Analgesic and neuropharmacological activities of the ethanolic extract of leaves of *Kalanchoe pinnata* (Lam.)Pers. in Swiss Albino mice

Submitted by

Sinthyia Ahmed

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Department of Pharmacy

East West University

A research paper submitted in partial fulfillment of the requirements for the award of the degree

Bachelor of Pharmacy

Under the Guidance of

Md. Razibul Habib

Lecturer

Department of Pharmacy

East West University

July, 2012

Declaration by the Research candidate

I, Sinthyia Ahmed, hereby declare that the dissertation entitled "**Analgesic and neuropharmacological activities of the ethanolic extract of leaves of *Kalanchoe pinnata* (Lam.) Pers.in Swiss Albino mice",**submitted by me to the Department ofPharmacy,East West University, in partial fulfillment of the requirements for the award of the degree of Bachelor of Pharmacy (B.PHARM) is a complete record of original research work carried out by me during the period 2011-2012 under the supervision and guidance of **Md.Razibul Habib,** Lecturer, Department of Pharmacy, East West University and it has not formed the basis for the award of any other Degree/Diploma/Fellowship or other similar title to any candidate of any University.

Place: Dhaka

Date:

Signature of the candidate

(Sinthyia Ahmed)

ID:2008-3-70-058

Thesis Certificate

This is to certify that the thesis entitled "Analgesic and neuropharmacological activities of the ethanolic extract of leaves of *Kalanchoe pinnata (*Lam.) Pers. in Swiss Albino mice" submitted to the Department of Pharmacy, East West University, in partial fulfillment of the requirements for the award of the degree of Bachelor of Pharmacy (B.PHARM) is a complete record of original research work carried out by Sinthyia Ahmed (ID. 2008-3-70-058) during the period 2011-2012 of her research in the Department of Pharmacy at East West University, under my supervision and guidance and the thesis has not formed the basis for the award of any other Degree/Diploma/Fellowship or other similar title to any candidate of any University.

Dhaka-1212

Date:

(Md.Razibul Habib)

Principle Supervisor

Lecturer

Department of Pharmacy

East West University

I, as the responsible academic Chairperson, to endorse the above statements:

(Dr. Sufia Islam)

Chairperson

Department of Pharmacy

East West University

43, Mohakhali C/A, Dhaka-1212

Date:

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July,2012 **Sinthyia Ahmed**

Author

List of Abbreviation

K.pinnataKalanchoe pinnata (Lam.) Pers.	
Gm	Gram
b.w.	Bodyweight
cm	Centimetre
COX	Cycloxygenase
EPM	Elevated Plus Maze
Mg	Milligram
i.p.	Intraperitionial
ICDDR,B	International Center for Diarrhoeal Disease and Research,Bangladesh
NSAIDs	Nonsteroidal anti-inflammatory drugs
GABA	Gamma amino butyric acid
p.o.	Per oral
SEM	Standard error mean
Et-OH	Ethanol
PGE2	Prostaglandin 2
CNS	Central nervous system
GABA	Gamma aminobutyric acid

Abstract

Kalanchoe pinnata(Lam.) Pers. (Family:Crassulacea), locally known as 'Pathorkuchi', is a medicinal herb, which has a good reputation in Bangladesh, India and many other countries of the world as a folk medicine for the treatment of a variety of diseases of different etiology such as infections, rheumatism and inflammation, traditional treatment for hypertensionand for the treatment of kidney stones. But till to date, sporadic attempts have been made for the scientific and methodical validation of these traditional claims. Therefore, the present study was designed to investigate analgesic, sedative and anxiolytic properties of ethanolic fractions of the leaf of *K.pinnata*.

Analgesic potential of ethanolic fractions of leaf of *K.pinnata*(Lam.) Pers.was evaluated for peripheral pharmacological actions using acetic acid-induced writhing and Formalin induced persistent pain test. The fraction, at the doses of 400 mg/kg body weight, displayed significant analgesic action in a dose dependent manner in the tested models.

In addition, sedative and anxiolytic properties of the ethanolic fractions of *K.pinnata*(Lam.) Pers.was investigated using rodent behavioural models, such as hole cross,open field and elevated plus-maze (EPM) test for anxiolytic potential, respectively. The fraction at dose 400 mg/kg, displayed a dose dependent suppression of motor activity, exploratory behaviour (in hole cross and open field tests). In EPM test, the extract increased exploration to and time spent by the treated mice in EPM open arms in a way similar to that of the reference anxiolytic drug Diazepam.

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Dedicated to my Family

CHAPTER 01: INTRODUCTION

1.1 Phytomedicine and its goal

Plants have been the basis of many traditional medicine systems throughout the world.Phytomedicine, popularly known as herbal medicine.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. The goals of using plants as sources of therapeutic agents are (Daniel *et al.,* 2001)

1)To isolate bioactive compounds for direct use as drugs, e.g. digoxin, digitoxin, morphine, reserpine, taxol, vinblastine, vincristine.

2)To produce bioactive compounds of novel or known structures as lead compounds for semisynthesis to produce patentable entities of higher activity and/or lower toxicity, e.g., metformin, nabilone, oxycodon (and other narcotic analgesics), taxotere, teniposide, verapamil, and miodarone, which are based, respectively, on galegine, Δ9- tetrahydrocannabinol,morphine, taxol, podophyllotoxin, khellin, and khellin;

3)To use agents as pharmacologic tools, e.g., lysergic acid diethylamide, mescaline, yohimbine; and

4)To use the whole plant or part of it as an herbal remedy, e.g., cranberry, echinacea, feverfew, garlic, etc.

