Analgesic And Neuropharmacological Evaluation of Ethyl Acetate Extract from *Kalanchoepinnata* leaves

A dissertation is submitted for the partial fulfillment of the course of Pharmaceutical Research (PHRM 404) of the Department of Pharmacy, East West University for the Degree of Bachelor of Pharmacy

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Declaration by the Research candidate

I, Md. JamilUddin, hereby declare that the dissertation entitled "**Neuropharmacological Evaluation of Ethyl Acetate Extract from *Kalanchoepinnata* leaves",** 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. RazibulHabib, 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

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Thesis Certificate

This is to certify that the thesis entitled "**Neuropharmacological Evaluation of ethyl Acetate Extract from *Kalanchoepinnata*leaves"**, 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 Md. JamilUddin (ID. 2007-3-70-045) during the period 2011-2012 of his 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.

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ABSTRACT

The analgesic and Neuropharmacologycal activity of acetic acid extract of leaves of *Kalanchoepinnata*(Lam.) Pers. was evaluated using an acetic acid-induced gastric pain model in Swiss albino mice. The acetic acid extract of leaves of *Kalanchoepinnata*demonstrated significant dose-dependent analgesic activity at all the tested doses of 100, 200, and 400 mg leaf extract/kg body weight in mice. Even at the lowest dose of 100 mg/kg body weight, the analgesic activity of leaf extract was comparable to that of a standard analgesic drug, aspirin, administered at 200 mg/kg body weight. The highest inhibition of writhing induced by acetic acid (47.5%) was observed with a dose of400 mg leaf extract/kg body weight, which was much greater than the inhibition obtained with aspirin (38.4%). At the lower dose of 250 mg bark extract/kg body weight, there was a decrease in the number of writhings compared to controls, but the decrease was not significant. The results suggest that acetic acid extract of leaves of *Kalanchoepinnata*and chloroform extract of barks have activities and validates of folk medicinal uses in Bangladesh for treatment of pain. It was however more efficacious against picrotoxininduced seizure where protection was observed in about one-quarter of mice, an effect which indicates that *k. pinnata* aqueous extract might produce its central nervous system depressant action as consequence of its GABAergic and less importantly, glycinergic transmission, since picrotoxin is a selective GABAA receptor antagonist (Rang et al 1996) while strychnine antagonizes the inhibitory spinal cord and brainstem reflexes of glycine (Yemitan et al 2001).

Key words:*Kalanchoepinnata*, Analgesic test, Writhing test, Formalin induced test, Neuropharmacology, Hole cross, Open field, EPM test, Result

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Introduction

Kalanchoepinnata(Lam.) Pers. (Family: Crassulaceae, local name: Pathorkuchi) is an herb foundubiquitously in Bangladesh having thick succulent leaves. Leaves have a variety of uses in the traditionalmedicinal system of Bangladesh. They are eaten for diabetes control, used as a diuretic and for dissolving ofkidney stones, and taken for respiratory tract infections, as well as applied to wounds, boils, and insect bites(Ghani, 2003). The leaves are used by traditionalmedicinalpractitoners for diabetes, and the bark as a purgative (Ghani, 2003).Phenolic components (Gaind and Gupta, 1973) and flavonoid glycosides (Gaind and Gupta, 1971) havebeen reported from *Kalanchoepinnata*. The leaf extract of the plant has been shown to significantly delay theonset of leishmaniasis in BALB/c mice when infected with *Leishmaniaamazonensis*(Da Silva *et al.,* 1995). Quercitrin (quercetin 3-*O*-a-L-rhamnopyranoside), obtained from an active aqueous extract of the plant has been identified as the active principle (Muzitano*et al.,* 2006). Effectiveness of an immunomodulatory extract of the plant has also been demonstrated against visceral leishmaniasis in BALB/c mouse model of infection with*Leishmaniachagasi*(Gomes *et al.,* 2010). Other activities reported for the plant or plant components include protection of mice against fatal anaphylactic shock (Cruz *et al.,* 2008), hepatoprotective activity in leaves (Yadav and Dixit, 2003), anti-tumor promoting activity of bufadienolides isolated from leaves of the plant (Supratman*et al.,* 2001) and isolation of insecticidal bufadienolides from leaves of the plant (Supratman*etal.,* 2000). The anti-diabetic and anti-obesity activity of *Lagerstroemia speciosa*as well as several phytochemical constituents isolated from the plant has been very well documented (Kakuda*et al.,* 1996; Suzuki *et al.,* 1999;Liu *et al.,* 2001; Hayashi *et al.,* 2002; Judy *et al.,* 2003; Hattori *et al.,* 2003; Liu *et al.,* 2005; Yamada *et al.,* 2008; Klein *et al.,* 2007; Bai*et al.,* 2008; Hou*et al.,* 2009; Sivakumar*et al.,* 2009; Ichikawa *et al.,* 2010).A phytochemical isolated from the plant, orobol 7-*O*-D-glucoside, has been reported to have inhibitory effectson human rhinoviruses replication (Choi *et al.,* 2010). Free radical scavenging and anti-inflammatory propertieshave been demonstrated in leaf extracts of the plant (Priya*et al.,* 2008). Ethyl acetate extract of leaves hasbeen shown to ameliorate cisplatin-induced nephrotoxicity in BALB/c mice (Priya*et al.,* 2007). Anti-fungal activity has been demonstrated with hot water as well as methanol extract of the plant against *Arthriniumsacchari*M001 and *Chaetomiumfunicola*M002 strains (Sato *et al.,* 2000). Xanthine oxidase inhibitors(valoneic acid dilactone and ellagic acid) have been isolated from leaves of the

plant (Unno*et al.*, 2004).Petroleum ether extract of seeds reportedly showed antibacterial activity against both Gram positive and Gramnegative bacteria (Sinhababu*et al.*, 1994).

In our ongoing survey on folk medicinal practices in Bangladesh (Rahmatullah*et al.*, 2009a; 2009b; 2009c), it was observed that leaves of *Kalanchoepinnata*were used by the folk medicinal practitioners in several parts of Bangladesh for treatment of rheumatoid arthritis and pain in the bones (unpublished observation). The barks of *Lagerstroemia speciosa*were also used as an analgesic (unpublished observation). The objective of the present study was to determine the analgesic potential of leaves of *Kalanchoepinnata* whether the experimental results validate the plants' folk medicinal uses.

Oxygen radicals, the products of some biochemical and physiological reactions, initiate cell signaling pathways, damage cellular lipids, proteins, and nucleic acids. Reactive oxygen species are pivotal for the onset of various conditions such as hypertension, atherosclerosis, cancer, and alzheimer's disease (Morris, 2005; Azadzoi et al., 2005). During normal aerobic metabolism, activated oxygen radicals, hydroxyl radicals and peroxynitrite. The *Kalanchoe*genus is a succulent perennial plant belonging to the class *Bryophyllum*. It grows naturally throughout the temperate areas of the world. In Africa, over 200 species have being identified and many of thesespecies have been used medicinally especially *Kalanchoepinnata*and *Kalanchoeintegra*(Figure 1A andB) which have been used traditionally for the treatment of many disease conditions like peptic ulcer, upper respiratory tract infections, coughs and as anti-infective in Ghana (Dokosi, 1998 and Torres-Santos et al., 2003). They grow widely along footpaths and forests in Ghanaand is called by various local names (for example, the Ewes call it 'aflatoga', Fantes -'eporow', Twi and Ga - 'egoro' and 'tamiwu' respectively). *Kalanchoe*is reported to contain considerable amounts of flavonoid and phenolic compounds (Gaind and Gupta, 1971; Adenike and Eretan, 2004). Despite its rich flavonoid and phenolic content, available literature indicates that no study has been carried out to investigate the antioxidant properties of this medicinal plant. The aim of this study is to determine the total flavonoid and phenolic content of two *Kalanchoe* species, namely, *K. pinnata*and *K. integra*and also to assess their antioxidant activity.

Fig: Pathorkuchi leaves (*Kalanchoepinnata*)

Traditional medicine involves the use of herbal medicine, animal parts and minerals. However, herbal medicines are the most widely used of the three. Herbal medicines contain an active ingredient, aerial or underground parts of plants as their petal or seeds materials or combinations thereof, whether in the crude state or as plant preparations. Furthermore, about 80% of the world population is dependent (wholly or partially) on plant-based drugs (WHO, 1996). In Nigeria and most developing countries of the world, rural and urban dwellers, literate or illiterate rely heavily on herbal preparations for the treatment of various diseases despite the availability of orthodoxmedicine (Nwabuise, 2002). *Bryophyllumpinnatum*(Lam.) (synonym: Kalanchoepinnata, Lam.; common names: Life plant, air plant (Mexican), love plant, Canterbury bells, Cathedral bells, e.t.c) is a perennial herb growing widely and used in folkloric medicine in tropical Africa, India, China, Australia and tropical America (Engler, 1926; Balzer et al, 1949). Classified as a weed(Oliver-Bever, 1983), the plant flourishes throughoutthe Southern part of Nigeria (Gill, 1992). A number of active compounds, including flavonoids, glycosides, steroids, bufadienolides and organic acids, have been identified in *Bryophyllumpinnatum*(Marriage and Wilson, 1971; Gaind and Gupta, 1972; 1974; Costa et al, 1995). In traditional medicine, the leaves of this plant have been reported to possess antimicrobial (Mehta and Bhat, 1952; Akinpelu, 2000; Oliver-Bever, 1983), antifungal (Misra and Dixit, 1979), anti-ulcer (Pal and Nag, 1991), anti-inflammatory and analgesic (Pal and Nag, 1989; 1992), and antihypertensive (Ojewole 2002) activities. The methanol extract of the leaf of the plant has also been reported to have histamine receptor (H1) antagonism in the ileum, peripheral vasculature and bronchial muscle (Pal et al, 1999)

Although, studies have shown the relative important effect of some medicinal plant on the central nervous system activities. (Dorr et al., 1971; Fujimori, 1995; Wakeel, 2004).. B. pinnatum has been used since 1921 in traditional medicine as an antipsychotic agent. Furthermore, Pal and Nag (1999) provided evidence for the neuropsychopharmacologic activitiesof the plant. The present study is therefore designed to further investigate the effects of the aqueous leaf extract of *B. pinnatum*on some central nervous**.** Kalanchoepinnata (Lamarck) Persoon (=Bryophyllumpinnatum) is a perennial medicinal herb, popularly used in Brazil and other parts of the world to treat various inflammatory diseases (Rossi-Bergmann et al., 1994).

