POTENTIAL ANALGESIC AND CYTOTOXIC ACTIVITIES OF THE PLANT *Typhonium trilobatum*

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DECLARATION

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

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Abstract

Typhonium trilobatum, a plant of Araceae family, containing many important chemical compounds was studied for its analgesic activity in three experimental models of nociception. The analgesic activity of crude chloroform extract of root, shoot and leaf, and the petrolium ether fraction of leaf, were evaluated by using acetic acid induced writhing method; formalin induced licking method and hot plate test on *Swiss albino* mice at doses of 200mg/kg and 400mg/kg body weight. In addition, cytotoxic activity of leaf, tuber and pet ether fraction was examined with brine shrimp lethality bioassay. The plant extract demonstrated a significant inhibition of writhing, licking and heat tolerance compared with the control group. The leaf extract showed 52.00% and 66.55%; tuber extract showed 38.57% and 53.78%, and the pet ether fraction of leaf showed 31.55% and 50.67% of inhibition of writhing during acetic acid induced writhing test at 200 mg/kg/dose and a bit of higher percentage of inhibition at 400 mg/kg/dose respectively. In addition, the percentages of inhibition were 35.67% and 41.67% for leaf extract for hot plate test at these dose levels. In case of formalin induced licking test, both the two group of mice showed more than 90% of inhibition of licking. Overall, the percentages of inhibition were 67.55%, 53.78% and 50.67% for the leaf, tuber and pet ether fraction of leaf. Morphine 5 mg/kg/dose was used as positive control and exhibited 76.0% inhibition compare to control. On the other hand, all the type of three different extracts showed the presence of cytotoxity over the brine shrimp, where vincristine sulfate was used as positive control. These results define that the extract possesses significant analgesic and cytotoxic activities that support to the ethnopharmacological uses of this plant. Hence, the obtained results in this project work provide a support for the use of this plant for medicinal purposes and encourage further investigation for more fruitful results.

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Abbreviation

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

1. INTRODUCTION

1.1 General introduction

Plants, plant parts and plant products of all descriptions, particularly those with medicinal properties, are invariably used as principal components or ingredients of various traditional medicines. The number of plants with medicinal properties in the subcontinent at present stand about 2000 (Chopra et al., 1958). More than 500 of such medicinal plants have so far been established as growing in Bangladesh (Yusuf et al., 1994). This number of the indigenous medicinal plants is increasing with the discovery and introduction of newer plants every day. In the traditional systems at the present time almost every plant and herb growing in the country has ascribed to it some medicinal virtues and is used as either principal therapeutic agent or as necessary associate (excipient) in medicinal preparations to increase the potency of principal ingredients (Ghani,1998).

Plants and humans are inseparable, because plants not only provide man with food, shelter and medicine, but also the life sustaining oxygen gas. Since disease, decay and death have always co-existed with life, the early man have to think about disease and its treatment at the down of human intellect. Thus the human race started using plant as a mean of treatment of diseases and injuries from the early days of civilization on earth and in its long journey from ancient time to modern age. Nowadays, plants and plant products are being used as effective therapeutic tools for fighting against diseases and other health hazards.

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

According to recorded history of human civilization, man was well aware of the medicinal properties of many plants growing around him more than 5000 years ago. The earliest mention of the medicinal use of plants was found in the Rig Veda (4500 -1600 BC), which reported that the Indo- Aryans used the Soma plant as a medicinal agent. The first Chinese pharmacopoeia containing a list of 135 different plant medicines with their uses and methods of preparation appeared around 1122 BC. More than 400 recipes of plant medicines used in Greek system of medicine were described by Hippocrates around 400 BC.

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

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

1.1.1 Medicinal plants

A medicinal plant is any plant which, is one or more of its origin, contains substance that can be used for therapeutic purposes or which is a precursor for synthesis of useful drugs. This definition of Medicinal plant has been formulated by World Health Organization (Sofowora, 1982).

Disease is as old as itself, and man has always been in search to cure disease. Medicinal plants and herbs have been in used for eradication of diseases and human suffering since antiquity. The plants that possess therapeutic properties or exert beneficial pharmacological effects on the animal body are generally designated as "Medicinal plants". Although there are no apparent morphological characteristics in the medicinal plants that make them distinct from other plants growing with them, yet they possess some special qualities or virtues that make them medicinally important. It has now been established that the plants which naturally synthesize

and accumulate some secondary metabolites, like alkaloids, glycosides, tannins, volatile oils and contain minerals and vitamins, possess medicinal properties (Ghani, 1998).

Medicinal plants constitute an important natural wealth of a country. They play a significant role in providing primary health care services to rural people. They serve as therapeutic agents as well as important raw materials for the manufacture of traditional and modern medicine. Substantial amount of foreign exchange can be possible to earn by exporting medicinal plants to other countries (Ghani, 1998).

1.1.2 History of medicinal plants

The history of the use of medicinal plants for alleviating diseases had its origin in the activities of the primitive man of the remote past. Selection of the medicinal plants by early man, without any prior knowledge about them, was largely best on intuition, guesswork or trial and error. Curiosity and search for food had contributed considerably to his knowledge about the plants and their virtues.

It appears that Babylonians (about 3000 years BC) were aware of a large number of medicinal plants and their properties. Some of the plants they used are still in use in almost the same manner and for the same purpose. As evident from the Papyrus Ebers (written in about 1500 BC), the ancient Egyptians possessed a good knowledge of the medicinal properties of hundreds of plants.

The Arabians Muslim physicians, like Al-Razi and IbneSina (9^{th} to12th century AD), brought about a revolution in the history of medicine by bringing new drugs of plant and mineral origin into general use. Enriching the original Greek system of medicine by the introduction of these new materials and knowledge they laid down the foundation stone of modern of western medicine.

The South American countries have provided the world with many useful medicinal plants, grown naturally in their forests and planted in the medicinal plant gardens. The medicinal plants

used by the Australian aborigines many centuries ago tremendously enriched the stock of the medicinal plants of the world. The current list of the medicinal plants growing around the world includes more than a thousand items (Ghani, 1998).

1.1.3 Plants for therapeutic purpose

The economic condition of the people of this subcontinent is not so well. They can hardly afford to spend much money for the prevention and cure of their diseases. As a result, about 70-80 percent of the population of these countries still has to depend on the indigenous systems for the maintenance of their health. Besides, many people believe that plants are less toxic and safer than manufactured drugs. They also believe that plants are more natural than manufactured drugs. In developing countries, medicinal plants often are more accessible than manufactured drugs (Ghani, 1998).

1.1.4 Plants for modern medicine

Plants have contributed and are still contributing to the development of modern synthetic drugs and medicines in a number of ways as stated below. Novel structures of biologically active chemical compounds, isolated from plant sources, often prompt the chemist to synthesize similar or better semi synthetic compounds. Synthetic drugs with similar or more potent therapeutic activity are often prepared by structural modification of the plant derived compounds with known biological activity. Various analogues and derivatives of plant constituents with similar or better pharmacological actions and therapeutic properties are often prepared by chemists for use as potent drugs.

Homatropine (a synthetic tropane alkaloid similar to atropine), syrosingopine a synthetic derivative of reserpine), chloroquine (a synthetic derivative of quinine), dihydromorphinone, methyl dihydromorphinone, oxymorphine, ethyl morphine and N-allylnormorphine (synthetic derivatives of morphine) are some of the examples of such synthetic drugs which plants have contributed indirectly. Even in this age of synthetic drugs, there are some naturally occurring drugs, such as the digitalis glycosides used in cardiac complications and the catharanthus

alkaloids used in cancers, which have no synthetic alternatives. In such cases, plants continue to remain as their principle and only sources (Ghani, 1998).

1.1.5 National status

Medicinal plants are resources of bioactive compounds and thus serve as important raw materials for drug production. They constitute a precious natural wealth of a country and contribute a great deal to its health care programs.

Being naturally gifted by a suitable tropical climate and fertile soil, Bangladesh possesses a rich flora of tropical plants. About 5000 species of phanerogams and pteridophytes grow in its forests, jungles, wastelands and road sides as indigenous, naturalized and cultivated plants. Out of them, more than a thousand have been claimed to contain medicinal and/or poisonous properties, of which 546 have recently been enumerated with their medicinal properties and therapeutic uses (Yusuf et al., 1994). In addition, while processing various medicinal properties, 257 of these medicinal plants have been identified as efficacious remedies for diarrhoeal diseases and 47 for diabetes. Medicinal plants and plant derived drugs play a very important role to the economy of tropical countries.

Bangladesh, being one of them and possessing such a rich flora of medicinal plants, should make serious efforts to derive maximum economic benefit from these plants by using them as raw materials for its indigenous drug manufacturing industries (Ghani, 1998).

1.1.6 International Status

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

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

1 In the United States, in 1980 alone, the consumer paid 8 billion dollars for prescriptions drugs in which the active ingredients are still derived from plants (Sofowora, 1982).
2 47% of some 300 million new prescriptions written by physicians in America in 1961 contained, as one or more active ingredients, a drug of natural origin (Farnsworth, 1966).
3 More than 47% of all drugs used in Russia are obtained from botanical sources. (Ampofo, 1979).

Table 1.1: Some common medicinal plants with their local name, scientific name and uses (Ghani, 2003).

Local name	Scientific name	Uses
Ulatkambal	*Abromaaugusta*	Emmenogogue; used in amenorrhoea and dysmenorrhoea.
Muktajhuri	*Acalyphaindica*	Expectorant, emetic, diuretic; used in bronchitis and asthma.
Apang	*Achyranthesaspera*	Purgative, diuretic, ecbolic, hypoglycemic; used in renal dropsy, piles, anasarca, boils and other skin eruptions.
Basak	*Adhatodazeylanica*	Expectorant, bronchodilator, used in cough, asthma, bronchitis, pneumonia, phthisis and respiratory problems.
Bel	*Aeglemarmelos*	Digestive, stomachic, laxative, astringent; used in constipation and dysentery.
Rashun	*Allium sativum*	Carminative, diuretic, hypotensive, used in indigestion, hypertension and diabetes.
Chhatim	*Alstoniascholaris*	Febrifuge, antiperiodic, astringent, anthelmintic, hypotensive; used in fever, hypertension, diarrhoea and dysentery.
Kalomegh	*Andrographispaniculata*	Febrifuge, alterative, stomachic, anthelmintic, cholagogue; used in liver diseases, colic, fever, diarrhoea and dyspepsia.
Shatamuli	*Asparagus racemosus*	Roots aphrodisiac, alterative, diuretic; promotes lactation; also used in diabetes.
Neem	*Azadirachtaindica*	Antiseptic; used in fevers, boils, ulcers, eczema and other skin diseases.
Nayantara	*Catharanthusroseus*	Used in blood cancer, Hodgkin's disease and diabetes.
Thankuni	*Centellaasiatica*	Leaf juice is used in cataract and other eye diseases; plant is used in dysentery, internal and external ulcers, convulsive disorders.
KaloDhutra	*Daturametel*	Narcotic, antispasmodic; leaves used in spasmodic asthma, colic, sciatica painful tumours, glandular inflammations.

