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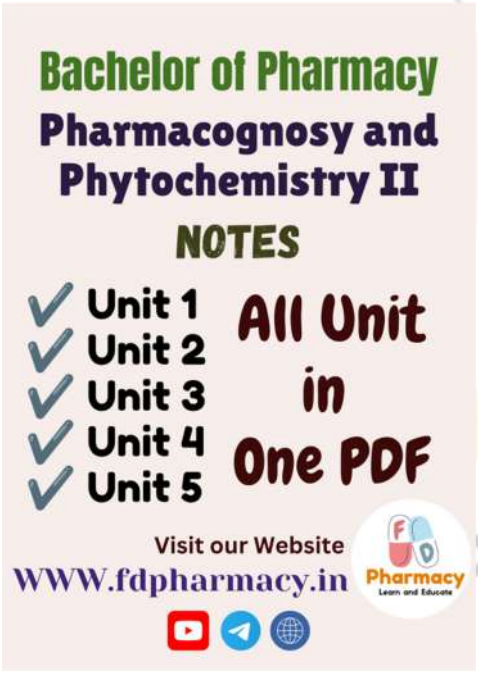
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PHARMACOGNOSY AND PHYTOCHEMISTRY - II

UNIT 3

TOPIC :

- Isolation, Identification and Analysis of Phytoconstituents

  a) Terpenoids: Menthol, Citral, Artemisin

Phytoconstituents

- Phytoconstituents are naturally occurring chemical compounds present in plants.
- These bioactive substances are responsible for the therapeutic, pharmacological, and physiological effects of medicinal plants.
- Phytoconstituents play a vital role in the development of modern medicines, cosmetic formulations, and nutraceuticals.

Examples:

- **Alkaloids** – e.g., Morphine, Quinine
- **Glycosides** – e.g., Digoxin
- **Flavonoids** – e.g., Quercetin
- **Tannins** – e.g., Catechin
- **Terpenoids** – e.g., Menthol
- **Phenolic compounds**, Resins, Volatile oils, etc.

Isolation of Phytoconstituents

- Isolation refers to the extraction and purification of bioactive compounds from plant materials such as leaves, roots, bark, stems, seeds, or flowers.

Purpose:

- To obtain pure phytochemicals for studying their chemical nature, biological activity, and potential use in medicine, cosmetics, and industries.

General Steps in Isolation:

1. Collection and Drying – Plant material is collected, cleaned, and shade-dried.
2. Grinding or Pulverization – Dried material is powdered to increase surface area.
3. Extraction – Solvent extraction using methods like maceration, percolation, Soxhlet extraction, or steam distillation.
4. Filtration and Concentration – The extract is filtered and concentrated under reduced pressure.
5. Purification – Using chromatography (TLC, column chromatography, HPLC, etc.) or crystallization.
6. Drying and Storage – Pure compounds are dried and stored for further analysis.

Identification of Phytoconstituents

- Identification involves determining the presence and nature of specific bioactive compounds in plant materials.

Methods Used:

1. **Physical Methods** – Color, odor, solubility, melting point, optical rotation.
2. **Chemical Tests** – Specific color reactions (e.g., Dragendorff's test for alkaloids, Keller–Killiani test for glycosides).
3. **Chromatographic Techniques** – TLC, Paper chromatography for compound separation and identification.
4. **Spectroscopic Techniques** – UV, IR, NMR, and Mass Spectrometry for structure elucidation.

Analysis of Phytoconstituents

- Analysis means performing qualitative and quantitative examination of plant constituents to determine their chemical composition and concentration.

Types of Analysis

1. **Qualitative Analysis:**
   - Detects the presence of various phytoconstituents.
   - Example: Mayer's and Dragendorff's reagents for alkaloids, Ferric chloride test for phenolics.
2. **Quantitative Analysis:**
   - Determines the amount or concentration of a particular compound.
   - Example: Spectrophotometric estimation, titrimetric methods, HPLC, GC, etc.

Purpose of Analysis

- To ensure quality control of herbal drugs.
- To standardize phytopharmaceutical formulations.
- To study structure–activity relationships (SAR) of compounds.

Terpenoids

- Terpenoids (also known as isoprenoids) are a large and diverse group of naturally occurring organic compounds formed from isoprene units (C_5H_8).
- They are one of the most important classes of secondary metabolites found in plants, responsible for aroma, flavor, color, and medicinal properties.
- They are commonly present in essential oils, resins, balsams, and plant pigments.
- Terpenoids are naturally occurring hydrocarbons and their oxygenated derivatives that are biosynthesized from isoprene (C_5H_8) units.
- Each terpenoid molecule is built up of repeating isoprene (C_5H_8) units joined in a head-to-tail manner.