Previous studies on the chemical composition of K. pinnata showed that bufadienolides, terpenoids and flavonoids are the main secondary metabolites of this species (Yamagishiet al., 1989; Costa et al., 1995). Our interest in K. pinnata is justified by its significant immunosuppressive effects, as well as its ability to protect against progressive infectionwith Leishmaniaamazonensis(Rossi-Bergmann et al., 1994; Da Silva et al., 1995).The leishmaniases are a complex of diseases caused by different species of the protozoan parasite Leishmania and are a major public health problem in many developing countries, where 350 million people live at risk of infection (WHO, 2005). There is no approved vaccine for clinicaluse. Despite a few research achievements, first-line chemotherapy is still based on pentavalentantimonials, developed more than 50 years ago, which are toxic and prone to drugresistance (Croft and Coombs, 2003). Recently, several natural products with antileishmanialactivity, including naphthoquinones, lignans, neolignans,alkaloids and triterpenoids have been reported (Chan-Bacab and Pen˜a-Rodriguez, 2001).

However, there have been few studies on the antileishmanial activity of the flavonoid class of natural polyphenols. These few studiesinclude that of luteolin, a common flavonoid in the human diet, which was recently described as a promising antileishmanial drug (Mittra et al., 2000). Proanthocyanidins also show antileishmanial activity, as well as modulatory effects on nitric oxide and tumor necrosis factoralpha release in RAW 264.7 cells (Kolodziej et al., 2001), and a methoxychalcone isolated from inflorescences of Piper aduncum(Piperaceae) reportedly has significant antileishmanial activity as well (Torres-Santos et al., 1999). Quercitrin, previously isolated from an activeflavonoid fraction of K. pinnataby our group, was an additional potent antileishmanial compound, with a low toxicity profile (Muzitano et al., 2006). Herein, we

describe a new flavonoid diglycoside and two other polar flavonoids from K. pinnataas well as their in vitro antileishmanial activity in comparison with three analogs: quercetin, quercitrin, and afzelin.

1.1 Plant description of kalanchoepinnata

Botanical Name: *Bryophyllumpinnatum*

Family Name: Crussulaceae

Sanskrit Name: Pashanabheda

Hindi Name: Patharchur

Common Names: Cathedral Bells, Air Plant (USA), Life Plant, Miracle Leaf, Goethe Plant and

Katakataka. Also called "Wonder of the World" in the English speaking Caribbean.'OliwaKaKahakai (Hawai'i), Mother Of Thousands, Herbe Mal Tete (Dominica) Never Dead, Parvu, HojaDelAire (Bolivia).

Synonym: Bryophyllumcalycinum, Bryophyllum pinnatum4,5,6.

1.2 Taxonomical tree

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Saxifragales

Genus: Kalanchoe

Section: Bryophyllum

Species: *K. pinnata*

The plant grows all over India in hot and moist areas, especially in Bengal. It is a succulent perennial plant that grows 1-1.5 m in height and the stem is hollow four-angled and usually branched. Leaves are opposite, decussate, succulent, 10-20 cm long. The lower leaves are simple, whereas, the upper ones 3-7 foliate and are long-petioled. They are fleshy dark green that are distinctively scalloped and trimmed in red. Leaf blade pinnately compound with 3-5 leaflets, 10-30 cm; petiolules 2-4 cm; leaflet blades oblong to elliptic, 6-8 X 3-5 cm, margin crenate with each notch bearing a dormant bud competent to develop into a healthy plantlet apex obtuse8. The leaves are furnished with rooting vegetative buds. Inflorescences terminal paniculate 10-40 cm. Flowers are many bell-like pendulous. Calyx tubular, 2-4 cm; Corolla reddish to purple, 5 cm, base sparsely ciliate; lobes ovate-lanceolate; stamens inserted basally on corolla; nectar scales oblong; follicles included in calyx and corolla tube. The fruit-pod with four septa and numerous, ellipsoid, smooth striate seeds within. The plant flowersin Nov-Mar and fruits in April 7,8,9 . It is astringent, sour in taste, sweet in the post digestive effect and has hot potency.

Figure: various plant parts of *k.pinnata*

Habitat: It is a succulent plant native to Madagascar. It is distinctive for the profusion ofminiature plantlets that form on the margins of its leaves, a trait it has in common with the othermembers of the Bryophyllum section of the Kalanchoegenus.It is a popular houseplant and hasbecome naturalized in temperate regions of Asia, the Pacific and Caribbean4.

Distribution: Kalanchoepinnata 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 and it is known as katakataka or kataka-taka which is also an adjective meaning astonishing or remarkable4,5.

Herbs as antibiotic: The development of drug resistance in human pathogens against commonly used antibiotics has necessiatated a search for new antimicrobial substances from other sources, including plants. Natural substances of plant origin have been used and are being used throughout the world for human and animal health care. The limitation of the synthetic medicines has created a challenge for the search of natural source of cure for many diseases. Plants used for traditional medicine contain a wide range of substances that are used to treat chronic as well as infectious diseases.

Herbal treatments are very effective in treating bacterial and other common childhood infections. Several herbs such as garlic, goldenseal, myrrh, usnea, and uvaursi have antibiotic effects. The difference between an antibiotic drug and an antibiotic herb is that the drug is an isolated constituent limited to the power of that one chemical, whereas the herb contains several constituents with a variety of healing properties, producing a synergistic effect.

The herb can actually kill only the bad bacteria while not harming the good; the drug does not have the wisdom to differentiate. Furthermore, most bacteria are not fooled by all isolated compound; Often the drug becomes ineffective or the cells mutate eventually to become resistant to the drug. The organic herb is nature's match for the bacteria.

Therapeutic Benefit

Much of the exploration and utilization of natural products as antimicrobials arise from microbial sources. It was the discovery of penicillin that led to later discoveries of antibiotics such as streptomycin, aureomycin and chloromycetin. Though most of the clinically used antibiotics are produced by soil microorganisms or fungi, higher plants have also been a source of antibiotics. Examples of these are the bacteriostatic and antifugicidal properties of Lichens, the antibiotic action of allinine in Allium sativum (garlic), or the antimicrobial action berberines in goldenseal (Hydrastiscanadensis)

Economic Benefit

Worldwide, there has been a renewed interest in natural products. This interest is a result of factors such as: consumer’s belief that natural products are superior; consumer’s dissatisfaction with conventional medicines; changes in laws allowing structure-function claims which results in

more liberal advertising; aging baby boomers; national concerns for health care cost. The potential for developing antimicrobials into medicines appears rewarding, from both the perspective of drug development and the perspective of phytomedicines. The immediate source of financial benefit from plants based antimicrobials is from the herbal products market.

Literature Review

Anthelmentic activity:

The roots of *K. pinnata*were subjected to petroleum ether, chloroform, methanol and aqueous solvent respectively for extraction and the *in-vitro* evaluation of anthelmentic activity was done against *Pheretimaposthuma*(Annelida) and *Ascardiagalli*(nematode). The results reveal that chloroform, methanolic and aqueous extract of *K. pinnata*have significant anthelmentic activitywhile petroleum ether does not show any activity against helminth. Methanolic extract of root of *K. pinnata*was found to be most effective as an anthelmentic as compared to other. The roots extract of *K. pinnata*not only demonstrated paralysis but also caused deaths of worms especially at higher concentrations of 100 mg/ml, in shorter time as compared to the reference drug, Piperazine citrate. Phytochemical analysis of the crude extracts revealed the presence of tannins which were shown to produce anthelmentic activity (Majaz et al., 2011).

Immunosuppressive effect:

The aqueous extract of *K. pinnata*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*showed a decreased ability to proliferate in response to both mitogen and to antigen *invitro.* Treatment with *K. pinnata*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. Theintraperitoneal 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).

Wound healing activity:

The extract of *K. pinnata*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 animalstreated with *K. pinnata*ethanolic extract compared to animals which received the control treatment and standard treatment. Significant progressive reduction inthe 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*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).

Antihypertensive activity:

The effects of aqueous leaf extract of *K. pinnata*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 catand also reduced the effect of adrenaline-induced elevation of blood pressure. It was concluded that the pharmacological basis for the use of *K. pinnata*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*leaf extract is potentially organotoxic whichnegates its use as a blood pressure lowering agent (Ghasi et al., 2011).

Hepatoprotective activity:

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 to be more effective than ethanolic extract (Yadav and Dixit, 2003).

Antinociceptive, anti-inflammatory and antidiabeticactivity:

In order to scientifically appraise some of the ethnomedical uses of *K. pinnata*leaves, a study was undertaken to investigate the antinociceptive, anti-inflammatory and antidiabetic properties of the plant's leaf aqueous extract in experimental animal models. *K.pinnata*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*leaf aqueous extractpossesses 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).

