Department of Pharmacy

Pharmacological Investigation of Dichloromethane Fraction of *Geodorum densiflorum* (Lam.) Schltr

BY

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Certificate

This is to certify that the thesis submitted to the Department of Pharmacy, East West University,Dhaka-1212, in partial fulfillment of the requirement for the Degree of Bachelor in Pharmacy, was carried out by Shammee Monira, ID: 2008-3-70-038.

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Abstract

Geodorum densiflorum (Family: Orchidacea), locally known as 'Shankhamul' in Bangladesh, is an endangered terrestrial orchid, which has long been used traditionally for various medicinal purposes in the Indian subcontinent. In this consequences, the present study was designed to investigate the analgesic and CNS activity properties of two different fraction (n-hexane and Dicloromethane soluble fraction, coded as HF and DCM respectively) of the root parts of *Geodorum densiflorum.*

Analgesic potential of the different fractions of the root parts of *G. densiflorum* was evaluated using acetic acid induced writhing method. The dichloromethane fraction of the methanolic extract of root part of *G. densiflorum* showed significant activity with 21.36 % ($p<0.05$) and 71.84 % ($P<0.05$) writhing inhibition in mice at a dose of 200 mg/kg body weight and 400 mg/kg body weight.The inhibitory response decreased in the order Diclofenac(85.44%) > n-hexane-400(75.73%) > n-hexane-200(25.25%) > DCM-400(21.36%). On the other hand the analgesic activity graph showed that the activity was dose dependent as in all test sample activity were high at 200 mg of dose than 400 mg of dose.

In addition, sedative and anxiolytic properties of the two different fractions of *G. densiflorum* were investigated using rodent behavioural models, such as Hole Cross, Open Field for sedative property and Elevated Plus-Maze (EPM) test and Hole Board for anxiolytic potential, respectively. DCM fractions, at the doses of 200 mg/kg and 400 mg/kg, displayed a dose dependent exploratory behaviour (in Hole Cross). In Open Field Test, DCM fraction at the dose of 200mg/kg showed statistically significant ($p<0.001$) exploratory behavior. In the Hole Board Test in mice, DCM fraction at doses of 200mg/kg BW and 400mg/kg BW showed anxiolytic activity which is statistically highly significant ($p<0.001$). In EPM test, DCM fraction with 200mg/kg BWand 400mg/kg BW doses significantly ($p < 0.05$) showed increased exploration and time spent by the DCM fraction treated mice in EPM open arms in a way greater than that of the reference anxiolytic drug Diazepam.

The results of the present study suggest that the root parts of *G. densiflorum* in general; DCM soluble fractions in particular, possess strong potential. Therefore, these findings may justify the use of this plant in folk medicine for the treatment of the aforementioned disease conditions. However, further studies are warranted to clearly understand the underlying mechanism of the observed activities in animal models.

Keywords: Analgesic, Anxiolytic, Sedative, *G. densiflorum*, Dichloromethane Fraction.

List of Abbreviation

BW	Body wieght
CNS	Central Nervous System
DCM	Dichloromethane
EtAc	Ethyl acetate
kg	Kilogram
mg	Milligram
NBF	*n*-butanol fraction
NHF	*n*-hexane fraction
STD	Standard
Sig	Significance
SEM	Standard Error Mean
SD	Standard Deviation

Contents

List of Tables

List of Figures

Dedicated to Almighty Allah and to My Parents

Chapter 01
Introduction

1.1 Medicine in the Ancient Time

All human societies have medical beliefs that provide explanations for birth, death, and disease. Throughout history, illness has been attributed to witchcraft, demons, astral influence, or the will of the gods. These ideas still retain some power, with faith healing and shrines still used in some places, although the rise of scientific medicine over the past millennium has altered or replaced mysticism in most cases.

Life in the ancient world was risky business. The perils of war, disease, famine and childbirth are a just a few examples of circumstances that contributed to a much lower average lifespan in the ancient world than we have in the modern era (Yeomans, 2005). People in ancient time were not less concerned about the prevention and cure of maladies than they are now, however, and entire cults, sanctuaries and professions dedicated to health dotted the spiritual, physical and professional landscapes of the ancient world (Yeomans, 2005).

The ancient Egyptians had a system of medicine that was very advanced for its time and influenced later medical traditions. The Egyptians and Babylonians both introduced the concepts of diagnosis, prognosis, and medical examination (Nunn & John, 2002). The Hippocratic Oath, still taken by doctors today, was written in Greece in the 5th century BCE. In the medieval era, surgical practices inherited from the ancient masters were improved and then systematized in Rogerius's The Practice of Surgery (Herwig et al., 2003) During the Renaissance, understanding of anatomy improved, and the invention of the microscope would later lead to the germ theory of disease (Nunn & John, 2002). These advancements, along with developments in chemistry, genetics, and lab technology (such as the x-ray) led to modern medicine.

Although there is no record to establish when plants were first used for medicinal purposes the use of plants as healing agents is an ancient practice. As far as records go, it appears that Babylonians (about 300 B.C) were aware of a large number of medicinal plants and their properties. Some of the plants, they used, are still in the same purpose. As evident from the papyrus Ebres (written in about 1500 B.C), the ancient Egyptians possessed a good knowledge of medicinal properties of hundreds of plants. Important plant drugs like Henbane (*Hyoscyamas spp.*), Mandrake (*Mandrogora officinarum*), Opium (latex of *Papaver somniferum* fruits), Pomegranate (*Punica granatum*), Castor oil (oil of *Ricinus communis* seeds), aloe (juice of Aloe spp.), Onion (*Allium cepa*) and many others were in common use

in Egypt about 4500 years ago. The use of plants for curing various human ailments figured in ancient manuscripts such as the Bible, the Rig-Vedas, the Iliad, and the Odyssey and the history of Herodotus (Breasted, 1991). The earliest mention of the medicinal use of plants in the Indian subcontinent is found in the Rig-Veda (4500-1600 B.C), which noted that the Indo-Aryans used the Soma plant (Amania muscaria, a narcotic and hallucinogenic mushroom) as a medicinal agent. The Vedas made many references to healing plant Sarpogondha (*Rawalfia serpentine*) while a comprehensive Indian herbal, the Charaka, Samhita cites more than 500 medicinal plants.

From the writings of Sumerians (4000 B.C) who inhabited by the river Tigris and Euphrates, we came to know that their medicine included opium, licorice and mustard (Bryan, 1930).

China also developed a large body of traditional medicine. Much of the philosophy of traditional Chinese medicine derived from empirical observations of disease and illness by physicians and reflects the classical Chinese belief (Bryan, 1930). The earliest known Chinese Pharmacopoeia appeared around 1122 B.C described the use of chaulmoogra oil to treat leprosy.

A towering figure in the history of Greek medicine was the physician Hippocrates (460 BC – 370 BC), considered the "father of modern medicine." Hippocrates and his followers were first to describe many diseases and medical conditions (History of medicine, 1992). The material medica of hippocrates consists of some 300-400 medicinal plants, which included opium, mint, rosemary, sage and verbena (Nunn & John, 2002). Aristotle, A Greek Philosopher (384-322 B.C) included an effort to catalogue the properties of the various medicinal herbs known at that time. The encyclopedic (1st century A.D) de Materia Medica was the forerunner of all modern pharmacopoeia and an authoritative text on botanical medicine.

The Arabs were influenced by, and further developed Greek, Roman and Indian medical practices. Galen, Hippocrates, Sushruta, and Charaka were pre-eminent authorities (Haque, 2004). Abu al-Qasim al-Zahrawi (Abulcasis), who some have called the father of modern surgery, wrote the Kitab al-Tasrif (1000), a 30-volume medical encyclopedia which was taught at Muslim and European medical schools until the 17th century (Haque,2004). In his book he included some numerous surgical instruments, hereditary pattern behind hemophilia, ectopic pregnancy etc (Zahoor & Huq, 1997). He also discusses the medicinal properties of

over thousands natural medicinal substances, most of these are botanical origin but drugs of animal and mineral origin also included.

Thus through experiences over thousands of years, human civilization came to know about herbs that are more powerful than others to heal disease. So people mainly started treatment with the use of substances from plant, animal and mineral sources. Today many plant-based and animal-based medicines are used. Minerals, including sulfur, arsenic, lead, copper sulfate and gold are also used in modern drugs. Isolation of natural analgesic drug morphine from the latex of *Papaver somniferum* capsules (Opium) in 1804 probably the first most important example of natural drug, which plant have directly contributed to modern medicine (Sarah, 2005). Isolation of other important plant-derived drug of modern medicine rapidly followed and many useful drugs have since been discovered and introduced into modern medicine. In addition to these natural drugs of modern medicine, plants have also contributed and are still contributing to the development of modern synthetic drug and medicines.

1.2 Medicines at Present

The fundamental concepts of Modern allopathic medicine have developed from traditional (Unani and Ayurvedic) medicines. The Traditional medicines are playing an important role in human society from the past centuries. Traditional medical practice illustrates the medical knowledge practices, which improved more than centuries ago within a variety of societies before the era of modern Allopathic or Homeopathic medication begins (Van, 2008). Ayurvedic medicine, traditional Chinese medicine, Unani, herbal as well as many other ancient medical practices from all over the world included in these medicines.

Historically, at the end of the twentieth century, a number of traditions came to dominate the practice of traditional medicine. Among all, the herbal medicine system of Ancient Greek and Ancient Roman sources, the Ayurvedic medicine system from India, traditional Chinese medicine, Unani-Tibb medicine and Shamanic Herbalism were the most dominant at the end of the twentieth century.

At present about 55% of present Allopathic or Homeopathic medications, which are using in the world, derived from plant or herb sources. Roughly 40% of plants provide active ingredients for modern drugs or medicines, and because of their use in traditional medicines, they came to the interest of researchers (Gossel et al., 2006). More than 120 active

ingredients presently isolated from the medicinal herbs. More than 8,000 active ingredients in the present pharmacopoeias extracted from plant sources (Imtiaz, 2001).

Among non-industrialized societies, the use of herbs to heal disease is almost universal. Peoples from countries in Latin America, Asia, and Africa are still using herbal products to fulfill their regular health related necessities (Van, 2008). As an example, nearly 75-80 percent of the population in Africa uses traditional medicine to fulfill their basic health related necessities. Another attention-grabbing thing is that presently, in United States, up to 158 million peoples use complementary medicines in their primary health related necessities (Imtiaz, 2001). Ethnobotany, ethnomedicine, and medical anthropology have included as the basic disciplines, which study these medicines.

With the development of Chemistry, the focus was to isolate and identify biologically active compounds from plants, as these were considered to be of greater value therapeutically and they would also provide the starting material for laboratory synthesis of new compounds of even

greater value. This approach to medicinal plant research led to the elucidation of the chemistry of Opium. From this plant, morphine was isolated and many derivatives have been synthesized and are being used medicinally (Gossel et al., 2006). Another example is *Vinca rosea*, which was used traditionally as atidiabetic drug was found to contain hypoglycemic alkaloid principles, and from further studies, it was found to contain anticancer principle vinca alkaloid in a high yield (Gossel et al., 2006).

With technological advancement, phytochemical studies of medicinal plants got a rapid place and the presence of many chemical compounds came into light. These plant-derived compounds often played an important role in directing laboratory synthesis of many new classes of drug molecules. For example, *Rauwolfia serpentina, which* was traditionally used for a variety of illnesses, revealed the presence of an antihypertensive and tranquilizing agent reserpine (Ghani, 2005). So systemic research on medicinal plants may open the door of many unknown therapeutic tools.

In recent years, the use of drugs or medications as well as search for new drugs and dietary supplements derived from traditionally used plant sources have accelerated much. As a result,

many researchers are studying and investigating on these medicines today to find out the proper remedial uses along with pharmacological effects (Van, 2008).

1.3 Medicinal Plants

A medicinal plant is any plant which, in one or more of its organ, contains substance that can be used for therapeutic purpose or which is a precursor for synthesis of useful drugs. The plants that possess therapeutic properties or exert beneficial pharmacological effects on the animal body are generally termed as "Medicinal Plants." Although there are no apparent morphological characteristics in the medicinal plants growing with them, yet they possess some special qualities or virtues that make them medicinally important (Kraus *et al*., 1981). It has now been established that the plants which naturally synthesis and accumulate some secondary metabolites, like alkaloids, glycosides, tannins, volatiles oils and contain minerals and vitamins, possess medicinal properties termed as medicinal plants.

1.4 Medicinal Plants in Global Healthcare

Plant-derived substances have recently become of great interest owing to their versatile applications. Medicinal plants are the richest bio-resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs (Prashant, 2011). The green plant is fundamental to all other life. The oxygen we breathe, the nutrients we consume, the fuel we burn and many of the most important materials we use are produced by plants. Plants represent the first stage in the evolution of living things. The study of disease and their treatment have been existing since the beginning of human civilization.

The importance of plants in search of new drugs is increasing with the advancements of medical sciences. For example, ricin, a toxin produced by the beans of *Ricinus communis*, has been found to be effective as a very potent antitumor drug, HIV inhibitory activity has been observed in some novel coumarins isolated from *Calophyllum lanigerum* (Spalding, 1991), Hypericin from Hypercium species is an anticancer agent. Taxol is another example of one of the most potent antitumor agent found from *Taxus bravifolia* (Gupta, 1992). These are very few examples of contribution of medicinal plant in global health care. Researchers today are emphasizing on evaluation and characterization of various plants and plant constituents against a number of diseases based on their traditional claims of the plants.

