This book describes the chemical and biological investigations of Erythrina variegata, a plant belonging to the family Fabaceae. The bark of E. variegata was extracted with methanol. The concentrated extract was then partitioned with n-hexane, carbon tetrachloride and chloroform. Investigation of Hexane soluble fraction of the methanolic extract yielded three compounds, EVB-1, EVB-2 and EVB-3 which were identified as Stigmasterol,Epilupeol,Alpinum isoflavone respectively.The crude extracts showed notable antibacterial and antifungal activity against sixteen microorganisms. The carbon tetrachloride, chloroform and aqueous soluble fractions were found to be moderate to highly inhibitory to microbial growth.The crude methanolic extract along with n-hexane, carbon tetrachloride, chloroform and aqueous soluble fractions of Erythrina variegata showed significant antimicrobial and cytotoxic activities. The significant results of cytotoxic screening support further studying for the development of anticancer drugs. Therefore, plant materials,especially the traditionally used medicinal plants can be potential sources of chemically interesting and biologically important drug candidate.

Khandokar Sadique Faisal

The author was born in 1987 at Kushtia. He finished his school and college with a magnificent academic result. At the age of seventeen he joined army, it makes him physically tough and mentally strong. He finished his B.Pharmacy from Northern University Bangladesh and joined as Lecturer there and also involved in research work.

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CONTENTS

RATIONALE OF THE WORK

The study of disease and their treatment have been existing since the beginning of human civilization. Norman R. Farnsworth of the University of Illinosis declared that, for every disease that affect mankind there is a treatment and cure occurring naturally on the earth. Plant kingdom is one of the major search areas for effective works of recent days. The importance of plants in search of new drugs is increasing with the advancements of medical sciences. For example, ricin, a toxin produced by the beans of *Ricinus communis*, has been found to be effectively couple to tumor targeted monoclonal antibiotics and has proved to be a very potent antitumor drug (Spalding, 1991; Gupta,1992). Further have the HIV inhibitory activity has been observed in some novel coumarins (complex angular pyranocoumarins) isolated from *Calophyllum lanigerum* (Kashman *et al*., 1992) and glycerrhizin (from Glycerrhiza species). Hypericin from *Hypercium* species) is an anticancer agent. Taxol is another example of one of the most potent antitumor agent found from *Taxus bravifolia*.

In fact, plants are the important sources of a diverse range of chemical compounds. Some of these compounds possessing a wide range of pharmacological activities are either impossible or to difficult to synthesize in the laboratory. A phytochemist uncovering these resources is producing useful materials for screening programs for drug discovery. Emergence of newer disease also leading the scientists to go back to nature for newer effective molecules.

Recently developed genetic engineering in plants has further increased their importance in the field of medicine, for example in the production of antibiotics by expression of an appropriate gene in the plant. By using these techniques it is possible to modify the activity or regulate the properties of the key enzymes responsible for the production of secondary metabolites. Thus by knowing the potential resources it is possible to increase the content of the important active compounds (Owen et al., 1992) and in the future, genes responsible for very specific biosynthetic processes may be encoded into host organism to facilitate difficult synthetic transformation.

Thus plants are considered as are of the most important and interesting subjects that should be explored for the discovery and development of newer and safer drug candidates.

Objective of the work

Bangladesh is a good source of the medicinal plants belonging to various families, including Fabaceae. The Fabaceaeous plants contain a wide range chemical and unique pharmacologically active compounds, including anti-inflammatory, anti-rheumatic, anti-diarrhoea and anti-emetic activities.

In Bangladesh there are about 40 plants belonging to the family Fabaceae. Although a large number of plants included in Fabaceae have been investigated all over the world, Fabaceae plants investigated in Bangladesh have to shown to have a wide range of secondary metabolites including cytotoxic compounds. Therefore an attempt has been taken to study the chemical constituents and biological activities of *Erythrina variegata*, a member of the Fabaceae family, growing in Bangladesh.

So, the main objective is to explore the possibility of developing new drug candidates from *Erythrina variegata* for the treatment of various diseases.

PRESENT STUDY PROTOCOL

Our present study was designed to isolate pure compounds as well as to observe biological activities of the isolated pure compounds with crude extract and their different fractions. The study protocol consisted of the following steps:

- Cold extraction of the powdered stem bark of the plant with methanol.
- Partitioning of the methanol crude extract with n-hexane, carbontetrachloride and chloroform.
- Fractionation of the n-hexane, carbon tetrachloride and chloroform extracts by column chromatography (CC).
- Isolation and purification of the pure compounds from different CC fractions by preparative thin layer chromatography (PTLC).
- Determination of the structure of the isolated compounds with the help of ^{1}H NMR, ^{13}C NMR, COSY, NOSY, HSQC HMBC spectroscopy and Mass (FAB) spectrometry.
- Observation of in vitro antimicrobial activity of crude extracts, CC fractions and pure compounds.
- Brine shrimp lethality bioassay and determination of LC_{50} for crude extract, CC fractions and pure compounds.

CHAPTER 1

INTRODUCTION

1.1. The Plant Family: Fabaceae

The plant under investigation is a glabrous tree *Erythrina variegata* belonging to the family Fabaceae. The Fabaceae are mostly herbs but include also shrubs and trees found in both temperate and tropical areas. Legumes are used as crops, forages and green manures. They also synthesis a wide range of natural products such as flavours, drugs poisons and dyes. They comprise one of the largest families of flowering plants, numbering some 650 genera and 18,000 species (Polhill & Raven,1981). This is just under a twelfth of all known flowering plants. The Fabaceae are generally divided into three subfamilies, which are sometimes raised to family rank. The Mimosoideae (58 genera, 3000 species) generally have small, radially symmetrical flowers, often with many stamens; the best – known genus is *Acacia*. In the caesalpinioideae (162 genera, 2000 species) the stamens are usually few in number, and the five petals are differentiated to some extent into an upper standard, two lateral wings, and two lower keel petals, which overlapthe outside of the wings. The Caesalpinioideae are mainly tropical woody plants, though some species of *Cassia* and the Judas tree *Cercis siliquastrum* are hardly cultivated ornamentals in temperate regions. In the Papilionoideae (450 genera,12000 species)the standard, wings, and keel are clearly differentiated, the wings overlapping the outside of the keel petals; there are ten stamens, nearly always joined together, either all ten together or nine joined and one separate. Economically the family is of appreciable importance as a source of edible fruits and many members of this are used in folk medicine for various purposes. On the basis of morphology and habitat, the Fabaceae is a very homogenous plant family (Le Thomas, 1969).

1.1.1. Characteristics of Fabaceae / Leguminosae family

The Fabaceae is an extremely diverse family. The major Characteristics of the family are given below

- Legumes are a significant component of nearly all-terrestrial biomes, on all continents (except Antarctica). Some are fresh-water aquatics, but there are no truly marine species
- The species within the family range from dwarf herbs of arctic and alpine vegetation to massive trees of tropical forest.
- The principal unifying feature of the family is the fruit, a pod, technically known as a Legume. The Legume is modified in many ways to facilitate dispersal by animals, wind and water. The fruit is a 1 or 2 compartment pod (legume) or a pod constricted between the seeds (loment).

Sub-families

The Family is usually divided into three sub families. These sub families are sometimes recognized as three separate families. The three subfamilies are generally identifiable by their flowers .The sub families are:

PAPILIONOIDEAE (PEA)

The Papilionoideae is the largest of the three subfamilies with about two-thirds of all the genera and species of the family. It is also the most widespread, extending further into temperate regions than the other two subfamilies.

The majority of the species are herbaceous, although there are some trees and shrubs, e.g. Laburnum and Gorse (*Ulex*).

For the most part the Papilionoideae are easily recognised by their characteristic papilionaceous (butterfly-like) flowers. The flower is irregular (zygomorphic) and is made up of five petals; two wing petals, and two petals partially fused together to form a boat-shaped keel. The keel encloses the stamens, which are not visible externally.

CAESALPINIOIDEAE

The majority of the Caesalpinioideae are tropical or subtropical trees and shrubs.

The flowers of the Caesalpinioideae are irregular (zygomorphic) with five petals, which are not differentiated into standard, wings and keel. The stamens are visible externally.

Several species in this subfamily are well-known tropical ornamentals such as Flamboyant (*Delonix regia*) and Barbados Pride (*Caesalpinia pulcherrima*). Alexandrian Senna (*Senna alexandrina*) is a commercially grown medicinal plant, known for its purgative qualities.

MIMOSOIDEAE

Like the Caesalpinioideae, the majority of the Mimosoideae are tropical or subtropical trees and shrubs.

The Mimosoideae are characterised by their small, regular (actinomorphic) flowers crowded together, generally into spikes or heads, which resemble a pom-pom. The stamens have become the most attractive part of the flower, the five petals inconspicuous. The leaves are predominately bipinnate.

Examples of genera within this subfamily are *Acacia* and *Mimosa*. Certain *Acacia* species are extremely important economically. An extract from the bark of the Golden Wattle (*Acacia pycnantha*) is used in tanning, several species, such as Australian Blackwood (e.g. *Acacia melanoxylon*) provide useful timbers and some (e.g. *Acacia senegal*) yield commercial gum arabic, which is used in a wide range of industrial processes.

1.1.2 Members of Fabaceae family

The plants belonging to the family Fabaceae, which are available all over the world, are shown in table 1.1. On the other hand, Fabaceae species available in Bangladesh are listed in table 1.2.

Table: 1.1. Fabaceaes species available in the world

Family	Genera	Botanical Names
Fabaceae	***Acacia***	*Acacia abyssinica*
		Acacia albida
		Acacia aneura
		Acacia angustissima
		Acacia auriculiformis
		Acacia baileyana
		Acacia berlandieri
		Acacia cognata
		Acacia constricta
		Acacia covenyi
		Acacia craspedocarpa

		Acacia cultriformis
		Acacia dealbata
		Acacia erioloba
		Acacia farnesiana
		Acacia glaucoptera
		Acacia greggii
		Acacia longifolia
		Acacia mangium
		Acacia mearnsii
		Acacia melanoxylon
		Acacia minuta
		Acacia nilotica
Family	**Genera**	**Botanical Names**
Fabaceae	***Acacia***	*Acacia notabilis*
		Acacia occidentalis
		Acacia pendula
		Acacia pennatula
		Acacia peuce
		Acacia retinodes
		Acacia rigidula
		Acacia salicina
		Acacia saligna
		Acacia schaffneri

		Acacia tetragonophylla
		Acacia vestita
		Acacia willardiana
	Albizia	*Albizia julibrissin*
	Amorpha	*Amorpha canescens*
		Amorpha fruticosa
	Baptisia	*Baptisia alba*
		Baptisia australis
	Bauhinia	*Bauhinia blakeana*
		Bauhinia forficata
		Bauhinia galpinii
		Bauhinia lunarioides
		Bauhinia monandra
		Bauhinia variegata
	Caesalpinia	*Caesalpinia cacalaco*
		Caesalpinia californica
		Caesalpinia gilliesii
		Caesalpinia mexicana
		Caesalpinia platyloba
Family	**Genera**	**Botanical Names**
Fabaceae	***Caesalpinia***	*Caesalpinia pulcherrima*
		Caesalpinia spinosa
	Calliandra	*Calliandra californica*

		Calliandra emarginata
		Calliandra eriophylla
		Calliandra haematocephala
		Calliandra tweedii
	Camoensia	*Camoensia scandens*
	Caragana	*Caragana arborescens*
	Cassia	*Cassia biflora*
		Cassia didymobotria
		Cassia excelsa
		Cassia fistula
		Cassia leptophylla
		Cassia nemophila
		Cassia phyllodinea
		Cassia splendida
	Cercis	*Cercis canadensis*
		Cercis canadensis var. mexicana
		Cercis chinensis
		Cercis occidentalis
		Cercis reniformis
		Cercis siliquastrum
	Cladrastis	*Cladrastis kentukea*
	Coronilla	*Coronilla varia*

	Cytisus	*Cytisus battandieri*
		Cytisus praecox
		Cytisus scoparius

Family	Genera	Botanical Names
Fabaceae	***Cytisus***	*Cytisus spachianus*
	Dalea	*Dalea bicolor*
		Dalea capitata
		Dalea formosa
		Dalea greggii
		Dalea pulchra
	Delonix	*Delonix decaryi*
	Dichrostachys	*Dichrostachys cinerea*
	Dolichos	*Dolichos lablab*
	Erythrina	*Erythrina abyssinica*
		Erythrina acanthocarpa
		Erythrina americana
		Erythrina bidwillii
		Erythrina caffra
		Erythrina coralloides

		Erythrina crista-galli
		Erythrina falcate
		Erythrina flabelliformis
		Erythrina herbacea
		Erythrina humeana
		Erythrina latissima
		Erythrina lysistemon
		Erythrina speciosa
		Erythrina sykesii
		Erythrina variegata
		Erythrina egglengii
		Erythrina gibbsae
		Erythrina graefferi
		Erythrina huillensis
Family	**Genera**	**Botanical Names**
Fabaceae	***Erythrina***	*Erythrina martii*
		Erythrina platyphllos
		Erythrina poianthes
		Erythrina princeps
		Erythrina purpusii
		Erythrina reticulata

		Erythrina suberifera
		Erythrina tomentosa
		Erythrina webberi
	Genista	*Genista aetnensis*
		Genista canariensis
		Genista monosperma
	Lotus	*Lotus bertholetii*
		Lotus corniculatus
		Lotus rigidus
	Maackia	*Maackia amurensis*
	Mimosa	*Mimosa dysocarpa*
	Prosopis	*Prosopis nigra*
		Prosopis pubescens
	Psorothamnus	*Psorothamnus spinosus*
	Robinia	*Robinia neomexicana*
	Senna	*Senna marilandica*
		Senna polyphylla
		Senna surattensis
		Senna wislizeni
	Sesbania	*Sesbania punicea*

	Sophora	*Sophora formosa*
		Sophora japonica
Family	**Genera**	**Botanical Names**
Fabaceae	***Sophora***	*Sophora secundiflora*
	Tamarindus	*Tamarindus indica*
	Thermopsis	*Thermopsis lupinoides*
		Thermopsis montana
		Thermopsis villosa
	Tipuana	*Tipuana tipu*
	Trifolium	*Trifolium fragiferum*
		Trifolium pratense
	Vigna	*Vigna caracalla*
	Wisteria	*Wisteria brachybotrys*
		Wisteria floribunda
		Wisteria frutescens
		Wisteria sinensis

TABLE: 1.2. FABACEAES species available in Bangladesh (Bangladesh National Herbarium, 2005)

