Pharmacological and biochemical investigations of methanolic extract of *Myristica fragrans* seeds

A dissertation submitted to the Department of Clinical Pharmacy and Pharmacology, Faculty of Pharmacy, University of Dhaka, for the partial fulfillment of the requirements for M. Phil. degree

Submitted By

Examination Roll: 02
Registration No.: 275
Session: 2011 – 12

Department of Clinical Pharmacy and Pharmacology
University of Dhaka

Declaration

Not any portion of this thesis paper entitled "Pharmacological and biochemical investigations of methanolic extract of *Myristica fragrans* seeds" has been submitted for another degree or qualification of University of Dhaka.

Researcher

Md. Shaiful Alam Bhuiyan
Examination Roll: 02
Registration No.: 275
Session: 2011 – 12
Department of Clinical Pharmacy and Pharmacology
University of Dhaka

Supervisor

Professor Dr. Md. Saiful Islam
Department of Clinical Pharmacy and Pharmacology
Faculty of Pharmacy
University of Dhaka

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Abstract

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The practice of herbal medicine begins from the very ancient periods of known human history. There is evidence of herbs having been used in the treatment of diseases and for revitalizing body systems in almost all ancient civilizations---the Indian, the Egyptian, the Chinese and even the Greek and Roman civilizations. Herbs act in almost magical and astonishing ways - spasms may relax, pains vanish, constipation overcome, nervousness recede, headaches disappear, colds be banished, allergies counteracted, fevers controlled, blood flow arrested.

The active constituents of the herb can enter the body in several ways. These include consuming the herb orally so as to be absorbed by the digestive system, application on the skin through medicinal poultices as well as cosmetics for being absorbed in the body through the pores, application on eyes through lotions and compresses; smelling the aroma through nose to enable the essential oil being absorbed in the bloodstream.

Finally, a word of caution that most herbs have little or no harmful side effects, some herbs may cause slightly undesirable reactions in some persons. Therefore, try only one herb at a time, beginning in small doses and wait and watch for side effects. If there are none, increase the use or dosage cautiously. Also, not all herbal applications are effective in every case in every person.

1.1 History of medicinal plants

Since disease, decay and death have always co-existed with life, the study of disease and their treatment must also have been contemporaneous with the dawn of the human intellect. The primitive man must have used as therapeutical agents and remedial measures those things which he was able to procure most easily. There is no authentic record of medicines used by the primitive man (Kirtikar & Bashu, 1987).

Illness, physical discomfort, injuries, wounds and fear of death had forced early man to use any natural substance that he could lay his hand on, without any resistance, for living the pains and sufferings caused by these abnormal conditions and for preserving his health against disease and death (Ghoni, 1998).

Primitive peoples in all ages have had some knowledge of medicinal plants, derived as the result of trial and error. There primitive attempts at medicine were based on intuition guess work, superstition or trial or error. Most savage people have believed that disease was due to the presence of evil spirits in the body and could be driven out by the use of poisonous or disagreeable substances calculated to make the body an unpleasant place in which to remain. The knowledge regarding the source and the use of the various products suitable for this purpose is usually restricted to the medicine men of the tribe. As civilization progressed the early physicians were guided in great part by these observations (Albert, 1972).

Rigveda (4500-1600 B.C.), which is the oldest book in the library of man supplies various informations on the medicinal use of the plants in the Indian subcontinent. It noted that Indo-Aryans used the Soma plant (*Amanita muscaria*, a narcotic and hallucinogenic mushroom) as medicinal agent. It is unfortunate that the Ayur Veda is no more available in

its original form but the most authentic and original texts considered as the renowned representatives of the original Ayur Veda, are the encyclopedic Agnivesha or Charaka Samhita and Sushruta Samhita (Mukherjee, 1992). The Sushruta Samhita attributed to Sushruta in the 6th century BC describes 700 medicinal plants, 64 preparations from mineral sources, and 57 preparations based on animal sources (Dwivedi, 2007).

The Indo-Aryans used the plant for sacrificial purposes and its juice is described in the ancient Aryan literature as stimulating beverage. The word oushadhi literally means heat producer. When the Indo-Aryans came to use the Soma plants for therapeutical purposes, they came to possess knowledge of the medicinal properties and uses of herbs and plants. Hence, Oushadhi applied to all herbs and medicinal plants. The Vedas made many references to healing plants including Sarpagondha (*Rauwolfia serpentine*), while a comprehensive Indian herbal, the Charaka Samhita, cites more than 500 medicinal plants (Kirtiker & Basu, 1987).

As far as records go, it appears that Babylonians (about 3000 years B.C.) were aware of a large number of medicinal plants and their properties. As evident from the Papyrus Ebers (about 1500 B.C.), the ancient Egyptians possessed a good knowledge of the medicinal properties of hundreds of plants. Many of the present day important plant drugs like henbane (*Hyoscyamus* spp.), mandrake (*Mandragora officinarum*), opium (latex of *Papaver somniferum* fruit), pomegranate (*Punica granatum*), castor oil (oil of *Riccinus communis* seeds), aloe (Juice of *Aloe* spp.), onion (*Allium cepa*) and many others were in common use in Egypt about 4500 years ago.

The first Chinese herbal book the Shennong Bencao Jing was compiled during the Han Dynasty but dates back to a much earlier date, possibly 2700 B.C. It lists 365 medicinal plants and their uses - including ma-Huang, the shrub that introduced the drug ephedrine to modern medicine. Succeeding generations augmented on the Shennong Bencao Jing, as in the Yaoxing Lun (Treatise on the Nature of Medicinal Herbs), a 7th century Tang Dynasty treatise on herbal medicine. The ancient Greeks and Romans made medicinal use of plants. Greek and Roman medicinal practices, as preserved in the writings of Hippocrates and - especially -Galen, provided the pattern for later western medicine. Hippocrates advocated the use of a few simple herbal drugs - along with fresh air, rest, and proper diet. Galen, on the other hand, recommended large doses of drug mixtures - including plant, animal, and mineral ingredients. The uses of plants for medicine and other purposes changed little in early medieval Europe. Many Greek and Roman writings on medicine, as on other subjects, were preserved by hand copying of manuscripts in monasteries. At the same time, folk medicine in the home and village continued uninterrupted, supporting numerous wandering and settled herbalists Avicenna's The Canon of Medicine (1025A.D.) lists 800 tested drugs, plants and minerals. Book Two is devoted to a discussion of the healing properties of herbs, including nutmeg, senna, sandalwood, rhubarb, myrrh, cinnamon, and rosewater. Baghdad was an important center for Arab herbalism, as was Al-Andalus between 800 and 1400 (Tapsell et al., 2006) (Castleman, 2002).

The Pen Tsao, the earliest known Chinease pharmacopoeia, appeared around 1122 B.C. attributed to the legendary Emperor Shen Nung, this authoritative work described the

use of Chaulmoogra oil (from the seed of *Hydnocarpus kurzii)* to treat leprosy (Ghoni, 1998).

The continuing importance of herbs for the centuries following the Middle Ages is indicated by the hundreds of herbals published after the invention of printing in the fifteenth century. Theophrastus' Historia Plantarum was one of the first books to be printed, but Dioscorides' De Materia Medica, Avicenna's Canon of Medicine and Avenzoar's pharmacopoeia were not far behind.

The practice of herbal medicine flourished most during the Greek civilization. When historical personalities, like Hippocrates (born in 460 B.C.) and Theophrastus (born in 370 B.C.) were practiced herbal medicine; Hippocrates is regarded as the father of medicine as he was distinguished physician, practicing and researching into herbal medicine. His Materia medica consists of some 300 – 400 medicinal plants. The far ranging scientific work of Aristotol (384-322 B.C.); a Greek philosopher, included an effort to catalog the properties of the various medicinal herbs known at that time. The Greek writer- physician Dioscorides (60 A.D.) who wrote the famous treatise De Materia Medica (published in 78 A.D.) which contained the description of 600 medicinal plants. Two of the 37 volumes of books were written by Pliny De Elder (23 - 70 A.D.), which included a large number of medicinal plants. Galen (131 - 200 A.D.) was the great Greek pharmacist and physician, who wrote about 500 volumes of books describing hundreds of recipes and formulations of medicinal preparations containing both plant and animal origin.

Allopathic and homeopathic systems of medicine today are based doctrine expatiated by Galen (Sofowora, 1982).

After the dark ages were over, there came the period of herbalists' and encyclopedist and the monasteries of Northern Europe produced vast compendiums of true and false information regarding plants, stressing in particular the medicinal value and folklore. It was about this time that the curious "Doctrine of Signatures" came into being. It was developed by Paracelsus (1490-1541 A.D.), a Swiss alchemist and physician. According to this superstitious doctrine all plants possessed some sign given by the Creator, which indicated the use for which they were intended. Thus a plant with heart shaped leaves should be heart ailments, the liver leaf with its three-logged leaves for liver troubles and so on. Many of the common names of our plants of today owe their origin this curious belief. Such names as heartsease, Solomon's seal, dogtooth violet and liverwort carry on the old superstition.

From this crude beginning the study of drug and drug plants has progressed until now pharmacognosy and pharmacology are essential branches of medicine (Albert, 1972).

1.2 Contribution of the medicinal plants to modern medicine

Plants remain the primary source of supply of many important drugs used in modern medicine. Modifications, improvements, sophistication and newer discoveries are continuously changing the type, quality, presentation and concept of medicinal preparations. The real changes that have taken place and taking place in medicines are those in their active ingredients, excipients and form of preparation. And these are the areas where plants

have been contributing enormously since the human race first discovered medicine to ensure its existence on earth.

Continued use of many plants from the progress of civilization and development of human knowledge, scientists promoted phytochemical and pharmacological research into them which resulted in the discovery, isolation and in many cases subsequent synthesis of a large number of modern medicinal substances. Some examples are - Ergot alkaloids, the popular oxytocic and antimigraine agents of modern medicine, were first isolated from the ergot of rye. The midwives of ancient Europe used ergot in childbirth to promote and strengthen uterine contractions (Taylor, 1965; Le Strange, 1977).

a) Morphine and other opium alkaloids, which are most potent analgesics and sedatives of modern medicine, are now commercially obtained from the capsules of Opium poppy, *Papaver somniferum*. Opium as a pain-killer and sleep inducing drug was well-known to the ancient (Taylor, 1965).
b) Atropine, hyoscyamine, hyoscine and other tropane alkaloids, which are used as sedative, anti-spasmodic, mydriatic and parasympatholytic agents in modern medicine, were isolated and are now produced from *Atropa,, Dhatura* and *Duboisia* spp. The plants have long been used in traditional medicine as pain killers and hypnotics.
c) Sennosides and other anthracene derivatives used in modern medicine as laxatives and purgatives are obtained from the leaves of *Cassia senna* (Sona pata), rhizomes of Rheum and barks of *Rhamnus* species. These plant parts have long been used in traditional medicine for their purgative properties.
d) Digoxin, digitoxin and some other most potent cardio tonic glycosides used in modern medicine were isolated and are now commercially produced from the leaves of *Digitalis purpurea*. The plant was traditionally used in the treatment of dropsy.
e) Vinblastine and vincristine, the most potent highly esteemed and most expensive anti-leukaemic drugs of modern anti-cancer therapy are obtained from the ornamental plant Madagascar periwinkle, *Catharanthus roseus* (Nayantara). The research into this plant was however promoted by its long use in traditional medicine for the treatment of diabetes.
f) Quinine, quinidine and other cinchona alkaloids, which are the most commonly used antimalarial substances of modern medicine, were isolated and ate now commercially produced from the barks of cinchona trees. Cinchona barks were traditionally used in the treatment of fevers long before their chemical constituents were discovered.
g) Reserpine, rescinnamine and other similar hypotensive and sedative substances now used in modern medicine were isolated and now produced from the roots of various *Rauwolfia* species. The roots have long been used in traditional medicine in the treatment of mental illness, insanity and insomnia.

There are many other such examples, which were results of research prompted by uses of medicinal plants in traditional medicine. This is how medicinal plant has contributed enormously over the years to the gradual development of modern medicine. This process of

contribution is still continuing significantly. Some of the plant-derived drugs of modern medicine are listed in the following table with their plant sources and therapeutic uses.

But don't have Herbal remedies and Home comforts until illness strikes. It also suggests preventive measures and recommends herbals to ward off illness, as well as giving instructions for making delightful herbal preparations that will scent and purify the home to ensure a healthy atmosphere.

1.3 Prospects of herbal drug research in Bangladesh

Herbs play a significant role, especially in modern times, when the damaging effects of food processing and over-medication have assumed alarming proportions. They are now being increasingly used in cosmetics, foods and teas as well as alternative medicines. The growing interest in herbs is a part of the movement toward change in life styles. This movement is based on the belief that the plants have a vast potential for their use as a curative medicine.

In Bangladesh, 5000 species of angiosperms are reported to occur (IUCN, 2003). The number of medicinal plants included in “Materia medica” of traditional medicine in this subcontinent at present stands as about 2,000. Since Bangladesh has an enormous resource of medicinal plants, majority of our population has to rely upon indigenous system of medication. 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 and as a result, traditional medicines usually exist side by side with western forms of health care.

Bioactive compounds deposited in medicinal plants can serve as important raw materials for pharmaceutical manufacturing. Therefore, well-judged and scientific investigation of this wealth can significantly contribute to the public health. Again, it was observed that developed countries mostly imports raw materials of valuable medicinal plants from developing countries. Where they are screened, analyzed and used in drug preparations, and returned as high priced medicines to developing countries. Thus, being available commodity of commerce, a country can also earn a good amount of foreign currency by exporting this natural wealth to other countries (Ghani et al., 2003).

The ancients knew that apple cleansed the blood, controlled high blood pressure and stimulated the flow of urine. Now, science has shown how apples lower sodium chloride, while increasing potassium and alkaline reserves.

Recent medical research has established onion as an effective preventive food against heart attack. They correct thrombosis and reduce blood cholesterol. Garlic juice dissolves accumulation of mucous in the sinus cavities, bronchial tubes, lungs and reduces the severity of asthmatic attacks. Blood sugar is controlled by amla. It stimulates production of hormone insulin. Scurvy resists treatment with synthetic vitamin C, yet responds quickly to certain vegetables and fruits like cabbage, lemon and amla.

Traditional herbal remedies have led scientists to the development of numerous 'Modern' drugs; from aspirin, tranquillizers and chywanprash to heart saving digitalis, establishing beyond doubt the efficacy of 'herbal medicine'.

Through herbal drug research we obtain their specific healing properties, how the herbs are useful in alleviating or preventing specific ailments; in most cases, the method of using and making herbal preparations.

1.4 Role of herbs in modern human society

The use of herbs to treat disease is almost universal among human societies. A number of traditions came to dominate the practice of herbal medicine at the end of the twentieth century:

1) The "classical" herbal medicine system, based on Greek and Roman sources

2) The Siddha and Ayurvedic medicine systems from various South Asian Countries

3) Chinese herbal medicine (Chinese herbalogy)

4) Traditional African medicine

5) Unani-Tibb medicine

6) Shamanic herbalism: a catch-all phrase for information mostly supplied from

South America and the Himalayas

7) Native American medicine.

Many of the pharmaceuticals currently available to physicians have a long history of use as herbal remedies, including opium, aspirin, digitalis, and quinine. The World Health Organization (WHO) estimates that 80 percent of the world's population presently uses herbal medicine for some aspect of primary health care (Fabricant and Farnsworth, 2001).

In addition to the use in the developing world, herbal medicine is used in industrialized nations by alternative medicine practitioners such as naturopaths. A 1998 survey of herbalists in the UK found that many of the herbs recommended by them were used traditionally but had not been evaluated in clinical trials. In Australia, a 2007 survey found that these Western herbalists tend to prescribe liquid herbal combinations of herbs rather than tablets of single herbs.

The use of, and search for, drugs and dietary supplements derived from plants have accelerated in recent years. Pharmacologists, microbiologists, botanists, and natural-products chemists are combing the Earth for phytochemicals and leads that could be developed for treatment of various diseases. In fact, according to the World Health Organization, approximately 25% of modern drugs used in the United States have been derived from plants (Barnes and Edzard, 1998).

The uses of plant species and herbs to treat medicine have made their lives easier because these types of medicine are cheap and also easy to obtain.

1.5 The study protocol

Our present study was designed to observe pharmacological activities of the crude extracts and their different partitioning fractions of the *Myristica fragrans* seeds. It consisted of the following steps:

1.5.1 Chemical investigation

1. Extraction of the plant *Myristica fragrans* for fifteen days with methanol.

2. Filtration of the crude methanolic extract by using the Markin cotton cloth and subsequently through the filter paper and solvent evaporation.

3. Fractionation of methanolic crude extract with Petroleum ether, n-Hexane and Chloroform by partition method.

4. Phytochemical screening for alkaloids, glycosides, tannins, flavonoids, fixed oils, steroids and volatile oils.

1.5.2 Pharmacological investigation

1. Evaluation of antioxidant activity of crude extract and different fractions by DPPH free radical scavenging method.

2. Brine shrimp lethality bioassay and determination of LC_{50} of crude extract and different fractions of *Myristica fragrans*.

3. Investigation of in-vitro antimicrobial activity of crude extract and different fractions of *Myristica fragrans* by Disc diffusion method.

4. Screening of anti-inflammatory activity of crude extract and different fractions of *Myristica fragrans* by Carrageenan induced inflammation on Long evans rat model.

5. Screening of anti-pyretic activity by Yeast induced fever on Long evans rat model.

6. Screening of peripheral analgesic activity of crude extract and different fractions of *Myristica fragrans* on Swiss albino mice model by acetic acid induced writhing method.

7. Screening of anti-hyperglycaemic activity by streptozotocin induced diabetes.

8. Screening of anti-diarrhoeal activity by castor oil induced diarrhoea.

2.1. A brief overview of the plant *Myristica fragrans*

Myristica fragrans is a small evergreen tree, usually 5–13 m (16–43 ft) tall, but occasionally reaching 20 m (66 ft). The alternately arranged leaves are dark green, 5–15 cm (2.0–5.9 in) long by 2–7 cm (0.8–2.8 in) wide with petioles about 1 cm (0.4 in) long. The species is dioecious, i.e. "male" or staminate flowers and "female" or carpellate flowers are borne on different plants, although occasional individuals produce both kinds of flower. The flowers are bell-shaped, pale yellow and somewhat waxy and fleshy. Staminate flowers are arranged in groups of one to ten, each 5–7 mm (0.2–0.3 in) long; carpellate flowers are in smaller groups, one to three, and somewhat longer, up to 10 mm (0.4 in) long.

Carpellate trees produce smooth yellow ovoid or pear-shaped fruits, 6–9 cm (2.4–3.5 in) long with a diameter of 3.5–5 cm (1.4–2.0 in). The fruit has a fleshy husk. When ripe the husk splits into two halves along a ridge running the length of the fruit. Inside is a purple-brown shiny seed, 2–3 cm (0.8–1.2 in) long by about 2 cm (0.8 in) across, with a red or crimson covering (an aril).

The seed of the plant is known as “nutmeg” and the arillus of the seed is called “mace”. Both nutmeg and mace contain many volatile oils. These oil constituents have a variety of individual pharmacological effects. The fruit contains ethereal oil-cells often with phenolic and myristicin; the seed and the aril are used for flavouring food.

Figure-2.1: *Myristica* leaf and seed

The **Myristicaceae** are a family of flowering plants. It is sometimes called the "nutmeg family", after its most famous member, *Myristica fragrans*, the source of the spices nutmeg and mace. The best known genera are *Myristica* and *Virola*.

The family consists of about 20 genera, with about 440 species of trees and shrubs found in tropical areas across the world. Most of the species are large trees that are valued in the timber industry. Other species include *Myristica argentea* (Papua), and *Myristica malabarica* in India, both used as adulterants in trade.

The nutmeg tree bears fruits which contain egg-shaped seeds wrapped in a red cover (aril), all year. The red cover is mace, which is another spice with a similar, but slightly more delicate flavour. Mace and its extract (oleoresin) are not a subject of this study. This study covers the aforementioned egg-shaped seed (nutmeg), in whole, broken or ground form.

2.2 Geographical distribution

Global distribution: The family is present in Europe, Asia, and the Americas.

Local distribution: Native of the Eastern Moluccas, Endemic to Indonesia and other countries in the South Pacific. Caribbean islands, India, Kerala, Tamil Nadu, Karnataka, Sri Lanka and Malaysia.

2.3 Classification of the plant

Taxonomical Hierarchy of *Myristica fragrans*

Rank	Scientific and common name
Kingdom	Plantae – Plants
Subkingdom	Tracheobionta – Vascular plants
Superdivision	Spermatophyta – Seed plants
Division	Magnoliophyta – Flowering plants
Class	Magnoliopsida – Dicotyledons
Subclass	Magnoliidae
Order	Magnoliales
Family	Myristicaceae – Nutmeg family
Genus	*Myristica* Gronov. – nutmeg
Species	*Myristica fragrans* Houtt. – nutmeg

2.4 Medicinal activities of the various parts of the plants

Common names: Jaiphol (Bengali)

Leaf: Less used in herbal medicine.

Bark: Less used in herbal medicine.

Flower: Dried flowers are used as antispasmodic, antiparasitic, anti-emetic, antioxidant, antirheumatic, carminative and antiseptic etc.

Seed: The nutmeg seeds are used as Analgesic, anti-inflammatory, anti-pyretic, anti-dontalgic, anti-emetic, antioxidant, anti-rheumatic, antiseptic, antispasmodic, cardiac, anti-parasitic, aphrodisiac, larvicidal, prostaglandin inhibitor, orexigenic, carminative, stomachic, stimulant (energetic), tonic (general neuro-muscular stimulant, digestive-gastric secretory and uterine-emmenagogue).

A nutmeg coarsely powdered and fried in til oil, until all the particles become brown, is very useful as an external application to relieve any rheumatic pain, neuralgia and sciatica. The oil should be cooled and strained before application.

Figure 2.2: Nutmegs

Root: Less used in herbal medicine.

2.5 Uses of *Myristica fragrans*

Essential oils

The essential oil obtained by steam distillation of ground nutmeg is used widely in the perfumery and pharmaceutical industries. This volatile fraction typically contains 60-80% d-camphene by weight, as well as quantities of d-pinene, limonene, d-borneol, l-terpineol, geraniol, safrol, and myristicin. In its pure form, myristicin is a toxin, and consumption of excessive amounts of nutmeg can result in myristicin poisoning. The oil is colourless or light yellow, and smells and tastes of nutmeg. It contains numerous components of interest to the oleochemical industry, and is used as a natural food flavouring

in baked goods, syrups, beverages, and sweets. It is used to replace ground nutmeg, as it leaves no particles in the food. The essential oil is also used in the cosmetic and pharmaceutical industries, for instance, in toothpaste, and as a major ingredient in some cough syrups. In traditional medicine, nutmeg and nutmeg oil were used for disorders related to the nervous and digestive systems.

Culinary uses

Nutmeg and mace have similar sensory qualities, with nutmeg having a slightly sweeter and mace a more delicate flavour. Mace is often preferred in light dishes for the bright orange, saffron-like hue it imparts. Nutmeg is used for flavouring many dishes, usually in ground or grated form, and is best grated fresh in a nutmeg grater.

2.6 Literature review

Methanolic extract have cytotoxic properties. From Iiterature review it has analgesic, anti-inflammatory and anti-pyretic properties. So, there is a strong reason of having anti-rheumatic activities are specifying below----

Three active components myristicin, licarin B and dehydrodiisoeugenol were isolated as active principles that have CNS depressant effects. In 400 mg doses, myristicin produced mild cerebral stimulation in human subjects. This effect is much less than that produced by 15 gm. of nutmeg powder, which was taken by people in order to describe its psychopharmacologic action. Removal of the volatile components of nutmeg eliminates the psychic action but not all of the side effects. It appears that myristicin does not reproduce the entire activities of whole nutmeg.

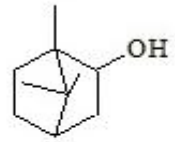
The isolation of 2 antimicrobial resorcinols, malabaricone B and malabaricone C, from the dried seeds of *Myristica fragrans* is described. Both compounds exhibited strong antifungal and antibacterial activities. Structure modification by methylation or reduction resulted in diminished activity. Nematicidal activity of *Myristica fragrans* oil is found against *Meloidogyne incognita.*

The seed contains myristicin, geraniol, diterpene, d-linalol, p-cymene, β- pinene, carotene as Vitamin-A, starch, reducing sugar. Seeds are rich in fatty acids -- lauric 4%; myristic 71.8%; palmitic, stearic 1.2% and linoleic 1.5%. The leaf, bark and flowers contain---- light brown volatile oil and stem contains a tannin mucilage complex.

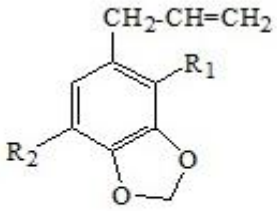
Essential oils of Myristicaceae have antifungal action and antimicrobial activity against *Streptococcus mutans*. The dark-red resin of the tree bark in some genera, such as *Virola*, contains several hallucinogenic alkaloids. Myristicin poisoning can induce convulsions, palpitations, nausea, eventual dehydration, and generalized body pain. It is also reputed to be a strong deliriant, and some fatal myristicin poisonings in humans have occurred.

Nutmegs have a strong, peculiar and delightful fragrance and a very strong bitter warm aromatic taste. During extraction deep yellowish fatty or waxy substances were found

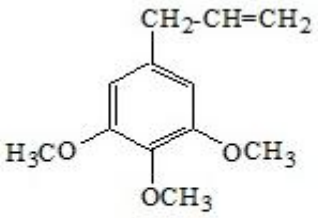
which removed through charcoal filtration. So from the data table, it is clear that volatile oils cannot saponify through alkaline potassium hydroxide but alkaline hydroxide saponifies fixed oils during saponification process.



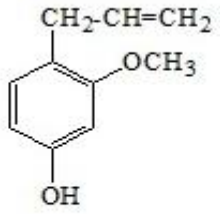
Bornenol



Safrole (R_1=R_2=H)
Myristicin (R_1=H, R_2=OCH_3)



Elemicin



Eugenol

Active chemical constituents provide anti-inflammatory and analgesic properties inhibiting prostaglandin synthesis and the symptomatic relief of rheumatoid arthritis is achieved. Myristicin, eugenol, borneol and elmicin are the constituents of oil produce such effect. But still unknown, if there is any effect to fight against the immune systems and provide remedy to such autoimmune disease.

Dried nutmeg and mace are used as spices. The essential oil (also called volatile oil) and the oleoresin are the major products of interest from the spice. Nutmeg is a stimulant, carminative, astringent and aphrodisiac. It is used in tonics and electuaries; and forms a constituent of preparations for dysentery, stomachache, flatulence, nausea, vomiting, malaria, rheumatism, sciatica and early stages of leprosy. Higher doses have a narcotic effect. Delirium and epileptic convulsions are found to occur. Mace is also used similarly. It is chewed for masking foul breath. It also prevents dental caries. Nutmeg has the following composition. 14.3% moisture, 7.5% protein, 36.4% ether extracts, 28.5% carbohydrates and 11.6% fibre. Mace, on the other hand, has following composition: 15.9% moisture, 6.5% protein, 24.4% ether extract, 47.8% carbohydrate and 3.8% fibre. It has 1.6% of mineral content with 0.18% calcium, 0.10% phosphorous and 12.6% of iron. It has 4 to15% of essential oil, 25% amylodextrin, reducing sugars, pectin and resins. The mace oil resembles nutmeg oil in odour, flavor and composition. Thus no distinction is made between them in trade (Anonymous, 1962).