The number of higher plant species (angiosperms and gymnosperms) on this planet is estimated at 250,000 (Ayensu & DeFilipps, 1978), with a lower level at 215,000 (Cronquist, 1981; Cronquist, 1988) and an upper level as high as 500,000 (Tippo & Stern, 1977; Schultes, 1972). Of these, only about 6% have been screened for biologic activity, and a reported 15% have been evaluated phytochemically (Verpoorte, 2000).

Medicinal plants have played an essential role in the development of human culture, for example religions and different ceremonies.(E.g. Dutura has long been associated with the worship of Shiva, the Indian god). Plants are directly used as medicines by a majority of cultures around the

world, for example Chinese medicine and Indian medicine. Many food crops have medicinal effects, for example garlic. Medicinal plants are resources of new drugs. It is estimated there are more than 250, 000 flower plant species. Studying medicinal plants helps to understand plant toxicity and protect human and animals from natural poisons. Cultivation and preservation of medicinal plants protect biological diversity, for example metabolic engineering of plants. Plant resources (E.g. Angiosperm, Gymnosperm, Seedless vascular plants, Bryophytes) for new medicine. The medicinal plants find application in pharmaceutical, cosmetic, agricultural and food industry. With onset of scientific research in herbals, it is becoming clearer that the medicinal herbs have a potential in today's synthetic era, as numbers of medicines are becoming resistant. According to one estimate only 20% of the plant flora has been studied and 60%of synthetic medicines owe their origin to plants.Ancient knowledge coupled with scientific principles can come to the forefront and provide us with powerful remedies to eradicate the diseases.

It was estimated that in 1991 in the United States, for every 10,000 pure compounds (most likely those based on synthesis) that are biologically evaluated (primarily *in vitro*), 20 would be tested in animal models, and 10 of these would be clinically evaluated, and only one would reach U.S. Food and Drug Administration approval for marketing. The time required for this process was estimated as 10 years at a cost of $231 million (U.S.) (Vagelos, 1991). Most large pharmaceutical manufacturers and some small biotechnology firms have the ability to screen 1,000 or more substances per week using high throughput *in vitro* assays. In addition to synthetic compounds from their own programs, some of these companies screen plant, microbial, and marine organisms.

Medicinal plants are resources of new drug. They have played an essential role in the development of human culture, for example religions and different ceremonies. (E.g. *Dutura* has long been associated with the worship of Shiva, the Indian God).Plants are directly used as medicines by a majority of cultures around the world, for example Chinese medicine and Indian medicine. Many food crops have medicinal effects, for example garlic.It is estimated there are more than 250, 000 flower plant species. Studying medicinal plants helps to understand plant toxicity and protect human and animals from natural poisons. Cultivation and preservation of medicinal plants protect biological diversity, for example metabolic engineering of plants. Plant

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1.2 History of herbal medicine

As this system of herbal medicine has been in use almost unchanged generation after generation throughout the ages for the treatment of various physical and psychological diseases, it is called traditional. Most of the times, the type, preparation, and uses of traditional medicines are largely influenced by folklore customs and the cultural habits, social practices, religious beliefs and, in many cases, superstitions of the people who prescribe or use them.

The earliest mention of traditional medicine is found in *Rigveda,* the oldest repository of knowledge in this subcontinent. Later *Ayurveda,* developed from the *Vedic* concept of life, became the important source of all systems of medical sciences. In course of time it became a part of culture and heritage of the people of the Indian subcontinent.

Traditional medicine involves the use of both material and non-material components. The material components invariably comprise parts or organs of plants and their products. They also consist of animal organs, minerals and other natural substances. The non-material components, which constitute important items of religious and spiritual medicines, include torture, charms, magic, incantations, religious verses, amulets and rituals like sacrifices, appeasement of evil spirits, etc.

Treatments in traditional medicine are carried out by internal and external application of medicaments, physical manipulation of various parts of the body, performing rituals, psychological treatment, and also by minor surgery. *Ayurvedic*medicinal preparations consist

mainly of plant materials in the form of powders, semi-solid preparations, decoctions, elixirs and distillates. Many of them also contain inorganic chemical substances, minerals and animal products. Alcoholic extracts and alcoholic solutions of the ingredients, tinctures and elixirs are also frequently used in *Ayurvedi*c medicine.

Whole plants or their powders or pastes or products and their extracts, infusions, decoctions and distillates constitute the major constituents of *Unani* medicine. Minerals, inorganic chemicals and animal products are also frequently used in preparing these medicines.

For hundreds of years, the medical knowledge in Bangladesh is termed as Ayurveda. Ayurveda remains an important system of medicine and drug therapy in Bangladesh. Plant alkaloids are the primary active ingredients of Ayurvedic drugs. Today the pharmacologically active ingredients of many Ayurvedic medicines are being identified and their usefulness in drug therapy being determined.

