

***IN VIVO* STUDY OF ANALGESIC AND CNS ACTIVITY OF THE AQUEOUS LEAF
EXTRACT OF *KALANCHOE PINNATA***



**A DISSERTATION SUBMITTED TO THE DEPARTMENT OF PHARMACY,
EAST WEST UNIVERSITY
IN THE PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE
OF BACHELOR OF PHARMACY**

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DECLARATION

I, do hereby declare that the research paper, entitled “*IN VIVO STUDY OF ANALGESIC AND CNS ACTIVITY OF KALANCHOE PINNATA (SEC. BRYOPHYLLUM CALYGINUM SALISB) LEAF ON SWISS ALBINO MICE*” submitted to the Department of Pharmacy, East West University, in partial fulfillment of the requirements for the degree of Bachelor of Pharmacy, is the outcome of the investigations performed by me, under the supervision of Md. Razibul Habib, Lecturer, Department of Pharmacy, East West University. I also declare that no part of this dissertation has been or is being submitted elsewhere for the award of any Degree/ Diploma.

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Analgesic and neuropharmacologic study of *K. pinnata*

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(Author)

LIST OF ABBREVIATIONS

<i>K. pinnata</i>	<i>Kalanchoe pinnata (Lam.) Pers</i>
AQ	Aqueous
E-OH	Ethanol
EA	Ethyl Acetate
i.p.	Intraperitoneal
p.o.	Per Oral
b.w.	Body Weight
SEM	Standard Error Mean
ICDDR,B	International Center for Diarrheal Disease and Research, Bangladesh
COX	Cyclooxygenase
GABA	Gamma aminobutyric acid

ABSTRACT

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CHAPTER ONE

1 INTRODUCTION

“A medicinal plant is any plant which, in one or more of its organ, contains substance that can be used for therapeutic purpose or which is a precursor for synthesis of useful drugs.” (Sofowora, 1982, Medicinal Plant and Traditional Medicine in Africa)...This definition of Medicinal Plant has been formulated by WHO (World Health Organization).

In Bangladesh, ninety percent of the medicinal plants are wild sourced (Ghani, 1998; SEDF & IC, 2003). Out of approximately 5,000 species of indigenous and naturalized phanerogamic and pteridophytic plants growing in the country, more than a thousand of them, including many food, vegetable, beverage, spice and ornamental plants, contain medicinally useful chemical substances (Mia, 1990). Growing in the forests, jungles, wastelands, and along roadsides, the types of medicinal plants in Bangladesh are varied. A total of 546 medicinal plants that occur in the country have been counted thus far (Yusuf et al., 1994). However, this list is not exhaustive since it is believed that many other medicinal plants also grow there, but have not yet been discovered or identified (Said, 1995; Ghani, 1998).

1.1 Phytomedicine in Global Health Care

Plants have been the basis of many traditional medicine systems throughout the world for thousands of years and continue to provide mankind with new remedies. Plantbased medicines initially dispensed in the form of crude drugs such as tinctures, teas, poultices, powders, and other herbal formulations, now serve as the basis of novel drug discovery (Jachak and Saklani, 2007). Phytomedicine, popularly known as herbal medicine, refers to the use of plant seeds, berries, roots, leaves, bark, or flowers for medicinal purposes. It has long reputation as “the people’s medicine” for its accessibility, safety and the ease with which it can be prepared. According to World Health Organisation (WHO), from 119 plant-derived medicines, about 74% are used in modern medicine in ways that correlate directly with their traditional uses. WHO also estimates that 4 billion people, 80% of the world's population, presently use herbal medicine for primary health care. Herbal medicine is a common element in Ayurvedic, Homeopathic, Naturopathic, Traditional oriental, Native American and Indian medicine. Even among

prescription drugs, at least 25% contain at least one compound derived from higher plants. The percentage might be higher if we include over-the-counter (OTC) drugs (Barrett et al., 1999).

In developing countries including Bangladesh, about 75% of the populations rely on different forms of traditional medicine for their primary health care (Matu and Staden, 2003). The high cost of imported conventional drugs and/ or inaccessibility to western health care facility, imply that traditional mode of health care is the main form of health care that is affordable and available to our rural people. On the other hand, even when western health facilities are available, traditional medicine is viewed as an efficient and an acceptable system from a cultural perspective. As a result, traditional medicines usually exist side-by-side with western forms of medicine.

1.2 Prospect of natural products and phytomedicine

Numerous methods have been utilised to acquire compounds for drug discovery, including isolation from plants and other natural sources, synthetic chemistry, combinatorial chemistry and molecular modeling. Despite the recent interest in molecular modeling, combinatorial chemistry and other synthetic chemistry techniques by pharmaceutical companies and funding organizations, natural products and particularly medicinal plants, remain an important source of new drugs, new drug leads and new chemical entities (NCEs). According to Newman et al. (2003), 61% of the 877 small-molecule NCEs introduced as drugs worldwide during 1981–2002 was inspired by natural products. These include: natural products (6%), natural products derivatives (27%), synthetic compounds with natural products-derived pharmacophore (5%) and synthetic compounds designed from natural products (natural products mimic, 23%) (Butler, 2004; Geysen et al., 2003; Lombardino and Lowe, 2004). New drugs derived from natural sources have been launched on the market during the last couple of years. These new drugs have received approval for the treatment of cancer, neurological diseases, infectious diseases, cardiovascular and metabolic diseases, immunological, inflammatory and related diseases, and genetic disorders, which encompass many of the common human diseases. Besides new drugs launched on the market from 2000 to date, there are a variety of new chemical entities from natural sources undergoing clinical trials, (Newman et al., 2003). However, the potential benefits of herbal medicines could lie in their high acceptance by patients, efficacy, relative safety and low costs (Thomas et al., 2001). Thus documentation of indigenous knowledge on the use of

plants and providing an inventory of useful plants from local flora can be a great help for accurate use of traditional medicines. Identification and isolation of the active constituents from traditionally used phyto-therapy can ensure the health care. In addition, herbal drugs can also be scientifically modified for better pharmacological activity and to establish safe and effective drugs.

1.3 Natural product research and drug discovery

Nature appears to be a therapeutic cornucopia to treat superfluity of diseases ranging from common cold to multifarious type of illness since the dawn of civilization. Overwhelming evidence has accumulated showing that natural products from plants, microorganisms and marine organisms comprise major portion of the total repertoire of the available therapeutic drugs. Products of natural origins are often called “natural products.” Natural products include: an entire organism (e.g., a plant, an animal, or a microorganism) that has not undergone any kind of processing or treatment other than a simple process of preservation (e.g., drying), part of an organism (e.g., leaves or flowers of a plant, an isolated animal organ), an extract of an organism or part of an organism, and exudates, and pure compounds (e.g., alkaloids, glycosides, sugars, flavonoids, coumarins, lignans, steroids, terpenoids, etc.) isolated from plants, animals, or microorganisms (Samuelsson, 1999). However, in most cases the term natural products refer to secondary metabolites, small molecules (mol wt <2000 amu) produced by an organism that are not strictly necessary for the survival of the organism (Cannell, 1998).

Natural products have played a key role in drug discovery research, as many medicines are either natural products or derivatives thereof. Indeed, it is estimated that about 40% of all medicines is either natural products or their semi-synthetic derivatives. This may not be surprising as herbal medicine is a tradition of healthcare since ancient times and natural extracts screening has been one of the roots of drug discovery research, where erythromycin and rifampicin (bacterial infections), statins (hyperlipidemia), quinines and artemesinin (malaria), paclitaxel, vinblastine and vincristine (cancer), are a few well-known natural products-based medicines. For bacterial infections, over 80% of all medicines in clinical use is either natural products or their derivatives, while for anticancer agents over 60% of all drugs is either natural products or derivatives thereof; examples of several potential lead molecules are vincristine, vinblastine, taxol, camptothecin, podophyllotoxin, combretastatins, etc which have been isolated from plants for successful use in cancer treatment (Newman and Cragg, 2007; Butler, 2004; Newman et al., 2000; Srivastava et

al., 2005). In earlier times, all drugs and medicinal agents were derived from natural substances, and most of these remedies were obtained from higher plants. Today, many new chemotherapeutic agents are synthetically derived, based on "rational" drug design. The study of natural products has advantages over synthetic drug design in that it leads optimally to materials having new structural features with novel biological activity. Not only do plants continue to serve as important sources of new drugs, but phytochemicals derived from them are also extremely useful as lead structures for synthetic modification and optimization of bioactivity. The starting materials for about one-half of the medicines we use today come from natural sources. Virtually every pharmacological class of drugs includes a natural product prototype. The future of plants as sources of medicinal agents for use in investigation, prevention, and treatment of diseases is very promising.

1.4 Approaches to natural product research and drug discovery

Different approaches to drug discovery from plants can be enumerated as: random selection followed by chemical screening, random selection followed by one or more biological assays, follow-up of biological activity reports, follow-up of ethnomedical (traditional medicine) use of plants, use of appropriate plant parts as such in powdered form or preparation of enriched / standardised extracts (herbal product development), use of a plant product, biologically potent but beset with other issues, as a lead for further chemistry, and single new compounds as drugs. The objective of the later approach is the targeted isolation of new bioactive plant products, i.e. lead substances with novel structures and novel mechanisms of action. This approach has provided a few classical examples, but the problem most often encountered here is not enough availability. The problem of availability can be overcome by semi-synthesis/synthesis or using tissue culture techniques (by genetically modifying the biosynthetic pathway of the compound of interest).

Drug discovery from plants involves a multidisciplinary approach combining botanical, ethnobotanical, phytochemical and biological techniques. The search for bioactive chemicals from the unstudied part of the plant kingdom can be conducted essentially with three methods (Cotton, 1996): the random method involves the collection of all plants found in a given area of study, phylogenetic targeting means the collection of all members of those plant families which are known to be rich in bioactive compounds, and the ethnobotanical approach is based on the

traditional knowledge of medicinal plant use. It has been suggested that the ethno-directed sampling is most likely to succeed in identifying drugs for use in the treatment of gastrointestinal, inflammatory and dermatological complaints. Strategies for research in the area of natural products have evolved quite significantly over the last couple of decades. These can be broadly divided into two categories:

Older approach

- Focused on chemistry of compounds from natural sources, but not on activity.
- Straightforward isolation and identification of compounds from natural sources followed by testing of biological activity in animal model.
- Chemotaxonomic investigation.
- Selection of organisms primarily based on ethnopharmacological information, folkloric reputations, or traditional uses.

