BIOASSAY OF DIFFERENT EXTRACT OF *Clerodendrum infortunatum* ON SOME VEGETABLE SEEDS WITH THEIR CHEMICAL INVESTIGATION

A Thesis

BY

MD. RAFIQUL ISLAM

Student No: 1005105

Session: 2010-2011

MASTER OF SCIENCE (M.S.)
IN
BIOCHEMISTRY AND MOLECULAR BIOLOGY

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY
HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR

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Approved as to the style and content by:

(Professor Dr. M. Afzal Hossain)
Supervisor

(Professor Dr. Balaram Roy)
Co-Supervisor

(Professor Dr. Bikash Chandra Sarker)

Chairman
Examination Committee
&
Chairman

Department of Biochemistry and Molecular Biology
Hajee Mohammad Danesh Science and Technology University
Dinajpur

Winter, 2011

DEDICATED TO

MY BELOVED PARENTS

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The Author

ABSTRACT

An experiment was conducted on bioassay of different extracts of leaves of *Clerodendrum infortunatum* for each vegetable crop such as, kolmishak, bhendi, and borboti with the attempt for chemical investigation on effective plant extract. The chloroform extract of *C. infortunatum* significantly increased and enhanced germination, growth of shoot length and root length of yard long bean and swamp cabbage seeds compared with control. But germination, growth of shoot length and root length of lady's finger is increased and enhanced by control treatment. The chloroform extract of C. infortunatum showed the highest germination at 7 days after sowing for these vegetable crops. Increased and enhanced but the lowest germination percentage (12.00%) was in T_1 at 2 DAS in swamp cabbage. Highest germination percentage was in T_2 at 7 DAS for long yard bean, swamp cabbage, lady's finger whereas lowest germination percentage was in T_1 at 2 DAS in those vegetables. Shoot length of swamp cabbage at different days after sowing influenced significantly by the effects of different extract. At 11 Days after sowing (DAS) with the leaf extract of *C. infortunatum* showed the highest shoot length 5.867 cm while the lowest shoot length (0.9267 cm) was recorded in control. Root length of swamp cabbage seedling at different days after sowing was lowest at 5 DAS and showed an increasing trend up to 11 DAS. Incase of lady's finger the germination percentage was highest found in T_c. At 5 DAS highest germination found in T_c (36.00). Shoot length of ladies finger at different days after sowing influenced significantly by the effects of different leaf extract but highest result found on control. Root length of lady's finger seedling at different days after sowing was enhanced by control treatment. TLC of chloroform extracts of *C. infortunatum* at hexane indicated that it contains five compounds. Separation of individual components and their structure determination by spectral study in progress which will be reported in due course.

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ABBREVIATIONS AND ACRONYMS

CI	: *Clerodendrum infortunatum*
DAS	: Days After Sowing
DMRT	: Duncan's Multiple Range Test
et al.	: and the others
IR	: Infrared Ray
MS	: Mass Spectroscopy
NMR	: Nuclear Magnetic Resonance
TLC	: Thin Layer Chromatography

CHAPTER 1

INTRODUCTION

Plant kingdom supplies food, fuel, fodder, shelter, wind breaker, raw material for cloth, medicine etc. for human being. There are different types of plants such as medicinal plants, fruit trees, herbal plants, flower plants etc. Different types of naturally occurring organic and bioorganic compounds have been isolated from them. Most of them have effective medicinal, insecticidal, pesticidal or toxic value. Plants are the richest source of renewable bio-active organic chemicals. The total number of plant chemicals exceeds 4000,000 out of these chemicals; secondary metabolites play a major role in the plants applied in agriculture field.

Synthetic chemicals, which are highly toxic, require careful handling, whereas botinaoicals, which form a part of the farmers habited, can be safely handled. Furthermore, botanicals will fit into the low input sustainable farming system preferred today. Researches on herbal plants have been conducted in an isolated fashion. Biologists, especially entomologists, have detected, extracted and demonstrated biologically active compounds, where as chemists have confined themselves to identifying the various compounds present in a plant with interest in their biological activity. This situation has helped developed countries to get clues from our work to exploit our natural resources for their commercial interests.

Clerodendrum infortunatum (common name Bhat; synonyms *Clerodendrum viscosum* Vent. and *Volkameria infortunata* Roxb.) is a perennial shrub belonging to the family Lamiaceae, also sometimes classified under Verbenaceae. The major compounds are sterols, sugars, flavonoids and saponins. Novel crystalline compounds such as clerodolone, clerodone, clerodol and a sterol designated clerosterol have been isolated from the root. A paste of leaves and roots are applied externally over skin diseases especially fungal infections and alopecia. Fresh leaves are given for diarrhoea, liver disorders and headache. (Duke, 2010).Various parts of the plant have been used by tribes in colic, scorpion string, snake bite, tumor and certain skin diseases (Nadkarni *et al.*, 2002) also used in Indian folk medicine as in the treatment of bronchitis, asthma, fever, diseases of the blood, inflammation, burning sensation and epilepsy (Kapoor, 2001). Fresh juice of the leaves has been used as vermifuge and in treatment of malaria (Goswami, 1998).

Clerodendrum infortunatum leaves on preliminary chemical analysis are found to contain saponin, clerodin (a bitter diterpene) (Chopra *et al.*, 1998) and some enzymes. Leaves also contain a fixed oil which consists of Glycerides of Lenoleic, oleic, stearic sand lignoceric acid (Kapoor, 2001). Traditionally the leaf and root are widely used as antidandruff, antipyretic, ascaricide, laxative, vermifuge, and in treatments of convulsion, diabetes, gravel, malaria, scabies, skin diseases, sore, spasm, scorpion sting, snake bite and tumor. (Sharma, 2001; Rahman and Zaman 1989). In Thai medicine the leaves and root are known to be diuretic; and used for treatment of intestinal infections and kidney disfunction; when boiled or ground with water, it is taking to increase milk secretion for post-labor. In many traditional practices the leaves and root are widely used as anti hyperglycemic. (Modi, 2010). The use of herbal medicine has become increasingly popular worldwide and medicinal plants are believed to be an important source of new chemical substances with potential therapeutic effects (Estakhr *et al.*, 2011).

From above reports it is clearly observed that plant extract especially *C. infortunatum* having biological activity are relatively safe to the user, non-target organisms and environment. They are cheaper and renewable.

Keeping this view in mind the research has been under taken as title "Bioassay of different extract of *Clerodendrum infortunatum* on some vegetable seeds with their chemical investigation" to assess the following objectives-

i) To check the bioassay of different extract of *Clerodendrum infortunatum* on some vegetable seeds.

ii) To isolate the different bioactive compounds from chloroform extract of *Clerodendrum infortunatum* and

iii) To determine the structure of isolated compound by (^{1}H-NMR, ^{13}C-NMR, IR, and MS study).

CHAPETR 2

REVIEW OF LITERATURE

Study on natural products is always an interesting target for scientists over decades, especially on plants. Historically, plants (fruits, vegetables, medicinal herbs, etc.) have provided a good source of a wide variety of compounds, such as phenolic compounds, nitrogen compounds, vitamins, terpenoids and some other secondary metabolites, which show valuable bioactivities, e.g., antioxidant, antidiabetic, anti-inflammatory, antitumor, antimutagenic, anti-carcinogenic, antibacterial, or antiviral activities. In many oriental countries (China, Japan, etc), the traditional herbal medicines have been widely used for thousands of years. Herbal plants have become the main object of chemists, biochemist, and pharmaceutics. Their research plays an important role for discovering and developing new drugs, which are having hopefully more effectiveness and no side actions like most modern drugs. Besides focusing on chemistry of compounds from any plants, the studies of herbal plant are based on folkloric reputation and traditional uses. Biological activity of different extracts of leaves of medicinal plants is relatively a new approach. In addition, the isolation and identification on these plants are due to the activities of their extracts and fractions. Therefore, literatures some way linking to the subject of interest from home and abroad are reviewed and outlined below under the following sections.

Sanyasi and Varma (2008) viewed that in Chittoor, Ananthapur districts of Andhra Pradesh and Southern India C. phlomidis is used for alleviating diseases of livestock by the local traditional herbal practitioners. Leaves are given orally twice daily to cure convulsive seizures and trypanosomosis infection until cured

An ethanolic extract of leaves (150 and 300 mg/kg, i.p.) was evaluated for analgesic activity in albino mice (either sex, 20 to 25 g) by Eddy's hot plate method. The extract at 300 mg/kg showed significant activity, supporting the folklore claim as analgesic (Srinivasa et al. 2007).

C. myricoides a species from Southern Africa was also tested positive for its antimalarial activity against both sensitive and resistant strains of *P. falciparum* with IC50< 30 µg/ml , it also showed 31.7% suppression in parasitaemia against cloroquine tolerant strain of *Plasmodium berghei* NK65 (Muregi *et al.* 2007).

Vadnere (2007) reported that, An aqueous extract (yield 7.9% w/w) of leaves was studied for anti-asthmatic activity in male albino mice (Swiss strain, 22 to 25 g). The effect of extract (2, 4, 10 mg/mL) on goat tracheal chain was also studied, indicating a significant activity at 4 and 10 mg/mL with the relaxant effect (depression of histamine receptor 1). The extract at dose levels of 25, 50 and 100 mg/kg, i.p. in milk-induced eosinophilia showed significantly at 100 mg/kg the antagonizing effect. In three-day treatment by the aqueous extract (25, 50 and 100 mg/kg, i.p.), the 100 mg/kg dose showed 73.25% protection of mast cell degranulation. The aqueous extract, when studied for capillary permeability (25, 50 and 100 mg/kg, i.p.), at 100 mg/kg dose level significantly decreased transmittance, indicating its effect on optical density of the eye. The overall study lends credence to the beneficial use of aqueous extract in the treatment of asthma and related conditions.

Anti infective compounds from natural resources are of great interest as the existing drugs are getting less effective due to increased tolerance of microorganisms. A number of species from the genus *Clerodendrum* were documented in ancient texts for their antimicrobial action. To validate these claims, research work was carried out with various Gram positive and Gram negative bacterial strains and also with fungal and viral pathogens.Twenty microliters of defatted methanolic (yield 4.4% w/w) and acetone (yield 1.7% w/w) extracts of stems and leaves (combined) were screened for five Gram-positive bacteria, seven Gram-negative bacteria and three fungi species by an agar diffusion method, respectively. Acetone extract was not active while the methanolic extract showed inhibition against Citrobacter freundii and Staphylococcus epidermidis (Vaghasiya and Chanda, 2007).

Rajasekaran and Kannan, (2006) stated that the preliminary phytochemical analysis of C. phlomidis extracts was not clear whether it is for methanolic or acetone extract. Furthermore, it was astonishing to note that although the data indicated the absence of alkaloids, tannins, cardiac glycosides, steroids, flavonoids and saponins, the authors concluded that the antimicrobial activity might be attributed to various active constituents present in either mono or combined way of them. Ethyl acetate and hexane extracts of leaves (yield 8.4% and 1.1% w/w) and stems (yield 3.21% and 0.52% w/w) at concentration of 1 mg/ml were screened for human pathogens and plant pathogens by poison plate technique, respectively. The leaf extract (particularly hexane extract) was

more active than stem extract on both pathogens. However, the stem extract was only inhibitory to plant pathogens. The study revealed that both extracts were more effective in controlling plant pathogens than human pathogens and could be utilized in pesticide formulations

An Experiment was conducted by Roy *et al.* (2006) on naturally occurring growth substances in aqueous extracts of some common weeds viz. Bothua (*Chenopodium album*), Bijli ghas (*Striga densiflora*), Shetdrone (*Leucus aspera*), Mutha (*Cyperus rotundus*), Chapra (*Eleusine indica*) and Khude anguli (*Digitaria ischaemum*) with the attempt for chemical investigation on effective extracts. Boiled and unboiled extracts of all the weed species under test significantly reduced and delayed germination of wheat and jute seeds compared with control. The effect of boiled and unboiled extracts of Bothua (*Chenopodium album*) sowed the lowest germination in seeds of wheat. The root and shoot length of wheat and jute were also decreased in presence of above mentioned weed extracts.