Herbal medicines have been developed to a remarkable standard by applying modern scientific technology in many countries, such as China, India, Bangladesh, Sri Lanka, Thailand and United Kingdom. Thus it is apparent that whatever progress, science might have made in the field of medicine over the years, plants still remain in the primary sources of supply of many important drugs used in modern medicine. Indeed the potential of obtaining new drugs from plant sources is so great that thousands of substances of plant origin are now being studied for activity against such formidable foes as heart disease, cancer and AIDS. This type of studies are sure to bring fruitful results, because of the fact that plant kingdom represents a virtually untapped reservoirs of new chemical compounds, some providing novel bases on which the synthetic chemist may build even more interesting structures. In this way modern medicines will continue to be enriched by the introduction of newer and potent drugs from plant sources. At present, thousands of plant metabolites are being successfully used in the treatment of variety of disease.

1.3 Trials and threats in drug discovery

Natural products scientists and pharmaceutical industries 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

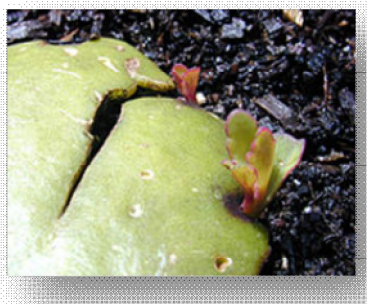
average period of 10 years and cost more than 800 million dollars (Dickson and Gagnon, 2004).Much of this time and money is spent on the numerous leads that are discarded during the drug discovery process. It is estimated that only one in 5000 lead compounds will successfully advance through clinical 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 much time and efforts.

CHAPTER 02:PLANT DETAILS

2.1 The Plant Family:Crassulaceae

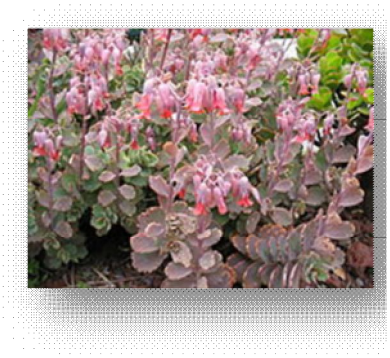
Crassulaceae are a family of dicotyledones also known as orpine family.This family store water in their succulent leaves. So they grow in dry and/cold areas where the water may be scarce. They are found worldwide but mostly occur in the northern hemisphere and southern Africa. The family includes about 1,400 species in 33 genera.Familiar species include the Jade plant, *Crassula ovata* and Florists Kalanchoe, *Kalanchoe blossfeldia*.

Table 2.1:Common species of Kalanchoe

Species name	Common name	Distribution	Picture
Kalanchoe pinnata	Cathedral bells	Asia, Australia, New Zealand, West Indies, Macaronesia, Mascarenes, Galapagos, Melanesia, Polynesia and Hawaii	
Kalanchoe beharensis	Felt plant	Mexico, southern Africa, Macaronesia, and the Himalayas	

Kalanchoe blossfeldiana	Flaming katy	Native to Madagascar and other countries	
Kalanchoe orgyalis	Copper spoon	Native to Madagascar	
Kalanchoe diagremontiana	Devil's backbone	Native to Madagascar	
Kalanchoe delagoensis	Mother of Millions	Native to Madagascar	

Kalanchoe fedtschenkoi	----------	Southern parts of the United States of America.



2.2 Introduction to *Kalanchoe Pinnata*(Lam.) Pers.

Taxonomic hierarchy of *Kalanchoe pinnata*(Lam.) Pers.

Kingdom: Plantae

Subkingdom: Tracheobionta

Division: Magnoliophyta

Superdivision: Spermatophyta

Class: Magnoliopsida

Subclass: Rosidae

Order: Rosales

Family: Crassulaceae

Genus: Kalanchoe Adans

Species: Kalanchoe pinnata (Lam.) Pers.

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

(National Plant Database. 2005.)

2.3 Plant Description

2.3.1 General Information

Kalancha pinnata is a perennial herb, up to 6 feet (1.8 m) tall, ornamental garden plant and medicinal herb. The color of the flower is red and the pendent flowers are on short, lateral branches on tall, upright, chandelier-like flower stalks. The individual flowers are tubular,1 inch (2.5 cm) long, enclosed in papery, inflated, green to reddish pink sepals, and have 4 red, narrowly triangular lobes. The flowers dry on the plant and gradually turn a light papery brown color. The leaves have scalloped, dark maroon margins and are green, succulent, opposite and mostly pinnately compound with 3 to 5 elliptic leaflets. New baby plants can form along the edges of the leaves.The plant grows wild in dry to moist areas at lower elevations.

2.3.2 Common Name

Pathorkuchi,Pather chat,Pathor-futti,Cathedral bells, Air Plant, Life Plant, Miracle leaf, Goethe plant and the Katakataka

2.3.3 Different parts of *K.pinnata*

Leaves: Leaves 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.Leaves are the most important part of the plant.

Fig 2.1: Leaves of *K.pinnata*

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.

Fig 2.2: Flower of *K.pinnata*

Fruit:A follicle.Fruits are seen rarely.

Fig 2.3: Fruit of *K.pinnata*

2.3.4 Habitat

The widespread naturalization of this plant can be traced to its popularity as a garden plant. It is a popular houseplant and has become naturalized in temperate regions of Asia, the Pacific and Caribbean.Itis commonly found in tropical and subtropical lowlands, moistconditions, sandy

orrocky soils; as a weed of cultivated lands, field borders, wetpasturelands, banks of irrigation ditches, gardens, roadsides and waste places.

