Isolation, Purification and Identification of Bio-Active Components with Potential Pesticidal Properties from Plant Extracts and Their Interaction with Transition Metal Ions

A Thesis submitted
in partial fulfilment of the requirements for the Degree of

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In

Chemistry

By

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THESIS CERTIFICATE

This is to certify that the research thesis entitled ***Isolation, Purification and Identification of Bio-Active Components with Potential Pesticidal Properties from Plant Extracts and Their Interaction with Transition Metal Ions*** submitted by ***Samuel Lallianrawna*** to Mizoram University, Tanhril, Aizawl, for the award of the degree of Doctor of Philosophy is a bonafide record of research work carried out by him under my supervision. The contents of this thesis, in full or in parts, have not been submitted to any other Institute or University for the award of any degree or diploma.

Aizawl 796 004
Date: 12. 12. 2013

Dr. Muthukumaran, R.
Research Supervisor

Declaration of the Candidate

I, Samuel Lallianrawna, a Ph.D. Scholar in Department of Chemistry, Mizoram University, Aizawl, do hereby solemnly declare that the subject matter of my thesis entitled **'ISOLATION, PURIFICATION AND IDENTIFICATION OF BIO-ACTIVE COMPONENTS WITH POTENTIAL PESTICIDAL PROPERTIES FROM PLANT EXTRACTS AND THEIR INTERACTION WITH TRANSITION METAL IONS'** is the bonafide record of the work done by me during my Ph.D. programme. I have duly worked on my Ph.D. thesis under the supervision of Dr. Muthukumaran, R., Associate Professor & Head, Department of Chemistry, Mizoram University, Aizawl. This is being submitted to the Mizoram University for the degree of Doctor of Philosophy in Chemistry and that I have not submitted this work to any other University or Institute for any other degree.

Dated: 13th December, 2013 **(SAMUEL LALLIANRAWNA)**

Candidate

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Samuel Lallianrawna

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ABBREVIATIONS

AA – *Ageratina adenophora*

ACN – Acetonitrile

ANOVA – Analysis of Variance

BHA – Butylated Hydroxyanisole

***d*-DMSO** – Deuterated Dimethyl Sulphoxide

DDT – Dichlorodiphenyltrichloroethane.

DPPH – 2,2-diphenyl-1-picrylhydrazyl

EG1 & EG2 – *Eupatorium glandulosum* 1 & Eupatorium *glandulosum* 2

EPA – Environment Protection Agency

EtOAc – Ethyl acetate

EtOH – Ethanol

FCR – Folin-Ciocalteu Reagent

FDA – Food and Drug administration

FT-IR – Fourier Transform Infra Red

GAE – Gallic Acid Equivalent

HMIS – Health Management Information Statistics

HOMO – Highest Occupied Molecular Orbital

HPLC – High Performance Liquid Chromatography

HPTLC – High Performance Thin Layer Chromatography

IC – Inhibition Concentration

IC1 & IC2 – *Ipomoea cairica* 1 & *Ipomoea cairica* 2

ICF – *Ipomoea cairica* Flowers

ICL – *Ipomoea cairica* Leaves

ICMR – Indian Council of Medical Research

LC – Lethal Concentration

LT – Lethal Time

LUMO – Lowest Unoccupied Molecular Orbital

MeOH – Methanol

MDA – Mass Drug Administration

MHz – Mega Hertz

NAPRALERT – Natural Product Alert

NHRM – National Rural Health Mission

NMR – Nuclear Magnetic Resonance

NTD – Neglected Tropical Disease

PDA – Photo Diode Array

ppm – Parts per million

QE – Quercetin Equivalents

ROS – Reactive Oxygen Species

RP-LC – Reversed Phase Liquid Chromatography

S.E. – Standard Error

SVBDCP – State Vector Borne Disease Control Programme

VLC – Vacuum Liquid Chromatography

UV-Vis – Ultra Violet/visible

CHAPTER 1

INTRODUCTION

1.1. Deforestation and Insect Pest Problem in Mizoram

Mizoram is one of the rich biodiversity hotspots in the eastern Himalayan region of India whose vegetation, according to the proposed classification, is tropical evergreen and semi-evergreen forest in the lower altitude hills; sub-tropical to mundane sub-tropical in the high hills (Rai, 2009). The total forest area is 15,825 km^2. Forests area spread around 500 to 2,157 m in altitude. However, these forests are exposed to various anthropogenic disturbances, e.g., shifting cultivation (largest factor), timber logging, extraction of fuel wood by poor/rural people, hunting and over-exploitation of resources, introduction of exotic species in river ecosystems, poisoning and other factors including progressive disempowerment of local communities, population growth and destabilization of Mizo traditional management systems and in fact urbanization in some parts of Mizoram like Aizawl (the state Capital). The loss of biodiversity, which have remain undetected or severely under-studied results in extinction and/or threatening of certain sub-species and varieties, while at the same time serves as a potential factor for resurgence of certain insect pests. As a result of environmental imbalance among the species, Mizoram is infested with insects/pests and the need to control these pests/insects is a real concern. As a result, use of cheap, safe, environmental friendly and biodegradable insecticides and/or pesticides is of utmost importance to the people living in the area as agents for vector control and pest management programme.

Currently, a variety of synthetic chemicals have been used extensively as pesticides by the farmers and rural people of Mizoram for protecting their agricultural crops and household purposes. These chemicals have been supplied and/or subsidized by the concerned departments,

i.e., the Department of Agriculture and the Department of Horticulture, Government of Mizoram to farmers and rural people.

Malaria is one of the major public health problems in Mizoram, transmitted by mosquito species, in a vigorous manner during monsoon seasons. It has been reported that out of 10.32 lakhs population, 29 deaths were reported during 2011, about 94.47% being due to *Plasmodium falciparam*. Among the 25 species of *anophelines* identified in Mizoram by taxonomic characters (Rita *et al*., 2009), *Anopheles dirus* and *Anopheles minimus* have been implicated as the primary malaria vectors in NE India, while an increasing number of other Anopheles species (NRHM HMIS report, 2009; Bhattacharyya *et al.*, 2010) have been reported as incriminated vectors. However, there is no specific data of cases and deaths reported due to other vector borne diseases such as dengue, filariasis, etc. About 80 to 100 metric tons of DDT (**Fig. 1.1**) is being used annually under SVBDCP (Mizoram Health & Family Welfare Department, 2012). It is interesting to note that these synthetic chemicals are administered over household and agricultural lands without proper precautionary measures and the people are not well informed of the potential short term and long term risks that may results from the usage of these deleterious pesticides.

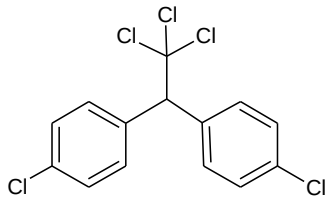


Figure 1.1. Dichlorodiphenyltrichloroethane (DDT)

1.2. Chemical Pesticides and their Environmental Impact

According to the British Pharmaceutical Codex (1979), pesticides are chemical substances used to destroy organisms that are nuisance or cause injury to crops, stored produce,

livestock or man. Most pesticides are applied to protect plant crops grown for food and clothing. Such pesticides include insecticides, fungicides, herbicides and rodenticides. In most cases, pesticides are subjected to legal control and any stability regulations covering such matters are manufacture, distribution, storage, labeling and used must be duly compiled with (British Crop Protection Control, 1972). Insecticides include substances that kill insects or related forms of animal life such as mites (Chukwu, 2001). Insecticides may be classified on the basis of their mode of action into stomach poisons, contact poisons, fumigants, ovicides and systemic insecticides. On the basis of their chemical types, they may be further classified as organochlorine compounds, organophosphorous compounds, carbamates, dinitro compounds, naturally occurring organic compounds and inorganic compounds (Burgess, 1990).

Chemical pesticides are now employed by practically all farmers to control a variety of pest organisms. These pesticides eventually end up with the produce that we consume and many people are concerned about the risks associated with their ingestion. Certain pesticides, particularly those based on organophosphates, are also toxic to humans. Furthermore, most synthetic chemicals that have been commercialized as herbicides are halogenated hydrocarbons with relatively long environmental half-lives and more suspicious toxicological properties than most natural compounds. The impact of synthetic pesticides on the environment is often greater than intended by those who used them. Over 98% of sprayed insecticides and 95% of herbicides reach a destination other than their target species, including non-target species, air, water, bottom sediments and food (Miller, 2004). Though there can be potential benefits using pesticides, inappropriate usage can counterproductively increase pest resistance and kill the natural enemies of pests. Many users are inadequately informed about potential short and long term risks, and the necessary precautions in the correct application of such toxic chemicals are not always followed

(Damalas *et al.*, 2011). Pesticides can contaminate unintended land and water bodies when they are sprayed aerially or allowed to run off fields, or when they drift away from production sites and storage tanks or are inappropriately discarded (Tashkent, 1998).

It is reported that some synthetic pesticides even contributed to the global warming leading to the depletion of ozone layer. Pesticide drift occur when pesticide suspended in the air as particles are carried by wind to other areas, potentially contaminating them (Reynolds, 1997). Pesticides that are applied to crops can volatilize and may be blown by winds into nearby areas, potentially posing a threat to wildlife (National Park Service, 2006).

Nitrogen fixation, which is required for the growth of higher plants, is hindered by pesticides in soil. The insecticides DDT, methyl parathion and especially pentachlorophenol have been shown to interfere with legume-rhizobium chemical signaling. Reduction of this symbiotic chemical signaling result in reduced Nitrogen fixation and thus reduced crop yields (Rockets & Rusty, 2007).

It has also been reported that widespread application of chemical pesticides can eliminate food sources needed by certain types of animals, causing the animals to relocate, change their diet or even starve. Some pesticides can bioaccumulate, or built up to toxic levels in the fat tissues of bodies of organisms that consume them over time, a phenomenon that impacts species high on the food chain especially hard (Cornell University, 2007).

In recent years, vector control programme have failed because of the ever increasing insecticide resistance developed by the target mosquitoes (WHO, 1992). It has also resulted in the development of resistance, an undesirable effect on non-target organisms and forested environmental and human health concern (Brown, 1986), which initiated a search for alternative

control measures. Recent studies indicated that there are now over 500 insect and mite species resistant to synthetic pesticides (Mann & Kaufman, 2012).

1.3. Biopesticides for the Control of Insects and Pests

Plants have always been a rich source of chemicals and drug/lead compounds for man. During the 20th century, a few of these natural compounds like nicotine, rotenone and pyrethrins have been used commercially as insecticides. Probably one of the most successful application of a plant based product as an insecticide is that of the pyrethroids. However, plants also produce thousands of other secondary metabolite compounds that are insecticidal and exhibit diverse modes of action like hormonal, neurological, nutritional or enzymatic (Rosenthal *et al.*, 1979). One of the most commonly studied plant for control of mosquitoes is *Azadirachta indica*, (Meliaceae) commonly known as Neem in India. Neem contains at least 35 biologically active principles, (Mulla *et al*, 1999) of which azadirachtin (AZA), a triterpenoid is the predominant insecticidal active ingredient expressed in the seeds, leaves, and other parts of the tree. Neem products containing azadirachtin and other ingredients possess antifeedant, ovipositional deterrence, repellency, growth disruption, sterility and larvicidal action against insects (Schmutterer, 1990). Neem based pesticides are now extensively employed for agricultural practice all over the world. Neem oil and other commercial preparations of Neem have been found as potential mosquito larvicide (Mittal *et al.*, 1995).

Insect pest management is facing the economic and ecological challenge worldwide due to the human and environmental hazards caused by majority of the synthetic pesticide chemicals. Though synthetic insecticides can target non-specifically all organisms in the field, and usage of synthetic pesticides are mostly limited in recent times, as most of the target pests have developed resistance towards them, due to their indiscriminate usage. In recent years, use of

environmentally benign and biodegradable natural pesticides of plant origin has received renewed attention as agents for vector control/pest management programme and has increasingly become the focus of those interested in the discovery of pesticides. Natural pesticides are generally pest specific, readily biodegradable and usually lack toxicity towards higher animals (Browers, 1992). More than 2000 plant species have been known to produce chemical factors and metabolites of value in pest control programmes and among these plants, products of some 344 species have been reported to have a variety of activity against mosquitoes (Chansang *et al*., 2005).

Natural pesticides are a cheap and safer alternative to commercial synthetic compounds. Unfortunately, these recipes are being neglected as advertising to use modern chemicals increases. It is still not old fashioned to use natural pesticides – rather it is a smart way of harnessing the natural resources that are readily available. Identification of novel effective pesticides of biological origin is essential to check the upsurging resistance developed by insect pests against chemical pesticides. Abayomi, (1993) and Bisht *et al.* (1994) observed that there is a strong need to investigate the chemical composition of many plants to determine their ability to be employed as fungicides or insecticides. Phytochemicals derived from plant sources can act as larvicide, insect growth regulators, repellent and ovipositor attractant and have different activities as observed by many researchers (Babu *et al.*, 1998; Venketachalam *et al.*, 2001). Many studies on plant extracts against mosquito larvae have been conducted around the world. Extracts or essential oils from plants may be alternative sources of mosquito larval control agents, as they constitute a rich source of bioactive compounds that are biodegradable into non-toxic products and potentially suitable for use in control mosquito larvae. In fact, many

researchers have reported the efficacies of plant extracts of essential oils against mosquito larvae (Sharma *et al*., 2006; Amer and Mehlhorn, 2006).

Study of larvicidal and adult emergence inhibition activity of ethanolic extract of *Centella asiatica* leaves against mosquito *C. quinquefasciatus* suggested the application of the leaf extract of *C. asiatica* as promising larvicide and adult emergence inhibitor against *Culex quinquefasciatus* in small volume aquatic habitats or breeding sites of limited size around dwellings (Rajkumar and Jebanesan, 2005).

Natural products, i.e. phytochemicals isolated from plants, offer a vast, virtually untapped reservoir of chemical compounds with many potential uses. One of these uses is in agriculture to manage pests and/or weeds with less risk than with synthetic compounds that are toxicologically and environmentally undesirable (Zhao-Hui *et al.*, 2010). Tens of thousands of secondary metabolites of plants have been identified and there are estimates that hundreds of thousands of these compounds exist. Some of the important secondary metabolites provide host plant defense against attacking insects as biopesticides in the pest management because they are environmentally benign, bio-degradable and specific to a particular organism and at the same time they pose low risk to non- target organisms, that includes beneficial predators and natural enemies. They also provide effective control against certain insect pests that have developed resistance towards synthetic insecticides as a result of indiscriminate usage of synthetic insecticides either individually or in combination. A study comparing biological pest control and use of pyrethroid insecticides for diamondback moths, a major cabbage *Brassica* family insect pest, showed that the insecticide application created a rebounded pest population due to loss of insect predators, where as the biocontrol did not show the similar effect (Muckenfuss *et al.*, 2005). In addition to their natural bioavailability and biodegradability, biopesticides are easily

marketable under the aegis of organic farming and as a result may fetch a much better commercial value for the agricultural produce. Furthermore, plants may biosynthesize specific phytochemical compounds as inherent resistance against pests that can act as antifeedant, antidigestive, ovicidal compounds. So, it is of interest to learn biocompatible and non-conventional natural pest resistance programme in identifying the potential phytochemicals that deter invertebrate predators and their application as alternative pest management strategies.

1.3.1. The Future of Biopesticides from Plants

The number of options that must be considered in the discovery and development of a natural product as a pesticide is larger than for a synthetic pesticide. Furthermore, the molecular complexity has rendered limited environmental stability and low activities of many biocides from plants, compared to synthetic pesticides. Moreover, advances in chemical and bio-technology are increasing the speed and ease with which man can discover and develop secondary compounds of plants as pesticides. These advances, combined with increasing need and environmental pressure, are greatly increasing in the interest in plant products as pesticides. Furthermore, the resistance acquired by pests and vectors against botanicals have not been reported so far (Jill, 1993; Hotez *et al*., 2004; Madhumathy *et al*., 2007).

However, natural pesticides usually take longer to exert biocidal action than synthetic pesticides. Insects are killed either by contact or by ingestion of the biocide. Some biocides only repel the insects by a strong/specific odour. Since most of the plant based products are not as effective as synthetic ones and do not produce rapid results, their employment for mosquito control on a large scale under epidemic conditions may not be acceptable. However, the application of indigenous plant based products by individual and communities can provide a prophylactic measure for protection against various mosquito- borne diseases. There is an urgent

need for promoting the use of herbal products through community based vector control programme (ICMR Bulletin, 2003).

1.3.2. Secondary Metabolites as a Source of Pesticides/Insecticides

Secondary metabolites, also referred to as phytochemicals or natural products, are organic compounds that are not directly involved in the growth, development, or reproduction of plants. They do not even directly participate in the respiration, translocation, protein synthesis, nutrient assimilation, photosynthesis, or differentiation. They apparently act as defence (against herbivores, microbes, viruses or competing plants) and/or signal compounds (to attract pollinating or seed dispersing animals), as well as protecting the plant from ultraviolet radiation and oxidants (Swain, 1977). Therefore, they represent adaptive characters that have been subjected to natural selection during evolution. This survival requirement for secondary metabolites to have highly diverse biological activities has led plants to accumulate a vast catalogue of compounds. Plant genomes are variously estimated to contain 20,000 – 60,000 genes, and perhaps 15 – 20% of these genes encode enzymes for the secondary metabolism (Kutchan, 2001). Phytochemicals, particularly the plant secondary metabolites can be extracted from various parts of plants (seeds, leaves, flowers, barks, etc.). In fact, these secondary compounds represent a large reservoir of chemical structures with biological activity. This resource is largely untapped for use as pesticides. These compounds tend to be more complex than the primary metabolites and includes pigments, antitumor agents, effectors of ecological competition/symbiosis and molecules of plant chemical defense such as alkaloids, phenolics, terpenoids and steroids. Many natural insecticides have known active control agents for a variety of insect pests. Soejarto and Farnsworth (1989) estimated that out of the 250000 species of flowering plants, only 5000 species had been thoroughly investigated according to

NAPRALERT database, leaving 98% of these species with the potential for phytochemical discovery. Approximately 2500 plants in 247 families had some toxic properties against insects (Heal *et al.*, 1950). Although primary metabolites are present in every living cell, secondary metabolites are expressed occasionally and are not of much significance for primary plant life but are however, widely distributed in plants and play a crucial role. Recently, such compounds have become the subject of increasing interest because of their practical significance for nutritive, medicinal, and cosmetic purposes, as well as their indisputable role in plant stress physiology.

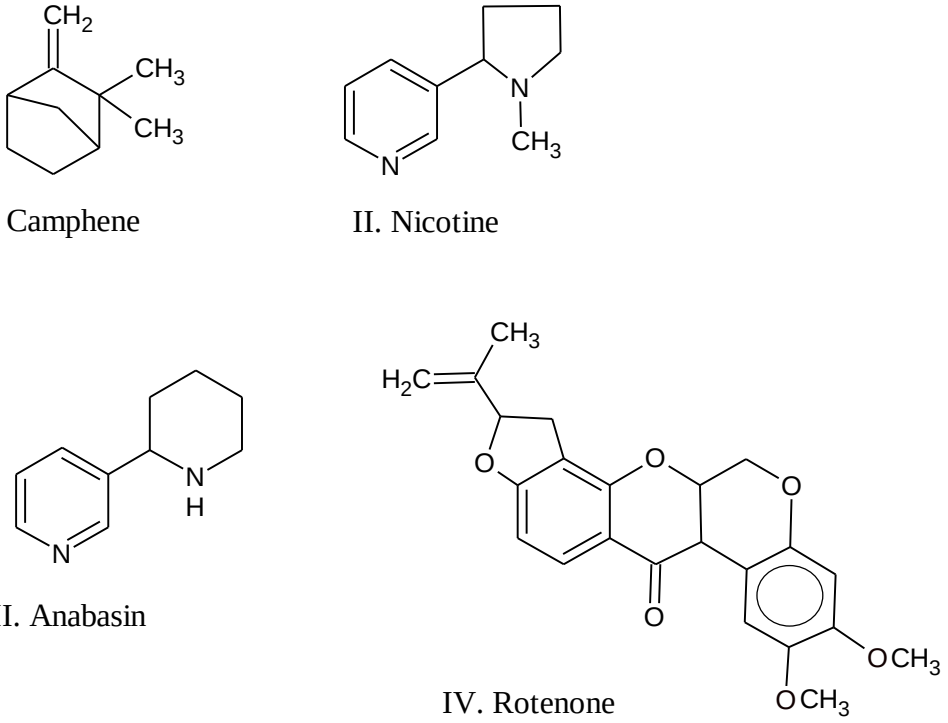
As plants detect an attack, they respond by the expression of toxins (phytochemicals) or enzymes to defend themselves. These are secondary metabolites, only produced when a need arises. Levels of these phytochemicals are modulated by many genotype – independent factors such as light, water, CO_2, oxygen and ozone, nutrient supply, pesticides, levels of diseases and herbivore damage. Once induced, they act as chemical signals to neighbouring plants to begin producing similar compounds. These phytochemicals have received considerable attention owing to their biosynthesis regulation in plants, their health effects in plants/animals and their insecticidal/pesticidal properties.

Essential oils and other major chemical constituents from the leaves of *Mentha spicata* had a significant toxic effect against early third-stage larvae of *C. quinquefasciatus, A. aegypti,* and *A. stephensi* with LC_{50} values of 62.62, 56.08, and 49.71 ppm and LC_{90} values of 118.70, 110.28, and100.99 ppm, respectively (Govindarajan *et al.*, 2011).

When acetone, chloroform, ethyl acetate, hexane and methanol extracts of *A. aspera* and *Ficus racemosa* L. were studied against the early fourth-instar larvae of *Culex quinquefasciatus* Say, all extracts showed modest larvicidal effects. However, the highest larval mortality was

found in the acetone extract of *Ficus racemosa* L. (Rahuman *et al*. 2008). They suggested that gluanol acetate that was present in the extract as quite potent new mosquito larvicidal compound.

Cynogenic glycosides and glucosinolates found in plants are pro-cyanogens proven to be not toxic themselves but are readily broken down to give off poison, some of which are volatile, when the plant is crushed. Hydrogen cyanide is a well-known poisonous gas released by cynogenic glycosides. The presence of cynogenic glycosides deters feeding by insects and other herbivores. Thus, these compounds are believed to be responsible for pest resistance (Ali, 2000). Figure 1.1 lists some plant produced compounds with insecticidal activity.



I. Camphene II. Nicotine

III. Anabasin

IV. Rotenone

Figure 1.2. Some Plant-Produced Compounds with Insecticidal Activity.

1.4. Plant Phenolics

1.4.1. Characteristic Features of Phenolics

Phenolics (**fig. 1.3.**) are aromatic benzene ring compounds with one or more hydroxyl groups produced by plants mainly for protection against stress. They are found throughout the plant

kingdom and are the most abundant secondary metabolites of plants with more than 8,000 phenolic structures currently known, ranging from simple molecules such as phenolic acids to highly polymerized substances such as tannins (Jin and Russel, 2010). Phenolics are uncommon in bacteria, fungi and algae and the class of phenols recorded are few: flavonoids are almost completely absent. Bryophytes are regular producers of polyphenols including flavonoids, but it is in the vascular plants the full range of polyphenol is found (Harborne, 1989; Swain *et al*., 1975). Plant phenolics include phenolics acids, flavonoids, tannins and the less common stilbenes and lignans. Several classes of phenolics have been categorized on the basis of their basic skeleton: C_6 (simple phenol, benzoquinones), C_6–C_1 (phenolic acids), C_6–C_2 (acetophenone, phenylacetic acid), C_6–C_3 (Hydroxycinnamic acids, coumarins, phenylpropanes, chromones), C_6–C_4 (naphthoquinones), C_6–C_1–C_6 (xanthones), C_6-C_2–C_6 (stilbenes, anthraquinones), C_6–C_3–C_6 (flavonoids, isoflavonoids), $(C_6$–$C_3)_2$ (lignans, neolignans), $(C_6$–C_3–$C_6)_2$ (biflavonoids), $(C_6$–$C_3)$n (lignins), $(C_6)_n$ (catechol melanins), $(C_6$–C_3–$C_6)_n$ (condensed tannins) (Harborne *et al*., 1980; Aoki *et al*., 2000; Hattenschwiler *et al*., 2000; Iwashina, 2000; Whiting, 2001; Lattanzio *et al*., 2003).

Flavonoids are the most abundant polyphenols in our diets. The basic flavonoid structure is the flavan nucleus, containing 15 carbon atoms arranged in three rings (C_6-C_3-C_6), which are labeled as A, B and C. Flavonoid are themselves divided into six subgroups: flavones, flavonols, flavanols, flavanones, isoflavones, and anthocyanins, according to the oxidation state of the central C ring. Their structural variation in each subgroup is partly due to the degree and pattern of hydroxylation, methoxylation, prenylation, or glycosylation.

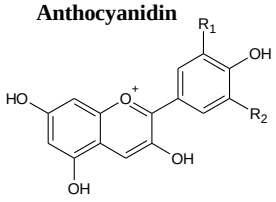
Phenolic acids can be divided into two classes: derivatives of benzoic acid such as gallic acid, and derivatives of cinnamic acid such as coumaric, caffeic and ferulic acid. Caffeic acid is

the most abundant phenolic acid in many fruits and vegetables, most often esterified with quinic acid as in chlorogenic acid, the major phenolic compound in coffee. (D'Archivio *et al*., 2007).

Tannins are another major group of polyphenols in our diets and usually subdivided into two groups: (1) hydrolysable tannins and (2) condensed tannins. Hydrolysable tannins are compounds containing a central core of glucose or another polyol esterified with gallic acid, also called gallotannins, or with hexahydroxydiphenic acid, also called ellagitannins (Khanbabaee and van Ree, 2001). Condensed tannins are oligomers or polymers of flavan-3-ol linked through an interflavan carbon bond. They are also referred to as proanthocyanidins because they are decomposed to anthocyanidins through acid-catalyzed oxidation reaction upon heating in acidic alcohol solutions. The structure diversity is a result of the variation in hydroxylation pattern, stereochemistry at the three chiral centers, the location and type of interflavan linkage, as well as the degree and pattern of methoxylation, glycosylation and galloylation (Koleckar *et al*., 2008).

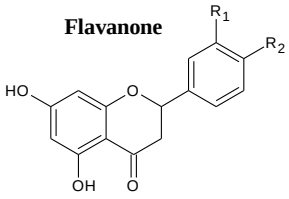
Phenolic metabolism in plants is a complex process resulting from the interaction of, at least, five different pathways. The glycolytic pathway that produces phosphoenolpyruvate; the pentose phosphate pathway that produces erythrose-4-phosphate; the shikimate pathway that synthesizes phenylalanine; the general phenylpropanoid metabolism that results in the activated cinnamic acid derivatives and the plant structural component lignin, and the diverse specific flavonoid pathways (Austin and Noel, 2003). They are but one category of the many secondary metabolites implicated in plant allelopathy.

Anthocyanidin



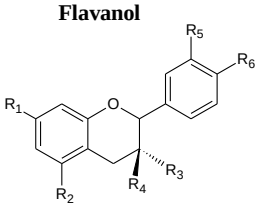
Delphinidin:R_1=R_2=OH
Cyanidin: R_1=OH,R_2=H

Flavanone



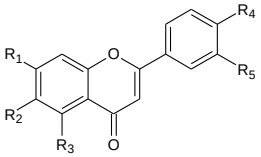
Hesperetin: R_1= OH, R_2= OCH_3
Naringenin: R_1= H, R_2= OH

Flavanol



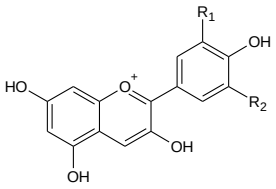
Catechin:R_1=R_2=R_4=R_5=R_6=OH,R_3=H
Epicatechin: R_1=R_2=R_3=R_5=R_6= OH, R_4= H

Flavone



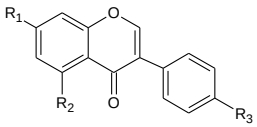
Apigenin: R_1=R_3=R_4= OH, R_2=R_5= H
Luteolin: R_1=R_3=R_4=R_5= OH, R_2=H

Flavonol



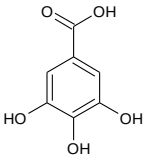
Quercetin: R_1=R_2=R_3=R_4= OH, R_5= H
Myricetin: R_1=R_2=R_3=R_4=R_5= OH

Isoflavone



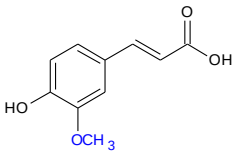
Genistein:R_1=R_2=R_3=OH
Diadzein: R_1=R_3= OH, R_2= H

Hydroxybenzoic acid

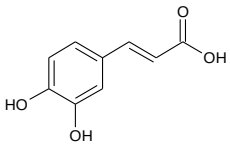


Gallic acid

Hydroxycinnamic acid

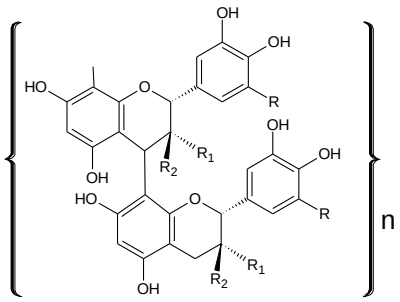


Ferulic acid



Caffeic acid

Proanthocyanidin



Procyanidin:R=H
Prodelphinidin: R= OH

Figure 1.3. Structure of Some Phenolic Compounds

1.4.2. Phenolic Compounds and Plant Defence

Phenolic compounds play a major role in the interaction of plants with their environment. They may attract insects, function as signal between plants (allelopathy), as signals between plants and symbiotic (N_2-fixing bacteria) or pathogenic organism (phytopathology), and they may protect plants against biotic stresses such as microbial pests and herbivores or abiotic stresses such as air pollution, heavy metal ions, UV-B radiation, etc. (Harborne, 1993). The induction of chemicals that deter or kill pests and pathogens represents one mean of self protection. The diversified array of phenolic compounds produced by plant tissues (several thousand different chemical structures have been characterised) belong to various classes, such as esters, amides and glycosides of hydroxycinnamic acids, glycosylated flavonoids especially flavonols, proanthocyanidins , polymeric lignin and suberin. They are the aromatic compounds that give plants their distinctive smells and encompass a wide variety of defense related compounds including flavonoids, anthocyanins, phytoalexins, tannins, lignin and furanocoumarins. The first demonstrated example from an early plant pathology literature of phenolic compounds providing disease resistance was the case of coloured onion scales accumulating sufficient quantities of catechol and protocatechuic acid to prevent the germination of *Colletotrichum circinans* spores (Walker and Stahmann, 1955). The role of plant phenolics in chemoecology, especially on the feeding behaviour of herbivores, has been recognised since 1959 when Fraenkel described phenolic compounds as 'trigger' substances which induce or prevent the uptake of nutrients by animal herbivores. They play a variety of roles in plants as they are the most abundant class of secondary metabolites. Ehrlich and Raven (1964) were among the first to propose a defined ecological role for plant secondary metabolites as defence agents against herbivorous insects.

1.4.3. Phenolics as Pesticidal/Insecticidal Molecules against Insects

It is widely documented that plants produce a wide variety of secondary metabolites as defensive mechanism against insect pests during attack (Dakora, 1995; Dakora and Phillips, 1996; Ndakidemi and Dakora, 2003). These include alkaloids, terpenoids, phenolic compounds, and many others (Ndakidemi and Dakora 2003). During the past few decades researchers have identified plant extracts highly effective against a wide range of plant pests (Dakora, 1995; Dakora and Phillips, 1996; Ndakidemi and Dakora 2003). The most active documented compounds have been isolated from the Neem tree (Egunjobi and Afolami, 1976; Badra and Eligindi, 1979; Thakur *et al*., 1981). Phenolic compounds extracted from different parts of Neem tree include: limonoids, phenols, tannins, nimbin, salanin, thionemone, azadirachtin and various flavonoids. These compounds have been found to exhibit nematicidal properties and are also reported to be highly effective against nematodes and insects (Badra & Eligindi, 1979; Thakur *et al*., 1981, Akhtar and Malik, 2000).

According to Levin (1971), one of the most important groups of the plant secondary constituents playing a defensive role against pests are phenolics. In fact, they are the biologically active secondary metabolites that influence insect growth and feeding (Cipollini *et al.*, 2008). The plant polyphenols are a very heterogeneous group, some universally and others widely distributed among plants, and often present in surprisingly high concentrations. Polyphenols, particularly flavonoids and tannins, have long been associated with plant defence against herbivores. These polyphenolic compounds disrupt pathogen metabolism or cellular structure but are often pathogen specific in their toxicity. A wide variety of plants produce toxins that are activated by ultraviolet light. The toxins are deleterious to certain vertebrae and invertebrate herbivores that feed on the plants due to rapid cell death from damage to the DNA. Study of

defensive role played by phenolics in preventing herbivory of fruit fly *Bactrocera dorsalis* (Tephritidae: Diptera) in mango shows that phenolics concentration between 42.37 and 53.12 mg/g in the peel of the mango is capable of defending the fruit from attack by the fruit fly (Verghese *et al.*, 2012). Most research concerning insect anti-feeding agents has shown the involvement of phenylpropanoids, flavonoids and lignans in the plant resistance mechanism against insects. Tannins, the protein complexing compounds and enzyme inhibitors, and may also affect the growth of insects.

1.5. Literature Review of the Selected Plants

(A) *Ipomoea cairica*

Figure 1.4. Photography of *Ipomoea cairica* Showing its Leaves and Flowers

- **Common name** **:** Mile a minute vine, Messina creeper, Cairo morning glory, Coast morning glory and Railroad creeper.
- **Family** **:** Convulvulaceae
- **Species** **:** *Ipomoea cairica*
- **Synonyms** **:** *Ipomoea palmata* Forssk, *Ipomoea stipulacea* (Jacq.)
- **Description:** It is a vinning perennial herb growing from a tuberous footstock. It is a prostrate creeper or twining into other vegetation and has large palmate leaves with 5 – 7 lobes with showy white to lavender colour flowers. Each fruit matures at about 1 cm across and contain hairy seeds**.**
- **Distribution:** Convulvulaceae comprise nearly 1650 predominantly tropical species. The genus Ipomoea with approximately 500 – 600 species comprises the largest no. of species within the Convulvulaceae (Austin & Huaman, 1996). This family is dominated by twining

and climbing woody or herbaceous plants that often have heart- shaped leaves and funnel-shaped flowers (Austin, 1997). The genus Ipomoea occurs in the tropics of the world although some species also reach temperate zones (Cao *et al*., 2005). The species of this genus are mainly distributed throughout the South and Central America countries and Tropical African territories (Austin & Huaman, 1996). One of the most noticeable characteristics of the Convulvulaceae is the existence of cells which secrete resin glycosides in the foliar tissues and in the roots of the plants. These glycoresins constitute one important chemotaxonomic marker of this family (Wagner, 1973) and are responsible for the purgative properties of some species of this family (Pereda-Miranda and Bah, 2003). Convulvulaceae are found throughout tropical and subtropical regions of the world.

- **Reported Biological Activity:** Alkaloids, phenolic compounds and glycolipids are the most common biologically active constituents from these plant extracts (Marilena *et al*., 2012). Indole alkaloids were isolated from the leaves of this species (Sharda and Kokate, 1979). The major constituents of the extracts were the coumarins scopoletin and umbelliferone and the lignans, arctigenin, matairesinol and tracheologenin (Lima and Braz-Filho, 1997). Arctigenin attributed for cytotoxicity and also for antioxidant and anti-inflammatory activities (Cho *et al*., 2004) as well as inhibition of replication of human immunodeficiency virus (Eich *et al*., 1996). Pinoresinol (tetrahydrofuran lignin) having antioxidant and Ca^{2+} antagonist properties (Paswka *et al.*, 2002). The essential oil possesses remarkable larvicadal properties of 100% mortality in the larvae of *Culex tritaeniorhynchus* (100 ppm), *Aedes aegypti* (120 ppm), *Anopheles stephensi* (120 ppm) and *Culex quinquefasciatus* (170 ppm). Aqueous extract showed anti-RSV (Respiratory syncytial virus) activity in vitro (Ma *et al*., 2002). Cytotoxicity against LNCaP cell line and significant activity against A549 cell line (Lin *et al*., 2008).

(B) *Ageratina adenophora*

Figure 1.5 Photography of *Ageratina adenophora* Showing its Leaves

- **Common Name :** Eupatory, sticky snakeroot, crofton weed and Mexical Devil
- **Local Name** : Bihar hlo, Nepal tlangsam, tlangsam suak, ai eng rim nam
- **Family :** Asteraceae
- **Species :** *Ageratina adenophora* (Spreng.)
- **Synonyms :** *Eupatorium glandulosum*, *Eupatorium adenophorum*
- **Description:** It is a perennial herbaceous shrub which may grow to 1 or 2 metres (3.3 or 6.6 ft) high with trailing purplish to chocolate-brown branches that strike roots upon contact with soil, resulting in dense thickets (Bess and Haramoto, 1958). The base of the plant is woody and densely clothed with stalked glandular hairs. Leaves are dark green, opposite, deltoid-ovate, serrate, and purple underneath, and each grows to about 10 cm in length. Flowers are borne terminally in compound clusters during spring and summer. The seed is an achene, varying from elliptic to oblanceolate, often gibbous, 1.5–2 mm long, 0.3–0.5 mm wide; with five prominent ribs and five to 40 pappi with slender scabrous bristles (Hickman, 1993). Each flowerhead is upto 0.5 cm in the diameter and creamy white in colour. They are followed by a small brown seed with a white feathery 'parachute' (Wolff and Mark, 1999)
- **Distribution:** *Ageratina adenophora* is a native of Mexico, but has been naturalized in many countries. It was introduced into several parts of the world as an ornamental in the nineteenth century and is now an established invasive weed in many subtropical regions of Asia (India, Nepal, China, the Philippines, Thailand, and Brunei), Oceania (Hawaiian Islands, Tahiti, New Zealand, Australia, and Papua New Guinea), Africa and Europe (Wagner *et al*., 1999).

It has flourished in areas which receive year-round rainfall, edging out native vegetation (Fuller, 1981). Its occurrence in tropical countries such as India, Nepal, the Philippines, and Thailand is limited to elevations between 1000 and 2000 m in the hills (Borthakur, 1977; Sharma and Chhetri, 1977).

- **Reported Biological activity:** 9-oxo-10,11 dehydroagerophorone responsible for development of lesions in the liver of mice (Oelrichs *et al.*, 1995). Analgesic activity from methanolic extracts of leaves (Mandal *et al*., 2005). Toxic property of the leaf extract on cuticular and sensilla of mustard aphid, Lipaphis erysimsi Kalt. (Sudip *et al*., 2005). It is usually not eaten by cattle, and can reduce the carrying capacity and productivity of invaded areas. It is also poisonous to livestock particularly toxic to horses. It is reported to be the cause of an acute pulmonary disease of horses which is known as "Tellabudgera horse disease" in Queensland and "Numinbah horse sickness" in New South Wales. This condition can be fatal enough if enough of the weed is consumed over a long period (Agnes *et al*., 2011). Anticoccidial activity against coccidian oocyst activity, a common parasite in poultry farms (Yang *et al*., 2012).

1.6. Phytochemical Characterization

1.6.1. Collection and Identification of Plant Species

The phytochemical investigation of plant involves different technique like extraction of plant material, separation and isolation of target chemical compounds and characterization of isolated compound. If the plant is known to contain volatile or thermolabile compounds, it may be advisable to snap-freeze the material as soon as possible after collection. A more common practice, however, is to leave the plant sample to dry on trays in air at ambient temperature and in a room with adequate ventilation.

The natural products of interest are usually small organic molecules (mol wt. < 2000 amu approx.). The most ideal phytochemical analysis consists of the fresh plant tissues and the other plant material that is under investigation should be plunged into boiling alcohol within minutes of collection. Alternatively, plants may be dried before extraction under controlled conditions or in shade to avoid occurrence of any chemical changes. It should be dried as quickly as possible, without using high temperature preferably in a good air draft.

The plant tissue free from contamination with other plants is an obvious point to watch. It is essential to use plant free from diseases i.e. which are not affected by viral, bacterial or fungal infection as signs of contamination may be linked to a change in the profile of metabolites occur and may seriously alter plant metabolism and unexpected results may be found. Contamination may also occur while collecting lower part of plant material for analysis. Parasitic fungi are known to contaminate the tree tissues. Again, mosses often grow in symbiosis with higher plants and it is sometimes difficult to obtain plant samples free from such litter. In higher plants, mixture of plants may, sometimes, be gathered erroneously. Two closely attached plants growing side by side may be assumed to be the same. Protection from direct sunlight is advised to minimize chemical alterations (and the formation of artifacts) induced by ultraviolet rays.

In phytochemical analysis, the botanical identity of the plant under study must be authenticated by an acknowledged authority at some stage during the investigation. Any features relating to the collection, such as the name of the plant, the identity of the part(s) collected, the place and date of collection should be recorded as part of a voucher (a dried specimen pressed between sheets of paper) deposited in a herbarium for the future reference. (Harborne, 1984).

1.6.2. Mode of Extraction

Prior to any isolation and purification work, a natural product has to be extracted (or released) from the biomass. Usually before extraction, plant samples are treated by milling, grinding and homogenization, which may be preceded by air-drying or freeze-drying. Plant material should be sliced into small pieces and distributed evenly using mechanical grinder, mortar and pestle, etc. to facilitate homogeneous drying and efficient extraction of the target compound. However, drying processes, including freeze-drying, can cause undesirable effects on the constituent profiles of plant samples, therefore, caution should be taken when planning and analyzing the medicinal properties of plants (Abascal *et al*., 2005). Extractions can be either selective (e.g., sequential process by increasing the polarity of the solvents) or total (e.g., using polar organic solvent to extract as many compounds as possible). A range of techniques, varying in cost and level of complexity, may be employed for extraction of plant material.

Regardless of the extraction technique applied, the resulting solution should be filtered in order to remove any remaining particulate matter. Plant extracts should not be kept in the solvent for long periods at room temperature, or in sunlight due to concomitant risk of artifact formation and decomposition or isomerization of extract constituents. Extracts can be concentrated at reduced pressure on a rotary evaporator or dried under a stream of nitrogen. If a rotary evaporator is used, it is advisable to keep the water bath temperature below 40 °C to prevent decomposition of thermolabile components.

Soxhlet extraction, using commercially available devices, is a convenient method for extraction of small to moderate volumes of plant material. As the extraction takes place in a closed system in which the solvent is continually recycled, the amount of solvent needed for Soxhlet extraction is minimal. In the most commonly used extractor, however, the heat needed to

drive the extraction will likely cause thermolabile constituents to form artifacts or decomposition products. Therefore though convenient, this method is not suitable when the target compounds are thermolabile.

1.6.3. Choice of Solvents

Factors that should be considered when choosing a solvent or solvent system for extracting plant material include the solubility of target constituents, safety, ease of working with the solvent, potential for artifact formation, and the grade and purity of the solvent. Following the principle of "like dissolves like", it is often possible to tailor the solvent of choice to maximize the yield of the compounds of interest, while minimizing the extraction of unwanted compounds. A careful review of literature related to the species and compound classes under investigation often saves time and effort. Even when little information is available regarding the secondary metabolites from a particular species, phytochemical reports on other related species of the genus or family can provide clues regarding which compound classes to expect, and which solvents and procedures may be used to isolate them. Comparison of total extract yields, yields of metabolites of interest, or efficacy of biological activity will indicate which method gives the best results.

The chemical method for obtaining organic constituents from dried plant tissue (heartwood, dried seed, root, leaves, flowers) is to continuously extract powdered material in a Soxhlet apparatus with a range of solvents, starting from petroleum ether and chloroform (to separate lipids and terpenoids) and then using alcohol and ethyl acetate (for more polar compounds). In most cases, mixtures of components will be present and it is necessary to separate those compounds by a suitable chromatography technique. As a standard precaution

against any loss of material, concentrated extract should be stored in the refrigerator and used for further investigation (Harborne, 1984).

1.6.4. Preliminary Phytochemical Analysis of Plant Extracts

Preliminary phytochemical group testing of the crude extracts is to detect the presence of chemical constituents like alkaloids, steroids, terpinoids, flavonoids, gums, reducing sugars, tannins, saponins, coumarin glycosides, anthraquinones, etc. Following reagents are widely used for different chemical group tests (Kokate, 1994): *Mayer's reagent, Liberman-Burchard reagent, Dragendroff's reagent, Fehling's solution A & B, Benedict's reagent* and *Molish's reagent.*

1.7. Isolation and Purification of Phytochemicals

The most important factor that has to be considered before designing an isolation protocol is the nature of the target compound present in the crude extracts or fractions. A crude natural product extract is literally a cocktail of compounds. Plant crude extracts usually contain large amounts of carbohydrates and/or lipoidal material and the concentration of the desired compounds (phenolics) in the crude extract may be low. To concentrate and obtain a phenol-rich fractions prior to the analysis, strategies including sequential extraction or liquid-liquid partitioning and/or solid phase extraction (SPE) based on polarity and acidity have been commonly employed. In general, elimination of lipoidal material can be achieved by washing the crude extract with non-polar solvents such as n-hexane (Ramirez-Coronel *et al*., 2004), dichloromethane (Neergheen *et al*., 2006) or chloroform (Zhang *et al*., 2008).

It is difficult to apply a single separation technique to isolate individual compounds from crude mixture. Hence, the crude extract is initially separated into various discrete fractions containing compounds of similar polarities or molecular sizes. The isolation and purification of plant constituents is mainly carried out using one or other, or a combination of four

chromatography technique like thin layer chromatography (TLC), column chromatography (CC), high performance liquid chromatography (HPLC) and gas liquid chromatography (GLC). The choice of technique depends largely on the solubility properties and volatilities of the compounds to be separated. Paper chromatography (PC) is also applicable to water-soluble plant constituents, namely the carbohydrates, amino acids, nucleic acid bases, organic acids and phenolic compounds.

TLC may be considered basically for qualitative identification and quantitative separation. In TLC, certain adsorbing material such as silica gel, alumina, cellulose etc. are supported as thin layers on glass or aluminium plates and offers separation by partition, and / or adsorption. It is also the method of choice for separating all lipid-soluble components, i.e. the lipids, steroids, carotenoids and chlorophyll.

CC, although, often labor-intensive and solvent-consuming, provides greater amounts of fractions for subsequent isolation and identification of pure substances. Typically-utilized column sorbents are RP-C18 (Queiroz *et al*., 2005), Sephadex LH-20 (Kandil *et al*., 2002) and to a less extent polyamide resin (Ranilla *et al*., 2007). Ethanol, methanol, acetone, and water and their combinations are commonly used as eluents. In particular, the isolation of proanthocyanidins (condensed tannins) is routinely carried out by employing Sephadex LH-20 column chromatography (Hagerman and Butler, 1980; Asquith *et al.*, 1983). Using LH-20 column chromatography, methanol is more commonly employed than ethanol to elute non-tannin compounds (Ek *et al*., 2006).

GLC finds its main application with volatile compounds, fatty acids, mono and sesquiterpenes, hydrocarbons and sulphur compounds, while less volatile compounds are usually separated by HPLC. Using HPLC technique, separation of non-polar, moderately polar and

highly polar substances is possible, depending upon the column and mobile phase that is being used. It is a rapid technique applicable to all kinds of chromatographic methods such as adsorption, partition, ion exchange, molecular exclusion and affinity chromatography (Jayaprakasam *et al*., 2004a; Henry *et al*., 2006). It is very important to note that there is a considerable overlap with the application of the above mentioned techniques and often a combination of PC and TLC, TLC and HPLC or TLC and GLC may be the best approach while separating a particular class of plant compounds. For preparative work, TLC is carried out and for isolation large diameter column is employed (Harborne, 1984).

1.8. **Determination of Molecular Structure**

In most cases of extraction and isolation of natural products, the final step is the identification of the compound or the conclusive structural elucidation of isolated compounds. However, the determination of molecular structure of compounds isolated from plants, fungi, bacteria, or other organisms is generally time consuming and sometimes can be "bottleneck" in the natural product research. There are many useful spectroscopic methods for getting useful information about chemical structures/functional groups, but the interpretation of these spectra normally requires specialists with detailed spectroscopic knowledge and wide experience in natural product chemistry.