Local name	Scientific name	Uses
Anantamul	*Hemidesmusindicus*	Alterative, sudurific, diuretic and blood purifier; used in abdominal tumours.
Kurchi	*Holarrhenaantidysenterica*	Antidysenteric, astringent, stomachic and anthelmintic. Fruits are hypoglycaemic.
Mehedi	*Lawsoniainermis*	Paste of leaves and bark is used in burns and scalds, dandruff and various other skin diseases. Decoction in jaundice.
Sarpagondha	*Raulwolfiaserpentina*	Roots are used as remedy for hypertension, insomnia, anxiety, excitement and insanity.
Ashoke	*Saracaasoca*	Strongly astringent and uterine sedative; used in menorrhagia, haemorrhoids and ulcers.
Arjun	*Terminaliaarjuna*	Bark is hypotensive, cardiac tonic, astringent and febrifuge; has tonic effect on liver cirrhosis.
Methi	*Trigonellafoenum-graecum*	Diuretic, carminative, emollient, tonic; used in menstrual disorders, diabetes, hypertension and sexual problems
Ashwagondha	*Withaniasomniferum*	Roots are used in headache, convulsions, insomnia, hiccup, coughs and dropsy.
Ada	*Zingiberofficinale*	Rhizome is carminative, stomachic, digestive; used in dyspepsia, vomiting, loss of voice, coughs, sore throat and fever

1.1.7 Bioactive Phyto-constituents in medicinal plants

Plants have been serving the animal kingdom as its sources of energy like foods, fuels as well as its means of shelter and substance since the very beginning of its existence on earth's surface habitable for the animals. In addition, to provide the animal kingdom its food, fuel and shelter, each of these plants have been synthesizing a large variety of chemical substances since their first day of life on earth. These substances include, in addition to the basic metabolites, phenol compounds, terpenes, steroids, alkaloids, glycosides, tannins, volatile oils, contain minerals,

vitamins and a host of other chemical substances referred to as secondary metabolites which are of no apparent importance to the plants own life. However, many of these compounds have prominent effects on the animal system and some possess important therapeutic properties which can be and have been utilized in the treatment and cure of human and other animal diseases since time immemorial. These secondary metabolites differ from plant to plant. Thus the plant kingdom provides a tremendous reservoir of various chemical substances with potential therapeutic properties (Chopra et al., 1956).

The chemical constituents, which are capable of influencing the physiological systems of the animal body by exerting some pharmacological actions, are designated as the active chemical constituents or simply active constituents.

Green leaves are the sites of a great deal of such chemical activity. The chemical substances with medicinal properties found in some plants are the products of such chemical process. The occurrence of the active chemical substances in all parts of the plant body or they may be accumulated in higher concentrations in some specific parts.

1.2. The plant family araceae

Araceae is a family of monocotyledonous flowering plants in which flowers are borne on a type of inflorescence called a spadix. The spadix is usually accompanied by, and sometimes partially enclosed in, a spathe or leaf-like bract. Also known as the arum family, members are often colloquially known as aroids. This family of 107 genera and over 3700 species is most diverse in the New World tropics, although also distributed in the Old World tropics and north temperate regions. ***Typhonium*** is a genus in the Araceae family. It is most often found growing in wooded areas. (Bown et al., 2000).

1.3 *Typhonium trilobatum*

Common name:

• Bengal Arum, Lobed Leaf *Typhonium*

• English Name: lobed leaf *typhonium*, Cypress Vine, Indian Pink, Cupid's Flower

• Tamil: karunai-k-kilanku, pitikarunai, karunai, karu karunai kilanku

• Bengali: Ghatkanchu, kharkon, ghet kachu

• Assamese: chema kachu

• Tribal name: kharbas, sarakao (Chakma), kalman (Garo).

Synonyms: Arum trilobatum, Arum orixense.

Description

This strange plant *Typhonium trilobatum* has very narrow 3 ft flower heads emerging before leaves in spring, then unfurl into only kind of narrow, with intricate maroon and cream patterning. When the leaves do appear, they're large and compound, similar to Cobra Lily, on a stalk that is light green and black-patterned. It emits a distinctive odour for a few hours when it first blooms, like most arums. Tubers are eaten in some tribal societies and the plant also has various medicinal uses.

This plant is a tuberous herb, with subglobose tuber up to 4 cm diam. Petiole 25-30 cm long; lamina hastate-subtrisect, segments all acuminate, front segment ovate, 8-18 cm long, lateral ones obliquely ovate, shorter, subbilobed at base. Peduncle is thin, 5-7 cm long; tube of spathe oblong, 2.5 cm long, lamina oblong-ovate-lanceolate, acuminate, 15 or more cm long, 5-7 cm broad, inside rose-purple. Spadix is nearly 15 cm long. Female inflorescence short-cylindric, about 7 mm long; male inflorescence 1.25-1.5 cm long, rose-pink, situated above the female. (Medicinal Plants Database of Bangladesh).

Flowering period

The month August.

1.3.1 Distribution

Typhonium trilobatum is seen at Chittagong Hill Tracts, Tangail, Sylhet and Dhaka in Bangladesh and throughout India, New Guinea, and Australia. (Govaerts, R. & Frodin, D.G., 2002).

1.3.2. Scientific Classification

Kingdom: *Planteae*

Subkingdom: *Viridaeplanteae*

Phylum: *Tracheophyta*

Subphylum: *Euphyllophytina*

Subclass: *Aridae*

Order: Alismatales

Family: *Araceae*

Subfamily: *Aroideae*

Genus: *Typhonium*

Species: *trilobatum*

Botanical name: *Typhonium trilobatum* (L.) Schott.

Figure 1.1 ***Typhonium trilobatum L.***

1.3.4 Traditional Uses

Plant parts used : Leaves, Fruits, seeds, bark, Roots.

The plant is hypnotic. Fresh corms are very acrid and a powerful stimulant; employed as a poultice in tumours. The corms are reported to relax the bowels and provide relief in haemorrhoids and piles. They are eaten with bananas to cure the stomach complaints. The Garo of Madhupur applies root paste locally on ulcer of cattle.

Applied as poultice on scirrhous tumors (fresh tuber is very acrid and a powerful stimulant). Eaten with bananas, the tubers relax the bowels and provide relief in haemorrhoids (tubers become innocuous on heating or drying.

1.4 Present study protocol

The potential of biological activities of *Typhonium trilobatum* was studied and the present study has been designed to observe the following bioactivities using in vivo experimental models.

- Collection and extraction of plant sample.
- Evaluation of analgesic activity of the extract using acetic acid-induced writhing test, formalin induced licking test and hot plate test.
- Evaluation of cytotoxic property of the crude extracts using Brine Shrimp Lethality bioassay.

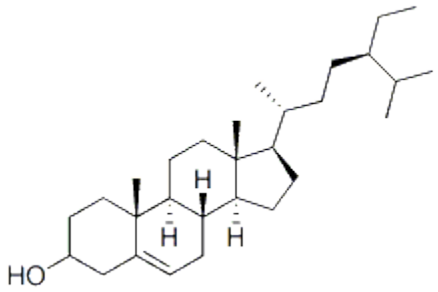
2. LITERATURE REVIEW

Typhonium is a genus of Araceae family, native to eastern and southern Asia, New Guinea, and Australia. The plants of this genus are mostly found to grow in wooded areas. Those have been valued in Ayurveda and Unani systems of medicine for possessing variety of therapeutic properties. Along with Typhonium, the other plants of the family araceae have therapeutic properties

2.1 Medicinal properties of Araceae

2.1.1 Phytohemical consitiuents

Tubers of *Typhonium trilobatum* contain a volatile acrid principle, β-sitosterol, two unidentified sterols and an unidentified crystalline compound (Ghani, 2003).



Some plants of the family araceae release calcium oxalate monohydrate (*Colocasia*).

Most of the plants of araceae family contain Calcium oxalate in leaves and fruits (Botanical Dermatology).

Antiproliferative and apoptosis inducing pheophorbides namely namely pheophorbide-a, pheophorbide-a′ , pyropheophorbide-a and methyl pyropheophorbide-a were identified among the chemical constituents isolated from *Typhonium flagelliforme*. (Choon-Sheen et al., 2008).

Lignanoids are found in the tubers of *Pinellia ternata.* (Ying-Ying et al., 2013).

Araceae contains a new type of cyanogenic constituent in which hydrocyanic acid is loosely bound. Cyanogenesis occurs in many araceous plants, that they contain triglochinin, and that the insrability of their cyanogenic compounds reported by Greshoff is conditioned by the presence of extremely active enzymes and not by loose binding of HCN. Storage of triglochinin is a character of a large family of Araceae. (Mayo et al., 1997)

The major chemical contents found in araceae are alkaloids, flavonoids, phenols and terpenoids. The rhizome yields 0.05% essential oil; of which the chief compounds are: 23.0% caryophyllene oxide, 19.9% geraniol, 19.4% eudesmol and 16.5% citronellyl OAc. Important compounds isolated from the seed cluster are kaempferol-3-O-glucoside (astragalin), kaempferol-3-O-glucuronide, heptatriacontanoic acid 2, 3-dihydroxypropyl ester, heptatriacontanoic acid 1, 3-dihydroxypropyl ester and pentatriacontanoic acid 1, 3-dihydroxypropyl ester. In addition, two major volatile oils, β-pinene and α pinene have also been isolated from the fruits and rhizomes. From the seed clusters two bioactive flavone glycosides were isolated, astragalin and kaempferol-3-Oglucuronide; and kaempferol-3-O-glucuronide were found to be a dominant compound which was distributed primarily in the pulp. The chemical 1,8-cineole is the major component in the leaf essential oil (25.4%) and rhizome oil (34%). In addition, β-pinene (15.1%), camphor (15.3%), carotol (7.3%), α-pinene (7.8%) and camphene (7.0%) were also present in leaf oil, whereas in the rhizome oil α-fenchyl acetate (13.1%), α-terpineol (9.6%), β-pinene (8.1%) and camphene (7.0%) were the other main constituents. (Mayo et al., 1997).

Indrayan et al., (2070) collected the essential oil of the rhizome of *Alpinia officinarum* by hydrodistillation and analysed by means of GLC and GC-MS in apolar and polar columns. Forty-nine compounds representing 99.21% of the oil have been identified. The major constituents of the essential oil of the rhizome are 1,8-cineole (55.39%), Δ^3-carene (8.96%), β-pinene (4.29%), camphene (2.81%), limonene (2.80%), isocaryophyllene (2.52%), camphor (2.35%), α-pinene (2.27%), ϒ-terpinene (2.23%) and ϒ-cadinene (2.17%). The oil shows antibacterial activity

against *Staphylococcus aureus* and *Bacillus subtilis* (Gram positive), *Escherichia coli, Klebsiella pneumoniae* and *Salmonella typhi* (Gram negative), and antifungal activity against *Candida albicans.*

All parts of the plant *Typhonium acetosella* contain calcium oxalate crystals, an irritant to the mouth and esophagus and toxic to animals. The Seeds from berry contains chemicals which inhibit germinations.

The results of various institutes and colleges in Malaysia and some countries have shown that the plant extract of Typhonium flagelliforme (juice) can destroy cancer cells. The results showed: Kill / inhibit pertumbumbuhan cancer cells, eliminating the adverse effects of chemotherapy, anti-virus and anti-bacterial. From the empirical test (based on experience) the treatment of cancer, some types of cancer that can be cured by using rodent tuber include: breast cancer, testicular cancer, prostate cancer, colon cancer, bone cancer, lung cancer and liver cancer. Tuber as a medicinal plant can be used whole, starting from the root, tuber, also leaves. The most common use is to eat all the plants in the form of fresh juice and drink immediately after processed. It is intended to retain as much as possible usefulness. (Cal Reid, 2013).

Aroids produce large amounts of oxalic acid, most of it being deposited as crystals of calcium oxalate. Raphide bundles such as agglomerations of large, needle-like crystals lying parallel to one another are the typical crystal form of the family. These raphide bundles usually occur singly, embedded in mucilage within large idioblasts. Other types of calcium oxalate crystals are found in aroids, such as druses, and in *Acorus* (*Acoraceae*), a genus lacking raphides, cells containing a solitary crystal are situated in rows accompanying fibres. According to Molisch (1918), who investigated *Amorphophallus rivieri, Caladium nymphaeifolium* (a variety of *Colocasia esculenta*), *Monstera deliciosa* and *Sauromatum guttatum* , aroids also tend to accumulate moderate to large amounts of soluble oxalates in leaves. In this respect they are similar to *Lemnaceae, Helobiae* such as *Stratiotes aloides* and *Vallisneria spiralis,* and *Zingiberales* (*Canna, Musa, Maranta*). Silica, aluminium and heavy metals are not known to be accumulated significantly by members of the family. Tubers of *Eminium spiculatum* and *Arisarum vulgare* were found not to contain soluble oxalic acid; tartaric and citric acid were detected in both species (Ahmed et al., 1968).