Nephroprotective and antioxidant activity:

Harlalka et al. (2007) evaluated the aqueous extract of *K. pinnata*for its protective effects on Gentamycin-inducednephrotoxicity in rats. It was observed that the aqueousextract of *K. pinnata*leaves significantly protects ratkidneys from Gentamycin-induced histopathologicalchanges. 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*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*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*leaf extract. In case of histopathological examination, control ratsshowed 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 theextract was found to reduce such changes in kidney histology induced by Gentamycin. *In-vitro* studies revealed that the *K. pinnata*leaf extract possesses

significant antioxidant as well as oxidative radical scavenging activities. Quercetin and kaemferol have been detected in the leaves of *K. pinnata*(Harlalka et al., 2007). Morales et al. (2006) suggested that quercetin hasa marked protective effect on cadmium-induced nephrotoxicity that results from an increaseMetallothionein, 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.

Antimicrobial activity:

The roots of *K. pinnata*were subjected to petroleum ether, chloroform, methanol and aqueous solvent respectively for extraction and *in vitro* evaluation ofantimicrobial activity was done against *Staphylococcusaureus, Escherichia coli, Pseudomonas aeruginosa*and*Candida albicans*. Methanolic extract of roots of *K.pinnata*was found to be most effective as antibacterial ascompare to others while none of extract showed theactivity against *C. albicans*(Quazi et al., 2011). Akinpelu(2000) in a study found that 60% methanolic leaf extractinhibits the growth of five out of eight bacteria used at anconcentration of 25 mg/ml. *Bacillus subtilis, E. coli,Proteus vulgaris, Shigelladysenteriae, S. aureus*werefound to be inhibited while *Klebsiellapneumoniae, P.aeruginosa*and C. *albicans*were found to resist theaction of the extract. Chemical investigation of thebioactive constituents from the leaf of *K. pinnata*resultedin the isolation of two new novel flavonoids; 5I Methyl 4I,5, 7 trihydroxyl flavone and 4I, 3, 5, 7 tetrahydroxy 5-methyl 5I-propenamine anthocyanidines. Theantimicrobial observation of the aforementionedcompounds could be responsible for the activity of *K.pinnata*and its use in herbal medicine in Nigeria (Okwuand Nnamdi, 2011).

Analgesic and anticonvulsant effects:

The analgesic effect of methylene chloride/methanol (1:1)(CH2Cl2/CH3OH) extract and its hexane, methylenechloride (CH2Cl2), ethyl acetate, *n*-butanol fractions andaqueous residue was evaluated using acetic acid,formalin and pressure test. The anticonvulsant effects ofthe CH2Cl2/CH3OH extract were also investigated onseizures induced by pentylenetetrazol (PTZ), strychninesulphate (STN) and thiosemicarbazide (TSC).CH2Cl2/CH3OH extract and its fractions administeredorally exhibited protective effect of at least 30% on thepain induced by acetic acid. The CH2Cl2 fraction at300 mg/kg showed a maximal effect of 78.49%.

TheCH2Cl2/CH3OH extract and its CH2Cl2 fraction at thedoses of 150 and 300 mg/kg significantly reduced the firstphase of pain induced by formalin while the secondphase was completely inhibited. The CH2Cl2 fractionproduced more than 45% reduction in the sensitivity topain induced by pressure. The CH2Cl2/CH3OH extractof *K. pinnata*significantly increased the latency period inseizures induced by PTZ and significantly reduced theduration of seizures induced by the three convulsantagents. The extract protected 20% of animals againstdeath in seizures induced by TSC and STN. Theseresults suggest a peripheral and central analgesicactivities as well as an anticonvulsant effect of the leavesof *K. pinnata*(Nguelefack et al., 2006)

Leishmaniasis activity:

Muzitano et al. (2009) carried out an investigation tostudy 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 theplants and were evaluated *in vivo* in murine model ofcutaneous leishmaniasis. Daily oral doses of quercetin 3- O-α-L-arabinopyranosyl, α-L-rhamnopyranoside, quercetin 3-O-α-L- rhamnopyranoside and free quercetin (16 mg/kgbody weight) were administered. It was observed that they were able to control the lesion growth caused by *Leishmaniaamazonensis*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 quercetinglucuronides 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.

Diuretic and anti-urolithiatic activity:

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.

Anti-tumor activity:

Five bufadienolides (1-5) isolated from the leaves of *K. pinnata*were examined for their inhibitory effects onEpstein-Barr virus early antigen (EBV-EA) activation inRaji cells induced by the tumor promoter, 12-Otetradecanoylphorbol-13-acetate. All bufadienolidesshowed inhibitory activity, and bryophyllin A (1) exhibitedthe most marked inhibition (IC50 = 0.4 microM) amongthe tested compounds. Bryophyllin C (2), a reductionanalogue of 1, and bersaldegenin-3-acetate (3) lackingthe orthoacetate moiety were less active. These resultsstrongly suggest that bufadienolides are potential cancerchemopreventive agents (Supratman et al., 2001).

Anti-allergic activity:

Cruz et al. (2008) reported on the protective effect of *K pinnata*in fatal anaphylactic shock, likewise a Th2-drivenimmunopathology and the identification of its activecomponent. *In vitro*, *K. pinnata*prevented antigen- induced mast cell degranulation and histamine release. Oral treatment with the quercitrin flavonoid isolated fromthe plant prevented fatal anaphylaxis in 75% of theanimals. These findings indicate that oral treatment with *K. pinnata*effectively down-modulates pro-anaphylacticinducing immune responses. Protection achieved withquercitrin, although not maximal, suggests that thisflavonoid is a critical component of *K. pinnata*extractagainst this extreme allergic reaction.

Neuropharmacological activity:

Effects of aqueous leaf extracts of *K. pinnata*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).

Antimutagenic activity:

Plant has potent antihistamine and antiallergic activity. The methanolextract of the leaves has also been reported to have histamine receptor (H1) antagonism in the ileum, peripheral vasculature and bronchial muscle and protect against chemically induced anaphylactic reactions and death by selectively blocking histamine receptors in the lungs . Quercetin-3-o-α-Larabinopyranosyl (1→2)-α-L-rhamnopyrano - side showed anti allergic activity in rats. Obaseiki- Ebor *et al* investigated that organic solvent extracts of leaves had inhibitory activity for His - to His + reverse-mutations induced by ethyl methanesulfonate acting on S. typhimurium TA100 or TA1002 and were also active against reversions induced by 4nitro-o-phenylenediamine and 2-aminofluorene in TA98. The alkaloidal/ water soluble and acid fraction had no appreciable antimutagenic activity.

Anti-ulcer activity:

Adesanwo*et al* in his study showed a significant reduction in incidence ofulceration and mean basal and histamine stimulated gastric acid secretion in a dose dependent manner thus justifying its use as an anti-ulcer agent in folklore medicine.

Uterine Contractility:

B. Gwehenberger*et al* characterise the phytotherapeutictocolytic effectof *B.Pinnatum*in vitro versus the conventional betamimetic, fenoterol, in human myometrium.

Contractility was measured in strips of term myometrium biopsied at caesarean section in 14 women and exposed to increasing concentrations of *B.Pinnatum*versus +/- oxytocin 1 U/l. Result state inhibition of spontaneous contraction was concentration dependent. *B.Pinnatum*increased contraction frequency by 91% at constant amplitude and inhibited oxytocin stimulated contractions by 20% at constant amplitude with slightly decreased frequency. Fenoterol decreased contraction by 50% with a significant decrease in frequency.

Toxic to cattle:

Mckenzie*et al* investigated that cardiac glycoside poisoning was produced incalves given flower heads of the hybrid *Bryophyllum Species* and found that for each plant (except *B. tubiflorum),* 2 calves were each given a single dose of 20 g wet weight per kg bodyweight. The resultsof the calf toxicity experiment with the amounts of bufadienolide measured in the plants suggests thatbryotoxins A, B and C probably account for the observed disease.

Anticancer:

Bryophyllin compounds have marked anticancer therapeutic value against cancer cells15. Bersaldegenin-1, 3, 5-orthoacetate inhibited cancer cell growth on several cancer lines.

Insecticidal, Fungitoxic and Phytotoxic activity:

Alabi*et al* studied to evaluate the fungitoxic and phytotoxic effects of extracts on the fungal pathogens inducing wilting on cowpea grown in Ago-Iwoye, South Western Nigeria. The extract reduces the Disease Infection Rate (DIR) in treated plants. *Sclerotiumrolfsiisacc*induced wilting of between 4 and 12% on cowpea seedlings treated with plant extract under field conditions while about 39.6% incidence of cowpea seedlings wilting was observed under control experiment on the same experimental plot. The extracts increased significantly the plant height, shelf life, relative water content and chlorophyll contents of the cowpea seedlings during both the wet and dry season. On the other hand, the extracts significantly reduced transpiration rate and stomata aperture of treated plant in both seasons. Furthermore, application of these extracts on the cowpea plants significantly enhanced the Leaf Area Index (LAI), number of branches and pods per plant, total dry matter per plant, weight per pod, 100 grains weight and grain yield in both season. The extracts also inhibited the release of current photosynthethates from treated plants thus maintaining the water status of plant and also making photosynthethates which can be oxidized to release energy needed for growth available to treated plants.

