Isolation and Purification of active principles from

Curcuma amada

A dissertation is 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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Declaration by the Research candidate

I, Kazi Md. Mahabubur Rahman, hereby declare that the dissertation entitled **"Isolation and Purification of active principles from *Curcuma amada*",** submitted by me to the Department of Pharmacy, East West University, in partial fulfillment of the requirements for the award of the degree of Bachelor of Pharmacy (B.PHRM) is a complete record of original research work carried out by me during the period 2011-2012 under the supervision and guidance of **Dr. Chowdhury Faiz Hossain,** Dean, Faculty of Sciences and Engineering and professor, Department of Pharmacy, East West University and Professor, Department of Pharmacy 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.

Place: Dhaka

Date: 31/05/2012

Signature of the candidate

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Thesis Certificate

This is to certify that the thesis entitled "**Isolation and Purification of active principles from *Curcuma amada***", submitted to the Department of Pharmacy, East West University, in partial fulfillment of the requirements for the award of the degree of Bachelor of Pharmacy (B.PHRM) is a complete record of original research work carried out by Kazi Md. Mahabubur Rahman (ID. 2008-3-70-002) during the period 2011-2012 of his research in the Department of Pharmacy at East West University, under my supervision and guidance and the thesis has not formed the basis for the award of any other Degree/Diploma/Fellowship or other similar title to any candidate of any University.

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Acknowledgement

At first I would like to thank my research supervisor **Dr. Chowdhury Faiz Hossain,** Dean, Faculty of Sciences and Engineering and Professor, Department of Pharmacy, East West University for his constant support and understanding throughout the progress of the project. Without his sincere help and supervision and valuable advice it would not have been possible for me to accomplish this study.

I acknowledge to **Dr. Sufia Islam,** Associate Professor and Chairperson, Department of Pharmacy for giving me the opportunities to use Laboratory and other facilities and for encouraging me to do this research work.

I also acknowledge to **Dr. Nasim Sultana** of Dhaka Laboratory, Bangladesh Council of Scientific and Industrial Research (BCSIR) for measurement of NMR spectra.

I gratefully thank to Dr. **Bushra khan,** Chief Scientist, and **Mrs. Hosne Ara,** Director, Bangladesh National Herbarium for free identification and Accession Number of my 32 plants and sharing their knowledge to solve my queries.

Then of course, I express my earnest thanks to the Lab instructors of Pharmacy Department for their constant help.

I am also grateful to **Md. Al-Amin, Graduate teaching assistant of East West University** for giving me very helpful knowledge about the research work.

My special thanks go to others reseach students like, **Mahmudul Hasan Chowdhury, Asif Mahmud Tunan, Ismail Siragee, Abu Sadat Md. Sayem, Fahima Khan**, who worked with me in the lab.

Last, I am greatful to my family for their constant financial support and encouragement to complete the research work.

Thank you


This research paper
is dedicated to my
Mother and Father

ABSTRACT

Extraction of *Curcuma amada* rhizome (800 gm) at room temperature by maceration with methanol yielded 85.8 gm (10.73% of dry weight) extract. The methanol extract of *Curcuma amada* showed significant antimicrobial activity at a concentration of 1mg/disc. The methanol extract of *Curcuma amada* was subjected to vacuum liquid chromatography (VLC) and five different fractions were collected using five different solvents; Fractions were- Fraction-1 (6.50 gm) eluted with 5000 ml n-hexane, Fraction-2 (14.2 gm) eluted 3000 ml Dichloromethane, Fraction-3 (7.60 gm) eluted with 5000 ml ethyl acetate, Fraction-4 (26.5 gm) eluted with 5000 ml acetone and Fraction-5 (23.4 gm) eluted with 5000 ml methanol. Fraction-1 of was separated by open column chromatography with silica gel and the fractions were collected by monitoring the Thin Layer Chromatography (TLC) which gave 60 fractions. Among them Fraction-1-31 yielded a colorless crystal, CADH-31 (554 mg). This compound was UV active and charring with MeOH and H_2SO_4 (9:1) gave dark brown color. Based on ^{1}H-NMR analysis, structure of CADH-31 is proposed as shown below (Fig-i). To the best of our knowledge the proposed structure of CADH-31 is novel. ^{13}CNMR, Mass spectroscopy, 1H-1H COSY, HMQC, HMBC and NOESY are required to confirm the proposed structure. Fraction-1-35 yielded another colorless crystal, CADH-35 (50mg). The Antioxidant test of this CADH-31 showed no free radical scavenging activity up to a concentration of 1mg/ml. The antimicrobial activity test of CADH-31 showed no antimicrobial activity up to a concentration of 100µg/disc. Then Fraction-2 was subjected to open Colum chromatography and 184 fractions were separated according to different color bands in the column and by monitoring the TLC. Further chemical separation and testing biological activities of these fractions are yet to be done.

Key words: Extraction, Chromatography, Antioxidant, Antimicrobial, Spectroscopy, etc.

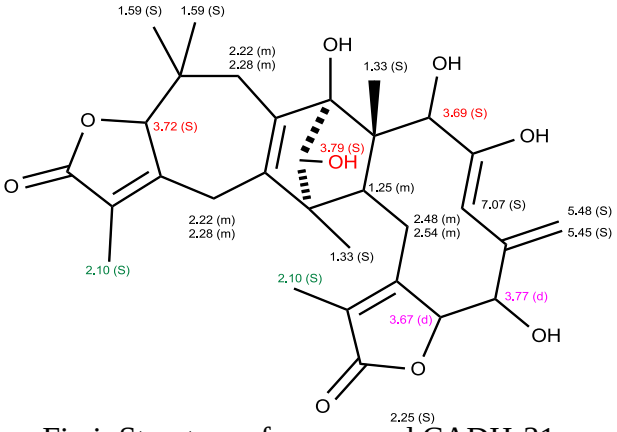

Fig i: Structure of compound CADH-31

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1.1 Introduction

Throughout the ages humans have relied on nature for their basic needs for the production of foodstuffs, shelter, clothing, means of transportation, fertilizers, flavors and not least, medicines. Nature has been a source of several medicines for treating various types of diseases in humans and animals for many years.

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

Plants have formed the basis for traditional medicine systems which have been used for thousands of years in countries such as China[1] and India.[2] The use of plants in the traditional medicine of many other cultures has been extensively documented. These plant-based systems continue to play an essential role in health care, and it has been estimated by the World Health Organization that approximately 80% of the world's inhabitants rely mainly on traditional medicines for their primary health care.[3] Plant products also play an important role in the health care systems of the remaining 20% of the population, mainly residing in developed countries.[4] In a study it has been shown that at least 119 chemical substances, derived from 90 plant species, can be considered as important drugs that are in use in one or more countries. Of these 119 drugs, 74% were discovered as a result of chemical studies directed at the isolation of the active substances from plants used in traditional medicine.[4]

Examples of traditional medicine providing leads to bioactive natural products abound. Suffice it to point to some recent confirmations of the wealth of this resource. Artemisine (1, Figure 1) is the antimalerial sesquiterpene from a Chinese medicinal herb *Artemisia annua* (wormwood) used in herbal remedies since ancient times.[5] Forskolin (2, Figure 1) is the antihypertensive agent from *Coleus forskohlii* Briq. (Labiatae), a plant whose use was described in ancient Hindu Ayurvadic texts.[6]

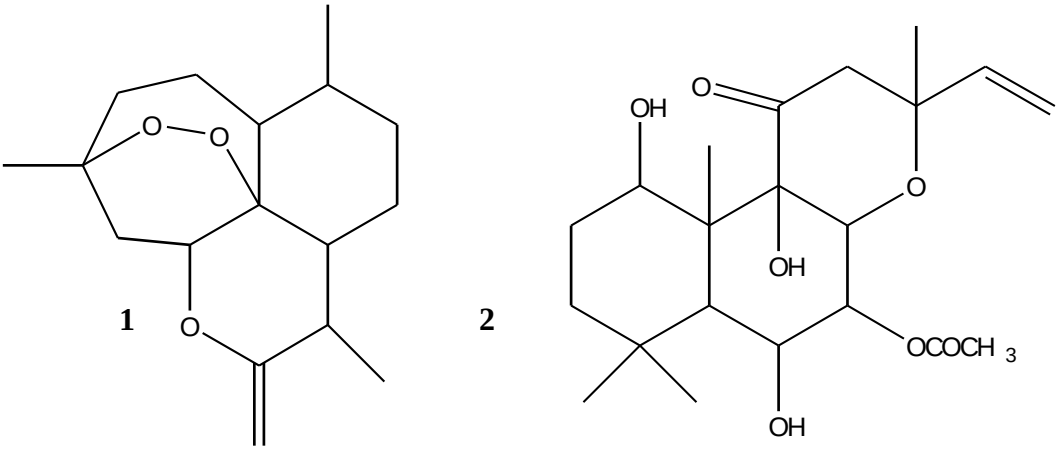


Figure: 1: Artemisinin (**1**) and Forskolin (**2**)

Paclitaxel (3, Figure 2) is the most recent example of an important natural product that has made an enormous impact on medicine. It interacts with tubulin during the mitotic phase of the cell cycle, and thus prevents the disassembly of the microtubules and their by interrupts the cell division.[7] The original target diseases for the compound were ovarian and breast cancers, but now it is used to treat a number of other human tissue proliferating diseases as well.[8]

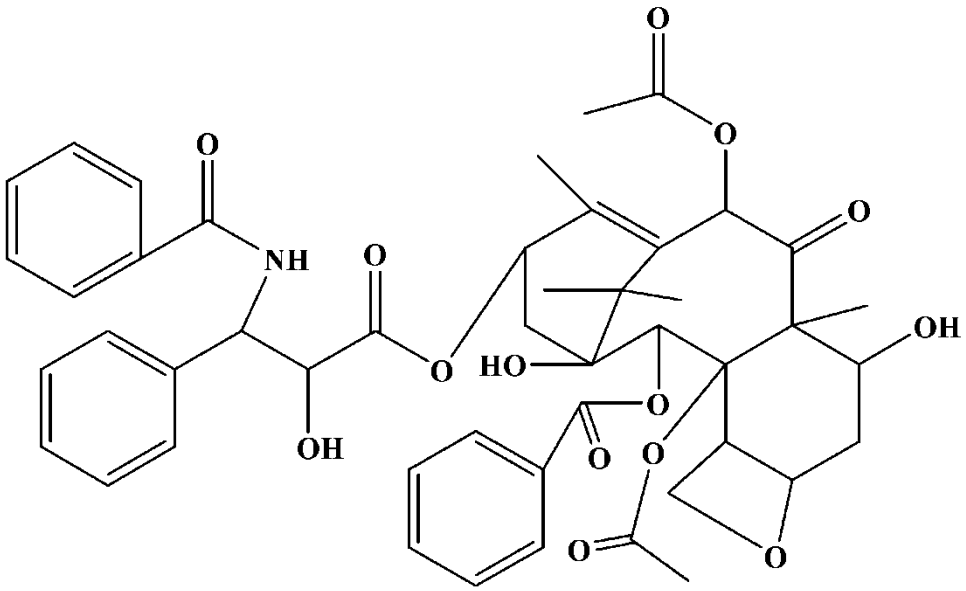
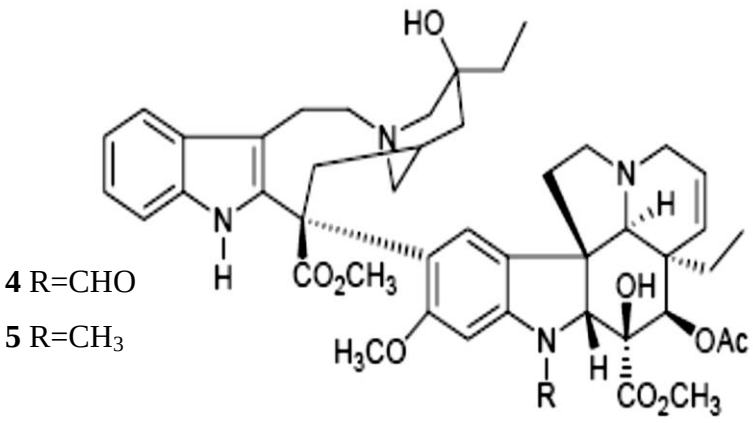


Figure 2: Paclitaxel (**3**)

A case of serendipity is the discovery of the so called vinca alkaloids, vincristine (4) and vinblastin (5), in *Catharanthus roseus*. A random screening program (conducted at Eli Lilly and Company) of plants with antineoplastic activity found these anticancer agents in the 40th of 200 plants examined. Ethnomedicinal information attributed an anorexigenic effect (i.e. causing anorexia) to an infusion from plant.[9]



4 R=CHO

5 R=CH_3

Fig 3: Vincristine (**4**) and Vinblastin (**5)**

In recent years, there is growing interest in the therapeutic use of natural products, especially those derived from plants, due to several reasons including 1.Conventional medicines have more side effects and ineffective in therapeutical use, 2. Abusive or incorrect use of synthetic drugs may cause many problems and side effects, 3. A huge number of population in the world not depend on conventional pharmacological treatment, and 4. Folk medicine and ecological awareness suggest that "natural" products are harmless.[10]

Within the next quarter century, the achievements of science and technology will be so great that, when brought to bear upon the mysteries of nature that have long puzzled us those mysteries will yield their secrets with amazing rapidity. It will be a fascinating and eventful period. We will not know only the causes of disease but the cures for most. Significant new drugs of plant origin and new methods of producing them will continue to be important parts of that service and thus Plants are considered as are of the most important and interesting subjects that should be explored for the discovery and development of newer and safer drug candidates.[10]

1.2 Objective of the work

Bangladesh has a rich source of a large number of medicinal plants belonging to various families like Zingiberaceae. Zingiberaceae or the Ginger family, is a family of flowering plants consisting of aromatic perennialherbs with creeping horizontal or tuberous rhizomes, comprising ca. 52 genera and more than 1300 species, distributed throughout tropical Africa, Asia, and the Americas. *Curcuma* (*Cúr-cu-ma*) is a genus of about 80 accepted species in the plant family Zingiberaceae that contains such species as turmeric and Mango-ginger. The name comes from Arabic *kurkum* (كركم) meaning "turmeric".[10] The species contain a wide range of pharmacologically active compounds which are very useful and effective against

various diseases like appetizer, antipyretic, aphrodisiac and laxative. It is useful in biliousness, itching, skin diseases, bronchitis, asthma, hiccough and inflammation due to injuries.[11] The rhizomes and roots are carminative and stomachic and in crushed pulp form they are applied over contusions, sprains and bruises for rapid healing.[12] Although some of these uses are based on old and new experiences and clinical data, many of them have no foundation what so ever. Therefore an attempt has been taken to study systematically the chemical constituents of *Curcuma amada* a member of the Zingiberaceae family, growing in Bangladesh and evaluate its pharmacological profiles. So, the main objective is to explore the possibility of developing new drug candidates from *Curcuma amada* for the treatment of various diseases.

1.3 Research of Traditional drug in Bangladesh

In rural areas medicinal plants have been being used as remedy for disease for a long time. They not only cure the disease but also provide an important role in the economy. Medicinal plants are cheap and easy to get to those people who knew it very well. Bioactive compounds are deposited in medicinal plants; it can serve as important raw materials for pharmaceutical manufacturing. They comprise a precious asset of a country and donate to its health care system.[13] Well-judged and scientific investigation of this wealth can significantly contribute to the public health. Besides being available commodity of commerce, a country can also earn a good amount of foreign currency by exporting this natural wealth to other countries. More than 500 of medicinal plants have so far been established as growing in Bangladesh. Almost all of these indigenous medicinal plants are extensively used in the preparation of unani, ayurvedic and homeopathic medicines in Bangladesh.[14]

A survey conducted in 1990 in different villages of Bangladesh shows that on average of 14% of people suffering illness approach qualified allopathic doctors, 29% contact unqualified village doctors, 10% contact mullahs, 29% contact quack and 19% contact homeopaths. The survey indicates an extensive use of medicinal plants, most of which are served in crude and substandard form, by our people. In fact, a survey report concluded that 39% of rural community members have knowledge about medicinal plants and 13% treat simple ailments with herbs.[14]

Traditional medicines are still manufactured in our country by following the age-old unscientific, traditional methods. Hundreds of indigenous medicinal plants are employed in different Ayurvedic and Unani commercial preparations without proper standardization,

quality control, evaluation and determination of the chemical nature, pharmacological and toxicological studies of the active components which are essential to utilize their therapeutic potential fully. Toxicity of the plants or plant extracts is coming to light with the advancement of science. Since Bangladesh is a country of low economic growth, a proper health care system can be established by supplying low cost medicines to its population. This may be possible only by developing standard drugs from our natural resources of medicinal plants. In order to achieve this goal, research and development of traditional medicines should be given the due priority. Besides, Bangladesh imports a large quantity of pharmaceutical raw materials including medicinal plants and semi-processed plant products to manufacture drugs. Each year a great deal of money is spent on this purpose.[14]

1.4 Phytochemistry

Phytochemistry is the name given to the study of the chemistry of plants. It is in the strict sense of the word the study of phytochemicals. These are chemicals derived from plants. Like animals, plants produce a wide variety of chemical compounds, called metabolites, as part of their normal life processes. These compounds perform different functions. For example, some enable plants to store energy in the form of sugar, whilst others are protective against disease or predators. The Phytochemistry Unit functions as a laboratory for herbal/plant collection and processing, sample preparation and chromatographic analysis of extracts, fractions and compounds isolated from herbs and medicinal plants.[15]

The Phytochemistry Unit plays a very important role in the collection of plants and has capabilities in the following areas:

1. Plant Sample Collection and Processing.
2. Plant Sample preparation for bioassay studies.
3. Phytochemical Screening.
4. Fractionation and Isolation of bioactive compounds using analytical techniques.
5. Preparation and Analysis of Standardized extracts by HPLC.

1.5 Selection, Collection, and Identification of Plant Material

1.5.1 Selection

The selection of a suitable plant for an isolation of a new drug that is pharmacologically active is very important consideration. There are several ways for the selection of plants

including traditional use, used of plants by the folk medicinal, used of plants for the isolation of drugs, chemical contents, toxicity, randomized selection or a combination of several criteria.[15] The most common strategy is careful observation of the use of natural resources in folk medicine in different cultures; this is known as ethnobotany or ethnopharmacology. Information on how the plant is used by an ethnic group is extremely important. The preparation procedure may give an indication of the best extraction method. On the basis of above selection ways; I have selected one spice for this research purpose.[16]

1.5.2 Collection

Collection of drugs from cultivated plants always ensures a true natural source and a reliable product. This may or may not be the case when drugs are collected from wild plants. Carelessness or ignorance on the part of the collector can result in complete or partial substitution. This is especially true when drugs are difficult to collect or the natural source is scarce. Many drugs are collected from wild plants, sometimes on a fairly extensive scale (traga-canth, senna) when collection is the vocation of the gatherer, and sometimes on a limited scale when collection is an avocation (podophyllum, hydrastis). Because drugs come from all over the world, collection areas are almost universal, and collectors may vary from uneducated natives to highly skilled botanists.[16]

The proper time of harvesting or collecting is particularly important because the nature and quantity of constituents vary greatly in some species according to the season. The most advantageous collection time is when the part of the plant that constitutes the drug is highest in its content of active principles and when the material will dry to give the maximum quality and appearance.[10] The plant concerned to this research paper has been collected at the beginning of winter (November, 2011).

