POTENTIAL ANALGESIC ACTIVITY OF *Alpinia nigra*

A DISSERTATION SUBMITTED TO THE BRAC UNIVERSITY IN PARTIAL FULFILMENT OF THE REQUIRMENTS FOR THE DEGREE OF MASTERS OF SCIENCE IN BIOTECHNOLOGY

SUBMITTED BY

MAHFUZUL KARIM

STUDENT ID: 12376007
SESSION: SUMMER-2012

DEPARTMENT OF MATHEMATICS AND NATURAL SCIENCES
BRAC UNIVERSITY
MOHAKHALI, DHAKA
BANGLADESH

MARCH, 2016

DECLARATION

This is to declare that the research work embodying the results reported in this thesis entitled " Potential Analgesic Activity of *Alpinia nigra*" submitted by Mahfuzul Karim, has been carried out by under joint supervision of Dr. M. Mahboob Hossain, Associate Professor, Biotechnology and Microbiology program, Department of Mathematics and Natural Sciences, BRAC University and Dr. Mohammad Shawkat Ali, Professor of Department of Clinical Pharmacy and Pharmacology, Faculty of Pharmacy, Dhaka University. It is further declared that the research work presented here is original and submitted in the partial fulfillment of the degree of Masters of Science in Biotechnology, BRAC University, Dhaka and has not be submitted anywhere else for a degree or diploma.

Mahfuzul Karim

Certified

Dr. M. Mahboob Hossain
Associate Professor
Mathematics and Natural Sciences
Department
BRAC University
Dhaka, Bangladesh

Dr. Mohammad Shawkat Ali
Professor
Clinical Pharmacy and Pharmacology
Department
Faculty of Pharmacy
Dhaka University
Dhaka, Bangladesh

Acknowledgement

All praises goes to most gracious, most merciful, almighty ALLAH who has given the author the ability to perform this thesis work successfully with sound condition.

The author wishes to express his gratitude to his honorable supervisor Dr. M. Mahboob Hossain, Associate Professor, Biotechnology and Microbiology program, Department of Mathematics and Natural Sciences, BRAC University, Dhaka for his inspiration, valuable opinion, constructive guidance, cordial behavior, valuable criticism and providing necessary facilities to accomplish research works.

The author feels it a proud privilege to express his deepest sense of gratitude, appreciation and indebtedness to his supervisor Dr. Mohammad Shawkat Ali, Professor of Department of Clinical Pharmacy and Pharmacology, Faculty of Pharmacy, Dhaka University, Dhaka for his generosity, materialistic support, careful guardianship, constructive criticism and encouragement throughout the course of the research work.

The author would like to express deep appreciation to, Dr. Mohammad Jafar Imam, Associate Professor, Pharmacy Discipline, Stamford University, Dhaka for paving his way into research area and for his generous cooperation and encouragement and timely assistance.

The author would like to express special thanks to his lab mate Mahmuda Bint Hossain, his friend Md. Imran Hossain and Tapan Kumar Mondol for their help and kind co-operation during this research work.

Finally the author would like to expresses his gratitude to all his well-wishers for their motivation, blessing, continuous inspiration and encouragement.

The Author

Abstract

Alpinia nigra, a plant of Zingiberaceae family, content many important medicinal compounds was studied in three experimental models of nociception. The analgesic activity of crude extracts of acetone-ethanol (1:1) of root, shoot and leaf ware evaluated by using acetic acid induced writhing method, formalin induced licking test and hot plate test on *Swiss albino* mice. In acetic acid induced writhing test, the root extract of *Alpinia nigra* at the doses of 200 mg/kg and 400 mg/kg body weight exhibited 58.2% and 62.2 % inhibition respectively. The shoot extract shows 53.8% inhibition at 200 mg/kg/dose and 60.7% inhibition at 400 mg/kg/dose. Whereas the leaf extract produced 44.0% inhibition at 200 mg/kg/dose and 50.7% inhibition at 400 mg/kg/dose. Morphine 5 mg/kg/dose used as positive control and exhibited 76.0% inhibition compare to control. *Alpinia nigra* caused a significant dose dependent inhibition of both the early (neurological, 0-5 min) and late (inflammatory, 15-30 min) phase of formalin induced licking. However, its antinociceptive effects were more pronounced against the late phase of this model of pain. The calculated licking response inhibitory effect of late phase for root, shoot and leaf were 89.4%, 84.2% and 90.9% at the dose of 200 mg/kg respectively. For 400 mg/kg/dose, licking response inhibition of root, leaf and shoot was 94.8%, 88.3% and 92.2% respectively in late phase. Morphine at the dose of 5 mg/kg per body weight exhibit licking response inhibition 99.2% in late phase. In hot plate test root extract at the dose of 200 mg/kg and 400 mg/kg showed significant increase in the percentage of tolerance time (pain reaction time). The peak effect was seen at 60 minutes, 77.3% and 46.5% of tolerance for 400 mg/kg/dose and 200 mg/kg/dose respectively as comparable to 82.1% at 60 minutes obtained from morphine (5 mg/kg/dose). But leaf extract showed tolerance time 59.1% and 52.2% at 60 minutes for 200 mg/kg and 400 mg/kg respectively. The results of the present study indicate that *Alpinia nigra* has significant analgesic potential, suggesting for the use of this plant for medicinal purposes and may be employed for further investigations.

Contents

List of Tables

List of Figures

Abbreviation

◦C	Degree Celsius
b.w.	Body weight
cm	Centimetre(s)
CNS	Central Nervous System
Conc.	Concentration
e.g	Such as
et al.	and others
g	Gram(s)
hr	Hour(s)
i.p.	Intraperitoneal
ICDDR, B	International Center for Diarrhoeal Disease and Research, Bangladesh
kg	Kilogram(s)
l	Liter(s)
LC_{50}	Lethal Concentration for 50% of the experimental animal
M	Meter(s)
mg	Milligram(s)
min	Minute(s)
ml	Milliliter(s)
p o	per os
sec	Second(s)
WHO	World Health Organisation
µg	Microgram(s)

CHAPTER ONE

INTRODUCTION

CHAPTER 1: Introduction

1.1. Introduction

The plants that have therapeutic properties or can introduce beneficial pharmacological effects on the body of patient are generally regarded as "Medicinal plants". Medicinal plants may be defined as a group of plants that possess some special properties or virtues that qualify them as article of drugs and therapeutic agent and are used for medicinal purposes (Ghani, 2003).

According to WHO consultative group on medicinal plants, "A medicinal plant is any plant which, in one or more of its organs contains, substances that can be used for therapeutic purposes or which, is a precursor for synthesis of useful drugs" (Sofowara, 1982). In our opinion, we should continue recognizing all those plants as medicinal which have been traditionally used over the years and are still being used for therapeutic purposes, some with spectacular reputation, until their efficacy is proved otherwise by scientific analysis and clinical evaluation.

Some generous estimates found that, almost 80% of the present day medicine are directly or indirectly obtained from plants (Myers, 1982). Surprisingly, this large quantity of modern drugs comes from less than 15% of the plants which are known to have been investigated pharmacologically, out of the estimated 500,000 species of higher plants growing on earth (Farnsworth and Morris, 1976). In developing countries including Bangladesh, about 75% of the populations relay on traditional medicines for their Primary health care (Matu and Staden, 2003).

According to recorded history of human civilization, man was well aware of the medicinal properties of many plants growing around him more than 5000 years ago. The earliest mention of the medicinal use of plants is found in the *Rig Veda* (4500-1600 BC), which reported that the Indo-Aryans used the Soma plant as medicinal agent. The famous *Papyrus Ebers* recorded the use of many plants as far that as in 1500 BC. Ayurvedic system of medicine described the use of 127 plants as curative agents as early

as in 1200 BC. The first Chinese Pharmacopoeia containing a list of 135 different medicinal plants with their uses and method of preparation appeared around 1122 BC. More than 400 recipes of plant medicines used in Greek system of medicine were described by Hippocrates around 400 BC. These are the starting materials from which modern medicine gradually evolved to reach the present state of its development (Ghani, 2003). The “De historia stipium” by Leonhart Fuchs (1542) is concerned with vegetable drugs and the primary object of the book was to provide reliable illustrated descriptions by with the plants used in medicine could be correctly identified (Wallis, 1985).

As therapeutic use of plants continued with the progress of civilization and development of human knowledge, scientists endeavored to isolate different chemical constituents from plants, put them to biological and pharmacological test and thus have been able to identify and isolate therapeutically active compounds, which have been used to prepare modern medicines.

The list of the plant derived medicinal substances occurring in modern medicine is very long now. About 100 such drugs of defined structures are in common use today throughout the world and about half of them are accepted as useful drugs in the industrialized countries (Ghani, 1998).

Plants, as part of their metabolic systems, biosynthesize a wide variety of secondary metabolites, most of which are biologically active, and can potentially be used to treat diseases. Bioactive natural products are those chemical compounds produced by living organisms that exert a biological effect on other organisms. This includes therapeutic activity for diseases of humans and animals, toxic activity responsible for causing human and animal disease, and selective, biodegradable toxicity to help combat pests that may adversely affect our endeavors to feed and otherwise service (e.g. protect cotton crops or plantations of timber used for construction, etc.) the human population (Colegate & Moleneux, 2008).

Plant contains compounds having interesting skeletons. Bioactivity guided phytochemical investigation of medicinal plants may yield newer chemical constituents of remarkable therapeutic interest. Occasionally, native lore provides clue to plants with pharmacologic activity. Curare was obtained from a South American plant long used by

natives to prepare arrow poison. *Rawolfia serpentina* was used for a variety of illnesses; only in recent years were its tranqulizing properties recognized in Western medicine and its active principle, reserpine, isolated (Goldstein et al., 1974).

Because of this inherent property of producing bioactive compounds, plants have long been used traditionally in the treatment of various ailments, and a number of ancient medicine systems, e.g. the Ayurvedic, the Unani, the traditional Chinese Medicine (TCM), predominantly rely significantly on the use of these medicinal plants. Plants still offer a great potentiality for drug search. Homatropine (a synthetic tropane alkaloid similar to atropine), syrosingopine (a synthetic derivative of reserpine), chloroquine (a synthetic derivative of quinine), dihydromorphinone, methyldihydromorphinone, oxymorphine, ethyl morphine and N-allylnormorphine (synthetic derivatives of morphine) are some of the example of such synthetic drugs which plants have contributed indirectly. Even in this age of synthetic drugs, there are some naturally occurring drugs, such as the Digitalis glycosides used in cardiac complications and the Catharanthus alkaloids used in cancers, which have no synthetic alternatives. In such cases, plants continue to remain as their principal and only sources (Ghani, 2003).

1.2. Current status of medicinal plants

1.2.1. National status

Medicinal plants constitute an important natural wealth of a country. They play a significant role in providing primary health care services to rural people. They serve as important therapeutic agents as well as important raw materials for the manufacture of traditional and modern medicines. Substantial amounts of foreign exchange can be saved and earned by commercial production of medicinal plants in a country and by exporting them to other countries. In this way indigenous medicinal plants play significant roles in the economy of a country. They constitute a precious natural wealth of a country and contribute a great deal to its health care programs.

Being naturally gifted by a suitable tropical climate and fertile soil, Bangladesh possesses a rich flora of tropical plants. About 5000 species of phanerogams and pteridophytes grow in its forests, jungles, wastelands and road sides as indigenous,

naturalized and cultivated plants. Out of them more than a thousand have been claimed tom possess medicinal and / or poisonous properties, of which 546 have recently been enumerated with their medicinal properties and therapeutic uses (Yusuf et al., 1994). In addition to possessing various medicinal properties, 257 of this medicinal plants have been identified as efficacious remedies for diarrhoeal diseases and 47 for diabetes. Medicinal plants and plant derived drugs play a very important role in the economy of tropical countries. Bangladesh, being one of them and possessing such a rich flora of medicinal plants, should make serious efforts to derive maximum economic benefit from these plants by using them as raw materials for its indigenous drug manufacturing industries (Ghani, 1998).

1.2.2. International status

An enumeration of the WHO from the late 1970s listed 21000 medicinal species (Penso, 1980). However , in China alone 40941 of 26092 native species are used as drugs in Chinese traditional medicine (Duke and Ayensu,1985). It is estimated that the number of plants species used for medicinal purposes in the world is more than 50000 .

How prominently plant derived drugs still feature in modern medicine can be accessed from the following facts:

- In the United States , in 1980 alone , the consumer paid 8 billion dollars for prescriptions drugs in which the active ingredients are still derived from plants (Sofowora, 1982).
- 47% of some 300 million new prescriptions written by physicians in America in 1961 contained, as one or more active ingredients, a drug of natural origin (Farnsworth, 1966).
- More than 47% of all drugs used in Russia are obtained from botanical sources. (Ampofo, 1979).

1.3. Review of *Alpinia nigra*

1.3.1. Taxonomic classification of *Alpinia nigra* (Gaertner) B. L. Burtt:

Kingdom: Plantae

Subkingdom: Tracheobionta

Division: Magnoliophyta

Class: Liliopsida

Subclass: Zingiberidae

Order: Zingiberales

Family: Zingiberaceae

Genus: *Alpinia*

Species: *Alpinia nigra*

Synonyms: *A. allughas* (Retz.) Rosc.

Local name: Jangli Ada, Tara (Chittagong).

Tribal name: Tara (Tanchangya), Kom Hing (Murong).

Other members of genus *Alpinia*:

Alpinia is the largest genus in the ginger family with about 230 species (Kress et al., 2005).

Fig. 1.1. *Alpinia nigra* plant

1.3.2. Distribution of *Alpinia nigra*

It is found to south-east Asia including Bhutan, China, India, Thailand, Burma and Sri Lanka. In India it is found mainly in the hillocks and riversides of northeastern states such as Assam, Mizoram and Tripura. In Bangladesh it is found by the side of the streams, canals and low lying areas (Ghani, 2003).

Fig. 1.2. Different parts of *Alpinia nigra* plant: (a) leave, (b) shoot, (c) root, (d) flower

1.3.3. Morphology of *Alpinia nigra*

Alpinia nigra is a biennial herbaceous plant. It is morphologically characterized by the presence of a rhizome, simple, wide-brim leaves protected by showy bracts, and terminal inflorescences (Qiao et al., 2001). It has a soft, leafy stem about 1.5–3 m high. Leaves are sessile or subsessile, elongated and pointed at the end. Its leaves are single cotyledons, shaped to look like a pike, about 7–9 cm wide and about 20–40 cm long. The fruit is a berry having many seeds, and the pericarp is thin and green when it is young, becoming black and brittle when it gets old (Roy et al., 2012).

1.3.4. Traditional uses of *Alpinia nigra*

The rhizome is used as an aphrodisiac, tonic, diuretic, expectorant, appetizer and analgesic. The rhizome, cooked or raw, has been traditionally acclaimed as a remedy for intestinal infections among the Mizo tribes of north-east India. Experimentally the crude extract was shown to be highly effective against the trematode *Fasciolopsis buski* (Roy et al., 2009).

In most tribal communities the root pounded and mixed with rice whisky is applied to skin for fungal infections, such as ringworm and melasma. The boiled green root is a potent carminative to reduce flatulence or dyspepsia. A root extract is taken daily for the treatment of gastric ulcers, and for the treatment of jaundice by the Chakmas. Its use as an antiinflammatory and analgesic agent has been supported by experiments in mice (Das and Biswas, 2012). Alcoholic extract of the leaves possess good antifungal and antibacterial properties (Dutta et al., 2010).

1.4. Aims and objectives

Since Bangladesh is a country of low economic growth, so scientific exploration and standardization of potential crude drugs is an urgent need to revolutionize our drug sector. Besides, Bangladesh imports a large quantity of pharmaceutical raw materials including medicinal plants and semi processed plant products to produce drugs and medicines. This huge foreign exchange can be saved if the manufacturers utilize the indigenous medicinal plants or their semi-processed products.

Zingiberaceae a medicinally important, ornamental, monocotyledonous family has potential members which possess many bioactive compounds against harmful microbes to deadly diseases like cancer by regulating the different signaling pathway systems. Several compounds have been discovered and found to deliver diversified biological efficacy either in vitro or in vivo against a range of diseases.

In the tribe Alpinieae, among which is found the Species *Alpinia nigra,* is extensively grown in Bangladesh. It is not only an edible vegetable, but also used in folk remedies to treat dyspepsia, gastric disease and insect bites. Previous phytochemical investigation revealed two flavone glycosides, astragalin and kaempferol-3-Oglucuronide, along with three dihydroxypropyl esters from the seed clusters of this plant (Qiao et al., 2000). Many pharmacological studies have reported that these compound possess several biological activities, including, antioxidant, antiprotozoal and Hepatoprotective effects (Karen et al., 2001). However there remains the opportunity to systematically search for every possible biological activity.

Thus the present work was designed to fulfill the following objectives:

- Collection and extraction of different parts of *Alpinia nigra* plant material.
- Investigation of analgesic activity of plant extract by using acetic acid induced writhing method, formalin induced licking test and hot plate test.

