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Phytochemical and Biological Investigation of the Plants Moringa Oleifera Lam. and Duranta Repens Linn

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PHYTOCHEMICAL AND BIOLOGICAL INVESTIGATION OF THE PLANTS *MORINGA OLEIFERA* LAM. AND *DURANTA REPENS* LINN.

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CERTIFICATE

This is to certify that the thesis entitled "**Phytochemical and Biological Investigation of the plants *Moringa oleifera* Lam. and *Duranta repens* Linn.**" submitted by **Farjana Nikkon** for the award of a Ph.D. (Science) degree of Rajshahi University, is absolutely based on her own work under our supervisions and that neither of this thesis nor any part of it has been submitted for any degree/deploma or other academic award anywhere before.

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DECLARATION

I do hereby declare that, the work presented in this thesis entitled "**Phytochemical and Biological Investigation of the Plants *Moringa oleifera* Lam. and *Duranta repens* Linn.**" submitted to the University of Rajshahi, Bangladesh, for the degree of **Doctor of Philosophy** in Science are the original research work of mine and neither of this thesis nor any part of it has been submitted previously for any degree or diploma anywhere.

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The Author

Summary

The plant *Moringa oleifera* Lam. belongs to the family Moringaceae is locally known as "Sajna" (Eng. Drumstick) found to grow everywhere in Bangladesh, India, Pakistan and other tropical and subtropical countries. It has various folk medicinal uses and effective against dysentery, diarrhoea, cholera, pneumonia, scabies, relieves the pain of gout and acute rheumatism etc. Isolation of two alkaloids, moringine and moringinine and an antibacterial substance, pterygospermin have been reported.

For chemical investigation, ethanolic extract of the dried powdered root barks of *Moringa oleifera* Lam. was successively fractionated with petroleum ether (40-60°C) and chloroform. The chloroform soluble fraction was subjected to a column of silica gel, eluted with n-hexane and ethyl acetate and their mixtures and rechromatography of the suitable portion afforded compound-**1** (31.4 mg) as needles, from petroleum ether, mp. 58-60°C. Compound-**1** was identified as deoxy-niazimicin (N-benzyl, S-ethyl thiocarbamate), a novel compound by analysis of its spectral data (IR, Mass and NMR).

The crude (chloroform, ethanol and petroleum ether) extracts and compound-**1** were tested for their biological activities (antibacterial, antifungal screening, MIC, brine shrimp lethality bioassay and acute toxicity test). For antibacterial activity both the crude extracts and compound-**1** showed mild to moderate activities against the tested Gram positive and Gram negative bacteria at concentrations of 30 μg/disc and 100 μg/disc. Crude chloroform extract and compound-**1** showed activity against *Shigella dysenteriae* at a lower concentration of 30 μg/disc.

The MIC of compound-**1** was found to be 32 μg/ml against *Staphylococcus aureus, Shigella dysenteriae* and *Shigella boydii.* Where as the other crude extracts gave higher MIC values.

In vitro antifungal activity was tested with all the crude extracts and compound-**1** against six pathogenic fungi at concentrations of 50 μg/disc and 100 μg/disc. Only chloroform extract and compound-**1** showed mild to moderate activity against some tested fungi at a concentration of 50 μg/disc.

Compound-**1** (LC_{50} value, 14.33 ppm) showed the highest activity than the other crude extracts in brine shrimp lethality bioassay experiment.

Toxicological study of the chloroform extract and compound-**1** was done to assess the margin of safety. No adverse effects on rats were observed in the hematological profiles and biochemical parameters. Histopathology of liver, kidney, heart and lung are within the normal range.

From the above study it can be concluded that the root barks of *Moringa oleifra* Lam. contains very potent bioactive principles and can be used in the indigenous system of medicine and hence the plant deserves extensive phytochemical and pharmacological work.

The plant *Duranta repens* Linn. (Eng: Golden dewdrop) locally called "Kata mehedi" belongs to the Verbenaceae family. This plant is found to grow wild around the globe and used in fancing in Bangladesh. The slightly poisonous fruits of this plant are used in the treatment of malaria and intestinal worms. The leaves are used in the treatment of abscess. This plant has remarkable insecticidal, larvicidal and antifeedant properties. Previous phytochemical studies on different parts resulted in isolation of triterpenoid, steroids and flavonoid type of compounds.

For chemical investigation, ethanolic extract of the stem of *D. repens* was successively fractionated with diethyl ether and chloroform. The chloroform extract showed 4 prominent spots on TLC. After column chromatography using suitable solvent system, the fractions containing one similar prominent spot and were combined. After evaporation of the solvent, the residue was subjected to PTLC seperation. The prominent band that glows under UV was scrapped off. After work up, compound-**2** (48 mg) was isolated as white amorphous powder, decomposed between 121-125°C. But from the IR, ^{1}H-NMR and ^{13}C-NMR spectral analysis, it was confirmed that the compound-**2** was not a single one but a mixture of two similar types of compounds. The major compound was designated as β-Amyrin and the minor one as 12-Oleanene-3β, 21β hydroxy-diol. These compounds were not be possible to separate due to their similar R_f values and used as compoud-**2** for all biological investigations.

In vitro antibacterial activity of crude ethanol, diethyl ether, chloroform extracts and compoud-**2** were determined against three Gram positive and three Gram negative bacteria at concentrations of 30 μg/disc and 100 μg/disc. At 30 μg/disc all the crude extracts and compound-**2** showed no zone of inhibition against all the test microorganisms. But at 100 μg/disc, they showed activities and chloroform extract gave comparatively better activity than the other tested materials.

The MIC of crude extracts and compound-**2** were determined against six pathogenic bacteria. Among the tested materials the chloroform extract showed lowest MIC against all the tested microorganisms.

In brine shrimp lethality bioassay, all the test samples were lethal to brine shrimp nauplii. The LC_{50} values of ethanol, diethyl ether and chloroform extracts were found to be 1.36 ppm, 1.06 ppm and 0.94 ppm, respectively, while the LC_{50} value of compound-**2** was 1.21 ppm. So, chloroform extract showed highest toxicity than the other crude extracts and compound-**2**.

The acute toxicity of chloroform extract and compound-**2** were carried out on Long Evan's rats. In chloroform extract treated rats, almost all the hematological and biochemical parameters were changed significantly and some degenerative changes of liver, heart and kidney tissues (except lung) were detected under microscope. But the changes that occur in hematological profiles of compound-**2** treated rats were statistically insignificant. Among the biochemical parameters, only SGPT and SGOT values were increased slightly. The increased value of SGPT and SGOT indicated that the compound-**2** was slightly toxic but from the histopathological examination, it was confirmed that compound-**2** had no toxic effect. The greater activity of chloroform extract than compound-**2** may be due to the synergic effect of the mixture of compounds present in the extract.

The insecticidal activity of crude stem extracts of *D. repens* and compound-**2** at different instars larvae and adult of *Tribolium castaneum* were examined after 24, 48 and 72 hours. All the crude extracts and compound-**2** caused larval and adult's mortality. The order of toxicity on different instars larvae and adult of *Tribolium castaneum* were chloroform extract> diethyl ether extract> ethanol extract> compoumd-**2**.

Cold ethanolic extract of dried fruits of *Duranta repens* was fractionated with chloroform and petroleum ether. The crude ethanol, chloroform and petroleum ether extracts were tested for their antibacterial, antishigellosis, MIC, brine shrimp lethality bioassay, insecticidal and larvicidal activity.

In vitro antibacterial activity of all fruit extracts of *D. repens* were performed against two Gram positive and three Gram negative bacteria at concentrations of 30 µg/disc and 200 µg/disc. At 30 µg/disc, only ethanol extract showed mild activity but at 200 µg/disc, all the crude extracts showed mild to moderate activity against all the microorganisms.

Antishigellosis activities of the fruit extracts were determined against five Gram negative bacteria at concentrations of 30 µg/disc and 200 µg/disc. The petroleum ether and chloroform extracts showed activity only at 200 µg/disc, while ethanol extract showed activity at both concentrations.

The MIC of fruit extracts were performed against two Gram positive and three Gram negative bacteria. The MIC of ethnolic extract was the lowest (32 µg/ml) against all the tested microorganisms than the petroleum ether and chloroform extract.

In brine shrimp lethality bioassay, ethanolic extract of the fruits showed the highest toxicity with LC_{50}, 0.49 ppm while the LC_{50} values of chloroform extract and petroleum ether extracts were 0.81 ppm and 1.21 ppm, respectively.

The insecticidal activities of ethanol, chloroform and petroleum ether extracts of the fruits of *D. repens* were tested against *Tribolium castaneum* (Harbst). All the test materials were proved to be toxic causing mortality of six instars larvae and adult. The order of toxicity was ethanol extract> chloroform extract> petroleum ether extract for both larval instars and adult.

The dose-mortality effect of ethanol, chloroform and petroleum ether extracts of fruits of *Duranta repens* were performed against 1st, 2nd, 3rd and 4th instars larvae of *Culex quinquefasciatus* Say. Among the tested extracts, the ethanol extract showed the highest larvicidal activity. The order of toxicity was ethanol extract> chloroform extract> petroleum ether extract for different instars of larvae.

After collection the fresh fruit of *Duranta repens*, solution at different concentrations were prepared with distilled water. Effect of 50% and 100% mortality of fruit juice on four instars larvae of *Culex quinquefasciatus* Say were observed at different time. From the experiment it was found that, with the increase of concentration of juice, the percentage of mortality was increased with the duration of time.

All these findings may support to the traditional use of *Duranta repens* Linn. in the indigenous system of medicine after dose adjustment and subacute and chronic toxicity tests. The above result was very much promising and the plant/ plant parts / fruits can be used as natural insecticide and natural larvicide.

Contents

List of Abbreviations

APCI	atmospheric pressure chemical ionization
^{13}C NMR	carbon-13 nuclear magnetic resonance
$CHCl_3$	chloroform
cm	centimeter
cm^{-1}	percentimeter
COSY	homonuclear correlation spectroscopy
$^{\delta}C$	carbon-13 chemical shift
$^{\delta}H$	proton chemical shift
δ (ppm)	chemical shift in parts per million
DC	differential count
dl	deciliter
DMSO	Dimethylsulfoxide
ESI	electron spray ionization
etal.	et alia (and others)
eV	electron volt
Fig.	Figure
GCMS	gas-chromatography/mass spectroscopy
gm	gram
H_2SO_4	sulfuric acid
HMBC	heteronuclear multiple-bond connectivity spectroscopy
HP	Hewlett Packard
HR	high resolution
hrs	hours
HSQC	heterounclease single-bound connectivity spectroscopy
Hz	hertz
^{1}H NMR	proton unclear magnetic resonance
i.p	intraperitoneally
In vacuo	with vacuum
IR	infrared absorption
J	coupling constant
kg	kilogram
lb/sq	pound per square
LC-MS	liquid chromatography/mass spectroscopy
LC_{50}	lethal concentration 50%

LH	lipopholic
lit	liter
$[M^+]$	molecular ion
m/z	mass-to-charge ratio
MeOH	methanol
mg	milligram
µg	microgram
µg/µl	microgram per microliter
µl	microliter
MHz	megahertz
MIC	minimum inhibitory concentration
min	minute
ml	mililiter
mm	milimeter
mp	melting point
MS	mass spectrometry or spectrum
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
Na_2SO_4	sodium sulfate
nm	nanometer
no.	number
OH	hydroxy
PDA	potato dextrose agar
PEG	polyethylene glycol
PPG	poly propylene glycol
ppm	parts per million
PTLC	preparative thin layer chromatography
R_f	migration distance relative to solvent front in thin-layer chromatography
rpm	rotation per minute
TC	total count
UV	ultraviolet absorption
v (cm^{-1})	infrared absorption frquency in reciprocal centimeters
χ^2	chi squared

Chapter-I
Introduction

Introduction

General

The study of plants arose in the effort of prehistoric man to utilize more effectively his vegetational environment as a source of food, clothing, shelter, fuel and medicine. Various primitive peoples of antiquity gathered much information about the growth and uses of plants, especially those of agricultural and medicinal value. Ancient manuscripts, pictures painted on the walls of tombs, plant remains found in caves and burial sites and carving in stone preserved through the ages have given modern scientists at least fragmentary insight into early man's knowledge and uses of plants (Fuller and Carothers, 1967). Green plants have mechanisms by which raw, inorganic materials of the air and soil are converted into those complex, organic compounds called foods, which are essential for the existence of all animal life. All the foods we eat comes from plants directly, or indirectly in the form of meat, fish, eggs and other animal products, which were formed at the expense of plant tissues eaten by animals. Three essential groups of foodstuffs are the carbohydrates, fats and proteins (Fuller and Carothers, 1967). Our widespread use of packaged, brand named medicines to help us combat everything from hay fever to heart disease has seldom led us to believe that plants could be involved. Plants have in fact given our modern Western pharmacopoeia some dozens of different medicinal compounds. The array of medicines derived from them is impressive and includes hypotensive drugs, analgesics, anaesthetics, anticancer and antiparasitic compounds, antiinflammatory drugs, steroids, laxatives, diuretics and many others (Lewington, 1990).

According to WHO estimate, around 80% of the world's 5.86 billion inhabitants depend on traditional medicines, majority of which use plants or their active principles (Gias, 1998). The WHO has emphasized the utilization of indigenous systems of medicines based on the locally available medicinal plants. Approximately one-third of all drugs used by common people are plant based and if bacteria and fungi are included then the picture is like that 60% of pharmaceuticals are of plant origin (Dymock *et al.*, 1976; World of Science, 1980). It is quite surprising that 25% of all prescriptions dispensed from pharmacies all over the 1959 to 1980 in USA contained plant extract or active

principles from plants. WHO gave official recognition to these medicines in a resolution adopted by the 30th World Health Assembly in 1977.

Necessity of Herbal Drug Research in Bangladesh

Nature has endowed Bangladesh with enormous plant resources. Many of these plants grow wild in jungles, forests, gardens and many of them are found lying every where in the fertile region of the country. In Bangladesh, the prospect of use of plant constituents as remedy for diseases is, therefore, very promising.

Approximately 80% of the population use plants for various healing purposes. In industrially developed countries almost 35% of drug contain active principle of natural origin and consumption of medicinal plants is increasing day by day around the world. The practice of traditional medicine in China is firmly established. Prolong use of modern medicine sometimes showed serious side effects. The research on plant material revealed that the activity of pure compound isolated from plant is not always increased, in many cases it is decreased but in combination with the other components present in the plant increase the activity. These findings ultimately attracted people to use traditional medicines instead of modern medicine. Recently, use of traditional medicine is increased tremendously in developed countries (Lewington, 1990).

The suitable weather and fertile soil has made Bangladesh a rich source of medicinal plants and about 500 species are being used in the purpose of traditional medication. About 80% of the total population are still live in the villages. Approximately 14% of them go to the qualified doctors (simple MBBS) and rests of the peoples are still dependent on the different types of traditional medicine practitioners (Mabud, 1990). Most of the practitioners use plant materials as such without knowing the side effect and toxicity and their preparation is substandard. This type of use sometimes causes serious health problem. To over come this problem, research on our enormous medicinal plant resources is necessary to maintain a safer traditional practice by determining their chemical entities and biological activity properly.

If we could use medicinal plants properly we could get medicines at low cost and then it might be possible to fulfil the demand of our medication. This will supply low cost medicine to our poor people and we could establish a better health care system. In order

to achieve this goal, research and development on the traditional medicines should be given the proper privilege. Because, following this trend, the countries like China, Japan, Sri-Lanka, India, Thailand and even UK had already developed a remarkable medication system. Thus their dependence on the allopathic drugs has been reduced to a great extent (Gemenden *et al.*, 1966). So to make the effective WHO slogan "Health for all by 21st century" for our country, we should make proper use of our traditional medicines.

The works cited about can by no means be said to be exhaustive considering the vast number of medicinal plants available in the country. A thorough compositional analysis, isolation of the active principles and search for hitherto undetected therapeutic properties of these plants is, therefore, of prime importance. Isolated efforts are being made along this direction. What is in need now is a coordinated approach on vast scale. This current work is just another step towards this goal.

Role of Plants as Insecticides

Plants are considered to be the most potent objects to human beings not only because of their support for food and shelter, but also because they provide almost all the requirements for the survival of the civilization. Within the past few decades the world advanced rapidly with remarkable development in pesticide technology and medicine, but there are still some problems especially in the field of pesticides for undesirable changes in the gene-pool for the presence of some mutagenic agents and also for increasing pesticide resistance in insects. So, a question has arisen for the sustainability and the survivability of the living beings on this planet with non-hazardous environment. Hence, a worldwide interest has created in the reevaluation and use of age-old traditional botanical agents (Heyde *et al.*, 1983).

Pest control is a major issue for underdeveloped agricultural countries. More than 2000 species of field and storage pests annualy destroy approximately one third of world's food production, valued US $100 billion among which highest losses (43% of potential production) occur in developing Asian countries (Ahmed and Grainge, 1986). Synthetic pesticides are the easy control technology for insect pests. Continuous or heavy usages of some pesticides has created serious problem such as direct toxicity to parasites, predators, pollinators, fish and man (Munakata, 1977; Pimentel, 1981), pesticides resistance

(Brown, 1968; Waiss *et al.*, 1981), susceptibility of crop plants to insect pests (Pimentel, 1977) and increased environmental and social coasts (Pimentel *et al.*, 1980). It has therefore become necessary to complement our reliance on synthetic pesticides with less hazardous and biodegradable substitutes.

Since plants may contain hundreds or even thousands of metabolites, there is currently a resurgence of interest in the vegetable kingdom as a possible source of new lead compounds for introduction into therapeutical screening programmes (Hostettmann *et al.*, 1995). Moreover botanicals, because of their low mamalian toxicity, have received much attention as control agents against stored grain pests. According to Feinstein (1952), over 2000 species of plants representing 170 odd families are said to have some insecticidal values. Due to biomagnification of the chemical insecticides used in agriculture and stored commodities, along with the problem of developing resistant strains of insects, botanicals are gaining importance as protectants day by day (Hasan *et al.*, 1990; Regnault *et al.*, 1993). In the rural areas of South Asia, including Bangladesh, farmers traditionally mix leaves, bark, seeds, roots or oils of certain plants with stored grains to keep them free from insect attacks. Such techniques have been inherited as part of the traditional culture (Sexena *et al.*, 1988). However, Several workers (Iwuala *et al.*, 1981; Ali *et al.*, 1983) have reported effective uses of plant materials as toxic substance against stored product pests.

The search for naturally occurring antifeedants against pests of yield crops and storage has been intensified. A number of investigators isolated, identified and screened compounds from parts of many botanical families for insect feeding deterrence and growth inhibition (Jurd and Manner, 1980; Saxena, 1983). Locally available plants and minerals have been widely used in the past to protect stored products against damage by insect infestation (Golob and Webley, 1980). The main advantage of botanicals is that they are easily produced by farmers in small-scale industries and are potentially less expensive. Moreover, botanical insecticides are broad-spectrum in pest control, many are safe to apply, unique in action and can be easily processed and used (Talukder and Howse, 1995).

Importance of Natural Product as Larvicides

Insects are undoubtedly the most widespread group and successful competitors of man. Of all the animals in the world, some 90% are insects (Hill,1997). Among the insects,

mosquitoes are probably the most important group in public health. They transmit harmful pathogens to man, causes severe and fatal diseases which create health problems and problems of their animals globally and particularly in the tropics (WHO, 1999 a,b).

Among the pathogenic diseases, malaria, filariasis, yellow fever, dengue fever and Japanese B-encephalitis are transmitted by mosquitoes which create severe public health problems in many South and South-East Asian countries including Bangladesh (Bang, 1985). Recently, It was reported that mosquitoes can transmited hepatitis B virus (Siemens, 1987). Hill (1997) pointed out that mosquito-borne disease now attacks 100 million people per year and more than 1 million dies.

In order to prevent the transmission of mosquito borne diseases and also to protect people from biting nuisance it is necessary to control mosquitoes for getting a healthy environment. But, today mosquito control is one of the major problems of the world in view of its vector nature. Various types of synthetic chemical insecticides are conventionally used to control vector mosquitoes as well as other medically and agriculturally important insects (Cheng and Hanlon, 1984; Schofield, 1993). Since 1793, the synthetic chemicals such as oil were used as pesticides to kill mosquito larvae and adults in Philadelphia, USA, on rain barrels. Howard in 1892 and Ross in 1960 (Kabir, 1987), recommended the use of kerosene and paraffin on mosquito infested waters for the control of mosquito larvae. The synthetic chemical insecticides which have been widely used all over the world during the past 40 years to control pests and mosquitoes are chlorinated hydrocarbons, organophosphates compounds, carbamates, pyrethroids, etc. (Setakana and Tan, 1991; Curtis, 1994). The most commonly used insecticides for vector mosquito control are DDT, dieldrin, lindane, chlordane and heptachlor among the chlorinated hydrocarbons; malathion, dibrom, fonthion, parathion, dichlorvos, EPN, abate, etc., among the organophosphates; carbaryl (Sevin) from the carbamates (WHO, 1995). In Bangladesh, lodophin phos was widely used as a mosquito larvicide but due to non-availability of this, DDVP and diazinon are being used as adulticides and larvicides, respectively with others (Hossain *et al.*, 1995).

Many mosquito species have already developed resistance against a number of chemical insecticides all over the world. Development of resistance to insecticides and widespread environmental pollution necessitate a continued search for alternative pest control as well

as vector control strategies (Pimental *et al.*, 1992; Mulrennan, 1995). Pest control experts for the last two decades or so have diverted their attention towards environmentally safer approaches, other than chemical approaches. For this they have been trying to explore several non-chemical approaches utilizing entomopathogens, insect natural enemies, semiochemicals, botanicals, etc. for the control of agriculturally and medically important insects including mosquitoes (Shonouda and Mehanney, 2000). Botanical insecticides derived from plants are promising alternatives to synthetic insecticides for the control of agriculturally and medically important insects. Because these products are biodegradable, they do not leave residues or byproducts to contaminate the environment, are non-toxic to non-target organisms and are specific in their action (Varma and Dubey, 1999). They can serve as ideal tools of integrated pest management (Morallo-Rejesus and Garcia, 1989). Moreover, they are easily produced by farmers and small-scale industries, and are potentially less expensive than chemical insecticides (Talukder and Howse, 1995). Vector control experts, especially those, who have been working on mosquito control using phytochemicals, already evaluated 344 plant species for their insecticidal, repellent, growth inhibiting, ovicidal and other type of activities and suggested to be advantageous for field use in mosquito larvae control programmers (Sukumar *et al.*, 1991).

Plants Investigated in this Study

Moringa oleifera Lam.

The plant *Moringa oleifera* Lam., locally known as 'Sajna' (English-drumstick) belongs to the monogeneric family Moringaceae. It is widely distributed throughout in Bangladesh and eaten as vegetables. Different parts of this plant are used as medicine in the treatment of a variety of human ailments.

The Plant Family Moringaceae

The family Moringaceae consists of 1 genera and 2 species, which are widely distributed in the Indo-Bangla subcontinent and cultivated through out the tropical belt (Kirtikar and Basu, 1984; Sastri, 1962). The members of the family are usually small to medium sized tree with long strangling branches and long cylindrical fruits (Ghani, 1998; Hooker, 1879).

Chemical Literature Review on the Moringaceae Family

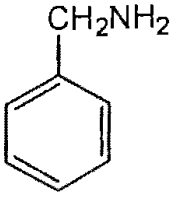
On the basis of the survey, a very short account on different types of compounds isolated from this family, which are medicinally important are given below.

Table-1 Chemical studies on *Moringa oleifera* Lam.

Name of compounds	Source and used part of plant	Reference
Moringine (1) Moringinine	Root bark of *Moringa oleifera* Lam.	Chopra, R.N., Indigenous Drugs of India, 364,1958.
Flavonoids	Whole plant extract of *Moringa oleifera* Lam.	Pankajamani, K.S. and Seshardri, T.S., *Proc.Ind.Acad. Sci.*, 36A,157-59, 1952; Nair, A.G.R. and subramanian, S.S., *Curr.Sci.* India, 31, 155-58, 1962.
Palmitic(2), stearic (3), behenic and oleic (4)acids	Seed oil from *Moringa oleifera* Lam.	Subba Rao, B.C., *et al., J. Ind. Chem., Soc.*,**30**, 477, 1953.
Pterygospermin $C_{22}H_{18}N_2O_2S$	Roots of *Moringa oleifera* Lam.	Kurup, P.A. and Rao, K.L., *Ind.J. Med. Res.*,**42**, 85-95,1954.
Myristic(5), plamitic(2), stearic(3), arachidonic(6), behenic, lignoceric(7), linoleic (8) and oleic (4) acids	Seed oil from *Moringa oleifera* Nimmo	Patel, K.C., *et al., Ind. J. Appl. Chem.*,**21**, 85, 1958.
Baurenol	Ethanol extract of *Moringa oleifera* leaves	Anjaneyulu, B. *et al., Ind. J. Chem.*, **3**, 273-78, 1965.

Name of compounds	Source and used part of plant	Reference
Aspartic acid(9), glutamic acid(10), serine(11), glycine(12), threonine(13), alanine(14), valine(15), leucine(16), isoleucine(17), histidine(18), lysine(19), arginine(20), phenylalanine(21), tryptophan(22), cysteine(23), methionine(24), β-carotene(25).	Ethanol and ether extract of leaves of *Moringa oleifera* Lam.	Das, J.M., *Curr. Sci.*, **34**, 374, 1965.
Sterols and terpenes	Barks of *Moringa oleifera* Lam.	Bhattacharjee, A.K. and Das, A. K., *Queart. J.Crude.Drug.Res.*, 9,1408, 1969.
Stearic(3), palmitic(2), oleic acid, ceryl alcohol and β-sitosterol(26)	Roots of *Moringa oleifera* Lam.	Gupta, A.P., *et al., Proc. Natl. Acad. Sci.*, India, 39 A, 393,1969.
Calcium, phosphorus and insoluble oxalate.	Leaves of *Moringa oleifera* Lam.	Singh, N., *et al., Ind. J. Med. Res.*, 57, 204, 1969.
Protein and fat	Seed of *Moringa oleifera* Lam.	Sengupta, A., *et al., Lloyd.*, 6, 666, 1971.
Iodine and Fluorine	Leaves of *Moringa oleifera* Lam.	Sengupta, S.R. and pal, B., *Ind.J. Nutr. Dietet.*, 8, 66 1971.
Vitamin C	Pods and leaves of *Moringa oleifera* Lam.	Dogra, P.D., *et al., Curr. Sci.*, **44**, 31, 1975.
Protein, fat and oleic acid(4)	Seeds of *Moringa concanensis*	Verma, S.G., *et al., Curr. Sci.*, **45**, 769,1976.
Alanine(14), arginine(20), glutamic acid(10), glycine(12), serine(11), threonine(13), valine(15), lysine(19), sucrose(27) and D-glucose(28)	Flower and fruit extracts of *Moringa oleifera* Lam.	Ramiah, N. and Nair, G.N., *J. Instn. Chem.*, **49**, 163, 1977.
4-hydroxymellein, vanillin, β-sitosterol(26), β-sitosterone(29) and octacosanoic acid	Ethanolic extract of stem of *Moringa oleifera* Lam.	Saluja *et al., Ind. J.Chem.*,**16 (B)**, 1044-45, 1978.
Calcium, phosphorus, iron, thiamine, ribo-flavin, niacin,vitamin-C, β-carotene(25), oxalic acid.	Leaves, fruits and flowers of *Moringa oleifera* Lam.	Gopalan, C., *et al., Ind. Coun. of Med. Res.*,**78**, 66, 1984.
4(alpha-L-rhamnosyloxy) phenylacetonitrile	Seeds of *Moringa oleifera* Lam.	Villasenor, I.M., *et al.,Carcino.*,**10** (6), 1085-7, 1989.
Niaziminin A (30) Niaziminin B(31)	Ethanol extract of leaves of *Moringa oleifera* Lam.	Faizi, S., *et al, J. Chem. Soc. Perkin. Trans.*, 1, 3237-41, 1992.

Name of compounds	Source and used part of plant	Reference
Squalene (32) , Cis-phytol (33), Trans-phytol (34)	Leaves of *Moringa oleifera* Lam.	Rashid, M.A. and Ahmed, M., *Bang. J. of Life Sci.*, 6, 45-48, 1994.
Niazirin and niazirinin	Leaves of *Moringa oleifera* Lam.	Faizi, S., *et al.,J. of Nat. Prod.*, 57, 1256-61, 1994.
4-[(4'-*O*-acetyl- alpha-L-rhamnosyloxy) benzyl] isothiocyanate	Ethanolic extract of *Moringa oleifera* Lam.	Gilani, A.H., *et al., Phyto. Res.*, 8, 87-91,1994.
Niazinin A(35) Niazinin B (36) Niazimicin (37)	*Moringa oleifera* Lam.	Faizi, S., *et al, Phyto.*, 38, 957,1995.
O-(Tri-*O*-acetyl-α-L-rhamnopyranoside) (*E*-), Me ester(38), *O*-(Tri-*O*-acetyl-α-L-rhamnopyranoside) (*Z*-), Me ester(39), *O*-(Tri-*O*-acetyl-α-L-rhamnopyranoside), Et ester(40).	*Moringa oleifera* Lam.	Murakami, A., *et al., Planta. Med.*, 64, 319, 1998.
O-ethyl-4-(α-L-rhamnosyloxy)benzyl carbamate , 4(α-L-rhamnosyloxy)-benzyl isothiocyanate, niazimicin(37) , niazirin, β-sitosterol(26), glycerol-1-(9-octadecanoate) , 3-*O*-(6'-*O*-oleoyl-β-D-gluco-pyranosyl)-β-sitosterol, β-sitosterol-3-*O*-β-D-glucopyranoside .	Seeds of *Moringa oleifera* Lam.	Guevara, A.P., *et al., Mutat. Res.*, 440, 181-8,1999.



(1)

$CH_3-(CH_2)_{14}-COOH$ **(2)**

$CH_3-(CH_2)_{16}-COOH$ **(3)**

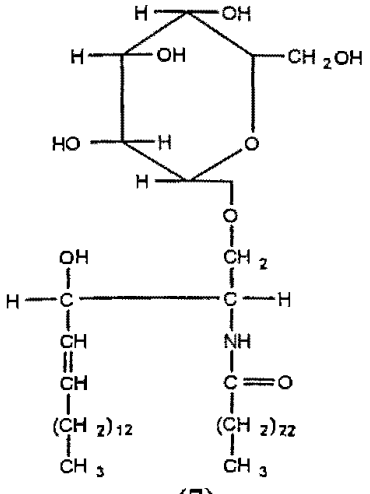
$CH_3-(CH_2)_7-CH=CH-(CH_2)_7-COOH$ **(4)**

$CH_3-(CH_2)_{12}-COOH$ **(5)**

$CH_3-(CH_2)_4-CH=CH-CH_2-CH=CH-CH_2-CH=CH-CH_2-CH=(CH_2)_3COOH$

(6)

$CH_3-(CH_2)_4-CH = CH - CH_2 - CH = CH\ (CH_2)_7-COOH$ (8)



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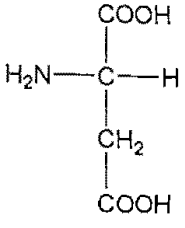

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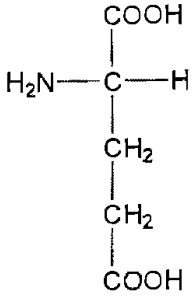

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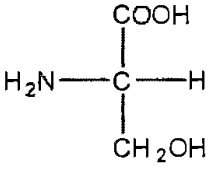

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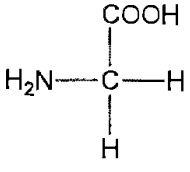

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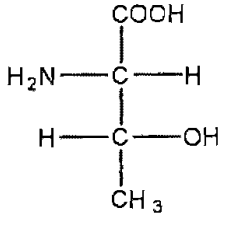

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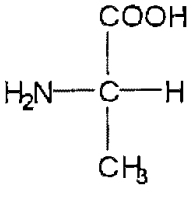

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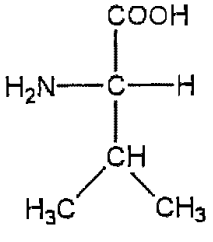

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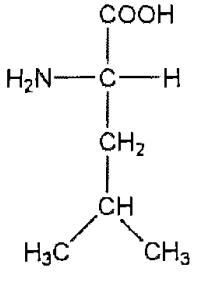

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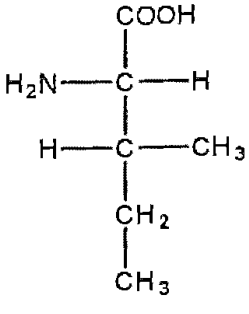

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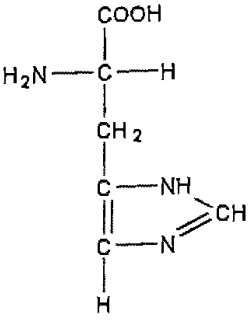

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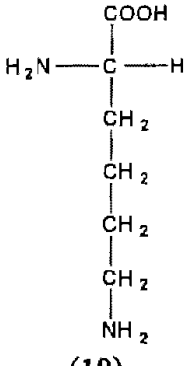

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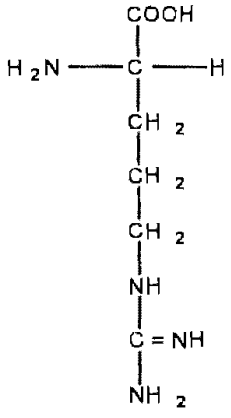

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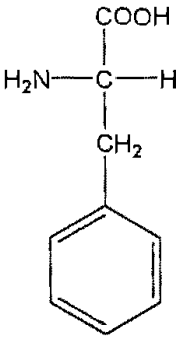

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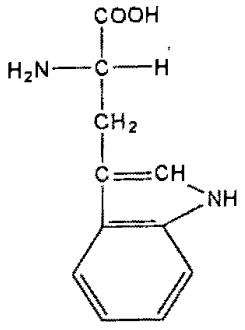

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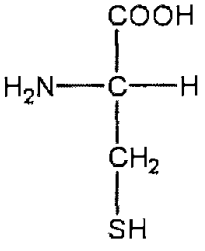

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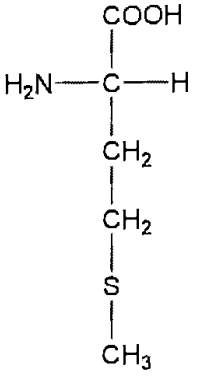

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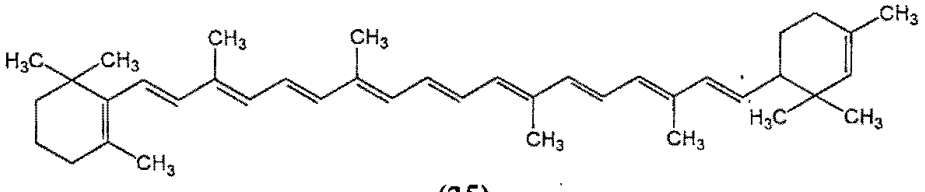

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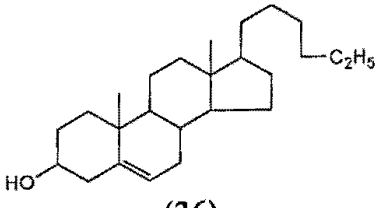

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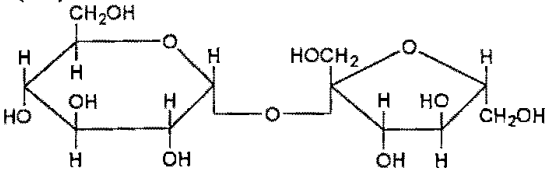

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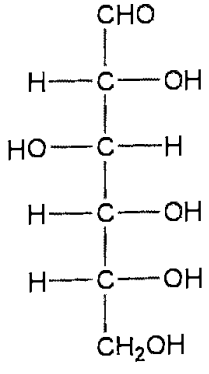

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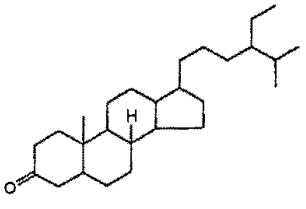

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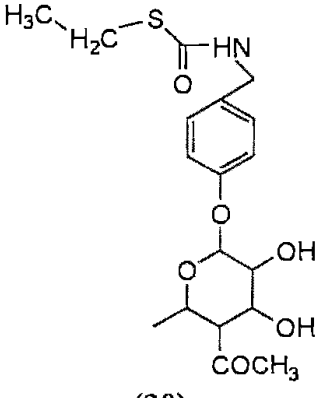

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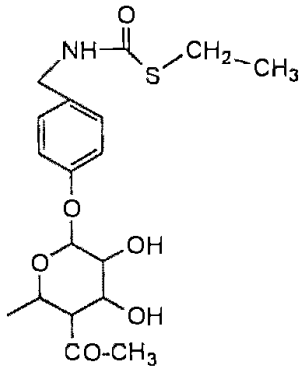

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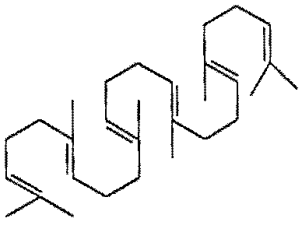

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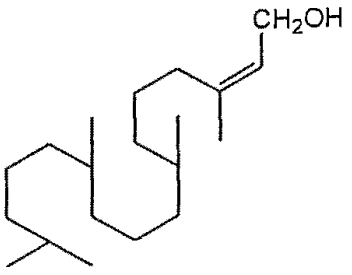

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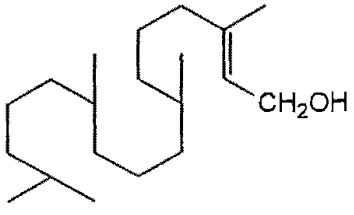

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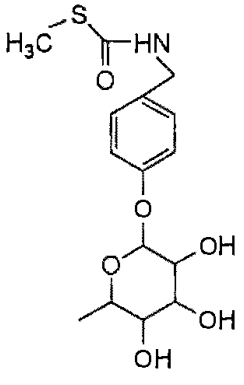

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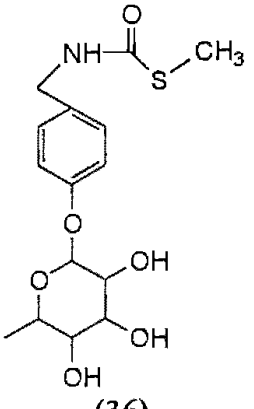

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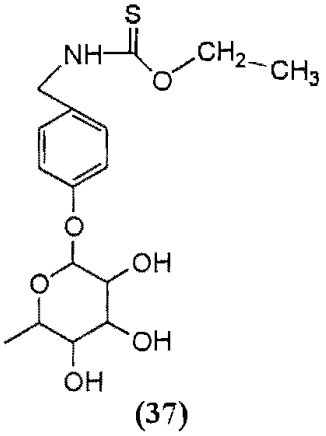

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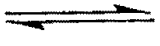

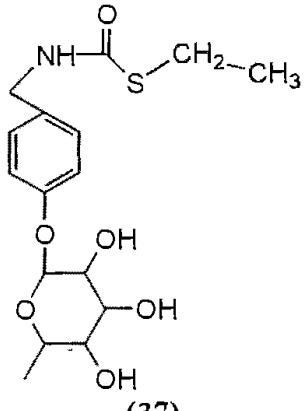

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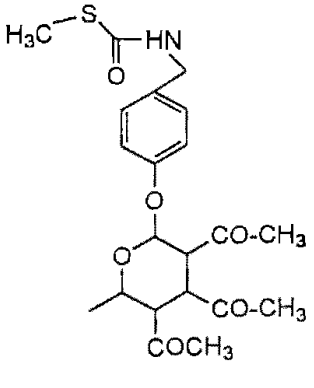

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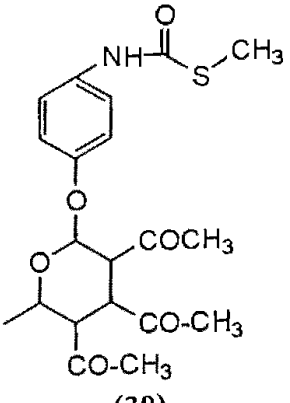

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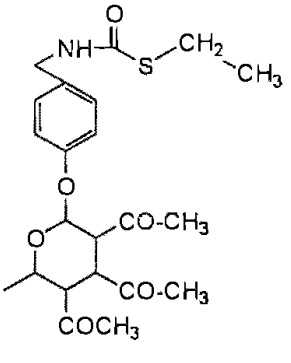

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Biological Literature Review on the Moringaceae Family

Antimicrobial Activity

Pterygospermin isolated from the roots of *Moringa oleifera* having antibacterial activity (Rao and George, 1949). Benzene, toluene and alcoholic extracts of the roots of *Moringa pterygosperma* were active against *Micrococcus pyogenes* (Kurup and Rao, 1952). Spirochin and pterygospermin also isolated from the root of *Moringa pterygosperma* were found to be effective in staphylococcal and streptococcal infections (Mukerji, 1953). Moreover a vibriocidal drug isolated from the root bark of *Moringa pterygosperma* was found to effective against *Vibrio cholerae* (Supta *et al.*, 1956).

Bark extract of *Moringa oleifera* showed antibacterial activity against *Mic. pyogenes, Var. aureus, B.subtilis, Dip. pneumoniae, Strep. pyogenes, Esch. coll, Sal. typhosa, Vib. comma* and *Sh. dysenteriae* and showed antifungal activity against *Microsporum gypseum, Trichophyton mentagrophytes, Candida albicans* and *Helminthosporium sativum* (Bhatnagar *et al.*,1961).Ether extract of leaves of *Moringa oleifera* showed bacteriostatic activity against *Staph. aureus* and *Sal. typhosa* and the juice from the leaves and stem bark inhibited *Staph. aureus* but not *Esch. coil* (Bhawasar *et al.*, 1965).

Ethanol extract of fruit and root bark of *Moringa oleifera* showed antibacterial, antifungal and antiviral activities. The fruit extract exerted only effect on vaccinia virus but was inactive against Ranikhet disease virus (Dhar *et al.*, 1968). The root extract of *Moringa oleifera* completely inhibited multiplication of vaccinia virus and also had inhibitory effect against replication of Ranikhet disease virus (Babbar *et al.*, 1970). Dhar *et al.*, (1973) reported that the ethanolic extract of the fruit, stem, stem bark and whole plant extract excluding the root of *Moringa oleifera* did not show antiviral activity against Ranikhet disease and vaccinia virus. The antimicrobial activity of the seeds of *Moringa oleifera* Lam. was also found (Eilert *et al.*, 1981). *Moringa oleifera* extract was found to inhibit the growth of *Trichophyton rubrum* and *T. mentagrophytes* and can be used in the treatment of subcutaneous phycomycosis in humans and animals (Nwosu and Okafor, 1995).

Hypotensive Action

Moringine and moringinine isolated from *Moringa pterygosperma* had hypotensive action on rats (Chopra and De, 1932). Aqueous and ethanolic extracts of leave of *Moringa pterygosperma* were also found to have hypotensive action on intravenous injection in anaesthetised dogs. The extracts stimulated the isolated rabbit's heart and frog's rectum abdomens muscle, had neuromuscular blocking action on phrenic nerve-diaphragm preparation and produced sedation in conscious animals (Siddiqi and Khan, 1968).

Singh *et al.*, (1976) reported the alcoholic extract of *Moringa oleifera* leaves caused an initial rise in blood pressure in mongrel dogs and cats followed by a gradual fall due to the presence of a potent adrenergic neurone blocking substance (s) in it. Dashputra *et al.*, (1977) also reprted that the leaf extract of *M. oleifera* had hypotensive and depressant action on the heart of dogs. Recently, Faizi *et al.*, (1998) reported that an ethanolic and aqueous extract of *Moringa oleifera* whole pods and their parts, namely, pulp and seed exhibited hypotensive activity on rats.

Toxic Effect

Pterygospermin isolated from the roots of *Moringa oleifera* having low toxicity on rats (Rao and George, 1949). A drug isolated from the root bark of *Moringa pterygosperma* was found to be entirely non-toxic in tested on rabbits (Supta *et al.*, 1956). Administration of methanolic extract of *M. oleifera* root extract in mice showed no alteration in hematological and biochemical parameters at low and moderate dose. But high dose affects liver and kidney functions and hematological parameters (Mazumder *et al.*, 1999).