1.5.3 Identification

It is advisable to attempt field identification of the samples collected. To aid taxonomic experts in confirming or refining the field identification, and as a permanent scientific record, voucher specimens (including reproductive organs, when feasible) should be prepared and deposited in herbaria, including at least one major institution and, if applicable, in a local herbarium in the source country. The voucher code should be retained for possible inclusion in publications resulting from the collection. A note card

affixed to the voucher specimen should include observations such as local uses of the species, its habitat, microenvironment (e.g., shaded vs. sunny location), state of overall plant health, stage in the reproductive cycle, and other facts that may be useful for future investigations. The collected *C. amada* has been identified by National Herbarium, Bangladesh and the voucher code is 37511.[15]

1.5.4 Harvesting

The mode of harvesting varies with each drug produced and with the pharmaceutical requirements of each drug. Some drugs may be collected by hand labor; however, when the cost of labor is an important factor, the use of mechanical devices is often more successful in economic production of the drug. With some drugs, where the skillful selection of plant parts is an important factor (digitalis), mechanical means cannot replace hand labor. The plant concerned to this research paper has been collected by hand labor.[10]

1.5.5 Drying

By drying the plant material, one removes sufficient moisture to ensure good keeping qualities and to prevent molding, the action of enzymes, the action of bacteria, and chemical or other possible changes. Drying fixes the constituents, facilitates grinding and milling, and converts the drug into a more convenient form for commercial handling. Proper and successful drying involves two main principles: control of temperature and regulation of air flow. Control of the drying operation is determined by the nature of the material to be dried and by the desired appearance of the finished product. The plant material can be dried either by the sun or by the use of artificial heat. With some natural products, such as vanilla, processes of fermentation or sweating are necessary to bring about changes in the constituents. Such drugs require special drying processes, usually called "curing." The rhizome of *C. amada* has been dried by sun drying.[16]

1.5.6 Grinding

Small quantities of plant material can be milled using a grinder or blinder or an electric spice mill, or in a mortar and pestle. Milling of large quantities of plant material is usually best carried out using industrial-scale comminution equipment. Grinding improves the

efficiency of extraction by increasing the surface area of the plant material. It also decreases the amount of solvent needed for extraction by allowing the material to pack more densely. Although it might seem that milling plant material to a fine powder would be ideal, if the particles are too fine, solvent cannot flow easily around them. Furthermore, the friction of milling generates heat (the finer the particle produced, the more heat), potentially causing volatile constituents to be lost, and thermolabile components to degrade and oxidize. Plants containing volatile components may be extracted by steam distillation of coarsely chopped plant material.[16]

1.6 Extraction of plant materials

For extraction, the plant materials should be properly authenticated. The choice of extraction procedure depends on the nature of the plant material and the components to be isolated. Dried materials are powdered before extraction, whereas fresh plants (leaves, etc) can be homogenized or macerated with a solvent such as alcohol. Solvent such as alcohol is also useful for stabilizing fresh leaves by dropping them into the boiling solvent. Alcohol is a general solvent for many plant constituents (most fixed oils excepted) and as such may givesproblems in the subsequent elimination of pigments, resins, etc. water immiscible solvents are widely used- Hexane, light petroleum(essential and fixed oil, steroids), ether and chloroform (alkaloids, quinones). The basification of the plants materials is required for the extraction of organic bases (e.g. alkaloids). For aromatic acids and phenols acidifications may be required. Extraction itself may be performed by repeated maceration with agitation, percolation, or by continuous extraction (e.g. in a Soxhlet extraction). Special methods for volatile oils (oils used in perfumery, such as oil of rose) are prepared by steam distillation, but many of the flower perfumes are extracted by enfleurage, by digestion in melted fats, by pneumatic methods or by means of solvents. In the enfleurage process, glass plates are covered with a thin layer of fixed oils or fat upon which the fresh flower are spread. The volatile oils gradually pass into the fat and the exhausted flowers are removed and replaced by a fresh supply. The pneumatic method, which is similar in principles to the enfleurage process, involves the passage of a current of a warm air through the flower. The air, laden with suspended flower oils, is then passed through a spray of melted fat until exhausted, when they are strained out and the perfume containing fat is allowed to cool. It will be seen that in each of the above processes the volatile oil has now been obtained in a fatty base. The volatile oil is obtained from this by three successive

extractions with alcohol. The alcoholic solutions may be put on the market as flower perfumes or the oil may be obtained in a pure form by recovery of the alcohol. Solvent extraction is based on the Soxhlet principle.[10]

1.7 Fractionation

A crude extract is a mixture of thousand compounds. It is difficult to isolate a single compound from the crude extracts by a single separation technique. Hence, the crude extract is initially separated into various discrete fractions containing compounds of similar polarities or molecular sizes. These fractions may be contiguous eluate from a chromatography column, e.g., vacuum liquid chromatography (VLC), column chromatography (CC), size-exclusion chromatography (SEC), solid-phase extraction (SPE), and etc. [15]For initial fractionation of any crude extract, it is advisable that, do not generate too many fractions, because it may spread the target compound over so many fractions, as a result, amount of target compound will be very small that will render the detection. For finer fractionation, often newly discovered detection technique, e.g., ultraviolet (UV), modern preparative, or semi preparative high-performance liquid chromatography (HPLC) can be used.[10]

1.8 Isolation

The most important factor that has to be considered before isolation is the nature of the target compound present in the crude extracts or fractions. The general features of the molecule that are helpful for isolation include solubility (hydrophobicity or hydrophilicity), acid-base properties, charge, stability, and molecular size. If isolating a known compound from the same or a new source, it is easy to obtain literature information on the chromatographic behavior of the target compound, and one can choose the most appropriate method for isolation without any major difficulty. However, it is more difficult to design an isolation protocol for a crude extract where the types of compounds present are totally unknown. In this situation, it is advisable to carry out qualitative tests for the presence of various types of compounds, e.g., phenolics, steroids, alkaloids, flavonoids, etc., as well as analytical thin-layer chromatography (TLC), HPLC, or GC.[10]

The nature of the extract can also be helpful for choosing the right isolation protocol. For example, a MeOH extract or fraction that contains polar compounds, it is advisable to use

reversed-phase HPLC (RP-HPLC), while for a Hexane extract or fraction that contains oils or nonpolar compounds are better deals with using Gas Chromatography.[15]

1.9 Solubility (Hydrophobicity or hydrophilicity)

The polarity of the extract as well as the compounds present in the extract can be determined by drying an aliquot of the mixture and trying to re-dissolve it in various solvents covering the range of polarities, e.g., water, MeOH. DMSO, acetonitrile (ACN), EtOAc, chloroform, dichloromethane, petroleum ether, n-hexane, etc.[15]

1.10 Acid-base properties

The acid base properties of the compounds in an extract is determined by partitioning the extract in aqueous solvents at a range of PH Value , typically 3, 7, and 10. It is necessary to adjust the aqueous solution with one or two drops of mineral acid or alkali (a buffer can also be used), followed by the addition of organic solvent and solvent extraction. Organic and aqueous phases are assessed, preferably by TLC, for the presence of compounds. This experiment can also provide information on the stability of compounds at various pH values.[15]

1.11 Chromatographic techniques used in the isolation

The chromatographic techniques used in the isolation of various types of natural products are classified into two categories:

A. Classical or older chromatographic techniques:

1. Thin-layer chromatography (TLC).
2. Preparative thin-layer chromatography (PTLC).
3. Open-column chromatography (CC).
4. Flash chromatography (FC).

B. Modern chromatographic techniques:

1. High-performance thin-layer chromatography (HPTLC).
2. Multiflash chromatography (e.g., Biotage).
3. Vacuum liquid chromatography (VLC).
4. Chromatotron,

5. Solid-phase extraction
6. Droplet countercurrent chromatography (DCCC).
7. High-performance liquid chromatography (HPLC).
8. Hyphenated techniques (e.g., HPLC-PDA, LC-MS, LC-NMR, LC-MS-NMR).[10]

1.12 Quantification

The yield of compounds at the end of the isolation and purification process is important in natural product research. An estimate of the recovery at the isolation stage can be obtained using various routine analytical techniques that may involve the use of a standard. In bioassay-guided isolation, the compound is monitored by bioassay at each stage, and a quantitative assessment of bioactivity of the compound is usually carried out by serial dilution method (see Note 3). Quantitative bioactivity assessment provides a clear idea about the recovery of the active compound(s) and also indicates whether the activity results from a single or multiple components. During the isolation process, if the activity is lost or reduced to a significant level, the possible reasons could be as follows:

1. The active compound has been retained in the column.
2. The active compound is unstable in the conditions used in the isolation process.
3. The extract solution may not have been prepared in a solvent that is compatible with the mobile phase, so that a large proportion of the active components precipitated out when loading on to the column.
4. Most of the active component(s) spread across a wide range of fractions, causing undetectable amounts of component(s) present in the fractions.
5. The activity of the extract is probably because of the presence of synergy among a number

of compounds, which, when separated, are not active individually. [10]

1.13 Structure Elucidation

The end point of the extraction and isolation of natural products is the identification of the compound or structure elucidation of the isolated compound. However, structure elucidation of compounds isolated from plants, fungi, bacteria, or other organisms is generally time consuming, and sometimes can be the "bottleneck" in natural product research. There are many useful spectroscopic methods of getting information about chemical structures, but

the interpretation of these spectra normally requires specialists with detailed spectroscopic knowledge and wide experience in natural product chemistry. If the target compound is known, it is often easy to compare preliminary spectroscopic data with literature data or to make direct comparison with the standard sample. However, if the target compound is an unknown and complex natural product, a comprehensive and systematic approach involving a variety of physical, chemical, and spectroscopic techniques is required. The following spectroscopic techniques are generally used for the structure determination of natural products:

1. Ultraviolet-visible spectroscopy (UV-vis): Provides information on chromophores present in the molecule. Some natural products, e.g., flavonoids, isoquinoline alkaloids, and coumarins can be primarily characterized from characteristic absorption peaks.
2. Infrared spectroscopy (IR): Determines different functional groups, e.g.,—C=O, —OH, —NH2, aromaticity, and so on, present in a molecule.
3. Mass spectrometry (MS): Gives information about the molecular mass, molecular formula, and fragmentation pattern. Most commonly used techniques are: electron impact mass spectrometry (EIMS), chemical ionization mass spectrometry (CIMS), electrospray ionization mass spectrometry (ESIMS), and fast atom bombardment mass spectrometry (FABMS).
4. NMR: Reveals information on the number and types of protons and carbons (and other elements like nitrogen, fluorine, etc.) present in the molecule, and the relationships among these atoms. The NMR experiments used today can be classified into two major categories:

    a. One-dimensional techniques: ^{1}H-NMR, ^{13}C-NMR, ^{13}C-DEPT, ^{13}C-PENDANT, ^{13}C- J mod., nOe-diff., and so on.

    b. Two-dimensional techniques: 1H-1H COSY, 1H-1H DQF-COSY, 1H-1H COSY-lr, 1H-1H NOESY, 1H-1H ROESY, 1H-1H TOCSY (or HOHAHA), 1H-13C HMBC, 1H-13C 1HMQC, 1H-13C HSQC, HSQCTOCSY, and the like.

In addition to the above-mentioned spectroscopic techniques, X-ray crystallographic techniques provide information on the crystal structure of the molecule, and polarimetry offers information on the optical activity of chiral compounds.[10]

1.14 Assays

Chemical, biological, or physical assays are necessary to pinpoint the target compound(s)

from a complex natural product extract. At present, natural product research is more focused on isolating target compounds (assay-guided isolation) rather than trying to isolate all compounds present in any extract. The target compounds may be of certain chemical classes, have certain physical properties, or possess certain biological activities. Therefore, appropriate assays should be incorporated in the extraction and isolation protocol.[10]

The following basic points should be borne in mind when carrying out assays of natural products:

1. Samples dissolved or suspended in a solvent different from the original extraction solvent must be filtered or centrifuged to get rid of any insoluble matter.
2. Acidified or basified samples should be readjusted to their original pH to prevent them from interfering with the assay.
3. Positive and negative controls should be incorporated in any assay.
4. Ideally, the assay should be at least semiquantitative, and/or samples should be assayed in a series of dilutions to determine where the majority of the target compounds reside.
5. The assay must be sensitive enough to detect active components in low concentration.[10]

1.14.1 Physical assays

Physical assays may involve the comparison of various chromatographic and spectroscopic behaviors, e.g., HPLC, TLC, LC-MS, LC-NMR, and so on, of the target compound with a known standard.

1.14.2 Chemical assays

Chemical assays involve various chemical tests for identifying the chemical nature of the compounds, e.g., Feds can be used to detect phenolics, Dragendorff s reagent for alkaloids, 2,2-diphenyl-l-picrylhydrazyl (DPPH) for antioxidant compounds, and so on.

1.14.3 Bioassays

Bioassays can be defined as the use of a biological system to detect properties (e.g., analgesic, anti-inflammatory, antiulcer, antibacterial, antifungal, anticancer, anti-HIV, antidiabetic, etc.) of a crude extract, chromatographic fraction, mixture, or a pure compound. Bioassays could involve the use of in vivo systems (clinical trials, whole animal

experiments), ex vivo systems (isolated tissues and organs), or in vitro systems (e.g., cultured cells). In vivo studies are more relevant to clinical conditions and can also provide toxicity data at the same time. Disadvantages of these studies are costs, need for large amount of test compounds/fractions, complex design, patient requirement, and difficulty in mode of action determination. In vitro bioassays are faster, and small amounts of test compounds are needed, but might not be relevant to clinical conditions. The trend has now moved from in vivo to in vitro. There are a number of biological assays available to assess various activities, e.g., Acetic acid induced writhing for analgesic, carrageenan induced paw edema for anti-inflammatory, and HCI induced gastric lesions for antiulcer, Disk diffusion for antimicrobials etc.[10]

1.15 Research on medicinal plants: Bioactivity guided approach

Plants, the molecular architect, still offer a great potentiality for drug research. Plants contain compounds having interesting skeletons. Bioactivity guided phytochemical investigation of medicinal plants may yield newer chemical constituents of remarkable therapeutic interest. Occasionally, native folklore provides clue to plants with pharmacological activity. Extensive phytochemical investigation and isolation of active component(s) in the pure form thus become necessary to avoid untoward effects and to ensure safe use of herbal medicines. With technological advancement, phytochemical studies of medicinal plants got a rapid pace and the presence of many chemical compounds came into light. These plant-derived compounds often played an important role in directing laboratory synthesis of many new classes of drug molecules. [10]

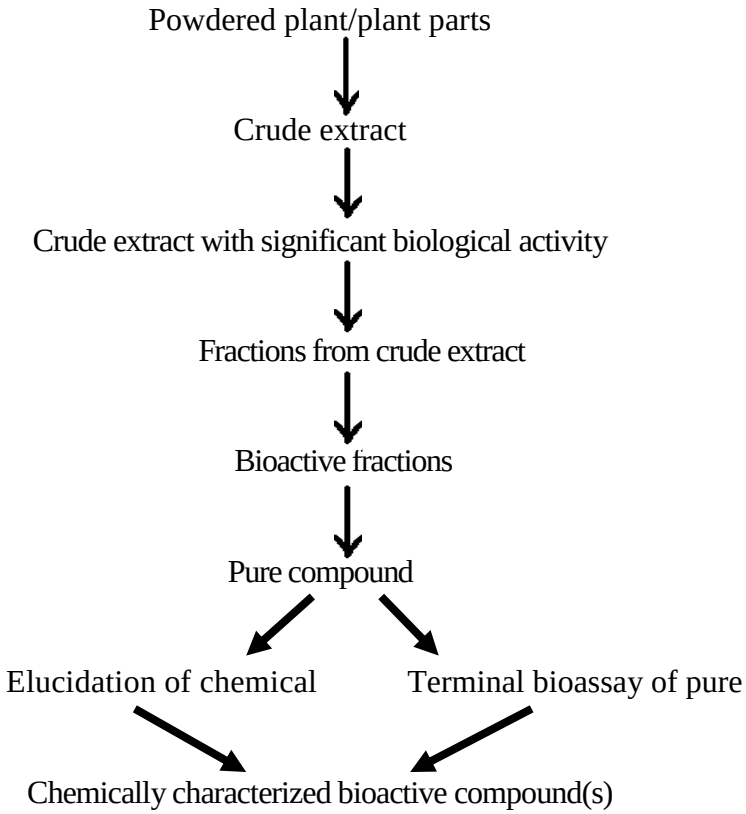
Bioactivity guided phytochemical approach, has three phases of investigation. First, biological activity is detected in crude material, and a bioassay system is set up to permit the identification of active fractions and discarding the inactive ones. Second, the crude material is fractionated by the most appropriate chemical procedures, all fractions are tested, and active fractions are further fractionated, and so on, until pure compounds are obtained. Third, the chemical structures of pure compounds are determined.

Sometimes phytochemical analysis of plants may yield such chemical constituents having no remarkable therapeutic interest. The crude drug containing several constituents may be found to be ineffective in case of therapy for which it was used traditionally. For example, *Vinca rosea*, once used traditionally as antidiabetic drug was found to contain hypoglycemic alkaloid principles in minute but it was found to contain anticancer principle *vinca* alkaloid in

a high yield. *Rauwolfia serpentine* which was traditionally used for a variety of illnesses, revealed the presence of an antihypertensive and tranquilizing agent reserpine. So, systemic research on medicinal plants may open the door of many unknown therapeutic tools.

It is evident from the above discussion that the search of plant constituents having therapeutic interest requires bioactivity studies with the crude extracts prior to phytochemical analysis. Only the bioactive extracts or fractions would be of interest for next phytochemical analysis. Without prior pharmacological studies, the phytochemical studies alone can provide the chemical constituents of the plant, but these may or may not have therapeutic value. So in medicinal plants research, bioactivity guided phytochemical approach might be a rational approach.[10]

Flow chart of Bioactivity Guided Phytochemical Approach:



1.16 Plant Metabolites: Primary and Secondary

Metabolites are organic compounds synthesized by organisms using enzyme-mediated chemical reactions called metabolic pathways. Primary metabolites have functions that are essential to growth and development and are therefore present in all plants. In contrast, secondary metabolites are variously distributed in the plant kingdom, and their functions are specific to the plants in which they are found. Secondary metabolites are often colored,

fragrant, or flavorful compounds and they typically mediate the interaction of plants with other organisms. Such interactions include those of plant-pollinator, plant-pathogen, and plant-herbivore.[17]

1.16.1 Primary Metabolites

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.[17]

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.[17]

1.16.2 Secondary Metabolites

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 as medicines. It is only since the late twentieth century that secondary metabolites have been clearly recognized as having important functions in plants. Research has focused on the role of secondary metabolites in plant defense.[17]

Biosynthetic pathways for the formation of secondary metabolites in plant has been shown below,

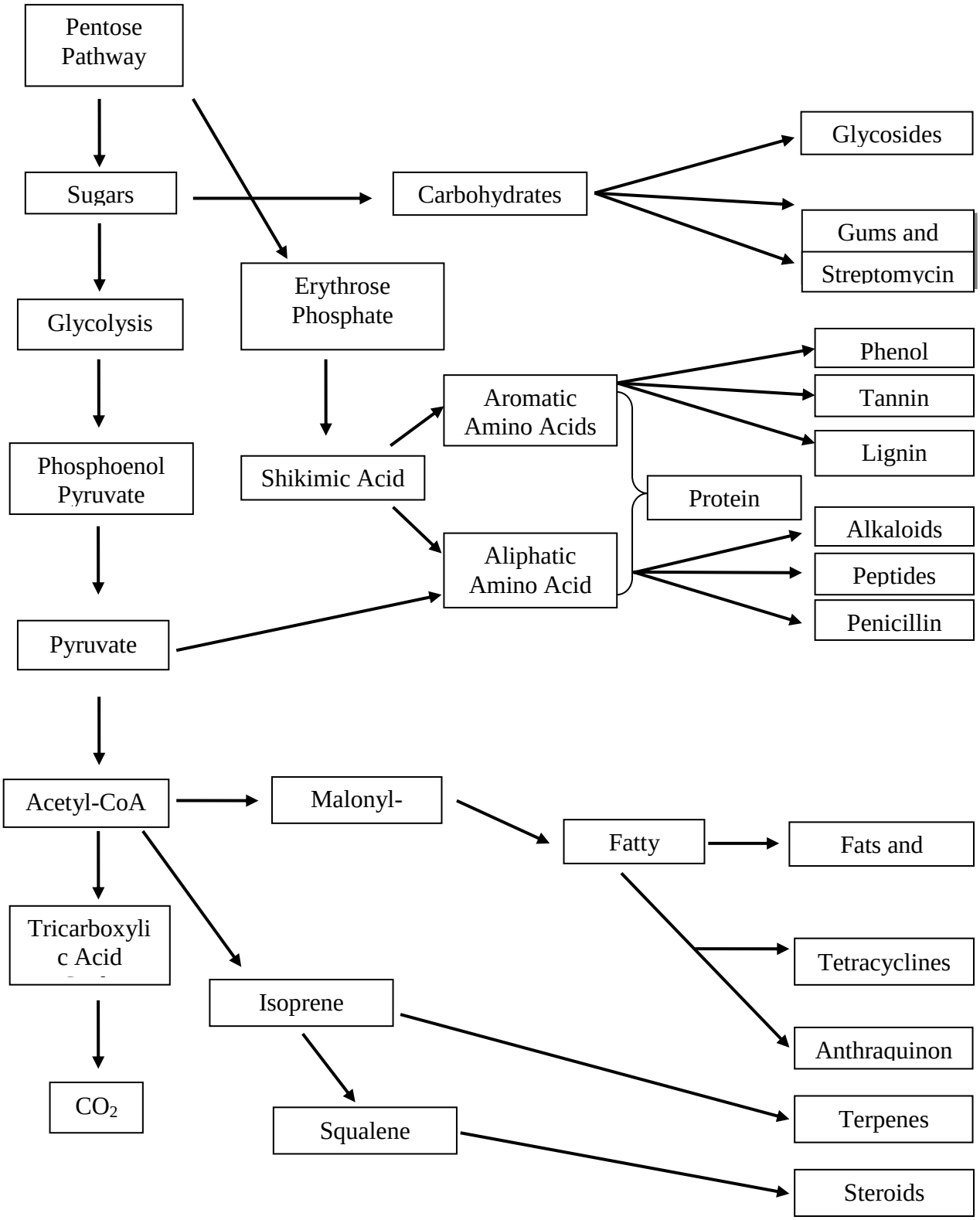


Fig 4: Interrelationships of biosynthetic pathways leading to secondary constituents in plant.[16]