Modern approach

- Bioassay-directed (mainly *in vitro*) isolation and identification of active lead compounds from natural sources.
- Production of natural products libraries.
- Production of active compounds by cell or tissue culture, genetic manipulation, natural combinatorial chemistry and so on.
- More focused on bioactivity.
- Introduction of the concepts of dereplication, chemical fingerprinting, and metabolomics.
- Selection of organisms based on ethnopharmacological information, folkloric reputations, or traditional uses, and also those randomly selected.

1.5 Challenges in drug discovery from medicinal plants

In spite of the success of drug discovery programmes from plants in the past 2–3 decades, future endeavors face many challenges. Natural products scientists and pharmaceutical industries will need to continuously improve the quality and quantity of compounds that enter the drug development phase to keep pace with other drug discovery efforts. The process of drug discovery has been estimated to take an average period of 10 years and cost more than 800 million dollars (Dickson and Gagnon, 2004). Much of this time and money is spent on the numerous leads that

are discarded during the drug discovery process. It is estimated that only one in 5000 lead compounds will successfully advance through clinical trials and be approved for use. In the drug discovery process, lead identification is the first step. Lead optimization (involving medicinal and combinatorial chemistry), lead development (including pharmacology, toxicology, pharmacokinetics, ADME and drug delivery), and clinical trials all take considerable time.

As drug discovery from plants has traditionally been time-consuming, faster and better methodologies for plant collection, bioassay screening, compound isolation and compound development must be employed (Koehn and Carter, 2005). Innovative strategies to improve the process of plant collection are needed, especially with the legal and political issues surrounding benefit-sharing agreements (Rosenthal, 2002). The design, determination and implementation of appropriate, clinically relevant, highthroughput bioassays are difficult processes for all drug discovery programmes (Knowles and Gromo, 2003; Kramer and Cohen 2004). The common problem faced during screening of extracts is solubility and the screening of extract libraries is many times problematic, but new techniques including pre-fractionation of extracts can alleviate some of these issues, (Koehn and Carter, 2005) Challenges in bioassay screening still remain an important issue in the future of drug discovery from medicinal plants. The speed of active compound isolation can be increased using hyphenated techniques like LC-NMR and LC-MS. Development of drugs from lead compounds isolated from plants, faces unique challenges. Natural products, in general, are typically isolated in small quantities that are insufficient for lead optimisation, lead development and clinical trials. Thus, there is a need to develop collaborations with synthetic and medicinal chemists to explore the possibilities of its semi-synthesis or total synthesis (Ley and Baxendale, 2002; Federsel, 2003). One can also improve the natural products compound development by creating natural products libraries that combine the features of natural products with combinatorial chemistry.

1.6 Opportunities in drug discovery from medicinal plants

Bio prospecting demands a number of requirements which should be co-coordinated, such as team of scientific experts (from all the relevant interdisciplinary fields) along with expertise in a wide range of human endeavours, including international laws and legal understanding, social sciences, politics and anthropology. In our context, Ayurveda and other traditional systems of medicine, rich genetic resources and associated ethno medical knowledge are key components

for sustainable bio prospecting and value-addition processes. For drug-targeted bio prospecting an industrial partner is needed, which will be instrumental in converting the discovery into a commercial product. Important in any bio prospecting is the drafting and signing of an agreement or Memorandum of Understanding that should cover issues on access to the genetic resources (biodiversity), on intellectual property related to discovery, on the sharing of benefits as part of the process (short term), and in the event of discovery and commercialization of a product (long term), as well as on the conservation of the biological resources for the future generations. When ethnobotanical or ethnopharmacological approach is utilised, additional specific requirements that relate to prior informed consent, recognition of Indigenous Intellectual Property and Indigenous Intellectual Property Rights as well as short- and long-term benefit sharing need to be taken into account (Patwardhan, 2005).

In order to screen thousands of plant species at one go for as many bioassays as possible, we must have a collection of a large number of extracts. Globally, there is a need to build natural products extract libraries. The extract libraries offer various advantages, such as reduction in cost and time for repeat collection of plants and availability of properly encoded and preserved extracts in large numbers for biological screening in terms of high-throughput screenings and obtaining hits within a short period. Such libraries could serve as a powerful tool and source of extracts to be screened for biological activities using high-throughput assays.

1.7 Medicinal plants of Bangladesh

Being naturally gifted by a suitable tropical climate and fertile soil, Bangladesh possesses a rich flora of tropical plants. Around 5000 species of phanerogams and pteridophytes grow in its forests, jungles, wastelands and roadsides as indigenous, naturalised and cultivated plants. Out of them, more than a thousand have been claimed to possess medicinal and / or poisonous properties, of which 546 have recently been enumerated with their medicinal properties and therapeutic uses (Ghani, 2003). In addition to possessing various other medicinal properties, 257 of these medicinal plants have been identified as efficacious remedies for diarrhoeal diseases and 47 for diabetes.

Medicinal plants are an accessible, affordable and culturally appropriate source of primary health care system in Bangladesh. Marginalised, rural and indigenous people, who cannot afford or access formal health care systems, are especially dependent on these culturally familiar,

technically simple, financially affordable and generally effective traditional medicines. As such, there is widespread interest in promoting traditional health systems to meet primary health care needs. This is especially true in this country, as prices of modern medicines spiral and governments find it increasingly difficult to meet the cost of pharmaceutical-based health care.

However, it has been observed that many other medicinal plants growing in the country have not been identified taxonomically and that there are many of them, which have not been chemically examined and no attention has yet been paid to characterize them from the pharmacognostic viewpoint. Thus, it is expected that the number of medicinal plants growing or available in Bangladesh may be more than what has so far been enumerated. It has further been observed that the countless herbs found in Bangladesh should be used for promotion of health and for fighting many diseases. Thus medicinal plants of Bangladesh hold good promises as potential resources for drug development.

However, in order to develop these medicinal plants as drugs, attempts should be first made to certainly identify them and preclinical studies on them should be carried out to establish their claimed therapeutic properties. These are very important because the biological activity of a plant or its preparation will assist on determining the therapeutic target of its development. Since the chemical constituents and pharmacological actions of most of these plants are known and as they are in current use in traditional medicines, their clinical evaluation can be undertaken.

1.8 The Plant Family- Crassulaceae

Crassulaceae, or the orpine family, are a family of dicotyledons. They store water in their succulent leaves. They are found worldwide, but mostly occur in the Northern Hemisphere and southern Africa, typically in dry and/or cold areas where water may be scarce. The family includes about 1,400 species in 33 genera.

No member of this family is an important crop plant, but many are popular for horticulture; many members have a bizarre intriguing appearance, and are quite hardy, typically needing only minimal care. Familiar species include the Jade plant or "friendship tree", *Crassula ovata* and "Florists' Kalanchoe", *Kalanchoe blossfeldia*.

Classification within the family is difficult because many of the species hybridize readily, both in the wild and in cultivation. Some older classifications included Crassulaceae in Rosales, but newer schemes treat them in the order Saxifragales.

1.9 The Genus *Kalanchoe*

Kalanchoe is a succulent perennial plant that grows 3-5 feet tall. Commonly known as 'air plant,' it has tall hollow stems, fleshy dark green leaves that are distinctively scalloped and trimmed in red, and bell-like pendulous flowers. *Kalanchoe* is botanically classified with two main Latin names which refer to the same plant: *Bryophyllum pinnatum* and *Kalanchoe pinnatum* (as well as various synonyms of both). This is the only *Kalanchoe* species found in South America, however, 200 other species of *Kalanchoe* are found in Africa, Madagascar, China and Java. A number of species are cultivated as ornamentals here in the U.S. and they are becoming popular tropical house plants. In Brazil the plant goes by the common names of *saião* or *coirama* and in Peru it is called *hoja del aire* (air plant) or *kalanchoe*.

The genus *Kalanchoe* includes more than 100 plants, but only a few are regularly seen in cultivation. *Kalanchoes* are native to arid areas, and they are popular succulents. Modern hybrids are valued for their interesting leaf-forms or for their flowers. Flowering *Kalanchoes* are available in red, pink, yellow, or white. Like many succulents, these are not difficult plants to grow, providing you are careful with the water, especially in the winter.

1.10 Introduction to *Kalanchoe pinnata*

1.10.1 Taxonomic hierarchy of *Kalanchoe pinnata*

Kingdom: Plantae-Plants

Subkingdom: Tracheobionta-Vascular plants

Superdivision: Spermatophyta-Seed plants

Division: Magnoliophyta-Flowering plants

Class: Magnoliopsida-Dicotyledons

Subclass: Rosidae

Order: Rosales

Family: Crassulaceae - Stonecrop family

Genus: *Kalanchoe* Adans. - Widow's Thrill

Species: *Kalanchoe pinnata* (Lam.) Pers.- Cathedral Bells

Botanical Name: *Kalanchoe pinnata* (Lam.) Pers

(National Plant Database. 2005.)

1.10.2 Plant Description

Kalanchoe pinnata (syn. *Bryophyllum calycinum*, *Bryophyllum pinnatum*, also known as the Air Plant, Life Plant, Miracle Leaf, Goethe Plant and the Katakataka (Filipino)) is a succulent plant native to Madagascar. It is distinctive for the profusion of miniature plantlets that form on the margins of its leaves, a trait it has in common with the other members of the *Bryophyllum* section of the *Kalanchoe* genus.

1.10.3 Common Names

Cathedral Bells, Miracle Leaf, Life Leaf, Live Forever, Mexican Loveplant, Air Plant, 'Oliwa Ka Kahakai (Hawai'i), Mother Of Thousands, Herbe Mal Tete (Dominica), Never Dead, Parvu, Wonder-Of-The-World (Trinidad)

Bengali/vernacular name: Patharkuchi; Koppata (Chittagong).

Tribal name: Rokkiapumbo (Marma), Gios (Chakma).

1.10.4 Identification

Leaves: Leaves often deciduous at anthesis, simple or pinnately compound, opposite or whorled, sometimes alternate above, margins crenate, serrate, or sometimes entire, often producing adventitious buds, petiolate or sessile.

Flowers: Flowers in 1-2 terminal cymes; sepals 4, sometimes inflated, connate into a tube or distinct; corolla tubular, constricted above the ovaries, 4 lobed; stamens (4)8; ovaries 4, slender and erect, ovules numerous.

Fruit: A follicle.



Leaf

Fruit

Flower

Figure 1.1: Various plant parts of *Kalanchoe pinnata*

1.10.5 Growing Conditions

Light: It prefers bright, sunny locations, especially in the summer growing season.

Water: Moderate watering throughout the summer and reduce watering in the winter.

Temperature: Prefer warmth. Growth is hampered if the temperature falls below 55°F.

Soil: An ordinary soil mix.