Family	Genera	Species	Local Names
Fabaceae	***Abrus***	*Abrus precatorius*	Kunch, Ratti
	Arachis	*Arachis hypogea*	Badam, Cheenabadam
	Butea	*Butea monosperma*	Polash, Kingsuk
	Caesalpinia	*Caesalpinia bonduc*	Nata Karanja
		Caesalpinia pulcherrima	Krishnachura, Radhachura
	Cajanus	*Cajanus cajan*	Arhar, Tur
	Abrus	*Abrus precatorius*	Kunch, Ratti
	Arachis	*Arachis hypogea*	Badam, Cheenabadam
	Butea	*Butea monosperma*	Polash, Kingsuk
	Caesalpinia	*Caesalpinia bonduc*	Nata Karanja
		Caesalpinia pulcherrima	Krishnachura, Radhachura
	Cajanus	*Cajanus cajan*	Arhar, Tur
	Cassia	*Cassia alata*	Dadmardan
Family	**Genera**	**Species**	**Local Names**
Fabaceae	***Cassia***	*Cassia fistula*	Bandar Lathi, Sonalu, Sondal
		Cassia obtusifolia	Chakunda, Goleski
		Cassia occidentalis	Kalkasunda
		Cassia sophera	Chhota kalkasunda
	Cicer	*Cicer arietinum*	Chhola, Boot, Chana

	Clitoria	*Clitoria ternatea*	Aparajita, Nila
	Crotalaria	*Crotalaria juncea*	Shone, Shonpat
		Crotalaria retusa	Bil-Jhunjhun, Atasi
		Crotalaria verrucosa	Jhanjhania, Bansan
	Cullen	*Cullen corylifolia*	Babchi, Buchki
	Dalbergia	*Dalbergia latifolia*	Sitsal
		Dalbergia volubilis	Ankilata
	Derris	*Derris trifoliata*	Pan Lata, Goali Lata
	Desmodium	*Desmodium gangeticum*	Salpani, Chalani
	Erythrina	*Erythrina variegata*	Mandar
	Indegofera	*Indegofera tinctoria*	Neel
	Lablab	*Lablab purpureas*	Shim, Sheemlata
	Lens	*Lens culinaris*	Masur, Musuri
	Mucuna	*Mucuna pruriens*	Alkushi, Bichchoti, Bilaiachra
	Pisum	*Pisum sativum*	Motor
	Pongamai	*Pongamai pinnata*	Karanja, Karamcha, Kangi
	Saraca	*Saraca asoca*	Ashok, Anganapria
	Tamarindus	*Tamarindus indica*	Tentul gachh
	Trigonella	*Trigonella foenum-graecum*	Methi shak, Methi

1.1.3. Medicinal Importance of Fabaceaes plants

Among the 18000 species of Fabaceae, only few are medicinally important. For many years some species of this family are being medicinally used by the indigenous people of Taiwan and southern China through the Philippines, Indonesia, Malaysia, Southeast Asia, India, islands in the Indian Ocean and all the way to tropical east Africa.. Recent data have shown that some species, of this family are being used in the treatments of arthritis, asthma, bronchitis, cancer, fever, colds, gastritis, leukemia, pain relief, parasites. The medicinal uses of Fabaceaes plants are listed in table 1.3.

Table: 1.3. Medicinal use of Fabaceae Plants (Kirtikar & Basu,1980)

Family	Genera	Species	Uses
Fabaceae	***Acacia***	*catechu*	Bark is astringent. Root is made into paste and applied on joints for rheumatism.
		nilotica	Flowers used for jaundice and palpitation. Leaf juice is taken for body dysentery. Powdered bark given for cute diarrhea. Extracts from root and bark are used as taenifuge and gum is used as emollient
		senegal	Gum demulcent and emollient used internally for intestinal mucosa and externally applied on inflammations.
	Albizia	*chinensis*	Infusion of the bark used as lotion for cuts, scabies and skin diseases.

	Butea	*monosperma*	Anti-pyretic, appetizer, aphrodisiac, blood purifier, diuretic, tonic viral hepatitis. Seed is anthelmintic. Gum is astringent.
	Bauhinia	*purpurea*	Purgative. Flowers and buds used for indigestion. Stem bark decoction used for body pain and fever. Paste from bark is given for cancerous growth in stomach.
	Bauhinia	*semlo*	Bark is astringent; used in diarrhea and dysentery. Decoction of leaf is used for headache and malaria.
	Caesalpinia	*pulcherrima*	Root decoction taken for fever, flowers used for asthma and bronchitis and as anti-pyretic and expectorant.

Family	**Genera**	**Species**	**Uses**
Fabaceae	***Cassia***	*fistula*	Flowers used for cough, diphtheria, constipation and edema.
	Dalbergia	*sissoo*	Leaves are bitter and stimulant, decoction is used in gonorrhea; root is astringent. Wood is also used in leprosy, boils, eruptions and to stop vomiting.

		sissoo	Leaves are bitter and stimulant, decoction is used in gonorrhea; root is astringent. Wood is also used in leprosy, boils, eruptions and to stop vomiting.
	Delonix	*regia*	Leaf decoction taken for constipation. Flowers are anthelmintic.
	Erithrina	*variegata*	Leaves contain a mixture of alkaloid, hyaphorine is present in leaves, seeds and bark. Apart from hyaphorine, betaine and choline and also found.
	Prosopis	*cineraria*	Flowers are beneficial against miscarriage.
	Saraca	*indica*	Bark is astringent; used in menorrhoea and uterine infections, also used for scorpion-sting.
	Tamarindus	*indica*	Leaf decoction used for throat infection. Intestinal worms and liver ailments. Flowers used as anti-viral against new castle disease virus and are astringent and sedative. Fruits used for loss of appetite, constipation and rheumatism.

1.1.4 Taxonomy of Fabaceae family

The Fabaceae are mostly herbs but include also shrubs and trees found in both temperature and tropical areas. They comprise one of the largest families of flowering plants, numbering some 650 genera and 18,000 species. The leaves are stipulate, nearly always alternate, and

range from pinnately or palmately compound to simple. Like the other legume families the petiole base is commonly enlarged into a pluvinus. The flowers are slightly to strongly perigynous, zygomorphic, and commonly in racemes, spikes, or heads. The perianth commonly consists of calyx and corolla of 5 segments each. The petals are overlapping (imbricate) in bud with the posterior petal (called the banner or flag) outermost (i.e., exterior) in position. The petals are basically distinct except for variable connation of the two lowermost ones called the keel petals. The lateral petals are often called the wings. The androecium most commonly consists of 10 stamens in two groups (i.e., the are diadelphous with 9 stamens in one bundle and the 10^{th} stamen more or less distinct). The pistil is simple, comprising a single style and stigma, and a superior ovary with one locule containing 2-many marginal ovules. The fruit is usually a legume. All members of this family have five-pelated flowers in which the superior ovary ripens to from a pod whose two sides spilt apart, releasing the seeds which are attached to one seam, alternately attached to one side or the other.

1.2. Information about the plant investigated

1.2.1. Description of the Plant *Erythrina variegata* (Kirtikar & Basu,1980)

A tree reaching 18 meter in height; bark thin, smooth, grey, armed with small conical dark-colored prickles. Leaves 15-30 cm. Long, deciduous. Flowers appearing before the leaves, in dense racemes, 10-23 cm. Long arranged in clusters of 1-3 on a puberulous or tomentose Calyx (before the expansion of the flower) tubular, 5- toothed at the tip, 2.5-3.2 cm. long, clothed with deciduous tomentum. Corolla bright red, 5-6.3 cm. Long; standard 2.5-3.8 cm. Broad; wings and keel-petals subequal, 1.3-2 cm. Long. Stamens much exserted. Pods 12.5-30 cm. Long.

1.2.2. BOTANICAL FEATURES OF *Erythrina variegata*

Scientific name : ***Erythrina variegata***

Family : **Fabaceae**

Bangla name : Palitamadar, Palitamandar, Paltemandar

English name : Indian coral-Tree, Mochi wood

Tree : 18 m. in height.

Leaves : 15-30 cm. Long, deciduous. Petioles, 10-15cm. Long, unarmed, readily disarticulating.

Calyx : Tubular, 5- toothed at the tip, 2.5-3.2 cm. long, clothed with deciduous tomentum, mouth very oblique, the upper segment subulate, the 2 lateral similar but smaller.

Corolla : Corolla bright red, 5-6.3 cm. Long; standard 2.5-3.8 cm. Broad; wings and keel-petals subequal, 1.3-2 cm. Long.

Flowers : Appearing before the leaves, in dense racemes, 10-23 cm. Long arranged in clusters of 1-3 on a puberulous or tomentose

Seeds : Seeds 4-8, subreniform, 2 by1 cm., brown.

(A)

(B)

(C) (D) (E)

Figure 1.1: - *Erythrina variegata* : (A) Whole Plant (B & E) Flower (C) Leave (D) Seeds

1.2.3. Distribution of *Erythrina variegata*

Erythrina variegata are available in coast forests from Bombay to Malabar, and from the sundribuns along the coast through Arakan and Tenasserim and in the Andamans and Nicobars; much planted for ornament.

In Bangladesh it is grown in all over the country.

1.2.4. Medicinal uses of *Erythrina variegata*

Different parts of *Erythrina variegata* are used traditionally as medicine by local people, some which are mentioned in table 1.4.

Table: 1.4. Ethnobotanical uses of different parts of *Erythrina variegata*.

Plant part	Taste	Medicinal/ Other Uses
Root	Bitter	Used as emmenagogue
Bark	Bitter	Bark alkaloids of *Erythrina variegata are* smooth muscle relaxant, CNS depressant, hydrocholeretic and anti-convulsants. Bark is astringent, febrifuge, anti-bilious and anthelmintic, and is used in ophthalmia and for skin problems e.g. leprosy and fever.(Chevallier, 1996)
Flowers	Acrid, bitter	Used in biliousness and ear troubles

Plant part	Taste	Medicinal/ Other Uses
Leaves	Acrid, Bitter, Pungent	Leaves are anthelmintic, stomachic, laxative, diuretic, galactagogue and emenagogue. Paste of leaves is applied externally to cure inflammations and to relieve pain in the joints; juice is used to relief earache and toothache; improve appetite, cure urinary discharge. Ethanolic extract of leaves is spasmogenic, diuretic and anticancer. (IJEB, 1968; 1980)

1.2.5. Reported biological works on *Erythrina variegata*

Extensive literature search revealed the following biological works on *E. Variegata in* **Table 1.5. Reported biological works on *Erythrina variegate***

* Methicillin resistant Staphylococcus aureus (MRSA)

Test sample	Investigation	Reference
Mupirocin	Anti MRSA*	Sato *et al.,* 2004 Tanaka *et al.,* 2002
Isoflavanone		

Protein	Protease inhibitory activities	Iwanaga *et al.,* 2005 Iwanaga *et al.,* 1999 Iwanaga *et al.,* 1998 Kimura *et al.*,1994
Protein	Cytotoxicity	Ohba *et al.,* 1998
Isoflavonoids	Antibacterial	Sato *et al.,* 2003
Erythrinin B	Inhibitors of Na+/H+ exchanger	Kobayashi *et al.,* 1997

1.3. Chemistry of the Fabaceae family

Literature review revealed that these genera contain saponins, stertols, phenols, tannins, flavonoids, and alkaloids. Among these chemical constituents flavonoids and alkaloids are the most prevalent ones.

Table: 1.6. Compounds reported from some species of Fabaceae

Plants	Compound isolated	Reference
Arachis hypogea	Tocopherol, triterpenoids, sterols, phenolic glycoside, caratenoids	WI, 1985
Butea monosperma	Kinnotannic acid, gallic acid, flavonoid,	Bandara et

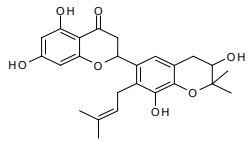
	glycosides, butin, butrin, isobutrin, plastrin, lupeol, beta-sitosterol, isoflavones, 5 methoxygenistein	al.,1990
Cassia obtusifolia	Anthraquinones and anthraquinone glycosides, chrysophanic acid rhein, emodin, gluco-obtusifolin, cascaroside, rubrofusarin, chrysophanol, questin, naphthalenic lactones	Acharya & chatterjee, 1974
Cassia fistula	anthraquinone glycosides like rhein, sennosides A and B flavones, sennosides, lupeol, beta-sitosterol, hexacosanol, tannin, flavonoid glycoside, aloe emodin, emodin, rhein and sennidin A and B.	Kaji et al., 1968
Clitoria ternatea	Glycosides of Kaempferol, stigmastone, beta and gamma-sitosterol, fixed oil, tannin, flavonol glycosides, hexacosanol, beta-sitosterol, anthoxanthin glucoside, tannin, taraxerone	Said et al., 1996

1.3.1. The important compounds, isolated from *Erythrina* Genus (Dictionary of Natural products, 2000)

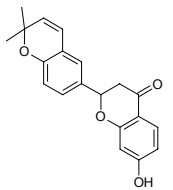
Different classes of compounds were isolated from Erythrina genus. These were alkaloids, glycosides, naphthoquinones & anthraquinones, flavonoids. Some of which are mentioned below:

1.3.1.1. Flavonoids

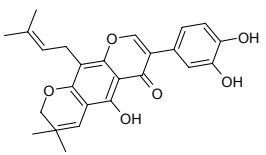
The flavonoid that had been isolated from this family