The carbohydrates stored by *Araceae* have been investigated many times. Pollard (1982) studied the distribution of kestose- and isokestose-types of sucrose fructosides in the basal parts of fresh monocotyledonous stems; no such oligofructans (oligofructosans) were present in *Acoraceae* (*Acorus gramineus*) or *Araceae* (*Arisaema atrorubens, Arisaema triphyllum*), *Dieffenbachia picta, Dieffenbachia maculata*) and *Peltandra virginica, Lemnaceae, Alismataceae, Dioscoreaceae* ,and *Sparganiaceae* (Dahlgren et al. 1985: 277). Sakai & Hayashi (1973) studied the distribution of starchy and sugary leaves in monocots. Japanese *Acoraceae* and *Araceae* belong to those taxa which temporarily store sugars and non-starchy polysaccharides, however, little if any starch, in the leaves. *Acorus gramineus, Amorphophallus konjac*, eight species of *Arisaema, Calla palustris, Colocasia esculenta, Lysichiton camtschatcensis* and *Pinellia ternata* were investigated. Starch was seen occasionally only in *Acorus gramineus* and in two species of *Arisaema*. Vegetative parts of the *Araceae* mainly store starch (Czaja 1978). In some taxa starch is accompanied by mucilages in considerable amounts, which usually consist mainly of glucomannans (Hegnauer 1986). Ohtsuki (1967) showed that subterranean parts of most *Acoraceae* and *Araceae* contain much starch but only a few amounts of glucomannans (*Acorus calamus, Arisaema atrorubens, Arisaema triphyllum, Arisaema japonicum,. Arisaema serratum, Arisaema thunbergii, Lysichiton camtschatcensis, Pinellia ternata, Pinellia tripartita, Amorphophallus campanulatus Amorphophallus paeoniifolius* and *Amorphophallus. Kiusianus).* In certain other species of *Amorphophallus* starch is partly (*Amorphophallus bulbifer*) or largely (*A. konjac, A. oncophyllus, A. variabilis*) replaced by glucomannans, which are located in giant idioblasts. Crude mucilages are always mixed with variable amounts of proteins and pectic and other substances. According to Amin (1955) and others, mucilages purified from taro tubers (in several cultivars) are essentially branched arabino-galactans with an approximate gal:arab ratio of 8:1 to 11:1; this is perhaps the mucilage of the raphide idioblasts. The mucilages isolated in 3–4% yields from tubers of *Eminium spiculatum* and *Arisarum vulgare* by Ahmed et al. (1968) were mixtures of pectic substances, hemicelluloses and true mucilages. Pectins The following remarks concern the occurrence of pectins in non-lignified cell walls. Jarvis et al. (1988) showed that dicots and part of the monocots have primary walls with more than 150 mg of galacturonans per gramme of cell wall preparations (high contents). Grasses and other *Commeliniflorae* had low (< 50 mg/g) galacturonan contents. *Alocasia* and *Lemna* (*Ariflorae*) and all investigated

Alismatiflorae and *Liliiflorae* belonged to the high-content group; some monocot taxa had intermediate (50–150 mg/g) galacturonan contents (Mayo et al., 1997).

2.1.2 Secondary metabolites

Saponins, phenolic compounds including flavonoids, cyanogenic glucosides, the widespread occurrence of constituents which cause skin irritation and painfully acrid sensations on mucous membranes (mouth, throat, eyes) and calcium oxalate raphides may be considered the key chemical characters of the family (Hegnauer 1986; Bown 1988).

2.1.3 Saponins

Saponins were shown to be present in a few numbers of taxa. Hegnauer (1986) stressed the lack of chemical knowledge about araceous saponins. The occurrence of steroidal saponins was mentioned for *Montrichardia* and *Pinellia* (Dahlgren & Clifford 1985) and for *Arales*. Hegnauer (1986) reported *Montrichardia arborescens* to be a rich source of steroidal sapogenins. His analytical method, however, was unreliable. Haemolytic and foam-producing substances, generally believed to be saponins, were shown to be present in many species. Saponins are probably present in some parts of *Alocasia odora* and *Arum italicum*, in *Acorus calamus* (*Acoraceae*), *Amorphophallus campanulatus* (= *A. paeoniifolius*) and *Colocasia esculenta*, and *Acorus calamus* (*Acoraceae*), several taxa of *Colocasia*, a species of *Philodendron* and *Symplocarpus foetidus* (Mayo et al., 1997).

2.1.4 Phenolic compounds

Bate-Smith (1968) investigated hydrolysed leaf extracts of 24 araceous plants. Ten of them contained procyanidins (formerly leucocyanidins), seven contained quercetin, six kaempferol, ten caffeic acid, seventeen *p*-coumaric acid, twelve sinapic acid and eleven contained ferulic acid; in the case of *Orontium aquaticum* the presence of scopoletin was indicated. In some taxa they are accompanied or even replaced by O-glycosides of flavonols or flavones. Sulphates of vitexin, isovitexin, vitexin 7-glucoside, chrysoeriolgalactoside and quercetin occur sporadically (*Philodendron ornatum, Culcasia saxatilis, Scindapsus pictus*).

Ellis et al., (1983) showed that the chemistry of the proanthocyanidins of the fruits of *Zantedeschia aethiopica* and *Z. rehmannii* varies with the taxon. They contain afzelechin,

catechin and (or) gallocatechin and their epimers as building blocks and consist either of pure procyanidins, mixtures of propelargonidins and procyanidins or mixtures of prodelphinidins and procyanidins. Since *p*-coumaric acid and ferulic acid are common in the family it is worth mentioning that they never seem to be bound to the cell wall polysaccharides. Harris & Hartley (1980) showed that *p*-coumaric, ferulic and diferulic acids occur combined with non-lignified cell walls in all investigated members of *Commelinidae* and in *Arecaceae, Philydraceae, Pontederiaceae* and *Haemodoraceae* in the strict sense. (Mayo et al., 1997).

2.1.5 Cyanogenic glucosides

Cyanogenic glucosides have been known since the work of Jorissen, Greshoff and Treub (Hegnauer 1986) to be rather common in the family. The distribution and chemistry of cyanogenesis (the ability to release Hydrogen Cyanide after injury) in aroids have been thoroughly discussed by Hegnauer (1986); one of three tested samples of *Gymnostachys anceps* was weakly and two were strongly cyanogenic (Kaplan et al., 1983).

Cyanogenesis is often extremely rapid in *Araceae* and in many instances the Hydrogen Cyanide is totally lost during the drying of plant parts. Triglochinin and the corresponding enzyme were shown to be the cause of cyanogenesis in *Alocasia macrorrhizos* (tribe *Colocasieae*), *Arum maculatum* (tribe *Areae*), *Pinellia tripartita* (tribe *Arisaemateae*), *Lasia spinosa* (subfamily *Lasioideae*) and *Dieffenbachia picta* (= *D. maculata*, tribe *Dieffenbachieae*) by Nahrstedt and his group (Hegnauer, 1986).

2.1.6 Treatment of malaria

Several plants have their uses both in the treatment of malaria and also in the treatment of their symptoms. Thus, whole plant decoctions of *Pistia stratiotes* are used in Togo to treat fevers and malaria infections (Frausin et al., 2015).

Some species of Araceae are not used specifically to treat malaria, but are used to treat symptoms often associated with malaria such as fevers and headaches. So, the indigenous Wayãpi in French Guyana treat fevers with a decoction of Tapi`Ykũ (*Philodendron linnaei* Kunth) (Grenand et al., 1987). Similarly, the Tukano tribes of southeast Colombia treat headaches by placing fresh inflorescences of madona lily (*Spathiphyllum floribundum)* on the

sick person´s forehead (Croat, 1994). *Culcasia lancifolia* has been used as traditional healers to treat headaches, fevers and vomiting (Lekana-Douki et al., 2011).

2.1.7 Antimicrobial activity

The test all extracts exhibited prominent antimicrobial activity against all tested organism, where chloroform extract shown the maximum zone of inhibition. The order of antimicrobial activity of all extract are, chloroform > Methanolic >Ethyl acetate against bacteria i.e. *Escherichia Coli, P. aeruginosa, Staphylococcus aureus and S. Epidermidis* and fungi i.e. *Candida albicans* and *Aspergillus niger*. (Saurav et al., 2012).

2.1.8 Anti-fungal activity

Anti-fungal activity was found against cultures of *Aspergillus niger* and *candida albicans*. (Sourav et al., 2012).

2.1.9 Wound healing activity

Methanolic and ethyl acetate extract were found greater wound healing activity than chloroform extract in terms of breaking strength in incision model and percentage wound healing contraction, period of epithelialization in excision model than that of other groups. (Sourov et al., 2012).

2.1.10 Analgesic, anti-inflammatory and anti-diarrheal activities

The plant extract demonstrated a significant inhibition of writhing ($P<0.01$) compared with the control group in acetic acid-induced writhing test in mice. The extract also significantly inhibited the xylene induced ear edema formation ($P<0.05$). In anti-diarrheal test, the extract significantly decreased the frequency of defecation and increased the mean latent period ($P<0.01$) in castor oil-induced diarrheal model mice at the doses of 250 and 500 mg/kg body weight. These results suggest that the extract possesses significant analgesic, anti-inflammatory and anti-diarrheal activities that support to the ethnopharmacological uses of this plant. (Khadem et al., 2012).

3. MATERIALS AND METHODS

3.1 Working Place

All the experiments of these investigations were carried out at the laboratories of the Department of Mathematics and Natural Sciences and the Department of Pharmacy, BRAC University and the Department of Pharmacy, Stamford University, Siddheswari, Dhaka, Bangladesh from June 2013 to May, 2014.

3.2 Plant Material

Typhonium trilobatum (L.) was selected for investigations and leaves and root part were used for investigations.

3.3 Preparation of Plant Extract for Experiments

3.3.1 Collection and the identification of the plant material

The plants were collected from Savar, Dhaka during June, 2013 and identified by the taxonomists of the National Herbarium, Mirpur, Dhaka, Bangladesh (**Accession No. 38368**), where a voucher specimen has been deposited for further reference.

3.3.2 Drying the Samples

The leaves, shoot and root were washed with water. Then the collected samples were dried for one month under shade at room temperature. Finally the leaves and seeds were dried in hot air woven at 35^0C for 24 hours. Before drying, the parts of the plant were cut into small pieces as necessary.

3.3.3 Grinding the Dried Samples

The dried samples were grounded to coarse powder with a blender and powdered samples were kept in clean closed glass containers for extraction. During grinding of sample, the blender was

thoroughly cleaned to avoid contamination with any other substance that was grounded previously. The dried grinded powder was weighed by rough balance.

3.3.4 Extraction and Fractionation of samples

The precise mode of extraction naturally depends on the texture and water content of the plant material being extracted and on the type of substance that is being isolated. Generally, cold as well as hot extraction procedures are being carried out. In this study cold extraction procedure was employed with chloroform.

About 200 g powder of root and 400 g powder of leaves were taken into separate clean, flat-bottomed glass container and soaked in 500 ml and 1000 ml of 50% chloroform. The container with its contents was sealed and kept for a period of 7 days accompanying occasional shaking and stirring. The whole mixture then underwent a coarse filtration by a piece of clean, white cotton material. Then it was filtered through Whatman 41 filter paper. The solvent was completely removed keeping open in room temperature and crude extract was dried. These crude extracts of leaf were fractionated with petroleum ether later. Both the crude leaf and tuber extract and the fraction of leaf were used for investigations.

3.4 Pharmacological Investigations of *Typhonium trilobatum*

3.4.1 List of Apparatus Used

Different types of apparatus are used for the extraction and experimentation which are given in Table 3.1.