CHAPTER TWO

LITERATURE REVIEW

CHAPTER 2: Literature review

2.1. Chemical constituents of *Alpinia nigra*

The major chemical contents are alkaloids, flavonoids, phenols and terpenoids (Nishat et al., 2012). The rhizome yields 0.05% essential oil; of which the chief compounds are: 23.0% caryophyllene oxide, 19.9% geraniol, 19.4% eudesmol and 16.5% citronellyl OAc. Important compounds isolated from the seed cluster are kaempferol-3-O-glucoside (astragalin,), kaempferol-3-O-glucuronide, heptatriacontanoic acid 2, 3-dihydroxypropyl ester, heptatriacontanoic acid 1, 3-dihydroxypropyl ester and pentatriacontanoic acid 1, 3-dihydroxypropyl ester (Qiao et al., 2000). In addition, two major volatile oils, β-pinene and α-pinene have also been isolated from the fruits and rhizomes. From the seed clusters two bioactive flavone glycosides were isolated, astragalin and kaempferol-3-O-glucuronide; and kaempferol-3-O-glucuronide were found to be a dominant compound which was distributed primarily in the pulp (Qiao et al., 2007). The chemical 1,8-cineole is the major component in the leaf essential oil (25.4%) and rhizome oil (34%). In addition, β-pinene (15.1%), camphor (15.3%), carotol (7.3%), α-pinene (7.8%) and camphene (7.0%) were also present in leaf oil, whereas in the rhizome oil α-fenchyl acetate (13.1%), α-terpineol (9.6%), β-pinene (8.1%) and camphene (7.0%) were the other main constituents (Kanjila et al., 2010).

Indrayan et al., (2007) collected the essential oil of the rhizome of *Alpinia officinarum* by hydrodistillation and analysed by means of GLC and GC-MS in apolar and polar columns. Forty-nine compounds representing 99.21% of the oil have been identified. The major constituents of the essential oil of the rhizome are 1,8-cineole (55.39%), Δ^3-carene (8.96%), β-pinene (4.29%), camphene (2.81%), limonene (2.80%), isocaryophyllene (2.52%), camphor (2.35%), α-pinene (2.27%), ϒ-terpinene (2.23%) and ϒ-cadinene (2.17%). The oil shows antibacterial activity against *Staphylococcus aureus* and *Bacillus subtilis* (Gram positive), *Escherichia coli, Klebsiella pneumoniae* and *Salmonella typhi* (Gram negative), and antifungal activity against *Candida albicans*.

Jian et al., (1998) isolated four diarylheptanoids and three flavonols were from the rhizomes of *Alpinia officinarum* and identified as 1, 7-diphenylhept-4-en-3-one, 7-(4"-hydroxy-3"-methoxyphenyl)-l-phenylhept-4-en-3-one, 5-hydroxy-7-(4"-hydroxy-3-methoxyphenyl)-1-phenyl-3-heptanone, 5-methoxy-7 (4"-hydroxy-3 methoxyphenyl)-1-phenyl-3-heptanone, rhamnocitrin, kaempferol-4'-methylether and galangin respectively. All of them (except first compound) appear the antioxidative activities *in vitro* by microassay for measurement of anti-lipid peroxidation which based on the mode of formation of microsomal lipid peroxidation induced by Fe^{2+}/ cysteine and principle of TBA reaction.

Akshaya et al., (2010) collected the essential oil of the rhizome of *Alpinia speciosa* K. Schum and isolated by hydrodistillation and analyzed by GC and GC/MS. Sixty-six compounds representing 95.98% of the oil were identified. The major constituents of the essential oil of the rhizome were terpinen-4-ol (15.4%), 1,8-cineole (11.1%) and T-cadinol (8.8%). The oil showed antibacterial activity against Micrococcus luteus, Streptococcus mutans, Bacillus subtilis, Staphylococcus aureus (Gram-positive), Salmonella typhi and Pseudomonas aeruginosa (Gram-negative) using the well diffusion method.

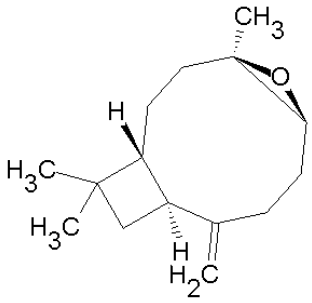

Fig. 2.1. Structure of caryophyllene oxide

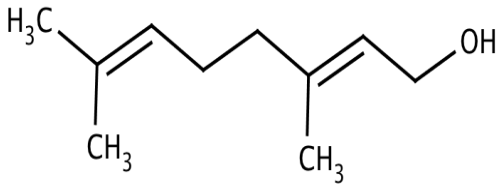

Fig. 2.2. Structure of geraniol

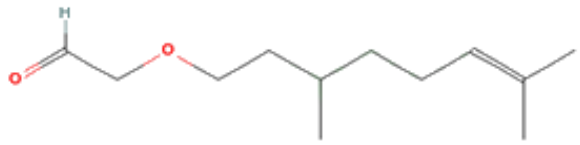

Fig. 2.3. Structure of citronellyl oxyacetaldehyde

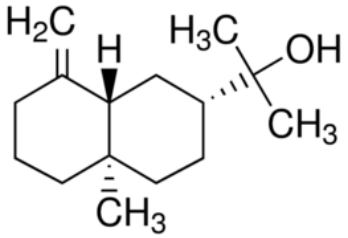

Fig. 2.4. Structure of eudesmol

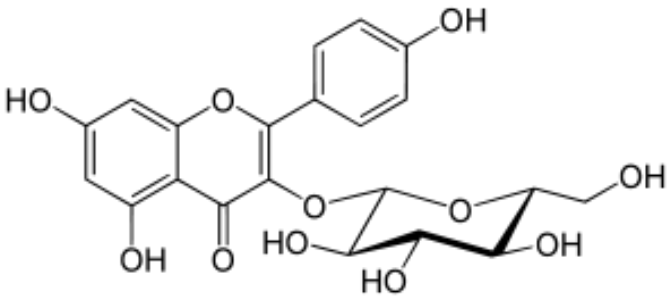

Fig. 2.5. Structure of kaempferol-3-O-glucoside

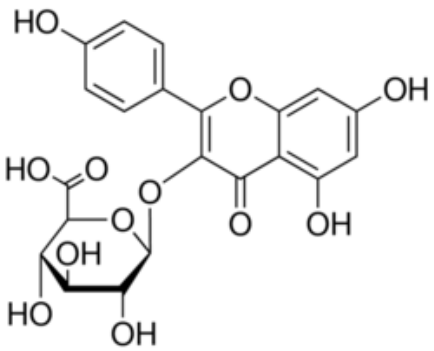

Fig. 2.6. Structure of kaempferol-3-O-glucuronide

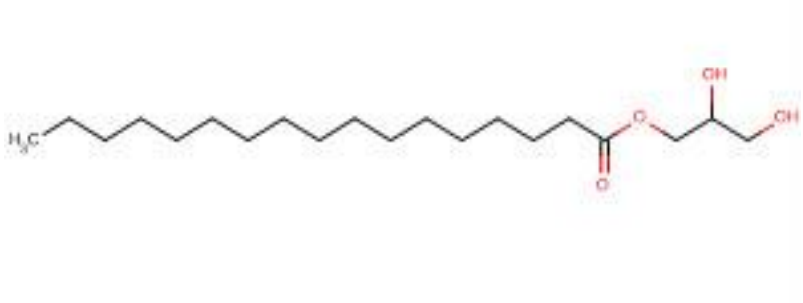

Fig. 2.7. Structure of heptadecanoic acid, 2,3-dihydroxypropyl ester

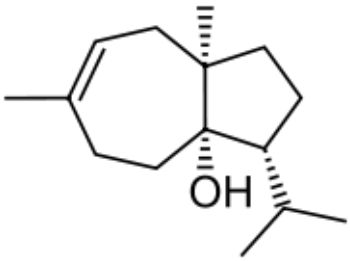

Fig. 2.8. Structure of carotol

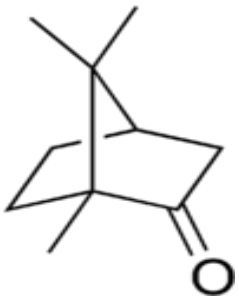

Fig. 2.9. Structure of camphor

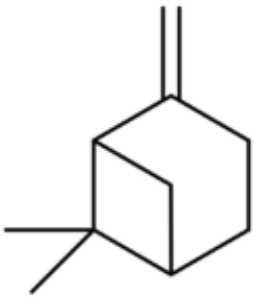

Fig. 2.10. Structure of β-pinene

Fig. 2.11. Structure of α-pinene

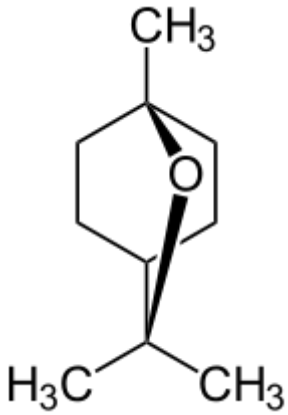

Fig. 2.12. Structure of 1,8-cineole

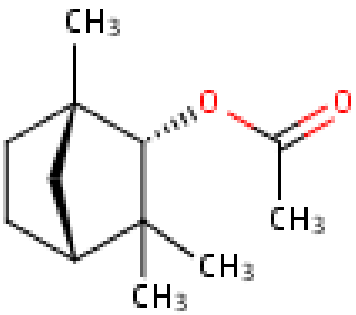

Fig. 2.13. Structure of alpha-fenchyl acetate

2.2. Pharmacological properties of *Alpinia nigra*

2.2.1. Analgesic properties

Das and Qais (2012) found analgesic activity of rhizome extract of *Alpinia nirga* on *Swiss albino* mice in the acetic acid-induced writhing test and radiant heat tail-flick method. The extract in doses of 200 and 400 mg/kg showed 37.31% ($p<0.001$) and 42.97% ($p<0.001$) inhibition of writhing respectively. The crude extract produced 35.75% and 39.98% elongation of tail flicking time 60 minutes after oral doses of 200 and 400 mg/kg body weight respectively.

Arambewelaa et al., (2004) examined the antinociceptive activity of hot water extract (HWE) and hot ethanol extract (HEE) of *Alpinia calcarata* rhizomes using rats and three models of nociception (tail flick, hot plate and formalin tests). Different concentrations of HWE (100, 250, 500, 750, 1000 mg/kg) and HEE (100, 250, 500, 750, 1000 mg/kg) were made and orally administrated to rats and the reaction times determined. The results showed that the extracts have marked dose-dependent antinociceptive activity, when evaluated in the hot plate and the formalin tests but not in the tail flick test. The antinociceptive effect was slightly higher in HEE than that of HWE. The antinociceptive effect was mediated via opioid mechanis

Acharya et al., (2001) evaluated the analgesic effect of extracts of *Alpinia galanga* (AG) rhizome in *Swiss albino* mice in three experimental models of nociception. For the hot-plate test, mice in the five groups with six in each received three different doses of ethanolic extracts of dried rhizome of AG suspended in 2% gum acacia orally, morphine subcutaneously and 2% gum acacia orally, respectively. For the hot-plate test after naloxone pretreatment, mice in the five groups received naloxone subcutaneously 30 min prior to the administration of vehicle or drugs. In the writhing test, writhes were induced by injecting acetic acid intraperitoneally in another 30 mice which were randomly allocated to five groups of six in each and received three different doses of ethanolic extracts of dried rhizome of AG suspended in 2% gum acacia, aspirin suspended in 2% gum acacia and 2% gum acacia orally, respectively. AG treatment significantly increased the latency period in the hot-plate test at all three doses at 30, 60, 90 and 120 min time intervals compared with control group ($P<0.05$ or $P< 0.01$). Naloxone pretreatment

significantly reduced the latency period in hot-plate test for both AG and morphine groups as compared with corresponding groups that did not receive naloxone pretreatment (P<0. 05 or P<0. 01) . AG at all doses significantly reduced the number of writhes compared with control group (P<0. 01).

Sulaiman et al., (2010) evaluated the analgesic and anti-inflammatory activities of the ethanol extract of *A. conchigera* rhizomes in mice and rats. The analgesic activity was elucidated using the acetic acid-induced writhing test, hot plate test, and formalin test, while the anti-inflammatory activity was determined using carrageenan-induced paw edema. The extract (30, 100, and 300 mg/kg) given intraperitoneally (i.p.) exhibited antinociceptive and anti-inflammatory activities in all tests used. The range of percentage of analgesia obtained for all doses of extract in the writhing test was 50–92%, and in the early and late phases of the formalin test was 25–62% and 63–98%, respectively. In addition, naloxone (5 mg/kg) given subcutaneously (s.c.) was found to reverse the extract (300 mg/kg)-induced antinociceptive activity in the writhing, hot plate, and formalin tests.

2.2.2. Anti-inflammatory properties

Das and Biswas (2012) found the extract of the rhizome of *Alpinia nirga* obtained by cold extraction of mixture of equal proportions of petroleum ether, ethyl acetate and methanol have anti-inflammatory activity. Rhizome of *Alpinia nirga* in Albino rats which was assessed by Carrageenin-Induced Paw Edema method. Alpinia nirga at doses of 200 and 400 mg/kg, caused significant inhibition of paw edema by 27.13% (p<0.001) and 34.97% (p<0.001) respectively, 4 hour after carrageenan administration.

Lee et al., (2009) tested the anti-inflammatory effects of *Alpinia officinarum* rhizomes on acute and chronic arthritis in SD rats. An 80% ethanolic extract showed acute anti-inflammatory activity that it reduced the edema volume in carrageenan-stimulated arthritis and inhibited NO generation in LPS-induced RAW 264.7 cells. In addition, this extract showed chronic anti-rheumatic and analgesic activities by suppressing the swelling volume, by recovering the paw withdrawal latency, and by inhibiting the

flexion scores in CFA-induced arthritis. Particularly, this medicine had potent meaningful effects on the second signal of the left hind paw in the form of an immunological reaction compared to its effects on the first signal in the right hind paw after the CFA treatment. This also shows an anti-psychiatric effect through control of the expression of the c-Fos protein of the brain hippocampus in CFA-stimulated arthritis. On the other hand, each fraction showed acute anti-inflammatory effects; the action of the EtOAc fraction may have resulted from the suppression of NO production.

Arawwawalaa et al., (2012) investigated the anti-inflammatory potential of *Alpinia calcarata* rhizomes using hot water extract (AWE) and hot ethanolic extract (AEE) by the carrageenan-induced paw oedema model in rats. All the tested doses of AWE and AEE (250, 500, 750, and 1000 mg/kg) produced a significant ($P \leq 0.05$) inhibition of the inflammation, most pronounced at 4 h after the injection of carrageenan. The anti-inflammatory effect induced by 500 mg/kg of AEE was superior than the reference drug, indomethacin at 4 h. Inhibition of histamine and prostaglandin synthesis is probable mechanisms by which *Alpinia calcarata* mediates its anti-inflammatory action.

Ghosh et al., (2011) evaluated the acute and chronic anti-inflammatory activities of root extract of *Alpinia galanga* in rodents. An extract of the root of *A. galanga* was prepared using absolute alcohol and distillation in a Soxhlet apparatus. The acute anti-inflammatory effects of this extract were evaluated using carrageenan-, bradykinin-, and 5-HT-induced rat paw edema. The chronic anti-inflammatory effects were evaluated using formaldehyde-induced rat paw edema. Inhibition of inflammation was seen to be 32.22% in carrageenan-induced, 37.70% in 5-HT-induced, and 35.21% in bradykinin-induced anti-inflammatory models. In chronic inflammatory model, a progressive inhibition of 34.73% (3 rd day), 37.50% (5 th day), 38.83% (7 th day), 44.66% (9 th day), 49.59% (11 th day), and 55.75% (13 th day) was observed with study compound. The efficacy was comparable with the standard drugs.

Ling et al., (2010) investigated the anti-inflammatory and analgesic effects of essential oil of fructus *Alpiniae zerumbet* in mice. The inflammatory models were produced by xylene, the ear edema and capillary permeability were assayed in mice. The analgesic effects were evaluated by writhing response induced by acetic acid and hot-plate procedure in mice. There were significantly analgesic effects of essential oil of fructus

Alpiniae zerumbet, and it could significantly inhibit ear edema and skin capillary permeability induced by xylene, there was significant difference compared with control group.

Rahman et al., (2012) analyzed essential oil isolated from *Alpinia calcaratawas* by using GC-MS on a combined GC-MS instrument. For evaluation of the anti-inflammatory property "carrageenan induced paw edema model" served as acute model. "Acetic acid induced writhing response model" was used to assess analgesic activity in mice. The major components of essential oils isolated from *Alpinia calcarata* were Camphene (3.86%), Betamyrcene (4.39%), Eucalyptol (14.05%), Linalol (2.48%), Pyrazine (1.72%), L-camphor (7.90%) and Berneol (5.67%). Intraperitoneal injection of essential oil isolated from *Alpinia calcarata* significantly ($P<0.05$) suppressed the paw edema induced by carrageenan in two different dose levels studies namely 400 mg/kg and 380 mg/kg. Intraperitoneal injection of essential oil also significantly attenuated the acetic acid induced writhing response in three different dose levels studies namely 200 mg/kg (significant at $P<0.05$), 400 mg/kg (significant at $P<0.01$) and 600 mg/kg (significant at $P<0.01$).