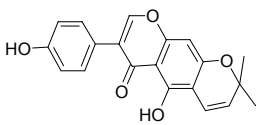
Abyssinoflavanone V **(1)**



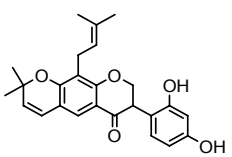
Abyssinone I; **(2)**



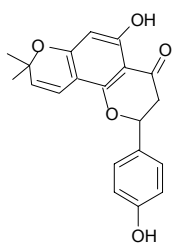
Auriculasin **(3)**



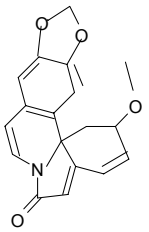
Alpinumisoflavone **(4)**



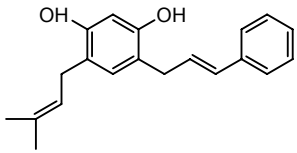
Bidwillon B; **(5)**



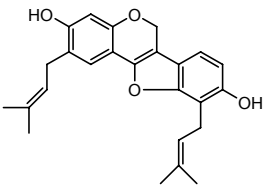
Citflavanone; **(6)**



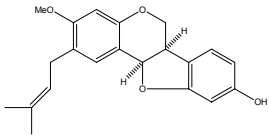
Crystamidine; **(7)**



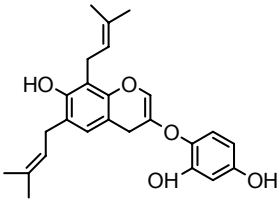
Eryvariestyrene **(8)**



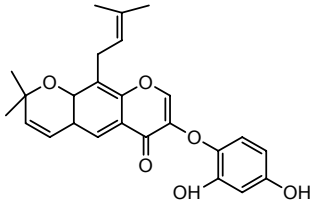
Erycristagallin **(9)**



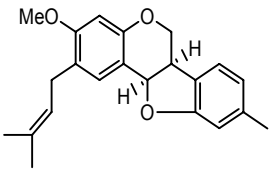
Orientanol B **(10)**



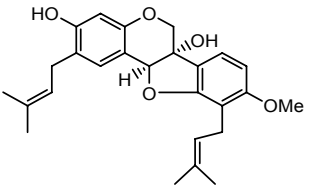
Eryvarin F **(11)**



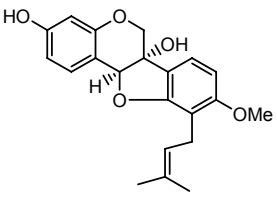
Eryvarin G **(12)**



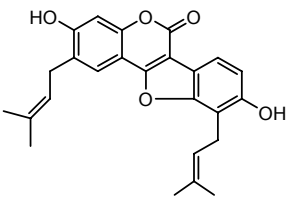
Orientanol B **(13)**



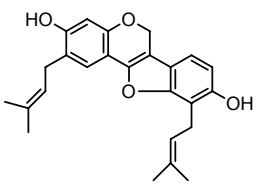
Erystagallin A **(14)**



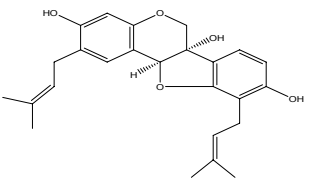
Cristacarpin **(15)**



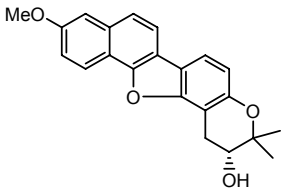
Sigmaidin K **(16)**



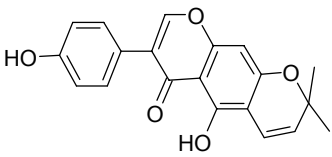
Erycristagallin **(17)**



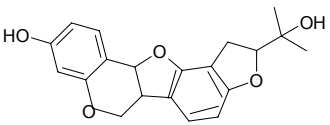
Calopocarpin **(18)**



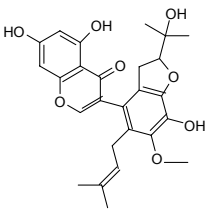
Eryvarin A **(19)**



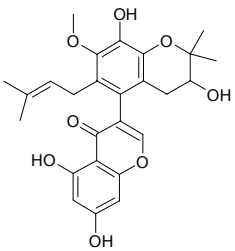
Erysenegalensein K **(20)**



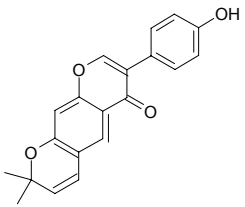
Erystagallin C **(21)**



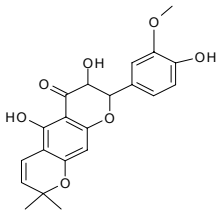
Erythbigenol A **(22)**



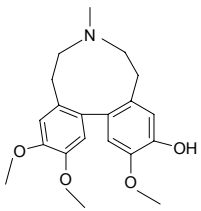
Erythbigenine A **(23)**



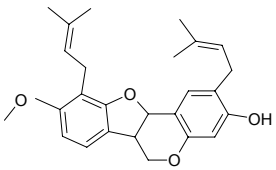
Erythrinin **(24)**



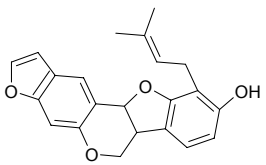
Eriotrinol **(25)**



Erybidine **(26)**



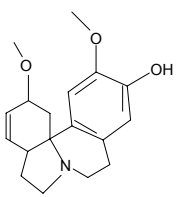
Ericristin **(27)**

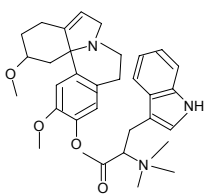


Erybraedin E **(28)**

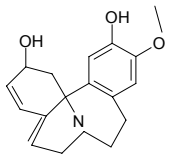
1.3.1.2. Alkaloids

A number of alkaloids have been isolated from Fabaceaes species. Most of the alkaloids are Pyridine, Piperidine, Pyrrolidine, Indole and Imidazole type. Some of the structures are given below:

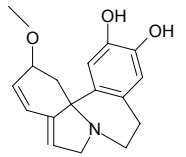




Erysodine **(29)**

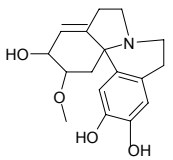


Erysodinophorine **(30)**

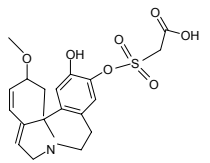


Erysoline **(31)**

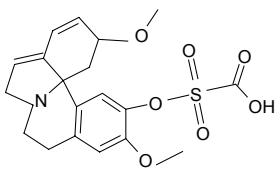
Erysopine **(32)**



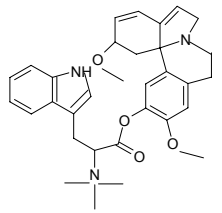
Erysopitine **(33)**



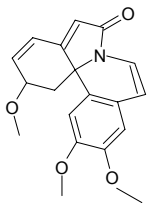
Erysothiopine **(34)**



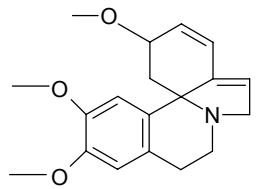
Erysothiovine **(35)**



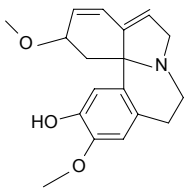
Erysophorine **(36)**



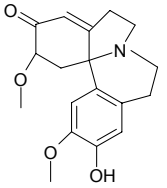
Erysotramidine **(37)**



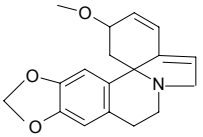
Erysotrine **(38)**



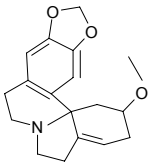
Erysovine **(39)**



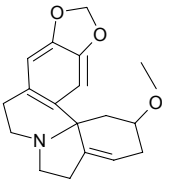
Erysotinone (+) **(40)**



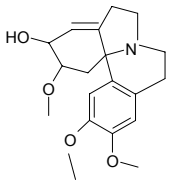
Erythraline **(41)**



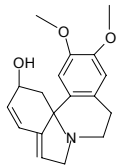
Erythramine **(42)**



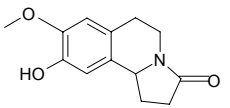
Erythramine **(43)**



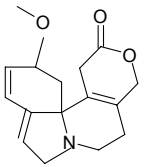
Erythratidine **(44)**



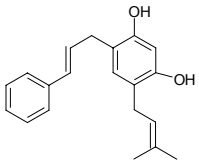
Erythravine **(45)**



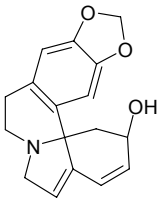
Erythrinarbine **(46)**



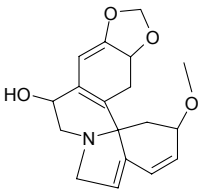
Erythrodine **(47)**



Eryvariestyrene **(48)**



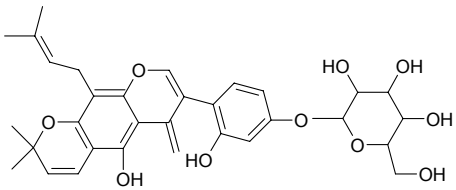
Erythrocarine **(49)**



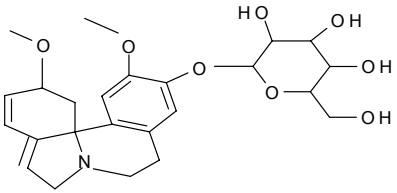
Erythrinine **(50)**

1.3.1.3. Glycosides

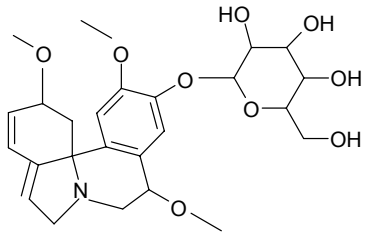
Glycosides isolated from Fabaceaes plants are of following types:



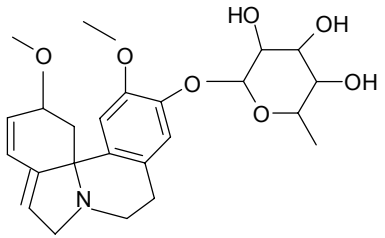
Auriculatin 4-O-β-D-Glucopyranoside **(51)**



Erysodine; 16-O-β-D-Glucopyranoside **(52)**



Erysodine; 11β-Methoxy, 16-O-β-D-Glucopyranoside **(53)**



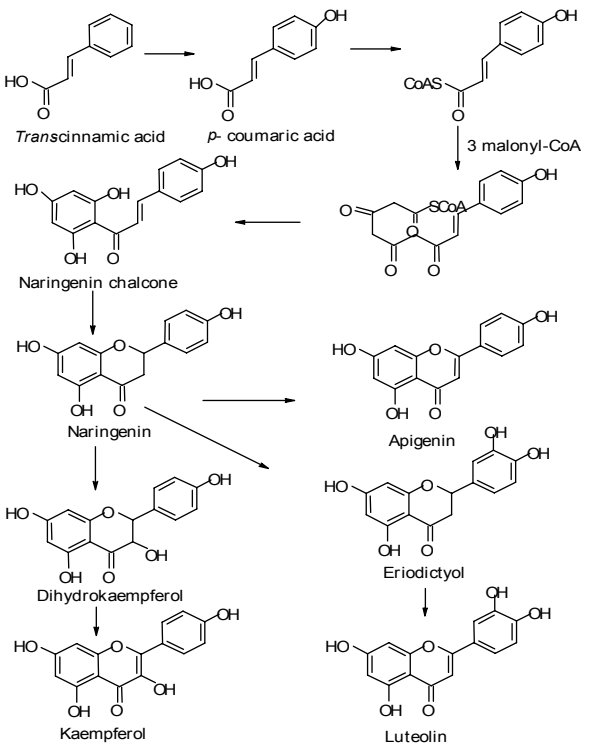
Erysodine; 16-O-α-L-Rhamnopyranoside **(54)**

1.4. Possible biosynthetic pathways of secondary metabolites

1.4.1. Biosynthesis of flavonoids

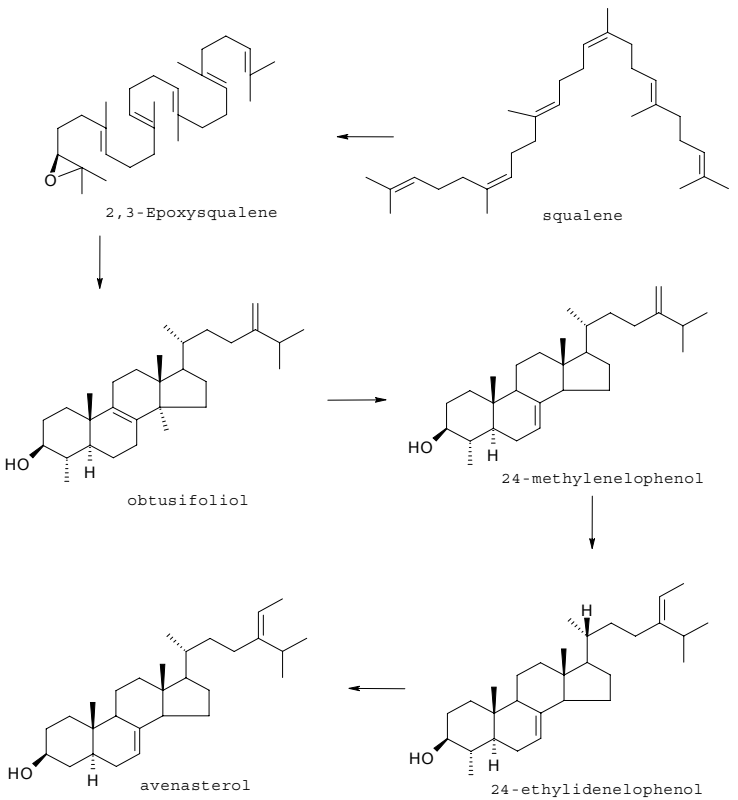
As flavonoides are commonly occurring constituents in *S. chelonoides* the biosynthesis of these compounds are described here. In the biosynthesis of flavonoids *trans*-cinnamic acid undergoes

aromatic hydroxylation to form *para*-coumaric acid (Scheme 1.3). It is further converted by the enzyme 4-coumarate-CoA ligase into *para*-coumaroyl-CoA. The later condenses with three molecules of malonyl-CoA to yield naringenin chalcone which is the precusor of different types of flavonoids and other related compounds (Heller and Forkmann, 1988; Dewick, 1990). Transformation of the stereospecific action of chalcone isomerase provides a flavone (eg. naringenin). Two different types of enzyme (dioxygenase and a mixed-function mono-oxygenase) are catalysts for the production of flavones (eg. apigenin). Dihydroflavonols (eg. dihydrokaempferol) are formed by direct hydroxylation of flavanones in the 3-position by the dioxygenase, flavanone 3-hydroxylase. Dihydroflavonols are biosynthetic intermediates in the formation of flavonols, catecheins, proanthocyanidins and anthocyanidins. Kaempferol is formed by flavonol synthase and a dioxygenase.



1.4.2 Biosynthesis of Steroidal compound

Sterols are the first steroid isolated from natures and the most common sterol in plants is β-sitosterol. Stigmasterol is closely related to β-sitosterol containing an additional double bond at position 22. Sterols are formed biosynthetically from isopentenyl pyrophosphate and involve the same sequence of reaction as terpenoid biosynthesis, where Squalene is an intermediate.



