated from *Alpinia calcarata* might possess significant anti-inflammatory activity and analgesic effect and could be a potential source for treatment of different inflammatory diseases.

Ratnasooriyaetal., (2006)[41] conducted a study on the effect of aqueous extract of the plant *Alpinia calcarata* rhizomes on reproductive competence of male rats. This study examined the effects of rhizomes of *Alpinia calcarata* (*Zingiberaceae*) on male sexual competence and fertility, using a hot water extract (HWE) and rats. Different doses of HWE (150, 250 and 500 mg/kg) were orally administrated to male rats and their sexual behaviour was monitored 3 hours later using receptive females. Fertility was determined in a separate group (with the highest dose) using a noncompetitive copulation test. In the sexual behaviour study, the HWE impaired the number of rats ejaculating and markedly prolonged the latency for ejaculation. Further, the number of rats mounting and intromitting, and the latencies for mounting and intromission were inhibited. Collectively, these observations indicate a strong aphrodisiac action. The other parameters remained unchanged indicating non-impairment in libido, sexual arousability, sexual vigour and sexual performance or penile erectile ability. However, a slight impairment was evident in sexual motivation (with the highest dose) in a partner preference test. In the fertility test, HWE induced profound oligozoospermia but fertility was uninhibited. The highest dose of HWE also elevated the serum testosterone level and the number of spontaneous penile erections rapidly and markedly. Further, the HWE was nontoxic. It is concluded that *Alpinia calcarata* rhizomes possess a strong and safe oral aphrodisiac activity.

3.1. AIM & OBJECTIVES

In recent year there has been tremendous increase in demand for herbal drugs because of its safety, efficacy and better therapeutic results. Due to its economic pricing as compared to synthetic or allopathic drugs, which have several therapeutic compilations.

Alpinia calcarata is also considered to be therapeutically important in traditional system of medicine.

Aim of the study to evaluate the anti-asthmatic, antioxidant and anti-inflammatory activities of ethanolic extract of *Alpinia calcarata* rhizome.

The objective study includes

- To study the anti-asthmatic effect of ethanolic extract of *Alpinia calcarata* rhizomes.
- To study the *in vitro* antioxidant activity of the ethanolic extract of *Alpinia calcarata* rhizomes.
- To study the *in vitro* anti-inflammatory activity of the ethanolic extract of *Alpinia calcarata* rhizomes.

4.1. PLAN OF WORK

1. Collection and authentification of plant material (rhizomes of *Alpinia calcarata*
2. Extraction of the plant material (Rhizomes of the plant *Alpinia calcarata*).
3. Conduction of the preliminary phytochemical screening and finding out the phytoconstituents present in the ethanolic extract of *Alpinia calcarata* rhizomes.
4. Performing of acute toxicity studies of ethanolic extract of *Alpinia calcarata* rhizomes (OECD 423).
5. Evaluation of the anti-asthmatic effect *of Alpinia calcarata* rhizomes
   - *In vivo* methods
     1. Histamine aerosol induced bronchoconstriction in guinea pigs.
     2. Milk induced leukocytosis and eosinophil count
   - *Ex vivo* methods
     1. Isolated guinea pig tracheal preparation.
6. Evaluation of the antioxidant study of *Alpinia calcarata* rhizomes
   - *In vitro* methods
     1. Hydrogen peroxide scavenging assay
     2. Reducing power assay
7. Evaluation of the anti-inflammatory study of *Alpinia calcarata* rhizomes
   - *In vitro* methods
     1. Protein denaturation method
     2. The rabbit red blood cell (RRBC) membrane stabilization method.

5.1. MATERIALS

5.1.1. Plant selected

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

5.1.2. Chemicals and reagents used

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

5.1.3. Drugs used

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

5.1.4. Instruments used for the study

- UV- Visible spectrophotometer- Jasco international
- Incubator- Rotek Instruments, B&C Industries, W. Vengola.
- Centrifuge- Rotek Instruments, B&C Industries, W. Vengola.
- Histamine chamber- Orchid Scientific Innovations India pvt. Ltd

5.1.5. Animals

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

5.2. METHODS

5.2.1. Collection and authentication of *Alpinia calcarata*

The dried rhizomes of the *Alpinia calcarata* were collected. The rhizomes were cleaned and shade dried and milled into coarse powder by a mechanical grinder.

5.2.2. Preparation of plant extract

The powdered rhizomes were extracted using ethanol by soxhlet extractor. In this process the powdered drug is placed into the extractor with ethanol as solvent. After extraction the extract was concentrated by evaporation then it was kept in a refrigerator for further use.[42, 43]

5.2.3. Preliminary phytochemical screening

The ethanolic extract of *Alpinia calcarata* rhizomes were subjected for the following chemical tests for the identification of various active constituents.[44, 45]

5.2.3.1. Detection of carbohydrates: -

- Molish test (General test)
  To 2-3 ml of aqueous extract, add few drops of α-naphthol solution in alcohol, shake and add conc. H_2SO_4 from a test tube. Violet ring is formed at the junction of two liquids.
- Test for reducing sugar
  (a)Fehling's test
  Mix 1 ml of fehling's A and 1 ml of fehling's B solution Boil for 1 min. Add equal volume of test solution. Heat in boiling water bath for5-10 min. First yellow, then brick red precipitate observed.
  (b) Benedict's test
  Mix equal volume of benedict's reagent and test solution in a test tube. Heat in boiling water bath for 5 min. Solution appears green, yellow or red depending on amount of reducing sugar present in test solution.

5.2.3.2. Detection of protein: -

- Biuret test (General test)
  3 ml of test solution add 4% NaOH and few drops of 1% $CUSO_4$ solution. Violet or pink color appear.
- Million's test (for proteins)
  Mix 2 ml of extract with million's reagent. white precipitate formed. warm precipitate turns brick red or the precipitate dissolves giving red colored solution.

5.2.3.3. Detection of proteins and amino acids: -

About 100 mg of extract was dissolved in 10 ml of distilled water and filtered through Whatman No.1 filter paper and the filtrate was subjected to tests for proteins and amino acids.

- Biuret test (General test)
  3ml of test solution add 4% NaOH and few drops of 1% $CUSO_4$ solution. Violet or pink color appear.
- Million's test (for proteins)

  Mix 2 ml of extract with millions reagent. White precipitate formed. Warm precipitate turns brick red or the precipitate dissolves giving red colored solution
- Ninhydrin test (for amino acid)
  About 2 drops of ninhydrin solution were added to 2 ml of test solution. Purple or bluish color appears

5.2.3.4. Detection for Fats and oils: -

- Solubility test
  Oils are soluble ether, benzene, chloroform but insoluble in 90% ethanol and in water. Hence filter paper get permanently stained with oils

5.2.3.5. Detection of steroid: -

- Salkowski reaction
  To 2 ml of extract, add 2 ml chloroform and 2 ml con H_2SO_4 shake well. Chloroform layer appear red and acid layer shows greenish yellow fluorescence.
- Liebermann – burchard reaction
  Mix 2 ml extract with chloroform. Add 1-2 ml of acetic anhydride and 2 drops of con H_2SO_4 from the side of test tube. First red, then blue finally green color appear.

5.2.3.6. Detection of glycoside: -

1) Cardiac glycoside

- Legal 's test (for cardiac glycoside)
  To aqueous or alcoholic extract add 1 ml pyridine and 1 ml sodium nitroprusside. pink to red color appears**.**
- Test for reducing sugar (for cardiac glycoside)
  To 2 ml extract add glacial acetic acid one drop 5% $Fecl_3$ and con H_2SO_4. Reddish brown color appears at junction of two liquid layer and upper layer appears bluish green.

2) Anthraquinone glycoside

- Bontragers test
  To 3 ml extract, dil. H_2SO_4 was added, boiled and filtered. To cold filtrate, equal volume of benzene or chloroform was added and shaken well. The organic solvent was separated and ammonia was added. The ammonical layer turns pink to red.

3) Saponin glycoside

- Foam test
  Shake the drug extract or dry powder vigorously with water. Persistant form observed.

- Heamolytic test
  Add drug extract or dry powder to one drop of blood placed on glass slide. Heamolytic zone appears

4) Cyanogenic glycoside

- Grignard reaction or sodium picrate test
  Soak a filter paper strip first in 10% picric acid, Then in 10% sodium carbonate, dry. In conical flask place moistened powdered drug. Cork it, place the above filter paper strip in the slit in cork. Filter paper turn brick red or maroon.