Roy *et al.* (2006) reported that banana plant extracts exerted a significant inhibition on seed germination of lettuce and the degree of inhibition increase with the increase in concentration of extract. The absolute concentration (100%) of extract showed the strongest inhibition of germination 74%) over control. The extracts from different parts of banana plants viz. rhizome, root, pseudo stem and leaves were observed inhibit the radical growth about 91%, 55%, 34%, and 78% respectively over control.

Isoacteoside, trichotomoside and jionoside D, three compounds isolated from *C. trichotomum*, when tested showed significant scavenging activity of intracellular reactive oxygen species produced by hydrogen peroxide suggesting their antioxidant properties (Chae *et al.* 2004, 2005, 2006).

Hong Gao *et al.* (2005) reported that Mammalian α-glucosidase inhibitory activity by *Terminalia chebula* Retz. fruits was investigated. The aqueous methanolic extract was found to have potent rat intestinal maltase inhibitory activity, whereas neither intestinal sucrase nor isomaltase activity was inhibited by this extract. Using bioassay-guided separation, three active ellagitannins were identified as chebulanin (1), chebulagic acid (2) and chebulinic acid (3) and were shown to possess potent intestinal maltase inhibitory activity, with the IC_{50} values of 690 μM, 97 μM and 36 μM, respectively. The intestinal

maltase inhibitory activities of 2 and 3 were even higher than that of 1,2,3,4,6-penta-*O*-galloyl-β-d-glucose (PGG) (4, IC_{50}=140 μM), which is a known potent α-glucosidase inhibitor. Comparison of the activities of 1–4, 1,2,3-*O*-trigalloyl-β-d-glucose (**5**), neochebulagic acid (6) and corilagin (**7**) suggested that the positions of chebulloyl and galloyl groups mostly affected the potency. Kinetic studies revealed that 2, 3, and 4 inhibited maltose-hydrolyzing activity of intestinal α-glucosidase, noncompetitively. This is the first report on mammalian α-glucosidase inhibition by 1, 2 and 3 isolated from *T. chebula* fruits. These results suggest a use of the extract of *T. chebula* fruits for managing Type 2 diabetes.

Regnault-Roger *et al.* (2005) reported the bioactivity of 22 essential oils from aromatic and medicinal plants was tested upon *Acanthoscelides obtectus*, Coleoptera; Bruchidae, a pest of kidney bean, *Phaseolus vulgaris*. The insecticidal effect was evaluated by determination of 24- and 48-hr LC_{50} and LC_{50} (from 1.50 mg/ dm^3 to more than 1000 mg/dm^3). Isoprenoids and phenylpropanoids were identified by gas chromatography. The most efficient essential oils were extracted from plants belonging to Labiatae.*Origanum marjorana* and *Thymus serpyllum* essential oils were the most toxic.

The anti-inflammatory activity of *C. trichotomum* leaves were checked in rat, mice and Raw 264.7 macrophage cells using experimental models with 1 mg/kg solution of 30% and 60% methanolic extracts of leaves. Experimental results concluded that inhibition by methanolic extract was comparable to that of the positive control in an acute inflammation model, while in the chronic model the extract showed 10% higher activity than the positive control. It also suppressed the levels of prostaglandin E2 (PGE2) in RAW 264.7 macrophage cells (Choi *et al.* 2004).

Dorsaz *et al.* (2004) stated that a new hydroquinone diterpenoid was isolated from *C. uncinatum* and was strongly fungi toxic to the spores of *Cladosporium cucumerinum.*

Singh (2004) studied the effect of neem oil as surface protectant at 0, 0.5, 1.0, 1.5 and 2.0% (w/w) 100g of lentil seeds against the pulse beetle, *Callosobruchus chinensis*. Observations of percentage of seed damage and insect adult mortality were performed periodically during summer, rainy and winter seasons. Neem cause significant reduction in seed damage and insect population in treated grains. During summer and winter seasons, the beetle populations were low having high mortality, whereas during rainy season the

population was high and low mortality. Average percentage damage caused by the pest progressively increased in the control with increase in time, and the damage in treated seeds was inversely proportional to the neem oil. All the concentrations were effective. The highest toxicity was recorded with 2.0 ml neem oil/100g seeds.

Kayode (2004) observed that the effect of 24 and 28-h *Colotropis procera* leaf extracts on the radicle and plumule growth of maize cultivars super 1, 2, 3 and 4 were studied. Both extracts showed considerable inhibitory effect on radicle and plumule growth of the cultivars. The severity of inhibition increased with an increase in the duration of the extraction. The growth of super I was the least inhibited then the growth of super 2, 3, and 4 by the extracts *C. procera* leaf when the growth and development of the radicle and plumule were compared.

Dayal *et al.*, (2003) carried out a study to evaluate the efficacy of some botanical insecticides and fungicides as protectant against *S. oryzae* infesting stored rice. The treatments were menthe oil (0.50 ml/kg), clove oil (0.5 ml/kg), turmeric powder (1.0 g/kg), mercury tablet (0.2w5 tablet/kg), DDVP [dichlorvos] (0.05ml/kg; encapsulated), camphor (0.5 g/kg), and control. Based on the cumulative percent mortality of adults, all treatments were significantly superior over the untreated control.

Organic and aqueous extracts of *C. colebrookianum* showed significant inhibition of lipid peroxidation *in vitro* and *in vivo* induced by $FeSO_4$-ascorbate in rats. Aqueous extracts showed strongest inhibitory activity over organic extracts. This lends scientific support to the therapeutic use of the plant leaves claimed in tribal medicine (Rajlakshmi *et al.* 2003).

Trematerra *et al.*, (2002) tested fruits, extracts and metabolites of chilli, (*C. annum)* var. acuminatum, typical of the geographic areas of the molise region (central Italy) in an area for their attractive/repellent activity against adults of saw-toothed grain beetle (*Tribolium castaneum).* According to the results obtained in the arena tests, whole fruits were attractive for all three insect species; cut fruits with the seeds, cut fruits without seeds and split seeds were attractive for *O. surinamensis;* cut fruits with seeds were repellent against *T. castaneum;* whole seeds and split seeds revealed a repellent activity against *S. oryzae* and *Tribolium castaneum.*

An aqueous leaf extract showed a moderate nematicidal activity against larvae of root-knot nematode Meloidogyne incognita and antifungal effect (43.58% inhibition) against Fusarium oxysporum f. sp. Cumini (Sharma and Trivedi, 2002).

Islam *et al.*, (2002) carried out an experiment with acetone, ethanol, methanol and water extracts of bitter gourd *(Momordica charantia),* karanja (P. *pinnata),* mehedi *(Lawsonia inermiY), urmoi (Salfizim indicum)* leaf and seed/bark to evaluate their direct toxicity against granary weevil, *S. granaries* at the concentration of *2.5, 5.0, 7.5* and 10.0%. The result showed that karanja and urmoi leaf and seed extracts were more toxic than those of the other two plants. Seed extracts of all the plants were slightly more toxic than the leaf extracts.

Patil (2002) conducted an experiment to study the effects of *Casuarina equisetifolia* leaf leachate (1.0, 22.5 and 5%) on the germination and growth of groundnut; soybean and green gram. These crops were kept at room temperature for 11days. Observation on germination percentage were recorded on the 6^{th} and 11^{th} days whereas observation on shoot length, root length, shoot dry weight and root dry weight were recorded on the 11^{th} day. Seed germination was not significantly affected by 1.0% leachate, but was reduced by higher leachate concentrations. Soybean treated with 5.0% leachate exhibited the highest reduction in seed germination (62.5%) and shoot growth (99.4%). The leachate at 1% stimulated root growth in green gram (19.64%) and groundnut (6.14%) but inhibited root growth in soybean (18.08%). Further increase in leachate concentration adversely affected root growth in all crops.

In various ancient literatures related to healthcare *Clerodendrum* have been reported for its antimalarial activities because of the presence of a bitter principle. Studies with different parasites support these ancient claims. The alcoholic extract of *C. phlomidis* showed antimalarial activity against *Plasmodium falciparum* with an IC50 value of 48 µg/ml (Simonsen *et al.*, 2001).

Rahman *et al.* (2001) conducted a bioassay to evaluate the seed oils of castor (*Ricinus communis*), neem (*Azadirachta indica*), pithraj (*Aphanamixis polystachya),* safflower and sesame against *Alphitobius diaperinus* (Panjab), Adult insects were fed on wheat grains with the oils at concentrations of 1, 2, 4 and 5%. Insect mortality was recorded at 24, 48, 72, and 96 hours after treatment (HAT). All seed oils exhibited significant repellent

property against *A. diaperinus*, with repellent increasing with rate and exposure time. The highest mean repellency was recorded in 5% pithraj oil (86.69%).

Rahman *et al.* (2001) observed that Ethanolic extract of Melgota is used for repellency, insecticidal activity against rice weevil *(Sitophilus oryzae)* with emphasis on chemical investigation. Fruits of Melgota *(Macaranga postulata)* were extracted on different solvents as in ethanol, acetone, petroleum ether, distilled water and the extracts were concentrated and dried. The ethanol extracts of Melgota (M. *postulata)* of different concentrations were investigated for their repellency and insecticidal activity against *S. oryzae.* Average mortality percentage indicated that the extracts caused significant mortality and repellency on the target insects and bioassays indicated that the toxic and repellent effect was proportional to the concentration and higher concentration has stronger effect. Mortality percentage at 0.25, 0.50, 0.75, 1.00, and 1.50 h after treatment (HAT) indicated that 4% solution showed the highest mortality (34.0%) in *S. oryzae* at 1.50 HAT compared to *pediculus humanus.* Mortality percentage showed parallel response to the level of concentration at different time intervals after treatment. 1 % fruit extract of Melgota (*M. postulata)* showed the lowest repellency 9.84 % in case of rice weevil. On other side, 2% showed 12.76% and 4% showed 22.43% respectively. TLC of crude ethanol extract of Melgota *(M. postulata)* showed six distinct compounds at visible light.

Rimando *et al.* (2001) conducted an experiment to study the several compounds from taichang native 1, allelopathic rice have been identified by the bioassay guided isolation method. They were azelaic acid, *p*-cumeric acid, ^{1}H indole carboxaldehyde, ^{1}H indole 3-carboxylic acid, ^{1}H indole 5-carboxylic acid and 1, 2-benzenedicarboxylic acid bis (2-ethylhexyl) ester. Among the allelopathic substances identified, *p*-cumeric acid, known allelochemical inhibited the germination of lettuce (*Lactuca sativa* L.) seedling at 1mM, but was active against barnyardgrass only at concentrations higher than 3mM.

Salas (2001) evaluated the efficacy of garlic based repellent, commercially known as Garlic Barrier, at 500, 750 and 1000 ml/ha on the reduction of whitelly B. lubucl populations on tomato. Results showed that Garlic Barrier at 500 and 750 ml/ha recorded the greatest egg population reduction in comparison with endosulfan and the control (untreated plot). The same treatments showed the lowest nymph populations. Since adults of B. tabaci were able to pose and lay eggs on the leaves, Garlic Barrier acted as an oviposition suppressor or deterrent rather than a repellent.

Singh *et al.,* (2001) tested leaf powder of Lantana, sadabahar neem, madar and tulsi at a dose rate 10 g/kg grain and oils of castor. neem and mahua 2ml/kg grain against pulse beetle.The result revealed that neem oil and neem leaf powder appeared to be most effective the minimize the damage.

Abe and Matsuda (2000) evaluated the methanol extracts of *M. charantia* leaves against four beetle species, viz. *Aulacophorafietnoralis, nigripennis, Epilachna admirabilis* and *E. boisduvali* feeding cucurbitaceous plants. The methanol extract of *M. charantia* leaves strongly deterred four beetle species from feeding. The methanol extract was partitioned between organic solvent and water, and the chloroform fraction, which showed a strong feeding deterrency. The chloroform fraction was chromatographed with a silica gel column, and momordicines I and 11 were isolated. *A. nigri p'enni . s* was strongly deterred from feeding by momordicines I and II at lower concentrations than those contained in *M. charantia* leaves. *A. femoralis* was deterred from feeding by momordicine II at a high concentration. The feeding of *E. admirabilis* and E. *boisduvali* was not affected by momordicines I and 11 alone. However, E. *admirabilis* and E. *boisduvali* were strongly deterred From feeding by mixtures of momordicines I and II, and momordicine 11 and other components.