Nutmegs contain 25-40 % of fixed oil, otherwise called *oleum myristiceae expressum*. It is a highly aromatic, orange-yellow coloured fat with the consistency of fat at room temperature. It has the odour and taste of nutmeg. It is composed of mainly trimyristin with a high proportion of essential oil. It is an ester of glycerol and tetradecanoic acid (myristic acid).

The other major sources of trimyristin are coconut oil and palm kernel oil. (Pursegl ove *et al.,* 1981, Anonymous, 1962). Studies by various groups have given different accounts regarding the proportion of fatty acids in cultivated and wild taxa of nutmeg. Similarly very little information is available on the proportion of amino acids in the leaves of cultivated and wild taxa. Maya *et al*., (2004) has reported variability in the concentration of essential oil constituents of East Indian and West Indian nutmeg. This has prompted for an in- depth study on the fatty acid and amino acid profile of cultivated and wild taxa of nutmeg.

Nutmeg was once considered an abortifacient, but may be safe for culinary use during pregnancy. However, it inhibits prostaglandin production and contains hallucinogens that may affect the fetus if consumed in large quantities.

3.1 Experimental design

Eight biological tests will be performed using the obtained plant extracts and fractions. The brief descriptions of the biological tests that will be performed are given below:

3.2 Laboratory animals

Swiss albino mice and Long evans rats will be used as model animal.

3.3 Preparation of methanolic extract from *Myristica fragrans* seed

The seed would be grinded to a fine powder and will be placed in a round bottomed flask (5 Litre). Sufficient quantity of solvent will be added to submerge the plant material. The container with its content was sealed by foil and kept for a period of 15 days accompanying occasional shaking and stirring. The methanolic extract will be evaporated to dryness with rota evaporator. The extract will be transferred to glass container of appropriate size. This will form the stock methanolic extract.

3.4 Phytochemical screening

Phytochemical screening will reveal the presence of alkaloids, glycosides, flavonoids, tannins, sterols, carbohydrates, amino acids, fixed oils, fats and volatile oils in the methanolic extract of the *Myristica fragrans* seed. (Ghani, 1997)

3.5 Antioxidant activity

The free radical scavenging activity of the extracts, based on the scavenging activity of the stable 1, 1-diphenyl-2- picrylhydrazyl (DPPH) free radical will be determined by the method described by Braca et. al. (2001). Plant extract (0.1 ml) will be added to 3 ml of 0.004% methanolic solution of DPPH. Absorbance at 517nm will be determined after 30 minutes and the percentage inhibition activity will be calculated from the following equation:

$$\% \text{ Inhibition} = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance of the control, and

A_1 is the absorbance of the extract or standard

The inhibition curves will be prepared and IC50 values will be obtained.

3.6 Brine shrimp lethality bioassay

Brine shrimp lethality test is a rapid and comprehensive bioassay for the bioactive compounds of natural and synthetic origin and is considered a useful tool for primary assessment of toxicity (Meyer et. al., 1982).

3.7 The in-vitro antimicrobial study

This study will be designed to investigate the anti-bacterial as well as anti-fungal spectrum of the crude extract and different fractions by observing the growth response. Minimum inhibitory concentration (MIC) required to kill the susceptible microbial strain is used to determine and to evaluate the extracts as potential source of antibiotic. The MIC test will not be performed as the anti-microbial activity of the plant extracts is mild. (Bauer et. al., 1966)

3.8 Anti-Inflammatory activity

Long evans rats weighing 50-100 gm would be fasted 16 hr prior to experiments, but will be supplied with water ad libitum. Carrageenan will be suspended in saline to make a 1 % (w/v) suspension. The suspension of carrageenan will be injected subcutaneously into the right hind paw 30 min after the test solutions has been administered orally. The inhibitory potency will be determined by 200 mg/kg and 400 mg/kg of the extract of *Myristica* comparing with 100 mg/kg of diclofenac sodium.

The volume of hind paw will be measured before the test solutions have been administered by insulin syringe and will be measured with a plethysmometer. The swelling hind paw volumes will be measured 30 min after the suspension of carrageenan has been administered and then up to 4 hr at intervals of 1 hr.

Control rats will be treated similarly receiving an oral dose of tween 80 and DMSO solution. The results will be expressed as the percent increase in hind paw volume due to swelling, as compared with the initial hind paw volume. (Ozaki *et. al*., 1988)

3.9 Anti-pyretic activity

The antipyretic efficacy of methanolic extract will be assessed using brewer's yeast induced pyrexia method in Long evans rats weighing 50-100 gm. Pyrexia will be induced by subcutaneous injection of 15 % w/v of brewer's yeast (10 ml/kg) in distilled water. The subcutaneous yeast injection will be given 30 min after the test solutions have been administered orally. Basal rectal temperature will be measured before the injection of yeast, by inserting digital clinical thermometer to a depth of 2 cm into the rectum. The rise in rectal temperature will be recorded 18 hr after yeast injection. Paracetamol 150 mg/kg body weight will be used as the standard antipyretic drug. Rectal temperature of animals will be noted at regular intervals following the respective treatments. The temperature will be measured at 1^{st}, 2^{nd}, 3^{rd} and 4^{th} hour after 18 hr of drug administration. (Amit Jaiswal *et. al*., 2011)

3.10 Peripheral analgesic activity

Mice would be randomly divided into ten groups of five animals that receive: (1) Tween 80 and DMSO solution orally; (2) diclofenac sodium (100 mg/kg) orally; (3) *Myristica fragrans* seed extract and different fractions of 200 mg/kg and 400 mg/kg orally.

Animals would be placed separately into a cage and allowed to acclimate for at least seven days. Mice will be given vehicle, diclofenac, methanolic extract and different fractions 40 minutes prior to induction of visceral pain. Visceral pain will be induced by intraperitoneal injection of 0.6 - 0.7% volume per volume (10 mL/kg) glacial acetic acid solution in normal saline. Animals will be returned to their cages and observed for writhing behavior, indicated by stretching of the abdomen with simultaneous stretching. The number of writhing responses will be counted for 15 minutes, starting directly after the acid injection. (Hayfaa *et. al*., 2013)

3.11 Anti-diabetic activity

Anti-hyperglycaemic activities of methanolic extract and different fractions of the *Myristica fragrans* seed will be tested in normoglycemic and streptozotocin induced diabetic mice.

Glucose tolerance property of methanol extract of *Myristica fragrans* seed would be determined as per the procedure previously described by Joy and Kuttan (1999) with minor modifications. In brief, fasted mice will be grouped into ten groups of five mice each. The various groups received different treatments like Group 1 will receive vehicle (3-4 drops of Tween 80 and few drops of DMSO in normal saline) that served as control, group 2 will receive standard drug (glibenclamide, 10 mg/kg body weight). Groups 3-10 will receive methanol extract and different fractions of *Myristica fragrans* seed at doses of 200 and 400 mg per kg body weight. Each mouse will be weighed and doses adjusted accordingly prior to administration of vehicle, standard drug and test samples. Then the animals were given glucose load orally (2 g/kg of body weight) at a certain dose 30 minutes after administration of crude extracts and their fractions, standard and control samples. Blood samples will be collected 30, 90 and 150 minutes after the glucose administration from tail vein. Blood glucose levels will be measured by glucose oxidase method (Venkatesh *et. al*., 2004).

3.12 Anti-diarrhoeal activity

The antidiarrhoeal activity of *Myristica fragrans* will be evaluated using castor oil induced diarrhoea in mice.

In overnight fasted mice that will induce diarrhoea by oral administration of castor oil (0.6 ml/mice). Test extracts will be given orally 30 minutes prior to castor oil administration. After 30 min administration of castor oil the diarrhoea will clinically apparent in all the animals of control, standard and test groups, for the next 4 hr.

Control mice will be treated similarly that they received an oral dose of tween 80 and DMSO solution. This will markedly reduce by 10 mg/ kg Loperamide. A similar marked reduction in the number of defecations over four hours will be tested with *Myristica fragrans* at the doses of 200 and 400 mg/kg. (Bass *et. al*., 1972)

Statistical analysis

Data would be presented as Mean ± SEM and analyzed by one way ANOVA followed by Dunnets t- test to determine the level of significance (Dunnet, 1987).

Methanol as solvent

Methanol is a kind of alcohols. Alcohol is a good solvent for many organic substances, both natural and synthetic. It dissolves important plant constituents such as resins, volatile oils, alkaloids, glycosides and neutral principles. They do not dissolve therapeutically inert plant materials like gums and starches.

4.1 Materials used

4.1.1 Glass wares

Materials	Source
Conical flasks (250 ml)	BDH Laboratory Equipments
Beakers (100 ml, 500 ml)	BDH Laboratory Equipments
Test tubes	BDH Laboratory Equipments
Funnels	BDH Laboratory Equipments
Measuring cylinders	BDH Laboratory Equipments
Pipettes	BDH Laboratory Equipments
Automatic pipette puller	Bel-Art Products, USA

4.1.2 Filter aids

1. Filter Paper (Whitman no. 1)
2. Normal Cotton

4.1.3 Equipments

Equipments	Source
Mechanical grinder	
Oven	Memmert
Spatula	
Electronic balance	Denver Instruments M-220
Refrigerator	Rangs electronics

4.2 Solvents

Methanol
Petroleum Ether
n-Hexane
Chloroform

4.3 Collection and preparation of the plant material

Myristica fragrans seeds were collected from herbal shop in Khilgaon at Dhaka. The seeds were washed properly and then air dried for several days. The pieces were then

ground into coarse powder in the Phytochemical Research Laboratory, Pharmacy Department, University of Dhaka, using high capacity grinding machine.

4.4 Extraction of the plant material

Preparation of the plant sample

The collected and identified *Myristica fragrans* seeds were dried in the sun. After complete drying, the sample was reduced to coarse powder separately with the help of a mechanical grinder and the powder was stored in a suitable container for extraction. The dried grinded powder weighed by rough balance.

Extraction of the plant material by methanol

About 800 gm. of the powdered material was taken in a clean, round bottomed flask (5 liters) and soaked in 2.5 liter of methanol. The container with its content was sealed by foil and kept for a period of 15 days accompanying occasional shaking and stirring. The whole mixture was then filtered through cloth filter and finally with a Whitman No.1 filter paper. The volume of the filtrate was then reduced using Rota evaporator at low temperature and pressure. The weight of the crude extract was 160 gm.

4.5 Yield determination

The dried extracts were weighed separately with the help of a digital balance and their yield was determined by using the following formula:

$$\% \text{ Yield} = \frac{\text{Wt. of particular extract}}{\text{Total amount of coarse powder}} \times 100$$

Total amount of coarse powder = 800 g

Weight of particular extract = 160 g

$$\text{Yield} = \frac{160}{800} \times 100 = 20\ \%$$

4.6 Solvent-solvent partitioning

Solvent-solvent partitioning was done using the protocol designed by Kupchan (Beckett et al., 1986). and modified by Van Wagenen. The crude extract (10 gm) was dissolved in 10% aqueous methanol. It was extracted with Petroleum Ether, then with n-Hexane and finally with Chloroform.

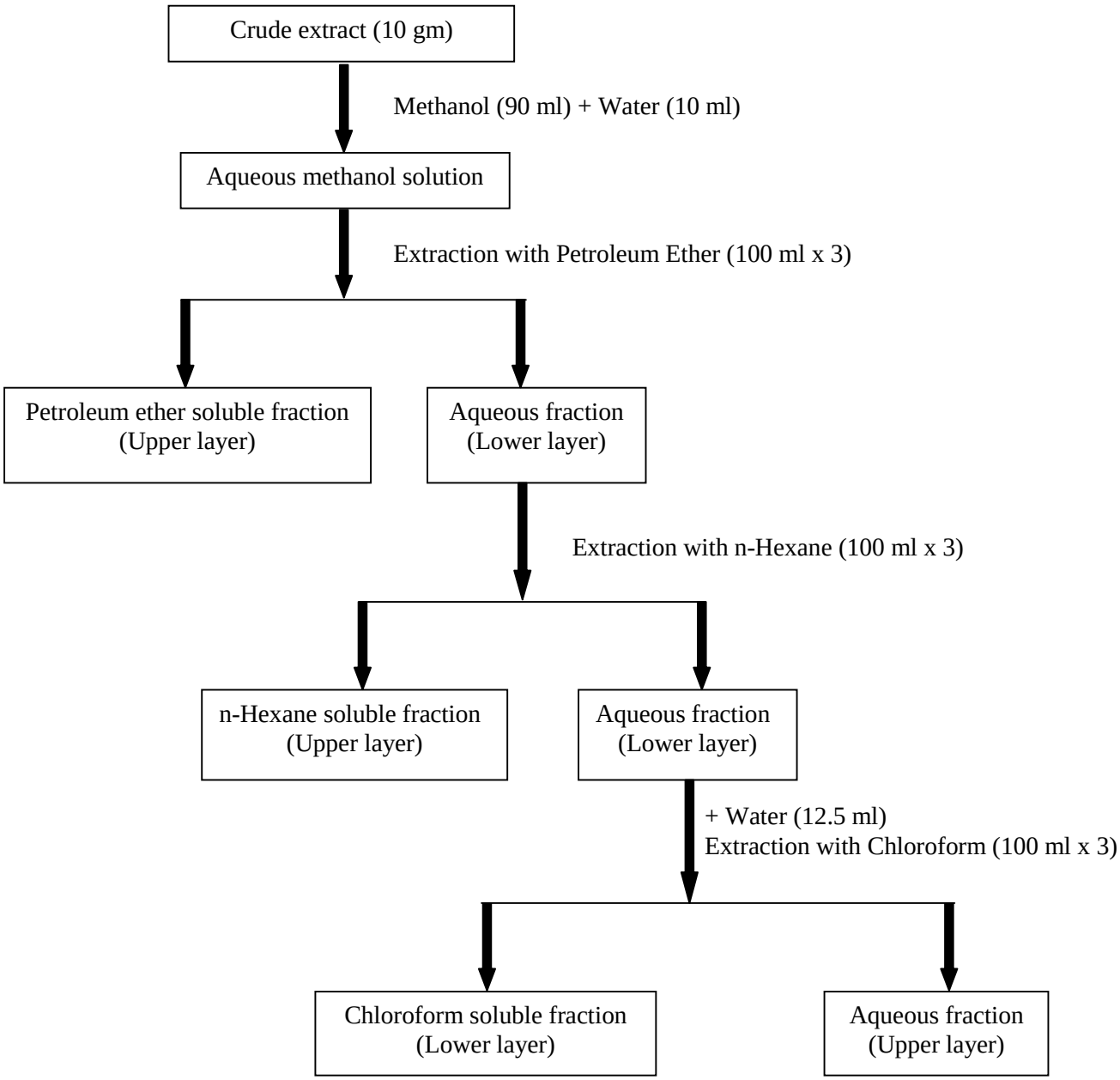


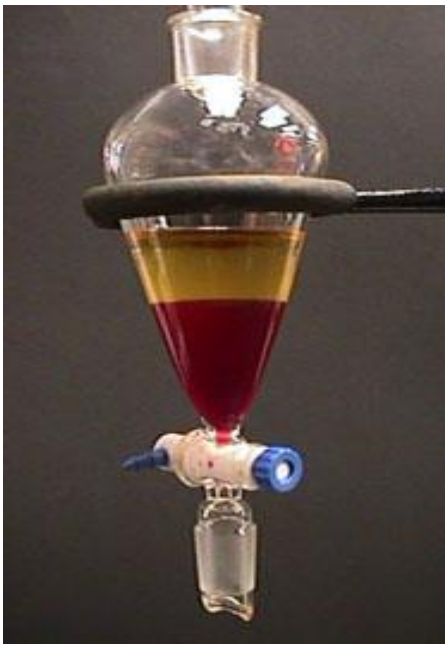
Figure 4.1: A schematic representation of the partitioning process

4.7 Preservation and storage

After the partitioned fractions were dried, the containers carrying the different dried fractionates were covered with aluminum foil and preserved in the refrigerator so that the extracts are free of any fungal or microbiological infection.

A.

B.



C.

D.

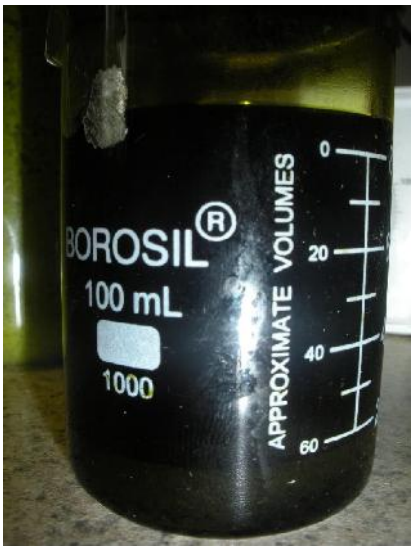


Figure 4.2:

A. Methanolic extract of *Myristica fragrans* seed

B. Partioning the extract

C. Rota evaporator

D. Dried extract

5.1Phytochemical screening

The Phytochemical investigation or screening is a process for the detection and evaluation of plant constituents through chemical analysis; phytochemical screening is co-related with phytochemical study. The compounds isolated through phytochemical study are applied on treated animal to find the pharmacological effects either beneficial or toxic material separated from the plant parts (Journal of pharmaceutical science, 1966).

In this research work, methanol extracts and different fractions of *Myristicafragrans* seeds were screened for carbohydrates, glycosides, saponins, flavonoids, tannins, steroids, alkaloids that have pronounced medicinal values.

5.2 Materials used for screening

5.2.1 Equipment

1. Test tube
2. Watch glass
3. Holder
4. Burner
5. Wood rack
6. Glass rod

5.2.2 Reagents and chemicals

1. Molisch's reagents (l0% napthol in alcohol) - for carbohydrate test.
2. Aqueous sodium hydroxide solution - for glycoside test.
3. Dilute sulfuric acid and sodium hydroxide solution - for glycoside test.
4. Fehling’s solution - for glycoside test.
5. 10% ammonia solution - for anthraquinone glycoside test.
6. Mayer's reagent (Potassium mercuric iodide solution) - for alkaloid tests.
7. Wagner's reagent (Solution of Iodine in KI) - for alkaloid tests.
8. Hagar's reagent (Saturated solution of picric acid) - for alkaloid tests.
9. Dragendorff's reagent (Bismuth subnitrate and acetic acid solution) – foralkaloid tests.
10. Tannic acid solution (l0%) - for alkaloid tests.
11. Conc. Hydrochloric acid - For flavonoid test.
12. Conc. Sulfuric acid - For steroid tests.
13. $FeCl_3$ (5%) - For tannin test.
14. Solvents – Methanol, Petroleum ether, n-Hexane and Chloroform.

5.3 Test compounds

1. Methanol extract of *Myristicafragrans*seeds.
2. Fractions of Petroleum ether, n-Hexane and Chloroform.

5.4 Preparation of sample solution

Small amounts of dried extracts were appropriately treated to prepare sample solution and then subjected to various phytochemical tests.

5.5 Phytochemical tests

Various phytochemical tests were performed for proper phytochemical screening. Those are mentioned below:

I. **Molisch'stest for carbohydrates:** Two drops of Molisch's reagent were added to about 5 mg of the extract in 5ml aqueous solution in a test tube. 1 ml of concentrate H_2SO_4 was allowed to flow down the side of the identical test tube so that the acid formed a layer beneath the aqueous solution without mixing it. A red ring was formed at the common surface of the two liquids; this indicated the presence of carbohydrate. On standing or shaking a solution was formed. Then the mixture was shaken and diluted with 5ml of distilled water. Dull violet precipitate was formed immediately.

II. **Fehling's test:** To 2 ml of extract of the equal volumes of Fehling's solutions A and B were added. Boiled for few minutes. A red or brick red precipitate was developed. It is the standard test for reducing sugars.

III. **Barfoed'stest:** 1 ml of extract of the plant material was taken in a test tube. 1ml of Barfoed's reagent was added and heated in a beaker of boiling water. Red ppt. of cuprous oxide was formed within two minutes if a monosaccharide was present.

IV. **Test for alkaloids:** A small volume of each extract was neutralized by adding 1 or 2 drops of dilute H_2SO_4 .This neutralized solution was treated with a very small amount of the following reagents and the respective color and / or precipitate formation was observed.

   a) **Mayer's reagent:** Formation of white or cream color precipitate indicated the presence of alkaloids.
   b) **Wagner's reagent:** Formation of brownish black ppt. indicated the presence of alkaloids.
   c) **Hager's reagent:** Formation of yellow crystalline ppt. indicates the presence of alkaloids.
   d) **Dragendorff's reagent:** Formation of orange or orange red ppt. indicates the presence of alkaloids.
   e) **Tannic acid solution (10%):** production of a dirty white or Blackish ppt. indicates the presence of alkaloids.

V. **General test for glycosides:** A small amount of extract was dissolved in 1ml of distilled water, and then few drops of aqueous Sodium hydroxide solution were added. A yellow color was developed in the presence of glycosides.

VI. **Test for glucosides:** A small amount of extract was dissolved in 1 ml of distilled water and alcohol then boiled with Fehling's solution. Any brick red precipitation was noted. Another portion of the extract was dissolved in water and alcohol then boiled with a few drops of dilute sulfuric acid. The acid was neutralized with sodium hydroxide solution and boiled with Fehling's solution. A brick red ppt. was produced in this experiment, which showed the presence of glucosides the extract.

VII. **Borntrager'stest of anthraquinoneglycosides:** 1 ml of sample solution was shaken with 5ml of chloroform in a test tube for at least 5 minutes then again shaken with an equal volume of 10% ammonia solution. A bright pink, red or violet color was developed in the aqueous (upper) layer in the presence of free anthraquinones.

VIII. **Test for saponins:** About 0.5 ml of extract was shaken vigorously with water in a test tube. If a frothing was produced and it was stable for 1 - 2 minutes and persisted on warming, it was taken as preliminary evidence for the saponin.

IX. **Test for flavonoids:** A few drops of conc. hydrochloric acid were added to small amount of extract. Immediate development of a red color indicated the presence of flavonoids.

X. **Test for steroids:** A small amount of extract was added with 2 ml of chloroform and then 1 ml of conc. sulfuric acid was carefully added from the side of the test tube. In presence of steroids, a reddish color was produced in chloroform layer.

XI. **$FeCl_3$ (5%) test for tannins:** About 0.5 ml of extracts was stirred with 10 ml distilled water. Production of a blue, blue-black, green or blue-green coloration or ppt. on the addition of $FeCl_3$ (5%) reagent was taken as evidence for the presence of tannins.

XII. **Lead subacetatetest for tannins:** 5 ml of sample solution of the plant material was taken in a test tube and few drops of a 1% solution of lead subacetate were added. A red or yellow ppt. was formed if tannin was present.

XIII. **Biuret's test for proteins:** To 1 ml of hot extract of the plant material 5-8 drops of 10% sodium hydroxide solution and 1-2 drops of 3% copper sulfate solution were added. A red or violet color was present if a protein was present.

XIV. **Saponification test for volatile oils, fixed oils and fats:** 0.2 g of sample was taken in a test tube. 3 ml of 5% potassium hydroxide solution was added. The tube was heated in a boiling water bath, shaking at frequent Intervals, for at least 45 to 60 minutes. A solution was formed, which on cooling becomes viscous, indicating positive saponification test for fixed oils. But formation of non-viscous emulsion was indicating negative saponification test for volatile oils.

XV. **Paper spot test for volatile oils and fixed oils:** One drop of oil was taken on a filter paper. Fixed oils form permanent staining upon the paper but volatile oils do not stain.

Phytochemical reports about chemical constituents of plants

Samples **Different Tests**	***Myristicafragrans* Seed extract and fractions**			
	Methanol extract	Petroleum ether fraction	n-Hexane fraction	Chloroform fraction
1. Test for alkaloid				
• Mayer's reagent	+	+	+	+
• Wagner's reagent	+	+	+	+
• Hager's reagent	+	+	+	+
• Dragendorff's reagent	+	+	+	+
• Tannic acid solution (10%)	+	+	+	+
2. Test for carbohydrate				
• Molisch's test	+	±	±	+
• Fehling's test	+	±	±	±
• Barfoed's test	+	±	±	±
3. Test for glycoside				
• General test	+	±	±	±
• Test for glucoside	±	-	-	-
• Anthraquinone glycoside test	±	-	-	-
• Frothing test (Saponin)	+	-	-	+
• Flavonoid test	+	-	-	±
4. Test for tannins				
• $FeCl_3$ test	±	-	-	-
• Lead subacetate test	+	-	-	-
5. Test for steroids				
• Salkowski test	+	+	+	±
6. Test for proteins				
• Biuret's test	+	±	±	±
7. Test for fixed oils and fats	+	+	+	±
8. Test for volatile oils	+	+	+	-

± = Trace; + = Present; - = Absent

Table:-5.1. Results of *Myristicafragrans* Seed extract and fractions.

6.1 Introduction

Free radicals occur oxidative damage to biomolecules and cause cancer, aging, visual impairments, neurodegenerative diseases, atherosclerosis and several other pathological events in living organisms. Antioxidants which scavenge free radicals are known to possess an important role in preventing these free radical induced diseases. There is an increasing interest in the antioxidants effects of compounds derived from plants, which could be relevant in relations to their nutritional incidence and their role in health and diseases. A number of reports on the isolation and testing of plant derived antioxidants have been described during the past decade. Natural antioxidants constitute a broad range of substances including phenolic or nitrogen containing compounds and carotenoids. Lipid peroxidation is one of the main reasons for deterioration of food products during processing and storage. Synthetic antioxidant such as Ascorbic acid, tert-butyl-1-hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and propyl gallate (PG) are widely used as food additives and pharmaceuticals to increase shelf life, especially liquid and lipid containing products by retarding the process of oxidation. However, BHT and BHA are known to have not only toxic and carcinogenic effects on humans, but abnormal effects on enzyme systems. Therefore, the interest in natural antioxidant, especially of plant origin, has greatly increased in recent years.

6.2 Principle

The free radical scavenging activities (antioxidant capacity) of the plant extracts on the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) were estimated by the method of Braca et al. (2001). 2.0 ml of a methanol solution of the extract at different concentration were mixed with 3.0 ml of a DPPH methanol solution (20 µg/ml). The antioxidant potential was assayed from the bleaching of purple colored methanol solution of DPPH radical by the extract as compared to that of tert-butyl-1-hydroxytoluene (BHT) and Ascorbic acid by UV spectrophotometer.