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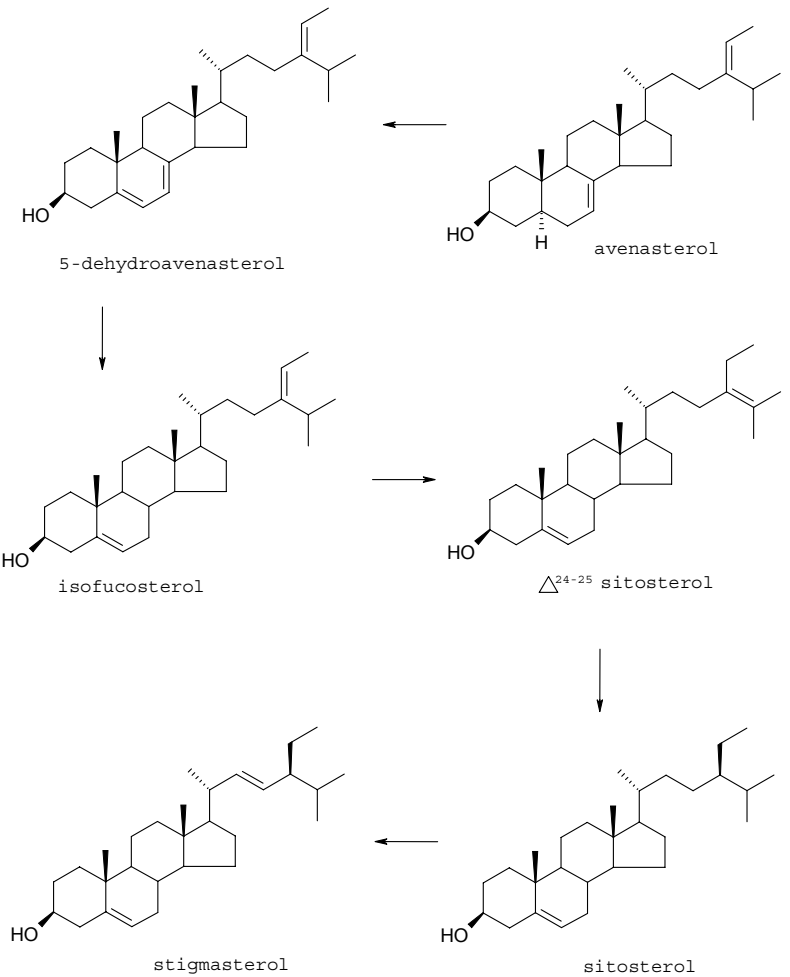

HO
5-dehydroavenasterol
HO
H
avenasterol
HO
isofucosterol
HO
Δ24-25 sitosterol
HO
stigmasterol
HO
sitosterol

CHAPTER 2

METHODS AND MATERIALS - CHEMICAL

2.1. Methods

The chemical investigation of a plant can be divided roughly into the following major steps:

a) Collection and proper identification of the plant materials

b) Preparation of plant sample

c) Extraction

d) Fractionation and isolation of compounds

e) Structural characterization of purified compounds

The last step will be discussed in Chapter-3. However, other steps will be presented here initially as general procedure and then in connection with concerned plants.

2.1.1. Collection and proper identification of the plant sample

At first with the help of a comprehensive literature review a plant was selected for investigation and then the whole plant/plant part(s) was collected from an authentic source and was identified by a taxonomist. A voucher specimen that contains the identification characteristics of the plant was submitted to the herbarium for future reference.

2.1.2. Plant material preparation

The stem bark of the plant was collected in fresh condition. It was sun-dried and then, dried in an oven at reduced temperature (not more than 50^0C) to make it suitable for grinding purpose. The coarse powder was then stored in air-tight container with marking for identification and kept in cool, dark and dry place for future use.

2.1.3. Extraction procedures

2.1.3.1. Initial extraction

Extraction can be done in two ways such as

a) Cold extraction

b) Hot extraction

a) Cold extraction : In cold extraction the powdered plant materials is submerged in a suitable solvent or solvent systems in an air-tight flat bottomed container for several days, with occasional shaking and stirring. The major portion of the extractable compounds of the plant material will be dissolved in the solvent during this time and hence extracted as solution.

b) Hot extraction : In hot extraction the powdered plant material is successively extracted to exhaustion in a Soxhlet at elevated temperature with several solvents of increasing polarity.

The individual extractives is then filtered through several means, e.g., cotton, cloth, filter paper etc. All the extractives are concentrated with a rotary evaporator at low temperature (40^0-50^0C) and reduced pressure. The concentrated extract thus obtained is termed as crude extract.

2.1.3.2. Solvent-solvent partitioning of crude extract

The crude extract is diluted with sufficient amount of aqueous alcohol (90%) and then gently shaken in a separating funnel with almost equal volume of a suitable organic solvent (such as petroleum ether) which is immiscible with aqueous alcohol. The mixture is kept undisturbed for several minutes for separation of the organic layer from the aqueous phase. The materials of the crude extract will be partitioned between the two phases depending on their affinity for the respective solvents. The organic layer is separated and this process is carried out thrice for maximum extraction of the samples. After separating of the organic phase, the aqueous phase thus obtained is successively extracted with other organic solvents, usually of the increasing polarity (such as carbon tetrachloride, dichloromethane, chloroform, ethylacetate, butanol etc). Finally, all the fractions (organic phases as well as the aqueous phase) are collected separately and evaporated to dryness. These fractions are used for isolation of compounds.

2.1.4. Isolation of compounds

Pure compounds are isolated from the crude and fractionated extracts using different chromatographic and other techniques. A brief and general description of these is given below.

2.1.4.1. Chromatographic techniques

Chromatographic techniques are the most useful in the isolation and purification of compounds from plant extracts. The advent of relatively new chromatographic media e.g. Sephadex and Polyamide, have improved the range of separations that can be performed.

2.1.4.1.1. Column Chromatography

Column Chromatography is the most common separation technique based on the principle of distribution (partition/adsorption) of compounds between a stationary and mobile phase.

A normal Chromatographic column is packed with silica gel (kiesel gel 60, mesh 70-230). A slurry of silica gel in a suitable solvent is added into a glass column of appropriate height and diameter. When the desired height of adsorbent bed is obtained, a few hundred millilitre of solvent is run through the column for proper packing of the column. After packing, the sample to be separated is applied as a concentrated solution in a suitable solvent or the sample is adsorbed onto silica gel (kiesel gel 60, mesh 70-230), allowed to dry and subsequently applied on top of the adsorbent layer. Then the column is developed with suitable solvent mixtures of increasing polarity. The elutes are collected either in test tubes or in conical flasks.

2.1.4.1.2. Vacuum Liquid Chromatography (VLC)

Vacuum Liquid Chromatography is a relatively recent separation technique which involves short column chromatography under reduced pressure, the column being packed with fine TLC grade silica (Kiesel gel 60H). Details of the method have been published by Pelletier *et al* (1986) and by Coll and Bowden (1986). This technique is used for the initial rapid fractionation of crude extracts.

The column is packed with silica gel (kiesel gel 60H) under vacuum. The size of the column and the height of the adsorbent layer are dependent upon the amount of extract to be analyzed. The column is initially washed with a non-polar solvent (petroleum ether) to facilitate compact packing. The sample to be separated was adsorbed onto silica gel (kiesel gel 60, mesh 70-230), allowed to dry and subsequently applied on top of the adsorbent layer. The column is then eluted with a number of organic solvents of increasing polarity and the fractions are collected.

2.1.4.1.3. Thin Layer Chromatography (TLC)

Ascending one-dimensional thin layer chromatographic technique is used for the initial screening of the extracts and column fractions and checking the purity of isolated compounds. For the latter purpose commercially available precoated silica gel (kiesel gel 60 PF_{254}) plates are usually used. For initial screening, TLC plates are made on glass plates with silica gel (kiesel gel 60 PF_{254}).

A number of glass plates measuring 20cm x 5cm are thoroughly washed and dried in an oven. The dried plates are then swabbed with acetone-soaked cotton in order to remove any fatty residue. To make the slurry-required amount of silica gel 60 PF_{254} and appropriate volume of distilled water (2ml/gm of silica gel) are mixed in a conical flask and the flask is gently shaken. The slurry is then evenly distributed over the plates using TLC spreader. After air drying the coated plates are subjected to activation by heating in an oven at 110^0c for 70 minutes (Stahl, 1969; Remington Pharmaceutical sciences, 1988). Table 2.1 shows the amount of silica gel required for preparing plates of varying thicknesses.

Table 2.1: **Amount of silica gel required preparing TLC plates of various thicknesses**

Size (cm x cm)	Thickness (mm)	Amount of silica gel/ plate (gm)
20 x 5	0.3	0.9
	0.4	1.2
	0.5	1.5

Cylindrical glass chamber (TLC tank) with airtight lid is used for the development of chromatoplates. The selected solvent system is poured in sufficient quantity into the tank. A smooth sheet of filter paper is introduced into the tank and allowed to soak in the solvent. The tank is then made airtight and kept for few minutes to saturate the internal atmosphere with the solvent vapour. A small amount of dried extract is dissolved in a suitable solvent to get a solution (approximately 1%)(Harborne, 1976; Touchstone and Dobbins, 1978). A small spot of the solution is applied on the activated silica plate with a capillary tube just 1 cm above the lower edge of the plate. The spot is dried with a hot air blower and a straight line is drawn 2 cm below the upper edge of the activated plate which marks the upper limit of the solvent flow.

The spotted plate is then placed in the tank in such a way as to keep the applied spot above the surface of the solvent system and the cap/lid is placed again. The plate is left for development. When the solvent front reaches upto the given mark, the plate is taken out and air-dried. The properly developed plates are viewed under UV light of various wavelengths as well as treated with suitable reagents to detect the compounds.

Preparative thin layer chromatographic technique is routinely used in separating and for final purification of the compounds. The principle of preparative TLC is same as that of TLC. Here larger plates (20cm x 20cm) are used. Table 2.2 shows the amount of silica gel required for preparing plates of varying thicknesses.

Table 2.2. : Amount of silica gel required preparing PTLC plates of various thicknesses

Size (cm x cm)	Thickness (mm)	Amount of silica gel/ plate (gm)
20 x 20	0.3	3.6
	0.4	4.8
	0.5	6.0

The sample to be analyzed is dissolved in a suitable solvent and applied as a narrow uniform band rather than spot. The plates are then developed in an appropriate solvent system previously determined by TLC. In some cases multiple development technique was adopted for improved separation. After development, the plates are allowed to dry and the bands of compounds are visualized under UV light (254 nm and 366 nm) or with appropriate spray reagents on both edges of the plates. The required bands are scraped from the plates and the compounds are eluted from the silica gel by treating with suitable solvent or solvent mixtures.

2.1.4.2. Solvent treatment

Solvent treatment is a process by which a compound consisting of the major portion of a mixture of compounds can be purified utilising selective solvent washing. Initially, a solvent or a solvent mixture in which the desired compound is practically insoluble and other components are soluble is chosen. The undesired components are separated with repeated washing with this solvent or solvent mixture. If required other solvent or solvent mixture can be used until a pure compound is obtained.

2.1.5. Visualization/ detection of compounds

Detection of compounds in TLC plates is a very important topic in analyzing extractives to isolate pure compounds. The following techniques are used for detecting the compounds in TLC/PTLC plates.

2.1.5.1. Visual detection

The developed chromatogram is viewed visually to detect the presence of coloured compounds.

2.1.5.2. UV light

The developed and dried plates are observed under UV light of both long and short wavelength (254 nm and 366 nm) to detect the spot/band of any compound. Some of the compounds appear as fluorescent spots while the others as dark spots under UV light.

2.1.5.3. Iodine chamber

The developed chromatogram is placed in a closed chamber containing crystals of iodine and kept for few minutes. The compounds that appeared as brown spots are marked. Unsaturated compounds absorb iodine. Bound iodine is removed from the plate by air blowing.

2.1.5.4. Spray reagents

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

a) Vanillin/H_2SO_4 (Stahl, 1966):
   1% vanillin in concentrated sulfuric acid is used as a general spray reagent followed by heating the plates to 100^0C for 10 minutes.

b) Modified Dragendorff's reagent (Touchstone and Dobbins, 1977):
Modified Dragendorff's reagent was used to detect alkaloids. Some coumarins also give a positive test with modified Dragendorff's reagent. The reagent is prepared by mixing equal parts (v/v) of 1.7% bismuth subnitrate dissolved in 20% acetic acid in water and a 40% aqueous solution of potassium iodide.

c) Ferric chloride/EtOH (*Dyeing Reagents for TLC and PC*, 1974):
Some of the phenolic compounds were detected by spraying the plates with ferric chloride (5% ferric chloride in absolute ethanol) reagent.

d) Perchloric acid reagent (Touchstone and Dobbins, 1978) :
2% aqueous perchloric acid produces brown spots with steroids after heating at 150^0C for 10 minutes.

e) Potassium permanganate reagent
Only the oxidizable compounds were detected by this reagent. After spraying with the reagent the compound appeared as yellow or pale yellow spot on the colored (color of permanganate) plate.

2.1.6. Determination of R_f (retardation factor) values

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

$$R_f \text{ value} = \frac{\text{Distance traveled by the compound}}{\text{Distance traveled by the solvent system}}$$

2.2. Chemical Investigation of experimental plants

The plant species belonging to Fabaceae family is investigated in this study.

Name of plant	Family	Plant part
Erythrina variegata	Fabaceae	Stem bark

Taxonomic hierarchy of the investigated plants (Wekepedia, 2005)

E. variegata	
Kingdom	Plantae
Phylum	Angiosperms
Class	Magnoliopsida
Subclass	Rosidae
Order	Fabales
Family	Fabaceae
Genus	*Erythrina*
Species	*Erythrina variegata*

Figure 2.1: Taxonomic hierarchy of the investigated plants

2.2.1. Collection and preparation of Plant Material

Plant sample of *Erythrina Variegata* was collected from Dhaka in August 2005. A voucher specimen has been deposited in University of Dhaka Herbarium (DUH accession no. 1). Bark of the plant was cut into small pieces and then air dried for several days. The pieces were then oven dried for 24 hours at considerably low temperature to effect grinding. The plant was then ground into a coarse powder.

