# Phytochemical and Biological Investigation of two Bangladeshi Medicinal plants, *Leucas aspera* and *Curcuma zedoria*

A dissertation submitted for the partial fulfillment of the course of Pharmaceutical Research (PHRM 404) of the Department of Pharmacy, East West University for the Degree of Bachelor of Pharmacy

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**Date of Submission:** May 31, 2012

# Dedication

*This Paper Is Dedicated To*

*My Parents*

# Declaration by the research candidate

I, Fahima Hassan (ID # 2008-1-70-059), hereby declare that the dissertation entitled **"Phytochemical and Biological Investigation of two Bangladeshi Medicinal plants, *Leucas aspera* and *Curcuma zedoria*",** submitted by me to the Department of Pharmacy, East West University, in the partial fulfillment of the requirement for the award of the degree of Bachelor of Pharmacy (Honors) is a genuine & authentic record of original research work carried out by me during 2011-2012 under the supervision and guidance of Dr. Chowdhury Faiz Hossain, Dean, Faculty of Sciences and Engineering & Professor, Department of Pharmacy, East West University and it has not formed the basis for the award of any other Degree/Diploma/Fellowship or other similar title to any candidate of any University.

**Date:** 31.05.2012

**Fahima Hassan**

ID# 2008-1-70-059

# Thesis Certificate

This is to certify that the thesis **"Phytochemical and Biological Investigation of two Bangladeshi Medicinal plants, *Leucas aspera* and *Curcuma zedoria*"** is submitted to the Department of Pharmacy, East West University, Mohakhali, Dhaka, in partial fulfillment of the requirements for the degree of Bachelor of Pharmacy (B. Pharm) was carried out by Fahima Hassan (ID # 2008-1-70-059) under our guidance and supervision and that no part of the proposal has been submitted for any other degree. We further certify that all sources of information and laboratory facilities availed of this connection are duly acknowledged.

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**ABSTRACT**

Extraction of *Leucas aspera* leaves (50.00 g) and *Curcuma zedoria* rhizome (1.00 kg) at room temperature by maceration with MeOH yielded 10.20 g (20.40% of dry weight) and 136.15 g (13.60% of dry weight) extracts, respectively. The MeOH extract of *Leucas aspera* was separated by open column chromatography with silica gel using step gradient of Hex-EtOAc-MeOH. The fractions were collected by monitoring with thin layer chromatography (TLC). Analysis of TLC of the MeOH extract and further column fractions did not show any characteristic spot of lignan derivatives. Elution with Hex-EtOAc (9:1) yielded Fr-3′ (0.85 g) and Fr-4 (0.55 g), respectively. On concentration of the solvent of Fr-3′ and Fr-4 yielded two colorless crystals LA-1 (12.00 mg) and LA-2 (7.00 mg), respectively. TLC analysis revealed that these compounds were UV inactive and charring with MeOH and $H_2SO_4$ (9:1) on TLC plate gave characteristic bright red color. This characteristic charring indicated the compounds were steroidal derivatives. Fr-16 (3.00 g), eluted with MeOH showed strong antioxidant activity. But further purification was not carried out due to absence of lignan derivatives as well as the small quantity. The MeOH extract of *Leucas aspera* did not show any significant antimicrobial activity up to a concentration of 1 mg/disc against *Escherichia coli, Staphylococcus aureus, Shigella dysentery, Salmonella typhi* and *Candida albicans*.

The MeOH extract of *Curcuma zedoria* showed significant antimicrobial activity. The zone of inhibitions were 12, 9.5, 8.5, 12 and 8 (mm) against *Escherichia coli, Bacillus subcutis, Shigella boydii, Salmonella paratyphi* and *Saccharomyces cereviceae,* respectively at a dose of 1000 µg/disc. The MeOH extract of *Curcuma zedoria* was subjected to vacuum liquid chromatography (VLC) and five different fractions were collected using five different solvents. The fractions were – Fr-1 (14.37 g, eluting solvent: n hexane), Fr-2 (20.28 g, eluting solvent dichloromethane or DCM), Fr-3 (16.50 g, eluting solvent EtOAc), Fr-4 (18.30 g, eluting solvent Acetone) and Fr-5 (9.87 g, eluting solvent MeOH). Among the five fractions Fr-1 and Fr-2 were subjected to open column chromatography. Fr-1 of *Curcuma zedoria* was separated by open column chromatography with silica gel using step gradient of Hex-EtOAc-MeOH. The fractions were collected by monitoring with TLC. Fr-1-42 yielded a colorless crystal, CZH-42 (60.00 mg). This compound was UV inactive and charring with methanol and $H_2SO_4$ (9:1) gave characteristic

bright red color which indicated the compound was a steroidal derivative. Structure of CZH-42 was elucidated by the analysis of $^{1}$H-NMR spectral data which revealed that the compound could be campesterol, a plant steroid. Further analysis such as $^{13}$C-NMR and Mass Spectroscopy (MS) are required for confirmation. As per our knowledge, based on the literature review, this is the first time report of champesterol isolation from *Curcuma zedoria*. CZH-42 showed no antioxidant activity. Fr-2 was then subjected to open column chromatography with silica gel using step gradient of Hex-EtOAc-MeOH and 11 fractions were obtained monitoring TLC. All of these fractions were mixtures of compounds which are needed to be separated by further column chromatography to isolate pure bio-active compounds. Bioactivities of these fractions are also needed to be evaluated.

**Key words**: Lignan, Steroid, Campesterol, VLC, TLC, Open column chromatography

## 1. INTRODUCTION

Nature has provided a complete store-house of remedies to cure all aliments of mankind. Medicinal plants are believed to be an important source of new chemical substances with potential therapeutic effects. Several of the current chemotherapeutic drugs like vinblastine, methotrexate, taxol, and so forth, were first identified in plants. Plant based medicines initially dispensed in the form of crude drugs such as tinctures, teas, poultices, powders, and other herbal formulations, now serve as the basis of novel drug discovery.[1, 2]

According to World Health Organization (WHO), from 119 plant-derived medicines, about 74% are used in modern medicine in ways that correlate directly with their traditional uses. WHO also estimates that 4 billion people, 80% of the world's population, presently use herbal medicine for primary healthcare. Herbal medicine is a common element in Ayurvedic, Homeopathic, Naturopathic, Traditional oriental, Native American and Indian medicine. Even among prescription drugs, at least 25% contain at least one compound derived from higher plants. The percentage might be higher if we include over-the counter (OTC) drugs.

In developing countries, the practice of medicine still relies heavily on plant and herbal extracts for the treatment of human ailments. Dietary agents consist of a wide variety of biologically active compounds that are ubiquitous in plants, and many of them have been used as traditional medicines. Some phytochemicals derived in spices and herbs as well as other plants possess substantial cancer preventive properties.[3]

There is a growing focus on the importance of medicinal plants and traditional health systems in solving the health care problems of the world. Current research in drug discovery from medicinal plants involves a multifaceted approach combining botanical, phytochemical, biological, and molecular techniques. Medicinal plant drug discovery continues to provide new and important leads against various pharmacological targets including cancer, HIV/AIDS, Alzheimer's, malaria, TB and pain. Recently introduced drugs from plant origin include Taxol, Podophyllotoxin, camptothecin, arteether, galantamine, nitisinone, and tiotropium.[4]

Population rise, inadequate supply of drugs, prohibitive cost of treatments, side effects of several allopathic drugs and development of resistance to currently used drugs for infectious diseases

have led to increased emphasis on the use of plant materials as a source of medicines for a wide variety of human ailments. As such herbal remedies have been used to cure a variety of disorders or conditions such as diabetes, cardiovascular problems, weight control, dermal infirmities, sexual malfunction, and of course cancer.

According to the World Health Organization, more than 70% of the world's population uses TM in order to fulfill their health necessities. The natural world has been providing life-saving antibiotics, nutritive supplements and our most potent anti-cancer drugs. The lush tropical rainforests and colorful coral reefs of our planet have long been a source of promise in the fight against cancer and other diseases.

Natural products, especially those from plants, have been a valuable source of new cancer drugs for many decades. Medicinal plants are the most exclusive source of life saving drugs for the majority of the world's population. The use of plant products in the treatment of cancer has been of recent interest .In the market; these products are offered as "natural products". Natural products appeared to be a promising source for new types of compounds to test for antitumor activity. The goals of using plants as sources of therapeutic agents are

- To isolate bioactive compounds for direct use as drugs, e.g., digoxin, digitoxin, morphine, reserpine, taxol, vinblastine, vincristine.
- To produce bioactive compounds of novel or known structures as lead compounds for semi synthesis to produce patentable entities of higher activity and/or lower toxicity, e.g., metformin, nabilone, oxycodon and other narcotic analgesics], taxotere, teniposide, verapamil, and amiodarone, which are based, respectively, on galegine, tetrahydrocannabinol, morphine, taxol, podophyllotoxin, and khellin.
- To use agents as pharmacologic tools, e.g., lysergic acid diethylamide, mescaline, yohimbine.[5]

### 1.1 Prospect of natural products and phytomedicine

Numerous methods have been utilized to acquire compounds for drug discovery, including isolation from plants and other natural sources, synthetic chemistry, combinatorial chemistry and molecular modeling. Despite the recent interest in molecular modeling, combinatorial chemistry and other synthetic chemistry techniques by pharmaceutical companies and funding organizations, natural products and particularly medicinal plants, remain an important source of new drugs, new drug leads and new chemical entities (NCEs).

According to Newman et al. (2003), 61% of the 877 small-molecule NCEs introduced as drugs worldwide during 1981–2002 was inspired by natural products. These include: natural products (6%), natural products derivatives (27%), synthetic compounds with natural products-derived pharmacophore (5%) and synthetic compounds designed from natural products (natural products mimic, 23%). These new drugs have received approval for the treatment of cancer, neurological diseases, infectious diseases, cardiovascular and metabolic diseases, immunological, inflammatory and related diseases, and genetic disorders, which encompass many of the common human diseases.

Over a 100 natural-product-derived compounds are currently undergoing clinical trials and at least 100 similar projects are in preclinical development (Table 1). Most are derived from leads from plants and microbial sources.[6]

**Table 1**

**1.2 Drugs based on natural products at different stages of development**

| Development stage | Plant | Bacterial | Fungal | Animal | Semi-synthetic | Total |
|---|---|---|---|---|---|---|
| Preclinical | 46 | 12 | 7 | 7 | 27 | 99 |
| Phase I | 14 | 5 | 0 | 3 | 8 | 30 |
| Phase II | 41 | 4 | 0 | 10 | 11 | 33 |
| Phase III | 5 | 4 | 0 | 4 | 13 | 26 |
| Pre-registration | 2 | 0 | 0 | 0 | 2 | 4 |
| Total | 108 | 25 | 7 | 24 | 61 | 225 |

## 1.3 Approaches to natural product research and drug discovery

Different approaches to drug discovery from plants can be enumerated as: random selection followed by chemical screening, random selection followed by one or more biological assays, follow-up of biological activity reports, follow-up of ethno-medical (traditional medicine) use of plants, use of appropriate plant parts as such in powdered form or preparation of enriched/standardized extracts (herbal product development), use of a plant product, biologically potent but beset with other issues, as a lead for further chemistry, and single new compounds as drugs. The objective of the later approach is the targeted isolation of new bioactive plant products, i.e. lead substances with novel structures and novel mechanisms of action. This approach has provided a few classical examples, but the problem most often encountered here is not enough availability. The problem of availability can be overcome by semi-synthesis/synthesis or using tissue-culture techniques (by genetically modifying the biosynthetic pathway of the compound of interest).

## 1.4 Older approach

- Focused on chemistry of compounds from natural sources, but not on activity.
- Straightforward isolation and identification of compounds from natural sources followed by testing of biological activity in animal model.
- Chemotaxonomic investigation.
- Selection of organisms primarily based on ethnopharmacological information, folkloric reputations, or traditional uses.

## 1.5 Modern approach

- Bioassay-directed (mainly *in vitro*) isolation and identification of active lead compounds from natural sources.
- Production of natural products libraries.

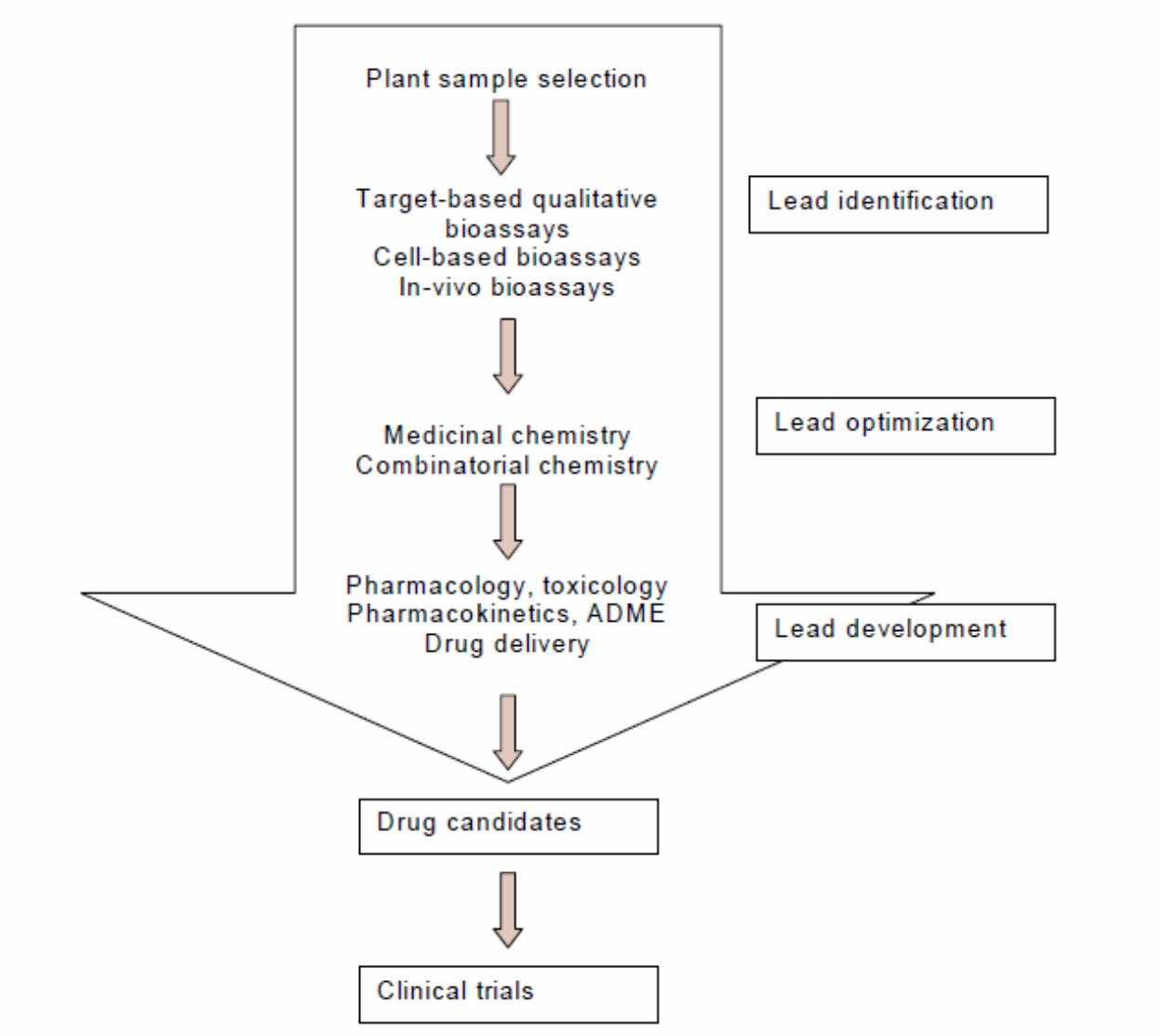



**Figure 1**: Steps involved in drug discovery from plants

- Production of active compounds by cell or tissue culture, genetic manipulation, natural combinatorial chemistry and so on.
- More focused on bioactivity.
- Introduction of the concepts of dereplication, chemical fingerprinting, and metabolomics.
- Selection of organisms based on ethnopharmacological information, folkloric reputations, or traditional uses, and also those randomly selected.[7]

### 1.6 Challenges in drug discovery from medicinal plants

In spite of the success of drug discovery programs from plants in the past 2–3 decades, future endeavors face many challenges. Natural products scientists and pharmaceutical industries will need to continuously improve the quality and quantity of compounds that enter the drug development phase to keep pace with other drug discovery efforts. The process of drug discovery has been estimated to take an average period of 10 years and cost more than 800 million dollars.[8] Much of this time and money is spent on the numerous leads that are discarded during the drug discovery process. It is estimated that only one in 5000 lead compounds will successfully advance through clinical trials and be approved for use. In the drug discovery process, lead identification is the first step. Lead optimization (involving medicinal and combinatorial chemistry), lead development (including pharmacology, toxicology, pharmacokinetics, ADME and drug delivery), and clinical trials all take considerable time.

As drug discovery from plants has traditionally been time-consuming, faster and better methodologies for plant collection, bioassay screening, compound isolation and compound development must be employed. [9] Innovative strategies to improve the process of plant collection are needed, especially with the legal and political issues surrounding benefit-sharing agreements.[10]

The design, determination and implementation of appropriate, clinically relevant, highthroughput bioassays are difficult processes for all drug discovery programs. The common problem faced during screening of extracts is solubility and the screening of extract libraries is many times problematic, but new techniques including pre-fractionation of extracts can alleviate some of these issues. Challenges in bioassay screening still remain an important issue in the future of drug discovery from medicinal plants. The speed of active compound isolation can be increased using hyphenated techniques like LC-NMR and LC-MS. Development of drugs from lead compounds isolated from plants, faces unique challenges. Natural products, in general, are typically isolated in small quantities that are insufficient for lead optimization, lead development and clinical trials. Thus, there is a need to develop collaborations with synthetic and medicinal chemists to explore the possibilities of its semi-synthesis or total synthesis.[11] One can also

improve the natural products compound development by creating natural products libraries that combine the features of natural products with combinatorial chemistry.

### 1.7 Opportunities in drug discovery from medicinal plants

Bioprospecting demands a number of requirements which should be co-coordinated, such as team of scientific experts (from all the relevant interdisciplinary fields) along with expertise in a wide range of human endeavours, including international laws and legal understanding, social sciences, politics and anthropology. In our context, Ayurveda and other traditional systems of medicine, rich genetic resources and associated ethnomedical knowledge are key components for sustainable bioprospecting and value-addition processes.

For drug-targeted bioprospecting an industrial partner is needed, which will be instrumental in converting the discovery into a commercial product. Important in any bioprospecting is the drafting and signing of an agreement or Memorandum of Understanding that should cover issues on access to the genetic resources (biodiversity), on intellectual property related to discovery, on the sharing of benefits as part of the process (short term), and in the event of discovery and commercialization of a product (long term), as well as on the conservation of the biological resources for the future generations. When ethnobotanical or ethnopharmacological approach is utilized, additional specific requirements that relate to prior informed consent, recognition of Indigenous Intellectual Property and Indigenous Intellectual Property Rights as well as short- and long-term benefit sharing need to be taken into account.

In order to screen thousands of plant species at one go for as many bioassays as possible, we must have a collection of a large number of extracts. Globally, there is a need to build natural products extract libraries. The extract libraries offer various advantages, such as reduction in cost and time for repeat collection of plants and availability of properly encoded and preserved extracts in large numbers for biological screening in terms of high-throughput screenings and obtaining hits within a short period. Such libraries could serve as a powerful tool and source of extracts to be screened for biological activities using high-throughput assays. [12]

## 1.8 Phytochemistry

Phytochemistry is in the strict sense of the word the study of phytochemicals. These are chemicals derived from plants. In a narrower sense the terms are often used to describe the large number of secondary metabolic compounds found in plants. Many of these are known to provide protection against insect attacks and plant diseases. They also exhibit a number of protective functions for human consumers.

Techniques commonly used in the field of phytochemistry are extraction, isolation and structural elucidation (MS,1D and 2D NMR) of natural products, as well as various chromatography techniques (MPLC, HPLC, LC-MS).