2.2.3. Anti-helmintic properties

Roy et al., (2009) tested ethanolic shoot extract of *Alpinia nigra*, *in vitro* to determine its antihelmintic efficacy in gastrointestinal trematode *Fasciolopsis buski*, using alterations in the activity of vital tegumental enzymes viz. acid phosphatase (AcPase), alkaline phosphatase (AlkPase) and adenosine triphosphatase (ATPase). Live adult *F. buskitreated in vitro* with different concentrations of the plant extract showed significant decline in the visible stain histochemically and enzyme activities. Quantitatively, the total enzyme activity of AcPase, AlkPase and ATPase was found to be reduced by 45, 41 and 43%, respectively compared to the control.

2.2.4. Thrombolytic properties

Mannan et al., (2011) investigated the thrombolytic activity, synergistic activity on thrombolysis in the aqueous extract of leavs of *Alpinia zerumbet, Alpinia nigra* and

Urena sinuata. Aqueous extract of *Alpinia zerumbet, Alpinia nigra* and *Urena sinuata* showed 43.85%, 42.83%, 26.40% thrombolytic activity and also showed 37.14%, 60.00%, 26.83% synergistic activity with streptokinase respectively. On the other hand streptokinase alone showed 81.20% thrombolytic activity. Among the plant extracts studied *Alpinia nigra* showed significant % of synergistic activity (60%) with compared to other plant extracts. *Alpinia nigra* extract showed synergistic activity with streptokinase on thrombolysis. *Alpinia zerumbet* and *Alpinia nigr*a also showed reasonable clot lytic property.

2.2.5. Antimicrobial properties

Das et al., (2012) investigated crude methanolic extract of rhizome of *Alpinia nigra* for its antibacterial and cytotoxic activities. Antibacterial activity of the extract was evaluated against various Gram-positive and Gram-negative bacteria using disk diffusion technique. For cytotoxic activity, brine shrimp lethality bioassay was performed to estimate LC50 values. The n-hexane, carbon tetrachloride, and dichloromethane soluble fractions of the crude methanolic extract of *Alpinia nigra* were subjected to antibacterial activity and brine shrimp lethality bioassay. The carbon tetrachloride soluble partitionate of the methanol extract exhibited mild to moderate antibacterial activity and strong cytotoxicity having LC50 of 0.832 μg/mL

Dutta and Sinha (2010) observed antimicrobial activity of some selected plants viz.; *T amarindus indica* L, *Emblica officinalis* Gaertn. Linn, *Alpinia nigra* (L) willd, *Azadiracta indica* A.Juss, *Cynodon dactylon* (L) Pers, *Dipteris wallichi* L, *Curcuma longa* L, *Chromolena odoratum* L, *Blechnum orientales, Centella asiatica* (L) urban. Methanol and Acetone extract of these plants were used against *E. coli, Staphyllococus sp, Klebsiella sp, Candida sp, Aspergillus sp,* using disc diffusion method. Zone of inhibition of Acetone extract of *Alpinia nigra* (10.0 mm) against *Candida sp, Azadiracta indica* (10.0 mm) against *Aspergillus sp, Apinia nigra* (12.5 mm) against *E. coli,* showed high antimicrobial activity compared to other plant extracts. For Methanol extract, the highest zone of inhibition was observed in *Azadiracta indica* (20.3 mm, 12.9 mm) against *Candida sp* and *Aspergillus sp,* respectively. Whereas *Centella asitica* (15.0 mm) showed highest zone of inhibition against *E. coli* and showed no zone of inhibition

against *Candida sp*. For *Staphyllococcus sp* the zone of inhibition was found average ranging from (6.0 mm to 9.8 mm) in both Acetone and methanol extract. In case of *Klebsiella sp* the highest zone of inhibition was found in case of *Alpinia nigra* (10.0 mm, 1 1.2 mm) for Methanol and Acetone extract.

2.2.6. Anti diarrheal properties

Chunping et al., (2006) studied the mechanism of *Alpiniac Officinarum* Rhlizoma (AOR) extracts on the ileum myoelectric activity of rabbits *in vitro*. The samples of isolated ileum segment were prepared, the contraction intensity were measured. Effects of RAO extracts on the contraction induced by BaCl_2, histamine and actions on 2-component contraction evoked by ACh in the rabbit ileum were studied. AOR extracts could significantly inhibit spontaneous constriction of ileum, exert an inhibitory effect on the contraction induced by BaCl_2, histamine. It also inhibited the inflow of incellular Ca~2+ induced by ACh. AO Rextracts could restrain the contraction on the rabbit ileum, this effect could be related to Ca~2+ channel, M-receptor, H-receptor and direct action.

2.2.7. Antiproliferative properties

Omoregie et al., (2013) assessed the potency of the extracts from lesser galangal, turmeric, and ginger against AML M5 to use the suitable fractions in neutraceuticals. Aqueous and organic solvent extracts from the leaves and rhizomes of lesser galangal and turmeric, and from the rhizomes only of ginger were examined for their antiproliferative activities against THP-1 AMoL cells *in vitro*. The turmeric leaf and rhizome extracts and ginger rhizome extracts in methanol also showed distinctive anticancer activities. The lesser galangal leaf methanol extract was subsequently separated into 13, and then 18 fractions using reversed-phase high-performance liquid chromatography. Fractions 9 and 16, respectively, showed the greatest antiproliferative activities. These results indicate that the use of plant extracts might be a safer approach to finding a lasting cure for AMoL. Further investigations will be required to establish the discriminatory tolerance of normal cells to these extracts, and to identify the compounds in these extracts that possess the antiproliferative activities.

CHAPTER THREE

MARERIALS AND METHODS

CHAPTER 3: Materials and methods

3.1. Working place

The experiments of the investigations were carried out at the laboratories of the Department of Pharmacy, BRAC University and Department of Pharmacy, Stamford University Bangladesh from June, 2013 to May, 2014.

3.2. Plant material

Alpinia nigra (Gaertner) B.L. Burtt. was selected for investigations and roots, shoots and leaves were used for research purpose.

3.3. Preparation of plant extract for experiments

3.3.1. Collection of the plant

The plant *Alpinia nigra* was collected from Savar, Dhaka district during last week of April, 2013.

3.3.2. Identification of the plant

The collected plant was identified by the taxonomist of National Herbarium, Mirpur, Dhaka, Bangladesh (Accession No. 38367) where a voucher specimen has been deposited for further reference.

3.3.3. Drying of the plant samples

The roots, shoots and leaves were washed with water and other adulterants were removed to get fresh sample. Then the collected samples were dried for seven days under shade at room temperature. Before drying, the samples were cut into small pieces as necessary.

3.3.4. Grinding of the dried samples

The dried samples were grounded to coarse powder with a mechanical grinder (Grinding Mill) and powdered samples were kept in clean closed glass containers for extraction. During grinding of sample, the grinder was thoroughly cleaned to avoid contamination with any other substance that was grounded previously. The dried grinded powder was weighed by rough balance.

3.3.5. Extraction of the dried powered samples

In this study cold extraction procedure was employed with the mixture of equal volume of acetone (500 ml) and ethanol (500 ml).

About 200 g powder of shoots, 200 g powder of leaves and 400 g powder of roots were taken into separate clean, flat-bottomed glass container and soaked in 500 ml and 1000 ml of 50% mixture of acetone and ethanol respectively. The container with its contents was sealed and kept for a period of 7 days accompanying occasional shaking and stirring. The whole mixture then underwent a coarse filtration by a piece of clean, white cotton material. Then it was filtered through Whatman 41 filter paper. The solvent was completely removed by rotary evaporator and crude extract was dried in waterbath. These crude extracts were used for investigations.

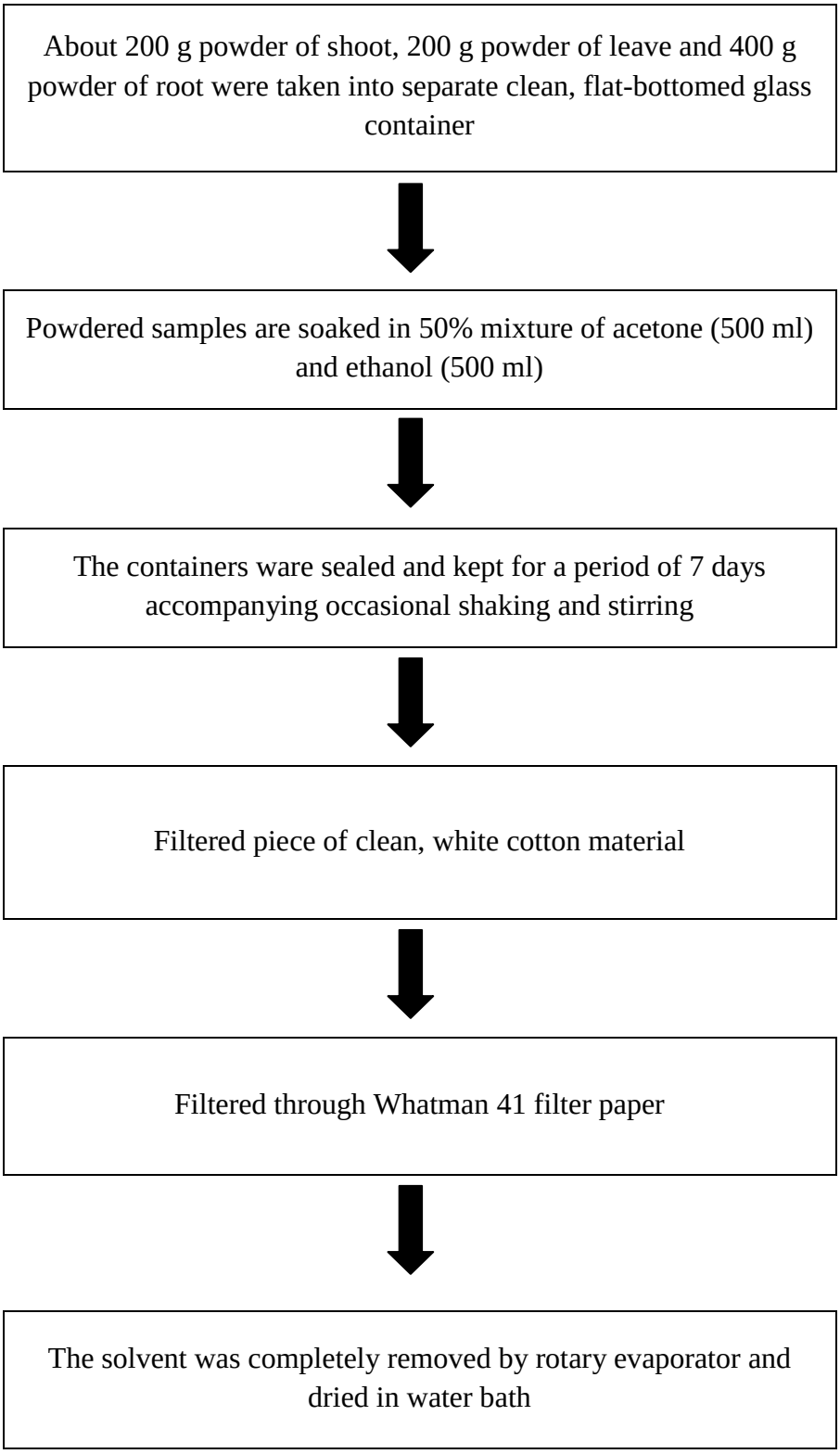

Fig. 3.1. Schematic representation of procedure for extraction of the dried powered samples of *Alpinia nigra*.

3.4. Pharmacological investigations of *Alpinia nigra*

3.4.1. Analgesic activity

Pain has been officially defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage. Pain acts as a warning signal against disturbances of the body and has a proactive function (Tripathi, 1999).

Analgesic means a drug that selectively relieves pain by acting in the CNS or on peripheral pain mechanisms, without significantly altering consciousness. So, analgesic activity means capacity of a substance to neutralize the pain sensation (Rang, 1991).

Analgesic drugs which are currently in use are either narcotics or non-narcotics which have proven side and toxic effects. To develop new synthetic compounds in this category is an expensive venture and again may have problems of side effects. On the contrary, many medicines of plant origin had been used and are in use successfully since long time without any serious effects.

3.4.2. Test for analgesic activity

The study of analgesic activity of the extract was performed in animal models for both central and peripheral mechanism of pain. For the screening of analgesic activity against peripheral mechanism of pain acetic acid induced writhing test and formalin induced paw lick tests were considered. On the other hand, to evaluate the analgesic activity against centrally mediated pain the hot-plate test was used.

Three models using albino mice were employed to study the analgesic effects:

1. Acetic acid induced writhing response (Koster et al., 1959)
2. Formalin-induced nociception test (Santos et al., 1997)
3. Hot-plate test (Eddy et al., 1953).

3.4.3. Experimental animal

For the experiment *Swiss albino* mice of 3-4 weeks of age, weighing between 20-25 gm, were collected from the Animal Research Branch of the International Center for Diarrheal Disease and Research, Bangladesh (ICDDR, B). Soft wood shavings were used as bedding of cages. Animals were maintained under standard environmental conditions (temperature: 24.0±1.0 ˚C, relative humidity: 55-65% and 12 hrs light/12 hrs dark cycle). Husk and excreta were removed from the cages every day. Pellets of mice foods, provided by ICDDR, B were given to the mice with water, ad libitam. The newly bought mice were given a week rest to get over the food and water restrictions incurred during transit and to get themselves adapted with the new environment of the laboratory, before being employed in any experiment (Hasan et al., 2009).

Fig. 3.2. *Swiss albino* Mice.

3.4.3.1. Identification of animals during experiment

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

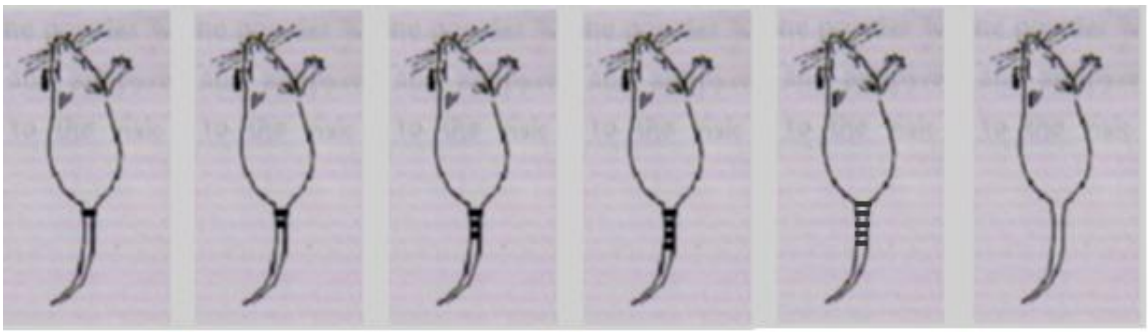

Fig. 3.3. Identification mark of mice.

3.4.4. Preparation of the test materials

3.4.4.1. Preparation of sample and standard

The sample was prepared by dissolving the extract of root, shoot and leaf of *Alpinia nigra* in normal saline with the help of small amount of Tween-80 (a suspending agent) for two doses (200 mg/kg body weight and 400 mg/kg body weight). 90 mg of the extract were measured and triturated by the addition of small amount of Tween-80. After proper mixing of extract and suspending agent, normal saline was slowly added. The final volume of the suspension was made up to 9 ml. To stabilize the suspension, it was stirred well by vortex mixture. From this solution 0.5 ml for 200 mg/kg dose and 1 ml for 400 mg/kg dose were used.

Morphine 10 mg/ml Carpuject was used as positive control. For the preparation of Morphine at the dose of 5 mg/kg body weight, 1 ml was taken and a suspension of 8 ml was made with normal saline. From this solution 0.1 ml was used per dose.

Fig. 3.4. Preparation of the solution of plant extract with the help of vortex apparatus.

3.4.4.2. Drugs and chemicals

Table 3.1. List of drugs and chemicals used for the analgesic activity test.

Serial No.	Drugs and Chemicals	Purpose	Source
1	Tween 80	Suspending agent & Vehicle	Sigma Aldrich
2	Saline	Vehicle	Opso saline
3	Morphine	Positive Control (Standard)	ACE Surgical Supply Company
4	Formalin	Writhing inducer	Sigma Aldrich
5	Acetic acid	Writhing inducer	Sigma Aldrich

3.4.5. Analgesic activity test by acetic acid induced writhing test

3.4.5.1. Mechanism of writhing test

Acetic acid is a pain stimulus. Intraperitoneal administration of acetic acid (0.7%) causes localized inflammation. Such pain stimulus causes the release of free arachidonic acid from tissue phospholipids by the action of phospholipase A_2 and other acyl hydrolases.