Table 3.1 List of Apparatus Used in the Experiment

Serial No.	Apparatus Name	Sources
1	Vortex Mixture	China
2	LC Oven	LAB-LINE USA
3	Syringe	Opsosaline Ltd. Bangladesh
4	Feeding Needle	Local made
5	Digital Weighing Balance	Denver Instrument Company USA
6	Cotton	India
7	Blender	NOWAKE, Japan
8	Aluminum Foil	DIAMOND, USA
9	Filter Paper	11cm DIA, China
10	Marker Pen	RED LEAF, Japan
11	Thermostatic Water Bath	Shanghai, China

3.4.2 List of Glasswares Used

Different types of glassware are used during experimentation; major wares are listed below-

Table 3.2 List of Glassware Used

Serial No.	Apparatus Name	Source
1	Volumetric Flask	Changdu, China
2	Test tube	Changdu, China
3	Beaker	Changdu, China
4	Funnel	Changdu, China
5	Measuring Cylinder	Changdu, China
6	Glass Rod	Changdu, China

3.4.3 Collection and Maintenance of Animals

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

Figure 3.1: Swiss Albino Mice

3.4.4 Identification of Animals during Experiment

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

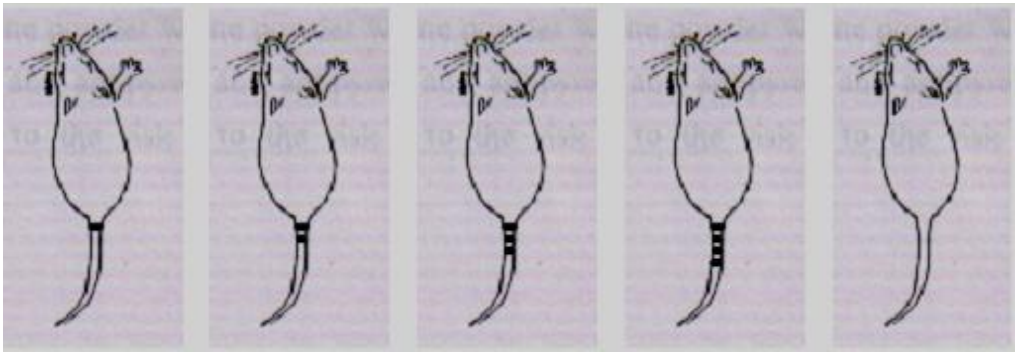


Figure 3.2 Identification of Animals

3.4.5 Preparation of Sample and Standard

The sample was prepared by dissolving the chloroform extract of *Typhonium trilobatum* with Tween 80 and diluted to distilled water at two doses (200 mg/kg body weight and 400 mg/kg body weight). 90 mg extract was measured and added with it 9 ml of tween-80 and mixing with the help of vortex apparatus. From this solution 0.50 ml for 200 mg/kg dose and 1 ml for 400 mg/kg dose were used.

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

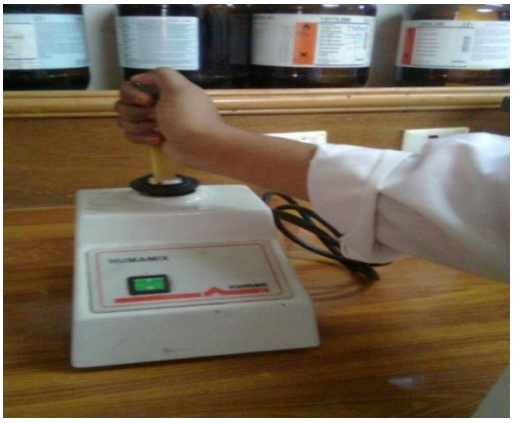


Figure 3.3: Preparation of the solution of plant extract with the help of vortex apparatus.

3.4.6 Tests for Analgesic Activity

The study of analgesic activity of the extract was performed in animal models for both central and peripheral mechanism of pain. For the screening of analgesic activity against peripheral mechanism of pain acetic acid induced writhing test was considered. On the other hand, to evaluate the analgesic activity against centrally mediated pain tail immersion test was used.

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

1. Acetic acid induced writhing response (Koster et al., 1959)
2. Formalin-induced nociception test (Santos *et al.,* 1999)
3. Hot-plate test - thermal nociception test (Eddy *et al*., 1953).

3.4.7 Drugs and Chemicals

Table 3.3 : List of Drugs and Chemicals Used for the analgesic activity test

Serial no.	Drugs and Chemicals	Purpose	Source
1	Morphine	Standard drug in acetic acid induced writhing test	ACE Surgical Supply Company
2	Acetic acid	Writhing inducer	Merck, Germany
3	Tween 80	Suspending agent	Merck, Germany
4	Distilled water	Vehicle	Laboratory Prepared

3.4.8 Acetic Acid Induced Writhing Test

The antinociceptive activity of the samples was studied using acetic acid-induced writhing model in mice (Ahmed et al., 2004; Hasan et al., 2009). The animals were divided into control, positive control and test groups with five mice in each group. The animals of test groups received test samples at the doses of 200 and 400 mg/kg body weight. Positive control group received standard drug morphine at the dose of 5 mg/kg body weight and vehicle control group was

treated with 1% Tween 80 in water at the dose of 10 ml/kg body weight. Test samples and vehicle were administered orally 30 min before intraperitoneal administration of 0.7% acetic acid but morphine was administered 15 min before injection of acetic acid. After an interval of 5 min, the mice were observed for specific contraction of body referred to as 'writhing' for the next 10 min (Meera et al., 2008).

Table 3.4 Experiment Profile to assess the effect of crude extract of *Typhonium trilobatum* acetic acid induced writhing of mice.

Animal Group	Treatment	No. of Animals	Dose	Route of Administration
Vehicle control	1% tween 80 in water	6	0.5 ml	P.O.
Standard	Morphine	6	5 mg/kg	I.P.
Group I	Leaf extract of *Typhonium trilobatum*	6	200 mg/kg	P.O.
Group II	Leaf extract of *Typhonium trilobatum*	6	400 mg/kg	P.O.
Group III	Tuber extract of *Typhonium trilobatum*	6	200 mg/kg	P.O.
Group IV	Tuber extract of *Typhonium trilobatum*	6	400 mg/kg	P.O.
Group V	Pet. Ether fraction of the leaf extract of Typhonium trilobatum	6	200 mg/kg	P.O
Group VI	Pet. Ether fraction of the leaf extract of Typhonium trilobatum	6	400 mg/kg	P.O

3.4.9 Counting of writhing and calculation

After inducing the plant extract and control every mice of all groups was observed carefully to count the number of writhing which made within 10 minutes.

Figure 3.4: Half Writhing Given by Mice

Figure 3.5: Full Writhing Given by Mice

Percentage of Inhibition = $\left(\frac{Writhing\ in\ Control - Writhing\ in\ Extract}{Writhing\ in\ Control}\right) \times 100$

3.4.10 Formalin induced licking test

The animals were divided into control, positive control and test groups with five mice in each group. Mice were orally pretreated with doses (200, 400 mg/kg, p.o.), and the control group received a similar volume of vehicle (Tween-80, 10 mL/kg, p.o.) 60 min prior the nociceptive agent. Morphine (5 mg/kg, i.p.) was used as the reference drug and administered 15 min before the nociceptive agent. Animals received 20 µl of a 2.5% formalin solution (0.92% formaldehyde) made up in saline, injected intraplantarly in the ventral surface of the right hind paw. Animals were observed from 0 to 5 min. (neurogenic phase) and 15–30 min. (inflammatory phase) and the number of licking of the injected paw was counted and considered as indicative of nociception (Santos ARS et al.,1999 and Santos ARS et al.,1997).

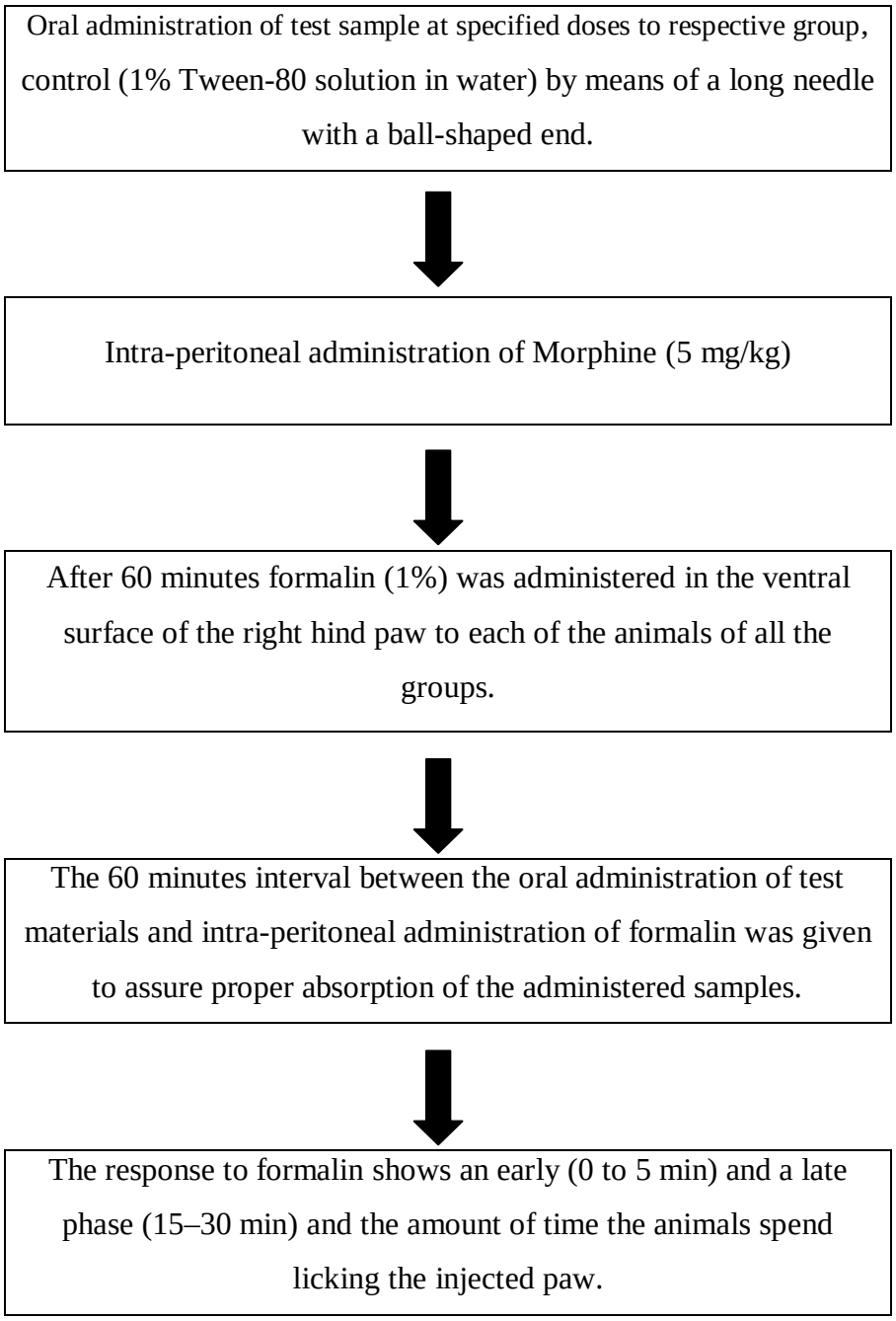


Figure 3.6 Schematic Diagram of Formalin Induced Licking Test procedure.

Percentage of Inhibition = $\left(\frac{Licking\ \ in\ Control - Lickinging\ \ in\ Extract}{Lickinging\ \ in\ Control}\right) \times 100$

3.4.11 Hot Plate Test

The hot plate test is a test of the pain response in animals, similar to the tail flick test was proposed by Eddy and Leimbach in 1953. It is used in basic pain research and in testing the effectiveness of analgesics by observing the reaction to pain caused by heat. The test is a behavioral model of nociception where behaviors such as jumping and hind paw-licking are elicited following a noxious thermal stimulus. Licking is a rapid response to painful thermal stimuli that is a direct indicator of nociceptive threshold. Jumping represents a more elaborated response, with a latency, and encompasses an emotional component of escaping (Espejo et al., 1993)

The hot plate test was used to measure the response latencies based on the procedure described by Eddy (1953). In these experiments, the hot plate was maintained at 50±0.5°c. The reaction time was recorded for animals pretreated with saline (10ml/kg 30 min before orally) as control or plant samples (200 mg/kg and 400 mg/kg orally, 30 min before), Morphine was used as a positive control group. Animals were placed into the hot plate chamber and the time of latency period between the zero point, when the animal was placed on the hot plate surface, and the time when animal licked its backed paw or jumped off to avoid thermal pain. The latent period of response was taken as the index of antinociception and was determined as pretreatment, 30, 60, 90, 120 min after the administration of test drugs and standard. In order to minimize the damage on the animal paws, the cut-off time was taken as 25 seconds.

