Studies of the effects of *Solanum sisymbriifolium* leaf on brine shrimp and different animal models

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A thesis report, submitted to the Department of Pharmacy, East West University, in partial fulfillment of the requirements for the degree of Bachelor of Pharmacy

ENDORSEMENT BY CHAIRPERSON

This is to certify that the dissertation entitled

"Studies of the effects of *Solanum sisymbriifolium* leaf on brine shrimp and different animal models"is a bonafide research work done by **Md. Shakhawat Hossan Bhuyan** under the guidance of **Mr. Apurba Sarker Apu,** Senior Lecturer, Department of Pharmacy, East West University, Dhaka.

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This is to certify that the dissertation entitled

"Studies of the effects of *Solanum sisymbriifolium* leaf on brine shrimp and different animal models"is a bonafide research work done by **Md. Shakhawat Hossan Bhuyan**, in partial fulfillment of the requirement for the Degree of Bachelor of Pharmacy.

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DECLARATION BY THE CANDIDATE

I hereby declare that this dissertation entitled

"Studies of the effects of *Solanum sisymbriifolium* leaf on brine shrimp and different animal models" is an authentic and genuine research work carried out by me under the guidance of **Mr. Apurba Sarker Apu,** Senior lecturer, Department ofPharmacy, East West University, Dhaka, Bangladesh.

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Dedicated

To

My Loving Parents

And

Research Supervisor

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ABSTRACT

Purpose: The purpose of this study was to evaluate the analgesic, neurophrmacological, anti-diarrheal and cytotoxic activity of the crude ethanolic leaf extract of *Solanum sisymbriifolium* and its *n*-hexane, chloroform, carbon tetrachloride and aqueous fractions.

Methods: The ethanolic extract was used to evaluate analgesic, neurophrmacological, and anti-diarrheal activities. The analgesic activity was measured by acetic acid induced writhing test. The neurophrmacological activity was evaluated by hole cross, hole board and elevated plus-maze test and the anti-diarrheal activity was assessed by castor oil induced anti-diarrheal activity test. Brine shrimp lethality bioassay was carried out for assessing the cytotoxicy of the crude extract and its various fractions. Data were analyzed by using SPSS.

Results: The extract showed highly significant ($p<0.001$) analgesic activity with % inhibitions of writhing response at doses 200 and 400 mg/kg were 33.72% and 94.40% respectively. The extract at low to medium dose showed significant ($p<0.05$) sedative effect while significant ($p<0.05$) CNS stimulatory effect was showed at higher doses in hole cross test. In hole board test, the extract showed highly significant ($p<0.001$) anxiolytic activity but result of elevated plus maze test was not statistically significant. The extract showed highly significant ($p<0.001$) anti-diarrheal activity. In brine shrimp lethality test, among the fractions chloroform fraction showed highest cytotoxic activity with LC_{50} value 13.97 µg/ml.

Conclusion: The results of the study clearly indicate the present of analgesic, neuropharmacological, anti-diarrheal and cytotoxic properties of the extract and its fractions.

Keywords: *S. sisymbriifolium*, analgesic, Hole cross, Hole board, Elevated plus-maze, anti-diarrheal, brine shrimp lethality bioassay.

Chapter: 1

INTRODUCTION

1. INTRODUCTION

For centuries, humankind has used extracts from plant sources or animal organs for the treatment of various diseases. Plants have always have been a good source of biologically active compounds or lead compounds (e.g. morphine, digitalis, quinine, nicotine and muscarine) (Patrick and Spencer, 2011). Many of these lead compounds are useful drugs in themselves (e.g. morphine and quinine) and others have been the basis for synthetic drugs (e.g. local anesthetics developed from cocaine) (Patrick and Spencer, 2011). Clinically useful drugs which have recently been isolated from plants including the anticancer agent paclitaxel from the yew tree, antimalarial agent artemisinin from a Chinese medicinal herb *Artemisia annua* (wormwood) (Patrick and Spencer, 2011) and vinblastin from *Catharanthus rosesus* (Farnsworth *et al.*, 1967). Moreover, many of antibiotics, vitamins and hormones are used today resulted from purification of such extracts and isolation and identification of their active principles (Korolkovas, 1988).

A medicinal plant is any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes, or which are precursors for chemo-pharmaceutical semi-synthesis (WHO, 2001).

Plant medicines are the most widely used medicines in the world today. Approximately, half of the world's 25 best-selling pharmaceutical agents are derived from plant origin (Arvigo and Balick, 1993). The World Health Organization (WHO) estimated that 80% of the population of developing countries still relies on traditional medicines, mostly plant drugs for their primary health care needs (UNDP, 1999). In a study it has been shown that about 74% of 119 plant-derived pharmaceutical medicines or biotechnology medicines are used in modern medicine in ways that correlate with their traditional use (Barrett *et al.*, 1999). Thus the use of traditional

medicine has become increasingly popular worldwide and medicinal plants are believed to be an important source of new chemical substances with potential therapeutic effects.

Bangladesh is a South Asian country where only 20% of the total populations are provided with modern healthcare services while the rest are dependent on traditional plant-based systems. Approximately 5,000 species of indigenous and naturalized phanerogamic and pteridophytic plants are growing in the country (Mia, 1990). But it is estimated that only 500 medicinal plant species had been recorded in Bangladesh out of approximately 1,900 species regarded as having medicinal value (Guerrero *et al*., 2004). So it is believed that many other medicinal plants are still there which have not yet been discovered or identified (Yusuf *et al.*, 1994).

Our planet shelters approximately 600,000 vegetables species, but less than 10% of them have been studied scientifically under the chemical and pharmacological aspects (Korolkovas, 1988). So research on plants based on their traditional uses has great importance in drug discovery as there is high scope for exhaustive studies to identify new medicinal plants and isolate active principle from them for threatening various diseases like HIV and cancer.

Pharmacology is the study of the therapeutic value and/or potential toxicity of chemical agents on biological systems. It targets every aspect of the mechanisms for the chemical actions of both traditional and novel therapeutic agents (Katzung, 2004). In its entirety, pharmacology embraces knowledge of the sources, chemical properties, biological effects and therapeutic uses of drugs. Pharmacological studies range from those that examine the effects of chemical agents on subcellular mechanisms, to those that deal with the potential hazards of pesticides and herbicides, to those that focus on the treatment and prevention of major diseases with drug therapy (Katzung, 2004).

For the success of a drug research program, choosing the right bioassay or test system in order to check different pharmacological activities of drug and plant extract is crucial. For this different *in vitro* (i.e. on isolated cells, tissues, enzymes or receptors) and/or *in vivo* (on animal) assays are usually carried out (Patrick and Spencer, 2011). *In vivo* assays are usually carried out to test whether the drug or plant extract has desired pharmacological activity. *In vivo* tests on animals often involve inducing a clinical condition in the animal to produce observable symptoms. The animal is then treated to see whether the drug alleviated the problem by eliminating the observed symptoms. Transgenic animals are often used in *in vivo* testing (Patrick and Spencer, 2011).

1.1 Plant Profile

Solanum sisymbriifolium is a traditional medicinal plant of the Solanaceae family.

1.1.1 Common name

Solanum sisymbriifolium (*S. sisymbriifolium*) from family Solanaceae is commonly known as Kanta begun, kantikari (bengla), Karnophuli (Chakma); Dense-thron bitter-apple, Red buttalo-bur, Sticky nightshade, Viscid nightshade (English); Doringtamatie, Wildetamatie (Afrikaans); Harinasubi (Japanese); Alco-chileo, Espina colorada, Mullaca espinudo, Ocote mullaca, Revienta caballo, Tomatillo, Tutia (Spanish); Jeweelie (Argentina); JoÃ£o bravo, Jua das queimadas (Portuguese); Jua de roca (Portuguese); Morelle de balbis (French); Raukenblatt-nachtschatten (Austria); Red buffalo-burr (British Isles); Blek taggborre (Swedish); Klebriger nachtschatten (German).

1.1.2 Taxonomic classification of *S. sisymbriifolium*

Kingdom	Plantae
Subkingdom	Tracheobionta

Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Sub class	Asteridae
Order	**S**olanales
Family	Solanaceae
Subfamily	*Solanoideae*
Tribe	*Solaneae*.
Genus	*Solanum*
Subgenus	*Leptostemonum*
Species	*Sisymbriifolium*
Botanical name	*Solanum sisymbriifolium* Lam.

1.1.3 Description

A viscoid and very prickly erect herb or under shrub(figure 1.1.3.1) up to 1.2 m tall with spreading branches, covered with stellate hairs. Prickles straight, yellowish, up to 0.7 cm or longer(Uddin,2010). Flowers (figure 1.1.3.2) up to 2 cm across. Calyx prickly, lobes narrowly triangular, much enlarged in fruit (up to 2 cm). Corolla up to 2 cm long, white to bluish-white. Filaments c. 2cm long, flattened, anthers c. 1 cm long, linear. Ovary and style glabrous. Stigma slightly 2-fid. Berry 1-2 cm across, globose, glabrous, scarlet, covered by enlarged and reflexed calyx lobes. (Uddin,2010).

Branchlets

Branchlets (figure 1.1.3.3) brown or green. Branchlet prickles 30–60 per decimetre, straight, broad-based or curved, broad-based, 1–12 mm long, 5–7 times longer than wide, glabrous. Branchlet hairs simple or stellate. Branchlet stellae dense, 0.3–0.5 mm diameter (Bean, 2012).

Branchlet stellae lateral rays 6–12, ascending or multiradiate. Branchlet stellae central ray 1.5–3 times as long as laterals, not gland-tipped or gland-tipped. Branchlet simple hairserect, straight, gland-tipped, 0.2–0.4 mm long (Bean, 2012).

Leaves

Leaves(figure 1.1.3.4) are ovate or broadly ovate, deeply lobed throughout. Leaf lobes are 4–6 on each side, acute and lobing index 2.5–11. Leaves are usually 8–12 cm long, 5.5–8.5 cm wide, and 1.4–1.8 times longer than broad (Bean, 2012). Leaf apex acute, base obtuse or cordate. Leaf oblique part 0–5 mm long, obliqueness index 0–5 percent. Petioles 2.1–4.6 cm long, 21–40% length of lamina, winged, and prickles present (Bean, 2012)

Upper leaf surface

Upper leaf surface (figure 1.1.3.4) green. Upper leaf surface prickles present on midvein and lateral veins, 9–30, straight, broad-based, 1–10 mm long. Upper leaf surface stellate hairs distributed throughout, hair density sparse to moderate density (Bean, 2012). Upper leaf surface stellae 0.3–0.5 mm apart, 0.3–0.5 mm across, stalks 0 mm long. Upper leaf surface stellae lateral rays 4–6, ascending. Upper leaf surface stellae central ray 1–3 times as long as laterals, not gland-tipped. Upper leaf surface simple hairs erect, straight, sparse, gland-tipped, 0.2–0.3 mm long. Upper leaf surface Type 2 hairs absent (Bean, 2012).

Lower leaf surface

Lower leaf surface (figure 1.1.3.4)green. Lower leaf surface prickles present on midvein and lateral veins, 7–20, straight, broad-based. Lower leaf surface stellate hair density moderate. Lower leaf surface stellae 0.3–0.6 mm apart, 0.4–0.6 mm diameter, stalks 0 mm long. Lower leaf

surface stellae lateral rays 4–7, ascending (Bean, 2012). Lower leaf surface stellae central ray 1–2 times as long as laterals, not gland-tipped. Lower leaf surface simple hairs erect, straight, sparse or moderate to dense, gland-tipped, 0.2–0.3 mm long. Lower leaf surface Type 2 hairs absent (Bean, 2012).

Inflorescence

Inflorescence supra-axillary, cymose (pseudo-racemose). Inflorescence common peduncle 15–52 mm long. Inflorescence rachis prickles present. Inflorescence 4–11-flowered, with some bisexual and some male flowers. Flowers 5-merous, all similar. Pedicels at anthesis 5–12 mm long, same thickness throughout, 0.5–0.7 mm thick at mid-point, prickles present. Calyx tube at anthesis 1.5–3 mm long (Bean, 2012). Calyx lobes at anthesis deltate, 5–6.5 mm long. Calyx prickles at anthesis present, 25–90 per flower, 1–7 mm long. Calyx stellae dense, transparent, 0.4–0.6 mm across, stalks 0 mm long, lateral rays 6–10. Calyx stellae central ray 2–3 times as long as laterals, not gland-tipped or gland-tipped. Calyx simple hairs present, Type 2 hairs absent. Corollawhite, 11–16 mm long, rotate or shallowly lobed, inner surface glabrous. Anthers8–10 mm long. Ovary with Type 2 hairs only. Functional style 15.5–17 mm long, protruding between anthers, with Type 2 hairs only or with stellate and Type 2 hairs. Functional style stellae c. 0.25 mm across, lateral rays c. 6, central ray 2–3 times as long as laterals (Bean, 2012).

Fruits

Fruiting (figure 1.1.3.6) calyx lobes more than half length of mature fruit, prickles 2–10 mm long. Mature fruits 1–3 per inflorescence, globular, 15–20 mm diameter, red. Mature fruits interior juicy, succulent (symon). Pedicels at fruiting stage 17–25 mm long, 1.1–1.9 mm thick at mid-point. Seeds pale yellow, 2.9–3.2 mm long (Bean, 2012).

1.1.4 Geographical distribution

S. sisymbriifolium is native to South America (Argentina, Brazil, Peru and Uruguay) (GRIN, 2007). But it is also distributed in North America (Canada, Mexico, the United States), Europe (Spain, the Netherlands), Asia (Bangladesh, India, China, Taiwan), Africa (South Africa, Congo, Swaziland), and Australasia (Australia, New Zealand).

Figure 1.1.3.1*:S. sisymbriifoliu*

Figure 1.1.3.2:Flowers of *S. sisymbriifolium*

***Figure 1.1.3.3*:** Branchlets of *S. sisymbriifolium*

Upper leaf surface Lower leaf surface

Figure 1.1.3.4:Leaves of *S. sisymbriifolium*

***Figure1.1.3.5*:**Fruits of *S. sisymbriifolium*

1.1.5 Habitat description

S. sisymbriifolium is found along roadsides and in waste places, landfills, and plowed fields both in its native South America (Hill and Hullley, 1995) as well as most of its nonnative range. In Australia it is found in shrubby eucalypt woodlands (Bean, 2012). It is able to succeed in any type of soil and soil pH, but requires moisture and thrives in peat and sandy soils. It is tolerant of low-light situations (PCN Control Group, 2004)

1.1.6 Reproduction

Sexual reproduction resulting in seeds is the predominant means of reproduction for *S. sisymbriifolium*(Hill and Hulley, 1995), but the species may also reproduce asexually by the growth of its rhizomes (Bean, 2004). It is believed to be self-incompatible (D'Arcy, 1974).

1.1.7 Lifecycle stages

When planted in the field, *S. sisymbriifolium* germinates in 2-4 weeks. It may grow slowly for the first 4-6 weeks, but growth following that period can be vigorous (PCN Control Group, 2004).

1.1.8 Chemical constituents of *S. sisymbriifolium*

Phytochemical studies

Phytochemical studies of different parts of the plant indicate the presence of alkaloids, flavonoids, steroids and tannins (Shilpi *et al.*, 2005).

Roots

Several alkaloids have been isolated from the roots, such as cuscohygrine (Evans and soman-abandhu, 1980) solacaproine (Maldoni, 1984), solamine, solasodiene and solasodine (Mazumdar, 1984). Isonuatigenin-3-*O*-β-solatriose, a steroidal saponin was also isolated from the roots of *S. sisymbriifolium* (Ferro *et al.*, 2005).

Fruits

Lignans (sisymbrifolin) and a C_{30} sterol (carpesterol) were isolated from the fruits of *S.sisymbriifolium* (Chakravarty *et al.*, 1996). Solasodine, a poisonous steroidal glycoalkaloid chemical compound, was also isolated from the dried fruits (Chauhan *et al.*, 2010).

Aerial part

The aerial part of the plant produces spirostane derivatives (Chakravarty *et al.*,1996).

1.1.9 Uses of *S sisymbriifolium* in traditional medicine

The plant is used in the treatment of hysteria, remitting fever, and stomachache (Uddin,2010).

Roots

The roots of *S. sisymbriifolium* are used in the treatment of hypertensive diseases in Paraguay and as diuretic, analgesic, contraceptive, antisyphilitic and hepatoprotective in Argentine.The roots have been reported as an emenagogue and fertility Argentine (Ferro *et al.*, 2005).The roots are also resistant to a number of strains of the bacterica wilt pathogen *Pseudomonas solanacearum* (Hill and Hulley, 1995).

Fruits

The fruits of *S. sisymbriifolium* are a source of solasodine, a glycoalkaloid used in the synthesis of corticosteroids and sex hormones, and a large component of oral contraceptives. The fruits are edible and are consumed regularly by indigenous birds (Hill and Hulley, 1995). Unripe fruit is used as a vegetable (Uddin,2010).

Flowers

Flowers are used in India as analgesic (Ferro *et al.*, 2005).

Leaves

Leaves are used as febrifuge in Peruand as diuretic in Brazil (Ferro *et al.*, 2005).

Aerial parts

Aerial parts are used in Argentine to treat diarrhea, infections of respiratory and urinary tracts (Ferro *et al.*, 2005).

1.2 Acetic Acid Induced Writhing Test

Pain is probably the most prevalent symptom in clinical practice, and characterization of pain is of major importance in the diagnosis and choice of treatment (Thumshirn *et al*., 1999). In the treatment of diseases associated with pain, the clinical effects typically guide the selection of the analgesics and titration of the dose. Several tests are available to evaluate the analgesic activities of drugs and plant extracts such as hot plate method, acetic acid induced writhing method, formalin induced pain method, tail flick method, and tail immersion method. Among them the acetic acid induced writhing test is more popular.