Plants are a source of large amount of drugs comprising to different groups such asantispasmodics, emetics, anti-cancer, antimicrobials, analgesics, anti-diarrheal etc. A large number of the plants are claimed to possess the antibiotic properties (e.g.; Penicillium notatum) in the traditional system and are used extensively by the tribal people worldwide. It is now believed that nature has given the cure of every disease in one way or another.

Medicinal components from plants play many important roles in traditional medicine. Their availability, historical use, religious belief make them more popular to people in all countries (Cowan, 1999). It is estimated that there are about 2,500,000 species of higher plants and the majority of these have not yet been investigated in detail for their pharmacological activities (Ram et al., 2003). In developing countries, about 80% of the population relies on traditional medicine for their primary health care (Matu & Staden, 2003).

Since Bangladesh has a vast resource of medicinal plants and majority of our population has to rely upon indigenous system of medication from economic point of view. The high cost of imported conventional drugs and inaccessibility to western health care facility, imply that traditional mode of health care is the only form of health care that is affordable and available to the rural people. On the other hand, even when western health facilities are available, traditional medicine is viewed as an efficient and an acceptable system from a cultural perspective (Munguti, 1997) and as a result, traditional medicine usually exist side by side with western forms of health care.

1.5 Medicinal Plants of Bangladesh

In Bangladesh Unani and Ayurvedic medicines were being prepared from plants following the age-old traditional methods available in literature. These herbal medicines may thus be termed as upgraded traditional herbal medicine or modern herbal medicines. Traditional Medicine is the medicine or treatment based on traditional uses of plants, animals or their products, other natural substances (including some inorganic chemicals), religious verses, cultural practices, and physical manipulations including torture. As this system of medicine has been in use almost unchanged generation after generation throughout the ages for the treatment of various physical and psychological diseases, it is called traditional (Munguti, 1997). Most of the times, the type, preparation, and uses of traditional medicines are largely influenced by folklore customs and the cultural habits, social practices, religious beliefs and, in many cases, superstitions of the people who prescribe or use them.

Being naturally gifted by a suitable tropical climate and fertile soil, Bangladesh possesses a rich flora of tropical plants. Around 5000 species of phanerogams and pteridophytes grow in its forests, jungles, wastelands and roadsides as indigenous, naturalised and cultivated plants (Munguti, 1997). Out of them, more than a thousand have been claimed to posses medicinal and poisonous properties, of which 546 have recently been enumerated with their medicinal properties and therapeutic uses (Ghani, 2003). In addition to possessing various other medicinal properties, 257 of these medicinal plants have been identified as efficacious remedies for diarrhoeal diseases and 47 for diabetes. Treatments in traditional medicine are carried out by internal and external application of medicaments, physical manipulation of various parts of the body, performing rituals, psychological treatment, and by minor surgery.

However, it has been observed that many other medicinal plants growing in the country have not been identified taxonomically and that there are many of them, which have not been chemically examined and no attention has yet been paid to characterise them from the pharmacognostic viewpoint (Munguti, 1997). Thus, it is expected that the number of medicinal plants growing or available in Bangladesh may be more than what has so far been enumerated. It has further been observed that the countless herbs found in Bangladesh should be used for promotion of health and for fighting many diseases. Thus medicinal plants of Bangladesh hold good promises as potential resources for drug development.

Table 1.1: Drugs of plant origin used in modern medicine of Bangladesh, (Ghani, 2005).

Common Name	Botanical Name	Use
Amla	Emblica officinalis	Vitamin - C, Cough , Diabetes, cold, Laxative, Hyperacidity.
Ashok	Saraca Asoca	Menstrual Pain, Uterine disorder, Deiabetes.
Bael / Bilva	Aegle marmelous	Diarrrhoea, Dysentry, Constipation.
Chiraita	Swertia Chiraita	Skin Desease, Burning sensation, Fever.
Kalmegh/ Bhui neem	Andrographis Paniculata	Fever, weekness, release of gas.
Long peeper / Pippali	Peeper longum	Appetizer, enlarged spleen ,

		Bronchities, Cold, antidote.
Pashan Bheda / Pathar Chur	Coleus barbatus	Kidny stone, Calculus.
Sandal Wood	Santalum Album	Skin disorder, Burning, sensation, Jaundice, Cough.
Satavari	Asparagus Racemosus	Enhance lactation, general weekness, fatigue, cough.
Senna	Casia augustifolia	General debility tonic, aphrodisiac.
Tulsi	Ocimum sanclum	Cough, Cold, bronchitis,expectorand
Pippermint	Mentha pipertia	Digestive, Pain killer
Henna/Mehd	Lawsennia iermis	Burning, Steam, Anti Imflamatary
Gritkumari	Aloe Verra	Laxative, Wound healing, Skin burns & care,Ulcer
Sada Bahar	Vincea rosea	Leaukamia, Hypotensiv, Antispasmodic , Atidot
Vringraj	Eclipta alba	Anti-inflamatory, Digestive, hairtonic
Neem	Azardirchata - indica	Sdedative, analgesic, epilepsy, hypertensive
Anantamool/sariva	Hemibi smus Indicus	Appetiser, Carminative, aphrodisiac, Astringent
Kantakari	Solanum Xanthocarpum	Diuretic, Antiinflamatory, Appetiser, Stomachic
Shankhamul	Geodorum denciflorum	Antidiabetic

1.6 Necessity of Studying Medicinal Plant

Medicinal plants have played an essential role in the development of human culture, for example religions and different ceremonies. (E.g. Dutura has long been associated with the worship of Shiva, the Indian god). Many of the modern medicines are produced indirectly from medicinal plants, for example aspirin. Plants are directly used as medicines by a majority of cultures around the world, for example Chinese medicine and Indian medicine. Many food crops have medicinal effects, for example garlic. Medicinal plants are resources of new drugs. It is estimated there are more than 250, 000 flower plant species (Ghani, 2003). Studying medicinal plants helps to understand plant toxicity and protect human and animals from natural poisons. Cultivation and preservation of medicinal plants protect biological diversity, for example metabolic engineering of plants.

Plant resources (E.g. Angiosperm, Gymnosperm, Seedless vascular plants, Bryophytes) for new medicine (Ghani, 2003). The medicinal plants find application in pharmaceutical, cosmetic, agricultural and food industry. With onset of scientific research in herbals, it is becoming clearer that the medicinal herbs have a potential in today's synthetic era, as numbers of medicines are becoming resistant. According to one estimate only 20% of the plant flora has been studied and 60%of synthetic medicines owe their origin to plants (Ghani, 2003). Ancient knowledge coupled with scientific principles can come to the forefront and provide us with powerful remedies to eradicate the diseases. To identify alternative and complementary medicine, to reduce the toxicity of drug therapy especially toxicity reduction of synthetic and semi synthetic drugs, to find the lead compound diversification to treat various diseases a good knowledge about medicinal plants is very important.

1.7 Challenges in Drug Discovery from Medicinal Plants

In spite of the success of drug discovery programs from plants in the past 2–3 decades, future endeavors face many challenges. Natural products scientists and pharmaceutical industries will need to continuously improve the quality and quantity of compounds that enter the drug development phase to keep pace with other drug discovery efforts. The process of drug discovery has been estimated to take an average period of 10 years and cost more than 800 million dollars (Dickson and Gagnon, 2004). Much of this time and money is spent on the numerous leads that are discarded during the drug discovery process. It is estimated that only one in 5000 lead compounds will successfully advance through clinical trials and be

approved for use. In the drug discovery process, lead identification is the first step. Lead optimization (involving medicinal and combinatorial chemistry), lead development (including pharmacology, toxicology, pharmacokinetics, ADME and drug delivery), and clinical trials all take considerable time.

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

1.8 Opportunities in Drug Discovery from Medicinal Plants

Bioprospecting demands a number of requirements which should be co-coordinated, such as team of scientific experts (from all the relevant interdisciplinary fields) along with expertise in a wide range of human endeavours, including international laws and legal understanding, social sciences, politics and anthropology. In our context, Ayurveda and other traditional systems of medicine, rich genetic resources and associated ethnomedical knowledge are key components for sustainable bioprospecting and value-addition processes(Patwardhan, 2005).

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

In order to screen thousands of plant species at one go for as many bioassays as possible, we must have a collection of a large number of extracts. Globally, there is a need to build natural products extract libraries(Patwardhan, 2005) . The extract libraries offer various advantages, such as reduction in cost and time for repeat collection of plants and availability of properly encoded and preserved extracts in large numbers for biological screening in terms of high-throughput screenings and obtaining hits within a short period. Such libraries could serve as a powerful tool and source of extracts to be screened for biological activities using high-throughput assays **(**Federsel, 2003).

Aim and Objective

The objectives of the present work were:

1. To carry out analgesic investigations of the root part of the DCM fraction of *G. densiflorum (Lam.) Schltr* using acetic acid-induced writhing for analgesic activity.
2. To evaluate sedative property of the DCM fraction of *G. densiflorum* using -

    - Hole cross method and
    - Open field method.

3. To explore the anxiolytic potential of the DCM fraction of *H. densiflorum* using –

    - Elevated plus maze test and
    - Hole board method

Rationale of the Work

During the last decades, the renewed interest in investigating natural products has led to the advent of several important drugs, such as the anticancer substances vinblastine, vincristine and taxol, or the anti-malarial agent artemisinin. Success in natural products research is conditioned by a careful plant selection, based on various criteria such as chemotaxonomic data, ethnomedical information, field observations or even random collection.

Scientific and methodical investigation of herbal plants has become a potential source for the discovery of lead compounds of high therapeutic value in terms of analgesic, anti-inflammatory, anticancer, anti-oxidant, anti-bacterial, anti- diarrheal activities. Drug discovery, including isolation from plants and other natural sources, synthetic chemistry, combinatorial chemistry and molecular modeling. Despite the recent interest in molecular modeling, combinatorial chemistry and other synthetic chemistry techniques, natural products and particularly medicinal plants, remain an important source of new drugs, new drug leads and new chemical entities.

Orchidaceae are cosmopolitan, occurring in almost every habitat apart from glaciers. A range of different approaches as described earlier has been employed to obtain lead compounds for drug discovery, antioxidant, cytotoxic, antibacterial and hepatoprtective activity of different orchid plants. From previous investigation the presence of different active compounds such as, glycoside, carbohydrate, falovonoid, steroid, polyphenol, alkaloid etc. were found in different plant under Orchidaceae family. *Geodorum densiflorum (Lam.) Schltr*., locally known as 'Shankhamul' in Bangladesh, is a medicinal herb, under Orchidaceae family and *Geodorum* genus. It has long been used in the Indian subcontinent as a folk medicine for the treatment of a variety of diseases of different etiology as analgesic, antiinflammatory, antioxidant, cytotoxic (Paresh and Chanda, 2008; Yusuf et al., 1994; Kirtikar and Basu, 1980; Habib et al.,2011).

However, only a few phytochemical and biological works of medicinal interest have so far been carried out on this plant to substantiate the above traditional claims. In view of this, the present study was designed to observe the central nervous system activity and analgesic activity of the DCM fraction of the respective plant *G. densiflorum (Lam) schltr*. The obtained result was compared to the n-hexane fraction of *G. densiflorum*.

Chapter 02
Plant Detail

2.1 The Plant Family-Orchidaceae

The family Orchidaceae is the most diverse and advanced of the angiosperms with over 800 genera and more than 25.000 species(Bell 1994). The word Orchid is derived from a Greek word Orchis meaning testicle because of the appearance of subterranean tubers of the genus Orchis (Batygina et al., 2003). Orchids are perennial herbs. The stems in most species, for example the genus *Bulbophyllum*, are thickened to form pseudobulbs that function in the storage of nutrients and water for use during dry periods. Orchids are either terrestrial (growing on soils), epiphytes (growing on plants but not parasitizing on them) or lithophytes (growing on rocks and sand grains). They produce dust like seeds (often thousands to a million) in dehiscent capsules that have three to six longitudinal slits. The seeds lack endosperms and so depend entirely on the establishment of a mycorrhizal association for their germination and establishment (Allen, 1992; Judd *et al*., 1999; Lambers *et al*., 1998; Sylvia, 1994).

The name comes from the Greek word (órkhis), literally meaning "testicle", because of the shape of the root. Orchid was introduced in 1845 by John Lindley in School Botany, due to an incorrect attempt to extract the Latin stem (orchis) from Orchidaceae (Stevens, 2008).

These flowers were previously called Orchis, Satyrion (Satyrion feminina), or "ballockwort" (Stevens, 2008).

2.1.1 Geographical Distribution

Orchidaceae are cosmopolitan, occurring in almost every habitat apart from glaciers. The world's richest concentration of orchid varieties is found in the tropics, mostly Asia, South America and Central America, but they are also found above the Arctic Circle, in southern Patagonia, and even two species of Nematoceras on Macquarie Island, close to Antarctica (K. jenny, 2011)

The following list gives a rough overview of their distribution:

- Tropical Asia: 260 to 300 genera
- Tropical America: 212 to 250 genera
- Tropical Africa: 230 to 270 genera
- Oceania: 50 to 70 genera

- Europe and temperate Asia: 40 to 60 genera
- North America: 20 to 26 genera

2.1.2 Botanical Features of Orchidaceae

Leaves: Orchids generally have simple leaves with parallel veins, although some Vanilloideae have a reticulate venation. They are normally alternate on the stem, often plicate, and have no stipules. Orchid leaves often have siliceous bodies called stegmata in the vascular bundle sheaths (not present in the Orchidoideae) and are fibrous (Orchid, 2010).