General Formula

- The general formula of terpenes = $(C_5H_8)_n$, where n = number of isoprene units.
- This is known as the Isoprene Rule.

Classification of Terpenoids

Type	Number of Isoprene Units	Carbon Atoms	Examples
Hemiterpenes	1	C_5H_8	Isoprene
Monoterpenes	2	$C_{10}H_{16}$	Menthol, Limonene, Camphor
Sesquiterpenes	3	$C_{15}H_{24}$	Farnesol, Artemisinin
Diterpenes	4	$C_{20}H_{32}$	Phytol, Retinol, Taxol
Sesterterpenes	5	$C_{25}H_{40}$	Geranylfarnesol
Triterpenes	6	$C_{30}H_{48}$	Squalene, Lanosterol
Tetraterpenes	8	$C_{40}H_{64}$	β-Carotene, Lycopene
Polyterpenes	Many	$(C_5H_8)_n$	Natural Rubber (Polyisoprene)

Biosynthesis

- Terpenoids are biosynthesized via two main pathways:
  1. Mevalonate Pathway (MVA pathway) – in cytoplasm.
  2. Non-Mevalonate Pathway (MEP or DOXP pathway) – in plastids.
- Both pathways form Isopentenyl pyrophosphate (IPP), the building block of terpenoids.

Therapeutic Uses

- Antimicrobial – e.g., Limonene, Camphor
- Antimalarial – e.g., Artemisinin
- Anticancer – e.g., Taxol
- Anti-inflammatory – e.g., Boswellic acid
- Expectorant & decongestant – e.g., Menthol
- Antioxidant & hepatoprotective – e.g., Squalene, β-carotene

Commercial Applications

- Used in pharmaceuticals (drugs, antiseptics, expectorants)
- Used in perfumery and cosmetic industries
- Flavoring agents in food and beverages
- Used in rubber and resin production
- Serve as vitamin precursors (Vitamin A, E, K)

Isolation, Identification, and Analysis of Menthol

- Menthol is a monoterpenoid alcohol obtained naturally from the essential oil of Mentha species (especially *Mentha arvensis*, *Mentha piperita*, and *Mentha viridis*).
- It is responsible for the characteristic cooling sensation and odor of peppermint oil.

Chemical Formula: $C_{10}H_{20}O$
Molecular Weight: 156.27
Nature: Crystalline solid with a pleasant peppermint odor and cooling taste.
Category: Terpenoid (specifically, monocyclic monoterpene alcohol)

Isolation of Menthol

Source:

- Menthol is isolated from **Peppermint oil** or **Japanese mint oil** obtained from *Mentha arvensis*.

Steps in Isolation:

Step 1: Extraction of Essential Oil

- Fresh Mentha leaves are subjected to steam distillation.
- The distillate obtained contains menthol and other volatile oils.
- The oil layer is separated from water.

Step 2: Cooling and Crystallization

- The oil is cooled at low temperature (around 0°C).
- Menthol crystallizes out because of its lower solubility in cold oil.
- The crystals are collected by filtration.

Step 3: Purification

- The crude menthol crystals are purified by recrystallization using solvents such as alcohol or acetone.
- The pure product is colorless, transparent, needle-shaped crystals with a strong peppermint odor.

Identification of Menthol

A. Physical Identification

1. **Appearance:** Colorless, hexagonal or needle-shaped crystals.
2. **Odor:** Pleasant peppermint odor.
3. **Taste:** Cooling and refreshing.
4. **Melting Point:** 41–44°C.
5. **Optical Rotation:** Menthol is optically active; natural menthol is the (-)-menthol (L-menthol) form with specific rotation about −50° to −51°.

B. Chemical Tests for Menthol

1. **Liebermann–Burchard Test:**
   - Reagent: Acetic anhydride + Concentrated sulfuric acid.
   - Observation: Blue to green coloration appears.
   - **Inference:** Confirms the presence of alcoholic group and terpenoid nature.
2. **Solubility Test:**
   - Menthol **is** insoluble in water, but freely soluble in alcohol, chloroform, and ether**.**
3. **Nitrate Test (for Alcohols):**
   - Menthol reacts with nitric acid forming menthyl nitrate**,** showing its alcoholic nature.
4. **Odor and Cooling Test:**
   - When applied on the skin or tongue, it produces a cooling **sensation** due to stimulation of cold-sensitive receptors (TRPM8 channels).

Analysis of Menthol

A. Qualitative Analysis

Used to confirm the presence of menthol in peppermint oil or formulations.