There are three major pathways in the synthesis of the eicosanoids from arachidonic acid. All the eicosanoids with ring structures, which is the prostaglandins, thromboxanes and prostacyclines, are synthesized via the cyclooxygenase pathway. The released prostaglandins, mainly prostacyclines (PGI_2) and prostaglandin-E have been reported to be responsible for pain sensation by exciting the Aδ-fibers. Activity in the A δ-fibers cause a sensation of sharp well localized pain (Rang et al., 1993).

Morphine used as the positive control in this method, acts by inhibition of prostaglandin synthesis. Any agent that lowers the number of writhing will demonstrate analgesia by inhibition of prostaglandin synthesis.

3.4.5.2. Experimental design of acetic acid induced writhing test

The acetic acid induced writhing method is an analgesic behavioral observation assessment method that demonstrates a noxious stimulation in mice. The test consists of injecting the 0.7% acetic acid solution intraperitoneally and then observing the animal for specific contraction of body referred as ‘writhing’ (Koster et al., 1959; Vogel and Vogel, 1997; Ahmed et al., 2001). The animals were divided into control, positive control and test groups with six mice in each group. The animals of test groups received test samples at the doses of 200 and 400 mg/kg body weight. Positive control group received standard drug morphine at the dose of 5 mg/kg body weight and vehicle control group was treated with 1% Tween 80. Test samples and vehicle were administered orally 30 min before intraperitoneal administration of 0.7% acetic acid but morphine was administered 15 min before injection of acetic acid. After an interval of 5 min, the mice were observed for specific contraction of body referred to as ‘writhing’ for the next 10 min (Meera et al., 2008).

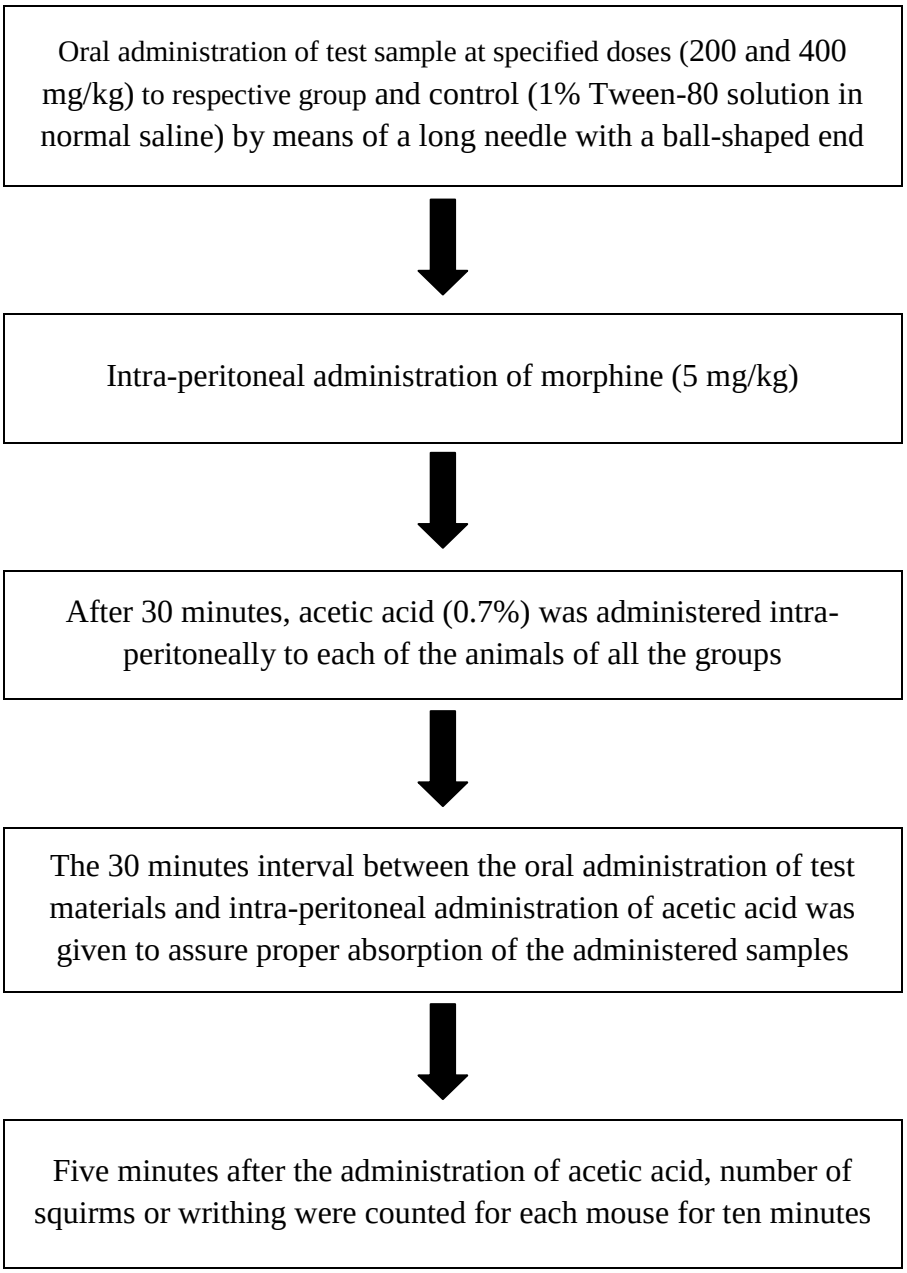

Fig. 3.5. Schematic representation of procedure for screening of analgesic property on mice by acetic acid induced method for *Alpinia nigra* extract.

Table 3.2. Experiment profile to assess the effect of crude extract of different parts of *Alpinia nigra* in acetic acid induced writhing test.

Animal Group	Treatment	No. of Animals	Dose	Route of Administration
Control	1% tween 80 in normal saline	6	10 ml/kg	P.O.
Positive Control (Standard)	Morphine	6	5 mg/kg	I.P.
Group I	Root extract of *Alpinia nigra*	6	200 mg/kg	P.O.
Group II	Root extract of *Alpinia nigra*	6	400 mg/kg	P.O.
Group III	Shoot extract of *Alpinia nigra*	6	200 mg/kg	P.O.
Group IV	Shoot extract of *Alpinia nigra*	6	400 mg/kg	P.O.
Group V	Leaf extract of *Alpinia nigra*	6	200 mg/kg	P.O.
Group VI	Leaf extract of *Alpinia nigra*	6	400 mg/kg	P.O.

Fig. 3.6. Oral administration of test sample.

Fig. 3.7. Intraperitoneal administration of acetic acid.

3.4.6. Analgesic activity test by formalin induced licking test

The formalin test in mice is a valid and reliable model of nociception and is sensitive for various classes of analgesic drugs. The procedure used was essentially the same as that described by Santos et al., 1997 and Santos et al., 1999. The animals were divided into control, positive control and test groups with six mice in each group. Mice were orally pretreated with test sample (200, 400 mg/kg, p.o.) and the control group received vehicle (1% Tween-80 solution in normal saline) 60 min prior the nociceptive agent. Morphine (5 mg/kg) was used as the reference drug and administered 15 min before the nociceptive agent. The noxious stimulus is an injection of dilute formalin (1%) under the skin of the dorsal surface of the right hind paw. The response involves moderate, continuous pain generated by injured tissue. In this way it differs from most traditional tests of nociception which rely upon brief stimuli of threshold intensity. The response to formalin shows an early and a late phase and the amount of time the animals spend licking the injected paw. Two distinct periods of high licking activity can be identified. Animal's received 20 µl of a 2.5% formalin solution made up in distilled water, injected intraplantarly in the ventral surface of the right hind paw. Animals were observed from 0 to 5 min (neurogenic phase) and 15–30 min (inflammatory phase) and the time spent licking the injected paw was recorded and considered as indicative of nociception.

3.4.6.1. Experimental design of formalin induced licking test

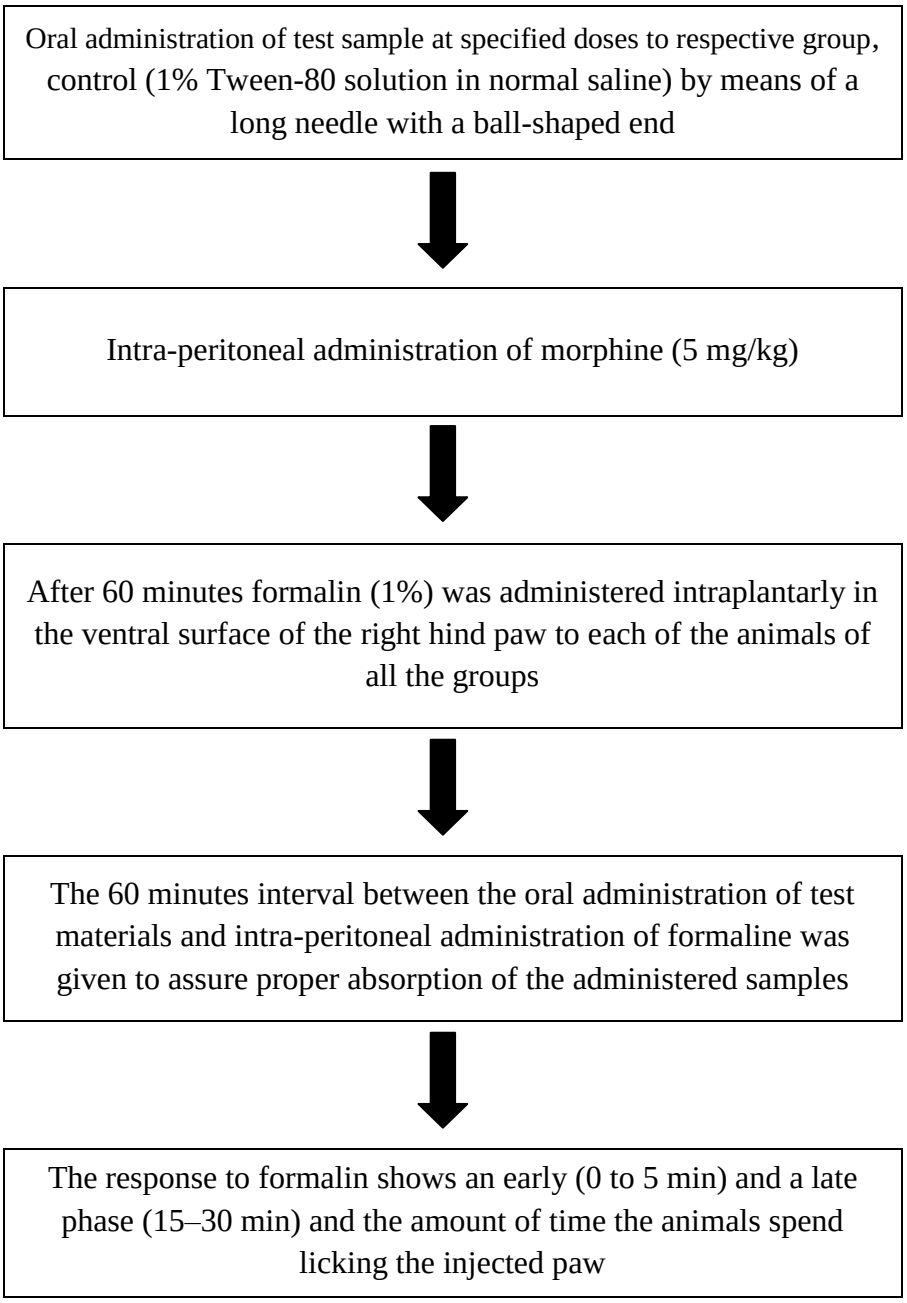

Fig. 3.8. Schematic representation of procedure for screening of analgesic property on mice by formalin induced licking test for *Alpinia nigra* extract.

Table 3.3. Experiment profile to assess the effect of crude extract of different parts of *Alpinia nigra* in formalin induced licking test.

Animal Group	Treatment	No. of Animals	Dose	Route of Administration
Control	1% tween 80 in normal saline	6	10 ml/kg	P.O.
Positive Control (Standard)	Morphine	6	5 mg/kg	I.P.
Group I	Root extract of *Alpinia nigra*	6	200 mg/kg	P.O.
Group II	Root extract of *Alpinia nigra*	6	400 mg/kg	P.O.
Group III	Shoot extract of *Alpinia nigra*	6	200 mg/kg	P.O.
Group IV	Shoot extract of *Alpinia nigra*	6	400 mg/kg	P.O.
Group V	Leaf extract of *Alpinia nigra*	6	200 mg/kg	P.O.
Group VI	Leaf extract of *Alpinia nigra*	6	400 mg/kg	P.O.

3.4.7. Analgesic activity test by hot plate test

The hot plate test is a test of the pain response in animals which measure the response latencies based on the procedure described by Eddy and Leimbach in 1953. It is used in basic pain research and in testing the effectiveness of analgesics by observing the reaction to pain caused by heat. The test is a behavioral model of nociception where behaviors such as jumping and hind paw-licking are elicited following a noxious thermal stimulus. Licking is a rapid response to painful thermal stimuli that is a direct indicator of nociceptive threshold. Jumping represents a more elaborated response, with a latency, and encompasses an emotional component of escaping (Espejo et al., 1993). In these experiments, the hot plate chamber temperature was maintained at 50±0.5°C. The reaction time was recorded for animals pretreated with vehicle (1% Tween-80 solution in normal saline) at 10 ml/kg, 30 min before as control and test sample (200, 400 mg/kg, p.o.). Morphine (5 mg/kg) was used as a positive control group. After placing the animals into the hot plate chamber, when animal licked its backed paw or jumped off to avoid thermal pain, the time of latency period was calculated. The latent period of response was taken as the index of antinociception and was determined as pretreatment, 30, 60, 90, 120 min after the administration of test drugs and standard. In order to minimize the damage on the animal paws, the cut-off time was taken as 25 seconds.

3.4.7.1. Experimental design of hot plate test

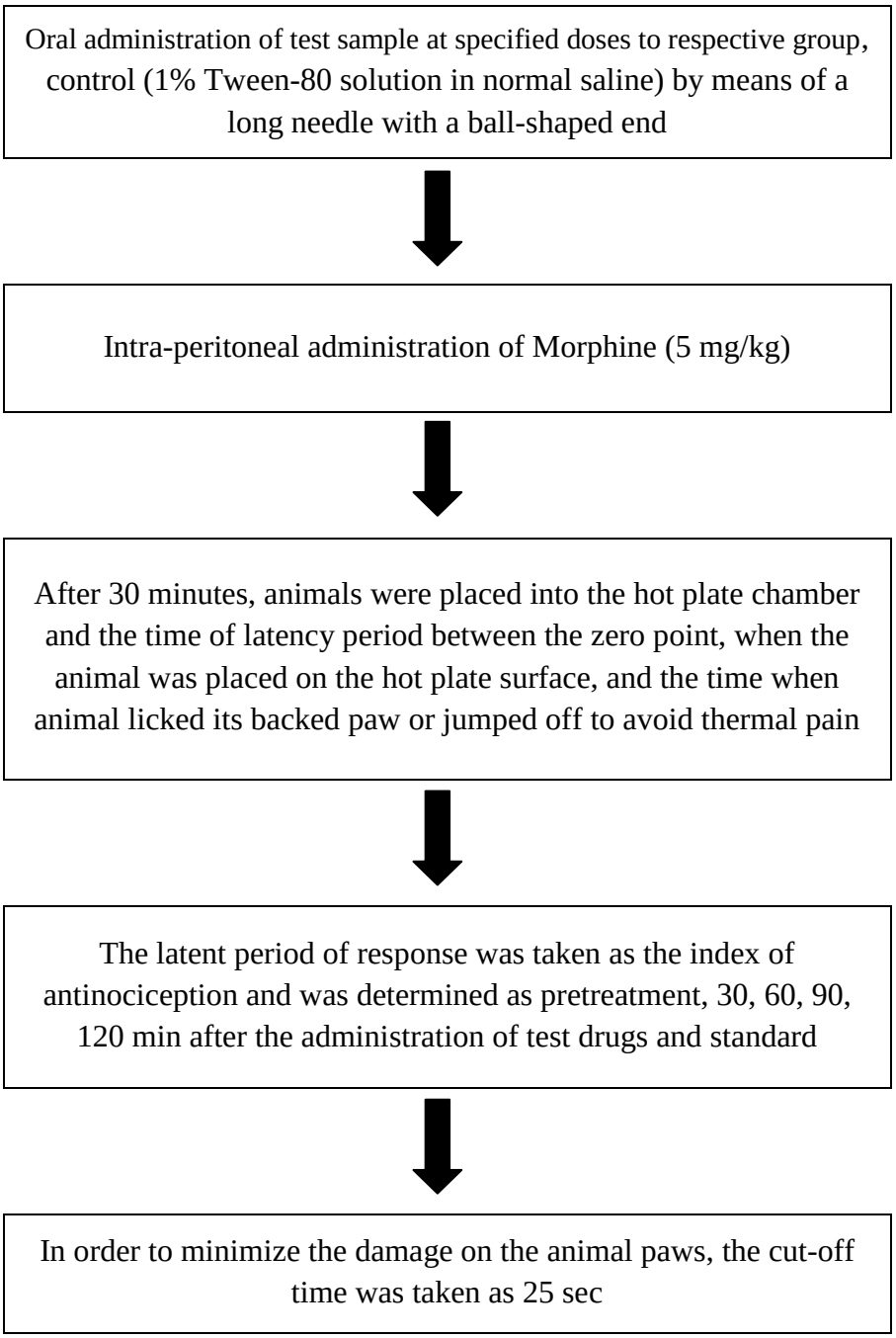

Fig. 3.9. Schematic representation of procedure for screening of analgesic property on mice by hot plate test for *Alpinia nigra* extract.