The acetic acid –induced writhing test in mice attributed visceral pain finds much attention of screening analgesic drugs (Hasan *et al*., 2010). This test is very sensitive and able to detect anti-nociceptive effects of compounds at dose levels that may appear inactive in other methods like tail flick test (Collier *et al*., 1968).Several researchers (Zulfiker *et al*., 2010; Podder *et al*., 2011)have used the method to identify the non-narcotic analgesic property of plant extracts asthe acetic acid induced writhing is inversely proportional to the non-narcotic analgesic property.

Pain sensation in acetic acid induced writhing method is elicited by triggering localized inflammatory response resulting release of free arachidonic acid from tissue phospholipid (Ahmed *et al*., 2006) via cyclooxygenase (COX), and prostaglandin biosynthesis (Duarte *et al*., 1988). In other words, the acetic acid induced writhing has been associated with increased level of prostanoids, particularly PGE_2 and $PGF_{2\alpha}$ in peritoneal fluids as well as lipoxygenase products (Derardt *et al*., 1980). The increase in prostaglandin levels within the peritoneal cavity then enhances inflammatory pain by increasing capillary permeability (Zakaria *et al*., 2008). The acetic acid induced writhing method was found effective to evaluate peripherally active

analgesics. The agent reducing the number of writhing will render analgesic effect preferably by inhibition of prostaglandin synthesis, a peripheral mechanism of pain inhibition (Duarte *et al.*, 1988).

However, this model may not be able to indicate the mechanism of analgesic effect of the extract because other agents such as antihistamines (Naik *et al.*, 2000) and myorelaxant (Koyama *et al.*, 1997) are able to reduce the pain induced by acetic acid.

1.3 Evaluation of Neuropharmacological Activity

Brain function and nervous system are the most important aspects of physiology that define the difference between humans and other species. Disorders of brain function and nervous system (central and peripheral) due to improper balance in neurotransmitter levels, whether primary and secondary, to malfunction of other systems, are a major concern of human society and a field in which pharmacological intervention plays a key role (Katzung, 2004).

Depression and anxiety are the most frequent mental disorders. Depression and anxiety are defined as the response of a subject to real or particular threats that may impair its homeostasis, this response may include physiological or/and behavioral. More than 20% of the adult population suffers from these conditions at some time during their life. The World Health Organization (WHO) estimated that about 450 million people worldwide suffer from a mental or behavioral disorder (WHO, 2001). Majority of such patients in the developing countries still rely on traditional healing practices and medicinal plants for treatment of these conditions (Magaji *et al.*, 2009). The inclusion of herbal medicines of proven safety and efficacy in the healthcare programs of developing countries is encouraged by the WHO because of the great potential they

possess in combating various diseases (Amos *et al*., 2001). So, the medical need for newer, better-tolerated and more efficacious treatments remains high.

Several animal model tests are available to evaluate the neuropharmacological activities of drugs and plant extracts in mice such as the open field test, elevated plus-maze (EPM) test, light dark model, Y- maze test, forced swimming test, hexobarbitone sleeping time test, thiopental-sodium induced sleeping time test, ketamine induced sleep test, hole board test, hole cross test. All these procedures are based upon the exposure of subject to unfamiliar aversive place.

1.3.1 Hole Cross Test

The hole cross test is used to determine the CNS stimulatory or depressive effect of test drug. Increased movement indicates stimulatory activity and decreased movement indicates depressive activity.

Several researchers (Akter *et al*., 2011;Subhan *et al*., 2008; Takagi *et al*., 1971; Robbins *et al*., 1977) have used the method to evaluate the exploratory behavior of different plant extracts on the experimental animals.

1.3.2 Hole Board Test

The hole board experiment is a measure of exploratory behavior in animals (File and Wardil, 1975) and is an accepted parameter for evaluating anxiety, emotionality and/ or responses to stress conditions in animals (Crawley, 1985).The test has been conceived to study the behavior of the experimental animal confronted with a new environment (head plunging stereotype) according to the method devised by Boissier *et al*., 1964.

Hole-board model indicated that head-dipping behavior was sensitive to changes in the emotional state of the animal, and suggested that the expression of an anxiolytic state in animals may be reflected by an increase in head-dipping behavior (Takeda *et al.*, 1998).The test enables the initial exploratory activity of the animal and its variations brought about by psychotropic elements of a drug to be unmistakably assessed. Exploration can be defined as a broad category of behavior, the consequences of which are to provide the organism with information about the exteroceptive environment (Nakama *et al.*, 1972). The principle of the test is that a novel situation of open field evokes in the animals a pattern of behavior characterized by exploration (head dipping through the holes), locomotion (ambulation past the holes) and emotional defecation. It has been considered that the exploration evoked under an unfamiliar environment is modified with physiological factors such as curiosity, fear and anxiety and the modulation of these factors after the administration of a drug (Nakama *et al.*, 1972).

Several researchers (Ashish *et al.*, 2010; Akter *et al.*, 2011; Woode *et al.*, 2009; File and Wardil, 1975) have used the method to evaluate the exploratory behavior of different plant extracts on the experimental animals.

1.3.3 Elevated Plus-Maze Test

Anxiety, a symptom that accompanies various central nervous system disorders and is also a disorder in itself, is characterized in humans by a tense and exhaustive physical alertness (Yadav *et al.*, 2008). Anxiety disorder is increasingly recognized as a highly prevalent and chronic disorder with onset during the teenage years with an incidence of 18.1% and a lifetime prevalence of 28.8% (Kessler *et al.*, 2005). This disorder accompanies most of the clinical conditions including cardiovascular disorder, thyroid disorders and post-partum condition

(Chatterjee *et al.*, 2010). Thus this disorder is associated with significant disability which has a negative impact on the quality of life (Kasper, 1998).

The regulation of anxiety is associated with function of the $GABA_A$ receptor system. Available evidence points to a major role for α2-containing $GABA_A$ receptors in modulating anxiety (Griebel *et al.*, 2003), although a recent study also suggests a possible implication for α3 and α5 subunit (Navarro *et al.*, 2002).Standard drug diazepam act selectively on $GABA_A$ receptors which mediate fast inhibitory synaptic transmission throughout the CNS. Benzodiazepines (BDZs) bind to the γ sub-unit of the $GABA_A$ receptor that causes an allosteric (structural) modification of the receptor results with an increase in $GABA_A$ receptor activity (Rang *et al.*, 2003). BDZs do not substitute for GABA, which bind at α sub-unit, but increase the frequency of channel opening events which leads to an increase in chloride ion conductance and inhibition of the action potential (Rang *et al.*, 2003). Thus these drugs exert their anxiolytic effect.

Pharmacotherapeutic approaches for the management of anxiety disorders include psychotropic drugs, but these agents are limited by their side-effects profile, the need for dietary precautions, and drug interactions (Baldessarini, 2001). Currently the most widely prescribed medications for anxiety disorders are Benzodiazepines **(**Emamghoreishi *et al.*, 2005), but the clinical applications of Benzodiazepines as anxiolytics are limited by their unwanted side effects. Regular use of benzodiazepines causes deterioration of cognitive functioning, addiction, psychomotor impairment, confusion, aggression, excitement, physical dependence and tolerance (Suresh and Anupam, 2006).

Therefore the development of new pharmacological agents from plant sources is well justified (Emamghoreishi *et al.*, 2005) and the use of medicinal plants for the symptomatic treatment of

anxiety has increased significantly in recent years (Carlini *et al.*, 2006). The use of herbal medications by physicians in Europe and Asia is becoming more common and researchers are exploring the traditional remedies to find a suitable cure for these mind affecting diseases **(**Rabbani *et al.*, 2004).

Several tests are available to evaluate the anxiolytic activities of drugs and plant extracts such as Y – Maze test, Elevated plus –maze test, Hexobarbitone sleeping time test, Light-dark model, Water maze, Hole board , Forced swimming test (FST). Among them the elevated plus maze test is more popular.

The elevated plus maze is a well-established and most widely used animal model for testing anxiolytic drugs (Kulkarni and Reddy, 1996). It is sensitive to both anxiogenic and anxiolytic drugs (Belzung and Griebel, 2001). The test also served to evaluate learning and memory in mice.

Although original validation of the EPM was performed in rats (Broadhurst *et al.*, 1987), but it has also been found to be selectively sensitive to the effects of anxiolytic and anxiogenic drugs in mice (Lister, 1987). The important variables of the elevated plus-maze test are: time spends in open arm as well as the number of entries in to these arms.

There is great diversity in possible applications of the elevated plus maze. The test can be used in prescreening of newly developed pharmacological agents for treatment of anxiety-related disorders can be carried out. The anxiolytic and anxiogenic effects of pharmacological agents, drugs of abuse and hormones can be investigated. The effects of reproductive senescence/aging and/or pre-, peri- or postnatal exposure to various stressors can be assessed (Alicia and Cheryl, 2007). Furthermore, beyond its utility as a model to detect anxiolytic effects of benzodiazepine-

related compounds, the elevated plus-maze can be used as a behavioral assay to study the brain sites (e.g., limbic regions, hippocampus, amygdala, dorsal raphe nucleus, etc.5,6) and mechanisms (e.g., GABA, glutamate, serotonin, hypothalamic–pituitary–adrenal axis neuromodulators, etc.1,3,7–12) underlying anxiety behavior (Alicia and Cheryl, 2007).

The open arm–closed arm approach in elevated plus-maze test for screening for anxiolytic effect has worked well in identifying the anxiolytic potential of benzodiazepine/GABAA receptor related agents while not being reliable in detecting anti-anxiety effects through unrelated mechanisms, e.g. 5-HT_{1A} partial agonists like buspirone (Rodgers *et al.*, 1997).

Several researchers ((Akindele *et al.*, 2010; Chatterjee *et al.*, 2010; Rabbani *et al.*, 2004) have used the method to evaluate the exploratory behavior of different plant extracts on the experimental animals.

1.4 Castor Oil Induced Anti-Diarrheal Activity Test

Gastrointestinal diseases particularly constipation and diarrhea are affecting 70% of the population worldwide (Ouyang and Chen, 2004). Diarrhea is characterized by increased frequency of bowel movement, wet stool and abdominal pain (Ezekwesili *et al.*, 2004). It is one of the leading causes of malnutrition and death in developing countries and prevails as an ailment underlying 1.5–2 million deaths among children under 5 years of age (WHO, 2009). Many synthetic chemicals like diphenoxylate, loperamide and antibiotics are available for the treatment of diarrhea but they have some side effects (Singh *et al.*, 2005). So, medicinal plants are usually preferred to treat gastrointestinal disorders, for example, constipation and diarrhea, because they contain multiple constituents with effect-enhancing and/or side effect-neutralizing potential, and, hence are considered relatively safe in prolonged use (Gilani and Rahman, 2005).

A range of medicinal plants with anti-diarrheal properties is widely used by traditional healers. However, the effectiveness of many of these antidiarrheal traditional medicines has not been scientifically evaluated. The plant *S. sisymbriifolium* is a typical example of a plant-based remedy used to treat diarrhea (Ferro *et al.*, 2005).

Various tests that are available to evaluate the antidiarrheal activities of drugs and plant extracts such as castor oil- induced diarrhea and magnesium sulphate induced diarrhea. Between them the castor oil- induced diarrhea test is widely used as the castor oil is cheap and readily available.

Castor oil, which was used to induce diarrhea in mice, mixed with bile and pancreatic lipase enzymes and liberated its most active component ricinoleic acid from the triglycerides upon oral administration. The liberation of ricinoleic acid from castor oil irritates the intestinal mucosa cause inflammation and release of prostaglandin and which results in stimulation of secretion of electrolyte and water (Gaginella *et al.*, 1975). Several other mechanisms have been previously proposed to explain the diarrheal effect of castor oil including inhibition of intestinal Na^+,K^+-ATPase activity to reduce normal fluid absorption (Gaginella and Bass, 1978), activation of adenylate cyclase or mucosal cAMP mediated active secretion (Capasso *et al.*, 1994), stimulation of prostaglandin formation (Galvez *et al.*, 1993), platelet activating factor and recently nitric oxide has been claimed to contribute to the diarrheal effect of castor oil (Mascolo *et al.*, 1996).

1.5 Brine Shrimp Lethality Bioassay

The pharmacological evaluation of substances from plants is an established method for the identification of lead compound, which can lead to the development of novel and safe medical agents. The *in vivo* lethality in a sample zoological organism can be used as a convenient monitor for screening and fraction in the discovery and monitoring of bioactive natural products.

The *in vivo* lethality tests available for screening of plant extract are either base on the effect of extract on *Artimia sailna* Leach or the inhibition of hatching of the cyst (encased embryos that are metabolically inactive (Migliore *et al*., 1997). But most researchers have preferred the use of brine shrimp lethality test for the primary screening purposes.

The brine shrimp lethality bioassay is rapid (24 h), simple (e.g., no aseptic techniques are required), safe, easily mastered, inexpensive, and requires small amounts of test material (2-20 mg or less) (Ghisalberti, 1993). The test was proposed by Michael *et al.*, 1956 and later developed by Vanhaecke *et al*., 1981 and Sleet and Brendel, 1983. It is based on the ability to kill laboratory-cultured *Artemia* nauplii brine shrimp. The test provides a front-line screen that can be backed up by more specific and more expensive bioassays once the active compounds have been isolated.In case of screening of plant extracts, the extracts are considered as bioactive when $LC_{50}$is 1000 µg/ml or less (Meyer *et al*., 1982).

The brine shrimp cytotoxicity assay is considered as a convenient probe for preliminary assessment of toxicity (Solís *et al*., 1993), and it has been used for detection of plant extract toxicity (McLauglin *et al.*, 1991), detection of fungal toxins (Harwig *et al*., 1971), heavy metals (Martínez *et al.*, 1998), cyanobacteria toxins (Jaki *et al*., 1999), pesticides (Barahona *et al*., 1999) and cytotoxicity testing of dental materials (Pelka *et al.*, 2000). The bioassay has also a good correlation with cytotoxic activity in some human solid tumors and with pesticidal activity (McLaughlin *et al.*, 1998). Since its introduction in 1982 (Meyer *et al.*, 1982), this *in vivo* lethality test has been successively employed for bioassay-guide fractionation of active cytotoxic and antitumor agents such as trilobacin from the bark of *Asimina triloba* (Zhao *et al.*, 1992), *cis*-annonacin from *Annona muricata* (Rieser *et al.*, 1996) and ent-kaur-16-en-19-oic acid from *Elaeoselinum foetidum* (Mongelli *et al.*, 2002).

1.6 Purpose of the Present Study

The purpose of this present study was to evaluate the analgesic, neurophrmacological, anti-diarrheal and cytotoxic activity of the crude ethanolic leaf extract of *S. sisymbriifolium* and its *n*-hexane, chloroform, carbon tetrachloride and aqueous fractions. Preliminary qualitative phytochemical screening reveals the presence of alkaloids, flavonoids, steroids and tannins in *S. sisymbriifolium* (Shilpi *et al.*, 2005). These constituents possess analgesic, neuropharmacological and anti-diarrheal properties which were previously reported by several researchers (Rajnarayana *et al*. 2001; Adeyemi *et al.*, 2006; Okudo *et al.*, 1989). In this study, an attempt has been made to correlate the observer results in different *in vivo* animal model systems with these phytochemical constituents.

Chapter: 2

LITERATURE REVIEW

2. LITERATURE REVIEW

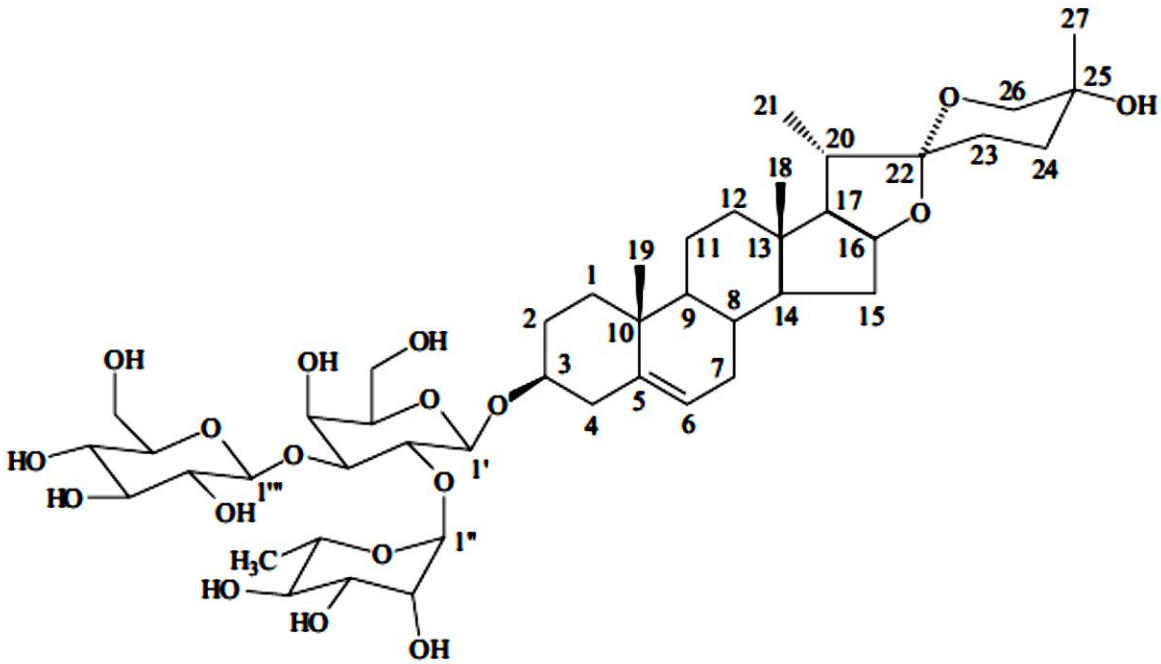
Phytochemical Studies

A neolignan and sterols in fruits of *Solanum sisymbriifolium*

A new neolignan, designated as sisymbrifolin and carpesterol, a rare C_{30} sterol, together with *β*-sitosterol and its *β*-D-glucoside, were isolated from the berries of *Solanum sisymbriifolium* (Chakravarty *et al.*, 1996).