Antitubercular Activity

Antitubercular activity of bark extract of *Moringa oleifera* Lam. was reported against *M. phlei* (Bhatnagar *et al.*, 1961). Oral administration of an ethanolic extract of *Moringa oleifera* showed a significant protective action on the levels of glutamic oxaloacetic transaminsae, glutamic pyruvic transaminase, alkaline phosphatase and bilirubin in the serum, lipids and lipid perxidation levels in liver. This observation was supplemented by histopatholigical examination of liver sections. The results of this study showed that

treatment with *Moringa oleifera* extracts or silymarin (as afeference) appear as to enhance the recovery from damage induced by antitubercular drugs (Pari *et al.*, 2002).

Antiinflammatory Activity

Alcoholic extract of *Moringa oleifera* root bark showed antiinflammatory activity aganist formalin-induced rat paw oedema, cotton pellet implantation and granuloma pouch in albino rats and also showed analgesic activity (Singh *et al.*, 1972). Eilert *et al.*, (1981) also reported that the leaves, fruits, barks and roots of *Moringa oleifera* Lam. has anti-inflammatory properties.

Anticancer and Antitumor Activity

Ethanolic extract of *Moringa oleifera* (whole plant excluding roots) showed anticancer activity against human and lymphocytic leukaemia in mice (Dhar *etal.*, 1973). Niazimicin isolated from seeds of *Moringa oleifera* have potent *in vivo* antitumor promoting activity in mouse. Three thiocarbamate (TC) and isothiocyanate (ITC)-related compounds, isolated from the leaves of *Moringa oleifera* are observed to be inhibitors of tumor promoter teleocidinB-4-incduced Epstein-Barr virus (EBV) activation in Raji cells (Guevara *et al.*, 1999).

Abortifacient

Administration of aqueous extract of *M. oleifera* Lam. to pregnant rats could not stimulate the uterus which remained non-receptive throughout the period of treatment, therefore, the fertilized eggs may not be welcomed by the unprepared uterus (Prakash *et al.*, 1987). An aqueous or ethanol leaves extract of *Moringa oleifera* has effects on foetal development and thus possess potential abortive effect on rats (Nath *et al.*, 1992).

Estrogenic and Antiprogestational Effect

Aqueous extract of *M. oleifera* Lam. caused biochemical and physiological alterations in the genital tract of female cyclic rats and increased glycogen contents, protein concentration, activity of acid and alkaline phosphatase and the total cholesterol level in all the organs at initial days of treatment. However, at longer days of treatment the values

revealed a significant depletion. The extract also showed estrogenic, anti-estrogenic, progestational and antiprogestational activities on rats (Shukla *et al.*, 1988).

Antioxidant

The protective effect of *Moringa oleifera* Lam. on hepatic marker enzymes, lipid peroxidation and antioxidants during antitubercular drug (isoniazid, rifampicin and pyrazinamide)- induced toxicity in rats were found that by decreasing liver lipid peroxides and enhancing antioxidants (Kumar and Pari, 2003).

Modulatory effects of a hydro-alcoholic extract of *Moringa oleifera* Lam. with reference to drug metabolising phase I and phase II (Glutathion-S-transferase) enzymes, anit-oxidant enzymes, glutathione content and lipid peroxidation in the liver of a Swiss albino mice was observed. A possible chemopreventive potential against chemical carcinogenesis on female Swiss albino mice was also observed (Bharali *et al.*, 2003). The water, aqueous methanol and aqueous ethanol extracts of freeze-dried leaves of *Moringa oleifera* Lam. were found to be a potential source of natural antioxidants due to their marked antioxidant activity (Siddhuraju *et al.*, 2003).

Miscellaneous

Spirochin and pterygospermin isolated from the root of *Moringa pterygosperma* were found to be effective in the treatment of intermittent fever, epilepsy, hysteria, dyspepsia and chronic rheumatism and Spirochin (Mukerji, 1953). *Moringa oleifera* in the form of glycol-propylene base known as *Sovanj* was clinically evaluated in patients of tuberculoid leprosy with uncomplicated trophic ulcers and did not show any significant improvement as compared to controls treated with only glycolpropylene dressings (Kundu and Ghosh, 1966).

Ethanol extract of fruit and root bark of *Moringa oleifera* showed antiprotozoal, anthelmentic, hypoglycemic and anticancer activities and the effect was also investigated on respiration and blood pressure. Both fruit and root bark extracts exerted contractile activity on guinea pig ileum (Dhar *et al.*, 1968). *Moringa oleifera* was also found to be popular for Senegalese remedy (Joseph, 1969). Seed extract of *Moringa oleifera* agglutinated blood cell of various animals (Sathe *et al.*, 1970). The antifertility activity of

roots and the spasmolytic and diuretic properties of the leaves, fruits, barks and roots of *Moringa oleifera* Lam. was found (Eilert *et al.*, 1981). Villasenor *et al.*,(1989) reported that 4(alpha-L-rhamnosyloxy) phenylacetonitrile, a genotoxic compound isolated from roasted seeds of *Moringa oleifera* Lam., showed the higher number of micronucleated polychromatic erythrocytes than that of the solvent control, dimethylsulfoxide, and approximates that of the positive control, tetracycline. Seed infusion of *Moringa oleifera* showed a significant antispasmodic activity on rats (Gilani *et al.*, 1994).

An aqueous leaf extract of *Moringa oleifera* was studied in the regulation of thyroid hormone status in adult Swiss rats and the hyperthyroidism effect has been suggested (Tahiliani *et al.*, 2000). The administration of the crude leaf extracts of *Moringa oleifera* along with high-fat diet decreased the serum, liver, and kidney cholesterol levels and increased serum albumin of obese patients (Ghasi *et al.*, 2000). The seeds of *Moringa oleifera* contain small storage proteins able to flocculate particles in suspension in water and was able to aggregate clay particles as well as Gram-positive and Gram-negative bacteria (Broin *et al.*, 2002). Seed extracts of *Moringa oleifera* Lam. have been proposed as an environment-friendly alternative, due to their traditional use for the clarification of drinking water or skin and mucosal disinfection in clinical settings (Suarez *et al.*, 2003).

Medicinal Uses of *Moringa oleifera* Lam.

The roots are carminative, stomachic, abortifacient, rubefacient and used as cardiac tonic and in the treatment of rheumatism, hysteria, and flatulence (Nadkarni, 1954). Root barks are used as fomentation to relieve spasm. Bark is considered to be an abortifacient. The fruits are used in the treatment of liver and spleen, in tetanus and paralysis (Chakravarty, 1975). Flowers are stimulant and aphrodisiac. Seed oil is applied externally in rheumatism (Chopra, *et al.*, 1956). Leaves are emetic and their juice with black pepper is used in headache. The poultice of leaves is used in reducing glandular swellings. The gum is given in dental caries and relief of otalgia and in headache (Jain and Tarafder, 1970). Seeds are used in venereal affection and to relieve the pain of gout and acute rheumatism (Sastri, 1962). The plant is considered to be useful by tribals (*Santals*) in burns, sores, epilepsy, adenitis, scrofulosa colli, erysipelas, scabies, retention of urine, haematuria, urinary gravel, cholera, dysentery, pneumonia, female sterility, snake bite, scorpion sting as also centipede and spider sting (Jain and Tarafder, 1970).

Duranta repens Linn.

The plant *Duranta repens* Linn. (Eng: Golden dewdrop) belongs to the family Verbenaceae is commonly known as pigeon berry and locally called 'Kata mehedi'. It is also grown as a hedge plant in various parts of our country. The plant is not browsed by cattle and is believed to be poisonous. However, birds feed on the fruits without difficulty (Nelson, 1996). Golden dewdrop forms a part of the coastal scrub community and contributes to soil and ecosystem stability. It is a popular ornamental used for accent plants in tropical and subtropical part of the world because of its profuse displays of flowers and fruits. The flowers of this plant attract butterflies and hummingbirds (Floridata 1999). It is sometimes grown in greenhouses in areas too cold for natural plants.

The Plant Family Verbenaceae

The Verbenaceae is a large plant family including herbs, shrubs or trees. It comprises of about 70 genera and 750 species. The following plant species of Verbenaceae family are available in the Indo-Pak Sub continent (David Prain, 1981; Kirtikar and Basu, 1980).

Table-2 Some plants of Verbenaceae family and their distribution.

Genus	Species	Distribution
Lantana	*Lantana indica* *Lantana trifolia* *Lantana camara* *Lantana aculeata*	Bangladesh, India, Ceylon, Baluchistan, Africa, America.
Lippia	*Lippia nodiflora* *Lippia geminata*	Throughout India, Ceylon, Baluchistan, Africa, and most tropical and subtropical regions.
Stachytarpheta	*Stachytarpheta indica*	Brazil, West Indies, Africa, America.
Verbena	*Verbena officinalis*	Bangladesh, North America, China, India.
Duranta	*Duranta repens* (syn: *Duranta plumieri)*	Bangladesh, India, Pakistan, China, America, Florida, Brazil and Australia.
Callicarpa	*Callicarpa arborea* *Callicarpa lanata* *Callicarpa cana* *Callicarpa macrophylla* *Callicarpa longifolia*	Bangladesh, India.
Tectona	*Tectona grandis*	Central India, Java, Philippine Islands, Burma.
Gmelina	*Gmelina arborea* *Gmelina asiatica*	Bangladesh, India, Australia.

Genus	Species	Distribution
Premna	*Premna coriacea* *Premna scandens* *Premna integrifolia* *Premna lengifolia* *Premna bengalensis* *Premna latifolia* *Premna flavescens* *Premna barbata* *Premna esculenta* *Premna herbacea* *Premna tomentosa*	Bangladesh, India, China, Fiji Islands, Ceylon, Nicobars.
Vitex	*Vitex trifolia* *Vitex negundo* *Vitex pubescens* *Vitex heterophylla* *Vitex peduncularis* *Vitex leucoxylon* *Vitex glabrata* *Vitex agnus-castus*	Bangladesh, India, Pakistan and Australia.
Clerodendron	*Clerodendron inerme* *Clerodendron neriifolium* *Clerodendron phlomoides* *Clerodendron serratum* *Clerodendron nutans* *Clerodendron infortunatum* *Clerodendron fragrans* *Clerodendron siphonanthus*	Bangladesh, India, Pakistan.
Holmskioldia	*Holmskioldia sanguinea*	India
Caryopteris	*Caryopteris wallichiana*	India
Symphorema	*Symphorema involucratum* *Symphorema polyandrum*	India
Sphenodesma	*Sphenodesma unguiculata*	Bangladesh
Congea	*Congea tomentosa*	Bangladesh
Avicennia	*Avicennia officinalis* *Avicennia alba* *Avicennia tomentosa*	India, Baluchistan, Ceylon.

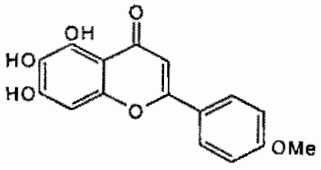
Chemical Literature Review on the Verbenaceae Family

Verbenaceae is a large family. Extensive chemical works have been carried out on Vernenaceae plants. So it was very difficult to survey the whole family. In this content, a very short account on different types of compounds isolated from the Duranta was cited below.

Table-3 Compounds isolated from the Verbenaceae family.

Name of compounds	Source	References
5,6,7-Trihydroxy-4'-methoxyflavone (**41**)	*Duranta repens* Linn.	Harborne, J.B. *et al., Phyto.*, **10**, 2850-2851, 1971.
Durantoside IV or Lamiidoside (**42**)	Leaves and fruits of *Duranta erecta*	Rao, C.B. *et al., Inid. J. Chem.*, **16**, 844, 1978.
β-Sitosterol, (**43**) *α*-amyrin, (**44**) scutellarein, 4-methoxy-scutellarein, scutellarein-7-*O*-rhamnoside, pectolinarigenin and pectolinarigenin-7-*O*-rutinoside.	Leaves of *Duranta Plumieri*	Makboul, A.M. and Abdel-Baki, A.M., *Fito.*, **52**, 219-220, 1981.
Duranterectoside A (**45**) Duranterectoside B (**46**) Duranterectoside C (**47**) Durantoside I (**48**) Durantoside II (**49**) Durantoside III (**50**) Lamiide (**51**) Lamiidoside (**42**) Duranterectoside D (**52**) Verbascoside (**53**)	Leaves and stems of *Duranta repens* and *Duranta erecta*	Takeda, Y. *et al.*, P*hyto.*, **39**,829-833,1995. Kuo, Y.H. *et al., Chem. pharm. Bull.*, **44**, 358, 1996.
12, 13- epoxyoctadec-cis-enoic acid (vernolic acid), 7-(2- octacyclopropen-1-yl) octanoic acid (malvalic acid) and 8- (octa-cyclopropen-1-yl) octanoic acid (sterculic acid).	Seed oil of *Duranta repens*	Hosamani, K.M., *J. of the oil Tech. Asso.*, India, **27**,162-164, 1995.
(22*E*,24*S*)-24-Ethyl-25-hydroxycholesta-4,22-dien-3-one.	Whole plant of *Duranta repens*	Ahmad, S. *et al., Fito.*, **69**,448-450, 1998.
Lupeol (**54**), *β*-sitosterol(**53**), Stigmasterol, (22*E*,24*α*)–24-ethly-3-oxo-cholesta-4,22 (23)-dien-25-ol. (**55**)	Whole plant of *Duranta repens*	Ahmed, S. *et al., Fito.*, **5**, 448-450, 1998.
Durantanin I (**56**) Durantanin II (**57**) Durantanin III (**58**)	Leaves of *Duranta repens*	Hiradae, S. *et al., Phyto.*, **52**,1223-1258, 1999.

Name of compounds	Source	References
3,4′,7-Trihydroxy-3′-(4-hydroxy-3-methylbutyl)-5,6-dimethoxyflavone. (**59**) 3,7-Dihydroxy-3′, -(4-hydroxy-3methylbutyl)-4′,5,6-trimethoxyflavone (**60**) Trans-clerodane diterpenoids 6 β-hydroxy-15,16-epoxy 5 β, 8 β, 9 β, 10 α-cleroda-3, 13 (16), 14-trien-18-oic acid; 6 β hydroxy-15,16-epoxy-5β, 8 β, 9 β, 10α-cleroda-3, 13, (16), 14-trien-oic acid.	*Duranta repens*	Anis I. *et al., Helv. Chim. Acta*, **84**, 649-655, 2001.
3′-(2-Hydroxy-3-methyl-3-butenyl)-2,4′-dimethoxyacetophenone; (**61**) 5,7-dihydroxy-3'-(2-hydroxy-3-methyl-3-butenyl)-3,6,4'-trimethoxyflavone); (**62**) 3,7-dihydroxy-3'-(2-hydroxy-3-methyl-3-butenyl)-5,6,4'-trimethoxyflavone; (**63**) 5-hydroxy-3,6,7,4'-tetramethoxyflavone; rosenonolactone; 6,7-dimethoxycoumarin, 5α, 8α-epidioxyergosta-6,22-dien-3β-ol ; 5α,8α-epidioxyergosta-6,9(11),22-trien-3β-ol.	*Duranta repens*	Anis, I. *et al., Chem. Pharm. Bull.*, **50**, 515-518, 2002.
6,7,8-trimethoxycoumarin; 5,4'-dihydroxy-3,6,7-trimethoxyflavone.	*Duranta repens*	Iqbal, K. *et al., Hetero.*, **60**, 151-157, 2003.



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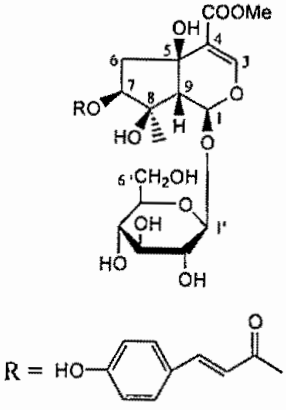

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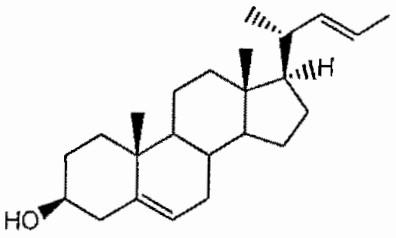

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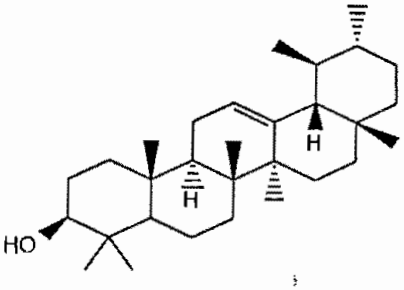

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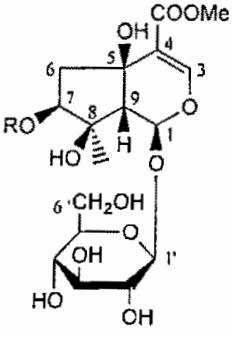

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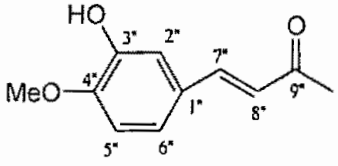

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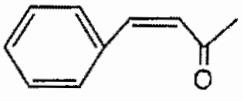

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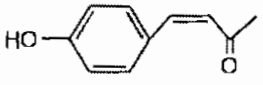

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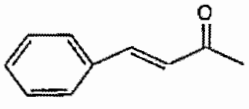

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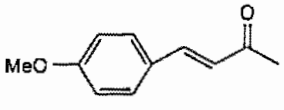

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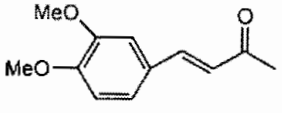

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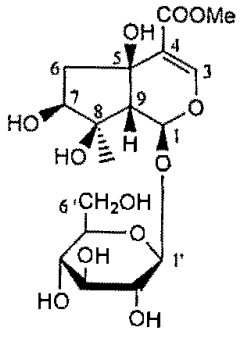

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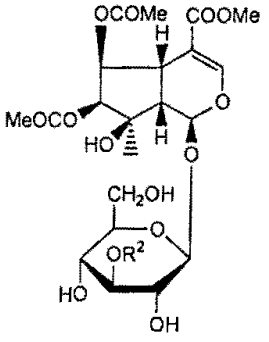

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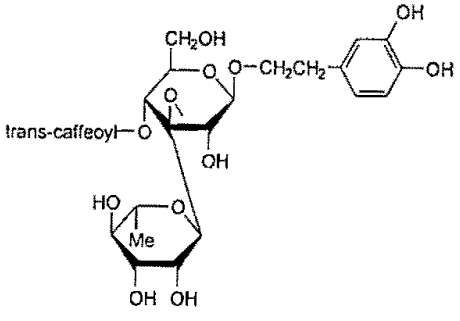

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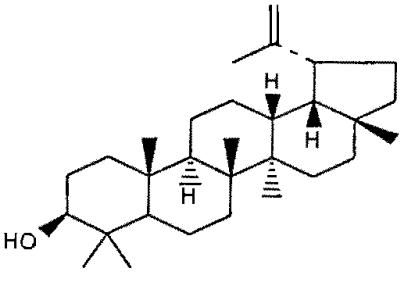

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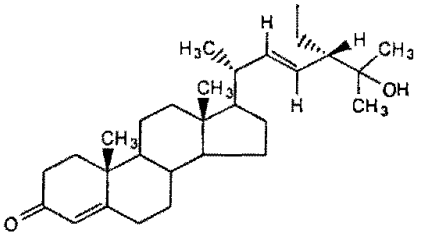

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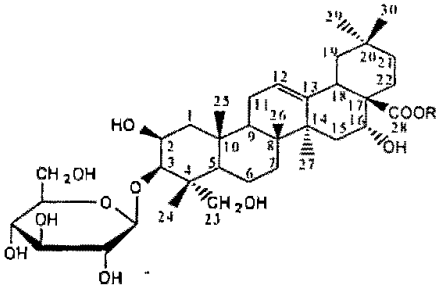
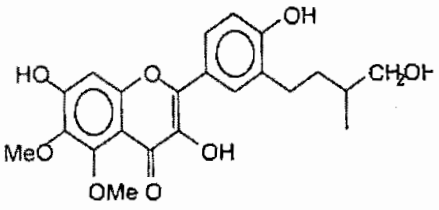


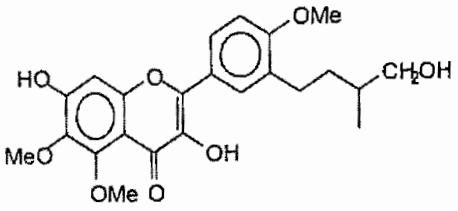
R =α-L-Rha-(1→3')-β-D-Api-(1→4)- α-L-Rha-(1→2')-β-L-Ara) (**56**)

R = α-L-Rha-(1→3)-β-D-Xyl-(1→4)-[β-D-Api-(1→3)]- α-L-Rha- (1→2)- β-L- Ara) (**57**)

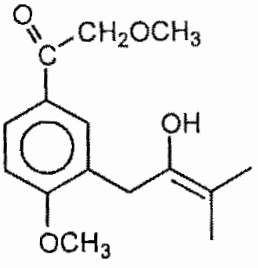
R = α-L-Rha-(1→3)-β-D-Xyl-(1→4)-α-L-Rha-(1→2)- β-L-Ara) (**58**)


OH
HO
CH2OH
MeO
OH
OMe O

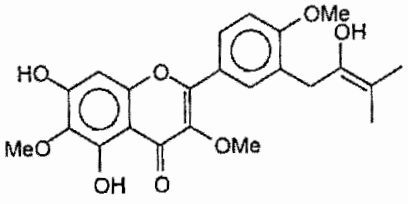
(59)


OMe
HO
CH2OH
MeO
OH
OMe O

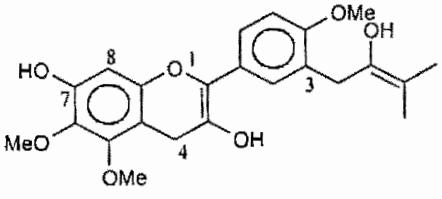
(60)


O
CH2OCH3
OH
OCH3

(61)


OMe
OH
HO
MeO
OMe
OH
O

(62)


OMe
OH
8
HO
1
7
3
MeO
4
OH
OMe

(63)

Biological Literature Review on the Verbenaceae Family

Insecticidal Activity

Rao *et al.*, (1978) reported that the fruit juice of *D. plumieri* has been used as larvicide. El-Naggar and Mosallam (1987) reported that the extracts from *Duranta repens* had antifeedant and insecticidal properties against the larave of *Culex pipiens* and *Spodoptera littoralis*, and against the adults of *Musca domestica* and *C. pipiens,* respectively.

Carvalho *et al.*, (2003) reported that the *Lippia sidoides* essential oil and its hydrolate have larvicidal action against the mosquito *Aedes aegypti,* causing an almost instantaneous mortality. Thymol, an alkylated phenol derivative and one of the major components of *L. sidoides* essential oil, was identified as the active principle responsible for the larvicidal action. These results suggest that the essential oil of *L. sidoides* is promising as larvicide against *A. aegypti* and could be useful in the search of newer, more selective, and biodegradable larvicidal natural compounds to be used in official combat programs and at home.

Kumar and Singh (1986) reported that, during the month of summer, spider mite, *Tetranychus macfarlanei* Baker and pritchard are potential pests of pumpkin, *Cucurbita moschata* Dutch. The extract of *Duranta repens* shown less mortality but having encouraging performance against this pest. The predatory and spider mite ratio 1:20 was very much suitable for mite management. So botanical pesticide and biological agent is the good component for integrated mite management and are eco-friendly to environment also.

Antimalarial Activity

Castro *et al.*, (1996) reported that the fruits of *duranta repens* was evaluated as antimalarials by oral and subcutaneous administration to mice infected with *Plasmodium berghei.* Significant antimalarial activity was observed in ethyl acetate and aqueous extract of fruits of *Duranta repens* [*D. erecta*].

Toxic Effect

Toxicity of acetone and petroleum ether extracts of leaves of *Duranta plumieri [D. erecta]* were studied on *Sitophilus oryzae.* For acetone extracts, mortalities increased

with increasing concentration and time of exposure. At various concentrations of acetone extract of *Durata plumieri,* the reduction in F1 progeny was recorded and it was ranged from 56-95%. But in case of petroleum extracts of *D. plumier*, the reduction rate in F1 progeny was recorded from 80-100% (El-Lakwah *et al.*, 1997a).

El-Lakwah *et al.,* (1997b) reported that the powdered fruits and extracts of *Duranta plumieri* [*D. erecta*] showed toxic effect on lesser grain borer, *Rhizopertha dominica* adults. Petroleum ether extracts of *D. plumieri* were more toxic against *R. dominica* adults than the acetone extracts. Plant powders of *D. plumieri* was less toxic than petroleum ether extracts. The effect of all materials on inhibition of FI-progeny was higher than on adult mortalities at all tested concentrations. Acetone extracts were moderately repellent at high concentrations, but the petroleum ether extracts showed very low repellency effects.

Antibacterial Activity

Dhembare and Sarla Sangle (2003) reported that the alcoholic extract of *Duranta plumieri* [*D. erecta*] was tested for their antibaterial activity against human pathogenic bacterial strains, *proteus, Pseudomonas, Klebsiella, E. coli* and *Staphylococcus aureus.* All the extracts showed antibacterial activities, more or less equal to the activity of the control (co-trimaxazole). However, *D. plumieri* was susceptible to *E.coli, Klebsiella* and *S. aureus.*

Hypotensive Action

Sajid *et al.,* (1996) reported that, the alcohol extracts of *Duranta repens* produced cardiac depression on isolated rabbit heart, and showed significant negative inotropic activity with negative chronotropic effects.

Medicinal Uses of *Duranta repens* Linn.

The wood is hard and useful for stakes and fuel. Ethyl acetate and aqueous extracts of leaves showed significant antimalarial activity when administered to mice (Castro *et al.*, 1996). The fruits are used in the treatment of malaria and intestinal worms (Whistler 2000). The leaves are used in the treatment of abscess (Xiao P, 1992).

Purpose of the Present Work

In the present investigation two plants *Moringa oleifera* Lam. and *Duranta repens* Linn. were selected for phytochemical and biological studies.

The plant *Moringa oleifera* (Family: Moringaceae) Lam. was chosen for investigation since it has a folkloric reputation. So far literature surveyed, *Moringa oleifera* Lam has tremendous uses in traditional medicines. For example, root-bark are used as astringent, anthelmintic, analgesic, useful in heart complains, eye diseases, dyspepsia, enlargement of the spleen, tuberculous glands in the neck, tumors and ulcers. The plant is also effective against burns, sores, epilepsy, scabies, cholera, dysentery and retention of urine ect. Previous phytochemical studies on different parts resulted in isolation of flavonoids, steroids, baurenol, pterygospermin, moringine and moringinine from this plant.

Versatile usefulness of the different parts of the plant in the folk medicine drew our attention to isolate its other bioactive principles along with the structure elucidation using physical methods of analysis (UV, IR, NMR and Mass etc). Different Biological screening (antimicrobial, antifungal activity, minimum inhibitory concentration, brine shrimp lethality bioassay and acute toxicity studies) of the crude extracts and isolated compounds. This will ultimately lead to use this important medicinal plant without having side effects and toxicity for particular disease

The plant *Duranta repens* Linn.(verbenaceae) was chosen for investigation because it has remarkable insecticidal, larvicidal and antifeedant properties. The slightly poisonous fruits of the plant afford a medicine for the treatment of malaria. The fruit juice can be used as a larvicide in ponds and swamps. Several workers reported the isolation of some compounds from this plant. Moreover, the leaves and fruits also showed insecticidal, larvicidal and antifeedant properties. But, there was no information on the toxicity of *D repens* against *T. castaneum* and *C. quenquifasciatus*.

The aim of the present work is to isolate the bioactive principles from the stem of the plant and evaluate the different biological screening such as antibacterial activity, MIC, brine shrimp lethality bioassay, acute toxicity study and insecticidal properties. Further more, elucidate the action of fruit extracts of *D. repens* Linn. on the rust-red flour beetle, *Tribolium castaneum* (Hervest). and larvicidal potentiality against the notorious filarial vector *Culex quinquefasciatus*.

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Chapter-II

General Methods and Materials

General Methods and Materials

Chemical Work

The phytochemical investigation of a plant involves the following five major steps:

a) Collection and proper identification of the plant samples

b) Preparation

c) Extraction

d) Isolation and purification of compounds and

e) Chemical characterization and structure determination of the isolated compounds.

Collection and Proper Identification of the Plant Sample

The whole plant/plant part(s) must be collected from an authentic source and identified by a taxonomist. A voucher specimen should be submitted to a herbarium for future reference.

Drying and Grinding

The plant materials (root bark, whole plant or fruit) should be air-dried followed by grinding into powder using a grinder (Hammer mill).

Extraction Procedures

Extraction can be done by two ways:

a) Cold extraction

b) Hot extraction

Cold Extraction

In cold extraction, the coarse powdered plant materials are submerged in a suitable solvent or solvent mixture in a flat bottom flask at room temperature and allowed to stand for several days with occasional shaking and stirring. When the concentrations of the extract is maximum, the content is then filtered. Evaporation of the solvent in *vacuo* in a rotary evaporator affords a crude extract (Vogel, 1978).

Hot Extraction

This extraction process is carried out at elevated temperature (usually reflux temp). In actual practice, this is done in a special apparatus, the Soxhlet Extractor (Quickfit, England). The apparatus ensures maximum extraction with a limited quantity of the solvent. Evaporation of solvent at low temperature (40°-50°C) and under reduced pressure in a rotary evaporator affords a crude extract (Bahl and Bahl, 1992).

Solvent Partitioning of Crude Extract

Organic compounds present in plant material can be isolated using suitable solvent or mixture of solvents in which the desired compounds are soluble. Selection of solvent / solvents actually depends on the type of compounds required to isolate. The crude extract is dissolved or mixed with distilled water and a suitable solvent, which is immiscible with water, is added to it in a separating funnel. The mixture is shaken and then kept undisturbed for several minutes for separation of the organic layer from the aqueous layer. All the organic extracts are dried over anhydrous Na_2SO_4 or $MgSO_4$ before it concentrated under reduced pressure in a rotary evaporator to afford crude extracts (Bahl and Bahl, 1992).

Isolation and Purification of Compounds

Chromatography is the most commonly used and convenient method for the separation, isolation and purification of compounds from the crude extracts. Various types of chromatographic techniques are used such as-

a. Thin layer chromatography (TLC)

b. Column chromatography and

c. Preparative thin layer chromatography (PTLC).

Thin Layer Chromatography (TLC)

This is the basic chromatographic technique operation on the principle of competitive resolution of samples between a stationary phase (silica adsorbent) and mobile phase (single solvent or combination of solvents). For the preparation of plates, a number of glass plates (20 cm ×5 cm) are thoroughly washed to remove any dirt present and dried in

a hot oven. The plates are then placed over an aligning tray specially made for TLC. The slurry (2 gm silica gel/plate) is then distributed uniformly over the carrier plates with help of a TLC spreader by adjusting the thickness of the layer. After air drying, the coated plates are activating by heating in a hot oven at 110°C for 70 minutes.

Cylindrical shaped glass chamber (TLC tank) with air tight glass lid is used for the development of a chromatoplate. The selected solvent system is poured into the tank and a smooth sheet of filter paper is laid and soaked in the solvent to saturate the internal atmosphere with the solvent vapor (Stahl, 1969).

A small amount of dried extract is dissolved in suitable solvent to get (1%) solution. A small spot with the solution is applied on the activated silica gel plate with a capillary tube just about 2 cm above the lower edge of the plate. The spot is then dried with an air blower. The spotted plate is then placed in saturated tank such a way as to keep the applied spot above the surface of the solvent and the lid is placed. The plate is left allowing the solvent system to creep up. When the solvent front reached up to 1 cm below the upper edge, the plate is taken out and dried with air blower and the spots on TLC plate is detected by spray the plate with spray reagent and under UV light (Harborne, 1976; Touchstone and Dobbins, 1978).

Column Chromatography

A column chromatography technique is used to separate the individual components from a mixture of organic compounds. It involves the principle of distribution (partition or adsorption) of components between a stationary and a mobile phase. The stationary phase is a macromolecule and the mobile phase is the selective organic solvent.

For packing the column, silica gel (60-120 mesh) is taken in a beaker and solvent is poured. The content is covered and kept at 20°C for at least 2 hrs, to make slurry. A glass column of required size is first plugged with a piece of clean cotton at the bottom and fitted with a stand. The stopcock of the column is opened and solvent is passed through the column. In the mean time the slurry is allowed to pour gently into the column. After settling, some solvent is allowed to drain out. However, care must be taken so that the solvent sufficiently covered the adsorption bed. The column is packed at 20°C to avoid cracking. After packing, the sample is applied to the column. The extract is mixed with a

a hot oven. The plates are then placed over an aligning tray specially made for TLC. The slurry (2 gm silica gel/plate) is then distributed uniformly over the carrier plates with help of a TLC spreader by adjusting the thickness of the layer. After air drying, the coated plates are activating by heating in a hot oven at 110°C for 70 minutes.

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small portion of the stationary phase (silica gel) using mortar and pestle in order to obtain a non-sticky free flowing mass. The amount thus obtained is placed on the packed column carefully in such a way that the upper layer of the bed is not disturbed.

The column is then run with solvent mixtures of increasing polarities. The flow is maintained at a constant rate (2 ml/min). Elutes are collected in different conical flasks. All organic extracts elutes are dried over anhydrous Na_2SO_4 or $MgSO_4$ (Touchstone and Dobbins, 1978; Srivastave and Srivatave, 1987).

Preparative Thin Layer Chromatography (PTLC)

Preparative TLC is applied to the separation and final purification of some compounds. Plates are prepared by coating glass plates (20×20 cm) to a thickness of 0.5 mm using a slurry made form 40 gm (for 6 plates) of silica gel (kiselgel 60 GF_{254}) and 85 ml of distilled water. The plates are allowed to air dry for a time and then activated in an oven at 110°C for 2-3 hours.

The sample to be separated is dissolved in a small amount of suitable solvent and applied to the plates as a uniform band 2cm from the bottom edge. The plates are developed to upper edge of plates with a suitable mobile phase. In some cases, a double or triple development technique is adopted for better separation of the bands. After development, the plates are allowed to dry and observed under UV light (254 nm and 366 nm). Non-UV active compounds are observed by spraying a small portion of both edges of the plate with an appropriate spray reagent. Bands of interest are scraped off from the plates with the help of a spatula and eluted from the silica matrix by dissolving in suitable solvent, removed the silica gel by filtration and obtained the compounds by evaporation of solvent in *vacuo*.

Detection of the Compounds

The following techniques are generally used to detect the compounds in TLC and PTLC plates (Bobbilt, 1963).

UV light

The developed and dried plates are exposed under UV light (254 mm and 366 nm) to observe quenching or fluorescing compounds.

Iodine Vapor

The developed chromatogram is exposed to iodine vapor in a closed jar containing few crystals of iodine. Brownish spots indicate resolution of the compounds.

Spray Reagents

Different types of spray reagents can be used depending upon the nature of compounds expected to be present in the crude extracts or fraction.

Vanillin in sulfuric acid: This reagent is prepared by dissolving vanillin (1gm) in conc. H_2SO_4 (100 ml) and used as general reagent for non-alkaloidal compounds. The plates sprayed with this reagent are heated at 110°C for 10-15 min to developed a color or ranges of colors developed with time, which, in turn, indicated the presence of higher alcohols, steroids, essential oils and terpens (Stahl, 1966).

Modified Dragendorff's reagent: This reagent is prepared by mixing equal parts (v/v) of 1.7% bismuth subnitrate dissolved in 20% acetic acid in water and 40% aqueous solution of potassium iodide. Usually orange-red color indicates the presence of alkaloids by this reagent (Touchstone and Dobbins, 1977).

Anisaldehyde-sulfuric acid: To prepare this reagent, conc. H_2SO_4 (10 ml) is added to glacial acetic acid (20 ml). This mixture is then poured into MeOH (170 ml) containing anisaldehyde (1 ml). The TLC plate is sprayed with this reagent and heated at 110°C for 10-15 min to visualize the spot.

Crystallization

Crystallization is the technique commonly used for the purification of solid organic compound. In this technique selection of solvent or solvent system is important. The good solvent is that in which the compound dissolved partially at room temperature and dissolved on boiling. The boiling solvent is then allowed to cool undisturbed till

crystallization is completed. The crystals are then separated from the mother liquor by filtration and dried. In another case when the compound is soluble at room temperature then another solvent is poured on to the boiling solvent until the proportion of the solvent is increased and allowed the solvent mixture to cool down until crystallization is completed (Bahl and Bahl, 1992).

Characterization of Isolated Compounds

To characterize each of the isolated compounds, physical and chemical properties as well as spectral data are fully studied.

Physical Characterization

The physical properties of the isolated compounds such as physical form, color, odor, solubility behavior, R_f value and melting point are studied carefully.

R_f Value

R_f value is characteristic of a compound in a specific solvent system. It helps in the identification of compounds. R_f value can be calculated by the following formula.

$$R_f \text{ value} = \frac{\text{Distance traveled by the sample}}{\text{Distance traveled by the solvent (solvent front)}}$$

Melting Point

A pure solid substance melts sharply at a definite temperature, while an impure substance will have a lower and indefinite melting point. Melting points are determined on melting point apparatus (PIC, India) and are uncorrected.

Spectroscopic Characterization

Different spectroscopic methods are used to elucidate the structure. Among the spectroscopic techniques Mass, IR, ^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{1}H COSY45°, HSQC, HMBC and others are carried out.

Infrared Spectra (IR)

IR spectrum is recorded with KBr discs, using the PERKIN ELMER 1600 FTIR spectrophotometer in the Department of Chemistry, University of Rajshahi, Bangladesh and the data are given in cm^{-1}.

Nuclear Magnetic Resonance (NMR)

^{1}H and ^{13}C NMR spectra were recorded on an AVANCE DRX500 Bruker spectrometer. The ^{1}H NMR spectra were recorded at 500.13 MHz and the ^{13}C NMR spectra recorded at 125.77 MHz, using the residual solvent peak as reference (**Table-1**).

Table-1 Chemical shifts (δ) of the deuterated NMR solvents used.

NMR solvents	**^{1}H (multiplicity)**	**^{13}C (multiplicity)**
Chloroform-d	7.27(1)	77.23 (3)
MeOH-d_4	4.87(1), 3.31 (5)	49.15 (7)
Pyridine-d_5	8.74 (1), 7.58 (1), 7.22(1)	150.35 (3), 135.91(3), 123.87 (5)

Mass Spectra (MS)

Mass spectral analysis conducted by LC/MS for preliminary screening of crude extracts and to determine mass of isolated compound using an Agilent LC-MS 1100 series which was controlled by HP chemstation LC-MS software at the Centre for Phytochemistry and Pharmacology, Southern Cross University. The ionization technique used was either atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI). GC-MS was also used for the non-polar fraction and isolated compounds at Centre for Phytochemistry and Pharmacology, Southern Cross University, Australia. In order to determine the accurate mass of novel isolated compounds High-Resolution Mass Spectroscopy was performed at University of Queensland, Australia. The EI were run on a Kratos MS25 RFA via direct insertion probe at 70 eV and source temperature of 200°C. PRK is used as reference for magnet scan accurate mass in EI. ESI were run on a Finnigan MAT 900 XL-Trap instrument with a Finnigan API III electrospray source, using MeOH as solvent and PPG or PEG as reference for accurate mass data, acquired by electric sector scan.

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Chapter-III

Study on *Moringa oleifera* Lam.

Botany of *Moringa oleifera* Lam.

Scientific name : *Moringa oleifera* Lam. (syn. *M. pterygosperma* Gaertn).

Bengali name : Sajna

English name : Drumstick, Indian Horse raddish.

Family : Moringaceae

Genus : Moringa

Habit : A small to medium sized tree, about 10 m. in height.

Leaves : A slender, sometimes 45 cm. long, usually 3-pinnate, thickened and articulated at the base; pinnae and pinnules opposite, leaflets 12-20 by 6-10 mm., petiolules of the lateral leaflets 1.5-2.5 mm., those of the terminal 3-6 mm. long.

Flowers : White, in large puberulous panicles.

Calyx : Lobes linear-lanceolate reflexed, puberulous outside, petals spathulate, veined.

Stamens : 5 fertile, alternating with 5-7 antherless ones, filaments villous at the base.

Ovary : Oblong, villous, style cylindric.

Pods : 45 cm. long, 9-ribbed.

Seeds : 3-angled, the angles winged.

Distribution : Throughout Bangladesh, India, Burma, Pakistan and in the Sub-Himalayan tract (Kirtikar and Basu, 1984; Hooker, 1879).

***Moringa oleifera* Lam.**

Root Barks of *Moringa oleifera* Lam.

Chemical Study

Experimental

Extraction, Isolation and Purification

Fresh root barks of *Moringa oleifera* Lam. were collected from the adjoining areas of Rajshahi University Campus, Bangladesh in March, 2001 and identified by Professor A.T.M. Nadiruzzaman, Department of Botany, University of Rajshahi and a voucher specimen (no. A Islam 3, collection date 02.05.1982) was deposited in the Department of Botany, University of Rajshahi.

The root barks were first washed with water to remove adhering dirt. Then cut into small pieces, dried in the sun for 7 days and finally in an oven for 36 hours at a temperature below 60°C. After complete drying, the dried materials were then pulverized into a coarse powder with the help of a grinding machine (FFC-15, China). The dried plant materials (1.5 kg) were then stored in an airtight container for further use.

The powdered plant materials (1.5 kg) were extracted with ethanol (5.0 lit) in an aspirator bottle, through occasional shaking and stirring for 7 days. The contents were pressed through the Tincture Press (Karlkolb, Scientific-Technical Suppliers, Frankfurt /m-Germany) to get maximum amount of extract. The extract was then filtered through Whatman No. 1 filter paper and the filtrate thus obtained was concentrated under reduced pressure at 50°C using rotary evaporator to afford a dark brownish syrupy residue (70.0 gm).

The concentrated ethanol extract (50.0 gm) was diluted with distilled water and taken in a separating funnel. Petroleum ether (40-60°C) (40 ml) was then added to the funnel. The funnel was shaken vigorously and allowed to stand for few minutes. The organic layer (upper layer) was then collected. The process was repeated three times. The organic layers were dried and concentrated in *vacuo* to afford a petroleum ether soluble fraction (4.0 gm). After petroleum ether extraction, the aqueous part was then extracted three times with chloroform (40 ml) each. The organic layers were dried and concentrated in *vacuo* to afford a chloroform soluble fraction (9.6 gm).

The chloroform extracts showed three prominent spots with other minor spots on TLC and were checked under UV, iodine vapor and vanillin-H_2SO_4 reagent. The chloroform extract (5.0 gm) was subjected to column silica gel and eluted with n-hexane, n-hexane with increasing portions of ethyl acetate and finally with ethyl acetate.

Among the 47 fractions, fraction 26-30 eluted with n-hexane-ethyl acetate (50:50) were combined and rechromatographed on silica gel-60 column eluting with n-hexane and chloroform with increasing portions of chloroform and finally with methanol. The fractions, which showed similar spots on TLC were combined and were subjected for further purification.

Among the 26 fractions, fraction 7-11 eluted with n-hexane-chloroform (75:25) showed similar single spot on TLC with a slight contamination with other compounds. The fractions were combined. The solvent was then evaporated off under reduced pressure to a certain volume and allowed to stand overnight. Few needle shaped crystals were appeared. Recrystallization from petroleum ether (40-60°C) afforded a new compound-**1** (31.4 mg). Schematic representation is given in **Fig-1**.