Figure 2.1: Orchid leaf (Stevens, 2001)

The structure of the leaves corresponds to the specific habitat of the plant. Species that typically bask in sunlight, or grow on sites which can be occasionally very dry, have thick, leathery leaves and the laminae are covered by a waxy cuticle to retain their necessary water supply. Shade species, on the other hand, have long, thin leaves (Orchid, 2010).

The leaves of most orchids are perennial, that is, they live for several years, while others, especially those with plicate leaves, shed them annually and develop new leaves together with new pseudobulbs, as in Catasetum. The leaves of most orchids are considered ornamental (Orchid, 2010).

Flowers: Orchidaceous are well known for the many structural variations in their flowers. Some orchids have single flowers sometimes with a large number of flowers. The orchid flower, like most flowers of monocots, has two whorls of sterile elements. The outer whorl has three sepals and the inner whorl has three petals. Orchid flowers with abnormal numbers of petals or lips are called peloric (Ramirez, 2007).

Fruits and Seeds: The ovary typically develops into a capsule that is dehiscent by three or six longitudinal slits, while remaining closed at both ends. The ripening of a capsule can take two to 18 months. The seeds are generally almost microscopic and very numerous, in some species over a million per capsule. After ripening, they blow off like dust particles or spores. They lack endosperm and must enter symbiotic relationships with various mycorrhizal basidiomyceteous fungi that provide them the necessary nutrients to germinate, so that all orchid species are mycoheterotrophic during germination and reliant upon fungi to complete their lifecycles (Ramirez, 2007).

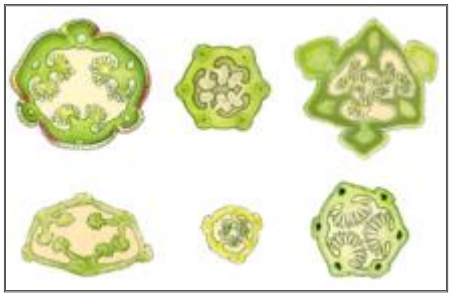

Figure 2.2: Cross-section of an orchid capsule, the longitudinal slits (King & Jenny, 2011)

As the chance for a seed to meet a fitting fungus is very small, only a minute fraction of all the seeds released grow into adult plants. In cultivation, germination typically takes weeks, while there is a report of one paphiopedilum that took fifteen years.

Stem and Roots: All orchids are perennial herbs, lack any permanent woody structure, and can grow according to two patterns:

Monopodial: The stem grows from a single bud, leaves are added from the apex each year and the stem grows longer accordingly. The stem of orchids with a monopodial growth can reach several metres in length, as in Vanda and Vanilla.

Sympodial: The plant produces a series of adjacent shoots which grow to a certain size, bloom and then stop growing, to be then replaced. Sympodial orchids grow laterally rather than vertically, following the surface of their support. The growth continues by development of new leads, with their own leaves and roots, sprouting from or next to those of the previous year, as in Cattleya. While a new lead is developing, the rhizome may start its growth again from a so-called 'eye', an undeveloped bud, thereby branching.

Terrestrial orchids may be rhizomatous or form corms or tubers. The root caps of terrestrials are smooth and white.Some sympodial terrestrials, such as Orchis and Ophrys, have two ubterranean tuberous roots. One is used as a food reserve for wintry periods, and provides for the development of the other one, from which visible growth develops (Orchid, 2010).

Figure 2.3: *Anacamptis lactea* showing the two tubers (Stevens, 2001)

With ageing, the pseudobulb sheds its leaves and becomes dormant. At this stage it is often called a backbulb. A pseudobulb then takes over, exploiting the last reserves accumulated in the backbulb, which eventually dies off, too. A pseudobulb typically lives for about five years (Orchid, 2010).

Reproduction:

Pollination: Orchids have developed highly specialized pollination systems, thus the chances of being pollinated are often scarce, so orchid flowers usually remain receptive for very long periods, and most orchids deliver pollen in a single mass. Each time pollination succeeds, thousands of ovules can be fertilized.

Pollinators are often visually attracted by the shape and colours of the labellum. The flowers may produce attractive odours. Although absent in most species, nectar may be produced in a spur of the labellum, on the point of the sepals or in the septa of the ovary, the most typical position amongst the Asparagales.After pollination, the sepals and petals fade and wilt, but they usually remain attached to the ovary.

Asexual reproduction: Some species, such as Phalaenopsis, Dendrobium and Vanda, produce offshoots or plantlets formed from one of the nodes along the stem, through the accumulation of growth hormones at that point. These shoots are known as keiki.

2.1.3 General uses of Orchidaceae

The scent of orchids is frequently analysed by perfumers (using headspace technology and gas-liquid chromatography) to identify potential fragrance chemicals.

The other important use of orchids is their cultivation for the enjoyment of the flowers. Most cultivated orchids are tropical or subtropical, but quite a few which grow in colder climates can be found on the market. Temperate species available at nurseries include Ophrys apifera (bee orchid), Gymnadenia conopsea (fragrant orchid), Anacamptis pyramidalis (pyramidal orchid) and Dactylorhiza fuchsii (common spotted orchid).

Orchids of all types have also often been sought by collectors of both species and hybrids. As such, many hundreds of societies and clubs worldwide have been established. These can be small, local clubs such as the Sutherland Shire Orchid Society, or larger, national organisations such as the American Orchid Society. Both serve to encourage cultivation and collection of orchids, but some go further by concentrating on conservation or research (Focho et al; 2010).

The term "botanical orchid" loosely denotes those small-flowered, tropical orchids belonging to several genera (not necessarily related to each other) that do not fit into the "florist" orchid category. A few of these genera contain enormous numbers of species. Some, such as Pleurothallis and Bulbophyllum, contain approximately 1700 and 2000 species, respectively, and are often extremely vegetatively diverse. The primary use of the term is among orchid hobbyists wishing to describe unusual species they grow, though it is also used to distinguish naturally occurring orchid species from horticulturally created hybrids (Focho et al;2010).

Use as Food

The dried seed pods of one orchid genus, Vanilla, are commercially important as flavoring in baking, for perfume manufacture and aromatherapy.

The underground tubers of terrestrial orchids [mainly Orchis mascula (early purple orchid)] are ground to a powder and used for cooking, such as in the hot beverage salep or in the Turkish frozen treat dondurma. The name salep has been claimed to come from the Arabic expression ḥasyu al-tha`lab, "fox testicles", but it appears more likely the name comes directly from the Arabic name saḥlab. The similarity in appearance to testes naturally accounts for salep being considered an aphrodisiac (Focho et al;2010).

The dried leaves of Jumellea fragrans are used to flavor rum on Reunion Island. Some saprophytic orchid species of the group Gastrodia produce potato like tubers and were consumed as food by native peoples in Australia and can be successfully cultivated, notably Gastrodia sesamoides. Wild stands of these plants can still be found in the same areas as early aboriginal settlements, such as Ku-ring-gai Chase National Park in Australia. Aboriginal peoples located the plants in habitat by observing where bandicoots had scratched in search of the tubers after detecting the plants underground by scent (Focho et al;2010).

Traditional Medicinal Uses

Orchids have been used in traditional medicine in an effort to treat many diseases and ailments. They have been used as a source of herbal remedies in China since 2800 BC.In the recent years, a number of studies have been published on anticancer activity of the chemical moscatilin, which is found in the stems of the orchid species Dendrobrium (Focho et al;2010).

2.2 Introduction to the genus *Geodorum*:

Geodorum is a genus of flowering plants from the orchid family, Orchidaceae. This is a genus of about 20 species widely distributed in tropical and subtropical areas with a single non-endemic species, *Geodorum densiflorum*, occurring in tropical and subtropical parts of Australia, in New South Wales extending south to the Macleay. Other states are Western Australia, Northern Territory, Queensland, New South Wales (Seidenfaden, 1983). The biological features of the members of the genus *Geodorum* are same as the family Orchidacea.

Table 2.1: Some examples of *Geodorum* genus (Dockrill, 1967).

Name of the species	Name of the species
G. appendiculatum	*G. neocaledonicum*
G. attenuatum	*G. nutans*
G. bicolor	*G. pacificum*
G. bracteatum	*G. pallidum*
G. candidum	*G. parviflorum*
G. citrinum	*G. pictum (Painted Orchid)*
G. densiflorum (Ground Gem Orchid)	*G. plicatum*
G. densifolium	*G. laoticum*
G. dilatatum	*G. laxiflorum*
G. duperreanum	*G. longifolium*
G. eulophioides	*G. pulchellum*
G. formosanum	*G. purpureum*
G. fucatum	*G. rariflorum*
G. siamense	*G. terrestre*

2.3 Introduction to *Geodorum densiflorum (Lam.) Schltr*

2.3.1 Taxonomical Classification of *Geodorum densiflorum (Lam.) Schltr*

Kingdom - Plantae

Subkingdom - Viridaeplantae

Infrakingdom - Streptophyta

Division - Tracheophyta

Subdivision - Spermatophytina

Infradivision - Angiospermae

Class - Magnoliopsida

Superorder - Lilianae

Order - Aspargales

Family - Orchidaceae

Genus - *Geodorum*

Species - ***Geodorum densiflorum (Lam.) Schltr***.

(ITIS Report, www.itis.gov)

.3.2 Botanical Description of *G. densiflorum*

Geodorum densiflorum have partly emergent pseudo-bulbs and broad pleated leaves that are distinctly stalked. All of the leaf stalks on a pseudo-bulb are enclosed together by several tubular bracts. Inflorescences are unbranched and characteristically nod in bud and flower, straightening and elongating as the capsules develop.

Figure 2.4: Aerial part with flower (Habib et al., 2011)

Figure 2.5: Cultivated plant of *G. densiflorum* (Habib et al., 2011)

The flowers are relatively small, crowded and in the native species remain semi-tubular with the segments not spreading widely. The labellum, which is stiffly but flexibly attached to the apex of the column foot, is 3-lobed with large lateral lobes, a short midlobe and a ridged or keeled callus. The column is short and broad with a short column foot (Garay,1974).

2.3.2.1 Common Names

Tosaka Meoto-Ran(Japanese common name) , Shankhamul (Bengali common name), Nodding Swamp Orchid (English common name).

2.3.2.2 Description of Each Individual Part of *G. densiflorum (Lam) Schltr*

G. densiflorum is a glabrous plant like other orchids. It is deciduous and grow during the spring and summer months, becoming deciduous in autumn and winter (Ramirez,2007). Description of each individual part of *G. densiflorum* is given below:

Leaves: leaves of *G. densiflorum* are 3-5 per shoot, deciduous annually, plicate, stalked, the petioles of all leaves form a pseudostem and enclosed together in 2-4 common sheathing bracts.

Roots and Stems: Roots of this orchid are filamentus. Stems are pseudo bulbous, subterranean or partially emergent, multinoded.

Peduncle and Rhachis: Peduncle is the distal part nodding in flower, straightening in fruit with few several tubular or semi-tubular sterile bracts. Peduncle is longer than rhachis. Rachis is the flowering part of the stalk, which is shorter than peduncle in *G. densiflorum.*

Flower: The floral bracts of this orchid are narrow, scarious, partially sheathing the base of the pedicel. Pedicle are shirt and merging with the ovary. Ovary of this orchid is short and straight. The flowers are resupinate, small, crowded, lasting 2-several days, opening sequentially, white, pink or reddish, pedicellate, scentless. The flower petals are subsimilar to the sepals, often asymmetric. Spur is absent in the flower. But pollinarim is present. Pollinarim has 2 pollinia, those are grooved, hard, waxy and sessile. Pollination of orchid flower usually occurs by insects. *Geodorum densiflorum* flowers in summer (wet season).

Capsule and Seeds: Capsules are dehiscent, glabrous, pendant; peduncle elongated in fruit; pedicel not elongated in fruit. Seeds are numerous, light coloured, winged. Chromosomal number of this orchid plant is 2n=52.

2.6.2.3 Ecology

Geodorum densiflorum is widespread in a range of habitats including rainforest, especially monsoonal vine thickets, rainforest margins, open forest, heathland and grassland, usually in well-drained soil, sometimes in sites that are seasonally moist (Geodorum densiflorum (Lam.) Schltr. 1919).

2.3.2.4 Reproduction

Reproduction in Geodorum is solely from seed. Seed dispersal takes 2-4 months from pollination and after fertilisation the peduncle straightens and the capsules develop in a pendant position. Apomixis is unknown in the genus (Ramirez,2007).

2.4 Research Done on *Geodorum* genus

2.4.1 Phytochemical Investigation of *Geodorum densiflorum* (Lam.) Schltr

Previous phytochemical investigation on *G. densiflorum* (Lam.) Schltr. was reported to investigate for the chemical constituents of methanol, ethyl acetate and petroleum ether extracts of the roots of Geodorum densiflorum. Phytochemical screening revealed the presence of carbohydrate, alkaloid, glycoside and steroid (Habib et al, 2011).

2.4.2 Phytochemical Investigation in *Geodorum* genus

Previous phytochemical investigation of the *Geodorum* genus was reported on Geodorum *laxiflorum* to investigate phytochemical property of methanolic extract of the respective orchid. This Phytochemical test confirmed the presence of flavonoid, polyphenols, strols and carbohydrates in methanolic extracts of the tuber (Besra et al, 2011).

2.4.3 Identification of Fungal Endophytes in Orchidacea Family

The previous study of fungal identification based on ITS sequences have precisely identified two important fungi belonging to trichomaceae members *Aspergillus terreus* and *Penicillium aculeatum* based on occurring as endophytes in orchid roots. Endophytes, are now considered as an important source of bioactive natural products, because they occupy unique biological niches as they grow in so many unusual environments. Endophytes colonizing photosynthetic orchids are recently studied (Kasmir et al, 2011).