5) Coumarin glycoside

- Coumarin glycoside have aromatic odour
- Alcoholic extract when made alkaline, shows blue or green fluorescence

5.2.3.7. Detection for flavonoids

- Shinoda test
  To dry powder or extract, add 5ml 95% ethanol, few drops con. Hcl and 0.5 g Magnesium turnings. Pink color observed.
- To small quantity of residue, add lead acetate solution. Yellow color precipitate is formed.
- Addition of increasing amount of NaOH to residue shows yellow coloration, which decolorizes after addition of acid.

5.2.3.8. Detection of alkaloid

Aqueous alcoholic and chloroform extract was evaporated separately. To residue dilute Hcl was added. Shaken well and filtered. With the filtrate the following test was performed.

- Dragondorff ‘s test
  To 2-3 ml. Filtrate, add few drops of dragondorff‘s reagent. Orange brown precipitate

- Mayers test
  To 2-3 ml of filtrate with few drops of mayer's reagent gives precipitate
- Hager's test
  2-3 ml filtrate with hager's reagent gives yellow precipitate
- Wagner's test
  2-3 ml of filtrate with wagner's reagent gives reddish brown precipitate

5.3. ACUTE TOXICITY STUDIES

Acute toxicity of *Alpinia calcarata* was done as per OECD guidelines 423. The substance was administered in a single dose by gavage using specially designed mice oral tube. Animals were fasted prior to dosing with food but not water withheld overnight. Following the period of fasting, the animals were weighed and the test substance was orally at a dose of 5, 50, 300 and 2000 mg/kg. The animals are observed continuously for first three hours, four any toxic manifestations like increased motor activity, salivation, acute convulsion, coma and death. Changes in the animal behavior should be noted before and after administration for 24hours. Treated animals are to be further observed for 14 days. If the extract does not produce mortality at the highest dose, then the $1/10^{th}$ or $1/20^{th}$ of the dose was selected for experiment.[46, 47]

5.4. EVALUATION OF ANTI ASTHMATIC ACTIVITY

5.4.1. *In vivo* anti-asthmatic activity

5.4.1.1. Histamine aerosol induced bronchoconstriction in guinea pigs

Histamine was dissolved in distilled water to prepare 0.2% w/v solution. Experimentally bronchial asthma was induced in guinea pigs by exposing histamine aerosol by a nebulizer in an aerosol chamber. The required time for appearance of preconvulsive dyspnoea produced by the histamine was noted for each animal. Each animal was placed in the histamine chamber and exposed to 0.2% histamine aerosol. The preconvulsion time (PCT), i.e. the time of aerosol exposure to the start of dyspnoea leading to the appearance of convulsion, was noted. As quickly as the preconvulsion dyspnoea (PCD) was recorded, the animals were removed from the chamber and positioned in fresh air for recover. This time for preconvulsive dyspnoea

was recorded as basal value. Guinea pigs were then allowed to recover from dyspnoea for 2 days. After that, the animals were allotted to four different groups of 4-5 animals per group. Animals in group 1 served as control and received carboxy methyl cellulose. The animals of group 2 and 3 were given, by oral intubation, 100 and 200 mg/kg of the plant extract, respectively, while group 4 received the standard drug - Chlorpheniramine maleate, intraperitoneally. After receiving the drugs, all the animals were again exposed to histamine aerosol in the chamber, one hour, four hours and 24 hours, to determine pre convulsive time (PCT).[48, 49, 50]

Percentage protection was calculated using the formula.

Percentage protection = $\frac{Eta-Etb}{Eta} \times 100$

Where *Eta* is the preconvulsion time after administration of drug and *Etb* is the preconvulsion time before administration of drug.

5.4.1.2. Milk induced leukocytosis and eosinophilia

Mice were divided into 4 groups with six in each group. Blood samples were collected from retro-orbital plexus. Group 1 served as control and received carboxy methyl cellulose solution, groups 2-3 received plant extract at (100-200 mg/kg) group 4 received dexamethasone at 50 mg/kg i.p. All the groups injected boiled and cooled milk (4 ml/kg, s.c.) 30 min after treatments. Total leukocyte and eosinophile count was done in each group before administration of test compound and 24 hours after milk injection. Difference in total leukocytes and eosinophile count before and after 24-hour drug administration was calculated.[51, 52]

5.4.2. *Ex vivo* anti-asthmatic activity

5.4.2.1. Isolated guinea pig tracheal preparation

Isolated guinea pig tracheal tissue was obtained by, Animals were sacrificed by cervical dislocation and carotid bleeding. The trachea was dissected out and transferred into a dish containing Krebs solution and cut crosswise between the section of the cartilage of the trachea and continuously ventilated and maintained at 37 + 0. 5°C.The adjourned trachea was allowed to make steady for at least 40 minutes. On equilibrium, the bath was supplied with Krebs solution for every

15 minutes Dose response curve of histamine (10 µg/ml) in plane Krebs solution and in 1 mg/ml of plant extract act in Krebs solution was taken. Percentage of maximum contractile response on ordinate and concentration of histamine on abscissa was plotted to record dose response curve of histamine, in absence and presence of plant extract.[48, 54]

5.5. *IN VITRO* ANTIOXIDANT ACTIVITY

5.5.1. Hydrogen peroxide scavenging

Hydrogen peroxide solution (20 Mm) was prepared with standard phosphate buffer (pH 7.4). Extract samples (25, 50, 100, 200 and 400 µg/ml) in distilled water were added to hydrogen peroxide solution (0.6 ml). Absorbance of hydrogen peroxide at 230 nm was determined after 10 minutes against a blank solution containing phosphate buffer without hydrogen peroxide. Ascorbic acid was used as the reference standard. The percentage scavenging of hydrogen peroxide of plant extract was calculated using the formula.[55]

$$\% \text{ Scavenged} = \frac{Ac - As}{Ac} \times 100$$

Where, *Ac* = Absorbance of control

As = Absorbance of sample

The experiments were performed in triplicates, and the results were expressed as Mean± S.E.M

5.5.2. Reducing power assay

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

experiments were performed in triplicate. Increased absorbance of reaction mixture indicates stronger reducing power.[56, 57]

5.6. *IN VITRO* ANTI-INFLAMMATORY ACTIVITY

5.6.1. Protein denaturation

A solution of 0.2% of bovine serum albumin (BSA) was prepared in tris buffer saline and pH was adjusted to 6.8 using glacial acetic acid. Test drug of different concentration (25, 50, 100, 200 and 400 µg/ml) was prepared using ethanol as solvent. 50 µl of each test drug was transformed to test tubes using micropipette. 5 ml of 0.2% w/v of BSA was added to the test tubes. The control consists of 5 ml of 0.2% w/v of BSA solution and 5µl alcohol. The test tubes were heated at 72^0 C for 5 min and then cooled for 10 min. The absorbance of these solution was determined using UV–visible spectrophotometer at 660nm. Diclofenac sodium was used as standard and treated similarly for determination of absorbance. The percentage inhibition of protein denaturation was calculated using the following formula.[58, 59]

Percentage of inhibition of denaturation = $\frac{Absorbance\ of\ control - Absorbance\ of\ extract}{Absorbance\ of\ control} \times 100$

5.6.2. The rabbit red blood cell membrane stabilization method

- Preparation of red blood cell suspension (RBCs suspension)

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

- Membrane stabilization test by hypotonicity induced haemolysis

The reaction mixture consists of 1 ml of test sample of different concentration (25, 50, 100, 200 and 400 µg/ml) in normal saline and 0.5 ml of 10% RBC suspension, 1 ml of 0.2 M phosphate buffer, 1 ml hypo saline were incubated at 37^0C for 30 minutes and centrifuged at 3000 rpm for 20 minutes and the haemoglobin content of the supernatant solution was estimated

spectrophotometrically at 560 nm. Diclofenac sodium was used as standard and a control was prepared without extract. The percentage of RBC haemolysis and membrane stabilization or protection was calculated using the following formula.[60, 61]

% Haemolysis = $\frac{Optical\ density\ of\ test\ sample}{Optical\ density\ of\ control} \times 100$

% Protection =100 - % Haemolysis

5.7. STATISTICAL ANALYSIS

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

6.1. COLLECTION & AUTHENTIFICATION OF *ALPINIA CALCARATA* RHIZOME

The rhizomes of *Alpinia calcarata* were collected and authentified.