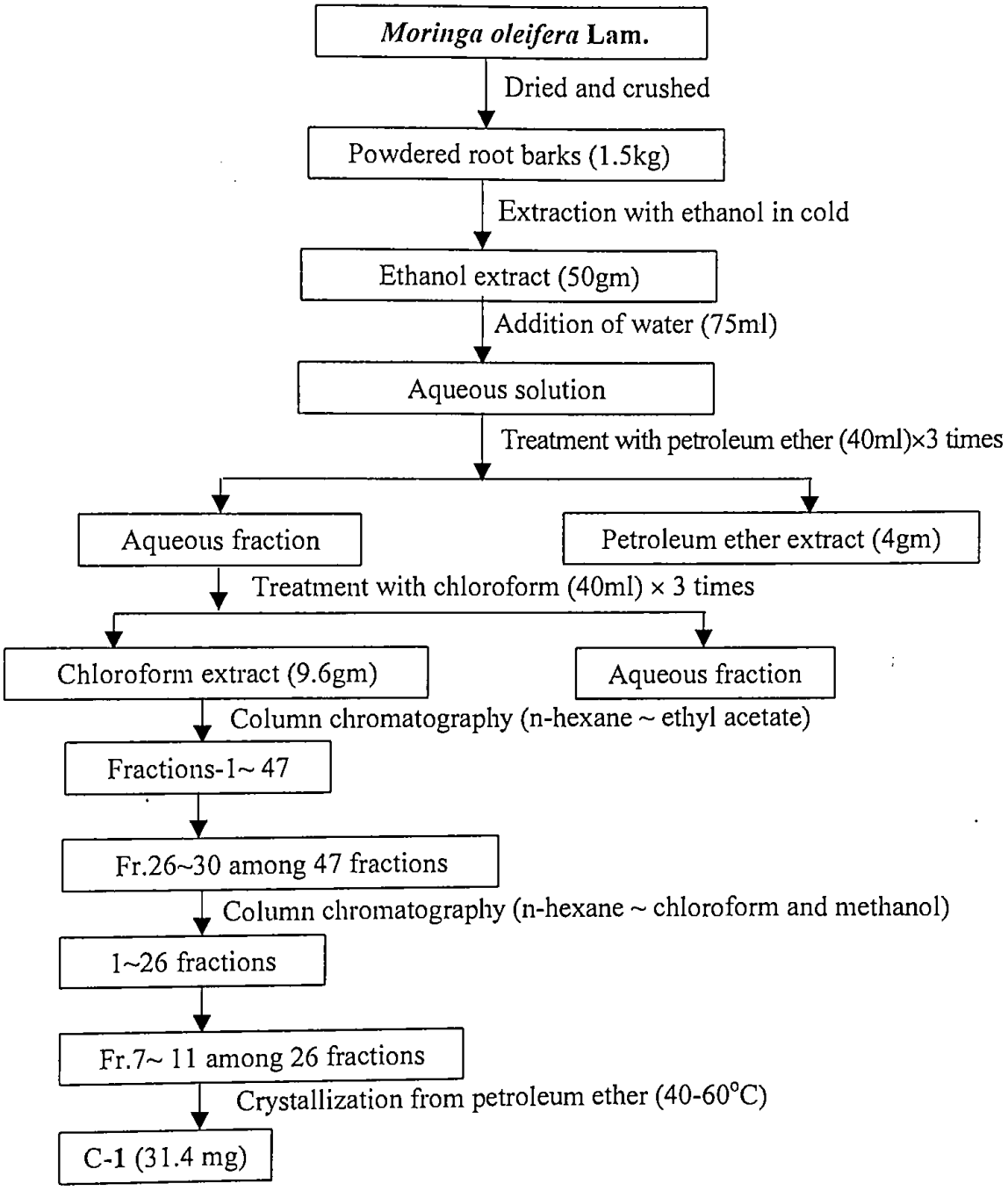


Fig-1 Extraction and isolation scheme of compound-1 from root bark of *Moringa oleifera* Lam.

Results and Discussion

Characterization of Compound-1

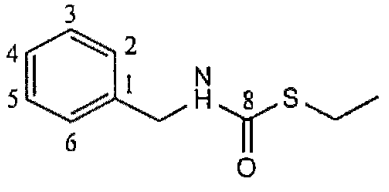
Compound-**1** was isolated from the chloroform fraction of ethanolic extract of *Moringa oleifera* Lam. as needles, mp. 58-60°C. The LCMS (APCI) showed a parent molecular ion $[M+H]^+$ peak at *m/z* 196 which corresponds to the molecular formula $C_{10}H_{13}NOS$. Other fragment ions were observed at *m/z* 166 which correspond to $[M+H]-CH_2CH_3-H$, *m/z* 132 corresponding to $[M+H]-SCH_2CH_3-3H$ and *m/z* 105 $[M+H]-COSCH_2CH_3-2H$, support the presence of a sulphur atom in the molecule (**Fig-2**). The IR spectrum of compound-**1** showed absorption bands at 3294, 2872 ~ 3032, 1639, 1525, 1217 and 1195 cm^{-1} indicating the presence of a mono-substituted benzene ring, an amino group and a carbonyl group (**Fig-3**). Moreover, preliminary chemical tests showed the presence of nitrogen and carbonyl group. The ^{13}C-NMR spectrum of compound-**1** showed the presence of ten carbon signals of which one accounts for a methyl group, two for methylene, five for methyne and one for a quaternary carbon atom (**Table-1** and **Fig-4**). The ^{1}H-NMR spectrum showed the typical signals for ethyl group, at δ_H 1.32 (t, *J*= 7.4 Hz) corresponds to the terminal CH_3 group and at δ_H 2.96 (q, *J*=7.4 Hz), due to the methylene group. The signal at δ_H 4.48 was assigned to an isolated methylene group which showed a coupling constant of 4.9 Hz, indicating the presence of an adjacent secondary amine. The NH proton appeared as a broad singlet at δ_H 5.63 that disappeared with D_2O (**Table-1** and **Fig-5**).

Table-1 ^{1}H and ^{13}C-NMR spectral data of compound-**1**.

Position	$^{\delta}$H	$^{\delta}$C
1	-	138.0
2	7.29, m	127.9*
3	7.35,m	129.0
4	7.29,m	127.8*
5	7.35,m	129.0
6	7.29,m	127.9*
7	4.48, 2H,d (*J*=4.9Hz)	45.5
8	-	167.6
9	2.96,2H, q (*J*=7.4Hz)	24.6
10	1.32,3H, t (*J*=7.4Hz)	15.9
NH	5.63, br s	

* These assignments may be interchanged.

The structure was further supported by analysis of its ^{1}H-^{1}H COSY and ^{1}H-^{13}C correlation spectra. From the ^{1}H-^{1}H COSY 45° spectrum, it was observed that the CH_2 protons at δ_H 4.48 correlate with benzene ring protons and NH proton. Hence, this methylene must be located between a benzene ring and a NH group. The other CH_2 group correlate with the terminal CH_3 group (**Fig-6**). Furthermore, the HMBC spectrum showed a ^{2}J and ^{3}J correlation between the CH_2 protons at δ_H 2.96 with the terminal CH_3 group carbon at δ_H 15.9 and the C=O carbon at δ_H 167.6, respectively (**Fig-7**). From the above spectral evidence the structure of compound-**1** was established as the deoxy-niazimicin (N-benzyl, S-ethyl thiocarbamate). Niazimicin was isolated previously from this plant (Guevara *et al.*, 1999) but it was the first report of isolation of the aglycone of deoxy-niazimicin from this plant (DNP, 2002).



Compound-**1** : N-benzyl, S-ethyl thiocarbamate

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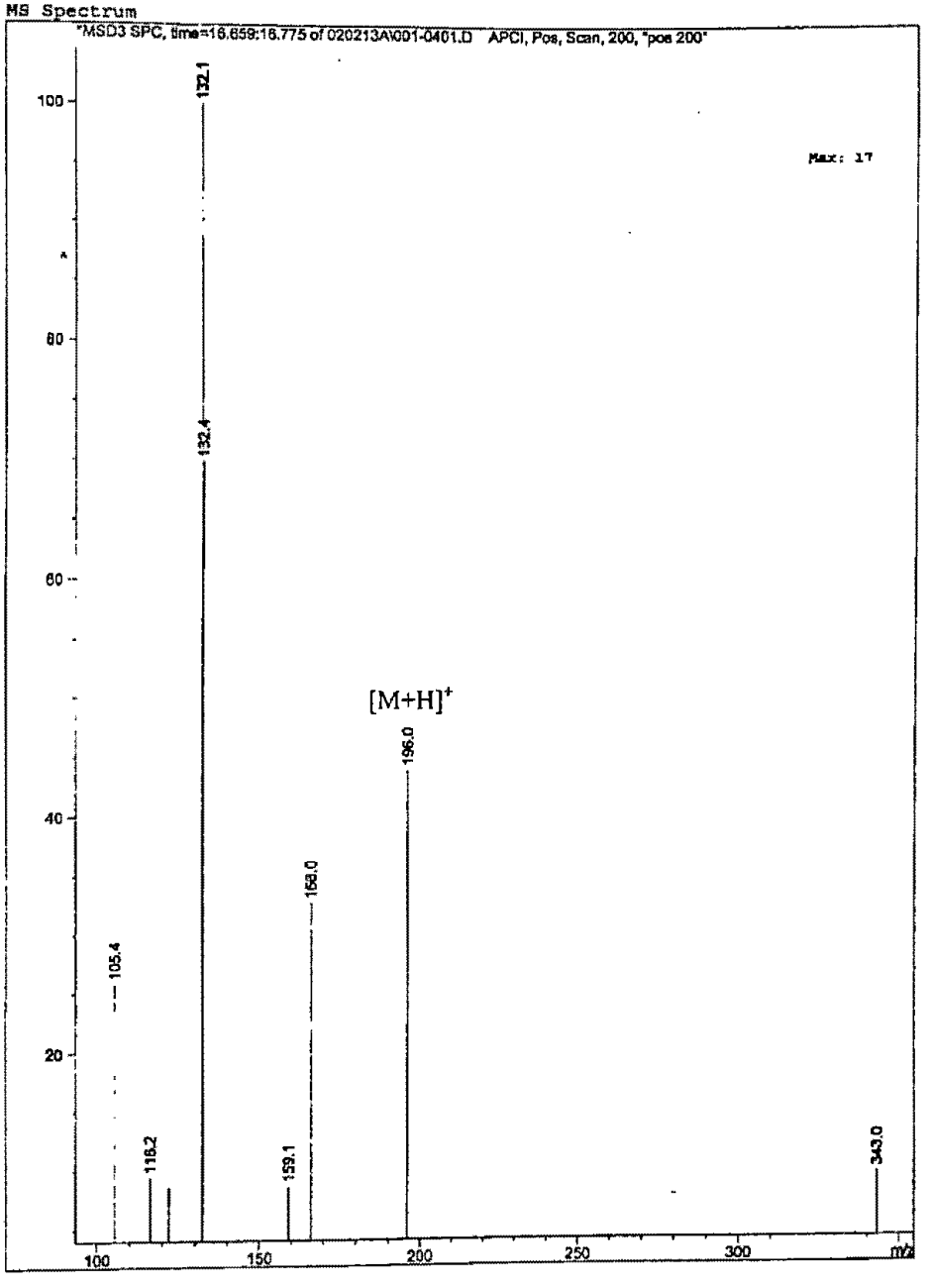

Fig-2 Mass spectrum of compound-1

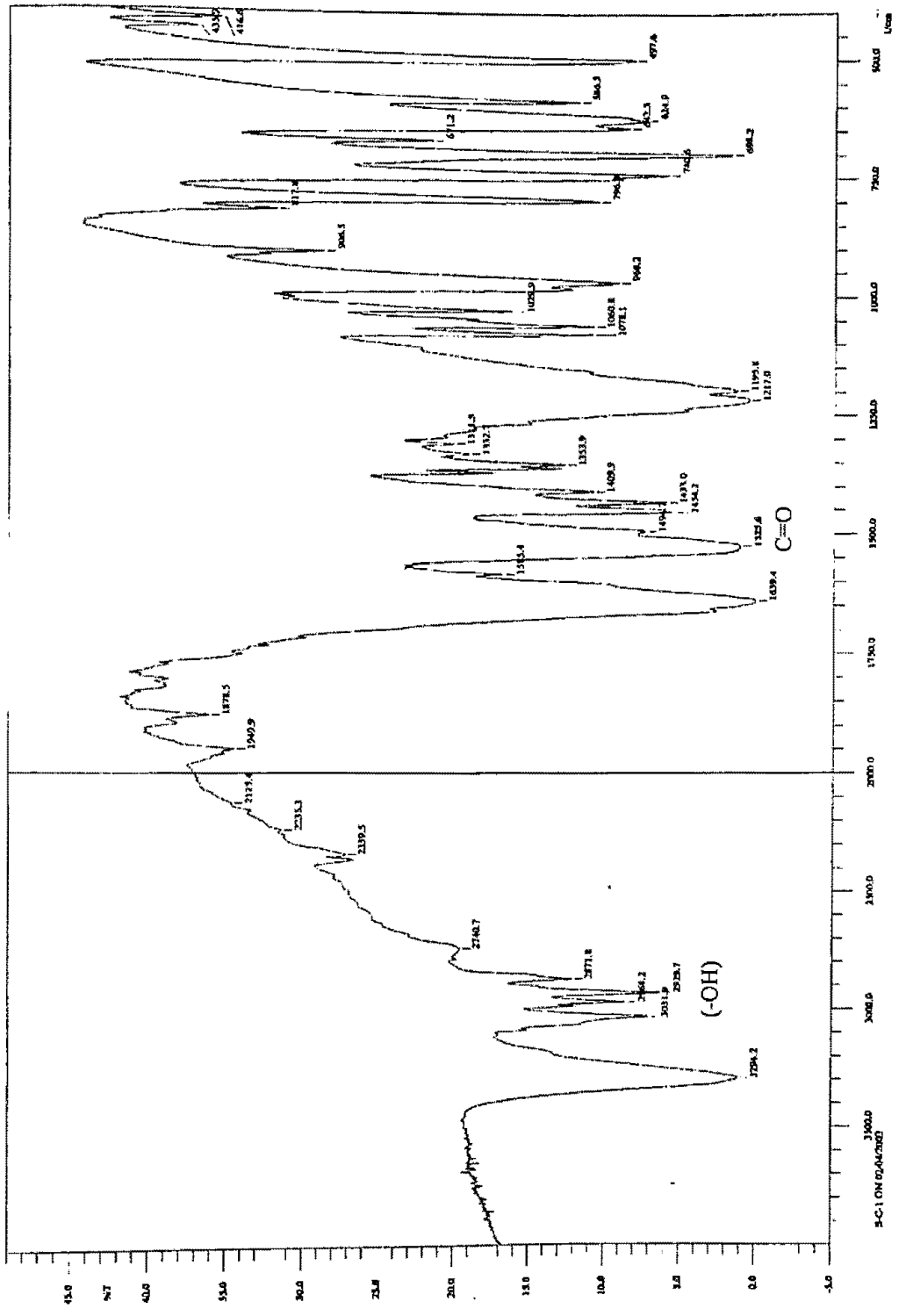

Fig-3 IR spectrum of compound-1

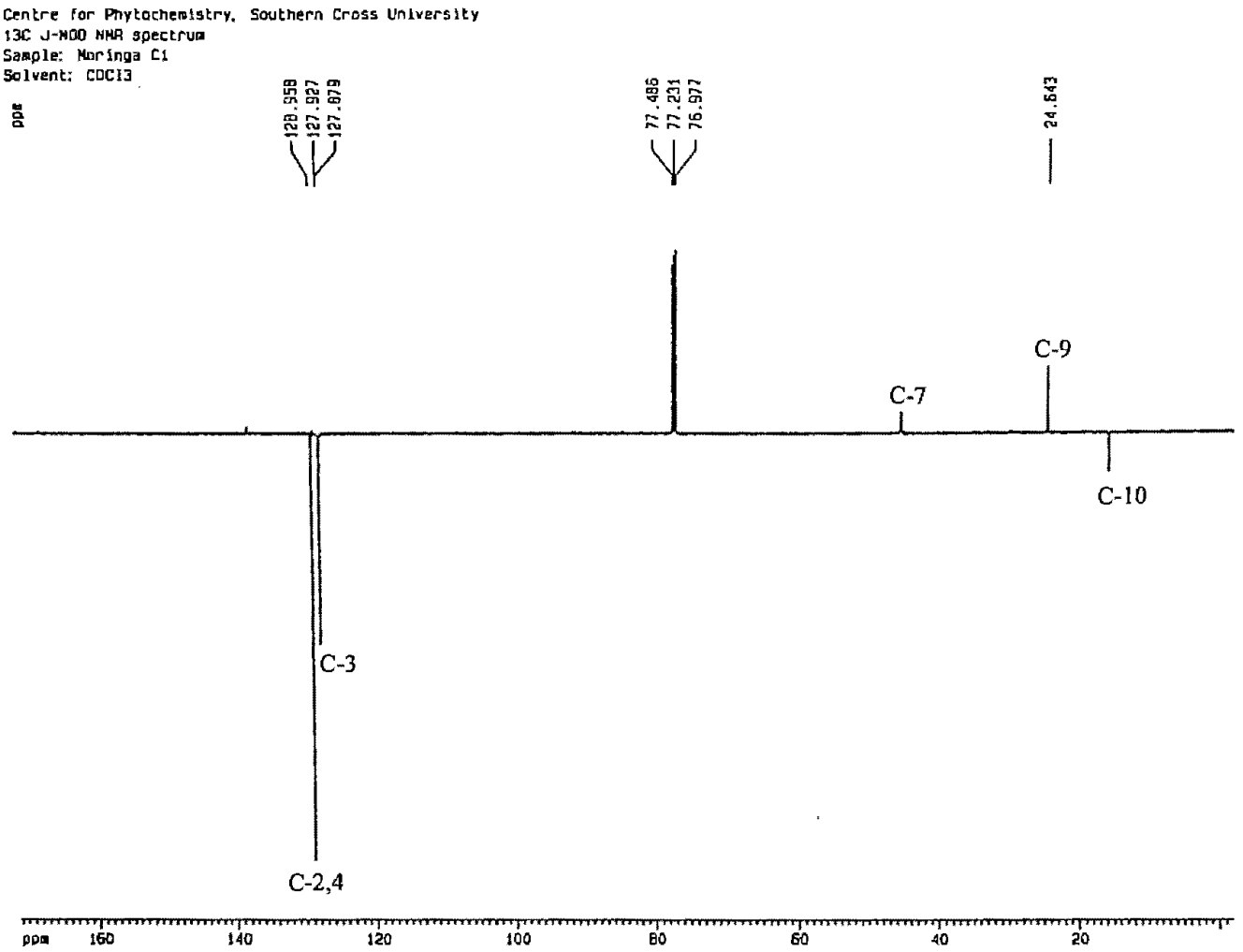

Fig-4 ^{13}C-NMR spectrum of compound-**1**

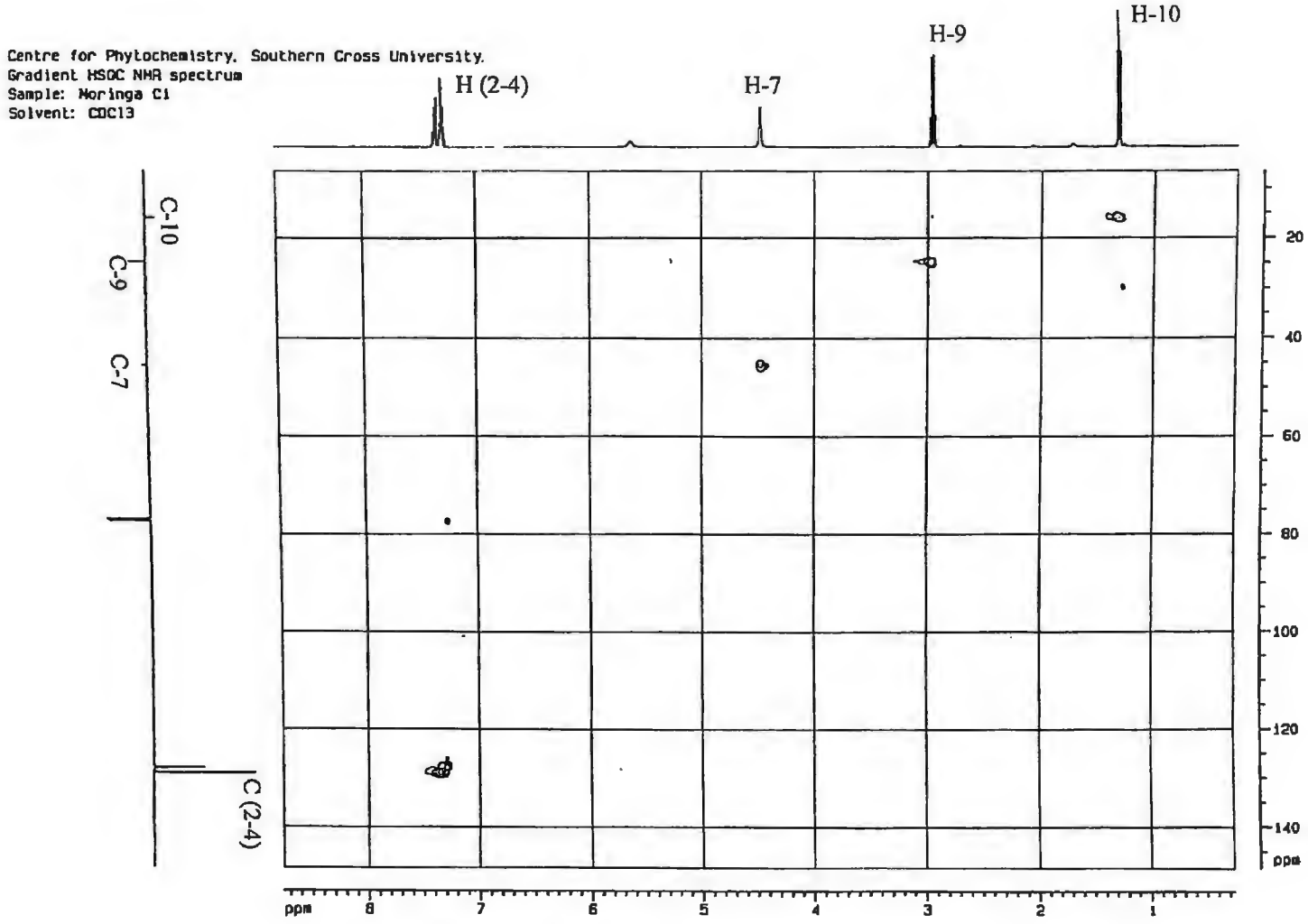

Fig-5 HSQC NMR spectrum of compound-1

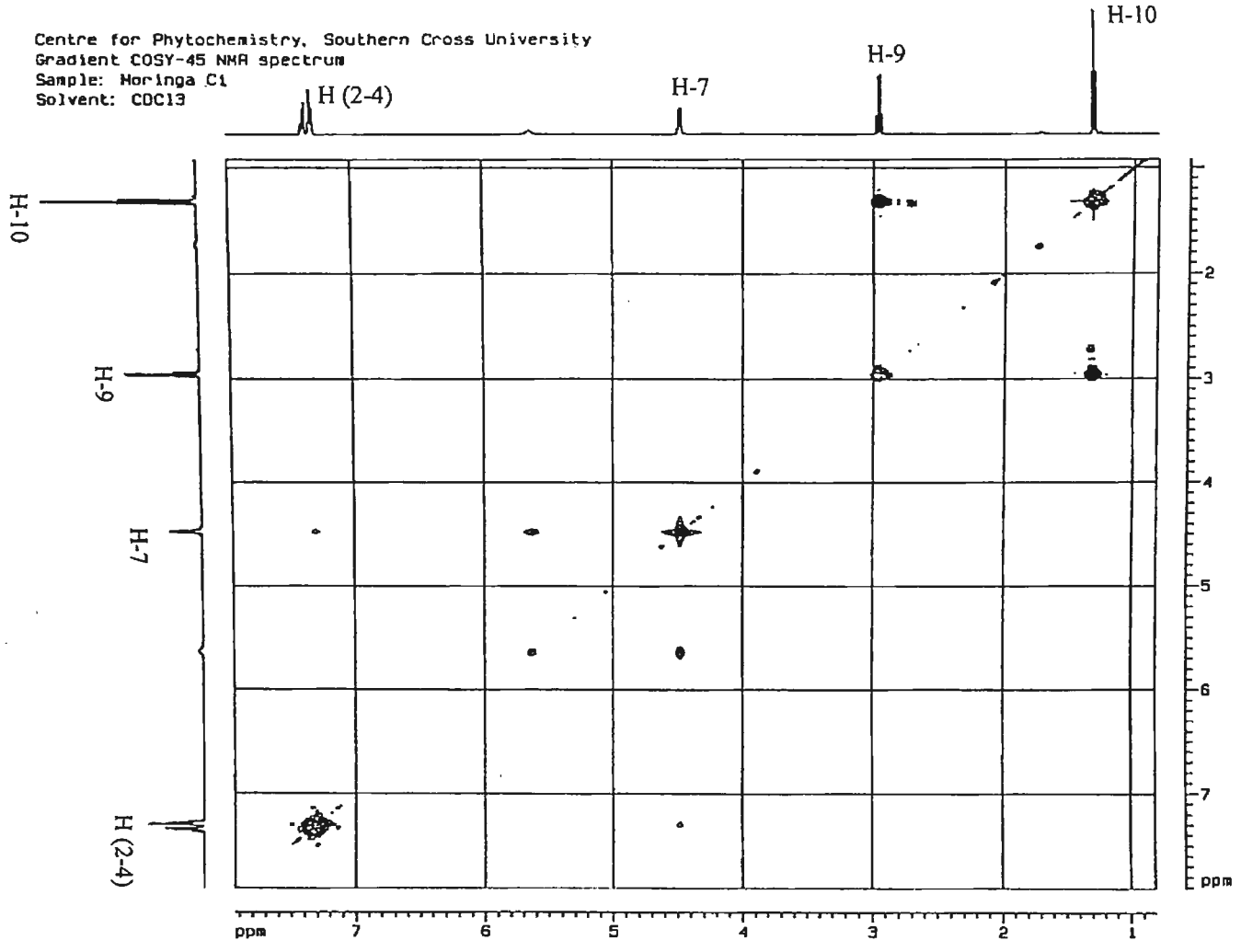

Fig-6 ^{1}H-^{1}H COSY-45^{0} NMR spectrum of compound-**1**

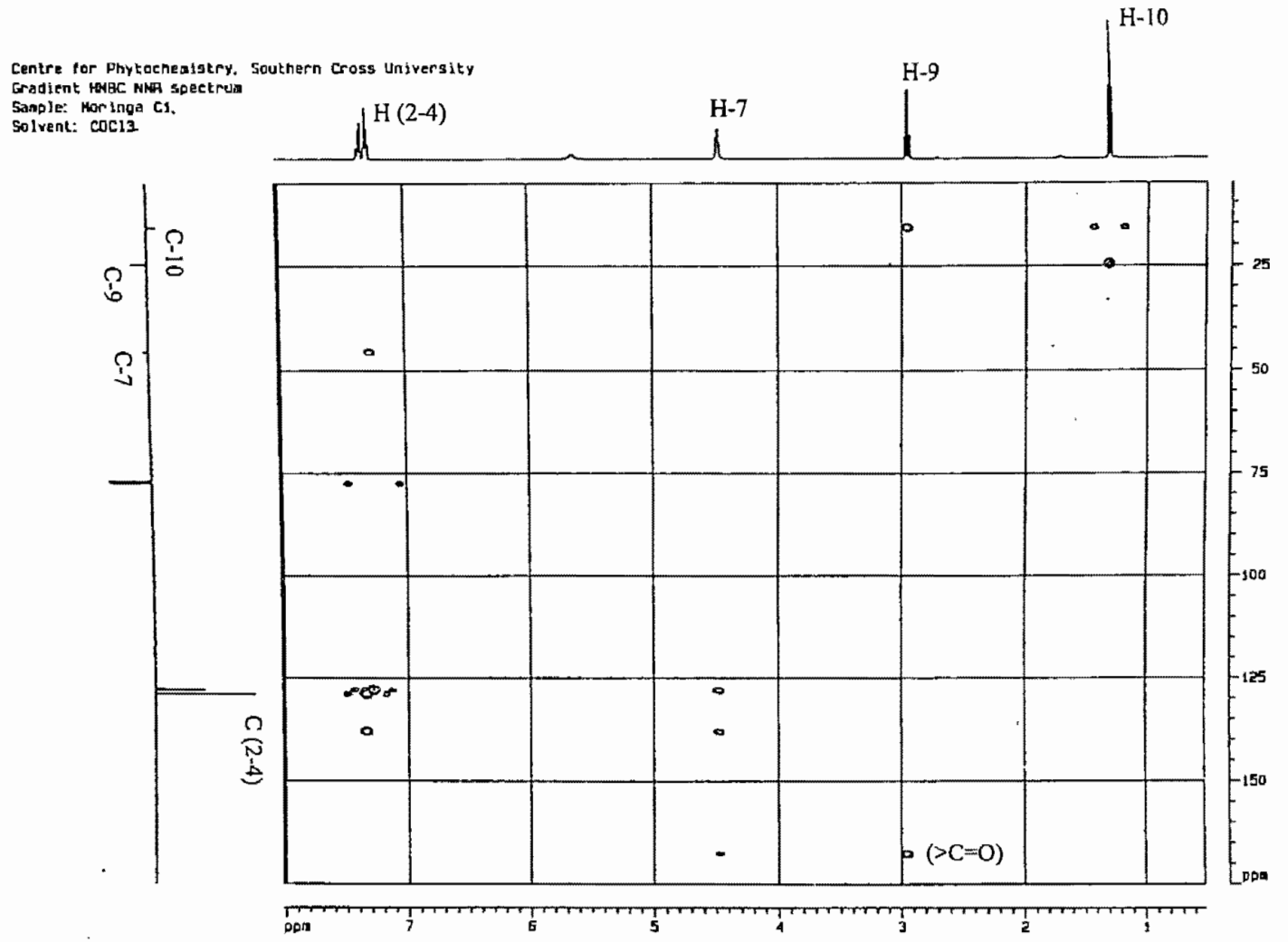

Fig-7 HMBC spectrum of compound-**1**

Properties of Compound-1

Needles ; mp. 58-60°C; IR (KBr): 3294, 2872~3032, 1639,1525, 1217, 1195 Cm^{-1}; ^{1}H-NMR(500 MHz, $CDCl_3$): δ7.29, m(H-2),δ7.35, m(H-3),δ7.29, m(H-4), δ7.35, m(H-5), δ7.29, m(H-6), δ4.48, 2H, d(*J*=4.9Hz, H-7), δ2.96, 2H, q(*J*=7.4Hz, H-), δ1.32, 3H, t(*J*=7.4Hz, H-10), δ5.63, br s(NH); ^{13}C-NMR (125 MHz, $CDCl_3$): 138.0 (C-1), 127.9* (C-2), 129.0 (C-3), 127.8* (C-4), 129.0 (C-5), 127.9* (C-6), 45:5 (C-7), 167.6 (C-8), 24.6 (C-9), 15.9 (C-10); LCMS (APCI) $[M+H]^+$ *m/z* 196 and other at *m/z* 166,132 and 105.

Conclusion

A novel compound-**1** named deoxy-niazimicin (N-benzyl S-ethyl thiocabamate) was isolated from the chloroform fraction of ethanol extract. A mixture of terpenoids, sitosterol and stigmasterol, long chain alkane and *β*-sitosterol with minor impurities were also isolated from this plant. But the amount was too small to obtain pure compound from the mixture.

Biological Study

Antibacterial Activity Test

Experimental

Plants have been a source of medicinal compounds since pre-historic time. All parts of plants were used in Ayurvedic, Unani and Allopathic systems of medicine for the treatment of human aliments such as wound, infections, typhoid, dysentery and number of skin diseases etc. Any chemical substance or biological agent that either destroy or suppress the growth of microorganism is called antimicrobial agent. Antimicrobial activity can be detected by observing the growth response of various microorganisms to the plant or parts of a plant extract, which is placed in contact with them. The susceptibility of microorganisms to antimicrobial agents can be measured *in vitro* by a number of techniques among which the disc diffusion method (Vander and Vlietnck, 1991) is widely acceptable for the preliminary evaluation of antimicrobial activity.

Test Organisms

Fourteen pathogenic bacteria (five Gram positive and nine Gram negative) were selected for the test (**Table-2**). These organisms were available in the microbiological research laboratory of Biochemistry and Molecular Biology department, University of Rajshahi. The pure cultures were collected from the Microbiological Laboratory of the Institute of Nutrition and Food Science (INFS) and Department of Microbiology, University of Dhaka, Bangladesh.

Table-2 List of test bacteria.

Gram positive	Gram negative
Bacillus subtilis *Bacillus megaterium* *Staphylococcus aureus* *Streptococcus-β-haemoliticus* *Sarcina lutea*	*Escherichia coli* *Shigella sonnei* *Shigella boydii* *Shigella dysenteriae* *Shigella flexneri* *Shigella shiga* *Salmonella typhi* *Pseudomonas aeruginosa* *Klebsiella species*

Sterilization Procedure

Antimicrobial screening was carried out in a laminar airflow unit and all types of precautions were highly maintained to avoid any contamination during the test. UV light has switched on before working in laminar hood for 1 hour to avoid any accidental contamination. Petridishes and other glassware were sterilized by autoclaving at a temperature of 121°C and pressure of 15 lb/sq inch for 20 minutes. Blank discs were first kept in a covered petridish and then subjected to dry heat sterilization at 180°C for 1 hour. Latter they were kept in laminar hood under UV light for 30 minutes.

Preparation of Sub-culture

The instant nutrient agar media was accurately weighted and then reconstituted with distilled water in a conical flask according to specification (28% w/v). It was then heated in water bath to dissolve the agar until a transparent solution was obtained.

The media prepared were then dispensed to a number of clean test tubes, each containing 5ml, to prepare slants. The slants were then plugged with cotton and sterilized in an autoclave. After sterilization, the test tubes were kept in an inclined position for solidification. These were then incubated at 37.5°C to ensure sterilization. Then the test organisms were transferred from the supplied pure culture to the agar slants with the help of an inoculating loop in an aseptic condition. For the growth of the test organism the inoculated slants were incubated at 37°C for 24 hours. These fresh cultures were used for sensitivity test within 2 to 3 days (Bauer *et al.*, 1951; Srivastava, 1984).

Preparation of the Test Plates

The test plates were prepared according to the following procedures:

1. A number of petridishes and test tubes were washed and sterilized by dry heat.
2. Nutrient agar media (20 ml) was poured in clean test tubes and plugged with cotton.
3. The test tubes were sterilized by autoclaving and allowed to cool at about 50°C.
4. The media in the test tubes were inoculated with fresh culture of the test bacteria by a sterile loop in aseptic condition and agitated to ensure uniform dispersion of organisms into the media.

5. Finally, the media was poured into sterile petridishes in aseptic condition. The petridishes were rotated several times, first clockwise and then anticlockwise to assure homogeneous distribution of test organisms. The depth of media into each petridish (120 mm diameter) was approximately 4 mm. After the media has cooled to room temperature, it was stored in a refrigerator at 4°C.

Preparation of Discs

Three types of discs were prepared for antibacterial screening. These are-

1. **Sample discs**

Sterilized (BBL, U.S.A) filter paper discs (5 mm in diameter) were prepared with the help of punch machine and were taken in a blank petridish. Sample solution of desired concentration was applied on the discs with the help of a micropipette in an aseptic condition.

2. **Standard discs**

These were used to compare the antibacterial activity of test material. In our investigation, Kanamycin (30 μg/ disc) was used as standard disc.

3. **Control discs**

These were used as negative control to ensure that the residual solvent and the filter paper were not active themselves.

Preparation of Test Sample

The crude ethanol, petroleum ether and chloroform extracts (3 mg each) were dissolved separately in methanol (600 μl) to get a concentration of 30 μg/6μl. Again 100 μg/10μl was obtained by dissolving the crude extracts (5 mg each) in methanol (500 μl).

Compound-**1** (3 mg) was dissolved in 600μl of methanol to get a concentration of 30 μg/6μl. Again 100 μg/10μl was obtained by dissolving compound-**1** (5 mg) in methanol (500 μl).

Placement of Discs and Incubation

The sample impregnated discs, control discs and standard antibiotic disc were placed gently on the solidified agar plates freshly seeded with the test organism with the help of a sterile forceps to assure complete contact with medium surface. The plates were then inverted and kept in a refrigerator for about 24 hrs at 4°C. Finally, the plates were incubated at 37°C for 12-18 hours.

Measurement of the Zone of Inhibition

After incubation, the antibacterial activity of the test agent was determined by measuring the diameter of zone of inhibition (mm). Zone of inhibition obtained by each sample was compared to the standard and control discs.

Results and Discussion

The crude ethanol, chloroform and petroleum ether extracts and compound-1 were used at two concentrations (30 μg/disc and 100 μg/disc). Standard antibiotic disc of Kanamycin (K-30 μg/disc) was used for comparison.

Compound-**1** showed moderate antibacterial activity against almost all Gram positive and Gram negative bacteria at both concentrations (30 μg/disc and 100 μg/disc). It showed prominent activity against *Staphylococcus aureus* (10 mm and 14 mm), *Shigella boydii* (12 mm and 15 mm), *Salmonella typhi* (10 mm and 13 mm), *Pseudomonas aeruginosa* (09mm and 12mm) and showed highest activity against *Shigella dysenteriae* (13mm and 16mm) at both concentrations (**Table-3** and **Fig-8, 9**).

At 30 μg/disc, chloroform extract gave mild activities against *Bacillus megaterium*, *Staphylococcus aureus*, *Shigella dysenteriae*, *Shigella flexneri*, *Shigella shiga* and *Shigella boydii* and produced zone of inhibition between 08 mm to 11 mm. But at 100 μg/disc, it gave moderate activities against almost all microorganisms and showed highest activities against *Shigella dysenteriae, Shigella flexneri, Shigella shiga* and *Shigella boydii* and produced zone of inhibition 14 mm. for each microorganisms (**Table-3** and **Fig-8, 9**).

Table-3 *In vitro* antibacterial activity of compound-**1** and crude extracts of *Moringa oleifera* Lam.

Zone of Inhibition (Diameter in mm)

Bacterial Strains	Compound-1		Crude extracts						Kana-mycin
			Chloroform		Ethanol		Pet-ether		
	30 µg/ disc	100 µg/ disc	30 µg/ disc	100 µg/ disc	30 µg/ disc	100 µg/ disc	30 µg/ disc	100 µg/ disc	30 µg/ disc
Gram positive									
Bacillus subtilis	00	08	00	08	00	00	00	00	20
Bacillus megaterium	08	12	08	10	00	00	00	00	18
Sarcina lutea	00	09	00	09	00	00	00	00	20
Staphylococcus aureus	10	14	09	13	00	10	00	08	30
Streptococcus-β-haemolyticus	00	10	00	10	00	00	00	00	24
Gram negative									
Escherichia coli	00	10	00	09	00	00	00	00	21
Klebsiella species	08	11	00	10	00	08	00	08	17
Pseudomonas aeruginosa	09	12	00	11	00	09	00	08	25
Shigella dysenteriae	13	16	10	14	00	11	00	10	20
Shigella flexneri	00	09	08	14	00	00	00	00	22
Shigella shiga	00	10	09	14	00	00	00	00	25
Shigella sonnei	00	09	00	00	00	00	00	00	19
Shigella boydii	12	15	11	14	00	10	00	08	30
Salmonella typhi	10	13	00	08	00	09	00	08	20

At 30 µg/disc, ethanol extract showed no activity against all microorganisms. But at 100 µg/disc, the ethanol extract showed mild activities against some Gram positive and Gram negative bacteria and it gave highest activity against *Shigella dysenteriae* (11 mm.) (**Table-3** and **Fig-8, 9**). The petroleum ether extract showed no activity against all microorganisms at 30 µg/disc. But at 100 µg/disc petroleum ether extract showed mild activities against *Staphylococcus aureus*, *Klebsiella species*, *Shigella dysenteriae*, *Shigella boydii* and *Shigella typhi* and produced zone of inhibition between 8 mm to 10 mm. (**Table-3** and **Fig-8, 9**). This investigation indicated that, compound-**1** possesses better antibacterial activities than the crude extracts at both concentrations and antibacterial activities were dose dependent. These findings may support to the traditional use of this plant in diarrhea and dysentery.

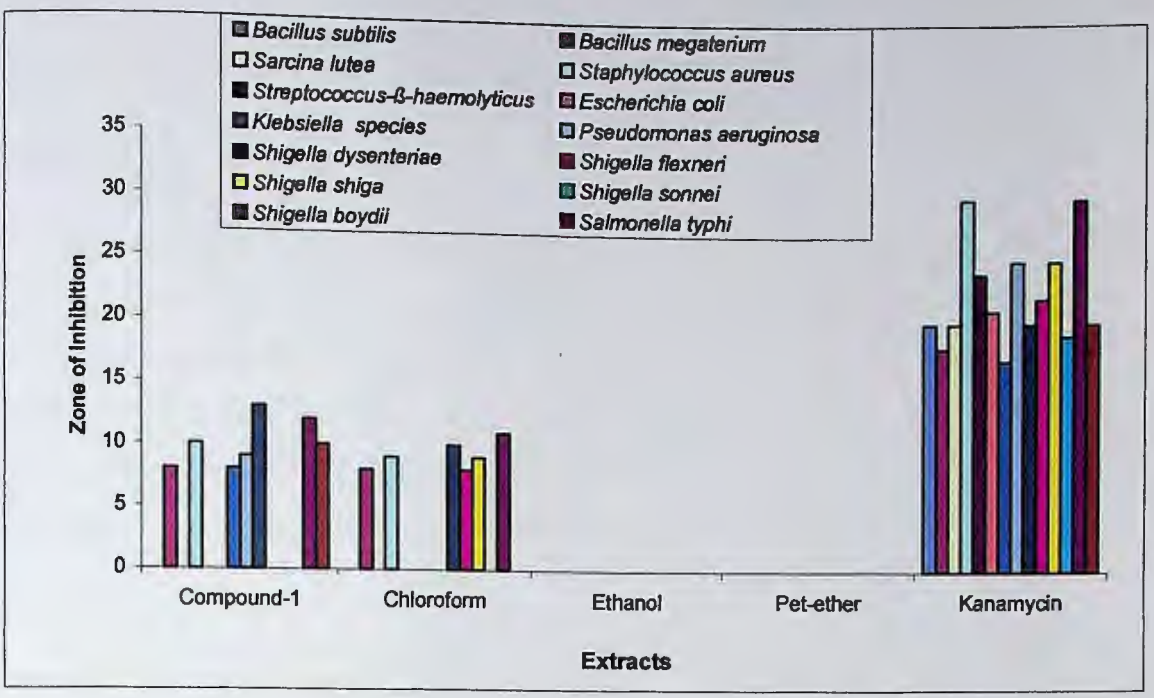

Fig-8 *In vitro* antibacterial activities of crude extracts and compound-**1** of *Moringa oleifera* Lam. (30 µg/disc).

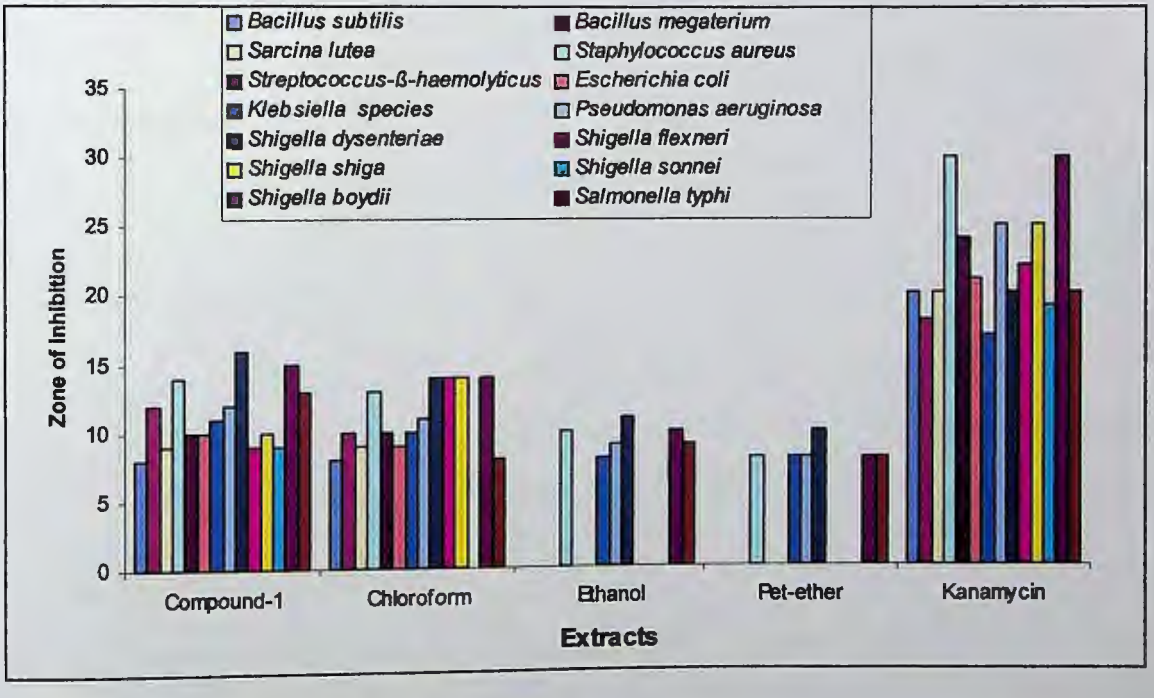

Fig-9 *In vitro* antibacterial activities of crude extracts and compound-**1** of *Moringa oleifera* Lam. (100 µg/disc).