2.4.4 In Vitro Germination and Microprpagation of *G. densiflorum* (Lam) Schltr

To investigate the in vitro germination of *G. densiflorum* (Lam) Schltr, seeds of the respective plant were aseptically cultured on 0.8% (w/v) agar solidified Murashige and Skoog (MS) and Phytamax (PM) media. Seeds germinated and formed light green globular structures on both the media (Besra et al, 2011).

2.4.5 In Vitro Antioxidant Study in *Geodorum* genus

Previous phytochemical investigation of the *Geodorum* genus was reported on *Geodorum laxiflorum* to investigate the anti-oxidant property of methanolic extract of the respective

orchid. In in vitro antioxidant study, extract has shown significant free-radical inhibiton activity (Besra et al, 2011).

2.4.6 In Vitro Antioxidant Study of *Geodorum densiflorum (Lam.) Schltr*

Previous phytochemical investigation on *G. densiflorum* (Lam) Schltr. was reported to investigate the anti oxidant property of methanol, ethyl acetate and petroleum ether extracts of the roots Geodorum densiflorum Linn. In vitro antioxidant activity of the extracts was performed using DPPH radical scavenging, nitric oxide (NO) scavenging, reducing power, CUPRAC, total antioxidant capacity, total phenol and total flavonoid content determination assays (Habib et al, 2011).

2.4.7 Cytotoxic Investigation of *Geodorum densiflorum* (Lam.) Schltr in Brine Shrimp

Previous phytochemical investigation on *G.densiflorum* (Lam.) Schltr. was reported to investigate the cytotoxic property of methanol, ethyl acetate and petroleum ether extracts of the roots *G. densiflorum.* All three extracts were subjected to brine shrimp lethality bioassay for possible cytotoxicity where a concentration dependent increment in percent mortality of brine shrimp nauplii was produced by the extracts indicating the presence of cytotoxic principles in these extractives (Habib *et al*, 2011).

2.4.8 In Vivo Hepatoprotective Activity of *Geodorum* genus

Previous investigation of the *Geodorum* genus was reported on *Geodorum laxiflorum* to investigate the hepatoprotective property of methanolic extract of the respective orchid. The methanolic extract of the respective plant was administered orally in the mice in two different dose to observe the hepatoprotective activity and compared with 100 mg kg-1 silymarin as standard. Methanolic extract showed prominent reduction in elevated hepatic marker enzymes and significant. In histopathological examination it is observed that extract has significant hepatoprotective activity (Besra et al, 2011).

2.4.9 Antibacterial Assay of *Geodorum densiflorum (*Lam.) Schltr

Previous investigation on *G.densiflorum* (Lam) Schltr. was reported to investigate the anti bacterial property of methanol, ethyl acetate and petroleum ether extracts of the roots *G. densiflorum*. The antibacterial assay was performed by using the disc diffusion method where

thirteen pathogenic bacteria were used as test organisms. Ethyl acetate extract displayed the highest antibacterial actions expressed in terms of diameter of zone of inhibition (Habib et al, 2011).

Chapter 03
Materials and Methods

3.1 Materials

The following materials were used during the course of pharmacological investigations-

3.1.1 Reagents, Chemicals, and Equipments Used for Analgesic Activity

Table 3.1: Reagents and chemicals for acetic acid induced writhing test

	Reagents & chemicals	Source
1	Acetic acid	Merck, Germany
2	Tween-80	BDH Chemicals Ltd
3	Diclofenac Na	Square pharmaceuticals
4	Normal saline solution (0.9% NaCl)	Beximco Infusion Ltd.

Table 3.2: Equipments for acetic acid induced writhing test

Equipments			
1	Rough balance	**6**	Glass rod
2	Electrical balance	**7**	Feeding needle
3	Vial	**8**	Syringe
4	Spatula	**9**	Others: Gloves, cotton, tissue
5	Pipette and pipette pumper		

3.1.2 Reagents, Chemicals and Equipments Used for CNS Activity

Table 3.3: Reagents and Chemicals Used for CNS Activity

Reagents and chemicals		Source
1	Diazepam (standard for anxiolytic activity)	Square pharmaceuticals
2	Tween 80 (as suspending agent)	BDH chemicals
3	Saline water (0.9% Nacl)	Beximco infusion LTD.

Table 3.4: Equipments used for CNS activity

Equipments			
1	Rough balance	**7**	Feeding needle
2	Electrical balance	**8**	Open field apparatus
3	Vial	**9**	Hole cross apparatus
4	Spatula	**10**	Hole board
5	Pipette and pipette pumper	**11**	Elevated plus maze
6	Glass rod	**12**	Others: cotton, tissue, gloves, handicam, stopwatch

3.2 Preparation of Plant Extract for Experiment

3.2.1 Collection of G*eodorum Densiflorum (Lam.) Schltr*

The fresh roots of the plant *G. densiflorum*, which is locally known as Shankhamul, was collected from the area of Savar, Dhaka district during the month October, 2011.

3.2.2 Drying of the Root

The collected roots of the plant(around 4 kilogram) were dried after cutting and slicing in the sun for about two weeks. In general the plant material should be dried at temperature below 30^0c to avoid the decomposition of thermolabile compounds. The roots were dried in the sun light thus chemical decomposition cannot take place.

3.2.3 Grinding of the Dried Roots

After drying, the roots were weighed in an electrical balance and the total weight was found to be 3.20 kg (three kilogram and two hundred gram). The dried roots were ground to coarse powder with a mechanical grinder. Before grinding of sample, the grinder was thoroughly cleaned to avoid contamination with any remnant of previously ground material or other foreign matter deposited on the grinder. Grinding improves the efficiency of extraction by increasing surface area. After grinding, the weight of the grinded root was measured and the weight was about 330 gm. All grinded roots were stored in an airtight container.

3.2.4 Maceration of Dried Powdered Sample

Principle of Maceration: In maceration (for fluid extract), whole or coarsely powdered plant-drug is kept in contact with the solvent in a stoppered container for a defined period with frequent agitation until soluble matter is dissolved. (P tiwari *et al*, 2011). The principle this process is that when a solid material comes in contact with a solvent, the soluble components in the solid material move to the solvent. Thus, solvent extraction of plant material results in the mass transfer of soluble active principle (medicinal ingredient) to the solvent, and this takes place in a concentration gradient. The rate of mass transfer decreases as the concentration of active principle in the solvent increases, until equilibrium is reached (J singh, 2009) Thereafter, there will be no more mass transfer of the active principle from plant material to the solvent.

Procedure: After getting the sample as dried powdered, the sample (330 gm) was then soaked in 800mL of methanol for seven days. This process is termed as maceration. A glass made jar with plastic cover was taken and washed thoroughly with ethanol and dried. Then the dried powder sample was taken in the jar. After that methanol (800mL) was poured into the jar up to 1-inch height above the sample surface as it can sufficiently cover the sample surface. The plastic cover with aluminium foil was closed properly to resist the entrance of air into the jar. This process was performed for seven days. The jar was shaked in several times during the process to get better extraction.

3.2.5 Filtration of the Extracts

After the extraction process the plant extracts was filtered with sterilized cotton filter. The cotton was rinsed with ethanol and fitted in a funnel. The filtrate was collected in a beaker. Then again it was filtered and this time What man's filter was used for getting more clear extract which would be useful making the sample more concentrate in Rotary Evaporation Technique. Then the filtrate was taken into a volumetric flask and covered with alumina foil paper and was prepared for rotary evaporation.

3.2.6 Sample Concentration by Rotary Evaporation Technique

Figure3.1: Rotary Evaporator in EWU Laboratory (IKA ®RV05 Basic, Biometra)

Principle: A rotary evaporator is a specially designed instrument for the evaporation of solvent (single-stage or straight distillation) under vacuum. The evaporator consists of a heating bath with a rotating flask, in which the liquid is distributed as a thin film over the hot wall surfaces and can evaporate easily. The evaporation rate is regulated by the heating bath

temperature, the size of flask, the pressure of distillation and the speed of rotation (Rosemary, 1998).

Procedure: After the filtration process two parts were obtained namely 'residue part' and 'filtered part or filtrate'. The filtered part, which contains the substance soluble in methanol, was putted into a1000ml round bottom flask (BOROSIL), and then the flask was placed in a rotary evaporator. The evaporation was done at 45^0c temperature. The number of rotation per minute was selected as 120 rpm. The pressure of the vacuum pumper machine was 6bar. The water flow through the distillation chamber was also provided in a satisfactory flow rate. When the evaporation seemed to be satisfactory, then the methanolic extract was collected in a 50mL beaker. The evaporator flask was rinsed by acetone. Then the beaker was covered with aluminum foil paper and kept for 60 minutes. Finally the concentrated methanolic plant extract was found and stored in the laboratory refrigerator from which the extract was used for many chemical investigations.

3.2.7 Solvent –solvent partitioning of methanolic extracts

3.2.7.1 Preparation of Mother Solution

Solvent–solvent partitioning of the crude aqueous methanolic extract of the root parts of *G. densiflorum* was done using the protocol described by Rahman et al. (1999) following the modiefied kupchan partitioning mthod. The concentrated methanolic plant extract obtained from rotary evaporation technique is the mother solution(35.67 gm) which was partitioned off successively by four solvents namely N-hexane, Dichoromethane, Ethyl Acetate, N-butanol.

3.2.7.2 Partitioning with *n*-hexane

Slurry of concentrated methanolic extract of *Geodorum densiflorum* was made with water. The slurry was taken in a separating 100 ml of the *n*-hexane was added to it and the funnel was shaken and then kept undisturbed. The organic portion was collected. The process was repeated thrice. The n-hexane fraction of root parts of the plants was found to be concentrated.

3.2.7.3 Partitioning with Dichloromethane

To the mother solution left after washing with *n*-hexane, 12.5 ml of distilled water was added and mixed. The mother solution was then taken in a separating funnel and extracted with 100 mL of dichloromethane. The Dichloro methane fraction (lower fraction) was collected .The process was repeated three times. The Dichloro methane fraction of root parts of the plants was found to be concentrated.

3.2.7.4 Partitioning with Ethyl acetate

To the mother solution that left after washing with *N*-hexane and Dichoromethane, 16 ml of distilled water was added and mixed uniformly. The mother solution was then taken in a separating funnel and extracted with 100mL of Ethyl Acetate. The process was repeated thrice. The Ethyl Acetate soluble fractions of root parts of the plant were collected together anf found to be concentrated.

3.2.7.5 Partitioning with N-butanol

To the remaining mother solution of concentrated methanolic extract of *Geodorum densiflorum* distilled water was added to make the slurry. The slurry was taken in a separating funnel and extracted with 1000 ml of N-butanol. The N-butanol fraction (lower fraction) was collected .The process was repeated thrice. The N-butanol fraction of root parts of the plants was found to be concentrated.

Table 3.6: Amount of fractions after fractionation of crude methanolic extract

Name of the plant	Part	Fraction	Weight (gm)
Local name- Shankhamul Scientific name- *G. densiflorum*	Root	*n*-Hexane fraction (HF)	9.62 gm
		Dichloromethane (DCM)	21.37gm
		Ethyl Acetate (EtAc)	6.67gm
		n-butanol fraction (BF)	8.65gm

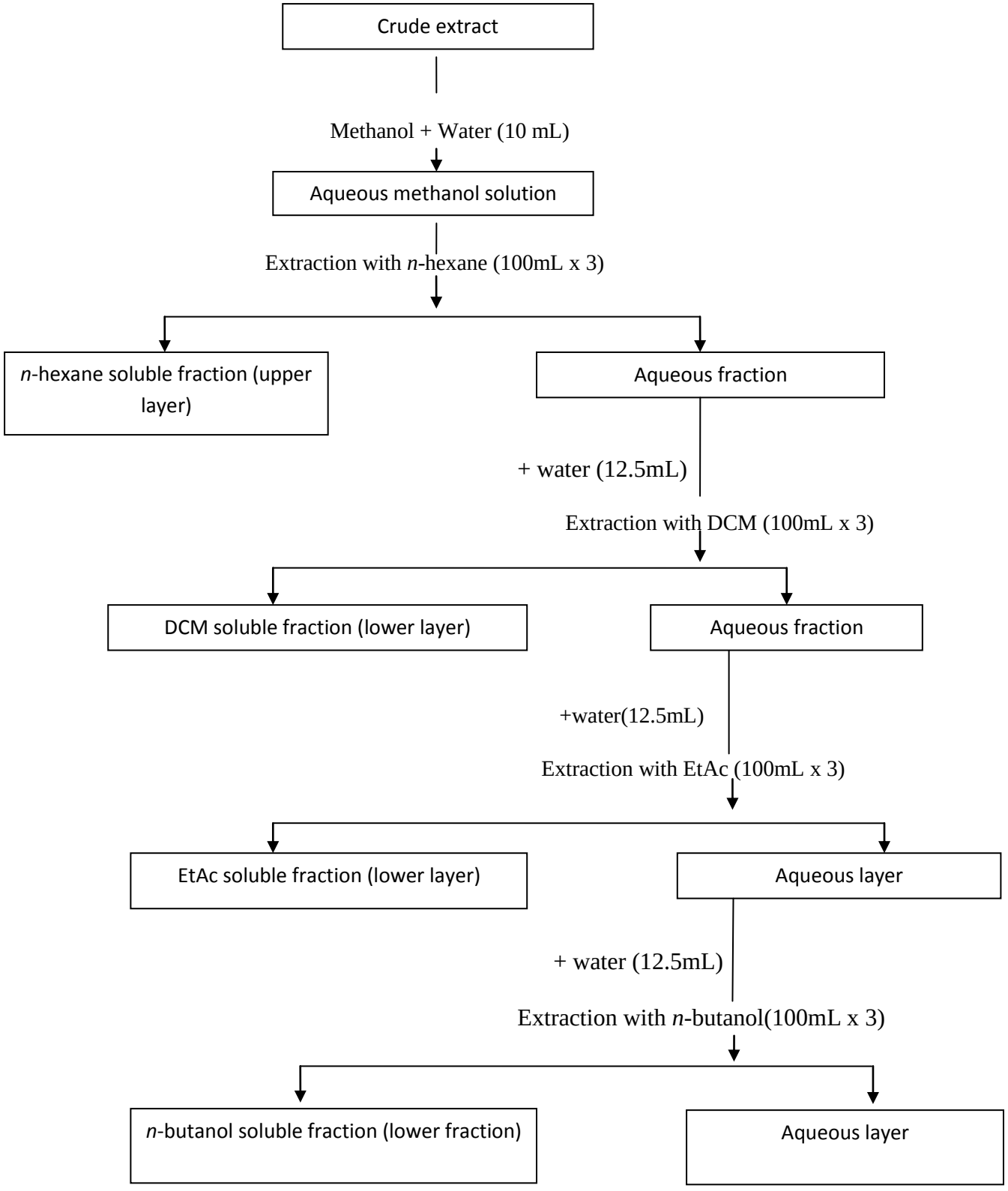

Figure 3.2: Schematic representation of the modified Kupchan Partitioning of methanolic crude extract of *G. densiflorum*