6.2. EXTRACTION OF PLANT MATERIAL

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

6.3. PRELIMINARY PHYTOCHEMICAL SCREENING OF ETHANOLIC EXTRACT OF *ALPINIA CALCARATA* RHIZOMES (EEAC)

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

Table No.3: Preliminary phytochemical analysis

Sl. No:	Constituents	Presence/absence
1	Carbohydrate	+
2	Proteins	+
3	Amino acids	–
4	Fats and oils	–

5	Steroids	+
6	Cardiac glycosides	+
7	Anthraquinone glycoside	–
8	Saponin glycosides	–
9	Cyanogenic glycosides	–
10	Coumarin glycosides	–
11	Flavonoids	+
12	Alkaloids	–
13	Tannins	+
14	Phenol	+

(+: presence, -: absence)

6.4. ACUTE TOXICITY STUDIES

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

6.5. EVALUATION OF ANTI ASTHMATIC ACTIVITY

6.5.1. *In vivo* anti asthmatic activity

6.5.1.1. Histamine aerosol induced bronchoconstriction in guinea pigs

Histamine was dissolved in distilled water to prepare 0.2% w/v solution. Experimentally bronchial asthma was induced in guinea pigs by exposing histamine aerosol by a nebulizer in an aerosol chamber. The required time for appearance of pre convulsive dyspnoea produced by the histamine was noted for each animal. Each animal was placed in the histamine chamber and exposed to 0.2% histamine aerosol. The preconvulsion time (PCT), i.e. the time of aerosol exposure to the start of dyspnoea leading to the appearance of convulsion, was noted. As quickly as the preconvulsion dyspnoea (PCD) was recorded, the animals were removed from the chamber and positioned in fresh air for recover. This time for preconvulsive dyspnoea was recorded as basal value. Guinea pigs were then allowed to recover from dyspnoea for 2 days. After that, the animals were allotted to four different groups of 4-5 animals per group. Animals in group 1 served as control and received carboxy methyl cellulose. The animals of group 2 and 3 were given, by oral intubation, 100 and 200 mg/kg of the plant extract, respectively, while group 4 received the standard drug - Chlorpheniramine maleate, intraperitoneally. After receiving the drugs, all the animals were again exposed to histamine aerosol in the chamber, one hour, four hours and 24 hours, to determine pre convulsive time (PCT).[48, 49, 50]

Percentage protection was calculated using the formula.

$$\text{Percentage protection} = \frac{Eta - Etb}{Eta} \times 100$$

Where *Eta* is the preconvulsion time after administration of drug and *Etb* is the preconvulsion time before administration of drug.

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

calculated from the latent period of convulsion. The maximum % protection of ethanolic extract of the plant was calculated as 60.79% at 200 mg/kg.

The standard drug used was chlorpheniramine maleate which showed significant % protection at time 1 hour and 4 hours. The plant extract at 100 mg/kg showed 43.2% protection at time 1 hour and the 100 mg/kg plant extract also showed 40.2% protection at time 24 hour and also showed 57.2% protection at time 4 hour. The control (Carboxy methyl cellulose) produced 10.9 % protection at time 1 hour and 12.3% protection at time 4 hour and 11.4% protection at time 24 hour. The plant extract at 200 mg/kg showed 48% at time 1 hour and 60.79% at time 4 hour and 44.3% at time 4 hour and 44.3% at 24 hours. The standard drug chlorpheniramine maleate possess 69.76% protection at time 1 hour and 78.3% at time 4 hour and 50.1% at time 24 hour.

The results of histamine aerosol induced bronchoconstriction in guinea pigs was shown in the table number 4 and figure number 3.

Table No.4: Histamine aerosol induced bronchoconstriction in guinea pigs

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

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

Figure No.3: Effect of ethanolic extract of *Alpinia calcarata* rhizomes against histamine induced bronchoconstriction in guinea pigs

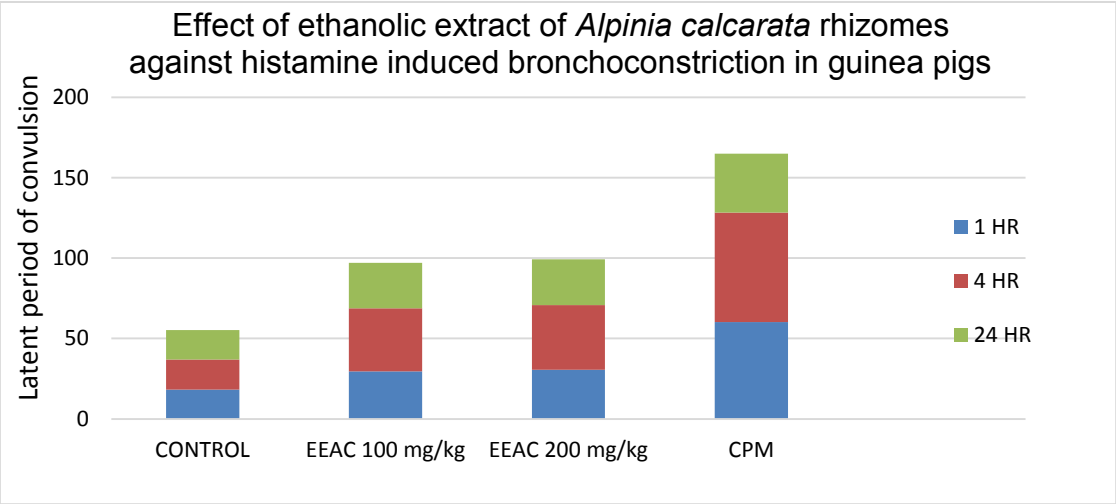
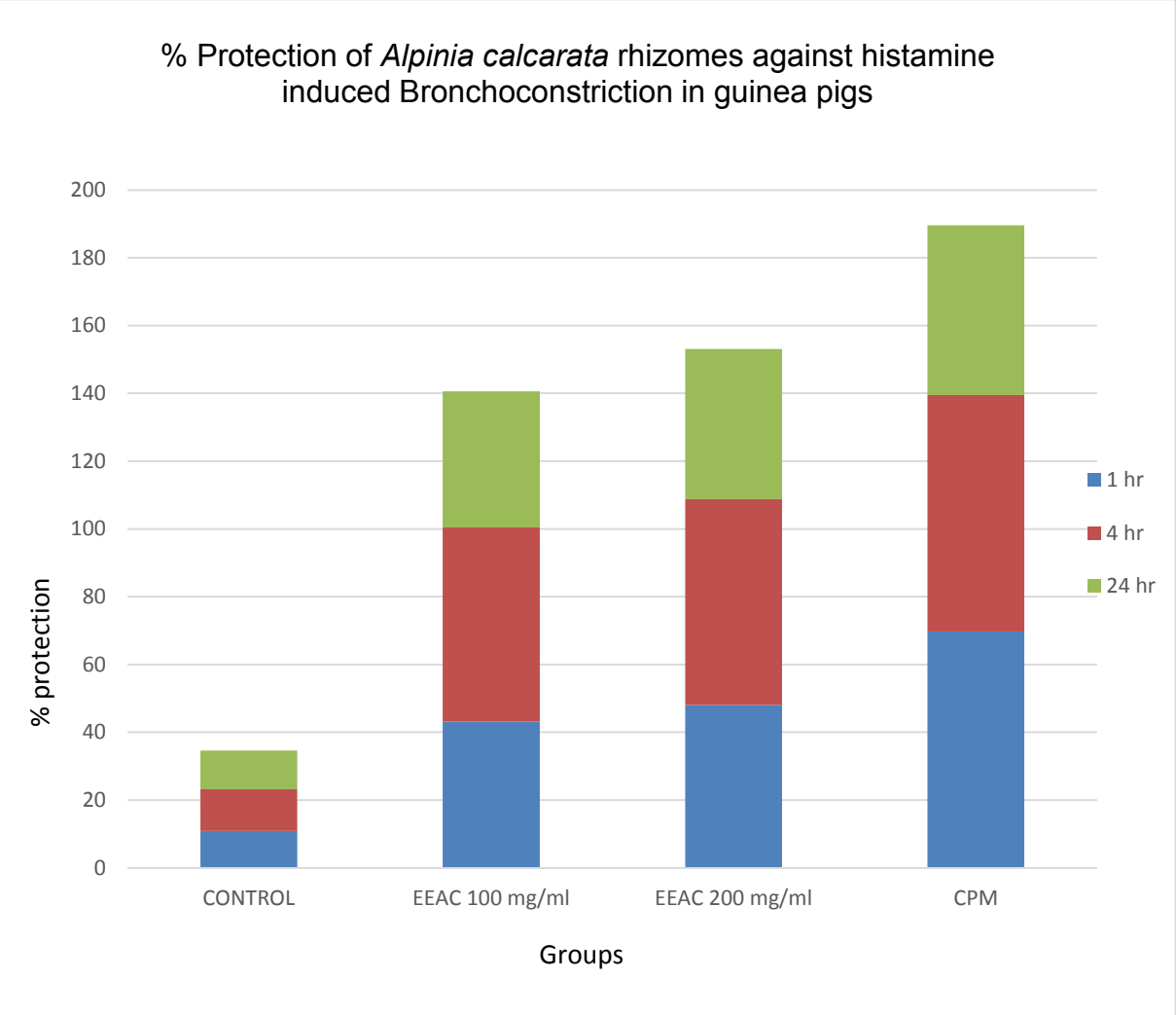