The following spectroscopic techniques are generally used for the structure elucidation of natural products:

i) Ultraviolet-visible spectroscopy (UV-vis): Provides information on chromophores present in the molecules. Some natural products, e.g., flavonoids, isoquinoline alkaloids, and coumarins, to name a few, can be primarily characterized (chemical class) from characteristics absorption peaks.

ii) Infrared spectroscopy (IR): Vibrational spectroscopies, Raman & FT-IR spectroscopy provide both the bonding as well as functional group (e.g., -C=O, -OH, -NH_2, aromacity, and so on) information of the phytochemicals under study.

iii) Mass spectrometry (MS): It is a method of determining the precise mass of a compound as it ascertains the mass to charge ratios of gaseous ions. It gives detailed information about the molecular mass, molecular formula, and the kind of fragmentation pattern the molecule may undergoes which directly provides information regarding the nature of bonding. Mass spectrometry has the advantage of high sensitivity, reproducibility, accuracy and minimal amount of sample required for mass analysis (Harwood *et al*., 1999). Mass spectral data can aid the interpretation of NMR spectral data by providing the molecular weight of molecular ion and the base ion those results from ionization induced rearrangements/fragmentation. Most commonly used techniques are: electron impact mass spectrometry (EIMS), chemical ionization mass spectrometry (CIMS), atmospheric pressure chemical ionization (APCI) mass spectrometry, electrospray ionization mass spectrometry (ESIMS), and fast atom bombardment mass spectrometry (FABMS).

iv) NMR Spectroscopy: NMR spectroscopy provides the most useful chemical insight regarding the structural features of the compound being analyzed. The fact that makes NMR spectroscopy a powerful tool is that the nuclei in the molecule interact with the surrounding electrons and with the other nuclei within the molecule. It reveals vital information of the number and types of protons and carbons (and other elements like nitrogen, fluorine, etc) present in the molecule, and the structural relationships among these atoms. The local chemical environment arising from the presence of various functional groups and substituent groups influence the 'local' electron density distribution. These local density distributions, in presence

of applied magnetic field, produce an induced magnetic field, which determines the resonance condition for magnetic nuclei of the chemical compound. The measurement of subtle yet distinct variations in these local electron density distributions, through chemical shift (δ) values for magnetic nuclei (^{1}H, ^{13}C, ^{15}N) provides the identification of specific functional groups and substituents, depending on the nature of electron-withdrawing or electron-donating properties of functional groups. The magnetic interactions between magnetic nuclei ($^{1}H - {}^{1}H$, $^{1}H - {}^{13}C$) in its immediate vicinity cause nuclear-nuclear spin-spin coupling (through bond, through space and chemical exchange) leading to the resolution of nuclear energy levels, which can be observed as splitting (coupling constant J) in the NMR spectrum (Claridge, 1999). From the measurement of spin-spin coupling, one can easily find out the number of interacting proton nuclei in a substituent group or adjacent carbon. Thus, measurement of various chemical shift and spin-spin couplings essentially maps out the molecular skeleton (structural features) of a chemical compound. The NMR experiments used today can be classified into two major categories:

(a) 1D NMR: ^{1}HNMR, ^{13}CNMR, ^{13}CDEPT.

(b) 2D-NMR: ^{1}H-^{1}H, COSY, ^{1}H-^{1}H, DQF- COSY, ^{1}H-^{1}H COSY-1r, ^{1}H-^{1}H NOESY, ^{1}H-^{1}H ROESY, ^{1}H-^{1}H TOCSY, ^{1}H-^{13}C HMBC, ^{1}H-^{13}C HMQC, ^{1}H-^{13}C HSQC, HSQC- TOCSY.

Simple 1D NMR spectrum contains so many resonance lines that may lead to the overlap of resonance lines and may cause confusion in the interpretation of NMR spectral data. Many pulse sequences have been developed over the period of time that provide a substantial increase in resolution such as DEPT, INEPT as well as 2D NMR techniques as mentioned above (Jayaprakasam *et al*., 2000; Jayaprakasam *et al*., 2004b). Extensive utilization of these techniques in the chemical investigations provides a more reliable way of extracting structural

information of organic compounds/ natural products, including subtle conformational changes (Claridge, 1999).

In addition, using single crystal X-ray diffraction, image of a molecule in space can be obtained by the image synthesis of the electron density around atoms of the molecule, by performing Fouries transform of structure factors (intensity of 'hkl' reflections inclusive of phase) (Satyajit, 2006).

1.9. **Interaction of Transition Metal ions with Natural Product Compounds**

The transition metal ions have tendency to form co-ordination compounds with Lewis bases with groups which are able to donate an electron pair. Phytochemicals containing one or more functional group(s) such as –OH, –COOH, –CHO, –SH and –NH– can interact with transition metal ions like Cu^{2+}, Fe^{2+}, Ni^{2+} and Mn^{2+} and form inorganic coordination complexes. Mandakmare and Narwade (1999) studied the determination of stability constants of Cu(II) chelates with some substituted coumarins at 0.1 M ionic strength pH metrically and Vyas *et al.*(2009) have synthesized Cu(II), Ni(II), Co(II) and Mn(II) metal complexes of hydroxy coumarin and reported their stability constants. The nature of coordination in the complex can be studied by using various spectroscopic techniques such as UV/VIS absorption, FT-IR, EPR and diffraction techniques like X-ray crystallography.

Metal ion plays important role in biological systems such as metalloproteins, metalloenzymes, metallochaperones, metal based drugs etc., which has lead to the development of a large collection of metal complexes with unique physical, chemical, magnetic, spectroscopic, and biological properties. Rational design, synthesis and characterization of inorganic coordination complexes for the study of the interaction of transition metal ions with natural product compounds may provide a wide range of information:

i) simple, direct, and rapid complex preparation procedure

ii) This will allow us to depict the atomic positions of constituent molecules as it is relatively easy to grow crystals of coordination complexes compared with crystallization of natural product compounds suitable for x-ray diffraction. In addition, the crystal structure may facilitate elucidation of the most probable configuration of natural product conformers

iii) It may constitute novel inorganic complexes with versatile physic-chemical properties besides it reveals diverse chelation capacity of natural product compounds with potential multiple binding sites

iv) Because of their structural analogy, these biomimetic inorganic complexes may also possess interesting biological activity

The properties of copper complexes are largely determined by the nature of ligands and donor atoms coordinated to the metal ion. The d^9 electronic configuration, typical of cupric ion, present in copper(II) complexes that exhibit the ligand field transitions (d-d transitions), are attributed to the intense coloured coordination complex species. In these complexes, the coordination number around copper varies from four to six, including four-coordinate square planar (sp), five-coordinate trigonal bipyramidal (tbp) or square pyramidal (sqpy) and six-coordinate octahedral (oh) geometries. In such molecular structures, normally a typical deviation from the ideal coordination geometries mostly resulting in tetragonal distortions is observed.

The ligand field (LF) environment of the metal site splits its d orbitals in energy and lifts the degeneracy of the d-orbitals. The highest energy orbitals are partially occupied, and these orbitals contribute to the optical and magnetic properties of complexes. When electromagnetic radiation of appropriate energy is applied (i.e., the near infrared [NIR] and visible spectral regions), the d-orbital electrons are excited from the filled d orbitals to the singly occupied or

unoccupied metal valence orbitals. These are the ligand field (LF) or d-d transitions. The d–d transitions are generally Laporte (parity) forbidden and appear as a broad composite weak band envelope centered at lower energy visible or visible-NIR region. These d-d transitions provide sensitive probes of the geometric and electronic structure of the coordination complex. For a number of ligand donor atoms, the transition metal ion complex also exhibits extremely intense, higher energy charge transfer (CT) absorption bands, which reflect highly covalent character of the ligand-metal bonds. Charge transfer transitions correspond to an electron being optically excited from filled ligand σ and π orbitals into the partially occupied d-orbital (d-orbital hole) on the metal center. The intensity and wavelength position of these CT bands can be affected by the geometry of metal site and the orientation of ligand-metal bonds.

CHAPTER 2
MATERIALS AND METHODS

2.1 MATERIALS

2.1.1. Selection of Plant Materials

In the present study, two wild plants abundantly found in Mizoram were selected. These are :

(1) Ipomoea cairica

(2) Ageratina adenophora

The above plants were collected from the areas in and around Mizoram University, Mizoram and were identified by Botanical Survey of India, Shillong, Meghalaya (No.BSI/ERC/2012/Plant identification/ dated 28-8-2013), coll no. 1 for *Ageratina adenophora* and coll no.2 for *Ipomoea cairica* respectively. Voucher specimens were kept in the Department of Botany, Pachhunga University College, Aizawl, Mizoram for future reference.

These plants were selected with an aim to provide the local people a safe, cheap and easily available plant based insecticides and/or pesticides for use in their households and agricultural lands because keen observation of these plants over ages reveals that these plants though abundantly found had not been attacked by most insect pests. It is therefore believed these properties might be attributed to the presence of some known /unknown bioactive compound(s) present in them which possess pesticidal and/or insecticidal properties. Hajra in 2002, reported the pesticidal properties of *Ageratina adenophora* against bunch caterpillar.

In Mizoram, there is no report on the traditional use of *Ipomoea cairica* as a medicine or as a pesticide and is considered only as a weed. However, *Ageratina adenophora* leaves are boiled and the water taken for kidney problem even though it is also considered as a noxious

weed. The juice of crushed leaves is also applied on fresh wounds (Sawmliana, 2013). However, their constituent phytochemicals possessing insecticidal or repellant property for vector programme have not well been studied, characterized and mass produced for commercial or local use.

2.1.2. Chemicals and Solvent Materials

All Chemicals were obtained from commercial sources and were of analytical grade. Gallic acid and ascorbic acid were purchased from Loba Chemie Pvt. Ltd., Mumbai and quercetin, from Sigma Aldrich, Bangalore. Sodium carbonate, sodium hydroxide, ferric chloride, potassium dichromate, lead acetate, sodium nitrite, aluminium chloride, sodium phosphate and ammonium molybdate were purchased from Himedia Laboratories Pvt. Ltd., Mumbai. Sulphuric acid was purchased from Merck Specialities Pvt. Ltd., Mumbai. All solvents used for phytochemical tests were used as purchased. However, solvents used for extraction and isolations were distilled prior to their used.

2.1.3. Chromatographic Materials

Silica gel mesh 60-120 (for CC) was purchased from SD fine Chemicals Ltd., Mumbai and mesh size 100-200 from Himedia Laboratories Pvt. Ltd., Mumbai. Sephadex LH-20 was procured from Sigma Aldrich, Bangalore. Silica gel G (for TLC) and Aluminium backed pre coated silica gel 60 F_{254} were purchased from Merck Specialities Pvt. Ltd., Mumbai. Solvents for HPLC analysis were of HPLC grade and were purchased from Himedia Laboratories Pvt. Ltd., Mumbai. Analytical grade solvents were used for HPTLC and TLC analysis.

2.2. METHODS

2.2.1 Methods of Extracts Preparation

The collected leaves of *A. adenophora* and leaves and flowers of *I. cairica* were cleaned, washed with distilled water and dried under shade with occasional shifting and then powdered with an electrical grinder. These powders were stored in air sealed brown bottles at ambient temperature. The dried, powdered materials of *A. adenophora* (3 kg) and *I. cairica* leaves (3.5 kg) and flowers (2.8 kg) were sequentially extracted starting with pet. ether/n-hexane to remove lipids, oils and fats followed by exhaustive extraction with $CHCl_3$ for removal of chlorophyll and its derivatives. The plant materials were then finally extracted continuously by soxhlet extractor and maceration with MeOH. Phenolics with only few hydroxyl groups are soluble in ether, $CHCl_3$, EtOAc, MeOH, and EtOH (Van Sumere, 1989). Methanol, ethanol, water, and alcohol-water mixtures are most commonly used for dissolving phenolic compounds for analytical purposes. The extractions were repeated for many times and the combined extracts were filtered and concentrated in a vacuo using a rotary evaporator at reduced pressure (22–26 mm Hg) and low temperature of 45°C to collect the crude extract and to remove the last traces of the solvents. A dark green coloured (for *I. cairica* leaves and *A. adenophora* leaves) and dark-red coloured semi- solid mass (for *I. cairica* flowers) were obtained after concentration. The concentrated extracts were kept in refrigerator at 4^oC for further use. The percentage yields of petroleum ether extract of *A. adenophora* and MeOH extract of *Ipomoea cairica* flowers were found to be 2.96 % w/w and 3.97 % w/w respectively. The pet. ether, $CHCl_3$ and MeOH extracts were subjected to preliminary phytochemical analysis to identify the presence of phytoconstituents. All the crude extracts obtained from selective extractions were also tested for their efficacy against the third instar larvae of test mosquito (*Culex quinquefasciatus* Say) at various concentrations.

2.2.2 Preliminary Phytochemical Analysis of Plant Extracts.

Pet. ether, $CHCl_3$ and MeOH extracts of the selected plants were tested by following standard procedures described below, for the presence of phytochemical constituents such as alkaloids, steroids, flavonoids, gums, reducing sugars, tannins, saponins, terpenoids, aminoacids, anthraquinones, cardiac glycosides and phenols. **Table 3.3** gives the results of preliminary phytochemical group testing of the crude extracts. Reagents used for the different chemical group tests were prepared as per standard protocols as follows:

- **Mayer's reagent:** Solution of 1.36gm of mercuric iodide in 60ml of water was mixed with a solution containing 5gm of potassium iodide in 20ml of water.
- **Libermann-Burchard reagent:** 5gm of acetic anhydride was mixed under cooling with 5ml of conc. H_2SO_4 and was added slowly to 50ml of absolute ethanol with cooling.
- **Dragendroff reagent:** 1.7gm basic bismuth nitrate and 20gm tartaric acid were dissolved in 80ml of water. This solution was mixed with a solution containing 16gm potassium iodide in 40ml of water.
- **Fehling's solution A:** 34.64gm copper sulphate was dissolved in a mixture of 0.5ml of sulfuric acid and sufficient water to produce 500ml.
- **Fehling's solution B:** 176gm of sodium potassium tartarate and 77gm of sodium hydroxide were dissolved in sufficient water to produce 500ml. Equal volumes of above solutions (Fehling's solution A and B) is mixed at the time of use.
- **Benedict's reagent:** 1.73gm of cupric sulphate, 1.73gm of sodium citrate and 10gm anhydrous sodium carbonate were dissolved in water and the volume is made up to 100ml with water.
- **Molish's reagent:** 2.5gm of pure α-naphthol was dissolved in 25ml of ethanol.

2.2.2.1. Test for Alkaloids: (Raaman, 2008)

- **Mayer's Test:** 1 to 2 ml of extract was taken in a test tube. 0.2 ml of dil. HCl and 0.1 ml of Mayer's reagent were added. Formation of yellowish buff colored precipitate gives positive test for alkaloid.
- **Dragendroff's Test:** 0.1ml of dil. HCl and 0.1 ml of Dragendroff's reagent were added in 2 ml solution of extract in a test tube. Development of orange brown colored precipitate suggested the presence of alkaloid.
- **Wagner's Test:** 2 ml of extract solution was treated with dil. HCl and 0.1 ml of Wagner's reagent. Formation of reddish brown precipitate indicated the positive response for alkaloid.
- **Hager's Test:** 2 ml of extract was allowed to react with 0.2ml of dil. HCl and 0.1 ml of Hager's reagent. A yellowish precipitate suggested the presence of alkaloid.

2.2.2.2. Test for Amino acids: (Trease & Evans, 1983)

- **Ninhydrin Test:** Extract solution was treated with Ninhydrin (Tri-ketohydrindene hydrate) at the pH range of 4 - 8. Development of purple color indicated the positive response for amino acids.

2.2.2.3. Test for Anthraquinones: (Kokate, 1994; Sofwara, 1982; Jeffrey, 2007)

- **Modified Borntrager's Test:** 5 ml of extract solution was hydrolyzed with dilute sulphuric acid and extracted with benzene. 1 ml of dilute ammonia was added to it. Rose pink coloration suggested the positive response for anthraquinones.

2.2.2.4. Test for Reducing sugars: (Kokate, 1994; Trease & Evans, 1983)

- **Fehling's test for free reducing sugar:** About 0.5 g of extract was dissolved in distilledwater and filtered. The filtrate was heated with 5 ml of equal volumes of Fehling's solution A and B.

Formation of a red precipitate of cuprous oxide was an indication of the presence of reducing sugars.

- **Benedict's Test:** To 5 ml of the extract solution, 5 ml of Benedict's solution was added in a test tube and boiled for few minutes. Development of brick red precipitate confirmed the presence of reducing sugars.

2.2.2.5. Test for Flavonoids: (Trease & Evans, 1983; Ayoola *et al.*, 2008)

- **Shinoda test:** About 0.5 g of each extract portion was dissolved in ethanol, warmed and then filtered. Three pieces of magnesium chips was then added to the filtrate followed by few drops of conc. HCl. A pink, orange, or red to purple colouration indicates the presence of flavonoids.
- **Sulfuric acid test:** A fraction of the extract was treated with concentrated H_2SO_4 and observed for the formation of orange colour.
- **NaOH test:** A small amount extract was treated with aqueous NaOH and HCl, observed for the formation of yellow orange colour.

2.2.2.6. Test for Gums:

- **Molisch's Test:** 2 ml of concentrated sulphuric acid was added to 2 ml of extract solution. Then it was treated with 15% α-naphthol in ethanol (Molisch's reagent). Formation of a red violet ring at the junction of two layers indicated the positive test for gums.

2.2.2.7. Test for Saponins: (Kokate, 1994; Trease & Evans, 1983)

- **Foam Test:** A small amount of extract was shaken with water and looked for the formation of persistent foam.

2.2.2.8. Test for Sterols: (Kokate, 1994; Trease & Evans, 1983)

- **Liebermann-Burchard test:** One ml extracts was treated with chloroform, acetic anhydride and added drops of H_2SO_4 and observed for the formation of dark pink or red colour.
- **Sulfuric acid test:** The fraction of extract was treated with ethanol and H_2SO_4 and observed for the formation of violet blue or green colour.

2.2.2.9. Test for Tannins: (Jeffrey, 2007; Kokate, 1994)

- **$FeCl_3$ Test:** 5 ml of extract solution was allowed to react with 1 ml of 5% ferric chloride solution. Greenish black coloration indicated the presence of tannins.
- **Pot. Dichromate Test:** 5 ml of the extract was treated with 1 ml of 10% aqueous potassium dichromate solution. Formation of yellowish brown precipitate suggested the presence of tannins.
- **Lead acetate Test:** 5 ml of the extract was treated with 1 ml of 10% lead acetate solution in water. Yellow color precipitation gave the test for tannins.

2.2.2.10. Test for Phenols: (Jeffrey, 2007; Kokate, 1994)

- **Ferric chloride test:** A fraction of extract was treated with 5% ferric chloride ,formation of deep blue colour confirms the presence of phenol
- **Liebermann's test:** The extracts was heated with sodium nitrite, add H_2SO_4 solution diluted with water and add excess of dilute NaOH and observed for the formation of deep red or green or blue colour.

2.2.2.11. Test for terpenoids: (Kokate, 1994; Trease & Evans, 1983)

- **Chloroform test:** The plant extract was taken in a test tube with few ml of chloroform and add concentrated sulfuric acid carefully to form a layer and observed for presence of reddish brown colour.
- **Liebermann-Burchard test:** 1 ml extracts was treated with chloroform, acetic anhydride and added drops of H_2SO_4 and observed for the formation of dark green colour.

2.2.2.12 Test for Glycosides: (Raaman, 2008)

- **Legal's test:** Dissolved the extract (0.1 g) in pyridine, added sodium nitroprusside reagent and made alkaline with NaOH solution. Pink to red colour solution indicates the presence of glycosides.
- **Borntrager's test:** The extract is hydrolyzed with concentrated HCl for 2 hours on a water bath and filtered and few ml of above filtrate was shaken with chloroform, chloroform layer was separated and added 10 % ammonia, formation of pink colour indicates the presence of glycosides.

2.3. LARVICIDAL ASSAY

2.3.1. Mosquito Larvicidal Efficacy of the Crude Extracts

Pet. ether, $CHCl_3$ and MeOH extracts of leaves and flowers of *I. cairica* and leaves of *A. adenophora* obtained by selective extraction were tested for their efficacy as a larvicide against third instar larvae of *Culex quinquefasciatus* (Say). The larvae of all the instars of *C. quinquefasciatus* were collected from Lengpui fish pond and Luangmual water tank, Aizawl, Mizoram during May–July 2013 and were reared in plastic and enamel trays (30×30×30 cm) containing tap water with the addition of artificial foods (brewer's yeast, dog biscuits, and algae collected from ponds in a ratio of 3:1:1) reared in the laboratory of Department of Chemistry,

Mizoram University. The plastic trays containing the test species were filled with tap water and were maintained at 26±2 0C and 76 – 84% relative humidity under photoperiod of 14L:10D cycles (WHO, 2005). Standard 1% stock solution of each extract was made by dissolving 400 mg of extract in 40 ml of ethanol (WHO, 2005).

Larvicidal activity against third instar larvae of *Culex quinquefasciatus* was carried out as per the procedure laid out by World Health Organisation (WHO, 1996). The stock solution of the extract was volumetrically diluted to 100 ml distilled water to obtain the test solutions of 50, 100, 200, 400 and 500 ppm. Four batches of early 25 third instar larvae were transferred by means of dropper to 250 ml test beakers each containing 100ml of test solutions of varying concentrations from 50 – 500 ppm and also to control and untreated solution. The control solution was made by mixing 99 ml of distilled water with 1 ml of ethanol, while the untreated solution contains 100 ml of distilled water only. Larval mortality was recorded after 24 hours and 48 hours of exposure, respectively, for determining the lethal concentrations, LC_{50} and LC_{90}. Percentage mortality was calculated from the average of four replicates.

Preliminary screening of the different plant extracts was carried out at higher concentrations of the plant extracts normally between 1000 – 500 ppm. The crude extracts that exhibit very low or no mortality between these concentrations were screened out. The data obtained were subjected to EPA probit analysis program version 1.5 in order to estimate the LC_{50} and LC_{90} values for all the selected crude extracts. Results of larvicidal efficacy for all the extracts at concentrations ranging from 50-500 ppm were presented in **table 3.4.**

2.3.2. Temporal Larvicidal Efficacy of Methanolic Extract of *Ipomoea Cairica* Flower

Lethal times, LT_{50} and LT_{90} values for the best larvicide i.e., MeOH extract of *Ipomoea caicica* flowers was determined by recording the mortality rate after every hour for a period of

24 hours for different concentrations. Here also, percentage mortality was calculated from the average of four replicates and the data obtained were subjected to EPA probit analysis program version 1.5 for determining LT_{50} and LT_{90} values. Slope and heterogeneity analysis, standard deviations or confidence intervals of the means of LT_{50} and LT_{90} values within a confidence limit of 95% were also calculated and reported. The results of lethal time determination for MeOH extract of *I. cairica* flower is produced in **table 3.5**

2.3.3. Statistical Analysis

All the experimental results of larvicidal studies were expressed as mean ± S.E. of four parallel measurements. Statistical analysis is estimated using one way ANOVA using Graphpad instat software. Results with $p<0.05$ were considered to be statistically significant.

2.4. SPECTRAL ANALYSIS OF EXTRACTS

2.4.1. UV-Visible Analysis of Crude Extracts

All the crude extracts of both the plants were analyzed with UV-Vis spectrophotometer (model Thermo Fischer Evolution 201, Finland) and the absorbance against wavelength produced in **figures 3.5.(A)-(I)**. UV-Vis spectroscopy is primarily used to detect electronic transitions arising from multiple bonds or phenyl ring moiety within molecules. It is also well known that molecules containing π-electrons or non-bonding electrons (n-orbital electrons) can absorb energy in the form of ultraviolet or visible light to elicit these electrons of ground molecular orbitals being promoted to higher antibonding molecular orbitals (Mehta, 2011). The more easily excited the electrons (i.e. lower energy gap between the HOMO and the LUMO) the longer the wavelength of electromagnetic radiation (EMR) it can absorb. UV spectrum records the wavelength of an absorption maximum, i.e. λ_{max} and the extent of interaction of the molecule

with EMR, i.e., molar absorptivity (molar extinction coefficient ε_{max}) as defined by the combined Beer-Lambert law (1):

$$Log(I_0/I) = \varepsilon.l.c \quad or \ \varepsilon = A/c.l \qquad \text{---------- (1)}$$

Where:

I_0 is the intensity of the incident light passing through a reference cell

I is the light transmitted through the sample solution

Log (I_0/I) is the absorbance (A) of the solution (optical density, OD)

C is the concentration of solute (in mol dm^{-3})

L is the path length of the sample (in cm)

ε is the molar absorptivity (extinction coefficient)

2.5. DETERMINATION OF TOTAL PHENOLIC CONTENT

Total phenolic content of MeOH extracts of *A.adenophora* leaves and *I. cairica* flowers and leaves were determined with Folin-Ciocalteu method (Singleton *et al*., 1999). This method is widely used to determine phenol content in plant extracts. It is accepted that the Folin Ciocalteu reagent (FCR) contains phosphomolybdic/phosphotungstic acid complexes and the chemistry behind the FC method counts on the transfer of electrons in alkaline medium from phenolic compounds and other reducing species to molybdenum, forming blue complexes that can be monitored spectrophotometet assay at 750–765 nm. The Folin-Ciocalteu reagent is sensitive to reducing compounds, polyphenols manifested as a blue colored complex. The phenolic compounds react with FCR only under basic conditions. The total phenol assay by FC method is convenient, simple, rapid and reliable (Huang *et al*., 2005; MacDonald-Wicks *et al*., 2006; Magalhaes *et al*., 2008). Standard solution (1mg/ml) of plant extract was prepared by dissolving 1mg of crude methanolic extract in 1ml of methanol. 3ml of samples were introduced into test

tubes, 0.15 ml of Folin Ciocalteu reagent and 0.3 ml of Na_2CO_3 (7%) were added. The tubes were mixed and allowed to stand for 30 minutes. The quantitative phenolic estimation was performed at absorption maximum of 765 nm by change in intensity of Folin-phenolic compounds complex. The total phenolics content was expressed as gallic acid equivalents (GAE) in mg per g of plant extract as calculated from standard gallic acid calibration curve. To prepare a calibration curve a concentration range of 0.05, 0.04, 0.03, 0.02, 0.01, 0.008, 0.005 and 0.001mg/ml of the gallic acid solutions were prepared to plot the standard calibration curve. Spectral measurements were taken after 1 hr at 725 nm by UV-Vis spectrophotometer (model Thermo Fischer Evolution 201, Finland) against reagent blank. The calibration curve of absorbance against concentration was plotted. 1mL of stock solution of each extract was transferred in 25 ml flask; similar protocol was adopted as described *vide supra* in the preparation of calibration curve. With the help of calibration curve, the phenolic concentrations of extracts were determined. The results were provided in **table 3.7(A).**

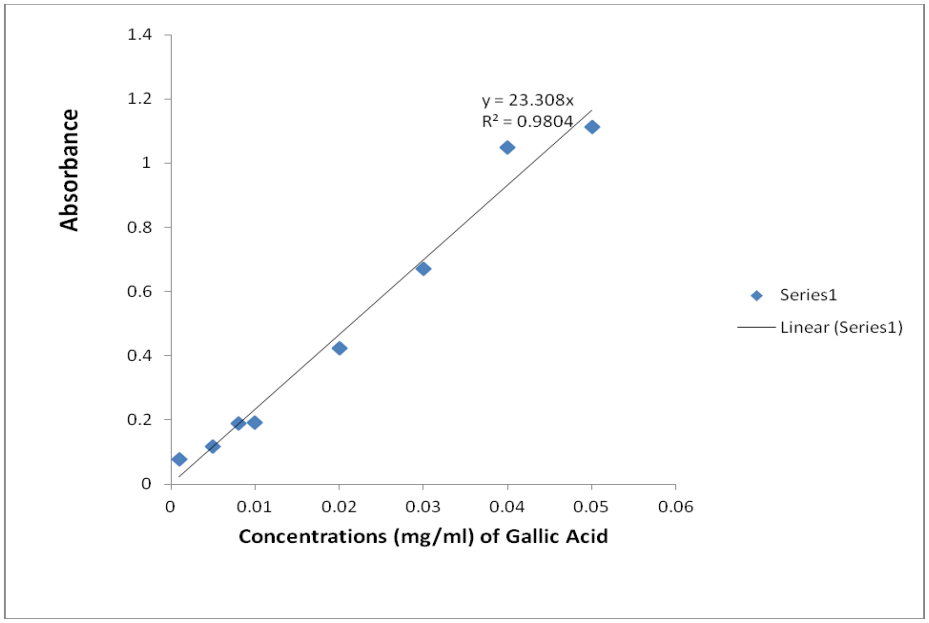


Figure 2.1 Calibration curve for gallic acid

2.6. DETERMINATION OF TOTAL FLAVONOID CONTENT

Total flavonoid contents of the extracts were determined using a colorimetric assay described by Zou *et al*., (2004). A mixture of 0.2 ml of extract and 0.15 ml of $NaNO_2$ (5% w/v) was first incubated at room temperature for 6 min. Next, 0.15 ml of $AlCl_3.6H_2O$ (10% w/v) was added to the mixture, which was then kept at room temperature for 6 min. Then, 0.8 ml of NaOH (10% w/v) was added and the absorbance of the mixture was measured at 510 nm after standing at room temperature for 15 min. To correct for background absorbance, each sample measurement was accompanied with a simultaneous reaction in which $AlCl_3.6H_2O$ was replaced with water. For the blank, the extracts were replaced with an equal volume of deionised water. A standard calibration curve was prepared with 0.01, 0.05, 0.1, 0.2, 0.4 and 0.6 mg/ml of quercetin (in deionised water). The total flavonoid content was expressed as the mg equivalents of quercetin (QE) per g of extracts. The results were shown in **table 3.5(B).**

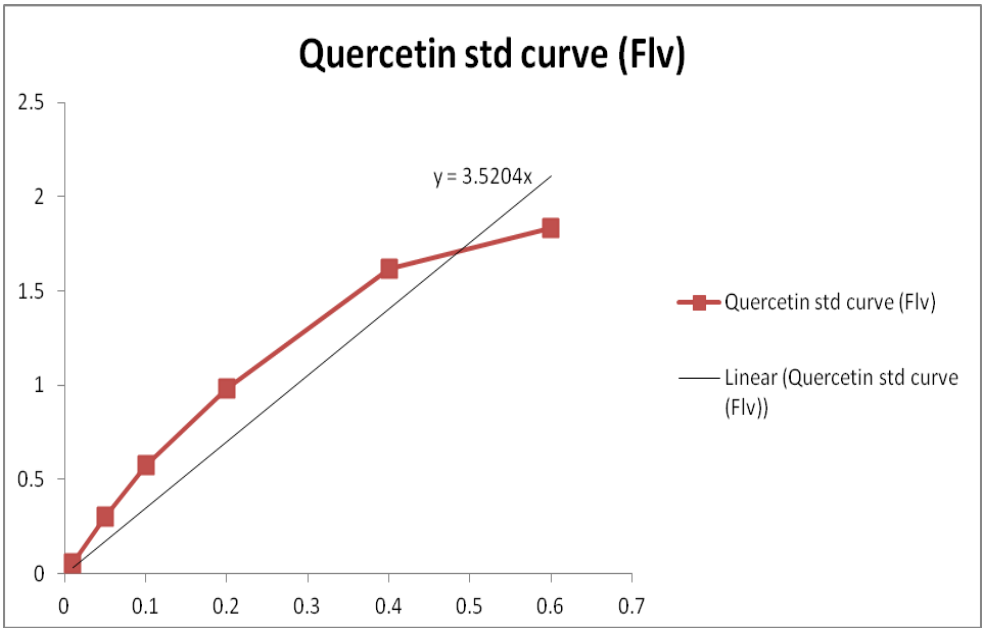


Figure 2.2. Calibration curve for Quercetin

2.7. DETERMINATION OF ANTIOXIDANT CAPACITY

2.7.1. Phosphomolybdate Assay

The total antioxidant capacity of plant extracts was determined by phosphomolybdate method using ascorbic acid as a standard (Umamaheswari & Chatterjee, 2008). An aliquot of 0.1 ml of sample solution was mixed with 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a water bath at 95°C for 90 min. After the samples were cooled down to room temperature, the absorbance of the mixture was measured at 695 nm against a blank on a Thermo Fischer UV-Vis spectrophotometer EVOLUTION 201. A typical blank contained 1 ml of the reagent solution and the appropriate volume of the solvent and incubated under the same conditions. The antioxidant capacity was estimated using following formula:

$$\text{Antioxidant Effect (\%)} = \left(\frac{\text{Control absorbance - sample absorbance}}{\text{Control absorbance}}\right) \text{X } 100$$

Table 3.7(C) shows the result of total antioxidant capacities exhibited by the methanol extracts of the plants.

2.7.2. DPPH Radical Scavenging Activity

DPPH **(**2,2-diphenyl-1-picrylhydrazil) radical scavenging was carried out according to the Blois method with slight modification (Blois, 1958). A standard stock solution of the plant extracts were made by dissolving 10 mg of plant extract in 10 ml of distilled water. 0.0005, 0.001, 0.005, 0.01, 0.025 & 0.05 mg/ml concentrations of each plant extracts were made. 3 ml of plant extract was then mixed with 1 ml of 0.1 mM DPPH solution (in MeOH) and the solution was made 6 ml with distilled water in a test tube. It was then vortexed and incubated at 37^0C for 30 minutes. Absorbance of the solution was then measured at 517 nm using Thermo Scientific

EVOLUTION 201 UV-Visible spectrophotometer. The percentage inhibition was calculated by comparing the absorbance values of the test samples with those of the controls (not treated with extract). The inhibition percentage (I) was calculated as radical scavenging activity as follows:

$$I\ (\%) = \left(\frac{\text{Control absorbance - sample absorbance}}{\text{Control absorbance}} \right) X\ 100$$

Lower absorbance of the reaction mixture indicates higher free radical scavenging activity [**Table 3.7(D)]**. The antioxidant activity of the extract was expressed as IC_{50} [**Table 3.7(E)]**. The IC_{50} value was defined as the concentration (µg/ml) of extract that inhibits the formation of DPPH radicals by 50%.

2.7.3. Statistical Analysis

Experimental results are expressed as mean ± S.E. of three parallel measurements. Statistical analysis is estimated using one way ANOVA followed by Dunnett's test. The values for $p < 0.05$ are considered as significant and values for $p<0.001$ as very significant.

2.8. ISOLATION AND PURIFICATION OF BIOACTIVE COMPOUND

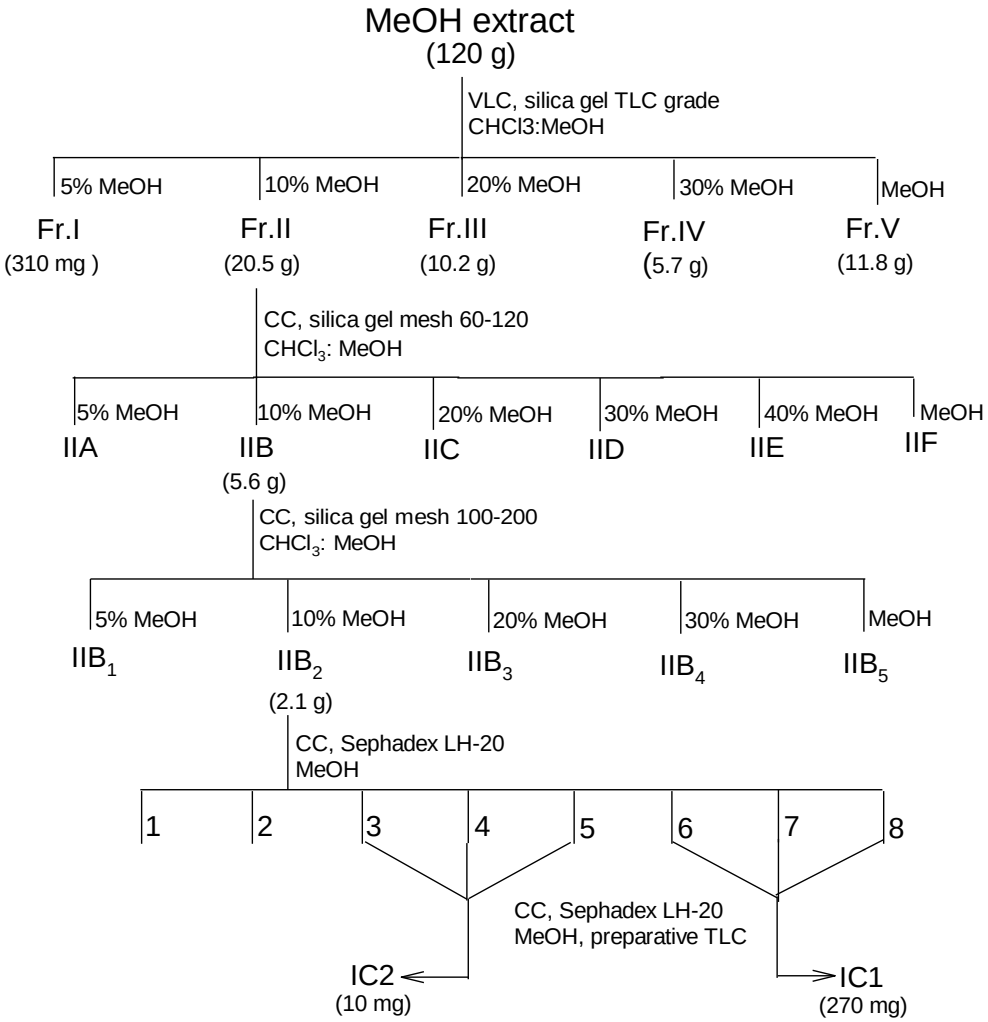
2.8.1. Fractionation of the Extracts

A. Methanol Extract of *Ipomoea cairica* Flower

Preliminary TLC studies of this extract showed phenolic and glycoside type of compounds. The extract was adsorbed on silica gel (mesh 100-200). The VLC column was packed with TLC grade silica and the adsorbed sample was introduced above the column followed by gradient elution starting with $CHCl_3$ with increment of MeOH by 5%, 10%, 20% 30% and 100%). The fractions collected were pooled into 5 major fractions.

Fraction II was concentrated in a vacuo under reduced pressure and column chromatography performed as per standard procedure (Lala, 1981) using silica gel (#60-120)

eluting with $CHCl_3$: MeOH gradient. The fractions collected were pooled together into 6 sub-fractions ($Fr.II_A$ – $Fr.II_F$) based on TLC profile.

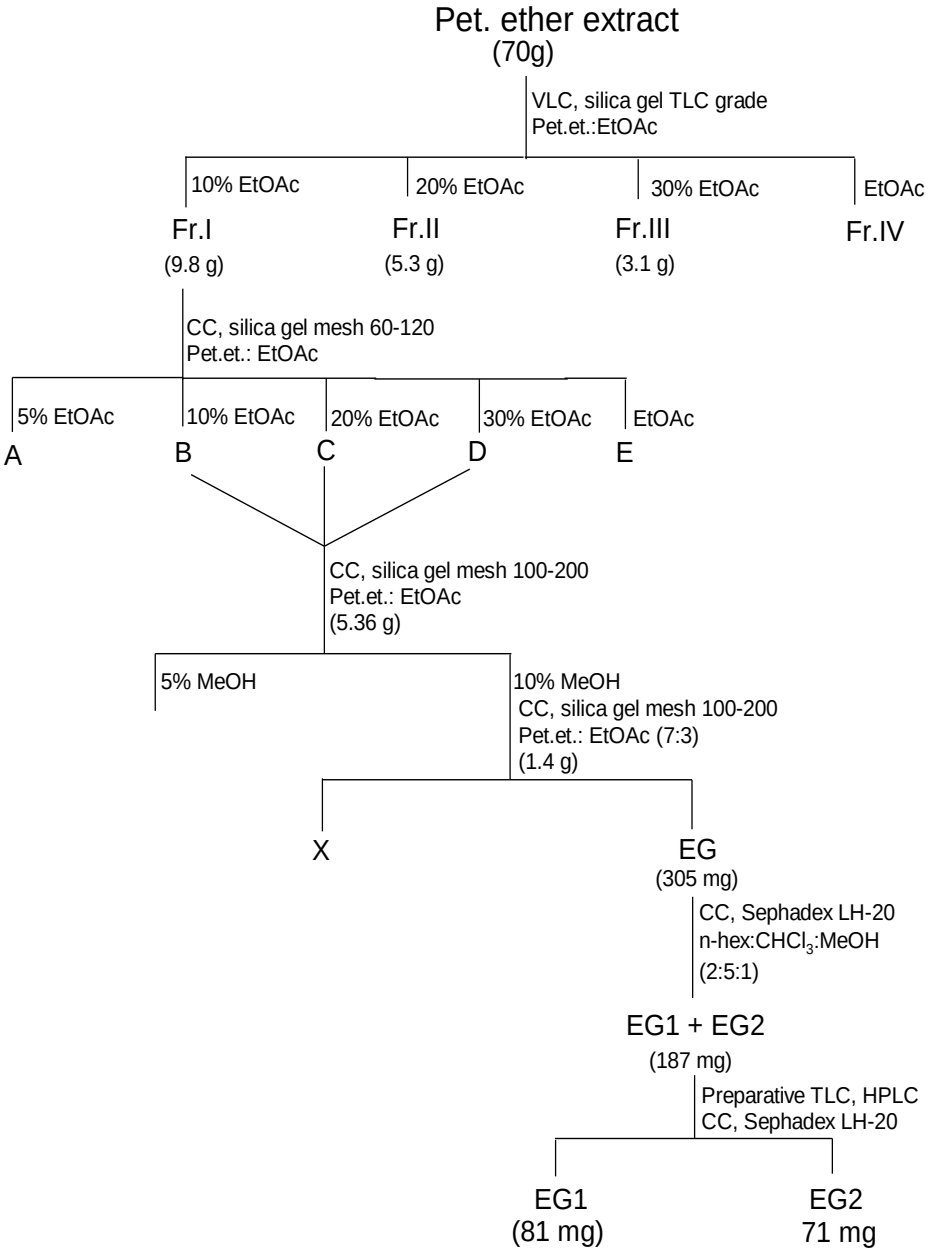


Scheme 2.1. Fractionation of MeOH extract of *I.cairica* and subsequent isolation of the compounds IC1 and IC2

B. Petroleum Ether Extract of *Ageratina adenophora* Leaves

TLC profiling and preliminary tests reveal the presence of terpenoids and sterol type of compounds. TLC was carried out using precoated silica gel-GF_{254} layer (Merck). Fractionation

of petroleum ether extract of *A.adenophora* leaves was also done as per the protocol of 2.8.1(A) (*vide supra*) with slight modification as shown below:



Scheme 2.2. Fractionation of Petroleum ether extract of *A.adenophora* and subsequent isolation of the compounds EG1 and EG2

2.8.2. Isolation of Compound IC1

Isolation and purification of the bioactive compound IC1 were carried out from the methanol extract of the flower of *Ipomoea cairica* after exhaustive fractionation by column chromatography.

(A) Development of Chromatogram by TLC

The TLC profiling of all the extracts were done by spotting the extracts in Aluminium precoated TLC silica gel 60 F_{254} plates activated at 110° C for 1hour (Stahl, 1969) and were eluted with the following solvent systems on trial and error basis:

1. $CHCl_3$:MeOH (9:1, 8:2, 7:3, 6:4)
2. $CHCl_3$:MeOH: H_2O (70:30:5, 60:40:5)
3. $CHCl_3$:MeOH: HCOOH (7:2:1, 7:3:1)
4. n-BuOH: AcOH: H_2O (4: 1:5)
5. Butanone : EtOAc: HCOOH: H_2O (3:5:1:1)
6. $CHCl_3$: MeOH: H_2O: 5% HCOOH (80:13:2:5)
7. EtOAc: AcOH: HCOOH: H_2O (100:11:11:27)
8. Toluene: EtOAc: HCOOH: MeOH (3:3:0.8:0.2, 3:3:0.6:0.4)

TLC Chromatograms were developed in chromatographic chambers using selected solvent systems at room temperature and at an angle of 70°. The plates were removed from the chamber after completion of the run and were allowed to dry in air. Spots on TLC were detected under UV light (254 and 366 nm) and by spraying with conc.H_2SO_4 followed by heating at 100 °C for 5 minutes and sprayed with Dragendorff reagent / 2% $FeCl_3$ solution to observe the presence of different class of compounds in the extracts (Horhammer *et al*., 1964).

(B) Separation and Isolation by Column Chromatography

10% MeOH (in $CHCl_3$) fraction of the methanol extract was dissolved in a minimum amount of methanol, adsorbed on silica gel to form a slurry, air dried till free flowing and chromatographed over silica column (using silica gel, 60-120 mesh size). The column was eluted successively with $CHCl_3$ and methanol of increasing order of polarity (10:0; 9:1; 8:2; 7:3; and 0:10). The solvent elution rate was adjusted at 40 drops per minute. Fractions of approximately 100 ml each were collected and monitored by TLC. Similar fractions were pooled together into 6 sub-fractions labelled as $Fr.II_A$ – $Fr.II_F$. Fraction II_B obtained from 10% methanol fraction was again re-chromatographed using silica gel 100-200 mesh size as adsorbent on a smaller column. Here again the 10% MeOH fraction showing three distinct spots on TLC plates was concentrated and re-chromatographed on Sephadex LH-20 column using methanol as eluent. Solvent elution rate was maintained at 4 drops per minute. Eight fractions were collected from the sephadex column. Fractions 6, 7 & 8 each giving dark spot on TLC plates at 254 nm and mobile phase toluene (3): EtOAc (3): HCOOH (0.6): MeOH (0.4) are coupled, dissolved in minimum amount of MeOH and re-chromatrographed on the same column using the same mobile phase. Compound code named IC1 was isolated from this fraction. IC1 was then washed with MeOH, evaporated and kept in a refrigerator for 5 days to enhance crystallization and purification.

2.8.3. Isolation of Compound IC2

Fractions 1,2,3,4 & 5 obtained from sephadex column described in section 2.8.4.(B) above were coupled. These fractions exhibit blue spots as major compound on TLC plates at 366 nm when eluted with $CHCl_3$(80): MeOH (13): H_2O (2): 5% HCOOH (5) as mobile phase. Repeated fractionation was carried out on the same sephadex column using the same eluent

giving six fractions A – F. Fractions D,E & F producing blue spot under long wavelength when chromatrographed again with the same mobile phase were coupled. These coupled fractions were again re-chromatographed on the same column using MeOH as mobile phase where flow rate is maintained at 2 drops per minute. Compound IC2 was isolated from the coupled fractions of 3, 4 & 5. It was further purified by preparative TLC using glass backed preparative plates 20 cm x 10 cm sizes of approximately 1mm thickness. The combined fraction above was eluted again with $CHCl_3$: MeOH :: 80:20. The distinct blue spot was scraped from the plate, and the silica gel was extracted overnight in methanol; the extracts were concentrated in a flash evaporator, followed by elution on Sephadex LH-20 column by isocratic elution using methanol as mobile phase. The collected fractions were coupled again in a beaker and dried. The isolated compound is code named IC2.

Figure 2.3. Photograph of column chromatography using silica gel as stationary phase (A) mesh 100-200 and (B) mesh 60-120

2.8.4. Isolation of Compounds EG1 and EG2

(A) Development of Chromatogram by TLC

Petroleum ether extract of *Ageratina adenophora* leaves was subjected to thin layer chromatography using silica gel F_{254} as stationary phase and various solvent systems were tried to optimize solvent system which gives better resolution. Detection was carried out by Anisaldehyde sulphuric acid reagent. For the present study, petroleum ether: ethyl acetate (9:1) and petroleum ether: chloroform: methanol (5:2:1) as mobile phase gave better resolution and its polarity can be increased by gradient increasing portion of ethyl acetate viz 8:2, 7:3 and in a similar manner subsequently on column chromatography. The chromatograms when developed in iodine chamber yielded five or six spots respectively that showed zones for steroidal nucleus with Liebermann – Buchard visualizing reagent.