2.1 Morphology of the plant

Mango-ginger is botanically related to neither mango nor ginger, but to turmeric (*Curcuma longa*). It is a unique spice having morphological resemblance with ginger *(Zingiber officinale)* but imparts a raw mango (*Mangifera indica*) flavor.[18] Morphologically mango-ginger plant is similar to turmeric, but has shorter crop duration of six months. The rhizomes are pale yellow inside with lighter color outside, have sweet smell of unripe mango when crushed. The crop comes up well in open conditions, but tolerates low levels of shade and therefore partially shaded situations can also be utilized for its cultivation. It can be well accommodated as an intercrop in coconut gardens and in rotation with other short duration crops like vegetables and also as a crop component in homesteads.[19] The plant grows to a height of 1 m (figure 5A). The leaves are long, oblong, lanceolate, radical, sheathed, and in tufts. Each plant bears 5 to 6 pairs of leaves. Mango ginger rhizomes (figure 5B and 5C) are fleshy, buff colored, 5–10 cm long, 2–5 cm in diameter and demarcated into nodes and internodes. At the rhizome nodes scaly leaves are arranged circularly giving the appearance of growth rings with scars on the surface. The rhizomes are branched, and the branching is sympodial. The rhizomes emulate a raw mango flavour and taste pungent.[18]

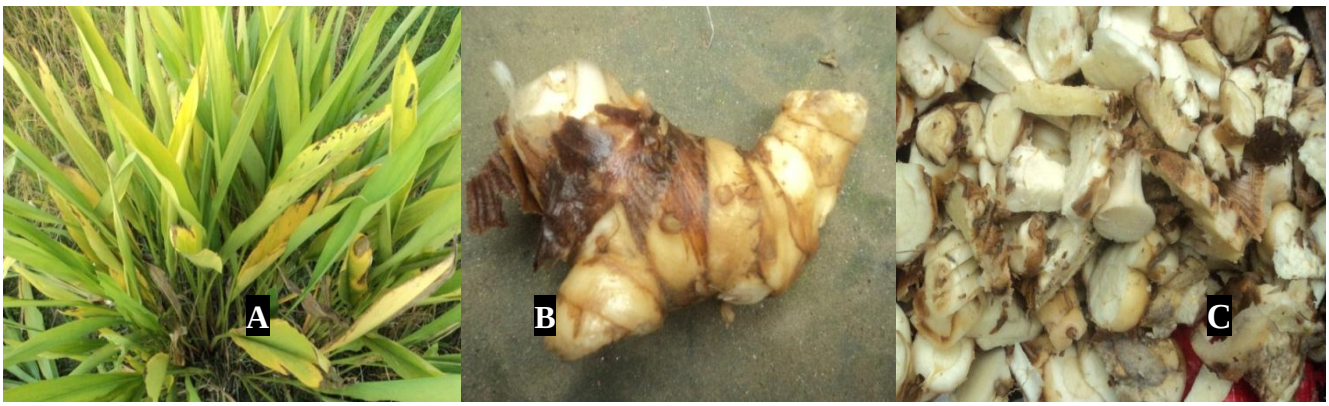


Figure 5: Mango ginger plant (A), rhizomes (B) and TS of rhizome (C).

2.2 Botany and taxonomy of *Curcuma amada*

The genus name Curcuma was coined by Linnaeus in 1753 in his Species Plantarum. The word probably derives from the Arabic word ‘kurkum’, which means yellow color. *Curcuma amada* Roxb. is commonly known as mango ginger. It is a perennial, rhizomatous, aromatic herb belonging to the family Zingiberaceae. This family is composed of 70–80 species of

rhizomatous annual or perennial herbs. The genus originated in the Indo-Malayan region, and is widely distributed in the tropics of Asia to Africa and Australia. [18]

The following Sankrit shloka explains the synonyms of mango ginger:

Darvibheda, Amragandha, Surabhidaru,

Karpurapadmapatra, Surimat and Surataraka. [18]

Table 1: The taxonomical hierarchy of mango ginger:[18]

Kingdom	Plantae
Sub kingdom	Tracheobionta – Vascular plants
Phylum	Spermatophyta – Seed plants
Sub phylum	Magnoliophyta – Flowering plants
Class	Liliopsida – Monocotyledons
Subclass	Zingiberidae
Order	Zingiberales
Family	Zingiberaceae – Ginger family
Genus	Curcuma L.
Species	Curcuma amada Roxb. – Mango ginger.

2.3 Occurrence and distribution

The geographical distribution of this genus ranges from India to Thailand, Indo-China, Malaysia, Indonesia and northern Australia. *C. amada* is found the wild in parts of West Bengal, and is cultivated in Gujarat, Uttar Pradesh, Kerala, Karnataka, Tamil Nadu and the north-eastern states. They originated in the Indo-Malayan region and distributed widely in the tropics from Asia to Africa and Australia. Out of 10 Curcuma species, 2 species, *C. amada* and *C. zedoaria*, are distributed throughout India in the wild and cultivated forms; 4 species, *C. aeruginosa*, *C. brog*, *C. caesia* and *C. sylvatica*, occur in wild conditions and distributed throughout north-eastern part of India.[18]

2.4 Use of mango ginger in food industry

Mango ginger has a typical exotic flavor of raw unripe mango. Therefore, it is used as a basic ingredient in pickles, preserves, candies, sauces, curries, salads and so on.[18]

2.5 Mango ginger and its use in traditional medicines (Ayurveda and Unani):

The ancient testimony for the use of plants as medicine was well documented in the treatise of Ayurveda and dates back to the pre-historic Vedic era. Accordingly, the medicinal properties of mango ginger are depicted in the following Sanskrit shloka:

Raw mango flavored ginger has a cooling effect on the body.

It aggravates Vata (An Ayurvedic principle necessary to co-ordinate the function of the nervous system).

It also pacifies deranged Pitta (An Ayurvedic principle that uses bile to direct digestion and hence metabolism into the venous system).

It cures all types of itching and skin diseases.[18]

Many species belonging to this genus have significant value as medicines, dyes and spices. Ayurveda, the oldest system of medicine in India, attributed multiple uses of rhizome as an appetizer, antipyretic, aphrodisiac and laxative. Additional health benefits of *C. amada* rhizome reported were biliousness, itching, skin diseases, asthma and inflammation due to injuries. According to the Unani systems of medicine, it is a diuretic, maturant, emollient, expectorant, antipyretic and appetizer. Moreover, several reports have demonstrated the *C. amada* rhizome's ability against inflammation in the mouth and ear, gleet, ulcers on the male sex organs, scabies, lumbago and stomatitis.[19]

2.6 Biological activities of Curcuma amada

Rhizome, being a storehouse of bioactive compounds, has extensive use. Mango ginger is used medicinally as a coolant, aromatic and astringent and to promote digestion. A rhizome paste has traditionally been used for healing of wounds, cuts and itching. The external use of the rhizome paste for sprains and skin diseases is also an old practice. The rhizome has carminative properties, as well as being useful as a stomachic. Very few reports are available on the aerial parts of the plant. However, a whole-plant paste with crushed long peppers (Piper longum) is reported to be effective for the treatment of piles, and a decoction of the rhizome with common salt is an effective treatment for colds and coughs and is used to improve blood quality. Topical use of native extract of leaves for contusions and sprains are also reported by several authors.[18]

2.7 Literature review of the biological activities of *C. amada*

2.7.1 Antibacterial activity

A novel and natural antibacterial compound (difurocumenonol), recently isolated from mango ginger, has high antibacterial activity against a wide spectrum of bacteria, including Gram-negative and Gram-positive bacteria (e.g. *Pseudomonas aeruginosa, Salmonella typhi, Klebsiella pneumoniae, Enterobacter aerogenes, Yersinia enterocolitica, Micrococcus luteus, Staphylococcus aureus, Enterococcus fecalis, Bacillus cereus, Bacillus subtilis* and *Listeria monocytogenes*). Tubers and essential oils of *C. amada* showed inhibitory activity against various pathogenic bacteria, including *Bacillus anthracis, B. mycoides, B. pumilus, B. subtilis, Pseudomonas mangiferae indicae, Salmonella paratyphi, Xanthomonas campestris* and *Vibrio cholerae*.[21] The chloroform extract of *C. amada* (mango ginger) rhizome was examined for its antibacterial activity. In the study a novel compound "Amadannulen" was identified and isolated from the *Curcuma amada* extract. Amadannulen showed antibacterial activity against both Gram-positive and Gram-negative bacteria. It also exhibited bactericidal activity against *M. luteus, B. cereus* and *B. subtilis* bacteria.[24]

2.7.2 Antifungal activity

In addition to antibacterial activity, the antifungal properties of mango ginger have been documented. The essential oils of *C. amada,* in addition to those of other plant species, show antifungal activity against sugarcane pathogenic fungi such as *Physalospora tucumanensis* (light and dark races), *Sclerotium rolfsii, Helminthosporium sacchari,subglutinans* and *Cephalosporium sacchari* at varying extract dilutions.[26] The volatile oil from mango ginger rhizomes has antifungal in nature. Myrcene (4.6%) and pinene (80.5%) are the major components of volatile oils responsible for antifungal activity against the wide range of fungi, viz. *Curvularia palliscens, Aspergillus niger, A. terreus, Fusarium moniliforme and F. falcatum.*[18]

2.7.3 Anti-inflammatory activity

The ethyl alcohol extract of mango ginger rhizome has anti-inflammatory activity in acute and chronic administration in albino rats. It has been reported that there is presence of

chemical compounds with hydroxyl, ester, carbonyl and olefin functional groups in ethyl alcohol extract. It was found to be significant at higher concentrations in acute carrageenan-induced rat paw edema model.[18]

2.7.4 Platelet aggregation inhibitory activity

Platelet aggregation inhibitory activity of ethyl acetate extract and acetone extract is reported to be very high compared to methanol extract.[19] The high platelet aggregation inhibitory activity of ethyl acetate, acetone and methanol extracts appears to be correlated with high phenolic content and to be concentration dependent.[25]

2.7.5 Cytotoxicity

Cytotoxicity of the hexane, chloroform, ethyl acetate, acetoneand methanol extracts of mango ginger towards both normaland cancer cell cultures were reported.All the extracts showed comparatively higher toxicitytowards cancer cells when compared with normal cells,which is a good indicator of the anticancer property ofextracts. Among the five extracts tested, the ethyl acetateextract showed greater toxicity, followed by chloroform,hexane, and acetone and methanol extract. The cytotoxicityresults of different extracts of mango ginger indicate that theextracts are less toxic towards the normal cell lines.[25]

2.7.6 Antioxidant activity

The antioxidant activity of aqueous methanol extract of mango ginger leaves and rhizomes by β-carotene bleaching method are reported. Leaf extract was more active than rhizomes extract. The antioxidant activity of sequential extracts of mango ginger with increasing polarity of solvents was reported. According to the report different antioxidant activity assays was done like DPPH radical scavenging activity, superoxide radical scavenging activity, metal chelating activity and lipid peroxidation activity. Among hexane, chloroform, ethyl acetate, acetone and methanol extracts, ethyl acetate and acetone extracts showed good DPPH radical scavenging activity. The non-polar extracts showed good lipid peroxidation inhibitory activity.[27]

Table 2: Antioxidant activity (AOA%) and total phenolic content (TPC) expressed as mg/g gallic acid equivalent on a dry weight basis.[20]

Plant	Plant part	AOA%	TPC mg/g gallic
Curcuma longa	Leaves	35.1	7.6
	Rhizomes	84.1	36.6
Curcuma amada	**Leaves**	**51.9**	**13.4**
	Rhizomes	**45.3**	**32.4**
Curcuma zeoderia	Leaves	24.2	6.8
	Rhizomes	40.2	28.7

2.7.7 Antiallergic activity

C. amada is reportedly used in various herbal preparations, including antiallergy formulations.[18]

2.7.8 Bio-pesticide

The usefulness of *C. amada* as an insecticide or pesticide was assessed in several studies. Ahmad and Ahmad (1992) evaluated the insecticidal, antioviposition and ovicidal properties of indigenous medicinal plants against the pulse beetle (*Callosobruchus chinensis* L.) infesting green gram (*Vigna radiata*). Of the seven plants tested, *C. amada* was highly effective, resulting in 100% adult mortality and a reduction in oviposition, even at 0.5% concentration. Different plant essential oils possessing diverse insecticidal properties were tested for repellency and direct toxicity toward laboratory bred houseflies (*Musca domestica* L.). Essential oils from *C. amada* showed 100% repellent activity. Insect mortality was 100%, even at 1% levels after 45 days. In addition, *C. amada* was highly effective in inhibiting the emergence of the F1 generation of weevils, as were other species of Zingiberaceae.[19]

2.7.9 Effects on lipids, triglycerides and cholesterol

Curcuma amada rhizomes markedly lowered blood cholesterol in rabbits. The effects of *C. amada* on the cholesterol level in hypercholesterolemic rabbits were investigated using

alcoholic and ether extracts given orally. Only ether extracts of *C. amada*, at 1 g orally, decreased blood cholesterol levels from 266 to 36 mg within 3 weeks; the blood cholesterol:phospholipid ratio decreased correspondingly. The results also showed that ether *C. amada* extracts lowered blood cholesterol in rabbits fed 1 g of cholesterol in olive oil daily for 12 days.[21]

2.7.10 Hypoglycemic and anti-hyperglycemic activity

Its rhizome has been used in treating various local ailments. The hypoglycemic and anti-hyperglycemic activity of the crude aqueous methanolic (1:4) extract of *Curcuma amada* was evaluated in normal and alloxan-induced diabetic mice. Administration of different doses (150-650 mg/kg b.w.) lowered blood glucose level in a dose- and time-dependent manner. Mild hypoglycemic activity was observed with all the administered doses, while the anti-hyperglycemic activity was found to be pronounced above the dose of 250 mg/kg b.w.[12]

2.7.11 Brine-shrimp lethal activity

The water extract of mango ginger rhizomes showed brine shrimp (*Artemia salina*) lethal activity. The lethality value (LC50=6,600μg, 24 h) was determined by a plot of the percentage of the shrimps killed against the concentrations of the extracts. The degree of lethality was found to be directly proportional to the concentration of the extract.[20]

2.7.12 CNS depressant and analgesic activity

A fraction obtained from ethanol extract of mango ginger rhizome exhibited CNS depressant and analgesic activity. The active fraction showed reduction in exploratory activity of barbiturate sleeping time, indicating CNS depressant activity. Further, it also showed reduction in acetic acid-induced writhing, tail-flick response, and carrageenan-induced inflammation, indicating potential anti-nociceptive activity.[21] Further, it also showed reduction in acetic acid–induced writhings, tail-flick response, and carrageenan-induced inflammation, indicating potential antinociceptive and antiphlogistic activity, respectively.[12]

2.7.13 Enterokinase inhibitory activity and anti-tubercular activity

The mango ginger also has enterokinase inhibitory activity and anti-tubercular activity. The labdane-type diterpenoid, labda-8 (17), 12-diene-15, 16-dial and its modified analogues have anti-tubercular properties.[18]

2.8 Chemistry of Curcuma Amada

2.8.1 Volatile constituents

There are many reports on the composition of mango ginger volatile oil. The mango flavor is mainly attributed to presence of car-3-ene and cis-ocimene among the 68 volatile aroma components present in the essential oil of mango ginger rhizome. The cis- and trans-hydroocimene, ocimene and myrcene were found to be the major compounds present in the volatile oils of *C. amada*, which indicates that the aroma of mango ginger is a mixture of characteristic compounds found in both raw mango and turmeric. The acetone extract of mango ginger is composed of colorless oil, curcumin, phytosterol and azulenogenic oil containing pinene, camphor, curcumene and ar-turmerone. There are more than 100 phytochemicals reported from fresh and dried extracts of *C. amada*.[18]

2.8.2 Curcuminoids in mango ginger

The well-known curcumin, demethoxycurcumin and bis-demethoxycurcumin are the major constituents from acetone extract of *C. amada*.[24]

2.8.3 Phenolic content in mango ginger extracts

The free phenolic acids (figure 4) present in mango ginger are caffeic (26%, 195 mg/g), gentisic (24%, 180 mg/g) and ferulic (20%, 150 mg/g) followed by gallic(10%, 75 mg/g), cinnamic (7%, 52.5 mg/g), protocatechuic(7%, 52.5 mg/g) and small amounts of syringic (4%,30 mg/g) and p-coumaric acids (2%, 15 mg/g). It also contains bound phenoliccompounds like ferulic acid (47%, 391.5 mg/g) andcinnamic acid (29%, 237 mg/g), p-coumaric acid (11%,95 mg/g), syringic acid (5%, 38.8 mg/g), caffeic acid (4%,30.7 mg/g), gallic acid (1%, 11.5 mg/g) and gentisic acid(1%, 4.9 mg/g).[18]

2.8.4 Terpenoid bioactive molecules

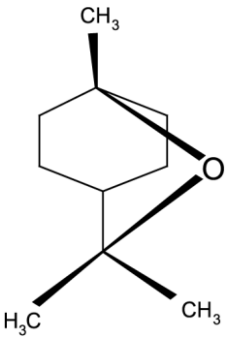
Three terpenoid bioactive compounds, viz. difurocumenonol,amadannulen and amadaldehyde (figure 5), weresuccessfully isolated and characterized from chloroformextract of *C. amada* rhizome. The bioactive compounds are antibacterial as well asantioxidant in nature with DPPH radical scavenging activity,superoxide radical scavenging activity, lipid peroxidationinhibitory activity, metal chelating activity and total reducingpower. The above bioactive compounds were alsopotential platelet aggregation inhibitors and toxic for cancer cell lines.[18]

2.8.5 Functional attributes of Curcuma amada starch

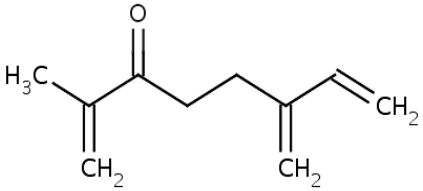
It appears that starch from mango ginger has potential functional properties. It has a distinct structural and biochemical features of its own. Mango ginger contains1.3% ash, 9.8% moisture and 45% starch with 43% amylose. Morphologically, mango ginger starch resembles ginger starch granules, but it differs by the absence of fissures on the surface and its X-ray diffractogram pattern. Scanning electron micrograph (SEM) revealed the variations in shape of granules that appeared as round, elliptic, irregular and polygonal. The solubility and water holding capacity of mango ginger starch was linear with increase in temperature. High amylose content and low solubility are interesting attributes of mango ginger starch that need to be explored for preparation of nutraceutical products of metabolic advantages.[18]

2.9 Reported compounds isolated from *C. amada*:

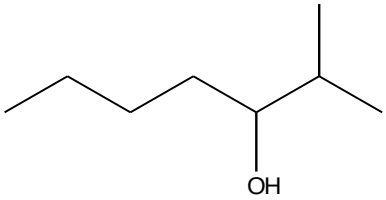
There are some structures of chemical compounds isolated from mango ginger:[21]



