17BTP305 A MEDICINAL PLANT BIOTECHNOLOGY 4H – 4C

Total hours/week: **L: 4 T:0 P:0** **Marks:** Internal:**40** External:**60** Total: **100**

Scope: The paper throws light on the alternative medicine from nature for the betterment of society.

Objective: It aims to impart knowledge to students about the use of medicinal plants and application techniques.

UNIT - I

Phytochemistry: Biosynthesis of primary and secondary metabolites - alkaloids, terpenoids, Phenolic compounds and coumarins. Classification of alkaloids and phenolic compounds.

UNIT - II

General extraction and isolation techniques: Alkaloids and phenolic compounds from plants. Techniques involved in extraction of phytochemicals – Perculation, Soxhlet extraction, reflux and other methods.

UNIT - III

Biotechnology of medicinal plants: Production of secondary metabolites from cultured plant cells, elicitation, immobilization and biotransformation. DNA bar coding. DNA finger-printing of medicinal plants – DNA isolation and fingerprinting techniques.

UNIT - IV

Bioactive studies: Anticancer, antidiabetic, anti-inflammatory, hepatoprotectives, antimicrobials from medicinal plants. Antioxidants of plant origin – Reactive Oxygen Species (ROS). Toxicity studies on medicinal plant products and herbal formulations.

UNIT - V

Pharmacognosy: Authentication of medicinal plants – Organoleptic and other pharmacognostic studies. Anatomical studies. Organic cultivation of medicinal plants

References

Harborne, J.B. (2013). *Phytochemical methods to modern techniques of plant analysis.* London: Chapman and Hall.

Irfan Khan, A., & Atitya Khanum. (2004). *Role of Biotechnology in medicinal and Aromatic plant* (Vols. 1-10). Hyderabad: Ukaaz Publications.

Slater, A., Scott, N.W., & Fowler, M.R. (2008). *Plant Biotechnology: The Genetic Manipulation of plants*. Oxford University press.

KARPAGAM ACADEMY OF HIGHER EDUCATION

DEPARTMENT OF BIOTECHNOLOGY
II M.Sc., BIOTECHNOLOGY – SEMESTER 3
LECTURE PLAN –MEDICINAL PLANT BIOTECHNOLOGY 17BTP305A

S.No	Lecture Duration (hr)	Topic to be covered	Support materials
		UNIT I	
1	1	Phytochemistry - Introduction	T1 Pg 1-10
2	1	Biosynthesis of Primary and Secondary Metabolites – Overview	T1 Pg: 141-145
3	1	Alkaloids – Synthesis, Significance and Uses	T1 Pg: 148-150
4	1	Terpenoids– Synthesis, Significance and Uses	T1 Pg: 150-155
5	1	Phenolic Compounds– Synthesis Significance, and Uses	T1 Pg: 156-158
6	1	Coumarins- Synthesis	T1 Pg: 175-179
7	1	Coumarins- Significance and Uses	T1 Pg: 175-179
8	1	Phenolic and courmarin analysis	T1 Pg: 175-179
9	1	Classification of Alkaloids	T1 Pg: 185
10	1	Classification of Phenolic Compounds	T3 pg: 471-484
11	1	Revision	
		Unit II	
12	1	General extraction and isolation techniques	T1 385-390
13	1	Alkaloids compounds from Plants	T1 Pg: 231-232
14	1	Phenolics compounds from Plants	T1 Pg: 239-242
15	1	Techniques involved in Phytochemical Extraction	T1 Pg: 405
16	1	Percolation	T1 Pg: 406
17	1	Soxhlet Extraction	T1 Pg: 409
18	1	Reflux Extraction	T1 PG: 433
19	1	Extraction Techniques in extraction	T1 Pg 391-393

UNIT III			
20	1	Biotechnology of medicinal Plants- Introduction and its Importance	T3 (46-48)
21	1	Secondary Metabolite Production from Cultured Plant Cells	T2 Pg: 5.1-5.4
22	1	Elicitation	T2 Pg: 5.5-5.8
23	1	Immobilization	T2 Pg: 5.9 – 5.11
24	1	Biotransformation	T2 Pg: 5.11- 5.17
25	1	DNA Barcoding of Medicinal Plants	T3: 385
26	1	DNA fingerprinting of Medicinal Plants	T3 Pg: 350
27	1	DNA Isolation – Fingerprinting Techniques	T3 Pg: 352-355
28	1	DNA Isolation – Fingerprinting Techniques in medicinal plants	T3 Pg: 352-355
29		Revision	
UNIT IV			
30	1	Screening of different properties from medicinal plants- Introduction	T3(129-130)
31		Bioactive studies from Plants	T3(129-132)
32	1	Anticancer drugs from Plants	T4 Pg: 569
33	1	Antidiabetic Drugs from Plants	T4 Pg: 512
34	1	Antiinflammatory compounds from Plants	T4 Pg:582
35	1	Hepatoprotective and antimicrobial activity from plants	T4 Pg: 578
36	1	Antimicrobial properties of screening from medicinal plants	T3 Pg 825-834
37	1	Antioxidants from Plants	T3 Pg: 557
38	1	Toxicity studies on Medicinal Plants	T2 Pg: 3.01 - 3.92; T3: 605
39	1	Herbal formulations of Drugs	T1: 454 – 461; T3: 645-650
40	1	Revisions	
UNIT V			
41	1	Pharmacognosy	T1 Pg: 276

42	1	Authentication of Medicinal Plants	T1Pg: 110-115
43	1	Organoleptic evaluation of Plants	T1 Pg: 29-56
44	1	Pharmacognostic studies	T3(129-132)
45	1	Anatomical studies of Plants	T1 Pg:69-87
46	1	Organic cultivation of Medicinal Plants- Introduction	T1 Pg: 557-558
47	1	Organic cultivation of Medicinal Plants	T1 Pg: 557-558
48	1	Revisions	

Reference Books

1. T1- BIREN SHAH (2009), Textbook of Pharmacognosy and Phytochemistry, 1st Edition; Elsevier India Limited.
2. T2 - C.K.KOKAT; A.P.PUROHIT (2008), PHARMACOGNOSY; 2008.42nd Edition; Nirali Prakashan Publishers.
3. T3- H.S.CHAWLA (2003), INTRODUCTION TO PLANT BIOTECHNOLOGY. Oxford Publishing Pvt. Ltd
4. T4: S.S.AGARWAL (2007), HERBAL DRUG TECHNOLOGY, University Press.

UNIT-I

SYLLABUS

Phytochemistry: Biosynthesis of primary and secondary metabolites- alkaloids, Terpenoids. Phenolic compounds and coumarins. Classification of alkaloids and phenolic compounds

A **metabolite** is an intermediate end product of metabolism. The metabolite is usually restricted to small molecules.

Metabolites have various functions, including fuel, structure, signaling, stimulatory and inhibitory effects on enzymes, catalytic activity of their own (usually as a cofactor to an enzyme), defense, and interactions with other organisms (e.g. pigments, odorants, and pheromones).

A **primary metabolite** is directly involved in normal "growth", development, and reproduction. Ethylene is an example of a primary metabolite produced in large-scale by industrial microbiology.

A secondary metabolite is not directly involved in those processes, but usually has an important ecological function. Examples include antibiotics and pigments such as resins and terpenes etc.

Secondary metabolism produces a large number of specialized compounds (estimated 200,000) that do not aid in the growth and development of plants but are required for the plant to survive in its environment.

Secondary metabolism is connected to primary metabolism by using building blocks and biosynthetic enzymes derived from primary metabolism. Primary metabolism governs all basic physiological processes that allow a plant to grow and set seeds, by translating the genetic code into proteins, carbohydrates, and amino acids.

Specialized compounds from secondary metabolism are essential for communicating with other organisms in mutualistic (e.g. attraction of beneficial organisms such as pollinators) or antagonistic interactions (e.g. deterrent against herbivores and pathogens). They further assist in coping with abiotic stress such as increased UV-radiation.

The broad functional spectrum of specialized metabolism is still not fully understood. In any case, a good balance between products of primary and secondary metabolism is best for a plant's optimal growth and development as well as for its effective coping with often changing environmental conditions.

Well known specialized compounds include alkaloids, polyphenols including flavonoids, and terpenoids. Humans use quite a lot of these compounds, or the plants from which they originate, for medicinal and nutraceutical purposes.

Secondary Metabolites types in plants

There is no fixed, commonly agreed upon system for classifying secondary metabolites. Based on their biosynthetic origins, plant secondary metabolites can be divided into three major groups.

1. Flavonoids and allied phenolic and polyphenolic compounds,
2. Terpenoids and
3. Nitrogen-containing alkaloids and sulphur-containing compounds.

Other researchers have classified secondary metabolites into following, more specific types.