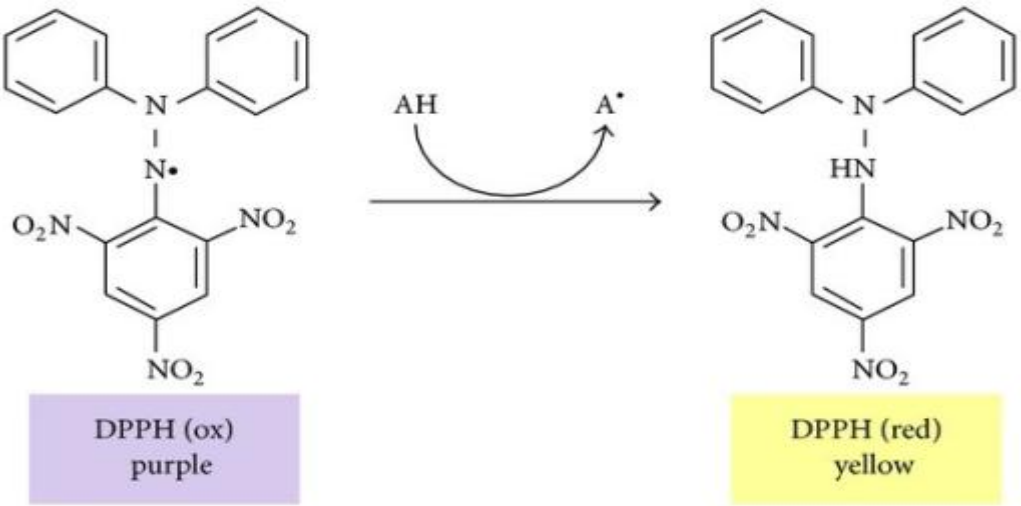


Figure 6.1: DPPH = 1,1-diphenyl-2-picrylhydrazyl

6.3 Materials and methods

DPPH was used to evaluate the free radical scavenging activity (antioxidant potential) of methanolic extract and various fractions of *Myristica fragrans.*

6.3.1 Materials

1) 1,1-diphenyl-2-picrylhydrazyl
2) Tert-butyl-1-hydroxytoluene
3) Distilled water
4) Methanol
5) UV-spectrophotometer
5) Beaker (100 ml and 200 ml)
6) Test tube
7) Light proof box
8) Pipette
9) Micropipette (50-200 µl)
10) Amber reagent bottle

6.3.2 Methods

- 2.0 ml of a methanol solution of the extract at different concentration (500 to 0.977 µg/ml) were mixed with 3.0 ml of a DPPH methanol solution (20 µg/ml).
- After 30 min reaction period at room temperature in dark place the absorbance was measured against at 517 nm against methanol as blank by UV spectrophotometer.
- Inhibition free radical DPPH in percent (I %) was calculated as follows:

$$(I\ \%) = (1 - A_{sample}/A_{blank})\ X\ 100$$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test material).

- Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted inhibition percentage against extract concentration.
- BHT and Ascorbic acid were used as positive control.

The entire procedure of the experiment is explained on figure 6.2 in the next page.

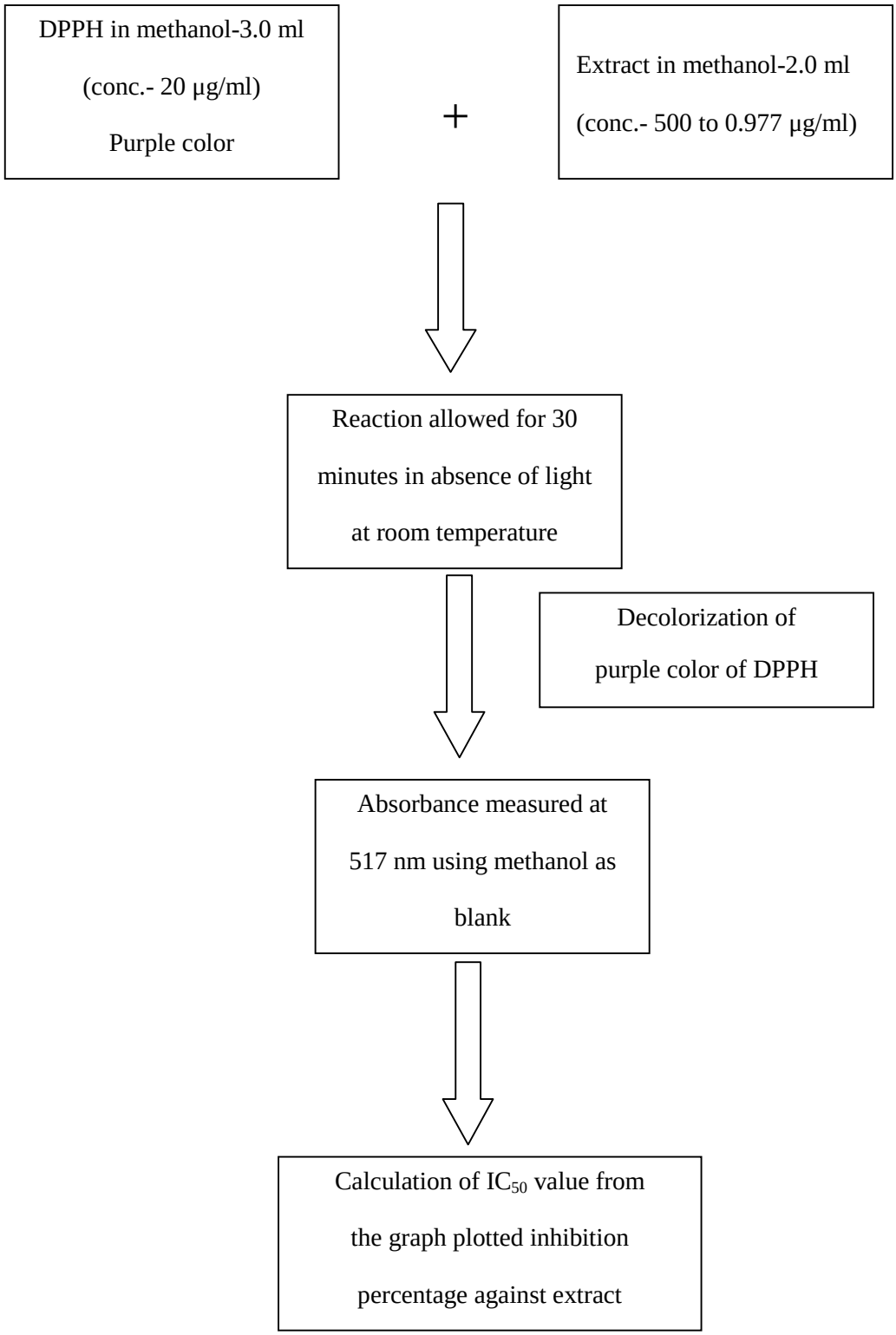


Figure 6.2: Schematic representation of the method of assaying free radical scavenging activity

6.4 Analytical data

Table 6.1: IC_{50} value of tert-butyl-1-hydroxytoluene (BHT)

Absorbance of Blank	Concentration (µg/ml)	Absorbance	% Inhibition	IC_{50} (µg/ml)
0.421	500	0.072	82.898	29.366
	250	0.109	74.109	
	125	0.139	66.983	
	62.5	0.175	58.432	
	31.25	0.210	50.119	
	15.625	0.242	42.518	
	7.813	0.281	33.254	
	3.306	0.312	25.891	
	1.953	0.336	20.190	
	0.976	0.373	11.401	

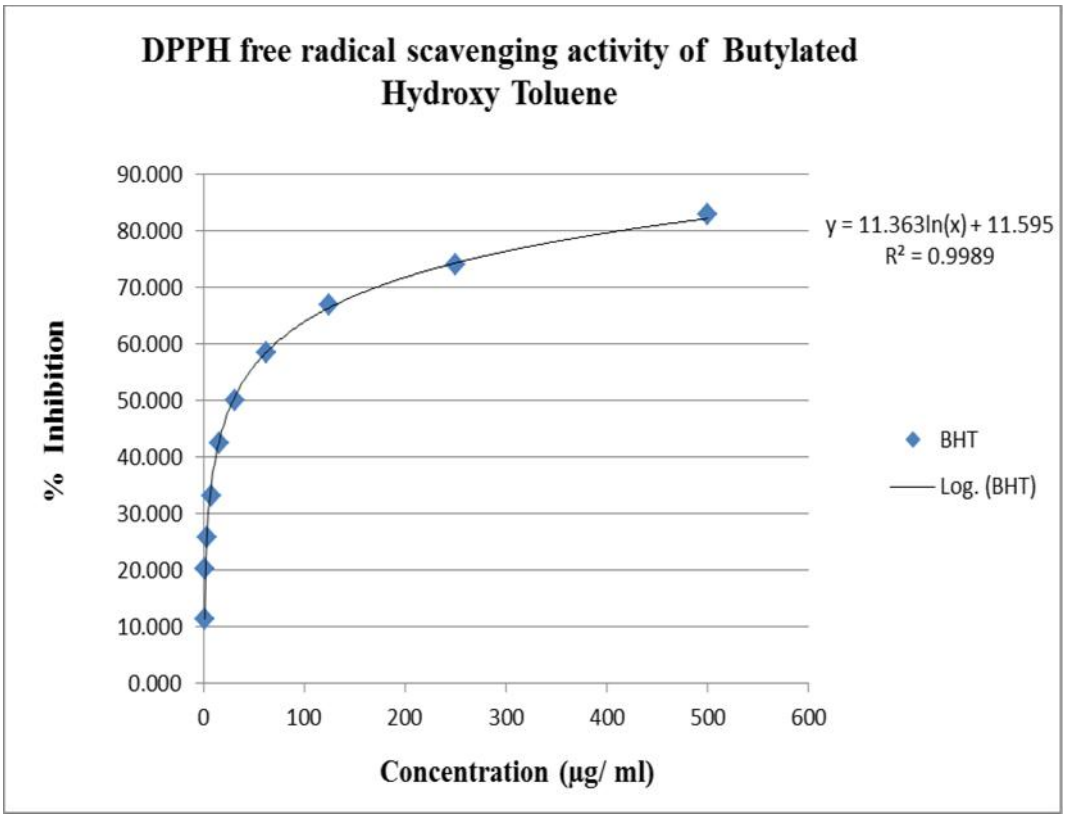


Figure 6.3: Plot of inhibition percentage vs Concentration of BHT

Table 6.2: IC_{50} value of Ascorbic acid

Absorbance of Blank	Concentration (µg/ml)	Absorbance	% Inhibition	IC_{50} (µg/ml)
0.421	500	0.011	97.387	4.512
	250	0.019	95.487	
	125	0.023	94.537	
	62.5	0.027	93.587	
	31.25	0.064	84.798	
	15.625	0.106	74.822	
	7.813	0.161	61.758	
	3.306	0.234	44.418	
	1.953	0.298	29.216	
	0.976	0.316	24.941	

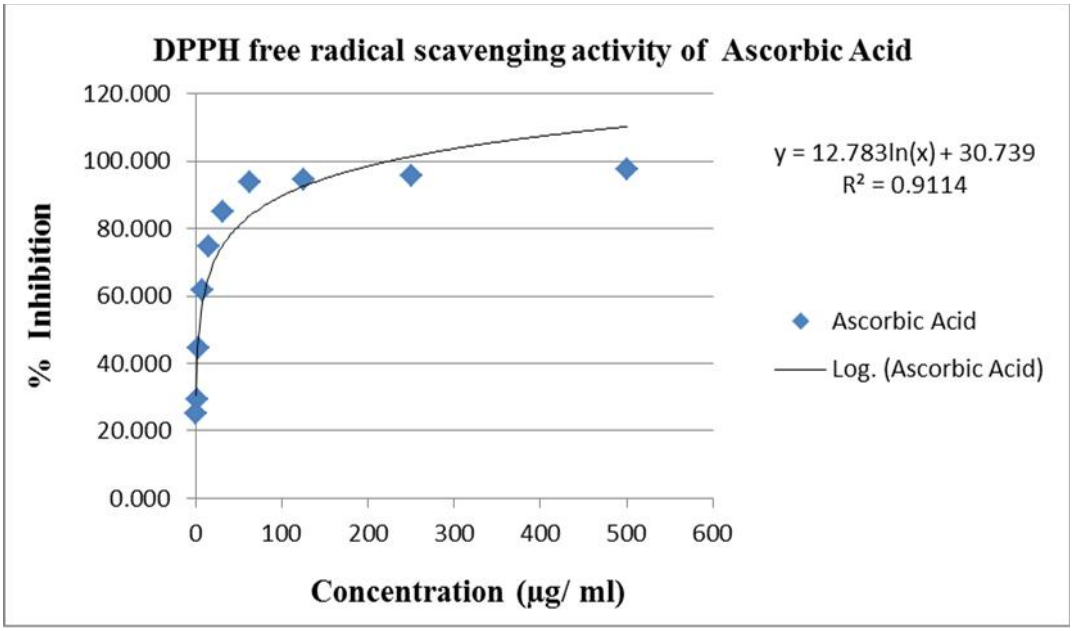


Figure 6.4: Plot of inhibition percentage vs Concentration of Ascorbic acid

Table 6.3: IC_{50} value of Methanolic extract of *Myristica fragrans*

Absorbance of Blank	Concentration (µg/ml)	Absorbance	% Inhibition	IC_{50} (µg/ml)
0.421	500	0.112	73.397	25.153
	250	0.118	71.971	
	125	0.126	70.071	
	62.5	0.137	67.458	
	31.25	0.188	55.344	
	15.625	0.212	49.644	
	7.813	0.240	42.993	
	3.306	0.312	25.891	
	1.953	0.348	17.340	
	0.976	0.379	9.976	

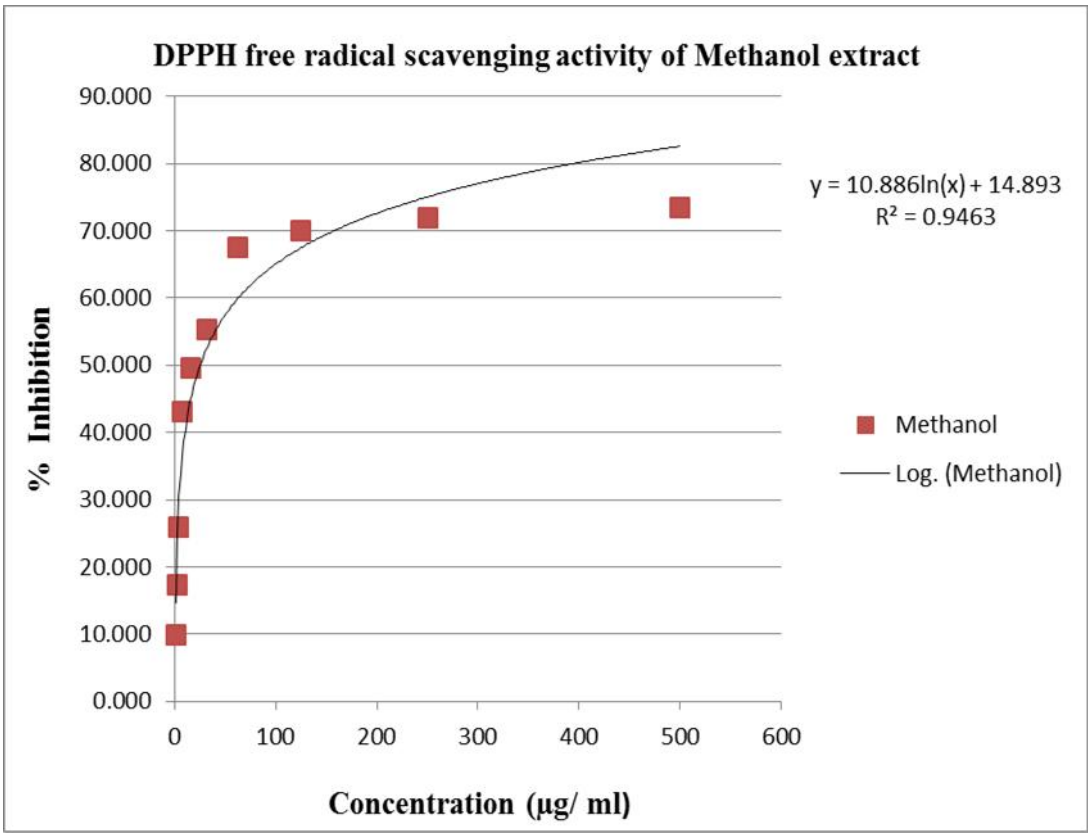


Figure 6.5: Plot of inhibition percentage vs Concentration of methanolic extract of *Myristica fragrans*

Table 6.4: IC_{50} value of Petroleum ether fraction of *Myristica fragrans*

Absorbance of Blank	Concentration (µg/ml)	Absorbance	% Inhibition	IC_{50} (µg/ml)
0.421	500	0.168	60.095	94.019
	250	0.181	57.007	
	125	0.195	53.682	
	62.5	0.206	51.069	
	31.25	0.231	45.131	
	15.625	0.252	40.143	
	7.813	0.331	21.378	
	3.306	0.357	15.202	
	1.953	0.378	10.214	
	0.976	0.394	6.413	

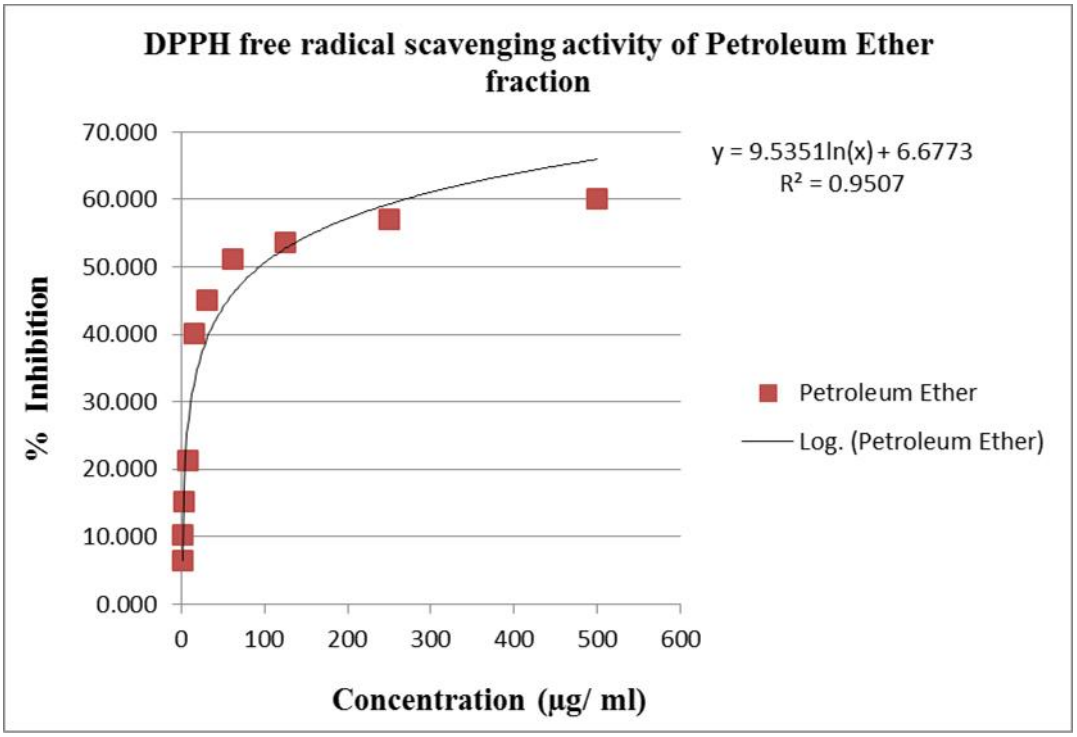


Figure 6.6: Plot of inhibition percentage vs Concentration of Petroleum ether fraction

Table 6.5: IC_{50} value of n-Hexane fraction of *Myristica fragrans*

Absorbanse of Blank	Concentration (µg/ml)	Absorbance	% Inhibition	IC_{50} (µg/ml)
0.421	500	0.174	58.670	119.321
	250	0.183	56.532	
	125	0.195	53.682	
	62.5	0.228	45.843	
	31.25	0.256	39.192	
	15.625	0.276	34.442	
	7.813	0.328	22.090	
	3.306	0.352	16.390	
	1.953	0.379	9.976	
	0.976	0.397	5.701	

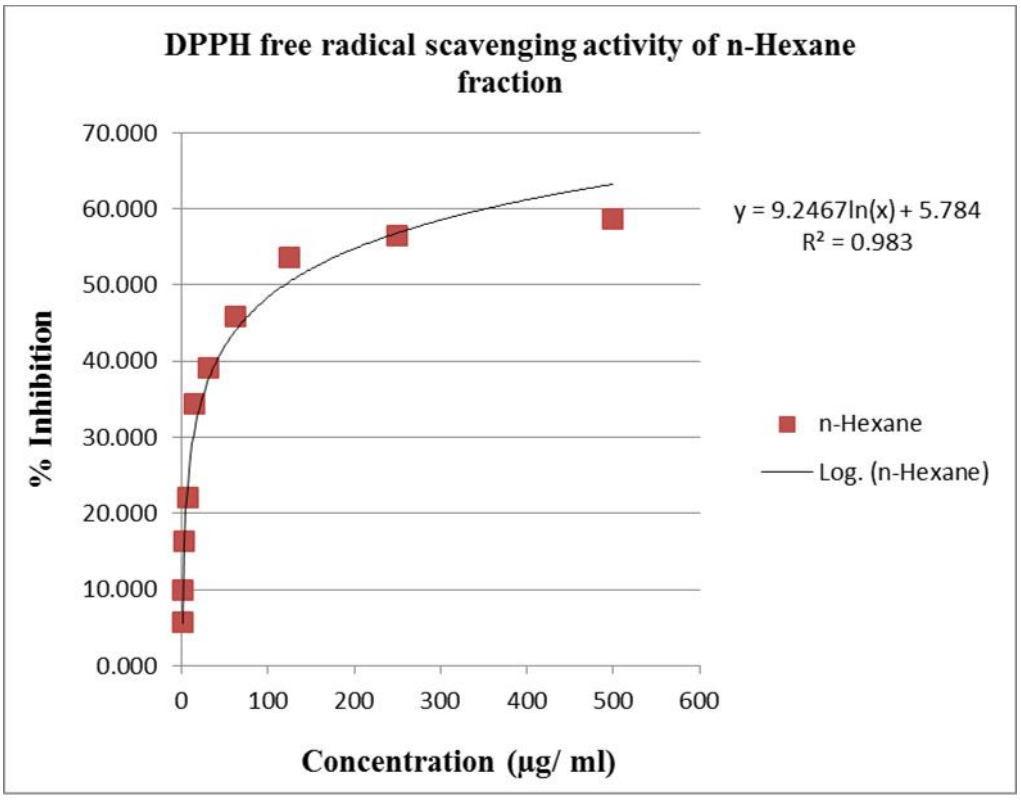


Figure 6.7: Plot of inhibition percentage vs Concentration of n-Hexane fraction

Table 6.6: IC_{50} value of Chloroform fraction of *Myristica fragrans*

Absorbanse of Blank	Concentration (µg/ml)	Absorbance	% Inhibition	IC_{50} (µg/ml)
0.421	500	0.117	72.209	16.879
	250	0.117	72.209	
	125	0.118	71.971	
	62.5	0.125	70.309	
	31.25	0.155	63.183	
	15.625	0.195	53.682	
	7.813	0.248	41.093	
	3.306	0.261	38.005	
	1.953	0.326	22.565	
	0.976	0.342	18.765	

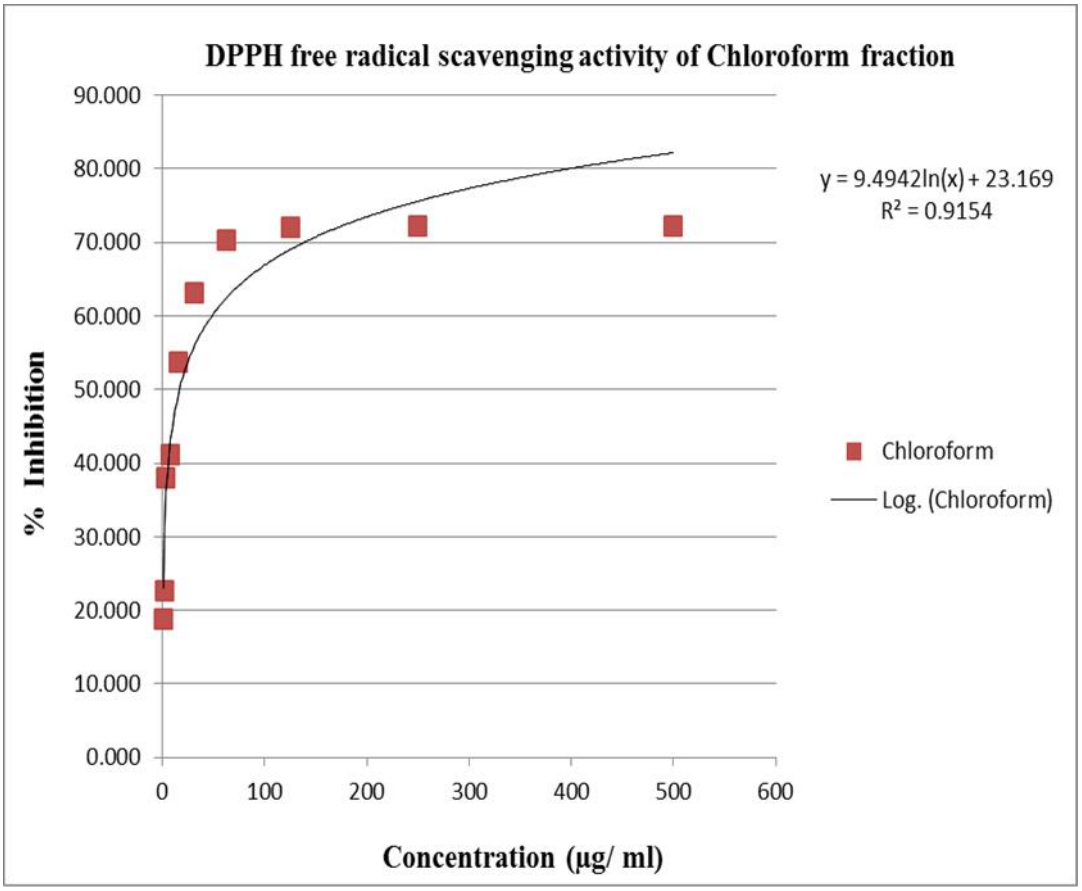


Figure 6.8: Plot of inhibition percentage vs Concentration of Chloroform fraction

Table 6.7: IC_{50} values of standard and different fractions of *Myristica fragrans*

Comparison	**IC_{50} (µg/ml)**
Ascorbic acid	4.512
Butylated hydroxy toluene	29.366
Methanolic extract	25.153
Petroleum ether fraction	94.019
n-Hexane fraction	119.321
Chloroform fraction	16.879

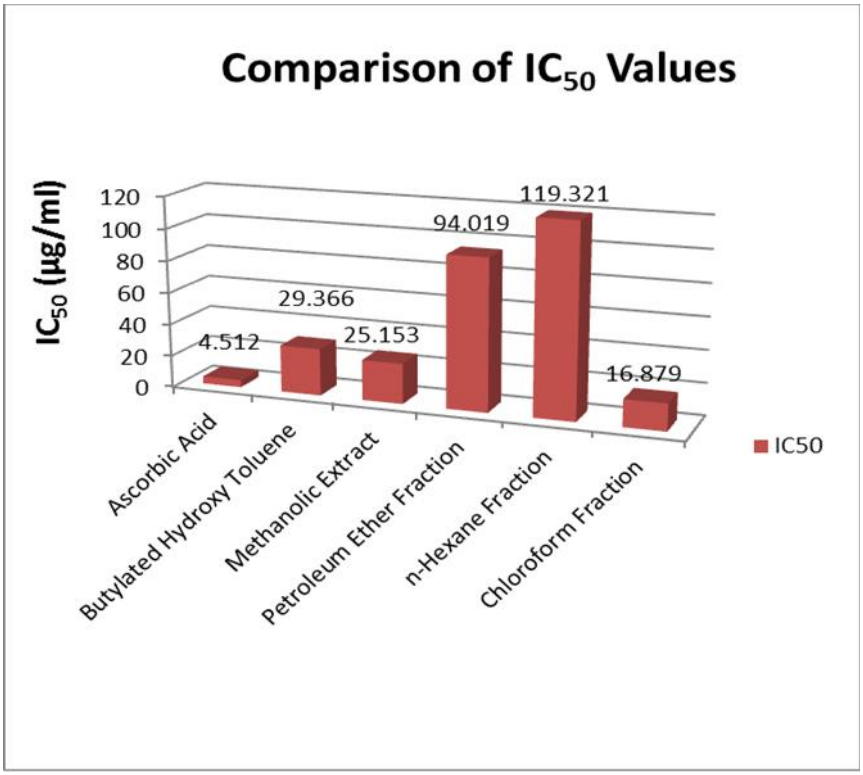


Figure 6.9: Plot of comparing IC_{50} values

6.5 Results and discussion

Different partitions of methanolic extract of *Myristica fragrans* were subjected to free radical scavenging activity by the method of DPPH free radical scavenging activity. Here, tert-butyl-1-hydroxytoluene (BHT) and ascorbic acid were used as reference standard. In this investigation, the chloroform fraction showed the highest free radical scavenging activity with IC_{50} value 16.879 µg/ml. At the same time the crude methanolic extract, petroleum ether fraction and n-hexane fraction also exhibited strong antioxidant potential having IC_{50} value 25.153 µg/ml, 94.019 µg/ml and 119.321 µg/ml respectively.