(B) Separation and Isolation by Column Chromatography

The crude petroleum ether extract of *Ageratina adenophora* leaves was subjected to column chromatography using glass column of internal diameter 450 mm and silica gel (mesh 60-120) as stationary phase. Column was eluted with 100% petroleum ether followed by gradient elution of increasing polarity with increment of EtOAc by 5%, 10%, 20% 30% and 100%). The combined fractions of fraction 10-50 collected from petroleum ether : EtOAc (9:1, 8:2 and 7:3) were pooled together and subjected to column chromatography using glass column of internal diameter 20 mm and silica gel (mesh 100-200) as stationary phase and eluted with same mobile phase system. The column was first eluted with 5% EtOAc in petroleum ether followed by 10% EtoAc. Fraction No. 10-20 showed same TLC pattern and combined and subjected to further purification by again column chromatography in column of 20 mm diameter and eluted with solvent system Petroleum ether : EtOAc (7:3). A total of 25 fractions were collected and the

combined fraction of fraction no. 11 – 25 yields two white compounds after solvent evaporation. These compounds were labeled EG1 and EG2

Separation of EG1 and EG2 in the mixture was quite difficult. They are assumed to be the mixture of β-sitosterol and stigmasterol. The only difference between the two compounds is the presence of C_{22}=C_{23} double bond in Stigmasterol and C_{22}-C_{23} single bond in β-Sitosterol, hence, the lack of practical difference in their R_f despite the use of several solvent systems. Furthermore, literatures have shown that sitosterol is difficult to be obtained in pure state. (Pollock & Stevens, 1965; Fieser & Fieser, 1962).

However, the separation of requisite purity was achieved by preparing TLC plates with Silica gel coated with silver nitrate (Si gel treated with silver nitrate dissolved in methanol as follows: 100 g Silica gel G stirred with 10 g silver nitrate dissolved in methanol (100 mL) to get an homogeneus slurry and then the solvent evaporated in a rotary evaporator. The argentic silica gel thus obtained was used to prepare the plates). A mobile phase hexane-acetate 83:17 was used to run the plate to get two developments labeled as EG1 and EG2.

(C) Test for Sterols on EG1 and EG2

From the results of preliminary phytochemical analysis of petroleum ether extract of *Ageratina adenophora* leaves, it was found that it gives positive result for the presence of sterols and terpenoids. Therefore, the isolated compounds EG1 and EG2 were tested by (a) Salkowski reaction and (b) Liebermann-Burchard reaction to confirm their steroidal nature:

(a) Salkowski Reaction: A few amount of the isolated compounds EG1 and EG2 were dissolved in $CHCl_3$ and 2-3 drops of conc. H_2SO_4 was added to each solution. It was found that both EG-1 and EG-2 formed reddish brown colour in the upper $CHCl_3$ layer indicating their steroidal character.

(b) Liebermann-Burchard reaction: Few amount of the isolated compounds EG1 and EG2 were again dissolved in $CHCl_3$ and 2-3 drops of conc. H_2SO_4 was added to each solution, followed by the addition of 1-2 drop of acetic anhydride. It was observed that both EG1 and EG2 solutions turned to violet blue and finally formed green colour which, once again confirm their steroidal character (Harborne, 1998)

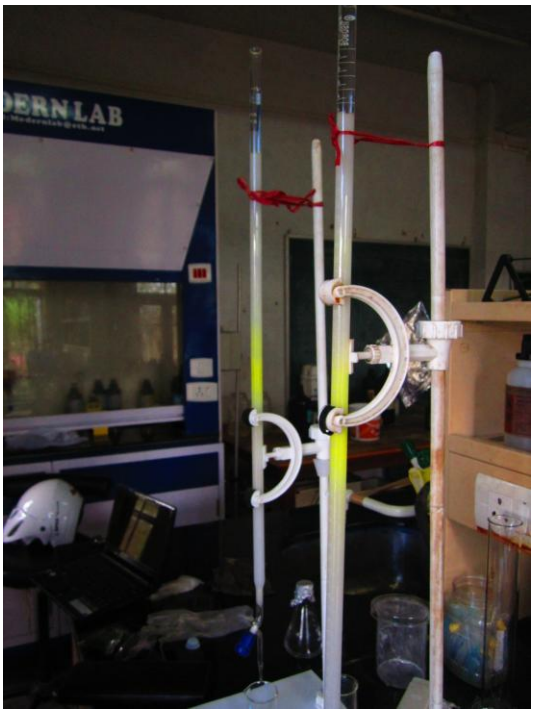


Figure 2.4. Photograph of column chromatography using sephadex LH-20 as stationary phase

2.8.5. HPTLC Analysis

(A) Method Development for Analysis by HPTLC

A novel method for HPTLC analysis of the methanolic crude extracts, petroleum ether extract of *Ageratina adenophora* leaves and isolated compound was developed**.** The instrument used was Camag Linomat V (semi-automatic spotting device) with Camag 100µl HPTLC syringe, Camag twin trough chambers (20 x 10cm), Camag TLC Scanner 3 and Camag WINCATS 4 Integration software. For all the HPTLC analysis, stationary phase used was

precoated silica gel 60 F_{254} plate (E. Merck, methanol washed, thickness 0.2mm, 10 x 10cm).The spotting parameters included start position of 15mm from bottom edge, band width of 8 mm, space between two bands 12mm and spraying rate of 150 nl/sec. The chromatographic conditions included ascending separation technique, twin trough chamber for plate development, chamber saturation time 4min and migration distance 10cm at a temperature of 25 ± 2°C. Detection was done in UV-visible range. Densitometric scanning was carried out in Absorbance/Reflectance mode using Mercury Lamp and Slit dimension 4 x 3mm.

(B) HPTLC Analysis of Compound IC1

The isolated compound labeled IC1 was dissolved in MeOH. HPTLC was performed on 10 cm × 10 cm commercially made aluminium backed plates coated with silica gel 60 F_{254} (E. Merck & Co Ltd, Mumbai, India). Standard solution of gallic acid and sample solution were applied to the plates as bands 8.0 mm wide, 30.0 mm apart, and 10.0 mm from the bottom edge of the same chromatographic plate by use of a Camag (Muttenz, Switzerland) Linomat V sample applicator equipped with a 100-L Hamilton (USA) syringe. Ascending development to a distance of 80 mm was performed at room temperature (28 ± 2°C), using toluene : ethyl acetate : formic acid : water 3 : 3 : 0.6 : 0.4. as mobile phase, in a Camag glass twin-trough chamber previously saturated with mobile phase vapor for 20 minutes. After development, the plate was dried with hair dryer and was observed after spraying with 2% $FeCl_3$ reagent as well as under UV radiation (365 nm). It was then scanned using Scanner 3 (CAMAG, Switzerland) at 280 nm using WinCATS 4 software using the deuterium lamp.

(C) HPTLC Analysis of Compound IC2 and MeOH extract of *Ipomoea cairica* Flower

Methanol extract of *Ipomoea cairica* flowers (10mg/ml) and isolated fraction IC2 residue (1mg/ml) was subjected to HPTLC (CAMAG, Switzerland) analysis. Extract and the isolated compound were spotted on a silica gel 60 F_{254} (Merck, Darmstadt, Germany) TLC plate. The

plate was dried with hair drier and then developed by using the solvent system chloroform: methanol (8:2 v/v) as mobile phase in a CAMAG- twin-trough glass chamber previously saturated with mobile phase vapor for 20 min. After developing the plate, it was dried at 105ºC for 15 min and then it was scanned using Scanner 3 (CAMAG, Switzerland) at 366 nm using WinCATS 4 software.

(D) HPTLC Analysis of EG1, EG2 and Pet. Ether Extract of *A. adenophora* Leaves

The isolated compounds EG1 and EG2 and Petroleum ether extract of *Ageratina adenophora* leaves were dissolved in chloroform and spotted onto 10 x 10 cm silica coated aluminum TLC plate (Merck F_{254}) with n-hexane : EtOAc (9:1, v/v) as mobile phase for sterol identification. Visualization of spots was afforded by spraying developed plates with 5% ethanolic H_2SO_4, followed immediately by 1% vanillin in ethanol. Spots developed after approximately 10 minutes of heating at 80–100ºC (John *et al*., 2004). Alternatively, the TLC plate was also developed for ±10 minutes, dried at room temperature and developed by dipping into a solution containing vanillin (15 g), EtOH (250 ml) and conc. H_2SO_4 (2.5 ml) for 15 s and dried at room temperature. Once dried, plate was heated at 100˚C for five min (Boukes *et al*., 2008).

2.8.6. HPLC Analysis

RP-LC is the dominant HPLC mode, especially for the separation of mixtures of organic compounds (Snyder *et al.*, 2010). In RP-LC, the column, packed with derivatised silica particles, acts as a non-polar retaining stationary phase. Support-based stationary phases such as octadecyl (C18), octyl (C8), phenyl-hexyl (Ph- C6) and cyano (CN) phases are commonly employed. The binary mobile phase is of mid to high polarity and consists of an aqueous phase, which may be slightly acidified, and an organic component. Acetonitrile and methanol are the preferred organic

solvents, whilst isopropanol and tetrahydrofuran may also be used (Snyder *et al*., 2010). RP-LC provides separation according to hydrophobicity – analyte molecules partition between the non-polar stationary phase and the polar mobile phase, leading to increased retention of the less polar analytes (Meyer, 2010; Snyder *et al.*, 2010).

HPLC analyses for all the isolated compounds were performed by employing Reversed Phase Water Ass HPLC (model no. 2998) system. This system is equipped with ì-Nova-Pak reverse phase C_{18} 8x10 RCM column (Water Ass), a PDA detector (Water 996 PDA detector, Water Ass) with Empower® software, for the separation, detection and data analysis respectively, at 27^0C and at a pressure of 2600 psi.

Following the absorption maxima in the UV-vis. spectra, the MeOH extract of *I. cairica* flower was analyzed for its RP-HPLC chromatogram in an isocratic mode using methanol as mobile phase at a flow rate of 0.2 ml per minute and an injection volume of 40 µL. PDA absorbance detections were performed at 220 nm, 280 nm, 325 nm and 620 nm. On the basis of the occurrence of λ_{max} in their UV-visible spectra, RP-HPLC analyses were also performed at for pet. ether extract of *A. adenophora*, at 280 nm, 325 nm and 620 nm for MeOH extract of *A. adenophora* and at 220 nm for methanol extract of *I. cairica* leaves. Here also, separations were done in the isocratic mode, using methanol as mobile phase at a flow rate of 0.2 ml min^{-1}; with an injection volume ("loop") of 40 µL.

(A) HPLC Analysis of IC1

For RP-HPLC analysis of the isolated compound, IC1 separation was done in the isocratic mode, using methanol as mobile phase at a flow rate of 0.1 and 0.2 mL min^{-1}; with an injection volume ("loop") of 40 µL. PDA detection was at done at 280 nm.

(B) HPLC Analysis of IC2

Isolated compound, IC2 was also analyzed using the same RP-HPLC system by isocratic elution using methanol as a mobile phase at a flow rate of 0.2 ml per minute. Here, detections were done at 280 nm.

(C) HPLC Analysis of EG1 & EG2

The isolated compounds EG1 and EG2 were analyzed by employing a column equipped with Zorbax 300SB-C18 5-µm column (250- × 4.6-mm i.d.) (Agilent, Palo Alto, CA) using absorbance detection at 202 nm with a variable wavelength PDA detector. The mobile phase consisted of ACN and water (95:5 v/v), filtered through a 0.2 µm membrane filter and degassed in an ultrasonic bath prior to use. The mobile phase was delivered at a flow rate of 0.2 ml/min in the isocratic mode.

2.9. STRUCTURAL CHARACTERIZATION OF ISOLATED COMPOUNDS

Several spectroscopic techniques are used in the identification and characterization of new natural product compounds, such as mass spectrometry (MS), nuclear magnetic resonance (NMR), ultraviolet spectroscopy (UV), infrared spectroscopy (IR), and X-ray crystallography. Melting points of the isolated compounds were also recorded. Melting points were determined on a melting point apparatus (JSGW, Model-3046).

2.9.1. UV-Visible Analysis

Solution of the isolated compound in methanol was scanned from 200 – 700 nm range at 1 nm interval in the ultraviolet/visible spectrophotometer (Thermo Fischer EVOLUTION 201) against solvent blank (Methanol and Acetonitrile). The spectrum was recorded, interpreted and compared with some obtained from literature.

2.9.2. Infra Red Spectroscopy Analysis

FT-IR spectroscopy is a form of Vibrational spectroscopy, and the FT-IR spectrum reflects both molecular structure and molecular environment. In this technique, the sample is irradiated with infrared radiation from an infrared source, and the absorption of this radiation stimulates vibrational motions by depositing quanta of energy into vibrational modes (Gomez *et al*., 2003; Sacksteder and Barry, 2001). FT-IR is used in the natural product chemistry for the identification of new compounds isolated from various plant species (Purohit *et al*., 1991) A molecule, when exposed to radiation produced by the thermal emission of a hot source (a source of IR energy), absorbs only at frequencies corresponding to its molecular modes of vibration in the region of the electromagnetic spectrum between visible (red) and short waves (microwaves) (Margarita and Quinteiro, 2000). These changes in vibrational motion give rise to bands in the vibrational spectrum; each spectral band is characterized by its frequency and amplitude (Sacksteder and Barry, 2001).

The FT-IR spectrum of each isolated compound was recorded in the using Thermo Fischer FT-IR spectrophotometer. A thin microfilm of the isolated compounds were prepared, placed in the optical path of the instrument and scanned over 4000 to 500 cm^{-1} frequency regions at 1 cm^{-1} interval. The absorption bands were obtained and recorded as frequency (cm^{-1}) against per cent transmittance (%T). The spectrums were interpreted to deduce the functional groups present in the compounds. Maximum of 32 scans were accumulated for each spectrum using the Horizontal Attenuated Total Reflection (HATR) device, using a Thermo Fischer FT-IR spectrometer (with apodization Happ-Genzel). The spectral data were processed using the IR solution Software Overview (Thermo Fischer) and Origin R 7SR1 Software. Each recorded spectrum was the result of 32 coadded scans.

2.9.3. NMR Analysis

Since the development of the high resolution NMR spectrometer in the 1950s, NMR spectra have been a major tool for the study of both newly synthesized and natural products isolated from plants, bacteria etc (Ulrike *et al*., 1998). NMR spectroscopy measures the resonances of magnetic nuclei such as ^{1}H, ^{13}C and ^{15}N that interact with an external magnetic field (Hatada and Kitayama 2004). It offers non-invasive structural analysis of metabolites in crude extracts, cell suspensions, intact tissues or whole organisms allowing in vivo analysis (Fan and Lane 2008). NMR spectra are unique and specific for each single compound (Verpoorte *et al*. 2008) and can be used to identify metabolites of biological origin of which no a-priori knowledge is needed (Fan and Lane 2008).

NMR analysis of the isolated compounds were done on samples prepared as solutions in *d*-DMSO for IC1 and deuterated chloroform ($CDCl_3$) for EG1 and $CDCl_3$ + 2-3 drops of d-DMSO for EG2 respectively, and the spectrum obtained using a 200 and 500 MHz NMR spectrophotometer. Hydrogen – hydrogen correlation analysis were performed to monitor the coupling of the various proton environments, within the chemical structure of the isolated compound.

^{13}Carbon NMR spectra were recorded at 125 MHz and ^{1}H NMR spectra at 500 MHz on a Bruker spectrometer (Germany). Chemical shifts were recorded in delta units from the internal standard of tetramethylsilane.

2.10. MOSQUITO LARVICIDAL EFFICACY OF THE ISOLATED COMPOUND, IC1

(A) Preparation of stock solution

A Standard 1% stock solution of the isolated compound IC1 was prepared by dissolving 100 mg of IC1 in 10 ml of EtOH as per WHO, 2005 guidelines with slight modification. Suitable

amount of sterilized distilled water were then added for the formulation of graded concentrations and these solutions were used for larvicidal bioassay.

(B) Mortality and dose–response larvicidal bioassay (LC_{50})

Required concentrations of each plant part solvent extracts (concentrations of 50, 100, 200, 400, and 500 ppm) were prepared through the mixing up of stock extract with variable amounts of sterilized distilled water. Each of the earlier prepared concentrations of solvent extracts were transferred into the sterile glass beakers (500 ml capacity).For bioassay test, third instar larvae of *C. quinquefasciatus* were divided into respective groups in four batches of 25 numbers in 249 ml of water and individually added with 1.0 ml of different concentrations (50, 100, 200, 400, and 500 ppm) of appropriate plant solvent extract. No food was provided during the treatment. Mortality was recorded after 24 and 48 h of post-exposure (WHO 1981). Dead larvae were identified when they failed to move after probing with a needle in the siphon or cervical region. The experiments were observed four times and conducted at 27±2°C and 80–90 % relative humidity. The untreated control was set up with 70% ethanol solvent. The corrected mortality was calculated by Abbott's formula (Abbott 1925).

(C)Time–response larvicidal bioassay (LT_{50})

One milliliter of standard stock solution of IC1 was added to 249 ml distilled water in a 500-ml plastic cup, which was shaken lightly to ensure a homogeneous test solution. Twenty-five specimens each of third instar larval stages of *C. quinquefasciatus* were divided into respective groups and placed in cups. No food was provided during the treatment. Lethal time was observed at the concentrations of 50,100, 200, 400and 500 ppm. Mortality was recorded at regular intervals of 1, 2, 3, 4, 5, 8, 12, 15, 16, 18, 20, 24, 28, 30, 32, 35, 36, 40 and 48 h of post-exposure in each concentration. Larvae were considered dead if they were incapable of rising to

the surface or did not show the characteristic dicing reaction when the water was disturbed (WHO 1981). The mean mortality number was recorded. Each experiment was performed in four replicates with a simultaneous control (1 ml 70 % EtOH in 249 ml water).

(C) Statistical analysis

The LC_{50} and $LC_{90,}$ values were calculated after 24 and 48 hours by EPA probit analysis program version 1.5 (Finney, 1979). Log probit was calculated to generate regression equation, LC_{50}, and LC_{90} values (95 % class intervals) with upper and lower fiducial limits, slope, intercept regression coefficient and chi-square values. Tukey's multiple range test ($P<0.05$) were used for taking statistical decisions on yield of plant extracts, dose and time mortality data (Snedecor and Cochran 1989). Results with $p<0.05$ were considered to be statistically significant.

2.11. INTERACTION OF TRANITION METAL IONS WITH PHYTOCHEMICALS

Complex 1

0.5 mM copper nitrate trihydrate was dissolved in minimum amount of methanol. To this azure solution, 0.5 mM dpt (diehylene triamine) was added dropwise resulting in a deep blue coloured solution. This solution turned as bluish-green in colour with the addition of 1 mM vanillic acid dissolved in methanol-acetonitrile mixture (50:50 , v/v) dropwise with slow stirring. The solution was filtered and was allowed to stand at RT for crystallization.

Complex 2

0.5 mM copper nitrate trihydrate was dissolved in 5 ml of acetonitrile. The solution turned pale blue and to this solution, 0.5 mM of dpt was added slowly dropwise leading to the formation of a deep blue coloured solution. A solution of 1 mM protocatechuic acid dissolved in a mixture of acetonitrile-methanol (4:1 ratio) was added dropwise with stirring to the deep blue coloured

copper complex solution. The resulting solution that exhibited a characteristic green colour was filtered and kept aside in cool area for crystallization.

Complex 3

0.5 mM copper nitrate trihydrate was dissolved in a mixture of acetonitrile and methanol (50:50, v/v). In this bluish green solution, a solution of 0.5 mM vanillic acid dissolved in a mixture of acetonitrile and methanol (50/50, v/v) was added dropwise slowly with constant stirring. The solution that turned green was filtered and kept at RT for crystallization.

Complex 4

0.5 mM copper nitrate trihydrate was dissolved in a mixture of acetonitrile and methanol (50:50, v/v). To the resulting bluish green solution, a solution of 1 mM protocatechuic acid dissolved in 10 ml of a mixture of acetonitrile and methanol (3:2 ratio) was added dropwise. Immediately, the solution turned into a characteristic red coloured solution. When the solution was then allowed to stand at room temperature for a minute or two, the solution precipitated out a red coloured solid. This red solid was filtered out and the remaining pale red coloured mother liquor was kept in cool room for crystallization.

Complex 5

To a solution of 0.5 mM copper nitrate trihydrate dissolved in a mixture of acetonitrile and methanol (50:50, v/v), 1 mM vanillic acid, dissolved in a mixture of acetonitrile and methanol (3:2 ratio) with a total volume of 10 ml, was added dropwise with stirring. After completing the addition of vanillic acid, the solution was kept aside. After few minutes, a red coloured precipitate was settling at the bottom of the reaction vessel. The mother liquor was carefully filtered and the resulting reddish yellow solution was kept for crystallization by slow evaporation.

Complex 6

The copper complex was prepared by 0.5 mM copper acetate dihydrate dissolved in 10 ml of methanol. To this turquoise green solution, a solution of 1 mM 1,10-phenanthroline dissolved in 10 ml of a mixture of acetonitrile and methanol (3:2 ratio) was added dropwise with constant stirring. The solution turned azure colour. To this azure blue colour solution, 0.5 mM vanillic acid dissolved in acetonitrile and methanol (3:2, v/v) was added dropwise with stirring. The solution that turned characteristic green colour with the addition of vanillic acid was filtered and it was allowed to stand at RT for crystallization.

Complex 7

0.5 mM copper nitrate trihydrate was dissolved in a minimum amount of methanol and a solution of 1 mM 1,10-phenanthroline dissolved in 10 ml solvent mixture of acetonitrile and methanol (3:2 ratio) was added dropwise with constant stirring. In the resulting azure blue colour solution, 0.5 mM protacatechuic acid, dissolved in a mixture of acetonitrile and methanol (4:1 ratio) was added dropwise and the resulting solution exhibited a characteristic olive green colour. This solution was filtered and kept in a cool shaded area for crystallization by slow evaporation.

CHAPTER 3

RESULTS AND DISCUSSIONS

3.1. Studies on Ethno medicinal Importance and Total Yield in Sequential Extraction

The medicinal plants tested, *I. cairica* and *A. adenophora* for the bioactivity against *C. quinquefasciatus* are shown in **Table 3.1.** The percentage yield in the sequential extraction of the two plants under study based on the polarity of the solvents used viz., non polar (Petroleum ether), medium polar ($CHCl_3$) and polar (MeOH) is provided (**Table 3.2**). The total yield of *I. cairica* and *A. adenophora* extract in the soxhlet extraction from 500 g of leaves/flowers was as follows: a) *A. adenophora* leaf - petroleum ether extract, 14.82 g; Chloroform extract, 11.68 g; and methanol extract, 5.33 g; b) *I. cairica* flower - petroleum ether extract, 3.96 g; chloroform extract, 11.96 g; and methanol extract, 19.35 g; and c) *I. cairica* leaf - petroleum ether extract, 1.25 g; chloroform extract, 16.45 g; and methanol extract, 6.58 g (**Table 3.2**). In the sequential extraction of test plant powders with petroleum ether, chloroform and methanol, *A. adenophora* leaf powder gave their lowest yield in methanol extraction (1.07%) while higher yield in petroleum ether (2.96%) and chloroform (2.34%) extraction ($p<0.0001$). In *I. cairica* leaf powder showed higher yield for chloroform extraction (3.29%) followed by methanol extraction (1.32%) and lowest yield for petroleum ether extraction (0.25%). Higher yield ($p<0.0001$) was observed for methanol (3.87%) and chloroform (2.39%) extraction in *I. cairica* flower powder. In overall, the yield percentage is significantly high ($p<0.0001$) in moderately polar solvents than non-polar solvents. Leaves of *A. adenophora* plant contain higher amount of non-polar compounds while flowers and leaves of *I. cairica* comprise moderately polar compounds, respectively (**Table 3.2**).

Solvents, such as methanol, ethanol, acetone, ethyl acetate, and their combinations have been used for the extraction of phenolics from plant materials, often with different proportions of water. Selecting the right solvent affects the amount and rate of polyphenols extracted (Xu & Chang, 2007). The classic mode of extraction naturally depends on the texture and water content of plant materials. In particular, methanol has been generally found to be more efficient in extraction of lower molecular weight polyphenols, while the higher molecular weight flavanols are better extracted with aqueous acetone (Metivier *et al*., 1980; Prior *et al*., 2001; Guyot *et al*., 2001; Labarbe *et al*., 1999). Ethanol is another good solvent for polyphenol extraction and is safe for human consumption (Shi *et al*., 2005)

The recovery of phenolic compounds from plant materials is also influenced by the extraction duration and temperature, which reflects the competing actions of solubilization and analyte degradation due to oxidation (Robards, 2003). An increase in the extraction temperature can promote higher analyte solubility by increasing both solubility and mass transfer rate. In addition, the viscosity and the surface tension of the solvents are decreased at elevated temperature, which facilitates the solvents to diffuse to the sample matrices, improving the extraction rate. However, many phenolic compounds are easily hydrolyzed and oxidized. Soxhlet extraction is found to be very convenient method for extraction of small to moderate volumes of plant material as the extraction takes place in a closed system in which the solvent is continually recycled, the amount of solvent needed is minimal, however the heat needed to drive the extraction is likely to cause thermolabile constituents to form artifacts or decomposition products. In our system, since maceration and soxhlet extraction are employed, the longer extraction duration (maceration) and high temperature (soxhlet) aggravate the possibility of oxidation of phenolics which may decreases the yield of phenolics in the extracts.

Table 3.1. List of Medicinal Plants Tested for Antioxidant Activity and Bioactivity Against *Culex quinquefasciatus*

Botanical name	Common name	Family	Medicinal property	Plant parts tested	Reference
Ipomoea cairica (L.) Sweet (Synonyms - *I. palmata* Forssk, *I. stipulacea* (Jacq.) *I. tuberculata*	Taiwan morning glory, Rheum Petunia, Cairica Dragon, Railway creeper, Palmate-leaved and Morning Glory.	Convolvulaceae	Antimicrobial, antioxidant, analgesic, spasmolitic, spasmogenic, hypoglycemic, hypotensive, anticoagulant, anti-inflammatory, psychotomimetic and anticancer activities and inhibition of HIV replication. Treatment for rheumatism, dysentery, constipation, fatigue, arthritis, hydrocephaly, meningitis, kidney ailments, body rash, cooling blood, strong bones; attending traumatic injury, fractures, rheumatic pain, amenorrhea and mosquito larvicidal activity.	Flower and leaf	Thomas *et al*. (2004) Ferreira *et al*. (2006) Meira *et al*. (2012)
A. adenophora (Spreng.) King & H.Rob. (Synonyms - *Eupatorium adenophorum*, *E. glandulosum*)	Eupatory, Sticky snakeroot, Crofton weed, and Mexican devil.	Asteraceae	Antimicrobial, antiseptic,blood coagulant,analgesic, antioxidant, antipyretic and enhancer of phenobarbitone induced sleep, anti-acetyl cholinesterase, mosquito larvicidal activity, and insect antifeedant.Treatment for cut or injury, diarrhea,dysentery and abortifacient.	Leaf	Shrestha & Dhillion, (2003); Yadav *et al*., (2010); Teodora & Ashlyn (2011); Rajeswary and Govindarajan (2013)

Table 3.2. The Percentage of Yield in the Sequential Extraction of Different Parts of *Ageratina adenophora* and *Ipomoea cairica*

Solvent	Ageratina adenophora		*Ipomoea cairica*			
	Leaf		Leaf		Flower	
	Yield (g)	Yield (%)	Yield (g)	Yield (%)	Yield (g)	Yield (%)
Petroleum ether	14.82	2.96a	1.25	0.25a	3.96	0.79a
Chloroform	11.68	2.34b	16.45	3.29b	11.96	2.39b
Methanol	5.33	1.07c	6.58	1.32c	19.35	3.87c

Means ± SE followed by different letters within the same column are significantly different (Tukey's test, P<0.0001)

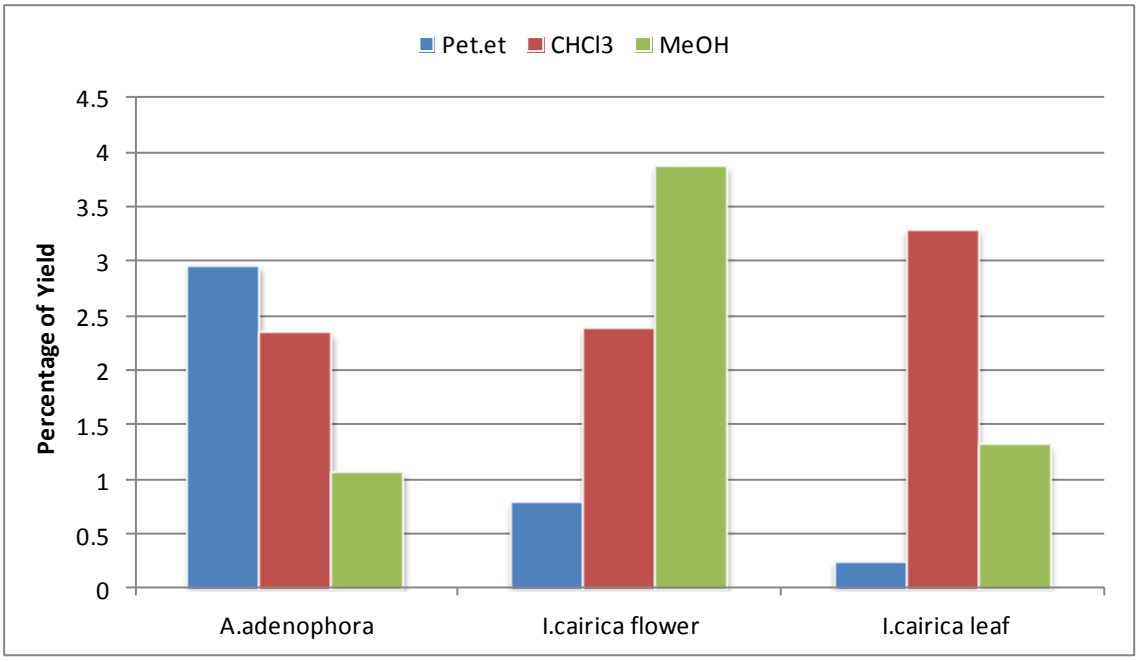


Figure 3.1. Bar Diagram of the Percentage of Yield in the Sequential Extraction of Different Parts of *A. adenophora* and *I. cairica*

3.2. Studies on Phytochemical Screening of Extracts

The results of phytochemical group tests for all the crude extracts of the two plants under study were provided in **table 3.3.** The results demonstrated the presence of alkaloids, sterols, flavonoids, reducing sugars, tannins, saponins, terpenoids, anthraquinones, glycosides and phenols. For *A. adenophora*, although, flavonoids, anthraquinones, terpenoids and glycosides are present in all the fractions, existence of steroids is confirmed only in the petroleum ether extract. Tannins, saponins, and phenols are present only in the methanol extract, while, reducing sugars are present in both $CHCl_3$ and MeOH fractions. In the leaves of *Ipomoea cairica*, terpenols and glycosides are present in its petroleum and $CHCl_3$ fractions, sterols in petroleum and MeOH fractions while reducing sugars and anthraquinones are exclusively present in the $CHCl_3$ fraction. The MeOH extract also contains alkaloids, flavonoids, tannins, saponins, anthraquinones and phenols. The flower extract of *Ipomoea cairica* contains sterols (all extracts), flavonoids, tannins, saponins and phenols (MeOH extract), reducing sugars ($CHCl_3$ and MeOH), and terpenoids and glycosides (both in petroleum and $CHCl_3$ fractions).

The presence of phenolic compounds may be the main reason why the methanolic extract of *Ipomoea cairica* flowers showed high antioxidant activity and remarkable larvicidal activity since phenolic compounds have traditionally been of great interest to humans because of their pronounced physiological, medicinal and pesticidal properties. Studies on larvicidal effect of methanol extract of *H. leucomela* against 2^{nd} and 3^{rd} instar larvae of *Aedes aegypti* concluded the observed activity could be due to the presence of the phenolic compounds and other secondary metabolites in the extract (Karthik *et al*., 2011). However the moderate larvicidal efficacy of the petroleum extract of *Ageratina adenophora* leaves may also be attributed due to the presence of steroids.

Table 3.3. Results of Phytochemical Screening of Plant Extracts

Sl. No.	Plant Constituents	Test/ Reagent	*Ipomoea cairica* leaves			*Ipomoea cairica* flower			*Ageratina adenophora* leaves		
			Pet ether.	$CHCl_3$	CH_3OH	Pet ether.	$CHCl_3$	CH_3OH	Pet ether.	$CHCl_3$	CH_3OH
1.	Alkaloids	Mayer	-	-	+	-	-	-	-	-	-
		Dragendorff	-	-	+	-	-	-	-	-	+
		Wagner	-	-	+	-	-	-	-	-	+
		Hager	-	-	+	+	-	-	-	-	+
2.	Sterols	Liebermann-Burchard	+	-	+	+	+	+	+	-	-
		H_2SO_4	+	-	+	+	+	+	+	-	-
3.	Flavonoids	Shinoda	+	+	+	-	-	+	+	+	+
		H_2SO_4	-	-	+	-	-	+	-	-	-
		NaOH	-	-	+	-	-	+	-	-	+
4.	Gums	Molisch	-	-	-	-	-	-	-	-	-
5.	Reducing sugars	Fehling	-	+	-	-	+	+	-	+	+
		Benedict	-	+	-	-	+	+	-	+	+
6.	Tannins	Pot. Dichromate	-	-	+	-	-	+	-	-	+
		Lead acetate	-	-	+	-	-	+	-	-	+
		$FeCl_3$	-	-	+	-	-	+	-	-	+
7.	Saponins	Foam	-	-	+	-	-	+	-	-	+
8.	Terpenoids	$CHCl_3$	+	+	-	+	+	-	+	+	+
		Liebermann-Burchard	+	+	-	+	+	-	+	+	+
9.	Anthraquinones	Borntrager									
		Upper layer	-	+	+	-	-	-	-	-	+
		$CHCl_3$ layer	-	+	+	-	-	-	-	-	+
10.	Glycosides	Legal	+	+	-	+	+	-	+	+	+
		Borntrager	+	+	-	+	+	-	+	+	+
11.	Phenols	$FeCl_3$	-	-	+	-	-	+	-	-	+
		Liebermann	-	-	+	-	-	+	-	-	+
12.	Amino acids	Ninhydrin	-	-	-	-	-	-	-	-	-

+Present; -Absent

3.3. Studies on Mosquito Larvicidal Efficacy of the Crude Extracts

TABLE 3.4(A). Mortality of Third Larval Instars of *C. quinquefasciatus* in Different Concentrations of Crude Leaf and Flower extracts of *I. cairica* and *A. adenophora* After 24 Hrs of Exposure

Plant species	Part used	Concentration (ppm)	Percent mortality ± Standard Error		
			Solvent fraction		
			Petroleum ether	Chloroform	Methanol
Ageratina adenophora	Leaf	50	0	0	0
		100	28.26 ± 1.55	0	0
		200	58.25 ± 3.45	32.65 ± 3.65	0
		400	80.17 ± 2.38	49.75 ± 1.98	7.38 ± 3.54
		500	88.35 ± 2.56*	78.45 ± 2.72*	49.32 ± 2.85*
		Control	0	0	0
Ipomoea cairica	Leaf	50	0	3.05 ± 0.65	0
		100	0	5.35 ± 0.75	0
		200	0	14.63 ± 1.27	0
		400	0	23.95 ± 2.65	9.75 ± 0.72
		500	1.00 ± 0.00	39.15 ± 2.15*	13.72 ± 0.58
		Control	0	0	0
	Flower	50	0	0	41.15 ± 2.85
		100	0	7.05 ± 0.31	46.65 ± 3.72
		200	0	19.18 ± 0.59	89.04 ± 3.65
		400	0	40.59 ± 1.64	100.00 ± 0.00
		500	3.85 ± 0.48	48.95 ± 1.55*	100.00 ±.00*
		Control	0	0	0

TABLE 3.4(B). Mortality of Third Larval Instars of *Culex quinquefasciatus* in Different Concentrations of Crude Leaf and Flower extracts of *I. cairica* and *A. adenophora* After 48 Hrs of Exposure

Plant species	Part used	Concentration (ppm)	Percent mortality ± Standard Error		
			Solvent fraction		
			Petroleum ether	Chloroform	Methanol
Ageratina adenophora	Leaf	50	0	0	0
		100	41.65 ± 1.65	0	0
		200	67.82 ± 2.95	41.85 ± 3.65	0
		400	100.00 ± 0.00	75.43 ± 2.72	38.64 ± 1.52
		500	100.00 ± 0.00*	100.00 ± 0.00*	55.66 ± 2.58*
		Control	0	0	0
Ipomoea cairica	Leaf	50	0	19.28 ± 1.67	0
		100	0	27.37 ± 1.75	0
		200	0	44.36 ± 2.68	0
		400	5.92 ± 0.38	54.55 ± 2.95	19.02 ± 0.22
		500	8.95 ± 0.26	77.27 ± 3.22*	25.82 ± 1.36
		Control	0	0	0
	Flower	50	0	0	95.00 ± 2.00
		100	0	9.38 ± 0.18	98.00 ± 3.10
		200	0	26.93 ± 1.15	100.00 ± 0.00
		400	5.63 ± 0.52	49.95 ± 2.62	100.00 ± 0.00
		500	13.85 ± 0.82	62.72 ± 1.95*	00.00 ± 0.00*
		Control	0	0	0

Each value represents mean of four values

* P<0.0001 compared to other concentrations in the same group

TABLE 3.4(C). Larvicidal Activity of Crude Extracts of *A. adenophora* and *I. cairica* Species Against 3rd Instar Larvae of *Culex quinquefaciatus* at Various Doses (Summary)

Plant species	Extracts	Percentage mortality at various concentrations[a]									
		After 24 hours (ppm)					After 48 hours (ppm)				
		500	400	200	100	50	500	400	200	100	50
Ageratina adenophora leaves	Pet. ether	88	80	58	28	0	100	100	67	41	0
	Chloroform	78	49	32	0	0	100	75	41	0	0
	Methanol	49	7	0	0	0	55	38	0	0	0
Ipomoea cairica leaves	Pet. ether	1	0	0	0	0	8	5	0	0	0
	Chloroform	39	23	14	5	3	77	54	44	27	19
	Methanol	13	9	0	0	0	25	19	0	0	0
Ipomoea cairica flower	Pet. ether	3	0	0	0	0	13	5	0	0	0
	Chloroform	48	40	19	7	0	62	49	26	9	0
	Methanol	100	100	89	46	41	100	100	100	100	100

Control – Nil mortality; [a]Mean value of four replicates

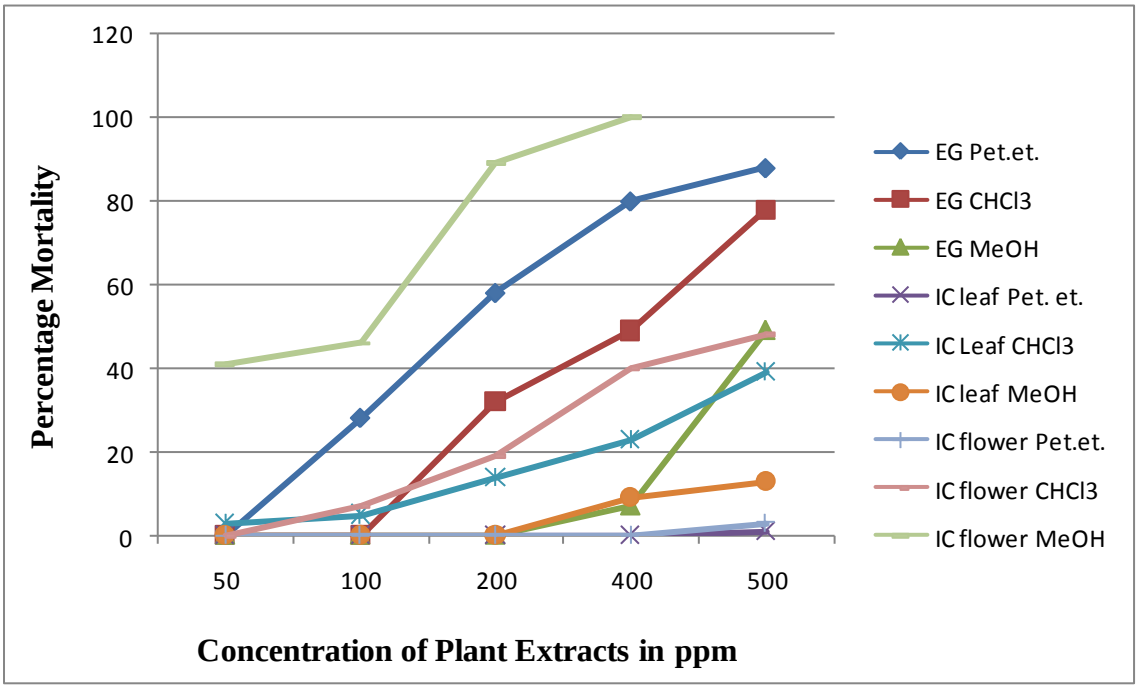


Figure 3.2(a). Graphical Representation of variation of Percentage Mortality with Concentration of Plant Extracts after 24 Hours of Exposure

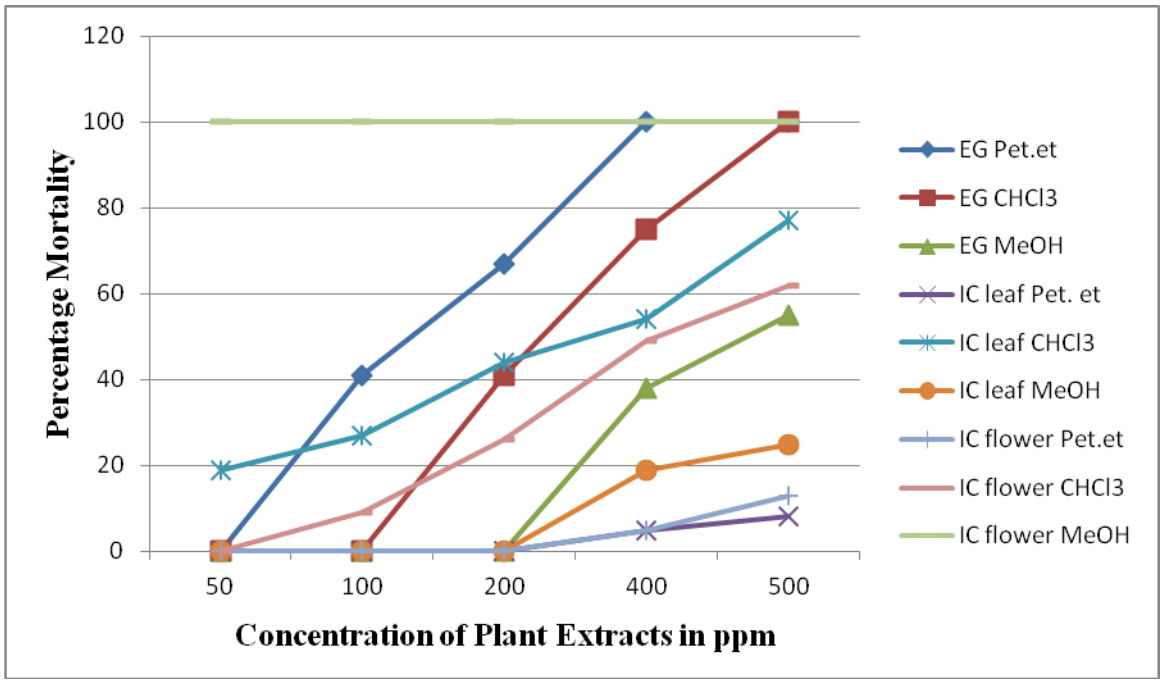


Figure 3.2(b). Graphical Representation of variation of Percentage Mortality with Concentration of Plant Extracts after 48 Hours of Exposure

Larvicidal activity of pet. ether, $CHCl_3$ and MeOH extracts of *Ageratina adenophora* leaves and *Ipomoea cairica* leaves and flowers obtained by selective sequential extraction are recorded and represented in Table 3.4. After 24 hours of exposure, the findings of the present investigation revealed that the methanolic extract of *Ipomoea cairica* flower possesses remarkable larvicidal activity against third instar larvae of *Culex quinquefasciatus* mosquito. The mortality values were significantly greater than the values of control and untreated solution. However, it is evident that not all extracts show activity; petroleum ether extract of *Ipomoea cairica* leaves exhibit negligible or no larvicidal activity at all, while petroleum ether extract of *I.cairica* flower exhibit low activity and petroleum ether extract of *A. adenophora* leaves exhibit relatively high activity against the test larvae. The activity of each extract exhibiting high, moderate or low larvicidal activity is directly related to the concentration of the extract, the higher the concentration the greater its activity.

The mortality of the third instar larval form with different polar and nonpolar solvent extracts of *I. cairica* and *A. adenophora* is presented in Table 3.4 and confirm their larvicidal activity against the LF vector, *C. quinquefasciatus*. The mortality rates at 500 ppm concentration were highest amongst all concentrations of the crude extracts tested against third instar larvae, and it was significantly higher ($p<0.05$) than the mortality rates at 50, 100, 200 and 400 ppm concentrations of crude plant extracts at 24 and 48 hours of exposure [**Table 3.4(C)**]. The mortality rate was higher in 48 hours bioassay than those in 24 hours. Among the three plant parts tested, the highest larvicidal activity (100%) was observed in methanol extract of *I. cairica* flower against *C. quinquefasciatus* after 24 hours and petroleum ether and chloroform extracts of *A. adenophora* leaf after 48 hours. Chloroform extracts of *I. cairica* flower and leaf showed 63 - 77% mortality after 48 hours at 500 ppm while petroleum ether extracts were not effective exhibiting only 9 - 14% mortality. At higher concentrations, the larvae showed restless movement for some time with abnormal wagging and then died. The rates of mortality were directly proportional to concentration (**Table 3.4 and Figure 3.2**).

Table 3.5(A_a) Log Probit and Regression Analysis of Third Larval Instars of *Culex quinquefasciatus* in Different Concentrations of Crude Leaf and Flower Extract of *A. adenophora* and *I. cairica* for 24 Hours (LC_{50})

Plant species	Solvent	LC_{50} (ppm)*	χ^2 (df=4)	95% Confidence limits		Slope ± SE	Intercept ± SE	R
				Lower limit	Upper limit			
Ageratina adenophora leaf	Petroleum ether	186.98a	9.36	132.47	257.46	14.97±2.34	-1.62 ± 0.87	0.98
	Chloroform	335.10b	17.23	217.45	597.38	3.55 ± 0.75	-3.97 ± 1.89	0.95
	Methanol	501.93c	7.81	485.33	526.90	2.91 ± 0.37	-35.43± 6.27	0.94
P value		<0.0001						
$F_{6,2}$ value		102476						
Ipomoea cairica leaf	Petroleum ether	0a	0	0	0	0	0	0
	Chloroform	905.78b	3.21	657.10	1546.37	4.25±1.37	0.18±0.58	0.94
	Methanol	884.38c	1.03	664.29	2997.04	1.62±0.23	-7.54± 3.64	0.95
P value		<0.0001						
$F_{6,2}$ value		122487						
Ipomoea cairica flower	Petroleum ether	0a	0	0	0	0	0	0
	Chloroform	512.74b	1.73	433.94	642.48	2.25±0.25	-1.10± 0.62	0.91
	Methanol	76.43c	21.27	19.28	130.58	2.83±0.65	-0.34± 1.35	0.96
P value		<0.0001						
$F_{6,2}$ value		9965.6						

Means ± SE followed by different letters within the same column are significantly different (Tukey's test, P<0.0001)

*LC_{50}: Lethal concentration that kills 50% of the treated larvae in parts per million;

Table 3.5(A_b) Log Probit and Regression Analysis of Third Larval Instars of *Culex quinquefasciatus* in Different Concentrations of Crude Leaf and Flower Extract of *A. adenophora* and *I. cairica* for 24 Hours (LC_{90})

Plant species	Solvent	LC_{90} (ppm)*	χ^2 (df=4)	95% Confidence limits		Slope ± SE	Intercept ± SE	R
				Lower limit	Upper limit			
Ageratina adenophora leaf	Petroleum ether	514.24a	9.36	351.19	1104.97	14.97±2.34	-1.62 ± 0.87	0.98
	Chloroform	768.36b	17.23	479.60	5896.89	3.55 ± 0.75	-3.97 ± 1.89	0.95
	Methanol	611.29c	7.81	570.53	692.75	2.91 ± 0.37	-35.43± 6.27	0.94
P value		<0.0001						
$F_{6,2}$ value		69515						
Ipomoea cairica leaf	Petroleum ether	0a	0	0	0	0	0	0
	Chloroform	5541.09b	3.21	2772.37	18923.78	4.25±1.37	0.18±0.58	0.94
	Methanol	1769.18c	1.03	1024.01	19834.83	1.62±0.23	-7.54± 3.64	0.95
P value		<0.0001						
$F_{6,2}$ value		617879						
Ipomoea cairica flower	Petroleum ether	0a	0	0	0	0	0	0
	Chloroform	1900.06b	1.73	1321.29	3309.41	2.25±0.25	-1.10± 0.62	0.91
	Methanol	216.26c	21.27	127.15	1884.36	2.83±0.65	-0.34± 1.35	0.96
P value		<0.0001						
$F_{6,2}$ value		95207						

LC_{90}: Lethal concentration that kills 90% of the treated larvae in parts per million;
χ^2: Chi-square;
df : degree of freedom;
SE : Standard Error;
R: regression coefficient.