***Primary metabolite***

A primary metabolite is a kind of metabolite that is directly involved in normal growth, development, and reproduction. Primary metabolites comprise many different types of organic compounds, including, but not limited to, carbohydrates, lipids, proteins, and nucleic acids. They are found universally in the plant kingdom because they are the components or products of fundamental metabolic pathways or cycles such as glycolysis, the Krebs cycle, and the Calvin cycle.

Because of the importance of these and other primary pathways in enabling a plant to synthesize, assimilate, and degrade organic compounds, primary metabolites are essential. Examples of primary metabolites include energy rich fuel molecules, such as sucrose and starch, structural components such as cellulose, informational molecules such as DNA (deoxyribonucleic acid) and RNA (ribonucleic acid), and pigments, such as chlorophyll. In addition to having fundamental roles in plant growth and development, some primary metabolites are precursors (starting materials) for the synthesis of secondary metabolites.

***Secondary metabolite***

Secondary metabolites are organic compounds that are not directly involved in the normal growth, development, or reproduction of an organism. Unlike primary metabolites, absence of secondary metabolites does not result in immediate death, but rather in long-term impairment of the organism's survivability, fecundity, or aesthetics, or perhaps in no significant change at all. Secondary metabolites are often restricted to a narrow set of species within a phylogenetic group. Secondary metabolites often play an important role in plant defense against herbivory and other interspecies defenses. Humans use secondary metabolites as medicines, flavorings, and recreational drugs. Examples include antibiotics and pigments such as resins and terpenes etc. Some antibiotics use primary metabolites as precursors, such as actinomycin which is created from the primary metabolite, tryptophan.

Secondary metabolites largely fall into three classes of compounds: alkaloids, terpenoids, and phenolics. However, these classes of compounds also include primary metabolites, so whether a compound is a primary or secondary metabolite is a distinction based not only on its chemical structure but also on its function and distribution within the plant kingdom.

Many thousands of secondary metabolites have been isolated from plants, and many of them have powerful physiological effects in humans and are used asmedicines. It is only since the late twentieth century that secondary metabolites have been clearly recognized as having important functions in plants.[12]

## 2. RATIONALE OF THE WORK

A range of different approaches as described earlier has been employed to obtain lead compounds for drug discovery, including isolation from plants and other natural sources, synthetic chemistry, combinatorial chemistry and molecular modeling. Despite the recent interest in molecular modeling, combinatorial chemistry and other synthetic chemistry techniques, natural products and particularly medicinal plants, remain an important source of new drugs, new drug leads and new chemical entities.

*Leucas aspera*, locally known as 'Dondokalash' in Bangladesh, *is* a medicinal herb, which has long been used in the Indian subcontinent as a folk medicine for the treatment of a variety of diseases of different etiology as analgesic, anti-inflammatory, antioxidant, cytotoxic, anticancer. Moreover literature review revealed that this plant contains various types of lignan. Lignan is important type of secondary plant metabolite and many lignan showed interesting biological activity, e.g. podophyllotoxin showed strong anticancer activity, manasssantins showed potent hypoxia inducible factor-1 (HIF-1) inhibitory activity etc. It was, therefore, the objective of this study to delineate and validate on scientific line some of the uses of the plant in folk medicine along with phytochemical study on the lignan content of the plant.

On the other hand, *Curcuma zedoria,* locally known as 'Shoti' is a well known ethnomedicinal plant that is also used in Ayurveda. Its use in the Indian traditional folk medicine is also well documented. It is used traditionally for the treatment of menstrual disorders, dyspepsia, vomiting and for cancer. Rural people use the rhizome for its rubefacient, carminative, expectorant, demulcent, diuretic and stimulant properties while the root is used in the treatment of flatulence, dyspepsia, cold, cough and fever. Moreover, *Curcuma zedoria* belongs to Zingiberaceae family. Previously a potent anti-ulcerant compound zirombune has been isolated from a plan of this family in our laboratory. Thus, it was our objective to isolate other potent bioactive metabolites of this plant and to elucidate the structures of purified metabolites by spectroscopic and spectrometric methods.

Thus, this was the purpose of the selection of these two medicinal plants in my research work.

## 3. MATERIALS AND EQUIPMENTS

### Drugs and chemicals

| No. | Drugs or chemicals | Company |
|---|---|---|
| 1. | Dimethylsulfoxide (DMSO) | Sigma Aldrich Laborchemikalein Gmbh |
| 2. | Hydrochloric acid | BDH chemicals |
| 3. | Amoxicillin | |
| 4. | Nutrient agar | Techno Pharmachem, Bahdurgarh, India |
| 5. | Silica get 60 (0.063-0.020mm) | Merck, Germany |

### Solvents

| No. | Solvents |
|---|---|
| 1. | N-hexane |
| 2 | Dichloromethane (DCM) |
| 3. | Ethyl acetate (EtOAC) |
| 4. | Acetone |
| 5. | Methanol |

### Instruments

| No. | Instruments |
|---|---|
| 1. | TLC plate precoated with Si60 F 254 |
| 2. | UV visible spectrum-SHIMADZU UV-1700 Pharma Spec UV-VIS Spectophotometer |

| | |
|---|---|
| 3. | 1H-NMR spectra- Ultra shield Bruker DPX 400 spectrometer |
| 4. | Analytical balance |
| 5. | Rotary evaporator |
| 6. | UV lamp |
| 7. | Autoclave |
| 8. | Hot air oven |
| 9. | Laminer air flow |
| 10. | Incubator |
| 11. | Refrigerator |
| 12. | Vortex machine |
| 13 | Micropipette and Micropipette tube |
| 14. | Hotplate |
| 15. | Burner |

**Equipments**

| **No.** | **Equipments** |
|---|---|
| 1. | Large beaker-1000 ml, Medium beaker-250 ml, Small beaker-50 ml |
| 2. | Spatula |
| 3. | Mice oral needle |
| 4. | 1m insulin syringe (50units) |
| 5. | Mortar and Pestle |
| 6. | Surgical tray |
| 7. | Aluminum foil paper |
| 8. | 5 ml vial |
| 9. | Small test tubes |

| | |
|---|---|
| 10. | Loop |
| 11. | 500ml, 1 liter and 2.5 liter container |
| 12. | Microorganism spreading glass rod |
| 13. | TLC tank |
| 14. | Pasteur pipette |

# CHAPTER 1

## 4. INTRODUCTION TO PLANT- *LEUCAS ASPERA*

### 4.1 *Plant name*

**Scientific name:** ***Leucas aspera***

**Local name:** Dondokalash, Dolkalash, Hulkasha, Dhronipushpi

**Family**: Lamiacea*e*

### 4.2 *Description*

*Leucas aspera* is an annual, branched, herb erecting to a height of 15-60 cm with stout and hispid acutely quadrangular stem and branches. Leaves are sub-sessile or shortly petiolate, linear or linearly lanceolate, obtuse, pubescent up to 8.0 cm long and 1.25 cm broad, with entire or crenate margin; petiole 2.5-6 mm long; flowers white, sessile small, in dense terminal or axillary whorls; bracts 6 mm long, linear, acute, bristle-tipped, ciliate with long slender hairs; calyx variable, tubular, 8-13 mm long; tube curved, contracted above the nutlets, the lower half usually glabrous and membranous, the upper half ribbed and hispid; mouth small, very oblique, not villous, the upper part produced forward; teeth small, triangular, bristle-tipped, ciliate, the upper tooth being the largest.

**Figure 2**: *Leucas aspera*

Corolla 1 cm long; tube 5 mm long and pubescent above, annulate in the middle; upper lip 3 mm long, densely white-woolly; lower lip about twice as long, the middle lobe obviate, rounded, the

lateral lobes small, subacute. Fruit nutlets, 2.5 mm long, oblong, brown, smooth, inner face angular and outer face rounded.

### 4.3 Habitant

Largely available in Bangladesh, India and Indonesia

### 4.4 Economic uses/values/harmful aspects

The plant is used traditionally as an antipyretic and insecticide. Flowers are valued as stimulant, expectorant, aperient, diaphoretic, insecticide and emmenagogue. Leaves are considered useful in chronic rheumatism, psoriasis and other chronic skin eruptions. Bruised leaves are applied locally in snake bites.[13]

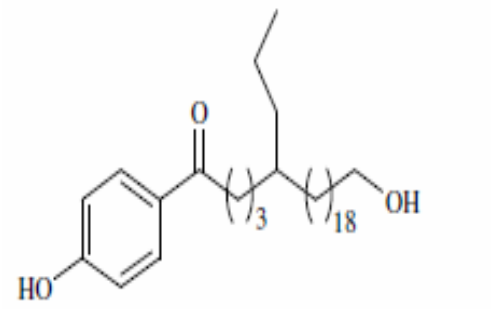
## 5. LITERATURE REVIEW OF *LEUCAS ASPERA*

### 5.1 Chemistry of *Leucas aspera*

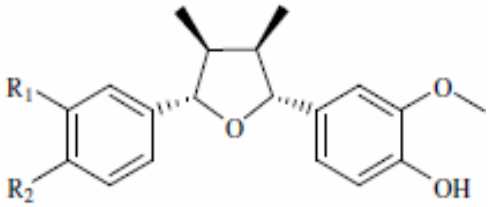
***Phenolic compounds***

Plant phenolics are a structurally diverse set of compounds responsible for organoleptic properties of plants. These are found to possess a wide range of therapeutic activity. They occur in plants in the form of simple phenolic acids or as complex structures associated with the oxygenated heterocyclic ring, such as benzoic acid derivatives, stilbenes, tannins, lignans, anthocyanins, flavonoids and coumarins.

4-(24′-hydroxy-1′oxo-5′-npropyltetracosanyl) - phenol (1) is found in the shoots of *Leucas aspera*. Lignans, another important class of plant phenolics, are formed as a result of dimerization of phenylpropanoid units at the central carbons of their side chains and generally occur in the root, stem, bark, fruit and seed parts of the plant. *Leucas aspera* contains eight lignans namely nectandrin B (2), (-)-chicanine (3), meso-dihydroguaiaretic acid (4), macelignan (5), myristargenol B (6), erythro-2-(4-allyl-2,6- dimethoxyphenoxy)-1-(4-hydroxy-3-methoxy phenyl) propan-1-ol (7), machilin C (8), (7R,8R)- and (7S,8S)-licarin (9) from the methanol extract of the whole plant of *Leucas aspera*.

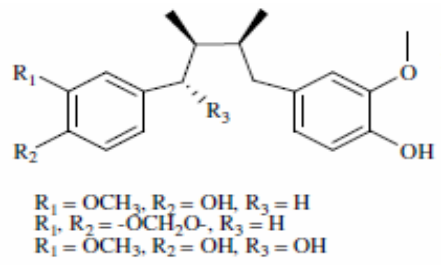



(1)




$R_1 = OCH_3, R_2 = OH$
$R_1, R_2 = -OCH_2O-,$

(2) & (3)

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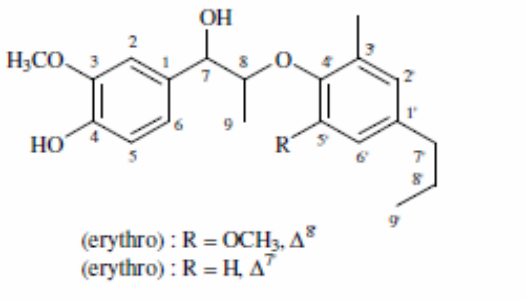


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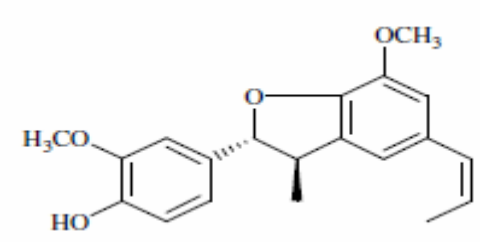
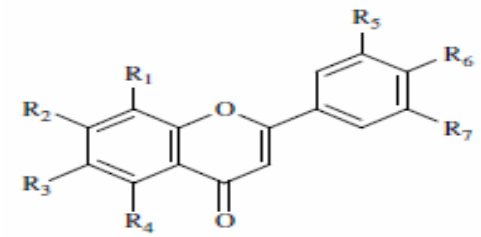



(9)

Flavonoids, another important class of phenolics featuring the linkage of two benzene rings by a chain of 3 carbon atoms, so as to form pyran or pyrone ring, play predominant role in plant physiology and serve as light screens, antioxidants, enzyme inhibitors, precursors of toxic substances and pigments. In the genus *Leucas*, many reports reveal the occurrence of flavonoids in the conjugated form (that is with sugar). However, free flavonoid 'baicalein' (10) was reported in the ethereal fraction of hydro methanolic extract of the flower of *Leucas*. *Aspera*. Sadhu et al. (2003) reported acacetin (11), chrysoeriol (12) and apigenin (13) from the *Leucas aspera.*




(10) $R_1$, $R_5$, $R_6$, $R_7$, =H, $R_2$, $R_3$, $R_4$=OH

(11) $R_1$, $R_3$, $R_5$, $R_7$=OH, $R_6$=$OCH_3$

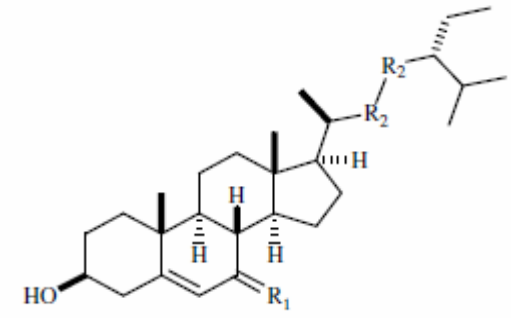
(12) $R_1$, $R_3$, $R_7$=H, $R_2$, $R_4$, $R_6$=OH, $R_5$=$OCH_3$

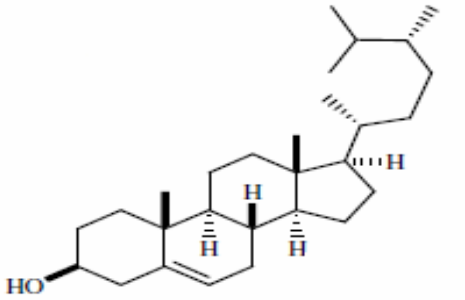
(13) $R_1$, $R_3$, $R_5$, $R_7$=H, $R_2$, $R_3$, $R_4$=OH

Coumarins, another class of plant phenolics, comprised of phenlypropanoid system, are found to be physiologically effective for animals as well as men. No coumarins have been found from *Leucas aspera*.

***Steroids***

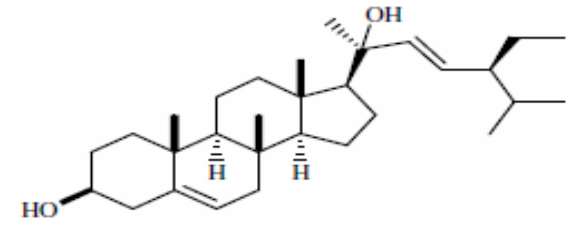
Sterols, structurally comprised of perhydrocyclopenta- (O)phenantherene ring system, are widely distributed in higher plants. Presence of ubiquitous phytosterol such as β-sitosterol (14), stigmasterol (15), campesterol (16), ursolic acid (17) and their derivatives have been reported in plants of genus *Leucas.*




(14) $R_1$=$H_2$, $R_2$=$CH_2$ (16)

(15) $R_1$=$H_2$, $R_2$=CH

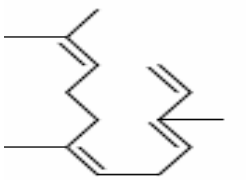



(17)

***Terpenes***

Terpenes constitute one of the largest and structurally diverse classes of plant secondary metabolites responsible for flavor, fragrance and bioactivity of the plants. Plants of genus *Leucas* are found to be rich in terpenes.

The essential oil fraction from the leaves and flowers of *Leucas aspera* were found to contain high amount of a-farnesene (18), α-thujene (19) and menthol (20). A new type of diterpenes, leucasperones A and B (21-22); leucasperols A and B, (23-24) has been reported from *Leucas aspera*. Maslinic acid (25), an oleanane-type triterpene, has also been reported from the plant. A lactone triterpene 3β, 16α-dihydroxyolean-28→13β-olide (26) was isolated from the benzene fraction of *Leucas aspera* root extract.[14]



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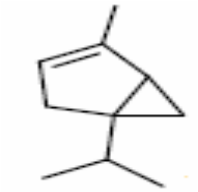

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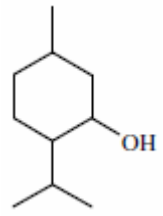


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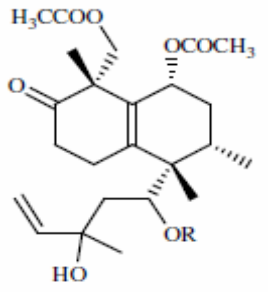
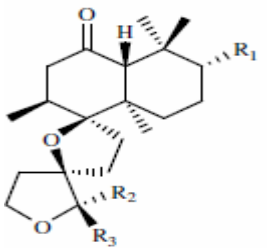


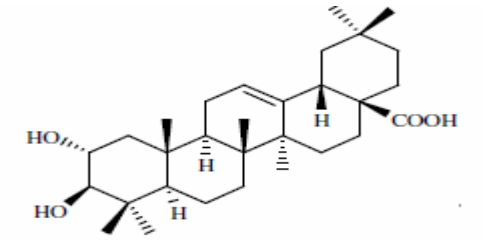

(21) R=COCH3

(22) R=H

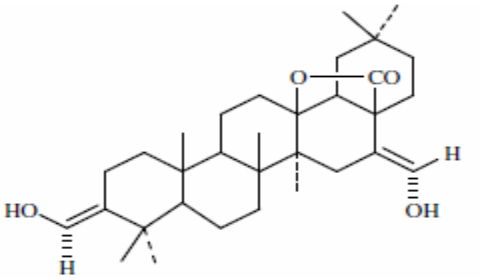



(23) $R_1$=OH, $R_2$=H, $R_3$=OH

(24) $R_1$=OH, $R_2$=OH, $R_3$=H

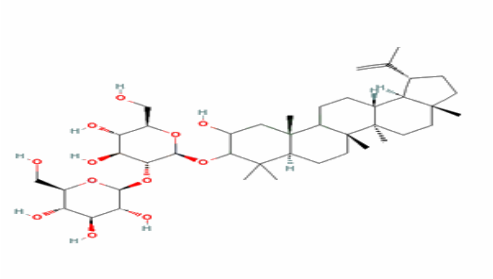



(25)




(26)

5, 7-dihydroxy-2-[14-methoxy-15-propyl phenyl]-4H-chromen-4-one (leucasin), a triterpene saponin was isolated from *Leucas aspera*.[15]

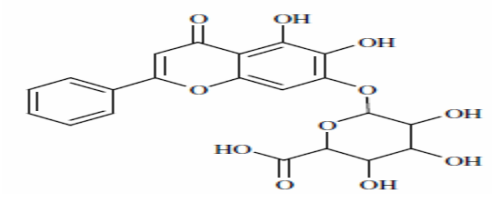


(27)

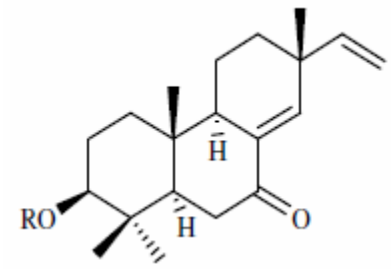
Leucolactone, isolated from the root of *Leucas aspera* has been characterized as 3β, 16α-dihydroxyoleanan-28→13β-olide.[16]

***Glycoside***

A flavonoidal glycoside, baicalin (28), from the fresh flower of *Leucas aspera*, showed significant biphasic RBC membrane stabilization activity against hypotonicity induced hemolysis. Further, the isopimarane-type diterpenoidal glycosides 'leucasperosides A, B, C' (29-31) and linifolioside (32) reported in *Leucas aspera*, exhibited inhibitory activity against PGE1 and PGE2 induced contractions in the guinea pig ileum. Flavonoidal glycoside apigenin 7-O-(6″-O-(p- coumaroyl) - β-Dglucoside) (81) has been isolated from the *Leucas aspera.*