7.1 Objective of brine shrimp lethality bioassay

Bioactive compounds are always toxic to living body at some higher doses and it justifies the statement that 'Pharmacology is simply toxicology at some lower doses and toxicology is simply pharmacology at some higher doses.' Brine Shrimp Lethality Bioassay (Meyer et al., 1982) is a rapid and comprehensive bioassay for the bioactive compounds of natural and synthetic origin. By this method, natural product extracts, fractions as well as pure compounds can be tested for their bioactivity. In this method, in vivo lethality in a simple zoological organism (Brine shrimp nauplii) is used as a favorable monitor for screening and fractionation in the discovery of new bioactive natural products. This bioassay indicates cytotoxicity as well as a wide range of pharmacological activities such as antimicrobial, antiviral, pesticidal and anti-tumor etc. of the compounds (Meyer et al., 1982).

Brine Shrimp Lethality Bioassay technique stands superior to other cytotoxicity testing procedures because it is rapid in process, inexpensive and requires no special equipment or aseptic technique. It utilizes a large numbers of organisms for statistical validation and a relatively small amount of sample. Furthermore, unlike other methods, it does not require animal serum.

7.2 Principle

Brine shrimp eggs are hatched in simulated sea water to get nauplii. By the addition of calculated amount of Dimethyl sulfoxide (DMSO), desired concentrations of the test sample is prepared. The nauplii are counted by visual inspection and are taken in vials containing 5 ml of simulated sea water. Then samples of different concentrations are added to pre-marked vials using micropipettes. Then the vials are left for 24 hour. Survivors are counted after 24 hours. These data are processed in Microsoft Excel to estimate LC_{50} values.

7.3 Materials

- Artemia salina leach (brine shrimp eggs)
- Sea salt (NaCl)
- Small tank with perforated dividing dam to hatch the shrimp
- Lamp to attract shrimps
- Pipettes, Micropipette
- Glass vials
- Test samples of experimental plants

Table 7.1: Test samples for the experiment

Code no.	Test sample	Amount (mg)
1	Crude methanolic extract of *Myristica fragrans*	4 mg
2	Petroleum ether fraction of *Myristica fragrans*	4 mg
3	n-Hexane fraction of *Myristica fragrans*	4 mg
4	Chloroform fraction of *Myristica fragrans*	4 mg

7.4 Experimental procedure

7.4.1 Preparation of seawater

38 gm sea salt (pure NaCl) was weighed, dissolved in one liter of distilled water and filtered off to get a clear solution.

7.4.2 Hatching of brine shrimps

Artemia salina leach (brine shrimp eggs) collected from pet shops was used as the test organism. Seawater was taken in the small tank and shrimp eggs were added to one side of the tank and then this side was covered. Two days were allowed to hatch the shrimp and to be matured as nauplii. Constant oxygen supply was carried out through the hatching time. The hatched shrimps were attracted to the lamp through the perforated dam and they were taken for experiment. With the help of a Pasteur pipette 10 living shrimps were added to each of the test tubes containing 5 ml of seawater.

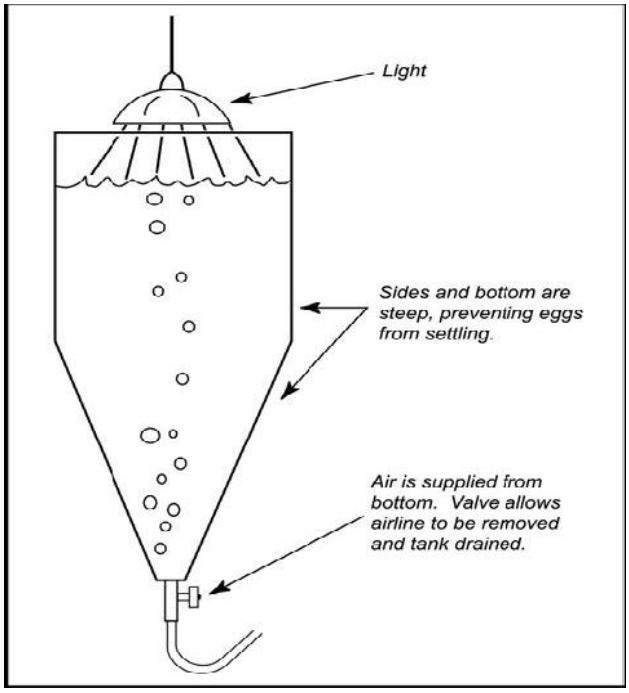


Figure 7.1: Brine shrimp Hatchery

7.4.3 Preparation of the test sample

Clean test tubes were taken. These test tubes were used for ten different concentrations (one test tube for each concentration) of test samples and ten test tubes were taken for ten concentrations of standard drug Vincristine and another one test tubes for control test. All the test samples (crude methanolic extract, Petroleum ether fraction, n-Hexane fraction and Chloroform fraction) of 4 mg were taken and dissolved in 200 µL of Di methyl sulfoxide (DMSO) in vials to get stock solutions. Then 100 µL of solution was taken in test tube each containing 5 ml of simulated seawater and 10 shrimp nauplii. Thus, final concentration of the prepared solution in the first test tube was 400 µg/ml. Then a series of solutions of varying concentrations were prepared from the stock solution by serial dilution method. In

each case 100 µL sample was added to test tube and fresh 100 µL DMSO was added to vial. Thus different concentrations were found in the different test tubes.

Table 7.2: Test sample concentration after dilution

Test tube	Concentration (µg/ml)	Test tube	Concentration (µg/ml)
1	400	6	12.5
2	200	7	6.25
3	100	8	3.125
4	50	9	1.563
5	25	10	0.781

7.4.4 Preparation of the positive control group

Positive control in a cytotoxicity study is a widely accepted cytotoxic agent and the result of the test agent is compared with the result obtained for the positive control. In the present study Vincristine sulphate used as the positive control. Measured amount of the Vincristine sulphate dissolved in DMSO to get an initial concentration of 400 µg/ml from which serial dilutions made using DMSO. Then the positive control solutions added to the pre marked vials containing ten living brine shrimp nauplii in 5 ml simulated sea water to get the positive control groups.

7.4.5 Preparation of the negative control

100 µL of DMSO was added to each of four pre marked glass vials containing 5 ml of simulated sea water and 10 shrimp nauplii to use as control groups. If the brine shrimps in these vials show a rapid mortality rate, then the test is considered as invalid as the nauplii died due to some reason other than the cytotoxicity of the compounds.

7.4.6 Counting of nauplii

After 24 hours, the vials were inspected using a magnifying glass and the number of survivors were counted. The percent (%) mortality was calculated for each dilution. The concentration- mortality data were analyzed statistically by using Microsoft Excel program. The effectiveness or the concentration mortality relationship of plant product is usually expressed as a median lethal concentration (LC50) value. This represents the concentration of the chemical that produces death in half of the test subjects after a certain exposure period.

7.5 Analytical data

Table 7.3: Effect of Vincristine Sulphate (Positive Control) on Shrimp nauplii

Concentration	Log C	Alive	Death	% Mortality	LC_{50} (µg/ ml)
400	2.602	0	10	100	0.510
200	2.301	0	10	100	
100	2.000	0	10	100	
50	1.699	0	10	100	
25	1.398	0	10	100	
12.5	1.097	0	10	100	
6.25	0.796	0	10	100	
3.125	0.495	2	8	80	
1.5625	0.194	3	7	70	
0.78125	-0.107	4	6	60	

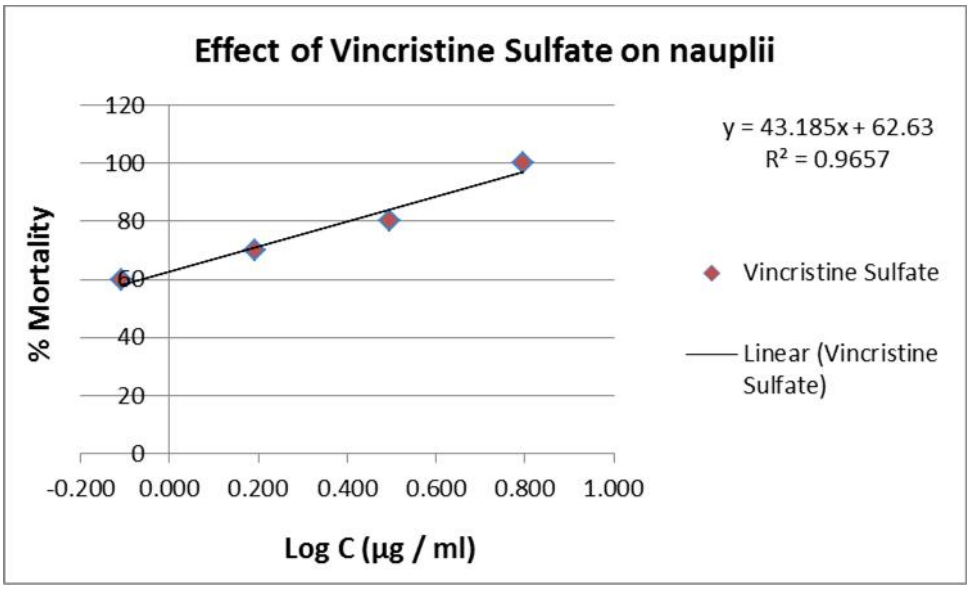


Figure 7.2: % Mortality vs. Log C for Vincristine sulfate

Table 7.4: Effect of crude methanolic extract of *Myristica fragrans* on shrimp nauplii

Concentration	Log C	Alive	Death	% Mortality	LC_{50} (µg/ ml)
400	2.602	0	10	100	2.079
200	2.301	0	10	100	
100	2.000	0	10	100	
50	1.699	0	10	100	
25	1.398	0	10	100	
12.5	1.097	0	10	100	
6.25	0.796	3	7	70	
3.125	0.495	4	6	60	
1.5625	0.194	6	4	40	
0.78125	-0.107	7	3	30	

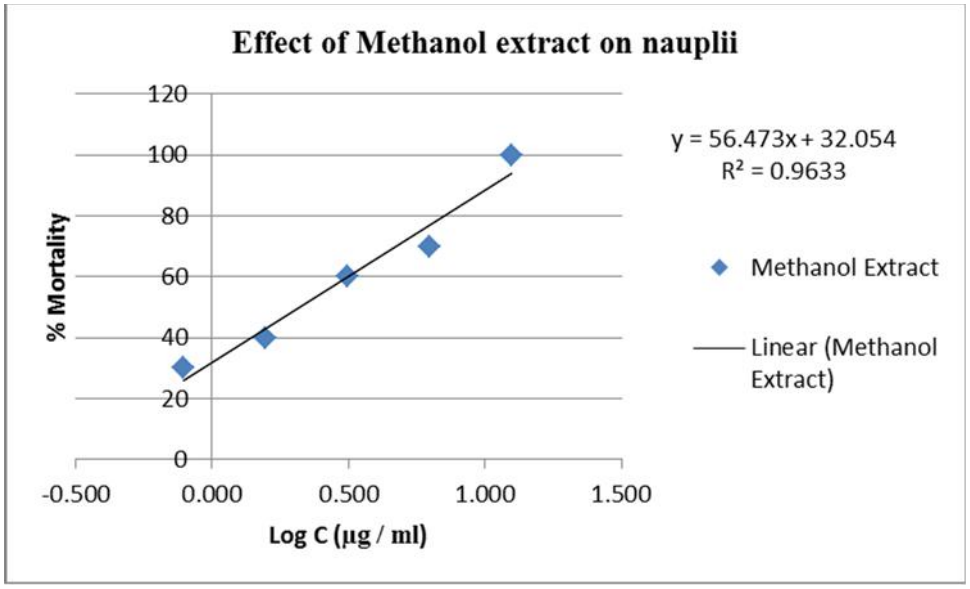


Figure 7.3: % Mortality vs Log C for the Crude methanolic extract

Table 7.5: Effect of Petroleum Ether fraction of *Myristica fragrans* on shrimp nauplii

Concentration	Log C	Alive	Death	% Mortality	LC_{50} (µg/ ml)
400	2.602	0	10	100	0.098
200	2.301	0	10	100	
100	2.000	0	10	100	
50	1.699	0	10	100	
25	1.398	0	10	100	
12.5	1.097	0	10	100	
6.25	0.796	0	10	100	
3.125	0.495	0	10	100	
1.5625	0.194	1	9	90	
0.78125	-0.107	2	8	80	

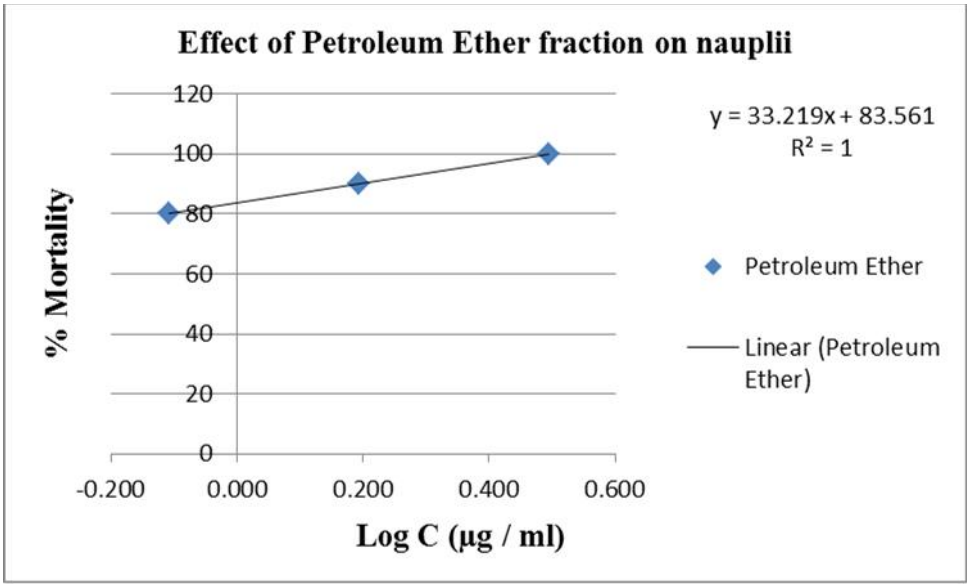


Figure 7.4: % Mortality vs Log C for the Petroleum ether fraction

Table 7.6: Effect of n-Hexane fraction of *Myristica fragrans* on shrimp nauplii

Concentration	Log C	Alive	Death	% Mortality	LC_{50} (µg/ ml)
400	2.602	0	10	100	0.818
200	2.301	0	10	100	
100	2.000	0	10	100	
50	1.699	0	10	100	
25	1.398	0	10	100	
12.5	1.097	0	10	100	
6.25	0.796	0	10	100	
3.125	0.495	0	10	100	
1.5625	0.194	3	7	70	
0.78125	-0.107	5	5	50	

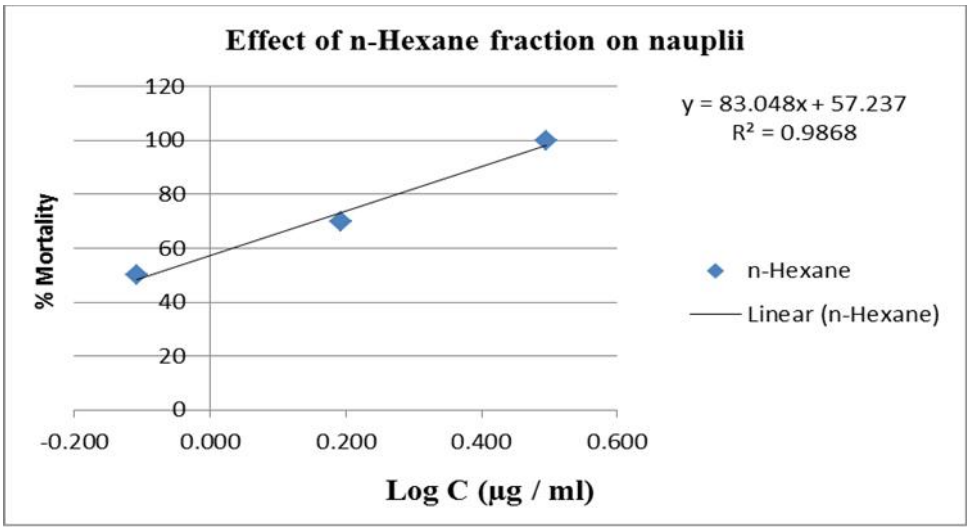


Figure 7.5: % Mortality vs Log C for the n-Hexane fraction

Table 7.7: Effect of Chloroform fraction of *Myristica fragrans* on shrimp nauplii

Concentration	Log C	Alive	Death	% Mortality	LC_{50} (µg/ ml)
400	2.602	0	10	100	0.276
200	2.301	0	10	100	
100	2.000	0	10	100	
50	1.699	0	10	100	
25	1.398	0	10	100	
12.5	1.097	0	10	100	
6.25	0.796	0	10	100	
3.125	0.495	2	8	80	
1.5625	0.194	3	7	70	
0.78125	-0.107	3	7	70	

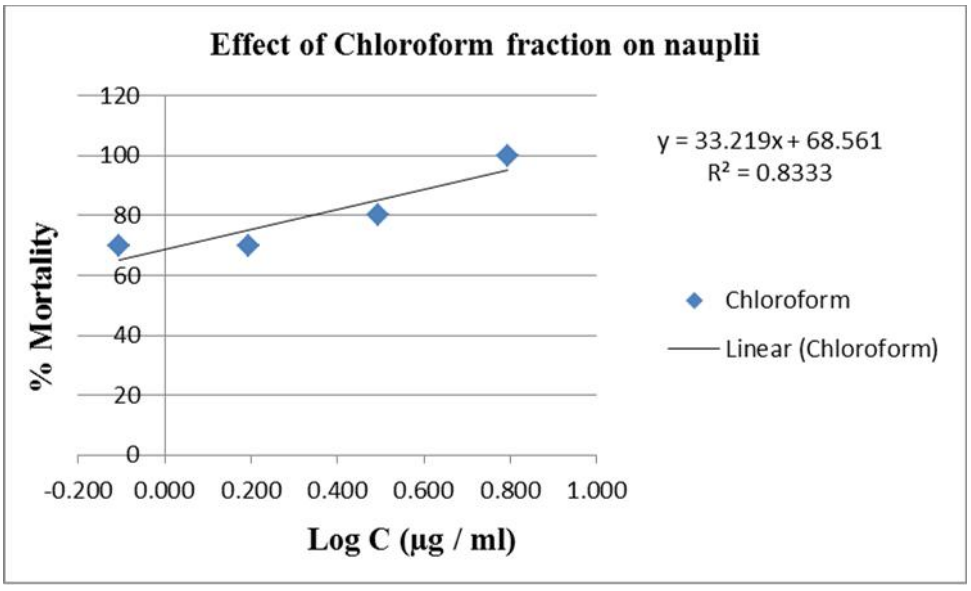


Figure 7.6: % Mortality vs Log C for the Chloroform fraction

Table 7.8: A brief overview of the cytotoxic activity of the different fractions of Methanolic extract of *Myristica fragrans*

Test Samples	**LC $_{50}$ (µg/ ml)**
Vincristine sulphate	0.510
Methanol extract	2.079
Petroleum ether fraction	0.098
n-Hexane fraction	0.818
Chloroform fraction	0.276

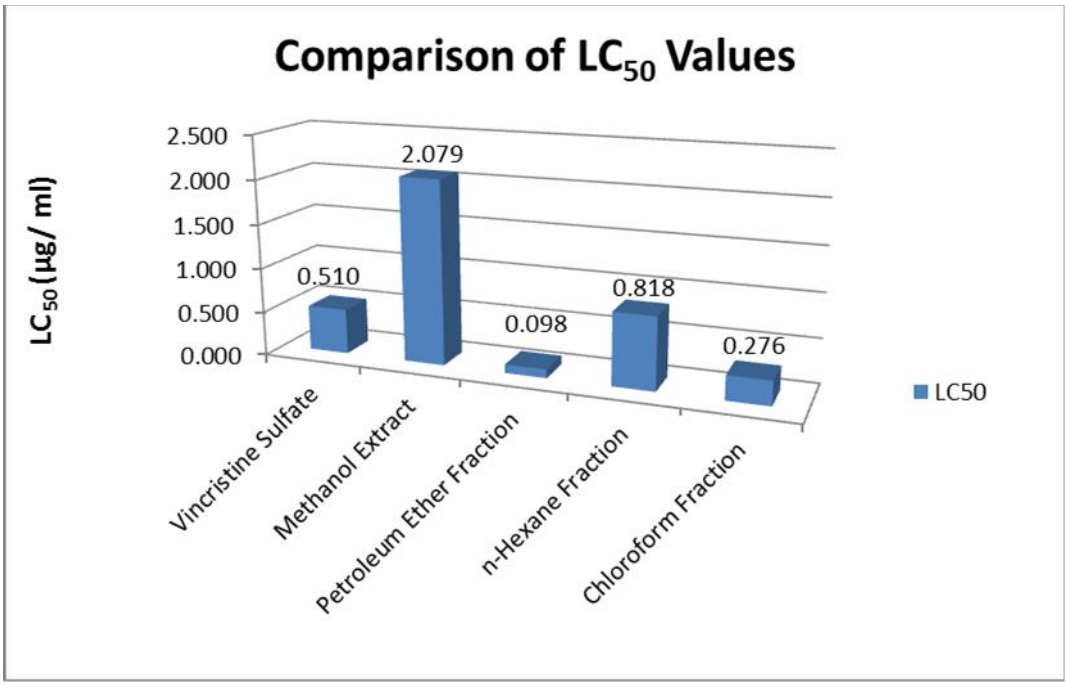


Figure 7.7: Comparison of LC_{50} values of the Test samples

7.6 Results and discussion

The different extracts of *Myristica fragrans* were subjected to brine shrimp lethality bioassay following the procedure of Meyer et al, (1982). The lethal concentration (LC_{50}) of the test samples after 24 hours was obtained by a plot of percentage of the shrimps died against the logarithm of the sample concentration (toxicant concentration) and the best fit line was obtained from the curve data by means of regression analysis. Vincristine sulphate (VS) was used as positive control and the LC_{50} was found as 0.510 µg/ml. Comparing with the positive control, test samples gave significant mortality and the LC_{50} values of the different fractions were significant. The crude methanolic extract, petroleum ether fraction, n-hexane fraction and chloroform fraction showed cytotoxic activity and LC_{50} were respectively 2.079 µg/ml, 0.098 µg/ml, 0.818 µg/ml and 0.276 µg/ml Petroleum ether and chloroform fractions have more cytotoxic property than Vincristine sulphate.

8.1 Introduction

Infectious disease is one of main causes of death accounting for approximately one-half of all deaths in tropical countries. Perhaps it is not surprising to see these statistics that the infectious disease mortality rates are actually increasing in developing countries, such as Bangladesh. This is alarming and it was once believed that we would eliminate infectious disease by the end of the millennium. The increases are attributed to increases in different infectious diseases. Other contributing factors are an increase in antibiotic resistance in nosocomial and community acquired infections. Furthermore, the most dramatic increases are occurring in the 25–45 year old age group.

These negative health trends call for a renewed interest in infectious disease in the medical and public health communities and renewed strategies on treatment and prevention. It is the last solution that would encompass the development of new antimicrobials.

The antimicrobial screening which is the first stage of antimicrobial drug research is performed to ascertain the susceptibility of various fungi and bacteria to any agent. This test measures the ability of each test sample to inhibit the in vitro fungal and bacterial growth. This ability may be estimated by any of the following three methods.

i) Disc diffusion method
ii) Serial dilution method
iii) Bioautographic method

But there is no standardized method for expressing the results of antimicrobial Screening. Some investigators use the diameter of zone of inhibition and/or the minimum weight of extract to inhibit the growth of microorganisms. However, a great number of factors viz., the extraction methods, inoculum volume, culture medium composition, pH, and incubation temperature can influence the results. Among the above mentioned techniques the disc diffusion is a widely accepted in vitro investigation for preliminary screening of test agents which may possess antimicrobial activity (Bauer et al., 1966). It is essentially a quantitative or qualitative test indicating the sensitivity or resistance of the microorganisms to the test materials.

8.2 Principle of disc diffusion method

In this classical method, antibiotics diffuse from a confined source through the nutrient agar gel and create a concentration gradient. Dried and sterilized filter paper discs (6 mm diameter) containing the test samples of known amounts are placed on nutrient agar medium uniformly seeded with the test microorganisms. Standard antibiotic (Ciprofloxacin) discs and blank discs are used as positive and negative control. These plates are kept at low temperature (4°C) for 24 hours to allow maximum diffusion of the test materials to the surrounding media (Barry, 1976). The plates are then inverted and incubated at 37°C for 24 hours for optimum growth of the organisms. The test materials having antimicrobial property inhibit microbial growth in the media surrounding the discs and thereby yield a clear, distinct area defined as zone of inhibition. The antimicrobial activity of the test agent

is then determined by measuring the diameter of zone of inhibition expressed in millimeter (Barry, 1976).

In the present study the crude extracts as well as fractions were tested for antimicrobial activity by disc diffusion method. The experiment is carried out more than once and the mean of the readings is required (Bauer et al., 1966).

8.3 Materials and methods

8.3.1 Apparatus and reagents

Refrigerator	Sterile forceps
Incubator	Nose mask, Hand gloves
Laminar air flow hood	Filter paper discs
Autoclave	Nutrient agar medium
Spirit burner	Sterile cotton
Inoculating loop	Micropipette
Petridishes	Chloroform
Screw cap test tubes	Ethanol

8.3.2 Test organisms

A variety of microbiological organisms were used for the experiment. These included both gram positive and gram negative bacteria and also fungi. In fact five strains of gram positive bacteria, eight strains of gram-negative bacteria and three strains of fungi was used for the experiment. The strains used for the experiment must be of pure culture. Otherwise the validity of the experiment will be under question. The bacterial and fungal strains used for the experiment were collected as pure cultures from the Institute of Nutrition and Food Science (INFS), University of Dhaka. The gram-positive, gram negative bacteria and the fungal strains used for the experiment are listed down in the table no 8.1.

