STANDARDIZATION
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Introduction

For pharmaceutical purposes, the quality of medicinal plant material must be as high as that of other medicinal preparations. The quality of a vegetable product depends on the geographical origin, time and stage of growth when collections have been done and post harvest handling. The raw material presently available to the industry is procured from more than one geographical region. The plant is usually collected without paying attention to the stage of maturity, dried haphazardly and stored for long periods under unsuitable conditions. The quality of crude drug, as such, is more often degraded.

Several pharmacopoeias including Indian Pharmacopoeia, British Pharmacopoeia, Japanese Pharmacopoeia and United States Pharmacopoeia do cover monographs and quality control tests for few of the medicinal plants used in their respective countries but basically these pharmacopoeias are designed to cater the chemical based medicines and pharmaceutical necessities by giving their standard test methods (Kohli, 1996).

World Health Organization (WHO) currently encourages, recommends and promotes traditional herbal remedies in National Health Care Programs, because such drugs are easily available at low costs, are comparatively safe and reliable. Plant materials and herbal remedies derived from them represent a substantial proportion of the global drug market and thus internationally recognized guidelines for their quality assessment are necessary. The WHO emphasized the need to ensure quality control of medicinal plants products by using modern techniques and applying suitable standards.

Directives on the analytical control of vegetable drugs must take account of the fact that the material to be examined has complex and inconsistent
composition. Therefore, the analytical limits cannot be as precise as for
the pure chemical compound. Vegetable drugs are inevitably inconsistent
because of their composition and hence the standardization may be
influenced by several factors such as age and origin, harvesting period,
method of drying and so on. To eliminate some of the causes of
inconsistency, one should use cultivated rather than wild plant which are
often heterogeneous in respect of the above factors and consequently in
their content of active principles.

Some other problems facing standardization of crude drugs include the
confusion existing over the identity of source material, the impossibility to
assay for a specific chemical entity when the bioactive ingredient is not
known and the problems posed by those preparations which contain
complex heterogeneous mixtures. The quality control of plant products is a
general requirement to be fulfilled. 'Quality' refers to the intrinsic value of
the drug, i.e., the amount of medicinal principles or active constituents
present. These constituents are classified into groups of nonprotoplasmic
cell contents. These groups include carbohydrates, glycosides, neutral
principles, acids, alkaloids, volatile oils, lipids, oleoresins, balsams,
steroids, amino acids and hormones.

Solubility profile, starting from petroleum ether to water, shows the wide
range of nonpolar and polar chemical constituents that the plant material
contains and that it may be a good source of active ingredients for
pharmacological evaluation. Ash values, on the other hand, are one of the
best physicochemical parameters for the evaluation of plant drug for its
purity, quality and strength.

To check any adulteration or non-deliberate mixing in the commercial
batches, specifications must be laid down for each herb (Raina, 1993;
Sarine, 1993).

From his first awakening, man has sought to fight and control diseases, and turned
to nature for inspiration and guidance. During thousands of years of early human
existence, many natural materials by instinct or intuition or trial and error got in use for combating human ailments. Thus, traditional system of medicines such as Ayurveda, Unani, Siddha, and Homoeopathy lean heavily on natural products, derived from higher plants, microbes or animals. Traditional healers have left voluminous treatises from which many formulations have been taken which are currently in use. Nonetheless, as the preparations of drugs have become commercial, an urgent need has arisen to have a quality control check on the various formulations to assure the consumer a supply of efficacious drugs. Traditional healing arts are generally based on a single medicinal plant drug or multiple drugs. In such circumstances, the medicinal plant used for the preparations of a drug should be authentic and genuine. The formulae used in some Ayurveda and Siddha drug preparations are taken even from palm leaf manuscripts. The consumers should be assured of the quality of the drugs. Hence, scientific methods of standardization are needed to confirm the authenticity of medicinal plants used in the preparation of a drug. To standardize or to evaluate a drug means to identify it and to determine its quality and purity. The identification of a crude drug can be established by actual collection of the drug from a plant that has been positively identified. Quality refers to the intrinsic value of the drug, i.e., the amount of medicinal principles or active constituents present. The evaluation or standardization of a crude drug involves a number of methods

**Organoleptic methods**

Organoleptic refers to the evaluation by means of the macroscopic appearance of the drug, its odor, taste and the ‘feel’ of the drug to touch.

**Pharmacognostical methods**

Pharmacognosy is an applied science, which deals with botanical, physicochemical and economical features of the crude drugs of plant origin.

**Phytochemical methods**

Phytochemistry has developed in recent years as a distinct discipline somewhere in between natural product organic chemistry and plant biochemistry and is closely related to both.
It is concerned with the enormous variety of organic substances that are accumulated by plants and deals with the chemical structures of these substances, their synthesis, turnover and metabolism, their natural contribution and their biological function.

Quality refers to the intrinsic value of the drug, i.e. the amount of medicinal principles present. The active constituents are carbohydrates, glycosides, tannins, lipids, flavonoids, coumarins, phenolic compounds, volatile oils, resins, steroids, alkaloids, peptide hormones, enzymes, and other proteins, vitamins, antibiotics, allergens and others.

A high grade of quality in a drug is of primary importance and efforts should be made to obtain and maintain this high quality in the plant drug.

Hence, qualitative and quantitative estimation of the phytochemical constituents will be very much useful in the standardization of crude drugs. In addition, the various chromatographic techniques such as thin layer, paper, column and HPLC techniques can be employed for the isolation of the particular phytochemical. Using modern spectroscopic techniques such as UV, IR, H-NMR, C-NMR, Mass, ORD and CD, the new compounds can be characterized and their structure determined.

The presence of a particular class of chemical compounds in the various species of the family of plants can be used as a chemotaxonomical marker. For many drugs, the chemical assay represents the best method of determining official potency (Gopalakrishan S, 2004).

EVALUATION OF HERBAL DRUGS

To evaluate a drug means to identify it and to determine its quality and purity.

The identity of a drug can be established by actual collection of the drug from a plant or animal, which has been positively identified. Research investigators must be absolutely certain of the origin of their samples; hence, "drug gardens" are frequently established institutions engaged in Pharmacognostical research.

Another method of identification is by comparing a representative unknown sample with a published description of the drug and with authentic drug samples.

Quality refers to the intrinsic value of the drug, i.e., the amount of medicinal principles or active constituents present. These constituents are classified into
groups of nonprotoplasmic cell contents. These groups include: carbohydrates, glycosides, neutral principles, tannins, lipids, volatile oils, resins and resin-combinations, steroids, alkaloids, peptide hormones, enzymes and other proteins, vitamins, antibiotics, biologics, allergens, and others.

A high grade of quality in a drug is of primary importance, and effort should be made to obtain and maintain this high quality. The most important means of accomplishing this include: (1) collection of the drug from the correct natural source at the proper time and in the proper manner; (2) preparation of the collected drug by proper cleaning, drying, and garbling; and (3) proper preservation of the cleaned, dried, pure drug against contamination with dirt, moisture, fungi, filth, and insects.

The evaluation of a drug involves a number of methods, which may be classified as follows: (1) Organoleptic, (2) Microscopic, (3) Biological, (4) Chemical, (5) Physical.

**Organoleptic evaluation**

Organoleptic (lit. "impression on the organs") refers to evaluation by means of the organs of sense, and includes the macroscopic appearance of the drug, its odor and taste, occasionally the sound or "snap" of its fracture, and the "feel" of the drug to the touch.

For convenience of description the macroscopic characteristics of a drug may be divided into four headings, viz.: (1) Shape and size, (2) Colour and external markings, (3) Fracture and internal colour, (4) Odour and taste. In some official crude drug monographs the entire macroscopic description consists of an organoleptic evaluation and is the only means of evaluation given.

Occurrence on the market. Vegetable drugs are brought into the market in various commercial forms. Crude drugs may be nearly entire as seeds, flowers, fruits, leaves and some roots and rhizomes; or they may be cut, broken, or sliced, as in woods, barks, many roots, and a few rhizomes. They may be more or less matted together, as in Chondrus and in baled leaves; they may be pressed together by hydraulic pressure giving the so-called pressed drugs; or they may be powdered and then molded into forms, as "rhubarb fingers." Sometimes the periderm is...
removed, as in roots (Russian Licorica), rhizomes (Ginger), and barks (sassafras). Mexican Sarsaparilla may come to the market in neat cylindrical rolls in which a mass of the bundled roots is tightly wrapped by many coils of long roots, or the roots may be in tightly packed bales, or it may be cut into short pieces, or be coarsely ground or finely powdered: each of these forms presents a very different appearance, yet all are the same drug.

Underground plant parts. Drugs derived from underground parts of the plant, such as rhizomes, roots, bulbs, corms, tubers, occur either (1) entire, (2) in longitudinal slices, (3) in oblique or transverse slices, (4) cut in small cubical pieces, or (5) broken into pieces. In shape they may be (1) cylindrical, as Sarsaparilla; (2) cylindraceous or subcylindrical or nearly cylindrical, as Podophyllum; (3) conical, as Aconite; (4) fusiform (enlarged in the middle and tapering toward the ends), ovoid (egg-shaped), or pyriform (pear-shaped), as Jalap; (5) terete (tapering gradually but nearly cylindrical); or (6) disk-shaped.

The parts may be simple or branched and are frequently curved and twisted. In the case of rhizomes, the direction of growth is often considered. This is usually horizontal but may be oblique and in a few cases is vertical. The direction may be roughly determined by the attachment of the roots and stem bases.

Sizes are given as to length and diameter and in the most convenient terms, either millimeters (mm) or centimeters (cm). In cases where the shape is conical, the diameter of both wide and narrow parts may be of importance.

The external colour varies from white (where the drugs have been deprived of the periderm) through yellowish gray, yellowish brown, reddish orange, to brownish black. It is often more or less gray from clay dust.

External markings may be classified as follows: (1) furrows, alternating ridges and valleys which are more or less parallel, well-defined, and usually due to the shrinkage of the internal parts caused by drying; (2) wrinkles, fine or delicate furrows; (3) annulations, transverse ring like markings; (4) fissures, splits extending into the tissues; (5) nodules, rounded outgrowths on the surface; (6) projections,
such as roots, stem bases and buds; (7) scars, such as leaf scars, stem-base scars, root scars, bud scars, bud-scale scars.

The fracture refers to the way the plant part breaks when subjected to sufficient pressure. Often the woody portion fractures in a different manner than the nonwoody portion. Terms used in describing fractures are as follow: (1) complete, breaking clean across; (2) incomplete, breaking only part way across; (3) short; a clean smooth break with a quick snap; (4) fibrous, a break accompanied by resistance and characterized by the projection of fibers from the broken surfaces; (5) splintery, breaking irregularly across into pieces with larger and smaller projecting edges and splinters; (6) brittle, easily broken, usually into many pieces when dropped into a hard surface; (7) tough, breaking with difficulty; (8) weak, breaking with little effort.

In many cases the nature of the fractured surface is as important as the fracture itself. Terms used to describe the fractured surface are as follow; (1) even, a smooth surface; (2) uneven, an irregularly broken surface; (3) granular, having a grain like appearance; (4) hard, a compact surface; (5) horny, a hornlike surface; (6) mealy, a surface characterized by powdering, usually due to an abundance of starch (starchy); (7) resinous, a smooth glossy surface; (8) conchoidal, a resinous surface characterized by having the surface curved in convex and concave fashion; (9) waxy, exhibiting a dull wax like surface. Other descriptive terms, as dull, smooth, rough, and the like, are also used in describing the fractured surface.

The colour of the fractured surface is known as the internal colour.

Barks. The bark refers to that portion of the woody exogenous stem or root that lies outside of the cambium ring. The majority of official barks have periderm present, yet in some cases, as sassafras and Ceylon cinnamon, this has been removed. In the entire state, barks occur in three shapes; (1) flat or transversely curved pieces; (2) single quills (rolled from one edge); (3) double quills (rolled from both edges). The most important measurement is the thickness of the bark. In the case of quills, both the diameter and the length should also be observed; in flat pieces, measurements of the length and breadth may be made.
Barks have two surfaces, an outer and an inner, and the official compendia describe both. The external colour of barks on both outer and inner surfaces usually varies from brownish gray to brownish black. The inner surface is usually lighter in colour than the outer surface and in some cases is almost white.

The markings on the outer surface of barks are often characteristic, such as (1) lenticels, (2) lichens with their apothecia, (3) corky ridges, warts, or prickles, (4) fissures, and (5) adhering mosses. The inner surface may be smooth or marked with fine parallel lines (striations) owing to the inner fibers of the phloem. Occasionally crystals are found on the inner surface of barks.

The fracture of barks may vary from short and weak to tough and fibrous. The examination of the fractured surface includes colour and the presence or absence of projecting bast fibers and stone cells. In many cases the nature of the fracture of the cortical region differs from that of the phloem region.

Woods. Wood refers to that part of the woody exogenous stem or root that lies inside the cambium ring. That portion of the wood which lies near the cambium ring and still functions in the vegetative process of the plant is known as the sapwood which is usually white; the inner layers of wood which have ceased to function in the transportation of sap form the heartwood which is often highly coloured.

Woods occur in the form of chips, rasplings, and shavings. Their external surfaces are usually striated from fibers or porous from vessels. The external colour varies widely. The fracture is usually tough and fibrous while the internal colour is the same as the external colour.

Leaves and flowers. Leaves and leaflets are described with the usual botanical terms. The length and width of the blade is the size recorded. The general shape of the blade, together with that of the apex, margin, and base or given. The external markings include the venation, the texture of the leaf, and the hairs or other characters of the upper and lower surfaces. The "feel" of the surface may be described as soft, harsh, smooth. The colour is included under the description of the surface.

Many of the leaves occur in a more or less crumpled condition (Belladonna Leaf. Hyoscyamus) and such specimens should be macerated in water and then spread.
out for study. Other leaves occur on the market in an entire state (Buchu, Coca). The fracture of leaves, unless particularly characteristic, is usually of no importance in identification.

Flowers, flower heads, and floral parts, such as stigmas, corollas and others, vary greatly as to shape. These shapes are described with the usual botanical terms. In the case of flower heads or inflorescences (Pyrethrum), the parts, such as receptacle, involucrum, ray-and disk-florets, are described individually.

**Fruits and seeds.** Fruits and seeds vary greatly as to shape. The usual forms are globular, ellipsoidal, ovoid, reniform, conical and other shapes. Two or three dimensions are to be noted as the case may be. Often only a portion of the fruit is utilized (Bitter Orange Peel). The usual markings of fruits consist of attachment scars and the scars or remains of various floral parts. Many of the fruits, especially the drupes, show a wrinkled pericarp surface. These wrinkles are often characteristically netted or reticulated. Seeds usually show a smooth or minutely pitted testa, hilum, and micropyle. Often ridges or furrows locating the hypocotyls or cotyledons are seen. **Colour** varies greatly and fracture is usually of little importance.

**Exudations, extractives and other drugs.** Those items sold as crude drugs which do not possess a definite histological (or cellular) structure may be products formed in the metabolic processes of the plant, they may be pathologic products, or they may have undergone some special pharmaceutic or physical treatment. This group includes items such as gums, resins, gum-resins, mucilages, oleoresins, inspissated juices, latex, tars, extracts, and the like. They may occur in (1) tears, small rounded masses formed naturally as the exudation hardens (Acacia); (2) cylindrical pieces (Gamboge); (3) rounded or flattened masses (Opium); (4) angular masses, broken up material that has hardened in the container (Aloe); and (5) agglutinated masses, harder portions massed with soft material (Benzoin). Often however, they occur in a state varying from a liquid in a semi plastic mass (Tolu Balsam). Only in the case of tears is the **size** of importance. In the case of solids, **fracture and fractured surface** are important, **colour** is characteristic, but significant **external markings** are usually absent.
Substances such as volatile and fixed oils, glycosides, alkaloids, and so forth are usually considered from the chemical standpoint because they have no morphologic characters. They will be considered in the drug monographs under the heading of active constituents. A miscellaneous group comprising such items as starch (Amylum), excrescences (Nutgall), trichomes (Lupulin), sclerotia (Ergot), diatom frustules (Purified Siliceous Earth), and other substances are of relative importance, and the usual characteristics of size, shape, colour, markings are determined either macroscopically or microscopically.

Odour and taste. The odour of a drug may be either distinct or indistinct, depending upon the amount of volatile constituents the drug possesses. General terms used in describing odour are aromatic, balsamic, spicy alliaceous, camphoraceous, terebinthinate, and others. These terms are comparative with other substances in nature. When no such correlation can be made and the odour is distinct. It is said to be characteristic.

Taste may be defined as a particular sensation excited by certain substances when these are brought into contact with special organs situated in portions of the epithelial layer of the mouth. Taste is that sense by which we perceive the characteristic or distinctive flavour of soluble substances when these are placed in the mouth and moistened with saliva. The taste of a substance is the quality or flavour of the substance perceived in this way. Substances may be classified according to taste into the following groups;

1. Those possessing a true taste, such as (a) Acid (Sour), (b) Saline (Salty), (c) Saccharine (Sweet), (d) Alkaline and (e) Bitter.
2. Those possessing no taste and hence are tasteless or insipid. This group includes all substances insoluble in the saliva.
3. Those possessing a characteristic odour, which gives a name to the so-called "taste". With this odour, a true taste may or may not be associated. Such tastes may be grouped broadly into those which are agreeable, including (a) aromatic, (b) balsamic, (c) spicy; and disagreeable, including (a) alliaceous, (b) camphoraceous, (c) terebinthinate.
4. Those imparting distinctive sensation to the tongue, exclusive of taste or touch. These sensations may or may not be associated with true taste. Such substances may be classified as: (a) mucilaginous, those producing a soft slimy feeling; (b) oily, those producing a bland smooth feeling; (c) stringent, those producing a contraction of the tissues of the mouth, causing a puckering feeling; (d) pungent, those producing a warm biting sensation; (e) acrid, those producing an unpleasant, irritating, tingling sensation; (f) nauseous, those tending to induce vomiting.