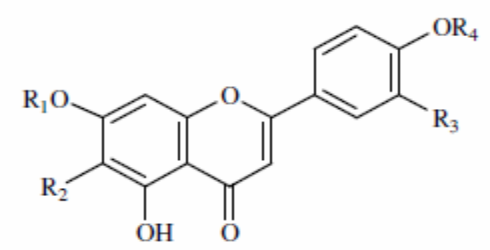


(28)



(29) R=Glc-Glc'-Glc'' (31) R=Glc- (Rha) Glc'

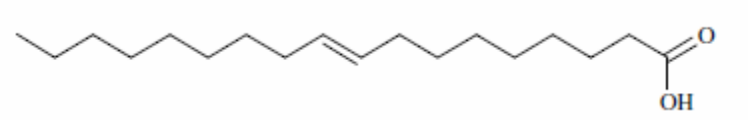
(30) R= Glc-Glc' (32) R=Glc-Rha




(33) $R_2$, $R_3$=H, $R_4$=CH3, $R_1$=-(6-*O*-*p*-coumaroyl) glucopyranoside

***Fatty acids***

Varying concentrations of oleic acid (34) and Linoleic acid (35) were also found in *Leucas aspera.*

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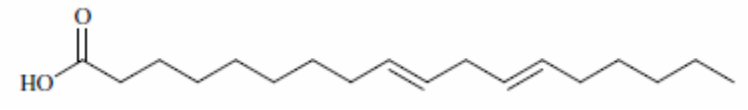
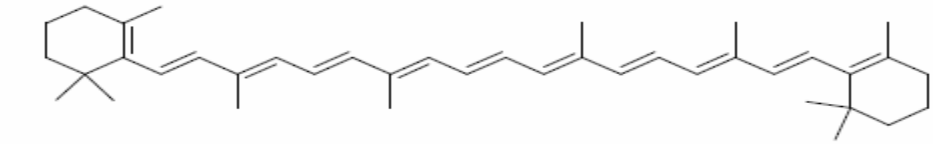



(35)

***Miscellaneous***

*Leucas aspera* is widely used in countryside as foods and also for nutritional requirement. It is reported to have high content (21.3%) of protein. *Leucas aspera* contains significant amounts of total carotenoid and β-carotene (36). Asperphenamate (37) and alkaloid nicotine (38) have also been reported in *Leucas aspera*. Long chain compounds nonatriacontane (39), 1-dotriacontanol (40), 1- hydroxytetratriacontan-4-one (41), 32-methyltetratriacontane (42) were reported in *Leucas aspera*. Aliphatic ketols namely 28-hydroxypentatriacontan- 7-one (43), 7-hydroxy-dotriacontan-2- one (44), 5-acetoxy-triacontane (45) were isolated from the shoots of *Leucas aspera.* Amyl propionate (46) and isoamylpropionate (47) were present in high concentration in the essential oil fraction of the leaf and flower parts of *Leucas aspera.*[14]



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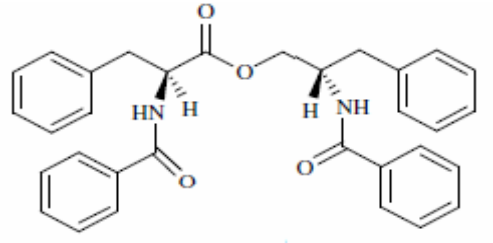


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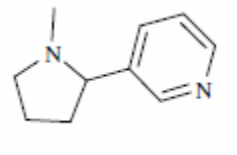


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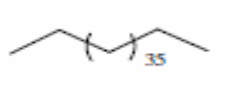


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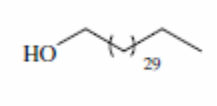


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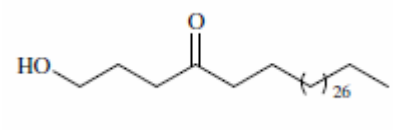


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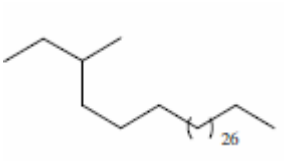


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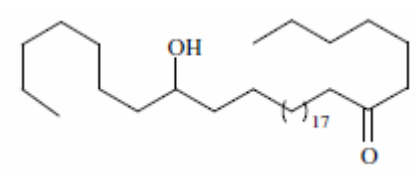


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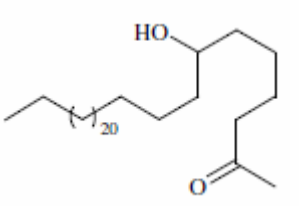


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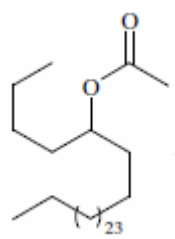


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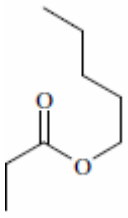


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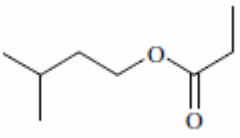



(47)

## 5.2 Pharmacological activity of *Leucas aspera*

### *Antioxidant activity*

The crude methanol extract of *Leucas aspera* leaves showed strong 1, 1-diphenyl-2-picrylhydrazyl (DDPH) and superoxide radical-scavenging activities compared to other polarity-based extracted fractions. It was selected for purification of the bioactive compound. The activity-guided repeated fractionation of the methanol extract by silica gel column chromatography yielded the bioactive compound isolated was 5, 7-dihydroxy-2-[14-methoxy-15-propyl phenyl]-4H-chromen-4-one (leucasin). Radical-scavenging potential and strong inhibition of lipid peroxidation in a liposome model were observed at a leucasin concentration of 40 ppm. Thus the antioxidant potency of leucasin could be the basis for alleged health-promoting potential.[15]

### *Hepatoprotective activity*

The cold methanolic extract of the whole plant of *Leucas aspera* was found to exhibit significant hepato protection in CCl4 induced liver damage.

### *Cytotoxicity*

Brine shrimp lethality assay is a rapid and inexpensive method used to correlate the cytotoxic and anti-tumor properties of natural and synthetic compounds. Various studies using this model showed that, the hydroalcoholic extract of *Leucas aspera* whole plant exhibited cytotoxicity (LC50 = 1,900 µg/ml) and this activity was more in the root extract (LC50 = 52.8 µg/ml).

### *Insecticidal and replant activity*

*Leucas aspera* leaves are used as mosquito repelant and as insecticide by countrified Indians. These claims were vindicated by extensive studies which indicated that *Leucas aspera* leaf extract exhibited significant larvicidal activity against first, second, third and fourth instar larvae of *Culex quinquefasciatus*. *Leucas aspera* leaf extract (4% solution) showed 90% death of the

fourth instar larvae and 100% death after 24 h were recorded for the third instar larvae of *Anopheles stephensi.* The petroleum ether extract of the leaves of *Leucas aspera* exhibited LC50 between 100 to 200 ppm against the fourth instar larvae of *C. quinquefasciatus*, *A. stephensi* and *Aedes aefypti.*

The highest larvicidal activity against I-IV instar of *C. quinquefasciatus* and *A. aefypti* was found in the hexane extract followed by chloroform and ethanol extracts. *A. stephensi* larval treatment with *Leucas aspera* leaf extract resulted in significant fall of its carbohydrate and DNA profile. Further, the highest mortality was seen during the moulting, melanization and tanning processes which are controlled by hormones. Hence, the above findings suggest that the larvicidal activity of the plant may be due to disturbance in hormonal and metabolic process of larvae.[14]

***Activity against inflammation and on mast cell degranuatlion***

The aqueous and alcoholic extracts of *Leucas aspera* were investigated for their action on experimental inflammation and on mast cell degranuatlion. Both the extracts exhibited significant anti-inflammatory action of acute and chronic inflammation. The mast cell drgranulation induced by proprancolol and Carbachol was effectively prevented by pretreatment with *Leucas aspera* extracts.The aqueous and alcoholic extracts of *Leucas aspera* were investigated for their action on experimental inflammation and on mast cell degranuatlion. Both the extracts exhibited significant antiinflammatory action of acute and chronic inflammation. The mast cell drgranulation induced by proprancolol and Carbachol was effectively prevented by pretreatment with *Leucas aspera* extracts.[17]

***Antimicrobial activity***

The aqueous extract of the leaves of *Leucas aspera* was found to be inactive against fungal strains- *Aspergillus flavus*, *Trichophyton rubrum*, *Aspergillus niger*. No inhibition against *Escherichia coli* and *Pseudomonas* aeruginosa were detected with 80% ethanolic extract. However, significant antimicrobial activity was reported for the alkaloidal fraction and the total MeOHextract the *Leucas aspera* flowers.[18]

***Miscellaneous activity***

In another study, the protective role of *Leucas aspera* against the snake (cobra) venom poisoning was studied in mice. This study revealed that *Leucas aspera* alcoholic extract treatment significantly improved the survival time which may be due to the stabilization of mast cells and inhibition of the secretion of platelet activating factor and histamine.[14]

# 6. EXTRACTION, SEPARATION & BIOLOGICAL TESTS- *LEUCAS ASPERA*

## 6.1 Collection and identification

The whole plant was collected from Sylhet district of Bangladesh in June 2012.The whole plant with leaves, stems and roots was collected and identified by experts in Bangladesh National Herbarium, Mirpur, Dhaka, where a Voucher specimen (Accession No:37512.) has been deposited for future reference.

## 6.2 Drying

The plant was thoroughly washed with water. All the plants were sliced into small pieces and spread on tray allowing shade drying. The plant parts were dried for the next 10 days.

## 6.3 Grinding and storage of the dried samples

The dried leaves were ground to coarse powder with a mechanical grinder (Grinding Mill). This process breaks the plant parts to smaller pieces thus exposing internal tissues and cells to solvents thus facilitating their easy penetration into the cells to extract the constituents. Then the powdered sample was kept in clean closed glass containers till extraction. During grinding of sample, the grinder was thoroughly cleaned to avoid contamination with any remnant of previously ground material or other extraneous matters deposited on the grinder. The weight of the total power was 250gm.

## 6.4 Extraction of the dried powdered sample

After collection 50gm of the powder was soaked in 350ml Methanol in a tightly closed jar for 3 days and was shaken several times a day for mixing properly. The powder (50gm) was extracted with Methanol at room temperature (350ml x 72 hrs x 3 times). The extracting solvent was filtered 3 times and the filtrate was concentrated in vaccuo by a rotary evaporator (50°C) yielding crude Methanol extract 10.18 g that is yield= 20.36%.

## 6.5 Thin layer chromatography (TLC) of MeOH extract

A fresh moisture free 25×25cm TLC plate was taken and cut in appropriate size on the basis of sample numbers to be loaded on the plate with the help of TLC cutter. The TLC plate was marketed 1cm upper from the bottom and 1cm lower from the upper side with pencil. Approximately 0.5-2 mg of the dried extract were diluted with 1 ml of the appropriate solvent then, approximately 2-6 (µl) aliquot of each dilution of the extract was carefully loaded individually onto the baseline of the TLC plates with the help of small capillary tube and the sample was allowed to dry. Different solvent systems were used as mobile phases, respectively on the basic of the nature of the compounds. The plate was placed in a closed chamber containing a mobile phase on the bottom. After run of mobile phase up to upper pencil mark, the plate was removed from the TLC tank. The plate was dried with the help of air of electric fan to evaporate the solvent. Once dried, the UV active compounds were observed by using UV lamp, here fluorescence compounds can also be observed. After detection of UV and fluorescence compounds, the plate was sprayed with charring reagent (10%$H_2SO_4$ in methanol). After spraying charring reagents, the plate was completely dried and heated at 100-120°C for the observation of organic compounds. After detection individual compounds, the Rf value and color of each compound was noted in the table.

### 6.6 Open Column Chromatography

***Slurry Preparation:***

At first, the methanol extract (6.42 g) of *Leucas aspera* was diluted with a very small amount of methanol and after that silica was added to it. They were mixed well until finely powdered. The slurry was left to dry for 24 hours.

***Packing of the Column:***

At first, a piece of cotton was placed at the bottom of the column. Then silica was added according to the amount calculated (40gm) with respect to the length and inner diameter of the column. Before stacking the silica on top of the cotton it was dissolved in hexane and then added to prevent the silica from cracking.

***Loading the Sample in the Column:***

After packing the column the methanol extract (6.42 g) of *Leucas aspera)* was stacked on top of the silica. For elution first pure n-hexane was used. After that the tap was used to drain the solvents out. 450 ml of n-hexane was recycled. Then 1260 ml of hexane –EtOAc (98:2) was recycled. Then elution was done with various hexane-ethyl acetate, ethyl acetate-methanol and methanol-acetic acid gradients.

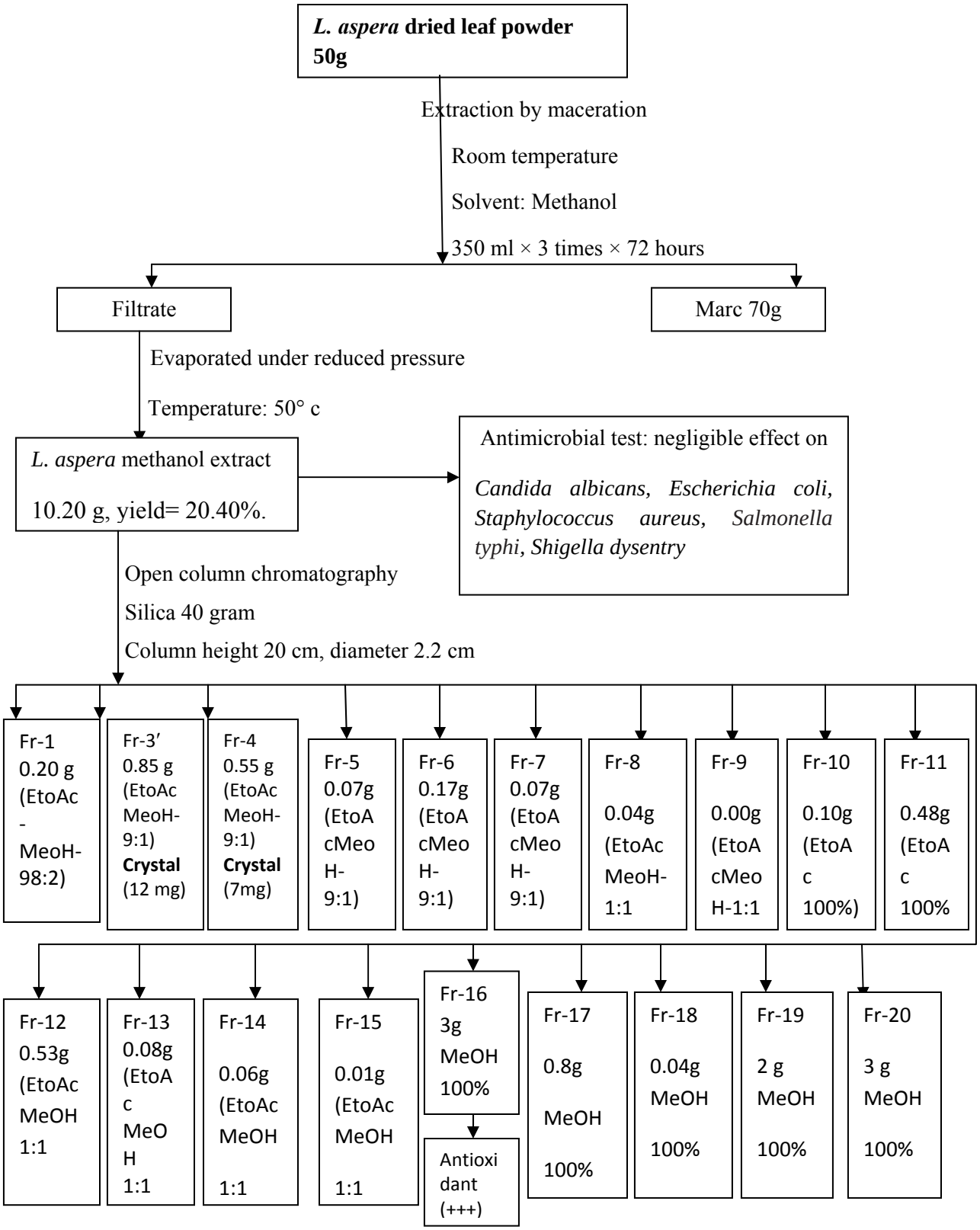
***Collection of the Fractions***

As the solvent passed through the column and came in contact with the extract, it caused different colored bands to appear in the silica. These bands were then collected as different fractions in 250 ml beakers. Thus total 20 fractions were collected naming Fraction-1, Fraction-2, Fraction-3, Fraction-4, Fraction-5, Fraction-6, Fraction-7, Fraction-8, Fraction-9, Fraction-10, Fraction-11, Fraction-12, Fraction-13, Fraction-14, Fraction-15, Fraction-16, Fraction-17, Fraction-18, Fraction-19 and Fraction-20.

***Analyzing the fractions by TLC***

Some of the fractions were analyzed by TLC. Different solvent systems were used as mobile phases, respectively on the basic of the nature of the compound. Charring was also done using same charring solution.

**Bioassay guided Isolation of Active Compounds from *Leucas aspera***



**6.7 Evaluation of Antibacterial activity:**

***Microorganisms***

The following microorganisms were used for disk diffusion method: *Candida albicans, Escherichia coli, Staphylococcus aureus, Salmonella paratyphi, Shigella dysentry*. The microbial species were collected from Department of Food and Nutrition, University of Dhaka. They were maintained on agar slant at 4˚C and sub-cultured on a fresh appropriate agar plate 24 prior to any antimicrobial test.

***Culture media***

Nutrient Agar (NA) was used for the activation of all bacteria and fungi. Agar medium was prepared by mixing 400m of distilled water with 11.2g agar and kept in autoclave.

***Bacterial solution preparation***

In 5 eppendrof tube 1000µl of .9% (.9g NaCl in 100ml distilled water) NaCl was taken by sterilizing the loop. Five types of microorganism were transferred into the five eppendrof tubes. Suspension of these test organisms was prepared using vortex machine.

***Disc diffusion method***

The antimicrobial activity of the tested samples (isolated compound, standard drugs and negative control) were assayed by the standard disc diffusion method. Tested samples were dissolved in methanol and injected into sterilized discs of 6mm in diameter. Nutrient Agar(NA) sterilized at 121°C by using autoclave and cooled to 60–70 °C were distributed to sterilized Petri dishes with a diameter of 9 cm (15 ml). After preparation of the suspensions of test organisms (C.F.U $1.6\times10^{5}$ organisms per ml), 100µL of suspension was added to each Petri dishes and distributed homogeneously. Dishes injected with tested materials were placed on the solid agar medium. Petri dishes were incubated at 37 ◦C for 24 h. In each plate 1 discs containing a dose of crude methanol extract of *Leucus aspera* (1000µg/disc) was applied. Then, on each plate a disc containing appropriate reference antibiotic (Amoxicillin 20µg/disc) disc and control (5µl methanol and DMSO (80:20)/disc) disc were applied. At the end of the period, inhibition zones formed on the NA were evaluated in millimeters. Studies were performed in duplicate, and the developing inhibition zones were compared with those of reference disks.

## 6.8 Evaluation of Antioxidant activity:

***Qualitative DPPH free radical scavenging activity assays***

For this test 0.02 % solution of radical-DPPH in methanol was prepared. This solution was taken in 19 vials; 2 ml in each. Very minute amount of all the fractions (Fraction-1 to Fraction-20) were then added to the vials and kept for 10 minutes. The whole process was done in a dark place. Compound with radical scavenging activity will show color change (yellow or brown) due to the discoloration of DPPH.

## 7. RESULTS AND DISCUSSION OF *LEUCUS ASPERA*

### 7.1 Thin Layer Chromatography (TLC)

**Table 2**

Thin Layer Chromatography (TLC-1) of Crude Methanol extract of *Leucas aspera*

| Plant Species | Compound | Mobile Phase | Total spot | Spot | Rf Value | Observation Under necked eye | UV Observa-tion | Observa-tion after charring | Quantity |
|---|---|---|---|---|---|---|---|---|---|
| *Leucas aspera* | MeOH Extract | Hex:EtOAc (1:1) | 3 | 1 | 0.73 | Brown | Deep Brown | Ash | *** |
| | | | | 2 | 0.48 | Purple brown | Brown | Black | ** |
| | | | | 3 | 0.42 | Green | Brown | Black | ** |
| | | | | 4 | 0.28 | Purple | Ash | Black | * |
| | | | | 5 | 0.14 | Purple | Ash | Black | ** |

 =No color, *** = Huge amount, ** =Moderate amount. * =small amount.