Table No.5: % Protection of the plant *Alpinia calcarata* rhizomes against histamine induced bronchoconstriction in guinea pig

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

Figure No.4: % Protection of the plant *Alpinia calcarata* rhizomes against histamine induced bronchoconstriction in guinea pigs



6.5.1.2. Milk induced leukocytosis and eosinophilia

6.5.1.2.1. Milk induced leukocytosis

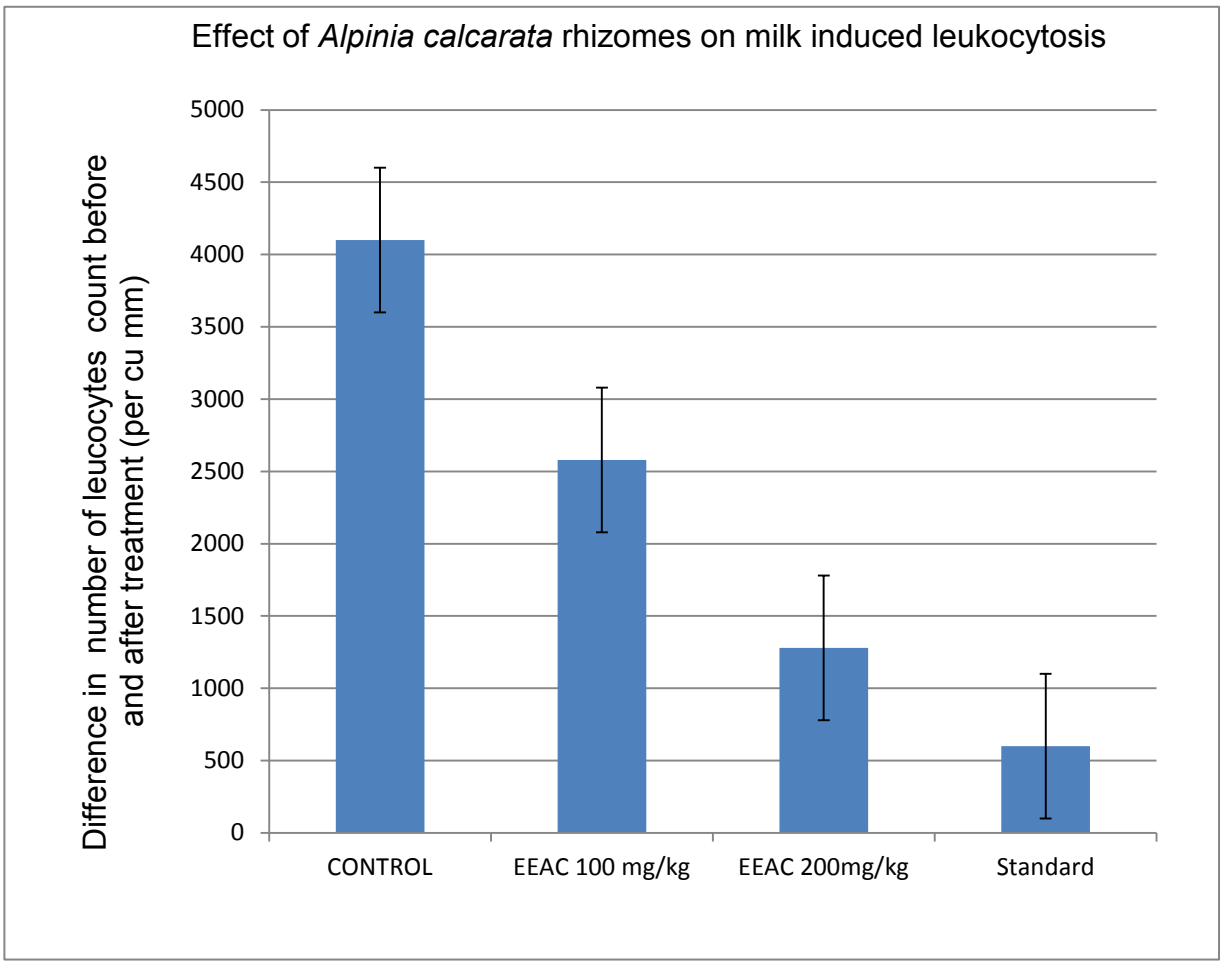
In the milk induced leukocytes the maximum increase in difference of leukocytes count was observed in control group (4100 ± 9) 24 hour after administration of milk. Groups of mice pretreated with ethanolic extract (200 mg/kg) showed significant activity. The ethanolic extract of plant *Alpinia calcarata*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

Table No.6: Effect of ethanolic extract of *Alpinia calcarata* rhizomes on milk induced leukocytosis

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

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

Figure No.5: Effect of *Alpinia calcarata* rhizomes on milk induced leukocytosis



6.5.1.2.2. Milk induced eosinophilia

In this study difference in number of eosinophilic count before and after treatment was studied. In control group there was maximum increase in difference in number of eosinophilic count (118± 1.414) was observed. The ethanolic extract of *Alpinia calcarata* (200 mg/kg) showed significant activity by less difference in number of eosinophilic count (53 ±1.434). The standard drug also exhibited significant activity by less difference in number of eosinophilic count (38 ±1.13). The ethanolic extract of plant (100 mg/kg) showed less significant activity.

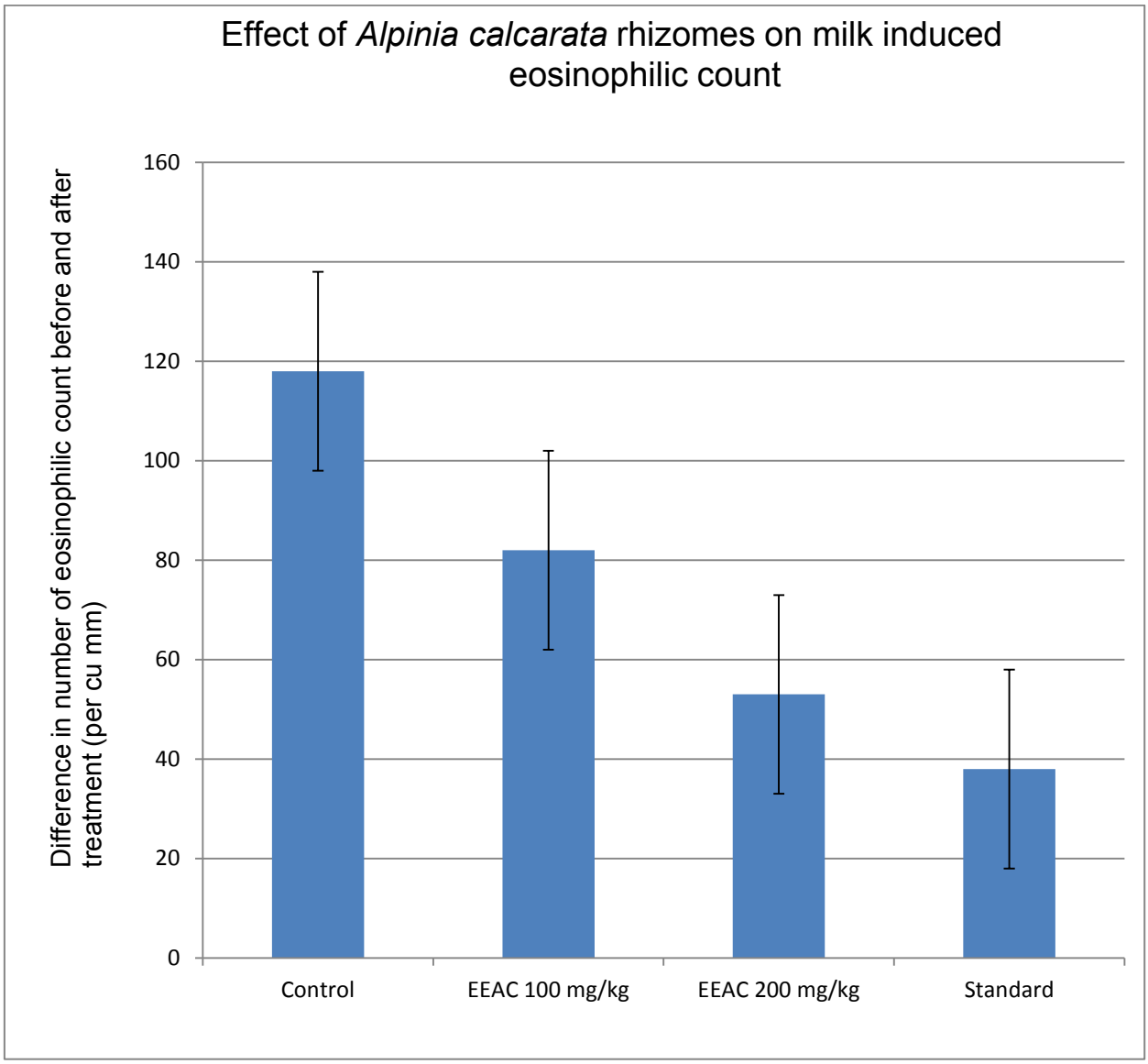
The results were expressed in the table number 7and figure number 6.