2.3.5 Distribution

The plant has become naturalized in temperate regions of Asia, Australia, New Zealand, West Indies, Macaronesia, Mascarenes, Galapagos, Melanesia, Polynesia, and Hawaii. In many of these, such as Hawaii, it is regarded as an invasive species. It is also widely distributed in the Philippines.

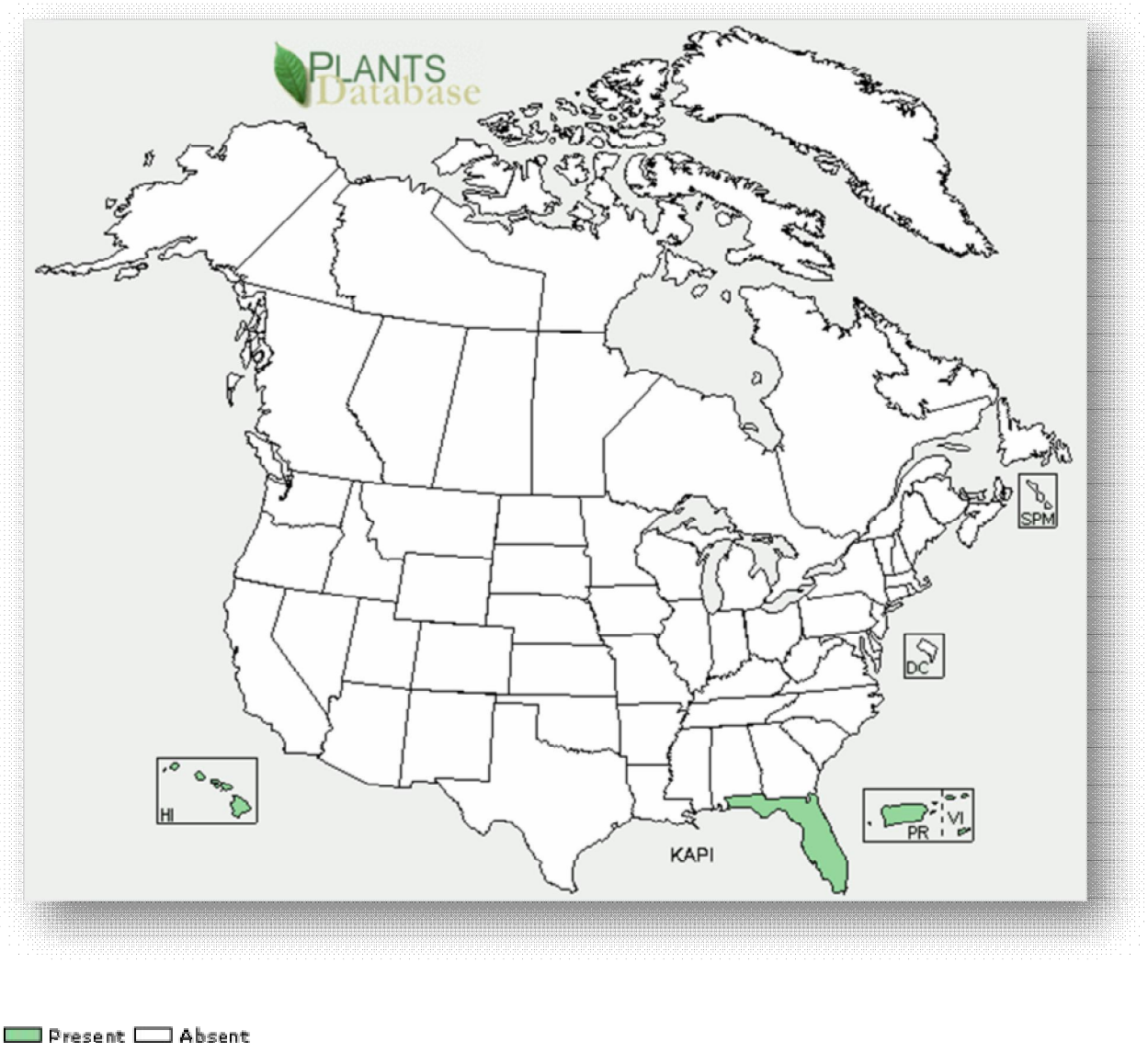


Fig 2.4: Global Distribution of *K.pinnata*(Lam.)Pers.(www.image.google.com)

2.4 Analgesic principles in medicinal plants

Investigation of herbal plants has become a potential source for the discovery of lead compounds of high therapeutic value in terms of analgesic 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.

Analgesics relieve symptoms of pain but hardly affect its underlying cause.Currently available analgesic 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.

2.5 Sedative and anxiolytic principles in medicinal plants

Many species of plants possessing activity on the central nervous system (CNS). In fact, they cover the whole spectrum of central activity such as psychoanaleptic, psycholeptic and psychodysleptic effects, and several of these plants are currently used in therapeutics to treat human ailments.

Many species of hallucinogenic (psychodysleptic) plants are used by humans throughout the world to achieve states of mind distortions; among those, a few have been used for therapeutic purposes, such as *Cannabis sativa* L., *Tabernanthe iboga* Baill. and the mixture of *Psychotria viridis* Ruiz and Pav. and*Banisteriopsis caapi* (Spruce ex Griseb.) C.V. Morton. Plants showing central psycholeptic activities, such as analgesic or anxiolytic actions (*Passiflora incarnata* L., *Valeriana* spp. and *Piper methysticum* G. Forst.), were also analysed.Finally, the use of crude or semipurified extracts of such plants instead of the active substances seemingly responsible for their therapeutic effect.