A new steroidal saponin from *Solanum sisymbriifolium* roots.

Isonuatigenin-3-*O*-β-solatriose was isolated from the roots of *Solanum sisymbriifolium*. Its structure was determined by spectroscopic method (Ferro *et al.*, 2005).



Isonuatigenin-3-*O*-β-solatriose

Antinociceptive activity of methanolic extract of *Solanum sisymbriifolium* Lamk.

Phytochemical investigation of the methanolic extract of the whole plant of *Solanum sisymbriifolium* indicated the presence of alkaloid, flavonoids, steroid and tannin (Shilpi *et al.*, 2005).

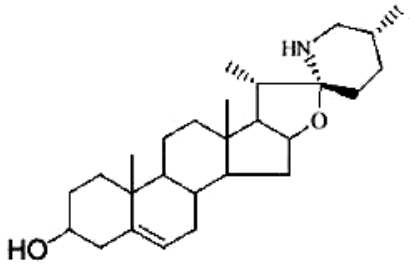
***Table2.1*:** Summary of the Phytochemical Studies on *S. sisymbriifolium*

Part of the plant	Findings	References
Berries	Isolation of sisymbrifolin (a new lignin) and carpesterol (a C_{30} sterol)	Chakravarty *et al.*, 1996
Roots	Isolation of Isonuatigenin-3-*O*-β-solatriose	Ferro *et al.*, 2005
Whole plant	Presence of alkaloid, flavonoids, steroid and tannin	Shilpi *et al.*, 2005

Pharmacological Studies

Anticonvulsant activity of solasodine isolated from *Solanum sisymbriifolium* fruits in rodents.

Solasodine, a poisonous steroidal glycoalkaloid chemical compound, was isolated from the dried fruits of *Solanum sisymbriifolium* showed potent anticonvulsant and CNS depressant activities (Chauhan *et al.*, 2010).



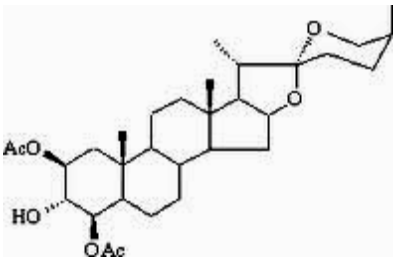
Solasodine

Isolation of hypotensive compounds from *Solanum sisymbriifolium* Lam.

The pharmacological action of fractionation of the crude hydroalcoholic root extract of *Solanum sisymbriifolium* Lam. was examined both in anaesthetized normotensive rats (lbarrola *et al*., 2000).Depression of locomotion, increase of breathing rate, increase in the gastrointestinal transit and piloerection was observed in anaesthetized normotensive rats. Hexanic and butanolic fractions induced remarkable hypotension activities in anaesthetized normotensive rats. Two compounds isolated from the butanolic fraction induced a significant decrease of the blood pressure, heart rate, amplitude of the ECG and breathing rate both systolic and diastolic, blood pressures were affected in a proportional mode (lbarrola *et al*., 2000).

Antihypertensive effect of nuatigenin-3-O-β-chacotriose from *Solanum sisymbriifolium* Lam. (Solanaceae) in experimentally hypertensive (ARH+DOCA) rats under chronic administration.

The hydro-ethanolic crude root extract, the *n*-butanol fraction and nuatigenin-3-O-[beta]-chacotriose, from *Solanum sisymbriifolium* Lam., was assessed in adrenal regeneration hypertension + deoxycorticosterone acetate (ARH+DOCA) rats, following a chronic administration. The oral administration showed a significant reduction of blood pressure value was induced in hypertensive animals (ARH+DOCA) in comparison to the control group receiving 1% saline solution (Ibarrola *et al.,* 2011). The latest demonstrated that the blood pressure-lowering effect, in clearly hypertensive animals, is reversible and depend upon administration of nuatigenin-3-0-[beta]-chacotriose [(B.sub.3-1]) (Ibarrola *et al.,* 2011).



Nuatigenin-3-O-β-chacotriose

On the nature and origin of the oxalate package in *Solanum sisymbriifolium* anthers. A detailed study was carried out in *Solanum sisymbriifolium* Lam. on the development of the circular cell cluster during crystal deposition, as well as the composition of the crystals (Burrieza *et al.*, 2010). TEM analysis revealed that the crystals originated simultaneously within the vacuoles in association with a paracrystalline protein. Prior to the appearance of protein within vacuoles, protein paracrystals were visible in both rough endoplasmic reticulum and vesicles with ribosome's on their membranes (Burrieza *et al.*, 2010). EDAX revealed that C, O, and Ca were the main elements, and K, Cl, Mg, P, S, and Si, the minor elements. X-ray powder diffraction of crystals detected the predominant presence of calcium oxalate, but also vestiges of calcite, quartz, and sylvite. The calcium oxalate coexisted in the three chemical forms, that is, whewellite, weddellite, and caoxite. Infrared spectrophotometry identified bands that characterize O-C-O, H-O, C-H bonds, all of calcium oxalate, and Si-O-Si, of quartz (Burrieza *et al.*, 2010).

Cardiovascular Action of Nuatigenosido from *Solanum sisymbriifolium*.

The pharmacological actions of nuatigenoside, isolated from the root of the herb *Solanum sisymbriifolium*. Lam (Solanceae), on cardiac system was investigated in rats (Derlis *et al.*, 2006). The study demonstrated the following results: Nuatigenosido lowered blood pressure in

rats, augmented the contractile force in the right atrium of a bullfrog, increased the overshoot amplitude in frog atrial myocytes, action potential durations were shortened, the calcium current (I_{Ca}) was increased, and the delayed outward potassium current was increased (Derlis *et al.*, 2006).

Molluscicidal activity of some Brazilian Solanum spp. (Solanaceae) against Biomphalaria glabrata.

Methanolic extracts of 13 Solanum species was tested for molluscicidal activity against *Biomphalaria glabrata*. According to the investigation the extract of fruits and aerial parts of *Solanum sisymbriifolium* showed significant positive molluscicidal activity (Silva *et al.*, 2005).

Cytotoxic effects of Argentinean plant extracts on tumor and normal cell lines.

A study was carried out to investigate the effects of 75 aqueous and methanol extracts from 41 Argentinean plant species on tumor and normal cell lines. From the study methanolic extract of flower of *Solanum sisymbriifolium* were found highly cytotoxic. It inhibited cell growth in a concentration-dependent manner (Mamone *et al*., 2011).

***Table 2.2*:** Summary of the pharmacological Studies on *S. sisymbriifolium.*

Part of the plant	Findings	References
Root	Hypotensive activity inanaesthetized normotensive rats	Lbarrola *et al.*, 2000
Whole plant	Significant positive molluscicidal activity	Silva *et al.*, 2005
Root	Lowered blood pressure, augmented the contractile force in the right atrium, increased the overshoot amplitude, shorten action	Derlis *et al.*, 2006

	potential durations	
Dried fruits	Potent anticonvulsant and CNS depressant activities.	Chauhan *et al.*, 2010
Whole plant	Composition of the crystals :C, O, and Ca were the main elements, and K, Cl, Mg, P, S, and Si, the minor elements	Burrieza *et al*., 2010
Root	Reduction of blood pressure value was induced in hypertensive animals	Lbarrola *et al.*, 2011
Whole plant	Methanolic extract of flower of *S. sisymbriifolium* were found highly cytotoxic	Mamone *et al.*, 2011

Chapter: 3

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Collection of Plant Material and Extraction

3.1.1 Materials and chemicals for extraction

Beaker, funnel, bottom glass container, filter paper, cotton, ethanol (BDH chemicals) and rotary evaporator (IKA, Germany).

3.1.2 Collection and identification of plants

The fresh leaves of the plant were collected from Agargaon, Dhaka in August, 2011 and identified by the taxonomist of Bangladesh National Herbarium, Mirpur, Dhaka as *S. sisymbriifloium*. A voucher specimen of the plant has been deposited (Accession No. DACB 35894) (figure 3.1.2) in the herbarium for further reference.

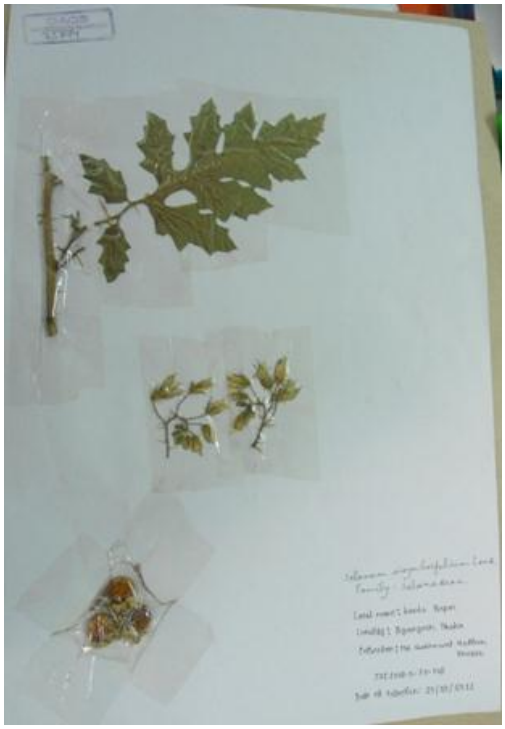


Figure 3.1.2*:* Identification plate of *S. sisymbriifolium*

3.1.3 Drying and pulverization

The collected leaves were thoroughly washed with water to remove adhering dirt and cut into small pieces. Then the small pieces were sun dried **(**figure 3.1.3) for 15 days. After complete drying, the dried leaves were pulverization into a coarse powder with the help of a locally fabricatedgrinding machine and were stored in an airtight container.

Figure 3.1.3*:* Drying of leaves and branches of *S. sisymbriifolium* under sunlight.

3.1.4 Extraction of the plant material

The plant powder (1kg) was extracted by cold extraction process using ethanol (2.5 liters) as solvent in a bottom glass container, through occasional shaking and stirring for 7 days. After 7 days, the extract was then filtered through cotton and filter paper(figure 3.1.4).

Figure3.1.4:Filtration of ethanolic extract of *S. sisymbriifolium* (leaf).

3.1.5 Drying of ethanolic extract

The filtrate was then dried and concentrated with using rotary evaporator (temperature 50 ˚C, rpm 120/minute) (figure 3.1.5).

Figure 3.1.5*:* Drying of extract using rotary evaporator.

3.1.6 Fractionation of the Ethanolic Extract

Materials

Separating funnel, measuring cylinder, beaker, pipette, and rotary evaporator.

Chemicals

Ethanol (BDH Chemicals), distilled water, *n*-Hexane (MERCK, Germany), carbon tetrachloride (MERCK, Germany), chloroform (MERCK, Germany), acetone (MERCK, Germany).

Method

A portion (20 g) of the concentrate ethanolic extract of *S. sisymbriifolium* was fractionated (figure 3.1.6)by the modified Kupchan partitioning method (Van Wagene *et al.*, 1993) into *n*-Hexane, carbon tetrachloride, chloroform and aqueous fractions. First the ethanolic extract (20 g) was dissolved in 100 ml of 10% aqueous ethanol (90ml ethanol+10ml water) and extracted three times with *n*-Hexane (100×3) by using a separating funnel. The *n*-Hexane soluble fraction formed the upper layerdue to lower density and polarity of *n*-Hexane (0.66 g/ml, 0.1) than ethanol (0.789 g/ml, 4.3) in water and collected in a beaker. The remaining aqueous phase was then increased in polarity to 20% water and extracted three times with carbon tetrachloride (100×3). The carbon tetrachloride soluble fraction formed the lower layer due to increase of density and polarity of carbon tetrachloride (1.587g/ml, 1.6) and collected in a separate beaker. The remaining aqueous phase was increased further in polarity to 40% water and extracted three times with chloroform (100×3). The chloroform soluble fraction formed the lower layer due to increase of solubility and polarity of chloroform (1.483 g/ml, 4.1) and collected in a separate beaker. The remaining aqueous fraction was collected in another separate beaker. The

subsequent evaporation of solvents from the beakers by using rotary evaporator (figure 3.1.5) afforded *n*-Hexane (4.584 g), carbon tetrachloride (3.873 g), chloroform (3.631 g) and aqueous (5.890 g) soluble crude materials. A Schematic diagram of the method of Fractionation of the ethanolic leaf extract is given below (figure 3.1.7).

Figure 3.1.6:Fractionation of the ethanolic leaf extract of *S. sisymbriifolium*

Kupchan and Tsou solvent-solvent partitioning

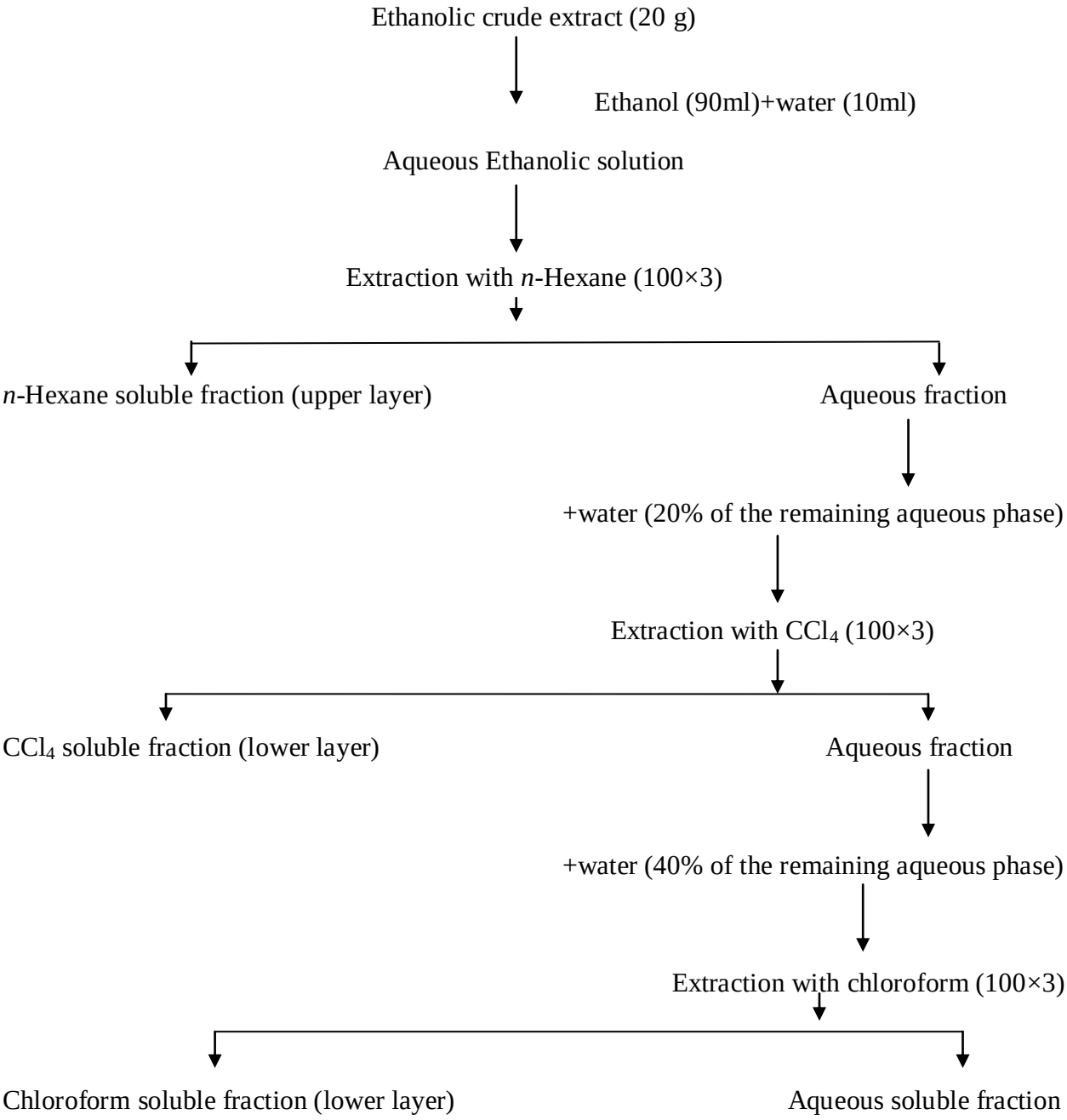


Figure 3.1.7:Schematic diagram of the method of Fractionation of the ethanolic leaf extract

3.2. Experimental Laboratory Animal

Twenty Swiss albino mice of either sex, weighing between 18-25 g, purchased from the Animal Research Branch of the International Center for Diarrheal Disease and Research, Bangladesh (ICDDR, B) in 19 January 2012 were used throughout the experiments. The animals were kept in cages having dimensions of 30×20×13 cm at research laboratory (Department of Pharmacy, East West University) in standard laboratory conditions (12 hours light or 12 hours dark cycle) after their purchase for adaptation with the new environment of the laboratory, before being employed in any experiment. Soft wood shavings were employed as bedding in the cages. They mice were fed with standard food (ICDDR, B formulated) and had free access to tab water. The animals were acclimatized to laboratory condition for two week prior to experimentation.