Propagation:

Many kinds of *Kalanchoe* will produce tiny plantlets along the leaf margins that can be individually potted up. These types include *K. pinnata*—the air plant. It should flower naturally in spring. Professional growers force *Kalanchoes* to bloom throughout the year (they are a short-day plant).

1.10.6 Global distribution

This is the only *Kalanchoe* species found in South America, however, 200 other species of *Kalanchoe* are found in Africa, Madagascar, China and Java.

A number of species are cultivated as ornamentals in the U.S. and they are becoming popular tropical house plants.

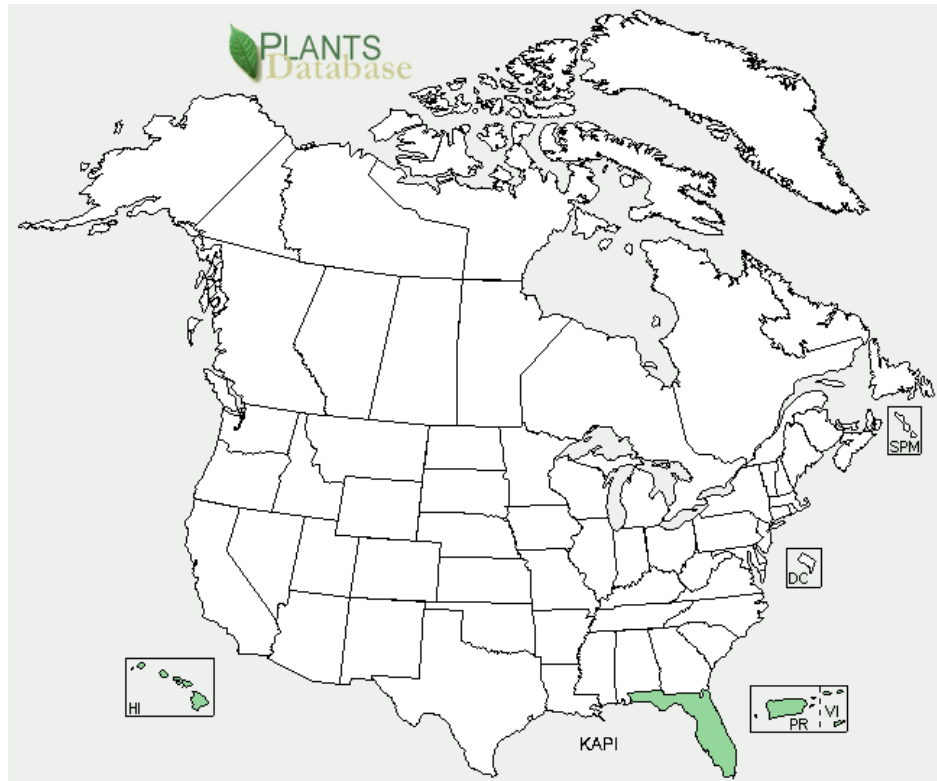


Figure 1.2: Global distribution of *Kalanchoe pinnata* (www.image.google.com).

1.10.7 Plant Chemicals

Kalanchoe is rich in alkaloids, triterpenes, glycosides, flavonoids, steroids and lipids. The leaves contain a group of chemicals called bufadienolides which are very active and have sparked the interest of scientists. They are very similar in structure and activity as two other cardiac glycosides, digoxin and digitoxin (drugs used for the clinical treatment of congestive heart failure and related conditions). *Kalanchoe*'s bufadienolides have demonstrated in clinical research to possess antibacterial, antitumorous, cancer preventative, and insecticidal actions.

The main plant chemicals found in *kalanchoe* include: arachidic acid, astragalgin, behenic acid, beta amyryn, benzenoids, beta-sitosterol, bryophollenone, bryophollone, bryophyllin, bryophyllin A-C, bryophyllol, bryophynol, bryotoxin C, bufadienolides, caffeic acid, campesterol,

cardenolides, cinnamic acid, clerosterol, clionasterol, codisterol, coumaric acid, epigallocatechin, ferulic acid, flavonoids, friedelin, glutinol, hentriacontane, isofucosterol, kaempferol, oxalic acid, oxaloacetate, palmitic acid, patuletin, peposterol, phosphoenolpyruvate, protocatechuic acid, pseudotaraxasterol, pyruvate, quercetin, steroids, stigmasterol, succinic acid, syringic acid, taraxerol, and triacontane.

P-coumaric, ferulic, syringic, caffeic and p-hydroxybenzoic acids, quercetin and kaempferol have been detected in leaves. Wax hydrocarbons (C25-35), wax alcohols (C26-36) and fatty acids are obtained from wax of leaves (Rastogi & Mehrotra, 1993). They have also been reported to contain fumaric acid, lipids, phenolic substances and a cytotoxic bufadienolide orthoacetate (Ghani, 2003). Cellular sap contains flavonoids. The plant extract also contains n-alkane, n-alkanol, α and β -amyrin and sitosterol in its unsaponifiable matter (Asolkar et al., 1992).

1.10.8 Biological Activity and Clinical Research

Many of kalanchoe's traditional uses can be explained by the clinical research conducted thus far on the plant. The traditional use for infectious conditions (both internally and externally) is supported by research indicating kalanchoe leaves have antibacterial, antiviral and antifungal activity. The leaf and leaf juice have demonstrated significant in vitro antibacterial activity towards *Staphylococcus*, *E. coli*, *Shigella*, *Bacillus* and *Pseudomonas*, including several strains of multi-drug resistant bacteria. A water extract of kalanchoe leaves (administered topically and internally) has been shown to prevent and treat leishmaniasis (a common parasitic disease in tropical countries which is transmitted by the bite of sand flies) in both humans and animals. In addition to its antibacterial properties, kalanchoe's traditional uses for upper respiratory conditions and coughs might be explained by research demonstrating that the leaf juice has potent anti-histamine and anti-allergic activity. In an in vivo study (with rats and guinea pigs) the leaf juice was able to protect against chemically induced anaphylactic reactions and death by selectively blocking histamine receptors in the lungs.

In another in vivo study scientists validated kalanchoe's use for gastric ulcers; a leaf extract protected mice from such ulcer-inducers as stress, aspirin, ethanol and histamine. Other in vivo research confirms that kalanchoe can reduce fevers, and provides anti-inflammatory, pain-relieving and muscle relaxant effects. Its anti-inflammatory effects have been partially attributed

to the immunomodulatory and immune suppressant effect documented by scientists in several studies. In several in vivo and in vitro studies, researchers reported that extracts of the leaf and/or juice suppressed various immune reactions, including those which trigger an inflammatory response as well as a histamine response. *Kalanchoe* has also shown sedative and central nervous system depressant actions in animal studies. These effects were attributed partially to the leaf extract demonstrating the ability to increase the levels of a neurotransmitter in the brain called GABA (gamma aminobutyric acid).

1.10.9 Worldwide Ethnomedical Uses

Leaves are diuretic, antilithic and insecticidal; applied to wounds, boils and bites of insects. It is useful in bronchial affections, kidney stones, blood dysentery, gout and jaundice. Juice of the warmed leaves is drunk for cough. Pounded leaves are applied to corns, burns and scalds. In Khagrachari leaf juice is given orally in jaundice. It is also given for indigestion and stomach pain. Pounded leaves soaked in water overnight and the mucilaginous water thus obtained is taken in the next morning in empty stomach for blood dysentery. Juice of the leaves along with sugar is given in gonorrhoea in Jointiapur of Sylhet (Yusuf et al. 2009). Plant extract possesses antifungal properties (Asolkar et al., 1992).

Table 1.1: Worldwide Ethnomedical Uses

Country	Purpose of use
Brazil	for abscesses, adenoids(infected), arthritis, athlete's foot, boils, bronchitis, bubos, burns, calluses, conjunctivitis, corns, coughs, dermatitis, dermatosis, earaches, eczema, edema, erysipelas, fever, glaucoma, headache, infections, inflammation, insect stings, intestinal problems, itch, kidney stones, lymphatic disorders, mouth sores, nervousness, respiratory infections, rheumatism, scurvy, skin problems, toothache, tuberculosis, tumor, ulcers, urinary insufficiency, wart, whooping cough, wounds, and as a sedative
Ecuador	for bruises, broken bones
Guatemala	for aches, diarrhea, pain, skin problems
India	for abdominal discomfort, boils, bruises, cholera, cuts, diabetes, diarrhea, dysentery, flatulence, headaches, kidney stones, indigestion, insect bites, scabies, sores, urinary insufficiency, wounds
Mexico	for eye infections, headaches, inflammation, menstrual disorders, pimples, wounds
Nicaragua	for aches, burns, childbirth, colds, coughs, fever, headache, pain, respiratory infections
Nigeria	for coughs, earaches, eczema, inflammation, pimples
Peru	for bacterial infections, boils, broken bones, bronchitis, cancer (lymphoma), conjunctivitis, coughs, earaches, eye infections, epilepsy, erysipelas, fever, gas, headache, heartburn, inflammation, intestinal problems, migraine, nausea, skin problems, sores, ulcers, urethritis
South America	for asthma, chest colds, earaches, headaches, sores, strains, tumors
USA	for chicken pox, fevers, stomachache
West Indies	for menstrual disorders, ulcers
Elsewhere	for arthritis, asthma, bruises, burns, constipation, diabetes, earaches, headaches, malnutrition, migraines, nephritis, paralysis, respiratory infections, rheumatism, sprains, swelling, ulcers, wound

1.10.10 Traditional Use in Bangladesh

Table 1.2: Traditional Use in Bangladesh

Location	Purpose of Use
Marma	The leaf is applied for muscle pain, scabies, boils, and rheumatism
Narshingdi	Juice from the leaves is taken during common cold
Kurigram	Juice from the leaves is mixed with water and sugar and taken 3 times a day after meal for 3-10 days for syphilis
Bogra	Shortness of breath, asthma
Chak	Pneumonia, respiratory difficulties, cough, mucus. One teaspoon of juice squeezed from the leaves is taken thrice daily for 7 days
Murong	Cough, mucus, respiratory problem. One teaspoon of leaf juice is mixed with 3 drops of honey and taken once daily for 5 days
Natore	Gastric ulcer, kidney stones, galls bladder stones. 1-2 cups of leaf juice is taken with honey 3 times a day
Noakhali	The green leaves are washed and then crushed on a shil-nora to extract juice. The juice is fed to patients for removing stomach stones
Tripura	Coughs, mucus, fever, sudden loss of consciousness (epilepsy-like), constipation, piles. 1. Juice squeezed from the leaves is taken two teaspoonfuls twice daily for coughs, mucus, fever, and sudden loss of consciousness. 2. Paste of leaves is applied to rectum for constipation and piles.
Khagrachari	leaf juice is given orally in jaundice
Sylhet	Juice of the leaves along with sugar is given in gonorrhoea

1.10.11 Contraindications

The plant should not be used in pregnancy. Though not supported by clinical research, it has traditionally been used during childbirth and may stimulate the uterus.