Class	Number of known metabolites	Examples
Alkaloids	21000	Cocaine, Psilocin, Caffeine, Nicotine, Morphine, Berberine, Vincristine, Reserpine, Galantamine, Atropine, Vincamine, Quinidine, Ephedrine, Quinine
Non-protein amino acids (NPAAs)	700	NPAAs are produced by specific plant families such as Leguminosae, Cucurbitaceae, Sapindaceae, Aceraceae and Hippocastanaceae. Examples: Azatyrosine, Canavanine
Amines	100	-
Cyanogenic glycosides	60	Amygdalin, Dhurrin, Linamarin, Lotaustralin,

Class	Number of known metabolites	Examples
		Prunasin
Glucosinolates	100	-
Alkamides	150	-
Lectins, peptides and polypeptides	2000	Concanavalin A
Terpenes	>15,000	Azadirachtin, Artemisinin, Tetrahydrocannabinol
Steroids and saponins	NA	These are terpenoids with a particular ring structure. Cycloartenol
Flavonoids and Tannins	5000	Luteolin, tannic acid
Phenylpropanoids, coumarins and lignans	2000	Resveratrol
Polyacetylenes, fatty acids and waxes	1500	-
Polyketides	750	-
Carbohydrates and organic acids	200	-

Tropane alkaloids are mainly found in the Solanaceae and include the anticholinergic drugs atropine, hyoscyamine, and scopolamine and the narcotic cocaine.

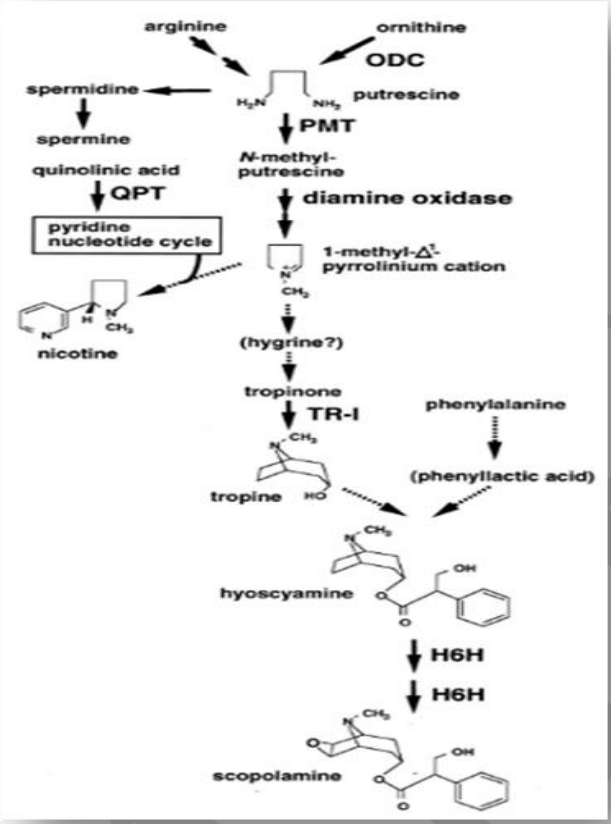
N-methylputrescine, the central precursor in tropane alkaloid biosynthesis, is also an intermediate in the nicotine pathway. N-methylputrescine is produced by the decarboxylation of ornithine or arginine by ornithine decarboxylase (ODC) or arginine decarboxylase (ADC), respectively.

Tropane alkaloids and nicotine biosynthesis are also closely related to polyamine metabolism. The first committed step in tropane/nicotine alkaloid biosynthesis is catalyzed by the SAM-dependent putrescine N-methyltransferase (PMT) which is highly homologous to spermidine synthase.

Methylputrescine is subsequently deaminated by a diamine oxidase, and spontaneous cyclization then forms the reactive N-methyl-Δ^1-pyrrolinium cation. The latter is thought to provide a precursor of the tropane ring or nicotinic acid to form nicotine, although details are not available.

PMT in nicotine biosynthesis is expressed specifically in the cortex and endodermis of tobacco root tips, whereas strong expression is seen in the xylem parenchyma and outer cortex cells in more differentiated parts of the root.

Hyoscyamine is produced by condensation of tropine and the phenylalaninederived intermediate (R)-phenyllactate. Hyoscyamine can be converted to its epoxide scopolamine via 6β-hydroxylhyoscyamine by a 2-oxoglutarate-dependent dioxygenase, hyoscyamine 6β-hydroxylase (H6H).H6H localizes in the pericycle in branch roots of several scopolamine-producing Solanaceae plants. Histochemical analysis using *H. niger* and *A. belladonna* H6H promoter::GUS fusion gene also showed that cell-specific expression of the H6H gene is controlled by (unknown) genetic regulation specific to scopolamine-producing plants but is absent in tobacco that does not produce scopolamine.



Biosynthesis of Plant Phenolics

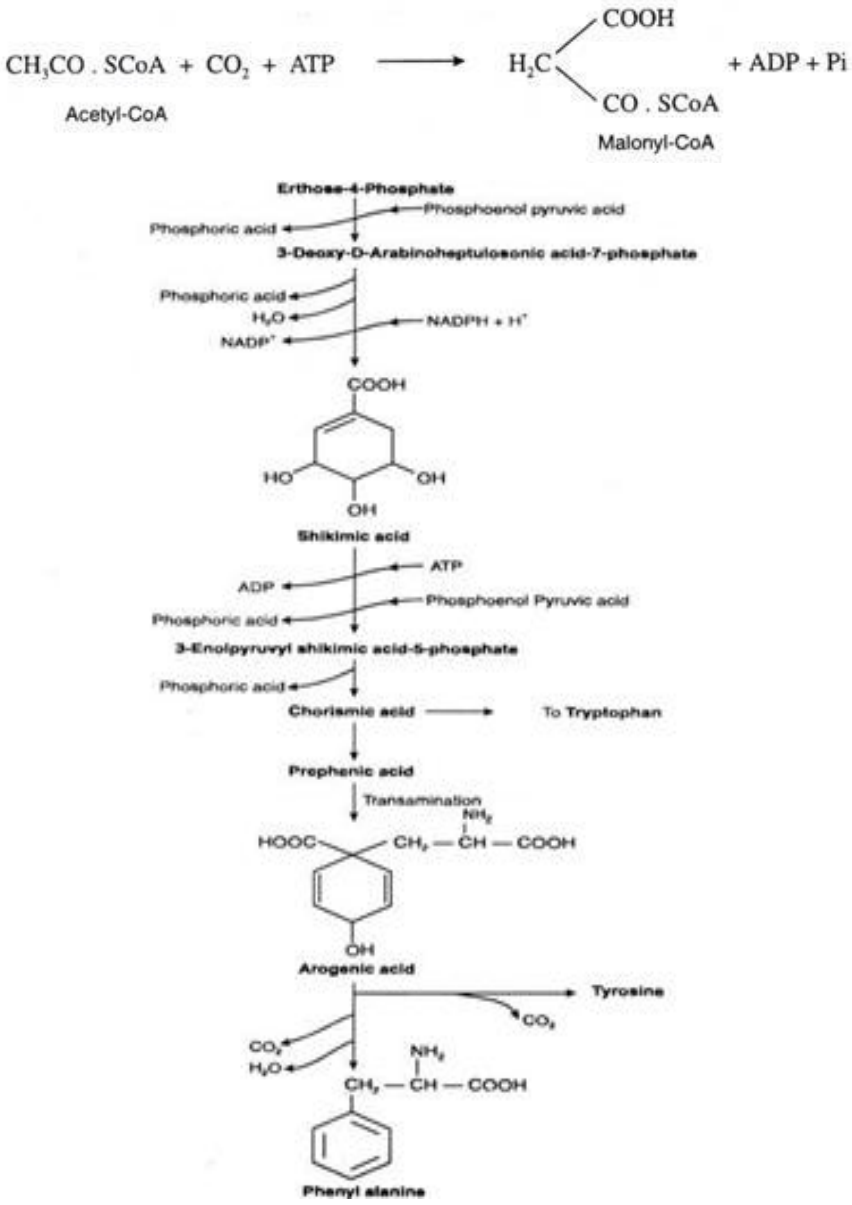
Except flavonoids, all other plant phenolics are biosynthesized in plants from a common biosynthetic intermediate, phenylalanine or its close precursor shikimic acid through shikimic acid pathway.