3.3 Method of identification and feeding of animals

It was difficult to observe the biological responses of mice at a time receiving same treatment. But it was necessary to observe the biological response of each animal in a group individually. Several methods were particularly well suited for identification cage mates. A felt tip marker was used for marking tails of the mice. Such mark usually disappears in 1 to 2 days. So, each of the mice in a group was marked few hours before the experiment at a sequence of 1 to 5 (figure 3.3.1). Feeing needle was used to feed leaf extract to mice (figure 3.3.2).

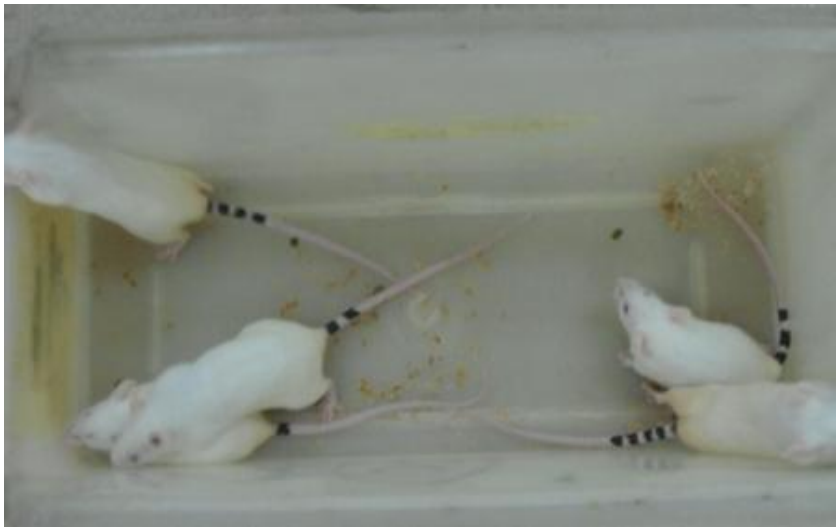


Figure 3.3.1: Identification of mice by tail marking.

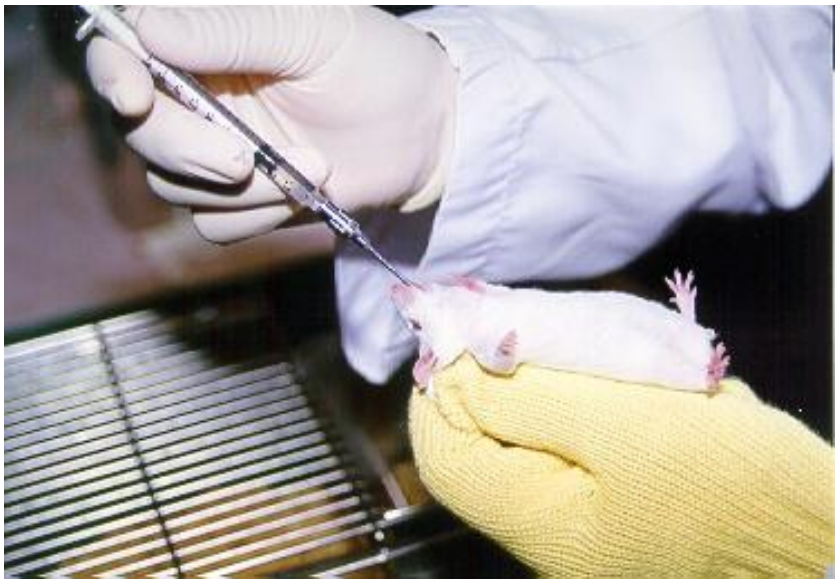


Figure 3.3.2: Feeding of *S. sisymbriifolium* leaf extract to mice.

3.4 Acetic Acid Induced Writhing Test

Materials

Mice cage, syringe (100 cc), syringe (5ml), feeding needle, stop watch, vial, beaker, vortex shaker.

Chemicals

Acetic acid (MERCK, Germany), Diclofenac sodium (Square Pharmaceutical Ltd., Bangladesh), Tween-80 (MERCK, Germany), Normal saline (Beximco infusion Ltd., Bangladesh), Methanol (Active Fine Chemicals Ltd., UK).

Method

The analgesic activity of crude ethanolic extract of *S. sisymbriifolium* (leaf) was evaluated using acetic acid induced writhing method in mice (Zulfiker *et al.,* 2010). Twenty Swiss mice of either sex were taken for this experiment. The experimental animals were randomly divided into four groups, each consisting of five animals. Group I was treated as 'control' which received 1% (v/v) Tween-80 in normal saline at a dose of 10 ml/ kg of average body weight (p.o.). Group II was treated as 'positive control' and was given standard drug diclofenac sodium at dose of 10mg/kg of average body weight (p.o.). Group III and Group IV were test groups and were treated with the extract at dose of 200 mg and 400 mg/kg of average body weight (p.o.), respectively. The test samples were prepared by dissolving the ethanolic extract in 1% Tween-80 in normal saline. The control vehicle and test samples were administered orally, 30 minutes prior to intraperitoneal administration (figure 3.4)of 0.7% v/v acetic acid solution (0.1ml/10g). The acetic acid was administered intraperitoneally to create pain sensation.

The diclofenac sodium was administered 15 minutes prior to acetic acid injection. Then the animals were placed inside the cages for observation. The number of writhing by each mouse was counted individually for a period of 20 minutes just 5 minutes after the intraperitonealadministration of 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

Figure 3.4: Intraperitoneal administration of acetic acid in mice.

incomplete writhing was considered as half-writhing. Accordingly, two half-writhing were taken as one full writhing (Al-amin *et al.*, 2011). The number of writhes in each treated group was compared to that of a control group.

$$\text{Movements inhibition}(\%)=\frac{\text{Mean No.of writhing(control)-Mean No.of writhing(test)}}{\text{Mean No.of writhing(control)}}\times 100$$

3.5 Evaluation of Neuropharmacological Activity

3.5.1 Hole Cross Test

Materials

Hole cross instrument (cage having a size of 30 × 20× 14 cm, a hole of 3 cm in diameter at a height of 7.5 cm on the wooden partition which is fixed in the middle of the cage), syringe (5ml), feeding needle, stop watch, vial, beaker, vortex shaker.

Chemicals

Diazepam (Square Pharmaceutical Ltd., Bangladesh), Tween 80 (MERCK, Germany), Normal saline (Beximco infusion Ltd, Bangladesh), Methanol (Active Fine Chemicals Ltd., UK).

Method

The method described by Takagi *et al.* (1971) was adopted for screening the sedative activity of crude ethanolic extract of *S. sisymbriifolium* (leaf). 20 Swiss mice of either sex were taken for this experiment. The experimental animals were randomly divided into four groups, each consisting of five animals. Group I was treated as 'control' which received 1% (v/v) Tween-80 in normal saline at a dose of 10 ml/ kg of average body weight (p.o.). Group II was treated as 'positive control' and was given crude Diazepam at dose of 1 mg/kg of average body weight (p.o.). Group III and Group IV were test groups and were treated with the extract at dose of 200 mg and 400 mg/ kg of average body weight (p.o.), respectively. The test samples were prepared by dissolving the ethanolic extract in 1% Tween-80 in normal saline. The control vehicle, positive control and extract were administered orally. After oral administration, each mouse was immediately placed in any of the two chambers of the cage. The number of passages of a mouse

through the hole from one chamber to anotherwas counted for a period of 3 minutes. The observation was conducted at 0, 30, 60, 90, and 120 minutes. The number of passages in each treated group was compared to that of a control group.

$$\text{Movements inhibition}(\%) = \frac{\text{Mean No. of writhing(control)} - \text{Mean No. of writhing(test)}}{\text{Mean No. of writhing(control)}} \times 100$$

3.5.2 Hole Board Test

Materials

Hole board apparatus (a wooden board having a size of 40 × 40 × 25 cm, contains total 16 holes eventually distributes on the floor each of 3 cm in diameter and elevates to the height of 25 cm), syringe (5ml), feeding needle, stop watch, vial, beaker, vortex shaker.

Chemicals

Diazepam (Square pharmaceutical Ltd., Bangladesh), Tween-80 (MERCK, Germany), Normal saline (Beximco infusion Ltd, Bangladesh), Methanol (Active Fine Chemicals Ltd., UK)

Method

The method described by Vogel (2002) and Woode *et al.* (2009) was adopted for testing the central nervous system activity of crude ethanolic extract of *S. sisymbriifolium* (leaf). 20 Swiss mice of either sex were taken for this experiment. The experimental animals were randomly divided into four groups, each consisting of five animals. Group I was treated as 'control' which received 1% (v/v) Tween-80 in normal saline at a dose of 10 ml/ kg of average body weight (p.o.). Group II was treated as 'positive control' and was given crude Diazepam at dose of 1mg/Kg of average body weight (p.o.). Group III and Group IV were test groups and were

treated with the extract at dose of 200 mg and 400 mg/ kg of average body weight (p.o.), respectively. The test samples were prepared by dissolving the ethanolic extract in 1% Tween-80 in normal saline. The control vehicle, positive control and extract were administered orally. 30 minutes after oral administration, each mouse was placed on the center of the board (facing away from the observer) and moved freely on the board (figure 3.5.1).The number of times of head dipping at the level of their eyes and the latency until the first entry (means time until the first head dipping after the mouse was placed on the board) were counted. A head dip into holes was used to indicate exploratory behavior. The number of head dipping was counted for a period of 5 minutes.

Figure 3.5.1: Mice on hole board instrument

3.5.3 Elevated Plus-Maze Test

The anxiolytic activity of ethanolic extract of *Solanum sisymbriifolium* (leaf) was evaluated using the elevated plus maze test (Itoh *et al.*, 1991).

Materials

Elevated plus maze apparatus (a wooden maze consists of two open arms 30 × 5 cm, cross with two closed arms 30× 5 × 25 cm and both closed and opened arms are connected with a central square 5 × 5cm. The floor and walls of the maze is painted black. The entire maze is elevated to the height of 40 cm above the floor), syringe (5ml), feeding needle, stop watch, test tube, vial, beaker, spatula, vortex shaker, digital video camera.

Chemicals

Diazepam (Square pharmaceutical Ltd., Bangladesh), Tween-80 (MERCK, Germany), Normal saline (Beximco infusion Ltd, Bangladesh), Methanol (Active Fine Chemicals Ltd., UK)

Method

20 Swiss mice of either sex were taken for this experiment. The experimental animals were randomly divided into four groups, each consisting of five animals. Group I was treated as 'control' which received 1% (v/v) Tween-80 in normal saline at a dose of 10 ml/ Kg of average body weight (o.p). Group II was treated as 'positive control' and was given crude Diazepam at dose of 1mg/Kg of average body weight (o.p). Group III and Group IV were test groups and were treated with the extracts at dose of 200 mg and 400 mg/ Kg of average body weight (o.p), respectively. The test samples were prepared by dissolving the ethanolic extract in 1% Tween-80 in normal saline. The control vehicle, positive control and extracts were administered orally at equal volume (0.5ml). The test was initiated thirty minutes after oral treatment by placing the mouse on the central platform of the maze, facing one of the open arms, and letting it move freely. The activities of each mouse for a period of 5 minutes were tapped by using a digital video camera. The digitized video of the each 5 min trial were later scored carefully and a

numbers of classical parameters were collected: (a) Open arm duration: the total amount of time the mouse spent in the open arms; (b) Closed arm duration: the total amount of time the mouse spent in the closed arms; (c) Open arm frequency: the frequency of mouse entry with all four paws into the open, unprotected arms; (d) Closed arm frequency: the frequency of mouse entry with all four paws into the closed, protected arms, and (e) Total number of entries in the arms. Entry into an arm was defined as the point when the mice place all four paws onto the arm. Likewise, different ethological measures were also quantified: (a) Rearing: number of times the mouse stood on its hind limbs; Grooming: number of times the mouse scratched various part of its body and Stretched attend posture (SAP): a body posture in which the mouse stretches forward and then retracts to its original position without moving the feet. The test was conducted in a sound attenuated room illuminated only by dim light and after each test; the maze was carefully cleaned up with wet tissue paper (10% ethanol solution).

3.6 Castor Oil Induced Anti-Diarrheal Activity Test

Anti-diarrheal activity of the ethanolic extract of leaf of *S. sisymbriifolium* was tested by using the model of castor oil induced diarrhea in mice.

Materials

Filter paper, syringe (5ml), feeding needle, stop watch, vial, beaker, vortex shaker.

Chemicals

Castor oil (BDH Chemical), Loperamide (Imotil®, Square Pharmaceutical Ltd., Bangladesh),Tween-80 (MERCK, Germany), Normal saline (Beximco infusion Ltd, Bangladesh).

Method

The antidiarrheal activity of the ethanolic extract of *S. sisymbriifolium* (leaf) was evaluated according to the method described by Shoba and Thomas (2001). According to the method, castor oil was administered orally to 20 Swiss mice of either sex to induce diarrhea. The mice were fasted for 24 hours with free access to water before the experiment. The experimental animals were randomly divided into four groups, each consisting of five animals. Group I was treated as 'control' which received 1% (v/v) Tween-80 in normal saline at a dose of 10 ml/ kg of average body weight (p.o.). Group II was treated as 'positive control' and was given standard antimotility drug loperamide in suspension at dose of 2 mg/kg of average body weight (p.o.). Group III and Group IV were test groups and were treated with the extract at dose of 200 mg and 400 mg/kg of average body weight (p.o.), respectively. The test samples were prepared by dissolving the ethanolic extract in 1% Tween-80 in normal saline. First, the control vehicle, standard drug and test samples were administered orally. After 30 minutes, 0.2 ml castor oil was administered to each mouse by a feeding needle. After administration of castor oil, each animal was placed in a separate beaker on a filter paper for observation. During an observation period of 2 hours, a numbers of parameters were recorded: (a) onset of dry stool (b) No. of wet stool (c) weight of wet stool (d) total weight of fecal output and (e) onset of wet stool.Diarrhea was defined by the presence of stool or any fluid material that stained the filter paper placed beneath the beaker. Time taken before the first defecation was the 'Latent period'. The number of stool or any fluid that stained the filter paper was counted at each successive hour during the 2 hours observation period. At the beginning of each hour new filter papers were placed for the old ones. The percent (%) inhibition of defecation was measured using the following formula.

$$\% \text{ inhibition of defecation} = [\frac{(A - B)}{A}] \times 100$$

A = Mean number of defecation caused by castor oil

B = Mean number of defecation caused by drug or extract

3.7 Brine Shrimp Lethality Bioassay

Materials

Artemia salina leach (Brine shrimp eggs), small glass tank with perforated dividing dam, table lamp, pipette, aquarium air pump (SB2488, Sovo), measuring cylinder, electric balance (SHIMADZU AY220 and SCALTEC SPB31), beaker (1000 ml), vortex shaker, micropipettes and vials.

Chemicals

Dimethyl sulfoxide (DMSO) (MERCK, Germany), Sodium hydroxide (NaOH) (MERCK, Germany), Sodium chloride pure (MERCK, Germany).

Method

Preparation of simulated seawater

To prepare sea water, 38g of sodium chloride (NaCl) was first weighed by using an electronic balance and then dissolved in distilled water. The volume of the solution was adjusted to 1000 ml by adding distilled water in a 1000 ml beaker. After that one or two drops of 1N NaOH (Sodium Hydroxide) was added to adjust the pH 8.4 of the solution.

Hatching of Brine Shrimp (Artemia salina)

The prepared simulated sea water was transferred in a small glass tank (figure 3.7.1) having several small holes of 0.5 cm in diameter at a height of 5 cm on the glass divider which is fixed in the middle of the tank. *Artemia salina* leaches (brine shrimp eggs) were added to one side of the tank and then that side was enclosed. The tank was kept under constant aeration by an electrical air pumper for 24 hours to hatch the shrimp and to be matured as nauplii. Light was also provided by a table lamp which was placed near the other side of the tank. The mature nauplii were attracted to the lamp through the perforated dam.

Figure 3.7.1: Hatching of *Artimia salina* in small glass tank.

Collection of Artemia salina Nauplii

For the collection of brine shrimp nauplii for test, positive control and negative control, 30 vials were taken, 10 for each purpose. In each vial, 5 ml of solution containing 10 living nauplii were required. To do that task, first each of the vials was filled with 2.5 ml of normal water and the

position of the water level was marked on each vial. Then water was removed from the vials and appropriate labeling was done. Hatched pre-matured nauplii from the hatching container were collected by using a dropper. Precautions were taken not to collect empty cysts and un-hatched eggs. To assure clean collection of only live nauplii, small amount of the hatching solution containing live nauplii were taken in a small beaker. After that with the help of the Pasteur pipette 10 living nauplii were added to each of the vials and the volume of the solution in the vials were adjusted to the prerecorded mark with fresh simulated sea water. The volume of the solution was below 5 ml at this point for the later addition of drugs or crude extracts.

Preparation of Control groups

Positive Control

For this purpose, 10 marked vials were taken in which 10 nauplii in 2.5 ml of simulated sea water were previously collected. For positive control, potassium permanganate ($KMnO_4$)was used as the standard drug. 25mg or 25000µg of $KMnO_4$ was dissolved into 5ml or 5000µl of simulated sea water previously prepared. So, the concentration of $KMnO_4$ in the parent solution was 5µg/µl. Then, 2µl of this solution was taken in first marked vial and the volume was adjusted to 5ml with simulated sea water. So, the concentration of $KMnO_4$ in the first test tube was 2µg/ml. Like this process, all the other test tubes were adjusted to the volume of 5ml with simulated sea water, containing 10 nauplii and different concentrations of $KMnO_4$ that are given bellow in Table 3.7. Then the test tubes were kept for 24 hours to observe the results.