Clinical usage

• The leaves are useful in burns, boils, bites of insects, congestive othalmia dysuria, diarrhoea,dysentery, impetigo, polyuria, plegmon, swellings, tuberculosis, ulcers and wounds

• The leaf juice 3 g, jeera 3 g and ghee 6 g is mixed and given for blood mixed diarrhoea

• The leaf poultice is applied on wounds, sprains, swellings and inflammations

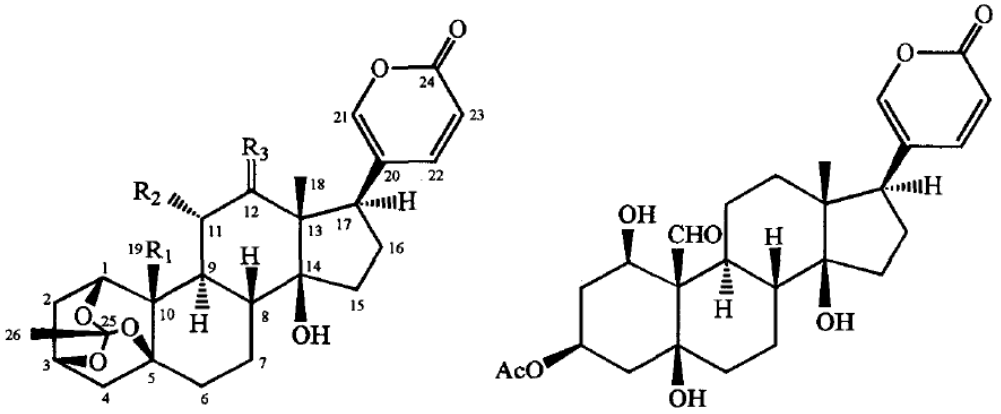
• The leaf juice is useful in cholera

• The leaf juice mixed with Kali Mirch is useful in blood oozing piles and haemorrhoids

• The leaf powder with Kali Mirch is also useful in inflammation, burning in urination and blockedurination and leprosy

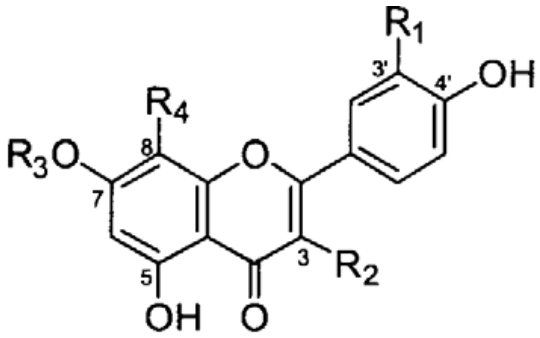
• The leaves roasted over fire are applied to places of wounds and surgical sutures in the skin toprevent discoloration of the skin

Chemical Constituents Isolated From KalanchoePinnata:



1 R_1= CHO; R_2= OH; R_3= H_2
2 R_1= CH_2OH; R_2= OH; R_3= H_2
4 R_1= CHO; R_2= H; R_3= H_2
5 R_1= CHO; R_2= OH; R_3= O

Fig. Structures of Compounds **1–5.**



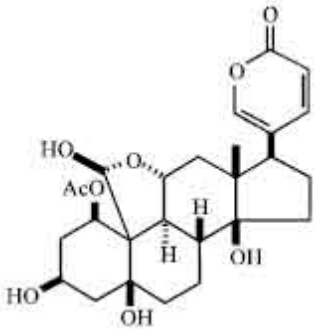


Fig 1.Bryophyllin

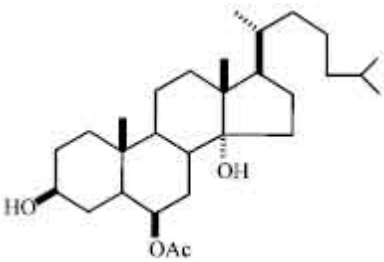


Fig 2 Bryophyllol

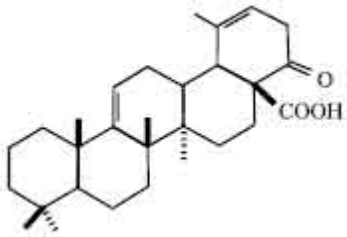
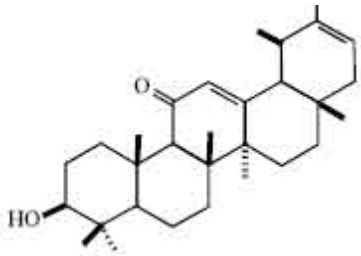
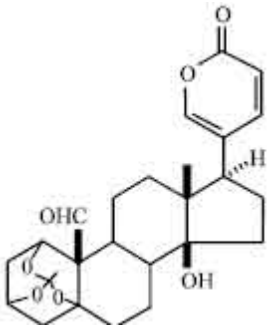


Fig 3 Bryophollone





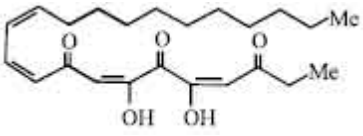


Fig 5 Bryophynol

Fig 6 Bersaldegenin

Fig 4 Bryophollenone

Chemical constituents: *B.Pinnatum is* rich in alkaloids, triterpenes, glycosides, flavonoids,cardienolides, steroids, bufadienolides and lipids. The leaves contain a group of chemicalscalled bufadienolides which are very active. Bufadienolides like bryotoxin A, B, C which are verysimilar in structure and activity as two other cardiac glycosides, digoxin and digitoxin and possessesantibacterial, antitumorous, cancer preventative and insecticidal actions .Bufadienolides-Bryophyllin A (bryotoxin); Bryophyllin B (Fig. 1); Bryophyllol (Fig. 2);Bryophollone (Fig. 3); Bryophollenone(Fig. 4); Bryophynol (Fig. 5).

Phenols, Phenylpropanoids and Flavanoids: Syringic acid, caffeic acid 10 , 4-hydroxy-3-methoxy-cinnamic acid, 4-hydroxybenzoic acid, p-hydroxycinnamic acid, paracoumaric acid, ferulic acid, protocatechuic acid, phosphoenolpyruvate, protocatechuic acid isolated from aerial parts ofplants. Leaves contains astragalin, 3,8-dimethoxy-4, 5, 7trihydroxyflavone, friedelin, epigallocatechin-3-osyringate, luteolin, rutin**,** kaempferol , quercetin , quercetin-3L-rhamonsido-L-arabino furanoside ;quercetin-3-Odiarabinoside, kaempferol-3-glucoside , kaempferol-3-O-α-L-arabinopyranosyl (1→2) α - L-rhamnopyranoside, quercetin-3-O-α-L-arabinopyranosyl (1→2)α -L-rhamnopyranoside and 4',5-dihydroxy-3',8-dimethoxy flavone-7O-β-D-glucopyranoside. Because of its restricted occurrence and its abundance in *B. Pinnatum,* flavonoid may be a chemical marker of the plant of high therapeutic potential.

Triterpenoids and Steroids: The plant contains α-amyrin, α-amyrinacetate, β-amyrin, β-amyrinacetate, bryophollenone , bryophollone , taraxerol, Ψ-taraxasterol , pseudo taraxasterol, 18-α-oleanane, friedelin, glutinol.Thecardienolide and steroidal contents includes β-sitosterol , bryophyllol, bryophynol , bryophyllinB (Antitumor) , bryophyllin A (bryotoxin C , bufadienolide1, 3, 5-orthoacetate) with potentcytotoxicity, a insecticidal bufadienolidebryophyllin C and bersaldegenin-3-acetate , bryotoxinA,bryotoxin B , bersaldegenin-1, 3, 5-orthoacetate, campesterol , 24-ethyl-25-hydroxycholesterol, isofucosterol , clionasterol, codisterol , peposterol, 22-dihydrobrassicasterol, clerosterol, 24- epiclerosterol, 24ethyl- desmosterol, 25-methyl-5α-ergost-24 -en-3-β-ol, ergosta-5-24 -dien-3-β-ol, 25-methyl-ergosta-5-24 -dien3-β-ol, 5α-stigmast-24-en-3-β-ol , (24s)-stigmast-25-en-3-β-ol, (24r)- 5α-stigmasta-7-25-dien-3-β-ol, (24s)-5α-stigmasta-7,25 dien-3-β-ol, 24(R)-stigmasta-5,25-dien-3β-ol, stigmasterol , patuletin , 3-O-(4-O-acetyl-α-L-rhamnopyranosyl)-7O-(2-O-acetyl-α-L-rhamnopyranoside) patuletin, 3-O-α-L-rhamno pyranosyl-7-O-(2-O-acetyl-α-L-rhamnopyranoside) patuletin, 3-O-(4-O-acetyl-α-L- rhamnopyranosyl)-7-Orhamno pyranosidepatuletin are isolated from aerialparts 17,18 .

Fatty Acids, Minerals and Others: Fatty acid fraction includes palmitic acid (89.3%), stearic acid (10.7%), traces of arachidic and behenic acid . Plant also contains HCN, oxalic acid , citric acid, isocitric acid, oxaloacetate, malic acid and succinic acid. The plant is rich in vitamins and aminoacids; ascorbic acid, riboflavin, thiamine, niacin, pyridoxine, glycine, cysteine, casein

hydrolysate, glutamic acid, protein hydrolysate, methionine, tyrosine, phenylalanine 19. Food contents are carbohydrates, protein, lipids, acids, iodine. The herb is good source of mineral elements such as Na, Ca, K, P, Mg, Mn, Fe, Cu, Zn. Sugar contents includes raffinose, lactose,

sucrose, glucose, galactose, fructose. Plant also contains alkaloids, tannins, phenanthrene derivatives: 2(9-decenyl)-phenanthrene, 2(9-undecenyl)-phenanthrene,alkanes (C 25-35), alkanols (C 26-34), ntriacontane, hentriacontane20.

Materials and Method

Plant material and extraction:

The leaves of *Kalanchoepinnata*were obtained from different places in Dhaka, Bangladesh in August, 2009. The plant was taxonomically identified by Mr.Manzur-ul-Kadir Mia, ex-Principal Scientific Officer and Curator of Bangladesh National Herbarium at Dhaka. The dried leaves of *Kalanchoepinnata*(leaves were air-dried in the shade for 144 hours) were grounded into a fine powder and were extracted with methanol at a ratio of 1:5 (w/v). After 24 hours, the mixture was filtered; filtrate was collected and the residue was again extracted with methanol at a ratio of 1:3 (w/v) for 24 hrs. Filtrates were combined and evaporated to dryness using rotary evaporator.