2.2.2. Extraction of the plant material

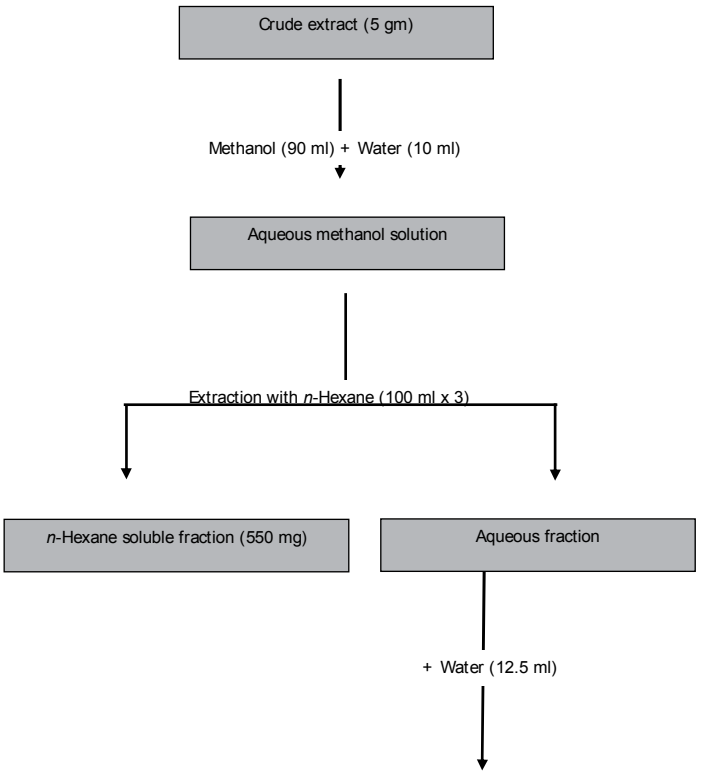
The air-dried and powdered plant material (750 gm) was extracted with methanol (300 ml) for 15 days at room temperature with occasional shaking and stirring. It was then filtered through a fresh cotton plug and finally with a Whatman No.1 filter paper. The volume of the filtrate was then reduced using a Buchii Rotavapor at low temperature and pressure. The weight of the crude extract was 5.2 gm.

2.2.3 Solvent – solvent partioning

Solvent–solvent partitioning was done using the protocol designed by Kupchan and modified by Wagenen *et al.* (1993). The crude extract (5 gm) was dissolved in 10% aqueous methanol. It was extracted with *n*-hexane, then with carbon tetrachloride and finally with chloroform. The whole partitioning process is schemetically shown in Figure 2.3. All the four fractions were evaporated to dryness. These were collected for further analysis.

2.2.4 Investigation of the *n*-hexane soluble fraction

The *n*-hexane soluble fraction of the methanol extract was subjected to TLC screening to see the type of compounds present in the extract. This revealed a considerable number of compounds which suggested for further fractionation. 750 mg of the *n*-hexane soluble fraction was subjected to column chromatography (CC) for fractionation. Then the column fractions were analysed by TLC. The fractions with satisfactory resolution of components were subjected to PTLC to obtain pure compounds.



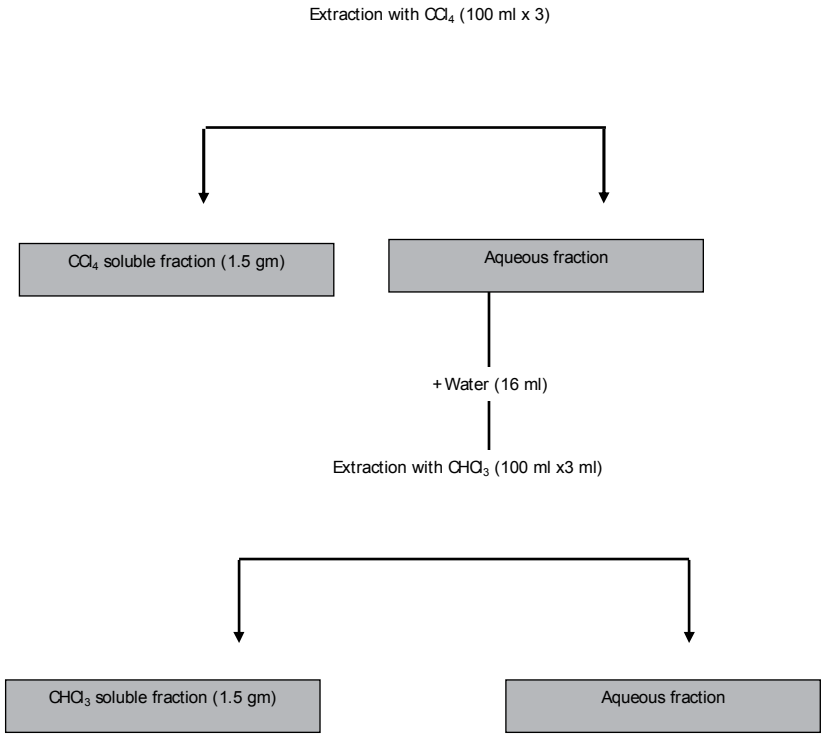


Figure-2.2: Schematic representation of the modified Kupchan partioning of methanolic crude extract of *Erythrina variegata*

2.2.5 Column Chromatography (CC) of *n*-hexane soluble fraction

The column was packed with silica gel (Kieselgel 60, mesh 70-230). Slurry of silica gel was added into a glass column having the length and diameter of 55 cm and 1.1 cm respectively. When sufficient height of the adsorbent bed was obtained, a few hundred millilitre of *n*-hexane was run through the column for proper packing of the column. The sample was prepared by adsorbing 374 mg of *n*-hexane extract onto silica gel (Kieselgel 60, mesh 70-230), allowed to

dry and subsequently applied on top of the adsorbent layer. The column was then eluted with *n*-hexane, followed by mixtures of *n*-hexane and ethyl acetate of increasing polarity, then by ethyl acetate and finally with ethyl acetate and methanol mixtures of increasing polarity. Solvent systems used as mobile phases in the analysis of petrol extract were listed in Table 2.3.

Table 2.3: Different solvent systems used for the column chromatographic analysis of *n*-hexane extract.

Fraction no.	Solvent systems	Volume collected (ml)
1	*n*-Hexane 100%	100
2	*n*-Hexane - Ethyl acetate (99 : 1)	50
3	*n*-Hexane - Ethyl acetate (98 : 2)	50
4	*n*-Hexane - Ethyl acetate (97 : 3)	50
5	*n*-Hexane - Ethyl acetate (96 : 4)	50
6	*n*-Hexane - Ethyl acetate (95 : 5)	50
7 to 9	*n*-Hexane - Ethyl acetate (94 : 6)	60
10 to 14	*n*-Hexane - Ethyl acetate (93 : 7)	100
15 to 24	*n*-Hexane - Ethyl acetate (90 : 10)	200
25 to 30	*n*-Hexane - Ethyl acetate (88 : 12)	200
31 to 45	*n*-Hexane - Ethyl acetate (85 : 15)	300
46 to 56	*n*-Hexane - Ethyl acetate (80 : 20)	200
57 to 66	*n*-Hexane - Ethyl acetate (75 : 25)	100
67 to 77	*n*-Hexane - Ethyl acetate (70 : 30)	200
78 to 82	*n*-Hexane - Ethyl acetate (60 : 40)	100
83 to 87	*n*-Hexane - Ethyl acetate (50 : 50)	100

88 to 92	*n*-Hexane - Ethyl acetate (40 : 60)	100
93 to 97	Ethyl acetate 100%	100
98 to 102	Ethyl acetate - Methanol (99 :1)	100
103 to 105	Ethyl acetate - Methanol (98 : 2)	60
106 -107	Ethyl acetate - Methanol (95 : 5)	50
108 -109	Ethyl acetate - Methanol (85 : 15)	50
110 -111	Ethyl acetate - Methanol (80 : 20)	50
112 -113	Ethyl acetate - Methanol (70 : 30)	50
114 -115	Ethyl acetate - Methanol (50 : 50)	50
116 -120	Methanol 100%	100

2.2.6 Analysis of column fractions by TLC

All the column fractions were screened by TLC under UV light and by spraying with vanillin-sulphuric acid reagent. A number of compounds were detected, which were purified from the different sub-fractions employing various techniques. A list of isolated compounds has been summarized in Table 2.

Table 2.4: A list of isolated compounds from *n*-hexane soluble fraction of methanolic extract.

Column Fractions	Mobile phases	R_f value	Amount (mg)	Yield * (%)	Code
30-36	Toluene : Ethyl acetate 95 : 5	0.33	4.0	0.002	EVB-1
40-44	Toluene : Ethyl acetate 90 :10	0.33	3.0	0.001	EVB-2
60-66	Toluene : Ethyl acetate 88 :12	0.533	4.0	0.002	EVB-3

* *calculated on total amount of plant.*

2.2.7 Isolation and purification of compounds from selected fractions

Isolation of compound EVB-1

The fractions 30 to 36 were bulked together as they showed similar TLC feature. After evaporation of solvents, colored crystals were appeared. It was washed with *n*-hexane and then with ethyl acetate in a sample vial. White needles of EVB-1 was obtained, which was found to be pure by TLC screening.

Isolation of compound EVB-2

The fractions 40-44 together as they showed similar TLC feature *i.e.* a distinct spot with same R_f value (0.33) was found in the solvent system Toluene : Ethyl acetate (90:10). It was then subjected to PTLC using the same solvent system. The desired band was scrapped and then eluted with distilled ethyl acetate to give EVB-2.

Isolation of compound EVB-3

The 60-66 together as they showed similar TLC feature *i.e.* a distinct spot with same R_f value (0.533) was found in the solvent system Toluene : Ethyl acetate (88:12). It was then subjected to PTLC using the same solvent system. The desired band was scrapped and then eluted with distilled ethyl acetate to give EVB-3.

2.2.8 Properties of the isolated compounds.

The physical properties of the isolated compounds and their reactions to vanillin-H_2SO_4 are summarized in the table 2.5.

Table 2.5: Properties of the isolated compounds from *n*-hexane soluble fraction.

Isolated Compounds	Physical form	Color	solubility						Color with Vanillin-H_2SO_4
			Hexane	EtOAc	$CHCl_3$	MeOH	Acetone	DMSO	
EVB-1	Needle shaped crystal	White	-	-	+	+	+	+	Purple
EVB-2	Amorphous Powder	White	-	+	+	+	+	+	Violet
EVB-3	Amorphous Powder	Pale yellow	Ps.	+	+	+	+	-	Yellow

+ indicates completely soluble; Ps. indicates partially soluble; - indicates not soluble

2.2.9 Instrumentation.

Melting points were recorded on an electrothermal melting point apparatus and paraffin oil bath. The ^1H NMR spectra were recorded using a Bruker AMX-400 (400 MHz) instruments. For NMR studies deuterated chloroform was used as solvent and the NMR instrument was locked at the solvent peak and the δ values for ^1H were referenced relative to the residual undeuterated solvent signal.

Chapter 3

Result and discussion- Chemical

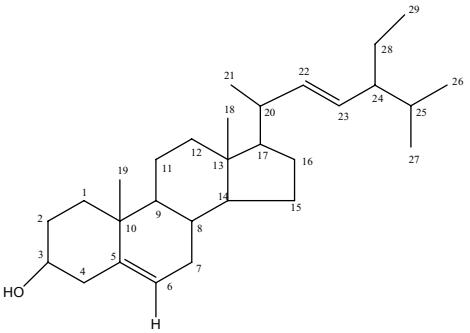
Repeated chromatographic separation and purification of the Hexane soluble fraction of a methanolic extract of the bark of *Erythrina variegata* afforded one Stigmasterol, Triterpene, Isoflavones. The structures of the isolated compounds were determined by extensive spectroscopic studies.

3.1 Characterization of EVB -1 as Stigmasterol (55):

Compound EVB-1 was obtained as needle shaped crystals. It was evident as a purple spot on TLC (Silica gel PF_{254}) when the developed plate was sprayed with vanillin-sulfuric acid followed by heating at 110°C for 5-10 minutes. The R_f value of the compound was 0.33 in toluene-ethyl acetate (95: 5) on Silica gel PF_{254} plate. It was found to be soluble in petroleum ether, methanol and chloroform. Its melting point was 160-164°C which is identical to that observed for stigmasterol (92) (Khan, R.I., 1991; Mandal, M. R., 1991).

The ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of EVB-1 (Table 3.1, Figure 3.1, 3.2, 3.3 & 3.4) revealed a one proton multiplet at δ 3.51, the position and multiplicity of which was indicative of H-3 of the steroidal nucleus. The typical signal for the olefinic H-6 of the steroidal skeleton was evident from a multiplet at δ 5.34 integrating one proton. The olefinic protons (H-22 and H-23) appeared as characteristic downfield signals at δ 5.16 and δ 5.03 respectively in the ^{1}H NMR spectrum. Each of the signal was observed as double-doublets (J = 15.0 Hz, 6.5 Hz) which indicated couplings with the neighbouring olefinic and methine protons. The spectrum further revealed signals at δ 0.67 and δ 1.00 (3H each) assignable to two tertiary methyl groups at C-13 and C-10, respectively.

The ^{1}H NMR spectrum showed two doublets centered at δ 0.83 (*J* = 6.0 Hz) and 0.85 (*J* = 6.0 Hz) which could be attributed to the methyl groups at C-25. The doublet at δ 0.91 (*J* = 6.4 Hz) was demonstrative of a methyl group at C-20. On the other hand, the triplet (*J* = 6.5 Hz) of three-proton intensity at δ 0.83 could be assigned to the primary methyl group attached to C-28. The above spectral features (Table 3.1) are in close agreement to those observed for stigmasterol (Khan, R.I., 1991). On this basis, the identity of EVB-1 was confirmed as stigmasterol.



EVB-1 **(55)**

Table 3.1 : Spectral data of EVB-1 and Stigmasterol (55) (Khan, R.I., 1991; Mandal, M. R., 1991) in $CDCl_3$.

Protons	EVB-1 (55) (δ_H in ppm in $CDCl_3$)	Stigmasterol (δ_H in ppm in $CDCl_3$)
H-3	3.51 m	3.52
H-6	5.3 m	5.32
Me-13	0.67	0.65
Me-10	1.00	1.00
Me-20	0.92 d (J = 6.4 Hz)	0.90
H-22	5.16 dd (J = 15.0, 6.5 Hz)	5.15
H-23	5.03 dd (J = 15.0, 9.0 Hz)	5.03
H-26	0.83*	0.83
H-27	0.84*	0.83
Me-28	0.81 t (J = 7.4 Hz)	0.81

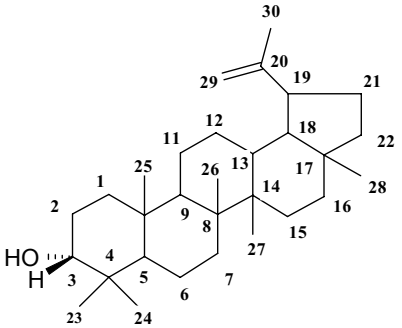
* Assignments are interchangeable

3.2 Characterization of EVB-2 as Epilupeol (56)

Compound EVB-2 was obtained as white crystal having melting point at 212-215°C. The compound was found as a black spot on TLC plate at 254 nm and appeared as a violet spot after treatment with vanillin-H_2SO_4 reagent followed by heating at 110°C for 5-10 minutes.

The ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of (Table 3.2 and Figure 3.5, 3.6, 3.7, 3.8 & 3.9) showed one triplet of one proton intensity at δ 3.37 typical for H-3. The spectrum displayed two singlet at δ 4.67 and δ 4.55 (1H each) assignable to protons at C-29. Doublet of double doublet at δ 2.28 assignable to proton at C-19. The spectrum displayed seven singlets at δ 0.95, 0.78, 0.84, 1.02, 0.93, 0.82 and 1.67 (3H each) assignable to protons of methyl groups at C-4 (H_3-23,

H_3-24), C-10 (H_3-25), C-8 (H_3-26), C-14 (H_3-27), C-17 (H_3-28) and C-20 (H_3-30) respectively. By comparing the ^{1}H NMR data of EVB-3 with that of previously published data (Aratanechemuge *et al.*, 2004), it was confirmed as epilupeol (Figure 3.2).



EVB-2 **(56)**

Table: 3.2. Comparison between ^{1}H NMR spectral data of EVB-2 and Lupeol (400 MHz, $CDCl_3$)

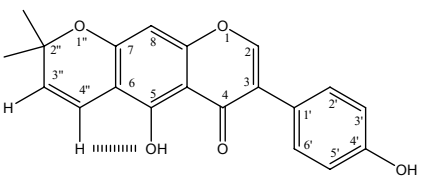
Protons	EVB-2 (δ_H-value, ppm)	Lupeol (δ_H-value, ppm)
3	3.37, 1H, t	3.20, 1H, dd
29	4.55, 1H, brs; 4.67,1H, brs	4.54, 1H, brs; 4.68,1H, brs
19	2.28, 1H, ddd	2.38, 1H, ddd
23	0.95, 3H, s	0.97, 3H, s
24	0.78, 3H, s	0.76, 3H, s
25	0.84, 3H, s	0.83, 3H, s
26	1.02, 3H, s	1.03, 3H, s
27	0.93, 3H, s	0.94, 3H, s
28	0.82 3H, s	0.79 3H, s
30	1.67, 3H, s	1.68, 3H, s

3.3 Characterization of EVB -3 as Alpinum isoflavone (57):

Compound EVB-3 was obtained as needle shaped crystals. It melted at 213 °C, which was identical to that reported for alpinum isoflavone (Olivares *et al.,* 1982). It was evident as a yellow spot on TLC (silica gel PF_{254}) when the developed plate was sprayed with vanillin-sulphuric acid followed by heating at 110 °C for 5-10 minutes. The R_f value of the compound was 0.533 in toluene-ethyl acetate (88:12) over silica gel PF_{254} plate. It was found to be soluble in ethyl acetate, chloroform, acetone and methanol.

The ^{1}H NMR spectrum of EVB-3, (400 MHz, $CDCl_3$, Fig. 3.10, 3.11 & 3.12, Table 3.3) revealed well resolved signals typical of an isoflavone nucleus having a pyran ring. Thus the ^{1}H NMR spectrum showed a pair of doublets (J = 10.6 Hz) centered at δ 5.53 and 6.60 and a sharp singlet of six proton intensity at δ 1.48. These were assigned to a 2,2-dimethylchromene ring system. The characteristic C-2 proton of the isoflavone skeleton was evident as a singlet at δ 7.83 (1H). The ^{1}H NMR spectrum also displayed a pair of doublets (J=8.5 Hz), each integrating for two protons, at δ 6.90 and 7.27, which were assigned to the H-3' & H-5' and H-2' & H-6' of the *para*-disubstituted aromatic nucleus.

The relatively upfield resonance (δ 6.90) of H-3' and H-5' suggested the presence of an oxygenated substituent at C-4'. This was substantiated by the presence of a broad singlet at δ 5.63 (1H), due to a hydroxyl group proton. The remaining two signals at δ 13.14 and 6.34 (each 1H) could be attributed to the chelated hydroxyl group proton at C-5 and H-18, respectively.



EVB-3 **(57)**

Table 3.3: ^{1}H NMR spectral data of EVB-3 and Alpinum isoflavone (57) (Olivares *et al.,* 1982)

Protons	EVB- 3 (57) (δ_H in ppm in $CDCl_3$)	Alpinum isoflavone (δ_H in ppm in $CDCl_3$)
H-2	7.83	7.72
H-5	13.14	13.20
H-8	6.34 s	6.23 s
H-2'	7.27 d (*J*= 8.5 Hz)	7.25 d (*J*= 8.5 Hz)
H-3'	6.90 d (*J*= 8.5 Hz)	6.80 d (*J*= 8.5 Hz)
H-4'	5.63	5.62
H-2''	1.48 s	1.40 s
H-3''	5.62 d (*J*= 10.0 Hz)	5.53d (*J*= 10.0 Hz)
H-4 ''	6.72 d (*J*= 10.0 Hz)	6.60 d (*J*= 10.0 Hz)

On the basis of the above spectral data and by comparison of these values with those reported for alpinum isoflavone, (Olivares *et al.,* 1982) the identity of EVB-3 was confirmed as alpinum isoflavone **(57)**. Although it has previously been reported from many plants (Chapman and Hall, 2001) this is the first report of its isolation from *Erythrina variegata.*

Chapter 4
Microbiological Investigation

4.1. Introduction

Herbal medicines in developing countries are commonly used for the traditional treatment of health problems (Martinez *et al.,* 1996). It is estimated, in developing countries, 80% of the population rely on traditional medicine for their primary health care (Esther and Staden, 2003). Owing to hot temperature and high humidity, the infections due to wounds are common in Bangladesh. For a developing country like Bangladesh, the therapy with synthetic antibiotic is not always possible due to their high cost. Additionally, the rapid development of drug resistant microbes has lead to the search of new antimicrobial agents especially from plant extracts to discover new chemical structures. The antimicrobial compounds from plants may inhibit bacterial growth by different mechanisms than those presently used antimicrobials and may have a significant clinical value in treatment of resistant microbial strains. In recent times, traditional medicine has served as an alternative form of health care and to overcome microbial resistance has led the researchers to investigate the antimicrobial activity of medicinal plants (Austin *et al.,* 1999).

4.1.1. Antimicrobial screening

The antimicrobial potency of the plant can be visualized by antimicrobial screening which measures the ability of a test sample to inhibit the *in vitro* microbial growth by any of the following three methods: a) Disc diffusion method b) Serial dilution method and c) Bioautographic method.

In 1966, Bauer *et al*. published a detailed description of a standardized single-disk method for performing the anti-microbial susceptibility test. This procedure has been widely accepted as the preferred reference method for bacterial susceptibility screening.

4.1.2. Principle of Disc Diffusion Method

In this classical method, antibiotics diffuse from a confined source through the nutrient agar gel and create a concentration gradient. Dried and sterilized filter paper discs (6 mm diameter) containing the test samples of known amounts are placed on nutrient agar medium uniformly seeded with the test microorganisms. Standard antibiotic (kanamycin) discs and blank discs are used as positive and negative control. These plates are kept at low temperature (4°C) for 24 hours to allow maximum diffusion of the test materials to the surrounding media (Barry, 1976). The plates are then inverted and incubated at 37°C for 24 hours for optimum growth of the organisms. The test materials having antimicrobial property inhibit microbial growth in the media surrounding the discs and thereby yield a clear, distinct area defined as **zone of inhibition**. The antimicrobial activity of the test agent is then determined by measuring the diameter of zone of inhibition expressed in millimetre (Bary, 1976; Bauer *et al*, 1966).

4.2. Experimental

4.2.1. Apparatus and Reagents

Filter paper discs	Petridishes	Inoculating loop
Sterile cotton	Sterile forceps	Spirit burner
Micropipette	Screw cap test tubes	Nose mask and Hand gloves
Laminar air flow hood	Autoclave	Incubator
Refrigerator	Nutrient Agar Medium	Ethanol
Chloroform		

4.2.2. Test Organisms

The microbial strains used for the experiment were collected as pure cultures from the Institute of Nutrition and Food Science (INFS), University of Dhaka. Both gram positive, gram-negative bacteria and fungi were taken for the test listed in the Table 4.1.

Table 4.1: List of Test microorganisms

Gram positive Bacteria	Gram negative bacteria	Fungi
Bacillus cereus	*Escherichia coli*	*Candida albicans*
Bacillus megaterium	*Pseudomonas aeruginosa*	*Aspergillus niger*
Bacillus subtilis	*Salmonella paratyphi*	*Sacharomyces cerevaceae*
Staphylococcus aureus	*Salmonella typhi*	
Sarcina lutea	*Shigella boydii*	
	Shigella dysenteriae	
	Vibrio mimicus	
	Vibrio parahemolyticus	

4.2.3. Test materials

Table 4.2: List of Test materials

Plant	Test Samples	Sample code
Erythrina variegata	1. Methanolic extract of the whole plant	MeOH
	2. Hexane soluble fraction of methanolic extract	Hex
	3. CCl_4 soluble fraction of methanolic extract	CT
	4. $CHCl_3$ soluble fraction of methanolic extract	C
	5. Aqueous soluble fraction of methanolic extract	AQ

4.2.4. Composition of Culture medium

Nutrient agar medium (DIFCO) was used in the present study for testing the sensitivity of the organisms to the test materials and to prepare fresh cultures

Table 4.3 : Composition of Nutrient agar medium

Ingredients	Amounts
Bacto peptone	0.5 gm
Sodium chloride	0.5 gm
Bacto yeast extract	1.0 gm
Bacto agar	2.0 gm
Distilled water q.s.	100 ml
pH	7.2-7.6 at 25°C

4.2.5. Preparation of medium

Specified amount of nutrient agar was taken in a conical flask and distilled water was added to it to make the required volume. The contents were heated in a water bath to make a clear solution. The pH (at 25 °C) was adjusted at 7.2-7.6 using NaOH or HCl. 10 ml and 5 ml of the medium was then transferred in screw cap test tubes to prepare plates and slants respectively. The test tubes were then capped and sterilized by autoclaving at 15-lbs. pressure at 121°C for 15 minutes. The slants were used for making fresh culture of microorganisms that were in turn used for sensitivity study.

4.2.6. Sterilization procedures

To avoid any type of contamination and cross contamination by the test organisms the antimicrobial screening was done in Laminar Hood and all types of precautions were strictly maintained. UV light was switched on an hour before working in the Laminar Hood. Petridishes and other glassware were sterilized by autoclaving at a temperature of 121°C and a pressure of 15-lbs./sq.inch for 15 minutes. Micropipette tips, cotton, forceps, blank discs were also sterilized by autoclave.

4.2.7. Preparation of subculture

In an aseptic condition under laminar air cabinet, the test organisms were transferred from the pure cultures to the agar slants with the help of a transfer loop to have fresh pure cultures. The inoculated strains were then incubated for 24 hours at 37°C for their optimum growth. These fresh cultures were used for the sensitivity test.

4.2.8. Preparation of the test plates

The test organisms were transferred from the subculture to the test tubes containing about 10 ml of melted and sterilized agar medium with the help of a sterilized transfer loop in an aseptic area. The test tubes were shaken by rotation to get a uniform suspension of the organisms. The microbial suspension was immediately transferred to the sterilized petridishes. The petridishes were rotated several times clockwise and anticlockwise to assure homogenous distribution of the test organisms in the media.

4.2.9. Preparation of discs

Measured amount of each test sample (specified in table 4.4) was dissolved in specific volume of solvent (methanol) to obtain the desired concentrations in an aseptic condition. Sterilized metrical (BBL, Cocksville, USA) filter paper discs were taken in a blank petridish under the laminar hood. Then discs were soaked with solutions of test samples and dried.

Table 4.4: Preparation of sample discs

Plant	Sample code	Sample	Dose (μg/disc)	Amount for 16 disc (mg)
Erythrina variegata	MeOH	Methanolic extract of the whole plant	400	6.4
	Hex	Hexane soluble fraction of methanolic extract	400	6.4
	CT	CCl_4 soluble fraction of methanolic extract	400	6.4
	C	$CHCl_3$ soluble fraction of methanolic extract	400	6.4
	AQ	Aqueous soluble fraction of methanolic extract	400	6.4

Standard Kanamycin (30 μg/disc) discs were used as positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antimicrobial agent with that of the test sample. Blank discs were used as negative controls which ensure that the residual solvents (left over the discs even after air-drying) and the filter paper were not active themselves.

4.2.10. Diffusion and Incubation

The sample, standard antibiotic and control discs were placed gently on the previously marked zones in the agar plates pre-inoculated with test microorganisms. The plates were then kept in a refrigerator at 4°C for about 24 hours to allow sufficient diffusion of the materials from the discs to the surrounding agar medium. The plates were then inverted and kept in an incubator at 37°C for 24 hours.

4.2.11. Determination of the Zone of Inhibition

After incubation, the antimicrobial activity of the test materials was determined by measuring the diameter of the zones of inhibition in millimetre using vernier calliper.

4.3 Results and Discussion of the test samples of *Erythrina variegata.*

The antimicrobial activities of extracts from *Erythrina variegata* were examined in the present study. The results were given in table 4.5. The zone of inhibition produced by methanolic crude extract (MeOH) of the bark, carbon tetrachloride, chloroform and aqueous soluble fraction of methanolic extract ranged from 10.9-15.2 mm , 9.2-12.9 mm , 11.2-16.4 mm and 12.7-16.6 mm respectively.

However, at a concentration of 400µg/disc the result of *n*-hexane soluble fraction of methanolic extract (Hex) showed poor activity against most of the test microorganisms. The growth of *E. coil* and *V. mimicus* (zone diameter 11.3 and 13.6 mm respectively) was moderately inhibited.

The methanolic crude extract (MeOH) of the bark of *Erythrina variegata* showed moderate to strong activity against most of the test organisms. The growth of *B. subtilis* (14.2 mm), *E.coli* (15.2 mm) and *A. niger* (14.1 mm) was strongly inhibited (Table 4.5).

The carbon tetrachloride partitionate of the methanolic extract at a concentration of 400 µg/ disc showed moderate activity against *B.cereus* (11.3 mm), *B. subtilis* (12.2 mm), *E.coli* (12.3), *P. aeruginosa* (11.1 mm), *S. dysenteriae* (12.6 mm) and *V. mimicus* (12.9 mm). However, the growth of fungi was weakly inhibited showing the zone of inhibition 10.2-10.8 mm.

The chloroform soluble fraction of methanolic extract showed strong activity against *B. cereus* (14.4 mm), *B. subtilis* (16.4 mm), *E.coli* (14.3 mm), *P. aeruginosa* (15.1 mm) and a fungi *A. niger* (15.2 mm).

The aqueous soluble fraction showed strong activity against gram positive bacteria namely *B.cereus* (15.4mm), *B. subtilis* (16.6 mm) and *S. Lutea* (14.5 mm). In case of gram negative bacteria only *P. aeruginosa* (16.2 mm) was strongly inhibited. The fraction also showed strong activity against *A. niger* (15.3 mm) and *S. cerevacae* (14.4 mm) and moderate activity against *C. albicans* (13.4 mm).