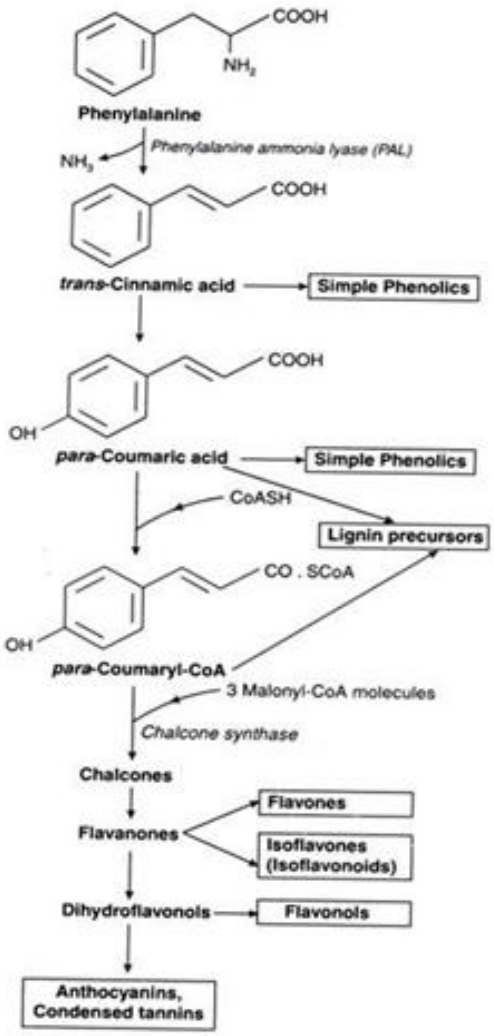
The starting metabolites in this pathway are erythose-4-phosphate and phosphoenol pyruvate which are intermediates of pentose phosphate pathway and glycolysis respectively.

In case of flavonoids which have $C_6 – C_3 – C_6$ carbon skelton, one aromatic ring and its side chain arises from phenyl alanine while the other aromatic ring arises from acetyl-CoA via malonic acid pathway.

The first step in the synthesis of phenolic compounds from phenylalaine in plants is deamination of phenylalanine by the enzyme phenylalanine ammonia lyase (PAL). The latter is most extensively studied enzyme of secondary metabolisim in plants.

Malonyl-CoA can be produced from acetyl-CoA in the presence of the enzyme acetyl-CoA carboxylase as follows:





Biosynthesis of Terpenoids

The 5-carbon isomers isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) are the fundamental building blocks used to synthesize key biological isoprenoids (terpenoids) including cholesterol and other steroids, carotenoids, saponins, and limonoids. Two metabolic pathways exist for the biosynthesis of isopentenyl pyrophosphate and dimethylallyl pyrophosphate:

- The **mevalonate pathway**, predominantly used by plants and in a few insect species
- The **non-mevalonate pathway** or methyl D-erythritol 4-phosphate (MEP) pathway, which occurs in plant chloroplasts, algae, cyanobacteria, eubacteria, and important pathogens such

as Mycobacterium tuberculosis and malaria parasites.

Mevalonate Pathway

The mevalonate pathway performs several key functions within cells and is an important central metabolic pathway in all higher eukaryotic cells.

The key isomers dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP) are produced via the mevalonate pathway from (R)-mevalonate and its subsequent phosphorylated metabolites (R)-mevalonate-5-phosphate and (R)-mevalonate-pyrophosphate.

DMAPP and IPP are further utilized in condensation reactions for the biosynthesis of isoprenoids. These isoprenoids are transformed to more complex, cyclised structures through steroid and terpenoid biosynthesis and are involved in protein prenylation and protein anchoring.

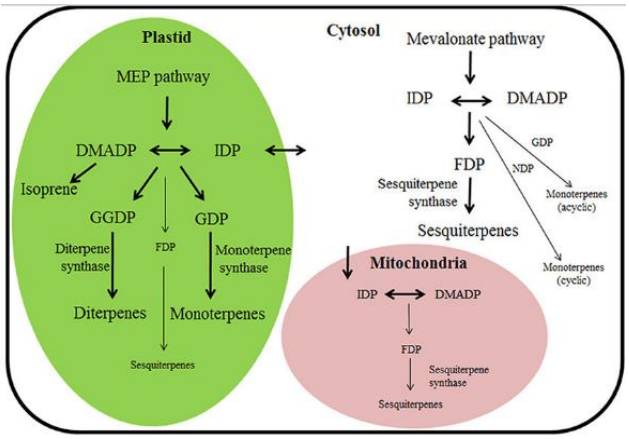
Mechanisms for feedback regulation of low-density lipoprotein receptors and enzymes involved in mevalonate biosynthesis ensure that sufficient mevalonate is available to generate the required quantity of DMAPP and IPP. The mevalonate pathway is of biomedical interest in certain types of cancer as well as heart disease, and a number of therapeutic drugs target this regulatory system.

Non-mevalonate (MEP) Pathway

The mevalonate-independent pathway for the biosynthesis of IPP and DMAPP was discovered in the 1990's and consists of eight enzyme-catalyzed reactions. Synonyms for this pathway are the non-mevalonate pathway, the 1-deoxy-D-xylulose-5-phosphate pathway (DXP (or DOXP) pathway), and the 2C-methyl-D-erythritol-4-phosphate pathway, (MEP pathway).

The MEP pathway starts with the condensation of pyruvate and D-glyceraldehyde-3-phosphate to 1-deoxy-D-xylulose-5-phosphate (DXP or DOXP). The key isomers DMAPP and IPP are subsequently formed via a series of enzymatic steps starting with the conversion of DXP to 2C-methyl-D-erythritol-4-phosphate (MEP).

Enzymes of this MEP pathway are attractive targets for the development of drugs targeting infectious diseases such as malaria and tuberculosis, because this pathway occurs in pathogenic prokaryotes but is absent in human metabolic pathways.



Non-mevalonate (MEP) Pathway

Despite the enormous structural differences between terpenoids, they are all derived from the same C_5 skeleton of isoprene. The terpenoidal backbone is synthesized from the two precursors: isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) through a different number of repeats, rearrangement and cyclization reactions.

Two distinct biosynthetic pathways for the formation of these universal precursors have been reported, the classical mevalonate (MVA) pathway and the most recently characterized 2C-methyl-D-erythritol-4-phosphate (MEP) pathway, also known as the 1-deoxy-D-xylulose- 5-phosphate (DXP) pathway.

The MVA pathway is present in eukaryotes (all mammals, the cytosol and mitochondria of plants, fungi), archaea, and some eubacteria while the non-mevalonate pathway occur in eubacteria, algae, cyanobacteria, and the chloroplasts of plants. The MVA pathway comprises seven enzymatic reactions to convert the precursor acetyl-CoA to IPP and DMAPP while the MEP pathway converts the starting materials, pyruvate and glyceraldehyde-3-phosphate, to IPP and DMAPP through eight enzymatic reactions.

The linear prenyl diphosphates such as geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), geranylgeranyl pyrophosphate (GGPP), and farnesyl geranyl pyrophosphate (FGPP) are synthesized from the two basic building blocks, IPP and DMAPP where a group of enzymes called prenyltransferases repeatedly add the active isoprene unit (IPP) to DMAPP or a prenyl diphosphate in consecutive head-to-tail condensations leading to the production of a range of

molecules with fixed lengths and stereochemistry. Geranyl pyrophosphate synthase (GPPS) and farnesyl pyrophosphate synthase (FPPS) catalyze the condensation of IPP and DMAPP to produce GPP (C_{10}) and FPP (C_{15}). Geranylgeranyl pyrophosphate synthase (GGPPS) and farnesyl geranyl pyrophosphate synthase (FGPPS) are responsible for formation of GGPP (C_{20}) and FGPP (C_{25}).

The precursors GPP, FPP, GGPP and FGPP, are cyclized and/or rearranged by different terpene synthase enzymes to produce the different classes of Terpenoids.

Biosynthesis of phenolic compounds

Phenolic compounds are the most studied secondary metabolites because of their considerable involvement in plant-environment interactions. They are molecules belonging to very diverse chemical families having in common an aromatic ring bearing at least one phenol hydroxyl substituent. Some phenolic compounds have several hydroxyl group substituents, which can undergo esterification, methylation, etherification or glycosylation. The molecular weight of phenolic compounds is variable, being lower in simple compounds, higher in those with complex structures, and higher still in polymerized tannins.

Phenolic compounds are classified according to:

– the nature and complexity of the carbonaceous skeleton;

– the degree of skeletal modification (degree of oxidation, hydroxylation, methylation, etc.);

– the link between the base unit and other molecules such as carbohydrates, lipids, proteins, or the link to other secondary metabolites, possibly polyphenols.