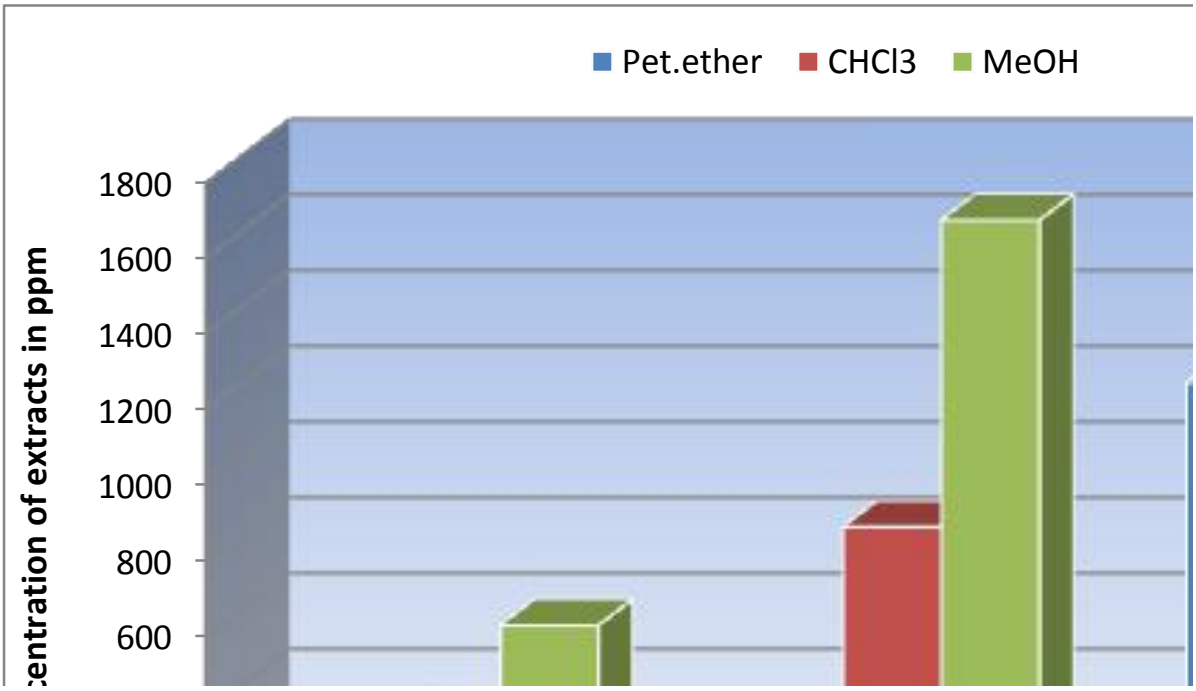


Figure 3.3(a). Bar Diagram showing LC_{50} values of the different extracts after 24 hours of exposure

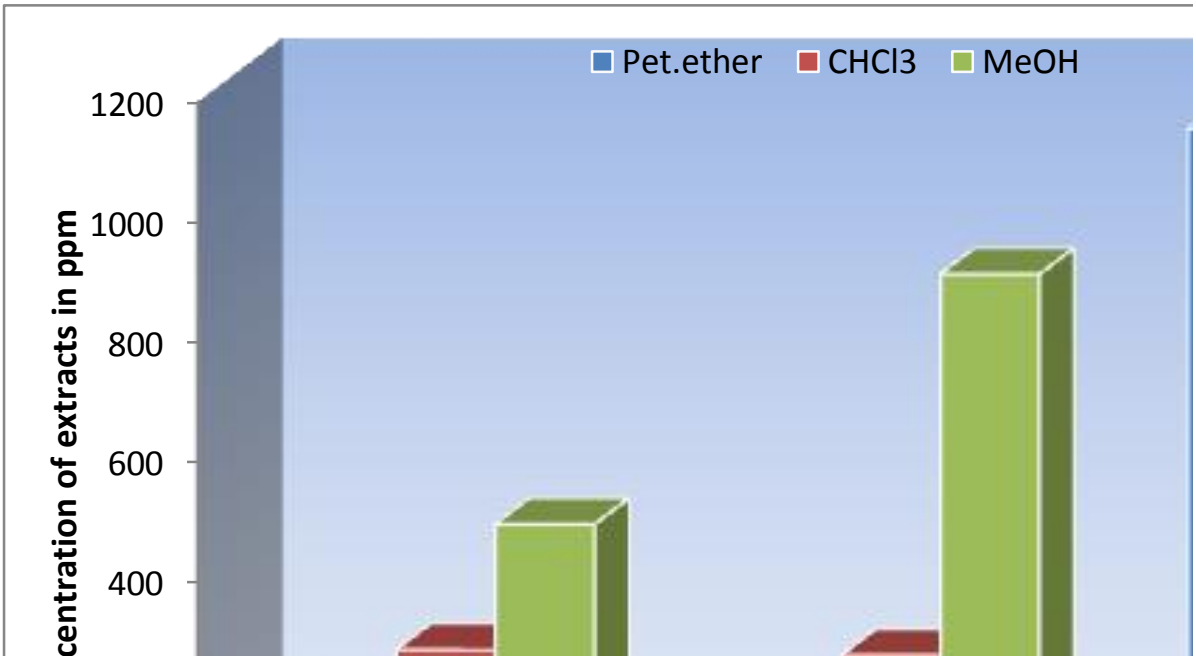


Figure 3.3(b). LC_{50} values of the different extracts after 48 hours of exposure

Table 3.5(B_a) Log Probit and Regression Analysis of Third Larval Instars of *Culex quinquefasciatus* in Different Concentrations of Crude Leaf and Flower Extract of *A. adenophora* and *I. cairica* for 48 Hours (LC_{50})

Plant species	Solvent	LC_{50} (ppm)*	χ^2 (df=4)	95% Confidence limits		Slope ± SE	Intercept ± SE	R
				Lower limit	Upper limit			
A. adenophora leaf	Petroleum ether	133.56a	19.79	82.30	204.99	6.75±1.04	-4.42±1.83	0.97
	Chloroform	245.80b	22.51	143.92	361.00	5.17±1.08	-7.37±2.64	0.94
	Methanol	463.87c	2.29	437.17	498.97	4.43±0.84	-13.00± 2.75	0.95
P value		<0.0001						
$F_{6,2}$ value		289728						
Ipomoea cairica leaf	Petroleum ether	1082.00a	0.46	711.00	127506.03	4.03±1.73	-7.24±4.60	0.92
	Chloroform	239.46b	7.92	141.44	503.32	4.83±1.07	1.56±0.62	0.98
	Methanol	656.37c	2.51	566.95	920.73	1.44±0.27	-8.61±2.84	0.91
P value		<0.0001						
$F_{6,2}$ value		3254370						
Ipomoea cairica flower	Petroleum ether	784.75a	0.05	613.15	5308.50	2.07±0.81	-11.50± 6.04	0.94
	Chloroform	383.07b	2.40	336.84	446.42	2.50±0.24	-1.46± 0.59	0.96
	Methanol	8.43c	0.67	0.007	21.72	5.70±2.26	3.07±1.50	0.99
P value		<0.0001						
$F_{6,2}$ value		4375.3						

Means ± SE followed by different letters within the same column are significantly different (Tukey's test, P<0.0001)

*LC_{50}: Lethal concentration that kills 50% of the treated larvae in parts per million;

Table 3.5(B_b) Log Probit and Regression Analysis of Third Larval Instars of *Culex quinquefasciatus* in Different Concentrations of Crude Leaf and Flower Extract of *Ageratina adenophora* and *Ipomoea cairica* for 48 Hours (LC_{90})

Plant species	Solvent	LC_{50} (ppm)*	χ^2 (df=4)	95% Confidence limits		Slope ± SE	Intercept ± SE	R
				Lower limit	Upper limit			
Ageratina adenophora leaf	Petroleum ether	259.85a	19.79	176.49	790.05	6.75±1.04	-4.42±1.83	0.97
	Chloroform	434.62b	22.51	308.23	1312.26	5.17±1.08	-7.37±2.64	0.94
	Methanol	718.06c	2.29	631.55	903.95	4.43±0.84	-13.00±2.75	0.95
P value		<0.0001						
$F_{6,2}$ value		602777						
Ipomoea cairica leaf	Petroleum ether	2248.11a	0.46	1064.79	13465305.00	4.03±1.73	-7.24±4.60	0.92
	Chloroform	1849.39b	7.92	734.03	54818.70	4.83±1.07	1.56±0.62	0.98
	Methanol	1208.64c	2.51	878.66	2683.58	1.44±0.27	-8.61±2.84	0.91
P value		<0.0001						
$F_{6,2}$ value		2439000						
Ipomoea cairica flower	Petroleum ether	1316.72a	0.05	824.87	55491.01	2.07±0.81	-11.50±6.04	0.94
	Chloroform	1245.03b	2.40	955.13	1814.43	2.50±0.24	-1.46±0.59	0.96
	Methanol	35.04c	0.67	3.34	52.81	5.70±2.26	3.07±1.50	0.99
P value		<0.0001						
$F_{6,2}$ value		76942						

Dose response - Log probit and regression analysis (LC_{50})

The LC_{50} of *A. adenophora* leaf, *I. cairica* flower and leaf ranged between 186.98 and 133.56 ppm, 0 and 784.75 ppm and 0 and 1082 ppm in petroleum ether extract; 335.10 and 245.80 ppm, 512.74 and 383.07 ppm and 905.78 and 239.46 ppm in chloroform extract; and 501.93 and 463.87 ppm, 76.43 and 8.43 ppm and 884.38 and 656.37 ppm in methanol extract for 24 and 48 h, respectively. Low LC_{50} and LC_{90} values and highest larvicidal activity were found in methanol extract of *I. cairica* flower followed by petroleum ether extract of *A. adenophora* leaf and finally by chloroform extract of *I. cairica* leaf. Chi-square value was significant at $P<0.0001$ level. Higher slope value (2.83±0.65 at 24 h; 5.70±2.26 at 48 h) and lower fiducial limits at 95 % of LC_{50} (19.28 – 130.58 ppm at 24 h; 0.007‑21.72 ppm at 48 h) were observed for methanol extract of *I. cairica* flower than those of other solvent extract. The same trend was noticed in petroleum ether extract of *A. adenophora* leaf as well as chloroform extract of *I. cairica* leaf (**Tables 3.5**). The results of regression analysis of crude extract of *I. cairica* and *A. adenophora* revealed that the mortality rate (Y) is positively correlated with the concentration of exposure (X) having a regression coefficient (R) close to 1 in each case. The results of log probit analysis (95% confidence level) revealed that LC_{50} and LC_{90} values gradually decreased with the exposure periods having the lowest value at 48 h of exposure to third instar larvae. The order of larvicidal action was observed to be of methanol extract of *I. cairica* flower > petroleum ether extract of *A. adenophora* leaf >chloroform extract of *I. cairica* leaf (**Tables 3.5**).

Table 3.5(C). Completely Randomized Three-way Factorial ANOVA Using Different Concentrations, Period of Exposure and Different Solvents as Variables for *Ipomoea cairica* Flower and *Ageratina adenophora* Leaf against Third Instar Larvae of *Culex quinquefasciatus*

.

Ipomoea cairica flower – three way factorial ANOVA					
Source of variation	Sum of squares	df	Mean square	F value	P value
Time (T)	29.47	1	29.47	60.14	0.0001
Solvent (S)	78.82	2	39.41	80.42	0.0001
Concentration (C)	73.75	4	49.52	101.06	0.0001
T × S	4.82	2	2.41	4.91	0.0104
T × C	10.38	4	2.59	5.28	0.0010
S × C	21.69	8	2.71	5.53	0.0001
T × S × C	9.84	8	1.23	2.51	0.0194
Residual	31.53	64	0.49		
Total	256.30	93			
Ageratina adenophora leaf – three way factorial ANOVA					
Source of variation	Sum of squares	df	Mean square	F value	P value
Time (T)	17.24	1	17.24	55.61	0.0001
Solvent (S)	55.62	2	27.81	89.70	0.0001
Concentration (C)	54.35	4	13.58	43.80	0.0001
T × S	2.16	2	1.08	3.48	0.0368
T × C	5.12	4	1.28	4.12	0.0049
S × C	12.35	8	1.54	4.96	0.0001
T × S × C	8.57	8	1.07	3.45	0.0023
Residual	20.92	64	0.31		
Total	251.65	125			

The result of the three-way factorial ANOVA [Table 3.5(C)] of crude extract of leaves of *A. adenophora* and flowers of *I. cairica* carried out at different concentrations (50 – 500 ppm), different time interval (24 and 48 h) and different solvents (petroleum ether, chloroform and methanol) revealed significant difference in larval mortality ($p<0.0001$). There was a significant effect of concentration ($F=101.06$ and 43.80; df=4, 64; $P<0.0001$), solvent ($F=80.42$ and 89.70; df=2, 64; $P<0.0001$), and time after application ($F=60.14$ and 55.61; df=1, 64; $P<0.0001$) on mortality of third instar larvae of *C. quinquefasciatus* in *I. cairica* flower and *A.*

adenophora leaf, respectively. There were also significant effect of all interactions including concentration×solvent (F=5.53 and 4.96; df=8, 64; P<0.0001), solvent×time (F=4.91 and 3.48; df=2, 64; P<0.03), concentration×time (F=5.28 and 4.12; df=4, 64; P<0.004), and concentration×solvent×time (F=2.51 and 3.45; df=8, 64; P<0.01) in *I. cairica* flower and *A. adenophora* leaf, respectively exhibiting significant difference in larval mortality [Table 3.5(C)].

3.4. Studies on Larvicidal Efficacy of Methanolic Extract of *I. cairica* flower with Time

TABLE 3.6(A). Time dependent larvicidal activity of methanolic flower extract of *I. cairica* and petroleum ether leaf extract of *A. adenophora* against third instar larvae of *C. quinquefaciatus*

Concentration (in ppm)	Time (in h)	% Mortality ± Standard Error	
		I. cairica- flower - methanol	*A. adenophora*- leaf - petroleum ether
500	1	6.56 ± 0.56	5.05 ± 0.05
	2	39.42 ± 0.32	13.15 ± 0.18
	3	49.75 ± 0.15	15.72 ± 0.11
	4	72.55 ± 0.05	20.75 ± 0.75
	5	100.00 ± 0.00*	23.00 ± 0.47
	15	-	40.15 ± 0.56
	18	-	67.25 ± 0.55
	20	-	74.85 ± 0.45
	24	-	88.95 ± 0.55
	30	-	100.00 ± 0.00*
400	1	0	0
	2	24.88 ± 0.64	0
	3	32.95 ± 0.17	0
	4	56.74 ± 0.44	15.99 ± 0.18
	8	100.00 ± 0.00*	27.75 ± 0.45
	16	-	43.85 ± 0.58
	20	-	68.80 ± 0.45
	24	-	80.82 ± 0.37
	35	-	100.00 ± 0.00*
	4	21.05 ± 0.75	7.05 ± 0.17

200	8	38.98 ± 0.55	16.65 ± 0.45
	12	52.16 ± 0.75	23.55 ± 0.68
	16	62.94 ± 0.44	32.65 ± 0.48
	20	85.48 ± 0.78	46.21 ± 0.56
	24	89.05 ± 0.16	58.39 ± 0.27
	28	100.00 ± 0.00*	58.05 ± 0.38
	36	-	60.00 ± 0.45
	48	-	67.45 ± 0.14*
100	4	0	0
	8	0	0
	12	0	0
	16	21.05 ± 0.05	7.16 ± 0.36
	20	34.76 ± 0.45	17.45 ± 0.22
	24	46.15 ± 0.56	28.45 ± 0.48
	28	58.72 ± 0.17	32.68 ± 0.18
	32	70.95 ± 0.27	35.95 ± 0.28
	36	79.44 ± 0.05	38.45 ± 0.38
	40	94.48 ± 0.38	40.66 ± 0.26
	48	100.00 ± 0.00*	41.33 ± 0.12*
50	4	0	0
	8	0	0
	12	0	0
	16	19.35 ± 0.45	0
	20	34.56 ± 0.32	0
	24	41.12 ± 0.96	0
	28	61.22 ± 0.68	0
	32	67.56 ± 0.38	0
	36	73.04 ± 0.18	0
	40	83.78 ± 0.31	0
	48	100.00 ± 0.00*	0

Each value represents mean of four values; * P<0.0001 compared to varied time in the same group
Control – Nil mortality

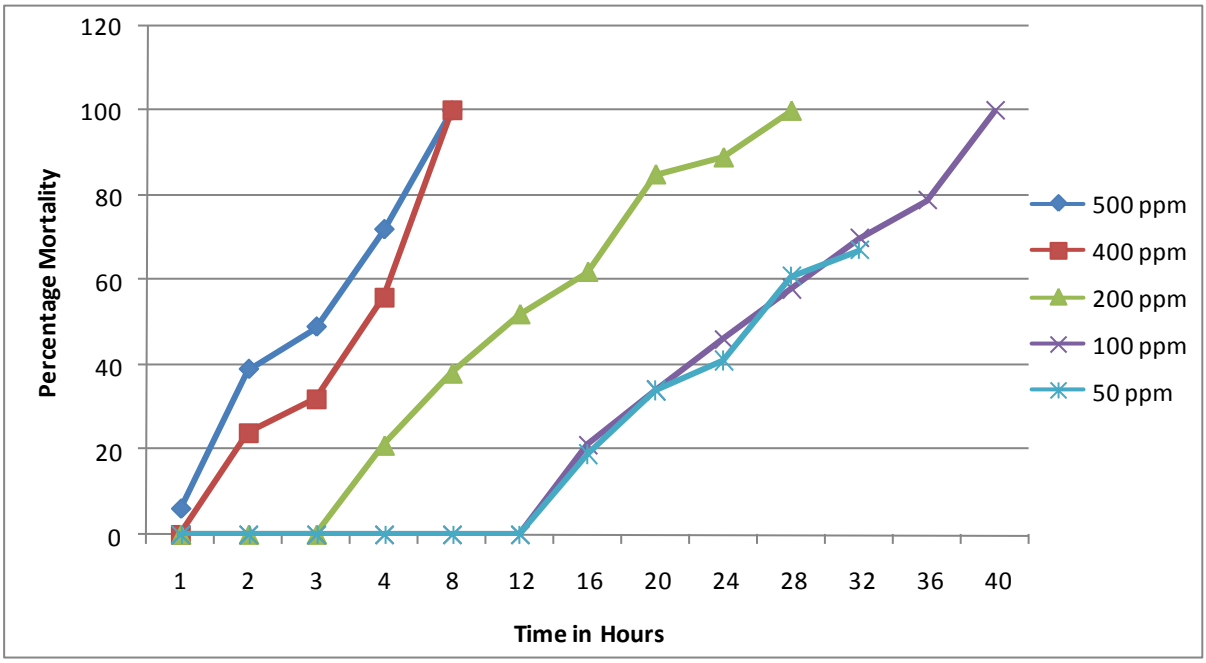


Figure 3.4(a). Variation of Percentage Mortality with Time for Different Concentrations of MeOH Extract of *I. cairica* Flowers

Table 3.6(B_a) Log Probit and Regression Analysis of Temporal Larvicidal Efficacy of Crude Methanolic Floral Extract of *I. cairica* and Petroleum Ether Leaf Extract of *A. adenophora* at Different Concentrations against Third Instar Larvae of *Culex quinquefaciatus* (LT_{50})

Concentration (ppm)	LT_{50} (h)*	95% Confidence limits		Slope ± SE	χ^2	df	Intercept ± SE	R
		Lower Limit	Upper Limit					
Methanolic floral extract of *Ipomoea cairica*								
500	2.51a	1.35	3.71	4.11 ± 0.92	24.02	4	3.35 ± 0.45	0.99
400	3.34b	2.39	4.96	4.65 ± 0.93	17.85	4	2.55 ± 0.50	0.98
200	9.53c	6.49	12.25	2.89 ± 0.48	25.24	6	2.16 ± 0.54	0.96
100	24.77d	22.75	26.07	6.05 ± 0.50	20.34	10	-3.39 ± 0.72	0.94
50	25.36d	23.57	27.19	5.58 ± 0.48	20.61	10	-2.84 ± 0.69	0.92
Petroleum ether leaf extract of *Ageratina adenophora*								
500	9.45a	6.60	13.57	2.04 ± 0.31	12.13	9	3.00 ± 0.32	0.96
400	12.77b	9.64	16.72	3.03 ± 0.44	13.70	8	1.64 ± 0.51	0.98
200	12.62b	9.68	16.27	3.05 ± 0.42	19.64	8	1.64 ±0.49	0.91
100	34.97c	30.27	43.15	4.84 ± 0.93	18.16	10	-2.47 ± 1.38	0.95
50	0d	0	0	0	0	0	0	0

Table 3.6(B_b) Log Probit and Regression Analysis of Temporal Larvicidal Efficacy of Crude Methanolic Floral Extract of *I. cairica* and Petroleum Ether Leaf Extract of *A. adenophora* at Different Concentrations against Third Instar Larvae of *Culex quinquefaciatus* (LT_{90})

Concentration (ppm)	LT_{90} (h)*	95% Confidence limits		Slope ± SE	χ^2	df	Intercept ± SE	R
		Lower Limit	Upper Limit					
Methanolic floral extract of *Ipomoea cairica*								
500	5.15	3.54	27.71	4.11 ± 0.92	24.02	4	3.35 ± 0.45	0.99
400	6.31	4.45	22.57	4.65 ± 0.93	17.85	4	2.55 ± 0.50	0.98
200	26.40	19.42	49.50	2.89 ± 0.48	25.24	6	2.16 ± 0.54	0.96
100	39.76	36.30	44.94	6.05 ± 0.50	20.34	10	-3.39 ± 0.72	0.94
50	43.01	38.86	49.44	5.58 ± 0.48	20.61	10	-2.84 ± 0.69	0.92
Petroleum ether leaf extract of *Ageratina adenophora*								
500	39.89	24.68	96.90	2.04 ± 0.31	12.13	9	3.00 ± 0.32	0.96
400	33.77	24.27	60.47	3.03 ± 0.44	13.70	8	1.64 ± 0.51	0.98
200	33.22	24.24	56.78	3.05 ± 0.42	19.64	8	1.64 ±0.49	0.91
100	64.30	49.66	118.28	4.84 ± 0.93	18.16	10	-2.47 ± 1.38	0.95
50	0	0	0	0	0	0	0	0

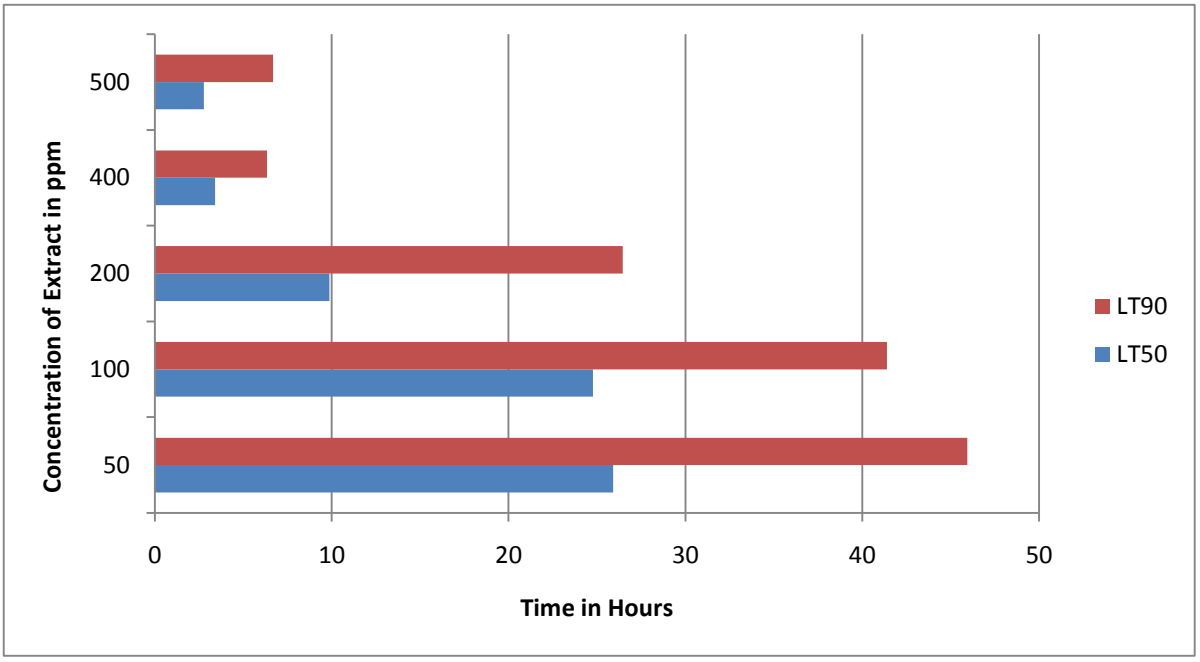


Figure 3.4(b). Variation of Lethal Time (LT_{50} & LT_{90}) with increase in Concentration

Time–response larvicidal bioassay (LT_{50})

Time-response larvicidal bioassay was performed in crude methanolic flower extract of *I. cairica* and petroleum ether leaf extract of *A. adenophora* at 50 – 500 ppm for 1-48 h against third instar larvae of *C. quinquefaciatus* (Table 3.6). The lethal time (LT_{50}) decreased as the concentration of the plant extracts was increased in the mosquito species (Table 3.6). In terms of lethal time to kill 50 % of the population of *C. quinquefaciatus*, 500 and 400 ppm of methanolic flower extract of *I. cairica* had minimum time, i.e., 2.51 and 3.34 h, followed by 200, 100, and 50 ppm (9.53 and 25.36 h, respectively). In case of petroleum ether leaf extract of *A. adenophora* the LT_{50} was 9.45 h in 500 ppm, 12.7 h in 200 and 400 ppm and 34.97 h in 100 ppm. The LT_{90} of methanolic flower extract of *I. cairica* and petroleum ether leaf extract of *A. adenophora* ranged between 5.51 and 43.01h and 39.89 and 64.30 h, respectively. Both the extracts at lower concentration had the longest time (24.77 ‑34.97 h, respectively) to cause 50% mortality (Table 3.6). Lethal times were also shorter in methanolic flower extract of *I. cairica*, particularly at higher dosages (500 and 400 ppm), which presented values ~3.8 times lower than those exhibited by petroleum ether leaf extract of *A. adenophora*. This could again be linked to the difference in toxicity noticed in the 1‑48 h of the experiment (Tables 3.6). Significant Chi-square value ($P<0.0001$ level), higher slope value, lower fiducial limits at 95 % of LT_{50} and positive correlation between mortality and time of exposure having a R value close to 1 in each case were observed (Table 3.6).

It was reported that essential oil of *Ipomoea cairica* Linn. possesses remarkable larvicidal properties as it could induce 100% mortality in the larvae of *Culex tritaeniorhynchus, Aedes aegypti, Anopheles stephensi* and *Culex quinquefasciatus* mosquitoes at concentrations ranging from 100 to 170 ppm (Thomas *et al*., 2004). Biological activities of

these plant essential oils is mainly attributed due to the presence of terpenoids which are the principal constituents of essential oils. In fact azadiractin, an insecticide found in seeds, leaves and other parts of neem tree is predominantly a triterpenoid (Ley *et al*., 1993).

After 24 hours of exposure, the methanolic extract of *Ipomoea cairica* flower showed remarkable larvicidal activity (LC_{50} = 76.99 ppm) against third instar larvae of *Culex quinquefasciatus* mosquito. The mortality values were significantly greater than the values of control and untreated solution. The time dependent larvicidal efficacy of methanolic extract of *Ipomoea cairica* flower also reveals a remarkable lethal time (LT_{50} = 25.91 hours ; LT_{90}= 45.95 for 50 ppm) even at low concentrations for a crude extract, a cocktail of compounds. The high Larvicidal efficacy of methanol extract of *Ipomoea cairica* flower may be attributed due to the presence of phenolics. The insecticidal property of plant phenolics is well documented. For an instance, a mitochondrial poison "Rotenone" isolated from the plant *Derris eliptica* is the most recognized example of toxic flavonoid (Mann and Kaufman, 2012). The flavones from the families Labitae, Umbelliferae and Compositae and tannins in all plants are other phenolic compounds known to possess insecticidal properties (Moore *et al*., 2006).

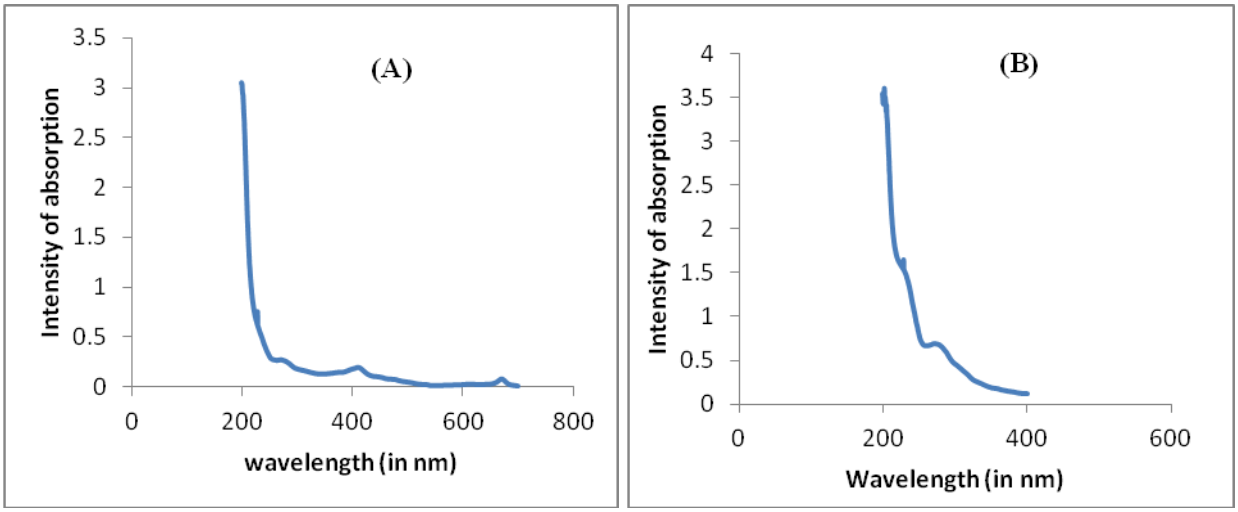
This perspective study aimed to determine the larvicidal activity of the selected plants against the 3rd instar larvae of *Culex quinquefasciatus* targeting the discovery of natural ecological friend, cost effective alternative to the harmful chemical insecticides.

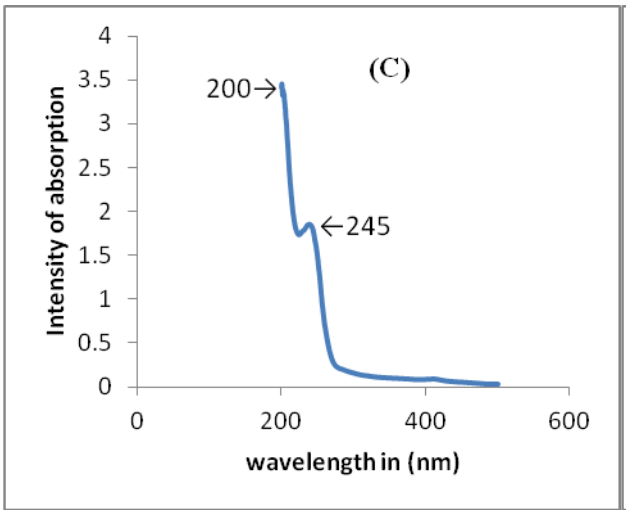
The use of synthetic organic insecticides in larvae control around the world has resulted in damage to the environment, pest resurgence and toxic effects on non-target organisms (Abudulai *et al.*, 2001). In addition, extensive use of chemical insecticides has made strains of the target insects resistant to most of them (Schaafsma, 1990) and so attention being diverted in favour of non-chemical methods for insect management.

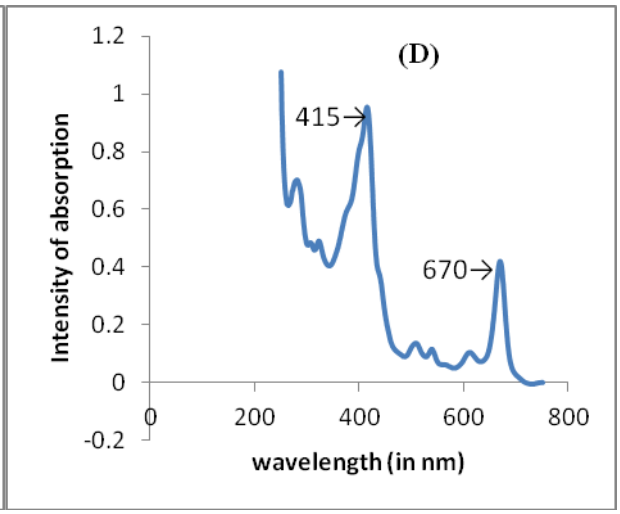
More than 2,000 species of plants are known to have insecticidal properties (Klocke, 1989) while others have reported the bioactivity of extracts and essential oils from various plants against agricultural pests (Nagpal *et al.,* 1996; Abdel-Hady *et al.,* 2005) and fortunately the plant derived insecticides encompasses an array of chemical compounds thus, the chance of insects developing resistance to such insecticides are less and also they are considered as ideal safe ecological friend insect controllers.

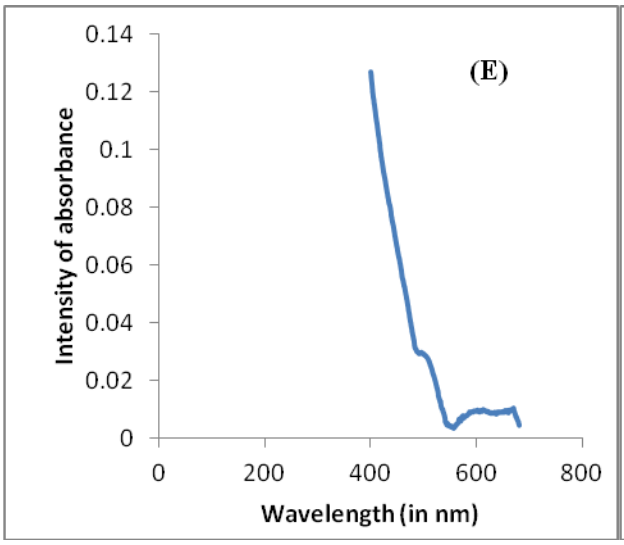
3.5. UV- Visible Spectral Analysis

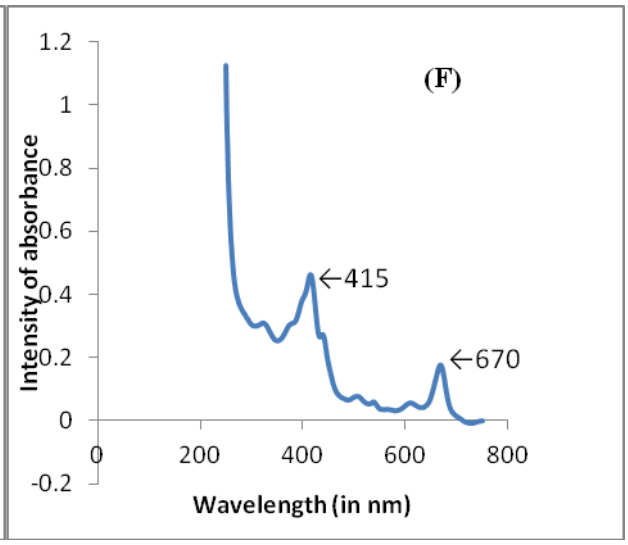
The UV-visible spectra were recorded between 700 nm - 200 nm for each extract (Pet. ether, $CHCl_3$ and MeOH) using a UV-visible Thermo Fischer EVOLUTION 201 Spectrophotometer. The maxima wavelength values were recorded for each extract. The chemical structure of phenolic compounds, specifically the aromatic ring, produces strong absorbance in the ultraviolet (UV) region associated with electronic transitions of the molecule. This provides a unique spectrum in addition to absorbance in the infrared (IR) region due to vibrational molecular motions.

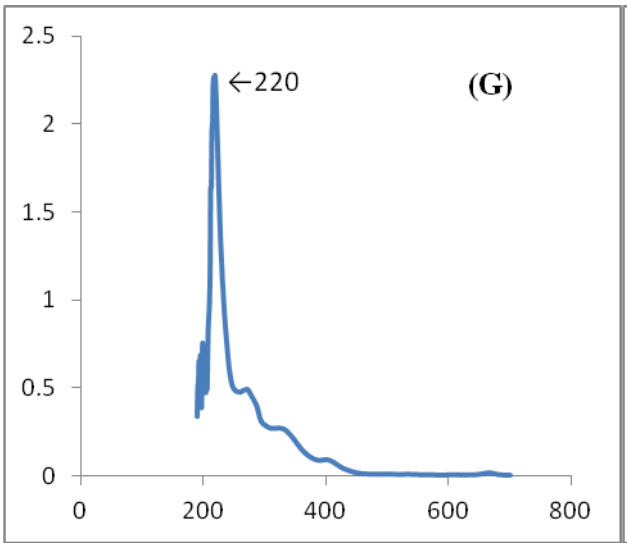


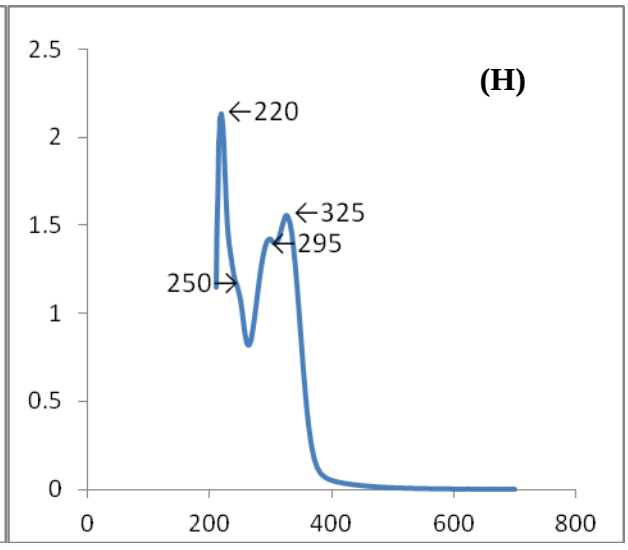

(C)
200→
←245
Intensity of absorption
wavelength in (nm)


(D)
415→
670→
Intensity of absorption
wavelength (in nm)


(E)
Intensity of absorbance
Wavelength (in nm)


(F)
←415
←670
Intensity of absorbance
Wavelength (in nm)


(G)
←220


(H)
←220
←325
←295
250→

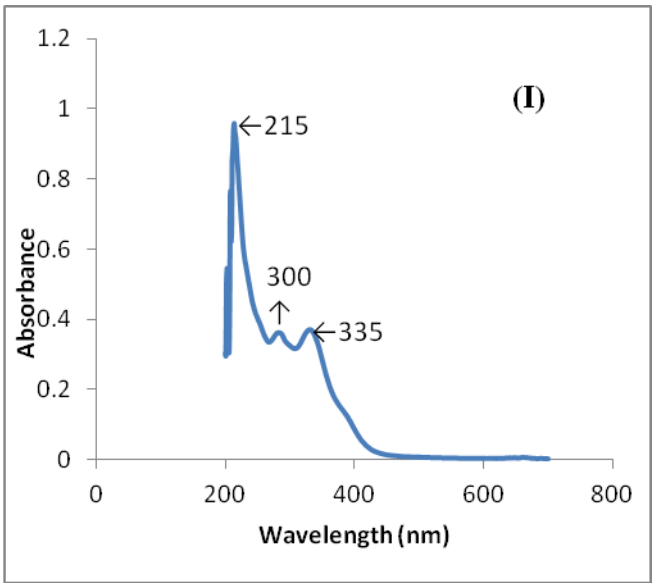


Figure 3.5. UV-Vis spectra of the Crude extracts

(A) Pet. ether extract of *Ipomoea cairica* leaves
(B) Pet. ether extract of *Ipomoea cairica* flowers
(C) Pet. ether extract of *Ageratina adenophora* leaves
(D) $CHCl_3$ extract of *Ipomoea cairica* leaves
(E) $CHCl_3$ extract of *Ipomoea cairica* flowers
(F) $CHCl_3$ extract of *Ageratina adenophora* leaves
(G) CH_3OH extract of *Ipomoea cairica* leaves
(H) CH_3OH extract of *Ipomoea cairica* flowers
(I) CH_3OH extract of *Ageratina adenophora* leaves

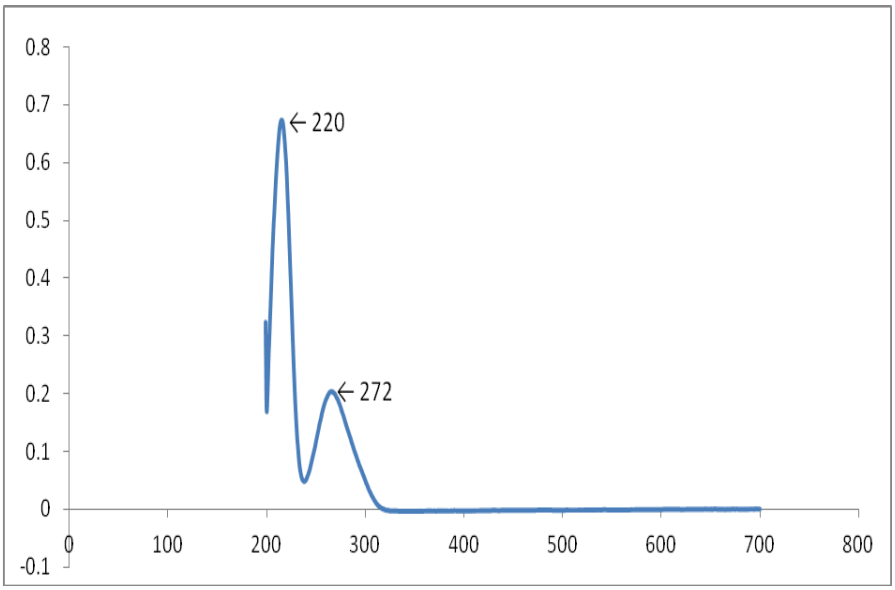


Figure 3.5(J). UV-Vis spectra of the Isolated Compound IC1

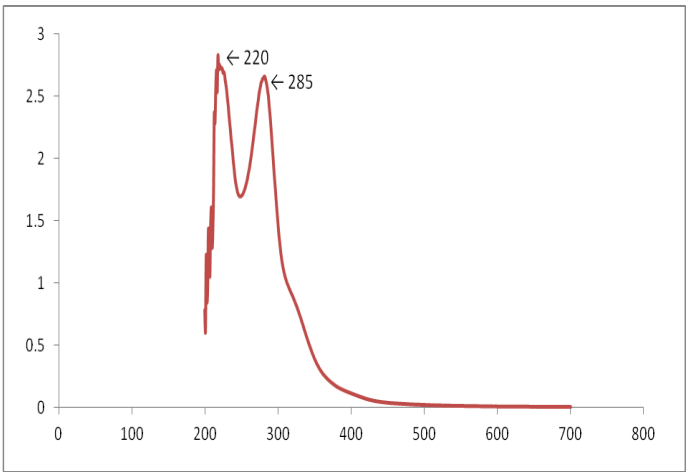


Figure 3.5(K). UV-Vis spectra of the Isolated Compound IC2

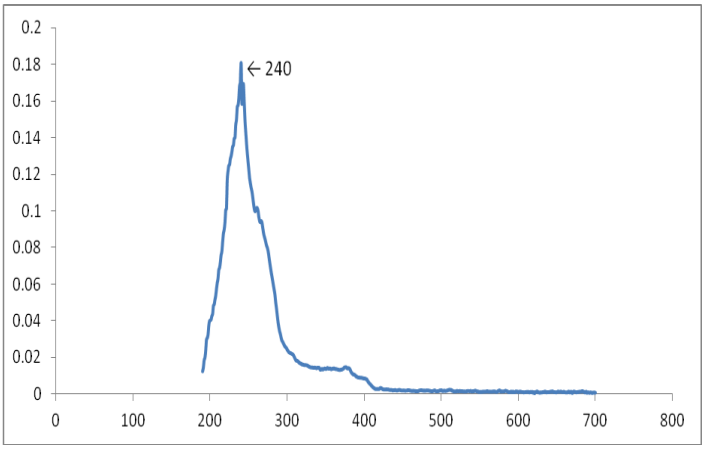


Figure 3.5(L). UV-Vis spectra of the Isolated Compound EG1

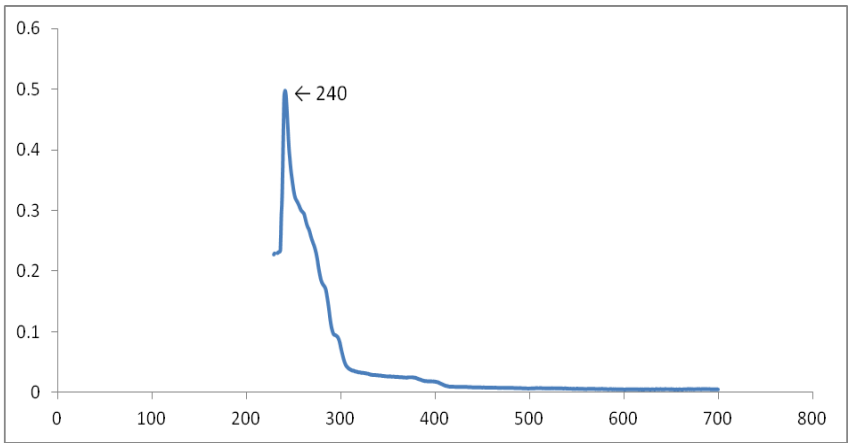


Figure 3.5(M). UV-Vis spectra of the Isolated Compound EG2

In general, UV-visible spectrophotometric assays provide simple and fast screening methods to quantify and/or identify classes of phenolic compounds in crude plant extracts. Given the intrinsic existence of conjugated double and aromatic bonds, every phenol exhibits a higher or lower absorption in ultraviolet (UV) or ultraviolet/visible (UV-VIS) region since phenolic compounds are aromatic and show intense absorption in the UV region of the spectrum. However, due to the complexity of the plant phenolics and different reactivity of phenols toward assay reagents, a broad spectrum of methods is used for assay of the constituents, leading to differing and often non-comparable results. The UV Spectrum showed λ_{max} at 250 nm and 295nm for π→π* and n→π* transition respectively indicates the presence of a chromophoric group probably (C=O) in figure 3.4(H).The π→π* and the n→π* transitions occurs in the near UV and visible region (180-700 nm). Molecule exhibits n→π* transition include molecules that contain -C=O, -N=O, -N=N-, -O-H, -O-R, -S-H, etc . The absorbance range of gallic acid is 250-350 nm (Maria and Daniela, 2009). For MeOH extract of *I.cairica* flower and isolated compound IC1, the maximum of absorption observed in the range 280-340 nm as a shoulder corresponds to gallic acid or a related phenolic compound.