Table 3.4. Experiment profile to assess the effect of crude extract of different parts of *Alpinia nigra* in Hot Plate Test.

Animal Group	Treatment	No. of Animals	Dose	Route of Administration
Control	1% tween 80 in normal saline	6	10 ml/kg	P.O.
Positive Control (Standard)	Morphine	6	5 mg/kg	I.P.
Group I	Root extract of *Alpinia nigra*	6	200 mg/kg	P.O.
Group II	Root extract of *Alpinia nigra*	6	400 mg/kg	P.O.
Group III	Leaf extract of *Alpinia nigra*	6	200 mg/kg	P.O.
Group IV	Leaf extract of *Alpinia nigra*	6	400 mg/kg	P.O.

CHAPTER FOUR

RESULTS

CHAPTER 4: Results

4.1. Investigation of analgesic activity by acetic acid induced writhing test

Table 4.1. Effect of root extract of mixture of equal proportions of acetone and ethanol of *Alpinia nigra* on acetic acid induced writhing.

Group	Writhing Count						Mean	SD	SEM	% of Writhing	% of Inhibition
	M1	M2	M3	M4	M5	M6					
Control Group	45	44	59	38	39	45	45.0	7.51	3.07	100.00	0.00
Positive Control	9	13	11	14	8	10	10.8	2.32	0.95	24.00	76.00
Group-1	22	14	12	26	21	18	18.8	5.23	2.14	41.78	58.22
Group-2	19	14	11	21	18	19	17.0	3.74	1.53	37.78	62.22

Control: 1% tween 80 in saline
Positive Control: Morphine (5 mg/kg)
Group-1: Root Extract (200 mg/kg)
Group-2: Root Extract (400 mg/kg)

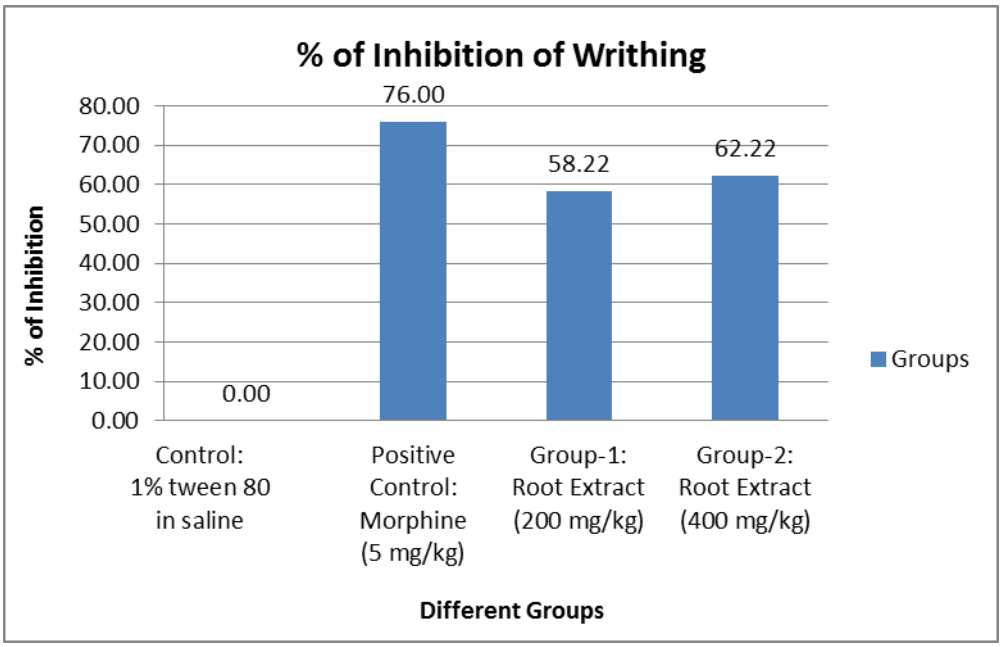

Fig. 4.1. Graphical presentation of the effect of root extract (acetone-ethanol, 1:1) of *Alpinia nigra* on mice by acetic acid induced writhing test.

Table 4.2. Effect of shoot extract of mixture of equal proportions of acetone and ethanol of *Alpinia nigra* on acetic acid induced writhing.

Group	Writhing Count						Mean	SD	SEM	% of Writhing	% of Inhibition
	M1	M2	M3	M4	M5	M6					
Control Group	45	44	59	38	39	45	45.0	7.51	3.07	100.00	0.00
Positive Control	9	13	11	14	8	10	10.8	2.32	0.95	24.00	76.00
Group-1	19	22	16	14	26	28	20.8	5.53	2.26	46.22	53.78
Group-2	15	18	13	22	17	21	17.7	3.44	1.41	39.33	60.67

Control: 1% tween 80 in saline
Positive Control: Morphine (5 mg/kg)
Group-1: Shoot Extract (200 mg/kg)
Group-2: Shoot Extract (400 mg/kg)

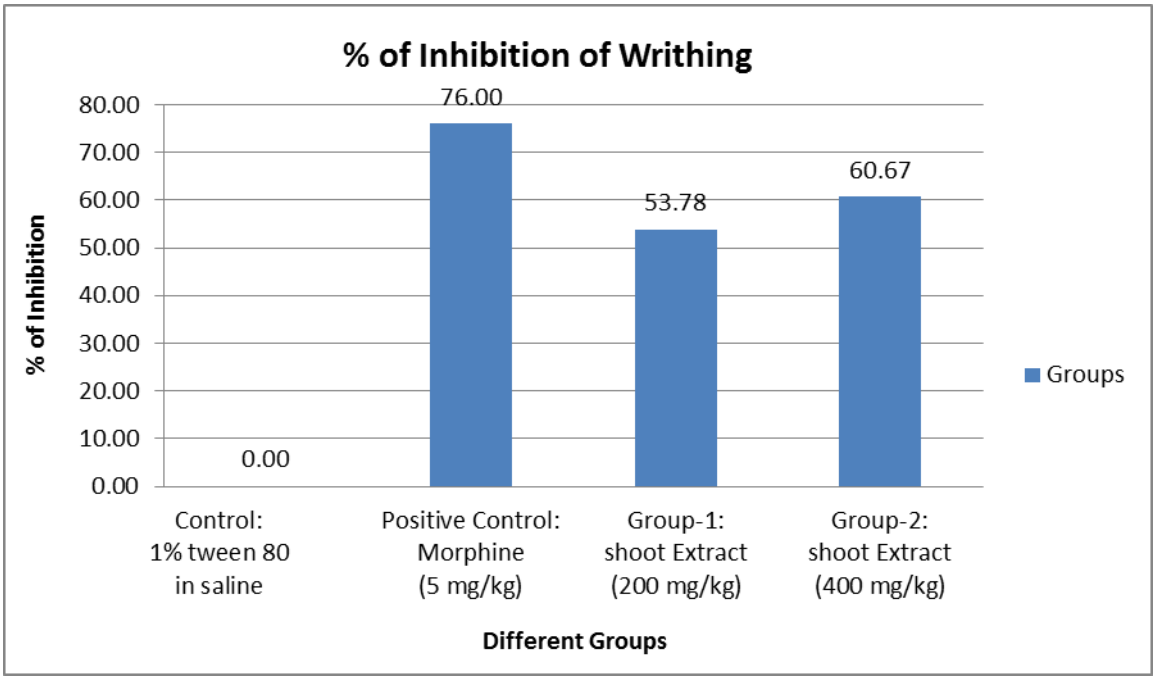

Fig. 4.2. Graphical presentation of the effect of shoot extract (acetone-ethanol, 1:1) of *Alpinia nigra* on mice by acetic acid induced writhing test.

Table 4.3. Effect of leaf extract of mixture of equal proportions of acetone and ethanol of *Alpinia nigra* on acetic acid induced writhing.

Group	Writhing Count						Mean	SD	SEM	% of Writhing	% of Inhibition
	M1	M2	M3	M4	M5	M6					
Control Group	45	44	59	38	39	45	45.0	7.51	3.07	100.00	0.
Positive Control	9	13	11	14	8	10	10.8	2.32	0.95	24.00	76.00
Group-1	18	28	26	19	31	29	25.2	5.42	2.21	56.00	44.00
Group-2	21	24	23	14	18	33	22.2	6.43	2.63	49.33	50.67

Control: 1% tween 80 in saline
Positive Control: Morphine (5 mg/kg)
Group-1: Leaf Extract (200 mg/kg)
Group-2: Leaf Extract (400 mg/kg)

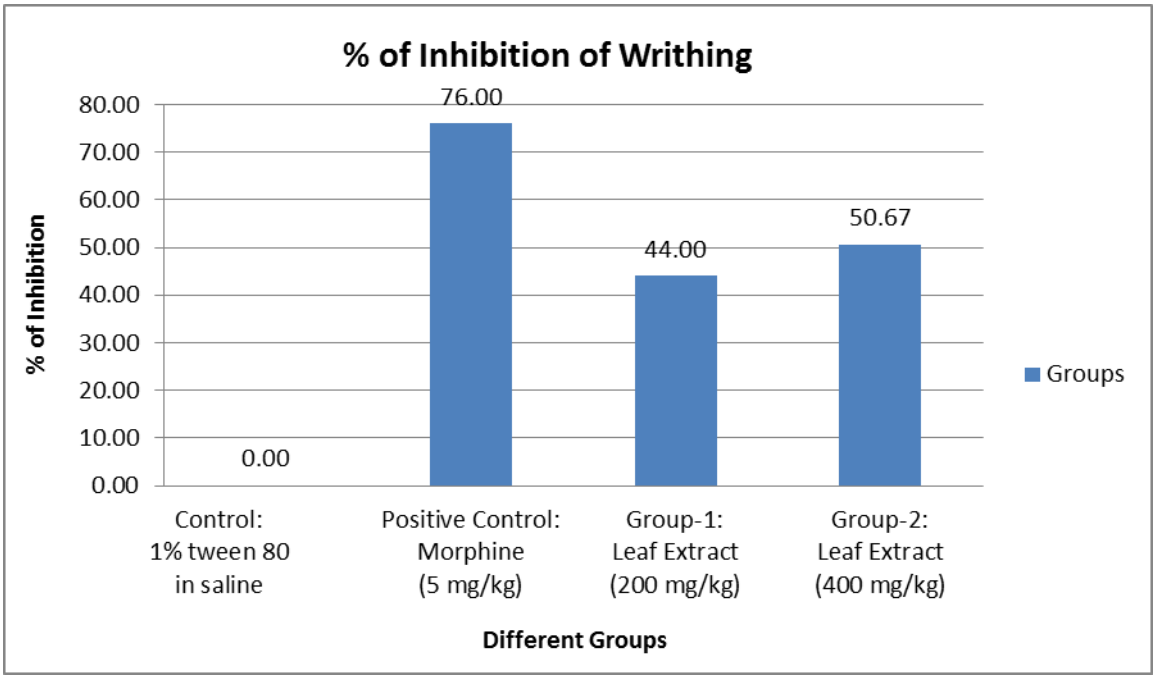

Fig. 4.3. Graphical presentation of the effect of leaf extract (acetone-ethanol, 1:1) of *Alpinia nigra* on mice by acetic acid induced writhing test.

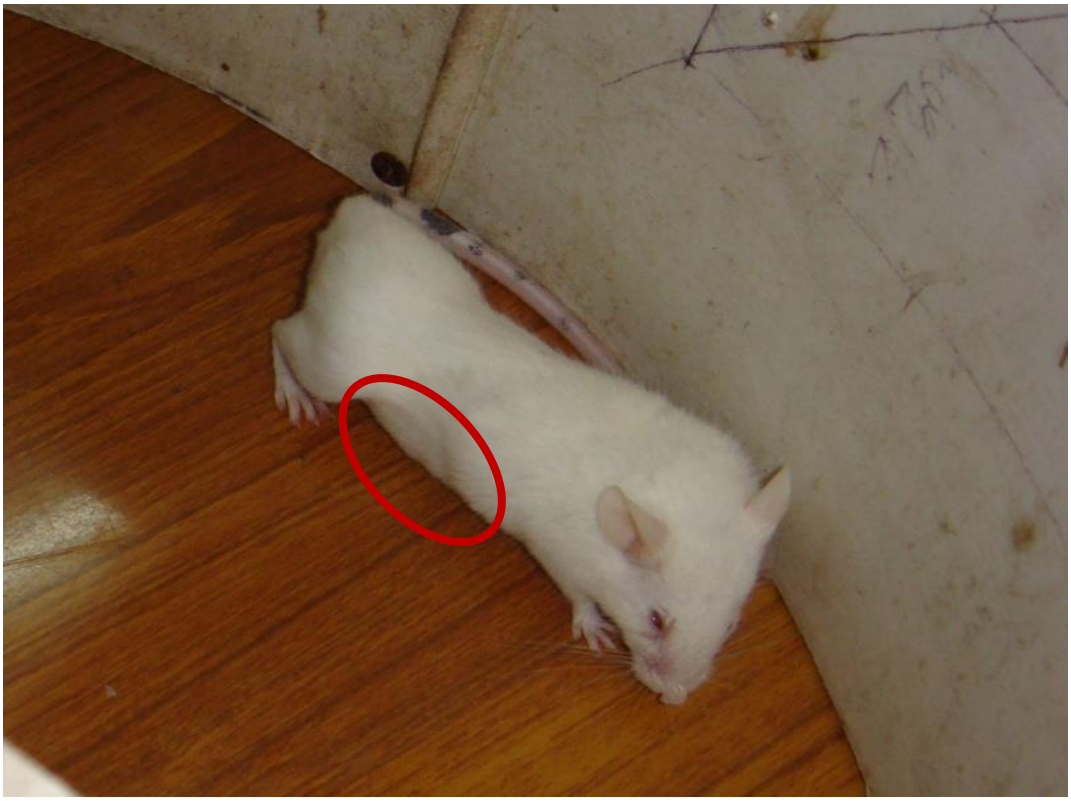

Fig. 4.4. Acetic acid induced writhing in mice (half writhing)

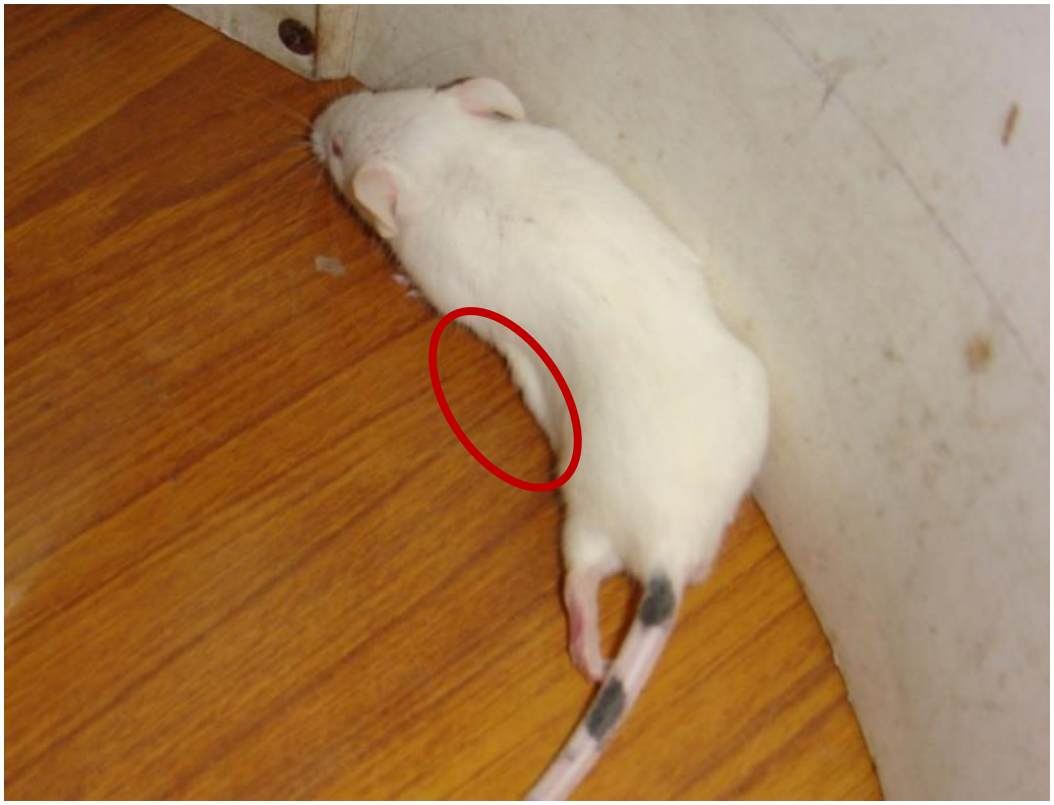

Fig. 4.5. Acetic acid induced writhing in mice (full writhing)

4.2. Investigation of analgesic activity by formalin induced licking test

Table 4.4. Effect of root extract of mixture of equal proportions of acetone and ethanol of *Alpinia nigra* on formalin induced licking.

