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PHARMACOGNOSY & PHYTOCHEMISTRY

UNIT – 3: ISOLATION, IDENTIFICATION & ANALYSIS

B. Pharmacy | 5th Semester | Subject Code: BP501T

★ Terpenoids: Menthol, Citral, Artemisinin
★ Glycosides: Glycyrrhetinic Acid, Rutin
★ Alkaloids: Atropine, Quinine, Reserpine, Caffeine
★ Resins: Podophyllotoxin, Curcumin

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INTRODUCTION: ISOLATION, IDENTIFICATION & ANALYSIS

Phytochemical analysis involves three key processes for each secondary metabolite: Isolation (extracting the compound from the plant matrix), Identification (confirming the identity of the compound), and Analysis/Estimation (quantifying the compound). These processes form the backbone of quality control, pharmacopoeial standards, and research in pharmacognosy.

Process	Definition & Purpose
ISOLATION	Separation of a pure phytoconstituent from the crude plant extract using physical and chemical methods; involves extraction, fractionation, and purification
IDENTIFICATION	Confirming the identity of the isolated compound using physical constants, chemical/colour reactions, chromatographic techniques (TLC, HPLC), and spectroscopic methods (UV, IR, NMR, MS)
ANALYSIS (Estimation)	Quantitative determination of the amount of a phytoconstituent in a given sample; essential for quality control and standardisation; includes gravimetric, titrimetric, colorimetric, HPLC, and spectrophotometric methods

GENERAL METHODS USED

- EXTRACTION METHODS: Maceration, Percolation, Soxhlet extraction, Steam distillation, Supercritical fluid extraction (SFE with CO_2), Ultrasound-assisted extraction, Cold pressing
- PURIFICATION: Liquid-liquid partitioning (using solvents of increasing polarity), Column Chromatography (silica gel, alumina, reverse-phase), Recrystallisation, Precipitation
- CHROMATOGRAPHIC IDENTIFICATION: Thin-Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC), Gas Chromatography (GC), Gas Chromatography-Mass Spectrometry (GC-MS)
- SPECTROSCOPIC IDENTIFICATION: UV-Vis spectroscopy, Infrared spectroscopy (IR/FTIR), Nuclear Magnetic Resonance (NMR – ^1H and ^{13}C), Mass Spectrometry (MS)
- PHYSICAL CONSTANTS: Melting point (MP), Boiling point (BP), Specific rotation ([α]D), Refractive index (RI), Specific gravity
- CHEMICAL/COLOUR TESTS: Group-specific reagents (Dragendorff's for alkaloids, $FeCl_3$ for phenolics, etc.)

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TERPENOIDS

TERPENOIDS

Terpenoids are derived from the isoprene unit (C5) via the MVA or MEP pathway. They include monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), etc.

1.1 MENTHOL

▶ Chemical Profile

Parameter	Details
Source	Mentha piperita (Peppermint), M. arvensis (Indian Mint/Japanese Mint – richest source); Family: Lamiaceae
Part Used	Aerial parts (leaves & stems) – steam distilled to get Mentha oil → Menthol crystallises on cooling
Chemical Formula	$C_{10}H_{20}O$; MW: 156.27; Monocyclic monoterpenol
Structure	1-Methyl-4-isopropylcyclohexan-3-ol; isopropyl-substituted cyclohexanol; 3 chiral centres → 8 possible stereoisomers; L(-)-Menthol = natural, cooling
Physical Properties	Colourless hexagonal crystals or granular powder; MP: 41–44°C (L-Menthol); BP: 212°C; $[\alpha]D^{20}$ = −50° (in alcohol); pleasant peppermint odour; slightly soluble in water; freely soluble in alcohol, chloroform, ether
Biosynthesis Pathway	MEP pathway → GPP (C10) → Limonene (Limonene synthase) → trans-Isopiperitenol → Pulegone → Menthone → L-Menthol (Menthone reductase – key step)

▶ Isolation of Menthol

ISOLATION PROCEDURE

Step 1: COLLECTION: Fresh aerial parts (leaves & stems) of Mentha arvensis collected at full bloom stage (maximum volatile oil content)

Step 2: STEAM DISTILLATION: Plant material is subjected to steam distillation (water distillation); temperature ~100°C; volatile oil + water vapour collected via condenser

Step 3: SEPARATION: Distillate collected in a Florentine flask / separator funnel; Mentha oil (lighter) separates from water (lower layer); drain water layer

Step 4: DEMENTHOLISATION (Cooling/Freezing): Crude Mentha oil (70–85% menthol) is chilled to −10 to −20°C in refrigerators or by passing through chilling coils; Menthol crystallises out (freezing point L-menthol = 42°C; crude mixture freezes at −15°C due to eutectic)

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Step 5: CENTRIFUGATION/FILTRATION: Crystallised menthol separated from the residual dementholised oil (which contains menthone, menthyl acetate etc.) by centrifugation or press-filtration using hydraulic press

Step 6: PURIFICATION/RECRYSTALLISATION: Crude menthol crystals dissolved in hot methanol or ethanol; filtered; cooled → purified menthol crystals obtained; repeat if needed

Step 7: VACUUM DISTILLATION (Optional): For pharmaceutical grade purity; distilled under vacuum to remove last traces of oil

Step 8: DRYING: Crystals dried at room temperature under air current; yield: 70–85% from Mentha arvensis oil

▶ Identification of Menthol

IDENTIFICATION TESTS

- PHYSICAL CONSTANTS: MP 41–44°C (IP); $[\alpha]D^{20}$ = −49° to −51° (L-menthol in ethanol); RI = 1.459–1.462; Specific gravity 0.890–0.910
- ODOUR & TASTE: Characteristic peppermint odour; cooling sensation on tongue and skin (activates TRPM8 cold receptors – threshold ~26°C)
- VANILLIN-SULPHURIC ACID TEST (Colour test): Spot menthol on TLC plate; spray with vanillin-H_2SO_4 reagent; heat at 105°C → VIOLET/PURPLE colour → positive for terpenoids
- TLC IDENTIFICATION: Stationary phase: Silica gel G; Mobile phase: Toluene:Ethyl acetate (95:5) or Hexane:Ethyl acetate (8:2); Detection: UV 254 nm (quenching) + Vanillin-H_2SO_4; Rf of L-Menthol ≈ 0.45–0.55; Compare with authentic menthol standard
- GC IDENTIFICATION (IP method): Column: Carbowax 20M or OV-17; Temperature programme 70–200°C; L-Menthol retention time compared to reference standard; GC-MS: m/z = 71 (base peak), 69, 55, 41; Molecular ion m/z 156 (weak)
- PHOSPHOMOLYBDIC ACID TEST: Spray TLC with phosphomolybdic acid reagent; heat → Blue spot for menthol
- IR SPECTRUM: Strong broad O-H stretch at 3300–3500 cm^{-1}; C-H stretch 2800–3000 cm^{-1}; C-O stretch 1050–1150 cm^{-1}
- ACETYLATION TEST: Menthol + Acetic anhydride → Menthyl acetate (BP 227°C); Saponification → back to menthol; confirms –OH group

▶ Analysis (Estimation) of Menthol

ANALYTICAL METHODS

- **GAS CHROMATOGRAPHY (IP Method – Primary):** Mentha oil dissolved in ethanol; GC with FID detector; Column: DB-WAX or equivalent; Carrier gas: N_2 or He; FID quantification; IP: Mentha piperita oil NLT 44% Menthol; Mentha arvensis oil NLT 70% Menthol
- **HYDROXAMIC ACID METHOD (Ester estimation – for menthyl acetate conversion):** Menthol esterified → menthyl acetate; treated with hydroxylamine HCl in alkaline conditions → Hydroxamic acid; + $FeCl_3$ → red-purple ferric hydroxamate complex; measured at 530 nm

- **GRAVIMETRIC METHOD:** Menthol crystallised at known temperature; crystals dried and weighed; crude purity assessment
- **HPLC METHOD:** Reverse-phase C18 column; Mobile phase: Acetonitrile:Water (70:30); Detection: RI (Refractive Index) detector (no UV chromophore); Quantified against external standard
- **REFRACTIVE INDEX (RI):** Measured using Abbe refractometer; RI = 1.459–1.462 (at 20°C for mentha oil); quick quality check