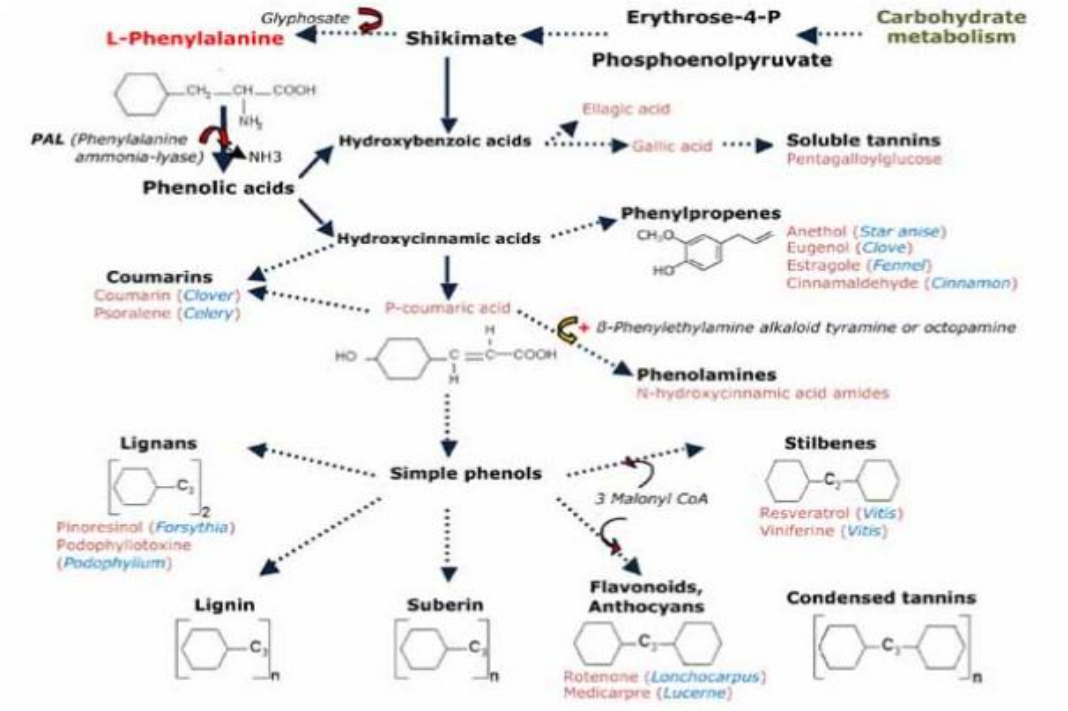
The phenolic compounds of plants include the simplest forms (hydroxybenzoic and hydroxycinnamic acids), condensed forms (tannins), and forms related to non-phenolic macromolecules (certain glucidic components of the pecto-cellulosic wall, cutin and suberin). Only two groups of phenolic compounds are mainly related to the plant walls: firstly, low-molecular-weight hydroxycinnamic acids related to various cell wall compounds, and secondly, lignins, which are polymers of monolignol units bound by oxidative coupling

The biosynthesis pathway of phenolic compounds is well characterized. Phenolic compounds are formed via the well-known shikimate pathway from simple sugars resulting from primary

metabolism.

Synthesis of phenylalanine begins with carbohydrates transforming into erythrose-4-phosphate (pentose phosphate pathway) and phosphoenolpyruvate (glycolysis), which enter into the shikimic acid metabolic pathway.

Phenylalanine deamination by phenylalanine ammonia-lyase (PAL) is the first crucial stage in the biosynthesis of the large majority of phenolic compounds. In addition to its involvement in the production of phenolic compounds, phenylalanine oxidation also leads to the formation of tyrosine, the precursor of catecholamines (dopamine and derivatives) which are abundant compounds in the banana tree and fruit.



Biosynthesis of Phenolic compounds and coumarins

Simple phenols (C6). These are compounds with one (monophenol-like catechin) or several phenolic groups (di, tri- and oligophenols): phenol, benzoquinone, pyrogallol, pyrocathecol, etc.

Phenolic acids (C_6-C_1 or C_6-C_3). These are benzoic or hydroxybenzoic acids (gallic acid, ellagic acid), and cinnamic or hydroxycinnamic acids such as caffeic, coumaric, ferulic, and chlorogenic acid. A group of small phenolic molecules is derived from the subclass of hydroxycinnamic acids

and is called phenylpropenes.

Flavonoids (C_6-C_3-C_6). These are present in plant vacuoles, where they are sometimes water-soluble or sometimes act as pigments. Flavonoids are the most abundant phenolic compounds in nature and are classified according to the degree of oxidation and unsaturation of their heterocyclic ring. Two classes of flavonoids can be distinguished: 4-oxoflavonoids and anthocyanidins .

Lignins (C_6-C_3)n. These are extremely complex phenolic polymers. Of the biopolymers, lignins rank second in abundance after cellulose. The synthesis of these compounds results from a three-dimensional polymerization of three basic phenolic molecules (called monolignols): coumarylic, coniferylic and sinapylic alcohol, corresponding respectively to p-coumaric, ferulic and sinapic acid. The complexity of lignins results from the potential association of these units *via* various chemical bonds, in a manner that is neither ordered nor repetitive, so as to generate an amorphous, hydrophobic polymer.

Tannins (C_6-C_3-C_6)n. These are found in several forms with different types of chemical reactivity and composition: water-soluble tannins, condensed tannins, catechic tannins and proanthocyanidins . Proanthocyanidins have a high molecular weight and are a group of condensed (chain dimers or oligomers) flavan-3-ols often related to cell walls.

The variability of phenolic compound classes in plants is far more complex than presented above. This is only intended to familiarize the reader with the classes of compounds relevant to the research topic

In plants, the phenolic composition varies considerably both qualitatively and quantitatively between species and between individuals of the same species. Some phenolic compounds are ubiquitous in plants (e.g. hydroxycinnamic acids), some are common (e.g. anthocyanins) and others are specific to certain families or species (e.g. isoflavonoids and stilbenes).

Besides plant variety, a wide range of biotic and abiotic stresses (physical, chemical and biological factors) both external and endogenous can influence the level of plant phenolic content both before and after harvest.

Changes in the level of plant phenolic content are effected through modulation of the phenolics metabolism: light (visible and UV), temperature, osmotic potential, plant nutrition, growth

regulators, biotic elicitors, the fruit maturation state at harvest, the photoperiod, moisture, processing, and storage.

This leads to an alteration of a very broad palette of functions, including the release or synthesis of stress metabolites involving polyphenols. For example, environmental stresses can affect photosynthesis and the assimilation of carbon required for growth, development and defense, leading to an influence on the final phenolics concentration in plant tissues. In the following sections, the influence of biotic and abiotic factors on phenolics synthesis is fully discussed.

Classification of Alkaloids:

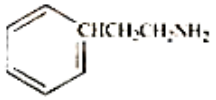
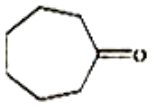
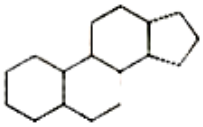
Alkaloids are basic nitrogen containing compounds. They are generally obtained from plants, animals and microorganisms and often demonstrate a marked physiological action. Alkaloids show greatly diverse structure and origins as well as pharmacological action.

The only thing that unites all these natural compounds under the term 'alkaloids' (alkali-like) is the nitrogen atom which is present in all of them. According to pharmacognosy, alkaloids are broadly classified into two classes depending upon whether the nitrogen is a part of a ring or not.

1] Non-Heterocyclic Alkaloids or Atypical Alkaloids:

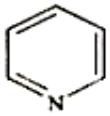
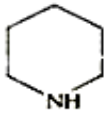
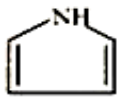
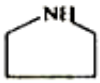
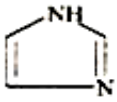
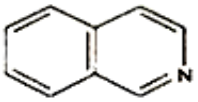
These are also sometimes called proto-alkaloids or biological amines. These are less commonly found in nature. These molecules have a nitrogen atom which is not a part of any ring

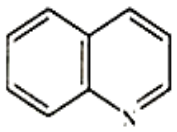
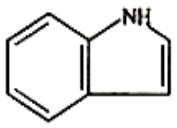
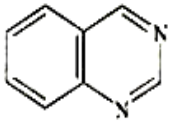
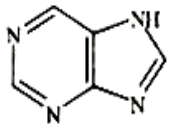
system. Examples of these include ephedrine, colchicine, erythromycin and taxol etc. Table below shows the chemical structure and biological significance of these compounds

S. No	Type of alkaloids	Structure	Source	Example	Use
A	NON-HETEROCYCLIC ALKALOIDS				
1	Phenyl ethyl amine		Ephedra	Ephedrine Pseudo ephedrine	Asthma
2	Tropolone		Colchicum	Colchicines	Gout & polypeptide
3	Steroidal		Kurchi Veratrum	Conessine Veratramine	Dysentery Hypertensive

2] Heterocyclic Alkaloids or Typical Alkaloids:

Structurally these have the nitrogen as a part of a cyclic ring system. These are more commonly found in nature. Heterocyclic alkaloids are further subdivided into 14 groups based on the ring structure containing the nitrogen.