3.3 Collection and Maintenance of Animals Used in Pharmacological Investigation Involving Animal Experimental Models

For the analgesic and CNS activity related experiments, Swiss Albino mice of either sex, 3-4 weeks of age, weighing between 20-25 gm, were collected from the Animal Research Branch of the International Center for Diarrhoeal Disease and Research, Bangladesh (ICDDR, B). They were housed in groups of 5, in plastic cages having dimension of (28×22×13 cm). Soft wood shavings were used as bedding of cages. Animals were maintained under standard environmental conditions (temperature: (24.0±1.0 ˚C), relative humidity: 55-65% and 12 hrs light/12 hrs dark cycle). Husk and excreta were removed from the cages every day. Pellets of mice foods, provided by ICDDR, B were given to the mice with fresh water *(*Hasan et al., *2009)*. The newly bought mice and rats 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.

Figure 3.3: Swiss Albino Mice (The Jackson laboratory, 2012)

3.4 Pharmacological Investigation of *G. densiflorum (Lam.) Schltr*

3.4.1 Test for Analgesic Activity

3.4.1.1 Experimental Design

Thirty experimental animals were randomly selected and divided into six groups denoted as experimental group *Geodorum densiflorum* n-hexane part (200mg, 400mg) and Dichloromethane part(200mg, 400mg) positive control group & negative control group. Each group of mouse was weighed properly & dose of the test sample & control materials was adjusted accordingly.

3.4.1.2 Preparation of Test Materials

The test sample of N-hexane and Dichloromethane was administered at dose 200 & 400 mg/kg body weight of mice of test groups. The test sample was prepared by calculating of mice body weight & was triturated in unidirectional way by the addition of 1.5 ml of distilled water. For proper mixing, small amount of suspending agent Tween-80 was slowly added. The final volume of the suspension was made 3 ml. To stabilize the suspension it was stirred well.

For the preparation of positive control group (10 mg/kg) Diclofenac is taken & a suspension of 3 ml is made and the vehicle control group was treated with 1% Tween 80 in water at the dose of 10 ml/kg body weight.

Table 3.7: Test sample used in evaluation of analgesic activity

Test sample	Group	Purpose	Dose	Root of administration
1% Tween-80 in saline	1	Control group	0.1 ml/10 gm	Oral
Diclofenac	2	Positive control	10mg/kg	Oral
Dichloromethane part	3	Test sample	200 mg/kg	Oral
	4		400 mg/kg	
n-hexane part	5	Test sample	200 mg/kg	Oral
	6		400 mg/kg	

3.4.1.3 Identification of Animals During Experiment

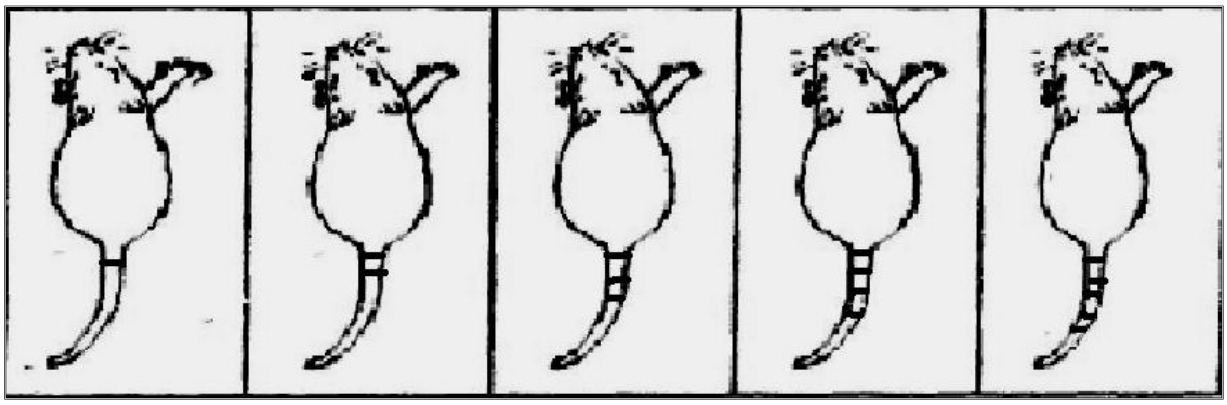

Figure3.4: Identification of test animals for analgesic property screening

Each group consists of five mice/rats and hence it is difficult to identify and observe at a time five mice/rats receiving same treatment. Thus, it was important to identify individual animal of a group during the treatment. The animals were individualized by marking: marked as M1=mice 1, M2=mice 2, M3=mice3, M4=mice 4 & M5=mice 5.

3.4.1.4 Acetic Acid-Induced Writhing Test

The analgesic activity of the samples was studied using acetic acid-induced writhing model in mice (Ahmed et al., 2004; Hasan et al., 2009) . the method described by (Koster et al., 1959). The animals were divided into control, positive control and test groups with five mice in each group. The animals of test groups received test samples at the doses of 200 and 400 mg/kg body weight. Positive control group received standard drug diclofenac at the dose of 10 mg/kg body weight and vehicle control group was treated with 1% Tween 80 in water at the dose of 10 ml/kg body weight. Test samples and vehicle were administered orally 30 min before intraperitoneal administration of 0.7% acetic acid but diclofenac was administered 15 min before injection of acetic acid. After an interval of 5 min, the mice were observed for specific contraction of body referred to as 'writhing' for the next 10 min. Intraperitoneal administration creates pain sensation to the experimental animals and they squirm their bodies at a regular interval termed as "writhing" (khatun et al, 2012).

After 5 minutes interval, the number of writhe (i.e. abdominal contractions and stretches) were counted and recorded for 10 minutes. Analgesic agents reduce the pain sensation which could be observed from reduced number of writhing compared to control group. As the

negative control group (normal saline group) contains no known compounds with analgesic properties (only saline and tween 80 present), the group's response (writhing) was considered as maximum and the percentage of writhing inhibion was minimum, for other groups % of writhing and % of writhin inhibition were calculated based on the following formula:

$$\%\ of\ writhing = \left(\frac{Mean\ writhing\ of\ control\ group - Mean\ writhing\ of\ the\ test\ group}{Mean\ writhing\ of\ the\ control\ group}\right) \times 100\%$$

$$\%\ of\ writhing\ inhibition\ =\ 100\ -\ \%\ of\ writhing$$

3.4.1.5 Procedure of the Experiment

1. The body weight of 30 mice (5X6=30) was weighed using a rough balance machine. From the average body weight of each group doses were calculated and prepared.
2. Test samples and vehicle were administered orally 30 min before intraperitoneal administration of 0.7% acetic acid and diclofenac was administered 15 min before injection of acetic acid.
3. After an interval of 5 min, each mouse of all groups were observed individually for specific contraction of body referred to as 'writhing' for the next 20 min. Full writhing was not always accomplished by the animal because sometimes the animals started to give writhing but they did not complete it. This incomplete writhing was considered as half writhing. Accordingly, two half was writhing, then taken as one full writhing.

3.4.2 Test for CNS Activity

3.4.2.1 Experimental Design

Thirty experimental animals were randomly selected and divided into six groups denoted as experimental group Geodorum densiflorum n-hexane part (200mg, 400mg) and Dichloromethane part(200mg, 400mg) positive control group & negative control group. Each group of mouse was weighed properly & dose of the test sample & control materials was adjusted accordingly.

3.4.2.2 Preparation of Test Material

The test sample of N-hexane and Dichloromethane was administered at dose 200 & 400 mg/kg body weight of mice of test groups. The test sample was prepared by calculating of mice body weight & was triturated in unidirectional way by the addition of 1.5 ml of distilled water. For proper mixing, small amount of suspending agent Tween-80 was slowly added. The final volume of the suspension was made 3 ml. To stabilize the suspension it was stirred well. For the preparation of positive control group (10 mg/kg) Diazepam is taken & a suspension of 3 ml is made and the vehicle control group was treated with 1% Tween 80 in water at the dose of 10 ml/kg body weight.

Table 3.8: Test sample used in evaluation of analgesic activity

Test sample	Group	Purpose	Dose	Route Of Administration
1% Tween-80 in saline	1	Control group	5mg/kg	Oral
Diazepam	2	Positive control	0.1 ml/10 gm	Oral
Dichloromethane part	3	Test sample	200 mg/kg	Oral
	4		400 mg/kg	
n-hexane part	5	Test sample	200 mg/kg	Oral
	6		400 mg/kg	

3.4.2.3 Identification of Animals During Experiment

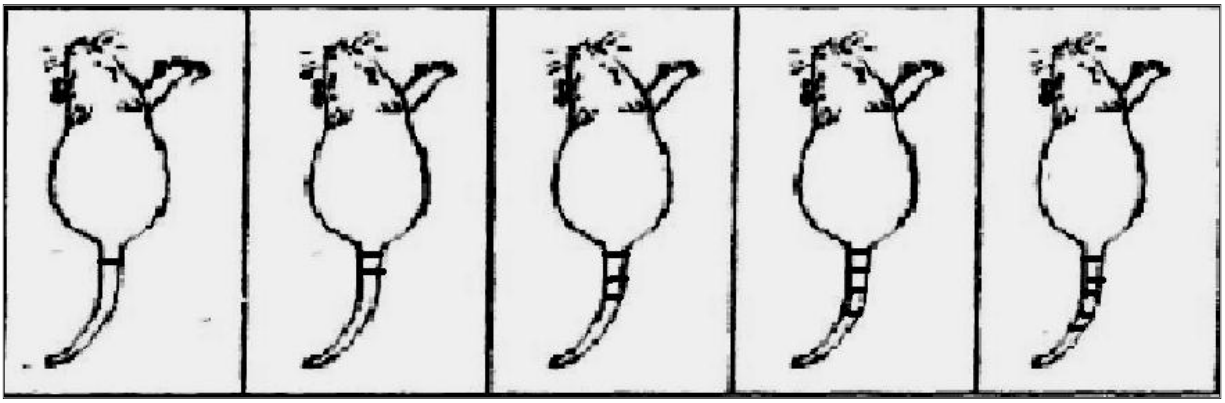

Figure 3.5: Identification of test animals for analgesic property screening

Each group consists of five mice/rats and hence it is difficult to identify and observe at a time five mice/rats receiving same treatment. Thus it was important to identify individual animal of a group during the treatment. The animals were individualized by marking: marked as M1=mice 1, M2=mice 2, M3=mice3, M4=mice 4 & M5=mice 5.

3.4.2.4 Open Field Test

Principle: The method described by Gupta *et al.* (1971) was adopted for this test. In open field test, the animals were divided into control, positive control, and test groups containing five mice each. The test groups received different fractions of the root parts of *G. densiflorum* at the doses of 200 mg/kg and 400 mg/kg body weight orally whereas the control group received vehicle (1% Tween 80 in water) And the positive control group received diazepam (1 mg/kg b.w.). The floor of an open field of half square meter was divided into a series of squares each alternatively colored black and white. The apparatus was constructed of white plywood and measured 72x72 cm with 40 cm wall. The frequency and duration of different behavior by the animals was counted for 3 min at 0, 30, 60, 90, and 120 min after oral administration of the test drugs and the standard (Gupta et al., 1971). The behaviours scored include: Line crossing, Center square entries, Center square duration, Rearing, Stretch attend postures, Grooming, Freezing, Urination, and Defecation.

Procedure:

At zero hour the plant extract (N-hexane 200/400 dose and DCM 200/400 dose) , diazepam and 1 % Tween solution were received orally by the test groups, positive group and control group respectively by a feeding needle with a ball shaped end.

1. At zero hours the frequency and duration scored by the animals was counted for a period of 3 minutes chemical interaction.
2. After 30, 60 & 90 minutes the frequency and duration scored by the animals was counted for a period of 3 minutes potentiality is present.