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3.5 Cytotoxic Activity: Brine Shrimp Lethality Bioassay

Brine shrimp lethality bioassay is considered as a useful tool for preliminary assessment of toxicity. It has also been suggested for screening pharmacological activities in plant extracts. It is

a rapid and comprehensive bioassay for the bioactive compounds of natural and synthetic origin (Michael et al., 1956). By this method, natural product extracts, fractions as well as pure compounds can be tested for their bioactivity. The method utilizes in vivo lethality in a simple zoological organism (Brine nauplii) as a convenient monitor for screening fractionation in the discovery of new bioactive natural products. Brine toxicity is closely correlated with 9KB(human nasopharyngeal carcinoma) cytotoxicity (P=0.036 and k=0.56). ED_{50} values for cytotoxicities are generally about one-tenth the LC_{50}values found in the brine shrimp test. Thus it is possible to detect and monitor the fractionation of the Cytotoxic, as well as 3PS (P_{388}) (in vivo murine leukemia) active extracts using the brine lethality bioassay (Meyer et al., 1982).

The brine shrimp assay is advantageous of being rapid (24hours), inexpensive and simple (e.g., no aseptic technique is required). It easily utilizes a large number of organisms for statistical validation and requires no special equipment and a relatively small amount of sample (2-20mg or less).

3.5.1 Materials

- Apparatus and glassware:
- *Artemia salina* Leach (Brine Shrimp eggs)
- *Electric balance*
- Filter paper
- Lamp
- Magnifying glasses
- Micropipette (5-50µl), (10-100µl), 1000 µl.
- Oxygen supply
- Pipettes (5,10ml)
- Small tank
- Spatula
- Test tubes with racks
- Volumetric flask (100ml)
- Yeast

- **Reagents**

- DMSO (dimethyl sulpoxide)
- Distilled water
- NaOH pellets
- NaCl
- Vincristine sulfate

3.5.2 Methods

Preparation of Seawater

38 gm sea salt i.e., NaCl (without iodine) was weighted, dissolved in one liter of distilled water adjusted to pH 8.5 using 1N NaOH and was filtered off to get clear solution.

Hatching of Brine Shrimp

Artemia salina leach (Brine shrimp eggs) collected from pet shops and used as the test organisms

Sea water was taken in the small tank and aerated (with oxygen pump) along with light supply on one side

After an hour, shrimps eggs were added to one side of the tank and the other side was covered.

After 24 hours, crushed yeast was added in the tank as food for the shrimps

Two days were allowed for the eggs to hatch and mature as nauplii

Constant oxygen supply was provided during the hatching period

The hatched shrimps were attracted to the light (phototaxis) and so nauplii freed from the egg shells were collected from the illuminated part of the tank

The nauplii were taken from the fish tank using a pipette and diluted in fresh clear sea water to increase visibility and 10 nauplii were taken carefully by capillary tube into each test tube.

Figure 3.7 Schematic diagram of the procedure of Hatching of Brine Shrimp

3.5.3 Preparation of samples

One milligram of each extract was added with 5 ml of seawater. Concentration was found 200 µg/ml. Then 50µl DMSO was added on these. Sample was prepared.

3.5.4 Dilution of samples

The solution was serial diluted to 100, 50, 25, 12.5, 6.25, 3.123, 1.563, µg/ml with seawater. Then 2.5 ml of plant extract solution was added to 2.5 ml of seawater containing 10 nauplii.

3.5.5 Preparation of control group

Control groups were used in cytotoxicity study to validate the test method and ensure that the results obtained were only due to the activity of the test agent and the effects of the other possible factors were nullified. Two types of control groups were used-

1. Positive control
2. Negative control

Preparation of positive control

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 sulfate was used. As vincristine is a very Cytotoxic alkaloid it was evaluated at very low concentration (40, 20, 10, 5, 2.5, 1.25, 0.625, µg/ml).

Preparation of negative control

Fifty micro liter of DMSO was added to each of three pre-marked test tubes containing 4.95 ml of seawater and 10 shrimp nauplii to use as control groups.

3.5.6 Adding of *Typhonium trilobatum* extract

Fifty milligram of each leaf, tuber and petroleum ether fraction of leaf extract were taken and dissolve in 5ml of DMSO and placed in 27 test tubes, each containing in 9.

3.5.7 Counting of nauplii

After 24 hrs (in 3 hrs time interval) the test tube were inspected using a magnifying glass against a black background and the number of survived nauplii in each tube was counted. From this data, the percentage (%) of mortality of the brine shrimp nauplii was calculated for each concentration using the following formula:

$$\frac{Nt}{No} \times 100$$

Where,

N_t =Number of killed nauplii after 24 hrs of incubation

N_o = Number of total nauplii transferred i.e., 10 nauplii

$$P_t = \left[\frac{(P0 - Pc)}{(100 - Pc)}\right] \times 100$$

Where,

P_0= Observed mortality and

P_c= Control mortality

The effectiveness of concentration-mortality relationship of plant product is usually expressed as medial lethal concentration (LC_{50}). This represents the concentration of the chemical that produces death in half of the subjects after a certain exposure time.

4. RESULTS

4.1 Investigation of analgesic activity by acetic acid induced writhing test

The acetic acid induced writhing method is an analgesic behavioral observation assessment method that demonstrates a noxious stimulation in mice. The test consists of injecting the 0.6% acetic acid solution intraperitoneally and then observing the animal for specific contraction of body referred as 'writhing'. A comparison of writhing was made between positive control (Morphine), control and test sample given orally 30 minutes prior to acetic acid injection.

Counting of Writhing

After feeding the plant extract and control every mice of all groups was observed carefully to count the number of writhing which made within 15 minutes.

Data of Analgesic Activity by Writhing Test of Extract

All the experimental data on Analgesic Activity by writhing are presented in the table.

Table 4.1: Analgesic Activity of chloroform extract of the leaf of *Typhonium trilobatum* on Mice

Group	Writhing Counting/15 min						Mean	% 0f writhing	% 0f Inhibition	SD	SEM
	M 1	M 2	M 3	M 4	M 5	M 6					
Control	45	44	59	38	39	45	45	100	0	7.51	3.06
Positive control	9	13	11	14	8	11	11	24.44	75.56	2.28	0.92
Group-1	23	26	20	20	19	20	21.6	48.00	52.00	5.20	2.12
Group-2	16	13	15	18	11	13	14.6	32.45	67.55	2.70	1.10

Control: 1% Tween 80 (10 ml/kg), **Positive Control:** Morphine (5 mg/kg),

Group-1: Extract (200 mg/kg), **Group-2:** Extract (400 mg/kg)

N=6

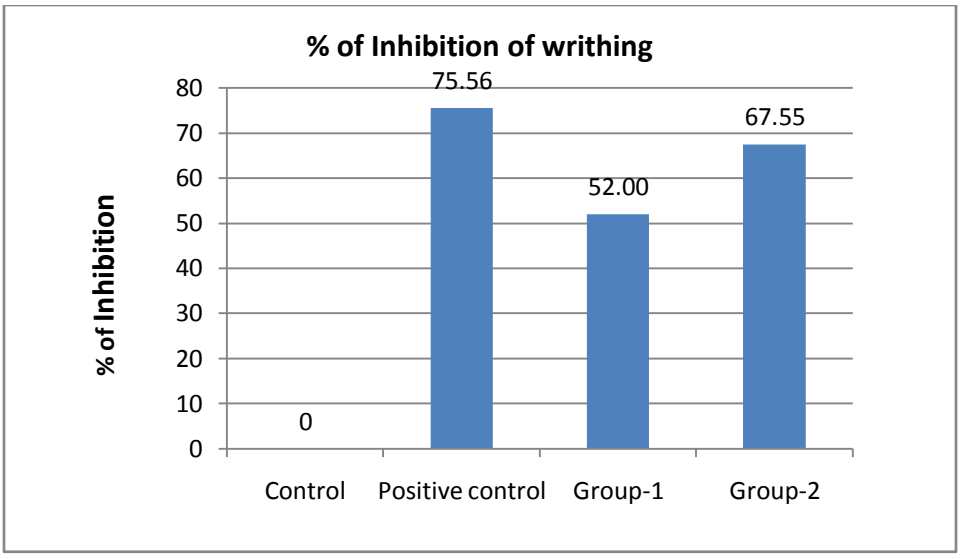

Fig 4.1: Graphical presentation of the effect of crude leaf extract of *Typhonium trilobatum* on mice by acetic acid induced writhing test.

Table 4.2 Analgesic Activity of chloroform extract of tuber of *Typhonium trilobatum*

Group	Writhing Counting						Mean	% of writhing	% inhibition	SD	SEM
	M 1	M 2	M 3	M 4	M 5	M6					
Control	45	44	59	38	45	45	45	100	0.00	7.51	3.35
Positive control	9	13	11	14	8	11	11	24.44	75.56	2.28	1.12
Group-1	30	27	26	28	31	23	27.6	61.33	38.67	3.21	1.43
Group-2	20	21	23	19	21	20	20.8	46.22	53.78	1.48	0.66

Control: 1% Tween 80 (10 ml/kg), **Positive Control :** Morphine (5 mg/kg),
Group-1: Extract (20 0mg/kg), **Group-2:** Extract (400 mg/kg) and N=6

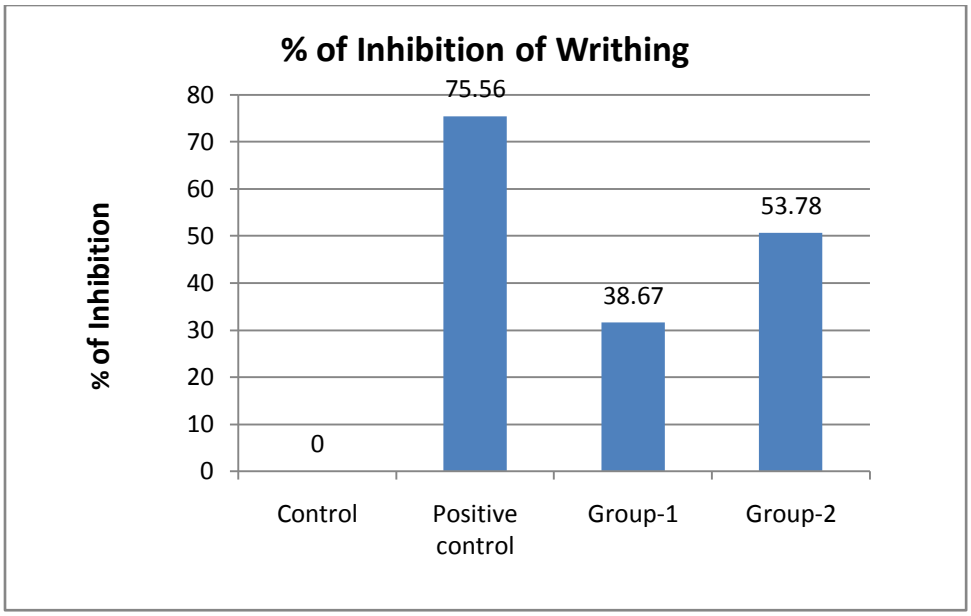

Figure 4.2 Graphical presentation of the effect of root extract of *Typhonium trilobatum* on mice by acetic acid induced writhing test

Table 4.3 Analgesic Activity of chloroform extract of the leaf (pet ether part) of *Typhonium trilobatum* on Mice

Group	Writhing Counting						Mean	% of Writhing	% ofInhibition	SD	SEM
	M1	M2	M3	M4	M5	M6					
Control	45	44	59	38	39	45	45	0.00	100.00	7.51	3.36
Positive control	9	13	11	14	8	11	11	24.44	75.56	2.28	1.02
Group-1	27	28	32	30	31	31	30.8	68.44	31.56	2.07	0.41
Group-2	21	27	26	19	18	21	22.2	49.33	50.67	4.08	0.82

Control: 1% Tween 80 (10 ml/kg), **Positive Control:** Morphine (5 mg /kg),
Group-1: Extract (200mg/kg), **Group-2:** Extract (400 mg/kg) and N=6