Table 8.1: List of gram positive, gram negative bacteria and fungi

Gram positive bacteria	**Gram negative Bacteria**	**Fungi**
1. Bacillus cereus	*1. Escherichia coli*	*1. Aspergillus niger*
2. Bacillus megaterium	*2. Pseudomonas aeruginosa*	*2. Sacharomyces cerevacae*
3. Bacillus subtilis	*3. Salmonella paratyphi*	*3. Candida albicans*
4. Sarcina lutea	*4. Salmonella typhi*	
5. Staphylococcus aureus	*5. Shigella boydii*	
	6. Shigella dysenteriae	
	7. Vibrio mimicus	
	8. Vibrio parahemolyticus	

Table 8.2: Test samples for the experiment

Code no.	Test sample	Amount (mg)
1	Crude methanolic extract of *Myristica fragrans*	8 mg
2	Petroleum ether fraction of *Myristica fragrans*	8 mg
3	n-Hexane fraction of *Myristica fragrans*	8 mg
4	Chloroform fraction of *Myristica fragrans*	8 mg

8.3.3 Composition of culture medium

Nutrient agar medium (DIFCO) was used in the present study for testing the sensitivity of the organisms to the test materials and to prepare fresh cultures.

Composition of nutrient agar medium (pH 7.2 -7.6 at 25º C)

Ingredients	Amount
Bacto peptone	0.5 gm
Sodium chloride	0.5 gm
Bacto yeast extract	1.0 gm
Bacto agar	2.0 gm
Distilled water	q.s. - 100 ml

8.3.4 Preparation of the medium

To prepare required volume of this medium, calculated amount of each of the constituents was taken in a conical flask and distilled water was added to it to make the required volume. The contents were heated in a water bath to make a clear solution. The pH (at 25º C) was adjusted at 7.2-7.6 using NaOH or HCl. 10 ml and 5 ml of the medium was then transferred in screw cap test tubes to prepare plates and slants respectively. The test tubes were then capped and sterilized by autoclaving at 15-lbs. pressure at 121º C for 20 minutes. The slants were used for making fresh culture of bacteria and fungi that were in turn used for sensitivity study.

8.3.5 Sterilization procedure

In order to avoid any type of contamination and cross contamination by the test organisms the antimicrobial screening was done in Laminar Hood and all types of precautions were highly maintained. UV light was switched on one hour before working in the Laminar Hood. Petridishes and other glassware were sterilized by autoclaving at a temperature of 121º C and a pressure of 15 lbs/sq. inch for 20 minutes. Micropipette tips, cotton, forceps, blank discs etc. were also sterilized by UV light.

8.3.6 Preparation of subculture

In an aseptic condition under laminar air cabinet, the test organisms were transferred from the pure cultures to the agar slants with the help of a transfer loop to have fresh pure cultures. The inoculated strains were then incubated for 24 hours at 37º C for their optimum growth. These fresh cultures were used for the sensitivity test.

8.3.7 Preparation of the test plate

The test organisms were transferred from the subculture to the test tubes containing about 10 ml of melted and sterilized agar medium with the help of a sterilized transfer loop in an aseptic area. The test tubes were shaken by rotation to get a uniform suspension of the organisms. The bacterial and fungal suspension was immediately transferred to the sterilized petridishes. The petridishes were rotated several times clockwise and anticlockwise to assure homogenous distribution of the test organisms in the media.

8.3.8 Preparation of discs

Measured amount of each test sample (specified in table 8.3) was dissolved in specific volume of solvent (Chloroform or methanol) to obtain the desired concentrations in an aseptic condition. Sterilized metrical (BBL, Cocksville, USA) filter paper discs were taken in a blank petridish under the laminar hood. Then discs were soaked with solutions of test samples and dried.

Table 8.3: Preparation of sample discs

Test samples	Dose (µg/disc)	Total amount (mg)
Crude methanolic extracts	400	8.0
Petroleum ether fraction	400	8.0
n-Hexane fraction	400	8.0
Chloroform fraction	400	8.0

Standard Ciprofloxacin (5 Pg/disc) discs were used as positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antimicrobial agent with that of produced by the test sample. Blank discs were used as negative controls which ensure that the residual solvents (left over the discs even after air-drying) and the filter paper were not active themselves.

8.3.9 Diffusion and incubation

The sample discs, the standard antibiotic discs and the control discs were placed gently on the previously marked zones in the agar plates pre-inoculated with test bacteria and fungi. The plates were then kept in a refrigerator at 4º C for about 24 hours upside down to allow sufficient diffusion of the materials from the discs to the surrounding agar medium. The plates were then inverted and kept in an incubator at 37º C for 24 hours.

8.3.10 Determination of the zone of inhibition

The antimicrobial potency of the test agents are measured by their activity to prevent the growth of the microorganisms surrounding the discs which gives clear zone of inhibition. After incubation, the Antimicrobial activities of the test materials were determined by measuring the diameter of the zones of inhibition in millimeter with a transparent scale.

Figure 8.1: Clear zone of inhibition

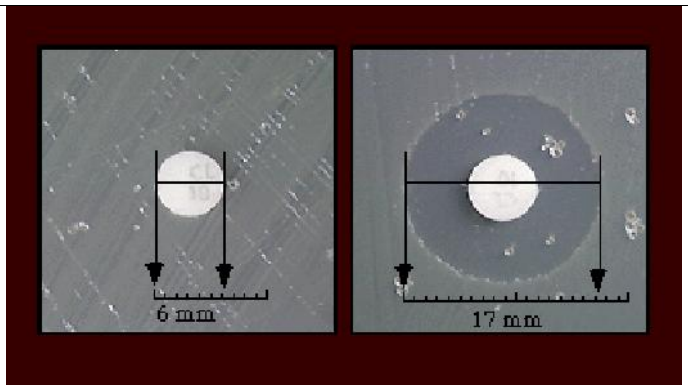


Figure 8.2: Determination of clear zone of inhibition

Table 8.4: Antimicrobial activity of test samples of *Myristica fragrans*

Test organism	**Diameter of zone of inhibition (mm)**				
	Ciprofloxacin	**Methanol extract**	**Petroleum ether fraction**	**n-Hexane fraction**	**Chloroform fraction**
Gram positive bacteria					
Bacillus cereus	45	-	-	-	9
Bacillus megaterium	45	-	-	-	9
Bacillus subtilis	45	-	-	-	10
Sarcina lutea	43	-	-	-	9
Staphylococcus aureus	45	-	-	-	9
Gram negative bacteria					
Escherichia coli	*42*	-	-	-	*9*
Pseudomonas aeruginosa	*43*	-	-	-	*9*
Salmonella paratyphi	*45*	-	-	-	*9*
Salmonella typhi	*44*	-	-	-	*9*
Shigella boydii	*44*	-	-	-	*8*
Shigella dysenteriae	*42*	-	-	-	*9*
Vibrio mimicus	*45*	-	-	-	*10*
Vibrio parahemolyticus	*45*	-	-	-	*9*
Fungi					
Aspergillus niger	*44*	-	-	-	*9*
Candida albicans	*44*	-	-	-	*9*
Sacharomyces cerevacae	*44*	-	-	-	*9*

8.4 Results and discussion

Antibacterial activity was compared with that of antibiotic ciprofloxacin which is considered popular in treatment of diseases caused by many types of bacterial species. The methanolic extract, petroleum ether fraction and n-hexane fraction did not show any remarkable antibacterial activity against the test organisms where ciprofloxacin gave antibacterial activity.

Chloroform fraction gave more distinct zone of inhibition for *Bacillus subtilis* and *Sarcina lutea* than other gram positive bacteria.

Chloroform fraction gave more remarkable zone of inhibition for *Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella dysenteriae* and *Vibrio mimicus* than other gram negative bacteria.

In comparison to Ciprofloxacin, chloroform fraction showed 20.45% zone of inhibition against fungi.

9.1 Principle

In this experiment, carrageenan-induced rat hind paw edema was used as the animal model of acute inflammation. Administration of Carrageenan in the sub-plantar region of rat's hind paw leads to the formation of edema in situ due to localized inflammation. About one hour prior to the administration of Carrageenan solution, experimental animals received test materials and standard anti-inflammatory drug at appropriate doses. The volume of rat's paw was measured each hour up to four hours by plethysmometer (Ugo Basile, Italy). The average percentage of increase in paw volume with time was calculated and compared against the control group. Percent inhibition was calculated using the formula-

$$\%\ inhibition = \frac{Vc - Vt}{Vc} x\,100$$

Where, V_C and V_t represent the average edema volume of control and treated animals respectively. (Ozaki *et. al*., 1988)

9.2 Experimental animal

Long-Evans rats (*Rattus sp.*) of either sex, weighing 50-100 gm were used. They were obtained from the ICDDR,B animal house. The animals were acclimatized in the environmental condition in the Animal House of Nutrition and Food Department of the University of Dhaka for three days providing rat food and water ad-libitum.

9.3 Preparation of test materials

9.3.1 Carrageenan solution (1%): Carrageenan (50 mg) was accurately weighed and then it was dissolved in WFI (Water for Injection) by gentle heating in a water bath. Finally the volume was adjusted up to 5 ml with WFI.

9.3.2 Control solution: 3-4 drops of Tween 80 and few drops of DMSO were mixed properly in the normal saline and the volume was made upto 4.0 ml.

9.3.3 Standard solution: Diclofenac sodium (100 mg) was accurately weighed and then it was dissolved in 5 ml normal saline (0.9% NaCl solution). Few drops of Tween 80 and DMSO were mixed in solution.

9.3.4 Test solutions: In order to obtain the dose of 200 mg/kg and 400 mg/kg body weight, 300 mg of the test samples were taken separately and each was finally made 8 ml suspension by means of Tween 80 and DMSO with normal saline.

9.4 Procedure

The animals were weighed and randomly divided into 5 groups of 5 rats in each group. Each group received a particular treatment as mentioned in the Table- 9.1. Diclofenac sodium at 100 mg/kg body wt. was used as standard anti-inflammatory agent. After 30 minutes of oral administration of test materials, 0.1 ml of 1% Carrageenan solution was injected into the sub-plantar surface of the right hind paw of each rat of every group. The paw volume was measured by plethysmometer (Ugo Basile, Italy) at 1, 2, 3 and 4 hours

after 30 minutes of carrageenan injection. Mean increase in paw volume were noted for the respective time intervals, thus edema volumes in control [(Ct-Co) control] and in groups treated with test materials [(Ct-Co) treated] were calculated. Percentage inhibition of paw edema was calculated by using the following formula:

% paw edema inhibition= [{(Ct-Co) control - (Ct-Co) treated}/ (Ct-Co) control] X100

Where, Co = paw volume at zero time (before carrageenan injection), Ct = paw volumes at t time. (Ct-Co) = paw edema.

Figure 9.1: Use of plethysmometer

Figure 9.2: Long Evans Rat

Table 9.1: Test samples used for the experiment

Group	Test Samples	Identification	Dose (mg/kg)*
I	Normal saline with few drops of Tween 80 and DMSO	Control group	0.1 ml/10 g of body wt
II	Diclofenac sodium	Standard group	100
III (A)	Crude methanol extract	Test group	200
III (B)	Crude methanol extract	Test group	400
IV (A)	Petroleum ether fraction	Test group	200
IV (B)	Petroleum ether fraction	Test group	400
V (A)	n-Hexane fraction	Test group	200
V (B)	n-Hexane fraction	Test group	400
VI (A)	Chloroform fraction	Test group	200
VI (B)	Chloroform fraction	Test group	400

Carrageenan was used as an inflammation inducer at a dose of 0.1 ml of 1% (w/v). The drug was injected in sub plantar region of hind paw of animal.

*all doses were given orally

9.5: Evaluation of anti-inflammatory activity

Table 9.2: Paw volume of rats receiving control (Normal saline) at different time intervals

Rat No.	Paw Volume (ml)				
	0 hour	1st hour	2nd hour	3rd hour	4th hour
1	0.25	0.38	0.56	0.62	0.64
2	0.24	0.39	0.57	0.64	0.66
3	0.29	0.46	0.64	0.72	0.73
4	0.27	0.39	0.59	0.64	0.67
5	0.26	0.43	0.57	0.66	0.69
Mean	0.262	0.410	0.586	0.656	0.678
Paw edema (Ct-Co)	Co = 0.262	0.148	0.324	0.394	0.416

** Where, Co= paw volume at zero time (before carrageenan injection),
Ct=paw volumes at t time. (Ct-Co)= paw edema

Table 9.3: Paw volume of standard group of rats at different time intervals

Rat No.	Paw Volume (ml)				
	0 hour	1st hour	2nd hour	3rd hour	4th hour
1	0.29	0.35	0.41	0.49	0.31
2	0.24	0.37	0.41	0.32	0.27
3	0.26	0.34	0.39	0.36	0.28
4	0.28	0.39	0.40	0.38	0.32
5	0.26	0.37	0.38	0.33	0.31
Mean	0.266	0.364	0.398	0.376	0.298
Paw edema (Ct-Co)	Co = 0.266	0.098	0.132	0.110	0.032

Table 9.4: Paw volume of rats receiving the crude methanolic extract (Single dose) III (A) of *Myristica fragrans* at different time intervals

Rat No.	Paw Volume (ml)				
	0 hour	1st hour	2nd hour	3rd hour	4th hour
1	0.29	0.36	0.43	0.49	0.41
2	0.24	0.32	0.34	0.31	0.25
3	0.25	0.37	0.46	0.41	0.37
4	0.26	0.36	0.45	0.36	0.29
5	0.25	0.34	0.29	0.25	0.20
Mean	0.258	0.350	0.394	0.364	0.304
Paw edema (Ct-Co)	Co =0.258	0.092	0.136	0.106	0.046

Table 9.5: Paw volume of rats receiving the crude methanolic extract (Double dose) III (B) of *Myristica fragrans* at different time intervals

Rat No.	Paw Volume (ml)				
	0 hour	1st hour	2nd hour	3rd hour	4th hour
1	0.22	0.33	0.36	0.30	0.31
2	0.24	0.33	0.38	0.31	0.28
3	0.30	0.38	0.41	0.35	0.32
4	0.28	0.37	0.40	0.34	0.30
5	0.27	0.35	0.41	0.33	0.29
Mean	0.262	0.352	0.392	0.326	0.300
Paw edema (Ct-Co)	Co = 0.262	0.090	0.130	0.064	0.038

Table 9.6: Paw volumes of rats receiving the petroleum ether fraction (Single dose) IV (A) of *Myristica fragrans* at different time intervals

Rat No.	Paw Volume (ml)				
	0 hour	1st hour	2nd hour	3rd hour	4th hour
1	0.29	0.35	0.43	0.51	0.43
2	0.24	0.38	0.41	0.40	0.32
3	0.33	0.42	0.46	0.41	0.37
4	0.31	0.41	0.45	0.42	0.33
5	0.28	0.37	0.41	0.39	0.36
Mean	0.290	0.386	0.432	0.426	0.362
Paw edema (Ct-Co)	Co = 0.290	0.096	0.142	0.136	0.072

Table 9.7: Paw volumes of rats receiving petroleum ether fraction (Double dose) IV (B) of *Myristica fragrans* at different time intervals

Rat No.	Paw Volume (ml)				
	0 hour	1st hour	2nd hour	3rd hour	4th hour
1	0.24	0.34	0.41	0.42	0.37
2	0.20	0.36	0.36	0.35	0.31
3	0.25	0.32	0.30	0.31	0.29
4	0.28	0.35	0.31	0.33	0.27
5	0.27	0.34	0.34	0.35	0.29
Mean	0.248	0.342	0.344	0.352	0.306
Paw edema (Ct-Co)	Co = 0.248	0.094	0.096	0.104	0.058

Table 9.8: Paw volumes of rats receiving n-Hexane fraction (Single dose) V (A) of *Myristica fragrans* at different time intervals

Rat No.	Paw Volume (ml)				
	0 hour	1st hour	2nd hour	3rd hour	4th hour
1	0.27	0.41	0.43	0.48	0.41
2	0.28	0.39	0.42	0.38	0.34
3	0.23	0.38	0.41	0.40	0.4
4	0.25	0.29	0.31	0.35	0.33
5	0.28	0.32	0.38	0.38	0.31
Mean	0.262	0.358	0.390	0.398	0.358
Paw edema (Ct-Co)	Co = 0.262	0.096	0.128	0.136	0.096

Table 9.9: Paw volumes of rats receiving n-Hexane fraction (Double dose) V (B) of *Myristica fragrans* at different time intervals

Rat No.	Paw Volume (ml)				
	0 hour	1st hour	2nd hour	3rd hour	4th hour
1	0.28	0.40	0.38	0.32	0.27
2	0.25	0.38	0.47	0.42	0.40
3	0.26	0.35	0.33	0.40	0.30
4	0.27	0.35	0.36	0.41	0.33
5	0.29	0.36	0.39	0.36	0.31
Mean	0.270	0.368	0.386	0.382	0.322
Paw edema (Ct-Co)	Co = 0.270	0.098	0.116	0.112	0.052

Table 9.10: Paw volumes of rats receiving chloroform fraction (Single dose) VI (A) of *Myristica fragrans* at different time intervals

Rat No.	Paw Volume (ml)				
	0 hour	1st hour	2nd hour	3rd hour	4th hour
1	0.26	0.35	0.38	0.33	0.31
2	0.21	0.33	0.34	0.39	0.37
3	0.25	0.34	0.39	0.44	0.35
4	0.28	0.38	0.41	0.42	0.34
5	0.27	0.34	0.46	0.43	0.32
Mean	0.254	0.348	0.396	0.402	0.338
Paw edema (Ct-Co)	Co = 0.254	0.094	0.142	0.148	0.084

Table 9.11: Paw volumes of rats receiving chloroform fraction (Double dose) VI (B) of *Myristica fragrans* at different time intervals

Rat No.	Paw Volume (ml)				
	0 hour	1st hour	2nd hour	3rd hour	4th hour
1	0.26	0.40	0.46	0.49	0.43
2	0.32	0.39	0.47	0.37	0.37
3	0.31	0.42	0.46	0.36	0.31
4	0.29	0.36	0.40	0.37	0.3
5	0.31	0.38	0.43	0.39	0.32
Mean	0.298	0.390	0.444	0.396	0.346
Paw edema (Ct-Co)	Co = 0.298	0.092	0.146	0.098	0.048

Table 9.12: Mean paw volume of rats receiving different extracts of *Myristica fragrans*

Group	Mean paw volume (ml) ± SEM*			
	1st hour	2nd hour	3rd hour	4th hour
1) Control	0.410 ± 0.015	0.586 ± 0.014	0.656 ± 0.017	0.678 ± 0.015
2) Standard	0.364 ± 0.009	0.398 ± 0.006	0.376 ± 0.030	0.298 ± 0.010
3) III (A)-Crude methanolic extract (Single dose)	0.350 ± 0.009	0.394 ± 0.034	0.364 ± 0.041	0.304 ± 0.038
4) III (B)-Crude methanolic extract (Double dose)	0.352 ± 0.010	0.392 ± 0.010	0.326 ± 0.009	0.300 ± 0.007

Group	Mean paw volume (ml) ± SEM*			
	1st hour	2nd hour	3rd hour	4th hour
5) IV (A)- Petroleum ether fraction (Single dose)	0.386 ± 0.013	0.432 ± 0.010	0.426 ± 0.022	0.362 ± 0.019
6) IV (B)- Petroleum ether fraction (Double dose)	0.342 ± 0.007	0.344 ± 0.020	0.352 ± 0.019	0.306 ± 0.017
7) V (A)- n-Hexane fraction (Single dose)	0.358 ± 0.023	0.390 ± 0.022	0.398 ± 0.022	0.358 ± 0.020
8) V (B)- n-Hexane fraction (Double dose)	0.368 ± 0.010	0.386 ± 0.023	0.382 ± 0.019	0.322 ± 0.022
9) VI (A)- Chloroform fraction (Single dose)	0.348 ± 0.009	0.396 ± 0.020	0.402 ± 0.020	0.338 ± 0.011
10) VI (B)- Chloroform fraction (Double dose)	0.390 ± 0.010	0.444 ± 0.013	0.396 ± 0.024	0.346 ± 0.024

*SEM= Standard error of mean

Table 9.13: Paw edema and percent inhibition of paw edema at different time intervals

Group	Paw edema (ml)				% Paw edema inhibition			
	1st hour	2nd hour	3rd hour	4th hour	1st hour	2nd hour	3rd hour	4th hour
1) Control	0.148	0.324	0.394	0.416				
2) Standard	0.098	0.132	0.110	0.032	33.784	59.259	72.081	92.308
3) III (A)-Crude methanol extract (Single dose)	0.092	0.136	0.106	0.046	37.838	58.025	73.096	88.942
4) III (B)-Crude methanol extract (Double dose)	0.090	0.130	0.064	0.038	39.189	59.877	83.756	90.865
5) IV (A)- Petroleum ether fraction (Single dose)	0.096	0.142	0.136	0.072	35.135	56.173	65.482	82.692
6) IV (B)- Petroleum ether fraction (Double dose)	0.094	0.096	0.104	0.058	36.486	70.370	73.604	86.058
7) V (A)- n-Hexane fraction (Single dose)	0.096	0.128	0.136	0.096	35.135	60.494	65.482	76.923
8) V (B)- n-Hexane fraction (Double dose)	0.098	0.116	0.112	0.052	33.784	64.198	71.574	87.500
9) VI (A)- Chloroform fraction (Single dose)	0.094	0.142	0.148	0.084	36.486	56.173	62.437	79.808
10) VI (B)- Chloroform fraction (Double dose)	0.092	0.146	0.098	0.048	37.838	54.938	75.127	88.462

According to the collected data, the crude methanolic extract, petroleum ether fraction, n-hexane fraction and chloroform fraction showed significant anti-inflammatory activity. The data was statistically evaluated.

9.6 Statistical evaluation of data

All values were expressed as the mean ± standard error of the mean (SEM) and the result were analyzed statistically by one way analysis of variance (ANOVA) followed by Dunnett's test by using SPSS ver.17. Where, the value of $P < 0.05$ was considered to be statistically significant.

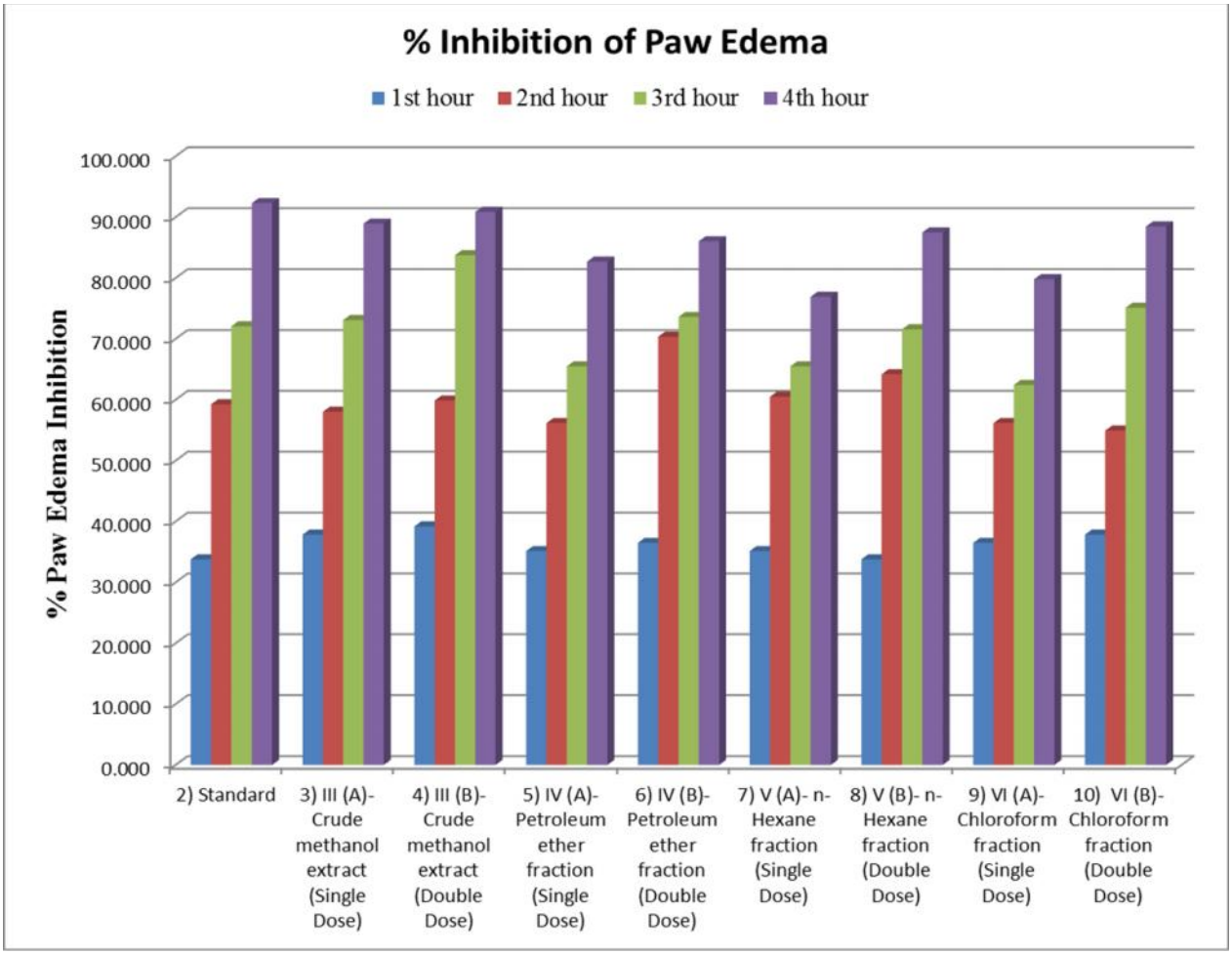


Figure 9.3: Comparison of % inhibition of paw edema in different groups

9.7 Results and discussion

Statistical data analysis confirmed that the crude methanolic extract, petroleum ether fraction, n-hexane fraction and chloroform fraction showed significant anti-inflammatory activity at a dose of 200 mg/kg and 400 mg/kg of body weight.

Carrageenan induced paw edema is a commonly used primary test for the screening of new anti-inflammatory agents. Inhibition of paw edema for the crude methanolic extract, petroleum ether fraction, n-hexane fraction and chloroform fraction were much significant in double doses than single dose comparing to the control.

The crude methanolic extract, petroleum ether fraction, n-hexane fraction and chloroform fraction were significantly decreased the paw edema in rats comparable to diclofenac sodium. Double dose had given better result than single dose.

10.1 Introduction

The present study was taken to evaluate the use of *Myristica fragrans* as an antipyretic in yeast induced pyrexia in Long evans rats. The study was carried out by the yeast induced pyrexia method.

Pyrexia or fever is defined as an elevation of body temperature. It is a response due to tissue damage, inflammation, malignancy or graft rejection. Cytokines, interleukin, interferon and Tumor Necrosis Factor α (TNF- α) are formed in large amount under this condition, which increase prostaglandin E2 (PGE2) which in turn triggers hypothalamus to elevate body temperature. Antipyretics are drugs which can reduce elevated body temperature.