B	HETEROCYCLIC ALKALOIDS				
1	Pyridine and		Lobelia	Lobeline	Spasmodic. Asthma
	Piperidine		Hemlock piper	Coniine Piperine	Gonorrhoea
2	Pyrrole and		Coca	Hygrine	C.N.S.
	pyrrolidine		Tobacco	Nicotine	Stimulant
3	Imidazole		Pilocarpus	Pilocarpine	Atropine antagonist
4	Isoquinoline		Opium	Papaverine Narcotine Morphine	Narcotic Analgesic
			Cephaelis	Emetine	Emetic

5	Quinoline		Cinchona	Quinine Quinidine	Antimaleria l Antiarrhyth emetic
6	Indole		Ergot Rauwe'fia Vinca Nux-vomica	Ergotamine Reserpine Vinblastin, Vincristin Strychnine	Oxytocic Hypertensi on Anticancer Insecticide
7	Quinazolin		Vasaka	Vasicine	Antitissue
8	Purine		Tea, Coffee Coca	Caffeine Theobromine	C.N.S. stimulant Asthma
9	Tropane		Datura Belladonna Coca	Atropine Hyosine Cocaine	Parasympat hetic depressant Parasympat hetic depressant Local anesthetics
10	Terpenoid		Aconitum	Aconitine	Bitter tonic
11	Aporphine		Peumus	Boldine	

Classification of Plant Phenolics

Plant phenolics may be classified into major categories on the basis of the no. of C-atoms and basic arrangement of carbon skeletons in their structure. The phenolics containing 6-10 C-atoms and basic carbon skeletons C_6, $C_6 - C_1$, $C_6 - C_2$, $C_6 - C_3$ and $C_6 - C_4$ as mentioned in the above table are sometimes grouped together as simple phenolic compounds or simple phenolics. Elaborations of basic carbon skeleton produce large array of phenolic compounds.

Table 24.2. Major Categories of Plant Phenolics.

No. of C-atoms	Basic carbon skeleton	Major category
6	C_6	Phenols
7	$C_6 - C_1$	Phenolic acids
8	$C_6 - C_2$	Phenylacetic acids, Hydroxy cinnamic acids, Phenyl propenes
9	$C_6 - C_3$	Coumarins, Isocoumarins, chromones
10	$C_6 - C_4$	Naphthoquinones
13	$C_6 - C_1 - C_6$	Xanthones, Stilbenes
14.	$C_6 - C_2 - C_6$	Anthraquinones
15.	$C_6 - C_3 - C_6$	Flavonoids
18.	$[C_6 - C_3]_2$	Lignans, Neolignans
30.	$[C_6 - C_3 - C_6]_2$	Biflavonoids
n	$[C_6 - C_3]_n$	Lignins
	$[C_6]_n$	Melanins
	$[C_6 - C_1 - C_6]_n$	Condensed tannins (Flavolans)

Possible Questions

Short questions

1. Describe the structure of alkaloids.
2. What is importance of extraction methodology?
3. Explain Terpenoids.
4. Explain pharmacognosy
5. Explain phytochemistry.
6. Write about the Terpenoids and its uses in medicinal plants.
7. What is phenols and its uses?
8. Write short note on tropane alkaloids?
9. Define phenolic compounds.
10. What is a metabolite?
11. Define a primary metabolite.
12. What is secondary metabolite?
13. What are coumarins?

Essay type questions

1. Explain the biosynthesis of alkaloids.
2. Give in detail about the biosynthesis of Tropane alkaloids and nicotine.
3. Explain in detail about the biosynthesis of phenolic compounds
4. Explain in detail about the berberine synthesis
5. Write in detail about the classification of alkaloids
6. Discuss the classification of phenolic compounds

UNIT-II

SYLLABUS

General extraction and Isolation Technique: Alkaloid and phenolic compounds from plants. Techniques involved in the extraction of phytochemical – Percolation, Soxhlet extraction, reflux and other methods.

Secondary metabolites are compounds which have no primary function in the life processes of the organism which synthesized them. Chemicals which are used in interspecific interactions are rarely used in life processes and so are considered to be secondary compounds. Some of the best studied secondary metabolites come from the plant kingdom.

Secondary metabolites are thought to be biologically active in organisms due mainly to their secondary nature. By the same token, primary compounds are used by every organism so that each individual should have the molecular machinery to breakdown these compounds.

Although more than 3,000 alkaloids have been isolated from about 4,000 species of plants, their function and the functions of other secondary compounds in plants is still a topic of debate. Some proposed functions in plants include:

- ✓ Alkaloids could function as nitrogen waste products like urea in mammals.
- ✓ Alkaloids could act as nitrogen storage reservoirs for use by the plant in nitrogen limiting situations.
- ✓ Secondary metabolites could serve as defense for the plant from parasites or other pathogens or herbivores.
- ✓ Secondary metabolites could serve as growth regulators.Secondary metabolites may be used to help the plant maintain ionic balance, especially when the roots take up ions.

  Although the debate on function continues, due to their biological activity in many animals, scientists have studied secondary compounds, including alkaloids, from the view that they are defense compounds.
- ✓ Plants are very well represented in the list of organisms which contain secondary metabolites which could be an effect of their life history. Animals are mobile and able to use this trait as a mechanism of defense against many predators. Plants, however, are relatively immobile and thus have had to develope alternative mechanisms for defense.

Secondary compounds, like alkaloids and phenols, have been of interest and have been studied for many years due to their effects (both physiological and psychological) on humans and

other animals. Morphine, an alkaloid from the opium poppy, was the first alkaloid to be isolated and crystalized.

Alkaloids in plants

The alkaloids represent a large group of chemical compounds found in many plants which are mainly identified by their nitrogen containing, heterocyclic rings. As may be inferred by their name, alkaloids are usually somewhat basic, with the nitrogen usually accepting the protons.

At physiological pH found in the cytosol and the vacuoles, the nitrogen is protonated making the alkaloid water soluble. As defensive compounds, alkaloids "advertise" their presence as severely bitter tasting.

For example, quinine is an alkaloid found in cinhona bark which tastes bitter. Actual toxicity, however, is dependant on the dosage involved. Solanine is present in tiny amounts in all eating potatos, but the concentration only reaches hazardous amounts in tubers which have grown andbeen exposed above the ground. Some alkaloids can prove to be fatal to some organisms but useful to others. Pyrrolizidine alkaloids, from weeds of genus Senecio.

Phenolics in plants

Phenolics are ubiquitously found across the plant kingdom, with ~10,000 structures identified to date.

With a few notable exceptions, phenolic compounds are synthesized from precursors produced by the phenylpropanoid pathway. Structurally, they share at least 1 aromatic hydrocarbon ring with 1 or more hydroxyl groups attached. The simplest compound with this structural motif is the phenol molecule, which itself does not occur in plants.

Phenolics range from simple low-molecular weight compounds, such as the simple phenylpropanoids, coumarins, and benzoic acid derivatives, to more complex structures such as flavanoids, stilbenes, and tannins. Of these, the flavonoids represent the largest, most diverse group, encompassing some 6000 compounds, all of which share a common underlying structure of two 6-carbon rings, with a 3-carbon bridge, which usually forms a 3rd ring. Flavanoids can then be subdivided according to modifications of this basic skeleton into chalcones, flavones, flavonols, flavanones, isoflavones, flavan-3-ols, and anthocyanins.

Curcumin

Curcumin, a curcuminoid polyphenol responsible for the bright yellow color of the Indian spice turmeric (*Curcuma longa* L.), has been utilized for centuries within the Ayurvedic system of medicine for the treatment of a whole host of ailments, including inflammation. Within the plant, curcumin is associated with potent suppression of bacteria, fungi, and viruses, with these effects also observed both in vitro and in animal models.

Resveratrol

The phytoalexin resveratrol (3, 4′, 5 trihydroxystilbene) is produced within a range of edible plants in response to tissue damage and environmental stressors such as fungal and viral attack. Antifungal effects have also been observed against human pathogenic fungi and antiviral effects against the herpes simplex virus.

Techniques involved in the extraction of phytochemical:

The active ingredients contained in the plants and that their action can be more effective, it is necessary to perform several procedures through which are extracted the active ingredients with the adequate solvents, selected according to the solubility and stability of the beneficial substances. Extraction methods allow to obtain products in pharmaceutical forms suitable for oral or external dosage according to the place of action recommended.

These preparations are known as: decoctions, infusions, fluid extracts, dense or dry (according to their content of liquids) and tinctures. They are also known as galenic formulations, in honor to Claudius Galen the precursor of preparing drugs from plants.

From these procedures have been developed extractive techniques that allow to obtain active ingredients in pure form for more sophisticated medications processing: in the form of pills, liquids, ointments, capsules, etc., but they have failed to displace the original preparations which have become more booming at present, because of being more innocuous and the lower unwanted reactions.

Pharmacopoeias have included within its specifications regulations scientifically based to ensure the quality of these preparations, which do not require an accurate control as the official drugs, although some care must be taken in terms of preservation and storage time. Immediate

use is preferred because of its ease of preparation and be available at any time from the medicinal plant.