Kalanchoe has documented immune modulating actions and should not be used chronically for long periods of time, or by those with a lowered immune system.

Drug Interactions:

- May potentiate barbiturates.
- May potentiate cardiac glycosides such as digoxin and digitoxin.
- May potentiate immunosuppressive medications.
- May potentiate CNS depressant medications.

1.11 Analgesic principles in medicinal plants

Scientific and methodical investigation of herbal plants has become a potential source for the discovery of lead compounds of high therapeutic value in terms of analgesic and anti-inflammatory activity. Ethno-pharmacological studies have become increasingly invaluable in the development of modalities for the management of pain and related disorders. Thus green pharmaceuticals have now received considerable attention and popularity in this area due to its availability, less side effects and economic feasibility compared to the orthodox medicine.

Pain is defined as a sensorial modality, primarily protective in nature, but often causes discomfort. It is the most important symptom that brings the patient to physician. Inflammation is a complex biological response of vascular tissues to harmful stimuli such as pathogens, damaged cells and irritants. It is also the protective attempt by the organism to the injurious stimuli as well as initiate healing process for the tissue and considered to be the major cause of rheumatoid arthritis (Fayyaz et al., 1998; Divya et al., 2009). Analgesics relieve symptoms of pain, but hardly affect its underlying cause. Currently available analgesic and anti-inflammatory drugs such as opiates and NSAIDs are not useful in all cases due to their adverse effects (Hasan et al., 2009).

Again, plant-derived secondary metabolites have, over the years, greatly contributed to our current understanding of the important mechanisms related to the process of inflammation, pain transmission and treatment. Furthermore, they have permitted us to characterise receptor types and identify endogenous ligands involved in the mechanism of nociception. Plants, such as *Papaver somniferum*, *Cannabis sativa* and those of the *Capsicum* and *Salix* species, have greatly accounted for the development of clinically relevant drugs which are useful for the management of pain disorders. The recent advances in our understanding of the mechanisms of action of the above plant-derived substances, together with use of molecular biology techniques, have greatly

accelerated attempts to identify promising targets for the discovery of new, safe, and efficient analgesic drugs. Despite the great progress that has occurred in the elucidation of pain transmission and despite decades of use, leaving aside its known undesirable side effects, morphine continues to be one of the most used drugs in clinical practice for the treatment of pain disorders.

Thus, safer and more efficacious analgesic and anti-inflammatory drugs are urgently needed. A search through the literature reveals that many potentially active antinociceptive plant-derived compounds have been identified. However, studies aiming at investigating their cellular and molecular mechanisms of action and well controlled clinical trials to prove their efficacy in humans are still lacking. Nevertheless, natural or synthetic substances that bind to vanilloid or cannabinoid receptors, or even those that are capable of modulating the endogenous ligands which bind to these receptors, are expected to soon appear to assist in the treatment of several pain disorders, including those of neuropathic or neurogenic origin (Calixto et al., 2001; Singh and Budhiraja, 2006; Scholten, 2006; Russo and Guy, 2006).

1.12 Neuropharmacologic principles of medicinal plant

Central nervous system (CNS) depressants are drugs that can be used to slow down brain activity. Signs of CNS depression include drowsiness, slower heart rate, and loss of motor skills, slower breathing, unclear speech, unclear thinking and unclear vision. Sometimes, there is a medical benefit to causing the central nervous system to calm down. Doctors frequently treat anxiety or sleep disorders by prescribing barbiturates such as Nembutal or benzodiazepines such as Valium and Xanax.

CNS depressants are also present, in relatively low doses, in over-the-counter cold medicines. CNS depressants include alcohol, opiates, pain medications and other common drugs of abuse. These are dangerous in their own right but doubly so when used in combination. Drinking while using CNS depressants (even with a prescription) can cause coma or death, as can using CNS depressants without a prescription or in a manner inconsistent with a prescription.

CNS depressants slow the brain by increasing the activity of a chemical in your brain called gamma aminobutyric acid (GABA). GABA is a neurotransmitter, a chemical that helps your

brain cells communicate with one another. GABA slows the brain, so the more it's working, the slower the brain works.

Central nervous system (CNS) stimulants are medicines that speed up physical and mental processes. Neuropharmacological potential of a medicinal plant is evaluated by some conventional tests like thiopental induced sleeping time, hole cross, hole board, open field, elevated plus MAZE test.

1.13 RATIONALE OF THE WORK

A range of different approaches as described earlier has been employed to obtain lead compounds for drug discovery, including isolation from plants and other natural sources, synthetic chemistry, combinatorial chemistry and molecular modeling. Despite the recent interest in molecular modeling, combinatorial chemistry and other synthetic chemistry techniques, natural products and particularly medicinal plants, remain an important source of new drugs, new drug leads and new chemical entities.

However, only a few phytochemical and biological works of medicinal interest have so far been carried out on this plant to substantiate the above traditional claims. It was, therefore, the objective of this study to delineate and validate on scientific line some of the uses of the plant in folk medicine along with phytochemical study on the leaves of *Kalanchoe pinnata* L.

In the present study, the leaves of *kalanchoe pinnata* were extracted with water, ethanol and ethyl acetate. All three fractions were subjected to the phytochemical and Pharmacological tests on laboratory animals. Hence, the specific objectives of the present work were:

- To carry out **analgesic** investigations on this plant using acetic acid-induced writhing and formalin induced persistent pain (Biphasic pain)
- To evaluate **sedative** property using hole cross, open field and **anxiolytic** potential using Elevated plus-maze (EPM) test, and
- To examine **antidiarrhoeal** properties of *Kalanchoe pinnata* using castor oil

In all cases of pharmacological tests, a major objective was to evaluate and establish the most active fraction of the aerial parts of *Kalanchoe pinnata* in respective testing models.

1.14 LIMITATION OF THE STUDY

The experiments designed and conducted in the present study are not, however, sufficient to arrive at a concrete conclusion about the results, since these are mainly preliminary screening and require extensive bioactivity-guided isolation and characterization of chemical constituents. It will not, therefore, be judicious to claim that a particular chemical group is surely responsible for a specific biological activity unless elaborate phytochemical investigation leading to isolation and characterization of the chemical constituents is undertaken.

Other limitation includes:

- Individual physiologic difference of test animal
- Individual psychological difference of test animal
- Difficult to identify any disease of the test animal
- Dose variation may occur
- Dose dumping may occur
- Data interpretation may be wrong
- Lack of fresh mice for each different test
- Animal maintenance is difficult
- Lack of sound proof room

CHAPTER TWO

2 LITERATURE REVIEW

2.1 T cell-suppressive flavonoids of *Kalanchoe pinnata*

The chemical composition and immunosuppressive potential of the flowers from *Kalanchoe pinnata* (Crassulaceae) were investigated. It was found that the aqueous flower extract was more active than the leaf extract in inhibiting murine T cell mitogenesis in vitro. Flavonoids isolated from the flower extract were identified and quantitated based on NMR and HPLC-DAD-MS analysis, respectively. Along with quercetin, four quercetin glycosyl conjugates were obtained, including quercetin 3-O-beta-D-glucuronopyranoside and quercetin 3-O-beta-D-glucopyranoside, which are described for the first time in *K. pinnata*. All flavonoids inhibited murine T cell mitogenesis and IL-2 and IL-4 production without cell toxicity. Findings show that *K. pinnata* flowers are a rich source of T-suppressive flavonoids that may be therapeutically useful against inflammatory diseases. (Coutinho MA et al. 2012 Feb)

2.2 Effects of light intensity on the distribution of anthocyanins in *K. pinnata*

This paper compares two medicinal species of *Kalanchoe*, which are often used interchangeably by the population, regarding the distribution of anthocyanins under the influence of four luminosity levels for 6 months. For the morphoanatomical analysis, the 6th stem node of each plant was sectioned. Usual histochemical tests revealed the presence of anthocyanins by cross sections of the stems, petioles and leaf blades. The petioles and leaf blades were submitted to the extraction with acidified methanol, and the anthocyanins were quantified by spectrophotometric readings. At the macroscopic level, it was noticed for both species a higher presence of anthocyanins in stems and petioles of plants under full sunlight. The microscopy of *K. brasiliensis* stems evidenced the deposition of anthocyanins in the subjacent tissue to the epidermis and cortex, which increased with light intensity. In *K. pinnata* a subepidermal collenchyma was observed, which interfered in the visualization of anthocyanins. In petioles and leaf blades of *K. brasiliensis* the deposition of anthocyanins was peripheral, and in *K. pinnata* it was also throughout the cortex. The quantification of anthocyanins in petioles showed in 70% of light higher averages than in 25%, but in leaf blades there were no significant results. This study

contributes to the pharmacognosy of *Kalanchoe* and it is sustained by the description of flavonoids as biological markers of the genus. (Cruz BP et al, 2012 March)

2.3 Myometrial relaxation by *Bryophyllum pinnatum*.

The use of preparations from *Bryophyllum pinnatum* (Lamarck) Oken (*Kalanchoe pinnata* (Lamarck) Persoon) in tocolysis is supported by clinical evidence. The effect of *B. pinnatum* leaf press juice and its chemical fractions on the response of human myometrial strips were studied. No data are available if the influence on myometrial strips of the juice differs from that of its components in the chemical fractions, in order to increase the pharmacological effect.

In vitro study to test the effect of repeated addition of *B. pinnatum* leaf press juice (BPJ) and its chemical components in several dilutions (undiluted, 1-10%) on myometrium strips hang up in a myograph chamber. Chemical analysis is including HPLC, MPLC with Sephadex LH-20 and TLC.

All test solutions are inhibiting contractility by reducing the amplitude and the area under the curve (AUC) of the contractions. Undiluted BPJ and its undiluted chemical fraction 4 are reducing most effective these two parameters: the amplitude was at 78% of the baseline (95% CI (77-89); $p < 0.05$) at the second addition of the BPJ and at 70% (95% CI (50-90); $p < 0.05$) of the first addition of fraction 4; the AUC was at 82% (95% CI (69-95); $p < 0.05$) of the baseline at the first addition of the press juice and at 51% (95% CI (27-74); $p < 0.05$) at the first addition of fraction 4. The BPJ decreased amplitude and AUC significantly faster and increased frequency significantly faster than the control. Fractions could be tentatively assigned to bufadienolids, flavonoids and cinnamic acids. Fraction 4, accounted for flavonoids, increased the frequency of the contractions most effectively: 557% of the baseline (95% CI (316-797); $p < 0.05$) at the first addition.