### 7.2 Open column chromatography

Methanol extract of Leucas aspera (6.42 gm) was subjected to flash silica gel (40 gm) column chromatography and eluted with n-hexane-ethylacetate (Hex-EtOAc), ethyl acetate-methanol and methanol-acetic acid gradients. 20 fractions of 100 ml each were collected: Hexane-EtOAc (97:3) (Fraction-1), Hexane-EtOAc (9:1) (Fraction-2 to Fraction-7), Hexane-EtOAc (1:1) (Fraction-8 to Fraction 9), EtOAc 100% (Fraction-10 to Fraction-11), EtOAc-Methanol (1:1) (Fraction-12 to Fraction-15), Methanol 100% (Fraction-16 to Fraction-20).

**Table 3**

Dry weights of the Combined Column Fractions

| Fraction No | Ratio of solvent used | Volume of fraction collected | Dry Weight (gm) |
|---|---|---|---|
| Fraction-1 | Hex-EtOAc (97:3) | 250 ml | 0.20 gm |
| Fraction-3′ | Hex-EtOAc (9:1) | 250 ml for each before combination of Fr-2+3 | 0.85 gm |
| Fraction-4 | Hex-EtOAc (9:1) | 175 ml | 0.55 gm |

| | | | |
|---|---|---|---|
| Fraction-5 | Hex-EtOAc (9:1) | 225 ml | 0.07 gm |
| Fraction-6 | Hex-EtOAc (9:1) | 250 ml | 0.17 gm |
| Fraction-7 | Hex-EtOAc (9:1) | 250 ml | 0.07 gm |
| Fraction-8 | Hex-EtOAc (1:1) | 160 ml | 0.04 gm |
| Fraction-9 | Hex-EtOAc (1:1) | 50 ml | 0.00 |
| Fraction-10 | EtOAc 100% | 50 ml | 0.10 gm |
| Fraction-11 | EtOAc 100% | 125 ml | 0.48 gm |
| Fraction-12 | EtOAc-Methanol (1:1) | 165 ml | 0.53 gm |
| Fraction-13 | EtOAc-Methanol (1:1) | 60 ml | 0.08 gm |
| Fraction-14 | EtOAc-Methanol (1:1) | 70 ml | 0.06 gm |
| Fraction-15 | EtOAc-Methanol (1:1) | 50 ml | 0.01 gm |
| Fraction-16 | Methanol 100% | 75 ml | 3 gm |
| Fraction-17 | Methanol 100% | 150 ml | 0.8 gm |
| Fraction-18 | Methanol 100% | 60 ml | 0.04 gm |
| Fraction-19 | Methanol 100% | 70 ml | 2 gm |
| Fraction-20 | Methanol-Aceticacid (99:1) | 40 ml | 3 gm |

Crystals were observed from the Fraction-3′ and Fraction- 4 which were named as LA-1 (12.00 mg) and LA-2 (7.00 mg) respectively. Further analysis using Infra-red spectroscopy, NMR, mass spectroscopy is required for structure elucidation. Rests of the fractions are mixtures and further separation is required for isolating pure bioactive compound.

**Figure 3**: Fraction 3′

**Figure 4**: Fraction- 4

### 7.3 TLC of column fractions

**Table 4**

| Fraction | Mobile Phase | Total spot | Spot | Rf Value | Observation Under necked eye | UV Observa -tion | Observation after charring | Quantity |
|---|---|---|---|---|---|---|---|---|
| Fraction-3′ | EtOAc-Hex (1:1) | 1 | 1 | 0.77 | - | - | Red | ** |
| Fraction-4 | EtOAc-Hex (1:1) | 1 | 1 | 0.88 | - | - | Red | *** |

-=No color, *** = Huge amount, ** =Moderate amount. * =small amount

**Figure 5:** TLC plate under UV

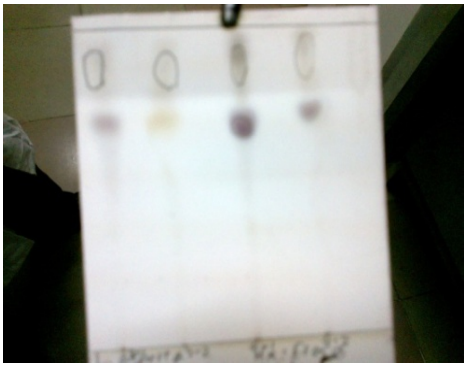


**Figure 6:** TLC plate after charring

### 7.4 Antimicrobial activity

Antimicrobial activity of methanol extract of Leucas aspera has been evaluated in vitro against *Candida albicans, Escherichia coli, Staphylococcus aureus, Salmonella typhi, Shigella dysentry* which are known to cause infections in humans. As summarized in the table, extract showed very little antimicrobial activity at a dose 1000 µg/disc against tested microorganisms. The highest inhibition zone diameter was 7 mm on *Candida albicans* and the lowest inhibition zone diameter was 6 on *Shigella dysentery*. The Reference drug penicillin showed highest zone of inhibition of 16 mm on *Staphylococcus aureus* and *Shigella dysentry* and lowest zone of inhibition of 14 mm on *candidas albicans and Escherichia coli at* 20µg/disk.

**Table 5**

***In vitro antimicrobioal activities of extract***

| samples | Concentration (µg/disc) | Inhibition zone diameter(mm) | | | | |
|---|---|---|---|---|---|---|
| | | Tested microorganisms | | | | |
| | | *Escherichia coli* | *Staphylococcus aureus* | *Shigella dysentry* | *Salmonella typhi* | *Candida albicans* |
| Extract | 1000 | 6.5 | 6.5 | 6 | - | 7 |
| Control | 10 | - | - | - | - | - |
| Amoxicillin | 20 | 14 | 16 | 16 | 15 | 14 |

- = No Inhibition

The results indicate that extract is not effective as an antimicrobial agent.

## 7.5Antioxidant test

**Table 6**

***Qualitative DPPH free radical scavenging activity of Leucas aspera***

| Plant | Compund | DPPH activity |
|---|---|---|
| Methanol extract of *Leucas aspera* | Fraction-1 | - |
| | Fraction-3′ | - |
| | Fraction-4 | - |
| | Fraction-5 | - |
| | Fraction-6 | - |
| | Fraction-7 | - |
| | Fraction-8 | - |
| | Fraction-9 | - |
| | Fraction-10 | - |
| | Fraction-11 | - |
| | Fraction-12 | - |
| | Fraction-13 | - |
| | Fraction-14 | - |
| | Fraction-15 | - |
| | Fraction-16 | +++ |
| | Fraction-17 | - |
| | Fraction-18 | - |
| | Fraction-19 | - |
| | Fraction-20 | - |

In the qualitative DPPH free radical scavenging activity test, Fraction-16 showed potent antioxidant activity. This fraction is a mixture and thus this fraction can be further separated to isolate the bioactive free radical scavenging compound. But other fractions did not show any antioxidant activity.

# CHAPTER 2

## 8. INTODUCTION TO PLANT- *CURCUMA ZEDORIA*

### 8.1 *Plant name*

**Scientific name:** ***Curcuma zedoria***

**Family**: Zingiberaceae

**Synonyms**: *Amomum latifolia* Lamik. (1692), *Curcuma officinalis* Salisb (1747), *Amomum zedoria* Christm (1779(, *Amomum zerumbet* Keon (1783), *Curcuma zerumbet* Roxb (1810).

**English name**: Zedorary

**Local name**:

- Shoti,
- Failla,
- Krachura (Sanskrit),
- Gandamatsi (Hindi) and
- Sutha (Bengali)

### 8.2 *Description*

Leafy rhizomatous herb, rhizome light yellow inside, sometimes brown tinged with age, with comphoraceous smell, some roots ending in ellipsoid tubers. Leaf tufts 1.0-1.2 m high. Leaves 4-6 in number, petioles 12-25 cm long, lamina oblong or narrowly oblong-lanceolate 20-60 X 9-5 cm, glabrous, acuminate at the apex, cuneate at the base , provided with a broad purple band along the midrib above. Spike radical, 14-19 x 5.5-6.0 cm, appearing before leaves on a 10-16 cm long peduncle, covered by 4 oblong sheaths. Fertile bracts 15-33, pale green, 3.5-5.0x 3.5 cm, glabrous, more or less rounded or obtuse at the apex, adnate to each other in the lower third, tip provided with short hairs, subtending 6-7 flowers, coma bracts 7-14, purplish-pink, up to 6.5 x 3.0 cm long, largest one more or less elliptic, obtuse, apiculate bracteoles obovate, folded, greenish-white, ciliate at the apex. Flowers exerted from the bracts. Calyx tubular, c 8 mm long white, obtusely 3 toothed or truncate, ciliate at the apex. Corolla tube funnel-shaped, c 27 mm

long, petals 3, white, laterals oblong-obtuse c 16 x10 cm, upper one hooded, spured at the apex, c 16 x 11 cm.

**Figure 7**: Curcuma zedoria

**Flowering:** April-May, Fruits are not usually formed

**Cromosome number**: 2n = 40

**Habitant**: Fallow lands by the road side and hill slopes

**8.3 *Distribution***:

Bhutan, India, Indonesia and Malaysia. In Bangladesh, this species is fairly common.

**8.4 *Economic uses/values/harmful aspects***

Rhizomes are expectorant, stomachic, cooing, diuretic, stimulant and carminative. Paste is used in leucoderma and piles; decoction mixed with pepper, cinnamon and honey is beneficial for cold, bronchitis and asthma and along with other ingredients is given in dropsy. *Shothi starch* prepared from the rhizomes is highly valued as an article of diet, especially for infants and convalescents. A red powder *abir* is prepared from powder rhizomes by treating it with a decoction of *sappon* wood.

**8.5 *Threats to species***: no immediate major threat is known. [19]

**8.6 *Ethnomedicinal or Traditional Uses***

*C. zedoaria* is a well known ethnomedicinal plant that is also used in Ayurveda. Its use in the Indian traditional folk medicine is also well documented. It is used traditionally for the treatment of menstrual disorders, dyspepsia, vomiting and for cancer. Rural people use the rhizome for its rubefacient, carminative, expectorant, demulcent, diuretic and stimulant properties while the root is used in the treatment of flatulence, dyspepsia, cold, cough and fever.

## 9. LITERATURE REVIEW OF *CURCUMA ZEDORIA*

### 9.1 Chemistry of *Curcuma zedoria*

*Curcuma zedoria* is reported to contain ashes, sugars, fibers, fat content, and protein with highest quantity of starch.

**The *general chemical composition of Curcuma zedoria***

**Table 7**

| Analysis % | Curcuma zedoria |
|---|---|
| Moisture | 8.82 |
| Ash | 0.33 |
| Fat | 0.03 |
| Protein | 0.52 |
| Starch | 86.84 |
| Fiber | 0.37 |
| Total sugars | 0.24 |
| Amylose (%) | 20.53 |

These contents may vary depending on season and geographical region.[20]

***Essential oil content in Curcuma zedoria***

The essential oil obtained by hydrodistillation of the rhizome of *C. zedoaria* native to north-east India has been analysed by gas chromatography (GC) and gas chromatography– mass spectrometry (GC-MS). Thirty-seven constituents representing about 87.7% of the total oil have been identified. Curzerenone (22.3%) was the major component, followed by 1,8-cineole (21) (15.9%) and germacrone (9.0%).The chemical investigation on essential oils of rhizomes of C. zedoaria, done by GC and GC-MS, revealed the presence of 1,8-cineole (18.5%), cymene (18.42%),a-phellandrene (14.9%) (27) and b-eudesmol (22) (10.6%).

The essential oil produced by hydrodistillation of *C. zedoaria* leaves was investigated by GC and GC-MS. Twenty-three compounds were identified, accounting for 75% of the oil. The oil of *C. zedoaria* was made up mainly of monoand sesquiterpenoids, monoterpene hydrocarbons (2.3%),

oxygenated monoterpenes (26%), sesquiterpene hydrocarbons (38%) and oxygenated sesquiterpenes (13.5%). The major constituents of the leaf oil were a-terpinyl acetate (8.4%), isoborneol (7%) and dehydrocurdione (9%).Chemical analysis of the volatile oil from *C. zedoaria* using GC-MS technique revealed the presence of b-tumerone (19.88%), 1,8-cineole (8.93%) and zingiberene (23) (7.84%) as major constituents. The essential oil of the dried rhizome was isolated using simultaneous steam distillation and solvent extraction and its fractions were prepared by silica gel column chromatography. In total, 36 compounds were identified in the essential oil, including 17 terpenes, 13 alcohols and 6 ketones. Epicurzerenone and curzerene were found in the first and second highest amounts (24.1 and 10.4 5) [21]

Jeng-Leun Mau. et all enlisted 36 essential oils derived from dried rhizomes of *Curcuma zedoria*.

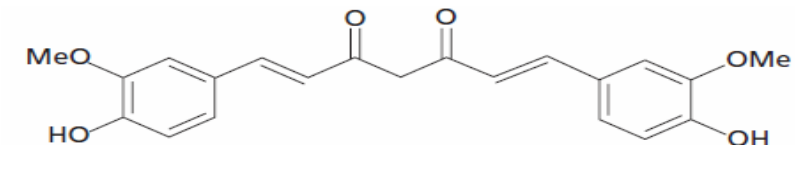
**Table: 8**

| No. | Constituent |
|---|---|
| 1. | α-Pinene |
| 2. | Camphene |
| 3. | β-Pinene |
| 4. | 1,8-Cineole |
| 5. | 2-Nonanone |
| 6. | 2-Decanone |
| 7. | Camphor |
| 8. | 2-Undecanone |
| 9. | β-Elemene |
| 10. | Elemene |
| 11. | b-Farnesene |
| 12. | α-Humulene |
| 13. | β-Himachalene |
| 14. | Zingiberene |
| 15. | α-Terpineol |
| 16. | α-Selinene |
| 17. | β-Selinene |

| | |
|---|---|
| 18. | β-Bisabolene |
| 19. | α-Curcumene |
| 20. | Germacrene B |
| 21. | α-Calacorene |
| 22. | Calarene |
| 23. | Curzerene |
| 24. | β-Elemenone |
| 25. | Epicurzerenone |
| 26. | Curdione |
| 27. | Elemol |
| 28. | Curzereneone |
| 29. | Spathulenol |
| 30. | α-Cadinol |
| 31. | Eudesmol |
| 32. | Curcumol |
| 33. | Isocurcumenol |
| 34. | Farnesol |
| 35. | Isospathulenol |
| 36. | 5-Isopropylidene-3,8-dimethyl-1(5H)-azulenone |

Thus it is evident that the plant contains a large amount of volatile oils.[22]

**Sesquiterpenoids from *Curcuma zedoaria* with structures**

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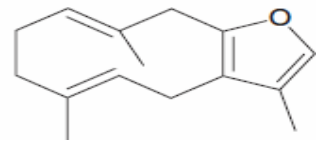


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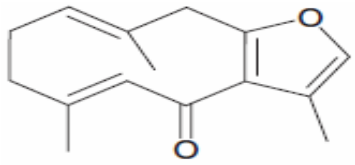

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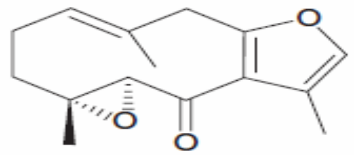

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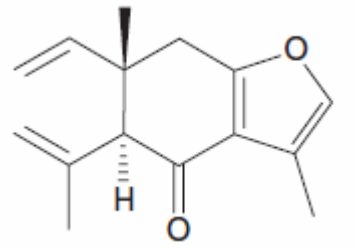

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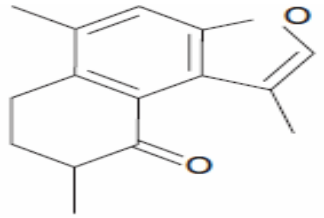

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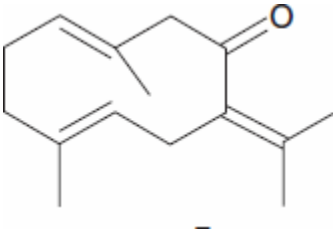

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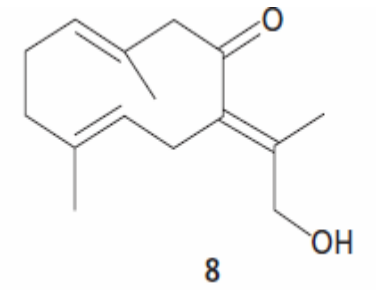

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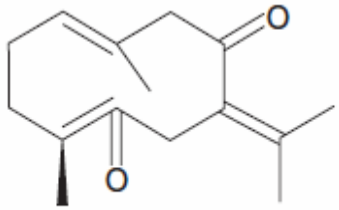

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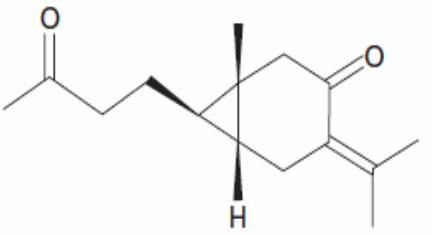

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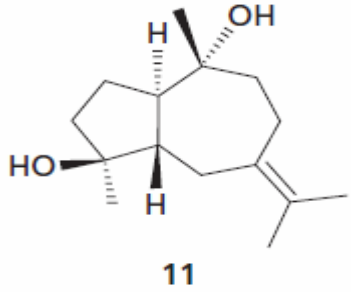

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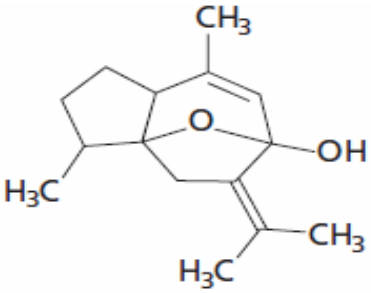

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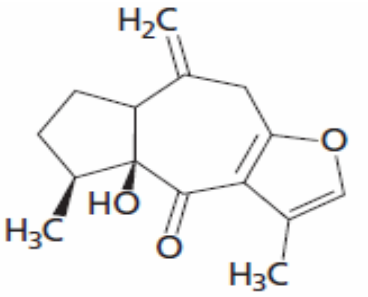

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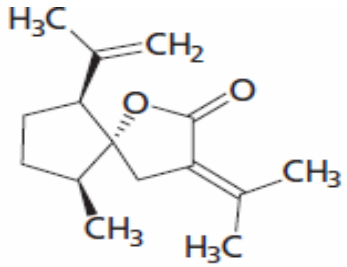


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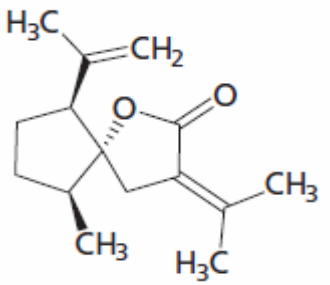


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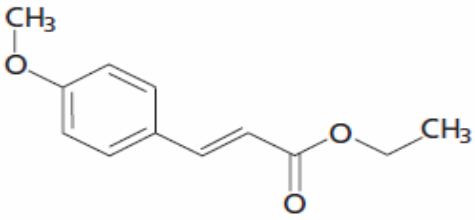


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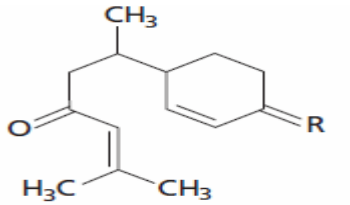


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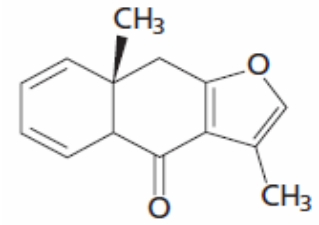
18 R = $CH_2$

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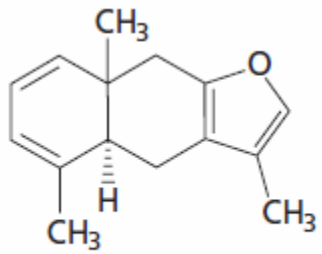