Determination of Minimum Inhibitory Concentration

Experimental

The minimum inhibitory concentration (MIC) is the lowest concentration of the test sample or drug at which it shows the highest activity against the growth of the pathogenic microorganisms and the culture will become turbid. Growth will not occur above the inhibitory level and the tube will remain clear through the large number of microorganism's presence in the tubes. The test was carried out by a serial tube dilution technique (Reiner, 1982) against Gram positive and Gram negative bacteria.

Test Microorganism

The following pathogenic bacteria were used for the determination of MIC of crude extracts and compound-1. The pure cultures were previously collected from the Microbiology Department, Dhaka University.

Table-4 List of bacteria used for determination of MIC.

Gram positive	Gram negative
Staphylococcus aureus	*Shigella dysenteriae*
Bacillus megaterium	*Shigella boydii*

Preparation of Inoculums

Fresh cultures of the test organisms were grown at 37.5°C for over night on nutrient agar media. Bacterial suspensions were then prepared in sterile nutrient broth media in such a manner so that the suspension contains approximately 10^7 cells/ml.

Preparation of the Sample Solution

The crude ethanol, chloroform and petroleum ether extracts and compound-**1** were measured accurately (2.048 mg each) and were taken in different vials. Then methanol (2 ml) was added to each of the vials and mixed well, to make sample solution whose concentration becames1024 µg/ml.

Procedure

1. Twelve autoclaved test tubes were taken, nine of which were marked 1, 2, 3, 4, 5, 6, 7, 8, 9 and the rest three were assigned as C_M (Media), C_{MS} (media + sample) and C_{MI} (media + inoculum).
2. To each of twelve test tubes, sterile nutrient broth media (1 ml) was added.
3. These test tubes were cotton plugged and sterilized in an autoclave for 15 minutes at 121°C temperature and 15lbs/sq. inch pressure.
4. After cooling, 1 ml of the sample solution was added to the 1st test tube and mixed well. Then 1 ml of this content was transferred to the 2nd test tube.
5. The content of the 2nd test tube was mixed uniformly. Again 1 ml of this mixture was transferred to the 3rd test tube. This process of serial dilution was continued up to 9th test tube.
6. Then 10 µl of properly diluted inoculum was added to each of the nine test tubes and mixed well.
7. To the control test tube C_{MS}, 1 ml of the sample was added, mixed well and 1 ml of this mixed content was discarded to check the clarity of the media in presence of diluted solution of the sample.
8. To the control test tube C_{MI}, 10µl of inoculum was added, to observe the growth of the organism in the media used.
9. The control test tube C_M, containing media only was used to confirm the sterility of the media.
10. All the test tubes were incubated at 37 °C for 18-24 hours.

Results and Discussion

The MIC of the crude ethanol, chloroform, petroleum ether extracts and isolated compound-1 were determined by serial dilution technique against *Staphylococcus aureus*, *Bacillus megaterium*, *Shigella dysenteriae* and *Shigella boydii*.

Table-5 MIC of crude extracts and compound-1 of *Moringa oleifera* Lam.

Minimum Inhibitory Concentration (µg/ml)

Sample	*Staphylococcus aureus*	*Bacillus megaterium*	*Shigella dysenteriae*	*Shigella boydii*
Chloroform extract	–	128	64	–
Ethanol extract	–	–	128	128
Petroleum ether extract	128	–	128	–
Compound-**1**	32	64	32	32

For the crude chloroform extract the growth was observed in the test tube containing 64 µg/ml of extract against *Bacillus megaterium* and for *Shigella dysenteriae* in the test tube containing 32 µg/ml of chloroform extract. The MIC for *Bacillus megaterium* and *Shigella dysenteriae* were 128 µg/ml and 64 µg/ml, respectively. No growths were observed against *Shigella boydii* and *Staphylococcus aureus* (**Table-5**). In case of ethanol extract, growths were first observed in the test tube containing 64 µg/ml of extract against *Shigella dysenteriae* and *Shigella boydii*. So, the MIC was 128 µg/ml for both microorganisms. No growths were found against *Bacillus megaterium* and *Staphylococcus aureus* in all test tubes (**Table-5**). For petroleum ether extract, the first sign of growths were observed against *Staphylococcus aureus* and *Shigella dysenteriae* in the test tube containing 64 µg/ml of extract and the MIC was found to be 128 µg/ml for both microorganisms. No growth was observed against *Shigella boydii* and *Bacillus megaterium* in all test tubes (**Table-5**). In case of compound-**1**, the first sign of growth was observed in the test tube containing 32 µg/ml of compound-**1** against *Bacillus megaterium* and the MIC was 64 µg/ml for *Bacillus megaterium*. But in case of *Shigella dysenteriae*, *Shigella boydii* and *Staphylococcus aureus* growth were observed in the test tube containing 16 µg/ml of compound-**1** and the MIC was 32 µg/ml against *Shigella dysenteriae*, *Shigella boydii* and *Staphylococcus aureus* (**Table-5**). From the above experiment it can be concluded that, the compound-**1** was biologically active than the crude extracts.

Antifungal Activity Test

Experimental

In vitro antifungal screening is a useful technique for the detection of new lead compounds for the development of potential bioactivity. Antifungal activity was performed by disc diffusion assay method (Vander, Vlietnck, 1991). Nystatin (50μg/disc) was used as standard.

Test Organisms

For antifungal activity test, the experimental fungi were collected from the Institute of Biological Science, University of Rajshahi, Bangladesh. The fungi were listed below-

Table-6 List of test fungi.

Candida albicans	*Aspergillus fumigatus*
Aspergillus niger	*Trichoderma species*
Aspergillus flavus	*Fusarium species*

Preparation of the Media

Potato dextrose agar (PDA) media was used to perform the antifungal activity test and for subculture of the test organisms. The composition of the media was as follow-

Table-7 Composition of PDA media.

Ingredient	Amount
Potato	20.0 gm
Dextrose	2.0 gm
Agar	2.0 gm
Distilled water q.s to	100.0 ml

The constituents of media were accurately weighed and dispersed in a conical flask with distilled water. It was heated in a water bath to dissolve the ingredients until a transparent solution was obtained. The pH of the media was adjusted to 5.6. The volume was adjusted by adding distilled water and sterilized in autoclave.

Preparation of Inoculum

The spore of isolated pure fungi were inoculated in screw capped tube containing equal amount of PDA media and incubated at 28°C for 5-7 days for the development of new pure culture that was used as inoculum.

Preparation of Test Sample

The crude ethanol, petroleum ether, chloroform extracts and compound-**1** (2.5 mg each) were dissolved separately in methanol (500 µl) to get a concentration of 50 µg/10µl. Again 5 mg of crude extracts and compound-**1** were dissolved in 500 µl of methanol separately to get a concentration of 100 µg/10µl. Sample solution of desired amounts were applied on the discs with the help of a micropipette in an aseptic condition. The discs were left for a few minutes in the same condition for complete removal of solvents.

Procedure

The test plates were prepared according to the following procedures:

1. Distilled water (10 ml) was poured in several clean test tubes and plugged with cotton.
2. The test tubes, a number of petridishes, glass rods, pieces of cottons and the media were sterilized by autoclave and then transferred to the laminar air flow cabinet.
3. Media (6 ml) was poured carefully in the medium sized peridishes in each. The petridishes were rotated several times, first clockwise and then anticlockwise for homogenous thickness. Then the media was allowed to cool and solidified at about 30°C.
4. The test tubes containing distilled water were inoculated with fresh culture of the test fungi and were shaken gently to form a uniform suspension of the organism because of their high prevalence sporulation process.

5. A piece of cotton was immerged in the test tubes with the help of individual glass rod then gentle rubbed the media and the cotton was discarded.

6. Finally, the plates were stored in a refrigerator (4°C) for overnight.

7. Preparation of the discs, placement of the discs, diffusion, incubation and measurement of zone of inhibition were almost same of the antibacterial screening. Here, only the incubation period was replaced by 48-72 hours at room temperature.

Results and Discussion

The antifungal activities of ethanol, chloroform, petroleum ether extracts and the compound-1 from root bark of *Moringa oleifera* Lam. was determined at concentrations of 50 µg/disc and 100 µg/disc against six pathogenic fungi. Nystatine (50 µg/disc) was used as a standard. The antifungal activity (diameter of the zone of inhibition, mm) of the crude extracts and pure compound were given in **Table-8** and **Fig-10, 11**.

Table-8 *In vitro* antifungal activities of the crude extracts and compound-1.

Zone of Inhibition (Diameter in mm)

Name of fungi	Compound-1		Crude extract						Nyst-atin
			Chloroform		Ethanol		Pet-ether		
	50 µg/ disc	100 µg/ disc	50 µg/ disc	100 µg/ disc	50 µg/ disc	100 µg/ disc	50 µg/ disc	100 µg/ disc	50 µg/ disc
Candida albicans	10	12	00	00	00	00	00	00	20
Aspergillus niger	00	10	00	00	00	00	00	00	22
Aspergillus flavus	10	13	09	14	00	10	00	09	19
Aspergillus fumigatus	09	11	00	00	00	09	00	00	22
Trichoderma species	00	09	12	15	00	12	00	10	25
Fusarium species	08	10	00	00	00	00	00	00	25

Compound-1 showed mild to moderate activity against *Candida albicans, Aspergillus flavus, Aspergillus fumigatus* and *fusarium species* at both concentrations. It showed no zone of inhibitions against *Aspergillus niger* and *Trichoderma species* at 50 µg/disc.

But at 100 µg/disc, compound-**1** showed mild activity against *Trichoderma species* (9mm), *Aspergillus niger* (10 mm), *fusarium species* (10 mm) and moderate activity against *Aspergillus fumigatus* (11 mm), *Candida albicans* (12 mm) and *Aspergillus flavus* (13 mm). Compound-**1** gave highest activity against *Aspergillus flavus* at both concentrations (**Table-8** and **Fig-10, 11**). Chloroform extract showed the positive sign of sensitivity against *Aspergillus flavus* (9 and 14 mm) and *Trichoderma species* (12 and 15 mm) at 50 µg/disc and 100 µg/disc, respectively. It showed no activity against *Candida albicans, Aspergillus niger, Aspergillus fumigatus* and *fusarium species* at both concentrations (**Table-8** and **Fig-10, 11**). Ethanol extract gave no activities against all fungi at 50 µg/ml. At 100 µg/disc ethanol extract gave mild to moderate activity against *Aspergillus flavus* (10 mm), *Aspergillus fumigatus* (9 mm) and *Trichoderma species* (12 mm) and no activity was found against *Candida albicans, Aspergillus niger* and *fusarium species* (**Table-8** and **Fig-10, 11**). Petroleum ether extract showed no activities against all fungi at 50 µg/ml. At 100 µg/disc petroleum ether extract showed mild activities against *Aspergillus flavus* (9 mm) and *Trichoderma species* (10 mm) but it showed no activities against *Candida albicans, Aspergillus niger, Aspergillus fumigatus* and *fusarium species* at same concentration (**Table-8** and **Fig-11**).

From the above investigation, it can be concluded that, compound-**1** showed better activities than the crude extracts and the antifungal activity may be dose dependants and sometime specific in action. So the compound-**1** was biologically active and can be consider as a good candidate for further investigation.

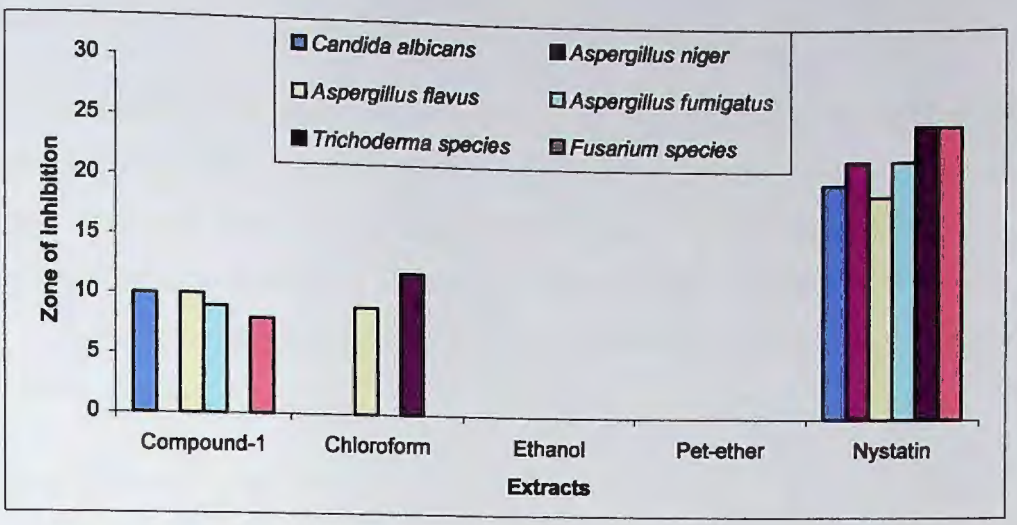

Fig-10 *In vitro* antifungal activities of the crude extracts and compound-1 of *Moringa oleifera* Lam. (50 µg/disc).

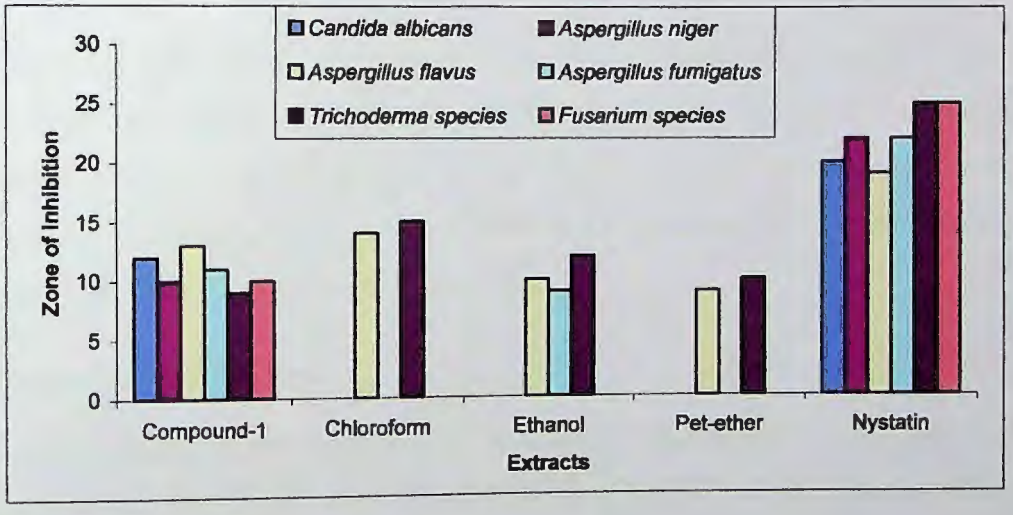

Fig-11 *In vitro* antifungal activities of the crude extracts and compound-1 of *Moringa oleifera* Lam. (100 µg/disc).

Brine Shrimp Lethality Bioassay

Experimental

Bioactive compounds can be bioassayed in a number of methods. Brine shrimp lethality bioassay (Meyer *et al.*, 1982) is recently developed method in this field. Extracts and isolated pure compounds from natural sources can be tested for their bioactivity by this method and the simple zoological organism (brine shrimp nauplii) was used for screening. This bioassay indicates toxicity as well as a wide range of pharmacological activities of the compounds.

Preparation of Brine Water

Pure NaCl (38 gm) was weighted accurately, dissolved in distilled water to make a volume of 1 litre and then filtered. The pH of the brine water was maintained using $NaHCO_3$ (pH, 8-9).

Hatching of Brine Shrimp

Toxicity bioassay was carried out using the eggs of the brine shrimp nauplii (*Artemia salina* L). Eggs were placed in one side of a small tank divided by a net containing 3.8% NaCl solution for hatching. In the other side of the tank, a light source was placed in order to attract the nauplii. Two days were allowed for the hatching of all the eggs and sufficient maturation of the nauplii for the experiment.

Preparation of Sample Solution

Crude ethanol, petroleum ether, chloroform extracts and compound-1 (3 mg each) were accurately measured and dissolved in 0.6 ml (600 µl) of DMSO to get a concentration of 5 mg/ml, for each of the samples. These samples were used as stock solution.

Procedure

The experiment was done into five groups for each sample. Each group contained two vials. 10 Brine shrimp nauplii were then placed in each vial. With the help of a micropipette 2, 4, 8, 20 and 40 µl of each sample were transferred from the stock solution in 5 different vials. NaCl solution (brine water) was added to each vial making the volume up to 5 ml. The final concentration of the samples, in the vials became 10, 20,

40,100 and 200 µg/ml, respectively. For each concentration, one vial containing 5 ml of the same volume of DMSO plus seawater and was used as control group.

Counting of Nauplii

After 24-hour of incubation, the vials were observed using a magnifying glass and the number of survivors in each vial were counted and noted. From this data, the percentage of mortality of the nauplii was calculated for each concentration and the LC_{50} values were determined using Probit analysis as described by Finney (1971).

Results and Discussion

Toxicity bioassay was carried out using brine shrimp nauplii eggs (*Artemia salina* L.) The crude ethanol, chloroform, petroleum ether extracts and compound-**1** isolated from root bark of *Moringa oleifera* Lam. were used for toxicity test.

Table-9 Toxicity of crude extracts and compound-**1** of *Moringa oleifera* Lam.

Extracts	LC_{50} (ppm)	95%Confidence limits (ppm)	Regression equation	χ^2 value
Ethanol	38.41	16.04 - 91.96	Y = 3.15 +1.05X	0.27
Pet-ether	56.01	25.03 - 125.32	Y = 3.15 +1.05 X	0.28
Chloroform	26.38	10.13 -68.70	Y= 3.65+0.95 X	0.28
Compound **1**	14.33	4.87- 42.13	Y= 3.75+1.08 X	8.91

From **Table-9,** it was revealed that all crude extracts and compound-**1** showed positive results indicating they were biologically active. In this bioassay, the mortality rate of brine shrimp was found to increased with the increase in concentration of the samples. Bioassay of isolated compound-**1** showed a significant activity with LC_{50} value of 14.33 ppm. The LC_{50} values of crude ethanol extract, chloroform extract and petroleum ether extracts were found to be 38.41 ppm, 26.38 ppm and 56.01 ppm, respectively, compared with the control DMSO. In the control group, no mortality of nauplii was observed. This effect support the previous findings published by (Guevara *et al.*,1999). This also explained the medicinal use of *Moringa oleifera* Lam. in traditional medicine. But the exact mechanism of the toxic action could not be explained by this preliminary test.

Acute Toxicity Study

Experimental

Toxicology is the aspect of pharmacology that deals with the adverse effect of bioactive substances on living organisms along with their diagnosis and clinical use. All drugs are toxic at higher doses and people vary greatly in their sensitivity to drugs. So in order to develop and establish the safety and efficacy level of a new drug, toxicity study is very essential experiment (Bertram, 1994). Acute toxicity of a drug is characterized by untoward reactions having serious symptoms and a short course of existence, which may follow the administration of a single dose (or an over dose) of the drug. The animals are also examined for sings of intoxication, lethargy behavioral modification and morbidity. A variety of parameters are monitored during this period and at the end of the study lessons and organs are examined for histopathological changes. During screening of new drug, acute toxicity tests are done to estimate the nature and extent of toxicity (Bertram, 1994).

Collection of Experimental Rats

For the purpose of the study 16 Long Evan's rats were collected from the Animal Resources Branch of International Center for Diarrhoeal Diseases and Research Bangladesh (ICDDR, B), Mohakhali, Dhaka.

Maintenance of the Rats

The rats were kept in properly numbered iron cages individually. They were given ideal food comprising the following ingredients per 100 gm of dried mixture.

Table-10 Balanced diet for the rats.

Ingredient	Amount (gm)
Ata (Flour)	40.0
Pulse powder	25.0
Skimmed milk powder	28.0
Soyabean oil	05.0
Salt mixture	01.0
Vitamin mixture	01.0

The diet supplied to each rat was about 20gm/ day, which was approximately isocaloric. They were kept in clean animal house with an optimal room temperature. The animals were maintained in this way before drug administration and continued up to the end of the experiment.

Grouping of the Rats

Weight of the individual rats was determined and they were grouped. Each group contains 4 rats. Control group-A continued to be fed the normal diet and received no vehicle. Group-B received vehicle only and acts as another controls. Experimental group-C and D both received normal diet plus crude chloroform extract and compound-**1**, respectively.

Table –11 Grouping of rats.

Group	No. of Rats	Average body weight (gm)	Sex	Average age (week)	Dose (i.p) mg/kg body wt./day
A	4	103.25	Male	4-7	Normal diet
B	4	104.25	Male	4-7	0.2ml of vehicle
C	4	103.75	Male	4-7	200 mg of $CHCl_3$ ext.
D	4	101.25	Male	4-7	5mg of Compound-**1**

Preparation of Sample Solution

Vehicles were prepared by dissolving the Tween-20 (5 drops) in distilled water in such a way that makes turbid suspensions. Chloroform extracts (581.0 mg) were measured and dissolved in distilled water (5.6 ml) with the help of Tween-20 in such a way that 0.2 ml contained 200 mg/kg body weight/day of chloroform extract. Again, compound-**1** (14.175mg) was accurately measured and dissolved in distilled water (5.6ml) with the help of Tween-20, so that 0.2ml contained 5mg/ kg body weight/day of compound-**1**.

Gross General Observation after Drug Administration

The rats in each group-B, C and D were injected intraperitoneally with vehicles, chloroform extract and compound-**1** (0.2 ml of each), respectively for 7 days. But control group-A fed on normal diet and received no vehicles. Then the control and experimental

rats were observed daily very keenly and notify their behavior, CNS excitation and depression, food intake, sativation, diarrhoea, muscular weakness and reflexes etc.

Changes of Body Weight

The body weights of each rat of groups-B, C and D were measured before and after the administration of the drugs. The change of body weights of each rat of group-A was measured after and before the experiment.

Hematological Profiles

The hematological profiles, such as total count of RBC and WBC, differential count of WBC, platelet counts, hemoglobin percentage and ESR (erythrocyte sedimentation rate) of the controls and experimental rats were done to check before and after administration of drugs intraperitoneally.

Procedure

1. Blood was drawn from the tail veins of all the rats before the commencement of drug administration.

2. Blood smears were made on glass slides and stained with Leishmen reagent to perform TC of RBC, TC of WBC, DC of WBC and platelet count. With the use capillary tubes blood was drawn from each rat to estimate the hemoglobin percentage and ESR. These were pre-hematological study on the rats (Schalm *et al.*, 1975).

   Post-hematological study was done on the 7^{th} day after the commencement of drug administration following the same procedure, as that was done on normal rats.

Biochemical Parameters of Blood

The biochemical parameters such as SGOT (Serum glutamic-oxaloacetic transaminase), SGPT (Serum glutamic pyruvic transaminase), SALP (Serum alkaline phosphatase), serum bilirubin, creatinine and urea were observed.

Procedure

On the 7th day of treatment, all the experimental and control rats were sacrificed and the blood were collected in plastic centrifuge tubes. These were then allowed to clot at 40°C for 4 hours. After clotting, the blood samples were centrifuged at 4000 rpm for 15 minutes using a WIFUNG centrifuge LABOR-50M. The clear straw color blood serum was then collected in vials with Pasteur pipette and stored at 20°C. Then the enzymes SGOT, SGPT, SALP and serum creatinine and urea were determined by using the procedures and reagents supplied by Boehringer Mannheim GmbH Diagnostica (King and Armstrong, 1934; Reitman and Frankel 1957; Coulomb and Favreau, 1963). Then the biochemical parameters of the experimental rat's blood were observed and compared the data with controls rats blood.

Histopathological Examination

Histopathology of liver, hear, lung and kidney were performed to observed any changes in the cellular structures (degradation and regeneration) of the rats received the chloroform extract and compound-1 for 7 consecutive days with respect to control groups.

Procedures

After sacrificing the control and experimental rats, the liver, kidney, heart and lungs were collected from different groups of rats and recommended technique for histopathological preparations were followed (Gurr, 1962). At first the tissues were cut into small pieces and immersed in 10% formalin for 3 days. The tissues were than dehydrated by passing through in graded series of ethyl alcohol. The tissues were then transferred in xylene for about 15 minutes to remove the alcohol. They were then roasted in saturated solution of paraffin wax by placing in an incubator at 60°C temperature for 15 minutes. Melted wax was poured into the paper boat. The tissues were then put in it and cooled in cold water. The block was attached to the block holder of the microtome machine. Serial sections were cut at 4-6μ thickness with the help of a rotary microtome machine. The sections were then stretching by heat (45°C) and of fixed by Mayer's albumin on the neat and clean slides, then the slides were dried for 20-24 hours. After drying, the slides were deparaffinized in xylene for 15 minutes and hydrated in descending grades of ethyl alcohol (10-15 minutes in each solution). The slides were then dipped in Haematoxylin

for nuclear staining and then counter stained with alcoholic eosin for cytoplasmic staining. Then the sections were dehydrated through different ascending grades of alcohol. The tissues were passed through xylene and finally mount in canadabalsam (Gurr, 1962). Then the slides were ready for observation under powerful microphotograph-camera Pentax MZ-M and were recorded by photographs.

Results and Discussion

The acute toxicity study was performed on normal rats by giving 200 mg/ kg body wt./day of chloroform extract and 5 mg/ kg body wt./day of compound-**1**, respectively for 7 consecutive days.

The rats of group-A, B, C and D showed no signs of tremor, convulsion and reflex abnormalities. No muscular numbness of the hind and fore legs, salivation or diarrhoea was observed. The body weights of all the rats were recorded before and after the treatment and the data were compared.

Table-12 Change of body weight of control rats and experimental rats.

Group of rats	Dose (i.p) mg/kg body wt./day	Body weight before drug treatment n=4 Mean±SD	Body weight after drug treatment n=4 Mean±SD	% change of body weight
A	Normal diet	103.25± 0.50	109.75± 1.26	6.3 %
B	0.2 ml of vehicle	104.25± 0.96	111.5± 2.38	6.9 %
C	200 mg/kg chloroform extract	103.75± 0.96	112± 1.41	7.9 %
D	5mg/kg compound-1	101.25± 1.50	107.75± 0.96	6.4 %

No significant changes in body weights of all the rats were observed. Percentage changes in body weights were due to normal body growth for the 7 days.

Hematological Profiles

The hematological profiles of the control rats and experimental rats were observed at 1st day and 7th day of treatment. The effects of chloroform extract and compound-1 on hematological profiles were compared with the control rats.

It was found that, in all rat groups, almost all hematological parameters were slightly increased after 7 days and there was no abnormality observed in both experimental group-C and D in comparison to control groups-A and B (**Table-13**).

Table-13 Hematological profiles of control group-A, B and experimental group-C and D.

Hematological parameter		Control Group-A		Control Group-B		Experimental Group-C		Experimental Group-D	
		1st day Mean + SD	7th da Mean + SD	1st day Mean + SD	7th da Mean + SD	1st day Mean + SD	7th da Mean + SD	1st day Mean + SD	7th da Mean + SD
Total RBC count (million/cu.mm)		4.95±1.52	5±0.08	4.8±0.18	5.05±0.06	4.85±0.13	5.1±0.16	4.75±0.06	5.25±0.06*
Total WBC count (Cells/cu.mm)		6600±182.57	7025±50.00	6775±125.83	7150±129.10	6875±95.74	7125±95.74	6500±81.65	7050±95.74
Differential count of WBC (%)	Neutrophil	54±1.83	54.75±2.06	54.5±1.73	56.75±1.50	54±1.83	54.75±2.06	54.5±1.73	56.75±1.50
	Lymphocyte	31.5±1.29	31.75±1.26	32.25±1.26	32.75±0.96	31.5±1.29	31.75±1.26	32.25±1.26	32.75±0.96
	Monocyte	4±0.82	4.75±0.96	3.75±0.50	4±0.82	4±0.82	4.75±0.96	3.75±0.50	4±0.82
	Eosinophil	1.5±0.54	1.75±0.50	1.75±0.50	1.75±0.50	1.5±0.54	1.75±0.50	1.75±0.50	1.75±0.50
Platelet count (no/u.mm)		302500± 12583.06	305000± 10000.00	308500± 9949.87	310000± 9128.71	298250± 2362.91	305000± 5773.50	305000± 5773.50	310000± 9128.71
Hemoglobin (mg/dL)		12.75±0.50	13.25±0.50	13±0.00	13.5±0.58	13±0.82	13.75±0.96	12.75±0.50	13±0.00
ESR (mm/1st hour)		13.75±1.26	14.25±1.26	14±0.82	14.75±0.50	14±0.82	14.5±1.29	14±0.82	14.25±1.26

*P<0.05, ** P<0.01, *** P<0.001 Vs control-A

• P<0.05, •• P<0.01, ••• P<0.001 Vs control-B

Biochemical Parameters

The biochemical parameters of blood e.g. SGPT, SGOT, SALP, bilirubin, creatinine and blood urea of both controls and experimental rats were determined at 7^{th} day after the experiment.

Table-14 Biochemical parameters of control group-A, B and experimental group-C and D.

Biochemical parameters	Control Group-A Mean ± SD	Control Group-B Mean±SD	Experimental Group-C Mean±SD	Experimental Group-D Mean±SD
SGPT (IU/L)	12.25± 0.50	12.50± 0.58	12.75± 0.50	12.50± 0.50
SGOT (IU/L)	14± 0.82	14.25± 0.50	14.75± 0	13.75± 0.50
SALP (IU/L)	10±0	10.25±0.50	10±0	10.5±0.58
Bilirubin (mg/dL)	0.37± 0.01	0.36± 0.01	0.37± 0.01	0.37± 0.01
Creatinine (mg/dL)	0.59± 0.01	0.59± 0.05	0.61± 0.03	0.60± 0.07
Blood urea (mg/dL)	17.75± 0.96	17.5± 2.08	17.75± 1.50	17.5± 1.29

The biochemical parameters of experimental rats group-C and D were changed slightly in comparison to control rats group-A and B. There was no abnormality found in both control and experimental rats and changed were statistically insignificant (**Table-14**). But there was some interesting differences observed between experimental group-C and D (**Fig-12** and **13**). Like SGOT and urea in chloroform treated rats group-C were increased from the control group but in case of compound-**1** treated rats group-D these two parameters were decreased. On the other hand changes in SALP between these two groups were opposite to each other (**Fig-12** and **13**).

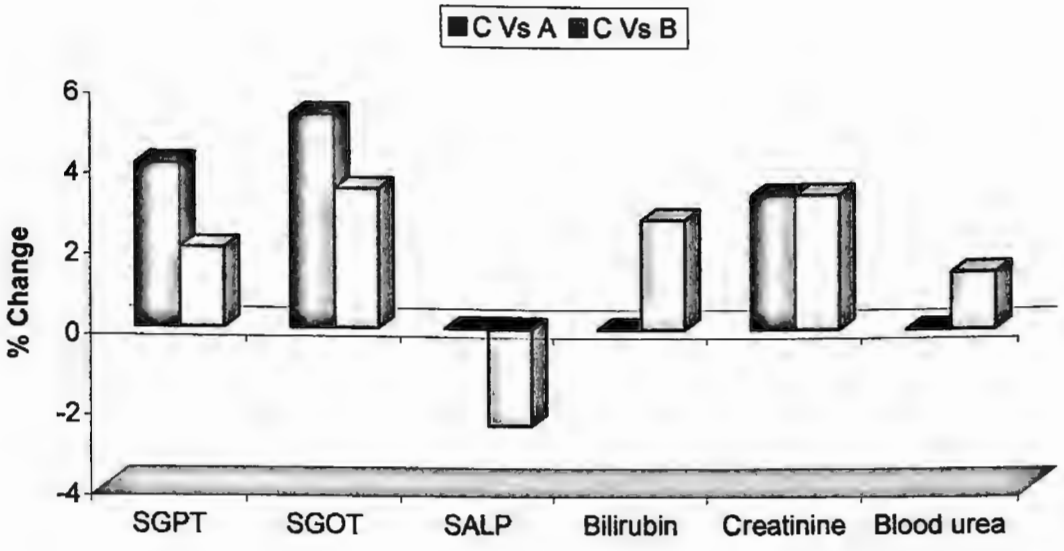

Fig-12 Percentage change of biochemical parameters of experimental rat group-C.

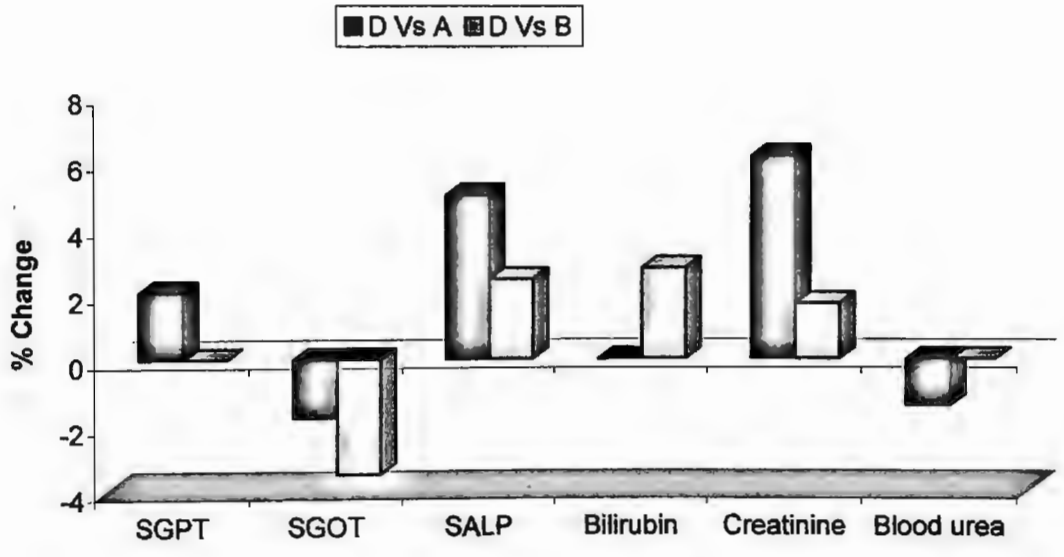

Fig-13 Percentage change of biochemical parameters of experimental rat group-D.

Histopathological studies

Histopathological examinations of liver, heart, lung and kidney of both the control rats groups-A and B were observed after 7 days of treatment. Again the histopathological studies of the same organs of experimental rats group-C and D were observed after intraperitoneal administration of the chloroform extract and compound-**1** for 7 consecutive days. No detectable changes in the histopathology of these organs were examined under microscope (**Table-15** and **Fig-14** to **29**).

Table-15 Histopathological changes in control and experimental rats (liver, heart, lung and kidney).

Group	Dose (i.p) mg/kg body weight/day	Histopathological changes observed			
		Liver	Heart	Lung	Kidney
A	Normal diet	NAD	NAD	NAD	NAD
B	0.2 ml vehicle	NAD	NAD	NAD	NAD
C	200mg of $CHCl_3$	NAD	NAD	NAD	NAD
D	5mg of Com-**1**	NAD	NAD	NAD	NAD

NAD means no abnormalities detected.

From the biochemical, hematological and histopathological examinations, it was confirmed that crude chloroform extract (200 mg/kg body weight/day) and compound-**1** (5 mg/kg body weight/day) had no toxic effect on cellular structure, i.e., they do not cause degeneration of the cells of these organs. Since, the isolated compound-**1** was not available enough for extensive toxicity study, so we did only acute toxicity test (7 days). However, further toxicological studies of compound-**1** (subacute and chronic toxicity) were needed in order to establish its safety. Therefore, these findings provide a support to the use of *Moringa oleifera* Lam. in indigenous system of medicine.

Fig-14 Microscopic view of liver tissues of control rat group-A after 7 days.

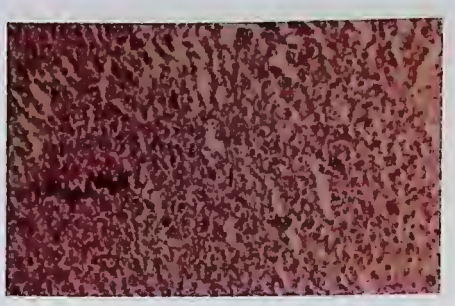

Fig-15 Microscopic view of liver tissues of control rat group-B after 7 days.

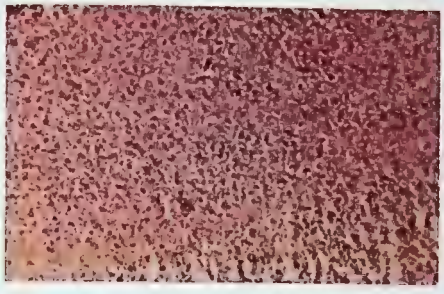

Fig-16 Microscopic view of liver tissues of experimental rat group-C after 7 days.

Fig-17 Microscopic view of liver tissues of experimental rat group-D after 7 days.

Fig-18 Microscopic view of heart tissues of control rat group-A after 7 days.

Fig-19 Microscopic view of heart tissues of control rat group-B after 7 days.

Fig-20 Microscopic view of heart tissues of experimental rat group-C after 7 days.

Fig-21 Microscopic view of heart tissues of experimental rat group-D after 7 days.

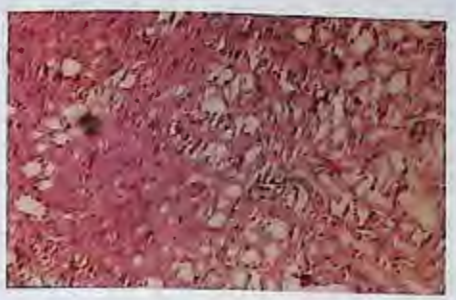

Fig-22 Microscopic view of kidney tissues of control rat group-A after 7 days.

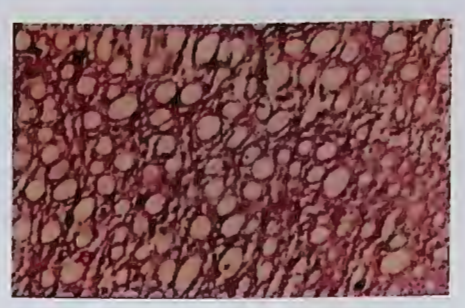

Fig-23 Microscopic view of kidney tissues of control rat group-B after 7 days.

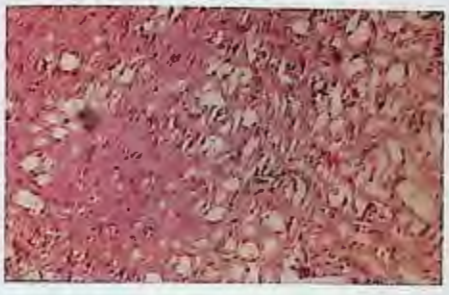

Fig-24 Microscopic view of kidney tissues of experimental rat group-C after 7 days.

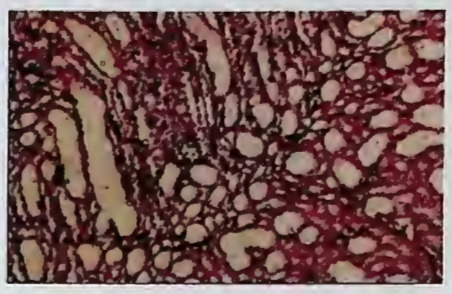

Fig-25 Microscopic view of kidney tissues of experimental rat group-D after 7 days.

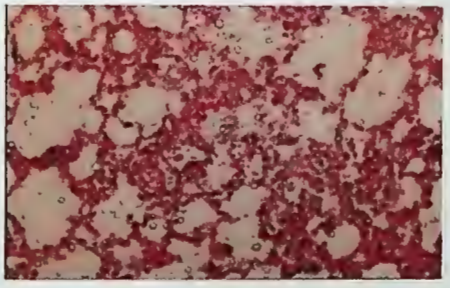

Fig-26 Microscopic view of lung tissues of control rat group- A after 7 days.

Fig-27 Microscopic view of lung tissues of control rat group- B after 7 days.

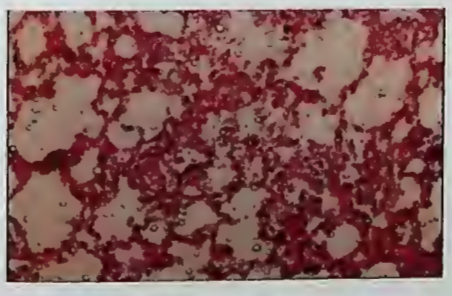

Fig-28 Microscopic view of lung tissues of experimental rat group-C after 7 days.

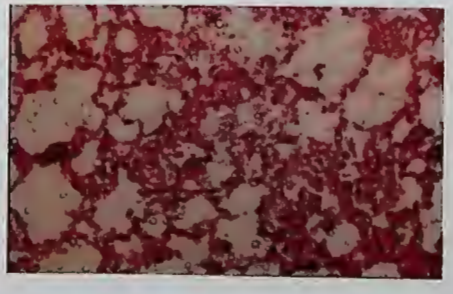

Fig-29 Microscopic view of lung tissues of experimental rats group-D after 7 days.

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Chapter-IV
Study on *Duranta repens* Linn.

A. Stem
B. Fruit

Botany of *Duranta repens* Linn.

Scientific name	: *Duranta repens* Linn.
Synonyms	: *Duranta plumieri* Jacq.
	: *Duranta erecta* Linn.
	: *Duranta ellisia* Jacq.
Bengali name	: Kata mehedi
English name	: Golden dewdrop, Skyflower, Pigeon berry.
Family	: Verbenaceae
Genus	: Duranta
Habit	: Shrubs, herbs or small tree usually 1 to 3 m. in height.
Leaves	: Opposite or whorled, toothed or entire.
Flower	: In long terminal or shorter axillary racemes, alternate on the rachis, short pedicelled in the axils of small bracts, bracteoles zero.
Sepals	: Connate in a tubular or sub-campanulate, truncate or minutely 5-toothed calyx, enlarged in fruit, embracing but free from drupe, often narrowed at the mouth.
Petals	: Five, connate in a short corolla, tube cylindric, limb oblique or not, lobes spreading.
Stamens	: Four, didynamous, included, anthers oblong, cells parallel.
Carpels	: Connate in a more or less completely 8-celled ovary, ovules 1 in each cell, style short, stigma oblique, unequally 4-lobed.
Fruit	: A succulent drupe, enveloped in the calyx, which is open or closed at the mouth, pyrenes 4, each 2-celled and 2-seeded. The yellow or yellow-orange fleshy fruits are ellipsoidal with five lobes and grow in hanging clusters. These fruits may contain up to eight nutlets.
Seeds	: Small, albumen zero.
Distribution	: Bangladesh, throughout India, Pakistan, China, America, Mexico, Florida, Australia and Brazil. (Little *et al.*, 1974; David Prain, 1981; Liogier, 1995; Whistler, 2000).

***Duranta repens* Linn.**

Fruits of *Duranta repens* Linn.

A. Stem of *Duranta repens* Linn.

Chemical Study

Experimental

Extraction, Isolation and Purification

Stem of *Duranta repens* Linn. were collected from the adjoining areas of Rajshahi University campus, during June 2001 and was identified by professor A.T.M. Nadiruzzaman, Department of Botany, University of Rajshahi, where a voucher specimen (no. Alam 78, collection date 19.09.1997) has been deposited.

The stems were cut into small pieces and sun dried for 7 days and finally in an oven for 36 hours at temperature below 60°C. Dried plant materials were then pulverized into a coarse powder with the help of a grinding machine (FFC-15, China). The dried plant material (1 kg) were then stored in an airtight container for further use.

The powdered dried plant materials (1kg) were extracted with ethanol (5.0 lit.) in an aspirator bottle, through occasional shaking and stirring for 7 days. The contents were pressed through the Tincture Press (Karlkolb, Scientific-Technical Suppliers, Frankfurt /m-Germany) to get maximum amount of extract. The extract was then filtered through Whatman No.1 filter paper and the filtrate thus obtained was concentrated under reduced pressure at 50°C using rotary evaporator to afford a dark greenish gummy residue (90 gm).