Chemicals

Glacial acetic acid was obtained from Sigma Chemicals, USA; diazepam was obtained from Square Pharmaceuticals Ltd., Bangladesh. All other chemicals were of analytical grade.

Experimental design:

The animals were divided into five groups. The first groups of animals were used for the behavioural changes and acute toxicity studies .The second groups were used for the exploratorybehaviour. The third groups were used for the benzodiazepam sleeping time. The fourth group were used also for the muscle relaxant tests. (Grp IV). The group which is group five was used for the study of anticonvulsant activity of the aqueous extract. Each group of animals was sub-divided into control and experimental groups, which was treated with the extract at sub-lethal doses of 50, 100, 200mg/kg bodyweight while the control received normal saline. The vehicle and the extracts were administered intraperitoneally before the experimentation.

Materials& Reagents:

1. Rotary evaporator (IKA RV05 basic, Biometra)
2. Closed test tube
3. beaker

4. Aluminium foil paper
5. spatula
6. pipette
7. Blender
8. Sodium Chloride
9. Distilled Water
10. Beaker
11. Cotton buds
12. Glass container for Isotonic Solution
13. Gas burner
14. Forceps
15. Labels
16. filter paper (Whatman 40)
17. permanent marking pen
18. aluminium foil paper
19. Plant extracts
20. 50 ml beakers
21. Electric balance (ELH 3000, Shimadzu, Japan)
22. pipette pumper
23. scissor
24. Ethyl acetate
25. n-Hexane
26. Acetic acid
27. Methanol
28. Dichloromethane
29. Sulfuric acid
30. Capillary tube
31. Pencil
32. Scale

Test Methods

Acetic Acid Induced Writhing Test:

The animals randomly divided into four groups consisted of 5 mice in each group. Group- I was kept ascontrol giving 1% Tween-80 in distilled water (10ml/kg b.w). Group II received Diclofenac-Na as standarddrug at a dose of 10mg/kg of body weight (p.o). Group-III and Group-IV received the test compoundacetic acid extract of *Kalanchoepinnata*at the doses of 200 mg/kg and 400 mg/kg of body weight (p.o)respectively. Thirty minutes after treatment, the mice were given an intraperitoneal (i.p.) injection of 0.7%acetic acid in a volume of 10ml/kg to induce the specific contraction of the body referred to as "writhing".The no. of writhings occurring between 5 and 15 minutes after acetic acid injection was recorded.*Kalanchoepinnata*leaves was examined using method as previously described (Deb *et al.,* 2010) with minor modifications. In the writhing test, pain was induced through intraperitoneal administration of 1% acetic acid at a dose of 10 ml 1% acetic acid/kg body weight. For experiments with *Kalanchoepinnata*leaves, mice were separated into five groups of five mice each. Group-I served as control and was administered vehicle (1% Tween 80 in water, 10 mg/kg body weight).

Formalin induced licking response model

Study design

Experimental animals were randomly selected and divided into three groups denotedas group-I, group-II and group-III consisting of 5 mice in each group. Each group received aparticular treatment i.e. control, positive control and a dose of the *k. pinnata*extract (Test group).Each animal was weighed properly and the dose of the test samples and control materials

were adjusted accordingly.

Methodology

Acetic acid induced pain model was performed according to the method described bySharma*et al*., (2010). 20µl of 1.0% v/v acetic acid was injected subcutaneously into the righthind paw of

mice. The time (in sec) spent in licking the paw and the biting responses of theinjected paw were taken as an indicator of pain response. The rats were observed for 30 minafter the injection of acetic acid, and the amount of time spent licking the injected hind pawwas recorded. The first 5 min post acetic acid injection is referred to as the early phase and theperiod between 15 and 30 min as the late phase. Extract (200 mg/kg, orally) and diclofenacsodium (0.5 mg/kg, i.p) were administered 30 min prior to formalin injection. Control animalsreceived 10 ml/kg of distilled water, orally.

Hole cross test:

The method was carried out as described by Takagi *et al.* (*8*).A steel partition was fixed in the middle of a cage of 30 × 20× 14 cm. A hole of 3 cm diameter was made at a height of7.5 cm in the center of the cage. The animals were dividedinto control, positive control, and test groups containing fivemice each. The test groups received acetic acid extract of *kolanchoepinnata*at doses of 200 and 400 mg/kg body weight orallywhereas the control group received vehicle (1% Tween80 in water). The number of passages of a mouse throughthe hole from one chamber to the other was counted for a period of 3 min at 0, 30, 60, 90, and 120 min after oral administration of both doses of the test drug.

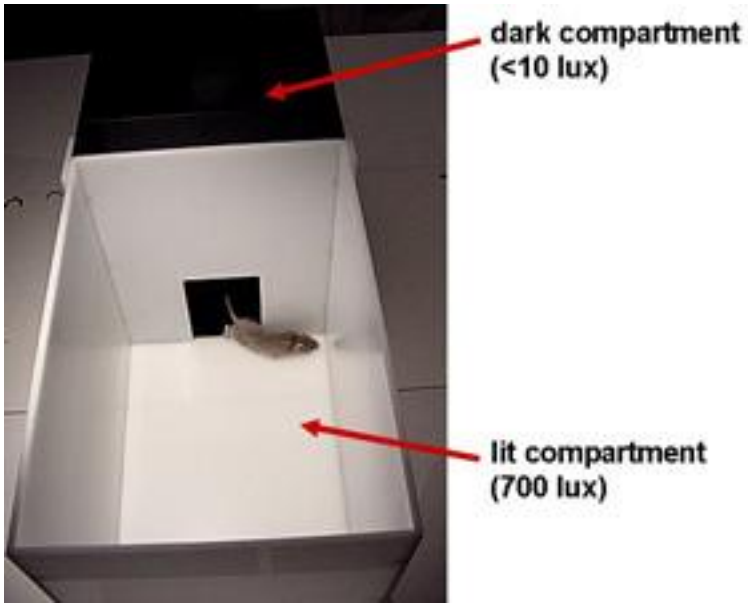


Figure: Hole test

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Open field test:

In the open field test, the animals were divided into control, positive control, and test groups containing five mice each. The test groups received acetic acid extract of the leaves of *chalanchoe pinnata*at doses of 400 mg/kg body weight orally whereas the control group received vehicle (1% Tween 80 in water). The floor of a half square meter open field (*9,10*) was divided into a series of squares each alternatively colored black and white. The apparatus had a 40 cm height wall. 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 both doses of the test drug.

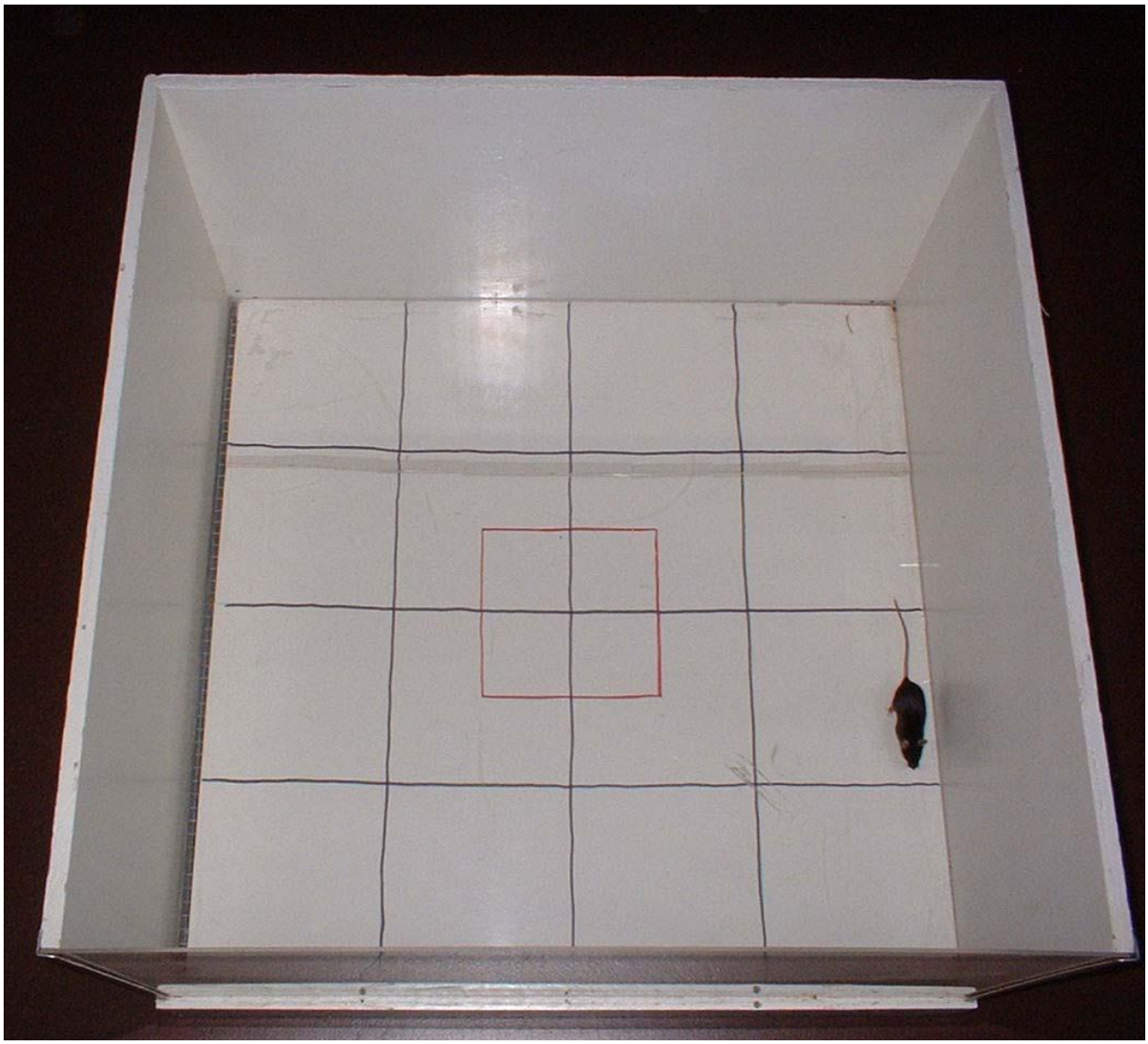

Figure: Open field test

The open field can be of different sizes; small (38 x 38 cm), or large (72 x 72 cm). The small open field can also serve as a hole board and as a test chamber for the novel object recognition task. The large open field is used for measuring anxiety and exploration as well as locomotion as it has a large center arena.