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.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.

2.6 Rationale of the work

Natural products,particularly medicinal plants, remain an important source of new drugs, new drug leads and new chemical entities.*Kalanchoe pinnata*, locally known as ‘Pathorkuchi’ in Bangladesh, is a medicinal herb, which has long been used in the Indian subcontinent as a folk medicine for the treatment of a variety of diseases of different etiology (Paresh and Chanda, 2008; Yusuf et al., 1994; Kirtikar and Basu, 1980;Okello and Ssegawa, 2007; Tabuti et al., 2003; Qaiser and Jafri, 1975; Mbazima et al.,2008; Mokgotho et al., 2009abc).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 pharmacologicalstudy on the leaves of *K.pinnata*.The specific objectives of the present work were-

1)To carry out analgesicinvestigations on this plant using acetic acid-induced writhing,formalin induced writhing test.

2)To evaluate sedativeproperty using hole cross, open field and anxiolyticpotential using Elevated plus-maze (EPM) test.

2.7 Limatations of the work

In vivoanalgesic tests might be enough to investigate for analgesic potential. However, some invitro tests (e.g. using COX enzyme and measuring biochemical mediators of pain) could support the *in vivo* data which in turn could make a good *in vivo-in vitro* correlation for strong claims in favour of analgesic activity. Like analgesic tests, this is also applicable to anti-inflammatory tests. Some other *in vivo* anti-inflammatory tests using serotonin, formalin, capsaicin, PGE2, xylene, cotton pellet etc. induced acute and chronic inflammatory models could have been strong supports to the existing findings. Moreover, several other *in vitro* models using isolated cells (e.g. RAW 264.7 macrophage cells) and measuring biochemical markers of inflammation might have been useful. 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
- Animal maintenance is difficult
- Lack of sound proof room
- Dose dumping may occur
- Data interpretation may be wrong
- Lack of fresh mice for each different test

CHAPTER 03: LITERATURE REVIEW

3.1 Phytochemical investigation of *Kalanchoe pinnata*(Lam.) Pers.

Kalanchoe pinnata(Lam.)Pers. contains a wide range of active compounds, including alkaloids, triterpenes, glycosides, flavonoids, steroids, bufadienolides, lipids and organic acids.The pharmacological studies are reviewed and discussed, focussing on activities as immunomodulator, CNS depressant, analgesic, antimicrobial, antiinflammatory, antiallergic, antianaphylactic, antileishmanial, antitumorous, antiulcerous, antibacterial, antifungal, antihistamine, antiviral, febrifuge, gastroprotective, immunosuppressive, insecticidal, muscle relaxant, sedative, anticancer. Now it becomes endangered plant which needs to be conserved as well as explored for its significant green chemistry.(Seema V. Pattewar ,2012).

3.2 Antihypertensive activity of *K.pinnata*(Lam.) Pers.

The effects of aqueous leaf extract of *K. pinnata*(Lam.)Pers.on the blood pressure of anaesthetized cats as well as on the liver and kidney status of the rabbit were investigated in this study. The results revealed that the extract produced a small fall in the blood pressure of the anaesthetized cat and also reduced the effect of adrenaline-induced elevation of blood pressure. It was concluded that the pharmacological basis for the use of *K. pinnata*(Lam.)Pers. among the Igbos of Nigeria to lower blood pressure was established by this study. However, the facts that the reduction in blood pressure produced is slight and the *K. pinnata*(Lam.)Pers.leaf extract is potentially organotoxic which negates its use as a blood pressure lowering agent (Ghasi et al., 2011).

3.3 Immunosuppressive effect of *K.pinnata*(Lam.) Pers.

The aqueous extract of *K.pinnata*(Lam.)Pers. leaves was found to cause significant inhibition of cell- mediated and humoral immune responses in mice. The spleen cells of animals pre-treated with *K.pinnata*(Lam.)Pers. showed a decreased ability to proliferate in response to both mitogen and to antigen in vitro. Treatment with *K.pinnata*(Lam.)Pers. also impaired the ability of mice to mount a delayed-type hypersensitivity reaction (DTH) to ovalbumin. The intravenous and topical routes of administration were the most effective by almost completely abolishing the DTH reaction. The intraperitoneal and oral routes reduced the reaction by 73 and 47% of controls, respectively. The specific antibody responses to ovalbumin were also significantly reduced by

treatment. Together, these observations indicate that the aqueous extract of K. pinnata possesses an immunosuppressive activity (Bergmann et al., 2006).

3.4 Wound healing activity of *K.pinnata*(Lam.) Pers.

The extract of *K.pinnata*(Lam.)Pers. was evaluated for its wound healing activity by using excision wound model in rats. On the 11th day wounding, there was a significant increase in the wound-healing activity in the animals treated with *K. pinnata*(Lam.)Pers. ethanolic extract compared to animals which received the control treatment and standard treatment. Significant progressive reduction in the wound area was observed by day 11 (86.3%) when compared to the control (68.0%) and standard (85.5%). The histological analysis showed that *K. pinnata*(Lam.)Pers. leaf extract exhibited significant wound healing potential. The wound healing exhibited by the extract may be attributed to the presence of steroid glycosides. The medicinal plant has been shown to have a significant quantity of bufadienolide, a steroidal aglycone which exists in the plant as steroidal glycoside (Nayak et al., 2010).