***Table 3.7*:** Preparation of positive control test solutions for brine shrimp lethality test.

SL #	Amount of $KMnO_4$ solution	Final Volume	Final Concentration
1	2 µl	5 ml	2 µg/ml
2	4 µl	5 ml	4 µg/ml
3	6 µl	5 ml	6 µg/ml
4	8 µl	5 ml	8 µg/ml
5	10 µl	5 ml	10 µg/ml
6	12 µl	5 ml	12 µg/ml
7	14 µl	5 ml	14 µg/ml
8	16 µl	5 ml	16 µg/ml
9	18 µl	5 ml	18 µg/ml
10	20 µl	5 ml	20 µg/ml

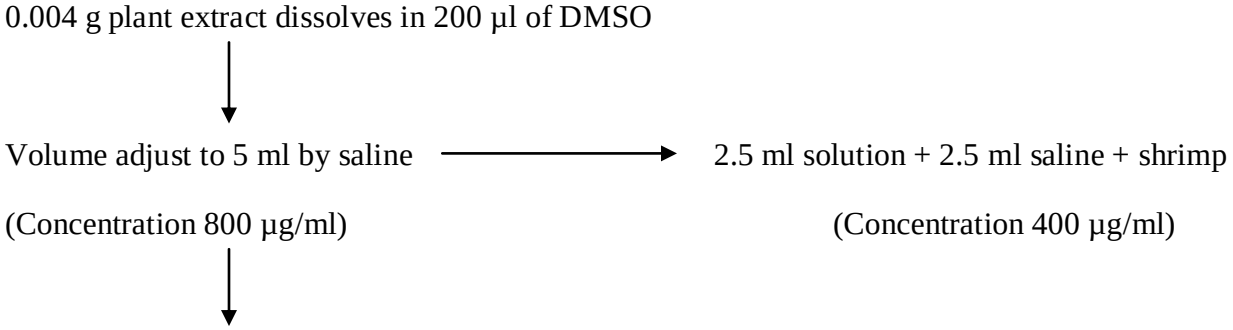
Negative control

For negative control, 10 marked vials were taken in which 10 nauplii in 2.5 ml of simulated sea water were taken previously. 200µl of DMSO was added to a vial and the volume of the vial was adjusted to 5ml using simulated sea water. So, the concentration of the stock solution was (40µl/ml). Then 2.5ml of the stock solution was added in the first marked vial and the volume of the vial adjusted to 5ml with simulated sea water. So, the concentration of DMSO in the first marked vial was 20µl/ml. After that the volume of stock solution was adjusted to 5ml using simulated sea water. 2.5ml of stock solution was added in the second marked vial and volume of the vial adjusted to 5ml with sea water. So, the concentration of DMSO in the second vial was 10µl/ml. Like this process, all the other marked vials were adjusted to the volume 5ml to get different concentrations of DMSO in solutions (5µl/ml, 2.5µl/ml, 1.250µl/ml, 0.62µl/ml,

0.313µl/ml,0.156µl/ml, 0.078µl/ml and0.039µl/ml). Finally these vials were kept for 24 hours to get the result of brine shrimp lethality test. The test was considered as invalid if the nauplii in the vials showed a rapid mortality rate and therefore conducted again.

Preparation of test Solutions of S. sisymbriifolium

For this purpose, 10 marked vials were taken in which 10 nauplii were previously collected and the volumes were adjusted to 2.5ml using simulated seawater. 4mg of the crude ethanolic leaf extract of *S. sisymbriifolium* was dissolve in 200µl of DMSO in a vial and the volume was adjusted to 5ml using simulated seawater. The concentration of the stock solution was 80µg/ml. Then 2.5ml of the stock solution was added in the first marked vial. So, the volume of the first marked vial was 5ml and the concentration of leaf extract of *S. sisymbriifolium* was 400µg/ml. After that the volume of stock solution was adjusted to 5ml using simulated sea water. 2.5ml of stock solution was added in the second marked vial. So the volume of the second vial was 5ml and the concentration of leaf extract was 200µg/ml. Like this process, all the other marked vials were adjusted to the volume 5ml to get different concentrations of leaf extract of *S. sisymbriifolium* in solution (100µg/ml, 50µg/ml, 25µg/ml, 12.5µg/ml, 6.25µg/ml,3.125µg/ml, 1.563µg/ml and0.781µg/ml). Finally these vials were kept for 24 hours to get the result of brine shrimp lethality bioassay. A schematic diagram of preparation of test solutions of *S. sisymbriifolium* leaf extractfor brine shrimp lethality test is given below (figure 3.7.2).



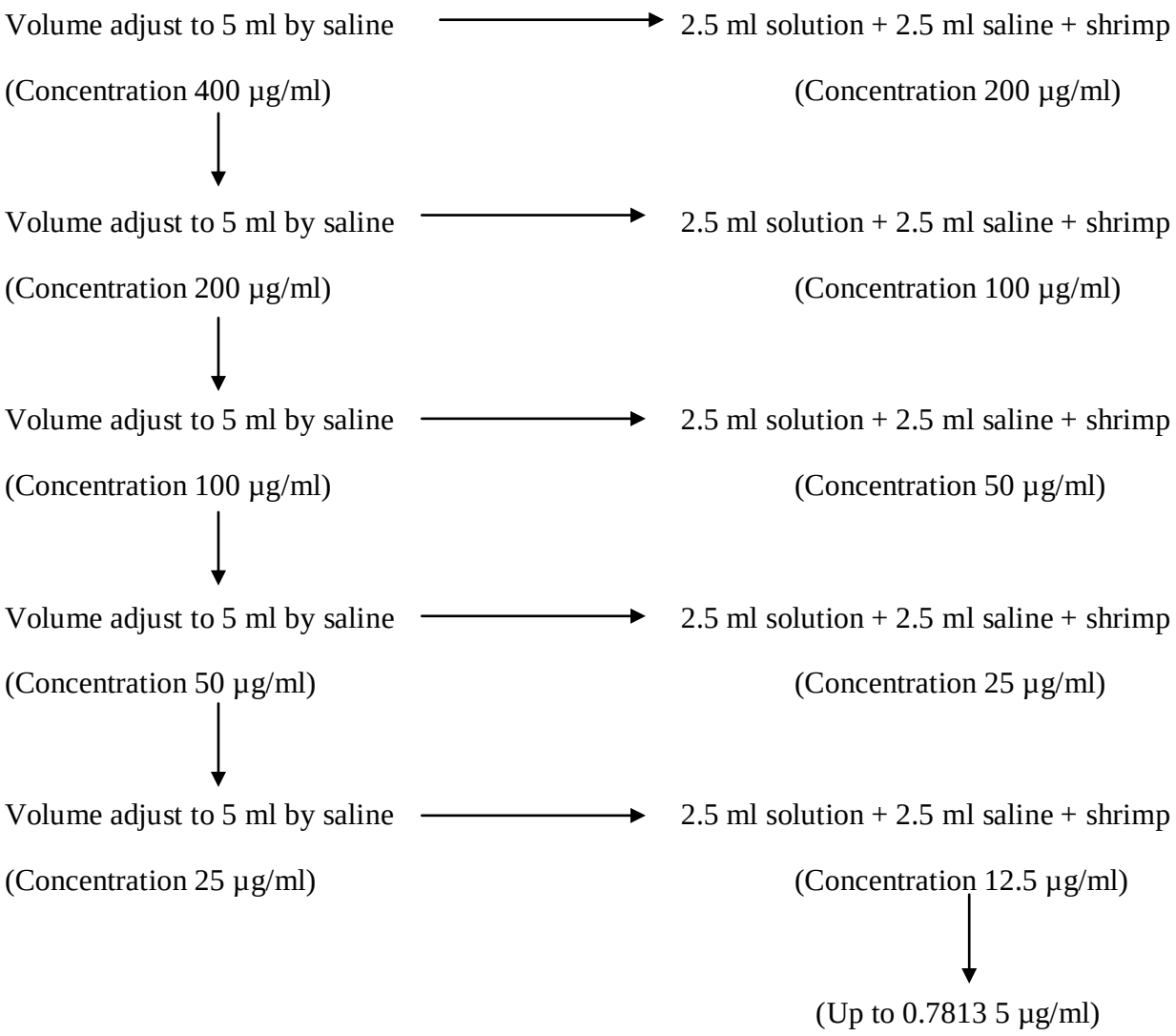


Figure 3.7.2: Schematic diagram of preparation of test solutions of *S. sisymbriifolium*leaf extractfor brine shrimp lethality test

3.8 Statistical Analysis

Data were presented as Mean±SEM (standard error of the mean). SPSS (statistical package for social science) for WINDOWS^{TM} (version 12.0) was applied for the analysis of data. The data was analyzed statistically by one-way ANOVA is followed by Dunnett t-test (2-sided). $p<0.05$

was taken to be the level of significance, $p<0.001$ was taken to be the level of highly significance.

p- Value determines the appropriateness of rejecting the null hypothesis in a hypothesis test. *p*-Valuesrange from 0 to 1. Smaller the *p*- value, the smaller the probability that rejecting the null hypothesis is a mistake.

For brine shrimp lethality test data analysis, Finney's statistical method of probit analysis (Finney, 1971) was used to calculate LC_{50} (the concentration of the extract that would kill 50% of brine shrimps within 24 h of exposure) with 95% confidence intervals. The analysis was done by Microsoft Excel 2007 from which equations were derived and these data were converted into charts, from which LC_{50} values for different extract of *S. sisymbriifolium* calculated.

Probit analysis is a specialized regression model of binomial response variables (e.g. in brine shrimp lethality test the variables are death or no death). It is commonly used in toxicology to determine the relative toxicity of chemicals to living organisms (Finney, 1971).

Chapter: 4

RESULTS

4. RESULTS

4.1 Acetic Acid Induced Writhing Test

Table 4.1: Effect of ethanolic leaf extract of *S. sisymbriifolium*(SSL) on acetic acid induced writhing in mice.

Group	Dose	Mean no. of writhing	% inhibition
Control (1% tween 80 in saline)	0.5ml/mice; p.o.	78.6±0.29	----
Positive Control (Diclofenac Sodium)	10mg/kg body weight; p.o.	1.5±0.16**	98.09
SSL200	200mg/kg body weight, p.o.	52.1±0.66**	33.72
SSL400	400mg/kg body weight; p.o.	4.4±0.64**	94.40

Values are expressed as Mean±SEM (n=5); **: $p<0.001$ dunnett t-test as compared to Control.

		Standard deviation	Standard error	95% confidence interval for mean	
Group	Mean		Mean	Lower bound	Upper bound
Control	78.60	0.65	0.29	77.79	79.41
Positive	1.50	0.35	0.15	1.06	1.93
SSL 200	52.10	1.47	0.66	50.26	53.93
SSL 400	4.40	1.43	0.64	2.62	6.17

In acetic acid-induced writhing test, the ethanolic extract of *S. sisymbriifolium* at both doses (200 and 400 mg/kg body weight) showed highly significant ($p<0.001$) inhibition of writhing response (table 4.1) induced by the acetic acid after oral administration in a dose dependant manner. The percent inhibitions of the writhing response at the doses 200 mg/kg and 400 mg/kg were 33.72% and 94.40% respectively.

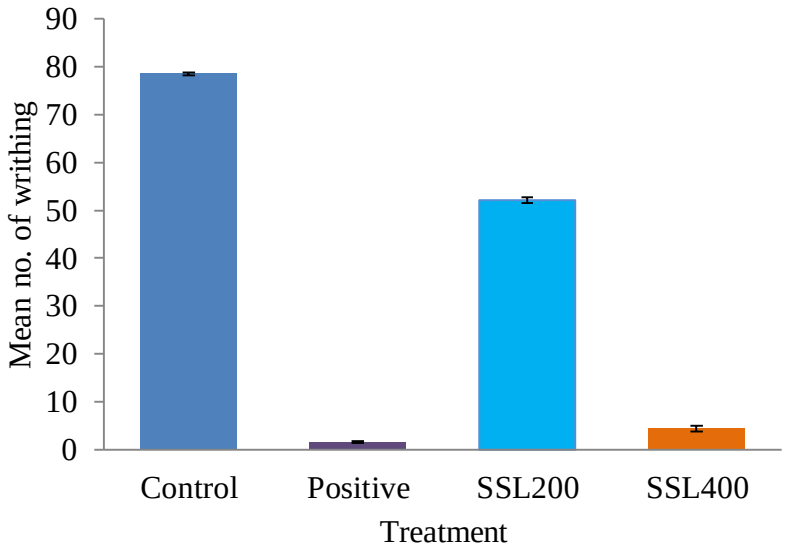


***Figure 4.1.1*:** Graphical presentation of the effect of ethanolic leaf extract of *S. sisymbriifolium* on mean number of writhing in acetic acid induced writhing test.

4.2 Evaluation of Neuropharmacological Activity

4.2.1 Hole Cross Test

Table 4.2.1:Effect of ethanolic leaf extract of *S. sisymbriifolium* on number of movement in hole cross test.

Groups	Dose	Mean number of movement				
		0 min	30 min	60 min	90 min	120 min
Control (1% tween 80 in saline)	0.5ml/mice, p.o.	10.0±0.71	8.6±0.60	7.2±0.58	7.0±0.55	6.2±0.37
Positive Control (Diazepam)	1mg/kg body weight; p.o.	7.8±0.74*	6.2±0.80*	5.8±0.74	4.2±1.16*	3.6±0.51*

SSL200	200mg/kg body weight; p.o.	5.8±0.37**	4.2±0.37**	4.8±0.49	5.8±0.37	6.2± 0.74
SSL 400	400mg/kg body weight; p.o.	3.6±0.51**	6.2±0.49*	8.0±0.89	4.0±0.63*	3.8±0.49*

Values are expressed as Mean±SEM (n=5); *: $p<0.05$, **: $p<0.001$ dunnett t-test as compared to Control.

In the hole cross test, the ethanolic leaf extract of *S. sisymbriifolium* at dose (200 mg/kg body weight) showed a decrease in locomotion activity in the test animals at the 2nd observation period (30 minutes) which was statistically highly significant ($p<0.001$) (table 4.2.1). But the extract at the same dose showed increase in activity from the 3rd observation period (60 minutes) and continued up to5th observation period (120 minutes)which were statistically not significant($p<0.05$(table 4.2.1).

The extract at dose (400 mg/kg body weight) showed significant ($p<0.05$) increase in locomotion activity from the second observation period (30 minutes) in the test animals and continuedup to3rd observation period (90 minutes)(table 4.2.1).But the extract at the same dose showed decrease in activity at the 4th (60 minutes) and 5th (240 minutes) observation periods which were also statistically significant ($p<0.05$)(table 4.2.1).The increase in locomotion activity showed at the 3rd observation period (90 minutes) was not statistically significant ($p<0.05$).

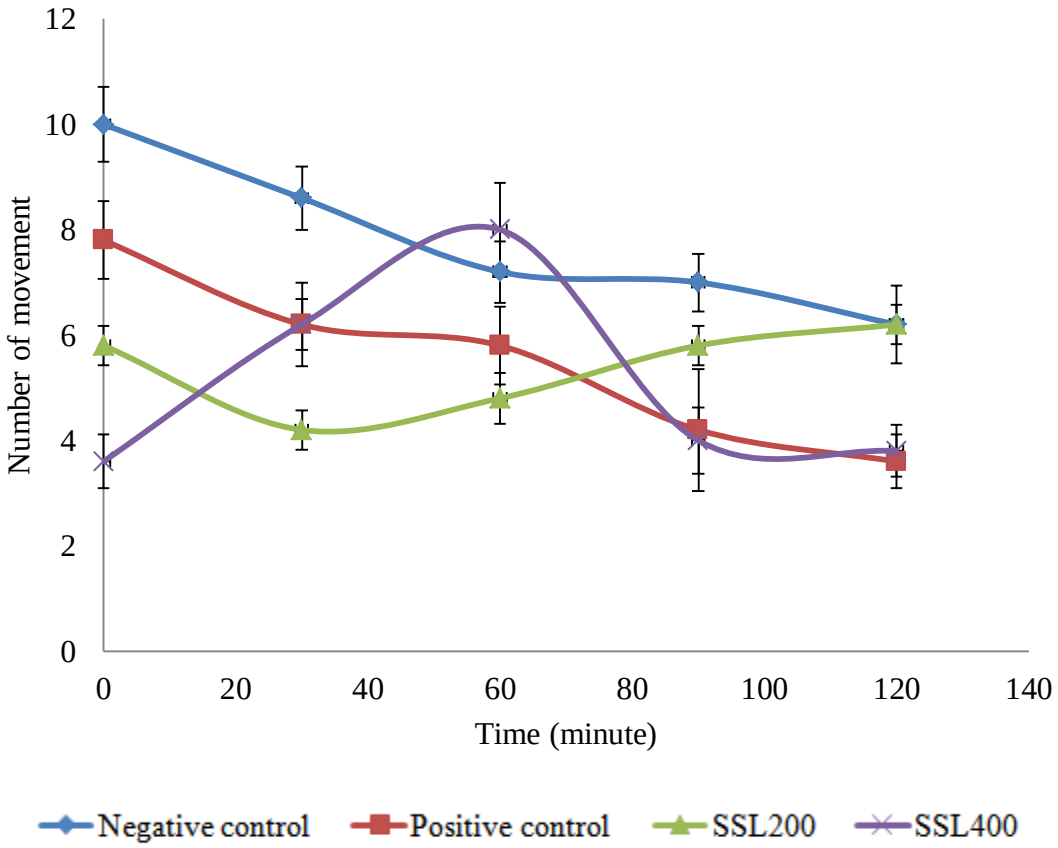


***Figure 4.2.1*:**Graphical presentation of the effect of ethanolic leaf extract of *S. sisymbriifolium* on number of movement in hole cross test.