A drug frequently gives more than one taste, the sensation usually being observed in consecutive order. The first taste noted is produced by the most soluble constituent.

Colour. The colour of drugs, whether they are whole, powdered, or in microscopic section, is determined by the Inter-Society Colour Council-National Bureau of Standards method. The colour names indicated in the official monographs are official standards of equal importance to the other organoleptic specifications.

Colour names are standardized, and each consists of a hue name, with or without one or two modifiers to describe the value (lightness) and chroma (strength) of the hue. For example, Reserpine is described as a "white or pale buff to slightly yellowish, odourless, crystalline powder", in reading colours, standard Munsell charts are used and the Munsell notation determines. This is then translated into colour nomenclature by the use of ISCC-NBS charts. The National Formulary IX includes a complete description of the application of the method.

Microscopic Evaluation/Microscopy

The microscope has been employed in the examination of drugs since 1847, which Schleiden used it in the examination of the sarsaparillas. In 1853 Schacht showed its value in the examination of textile fibres.

The microscope is not only essential to the study of adulterants in powdered plant and animal drugs, but is indispensable in the identification of the pure powdered drug. Those sections of the official monograph headed histology and powder deal almost exclusively with the microscopic appearance of the drug in sectional view and powdered form.
Plant parts are made up of tissues, each of which performs a definite function essential to the life of the plant. The histology refers to the character and arrangement of these tissues, as they are present in the drug. Some drugs have no cellular structure (acacia, rosin); some are composed of microscopic units, such as spores (lycopodium or hairs (lupulin, kamala); with others a knowledge of structure would not be particularly helpful, for they are easily identified macroscopically (eriodictyon); however, many drugs possess a characteristic structure helpful in identification of the drug. Histological studies are made from very thin transverse or longitudinal (radial and tangential) sections properly mounted in suitable stains, reagents, or mounting media.

Powdered drugs possess very few macroscopic features of identification outside of colour, odour and taste, hence the microscopic characteristics are very important. In the powdered drug (which should be reduced to not less than a No. 40 powder) the cells are mostly broken, except those with lignified walls, but the cell contents (starch, calcium oxalate crystals, aleurone, etc.) are scattered in the powder and become very evident in the mounted specimen.

To prepare a mount, transfer a suitable quantity of the powder to 2 or 3 drops of reagent or mounting medium on the clean slide and mix it well with the liquid. Place the cover glass on the specimen and, by light pressure, move it in a rotary motion to insure an even distribution of the powder under it. A properly prepared mount will just fill the space between slide and cover glass and will be slightly opaque.

The proper reagent or mounting medium to be used depends on the characteristic tissue element or cell content to be studied; starch is the best examined in water mount; lignified tissue such as bast fibres, stone cells and tracheae in a phloroglucinol-hydrochloric acid mount; calcium oxalate, leaf epidermal tissue, trichomes, etc., in a chloral hydrate mount. Special test reagents such as iodine t.s., zinc chloride t.s., ferric chloride t.s., etc., are also used where occasion demands.

Not only is the microscope useful in the study of the histological elements of the drugs and in the detection of adulterants, from the histological standpoint, but it
can be used for a quantitative microanalysis of admixed or adulterated powders. This is done by counting a specific histological feature in a measured quantity of the unknown powder and comparing the count with that obtained of the same feature in a known standard sample, which sample may be the designated powder itself, or of the adulterant, or of any one of the mixture of powders. Similar methods are used for the counting of mold filaments, mold spores, bacteria, etc.

Microchemistry

Microchemistry encompasses the study of constituents by the application of chemical reagents to microscopic sections of the drug or to a small quantity (a few milligrams) of the powdered drug. Microchemistry affords a means by which the constituents of many drugs may be isolated and identified (Claus Edward P a 1961).

Quantitative microscopy

Powdered drugs or adulterants, which contain a constant number, area or length of characteristic particles/mg (e.g., starch grains, epidermis, trichome ribs respectively) can be determined quantitatively by microscopy using lycopodium spores as an indicator diluent. In addition to the simple measurement of tissues, cells and cell contents by means of the micrometer eyepiece or camera lucida. It is possible to estimate the percentage of foreign organic matter in many powdered drugs by lycopodium spore method, which was described by Wallis and is described in Appendix XIII of the BP (1973). Other microscopical determinations, which may usually be made in certain cases, are vein-islet numbers, palisade ratio, stomatal number and stomal indices (Evans a, 1997).

*Lycopodium spore method*

Wallis showed that lycopodium spores are exceptionally uniform in size (about 25 μm) and that 1 mg of lycopodium contains an average of 94,000 spores. The number of spores mg⁻¹ was determined by direct counting and by calculations based on specific gravity and dimensions of the spores. The methods gave values in good agreement. These facts make possible to evaluate many powdered drugs, provided that they contain one of: (1) well-defined particles which may be counted (e.g., pollen grains or starch grains); or (2) single-layered tissues or cells the area
of which, may be traced at a definite magnification and the actual area calculated; or (3) characteristic particles of uniform thickness, the length of which can be measured at a definite magnification and the actual length calculated. Whichever method is adopted, mounts containing a definite proportion of the powder and lycopodium are used and the lycopodium spores are counted in each of the fields in which the number or area of the particles in the powder is determined. The method is somewhat laborious and has not been subjected to statistical assessment. Classical examples of drugs to which it was applied are senna and linseed (area measurements, nux-vomica (trichome-rib lengths) and pyrethrum and ginger (counts).

Leaf measurements
A number of leaf measurements are used to distinguish between some closely related species not easily characterized by general microscopy.

Palisade ratio
The average number of palisade cells beneath each upper epidermal cell is termed the palisade ratio. Quite fine powders can be used for the determination.

Method
Pieces of leaf about 2 mm square, or powder, are cleared by boiling with chloral hydrate solution, mounted and examined with a 4 mm objective. A camera lucida or other projection apparatus is arranged so that the epidermal cells and the palisade cells lying below them may be traced. First a number of groups each of four epidermal cells are traced and their outlines inked in to make them more conspicuous. The palisade cells lying beneath each group are then focused and traced. The palisade cells in each group are counted, those being included in the count which are more than half covered by the epidermal cells; the figure obtained divided by 4 gives the palisade ratio of that group. The range of a number of groups from different particles should be recorded.

Stomatal number
The average number of stomata per square millimeter of epidermis is termed the stomatal number. In recording results the range as well as the average value
should be recorded for each surface of the leaf and the ratio of values for the two surfaces.

*Method*

Fragments of leaf from the middle of the lamina are cleared with chloral hydrate solution or chlorinated soda. Timmerman counted the number of stomata in 12-30 fields and from a knowledge of the area of the field was able to calculate the stomatal number; the camera lucida method described for vein-islet numbers may also be used, the position of each stoma being indicated on the paper by a small cross.

Using fresh leaves, replica of leaf surface may be made which are satisfactory for the determination of stomatal number and stomatal index. An approximate 50% gelatin and water gel is liquefied on a water-bath and smeared on a hot slide. The fresh leaf is added the slide is inverted and cooled under a tap and after about 15-30 min the specimen is stripped off. The imprint on the gelatin gives a clear outline of epidermal cells, stomata and trichomes.

**Stomatal index**

The percentage proportional of the divisions of the epidermis of a leaf, which have been converted into stomata, is termed the *stomatal index.*

\[ I = \frac{S \times 100}{E} \times 100 \]

Where \( S \) = number of stomata per unit area and \( E \) = number of ordinary epidermal cells in the same unit area. While stomatal number varies considerably with the age of the leaf, stomatal index is highly constant for a given species and may be determined on either entire or powdered samples. It is employed in the *BP* and *EP* to distinguish leaflets of Indian and Alexandrian senna.

*Method*

Pieces of leaf other than margin or midrib are suitably cleared and mounted, and the lower surface examined by means of a microscope with a 4 mm objective and an eyepiece containing a 5 mm square micrometer disc. Counts are made of the numbers of epidermal cells and of stomata (the two guard cells and ostiole being considered as one unit) within the square grid, a cell being counted if at least half of its area lies within the grid. Successive adjacent fields are examined until about
400 cells have been counted and the stomatal index value calculated from these figures. The stomatal index may be determined for both leaf surfaces.

Vein-islet number

The term 'vein-islet' is used to denote the minute area of photosynthetic tissue encircled by the ultimate divisions of the conducting strands. The number of vein-islets $\text{mm}^2$ calculated from four contiguous square millimeters in the central part of the lamina, midway between the midrib and the margin is termed the vein-islet number. When determined on whole leaves, the area examined should be from the central part of the lamina, midway between the margin and midrib.

Method

Boiling in chloral hydrate solution in a test-tube placed in a boiling-water-bath may clear many leaves. Those, which are difficult to clear in this way, may, after soaking in water, be treated successively with sodium hypochlorite to bleach 10% hydrochloric acid to remove calcium oxalate, and finally chloral hydrate. A camera Lucida or projection apparatus is set up and by means of a stage micrometer the paper is divided into squares of 1 $\text{mm}^2$ using a 16 mm objective. The stage micrometer is then replaced by the cleared preparation and the veins are traced in four contiguous squares, either in a square 2 mm $\times$ 2 mm or a rectangle 1 mm $\times$ 4 mm. When counting, it is convenient to number each vein-islet on the tracing. Each numbered area must be completely enclosed by veins, and those which are incomplete are excluded from the count if cut by the top and left hand sides of the square or rectangle but included if cut by the other two sides.

Veinlet termination number

Hall and Melville (1951) determined veinlet termination number, which they defined as 'the number of veinlet terminations per $\text{mm}^2$ of leaf surface. A vein termination is the ultimate free termination of a veinlet or branch of a veinlet'. By this character they distinguished between Peruvian and Bolivian coca leaves and between Alexandrian and Tinnevellys senna leaflets.
One practical difficulty in the measurement of vein-islet and veinlet-termination numbers is deciding exactly where, and if, a veinlet termination. This may appear to vary according to the preliminary treatment a leaf has received. At present, of the above leaf measurements, only stomatal index is employed officially. With the increasing number of whole herbs and leaves now being introduced into the European and British Pharmacopoeias and the need for standardization of the many herbal products of interest world-wide, a further investigation of the possible usefulness of these leaf measurements might prove rewarding (Evans b, 1997).

Physical Evaluation

The application of typical physical constants such as viscosity of drugs containing gums, swelling factor or mucilage containing materials, froth number of saponin drugs, determination of extractive values, ash values and volatile content of crude drugs are of some significance for the evaluation of drugs. However, physical constants such as boiling point, freezing point, absorption coefficient (i.e. ability of the substance to absorb some specific compounds), refractive index, optical rotation, spectroscopic analysis, fluorescence analysis and radioimmuno assays are applied more frequently to the active principles of drugs, such as alkaloids, volatile oils, fixed oils and others. Some of the physical methods employed for determining the quality and purity of drugs may be considered under the following subheadings:

(a) Foreign matter Crude vegetable drugs are difficult to get in absolutely pure condition and thus pharmacopoeias prescribe limit to the percentage of the presence of other parts of the plant or any other organic material which may include animal excreta, insects or moulds. For the determination of foreign matter, weighed quantity (25-100g) of the drug is taken and spread in thin layer on a paper. The foreign matter is picked up and weighed. If the percentage of the foreign matter exceeds the prescribed pharmacopoeial limits, the sample is declared substandard. Sometimes the crude drugs get contaminated with certain microbes like bacteria, fungi and moulds. Such contaminations can lead to certain toxicities, which are not actually attributed to the crude drugs. Under such
circumstances the drugs may have to be subjected to sterilization in special equipment by treatment with ethylene oxide.

(b) Moisture content Presence of moisture in a crude drug can lead to its deterioration due to either activation of certain enzymes or growth of microbes. Moisture content can be determined by heating the drug at 105°C to constant weight and calculating the loss of weight. Moisture in a crude drug can also be determined by distilling the drug in presence of water-immisble solvent like toluene or xylene and from the distillate thus collected level of water can be measured. Karl Fischer method is a standard procedure for determining moisture content (Handa & Kapoor, 1989).

The most extensively employed chemical method for water determination is probably the Kark Fisher procedure. It is used in the BP and is particularly applicable for the expensive drugs and chemicals containing small quantities of moisture. Dry extracts of alkaloid-containing drugs, alginic acid, alginates and fixed oils (e.g., castor oil for parenteral use, BP) may usefully be evaluated. For crude drugs such as digitalis and ipecacuanha the powdered material can be first exhausted of water with a suitable anhydrous solvent (dioxan) and an aliquot taken for titration.

The reagent consists of a solution of iodine, sulphur dioxide and pyridine in dry methanol. This titrated against a sample containing water, which causes a loss of the dark brown colour. At the end point when no water is available, the colour of the reagent persists. The basic reaction is reduction of iodine by sulphur dioxide in the presence of water (Chaudhri, 1996).

(c) Extractive values In crude drugs sometimes the active chemical constituents cannot be determined and thus the water, alcohol or ether soluble extractive values are determined for evaluation of such drugs. Water-soluble extractive values in case of drugs aloe, gentian and liquorice are given in pharmacopoeias. Similarly alcohol soluble extractive values in cases of drugs like ginger, valerian and quillaia are important and other soluble material is determined in drugs like capsicum, maleferm, etc. All such extracts are prepared in an appropriate solvent either by maceration or by a continuous extraction process.
(d) Volatile content The efficacy of volatile oil containing drugs like cardamom, cinnamon, clove, fennel, dill etc. depends upon the amount of the volatile content present in the drug. Weighed quantity of the drug is boiled with water in a round-bottomed flask fitted with Clevenger apparatus. The distillate collected in graduated receiving tube separates into volatile oil which being lighter than water remains on the top and the amount thus collected is read from the tube.

(e) Ash values The total ash content is determined by incinerating a known quantity of the air-dried crude in a silica crucible and the residual ash is weighed to calculate the percentage of the ash on the basis of initial dry weight of the drug sample. Total ash of unpeeled drug is more than the peeled drug. Although total ash values are of little significance in the drug evaluation because of wide range of variations observed in different samples of the same drug but still very high ash values are indicative of contamination, adulteration or carelessness in preparing the drug for the market.

The total ash obtained from 2-4 g of air-dried drug when boiled for 5 minutes with 25 ml of dilute hydrochloric acid leaves behind acid insoluble ash. Determination of acid insoluble ash is of significance as the higher content of acid insoluble ash indicates possibility of sand being mixed with the drug. Pharmacopoeial limits for acid insoluble ash vary from 0.5 per cent (agar) to 12 per cent (hyoscyamus). Drugs like hyoscyamus with glandular trichomes have a capacity of retaining clay and thus the acid insoluble ash value is higher in such cases. If no limit of acid insoluble ash is given for a crude drug in the pharmacopoeial monograph this value should not exceed 2 per cent.

(f) Refractive index For the evaluation of volatile and fixed oils, measurements of refractive index is of significance. The refractive index changes if particular oil is mixed with another oil. The refractive index is defined as the ratio of the velocity of light in vacuum to velocity in the substance, and is the ratio of the sine of the angle of incidence to the sine of the angle of refraction. It is measured by means of a refractometer.

(g) Optical rotation Many substances of biological origin can rotate the plane of polarized light either to the left side. Such compounds are said to be optically
active and are designated as dextrorotatory or laevorotatory, as the beam is deflected to the right or to the left, respectively. The extent of rotation is expressed in degrees, plus (+) indicating rotation to the right, minus (-) indicating deflection to the left. A polarimeter is used to measure optical rotation. Most volatile oils contain optically active components and the direction of the rotation, as well as its magnitude, is a useful criterion of purity.

(h) Rf values Thin layer chromatography (TLC) has become increasingly popular for both the qualitative and quantitative evaluation of drugs. Rf value refers to the ratio of distance moved by the solute to the distance moved by the solvent on a thin layer of an adsorbent. Rf value of a compound is characteristic and can be used to identify the component by comparison with the reference standard. The intensity of the colour of spot of the compound under test can be utilized for quantitative estimation of the principle in the drug (Handa & Kapoor, 1989).

Chemical Evaluation

Determination of active constituent in a drug by chemical process is referred to as chemical evaluation. Vitali-Morin reaction for example, is one for determining tropane alkaloids in datura, belladonna and stramonium. For detecting and estimating emetine in ipecac, potassium chlorate and hydrochloric acid are used. Ammonium vanadate and cleremic acid are used for detecting strychnine in nux vomica. Borntrager's test is employed for detecting anthraquinone glycosides in purgative drugs like senna, rhubarb, cascara and aloe. A number of quantitative physico-chemical constants like acid value, iodine value, saponification value, ester value etc. are employed for fixed oils and fats. Determination of methoxyl value and volatile acidity are important for gums. Evaluation of alkaloid containing drugs can be done by determining total alkaloidal content through acid-base titration or by nonaqueous titrations of alkaloidal salts. Chemical evaluation of crude drugs is more appropriate because this identifies as well as estimates the phytoconstituents present in a drug (Kokate et al, 2003).

Quantitative Chemical Tests A number of quantitative chemical tests; acid value, iodine value, saponification value, ester value, unsaponifiable matter, acetyl value, volatile acidity are mainly applicable to fixed oils. Some of these tests are also
useful in the evaluation of resins (acid value, sulphated ash), balsams (acid value, ester value, saponification value), volatile oils (acetyl value, ester value) and gums (methoxyl determination, volatile acidity) (Evans c, 1997).

Assays
A crude drug may be assayed for a particular group of constituents- for example, the total alkaloids in belladonna or the total glycosides of digitalis. Alternatively, it may be necessary to evaluate specific components-for example, the Reserpine content, as distinct from the total alkaloid content, of Rouwolfia spp. Biological assays, which can be time-consuming, were at one time employed for the assay of those potent drugs (e.g. digitalis) for which no other satisfactory assay was available. In pharmacopoeias these have now been largely replaced by chemical and physical assays for routine standardization. However, the biological assay remains important for screening plant materials and their fractionated extracts in the search for new drugs. In this respect there is a role for simple biological assays (e.g. brine shrimp toxicity), which can be carried out by the phytochemist without the specialist procedures used by pharmacologist. Spectrometric methods, particularly in conjunction with chromatography, are finding increasing use.