The concentrated ethanolic extract (50 gm) was diluted with distilled water and taken in a separating funnel. 100 ml of diethyl ether was then added and shaken vigorously and allowed to stand for few minutes. The diethyl ether layer (upper layer) was then collected. The process was repeated three times. The organic layers dried over an anhydrous Na_2SO_4 and concentrated in *vacuo* to afford a diethyl ether soluble fraction (20.8 gm).

After diethyl ether extraction, the aqueous part was then transferred again in a separating funnel and chloroform (100 ml) was added. The funnel was shaken and allowed to stand for layer separation. The organic layer was collected. The process was repeated three

times. The organic layers were dried and concentrated in *vacuo* to afford a chloroform soluble fraction (15.6 gm).

The chloroform extract showed 4 prominent spots with other minor spots on TLC. The spots were checked under UV, iodine vapor and vanillin-H_2SO_4 acid spray reagent. The chloroform extract (5 gm) was subjected to a column of silica gel and was eluted with n-hexane, n-hexane with increasing portions of ethyl acetate and finally with ethyl acetate. Elutes were then collected in a number of conical flasks marked from 1 to 33.

Among 33 fractions, fraction 8-15 eluted with n-hexane-ethyl acetate (2:1) showed similar spots on TLC and gave radish brown colored spot with vanillin-H_2SO_4 reagent and were combined. The combined fraction was then subjected to PTLC using the solvent system n-hexane-ethyl acetate (5:1) and observed under UV light (254nm and 366nm range). The band which glow under UV light was scrapped off and eluted with ethyl acetate and evaporated off under reduced pressure to afford a compound-**2** (48mg) as amorphous powder.

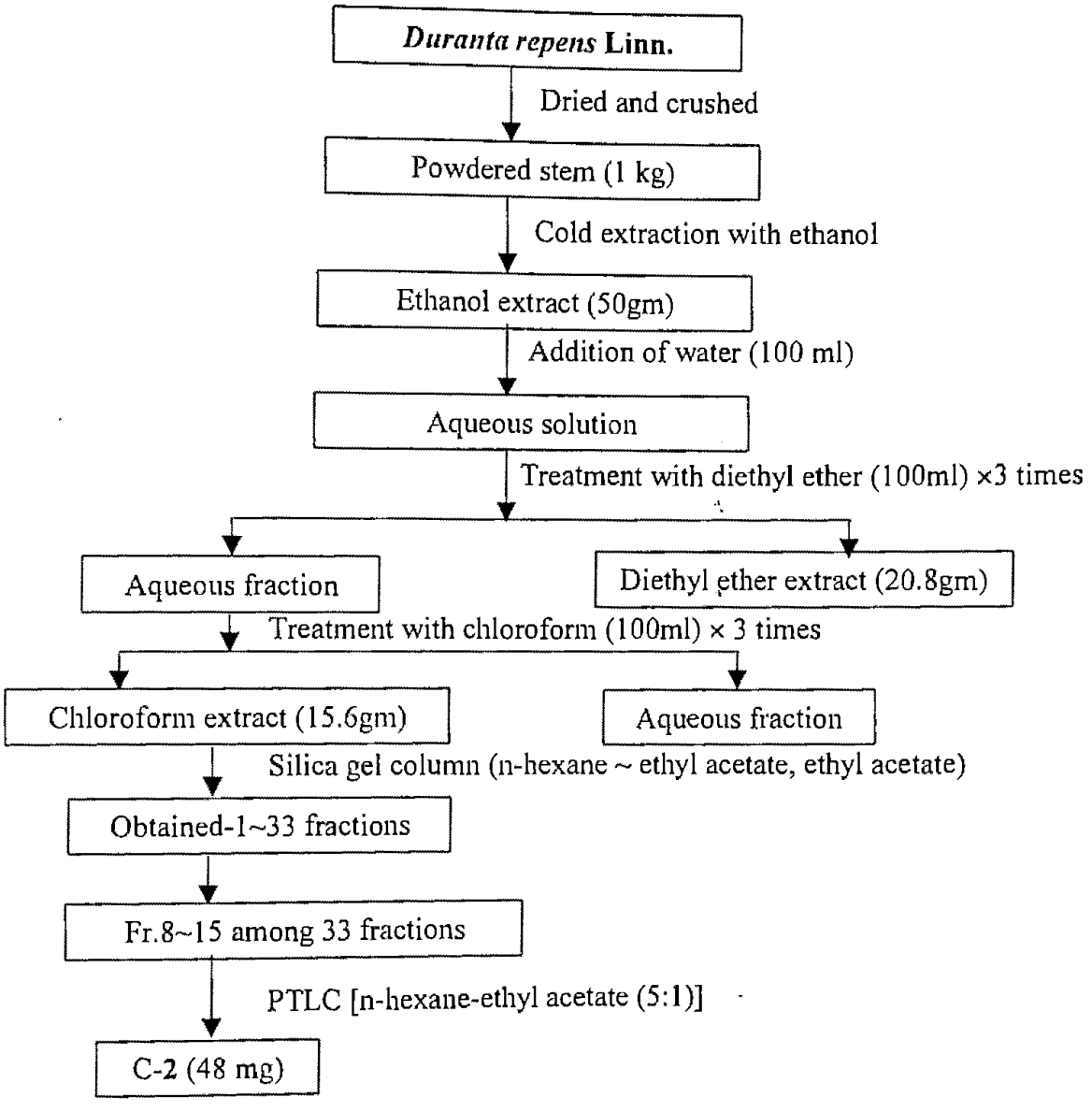


Fig-1 Extraction and isolation scheme of compound-2 from the stem of *Duranta repens* Linn.

Results and Discussion

Characterization of Compound-2

Compound-**2** was isolated from the chloroform fraction of ethanol extract of the stem of *Duranta repens* Linn. as white amorphous powder, decomposed between 121-125°C. IR spectrum of compound-**2** showed O-H stretching band between 3445-3888 cm^{-1} and C=O stretching vibration at 1099 cm^{-1}. C-H and >C=C-H stretching vibrations observed between 2877~2924 cm^{-1} and >C=C< stretching showed a strong band at 1689 cm^{-1} (**Fig-2**). TLC of compound-**2** showed one spot and mass spectrum of this compound did not show prominent molecular ion peak. Analysis of ^{1}H-NMR and ^{13}C-NMR spectrum, it was also found that the compound-**2** was not a single one but a mixture of two similar types of compound. The major compound was identified as *β*-Amyrin and the minor one as 12-Oleanene 3*β*, 21*β*-hydroxy-diol.

Characterization of *β*-Amyrin

β-Amyrin, a well know triterpene obtained as a major compound whose ^{1}H and ^{13}C spectral data was identical with the literature data reported by Gibbons, 1994. In its ^{1}H-NMR, H-3 proton appeared at δ3.23 as doublets of a doublet with a *J* values of 4.8 and 11.0 Hz. Olefinic H-12 proton appeared at δ5.30 as triplet with a coupling constant of 3.6 Hz. Moreover, H-18 proton appeared at δ2.01 as doublets of a doublet with a *J* values of 5.0 and 15.0 Hz. Eight methyl group protons appeared between δ0.77 to 0.94. Comparison of ^{1}H-NMR with the reported data of *β*-Amyrin is given in **Table-1** (**Fig-3**).

Table-1 ^{1}H-NMR data of compound-**2** (*β*-Amyrin).

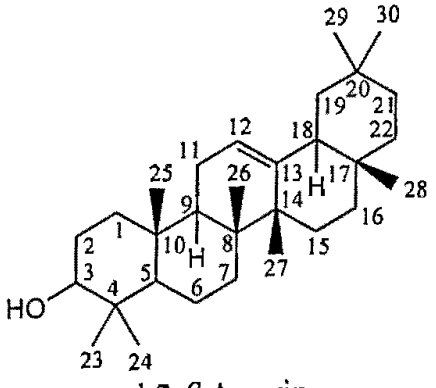
Protons	**Authentic *β*-Amyrin ($^{\delta}$H)**	**Isolated *β*-Amyrin ($^{\delta}$H)**
H-3	3.23 (1H,dd, *J*=5.2 and 10.8Hz)	3.23 (1H,dd, *J*= 4.8 and 11.0Hz)
H-5	2.21 (1H,br d, *J*= 10.6)	2.21 (1H,br d, *J*= 10.6Hz)
H-9	--	1.50~1.60 (m)
H-11	--	1.88~1.90 (m)
H-12	5.14 (1H,t, *J*= 3.6Hz)	5.30 (1H,t, *J*= 3.6Hz)
H-18	--	2.01 (1H,dd, *J*= 5.0 and 15.0Hz)
Me-23~30	0.80~1.80 (1 H, s)	0.77~0.92 (3H, s)

Almost all the ^{13}C-NMR data were identical with the reported β-Amyrin. C-3 carbon appeared at 38.2 (38.7) ppm. The olifinic carbon at C-12 and C-13 were appeared at 124.2 (121.9) and 145.5 (145.1) ppm, respectively. Moreover, the eight methylene carbon signals were appeared between 17.0~33.7 ppm (**Table-2** and **Fig-4, 5, 6**).

Table-2 ^{13}C-NMR data of compound-**2** (β-Amyrin).

No of carbon	Authentic β-Amyrin ($^{\delta}$C)	Isolated β-Amyrin ($^{\delta}$C)	No of carbon	Authentic β-Amyrin ($^{\delta}$C)	Isolated β-Amyrin ($^{\delta}$C)
C-1	38.7	38.2	C-16	27.0	29.6
C-2	27.3	28.8	C-17	32.5	38.6
C-3	72.9	80.6	C-18	47.4	49.5
C-4	38.8	40.3	C-19	46.8	40.4
C-5	55.5	54.3	C-20	31.1	32.1
C-6	18.4	19.9	C-21	34.8	43.6
C-7	32.8	33.9	C-22	32.7	35.4
C-8	38.8	40.3	C-23	28.4	29.7
C-9	47.7	49.1	C-24	15.5	17.0
C-10	37.6	35.5	C-25	15.6	17.1
C-11	23.5	24.8	C-26	16.8	18.5
C-12	121.9	124.2	C-27	26.0	25.1
C-13	145.1	145.5	C-28	28.4	29.7
C-14	41.7	40.2	C-29	33.3	33.7
C-15	26.2	25.7	C-30	23.7	22.7

Comparison of ^{1}H- and ^{13}C NMR data with the reported data it was confirmed that, one of the component of compound-**2** was β-Amyrin. So far literature surveyed it was found that α-Amyrin was isolated earlier from this plant but it was first report of isolation of β-Amyrin from the plant *Duranta repens* Linn.



Compound-**2** β-Amyrin

Characterization of 12-Oleanene 3β,21β-hydroxy-diol:

A proton geminal to a hydroxyl group at H-3 appeared at δ3.27 as a doublets of a doublet with a *J* value of 11.0 and 4.8 Hz indicating the coupling with its neighbouring two proton geminally disposed on C-2 position. H-5 proton resonates at 0.74 and appeared as br.dd with a *J* value of 11.0 and 4.8Hz coupled with geminally disposed of two protons on C-6. The olefinic proton on C-12 showed low field signal at δ 5.26 and appeared as triplet with a *J* value of 10.6 Hz coupled with two geminally disposed protons on its neighbour at C-11 position. Another proton geminal to an hydroxyl group on C-21 appeared at δ3.27 as doublets of a doublet with a *J* value of 4.8 and 10.6 Hz. Moreover, eight methyl group singles are appeared between δ 0.80 to δ 1.15 and these data were almost identical with the published data (Rahman, 2002) except the value of H-21 proton (**Table-3** and **Fig-3**).

Table-3 ^{1}H NMR data of compound-**2** (12-Oleanene-3β, 21β-hydroxy-diol).

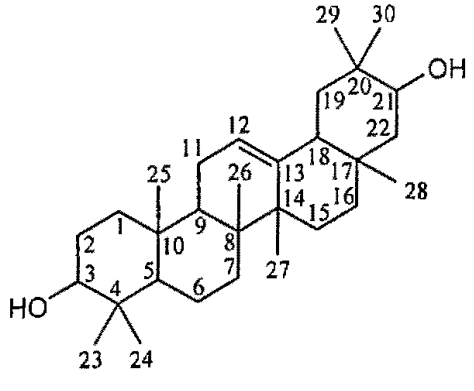
Protons	Authentic sample 12-Oleanene-3β,22β-diol ($^{\delta}$H)	Isolated compound 12-Oleanene-3β,21β-hydroxy-diol ($^{\delta}$H)
H-3	3.23 (dd, *J*= 10.8 and 5.2Hz)	3.27 (1H,dd, *J* = 11.0 and 4.8Hz)
H-5	0.75 (br d, *J*= 10.1 Hz)	0.74 (1H,br dd, *J* = 11.0 and 4.8Hz)
H-9	1.55 (1H, m)	1.55 (1H, m)
H-11	1.89 (1H, m)	1.89 (1H, m)
H-12	5.26 (t, *J* = 3.6 Hz)	5.26 (1H, t, *J* = 3.6Hz)
H-18	2.11 (br d, *J* = 12.8Hz)	2.83 (1H,dd, *J* = 4.8 and 10.6Hz)
H-21	1.45 (d, *J* = 5.6 Hz)	3.27 (1H, dd, *J* = 4.8 and 10.0 Hz)
H-22	3.44 (t, *J*= 5.2Hz)	2.19 (1H,bd, *J* = 10.6Hz)
Me-23~30	0.80 ~0.92, s	0.80 ~ 0.92, s

From the ^{13}C-NMR data of isolated compound, it was found that almost all the ^{13}C-NMR signals were identical with the published data except C-21 carbon. C-3 and C-21 carbon resonates at low field at 80.5 and 80.2 ppm, respectively. C-21 carbon signal correlated with H-21 appeared at δ3.27 as doublets of a doublet with a *J* value of 4.8 and 10.0Hz confirm the presence of a OH group at C-21 carbon. Moreover another two low field carbon signals were appeared at 127.4 and 139.5 ppm were assigned to be the olifinic carbon signals. One of which was a methyne and another was a quaternary carbon. Moreover seven methyl carbons were appeared between 17.1~34.6 ppm ((**Table-4** and **Fig- 4, 5, 6**).

Table-4 ^{13}C-NMR data of compound-**2** (12-Oleanene-3β, 21β hydroxy-diol).

No of carbon	Authentic sample 12-Oleanene-3β,22β-diol ($^{\delta}$C)	Isolated compound 12-Oleanene-3β, 21β hydroxy-diol ($^{\delta}$C)	No of carbon	Authentic sample 12-Oleanene-3β,22β-diol ($^{\delta}$C)	Isolated compound 12-Oleanene-3β, 21β hydroxy-diol ($^{\delta}$C)
C-1	38.9	38.5	C-16	28.4	29.2
C-2	27.5	28.8	C-17	37.6	38.6
C-3	79.2	80.5	C-18	45.0	42.7
C-4	39.0	40.2	C-19	46.4	47.4
C-5	55.5	55.8	C-20	30.7	32.2
C-6	18.6	19.2	C-21	41.7	44.2
C-7	33.1	33.3	C-22	76.9	80.2
C-8	42.3	40.4	C-23	15.8	18.5
C-9	47.9	49.2	C-24	28.3	24.6
C-10	37.2	35.4	C-25	15.8	17.1
C-11	23.7	24.6	C-26	17.2	16.9
C-12	122.7	127.4	C-27	25.6	25.2
C-13	144.1	139.5	C-28	20.2	22.7
C-14	39.9	39.3	C-29	28.4	27.4
C-15	26.1	25.0	C-30	33.0	34.6

For 12-Oleanene 3β, 21β hydroxy-diol other carbon signal were in accord with structure assigned. From its ^{1}H and ^{13}C-NMR data it can be concluded that the minor compound in the mixture was 12-Oleanene 3β, 21β hydroxy-diol and was the first report of isolation from *Duranta repens* Linn.



Compound-**2** 12-Oleanene 3β, 21β-hydroxy-diol

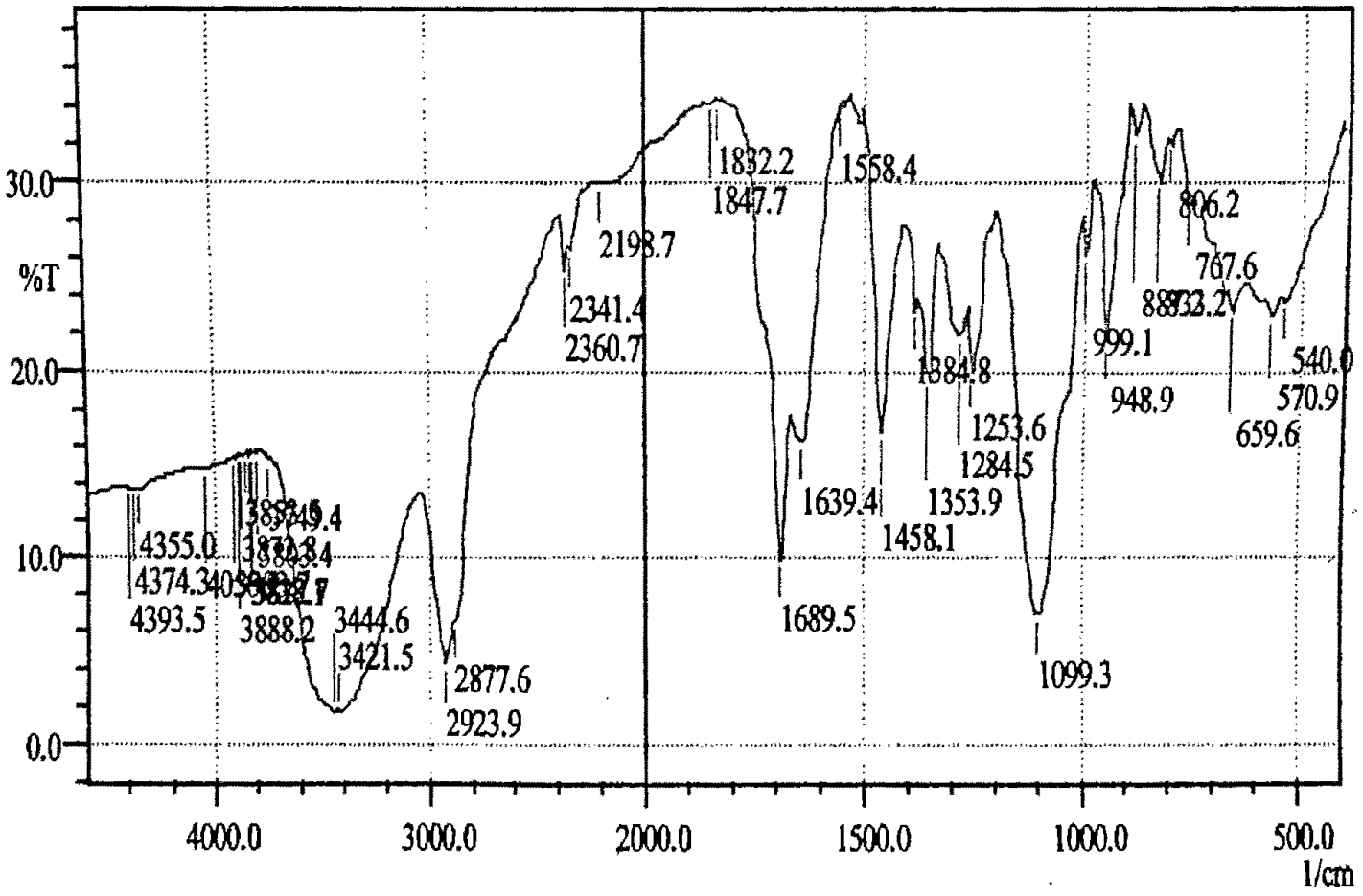


Fig-2 IR spectrum of compound-2

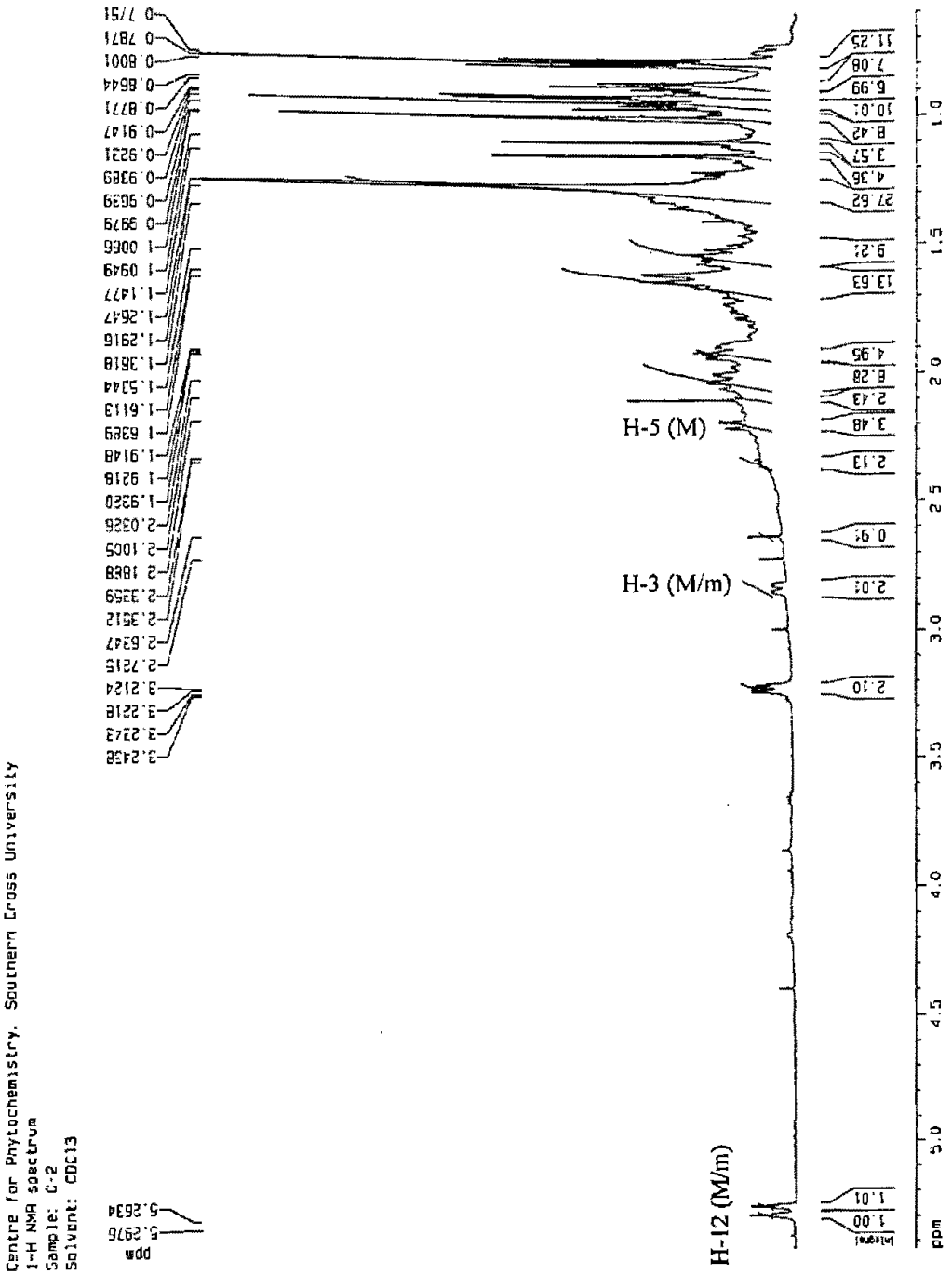


Fig-3 ^{1}H-NMR spectrum of compound-2

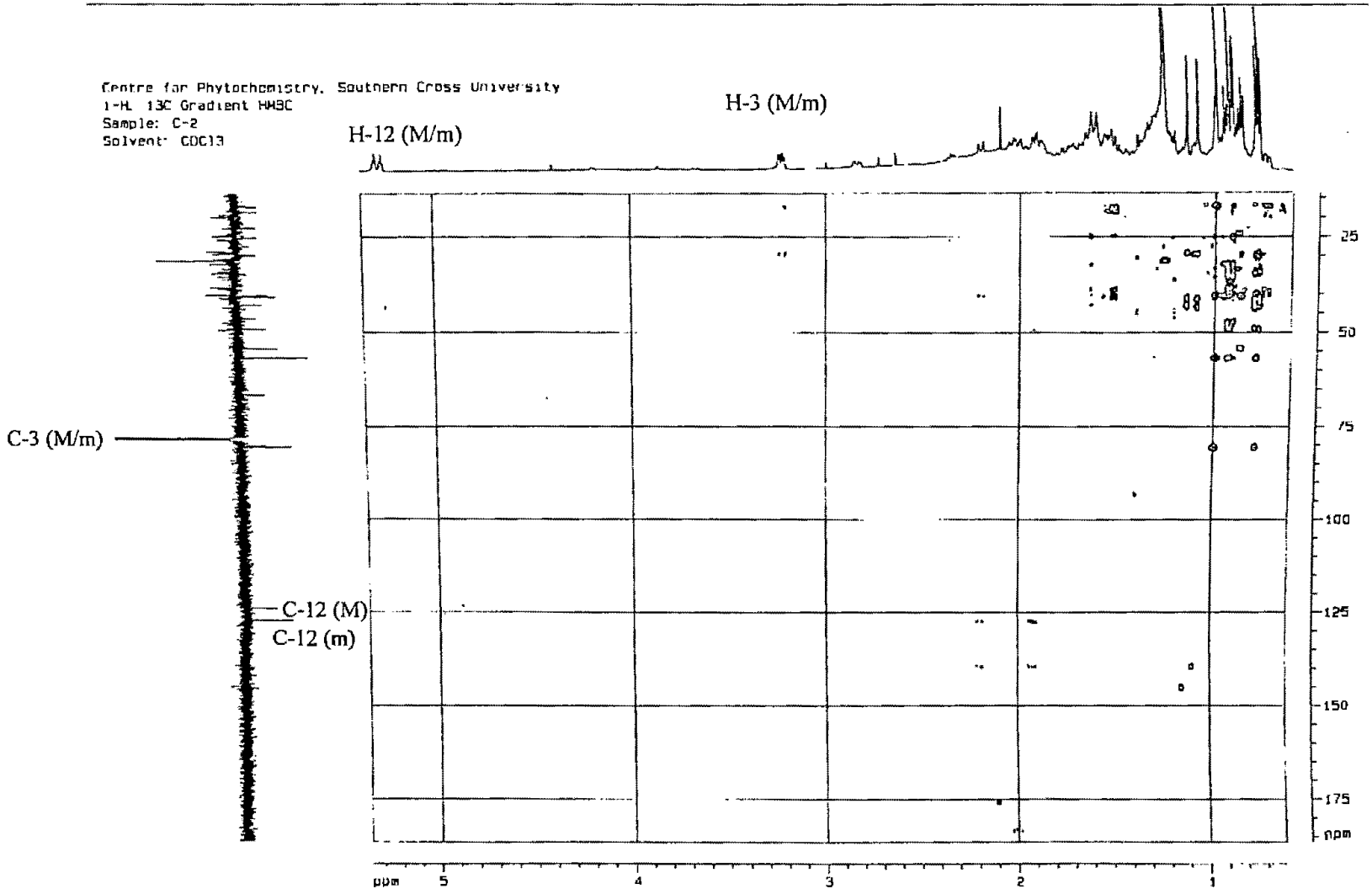


Fig-4 HMBC spectrum of compound-2

Fig-5 JMOD spectrum of compound-2

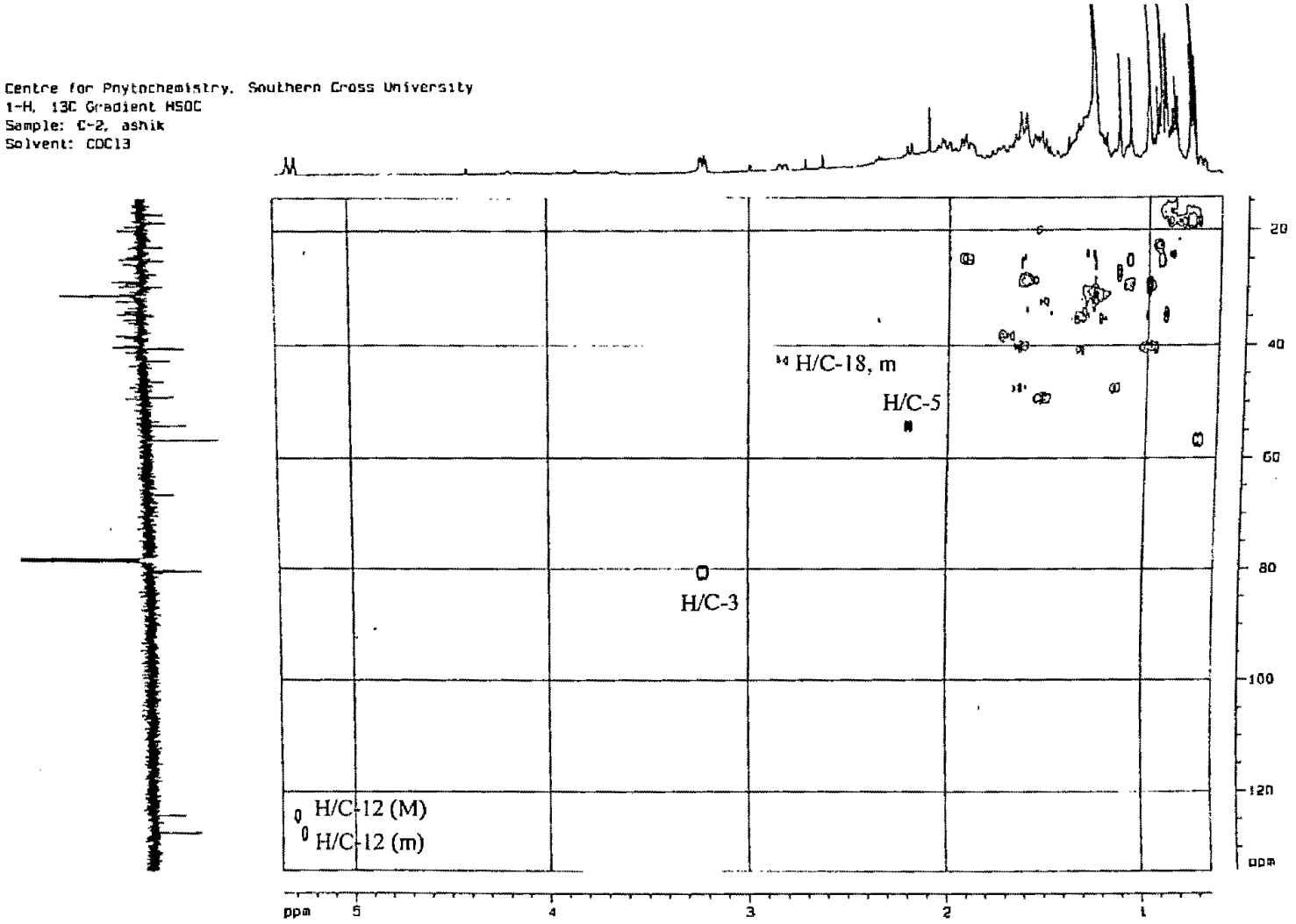


Fig-6 HSQC spectrum of compound-2

Properties of Compound-2

β-Amyrin: White amorphous powder; decomposed between: 121-125°C; IR (KBr): 1099,1689, 2877~2924, 3445~3888 cm^{-1}; ^{1}H-NMR (500 MHz, $CDCl_3$): δ3.23, dd, 1H, (*J*=4.82 11.0Hz,H-3), δ2.21, brd, 1H, (*J*=10.6Hz, H-5), δ1.50~1.60, m, (H-9), δ1.88-1.90, m, (H-11), δ5.30, t, 1H (*J*=3.6Hz, H-12), δ2.01, 1H, dd, (*J*= 5.0 and 15.0 Hz, H-18), CH_3: δ0.77, δ0.79, δ0.86, δ0.88, δ0.91, δ0.92 (3H,s); ^{13}C-NMR (125 MHz, $CDCl_3$): 38.2 (C-1), 28.8 (C-2), 80.6 (C-3), 40.3 (C-4), 54.3 (C-5), 19.9 (C-6), 33.9 (C-7), 40.3 (C-8), 49.1 (C-9), 35.5 (C-10), 24.8 (C-11), 124.2 (C-12), 145.5 (C-13), 40.2 (C-14), 25.7 (C-15), 29.6 (C-16), 38.6 (C-17), 49.5 (C-18), 40.4 (C-19), 32.1 (C-20), 43.6 (C-21), 35.4 (C-22), 29.7 (C-23), 17.0 (C-24), 17.1 (C-25), 18.5 (C-26), 25.1 (C-27), 29.7(C-28), 33.7 (C-29), 22.7 (C-30).

12-Oleanene 3β, 21β-hydroxy-diol: White amorphous powder; decomposed between: 121-125°C; IR (KBr): 1099,1689, 2877~2924, 3445~3888 cm^{-1}; ^{1}H-NMR (500 MHz, $CDCl_3$,): δ3.27, 1H, dd (*J*=11.0 and 4.8 Hz, H-3), δ0.74, 1H, br dd (*J*=11.0 and 4.8 Hz, H-5), δ1.55,1 H, m (H-9), δ1.89, 1H,m (H-11), δ5.26, 1H,t (*J*=3.6 Hz, H-12), δ2.83,1H,dd (*J*=4.8 and 10.6Hz,H-18), δ3.27, 1H,dd (*J*=4.8 and 10Hz,H-18), δ2.19,1H,bd (*J*=3.6Hz,H-22), CH_3: 0.80, 1.01,0.96, 0.99, 1.15, 0.89, 1.09 and 0.92 (3H,s); ^{13}C-NMR (125 MHz, $CDCl_3$): 38.5 (C-1), 28.8 (C-2), 80.5 (C-3), 40.2 (C-4), 55.8 (C-5), 19.2 (C-6), 33.3 (C-7), 40.4 (C-8), 49.2 (C-9), 35.4 (C-10), 24.6 (C-11), 127.4 (C-12), 139.5 (C-13), 39.3 (C-14), 25.0 (C-15), 29.2 (C-16), 38.6 (C-17), 42.7 (C-18), 47.4 (C-19), 32.2 (C-20), 80.2 (C-21), 54.2 (C-22), 18.5 (C-23), 24.6 (C-24), 17.1 (C-25), 16.9 (C-26), 25.2 (C-27), 27.4 (C-29), 34.6 (C-30).

Conclusion

From spectral analysis compound-**2** was found to be a mixture of two compounds, but their separation was not possible due to similar R_f value and used as such for all the biological investigations. A steroidal glycoside with some impurities was also isolated from this fraction. But the amount was too small to be separated and proceed for further investigation.

Biological Study

Antibacterial Activity Test

Experimental

In vitro antimicrobial activity can be detected by observing the growth of various microorganisms to the plant or parts of a plant extract, which was placed in contact with them. In the study disc diffusion method (Vander and Vlietnck, 1991) was used for the preliminary evaluation of antimicrobial activity of the crude extracts and compound-**2**.

Test Organisms

Six pathogenic bacteria (three Gram positive and three Gram negative) were selected for the test.

Table-5 List of test bacteria.

Gram positive	Gram negative
Bacillus subtilis	*Escherichia coli*
Staphylococcus aureus	*Shigella dysenteriae*
Streptococcus-β-haemolyticus	*Klebsiella species*

Preparation of Test Sample

Crude ethanol, diethyl ether and chloroform extracts (3 mg each) were dissolved in methanol (600 µl) to get a concentration of 30 µg/6µl. Again 100 µg/10µl was obtained by dissolving the crude extracts (5 mg each) in methanol (500 µl). Compound-**2** (3mg) was dissolved in 600 µl of methanol to get a concentration of 30 µg/6µl. Again 100 µg/10µl was obtained by dissolving compound-**2** (5 mg) in methanol (500 µl).

Procedures were same and described in **Chapter-III**.

Results and Discussion

The different crude extracts and compound-**2** isolated from the stem of *Duranta repens* Linn. were tested for their *in vitro* antibacterial activity against three Gram positive and three Gram negative bacteria. Kanamycin (30 µg/disc) was used as standard. In this antibacterial screening, the crude extracts and compound-**2** were used at concentrations of 30 µg/disc and 100 µg/disc.

Table-6 *In vitro* antibacterial activity of crude stem extracts and compound-2 of *Duranta repens* Linn.

Zone of Inhibition (Diameter in mm)

Bacteria	Ethanol extract		Diethyl ether extract		Chloroform extract		Compound-2		Kanamycin 30 µg/disc Standard
	30 µg/disc	100 µg/disc	30 µg/disc	100 µg/disc	30 µg/disc	100 µg/disc	30 µg/disc	100 µg/disc	
Gram positive									
Bacillus subtilis	0	10	0	11	0	10	0	10	21
Staphylococcus aureus	0	09	0	09	0	10	0	09	23
St.β-haemoliticus	0	11	0	10	0	14	0	13	23
Gram negative									
Escherichia coli	0	11	0	11	0	12	0	12	22
Klebsiella species	0	12	0	14	0	14	0	13	24
Shigella dysenteriae	0	08	0	08	0	13	0	11	23

The crude ethanol, chloroform, diethyl ether extracts and compound-2 showed no zone of inhibition against both Gram positive and Gram negative bacteria at a concentration of 30 µg/disc. But at 100 µg/disc they showed mild to moderate activity against all the microorganisms (**Table-6** and **Fig-7, 8**). The crude ethanol extract showed mild activity against *St.β-haemoliticus* (11mm.) and *Klebsiella species* (12 mm) in comparison with standard Kanamycin (30 µg/disc). But the crude diethyl ether extract gave mild activity against *Bacillus subtilis* and *E. coli* (11mm.) and moderate activity against *Klebsiella species* (14 mm) as compared with Kanamiycin. Among the tested extracts, the chloroform extract gave better activity against *St.β-haemoliticus* (14 mm.), *Klebsiella species* (14 mm.) and *Shigella dysenteriae* (13 mm.) than the ethanol and diethyl ether extracts (**Table-6** and **Fig-8**). Compound-2 exhibited activity against all Gram positive and Gram negative bacteria at 100 µg/disc. It showed comparatively lower activity than the chloroform extract against *St.β-haemoliticus* and *Klebsiella species* and produced same zone of inhibition 13mm (**Table-6** and **Fig-8**). In comparison with standard Kanamycin (30 µg/disc), the activities of the crude extracts and compound-2 were not promising but at higher concentration antibacterial activity was better.

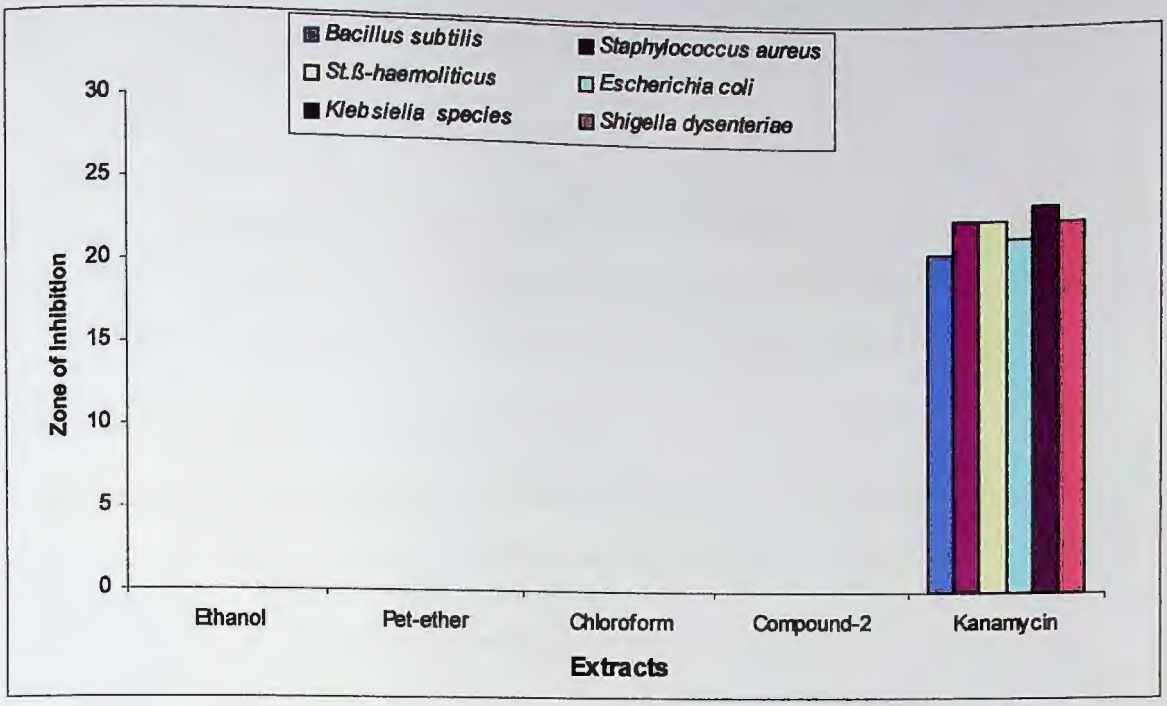


Fig-7 *In vitro* antibacterial activity of stem extracts and compound-**2** of *Duranta repens* Linn. (30µg/disc).

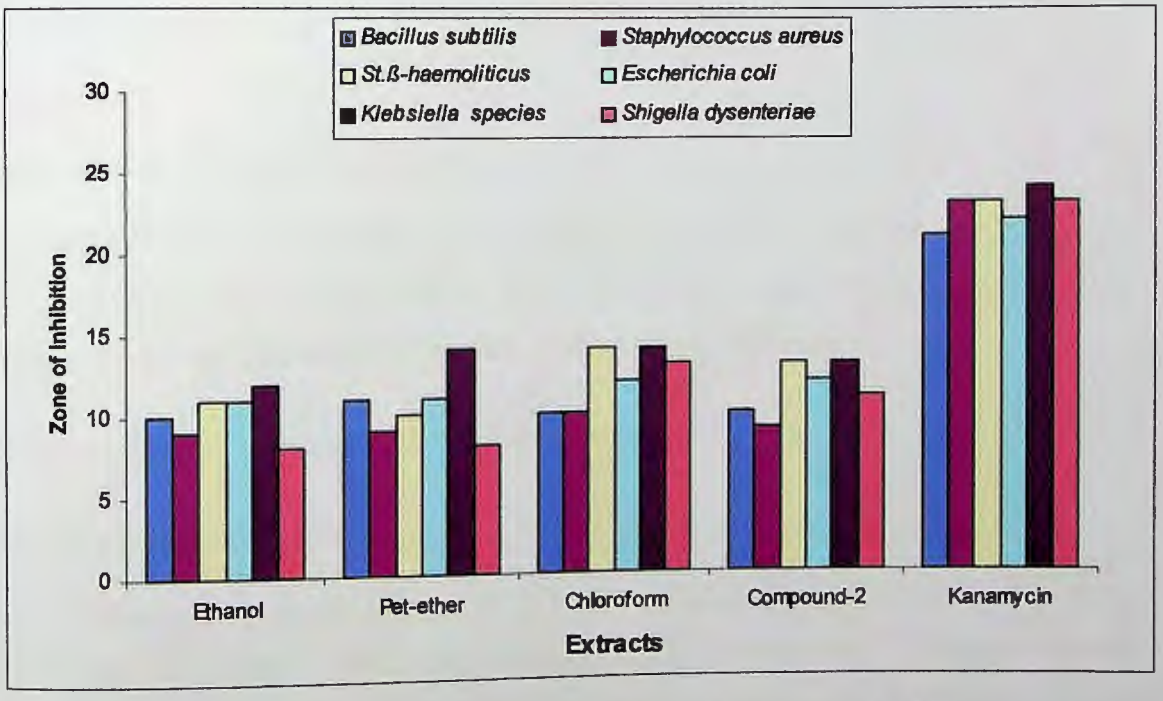


Fig-8 *In vitro* antibacterial activity of stem extracts and compound-**2** of *Duranta repens* Linn. (100µg/disc).

Determination of Minimum Inhibitory Concentration

Experimental

The minimum inhibitory concentration (MIC) is the lowest concentration of the test sample or drug at which it shows the highest activity against the growth of the pathogenic microorganisms. The test was carried out by a serial tube dilution technique (Reiner, 1982).