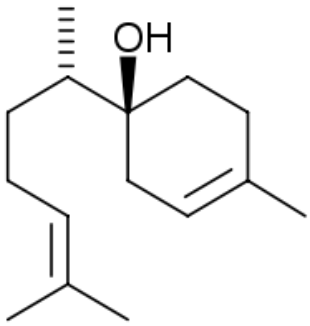
1,8-cineole



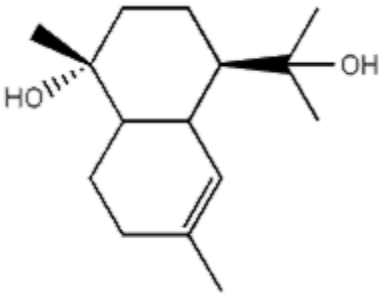
2-methyl-6-methylene-octa-1,7-dien-3-one



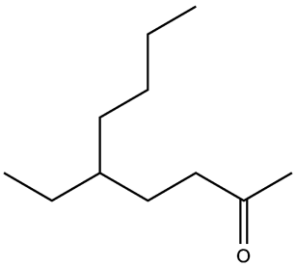
2-methylheptan-3-ol



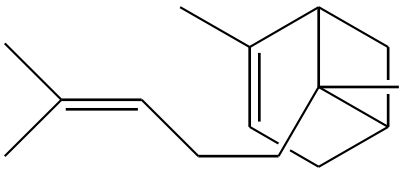
14-hydroxy-alpha-muurolol



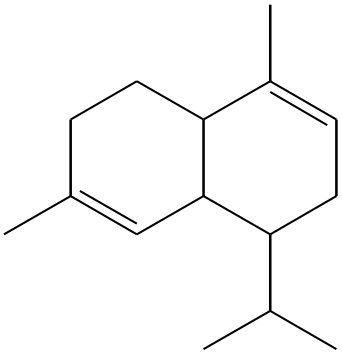
Beta-Bisabolol



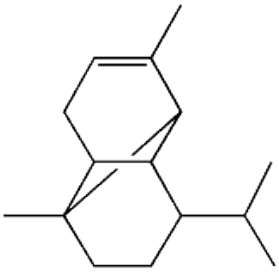
2-nonanone



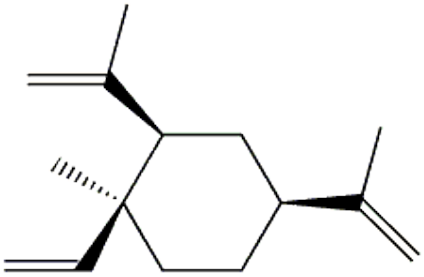
alpha-trans-bergamotene



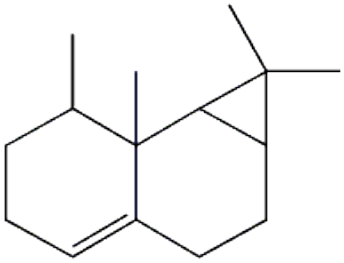
alpha-Muurolene



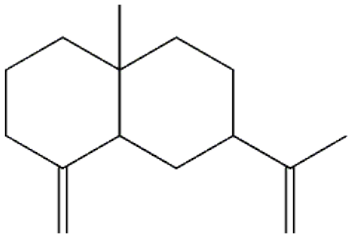
alpha-copaene



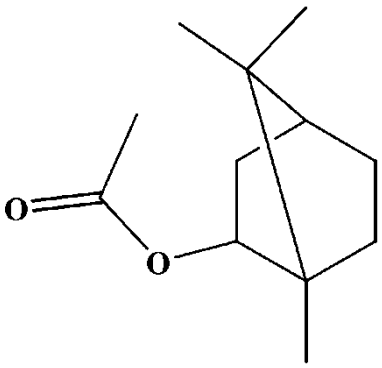
beta-elemene



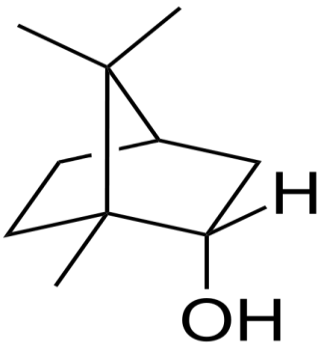
beta-gurjunene



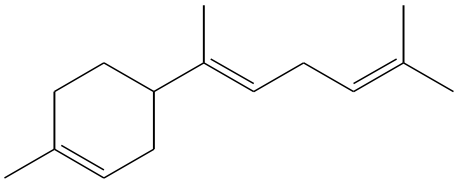
beta-selinene



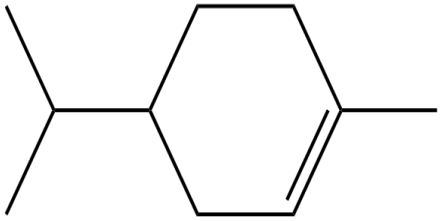
Bornyl Acetate



Borneol



bisabolene



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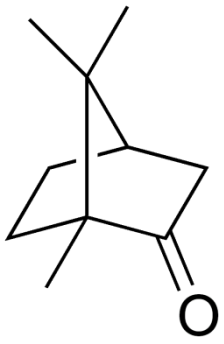

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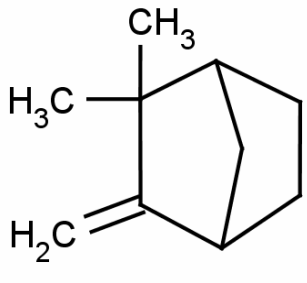

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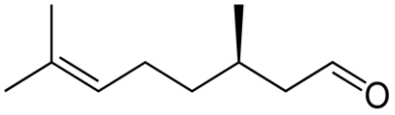
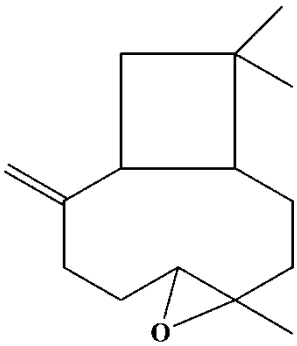


Citronellal-2D-skeletal



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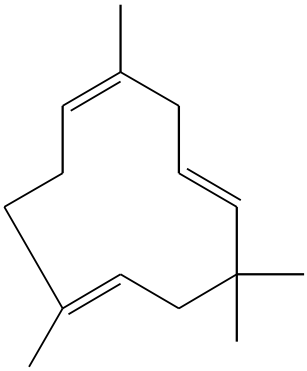

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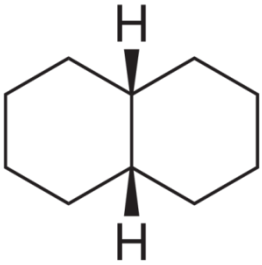

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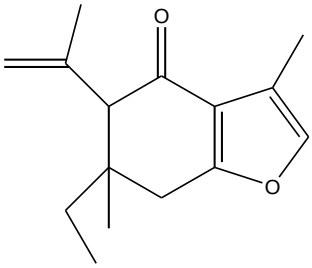

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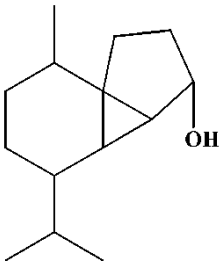

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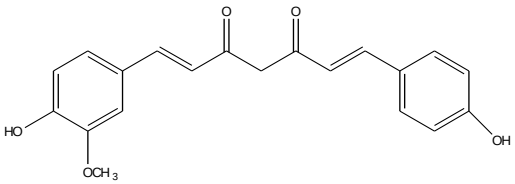
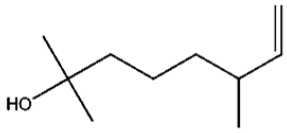


Demethoxycurcumin



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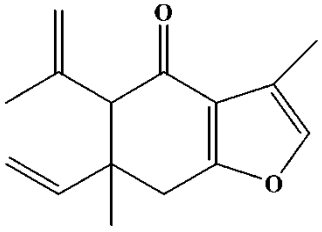

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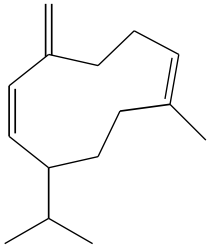

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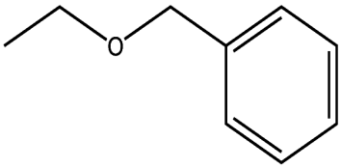

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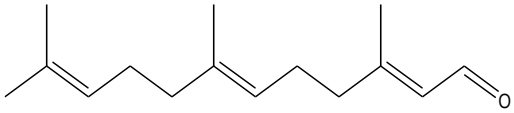
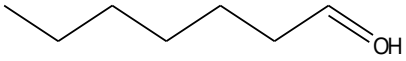
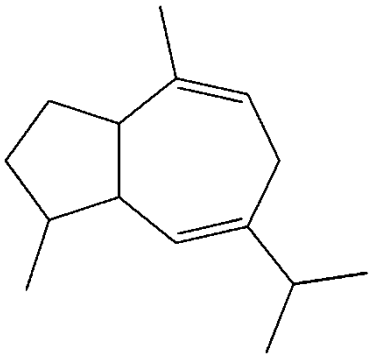


Farnesal



Hept-1en-1ol



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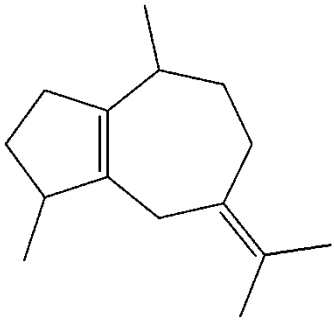
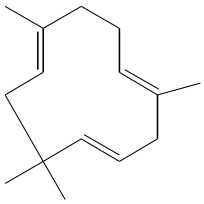


Guaiene

Hexadecyl



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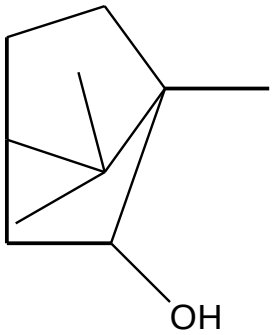
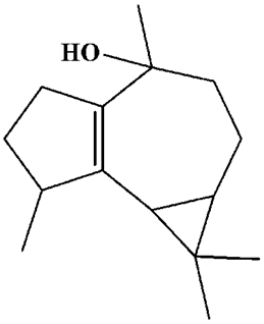


Isoborneol



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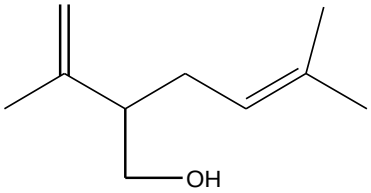

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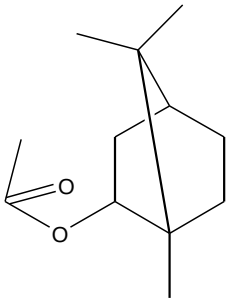

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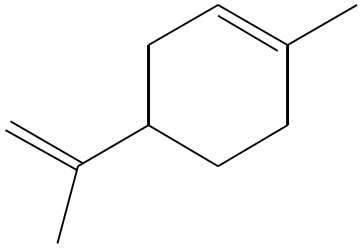
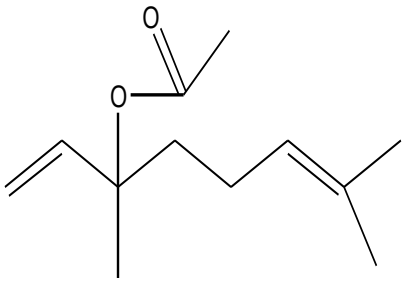
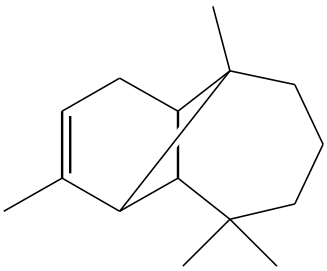


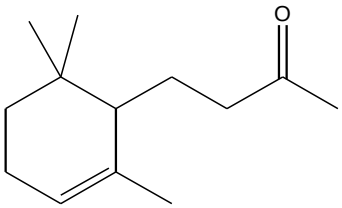
Limonene



Linalyl acetate



Longipinene



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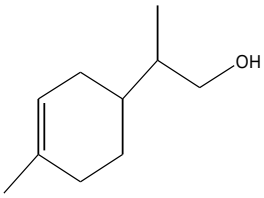
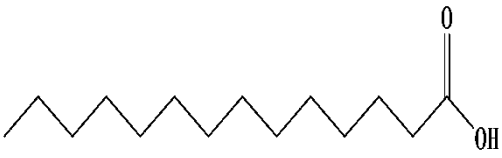
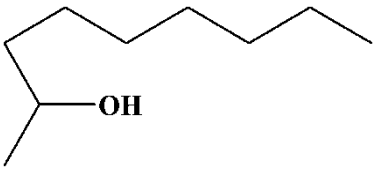


Menth-2en-9ol



Myristic acid

Nonan-2-One



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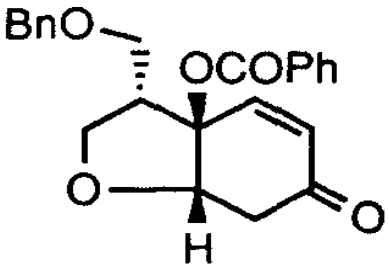

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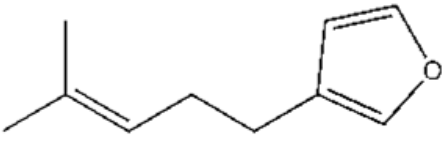

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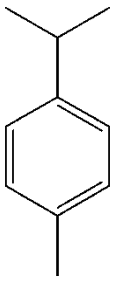

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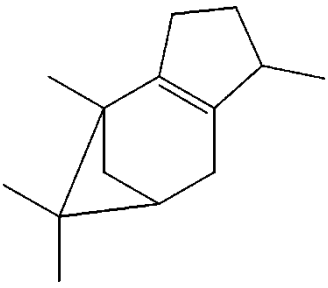

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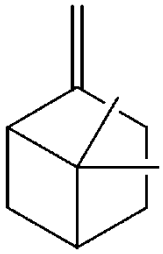

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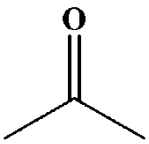

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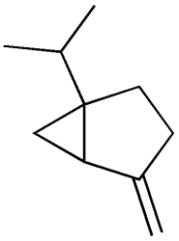

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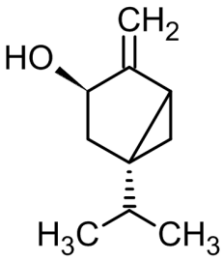

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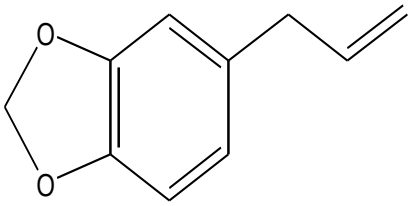

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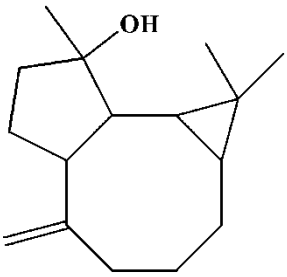

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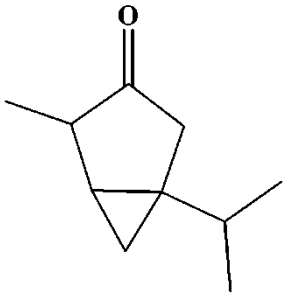

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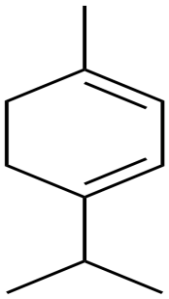

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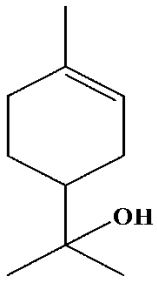

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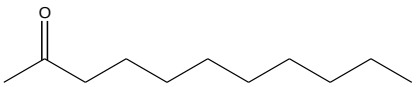

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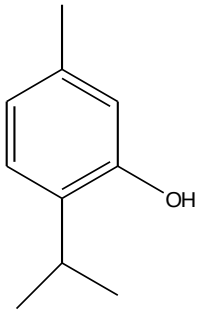

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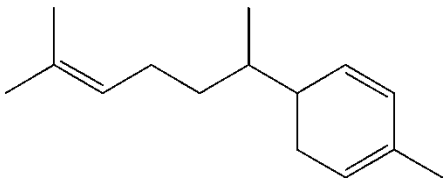

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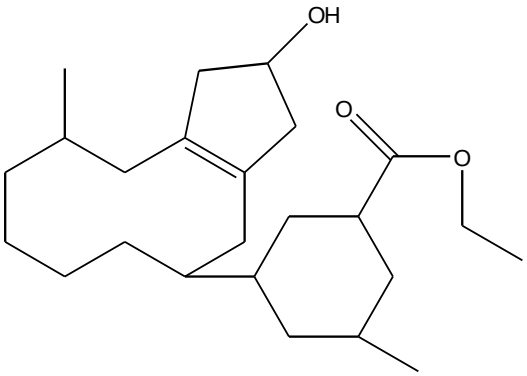

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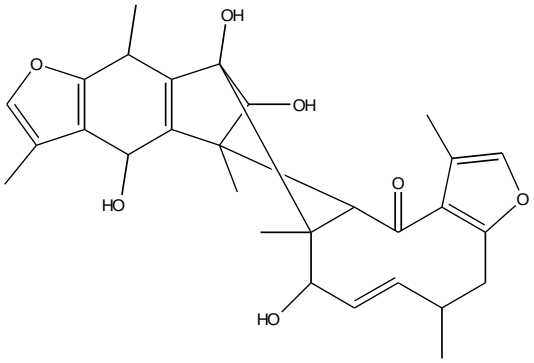

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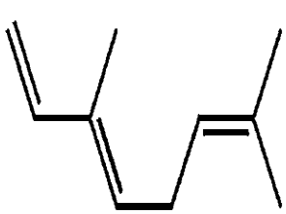
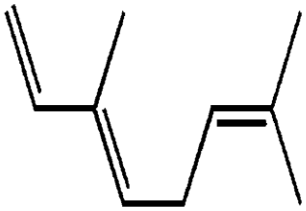


trans-hydroocimene



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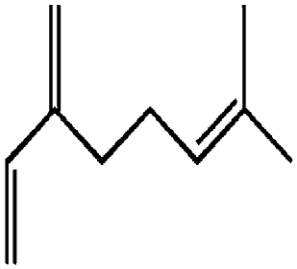
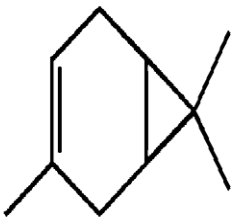
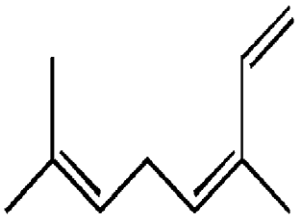


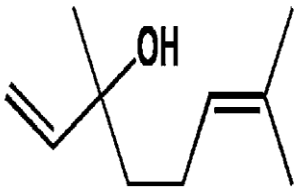
myrcene



car-3-ene



cis-ocimene



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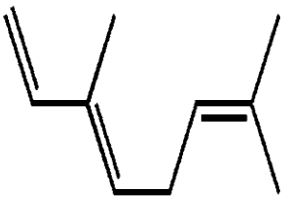

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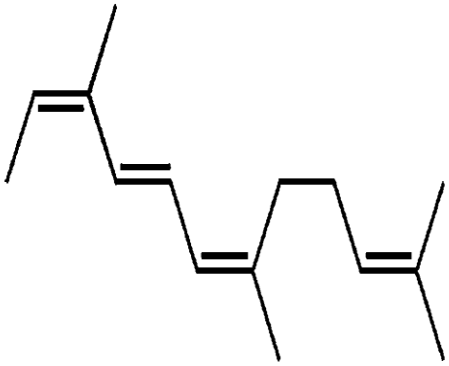
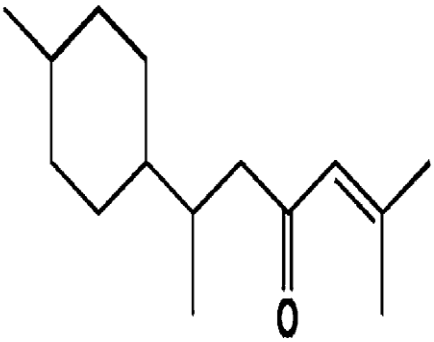
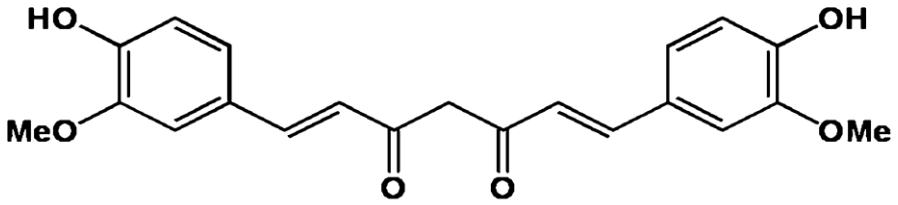