The UV-visible spectral analysis of IC1 showed λ_{max} at 220 nm and 272 nm. These are attributed due to the presence of chromophoee group such as -C=O in the molecule. The presence of an absorbance band at a particular wavelength often is a good indicator of the presence of a chromophore. However, the position of the absorbance maximum is not fixed but depends partially on the molecular environment of the chromophore but also the polarity of solvent in which the sample might have been dissolved. Other parameters, such as pH and temperature, also may cause changes in both the intensity and the wavelength of the absorbance maxima.

Compounds which can readily undergo $\pi \rightarrow \pi^*$ transitions include conjugated dienes, trienes, and conjugated α,β-unsaturated ketones, esters or lactones, with structural features of the types C=C–C=C, C=C–C=C–C=C, or C=C–C=O, respectively. They also include aromatic rings, such as the phenolic ring. All such conjugated compounds absorb UV radiation strongly in the wavelength range between 220 and 350 nm

All phenolic compounds exhibit intense absorption in the UV region of the spectrum and those that are coloured absorb strongly in the visible region as well. Each class of phenolic compounds has distinctive absorption characteristics. For example, phenols and phenolic acids show spectral maxima in the range 250-290 nm; cinnamic acid derivatives have principal maxima in the range 290-330 nm; flavones and flavonols exhibit absorption bands of approximately the same intensity at about 250 and 350 nm; chalcones and aurones have an absorption peak of great intensity above 350 nm and a much less intense band at 250 nm; anthocyanins and betacyanins show rather similar absorption in visible region (475-560 nm and 535-545 nm, respectively) and a subsidiary peak at about 270-275 nm (Harborne, 1964; Mabry *et al*., 1970).

3.6. Determination of Total Phenolic & Flavonoid Contents and Anti-oxidant Activity

Natural products are derived from the phenomenon of biodiversity in which the interactions among organisms and their environment formulate these diverse complex chemical entities within the organisms that enhance their survival and competitiveness (Nurmikko *et al.*, 2007), they are the main source for the majority of FDA-approved agents and are continued to be one of the major sources of inspiration for future drug discovery (Bhuwan *et al.*, 2011). Phenolic compounds (phenolic acids, flavonoids, quinones, coumarins, lignans, tannins) are one of the main free radical scavenging molecules in plants (Cai *et al.*, 2003; Zheng and Wang,

2001). Epidemiological studies have shown that many of these compounds possess anti-inflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial and antiviral activities (Owen *et al.*, 2000; Sala *et al.*, 2002; Cushnie and Lamb, 2005; El-Hela *et al*, 2011). Numerous physiological and biochemical processes in human body may generate oxygen entered free radicals and other reactive oxygen species as by-products, the overproduction of such free radicals can cause oxidative damage to biomolecules as lipids, proteins and even DNA, eventually leading to many chronic diseases, such as atherosclerosis, cancer, diabetes, aging, and other degenerative diseases in humans (Halliwell, 1994; Niki, 1997; Poulson *et al*, 1998).

Plant phenolics are a major group of compounds acting as primary antioxidants or free radical scavengers. Plant extracts with high phenolic contents also show high flavonoid content as reported for other plant species (Makepeace *et al*., 1985). ROS (reactive oxygen species) have been considered to cause harm to living organisms and thus play a significant role in many human diseases such as arthritis, myocardial infarction , atherosclerosis, diabetes mellitus and cancer (Elzaawely and Tawata, 2012; Gupta *et al*, 2007).

Table 3.7(A) Total Phenol Determination

Absorbance 765 nm											**Amount of Phenol content (mg/g GAE)**		
Standard Gallic acid (mg/ml)								**Test (0.01 mg/ml)**					
0.001	0.005	0.008	0.01	0.02	0.03	0.04	0.05	MeOH ext. *A.adenophora* leaves	MeOH ext. *I. cairica* flowers	MeOH ext. *I. cairica* leaves	MeOH ext. *A.adenophora* leaves	MeOH ext. *I. cairica* flowers	MeOH ext. *I. cairica* leaves
0.078	0.116	0.189	0.193	0.423	0.671	1.05	1.112	0.070	0.135	0.083	300	579	356

The total phenolic contents in the examined plant extracts using the Folin-Ciocalteu's reagent is expressed in terms of gallic acid equivalent (the standard curve equation: y = 23.308x, r^2 = 0.9804). The concentration-absorbance calibration curve for 8 sequentially and independently prepared stock standards of gallic acid solution is illustrated in Figure 2. The measured absorbance values at 765 nm for the indicated concentration of gallic acid solutions are in the range of 0.08 to 1.16. Within this range of concentrations (0.001 to 0.05 mg/ml), the calibration curve of gallic acid has clearly exhibited linearity (Figure 2.1).

The total phenolic contents in the examined extracts ranged from 300 to 579 mg GAE/g. The highest amount of total phenolics was found in methanol extract of *Ipomoea cairica* flower, followed by methanol extract of *Ipomoea cairica* leaves, while the methanol extract *Ageratina adenophora* leaves has lowest phenolic content [Table 3.4(A)]. The total amount of phenolics content in MeOH extracts of *I. cairica* flower, *I. cairica* leaves and *A.adenophora* leaves were determined to be 579 mg/g, 356 mg/g and 300mg/g equivalents of gallic acid respectively in comparison to the value for gallic acid (581 mg/g). The total phenolic contents in the extracts of the two plant species under study depends on the type of extract, i.e. the polarity of solvent used in extraction. Therefore, high concentration of these compounds in the MeOH extracts may be attributed due to high solubility of phenols in polar solvents like MeOH (Mohsen and Ammar, 2008; Zhou and Yu, 2004).

Table 3.7(B) Total Flavonoid Determination

Absorbance 510 nm									**Amount of Flavonoid content (mg/g QE)**		
Standard Quercetin (mg/ml)						**Test (1mg/ml)**					
0.01	0.05	0.1	0.2	0.4	0.6	MeOH ext. *A.adenophora* leaves	MeOH ext. *I. cairica* flowers	MeOH ext. *I. cairica* leaves	MeOH ext. *A.adenophora* leaves	MeOH ext. *I. cairica* flowers	MeOH ext. *I. cairica* leaves
0.058	0.302	0.574	0.983	1.617	1.832	1.798	2.276	0.638	510	646	181

The concentration of flavonoids in various plant extracts of the plant species under study were determined using spectrophotometric method with aluminum chloride. The content of flavonoids was expressed in terms of Quercetin equivalent (mg of QE/g of extract) using the standard curve equation: $y = 3.5204x$, $r^2 = 0.999$).

The calibration curve for 6 sequentially and independently prepared stock standard solutions of quercetin that depicts the concentration of quercetin against the absorbance, as presented in Figure 2.2. The absorbance value increased proportionately while increasing the concentration of quercetin from 0.01 to 0.6 mg/ml. A slight deviation from the linearity seemingly occurred at the higher concentration region of quercetin calibration plot. Nevertheless, for our estimation purposes, the calibration plot was employed to ascertain the total flavonoid content of the polar methanolic extract.

The concentration of flavonoids in the plant extracts ranged from 181 to 646 mg/g. The highest amount of total flavonoids was also found in methanol extract of *Ipomoea cairica* flower, followed by methanol extract of *Ageratina adenophora* leaves, while the methanol extract of *Ipomoea cairica* leaves has lowest flavonoid concentration [Table 3.4(B)] The total amount of flavonoids content present in the MeOH extracts of *I. cairica* flower, . *A.*

adenophora leaves and *I. cairica* leaves were 646 mg/g, 510 mg/g and 181 mg/g quercetin equivalents respectively in comparison to the value for quercetin (581 mg/g) [Table 3.4(B)]. The concentration of flavonoids in plant extracts depends on the polarity of solvents used in the extract preparation (Min and Chun-Zhao, 2005). Several reports have conclusively provided the correlation between antioxidant activity and the amount of total phenolics / total flavonoids (Negro *et al*., 2003; Ramadeep and Geoffrey, 2005; Anna *et al*., 2003).

Flavonoids are class of secondary plant metabolites endowed with significant antioxidant and chelating attributes. Antioxidant activity of flavonoids depends on the structure and substitution pattern of hydroxyl groups (Sharififar *et al*., 2008). Methanolic extracts from *I. cairica* flowers, *A. adenophora* leaves and *I. cairica* leaves have high concentration of total phenols [**Table 3.5(A)**] and flavonoids [**Table 3.5(B)**], which is in correlation with intense antioxidant activity of these extracts

The concentration of the phenolic compounds in the plant is also regarded as a key factor in deterrence for insect as an anti-feedant agent and it is the accumulation of phenols in selective parts of the plant that represents a feeding barrier. The effectiveness of phenolics as a resistance factor to animal feeding is enhanced, as aforementioned, with the oxidation that is leading to polymerisation, which alters digestibility, palatability and nutritional value (Ananthakrishnan, 1997; Lattanzio *et al*., 2005; Harborne, 2001; Simmonds, 2003; Harmatha and Dinan, 2003). In addition, plants may be unsuitable as hosts for fungal pathogens because of pre-formed antifungal phenolics and/or induced defence phenolics synthesised in response to biotic stress, as part of an active defence response, when a pathogen manages to overcome constitutive defence barriers (Lattanzio *et al*., 2006; Treutter, 2006).

Table 3.7(C). Determination of Total Antioxidant Capacity of MeOH Extract of *Ageratina adenophora* and *Ipomoea cairica* using Ascorbic acid as Standard

Concentration mg/ml	Absorbance				% TAC		
	Control	ICL	AA	ICF	ICL	AA	ICF
0.05	0.312	0.005	0.016	0.008	98.34	94.87	97.44
0.1		0.007	0.019	0.011	97.76	93.91	96.47
0.25		0.009	0.041	0.018	97.11	86.85	94.23
0.50		0.016	0.107	0.035	94.87	65.70	88.78
0.75		0.024	0.186	0.057	92.30	40.38	81.73
1.00		0.032	0.259	0.065	89.74	16.98	79.17

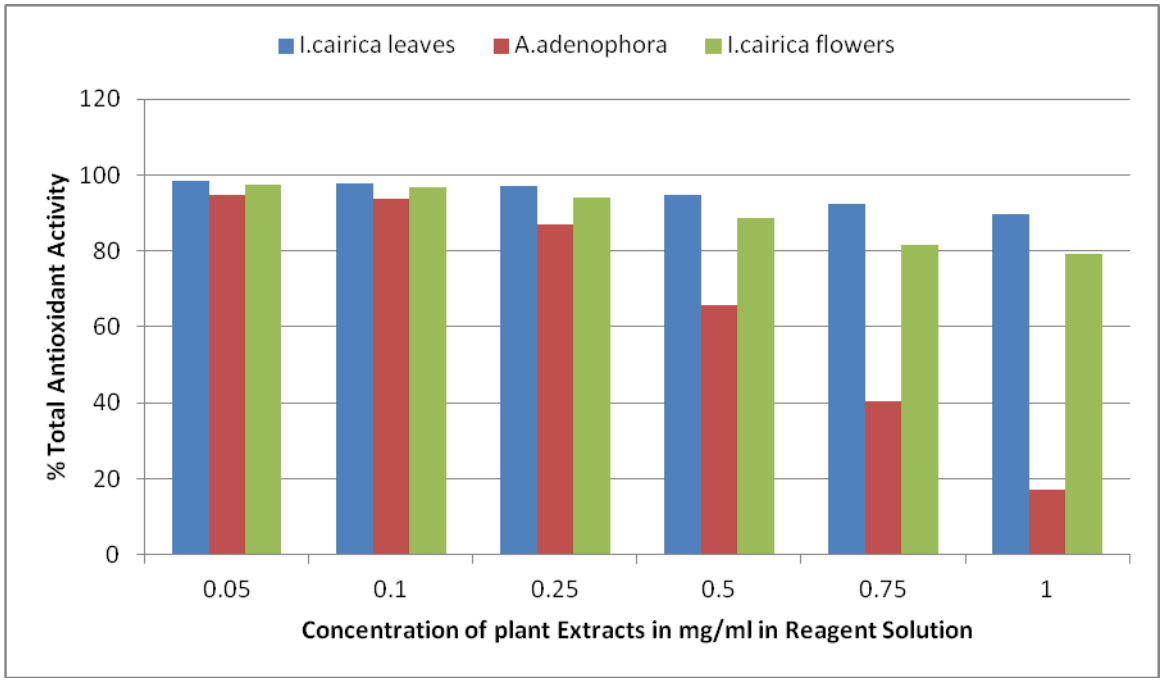


Figure 3.6(a): Bar graph showing Percentage of Total Antioxidant inhibition Capacity of Phosphomolybdate Free Radical by the MeOH extracts of the Plants at Various Concentrations

The phosphomolybdate method is a quantitative method for determining the total antioxidant capacity (TAC), which is expressed as ascorbic acid equivalents. This method gives a combined measure of the antioxidant activity of the range of chemically diverse phenolics and flavonoids present in the methanolic extract of *A. adenophora* leaves as determined by the formation of the reduced phosphomolybdate complex (695 nm) as indicated in Table 3.7(C). The total antioxidant capacity of MeOH extracts of *I. cairica* flowers, *A. adenophora* leaves and *I. cairica* leaves ranges from 79.17 to 97.44%, 16.98 to 94.87% and 89.74 to 98.34% of standard ascorbic acid at concentrations ranging from 0.05 to 1 mg/ml of the plant extract in methanol.

Table 3.7(D) Determination of DPPH radical scavenging activity by MeOH extract of *Ageratina adenophora* and *Ipomoea cairica* using BHT as standard

Concentration mg/ml	Absorbance				% Inhibition		
	Control	ICL	AA	ICF	ICL	AA	ICF
0.0005	0.074	0.072	0.059	0.059	2.70	20.27	20.27
0.001		0.046	0.045	0.035	37.84	39.19	52.70
0.005		0.037	0.024	0.019	50.00	67.57	74.32
0.01		0.016	0.020	0.008	78.38	72.97	89.19
0.025		0.015	0.018	0.007	79.73	75.67	90.54
0.05		0.013	0.016	0.005	82.43	78.38	93.24

Lower absorbance of the reaction mixture indicates higher free radical scavenging activity

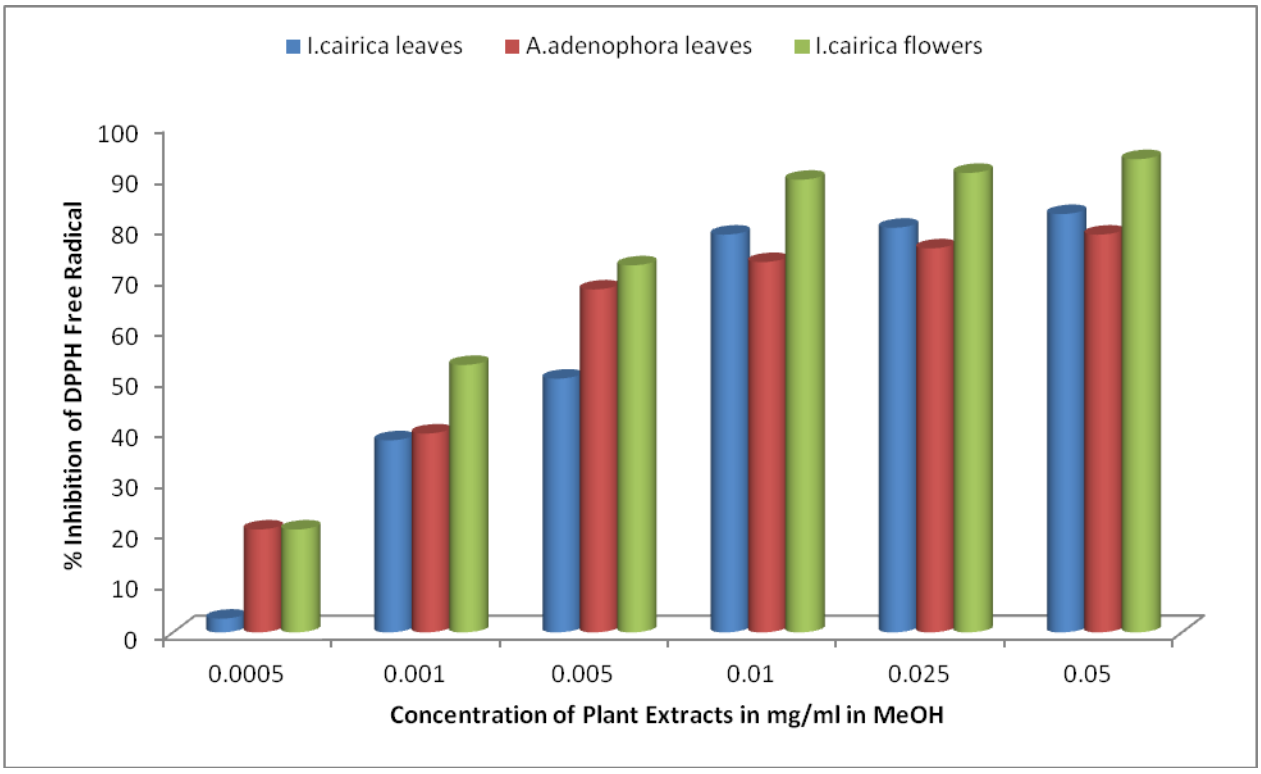


Figure 3.6(b): Bar graph showing percentage inhibition of DPPH free radical by the MeOH extracts of the plants at various concentrations

Table 3.7(E). DPPH free radical scavenging activities of MeOH extracts of *I.cairica* leaves, *A.adenophora* leaves and *I.cairica* flowers expressed in terms of IC_{50} and IC_{99} values in µg/ml

Plant part/species	IC_{50} µg/ml	95% confidence limits		IC_{99} µg/ml	95% confidence limits	
		Lower Limit	Upper Limit		Lower Limit	Upper Limit
I.cairica leaves	**5.0**	3.0	13.0	**5050.0**	760.0	10790.7
A.adenophora leaves	**3.0**	1.0	6.0	**2327.0**	301.0	5478.3
I.cairica flowers	**1.0**	1.0	3.0	**153.0**	44.0	278.8

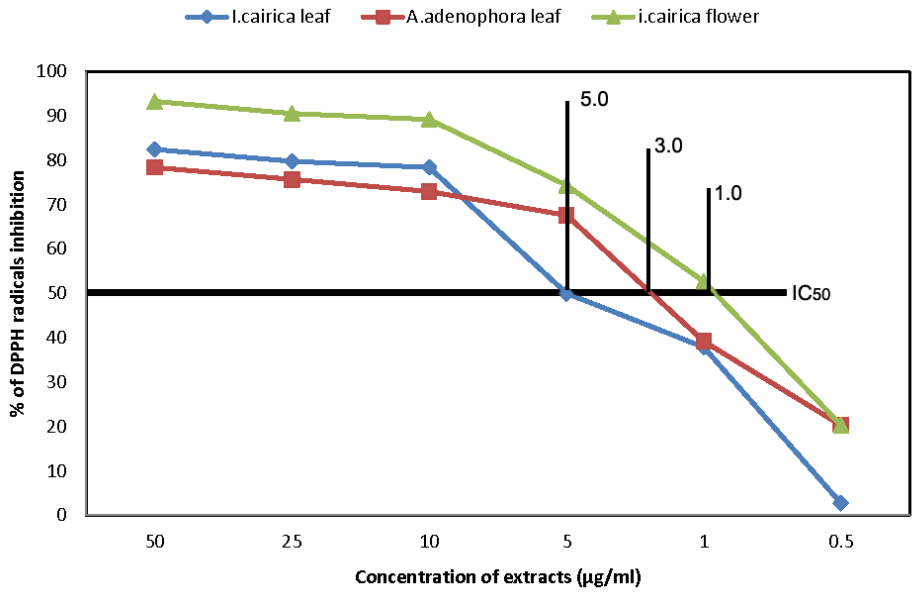


Figure 3.6(c). Antioxidant (DPPH scavenging) activity of investigated plant extracts presented as percentage of DPPH radicals inhibition and IC_{50} values (µg/ml).

The antioxidant activities of MeOH extracts of *A. adenophora* leaves, *I. cairica* flowers as well as leaves were determined using a methanol solution of DPPH reagent. DPPH is very stable organic molecular free radical. The unpaired electron of DPPH is predominantly situated on one of the hydrogen of hydrazine moiety and it is extensively delocalised over phenyl rings that are attached with the nitrogens of hydrazine moiety. Unlike free radicals generated *in vitro* such as the hydroxyl radical and superoxide anion radical ($O2^{-\bullet}$), DPPH has a unique advantage of not being perturbed by unwanted side reactions, such as metal ion chelation and enzyme inhibition. A freshly prepared DPPH solution exhibits a deep purple colour with an absorption maximum at 517 nm. This purple colour in general, fades when antioxidant molecules quench DPPH free radicals (i.e. by providing hydrogen atoms or by electron donation, conceivably via a free-radical attack on the DPPH molecule) and convert them into a colourless/bleached product (i.e. 2,2-diphenyl-1-hydrazine, or a substituted analogous hydrazine), resulting in a decrease in absorbance at 517 nm band (Amarowicz *et al*., 2003).

The antioxidant activities of methanol extracts from *A. adenophora* leaves, *I. cairica* flowers and leaves were expressed in terms of percentage of inhibition [Table 3.7(D)] and IC_{50} values in μg/ml [Table 3.7(E)]. BHA was used as the standard compound, parallel to examination of the antioxidant activity of plant extracts, the values for the standard compound were obtained and compared to the values of the antioxidant activity.

The examination of antioxidant activities of methanol extracts from *A. adenophora* leaves, *I. cairica* flowers and leaves showed different values. Several concentrations ranging from 0.0005 - 0.05 mg/mL of the plant extracts were tested for their antioxidant activity. It was observed that the DPPH free radicals were scavenged by the test extracts in a concentration dependent manner. The obtained values were in a range from 2.70 % inhibition for 0.0005 mg/ml concentration of *I. cairica* leaves to 93.24% inhibition for 0.05 mg/ml concentration of *I.cairica* flowers. The largest capacity to neutralize DPPH radicals was found for methanolic extract of *I.cairica* flowers which neutralized 50% of free radicals at the concentration of 1.00 μg/ml. A moderate activity was found for methanol extract of *A. adenophora* leaves which neutralized 50% of free radicals at the concentration of 3.00 μg/ml. The lowest capacity to inhibit DPPH radicals was determined for MeOH extract of *I.cairica* leaves for which the IC_{50} was calculated to be 4.00 μg/ml. In comparison to IC_{50} values of BHA, MeOH extract from *I.cairica* flowers manifested the strongest capacity for neutralization of DPPH radicals.

The antioxidant activity may be due to the presence of phenolic hydroxyl or methoxyl groups, flavone hydroxyl, keto groups, free carboxylic groups, quinones and other structural motifs (Patt and Hudson, 1990, Demiray *et al*., 2009). The commercially available synthetic antioxidants have been suspected of causing or instigating negative health effects, so strong restrictions imposed over their application and there is an urgent trend to substitute them with naturally occurring antioxidants (Hosny and Rosazza 2002; Molyneux, 2004) while the intake

of natural antioxidants has been associated with the concomitant reduced risks of cancer, cardiovascular disease, diabetes, and other diseases related with age as they have the advantage of being almost devoid of side effects (Yang *et al,* 2001; Sun *et al*, 2002).

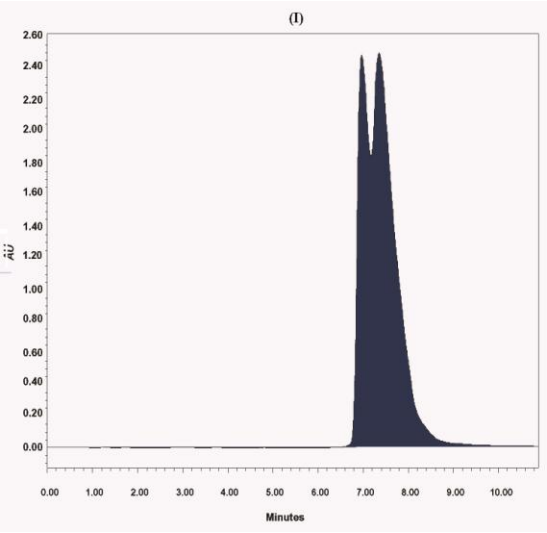
It is important to substitute synthetic antioxidants with naturally occurring safer antioxidants as the synthetics have been suspected of causing or provoking unfavorable side effects, while stronger restrictions are encountered on their application (Molyneux, 2004). Several reports have conclusively shown a correlation between the antioxidant activity and amount of total phenolics or total flavonoids (Negro *et al*., 2003; Ramadeep and Geoffrey, 2005). Plant extracts with high phenolic contents also possess high flavonoid content as reported for other plant species (Sun *et al*., 2002; Elzaawely and Tawata 2012). In our findings, the extracts that exhibit the highest antioxidant activity have the highest concentration of phenols. Therefore, the phenolic content of plants may contribute directly to their antioxidant action (Tosun *et al*., 2009). A significant linear correlation was found between the concentration of phenolic compounds [**Table 3.7(A)** & **3.7(E)**] and the antioxidant activity of MeOH extracts of the plants. The values for the concentration of phenolic compounds [**Table 3.7(A)**] and antioxidant activity of different plant extracts [**Table 3.7(E)**] proved a significant linear correlation. Numerous investigations of the antioxidant activity of plant extracts have confirmed a high linear correlation between the values of phenol concentration and antioxidant activity (Borneo *et al*., 2008; Katalinic *et al*., 2004).

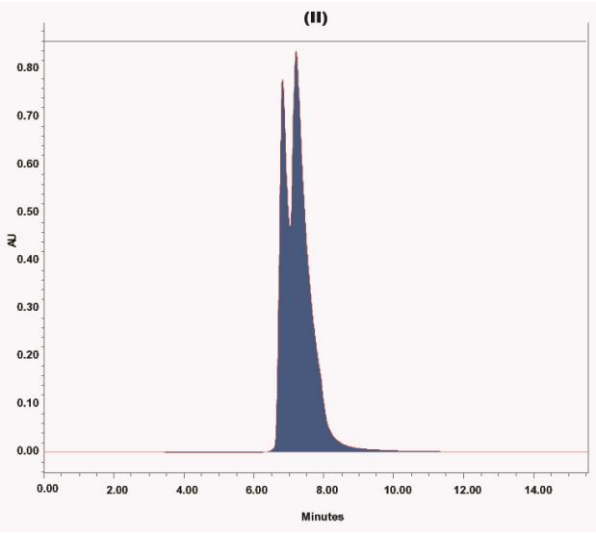
Aerobic organisms consume a large amount of molecular oxygen to maintain cellular metabolic processes. ROS are the ramification of various metabolic processes for which the terminal electron acceptor is molecular oxygen (O_2) that acts as a thermodynamic sink. ROS (reactive oxygen species) have been generally accepted to cause harm to living organisms and

thus, the induced oxidative stress due to the formation of ROS is attributed to the damage of biological systems in the body, promoting the development of various diseases such as cancer, atherosclerosis, myocardial infarction, diabetes mellitus, arthritis and also accelerating the aging process (Halliwell, 1994; Moskovitz *et al*., 2002). The regular intake of natural antioxidants, however, has been associated with the reduced risks of cancer, cardiovascular disease, diabetes, and other degenerative diseases associated with aging, have the advantage of being almost devoid of harmful side effects (Ramarathnam *et al*., 1995). On the basis of the results, it can be concluded that methanol extracts of the two plants had a powerful *in vitro* antioxidant capacity against various antioxidant system in a dose dependent manner.

3.7. HPLC Analysis of the Crude Extracts and Isolated Compounds

HPLC analysis of crude plant extracts and isolated compounds employing a Waters HPLC-PDA system applying isocratic elution with methanol (for methanolic flower extract of *I.cairica*) and acetonotrile(95):water(5) (for pet ether extract of *A. adenophora* leaf) are displayed as below:





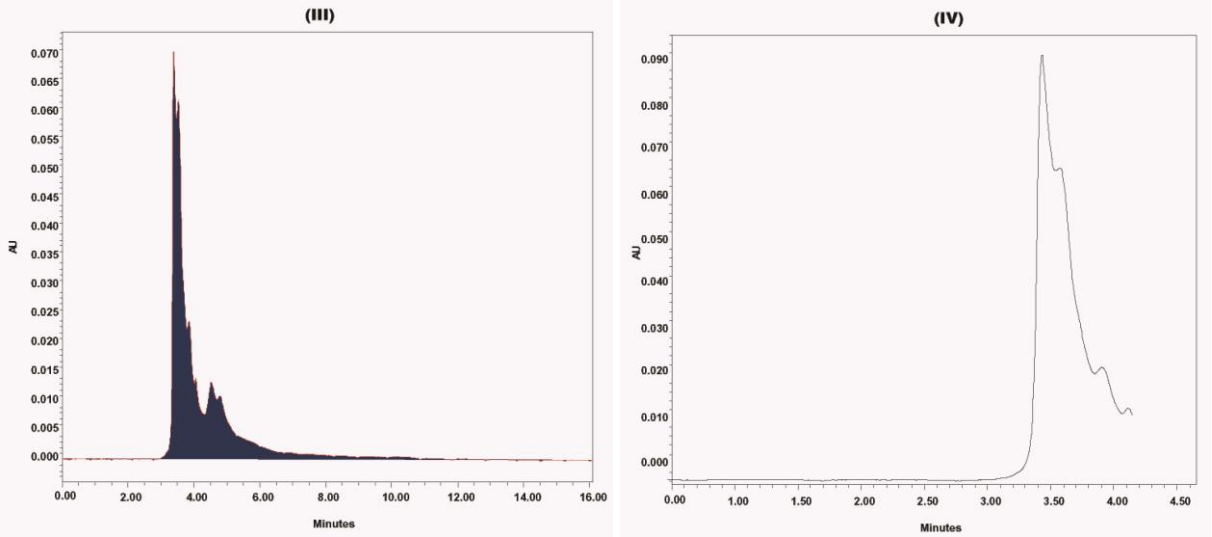


Figure 3.7(A). HPLC Chromatogram of MeOH Extract of *Ipomoea cairica* Flower at (I) 220 nm, (II) 280 nm, (III) 325 nm and (IV) 620 nm respectively at a flow rate of 0.2 ml per minute

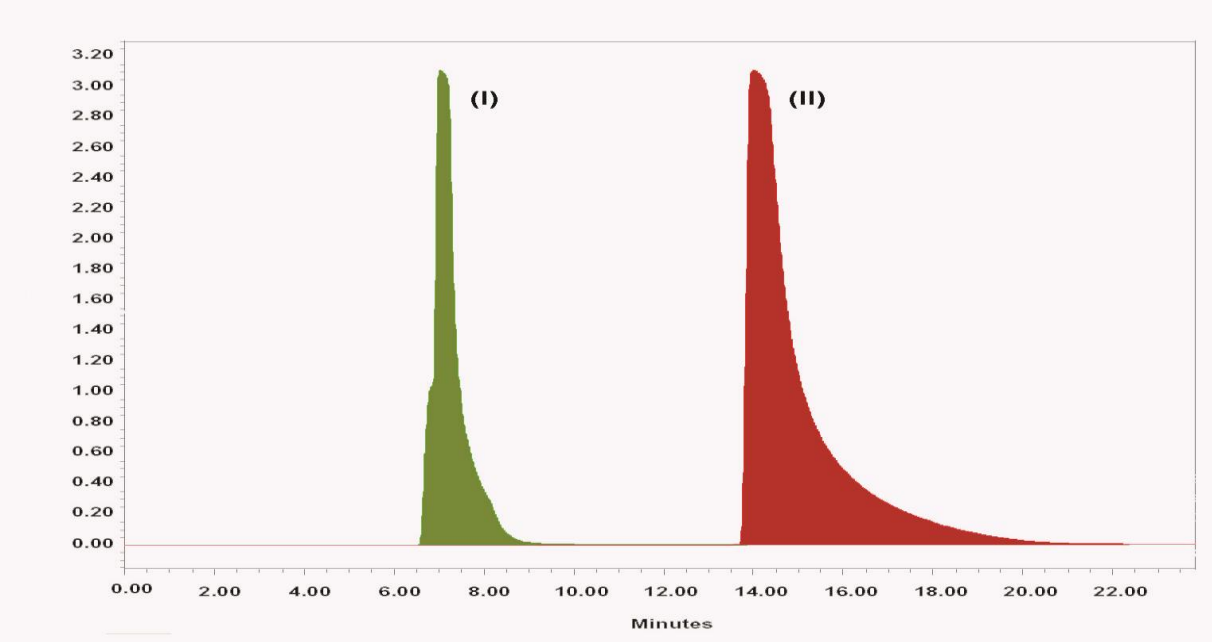


Figure 3.7(B). HPLC Chromatogram of Isolated Compound IC-1 at 280 nm: (I) At a flow rate of 0.2 ml per minute, (II) At a flow rate of 0.1 ml per minute.

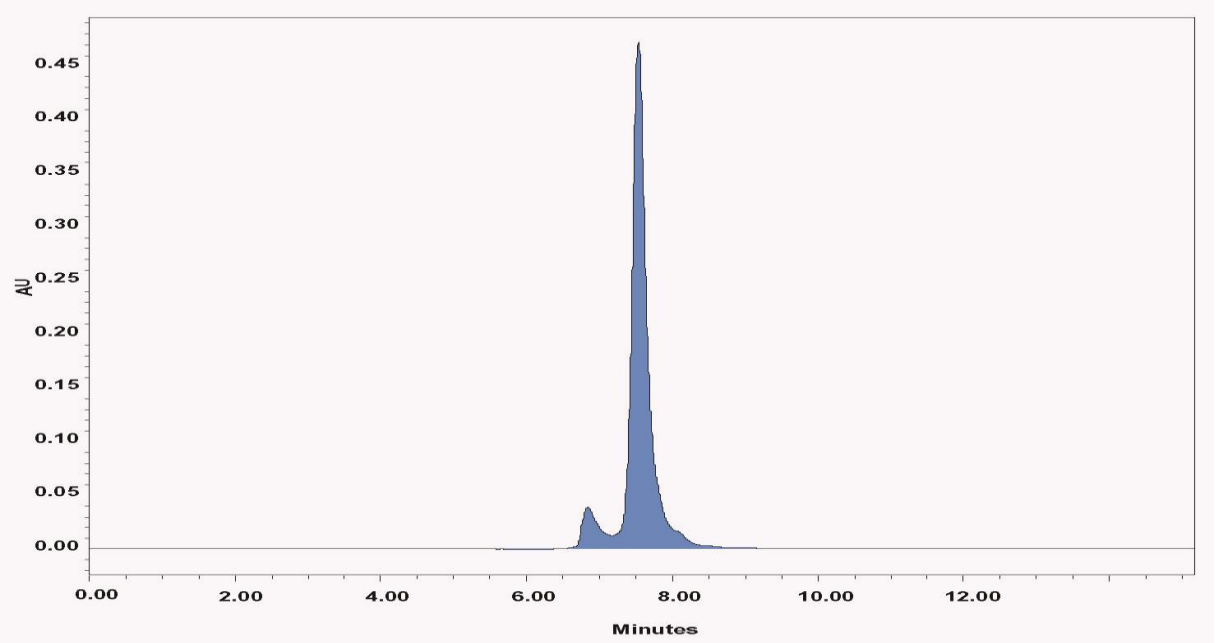


Figure 3.7(C). HPLC Chromatogram of Isolated Compound IC-2 at 280 nm at a flow rate of 0.2 ml per minute.

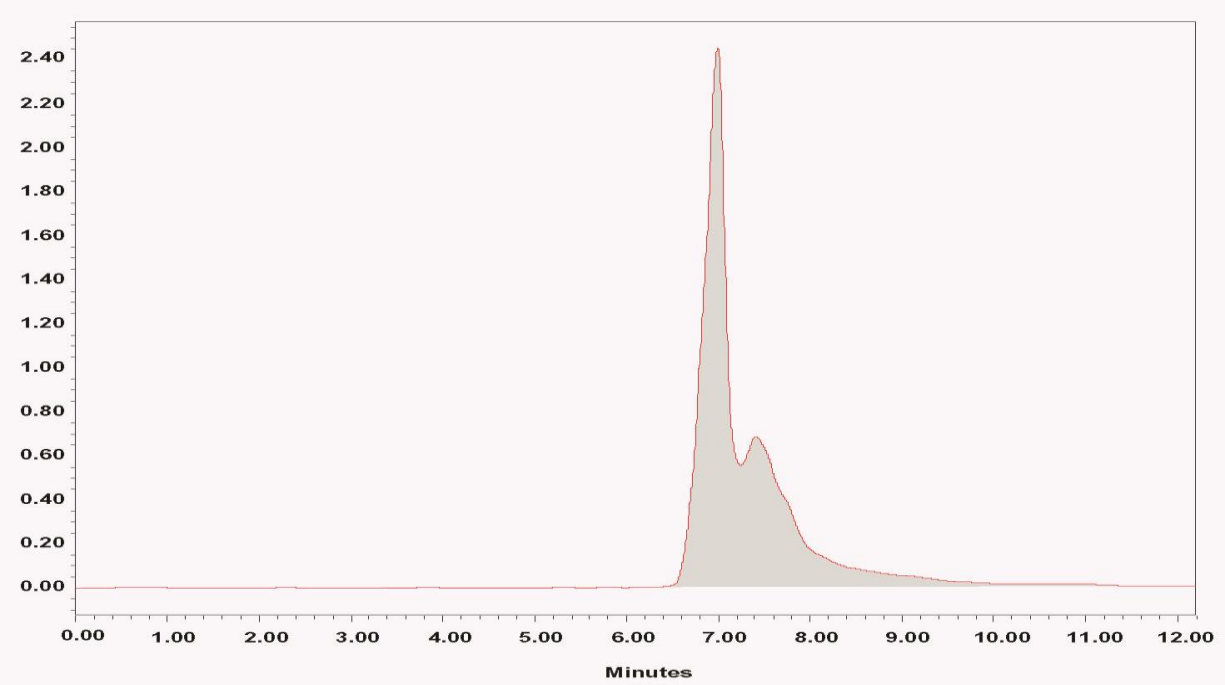


Figure 3.7(D). HPLC Chromatogram of MeOH Extract of *Ipomoea Cairica* Leaves at 220 nm at a flow rate of 0.2 ml per minute.

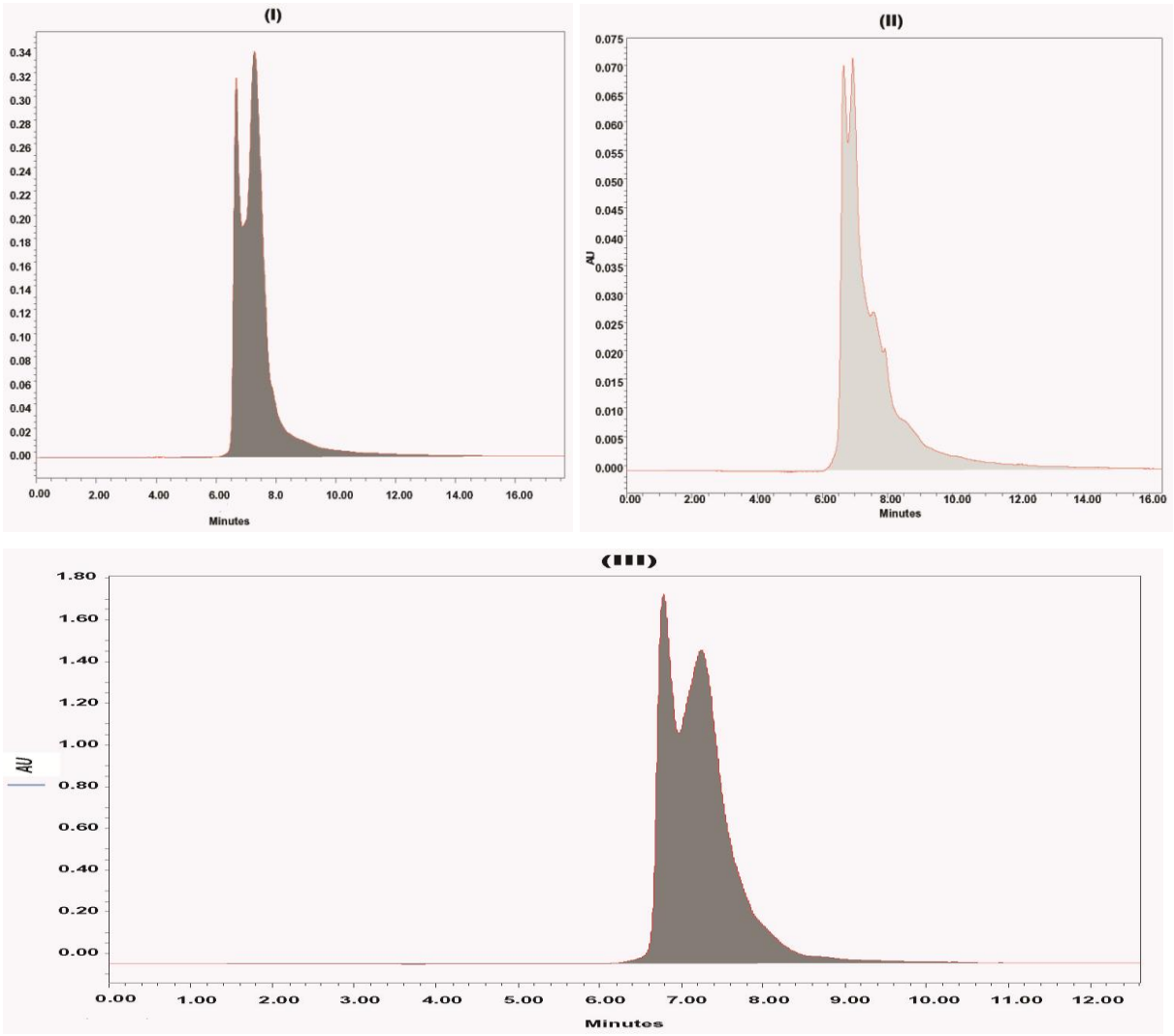


Figure 3.7(E). HPLC Chromatogram of MeOH Extract of *Ageratina adenophora* Leaves at (I) 280 nm, (II) 325 nm and (III) 620 nm at a flow rate of 0.2 ml per minute.

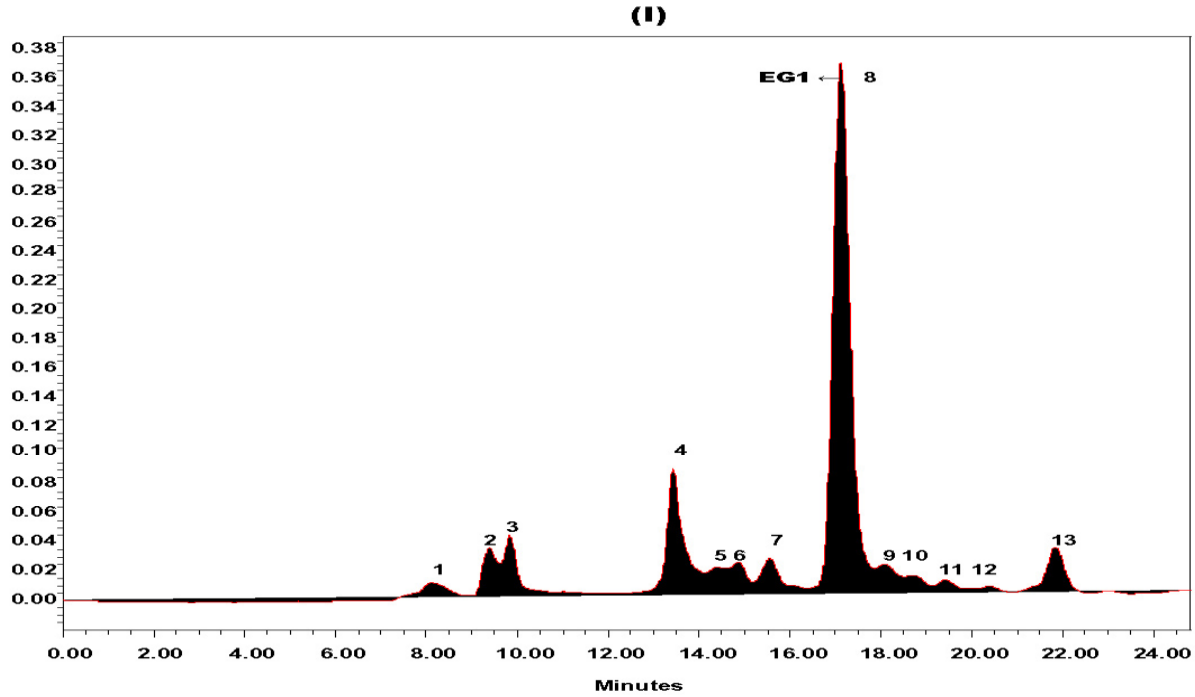


Figure 3.7(F). HPLC Chromatogram of Petroleum ether Extract of *Ageratina adenophora* Leaves at a flow rate of 0.2 ml per minute.

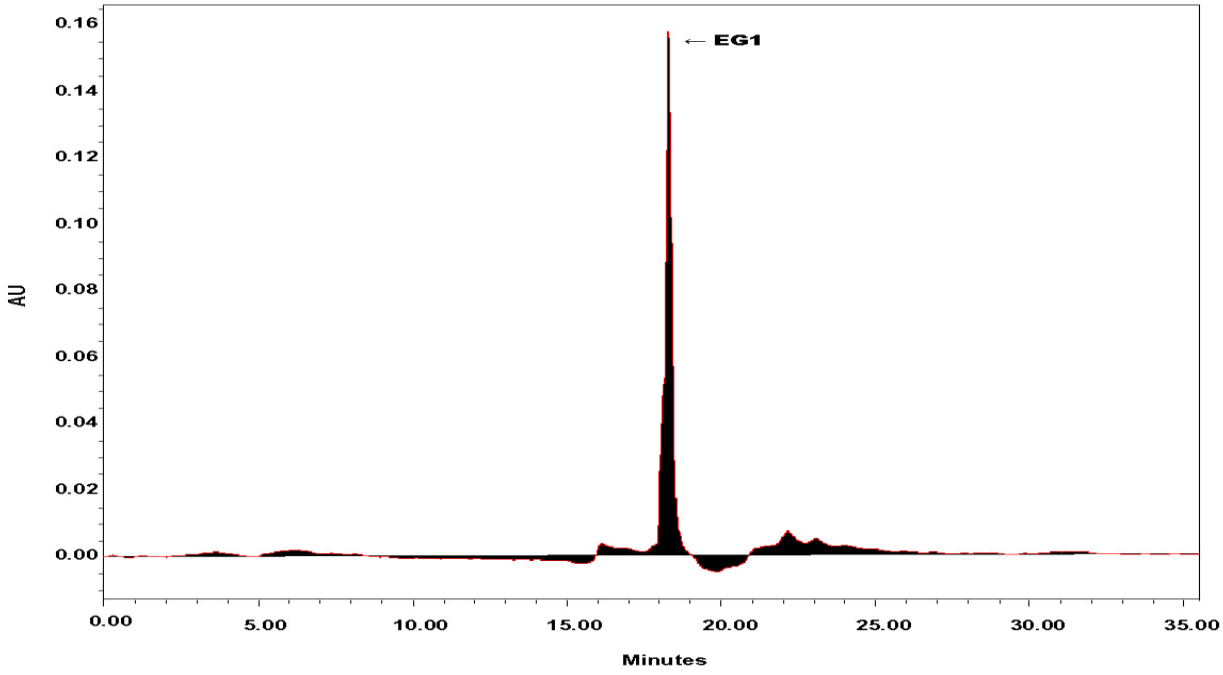


Figure 3.7(G). HPLC Chromatogram of Isolated Compound EG1 from Petroleum ether Extract of *Ageratina adenophora* Leaves at a flow rate of 0.2 ml per minute.