Test Microorganism

Five pathogenic bacteria (**Table-7**) were used for the determination of MIC. The pure culture was previously collected from the Microbiology Department, Dhaka University.

Table-7 List of pathogenic bacteria used for determination of MIC.

Gram positive	Gram negative
Staphylococcus aureus	*Escherichia coli*
Bacillus subtilis	*Klebsiella species*
St.β-haemoliticus	

Preparation of the Sample Solution

The crude extracts and compound-**2** were measured accurately (2.048 mg each) and were taken in different vials. Then methanol (2 ml) was added to each of the vials and mixed well to make sample solution whose concentration became 1024μg /ml.

Procedures were same and described in **Chapter-III.**

Results and Discussion

The minimum inhibitory concentrations of the crude ethanol, chloroform, diethyl ether extracts and compound-**2** were determined by serial tube dilution technique against *Bacillus subtilis* , *Staphylococcus aureus, St. β-haemoliticus, Escherichia coli* and *Klebsiella species.*

Table-8 MIC of crude extracts and compound-**2** of *Duranta repens* Linn.

Minimum Inhibitory Concentration (μg/ml)

Extracts	Name of Bacterial Strains				
	Bacillus subtilis	*Staphylococcus aureus*	*St .β-haemoliticus*	*Escherichia coli*	*Klebsiella species*
Ethanol	128	128	128	64	64
Diethyl ether	128	64	128	64	32
Chloroform	64	64	64	64	32
Compound-**2**	64	128	128	64	32

The MIC of ethanol extract was 64 μg/ml for *E. coli* and *Klebsiella species*. So the first sign of growth of *E. coli* and *Klebsiella species* were observed in the test tube containing 32 μg/ml of ethanol extract. But the MIC of ethanol extract was 128 μg/ml for *Bacillus subtilis, Staphylococcus aureus* and *St. β-haemoliticus.* So the first sign of growth of these microorganisms were observed in the test tube containing 64 μg/ml of ethanol extract (**Table-8**). In case of diethyl ether extract, the first sign of growth of *Bacillus subtilis* and *St .β-haemoliticus* were observed in the test tube containing 64 μg/ml of diethyl ether extract. So the MIC was 128 μg/ml against both *Bacillus subtilis* and *St. β-haemoliticus.* The first sign of growth of *Staphylococcus aureus* and *E. coli* were observed in the test tube containing 32 μg/ml of diethyl ether extract and the MIC was 64 μg/ml for both microorganisms. The growth of *Klebsiella species* was observed in the test tube containing 16 μg/ml of extracts and the MIC of *Klebsiella species* was 32 μg/ml (**Table-8**).

For chloroform extract, the growth of *Bacillus subtilis, Staphylococcus aureus, St .β-haemoliticus* and *E. coli* were first observed in the test tube having 32 μg/ml of chloroform extract. So the MIC was 64 μg/ml for these microorganisms. But in case of *Klebsiella species* the MIC was 32 μg/ml. So the first sign of growth was observed in test tube containing 16 μg/ml of chloroform extract (**Table-8**).

In case of compound-**2** the first sign of growth of *Staphylococcus aureus* and *St .β-haemoliticus* were observed in the test tube containing 64 μg/ml of compound-**2**. So

the MIC was 128 μg/ml for *Staphylococcus aureus* and *St .β-haemoliticus* both. But the first sign of growth of *Bacillus subtilis* and *E. coli* were observed in the test tube containing 32 μg/ml of compound-**2** and the MIC was 64 μg/ml for both of microorganisms. For *Klebsiella species,* the growth was observed in the test tube containing 16 μg/ml of compound-**2** and the MIC was 32 μg/ml (**Table-8**).

From the above experiment, it can be concluded that, chloroform extract showed comparatively better MIC than the other crude extracts and compound-**2**.

Brine Shrimp Lethality Bioassay

Experimental

Brine shrimp lethality bioassay (Meyer *et. al.*, 1982) is an important method by which toxicity as well as a wide range of pharmacological activities of the compounds can be bioassayed. Toxicity bioassay was carried out using the eggs of the brine shrimp nauplii (*Artemia salina* L).

Preparation of Sample Solution

Crude ethanol, diethyl ether, chloroform extracts and compound-**2** (3mg each) were accurately measured and dissolved in 0.6 ml (600 μl) of DMSO to get a concentration of 5mg/ml. For each of the samples, which was used as stock solution-A. From the stock solution-A, 50 μl was taken and diluted upto 1 ml with brine water to obtain a concentration of 0.25 μg/μl and this was indicated as stock solution-B.

From the stock solution-B, 5, 10, 20 and 40 μl were placed in 4 different vials and brine water was added to each vial making the volume up to 5 ml. The final concentration of the samples, in the vials became 0.25, 0.50, 1.0 and 2.0 μg/ml, respectively. Brine shrimp nauplii 10 in number were then placed in each vial. For each concentration, one vial containing same volume of DMSO plus 4 ml seawater was used as control group.

Procedures were same and described in **Chapter-III.**

Results and Discussion

In brine shrimp lethality bioassay, the crude ethanol, chloroform, diethyl ether extracts and compound-2 isolated from the stem extracts of *Duranta repens* Linn. showed positive results, indicating that they were biologically active. From this experiment it was revealed that each of the test samples showed different mortality rates at different concentrations.

Table-9 Toxicity of crude stem extracts and compound-2 against brine shrimp nauplii.

Extracts	LC_{50} (ppm)	95%Confidence limits (ppm)	Regression equation	χ^2 value
Ethanol	1.36	0.73-2.50	Y= 2.85+1.90 X	0.68
Diethyl ether	1.06	0.68-1.67	Y= 2.65+2.32X	0.59
Chloroform	0.94	0.64-1.37	Y= 2.39+2.75 X	1.67
Compound-2	1.21	0.69- 2.10	Y = 2.79+2.03X	0.13

From **Table-9** it is clear that all the crude extracts and compound-2 showed toxicity. The LC_{50} values of ethanol, diethyl ether and compound-2 were greater than 1 and was 1.36 ppm, 1.06 ppm and 1.21 ppm, respectively. But the chloroform extract showed highest toxicity and the LC_{50} value was 0.94 ppm, which may provide some explanation for the use of this plant as larvicide and insecticide.

Acute Toxicity Study

Experimental

The acute toxicity of the crude chloroform extract and compound-2 were observed on Long Evan's rats. The rats were collected from the Animal Resources Branch of International Center for Diarrheal Diseases and Research, Bangladesh (ICDDR,B), Mohakhali, Dhaka.

Grouping of the Rats

Weight of the individual rats was determined and they were grouped. The control group-E fed on normal diet and received no vehicle. Another control group- F received vehicle only. Group-G and H received crude chloroform extract (5 mg/kg body wt./day) and compound-2 (2 mg/kg body wt./day), respectively. Each group contains 4 rats.

Table-10 Grouping of rats.

Group	No. of Rats	Average body weight (gm)	Sex	Average age (week)	Dose (i.p) mg/kg body wt./day
E	4	103.25	Male	4-7	Normal diet
F	4	104.25	Male	4-7	0.2 ml of vehicle
G	4	103.75	Male	4-7	5 mg of $CHCl_3$ ext.
H	4	100.50	Male	4-7	2 mg of Compound-**2**

Preparation of Sample Solution

For the rat group-G, chloroform extract (14.525 mg) were accurately measured and dissolved in 5.6 ml of water with the help of Tween-20 in such a way that 0.2 ml contained 5 mg/kg body weight/day of chloroform extract.

Again for group-H, compound-**2** (5.628 mg) was measured and dissolved in 5.6 ml of water with the help of Tween-20 in such a way that 0.2 ml contained 2 mg/kg body weight/day of compound-**2**.

Gross General Observation after Drug Administration

The rats in each group-F, G and H were injected i.p with vehicles, chloroform extract and compound-**2** (0.2ml of each) respectively, for 7 days and control group-E fed on normal diet and received no vehicle. After the drug treatment the control and experimental rats were observed daily and notify their changes.

Changes of Body Weight

The body weights of each rat of groups-E, F, G and H were measured before and after the experiment and were recorded.

Hematological Profiles

The hematological profiles such as TC of RBC and WBC, DC of WBC, platelet count, percentage of hemoglobin and ESR of the controls and experimental rats were done at 1st day and 7th day after administration of drugs i.p.

Procedures were same and described in **Chapter-III.**

Biochemical Parameters of Blood

The biochemical parameters such as SGOT (Serum glutamic oxaloacetic transaminase), SGPT (Serum glutamic pyruvic transaminase), SALP (Serum alkaline phosphatase), bilirubin, creatinine and urea were observed on the 7th day after sacrificing the control and experimental rats.

Procedures were same and described in **Chapter-III.**

Histopathological Examination

After sacrificing the control and experimental rats, histopathology of liver, hear, lung and kidney were performed to observe any changes in the cellular structures (degradation and regeneration) of the experimental rats as compared with control rats. Histopathological examinations were done under high power microphtograph-camera Pentax MZ-M and were recorded by photographs.

Procedures were same and described in **Chapter-III.**

Results and Discussion

The acute toxicity study was performed using crude chloroform extract (5 mg/kg body wt./day) and compound-**2** (2 mg/kg body wt./day) for 7 consecutive days. The rats were kept under close observation throughout the experiment. The rats of group-E, F and H showed no signs of abnormalities. But in case of rat group-G, muscular numbness of the hind and fore legs, salivation and diarrhoea were observed. The change of body weights of control rats and experimental rats were observed and compared (**Table-11**).

Table -11 Change of body weights of controls and experimental rats.

Group of rats	Dose (i.p) mg/kg body weight/day.	Body weight before drug treatment n=4 Mean±SD_1	Body weight after drug treatment n=4 Mean±SD_2	% change of body weight
E	Fed on normal diet	103.25± 0.50	109.75± 1.26	6.3
F	0.2 ml of vehicle	104.25± 0.96	111.5± 2.38	6.9
G	5 mg/kg chloroform extract	103.75± 4.57	105± 2.94	1.2
H	2 mg/kg compound-2.	100.5± 3.11	109.25± 6.50	8.7

From the above table, it is found that, the percentage change in body weight of rats group-E, F and H were normal and insignificant. But in case of experimental rat group-G, the food intake per day was slightly lower than the other rat groups, because some abnormalities were observed in rat group-G.

Hematological Profiles

The hematological profiles of control rats and experimental rats were observed at 1st and 7th day of treatment. The hematological profiles of experimental rats were compared with control rats to check the disorders after i.p. administration of crude chloroform extract and compound-2.

In rat group-G almost all heamatological parameters were decreased significantly after 7 days. But only the eosinophil value was increased significantly. This indicated that the crude chloroform extract inhibited the maturation of the blood cells and showed toxic effect (**Table-12**). But in rat group-H, almost all hematological parameters were slightly increased after 7 days and no abnormalities were found in this group (**Table-12**).

Table-12 Hematological parameters of control group-E, F and experimental group-G and H.

Hematological parameter		Control Group-E		Control Group-F		Experimental Group-G		Experimental Group-H	
		1st day Mean + SD	7th da Mean + SD	1st day Mean + SD	7th da Mean + SD	1st day Mean + SD	7th da Mean + SD	1st day Mean + SD	7th da Mean + SD
Total RBC count (million/cu.mm)		5.525± 1.52	5± 0.08	4.8± 0.18	5.05± 0.06	4.825±0.10	3.95±0.06 *** •••	4.75±0.13	5.0±0.17
Total WBC count (Cells/cu.mm)		6600± 182.57	7025± 50.00	6775± 125.83	7150± 129.10	6850±57.74	5950±57.74*** •••	6875±95.74	7150±129.10
Differential count of WBC (%)	Neutrophil	54± 1.83	54.75± 2.06	54.5± 1.73	56.75± 1.50	55.0±0.82	52.5±0.58 **	54.75±0.96	55.00±0.82
	Lymphocyte	31.5± 1.29	31.75± 1.26	32.25± 1.26	32.75± 0.96	31.25±1.26	30.5±0.58 **	32.5±0.58	32.5±0.58
	Monocyte	4± 0.82	4.75±± 0.96	3.75± 0.50	4± 0.82	3.75±0.50	3.25±0.50 *	3.5±0.58	4.0±0.82
	Eosinophil	1.5± 0.58	1.75± 0.50	1.75± 0.50	1.75± 0.50	1.5±0.58	3.0±0.00 ** ••	1.5±0.58	2.0±0.00
Platelet count (no/u.mm)		302500 ± 12583.06	305000 ± 10000.00	308500 ± 9949.87	310000 ± 9128.71	296250 ± 4787.14	252500 ± 5000.00 *** •••	302500 ± 2886.75	305000±0.00
Hemoglobin (mg/dL)		12.75± 0.50	13.25± 0.50	13± 0.00	13.5± 0.58	12.5±0.58	12.25±0.50 * •	12.75±0.50	13.00±0.00
ESR (mm/1st hour)		13.75± 1.26	14.25± 1.26	14± 0.82	14.75± 0.50	13.75±0.96	13.0±0.00 •••	13.5±0.58	14.0±0.82

*P<0.05, ** P<0.01, *** P<0.001 Vs control-E

• P<0.05, •• P<0.01, ••• P<0.001 Vs control-F

Biochemical Parameters of Blood

The biochemical parameters of blood such as SGOT, SGPT, SALP, serum bilirubin, creatinine and urea were observed on the 7th day after sacrificing the control and experimental rats.

Table-13 Biochemical parameters of control rats group-E, F and experimental rat group-G and H.

Biochemical parameters	Control Group-E Mean ± SD	Control Group -F Mean±SD	Experimental Group-G Mean±SD	Experimental Group-H Mean±SD
SGPT (IU/L)	12.25±0.50	12.50±0.58	18.00±0.82*** •••	13.50±0.58* •
SGOT (IU/L)	14.00±0.82	14.25±0.50	19.50±0.58*** •••	15.50±0.58* •
SALP (IU/L)	10.00±0.00	10.25±0.50	11.75±0.50*** ••	10.50±0.58
Bilirubin (mg/dL)	0.37±0.01	0.36±0.01	0.38±0.00*	0.37±0.00
Creatinine (mg/dL)	0.59±0.01	0.59±0.05	0.60±0.06	0.57±0.06
Blood urea (mg/dL)	17.75±0.96	17.50±2.08	20.50±0.58** •	17.50±0.58

*P<0.05, ** P<0.01, *** P<0.001 Vs control Group-E

• P<0.05, •• P<0.01, ••• P<0.001 Vs control Group-F

The biochemical parameters such as SGPT, SGOT, SALP, bilirubin and blood urea of experimental rat group-G were increased significantly. But the creatinine value was increased insignificantly (**Table-13**). In case of experimental rat group-H, the values of SGPT and SGOT were increased significantly while the other parameters were changed insignificantly (**Table-13**). There are some negligible differences observed between experimental group-G and H. The values of creatinine and blood urea in chloroform treated group-G were increased but in compound-2 treated group-H, these two parameters were decreased from the control group-E and F (**Fig-9** and **10**).

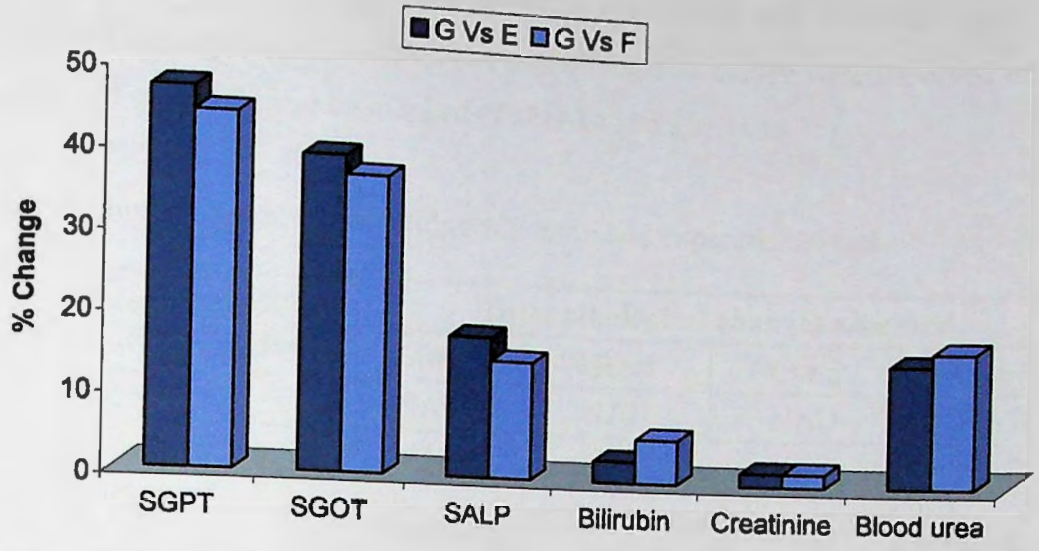


Fig-9 Percentage change of biochemical parameter of experimental rats group-G

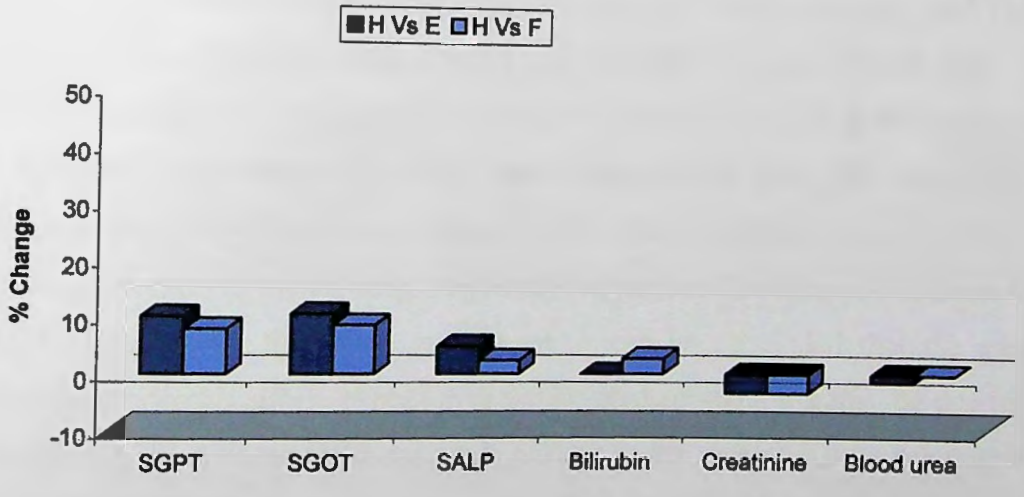


Fig-10 Percentage change of biochemical parameter of experimental rats group-H

Histopathological Studies

After 7 days of treatment, all the control and experimental rats were sacrificed and histopathological examinations of the liver, heart, lung and kidney were observed under microscope and recorded by photographed (**Table-14** and **Fig-11** to **26**).

Table-14 Histopathological examinations of control and experimental rats.

Rat Group	Dose (i.p) mg/kg body weight/day	Histopathological changes observed			
		Liver	Heart	Lung	Kidney
E	Normal diet	NAD	NAD	NAD	NAD
F	0.2ml of vehicle	NAD	NAD	NAD	NAD
G	5 mg/kg chloroform extract	Hyperplasia	Degenerative change	NAD	Hyperplasia
H	2 mg/kg compound-**2**	NAD	NAD	NAD	NAD

NAD indicates no abnormality detected.

In rat group-H, no abnormalities in the histopathology of liver, heart, lung and kidney were detected in comparisons with control group-E and F under microscope. This indicated that, compound-**2** had no toxic effect in this dose (2 mg/kg body weight/day) on cellular structure. But when the slides of these tissues of rat group-G were examined under microscope, some degenerative changes of cells were detected. This indicated that, the chloroform extracts (5 mg/kg body weight/day) of stem of *Duranta repens* Linn. were found to be toxic. From the above experiment, it can be concluded that the greater activity of the crude chloroform extract than the isolated compound-**2** may be due to the synergic effect of the mixture of compounds present in the extract. These findings may support to the traditional use of *Duranta repens* Linn. in indigenous system of medicine after subacute and chronic toxicity test and after dose adjustment.

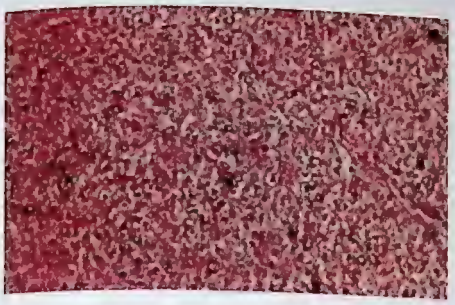


Fig-11 Microscopic view of liver tissues of control rat Group-E after 7 days.

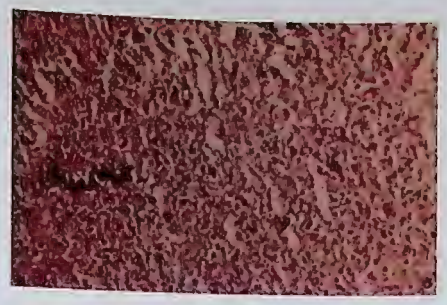


Fig-12 Microscopic view of liver tissues of control rat Group-F after 7 days.

Fig-13 Microscopic view of liver tissues of experimental rat group-G after 7 days.

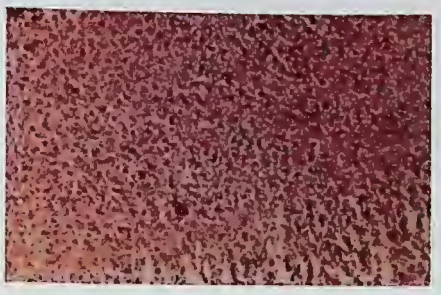


Fig-14 Microscopic view of liver tissues of experimental rat group-H after 7 days.

Fig-15 Microscopic view of heart tissues of control rat group-E after 7 days.

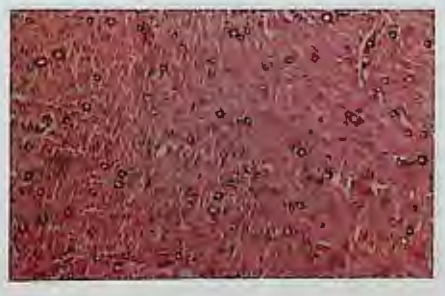


Fig-16 Microscopic view of heart tissues of control rat group-F after 7 days.

Fig-17 Microscopic view of heart tissues of experimental rats group-G after 7 days.

Fig-18 Microscopic view of heart tissues of experimental rats group-H after 7 days.

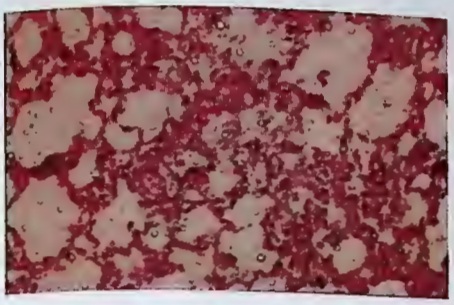


Fig-19 Microscopic view of lung tissues of control rat group-E after 7 days.

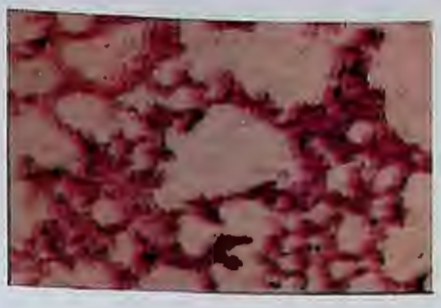


Fig-20 Microscopic view of lung tissues of control rat group-F after 7 days.

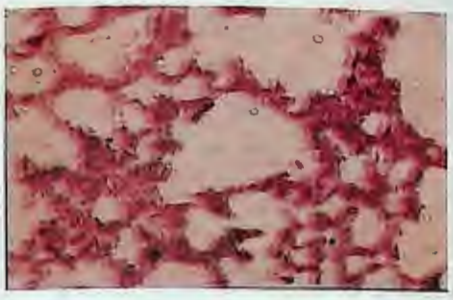


Fig-21 Microscopic view of lung tissues of experimental rat group-G after 7 days.

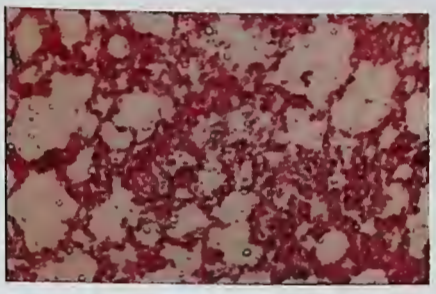


Fig-22 Microscopic view of lung tissues of experimental rat group-H after 7 days.

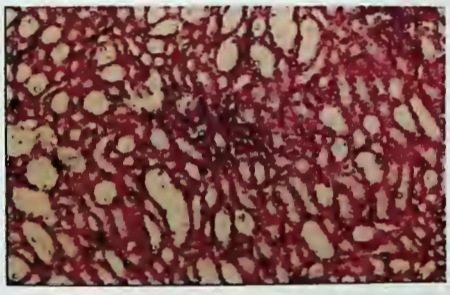


Fig-23 Microscopic view of kidney tissues of control rat group-E after 7 days.

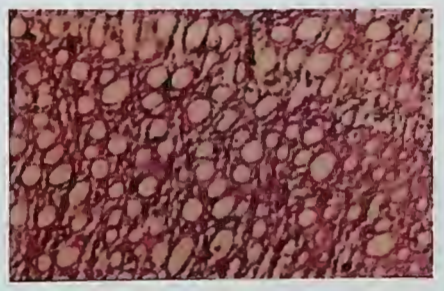


Fig-24 Microscopic view of kidney tissues of control rats group-F after 7 days.

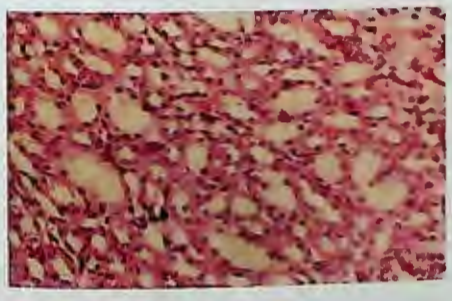


Fig-25 Microscopic view of kidney tissues of experimental rat group-G after 7 days.

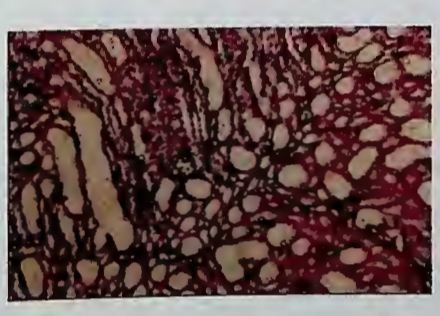


Fig-26 Microscopic view of kidney tissues of experimental rat group-H after 7 days.

Insecticidal Activity Test

Experimental

A large number of insects including many species of beetles and weevils attack stored products. Among them *Tribolium* (Coleoptera: Tenebrionidae) is a major pest and world wide distributed (Good,1936; Cotton,1947). *Tribolium* live on cracked grain or breakfast food or meal (Chapman, 1931), rice, dried fruit, bleached and unbleached white flour, cornmeal, barley flour and oatmeal (Chittenden, 1896). They also feed on chocolate, spices, peppers, peas oilseeds, semolina, coffee, cocoa, beans, various kinds of nuts and sometimes feed on specimen in insect collection (Good, 1933).

In Bangladesh, *T. castameum* is abundantly found associated with stored grain of different cereals. Dyte (1970) and Rowlands (1975) reported that almost all of the strains of *T.castaneum* have become resistant to malathion and almost all organophosphorus insecticides. The occurrence of resistance in different strains of *T. castaneum* has given an extra impetus to search for alternative way for the control of this pest. In view of this, the present investigation deal with the effects of crude extracts and compound-**2** of *Duranta repens* L. on *Tribolium castaneum* (Herbst).

Origin of Beetles

The insects *Tribolium castaneum* (Herbst) used in the experiment were obtained from a culture maintained in the Entomology Laboratory, Department of Zoology and IPM Laboratory, Institute of Biological Sciences, University of Rajshahi, Bangladesh.

Culture of the Beetles

Cultures were maintained in 1 lit. glass jar containing food medium. A filter paper was placed inside each jar for easy movement of the insects. The jar was covered with a piece of cloth and kept in an incubator at $30\pm0.5^{\circ}C$.

Food Medium

A standard mixture of whole-wheat flour and powered brewers yeast in the ratio of 19:1 (Park and Frank, 1948) was used as food medium. Both flour and the powdered dry yeast were previously passed through a 250-micrometer aperture sieve and mixed thoroughly

using an electric blender. The food medium was sterilized in an oven at 120°C for 6 hours. Food was not used until at least 15 days after sterilization to allow its moisture content to equilibrate with that of environment.

Collection of Eggs

About one hundred beetles were placed in a 500 ml-beaker containing food medium. The beaker was covered with a piece of cloth and kept in an incubator at 30±5°C. On the following day the eggs were collected by sieving the food medium using 500 and 250-micrometer aperture sieves (Khan and Selman, 1981). Eggs were transferred to glass petridish (9 cm in diameter) and incubated at 30±0.5°C.

Collection of Newly Hatched Larvae

Larvae emerged after 3 days in that condition. Newly hatched larvae were then collected with a fine camel hair brush and transferred to fresh food medium.

Determination of Larval Instars

Most larvae had six instars as reported by Good (1936). The second, third, fourth, fifth and sixth instars larvae were obtained form the larval culture on the 3rd, 6th, 9th, 12th and 16th day from the hatching, respectively. While the newly hatched larvae was used as first instar (Mondal, 1984a). Every three days the food medium was changed by fresh one to avoid conditioning by the larvae (Park, 1935; Mondal, 1984b).

Collection of Adults

A huge number of flour beetles were thus rared to get a regular supply of the newly formed adults. When sufficient adults produced in the sub-cultures they were collected from the food medium. Some pieces of filter paper were kept inside the beaker on the food. Adults crawled upon the paper and then the paper was taken out with a forceps. Beetles were then collected in a small beaker (50ml) with the help of a fine camel hair brush.

Precaution

All glasswares and sieves were regularly cleaned using washing liquid detergent and sterilized on an oven at 120°C for six hours. The working bench and other equipments were also cleaned before used.

Preparation and Application of Doses

Residual film method (Busvine, 1971) was used to test the mortality rate of larvae and adults of *Tribolium castaneum* (Herbst). Stem ethanol extract (350 mg), chloroform extract (80 mg) and diethyl ether extracts (250 mg) were dissolved separately in 10 ml of ethanol, chloroform and diethyl ether to get concentrations of 35000 µg/ml, 8000 µg/ml and 25000 µg/ml, respectively. Again compound-**2** (320 mg) was dissolved in 10 ml of chloroform to get a concentration of 32000 µg/ml. Then desired serial dilutions were prepared from the each stock solution using corresponding solvents. Various concentrations (1ml) of each extract were applied to petridishes (5cm diameter). After evaporation of solvents and drying off the dishes at room temperature 30 randomly selected larvae and adults were transferred to each dish and left without food for 24, 48 and 72 hours. Control dishes treated as above, but with solvents only. Those insects (larvae and adult) that did not move when prodded gently with a brush were considered as dead. All experiments were conducted separately under laboratory condition at 30°C.

Statistical Analysis

The mortality data of different larvae instars and adults were corrected by Abbott's formula (1925). The median lethal doses LC_{50} for each instar and stage were calculated by Probit analysis as described by Finney (1971).

Results and Discussion

The result of dose-mortality of crude stem extracts of *Duranta repens* Linn. and compound-**2** against *T.castaneum* were presented in **Table-15** and **Fig-27** to **54**. All the extracts and compound-**2** caused larval and adults mortality. The results showed that the chloroform extract of the stem of *D. repens* was more toxic to both larvae and adults of *T. castaneum* than the ethanol, diethyl ether extracts and compound-**2**. In control groups,

there was no mortality even without food for 3 days. But in experimental groups, at longer exposure (72 hr.) of the insects on all the extracts and compound-2 resulted in higher mortality rate. The increase in mortality with increase in exposure period could be due to several factors, which may be acting separately or jointly. For example, the uptake of the active moiety of extracts could be time dependent or it could get converted into more toxic metabolites in the larval integument and as well as alimentary canal, regulating in time-dependent effects. **Table-15** also demonstrated that toxicity of the plant extracts decreased with the increase of age of the larvae. For the stem extracts, the 1st instar larvae were most susceptible to chloroform (LC_{50} value, 9.13 $\mu g/cm^2$) followed by diethyl ether (LC_{50} value, 61.01 $\mu g/cm^2$), ethanol (LC_{50} value, 77.61$\mu g/cm^2$) and compound-2 (LC_{50} value, 90.59 $\mu g/cm^2$) than the older larval instars. **Fig-27** to **54** represents Probit regression line for the mortality of 1st to 6th instar larvae and adults of *T. castaneum* after 24, 48 and 72 hours. **Table-15** also demonstrates that compound-2 showed lowest activity against both larvae (1st to 6th instar) and adults of *T. castaneum* than the all crude extracts. But in average, all crude extracts of stem and compound-2 were found to more toxic to the all larval instars than the adults and the LC_{50} values for larvae were lower that those of adults. This might be due to relatively small amount of extracts were consumed and was quite sufficient to kill them in a large number. The order of toxicity on different instars larvae and adult of *T. castaneum* were chloroform extract> diethyl ether extract> ethanol extract> compound-2.

The present result was similar to Khanam *et al.*, (1990) who reported toxic effect of Royna (*Aphanamixis polystachya)* against *T. confusum.* This result is also similar to the findings of Khalequzzamen *et al.* (1988) and Jahan *et al.* (1989) who reported the insecticidal properties of tobaco (*Nicotiana tobactam)* leaf and bishkatali (*Polygonum hydropiper)* against *T. confusum* larvae, respectively. This result indicated that like other plant oils and extracts, stem extracts of *D. repens* may be used in the control of *T. castaneum* population with integrated pest management system which seems to be economically feasible and ecologically sound. This study also confirmed the validity of traditional use of Kata mehedi (*D. repens)* against stored grain pests.

Table-15 Insecticidal activity of crude stem extracts and compound-**2** of *D. repens* against *Tribolium castaneum* (Herbst).

Plant extract	Exposure time (h)	1st instar	2nd instar	3rd instar	4th instar	5th instar	6th instar	Adult (µg/cm^2)
Chloro-form	24	22.7	32.4	36.6	86.0	117.1	136.1	149.0
	48	13.4	19.9	25.4	59.0	61.6	64.2	117.8
	72	9.1	11.5	17.5	43.8	50.5	54.5	90.5
Diethyl ether	24	110.5	143.0	346.5	336.8	476.1	543.3	633.5
	48	79.6	96.4	230.0	244.8	372.9	402.0	448.3
	72	61.0	85.7	129.7	166.8	274.0	332.0	334.2
Ethanol	24	180.6	289.0	352.4	403.07	432.8	429.1	502.0
	48	106.4	257.0	205.0	300.3	305.6	355.6	414.0
	72	77.6	215.2	183.0	206.6	250.9	299.0	313.2
Com-**2**	24	442.5	565.6	1131.0	1131.0	1537.1	1964.1	2579.9
	48	150.5	253.4	352.6	352.5	465.1	486.0	774.1
	72	90.6	107.7	127.3	127.3	170.2	321.5	504.7

Values were based on three concentrations with 30 insects each.

Control group (solvent) showed no mortality even after 3 days without food.

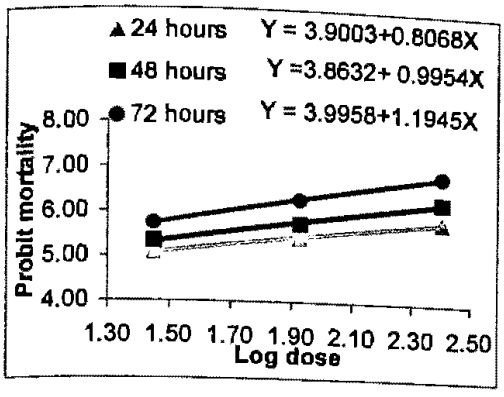


Fig-27 Probit regression line for the mortality of 1st instar larvae of *T.castaneum* treated with chloroform extracts of *Duranta repens* L.

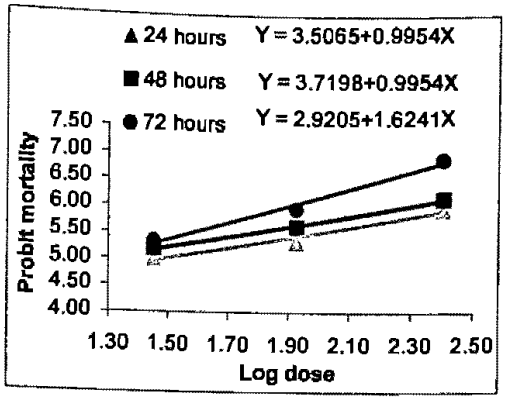


Fig-28 Probit regression line for the mortality of 2nd instar larvae of *T.castaneum* treated with chloroform extracts of *Duranta repens* L.

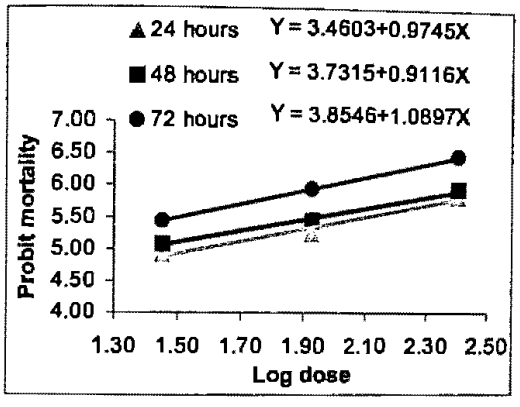


Fig-29 Probit regression line for the mortality of 3rd instar larvae of *T.castaneum* treated with chloroform extracts of *Duranta repens* L.

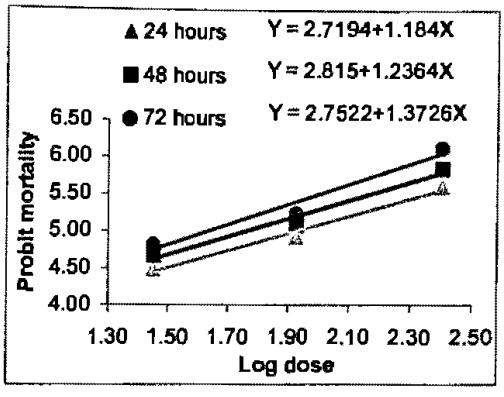


Fig-30 Probit regression line for the mortality of 4th instar larvae of *T.castaneum* treated with chloroform extracts of *Duranta repens* L.

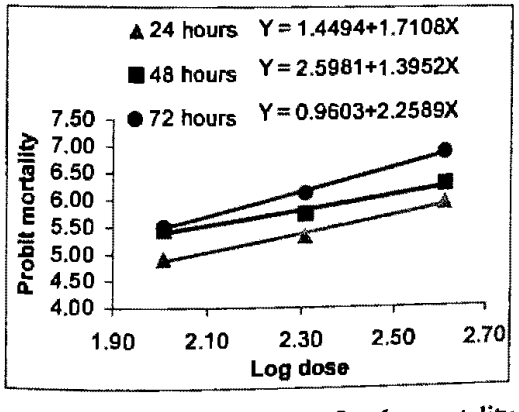


Fig-31 Probit regression line for the mortality of 5th instar larvae of *T.castaneum* treated with chloroform extracts of *Duranta repens* L.

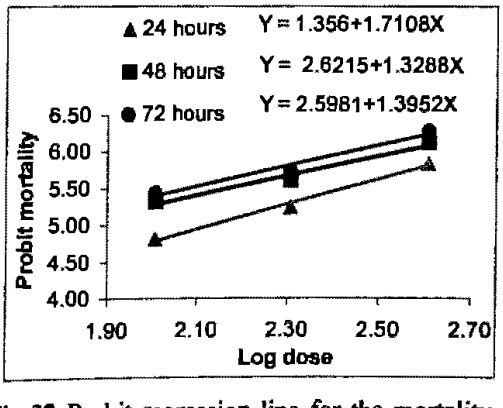


Fig-32 Probit regression line for the mortality of 6th instar larvae of *T.castaneum* treated with chloroform extracts of *Duranta repens* L.

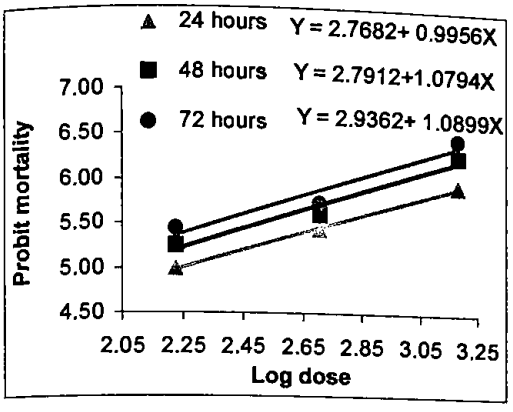


Fig-33 Probit regression line for the mortality of 1st instar larvae of *T.castaneum* treated with ethanol extracts of *Duranta repens* L.

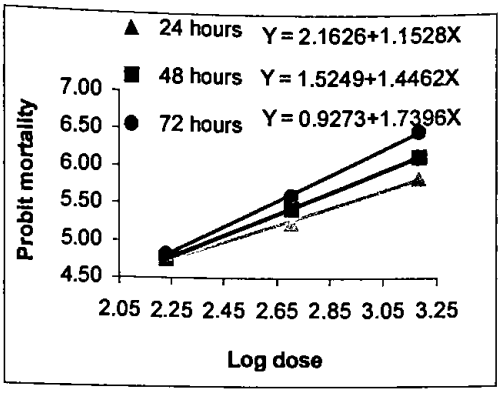


Fig-34 Probit regression line for the mortality of 2nd instar larvae of *T.castaneum* treated with ethanol extracts of *Duranta repens* L.

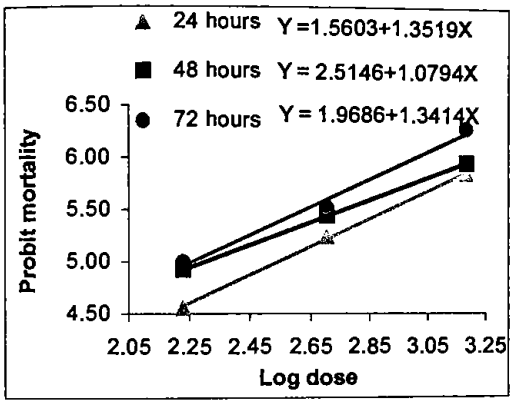


Fig-35 Probit regression line for the mortality of 3rd instar larvae of *T.castaneum* treated with ethanol extracts of *Duranta repens* L.

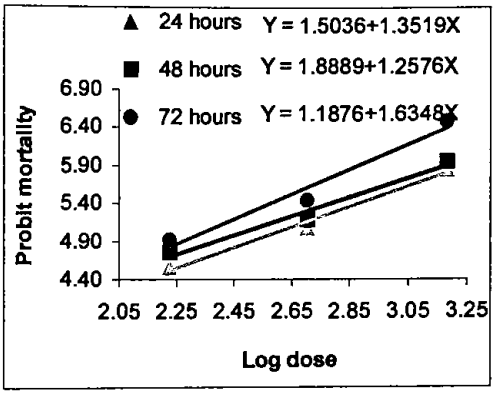


Fig-36 Probit regression line for the mortality of 4th instar larvae of *T.castaneum* treated with ethanol extracts of *Duranta repens* L.

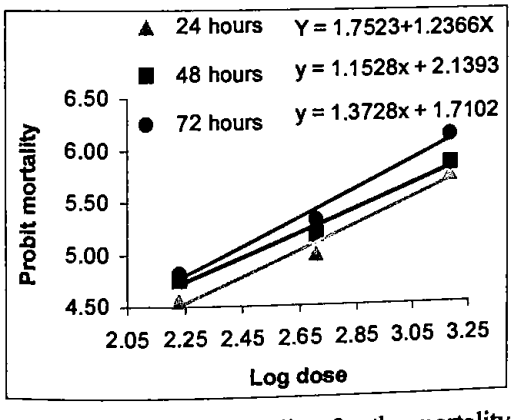


Fig-37 Probit regression line for the mortality of 5th instar larvae of *T.castaneum* treated with ethanol extracts of *Duranta repens* L.