Out of all the samples, aqueous soluble fraction of the methanolic extract showed best result in terms of both zone size and spectrum of activity. Reversed phase chromatographic technique

can be used to separate and purify the bioactive constituents from the polar aqueous soluble fraction of methanolic extract.

Table 4.5: Antimicrobial activity of test samples of *E. variegata*

Test microorganisms	Diameter of zone of inhibition (mm)					
	MeOH	**Hex**	**CT**	**C**	**AQ**	**Std.**
Gram positive bact.						
Bacillus cereus	13.1	7.3	11.3	14.4	15.4	33.9
Bacillus megaterium	10.9	6.7	9.2	11.5	13.2	38.5
Bacillus subtilis	14.2	8.3	12.2	16.4	16.6	35.5
Staphylococcus aureus	12.3	7.2	10.7	13.1	14.6	31.3
Sarcina lutea	12.5	6.6	10.1	13.8	14.5	25.5
Gram negative bact.						
Escherichia coli	15.2	11.3	12.3	14.3	13.8	35.6
Pseudomonas aeruginosa	13.5	7.7	11.1	15.1	16.2	36.2
Salmonella paratyphi	13.6	7.1	10.1	13.3	13.8	26.6
Salmonella typhi	11.2	8.3	10.5	13.1	13.7	20.5
Shigella boydii	12.9	7.6	10.3	12.6	12.7	26.2
Shigella dysenteriae	11.9	8.5	12.6	12.9	13.8	32.5
Vibrio mimicus	13.9	13.6	12.9	12.9	12.7	31.1
Vibrio parahemolyticus	12.2	6.9	10.2	12.6	13.6	31.2
Fungi						
Candida albicans	12.7	7.2	10.3	11.2	13.4	36.9
Aspergillus niger	14.1	6.8	10.8	15.2	15.3	26.2
Sacharomyces cerevacae	11.8	6.5	10.2	13.7	14.4	29.9

MeOH: Methanolic extract of the whole plant

Hex: Hexane soluble fractions of the methanolic extract

CTSF : Carbon tetrachloride soluble fractions of the methanolic extract

CFSF : Chloroform soluble fractions of the methanolic extract

AQF : Aqueous soluble fractions of the methanolic extract

Std : Standard kanamycin disc (30µg)

In conclusion, the obtained results confirmed the presence of anti-microbial properties of *Erythrina. variegata* which supports the traditional use of this plant in various diseases caused by pathogenic microorganisms. Bioactiviy guided isolation can be carried out to separate bioactive metabolites.

CHAPTER 5

BRINE SHRIMP LETHALITY BIOASSAY

Pharmacology is simply toxicology at a lower dose, and toxicology is simply pharmacology at a higher dose. Bioactive compounds are almost always toxic in high doses. The *in vivo* lethality in a simple zoologic organism can be used as a convenient monitor for screening and fractionation in the discovery and monitoring of bioactive natural products. Meyer *et al*., 1982 focused on *Artemia salina* as a test organism and developed a protocol for Brine shrimp lethality bioassay to monitor cytotoxicty of a compound.

5.1 Brine Shrimp Lethality Bioassay

5.1.1. Principle ***(Meyer* et al.*, 1982)***

Brine shrimp eggs are hatched in simulated sea water to get nauplii. Sample solutions are prepared by dissolving the test materials in pre-calculated amount of DMSO. Ten nauplii are taken in vials containing 5 ml of simulated sea water. The samples of different concentrations are added to the premarked vials with a micropipette. The assay is performed using three replicates. Survivors are counted after 24 hours. These data are processed in a simple program for probit analysis to estimate LC_{50} values with 95% confidence intervals for statistically significant comparisons of potencies.

5.1.2. Materials

- *Artemia salina* leach (brine shrimp eggs)
- Sea salt (NaCl)
- Small tank with perforated dividing dam to hatch the shrimp
- Lamp to attract shrimps
- Pipettes (5, 25ml) and Micropipette (5-40µl)
- Glass vials
- Magnifying glass
- Test samples of experimental plants (Table 5.1)

Table 5.1 : Test samples of experimental plants

Plant	Test samples	Measured Amount (mg)
Erythrina variegata	Hexane soluble fraction of methanolic extract	4.00
	CCl_4 soluble fraction of methanolic extract	4.00
	Chloroform soluble fraction of methanolic extract	4.00
	Aqueous soluble fraction of methanolic extract	4.00

5.1.3. Procedure

5.1.3.1. Preparation of seawater

38 gm sea salt (pure NaCl) was weighed, dissolved in one litre of distilled water and filtered off to get clear solution.

5.1.3.2. Hatching of brine shrimps

Artemia salina leach (brine shrimp eggs) collected from pet shops was used as the test organism. Seawater was taken in the small tank and shrimp eggs were added to one side of the tank and then this side was covered. Two days were allowed to hatch the shrimp and to be matured as nauplii. Constant oxygen supply was provided throughout the hatching time. The

hatched shrimps were attracted to the lamp through the perforated dam and with the help of a pasteur pipette 10 living shrimps were added to each of the vials containing 5 ml of seawater.

5.1.3.3 Preparation of test solutions

Measured amount (Table5.1) of each sample was dissolved in 100μl of DMSO. A series of solutions of lower concentrations were prepared by serial dilution with DMSO. From each of these test solutions 50 μl were added to premarked glass vials/test tubes containing 5 ml of seawater and 10 shrimp nauplii. So, the final concentration of samples in the vials/test tubes were 400 μg/ml, 200 μg/ml, 100 μg/ml, 50 μg/ml, 25 μg/ml, 12.5 μg/ml, 6.25 μg/ml, 3.125 μg/ml, 1.5625μg/ml, 0.78125μg/ml for 10 dilutions.

5.1.3.4. Preparation of controls

Vincristine sulphate served as the positive control. 0.2mg of vincristine sulphate was dissolved in DMSO to get an initial concentration of 20 μg/ml from which serial dilutions were made using DMSO to get 10 μg/ml, 5 μg/ml, 2.5μg/ml, 1.25 μg/ml, 0.625 μg/ml, 0.3125 μg/ml, 0.15625 μg/ml, 0.078125 μg/ml, 0.0390 μg/ml. The control groups containing 10 living brine shrimp nauplii in 5 ml simulated sea water received the positive control solutions.

As for negative control, 30 μl of DMSO was added to each of three premarked glass vials containing 5 ml of simulated seawater and 10 shrimp nauplii. The test was considered invalid if the negative control showed a rapid mortality rate and therefore conducted again.

5.1.3.5. Counting of Nauplii and Analysis of Data

After 24 hours, the vials were inspected using a magnifying glass and the number of survivors were counted. The percent (%) mortality was calculated for each dilution. The concentration-mortality data were analysed statistically by using Microsoft excel. The effectiveness or the concentration-mortality relationship of plant product is usually expressed as a median lethal

concentration (LC_{50}) value. This represents the concentration of the chemical that produces death in half of the test subjects after a certain exposure period.

5.2 Results and Discussion of the test samples of *Erythrina variegata*

Following the procedure of Meyer (Meyer *et al.*, 1982) the lethality of *n*-hexane (Hex), CCl_4 (CT), $CHCl_3$ (C) and aqueous soluble fraction (AQ) of the methanolic extract to brine shrimp were investigated.

Table 5.2 gives the results of the brine shrimp lethality after 24 hours exposure to all the samples and the positive control, vincristine sulphate. The positive control, compared with the negative control (sea water) was lethal, giving significant mortality to the shrimp.
The lethal concentration LC_{50} of the test samples after 24 hr. was obtained by a plot of percentage of the shrimps killed against the logarithm of the sample concentration (toxicant concentration).and the best-fit line was obtained from the curve data by means of regression analysis.

Table 5.2: Results of the test samples of *Erythrina variegata*

Sample	LC_{50} (µg/ ml)	Regression equation	R^2
Vincristine sulphate (Std.)	0.3229	y = 29.797x + 64.628	0.927
Hex	4.67	y = 25.971x + 32.603	0.9605
CT	36.68	y = 18.925x + 20.393	0.9428
C	7.733	y = 33.421x + 20.31	0.9532
AQ	14.289	y = 32.414x + 12.566	0.9123

The degree of lethality was directly proportional to the concentration of the extract ranging from significant with the lowest concentration (0.78125µg/ml) to highly significant with the highest concentration (400µg/ml). Maximum mortalities took place at a concentration of

400µg/ml, whereas least mortalities were at 0.78125 µg/ml concentration. In other words, mortality increased gradually with the increase in concentration of the test samples.

LC_{50} obtained from the best-fit line slope were 4.67, 36.68, 7.733 and 14.289 µg/ml (Fig. 6.2, 6.3, 6.4, 6.5 & 6.6) for Hex, CT, C and AQ respectively. In comparison with positive control (vincristine sulphate), the cytotoxicity exhibited by hexane and chloroform soluble fraction of methanolic extract were promising.

Table 5.3 : Effect of Hexane (Hex) and carbontetra chloride (CT) soluble fractions of methanolic extract on brine shrimp nauplii

Conc. (µg/ml)	Log C	% Mortality		LC_{50} (µg/ml)		Vincristine Sulphate			
		Hex	**CT**	**Hex**	**CT**	Conc. (µg/ml)	Log C	% Mortality	LC_{50} (µg/ml)
400	2.602	100	70	0.564	36.68	40	1.602	100	0.3229
200	2.301	100	60			20	1.301	100	
100	2	80	60			10	1.000	100	
50	1.699	80	50			5	0.698	90	
25	1.398	60	50			2.5	0.397	80	
12.5	1.097	60	40			1.25	0.096	80	
6.25	0.796	50	40			0.625	-0.204	60	
3.125	0.495	50	30			0.3125	-0.505	50	
1.563	0.194	40	30			0.15625	-0.806	40	
0.781	-0.107	30	10			0.078125	-1.107	20	

Table 5.4 : Effect of Chloroform (C) and aqueous (AQ) soluble fractions on brine shrimp nauplii

Conc. (μg/ml)	Log C	% Mortality		LC_{50} (μg/ml)		Vincristine Sulphate			
		C	**AQ**	**C**	**AQ**	Conc. (μg/ml)	Log C	% Mortality	LC_{50} (μg/ml)
400	2.602	100	100	7.733	14.289	40	1.602	100	0.3229
200	2.301	100	100			20	1.301	100	
100	2	90	80			10	1.000	100	
50	1.699	80	50			5	0.698	90	
25	1.398	80	50			2.5	0.397	80	
12.5	1.097	50	40			1.25	0.096	80	
6.25	0.796	40	40			0.625	-0.204	60	
3.125	0.495	30	40			0.3125	-0.505	50	
1.563	0.194	30	20			0.15625	-0.806	40	
0.781	-0.107	20	10			0.078125	-1.107	20	

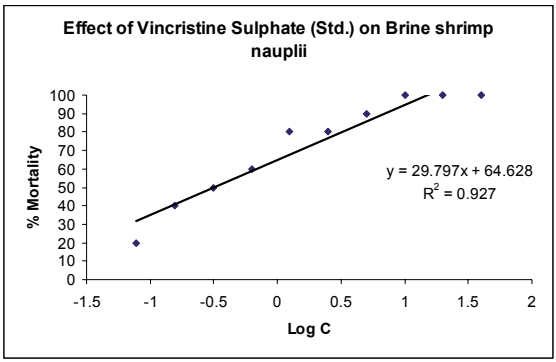


Figure 5.1: Effect of vincristine sulphate on Brine shrimp

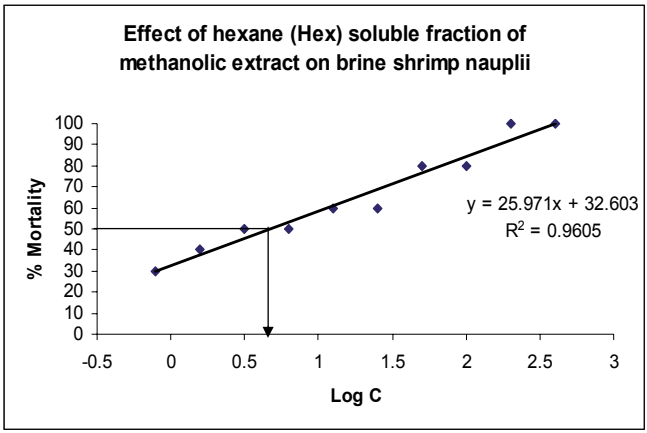


Figure 5.2: Effect of Hexane (Hex) soluble fraction of methanolic extract on brine shrimp nauplii

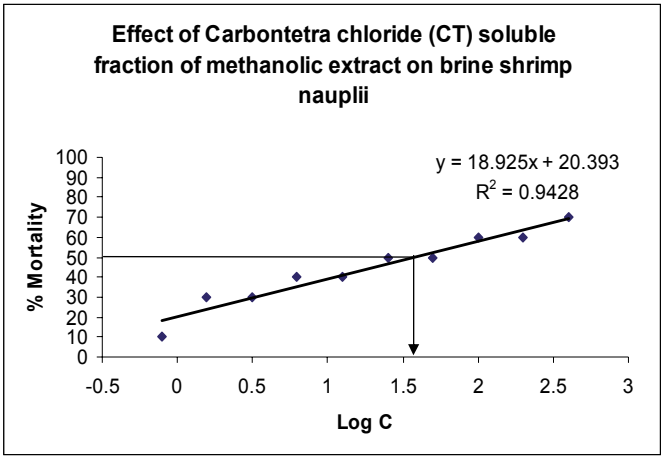


Figure 5.3: Effect of carbon tetra chloride (CT) soluble fraction of methanolic extract on brine shrimp nauplii

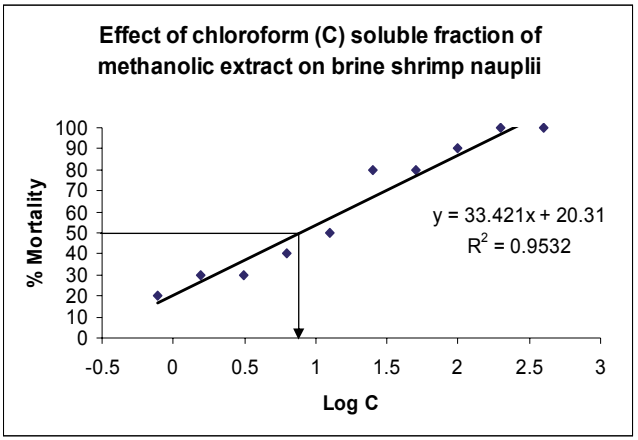


Figure 5.4: Effect of chloroform (C) soluble fraction of methanolic extract on brine shrimp nauplii

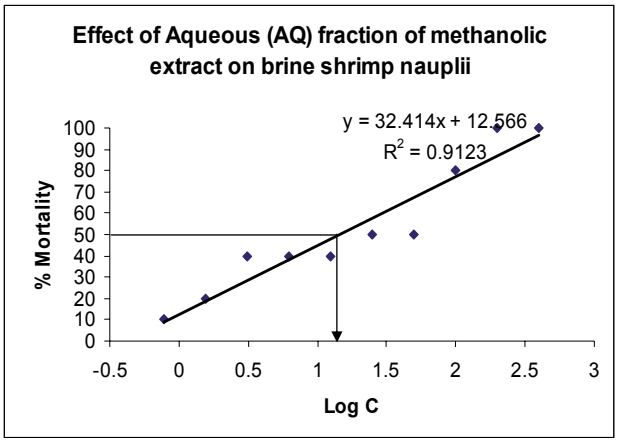


Figure 5.5: Effect of Aqueous (AQ) fraction of methanolic extract on brine shrimp nauplii

Conclusion

Successive chromatographic separation and purification of the Hexane soluble fraction of a methanolic extract of *Erythrina variegata* yielded a total three compounds. The structures of the compounds were elucidated as Stigmasterol **(55)**, Epilupeol **(56)**, Alpinum isoflavone **(57)**.