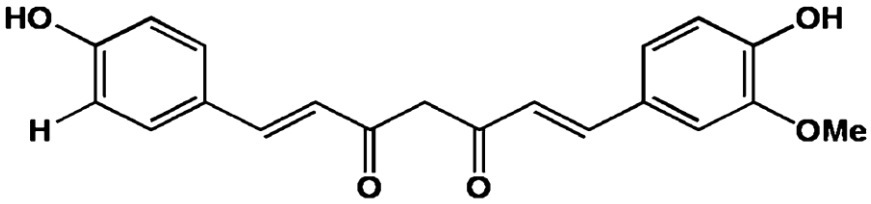
(z)-farnesene



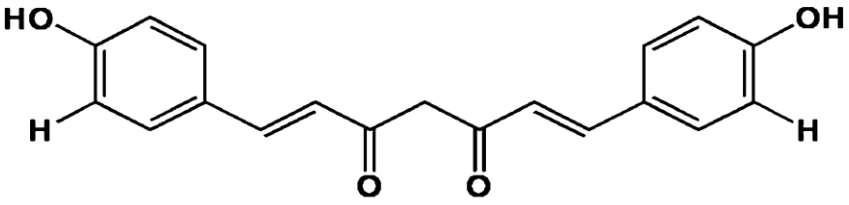
turmerone



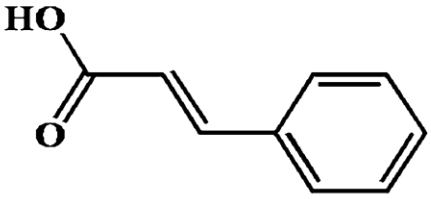
Curcumin



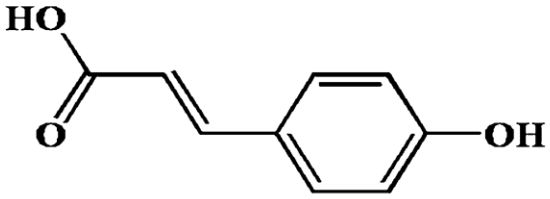
Bismethoxycurcumin



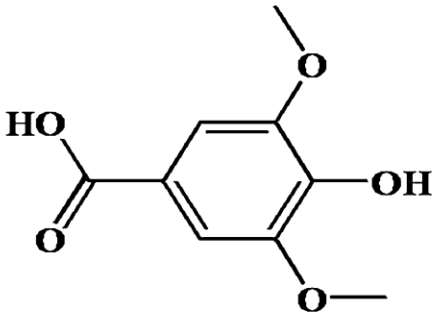
Bisdemethoxycurcumin



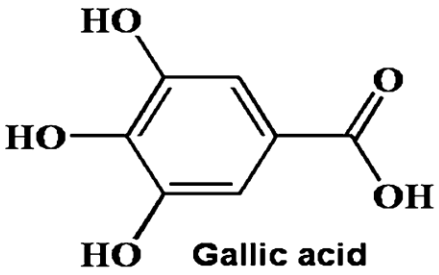
Cinnamic acid



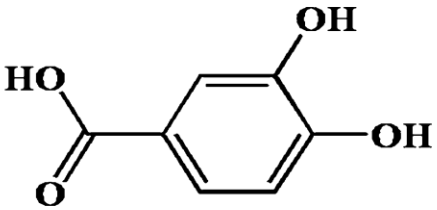
P-coumaric acid



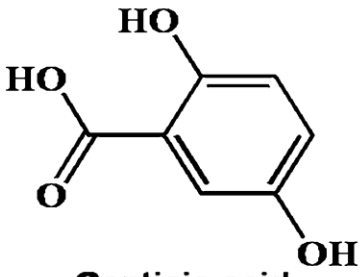
Syringic acid



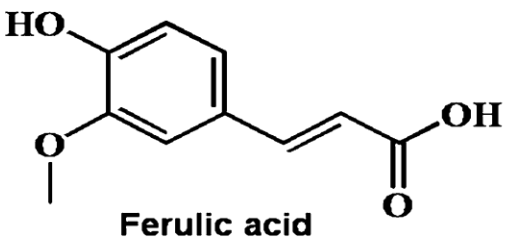
Gallic acid



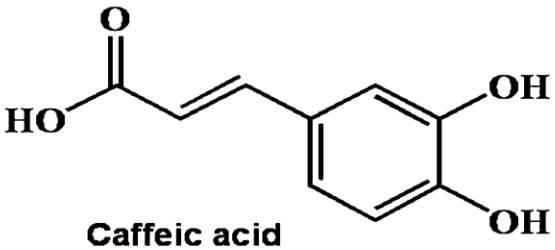
Protocatechuic acid



Gentisic acid



Ferulic acid



Caffeic acid

Materials: Chemical and Biological

3.1 Materials for chemical investigation

- **Table 3: Glass wares**

Sl. No.	Materials	Source
1.	Column (Size 16 inches X 1.5 inches)	BDH Laboratory Equipments
2.	Thin layer chromatographic (TLC) tank	Merck Germany
3.	Thin layer chromatographic plates (20cm x 20cm)	Merck Germany
5.	Conical flasks	BDH Laboratory Equipments
6.	Beakers	BDH Laboratory Equipments
7.	Test tubes	BDH Laboratory Equipments
8.	Funnels	BDH Laboratory Equipments
9.	Measuring cylinders	BDH Laboratory Equipments
10.	Pipettes etc.	BDH Laboratory Equipments
11.	Pasteur pipettes	BDH Laboratory Equipments

- **Table 4: Solvents**

Sl. No.	Materials	Source
1.	Ethanol	Merck Germany
2.	Hexane	Merck Germany
3..	Chloroform	Merck Germany
4.	Methylene chloride	Merck Germany and Active Fine Chemical
5.	Ethyl acetate	Merck Germany and Active Fine Chemical
6.	Acetone	Merck Germany
7.	Methanol	Merck Germany and Active Fine Chemical

- **Table 5: Equipments**

Sl. No.	Equipments	Source
1.	Rotary vacuum evaporator	IKA
2.	Electronic balance	Denver Instruments M-220
5.	Grinding machine	University Instruments Lab
6.	Oven (0°C-210°C)	Gallen Kamp Hotbox
7.	Solvent distillation plant	University Instruments Lab
8.	Distilled water plant	University Instruments Lab

- **Table 6: Spray reagents and filter aids**

	Spray reagents		Filter aids
1.	Charring solution (90% MeOH with 10% H_2SO_4)	1.	Cotton (normal wool)
		2.	Filter paper (Whatman No.1)

- **Table 7: Chromatographic materials**

SL.	Equipments	Source
1.	VLC grade Silica gels	Merck, Germany
2.	Column grade Silica gel	Merck, Germany
3.	Pre-coated, aluminum base (60 F-254) 20 cm X 20 cm TLC plates	Merck, Germany

3.2 Materials for pharmacological Investigation

- **Table 8: Test materials**

Sl. No.	Test materials
1.	Methanolic crude extract of the rhizome of *C. amada*
2.	Chloroform fraction of rhizome of *C. amada*
3.	n-Hexane fraction of rhizome of *C. amada*

3.3 Table 9: Material for microbial investigation

Sl. No.	Apparatus and Reagents
1.	Filter paper discs
2.	Petri dishes
3.	Inoculating loop
4.	Sterile cotton
5.	Sterile forceps
6.	Spirit burner
7.	Micropipette
8.	Screw cap test tubes
9.	Nose mask and Hand gloves
10.	Laminar air flow hood
11.	Autoclave
12.	Incubator
13.	Refrigerator
14.	Nutrient Agar Medium
15.	Ethanol
16.	Chloroform

- **Table 10: Test materials for microbial test**

Test samples
Isolated compound from n-hexane fraction(CADH-31)

- **Test Organisms**

The bacterial and fungal strains used for the experiment were collected as pure cultures from the university microbiology laboratory.

- **Culture medium and their composition**

The following media is used normally to demonstrate the antimicrobial activity and to make subculture of the test organisms.

- **Table 11: Composition of nutrient agar medium**

Ingredients	**Amounts**
Peptone	0.5 gm
Sodium chloride	0.5 gm
Yeast extract	1.0 gm
Agar	2.0 gm
Distilled water q.s.	100 ml
p^H	7.2 $\pm$ 0.1 at 25^0C

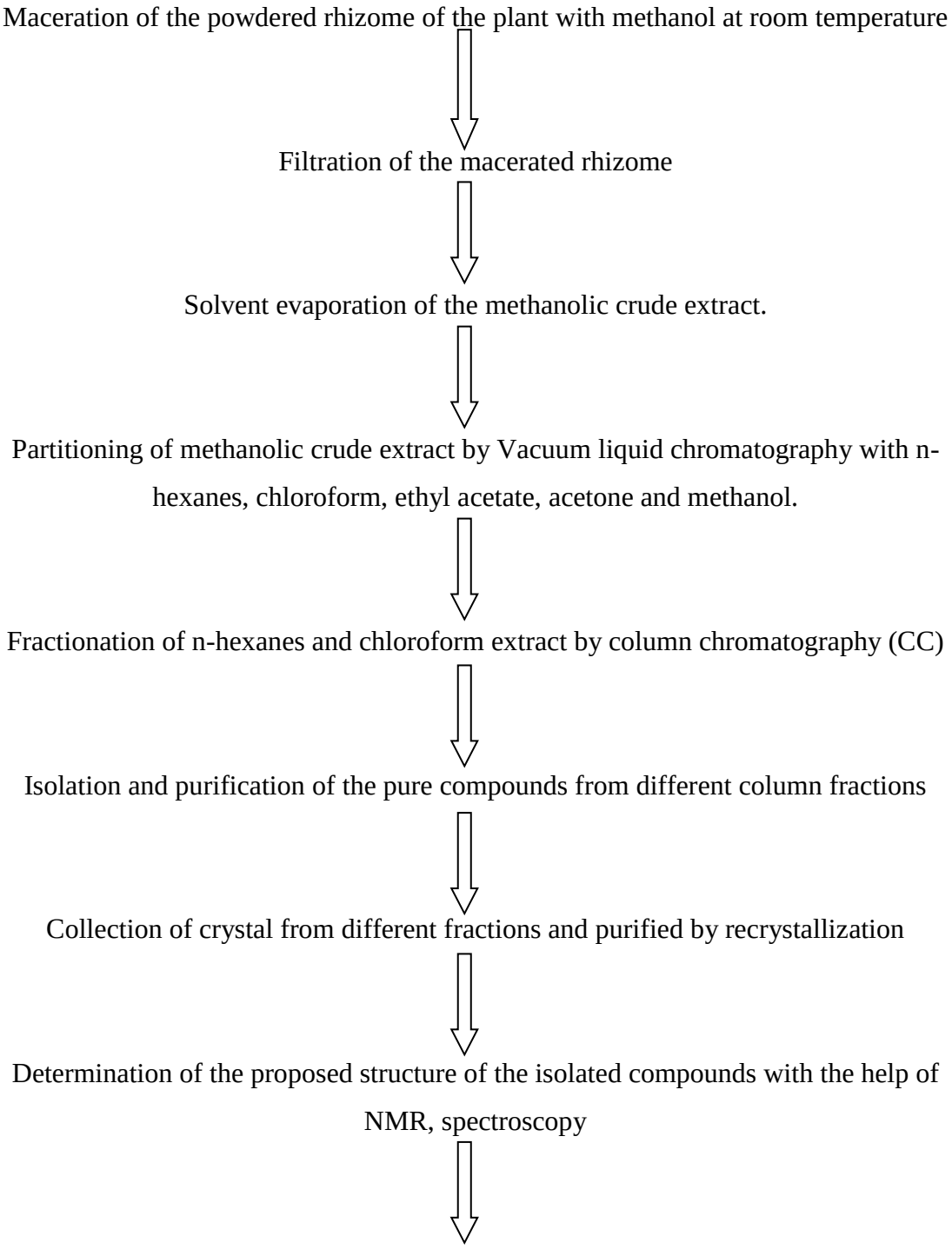
- **Table 12: Composition of Nutrient broth medium**

Ingredients	**Amounts**
Beef extract	0.3gm
Peptone	0.5gm
Distilled water q.s.	100ml
p^H	7.2 ±0.1 at 25^0C

Phytochemical Investigation

4.0 Study Protocol

Our present study was designed to isolate pure compounds as well as to observe biological activities of the isolated pure compounds with crude extract and their different fractions of the rhizome of the plant Curcuma amada (Family: Zingiberaceae). The study protocol consisted of the following steps:



Observation of *in vitro* antimicrobial activity of isolated pure compound

4.1 Phytochemical investigation

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

a) Collection and proper identification of the plant materials

b) Preparation of plant sample

c) Extraction

d) Fractionation and isolation of compounds

e) Structural characterization of purified compounds.

- **Experimental plants**

The plant species belonging to Zingiberaceae family has been investigated in this study is *Curcuma amada*. The plant part used in this investigation is only the rhizome of *Curcuma amada.*

4.1.1 Collection and identification of the plant sample

Plant sample rhizome of *Curcuma amada* was collected from Panchagarh, Bangladesh, in November 2011 and authentication of the plant sample has been confirmed by the taxonomist (Mrs. Hosne Ara) of the National Herbarium of Bangladesh which accession Number is 37511.

4.1.2 Preparation of plant material

The rhizome (after cutting into small pieces) was sun dried for several days. The dried samples were then ground in coarse powder using high capacity grinding machine in the spice mill. The coarse powder was then stored in air-tight container with marking for identification and kept in cool, dark and dry place for use.

4.1.3 Extraction procedure

About 800g of stem bark, the powdered material was taken in separate clean, plastic container and dissolved it in methanol for cold extraction process (2200ml×72hrs×3times). The whole mixture was then filtered through Whatman No.1 filter paper and the filtrate thus

obtained was concentrated in vaccuo at 45°C by a rotary evaporator. The concentrated extract was then air dried to solid residue. The weight of the crude methanolic extract was obtained 85.808gm. The percent yield was 10.73%.

4.1.4 Fractionation of Extract of rhizome of *Curcuma amada* through VLC

80gm of methanolic crude extract of rhizome of *Curcuma amada* was mixed with 75gm of silica (60grade and Mesh size was 70-230µm) and 5.5gm of crude extract was kept for future use. The mixture forms slurry which has been kept open overnight for drying the slurry. Silica for stationary phase was taken with mesh size of 20-60µm.

4.1.5 Investigation of hexane fractions by chromatographic methods and compound isolation

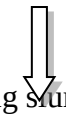
The fractions were subjected to TLC screening to see the type of compounds present in the extract. This revealed a considerable number of compounds and the finding suggested for further fractionation. Thus decision was taken to take further investigation of n hexane fraction of methanolic crude extract of stem bark of *C. amada.*

4.1.6 Preparation of Column Chromatography of n-Hexane fraction

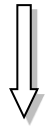
4.1.6.1 Column packing and Fractionation of sample

Chromatographic techniques are the most useful in the isolation and purification of compounds from plant extracts. Column Chromatography is the most common separation technique based on the principle of distribution (partition/adsorption) of compounds between a stationary and Mobile phase.

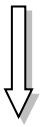
A glass column (46cm X 3cm) was cleaned and dried. Cotton pad was placed at the bottom of the column. The column was packed with 107gm of column grade silica (60grade, Mesh size was 70-230µm).



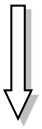
The silica, added to the column by making slurry with n-hexane, was allowed to settle down of appropriate height and slowly keeping the column outlet closed.



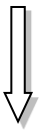
When the desired height of adsorbent bed was obtained, the column was flashed with three column volumes of n-hexane.



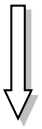
The n Hexane fraction (6.0gm) was dissolved in small quantity of n-hexane and mixed until the extract was dissolved.



Then sample was introduced onto the column as liquid application of sample.



The applied sample was eluted by a mixture of solvents comprising n-hexane, ethyl acetate and methanol with increasing polarity. This was done by allowing the flow of at a fixed rate of 15-20 drops per minute.



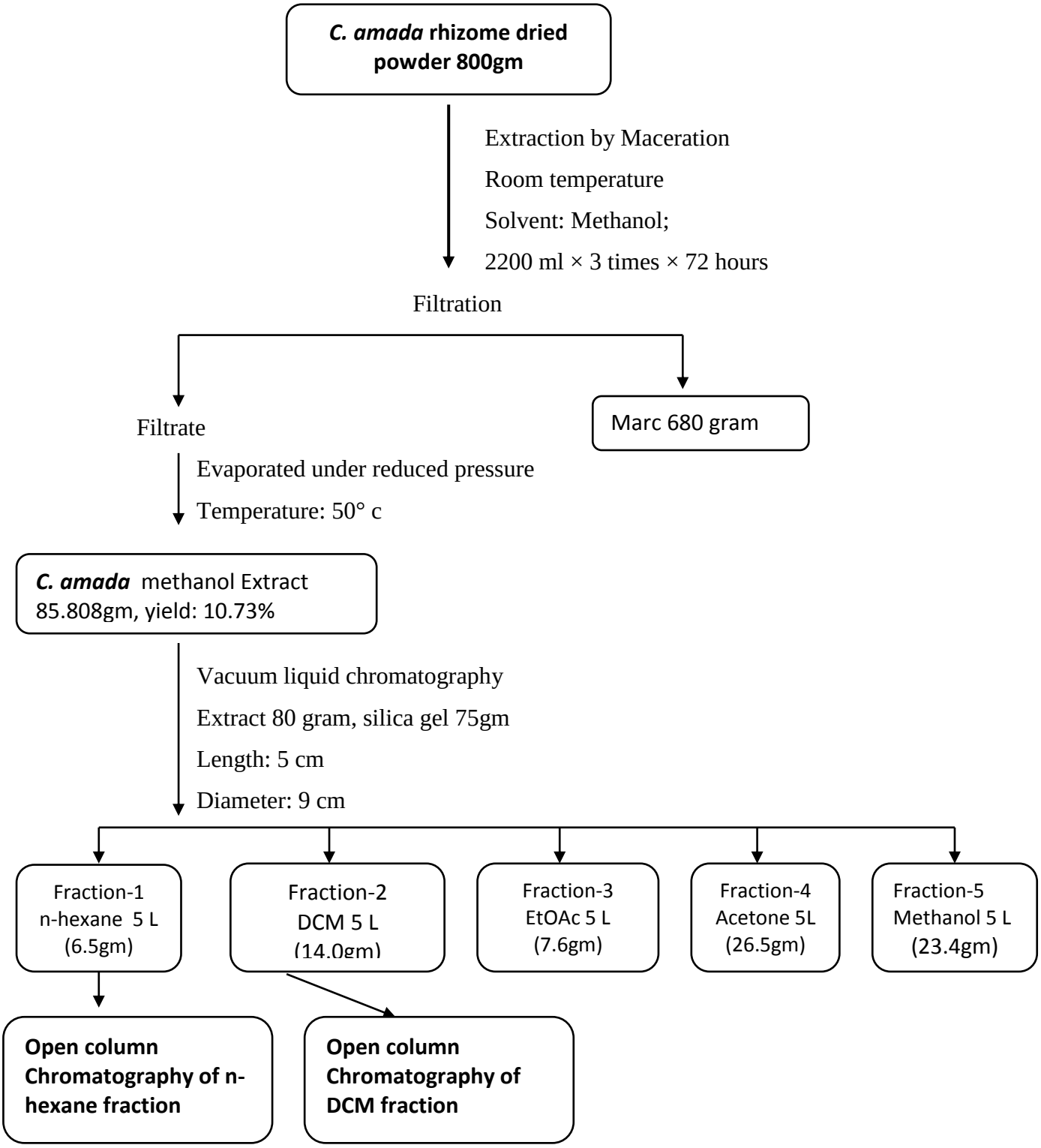
The Elutes were collected in beaker.