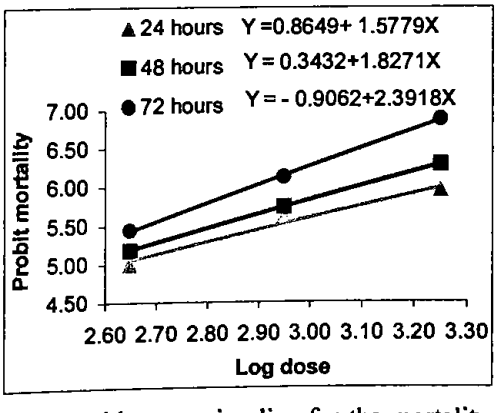


Fig-38 Probit regression line for the mortality of 6th instar larvae of *T.castaneum* treated with ethanol extracts of *Duranta repens* L.

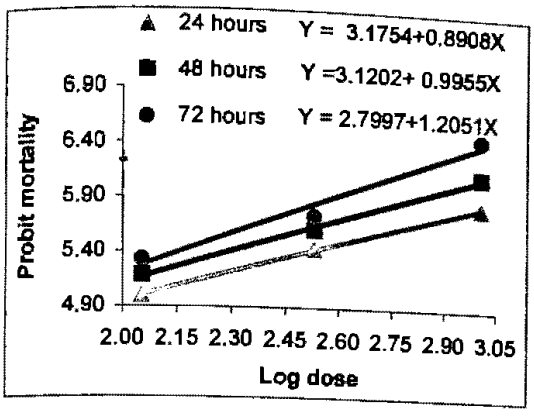


Fig-39 Probit regression line for the mortality of 1st instar larvae of *T.castaneum* treated with diethyl ether extracts of *Duranta repens* L.

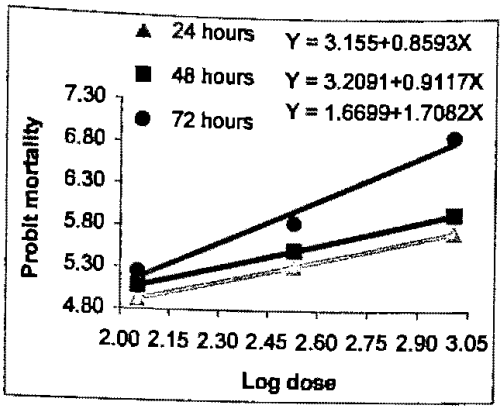


Fig-40 Probit regression line for the mortality of 2nd instar larvae of *T.castaneum* treated with diethyl ether extracts of *Duranta repens* L.

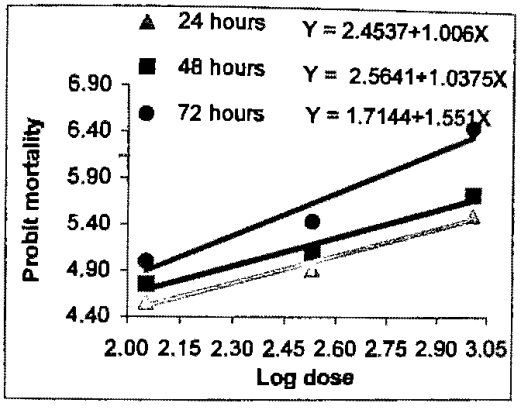


Fig-41 Probit regression line for the mortality of 3rd instar larvae of *T.castaneum* treated with diethyl ether extracts of *Duranta repens* L.

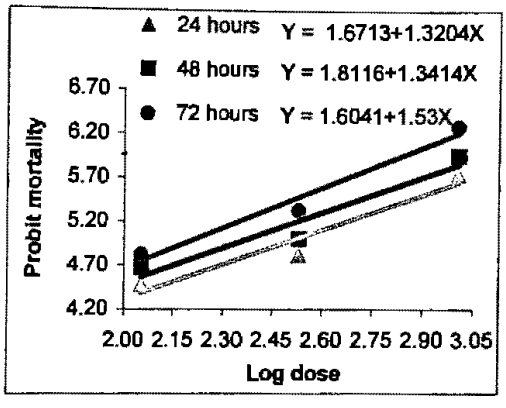


Fig-42 Probit regression line for the mortality of 4th instar larvae of *T.castaneum* treated with diethyl ether extracts of *Duranta repens* L.

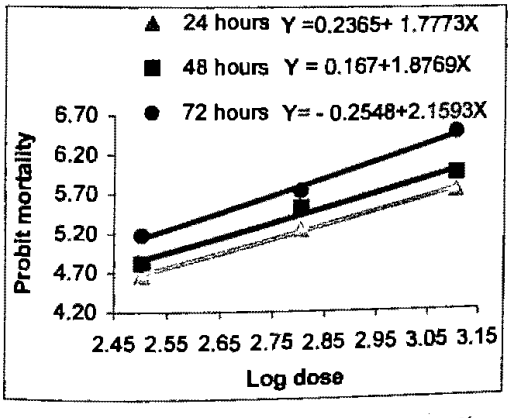


Fig-43 Probit regression line for the mortality of 5th instar larvae of *T.castaneum* treated with diethyl ether extracts of *Duranta repens* L.

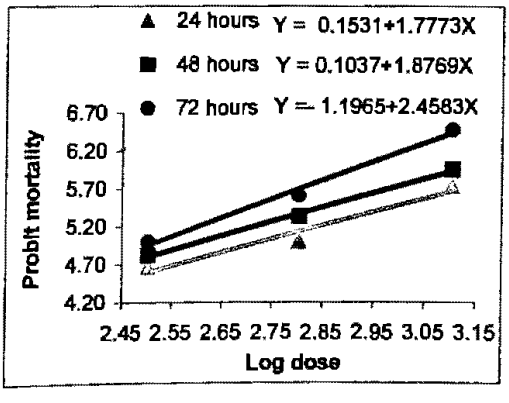


Fig-44 Probit regression line for the mortality of 6th instar larvae of *T.castaneum* treated with diethyl ether extracts of *Duranta repens* L.

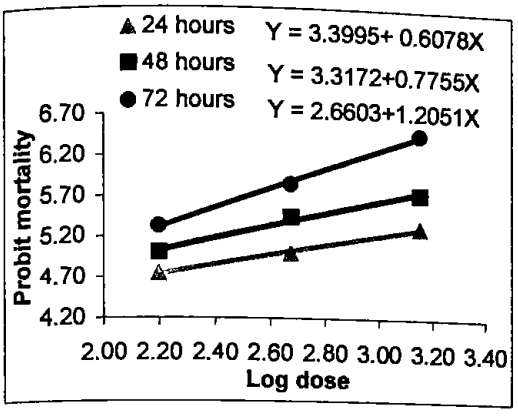


Fig-45 Probit regression line for the mortality of 1st instar larvae of *T.castaneum* treated with compound-2 of *Duranta repens* L.

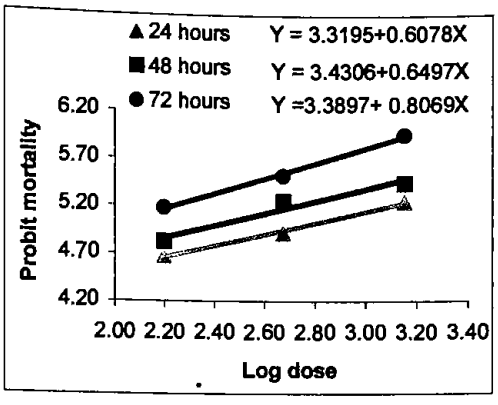


Fig-46 Probit regression line for the mortality of 2nd instar larvae of *T.castaneum* treated with compound-2 of *Duranta repens* L.

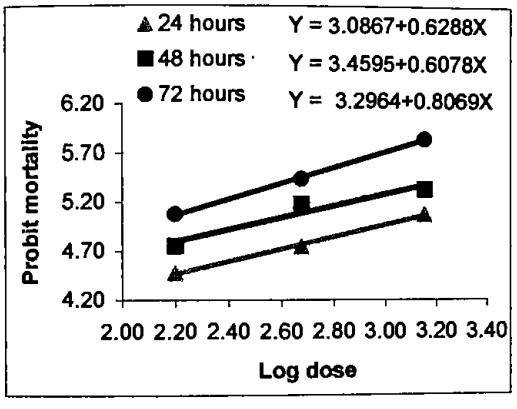


Fig-47 Probit regression line for the mortality of 3rd instar larvae of *T.castaneum* treated with compound-2 of *Duranta repens* L.

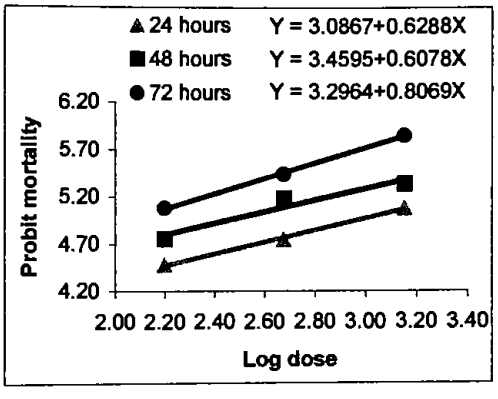


Fig-48 Probit regression line for the mortality of 4th instar larvae of *T.castaneum* treated with compound-2 of *Duranta repens* L.

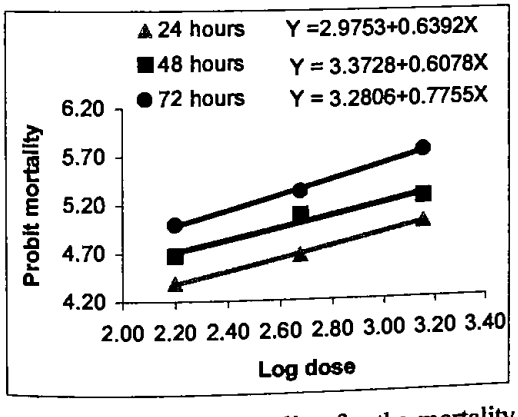


Fig-49 Probit regression line for the mortality of 5th instar larvae of *T.castaneum* treated with compound-2 of *Duranta repens* L.

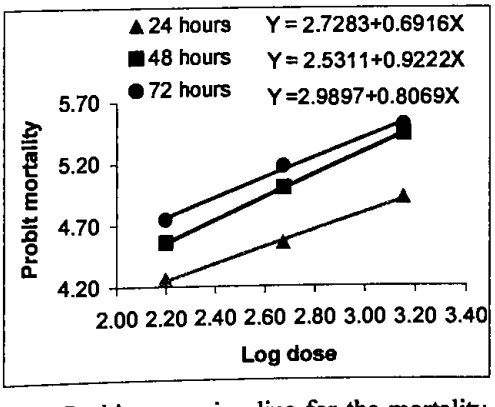


Fig-50 Probit regression line for the mortality of 6th instar larvae of *T.castaneum* treated with compound-2 of *Duranta repens* L.

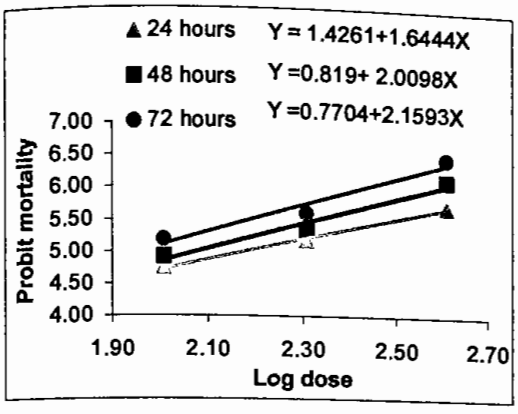


Fig-51 Probit regression line for the mortality of adult of *T.castaneum* treated with chloroform extracts of *Duranta repens* L.

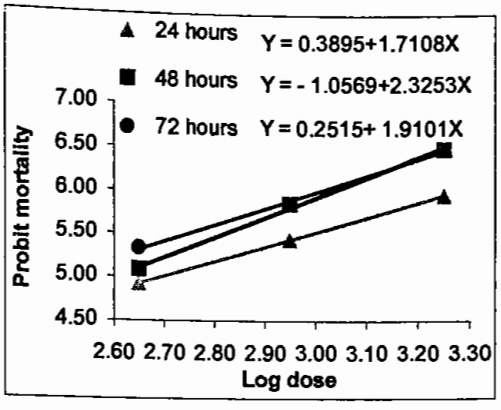


Fig-52 Probit regression line for the mortality of adult of *T.castaneum* treated with ethanol extracts of *Duranta repens* L.

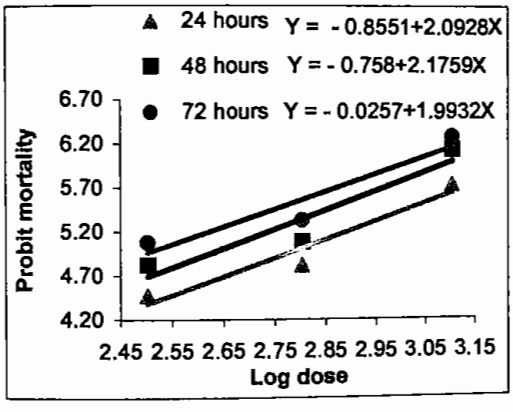


Fig-53 Probit regression line for the mortality of adult of *T.castaneum* treated with diethyl ether extracts of *Duranta repens* L.

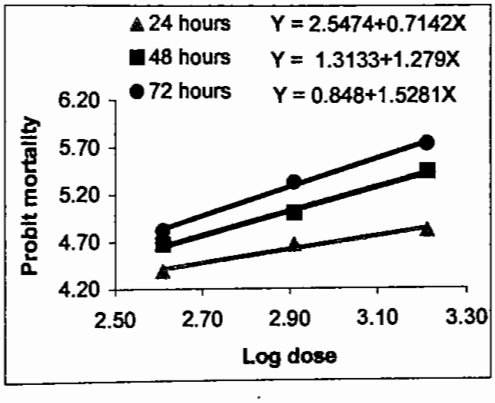


Fig-54 Probit regression line for the mortality of adult of *T.castaneum* treated with compound-2 of *Duranta repens* L.

B. Fruits of *Duranta repens* Linn.

Experimental

Collection and Extraction

Fruits of *Duranta repens* Linn were collected from the Botanical garden of Rajshahi University campus during November, 2001 and was identified by Professor A.T.M. Naderuzzaman, Department of Botany, University of Rajshahi, where a voucher specimen (no. Alam 78, collection date 19.09.1997) has been deposited.

Duranta repens fruits were sun dried and pulverized into a coarse powder. The ground fruits (950 gm) were extracted with ethanol and after evaporation under reduced pressure at 50°C affords a semisolid mass (42.0 gm). The concentrated ethanolic extract (30.0 gm) was diluted with distilled water and taken in a separating funnel. 30 ml of petroleum ether was then added and shaken vigorously and allowed to stand for few minutes. The petroleum ether layer (upper layer) was then collected. The process was repeated three times. The organic layers dried over an anhydrous Na_2SO_4 and concentrated in *vacuo* to afford a petroleum ether soluble fraction (6.0 gm).

After petroleum ether extraction, the aqueous part was then transferred again in a separating funnel and chloroform (30 ml) was added. The funnel was shaken and allowed to stand for layer separation. The organic layer was collected. The process was repeated three times. The organic layer were dried over and concentrated in *vacuo* to afford a chloroform soluble fractions (8.0 gm).

All crude extracts were run on pre-coated silica gel plate using n-hexane and ethyl acetate (2:1 and 1:1) as the mobile phase and vanillin-H_2SO_4 reagent was used as spray reagent. Crude ethanol extract gave positive test for steroids and glycosides. The petroleum ether extracts gave positive tests for steroids and flavonoids. The chloroform extract also gave positive tests for steroids and mainly flavonoids according to Harborne's method (1984).

The crude ethanol, chloroform and petroleum ether extracts of fruits of *Duranta repens* Linn. were then tested for their biological activities such as antibacterial, antishigellosis activity, MIC, brine shrimp lethality bioassay, insecticidal activity against *T. castaneum* and larvicidal activity test against mosquito larvae of *Culex quinquefasciatus*.

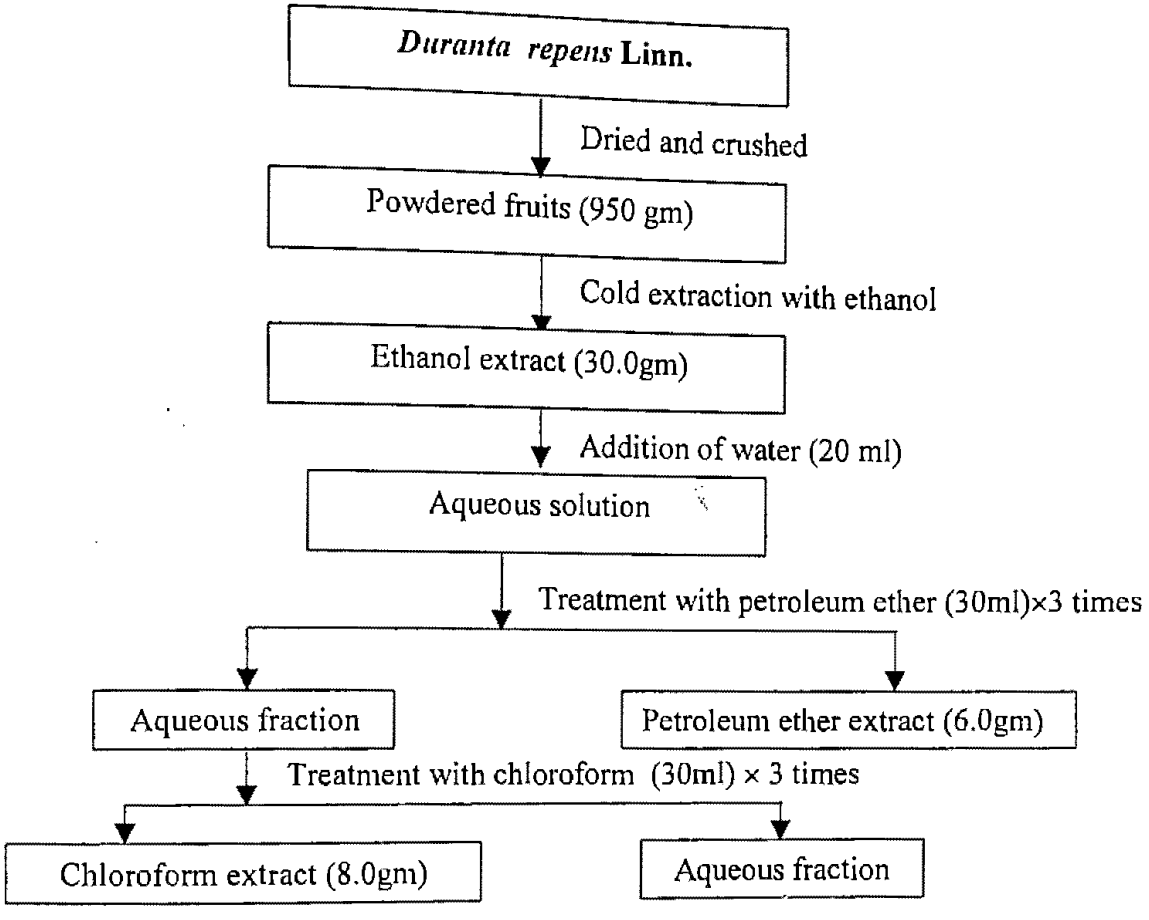


Fig-55 Extraction scheme of fruits of *Duranta repens* Linn.

Biological Study

Antibacterial Activity Test

Experimental

In vitro antimicrobial activities of the crude ethanol, chloroform and petroleum ether extracts were performed by disc diffusion method (Vander and Vlietnck, 1991).

Test Organisms

Five pathogenic bacteria (two Gram positive and three Gram negative) were selected for the test. Nutrient agar media was used for the test. Ciprofloxacin (30 µg/disc) was used as a standard disc.

Table-16 List of test bacteria.

Gram positive	Gram negative
Bacillus subtilis	*Escherichia coli*
Streptococcus-β-haemoliticus	*Pseudomonas aeruginosa*
	Klebsiella species

Preparation of Test Sample

Crude ethanol, petroleum ether and chloroform extracts (3 mg each) were dissolved in methanol (600 µl) to get a concentration of 30 µg/6µl. Again 5 mg of ethanol, petroleum ether and chloroform extracts were dissolved in 250 µl of methanol to get a concentration of 200 µg/10µl.

All the procedures were same and described in **Chapter-III.**

Results and Discussion

The antibacterial activity of petroleum ether, chloroform and ethanol extracts of fruits of *Duranta repens* Linn. were tested against two Gram positive and three Gram negative bacteria. Ciprofloxacin (30 µg/disc) was used as standard. At 30 µg/disc, the crude petroleum ether extracts showed no zone of inhibition. But at 200 µg/disc, the petroleum ether gave mild to moderate activities against *Streptococcus-β-haemolyticus*(11 mm.), *Escherichia coli* (12 mm.), *Psedomonus aeruginosa* (13 mm.) and *Klebsiella species* (11 mm.) (**Table-17** and **Fig-56, 57**).

Table-17 *In vitro* antibacterial activity of the fruit extracts of *Duranta repens* .

Name of Bacterial Strains	Petroleum ether		Chloroform		Ethanol		Cipro-floxacin 30 µg/ disc
	30 µg/disc	200 µg/disc	30 µg/disc	200 µg/disc	30 µg/disc	200 µg/disc	
Gram Positive							
Bacillus subtilis	00	10	00	13	09	18	26
Streptococcus β-haemolyticus	00	11	00	12	10	17	25
Gram Negative							
Escherichia coli	00	12	00	14	00	20	26
Pseudomomas aeruginosa	00	13	00	15	09	19	24
Klebsiella species	00	11	00	14	10	21	25

The chloroform extract (30 µg/disc) showed no zone of inhibition but at 200 µg/disc it showed some activities against *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella species* and produced zone of inhibition between 13 to 15 mm. (**Table-17** and **Fig-56, 57**). In case of ethanol extract, it showed activity at both concentrations. At 30 µg/disc ethanol extract gave mild activities against *Streptococcus-β-haemolyticus* (10 mm.) and *Klebsiella species* (10mm.). But at 200 µg/disc the ethanol extract showed higher activities against *Bacillus subtilis* (18 mm.), *Escherichia coli* (20 mm.), *Pseudomonas aeruginosa* (19 mm.) and *Klebsiella species* (21mm.) (**Table-17** and **Fig-56, 57**) as compared with the standard Ciprofloxacin (30 µg/disc). Among the tested materials, the ethanol extract gave comparatively better activity than the petroleum ether and chloroform extract.

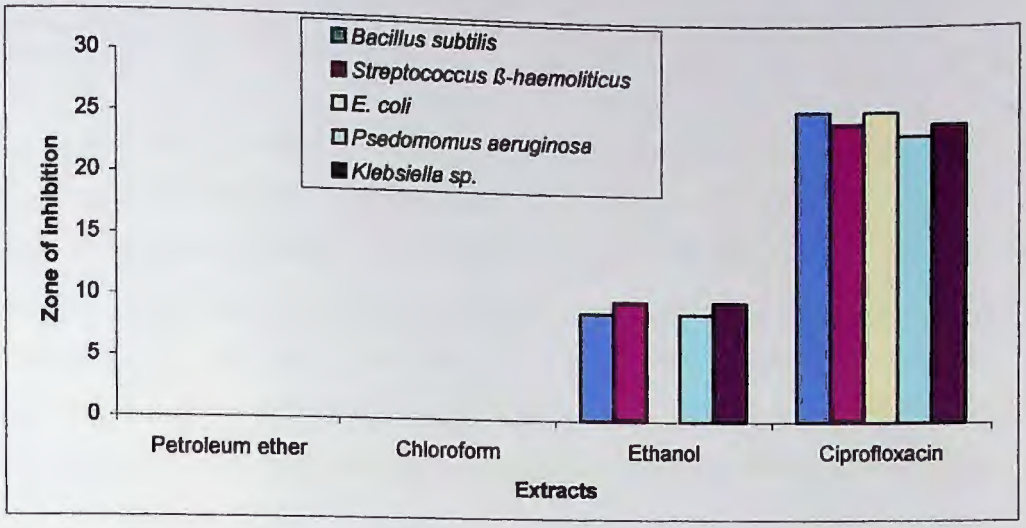


Fig-56 *In vitro* antibacterial activity of fruit extracts of *Duranta repens* Linn.(30µg/disc).

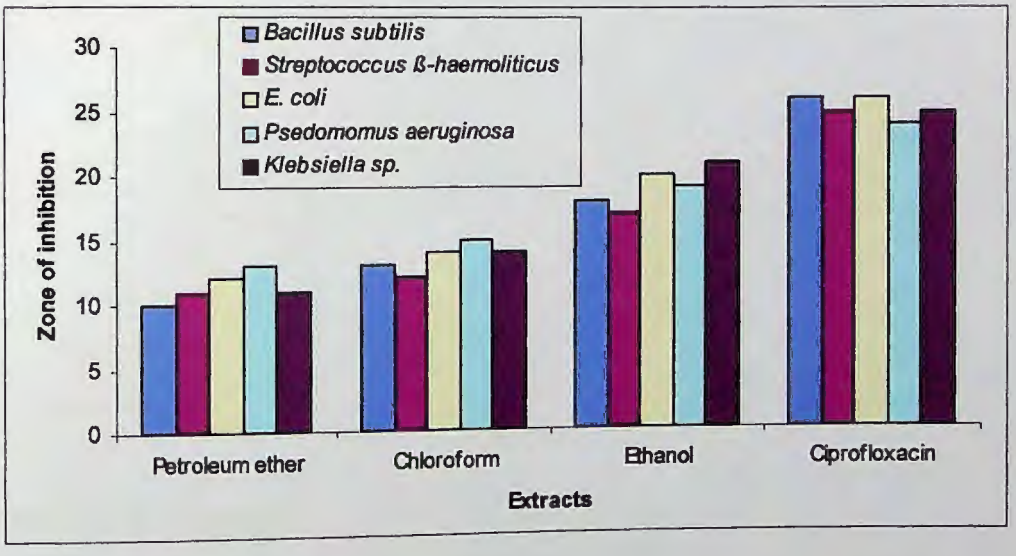


Fig-57 *In vitro* antibacterial activity of fruit extracts of *Duranta repens* Linn. (200µg/disc).

Antishigellosis Activity Test

Experimental

Shigellosis or bacillary dysentery is endemic throughout the world and account for about 15% of diarrhea-associated deaths among the children worldwide (Victora *et al.*, 1993). In Bangladesh, *shigella* infection is most frequent and the fatality rate between the children is 3.5% (Stoll *et al.*,1982). The pathogens responsible for this fatal disease are growing resistance day by day in Bangladesh. For to fight against the resistance of microbes, scientists all over the world have been searching new and potent bioactive principles from plants and in continuation of this search we studied the fruit of *Duranta repens.*

Test Organisms

In vitro antishigellosis activities of the fruit extracts of *Duranta repens* Linn. were performed on five Gram negative bacteria by disc diffusion method (Vander and Vlietnck,1991).

Table-18 List of test bacteria.

Gram negative
Shigella boydii
Shigella shiga
Shigella dysenteriae
Shigella flexneri
Shigella sonnie

Preparation of Test Sample

Crude ethanol, petroleum ether and chloroform extracts (3 mg each) were dissolved in methanol (600 µl) to get a concentration of 30 µg/6µl. Again 200 µg/10µl was prepared by dissolving 5 mg of ethanol, petroleum ether and chloroform extracts in 250 µl of methanol. Ciprofloxacin (30µg/disc) was used as standard disc.

Procedures were described in **Chapter-III.**

Results and Discussion

In vitro antishigellosis activity of the fruit extracts of *Duranta repens* Linn. were tested against five Gram negative *Shigella* bacteria. Ciprofloxacin (30µg/disc) was used as standard disc for comparison the data. All the crude extracts of *D. repens* showed activity against *Shigella* bacteria.

Table-19 *In vitro* antishigellosis activity of the fruit extracts of *Duranta repens.*

Zone of Inhibition (Diameter in mm)

Name of Bacterial Strains	Petroleum ether		Chloroform		Ethanol		Ciprofloxacin 30µg/disc
	30 µg/disc	200 µg/disc	30 µg/disc	200 µg/disc	30 µg/disc	200 µg/disc	
Shigella boydii	00	11	00	13	09	20	26
Shigella shiga	00	10	00	13	10	19	25
Shigella dysenteriae	00	12	00	14	09	22	26
Shigella flexneri	00	10	00	13	08	18	24
Shigella sonnie	00	11	00	12	00	20	25

The petroleum ether extracts showed no inhibitory zones at 30 µg/disc. At 200 µg/disc petroleum ether showed mild activities against all tested bacteria and it showed highest activity against *Shigella dysenteriae* and produced zone of inhibition 12mm. (**Table-19** and **Fig-58, 59**). At 200 µg/disc the chloroform extract gave moderate activities against all *Shigella* bacteria and it showed highest activity against *Shigella dysenteriae* (14mm.) The chloroform extract showed no activities at 30 µg/disc (**Table-19** and **Fig-58, 59**). On the other hand, the ethanol extract showed zone of inhibition at both concentrations. At 30 µg/disc ethanol extract gave mild activity against almost all bacteria and it gave highest activity against *Shigella shiga* (10mm.). But it showed better activity against all *Shigella* bacteria at 200 µg/disc and produced zone of inhibition between 18 to 22mm. The ethanol extract also gave intense activity against *Shigella dysenteriae* (22mm) at 200 µg/disc (**Table-19** and **Fig-58, 59**).

From the above two types of antibacterial activity test, it can be concluded that, such activities may support to the traditional use of the fruits of *Duranta repens* Linn. in the treatment of infection and intestinal worms.

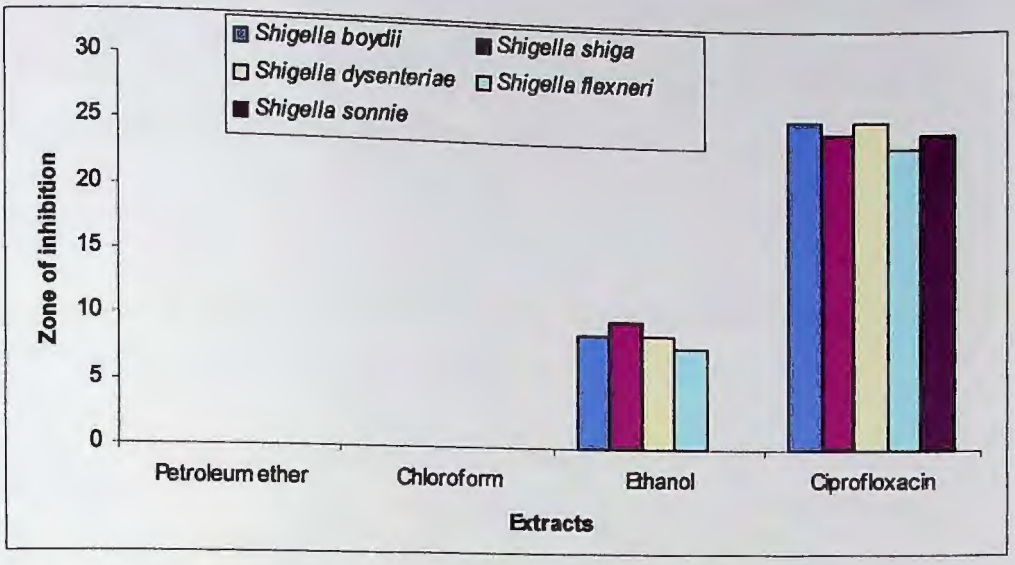


Fig-58 *In vitro* antishigellosis activity of fruit extracts of *Duranta repens* Linn.(30µg/disc).

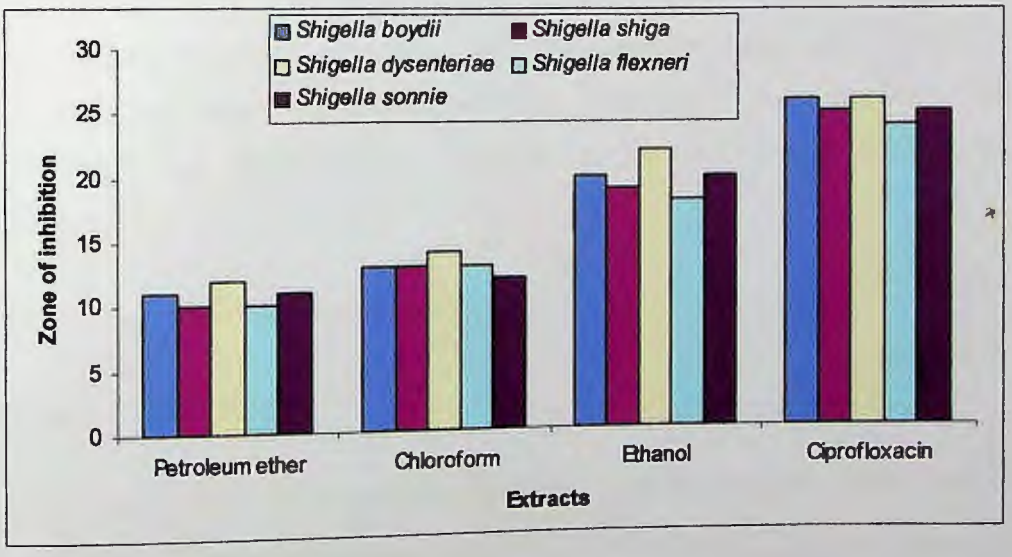


Fig-59 *In vitro* antishigellosis activity of fruit extracts of *Duranta repens* Linn. (200µg/disc).

Determination of Minimum Inhibitory Concentration

Experimental

Minimum inhibitory concentration of the fruit extracts of *Duranta repens* Linn. were carried out by a serial tube dilution technique (Reiner, 1982).

Test Organism

The following five pathogenic bacteria were used for the determination of MIC of crude extracts.

Table-20 List of pathogenic bacteria used for determination of MIC.

Gram positive	Gram negative
Streptococcus β-haemoliticus	*Shigella dysenteriae*
Bacillus subtilis	*Shigella boydii*
	Escherichia coli

Preparation of the Sample Solution

The crude ethanol, petroleum ether and chloroform extracts were measured accurately (2.048 mg each) and were taken in different vials. Then 2 ml of methanol was added to each of the vials and mixed well to make the sample solution whose concentration became 1024 μg /ml.

All the procedures were described in **Chapter-III.**

Results and Discussion

The MIC of fruit extracts of *Duranta repens* Linn. was determined against two Gram positive and three Gram negative bacteria.

For petroleum ether extract, the first sign of growth of all microorganisms were observed in the test tube containing 64 μg/ml of extract and the MIC of petroleum ether extract against five microorganisms were calculated and found to be 128 μg /ml **(Table-21).**

Table-21 MIC of the fruit extracts of *Duranta repens* Linn.

Minimum Inhibitory Concentration (µg/ml)

Extracts	Name of Bacterial Strains				
	Streptococcus β-haemolyticus	*Bacillus subtilis*	*Shigella boydii*	*Eschericia coli*	*Shigella dysenterie*
Petroleum ether	128	128	128	128	128
Chloroform	128	64	64	64	64
Ethanol	32	32	32	32	32

In chloroform extract, the first sign of growth of was observed in the test tube containing 64 µg/ml of extract and the MIC value of chloroform extract was 128 µg/ml against *Streptococcus β-haemolyticus*. In case of microorganisms *Bacillus subtilis, Shigella boydii, Eschericia coli* and *Shigella dysenterie* the growth were observed in the test tube containing 32 µg /ml of chloroform extract and the MIC for these microorganisms were 64 µg /ml (**Table-21**). On the other hand, the MIC of ethanol extract against all microorganisms was 32 µg/ml. So the first sign of growth of all microorganisms were observed in the test tube containing 16 µg /ml of the ethanol extract (**Table-21**).

From the above results it can be concluded that, among the tested extracts, ethanol extract was biologically more active having lowest MIC and the plant *Duranta repens* Linn. could be a good candidate for more work as antibiotic agents.

Brine Shrimp Lethality Bioassay

Experimental

Brine shrimp lethality bioassay (Meyer *et. al.*, 1982) was used for toxicity test. For hatching, shrimp eggs were kept in 3.8% brine water with a constant oxygen supply for 48 hours. The mature nauplii were then used in the experiment.

Preparation of Sample Solution

Crude ethanol, petroleum ether and chloroform extracts (3mg each) were accurately measured and dissolved in 0.6 ml (600 µl) of DMSO to get a concentration of 5mg/ml,

which was used as stock solution-A. From the stock solution-A, 50 µl was taken and diluted upto 1 ml with brine water to obtain a concentration of 0.25 µg/µl and this was indicated as stock solution-B. From the stock solution-B, 5, 10, 20 and 40 µl were placed in 4 different vials and NaCl solution was added to each vial making the volume up to 5 ml. The final concentration of the samples, in the vials became 0.25, 0.50, 1.0 and 2.0 µg/ml, respectively. For each concentration, 10 brine shrimp nauplii were then placed in 4 different vials. For each concentration, one vial containing same volume of DMSO plus 4 ml brine water was used as control group.

Procedures were described in **Chapter-III**.

Results and Discussion

The toxicity of crude ethanol, chloroform and petroleum ether extracts of fruit of *Duranta repens* Linn. were observed against brine shrimp nauplii.

Table-22 Toxicity of fruit extracts of *Duranta repens* against brine shrimp nauplii

Extracts	LC_{50} (ppm)	95%Confidenc limits (ppm)	Regression equation	χ^2 value
Petroleum ether	1.21	0.69-2.10	Y=2.79 +2.03X	0.13
Chloroform	0.81	0.55-1.19	Y=2.55+2.69X	0.55
Ethanol	0.49	0.28-0.85	Y=3.69+1.89 X	5.26

All the crude extracts showed toxicity against *Artemia salina* Leach (brine shrimp eggs). The LC_{50} values of petroleum ether, chloroform and ethanol extracts were found to be 1.21 ppm, 0.81 ppm and 0.49 ppm, respectively. So the ethanol extract was found to be more toxic than the chloroform and petroleum ether extracts (**Table-22**). This finding may support to the traditional use of fruits of *Duranta repens* Linn. as insecticides and larvicides against pests and mosquito larvae, respectively.

Insecticidal Activity Test

Experimental

Residual film method (Busvine, 1971) was used to test the fruit extracts of *Duranta repens* Linn. on mortality rate of larvae and adults of *Tribolium castaneum* (Herbst).

Preparation of Doses

The crude ethanol (40.0 mg), chloroform (60.0 mg) and petroleum ether (70.0 mg) extracts were dissolved in 10 ml of ethanol, chloroform and petroleum ether to get concentrations of 4000 µg/ml, 6000 µg/ml and 7000 µg/ml, respectively. The desired serial dilutions were prepared from the stock solutions using corresponding solvents.

All the procedures were same and described in **Chapter-IV (A)**.

Results and Discussion

The insecticidal activities of ethanol, chloroform and petroleum ether extracts of fruit of *Duranta repens* were tested against *Tribolium castaneum* (Herbst). All the test materials were proved to be toxic causing mortality of *T. castaneum.* Among larvae, first instar larvae were highly susceptible to extracts than the older instar larvae. In control groups, there was no mortality even without food for 3 days. But in experimental groups, at longer exposure (72 hr.) of the insects on all the extracts resulted in higher mortality rate. **Table-23** also demonstrated that, toxicity of the plant extracts decreased with the increase of age of the larvae. The ethanol extract of fruit showed better toxicity against both larvae and adults of *T. castaneum.* For the fruit extracts, the 1st instar larvae were most susceptible to ethanol (8.31 µg/cm^2) followed by chloroform (22.47 µg/cm^2) and petroleum ether (31.38 µg/cm^2) than the older larval instars. The ethanol, chloroform and petroleum ether extracts of fruit were found to more toxic to the 1st larval instar than the adults and the LC_{50} values for the adults at 72 hour exposure were 65.93 µg/cm^2, 149.34 µg/cm^2 and 152.13µg/cm^2, respectively. **Fig-60** to **80** indicated the Probit Regression line for the mortality of fruit extracts after 24, 48 and 72 hours.

The present result of the experiment indicated that the fruit extracts of *D. repens* may be used in the control of *T. castaneum* population with integrated pest management system which seems to be economically feasible and ecologically sound. However, more research should be directed towards isolation of bioactive compounds as well as field trials must be conducted before these extracts were used in grain storage.

Table-23 Insecticidal activity of fruit extracts of *D .repens* against *Tribolium castaneum* (Herbst).

Plant extract	Exposure time (h)	1st instar	2nd instar	3rd instar	4th instar	5th instar	6th instar	Adult ($\mu g/cm^2$)
Ethanol	24	26.7	30.2	31.8	38.3	60.0	84.6	118.5
	48	19.5	21.5	23.5	27.6	41.1	60.3	89.5
	72	8.3	13.8	17.7	19.4	27.6	38.7	66.0
Chloroform	24	40.0	53.5	63.4	92.3	137.3	225.9	238.3
	48	29.1	42.0	49.0	65.8	88.8	156.3	181.0
	72	22.5	31.8	36.8	43.0	62.3	95.3	149.3
Petroleum ether	24	58.7	66.3	79.6	93.2	140.6	189.5	242.0
	48	45.1	48.8	63.4	73.9	102.3	140.6	195.5
	72	31.4	34.4	52.8	59.8	75.4	102.3	152.1

Values were based on three concentrations with 30 insects each.

Control group (solvent) showed no mortality even after 3 days without food.

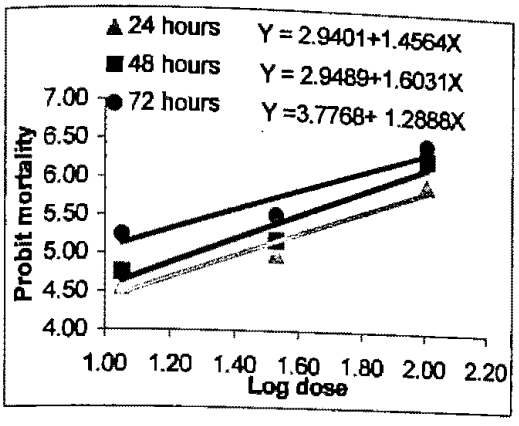


Fig-60 Probit regression line for the mortality of 1st instar larvae of *T.castaneum* treated with ethanol extract after 24, 48 and 72 hours.

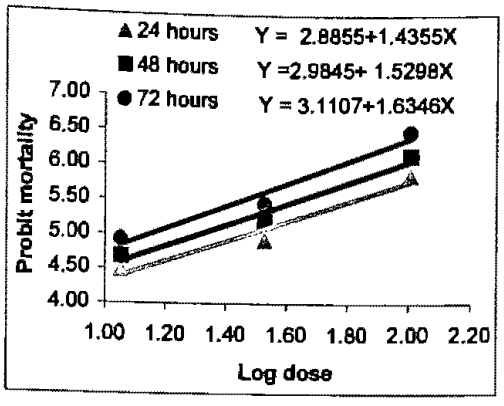


Fig-61 Probit regression line for the mortality of 2nd instar larvae of *T.castaneum* treated with ethanol extract after 24, 48 and 72 hours.

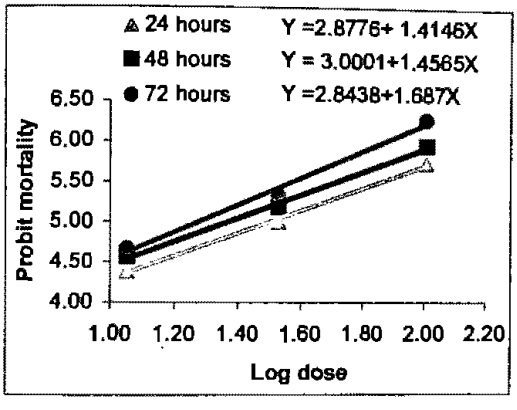


Fig-62 Probit regression line for the mortality of 3rd instar larvae of *T.castaneum* treated with ethanol extract after 24, 48 and 72 hours.

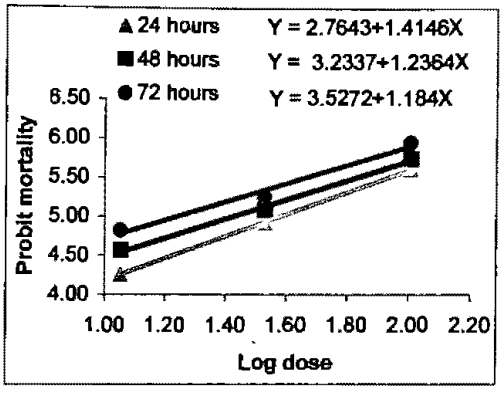


Fig-63 Probit regression line for the mortality of 4th instar larvae of *T.castaneum* treated with ethanol extract after 24, 48 and 72 hours.