CITRAL

▶ Chemical Profile

Parameter	Details
Sources	Lemon grass oil (Cymbopogon citratus / C. flexuosus – 70–80% citral), Lemon oil (Citrus limon – 5–7%), Lime oil, Verbena oil, Litsea cubeba oil (70–80%)
Chemical Formula	$C_{10}H_{16}O$; MW: 152.23; Acyclic monoterpene aldehyde
Structure	Mixture of two geometric isomers: GERANIAL (Citral-a, trans/E-isomer, ~60%) + NERAL (Citral-b, cis/Z-isomer, ~40%); both are 3,7-dimethyl-2,6-octadienal; differ only in geometry at C2=C3 double bond; Geranial has stronger lemon odour
Physical Properties	Pale yellow mobile liquid; BP: 228°C (geranial), 225°C (neral); $[\alpha]D = 0°$ (achiral – both isomers are achiral despite double bonds); RI = 1.486–1.489; Specific gravity 0.885–0.895; Characteristic strong lemon odour; insoluble in water; miscible with alcohol, oils
Biosynthesis	MEP pathway → GPP (C10, Geranyl Pyrophosphate) → Geraniol (Geraniol dehydrogenase) → Geranial (Citral-a); Nerol → Neral (Citral-b)

▶ Isolation of Citral

ISOLATION PROCEDURE FROM LEMONGRASS

Step 1: COLLECTION: Fresh or dried lemongrass (Cymbopogon citratus/flexuosus) harvested at full growth

Step 2: STEAM DISTILLATION: Plant material subjected to steam distillation; lemongrass oil (pale yellow) collected

Step 3: SEPARATION FROM WATER: Oil layer separated using separating funnel; dried over anhydrous Na_2SO_4

Step 4: BISULFITE ADDITION COMPOUND METHOD (Chemical isolation): Crude lemongrass oil treated with saturated $NaHSO_3$ (sodium bisulfite) solution with stirring; Citral (aldehyde) reacts selectively: Citral + $NaHSO_3$ → Water-soluble Citral-bisulfite addition compound (precipitate); Non-aldehyde terpene hydrocarbons do NOT react → remain in oil layer; Mixture filtered – citral bisulfite = solid precipitate; Terpene-rich oil = filtrate

Step 5: DECOMPOSITION OF BISULFITE COMPOUND: Citral-bisulfite compound treated with dilute Na_2CO_3 solution OR dilute H_2SO_4 → releases free Citral: Citral-$NaHSO_3$ + Na_2CO_3 → Citral + Na_2SO_3 + $NaHCO_3$

Step 6: EXTRACTION: Liberated Citral extracted into diethyl ether or ethyl acetate; Aqueous layer discarded; Organic layer washed with water, dried over Na_2SO_4

Step 7: DISTILLATION: Solvent removed by rotary evaporation; Citral further purified by vacuum fractional distillation; BP 228°C (geranial) / 225°C (neral) at atmospheric pressure

Step 8: SEPARATION OF ISOMERS (if required): Geranial and Neral can be separated by: (a) Preparative GC; (b) Fractional crystallization of their semicarbazones (derivatives with different MP)

▶ Identification of Citral

IDENTIFICATION TESTS

- **PHYSICAL CONSTANTS:** BP 228°C; RI 1.486–1.489; SG 0.885–0.895; lemon odour
- 2,4-DINITROPHENYLHYDRAZINE (2,4-DNP) TEST: Citral + 2,4-DNP reagent → Yellow-orange precipitate (2,4-dinitrophenylhydrazone); MP of citral DNP derivative ~75°C; confirms aldehyde group (–CHO)
- **SODIUM BISULFITE TEST:** Citral + sat. $NaHSO_3$ → White crystalline bisulfite adduct; soluble in water; regenerates citral on treatment with Na_2CO_3
- **TOLLEN'S REAGENT (Silver Mirror):** Citral + Tollen's reagent (ammoniacal $AgNO_3$) → Silver mirror OR silver precipitate; confirms aldehyde
- **FEHLING'S TEST:** Citral (unsaturated aldehyde) gives WEAK/NEGATIVE Fehling's test (unlike aliphatic aldehydes) → useful for distinguishing
- **TLC:** Silica gel G; Mobile: Hexane:EtOAc (9:1); Detection: Anisaldehyde-H_2SO_4 reagent → Yellow-orange spots for Geranial (Rf ~0.35) and Neral (Rf ~0.30); UV: weak absorbance
- **GC-MS:** Two peaks – Neral (earlier): m/z 152 (M^+), 137, 109, 69, 41; Geranial (later): same fragmentation; Ratio ~40:60 (neral:geranial) confirms identity
- **UV SPECTROSCOPY:** Citral shows absorption at 238 nm ($n \rightarrow \pi^*$ transition of α,β-unsaturated aldehyde) in ethanol

▶ Analysis (Estimation) of Citral

ANALYTICAL METHODS

- **GAS CHROMATOGRAPHY (Official method):** FID detector; Column: DB-5 or CP-Wax; Total citral content = Neral (%) + Geranial (%); Lemongrass oil IP: NLT 75% citral; Litsea cubeba oil: 70–80% citral
- **HYDROXYLAMINE HYDROCHLORIDE METHOD (BP/IP):** Citral (aldehyde) + $NH_2OH \cdot HCl$ → Citral oxime + HCl; HCl liberated titrated with standard NaOH (0.1 M); Endpoint: pH indicator or potentiometry; Calculate % citral from moles of NaOH consumed; Formula: % Citral = (V × M × MW / 10 × W) × 100; V = volume NaOH (mL), M = molarity, W = weight of oil (g)
- **COLORIMETRIC METHOD:** Citral reacts with p-anisidine in acetic acid; forms yellow chromogen; absorbance at 380 nm; quantified against standard curve
- **UV SPECTROPHOTOMETRY:** Absorbance at 238 nm in ethanol; Beer-Lambert law applied; Reference standard citral
- **BISULFITE BACK-TITRATION (Pharmacopoeial):** Citral reacts with excess $NaHSO_3$; residual bisulfite back-titrated with iodine solution

ARTEMISININ (Qinghaosu)

▶ Chemical Profile

Parameter	Details
Source	Artemisia annua Linn. (Sweet Wormwood / Qinghao); Family: Asteraceae; Parts: Leaves and flowering tops (highest artemisinin in leaves just before flowering, 0.01–0.8%)
Chemical Formula	$C_{15}H_{22}O_5$; MW: 282.33; Sesquiterpene lactone endoperoxide
Structure	Tricyclic sesquiterpene; contains 1,2,4-trioxane ring (three-oxygen endoperoxide bridge – most critical feature); also contains a lactone group; six stereocentres; $[\alpha]D^{20}$ = +66° (in ethanol); all-trans configuration at ring junctions
Physical Properties	Colourless needles/white crystalline powder; MP: 152–157°C; Solubility: freely soluble in acetone, ethyl acetate, chloroform; slightly soluble in ethanol; INSOLUBLE in water; Unstable to heat (>150°C) and alkali (endoperoxide cleaved)
Biosynthesis	MVA pathway → FPP (C15, Farnesyl PP) → Amorpha-4,11-diene (Amorphadiene Synthase – ADS) → Artemisinic aldehyde → Artemisinic acid (CYP71AV1) → Dihydroartemisinic acid → Artemisinin (non-enzymatic photochemical step – singlet oxygen endoperoxidation in planta)

▶ Isolation of Artemisinin

ISOLATION PROCEDURE

Step 1: COLLECTION: Leaves + flowering tops of A. annua at early flowering stage (highest artemisinin); dried at <40°C to prevent degradation of the heat-labile endoperoxide

Step 2: SOLVENT EXTRACTION (Preferred): Dried powdered plant material (500 g) extracted with n-HEXANE or PETROLEUM ETHER (40–60°C) using Soxhlet apparatus (6–8 hours); Artemisinin is preferentially extracted by non-polar solvents; OR: Ethanol or methanol can be used (give more impure extracts); Preferred industrial: Hexane or ethyl acetate

Step 3: FILTRATION & EVAPORATION: Hexane extract filtered; solvent removed by rotary evaporation under vacuum (below 40°C to protect endoperoxide); Green waxy residue obtained

Step 4: DE-FATTING (Petroleum ether wash): Residue treated with cold petroleum ether (40–60°C) to remove chlorophyll, waxes and non-polar lipids; filter → filtrate (lipid impurities) + residue (artemisinin-enriched)

Step 5: COLUMN CHROMATOGRAPHY (Silica Gel): Residue dissolved in hexane; loaded on silica gel (100–200 mesh) column; Gradient elution: Hexane → Hexane:Ethyl acetate (9:1 → 8:2 → 7:3); Fractions collected; monitor by TLC