Spectroscopic analysis
The electromagnetic vibrations utilized in spectroscopic analysis can be roughly divided, according to wavelength, into the ultraviolet (185-380 nm), the visible (380-780 nm), the near-infrared (780-3000 nm) and the infrared (3-40 μm) regions. In spectroscopic analysis we are concerned with the capacity of certain molecules to absorb vibrations at specific wavelengths. Thus, the butenolide side-chain of cardiac glycosides is responsible for a strong absorption at 215-220 nm, the conjugated double bonds of lycopene (a pigment of tomatoes and other fruits) gave rise to the absorption of light at a wavelength of 470 nm, thus giving a red colour, and the carbonyl group of ketones, arboxylic acids and esters is responsible for a strong absorption in the infrared at about 5.7-6.1 μm. In the ultraviolet and visible regions the characteristic absorption spectrum of a molecule is produced by changes in the electronic energy levels associated
with various chromophoric groups within the molecule. These changes involve the absorption of relatively high amounts of energy (in precise quanta), and they are also accompanied by changes in vibrational and rotational energy changes within the molecule. The result is a band absorption spectrum showing no sharp defined peaks. By comparison, the absorption spectrum of molecule in the infrared region is much more complex, because here the energies involved are too small to bring about electronic transitions but large enough to produce numerous vibrational and associated rotational energy changes. Each of these changes is associated with a characteristic wavelength and the spectrum shows a much finer structure than in the visible or ultraviolet regions. The infrared spectrum of a molecule can be divided into the 'fingerprint' region (7-11 μm), which is characteristic of the molecule under examination but in which it is difficult to assign peaks to specific vibrations, and the remainder of the spectrum, in which many functional groups can be recognized. The BP employs the comparison of infrared spectra of phytochemicals (pilocarpine, physostigmine, etc.) with European Pharmacopoeia Chemical Reference substances (EPCRS) as a test of identity.

The BP uses ultraviolet absorption characteristics as standards for benzylpenicillin, lanatoside C and a number of alkaloids—for example, morphine, reserpine, cocaine, colchicines and tubocurarine chloride.

If light of a particular wavelength is passed through a solution of a substance, the transmission \( T = \frac{I}{I_0} \), where \( I_0 \) is a measure of the light reaching the detector (a photoelectric cell) when solvent alone is used in the light path and \( I \) is the light reaching the detector when a solution of the substance under investigation is examined. \( T \) is measured in experiments but the most useful value is \( \log_{10} \left( \frac{I_0}{I} \right) \), the decimal optical density or simply the optical density \( (E) \). The optical density, but other transmission, is proportional to the number of absorbing units in the light-path. For solutions this is Beer's law. The absorption spectrum of a pure substance under defined conditions of solvent and temperature is a set of values of \( E \) observed at different wavelengths in solution of unit concentration (1 mol⁻¹) when the thickness of the layer traversed by the light is 1 cm. Alternatively, another solution of known strength may be used. For example, for a 1% solution with a
layer thickness of 1 cm the optical density is indicated by $E_{1cm}^{1%}$. Such absorption spectra are valuable for the identification, determination of the structure and purity and analysis of compounds. Some substances will absorb ultraviolet light of certain wavelength and during the period of excitement re-emit light of longer wavelength and often in the visible region. This is fluorescence, and the fluorescence spectrum is characteristic for those substances, which exhibit the phenomenon. The applications of fluorescence analysis are discussed below.

For the quantitative evaluation of a substance, a standard curve is first prepared by measuring the optical densities of a series of standard solutions of the pure compound by the use of light of a suitable wavelength, usually that at which the compound gives an absorption maximum. The solutions must be sufficiently dilute to obey Beer's law. The optical density of the solution to be evaluated is then determined and its composition ascertained from the standard curve. Individual components of a mixture can be determined by ultraviolet absorption maxima. Thus, for strychnine and brucine the reported $E_{1cm}^{1%}$ values at the wavelengths ($\lambda$) indicated are:

Strychnine 322 at 262 nm and 5.16 at 300 nm
Brucine 312 at 262 nm and 216 at 300 nm

By measurement of the extinctions of the mixed alkaloid solution at the above wavelengths, a two-point spectrophotometric assay is available for the determination of both alkaloids. This method, official in the BP (1980) for the assay of nux vomica seeds and preparations, replaced the older, chemical method. A similar type of assay is employed by the EP for quinine-type alkaloids and cinchonine-type alkaloids in cinchona bark; measurements are made at 316 and 348 nm. Occasionally it is useful to examine the ultraviolet spectrum of a more complex mixture; thus, the USP includes an ultraviolet absorbance test for the absence of foreign oils of lemon and orange and the BP an extinction limit test between 268 and 270 nm for castor oil.

In most cases it is essential that no interfering substances are present during the measurements; these can be particularly troublesome in the ultraviolet region, particularly with the materials extracted from thin-layer and paper chromatograms.
For this reason, if pure solutions are not available for analysis, some form of
colourimetric analysis is often preferable, particularly if the reaction used to
produce the colour is highly specific for the compound under consideration.
Colorimetric analyses can be carried out with a suitable spectrophotometer-most
instruments which operate in the ultraviolet range also have facilities in which
suitable filters are used to select the correct wavelength of light required are quite
satisfactory for most purposes. In these instruments a simple light-source is used
and, between the lamp and the solution to be analyzed, a filter is placed which
transmits that range of wavelengths absorbed by the compound under test (i.e., a
colour complimentary to that of the solution under test). The transmitted light is
recorded by a photoelectric cell and the composition of the solution determined by
a photoelectric cell and the composition of the solution determined by reference to
a standard curve.

Characteristic absorption maxima from the more complex infrared spectra can also
be utilized in quantitative analysis in the same way as ultraviolet and visible
absorptions. Mixtures of substances can also be evaluated; thus, by
measurements at 9.80, 9.15 and 9.00 μm it is possible to evaluate separately the
25β- and 25α-epimeric steroidal sapogenins present in plant extracts.

Fluorescence analysis
Many substances, e.g., quinine in solution in dilute Sulfuric acid, when suitably
illuminated, emit light of a different wavelength or colour from that which falls on
them. This emitted light (fluorescence) ceases when the exciting light is removed.
Analytical tests based on fluorescence in daylight are not much used, as they are
usually unreliable, owing to the weakness of the fluorescence effect. An exception
to this is the well-known umbelliferous test, which can be applied to ammoniacum,
galbanum and sclereids. A strongly fluorescent solution of umbelliferone can be
prepared by boiling galbanum with acid and filtering into an excess of alcoholic
ammonia. Other fluorescent solutions are those of quinine (in dilute acid), aesculin
(by infusion horse chestnut bark), chlorophyll (from nettle or parsley leaves), β-
naphthol (dissolved in alkali) and aqueous solutions of the dyes eosin and
fluorescein. A very important generalization made by Stokes in 1825 stated that 'in
fluorescence the fluorescent light is always of greater wavelength than the excited light'. Light rich in short wavelength is very active in producing fluorescence and for this reason strong ultraviolet light (such as can be obtained from a tungsten arc or mercury vapour lamp) produces fluorescence in many substances, which do not visibly fluoresce in daylight. Fluorescence lamps are usually fitted with a suitable filter, which eliminates visible radiation from the lamp and transmits ultraviolet radiation of the desired wavelength. Convenient long- and short-wave ultraviolet hand lamps are available for chromatographic observations; it is most important that the eyes are properly protected in the presence of ultraviolet radiation.

For examination, solids may be placed directly under the lamp, whereas liquids may be examined in non-fluorescent dishes or test tubes or after spotting on to filter paper. Many alkaloids in the solid-state show distinct colours, e.g., aconite (light blue), berberine (yellow) and emetine (orange). Pieces of cinchona bark when placed under the lamp show a number of luminous yellow patches and a few light blue ones. If the inner surface of the bark is touched with dilute sulfuric acid the spot immediately turns blue. Ipecacuanha root has a brightly luminous appearance wherever the wood is exposed, while the wood of hydrastis rhizome shines golden yellow. Areca nuts when cut show a light blue endosperm. Slices of calumba appear intensely yellow, with the cambium and phloem distinguished by their dark-green colour. Precipitated and prepared chalks may readily be distinguished from one another.

Most oils, fats and waxes show some fluorescence when examined in filtered ultraviolet light. In general, fixed oils and fats fluoresce least, waxes more strongly and mineral oils (paraffins) most of all.

Powders may be examined macroscopically as above or microscopically by means of a fluorescence microscope. In connection with powdered drugs may be mentioned the detection of ergot in flour, of cocoa shells in powdered cocoa, and rumex in powdered gentian. Different varieties of rhubarb may be distinguished from one another. The BP 1973 included a fluorescence test on the entire or powdered drug for the detection of rhapontic rhubarb but this is now replaced by a TLC test.
The location of separated compounds on paper and thin-layer chromatograms by
the use of ultraviolet light has been extensively employed. With plant extracts it is
often worthwhile to examine the chromatogram in ultraviolet light even if the
constituents that one is investigating are not themselves fluorescent. In this way
the presence of fluorescent impurities may be revealed which, if otherwise
undetected, could interfere with a subsequent absorption analysis. Sometimes
fluorescence-quenching can be employed to locate non-fluorescent substances on
thin-layer chromatograms. For this, an ultraviolet fluorescent background is
produced by the incorporation of a small amount of inorganic fluorescent material
into the thin layer. The separated substances cause a local quenching of the
background fluorescent and the therefore appear as dark spots on a coloured
background.

Quantitative fluorescence analysis. This technique utilizes the fluorescence
produced by a compound in ultraviolet light for quantitative evaluation. The
instrument employed is a fluorimeter and consists of a suitable ultraviolet source
and a photoelectric cell to measure the intensity of the emitted fluorescent light.
Within certain limits of concentration the intensity of the fluorescence for a given
material is related to its concentration. It is usual to select a narrow range of
wavelength for measurement by inserting a filter between the fluorescing solution
and the photoelectric cell. The concentration of a substance in solution is obtained
by reference to a standard curve prepared by subjecting standard solution to the
fluorimetric procedure. With plant extracts it is important to ascertain that (1) the
substance being determined is the only one in the solution producing a
fluorescence at the measured wavelength and (2) there are no substances in the
solution which absorb light at the wavelength of the fluorescence. Refined
instruments are now available in which the fluorescence spectrum is automatically
analysed and in which the wavelength of the incident radiation can also be varied.
Quinine can be conveniently assayed by the measurement of the blue
fluorescence (366 nm) produced by irradiation of the alkaloid in a dilute
hydrochloric acid solution at about 450 nm. The method can be used for the assay
of quinine in the presence of other alkaloids (e.g., strychnine). Alexandrian senna
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has been assayed by the measurement of the fluorescence produced in the Borntrager reaction under specified conditions. The hydrastine content of hydrastis root may be determined by oxidizing an extract of the drug with nitric acid and measuring the fluorescence of the hydrastinine; by this method berberine and canadine, other alkaloids of hydrastis, are excluded from the assay. Emetine and papaverine may be determined fluorimetrically after oxidation with acid permangnate and noscapine after oxidation with persulphate. Fluorimetric methods have also been published for the estimation of the ergot and rauwolfia alkaloids, umbelliferone, aflatoxin and a number of drugs in body fluids (Evans c, 1997).

Biological evaluation

The pharmacological activity of certain drugs has been applied to their evaluation and standardization. Assays on living animals as well as on intact or excised organs often indicate the strength of the drug or its preparations. Since living organisms are used, the assays are called biologic assays or bioassays. Although the determination of pharmacological activity is not strictly within the province of Pharmacognosy, a brief summary is presented here of the organisms used and the methods employed in the biologic evaluation of certain drugs.

1. Bacteria. Such as Salmonella typhi and Staphylococcus aureus, are a means of determining the phenol coefficient or antiseptic value of certain drugs in accordance with the U.S Food and Drug Administration (FDA) method. A definite quantity of a twenty-four-hour broth culture of bacteria is brought into contact with the specified dilution of the antiseptic agent for a definite length of time. If the bacteria are killed during the test, the antiseptic agent meets the required standard.

2. Microbiological methods employing living bacteria, yeasts, and molds were formerly employed for assaying many vitamins. Lactobacillus casei has been used in the assay of riboflavin, biotin, and pantothenic acid; Lactobacillus arabinosus in the assay of nicotinic acid; lactobacillus plantarum in the assay of calcium pantothenate; Latobacillus leichmannii in the activity assay of vitamin B\textsubscript{12} (Cyanocobalamin). A yeast, Saccharomuces cerevisix, was useful in the thiamin assay while a mold, Neurospora sitophila, was suitable in the pyridoxine assay. With the exception of the vitamin B\textsubscript{12} Assay described in the USP XVI which
employes *Lactobacillus leichmanii* as the test organism, most of the other microbiological vitamin assays have been replaced by spectrophotometric or fluorometric methods. However, microbiological assays are presently used to determine the activity of the antibiotic drugs. *Micrococcus flavus, Sarcinaflava, Sarcina lutea, Staphyloccus areus, Klebsiella pneumoniae, Staphylococcus albus, Saccharomyces cerevisix* are some of the microorganisms used in the assay of antibiotics. Mice are the test animals utilized in the safety test for Rabies Vaccine, Diphtheria Toxoid, and other biologics. The rat "line" test is an alternative assay method, which may be employed for the assay of vitamin D preparations. Rats are also used in the assay of pressor substances (Vasopressin Injection).

3. Living microscopic animal organisms, such as *Daphnia* species, which are transparent in a microscopic mount, can be observed while being treated with specific drugs, *i.e.*, Cascara Sagrada, Digitalis. A dilute extract of the drug is added to the mounting fluid, it is absorbed to some extent by the minute animal, and the pharmacological effects are noted. With Cascara Sagrada a marked cathartic action with rapid evacuation of the alimentary tract may be observed; with Digitalis the effect on the heart may be vividly portrayed. By quantitative methods the quantity of the drug can be gauged. The Craw unit, formerly used to denote the activity of Veratrum Viride, was based on the cardiac depressant effects of the drug on *Daphnia magna*.

4. Goldfish were proposed for the standardization of Digitalis and similar cardiac stimulant drugs. The fish were placed in known high dilutions of the drug, kept at a standard temperature, and were transferred from one dilution to another of slightly greater concentration until a dilution was reached that killed the fish within a certain length of time.

5. Frogs were the official test animals for standardizing Digitalis and other members of this group of drugs until 1947. The properly prepared dilution of the extract of the drug was compared with a similar preparation of standard Digitalis. These dilutions in known quantity were injected into the ventral lymph sacs of frogs and a comparison was made of the exposed heats. The end-point was stoppage of the heart in systole.
6. Rats or mice suffering from avitaminose induced as a result of experimental feeding with vitamin-deficient diets frequently serve as test animals in the biological standardization of vitamins. Results are compared with normal, healthy animals. The rat "line test" is employed for the assay of vitamin D preparations.

7. Guinea pigs are used to test the toxicity and the antigenicity of certain biological products such as Diagnostic Diphtheria Toxin and Tetanus Toxoid. They are also employed in the USP test to determine oxytocic activity. Aconite was formerly tested on guinea pigs by injecting a diluted tincture subcutaneously. The time of death was noted and a comparison made with a suitably diluted solution of aconite.

8. Posterior Pituitary Injection, formerly standardized by noting the degree of contraction of isolated guinea pig uterus muscle, is now assayed on young adult domestic chickens. The standard preparation and the assay preparation are injected intravenously by cannulation into the exposed ischiatic artery and the corresponding changes in blood pressure are noted. Ergot was formerly standardized by noting the change in colour from red to blue of the combs of white leghorn cocks after the animals had been injected with various dilutions of extracts of the drug. A standard solution of ergotoxine ethanesulfonate similarly injected into another group of animals served as the control. This method is still employed by some pharmaceutical companies to correlate results with the newer chemical assay.

9. The present pharmacopoeial method of assaying Digitalis utilizes pigeons. A definite quantity of a known dilution of the drug is transfused through the alar vein into the blood stream of the pigeon and the lethal effects are noted and compared with a similar dilution of the standard Digitalis preparation.

10. Cats were formerly employed in standardization of Digitalis in a manner similar to that described for pigeons. They are currently employed in the USP Depressor substances Test. The cat's eye test is of present interest in evaluating mydriatic drugs such as atropine. A highly dilute aqueous solution is dropped into the cat's eye and the degree of mydriasis is noted.
11. Curare alkaloids, particularly d-Tubocurarine Chloride, are injected into rabbits to ascertain the amount necessary to produce a "head-drop" indicative of muscle relaxation. Rabbits are invaluable in determining the presence of pyrogens in parenteral solution of the antibiotics.

12. Dogs are the test animals now utilized in assaying Veratrum Viride preparations. The carotid sinus reflex unit (CSR Unit) is the amount necessary to elicit the carotid sinus blockage in an anesthetized dog. Thirteen CSR units equal the activity of one Craw unit. In the past Cannabis was standardized on dogs by noting the degree of incoordination in the dog after administration of a known quantity of an extract of the drug.

13. Humans have long offered a valuable means of noting the activity of drugs. Recently, drugs such as Liver, stomach, and other preparations suitable for the oral treatment of pernicious anemia have been officially standardized on humans. A series of different animals may be necessary for the complete evaluation of one drug or preparation; for instance, Corticotropin Injection requires adult cats for the vasopressin activity and rats for the assay.

Before a new product is introduced on the market it must be proven both safe and effective in preliminary pharmacological tests on animals and in quantitative clinical tests on humans (Claus Edward P b, 1961).