The antipyretic efficacy of methanolic extract and other fractions will be assessed using brewer's yeast induced pyrexia method in Long Evans rats. Pyrexia will be induced by subcutaneous injection of 15 % w/v of brewer's yeast (10 ml/kg) in distilled water. Basal rectal temperature will be measured before the injection of yeast, by inserting digital clinical thermometer to a depth of 2 cm into the rectum. The rise in rectal temperature will be recorded 18 hr after yeast injection. Paracetamol 150 mg/kg body weight will be used as the standard antipyretic drug. Rectal temperature of animals will be noted at regular intervals following the respective treatments. The temperature will be measured at 1^{st}, 2^{nd}, 3^{rd} and 4^{th} hour after 18 hr of drug administration. (Amit Jaiswal *et. al*., 2011)

10.2 Experimental animal

Long evans rats weighing 50-100 gm were used for the present study. They were obtained from the Animal farm of ICDDR,B and maintained in the animal house for experimental purpose under controlled conditions of temperature (23 ± 2ºC), humidity (50 ± 5%) and 12-hr light-dark cycles. All the animals were acclimatized for seven days before the study. The animals were randomized into experimental and control groups and housed individually in sanitized polypropylene cages containing sterile paddy husk as bedding. They were fed pellets as basal diet and water ad libitum.

Figure 10.1: Long evans rat

10.3 Preparation of test materials

10.3.1 Test solutions: In order to administer the crude extract at doses of 200 mg/kg body wt and 400 mg/kg body wt of rat, 300 mg of the extracts were measured respectively and triturated in unidirectional way by adding of 3-4 drops of Tween 80 and few drops of DMSO. After proper mixing of extract, suspending agent and DMSO, normal saline was slowly added. The final volume of the suspension was made up to 8.0 ml. To stabilize the suspension, it was stirred well by vortex mixture.

10.3.2 Control solution: For the preparation of control, 3-4 drops of Tween 80 and few drops of DMSO were mixed in normal saline. The final volume of the suspension was made up to 4.0 ml.

10.3.3 Standard solution: For the preparation of paracetamol at the dose of 150 mg/kg body weight, 500 mg of paracetamol tablet was dissolved in 40.0 ml normal saline. Few drops of Tween 80 and DMSO were mixed in solution.

10.3.4 Brewer's yeast suspension: 15 gm of brewer's yeast were suspended in 100 ml of hot distilled water.

10.4 Pyrexia induced by Brewer's yeast suspensions in rats

Antipyretic activity on rats was studied with fever induced by 15% brewer's yeast. Healthy Long evans rats weighing about 50-100 gm were taken. They were fasted overnight with water ad libitum before inducing pyrexia and just before induce pyrexia animals were allowed to stay in the cage for some time and after that their basal rectal temperature were measured by using a clinical digital thermometer by insertion of thermometer to a depth of 2 cm into the rectum. After taking the temperature Pyrexia was induced by injecting subcutaneously 15% w/v suspension of Brewer's yeast in distilled water at a dose of 10 ml/kg body weight in the back below the nape of the neck. The site of injection was massaged in order to spread the suspension beneath the skin and returned to their cage and allowed to feed. After 18 hrs of Brewer's yeast injection the rise in rectal temperature was recorded. Only rats which were shown an increase in temperature, at least 0.6º C (or 1º F), was used for further experiment. The animals were divided into 10 groups, each group contain five animals. The control, standard and test extracts were administered orally to the animals before 30 minutes of yeast injection. After the drug was administered, the temperature of all the rats in each group was recorded at 1 hr, 2 hr, 3 hr and 4 hr. The mean temperature was found out for each group and compared with the value of standard drug. The obtained data were statistically evaluated and the P value elucidated to determine the statistical significance.

10.5 Analytical data

Table 10.1: Test materials used in the evaluation of analgesic activity of crude extract and its different fractions of *Myristica fragrans*.

Group	Test Samples	Identification	Dose (mg/kg)*
I	Normal saline with 3-4 drops of Tween-80 and few drops of DMSO	Control group	0.1ml/10 g of body wt
II	Paracetamol	Standard group	150
III (A)	Crude methanolic extract	Test group	200
III (B)	Crude methanolic extract	Test group	400
IV (A)	Petroleum ether fraction	Test group	200
IV (B)	Petroleum ether fraction	Test group	400
V (A)	n-Hexane fraction	Test group	200
V (B)	n-Hexane fraction	Test group	400
VI (A)	Chloroform fraction	Test group	200
VI (B)	Chloroform fraction	Test group	400

*All doses were given orally.

Rectal temperature determination

Table 10.2: Effect of normal saline (control) on yeast induced pyrexia in rats at different time intervals

Rat No.	Rectal Temperature (^{0}F) Before Yeast Injection	Rectal Temperature (^{0}F) After 18 hours of Yeast Injection				
		0 hour	1st hour	2nd hour	3rd hour	4th hour
1	99.3	101.6	101.8	101.7	101.7	101.6
2	98.7	100.9	101.3	101.5	101.4	101.4
3	96.7	99.4	100.8	100.6	100.5	100.3
4	98.5	99.7	101.6	101.2	101.2	101.0
5	99.1	100.8	101.7	101.6	101.3	101.1
Mean	98.46	100.48	101.44	101.32	101.22	101.08

Table 10.3: Effect of paracetamol (standard) on yeast induced pyrexia in rats at different time intervals.

Rat No.	Rectal Temperature (^{0}F) Before Yeast Injection	Rectal Temperature (^{0}F) After 18 hours of Yeast Injection				
		0 hour	1st hour	2nd hour	3rd hour	4th hour
1	94.8	98.1	98.0	97.8	97.5	97.4
2	94.5	99.5	99.2	98.9	95.6	95.8
3	99.5	100.4	100.1	98.8	96.4	96.3
4	98.6	99.4	99.2	99.1	98.9	98.7
5	97.8	98.5	98.5	98.4	98.1	97.7
Mean	97.04	99.18	99.00	98.60	97.3	97.18

Table 10.4: Effect of methanolic extract (200 mg/kg body weight) on yeast induced pyrexia in rats at different time intervals.

Rat No.	Rectal Temperature (^{0}F) Before Yeast Injection	Rectal Temperature (^{0}F) After 18 hours of Yeast Injection				
		0 hour	1st hour	2nd hour	3rd hour	4th hour
1	96.2	98.2	98.2	98.1	95.4	95.5
2	94.7	94.6	95.3	97.8	97.4	97.5
3	94.9	94.7	95.8	98.3	96.7	96.6
4	98.2	98.4	98.6	98.5	97.5	97.5
5	98.4	98.6	98.7	97.9	98.3	98.4
Mean	96.48	96.9	97.32	98.12	97.06	97.10

Table 10.5: Effect of methanolic extract (400 mg/kg body weight) on yeast induced pyrexia in rats at different time intervals.

Rat No.	Rectal Temperature (^{0}F) Before Yeast Injection	Rectal Temperature (^{0}F) After 18 hours of Yeast Injection				
		0 hour	1st hour	2nd hour	3rd hour	4th hour
1	98.2	98.9	98.8	98.7	97.5	96.2
2	99.8	100.5	100.6	99.9	98.3	97.9
3	97.5	98.4	97.9	97.9	98.2	95.7
4	97.8	98.7	98.6	98.7	98.6	96.8
5	98.3	99.2	99.3	99.4	99.5	99.5
Mean	98.32	99.14	99.04	98.92	98.42	97.22

Table 10.6: Effect of petroleum ether fraction (200 mg/kg body weight) on yeast induced pyrexia in rats at different time intervals.

Rat No.	Rectal Temperature (^{0}F) Before Yeast Injection	Rectal Temperature (^{0}F) After 18 hours of Yeast Injection				
		0 hour	1st hour	2nd hour	3rd hour	4th hour
1	97.9	96.9	96.8	96.7	96.9	96.7
2	99.4	98.7	98.5	97.8	97.9	97.8
3	98.7	99.8	99.7	99.3	99.4	99.1
4	99.5	99.7	99.7	99.8	99.7	99.5
5	98.6	98.7	98.6	98.4	98.8	98.7
Mean	98.82	98.76	98.66	98.40	98.54	98.36

Table 10.7: Effect of petroleum ether fraction (400 mg/kg body weight) on yeast induced pyrexia in rats at different time intervals.

Rat No.	Rectal Temperature (^{0}F) Before Yeast Injection	Rectal Temperature (^{0}F) After 18 hours of Yeast Injection				
		0 hour	1st hour	2nd hour	3rd hour	4th hour
1	96.8	96.9	96.8	96.7	96.6	96.4
2	96.8	96.8	96.5	96.6	95.3	92.4
3	97.2	98.4	98.4	98.5	98.2	98.3
4	98.3	98.1	98.3	98.1	97.4	96.3
5	97.5	97.2	97.4	97.3	97.1	94.2
Mean	97.32	97.48	97.48	97.44	96.92	95.52

Table 10.8: Effect of n-hexane fraction (200 mg/kg body weight) on yeast induced pyrexia in rats at different time intervals.

Rat No.	Rectal Temperature (^{0}F) Before Yeast Injection	Rectal Temperature (^{0}F) After 18 hours of Yeast Injection				
		0 hour	1st hour	2nd hour	3rd hour	4th hour
1	97.3	98.5	98.1	98.3	97.6	97.5
2	99.4	99.6	99.5	99.2	98.8	98.9
3	98.1	99.4	99.2	98.9	98.7	98.3
4	97.8	99.2	99.3	98.8	98.7	98.7
5	98.3	99.4	99.6	97.8	97.7	97.2
Mean	98.18	99.22	99.14	98.60	98.30	98.12

Table 10.9: Effect of n-hexane fraction (400 mg/kg body weight) on yeast induced pyrexia in rats at different time intervals.

Rat No.	Rectal Temperature (^{0}F) Before Yeast Injection	Rectal Temperature (^{0}F) After 18 hours of Yeast Injection				
		0 hour	1st hour	2nd hour	3rd hour	4th hour
1	98.6	97.2	97.1	97.3	97.2	96.4
2	98.7	98.8	98.7	98.6	98.5	97.8
3	96.2	96.0	96.2	96.3	95.7	93.7
4	98.8	98.6	98.8	98.7	98.6	98.5
5	97.6	97.7	97.4	97.7	97.5	97.3
Mean	97.98	97.66	97.64	97.72	97.5	96.74

Table 10.10: Effect of chloroform fraction (200 mg/kg body weight) on yeast induced pyrexia in rats at different time intervals.

Rat No.	Rectal Temperature (^{0}F) Before Yeast Injection	Rectal Temperature (^{0}F) After 18 hours of Yeast Injection				
		0 hour	1st hour	2nd hour	3rd hour	4th hour
1	98.6	97.2	97.5	97.3	96.6	96.1
2	97.8	97.4	97.8	97.6	97.7	96.8
3	98.8	96.8	96.7	96.8	96.9	96.7
4	97.7	97.8	97.9	97.7	97.6	97.8
5	98.7	98.6	98.5	98.3	98.1	97.9
Mean	98.32	97.56	97.68	97.54	97.38	97.06

Table 10.11: Effect of chloroform fraction (400 mg/kg body weight) on yeast induced pyrexia in rats at different time intervals.

Rat No.	Rectal Temperature (^{0}F) Before Yeast Injection	Rectal Temperature (^{0}F) After 18 hours of Yeast Injection				
		0 hour	1st hour	2nd hour	3rd hour	4th hour
1	98.0	98.6	98.5	98.3	98.4	95.6
2	98.2	98.8	98.7	98.6	98.5	97.8
3	95.7	98.3	98.2	98.4	98.3	98.6
4	96.8	96.4	96.6	96.7	96.8	97.3
5	97.4	97.2	97.4	97.3	97.9	97.7
Mean	97.22	97.86	97.88	97.86	97.98	97.40

Table 10.12: Antipyretic effect of *Myristica fragrans* on Long evans rats

Group	Rectal temperature (^{0}F) before yeast Injection	Rectal temperature (^{0}F) after 18 hours of yeast injection				
		0 hour	1 hour	2 hour	3 hour	4 hour
1) Control	98.46 ± 0.462	100.48 ± 0.407	101.44 ± 0.181	101.32 ± 0.198	101.22 ± 0.198	101.08 ± 0.222
2) Standard	97.04 ± 1.013	99.18 ± 0.404	99.00 ± 0.356	98.60 ± 0.230	97.30 ± 0.589	97.18 ± 0.515
3) Methanolic extract (200 mg/kg)	96.48 ± 0.787	96.90 ± 0.921	97.32 ± 0.732	98.12 ± 0.128	97.06 ± 0.486	97.10 ± 0.491
4) Methanolic extract (400 mg/kg)	98.32 ± 0.397	99.14 ± 0.364	99.04 ± 0.450	98.92 ± 0.341	98.42 ± 0.325	97.22 ± 0.678
5) Petroleum ether fraction (200 mg/kg)	98.82 ± 0.292	98.76 ± 0.521	98.66 ± 0.532	98.40 ± 0.549	98.54 ± 0.512	98.36 ± 0.502
6) Petroleum ether fraction (400 mg/kg)	97.32 ± 0.278	97.48 ± 0.325	97.48 ± 0.384	97.44 ± 0.376	96.92 ± 0.481	95.52 ± 1.015
7) n-Hexane fraction (200 mg/kg)	98.18 ± 0.448	99.22 ± 0.239	99.14 ± 0.315	98.60 ± 0.187	98.30 ± 0.284	98.12 ± 0.310
8) n-Hexane fraction (400 mg/kg)	97.98 ± 0.494	97.66 ± 0.508	97.64 ± 0.495	97.72 ± 0.443	97.50 ± 0.526	96.74 ± 0.833
9) Chloroform fraction (200 mg/kg)	98.32 ± 0.235	97.56 ± 0.306	97.68 ± 0.294	97.54 ± 0.246	97.38 ± 0.275	97.06 ± 0.344
10) Chloroform fraction (400 mg/kg)	97.22 ± 0.452	97.86 ± 0.458	97.88 ± 0.389	97.86 ± 0.367	97.98 ± 0.312	97.40 ± 0.497

10.6 Statistical analysis

All values were expressed as the mean ± standard error of the mean (SEM) and the result were analyzed statistically by one way analysis of variance (ANOVA) followed by Dunnett's test by using SPSS ver.17. Where, the value of $P < 0.05$ was considered to be statistically significant. The Dunnets t-test was carried out and the P-value determined for the tests.

Statistical evaluation of the data obtained from the crude methanolic extract and other fractions of *Myristica fragrans* at both 200 mg/kg and 400 mg/kg dose showed significant anti-pyretic activity after 18 hours and onward of its administration in rats.

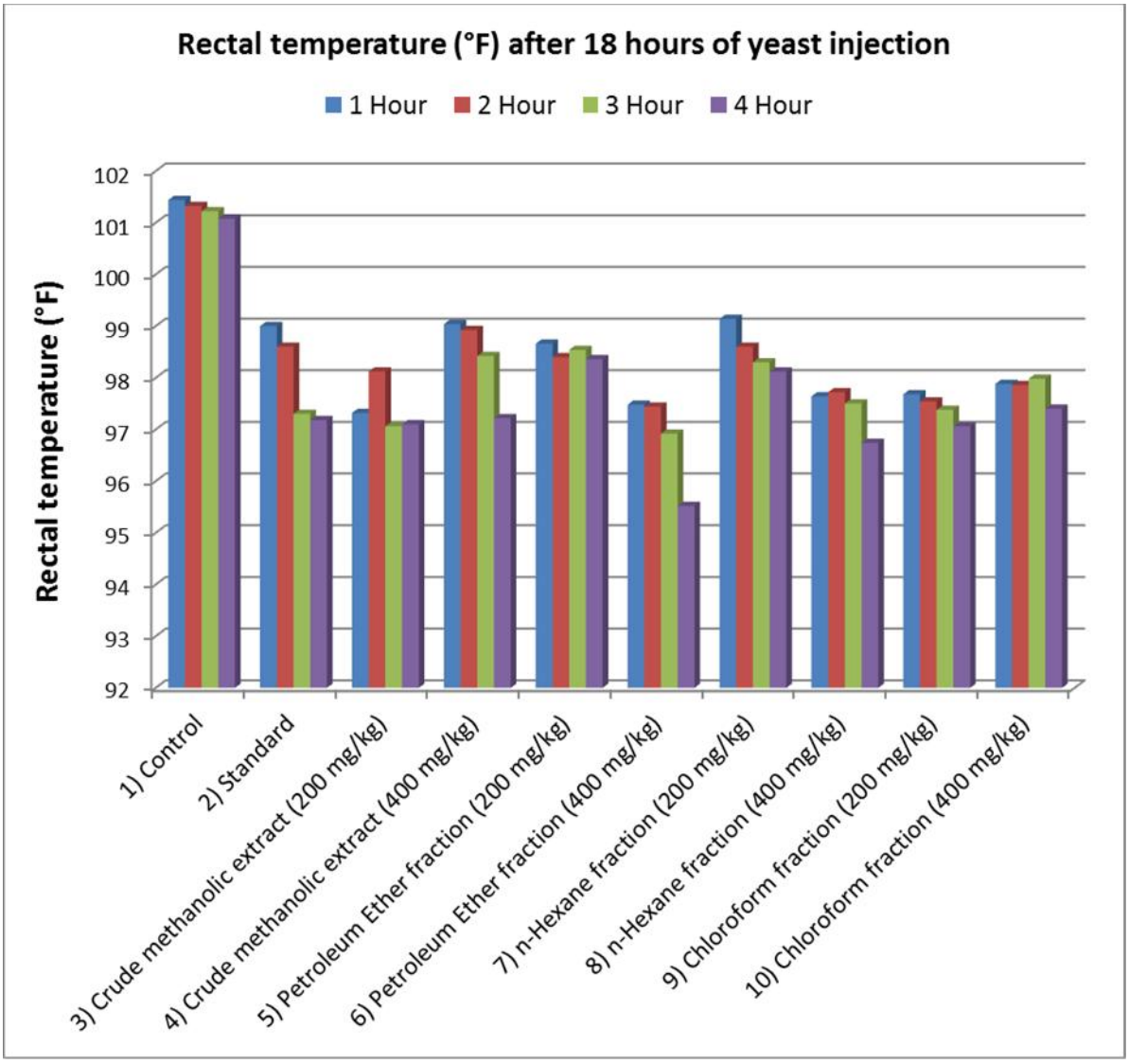


Figure 10.2: Comparison of Rectal temperature (^{0}F) after 18 hours of yeast injection

10.4 Results and discussion

Statistical analysis revealed that the effect of methanolic extract of *Myristica fragrans* and its fractions of petroleum ether, n-hexane and chloroform had showed significant lowering of the body temperature up to 4 hr at doses of 200 mg/kg and 400 mg/kg.

Brewer's yeast induced pyrexia is used to test for the screening of new anti-pyretic agents. Inhibition of pyrexia for the crude methanolic extract, petroleum ether fraction, n-hexane fraction and chloroform fraction is statistically significant comparing to the control.

The crude methanolic extract, petroleum ether fraction, n-hexane fraction and chloroform fraction were significantly decreased the pyrexia in rats comparable to paracetamol.

11.1 Principle

Analgesic activity was evaluated by acetic acid induced Writhing method. Visceral pain will be induced by intraperitoneal injection of 0.7% volume per volume (10 mL/kg) glacial acetic acid solution in normal saline. As a result, the animals squirms their body at regular interval out of pain. This squirm or contraction of the body is termed as “writhing”. As long as the animals feel pain, they continue to give writhing. Each writhing is counted and taken as an indication of pain sensation. Any substance that has got analgesic activity is supposed to lessen the number of writhing of animals within a given time frame and with respect to the control group. The writhing inhibition of positive control was taken as standard and compared with test samples and control. Mice will be given vehicle, diclofenac sodium, crude methanolic extract and fractions of extract, 40 minutes prior to induction of visceral pain. According to this principle crude methanolic extract, petroleum ether fraction, n-hexane fraction and chloroform fraction were introduced to analgesic testing at two different doses: Dose 200 mg/kg and 400 mg/kg of body weight.

Animals in the cages were observed for writhing behavior, indicated by stretching of the abdomen with simultaneous stretching. The number of writhing responses will be counted for 15 minutes, starting directly after the acid injection. (Hayfaa et al., 2013)

11.2 Experimental animal

Swiss albino mice of either sex, aged 4-5 weeks and weighed 30-35 gm, obtained from the Animal farm of ICDDR,B. They were kept in suitable environmental condition and fed mice food and water ad-libitum in the Animal House of Nutrition and Food Department of the University of Dhaka. As these animals are very sensitive to environmental changes, they were acclimatized before the test for at least 3-4 days in the environment where the experiment will take place.

Figure 11.1: Swiss albino mice

11.3 Experimental design

50 experimental animals were randomly selected, weighed and divided into ten groups denoted as group-I, group-II, group-III(A-B), group- IV(A-B), group- V(A-B), and group VI(A-B), consisting of 5 mice in each group. Each group received a particular

treatment. Prior to any treatment, each mouse was weighed properly and the doses of the test samples and control materials were adjusted accordingly. Mice will be given vehicle, diclofenac sodium, crude methanolic extract and fractions of extract, 40 minutes prior to induction of visceral pain.

As it was difficult to observe the biologic response of 5 mice at a time receiving same treatment, it was necessary to identify individual animal of a group during the treatment. The animals were individualized in the following way (Figure 11.2). And marked as M1=Mice 1, M2=Mice 2, M3=Mice 3, M4=Mice 4 and M5=Mice 5.

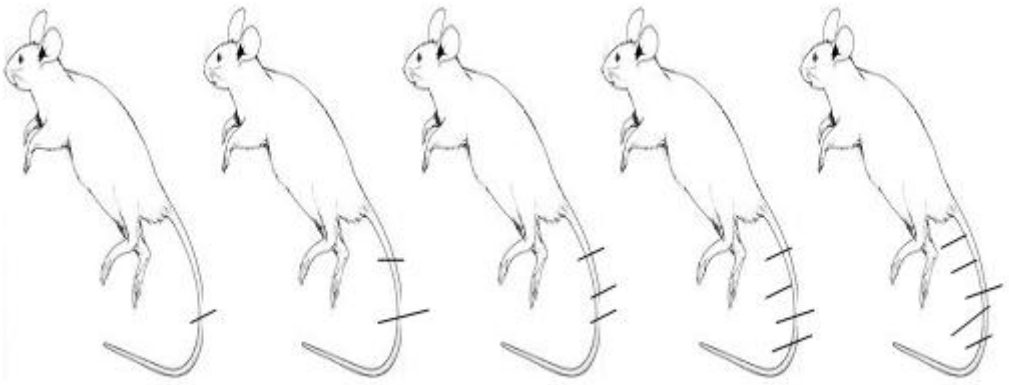


Figure 11.2: Numbering of mice

11.4 Preparation of test materials

11.4.1 Test solutions: In order to administer the extract at doses of 200 mg/kg body wt and 400 mg/kg body wt of mice, 240 mg of the extract were measured respectively and triturated in unidirectional way by adding of small amount of DMSO (a suspending agent) and 3-4 drops of Tween 80. After proper mixing of extract and suspending agent, normal saline was slowly added. The final volume of the suspension was made up to 8.0 ml. To stabilize the suspension, it was stirred well by vortex mixture.

11.4.2 Standard solution: For the preparation of Diclofenac sodium solution at the dose of 100 mg/kg body weight, 50 mg of Diclofenac sodium was taken and a suspension of 5.0 ml was made. Few drops of Tween 80 and DMSO were mixed in solution.

11.4.3 Control solution: 3- 4 drops of Tween 80 and few drops of DMSO were mixed properly in the normal saline and the volume was made upto 4.0 ml.

11.4.4 Acetic acid solution: For the preparation of acetic acid solution, 0.7 ml glacial acetic acid was diluted with 100 ml distilled water.

Figure 11.3: Oral administration of the drug

Table 11.1: Test materials used in the evaluation of analgesic activity of crude extract and its different fractions of *Myristica fragrans*.

Group	Test Samples	Identification	Dose (mg/kg)*
I	Normal saline with 3 – 4 drops of Tween 80 and few drops of DMSO	Control group	0.1ml/10 g of body wt
II	Diclofenac sodium	Standard group	100
III (A)	Crude methanolic extract	Test group	200
III (B)	Crude methanolic extract	Test group	400
IV (A)	Petroleum ether fraction	Test group	200
IV (B)	Petroleum ether fraction	Test group	400
V (A)	n-Hexane fraction	Test group	200
V (B)	n-Hexane fraction	Test group	400
VI (A)	Chloroform fraction	Test group	200
VI (B)	Chloroform fraction	Test group	400

*All doses were given orally. Glacial Acetic Acid 0.7% was administered at a dose of 0.1ml/10 gm. of body weight to each mouse.

11.5 Procedure

- All animals were weighed.
- At zero hour, test samples, control and diclofenac sodium were administered orally by means of a long needle with a ball shaped end.
- After 40 minutes, glacial acetic acid (0.7% v/v) was administered intraperitoneally to each of the animals of all the groups.
- Five minutes after the administration of acetic acid, number of squirms or writhing were counted for each mouse for ten minutes.

11.6 Counting of writhing

Each mouse of all groups were observed individually for counting the number of writhing, that made in 10 minutes commencing just 5 minutes after the Intra-peritoneal administration of glacial acetic acid solution. 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 writhing were taken as one full writhing.

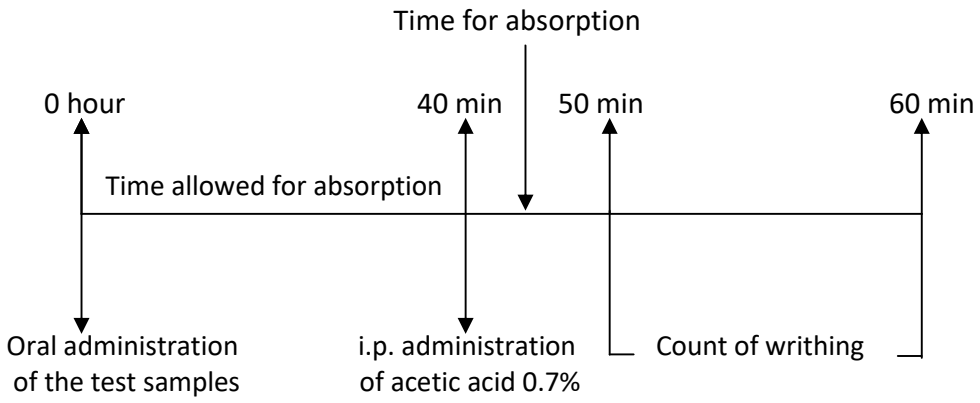


Figure 11.4: Schematic diagram of the induction and counting of writhing. The different test samples were subjected to screening for analgesic activity by glacial acetic acid induced writhing inhibition method. The test was performed by taking samples at doses 200 mg/kg and 400 mg/kg body weight.