Leaf juice of *B. pinnatum* and its flavonoid fraction are most effective in relaxing myometrial strips by inducing frequency. (Wächter R et al., 2011 Dec 15)

2.4 Mast cell inactivation and prevention of allergic airway disease by *K. pinnata*

Aqueous extract of *Kalanchoe pinnata* (Kp) have been found effective in models to reduce acute anaphylactic reactions. In the present study, the effect of Kp and the flavonoid quercetin (QE) and quercitrin (QI) was investigated on mast cell activation in vitro and in a model of allergic airway disease in vivo. Treatment with Kp and QE in vitro inhibited degranulation and cytokine production of bone marrow-derived mast cells following IgE/FcεRI crosslinking, whereas treatment with QI had no effect. Similarly, in vivo treatment with Kp and QE decreased development of airway hyperresponsiveness, airway inflammation, goblet cell metaplasia and production of IL-5, IL-13 and TNF. In contrast, treatment with QI had no effect on these parameters. These findings demonstrate that treatment with Kp or QE is effective in treatment of allergic airway disease, providing new insights to the immunomodulatory functions of this plant. (Cruz EA et al.,)

2.5 Wound healing potential of ethanolic extract of *K. pinnata*

The extract of *K. pinnata* was evaluated for its wound healing activity by using excision wound model in rats. On day 11, animals treated with the ethanolic leaf extract exhibited 86.33% reduction in the wound area, compared to petroleum jelly treated control (69.36%) and the mupirocin treated standard (85.49%). The hydroxyproline content of extract treated animals was higher, as compared to control and the standard groups. Histological analysis was also consistent with the proposal that *K. pinnata* leaf extract exhibits significant wound healing potential. The increased rate of wound contraction and hydroxyproline content in the extract treated animals supports the claims made by traditional healers of the benefits obtained from the medicinal use of *K. pinnata*. (Nayak BS et al.,)

2.6 Bioactive molecules in *K. pinnata*

Kalanchoe pinnata (Lam.) Pers. (syn. *Bryophyllum pinnatum*; family Crassulaceae) is a popular plant used in traditional medicine in many temperate regions of the world and particularly in South America. In Guyana, the leaves are traditionally used as an anti-inflammatory and antiseptic to treat coughs, ulcers, and sores. The purpose of this study was to implement a method for targeting and identifying molecules with antimicrobial activity, which could replace

chemical preservatives in cosmetic applications. The leaves were extracted by a method based on pressurized liquid extraction (PLE), using different solvents. A study of antimicrobial activity and cytotoxicity tests were performed to select the most interesting extract. To isolate one or more active molecules, the selected crude extract was fractionated by centrifugal partition chromatography (CPC) and then antimicrobial activity and cytotoxicity of each fraction were tested under the same procedure. The last step consisted of identifying the main compounds in the most active fraction by LC-MS/MS. (El Abdellaoui S, et al.,)

2.7 Oral metabolism and efficacy of *K. pinnata* flavonoids

Leishmaniasis is a parasitic disease that threatens 350 million people worldwide. In a search for new antileishmanial drugs, the *in vitro* activity of flavonoids from *Kalanchoe pinnata* (Crassulaceae) was previously demonstrated in infected cells. In order to demonstrate the safety and oral activity of *K. pinnata*, flavonoids were evaluated *in vivo* in a murine model of cutaneous leishmaniasis. Daily oral doses of quercetin 3-O- α -L-arabinopyranosyl (1 \rightarrow 2)- α -L-rhamnopyranoside, quercetin 3-O- α -L-rhamnopyranoside, and free quercetin (16 mg/kg body weight) all were able to control the lesion growth caused by *Leishmania amazonensis* and to significantly reduce parasite load. These flavonoids were as effective as the crude *K. pinnata* aqueous extract given at 320 mg/kg body weight. HPLC-DAD-MS analysis of the plasma of extract-treated mice suggested that quercetin and quercetin glucuronides are the main metabolites of *K. pinnata* quercetin glycosides. The results indicate that *K. pinnata* quercetin glycosides are important active components of the aqueous extract and that they possess potent oral efficacy against cutaneous leishmaniasis. (Muzitano MF, et al.,)

2.8 Effectiveness of immunomodulatory extract of *K. pinnata*

The study, shows the effectiveness of *Kalanchoe pinnata* (Kp) against visceral leishmaniasis, using the BALB/c mouse model of infection with *Leishmania chagasi*. Mice receiving oral daily doses of Kp (400 mg/kg) for 30 days displayed significantly reduced hepatic and splenic parasite burden, when compared with untreated animals. Protectiveness was accompanied by a reduction in parasite-specific IgG serum levels, and impaired capacity of spleen cells to produce IL-4, but not IFN- γ and nitric oxide upon antigen recall *in vitro*. The reference drug Pentostam (72 mg/kg) given by the intra-peritoneal route on alternate days produced an anti-leishmanial effect similar to oral Kp. Findings show that the oral efficacy of Kp, seen previously in murine

cutaneous leishmaniasis, extends also to visceral leishmaniasis caused by *L. chagasi*, a difficult to treat and lethal disease of man. (Gomes DC et al.,)

2.9 Protection against fatal anaphylactic shock by *K. pinnata*

An investigation was performed on the protective effect of Kp in fatal anaphylactic shock, likewise a Th2-driven immunopathology, and the identification of its active component. Mice daily treated with oral Kp during hypersensitization with ovalbumin were all protected against death when challenged with the allergen, as compared with the 100% mortality in the untreated group. A single intraperitoneal dose 3 h prior to challenge was partially effective. Oral protection was accompanied by a reduced production of OVA-specific IgE antibodies, reduced eosinophilia, and impaired production of the IL-5, IL-10 and TNF-alpha cytokines. In vitro, Kp prevented antigen-induced mast cell degranulation and histamine release. Oral treatment with the quercitrin flavonoid isolated from Kp prevented fatal anaphylaxis in 75% of the animals. These findings indicate that oral treatment with Kp effectively downmodulates pro-anaphylactic inducing immune responses. Protection achieved with quercitrin, although not maximal, suggests that this flavonoid is a critical component of Kp extract against this extreme allergic reaction. (Cruz EA et al.,)

2.10 Antileishmanial activity of flavonoid of *K. pinnata*.

The aqueous leaf extract from the medicinal plant *K. pinnata* (Crassulaceae) afforded a kaempferol di-glycoside, named kapinnatoside, identified as kaempferol 3-O-alpha-L-arabinopyranosyl (1-->2) alpha-L-rhamnopyranoside (1). In addition, two unusual flavonol and flavone glycosides already reported, quercetin 3-O-alpha-L-arabinopyranosyl (1-->2) alpha-L-rhamnopyranoside (2) and 4',5-dihydroxy-3',8-dimethoxyflavone 7-O-beta-D-glucopyranoside (3), have been isolated. Their structures were determined via analyses of mono and bi-dimensional (1)H and (13)C NMR spectroscopic experiments and HR-MALDI mass spectra. Because of its restricted occurrence and its abundance in *K. pinnata*, flavonoid (2) may be a chemical marker for this plant species of high therapeutic potential. The three flavonoids were tested separately against *Leishmania amazonensis* amastigotes in comparison with quercitrin, quercetin and afzelin. The quercetin aglycone - type structure, as well as a rhamnosyl unit linked at C-3, seems to be important for antileishmanial activity. (Muzitano MF et al.,)

2.11 Effectiveness of oral *K. pinnata* on leishmaniasis.

Leishmaniasis is an extremely difficult disease to treat. Previously, it was shown that oral *Kalanchoe pinnata* (Kp) leaf extract is strongly effective against murine leishmaniasis. Here, it is shown that the serum levels of alanine-aminotransferase (ALT), aspartate-aminotransferase (AST), urea and alkaline phosphatase were unchanged in mice orally treated with supraoptimal Kp doses for 30 days, indicating the absence of chronic toxicity to the liver, heart or kidney. Additionally, evidence is presented that human leishmaniasis may also be controlled with oral Kp. A 36-year-old man with an active cutaneous leishmaniasis was orally treated with 30 g wet weight of Kp leaves/day for 14 days. During the Kp treatment, the lesion stopped growing and slightly decreased. No adverse reactions or toxicity was observed. (Torres-Santos EC, et al.,)

2.12 Hepatoprotective activity of leaves of *K. pinnata* Pers.

Kalanchoe pinnata Pers. is naturalized throughout the hot and moist parts of India. Juice of the fresh leaves is used very effectively for the treatment of jaundice in folk medicines of Bundelkhand region of India. The juice of the leaves and the ethanolic extract of the marc left after expressing the juice were studied in rats against CCl₄-induced hepatotoxicity. The test material was found effective as hepatoprotective as evidenced by in vitro, in vivo and histopathological studies. The juice was found more effective than ethanolic extract. (Yadav NP et al.,)

2.13 Anti-tumor promoting activity of bufadienolides from *K. pinnata*

Five bufadienolides (1-5) isolated from the leaves of *Kalanchoe pinnata* and *K. daigremontiana* x *tubiflora* (Crassulaceae) were examined for their inhibitory effects on Epstein-Barr virus early antigen (EBV-EA) activation in Raji cells induced by the tumor promoter, 12-O-tetradecanoylphorbol-13-acetate. All bufadienolides showed inhibitory activity, and bryophyllin A (1) exhibited the most marked inhibition (IC₅₀ = 0.4 microM) among the tested compounds. Bryophyllin C (2), a reduction analogue of 1, and bersaldegenin-3-acetate (3) lacking the orthoacetate moiety were less active. These results strongly suggest that bufadienolides are potential cancer chemopreventive agents. (Supratman U et al.,)

2.14 New insecticidal Bufadienolide, Bryophyllin C from *K. pinnata*

Two insecticidal bufadienolides (1 and 2) were isolated from a methanol extract of the leaves of *Kalanchoe pinnata* by bioassay-guided fractionation. Compound 1 was identified as known bryophyllin A (bryotoxin C). The structure of new bufadienolide 2, named bryophyllin C, was determined by spectroscopic methods and the chemical transformation of 1. Compounds 1 and 2 showed strong insecticidal activity against third instar larvae of the silkworm (*Bombyx mori*), their LD50 values being evaluated as 3 and 5 microg/g of diet, respectively. (Supratman U et al.,)