Step 6: TLC MONITORING: Each fraction spotted on TLC (Hexane:EtOAc 8:2); detected with Vanillin-H_2SO_4 → Artemisinin appears as BLUE/VIOLET spot; Rf ≈ 0.40–0.50

Step 7: POOLING & RECRYSTALLISATION: Artemisinin-rich fractions pooled; solvent evaporated; residue dissolved in hot acetone or ethyl acetate; cooled to −5°C → artemisinin crystallises as white needles

Step 8: DRYING: Crystals filtered and dried at room temperature under vacuum; Final purity checked by HPLC; Yield: 0.5–1.5% of dry plant material

▶ Identification of Artemisinin

IDENTIFICATION TESTS

- **PHYSICAL CONSTANTS:** MP 152–157°C (sharp); $[\alpha]D^{20}$ = +66° in ethanol; Insoluble in water
- **TLC:** Silica gel G; Mobile phase: Hexane:EtOAc (8:2) or Petroleum ether:Ethyl acetate (3:1); Spray: Anisaldehyde-H_2SO_4 OR Vanillin-H_2SO_4; Heat at 110°C → BLUE-VIOLET spot; Rf ≈ 0.40–0.55
- **IRON(II) SULPHATE TEST (Endoperoxide detection):** Artemisinin + $FeSO_4$ in H_2SO_4 → Brown ring; Endoperoxide decomposed by Fe^{2+} (Fenton-like reaction) → free radicals → brown colour; Specific for peroxide-containing compounds
- **POTASSIUM PERMANGANATE TEST:** Artemisinin decolourises dilute $KMnO_4$ solution → confirms reducing character of the endoperoxide
- **HPLC-UV:** C18 reverse-phase column; Mobile: Acetonitrile:Water (60:40); Artemisinin absorbs weakly at 210 nm (no strong UV chromophore – contains no aromatic/conjugated system); Retention time compared to reference standard
- **GC-MS:** After chemical derivatisation (reduction to dihydroartemisinin or trimethylsilyl ether); m/z = 282 (M^+), 264, 246, 209, 163, 71 (characteristic fragments)
- **IR SPECTRUM:** Strong C=O stretch of lactone at 1740 cm^{-1}; C–O–O stretch at 1050–1100 cm^{-1} (peroxide); absence of O-H and C=C conjugated system
- **^{1}H-NMR ($CDCl_3$):** δ 5.8 (s, 1H, H-12 – hemiacetal CH); δ 3.4 (m, 1H, H-9); δ 2.0–1.0 (multiple CH, CH_2 protons); Characteristic peroxide methine at δ 5.8
- **HORECKER'S TEST (Peroxide test):** Artemisinin + KI + starch → BLUE colour (due to iodine liberation from KI by peroxide) → confirms endoperoxide

▶ Analysis (Estimation) of Artemisinin

ANALYTICAL METHODS

- **HPLC-UV METHOD (WHO / IP standard):** Reverse-phase C18 column (250 × 4.6 mm); Mobile phase: Acetonitrile:0.01M H_3PO_4 (60:40); Detection: UV 210 nm (low UV – requires careful baseline); Flow rate: 1 mL/min; External standard calibration; NLT 98.0% purity (pharmaceutical grade)
- **HPLC with ELSD (Evaporative Light Scattering Detector):** More sensitive than UV for artemisinin (weak UV); used for raw plant extracts
- **COLORIMETRIC METHOD ($NaBH_4$ reduction):** Artemisinin reduced by $NaBH_4$ → Dihydroartemisinin; then treated with p-dimethylaminobenzaldehyde (DMAB) + HCl → Pink/red colour at 535 nm; Quantified by Beer-Lambert law
- **COLORIMETRIC ($KMnO_4$ method):** Artemisinin reacts with $KMnO_4$; absorbance decrease measured at 525 nm; proportional to artemisinin content

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- **GC METHOD:** After derivatisation; FID detector; used for plant extract screening
- **NMR QUANTIFICATION (qNMR):** ^{1}H-NMR with internal standard (e.g., dimethyl sulphone); absolute quantification; used for reference standard characterisation

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GLYCOSIDES

GLYCOSIDES

Glycosides are compounds formed by condensation of a sugar (glycone) with a non-sugar (aglycone). Hydrolysis releases the pharmacologically active aglycone. The sugar part improves water-solubility. Two important glycoside aglycones studied here are Glycyrrhetinic Acid (triterpenoid) and Rutin (flavonol glycoside).

GLYCYRRHETINIC ACID (Enoxolone)

▶ Chemical Profile

Parameter	Details
Source	Glycyrrhiza glabra Linn. (Liquorice); Family: Leguminosae; Part: Dried roots and rhizomes; Glycyrrhetinic acid = aglycone of Glycyrrhizin
Chemical Formula	$C_{30}H_{46}O_4$; MW: 470.7; Pentacyclic oleanane-type triterpenoid acid
Structure	Oleanane skeleton (olean-12-en-3β-ol-30-oic acid); five fused six-membered rings (A/B/C/D/E); C11-keto group; Δ12 double bond (C12=C13); 3β-OH group; 30-COOH; 18β-configuration (differs from 18α-epimer – Ursolane type); Obtained from Glycyrrhizin by hydrolysis of two glucuronic acid sugars
Physical Properties	White or slightly yellow crystalline powder; MP: 296–298°C; $[\alpha]D^{20}$ = +86° (in pyridine); Insoluble in water; soluble in ethanol, methanol, acetone; slightly soluble in chloroform
Relation to Glycyrrhizin	Glycyrrhizin = Glycyrrhetinic acid + 2 Glucuronic acid residues (glycoside bond at C3-OH); Acid hydrolysis releases aglycone

▶ Isolation of Glycyrrhetinic Acid

ISOLATION PROCEDURE

Step 1: PREPARATION OF GLYCYRRHIZIN FIRST: Liquorice root powder (coarse) exhaustively extracted with hot water or 70% ethanol by percolation or Soxhlet; Aqueous extract concentrated under vacuum

Step 2: PRECIPITATION OF GLYCYRRHIZIN: Concentrated aqueous extract acidified with dilute HCl or H_2SO_4 (pH 1–2); Glycyrrhizin (ammonium salt, soluble in water) precipitates as free acid on acidification; precipitate filtered

Step 3: ACID HYDROLYSIS OF GLYCYRRHIZIN TO GLYCYRRHETINIC ACID: Glycyrrhizin dissolved in 10% H_2SO_4; Refluxed at 100°C for 4–6 hours; Two glucuronic acid molecules cleaved → Glycyrrhetinic acid liberated + 2 Glucuronic acid in solution

Step 4: EXTRACTION OF AGLYCONE: After hydrolysis mixture cooled; Glycyrrhetinic acid precipitates or extracted with butanol or ethyl acetate (pH adjusted to 3–4); Organic layer separated; washed with water

Step 5: COLUMN CHROMATOGRAPHY: Extract concentrated; loaded on silica gel column; Eluted with Hexane:EtOAc gradient (8:2 → 6:4 → 4:6); Fractions monitored by TLC

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Step 6: RECRYSTALLISATION: Glycyrrhetinic acid-rich fractions pooled; evaporated; recrystallised from ethanol or methanol → white/yellowish crystals; Repeat recrystallisation for high purity

Step 7: DRYING: Crystals dried under vacuum at 60°C; MP check: 296–298°C confirms identity; Yield ~0.5–2% from root

▶ Identification of Glycyrrhetinic Acid

IDENTIFICATION TESTS

- **PHYSICAL CONSTANTS:** MP 296–298°C; $[\alpha]D^{20}$ = +86° (in pyridine); Colourless to slightly yellowish crystals
- **TLC:** Silica gel G; Mobile: Chloroform:Methanol:Acetic acid (95:5:0.5) or Toluene:EtOAc:Formic acid (5:4:1); Detection: Anisaldehyde-H_2SO_4 spray + heat at 110°C → VIOLET/PURPLE spot; Rf ≈ 0.5–0.6; Liebermann-Burchard spray → blue-green (triterpenoid)
- **LIEBERMANN-BURCHARD TEST:** Glycyrrhetinic acid dissolved in $CHCl_3$; add Liebermann-Burchard reagent (Acetic anhydride + H_2SO_4) → Blue→Green→Brown colour change; Positive for pentacyclic triterpenoids and steroids
- **FOAM TEST (Saponin precursor check):** Glycyrrhizin (parent saponin) aqueous solution shaken vigorously → Persistent foam (due to saponin character); Glycyrrhetinic acid itself: less foaming (no sugar part)
- **BALJET TEST:** Dissolve in ethanol; add Baljet's reagent (Picric acid + NaOH 1:1) → Orange/Red colour; positive for triterpenoids with α,β-unsaturated lactone
- **$FeCl_3$ TEST:** Glycyrrhetinic acid + $FeCl_3$ → no significant colour (no phenolic –OH at ring A in glycyrrhetinic acid); differentiates from phenolic triterpenoids
- **HPLC:** C18 column; Mobile: Acetonitrile:0.1% phosphoric acid (85:15); UV 254 nm; Retention time compared to reference; peak purity by PDA detector
- **MS (FAB-MS or ESI-MS):** $[M+H]^+$ = 471.3 Da; fragmentation at C3-OH and C12=C13 double bond; characteristic fragments m/z 453 (M+H-H_2O), 247 (D-ring fragment)