Isjima *et al.* (2000) reported that aqueous extract of aerial part of buckwheat inhibited germination or seedling growth of lettuce and other weeds.

Rani (1999) viewed that a successive methanolic extract (yield 7.5% w/w) of leaves showed no mortality till an oral dose of 1 g/kg. The methanolic extract at doses of 200, 400, 600 and 800 mg/kg was evaluated for castor oil-induced diarrhea, gastrointestinal motility and prostaglandin E2-induced enteropooling in albino rats (Wistar strain, 180 to 200 g, either sex). The methanolic extract at 600 and 800 mg/kg showed significant inhibition of defecation frequency and decrease in propulsion of the charcoal meal through gastrointestinal tract. The extract also significantly inhibited prostaglandin E2-induced enteropooling in almost all the dose levels. The mechanism appears to be spasmolytic and anti-enteropooling. Although the extract has shown only the presence of steroids, alkaloids and flavonoids, the authors concluded that the activities of the extract might be due to tannins, which is controversial.

Masuda *et al.* (1999) stated that, antioxidant compounds are responsible for scavenging free radicals, which are produced during normal metabolism or uring adverse conditions that can be harmful to biological systems and leading to death of an organism. Species like *C. inerme* have been used as antioxidant drugs in various indigenous systems of medicines.

The alcoholic extract of roots of *C. serratum* showed a significant anti-inflammatory activity in carrageenan and also in the cotton pellet model in experimental mice, rats and rabbits (Narayanan *et al.* 1999).

Thomas and Callaghan (1999) studied the effects of garlic and lemon peel extracts against C. *pipiens* larvae. Both garlic and lemon were found to be toxic to mosquitoes. Garlic was more persistent than lemon, with no significant differences in mortality rate between fresh and 4.5-day-old treatments. The addition of food to the bioassays increased toxicity of both lemon and garlic and represented the situation in the field more closely, where food would be available to the larva.

Pande *et al.* (1998) conducted an experiment and found that the leaves of *Pnunus amygdalus* were extracted in water on test crops of wheat and finger millet. In bioassays, the compound inhibited the germination of wheat.

Rahman (1998) treated the wheat grains with 5% powder of pithraj, castor and neem seed against *Sitophilus oryzae.* By releasing insect on 20 days after treatment the inhibition rate of F_1 progeny of the insect was found to be 24.89, 32.19and 40.06% for pithraj, castor and neem respectively.

Ghanch (1998) stated that leaf extract are used to control green worms (Heliothis sp.).

Jowar (sorghum) seeds are treated at the spike forming stage with leaf juice of C. multiflorum to protect from fungal infections (Sporisorium sp.) (Parmar 1997).

Dried, aerial parts of *C. inerme* showed potent antiviral activity against Hepatitis B virus with an ED50 value of 16 µg/ml (Mehdi *et al.* 1997).

Bhatt *et al.* (1997) reported that the allelopathic influences of *Terminalia belerica* Roxh., *T. chebula* Retz., and *T. tomentosa* Wight & Arn were tested by growing crops of *Eleusine*

coracana, Brassica campestris, Hordeum vulgare, and *Glycine max* in rhizosphere soil collected from woodlots or on field soil either mulched with dry leaves or fruit pulp of *Terminalia* spp. Growth of the tested crops was depressed significantly in all the growth media. Percent germination and radicle extension of all test crops was also significantly reduced by exposure to aqueous extracts of dried leaves and fruit pulp of *Terminalia* spp. in bioassay studies. Moreover, total ground cover of woodlots remained significantly low as compared to biomass of control plots, though there was no competition for growth resources like moisture, light and soil nutrients between control plots and understory plots. Laboratory and field tests indicated that all of the tree crops examined were phytotoxic to growing food crops as well as to understory vegetation.

Raguraman *et al.*, (1997) studied Oils obtained from neem, *Azadirachta indica* seed kernel, the Himalayan cedar wood, *Cedrus deodara* and their combination (1:1) each at 3, 2 and 1% concentrations were tested against adults of pulse beetle. Cedar wood oil exhibited highest fumigant potential at 3, 2 and 1% concentrations showing corrected inhibition (knock-down) of 100, 100 and 96%, respectively. While neem oil + cedar wood oil (1:1) at 3, 2 and 1% concentrations inhibited 96, 84 and 51.66%.

Rahaman *et al.* (1996) conducted an experiment on growth factors in aqueous extracts of some common weeds viz. Kanyanotey (*Amarathus spinosus* L.), Durba (*Cynodon dactylon*), Lazzabati (*Mmosa pudica*), Mutha (*Cyperus rotundus*), Shama (*Echinochloa crusgalli*), Baradudhia (*Euphorbia hirta*) and Haldemutha (*Cyperus esculentus*). Boiled and unboiled extracts of katanotey, durba and lazzabati reduce the germination early growth of rice while all the 7 weed species inhibited germination and growth of jute. The extracts of all weed delayed the germination of rice and jute seeds.

Two flavonoids from roots of *C. infortunatum*, cabruvin and quercetin, showed strong antifungal activity. The former showed activity against *Alternaria carthami* and *Helminthosporin oryzae*, the latter against *Alternaria alternate* and *Fusarium lini* at concentrations of 200, 500 and 1000 mg/ml (Roy *et al.* 1996).

Misra *et al.* (1995) reported that hexane extracts of *C. colebrookianum* at concentrations of 1000 and 2000 ppm showed strong antibacterial activities against various Gram positive and Gram negative pathogens such as *S. aureus*, *Staphylococcus haemolyticus*, *E. coli*, *Pseudomonas aeruginosa.*

Chou (1993) observed that rice seedling growing poorly in the decomposed straw and soil mixture in pot experiment. He found dark brown roots with abnormal and enlarged cells in the retarded plants. Furthermore, he observed an increase in phytotoxicity with increased straw level, and the toxicity of aqueous extracts of decomposing rice residue was still persistent after the weeks of decomposition. He also extracted the straw and soil mixture with ethanol, purified and identified the phytotoxicity compounds to be *p*-hydrobenzoic, syringic, vanillic, ferulic, acetic,o-hydrophenylacetic, propinic, and butyric acid.

Hakim *et al.* (1991) found that maize dry matter accumulation reduced and tissue N concentration was increased in intercrop culture with wingbean (*Phosphocarpus tetragonolobus* L). These results indicated that N stress did not because the decreased growth of maize when grown with wingbean on maize is occurred.

Bhagore (1991) observed that leaf paste is applied to infested hooves to give a relief for the animals and reportedly cures foot and mouth diseases and secondary infections

Yang and Fustuhara (1991) found that when soybean callus and cultured bottle, the allelopathic effect was so intense that the growth rates of the soybean calli were reduced by more than 100-fold many experimental conditions. Further studies showed that the inhibitory effect was from volatile compounds which were produced by rice callus.

Khan *et al.* (1991) observed that an ethanolic extract of leaves was evaluated for antiviral activity against sunnhemp rosette virus (SRV) on Cyamopsis tetragonoloba. The virus inhibitory activity was 29% with no significant antiviral response.

Alam *et al.* (1990) demonstrated that aqueous extract of fresh leaves at 0.05, 1.0, 1.5 and 2.0% (w/w) of purple nutsedge significantly reduced the present germination, shoot and root lengths of wheat crops. At the highest level of 2% extract, the shoot growth was reduced by 33% and root by 40%. The reductions in shoot and root growth may possibly be due to release of water-soluble compounds affecting the growth. Interference by yellow nutsedge (*Cyperus esculentus* L.) markedly reduced crop yield.

Aliotta *et al.* (1990) studied an experiment of the chloroform extracts of scarlet pimpernel (*Anagallis avernesis*) on germination of radish and lettuce seeds. They found that although germination was affected, but root growth and dry weight of both and lettuce were

markedly inhibited. The phytotoxicity constituent isolate from chloroform extracts was a triterpinoid.

Putnum *et al.* (1990) reported that have drilled rye into field plots during early October, over wintered and killed with glyphosate (N-phosphonomethylglycine) when it attained about 5 metrictons/ha, and evaluated its weed control capacity in vegetable and fruit cropping systems over a 10-year period. Their experiments indicated that living rye has strong interference ability against weeds, providing excellent weeds control prior to annual crop establishment or provides soil cover during dormant period in perennial crops.

An experiment was conducted by Meissner N. and Beyers (1989) on the growth pattern of young carrot, cucumber, lattuce, maize, squash, onion, radish, sunflower and tomato plants was affected when grown in *Cyanodon dactylon* infested soil. Shoot growth on all the species was reduced. Reduction of plant height occurred in all species, except for sunflower, which was spindly in appearance.

Walker *et al.* (1989) conducted that decaying sweet potato plant residues incorporated into soil caused significantly inhibition of growth of sweet potato vine cutting and cowpea plants.

Cheema *et al.* (1988) reported that wheat straw aqueous extract caused 15-20% inhibition of germination of cotton. But stimulated shoot and roots growth dry matter production. Extract of decomposition field residues of barley, rye, broadbean (*Vicia faba*), wheat, vetch and Sudan grass (*Sorghum sudanese*) were found to be toxic to lettuce seedlings.

Lopes *et al.* (1987) studied that the effects of aqueous extracts of shoots and roots of *Cyperus* on germination and seedling growth of rice. About 95% germination of rice seeds was obtained in all cases.

Kil and Lee (1987) reported that aqueous extracts of young top of *Crysynthamum moriflorum* significantly inhibited the germination and seedling growth of six flowering plants: *Callistephus chinesis*, *Cosmos bipinnata*, *Tagetes electa*, *Petunia bybrida*, *Celosia cristata*, *Salvia spendens* and *Portulaca grandiflora*. Allelochemicals related to this phenomenon here salicyclic, ferulic, vanillc, gallic and caffeic acids.

Sarma and Nathawat (1987) reported that seeds of 4 crops were germinated in the presence of 50, 100 and 200 mg *Argemone maxicana* plant powder /100 ml water. Increasing the amount of the power decreased percentage germination in *Raphnus sativus* and *Penisetum typhoides* but effects on germination of wheat and *Brasica campestris* var. sarson were inconsistent; Shoot length of the 4 species was decreased. Root length in Wheat and *Brassica napus* var. *glauca* decreased with increasing amount of the powder. Root length in *Pennisetum americanum* decreased with up 100mg powder. Root length in *Raphanus sativus* increased with 50 g, was not affected with 100 mg and was decreased with 200 mg.

Oleswzek and Zurysta (1987) reported that seed germination and seedling growth were suppressed by water and alcohol extracts of alfalfa roots. Medicagenic and glycosides were found to be the inhibitors.

Alsaadawi *et al.* (1985) conducted a screening experiment to examine the activity of sorghum root exudates of 100 cultivars to inhibit germination and seedling development of pigweed (*Amaranthus retroflexus*) in a sand culture medium. A high variability was observed in the ability of the test cultivars to alter seed germination and/or seedling growth of the weed. They found in 82% of the control reduction in seed germination in 25 cultivars. They also observed 10 cultivars inhibiting *Amaranthus retroflexus* growth by more than 79% of control.

Bansal and Singh (1984) reported that root, shoot; leaf and flower extracts of *Phalaris minor* mixes with soil were studied for their effect on the rice seedling growth. All *Phalaris minor* plant parts decreased rice root dry weight compared with the control. Pope *et al.* (1984) reported that root exudates of *Portulaca olerecea* significantly reduced soybean height. Leather (1983) found that several varieties of sunflower (*Helianthus annus* L.) were more allelopathic to broadleaf weeds than the native wide sunflower.

Putnam Defrank (1983) found that the addition of sunflower residues to the soil reduced the seed germination of *Amaranthus retroflexo* and percentage of inhibition increased with increased with increased concentration of total phenolics in that sunflower residue.

Saleem and Fawusi (1983) found in greenhouse and laboratory experiments that aqueous extracts of purple nutsedge completely inhibited root development and reduced growth of aerial part of rice crop.