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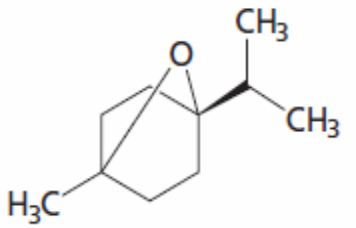


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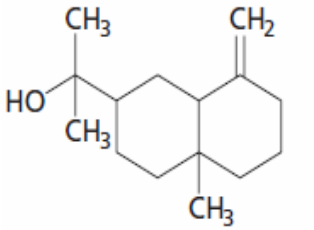


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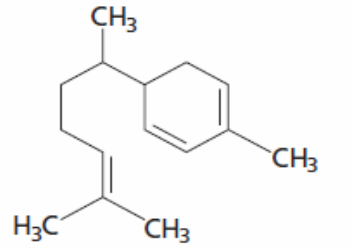


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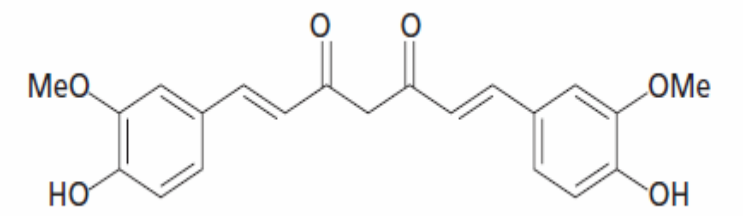


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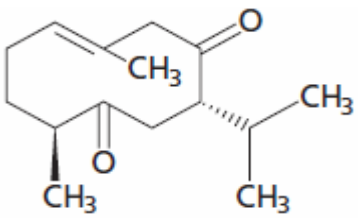


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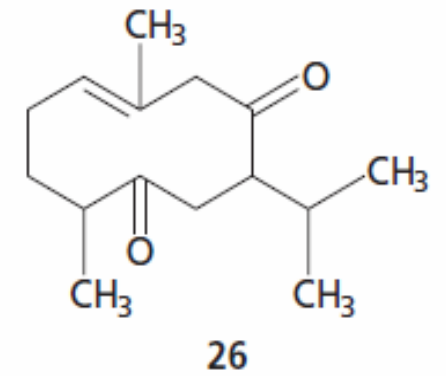


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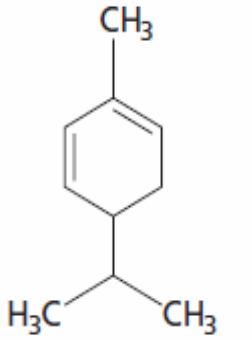
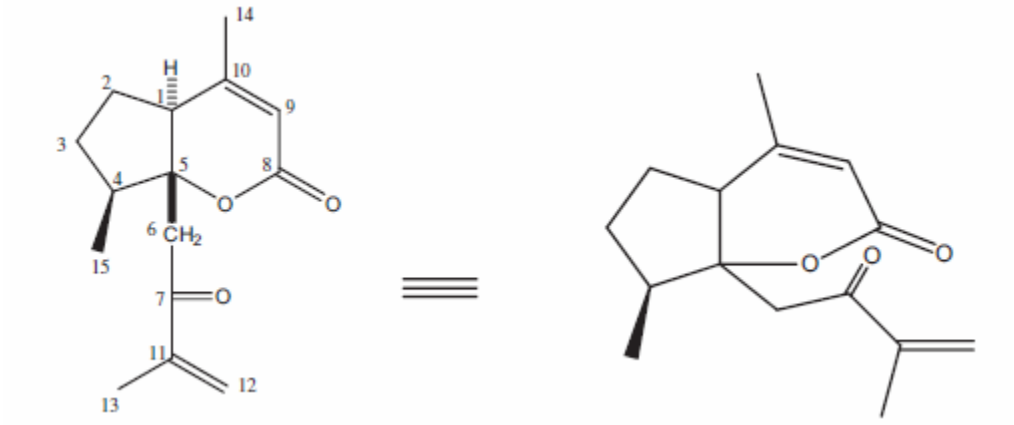



27

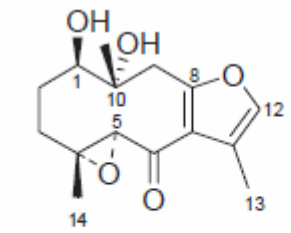
Structures of the biologically active compounds isolated from C. zedoaria. **1**, Cucrumin; **2**, furanodiene; **3**, furanodienone; **4**, zedorone; **5**, curzerenone; **6**, curzeone; **7**, germacrone; **8**, 13-hydroxygermacrone; **9**, dihydrocurdione; **10**, curcumenone; **11**, zedoaronediol; **12**, curcumenol; **13**, zedoarol; **14**, curcumanolide-A; **15,** curcumanolide-B; **16**, ethyl para-methoxycinnamate; **17, 18**, β-turmerone; **19**, epicurzerenone; **20**, curzerene; **21**, 1,8-cineole; **22**, β-eudesmol; **23**, zingiberene; **24**, dihydrocurcumin; **25**, curdione; **26**, neocurdione; **27**, α-phellandrene [21]

Other sesquiterpenoid found from *Curcuma zedoria* is curcuzedoalide.[23]

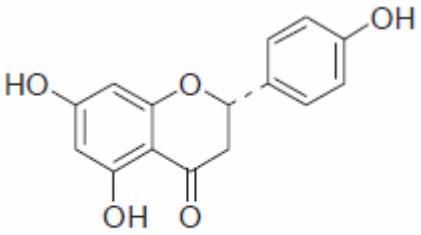



Curcuzedoalide

Another study describes isolation and structural determination of a new sesquiterpenoid **1**, named as curcuzederone, along with a known flavonoid, naringenin (**2**).[24]

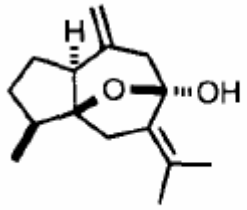



1

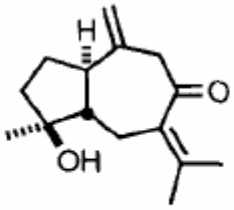



2

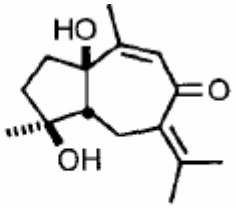
Other than all these sesquiterpenoids, some other hepatoprotective sesquiterpenoids are also found in *Curcuma zedoria*.[25]




isocurcumenol




isoprocurcumenol




aerugidiol

Aneweudesmane-type sesquiterpene, zedoarofuran, and six new guaiane or secoguaiane-type sesquiterpenes, 4-epicurcumenol, neocurcumenol, gajutsulactones A and B and zedoarolides A and B, were isolated from the aqueous acetone extract of *C. zedoaria* rhizome togetherwith 36 known sesquiterpenes and two diarylheptanoids. Their stereostructures were elucidated on the basis of chemical and physicochemical evidence. Two guaiane derivatives were isolated from the rhizomes of *C. zedoaria*. Their structures, zedoalactone A and zedoalactone B, were established by 1H and 13C NMR spectroscopic studies and by comparison with closely related compounds Zedoarol (13), 13-hydroxygermacrone and curzeone were isolated and structurally elucidated by Shiobara et al. Curcumin, dihydrocurcumin (24), tetrahydrodemethoxycurcumin and tetratetrahydrobisdemethoxycurcumin were isolated together with two bisabolane-type sesquiterpenes from 80% aqueous acetone extract of the rhizome of *C. zedoaria*.[21]

Bioassay-directed fractionation of an EtOH extract of *Curcuma zedoaria* led to isolation of three additional novel compounds, 3, 7-dimethylindan-5-carboxylic acid curcolonol and guaidiol.[26]

## 9.2 Pharmacological activity of *Curcuma zedoria*

### *Cytotoxicity*

Study isolated an active curcuminoid, demethoxycurcumin, together with curcumin and bisdemethoxycurcumin. The curcuminoids demonstrated cytotoxicity against human ovarian cancer OVCAR-3 cells.

### *Antimicrobial Activity*

The essential oils epicurzerenone and curdione were evaluated for potential antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonasa aeruginosa*, *Vibrio parahaemolyticus*, *Salmonella typhimurium* and *Bacillus cereus*. *V. parahaemolyticus* was sensitive to the presence of the essential oil, while the most resistant strain appeared to be *E. coli*. Based on 3- (4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay, nitroblue tetrazolium (NBT) reduction and cell morphology, the essential oil of *C. zedoaria* could inhibit the proliferation of human promyelocytic leukemia HL-60 cells. These results suggest that the essential oil has the antimicrobial activity against some of Gram- positive and negative pathogenic microorganisms and the components of the extract lead to the apoptosis of human cancer cell line. [27]

### *Inhibitors of Macrophage TNF-α Release*

1, 7-bis (4-hydroxyphenyl)-1, 4, 6-heptatrien-3-one (*1*), procurcumenol (*2*) and epiprocurcumenol (*3*) inhibite the production of TNF-α by lipopolysaccharide (LPS)-activated macrophages from the results of bioassay. These results imply that the traditional use of *C. zedoaria* rhizome as anti-inflammatory drug may be explained at least in part, by the inhibition of TNF-α production.[28]

### *Anti-inflammatory potency*

Dehydrocurdione, a sesquiterpene isolated from zedoary, was tested for in vivo and in vitro antiinflammatory actions. Oral administration of dehydrocurdione (40 to 200 mg/kg) mitigated the writhing reflex induced by acetic acid and the fever elicited by baker's yeast. A higher dose (200 mg/kg) of dehydrocurdione was required to inhibit the carrageenan-induced paw edema.

Oral administration of dehydrocurdione at 120 mg/kg/day for 12 days significantly reduced chronic adjuvant arthritis.[29]

Another study showed Anti-inflammatory sesquiterpene furanodiene and furanodienone suppresses the TPA-induced inflammation of mouse ears by 75% and 53%, respectively, at a

dose of 1.0 µmol. Their activities are comparable to that of indomethacin, the normally used anti

inflammatory agent.[30]

***Inhibitory potency***

β-Turmerone and ar-turmerone, sesquiterpenoids isolated from the rhizome of *Curcuma zedoaria*, inhibited lipopolysaccharide (LPS)-induced prostaglandin $E_2$ production in cultured mouse macrophage cell. In addition, these compounds exhibited inhibitory effects on LPS-induced nitric oxide production in the cell system.[31]

***Antimicrobial / Mouthrinse Activity***

Study comparing the antimicrobial activity of *Curcuma zedoria* against *Streptococcus mutans, Enterococcus faecalis, Staphylococcus aureus* and *Candida albicans* with five commercial mouthrinses showed it to be comparable to that of commercial products. Its incorporation into a mouthrinse could be an alternative for improving the antimicrobial efficacy of the oral product.[32]

***Antitumor / Genotoxicity / Anticlastogenic***

Antitumor, genotoxicity and anticlastogenic activities of polysaccharide from *Curcuma zedoaria*: Study suggests the CZ-I-III, the polysaccharide fraction from CZ, decreases tumor size of mouse and prevents chromosomal mutation.[33]

***Effect against tumor progression and immunomodulation***

The aim of the work was to study the effect of the crude extract of *Curcuma zedoaria* on peripheral blood cells and tumor progression in C57Bl/6J mice injected with B16F10 murine melanoma cells. The intraperitoneal therapy showed a significant increase in total white and red blood cell counts, a decrease in peritoneal cell number and tumor volume reduction, whereas the oral administration revealed a noteworthy augmentation only in total leukocyte count. These results contribute to evaluate the importance of alternative treatments that employ phytotherapic compounds against tumor progression and its possible immunomodulation.[34]

***Uterine Myoelectric Effect***

Study showed *C. zedoaria* has an exciting effect on the smooth muscle of uterus in rats, with a mechanism that may be associated with M- and alpha-receptors.[35]

***Antioxidant activity***

The compound responsible for better antioxidant properties (scavenging effect on 1, 1-diphenyl-2-picrylhydrazyl radical) might be 5-isopropylidene-3, 8-dimethyl-1(5H)-azulenone in *Curcuma zedoria*.[22]

***Anti-angiogenesis effect***

Essential oil, a fat-soluble fraction of *Curcuma zedoaria*, presented anti-angiogenic activity *in vitro* and *in vivo*, resulting in suppressing melanoma growth and lung metastasis. And this was associated with down-regulating MMPs.[36]

***Analgesic Activity***

In analgesic activity screening, the chloroform and methanol extracts showed significant activity on Swiss albino mice. Oral administration of the crude extracts at a dose of 400 mg/kg body weight, exhibits 29.5 and 38.0 % inhibition of acetic acid induced writhing in mice, respectively while the hexane extract shows weak analgesic activity in the experiment.[37]

## 10. EXTRACTION, SEPARATION & BIOLOGICAL TESTS - *CURCUMA ZEDORIA*

### 10.1 Collection and identification

The whole plant was collected from Panchagarh district of Bangladesh in November 2012.The whole plant with leaves, stems and roots was collected and identified by experts in Bangladesh National Herbarium, Mirpur, Dhaka, where a Voucher specimen (Accession No. 37516) has been deposited for future reference.

### 10.2 Drying of the rhizome

The plant was thoroughly washed with water. Aerial parts were discarded and rhizoid the parts were sliced into small pieces and spread on tray allowing shade drying. The rhizomes were dried for the next 10 days.

### 10.3 Grinding and storage of the dried samples

The dried parts were ground to coarse powder with a mechanical grinder (Grinding Mill). This process breaks the plant parts to smaller pieces thus exposing internal tissues and cells to solvents thus facilitating their easy penetration into the cells to extract the constituents. Then the powdered sample was kept in clean closed glass containers till extraction. During grinding of sample, the grinder was thoroughly cleaned to avoid contamination with any remnant of previously ground material or other extraneous matters deposited on the grinder. The weight of the total power was 1200gm.

### 10.4 Extraction of the dried powdered sample

After collection 1000gm of the powder was soaked in 2500ml Methanol in two tightly closed jar for 3 days and was shaken several times a day for mixing properly. The powder (1000gm) was extracted with Methanol at room temperature (2500ml x 72 hrs x 3 times). The extracting solvent

was filtered 3 times and the filtrate was concentrated in vaccuo by a rotary evaporator (50°C) yielding crude Methanol extract 136.15 g that is yield= 13.6%.

### 10.5 TLC of MeOH extract

Following the same method described before TLC was performed of the crude methanol extract of *Curcuma zedoria*. Sample was diluted with DCM. Different solvent systems were used as mobile phases, respectively on the basic of the nature of the compound. Charring was also done using same charring solution.

### 10.6 Vacuum liquid chromatography (VLC)

***Slurry preparation***

From the 136 gm methanol extract of *Curcuma zedoria*, 125gm was taken for slurry preparation. Left 1 gm was kept in a test tube for further use. Slurry was prepared mixing 90gm silica (0.063-0.020mm) and 125 gm extract using a few ml of dichloromethane (DCM). Thus the weight of slurry was 125+90=215 gm which was found to be 200 gm after one night drying.

***Procedure of performing VLC***

For VLC a Bruckner funnel was taken and an 11 cm filter paper was placed over it. The total 200 gm slurry was placed above the filter paper. Some n-hexane was poured to set the slurry in the funnel perfectly. Then another filter paper was placed above the slurry. Slowly 5 liter of n-hexane was poured into the funnel through the slurry. Then 5 liter of Dichloromethane (DCM), 5 liter of Ethyl-acetate, 5 liter of Acetone and 5 liter of Methanol was poured into the Bruckner funnel. Then all the fractions such as n-hexane fraction, DCM fractions were concentrated by evaporating the solvents using Rotary evaporator. A very low temperature (50°) was used so that the thermolabile bioactive constituents do not get destroyed. After evaporation all the fractions was collected in 50 ml beakers. The n-hexane fraction was named N-hexane-**Fraction -1**, DCM fraction-**Fraction-2**, Ethyl-acetate fraction- **Fraction-3,** Acetone fraction- **Fraction-4** and Methanol fraction- **Fraction-5.** Al the five fractions were weighed and stored. Their weights were Fraction -1=14.37 gm, Fraction-2=20.28 gm, Fraction-3=16.5 gm, Fraction-4=18.3 gm and Fraction-5=9.87 gm.

***Thin layer chromatography of the VLC fractions of Curcuma zedoria***

Following the same method described before second TLC was performed of all five fractions (n-hexane fraction, DCM fraction, Ethyl-acetate fraction, Acetone fraction and Methanol fraction. Samples were diluted with DCM. Charring was also done using same charring solution.

## 10.7 Column Chromatography of VLC fractions

### 10.7.1 Equipments:

1. Column
2. Pipettes
3. Beaker
4. Ring Stand
5. Foil Paper

### 10.7.2 Solvents:

1. Hexane
2. Ethyl acetate

### 10.7.3 Column chromatography of Fraction-1

***Packing of the Column:***

At first, a piece of cotton was placed at the bottom of the column. Then silica was added according to the amount calculated (110gm) with respect to the length and inner diameter of the column. Before stacking the silica on top of the cotton it was dissolved in hexane and then added to prevent the silica from cracking.

***Loading the Sample in the Column:***

After packing the column the n-hexane fraction (14 g) of *Curcuma zedoria* was stacked on top of the silica. For elution first pure n-hexane was used. After that the tap was used to drain the solvents out. Then the solvents were recycled again and again until the previous colored solvent was obtained.300 ml of n-hexane was recycled. Then elution was done with hexane and ethyl acetate gradients. Finally pure ethyl acetate was used to elute rest of the constituent component from the slurry.

***Collection of the Fractions:***

As the solvent passed through the column and came in contact with the extract, it caused different colored bands to appear in the silica. These bands were then collected as different fractions in beakers in amounts of 100 ml. Then the collected fractions were allowed to evaporate and preserved for later use. Fraction-1-42 was crystallized to get colorless crystal (60 mg)

***Analyzing the CZH-42***

After collecting the Fraction-1-42 crystal or CHZ-42, it was analyzed by TLC with the solvent systems; hexane: ethyl acetate (9.5:0.5) ratio to determine their purity.

### 10.7.4 Column chromatography of Fraction-2

***Slurry Preparation:***

At first, the DCM fraction (20.28 g) of *Curcuma zedoria* was dissolved in DCM and after that silica was added to it. They were mixed well until finely powdered. The slurry was left to dry for 24 hours.

***Determining solvent system by TLC:***

TLC of fraction-2 or DCM fraction was done using three solvent systems. The solvent systems were 20% ethyl-acetate, 50% ethyl-acetate and DCM: Methanol in (100:1) ratio. From the three solvent system 20% ethyl-acetate was preferred to use as the first solvent to elude the slurry.

***Packing of the Column:***

At first, a piece of cotton was placed at the bottom of the column. Then silica was added according to the amount calculated (90gm) with respect to the length and inner diameter of the column. Before stacking the silica on top of the cotton it was dissolved in hexane and then added to prevent the silica from cracking.