**Immonoassays**

Such assays are highly sensitive and usually very specific and have been developed as a powerful analytical tool for the quantitative determination of many compounds in biological fluids.

**Radiimmunoassays (RIA).** The assay depends on the highly specific reaction of antibodies to certain antigens. There are various modifications of the technique and it is the saturation method that has been developed for phytoanalysis. Usually the relatively small molecules (below MW 1000) constituting the secondary plant metabolites are not involved in such immuno responses but when bound covalently to protein carriers, as haptens, they do become immunogenic. (Haptens are molecules, which combine with antibodies but do not stimulate their production unless linked to a carrier molecule). If such a hapten is prepared in the labeled...
condition (e.g., $^3$H- or $^{125}$I labelled) with a known specific activity, mixed with an unknown amount of unlabelled hapten and added to a limited amount of antibody in the form of a serum, then there will be competition between the labeled and unlabelled antigen for the restricted number of binding sites available. This results in some bound and unbound hapten; these can be separated and a determination of the radioactivity in either fraction, with reference to standard curve, enables the amount of unlabelled antigen to be calculated. The antiserum is raised in suitable animals (e.g., rabbits). Following the rapid development of RIA procedures in clinical analyses, and largely owing to the work of Weiler, Zenk and colleagues in Germany since 1976, the method has been satisfactorily applied to a range of plant medicine.

RIA has the advantage that only small amounts of plant material are required; it is usually specific for a single or small range of metabolites; relatively crude, unprocessed plant extracts can usually be used; and the process can be mechanized. Thus, it is an efficient tool for the screening of large numbers of plants, some 200-800 specimens being assayed in 1 day. For the application of the method to the selection of highly yielding stains of *Digitalis* and *Solanum*. With herbarium material, assays can be performed on quantities of sample ranging from 0.5 mg to a few milligrams and in the examination of individual plants, structures as small as anther filaments (e.g., in digitalis) can be accommodated.

Possible disadvantages of the method are the considerable specialized expertise required to set up the assays and the possibility of cross-reaction with components of the plant extract other than those under investigation. Problems arising from the latter need to be ascertained before the assay. The RIA for hyoscine, e.g., is highly specific but norhyoscine will react even more strongly; the cross-reaction with 6-hydroxyhyoscyamine is considerably less, and with hyoscyamine, very much less. Similarly, in the assay for solasodine, tomatidine, if present, will cross-react.

**Enzyme-linked immunosorbent assays (ELISA).**

In this method, competition for an immobilized antibody takes place with a modified form of the compound under analysis that has an enzyme bound to it. Release of the compound-enzyme from the binding site and determination of the enzyme
activity enables the original solution to be quantified. As with RIA's ther method is very sensitive; thus for the pyrrolizidine alkaloid retronecine it can be measured in the parts per billion range and one sclerotium of ergot is detectable in 20 kg of wheat (Evans c 2002).

**Chromatography study of drugs**

In recent years the study of chromatography has become prominent as a means of separating and analyzing organic and inorganic materials. Large amounts as well as microquantities may be employed, and the analysis may be conducted either qualitatively or quantitatively. Chromatographic methods often prove more effective than other means of separation and identification of solutions of drugs principles. Such techniques are finding wide application in research in Pharmacognosy involving the determination of identity and purity of drugs and derivatives of natural origin as well as in pharmaceutic research and manufacturing processes.

**Chromatography** is defined as a method of analysis in which the flow of a solvent (liquid or gaseous) promotes the separation of substances by differential migration from a narrow initial zone in a porous sorptive medium. The two major subdivisions are solution chromatography and gas chromatography.

- Column chromatography, paper chromatography, and thin-layer chromatography are forms of solution chromatography; the terms relate to the type of sorption system. Both are further divided according to the manner in which the separations occur.

For practical purposes, chromatography is defined as a procedure by which drug principles as well as inert materials occurring in drugs and pharmaceutic preparations are separated by fractional extraction, or adsorption, or ion exchange, or other means on a porous solid by the use of a flowing solvent. The materials separated in this manner can be identified by analytical methods.

Four types of chromatography are generally employed in the USP assay and tests; column, paper, thin-layer, and gas. High-pressure liquid chromatography is a type of column chromatography that requires specialized apparatus. In Pharmacognosy, column chromatography is used where large quantities of the
material to be tested are available. However paper chromatography and thin-layer chromatography are preferred for purposes of identification because of their selectivity, convenience, and adaptability to small quantities of material.

Column chromatography

Column chromatography has been defined as "uniform percolation of a fluid through a column of more or less finely divided substance which selectively retards certain components of the fluid."

In the column method, many adsorbents have been used such as sucrose, talc, calcium or sodium carbonate, activated alumina, silicic acid, and fuller's earth. Solvents (called eluants) include petroleum ether, carbon tetrachloride, carbon disulfide, ether, acetone, alcohol, water, and mixtures of acids and bases in water, alcohol, and pyridine. The adsorbent is packed uniformly into a suitable glass or quartz tube, and the solution of the drug or substance in a small amount of eluant is passed through the column. This method is sometimes known as adsorption chromatography. According to their adsorption coefficients, the various constituents in the drug or substance are removed from the solution and are adsorbed in transverse bands at varying distances from the top of the column. Each component progresses downward at a characteristic rate. Upon drying, the resulting column consists of a series of bands of separated components and is known as the chromatogram. This is removed from the tube as a single column and then divided into the separated bands for individual analysis.

A modification of the column method known as a flowing chromatogram depends upon a larger quantity of the solution to be eluted being introduced into the tube. The liquid is allowed to flow continuously through the column until the separated substance appears in the discharged solution (eluate). It is then identified either as the solution or as the evaporated material.

Still another modification is known as partition chromatography. In this process, two immiscible liquids are used: one representing an immobile phase, the other a mobile phase. The immobile phase is adsorbed on a solid adsorbent, thereby presenting a large surface area to the flowing solvent or mobile phase. The large number of liquid-liquid contacts thus the substance and by a reference substance
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is known as the Rf value. The Rf values for many compounds have been established and have been tabulated for reference by laboratory workers. The resulting spots or pattern of distribution on the paper is called the chromatogram. By a study of the design (colour, size, shape, visibility under white or ultraviolet light) of the markings on the chromatogram and by comparing the similar chromatograms of the known achieved promote a highly efficient separation process.

Relatively recent and advances in chromatographic systems which future high inlet pressures and high sensitivity are known variously as high-pressure, high-speed, high-performance, high-efficiency, or high-sensitivity liquid chromatography. In all cases, the technology employed is based upon the use of small-bore (2-5 mm) columns filled with small practical (3-5 micro m) pickings, which permit a rapid equilibrium between the mobile and stationary phases. The technique also requires high-pressure pumping system capable of exerting pressures up to 5000 pounds per square inch on the mobile phase in order to achieve flow rates through the column of about 1 ml per minute. Since small samples (<20 micro g) are often employed, sensitive detectors are mandatory. This type of chromatography effects high-speed separations comparable to those yielded by gas chromatography but has the advantage of permitting separation of nonvolatile or heat-labile materials. Since many drugs fall into these categories, high-pressure liquid chromatography has become an important analytical method in Pharmacognosy.

Paper chromatography

Because of the simplicity of the apparatus and the ease with which a large number of analyses can be conducted simultaneously, paper chromatography is more popular than column chromatography as a general screening procedure. Two techniques may be employed: the descending and the ascending, depending upon whether the mobile phase moves downwards or upwards on the chromatographic paper. The ratio of the distance traveled on the paper sheets by the test substance to the distance traveled by the front of the mobile phase, from the point of application of the test substance, is termed as the Rf value of the
PhD Thesis

substance. The ratio between the distance traveled by the drug or substance, identification is indicated. Verification may be accomplished by treating the spots with characteristics reagents. A modification of the paper chromatographic method is the two-dimensional separation of a sample by using another solvent on the same chromatogram. Another chamber is prepared, the chromatogram is turned at right angle, and the entire process is repeated using the second solvent.

Thin-layer chromatography

Thin-layer chromatography is a modification of the above types of chromatography that is particularly adaptable to the analysis of small amounts of the substance. The method was originated in 1956 by a German pharmacognist, Professor Egon Stahal. It is characterized by the application of dry, finely powdered adsorbent in a thin, uniform layer to a glass plate. This coated plate is comparable to an open chromatographic column, and the separation of the components in the test material is based on adsorption, partition, or a combination of both, depending on the adsorbents and the solvents used. Positive identification of a component is made by comparing the resulting spots of identical Rf value and of equal magnitude representing the unknown sample and the known reference material in the same manner as for paper chromatography.

For two-dimensional thin-layer chromatography, the glass plate is turned at right angle and again subjected to the same conditions as previously in another chamber and with a different solvent.

Gas chromatography

Gas chromatography is a specialized chromatographic method wherein the moving phase is gas. Two types are in use: gas-liquid chromatography and gas-solid chromatography, the difference being the type of stationary phase over which the gas flows. In gas-liquid chromatography, the immobile liquid phase consists of a thin film adhering to an inert, finely divided, solid support that does not retard the drug mixture from passing through. Chromatographic siliceous earth, crushed firebrick, or even the inner surface of a small diameter tube may be employed as the solid support for the stationary phase. In gas-solid chromatography, the immobile phase is represented by an active solid adsorbent, such as alumina,
silica gel, or carbon. When the substance to be analyzed is vaporized and introduced into the moving gas phase, it is carried into the column and is distributed between the gas and the stationary phase (liquid or solid) on which it will be dissolved or adsorbed. The efficiency of this method depends on a number of factors: the specific solute, the type of liquid solvent or solid adsorbent, the total amount of solvent or adsorbent, the temperature at which the procedure is conducted, and the rate of gas flow. The material to be chromatographed must be subject to being vaporized or finely divided in the form of a mist or spray.

The gas chromatographic apparatus must be utilized carefully to obtain precise results. A recording device is generally used in conjunction with it to obtain a permanent record of the results.

As stated earlier, paper chromatography and thin-layer chromatography are the methods of choice of investigators who are screening large numbers of drug plants from all parts of the world for active principles. By this means of separation and identification, alkaloids can readily be determined in alcoholic or aqueous extracts of plant materials. Similarly, the presence of various other types of constituents can also be substantiated.

Counter-current methods and electrophoretic methods of analysis are also utilized in drug analysis. Since these methods are based on essentially the same fundamental principles as chromatographic techniques, but are ordinarily less efficient and somewhat cumbersome to apply, they are usually reserved for highly specialized analytical applications. As a consequence, they are not discussed further here (Claus Edward P c, 1961).
REFERENCES


MATERIALS AND METHODS

The roots of *Aralia cachemirica* (araliaceae) and whole plant of *Swertia tetragona* (Gentianaceae) were collected from Aharbal region of Kashmir (J&K) in presence of Prof. A.R. Naqshi, taxonomist, dept. of taxonomy, university of Kashmir. The collected material were cleaned and dried in shade at the collection site and cut into small pieces. Herbariums of *Aralia cachemirica* and *Swertia tetragona* were also prepared and designated as AR-1 and SW-1 respectively and the same were deposited in the dept. of Pharmaceutical sciences, university of Kashmir.

PARAMETERS FOR STANDARDIZATION

The following parameters were taken:

1) Macroscopic characters
2) Microscopic characters
3) Successive extractions
   a) Petroleum ether
   b) Benzene
   c) Chloroform
   d) Ethyl Acetate
   e) Methanol
4) Cold extractions
   a) Petroleum ether
   b) Benzene
   c) Chloroform
   d) Ethyl Acetate
   e) Methanol
5) Water soluble extractive
6) Alcohol soluble extractive
7) Ash value
   a) Total ash
   b) Acid insoluble ash
   c) Water soluble ash
8) Foreign matter
9) Fluorescent analysis
10) Powdered drug reaction with different chemicals
11) Phytochemical investigation

**OBSERVATIONS**

*Aralia cachemirica*

1) **Macroscopic Characters**
   - Shape: cylindrical
   - Size: 3.8-5 cm long
   - Surface: rough
   - Texture: rough
   - Colour: yellowish brown
   - Odour: aromatic and characteristic
   - Taste: aromatic and pungent

2) **Microscopic characters (Powder Characteristics)**
   A pale brownish yellow powder with a faint, characteristic odour and a mucilaginous, slightly acrid taste.
   
   The diagnostic characters are:
   
   a) Trachieds and trachiedal vessels, which occur singly but more commonly are found in groups. They have moderately thickened lignified walls and numerous bordered pits. The vessels have somewhat oblique end walls and are usually larger than the trachiedal vessels. Fragments of pitted vessels also occur in the powder.
   
   b) The abundant fragments of reddish brown cork composed of 3-4 layers of thick walled cells which in surface view are polygonal and more or less isodiametric. They are strongly lignified. Occasionally portions of phelloderm is attached to the cork. Cells of phelloderm are parenchymatous.
   
   c) Oleoresin droplets, yellowish in colour are seen scattered throughout the powder.
Aralia cachemirica

Aralia cachemirica (Crude drug)
T.S. Of *A.cachemirica* root showing cork cells, phelloderm and oleo resin cells.

T.S. Of *A.cachemirica* showing oleoresin ducts and xylem vessels
Trechiedal vessels of *A. Cachemirica*

Cork cells of *A. Cachemirica* root in surface view
d) Group of fibres are found occasionally. The walls of these individual fibres are non-lignified, moderately thickened and have slit shaped pits.

e) The calcium oxalate crystals are found scattered and in small groups in some parenchymatous cells. They are irregularly prismatic and show considerable variation in size.

f) Starch granules, both simple and compound are found in moderate quantity. Individual starch granules are spherical and have a well marked hilum in the form of simple split.

Table-2.1 Hot extractive (successive) (%w/w) values of *Aralia cachemirica*

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Petroleum ether</th>
<th>Chloroform</th>
<th>Acetone</th>
<th>Methanol</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10.105</td>
<td>3.334</td>
<td>4.588</td>
<td>3.386</td>
<td>12.342</td>
</tr>
<tr>
<td>Mean</td>
<td>10.015</td>
<td>3.432</td>
<td>4.224</td>
<td>3.385</td>
<td>13.794</td>
</tr>
</tbody>
</table>

Table-2.2. Hot extractive (individually) extractive (%w/w) values of *Aralia cachemirica*

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Petroleum ether</th>
<th>Chloroform</th>
<th>Acetone</th>
<th>Methanol</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10.105</td>
<td>4.676</td>
<td>7.348</td>
<td>14.665</td>
<td>34.434</td>
</tr>
<tr>
<td>2</td>
<td>9.620</td>
<td>4.712</td>
<td>7.420</td>
<td>14.865</td>
<td>34.932</td>
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<tr>
<td>3</td>
<td>10.320</td>
<td>4.672</td>
<td>7.460</td>
<td>13.876</td>
<td>34.467</td>
</tr>
<tr>
<td>Mean</td>
<td>10.015</td>
<td>4.686</td>
<td>7.409</td>
<td>14.468</td>
<td>34.611</td>
</tr>
</tbody>
</table>
Table-2.3. Cold extractive (individually) extractive (% w/w) values of *Aralia cachemirica*

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Petroleum ether</th>
<th>Chloroform</th>
<th>Acetone</th>
<th>Methanol</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.574</td>
<td>0.432</td>
<td>0.345</td>
<td>0.380</td>
<td>1.753</td>
</tr>
<tr>
<td>2</td>
<td>0.563</td>
<td>0.455</td>
<td>0.342</td>
<td>0.365</td>
<td>1.864</td>
</tr>
<tr>
<td>3</td>
<td>0.521</td>
<td>0.412</td>
<td>0.336</td>
<td>0.324</td>
<td>1.748</td>
</tr>
<tr>
<td>Mean</td>
<td>0.552</td>
<td>0.433</td>
<td>0.341</td>
<td>0.356</td>
<td>1.788</td>
</tr>
</tbody>
</table>

Table-2.4. Ash values of *Aralia cachemirica*

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Total Ash</th>
<th>Acid Insoluble Ash</th>
<th>Water Soluble Ash</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.322</td>
<td>1.802</td>
<td>1.224</td>
</tr>
<tr>
<td>2</td>
<td>4.010</td>
<td>1.690</td>
<td>1.231</td>
</tr>
<tr>
<td>3</td>
<td>4.166</td>
<td>1.746</td>
<td>1.227</td>
</tr>
<tr>
<td>4</td>
<td>4.245</td>
<td>1.864</td>
<td>1.341</td>
</tr>
<tr>
<td>5</td>
<td>4.545</td>
<td>1.743</td>
<td>1.289</td>
</tr>
<tr>
<td>Mean</td>
<td>4.257</td>
<td>1.769</td>
<td>1.262</td>
</tr>
</tbody>
</table>

Table-2.5. Foreign matter

<table>
<thead>
<tr>
<th>Wt. of Crude Drug (g)</th>
<th>Wt. of drug after removal of foreign matter (g)</th>
<th>Wt. of foreign matter (g)</th>
<th>Foreign matter (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>198.5</td>
<td>1.50</td>
<td>0.75</td>
</tr>
<tr>
<td>200</td>
<td>199.0</td>
<td>1.00</td>
<td>0.50</td>
</tr>
<tr>
<td>200</td>
<td>198.4</td>
<td>1.60</td>
<td>0.80</td>
</tr>
<tr>
<td>Mean</td>
<td>198.6</td>
<td>1.36</td>
<td>0.68</td>
</tr>
</tbody>
</table>
Table-2.6: Determination of resin, tannin and fat content in *Aralia cachemirica*

<table>
<thead>
<tr>
<th>S.NO.</th>
<th>Resin</th>
<th>Tannin</th>
<th>Fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.64</td>
<td>0.87</td>
<td>2.88</td>
</tr>
<tr>
<td>2</td>
<td>8.78</td>
<td>0.74</td>
<td>2.68</td>
</tr>
<tr>
<td>3</td>
<td>9.27</td>
<td>0.68</td>
<td>2.65</td>
</tr>
<tr>
<td>Mean</td>
<td>9.23</td>
<td>0.76</td>
<td>2.73</td>
</tr>
</tbody>
</table>