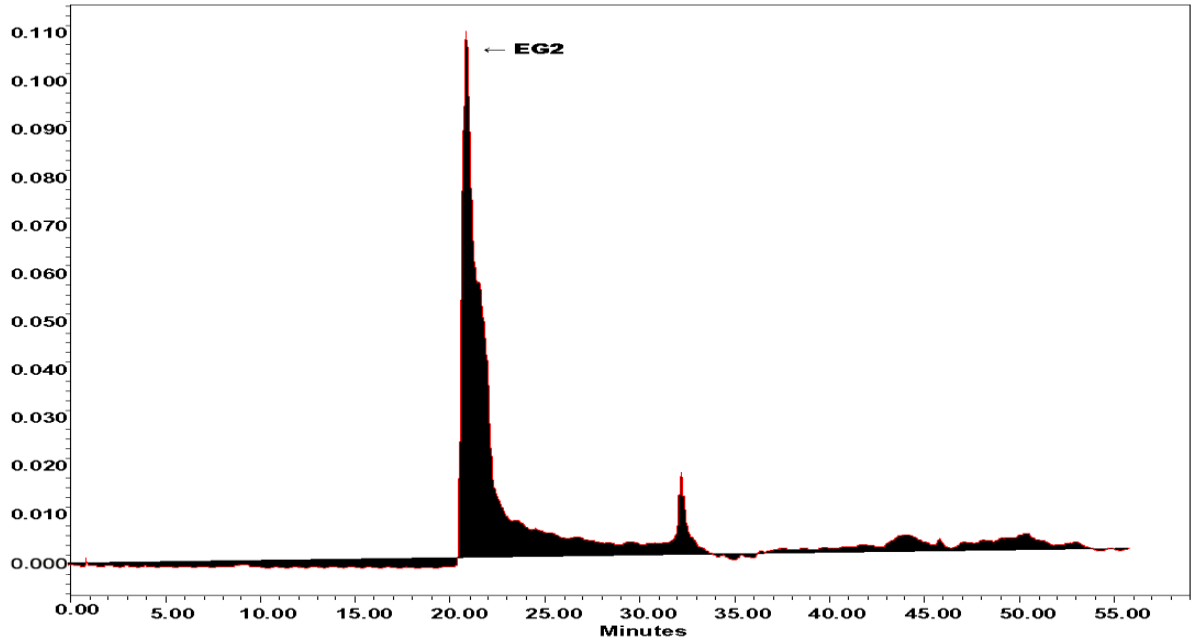


Figure 3.7(H). HPLC Chromatogram of Isolated Compound EG2 from Petroleum ether Extract of *Ageratina adenophora* Leaves at a flow rate of 0.2 ml per minute.

HPLC currently is considered as a versatile and reliable technique for analysis of phenolic compounds during their isolation from crude extracts. A diverse collection of stationary and mobile phases variable degree of polarity are available for the analysis of phenolics including anthocyanins, proanthocyanidins, hydrolysable tannins, flavonols, flavan-3-ols, flavanones, flavones, and phenolic acids in different plant extract and food samples (Yanagida *et al*., 2002; Mertz *et al*., 2007). Moreover, HPLC techniques offer a unique opportunity to analyze simultaneously all components of interest together with their possible derivatives or degradation products (Sakakibara *et al*., 2002; Downey and Rochfort, 2008). The introduction of reversed-phase (RP) columns has considerably enhanced HPLC separation of different classes of phenolic compounds and RP C-18 columns are almost exclusively employed.

PDA is the most prevalent method for detection since it allows for simultaneous scanning of a range of wavelengths, real time, of UV/VIS spectra of all solutes passing through the detector, giving more information of compounds in complex mixtures such as a plant crude extract (Jin Dai and Russel, 2010).

The HPLC chromatogram of MeOH extract of *I.cairica* flowers indicated the presence of the phenolic acid IC1, at a retention time of 7.2 minutes and unknown phenolic compound IC2, at a retention time of 7.6 minutes in the crude extract when eluted with methanol in an isocratic mode at a flow rate of 0.2 ml/minute. The decreased flow rate of 0.1 ml/minute from 0.2 ml/minute also shows that the retention time increased with decrease in flow rate (from 7.2 to 14.50) as in the case for IC1 [figure 3.7(B)]. In our experiments, RP columns are used because phenolic compounds are weak acids that can be separated as neutral, relatively hydrophobic compounds in a weak acid matrix (Harnly *et al.,* 2007).

The multiple conjugate double and aromatic bonds make phenolic compounds strong chromophores, exhibiting absorption in the UV or UV-VIS region (Harnly *et al*., 2007; Stalikas, 2007). A PDA detector allows recording of the UV-VIS spectra of each chromatographic peak. Each chromatographic peak may then be attributed to a polyphenol subclass, since each subclass exhibit a characteristic UV-Vis absorption maxima (De Rijke *et al.,* 2006)

The HPLC chromatogram of petroleum ether extract of *Ageratina adenophora* leaves indicated the presence of the sterol EG1 and EG2 in the crude extract [Figure 3.7(F)]. EG1 had a retention time of 17.3 minutes and EG2 had a retention time of 22.1 minutes. Many unknown peaks were found to exist in the chromatograms. In this work, a method based on reversed phase HPLC separation combined with PDA detection has been developed for phytosterol analysis in *Ageratina adenophora*. An isocratic elution was chosen since it is simple, requires

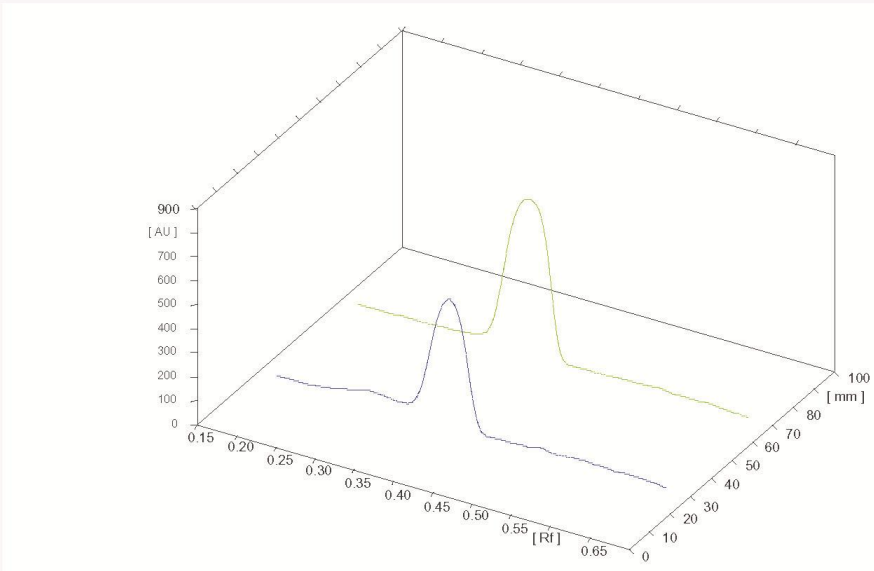
only one pump and minimizes the variation of baseline and ghost peaks. For RP-HPLC, various columns are available, but a C_{18} column (250 × 4.6 mm, 5 µm i.d.) was preferred because its peak shape and resolution are better. Among various mobile phases employed, acetonitrile and water (95:5 v/v) was found to be suitable for analysis of EG1 and EG2. Further, a flow rate of 0.2 ml/min and an injection volume of 20 µl along with UV detection at 240 nm provided the optimal conditions for analysis of these phytosterols.

3.8. HPTLC Analysis of the Crude Extracts and Isolated Compounds

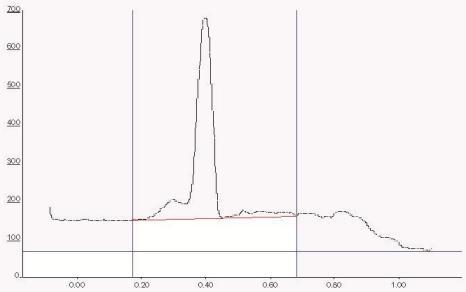
The high performance thin layer chromatogram (HPTLC) of the methanolic extracts of *Ipomoea cairica* flowers, petroleum ether extract of *Ageratina adenophora* leaves and the isolated compounds were presented in figure 3.8. and figure 3.9 respectively. Various mobile phases were tested to get the best eluent for obtaining distinctive spots without tailing in the developed TLC plates (silica gel F_{254}). For spot identification, separation and subsequent isolation of IC1 a mobile phase of toluene: EtOAc: HCOOH: MeOH in the ratio of 3: 3: 0.6: 0.4 was found to be most suitable while $CHCl_3$(80): MeOH(13): H_2O(2): 5%HCOOH(5) and and $CHCl_3$: MeOH (8:2) were selected for IC2.

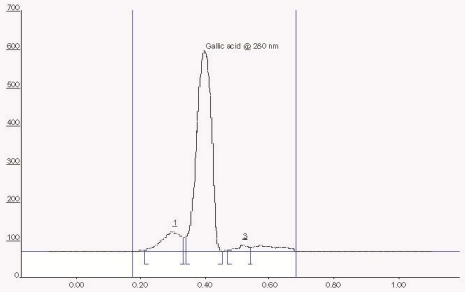
For development of chromatogram and subsequent isolation of EG1 and EG2 from petroleum ether extract of *A. adenophora* leaf, a mobile phase of pet ether(9): EtOAc(1) was found to be most suitable.

winCATS Planar Chromatography Manager
All tracks at WavelengthSc4



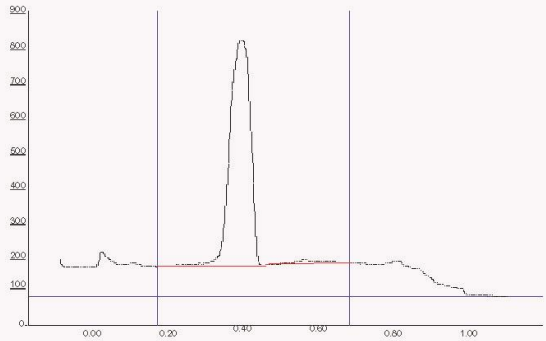
Track 1, ID: Standard Gallic acid

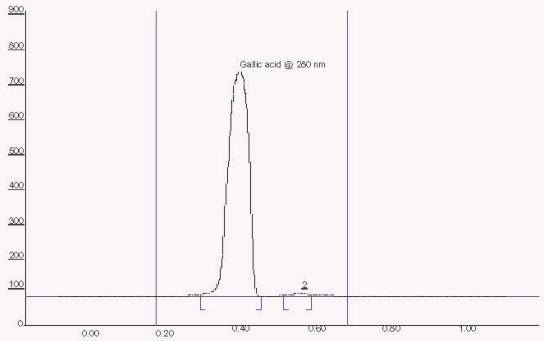




Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.21	1.4	0.29	50.2	8.47	0.33	38.4	2051.4	10.91	unknown *
2	0.33	38.5	0.39	524.2	88.55	0.44	0.4	16376.4	87.08	Gallic acid
3	0.46	2.2	0.51	17.6	2.98	0.53	8.6	377.7	2.01	unknown *

Track 2, ID: IC-1





Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.29	6.8	0.39	647.0	98.07	0.44	1.0	23732.0	98.43	Gallic acid (IC-1)
2	0.50	5.2	0.55	12.8	1.93	0.57	6.4	377.8	1.57	unknown *

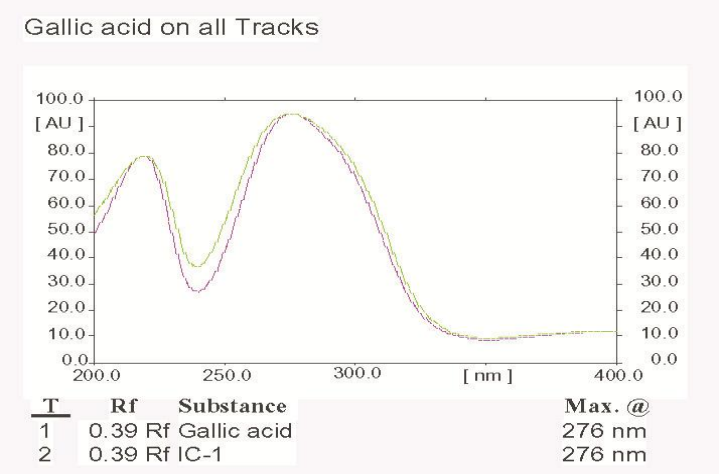


Figure 3.8(A) Chromatogram showing winCATS planar chromatography HPTLC analysis of Isolated compound IC-1 and standard gallic acid.

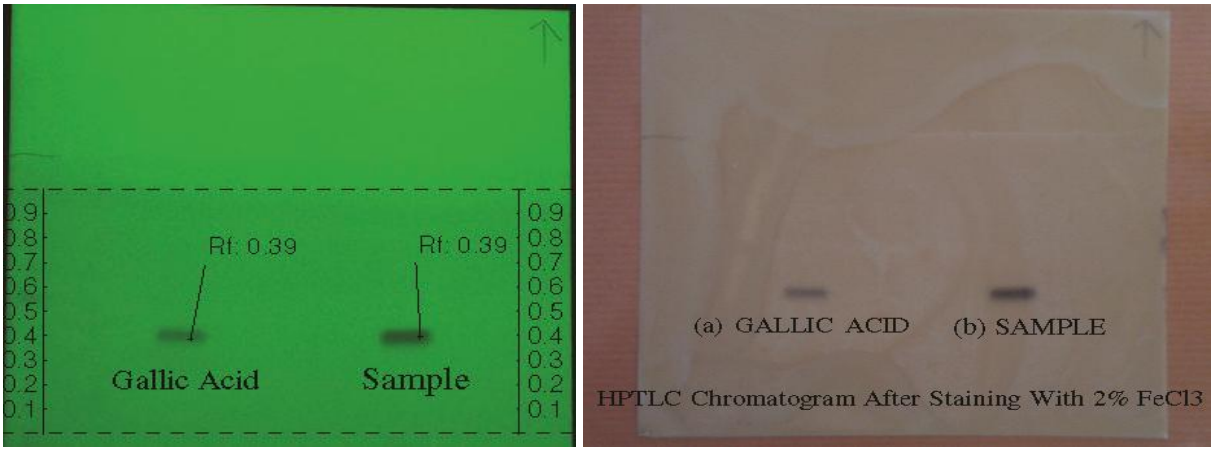


Figure 3.8(B): Photograph of developed TLC plate showing chromatography of Isolated compound gallic acid (a) and IC-1 (b).

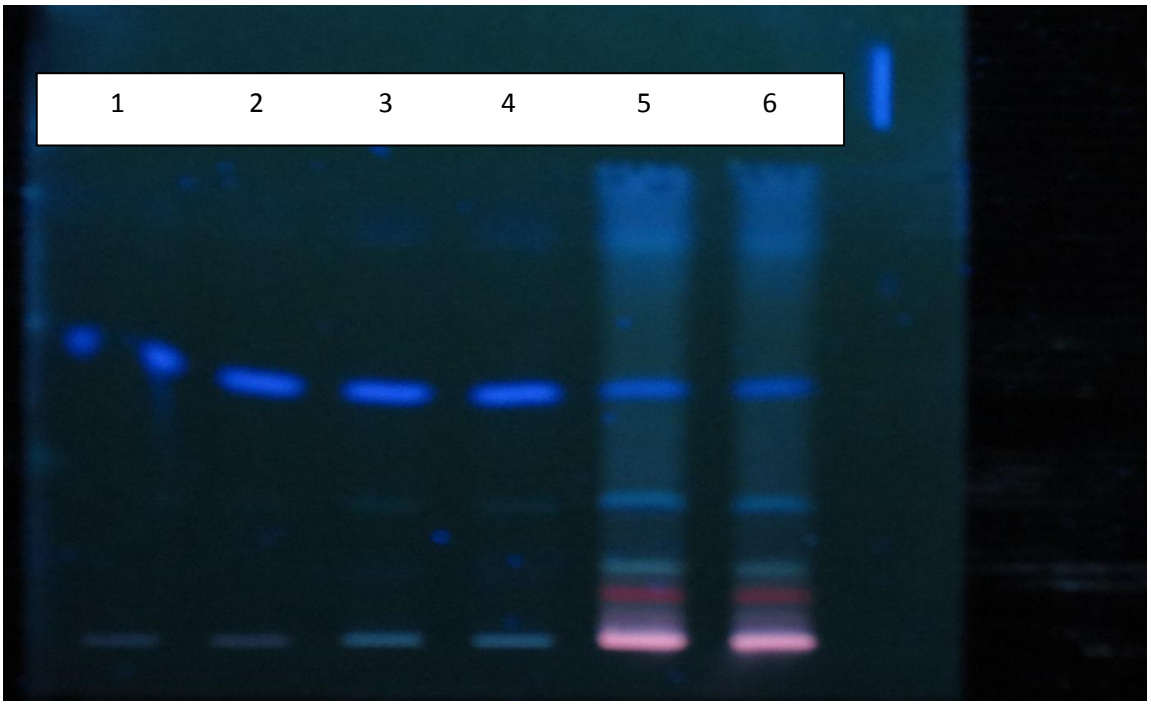


Figure 3.9(a). Photograph of developed TLC plate showing chromatography of Isolated compound EG1 (1, 2), EG2 (3,4) and petroleum ether extract of leaf powder of *Ageratina adenophora* (5, 6)

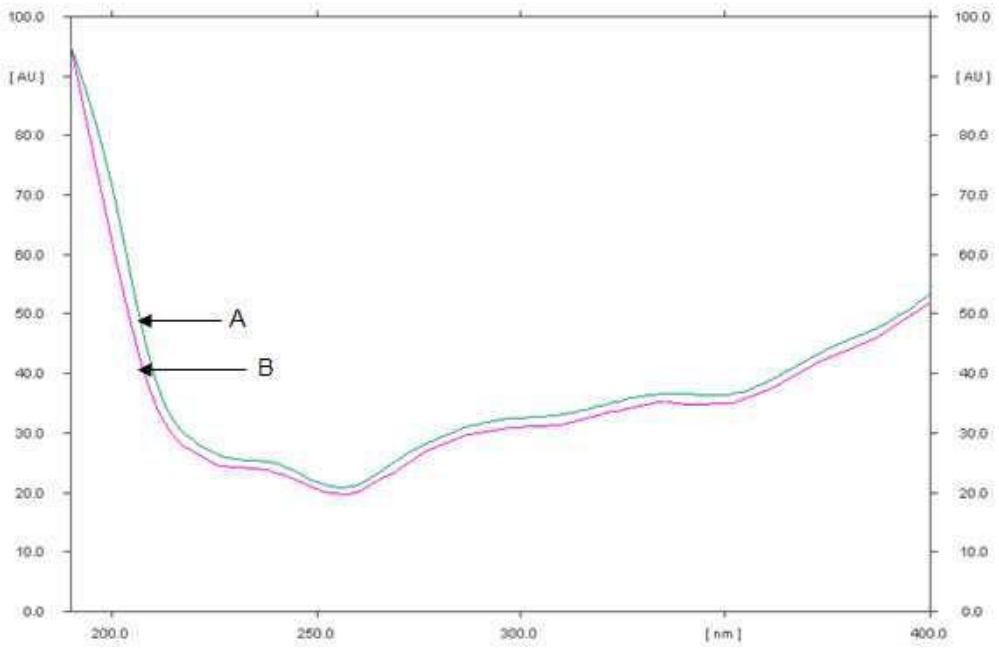


Figure 3.9(b). Overlay of spectra of stigmasterol standard solution (A) and isolated compound EG1 (B) from petroleum ether extract of *A.adenophora* leaf powder.

HPTLC is a rudimentary separation technique which is adequate for the sample clean up, purification, qualitative assays and preliminary estimation for phytochemical studies. $CHCl_3$ was used as a solvent for phytosterols due to its non polar nature and has been shown to be very efficient in dissolving extracts (Boukes *et al*., 2008). The presence of phytosterols in the petroleum ether extract of *Ageratina adenophora* leaves was confirmed by HPTLC followed by qualitative test using Liebermann-Burchard reagent and Salkowski test. The HPTLC chromatogram of petroleum ether extract of *Ageratina adenophora* leaves yielded a characteristic pattern of spots when observed under long wavelength of 366 nm [Figure 3.9(a)]. Five different spots could be reliably distinguished on the TLC plates with colors and R_f values as indicated in figure 3.9(a), chromatogram no. 5&6. The blue spot at R_f 0.52 corresponds with the migration distance and spot color obtained with authentic beta-sitosterol.

The extract had the same R_f value for EG1 and EG2, this confirms the presence of these phytosterol in the extract, however it does not mean that these are not the only phytosterols present in this extract. Phytosterols are very similar in structure and because of these similarities; EG1 and EG2 were the only phytosterols observed in the extracts and also it may be difficult for HPTLC to separate them efficiently. In addition to the similarity in structures, the molecular weight of phytosterols is very close together with EG1 having a molecular weight of 412.69 g/mol which is lower than that of EG2 (414.72 g/mol). With these results we can conclude that EG2 is also more polar phytosterol than EG1 which may be the reason why it was identifiable in the extracts more so than EG1.

3.9. FT-IR Analysis of the Isolated Compounds Spectral Studies

Results of Fourier transform infra red spectrometer analysis of the isolated compounds **IC1**, **EG1** and **EG2** were provided in figures 3.10(a), 3.10(b) and 3.10(c) respectively.

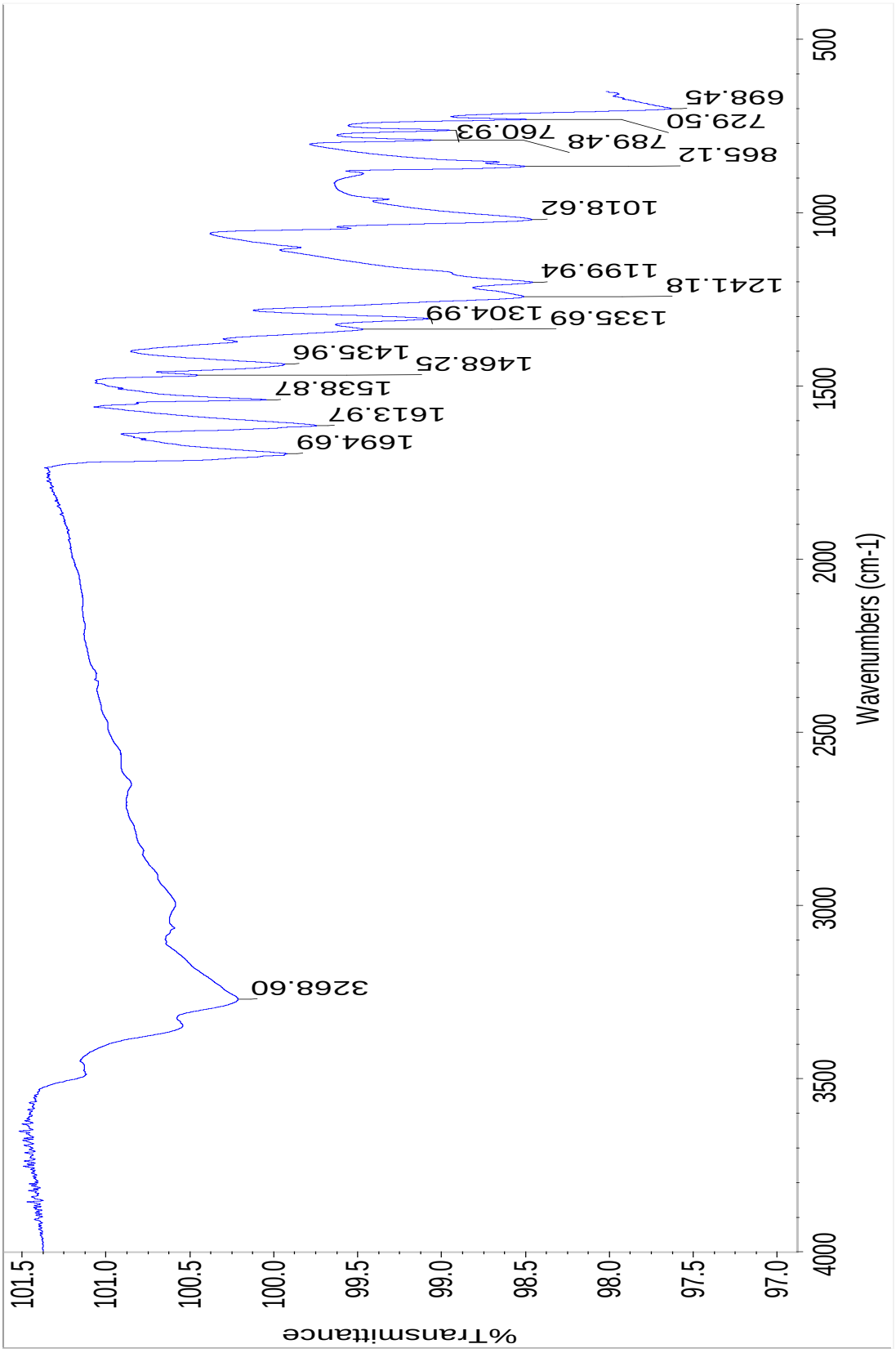


Figure 3.10.(a). FT-IR Spectral Data of Isolated Compound IC-1 from Methanol Extract of *Ipomoea cairica*

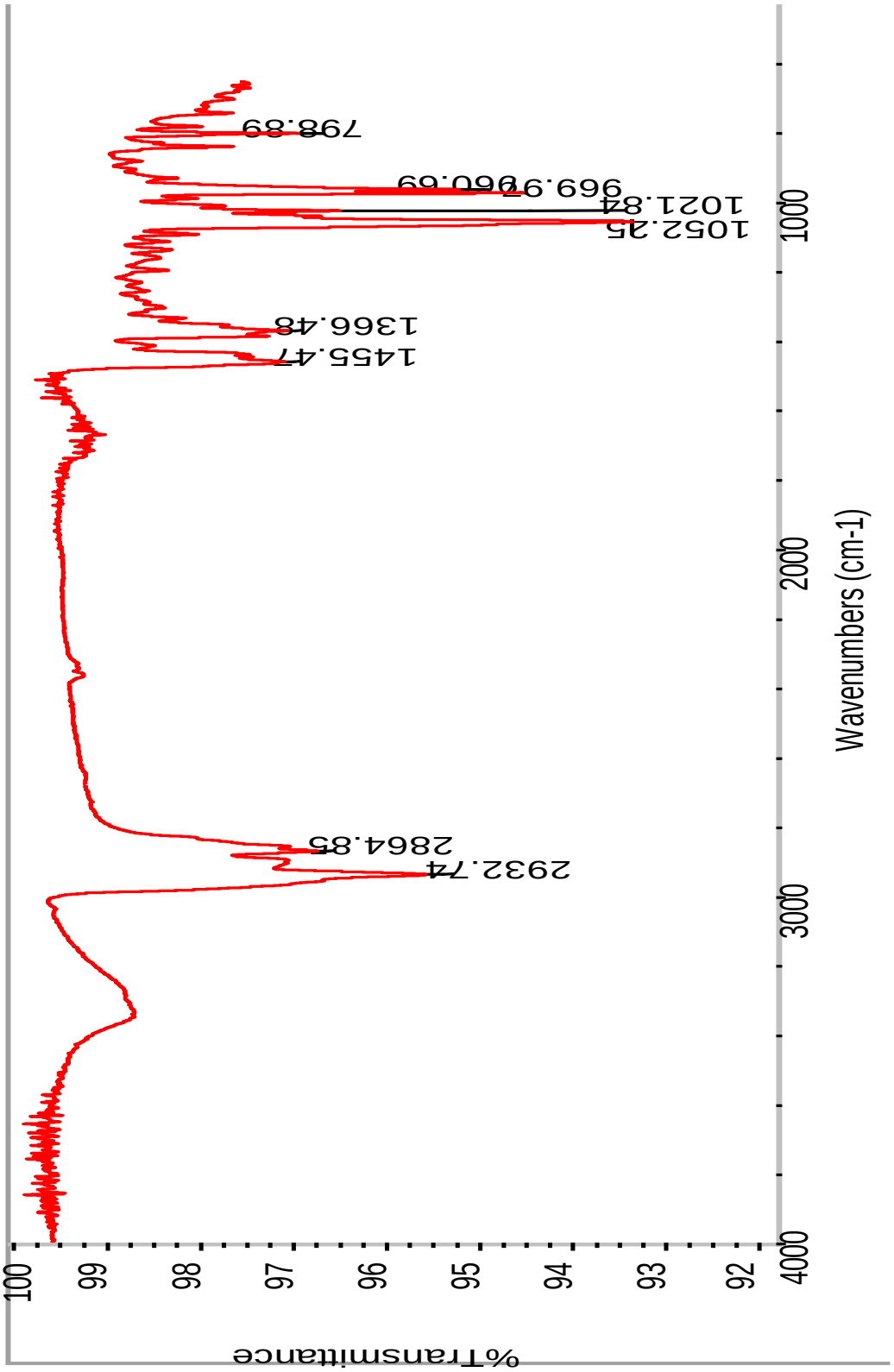


Figure 3.10.(b). FT-IR Spectral Data of Isolated Compound EG-1 from Pet Ether Extract of *Ageratina*

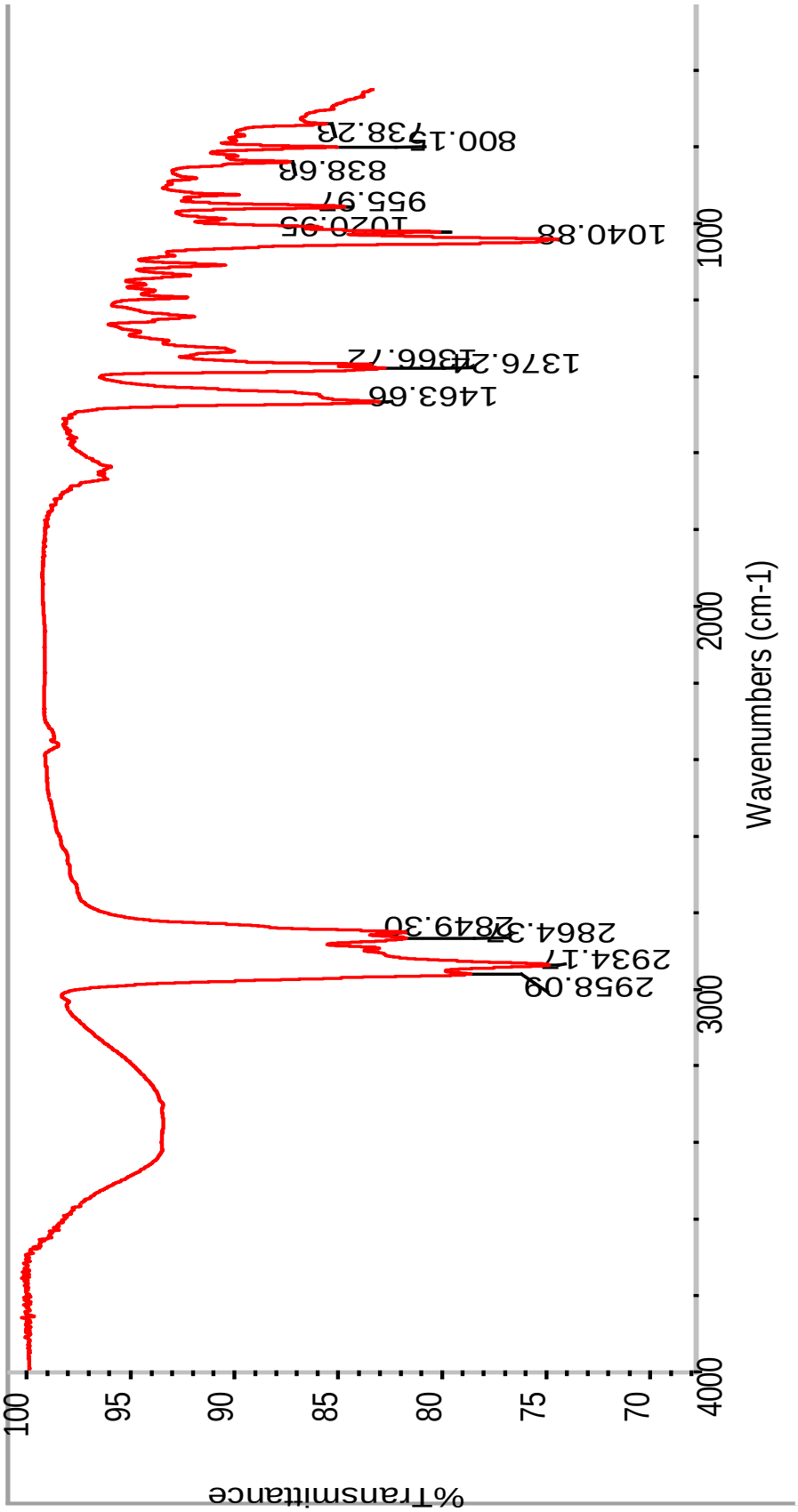


Figure 3.10.(c). FT-IR Spectral Data of Isolated Compound EG-2 from Pet Ether Extract of *Ageratina adenophora*

Table 3.8(A). Interpretation of IR Spectrum of Isolated Compound IC1

υ(cm^{-1})	**Nature**	**Inference**
3268.60	*s,board*	*OH- stretching*
1694.69	*s,sharp*	*C=O stretching*
1613.97	*s,narrow*	*C=C stretching*
1018.62	*s,broad*	*C-O stretching*

IR spectrum of IC1 exhibited an intense vibrational band centered at ≈ 3268.60 cm^{-1} that may be indicative of the presence of the -OH group (O–H stretching) and the broadness of the peak may be due to hydrogen bonding interactions. The IR frequency band at 1694.69 cm^{-1} that may indicate the presence of a carbonyl group (C=O stretching). The IR absorption band at 1613.97 cm^{-1} may be due to the presence of C=C group frequency and also as aresult of extended conjugation as in aromatic ring and at 1018.62 cm^{-1} may be assigned to molecular vibrations of carbonyl C-O single bond. The strong and broad band between 3600 – 2500 cm^{-1} and the strong and narrow peak at 1694.69 cm^{-1} could be assigned to be due to stretching vibration of -OH group and carbonyl group respectively, which indicated that carboxyl group, existed in the Isolated compound IC-1

The bands in the 3400-3500 cm^{-1} region are assigned to different kinds of -OH functional groups (from carboxyl or phenols) and those between 2800 and 2980 cm^{-1} may belong to stretching vibrations of the aliphatic and aromatic – C-H stretching frequency. The peaks at 1694.69 cm^{-1} and 1241.18 cm^{-1} indicate the presence of carboxylic groups. The aromatic character of the compound may also confirmed by the absorption bands at 1600-1620 cm^{-1}, along with the intense absorption at 864–868 (here 865.12) cm^{-1} that may be assigned to the aromatic C-H out of plane bending vibrational frequencies. The bands between 1200 and 1270

cm^{-1} (here 1241.18) represent the C-O deformation vibrations of phenols and carboxyl group (Mattoli *et al*., 2006). In alcoholic extracts there are absorption peaks in the region 1300-1800 cm^{-1}, more than in hexane, e.g. at 1558, 1517 and1467 cm^{-1}, as well in the region 1380-1450 cm^{-1}. Carbonyl groups have specific signals at 1743 cm^{-1}.

Table 3.8(B) Interpretation of IR Spectrum of Isolated Compound EG1

υ(cm^{-1})	**Nature**	**Inference**
1366.48	s,narrow	-OH deformation
1455.47	b, weak	cyclic $[-CH_2-]_n$
1656	s, weak	C=C stretching
3434	s, broad	O-H stretching

Table 3.8(C) Interpretation of IR Spectrum of Isolated Compound EG2

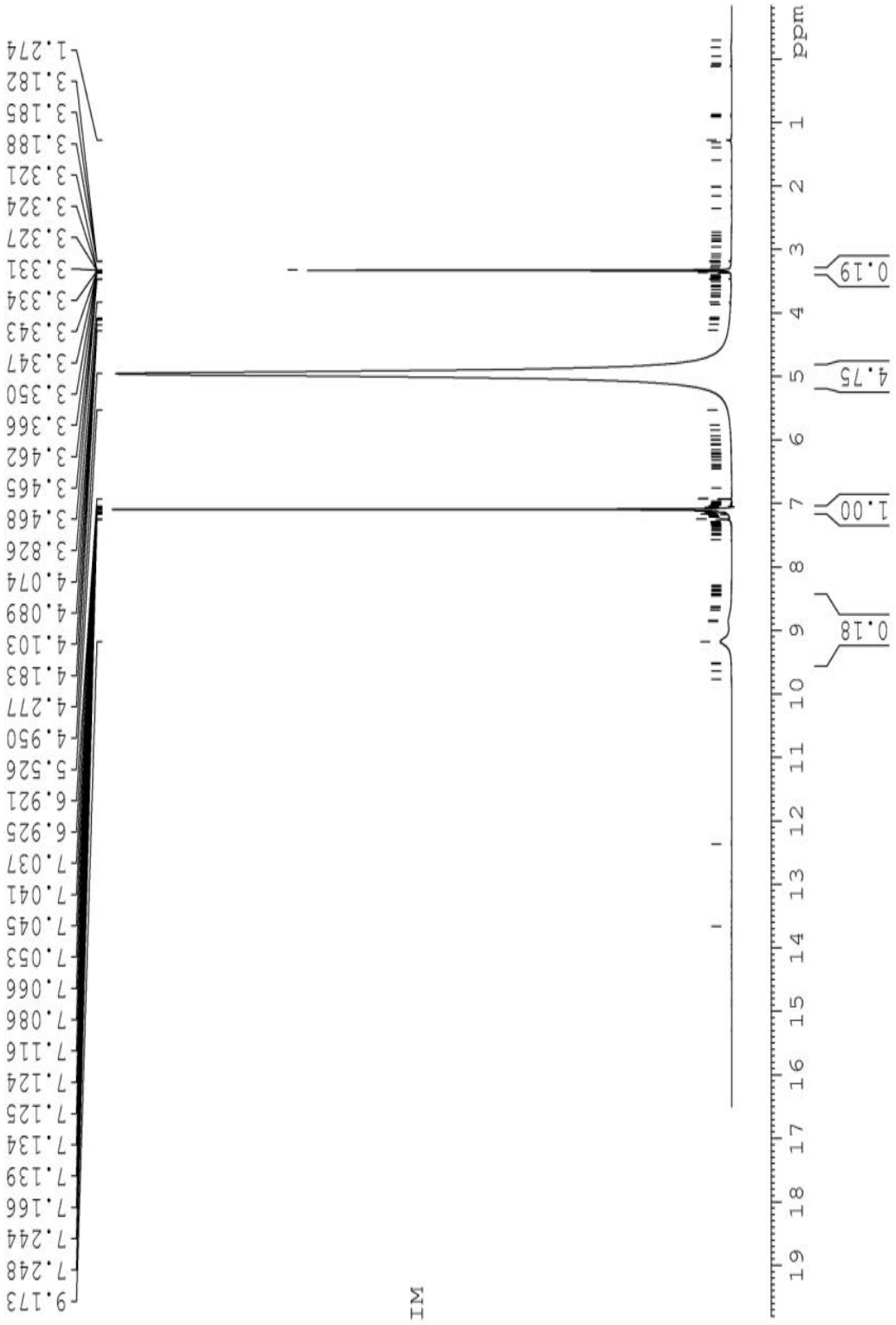
υ(cm^{-1})	**Nature**	**Inference**
1048.88	s, weak	cycloalkane
1376.2	weak	-OH deform
1463.6	b, weak	cyclic $[-CH_2-]_n$
1650	s,strong	presence of –OH

In the IR spectrum of EG1, the intense band at 2932.74 cm^{-1} and the band at 2864.85 cm^{-1} may be assigned to asymmetrical C-H and symmetrical C-H stretching frequencies of alkyl substituents such as $–CH_3$ groups and the medium band at 1455.47 cm^{-1} may be due to the asymmetrical bending vibrations. The moderate intense band at 798.86 cm^{-1} may be attributed to the rocking movement of methylene part.

3.10. ^{13}C and ^{1}H Analysis Studies of the Isolated Compounds.

Routine ^{1}H and ^{13}C NMR spectra were obtained on a Bruker spectrometer. The samples were dissolved in 0.8 ml of deuterated solvent (CD_3OD for IC1, $CDCl_3$ for EG2 and $CDCl_3$ + 2-3 drops of d-DMSO for EG1 respectively).

Figure 3.11.1 (A) One Dimensional ^{1}H NMR Spectra of IC-1 Isolated from MeOH Extract of *Ipomoea cairica* Flower



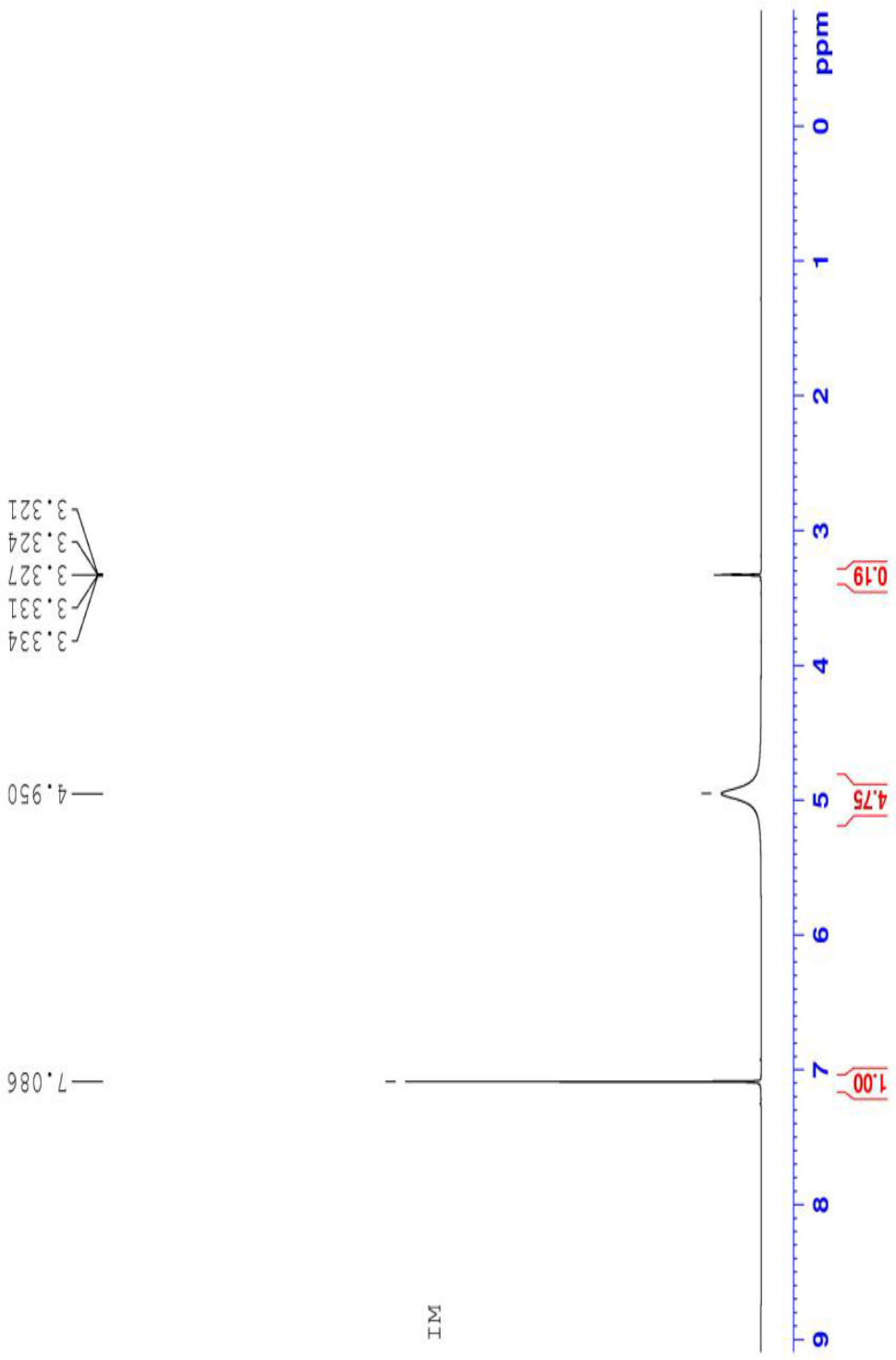


Figure 3.11.1 (B) One Dimensional ^{1}H NMR Spectra of IC-1 Isolated from MeOH Extract of *Ipomoea cairica* Flower (Expanded) at 0 – 9 ppm

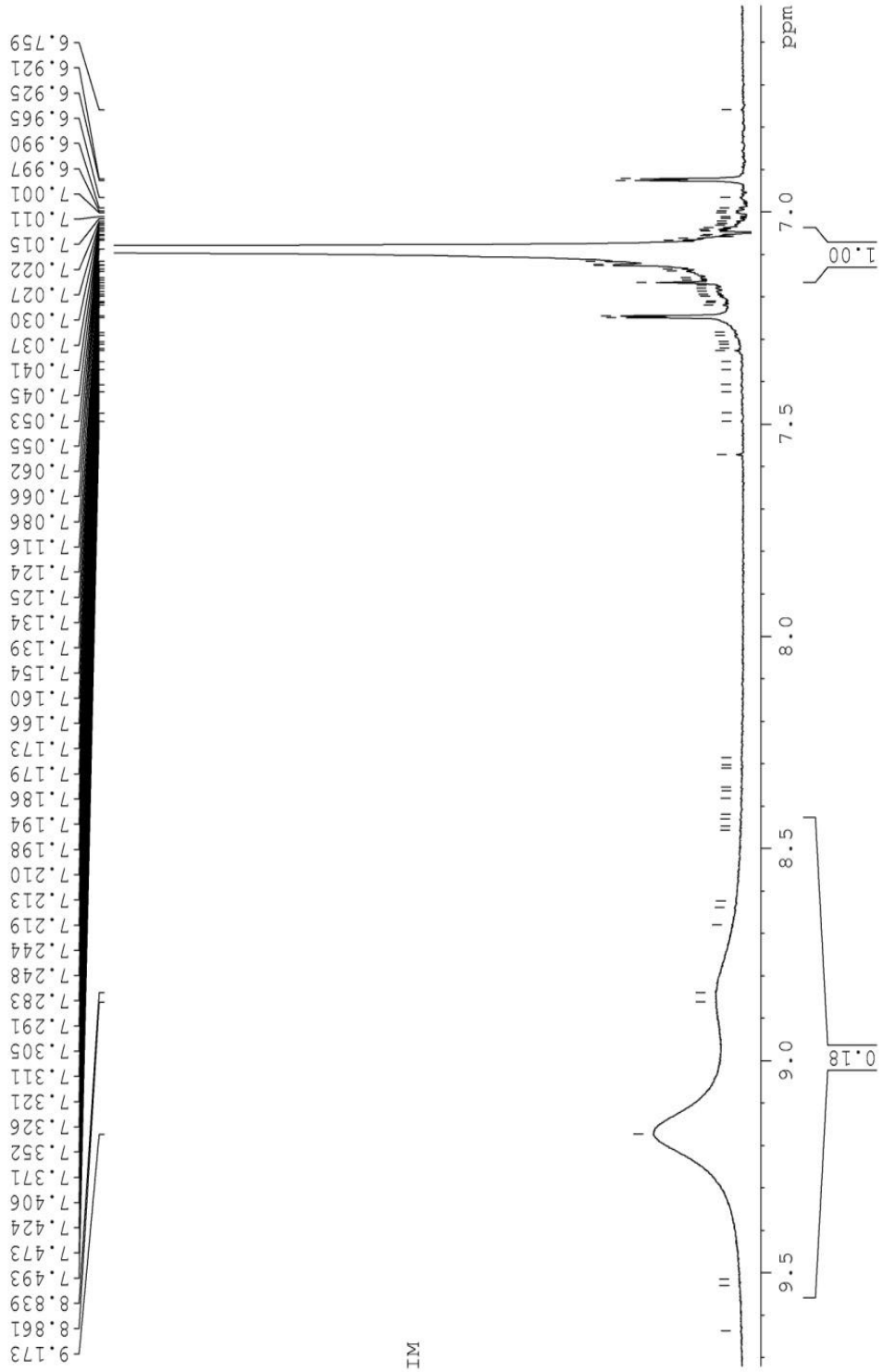


Figure 3.11.1 (C) One Dimensional ^{1}H NMR Spectra of IC-1 Isolated from MeOH Extract of *Ipomoea cairica* Flower (Expanded Up Field) 6.5ppm to 9.5 ppm