Group		Licking Count						Mean	SD	SEM	% of Licking	% of Inhibition
		M1	M2	M3	M4	M5	M6					
Control Group	Early phase	120	112	115	118	121	117	117.2	3.31	1.35	100.00	0.00
	Late phase	99	94	97	96	97	95	96.3	1.75	0.71	100.00	0.00
Positive Control	Early phase	40	41	39	41	37	39	39.5	1.52	0.62	33.71	66.29
	Late phase	1	2	1	0	0	1	0.8	0.75	0.31	0.83	99.17
Group-1	Early phase	62	69	52	57	61	71	62.0	7.16	2.92	52.91	47.09
	Late phase	8	10	8	9	12	14	10.2	2.40	0.98	10.60	89.40
Group-2	Early phase	56	62	65	51	58	59	58.5	4.85	1.98	49.91	50.08
	Late phase	4	8	5	3	6	4	5.0	1.79	0.73	5.19	94.81

Control: 1% tween 80 in saline
Positive Control: Morphine (5 mg/kg)
Group-1: Root Extract (200 mg/kg)
Group-2: Root Extract (400 mg/kg)

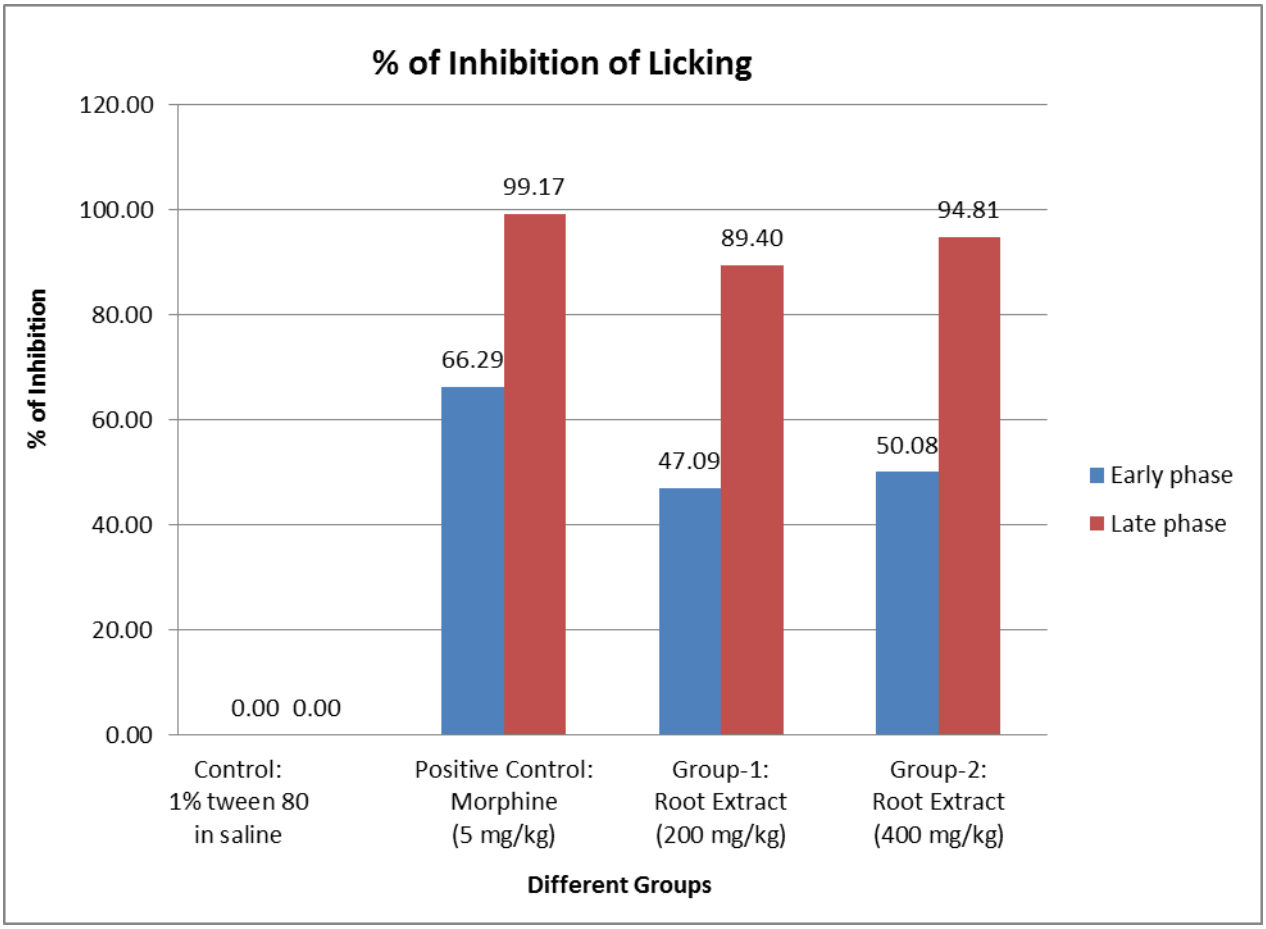

Fig. 4.6. Graphical presentation of the effect of root extract (acetone-ethanol, 1:1) of *Alpinia nigra* on mice by formalin induced licking test.

Table 4.5. Effect of shoot extract of mixture of equal proportions of acetone and ethanol of *Alpinia nigra* on formalin induced licking.

Group		Licking Count						Mean	SD	SEM	% of Licking	% of Inhibition
		M1	M2	M3	M4	M5	M6					
Control Group	Early phase	120	112	115	118	121	117	117.2	3.31	1.35	100.00	0.00
	late phase	99	94	97	96	97	95	96.3	1.75	0.71	100.00	0.00
Positive Control	Early phase	40	41	39	41	37	39	39.5	1.52	0.62	33.71	66.29
	Late phase	1	2	1	0	0	1	0.8	0.75	0.31	0.83	99.17
Group-1	Early phase	74	82	89	79	77	83	80.7	5.24	2.14	68.85	31.15
	late phase	14	16	9	19	21	12	15.2	4.45	1.82	15.78	84.22
Group-2	Early phase	62	72	78	69	61	75	69.5	6.89	2.81	59.30	40.70
	late phase	9	8	12	6	15	18	11.3	4.55	1.86	11.73	88.26

Control: 1% tween 80 in saline
Positive Control: Morphine (5 mg/kg)
Group-1: Shoot Extract (200 mg/kg)
Group-2: Shoot Extract (400 mg/kg)

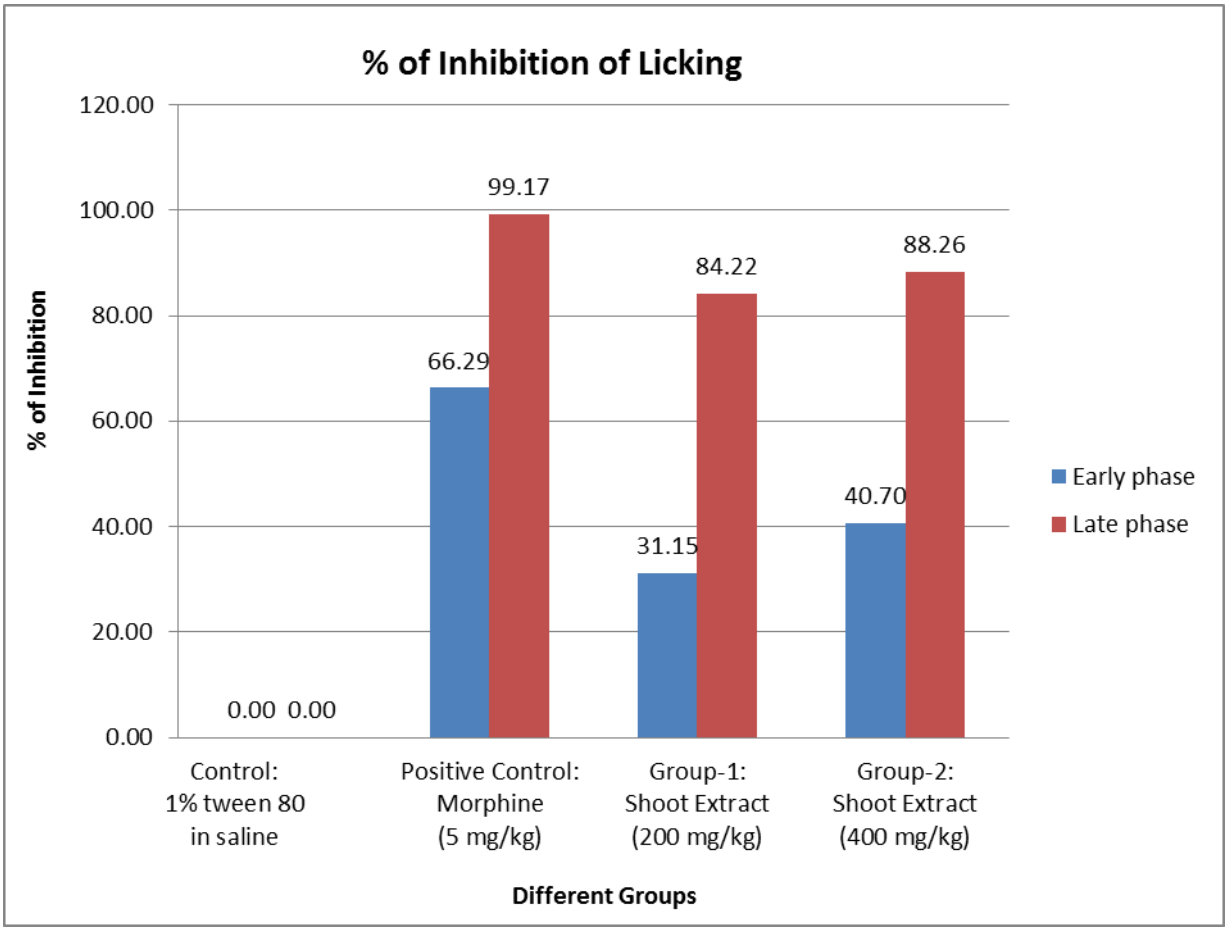

Fig. 4.7. Graphical presentation of the effect of shoot extract (acetone-ethanol, 1:1) of *Alpinia nigra* on mice by formalin induced licking test.

Table 4.6. Effect of leaf extract of mixture of equal proportions of acetone and ethanol of *Alpinia nigra* on formalin induced licking test.

Group		Licking Counting						Mean	SD	SEM	% of Licking	% of Inhibition
		M1	M2	M3	M4	M5	M6					
Control Group	Early phase	120	112	115	118	121	117	117.2	3.31	1.35	100.00	0.00
	late phase	99	94	97	96	97	95	96.3	1.75	0.71	100.00	0.00
Positive Control	Early phase	40	41	39	41	37	39	39.5	1.52	0.62	33.71	66.29
	Late phase	1	2	1	0	0	1	0.8	0.75	0.31	0.83	99.17
Group-1	Early phase	63	69	58	61	64	72	64.5	5.17	2.11	55.03	45.97
	late phase	7	9	10	8	11	13	9.7	2.16	0.88	10.07	90.93
Group-2	Early phase	58	61	64	50	61	63	59.5	5.09	2.08	50.76	49.23
	late phase	8	9	7	5	10	6	7.5	1.87	0.76	7.78	92.22

Control: 1% tween 80 in saline
Positive Control: Morphine (5 mg/kg)
Group-1: Leaf Extract (200 mg/kg)
Group-2: Leaf Extract (400 mg/kg)

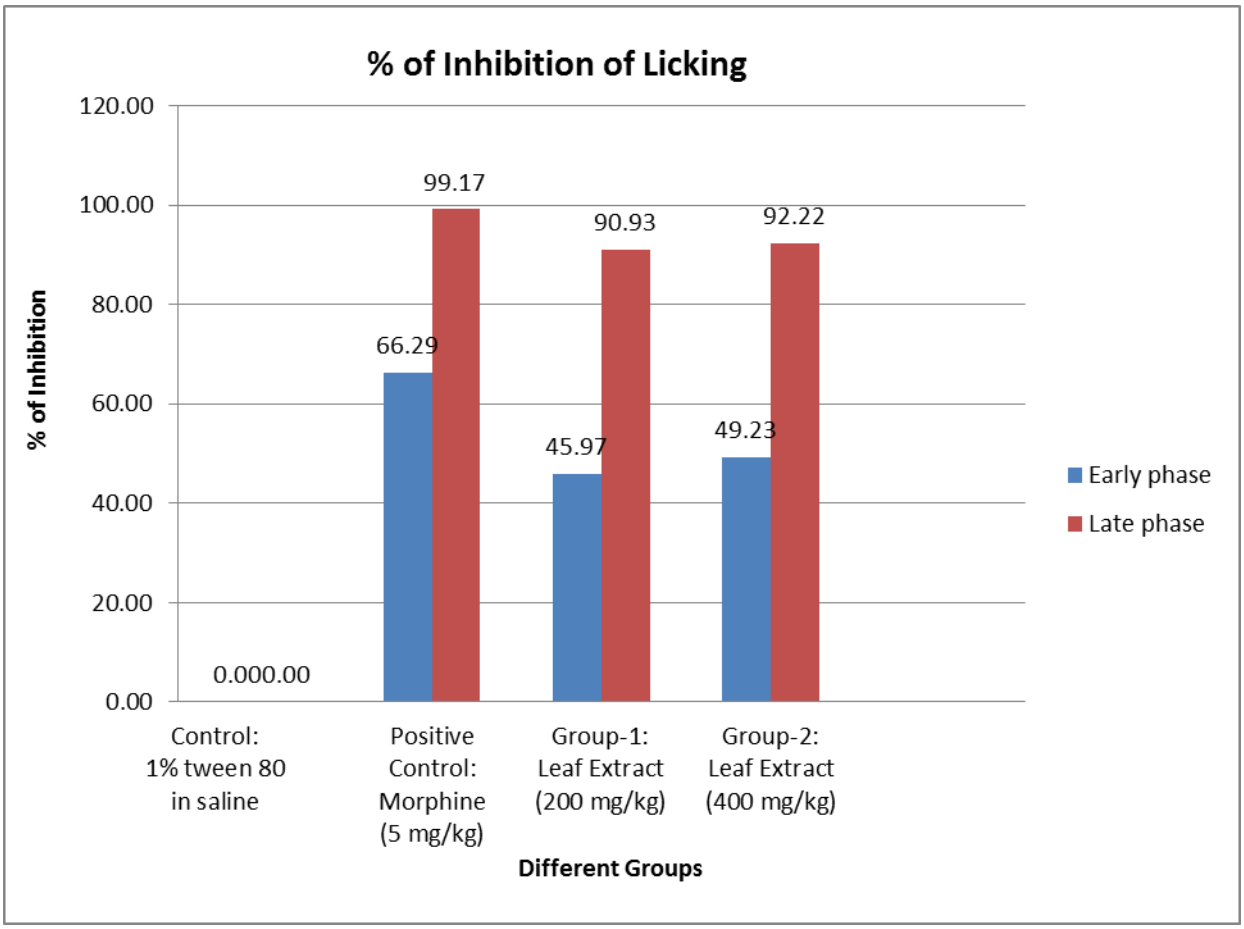

Fig. 4.8. Graphical presentation of the effect of leaf extract (acetone-ethanol, 1:1) of *Alpinia nigra* on mice by formalin induced licking test.

4.3. Investigation of analgesic activity by hot plate test

Table 4.7. Effect of root extract of mixture of equal proportions of acetone and ethanol of *Alpinia nigra* on hot plate test.