Table-2.7: Fluorescent analysis

<table>
<thead>
<tr>
<th>Treatment of the Drug Powder</th>
<th>OBSERVATION UNDER</th>
<th>Ordinary Light</th>
<th>UV (254 nm)</th>
<th>UV (366 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry powder as such</td>
<td></td>
<td>Buff colour</td>
<td>Greyish green</td>
<td>Greyish green</td>
</tr>
<tr>
<td>Conc. H₂SO₄</td>
<td></td>
<td>Dark brown</td>
<td>Dark green</td>
<td>Black</td>
</tr>
<tr>
<td>Conc. H₂SO₄ + water</td>
<td></td>
<td>Dark brown</td>
<td>Dark green</td>
<td>Dark green</td>
</tr>
<tr>
<td>Conc. HCl</td>
<td></td>
<td>Light yellow</td>
<td>Lemon green*</td>
<td>Green</td>
</tr>
<tr>
<td>Conc. HCl + water</td>
<td></td>
<td>Light yellow</td>
<td>Light green</td>
<td>Green</td>
</tr>
<tr>
<td>Conc. HNO₃</td>
<td></td>
<td>Light brown</td>
<td>Light green</td>
<td>Yellowish green</td>
</tr>
<tr>
<td>Conc. HNO₃ + water</td>
<td></td>
<td>Light yellow</td>
<td>Light green</td>
<td>Light green</td>
</tr>
<tr>
<td>Acetic Acid</td>
<td></td>
<td>Cream yellow</td>
<td>Light green</td>
<td>Parrot green</td>
</tr>
<tr>
<td>Methanol</td>
<td></td>
<td>Faint yellow</td>
<td>Faint green</td>
<td>Greyish white*</td>
</tr>
<tr>
<td>Ethanol</td>
<td></td>
<td>Faint yellow</td>
<td>Faint green</td>
<td>Greyish white*</td>
</tr>
<tr>
<td>Chloroform</td>
<td></td>
<td>colourless</td>
<td>Greyish green</td>
<td>Light green</td>
</tr>
<tr>
<td>Petroleum ether</td>
<td></td>
<td>Light green</td>
<td>colourless</td>
<td>pink*</td>
</tr>
<tr>
<td>Dist. Water</td>
<td></td>
<td>Light yellow</td>
<td>Light green</td>
<td>Parrot green*</td>
</tr>
<tr>
<td>10% NaOH</td>
<td></td>
<td>Yellowish brown</td>
<td>Dark green</td>
<td>Dark green*</td>
</tr>
<tr>
<td>5% Iodine</td>
<td></td>
<td>Blackish brown</td>
<td>black</td>
<td>Black</td>
</tr>
<tr>
<td>Picric Acid</td>
<td></td>
<td>yellow</td>
<td>Lemon green</td>
<td>Dark green</td>
</tr>
<tr>
<td>FeCl₃ solution</td>
<td></td>
<td>brown</td>
<td>Light green</td>
<td>Blackish green</td>
</tr>
<tr>
<td>NH₃ Solution</td>
<td></td>
<td>Light yellow</td>
<td>Light green</td>
<td>Light green</td>
</tr>
</tbody>
</table>

*Shows prominent fluorescence

Table-2.7: Powdered drug reaction with different chemicals
<table>
<thead>
<tr>
<th>Treatment with chemicals</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc. Hydrochloric acid</td>
<td>Light Yellow</td>
</tr>
<tr>
<td>Conc. Nitric acid</td>
<td>Light brown</td>
</tr>
<tr>
<td>Conc. Sulphuric acid</td>
<td>Dark brown</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>Cream colour</td>
</tr>
<tr>
<td>Picric acid</td>
<td>Yellow</td>
</tr>
<tr>
<td>Iodine solution 5%</td>
<td>Blackish brown</td>
</tr>
<tr>
<td>5% Sodium hydroxide</td>
<td>Yellowish brown</td>
</tr>
</tbody>
</table>

Table-2.8. Phytochemical investigations

<table>
<thead>
<tr>
<th>Tests</th>
<th>Pet. Ether Extract</th>
<th>Benzen e Extract</th>
<th>Chloroform Extract</th>
<th>Ethyl acetate Extract</th>
<th>Methanol Extract</th>
<th>Aqueous Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Glycosides</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tannins</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Proteins</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Phenols</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Acids</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Fixed oil &amp; fats</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

- Negative, + slightly positive, ++ moderately positive, +++ strongly positive
**Swertia tetragona**

1) **Macroscopic Characters**

Shape: irregular  
Surface: Rough, wrinkled  
Colour: greenish yellow  
Odour: slight  
Taste: bitter  

2) **Microscopic characters (Powder Characteristics)**

A greenish yellow powder with a faint, characteristic odour and a very bitter taste typical of the family Gentianaceae. The diagnostic characters are:

a) The xylem vessels which occurs in groups but sometimes singly accompanied by thin walled xylem parenchyma. Vessels are lignified and are with spiral or annularly thickening. Fragments of the same vessels are also seen in the powder.

b) Presence of non lignified long fibres, some with one end swollen and have a well marked lumen. Fragments of the same fibres are also visible.

c) A few prisms of calcium oxalate, are found scattered. They vary considerably in size and are irregularly shaped.

d) Unicellular trichomes from the leaves with tapered tips are found. Some epidermal cells having remains of trichomes are also visible.

e) Starch grains are simple and few. They are spherical in shape and have a well marked hilum.

f) Fragments of leaf lamina in surface view show epidermis composed of cells with a slightly sinuous outline, anisocytic stomata which are not numerous. The underlying palisade cells are fairly small and tightly packed.
Swertia tetragona

Swertia tetragona (Crude drug)
T.S. Of *Swertia tetragona* showing epidermis, phloem and xylem

Calcium oxalate crystals of *Swertia tetragona*
Group Of Xylem vessels (lignified) of Swertia tetragona

Parenchymatous cells of spongy mesophyll of leaf of Swertia tetragona
Calcium oxalate crystals of *A. Cachemirica*

A Fibre, vessel element and crystal of *A. Cachemirica* root
### Table- 2.10: Hot extractive (successive) values (%w/w) of *Swertia tetragona*

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Petroleum ether</th>
<th>Chloroform</th>
<th>Acetone</th>
<th>Methanol</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.541</td>
<td>4.256</td>
<td>4.216</td>
<td>5.210</td>
<td>10.215</td>
</tr>
<tr>
<td>3</td>
<td>7.942</td>
<td>4.112</td>
<td>4.102</td>
<td>6.102</td>
<td>10.714</td>
</tr>
<tr>
<td>Mean</td>
<td>8.229</td>
<td>4.339</td>
<td>4.319</td>
<td>5.558</td>
<td>10.018</td>
</tr>
</tbody>
</table>

### Table- 2.11: Hot extractive (individually) extractive (%w/w) values of *Swertia tetragona*

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Petroleum ether</th>
<th>Chloroform</th>
<th>Acetone</th>
<th>Methanol</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.541</td>
<td>5.216</td>
<td>6.460</td>
<td>13.216</td>
<td>28.467</td>
</tr>
<tr>
<td>2</td>
<td>8.206</td>
<td>5.640</td>
<td>6.518</td>
<td>14.814</td>
<td>29.204</td>
</tr>
<tr>
<td>3</td>
<td>7.946</td>
<td>4.912</td>
<td>7.010</td>
<td>14.814</td>
<td>29.686</td>
</tr>
<tr>
<td>Mean</td>
<td>8.229</td>
<td>5.256</td>
<td>6.662</td>
<td>14.165</td>
<td>29.119</td>
</tr>
</tbody>
</table>

### Table- 2.12: Cold extractive (individually) extractive (%w/w) values of *Swertia tetragona*

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Petroleum ether</th>
<th>Chloroform</th>
<th>Acetone</th>
<th>Methanol</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.618</td>
<td>0.562</td>
<td>0.411</td>
<td>1.021</td>
<td>1.942</td>
</tr>
<tr>
<td>2</td>
<td>0.722</td>
<td>0.604</td>
<td>0.484</td>
<td>1.114</td>
<td>1.871</td>
</tr>
<tr>
<td>3</td>
<td>0.741</td>
<td>0.602</td>
<td>0.387</td>
<td>0.846</td>
<td>1.855</td>
</tr>
<tr>
<td>Mean</td>
<td>0.693</td>
<td>0.589</td>
<td>0.427</td>
<td>0.993</td>
<td>1.889</td>
</tr>
</tbody>
</table>
Table 2.13: Ash values of *Swertia tetragona*

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Total Ash</th>
<th>Acid Insoluble Ash</th>
<th>Water Soluble Ash</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.521</td>
<td>2.261</td>
<td>1.842</td>
</tr>
<tr>
<td>2</td>
<td>6.166</td>
<td>2.143</td>
<td>1.719</td>
</tr>
<tr>
<td>3</td>
<td>5.722</td>
<td>2.742</td>
<td>1.642</td>
</tr>
<tr>
<td>Mean</td>
<td>6.469</td>
<td>2.382</td>
<td>1.734</td>
</tr>
</tbody>
</table>

Table 2.14: Foreign matter

<table>
<thead>
<tr>
<th>S.No</th>
<th>Wt. of Crude Drug (g)</th>
<th>Wt. of drug after removal of foreign matter (g)</th>
<th>Wt. of foreign matter (g)</th>
<th>Foreign matter (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>200.008</td>
<td>198.214</td>
<td>1.794</td>
<td>0.897</td>
</tr>
<tr>
<td>2</td>
<td>200.022</td>
<td>199.021</td>
<td>1.001</td>
<td>0.500</td>
</tr>
<tr>
<td>3</td>
<td>200.078</td>
<td>198.617</td>
<td>1.401</td>
<td>0.700</td>
</tr>
<tr>
<td>Mean</td>
<td>200.016</td>
<td>198.6</td>
<td>1.36</td>
<td>0.68</td>
</tr>
</tbody>
</table>

Table 2.15: Determination of resin, tannin and fat content in *Swertia tetragona*

<table>
<thead>
<tr>
<th>S.NO.</th>
<th>Resin</th>
<th>Tannin</th>
<th>Fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.12</td>
<td>4.87</td>
<td>1.56</td>
</tr>
<tr>
<td>2</td>
<td>3.78</td>
<td>4.74</td>
<td>1.68</td>
</tr>
<tr>
<td>3</td>
<td>2.82</td>
<td>4.68</td>
<td>1.65</td>
</tr>
<tr>
<td>Mean</td>
<td>3.24</td>
<td>4.76</td>
<td>1.63</td>
</tr>
</tbody>
</table>
Table 2.16: Fluorescent analysis

<table>
<thead>
<tr>
<th>Treatment of the Drug Powder</th>
<th>OBSERVATION UNDER</th>
<th>Ordinary Light</th>
<th>UV (254 nm)</th>
<th>UV (366 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry powder as such</td>
<td>Greenish yellow</td>
<td>Greyish green</td>
<td>Greyish green</td>
<td></td>
</tr>
<tr>
<td>Conc. H₂SO₄</td>
<td>Greenish black</td>
<td>Greenish black</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conc. H₂SO₄ + water</td>
<td>Greyish green</td>
<td>Green</td>
<td>Yellowish green</td>
<td></td>
</tr>
<tr>
<td>Conc. HCl</td>
<td>Dark green</td>
<td>Green</td>
<td>Yellowish green*</td>
<td></td>
</tr>
<tr>
<td>Conc. HCl + water</td>
<td>Yellowish green</td>
<td>Green</td>
<td>Yellowish green*</td>
<td></td>
</tr>
<tr>
<td>Conc. HNO₃</td>
<td>Straw yellow</td>
<td>Green</td>
<td>Brownish yellow*</td>
<td></td>
</tr>
<tr>
<td>Conc. HNO₃ + water</td>
<td>Straw yellow</td>
<td>Green</td>
<td>Green*</td>
<td></td>
</tr>
<tr>
<td>Acetic Acid</td>
<td>Greyish green</td>
<td>Light green</td>
<td>Yellowish green</td>
<td></td>
</tr>
<tr>
<td>Methanol</td>
<td>Yellowish green</td>
<td>Yellowish green</td>
<td>Pinkish yellow*</td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>Dark green</td>
<td>Yellowish green</td>
<td>Red*</td>
<td></td>
</tr>
<tr>
<td>Chloroform</td>
<td>Light green</td>
<td>Yellowish green</td>
<td>Pinkish red*</td>
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</tr>
<tr>
<td>Petroleum ether</td>
<td>No change</td>
<td>Light pink</td>
<td>Red*</td>
<td></td>
</tr>
<tr>
<td>Dist. Water</td>
<td>Straw yellow</td>
<td>Light green</td>
<td>Green*</td>
<td></td>
</tr>
<tr>
<td>10 % NaOH</td>
<td>Brownish black</td>
<td>Light green</td>
<td>Dark green</td>
<td></td>
</tr>
<tr>
<td>5% Iodine</td>
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<td>Dark green</td>
<td>Black</td>
<td></td>
</tr>
<tr>
<td>Picric Acid</td>
<td>Greenish yellow</td>
<td>Yellowish green</td>
<td>Dark green</td>
<td></td>
</tr>
<tr>
<td>FeCl₃ solution</td>
<td>Dark green</td>
<td>Dark green</td>
<td>Dark green</td>
<td></td>
</tr>
</tbody>
</table>

*Shows prominent fluorescence

Table 2.17: Powdered drug reaction with different chemicals

<table>
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<th>Treatment with chemicals</th>
<th>Observation</th>
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<tbody>
<tr>
<td>Conc. Hydrochloric acid</td>
<td>Dark green</td>
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<tr>
<td>Conc. Nitric acid</td>
<td>Straw yellow</td>
</tr>
<tr>
<td>Conc. Sulphuric acid</td>
<td>Greenish black</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>Greyish green</td>
</tr>
<tr>
<td>Picric acid</td>
<td>Greenish yellow</td>
</tr>
<tr>
<td>Iodine solution 5%</td>
<td>Greenish black</td>
</tr>
<tr>
<td>5% Sodium hydroxide</td>
<td>Brownish black</td>
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# Table 2.18: Phytochemical investigations

<table>
<thead>
<tr>
<th>Tests</th>
<th>Pet. Ether Extract</th>
<th>Benzene Extract</th>
<th>Chloroform Extract</th>
<th>Ethyl Acetate Extract</th>
<th>Methanol Extract</th>
<th>Aqueous Extract</th>
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<td>Glycosides</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tannins</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>++</td>
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<tr>
<td>Carbohydrates</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Phenols</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Acids</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Fixed oil &amp; fats</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

- Negative, + slightly positive, ++ moderately positive, +++ strongly positive
PHYTOCHEMISTRY
MATERIAL AND METHODS
Apparatus, Chemicals and Instruments

1. All the chemicals and reagents were obtained from s.d. fine chemicals and were of Analytical Reagent (AR) grade.

2. Silica gel (60-120 mesh) and silica gel-G, obtained from s.d. fine chemicals, were used for the column chromatography and thin layer chromatography (TLC), respectively.

3. Anhydrous sodium sulphate was used for drying organic solvent petroleum ether (60-80°C) and chloroform.

4. Melting points were determined on Perfit melting point apparatus and are uncorrected.

5. All the weighing were done on single pan Mettler Balance.

6. Rf values were taken using TLC plates, solvent chamber and iodine chamber.

7. Ultra-violet (UV) spectra were recorded on Lambda-Bio 20 Spectrophotometer in CCl₄.

8. Fourier Transforms Infra-Red (FT-IR) spectra were recorded on Bio-Rad FT Spectrophotometer using CCl₄ as solvent vehicle. \( v_{\text{max}} \) values are given in cm⁻¹.

9. ¹H-NMR spectra were screened on Advance DRY 400, Bruker Spectrospin 400-MHz instrument using CDCl₃/dmso as solvent and TMS as an internal standard. Chemical shifts are given in δ (ppm) scale with tetramethyl silane (TMS) as internal standard. Coupling constants (J values) are expressed in Hz. Notation used throughout as s=singlet, d=doublets, dd-double doublets, t=triplet, m=multiplet, brs=unresolved broad singlet.

10. ¹³C FT-NMR Spectra were recorded on Advance DRY 400, Bruker Spectrospin 100-MHz with TMS as an internal standard in 5 mm spinning tubes at 27°C.

11. The FAB mass spectra were recorded on a JEOL SX 102/DA – 6000 Mass Spectrometer / Data System using Argon / Xenon (6 KV, 10 mA) as the FAB gas. The accelerating voltage was 10 KV and the spectra were recorded at room temperature. M-Nitrobenzyl alcohol (NBA) was used as the matrix unless
specified otherwise. The matrix peaks may appear at m/z 136, 137, 154, 289, 307 in the absence of any metal ions.

12. The plant material *Aralia cachemirica* (roots) and *Swertia tetragona* (whole plant) were collected from Aharbal region of kashmir in presence of Dr. A.R.Naqshi, taxonomist, department of taxonomy, University of Kashmir.

Packing of Column: The lower end of column was plugged with absorbent cotton, over which a piece of filter paper was placed. The column was then half filled with petroleum ether, silica gel was added in small portions and allowed to settle down gently until the necessary length of column was attained. All the air bubbles were allowed to escape by running the column continuously with solvent. The silica gel slurry of the crude drug extract was packed in the column and then eluted successively in order of increasing polarity with different solvents. The development and elution of the column was carried with successive series of solvents in various combinations, viz., petroleum ether (100%), benzene (100%), benzene - chloroform (1:1); chloroform (100%), chloroform-methanol (99.5:0.5, 99:1, 98:2, 4:1).

Homogeneity of the Fractions: The fractions collected were subjected to thin layer chromatography to check homogeneity of various fractions. Chromatographically identical fractions were combined and concentrated.