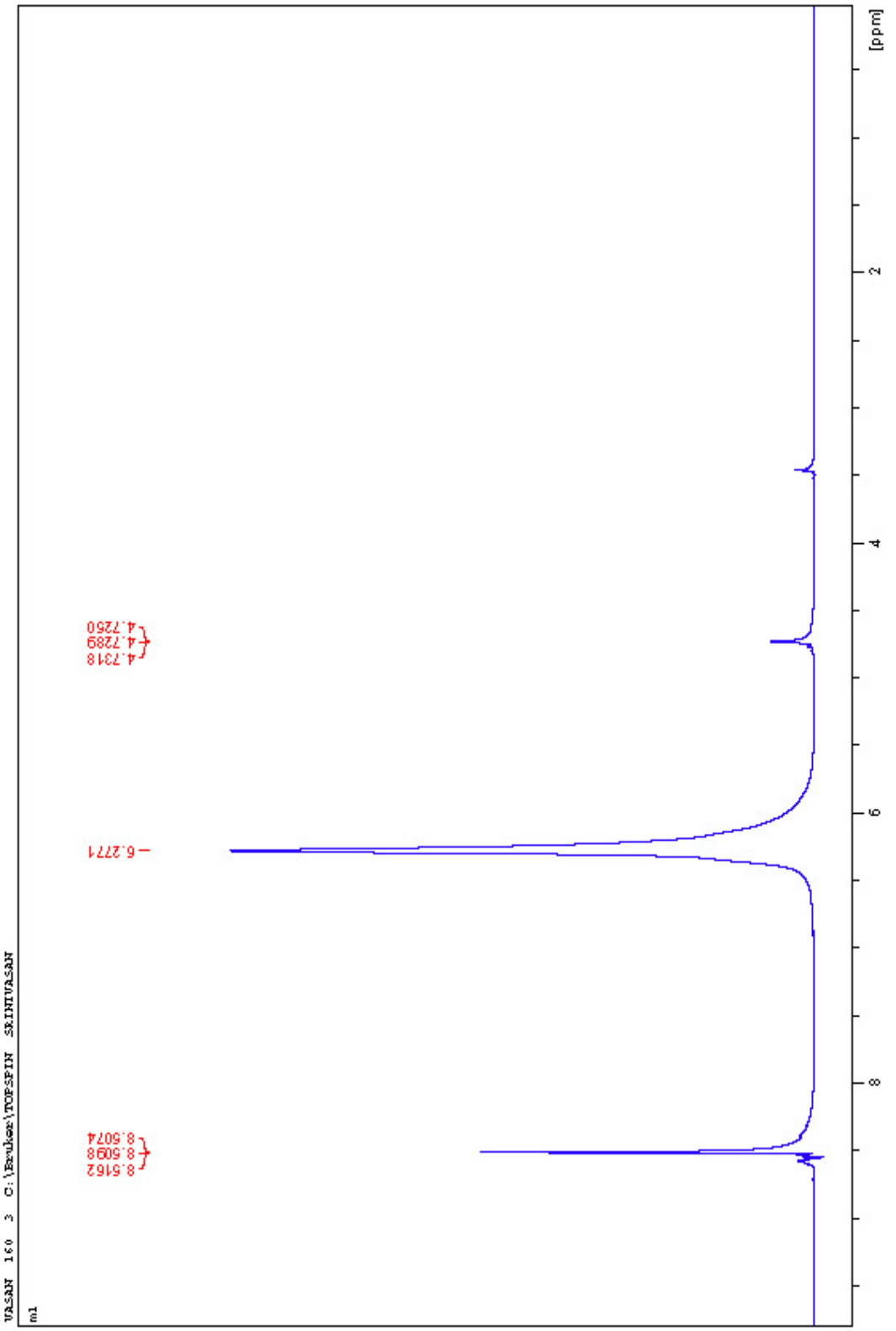


Figure 3.11.1 (D) One Dimensional ^{1}H NMR Spectra of IC-1 Isolated from MeOH Extract of *Ipomoea cairica* Flower (Topspin) 0 ppm to 9 ppm

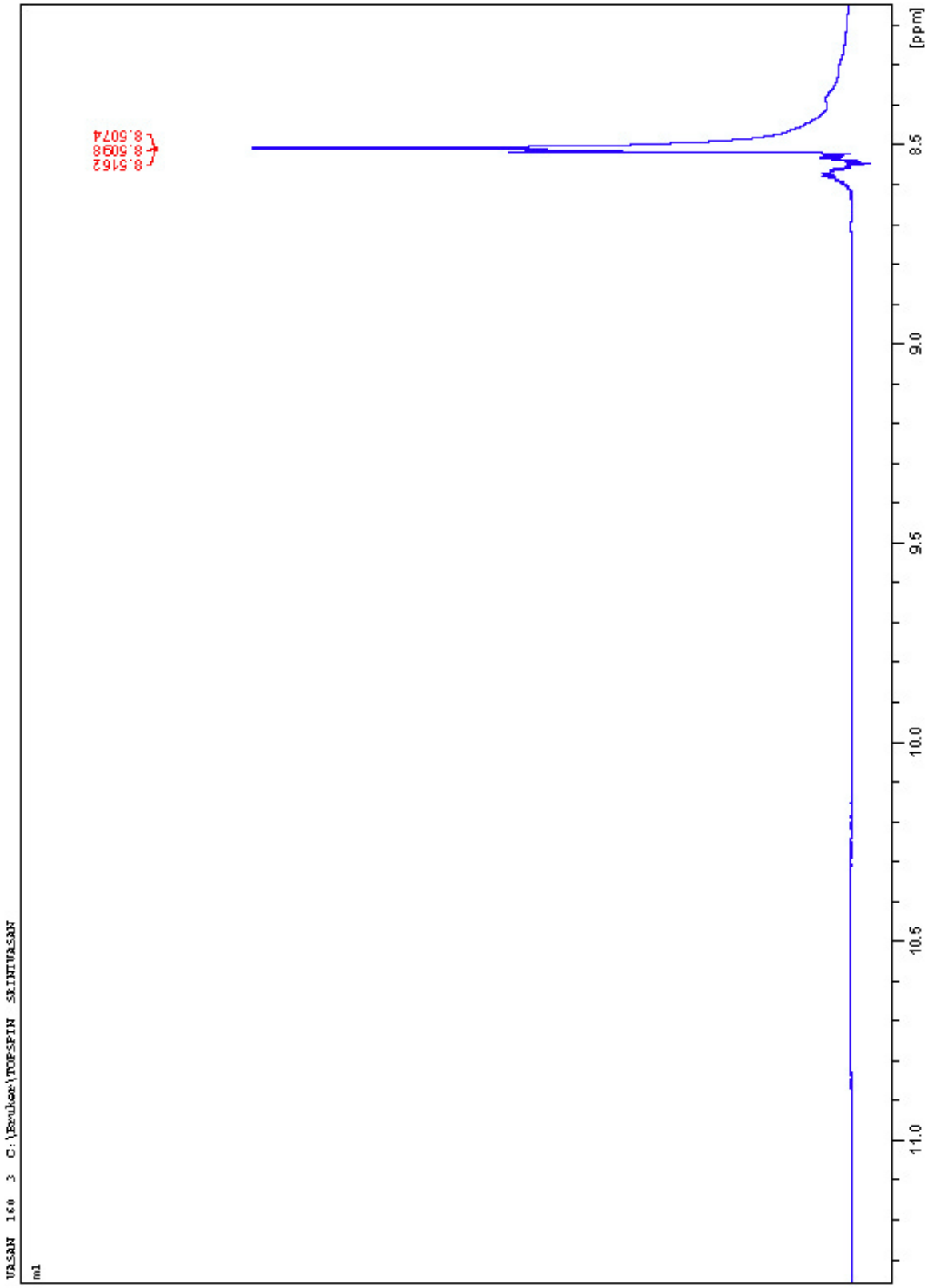


Figure 3.11.1 (E) One Dimensional ^{1}H NMR Spectra of IC-1 Isolated from MeOH Extract of *Ipomoea cairica* Flower (Topspin) 8 ppm to 9 ppm

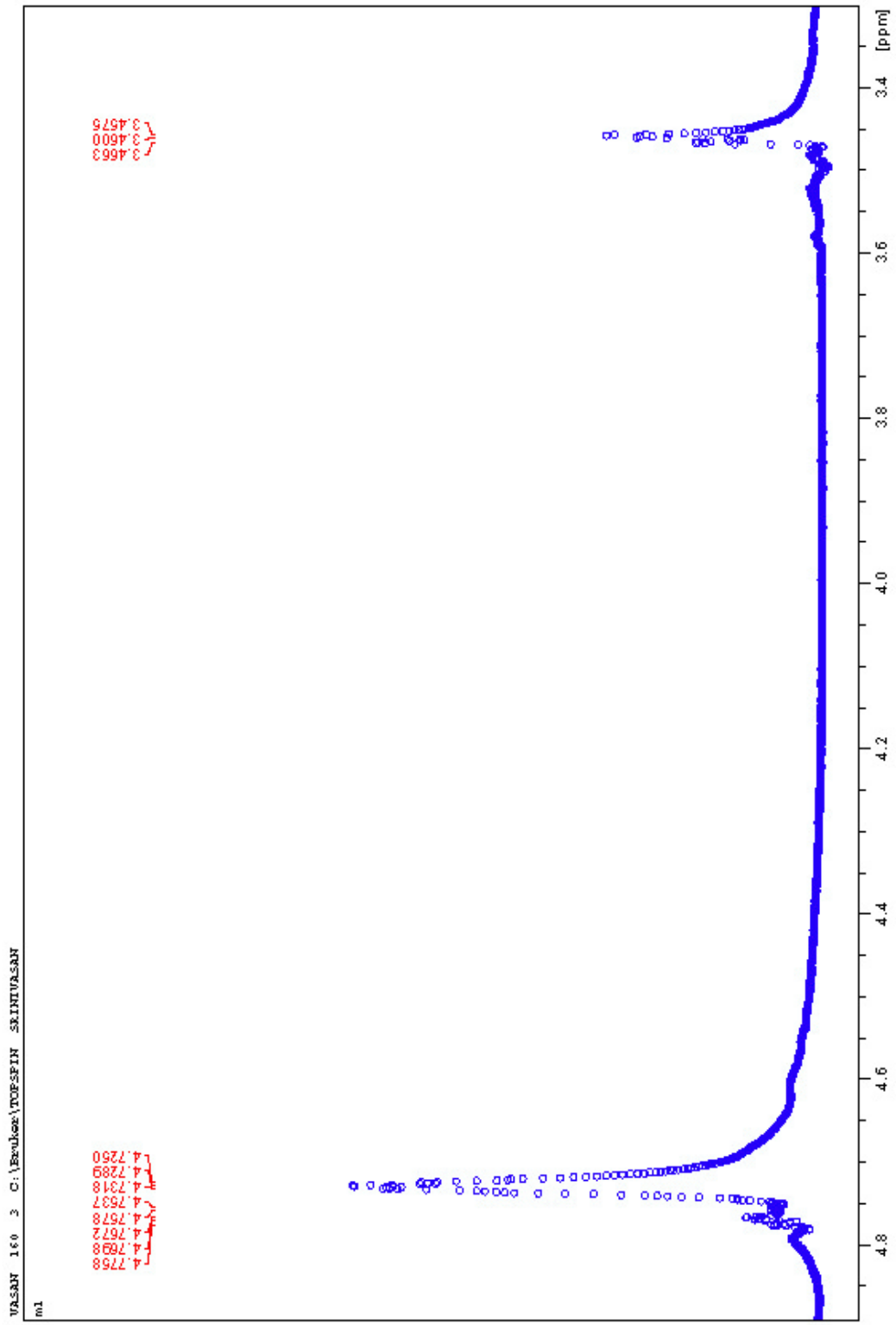


Figure 3.11.1 (F) One Dimensional ^{1}H NMR Spectra of IC-1 Isolated from MeOH Extract of *Ipomoea cairica* Flower (Topspin) 3.4 ppm to 4.8 ppm

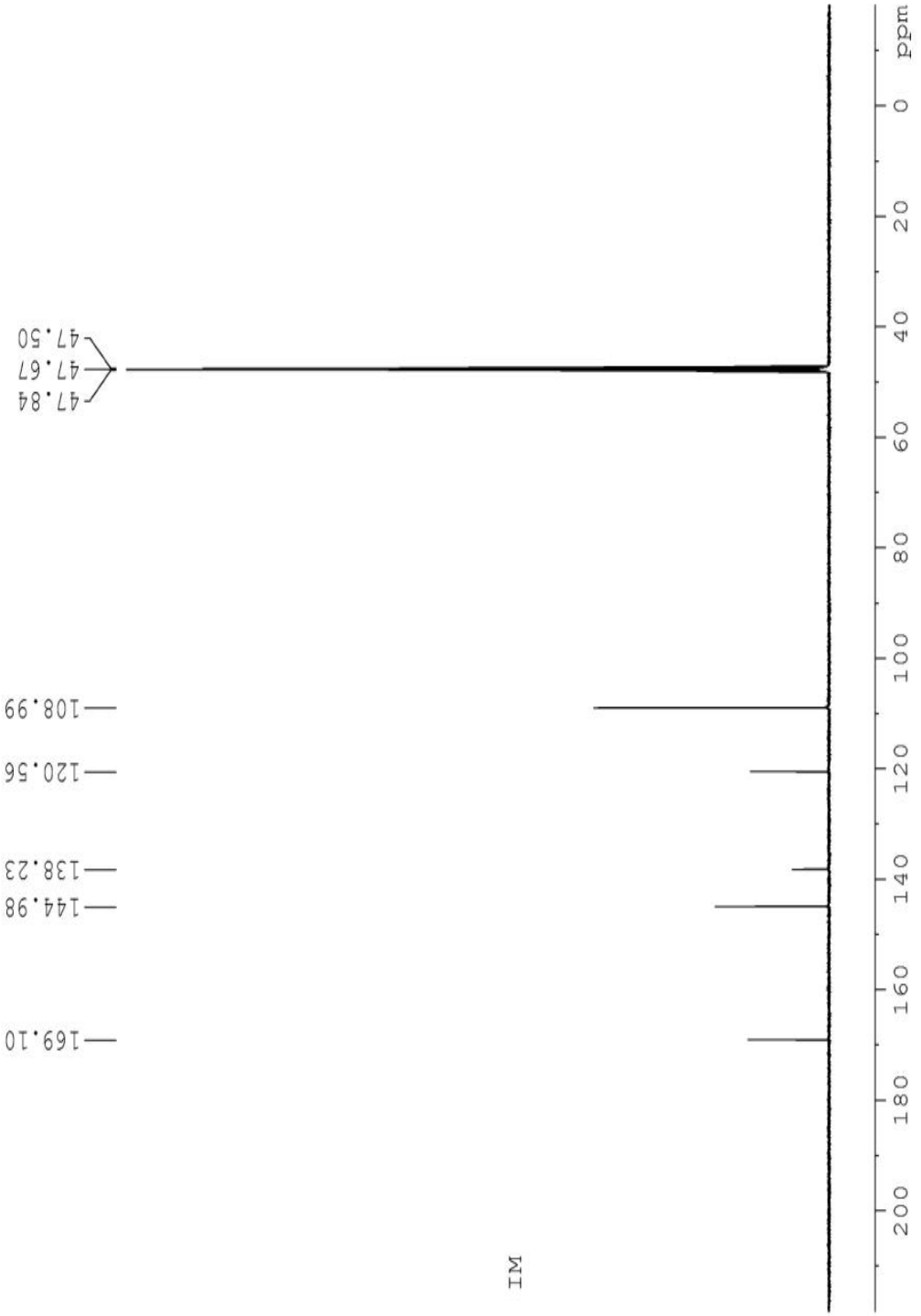


Figure 3.11.1 (G) One Dimensional ^{13}C NMR Spectra of **IC-1** Isolated from MeOH Extract of *Ipomoea cairica* Flower (0 ppm to 200 ppm)

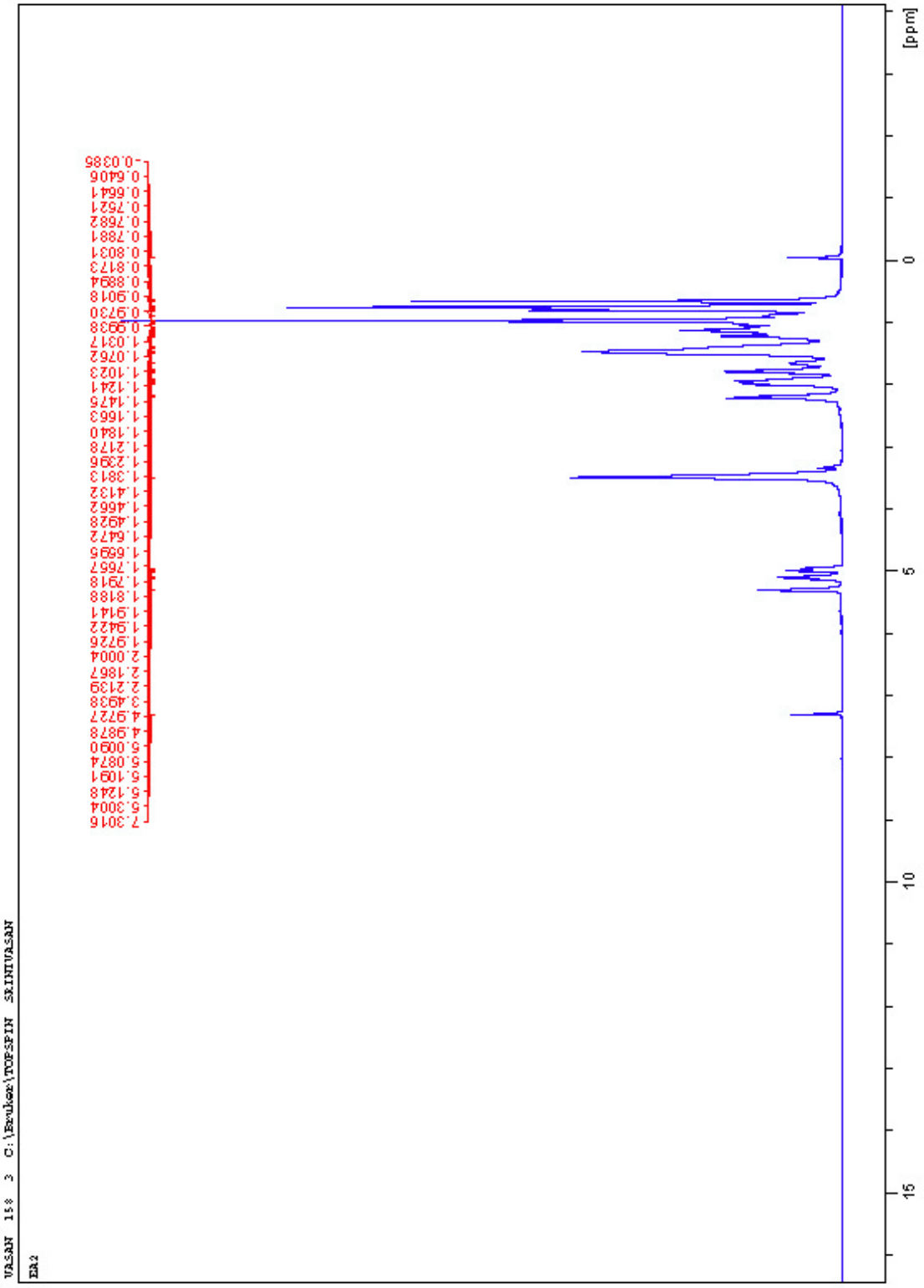


Figure 3.11.2 (A) One Dimensional ^{1}H NMR Spectra of **EG1** Isolated from Petroleum Ether Extract of *Ageratina adenophora* Leaves (0 ppm to 15 ppm)

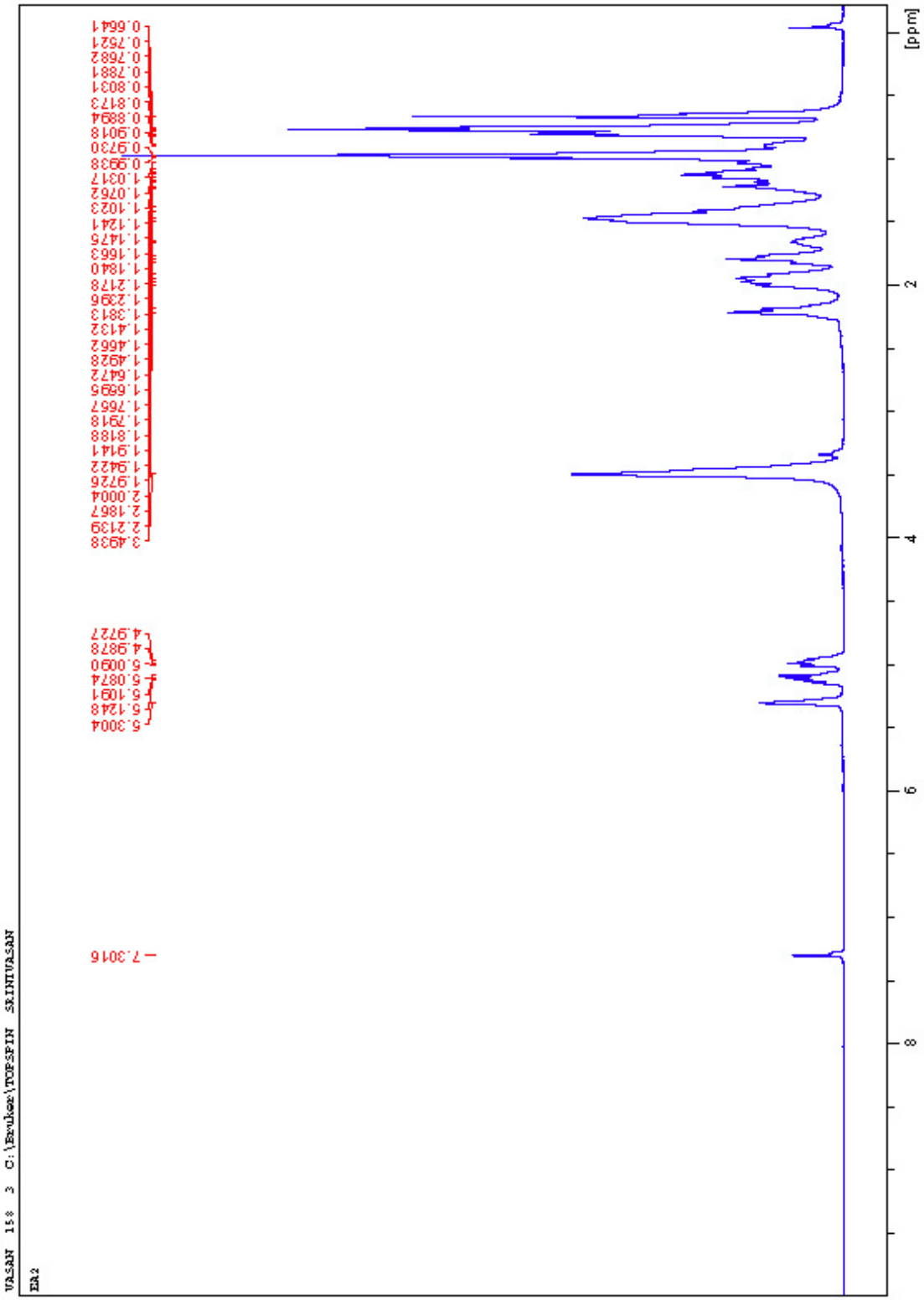


Figure 3.11.2 (B) One Dimensional ^{1}H NMR Spectra of **EG1** Isolated from Petroleum Ether Extract of *Ageratina adenophora* Leaves (Expanded Up Field) 0 ppm – 8 ppm

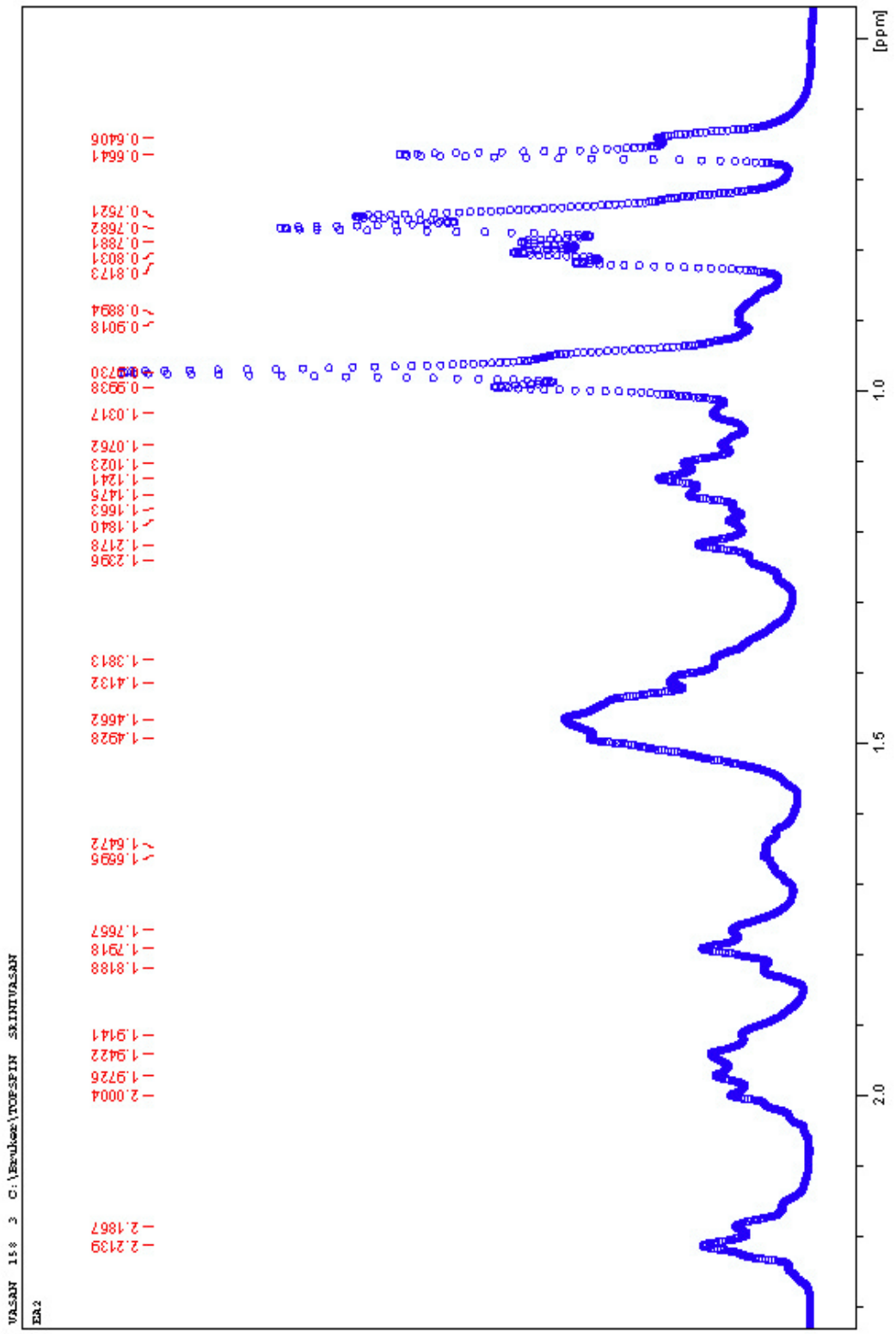


Figure 3.11.2 (C) One Dimensional ^{1}H NMR Spectra of **EG1** Isolated from Petroleum Ether Extract of *Ageratina adenophora* Leaves (resolved) 0 ppm – 2.5 ppm

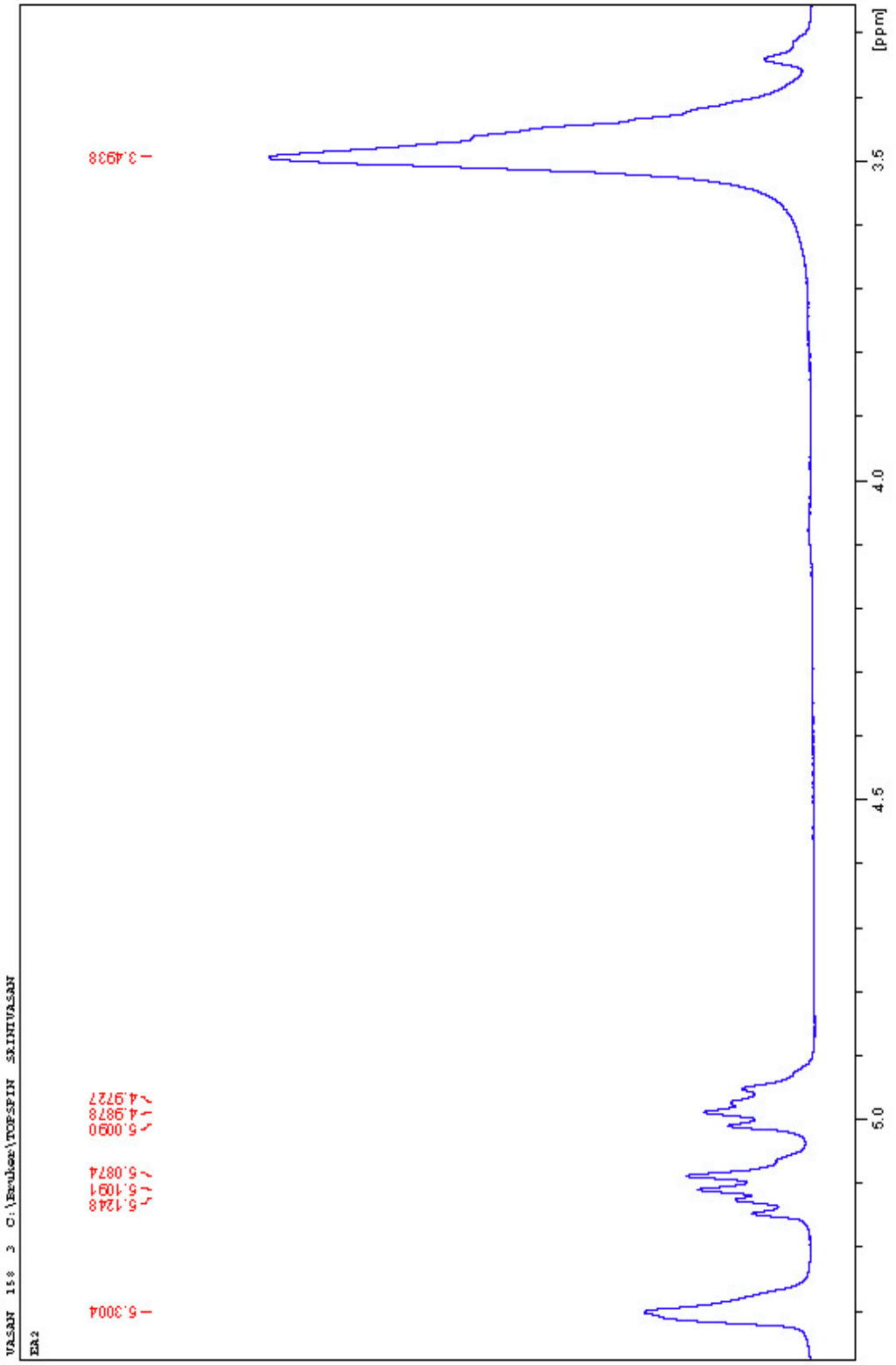


Figure 3.11.2 (D) One Dimensional ^{1}H NMR Spectra of **EG1** Isolated from Petroleum Ether Extract of *Ageratina adenophora* Leaves (resolved) 3 ppm – 6 ppm

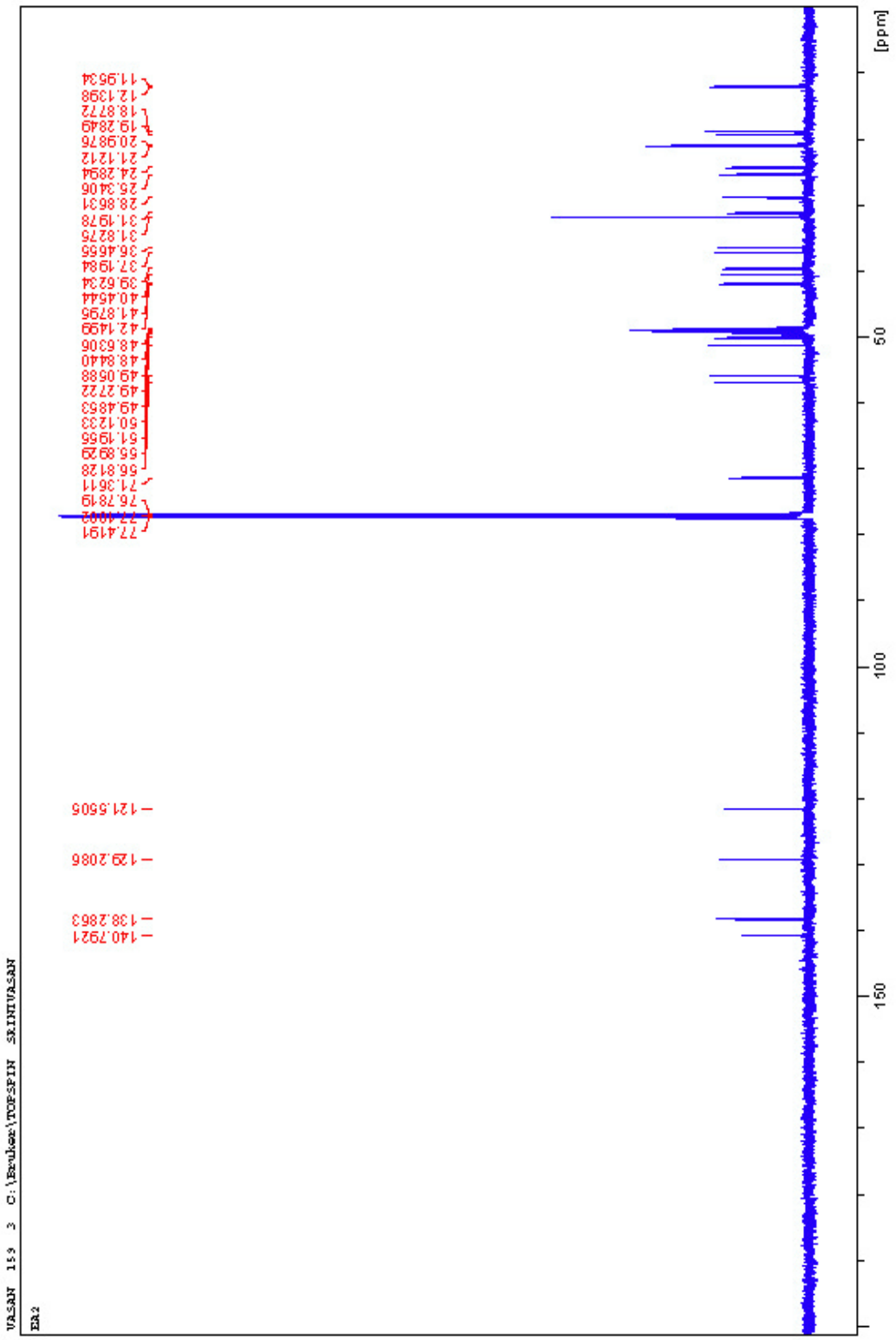
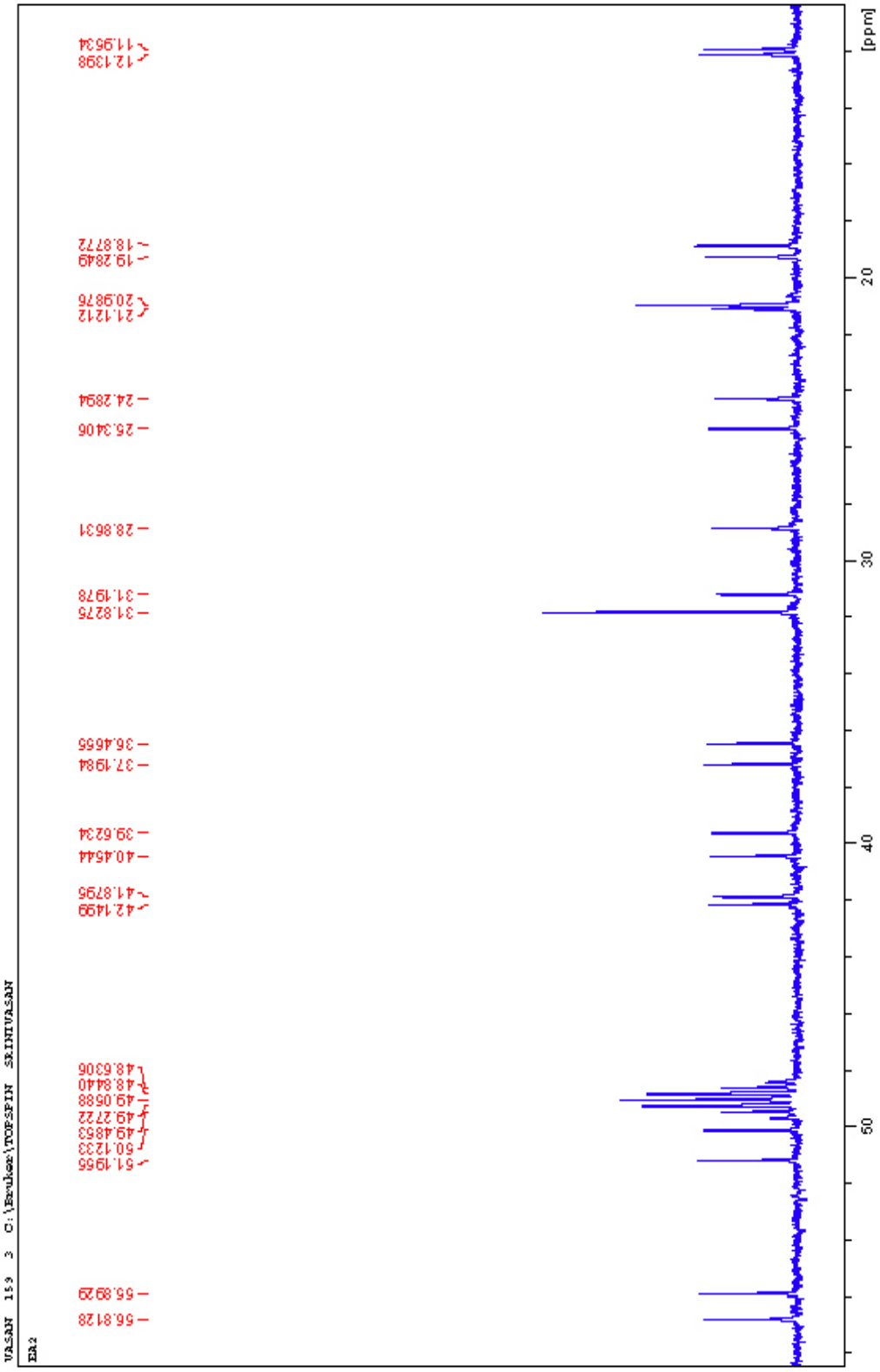


Figure 3.11.2 (E) One Dimensional ^{13}C NMR Spectra of **EG1** Isolated from Petroleum Ether Extract of *Ageratina adenophora* Leaves .

Figure 3.11.2 (F) One Dimensional ^{13}C NMR Spectra of **EG1** Isolated from Petroleum Ether Extract of *Ageratina adenophora* Leaves (Expanded Up Field) at 0-60 ppm



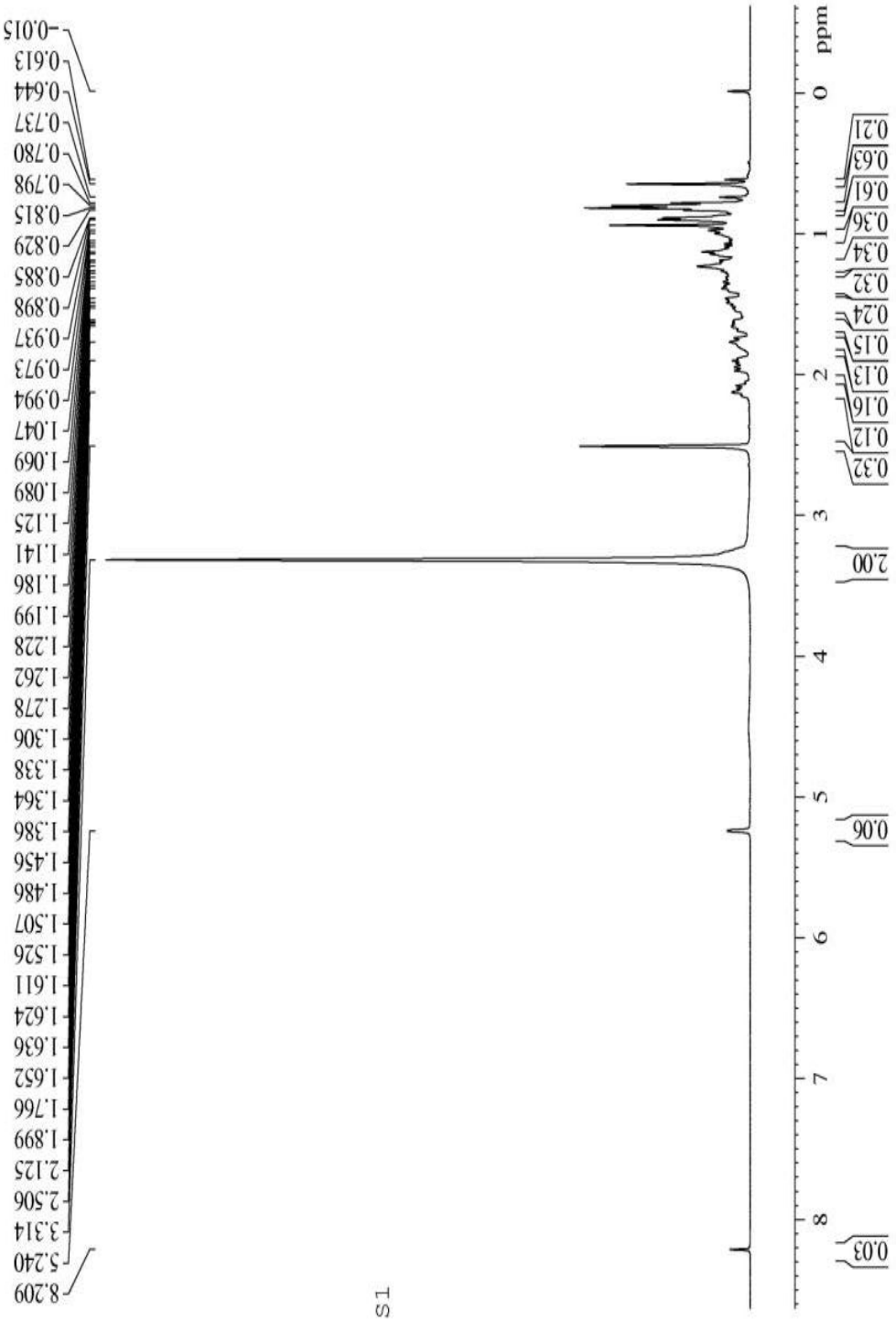


Figure 3.11.3 (A) One Dimensional ^{1}H NMR Spectra of **EG2** Isolated from Petroleum Ether Extract of *Aeratina adenophora* Leaves (0 ppm to 9 ppm)

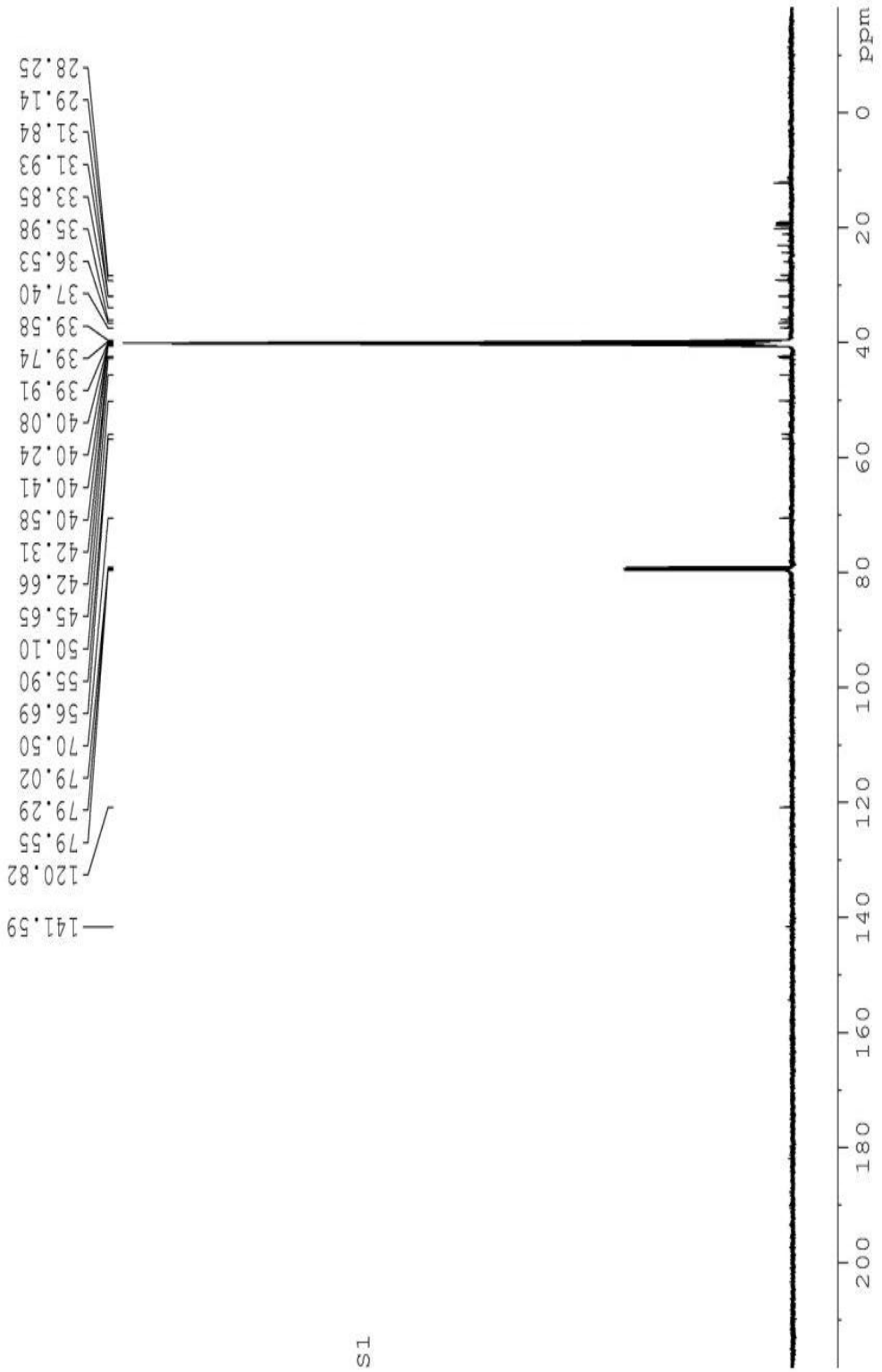


Figure 3.11.3 (B) One Dimensional ^{13}C NMR Spectra of **EG2** Isolated from Petroleum Ether Extract of *Ageratina adenophora* Leaves

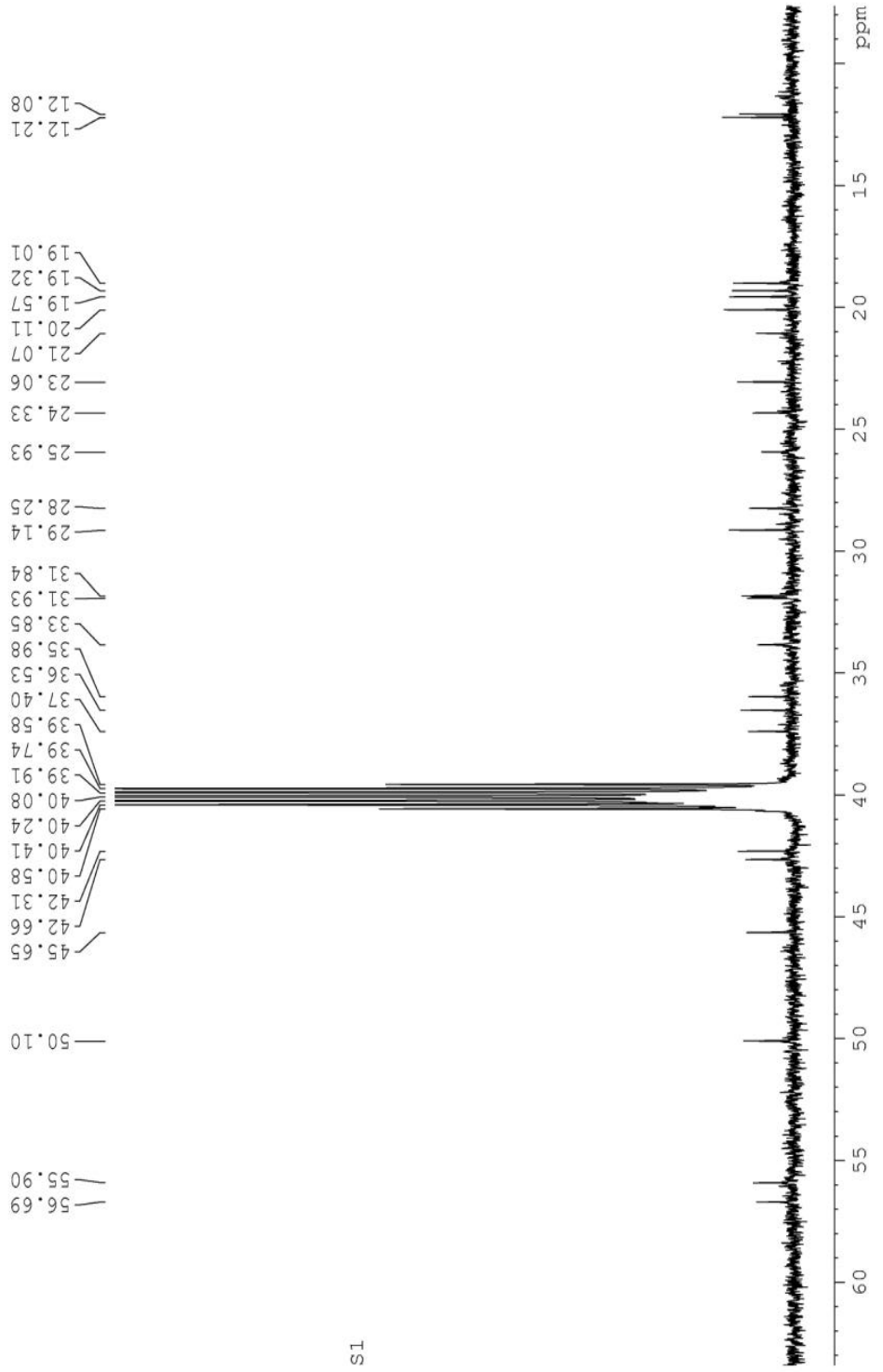


Figure 3.11.3 (C) One Dimensional ^{13}C NMR Spectra of **EG2** Isolated from Petroleum Ether Extract of *Ageratina adenophora* Leaves (Expanded Up Field) at 0-60 ppm

Table 3.9(A). ^{1}H NMR and ^{13}C chemical shifts values for IC1 recorded in MeOD

^{1}H	^{13}C
3.32	47.6
4.95	108.9
6.27	120.56
7.08	138.23
8.5	144.98
	169.10

Table 3.9(B). ^{1}H NMR and ^{13}C chemical shifts values for EG1 recorded in $CDCl_3$ + 2-3 drops of d-DMSO

^{1}H	^{13}C
0.66	11.95
1.03	19.28
2.18	25.34
3.49	36.40
4.98	50.12
5.30	71.36
7.30	77.1
	121.5
	129.2
	138.28
	140.79

Table 3.9(C). ^{1}H NMR and ^{13}C chemical shifts values for EG2 recorded in $CDCl_3$

^{1}H	^{13}C
0.61	28.25
0.88	31.84
0.93	35.98
1.04	40.08
1.12	45.55
1.08	50.10
2.12	55.90
3.31	70.5
5.24	79.29
8.20	120.82
	141.59

Although ^{1}H resonance peaks of phenolic protons are similar to that of an aliphatic alcohol, its resonance peak position, in general, is shifted downfield compared with alcoholic proton resonance features. The resonance frequency of the phenolic proton would be further shifted to higher frequency range (relatively higher δ value) due to intramolecular hydrogen bonding when an appropriate functional group that facilitates hydrogen bonding exists at the ortho position to the aromatic alcohol (phenol) group. As hydrogen bonding withdraws electron density and concomitantly shifts the proton resonance frequency downfield with the decrease in the electron density at the immediate vicinity of proton. Furthermore, the resonance position of labile protons also varies as a result of the degree of broadening, depending on the rate of proton exchange under suitable conditions. An organic compound that possesses labile protons that exhibits an intermediate rate of exchange whose broad resonance features can be simplified with the removal of labile resonance absorption features, by mixing with D2O or few drops of acidified solvent.