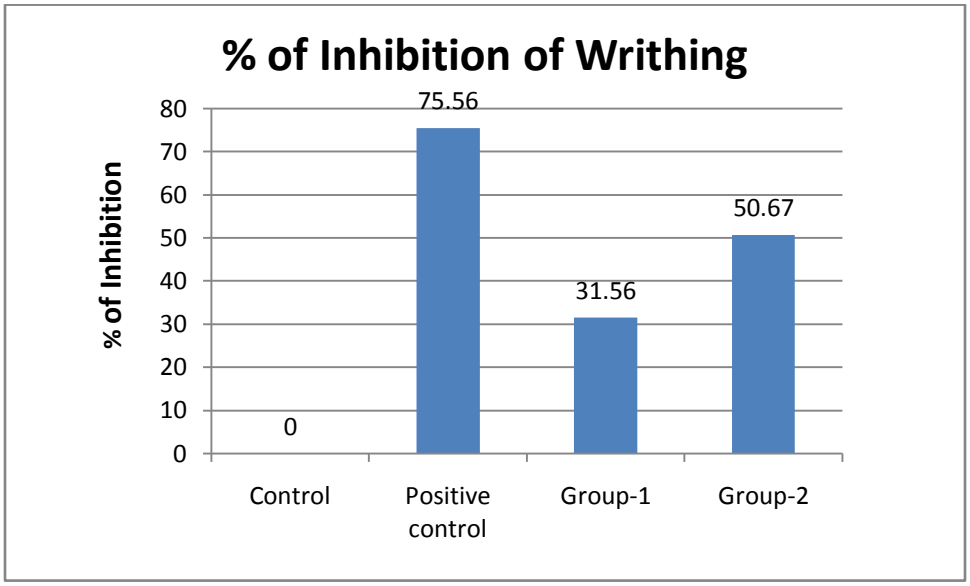

Figure 4.3 Graphical presentation of the effect of pet ether fraction of leaf extract of *Typhonium trilobatum* on mice by acetic acid induced writhing test

Among the three extract, the dose 400mg/kg gives the better result. The percentage of inhibition are 67.55%, 53.78% and 50.67% for the leaf, tuber and pet ether fraction of leaf. Among them, the crude leaf extract gives the better result than other. This is shown as a pie chart.

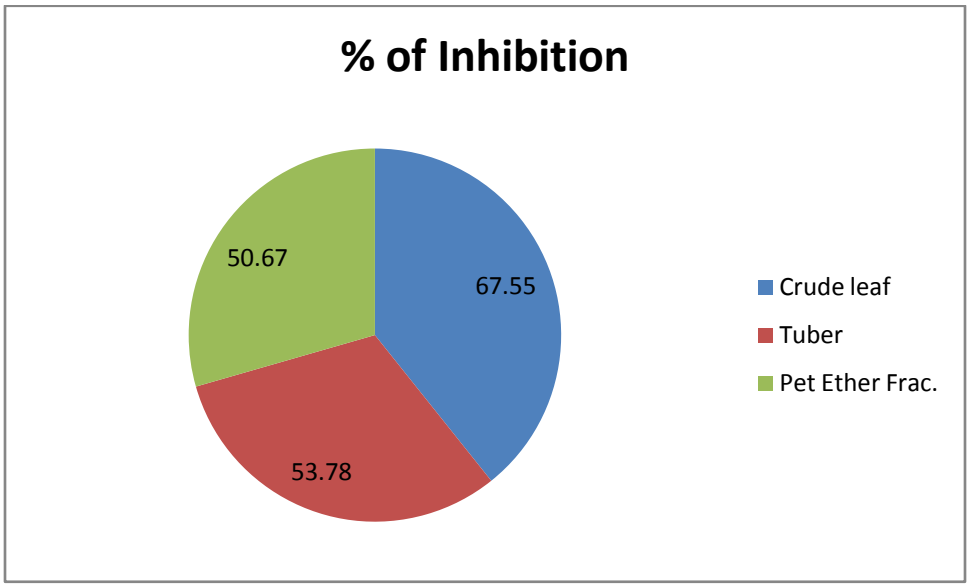

Fig 4.4 Comparison of percentage of inhibition of three extracts (400 mg/kg).

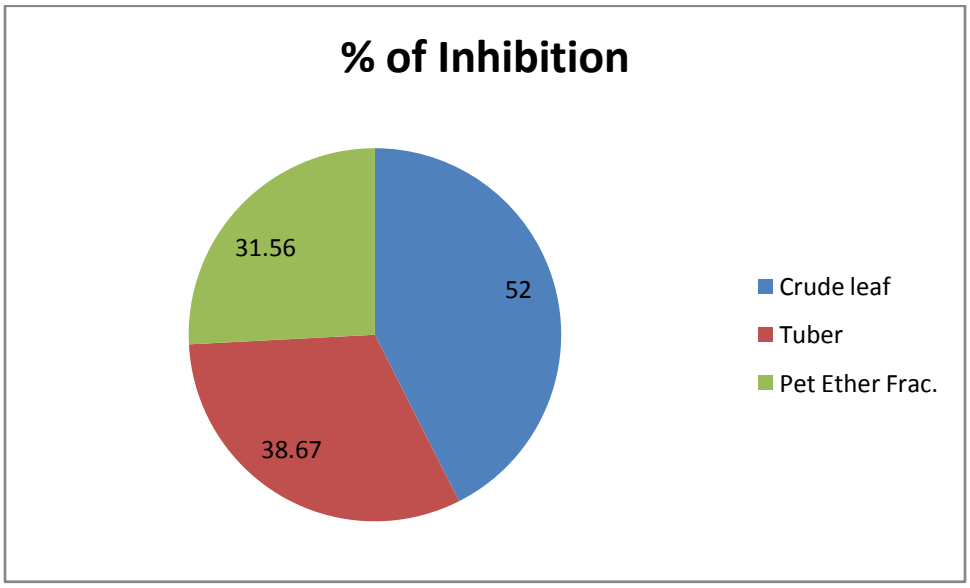

Fig 4.5 Comparison of percentage of inhibition of three extracts (200 mg/kg).

4.2 Investigation of analgesic activity by hot plate test

The hot plate test was used to measure the response latencies based on the procedure described by Eddy and Leimbach (1953). In the present study, the hot plate was maintained at 50±0.5°C. The reaction time was recorded for animals pretreated with saline (10 ml/kg 30 min before orally) as control or EEGO (200 mg /kg and 400 mg /kg orally, 30 min before), Morphine was used as a positive control group. Animals were placed into the hot plate chamber and the time of latency period between the zero point, when the animal was placed on the hot plate surface, and the time when animal licked its backed paw or jumped off to avoid thermal pain. The latent period of response was taken as the index of antinociception and was determined as pretreatment at 30, 60, 90, 120 min after the administration of test drugs and standard. In order to minimize the damage on the animal paws, the cut-off time was taken as 25 seconds.

In the hot plate test there were no significant differences in the antinociceptive effect of 200 and 400 mg/kg in the mean±SEM value for nociception. The antinociceptive effect of EEGO was found dependent. Administration of Morphine at a dose of 5 mg/kg demonstrated a significant antinociceptive effect when compared with control group.

Data Analysis of Extract

The Analgesic Activity Tests results by Hot Plate Test are given in the following Tables

Table 4.4: Primary Data Table for Hot Plate Test for Leaf Extract 0f *Typhonium trilobatum*

Group	Time (min)	Tolerance Time						Mean	SD	SEM
		1M	2M	3M	4M	5M	6M			
Control	**0**	15.00	5.57	5.35	8.02	5.69	5.33	7.49	3.48	0.70
	30	9.30	7.23	14.09	8.48	10.36	12.30	10.29	2.31	0.46
	60	14.69	13.45	3.27	3.37	2.83	10.37	7.99	5.01	1.00
	90	15.33	8.16	11.74	8.23	5.65	8.85	9.66	3.09	0.62
	120	14.85	10.39	14.02	15.89	7.97	7.99	11.85	3.22	0.64
Positive Control	**0**	12.01	14.56	15.04	7.63	9.00	11.98	11.70	2.69	0.52
	30	19.83	18.04	20	20	18.83	13.45	18.36	2.31	0.46
	60	14.66	7.96	7.37	20	16.55	16.46	13.83	4.64	0.93
	90	15.51	10.31	9.82	20	11.00	19.83	14.41	4.31	0.86
	120	20	8.99	17.46	20	16.87	12.81	16.02	3.96	0.79
Group-1	**0**	9.88	11.02	8.88	5.58	6.00	18.03	9.9	4.13	0.85
	30	4.21	2.00	12.07	5.68	7.00	5.02	5.99	3.11	0.62
	60	7.67	10.31	7.00	6.00	8.00	7.31	7.71	1.32	0.26
	90	16.03	5.28	6.46	12.01	11.81	19.03	11.77	4.85	0.97
	120	20	13	15.27	6.88	17.63	16.61	14.9	4.17	0.83
Group-2	**0**	14.22	19.83	15.47	18.09	20.00	18.63	17.70	2.16	0.43
	30	19.88	19.88	10.02	12.04	12.31	13.31	14.57	3.88	0.78
	60	15.81	16.71	13.41	20.00	13.87	15.63	15.90	2.16	0.43
	90	18.71	18.05	12.02	13.44	19.11	13.00	15.72	2.95	0.59
	120	19.00	19.87	19.68	20.00	9.88	6.67	15.85	5.44	1.09

Control: 1%tween 80, 10ml/kg, **Positive Control:** Morphine (5 mg/kg),

Group-1: Extract (200 mg/kg), **Group-2:** Extract (400 mg/kg) , N=6

Table 4.5: Secondary Data Table for Hot Plate Test for Leaf Extract

Test Group	Dose mg/kg	0 min	30 min	60 min	90 min	120 min
Control	1	7.49±0.70	10.29±0.46	7.99±1.00	9.66±0.62	11.85±0.64
Positive Control	5	11.70±0.52	18.36±0.46	13.83±0.93	14.41±0.86	16.02±0.79
Group-1	200	9.9±0.85	5.99±0.62	7.71±0.26	11.77±0.97	14.9±0.83
Group-2	400	17.70±0.43	14.57±0.78	15.90±0.43	15.72±0.59	15.85±1.09

Values are expressed as Mean ± SEM, (n=6)

Table 4.6: Percentage of Inhibition of temperature tolerance by mice

Substances	% of Inhibition		
	30 min	60 min	90 min
Control	0.00	0.00	0.00
Positive Control	71.8	73.02	45.06
Group-1	35.67	35.76	25.44
Group-2	41.67	98.99	33.75

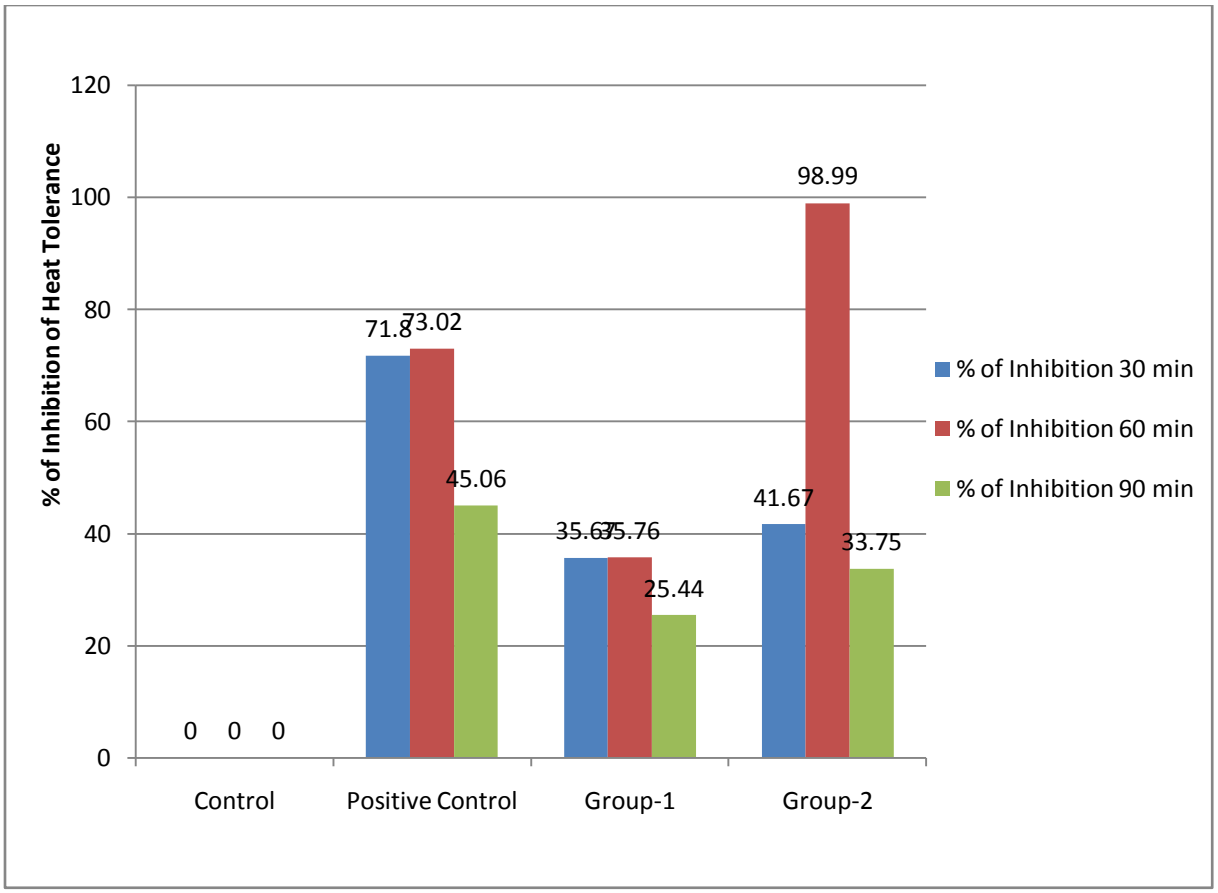

Figure 4.6: Graphical presentation of the effect of Leaf extract of *Typhonium trilobatum* on mice by Hot Plate Test.