4.2.2 Hole Board Test

Table 4.2.2.1: Effect of ethanolic leaf extract of *S. sisymbriifolium* on no. of head dipping in hole board test.

Group	Dose	Mean no. of head dipping in 5 minutes
Control (1% tween 80 in saline)	0.5 ml/ mice; p.o.	44.8±1.24
Positive control (Diazepam)	1 mg/ Kg body weight; p.o.	29.6±0.93**

SSL200	200 mg/ Kg body weight; p.o.	65.6±0.68**
SSL400	400 mg/ kg body weight; p.o.	35.8±0.80**

Values are expressed as Mean±SEM (n=5); **: $p<0.001$ dunnett t-test as compared to Control.

		Standard deviation	**Standard error**	**95% confidence interval for mean**	
Group	**Mean**		**Mean**	**Lower bound**	**Upper bound**
Control	44.80	2.77	1.24	41.35	48.25
Positive	29.60	2.07	0.93	27.03	32.17
SSL200	65.60	1.52	0.68	63.72	67.48
SSL400	35.80	1.79	0.80	33.58	38.02

The effect of ethanolic leaf extract of *S. sisymbriifolium* upon the hole board test was performed by measuring the number of head dipping. The ethanolic extract of *S. sisymbriifolium* at dose (200 mg/kg body weight) showed an increase number in head dipping (65.6±0.68) behavior compared with the control group which was statistically highly significant ($p<0.001$) (table 4.2.2.1). But the extract at dose (400 mg/kg body weight) showed a decrease number in head dipping (35.8±0.80) behavior compared with the control group which was also statistically highly significant ($p<0.001$) (table 4.2.2.1).

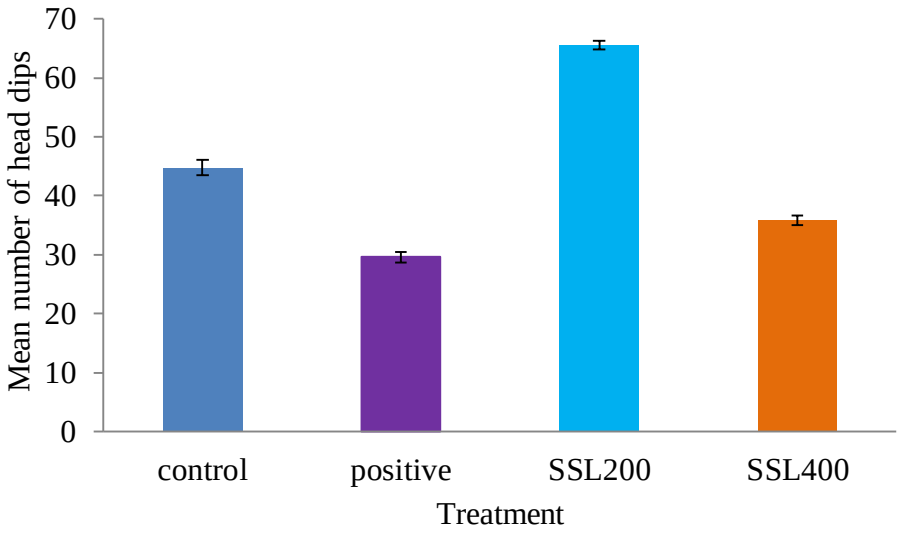


***Figure 4.2.2.1*:** Graphical presentation of the effect of ethanolic leaf extract of *S. sisymbriifolium* on mean number of head dips in hole board test.

Table 4.2.2.2: Effect of ethanolic leaf extract of *S. sisymbriifolium* onlatency until the first head dipping in hole board test.

Group	**Dose**	**Mean latency until the first head dipping (second)**
Control (1% tween 80 in saline)	0.5 ml/ mice; p.o.	14.8±0.37
Positive control (Diazepam)	1 mg/ Kg body weight; p.o.	2.0±0.45**
SSL200	200 mg/ Kg body weight; p.o.	16.2±0.66
SSL400	400 mg/ kg body weight; p.o.	6.4±0.40**

Values are expressed as Mean±SEM (n=5); **: $p<0.001$ dunnett t-test as compared to Control.

Group	Mean	Standard deviation	Standard error	95% confidence interval for mean	
			Mean	Lower bound	Upper bound
Control	14.80	0.84	0.37	13.76	15.84
Positive	2.00	1.00	0.45	0.76	3.24
SSL200	16.20	1.48	0.66	14.36	18.04
SSL400	6.40	0.89	0.40	5.29	7.51

In hole board test, the ethanolic leaf extract of *S. sisymbriifolium* at dose (200 mg/kg body weight) showed an increase in latency until the first head dipping (16.2±0.66) behavior compared with the control group which was statistically not significant ((p<0.05) (table 4.2.2.2). But the extract at dose (400 mg/kg body weight) showed a decrease in latency until the first head dipping (6.4±0.40) behavior compared with the control group which was also statistically highly significant (p<0.001) (table 4.2.2.2).

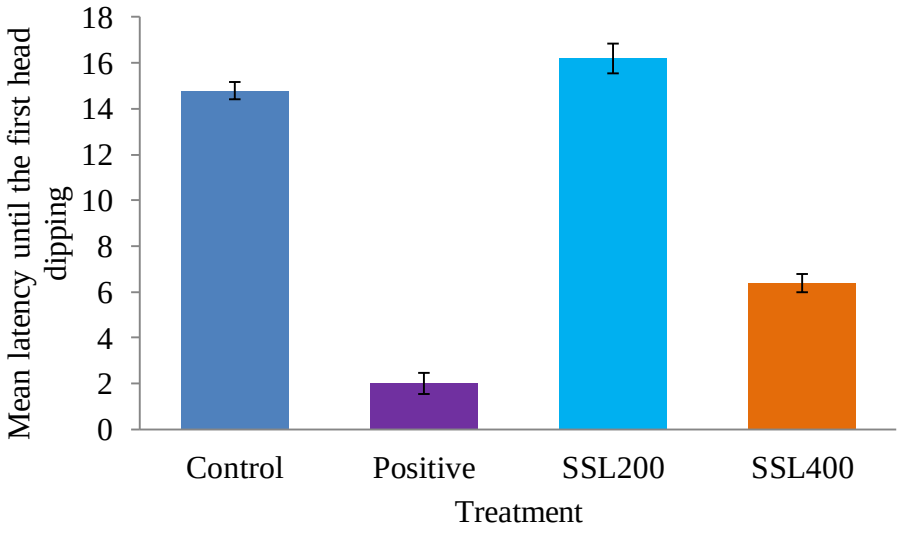


***Figure 4.2.2.2*:** Graphical presentation of the effect of ethanolic leaf extract of *S. sisymbriifolium* on mean latency until the first head dipping in hole board test.

4.2.3 Elevated Plus-Maze Test

Table 4.2.3:Effect of ethanolic leaf extract of *S. sisymbriifolium* on mean number of entries and mean no. of stretch attend postures, grooming and rearing in elevated plus-maze model.

Group	Dose	Mean no. of entry in (counts/5minutes)			Mean no. of stretch attend postures	Mean no. of grooming	Mean no. of rearing
		Open arm	Close arm	Center			
Control (1% tween 80 in saline)	0.5 ml/ mice; p.o.	1.2±0.49	9.4±1.91	4.2±0.20	9.2±1.42	3.8±0.58	14.2±1.66
Positive control (Diazepam)	1 mg/ Kg body weight; p.o.	1.6±0.81	12.0±3.03	4.6±0.75	8.6±2.29	6.2±0.80*	9.4±3.37
SSL200	200 mg/ Kg body weight; p.o.	0.8±0.37	15.4±0.74	4.6±0.60	8.2±1.11	0.8±0.37*	18.0±1.79
SSL400	400 mg/ kg body weight; p.o.	0.8±0.37	9.0±2.92	4.0±1.70	11.6±2.73	0.6±0.40*	10.0±3.03

Values are expressed as Mean±SEM (n=5); *: $p<0.05$, **: $p<0.001$ dunnett t-test as compared to Control.

In elevated plus-maze test (EPM), the ethanolic leaf extract of *S. sisymbriifoliumat* at both doses (200 and 400 mg/kg body weight) showed decrease mean number of entries into the open arm compare to control which were statistically (p<0.05) not significant (table 4.2.3). The extract at dose (200 mg/kg body weight) showed an increase mean number of entries into close arm which was statistically not significant (p<0.05) whereas the extract at dose (400 mg/kg body weight) showed decrease mean number of entries into close arm which was also statistically (p<0.05) not significant (table 4.2.3).

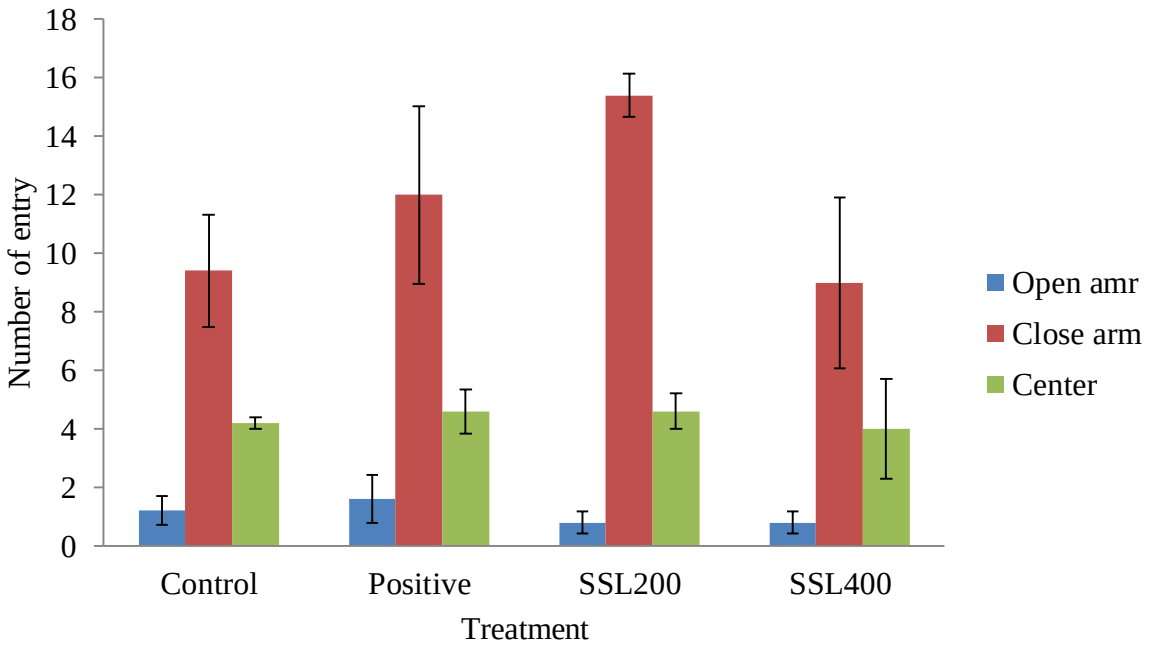


***Figure 4.2.3.1*:** Graphical presentation of the effect of ethanolic leaf extract of *S. sisymbriifolium* on number of entry into arms in elevated plus-maze test.Values are expressed as Mean±SEM (n=5).

Table 4.2.3.1:Effect of ethanolic leaf extract of *S. sisymbriifolium* on mean number of stretch attend postures in elevated plus-maze model.

Group	Dose	Mean no. of stretch attend postures
Control (1% tween 80 in saline)	0.5 ml/ mice; p.o.	9.2±1.42
Positive control (Diazepam)	1 mg/ Kg body weight; p.o.	8.6±2.29
SSL200	200 mg/ Kg body weight; p.o.	8.2±1.11
SSL400	400 mg/ kg body weight; p.o.	11.6±2.73

The extract at dose (200 mg/kg body weight) showed decrease mean number of stretch attend postures whereas the extract at dose (400 mg/kg body weight) showed an increase mean number of stretch attend postures. The both data were statistically not significant (p<0.05) (table 4.2.3.1).

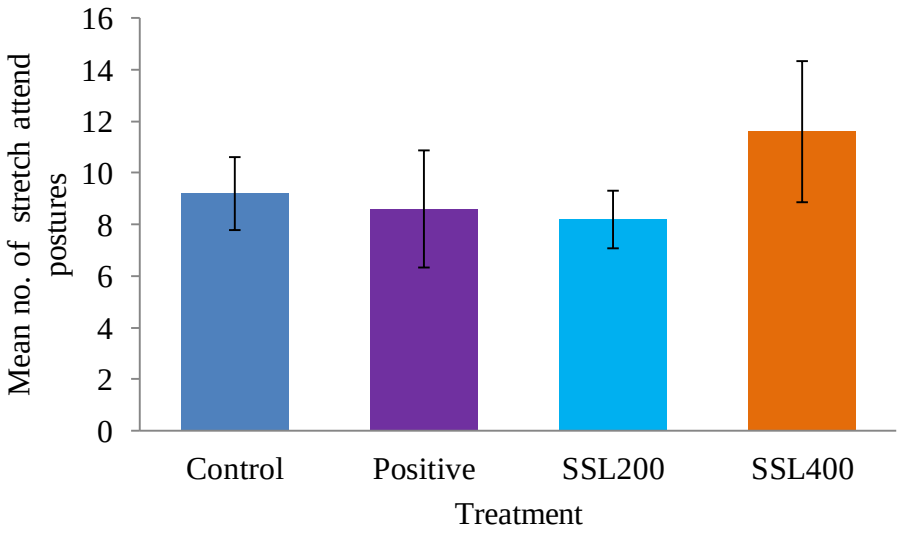


***Figure 4.2.3.2*:** Graphical presentation of the effect of ethanolic leaf extract of *S. sisymbriifolium* on mean number of stretch attends postures in elevated plus-maze test.

Table 4.2.3.2:Effect of ethanolic leaf extract of *S. sisymbriifolium* on mean number of grooming in elevated plus-maze model.

Group	Dose	Mean no. of grooming (counts/5minutes)
Control (1% tween 80 in saline)	0.5 ml/ mice; p.o.	3.8±0.58
Positive control (Diazepam)	1 mg/ Kg body weight; p.o.	6.2±0.80*
SSL200	200 mg/ Kg body weight; p.o.	0.8±0.37*
SSL400	400 mg/ kg body weight; p.o.	0.6±0.40*

Values are expressed as Mean±SEM (n=5); *: $p<0.05$, **: $p<0.001$ dunnett t-test as compared to Control.

Group	Mean	Standard deviation	Standard error Mean	95% confidence interval for mean	
				Lower bound	Upper bound
Control	3.80	1.30	0.58	2.18	5.42
Positive	6.20	1.79	0.80	3.98	8.42
SSL 200	0.80	0.84	0.37	-0.24	1.84
SSL 400	0.60	0.89	0.40	-0.51	1.71

The ethanolic leaf extract of *S. sisymbriifoliumat* at both doses (200 and 400 mg/kg body weight) showed decrease mean number of grooming compare to control which were statistically ($p<0.05$) significant (table 4.2.3.2).

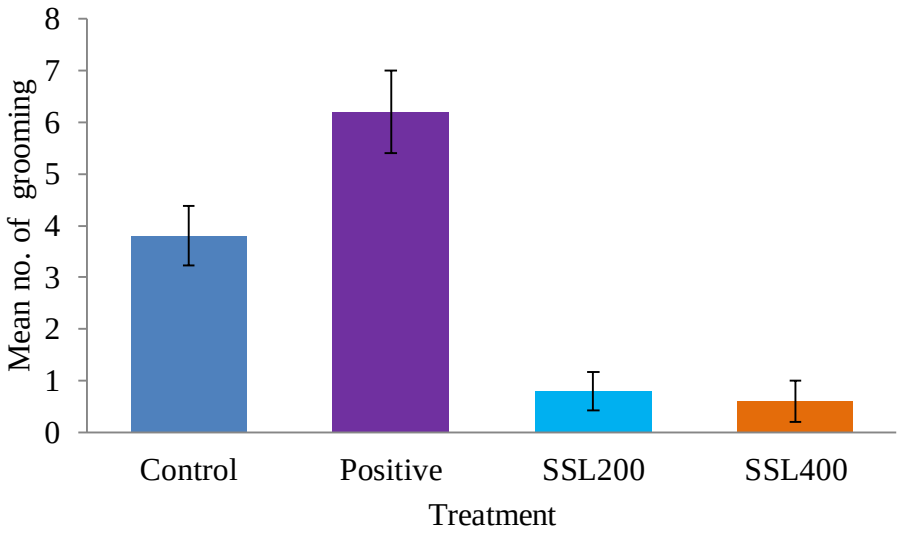


***Figure 4.2.3.3*:** Graphical presentation of the effect of ethanolic leaf extract of *S. sisymbriifolium* on mean number of grooming in elevated-plus maze test.

Table 4.2.3.3:Effect of ethanolic leaf extract of *S. sisymbriifolium* on mean number of rearing in elevated plus-maze model.

Group	**Dose**	**Mean no. of rearing (counts/5minutes)**
Control (1% tween 80 in saline)	0.5 ml/ mice; p.o.	14.2±1.66
Positive control (Diazepam)	1 mg/ Kg body weight; p.o.	9.4±3.37
SSL200	200 mg/ Kg body weight; p.o.	18.0±1.79
SSL400	400 mg/ kg body weight; p.o.	10.0±3.03

Values are expressed as Mean±SEM (n=5); *: $p<0.05$, **: $p<0.001$ dunnett t-test as compared to Control.