2.15 Potent lymphocyte suppressive activity of fatty acid fraction of *K. pinnata*

Almeida AP et al. attempted to identify the immunosuppressive substances present in KP guided by the lymphoproliferative assays. From the ethanolic extract was purified a fraction (KP12SA) twenty-fold more potent to block murine lymphocyte proliferation than the crude extract. Chemical analysis by ¹H- and ¹³C-NMR, IR and GC-MS of KP12SA (methylated sample) showed 89.3% of palmitic acid (C16), 10.7% of stearic acid (C18) and traces of arachidic (C20) and behenic acids (C22). This study provides evidence that fatty acids present in *Kalanchoe pinnata* may be responsible, at least in part, for its immunosuppressive effect in vivo. (Almeida AP et al.,)

2.16 Anti-leishmanial effect of *Kalanchoe* mediated by nitric oxide intermediates

Da-Silva SA et al. reported on the mode of action of Kp, particularly on the induction of nitric oxide (NO) production by macrophages. They observed that Kp has no direct inhibitory activity on extracellular promastigotes, but effectively decreases the intracellular amastigote growth in a dose-related fashion. A 58% reduction in amastigote growth induced by 500 micrograms/ml Kp was associated with a 6-fold increase in the production of NO by the macrophages. IFN-gamma synergistically enhanced the NO-stimulating effect of Kp in culture. Co-treatment with the inducible NO synthase enzyme inhibitor L-NG-monomethyl-arginine abolished the antileishmanial effect of Kp in vitro and in *L. amazonensis*-infected BALB/c mice. These results indicate that the protective effect of Kp in leishmaniasis may not be due to a direct effect on the parasite itself but rather to activation of the reactive nitrogen intermediates pathway of macrophages. (Da-Silva SA et al.,)

2.17 Summary of Literature Review

Patharkuchi has lots of medicinal properties. Generally, juice made from the leaves of this plant is used in the herbal treatment of cold, cough, diabetes, diuretic and blood dysentery. A thick paste of these leaves is also applied on wounds. It helps them to heal faster. The therapeutic use the plant *Kalanchoe pinnata* possess are-

- Anthelmentic
- Immunosuppressive
- Wound healing
- Hepatoprotective
- Antinociceptive
- Anti-inflammatory
- Antidiabetic
- Nephroprotective
- Antioxidant activity
- Antimicrobial activity
- Analgesic
- Anticonvulsant
- Neuropharmacological
- Antipyretic.

CHAPTER THREE

3 MATERIALS AND METHODS

3.1 Collection of Plant Material

The plant was collected from Forests of Chittagong Hill Tracts in October 2011 when leaves were in their maximum densities. The plant leaves were thoroughly washed with water and were dried in a hot air oven at room temperature for 7 days and at 40°C for the next 2 days.

3.2 Identification

The plant was identified and authenticated by Associate Prof. Dr. Shaikh Bokhtear Uddin (Department of Botany, University of Chittagong, Bangladesh) in October 2011.

3.3 Preparation of plant extract

The dried leaves were coarsely powdered and about 1,000 g of powdered material was macerated with water, ethanol and ethyl acetate at room temperature for a period of 7 days with occasional shaking and stirring. The whole mixture was filtered and the filtrate thus obtained was concentrated using a rotary evaporator (Bibby RE200, Sterlin Ltd., UK) to get a viscous mass. The viscous mass was kept at room temperature under a ceiling fan to get a dried extract (about 10%).

3.4 Collection and Maintenance of Animals

For the analgesic, sedative and anxiolytic and experiments, Swiss Albino mice of either sex, 3-4 weeks of age, weighing between 20-25 g, were collected from the Animal Research Branch of the International Center for Diarrhoeal Disease and Research, Bangladesh (ICDDR, B). They were housed in groups of 5, in plastic cages having dimension of (28×22×13 cm). Soft wood shavings were used as bedding of cages. Animals were maintained under standard environmental conditions (temperature: (24.0±1.0 °C), relative humidity: 55-65% and 12 hrs light/12 hrs dark cycle). Husk and excreta were removed from the cages every day. Pellets of mice foods, provided by ICDDR, B were given to the mice with fresh water *ad libitum*. The newly bought mice were given a week rest to get over the food and water restrictions incurred during transit

and to get themselves adapted with the new environment of the laboratory, before being employed in any experiment (Hasan et al., 2009).

Animal feed: Mouse-pellets supplied by ICDDR, B and wheat.

Material used for animal housing: Plastic cages having a dimension of (28×22×13) cm; Soft wood Shaving

Identification of animals during experiment

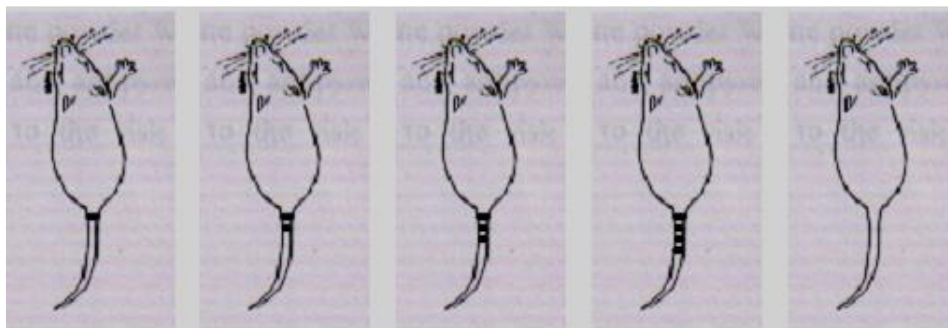


Figure 3.1: Marks used for identifying experimental animals.

Each group consists of five mice/rats and hence it is difficult to identify and observe at a time five mice/rats receiving same treatment. Thus it was important to identify individual animal of a group during the treatment. To denote individual animal, they were marked or coded I, II, III, IIII and none (for no. five) on their tails.

3.5 Tests for analgesic activity

Drugs and chemicals

Table 3.1: List of drugs and chemicals used in analgesic activity tests and their sources

Drugs and Reagents	Purpose	Source
Diclofenac-Na	Standard drug in acetic acid induced writhing test	Square pharmaceuticals Ltd., Banglaesh
Tween 80	Suspending agent	Merck, Germany
Acetic acid	Writhing reflex inducer	Merck, Germany

3.5.1 Acetic acid-induced writhing test

The antinociceptive activity of the samples was studied using acetic acid-induced writhing model in mice (Ahmed et al., 2004; Hasan et al., 2009). The animals were divided into control, positive control and test groups with five mice in each group. The animals of test groups received test samples at the doses of 400 mg/kg body weight. Positive control group received standard drug diclofenac at the dose of 10 mg/kg body weight and vehicle control group was treated with 1% Tween 80 in water at the dose of 10 ml/kg body weight. Test samples and vehicle were administered orally 30 min before intraperitoneal administration of 0.7% 0.1 ml/10 gm acetic acid solution but diclofenac was administered 15 min before injection of acetic acid. After an interval of 5 min, the mice were observed for specific contraction of body referred to as 'writhing' for the next 10 min (Meera et al., 2008). Reduction in the number of writhes compared to the control groups was considered as evidence of analgesic effect. The percent inhibition (% analgesic activity) was calculated as percent inhibition using following equation:

$$\% \text{ inhibition} = \{(A-B)/A\} \times 100$$

3.5.2 Formalin induced persistent pain (Biphasic pain)

Formalin induced pain model was performed according to the method described by Sharma *et al.*, (2010). Swiss Albino mice were acclimated after arrival for 1-2 weeks in a temperature controlled room with a 12 hour light/dark cycle and allowed free access to standard laboratory chow and water. On day of experiment non fasted animals were weighed and dosed as per randomization; test compounds were dosed orally 1 hour before formalin challenge. Formalin was injected into the dorsal lateral surface of the left hind paw and the time spent for licking and biting in seconds by each animal at 5 minute interval is recorded for 40 min immediately after formalin injection in the following order of the time intervals: 0-5, 5-10, 10-15, 15-20, 20-25, 25-30, 30-35, 35-40. The first phase score is the sum of time spent for licking and biting in seconds from 0-5 min. the second phase score is the sum of time spent for licking and biting in seconds from 16-30 min., data was presented as mean \pm SEM % reversal was calculated by following formula,

$$\% \text{ reversal} = 100 - \left(\frac{\text{average response of test drug}}{\text{average response of vehicle}} \times 100 \right)$$

3.6 Tests for sedative and anxiolytic activity

Drugs and chemicals

Table 3.2: List of drugs and chemicals used in sedative and anxiolytic activity tests and their sources:

Drugs and Reagents	Purpose	Source
Diazepam	Standard sedative-anxiolytic drug	Square Pharmaceuticals Ltd., Bangladesh
Tween 80	Suspending agent	Merck, Germany

3.6.1 Hole cross test

The method was carried out as described by Takagi et al. (1971). A steel partition was fixed in the middle of a cage having a size of 30 × 20 × 14 cm. A hole of 3 cm diameter was made at a height of 7.5 cm in the centre of the cage. The animals were divided into control, positive control, and test groups containing five mice each. The test groups received different fractions of the leaves of *Kalanchoe pinnata* at the doses of 400 mg/kg body weight orally whereas the vehicle control and positive control groups received vehicle (1% Tween 80 in water) and the standard drug diazepam (1 mg/kg b.w.) respectively. The number of passage of a mouse through the hole from one chamber to other was counted for a period of 3 min at 0, 30, 60, 90 and 120 min after oral administration of the test drugs and the standard.

3.6.2 Open field test

In open field test, the animals were divided into control, positive control, and test groups containing five mice each. The test groups received different fractions of the aerial parts of *Kalanchoe pinnata* at the doses of 200 mg/kg and 400 mg/kg body weight orally whereas the control group received vehicle (1% Tween 80 in water). Like hole cross test, animals in positive control group received diazepam (1 mg/kg b.w.). The floor of an open field of half square meter was divided into a series of squares each alternatively colored black and white. The apparatus had a wall of 40 cm height. The number of squares visited by the animals was counted for 3 min at 0, 30, 60, 90, and 120 min after oral administration of the test drugs and the standard (Gupta et al., 1971).

3.6.3 Elevated plus-maze (EPM) test

The EPM apparatus consists of two open arms (5×10 cm) and two closed arms (5×10×15 cm) radiating from a platform (5×5 cm) to form a plus-sign figure. The apparatus was situated 40 cm above the floor (Lister, 1987). The open arms edges were 0.5 cm in height to keep the mice from falling and the closed-arms edges were 15 cm in height. Sixty minutes after administration of the test drugs, each animal was placed at the center of the maze facing one of the enclosed arms. During the 5-min test period, the number of open and enclosed arms entries, plus the time spent in open and enclosed arms, was recorded (Pellow and File, 1986). Entry into an arm was defined as the point when the animal places all four paws onto the arm. The procedure was conducted in a sound attenuated room; observations made from an adjacent corner (Braida et al., 2008; Braida et al., 2009).