Chaturvedi *et al.* (1983) studied the effect of decoction and alcoholic extract of C. phlomidis on adrenaline-induced hyperglycemia and alloxan-induced diabetics in rats, in which the alcoholic extract had a more significant inhibitory effect. Both decoction and alcoholic extract brought down the blood sugar levels effectively and exhibited the same degree of action in alloxan-induced diabetic rats. In a comparative study between the immediate effect (hourly basis) and long term effect (30 days) of decoction in normal rats, C. phlomidis produced comparable fall in blood sugar both on immediate as well as on long term use.

Bhatia *et al.* (1982) studied the effects of common lamb's quarters on wheat and found stimulating effect on growth of wheat.

Datta and Ghosh (1982) studied the effects of nettle leaf goose foot (*Chenopodium murale* L.) on mustard (*Brassica juncea*) and found phytotoxicity effects of the growth of mustard. An oily residue having carboxyl and hydroxyl functions and a solid residue containing oxalic acid were probably involved in the phytotoxicity of this weed. Root exudates of *Phalaris minor* and *Chenopodium murale* decreased shoot and ear length and dry matter production of wheat.

Irons and Burnside (1982) reported that when a 2% (w/w) mature sunflower leaves mixed with the soil reduced emergence and growth of soybean, sorghum and sunflower. The root exudates of sunflower inhibited sunflower emergence, height, fresh weight and dry weight. Aqueous extracts from fresh and dried roots and roots exudates of the Chinese cabbage inhibited growth of mustard.

Lovett and Jessop (1982) studied a range of twelve crop plants (four cereals, five legumes, and three oil seeds) and found that produced chemicals, which significantly reduced the early growth of wheat under controlled and field conditions.

Patterson (1981) reported that the effect of extracted secondary metabolites from Johnson grass (*Sorghum helpense*) on germination, growth and development of various crops. Phytol, vanillin, 3-methoxy 4-hycroxynitrobezene and 2, 6-dimethoxybenzoquinone was isolated from palmer amaranth which inhibited germination and growth of onion and wheat reported by Menges (1988).

Tripathi *et al.* (1981) were reported that the strongest inhibitory effect of aqueous extracts of *Eupatorium adenophorum* on wheat seed germination, radicale and plumule growth.

Drost and Doll (1980) conducted that the foliage residues of yellow nutsedge were very inhibitory to root and shoot growth of corn and soybean.

Meissner *et al.* (1980) reported that growth pattern of young carrot, cucumber, lettuce, maize, squash, onion, radish, sunflower, and tomato plants affected when grown in *Cynodon dactyllon* infested soil.

Essential oil obtained from leaves of the plant showed antifungal activity against variety of fungal species such as *Alternaria* species, *Aspergillus* species, *Cladosporium herbarum*, *Cunnimghamella echinulata*, *Helminthosporium saccharii*, *Microsporum gypseum*, *Mucor mucedo*, *Penicillium digitatum*, *Rhizopus nigricans*, *Trichophyton rubrum* and *Trichothecium roseum* (Sharma and Singh, 1979).

Justification of the Research:

From the above reviews, it showed that so many works were done by many researchers on herbal concepts, such as, growth regulator, botanical herbicides, botanical insecticides, pharmaceutical uses, etc. and these plants were medicinal plants (mainly), some weeds, some fruit trees and other plants also. But no more work was done on biological activity of different extracts of *Clerodendrum infotunatum* with chemical investigation. By this research we will be able to know how plant extract/herbal plant extracts effect on the growth of the plant or crops etc.

CHAPTER 3

MATERIALS AND METHODS

3.1. Experimental Site

The experiment was conducted at the research laboratory of the Department of Biochemistry and Molecular Biology, Hajee Mohammad Danesh Science and Technology University, Dinajpur, Bangladesh, during January 2010 to December 2011 for the bioassay and chemical investigation on chloroform extract of *Clerodendrum infortunatum.*

3.2 Plant Material

Fig.:3.1. A branch of Bhat plant

3.2.1 Scientific classification

Kingdom: Plantae
Unmarked: Angiospermae
Unmarked: Udicots
Unmarked: Asteris
Order: Lamialis
Family: Lamiaceae
Genus: *Clerodendrum*
Species: *C. infortunatum*

3.2.2 Collection of *Clerodendrum infortunatum*

Clerodendrum infortunatum leaves were collected from the Village- Knoukhair, Upazila-Chirirbandar, and District- Dinajpur. The plant was identified by the Bangladesh National Herbarium, and a herbarium was preserved at the Laboratory of the Department of Biochemistry and Molecular Biology, Hajee Mohammad Danesh Science and Technology University, Dinajpur, Bangladesh. *Clerodendrum infortunatum* (common name Bhat; synonyms *Clerodendrum viscosum* Vent. and *Volkameria infortunata* Roxb.) is a perennial shrub belonging to the family Lamiaceae, also sometimes classified under

Verbenaceae. It is the type species among ~400 species of *Clerodendrum*. It is one of the most well-known natural health remedies in traditional practices. *C. infortunatum* is a flowering shrub or small tree, and is so named because of its rather ugly leaf. The stem is eresct, 0.5–4 m high, with no branches and produce circular leaves with 6 inch diameter. Leaves are simple, opposite; both surfaces sparsely villous-pubes-cent, elliptic, inflorescence in terminal, peduncled, few-flowered cyme; flowers white with purplish pink or dull-purple throat, pubescent. Fruit berry, globose, turned bluish-black or black when ripe, enclosed in the red accrescent fruiting-calyx. The fruits are attractive dark metallic blue drupes, about a half inch in diameter. Fruit usually with 4 dry nutlets and the seeds may be with or without endosperm. It flowers from April to August (Jayaweera, 1982).

3.3 Selection and Collection of Summer Vegetables

The following summer vegetables were selected due to their short life and growth period and also available in sub-tropical countries. The seeds of these vegetables were collected from the Dinajpur seed market. The purity percentages and germination percentages of these seeds were 95 and 90, respectively.

The following vegetable crops were selected:

Sl. No.	Bangla name	English name	Scientific name	Family
1.	Bhendi	Lady's finger/okra	*Hibiscus esculentus*	Malvaceae
2.	Kolmishak	Swamp cabbage	*Impoea aquatica*	Convolvulaceae
3.	Borboti	Yard long bean	*Vigna unguiculata*	Leguminosae

3.4 Preparation of Water extract of *Clerodendrum infortunatum* leaves

Newly grown, fresh, green leaves of *Clerodendrum infortunatum* were collected for preparing the water extract. The leaves were weighed 4kg and they were cut into small pieces and water was added. The mixture was homogenized with Blender and 2.5 kg leaf-pastes were obtained. The paste was suspended in 2 liter of water and then filtered through a filter paper. Finally, a total of 3.75 Liter of extract was obtained.

3.5 Preparation of chloroform extract of *Clerodendrum infortunatum* leaves

Six kg of fresh and green leaves were collected. The leaves were cleaned and sundried for 7 days, then it was dried 48 hours at 70^0c by oven. The leaves were then making powder

by Blender Machine and obtained 1.5 kg leaves powder. The powder then dissolved in 4 liter absolute chloroform (96%) and kept for 72 hours. These suspensions were filtered with thin and clean cloth and filtered by filter paper. The suspensions were dried by a model vacuum evaporator (BUCHI Rota vapor R-114) connected with water bath B-480 at 70 ^{0} C. The dried extract was weighed by digital balance. The total weight of leaves chloroform extract obtained 185 gm.

3.6 Preparation of ethanol extract of *Clerodendrum infortunatum* leaves

Six kg of fresh and green leaves were collected. The leaves were cleaned and sundried for 7 days, and then it was dried 48 hours at 70^{0}c by oven. The leaves were then making powder by Blender Machine and obtained 1.5 kg leaves powder. The powder then dissolved in 4 liter absolute ethanol (96%) and kept for 72 hours. These suspensions were filtered with thin and clean cloth and filtered by filter paper. The suspensions were dried by a model vacuum evaporator (BUCHI Rota vapor R-114) connected with water bath B-480 at 70^{0}C. The dried extract was weighed by digital balance. The total weight of leaves ethanol extract obtained 105 gm.

3.7 Treatments under Investigation for the Study of Lady's finger, Swamp cabbage and Yard long bean seeds germination

The herbal plant extracts were investigated as following sequential treatments:

a) Water or control T_c

b) Aqueous extract of *C. infortunatum* T_1

c) Chloroform extract of *C. infortunatum* T_2

d) Ethanol extract of *C. infortunatum* T_3

3.8 Set up for the Investigation of Vegetable Crop Seeds

Petridish experiment was done for lady's finger, swamp cabbage and yard long bean seeds for the observation of germination percentage; shoot growth and root growth, plant height etc. For this experiment, clean petridish with two sheets filter papers were used.

For the investigation of germination percentage, growth and development of vegetable seeds, fifteen ml of each aqueous extract was put in each petridish. In control, only distilled water was used and amount of distilled water was also same. Then twenty five seeds of each vegetable crop were kept in each petridish and each treatment was replicated into three times. The petridishes were kept in natural diffused light under laboratory conditions at 29±2°c temperature and relative humidity of 85±5% after placing. 5 ml of

water was used per day per petridish to keep constant moisture (Dubey, 1973). In control, only water was added if necessary per day per petridish.

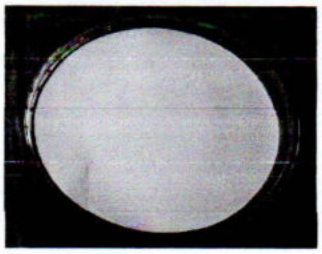

Fig.: 3.2. Two sheets filter paper in petridish

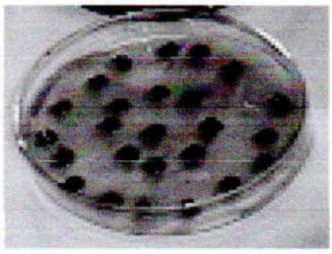

Fig.: 3.3. 25 Seeds in petridish

In this experiment, all subsequent observations were recorded and it was started from 31^{sth} January, 2011. After setting the experiment, the germination percentages, shoot length, root length and completion of germination were recorded. Effects of different treatments on morphology of seedlings were also recorded. The data were subjected to analyze the co-efficient of variance and means were compared by the DMRT method.

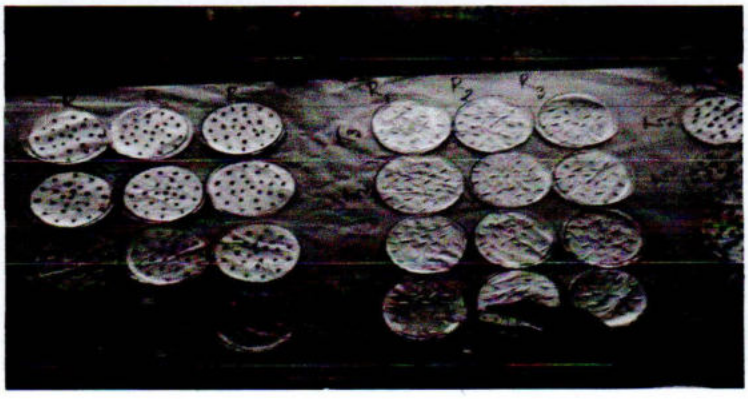

Fig.: 3.4. Seeds setup for the investigation

3.9 Technique for Shoot and Root Growth Measurement:

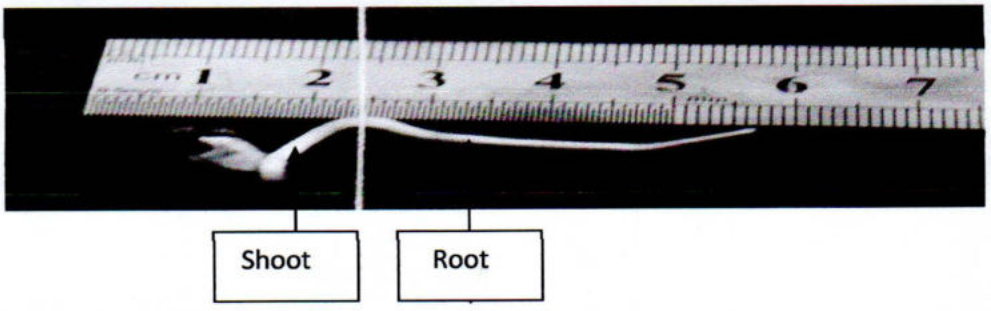

Fig.: 3.5. Technique for shoot and root growth measurement of a seedling

Ten healthy seedlings were taken from each replication of all treatments for measurement of shoot and root length. Each replication of individual treatments was averaged the root and shoot lengths measured individual treatment finally.