Prior to extraction treatment the plant must be cleaned carefully to avoid contamination with other plants or foreign mechanical particles. The aim is the extraction of substances using an appropriate solvent called menstruum solvent.

These extractions are distinguished from real solutions in which suspended substances are present.

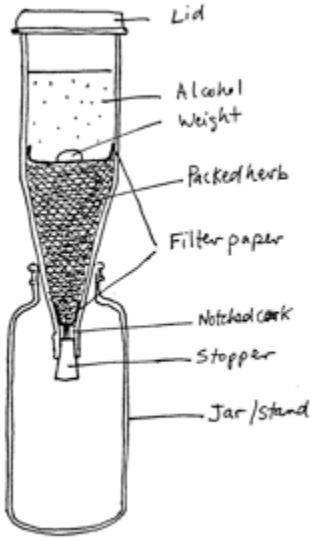
The main extraction methods are:

- Maceration
- Percolation
- Digestion
- Infusion
- Decoction

To get the **maceration** process the plant material is placed in peaces or powder, depending on convenience, in a container full of menstruum and let stand for three or more days, shake frequently until complete extraction of plant material. After this, the material is strained and the remaining solid is squeezed to remove all the remaining liquid. The obtained liquid is clarified by decantation or filtration. The **maceration** is performed at room temperature and liquids that are most frequently used are water and alcohol, or both combination, although can also be used red or white wines. **The maceration** in water should not be prolonged for too long as this can present fungal contamination, which does not occur in alcohol orhydroalcoholic solutions. Total maceration time depends on the type of plant or the part thereof, or the active ingredient to extract.The most commonly used ratio is 1:20 herb/ liquid by Drug analysis methods

The percolation is the most common procedure for the preparation of tinctures and fluid extracts. The percolator is a conical vessel with a top opening in which is placed a circular drilled lid allowing the pass of liquid and subjecting the materials placed on it to a slight pressure. The bottom has an adjustable closure to allow passage of the fluid at a convenient rate.

- The plant material is moistened prior to their placement in the percolator with a proper amount of menstruum, it´s placed in a sealed container and leave stand for approximately four hours.
- After that time the plant material must be conveniently placed in the percolator so as to allow the even passage of fluid and the complete contact with the plant material. The percolator must be filled with liquid and covered up.
- The bottom outlet is opened until get a regular dripping and then closes. More menstruum is added to cover all the material and must stand to soak in the percolator closed for 24 hours. After this time leave it to drip slowly and added enough menstruum to a proportional volume of 3/4 of the total volume required for the final product.
- The wet mass is pressed to extract the maximum residual fluid retained and supplemented with sufficient menstruum to get the proper proportion, it´s filtered or clarified by decantation.



- Once the powder is properly moistened it is time to pack it into the percolator. Start by finding a cork that fits into the end of the percolator. Cut 3/8 to 1/2 inch off of the end of the cork and notch it with a knife in the shape shown in the illustration.

- Push it just inside the neck of the cone and follow it up with the rest of the cork thereby sealing the bottom of the percolator. Set the percolator on its stand or into the neck of a honey jar or two quart canning jar.
- Cut and form a small cup shape with the filter paper, add some moistened herb to the cup and gingerly position it down into the tip of the cone. (The bottle is now up side down with the neck sitting inside the jar). Pack this down until it pushes up against the notched cork. Add more herb and pack it down onto the rest in one-inch layers until you have added all four ounces of herb.

The degree of compression used in packing will depend upon the properties of viscosity, adhesion, and capillarity of the herb that is used. Percolation will not work with every herb. Mucilaginous herbs like Comfrey root or resins like myrrh will clog the cone and obstruct the process. If this happens simply dump the marc into a jar and proceed to make a standardized maceration. Next time try a lighter packing process and see if this solves the problem. With experience one can begin to predict the viscosity of a new herb from the consistency of the moistened herb.

Cut a circle of filter paper slightly larger than the diameter of the top of the percolator, insert it into the percolator so it sits just on top of the herb. Weigh the paper down with a pebble or an official glass percolator weight. This keeps the herb from being washed out when the menstruum is added. Set the apparatus up in an area with good lighting and add 8 ounces or so of the menstruum. Watch the column carefully. The solvent should soak down into the herb gradually and evenly. If the packing is irregular or loose in some areas the menstruum will flow through those areas first and fail to exhaust the rest of the herb properly. Loosen the stop cork until a few drops percolate out, reseal the cork, and cover the top of the percolator. The solvent should completely saturate the herb and be left to macerate for 12 to 48 hours depending on the density of the herb.

After macerating it is time to begin the percolation. The top of the percolator should be filled with a measured amount of menstruum. If there is not enough room to hold all of the menstruum (20 ounces total for 4 ounces of herb) care must be taken to ensure that the level of

solvent does not drop down to the top of the herb before all of the menstruum is added. If the powder dries fissures may form that will short cut the percolation when more solvent is added. The cork is loosened just enough that a controlled rate of flow is established. Around sixty drops per minute is ideal. If the rate is too fast tighten the cork, if the rate is too slow loosen the cork, and the cork is completely removed and the rate is still too slow there are two options. You can simply wait out a slow drip rate or dump the works into a jar and macerate as described above for two weeks. Next time lighten up on the packing process. Ideally in a 1:5 tincture the solvent should exhaust the constituents of the herb completely and you will recover five times the weight of the herb in fluid ounces of tincture. There is nothing left in the marc except compost.

Digestion is a form of maceration with slight warming during the extraction process, provided that the temperature does not alter the active ingredients of plant material and so there is greater efficiency in the use of menstruum. The most used temperatures are between 35 ° and 40 ° C., although can rise to no higher than 50 ° C. This process is used with the tougher plant parts or those that contain poorly soluble substances. We introduce the parts to extract in a container with the liquid pre-heated to the indicated temperatures, is maintained for a period that may vary between half an hour and 24 hours, shaking the container regularly.

An **infusion** is a dilute solution of easily soluble constituents of the raw drug. The aromatic drugs, to prevent volatile oils evaporate at other temperatures. The infusion is carried out by immersing the plants parts to use in an amount of boiling water, allowed to stand 15 minutes and then filtered through a filter or filter paper. There are in the market many herbal drugs properly packed in filter paper envelopes suitable for this process without using additional utensils. Overall doses are approximately one gram of plant per 10 cc of water. Here are some examples of **infusions** of medicinal plants with healing properties or health benefits. Click on the plant to see more details of this.

The **decoction** is used for active ingredients that doesn´t modify with temperature. In this process the drug is boiled in water for 15 to 60 minutes (depending on the plant or the active ingredient to extract), it´s cooled, strained and added enough cold water through the drug to obtain the desired volume. Depending on the consistency of the parts to

extract, **decoction** times will be more or less long; generally roots, leaves, flowers and leafy stems are boiled in water for about 15 minutes, while the branches and other hard parts can require up to an hour, during this time the evaporated water must be replacing. Once the decoction is done it´s necessary to filter the liquid through a cloth, squeezing very well the obtained liquid. Doses are similar to the infusion ones, ie a plant part per ten of water, except for the plants with high mucilage content in this case will be 1/20 to prevent the solution takes much viscosity. The **decoctions** are prepared for using in the moment and shouldn't be stored for more than 24 hours.

A **Soxhlet Extractor** has three main sections: A percolator (boiler and reflux) which circulates the solvent, a thimble (usually made of thick filter paper) which retains the solid to be extracted, and a siphon mechanism, which periodically empties the thimble.

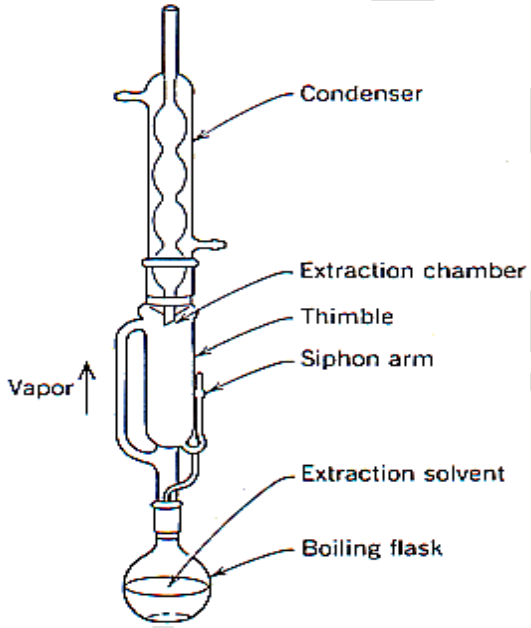
Assembly

1. The source material containing the compound to be extracted is placed inside the thimble.
2. The thimble is loaded into the main chamber of the Soxhlet extractor.
3. The extraction solvent to be used is placed in a distillation flask.
4. The flask is placed on the heating element.
5. The Soxhlet extractor is placed atop the flask.
6. A reflux condenser is placed atop the extractor.