3.7 Statistical analysis

Statistical analysis for animal experiments was carried out using oneway analysis of variance (ANOVA) followed by Dunnet's multiple comparison tests using SPSS 11.5 for windows. The results obtained were compared with the vehicle control group. *p* values < 0.05, 0.01 and 0.001 were considered to be statistically significant (Hasan et al., 2009; Ahmed et al., 2004).

CHAPTER FOUR

4 RESULT AND DISCUSSION

4.1 Results

4.1.1 Acetic acid-induced writhing test

Table 4.1 shows the effect of the aqueous extract of *K. pinnata* and Diclofenac Sodium on the number of writhing and percent inhibition in comparison with the control.

Table 4.1: Acetic acid-induced writhing test

Group	No. of writhing	% inhibition
Positive	0.1 ± 0.112***	99.70674
Negative	34.10 ± 7.478	0
<i>K. pinnata</i> Aq	1.1 ± 0.447***	96.77419

***the mean difference is significant at the 0.001 level.

Figure 4.1 shows the effects of the extract on acetic acid induced writhing in mice. Oral administration of the extract significantly ($p < 0.001$) inhibited writhing response induced by acetic acid which was comparable to the reference drug.

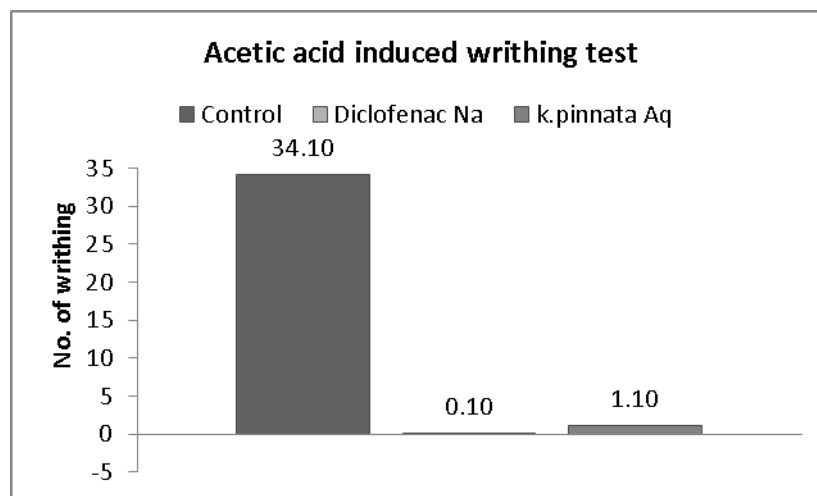


Figure 4.1: Acetic acid induced no. of writhing in mice.

4.1.2 Formalin induced persistent pain (Biphasic pain)

Table 4.2 shows the effect of the aqueous extract of *K. pinnata* and Diclofenac Sodium on formalin induced pain response and percent inhibition in comparison with the control.

Table 4.2: Formalin induced analgesic test

Group	Early phase	% inhibition	Late phase	% inhibition
Positive	42.20 ± 2.219***	61.78	25.4 ± 1.924**	58.36
Negative	110.4 ± 6.130	0	61.00 ± 4.472	0
<i>K. pinnata</i> Aq	58.60 ± 6.048**	46.92	36.00 ± 5.788**	40.98

***the mean difference is significant at the 0.001 level.

**the mean difference is significant at the 0.01 level.

Aqueous extract of *K. pinnata* (400 mg/kg, p.o.) significantly suppressed formalin induced pain response in mice, with a potent effect on either of the phases . (Figure 4.2).

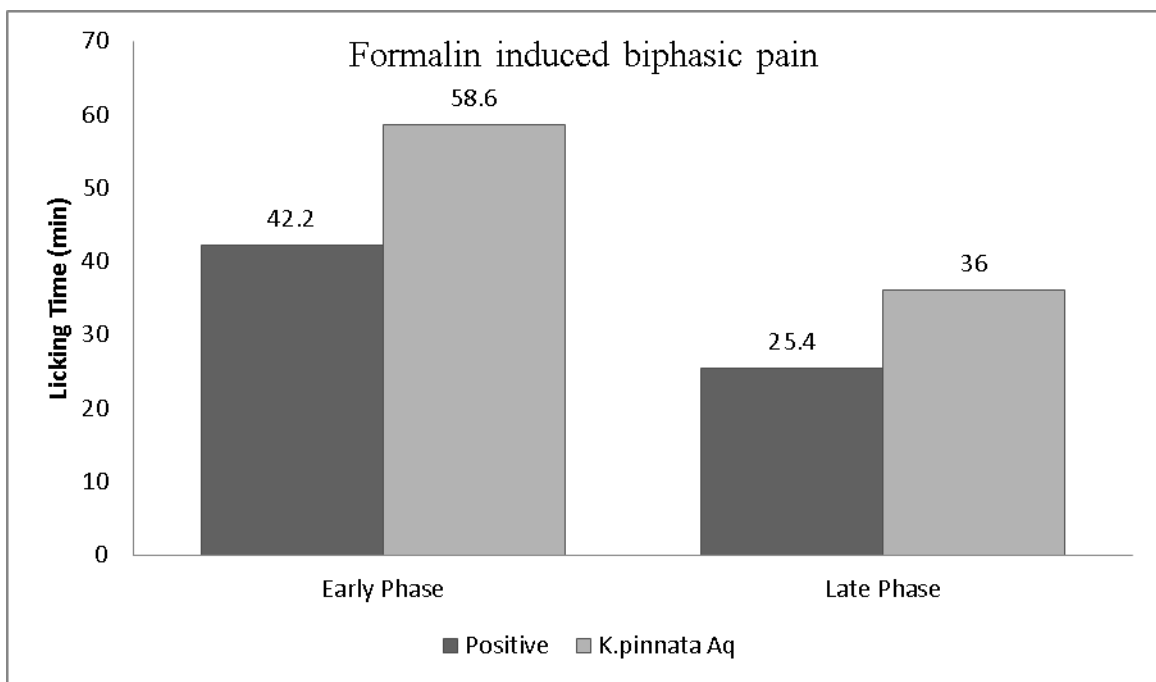


Figure 4.2: : Formalin induced licking time

4.1.3 Hole cross test

Table 4.3 shows the effect of the aqueous extract of *K. pinnata* and Diazepam on inhibition of locomotion activity (no. of hole crossed) in comparison with the control.

Table 4.3: Hole Cross test

Minute	Control	Diazepam	<i>K.pinnata</i> Aq
0	18.60 ± 0.570	12.80 ± 3.090	8.80 ± 2.559*
30	9.20 ± 2.329	6.60 ± 1.151	7.40 ± 3.347
60	5.40 ± 0.671	4.40 ± 1.483	4.60 ± 2.49
90	5.40 ± 0.570	4.60 ± 0.837	4.60 ± 1.987
120	4.60 ± 0.570	3.80 ± 1.917	3.80 ± 1.342

*the mean difference is significant at the 0.05 level.

At the dose of 400 mg/kg body weight produced a significant ($p < 0.01$) decrease of locomotion from its initial value during the period of the experiment (Figure 4.3). Maximum suppression of locomotor activity was displayed at the last three periods of time, which was comparable to the reference drug diazepam.

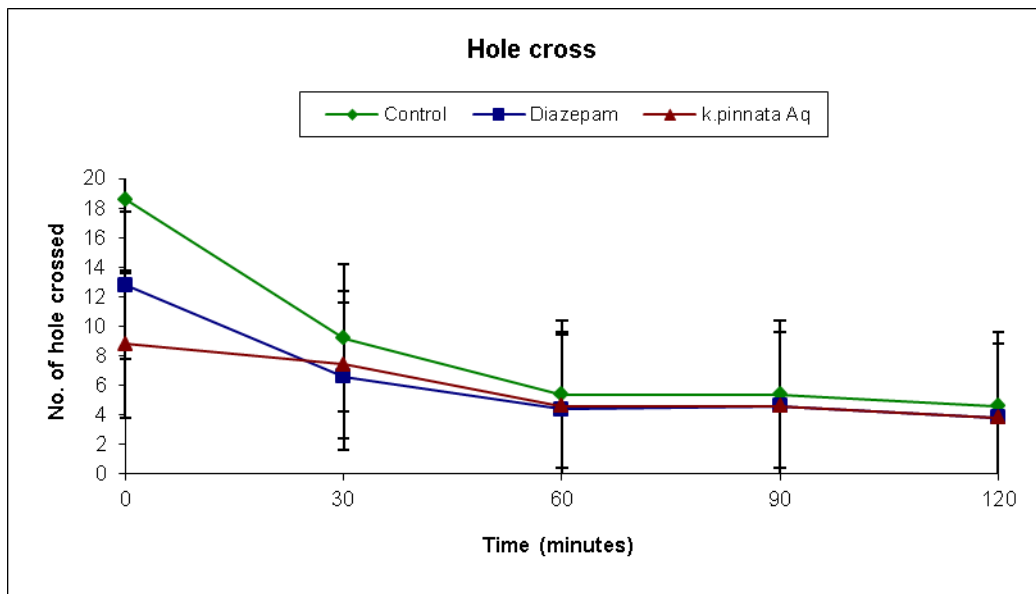


Figure 4.3: No. of hole crossed by the mice

4.1.4 Open field test

Table 4.4 shows the effect of the aqueous extract of *K. pinnata* and Diazepam on inhibition of locomotion activity (number of squares traveled in open field) in comparison with the control.

Table 4.4: : Open field test

Minute	Control	Diazepam	<i>K.pinnata</i> Aq
0	121.20 ± 7.652	110.40 ± 5.943	97.20 ± 5.825*
30	60.20 ± 3.435	56.20 ± 7.012	43.80 ± 2.434*
60	40.60 ± 4.207	36.60 ± 3.616	32.20 ± 2.162
90	29.40 ± 2.864	25.80 ± 2.302	20.00 ± 0.791*
120	28.80 ± 4.866	24.80 ± 8.863	26.40 ± 3.054

*the mean difference is significant at the 0.05 level.

The number of squares traveled by the mice was suppressed significantly in the second observation period at dose levels (400 mg/kg body weight) of the aqueous extract from the leaves of *K.pinnata*. The results were statistically significant (Figure 4.4).