Group	Time	Tolerance Time (pain reaction time) in second						Mean	SD	SEM	% of tolerance time
		M1	M2	M3	M4	M5	M6				
Control Group	0 min	12.38	5.57	9.35	8.02	14.69	10.33	10.06	3.22	1.31	0.00
	30 min	9.30	7.23	14.09	8.48	10.36	12.30	10.29	2.53	1.03	0.00
	60 min	14.69	13.45	8.27	11.37	6.83	10.37	10.83	2.99	1.22	0.00
	90 min	15.33	8.16	11.74	8.23	5.65	8.85	9.66	3.39	1.38	0.00
	120 min	14.85	10.39	11.02	12.89	7.97	9.99	11.19	2.40	0.98	0.00
Positive Control	0 min	9.34	14.56	15.04	8.40	7.45	11.98	11.13	3.22	1.32	10.66
	30 min	19.83	18.04	20.21	14.38	18.83	16.45	17.96	2.21	0.90	74.45
	60 min	16.66	22.96	18.37	24.34	16.55	19.46	19.72	3.26	1.33	82.12
	90 min	15.51	10.31	11.82	20.00	13.40	19.83	15.15	4.08	1.66	56.78
	120 min	20.00	12.99	17.46	20.00	16.87	14.81	17.02	2.80	1.14	52.18
Group-1	0 min	10.14	11.60	6.98	7.84	9.34	16.40	10.38	3.37	1.38	3.25
	30 min	15.20	13.40	8.10	10.80	21.30	16.10	14.15	4.57	1.87	37.47
	60 min	11.10	16.20	18.14	9.45	23.20	17.10	15.87	4.99	2.04	46.49
	90 min	10.23	14.30	18.40	19.20	8.56	9.12	13.30	4.72	1.93	37.70
	120 min	16.23	7.81	19.60	11.23	16.18	17.40	14.74	4.37	1.78	31.80
Group-2	0 min	10.10	13.19	15.90	9.25	7.14	7.57	10.53	3.41	1.39	4.66
	30 min	18.90	12.10	16.02	21.30	14.40	13.12	15.97	3.54	1.44	55.18
	60 min	16.40	18.10	26.70	19.70	14.20	20.10	19.20	4.27	1.75	77.29
	90 min	10.40	14.60	16.70	22.50	8.10	21.30	15.60	5.75	2.35	61.49
	120 min	9.10	13.00	18.74	21.30	14.60	18.40	15.86	4.47	1.82	41.77

Control: 1% tween 80 in saline
Positive Control: Morphine (5 mg/kg)
Group-1: Root Extract (200 mg/kg)
Group-2: Root Extract (400 mg/kg

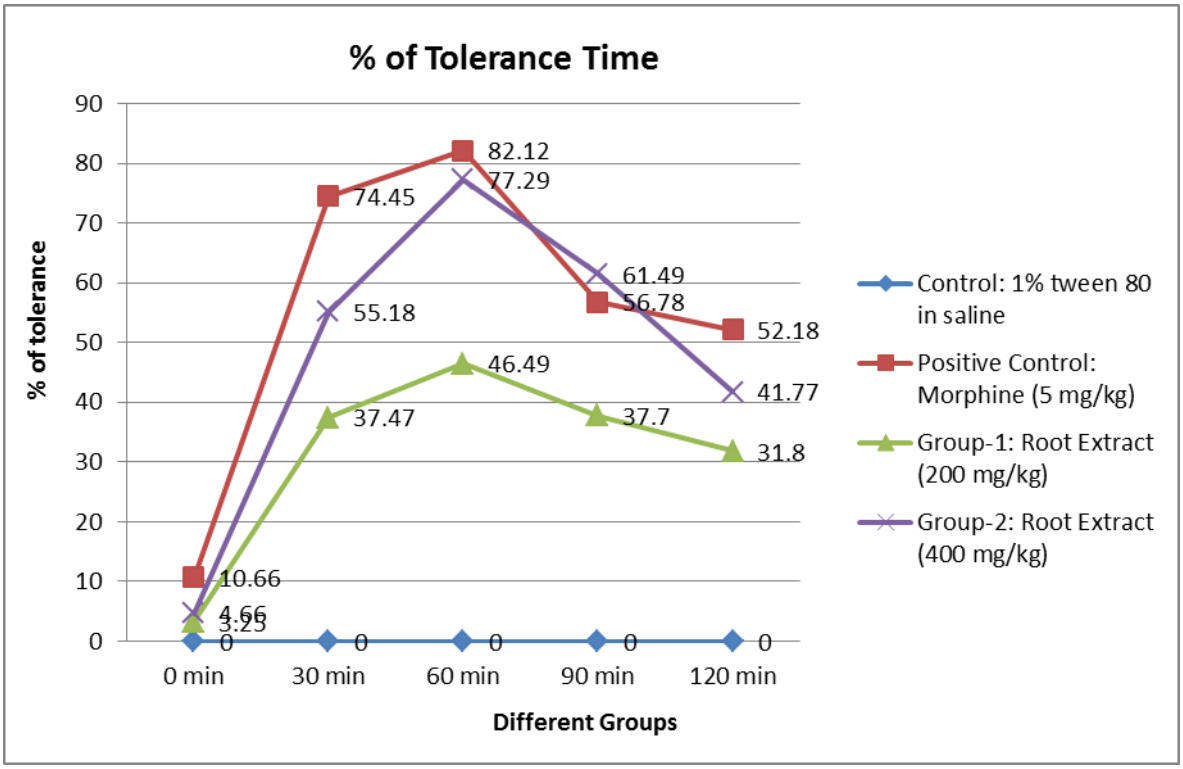

Fig. 4.9. Graphical presentation of the effect of root extract (acetone-ethanol, 1:1) of *Alpinia nigra* on mice by hot plate test.

Table 4.8. Effect of leaf extract of mixture of equal proportions of acetone and ethanol of *Alpinia nigra* on hot plate test.

Group	Time	Tolerance Time (pain reaction time) in second						Mean	SD	SEM	% of tolerance time
		M1	M2	M3	M4	M5	M6				
Control Group	0 min	12.38	5.57	9.35	8.02	14.69	10.33	10.06	3.22	1.31	0.00
	30 min	9.30	7.23	14.09	8.48	10.36	12.30	10.29	2.53	1.03	0.00
	60 min	14.69	13.45	8.27	11.37	6.83	10.37	10.83	2.99	1.22	0.00
	90 min	15.33	8.16	11.74	8.23	5.65	8.85	9.66	3.39	1.38	0.00
	120 min	14.85	10.39	11.02	12.89	7.97	9.99	11.19	2.40	0.98	0.00
Positive Control	0 min	9.34	14.56	15.04	8.40	7.45	11.98	11.13	3.22	1.32	10.66
	30 min	19.83	18.04	20.21	14.38	18.83	16.45	17.96	2.21	0.90	74.45
	60 min	16.66	22.96	18.37	24.34	16.55	19.46	19.72	3.26	1.33	82.12
	90 min	15.51	10.31	11.82	20.00	13.40	19.83	15.15	4.08	1.66	56.78
	120 min	20.00	12.99	17.46	20.00	16.87	14.81	17.02	2.80	1.14	52.18
Group-1	0 min	11.10	12.30	6.12	8.14	9.56	14.50	10.29	3.00	1.22	2.29
	30 min	13.17	18.19	8.54	11.31	19.34	18.70	14.88	4.50	1.84	44.51
	60 min	11.45	17.50	19.60	10.20	21.60	18.56	16.49	4.61	1.88	52.22
	90 min	11.34	13.45	18.45	16.56	7.89	9.87	12.93	4.03	1.65	33.82
	120 min	16.23	7.81	19.60	11.23	16.18	17.40	14.74	4.37	1.78	31.80
Group-2	0 min	12.40	13.19	15.90	9.25	7.14	7.57	10.91	3.48	1.42	8.47
	30 min	17.60	12.10	16.02	21.30	14.40	13.12	15.76	3.36	1.37	53.08
	60 min	16.40	18.10	21.50	19.70	14.20	13.45	17.23	3.14	1.28	59.05
	90 min	10.10	14.60	16.70	13.87	8.10	21.30	14.11	4.71	1.92	46.08
	120 min	11.80	9.76	18.74	17.67	14.60	18.45	15.17	3.76	1.54	35.63

Control: 1% tween 80 in saline
Positive Control: Morphine (5 mg/kg)
Group-1: Leaf Extract (200mg/kg)
Group-2: Leaf Extract (400mg/kg)

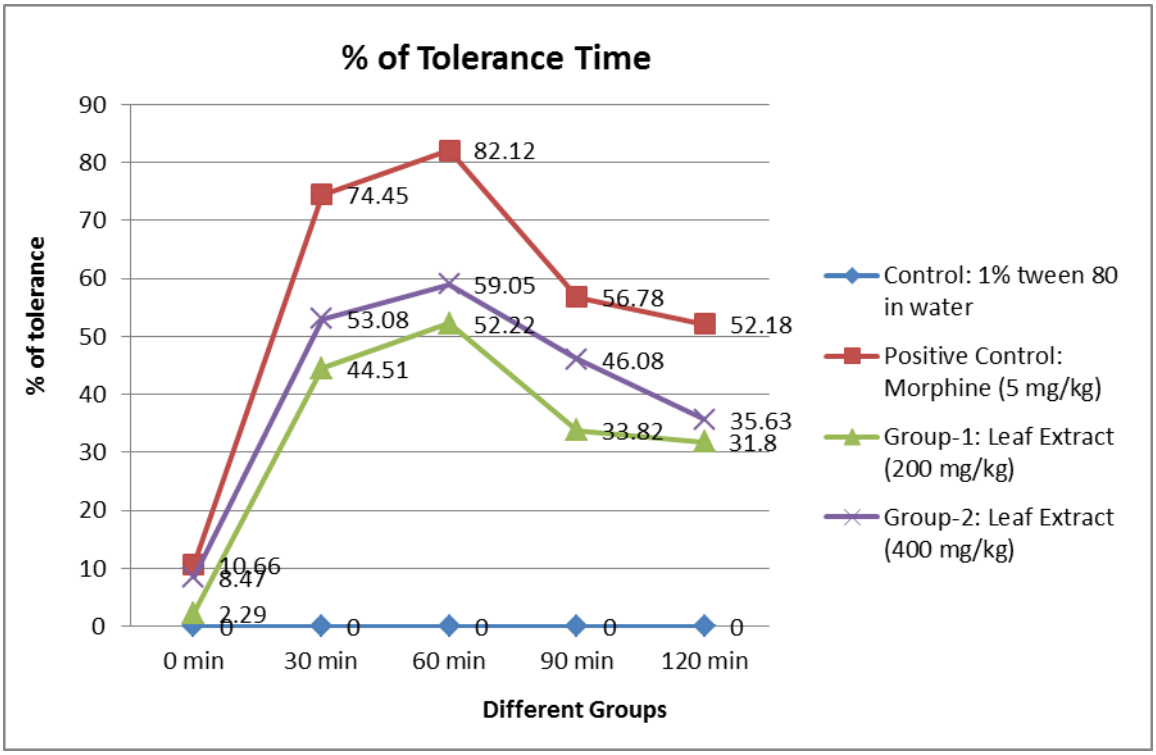

Fig. 4.10. Graphical presentation of the effect of leaf extract (acetone-ethanol, 1:1) of *Alpinia nigra* on mice by hot plate test.

Fig. 4.11. Mice is placing on hot plate

Fig. 4.12. Mice on hot plate

CHAPTER FIVE

DISCUSSION

CHAPTER 5: Discussion

5.1. Discussion

Nociception is the mechanism where noxious peripheral stimuli are transmitted to the central nervous system and is a component of pain. There are two components of pain either or both of which may be involved in pathological pain states. They include; the peripheral nociceptive afferent neuron which is activated by noxious stimuli and the central mechanism by which the afferent input generates a pain sensation (Rang et al., 2003). The stimulus may be thermal (Tail immersion and hot plate test), electrical (Paw, tail or dental pulp stimulation), mechanical (tail and paw pressure tests) or chemical (acetic acid writhing or formalin test) (George et al., 2009).

In this study, acetic acid induced writhing test, formalin Induced licking test and hot plate methods were used to elucidate central and peripheral anti-nociceptive effects.

5.2. Acetic acid induced writhing test

Acetic acid induced writhing response is a sensitive procedure to evaluate peripherally acting analgesics. Taber et al., (1969) earlier observed that certain chemicals have the ability to induce writhing response in laboratory animals. Writhing reflex or abdominal constrictions was produced in this study by intraperitoneal injection of 0.7% glacial acetic acid in mice. This model represents pain sensation by triggering localized inflammatory response which is thought to be mediated by peripheral mast cell (Ronaldo et al., 2000), acid sensing ion channels (Voilley, 2004) and the prostaglandin pathway (Hossain et al., 2006)

The crude extract of mixture of equal proportions of acetone and ethanol of different parts of *Alpinia nigra* displayed dose dependent analgesic activity against acetic acid induced writhing test on *Swiss albino* mice. The Root extract of *Alpinia nigra* at the doses of 200 mg/kg and 400 mg/kg body weight exhibited 58.2% and 62.2 % inhibition respectively. The shoot extract shows result like root extract 53.8% inhibition at the dose of 200 mg/kg and 60.7% inhibition at 400 mg/kg/dose. The leaf extract shows lower

result then root extract and shoot extract, 44.0% inhibition at 200 mg/kg/dose and 50.7% inhibition at 400 mg/kg/dose. Morphine 5 mg/kg used as positive control and exhibited 76.0% inhibition compared to control. The abdominal constriction in writhing is related to the sensitization on antinoceptive receptors to prostaglandins. Here pain is generated indirectly via endogenous mediators like prostaglandins which in turn cause the stimulation of peripheral nociceptive neurons. The neuronal fibres are known to be sensitive to both narcotics and non-steroidal anti-inflammatory drugs (Collier, et al., 1968 and Onansanwo and Elegbe, 2006). The extract produced analgesic effect which may be due to the inhibition of synthesis of prostaglandin. *Alpinia nigra* contains alkaloid which may be responsible for the analgesic activity (Nishat et al., 2012).

5.3. Formalin induced licking test

Formalin test is a pain model which assesses the animal responds to moderate continuous pain generated by injured tissue (Ttjolsen et al., 1992). The effect of drugs on licking in early and late phase represent antinociceptive action on sensory receptor stimulation and anti-inflammatory action respectively (Dubuission and Dennies, 1977; Hunskaar and Hole, 1987). Drugs which acts centrally inhibit both phase of pain in this model while peripherally acting drugs only inhibit the late phase (Santos et al., 1997)

The experimental result presented in table 4.4, table 4.5, and table 4.6. represents the oral administration of extract of mixture of acetone and ethanol (1:1) of different parts of *Alpinia nigra* caused a significant dose (200 mg/kg and 400 mg/kg) dependent inhibition of both the early (neurological, 0-5 min) and late (inflammatory, 15-30 min) phase of formalin induced licking. However, its antinociceptive effects were more pronounced against the second phase of this model of pain. The calculated licking response inhibitory effect of late phase for root, shoot and leaf were 89.4%, 84.2% and 90.9% at the dose of 200 mg/kg respectively. For 400 mg/kg dose, licking response inhibition of root, leaf and shoot was 94.8%, 88.3% and 92.2% respectively in late phase. The root extract in early phase at the dose of 200 mg/kg and 400 mg/kg body weight was found to have licking response inhibitory effect 47.1% and 50.1%. The shoot extract shows 31.2% and 40.7% inhibition of licking. The leaf extract at the dose of 200 mg/kg and 400 mg/kg body weight was found to have licking response inhibitory effect 46.0% and 49.2%

respectively. Morphin used as standard, administrated intra-peritonealy at the dose of 5 mg/kg per body weight exhibit licking response inhibitory effect 66.3% and 99.2% in early and late phase respectively.

5.4. Hot plate test

The hot plate model has been used for the study of centrally acting analgesia (antinociception) (Woolfe and MacDonald, 1994). Here the nociceptors seem to be sensitized by sensory nerves and the involvements of endogenous substances such as prostaglandins (PGs) are minimized (Bachlav et al., 2009). In the hot plate models, the tolerance to thermal stimulus manifested by increase in the pain reaction time indicates the level of antinociception induced by extract or drug (Ramadran and Basinath, 1986).

In this study, crude extract of mixture of equal proportions of acetone and ethanol of root and leaf of *Alpinia nigra* was compared to the control group in hot plate models. The effect of the extract was comparable to the reference drug morphine (5 mg/kg) which exhibit percentage of tolerance time (pain reaction time) 82.1% at 60 minutes. Root extract at the dose of 200 mg/kg and 400 mg/kg showed significant increase in the percentage of tolerance time. The peak effect was seen at 60 minutes, 77.3% and 46.5% of tolerance for 400 mg/kg and 200 mg/kg respectively. But leaf extract showed lower percentage of tolerance time, 59.1% and 52.2% at 60 minutes for 400 mg/kg and 200 mg/kg respectively. The highest antinociceptive effect (4.7% - 0 min, 55.2% - 30 min, 77.3% - 60 min, 61.5% - 90 min and 41.8% - 120 min,) was observed in the 400 mg/kg root extract treated group which lasted for whole study period. According to Vogel and Vogel (1997), increase in stress tolerance capacity of animals in hot plate model indicates the possible involvement of higher center. The above assertions suggest that the activity of *Alpinia nigra* in increasing the pain reaction time in these models may have been mediated through central nervous system.