3.5 Hepatoprotective activity of *K.pinnata*(Lam.) Pers.

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 hepatotoxicity. The test material was found effective as hepatoprotective as evidenced by in vitro, in vivo and histopathological studies. The juice was found to be more effective than ethanolic extract (Yadav and Dixit, 2003).

3.6 Antinociceptive,anti-inflammatory and antidiabetic activity of*K.pinnata*(Lam.) Pers.

In order to scientifically appraise some of the ethnomedical uses of *K.pinnata*(Lam.)Pers.leaves, a study was undertaken to investigate the antinociceptive, antiinflammatory and antidiabetic properties of the plant's leaf aqueous extract in experimental animal models. *K. pinnata*(Lam.)Pers.leaf aqueous extract (BPE, 25 to 800 mg/kg i.p.) produced significant ($P < 0.05$ to 0.001) antinociceptive effects against thermally- and chemically-induced nociceptive pain stimuli in mice. The plant extract (BPE, 25 to 800 mg/kg p.o. or i.p.) also significantly ($P < 0.05$ to 0.001) inhibited fresh egg albumin-induced acute inflammation and caused significant

($P < 0.05$ to 0.001) hypoglycaemia in rats. The results of this experimental animal study suggest that *K.pinnata*(Lam.)Pers. leaf aqueous extract possesses antinociceptive, anti-inflammatory and hypoglycaemic properties. The different flavonoids, polyphenols, triterpenoids and other chemical constituents of the herb are speculated to account for the observed antinociceptive, anti-inflammatory and antidiabetic properties of the plant (Ojewole, 2005).

3.7 Nephroprotective and antioxidant activity of *K.pinnata*(Lam.) Pers.

Harlalka et al. (2007) evaluated the aqueous extract of *K pinnata* (Lam.)Pers.for its protective effects on Gentamycin-induced nephrotoxicity in rats. It was observed that the aqueous extract of K. pinnata leaves significantly protects rat kidneys from Gentamycin-induced histopathological changes. Gentamycin-induced glomerular congestion, peritubular and blood vessels congestion, epithelial desquamation, accumulation of inflammatory cells and necrosis of the kidney cells were found to be reduced in the group receiving the leaf extract of *K. pinnata*(Lam.)Pers. along with Gentamycin. Urine creatinine, serum creatinine, blood urea, blood urea nitrogen and the weights of the kidneys were found to be significantly increased in rats treated with only Gentamycin; whereas the treatment with the aqueous extract of *K. pinnata*(Lam.)Pers.was found to protect the rats from such effects of Gentamycin. The volume of urine was found to be significantly increased in the rats treated with *K. pinnata*(Lam.)Pers.leaf extract. In case of histopathological examination, control rats showed normal glomerular and tubular histology whereas Gentamycin was found to cause glomerular, peritubular and blood vessel congestion and result in the presence of inflammatory cells in kidney sections from the Gentamycin-treated group. Concurrent treatment with the extract was found to reduce such changes in kidney histology induced by Gentamycin. In-vitro studies revealed that the *K. pinnata*(Lam.)Pers. leaf extract possesses significant antioxidant as well as oxidative radical scavenging activities. Quercetin and kaemferol have been detected in the leaves of *K. pinnata*(Lam.)Pers. (Harlalka et al.,2007). Morales et al. (2006) suggested that quercetin has a marked protective effect on cadmium-induced nephrotoxicity that results from an increase Metallothionein, a small cysteine-rich protein and eNOS (endothelial nitric oxide synthase) expression and the inhibition of COX-2 (cyclooxygenase-2) and iNOS (inducible nitric oxide synthase) expression.

3.8 Antimicrobial activity of *K.pinnata*(Lam.) Pers.

The roots of *K. pinnata*(Lam.)Pers.were subjected to petroleum ether, chloroform, methanol and aqueous solvent respectively for extraction and in vitro evaluation of antimicrobial activity was done against Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa and Candida albicans.Methanolic extract of roots of *K.pinnata* was found to be most effective as antibacterial as compare to others while none of extract showed the activity against C. albicans (Quazi et al., 2011). Akinpelu (2000) in a study found that 60% methanolic leaf extract inhibits the growth of five out of eight bacteria used at a concentration of 25 mg/ml. Bacillus subtilis, E. coli, Proteus vulgaris, Shigella dysenteriae, S. aureus were found to be inhibited while Klebsiella pneumoniae, P. aeruginosa and C. albicans were found to resist the action of the extract. Chemical investigation of the bioactive constituents from the leaf of K. pinnata resulted in the isolation of two new novel flavonoids; 5 I Methyl 4 I , 5, 7 trihydroxyl flavone and 4 I , 3, 5, 7 tetrahydroxy 5- methyl 5 I -propenamine anthocyanidines. The antimicrobial observation of the aforementioned compounds could be responsible for the activity of *K. pinnata* and its use in herbal medicine in Nigeria (Okwu and Nnamdi, 2011).