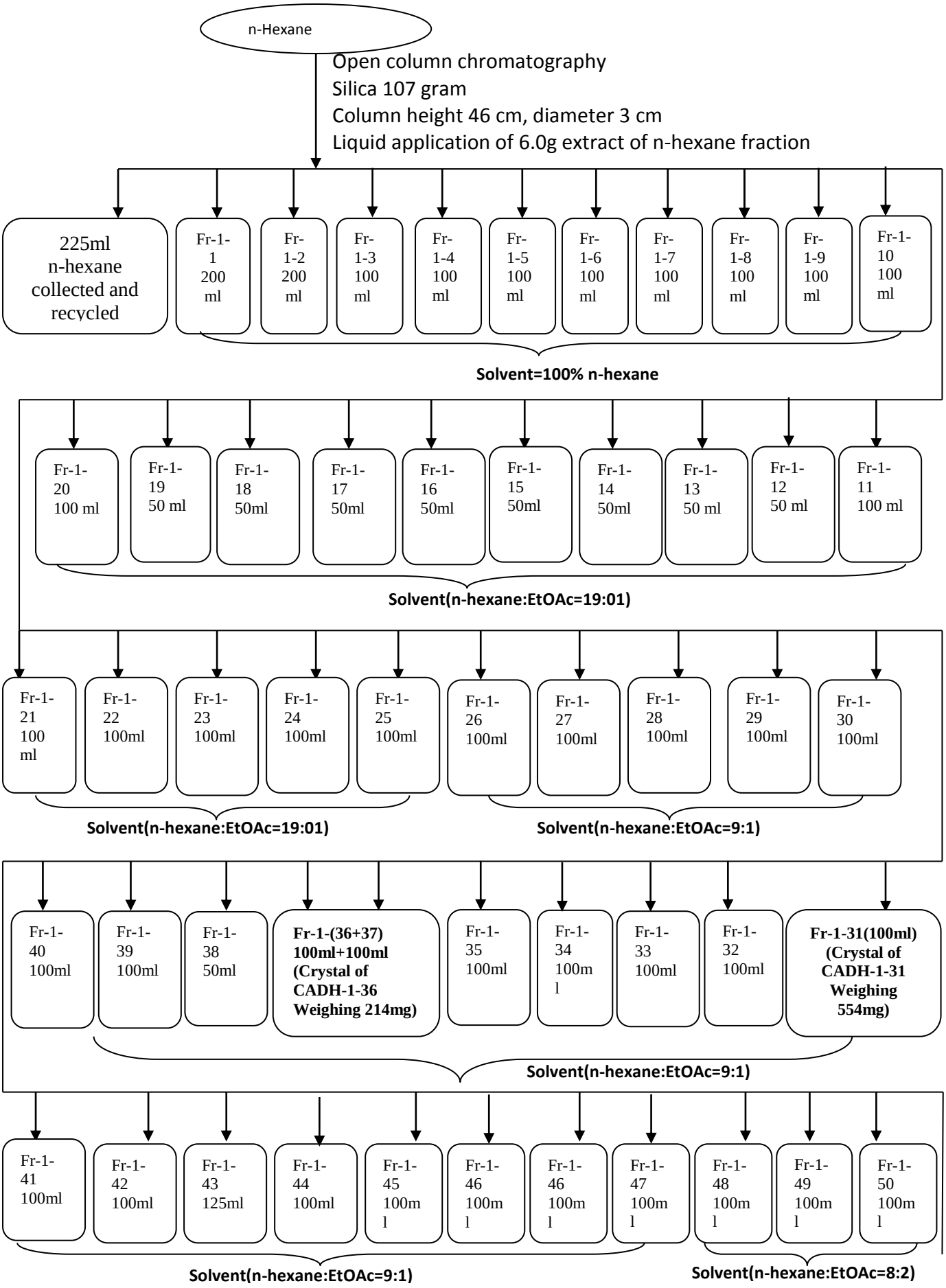
4.1.7 Preparation of Column Chromatography of Dichloromethane fraction

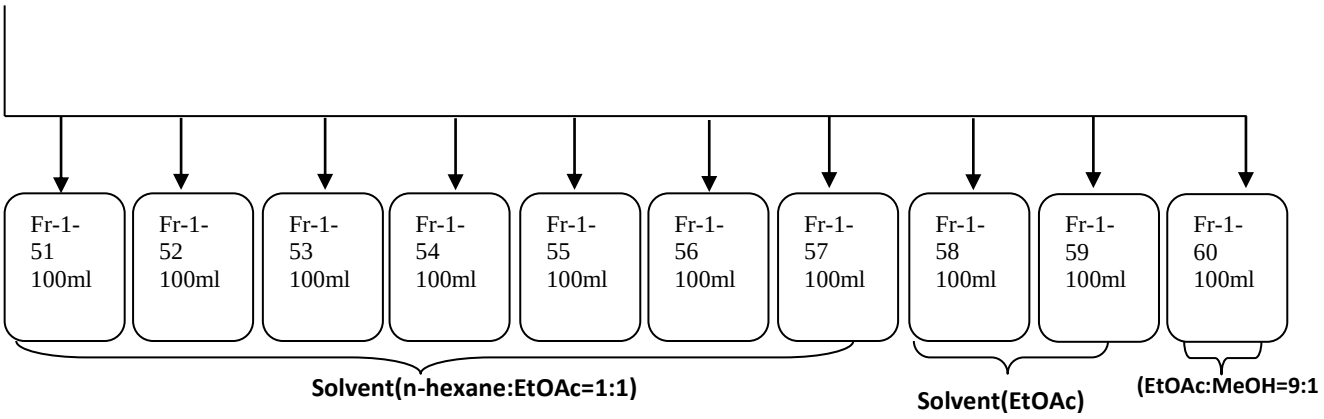
4.1.7.1 Column packing and Fractionation of sample

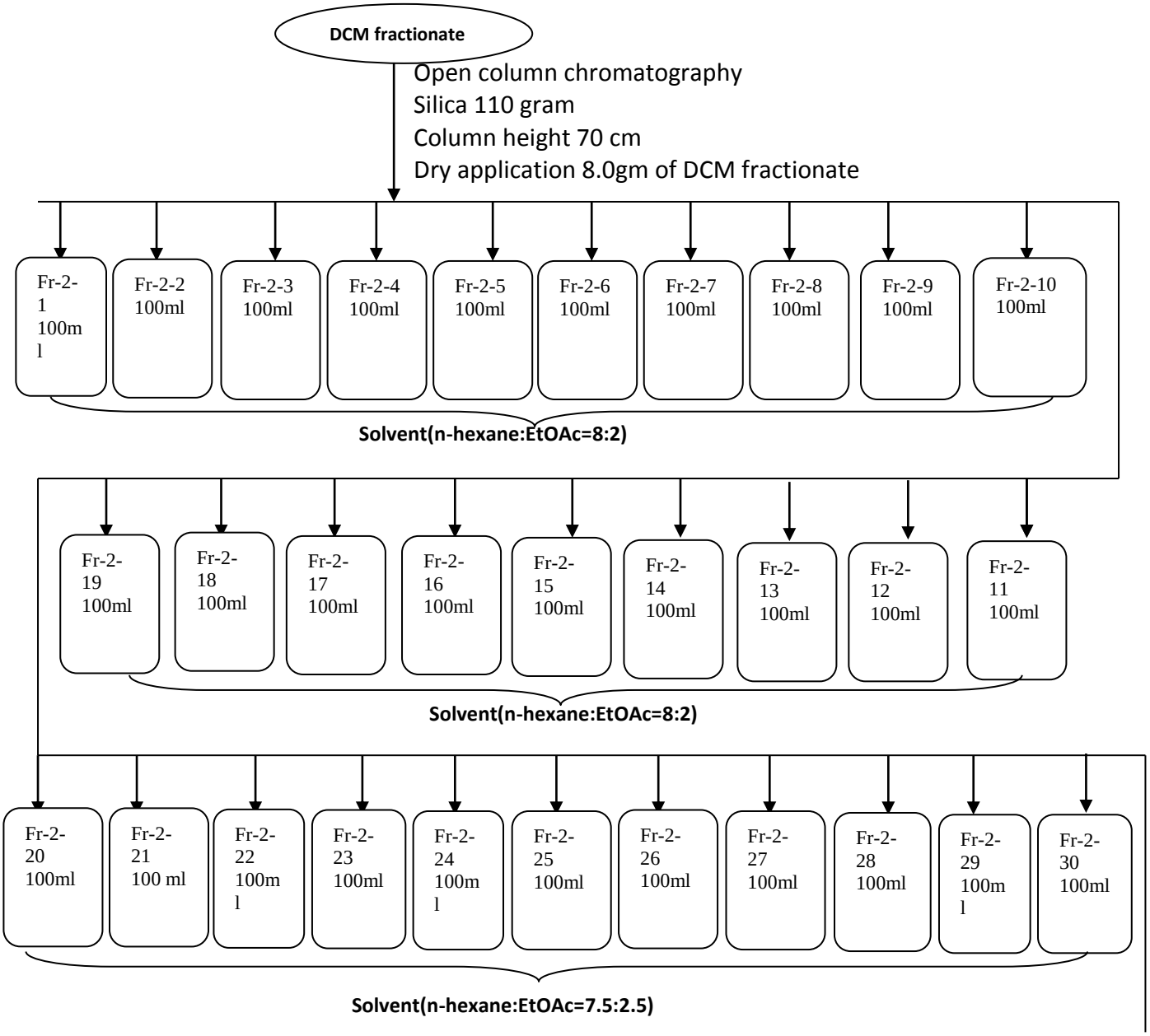
A glass column (46cm X 3cm) was cleaned and dried. Cotton pad was placed at the bottom of the column. The column was packed with 100gm of column grade silica (60grade, Mesh size was 70-230µm). Then further processing was as same as followed for n-hexane fraction.

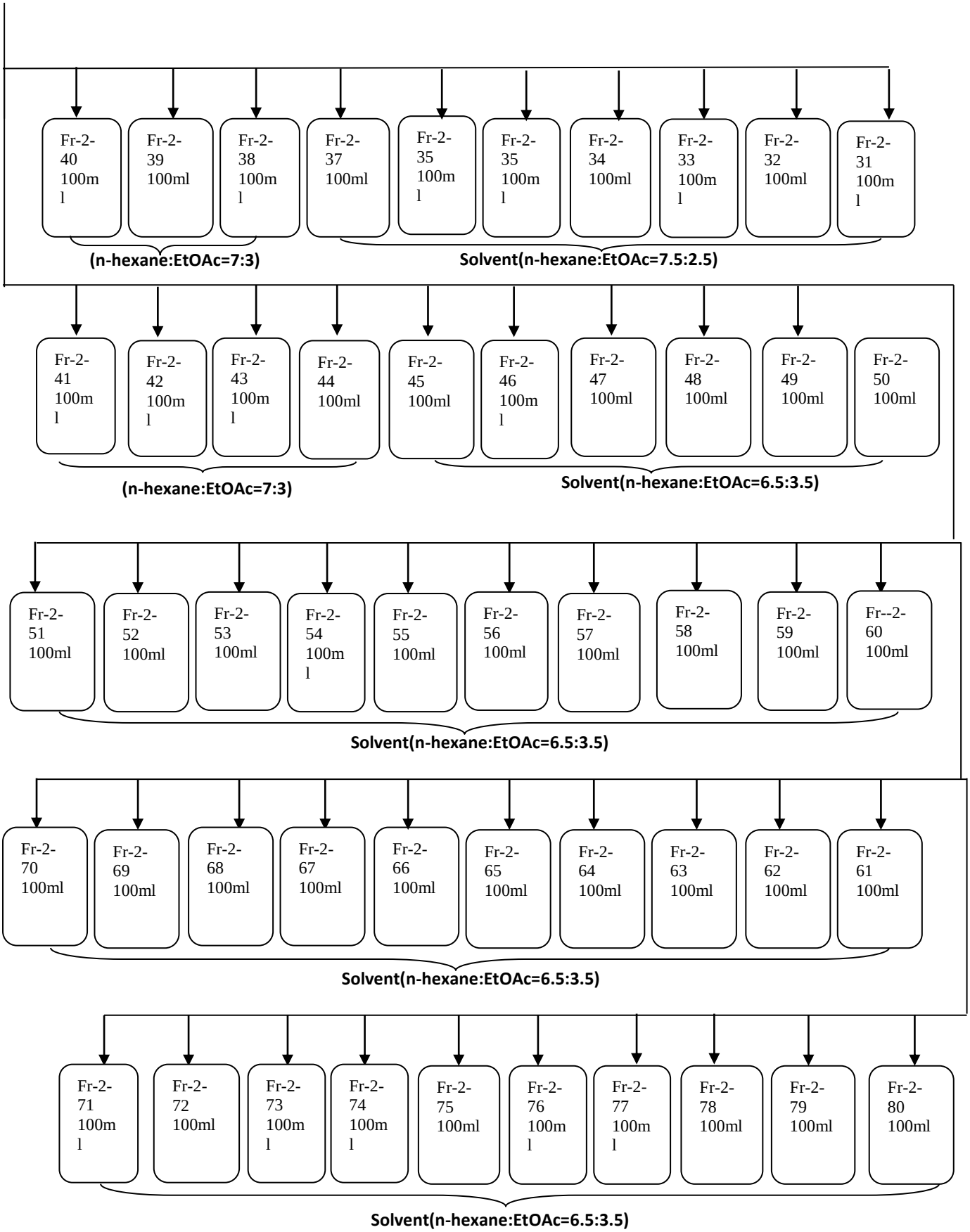
A schematic representation of Vacuum Liquid Chromatographic separation and column chromatography of N-hexane and DCM fraction has been shown below:




n-Hexane
Open column chromatography
Silica 107 gram
Column height 46 cm, diameter 3 cm
Liquid application of 6.0g extract of n-hexane fraction
225ml n-hexane collected and recycled
Fr-1-1 200 ml
Fr-1-2 200 ml
Fr-1-3 100 ml
Fr-1-4 100 ml
Fr-1-5 100 ml
Fr-1-6 100 ml
Fr-1-7 100 ml
Fr-1-8 100 ml
Fr-1-9 100 ml
Fr-1-10 100 ml
Solvent=100% n-hexane
Fr-1-20 100 ml
Fr-1-19 50 ml
Fr-1-18 50ml
Fr-1-17 50ml
Fr-1-16 50ml
Fr-1-15 50ml
Fr-1-14 50ml
Fr-1-13 50 ml
Fr-1-12 50 ml
Fr-1-11 100 ml
Solvent(n-hexane:EtOAc=19:01)
Fr-1-21 100 ml
Fr-1-22 100ml
Fr-1-23 100ml
Fr-1-24 100ml
Fr-1-25 100ml
Fr-1-26 100ml
Fr-1-27 100ml
Fr-1-28 100ml
Fr-1-29 100ml
Fr-1-30 100ml
Solvent(n-hexane:EtOAc=19:01)
Solvent(n-hexane:EtOAc=9:1)
Fr-1-40 100ml
Fr-1-39 100ml
Fr-1-38 50ml
Fr-1-(36+37) 100ml+100ml (Crystal of CADH-1-36 Weighing 214mg)
Fr-1-35 100ml
Fr-1-34 100ml
Fr-1-33 100ml
Fr-1-32 100ml
Fr-1-31(100ml) (Crystal of CADH-1-31 Weighing 554mg)
Solvent(n-hexane:EtOAc=9:1)
Fr-1-41 100ml
Fr-1-42 100ml
Fr-1-43 125ml
Fr-1-44 100ml
Fr-1-45 100ml
Fr-1-46 100ml
Fr-1-46 100ml
Fr-1-47 100ml
Fr-1-48 100ml
Fr-1-49 100ml
Fr-1-50 100ml
Solvent(n-hexane:EtOAc=9:1)
Solvent(n-hexane:EtOAc=8:2)


Fr-1-51 100ml
Fr-1-52 100ml
Fr-1-53 100ml
Fr-1-54 100ml
Fr-1-55 100ml
Fr-1-56 100ml
Fr-1-57 100ml
Fr-1-58 100ml
Fr-1-59 100ml
Fr-1-60 100ml
Solvent(n-hexane:EtOAc=1:1)
Solvent(EtOAc)
(EtOAc:MeOH=9:1