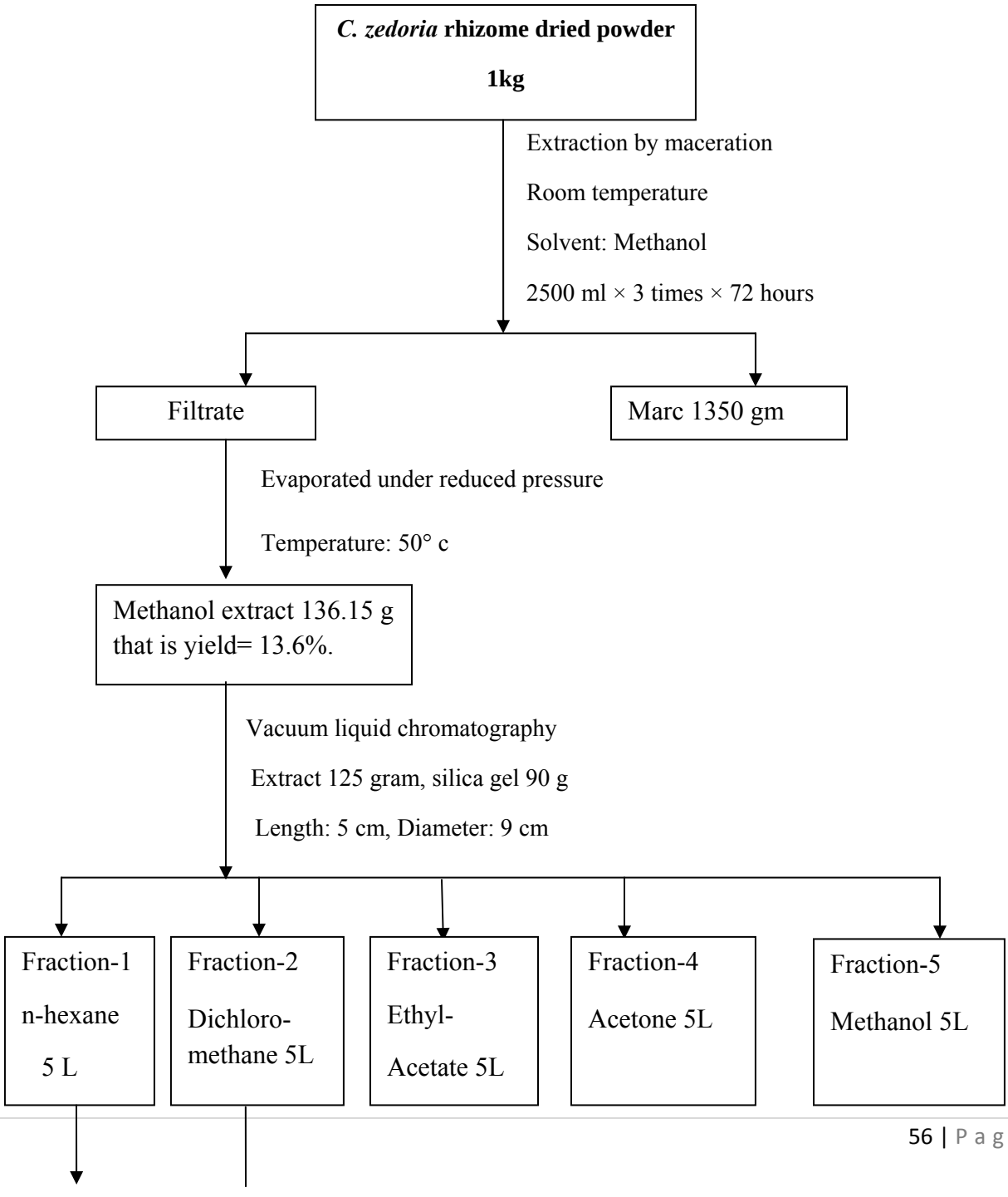
***Loading the Sample in the Column:***

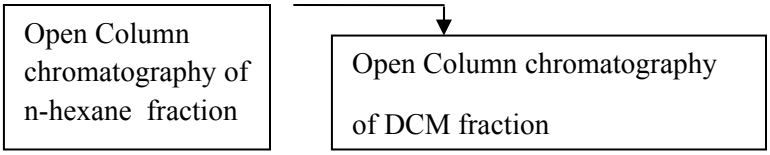
After packing the column the DCM fraction of *Curcuma zedoria* was stacked on top of the silica. For elution first 20% Ethyl-acetate was used. After that the tap was used to drain the solvents out. Then elution was done with various hexane and ethyl acetate gradients such as 40%, 50%, 60%, 75%, 80%, and 90%. Finally pure ethyl acetate was used to elute rest of the constituent component from the slurry.

***Collection of the Fractions:***

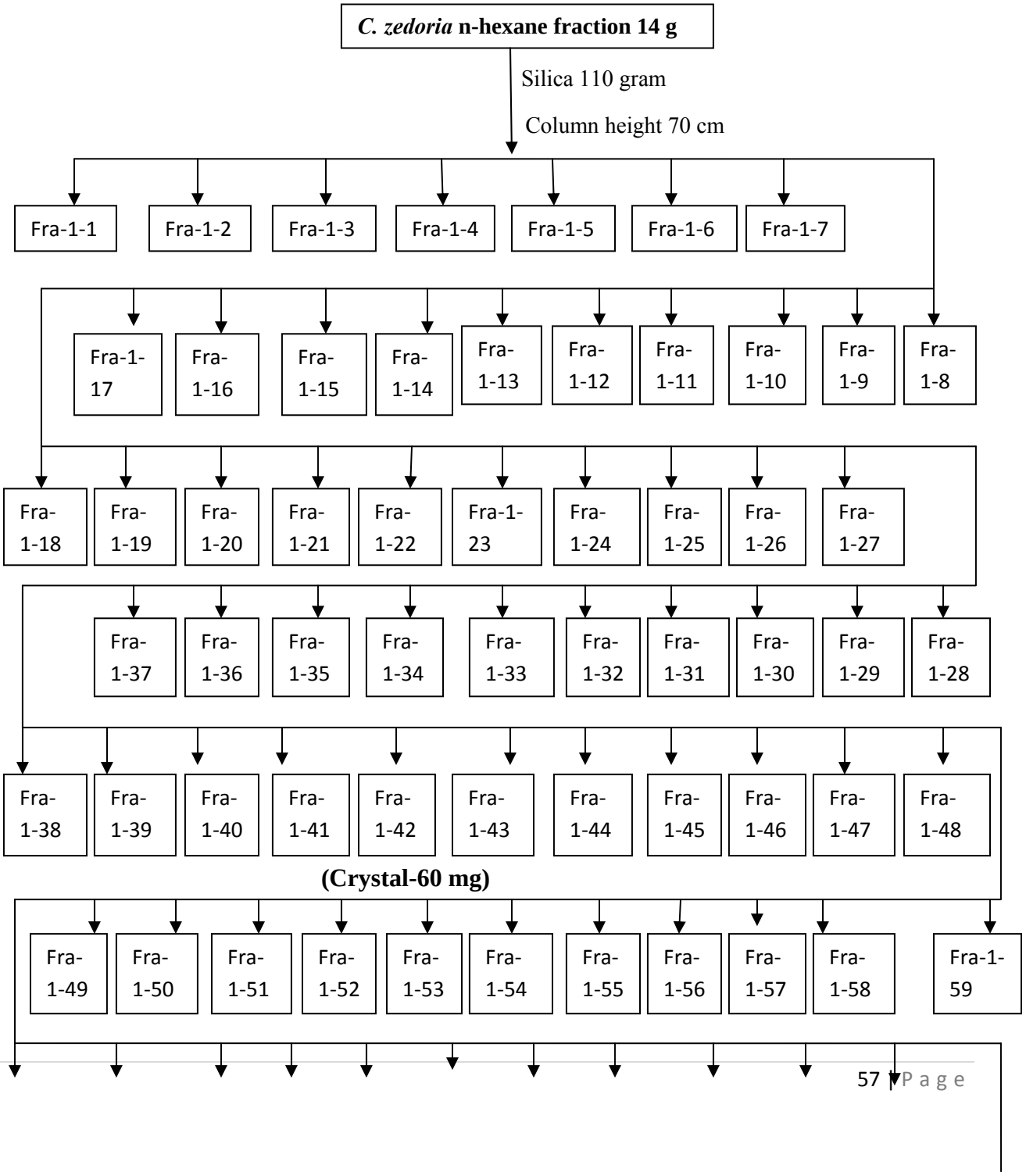
As the solvent passed through the column and came in contact with the extract, it caused different colored bands to appear in the silica. These bands were then collected as different fractions in beakers in amounts of 200 ml. Thus total 15 fractions were collected naming Fraction-2-1, Fraction-2-2, Fraction-2-3, Fraction-2-4, Fraction-2-5, Fraction-2-6, Fraction-2-7, Fraction-2-8, Fraction-2-9, Fraction-2-10, Fraction-2-11, Fraction-2-12, Fraction-2-13, Fraction-2-14 and Fraction-2-15.

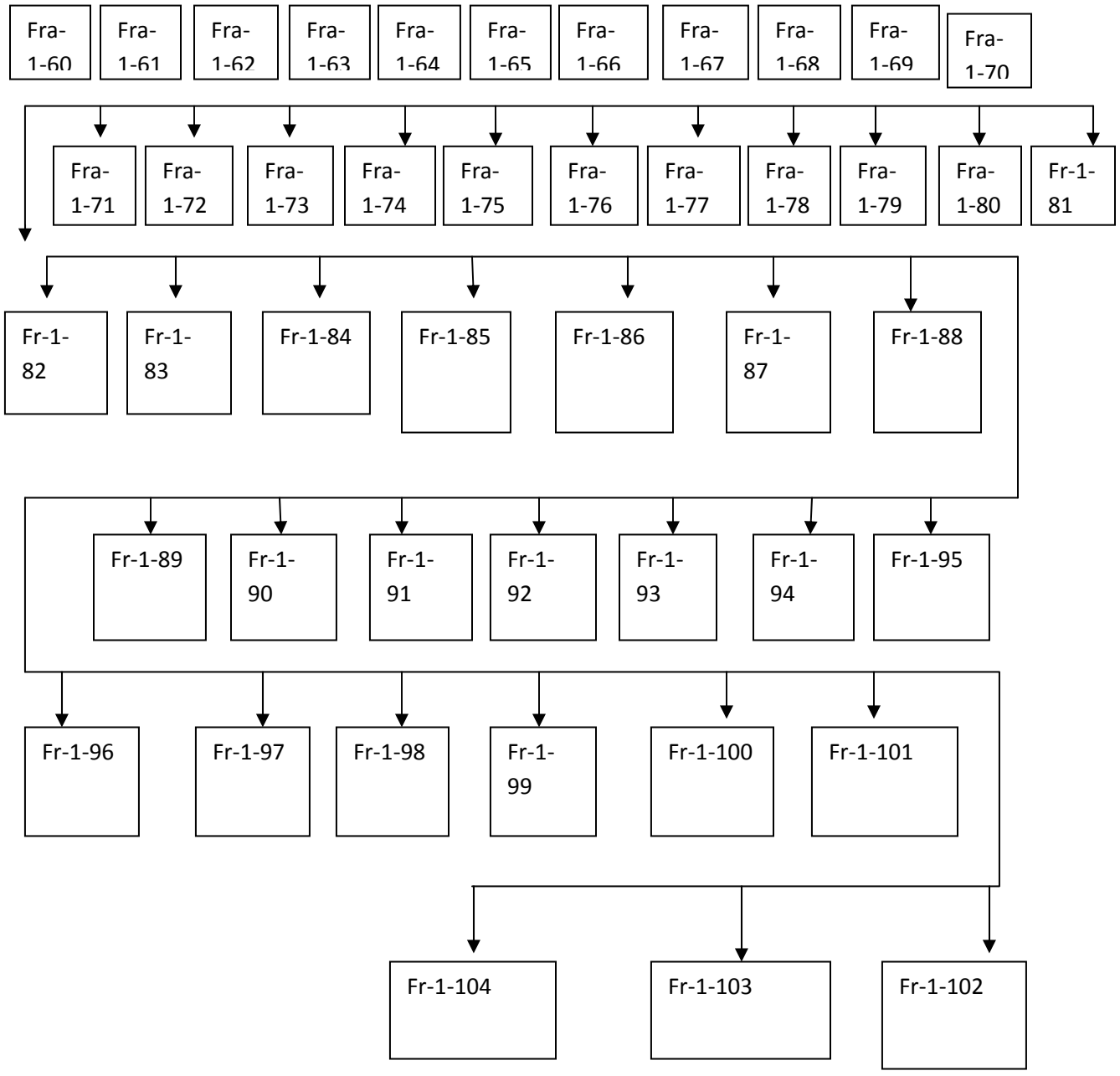
**Bioguided Isolation of Active Compounds from *Curcuma zedoria***






**Open column chromatography of N-hexane fraction of *Curcuma zedoria***




Fra-1-60
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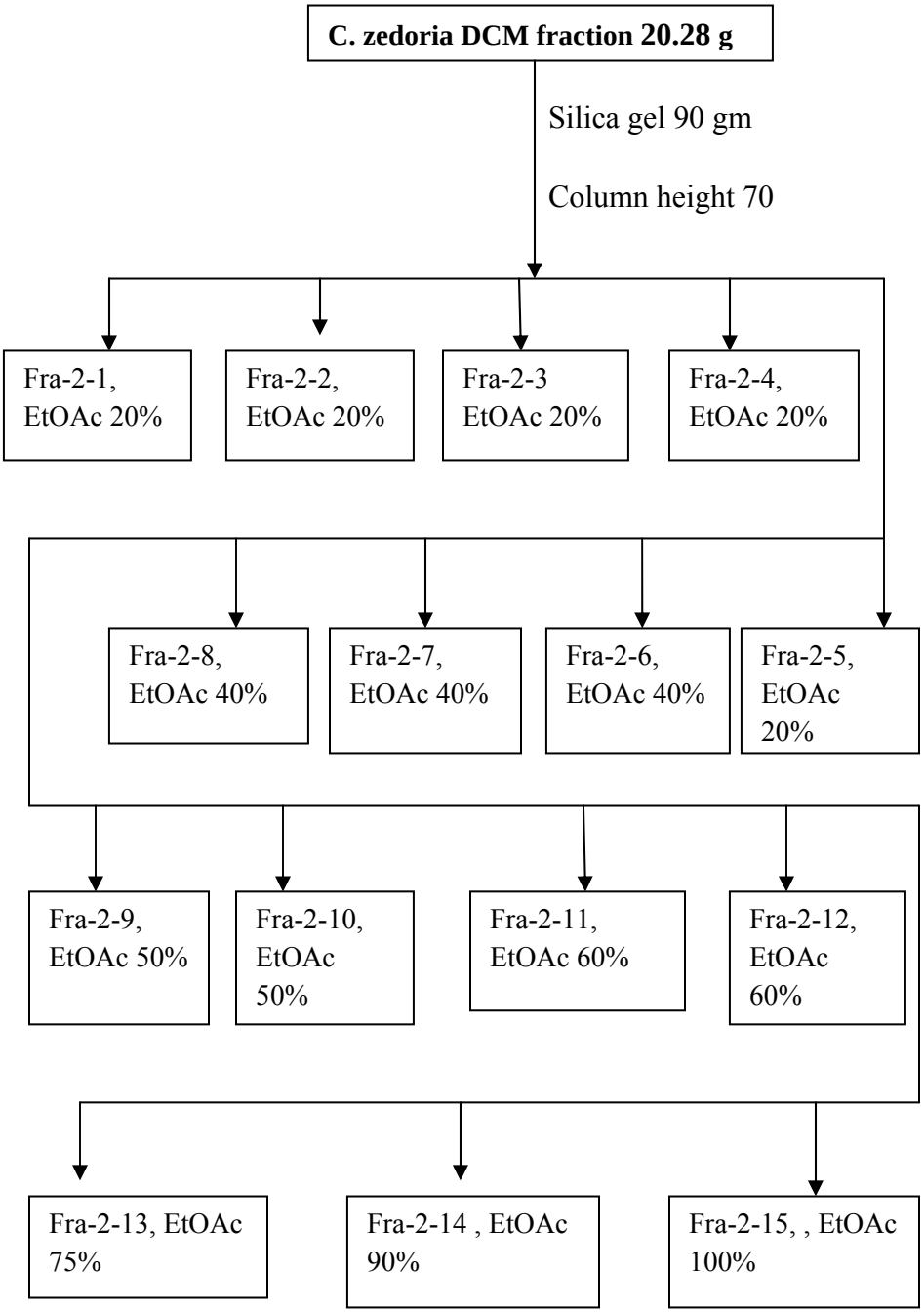
**Open column chromatography of DCM fraction of *Curcuma zedoria***



**10.8 Evaluation of Antibacterial activity:**

***Microorganisms***

The following microorganisms were used for disk diffusion method: *Bacillus subcutis, Escherichia coli, Saccharomyces cereviceae, Salmonella paratyphi, Shigella boydii*. The microbial species were collected from Department of Food and Nutrition, University of Dhaka. They were maintained on agar slant at 4˚C and sub-cultured on a fresh appropriate agar plate 24 prior to any antimicrobial test.

***Culture media***

Nutrient Agar (NA) was used for the activation of all bacteria and fungi. Agar medium was prepared by mixing 200m of distilled water with 5.6g agar and kept in autoclave.

***Bacterial solution preparation:***

In 5 eppendrof tube 1000µl of .9% (.9g NaCl in 100ml distilled water) NaCl was taken by sterilizing the loop. Five types of microorganism were transferred into the five eppendrof tubes. Suspension of these test organisms was prepared using vortex machine.

***Disc diffusion method***

The antimicrobial activity of the tested samples (isolated compound, standard drugs and negative control) were assayed by the standard disc diffusion method. Tested samples were dissolved in methanol and injected into sterilized discs of 6mm in diameter. Nutrient Agar(NA) sterilized at 121°C by using autoclave and cooled to 60–70 °C were distributed to sterilized Petri dishes with a diameter of 9 cm (15 ml). After preparation of the suspensions of test organisms (C.F.U $1.6\times10^5$ organisms per mL), 100µL of suspension was added to each Petri dishes and distributed homogeneously. Dishes injected with tested materials were placed on the solid agar medium.

Petri dishes were incubated at 37 °C for 24 h. In each plate 3 discs containing 3 different dose of Fraction-1-42 crystal or compound-1 (50µg/disc, 100µg/disc, and 200µg/disc) was applied. Then, on each plate a disc containing appropriate reference antibiotic (Amoxicillin 20µg/disc) disc and control (5µl methanol and DMSO (80:20)/disc) disc were applied. At the end of the period, inhibition zones formed on the NA were evaluated in millimeters. Studies were performed in duplicate, and the developing inhibition zones were compared with those of reference disks.

**10.9 Evaluation of Antioxidant activity:**

***Qualitative DPPH free radical scavenging activity assays***

For this test 0.02 % solution of radical-DPPH in methanol was prepared. 2 ml of this solution was taken in a vial. Very minute amount of Fraction-1-42 crystal or compound-1 was then added to the vial and kept for 10 minutes. The whole process was done in a dark place. Compound with radical scavenging activity will show color change (yellow or brown) due to the discoloration of DPPH.

## 11. RESULTS AND DISCUSSION OF *CURCUMA ZEDORIA*

### 11.1 Thin Layer Chromatography

**Table 9**

Thin Layer Chromatography (TLC-1) of Crude methanolic extract *Curcuma zedoria*

| Plant Species | Compound | Mobile Phase | Total spot | Spot | Rf Value | Observa-tion Under necked eye | UV Observa-tion | Observa-tion after charring | Quantity |
|---|---|---|---|---|---|---|---|---|---|
| Curcuma zedoria rhizomes | MeOH Extract | Hex:EtAc:Aceitic acid (10:50:1) | 1 | 1 | 0.90 | Brown | Deep Brown | Black | *** |
| | | | | 2 | 0.66 | Light brown | Purple Brown | Black | ** |
| | | | | 3 | 0.13 | Yellow | Brown | Ash | * |

- =No color, *** = Huge amount, ** =Moderate amount. * =small amount.