4.3 Investigation of analgesic activity by formalin induced licking test

The results show that the Chloroform extract from *Typhonium ttrilobatum* (200 mg/kg and 400 mg/kg orally) caused a significant dose dependent inhibition of both the neurological (0-5 min.) and inflammatory (15-30 min.) phase of formalin induced licking.

Table 4.7 : Effect of leaf extract of Typhonium trilobatum on Formalin Induced licking test

Group	Phases	Licking Counting						Mean	% of inhibition	SD	SEM
		M 1	M 2	M 3	M 4	M 5	M6				
Control	Phage-1	120	112	115	118	121	117	117.2	0	3.7	0.74
	Phage-2	99	94	97	96	97	97	96.60	0	1.81	0.36
Positive control	Phage-1	40	41	39	41	37	39	39.6	66.00	1.67	0.33
	Phage-2	0	0	0	0	0	0	0	100	0	0
Group-1	Phage-1	70	71	73	75	79	73	73.60	37.20	3.58	0.72
	Phage-2	0	0	0	0	1	0	0.20	99	0.45	0.09
Group-2	Phage-1	45	46	46	41	40	43	43.60	62.28	2.88	0.58
	Phage-2	0	0	0	0	0	0	0	100	0	0

Control: 1% Tween 80 (10 ml/kg), **Positive Control:** Morphine (5 mg/kg),

Group-1: Extract (200 mg/kg), **Group-2:** Extract (400 mg/kg) , N=6

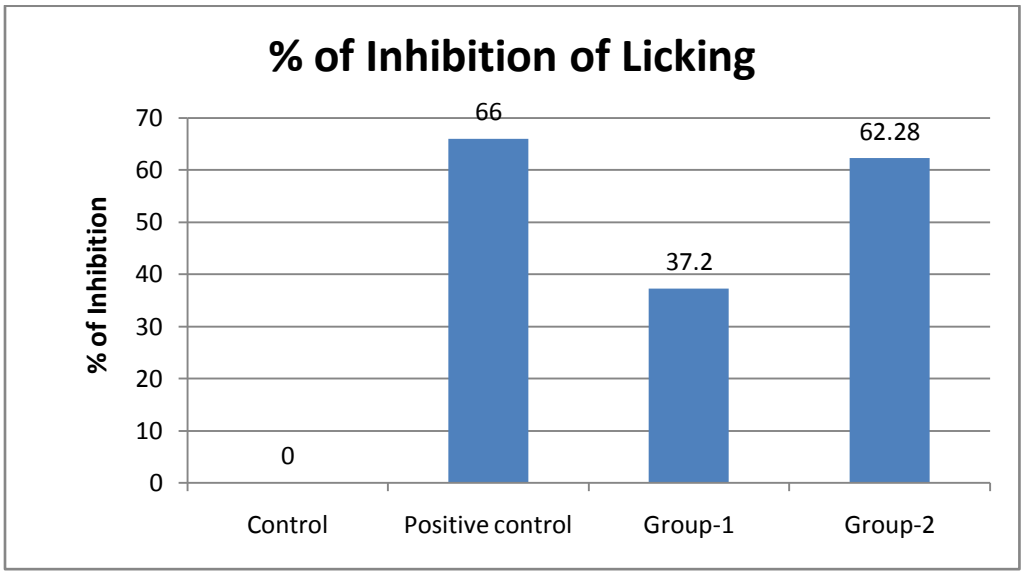

Fig 4.7 Graphical presentation of the effect of leaf extract of *Typhonium trilobatum* on mice by formalin induced licking test.

Table 4.8: Effect of tuber extract of *Typhonium trilobatum* on Formalin Induced licking test

Group	Phases	Licking Counting						Mean	% of inhibition	SD	SEM
		M 1	M 2	M 3	M 4	M 5	M 6				
Control	Phage-1	120	112	115	118	121	117	117.2	0	3.7	0.74
	Phage-2	99	94	97	96	97	97	96.6	0	1.81	0.36
Positive control	Phage-1	40	41	39	41	37	39	39.6	66	1.67	0.33
	Phage-2	0	0	0	0	0	0	0	100	0	0
Group-1	Phage-1	71	65	62	68	64	66	66.00	43.69	3.54	0.71
	Phage-2	2	1	0	2	3	1	1.40	98.55	1.14	0.23
Group-2	Phage-1	49	43	48	45	47	46	46.4	60.41	2.41	0.48
	Phage-2	0	1	2	3	0	1	1.20	98.75	1.30	0.26

Control: 1% Tween 80 (10 ml/kg), **Positive Control :** Morphine (5 mg/kg),

Group-1: Extract (200 mg/kg), **Group-2:** Extract (400 mg/kg)

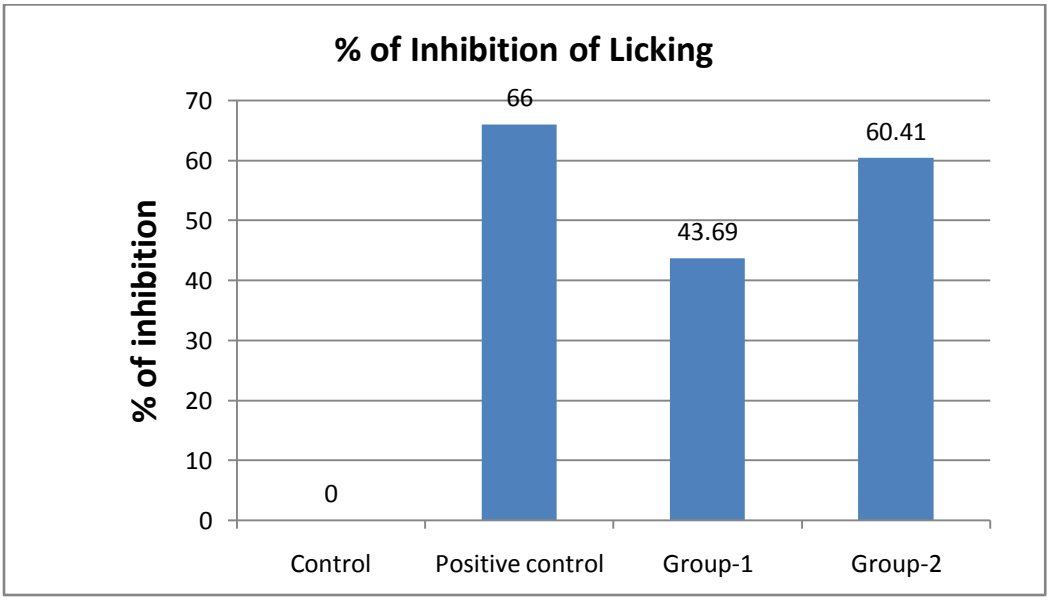

Fig 4.8 Graphical presentation of the effect of tuber extract of *Typhonium trilobatum* on mice by formalin induced licking test.

Table 4.9: Effect of leaf extract (pet ether fraction) of *Typhonium trilobatum* on Formalin Induced licking test

Group	Phases	Licking Counting						Mean	% of inhibition	SD	SEM
		M 1	M 2	M 3	M 4	M 5	M 6				
Control	Phage-1	120	112	115	118	121	117	117.2	0	3.7	0.74
	Phage-2	99	94	97	96	97	97	96.6	0	1.81	0.36
Positive control	Phage-1	40	41	39	41	37	37	39.6	66	1.67	0.33
	Phage-2	0	0	0	0	0	0	0	100	0	0
Group-1	Phage-1	50	53	48	47	47	48	49	58.19	2.55	0.51
	Phage-2	2	0	0	0	0	1	0.5	99.59	0.89	0.18
Group-2	Phage-1	38	41	43	40	37	39	39.8	66.04	2.39	0.48
	Phage-2	2	1	0	1	0	0	0.66	99.17	0.84	0.17

Control: 1% Tween 80 (10 ml/kg), **Positive Control :** Morphine (5 mg/kg),

Group-1: Extract (200 mg/kg), **Group-2:** Extract (400 mg/kg)

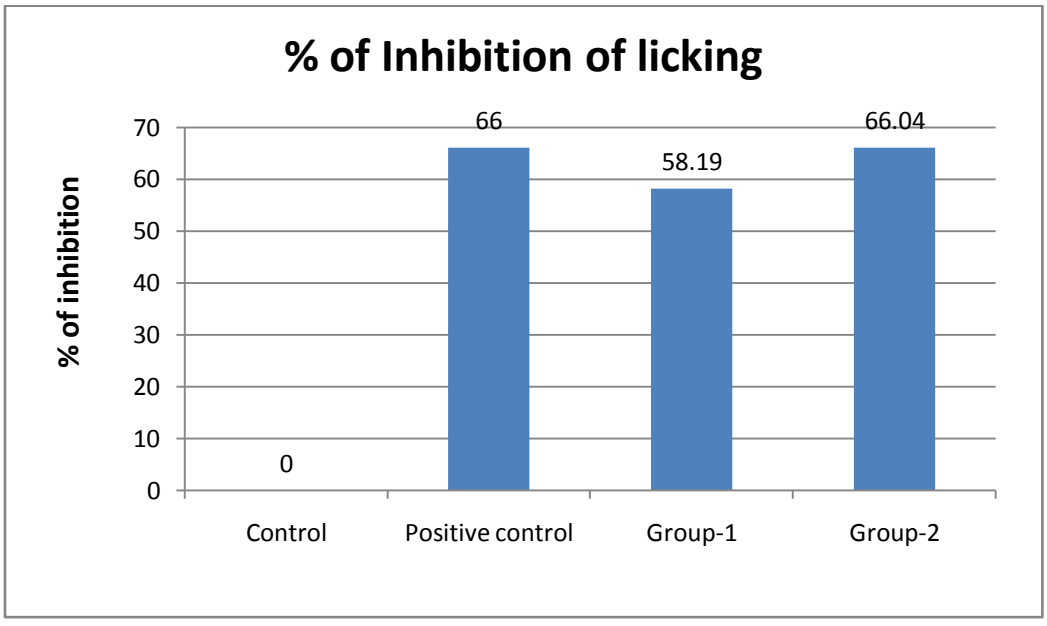

Fig 4.9 Graphical presentation of the effect of pet ether fraction of leaf extract of *Typhonium trilobatum* on mice by formalin induced licking test.

4.4 Investigation of cytotocix activity by brine shrimp lethality bioassay

Pharmacology is simply toxicology at a lower dose, and toxicology is simple pharmacology at a higher dose. Upon being placed in sea water the eggs hatched within 48 hours to provide large number of larvae (nauplii) for experimental use. After 24 hours of inoculation of brine shrimps in test tubes containing respective concentration of extracts survivors are counted. These data are processed to estimate LC_{50} values with 95% confidence intervals for satistically significant comparisons of potencies. Researcher have observed that ED_{50} values for cytotoxicities are generally about one-tenth of the LC_{50} values found in the brine shrimp tests (Jerry *et al.*, 1999).