3.10 Identification of Effective Extracts

The collected data were analyzed statistically for comparing the difference of means. When the treatments were significant, further analyses for LSD and DMRT were performed for result interpretation.

3.11 Chemical investigation of effective extract

3.11.1 Making Powder of Effective *Clerodendrum infortunatum*

About 5 kg of fresh leaves of plants were collected and sun dried for 7 days. It was then grinding by using grinder to make powder.

3.11.2 Isolation of Crude Compounds from *C. infortunatum* Plant Using Chloroform

For isolation of crude compounds of the individual herbal plant, 100 gm of the leaves of effective herbal plant's powder was taken in a 2.5 liter reagent bottle and 250 ml chloroform was added to it. It was then kept 72 hour with regular interval of shaking. After 72 hours it was filtered by using Whatman filter paper No.1. The extracts were collected in 500 ml reagent bottle and 200 ml of chloroform was added to the residue again, the reagent bottle was again kept for next 72 hours with also regular interval of shaking. After 72 hours it was then filtered. The extracting processes were repeated for at least three times. The chloroform extracts of individual plant were combined together. The solvent was evaporated by using thin film rotary Evaporator under reduced pressure.

3.11.3 Examination of Crude Extracts or Crude compounds by Thin Layer Chromatography (TLC)

3.11.3.1 Thin Layer Chromatography (TLC)

Thin Layer Chromatography (TLC) is one of the most important techniques, by which we are able to detect or identify the presence the number of compounds or number of components present in a crude extract or crude compound in which R_f value of each component was calculated by using this formula:

$$R_f = \frac{\text{Distance traveled by the component}}{\text{Distance traveled by the solvent front}}$$

Thin Layer Chromatography (TLC) was carried on glass plates (slides) coated with silica gel G type 60 (BDH, England).

3.11.3.2 Procedure for Preparation of TLC Plates

Slurry was prepared by the slow addition with shaking 30 gm of absorbent (silica gel) to 100 ml of chloroform in a wide-racked capped bottle. A pair of microscopic slides was held together and dipped into the slurry, slowly withdrawn and allowed to drain momentarily while held over the bottle. The slides were parted carefully and placed horizontally in a rack; it was then dried in sunlight or in oven at 30-40^{0}C for 10-15 minutes (Furniss *et al.*, 1989).

3.11.3.3 Procedure for doing TLC

The crude extract was dissolved in the appropriate solvent and the solution of the compounds was then spotted with thin glass capillary tube at one end of the plate. The plate was then placed vertically with the spotted end downward in a solvent tank. Crude extracts were checked by TLC by the following way:

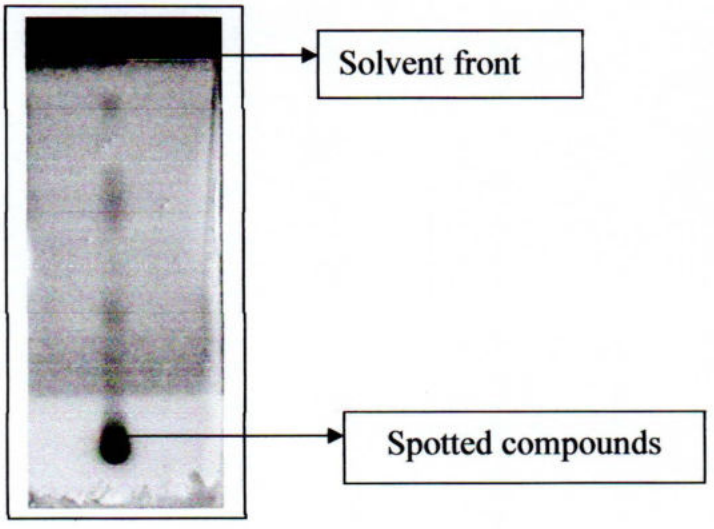

Fig.: 3.6. Thin Layer Chromatographic Plate

3.11.4 Preparation of Solvent Tank and its Working Principle

A 250 ml wide mouth reagent bottle was used as solvent tank, containing desired single solvent or mixture of solvents as the mobile phase, so that the spots did not immerse in the

solvent. After running the solvent to appropriate distances (which was designated as solvent front). The plates were taken out and allowed to dry in air for 10-20 minutes. Subsequently, the plates were developed in iodine vapor in an iodine tank. The mixture of the polar and the non-polar solvent were used in a solvent tank as mobile phase.

3.11.5 Preparation of Iodine Tank and its Working Principle

A 250 ml wide mouth reagent bottle containing little amount of iodine crystal was used as iodine tank. After drying the TLC plate from the solvent tank, it was then placed with the spotted end downward in the iodine tank. Where the desired compound reacts with iodine vapor forming a complex compound and was indicated as yellowish spot for each compound.

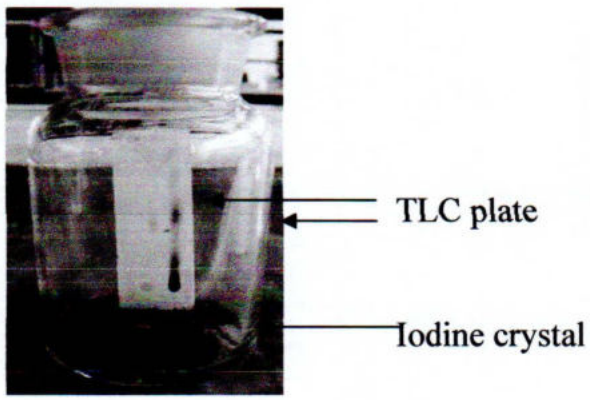

Fig.: 3.7. An iodine tank

3.12 Preparation of Preparative Thin Layer Chromatography

Preparative thin layer chromatography was carried out on preparative glass plates coated with silica gel G type 60 (Merck). The glass plates were cleaned with strong soda water and made completely free from wastes. The plates were then washed with distilled water and dried in an oven. Five glass plates (25 cm × 23 cm) were then placed on a bench made of steel and provided with arrangements so that the plates could be held very tightly. Slurry was made by vigorously shaking the coating materials with required amount of water (silica gel 70 gm evenly mixed with 140 ml of distilled water) in a 500 ml Stoppard conical flask. The slurry was then transferred to the open spreader which was placed at one end on the bench in such away that the coating of thickness 0.50 mm could be obtained. The spreader was then drawn across in the plates uniformly to the other end of the bench. The plates were then left in position until their surfaces become completely mat and were further allowed to stand overnight when the plates were finely coated. Allowing

them to stand for 48 hours at room temperature and then those were ready for use. Preparative thin layer chromatography was used to separate different components of a mixture after establishing the solvent system for TLC. The solution was placed along a straight line vertically at right end to left of the plate by means of glass capillary tube. The solvent was then allowed to vertically in a large solvent (same ratio) tank containing the solvent used as the mobile phase. After the mobile phase had moved over appreciable distance, the plates were taken out and dried in air. The appropriate zones corresponding to different R_f values were detected by exposing one side of the plate in iodine vapor with the rest of the plates surface covered by a clean glass plates. The relevant zone/ zones were significantly indicated compound were cut out from the plates and extracted separately with appropriate solvent. The solvent was then removed under reduced pressure to get the desired compound.

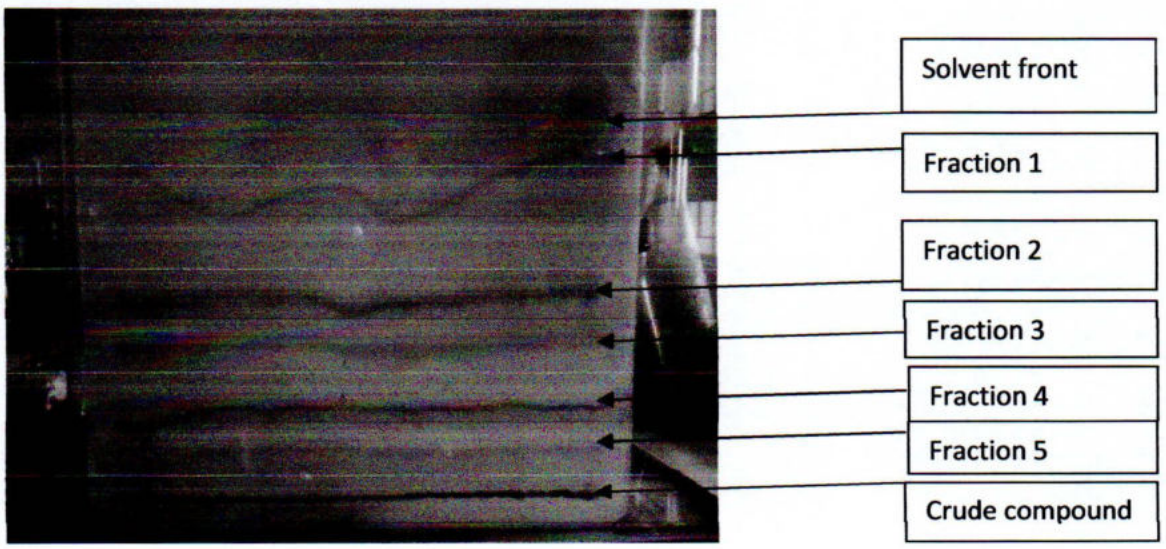

Fig.: 3.8. A preparative TLC plate

3.13 Tests for Sterol

After purification of different crude compounds, the isolated and purified compounds were subjected to test for sterol by following reaction.

3.13.1 Salkowaski reaction

A small amount of compound was taken and dissolved in chloroform and a few drops of concentrated sulfuric acid were added to it. A radish color development indicating the presence of sterol.

3.13.2 Lieberman-Burchard Reaction

A small amount of compound was taken and dissolved in chloroform and a few drops of concentrated sulfuric acid were added to it followed by 2-3 drops of acetic anhydride. A slightly greenish color developed indicating the presence of sterol.

CHAPTER 4

RESULTS AND DISCUSSION

The results showed that growth regulatory activity in different extracts of *Clerodendrum infortunatum* plant (fresh and clean leaf) for germination, root and shoot growth of three vegetables viz yard long bean, swamp cabbage and lady's finger. The result of the present study has been presented in tables and Figures along with adequate discussion in this chapter.

4.1 Effect of Different extracts of *Clerodendrum infortunatum* on Yard long bean

4.1.1 Germination percentage

The germination percentage was counted in 2st, 4rd and 7th days presented in Table 4.1. In 2st day, the highest germination percentage was found in T_2 (30.67%) which was followed by T_c and T_3 and the lowest germination percentage was recorded in T_1 (22.67%), respectively. In 4rd day, the highest germination percentage was found in T_c (58.67%) which was followed by T_2 and T_3 and the lowest germination percentage was recorded in T_1 (38.67%), respectively. In 7th day, the highest germination percentage was found in T_2 (81.33%) and the lowest germination percentage was recorded in T_1 (66.67%), respectively.

Table 4.1. Effect of different extract of *Clerodendrum infortunatum* on germination percentage of long yard bean

Treatments	Germination %		
	2nd day	**4th day**	**7th day**
T_c	29.33 ab	58.67 a	80.00 a
T_1	22.67 bc	38.67 b	66.67 b
T_2	30.67 a	54.67 a	81.33 a
T_3	20.00 c	46.67 ab	66.67 b
Lsd (0.05)	7.173	13.84	10.98

Means followed by the same letter(s) did not differ significantly at 5% level by DMRT.