3.4.2.5 Hole Cross Test

Principle: The method was carried out as described by Takagi et al. (1971). A steel partition was fixed in the middle of a cage having a size of 30 × 20 × 14 cm. A hole of 3 cm diameter was made at a height of 7.5 cm in the centre of the cage. The animals were divided into positive control, control, and test groups containing five mice each. The test groups received four different fractions of the root parts of *G. densiflorum* at the doses of 200 mg/kg and 400 mg/kg body weight orally whereas the vehicle control and positive control groups received vehicle (1% Tween 80 in water) and the standard drug diazepam (1 mg/kg b.w.) respectively. The number of passage of a mice through the hole from one chamber to other was counted for a period of 3 min at 0, 30, 60, 90 and 120 min after oral administration of the test drugs and the standard.

Procedure:

1. At zero hour the plant extract (N-hexane 200/400 dose and DCM 200/400 dose), diazepam and 1% Tween solution were received orally by the test groups, positive group and control group respectively by a feeding needle with a ball shaped end .
2. At zero hours the number of passage of a mouse through the hole from one chamber to other was counted for a period of 3 minutes chemical interaction.
3. After 30, 60 & 90 minutes the number of passages of a mouse through the hole from one chamber to other was counted for a period of 3 minutes potentiality is present.

3.4.2.6 Elevated Plus-Maze (EPM) Test

Principle: The EPM apparatus consists of two open arms (5×10 cm) and two closed arms (5×10×l5 cm) radiating from a platform (5×5 cm) to form a plus-sign figure. The apparatus was situated 40 cm above the floor (Lister, 1987). The open arms edges were 0.5 cm in height to keep the mice from falling and the closed-arms edges were 15 cm in height. Thirty minutes after administration of the test drugs, each animal was placed at the center of the maze facing one of the enclosed arms. During the 5-min test period, the frequency of open and enclosed arms entries, the time spent in open and enclosed arms, frequency and duration of centre place entries, frequency of rearing and grooming were recorded (Pellow and File, 1986). Entry into an arm was defined as the point when the animal places all four paws onto the arm. The procedure was conducted in a sound attenuated room; observations made from an adjacent corner (Braida et al., 2008;Braida et al., 2009).

Procedure:

1. At zero hour, the plant extract (N-hexane 200/400 dose and DCM 200/400 dose), diazepam and 1% Tween solution were received orally by the test groups, positive group and control group respectively by a feeding needle with a ball shaped end.
2. After 30 minutes of administration of test drugs each animal was placed at the center of the maze facing one of the enclosed arms.
3. During the 5-min test period, the frequency of open and enclosed arms entries, the time spent in open and enclosed arms, frequency and duration of centre place entries, frequency of rearing and grooming were recorded.

3.4.2.7 Hole Board Test

Principle: Hole board test is a generally used method for screening the potential anxiolytic character of drugs. The test is based on the theory, that head-dipping activity of the animals is inversely proportional to their anxiety state. There are sixteen holes in the board, and was first described by Christchurch's David L Smith(1991). At first head-dip was measured; and found the proportion of animals with short latency was significantly increased in moderately and highly aversive environments. It was fulfilled, that the inverse relation between anxiety state and head-dipping activity is true only in a certain range of anxiety level. In more

aversive situations, then the anxiety level of the animals is high. Count the total number of dipping.

Procedure:

1. At zero hour, the plant extract (N-hexane 200/400 dose and DCM 200/400 dose), diazepam and 1% Tween solution were received orally by the test groups, positive group and control group respectively by a feeding needle with a ball shaped end.
2. After 30 minutes of administration the mouse was placed at the middle of the hole board.
3. The time of first head dipping and the total number of head dipping during the 5-min test period was counted.

3.5 Statistical analysis

Values are expressed as mean ± SEM from 5 animals of each group. Statistical analysis carried out by One way ANOVA(analysis of variance) using Dunnett t test.

Chapter 04
Results & Discussion

4.1 Analgesic Activity by Acetic Acid Induced Writhing Method

Analgesic activity of the methanolic extract of the root part of the plant *G. densiflorum* studied in different doses (200 and 400 mg/Kg body weight) levels of DCM fraction of the extract, using acetic acid induced writhing. The extract produced % inhibition of writhing at doses of 200 and 400 mg/kg body weight respectively (Table 4.1 and Fig. 4.1). The result was found to statistically significant.

Table 4.1: Analgesic activity of methanolic crude extracts of *G. densiflorum*

Animal Group	Writhing count					Mean writhing ± SEM	% of writhing	% of inhibition
	M1	M2	M3	M4	M5			
1.Negative Control **1% tween 80 in saline water**	19	21	23	21	19	20.6±1.3784	100	0
2.Standard (DICLOFENAC)	2	4	2	5	2	3±1.3784	14.56*	85.44
3.DCM-200	14	17	18	12	20	16.2±1.3784	78.64***	21.36
4.DCM-400	5	3	4	8	9	5.8±1.3784	28.16*	71.84
5.*n*-hexane-200	17	15	13	18	14	15.4±1.3784	74.75**	25.25
6.*n*-hexane-400	5	4	7	6	3	5±1.3784	24.27*	75.73

****p*<0.001; ***p*<0.01; ****p*<0.05; F=55.916**
*** The mean difference is highly significant at the 0.001 level, ** very significant at the 0.01 level and ***significant at the 0.05 level.**
a Dunnett t-tests treat one group as a control, and compare all the groups against it.

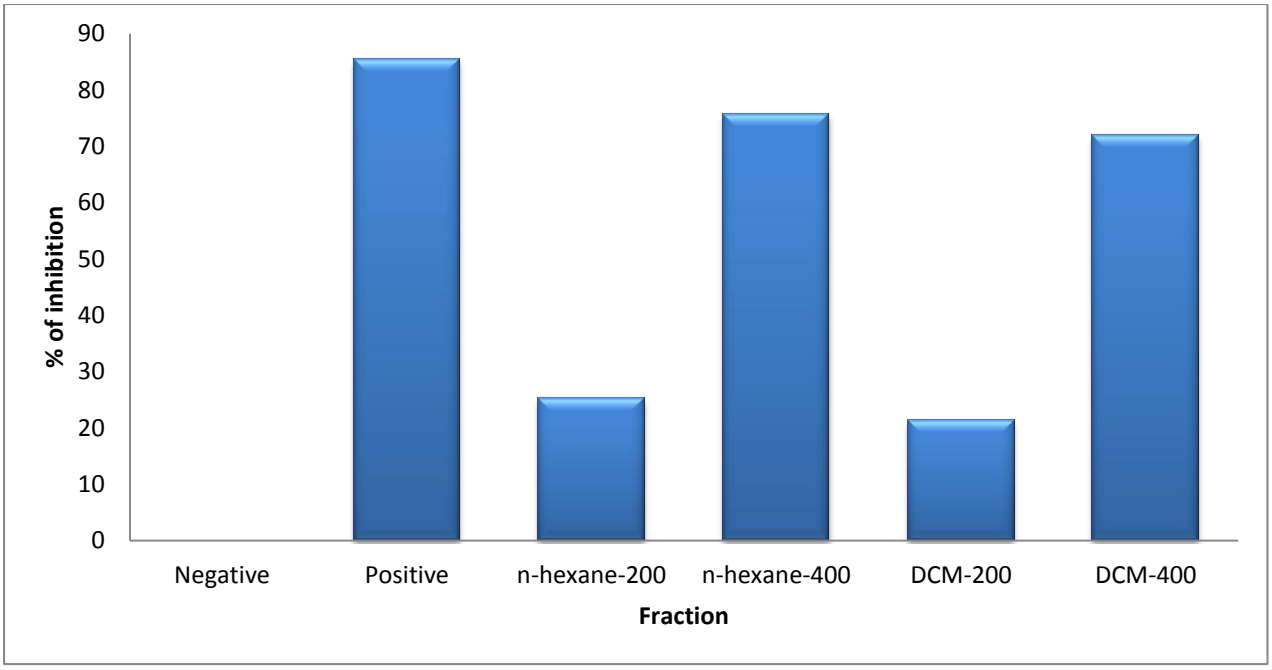

Figure 4.1: % of writhing inhibition induced by different fraction of *G. densiflorum* and Diclofenac

The dichloromethane fraction of the methanolic extract of root part of *G. densiflorum* showed significant activity with 21.36 % ($p<0.05$) and 71.84 % ($P<0.001$) writhing inhibition in mice at a dose of 200 mg/kg body weight and 400 mg/kg body weight respectively. On the other hand the *n*-hexane fraction showed significant analgesic activity of 25.25% ($p<0.01$) and 75.73% ($p<0.001$) writhing inhibition at a dose of 200 mg/kg and 400 mg/kg respectively. Diclofenac at a dose of 1 mg/kg body weight was used as positive control in this investigation that showed 85.44% ($p<0.001$) inhibition. The inhibitory response decreased in the order Diclofenac (85.44%) > n-hexane-400(75.73%) > n-hexane-200(25.25%) > DCM-400(21.36%).

The result obtained by Dunnett method, all group compared against control group. A difference between group 1 (control) & group 2 (standard) has been found with p value = 0.001; group 1 (control) & group 3 (DCM-200 mg/kg) (p=0.016); group 1 (control) & group 4 (DCM-400 mg/kg) (p = 0.001). On other hand, a difference between group 1 (control) & group 5 (n-hexane-200 mg/kg) (p=0.004); group 1 (control) & group 6 (n-hexane-400 mg/kg) (p=0.001).The result indicates that both higher dose of the experimental plant extract showed analgesic activity which is statistically significant ($p<0.001 – 0.05$).

4.1.1 Overview on Analgesic Activity

Geodorum densiflorum has not been subjected to pharmacological investigations so far for analgesic screening to provide scientific justification to its traditional claim in various pain. The present study has established analgesic potential of *G. densiflorum* using acetic acid-induced writhing test for visceral pain.

Acetic acid-induced writhing in mice is a model of visceral pain which is highly sensitive and useful for screening peripherally acting analgesic drugs. Acetic acid-induced writhing method is not only simple and reliable but also affords rapid evaluation of peripheral type of analgesic action. This model represents pain sensation by triggering localized inflammatory response. Such pain stimulus leads to the release of free arachidonic acid from tissue phospholipid by the action of phospholipase A2 and other acyl hydrolases (Ahmed et al., 2004). The prostaglandins, mainly prostacyclin and prostaglandin-E have been reported to be responsible for pain sensation by exciting the A-fibres. Activities in the A ∂-fibres cause a sensation of sharp well localized pain. Any agent that lowers the number of writhing will demonstrate analgesia preferably by inhibition of prostaglandin synthesis, a peripheral mechanism of pain inhibition (Ferdous et al., 2008). Additionally, local peritoneal receptors are postulated to be partly involved in the abdominal constriction (writhing) response. This method has been associated with prostanoids in general, i.e. increases levels of PGE2 and PGF2α in peritoneal fluids as well as lipoxygenase products (Ochi et al., 2000). It was found that extractives significantly inhibited the acetic acid induced writhing response. The abdominal constriction is related to the sensitisation of nociceptive receptors to prostaglandins. It is therefore possible that extract produced analgesic effect which may be due to the inhibition of synthesis or action of prostaglandin.

G. densiflorum plant extracts caused dose-dependent antinociception against chemicaly induced pain in mice. DCM fraction of the root part of the plant at the dose of 400 mg/kg body weight was found to exhibit 71.84 % writhing response inhibitory effect (Table 4.1) and *n*-hexane fraction of the root part of the plant at the dose of 400 mg/kg body weight was found to exhibit 75.73% writhing response inhibitory effect. Which values are statistically significant.

G. densiflorum is reported to contain steroids, alkaloids, glycosides, flavonoids, tannin, glucosides, carbohydrate (Habib et al., 2011). These compounds may be responsible for the

analgesic activity. Recent studies suggest that the inflammatory tissue damage is due to the liberation of reactive oxygen species form phagocytes invading the inflammation sites (Parke and Sapota, 1996). Many natural and synthetic antioxidants are in use to prevent the lipid peroxidation. There is also reports on the role of flavonoid, a powerful antioxidant (Brown and Rice-Evans, 1998; Vinson et al., 1995), in analgesic activity (Adedapo et al., 2008; Parmar and Ghosh, 1978; Mutalik et al., 2003; Venkatesh et al., 2003) primarily by targeting prostaglandins (Rajnarayana et al. 2001; Galati et al., 1994; Rao et al., 1998). Carotenoids are also reported to possess antioxidant action (Stahl and Sies, 2003; Duh et al., 1999; Veeru et al., 2009). So it can be assumed that their Cyclooxygenase (COX) inhibitory activity and antioxidant activity may reduce the production of free arachidonic acid from phospholipid or may inhibit the enzyme system, which is responsible for the synthesis of prostaglandins, and ultimately relieve pain-sensation.

4.2. CNS Activity Test

4.2.1 Evaluation of sedative property using Hole Cross Method

The sedative property of the methanolic extract of the root part of the plant *G. densiflorum* studied in different doses (200 and 400 mg/Kg body weight) levels of DCM fraction of the extract, using hole board method. The average and standard error mean of crossing the hole by the dose of 200 mg/kg and 400 mg/kg respectively have been showed in Table 4.2 and Fig. 4.2. The result was found to statistically highly significant.