Table 4.10 Effect of leaf extract on brine shrimp lethality bioassay

Concentration (µg/ml)	Total Population	Survivors	Dead	Mortality %
320	10	5	5	50
160	10	5	5	50
80	10	7	3	30
40	10	7	3	30
20	10	8	2	20
10	10	8	2	20
5	10	8	2	20
2.5	10	8	2	20
1.25	10	10	0	0

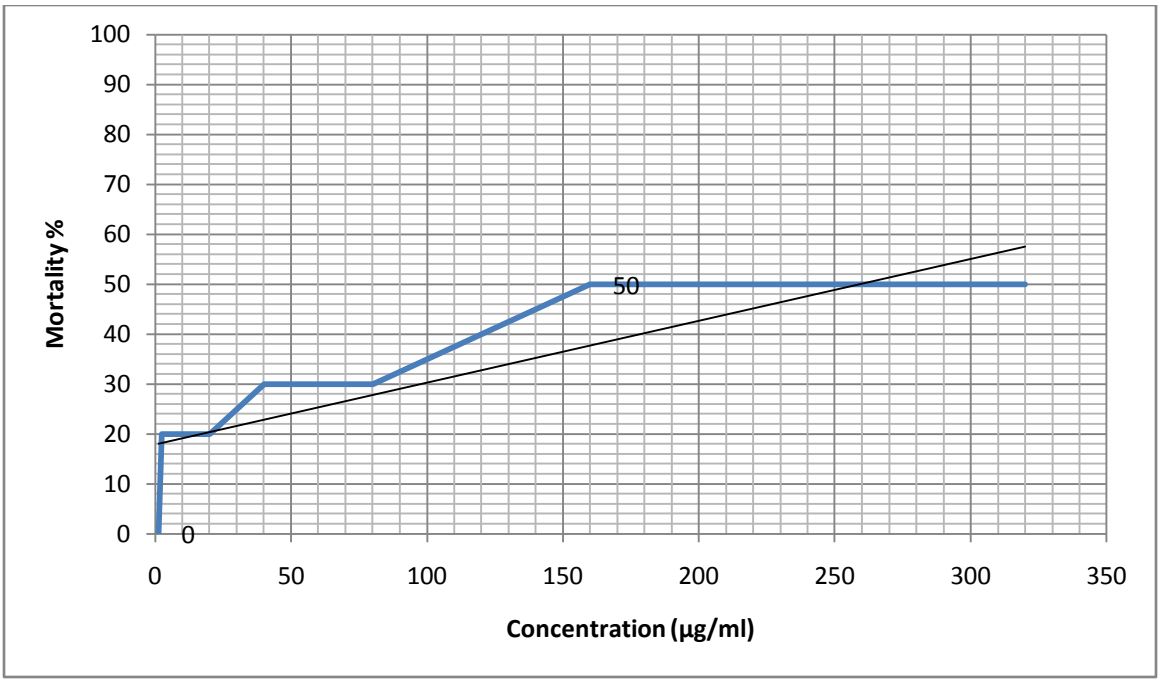

Figure 4.10 Graphical presentation of mortality by the leaf extract on brine shrimp lethality bioassay.

Table 4.11 Effect of tuber extract on brine shrimp lethality bioassay

Concentration (µg/ml)	Total Population	Survivors	Dead	Mortality %
320	10	3	7	70
160	10	5	5	50
80	10	5	5	50
40	10	6	4	40
20	10	6	4	40
10	10	6	4	40
5	10	7	3	30
2.5	10	7	3	30
1.25	10	8	2	20

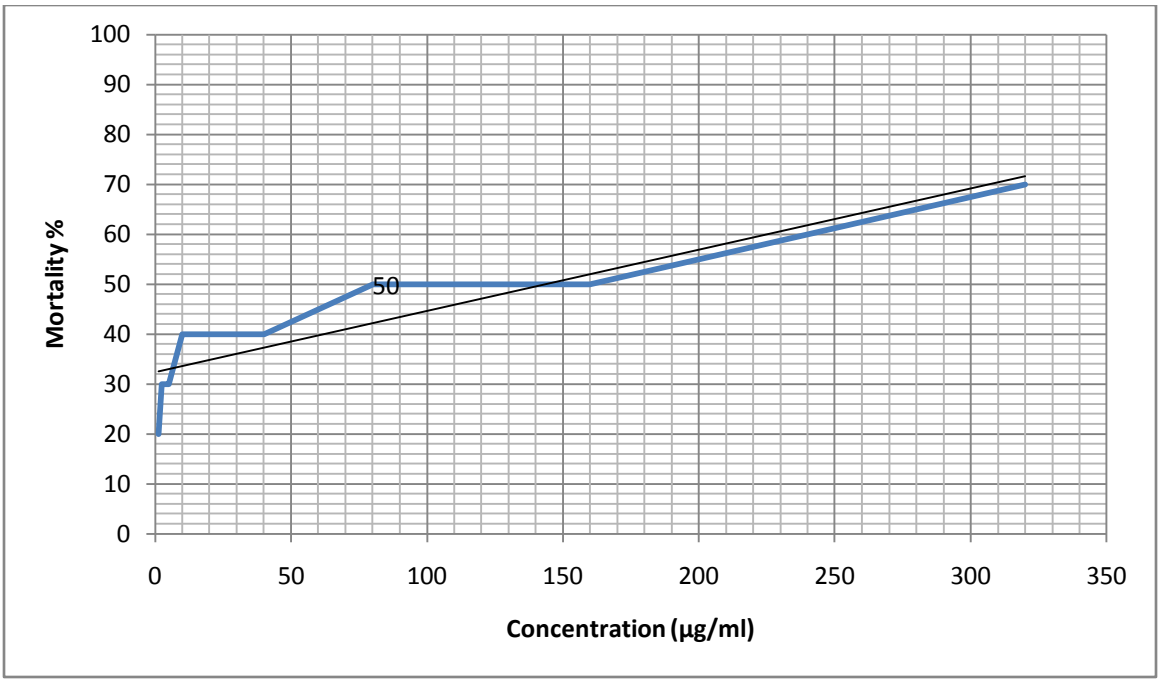

Figure 4.11 Graphical presentation of mortality by the tuber extract on brine shrimp lethality bioassay.

Table 4.12 Effect of pet ether fraction of leaf extract on brine shrimp lethality bioassay

Concentration (µg/ml)	Total Population	Survivors	Dead	Mortality %
320	10	7	3	30
160	10	7	3	30
80	10	7	3	30
40	10	8	2	20
20	10	8	2	20
10	10	9	1	10
5	10	9	1	10
2.5	10	9	1	10
1.25	10	10	0	0

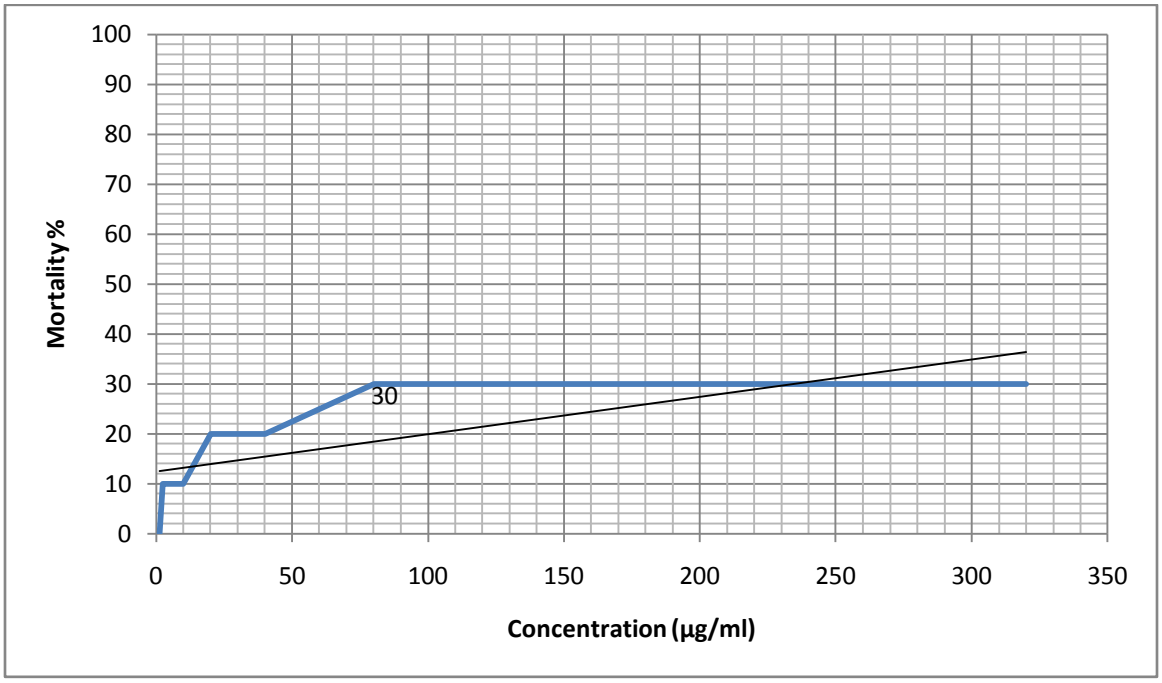

Figure 4.12 Graphical presentation of mortality by the pet-ether fraction of leaf extract on brine shrimp lethality bioassay.

5. DISCUSSION

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

5.1 Acetic acid induced writhing test

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

The plant *Typhonium trilobatum* caused dose dependent analgesic against chemical induced pain in mice. The chloroform extract of *Typhonium trilobatum* at the dose of 200 mg/kg and 400 mg/kg body weight was found exhibit writhing response inhibitory effect. The abdominal constriction is related to the sensitization on antinoceptive receptors to prostaglandins. The extract produced analgesic effect which may be due to the inhibition of synthesis of prostaglandin. *Typhonium trilobatum* contains alkaloid which may be responsible for the analgesic activity (Khadem *et al*., 2012). The percentages of inhibition are 67.55%, 53.78% and 50.67% for the leaf, tuber and pet ether fraction of leaf.

5.2 Hot Plate Test

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

The hot plate tests are widely used for assessing central analgesic activities. Aroids agents exhibit their analgesic effects both via supra spinal and spinal receptors. In this study, the *T. trilobatum* exhibited good analgesic activity compared to morphine in hot plate test. The doses were 200 mg/kg and 400 mg/kg of chloroform extract of leaf of *T. trilobatum.* The rate was then compared to the control group in hot plate models. In hot plate test, the result was not so significant for 200 mg/kg dose of leaf extract, but for 400 mg/kg dose, the result was significant compared to the control.

5.3 Formalin Induced Licking Test

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

In formalin-induced licking behavior was significantly attenuated by the plant at a dose of 200 and 400 mg/kg, compared with control. The licking behaviour induced by the injection of formalin in the late phase was more actively and significantly attenuated by plant extract at all test doses (200 and 400 mg/kg). (Khadem *et al.,* 2012).

5.4 Cytotoxic Activity

The degree of lethality shown by the extract was found to be directly proportional to the concentration of the extracts ranging from the lowest concentration (1.25 µg/ml) to the highest concentration (320 µg/ml). This concentration dependent increment in percent mortality of brine shrimp nauplii produced by extract indicates the presence of cytotoxic activity in the plant.

There is no mortality in the negative control groups indicating the test as a valid one and the result obtained are only due to the activity of the test agents. (Jerry *et al*., 1998).

5.5 Conclusion

Based on the result of present study, it can be concluded that the leaves and tuber parts of chloroform extract of *Typhonium trilobatum* possesses remarkable analgesic and cytotoxic effects. These compounds may be responsible for the bioactivities. Since the extract is reported to contain a myriad array of compounds, it is difficult to ascribe these observed activities to any specific group of compounds. Hence, further studies are suggested to be undertaken to pinpoint the exact compound(s) and to better understand the mechanism of such actions scientifically.

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Appendix

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

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

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

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

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

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

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

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