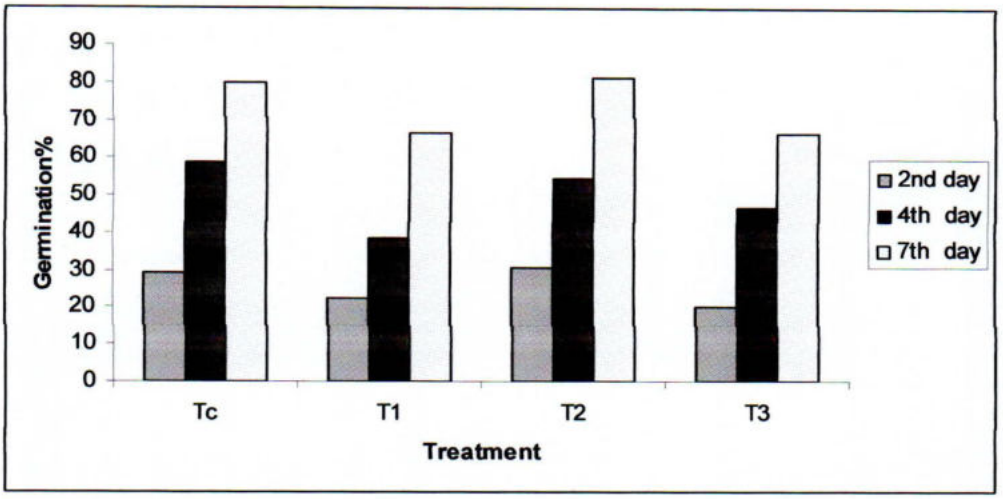

Fig.:4.1. Effect of different extract of *Clerodendrum infortunatum* on germination percentage of long yard bean

4.1.2 Shoot length

Shoot length of long yard bean at different days of experiment was influenced significantly by the effects of different leaf extract (Table 4.2). At 5 Days after sowing (DAS) with the Chloroform extract of *Clerodendrum infortunatum* was the highest shoot length (2.603 cm) whereas the lowest shoot length (1.023 cm) was recorded in T_3 treatment. The highest shoot length of yard long bean seedling was found in T_2 i.e. Chloroform leaf extract of *Clerodendrum infortunatum* (3.573 cm) at 8 DAS that was statistically similar to others. At 11 DAS the highest shoot length was recorded in T_c (4.533 cm) and the lowest was found in T_1 (2.527 cm), respectively. At 14 DAS, the highest shoot length was found in T_2 i.e. Chloroform leaf extract of *Clerodendrum infortunatum* (4.580 cm) followed by T_1 and T_3. On the other hand the lowest shoot length was recorded in T_2 (2.883 cm). Possibly due to presence of growth regulator or other bioactive substances in the chloroform extract of *Clerodendrum infortunatum* shoot growth of long yard bean was gradually increased.

Table 4.2. Effect of different extract of *Clerodendrum infortunatum* on Shoot length of long yard bean

Treatments	Shoot length of (cm)			
	5th day	**8th day**	**11th day**	**14th day**
T_c	1.363 bc	1.687 b	4.533 a	4.143 a
T_1	1.640 b	2.393 b	2.527 a	2.883 a
T_2	2.603 a	3.573 a	3.513 a	4.580 a
T_3	1.023 c	2.030 b	2.970 a	3.250 a
Lsd (0.05)	0.4853	0.9371	2.247	1.854

Means followed by the same letter(s) did not differ significantly at 5% level by DMRT.

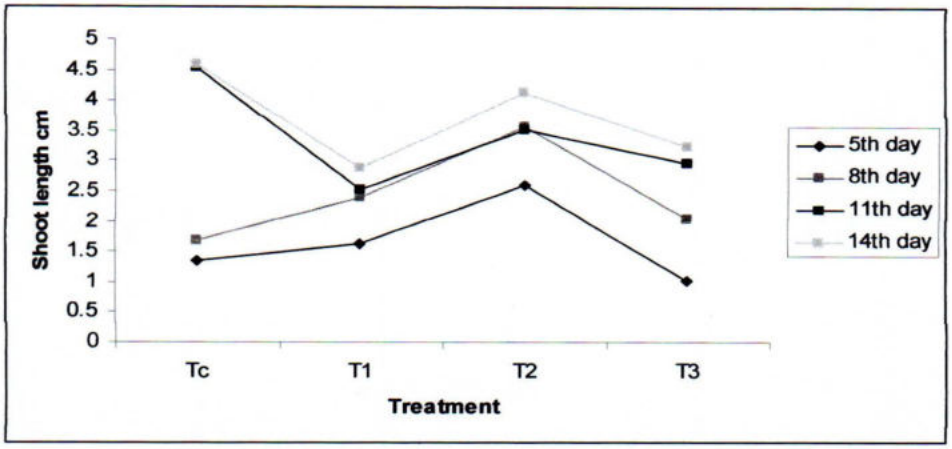

Fig.:4.2. Effect of different extract of *Clerodendrum infortunatum* on shoot length of long yard bean

4.1.3 Root length

Root length of long yard bean at different days after seed setting was influenced significantly by the effects of different stem extract (Table 4.3). At 5 Days after sowing (DAS) with the Chloroform leaf extract of *Clerodendrum infortunatum* showed the highest root length (1.983 cm) which was similar to T_c and T_3 treatments, whereas the lowest root length (1.14 cm) was recoded in T_1 treatment. The highest root length of yard long bean seedling was found in T_2 i.e. Chloroform leaf extract of *Clerodendrum infortunatum* (2.847 cm) at 8 DAS that was statistically similar to others. At 11 DAS the highest root length was recorded in T_2 (4.437 cm) and the lowest was found in T_1 (1.417 cm), respectively. At 14 DAS, The highest root length of long yard bean was found in T_2 i.e. Chloroform leaf extract of *Clerodendrum infortunatum* (4.967 cm) followed by T_c and T_3. On the other hand the lowest root length was recorded in T_1 (1.873 cm). Due to presence of some bioactive substances in the chloroform extracts of *Clerodendrum infortunatum*, root growth was increased in long yard bean seedling.

Table 4.3. Effect of different extract of *Clerodendrum infortunatum* on root length of long yard bean

Treatments	Root length (cm)			
	5th day	**8th day**	**11th day**	**14th day**
T_C	1.147 b	2.770 ab	3.507 a	4.667 a
T_1	1.140 b	1.600 c	1.417 b	1.873 b
T_2	1.983 a	2.847 a	4.437 a	4.967 a
T_3	1.517 b	1.693 bc	1.770 b	2.643 b
LSD (0.05)	0.4377	1.111	1.092	1.680

Means followed by the same letter(s) did not differ significantly at 5% level by DMRT

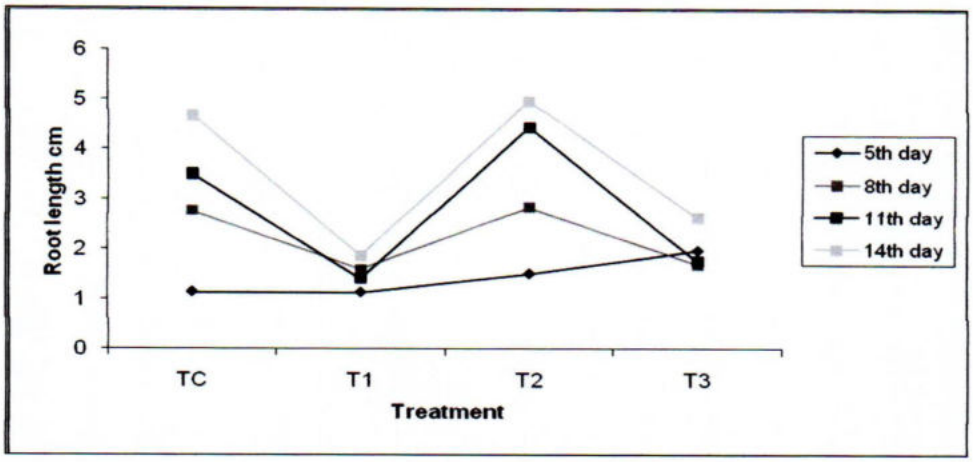

Fig.: 4.3. Effect of different extract of *Clerodendrum infortunatum* on root length of long yard bean

4.2 Effect of different extract of *Clerodendrum infortunatum* on Swamp cabbage

4.2.1 Germination percentage

The germination percentage was counted in 2st, 4rd and 7th days presented in table 4.4 In 2st day, the highest germination percentage was found in T_1 (36.0%) which was followed by T_2 and T_3 and the lowest germination percentage was recorded in T_1 (13.33%), respectively. In 4rd day, the highest germination percentage was found in T_c (53.33%) which was followed by T_2 and T_3 and the lowest germination percentage was recorded in T_1 (33.33%), respectively. In 7th day, the highest germination percentage was found in T_2 (80.0%) and the lowest germination percentage was recorded in T_1 (61.33%), respectively.

Table 4.4. Effect of different extract of *Clerodendrum infortunatum* on germination percentage of swamp cabbage

Treatments	Germination %		
	2st day	4rd day	7th day
T_c	36.00 a	53.33 a	76.00 ab
T_1	13.33 b	33.33 b	61.33 b
T_2	32.00 a	52.00 a	80.00 a
T_3	17.33 b	37.33 b	62.67 ab
Lsd (0.05)	12.49	11.99	17.67

Means followed by the same letter(s) did not differ significantly at 5% level by DMRT.

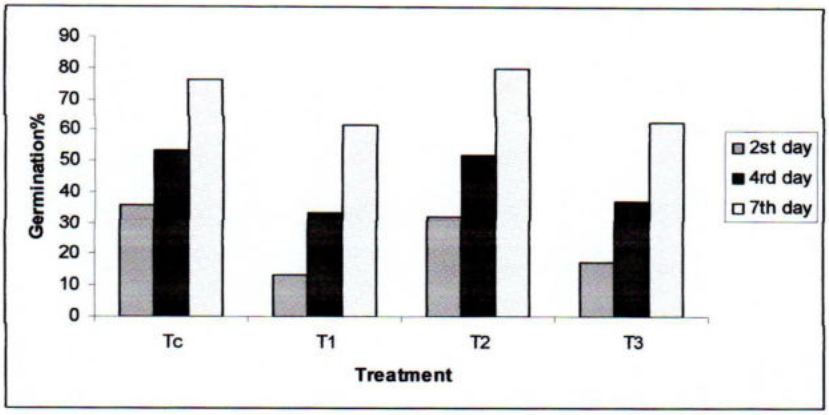

Fig.4.4. Effect of different extract of *Clerodendrum infortunatum* on germination percentage of swamp cabbage

4.2.2 Shoot length

Shoot length of swamp cabbage at different days after sowing influenced significantly by the effects of different leaf extract (Table 4.5). At 5 Days after sowing (DAS) with the Chloroform extract of *Clerodendrum infortunatum* was the highest shoot length (1.653 cm) whereas the lowest shoot length (1.233 cm) was recorded in T_1 treatment. Other treatments showed more or less moderate statistical results at the same time. The highest shoot length of yard long bean seedling was found in T_2 i.e. Chloroform leaf extract of *Clerodendrum infortunatum* (2.267 cm) at 8 DAS that was statistically similar to others. At 11 DAS the highest shoot length was recorded in T_2 (4.583 cm) and the lowest was found in T_1 (1.503 cm), respectively. Possibly due to presence of growth regulator or other bioactive substances in the chloroform extract of *Clerodendrum infortunatum* shoot growth of swamp cabbage was gradually increasing.

Table 4.5. Effect of different extract of *Clerodendrum infortunatum* on Shoot length of swam cabbage

Treatments	Shoot length of (cm)		
	5th day	**8th day**	**11th day**
T_c	1.493 ab	2.400 a	2.840 a
T_1	1.233 b	2.250 a	3.107 b
T_2	1.653 a	2.267 a	4.583 a
T_3	1.460 ab	1.790 a	1.503 c
Lsd (0.05)	0.3222	1.003	0.8640

Means followed by the same letter(s) did not differ significantly at 5% level by DMRT.