▶ Analysis (Estimation) of Glycyrrhetinic Acid

ANALYTICAL METHODS

- **HPLC (Official / Most Accurate):** Reverse-phase C18 (250 × 4.6 mm, 5 µm); Mobile: MeOH:Water:Acetic acid (88:11:1); Detection: UV 254 nm; RT compared to reference standard; Linearity: 50–500 µg/mL; LOD ~1 µg/mL; Purity NLT 95% for pharmaceutical grade
- **COLORIMETRIC METHOD (Vanillin-H_2SO_4):** Dissolve in EtOH; add vanillin + conc. H_2SO_4; colour develops at 60°C; measure at 530 nm; calibration curve using pure glycyrrhetinic acid standard
- **SPECTROPHOTOMETRIC (UV):** Dissolved in ethanol; measure at 254 nm (Δ12-13 double bond absorption); quantified using molar extinction coefficient
- **TITRATION (for triterpenoid acid):** Dissolve in ethanol; titrate with standardised NaOH (0.1 M); phenolphthalein indicator; calculate based on one –COOH group per molecule (MW 470.7); % purity calculated

RUTIN (Quercetin-3-O-Rutinoside)

▶ Chemical Profile

Parameter	Details
Sources	Ruta graveolens (richest – leaves); Buckwheat (Fagopyrum esculentum); Citrus peel; Sophora japonica (flower buds – major commercial source); Capparis spinosa; Tobacco; Tea
Chemical Formula	$C_{27}H_{30}O_{16}$; MW: 610.52; Flavonol glycoside
Structure	Quercetin (3,5,7,3',4'-pentahydroxyflavone aglycone) + Rutinose (disaccharide: 6-O-α-L-Rhamnopyranosyl-β-D-glucopyranose) attached at C3-OH of quercetin; O-glycosidic bond; Yellow crystalline powder
Physical Properties	Yellow needles/crystals; MP: 214–215°C (anhydrous) or 125°C (with $3H_2O$ crystal water); $[\alpha]D^{20}$ = −39.4° (in water); UV absorption: 257 nm (Band II) and 354 nm (Band I) in methanol; slightly soluble in water (cold), more soluble in hot water; freely soluble in methanol; insoluble in chloroform, petroleum ether
Biosynthesis	Shikimic acid + Acetate-Malonate pathway → Naringenin → Dihydrokaempferol → Dihydroquercetin → Quercetin (aglycone) → Rutin (glycosylation: Quercetin + UDP-Glucose + UDP-Rhamnose → Rutin, catalysed by Flavonol 3-O-glucosyltransferase and rhamnosyltransferase)

▶ Isolation of Rutin

ISOLATION FROM SOPHORA JAPONICA FLOWER BUDS (Commercial Method)

Step 1: COLLECTION: Dried flower buds of Sophora japonica (Japanese Pagoda tree); highest rutin content 20–30%

Step 2: BOILING WATER EXTRACTION: Dried powder (500 g) boiled with 5 L of distilled water for 30–45 minutes; Rutin is water-soluble (particularly in hot water); repeated 2–3 times; extracts combined

Step 3: FILTRATION: Hot extract filtered through Buchner funnel while hot (Rutin crystallises on cooling); Filtrate collected

Step 4: ACIDIFICATION: Filtrate cooled to room temperature; Acidified to pH 3–4 with dilute HCl or H_2SO_4; Rutin precipitates as yellow crystals (solubility decreases markedly in cold acid)

Step 5: COLLECTION OF CRUDE RUTIN: Yellow crystalline precipitate collected by vacuum filtration; washed with cold water

Step 6: ALTERNATIVE – ALCOHOL EXTRACTION: Dried Ruta graveolens leaves extracted with 70% ethanol by Soxhlet (for smaller scale lab isolation); Ethanol extract concentrated; water added → Rutin precipitates (anti-solvent precipitation)

Step 7: PURIFICATION BY RECRYSTALLISATION: Crude rutin dissolved in hot 30% ethanol or dimethylformamide (DMF); solution filtered hot; cooled slowly → pure rutin crystallises as yellow needles

Step 8: DECOLOURISATION (if needed): Dissolve in water with activated charcoal; filter hot → removes green/brown pigments; recrystallise

Step 9: DRYING: Crystals dried at 60°C; MP 214–215°C; Yield: 20–28% from Sophora; commercial rutin >95% purity

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▶ Identification of Rutin

IDENTIFICATION TESTS

- **PHYSICAL CONSTANTS:** MP 214–215°C; $[\alpha]D^{20}$ = −39.4°; Yellow needles; UV λmax: 257 nm and 354 nm in MeOH
- **SHINODA TEST (Cyanodin Test):** Rutin dissolved in methanol; add Mg turnings + few drops conc. HCl → Magnesium reduces flavonoid → PINK to RED colour (cherry red); Flavonoid identification test
- **FERRIC CHLORIDE TEST:** Rutin solution + $FeCl_3$ → Black-green precipitate or dark-green colour; Positive for ortho-dihydroxy phenols (catechol B-ring of quercetin)
- **MOLISCH TEST:** Rutin dissolved in alcohol; add α-naphthol + conc. H_2SO_4 carefully → Violet ring at interface; Positive for glycoside (sugar portion – rutinose)
- **ACID HYDROLYSIS TEST:** Rutin + dil. HCl (reflux 2 hours) → Quercetin (yellow precipitate – aglycone) + Rutinose (sugar – detected by Fehling's solution: brick red ppt, confirms reducing sugar after hydrolysis of rutinose)

IDENTIFICATION TESTS (Continued)

- **TLC:** Silica gel 60 F_{254} plates; Mobile: n-Butanol:Acetic acid:Water (4:1:5, upper layer – BAW); Detection: UV 366 nm → yellow-green fluorescence; Spray: Naturstoff reagent (NPR) + PEG → Bright yellow-green fluorescence at 366 nm; Rf ≈ 0.30–0.35; Compare with reference rutin
- **HPLC:** C18 column; Mobile: Methanol:0.5% glacial acetic acid in water (45:55); UV: 254 nm or 354 nm; Retention time compared to rutin reference standard; PDA detector for peak purity
- **UV SPECTROSCOPY (Flavonoid characterisation):** In methanol: Bands at 257 nm (Band II, benzene ring A) and 354 nm (Band I, cinnamoyl ring B); $AlCl_3$ shift: Band I shifts to ~420 nm (chelation with 5-OH and C4=O); NaOMe shift: Band I bathochromic shift (ionisation of 4'-OH); confirms flavonol glycoside
- **MASS SPECTROMETRY:** ESI-MS: $[M\text{-}H]^-$ = 609.1; MS/MS fragmentation: m/z 301 (quercetin aglycone), 271, 179; confirms quercetin aglycone + rutinose

▶ Analysis (Estimation) of Rutin

ANALYTICAL METHODS

- **ALUMINIUM CHLORIDE COLORIMETRIC METHOD (Most used):** Rutin sample dissolved in methanol (1 mg/mL); take 1 mL + 1 mL $AlCl_3$ (2% in methanol) + 1 mL sodium acetate (120 mg/mL) + 6 mL water; Incubate 30 min at RT; Read absorbance at 415 nm; Quercetin used as standard (calibration curve); Report as % quercetin equivalent or % rutin equivalent; LOD ~0.1 µg/mL
- **HPLC METHOD (Official):** C18 (250 × 4.6 mm); Mobile: Methanol:0.5% acetic acid (45:55); Flow: 1 mL/min; Detection: UV 354 nm; Rutin NLT 95.0% (pharmaceutical grade); Linearity 10–200 µg/mL

- **FOLIN-CIOCALTEU (Total Polyphenol – indirect):** Used for plant extracts; not specific for rutin alone; gives total polyphenol content expressed as gallic acid equivalents
- **SPECTROPHOTOMETRIC (Direct):** Dissolve in methanol; absorbance at 354 nm (Band I of rutin); molar absorptivity ε = 18,000–19,000 L/mol/cm; apply Beer-Lambert law

ALKALOIDS

ALKALOIDS

Alkaloids are basic nitrogen-containing compounds. General principle of isolation exploits their basic nature: alkaloids form salts with acids (water-soluble) and are liberated as free bases (organic solvent-soluble) with alkali.