3.9 Analgesic and anticonvulsant effects of *K.pinnata*(Lam.) Pers.

The analgesic effect of methylene chloride/methanol (1:1) (CH2Cl2/CH3OH) extract and its hexane, methylene chloride (CH2Cl2), ethyl acetate, n-butanol fractions and aqueous residue was evaluated using acetic acid, formalin and pressure test. The anticonvulsant effects of the CH2Cl2/CH3OH extract were also investigated on seizures induced by pentylenetetrazol (PTZ), strychnine sulphate (STN) and thiosemicarbazide (TSC). CH2Cl2/CH3OH extract and its fractions administered orally exhibited protective effect of at least 30% on the pain induced by acetic acid. The CH2Cl2 fraction at 300 mg/kg showed a maximal effect of 78.49%. The CH2Cl2/CH3OH extract and its CH2Cl2 fraction at the doses of 150 and 300 mg/kg significantly reduced the first phase of pain induced by formalin while the second phase was completely inhibited. The CH2Cl2 fraction produced more than 45% reduction in the sensitivity to pain induced by pressure. The CH2Cl2/CH3OH extract of *K. pinnata* significantly increased the latency period in seizures induced by PTZ and significantly reduced the duration of seizures induced by the three convulsant agents. The extract protected 20% of animals against death in seizures induced by TSC and STN.These results suggest a peripheral and central analgesic activities as well as an anticonvulsant effect of the leaves of *K. pinnata* (Nguelefack et al.,2006).

3.10 Leishmaniasis activity of *K.pinnata*(Lam.) Pers.

Muzitano et al. (2009) carried out an investigation to study the effect of *K. pinnata* on cutaneous leishmaniasis. In order to demonstrate the safety and oral activity of *K. pinnata*, different flavonoids were extracted from the plants and were evaluated in vivo in murine model of cutaneous leishmaniasis. Daily oral doses of quercetin 3- O--L-arabinopyranosyl, -L-rhamnopyranoside, quercetin 3-O--L- rhamnopyranoside and free quercetin (16 mg/kg body weight) were administered. It was observed that they were able to control the lesion growth caused by Leishmania amazonensis and significantly reduce the 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. These results indicate that quercetin glycosides are important active components of the aqueous extract and that they possess potent oral efficacy against cutaneous leishmaniasis.

3.11 Diuretic and anti-urolithiatic activity of *K.pinnata*(Lam.) Pers.

Patil et al. (2009) studied the diuretic and anti-urolithiatic activity of *K. pinnata*. Hydroalcoholic extract of leaves of *K. pinnata* was administered to male Wistar rats orally and intraperitonially. The effect of the extract on urine output was determined by comparing the urine volume collected by keeping the individual animals in metabolic cages. Calcium oxalate urolithiasis was induced in rats by giving ethylene glycol orally for 7 days and the effect of the extract was observed by its concurrent administration. The extract was found to have significant diuretic and anti-urolithiatic activity and the intraperitonial administration of the extract gave more potent diuretic effect.

3.12 Anti-tumor activity of *K.pinnata*(Lam.) Pers.

Five bufadienolides (1-5) isolated from the leaves of *K. pinnata*(Lam.)Pers.were examined for their inhibitory effects on Epstein-Barr virus early antigen (EBV-EA) activation in Raji cells induced by the tumor promoter, 12-Otetradecanoylphorbol-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

resultsstrongly suggest that bufadienolides are potential cancer chemopreventive agents (Supratman et al., 2001).

3.13 Anti-allergic activity of *K.pinnata*(Lam.) Pers.

Cruz et al. (2008) reported on the protective effect of *K pinnata*(Lam.)Pers. in fatal anaphylactic shock, likewise a Th2-driven immunopathology and the identification of its active component. In vitro, *K. pinnata*(Lam.)Pers.prevented antigen(Biswas et al. 1261) induced mast cell degranulation and histamine release. Oral treatment with the quercitrin flavonoid isolated from the plant prevented fatal anaphylaxis in 75% of the animals. These findings indicate that oral treatment with *K. pinnata*(Lam.)Pers. effectively down-modulates pro-anaphylactic inducing immune responses. Protection achieved with quercitrin, although not maximal, suggests that this flavonoid is a critical component of *K. pinnata*(Lam.)Pers. extract against this extreme allergic reaction.

3.14 Neuropharmacological activity of *K.pinnata*(Lam.) Pers.

Effects of aqueous leaf extracts of *K. pinnata*(Lam.)Pers.on some neuropharmacological activities were studied in mice. The extract was found to produce a profound decrease in exploratory activity in a dose-dependent manner. It also showed a marked sedative effect as evidenced by a significant reduction in gross behaviour and potentiation of pentobarbitone-induced sleeping time. It delayed onset in strychnine-and picrotoxin-induced convulsion (seizures) respectively with the protective effect being significantly higher in picrotoxin- than strychnine-induced convulsion. It also decreases the rate of picrotoxininduced mortality in mice with LD50 of 641 mg/kg. The totality of these effects showed that the extract possesses depressant action on the central nervous system (Salahdeen and Yemitan, 2006).

CHAPTER 04:METHODS AND MATERIALS

4.1 Preparation of plant extract for experiment

4.1.1 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.

4.1.2 Identification

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

4.1.3 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%).