DCM fractionate
Open column chromatography
Silica 110 gram
Column height 70 cm
Dry application 8.0gm of DCM fractionate
Fr-2-1 100ml
Fr-2-2 100ml
Fr-2-3 100ml
Fr-2-4 100ml
Fr-2-5 100ml
Fr-2-6 100ml
Fr-2-7 100ml
Fr-2-8 100ml
Fr-2-9 100ml
Fr-2-10 100ml
Solvent(n-hexane:EtOAc=8:2)
Fr-2-19 100ml
Fr-2-18 100ml
Fr-2-17 100ml
Fr-2-16 100ml
Fr-2-15 100ml
Fr-2-14 100ml
Fr-2-13 100ml
Fr-2-12 100ml
Fr-2-11 100ml
Solvent(n-hexane:EtOAc=8:2)
Fr-2-20 100ml
Fr-2-21 100 ml
Fr-2-22 100ml
Fr-2-23 100ml
Fr-2-24 100ml
Fr-2-25 100ml
Fr-2-26 100ml
Fr-2-27 100ml
Fr-2-28 100ml
Fr-2-29 100ml
Fr-2-30 100ml
Solvent(n-hexane:EtOAc=7.5:2.5)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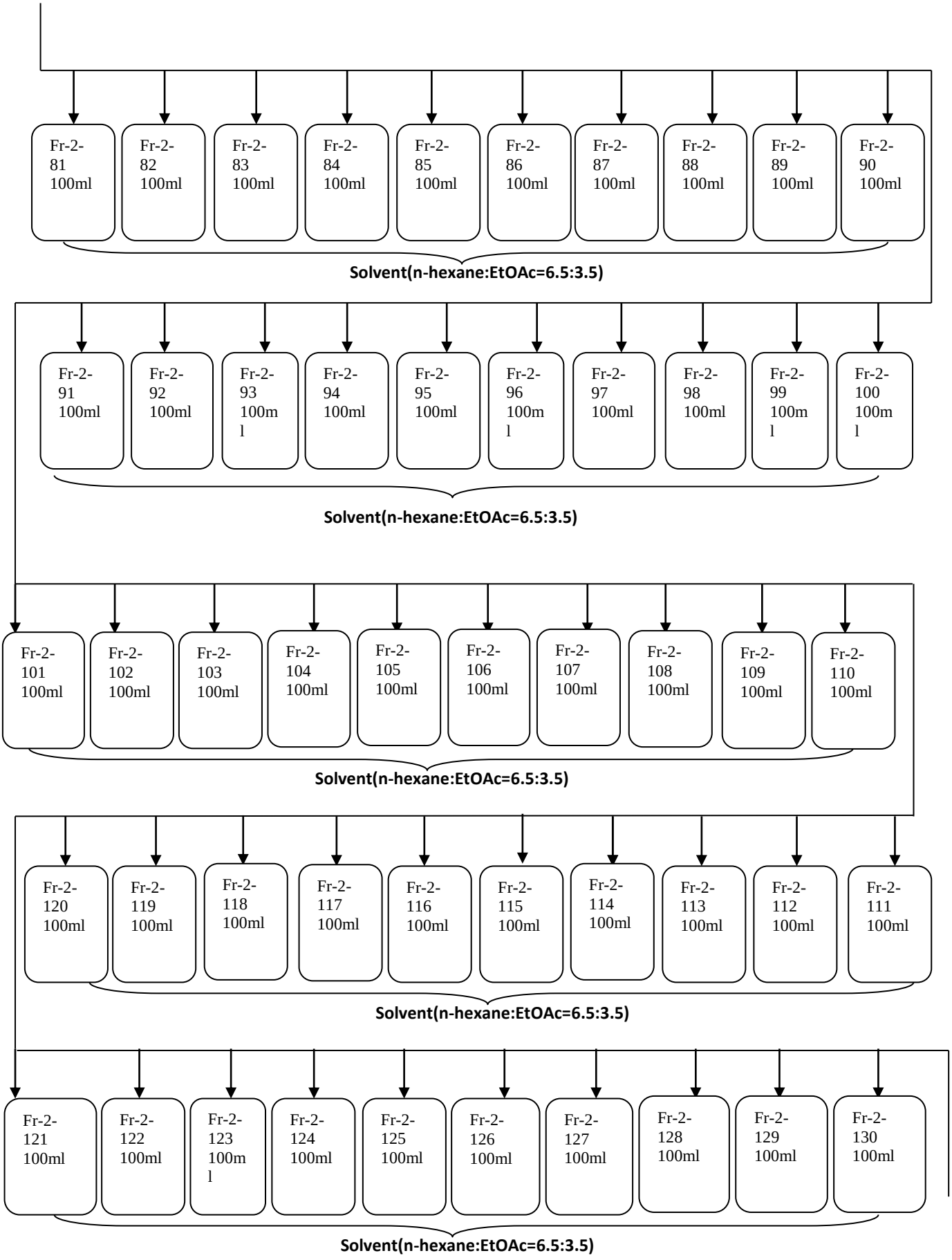
Fr-2-39 100ml
Fr-2-38 100ml
Fr-2-37 100ml
Fr-2-35 100ml
Fr-2-35 100ml
Fr-2-34 100ml
Fr-2-33 100ml
Fr-2-32 100ml
Fr-2-31 100ml
(n-hexane:EtOAc=7:3)
Solvent(n-hexane:EtOAc=7.5:2.5)
Fr-2-41 100ml
Fr-2-42 100ml
Fr-2-43 100ml
Fr-2-44 100ml
Fr-2-45 100ml
Fr-2-46 100ml
Fr-2-47 100ml
Fr-2-48 100ml
Fr-2-49 100ml
Fr-2-50 100ml
(n-hexane:EtOAc=7:3)
Solvent(n-hexane:EtOAc=6.5:3.5)
Fr-2-51 100ml
Fr-2-52 100ml
Fr-2-53 100ml
Fr-2-54 100ml
Fr-2-55 100ml
Fr-2-56 100ml
Fr-2-57 100ml
Fr-2-58 100ml
Fr-2-59 100ml
Fr--2-60 100ml
Solvent(n-hexane:EtOAc=6.5:3.5)
Fr-2-70 100ml
Fr-2-69 100ml
Fr-2-68 100ml
Fr-2-67 100ml
Fr-2-66 100ml
Fr-2-65 100ml
Fr-2-64 100ml
Fr-2-63 100ml
Fr-2-62 100ml
Fr-2-61 100ml
Solvent(n-hexane:EtOAc=6.5:3.5)
Fr-2-71 100ml
Fr-2-72 100ml
Fr-2-73 100ml
Fr-2-74 100ml
Fr-2-75 100ml
Fr-2-76 100ml
Fr-2-77 100ml
Fr-2-78 100ml
Fr-2-79 100ml
Fr-2-80 100ml
Solvent(n-hexane:EtOAc=6.5:3.5)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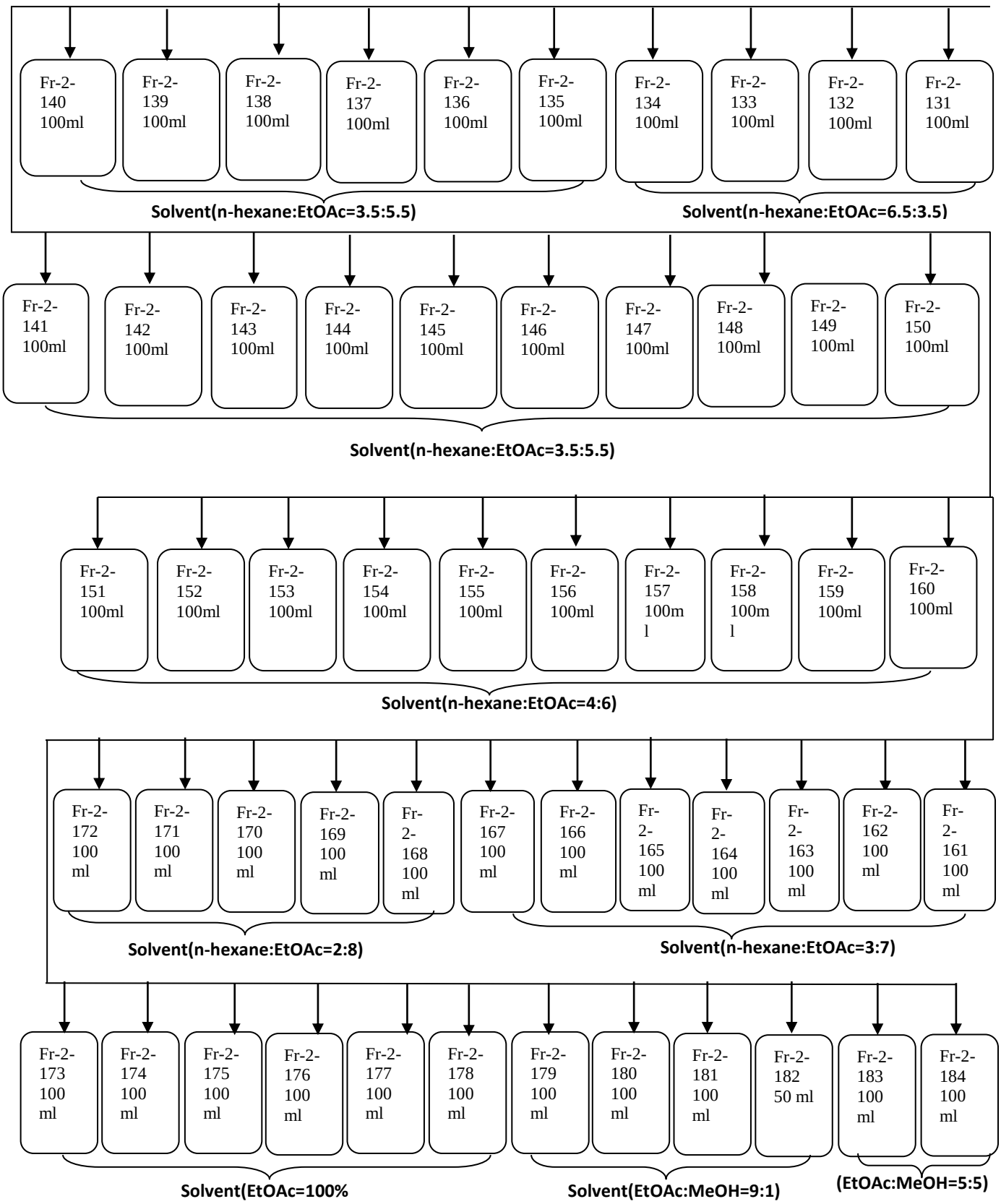
Fr-2-83 100ml
Fr-2-84 100ml
Fr-2-85 100ml
Fr-2-86 100ml
Fr-2-87 100ml
Fr-2-88 100ml
Fr-2-89 100ml
Fr-2-90 100ml
Solvent(n-hexane:EtOAc=6.5:3.5)
Fr-2-91 100ml
Fr-2-92 100ml
Fr-2-93 100ml
Fr-2-94 100ml
Fr-2-95 100ml
Fr-2-96 100ml
Fr-2-97 100ml
Fr-2-98 100ml
Fr-2-99 100ml
Fr-2-100 100ml
Solvent(n-hexane:EtOAc=6.5:3.5)
Fr-2-101 100ml
Fr-2-102 100ml
Fr-2-103 100ml
Fr-2-104 100ml
Fr-2-105 100ml
Fr-2-106 100ml
Fr-2-107 100ml
Fr-2-108 100ml
Fr-2-109 100ml
Fr-2-110 100ml
Solvent(n-hexane:EtOAc=6.5:3.5)
Fr-2-120 100ml
Fr-2-119 100ml
Fr-2-118 100ml
Fr-2-117 100ml
Fr-2-116 100ml
Fr-2-115 100ml
Fr-2-114 100ml
Fr-2-113 100ml
Fr-2-112 100ml
Fr-2-111 100ml
Solvent(n-hexane:EtOAc=6.5:3.5)
Fr-2-121 100ml
Fr-2-122 100ml
Fr-2-123 100ml
Fr-2-124 100ml
Fr-2-125 100ml
Fr-2-126 100ml
Fr-2-127 100ml
Fr-2-128 100ml
Fr-2-129 100ml
Fr-2-130 100ml
Solvent(n-hexane:EtOAc=6.5:3.5)


Fr-2-140 100ml
Fr-2-139 100ml
Fr-2-138 100ml
Fr-2-137 100ml
Fr-2-136 100ml
Fr-2-135 100ml
Fr-2-134 100ml
Fr-2-133 100ml
Fr-2-132 100ml
Fr-2-131 100ml
Solvent(n-hexane:EtOAc=3.5:5.5)
Solvent(n-hexane:EtOAc=6.5:3.5)
Fr-2-141 100ml
Fr-2-142 100ml
Fr-2-143 100ml
Fr-2-144 100ml
Fr-2-145 100ml
Fr-2-146 100ml
Fr-2-147 100ml
Fr-2-148 100ml
Fr-2-149 100ml
Fr-2-150 100ml
Solvent(n-hexane:EtOAc=3.5:5.5)
Fr-2-151 100ml
Fr-2-152 100ml
Fr-2-153 100ml
Fr-2-154 100ml
Fr-2-155 100ml
Fr-2-156 100ml
Fr-2-157 100ml
Fr-2-158 100ml
Fr-2-159 100ml
Fr-2-160 100ml
Solvent(n-hexane:EtOAc=4:6)
Fr-2-172 100 ml
Fr-2-171 100 ml
Fr-2-170 100 ml
Fr-2-169 100 ml
Fr-2-168 100 ml
Fr-2-167 100 ml
Fr-2-166 100 ml
Fr-2-165 100 ml
Fr-2-164 100 ml
Fr-2-163 100 ml
Fr-2-162 100 ml
Fr-2-161 100 ml
Solvent(n-hexane:EtOAc=2:8)
Solvent(n-hexane:EtOAc=3:7)
Fr-2-173 100 ml
Fr-2-174 100 ml
Fr-2-175 100 ml
Fr-2-176 100 ml
Fr-2-177 100 ml
Fr-2-178 100 ml
Fr-2-179 100 ml
Fr-2-180 100 ml
Fr-2-181 100 ml
Fr-2-182 50 ml
Fr-2-183 100 ml
Fr-2-184 100 ml
Solvent(EtOAc=100%
Solvent(EtOAc:MeOH=9:1)
(EtOAc:MeOH=5:5)

4.1.8 Analysis of Column Chromatography Fractions by TLC

All the column fractions were screened by TLC under UV light and by spraying with Methanol-sulphuric acid reagent.

4.1.8.1 Chamber saturation

Glass chamber having cylindrical and rectangular shapes and solvent tanks with airtight lid were taken for the development of plates. To saturate the interior environment-developing solvents were introduced separately into the TLC tanks with closing the lid tightly and kept undisturbed for 20 minutes. A filter paper touching the mobile phase was placed inside the tank for ensuring appropriate saturation of the chambers.

4.1.8.2 Detection, isolation and purification of compounds from the selected Column Chromatographic fractions

The developed plates were examined under UV lamp. The sample was UV active and appeared black after spraying with Methanol-sulphuric acid reagent**.**

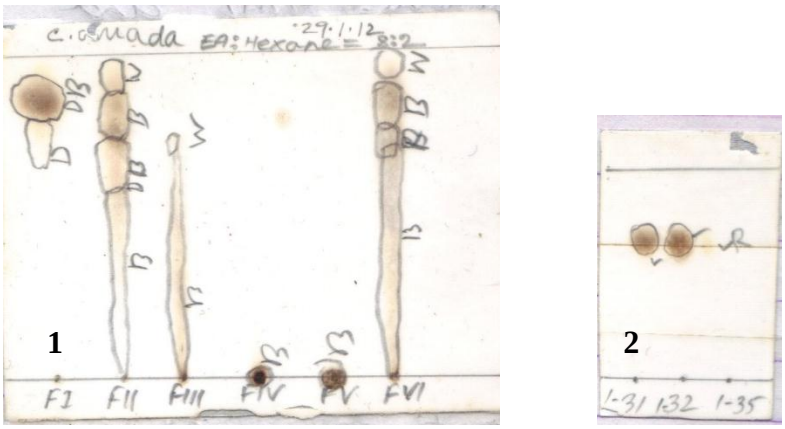


Fig 6: TLC of fractions: 1. VLC fractions; 2. Pure compounds

4.1.9 The physical properties of the isolated compounds

The compound was white in color and crystalline in nature**.**

4.2 The [1]H-NMR analysis of sample CADH-31

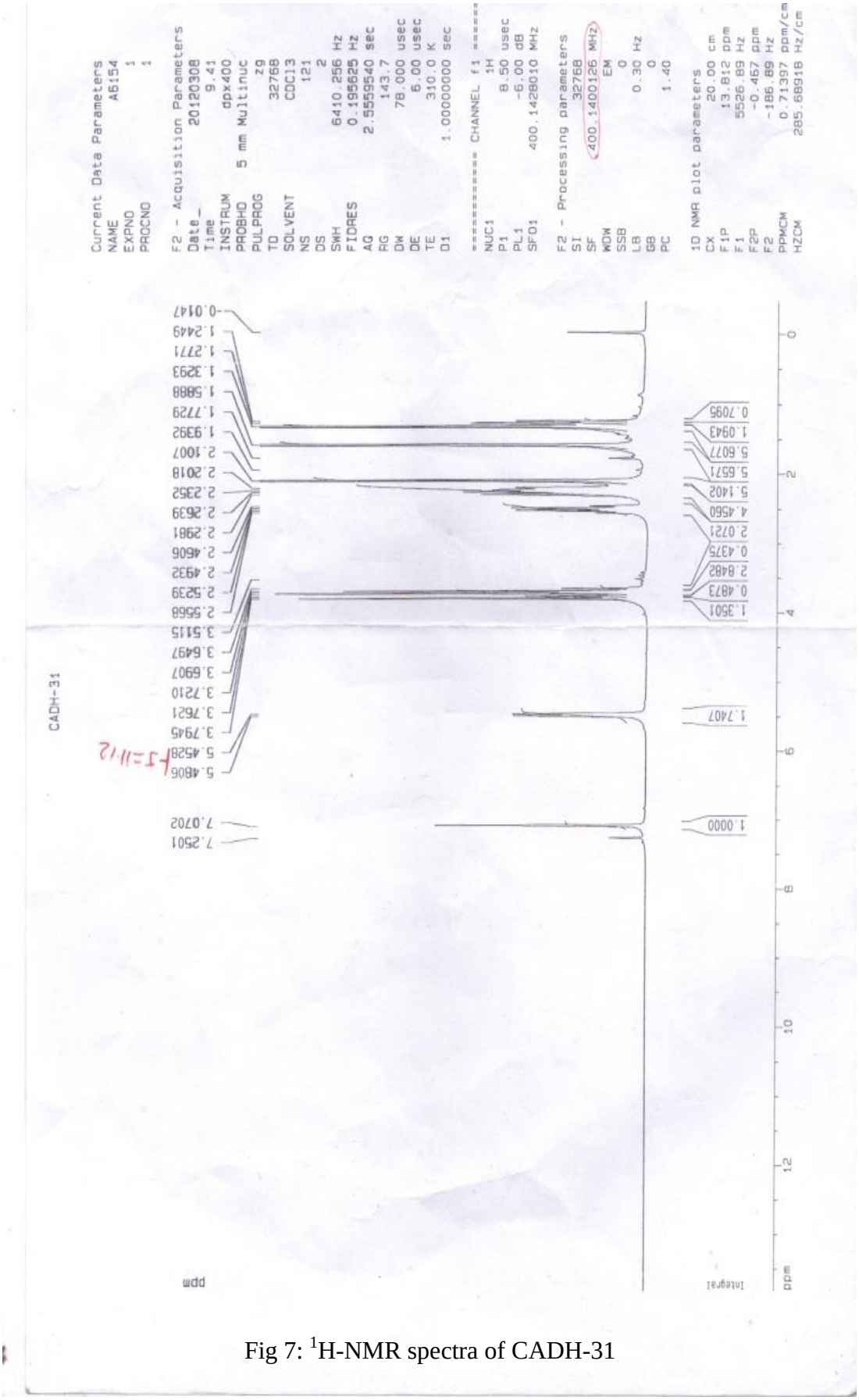


Fig 7: [1]H-NMR spectra of CADH-31

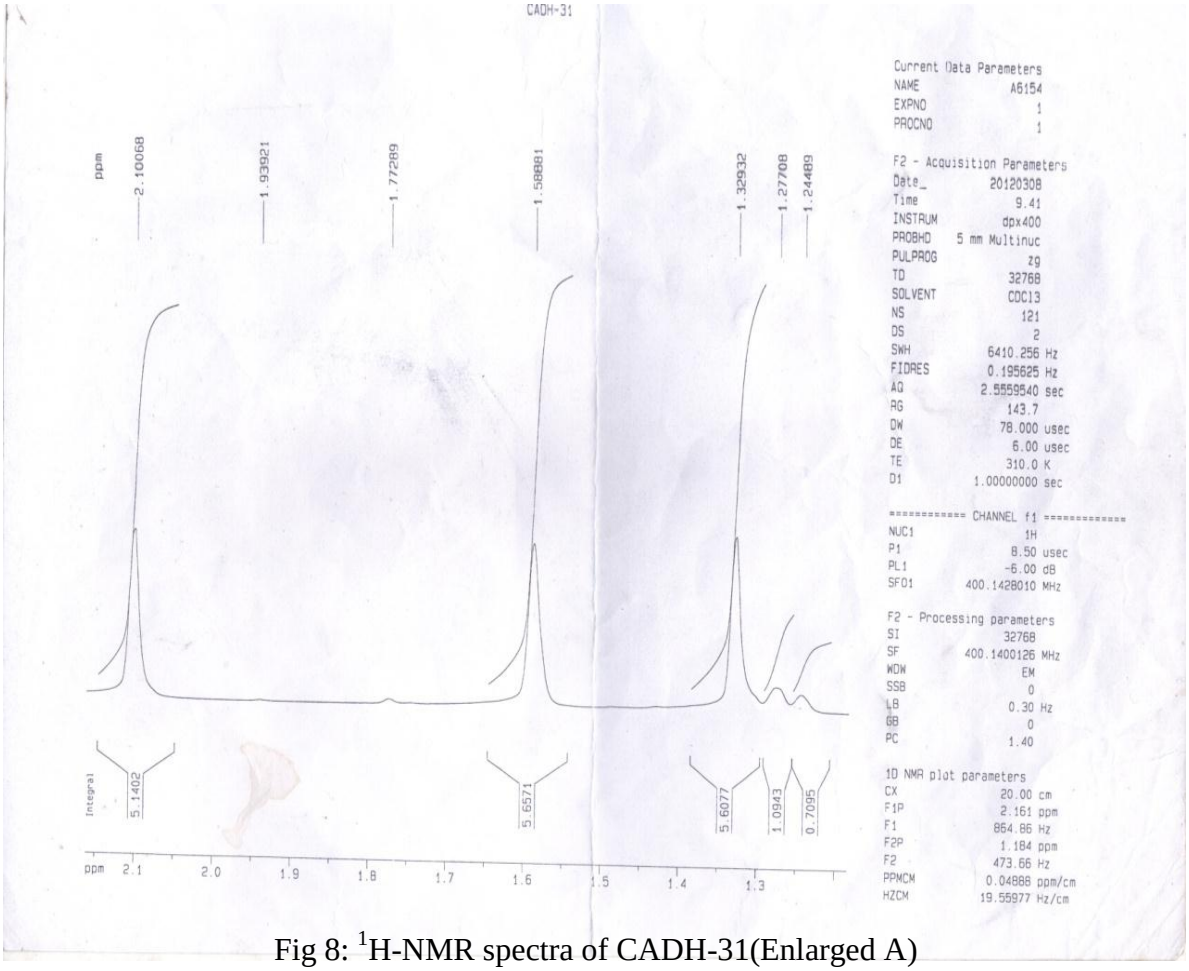


Fig 8: ^{1}H-NMR spectra of CADH-31(Enlarged A)