5.5. Conclusion

Based on the result of present study, it can be concluded that the crude extract of mixture of equal proportions of acetone and ethanol of root, shoot and leaf of *Alpinia nigra* possesses remarkable analgesic effect. Various phytochemicals have been proved as evident for the analgesic activity of *Alpinia nigra*. These compounds may be responsible for the bioactivities. Since the extract is reported to contain a myriad array of compounds, it is difficult to ascribe these observed activities to any specific group of compounds. Hence, further studies are suggested to be undertaken to pinpoint of the exact compound(s) and to better understand the mechanism of such actions scientifically.

References

Acharya, S.D., Ullal, S.D., Padiyar, S., Rao, Y.D., Upadhyaya, K., Pillai, D., Raj, V. (2001) Analgesic effect of extracts of *Alpinia galanga* rhizome in mice. *Journal of Chinese Integrative Medicine*, 9(1): 100-104

Ahmed, F., Selim, M.S.T., Das, A.K., Choudhuri, M.S.K. (2004) Anti-inflammatory and antinociceptive activities of *Lippia nodiflora Linn. Pharmazie,* 59: 329-33

Ahmed, S., Rahman, A., Mathur, M., Athur, M and Sultana, S. (2001) Anti-tumor promoting activity of Asteracantha longifolia against experimental hepato carcinogenesis in rats. *Food. Chem. Toxicol* 39(1): 19-28

Ampofo, O. (1979) The practice of phytotherapy in Ghana, In A. Sofowora (Ed.), *African Medicinal Plants*, University of Ife Press, Nigeria.

Arambewela, L.S.R., Arawwawala, L.D.A.M., and Ratnasooriya, W.D. (2004) Antinociceptive activities of aqueous and ethanolic extracts of *Alpinia calcarata* rhizomes in rats. *Journal of Ethnopharmacology*, 95(3): 311–316

Arawwawalaa, L.D.A.M., Arambewelaa, L.S.R., and Ratnasooriyab, W.D. (2012) *Alpinia calcarata* Roscoe: A potent antiinflammatory agent. *Journal of Ethnopharmacology*, 139 (3): 889–892

Asolkar, L.V., Kakkar, K.K., and Chakre, O.J. (1992) Secondary Supplement to Glossary of Indian Medicinal Plants with Active Principles, Publications and Information Directorate. *Council of Scientific and Industrial Research*.

Chunping, T., Tao, J., Yuanfen, C., Shaoyi, C., and Chaoyan, Y. (2006) The effects of *Alpinia officinarum* Rhizoma extracts on the activity of isolated ileum musle in rabbits. *Section of Chinese Materia Medica Pharmacology, Guangdong College of Pharmacy, Guangzhou*. Pharmacology and Clinics of Chinese Materia Medica.

Colegate, M.S. and Molyneux R.J. (2008) Bioactive natural products: detection, isolation, and structural determination. 2nd ed. *CRC Press*. Pp. 1-3

Collier, H.O., Dinneen, L.C., Johnson, C.A., Schneider, C. (1968) The Abdominal Constriction Response and its suppression by analgesic drugs in mouse. *British Journal of Pharmacology,* 32: 295-310

Das, B. N. and Qais, N. (2012) Antibacterial and cytotoxic activities of the rhizome extract of *Alpinia nigra*. *International journal of comprehensive pharmacy,* 3(01): 01-03

Das, B.N. and Biswas, B.K. (2012) Anti-inflammatory activity of the rhizome extract of *Alpinia nigra*. *International Research Journal of Pharmaceuticals,* 2(3): 73–76

Das, B.N. and Qais, N. (2012) Analgesic activity of the rhizome extract of Alpinia nigra. *IJPI's Journal of Pharmacognosy and Herbal Formulations,* 2(9): 28–32

Dubuisson, D. and Dennis, S.G., (1977) The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. *Pain* 4:161–177

Duke, J.A. and Ayensu, E.S. (1985) Medicinal plants of China. Vol. 1 & 2. *Algonac, USA, Reference Publications* (Medicinal Plants of the World 4).

Dutta, M. and Sinha, M. (2010) Antimicrobial Activity of Some Selected Plants from Southern Assam. *Assam University Journal of Science & Technology : Biological and Environmental Sciences,* 6(01): 58-65

Eddy, N.B., and Leimbach, D. (1953) Synthetic analgesics. II. Dithienylbutenyl- and dithienylbutylamines. *Journal of Pharmacology and Experimental Therapeutics*, 107 (3): 385–393

Espejo, E., and Mir, D. (1993) Structure of the rat's behaviour in the hot plate test. *Behavioural Brain Research* 56 (2): 171–176

Farnsworth, N.R. (1966) Biological and Phytochemical screening of plants. *Journal of pharmaceutical science.* 55 (3): 225 – 276

Farnsworth, N.R. and Morris, R.W. (1976) Higher plants–the sleeping giant of drug development. *Am. J. Pharm*. 148: 46–52

George, K.A, Eric, W., David, D.O., George, A.K (2009) Antinociceptive Effects of *Newboulia laveis* (P. Beauv) Stem Bark Extract in Rat Model. *Pharmacog. Mag.* 17:49-54

Ghani, A. (1998) Medicinal Plants of Bangladesh. *The Asiatic Society of Bangladesh*. 3rd ed. Pp. 4-19

Ghani, A. (2003) Medicinal Plants of Bangladesh with chemical constituents and uses. *Asiatic Society of Bangladesh*. 2nd edition. Pp. 1-8

Ghosh, A. K., Banerjee, M. and Bhattacharyya, N. K. (2011) Anti-inflammatory activity of root of *Alpinia galanga* willd. *Original article,* 2(3): 139-143

Goldstein, A., Lewis, A, and Kalman, S. M (1974) Principles of Drug Action : The Basis of Pharmacology. *K.S. Krishnan Marg, New Delhi*. 2nd ed. Pp.729-750

Hasan, S.M.R., Jamila, M., Majumder, M.M., Akter, R., Hossain, M.M., Mazumder, M.E.H., Alam, M.A., Jahangir, R., Rana, M.S., Arif, M. and Rahman, S. (2009) Analgesic and antioxidant activity of hydromethanolic extract of *Mikania scandens* (L.) willd. leaves. *Am. J. Pharmacol. Toxicol*., 4,1-7

Hossain, M.M., Ali, M.S., Saha, A and Alimuzzaman, M. (2006) Antinociceptive activity of whole plant ext-racts of Paederia foetida. *Dhaka University Journal of Pharmaceutical Science*, 5, 67-69

Hunskaar, S. and Hole, K. (1987) The formalin test in mice: Dissociation between inflammatory and non-inflammatory pain. *Pain*, 30: 103-114

Indrayan, A. K., Garg, S. N., Rathi, A. K. and Sharma, V. (2007) Chemical composition and antimicrobial activity of the essential oil of *Alpinia officinarum* rhizome. *Indian Journal of Chemistry -Section B*., 46: B

Indrayana, A. K., Tyagia, P.K., and Agrawala, N. K. (2010) Chemical Composition and Antimicrobial Activity of the Essential Oil of *Alpinia speciosa* K. Schum. Rhizome From India. *Journal of Essential Oil Research,* 22(2): 179-182

Jian, S., Huyi, Z., Bo, X., and Jingxian, P. (1998) The antioxidative constituents of rhizomes of *Alpinia officinarum. National Key Laboratories of Natural and Biomimetic Drugs, Beijing.*

Kanjila, P.B., Kotoky, R. and Couladis, M. (2010) Essential oil composition of leaf and rhizome oil of *Alpinia nigra* (Gaertner) B.L.Burtt. from northeast India. *Asian Journal of Traditional Medicines,* 22 (4): 358–359

Karen, A.O., Andrea, J.D., Paul. W.N., et al. (2001) Flavonoid glucuronides are substrates for human liver L-glucuronidase. *FEBS Letters;* 503: 103-106

Koster, R., Anderson, M., and Beer, E.J. (1959) Acetic acid for analgesic screening. *Federation Proceeds* 18: 412–416

Kress, W.J., Liu, A.Z., Newman, M., and Li, Q.J. (2005) The molecular phylogeny of *Alpinia* (Zingiberaceae): a complex and polyphyletic genus of gingers. *American Journal of Botany,* 92(1): 167-78

Lee, J.S., Kim, K.A., Jeong, S.H., Lee, S.G., Park, H.J., Kim, N.J., and Lim, S. (2009) Anti-inflammatory, anti-nociceptive, and anti-psychiatric effects by the rhizomes of *Alpinia officinarum* on complete Freund's adjuvant-induced arthritis in rats. *Journal of Ethnopharmacology,* 126 (2): 258–264

Ling, T., Xiang, S., Jiao, P., and Shuai, B. (2010) Experimental study on the anti-inflammatory and analgesic effects in vivo of essential oil from fructus *Alpiniae zerumbet* in mice. *Guiyang Medical college, Guizhou, Guiyang, China.*

Mannan, A., Ahmed, A.M.A., Sharmin, F., Fatema, T. and Sikder, M.O.F. (2011) *In vitro* thrombolytic assay *of Alpinia zerumbet, Alpinia nigra* and *Urena sinuate. International Journal of Research in Phytochemistry and Pharmacology,* 1(3): 187-191

Matu, E.N. and Staden, J. (2003) Antibacterial and antiinflammatory activities of some plants used for medicinal purposes in Kenya. *J. Ethnopharm*. 87: 35-41

Meera S., Kumar N.S. and Guptatyam V.S.S. (2008) Screening of anti arthritic, anti inflammatory and analgesic activity of a polyherbal formulation. *International Journal of Pharmaceutics.* 4:398–402

Meyer, B.N, Ferringni, N.R., Puam, J.E., Lacobsen, L.B., Nichols, D.E. and McLaughlin, J.L. (1982) Brine shrimp: a convenient general bioassay for active constituents. *Planta Med.* 45: 31-32

Nishat, F.S., Nilima, N., and Saikia, B.M. (2012) Phytochemical analysis of *Lasia spinosa* and *Alpinia nigra*, potential medicinal plants of Assam. *Phytochemistry* 4 (3): 170–173

Omoregie, S.N., Omoruyi, F.O., Wright, V.F., Jones, L., and Zimba, P.V. (2013) Antiproliferative Activities of Lesser Galangal (*Alpinia officinarum* Hance Jam1), Turmeric (*Curcuma longa* L.), and Ginger (*Zingiber officinale* Rosc.) *Against Acute Monocytic Leukemia Journal of Medicinal Food,* 16(7): 647-655

Onasanwo, S.A. and Elegbe, R.A. (2006) Antinociceptive and Anti-Inflammatory Properties of the Leaf Extract of *Hedranthera barteri* in Rats and Mice. *African J. of Biomedical Research,* 2: 109-118

Penso, G. (1980) WHO inventory of medicinal plants used in different countries. Geneva, Switzerland, WHO.

Qiao, C., Xu, L., and Wang, Z. (2001) Pharmacognostical studies on the rhizome and root of *Alpinia nigra*. *Zhong Yao Cai*, 24(7): 486–487

Qiao, C.F., Wang, Z., Dong, H., Xu, L. and Hao, X. (2000) The chemical constituents of blackfruit galangal (*Alpinia nigra*). *Chinese Traditional and Herbal Drugs* 31(6): 404–405

Qiao, C.F., Han, Q., Song, J., Wang, Z., Xu, L., & Xu, H. (2007) HPLC determination of two bioactive flavone glycosides and GC-MS analysis of volatile oil constituents in *Alpinia nigra*. *Asian Journal of Traditional Medicines*, 2(3): 85–91

Rahman, M., Rahman, A., Hashem, M. A.,Ullah, M.,Afroz, S.,andChaudhary, V. (2012) Anti-Inflammatory, Analgesic And Gc – Ms Analysis Of Essential Oil *Of Alpinia Calcaratarhizome International Journal of Pharma and Bio Sciences*, 3(4): 55 – 63

Ramadran, K. and Basinath, L. (1986) A Critical Analysis of the Experimental Evaluation of Nociceptive Reactions in Animals. *Pharm. Research,* 3:253-270

Rang, H. P., Bevan, S. J. and Dray, A. (1991) Chemical activation of nociceptive peripheral neurones. Br. *Med. Bull*.47:534

Rang, H. P., Dale, M., M. (1993) Pharmacology. 2nd ed. UK: *Churchill Livingstone Publisher;* Pp. 706-711

Rang, H.P., Dale, M.M., Ritter, J.M. and More, P.K. (2003) Pharmacology, Fifth Edition, *Elsevier Science Ltd*, New Delhi, Pp 562-569

Ronaldo, A.R, Mariana, L.V, Sara M.T, Adriana, B.P.P., Steve P, Ferreira SH and Fernando Q.C. (2000) Involvement of resident macrophages and mast cells in the writhing nociceptive responseinduced by zymosan and acetic acid in mice. *European Journal Pharmacology,* 387: 111-118

Roy, B. and Swargiary, A. (2009) Anthelmintic efficacy of ethanolic shoot extract of *Alpinia nigra* on tegumental enzymes of *Fasciolopsis busk*i, a giant intestinal parasite. *J Parasit Dis*, 33(1&2): 48–53

Roy, B., Dasgupta, S. and Tandon, V. (2009) Ultrastructural observations on *Fasciolopsis buski* and its alterations caused by shoot extract of *Alpinia* nigra. *Microscopy Research and Technique* 72(2): 61–66

Roy, B., Swargiary, A. and Giri, B.R. (2012) *Alpinia Nigra* (Family Zingiberaceae): an anthelmintic medicinal plant of north-east India. *Advances in Life Sciences*, 2(3): 39–51

Santos, A.R.S., Calixto J.B. (1997) Further evidence for the involvement of tachykininreceptor subtypes in formalin and capsaicin models of pain in mice. *Neuropeptides*, 31:381-9

Santos, A.R.S., Miguel, O.G., Yunes, R.A. and Calixto, J.B. (1999) Antinociceptive properties of the new alkaloid, cis-8, 10-di-N-propyllobelidiol hydrochloride dihydrate isolated from *Siphocampylus verticillatus*: evidence for the mechanism of action. *Journal of Pharmacology and Experimental Therapeutics*, 289 , pp. 417–42

Sofowara, E.A. (1982) Medicinal Plants and Traditional Medicines in Africa. Nigeria: *John Wiley and Sons Ltd*, Pp. 64–79

Sulaiman, M. R., Zakaria, Z. A., Mohamad, A. S., Ismail, M., Hidayat, M. T., Israf, D. A. and Adilius M. (2010) Antinociceptive and anti-inflammatory effects of the ethanol extract *of Alpinia conchigera* rhizomes in various animal models. *Pharmaceutical Biology*. 48(8): 861-868

Taber, R.L., Greenhouse, D.D., Randel, J.K. and Irwin, S. (1969) Agonist and Antagonist Interaction of Opioid and Acetic Acid-Induced Abdominal Stretching in Mice. *J. Pharm. Exp. Ther,* 169: 29-38

Tjolsen, A., O.G. Berge, S. Hunskaar, J.H. Rosland and K. Hole, (1992) The formalin test: An evaluation of the method. *Pain,* 51: 5-17

Tripathi, K.D. (1999) Essentials of Medical Pharmacology. 4 th edn., *Jaypee Brothers Medical Publishers (P) Ltd.,* New Delhi, India. P-432

Vogel, H.G. and Vogel, W.H. (1997) Drug Discovery and Evaluation. 1st Ed., *Springer-Verlag, Berlin,* Heidelberg, New York pp: 376- 377

Voilley N. (2004) Acid-sensing ion channels (ASICs): new targets for theanalgesic effects of non-steroid anti-inflammatory drugs (NSAIDs). Curr. Drug Targets. *Inflamm. Allergy* 3, 71–79

Wallis, T.E. (1985) Text Book of Pharmacogonosy. *CBS Publishers and Distributors, Delhi.* 3rd ed.

Woolfe, G. and MacDonald, A.D (1994) The Evaluation of Analgesic Action of Pethidine Hydrochloride. *Journal Pharmacol. and Exp. Ther.*, 80:300

Yusuf, M., Chowdhury, J.U., Wahab, M.A. and Begum, J. (1994) Medicinal plants of Bangladesh. *Bangladesh council of Scientific and Industrial Research*, Dhaka-1205, Bangladesh, pp. 340

Appendix

1. Arithmetic Mean ($\overline{X}$) = $\frac{\sum X}{n}$

Where, ΣX = Summation of Observed Value
n = No. of Observation

2. Standard Deviation (SD) = $\sqrt{\frac{\Sigma(X - \overline{X})^2}{n}}$

Where, X = individual Value
$\overline{X}$ = Mean Value
n = No. of Observation

3. Standard Error Mean (SEM) = $\frac{SD}{\sqrt{n}}$

Where, SD = Std. Deviation
n = No. of Observation

4. Percentage of licking or writhing = $\frac{Test}{Control} \times 100\%$

5. Percentage of Inhibition (licking or writhing) = $\frac{Test - Control}{Control} \times 100\%$