### 11.2 Vacuum Liquid Chromatography (VLC):

**Table 10**

Vacuum Liquid Chromatography of methanolic extract of *Curcuma zedoria* (125g)

| Name of the fraction | Code | Amount (g) | Yeild (%w/w) | Physical appearance |
|---|---|---|---|---|
| Fraction-1: N-hexane soluble fraction | Fraction-1 | 14.7 g | 11.76% | Light reddish brown liquid oil like appearance |
| Fraction-2: DCM soluble fraction | Fraction-2 | 20.28 g | 16.224 % | Deep brown thick sticky substance |
| Fraction-3: EtOAC soluble fraction | Fraction-3 | 16.5 g | 13.2% | Deep brown thick sticky substance |

| | | | | |
|---|---|---|---|---|
| Fraction-4: Acetone soluble fraction | Fraction-4 | 18.3 g | 14.64% | Deep brown muddy Structure |
| Fraction-5: MeOH soluble fraction | Fraction-5 | 9.87 g | 7.896 % | Blackish thick muddy structure |

### 11.3 Thin Layer Chromatography

**Table 11**

Thin Layer Chromatography (TLC-2) of five fractions of *Curcuma zedoria*

| Frac-tion | Compound | Mobile Phase | Total spot | Spot | Rf Value | Observation Under necked eye | UV Observa-tion | Observation after charring | Quantity |
|---|---|---|---|---|---|---|---|---|---|
| Fraction-1 | n-hexane fraction | EtOAc-Hex (8:2) | 1 | 1 | 0.37 | Yellowish | Brown | Deep blackish brown | *** |
| | | | | 2 | 0.51 | Yellowish | Brown | Deep blackish brown | *** |
| | | | | 3 | 0.69 | Brown | Purple brown | Ash | * |
| Fraction-2 | DCM-fraction | EtOAc-Hex (8:2) | 1 | 1 | 0.23 | Yellow | Purple | Ash | * |
| | | | | 2 | 0.46 | Yellow | Purple | Ash | * |
| | | | | 3 | 0.62 | Brown | Purple brown | Ash | ** |
| Fraction-3 | EtOAc fraction | EtOAc-Hex (8:2) | 1 | 1 | 0.30 | Yellow | Deep brown | Ash | ** |
| | | | | 2 | 0.46 | Yellow | Deep brown | Ash | ** |
| | | | | 3 | 0.46 | Brown | Deep brown | Ash | ** |
| | | | | 4 | 0.93 | - | Purple | - | * |
| Fraction-4 | Acetone fraction | EtOAc-Hex (8:2) | 1 | 1 | 0 | Brown | Deep brown | Black | *** |
| Fraction-5 | Methanol fraction | EtOAc-Hex (8:2) | 1 | 1 | 0 | Brown | Deep brown | Black | *** |

-- =No color, *** = Huge amount, ** =Moderate amount. * =small amount

### 11.4 Column chromatography of n-hexane fraction or Fraction-1

n-hexane fraction or Fraction-1 (14.37g) obtained by VLC was subjected to flash silica gel (110 gm) column chromatography and eluted with pure n-hexane and n-hexane-ethylacetate (Hex-EtOAc), gradient. 104 fractions of 100 ml each were collected: Hexane (Fraction-1-1 to Fraction-1-32), 10% EtOAc (Fraction-1-33 to Fraction-1-40), EtOAc 20% (Fraction-1-33 to Fraction-1-40), EtOAc 20% (Fraction-1-41 to Fraction-1-74), EtOAc 30% (Fraction-1-75 to Fraction-1-88), EtOAc 40% (Fraction-1-89 to Fraction-1-93), EtOAc 50% (Fraction-1-94 to Fraction-1-96), EtOAc 55% (Fraction-1-97 to Fraction-1-99), EtOAc 70% (Fraction-1-100 to Fraction-1-102) and EtOAc 100% (Fraction-1-103 to Fraction-1-104).

Crystals were observed in Fraction-1-42.The purity of the crystal was determined by TLC. The crystal was white with an amount of 60 mg. The crystal was then sent for NMR spectroscopy for structure elucidation.

### 11.5 Thin Layer Chromatography

**Table 12**

Thin Layer Chromatography (TLC-1) of Fraction-1-42 crystal of *Curcuma zedoria*

| Compound | Mobile Phase | Total spot | Spot | Rf Value | Observation Under necked eye | UV Observa-tion | Observation after charring | Quantity |
|---|---|---|---|---|---|---|---|---|
| Fraction-1-42 | hexane : ethyl acetate (9.5:0.5) | 1 | 1 | 0.35 | - | - | Red | *** |

- =No color, *** = Huge amount, ** =Moderate amount. * =small amount

### 11.6 Column chromatography of DCM fraction or Fraction-2

DCM fraction or Fraction-2 (20.28 gm) obtained by VLC was subjected to flash silica gel (90 gm) column chromatography and eluted with various n-hexane-ethylacetate (Hex-EtOAc) gradients. 15 fractions of 200 ml each (except Fraction-2-1) were collected: EtOAc 20% (Fraction-2-1 to Fraction-2-5), EtOAc 40% (Fraction-2-6 to Fraction-2-8), EtOAc 50% (Fraction-2-9 to Fraction-2-10), EtOAc 60% (Fraction-2-11 to Fraction-2-12), EtOAc 75% (Fraction-1-13) and EtOAc 90% (Fraction-2-14) and EtOAc 100% (Fraction-2-15).

### 11.7 Antimicrobial test

Antimicrobial activity of methanol extract of *Curcuma zedoria* has been evaluated in vitro against *Bacillus subcutis, Escherichia coli, Saccharomyces cereviceae, Salmonella paratyphi, Shigella boydii* which are known to cause infections in humans. As summarized in the table, extract showed very little antimicrobial activity at a dose 1000 µg/disc against tested microorganisms. The highest inhibition zone diameter was 12 mm on *Escherichia coli* and *Salmonella paratyphi* and the lowest inhibition zone diameter was 8 on *Saccharomyces cereviceae*. The Reference drug Amoxicillin showed highest zone of inhibition of 15 mm on *Escherichia coli* and lowest zone of inhibition of 14 mm on *Shigella boydii* at 20µg/disk.

**Table 13**

Antimicrobial activity of *Curcuma zedoria*

| Samples | Concentration (µg/disc) | Inhibition zone diameter(mm) | | | | |
|---|---|---|---|---|---|---|
| | | Tested microorganisms | | | | |
| | | *Escher-ichia coli* (mm) | *Bacillus subcutis* (mm) | *Shigella boydii* (mm) | *Salmonella paratyphi* (mm) | *Saccharomyces cereviceae* (mm) |
| Extract | 1000 | 12 | 9.5 | 8.5 | 12 | 8 |
| Amoxicillin | 20 | 15 | 14 | 12.5 | 14 | – |

- = No Inhibition

### 11.8 Antioxidant test

**Table 14**

***Qualitative DPPH free radical scavenging activity***

| Plant | Compund | DPPH activity |
|---|---|---|
| Curcuma zedoria | Fraction-1-42 | - |

Antooxident activity = +, Weak; ++, Intermediate; +++, strong. -, No activity

## 11.9 Spectral analysis

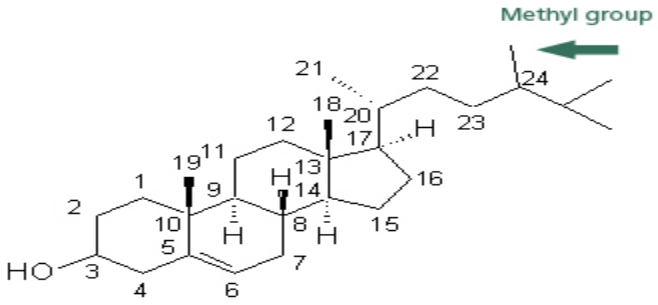
$^1$HNMR was used to elucidate the structure of CZH-42 crystal was obtained as colourless small sized crystals.

The report was like below-

δ ( $CDCl_3$ ,400 MHz) : 5.33(1H, brs, H-6) , 3.50 (1H, m, H-3), 2.2-2.38 (2H, m, J=17.1), 1.98 (3H, s), 1.81-1.88 (3H, m, 10.0), 1.41-1.62 (12H, m, J=16.9), 1.24 (5H, s), 1.14 (3H, s), 1.00 (6H, s), 0.91 (3H, d, J=5.1), 0.82 (6H, d, J=7.5), 0.81(3H, d, J=7.9)

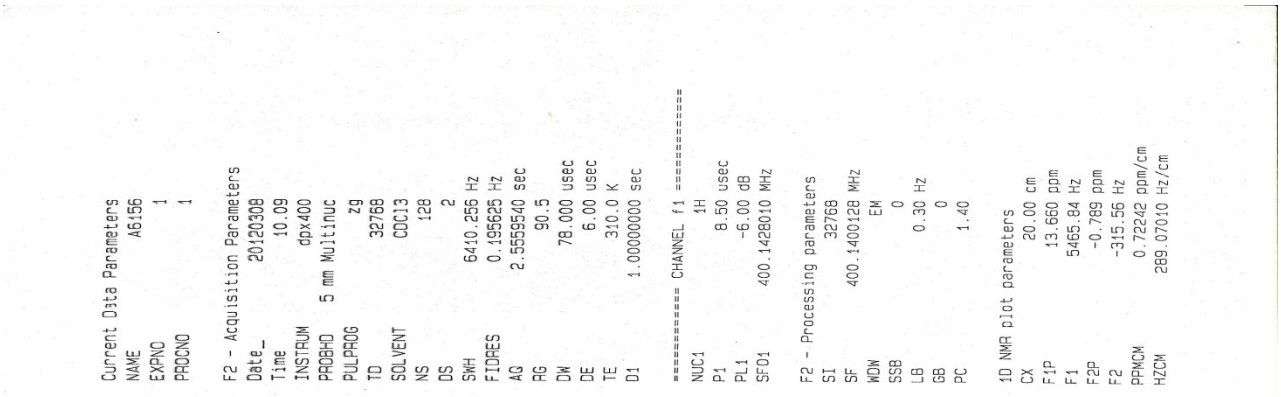
TLC analysis of CZH-42 with mobile phase EtOAc-MeOH (9.5:0.5) revealed that compound is UV inactive but after charring it gives a bright red colour. This clearly reveals that the metabolite is a steroidal compound. Now, from the $^1$HNMR report it is assumed that this compound may be Campesterol.

$^1$NMR data showed 6 methyl signals at δ 1.00 ($CH_3$ x 2), 0.91 ($CH_3$), 0.82 9 ($CH_3$ X 2) and 0.81 ($CH_3$). Among these methyl resonances four methyl peaks appeared as doublet (δ 0.91, 0.82 ($CH_3$ X 2) and 0.81) which indicates that these methyl groups can be attached to a tertiary group of the compound. Other two methyl signals appeared as singlets which indicate that these methyl groups are attached to quaternary carbon of the compound. The NMR spectral analysis also revealed that the presence of a oxymethylene proton by downfield shifted proton resonance at δ 3.0 (1H).Besides the CZH-42 also gave a signal of olefinic proton at δ 5.33 (1H).These analysis clearly indicates that CZH-42 should be campesterol, a steroid.




Champesterol

$^{1}$HNMR, $CDCl_3$, 400 MHz, Bruker NMR instrument



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PROCNO                     1

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PULPROG                zg
TD                     32768
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FIDRES            0.195625 Hz
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RG                      90.5
DW                78.000 usec
DE                 6.00 usec
TE                  310.0 K
D1                1.00000000 sec

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P1                       8.50 usec
PL1                     -6.00 dB
SFO1              400.1428010 MHz

F2 - Processing parameters
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WDW                       EM
SSB                        0
LB                    0.30 Hz
GB                         0
PC                      1.40

1D NMR plot parameters
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F2P                 -0.789 ppm
F2                  -315.56 Hz
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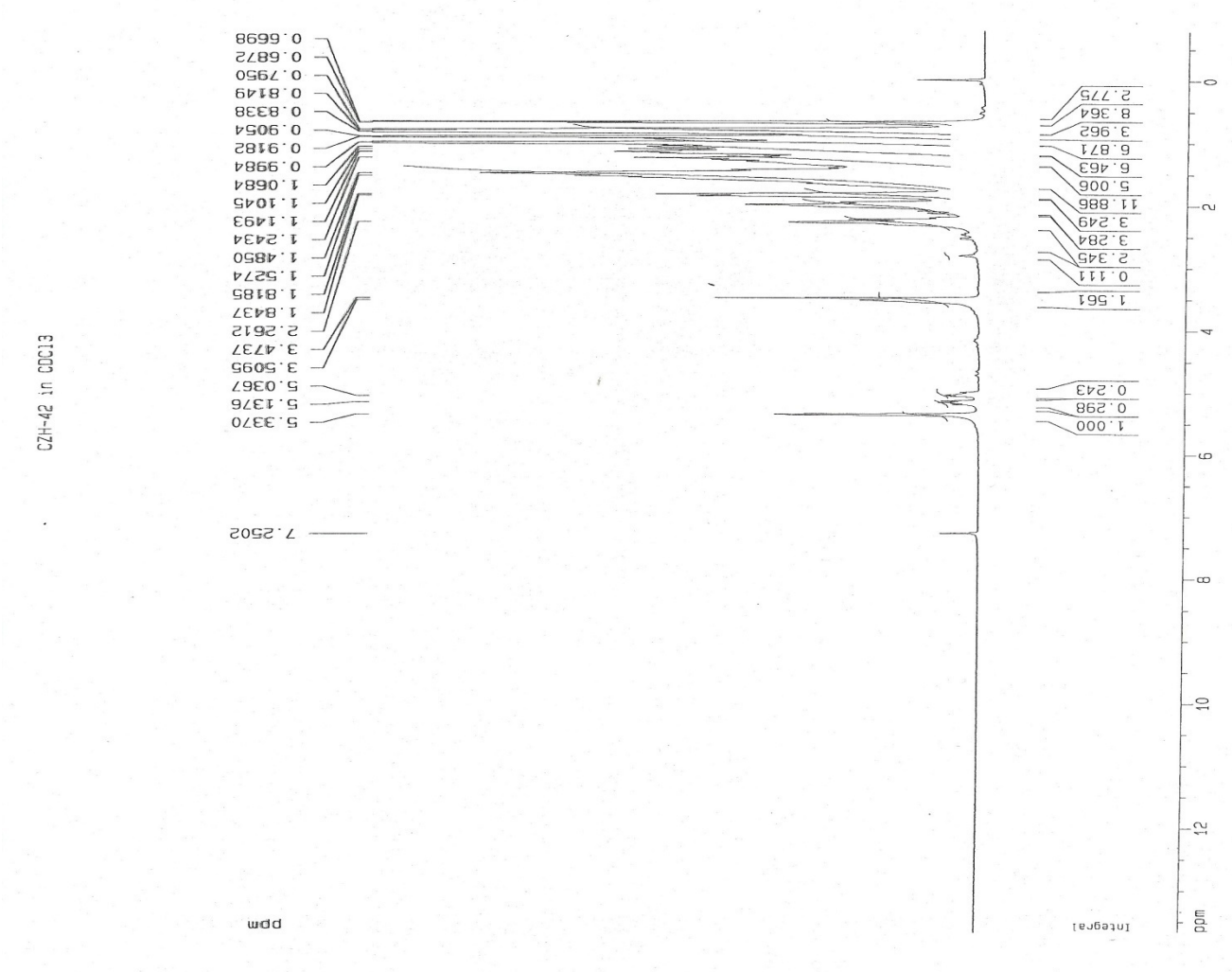
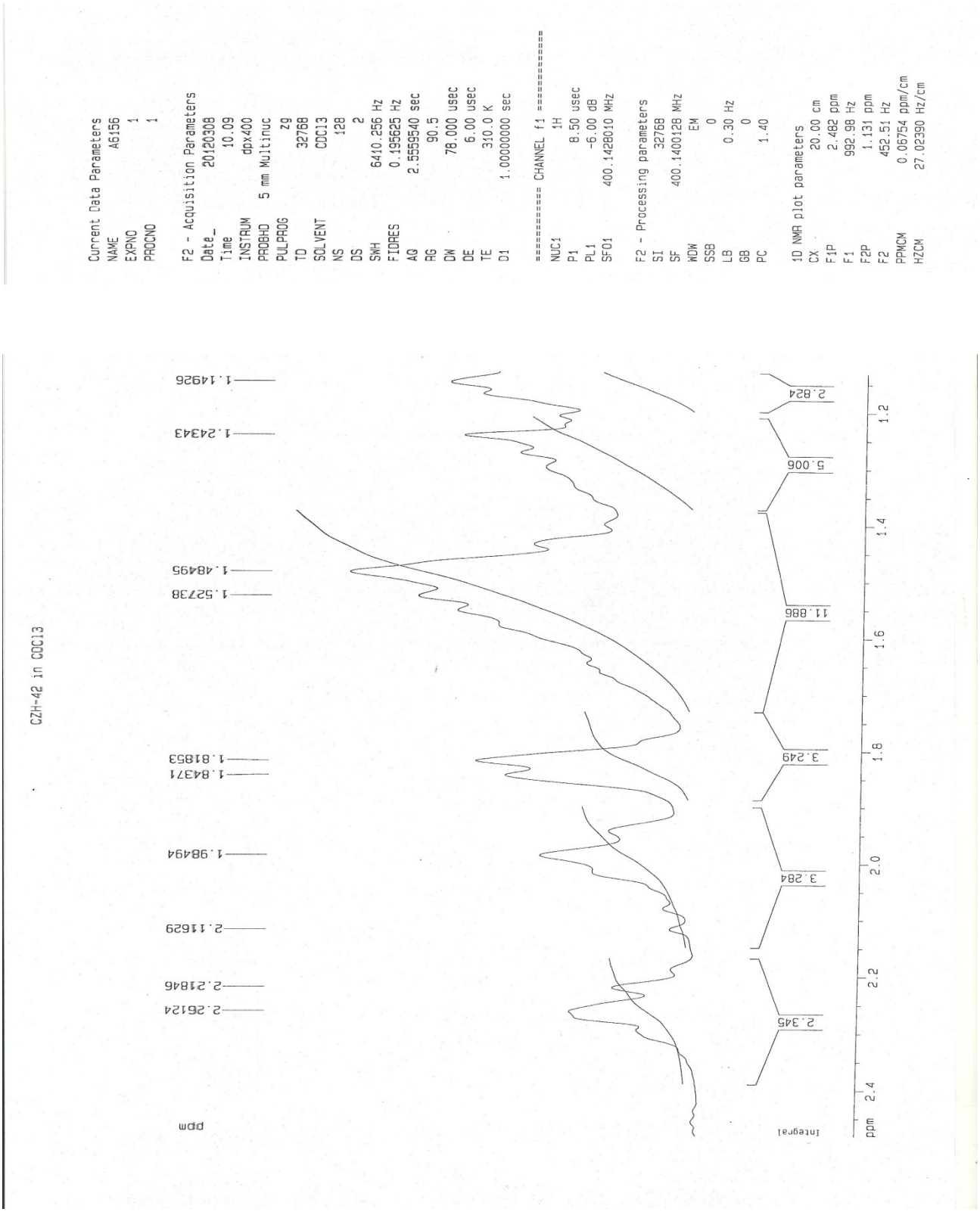
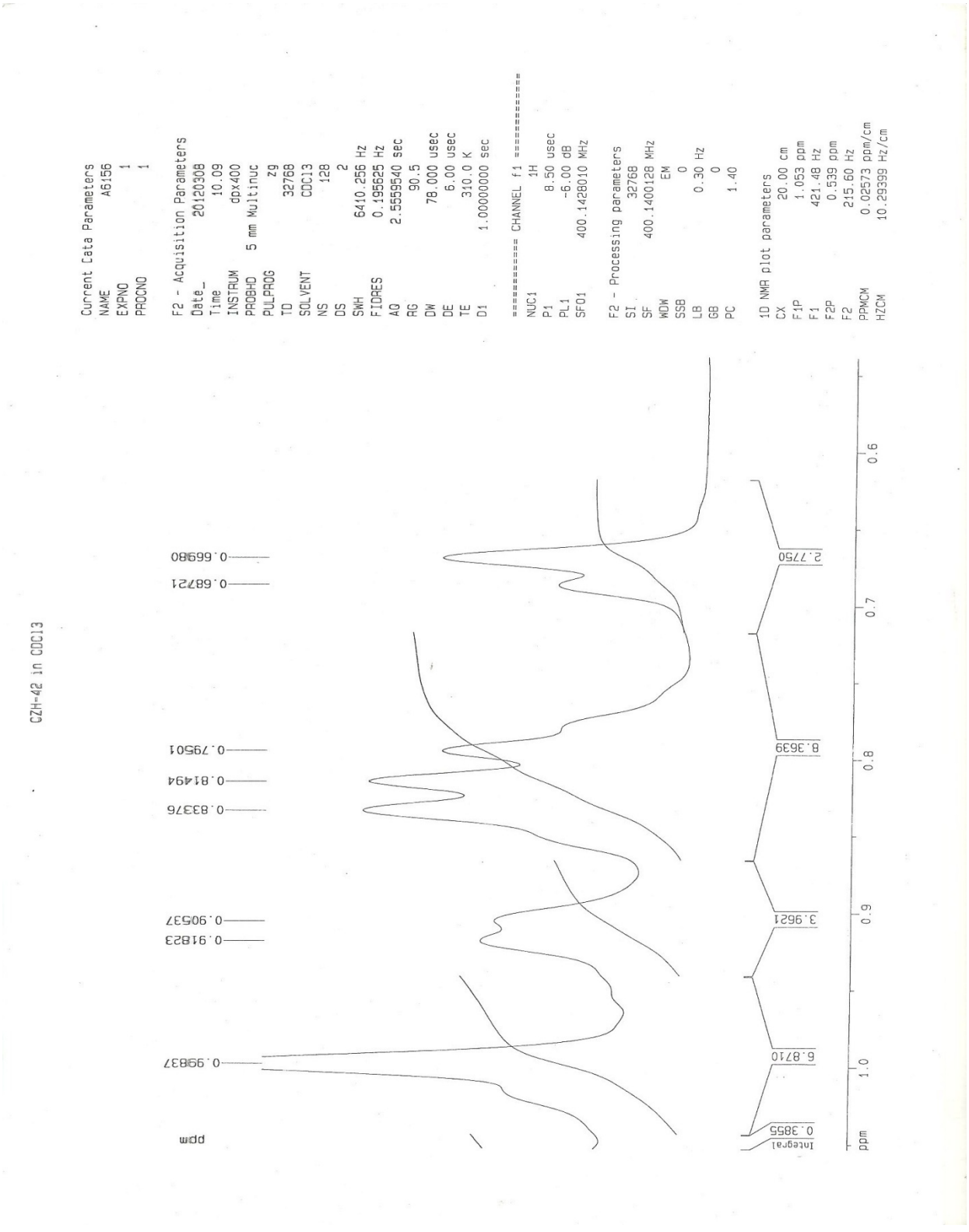