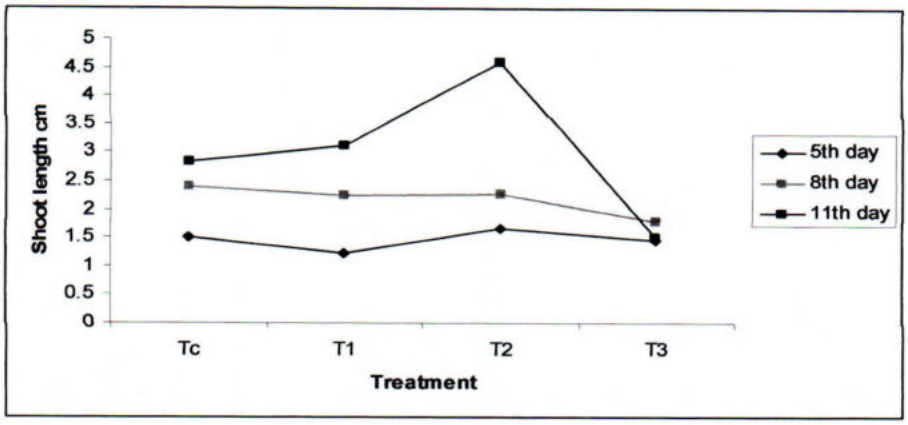

Fig.4.5. Effect of different extract of *Clerodendrum infortunatum* on shoot length of swamp cabbage

4.2.3 Root length

Root length of swamp cabbage at different days after sowing influenced significantly by the effects of different stem extract (Table 4.6). At 5 Days after sowing (DAS) with the Chloroform leaf extract of *Clerodendrum infortunatum* was the highest root length on T_c (1.273 cm) which was similar to T_2 and T_3 treatments, whereas the lowest root length (0.7667 cm) was recorded in T_1 treatment. The highest root length of yard long bean seedling was found in T_2 i.e. Chloroform leaf extract of *Clerodendrum infortunatum* (3.057 cm) at 8 DAS that was statistically similar to others. At 11 DAS the highest root length was recorded in T_2 (5.500 cm) and the lowest was found in T_1 (0.9633 cm), respectively. Similarly due to presence of some bioactive substances in the chloroform extracts of *Clerodendrum infortunatum* root growth was also enhanced.

Table 4.6. Effect of different extract of *Clerodendrum infortunatum* on root length of swamp cabbage

Treatments	Root length of (cm)		
	5th day	**8th day**	**11th day**
T_c	1.273 a	2.793 a	4.380 b
T_1	0.7667a	0.8633 b	0.9633 c
T_2	1.247 a	3.057a	5.500 a
T_3	1.047 a	2.837a	4.417 b
Lsd (0.05)	0.6158	0.4476	0.6286

Means followed by the same letter(s) did not differ significantly at 5% level by DMRT.

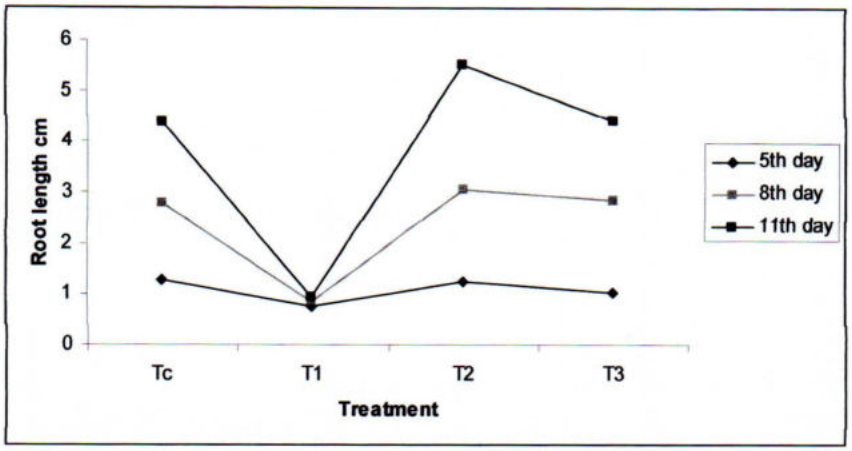

Fig.:4.6. Effect of different extract of *Clerodendrum infortunatum* on root length of swamp cabbage

4.3 Effect of Different extracts of *Clerodendrum infortunatum* on lady's finger

4.3.1 Germination percentage

The germination percentage was counted in 2st, 4rd and 7th days presented in table 4.7 In 2st day, the highest germination percentage was found in T_c (36.00%) which was followed by T_3 and T_2 and the lowest germination percentage was recorded in T_1 (12.00%), respectively. In 4rd day, the highest germination percentage was found in T_c (50.67%) which was followed by T_3 and T_2 and the lowest germination percentage was recorded in T_1 (29.33%). In 7th day, the highest germination percentage was found in T_2 (64.00%) and the lowest germination percentage was recorded in T_1 (44.00%), respectively.

Table 4.7. Effect of different extract of *Clerodendrum infortunatum* Germination percentage of lady's finger

Treatments	Germination %		
	2st day	4rd day	7th day
T_c	36.00 a	50.67 a	61.33 a
T_1	12.00 b	29.33 c	44.00 b
T_2	16.00 b	41.33 b	64.00 a
T_3	29.33 a	49.33 ab	58.67 a
Lsd (0.05)	10.32	8.935	6.388

Means followed by the same letter(s) did not differ significantly at 5% level by DMRT.

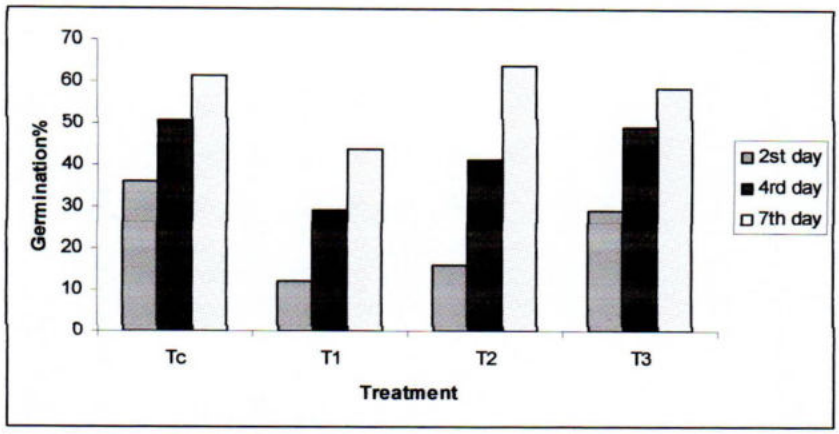

Fig.4.7. Effect of different extract of *Clerodendrum infortunatum* on germination percentage of lady's finger

4.3.2 Shoot length

Shoot length of ladies finger at different days after sowing influenced significantly by the effects of different leaf extract (Table 4.8). At 5 Days after sowing (DAS) the highest shoot length on T_c (1.637 cm) whereas the lowest shoot length (1.467 cm) was recorded in T_1 treatment. Other treatments showed more or less moderate statistical results at the same time. The highest shoot length of yard long bean seedling was found in (3.667 cm) at 8 DAS and lowest in T_c. At 11 DAS the highest shoot length was recorded in T_c (5.737 cm) and the lowest was found in T_1 (0 cm), respectively.

Table 4.8. Effect of different extract of *Clerodendrum infortunatum* Shoot length of lady's finger

Treatments	Shoot length of (cm)		
	5th day	**8th day**	**11th day**
T_c	1.637 a	3.667 a	5.737 a
T_1	1.467 b	1.750 b	0.000 d
T_2	1.617 a	3.563 a	5.380 b
T_3	1.527 b	3.280 a	5.163 c
Lsd (0.05)	0.08935	0.6656	.1548

Means followed by the same letter(s) did not differ significantly at 5% level by DMRT.

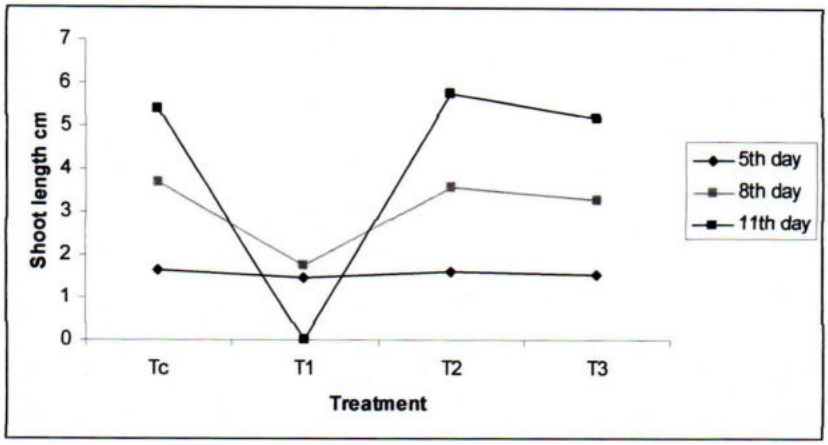

Fig.:4.8. Effect of different extract of *Clerodendrum infortunatum* on shoot length of lady's finger

4.3.3 Root length

Root length of lady's finger at different days after seed setting was influenced significantly by the effects of different stem extract (Table 4.9). At 5 Days after sowing (DAS) with control gave the highest root length (1.350 cm) which was similar to T_2 and T_3 treatments, whereas the lowest root length (1.257 cm) was recorded in T_1. The highest root length of yard long bean seedling was found in T_2 i.e. Chloroform leaf extract of *Clerodendrum infortunatum* (3.177 cm) at 8 DAS that was statistically similar to others. At 11 DAS the highest root length was recorded in T_c (5.300 cm) and the lowest was found in T_1 (0 cm), respectively.

Table 4.9. Effect of different extract of *Clerodendrum infortunatum* Root length of lady's finger

Treatments	Root length of (cm)		
	5th day	**8th day**	**11th day**
T_c	1.353 a	3.017 a	5.300a
T_1	1.257 a	1.330 b	0.0000 d
T_2	1.340 a	3.177 a	5.033 b
T_3	1.303a	2.617 a	4.733 c
Lsd (0.05)	0.6318	0.7120	0.2364

Means followed by the same letter(s) did not differ significantly at 5% level by DMRT.

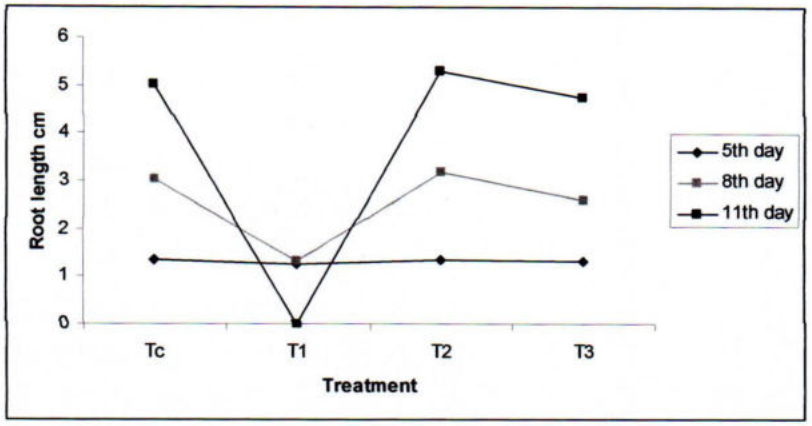

Fig. 4.9. Effect of different extract of *Clerodendrum infortunatum* on root length of lady's finger

4.4 Chemical Investigation

The results in this experiment indicates that the different extracts of *Clerodendrum infortunatum* have increasing or inhibitory activity on germination parameters like time to get fast germination, , coefficient of germination, germination percentages and increasing on root and shoot length or early growth of vegetables. It is very interesting that the increasing tendency of germination parameters roots and shoots length in chloroform extracts of *Clerodendrum infortunatum*. It is a great interesting challenge for the farmer's of our country as well as me that why and which compound is responsible for this type of activity. For this reason I have isolated the crude compounds from the powder of respective leave of herbal plants with different non-polar and polar solvents like chloroform and ethanol etc.