A resonance peak resulting from dissolved H_2O will appear, generally, in deutrated solvents irrespective of the polarity of solvents. Thus, for methanol-d_4, resonance signal due to the dissolved H_2O appears at δ ~ 4.75 – 4.85 ppm. In addition, the residual protons of methanol-d_4 also exhibit a resonance peak at 3.30 ppm, whilst, the residual protons of chloroform-d absorb at 7.26 ppm. Similarly resonance signal arising from ^{13}C nuclei of solvent molecules can be revealed in the corresponding ^{13}C-NMR spectral data. For example, ^{13}C peaks of methanol-d4 will be present at 49.0 ppm, while ^{13}C nuclei of chloroform-d will be absorbing at 77.0 ppm.

The identity of the bioactive compound, IC1, was established as ***gallic acid*** from ^{1}H and ^{13}C NMR along with infrared spectral data [ν: 3268, 1694, 1613, 1538, 1436, 1242 cm^{-1}]

as the experimentally observed molecular spectral data commensurate with the examined literature data for gallic acid [λ_{max} (MeOH) 272 nm, ^{1}H-NMR (DMSO-d_6) 6.91(^{1}H, s, H-2, 6), ^{13}C-NMR (DMSO-d_6): 121.0 (C-1), 109.0 (C-2 & C-6), 145.9 (C-3 & C-5), 138.3 (C-4), and 168.0 (C-7)] (Eldahshan, 2011). The very broad ^{1}H NMR signal arising from H-bonded phenolic protons are observed with two prominent chemical shift values 9.17 and 8.86 ppm. Upon addition of a drop of water, the broad resonance feature disappears and a sharp singlet is observed at 8.5 ppm that corresponds to o-substituted phenolic protons.

3.11. Characterization of Isolated Compound, IC1

Physical Examination:

Colour/Nature : White or pale pink colored double growth crystal
Melting point : 257-263°C
Solubility : Soluble in acetone and methanol but insoluble in benzene or in $CHCl_3$
It can react with ferric chloride and then appear blue-black colour.

This compound was obtained as white or pale pink colored double growth crystal from 10% MeOH (in $CHCl_3$) fraction from silica gel column which when eluted in Sephadex LH-20 column using MeOH (as eluent) yielded the copmpound. It has an R_f value of 4.8 on silica gel TLC plates at 254 nm and a mobile phase toluene (3): EtOAc (3): HCOOH (0.6): MeOH (0.4). It also has a retention time of 7.2 minutes in HPLC analysis (MeOH isocratic).

The n → π* transition of isolated ketonic C = O group typically occurs at 275 – 295 nm (Stern and Tinmons, 1970). On α,*β*–alkenyl conjugation, the absorption peak suffers a bathochromic shift, becomes broadened and centres on 300 – 320 nm wavelength region — with the wavelength of maximum absorption, λ_{max}, lying close to 300 nm (Kemp, 1978). This was encountered in the Ultraviolet absorption spectrum of IC1 (λ_{max} = 272nm). Thus suggesting the presence of a carbonyl function in the molecule, which was confirmed by the infrared

absorption peak at 1694.7 cm^{-1} and ^{13}C chemical shift at 169.10 ppm. The infrared and ^{13}C absorption spectra further confirmed the presence of C = C double bond in the molecular structure of IC1, and as well indicated that it is in conjugation with the carbonyl group.

Results of the spectral analysis coupled with the comparison of the melting point and spectral data confirmed that the compound **IC1** is gallic acid (3,4,5-trihydroxybenzoic acid)

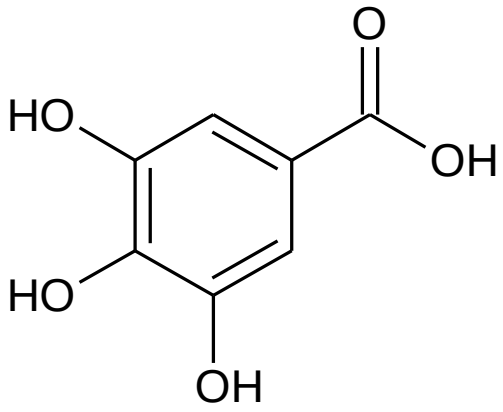


Figure 3.12. Photograph and Structure of IC1 (Gallic acid) Isolated from MeOH Extract of *Ipomoea cairica* flower

3.12. Characterization of Isolated Compound, EG1

Physical Examination:

Colour/Nature : White powder

Melting point : 172-175 °C

Solubility : highly soluble in $CHCl_3$

This compound was obtained as white powder from 10% EtOAc fraction from column with retention time of 17.30 minutes in HPLC analysis. The EI mass spectrum showed the molecular ion peak at m/z 412 consistent with the molecular formula $C_{29}H_{48}O$. On subjection to IR spectroscopic analysis, the observed absorption bands are 3434 cm^{-1} which may be assigned due to characteristic O-H stretching. Absorption at 2932.7 cm^{-1} and 2864.8 cm^{-1} may be assigned due to aliphatic C-H stretching. The absorption band at 1656 cm^{-1} is typical of C-C

double bond stretching, however this band is weak. Other absorption peaks include 1455.47 cm^{-1} which may be due to cyclic $[–CH_2–]_n$ bending frequencies, absorption at 1366.48 cm^{-1} assigned due to –OH deformation.

In the ^1H NMR spectrum of **EG1**, proton appeared as a triplet of a double doublet (tdd) at δ 3.49. Another sp2 methine proton was represented by a triplet signal at δ 5.36. The multiplet signal at δ 3.52 was assigned to the resonance of methine proton.

The ^{13}C NMR spectra revealed 34 signal peaks out of which strong peaks at 76.78, 77.1 and 77.42 are solvent ($CDCl_3$) peaks. The alkene carbons appeared at 121.5, 129.2, 138.3 and 140.8. Recognizable signals at 140.8 and 138.3 (i.e. downfields) may be assigned due to double bonded carbon atoms while 129.2 and 121.5 may also be assigned due to C = C carbon atoms respectively.

Figure 3.13. Photofraph of EG1 & EG2 Isolated from Petroleum ether Extract of *Ageratina adenophora* Leaves

3.13. Characterization of Isolated Compound, EG2

Physical Examination:

Colour/Nature : White powder
Melting point : 172-175 °C
Solubility : highly soluble in $CHCl_3$

This compound was obtained as white powder from 10% EtOAc fraction from column with retention time of 22.1 minutes in HPLC analysis when eluted with ACN: Water (95:5) at a flow rate of 0.2 ml/min. The IR spectrum showed a broad strong band at around 3200-3500 cm^{-1} which may be assigned due to the presence of a hydroxyl group. The absorption band at around 1650 cm^{-1} was typical of C-C double bond. Other absorption peaks include 1463.6 cm^{-1} which may be due to $–CH_2–$, absorption at 1376.2 cm^{-1} assigned due to –OH deformation, peak at 1040.88 cm^{-1} which may be due to cycloalkane (Kamboj and Saluja, 2010). This was further supported by the presence of a pair of doublets at δ 5.14 and 5.02 in the 1H-NMR spectrum, which is the characteristic of the sp2 methine protons at carbons of the side chain, respectively. Another sp2 methine proton was represented by a triplet signal at δ 5.36. The multiplet signal at δ 3.52 was assigned to the resonance of methine proton.

Mixture of EG1 and EG2 was obtained as a white amorphous solid. It gave positive color tests for sterols in the Salkowski and Liebermann-Burchard reactions. It gave one spot on TLC chromatograms.

Phytosterols are typical constituents of plant cell walls (Marangoni and Poli, 2010), they are members of the family ‘terpene’ which includes more than 100 different phytosterols and more than 4000 other types of triterpenes plant membranes contain several types of phytosterols that are similar in structure to cholesterol but a methyl or ethyl group at C-24. They have the same basic function as cholesterol does in animals and that is in the structure and function of cell membranes (Nair *et al*., 2006; Moreau *et al*., 2002).

Currently 2D - NMR analysis of these two sterols (EG1 & EG2) to ascertain the exact position of carbons and hydrogens in their molecules by carbon-carbon and H-H exchange study is under progress.

3.14. Studies on Larvicidal Activity of Isolated Compound, IC1

Table 3.10(a). Mortality of Third Larval Instars of *Culex quinquefasciatus* in Different Concentrations of IC1 isolated from MeOH extract of *Ipomoea cairica* flowers

Concentration (ppm)	Percent mortality ± Standard Error	
	24 Hours	48 Hours
10	4.00±0.82	8.00±0.82
20	14.00±0.58	20.00±0.82
30	21.00±0.50	27.00±0.50
40	27.00±0.96	36.00±0.82
50	36.00±1.41	45.00±0.96
75	48.00±0.82	57.00±0.96
100	58.00±0.58	67.00±0.96
200	68.00±0.82	78.00±1.30

Table 3.10(b). Log Probit and Regression Analysis of Third Larval Instars of *Culex quinquefasciatus* in Different Concentrations of Compound IC1 Isolated from Methanolic Flower Extract *Ipomoea cairica* for 24 Hours and 48 Hours

Period of Exposure (Hours)	LC_{50} (ppm)	95% Confidence limits (LCL – UCL)[a]	LC_{90} (ppm)	95% Confidence limits (LCL – UCL)	χ^2	Slope ± SE	Intercept ± SE
24	88.76	76.67 – 105.32	487.7	347.95 – 780.23	3.15	1.73±0.15	1.62±0.27
48	63.62	55.68 – 73.34	340.0	254.45 – 501.36	1.57	1.76±0.15	1.82±0.26

[a]LCL-lower confidence limit; UCL-upper confidence limit

The mortality of the third instar larval form with the compound, IC1 isolated from methanolic floral extracts of *I. cairica* is presented in Table 3. and confirm its larvicidal activity against the LF vector, *C. quinquefasciatus*. The mortality rate at 200 ppm concentration was found to be highest amongst all concentrations of the compound tested

against third instar larvae, and it was significantly higher ($p<0.05$) than the mortality rates at 10, 20, 30, 40, 50, 75 and 100 ppm concentrations of the isolated compound **IC1** at 24 and 48 hours of exposure (Table 3.). The mortality rate was higher in 48 hours bioassay than those in 24 hours. At higher concentrations, the larvae showed restless movement for some time with abnormal wagging and then died. Higher percentages of mortality were observed at higher concentrations of the isolated compound (Table 3.). The LC_{50} (88.76 ppm for 24 hours exposure & 63.62 for 48 hours exposure) value was however found to be lower than the methanolic floral extract (76.43 ppm for 24 hours exposure & 8.43 ppm for 48 hours exposure) of the parent plant. This clearly explains that the isolated compound IC1 greatly contribute to the larvicidal potential of the plant. Thus it may be proposed that high larvicidal activity of *I. cairica* flower against third instar larvae of *C. quinquefasciatus* may be attributed to synergistic action of IC1 with other bioactive compound(s) present in the plant. Thus the activity of IC1 against the mosquito larvae (LC_{50} = 88.76 ppm for 24 hours exposure & 63.62 for 48 hours exposure) may be attributed to the additive or synergistic or blend effect of many/some IC1 with other bioactive compound(s) present in the plant. Such an effect has been previously observed with some essential oils extracted from plants where the activity was due to the combination of the major constituents, none of which was found to exhibit significant activity, individually (Papachristos *et al*., 2004; Omolo *et al*., 2005)

TABLE 3.10(c). Time dependent larvicidal activity of Compound IC1 Isolated from Methanolic Floral Extract of *Ipomoea cairica* Against Third Instar Larvae of *Culex quinquefaciatus*

Concentrations (ppm)	Time (in h)	% Mortality		Concentrations (ppm)	Time (in h)	% Mortality
	4	-			4	0.00
	8	-			8	2.00

10	12	-	20	12	5.00
	16	1.00		16	8.00
	20	3.00		20	11.00
	24	4.00		24	14.00
	28	4.00		28	14.00
	32	6.00		32	17.00
	36	6.00		36	17.00
	40	8.00		40	19.00
	44	8.00		44	19.00
	48	8.00		48	20.00
30	4	1.00	40	4	4.00
	8	3.00		8	6.00
	12	6.00		12	13.00
	16	11.00		16	17.00
	20	17.00		20	21.00
	24	21.00		24	27.00
	28	22.00		28	29.00
	32	25.00		32	31.00
	36	25.00		36	32.00
	40	26.00		40	35.00
	44	26.00		44	36.00
	48	27.00		48	38.00
Concentrations (ppm)	Time (in h)	% Mortality	Concentrations (ppm)	Time (in h)	% Mortality
50	4	7.00	75	4	10.00
	8	10.00		8	13.00
	12	15.00		12	16.00
	16	19.00		16	27.00
	20	25.00		20	31.00
	24	31.00		24	39.00
	28	37.00		28	45.00
	32	40.00		32	47.00
	36	41.00		36	50.00
	40	43.00		40	52.00
	44	43.00		44	55.00
	48	45.00		48	58.00
100	4	15.00	200	4	18.00
	8	20.00		8	25.00
	12	31.00		12	30.00
	16	41.00		16	45.00
	20	49.00		20	49.00

	24	54.00			24	52.00
	28	58.00			28	68.00
	32	63.00			32	70.00
	36	65.00			36	72.00
	40	66.00			40	75.00
	44	67.00			44	76.00
	48	67.00			48	78.00

Table 3.10(d). Log Probit and Regression Analysis of Time Dependent Larvicidal Efficacy of Compound IC1 Isolated from Methanolic floral extract of *Ipomoea cairica* at different concentrations against third instar larvae of *Culex quinquefaciatus*

Concentration (ppm)	**LT_{50} (Hours)**	**95% Confidence limits**		**Slope ± SE**	**χ^2**	**Intercept ± SE**
		Lower Limit	**Upper Limit**			
10	∞	-	-	-	-	-
20	∞	-	-	-	-	-
30	∞	-	-	-	-	-
40	69.37	52.39	113.50	1.53±0.25	0.25	2.18±0.36
50	55.08	44.59	75.48	1.47±0.21	1.47	2.44±0.30
75	38.22	31.93	48.35	1.62±0.21	4.2	2.44±0.3
100	22.32	18.77	26.62	1.50±1.17	1.48	2.97±0.24
200	18.22	15.48	21.14	1.74±0.18	6.6	2.80±0.24
Concentration (ppm)	**LT_{90} (Hours)**	**95% Confidence limits**		**Slope ± SE**	**χ^2**	**Intercept ± SE**
		Lower Limit	**Upper Limit**			
10	∞	-	-	-	-	-
20	∞	-	-	-	-	-
30	∞	-	-	-	-	-

40	476.76	233.54	1898.24	1.53±0.25	0.25	2.18±0.36
50	410.1	226.92	1145.98	1.47±0.21	1.47	2.44±0.30
75	236.61	146.63	522.61	1.62±0.21	4.2	2.44±0.3
100	159.2	106.95	293.55	1.50±1.17	1.48	2.97±0.24
200	98.78	74.00	149.74	1.74±0.18	6.6	2.80±0.24

Time-response larvicidal bioassay was performed with compound IC1 isolated from methanolic flower extract of *I. cairica* at concentrations ranging from 10 – 200 ppm for 1-48 hours against third instar larvae of *C. quinquefaciatus* [Table 3.10(c)]. The LT_{50s} of the compound IC1 isolated from MeOH extract of *I. cairica* flower leaf ranged between 18.22 hours to 69.37 hours for decreasing concentration ranging from 200 to 40 ppm where as LT_{90s} ranges between 98.78 hours to 476.76 hours of the same concentration range of the test compound. Low LT_{50} and LT_{90} values and highest larvicidal activity were found for higher concentration of the test compound. The lethal time (LT_{50}) decreased as the concentration of the test compound was increased in the mosquito species [Table 3.10(d)]. In terms of lethal time to kill 50 % of the population of *C. quinquefaciatus*, 200 ppm of IC1 had minimum time, i.e., 18.22 hours and 98.78 hours for killing 90 % of the test population, followed by 100, 75, 50 and 40 ppm [Table 3.10(d)]. Lethal times were also shorter particularly at higher dosages (200 and 100 ppm), which presented values ~3.8 times lower than those exhibited at lower concentrations. This could again be linked to the difference in toxicity noticed in the 1‑48 h of the experiment [Table 3.10(d)]. Chi-square value was significant at $P<0.0001$ level. Higher slope value (1.74±0.18 at 200 ppm) and lower fiducial limits at 95 % of LT_{50} (15.48 – 21.14 hours at 200 ppm) were observed. The results of regression analysis revealed that the mortality rate (Y) is positively correlated with the concentration of exposure (X) having a regression coefficient (R) close to 1 in each case. The results of log probit analysis (95% confidence level)

revealed that LT_{50} and LT_{90} values gradually decreased with concentrations having the lowest value at 200 ppm of exposure to third instar larvae.

Larviciding is a successful way of reducing *C. quinquefasciatus* mosquito densities in their breeding places before they emerge into adults. Larviciding largely depends on the use of synthetic chemical insecticides – organochlorine (DDT), organophosphates (malathion, temephos and fenthion), synthetic pyrethroids (deltamethrin), insect growth regulators (diflubenzuron and methoprene) etc. Although effective, their repeated use has disrupted natural biological control systems and sometimes resulting in the widespread development of resistance. Insecticide-resistant populations of C. *quinquefasciatus* have been reported worldwide (Kaushal *et al*., 2011; Norris and Norris 2011) and is of concern for the NTD Program if insecticide-based control methods are considered as an intervention in the future (Curtis and Pasteur 1981; Khayrandish and Wood 1993; Jones *et al*., 2012). A high level of DDT and malathion resistance was observed in *C. quinquefasciatus*, which may be correlated with the use of DDT and malathion for vector control in Northeast India for many years. The use of DDT is discontinued in most parts of India due to development of resistance in vector populations. However, it is still being used for control of Kala-azar vector and some parts of Northeastern India for malaria vectors especially Mizoram (Sarkar *et al*., 2009a,b). Since insecticide resistance threatens to contribute towards the reintroduction of vector borne diseases in many parts of the world, efforts have been focused on finding an alternative form of mosquito control. MDA is now accepted as a global program to eliminate lymphatic filariasis. There is a general consensus that the time for elimination can be drastically reduced if MDA is coupled with appropriate vector control measures (Addis, 2013).

3.15. Interaction of Transition Metals with the Bioactive Compounds

The possibility of interaction of the isolated compounds with transition metal ions was studied by reaction the compound(s) with salts of transition metal ions. Various salts of Copper were experimented on the possibility of their interaction with Protocatechuic acid (VA) previously isolated from *A.adenophora* leaves. It was observed that a 1:1 ratio molar concentration of $CuNO_3$ with VA dissolved in acetonitrile with few drops of EtOAc yielded a light green colour solution. When the solution was kept in a dry air conditioned environment for one month, few microcrystals emerged. The tentative structure of this inorganic crystal is

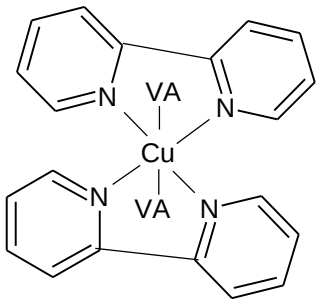
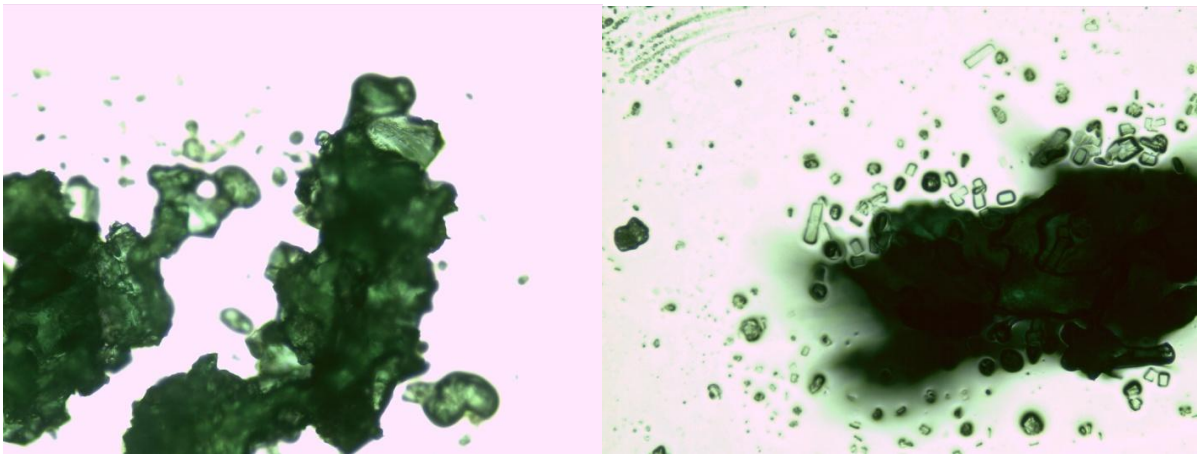


Figure 3.14(a). Putative Molecular Structure of Complex 7

However, growth of single crystal due to interaction of IC1 and PA with Cu^{2+} salts, suitable for X-ray diffraction (XRD) and subsequent characterization using XRD technique is currently under progress.



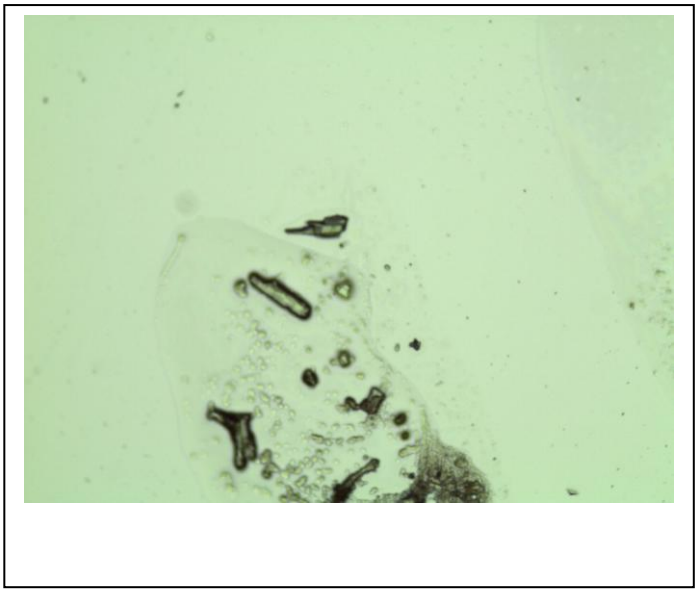
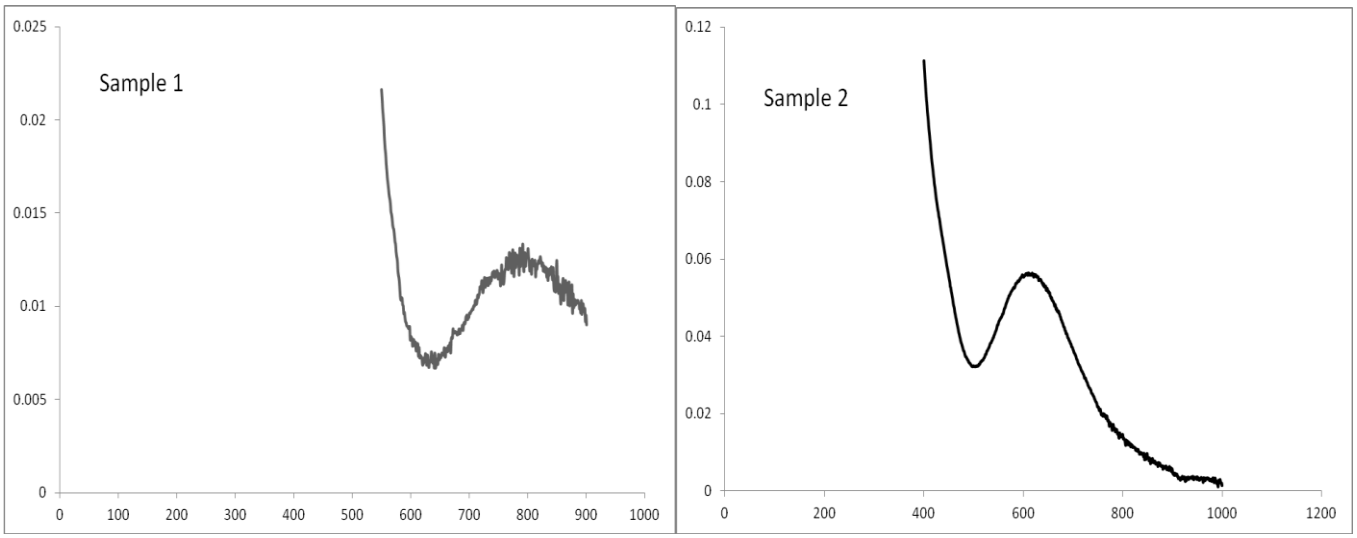


Figure 3.14(b). Microcrystals and Twin Crystals of Complex 7



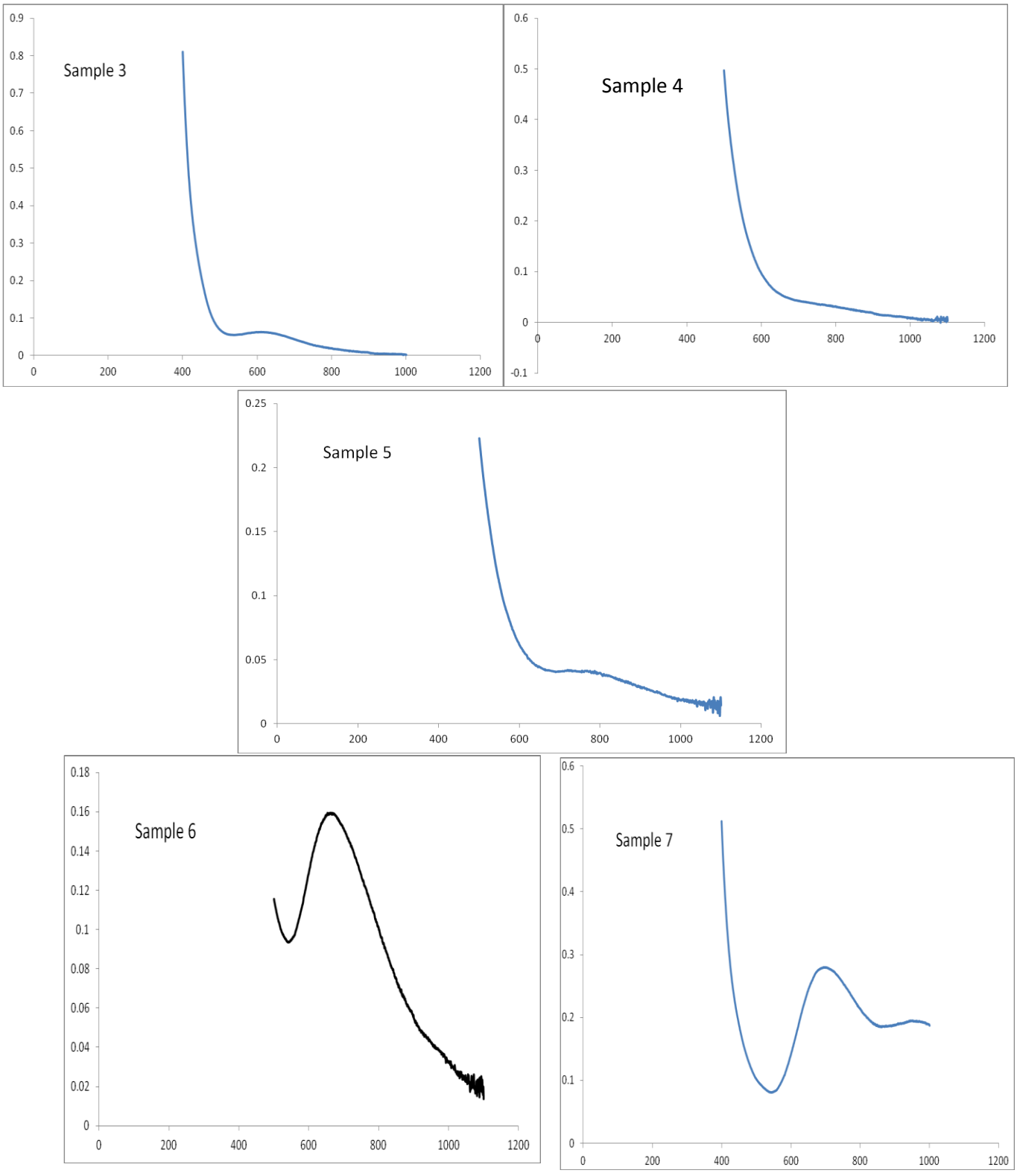


Figure 3.14(c). UV/Visible Spectra of Complexex 1-7.

The goals of molecular spectroscopy of the coordination complexes are to determine the metal-site coordination geometry as well as electronic structures and to develop the structure-function correlations. For complex **1**, the optical spectrum measured in methanolic solution, displayed higher energy bands at 361 and 425 nm in the UV-visible region, corresponding to the ligand-to-metal charge transfer (LMCT) transitions. The absorption band at 768 nm (13, 020 cm^{-1}) might be assigned to a d-d transition and it reflects a distorted square pyramidal geometry around copper(II) ion. The absorption spectrum of complex **2** in MeOH is shown in figure a. A broad band (figure a) due to the d-d transition is observed in the visible region (at 611 nm) at 16, 366.6 cm^{-1}, a feature characteristic of many Cu(II) square pyramidal systems. The presence of a single d–d band may be attributed to a relatively symmetric nature of the ligand field. Complex **3** in methanol exhibited a very broad absorption band in the visible region with a weak maximum at 599 nm (16, 694.5 cm^{-1}), indicative of a d-d transition. The position of these maxima is consistent with pentacoordinated copper(II) chromophores.

The ligand field spectra of complex **4** and **5** recorded in the solution state, exhibited a very weak and broad spectral features. Both spectra are characterized by the presence of d-d transitions around 800 nm (for complex **4** at 792 nm [12, 626 cm^{-1}] and complex **5** at 800 nm [12, 500 cm^{-1}]) and it is indicative of copper(II) ions in a distorted square planar ligand field environments. For complex **6** in methanol d-d transitions was observed at 653 nm (15, 314 cm^{-1}). The observation of a weak d-d transition is typical for square- pyramidal copper(II) complexes. The electronic absorption spectra for five coordinated copper(II) complexes normally fall into two general categories: (i) Square-pyramidal complexes typically exhibit a high-energy absorption band in the visible region with a low-energy shoulder. (ii) While, for trigonal-bipyramidal complexes a low-energy absorption band with a high-energy shoulder in

the visible region is observed. For complex **7** in methanol solution, the main optical features are at 362 nm (27, 472 cm^{-1}) and 698 nm (14, 327 cm^{-1}) with weak shoulder at 962 nm (10, 395 cm^{-1}), corresponding to the ligand-to-metal charge transfer (LMCT) transition and the d-d transitions, respectively. As reported earlier, such spectroscopic behaviour (high-energy absorption band in the visible region with a low energy shoulder) may be ascribed to square-pyramidal copper(II) complexes. In addition, complex 7, also provided few twinned crystals along with overshoots when the mother liquor of complex 7 was allowed to stand for crystallization at room temperature. Along with the twinned crystals, few microcrystalline solids were present and when viewed under a polarized optical microscope, few crystals can be clearly shown as illustrated in the diagram. (Fig.). These crystalline materials were redissolved in methanol:acetonitrile mixture and kept for recrysallization.

CHAPTER 4

SUMMARY AND CONCLUSIONS

The present study incorporated in the thesis entitled "***Isolation, purification and identification of bio-active components with potential pesticidal properties from plant extracts and their Interaction with Transition Metal Ions***" deals with the exploration of phytochemical, antioxidant and bio-pesticidal investigations and further isolation of the bioactive compound from selected two wild plants which are considered as noxious weeds and abundantly found in Mizoram and distributed worldwide.

The first section focused on the biodiversity of North-East India with special reference to Mizoram, deforestation and insect/pest problem especially vector borne and problems encountered by use of synthetic pesticides. It also emphasizes on the use of natural pesticides as an alternative and/or substitute to chemicals pesticides owing to their safety, availability and biodegradability.

Quantitative estimation of the total phenolic content of MeOH extracts of the selected plants done by Folin-Ciocalteu's method showed that there was a great difference on the quantities of phenolic compounds depending on the plant species and part of the plant used. The highest percent of total phenolic compounds was calculated for MeOH extract of *Ipomoea cairica* flowers while the lowest was calculated for MeOH extract of *Ageratina adenophora* leaves. Determination of flavonoid content also reveals highest flavonoid content for MeOH extract of *Ipomoea cairica* flowers and lowest for MeOH extract of its leaves.

The *in vitro* and free radical scavenging potentials of methanol extracts of *Ageratina adenophora* leaves and *Ipomoea cairica* leaves and flowers was noted by measuring total phenol

and flavonoid content, free radical scavenging activities such as DPPH radical and phosphomolybdate assay. The results obtained in this comparative study clearly demonstrated that all the tested extracts of both the plants showed antioxidant activities at different magnitudes of potency. The highest radical scavenging activity observed for the methanolic extract of *I. cairica* flowers, while its leaves has shown the least activity. The decreasing order of radical scavenging activities among the extracts assayed through DPPH and phosphomolybdate assay methods were found to be in the order *I. cairica* flower > *A. adenophora* leaves > *I. cairica* leaves. The highest amount of flavonoids content per gram was found in *I. cairica* flowers. Accordingly, phenol content was found in the order: *I. cairica* flowers > *A. adenophora* leaves > *I. cairica* leaves. The study conclude that methanol extract of *I. cairica* flowers might be valuable antioxidant obtained from natural sources and may have application in health and food industry. It is noticed that the highest concentration of phenolic compounds in the extracts were obtained using solvents of high polarity; the methanolic extract manifested greater power of extraction for phenolic compounds from the plants under study. The high contents of phenolic compounds and significant linear correlation between the values of the concentration of phenolic compounds and antioxidant activity indicated that these compounds contribute to the strong antioxidant activity. The free radical scavenging activities as observed in the above experiments clearly suggests that it might be due to the presence of gallic acid in the plant extract.

HPTLC method has been developed for qualititative estimation of gallic acid (IC1) present in the dried flower of *I.cairica* and EG1 and EG2 in the dried leaves of *A. adenophora*. This method is rapid, simple, accurate and specific. The method used in this work resulted in good peak shape and enabled good resolution of ***gallic acid*** (IC1), EG1 and EG2 from other constituents of the plant materials. There was no interference with the IC1, EG1 and EG2 peaks

from other constituents present in the two plants. The HPTLC images shown in figure 3.7 and 3.8 indicate that all sample constituents were clearly separated without any tailing and diffuseness.

The HPLC technique developed for determination of the isolated compounds from the solvent fractions of the plants under study precise, specific, accurate and robust. The proposed method developed also be used for qualitative as well as quantitative analysis of the compounds from the plants they had been isolated. This technique provides us with the UV-Visible spectrum of the different compounds detected. In the absence of authentic standards, these spectra only provide the elucidation of the compound skeletal strucutre. Therefore, this method must be coupled to MS and/or NMR in order to identify completely the compounds detected and provide some insight into their structure.

Study of the larvicidal activity reveals that the activities exhibited by the crude extracts of *A. adenophora* and *I. cairica* against third instar larvae of *C. quinquefasciatus* has been well established in the laboratory condition. The highest mortality was recorded in 500 ppm crude flower and leaf extract of *I. cairica* and *A. adenophora* at 24 - 48 h. Among the polar and nonpolar solvent extracts, the highest efficacy as a larviciding agent against third instar larval form is found in methanolic flower extract and chloroform leaf extract of *I. cairica* and petroleum ether and chloroform leaf extracts of *A. adenophora* which definitely suggests that any secondary metabolites of the plant are responsible for larval mortality. It is clear from the above finding that both the weedy plants can serve as a potent larvicide against *C. quinquefasciatus* larvae. The bioactive solvent extract responsible for mortality was isolated and the lethal concentration as well as lethal time was determined. Further analysis to isolate the active compound for larval control is under way in our laboratory. The study suggests that the active

ingredients of the petroleum ether and chloroform leaf extracts of *A. adenophora and* methanol flower extract and chloroform leaf extract of *I. cairica* should be identified and utilized, if possible, in preparing commercial product formulation as a mosquito larvicide.

Natural products with herbicidal, insecticidal or fungicidal activity have been shown to be an important source of lead compounds in the past and we can probably expect more new interesting compounds to be discovered in the future. Many plants possessing pesticidal properties are found abundantly in different climatic zones in India but remain unexploited not only for traditional preparations but also for commercial formulations, probably because the process of isolation, synthesis and formulation of phytochemicals is long and expensive (Jaglan *et al*., 1997). Plants are proven source of bio-pesticides possessing valuable chemicals. The pesticidal value of these plants lies in some chemical substances that produce a definite physiological action on the target insect pests. Plant products are biodegradable leaving no residues on plants; effective against insects, plant pathogens, nematodes and non-insect pests; and cheaper, non-polluting, easy to prepare and compatible with several biopesticides and synthetic pesticides. They are therefore recommended for large scale application in agriculture in general and plant protection in particular (Gahukar, 2010). However, good lead structures are uncommon and hard to find, but the search is worthwhile as the best ones can bring novelty, in terms of structure and mode of action.

Further bioactivity guided isolation of bioactive constituent from the MeOH extract of *Ipomoea cairica* flowers led to the isolation of two phenolic acids, IC1 and IC2 where as two sterols EG1 and EG2 were isolated from pet. ether extract of *A.adenophora* leaves. It can be concluded that the larvicidal activity of this plant may be totally or partly due to the presence of these two steroids. The Larvicidal potential of the isolated compound IC1 from MeOH extract of

Ipomoea cairica flower was tested for its larvicidal activity against the 3rd instar larvae of *Culex quinquefasciatus* as per the method laid down by WHO (1996). The result showed remarkable activity against the test insect. Comparison of the obtained results of total phenolic compounds and larvicidal activity revealed that there was a linear correlation between larvicidal effect indicated as LC_{50} and total phenolic content of the investigated plants, suggesting that the phenolic compounds have significant larvicidal activity.

Today, environmental safety is considered to be of paramount importance. An insecticide does not need to cause high mortality on target organisms in order to be acceptable but should be eco-friendly in nature. Phytochemicals may serve as these are relatively safe, inexpensive and is readily available in many parts of the world. Several plants are used in traditional medicines for the mosquito Larvicidal activities in many parts of the world. According to Bowers *et al* (1995), the screening of locally available medicinal plants for mosquito control would generate local employment, reduce dependence on expensive and imported products, and stimulate local efforts to enhance the public health system. The ethno-pharmacological approaches used in the search of new bioactive toxins from plants appear to be predictive compared to the random screening approach. The recently developed new isolation techniques and chemical characterization through different types of spectroscopy and chromatography together with new pharmacological testing have led to an interest in plants as the source of new larvicidal compounds. Synergistic approaches such as application of mosquito predators with botanical blends and microbial pesticides will provide a better effect in reducing the vector population and the magnitude of epidemiology.

In conclusion, the isolated compound, IC1 (***gallic acid***) from MeOH floral fraction of *I. cairica* seem to be responsible for biological properties such as larvicidal activity and free radical scavenging activity, while EG1 and EG2 isolated from Petroleum ether fraction of *A. adenophora* leaf may attribute to its larvicidal activity. Owing to their bioavailability and worldwide distribution both the plants may be used as a source of larvicide and natural antioxidant, a good biocide and nutraceutical herb for mankind. Further research on these plants is currently under progress in the area of biopesticides to isolate more bioactive components and ascertain SAR of the plants as a larvicide against mosquito vectors.

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LIST OF PUBLICATIONS

1. **Samuel Lallianrawna**, R. Muthukumaran, Vanlalhruaii Ralte, G. Gurusubramanian, N. Senthil Kumar. Determination of Total Phenolic Content, Total flavonoid Content and Total Antioxidant capacity of *Ageratina adenophora* (Spreng.) King & H. Rob., ***Science Vision***, **13(4) (2013):**

2. **Lallianrawna Samuel**, Lalrotluanga, Rajendra Bose Muthukumaran, Guruswami Gurusubramanian, Nachimuthu Senthilkumar. Larvicidal activity of *Ipomoea cairica* (L.) Sweet and *Ageratina adenophora* (Spreng.) King & H. Rob. plant extracts against arboviral and filarial vector, *Culex quinquefasciatus* Say (Diptera: Culicidae)

LIST OF CONFERENCE PAPERS

1. **Samuel Lallianrawna**, Muthukumaran R., (2013). Efficacy of *Ipomoea cairica* against third instar larvae of *Culex quinquefasciatus* (Say.) mosquito. *Proceedings of National Conference on Frontier Areas in Chemistry* (NCFAC-2013), 23 & 24 October, 2013, Department of Chemistry, Shri Shivaji Science College, Amravati, Maharashtra, India.

LIST OF CONFERENCE AND WORKSHOP ATTENDED

1. National seminar cum Training Programme on Green & Environmental Chemistry, March 30, 2011. Organized by Department of Chemistry, Mizoram University, Aizawl.
2. UGC Sponsored Short Term Course on Computer Application, December 12-16, 2011. Organized by Academic Staff College, Mizoram University, Aizawl.
3. National Level Workshop cum Training Programme on Recent Advances in Medicinal & Aromatic Plants, April 11-25, 2011. Organized by Department of Horticulture, Medicinal & Aromatic Plants, Mizoram University, Aizawl.
4. DBT Sponsored 1st Summer School cum Workshop, May 23-27, 2011. Organized by Institutional Biotech Hub, Department of Pharmacy, Regional Institute of Paramedical & Nursing Sciences, Aizawl.

5. International Conference on Advances in Environmental Chemistry (AEC 2011), November 16-18, 2011. Organized by Department of Chemistry, Mizoram University, Aizawl.
6. National Seminar on Green Chemistry for Greener Environment, November 26-27, 2012. Organised by Department of Chemistry, Pachhunga University College, Aizawl.
7. One Day Seminar on Animal Welfare & Ethics, June 6, 2013. Organized by Department of Pharmacy, Regional Institute of Paramedical & Nursing Sciences, Aizawl.