Apparatus

The open field apparatus was constructed of white plywood and measuredapparatus. Blue lines were drawn on the floor with a marker and were visible through the clear Plexiglas floor. The lines divided the floor into sixteen 18 x 18 cm squares. A central square (18 cm x 18 cm) was drawn in the middle of the open field (Brown, Corey, & Moore, 1999). The central square is used because some mouse strains have high locomotor activity and cross the lines of the test

chamber many times during a test session. Also, the central square has sufficient space surrounding it to give meaning to the central location as being distinct from the outer locations (Carrey, McFadyen, & Brown, 2000).

The maze was located in a 1.8 x 4.6 m test room and lit by a 60-watt red lamp for background lighting. The open field maze was cleaned between each mouse using 70 % ethyl alcohol. Behavior was scored with Hindsight for MS-dos (ver 1.5), and each trial was recorded for latter analysis, using a video camcorder (Hitachi, VM-7500LA) positioned above the apparatus. Measures of line crosses were obtained with an automated camera-based computer tracking system (Limelight, Actimetrics) on an IBM PC computer with the camera fixed to the ceiling, 2.1 m above the apparatus.

Procedure

Mice were carried to the test room in their home cages and were handled by the base of their tails at all times. Mice were placed into the center, or one of the four corners of the open field and allowed to explore the apparatus for 5 minutes. After the 5 minute test, mice were returned in their home cages and the open field was cleaned with 70 % ethyl alcohol and permitted to dry between tests. To assess the process of habituation to the novelty of the arena, mice were exposed to the apparatus for 5 minutes on 2 consecutive days.

Behaviours scored

The behaviours scored (Brown et al, 1999) included:

1. Line Crossing: Frequency with which the mice crossed one of the grid lines with all four paws.
2. Center Square Entries: Frequency with which the mice crossed one of the red lines with all four paws into the central square.
3. Center Square Duration: Duration of time the mice spent in the central square.
4. Rearing: Frequency with which the mice stood on their hind legs in the maze.
5. Stretch Attend Postures: Frequency with which the animal demonstrated forward elongation of the head and shoulders followed by retraction to the original position.
6. Grooming: Duration of time the animal spent licking or scratching itself while stationary.
7. Freezing: Duration with which the mouse was completely stationary.
8. Urination: number of puddles or streaks of urine.

9. Defecation: number of fecal boli produced.

Each animal was then given a score for total locomotor activity that was calculated as the sum of line crosses and number of reaction.

Why open field test is important:

The Open Field Test (Walsh & Cummins, 1976) provides simultaneous measures of locomotion, exploration and anxiety. The number of line crosses and the frequency of rearing are usually used as measures of locomotor activity, but are also measures of 4

exploration and anxiety. A high frequency of these behaviours indicates increased locomotion and exploration and/or a lower level of anxiety. The number of central square entries and the duration of time spent in the central square are measures of exploratory behaviour and anxiety. A high frequency/duration of these behaviours indicates high exploratory behaviour and low anxiety levels.

Stretch attend postures are "risk-assessment" behaviours which indicate that the animal is hesitant to move from its present location to a new position (Blanchard, Griebel & Blanchard, 2001) and thus a high frequency of these postures indicates a higher level of anxiety. Grooming behaviour is a displacement response and is expected to be displayed in a novel environment (Espejo, 1997). Therefore grooming behaviour should decrease with repeated exposure to the testing apparatus. Defecation and urination are often used as measures of anxiety, but the validity of defecation as a measure of anxiety has been questioned (Lister, 1990). Hall (1934) describes defecation and urination as indices of anxiety in rodents. He argues that the animal will have reduced locomotion in a novel environment but the autonomic nervous system will be activated which will increase defecation in this noxious arena. However, Bindra & Thompson (1953) argue that there is no significant relation between fearfulness and urination and defecation as measured in the Open Field test. Nevertheless, Bindra & Thompson (1953) agree that defecation and urination in a novel environment are signs of emotionality, which is not tobe equated with fearfulness or timidity.

Factor Analysis

Several dependent variables measured in the open field correlate significantly with one another, such as: line crosses and rearing, as well as line crosses and central square activity (Walsh & Cummins, 1976). Factor analysis of open field behaviour generally yields 3 factors but the names of these factors vary. Jahkel et al. (2000) identified 3 factors: **Activity** (53.5% of the variance) (line crosses, time active); **Exploration** (15.5% of the variance) (Center squares crossed, time in center); and **Irritation** (9.5% of the variance) (time passive). Ramos et al. (1997) also identified 3 factors: **Anxiety or Approach/Avoidance** (36.6% of variance) (locomotion in center squares); **Locomotor Activity** (30.3% of variance) (total lines crossed, lines crosses in outer squares) and **Defecation** scores (18.3% of variance). Crusio & Schwegler (1987) also found 3 factors: **Locomotion** (rearing, lines crossed); **Grooming** (grooming frequency and duration); and **Defecation** (sniffing, defecates). These studies did not always

measure the same behaviours and the different factors identified indicate that the definitive study of the open field test has yet to be conducted.

Repeated Exposure

Repeated exposure to the open field apparatus results in time dependent changes in behaviour (Choleris, et al., 2001). At first, when the apparatus is novel to the animals, more fear-related behaviours (such as stretch attends and activity in the corners and walls of the open field) are displayed. However, with repeated trials, more exploration and locomotor activity (such as rearing and line crosses as well as more central square activity) is observed. There are, however, strain differences in behaviour after repeated testing in the open field. With repeated exposure, some strains show increased activity while others show habituation and decreased activity levels and others show no change (Bolivar et al. 2000).

EPM test

The method initially suggested by Handley and Mithaniwas employed with minor modifications (*12*). Theapparatus consists of two open arms (5 × 10 cm) and twoclosed arms (5 × 10 × l5 cm) radiating from a platform(5 × 5 cm) to form a plus-sign figure. The apparatus wassituated 40 cm above the floor. The open arms edgeswere 0.5 cm in height to keep the mice from fallingand the closed-arms edges were 15 cm in height. Sixtyminutes after administration of the test drugs, eachanimal was individually placed in the center of the EPMand were allowed 5 min for free exploration. Next, thenumber of open and enclosed arm entries, and time spenton open arms were manually registered (*13*). Entry intoan arm was defined as the point when the animal placedall four paws onto the arm. The percentage of open armentries (100 × open/total entries) and the percentageof time spent in the open arms (100 × open/(open +enclosed)) were calculated for each animal. Observationsmade from an adjacent corner produced significant ($p< 0.001$, $p < 0.05$) decreases of locomotion from itsinitial value during the period of the experiment (Table1).

Maximum suppression of locomotor activity wasdisplayed at the dose of 400 mg/kg body weight, whichwas comparable to the reference drug diazepam.

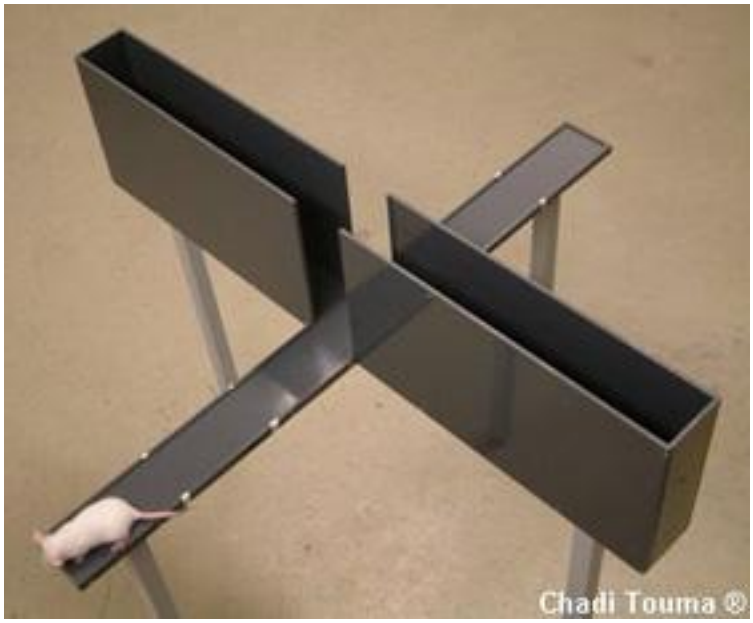

Figure: EPM test

Statistical analysis:

Experimental values are expressed as Mean ± SEM. Independent Sample t-test was carried out for statistical comparison. Statistical significance was considered to be indicated by a P value < 0.05 in all cases.