GENERAL PRINCIPLE OF ALKALOID ISOLATION: Plant material → Extract with dilute acid (alkaloids form water-soluble salts) → Basify with NaOH/NH_3 → Free base alkaloid extracted with organic solvent (chloroform, ethyl acetate) → Purified by column chromatography → Recrystallised. OR: Extract with organic solvent (if free base) → Shake with dilute acid (partition extraction) → Basify → Re-extract with solvent.

ATROPINE

▶ Chemical Profile

Parameter	Details
Source	Atropa belladonna (Belladonna leaf/root), Datura stramonium, Hyoscyamus niger; Family: Solanaceae
Chemical Formula	$C_{17}H_{23}NO_3$; MW: 289.37; Tropane alkaloid
Structure	(±)-Hyoscyamine = Racemic mixture of L- and D-Hyoscyamine; Ester of Tropine (bicyclic aminoalcohol) + Tropic acid; Tertiary amine (N-methyl); pKa = 9.7; L-hyoscyamine (natural) racemises to Atropine (DL) during isolation
Physical Properties	White crystalline powder; MP: 115–118°C (Atropine base); Very bitter; [α]D = 0° (racemic); Freely soluble in alcohol, chloroform; Slightly soluble in water; Atropine sulphate: freely soluble in water

▶ Isolation of Atropine

ISOLATION PROCEDURE

Noteskarts

Step 1: EXTRACTION: Dried belladonna root (powdered) extracted with 70% ethanol or dilute H_2SO_4 (pH 2–3) → Alkaloids dissolve as sulfate salts; repeat extraction; combine extracts; filter

Step 2: BASIFICATION: Filtrate made alkaline with NH_3 solution or 10% NaOH (to pH 10–11) → Free alkaloid bases liberated from salts

Step 3: SOLVENT EXTRACTION: Free bases extracted with chloroform ($CHCl_3$) 3–4 times in separating funnel; chloroform layer contains atropine, hyoscine, and other tertiary alkaloids; aqueous layer discarded

Step 4: ACID STRIPPING: Chloroform extract shaken with 5% H_2SO_4 → alkaloids back into aqueous acid layer; allows removal of non-basic co-extractives; Aqueous acid layer collected

Step 5: BASIFICATION & RE-EXTRACTION: Acid aqueous layer basified with NH_3 → Free bases; Re-extracted with chloroform

Step 6: COLUMN CHROMATOGRAPHY: Chloroform extract concentrated; loaded on alumina (basic, Brockmann Grade II) column; Eluted with $CHCl_3$ → $CHCl_3$:MeOH (99:1 → 95:5); Atropine elutes first; Scopolamine elutes later

Step 7: SALT FORMATION: Atropine free base dissolved in ethanol; treated with dilute H_2SO_4 → Atropine sulfate salt (water-soluble); crystallised from ethanol/water

Step 8: RECRYSTALLISATION: From ethanol-water or acetone; Atropine sulfate: White crystals; MP of atropine base: 115–118°C

IDENTIFICATION TESTS – ATROPINE

- PHYSICAL: MP 115–118°C (base); $[\alpha]D = 0°$ (racemic); Bitter taste
- VITALI-MORIN TEST (Most specific): Atropine evaporated with fuming HNO_3 on water bath → residue (trinitro derivative); add acetone + KOH → VIOLET colour → fades to red → confirms tropane esters; Distinguishes from other alkaloids
- MAYER'S REAGENT: Atropine + Mayer's reagent (K_2HgI_4) → Cream/White precipitate; General alkaloid test
- DRAGENDORFF'S REAGENT: Atropine + Dragendorff's ($KBiI_4$) → Orange-red precipitate; General alkaloid test
- MANDELIN'S REAGENT: Atropine + Mandelin's (ammonium metavanadate in H_2SO_4) → Reddish-brown (belt test)
- TLC: Silica gel; Mobile: $CHCl_3$:MeOH:NH_3 (85:15:1); Detection: Dragendorff's spray → Orange spot; Rf ≈ 0.40–0.50
- HPLC: C18 column; Mobile: Phosphate buffer (pH 3):MeCN (75:25); UV: 210 nm; compare with standard

ANALYSIS (ESTIMATION) OF ATROPINE

- **HPLC (IP/USP):** C18 column; Mobile phase: Acetonitrile:0.02M KH_2PO_4 adjusted to pH 3.0 (25:75); UV 210 nm; external standard; NLT 98.5% (atropine sulfate)
- **NON-AQUEOUS TITRATION (BP):** Atropine sulfate dissolved in glacial acetic acid; titrate with 0.1 M $HClO_4$; endpoint: potentiometric or crystal violet indicator; 1 mL 0.1M $HClO_4$ = 17.42 mg atropine sulfate
- **UV SPECTROPHOTOMETRY:** In 0.1N HCl; λmax at 251, 257, 262 nm (weak aromatic absorption of tropic acid moiety); Beer-Lambert applied

QUININE

Parameter	Details
Source	Cinchona ledgeriana, C. succirubra, C. officinalis; Family: Rubiaceae; Part: Bark (Cinchona bark)
Formula	$C_{20}H_{24}N_2O_2$; MW: 324.42; Quinoline alkaloid (quinuclidine + quinoline ring system)
Physical Properties	White amorphous powder/crystals; MP: 177°C; $[\alpha]D^{20} = -169°$ (in EtOH); Sparingly soluble in water; Quinine sulfate: soluble in water; Strongly laevorotatory; Fluorescent (blue fluorescence in dilute H_2SO_4)

ISOLATION OF QUININE FROM CINCHONA BARK

Step 1: Bark powdered and extracted with Lime (CaO) and petroleum benzin; CaO liberates alkaloid bases from salts; petroleum benzin dissolves free alkaloid bases

Step 2: Petroleum benzin extract treated with dilute H_2SO_4 → Alkaloids extracted into aqueous acid layer as sulfate salts; Non-alkaloid lipids remain in petroleum layer

Step 3: Aqueous sulfate layer basified with NaOH → Free bases precipitate or re-extracted with $CHCl_3$

Step 4: FRACTIONAL PRECIPITATION: Quinine sulfate (less soluble) crystallised preferentially by acidifying the extract to pH 5.5–6.0; Quinine sulfate crystallises first; Filter → crude quinine sulfate

Step 5: RECRYSTALLISATION from dilute H_2SO_4 / ethanol; Pure quinine sulfate obtained; For free base: basify → extract with ether/$CHCl_3$ → recrystallise

IDENTIFICATION – QUININE

- **THALLEIOQUIN TEST (Most specific):** Quinine in dilute H_2SO_4 + Br_2 water → then excess NH_3 → EMERALD GREEN (thalleioquin) → specific for quinoline alkaloids with $-OCH_3$ at C6
- **HERAPATHITE TEST:** Quinine + I_2 + H_2SO_4 → Green crystalline precipitate (quinine periodide) = Herapathite
- **BLUE FLUORESCENCE:** Dilute quinine sulfate in 0.1M H_2SO_4 shows BLUE fluorescence under UV 366 nm; Very sensitive (1:100,000 dilution); Specific and characteristic
- **MAYER'S/DRAGENDORFF'S:** Cream/orange precipitate (general alkaloid test)
- **TLC:** Silica gel; $CHCl_3$:MeOH:NH_3 (85:14:1); Dragendorff's or UV 254+366 nm; Rf quinine ≈ 0.50

ANALYSIS – QUININE

- **HPLC (Official):** C18 column; Mobile: MeCN:0.05M H_2SO_4 (15:85); UV 235 nm and 316 nm; Distinguish from quinidine, cinchonine, cinchonidine
- **TITRIMETRIC (Non-aqueous):** Dissolved in glacial acetic acid; titrate with 0.1M $HClO_4$ (two equivalents for two basic nitrogens); 1 mL 0.1M $HClO_4$ = 16.22 mg quinine
- **UV FLUORIMETRY:** Extremely sensitive; Excitation 350 nm, Emission 450 nm; LOD 0.1 ng/mL; Used in plasma level monitoring

RESERPINE

Parameter	Details
Source	Rauvolfia serpentina (Indian Snakeroot); Family: Apocynaceae; Part: Root
Formula	$C_{33}H_{40}N_2O_9$; MW: 608.68; Indole alkaloid (yohimbane skeleton; MIA type)
Physical Properties	White/pale buff crystalline powder; MP: 264–265°C (decomposes); $[\alpha]D^{20}$ = −118° (in $CHCl_3$); Freely soluble in $CHCl_3$, glacial AcOH; slightly soluble in EtOH; very slightly soluble in water; Sensitive to light (darkens); Acid-labile

ISOLATION OF RESERPINE

Step 1: Dried root powdered and treated with NH_3 solution + methanol (or chloroform) → alkaloids extracted as free bases; repeat 3×; combine

Step 2: Methanolic extract concentrated; dissolved in dilute H_2SO_4 (pH 3–4); filtered (removes chlorophyll etc.)