Group	Mean	Standard deviation	Standard error Mean	95% confidence interval for mean Lower bound	Upper bound
Control	14.20	3.70	1.66	9.60	18.80
Positive	9.40	7.54	3.37	0.42	18.76
SSL 200	18.00	4.00	1.79	13.03	22.97
SSL 400	10.00	6.78	3.03	1.58	18.42

The extract at dose (200 mg/kg body weight) showed an increase mean number of rearing whereas the extract at dose (400 mg/kg body weight) showed decrease mean number of rearing. The both data were statistically not significant (p<0.05) (table 4.2.3.3).

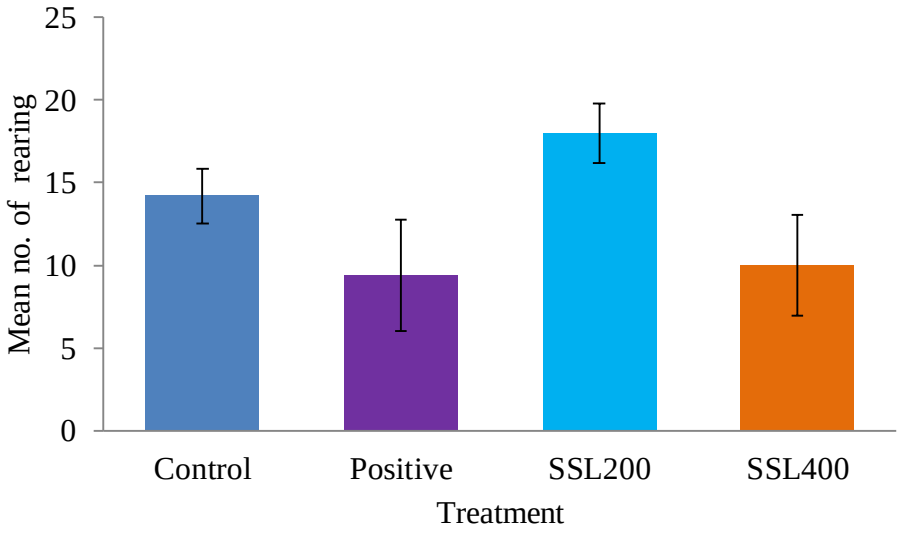


***Figure 4.2.3.4*:** Graphical presentation of the effect of ethanolic leaf extract of *S. sisymbriifolium* on mean number of rearing in elevated plus-maze test.

4.3 Castor Oil Induced Anti-Diarrheal Activity Test

Table 4.3.1: Effect of ethanolic leaf extract of *S. sisymbriifolium* on total latent period, mean no. of stool and total weight of faecal output in castor oil- induced anti-diarrheal activity test.

Group	Dose	Total latent period (min)	Mean no. of stool	Total weight of faecal output
Control	0.5 ml/mice; p.o.	42.8±1.16	14.4±1.75	0.935±0.02
Positive	2 mg/kg body weight; p.o.	95.2±1.71**	8.6±0.75*	0.731±0.03*
SSL200	200 mg/kg body weight; p.o.	56.8±1.24**	9.6±0.81*	0.768±0.04*
SSL400	400 mg/kg body weight; p.o.	7.2±0.86**	6.6±0.75**	0.731±0.03**

Values are expressed as Mean±SEM (n=5); *: $p<0.05$, **: $p<0.001$ dunnett t-test as compared to Control.

Table 4.3.2: Effect of ethanolic leaf extract of *S. sisymbriifolium* on total latent period in castor oil- induced anti-diarrheal activity test.

Group	Dose	Total latent period (min)
Control (1% tween 80 in saline)	0.5 ml/ mice; p.o.	42.8±1.16
Positive control (Loperamide)	2 mg/ Kg body weight; p.o.	95.2±1.71**
SSL200	200 mg/ Kg body weight; p.o.	56.8±1.24**
SSL400	400 mg/ kg body weight; p.o.	7.2±0.86**

Values are expressed as Mean±SEM (n=5); **: $p<0.001$ dunnett t-test as compared to Control.

Group	Mean	Standard deviation	Standard error mean	95% confidence interval for mean	
				Lower bound	Upper bound
Control	42.80	2.59	1.16	39.59	46.01
Positive	95.20	3.83	1.71	90.44	99.96
SSL200	56.80	2.77	1.24	53.35	60.25
SSL400	7.20	1.92	0.86	4.81	9.59

In castor oil- induced diarrhea test, the ethanolic leaf extract of *S. sisymbriifolium* showed highly significant antidiarrheal activity in a dose dependent manner. The extract at a dose (200 mg/kg body weight) showed an increase mean latent period for diarrhea episode (0.95 hour) (table 4.3.2) which was statistically highly significant ($p<0.001$) compare to control (0.71 hour). But the extract at another dose (400 mg/kg body weight) caused a decrease in latent period for diarrhea episode (0.12 hour) which was also statistically highly significant ($p<0.001$) compare to control (table 4.3.2).

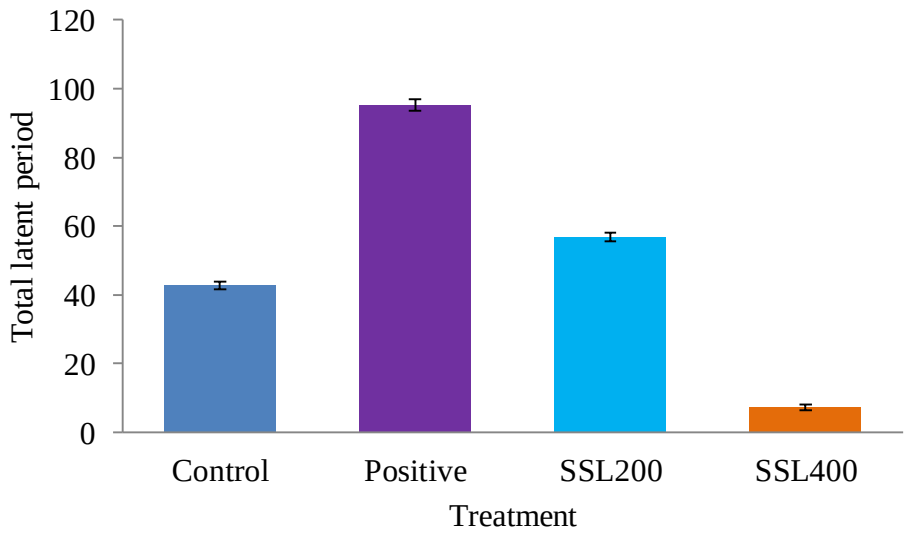


Figure 4.3.1:Graphical presentation of the effect of ethanolic leaf extract of *S. sisymbriifolium* on total latent period in castor oil induced anti-diarrheal activity test.

Table 4.3.3: Effect of ethanolic leaf extract of *S. sisymbriifolium* on mean no. of stool in castor oil- induced anti-diarrheal activity test.

Groups	Dose	Mean no. of stool
Control (1% tween 80 in saline)	0.5 ml/mice; p.o.	14.4±1.75
Positive control(loperamide)	2 mg/kg body weight; p.o.	8.6±0.75*
SSL	200 mg/kg body weight; p.o.	9.6±0.81*
SSL	400 mg/kg body weight; p.o.	6.6±0.75**

Values are expressed as Mean±SEM (n=5); *: $p<0.05$, **: $p<0.001$ dunnett t-test as compared to Control.

Group	Mean	Standard deviation	Standard error mean	95% confidence interval for mean	
				Lower bound	Upper bound
Control	14.40	3.91	1.75	9.54	19.26
Positive	8.60	1.67	0.75	6.52	10.68
SSL200	9.60	1.82	0.81	7.34	11.86
SSL400	6.60	1.67	0.75	4.52	8.68

In castor oil- induced diarrhea test, the ethanolic leaf extract of *S. sisymbriifolium* at both doses (200 and 400 mg/kg body weight) showed a significant ($p<0.05$) decrease in mean number of stool in a dose dependent manner (table 4.3.3). The extract at the dose (400 mg/kg body weight) showed a mean number of stools (6.6±0.75) which was statistically highly significant ($p<0.001$) compare to control (14.4±1.75) (table 4.3.3).

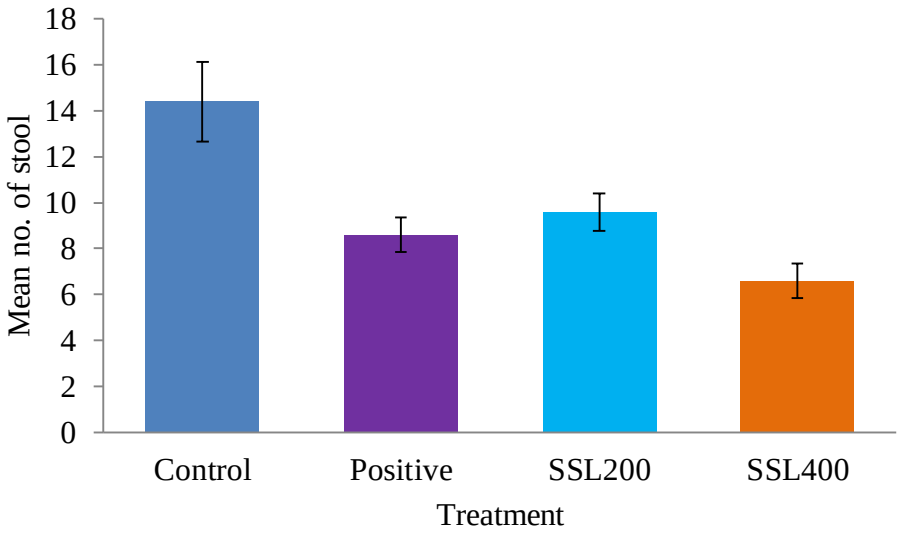


***Figure 4.3.2*:**Graphical presentation of the effect of ethanolic leaf extract of *S. sisymbriifolium* on mean number of stool in castor oil induced anti-diarrheal activity test.

Table 4.3.4: Effect of ethanolic leaf extract of *S. sisymbriifolium* on total weight of faecal output in castor oil- induced anti-diarrheal activity test.

Groups	Dose	Total weight of faecal output
Control (1% tween 80 in saline)	0.5 ml/mice; p.o.	0.935±0.02
Positive control(loperamide)	2 mg/kg body weight; p.o.	0.731±0.03*
SSL	200 mg/kg body weight; p.o.	0.768±0.04*
SSL	400 mg/kg body weight; p.o.	0.731±0.03**

Values are expressed as Mean±SEM (n=5); *: $p<0.05$, **: $p<0.001$ dunnett t-test as compared to Control.

Group	Mean	Standard deviation	Standard error mean	95% confidence interval for mean	
				Lower bound	Upper bound
Control	0.935	0.044	0.020	0.880	0.990

Positive	0.731	0.063	0.028	0.652	0.809
SSL200	0.768	0.081	0.036	0.668	0.868
SSL400	0.731	0.067	0.030	0.647	0.815

In castor oil- induced diarrhea test, the ethanolic leaf extract of *S. sisymbriifolium* at both doses (200 and 400 mg/kg body weight) showed a significant ($p<0.05$) decrease in total weight of faecal output in a dose dependent manner (table 4.3.4). The extract at the dose (400 mg/kg body weight) showed a total weight of faecal output (0.731±0.03) which was statistically highly significant ($p<0.001$) compare to control (0.935±0.02) (table 4.3.4).

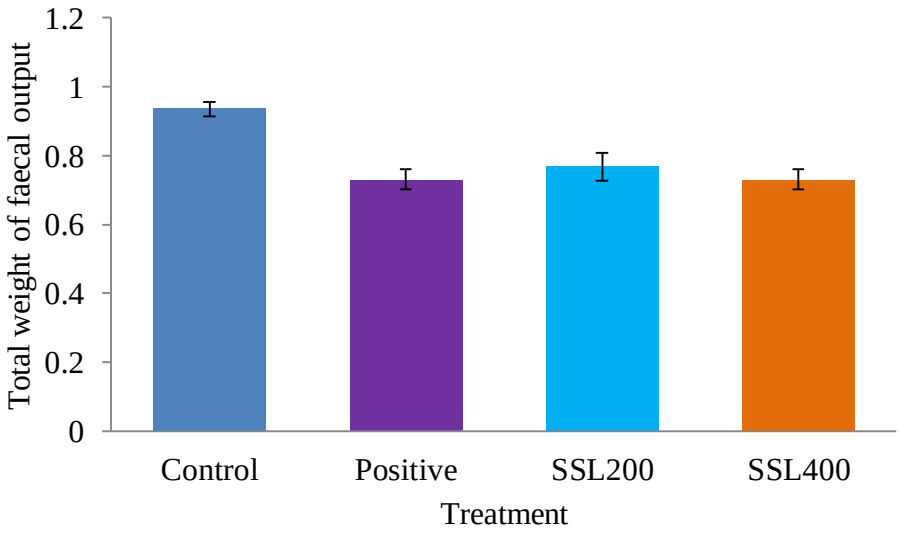


***Figure 4.3.3*:** Graphical presentation of the effect of ethanolic leaf extract of *S. sisymbriifolium* on total weight of faecal output in castor oil induced anti-diarrheal activity test.

4.4 Brine Shrimp Lethality Bioassay

Table 4.4:LC_{50} Values *S. sisymbriifolium* crude leaf extract and *n*-hexane, chloroform, carbon tetrachloride and aqueous fraction of the crude extract compared to $KMnO_4$.

Test Compound	LC_{50} (µg/ml)	Best Fit Equation	R^2
$KMnO_4$	11.898	y = 5.181x - 0.572	0.851
SSL (crude ethanol)	61.66	y = 3.781x - 1.767	0.846
SSL (*n*-hexane fraction)	38.90	y = 5.049x - 3.026	0.886
SSL (chloroform fraction)	13.97	y = 3.391x + 1.117	0.899
SSL (carbon tetrachloride fraction)	203.33	y = 3.05x - 2.04	0.769
SSL (aqueous fraction)	247.638	y = 3.753x - 3.984	0.756

$KMnO_4$: potassium permanganateSSL: *S. sisymbriifolium* leaf

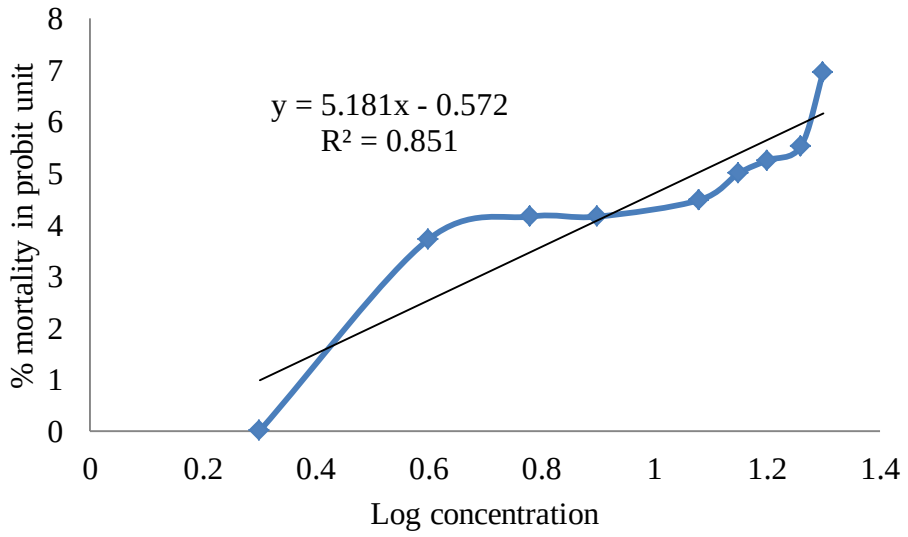


***Figure 4.4.1*:**Graphical presentation of percentage of mortality in probit unit versus log concentration curve of $KMnO_4$ in Brine Shrimp Nauplii.

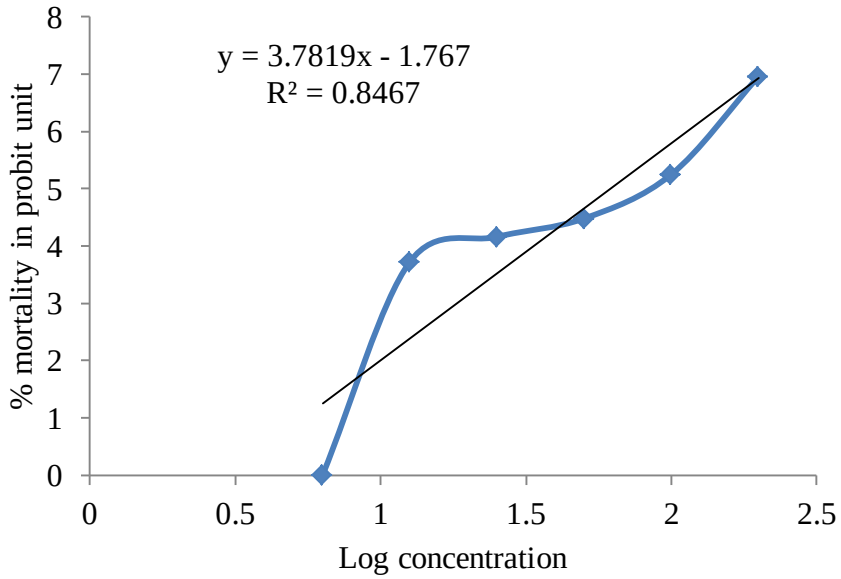


***Figure 4.4.2*:**Graphical presentation of percentage of mortality in probit unit versus log concentration curve of crude ethanolic leaf extract of *S. sisymbriifolium* in Brine Shrimp Nauplii.