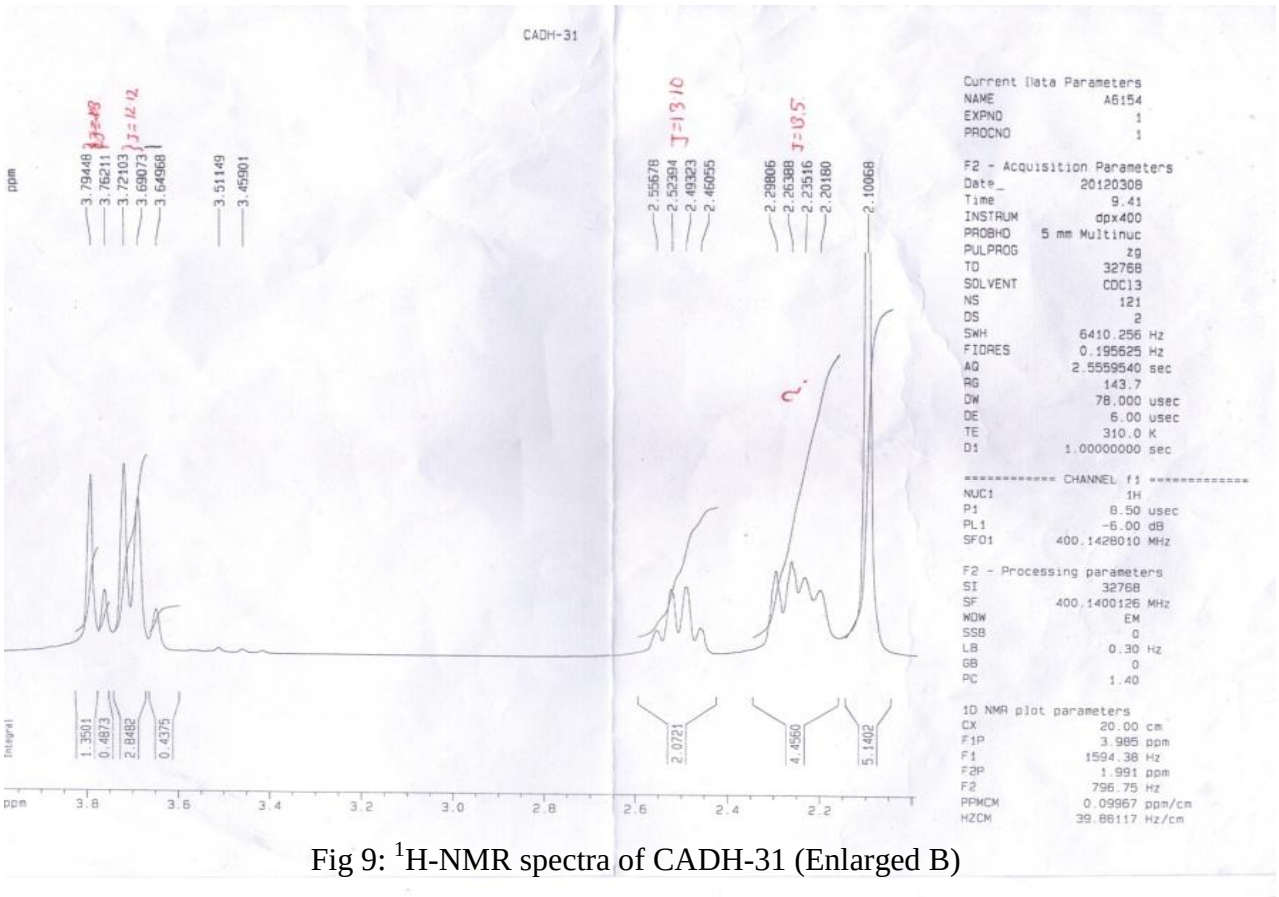
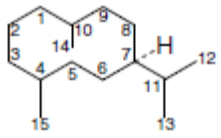


Fig 9: ^{1}H-NMR spectra of CADH-31 (Enlarged B)

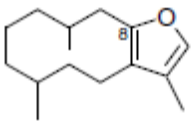
4.2.1 Discussion

The proton NMR spectral analysis of CADH-31 showed six methyl signals at δ-1.26 (CH_3 x 2,m), 1.59(CH_3 x 2,S), and 2.10 (CH_3 x 2,S). In ^{1}H-NMR spectra five oxymethyne peaks appeared among which three of these oxymethyne proton resonances appeared at δ-3.69, 3.72,3.79 as singlate and remaining two oxymethyne proton signals at δ-3.69,d and 3.77,d appeared as doublets.

A characteristic exomethylene protons are appeared at δ-5.45(s) and d-5.48(s). Besides, the NMR also showed three methylene protons at δ-2.22 (2H,m), 2.28(2H, m), 2.48(1H)m, 2.54(1H)m and one methyne proton at δ -1.25 (1H, m). Based on the above analysis of the ^{1}H-NMR spectrum, I proposed the following structure of CADH-31 as show in the figure which is a sesquiterpene derivative of furanogermacrane type.



Germacrane
1,7-Dimethyl-4-(1-methyl-ethyl)cyclodecane, 9CI



Furanogermacrane
4,5,6,7,8,9,10,11-Octahydro-3,6,10-trimethylcyclodeca[*b*]-furan, 9CI

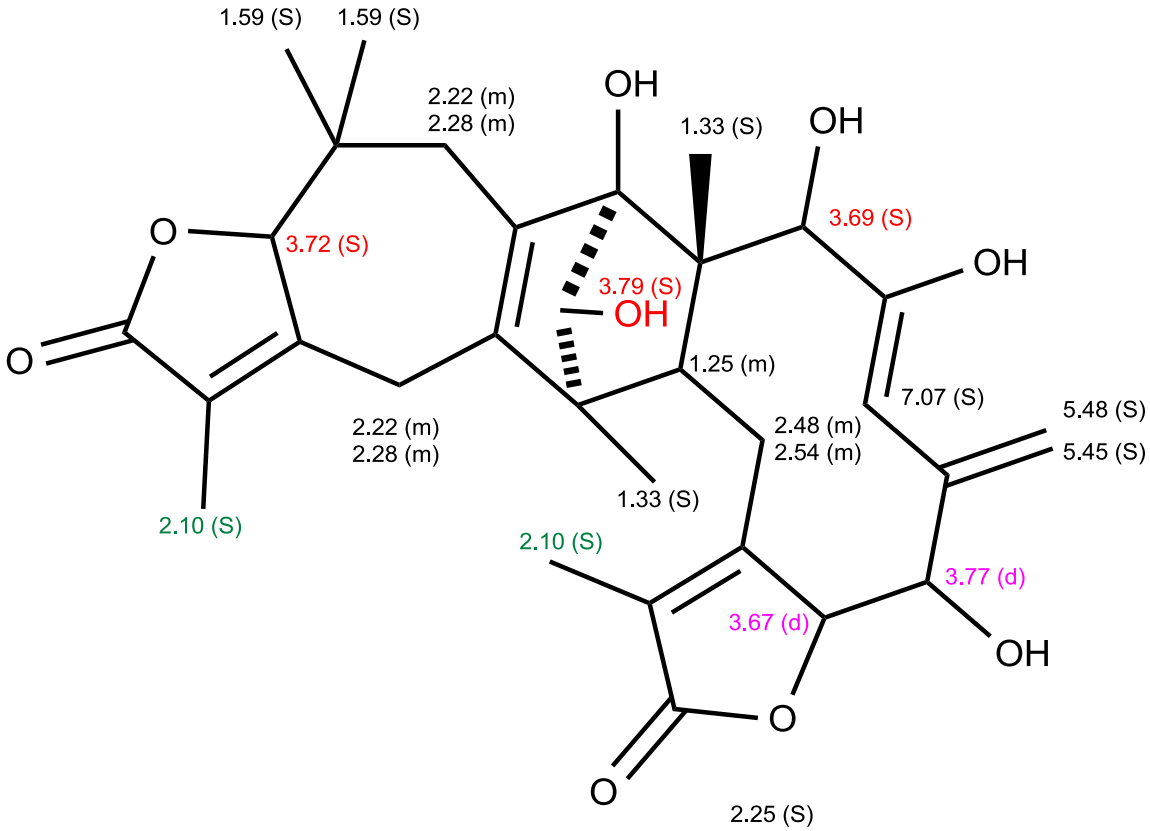
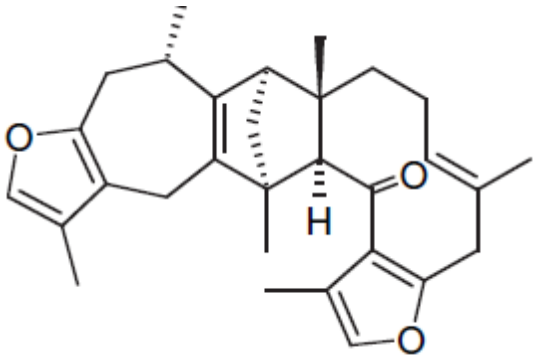
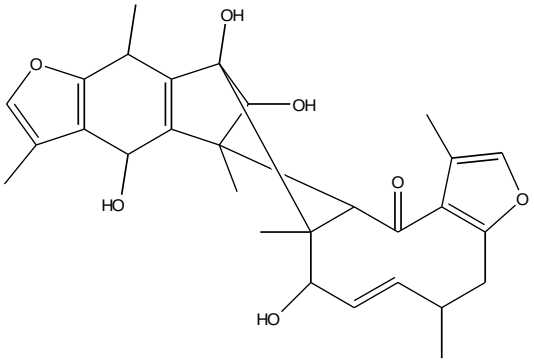


Fig 10: Structure of CADH-31

To the best of our knowledge it is a novel compound. Two relevant furanogermacrane type compound isolated from the genus of *Curcuma*.



Difurocumenone



Difurocumenonol

As it is far more difficult to analyse the compound structure from only ^{1}H-NMR, so our sample has been sent to United Kingdom for further spectroscopic analysis. So the structure can be confirmed after having the ^{13}C-NMR data with other spectroscopic analysis like High resoulation mass spectroscopy, etc.

Experimental

Biological investigation

5.0 Pharmacological evaluation of the crude extract and different fraction of *C. amada*

5.1 Objectives

As the present study was designed to be 'Bioactivity guided', preliminary pharmacological investigations of the extracts of the plant and various fractions, were very crucial in the perspective of the entire work and the findings of the preliminary study was to direct the next step of the work. This would also help to rationalize the folklore use of the plant concerned.

As the plant *Curcuma amada* is biologically very important plant, the whole biological investigation of the plant was guided by methods developed for evaluation of analgesic, and antimicrobial activity. In this study, the evaluation of pharmacological activity was primarily focused on the pain and antimicrobial effect.

5.2 Biological investigations observed in this study

- **Anti- Microbial activity.**

5.3 Antimicrobial Screening

5.3.1 Introduction

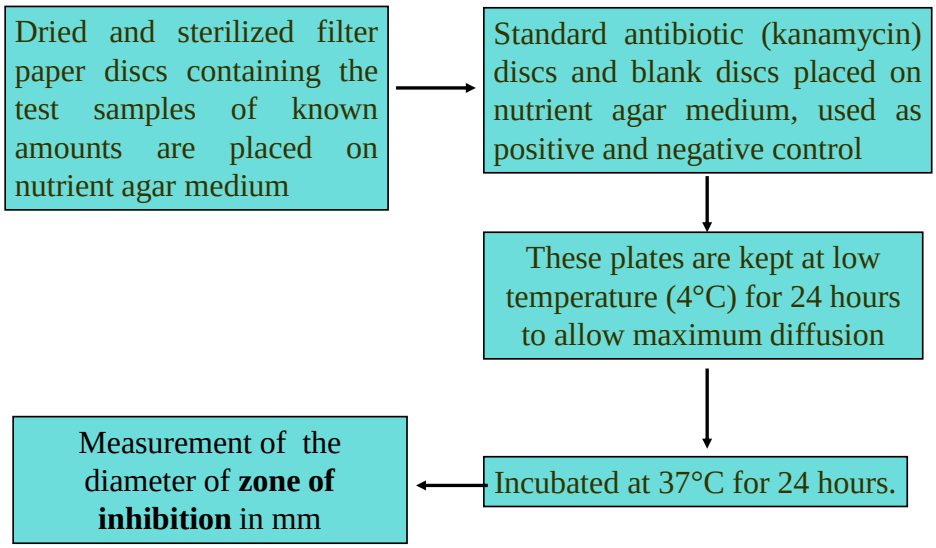
Bacteria and fungi are responsible for many infectious diseases. The increasing clinical implications of drug resistant fungal and bacterial pathogens have lent additional urgency to antimicrobial drug research. The antimicrobial screening which is the first stage of antimicrobial drug research is performed to ascertain the susceptibility of various fungi and bacteria to any agent. This test measures the ability of each test sample to inhibit the *in vitro* fungal and bacterial growth. This ability may be estimated by any of the following three methods.

i) Disc diffusion method
ii) Serial dilution method
iii) Bioautographic method

But there is no standardized method for expressing the results of antimicrobial screening. Some investigators use the diameter of zone of inhibition and/or the minimum weight of extract to inhibit the growth of microorganisms. However, a great number of factors viz., the extraction methods, inoculums volume, culture medium composition and PH and incubation temperature can influence the results.

Among the above mentioned techniques the disc diffusion is a widely accepted *in vitro* investigation for preliminary screening of test agents which may possess antimicrobial activity. It is essentially a quantitative or qualitative test indicating the sensitivity or resistance of the microorganisms to the test materials. However, no distinction between Bacteriostatic and Bactericidal activity can be made by this method.

Procedure Of Antimicrobial Investigation



5.3.2 Principle of Disc Diffusion Method

Solutions of known concentration (μg/ml) of the test samples are made by dissolving measured amount of the samples in calculated volume of solvents. Dried and sterilized filter paper discs (6 mm diameter) are then impregnated with known amounts of the test substances using micropipette. Discs containing the test material are placed on nutrient agar medium uniformly seeded with the test microorganisms. Standard antibiotic discs and blank discs (impregnated with solvents) are used as positive and negative control. These plates are then kept at low temperature (4 ^{0}C) for 24 hours to allow maximum diffusion. During this time dried discs absorb water from the surrounding media and then the test materials are dissolved

and diffused out of the sample disc. The diffusion occurs according to the physical law that controls the diffusion of molecules through agar gel. As a result there is a gradual change of test materials concentration in the media surrounding the discs.

The plates are then incubated at 37 ^{0}C for 24 hours to allow maximum growth of the organisms. If the test materials have any antimicrobial activity, it will inhibit the growth of the microorganisms and a clear, distinct zone of inhibition will be visualized surrounding the medium. The antimicrobial activity of the test agent is determined by measuring the diameter of zone of inhibition expressed in millimeter.The experiment is carried out more than once and the mean of the readings is required.

In the present study the purified compound was tested for antimicrobial activity by disc diffusion method.

5.3.3 Preparation of medium

To prepare required volume of this medium, calculated amount of the constituent was taken in a conical flask and distilled water was added to it to make the required volume. The contents were heated in a water bath to make a clear solution. The P^H (at 25 ^{0}C) was adjusted at 7.2 – 7.6 using NaOH or HCl. 10 ml and 5 ml of the medium was then transferred in screw cap test tubes to prepare plates and slants respectively. The test tubes were then capped and sterilized by autoclaving at 15-lbs. pressure/sq. inch at 121 ^{0}C for 20 minutes. The slants were used for making fresh culture of bacteria and fungi that were in turn used for sensitivity study.

5.3.4 Sterilization procedures

In order to avoid any type of contamination and cross contamination by the test organisms the antimicrobial screening was done in Laminar Hood and all types of precautions were highly maintained. UV light was switched on one hour before working in the Laminar Hood. Petri dishes and other glassware's were sterilized by autoclaving at a temperature of 121 ^{0}C and a pressure of 15-lbs./sq. inch for 20 minutes. Micropipette tips, cotton, forceps, blank discs etc. were also sterilized.

5.3.5 Preparation of subculture

In an aseptic condition under laminar air cabinet, the test organisms were transferred from the pure cultures to the agar slants with the help of a transfer loop to have fresh pure cultures. The inoculated strains were then incubated for 24 hours at 37 ^{0}C for their optimum growth. These fresh cultures were used for the sensitivity test.

5.3.6 Preparation of the test plates

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

5.3.7 Preparation of discs

Three types of discs were used for antimicrobial screening.

- **Standard discs**

These were used as positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antimicrobial agent with that of the test sample. In this investigation, amoxycillin (30μg/disc) disc was used as the reference.

- **Blank discs**

These were used as negative controls which ensure that the residual solvent (left over the discs even after air-drying) and the filter paper were not active themselves.

- **Preparation of sample discs with test samples**

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

5.3.8. Diffusion and Incubation

The sample discs, the standard antibiotic discs and the control discs were placed gently on the previously marked zones in the agar plates pre-inoculated with test bacteria and fungi. The plates were then kept in a refrigerator at 4 ^{0}C for about 24 hours upside down to allow sufficient diffusion of the materials from the discs to the surrounding agar medium. The plates were then inverted and kept in an incubator at 37 ^{0}C for 24 hours.

5.4 Determination of antimicrobial activity by the zone of inhibition, Result

The antimicrobial potency of the test agents are measured by their activity to prevent the growth of the microorganisms surrounding the discs which gives clear zone of inhibition. After incubation, the Antimicrobial activities of the test materials can be determined by measuring the diameter of the zones of inhibition in millimeter with a transparent scale. But the showed negative effect and didn't give any zone of inhibition.

5.5 Discussion

The compound doesn't have any antibacterial activity because according to the positive control and negative control the sample does not show any zone of inhibition. But it may be due to the contaminated culture or other mistake due to experimental procedure. But it may show any other pharmacologically active properties because literature review showed that this plant has various traditional use as well as a wide variety of pharmacological properties.

6.0 Conclusion:

Curcuma amada Roxb. (Family *Zingiberaceae*) commonly known as "mango ginger" is an important species of *Curcuma*. The rhizomes are more popular in traditional medicines to treat many diseases.

There are many pharmacological activity evaluated by the extract of rhizomes and many chemical constituents already isolated from the rhizomes of this plant by using small quantity of extract. Nevertheless, there are some biologically active chemical constituents were isolated including amadannulen, amadaldehyde (antimicrobial agents), and difurocumenonol (antituberculosis agent).

As the rhizomes exerted so many pharmacological activities, the chemical constituents were isolated using small quantity of extract and limited biologically active compounds have been isolated, therefore our study is isolation and purification of active principles from 80gm MeOH extract of rhizomes of *Curcuma amada*.

The extraction was carried out by maceration of powder in MeOH at room temperature and the extract was concentrated in vaccuo by a rotatory evaporator at 50^{o} C. A total of 85.8 gm extract was obtained from 800 gm dried powder (Yield 10.73%).

The crude extract was subjected to Thin Layer Chromatography (TLC) to see the quality of chemical constituents of extract. For fractionation the nonpolar to polar compounds, Vacuum Liquid Chromatography (VLC) was used using Hex, $CHCl_3$, EtOAc, Acetone and MeOH as solvent. A total of five fractions were obtained, which are fraction-1 (Hex), fraction-2 ($CHCl_3$), fraction-3 (EtOAc), fraction-4 (Acetone), and Fraction-5 (MeOH). All fractions were subjected to TCL to choose the fractions with more desirable chemical constituents and fraction-1 and fraction-2 were selected for column chromatography.

Fraction-1 (6.5gm) was subjected to silica gel (107gm) open column chromatography and eluted with hexanes-ethyl acetate, ethyl acetate-methanol step gradients. A total of 60 fractions were obtained. The fraction-31 to fraction-37 was crystals with small amount of impurities. These fractions were crystallized by hexanes to get colorless crystals. The fraction-31 to fraction-33 showed same Rf value and the fraction-35 to fraction-37 showed same Rf value with only one spot in analytical TCL plates. The fraction-31 to fraction-33 and fraction-35 to fraction-36 were pooled and named as CADH-31 and CADH-36. The compound CADH-31 was subjected to ^{1}H NMR. The result of ^{1}H NMR and the analytical TLC suggested that the compound CADH-31 is a polyketides group containing sesquiterpene, but proton NMR didn't match with compounds isolated from Curcuma

species. The compound CADH-36 was sent to United Kingdom for Nuclear Magnetic Spectrophotometric analysis.

The fraction-2 (14.0gm) was subjected to silica gel (100gm) open column chromatography and eluted with hexanes-ethyl acetate, ethyl acetate-methanol step gradients. A total of 184 fractions were obtained.

The compound CADH-31 was used for antimicrobial screening using Disk diffusion method and amoxicillin was used as positive control. In this screening, the compound showed no antimicrobial activity.

From the above statement, it can be concluded that the compound CADH-31 needs more spectroscopic analysis for the conformation of the compound and it could be a new lead compound. The isolated compounds need to conduct other biological activity tests on the basis of literature review to get active principles. All the fractions should combine on the basis of analytical TLC and further isolation and purification processes and biological activity tests must be conducted to get more biologically active potent drug leads.

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