$^{1}$HNMR, $CDCl_3$, 400 MHz, Bruker NMR instrument




$^{1}$HNMR, $CDCl_3$, 400 MHz, Bruker NMR instrument




## 12. BIOSYNTHETIC PATHWAY OF CHOLESTEROL

***Cholesterol synthesis 1***

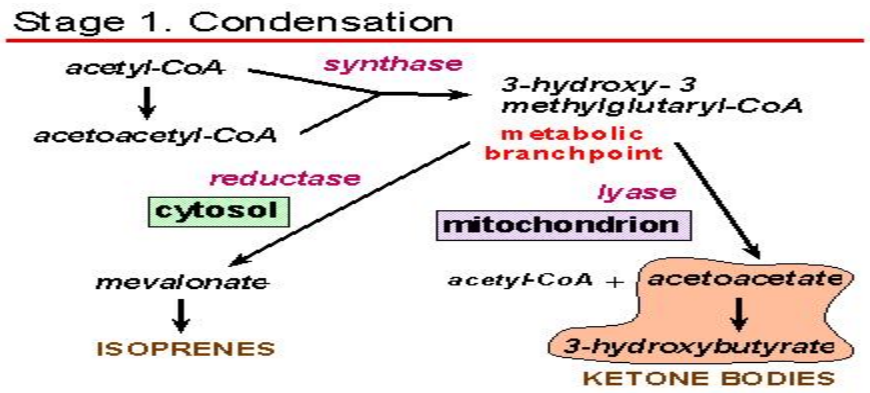



**Figure 8**: Condensation

Acetyl-CoA is the sole carbon building block for cholesterol synthesis. The intermediate 3-hydroxy- 3-methylglutaryl-CoA (HMG-CoA) sits at an important metabolic branch point: in the cytosol its fate is to the synthesis of polyisoprenes while in the mitochondrion it is the precursor of ketone bodies.

***Cholesterol synthesis 2***

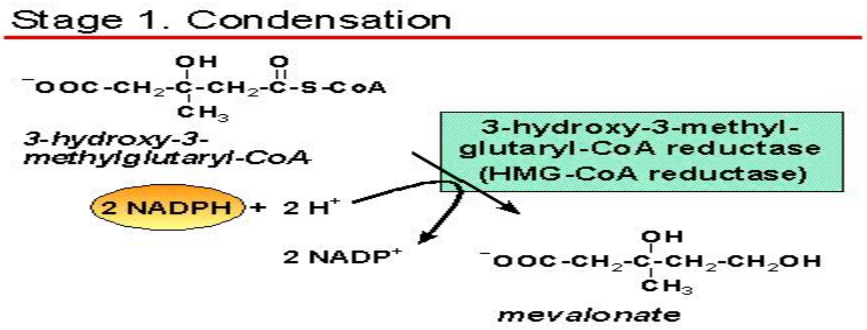



**Figure 9**: Condensation

The enzyme that catalyzes the reduction of 3-hydroxy-3- methylglutaryl-CoA in the cytosol, 3-hydroxy-3- methylglutaryl-CoA reductase (HMG-CoA reductase), is the major site of regulation in the synthesis of cholesterol and other polyisoprenes.

***Cholesterol synthesis 3***

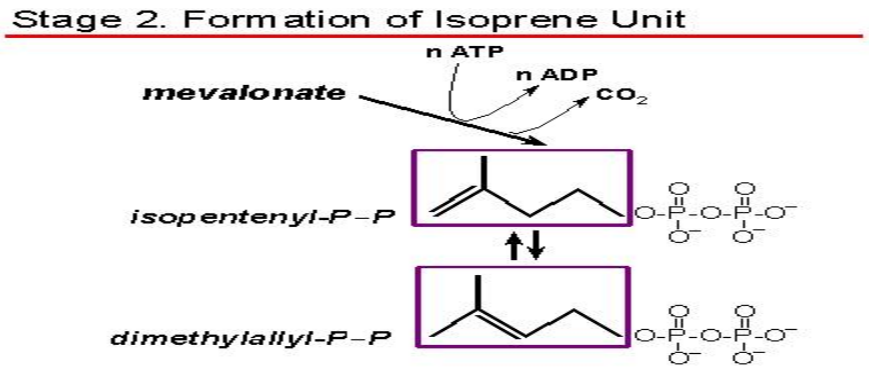



**Figure 10**: Formation of Isoprene unit

In the boxes are the two simple, 5-carbon isoprene structures that are found in ALL sterols, natural rubber, and many other natural products.

***Cholesterol synthesis 4***

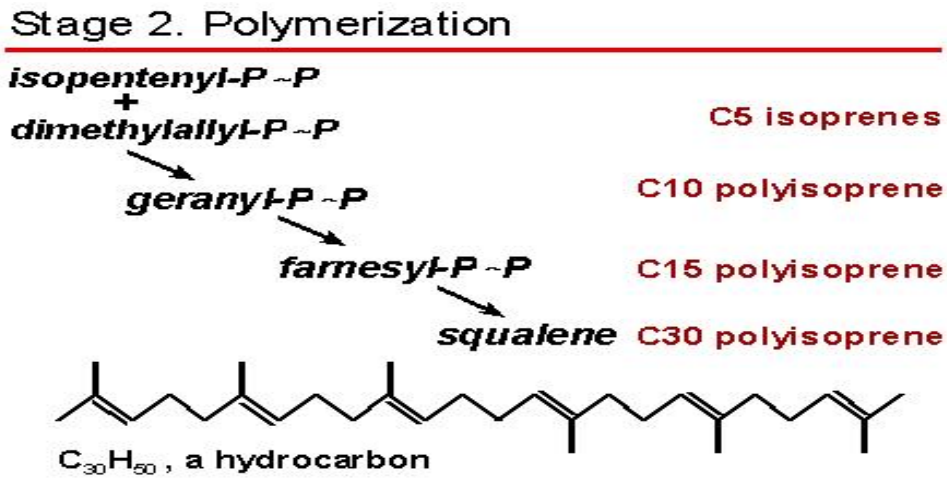



**Figure 11**: Polymerization

Polymerization usually occurs with additions of 5 carbons. Infrequently, fusions of larger structures take place, such as the formation of a molecule of squalene from two molecules of farnesyl-pyrophosphate. Squalene is an unusual biological compound, in that it is a pure hydrocarbon (only carbons and hydrogens).

***Cholesterol synthesis 5***

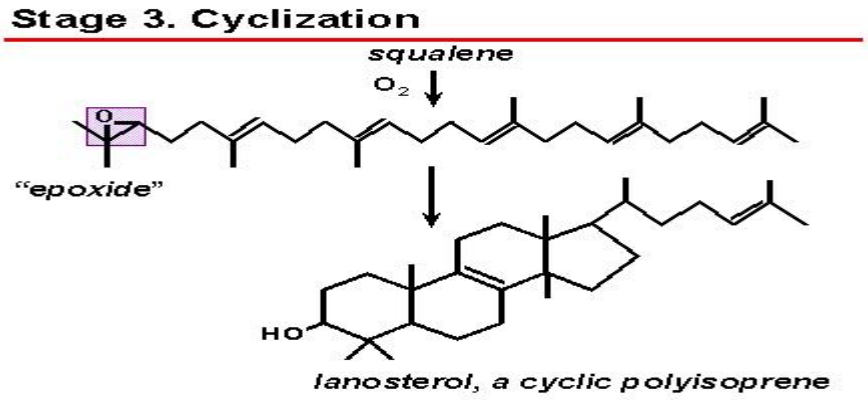



**Figure 12**: Cyclization

The cyclization of squalene to lanosterol is a remarkable series of molecular rearrangements.

***Cholesterol synthesis 6***

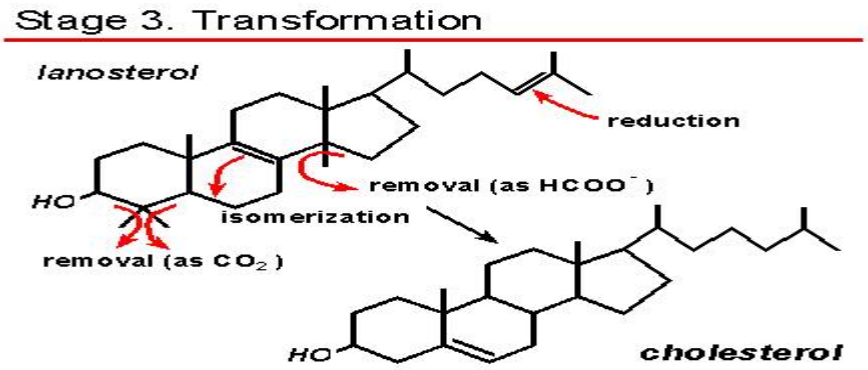



**Figure 13**: Transformation

The loss of the methyl group at carbon-14 as HCOO- (formate, anion of formic acid) yields a substrate for one-carbon metabolism that utilizes tetrahydrofolate-bound intermediates.

## 13. BIOSYNTHETIC PATHWAY OF CAMPESTEROL

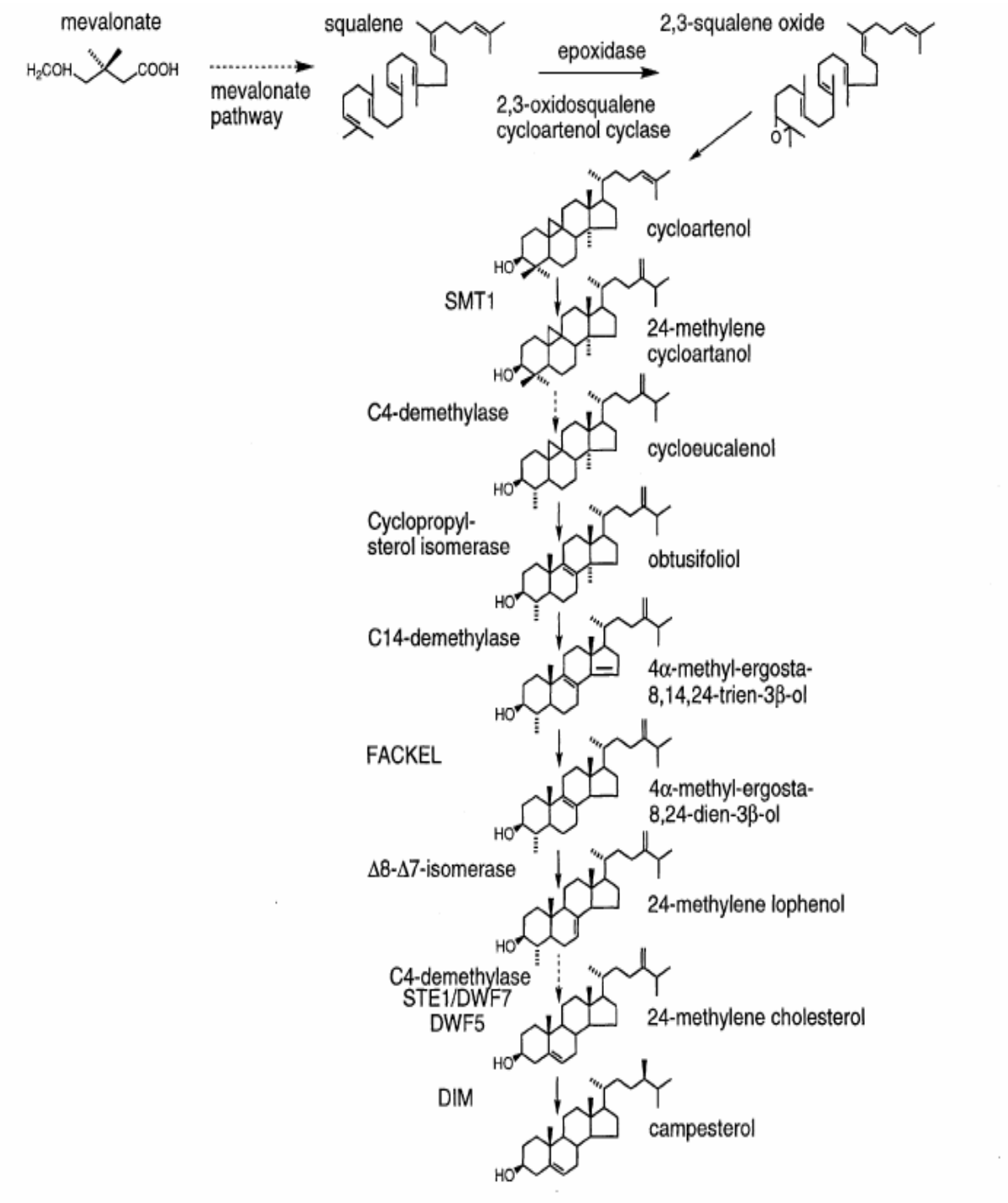



**Figure 14**: Biosynthetic pathway of Campesterol

## 14. CONCLUSION

From ancient to modern times, herbs and other plants have been used as medicinal agents,

first only on a folkloric basis and later developed on a scientific basis into single agent drugs. Phytomedicine, popularly known as herbal medicine, refers to the use of plant seeds, berries, roots, leaves, bark, or flowers for medicinal purposes. It has long reputation as "the people's medicine" for its accessibility, safety and the ease with which it can be prepared.

*Leucas aspera* (Family: Lamiaceae), locally known as 'Dondokalash' and *Curcuma zedoria* (Family: Zingiberaceae), are two medicinal herb, which have a good reputation in Bangladesh, India and many other countries of the world as a folk medicine for the treatment of a variety of diseases of different etiology. *Leucas aspera* plant's flowers, seeds, roots, berries, bark or leaves are recognized for treating nasal congestion, coughing, cold, headache and fever. The juices of the flower are known for treat sinusitis, as well as headaches. The juice of the flowers can also be used to treat intestinal worms in children. *Leucas aspera* has many medical benefits including: antifungal, prostaglandin inhibitory, antioxidant, antimicrobial, antinociceptive and cytotoxic. *Leusas aspera* has also been used as a snake bite antidote by applying bruised leaves to snake bites. This same technique can also be used to treat scorpion bites. The leaves of the *Leucas aspera* can be used to help treat chronic rheumatism. It is also an antipyretic.

On the other hand, *Curcuma zeroria*, commonly known as 'Shoti' is recognized for its effects on inflammation, wounds, skin ailments, pain, body stimulation, blood purification, respiratory disorders, digestion, liver condition, body temperature, urinary tract infections, tumor and cancer. But till to date, sporadic attempts have been made for the scientific and methodical validation of these traditional claims. Therefore, the present study was designed to isolate the bioactive compounds responsible for such activities, using different separation techniques. Moreover, a complete presentation of the literature review on the Ethno-medicinal, Phytochemical studies of these two plants have been presented along with Antioxidant activity and Antimicrobial activity tests.

Extraction of *Leucas aspera* leaves (50.00 g) and *Curcuma zedoria* rhizome (1.00 kg) at room temperature by maceration with MeOH yielded 10.20 g (20.40% of dry weight) and 136.15 g (13.60% of dry weight) extracts, respectively.

The MeOH extract of *Leucas aspera* was separated by open column chromatography with silica gel using step gradient of Hex-EtOAc-MeOH. The fractions were collected by monitoring with thin layer chromatography (TLC). Analysis of TLC of the MeOH extract and further column fractions did not show any characteristic spot of lignan derivatives. Elution with hexane-EtOAc (9:1) yielded Fr-3′ (0.85 g) and Fr-4 (0.55 g), respectively. On concentration of the solvent of Fr-3′ and Fr-4 yielded two colorless crystals LA-1 (12.00 mg) and LA-2 (7.00 mg), respectively. TLC analysis revealed that these compounds were UV inactive and charring with MeOH and $H_2SO_4$ (9:1) on TLC plate gave characteristic bright red color. This characteristic charring indicated the compounds were steroidal derivatives. Fr-16 (3.00 g), eluted with MeOH showed strong antioxidant activity. Further analysis using Infra-red spectroscopy, NMR, mass spectroscopy is required for structure elucidation. Fr-16 and rests of the fractions are mixtures and further separation is required for isolating pure bioactive compound. In the study, no lignin was found from this plant. The plant *Leucas aspera* showed no significant antimicrobial activity against *Escherichia coli, Staphylococcus aureus, Shigella dysentery, Salmonella typhi* and *Candida albicans*.

The MeOH extract of *Curcuma zedoria* showed significant antimicrobial activity. The zone of inhibitions were 12, 9.5, 8.5, 12 and 8 (mm) against *Escherichia coli, Bacillus subcutis, Shigella boydii, Salmonella paratyphi* and *Saccharomyces cereviceae,* respectively at a dose of 1000 µg/disc, MeOH extract of *Curcuma zedoria* was analyzed by TLC to get primary idea about its content. As crude extract is a mixture of thousand compounds, initially separating the extract into various discrete fractions will help to isolate pure compounds easily. Thus the crude methanol extract of *Curcuma zedoria* was subjected to vacuum liquid chromatography and five different fractions were collected using five different solvents; The fractions were – Fr-1 (14.37 g, eluting solvent: n hexane), Fr-2 (20.28 g, eluting solvent dichloromethane or DCM), Fr-3 (16.50 g, eluting solvent EtOAc), Fr-4 (18.30 g, eluting solvent Acetone) and Fr-5 (9.87 g, eluting solvent MeOH). Among the five fractions Fr-1 and Fr-2 were subjected to open column chromatography. Fr-1 of *Curcuma zedoria* was separated by open column chromatography with silica gel using step gradient of Hex-EtOAc-MeOH. The fractions were collected by monitoring with TLC. Fr-1-42 yielded a

colorless crystal, CZH-42 (60.00 mg). The physical appearance of the crystal was pure white. Analysis by TLC revealed the compound is UV inactive and shows red color upon charring Structure of CZH-42 was elucidated by the analysis of $^{1}$H-NMR spectral data which revealed that the compound could be campesterol, a plant steroid. Further analysis such as $^{13}$C-NMR and Mass Spectroscopy (MS) are required for confirmation. As per our knowledge, based on the literature review, this is the first time report of champesterol isolation from *Curcuma zedoria.* CZH-42 showed no antioxidant activity.

Fr-2 was then subjected to open column chromatography with silica gel using step gradient of Hex-EtOAc-MeOH and 11 fractions were obtained monitoring TLC. All of these fractions were mixtures of compounds which need to be separated further by column chromatography to isolate pure bio active compound. Bioactivities of these fractions are also needed to be tested. Moreover, further studies are warranted to clearly understand the underlying mechanism of the observed bioactivities and to isolate the active phytochemical constituent (s) responsible for such activities in animal models.

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