Extract preparation:

The plant extract was prepared by blending and macerating 500g of the fresh leaves of *kalanchoe pinnata* with 100ml of distilled water and was kept at 40oC for 24 hours for extraction to take place. The resulting mixture was filtered. The concentration of the extract recovered from the filtrations was computed using the expression:

Concentration = (X – Y)/ Z g/ml

Where X = Weight of fresh leaves before blending

Yg = Weight of leaves after filtration

Zml = Volume of water after filtration

Fresh preparation was used for each experimental run.

Animals

. Swiss albino mice of either sex, weighing 22-28 g, obtained from the Animal Resource Division, International Center for Diarrheal Disease and Research, Bangladesh (ICDDRB), were used throughout the experiments. All animals were kept in standard environment condition, had free access to standard.

Chemicals :

Glacial acetic acid was obtained from Sigma Chemicals, USA; diazepam was obtained from Square Pharmaceuticals Ltd., Bangladesh. All other chemicals were of analytical grade.

Animals

. Swiss albino mice of either sex, weighing 22-28 g, obtained from the Animal Resource Division, International Center for Diarrheal Disease and Research, Bangladesh (ICDDRB), were used throughout the experiments. All animals were kept in standard environment condition, had free access to standard food (ICDDRB formulated) and water *ad libitum.* Prior to experiments, all the animals were acclimatized for one week at ambient temperature.

Figure: Albino swiss mice

Experimental design:

The animals were divided into five groups. The first groups of animals were used for the behavioural changes and acute toxicity studies .The second groups were used for the exploratory behaviour. The third groups were used for the benzodiazepam sleeping time. The fourth group were used also for the muscle relaxant tests. (Grp IV). The group which is group five was used for the study of anticonvulsant activity of the aqueous extract. Each group of animals was sub-divided into control and experimental groups, which was treated with the extract at sub-lethal doses of 50, 100, 200mg/kg bodyweight while the control received normal saline. The vehicle and the extracts were administered intra peritoneally before the experimentation

RESULT:

Acetic Acid induced writhing test

Table: No. of writhing

	No. of writhing	% inhibition
POSITIVE	0.1±0.112***	99.70674
NEGATIVE	34.10±7.478	0
K. pinnata EA	14.80±4.186*	56.59824

The effect of the ethyl acetate extract of *k. pinnata* is comparable with standard and in some cases more effective than the standard.

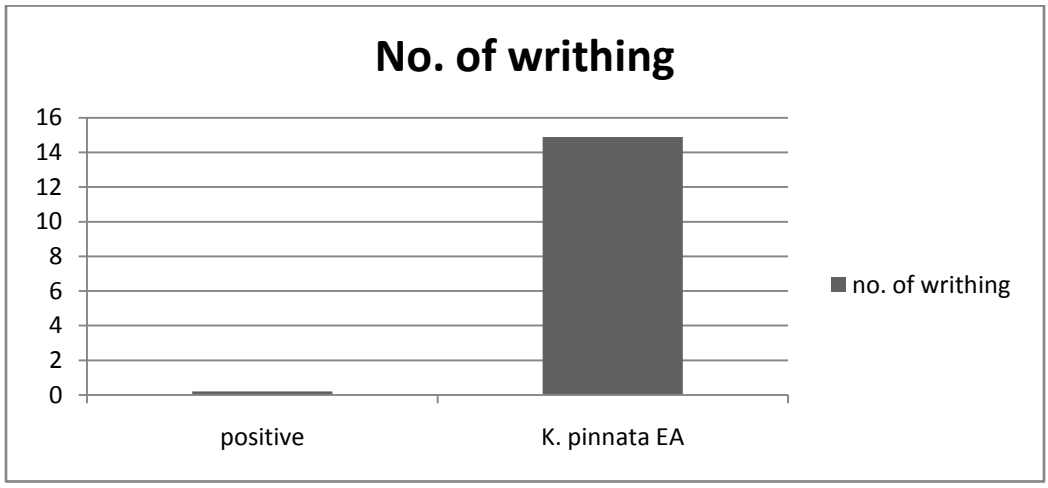

Figure:No. of writhing

Formalin induced biphasic pain

Table: licking time in early phase and late phase

	Early phase	% inhibition	Late phase	% inhibition
positive	42.20 ± 2.219***	46.96429	25.4 ± 1.924**	24.O5822
negative	110.4 ± 6.130	0	61.00 ± 4.472	0
K. pinnata EA	83.20 ± 2.71	20.35714	56.2 ± 3.156	38.61301

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

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

The effect of the ethyl acetate extract of k. pinnata is shown in the graph.

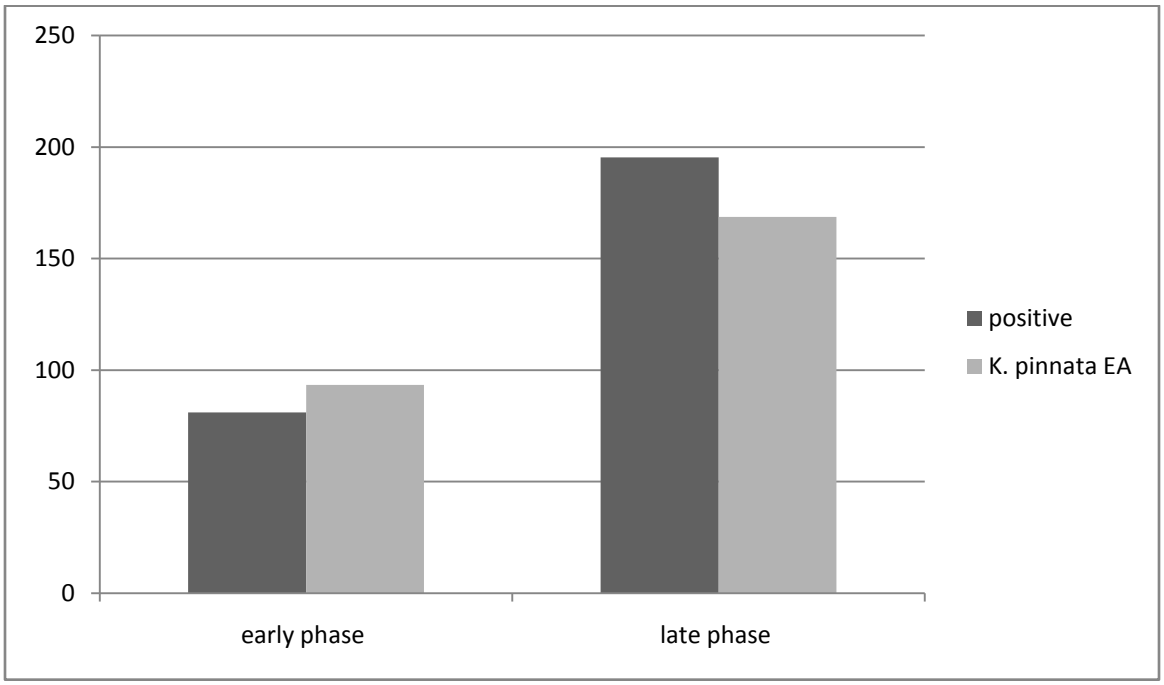

Figure: licking time of the early phase and late phase of the treated mice

HOLE CROSS test

Table : Number of Hole crossed by the mice

Minute	Control	Diazepam	K.pinnata EA
0	18.60±0.570	12.80±3.090	11.40±1.643
30	9.20±2.329	6.60±1.151	5.60±1.440
60	5.40±0.671	4.40±1.483	2.60±1.351
90	5.40±0.570	4.60±0.837	1.40±0.837
120	4.60±0.570	3.80±1.917	0.80±0.224

The effect of the ethyl acetate extract of *k. pinnata* is comparablefrom the second interval with standard and in some cases more effective than the standard.

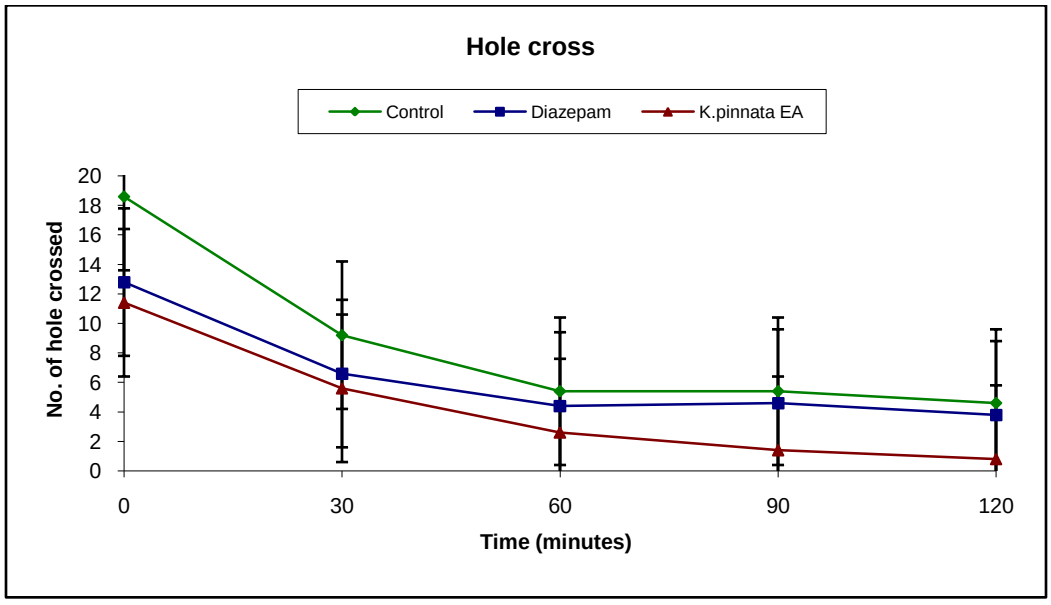

Figure: Number of Hole crossed by the mice

Open Field

Table: Number of square travelled by the mice

Minute	Control	Diazepam	K.pinnata EA
0	121.20±0.7.652	110.40±5.943	85.00±5.099
30	60.20±3.435	56.20±7.012	31.80±2.104
60	40.60±4.207	36.60±3.616	25.00±1.969
90	29.40±2.864	25.80±2.302	20.00±0.791
120	28.80±4.866	24.80±8.863	22.40±2.797

The effect of the ethyl acetate extract of *k. pinnata* is comparable from the second interval with standard and in some cases more effective than the standard.