Operation

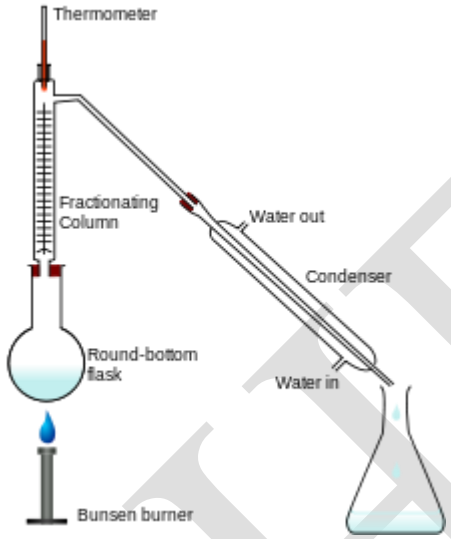
The solvent is heated to reflux. The solvent vapour travels up a distillation arm, and floods into the chamber housing the thimble of solid. The condenser ensures that any solvent vapour cools, and drips back down into the chamber housing the solid material. The chamber containing the solid material slowly fills with warm solvent. Some of the desired compound dissolves in the warm solvent. When the Soxhlet chamber is almost full, the chamber is emptied by the siphon. The solvent is returned to the distillation flask. The thimble ensures that the rapid motion of the solvent does not transport any solid material to the still pot. This cycle may be allowed to repeat many times, over hours or days.

During each cycle, a portion of the non-volatile compound dissolves in the solvent. After many cycles the desired compound is concentrated in the distillation flask. The advantage of this system is that instead of many portions of warm solvent being passed through the sample, just one batch of solvent is recycled. After extraction the solvent is removed, typically by means of a rotary evaporator, yielding the extracted compound. The non-soluble portion of the extracted solid remains in the thimble, and is usually discarded.



Reflux is a technique involving the condensation of vapors and the return of this condensate to the system from which it originated. It is used in industrial and laboratory distillations. It is also used in chemistry to supply energy to reactions over a long period of time.Reflux in laboratory distillation.

Laboratory apparatus using reflux to supply energy to chemical reactions. An Erlenmeyer flask is used as a receiving flask. Here the distillation head and fractionating column are combined in one piece.



The apparatus shown in the diagram represents a batch distillation as opposed to a continuous distillation. The liquid feed mixture to be distilled is placed into the round-bottomed flask along with a few anti-bumping granules, and the fractionating column is fitted into the top. As the mixture is heated and boils, vapor rises up the column.

The vapor condenses on the glass platforms (known as plates or trays) inside the column and runs back down into the liquid below, thereby refluxing the upflowing distillate vapor. The hottest tray is at the bottom of the column and the coolest tray is at the top. At steady state conditions, the vapor and liquid on each tray is at equilibrium.

Only the most volatile of the vapors stays in gaseous form all the way to the top. The vapor at the top of the column then passes into the condenser, where it cools until it condenses into a liquid. The separation can be enhanced with the addition of more trays (to a practical limitation of heat, flow, etc.).

The process continues until all the most volatile components in the liquid feed boil out of the mixture. This point can be recognized by the rise in temperature shown on the thermometer. For continuous distillation, the feed mixture enters in the middle of the column.

Possible Questions

Short questions

1. What is importance of extraction methodology?
2. Describe the other techniques of extraction of medicinal plants.
3. Write a note on Infusion.
4. Write a note on decoction.
5. Write a note on Percolation.
6. Write about the maceration.
7. What is best extraction procedure and justify it?
8. Write short note types of percolation?
9. Define digestion.
10. What is a reflux extraction?

Essay type questions

1. Describe percolation techniques in detail.
2. Highlight the reflux extraction methodology?
3. Expalin about the soxhlet extraction?
4. Explain about the maceration and decoction extraction of plants?
5. Write in detail about the infusion techniques?
6. Discuss the different techniques of extraction procedures.

UNIT-III

SYLLABUS

Biotechnology of medicinal plants: Production of secondary metabolite from cultured cells, elicitation, immobilization and biotransformation, DNA Bar coding, DNA Fingerprinting of medicinal plants – DNA Isolation and Fingerprinting techniques.

Production of Secondary Metabolites:

The process of in vitro culture of cells for the large scale production of secondary metabolites is complex, and involves the following aspects:

1. Selection of cell lines for high yield of secondary metabolites.
2. Large scale cultivation of plant cells.
3. Medium composition and effect of nutrients.
4. Elicitor-induced production of secondary metabolites.
5. Effect of environmental factors.
6. Biotransformation using plant cell cultures.
7. Secondary metabolite release and analysis.

1. Selection of Cell Lines for High Yield of Secondary Metabolites:

The very purpose of tissue culture is to produce high amounts of secondary metabolites. However, in general, majority of callus and suspension cultures produce less quantities of secondary metabolites. This is mainly due to the lack of fully differentiated cells in the cultures.

Some special techniques have been devised to select cell lines that can produce higher amounts of desired metabolites. These methods are ultimately useful for the separation of producer cells from the non-producer cells. The techniques commonly employed for cell line selection are cell cloning, visual or chemical analysis and selection for resistance.

Cell Cloning:

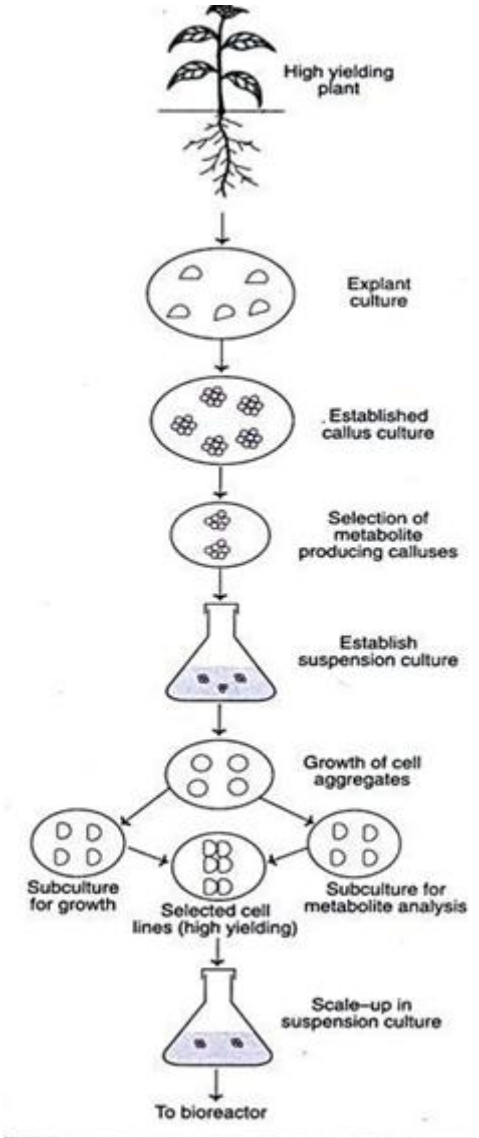
This is a simple procedure and involves the growth of single cells (taken from a suspension cultures) in a suitable medium. Each cell population is then screened for the secondary metabolite formation. And only those cells with high-yielding ability are selected and maintained by sub-cloning.

Single cell cloning:

There are certain practical difficulties in the isolation and culture of single cells.

Cell aggregate cloning:

Compared to single cell cloning, cell aggregate cloning is much easier, hence preferred by many workers. A schematic representation of cell aggregate cloning for the selection of cells yielding high quantities of secondary metabolites is given in Fig. 42.9. A high yielding plant of the desired metabolite is selected and its explants are first cultured on a solid medium. After establishing the callus cultures, high metabolite producing calluses are identified, and they are grown in suspension cultures.



Selection of cell lines for high yield of secondary metabolites

Cell aggregates from these cultures are grown on solid medium. The freshly developed cell aggregates (calluses) are divided into two parts. One half is grown further, while the other half is used for the quantitative analysis of the desired metabolite produced. The cell lines with high yield of secondary metabolites are selected and used for scale-up in suspension cultures. This is followed by large scale tissue culture in a bioreactor.

Visual or Chemical Analysis:

A direct measurement of some of the secondary metabolites produced by cell lines can be done either by visual or chemical analysis. Visual identification of cell lines producing coloured secondary metabolites (pigments e.g., β-carotene, shikonin) will help in the selection of high-yielding cells. This method is quite simple and non-destructive. The major limitation is that the desired metabolite should be coloured.

Certain secondary metabolites emit fluorescence under UV light, and the corresponding clones can be identified. Some workers use simple, sensitive and inexpensive chemical analytical methods for quantitative estimation of desired metabolites. Analysis is carried out in some colonies derived from single cell cultures. Radioimmunoassay is the most commonly used analytical method. Micro spectrophotometry and fluorescent antibody techniques are also in use.