4.2 Tests For analgesic activity

Principle

In this method (Koster *et al*., 1959; Whittle, 1964; Vogel & Vogel, 1997; Ahmed *et al*., 2001) acetic acid is administered intra-peritoneal to the experimental animals to create pain sensation. As a result, the animals squirms their body at regular interval out of pain. This squirm/contraction of the body is termed as “writhing”. As long as the animals feel pain, they continue to give writhing. Each writhing is counted and taken as an indication of pain sensation. Any substance that has got analgesic activity is supposed to lessen the number of writhing of animals within in a given time frame and with respect to the control group. The writhing inhibition of positive control was taken as standard and compared with test samples and control. As positive control, any standard NSAID drug can be used. In the present study, Diclofenac was used as standard.

Experimental Animal

Swiss-albino mice of either sex, aged 4-5 weeks, brought from the Animal Resource Branch of the International Centre for Diarrhoeal Diseases and Research, Bangladesh (ICDDR, B) were used for the experiment.

Animal feed: 'Mouse-pellets' supplied by ICDDR, B Dhaka.

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

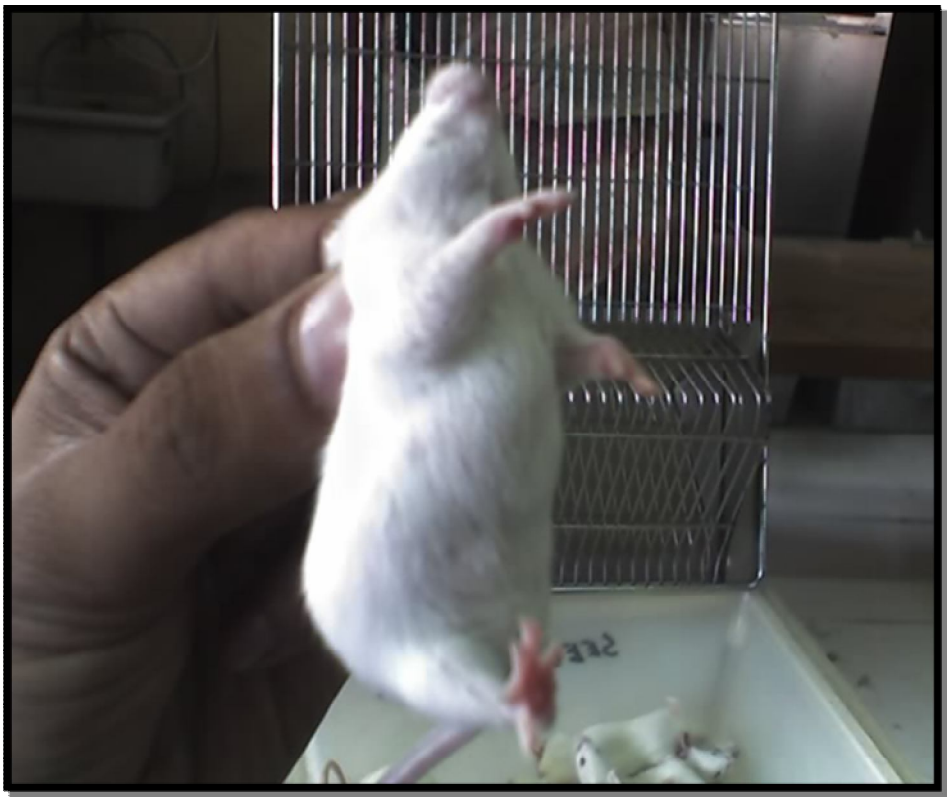


Fig 4.1: Swiss Albino mice

Identification of animals during experiment

Fifty experimental mice were randomly selected and divided into ten groups denoted as group-I, group-II, group-III, group- lV and group V consisting of 5 mice in each group. Each group received a particular treatment i.e. control, standard and two different doses of the extract of ethalonic fraction. Prior to any treatment, each mouse was weighed properly and the doses of the test samples and control materials were adjusted accordingly. It is difficult to identify and

observe at a time five mice receiving same treatment. Thus it was important to identifyindividual animal of a group during the treatment. To denote individual animal, they were marked or coded I, II, III, IIII and no marks on their tails.

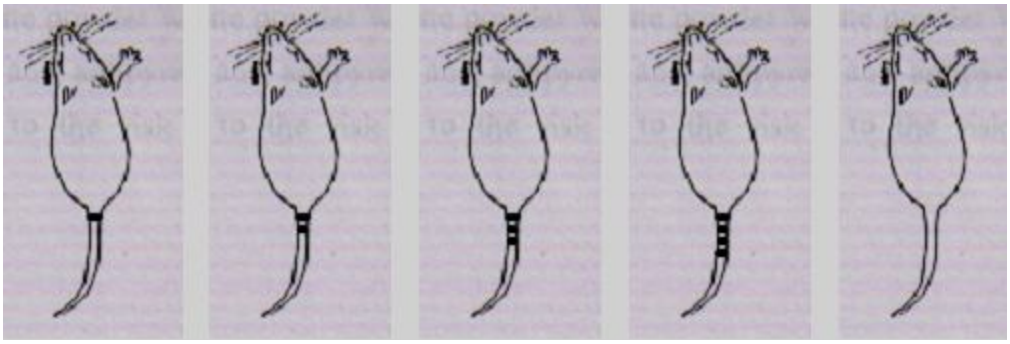


Fig 4.2: Marks used for identifying experimental mice

4.2.1Test for analgesic activity

Table 4.1: Drugs and chemicals for analgesic test