Table 4.2: Data of CNS depressant activity by hole cross method

Groups	**Average ± SEM**				
	0 min	**30 min**	**60 min**	**90 min**	**120 min**
1.Control group :1%tween in saline	6.2±0.3637	7.4±0.3637	7±0.3637	8±0.3637	7.2±0.3637
2.Positive control: diazepam 1mg/kg)	5.6±0.3637	5.6±0.3637	6.4±0.3637	6.2±0.3637	6.4±0.3637
3.DCM 200mg/kg	5.2±0.3637	5±0.3637	4.6±0.3637	6.8±0.3637	7±0.3637
4.DCM 400mg/kg	3.6±0.3637	3.6±0.3637	4±0.3637	3.2±0.3637	4±0.3637
5.n-hexane 200mg/kg	5±0.3637	4.8±0.3637	5±0.3637	5±0.3637	4.8±0.3637
6.n-hexane 400mg/kg	6.4±0.3637	6.2±0.3637	6±0.3637	6.2±0.3637	5.8±0.3637

***p<0.001; **p<0.01; ***p<0.05; F=21.352**
*** The mean difference is highly significant at the 0.001 level, ** very significant at the 0.01 level and ***significant at the 0.05 level.**
a Dunnett t-tests treat one group as a control, and compare all the groups against it

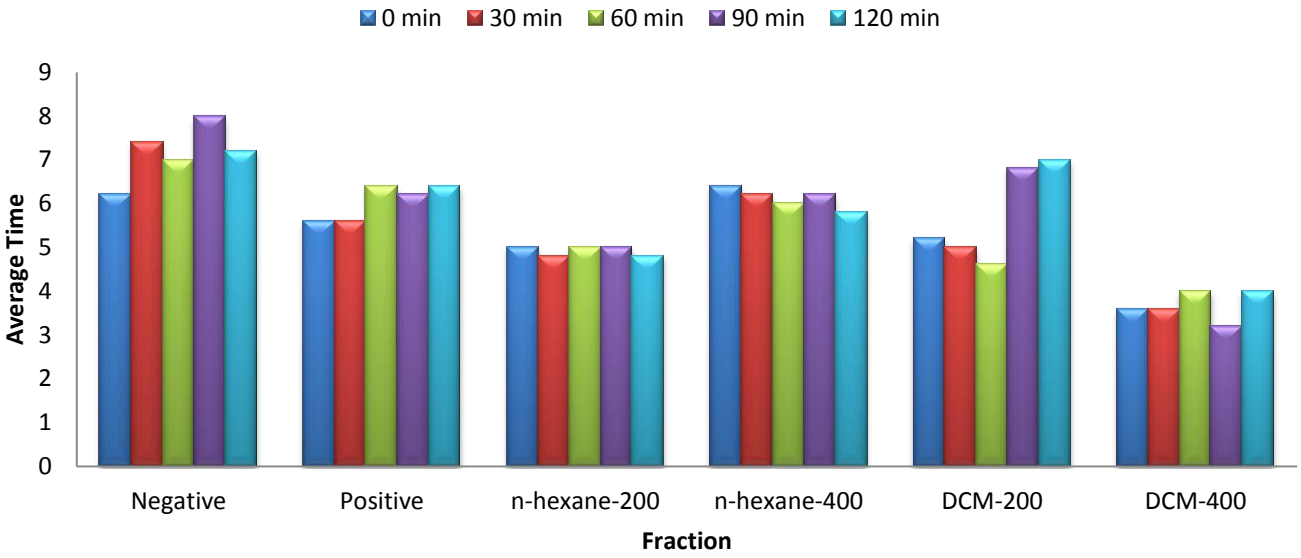

Figure 4.2: Graphical representations of CNS depressant action by hole cross method

The DCM fractions, at 200 mg/kg and 400mg/kg body weight doses, produced highly significant (p< 0.001-0.05) decrease of locomotion from its initial value during the period of experiment (Figure 4.2). Maximum suppression of locomotor activity was displayed by DCM fraction at a dose of 400mg/kg and *n*-hexane fraction at a dose of 200mg/kg. The result obtained by Dunnett method, all group compared against control group. A difference between group 1 (control) & group 2 (standard) has been found with p value = 0.021; group 1 (control) & group 3 (DCM-200 mg/kg) (p = 0.003); group 1 (control) & group 4 (DCM-400 mg/kg) (p = 0.000). On other hand, a difference between group 1 (control) & group 5 (n-hexane-200 mg/kg) (p = 0.000); group 1 (control) & group 6 (n-hexane-400 mg/kg) (p = 0.035).The result indicates that both high & low dose of the experimental plant extract shows dose depended suppression of motor activity which is statistically significant (p<0.001-0.05).

4.2.2 Evaluation of sedative property using Open field Method

The sedative property of the methanolic extract of the root part of the plant *G. densiflorum* studied in different doses (200 and 400 mg/Kg body weight) levels of DCM fraction of the extract, using open field method. The average and standard error mean of line crossing by the dose of 200 mg/kg and 400 mg/kg respectively have been showed in Table 4.3 and Fig. 4.3. The result was found to statistically highly significant.

Table 4.3: Data of CNS depressant activity by open field

Groups	**AVERAGE ± SEM**				
	0 min	30 min	60 min	90 min	120 min
1.Control group 1%tween in saline	113.8 ±7.89785	72±7.89785	93.2±7.89785	85.8±7.89785	79.6±7.89785
2.Positive control diazepam (1mg/kg)	119±7.89785	116.8±7.89785	99.4±7.89785	89.6±7.89785	93.2±7.89785
3.DCM-200mg	26.4±7.89785	31.6±7.89785	28±7.89785	30.8±7.89785	30.6±7.89785
4.DCM-400mg	103.8±7.89785	113.2±7.89785	85±7.89785	131.8±7.89785	84.6±7.89785
***5.n*-hexane 200mg/kg**	49±7.89785	56.6±7.89785	54.5±7.89785	39.4±7.89785	60±7.89785
***6.n*-hexane 400mg/kg**	64.4±7.89785	59.8±7.89785	60.2±7.89785	50.4±7.89785	62.6±7.89785

***p<0.001; **p<0.01; ***p<0.05; F=29.835**
*** The mean difference is highly significant at the 0.001 level, ** very significant at the 0.01 level and ***significant at the 0.05 level.**
a Dunnett t-tests treat one group as a control, and compare all the groups against it.

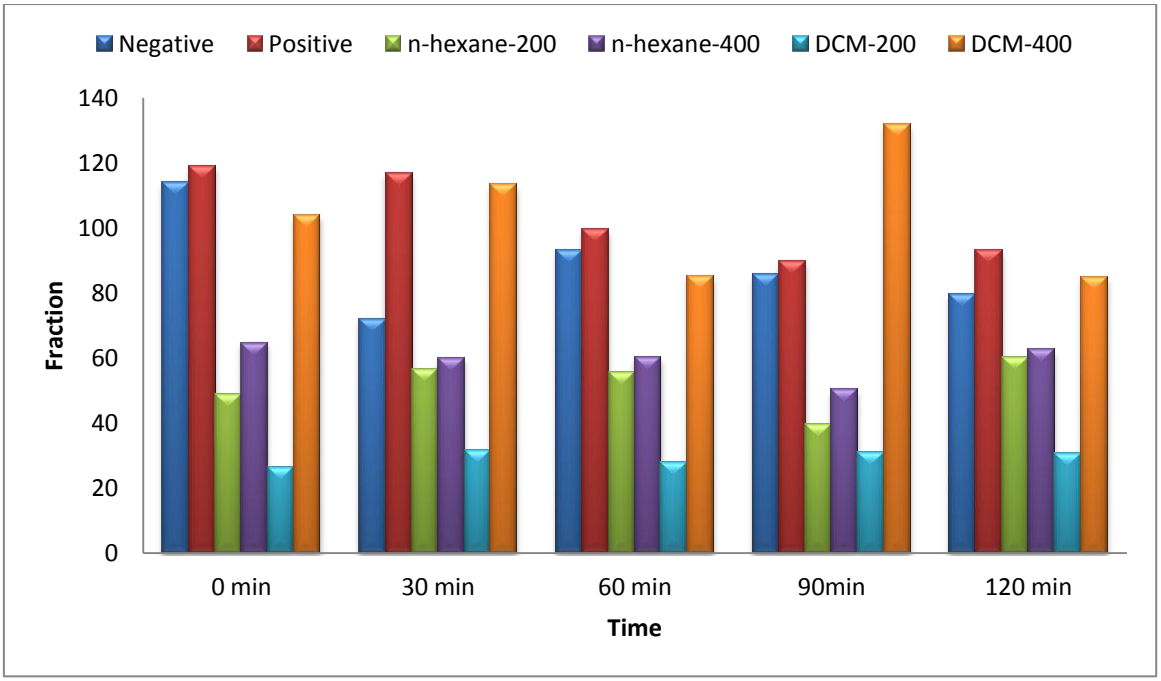

Figure 4.3: Graphical representations of CNS depressant action by open field method

The number of line crossing by the mice was suppressed significantly from the third observation period at the dose levels of 400mg/kg BW of the DCM fraction of the root part of *G. densiflorum.* Similarly at 400mg/kg BW dose level, *n*-hexane fraction suppressed the number of line crossing by the mice from the second observation period. The results were dose dependent and statistically significant (Figure 4.3).

The result obtained by Dunnett method, all group compared against control group. A difference between group 1 (control) & group 2 (standard) has been found with *p* value = 0.251; group 1 (control) & group 3 (DCM-200 mg/kg) (p = 0.001); group 1 (control) & group 4 (DCM-400 mg/kg) (*p* = 0.247). On other hand, a difference between group 1 (control) & group 5 (n-hexane-200 mg/kg) (*p* = 0.001); group 1 (control) & group 6 (n-hexane-400 mg/kg) (*p* = 0.005). The result indicates that the lower dose (200mg/kg) of DCM fraction showed dose depended suppression of motor activity which is statistically significant.And both high & low dose of *n*-hexane fraction shows dose depended suppression of motor activity which is statistically significant (p<0.001-0.05). On the other hand

4.2.3 Evaluation of Anxiolytic Property Using Hole Board Method

Anxiolytic property of the methanolic extract of the root part of the plant *G. densiflorum* studied in different doses (200 and 400 mg/Kg body weight) levels of DCM fraction of the extract, using hole board method. The extract produced % inhibition of head dipping at doses of 200 and 400 mg/kg body weight respectively (Table 4.2 and Fig. 4.2). The result was found to statistically highly significant.

Table 4.4: Data of CNS depressant activity by hole board method

Animal Group	Frequency of Deeping					Mean±SEM	% of frequency	% of inhibition
	M1	M2	M3	M4	M5			
Negative Control 1% tween 80 in saline water	70	67	64	60	62	64.6±2.1229	100	0
Standard (Diazepam)	35	32	38	34	39	35.6±2.1229	55.1*	44.9
Dcm-200	18	19	18	22	21	19.6±2.1229	30.34*	69.66
Dcm-400	55	45	50	49	57	51.2±2.1229	79.26*	20.74
n-hexane-200	44	46	51	48	49	47.6±2.1229	73.7*	26.3
n-hexane-400	68	67	71	75	70	70.2±2.1229	108.7	-8.7

***p<0.001; **p<0.01; ***p<0.05; F=154.350**
*** The mean difference is highly significant at the 0.001 level, ** very significant at the 0.01 level and ***significant at the 0.05 level.**
a Dunnett t-tests treat one group as a control, and compare all the groups against it.

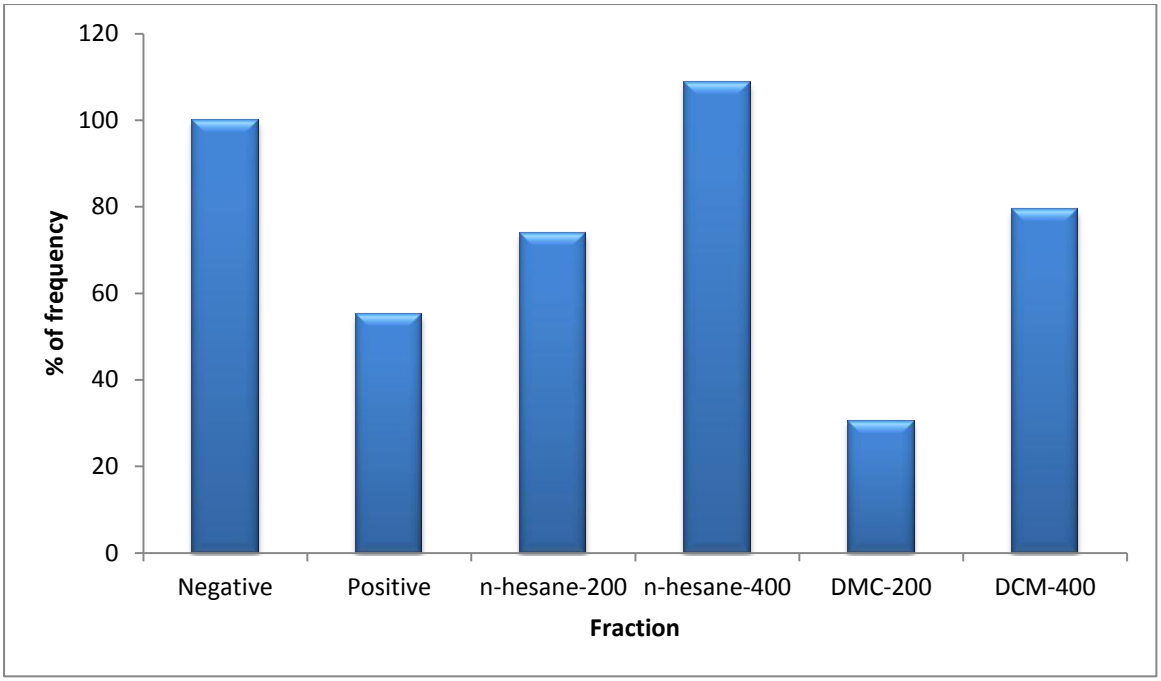

Figure 4.4: Graphical representation of % of head dipping frequency

In the vehicle treated control group, the mean number of head dipping by mouse was 64.6. Diazepam as a reference standard showed decrease in number of head dipping by 55.1%. Administration of DCM fraction of root part of *G. densiflorum* at 200mg/kg showed decrease in number of head dipping(30.34%) and 400 mg/kg dose showed increase in number of head dipping (79.26). on the other hend, n-hexane fraction showed 73.7% and 108.7% of frequency of head dipping at the dose of 200mg and 400mg res[ectively.