Table No.7: Effect of ethanolic extract of *Alpinia calcarata* rhizomes on milk induced eosinophilia

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

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

Figure No.6: Effect of *Alpinia calcarata* rhizomes on milk induced eosinophilic count



6.5.2. *Ex vivo* anti-asthmatic study

6.5.2.1. Isolated guinea pig tracheal preparation

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

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

Figure No.7: Concentration Response Curve of Histamine using Guinea pig tracheal preparation (In Absence of Plant Extract).

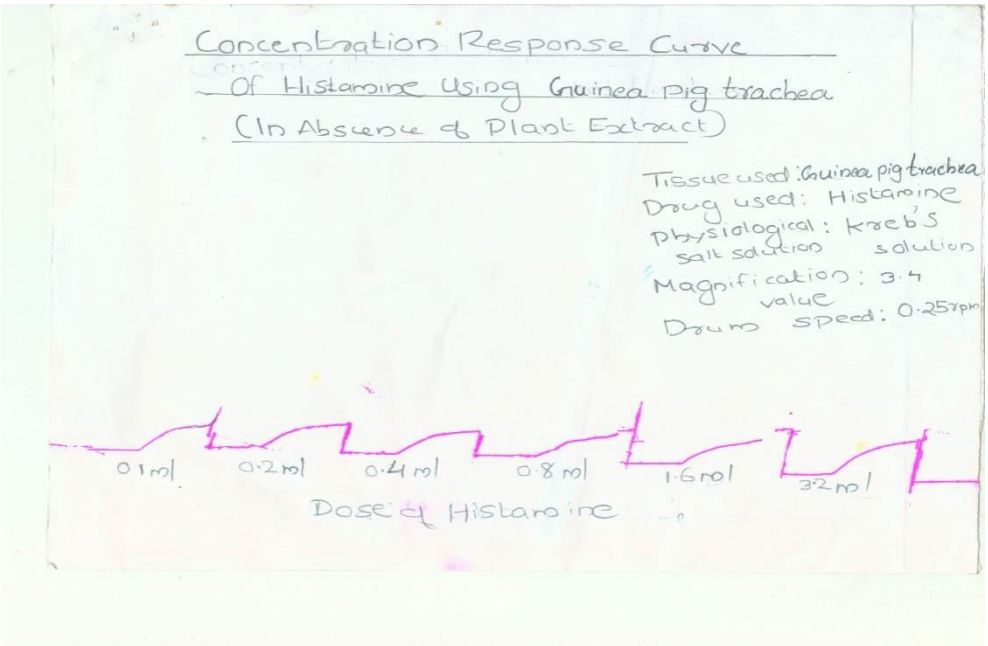
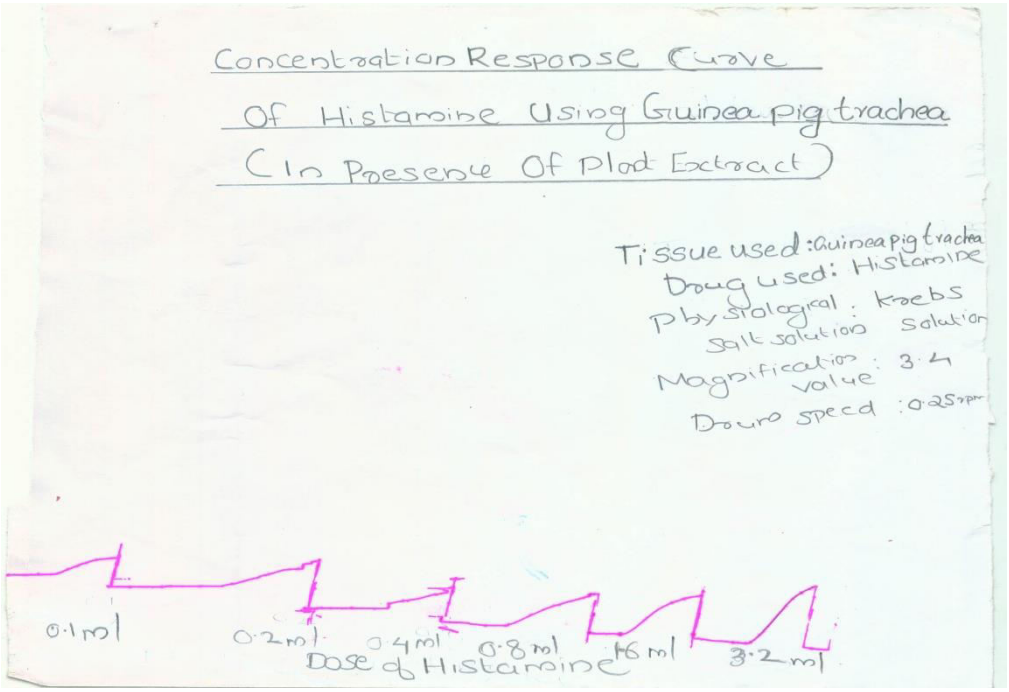


Figure No.8: Concentration Response Curve of Histamine using Guinea pig tracheal preparation (In Presence of Plant Extract).



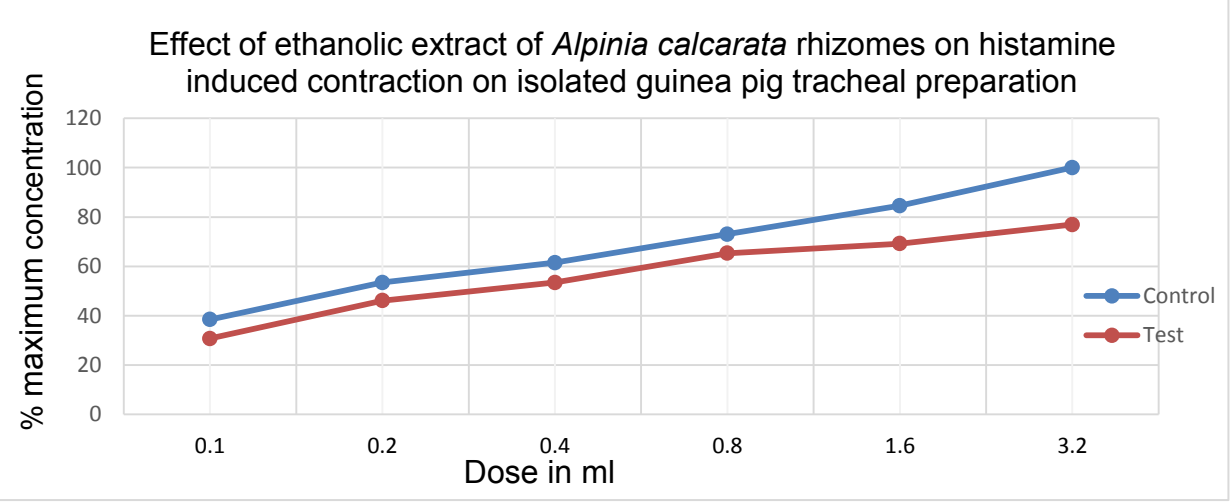
The results were expressed in table number 8 and the effect of *Alpinia calcarata* rhizomes on histamine induced contraction on isolated guinea pig tracheal is shown in figure number 9