Step 3: Basified with NaOH to pH 10 → alkaloid bases liberated; extracted with $CHCl_3$

Step 4: $CHCl_3$ extract washed with water, dried over Na_2SO_4; concentrated

Step 5: ALUMINA COLUMN CHROMATOGRAPHY: Load on neutral Al_2O_3 column; elute with $CHCl_3$ (Reserpine elutes early); $CHCl_3$:MeOH gradient; Monitor fractions by TLC

Step 6: RECRYSTALLISATION: Reserpine-rich fractions crystallised from $CHCl_3$-MeOH (1:1) → Pale yellowish-white crystals; Light-sensitive – protect from UV

IDENTIFICATION – RESERPINE

★ PHYSICAL: MP 264–265°C (dec.); $[\alpha]D^{20}$ = −118°; Pale yellow crystals

★ MANDELIN'S TEST: Reserpine + Mandelin's reagent (ammonium vanadate in H_2SO_4) → ORANGE-RED colour; specific for reserpine

★ MARQUIS REAGENT (H_2SO_4 + formaldehyde): Reserpine → YELLOW-ORANGE → purple; useful screening

★ POTASSIUM DICHROMATE + H_2SO_4: Reserpine → orange-yellow → green change

★ FLUORESCENCE: Reserpine solutions in $CHCl_3$ show YELLOW fluorescence under UV 366 nm

★ TLC: Silica gel; $CHCl_3$:MeOH (9:1); Dragendorff's or UV 366 nm; Rf ≈ 0.45–0.55

★ HPLC: C18; MeCN:phosphate buffer pH 3 (50:50); UV 268 nm or 295 nm

ANALYSIS – RESERPINE

- **HPLC (BP/USP):** C18; Mobile: MeOH:0.01M KH_2PO_4 (65:35); UV 268 nm; NLT 99.0% (pharmaceutical); Sensitive – LOD ~10 ng/mL
- **COLORIMETRIC (Marquis):** Reserpine + formaldehyde-H_2SO_4 → colour measured at appropriate wavelength; linear calibration
- **NON-AQUEOUS TITRATION:** $HClO_4$ in glacial acetic acid; potentiometric endpoint

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CAFFEINE

Parameter	Details
Source	Camellia sinensis (Tea – 1–4%), Coffea arabica (Coffee beans – 1–3%), Cola nitida (Cola nuts), Paullinia cupana (Guarana), Theobroma cacao (Cocoa – trace); Family: Various
Formula	$C_8H_{10}N_4O_2$; MW: 194.19; Methylxanthine (Purine alkaloid); 1,3,7-Trimethylxanthine
Physical Properties	White silky needles or powder; MP: 235–237°C; sublimes at ~180°C; [α]D = 0° (achiral); Slightly bitter; Soluble in water (2 g/100 mL at 25°C, more at 80°C), freely in $CHCl_3$; Sparingly in ethanol; NOT precipitated by most alkaloid reagents (Mayer's etc.) due to non-basic nature (pKa ≈ 14 – practically non-basic)

ISOLATION OF CAFFEINE FROM TEA

Step 1: Dried tea leaves (50 g) boiled with water (500 mL) + CaO (calcium oxide, 15 g) for 30–45 min; CaO precipitates tannins as insoluble calcium tannate (removes interfering tannins); Caffeine remains in solution

Step 2: Filter while HOT; filtrate collected; tannin-free caffeine solution obtained

Step 3: CONTINUOUS EXTRACTION: Hot aqueous filtrate extracted with chloroform in a continuous liquid-liquid extractor or repeated 3× separating funnel with $CHCl_3$ (caffeine is soluble in $CHCl_3$); $CHCl_3$ layers combined

Step 4: Chloroform extract washed with dilute NaOH (removes any phenolic acids), then water; dried over anhydrous Na_2SO_4

Step 5: EVAPORATION: $CHCl_3$ evaporated on water bath → crude caffeine residue (pale green from chlorophyll)

Step 6: SUBLIMATION PURIFICATION (Classical method): Crude caffeine placed in sublimation apparatus; heated to 175–180°C; vacuum applied; Caffeine sublimes and collects on cold finger as white crystalline needles; Very pure product; OR: Recrystallise from ethanol or hot water

Step 7: CHARCOAL DECOLOURISATION: Dissolve in hot water + activated charcoal; filter hot → colourless solution; cool → white caffeine crystals

IDENTIFICATION – CAFFEINE

- **PHYSICAL:** MP 235–237°C; Sublimation characteristic; White silky needles; Bitter taste
- **MUREXIDE TEST (Most specific – Paraxanthine test):** Caffeine + dilute HNO_3 on water bath → evaporate → residue (xanthine derivative) + NH_3 vapour → PURPLISH-RED colour (Murexide) → specific for purine alkaloids (caffeine, theophylline, theobromine)
- **TANNIC ACID TEST:** Caffeine is NOT precipitated by tannic acid (unlike most alkaloids) → useful NEGATIVE test to differentiate from other alkaloids
- **MAYER'S / DRAGENDORFF'S:** Caffeine gives weak or NO precipitate (non-basic; no nitrogen protonation at physiological pH) → useful differentiating property
- **TLC:** Silica gel; Mobile: $CHCl_3$:MeOH (9:1) or $CHCl_3$:Acetone (4:1); Detection: Dragendorff's (faint orange) OR UV 254 nm (strong quenching – aromatic purine ring); Rf ≈ 0.35–0.50

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- **UV SPECTROSCOPY:** λmax 272 nm in water (purine chromophore); ε = 9800; Strong absorption – useful for quantitation
- **IR SPECTRUM:** Strong C=O at 1700 and 1659 cm^{-1} (two carbonyl groups); C–N stretch 1550–1600 cm^{-1}; aromatic C=C

ANALYSIS – CAFFEINE

- **UV SPECTROPHOTOMETRY (Simplest/Official):** Dissolve in 0.1N H_2SO_4; measure at 272 nm; ε = 9800 L/mol/cm; Beer-Lambert law: A = εcl; highly specific and linear
- **HPLC (USP/BP):** C18 column; Mobile: 0.05M KH_2PO_4 (pH 4.5):MeOH (80:20); UV 254 nm; Retention time vs reference; Purity NLT 98.5% (anhydrous basis)
- **TITRIMETRIC (Non-aqueous – if applicable):** Caffeine is too weakly basic for direct $HClO_4$ titration; alternative – weigh precisely, dissolve, measure UV
- **COLORIMETRIC (Chlorogenic acid method):** Caffeine extracted into $CHCl_3$; back-extracted into water; add Nessler's reagent → colour measured (for plant extract screening)

RESINS / RESINOUS CONSTITUENTS

RESINS / RESINOUS PHYTOCONSTITUENTS

Resins are complex mixtures of non-volatile compounds. Podophyllotoxin is a lignan from Podophyllum resin, and Curcumin is the active phenolic diarylheptanoid from Curcuma (turmeric oleoresin). Both are pharmacologically significant resinous constituents.