4.5 TLC (Thin Layer Chromatography) of *Chloroform* Extract of *Clerodendrum infortunatum*

The TLC (Thin Layer Chromatography) of chloroform extract of *Clerodendrum infortunatum* was showed distinctly five compounds at Hexane: Ethylacetate (5:1 v/v). This result suggested that it contained five distinct compounds, designated as R_1, R_2, R_3, R_4 and R_5 respectively. These compounds are detected in iodine tank and the following R_f value were calculated by using the formula (Furniss *et al.*, 1989).

$$R_f = \frac{\text{Distance traveled by the component}}{\text{Distance traveled by the solvent front}}$$

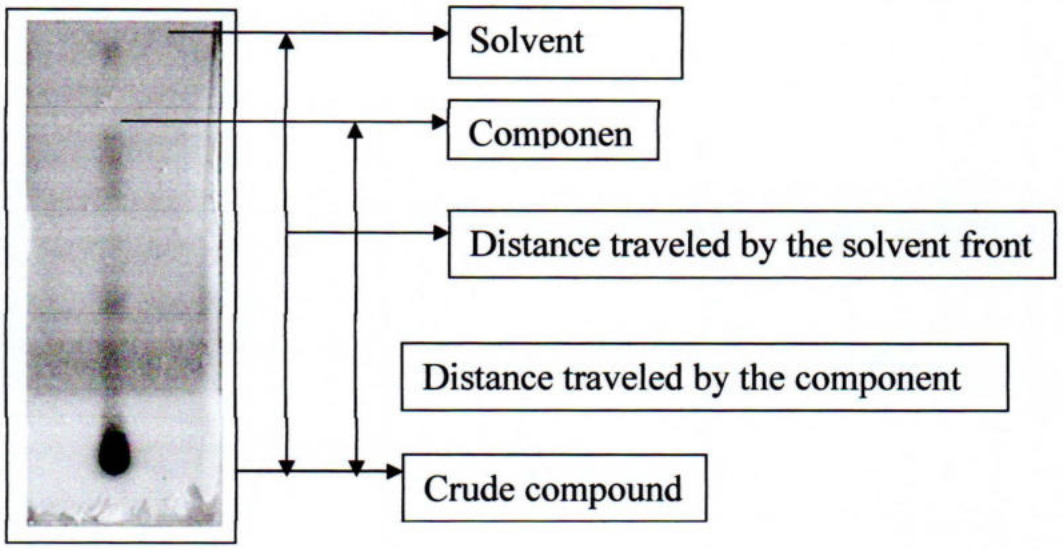

Fig.:4.10. R_f value determination process

Table-4.10. R_f values of detected components of *Clerodindrum infortunatum* (CI)

Name of the Plant Species	Ratio(hexane:ethylacetate)	Detected component	R_f value
Clerodindrum infortunatum	5:1	R_1	0.86
		R_2	0.71
		R_3	0.52
		R_4	0.49
		R_5	0.43

From the Table 4.10 the higher R_f value (0.86) indicates the most non-polar compound and lower R_f value (0.43) indicates the most polar compound.

4.6 Separation of individual fractions by preparative TLC

Five fractions were individually Separation by preparative TLC of solvent system (hexane: ethylacetate 5:1 v/v). 20X20 cm wide and 0.50mm thick Preparative TLC plate (Merck, Germany) was used for this purpose. Preparative TLC was used to separate different components of a mixture after establishing the solvent system for TLC. The solution was placed along a straight line vertically at right end to left of the plate by means of glass capillary tube. The solvent was then allowed to vertically in a large solvent (same ratio) tank containing the solvent used as the mobile phase so that the line containing the mixture stayed half inch above the solvent level in the tank. After the mobile phase moved over

appreciable distance, the plates were taken out and dried in air. The appropriate zones corresponding to different Rf values were detected by exposing one side of the plate in iodine vapor with the rest of the plates surfaces covered by a clean glass plates. The relevant zone/ zones were significantly indicated compound were cut out from the plates and extracted separately with appropriate solvent. The solvent then removed under reduced pressure to get the desired compound.

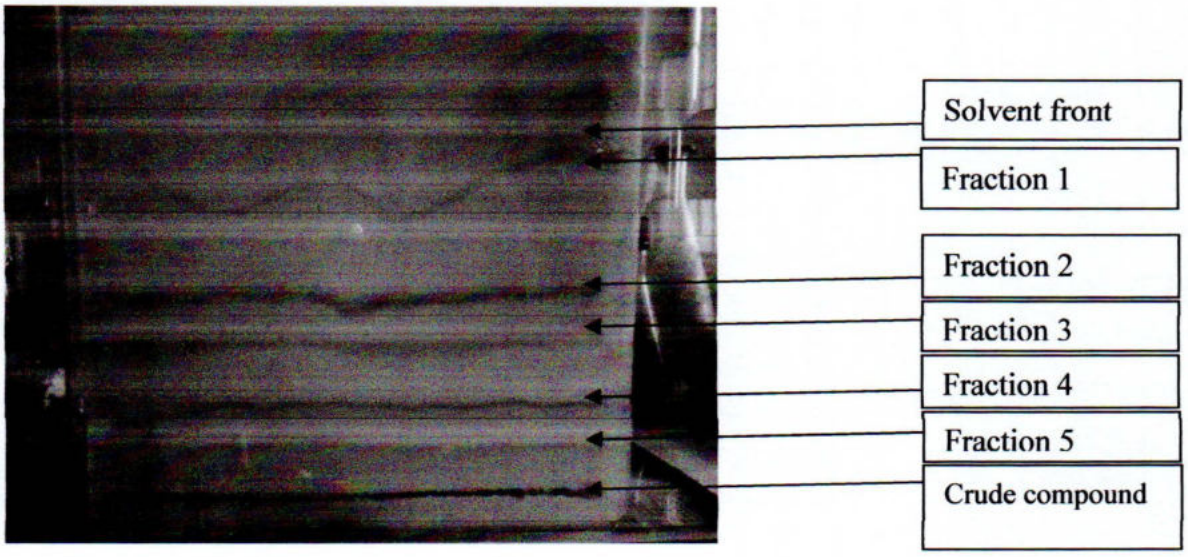

Fig.: 4.11. A preparative TLC plate

The amount of all fractions after complete separation was as follows-

1. Fraction-1: 17 mg.
2. Fraction-2: 7 mg.
3. Fraction-3: 5 mg.
4. Fraction-4: 5.3 mg.
5. Fraction-5:4.1mg.

4.7 Conformation of separation

The separated compounds were compared with crude in one TLC plate.

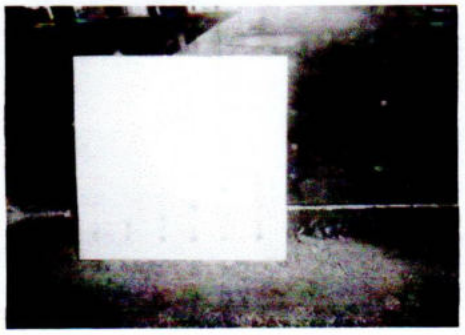

Fig. 4.12.TLC comparing crude with separated compounds

4.8 Chemical test for sterol for isolated fraction

The chemical tests for different fractions were carried out by the method of Finar et al. 1985. The results of the different chemical tests were presented in tabular form. Of the entire fractions only fraction 1 were showed both Salkowaski and Liebermann-Burchard reaction positive, which was indicated that fraction 1 may be sterol type of compound or compounds.

Table No 4.11 Chemical tests for sterol[16] for isolated fractions

Name of fraction	Salkowaski reaction	Liebermann-Burchard reaction
Fraction 1	+ve	+ve
Fraction 2	-ve	-ve
Fraction 3	-ve	-ve
Fraction 4	-ve	-ve
Fraction 5	-ve	-ve

Finally we can concluded that the chloroform extracts of *Clerodendrum infortunatum* enhanced the germination %, shoot and root length of swamp cabbage and lady's finger in all respects comparison with other treatments. So, it is no doubt to say that chloroform extracts of *Clerodendrum infortunatum* may contain growth regulatory or other bio-active substances. Separation of individual fractions and structure determination of active fraction is most essential for interest of research as well as for the interest of the part o this research is going on, which will be reported in due course.

CHAPTER 5

SUMMARY

The experiment was conducted at research laboratory, Department of Agricultural Chemistry, Hajee Mohammad Danesh Science and Technology University, Dinajpur, Bangladesh, during August 2011- February 2012 for the study of bioassay of different extract of *Clerodendrum infortunatum* on some vegetable seeds with their chemical investigation. Bhat plants were selected because these types of plants are available in our country and grow everywhere. The leaves of the selected plants were collected from the Village- Knoukhair, Upazila-Chirirbandar, District- Dinajpur. The seeds of the selected vegetables were collected from Dinajpur seed market. The purity percentages and germination percentages of these seeds were 95 and 90, respectively.

The highest germination percentage (81.33%) was at 7 DAS found in yard long bean seeds treated with chloroform extract (T_2) where the lowest germination percentage (12.00%) was in T_1 at 2 DAS in swamp cabbage. Highest germination percentage was in T_2 at 7 DAS for long yard bean, swamp cabbage, lady's finger whereas lowest germination percentage was in T_1 at 2 DAS in those vegetables.

In 2st day, the highest germination percentage was found in T_2 (30.67%) which was followed by T_c and T_3 and the lowest germination percentage was recorded in T_1 (22.67%), Incase of yard long bean. In 4rd day, the highest germination percentage was found in T_c (58.67%) which was followed by T_2 and T_3 and the lowest germination percentage was recorded in T_1 (38.67%), respectively. In 7th day, the highest germination percentage was found in T_2 (81.33%) and the lowest germination percentage was recorded in T_1 (66.67%), respectively.

At 5 Days after sowing (DAS) with the Chloroform extract of *Clerodendrum infortunatum* was the highest shoot length (2.603 cm) whereas the lowest shoot length (1.023 cm) was recorded in T_3 treatment incase of yard long bean. The highest shoot length of yard long bean seedling was found in T_2. At 11 DAS the highest shoot length was recorded in T_c (4.533 cm) and the lowest was found in T_1 (2.527 cm), respectively. At 14 DAS, the highest shoot length was found in T_2 i.e. Chloroform leaf extract of *Clerodendrum*

infortunatum (4.580 cm) followed by T_1 and T_3. On the other hand the lowest shoot length was recorded in T_2 (2.883 cm).

Shoot length of swamp cabbage at different days after sowing influenced significantly by the effects of different extract. At 11 Days after sowing (DAS) with the leaf extract of Bhat showed the highest shoot length (5.867) whereas the lowest shoot length (0.9267 cm) was recorded with aqueous . The increasing tendency of shoot length in chloroform extract of Bhat treated seedlings might be due to the presence of some growth regulatory materials.

Root length of swamp cabbage seedling at different days after sowing was lowest at 5 DAS and showed an increasing trend up to 11 DAS. At 11 Days after sowing (DAS) seed treated with chloroform extract showed the best result (5.500 cm) whereas aqueous extract showed the lowest root length (0.9633 cm). Other treatments showed moderately similar results for the same days after sowing.

Shoot length of lady's finger at different days after sowing influenced significantly by the effects of different extract. At 11 Days after sowing (DAS) with control showed the highest shoot length (5.737) whereas the lowest shoot length (0.0000 cm) was recorded with aqueous extract. Root length of lady's finger seedling at different days after sowing was lowest at 11 DAS and showed an increasing trend up to 11 DAS by control treatment. At 11 Days after sowing (DAS) seed treated with chloroform extract showed the best result (5.300 cm) whereas aqueous extract showed the lowest root length (0.000 cm). Other treatments showed moderately similar results for the same days after sowing.

The TLC of chloroform extract of Bhat had distinctly five compounds as R_1, R_2, R_3, R_4 and R_5 respectively. The intensity of non polar compound like R_1 was too much high comparison to others. The separated fractions are 17 mg, 7 mg, 5 mg, 5.3 mg and 4.1mg respectively. Among the separated compounds only R_1 contained sterol.

CHAPTER 5

CONCLUSION

This study interestingly indicated that herbal plant extracts have also strong biological activity in the field of agriculture. From this small scale study we may conclude that;

i. Chloroform extract of Bhat significantly enhance the germination of some vegetable crops.
ii. It needs pot experiment as well as field experiment for better conclusion.
iii. Leaves of Bhat may contain some growth promoting and other bio-active substances like sterol.
iv. It needs structure determination by spectral study of isolated compounds.

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