1. **Chromatographic Methods:**
    - **Thin Layer Chromatography (TLC):** Menthol shows specific Rf value when compared with standard.
    - **Gas Chromatography (GC):** Commonly used to separate and identify menthol in essential oils.
2. **Spectroscopic Methods:**
    - **IR Spectrum:** Shows characteristic –OH stretching at 3200–3500 cm^{-1} and C–H stretching around 2900 cm^{-1}.
    - **NMR Spectrum:** Confirms the structure of menthol (monoterpene alcohol).

B. Quantitative Analysis

Used to determine the amount of menthol in peppermint oil.

1. **Gas Chromatography (GC):**
    - Most accurate and widely used method.
    - Menthol content is determined by comparing **peak area** with that of standard menthol.
2. **Polarimetry:**
    - Measures **optical rotation** to determine the proportion of **L-menthol** (natural) and **D-menthol** (synthetic).
    - Used for assessing **purity and quality control**.
3. **Titrimetric Methods:**
    - Menthol can be acetylated and the **acetyl value** determined to estimate menthol concentration indirectly.

Uses of Menthol

- **Pharmaceutical:**
    - Cooling agent in creams, balms, cough drops, and inhalations.
    - Local anesthetic and antipruritic (relieves itching).
- **Cosmetic:**
    - Used in toothpaste, mouthwash, shaving creams, and perfumes.
- **Industrial:**
    - Used in flavoring of foods, beverages, and tobacco products.

Isolation, Identification and Analysis of Citral

Citral is a monoterpenoid aldehyde that gives a lemon-like odor to several essential oils such as lemongrass, lemon, and orange oils.

It exists as a mixture of two geometric isomers:

- **Geranial (Citral A)** – trans-isomer
- **Neral (Citral B)** – cis-isomer

Both isomers possess the same molecular formula but differ in structure and slightly in odor.

Chemical Information

Property	Description
Chemical name	3,7-Dimethyl-2,6-octadienal
Molecular formula	$C_{10}H_{16}O$
Molecular weight	152.23 g/mol
Type	Monoterpene aldehyde
Appearance	Pale yellow liquid with strong lemon odor
Boiling point	228°C
Solubility	Soluble in alcohol and ether, insoluble in water

Isolation of Citral

Citral is mainly isolated from Lemongrass oil (Cymbopogon citratus), where it is present in 70–85% concentration.

Steps of Isolation

Step 1: Extraction of Essential Oil

- Fresh lemongrass leaves are subjected to steam distillation.
- The distillate obtained separates into an oil layer (citral-rich) and a water layer.
- The oil layer is collected as lemongrass oil.

Step 2: Fractional Distillation

- The crude oil is subjected to fractional distillation under reduced pressure.
- Citral, having a boiling point around 228°C, is obtained as a separate fraction.

Step 3: Purification

- The citral fraction is treated with sodium bisulfite solution, forming a bisulfite addition compound.
- The compound is then regenerated by acid treatment (dilute sulfuric acid), giving pure citral.

Step 4: Drying and Storage

- The purified citral is dried over anhydrous sodium sulfate and stored in airtight amber bottles to prevent oxidation.

Identification of Citral

A. Physical Characteristics

Property	Observation
State	Yellow liquid
Odor	Strong lemon-like fragrance
Boiling point	228°C
Optical activity	Optically inactive
Solubility	Soluble in alcohol and ether; insoluble in water

B. Chemical Tests

1. Schiff's Test
   - Reagent: Schiff's reagent (rosaniline hydrochloride + sodium bisulfite)
   - Observation: Pink or magenta coloration
   - Inference: Presence of aldehyde group
2. Tollen's Test (Silver Mirror Test)
   - Reagent: Ammoniacal silver nitrate solution
   - Observation: Formation of silver mirror on test tube wall
   - Inference: Confirms aldehyde group
3. 2,4-Dinitrophenylhydrazine (2,4-DNP) Test
   - Reagent: 2,4-DNP reagent
   - Observation: Formation of yellow to orange precipitate
   - Inference: Confirms carbonyl group (C=O)
4. Fehling's Test
   - Observation: No red precipitate formed
   - Inference: Citral is an α,β-unsaturated aldehyde, hence does not reduce Fehling's solution.

Analysis of Citral

A. Qualitative Analysis

Used to confirm the presence of citral and its purity in essential oils.

1. **Thin Layer Chromatography (TLC)**
   - Stationary phase: Silica gel plate
   - Mobile phase: Hexane : Ethyl acetate (9:1)
   - Detection: Spray with 2,4-DNP reagent → yellow-orange spot confirms citral
   - Rf value: Matches with that of standard citral
2. **Gas Chromatography (GC)**
   - Separates Neral and Geranial isomers as two distinct peaks.
   - Used to identify and estimate citral content in lemongrass oil.

B. Quantitative Analysis

Used to determine the concentration of citral in essential oils or formulations.