PODOPHYLLOTOXIN

▶ Chemical Profile

Parameter	Details
Source	Podophyllum emodi Wall. (Indian Podophyllum, Himalayan May Apple) and P. peltatum Linn. (American May Apple); Family: Berberidaceae; Part: Rhizome and roots
Chemical Formula	$C_{22}H_{22}O_8$; MW: 414.41; Cyclolignan (Aryltetralin lactone lignan)
Structure	Aryltetrahydronaphthalene skeleton (aryltetralin); 4 stereocentres (C1, C2, C3, C4); trans-lactone ring fusion (C4–C4a); methylenedioxy group (C3',C4'); three methoxy groups; C4 has β-configuration (critical for activity); contains a γ-butyrolactone ring
Physical Properties	White to off-white crystalline powder; MP: 183–184°C; $[\alpha]D^{20} = -132°$ (in $CHCl_3$); Freely soluble in $CHCl_3$, acetone, EtOH; Practically insoluble in water; Sensitive to alkali (forms dehydro derivative); Photostable
Biosynthesis	Shikimic pathway → Phenylalanine → Ferulic acid/Coniferyl alcohol → Dimerization → Matairesinol → Yatein → Podophyllotoxin (via enantioselective oxidative coupling by dirigent proteins; CYP450 epoxidase)

▶ Isolation of Podophyllotoxin

ISOLATION PROCEDURE

Step 1: DRYING: Rhizomes of Podophyllum emodi dried at <45°C; powdered (coarse 20 mesh)

Step 2: SOLVENT EXTRACTION: Powdered rhizome extracted with 70% ETHANOL or METHANOL (Soxhlet or percolation, 72 hours); ethanol dissolves podophyllotoxin and other polar lignans; Extracts combined

Step 3: CONCENTRATION: Ethanolic extract concentrated to ~1/5 volume under vacuum (rotary evaporation, <40°C) to avoid degradation

Step 4: DEFATTING with PETROLEUM ETHER: Concentrated extract diluted with water (1:1); shaken with petroleum ether (40–60°C) 3× → removes chlorophyll, fats, waxes; petroleum ether layer discarded; aqueous ethanol layer retained

Step 5: CHLOROFORM EXTRACTION: Aqueous ethanol layer extracted with chloroform ($CHCl_3$) 3–4×; $CHCl_3$ dissolves podophyllotoxin preferentially; $CHCl_3$ extracts pooled

Step 6: COLUMN CHROMATOGRAPHY (Main separation): $CHCl_3$ extract concentrated and loaded on SILICA GEL column (100–200 mesh); Gradient elution: Hexane:EtOAc (7:3 → 1:1 → 0:1); Monitor fractions by TLC (Vanillin-H_2SO_4); Podophyllotoxin elutes in ~60–70% EtOAc in hexane fractions

Step 7: TLC MONITORING: Silica gel; Hexane:EtOAc (1:1); Spray Vanillin-H_2SO_4 + heat → Blue-grey spot; Rf ≈ 0.35–0.45

Step 8: RECRYSTALLISATION: Podophyllotoxin fractions pooled; evaporated; recrystallised from ethanol or $CHCl_3$-methanol (1:2) → White needles; MP 183–184°C; Yield: ~3–5% of rhizome powder

IDENTIFICATION – PODOPHYLLOTOXIN

- **PHYSICAL:** MP 183–184°C; $[\alpha]D^{20} = -132°$; White crystals; Insoluble in water
- **TLC:** Silica gel; Mobile: Hexane:EtOAc (1:1) or $CHCl_3$:MeOH (19:1); Detection: Vanillin-H_2SO_4 (heat) → Blue-grey spot; UV 254 nm (quenching – aromatic ring); Rf ≈ 0.40
- **FERRIC CHLORIDE TEST:** Podophyllotoxin + $FeCl_3$ solution → Blue-green colour (phenolic groups of the aryl ring are partially phenolic); less intense than simple phenols
- **ANISALDEHYDE-H_2SO_4 REAGENT (Spray on TLC):** Blue-grey to brown-violet colour on heating at 110°C; characteristic of lignan-type compounds
- **HPLC:** C18; Mobile: MeCN:H_2O (50:50) or MeOH:water (60:40); UV 290 nm (characteristic aromatic/lactone chromophore); Retention time vs reference; PDA detector for peak purity

ANALYSIS – PODOPHYLLOTOXIN

- **HPLC (Most accurate):** C18 (250 × 4.6 mm); Mobile: Acetonitrile:Water (50:50) or MeOH:Water (60:40); UV 290 nm; External standard calibration; Linearity 10–200 µg/mL; LOD ~0.5 µg/mL; Used for QC of Podophyllum resin (Podophyllin – NLT 40% podophyllotoxin and related lignans)
- **COLORIMETRIC:** Podophyllotoxin + $FeCl_3$-HCl reagent → colour at specific wavelength; alternative for plant extract screening

- **UV SPECTROPHOTOMETRY:** λmax 290 nm in MeOH; Molar absorptivity ε ≈ 4,100 L/mol/cm; Beer-Lambert law applied; less specific than HPLC

CURCUMIN

▶ Chemical Profile

Parameter	Details
Source	Curcuma longa Linn. (Turmeric); Family: Zingiberaceae; Part: Dried rhizome; Curcumin = principal yellow pigment (2–5% of dry rhizome)
Chemical Formula	$C_{21}H_{20}O_6$; MW: 368.38; Diarylheptanoid (Phenylpropanoid-polyketide)
Structure	Bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione; Symmetric structure: Two ferulic acid units joined by a heptadiene-dione bridge; Two enol-keto tautomers (keto form predominates in acidic/neutral, enol form in alkaline); Contains two phenolic –OH, two $–OCH_3$, and two α,β-unsaturated ketone groups (Michael acceptors)
Curcuminoid Mixture	Turmeric yields curcuminoids: Curcumin I (Curcumin, ~77%), Curcumin II (Demethoxycurcumin, ~17%), Curcumin III (Bisdemethoxycurcumin, ~3–5%)
Physical Properties	Bright orange-yellow crystalline powder; MP: 183°C; [α]D = 0° (achiral – symmetric molecule); λmax: 425–430 nm (yellow – responsible for turmeric colour); Freely soluble in acetone, EtOH, DMSO; Practically insoluble in water at pH < 6.5; Soluble in alkaline conditions (phenolate forms); Photodegradable (sensitive to UV light and sunlight); Sensitive to alkaline conditions
Biosynthesis	Shikimic pathway (two units of ferulic acid/p-coumaroyl-CoA) + Polyketide (malonyl-CoA) → Curcumin via Curcumin Synthase (diketide-CoA synthase type)

▶ Isolation of Curcumin

ISOLATION PROCEDURE

Step 1: DRYING & POWDERING: Fresh turmeric rhizomes dried at 40–50°C; powdered to 40 mesh

Step 2: DEFATTING (OPTIONAL): Extracted first with petroleum ether or hexane (to remove fixed oils, resins, volatile oil); petroleum ether discarded; powder dried

Step 3: ETHANOL EXTRACTION (Primary): Defatted powder extracted with 95% ETHANOL or ACETONE (Soxhlet extraction, 6–8 hours); Curcuminoids are soluble in polar organic solvents; Combined ethanol extracts

Step 4: CONCENTRATION: Ethanol extract filtered; concentrated to ~1/5 volume by rotary evaporation (40°C); dark orange concentrate

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Step 5: LIQUID-LIQUID PARTITIONING: Concentrate suspended in water; extracted with ETHYL ACETATE (3×); Curcumin preferentially into ethyl acetate layer (more polar solvent better for curcumin); ethyl acetate layers combined

Step 6: COLUMN CHROMATOGRAPHY: Ethyl acetate extract concentrated; loaded on silica gel column (60–120 mesh); Elution with Hexane:EtOAc gradient (9:1 → 7:3 → 1:1); Three curcuminoids separate: Curcumin I (major) elutes in ~40% EtOAc; Demethoxycurcumin elutes slightly later; TLC monitoring: Hexane:EtOAc 1:1

Step 7: TLC MONITORING: Silica gel 60 F_{254}; Hexane:EtOAc (1:1); Detection: UV 366 nm → YELLOW-ORANGE fluorescence; Curcumin: Rf ≈ 0.55–0.65; Demethoxycurcumin: slightly lower Rf

Step 8: RECRYSTALLISATION: Curcumin-rich fractions combined; evaporated; recrystallised from ethanol or acetone-water → Bright orange-yellow crystals; MP 183°C; High-purity curcumin (>95% curcumin I) requires multiple recrystallisations or preparative HPLC