Table 11.2: Screening of analgesic activity by counting the number of writhing after intraperitoneal administration of 0.7% glacial acetic acid

Animal Group	**Writhing count**					**Average**	**% Writhing**	**% Inhibition**
	M-1	**M-2**	**M-3**	**M-4**	**M-5**			
Control	26	28	25	27	29	27	100	
Standard	6	5	8	6	7	6.4	23.70	76.30
Crude methanolic extract (Dose- 200 mg/kg)	10	12	12	14	16	12.8	47.41	52.59
Crude methanolic extract (Dose- 400 mg/kg)	12	8	10	14	13	11.4	42.22	57.78
Petroleum ether fraction (Dose- 200 mg/kg)	10	11	12	10	13	11.2	41.48	58.52
Petroleum ether fraction (Dose- 400 mg/kg)	9	10	10	11	13	10.6	39.26	60.74
n-Hexane fraction (Dose- 200 mg/kg)	10	11	14	13	12	16	59.26	40.74
n-Hexane fraction (Dose- 400 mg/kg)	13	9	10	11	15	11.6	42.96	57.04
Chloroform fraction (Dose- 200 mg/kg)	12	16	17	13	14	14.4	53.33	46.67
Chloroform fraction (Dose- 400 mg/kg)	11	14	10	16	12	12.6	46.67	53.33

Table 11.3: Analgesic activity of crude methanolic extract and its different fractions of *Myristica fragrans*

Group	**Number of Writhing (Mean ± SEM)**	**% of Inhibition of Writhing**
I. Control	100 ± 0.707	00.00
II. Standard	23.70 ± 0.509	76.30
III (A). Crude methanolic extract (Dose- 200 mg/kg)	47.41 ± 1.019	52.59
III (B). Crude methanolic extract (Dose- 400 mg/kg)	42.22 ± 1.077	57.78
IV (A). Petroleum ether fraction (Dose- 200 mg/kg)	41.48 ± 0.583	58.52
IV (B). Petroleum ether fraction (Dose- 400 mg/kg)	39.26 ± 0.678	60.74
V (A). n-Hexane soluble fraction (Dose- 200 mg/kg)	59.26 ± 0.707	40.74
V (B). n-Hexane soluble fraction (Dose- 400 mg/kg)	42.96 ± 1.077	57.04
VI (A). Chloroform soluble fraction (Dose- 200 mg/kg)	53.33 ± 0.927	46.67
VI (B). Chloroform soluble fraction (Dose- 400 mg/kg)	46.67 ± 1.077	53.33

11.7 Statistical analysis

All values were expressed as the mean ± standard error of the mean (SEM) and the result were analyzed statistically by one way analysis of variance (ANOVA) followed by Dunnett's test by using SPSS ver.17. Where, the value of $P < 0.05$ was considered to be statistically significant. The Dunnets t-test was carried out and the P-value determined for the tests.

Statistical evaluation of the data obtained from the crude methanolic extract and other fractions of *Myristica fragrans* at both 200 mg/kg and 400 mg/kg dose showed significant inhibition of writhing and onward of its administration in mice.

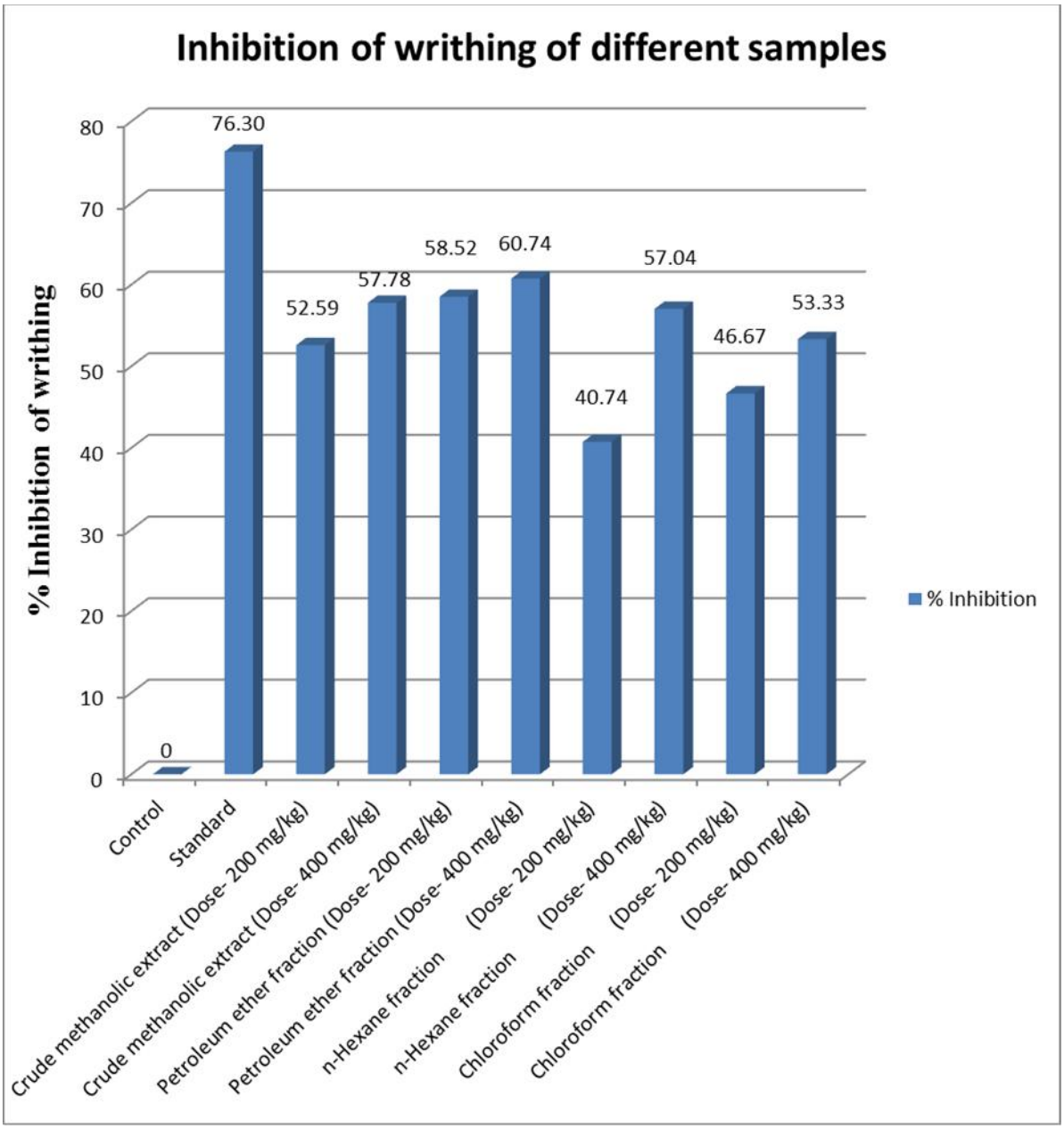


Figure 11.5: Comparison of % inhibition of writhing by different samples

11.8 Results and discussion

The effect of methanolic extract of *Myristica fragrans* and its fractions of petroleum ether, n-hexane and chloroform on mice had showed that the extract at doses of 200 mg/kg and 400 mg/kg given significant writhing according to statistical analysis.

Acetic acid induced writhing is used to test for the screening of new analgesic agents. Inhibition of writhing for the crude methanolic extract, petroleum ether fraction, n-hexane fraction and chloroform fraction is statistically significant in double doses than single dose comparing to the control.

The crude methanolic extract, petroleum ether fraction, n-hexane fraction and chloroform fraction were significantly decreased the writhing in mice comparable to diclofenac sodium. Double dose had given better result than single dose.

12.1 Principle

Anti-hyperglycaemic activities of methanolic extracts of the *Myristica fragrans* seed will be tested in normoglycemic and streptozotocin induced diabetic mice.

Glucose tolerance property of methanol extract of *Myristica fragrans* seed would be determined as per the procedure previously described by Joy and Kuttan (1999) with minor modifications. In brief, fasted mice will be grouped into ten groups of five mice each. The various groups received different treatments like Group 1 will receive vehicle (3-4 drops of Tween 80 and few drops of DMSO in normal saline) that served as control, group 2 will receive standard drug (glibenclamide, 10 mg/kg body weight). Groups 3-10 will receive methanol extract of *Myristica fragrans* seed and other fractions at doses of 200 and 400 mg per kg body weight. Each mouse will be weighed and doses adjusted accordingly prior to administration of vehicle, standard drug, and test samples. All mice will be orally administered 2 g glucose/kg of body weight after 30 minutes of oral administration of test solutions. Blood samples will be collected 30, 90 and 150 minutes after the glucose administration from tail vein. Blood glucose levels will be measured by glucose oxidase method (Venkatesh *et al*., 2004).

12.2 Experimental animal

Swiss-albino mice of either sex, aged 4-5 weeks and weighed 30-35 gm, obtained from the Animal farm of ICDDR,B. They were kept in suitable environmental condition and fed formulated rodent food and water (ad-libitum) in the Animal House of Nutrition and Food Department of the University of Dhaka. As these animals are very sensitive to environmental changes, they are kept before the test for at least 3-4 days in the environment where the experiment will take place.

Figure 12.1: Swiss albino mice

12.3 Experimental design

50 experimental animals were randomly selected and divided into ten groups denoted as group-I, group-II, group-III(A-B), group- IV(A-B), group- V(A-B), and group VI(A-B), consisting of 5 mice in each group. Each group received a particular treatment. Prior to any treatment, each mouse was weighed properly and the doses of the test samples and control materials were adjusted accordingly. As it was difficult to observe the biologic

response of 5 mice at a time receiving same treatment, it was necessary to identify individual animal of a group during the treatment. The animals were individualized in the following way (Figure 12.2). And marked as M1=Mice 1, M2=Mice 2, M3=Mice 3, M4=Mice 4 and M5=Mice 5.

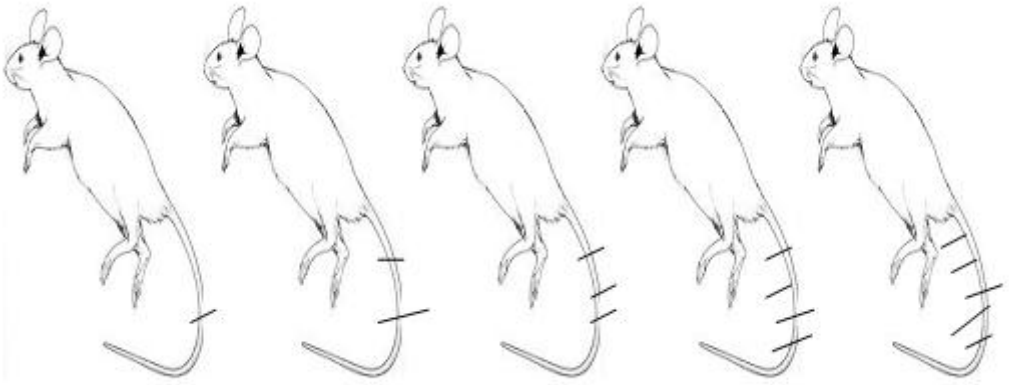


Figure 12.2: Numbering of mice

12.4 Preparation of test materials

12.4.1 Test solutions: In order to administer the extract at doses of 200 mg/kg body wt and 400 mg/kg body wt of mice, 240 mg of the extract were measured respectively and triturated in unidirectional way by adding of small amount of DMSO (a suspending agent) and 3 -4 drops of Tween 80. After proper mixing of extract and suspending agent, normal saline was slowly added. The final volume of the suspension was made up to 8.0 ml. To stabilize the suspension, it was stirred well by vortex mixture.

12.4.2 Standard solution: For the preparation of Glibenclamide solution at the dose of 10 mg/kg body weight, 10 mg of Glibenclamide was taken and a suspension of 4.0 ml was made. Few drops of Tween 80 and DMSO were mixed in solution.

12.4.3 Control solution: 3-4 drops of Tween 80 and few drops of DMSO were mixed properly in the normal saline and the volume was made upto 4.0 ml.

12.4.4 Glucose solution: For the preparation of glucose solution at the dose of 2 g/kg body weight, 10 g glucose was dissolved in 100 ml of distilled water.

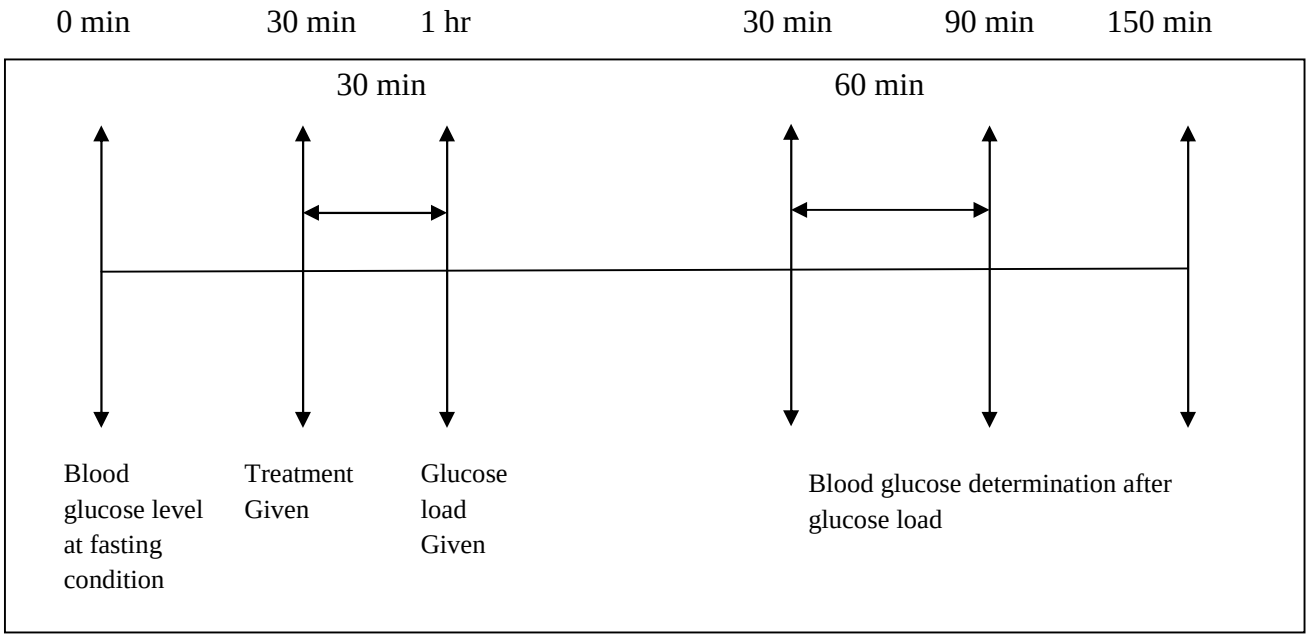
Figure 12.3: Oral administration of the drug

Table 12.1: Test materials used in the evaluation of analgesic activity of crude extract and its different fractions of *Myristica fragrans*.

Group	Test Samples	Identification	Dose (mg/kg)*
I	Normal saline with 3-4 drops of Tween 80 and few drops of DMSO	Control group	0.1ml/10 g of body wt
II	Glibenclamide	Standard group	10
III (A)	Crude methanolic extract	Test group	200
III (B)	Crude methanolic extract	Test group	400
IV (A)	Petroleum ether fraction	Test group	200
IV (B)	Petroleum ether fraction	Test group	400
V (A)	n-Hexane fraction	Test group	200
V (B)	n-Hexane fraction	Test group	400
VI (A)	Chloroform fraction	Test group	200
VI (B)	Chloroform fraction	Test group	400

12.5 Oral glucose tolerance test in *Swiss albino* mice

In this method, crude methanolic extract and different fractions of *Myristica fragrans* (i.e. petroleum ether fraction, n-hexane and chloroform fraction) had administered orally to the experimental animals at the fasting condition. Then the animals were given glucose load at a certain dose 30 minutes after administration of crude extracts and their fractions, standard and control samples. The blood glucose levels of the experimental animals were measured by using a glucometer (AccuChek) and Glucose oxidase-peroxidase reactive strips at 30 minutes, 90 minutes and 150 minutes interval. The hypoglycemic effect of the test samples were then compared with relative to that of control (vehicle containing Tween 80 and DMSO in normal saline) and standard (vehicle containing Glibenclamide) group.



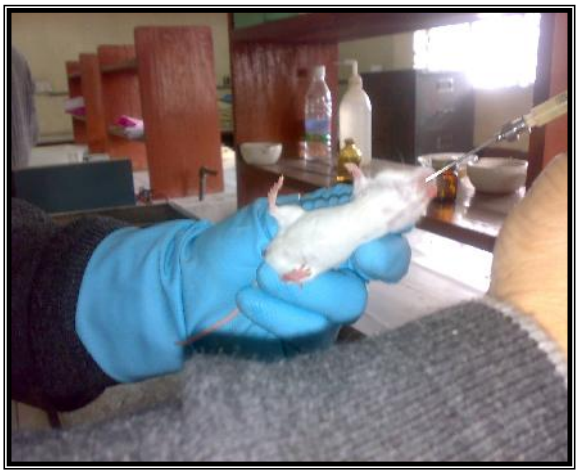
12.6: Procedure

1. The animals were weighed and randomly divided into ten groups consisting of 5 mice in each group.
2. To perform the glucose tolerance test, mice were kept fasting overnight.
3. Blood glucose level of each mouse under fasting condition was measured using glucometer and glucose oxidase-peroxidase reactive strips and it was denoted as 0 min record.
4. Blood sample were collected by cutting the tail tips with a sharp scissor.
5. Blood samples were exposed to glucose test strips and blood glucose level was visualized in the glucometer.
6. After collection of blood the tail tips were exposed to Povidone iodine solution to counteract the possibility of infection and inflammation.
7. Among the mice groups, group I was considered as control receiving 0.30 ml of Tween 80 and DMSO in normal saline each, group II was considered as standard receiving 0.12 ml glibenclamide each, group III-VI was kept as test groups receiving crude methanolic extract, petroleum ether fraction, n-hexane fraction and chloroform fraction of 200 mg/kg and 400 mg/kg of each respectively.
8. The extracts, standard and control samples were given orally with the help of feeding needle.
9. All the groups were administered 2g/kg body weight of glucose solution orally after 30 minutes of receiving the test samples.
10. Blood glucose levels were again measured using glucometer at 30 min, 90 min and 150 min after glucose administration.
11. Then the result of hypoglycemic effects of the test sample is compared in relative to control and standard groups.

A.

B.

C.



D.

Figure 12.4: (A) Housing of test animals,
(B) Holdling of test animal,
(C) Oral administration of test sample and
(D) Estimation of blood glucose level

Table 12.2: Screening of Anti-diabetic activity by different test samples

Group	Mice	0 min	Mean	30 min after glucose load	Mean	90 min after glucose load	Mean	150 min after glucose load	Mean
1) Control	M1	8.3	8.02	14.8	14.6	13.2	13.68	12.3	12.68
	M2	7.8		15.0		14.5		13.6	
	M3	8.0		14.4		13.3		12.5	
	M4	8.1		14.3		13.6		12.2	
	M5	7.9		14.5		13.8		12.8	
2) Standard	M1	9.5	9.74	9.4	9.20	4.7	5.12	4.7	4.66
	M2	10.5		10.8		5.5		4.5	
	M3	11.2		9.8		4.9		4.8	
	M4	8.8		8.1		5.2		4.6	
	M5	8.7		7.9		5.3		4.7	
3) Crude methanolic extract Dose 200 mg/ Kg	M1	9.0	8.26	12.6	12.6	5.9	5.64	5.6	5.22
	M2	7.1		11.4		5.6		5.3	
	M3	8.3		13.2		5.3		5.2	
	M4	8.5		12.4		5.6		5.1	
	M5	8.4		13.4		5.8		4.9	
4) Crude methanolic extract Dose 400 mg/ Kg	M1	8.2	8.72	12.2	12.26	5.7	5.52	4.3	4.90
	M2	9.0		11.6		5.6		4.8	
	M3	8.7		12.6		5.8		4.6	
	M4	9.1		12.1		5.3		5.2	
	M5	8.6		12.8		5.2		5.6	
5) Petroleum ether fraction Dose 200 mg/ Kg	M1	8.0	9.58	10.4	12.36	4.3	5.08	4.6	4.82
	M2	10.4		13.6		5.5		5.2	
	M3	9.5		11.8		4.6		4.7	
	M4	10.2		12.8		5.8		4.5	
	M5	9.8		13.2		5.2		5.1	
6) Petroleum ether fraction Dose 400 mg/ Kg	M1	9.7	8.88	10.8	10.24	4.9	4.66	4.6	4.62
	M2	8.7		8.8		4.6		4.7	
	M3	8.9		10.4		4.7		4.6	
	M4	8.5		11.5		4.5		4.7	
	M5	8.6		9.7		4.6		4.5	
7) n-Hexane fraction Dose 200 mg/ Kg	M1	5.9	6.84	9.8	11.40	4.9	5.42	4.8	5.26
	M2	8.7		12.2		5.6		5.2	
	M3	6.5		10.8		4.8		4.7	
	M4	6.7		11.5		5.6		5.7	
	M5	6.4		12.7		6.2		5.9	

Group	Mice	0 min	Mean	30 min after glucose load	Mean	90 min after glucose load	Mean	150 min after glucose load	Mean
8) n-Hexane fraction Dose 400 mg/ Kg	M1	7.8	7.26	9.8	11.24	4.8	5.14	4.7	4.98
	M2	6.9		10.6		5.1		5.0	
	M3	7.2		11.4		5.2		4.9	
	M4	7.1		12.1		4.9		5.1	
	M5	7.3		12.3		5.7		5.2	
9) Chloroform fraction Dose 200 mg/ Kg	M1	6.3	6.84	13.7	12.86	5.1	4.78	4.2	4.56
	M2	7.0		12.7		4.2		4.6	
	M3	6.6		11.4		4.6		4.5	
	M4	6.8		12.9		4.7		4.8	
	M5	7.5		13.6		5.3		4.7	
10) Chloroform fraction Dose 400 mg/ Kg	M1	8.5	7.86	11.4	12.38	4.3	4.60	4.2	4.42
	M2	7.2		12.2		4.2		4.3	
	M3	7.6		13.1		4.7		4.8	
	M4	7.9		12.5		5.2		4.5	
	M5	8.1		12.7		4.6		4.3	

Table 12.3: Anti-diabetic activity of crude methanolic extract and its different fractions of *Myristica fragrans*

Group	% lowering of glucose		
	After 30 minutes	After 90 minutes	After 150 minutes
1) Control			
2) Standard	36.99	62.57	63.25
3) Crude methanolic extract (Dose 200 mg/Kg)	13.70	58.77	58.83
4) Crude methanolic extract (Dose 400 mg/Kg)	16.03	59.65	61.36
5) Petroleum ether fraction (Dose 200 mg/Kg)	15.34	62.87	61.99
6) Petroleum ether fraction (Dose 400 mg/Kg)	29.86	65.94	63.56
7) n-Hexane fraction (Dose 200 mg/Kg)	21.92	60.38	58.52
8) n-Hexane fraction (Dose 400 mg/Kg)	23.01	62.43	60.73
9) Chloroform fraction (Dose 200 mg/Kg)	11.92	65.06	64.04
10) Chloroform fraction (Dose 400 mg/Kg)	15.21	66.37	65.14

12.7 Statistical analysis

All values were expressed as the mean ± standard error of the mean (SEM) and the result were analyzed statistically by one way analysis of variance (ANOVA) followed by Dunnett's test by using SPSS ver.17. Where, the value of $P < 0.05$ was considered to be statistically significant. The Dunnets t-test was carried out and the P-value determined for the tests.

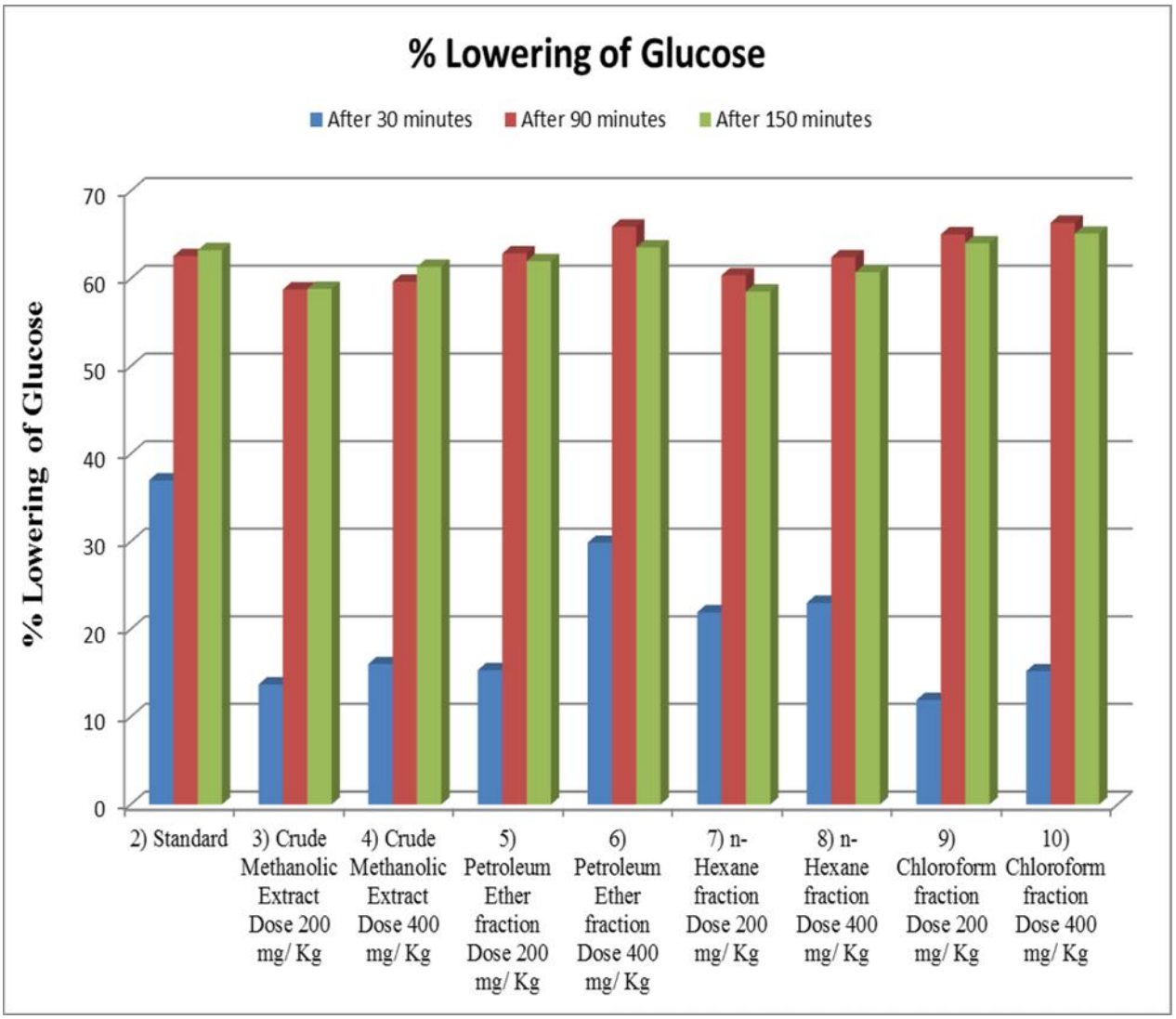


Figure 12.5 : Comparison of % lowering of blood glucose level

12.8 Result and discussion

The effect of methanolic extract of *Myristica fragrans* and its fractions of petroleum ether, n-hexane and chloroform on elevated blood glucose in mice had showed that the extract at doses of 200 mg/kg and 400 mg/kg caused significant lowering of the blood glucose.