Table No.8: Effect of ethanolic extract of *Alpinia calcarata* rhizomes on histamine induced contraction on isolated guinea pig tracheal preparation

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

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

Figure No.9: Effect of ethanolic extract of *Alpinia calcarata* rhizomes on histamine induced contraction on isolated guinea pig tracheal preparation



6.6. *IN VITRO* ANTIOXIDANT ACTIVITY

6.6.1. Hydrogen peroxide scavenging

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

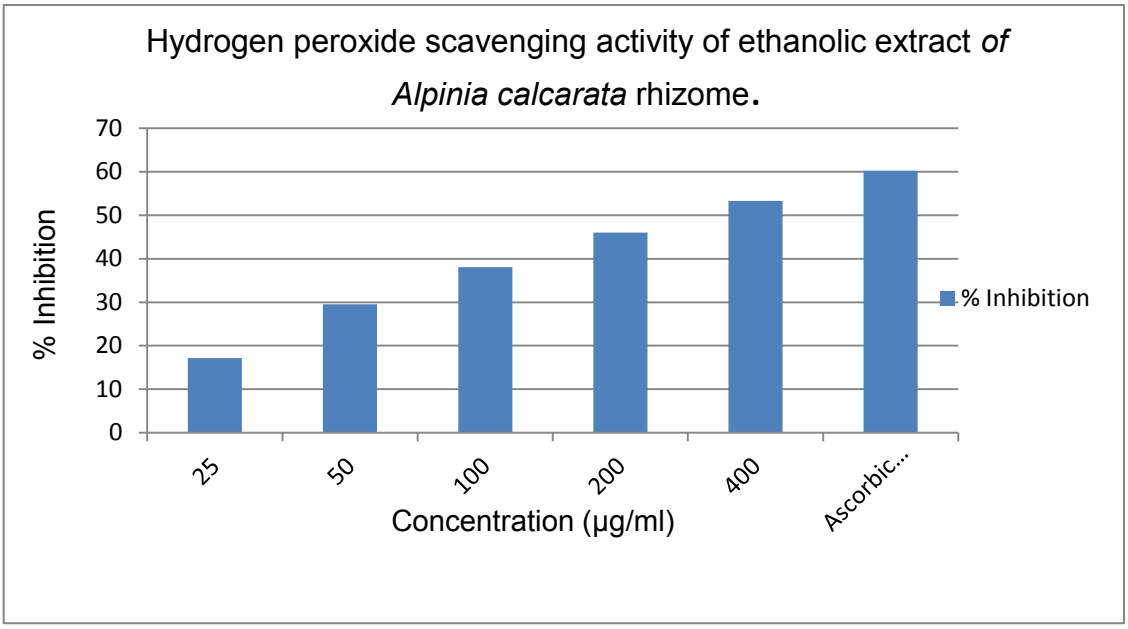
The hydrogen peroxide scavenging effect of ethanolic extract was shown in table number 9.

Table No.9: Hydrogen peroxide scavenging activity of ethanolic extract of *Alpinia calcarata* rhizomes

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

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

Figure No.10: Hydrogen peroxide scavenging activity of ethanolic extract of *Alpinia calcarata* rhizome.



6.6.2. Reducing power assay

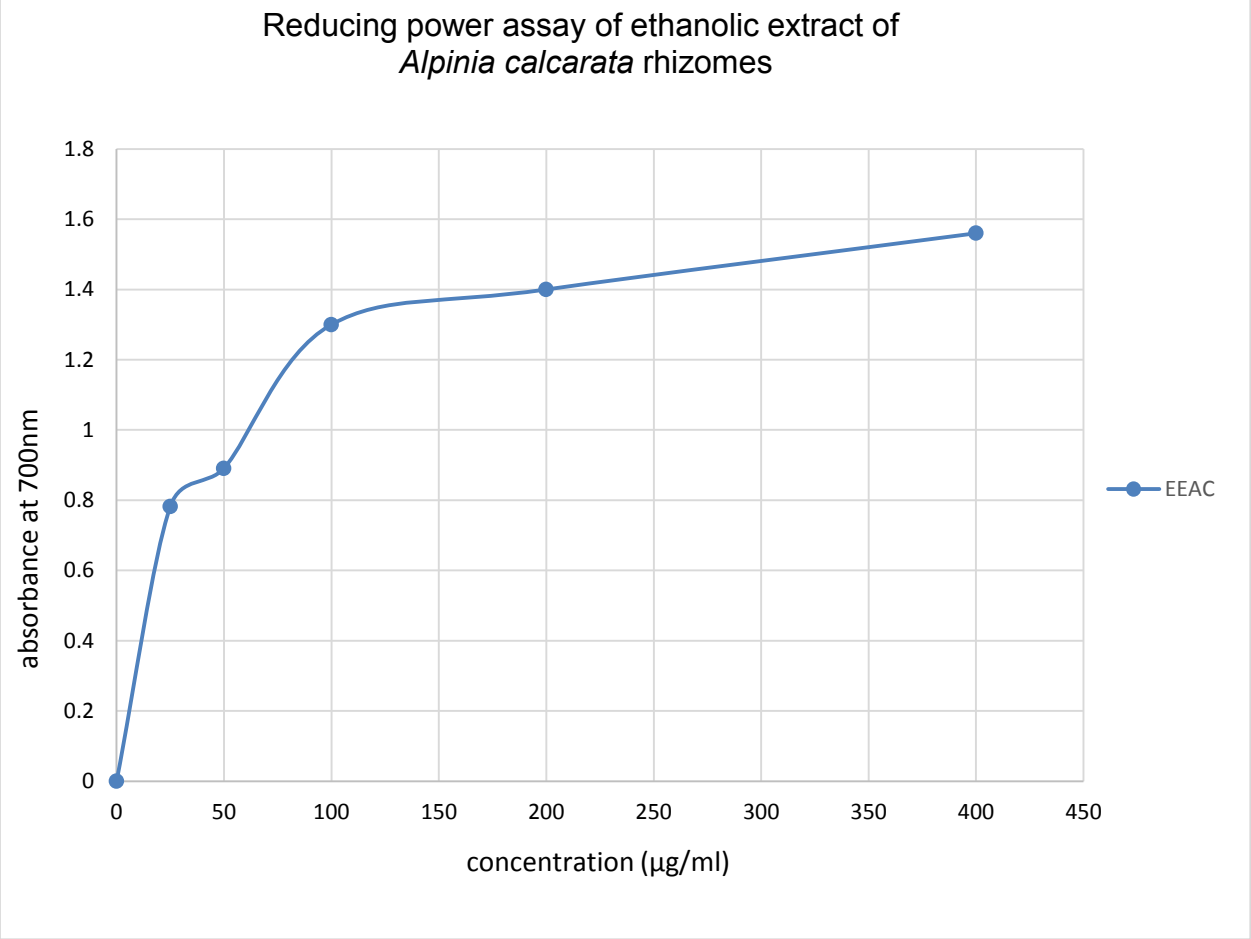
Increase in absorbance of the extract indicates the reducing power of the test sample. Reducing power of ethanolic extract of *Alpinia calcarata* rhizomes increased with increasing concentration. Results are expressed below

Table No.10: Reducing power activity of ethanolic extract of *Alpinia calcarata* rhizomes

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

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

Figure No.11: Reducing power assay of ethanolic extract of *Alpinia calcarata* rhizomes