Preparation of Plates: Silica gel-G (35 gl was mixed with 80 ml of distilled water by trituratiuon method in a glass pestle and mortar to form a fine thin cream. Thickness of 0.25 mm was achieved by means of an applicator. The plates were allowed to dry in air at room temperature.

Activation of Plates: The dried plates were kept in an oven at 110°C for an hour and were stored in a dessicator.

Equilibration of the Chromatographic Chamber: About 1 cm height of the solvent was taken in a clean dry chamber, after the walls of chamber were lined with a strip of filter paper impregnated with the solvent system. The chamber was closed and allowed to saturate with vapours of solvent.
Application of Spots: The base line was marked at about 1.5 cm above from the lower edge. The dissolved fractions were spotted on the plates with the fine capillary tubes and then allowed to dry in air.

Development of Chromatogram: The spotted plates were kept in the chromatographic chambers containing the solvent mixtures. The chambers were covered with greased glass plates. The solvent system was allowed to ascend up to \( \frac{3}{4} \) the length of the plate.

Solvent Systems for TLC Plates: Petroleum ether: Benzene (1: 1 v/v); Pet. Ether: Benzene (3: 1 v/v); Benzene: Benzene: Chloroform (3: 1 v/v); Benzene: Chloroform (1: 1 v/v); Chloroform + Methanol (99: 1 v/v); Chloroform + Methanol (95: 5 v/v); Chloroform + Methanol (3: 1 v/v).

Detection of TLC Spots: The air-dried plates were viewed in ultra-violet light chamber to look for the coloured fluorescent spots if any. Freshly prepared ceric-ammonium sulphate (CAS) was used as a spray reagent for location of spots. The reagent was carefully sprayed on the plates and were subsequently heated in an oven at 110°C for 10-15 minutes.
EXPERIMENTAL PHYTOCHEMISTRY OF A. CACHERMIRA

Extraction: The roots of Aralia cachemirica (Araliaceae) were dried under shade. Dried and powdered roots were weighed (2.5 kg) and then extracted exhaustively with petroleum ether in soxhlet apparatus. The extract was concentrated to yield brownish yellow coloured mass. It was subjected to phytochemical screening for determining the presence of different chemical constituents as given in standardization of Aralia cachemirica.

Isolation: The viscous brownish yellow mass was adsorbed on silica gel (60-80 mesh) for preparation of slurry. It was dried on water bath and then air dried. After complete drying, it was packed in glass column and then subjected to elution by different solvents ranging from petroleum ether to methanol in order of increasing polarity to isolate the following compounds.

AC-1: Elution of column with petroleum ether furnished white amorphous powder of AC-1 (Fraction No. 16-52), recrystallized with Hexane, yield = 0.36%, M.P. 135.4°C, Rf = 0.3, Chloroform: petroleum ether (1:9)

IR vmax (CCl4) 3300,2950,1645,1410,1315,1280,1130,795 cm⁻¹.

¹H NMR (CDCl₃): δ 5.69 (1H, m, H-7), 5.03(1H, m, H-9), 4.94 (1H, m, H-6), 4.91 (1H, m, H-10), 2.14 (2H, m, H₂-8), 1.95 (2H, m, H₂-2), 1.84 (2H, m, H₂-5), 1.58(2H, m, H₂-11), 1.25(12H, brs, 6 x CH₂), 1.04(2 H, m, CH₂), 0.99(2H,m, CH₂), 0.87(3H,t, j=6.1 Hz, Me-18)

¹³C NMR (CDCl₃): δ 180.69 (C-1), 146.26(C-7), 127.54 (C-9), 123.61 (C-6), 113.04 (C-10), 56.61 (C-8), 55.75 (C-5), 47.91 (C-2), 43.32 (C-11), 40.60 (C-12), 39.54(C-13), 37.85 (C-14), 35.56 (C-3), 31.73 (C-4), 28.83 (C-15), 25.90(C-16, C-17), 18.98(C-18),

+ve ion FAB MS m/z (rel.int.) : 280[M]+ (C₁₈H₂₂O₂) (26.7), 363(8.9), 265(43.1), 239(57.8), 165(15.3), 143(16.2).

AC-4: Elution of column with petroleum ether: chloroform (90:10) furnished crystalline compound (AC-4), recrystallized with methanol, yield = 1.36%, M.P. 142.8°C, Rf = 0.5, Benzene: petroleum ether (1:9)
IR\textsubscript{\text{v(max)}} (CCl\textsubscript{4}) \text{3280, 2930, 2850, 1693, 1453, 1262, 1183, 996, 915, 839 cm}^{-1}.

TABLE\textsuperscript{-1}\textsuperscript{H} AND \textsuperscript{13}C NMR SPECTRAL DATA OF COMPOUND AC-4

<table>
<thead>
<tr>
<th>POSITION</th>
<th>\textsuperscript{1}HNMR</th>
<th>\textsuperscript{13}C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ALPHA</td>
<td>BETA</td>
</tr>
<tr>
<td>1</td>
<td>1.98M</td>
<td>1.84M</td>
</tr>
<tr>
<td>2</td>
<td>2.18M</td>
<td>2.04M</td>
</tr>
<tr>
<td>3</td>
<td>1.29M</td>
<td>0.85M</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>1.78dd(3.6,6)</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>1.65m</td>
<td>1.52m</td>
</tr>
<tr>
<td>7</td>
<td>2.35dd(2.4,5,5)</td>
<td>2.31dd(1.5,5,5)</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>2.63brs</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>-</td>
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<tr>
<td>11</td>
<td>1.70m</td>
<td>1.65m</td>
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<tr>
<td>12</td>
<td>1.52m</td>
<td>1.47m</td>
</tr>
<tr>
<td>13</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>5.14brs</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>5.72dd(10.8)</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>4.94d(10.8)</td>
<td>4.88d(4.2)</td>
</tr>
<tr>
<td>17</td>
<td>-</td>
<td>-</td>
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<tr>
<td>18</td>
<td>0.99brs</td>
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<td>19</td>
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<td>-</td>
</tr>
<tr>
<td>20</td>
<td>1.29brs</td>
<td>-</td>
</tr>
</tbody>
</table>

ESI MS, m/z: 302(M\textsuperscript{+}) (C\textsubscript{20}H\textsubscript{30}O\textsubscript{2})
AC-25: Elution of column with chloroform: methanol (98:2) furnished white amorphous powder of AC-25 (Fraction No. 1-52), recrystallized with methanol, yield = 1.26%, M.P 226.6, Rf = 0.5, Chloroform: ethyl acetate (8:2)

IR$_{\text{vmax}}$ (CCl$_4$) 3410, 2926, 2850, 1697, 1645, 1539, 1472, 1370, 1250, 1120 cm$^{-1}$.

| TABLE: $^1$H AND $^{13}$C NMR SPECTRAL DATA OF COMPOUND AC-25 |
|---|---|---|
| POSITION | $^1$HNMR | $^{13}$C |
|  | ALPHA | BETA |  |
| 1 | 1.76m | 1.86m | 37.25 |
| 2 | 1.86m | 1.83m | 23.60 |
| 3 | 1.29m | 1.47m | 41.20 |
| 4 | - | - | 47.51 |
| 5 | 2.25m | - | 56.81 |
| 6 | 0.96m | 1.76m | 25.96 |
| 7 | 1.53m | 1.47m | 42.50 |
| 8 | - | 1.97brs | 55.86 |
| 9 | 2.10m | - | 55.05 |
| 10 | - | - | 38.60 |
| 11 | 1.29m | 1.30m | 38.73 |
| 12 | 1.99m | 1.86m | 28.16 |
| 13 | - | - | 44.31 |
| 14 | 1.53m | 1.15m | 39.35 |
| 15 | 1.29brs | 1.33br | 38.73 |
| 16 | 0.82(5.2) | - | 17.39 |
| 17 | - | - | 179.05 |
| 18 | 1.38brs | - | 18.29 |
| 19 | 1.18brs | - | 14.77 |
| 20 | 0.88 | - | 21.30 |

ESI MS. m/z: 306(M$^+$) (C$_{20}$H$_{34}$O$_2$)
AP-27: Elution of column petroleum ether furnished colourless crystals of AP-27 (Fraction No. 30-55), recrystallized with methanol, yield = 0.36%, M.P. 85-86°C, Rf = 0.4, petroleum ether: benzene (17:3)

IR\text{\tiny max} (\text{CCI}_4) 3410, 3380, 2950, 2872, 1755, 1725, 1642, 1458, 1173 cm\textsuperscript{-1}.

\textsuperscript{1}H NMR (DMSO-\text{d}_6) : \( \delta \) 5.126 (1H, m, H -22), 5.13 (1H, m, ), 4.50 (1H, d, H\textsubscript{2}2b ), 4.25 (1H, brm, w 1/2 =18.1 Hz, H-10a), 4.00 (1H, d, j=16.5 hz, H-17a), 3.52 (1H, brs, w\textsubscript{1/2} 18.5 Hz, H-5a), 1.54 (6H, brs, Me-38, Me-39), 1.24 (6H, brs, Me-33, Me-35), 0.95 (3H, d, j=6.5 Hz, Me-36), 0.83 (3H, d, j=6.5 Hz, Me-40), 0.68 (3H, t, J=6.1 Hz, Me-1)

\textsuperscript{13}C NMR (\text{CDCl}_3):

<table>
<thead>
<tr>
<th>Position</th>
<th>( \delta )</th>
<th>Position</th>
<th>( \delta )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11.62</td>
<td>21</td>
<td>45.08</td>
</tr>
<tr>
<td>2</td>
<td>25.38</td>
<td>22</td>
<td>127.45</td>
</tr>
<tr>
<td>3</td>
<td>66.43</td>
<td>23</td>
<td>140.92</td>
</tr>
<tr>
<td>4</td>
<td>35.95</td>
<td>24</td>
<td>42.01</td>
</tr>
<tr>
<td>5</td>
<td>61.51</td>
<td>25</td>
<td>33.28</td>
</tr>
<tr>
<td>6</td>
<td>36.81</td>
<td>26</td>
<td>120.22</td>
</tr>
<tr>
<td>7</td>
<td>56.20</td>
<td>27</td>
<td>137.82</td>
</tr>
<tr>
<td>8</td>
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Jamia Hamdard

128
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ESI MS: m/z (rel.int.) : 635[M]⁺ (C₄₀H₇₅O₅) (5.8), 5054(7.0), 405(3.5) 386(3.1), 373(23.5), 342(21.3), 328(4.5), 300(8.2), 292(4.3), 262(2.3)258(1.0), 249(8.3), 230(1.5), 221(1.6), 193(5.1), 153(4.3) 125(1.4), 85(1.0)
AM-1: Elution of column with chloroform: methanol (90:10) furnished white crystals of AM-1 (Fraction No. 16-52), recrystallized with methanol, yield = 0.36%, M.P. 201°C, Rf = 0.3, toluene ethyl acetate (8:2)

\[ \text{IR}_{\text{vmax}} \text{ (CCl}_4\text{)} \ 3500, 3460, 3320, 2950, 2845, 1310, 1215, \text{ cm}^{-1}. \]

\[^{1}\text{H NMR} \text{ (DMSO-d}_6\text{)} : \delta 5.17 (1\text{H, brs, H-1'}), 5.04 (1\text{H, d, } j=5.1 \text{ Hz, H-1}), 4.50 (1\text{H, d, } j=7.5 \text{ Hz, H-5'}), 4.39 (1\text{H, d, } j=9.9 \text{ Hz, H-5}), 4.36 (1\text{H, m, H-4'}), 4.10 (1\text{H, m, H-4}), 3.87 (1\text{H, m, H-2'}), 3.77 (1\text{H, m, H-21}), 3.55 (2\text{H, brs, H-3, H-3'}), 3.45 (1\text{H, d, } j=6.1 \text{ Hz, H-26a}), 3.40 (1\text{H, d, } j=6.1 \text{ Hz, H-26b}), 3.39 (1\text{H, d, } j=5.1 \text{ Hz, H-26a}), 3.15 (1\text{H, d, } j=5.1 \text{ Hz, H-26b}) \]

\[^{13}\text{C NMR} \text{ (CDCl}_3\text{)} : \delta 91.85 (C-1), 74.39 (C-2), 72.46 (C-3), 72.96 (C-4), 77.17 (C-5), 62.21 (C-6), 104.16 (C-1'), 72.96 (C-2'), 70.66 (C-3'), 72.96 (C-4'), 82.62 (C-5'), 60.62 (C-6') \]

\[ +\text{ve ion FAB MS m/z (rel.int.)} : 280[M]^+ (\text{C}_{18}\text{H}_{32}\text{O}_2) (26.7), 363(8.9), 265(43.1), 239(57.8), 165(15.3), 143(16.2). \]

AM-2: Elution of column with chloroform: methanol (95:5) furnished colourless crystals of AM-2 (Fraction No. 5-15), recrystallized with methanol, yield = 0.12%, M.P. 185°C, Rf = 0.5, toluene:ethyl acetate (8:2)

\[ \text{IR}_{\text{vmax}} \text{ (KBr) : 3540, 3380, 3275, 2950, 2835, 1364, 1208, 1134, 965 cm}^{-1}. \]

\[^{1}\text{H NMR} \text{ (DMSO-d}_6\text{)} : \delta 5.17 (1\text{H, dd, } j=2.5, 3.2 \text{ Hz, H-4'}), 5.06 (1\text{H, d, } j=6.0 \text{ Hz, H-1'}), 4.81 (1\text{H, m, H-65}), 4.77 \]

\[^{13}\text{C NMR} \text{ (CDCl}_3\text{)} : \]

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Jamia Hamdard
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**ESI MS: m/z (rel.int.):** $422[M^+]$ (C$_{12}$H$_{22}$O$_{11}$)

_Jamia Hamdard_
AM-4: Elution of column with chloroform:methanol (8:2) furnished colourless crystals of AM-4 (Fraction No. 10-28), recrystallized with methanol, yield = 0.15%, M.P. 220°C, Rf = 0.25, toluene:ethyl acetate (8:2)

$\text{IR}_{\text{max}}$ (KBr): 3350, 2955, 2845, 1640, 1470, 1265, 1080 cm$^{-1}$.

$^1$H NMR (DMSO-d$_6$): $\delta$ 5.13 (1H, m, H-6), 3.52 (1H, dd, J = 8.5, 5.1 Hz, H-3$\alpha$), 2.56 (2H, brs, H$_2$-7), 2.50 (1H, m, H-1a), 2.26 (2H, m, H$_2$-2), 2.18 (2H, m, H-9$\alpha$, H-8$\alpha$), 2.01 (2H, m, H$_2$-11), 1.77 (3H, m, H$_2$-1b, H-14$\alpha$, H-18$\alpha$), 1.52 (6H, brs, H$_2$-12, H$_2$-13, H$_2$-15), 1.25 (6H, brs, H$_2$-16, H$_2$-17, H$_2$-19), 1.23 (3H, brs, Me-22), 1.22 (6H, brs, Me-23, Me-21), 1.11 (3H, d, j = 6.1 Hz, Me-24), 0.96 (3H, d, j = 6.2 Hz, Me-25), 0.88 (6H, m, Me-20, Me-26)

$^{13}$C NMR (CDCl$_3$):

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ESI MS: m/z (rel.int.): 376[M]$^+$ (C$_{26}$H$_{46}$O)
MASS SPECTRUM OF AC-1
1H NMR SPECTRUM OF AC-1

Current Data Parameters
NAME proton
EXPMO 167
PROCNO 1

RF - Acquisition Parameters
Date 990000
Time 12.40
INSTRIN cp300
PROBMD 9mm Dual ZB
PULPROB 2
TD 32768
SOLVENT D2O
N9 16
DS 0
SNM 6992.008 Hz
FTOMES 0.274 kHz Hz
AD 1.0193508 mmr
RB 90.5
DM 55.600 usec
DE 4.80 usec
TE 300.0 kHz
DI 5.0000000 usec
P1 11.00 usec
OE 4.80 usec
SFOL 300.1542018 MHz
NCH 1
PL1 0.00 dB

FP - Processing parameters
BT 32768
SF 200.1300045 MHz
WMN EK
SSB 0
LB 1.00 Hz
OB 0
PC 1.40

1D NMR plot parameters
DX 20.00 cm
F1P 10.000 ppm
F1 3001.30 Hz
F2P 0.950 ppm
F2 150.06 Hz
PPHM 0.47727 ppm/cm
HZCM 143.24385 Hz/cm
IR SPECTRUM OF AC-1

File #1: ZAC1

View Mode: Overlay
Analysis Info

Analysis Name: KAS00076.d
Sample Name: AC-4
Comment:

Acquisition Info

Acquisition Date: 08/17/04 11:55:01
Method: GERD Default.ms
Operator: Administrator
Instrument: esquire3000_00037

Acquisition Parameters

- Ion Source Type: ESI
- Scan Begin: 50 m/z
- Capillary Exit: 113.5 Volt
- Mass Range: Std/Normal
- Blade End: 900 m/z
- Scan Type: Normal
- Ion Polarity: Positive
- Averages: 5 Spectra
- Trap Drive: 36.3
- Alternating Ion: Off
- Retention Time: 222 μs
- Mass: 358.1

Mass Spectrum of AC-4

MASS SPECTRUM OF AC-4
## Display Report

### Analysis Info

- **Analysis Name**: KAS000008.d
- **Sample Name**: AC-25
- **Acquisition Date**: 08/26/04 12:04:44
- **Method**: GERD Default.ms
- **Operator**: Administrator
  - Instrument: esquire3000_00037

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### Mass Spectrum of AC-25

- Mass spectrum plotted from 0.0 to 600 m/z, with peaks at 301.0, 365.0, and 625.3.
13C NMR SPECTRUM OF AC-25