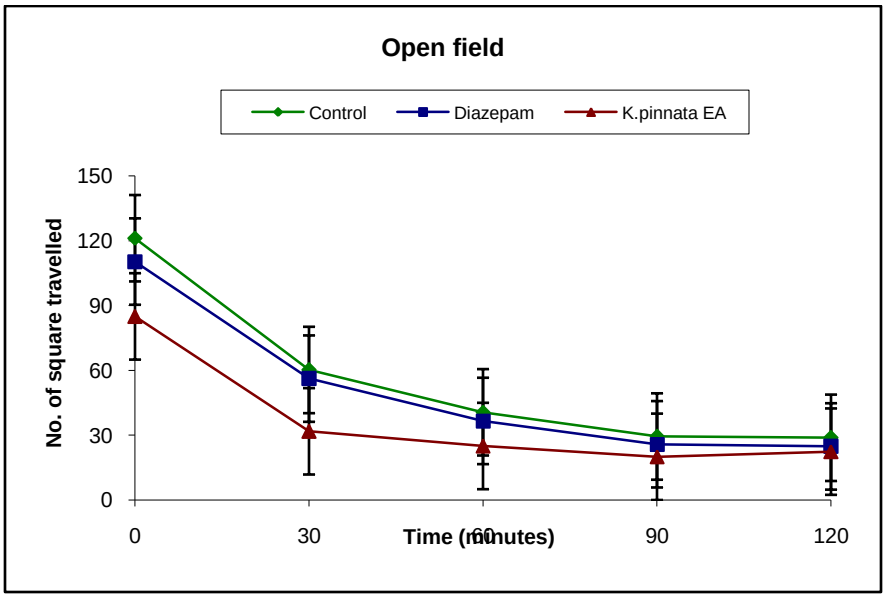

Figure: Number of square travelled by the mice

EPM

Table : % number of entry and time spent in the open arm by the mice

	% 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
K. pinnata EA	17.00±3.687	2.71±0.346

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

The effect of the ethyl acetate extract of *k. pinnata* is comparable with standard and in some cases more effective than the standard.

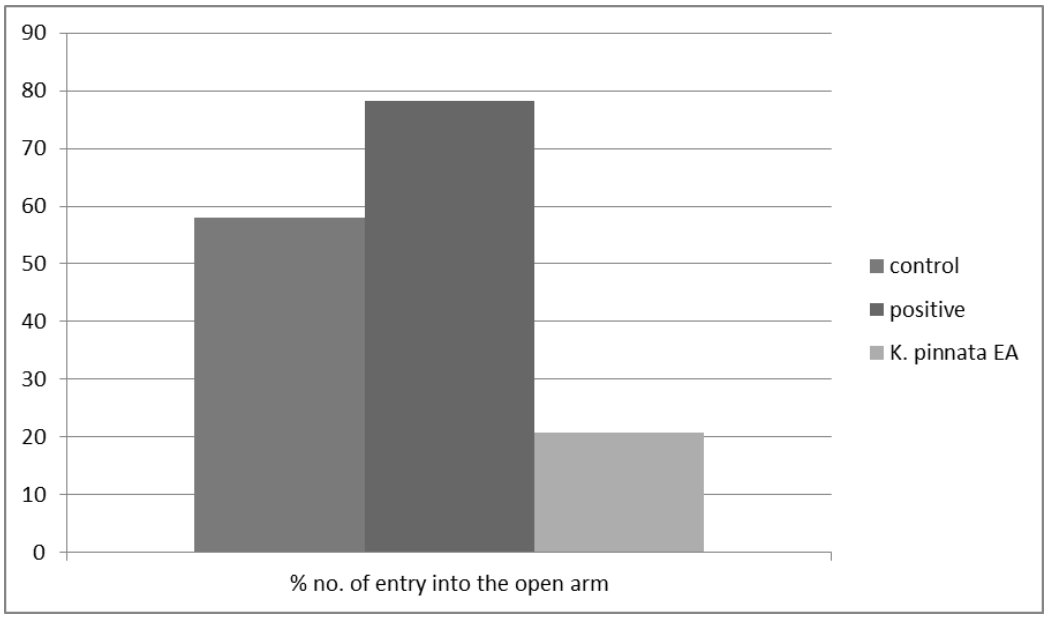

Figure: % number of entry into the open arm

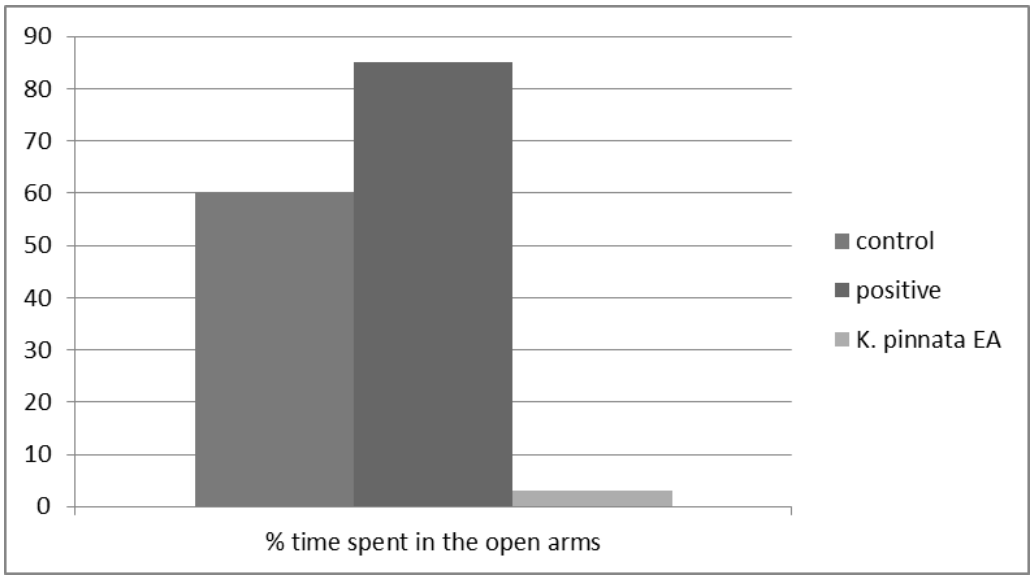

Figure: % time spent by the mice in the open arm

Discussion

Acetic acid induced writhing is the main point for finding new peripherally active analgesic drugs (Hasan *et al*., 2010). Pain sensation in writhing method is due to abdominal constrictions associated with irritation of peritoneal cavity by acetic acid. Prolonged acetic acid induced irritation lead to increase levels of prostaglandins (PGE2 and PGF2α) biosynthesis via cyclooxygenase (COX) and lipoxygenase products in peritoneal fluids followed by increase levels of free arachidonic acid secretion from tissue phospholipid. These increase levels of prostaglandins and lipoxygenase products enhance inflammatory pain by increasing capillary permeability in peritoneal cavity of abdomen (Zulfiker *et al*., 2010; Zakaria *et al*., 2008). The analgesic agents reduced the number of writhing preferably by inhibition of synthesis of prostaglandins and lipoxygenase products (Ferdous *et al*., 2008). The crude extract showed significant analgesic action compared to the reference drug aspirin by reducing the number of acetic acid induced writhing in mice at dose of 400 mg/kg. Thus, the results indicate that the significant pain reduction of the plant extract might be due to the presence of peripherally active analgesic principles. Similarly in chimney, climbing and inclined screen tests, there was a significant loss of coordination and decrease muscle tone in animals treated intraperitoneally

with *k. pinnata* aqueous extract in a dose dependent fashion (Table 2). The treatment with *k. pinnata* extract, is also able to cause a dose related delay of the onset in tonic and picrotoxin, even if it was unable to prevent convulsion, an inhibition of mortality was also observed with 400mg/kg The result of the present study indicates that the crude extract of the *k. pinnata* leaf produced a significant alterations in general behaviour pattern, reduction in spontaneous mortality, potentiation of pentobarbitone-induced sleeping time in a dose dependent fashion. This is similar with the findings of Fujimori (1995) who proposed that the enhancement of barbital hypnosis is a good index of CNS depressant activity (Fujimori, 1995). The extract also produces a significant decrease in exploratory behaviour pattern as evident from the results of head-dip, climbing, and evasion tests. Furthermore the aqueous extract of *K. Pinnata* produces minor anticonvulsant effect by delaying seizure produced by strychnine and picrotoxin. It was however more efficacious against picrotoxinb induced seizure where protection was observed in about one-quarter of mice, an effect which indicates that *B. pinnatum* aqueous extract might produce its central nervous system depressant action as consequence of its GABAergic and less importantly, glycinergic transmission, since picrotoxin is a selective GABAA receptor antagonist (Rang et al 1996) while strychnine antagonizes the inhibitory spinal cord and brainstem reflexes of glycine (Yemitan et al 2001).

Conclusion

The acetic acid leaf extract of *kalanchoe pinnata* showed a significant analgesic activity in writhing test and a beneficial preventive effect in neuropharmacological test(open field test, maze test and hole tet) . However, further study is needed in or der to understand the precise mechanism. Studies with pure active compounds of the extract must be conducted for further pharmacological and toxicological characterization.

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