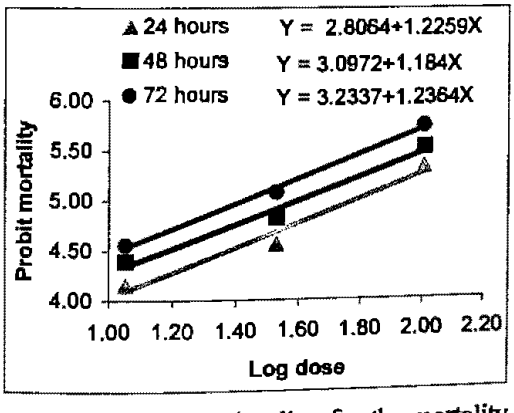


Fig-64 Probit regression line for the mortality of 5th instar larvae of *T.castaneum* treated with ethanol extract after 24, 48 and 72 hours.

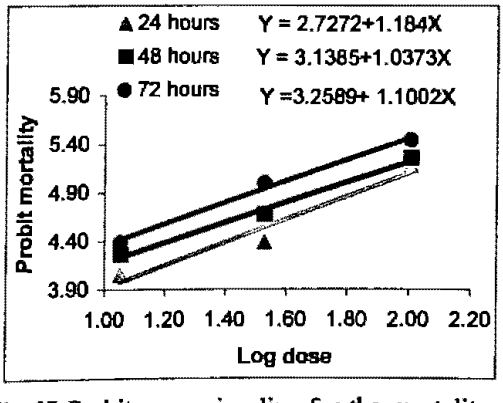


Fig-65 Probit regression line for the mortality of 6th instar larvae of *T.castaneum* treated with ethanol extract after 24, 48 and 72 hours.

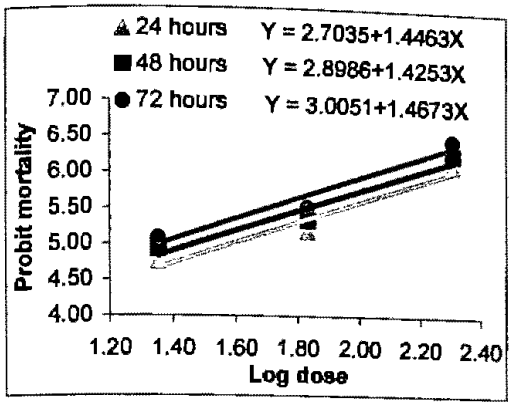


Fig-66 Probit regression line for the mortality of 1st instar larvae of *T.castaneum* treated with chloroform extract after 24, 48 and 72 hours.

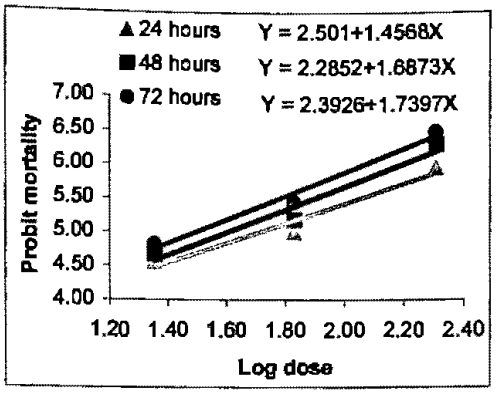


Fig-67 Probit regression line for the mortality of 2nd instar larvae of *T.castaneum* treated with chloroform extract after 24, 48 and 72 hours.

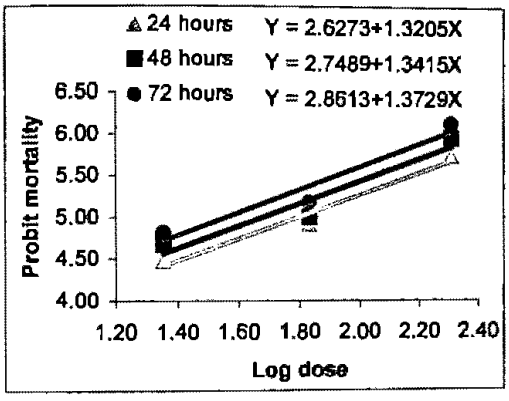


Fig-68 Probit regression line for the mortality of 3rd instar larvae of *T.castaneum* treated with chloroform extract after 24, 48 and 72 hours.

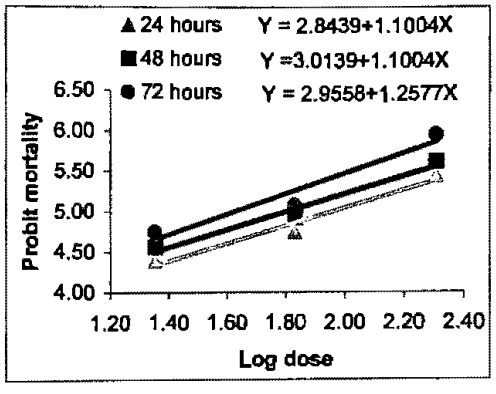


Fig-69 Probit regression line for the mortality of 4th instar larvae of *T.castaneum* treated with chloroform extract after 24, 48 and 72 hours.

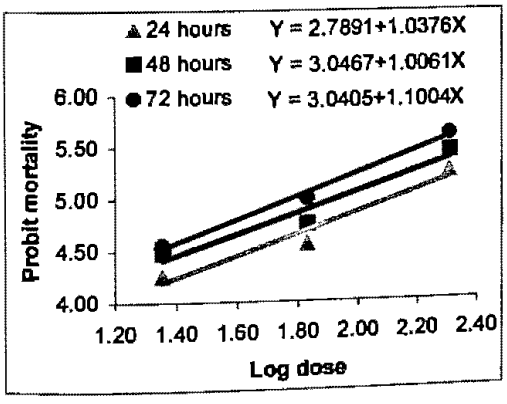


Fig-70 Probit regression line for the mortality of 5th instar larvae of *T.castaneum* treated with chloroform extract after 24, 48 and 72 hours.

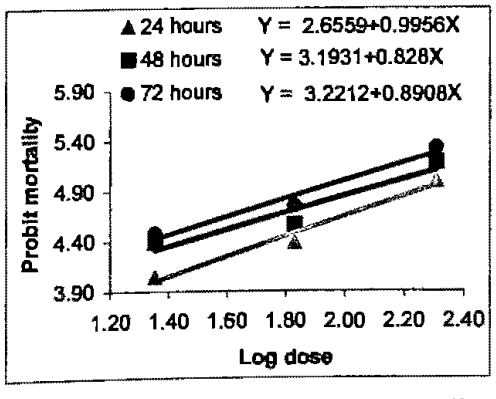


Fig-71 Probit regression line for the mortality of 6th instar larvae of *T.castaneum* treated with chloroform extract after 24, 48 and 72 hours.

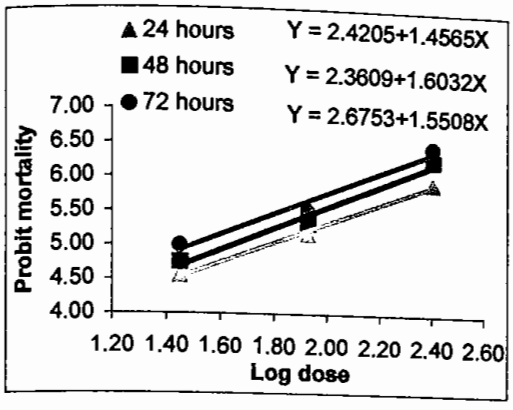


Fig-72 Probit regression line for the mortality of 1st instar larvae of *T.castaneum* treated with petroleum ether extract after 24, 48 and 72 hours.

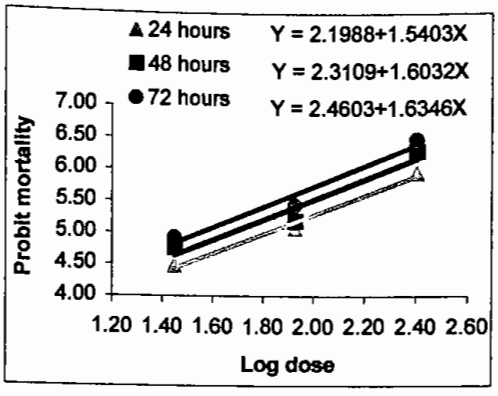


Fig-73 Probit regression line for the mortality of 2nd instar larvae of *T.castaneum* treated with petroleum ether extract after 24, 48 and 72 hours.

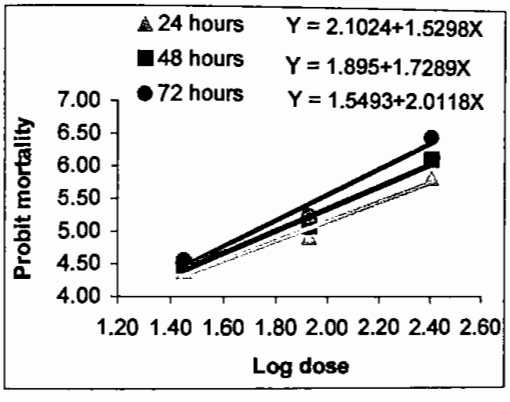


Fig-74 Probit regression line for the mortality of 3rd instar larvae of *T.castaneum* treated with petroleum ether extract after 24, 48 and 72 hours.

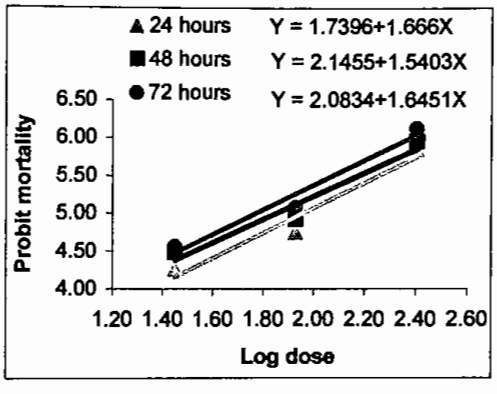


Fig-75 Probit regression line for the mortality of 4th instar larvae of *T.castaneum* treated with petroleum ether extract after 24, 48 and 72 hours.

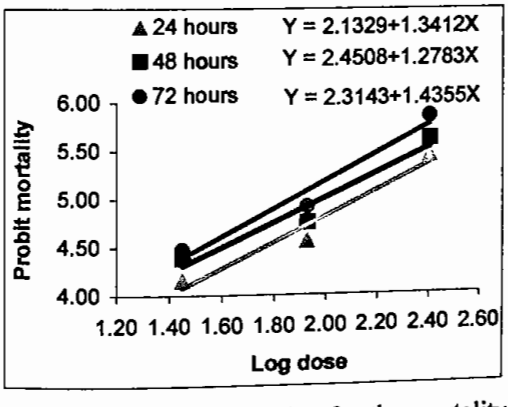


Fig-76 Probit regression line for the mortality of 5th instar larvae of *T.castaneum* treated with petroleum ether extract after 24, 48 and 72 hours.

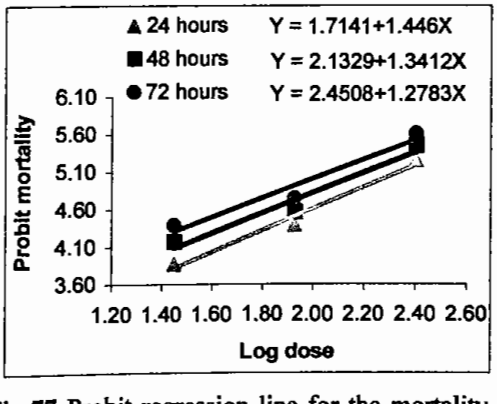


Fig-77 Probit regression line for the mortality of 6th instar larvae of *T.castaneum* treated with petroleum ether extract after 24, 48 and 72 hours.

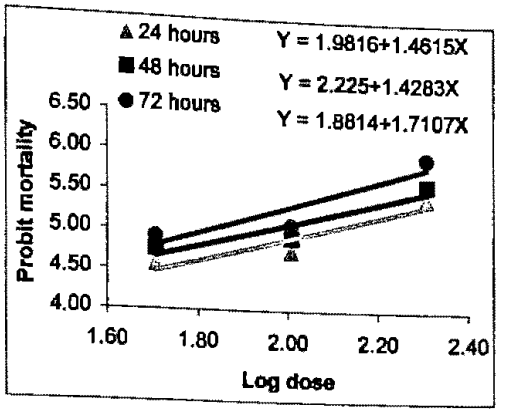


Fig-78 Probit regression line for the mortality of adult of *T.castaneum* treated with ethanol extract after 24, 48 and 72 hours.

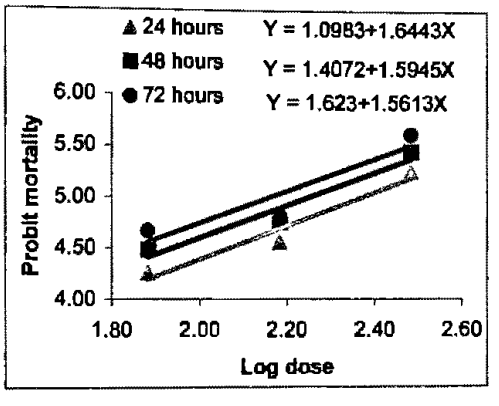


Fig-79 Probit regression line for the mortality of adult of *T.castaneum* treated with chloroform extract after 24, 48 and 72 hours.

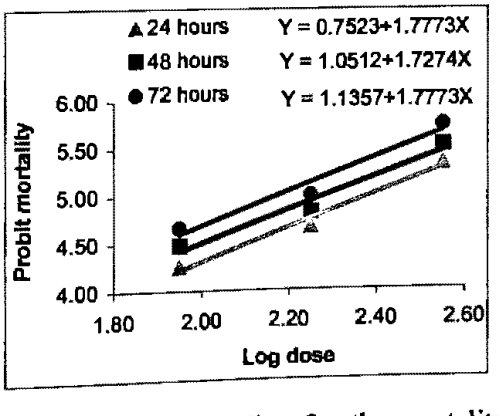


Fig-80 Probit regression line for the mortality of adult of *T. castaneum* treated with petroleum ether extract after 24, 48 and 72 hours.

Larvicidal Activity Test

Experimental

In Bangladesh, altogether 113 species of mosquitoes, including 34 anophelines and 79 Culicines, have so far been recorded (Ahmed, 1987). It belongs to the order Diptera, and is the members of a single family Culicidae. There are more than 3,268 species of mosquitoes, classified in 34 genera (Knight, 1978). Among the genus, *Culex quinquefasciatus* Say is widely distributed in urban and rural areas in Bangladesh and are predominant throughout the year. They grow abundantly in damp marshy places all over the country, especially in the drain water in urban area (Hossain *et.al.*, 1998).

C. quinquefasciatus is a potential vectors of human lymphatic filariasis in Bangladesh and all over the globe (Ahmed, 1994). Recently it has been found in Mirpur area in Dhaka City (Ahmed *et. al.*, 1986). But in recent years, mosquito control programmed has been suffering from failures because of the ever-increasing insecticide resistance. In Bangladesh, *C. quinquefasciatus* is totally resistant to diazinon, fenitrothion, malathion, and primiphos-methyl and DDT (WHO, 1992).

Plant products or plant-derived compounds were promising alternatives to synthetic insecticides in controlling insect pests (Rahuman *et al.*, 2000). In view of these the fruits of *Duranta repens* Linn. was chosen on the basis of their previously documented poisonous or other bioactivities and used for elucidating their insecticidal potentiality against *C. quinquefasciatus* under laboratory conditions.

Initial Stock Collection

A large number of pupae were collected from the pesticide free, breeding grounds in the villages adjunct to the Rajshahi University Campus with the use of long handled dipper fry pan. After collection they were cleaned with distilled water and kept in a number of 500 ml glass beakers each containing approximately 300 ml distilled water and finally, these beakers were taken to the laboratory for raising healthy mosquito colonies.

Adult Rearing

Pupae were placed inside a standard mosquito cage (12 inch square and 9 inch deep). After emergence, adults were normally fed on an artificial diet (10% sucrose solution) soaked in cotton wool circle placed on petridishes. During the pre-oviposition period (2-3 days after emergence) the female mosquitoes inside the cages were fed, in addition to sucrose solution, on blood meals from domestic chickens, twice a week before egg laying because the blood meals provide them with nutrients which are necessary for eggs maturation (Snow, 1990). During blood feeding, a healthy chicken with tied wings, legs and feathers removed from the abdomen were kept in the rearing cage in a dark place at day time or night for 3-5 hours. The oviposition of females took place within 2-3 days of blood meal. The females laid eggs in rafts on the surface of water in glass beakers. After oviposition the number of eggs per raft was recorded and each raft was introduced separately into glass containing water for hatching.

Larval Rearing

To ensure a constant supply of test larvae different instars, viz. 1^{st}, 2^{nd}, 3^{rd} and 4^{th} for insecticidal bioassays they were reared after hatching using standardized rearing methods, partially modified from Gerberg (1970). The larvae were supplied with appropriate amounts of powdered dry yeast (Red Star®) with sucrose granules at a ratio 1:3, which dissolved in rearing water. While feeding the larvae, the amount of dry yeast was regulated with utmost care since an excessive use of yeast without renewing the water daily may from a thin film of yeast on water surface and cause natural mortality (Bar-Zeev, 1959). Moreover, to avoid overcrowding the larvae were transferred to separate beakers, limiting the numbers to approximate 150-200 in each beaker. This ensured healthy individuals for bioassay experiments. The larval instars were determined by simply observing the length of exuviae or counting the number of exuviae floating on the surface of water. Mosquito colonies were maintained in the laboratory at a room mean temperature of $27\pm 1^{\circ}C$, 40-60% relative humidity and a 12:12 h light: dark photoperiod.

Preparation of Sample

The main stock solutions were prepared by dissolving 10 mg of ethanol, 20 mg of chloroform and 50 mg of petroleum ether in 1 ml of DMSO to get concentrations of 10000 µg/ml, 20000 µg/ml and 50000 µg/ml, respectively.

Bioassay Procedure

The larvicidal effect of fruit extracts of *Duranta repens* Linn. were determined by the WHO standard procedure (WHO, 1960) using *C. quinquefasciatus* as the test subject under laboratory conditions. Twenty-five laboratory reared 1^{st}, 2^{nd}, 3^{rd}, and 4^{th} instar larvae were dipped into 100 ml glass beakers, containing 50ml of distilled water to which the required volumes of stock solutions were added using micro-pipettes to get the desired test concentrations (w/v) of the extracts. Three replications were made for each concentration of the test products and three types of control were maintained: one of distilled water alone; one of distilled water plus solvent (DMSO) and the last one of distilled water plus food medium. Each replication used 25 larvae. Control insects were similarly raised. The experiment was performed under laboratory conditions at 27± 1°C and 40-60 % relative humidity. Granular dry yeast was supplied as a larval food during the test period.

Analysis of Data

For toxicity tests, the cumulative mortality data were corrected by Abbott's (1925) formula. Data were then subjected to Probit analysis (Busvine, 1971) for the determination of LC_{50} values.

Results and Discussion

The dose-mortality effect of ethanol, chloroform and petroleum ether extracts of fruits of *Duranta repens* Linn. were performed against 1^{st} ,2^{nd} ,3^{rd} and 4^{th} instar larvae of *C. quinquefasciatus*. From the **Table-24** and **Fig-81** to **92,** it was clear that all the crude extracts were effective against mosquito larvae. Mortality percentages were directly proportional to the level of concentration and time after treatment. The Probit regression line showed that the larvae mortality was positively co-related with concentration and

time in all the cases. Among the tested extracts, the ethanol extract showed the highest toxicity and consequently, the lowest LC_{50} values (8.51 $\mu g/cm^2$, 12.17 $\mu g/cm^2$, 14.37 $\mu g/cm^2$ and 19.70 $\mu g/cm^2$) in all instars. On the other hand, the LC_{50} values of chloroform extract and petroleum ether extract were 37.78 $\mu g/cm^2$, 42.53 $\mu g/cm^2$, 48.83 $\mu g/cm^2$ and 80.69 $\mu g/cm^2$ and 61.63 $\mu g/cm^2$, 124.28 $\mu g/cm^2$, 161.11 $\mu g/cm^2$ and 268.94 $\mu g/cm^2$, respectively. With an increase in exposure time, the LC_{50} values of extracts decreased in all the instars tested. The increase in mortality with increase in exposure period could be due to several factors, which may be acting separately or jointly. For example, the uptake of the active moiety of the compound could be time dependent, leading to a progressive increase in the titre of the plant-derived compounds tested and its effect on the larval body. The results obtained in the present investigation, proved that the ethanol extract contain potential bioactive principles, which gave promising effects, than the other extracts tested.

Table-24 Larvicidal activity of fruit extracts of *D. repens* against *Culex. quinquefasciatus* larvae.

Plant Extracts	larval stage	Exposure time (h)	LC_{50} $\mu g/cm^2$
Ethanol	1st instar	3	30.03
		6	14.05
		12	8.51
	2nd instar	6	51.84
		12	22.53
		24	12.17
	3rd instar	12	38.90
		24	14.37
	4th instar	12	72.79
		24	19.70
Chloroform	1st instar	6	90.81
		12	59.04
		24	37.78
	2nd instar	12	88.26
		24	70.76
		48	42.53
	3rd instar	24	121.26
		48	48.83
	4th instar	24	154.53
		48	80.69
Petroleum ether	1st instar	12	245.58
		24	103.82
		48	61.63
	2nd instar	24	325.85
		48	124.28
	3rd instar	24	492.88
		48	161.11
	4th instar	24	858.05
		48	268.94

Values were based on three concentrations with 25 insects each.
Control groups showed no mortality.

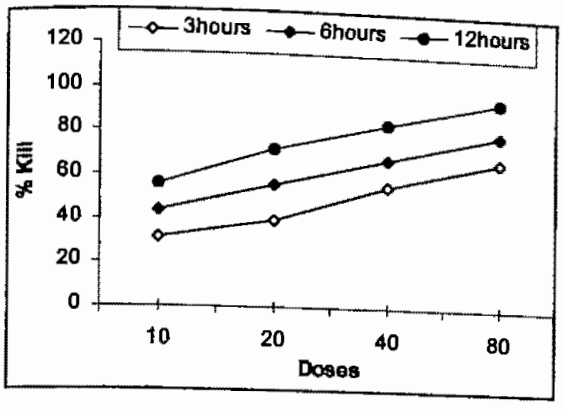


Fig-81 Effect of ethanol fruit extracts of *Duranta repens* on the mortality of 1st instar larvae of *C.quinquefasciatus* after 3, 6 and 12 hours.

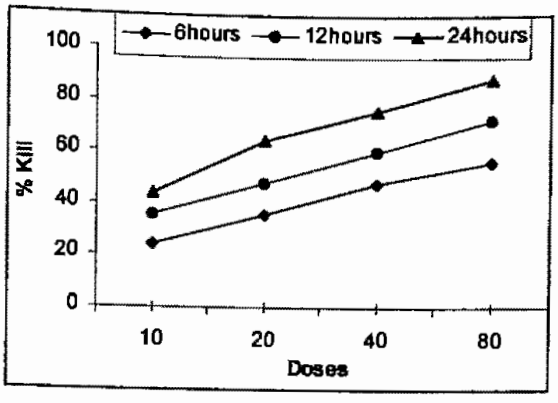


Fig-82 Effect of ethanol fruit extracts of *Duranta repens* on the mortality of 2nd instar larvae of *C.quinquefasciatus* after 6, 12 and 24 hours.

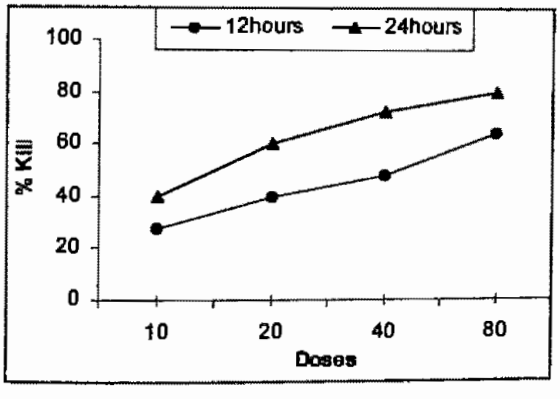


Fig-83 Effect of ethanol fruit extracts of *Duranta repens* on the mortality of 3rd instar larvae of *C.quinquefasciatus* after 12 and 24 hours.

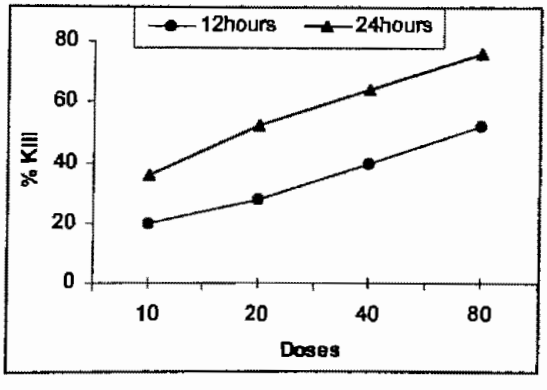


Fig-84 Effect of ethanol fruit extracts of *Duranta repens* on the mortality of 4th instar larvae of *C.quinquefasciatus* after 12 and 24 hours.

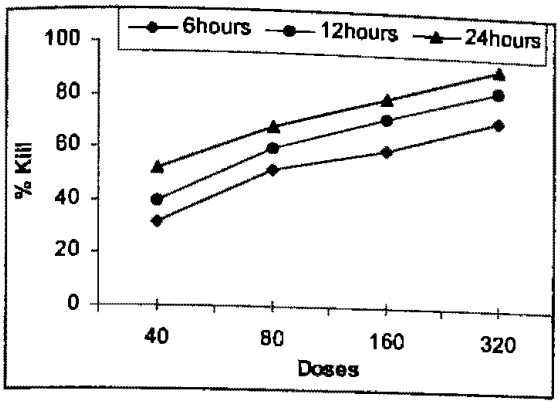


Fig-85 Effect of chloroform fruit extracts of *Duranta repens* on the mortality of 1st instar larvae of *C.quinquefasciatus* after 6, 12 and 24 hours.

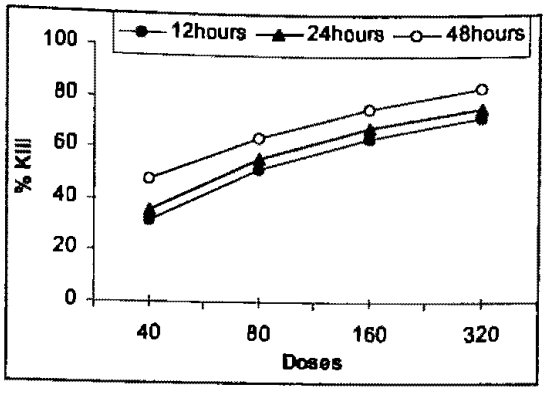


Fig-86 Effect of chloroform fruit extracts of *Duranta repens* on the mortality of 2nd instar larvae of *C.quinquefasciatus* after 12, 24 and 48 hours.

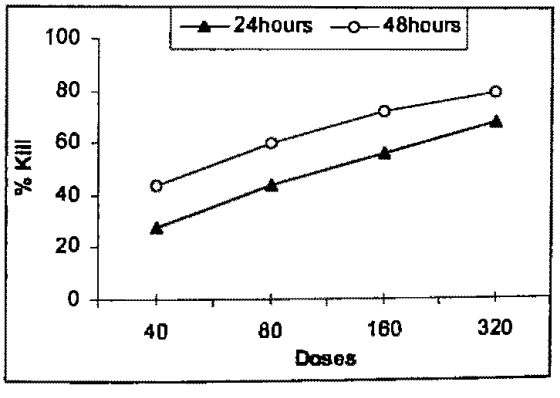


Fig-87 Effect of chloroform fruit extracts of *Duranta repens* on the mortality of 3rd instar larvae of *C.quinquefasciatus* after 24 and 48 hours.

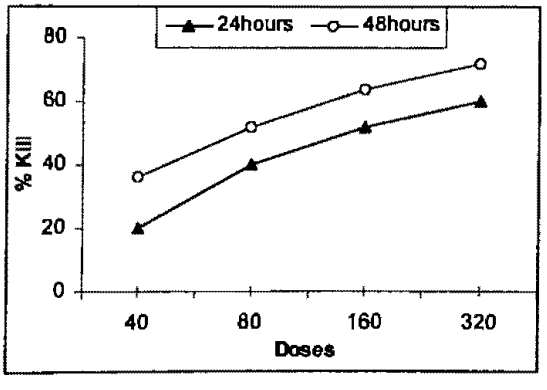


Fig-88 Effect of chloroform fruit extracts of *Duranta repens* on the mortality of 4th instar larvae of *C.quinquefasciatus* after 24 and 48 hours.

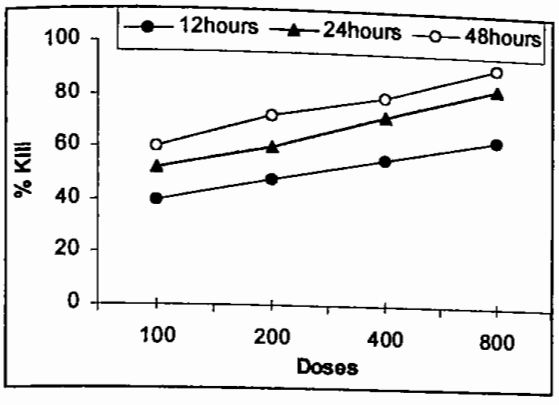


Fig-89 Effect of petroleum ether fruit extracts of *Duranta repens* on the mortality of 1st instar larvae of *C.quinquefasciatus* after 12, 24 and 48 hours.

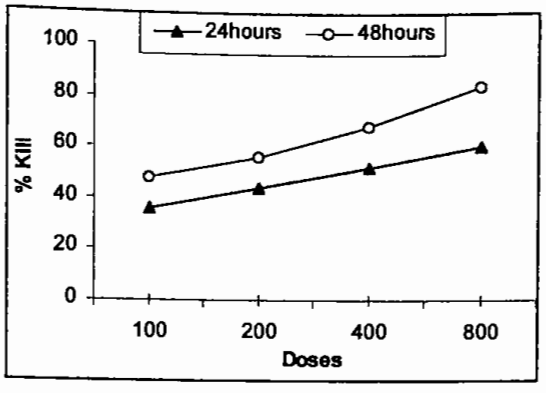


Fig-90 Effect of petroleum ether fruit extracts of *Duranta repens* on the mortality of 2nd instar larvae of *C.quinquefasciatus* after 24 and 48 hours.

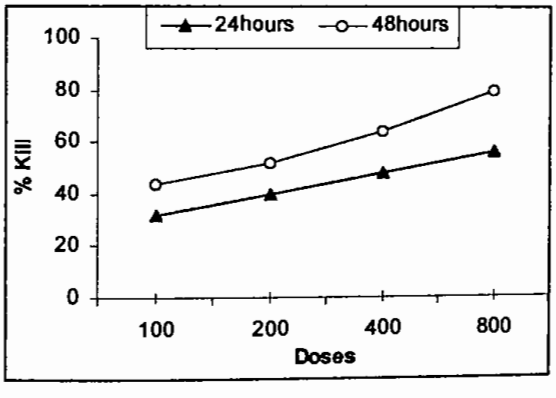


Fig-91 Effect of petroleum ether fruit extracts of *Duranta repens* on the mortality of 3rd instar larvae of *C.quinquefasciatus* after 24 and 48 hours.

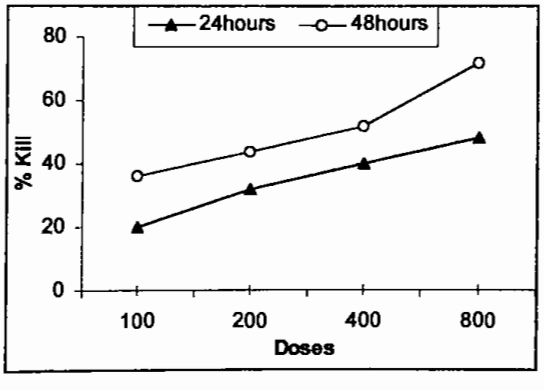


Fig-92 Effect of petroleum ether fruit extracts of *Duranta repens* on the mortality of 4th instar larvae of *C.quinquefasciatus* after 24 and 48 hours.

Larvicidal Activity Test of Fresh Fruit

Experimental

After collections the fresh fruits (950 gm) of *Duranta repens* Linn., the plant materials were washed gently with tap water to avoid foreign particles. Fresh fruits were then crushed in a mortar and pestle, filtered through Whatman no. 1 filter paper and thus obtained an orange color clear juice (200 ml). Then 1%, 2%, 4%, 10%, 25% of test solutions (v/v) were prepared by distilled water.

Bioassay Procedure

The larvicidal effect of fresh fruits of *D. repens* Linn. were determined by the method of Carvalho *et al.*, (2003). 25 ml of 1%, 2%, 4%, 10%, 25% and 100% of test solutions were taken into 100 ml of glass beakers. Twenty laboratory reared 1^{st}, 2^{nd}, 3^{rd} and 4^{th} instar larvae were placed on a filter paper separately and allowed to stand for few minutes. After the removal of water, the larvae were collected with a fine pointed camel hair brush and transferred into glass beakers. The experiment was performed under laboratory conditions at 27± 1°C and 40-60 % relative humidity. Finally for each concentration, 50% and 100% of mortality were detected and recorded by timing.

Results and Discussion

Effect of 50% and 100% mortality of fruit juice of *Duranta repens* Linn. on 1^{st}, 2^{nd}, 3^{rd} and 4^{th} instar larvae of *Culex quinquefasciatus* were presented in **Table-25** and **Fig-93** to **96**. From the table it is evident that, with an increase in concentration of juice, the percentage of mortality depend on duration of time. The mortality rate also depends on instar of larvae. At lower (1%) concentration, 100% mortality of 1^{st} instar larvae occurs at 15 hours, where as at higher (100%) concentration, it occurs at only 35 minutes. On the other hand, the duration of time for 100% mortality at lower concentration for 2^{nd}, 3^{rd} and 4^{th} instar larvae were 24 hours, 26 hours and 28 hours, respectively. But at higher concentration, 100% mortality occurs after 1.30 hour, 1.45 hour and 2 hours for 2^{nd}, 3^{rd} and 4^{th} instar larvae, respectively. The uptake of the active moiety of the compound could be time dependent, leading to a progressive increase in the titre of the plant-derived compounds tested and its effect on the larval body. The above results, was very much promising. However, more comprehensive experiments were solicited in the future and if possible to isolate their bioactive principle and produce their active analogues synthetically, which might prove more effective and more economical.

Table-25 Effect of fruit juice of *Duranta repens* Linn. on *Culex quinquefasciatus* larvae at different instars.

Dose	Amount	No. of Larvae	Larval instar	% of mortality	Time
1%	25 ml	20	1st instar	50% 100%	9 hours 15 hours
2%	25 ml	20	1st instar	50% 100%	7 hours 13 hours
4%	25 ml	20	1st instar	50% 100%	6 hours 11 hours
10%	25 ml	20	1st instar	50 % 100%	3 hour 6 hours
25%	25 ml	20	1st instar	50% 100%	1 hour 3 hour
100%	25 ml	20	1st instar	50 % 100%	15 min 35 min.
1%	25 ml	20	2nd instar	50% 100%	11 hours 24 hours
2%	25 ml	20	2nd instar	50% 100%	8 hours 21 hours
4%	25 ml	20	2nd instar	50% 100%	6 hours 19 hours
10%	25 ml	20	2nd instar	50 % 100%	4 hour 11 hours
25%	25 ml	20	2nd instar	50% 100%	2 hour 6 hour
100%	25 ml	20	2nd instar	50 % 100%	20 min 1.30 hours.
1%	25 ml	20	3rd instar	50% 100%	12 hours 26 hours
2%	25 ml	20	3rd instar	50% 100%	8-9 hours 24 hours
4%	25 ml	20	3rd instar	50% 100%	7-8 hours 20 hours
10%	25 ml	20	3rd instar	50 % 100%	5 hour 12 hours
25%	25 ml	20	3rd instar	50% 100%	3 hour 8 hour
100%	25 ml	20	3rd instar	50 % 100%	25 min 1.45 hours.
1%	25 ml	20	4th instar	50% 100%	12-13 hour 28 hour
2%	25 ml	20	4th instar	50% 100%	12 hour 27 hour
4%	25 ml	20	4th instar	50% 100%	10 hour 24 hour
10%	25 ml	20	4th instar	50% 100%	6 hour 15 hour
25%	25 ml	20	4th instar	50% 100%	4 hour 9.30 hour
100%	25 ml	20	4th instar	50 % 100%	35 min 2 hours

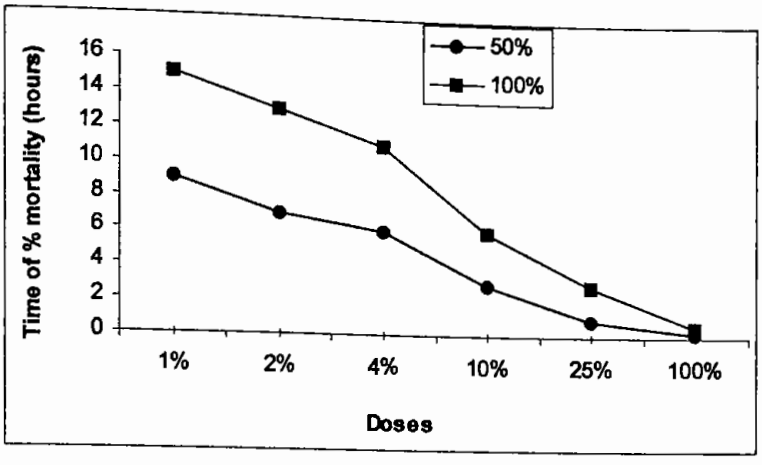


Fig-93 Effect of 50% and 100% mortality of fruit juice of *Duranta repens* against 1st instar larvae of *C. quenquifasciatus.*

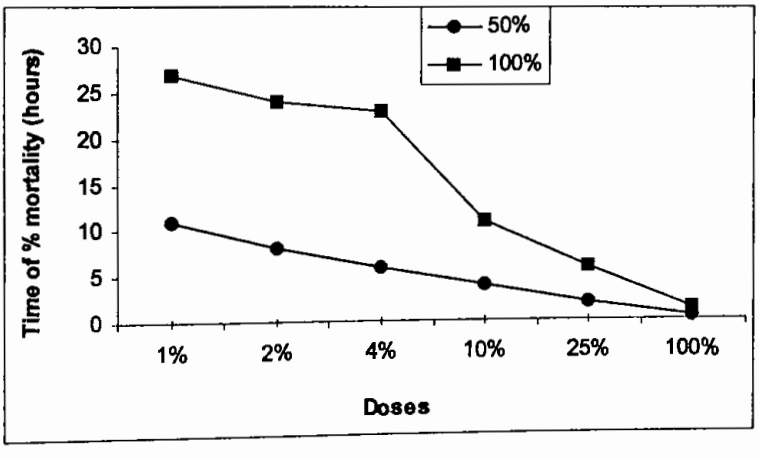


Fig-94 Effect of 50% and 100% mortality of fruit juice of *Duranta repens* against 2nd instar larvae of *C. quenquifasciatus.*

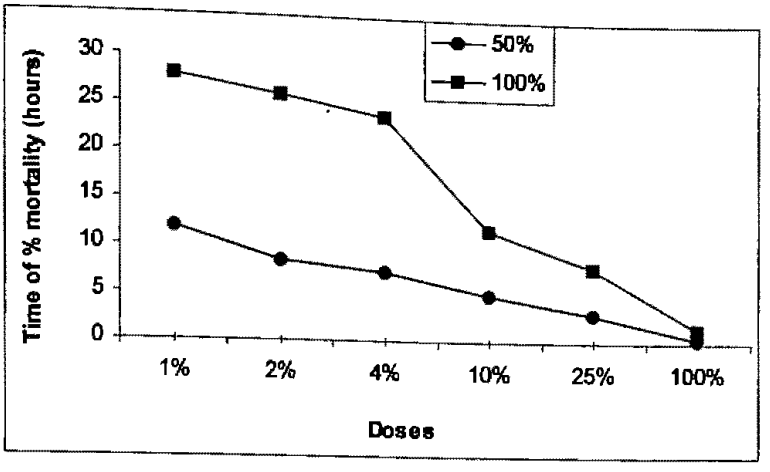


Fig-95 Effect of 50% and 100% mortality of fruit juice of *Duranta repens* against 3rd instar larvae of *C. quenquifasciatus.*

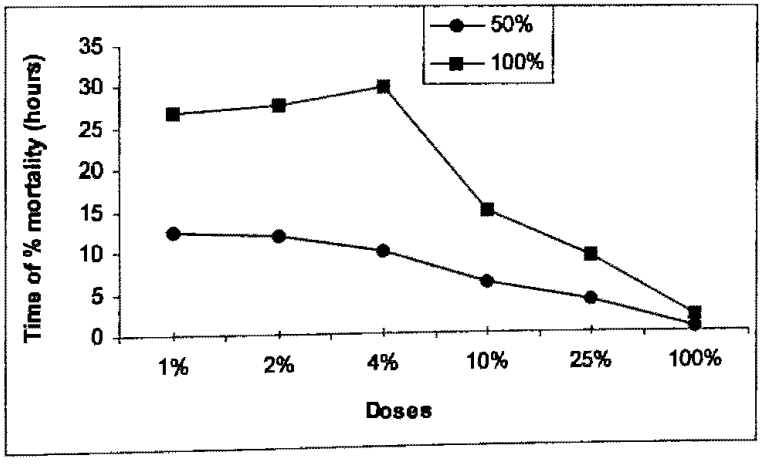


Fig-96 Effect of 50% and 100% mortality of fruit juice of *Duranta repens* against 4th instar larvae of *C. quenquifasciatus.*

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Appendix

Statistical Calculation

The results of study of biochemical parameters (toxicology), pharmacological tests have been expressed in terms of Mean ± Standard deviation ($\bar{X} \pm$ SD) and the success or failure was confirmed by the test of significance at different levels.

Mean or Average ($\bar{X}$)

$$\bar{X} = \frac{\sum x_i}{n}$$

X_i = Individual values (observations) and N= Number of observations.

Standard Deviation (SD)

$$SD = \sqrt{\frac{\sum (Xi - \bar{X})^2}{n-1}}$$

$\sum (X_i - \bar{X})^2$ = Summation of squares of individual deviations from the mean values.

n = Number of observations.

Significance of Difference (t)

The significance of difference, 't' between two means was calculated by using the following formula:

$$t = \frac{\bar{X}_1 - \bar{X}_2}{SD}$$

X_1 and X_2 = Means of two separate sets of experiments

Sd = Standard error of two means taken together.

Standard Error (Sd)

The standard error of two means obtained from separate sets of similar experiments were calculated from the formula:

$$Sd = \sqrt{\frac{(SD_1)^2}{n_1} + \frac{(SD_2)^2}{n_2}}$$

Degree of Freedom (DF)

$DF = n_1 + n_2 - 2$

n_1 and n_2 are number of two sets of observations.

List of Publications

1. Farjana Nikkon, Zahangir Alam Saud, M. E. Haque, G. Karagianis, M. A. Mosaddik; Isolation of Aglycone of Deoxy-niazimicin from *Moringa oleifera* Lam. and its Cytotoxicity; *Revista Latinoamericana De Quimica*; **31 (1)**, 5-9, 2003.

2. Farjana Nikkon, Zahangir Alam Saud, M. Habibur Rahman and Md. Ekramul Haque; *In vitro* Antimicrobial Activity of the Compound Isolated from Chloroform Extract of *Moringa oleifera* Lam.; *Pakistan Journal of Biological Sciences*, **6 (22)**, 1888-90, 2003.

3. Farjana Nikkon, M. Ashik Mosaddik and M. Ekramul Haque; Antimicrobial Activity and Cytotoxicity of *Duranta repens* Linn.; *Fitoterapia*, 2004 (Submitted).

4. Farjana Nikkon, M. Ashik Mosaddik and M. Ekramul Haque; Pesticidal Evaluation of Stem and Fruit Extracts of *Duranta repens* Linn. Against *Tribolium castaneum* (Herbst); *Insect Science and its Application*, 2005 (Submitted).