The result obtained by Dunnett method, all group compared against control group. A difference between group 1 (control) & group 2 (standard) has been found with p value = 0.000; group 1 (control) & group 3 (DCM-200 mg/kg) (p = 0.000); group 1 (control) & group 4 (DCM-400 mg/kg) (p = 0.000). On other hand, a difference between group 1 (control) & group 5 (n-hexane-200 mg/kg) (p = 0.000); group 1 (control) & group 6 (n-hexane-400 mg/kg) (p = 0.057).The result indicates that both high & low dose the experimental plant extract except n-hexane-400mg dose showed anxiolytic activity which is statistically significant ($p<0.05$).

4.2.4 Evaluation of Anxiolytic Property Using Elevated Plus Maze (EPM) Test

Anxiolytic property of the methanolic extract of the root part of the plant *G. densiflorum* studied in different doses (200 and 400 mg/Kg body weight) levels of DCM fraction of the extract, using EPM test.

Table 4.5: Data of CNS depressant activity by EPM test

Group	Mean ± SD (counts/5minutes)					
	Open arm duration	**Open arm frequency**	**Close arm duration**	**Close arm frequency**	**Centre square duration**	**Centre square frequency**
1.Control (1% tween in saline)	2.6±3.71	0.8±1.09	257.8±23.10	8.4±3.13	43±25.37	9.4±2.88
2.Positive control	2.4±5.37	0.2±0.45	261.8±17.58	7.6±4.04	35±13.28	6.8±3.77
3.DCM-200	20.2±15.07	5.8±3.11	177.6±56.41	7.8±1.92	60±23.47	9.8±2.28
4.DCM-400	3.4±4.72	1.4±20.07	202.6±119.03	61.2±127.36	23.4±26.87	2.8±2.77
5.*n*-hexane-200	4.2±9.39	0.6±1.34	249.8±40.42	6.4±3.58	46.8±31.44	5.63.91±3.91
6.*n*-hexane-400	7.4±12.99	0.8±1.30	162.8±69.14	7.6±4.09	68.8±31.84	8±4
	F=2.570 *P*=0.053	F-6.959 *P*=0.001	F=5.089 *P*-0.003	F=2.829 *P*=0.038	F=4.648 *P*=0.004	F=3.078 *P*=0.028

***p<0.001; **p<0.01; ***p<0.05; F=154.350**
*** The mean difference is highly significant at the 0.001 level, ** very significant at the 0.01 level and ***significant at the 0.05 level.**
a Dunnett t-tests treat one group as a control, and compare all the groups against it.

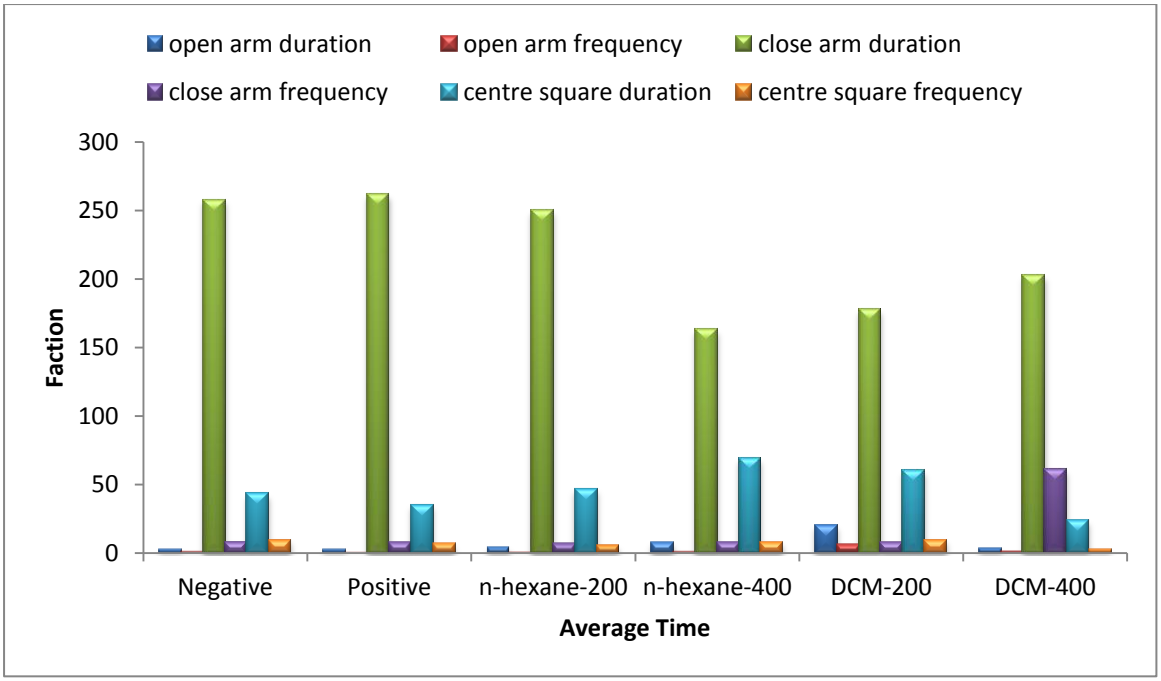

Figure 4.5: Graphical representation of % of head dipping frequency

DCM fraction treatment, at 200mg/kg b.w. and 400 mg/kg b.w., significantly increased the frequency of entries (Table 4.5) of mice into the open arms, and the duration of time spent (Table 4.5) in the open arms of the elevated plus-maze compared to the reference anxiolytic drug Diazepam (1mg/kg b.w.), which indicates the significant anxiolytic activity of the fraction. DCM fraction at 400 mg/kg b.w. also significantly increased the frequency of entries (Table 4.5) of mice into the close arms, and decreased the duration of time spent (Table 4.5) in the close arms of the elevated plus-maze compared to the reference anxiolytic drug Diazepam (1mg/kg b.w.). DCM fraction at the doses level of 400mg/kg also decreased the frequency and duration of centre square entries compared to reference anxiolytic drug Diazepam(1mg/kg b.w.). The result (*p* value and F) obtained from one way ANOVA test are shown in Table 4.5.

4.2.5 Overview on CNS Activity

Anxiety is unpleasant feeling of apprehension or fearful concern. It can be a normal, reasonable and expected response to a stressful situation or perceived danger or it may be an excessive, irrational state that signifies a mental disorder. The situation induces anxiety behavior in mice is triggered by two factors, i.e., individual testing as the animal was separated from its social group and agoraphobia, as the arena is very large, relative to the animals breeding or the natural environment. In such situations mice show thigmotaxic

behavior identified by spontaneous preference to the periphery of the apparatus and reduced ambulation (Nimbal et al., 2011). Mice demonstrate anxiety, fear and curiosity when placed in a new environment, and an overall assessment of behavior could be determined through the observation of freezing, grooming (fear), rearing, head dips (curiosity) and the number of fecal boli . Anxiolytic treatment (i.e: treating with diazepam, thiopental) decreases this anxiety induced inhibition of exploratory behavior.

Diazepam is in a group of drugs called benzodiazepines. Diazepam is used to treat anxiety disorders, it affects chemicals in the brain that may become unbalanced and cause anxiety. Diazepam works by acting on receptors in the brain called GABA receptors. This causes the release of a neurotransmitter called GABA in the brain. Neurotransmitters are chemicals that are stored in nerve cells in the brain and nervous system. They are involved in transmitting messages between the nerve cells. GABA is a neurotransmitter that acts as a natural 'nerve-calming' agent. It helps keep the nerve activity in the brain in balance, and is involved in inducing sleepiness, reducing anxiety and relaxing muscles. As diazepam increases the activity of GABA in the brain, it increases its calming effect and results in sleepiness, a decrease in anxiety and relaxation of muscles.

The sedative property of DCM fraction of *G. densiflorum* was carried out by Hole Cross test and Open Field test. The extract significantly ($p < 0.001$- 0.05) displayed a dose dependent suppression of motor activity and exploratory behaviour in this two test; results are shown in Table 4.2 and 4.3 and in Figure 4.2 and 4.3. Again anxiolytic activity of DCM fraction of the respective plant was carried out by Hole Board test and EPM test. In these tests the extract also showed significant ($p<0.001$-0.05)) decrease in locomotor activity.

Since locomotor activity is a measure of the level of excitability of the CNS (Mansur et al., 1980), this decrease in spontaneous motor activity could be attributed to the sedative effect of the plant extracts (Rakotonirina et al., 2001; Ozturk et al., 1996).

Phytochemical analyses of different fraction of the methanol, ethyl acetate and petroleum ether extract of root parts of *G. densiflorum* revealed the presence of carbohydrates, alkaloid, glycoside, flavonoids, steroid etc (Habib et al., 2011).So the observed bioactivities may be attributed to flavonoid, glycoside or steroid compounds. However, many flavonoids were found to be ligands for the gamma aminobutyric acid type A (GABAA) receptors in the central nervous system (CNS); which led to the hypothesis that they act as benzodiazepine-

like molecules. Thus the sedative and anxiolytic effects observed might be due to the interaction of flavonoids with the GABA/ benzodiazepine receptor complex in brain (Trofimiuk et al., 2005). This is supported by their behavioral effects in animal models of anxiety, sedation and convulsion (Trofimiuk et al., 2005; Marder and Paladini, 2002). Electrophysiological experiments with flavone and flavanone derivatives have shown that some of them can modulate GABA-generated chloride currents, either positively or negatively. Due to the increased knowledge of the diversity of GABAA receptor subtypes, the number of studies with cloned receptors of defined subunit composition has recently risen, and experiments with some natural and synthetic flavones and flavonones have shown that they can modulate gamma aminobutyric acid (GABA)-generated chloride currents, either positively or negatively (Johnston, 2005; Goutman et al., 2003; Campbell et al., 2004; Kavvadias et al., 2004; Hall et al., 2005). Thus decreased spontaneous motor activity could be attributed to the CNS depressant activity of the root parts of *G. densiflorum.*

The results of the investigations of the present study lend partial scientific support in favor of the traditional uses of *Geodorum densiflorum* as traditional use varies among the local practitioners and the scientific evidence that support the traditional uses is not well established. Therefore, this plant deserves extensive and well-designed pharmacological and phytochemical test for their effective use in medical science.

Chapter 05
Conclusion

Natural products, specially those of plant origin, have been a promising source of new lead compound for drug discovery for ages. Bangladesh is blessed with rich floristic resources, where a large number of plants still remain unexplored. So well designed, systematic and objective research in this area might benefit our people who have been deluged with superfluity of diseases, and who lack technological and economic resources to cope up with them with orthodox medicine.

Based on the results of the present study, it can be proposed that the root part of *G. densiflorum* in general dichloromethane soluble fractions in particular, has strong analgesic, properties. Again, these findings may justify scientifically the basis for the use of this plant in folk medicine for the treatment of a wide range of disease of different etiology such as, skin cancer, snake bite, headache, fever, inflammation etc. These results also lend support to the relevant phytochemical and pharmacological works carried out so far on G. densiflorum. However, further studies are suggested to be undertaken to understand the underlying mechanism of the observed activities and to isolate, purify and characterise active phytochemical ingredient(s) responsible for these bioactivities in animal models.

The future goal of this study is to identify effective, cheap and available modalities to cope up with the upsurge of the dangers of diseases of different aetiology in Bangladesh. Approaches may be developed to prevent and/or treat illness easily and effectively with readily available and cheaper resources. This research may be a platform for further investigation in this area. It is likely to show directions for the researchers to find ways out to save our lay people from the curse of diseases. Future endeavours in this area may open up exciting new therapeutic avenues.

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Appendix

7.1 Equations

1. $\%\ \boldsymbol{of\ writhing} = \left(\frac{(\boldsymbol{Mean\ writhing\ of\ control\ group - Mean\ writhing\ of\ the\ test\ group})}{\boldsymbol{Mean\ writhing\ of\ the\ control\ group}}\right) \times \mathbf{100}\%$

2. $\%\ \boldsymbol{of\ writhing\ inhibition} = \mathbf{100} - \%\ \boldsymbol{of\ writhing}$

7.2 Definition

Analysis of Variance (ANOVA): A method of significance testing based on the ratio of between-groups variance to within-groups variance. This method is used in statistical analysis if the dependent variable is continuous and the independent variables are all categorical (i.e. nominal, dichotomous, or ordinal etc.). If there is one independent variable, the method is usually called one-way ANOVA. If there is more than one independent variable, the method is usually *N*-way ANOVA, with *N* representing the number of independent variables.

Dunnett's Test: Sometimes experiments are designed to compare several groups with a control group but not among each other. Thus a multiple comparison against a single control group is Dunnett's test. This test is suitable for small number of comparisons. Here the observed differences in means from all possible pairs are compared with the calculated statistic at some specific α (usually 0.05). If any difference in means is greater than the calculated statistic, the difference is marked as significant at α level.

***p* Value:** It is the probability of being wrong when asserting that a true difference exists between the different treatment groups. It is used to assess the degree of dissimilarity between two or more groups of observations. It indicates the probability of occurrence of the difference by chance.

Standard Deviation (SD): The square root of variance.

Standard Error of Mean (SEM): The standard deviation (SD) of all sample means, rather than of individual observations. The SEM is calculated as the observed SD of the sampled data divided by the square root of the number of observation in the sample.