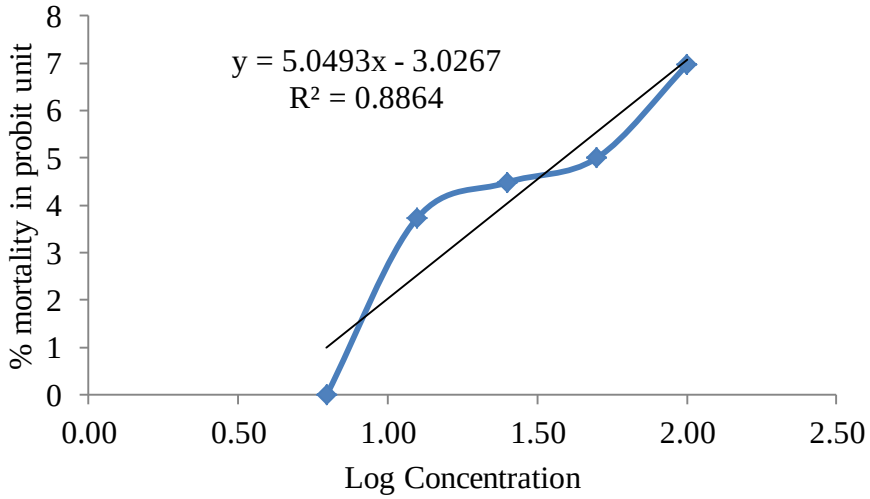


***Figure 4.4.3*:**Graphical presentation of percentage of mortality in probit unit versus log concentration curve of *n*-hexane fraction of *S. sisymbriifolium*extract in Brine Shrimp Nauplii.

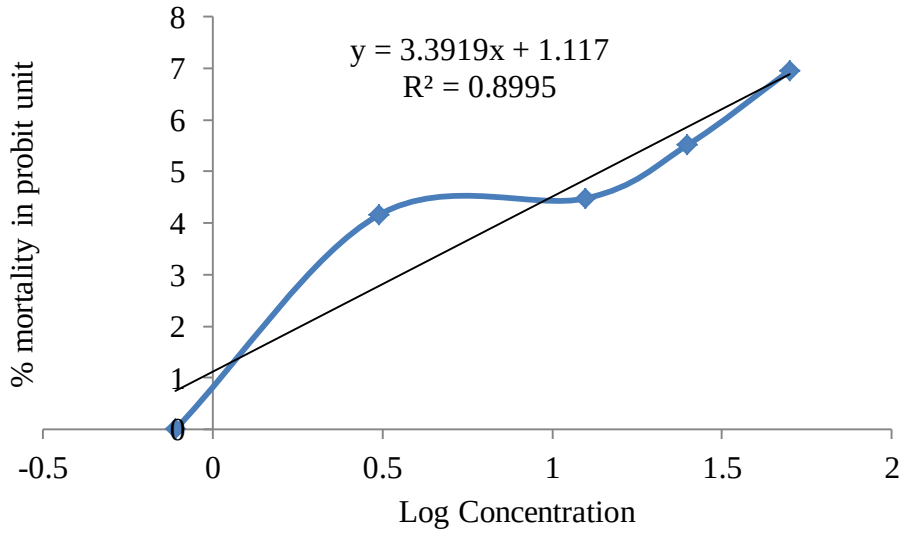


***Figure 4.4.4*:**Graphical presentation of percentage of mortality in probit unit versus log concentration curve of chloroform fraction of *S. sisymbriifolium*extract in Brine Shrimp Nauplii.

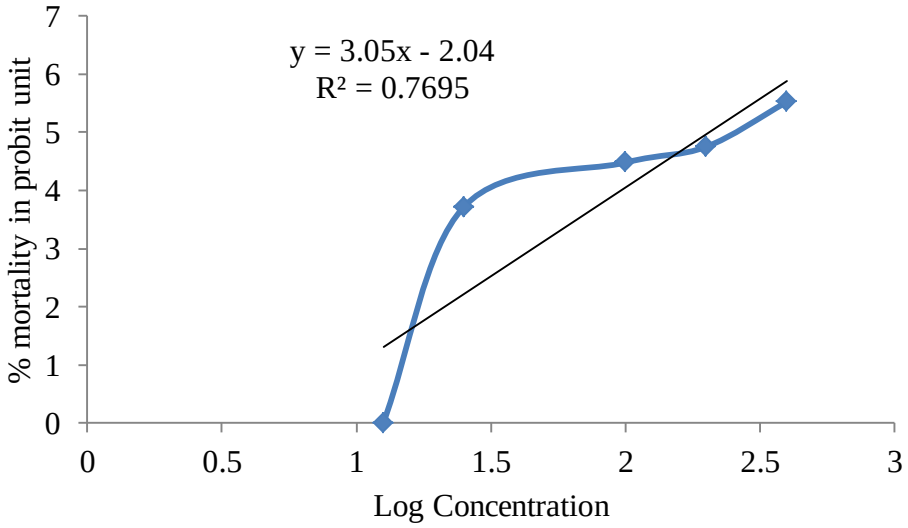


***Figure 4.4.5*:**Graphical presentation of percentage of mortality in probit unit versus log concentration curve of carbon tetrachloride fraction of *S. sisymbriifolium* leaf extract in Brine Shrimp Nauplii.

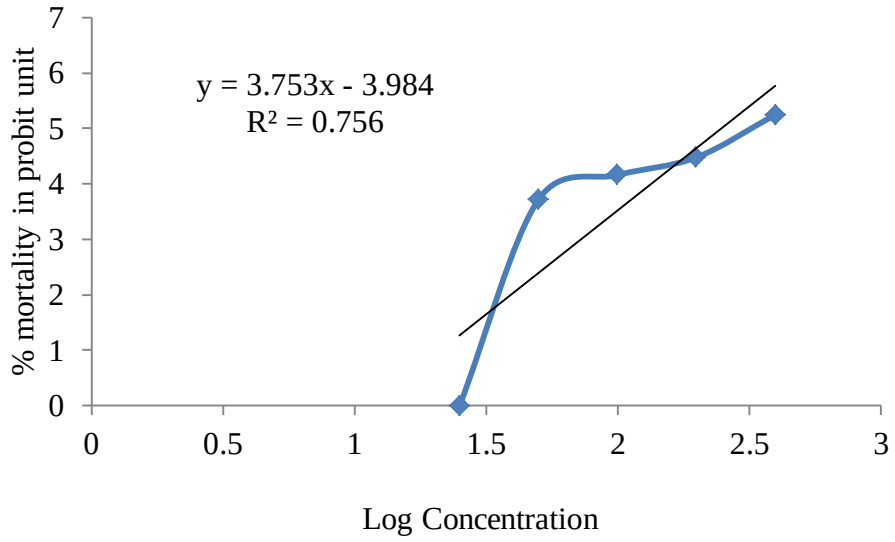


***Figure 4.4.6*:**Graphical presentation of percentage of mortality in probit unit versus log concentration curve of aqueous fraction of *S. sisymbriifolium* leaf extract in Brine Shrimp Nauplii.

In the brine shrimp lethality test, the LC_{50} values of the crude ethanolic leaf extract of *S sisymbriifolium* and its *n*-hexane, chloroform, carbon tetrachloride, aqueous fractions were found to be 61.660µg/ml, 38.900 µg/ml, 13.970 µg/ml, 203.330 µg/ml and 247.638 µg/ml respectively (table 4.4). Among all this values the chloroform and *n*-hexane fractions showed lowest LC_{50} values 13.970 and 38.900 µg/ml respectively. The highest LC_{50} values were showed by the carbon tetrachloride (203.330 µg/ml) and aqueous (247.638 µg/ml) fraction of the plant extract(table 4.4). The positive control $KMnO_4$ showed LC_{50} value of 11.898 µg/ml(table 4.4.).

Chapter: 5

DISCUSSION

5. DISCUSSION

5.1 Acetic Acid Induced Writhing Test

The crude ethanolic extract of *S. sisymbriifolium* showed significant ($p<0.05$; $p<0.001$) analgesic action compared to the negative control at two dose levels i.e. 200 and 400 mg/kg body weight (table 4.1). The significantpain reduction by the plant extract might be due to the presence of analgesic principles acting with the prostaglandin pathways.

Preliminary qualitative phytochemical screening reveals the presence of alkaloids, flavonoids, steroids and tannins in *S. sisymbriifolium* (Shilpi *et al.*, 2005).Flavonoids were reported to have a role in analgesic activity primarily by targeting prostaglandins (Rajnarayana *et al.* 2001; Rao *et al*., 1998). There are also reports on the role of tannins in anti-nociceptive activity (Vanu *et al*., 2006). Besides alkaloids are well known for their ability to inhibit pain perception (Uche *et al*., 2008). Findings in this study justify the use of *S. sisymbriifolium* as an analgesic in Indian traditional medicine (Ferro *et al*., 2005). So, further detailed investigations are needed to determine the actual mechanism by which the plant shows its non-narcotic analgesic activity.

5.2 Evaluation of Neuropharmacological Activity

5.2.1 Hole Cross Test

In the hole cross test, the ethanolic leaf extract of *S. sisymbriifolium* showed significant ($p<0.05$) decrease in locomotion activity at low dose (200 mg/kg body weight) whereas it showed significant ($p<0.05$) increase in locomotion activity at higher dose (400 mg/kg body weight) (table 4.2.1). This indicates that low to medium doses of the plant extract had sedative effect while higher dose had CNS stimulatory effect.

The sedative effect of the plant extract at low to medium doses may be due to interaction with central presynaptic α_2A-receptor as partial agonist by decreasing noradrenergic drive resulting in CNS depression, as decrease in central sympathetic drive is related to depression (Barar, 2007). Gamma-amino-butyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system. CNS depressant drugs mainly exert their action through GABAA receptor (Kolawole *et al*., 2007). So, the extract of *S. sisymbriifolium* may acts by hyperpolarization of the CNS via GABA receptor or benzodiazepine receptor located adjacent to the GABA receptor.

The CNS stimulatory effect of the plant extract at higher doses may involve the stimulation of norepinephrine, dopamine, serotonin and nicotinic cholinergic systems in the CNS (Kurkin and Zapesochnaya, 1986). The extract may also increase the permeability of the blood-brain barrier to precursors of dopamine and serotonin and also improve cerebral circulation (Saratikov *et al*., 1978).

However, further studies are needed to determine the exact phytoconstituents that are responsible for the neuropharmacological activities of the ethanolic leaf extract of *S. sisymbriifolium.*

5.2.2 Hole Board Test

The crude ethanolic extract of *S. sisymbriifolium*at dose (200 mg/kg body weight) showed highly significant (p<0.001) increase number of head dipping compared to the negative control(table 4.2.2.1). The increase number of head dipping by the plant extract in hole board test is not only a representation of hyperactivity but also a reflection of anxiolytic effect of the extract. The mechanism of anxiolytic action of the extract may involve as action on GABAergic transmission; however further studies are needed to ascertain this (File and Pillow, 1985).

The ethanolic extract of *S. sisymbriifolium* at higher dose (400 mg/kg body weight) showed highly significant (p<0.001) decrease in both the number of head dipping and the latency until the first head dipping(table 4.2.2.1).The decrease in number of head dips reveals sedative behavior (File and Pellow, 1985) and is thus a measure of CNS depressant activity of the plant extract (Viswanatha *et al.*, 2006). It also indicates a decrease in the curiosity or exploratory behavior of the test animal (Suba *et al.*, 2002).

Earlier reports on the chemical constituents of plants and their pharmacology suggest that plant containing flavonoids, saponins, and tannins possess activity against many CNS disorders (Adeyemi *et al.*, 2006).Preliminary qualitative phytochemical screening reveals the presence of alkaloids, flavonoids, steroids and tannins in *S. sisymbriifolium* (Shilpi *et al.*, 2005).It is possible that the mechanism of anxiolytic and sedative effects of *S. sisymbriifolium* could be due to the binding of any of these phytochemicals to the $GABA_A$-BZDs complex.

It is possible that the sedative activity of ethanolic extract of *S. sisymbriifolium*is mediated by GABAergic pathway, since GABAergic transmission can produce profound sedation in mice (Gottesmann, 2002). The inhibitory action of GABA consists in the opening of chloride channels to allow hyperpolarizing the membrane, leading to CNS depression and resulting in sedative and hypnosis activity. Glutamate and GABA are quantitatively the most important excitatory and inhibitory neurotransmitters, respectively, in the mammalian brain (Rang *et al.*, 2003). Thus, receptors for these two neurotransmitters are regarded as important targets for psychotropic drugs.

So the data lead to conclude that the crude ethanolic extract of *S. sisymbriifolium* possess potential sedative effects on the central nervous system at higher doses.

5.2.3 Elevated Plus-Maze Test

In elevated plus-maze test, the ethanolic leaf extract of *S. sisymbriifoliumat* at both doses (200 and 400 mg/kg body weight) showed decrease mean number of entries into the open arm compare to control which were statistically (p<0.05) not significant(table 4.2.3). But the extract showed decrease mean number of grooming at both doses (200 and 400 mg/kg body weight) compare to control which were statistically (p<0.05) significant (table 4.2.3.2).

5.3 Castor Oil Induced Anti-Diarrheal Activity Test

The crude ethanolic extract of *S. sisymbriifolium* showed significant (p<0.05) antidiarrheal activity compared to the negative control at both the doses (200 and 400 mg/kg body weight) in a dose dependent manner throughout the observation period (table 4.3.1). The antidiarrheal activity was characterized as decreased frequency of defecation and total weight of faecal output and increase mean latent period.

It has been shown that E type of prostaglandins cause diarrhea in experimental animals as well as human beings(Eakins *et al*., 1972). Their mechanism has been associated with dual effects on gastrointestinal motility as well as on water and electrolyte transport (Dajani*et al*., 1975). PGE_2 also inhibit the absorption of glucose, a major stimulus to intestinal absorption of water and electrolytes(Jaffe *et al.*, 1979). From the acetic acid induced writhing test it has been found that the extract exhibited significant analgesic activity. Based on the observation, it seems that the anti-diarrheal effect of the ethanolic leaf extract of *S. sisymbriifolium* may be due to the inhibition of prostaglandin biosynthesis or by decreasing the peristaltic movement.

Preliminary qualitative phytochemical screening reveals the presence of alkaloids, flavonoids, steroids and tannins in *S. sisymbriifolium* (Shilpi *et al.*, 2005).Tannins can evoke an antidiarrheal

effect since these substances may precipitate proteins of the enterocytes; reduce peristaltic movement and intestinal secretions (Okudo *et al.*, 1989). The anti-diarrheal activity of flavonoids has been ascribed to their ability to inhibit intestinal motility and hydro electrolytic secretion (Giulia *et al.*, 1993) which is known to be altered in this intestinal condition. *In vitro* and *in vivo* experiments have shown that flavonoids are able to inhibit the intestinal secretory response, induced by prostaglandin E_2(Sanchez *et al*., 1997). In addition, flavonoids possess antioxidant properties (Su *et al.*, 2000) which are presumed to be responsible for the inhibitory effects exerted upon several enzymes including those involved in the arachidonic acid metabolism (Mora *et al.*, 1990).

On the basis of the result of castor oil induced diarrhea, it can be concluded that the crude ethanolic leaf extract of *S. sisymbriifolium* possesses significant ($p<0.05$) anti-diarrheal activity which may due to the inhibitory effect both on gastrointestinal propulsion and fluid secretion. The data obtained is consistent with the use of the plant as a non specific anti-diarrheal agent in folklore medicine. Further detailed investigations are needed to determine the actual mechanism by which the plant shows its anti-diarrheal activity.

5.4 Brine Shrimp Lethality Bioassay

In brine shrimp lethality test, plant extracts are considered as bioactive when $LC_{50}$is 1000 µg/ml or less (Meyer *et al.*, 1982). So, from the results of the brine shrimp lethality bioassay it can be well predicted that the crude ethanolic leaf extract of *S sisymbriifolium* and its fractions are biologically active and have considerable cytotoxic potency.

The lowest LC_{50} values of the chloroform (13.970 µg/ml) and *n*-hexane (38.900 µg/ml) fractions of the extract (table 4.4)indicate the presence of potential candidates of cytotoxic compounds.

The LC_{50} value of crude ethanolic leaf extract was 61.660µg/ml(table 4.4) which is also a potential candidate of cytotoxic compounds. However, varying degree of lethality was observed with exposure to different dose levels of the test samples. The degree of lethality was directly proportional to the concentration ranging from significant with the lowest concentration (0.781µg/ml) to highly significant with the highest concentration (400µg/ml). In other words, mortality increased gradually with the increase in concentration of the test samples.

However, further investigations using carcinoma cell line are necessary to isolate the active compound(s) responsible for the cytotoxic activity.

Chapter: 6

CONCLUSION

6. CONCLUSION

The results obtained in this study indicate that the ethanolic leaf extract of *S. sisymbriifolium*and its *n*-hexane, chloroform, carbon tetrachloride and aqueous fractions possess bioactive principles that havesignificant analgesic, neuropharmacological, anti-diarrheal and cytotoxic activities in different *in vivo* animal model systems. The medicinal values of the plant leaves may be related to their constituent phytochemicals. So, further detailed investigations are needed to isolate and identify the active compounds present in the plant extract and its various fractions and their efficacy need to be done. It will help in the development of new novel and safe drugs for the treatment of various diseases.

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