IDENTIFICATION – CURCUMIN

- **PHYSICAL:** MP 183°C; Bright orange-yellow powder; λmax 425–430 nm (ethanol); $[\alpha]_D$ = 0° (achiral)
- **BORIC ACID TEST (ROSOCYANINE TEST – Most specific):** Curcumin + boric acid in acetic acid/oxalic acid → ROSOCYANINE (red colour in acidic condition); Curcumin + boric acid then alkalify → Blue-green (boronate complex); Specific test for curcumin; In presence of boric acid, two curcumin molecules chelate one boron → red rosocyanine
- **FERRIC CHLORIDE TEST:** Curcumin solution + $FeCl_3$ → Dark GREEN to BROWNISH-GREEN colour; Phenolic –OH groups react with Fe^{3+}
- **ALKALINE TEST (pH indicator):** Curcumin is yellow in acidic/neutral; ORANGE-RED/BROWN in alkaline (pH > 7.5); Turns red paper: Turmeric paper in NaOH → red (Turmeric paper test for borates)
- **TLC:** Silica gel; Hexane:EtOAc (1:1) or $CHCl_3$:MeOH (95:5); Detection: (a) UV 366 nm → yellow fluorescence; (b) Anisaldehyde-H_2SO_4 → yellow-orange; Rf curcumin ≈ 0.55–0.65

ANALYSIS – CURCUMIN

- **UV-VIS SPECTROPHOTOMETRY (Most common for plant extracts):** Dissolve in ethanol; measure at 425 nm; standard curve from pure curcumin; Beer-Lambert: $A = \varepsilon cl$; ε = 55,000; Highly sensitive (detect µg levels); NLT 90% total curcuminoids (as curcumin) in standardised extract
- **HPLC (USP/Most specific):** C18; MeOH:0.1% acetic acid (65:35); UV 425 nm; Separates all three curcuminoids: Bisdemethoxycurcumin (RT ~6 min), Demethoxycurcumin (RT ~8 min), Curcumin (RT ~10 min); Quantify each individually; Standardised C. longa extract: NLT 95% total curcuminoids
- **COLORIMETRIC (BORIC ACID METHOD):** Sample + oxalic acid + boric acid in glacial acetic acid → Rosocyanine; Absorbance at 540 nm; Used for plant extract screening (less specific)
- **FOLIN-CIOCALTEU (Polyphenol method):** Measures total polyphenol content; less specific but quick screening

EXAM-ORIENTED MCQs – PHARMACOGNOSY & PHYTOCHEMISTRY UNIT 3

Q1. Menthol is isolated from Mentha oil by which primary process?
a) Steam distillation only b) Solvent extraction c) Freezing/Cooling to crystallise menthol d) Column chromatography
✓ Answer: c) Freezing/Cooling to crystallise menthol (Dementholisation at −10 to −20°C)

Q2. Citral is chemically isolated from lemongrass oil by treatment with:
a) NaOH b) Sodium Bisulfite ($NaHSO_3$) – bisulfite addition c) HCl d) $FeCl_3$
✓ Answer: b) Sodium Bisulfite ($NaHSO_3$) – bisulfite addition compound formation (selective for aldehydes)

Q3. Which test is MOST SPECIFIC for identification of Citral?
a) Mayer's test b) Shinoda test c) 2,4-Dinitrophenylhydrazine test (→ yellow-orange precipitate) d) Vitali-Morin test
✓ Answer: c) 2,4-Dinitrophenylhydrazine test (→ yellow-orange precipitate) – confirms aldehyde group

Q4. The unique structural feature responsible for Artemisinin's antimalarial activity is:
a) Lactone ring b) 1,2,4-Trioxane endoperoxide bridge c) Carbonyl group d) Double bond
✓ Answer: b) 1,2,4-Trioxane endoperoxide bridge

Q5. Detection of Artemisinin's endoperoxide in identification can be done by:
a) Shinoda test b) KI + Starch → Blue colour c) Mayer's reagent d) Liebermann-Burchard
✓ Answer: b) KI + Starch → Blue colour (confirms peroxide; also $FeSO_4$ test)

Q6. Glycyrrhetinic acid is obtained from Glycyrrhizin by:
a) Alkaline hydrolysis b) Enzymatic hydrolysis with glucuronidase c) Acid hydrolysis (H_2SO_4) d) Steam distillation
✓ Answer: c) Acid hydrolysis (H_2SO_4) – cleaves two glucuronic acid sugar units from the triterpenoid aglycone

Q7. The Liebermann-Burchard test gives which colour with triterpenoids like Glycyrrhetinic acid?
a) Red b) Yellow c) Blue → Green d) Pink
✓ Answer: c) Blue → Green (then brown on standing)

Q8. Rutin isolation from Sophora japonica involves acid precipitation at which pH?
a) pH 7–8 b) pH 3–4 c) pH 10–11 d) pH 1–2
✓ Answer: b) pH 3–4 (cold dilute HCl reduces solubility of Rutin → yellow crystalline precipitate)

Q9. The Naturstoff Reagent (NPR) gives which colour with Rutin under UV 366 nm?
a) Red fluorescence b) Yellow-green fluorescence c) Blue fluorescence d) Purple
✓ Answer: b) Yellow-green fluorescence (characteristic of flavonoids – Naturstoff/DPBA reagent)

Q10. The MOST SPECIFIC test for identification of Atropine is:
a) Mayer's test b) Dragendorff's test c) Vitali-Morin test (violet colour) d) Murexide test
✓ Answer: c) Vitali-Morin test (specific for tropane esters → violet colour with KOH + acetone)

Q11. Caffeine is isolated from tea by boiling with CaO. The purpose of CaO is:
a) Liberate caffeine as free base b) Precipitate tannins as calcium tannate (remove interfering tannins) c) Increase solubility of caffeine d) Act as catalyst

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✔ **Answer: b) Precipitate tannins as calcium tannate (remove interfering tannins – otherwise tannins precipitate caffeine)**

Q12. The MOST SPECIFIC test for Caffeine is:

a) Mayer's test b) Dragendorff's test c) Vitali-Morin test d) Murexide test (purple-red colour with NH_3)

✔ **Answer: d) Murexide test (purple-red colour with NH_3 – specific for purine alkaloids)**

Q13. Caffeine gives NEGATIVE result with Mayer's and Dragendorff's reagents because:

a) It has no nitrogen b) It is non-basic (pKa ~14) and cannot be protonated to form ionic precipitate c) It is a protein d) It is volatile

✔ **Answer: b) It is non-basic (pKa ~14) and cannot be protonated to form ionic precipitate with alkaloid reagents**

Q14. Podophyllotoxin is detected on TLC by which spray reagent?

a) Dragendorff's b) $FeCl_3$ only c) Vanillin-H_2SO_4 (→ blue-grey spot on heating) d) KOH

✔ **Answer: c) Vanillin-H_2SO_4 (→ blue-grey to brown-violet spot on heating at 110°C) – characteristic of lignans**

Q15. The Boric Acid (Rosocyanine) test is SPECIFIC for:

a) Alkaloids b) Tannins c) Curcumin (diarylheptanoid) d) Cardiac glycosides

✔ **Answer: c) Curcumin (diarylheptanoid) → Rosocyanine (red colour in acidic) – highly specific for curcumin**

Q16. Quinine shows intense BLUE fluorescence in:

a) Concentrated H_2SO_4 b) Dilute H_2SO_4 (0.05M) under UV 366 nm c) NaOH solution d) Chloroform

✔ **Answer: b) Dilute H_2SO_4 (0.05M) under UV 366 nm – very sensitive; detectable at 1:100,000 dilution**

Q17. HPLC detection of Curcumin is performed at which wavelength?

a) 254 nm b) 272 nm c) 290 nm d) 425 nm

✔ **Answer: d) 425 nm (characteristic absorption of curcumin chromophore – conjugated diarylheptanoid)**

Q18. UV spectrophotometric estimation of Caffeine is performed at:

a) 254 nm b) 272 nm ($\varepsilon = 9800$) c) 290 nm d) 425 nm

✔ **Answer: b) 272 nm ($\varepsilon = 9800$ L/mol/cm – purine chromophore of caffeine)**

Q19. Reserpine is sensitive to:

a) Heat only b) Acids c) Light (photodegrades) and its pale yellow crystals darken on exposure d) Alkaline conditions only

✔ **Answer: c) Light (photodegrades) – stored in dark; also acid-labile**

Q20. The Shinoda test (Mg + HCl) gives PINK to RED colour and is specific for:

a) Alkaloids b) Tannins c) Flavonoids (Rutin – flavonol glycoside) d) Cardiac glycosides

✔ **Answer: c) Flavonoids (Rutin – flavonol glycoside) → Magnesium reduces flavonoid → cyanidin-type colour**

BEST OF LUCK FOR EXAMS!

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