Streptozotocin induced diabetic mice is commonly used for primary test of the screening of new anti-diabetic agents. Lowering of glucose for the crude methanolic extract, petroleum ether fraction, n-hexane fraction and chloroform fraction were much significant in double doses than single dose comparing to the control.

The crude methanolic extract, petroleum ether fraction, n-hexane fraction and chloroform fraction were significantly decreased glucose level in mice comparable to glibenclamide. Double dose had given better result than single dose.

13.1 Principle

The antidiarrhoeal activity of *Myristica fragrans* will be evaluated using castor oil induced diarrhoea and intestinal fluid accumulation tests in mice.

In overnight-fasted mice that will induce diarrhoea by oral administration of castor oil (0.6 ml/mice). Test extracts will be given orally 30 minutes prior to castor oil administration. After 30 min administration of castor oil the diarrhoea will clinically apparent in all the animals of control, standard and test groups, for the next 4 hr.

Control rats will be treated similarly that they received an oral dose of tween 80 and DMSO solution. This will markedly reduce by 10 mg/kg Loperamide. A similar marked reduction in the number of defecations over four hours will be tested with *Myristica fragrans* at the doses of 200 and 400 mg/kg. (Bass *et al*., 1972)

13.2 Experimental animal

Swiss albino mice of either sex, weighing 30-35 gm were used. They were obtained from the ICDDR,B animal house. The animals were kept in suitable environmental condition, given standard mice food and water ad-libitum in the Animal House of Nutrition and Food Department of the University of Dhaka. The animals were acclimatized in the environmental condition for 3-4 days before testing.

13.3 Experimental design

50 experimental animals were randomly selected, weighed and divided into ten groups denoted as group-I, group-II, group-III(A-B), group- IV(A-B), group- V(A-B), and group VI(A-B), consisting of 5 mice in each group. Each group received a particular treatment. Prior to any treatment, each mouse was weighed properly and the doses of the test samples and control materials were adjusted accordingly. As it was difficult to observe the biologic response of 5 mice at a time receiving same treatment, it was necessary to identify individual animal of a group during the treatment. The animals were individualized in the following way (Figure 13.1). And marked as M1=Mice 1, M2=Mice 2, M3=Mice 3, M4=Mice 4 and M5=Mice 5.

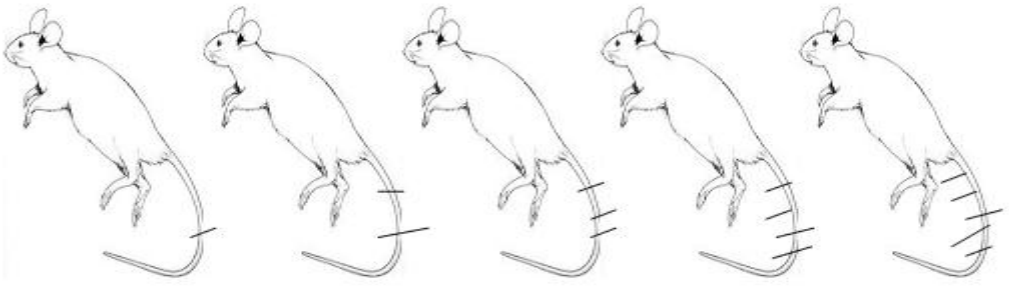


Figure 13.1: Numbering of mice

13.4 Preparation of test materials

13.4.1 Control solution: 3-4 drops of Tween 80 and few drops of DMSO were mixed properly in the normal saline and the volume was made upto 4.0 ml.

13.4.2 Standard solution: Loperamide HCl (10 mg) was accurately weighed and then it was dissolved in 5 ml normal saline (0.9% NaCl solution). Few drops of Tween 80 and DMSO were mixed in solution.

13.4.3 Test solutions: In order to obtain the dose of 200 mg/kg and 400 mg/kg body weight, 240 mg of the test samples were taken separately and each was finally made into 8 ml suspension by means of few drops of Tween 80, DMSO and normal saline. To stabilize the suspension, it was stirred well by vortex mixture.

13.5 Procedure

1. The animals were numbered, weighed and randomly divided into ten groups of 5 mice in each group.
2. To perform the anti-diarrhoeal test, mice were kept fasting overnight.
3. The crude methanolic extract, different fractions of the extract, standard and control samples were given orally. Each group received a particular treatment as mentioned in the Table- 13.1. Loperamide at 10 mg/kg body wt. was used as standard anti-diarrhoeal agent.
4. Each mouse of all the groups were administered 0.6 ml castor oil orally 40 minutes after receiving the treatment.
5. Faecal output was counted of each mouse at 1^{st} hr, 2^{nd} hr, 3^{rd} hr and 4^{th} hr.

Table 13.1: Test samples used for the experiment

Group	Test Samples	Identification	Dose (mg/kg)*
I	Normal saline with few drops of Tween 80 and DMSO	Control group	0.1ml/10 g of body wt
II	Loperamide HCl	Standard group	10
III (A)	Crude methanolic extract	Test group	200
III (B)	Crude methanolic extract	Test group	400
IV (A)	Petroleum ether fraction	Test group	200
IV (B)	Petroleum ether fraction	Test group	400
V (A)	n-Hexane fraction	Test group	200
V (B)	n-Hexane fraction	Test group	400
VI (A)	Chloroform fraction	Test group	200
VI (B)	Chloroform fraction	Test group	400

Castor oil was used as a purgative at a dose of 0.6 ml

*all doses were given orally

13.6: Evaluation of anti-diarrhoeal activity

Table 13.2: Evaluation of different test samples and diarrhoeal activity

Group	Mice No.	No. of diarrhoeal faeces				Total No. of diarrhoeal faeces	Average
		1st Hour	2nd Hour	3rd Hour	4th Hour		
1) Control	M1	6	4	3	2	15	17.4
	M2	6	6	2	3	17	
	M3	7	5	3	3	18	
	M4	7	6	4	2	19	
	M5	6	5	3	4	18	
2) Standard	M1	0	1	2	1	4	2.8
	M2	1	1	1	0	3	
	M3	1	0	1	0	2	
	M4	0	1	0	1	2	
	M5	1	0	1	1	3	
3) Crude methanolic extract (Dose 200 mg/Kg)	M1	5	2	3	2	12	8.8
	M2	3	2	2	1	8	
	M3	2	3	2	1	8	
	M4	2	2	3	1	8	
	M5	2	3	2	1	8	
4) Crude methanolic extract (Dose 400 mg/Kg)	M1	2	2	1	1	6	7.2
	M2	3	2	1	1	7	
	M3	3	1	2	0	6	
	M4	3	2	1	1	7	
	M5	4	3	2	1	10	
5) Petroleum ether fraction (Dose 200 mg/Kg)	M1	3	2	1	1	7	7.0
	M2	3	2	1	0	6	
	M3	3	3	1	1	8	
	M4	2	2	1	1	6	
	M5	3	3	1	1	8	
6) Petroleum ether fraction (Dose 400 mg/Kg)	M1	2	1	0	2	5	6.6
	M2	3	2	1	1	7	
	M3	4	2	2	1	9	
	M4	3	2	2	1	8	
	M5	2	1	1	0	4	
7) n-Hexane fraction (Dose 200 mg/Kg)	M1	4	2	1	1	8	7.4
	M2	3	2	2	1	8	
	M3	4	2	1	0	7	
	M4	2	3	1	1	7	
	M5	3	2	2	0	7	

Group	Mice No.	No. of diarrhoeal faeces				Total No. of diarrhoeal faeces	Average
		1st Hour	2nd Hour	3rd Hour	4th Hour		
8) n-Hexane fraction (Dose 400 mg/Kg)	M1	3	1	1	0	5	5.0
	M2	2	1	1	1	5	
	M3	1	2	1	0	4	
	M4	2	1	2	1	6	
	M5	1	2	1	1	5	
9) Chloroform fraction (Dose 200 mg/Kg)	M1	4	2	3	2	11	9.4
	M2	4	2	1	1	8	
	M3	5	3	2	1	11	
	M4	4	2	2	0	8	
	M5	5	2	1	1	9	
10) Chloroform fraction (Dose 400 mg/Kg)	M1	2	4	1	3	10	8.2
	M2	3	3	1	1	8	
	M3	3	4	1	1	9	
	M4	2	2	1	2	7	
	M5	3	2	1	1	7	

Table 13.3: Effect of crude methanol extract and different fractions on castor oil induced diarrhoea in mice

Test samples	Number of diarrhoeal faeces (Mean± SEM)	% Reduction of diarrhoea
Control (0.1 ml/ 10 g)	17.4 ± 0.68	0
Standard (Loperamide HCl) 10 mg/Kg	2.8 ± 0.37	83.91
Crude methanol extract (Dose 200 mg/Kg)	8.8 ± 0.80	49.43
Crude methanol extract (Dose 400 mg/Kg)	7.2 ± 0.73	58.62
Petroleum ether fraction (Dose 200 mg/Kg)	7.0 ± 0.45	59.77
Petroleum ether fraction (Dose 400 mg/Kg)	6.6 ± 0.93	62.07
n-Hexane fraction (Dose 200 mg/Kg)	7.4 ± 0.24	57.47
n-Hexane fraction (Dose 400 mg/Kg)	5.0 ± 0.32	71.26
Chloroform fraction (Dose 200 mg/Kg)	9.4 ± 0.68	45.98
Chloroform fraction (Dose 400 mg/Kg)	8.2 ± 0.58	52.87

13.7 Statistical analysis

All values were expressed as the mean ± standard error of the mean (SEM) and the result were analyzed statistically by one way analysis of variance (ANOVA) followed by Dunnett's test by using SPSS ver.17. Where, the value of $P < 0.05$ was considered to be statistically significant. The Dunnets t-test was carried out and the P-value determined for the tests.

Statistical evaluation of the data obtained from the crude methanolic extract and other fractions of *Myristica fragrans* at both 200 mg/kg and 400 mg/kg dose showed significant anti-diarrhoeal activity and onward of its administration in mice.

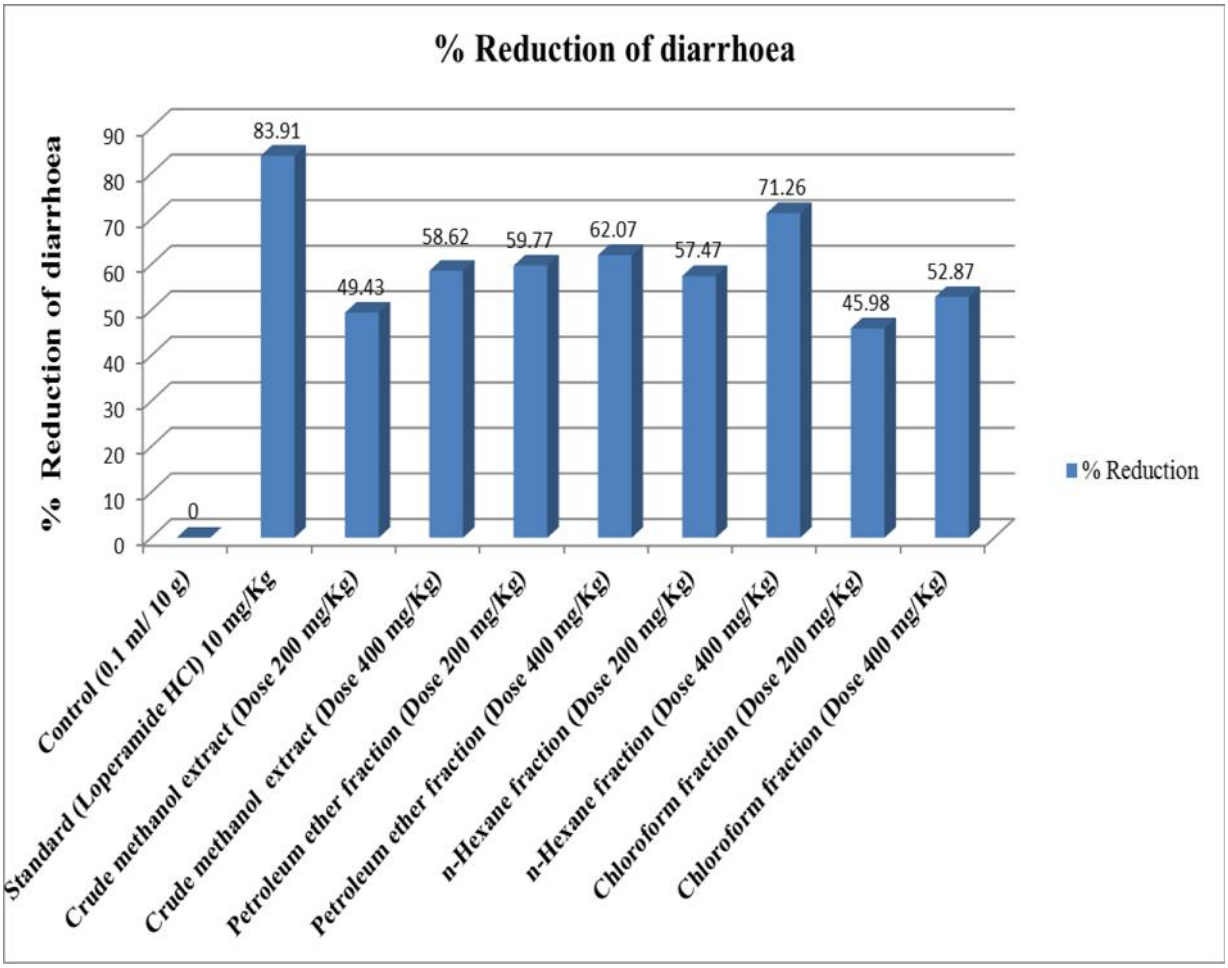


Figure 13.2: Comparison of % reduction of diarrhoea in different groups

13.8 Results and discussion

Statistical data analysis confirmed that the crude methanolic extract, petroleum ether fraction, n-hexane fraction and chloroform fraction showed significant anti-diarrhoeal activity at a dose of 200 mg/kg and 400 mg/kg body weight.

Castor oil induced diarrhoea is commonly used primary test for the screening of new anti-diarrhoeal agents. Reduction of diarrhoea for the crude methanolic extract, petroleum ether fraction, n-hexane fraction and chloroform fraction were much significant in double doses than single dose comparing to the control.

The crude methanolic extract, petroleum ether fraction, n-hexane fraction and chloroform fraction were significantly reduced faecal output in mice comparable to Loperamide HCl. Double dose had given better result than single dose.

Bangladesh is a developing country with a very poor health care system. Less than 50% of the populations have access to basic health care services and the utilization of Government health care facilities are very poor. So, the introduction of herbal medicine in the national health care system may help the nation.

Plants are most valuable sources of biologically active products, which could provide main chemical constituents that are of prime importance in the fight against diseases, pain, inflammation, infections, fever, diarrhoea and diabetes etc. The seed of *Myristica fragrans has* many pharmacological properties, which are directly related to the chemical constituent of the plant. Thus, the selected plants analyzed under this research work have some direct therapeutic effects. In that contrast, an attempt was made to investigate the phytochemical constituents, biological as well as pharmacological properties.

The hexane fraction contains myristicin, an anti-inflammatory principle, licarin-B and dehydro diisoeugenol which exhibited CNS depressant properties. The extracts of nutmeg decreased kidney prostaglandin levels in rats. They also inhibited platelet aggregation (due to eugenol and isoeugenol). The anti-inflammatory activity observed in carrageenan- induced oedema in rats and enhanced vascular permeability in mice, are attributed to myristicin. It also gives anti-pyretic property.

Mace also activates hepatic detoxification process. Monomeric and dimeric phenyl propanoids (myristicin, dehydro diisoeugenol) from mace, on p.o. administration in mice, produced suppression of lipid peroxidation in liver.

The seed of *Myristica fragrans* possesses antioxidant, cytotoxic, anti-microbial effect as well as other beneficial pharmacological potentialities, the disease related to these properties can be cured by the proper utilization of the plant that would be very much beneficial to our country people.

A Al-Shammary Hayfaa, AA Malik Al-Saadi Sahar and M Al-Saeidy Awatif, **Evaluation of analgesic activity and toxicity of alkaloids in *Myristica fragrans* seeds in mice,** *Journal of Pain Research, July 2013 Volume 2013:6, Pages 611—615*

Ahmed M1, Shikha HA, Sadhu SK, Rahman MT, Datta BK. **Analgesic, diuretic, and anti-inflammatory principle from *Scoparia dulcis***. Pharmazie. 2001 Aug; 56(8):657-660.

A. Kumar1*, R. Ilavarasan2, T. Jayachandran1, M. Deecaraman1, P. Aravindan1, N. Padmanabhan1 and M. R. V.Krishan1, **Anti-diabetic activity of Syzygium cumini and its isolated compound against streptozotocin induced diabetic rats**, *Journal of Medicinal Plants Research* Vol. 2(9), pp. 246-249, September, 2008.

Albert, V. & Struwe, L., **Journal of Medicinal and Aromatic Plant Sciences**, 20 (3): 740–741.

Alluri V. Krishnaraju1, Tayi V. N. Rao1, Dodda Sundararaju1, Mulabagal Vanisree2, Hsin-Sheng Tsay2, and Gottumukkala V. Subbaraju1*, **Biological Screening of Medicinal Plants Collected from Eastern Ghats of India Using Artemia salina (Brine Shrimp Test),** *International Journal of Applied Science and Engineering*, 2006. 4, 2: 115-125

Amit Jaiswal*1, Rajesh Kumar1, Kumar Abhinav1, Avanish Kumar2, **Antipyretic Activity Of *Platycladus Orieantalis* Leaves Extract In Rat**, *International Journal of Applied Biology and Pharmaceutical Technology*, Volume: 2: 250- 252, Issue-1: Jan-Mar -2011

Apurba Sarker Apu, Shakhawat Hossan Bhuyan, Shamina Saiyara Prova, and Md. Abdul Muhit, **Anti-inflammatory activity of medicinal plants native to Bangladesh: A review**, *Journal of Applied Pharmaceutical Science* 02 (02); 2012, 07-10

Bakhru.H.K. **Herbs That Heal**, Orient paper Backs (a division of vision books pvt. ltd.)

Bakhru.H.K. **Foods That Heal**, Orient paper Backs (a division of vision books pvt. ltd.)

Bauer AW, Kirby WMM, Sherris JC & Turck M. **Antibiotic susceptibility testing by a standardized single Disk method**. *Amer. I. Clin. Pathol*. 45:493-6, 1966.

Barry AL. **Principles and Practice of Microbiology**. 3rd ed. Philadelphia: Lea & Fabiger; 1976.

Beckett. M. and Stenlake, O.I. **Modified Kupchan Partition**. *J Med Chem*. 52: 1525-1529.

Brady & Taylor, 1988, **Pharmacognosy**, 9th edition, Lea & Febiser, Philadelphia.

B. Mahesh and S. Satish, **Antimicrobial Activity of Some Important Medicinal Plant Against Plant and Human Pathogens**, *World Journal of Agricultural Sciences* 4 (S): 839-843, 2008

Chitme HR1, Chandra M, Kaushik S. **Studies on anti-diarrhoeal activity of *Calotropis gigantea* R.Br. in experimental animals**. , 2004 Feb 25; 7(1):70-75.

Chung-Tei Chou, **The Anti-inflammatory Effect of an Extract of *Tripterygium wilfordii Hook F* on Adjuvant-induced Paw Oedema in Rats and Inflammatory Mediators Release**, *Phytotherapy Research,* Volume 11, Issue 2, pages 152–154, March 1997.

C.P. Khare, **Indian Medicinal Plants**, Springer, page:429

Drabu S1,Chaturvedi S1 and Sharma M2, **Analgesic Activity of Methanolic Extract from the Fruits of *Trichosanthus Dioica***, *International Journal of Research in Pharmaceutical and Biomedical Sciences* , Vol. 3 (1) ,Jan – Mar 2012

Fatope , M.O. , Ibrahim, H, Takeda, Y. **Screening of Higher Plants Reputed as Pesticides Using the Brine Shrimp Lethality Assay**, *Pharmaceutical Biology*, 31(4):250-254 · January 1993.

Forrest, J.E. & Heacock, R.A. (1972), ***Nutmeg and mace, the psychotropic spices from Myritica fragrans,*** Lloydia, 35(4), 440 – 449.

G.E.Trease and W.C.Evans, 1972, **Pharmacognosy**, Bailliere Tindall, London.

Ghani, A, 2012, **Medicinal plants of Bangladesh with Chemical Constituents and Uses**, Asiatic society of Bangladesh, Dhaka.

Ghani, A, 1997, **Practical Phytochemistry**, Jahangirnagar University, Savar, Dhaka.

Ghani, A, 1990, **Traditional Medicine**, Jahangirnagar University, Savar, Dhaka.

Ghani, A, 1976, **Practical Phytochemistry**, Adeyl Brothers, Banglabazar, Dhaka.

Joy, K.L., and R.J. Kuttan, 1999., **Anti-diabetic activity of *Picrorrhiza kurroa* extract. Journal of Ethnopharmacology**, 67: 143-148.

Kadhim M. Ibrahim, Rana K. Naem* and Amaal S. Abd-Sahib, **Antibacterial Activity of Nutmeg (*Myristica fragrans*) Seed Extracts Against Some Pathogenic Bacteria**, *Journal of Al-Nahrain University*, Vol.16 (2), July, 2013, pp.188-192.

Katiyar et al., **Evaluation Of Anti-Diarrhoeal Potential Of Leaf Extracts Of *Ocimum Sanctum* Linn. In Expetimental Rats**, *International Journal of Pharmaceutical Sciences and Research (IJPSR),* 2015; Vol. 6(11): 4804-4809.

Kirtikar KR, Basu BD, **Indian Medicinal Plants**. 2nd ed. Vol 1-IV, Deharadun; 1987

LIU Xin1, TANG Yu-Ping1∗, LIU Rui1, JIN Yi1, GUO Jian-Ming1, ZHAO Jin-Long1,DING Shao-Xiong2, LIN Xiang-Zhi3, LIN Ru-Rong3, DUAN Jin-Ao1∗, **Antipyretic and anti-inflammatory activities of *Thais luteostoma* extracts and underlying mechanisms**, *Chinese Journal of Natural Medicines*, 2015, 13(3): 192−198.

M Gupta, UK Mazumdar, T Sivahkumar, MLM Vamis, S Karki, R Sambathkumar, L Manikandan, **Antioxidant And Anti-Inflammatory Activities Of *Acalypha Fruticosa***, Nigerian Journal of Natural Products and Medicine Vol.7 2003: 25-29.

Matthews, W.S.A., Pickering, G.R. & Robinson, F.U. (1974), **The distillation and composition of nutmeg oils,** *VI. Proc. Int. Congr. Essential Oils*, San Francisco, CA.

Meyer BN1, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DE, McLaughlin JL. **Brine shrimp: a convenient general bioassay for active plant constituents.** *Planta Med.* 1982 May;45(5):31-34.

Monic shah, Pradeep Kumar Reddy P, Yerram raju Behara, **Anti-diarrhoeal activity of *Toddalia asiatica* (l) in castor oil induced diarrhoea in rats,** *International Journal of Research in Pharmacology and Pharmacotherapeutics*, Vol 1 (1), 2012, 5-9.

Muhammad Shaiful Alam Bhuiyan[1], Md. Sohel Rana[2], Mohammed Rahmatullah[1]*, **Preliminary phytochemical screening of five plant parts used in Bangladesh for treatment of rheumatoid arthritis,** *Advances in Natural and Applied Sciences***,** 9(13) September 2015, Pages: 15-21

N. R. Pillai And L. Lillykutty, **Anti-diarrhoeal potential of Myristica fragrans seed extracts**, *Ancient Science of Life,* Vol No. XI No.1 & 2, July & October 1991, Pages 74- 77.

Panda, S.K., **Screening Methods In The Study Of Antimicrobial Properties Of Medicinal Plants**, *International Journal of Bio-Technology and Research (IJBTR*) 06/2012; 2(2):1-35.

Rancy Ann Thomas, Krishnakumari, **Phytochemical profiling of Myristica fragrans seed extract with different organic solvents,** *Asian J Pharm Clin Res*, Vol 8, Issue 1, 2015, 303-307.

S. M. Raquibul Hasan1, Md. Mokarram Hossain1, Raushanara Akter2, Mariam Jamila3, Md. Ehsanul Hoque Mazumder1* and Shafiqur Rahman4, **DPPH free radical scavenging activity of some Bangladeshi medicinal plants,** *Journal of Medicinal Plants Research*, Vol. 3(11), pp. 875-879, November, 2009.

Shanthun-Al-Arefin, Shahnaz Rahman, Shiblur Rahman, Mahfuza Akter, Mahmuda Munmun, Marjina Akter Kalpana, Sharmin Jahan, Md. Shaiful Alam Bhuiyan, Mohammed Rahmatullah, **Scientific validation of folk medicinal uses in Bangladesh of *Piper betle* L. leaves to alleviate pain and lower blood sugar**. *Advances in Natural and Applied Sciences*, 6(8): 1496-1502, 2012.

Sarder Fahim Hossain1, Md. Siddiqul Islam2, Salma Parvin2, Tahiatul Shams1,Mohammad Fahim Kadir1, S. M. Ashraful Islam3, A. G. M. Mostofa4, Muhammad Shahdaat Bin Sayeed4, **Antimicrobial Screening and Brine Shrimp Lethality Bioassay of *Calotropis gigantea* (Fam: Asclepiadaceae)** , *J. Nat. Prod. Plant Resour*., 2012, 2 (1): 49-59 .

Sofowara, A. 1982, **Medicinal Plants and Traditional Medicine in Africa**, *John Wiley and Sons Ltd., New York, USA.*

Sonali Phulsagar1, Manjunath Dundi2 , Shalini Bhagwat3, Yogesh Girigaon, **An Inside Review Of *Myristica Fragrans* Houtt.** , **A Potential Medicinal Plant Of India**; *Valley International Journals*, Vol 1| Issue 09 | Pg: 500-513, 2014.

Truitt, Jr., E.B. **The pharmacology of myristicin and nutmeg,** *Ethnopharmacol. Search Psychoact. Drugs (Proc. Symp.)*, 1979, pp. 215-229.

Vasundra Devi , Divya Priya , **Antipyretic activity of ethanol and aqueous extract of root of *Asparagus racemosus* in yeast induced pyrexia,** *Asian J Pharm Clin Res*, Vol 6, Suppl 3, 2013, 190-193.

Venkatesh, S., G.D. Reddy, Y.S.R. Reddy, D. Sathyavathy and B.M. Reddy, 2004. **Effect of *Helicteres isora* root extracts on glucose tolerance in glucose induced hyperglycemic rats**. Fitoterapia, 75: 364-367.

Venkatesan N1, Thiyagarajan V, Narayanan S, Arul A, Raja S, Vijaya Kumar SG, Rajarajan T, Perianayagam JB**., Anti-diarrhoeal potential of Asparagus racemosus wild root extracts in laboratory animals.** *J Pharm Pharm Sci*, 2005 Feb 25; 8(1):39-46.

Websites www. Plantillustrations.org

www. Wikipedia.com

Yukihiro Ozaki, Soekeni Soedigdo, Yoke Rosina Wattimena And Asep Gana Suganda, **Anti-inflammatory Effect of Mace, Aril of *Myristica fragrans* HOUTT., and Its Active Principles**, *Jpn J Pharmacol*. 1989 Feb; 49 (2):155-163.