Acquisition Parameters:
- Sample: c00000
- Time: 15.80
- INSTRUM: cdx300
- PROBID: 5mm Qutl 28
- PULPROG: xgrad
- TD: 16364
- SOLVENT: CDCl3
- NS: 1546
- DB: 0
- SNR: 28882.903 Hz
- FIDRES: 1.14943B Hz
- AG: 0.3590-42 sec
- NW: 9152
- DW: 29.900 usec
- DE: 4.50 usec
- TE: 300.0 K
- DI: 0.0300000 sec
- PL12: 17.80 usec
- CPMAS: wait16
- POPG: 80.00 usec
- SF02: 300.133013 MHz
- NUC2: 1H
- PL2: 0.00 dB
- D1: 1.00000000 sec
- P1: 5.40 usec
- DF: 4.30 usec
- SF01: 75.476752 Hz
- NUC1: 13C
- PL1: 0.00 dB

Processing Parameters:
- C1: 16384
- SF: 75.4681708 MHz
- KDW: 1H
- SSB: 0
- LB: 10.00 Hz
- GB: 0
- PC: 1.40

1D NMR plot parameters:
- CX: 20.00 cm
- F1P: 220.000 ppm
- F1: 16803.00 Hz
- F2P: 0.000 ppm
- FP: 0.00 Hz
- PH: 16.60000 ppm/cm
- HZCM: 850.14800 Hz/cm
IR SPECTRUM OF AC-25

File #1: ZAC25

Number of Scans: 16

Comment: Bio-Rad FTS
MASS SPECTRUM OF AM-1
1H NMR SPECTRUM OF AM-I

Current Data Parameters
NAME proton
EXPN 112
PROGH 1

F2 - Acquisition Parameters
Date_ 500000
Time 13.28
INSTRUM dxx300
PULPROG 5mm Dual 25
TD 15384
SOLVENT DMSO
MS 6
DS 0
B0 0.002.805 Hz
FICRES 0.548877 Hz
AQ 0.8110004 Hz
AG 20.2
DM 55.500 user
DE 4.50 user
TE 300.0 K
D1 5.0000000 sec
P1 11.55 user
DE 4.50 user
SFQ 300.132016 kHz
MUC1 1H
PL1 0.00 aq

F2 - Processing parameters
SF 32788
SF 300.130000 kHz
MDW EM
SSB 0
LB 1.00 Hz
BB 0
PC 1.40

1D NMR plot parameters
tK 22.00 cm
F1P 10.030 ppm
P1 5001.30 Hz
PP -0.500 ppm
NF -150.00 Hz
PHCM 0.47727 ppm/cm
HZCM 143.24386 Hz/cm
13C NMR SPECTRUM OF AM-1

Current Data Parameters
NAME  13C
EXPNO  201
PROCNO  1

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Date  200000
Time  13.28
INSTRUM  dpx300
PRF 500 (Dual ZG)
PULPROG  zgdc
TO  163B4
SOLVENT  CH2Cl2
NS  2004
DS  0
SW  100.22222 Hz
FIDRES  1.146838 Hz
AQ  0.4330452 sec
AT  7256.2
M  56.560 usec
DQ  4.50 usec
TF  300.0 K
ds  0.0000000 sec
PL12  17.00 db
QPQ 106
PPQ  20.00 usec
SFQ  300.1330015 MHz
MUQ  10
PLS  0.00 db
D1  1.00000000 sec
P1  0.40 usec
D2  4.50 usec
SFQ  75.4767781 MHz
MUC  10
PL1  0.00 db

F2 - Processing parameters
S1  163B4
SF  75.4767784 MHz
HOM  CH
SSB  c
LS  8.00 Hz
GB  C
PC  1.00

1D NMR plot parameters
 CSI  20.00 cm
FSP  200000 ppm
F1  10083.05 Hz
F2  0.00 ppm
PM  0.00 Hz
PPCM  10.20000 ppm/cm
HIDK  754.15777 Hz/cm
IR SPECTRUM OF AM-1
### Display Report

**Analysis Info**
- **Analysis Name**: SL000057.d
- **Sample Name**: AM-2
- **Comment**

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**Mass Spectrum of AM-2**

![Mass Spectrum Graph](image)
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PROCNO 1

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SOLVENT DMSO
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SN 18032.363 Hz
TFREE 1.12455 Hz
AQ 0.4302462 sec
AQ 7296.2
DE 26.660 usec
DR 4.50 usec
TE 306.0 K
d11 0.00E000usec
PL12 17.80 db
CPMG8 90.00 usec
SFDS 200.120018 Hz
NUC1 1
PL3 0.00 db
CS 1.00000000 sec
F1 9.40 usec
DE 4.50 usec
PS1 75.4767765 Hz
NUC1 13C
PL1 0.00 db

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SI 18384
SF 75.4677837 Hz
WDM EB
SSB 0
LB 10.00 Hz
SB 0
PC 1.00

10 NMR plot parameters
cx 0.30 cm
fwp 205.00 nm
f1 200.00 ppm
f2 25093.56 Hz
f2 0.00 ppm
f2 0.00 Hz
TMQ 10.0000 ppm/cm
MOM 754.67769 Hz/cm
IR SPECTRUM OF AM-2
Analysis Info
Analysis Name: SL000059.d
Sample Name: AM-4
Comment:

Acquisition Info
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 Operator: esquire3000_00037
 Instrument: GERD Default.m

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- Scan Begin: 50 m/z
- Capillary Exit: 100.3 Volt
- Mass Range: Std/Normal
- Ion Polarity: Positive
- Alternating Ion: off
- Repetition Time: 200000 μs
- Auto MS/MS: off

Ion Source Type: ESI
Scan Begin: 50 m/z
Capillary Exit: 100.3 Volt
Mass Range: Std/Normal
Ion Polarity: Positive
Alternating Ion: off
Repetition Time: 200000 μs
Auto MS/MS: off

MASS SPECTRUM OF AM-4
### 13C NMR Spectrum of AM-3

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MASS SPECTRUM OF AP-27
IR SPECTRUM OF AP-27
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- Sample Name: SW-1
- Method: GERD Default.ms
- Instrument: esquire3000_00037

Acquisition Parameter
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- State/End: SIMs 1
- Ion Polarity: Positive
- Averaging: 5 Spectra
- Trap Drive: 33.7
- Alternating Ion: off
- Rotary/Station Time: 140 μs
- Auto MS/MS: off

**MASS SPECTRUM OF SW-1**
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1H NMR spectrum of SW-1
IR SPECTRUM OF SW-1
Analysis Info
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Sample Name: SW-2
Comment:

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MASS SPECTRUM OF SW-2
IR SPECTRUM OF SW-2
**Analysis Info**

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- **Sample Name:** SW-3

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$^{13}$C NMR SPECTRUM OF SW-3

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IR SPECTRUM OF SW-3
C NMR SPECTRUM OF SW-4
IR SPECTRUM OF SW-4
RESULTS AND DISCUSSIONS:

AC-1: Compound AC-1, namely linoleic acid was obtained as an amorphous powder from petroleum ether (15-31) eluants. Compound responded positively to bromine water test for unsaturation and gave effervescence with sodium bicarbonate supporting the acidic nature of the compound. Its IR showed absorption bands at (3300 cm\(^{-1}\)), unsaturation (1690 cm\(^{-1}\)) and long chain aliphatic nature (795 cm\(^{-1}\)).

ESI Mass spectrum showed a molecular ion peak at m/z 280, which correspond to molecular formula of an aliphatic acid. It showed two double-bond equivalents, which were adjusted, in two olefinic linkages in the compound.

\(^1\)H NMR of AC-1 showed four downfield multiplets, one proton each, at $\delta$ 5.69, 5.03, 4.94 and 4.91 that were assigned to four olefinic protons at H-7, H-9, H-6 and H-16 respectively. Another set of 4 multiplets, two protons each, at $\delta$ 2.14, 1.95, 1.84 and 1.58 correspond to eight methylenic protons at H-8, H-2, H-5 and H-11, respectively. Rest of the methylene protons appeared at $\delta$ 1.25 (6xCH\(_2\)), 1.04(CH\(_2\)) and 0.99 (CH\(_2\)). Terminal methyl protons appeared as a three-proton triplet at $\delta$ 0.87(j = 6.1 Hz)

\(^{13}\)C NMR of AC-1 showed seventeen signals. The important ones are those for C-1 (carboxylic carbon)(5180-69) and olefinic methylene carbons C-7, C-9, C-6 and C-10 at $\delta$ 146.26, 127.54, 123.61 and 113.04 respectively. Rest of the saturated methylene carbons appeared between $\delta$ 56.61-25.90. Terminal methyl carbon at C-18 appeared at $\delta$ 18.98.

On the basis of above chemical reactions and spectral details the structure of AC-1 has been elucidated as linoleic acid.

\[
\text{CH}_3 (\text{CH}_2)_{7}\text{CH}=\text{CH}-\text{CH}_2\text{CH}=\text{CH} (\text{CH}_2)_{4}\text{-COOH}.
\]

AC-4: Compound AC-4, was obtained as a crystalline compound from chloroform: petroleum ether (10:90) eluants. Compound responded positively to bromine water test for unsaturation and gave effervescence with sodium bicarbonate supporting
the acidic nature of the compound. Its IR showed absorption bands at (3280 cm⁻¹), unsaturation (1693 cm⁻¹).

ESI Mass spectrum showed a molecular ion peak at m/z 302, which correspond to molecular formula of a diterpenic acid, C₂₀H₃₀O₂. It indicated five double bond equivalents two of that were adjusted in two olefinic linkages and three in a tri-cyclic carbon framework.

The ¹H NMR of AC-4 showed one proton downfield signal at δ 5.14 assignable to olefinic methine proton at C-14. Another one-proton double-doublet at δ 5.72 (j=10.8 Hz) arose due to olefinic methylene proton at C-15. Two olefinic primary methylene protons at C-16 appeared as doublet signals at δ 4.94 (j = 10.8 Hz 4.) and 4.88 (4.2 Hz ). Three one proton double-doublets at δ 2.35 (2.4, 5.5 Hz), 2.31 (1.5, 5.5 Hz) and 1.78 (3.6, 6.0 Hz) were assigned to H-7α, H-7β methylene and H-5 methine protons. The methylene protons resonated between δ 1.98-0.85 as multiplets. Three three-protons broad signals at δ 0.99, 0.65 and 1.29 were assigned to C-18, C-19 and C-20 methine proton respectively. A broad signal at δ 2.63 arose due to H-9 methine proton.

¹³C NMR spectrum of AC-4 showed twenty signals that mainly consisted of a carboxylic carbon at C-17 (δ184.83) and four olefinic carbon signals for C-8, C-14, C-15 and C-16 at δ137.93, 127.96, 147.14 and 112.91, respectively. Rest of methylene carbons resonated between δ38.51-19.22. Three quaternary carbons at C-4, C-10 and C-13 appeared at δ 37.90, 29.21 and 44.04. Three methyl carbons viz. C-18, C-19 and C-20 appeared at δ 24.08, 13.79 and 19.56 respectively.

On the basis of the above give discussion the structure of AC-4 has been elucidated as Primar-8 (14), 15-diene-17-oic acid.

AC-25: Compound AC-25, was obtained as effervescence with sodium bicarbonate. The IR spectrum of AC-25 showed absorption band for carboxylic group (3410 cm⁻¹). ESI mass spectrum of AC-25 showed a molecular ion peak at m/z 306, which corresponded, to molecular formula of a diterpenic acid C₂₀H₃₄O₂. It depicted three double bond equivalents that were adjusted in a tri-cyclic carbon.
framework. The HNMR of AC-2 showed one-proton broad singlet at δ 1.97 due to a β-oriented methine proton at C-8. Another methine proton AC-19 appeared as a multiplet at δ 2.10. All the methylene proton resonated between δ 1.99-0.96 as multiplets. A three-proton triplet (J=5.2Hz) at δ 0.82 and three three-proton broad singlets at δ 1.38, 1.18 & 0.88 were assigned to C-16, C-18, C-19 and C-20 methyl proton respectively.

13CNMR of AC-25 showed twenty signals. Carboxylic carbon at C-17 appeared at δ 179.05 while as four methyl carbon at C-16, C-18, C-19 and C-20 appeared at δ 17.39, 18.29, 14.77 and 21.30 respectively. Three quaternary carbons C-4, C-10 and C-13 appeared at δ 47.51, 36.60 and 44.31 while as all the methinic carbons at C-5, C-8 and C-9 resonated at δ 56.81, 55.86 and 55.05 respectively.

On the basis of above spectral data and discussion, the structure of AC-25 was elucidated as primar-17-oic acid.

AM-1: Compound AM-1, designated as glucopyranosyl-O-1→4 glucopyranoside was obtained as a crystalline compound from chloroform:methanol(90:10) eluants. Its IR spectrum exhibited absorption bands at (3500, 3460 cm⁻¹) and glycosidic linkage (2845 cm⁻¹). Its ESI MS spectrum showed a molecular ion peak at m/z 422 which correspond to molecular formula of C₁₂H₂₂O₁₁ of a disaccharide.

The ¹H NMR of AM-1 exhibited a broad singlet and a doublet, one proton each at δ 5.17 and 5.04 (J=5.1 Hz) assigned to H-1 and H-1 anemic protons respectively. Oxygenated methine protons appeared as two one proton doublets at δ 4.50 (J=7.5 Hz) and δ 4.39 (J=9.9 Hz) corresponding for H-5 and H-5. The carbinol protons resonated between δ 4.36 and 3.55. Four one-proton doublets at δ 3.45 (J=6.1 Hz), 3.40 (6.1Hz), 3.39 (J=5.1Hz) and 3.15 (J=5.1Hz) were assigned to H₂-6a, H₂-6b, H₂-6a', H₂-6b' hydroxyl methylene protons respectively.

¹³C NMR spectrum of AM-1 confirmed a disaccharide compound as it displayed two anomeric carbon signal at δ 91.85(C-1) and 104.16 (C-1'). Comparison of the melting point of AM-1 and the authentic sample along with a co TLC pattern confirmed AM-1 to be a disaccharide. Its structure has been designated as glucopyranosyl-O-1→4 glucopyranoside (sucrose).
AM-2: Compound AM-2, designated as glucopyranosyl-O-(1→1) fructofuranoside was obtained as a crystalline compound from chloroform:methanol (95:5) eluants. Its IR spectrum exhibited absorption bands at (3510,3380 cm⁻¹) and glycosidic linkage (2835 cm⁻¹). Its ESI MS spectrum showed a molecular ion peak at m/z 422 which correspond to molecular formula of C₁₂H₂₂O₁₁ of a disaccharide.

The ¹H NMR of AM-2 exhibited one proton double doublet at δ 5.06 (j=2.5, 3.2 Hz) and one proton doublet at δ 5.06 (j=6.0 Hz) corresponding to methine proton at C-4 and the anomeric proton at C-1. A one proton multiplet at δ 4.81 was assigned to oxygenated methine proton H-5. The carbinol protons H-4, H-3, H-2, H-2' and H-3' appeared as five one proton multiplets at δ 4.77, 4.74, 4.11, 3.91 and 3.88 respectively. Six one-proton doublets at δ 3.49 (j=6.0 Hz), 3.45 (6.0 Hz), 3.21 (j=6.0 Hz), 3.18 (j=6.0 Hz), 3.14 (j=9.0 Hz) and 3.11 (j=9.0 Hz) were assigned to H₂-6a, H₂-6b, H₂-5'a, H₂-5'b, H₂-6'a, H₂-6'b hydroxy methylene protons.

¹³C NMR spectrum of AM-2 displayed anomeric carbons C-1 and C-1' at δ 104.03 and 91.74 respectively where as the oxygenated methylene carbons C-5', C-6 and C-6' resonated between δ 60.10-62.10. Comparison of the melting point of AM-2 and the authentic sample along with a co TLC pattern confirmed AM-2 to be a disaccharide (sucrose). Its structure has been designated as glucopyranosyl-O-(1→4) fructofuranoside (Maltose).

AM-4: Compound AM-4, was obtained as a crystalline compound from chloroform: methanol (80:20) eluants. Its IR spectrum exhibited absorption bands for hydroxyl group (3350 cm⁻¹), unsaturation (1640 cm⁻¹) and long aliphatic side chain unsaturation (1080 cm⁻¹). ESI MS spectrum showed a molecular ion peak at m/z 376 corresponding to molecular formula of a bicyclic homosesquiditerpenic derivative C₂⁹H₄₄O which was supported by ¹H and ¹³C NMR data also.

The ¹H NMR spectrum of of AM-4 exhibited one proton double multiplet at δ 5.13 assigned to the vinylic proton H-6 while as the H-3 carbinol proton appeared as a double doublet at δ 3.82 (j=8.5, 5.1 Hz) placed in δ orientation. A two proton broad signal at δ 2.56 and two six proton broad signals at δ 1.52 and 1.25 were
assigned to H₂-7, H₂-12, H₂-13, H₂-15, H₂-16, H₂-17 and H₂-19 methylene protons respectively. Rest of the methylene protons resonated between δ 2.50-1.77. A three-proton broad singlet at δ 1.23 and two six proton singlets at δ 1.22 and 0.88 were assigned correspondingly to Me-24 and Me-25 protons respectively. Further evidences in support of the structure were provided by the ¹³CNMR spectrum of AM-4 that displayed a 26 carbons signals. Important signals at δ 140.81 and 127.13 were assigned to vinylic carbons C-5 and C-6 while the carbinolic carbon C-3 appeared at δ-72.08.

On the basis of the above discussions the structure of AM-4 was elucidated as 9-(25,26-dimethyl decanyl)-21,22,23,24-tetramethyl-3-hydroxyperhydronaphthal-5-ene. This is a new phytoconstituent isolated from the plant.