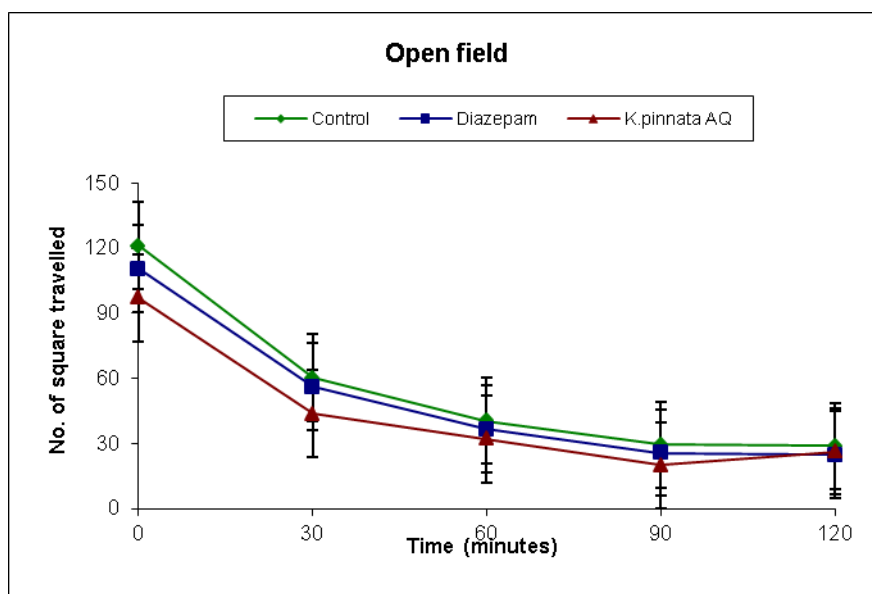


Figure 4.4: No. of square travelled by the mice in open field

4.1.5 Elevated plus-maze (EPM) test

Table 4.5 shows the effect of the aqueous extract of *K. pinnata* and Diazepam on the increase of the percent no. of entry and percent time spent as well (EPM) in comparison with the control.

Table 4.5: EPM test of the mice

Group	% no. of entry into the open arm	% time spent in the open arms
Positive	76.28 ± 1.847**	79.39 ± 5.749*
Negative	55.88 ± 2.133	51.93 ± 8.243
<i>K. pinnata</i> Aq	80.62 ± 9.511**	85.94 ± 4.60

*the mean difference is significant at the 0.01 level.

**the mean difference is significant at the 0.001 level.

The aqueous extract of *K. pinnata* at the dose of 400 mg/kg body weight, significantly increased the percentage of entries (Figure 4.5.1) of mice into the open arms, and the percentage of time spent (Figure 4.5.2) in the open arms of the EPM.

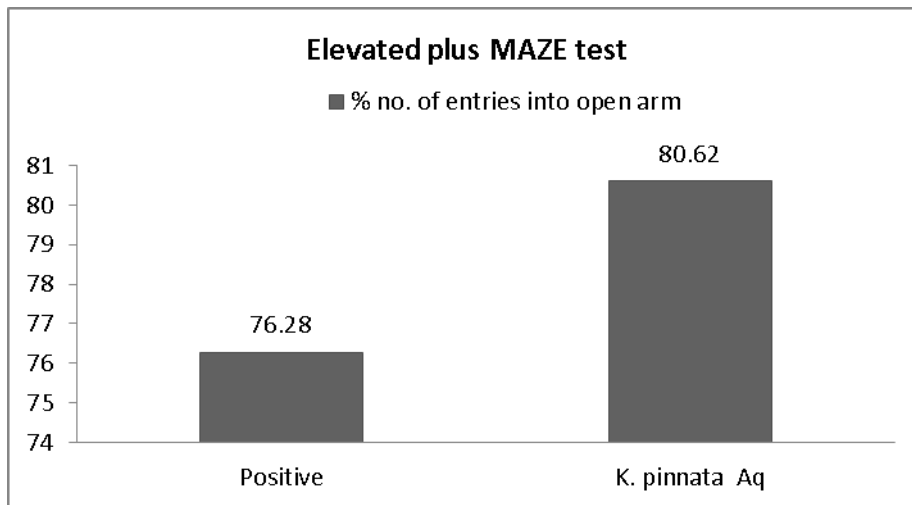


Figure 4.5: EPM % no. of entries into open arm

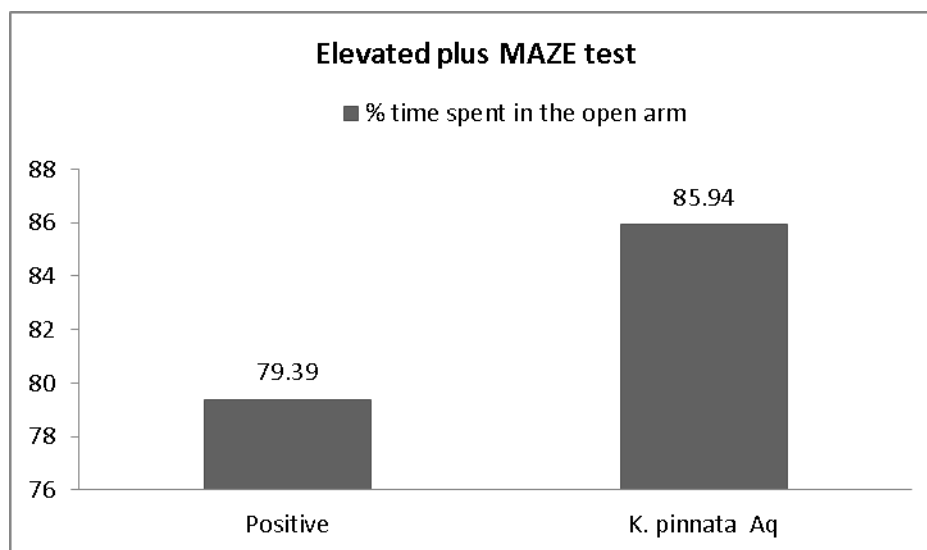


Figure 4.6: EPM % time spent in open arm

4.2 Discussion

The acetic acid induced writhing response is a sensitive procedure to evaluate peripherally acting analgesic. The response is thought to be mediated by the prostaglandin pathways, peritoneal mast cells and acid sensing ion channels (Hossain *et al.*, 2006; Ronaldo *et al.*, 2000; Voilley, 2004). Intraperitoneal administration of acetic acid releases prostaglandins and sympathomimetic mediators like PGE₂ and PGF₂ α and their levels increase in the peritoneal fluid of the acetic acid induced mice (Deraedt R *et al.*, 1980). The abdominal constrictions produced after administration of acetic acid is related to sensitization of nociceptive receptors to prostaglandins. It is therefore possible that the extract exerts its analgesic effect by inhibiting the synthesis or action of prostaglandins which may be due to phytochemicals present in the extract. Moreover, since the extract inhibited both peripheral and central mechanisms of pain, it is possible that the extract acted on opioid receptor (Elisabetsky E *et al.*, 1995; Pal S *et al.*, 1999). Therefore, the significant pain reduction of the plant extract may be due to the presence of analgesic principles acting with the prostaglandin pathways or interfering with other mediators responsible for peripheral pain.

The formalin test is another reliable model of analgesic which is better correlated with clinical pain (Tjolsen A *et al.*, 1992; Ghannadi A *et al.*, 2005). This method elucidates central and peripheral activities. The response of early phase is supposed to represent a direct chemical

stimulation of pain, due to the irritant effect of formalin on sensory C fibers (Hunnskaar *et al.*, 1985; Tjolsen *et al.*, 1992). The late phase response is most likely secondary to the development of an inflammatory response and the release of allergic mediators (Hunnskaar & Hole 1987). Inhibition of licking response of the test drugs in the early phase and late phase signifying the analgesic effect of the extract in the formalin test.

Results of the study demonstrated that aqueous extract of *K. pinnata* leaf exerts potential analgesic effect in experimental animal models which support the claims by traditional medicine practitioners. On the basis of the results, it can be used as a good source of analgesic drugs. However, pharmacodynamic studies should be undertaken to establish the mechanism of action of the plant extracts contributing in nociception. Phytochemical investigation is also proposed in order to isolate the active fraction and eventually the pure compound.

The study on locomotor activity, as measured by hole cross and open field tests, showed that aqueous extract from the leaves of *K. pinnata* decreased the frequency and the amplitude of movements. Since locomotor activity is a measure of the level of excitability of the CNS (Mansur RM, Martz W, Carlini EA, 1980), this decrease in spontaneous motor activity could be attributed to the sedative effect of the plant extracts (Rakotonirina VS *et al.*, 2001). The 400mg/kg doses significantly decreased locomotion in mice. The locomotor activity lowering effect was evident at the 2nd observation (30 min) and continued up to the 5th observation period (120 min). The results were statistically significant (Figures 2 and 3).

However, the anxiolytic effect was evidenced by the EPM test that has been recognized as a valuable model able to predict anxiolytic effects of drugs in rodents (Perez RM *et al.*, 1998). The anxiolytic effect is observed when the experimental drug increases open arms entries without altering the total number of arm entries (Barrett JE., 1991). The aqueous extract at 400 mg/kg body weight, in mice, display a significant increase in the percentage of entries into open arms, showed a significant increase in the percentage of time spent in the open arms of the maze. This was slightly larger than the effects observed following treatment with the reference anxiolytic drug diazepam. These results could indicate an anxiolytic-like activity of the aqueous extract from the leaves of *K. pinnata*.

GABAA-benzodiazepine receptors are the most abundant inhibitory receptor (Squires RF, Braestrup C.,1977) system in the CNS and binding of a benzodiazepine agonist to its recognition site results in increased chloride ion flux (Trofimiuk, 2005) which in turn hyperpolarizes the postsynaptic membrane at a level below that at which spike generation is possible and for this reason some GABAA agonists are frequently used for their hypnotic effects. The compounds identified from the leaves of *K. pinnata* contain P-coumaric, ferulic, syringic, caffeic and p-hydroxybenzoic acids, quercetin and kaempferol, act as GABAA agonists and this agonistic property could be attributed to the CNS depressant effect of *K. pinnata* leaves although there is no consensus about which substances are exactly responsible for these effects. However, further studies are necessary to evaluate the contribution of other substances that are isolated for the activity observed, because it still remains to be determined which components exactly were responsible for these effects.

5 CONCLUSION

The aqueous extract of *K. pinnata* was evaluated in the formalin and acetic acid-induced writhing test for its analgesic activity and result showed remarkable analgesic potential. Various phytochemical constituents like alkaloids, triterpenes, glycosides, flavonoids, steroids and lipids present in the plant, as evident from phytochemical analyses, may be responsible for the observed bioactivities. The results from the experiments confirmed that the aqueous extract from *K. pinnata* leaves possesses a strong sedative and anxiolytic potential. Therefore, I advance the suggestion that this extract may fulfill the therapeutic need for the treatment of anxiety, related neuropsychiatric disorders and pain sensation. However, further studies would be necessary to evaluate the contribution of other substances for the activity showed as it still remains to be determined which components were exactly responsible for these effects.

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