6.7. *IN VITRO* ANTI-INFLAMMATORY ACTIVITY

6.7.1. Protein denaturation

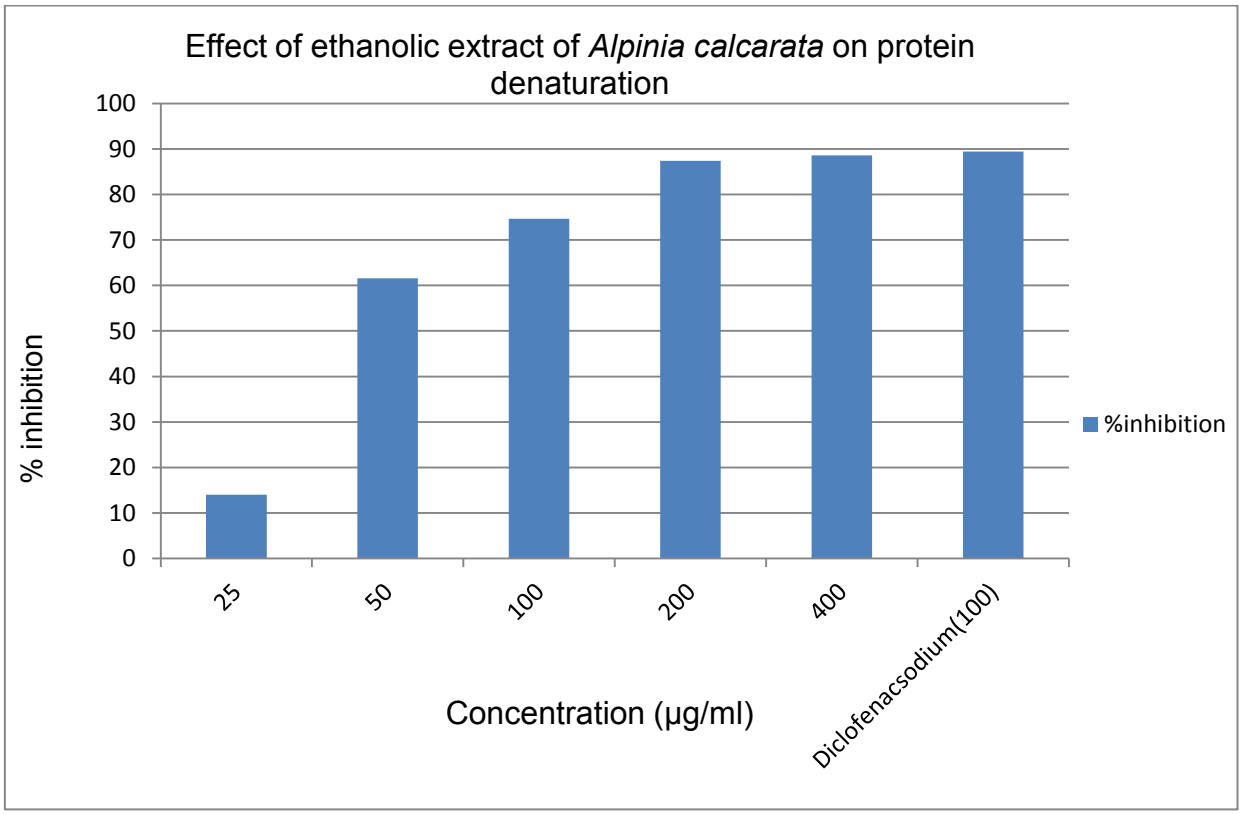
As part of the evaluation of anti-inflammatory activity, ability of plant extract on protein denaturation was studied. It was effective in inhibiting heat induced protein denaturation. Diclofenac sodium a standard anti-inflammatory agent possesses maximum % inhibition. The ethanolic extract of the plant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

Table No.11: Effect of ethanolic extract of *Alpinia calcarata* on protein denaturation

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

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

Figure No 12: Effect of ethanolic extract *of Alpinia calcarata* on protein denaturation.



6.7.2. Rabbit red blood cell membrane stabilization method

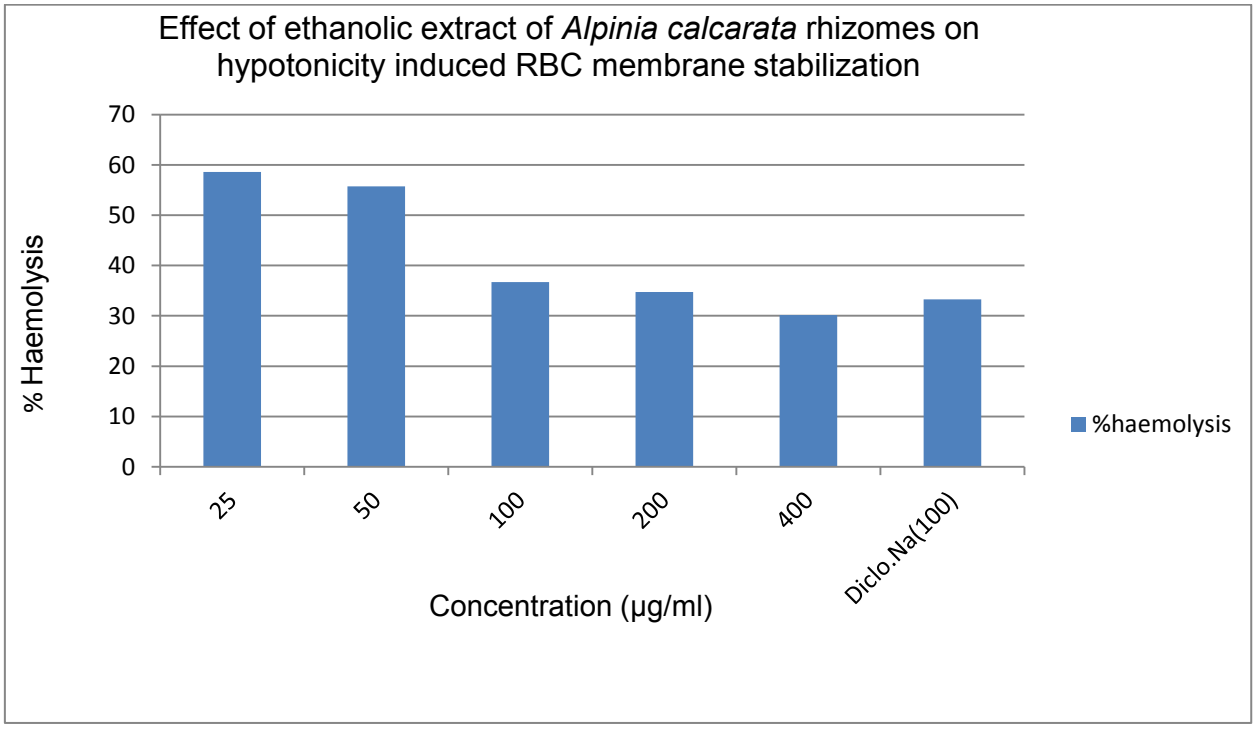
The ethanolic extract of *Alpinia calcarata* rhizome had shown significant inhibition of haemolysis or the active RBC membrane stabilization comparing to diclofenac sodium the reference standard. The maximum percentage protection shown by test extract was 69.82% at 400 µg/ml and minimum percentage protection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

Table No.12: Effect of ethanolic extract of *Alpinia calcarata* rhizomes on hypo tonicity induced RBC membrane stabilization.

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

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

Figure No.13: Effect of ethanolic extract of *Alpinia calcarata* rhizomes on hypo tonicity induced RBC membrane stabilization



6.8. DISCUSSION

6.8.1. Preliminary phytochemical screening of ethanolic extract of *Alpinia calcarata* rhizome.

The ethanolic extract of *Alpinia calcarata* rhizomes were subjected to phytochemical screening. The result indicated that, rhizome extract shows the presence of carbohydrate, proteins, cardiac glycosides, flavonoid, tannins and phenol. The knowledge of chemical constituents of plant is desirable because such information will be valuable for synthesis of complex chemical substances and to screen for biological activities.

Phytochemicals are natural bioactive compounds found in plants. They are mainly two groups, which are primary and secondary metabolites. Primary metabolites are sugars, amino acids, proteins and chlorophyll while secondary metabolites consist of alkaloids, terpenoids and phenolic compounds. The beneficial medicinal effect of plant material results from the combination of secondary

metabolites present in the plant. So the systematic screening of plant species is necessary for the purpose of discovering new bioactive compounds.

The phenolic compounds and flavonoids are secondary metabolites in plants having antioxidant activity. They have wide range of biological activities as cardio protection, cell proliferation and anti-aging. Tannins are potential toxic agents to fungi, bacteria and viruses in plants. They are currently investigated for human medicinal use, in order to reduce the risk of coronary heart diseases.