1. **Gas Chromatography (GC):**
   - Most reliable and accurate method.
   - The area under the peaks corresponding to Neral and Geranial gives the percentage of citral.
2. **Spectrophotometric Method:**
   - Reaction with 2,4-DNP reagent forms a yellow compound.
   - The absorbance is measured at λmax 360 nm, and concentration is calculated from a calibration curve.

Uses of Citral

Category	Uses
Pharmaceutical	Used as an intermediate for Vitamin A, ionone, and menthol synthesis.
Flavoring agent	Imparts lemon flavor to food, beverages, and oral care products.
Perfumery	Used in lemon-scented perfumes, soaps, and detergents.
Antimicrobial	Exhibits antibacterial and antifungal activity.
Cosmetic	Used in lotions, creams, and deodorants for fragrance.

Isolation, Identification, and Analysis of Artemisinin

- Artemisinin is a sesquiterpene lactone with a peroxide bridge, isolated from the plant *Artemisia annua* (commonly known as Sweet Wormwood or Qinghao).
- It is one of the most important antimalarial compounds, effective against *Plasmodium falciparum*, including drug-resistant strains.

Isolation of Artemisinin

Source:

- *Artemisia annua* (Family: Asteraceae)
- Active constituent: Artemisinin
- Plant part used: Leaves and flowering tops

Steps for Isolation:

1. **Collection and Drying:**
   Fresh leaves of *Artemisia annua* are collected, cleaned, and shade-dried.
2. **Powdering:**
   The dried leaves are finely powdered to increase surface area for extraction.
3. **Extraction:**
   - The powdered material is extracted with n-hexane or petroleum ether using Soxhlet extraction or maceration method.
   - The solvent dissolves the non-polar terpenoids, including artemisinin.
4. **Filtration and Concentration:**
   - The extract is filtered and concentrated under reduced pressure (using rotary evaporator) to remove solvent.
5. **Purification:**
   - The crude extract is purified by column chromatography using silica gel as stationary phase.
   - The mobile phase may be hexane:ethyl acetate (3:1).
   - Fractions containing artemisinin are collected based on TLC results.
6. **Crystallization:**
   - The purified fractions are concentrated and allowed to crystallize from ethanol or acetone to yield pure artemisinin.

Identification of Artemisinin

Identification confirms the presence and purity of artemisinin using physical, chemical, and instrumental methods.

A. Physical Characteristics:

- Color: Colorless crystals
- Melting point: 156–158°C
- Solubility: Insoluble in water; soluble in organic solvents like chloroform and ethanol
- Odor: Mild characteristic smell

B. Chemical Tests:

1. **Peroxide Test:**
    - Artemisinin gives a positive test due to the presence of an endoperoxide bridge.
    - When treated with potassium iodide and starch solution, a blue color appears, confirming peroxide group.
2. **TLC (Thin Layer Chromatography):**
    - Stationary phase: Silica gel G
    - Mobile phase: Hexane:ethyl acetate (3:1)
    - Detection: UV light (254 nm) or iodine vapor
    - Rf value of artemisinin ≈ 0.4–0.5

C. Spectroscopic Identification:

- UV-Visible Spectroscopy: Peak around 252 nm
- IR Spectroscopy:
    - Peroxide group (O–O stretch): 880–890 cm^{-1}
    - Lactone C=O group: 1735–1750 cm^{-1}
- Mass Spectrometry: Molecular ion peak at m/z 282
- NMR (^{1}H and ^{13}C): Confirms the sesquiterpene lactone structure.

Analysis of Artemisinin

A. Qualitative Analysis:

- Confirms the presence of artemisinin in plant extract using TLC or HPTLC.
- TLC fingerprinting helps compare the extract with a standard artemisinin sample.

B. Quantitative Analysis:

1. Spectrophotometric Method:
    - Based on reaction of artemisinin with alkali and measurement of absorbance at 252 nm.
    - Concentration is calculated using Beer-Lambert's law.
2. HPLC Method (High-Performance Liquid Chromatography):
    - Column: C18 reverse-phase
    - Mobile phase: Acetonitrile : Water (65:35)
    - Detector: UV detector at 252 nm
    - Flow rate: 1.0 mL/min
    - Retention time: About 6–8 minutes
    - Quantifies the amount of artemisinin in extracts accurately.
3. GC-MS Analysis:
    - Used to determine the molecular weight and purity of artemisinin.

Applications of Artemisinin

- Antimalarial: Effective against all *Plasmodium* species, especially resistant *P. falciparum*.
- Antiparasitic: Shows activity against schistosomiasis and other parasitic infections.
- Anticancer potential: Under research for cytotoxic activity on cancer cells.