AP-27: Compound AP-27 was obtained as a crystalline compound from chloroform-methanol (90:10) eluants. It responded positively to tetranitromethane and bromine water test for unsaturation. Its IR spectrum exhibited absorption bands for hydroxyl group (3410, 3380 cm⁻¹), carbonyl group (2872, 1725 cm⁻¹), lactone ring (1755 cm⁻¹) and unsaturation (1642 cm⁻¹). Its ESI MS spectrum showed a molecular ion peak at m/z 635 consistent within the molecular formula C₄₀H₇₅O₅ which was supported by ¹³C NMR data also. Other diagnostic peaks appearing at m/z 230 [M-C₁₂H₂₂O₄]⁺, 405 [M-230]⁺ and 505 [M-C₁₇H₁₄O₂]⁺ supported the presence of a di hydroxy hexyl substituted lactone ring in the compound AR-27. Ion fragmentation at m/z 85 [C₉H₁₄]⁺, 125 [C₉H₁₇]⁺, 153 [C₁₁H₂₃]⁺, 193 [C₁₄H₂₈]⁺, 221 [C₁₆H₃₈]⁺, 249 [C₁₈H₃₃]⁺ and 386 [M-249]⁺ suggests the presence of a C₁₈ unit with two vinylic linkage in the compound. C₁₇, C₁₉ and C₁₆, C₁₇ fissions led to appearance of fragment ion peaks at m/z 373[M-C₁₉H₃₄]⁺, 343[M-C₂₀H₃₆O]⁺, 292 [M-343] and 262 [M-373] that suggested the presence of hydroxyl group on C-17.

The ¹H NMR spectrum of AP-27 exhibited two one proton multiplet signals for vinylic H-22 and H-26 at δ 5.26 and 5.13 respectively. Two one proton broad signals centered at δ 4.25 (was assigned to the vinylic proton H-6 while as the H-3 carbinol proton appeared as a double doublet at δ 3.82 (j=8.5, 5.1 Hz) placed in δ
orientation two proton broad signal at $\delta$ 2.56 and two six proton broad signals at $\delta$ 1.52 and 1.25 ($\omega_{1/2} = 18.1$ Hz) and 3.52 ($\omega_{1/2} = 18.5$ Hz) were assigned correspondingly to H-10$\alpha$ and H-5$\alpha$ carbinol protons. Another carbinol proton at C-17 appeared as a one-proton doublet at $\delta$ 4.0 ($j=16.5$ Hz). High coupling constant value supported the $\alpha$-orientation of H-17 proton. Two six proton singlets at $\delta$ 1.54 and 1.24 were attributed to Me-38 and Me-39 and Me-33$'$ Me-35 secondary methyl protons. Other four secondary methyl protons Me-36 and Me-37, Me-32 and Me-40 appeared as doublets, three proton each at $\delta$ 0.95 ($j=6.5$ Hz) $\delta$ 0.91 ($j=6.1$ Hz), $\delta$ 0.86 ($j=6.3$ Hz) and $\delta$ 0.83 ($j=6.5$ Hz) respectively. The primary methyl protons of Me-1 appeared as a three-proton triplet at $\delta$ 0.68 ($j=6.1$ Hz).

The $^{13}$C NMR spectrum of AP-27 displayed forty signals. Vinylic carbon signals appearing at $\delta$ 127.45, 140.92, 120.22 and 137.82 were correspondingly assigned to C-22, C-23, C-26 and C-27 where as the carbonyl carbon C-34 at $\delta$-172.12. The carbinol carbons exhibited signals at $\delta$ 68.43, 61.51 and 64.5 and were assigned to C-3, C-5 and C-17 respectively whereas the oxygenated carbon C-10 appeared at $\delta$ 11.62.

On the basis of the above discussions the structure of AP-27 has been designated as 3,11,15,19,23,27,31-hepta methyl-3$\alpha$, 5$\beta$, 17$\beta$-tri hydroxy-ditriacont-22,26-diene-10,34-olide. This is the first report of this phytoconstituent.

SW-1: Elution of column with petroleum ether furnished white crystalline powder of SW-1 (Fraction No. 41-72), recrystallized with chloroform: methanol (1:1), yield = 0.12%, M.P. 160°C, Rf = 0.4, benzene:petroleum ether(3:1)

$\text{IR}_{\text{w}}$ max 3490, 2950, 2855, 1640, 1470, 1310, 1250, 1065, 850 cm$^{-1}$

$^{1}$H NMR (DMSO-$_d_6$): $\delta$ 5.26 (1H, brs, H -6), 3.58(1H, brs, $W_{1/2}$ 8.35 Hz, H-3$\beta$), 0.95(3H,d, $j=8.1$Hz, H3-20), 0.91(3H,d, (1H, m, H -6), 4.91 (1H, m, H-10), 2.14(2H, m, H2-8), 1.95 (3H,d,J=6.1Hz, Me-21), 0.84(3H, brs, H3-19), 0.65(3H, brs, H3-18).

$^{13}$C NMR (DMSO- $d_6$): $\delta$ 119.94 (C-6), 69.77(C-3), 55.98 (C-14), 55.33 (C-17), 49.50 (C-9), 45.06 (C-8), 41.97 (C-13), 36.71 (C-10), 36.71 (C-4), 35.16 (C-7),
33.23(C-1), 31.13(C-5) 27.61 (C-2) 27.39 (C-11), 25.61 (C-12), 23.55 (C-15), 22.48 (C-16), 20.36(C-19), 19.31(C-20), 18.76(C-21), 11.13(C-18)

+ve ion FAB MS m/z (rel.int.) : (C_{21}H_{34}O); [M]^{+}m/z 302(9.6), 284(11.6), 269(5.6), 254(100), 152(10.5), 138(5.6)

SW-2 : Elution of column with petroleum ether furnished white crystalline powder of SW-1 (Fraction No. 41-72), recrystallized with chloroform:methanol (1:1), yield = 0.12%, M.P. 220°C, R_f = 0.25, benzene:petroleum ether(3:1)

IR_v_max 3461, 2936, 2936, 1642, 1474, 1419, 1375, 1019, 853 cm^{-1}

^1H NMR (DMSO-d_6): δ 5.27(1H, brs, H -7), 4.39(1H, brs, W_{1/2} 9.1 Hz, H-6β), 2.50(3H,brrs, W_{1/2} 9.1, H-β), 2.50(3H,brrs,Me-21), 1.20 (3H, brs, Me-18), 0.96 (3H, brs, Me-19), 0.91(3H,t,J=6.5Hz, Me-17), 0.84(3H, d, j=6.1Hz, Me-22), 0.67(3H, brs, Me-20).

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Jamia Hamdard
SW-3: Elution of column with petroleum ether:chloroform (1:1) furnished white crystalline powder of SW-3 (Fraction No. 41-72), recrystallized with chloroform:methanol (1:1), yield = 0.014%, M.P. 191°C, Rf = 0.6, benzene:petroleum ether(3:1)

IR_{vmax} 3510,2950, 2845, 1710, 1620, 1550, 1280, 1170, 1105, 980, 830 cm^{-1}

^{1}H NMR (DMSO-d_{6}): \delta 7.26 (1H,cl, j=8.7 Hz, H-2), 6.62 d, j=8.7Hz, H-2, 6.62(1H,d,j=8.7Hz-H-3), 6.65 (1H,b.s. H-12), 6.34 (1H,b.s. H-9), 2.19 (3H,b.s.Me-14).

^{13}C NMR (DMSO-d_{6}): \delta 181.75(C-7), 166.84(C-11), 161.75(C-10), 157.10(C-5), 151.69(C-13), 137.02(C-1), 123.73(C-4), 109.15(C-6 101.88(C-8), 97.11(C-2), 97.11(C-12), 92.67(C-3), 55.86(omc), 28.60(C-14)

+ve ion FAB MS m/z (rel.int.): 282[M]^+(C_{15}H_{22}O_{5})

SW-4- Stigmast-5, 22-dien-3β-ol Elution of the column with CHCl_{3} furnished colourless needles of SW-4, recrystallized from CHCl_{3}-MeOH (1:1), Yield =75 mg (0.0025% Yield), Rf = 0.58 (C_{6}H_{10}), m.p. = 115-116^\circ

UV \lambda_{max} (MeOH) 205.5 mm. (Log e 5.2),

IR Vmax (KBr) 3450, 2965, 2870, 1640, 1640, 1475, 1390, 1075,1035, 985cm^{-1}.

^{1}H NMR (CDCl_{3}), \delta 5.33 (1H, b.s, H-6), 5.13 (1H, dd, J=8.10, 8.0 Hz, H-22), 5.04 (1H, dd, J = 8.0, 7.8 Hz, H-23), 3.50 (1H, m, W/2 = 16.50 Hz, H-3α), 2.70 (1H, m, H-20), 2.62 (1H, m, H-24), 2.26 (2H, m, H_{2}-1), 1.99 (2H, m, H_{2}-1), 1.84 (2H, m, H_{2}-1), 1.81 (1H, m, H_{2}-2) 1.481 (4 H, m, 2xH_{2}), 1.25 (3H, b.s, Me-19), 1.00 (3H, d, J = 6.0H, M-21), 0.84 (3H, d, J = 6.6 Hz, Me-26), 0.80 (3H, d, J = 7.3 Hz, Me-29, 0.69 (3H, d, J = 6.20 Hz, Me-27), 0.67(3H, b.s, Me-18)
$^{13}$C NMR (CDCl$_3$) OF SW-4

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<th>C</th>
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ESI MS m/z (rel.int.) 4.2[M+] $^{13}$, (C$_{29}$H$_{48}$O) (41.4), 396(20.0), 350(10.0), 369(11.0), 351(15.11), 329(11.3), 314(31.7), 281(11.2), 273(21.3), 271(22.9), 255(49.8), 213(32.0), 198(15.4), 192(6.1), 179(10.7), 174(12.8), 164(22.7), 160(29.5), 149(17.7), 146(36.6), 138(10.6), 124(29.9), 120(19.6), 106(48.2), 83(73.5), 81(72.8), 72(23.1), 69(71.2), 55(100)

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RESULTS AND DISCUSSIONS

SW-1: Compound SW-1, was obtained as colorless crystalline compound from petroleum ether eluants. Its IR spectrum exhibited absorption bands for hydroxyl group (3490 cm⁻¹) and unsaturation (1640 cm⁻¹). The ESI MS spectrum showed a molecular ion peak at m/z 302 which correspond to molecular formula of a pregnane derivative C₂₁H₃₄O which was supported by the ¹³C NMR data also. Other diagnostically important peaks at m/z 284[M-H₂O]⁺, 269[284-Me]⁺ and base peak at m/z 254[269-Me]⁺ suggested the presence of a hydroxyl group and methyl groups in the compound SW-1 [scheme-1]. Hydroxyl group was placed at C-3 in the α- orientation due to ¹H NMR data that suggested a β- carbinol proton at δ 3.58 with half width of 8.35 Hz. Two fragment ions at m/z 138 and 152 arouse due to C₆₋₇-C₉₋₁₀ fission and C₇₋₈-C₉₋₁₀ fission, respectively and suggested the presence of a vinylic linkage between C₅ and C₆ that was further supported by the vinylic signal at δ 5.26 for H-6 in ¹H NMR and at δ 131.13 and 119.94 for C-5 and C-6 in ¹³C NMR spectra.

The ¹H NMR spectrum of SW-1 displayed two three-proton doublets at δ 0.95 (j= 8.1 Hz) and 0.91 (j= 6.1 Hz) assigned to Me-20 and Me-21 methyl protons, respectively. Two broad signals, integrating of three-protons each, appearing at δ 0.84 and 0.65 were assigned correspondingly to Me-19 and Me-18 methyl protons.

¹³C NMR spectrum further supported the presence of pregnane nucleus by displaying 21 signals. The carbinol carbon C-3 appeared at δ 69.77 while as the methyl carbons C-18, C-19, C-20 and C-21 appeared at δ111.37, 20.36, 19.31, and 18.76 respectively.

On the basis of above discussion the structure of SW-1 has been elucidated as 4-methyl, pregn-5-ene-3-ol. This is the first report of a pregnane derivative from the plant.

SW-2: Compound SW-2 was obtained as a crystalline colourless compound from petroleum ether:chloroform (80:20) eluants. Its IR spectrum exhibited absorption bands for hydroxylic group (3461 cm⁻¹), unsaturation (1642 cm⁻¹) and a long chain side chain (1019, 831 cm⁻¹). The ESI MS of SW-2 showed a molecular ion peak at
m/z 320 corresponding molecular formula of a bicyclic homoditerpene derivative C_{22}H_{40}O that was supported by its H and C NMR data. The 1H-NMR spectrum of SW-2 displayed a broad signal at δ 5.27 assigned to vinylic H-7 proton and another one-proton broad singlet centered at δ 4.39 with half width of 9.1 Hz was assigned to β-oriented carbinol proton H-6. Four three - protons broad singlets at δ 2.50, 1.20, 0.96 and 0.67 were assigned to Me-21, Me-18, Me-19, and Me-20 methyl protons, respectively. A three proton triplet at δ 0.91(j=6.5 Hz) and a three −proton doublet at 50.84(j=6.1 Hz) were assigned correspondingly to Me-17 and Me- 22 methyl protons. Further evidences in support of the structure came from the C NMR data of SW-2 that showed 22 signals. The vinylic carbon signals appeared at δ 119.95(C-7) and 141.36 (C-8) where as the carbinol carbon C-6 appeared at the δ 69.77.

On the basis of above discussion the structure of SW-2 has been elucidated as 9-(22-methyl hexyl)-18,19,20,21-tetramethyl-6-hydroxy perhydronapthal-7-ene. This constituent is reported for the first time from the plant.

SW-3:Compound SW-3, was obtained as a crystalline compound from petroluim ether:chloroform (40:60) eluants. Its IR spectrum exhibited absorption bands for hydroxyl group (3510 cm^{-1}), carbonyl group (2845, 1710 cm^{-1}) and highly conjugated systemn (1170, 1105, 980 cm^{-1}). The ESI MS spectrum showed a molecular ion peak at m/z 282 consistent with the molecular formula C_{15}H_{22}O_{5}.

The 1H-NMR spectrum of SW-3 displayed two ortho-ortho coupled doublets at δ 7.26 (j= 8.7 Hz) and 6.62 (j= 8.7 Hz) assigned to H-2 and H-3 aromatic methine protons respectively. Other two methine proton signals appearing as two one-proton broad singlets at δ 6.65 and 6.34 were assigned correspondingly to H-12 and H-9. A three-proton broad signal due to Me-14 methyl protons appeared at δ 2.19. Further evidences in support of the structure came from the C NMR data of SW-3 that displayed three downfield signals at δ 181.75, 166.84, 161.75 correspondingly assigned to C-7 ketonic carbon and C-11 and C-10 carbinol carbons. The aromatic carbons resonated between δ 157.10-92.67. The C-15
methoxy carbon and the C-14 methyl carbon appeared at 855.86 and 28.60 respectively.

On the basis of above discussion the structure of SW-3 has been elucidated as 4-methyl-5 methoxy -10,11 dihydroxy xanthone. This xanthone derivative is reported for the first time from this plant.

Compound SW- 4, positive to Liebermann-Burchard test, had a molecular ion peak at m/z 412(C_{29}H_{46}O). Its IR spectrum showed absorption bonds for hydroxyl group (3450 cm\(^{-1}\)) and olefinic linkage(1640 cm\(^{-1}\)). Its mass spectrum followed the characteristic fragmentation pattern generating is typical ion peaks of diagnostic importance at m/z 273[M-side chain, [C_{10}H_{19}]^+, 271[271-2H]^+, 255[273-H_2O]^+, 213[255-ring D]^+, and 198[213-Me]^+. The ion peaks at m/z 83[C_{2,3-C_{6,10}-C_{7,8} fission}]^+, 72[C_{1,10},C_{4,5} fission]^+, 138[C_{7,8}-C_{9,10} fission]^+, 120[138-H_2O]^+, 124[138-CH_2]^+, and 106[124-H_2O]^+ indicated the existence of the olefinic linkage in group B at \(\Delta^5\) and hydroxyl group in ring A, placed at C-3 on the basis of biognetic considerations. The saturated nature of ring C was inferred from the ion peak appearing at m/z 164[C_{8,14-C_{9,11} fission}]^+, 146[164-H_2O]^+, 149[164-Me]^+, 178[C_{8,14-C_{11,12} fission}]^+, 160[178-H_2O]^+,192[C_{8,14-C_{12,13} fission}]^+, and 174[192-H_2O]^+. From the evidences it was concluded the molecule was a steroid possessing one olefinic linkage in steroidal carbon framework and another in the side chain.

The \(^1\)H NMR spectrum of 4 exhibited three one-proton downfield signals as a broad singlet at \(\delta 5.33\) and as double doublet at \(\delta 5.13\) (J=8.10,8.0 Hz) and 5.04(J=8.0, 7.8 Hz) assigned to H-6, H=22 and H=23, respectively. A one proton multiplet at \(\delta 3.50\) with half-width of 16.50 Hz was attributed to C-3 carbinol proton magnetically interacting with CH\(_2\)-2 and CH\(_2\)-4. The C-18 and C-19 tertiary methyl signals, appeared as three-proton broad signals at \(\delta 0.67\) and 1.25, respectively. Four doublets at \(\delta 1.00\)(J=6.0 Hz), 0.84(J=6.6 Hz), 0.89(J=6.20 Hz), 0.80(J=7.3 Hz) integrating for C-21, C-26 and C-27 secondary methyl and C-29 primary methyl functionality's.
methoxy carbon and the C-14 methyl carbon appeared at 855.86 and 28.60 respectively.

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The presence of methyl resonance between $\delta$ 1.25-0.67 indicated the existence of these groups at saturated carbons. The $^{13}$C NMR spectrum of CO4 showed 29 carbon signals and the signals at $\delta$ 140.72(C-5), 121.57(C-6), 139.26(C-22) and 129.22 (C-23) were assigned to olefinic carbons. The C-3 carbinol carbon appeared at $\delta$ 71.64. The evidences led to formulate the structure of CO-4 as stigmast-5, 22-dien-3β-ol.