6.8.2. Histamine aerosol induced bronchoconstriction in guinea pigs

Histamine is one of the major inflammatory mediators in the immediate phase of asthma, causing airway hyper responsiveness and bronchial airway inflammation. Histamine induced bronchodialators is the traditional immunological model of antigen induced airway obstruction. Histamine when inhaled causes hypoxia and leads to convulsion in guinea pigs and causes very strong smooth muscle contraction, profound hypotension and capillary dialation in the cardiovascular system. A prominent effect caused by histamine leads to severe bronchoconstriction in the guinea pigs that causes convulsion and leads to death. Bronchodialators can delay the occurrence of these symptoms. In this histamine induced bronchospasm the ethanolic extract of the plant *Alpinia calcarata* rhizomes showed significant activity and increase in dose of extract increase % protection. The maximum % protection shown by the plant extract was 60.79 % observed at 200 mg/kg for bronchorelaxant study comparable to that of standard drug chlorpheniramine maleate 78.3 %.

The results of the study confirmed the bronchodialotor properties of the plant, justifying its traditional claim in the treatment of asthma.

6.8.3. Milk induced leukocytosis and eosinophilia

In the present investigation ethanolic extract of the plant *Alpinia calcarata* rhizomes (100, 200 mg/kg) was evaluated for the management of asthma using milk induced leukocytosis and eosinophilia in mice. Asthma involves various types of mediator in pathology. It was demonstrated that potential administration of milk produces a marked increase in leukocytes and eosinophils count after 24 hours of its

administration. Leukocytes during asthmatic inflammation release the inflammatory mediators like cytokines, histamine and major basic protein, which promote the ongoing of inflammation. The infiltration of leukocytes potentiates the inflammatory process by the release of reactive oxygen species into the surrounding tissue, resulting in increased oxidative stress and associated with many pathological features of asthma. In this study we observed that leukocyte count was decreased in mice treated with ethanolic extract of plant at doses 100 and 200 mg/kg significantly as compared to control group. Results suggests that the ethanolic extract of the plant *Alpinia calcarata* rhizomes decreases milk induced leukocytes count by normalizing oxidative stress. An abnormal increase in peripheral eosinophil to more than 4% of total leukocytes count is termed as eosinophil. In asthmatic patient there is an increase in eosinophilic count and mucus hypersecretion and airway hyper reactivity were stimulated. Eosinophils infiltrating the airway also have an effect on mucus secretion by epithelial goblet cell. In our study it was observed that the ethanolic extract of *Alpinia calcarata* rhizomes at doses 100 and 200 mg/kg significantly decreased milk induced eosinophils count.

The ethanolic extract of *Alpinia calcarata* rhizomes decrease the leukocyte count by normalizing oxidative stress and decrease in eosinophil count may reduce type I hypersensitivity in asthma. In conclusion ethanolic extract of the plant *Alpinia calcarata* rhizomes is effective in management of asthma.

6.8.4. Isolated guniea pig tracheal preparation

It was observed that the ethanolic extract of *Alpinia calcarata* rhizomes inhibits contraction produced by histamine in these tissue preparations. Histamine (10 µg/ml) was taken in different dose level and concentration response curve was plotted. Study revealed that the ethanolic extract of the plant *Alpinia calcarata* rhizome exhibit significant percentage decreased contraction at concentration 0.8 mg/ml.

6.8.5. Hydrogen peroxide scavenging

The ethanolic extract of *Alpinia calcarata* rhizomes showed good dose dependent hydrogen peroxide scavenging activity. Hydrogen peroxide (H_2O_2), a biologically relevant, non- radical oxidizing species, may be formed in tissue through

oxidative processes. Although hydrogen peroxide is a weak oxidizing agent it can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Hydrogen peroxide itself is not reactive, but can generate hydroxyl radical (OH) (via fenton reaction) in the cells resulting in initiation and propagation of lipid peroxidation. Thus the removal of H_2O_2 is very important for antioxidant defence in cell or food systems. Hydrogen peroxide can cross cell membranes and may oxidize a number of compounds. The ability of the extracts to quench OH^- seems to be directly related to the prevention of the lipid peroxidation and appears to be moderate scavenger of active oxygen species, thus reducing rate of chain reaction.

As a conclusion the ethanolic extract of the *Alpinia calcarata* rhizomes showed hydrogen peroxide activity as compared to standard ascorbic acid (100 µg/ml).

6.8.6. Reducing power assay

The reducing power of the extract, which may serve as a significant reflection of antioxidant activity, was determined using reducing power assay, where by the yellow colour of the test solution changes to various shades of green and blue is depending upon the reducing power of each compound. The presence of radicals (ie, antioxidant) causes the conversion of the Fe^{3+}/ ferricyanide complex $[Fe^{3+}(CN^-)_6]$ used in this method to the ferrous form (Fe^{2+})/ ferrocyanide complex $[Fe^{2+}(CN^-)_6]$. Therefore, by measuring the formation of Pearls Prussian blue spectrophotometrically, the Fe^{2+}concentrationcan be monitored, a higher absorbance indicates a higher reducing power. The increased reducing power in the extracts indicates that some components in the extract were electron donors that could react with the free radicals to convert them into more stable products to terminate radical chain reaction. Antioxidant compounds are able to donate electrons to reactive radicals, reducing them into more stable unreactive species.

The reducing power of the ethanolic extract of *Alpinia calcarata* rhizomes increased with increasing concentration.

6.8.7. Protein denaturation method

Denaturation of protein is a well-documented cause of inflammation. Production of auto antigens may be due to the denaturation of tissue protein. Agents that can prevent protein denaturation therefore would be worthwhile for anti-inflammatory drug development. The mechanism of denaturation probably involves the alteration electrostatic hydrogen, hydrophobic and disulphide bonding. It has been reported that one of the features of several non-steroidal anti-inflammatory drugs in their ability to stabilize heat treated protein at physiological pH.

The ethanolic extract of the *Alpinia calcarata* rhizomes exhibited concentration dependant inhibition of protein denaturation. Therefore, from the study it can be concluded that the rhizomes of the plant extract possess marked in vitro anti-inflammatory effect.

6.8.8. Rabbit red blood cell membrane stabilization method

The ethanolic extract of plant exhibit membrane stabilization property by inhibiting hypotonicity which effectively indicates the anti-inflammatory property.

The viability of the cell depends up on the integrity of their membrane exposure of RBC to hypotonic medium, injurious substance results in the lysis of its membrane accompanied by haemolysis and oxidation of haemoglobin. Compounds with membrane stabilizing properties are well known for their ability to their ability to interfere with the early phase of inflammatory reactions.

Alpinia calcarata is a rhizomatous perennial herb belonging to family *zingiberaceae*. It was traditionally used for the treatment of asthma, bronchitis, rheumatoid arthritis, stomachic disease, diabetes and heart diseases.

The study includes the phytochemical and pharmacological investigation of the ethanolic extract of *Alpinia calcarata* rhizomes. The powdered rhizomes were extracted by means of soxhlet using the solvent ethanol.

Preliminary phytochemical screening revealed the presence of carbohydrate, cardiac glycoside, protein, alkaloids, steroids, flavonoids, tannins and phenolic compounds. These constituents may be represented the presence for biological activities of the plant. The acute toxicity study revealed that there is no mortality with the ethanolic extract of *Alpinia calcarata* rhizomes up to the dose level of 2000 mg/kg.

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

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

Herbal formulations used in the treatment of asthma include some anti-stress herbs to enable adoption to stress since excessive stress or nervous debility may aggravate symptoms of asthma. The normalization effect of an adaptogen can be observed in milk induced leukocytosis after administration of milk. Also eosinophil play a pivotal role in the pathogenesis of allergic disorders. The plant extract showed marked protection against eosinophil cell count, which is a hallmark of allergic asthma as compared to control group. The reduction in eosinophil count means inhibition in eosinophil cell recruitment and inhibition of interleukins such as IL-4, IL-5

and IL-13 which play important role in eosinophil cell count. So the milk induced leukocytosis and eosinophilic study revealed anti asthmatic property of the plant extract.

Histamine contracts the tracheal-bronchial muscle of guinea pig, goat, horse, dog and man. Guinea pig trachea is used for the screening of anti-asthmatic activity. The H_1 receptor after stimulation produces well-ordered dose related contraction of isolated guinea pig trachea. In current study *Alpinia calcarata* significantly inhibited histamine induced contraction of isolated guinea pig trachea preparation indicating its H_1 receptor antagonist activity and supports anti asthmatic property of the plant.

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

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

CONCLUSION

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

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