The crude methanolic extract along with *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble fractions of *Erythrina variegata* showed significant antimicrobial and cytotoxic activities, which can be further screened against various diseases in order to find out their unexplored efficacy. The significant results of cytotoxic screening support further studying for the development of anticancer drugs.

Therefore, plant materials, especially the traditionally used medicinal plants can be potential sources of chemically interesting and biologically important drug candidate.

Bibliography

Agarawal, P.K. and Jain D.C. (1992). ^{13}C NMR spectroscopy of oleananetriterpenoids. *Progress in NMR Spectroscopy* 24, 1-90.

Alkofahi, A., Smith, D. L. McLaughlin, J. L (1988) *Experientia,* 44, 83.

Antoniolli AR., Marchioro M, Blank M. D., Mourao R. H. (2005). Anti-nociceptive activity of the aqueous extract of Erythrina velutina leaves. *Fitoterapia* 76, 143-280

Austin *et al., Proceedings of the National Academy of Sciences of the United States of America*, 1999, 96, 1152–1156

Baker *et al*, *Biodiversity: New leads for pharmaceutical and agrochemical industries,* The Royal Society of Chemistry, Cambridge, UK, 2000, 66-72

Bangladesh National Herbarium, Data base, 2005.

Barham, D. and Trinder, P., *Analyst*, 1972, 97, 142

Barry, A. L., *Principle and practice of Microbiology*, 1976; 3rd edition; lea & Fabger, Philadelphia

Bouer, A.W., Kirby W.M.M., Sherries J.C., Truck M. (1966). Antibiotic susceptibility testing by standard single disc diffusion method. *American Journal of Clinical Pathology* 45, 426-493.

Bremer, K., 1994. *Asteraceae: Cladistics and Classi.cation*. Timber Press, Portland.

Brown, D.A. *et al.*, *Phytochemistry*, 1978, 17, 1995 (isol, pmr)

Chandrasekharan, S. *et al.*, *J. Indian Chem. Soc.*, 1968, 45, 208

Chatterjee, A. *et al.*, *Tetrahedron*, 1970, 26, 1859

Chatterjee, S.; Das, S.N.; *Indian Drugs* 1999, 36(2), 140-141.

Chevallier. A., *The Encyclopedia of Medicinal Plants* Dorling Kindersley. London 1996 ISBN 9-780751-303148

Chopra. R. N., Nayar. S. L. and Chopra. I. C. *Glossary of Indian Medicinal Plants (Including the Supplement).* Council of Scientific and Industrial Research, New Delhi. 1986

Cowan, M.M. (1999). Plant products as antimicrobial agents. *Clinical Microbiology Review* 12, 546-582.

Dulcie A. Mulholland, Bret Parel and Philip H. Coombes, *Current Organic Chemistry,* 4(10), 2000.

Emerenciano *et al*., *Biochemical Systematics and Ecology*, 2001, 29, 947

Fararh *et al*., Research in veterinary science, 2002, 73, 279

Farzami *et al*., *Journal of Ethnopharmacology*, 2003, 89, 47

Fernando. *et al*., *Phytochemistry*, 2005, 66, 345

Geissman, T. A., 1963. *Flavonoid compounds, tannins, lignins and related compounds*, p. 265. In M. Florkin and E. H. Stotz (ed.), Pyrrole pigments, isoprenoid compounds and phenolic plant constituents, vol. 9. Elsevier, New York.

Gennaro, A. *et al*., *Phytochemistry*, 1972, 11, 1515

Gole *et al*., *International Journal of Pharmacognosy*, 1997, 35(5).

Govindachari, T.R. *et al*., *J.C.S.*,1956, 629

Govindachari, T.R. *et al*., *J.C.S.*,1957, 545-548

Gray *et al*., *Journal of nutrition*, 2000, 130, 15

Gray, A.M., Flatt, P.R., *Journal of endocrinology*, 1998, 157, 259

Gray, A.M., Flatt, P.R., *Journal of endocrinology*, 1999, 160, 409

Gunatilaka *et al*., *Phytochemistry*, 1983, 22, 991

Gupta, A.K. *et al*., *Planta Med.*, 1980, 38, 174

Hennessy, E.F. (1972). South African *Erythrina's*. Natal Branch of the Wildlife Protection and Conservation of South Africa, Natal.

Hutchings, A., Scott, A.H., Lewis, G., Cunningham, A. B. (1996). Zulu Medicinal Plants, An Inventory, University of Natal Press, Scottsville.

Ikan, 1991: *Natural products laboratory guide*, Academic press Inc., Harcourt Brace, Javonovich publishers, New York, USA.

Internet 1: http://www.floridata.com/ref/E/eryt_var.cfm

IUBMB (International Union Of Biochemistry And Molecular Biology), 2005;
http://www.chem.qmul.ac.uk/iubmb/

Iwanaga S, Nagata R, miyamoto A, Kouzuma Y, Yamasaki N, Kimura M. (1999). Conformation of the primary binding loop folded through an intramoleucular interaction contributes to the strong chymotrypsin inhibitory activity of the chymotrypsin inhibitor from *Erythrina variegata* seeds. *J Biochem (Tokyo)*. 126, 162-7.

Iwanaga S, Yamasaki N, Kimura M (1998). Chymotrypsin inhibitor from *Erythrina variegata* seeds: involvement of amino acid residues within the primary binding loop in petent inhibitory activity toward chymotrypsin. *J Biochem (Tokyo)*. 124, 663-9.

Iwanaga S, Yamasaki N, Kimura M, Kouzuma Y. (2005). Contribution of conserved Asn residues to the inhibitory activities of Kunitz- type protease inhibitors from plants. Biosci Biotechnol Biochem. 69, 220-3.

Khan, R.I.; *Natural Product : A Laboratory Guide*, 2nd Ed. Academic Press, N.Y., USA, 1991.

Khin *et al.*, *Toxicol Appl Pharmacol*. 1978,45(3),723-8

Kimura M, Kouzuma Y, Abe K, Yamasaki N. (1994). On a Bowman- Birk family proteinase inhibitor from *Erythrina variegata* seeds. *J Biochem (Tokyo)*. 115, 369-72.

King, T.J. *et al.*, *Phytochemistry*, 1983, 22, 307 (cryst struct)

Kirtikar, k.R. and Basu, B.D. ; *Indian Medicinal Plants*, 1980, Vol. 1; 2nd ed. p533-565. Published by B. singh and M, P. Singh, India.

Kobayashi M, Mahmud T. Yoshioka N, Shibuya H, Kitagawa I. (1997). Indonesian medicinal plants. XXI. Inhibitors of Na+/ H+ exchanger from the bark of Erythrina variegata and the roots of Maclura cochinchinensis. *Chem Pharm Bull* (Tokyo) 45,1615-9.

Kobori *et al.*, *Cell Death Differ.* 2004 ,11(1),123-30.

Kundu, A.B. *et al.*, *Phytochemistry*, 1985, 24, 2123

Kuo, Y.H. and Chu, P.H., *Journal of the Chinese chemical society*, 2002, 49, 269

Kupchan and modified by Wagenen *et al.* (1993)

Leal *et al.*, *J Ethnopharmacol.* 2000 ,70(2):151-9.

Lester, A. Mitscher *et al., Lloydia,* 1972, 35(2), 157-166.

Liu *et al*., *Zhong Yao Cai*. 2000,23(7):407-9.

McClatchey, W.(1996).The ethnopharmacopoeia of Rotuma. *Journal of Ethnopharmacology* 50, 147-156.

Macedo *et al*., *Mem Inst Oswaldo Cruz.* 1997,92(4):565-70.

MacLachlan, L.K. *et al*., *Phytochemistry*, 1982, 21, 2426 (struct).

Maghrani *et al*., *Journal of Ethnopharmacology*, 2004, 91, 309.

Mansour *et al*., *Toxicology*, 2002, 170, 221.

Mason, T. L., and B. P. Wasserman. *Phytochemistry,* 1987, 26, 2197.

Markham, K.R., Mabry, T.J. and Swift T.W. (1968). New isoflavones from the genus *Baptisia* (Leguminosae). *Phytochemistry* 7, 803-808.

Martinez, M.J., Bentancourt J., Alanso-Gonzalea N., Jauregui A. (1996). Screening of some Cuban medicinal plants for antimicrobial activity. *Journal of Ethnopharmacology* 52, 171-174.

Matu, E.N. and Staden, J.V. (2003). Antibacterial and anti-inflammatory activities of some plants used for medicinal purposes in Kenya. *Journal of Ethnopharmacology* 87, 35-41.

Matu, N.E. and Staden J.V. 2003. Antibacterial and anti inflammatory activities of some plants used for medicinal purposes in Kenya. *Journal of Ethnopharmacology* 87, 35-41.

Meyer, B. N., N. R. Ferrigni, J. E. Putnam, J. B. Jacobsen, D. E., Nicholsand J. L., Mclaughlin, (1982). Brine shrimp; a convenient general bioassay for active plant constituents. *Planta medica,* 45, 31-34.

Misra, L.N. *et al*., *Tetrahedron*, 1985, 41, 5353

Morales *et al*., *Journal of the Chilean Chemical Society*, 2003, 48, 2

Mors *et al., Toxicon.* 1989, 27(9),1003-9

Munguti, K., 1997. Indigenous knowledge in the management of malaria and visceral leishmaniasiss among the Tugen of Kenya. *Indigenous Knowledge and Development Monitory* 5, 10-12.

Nkengfack, A. E., Vouffo, T.W., Vardamides, Fomum, Z.T., Bergendorff, O., Olov Sterner, O.(1994). Sigmoidins J and K, Two New Prenylated Isoflavonoids from *Erythrina sigmoidea. J. Nat. Prod.* 57, 1172-1177.

Nwosu M. O. (1999). Herbs for mental disorders. *Fitoterapa.*70, 58-53.

Ohba H, Nishikawa M, Kimura M, Yamasaki N, Moriwaki S, Itoh K. (1998). Cytotoxicity induced by Erythrina variegata serine proteinase inhibitors in tumor heatopoietic stem cell lines. *Biosci Biotechnol Biochem.* 62,1166-70.

Olivares, E.M., Lwande, W. Monache F.D. and Bettolo G.B.M. (1982). A pyrano-isoflavone from seeds of *Milletia thonningii. Phytochemistry* 21, 1763-1765.

Payne, L. (1991) The alkaloids of *Erythrina,* clonal evaluation and metabolic fate. PhD thesis, Department of Chemistry, Louisiana State University.USA.

Polonsky, J. *et al.*, *J.A.C.S.*, 1978, 100, 7731 (isol, pmr).

Pushparaj *et al.*, *Journal of Ethnopharmacology*, 2000, 72, 69.

Ram, A.J., Raju V.R.R., Bhakshu Md. L., 2003. In vitro antimicrobial activity of certain medicinal plants from Eastern Ghats, India, used for skin diseases. *Journal of Ethnopharmacology* 90, 353-357.

Rashid, M.A. (1992); Ph.D. thesis, University of Strathclyde, U.K.

Roberts, M. (1990). Indigenous Healing Plants. Southern Book, Pretoria.

Sarg *et al.*, *Sci. Pharm.,* 1981, 49, 262

Saris *et al.*, *Clinical chemistry*, 1978, 24, 720

Sato M, Tanaka H, Fujiwara S, Hirata M, Yamaguchi R, Etoh H, Tokuda C. (2003). Antibacterial property of isoflavonoids isolated from Erythrina variegata against cariogenic oral bacteria. *Phytomedicine.* 10, 427-33.

Sato M, Tanaka H, Yamaguchi R, Kato K, Etoh H. (2004). Synergistic effects of mupirocin and an isoflavanone isolated from Erythrina variegata on growth and recovery of methicillin-resistant Staphylococcus aureus. *International Journal of Antimicrob Agents.* 24, 241-6.

Tanaka H, Sato M, Fujiwara S, Hirata M, Etoh H, Takeuchi H. (2002). Antibacterial activity of isoflavonoids isolated from Erythrina variegata against methicillin- resistant Staphylococcus aureus. *Lett Appl Microbiol*. 35, 494-8.

Thakur V.D.,and Mengi S.A., J Ethnopharmacol. 2005, in press.

Thyagarajan *et al.*, *Indian J Med Res.* 1982 Dec;76 Suppl:124-30.

Trease and Evans, *Pharmacognosy,* 1996, 14th Ed, Published by WB Saunders Co. Ltd. London.

Van Rensburg, T.J. F., 1982. Coral Tree, Tree of the Year. Pretoria Directorate of Forestry, Pretoria.

Wagenen et al., *Journal of Organic Chemistry*, 1993, 58, 335.

Wagner *et al*., *Planta Med.* 1986, (5), 370-4.

Yahara *et al*., *Chem. Pharm. Bull*, 1994, 5, 839

Yahara *et al*., *Pytochemistry.*, 1997, 74, 131

Yenesew, A., Midiwo,J.O., Miessner, M., Heydenreich, M., Peter, M.G.(1998). Two prenylated flavanones from stem bark of *Erythrina burttii.* Phytochemistry 48, 1439-1443 .

Yeung, Him-Che. *Handbook of Chinese Herbs and Formulas.* Institute of Chinese Medicine, Los Angeles 1985.

Zhang *et al., Acta Pharm.Sci.,* 2004,25,191.

Zhang *et al*., *Yaoxue Xuebao*, 1996, 31, 196.