Selection for Resistance:

Certain cells resistant to toxic compounds may lead to the formation of mutant cells which can overproduce a primary metabolite, and then a secondary metabolite. Such mutants can be selected and used to produce the desired metabolite in large quantities. One example is described.

Cell lines selected for resistance of 5-methyl- tryptophan (analogue of tryptophan) produce strains which can overproduce tryptophan. These tryptophan overproducing strains can synthesize 10-50 times higher levels of the natural auxin namely indole acetic acid (Note: The secondary metabolite indole acetic acid is derived from the primary metabolite tryptophan).

2. Large Scale (Mass) Cultivation of Plant Cells:

In order to achieve industrial production of the desired metabolite, large scale cultivation of plant cells is required. Plant cells (20-150 µm in diameter) are generally 10-100 times larger than bacterial or fungal cell. When cultured, plant cells exhibit changes in volumes and thus variable shapes and sizes. Further, cultured cells have low growth rate and genetic instability. All these aspects have to be considered for mass cultivation of cells.

The following four different culture systems are widely used:

1. Free-cell suspension culture

2. Immobilized cell culture

3. Two-phase system culture

4. Hairy root culture.

Free-cell Suspension Culture:

Mass cultivation of plant cells is most frequently carried out by cell suspension cultures. Care should be taken to achieve good growth rate of cells and efficient formation of the desired secondary metabolite. Many specially designed bioreactors are in use for free-cell suspension cultures.

Some of these are listed below:

i. Batch bioreactors

ii. Continuous bioreactors

iii. Multistage bioreactors

iv. Airlift bioreactors

v. Stirred tank bioreactors.

Two important aspects have to be considered for good success of suspension cultures.

1. Adequate and continuous oxygen supply.

2. Minimal generation of hydrodynamic stresses due to aeration agitation.

Immobilized Cell Cultures:

Plant cells can be made immobile or immovable and used in culture systems. The cells are physically immobilized by entrapment. Besides individual cells, it is also possible to immobilize aggregate cells or even calluses. Homogenous suspensions of cells are most suitable for immobilization.

Surface immobilized plant cell (SIPC) technique efficiently retains the cells and allows them to grow at a higher rate. Further, through immobilization, there is better cell-to-cell contact, and the cells are protected from high liquid shear stresses. All this helps in the maximal production the secondary metabolite.

The common methods adopted for entrapment of cells are briefly described:

1. Entrapment of cells in gels:

The cells or the protoplasts can be entrapped in several gels e.g., alginate, agar, agarose, carrageenin. The gels may be used either individually or in combination. The techniques employed for the immobilization of plant cells are comparable to those used for immobilization of microorganisms or other cells.

2. Entrapment of cells in nets or foams:

Polyurethane foams or nets with various pore sizes are used. The actively growing plant cells in suspension can be immobilized on these foams. The cells divide within the compartments of foam and form aggregates.

3. Entrapment of cells in hollow-fibre membranes:

Tubular hollow fibres composed of cellulose acetate silicone polycarbonate and organized into parallel bundles are used for immobilization of cells. It is possible to entrap cells within and between the fibres. Membrane entrapment is mechanically stable. However, it is more expensive than gel or foam immobilization.

Bioreactors for Use of Immobilized Cells:

Fluidized bed or fixed bed bioreactors are employed to use immobilized cells for large scale cultivation. In the fluidized-bed reactors, the immobilized cells are agitated by a flow of air or by

pumping the medium. In contrast, in the fixed-bed bioreactor, the immobilized cells are held stationary (not agitated) and perfused at a slow rate with an aerated culture medium.

Biochemicals produced by using immobilized cells:

A selected list of the immobilized cells from selected plants and their utility to produce important bio-chemicals.

Plant culture species	*Immobilization method*	*Substrate*	*Product*
Catharanthus roseus	Entrapment in agarose	Cathenamine	Ajmalicine
Digitaiis lanata	Entrapment in alginate	Digitoxin	Digoxin
Capsicum frutescens	Entrapment in polyurethane foam	Sucrose	Capsaicin
Catheranthus roseus	Entrapment in alginate, agarose, carrageenin	Sucrose	Ajmalicine
Petunia hybrida	Entrapment in hollow fibres	Sucrose	Phenolics
Morinda citrifolia	Entrapment in alginate	Sucrose	Anthraquinone
Solanum aviculare	Attachment polyphenylene beads	Sucrose	Steroid glycosides
Glycine max	Entrapment in hollow fibre	Sucrose	Phenolics

Two-phase System Culture:

Plant cells can be cultivated in an aqueous two phase system for the production of secondary metabolites. In this technique, the cells are kept apart from the product by separation in the bioreactor. This is advantageous since the product can be removed continuously. Certain polymers (e.g., dextran and polyethylene glycol for the separation of phenolic compounds) are used for the separation of phases.

Hairy Root Culture:

Hairy root cultures are used for the production of root-associated metabolites. In general, these cultures have high growth rate and genetic stability. For the production of hairy root cultures, the explant material (plant tissue) is inoculated with the cells of the pathogenic bacterium, Agrobacterium rhizogenes. This organism contains root-inducing (Ri) plasmid that causes genetic transformation of plant tissues, which finally results in hairy root cultures. Hairy roots produced by plant tissues have metabolite features similar to that of normal roots.

Hairy root cultures are most recent organ culture systems and are successfully used for the commercial production of secondary metabolites. A selected list of the plants employed in hairy root cultures and the secondary metabolites produced.

Plant species	*Secondary metabolite(s)*
Nicotiana tabacum	Nicotine, anatabine
Atropa belladonna	Atropine
Datura stramonium	Hyoscyamine
Lithospermum erythrorhizon	Shikonin
Catharanthus roseus	Ajmalicine, serpentine
Cinchona ledgeriana	Quinine alkaloids
Mentha vulgaris	Monoterpenes
Solanum laciniatum	Steroid alkaloids

3. Medium Composition and Effect of Nutrients:

The in vitro growth of the plant cells occurs in a suitable medium containing all the requisite elements. The ingredients of the medium effect the growth and metabolism of cells. For optimal production of secondary metabolites, a two-medium approach is desirable.

The first medium is required for good growth of cells (biomass growth) while the second medium, referred to as production medium promotes secondary metabolite formation. The effect of nutrients (carbon and nitrogen sources, phosphate, growth regulators, precursors, vitamins, metal ions) on different species in relation to metabolite formation are variable, some of them are briefly described.

Effect of Carbon Source:

Carbohydrates influence the production of phytochemicals.

Some examples are given below:

1. Increase in sucrose concentration (in the range 4-10%) increases alkaloid production in Catharanthus roseus cultures.
2. Sucrose is a better carbon source than fructose or galactose for diosgenin production by Dioscorea deltoidea or Dalanites aegyptiaca cultures.
3. Low concentration of sucrose increases the production of ubiquinone-10 in tobacco cell cultures.

Effect of Nitrogen Source:

The standard culture media usually contain a mixture of nitrate and ammonia as nitrogen source. Majority of plant cells can tolerate high levels of ammonia. The cultured cells utilize nitrogen for

the biosynthesis of amino acids, proteins (including enzymes) and nucleic acids. The nitrogen containing primary metabolites directly influence the secondary metabolites.

In general, high ammonium ion concentrations inhibit secondary metabolite formation while lowering of ammonium nitrogen increases. It is reported that addition of KNO_3 and NH_4NO_3 inhibited anthocyanin (by 90%) and alkaloid (by 80%) production.

Effect of Phosphate:

Inorganic phosphate is essential for photosynthesis and respiration (glycolysis). In addition, many secondary metabolites are produced through phosphorylated intermediates, which subsequently release the phosphate e.g., phenylpropanoids, terpenes, terpenoids. In general, high phosphate levels promote cell growth and primary metabolism while low phosphate concentrations are beneficial for secondary product formation. However, this is not always correct.

Increase in phosphate concentration in the medium may increase, decrease or may not affect product formation e.g.:

1. Increased phosphate concentration increases alkaloid (in *Catharanthus roseus*), anthraquinone (in *Morinda citrifolia*) and diosgenin (in *Dioscorea deltoidea*) production.

2. Decreased phosphate level in the medium increases the formation of anthocyanins and phenolics (in *Catharanthus roseus*), alkaloids (in *Peganum harmala*) and solasodine (in *Solanum lanciatum*).

3. Phosphate concentration (increase or decrease) has no effect on protoberberine (an alkaloid) production by Berberis sp.

Effect of Plant Growth Regulators:

Plant growth regulators (auxins, cytokinins) influence growth, metabolism and differentiation of cultured cells. There are a large number of reports on the influence of growth regulators for the production of secondary metabolites in cultured cells. A few examples are given.

1. Addition of auxins (indole acetic acid, indole pyruvic acid, naphthalene acetic acid) enhanced the production of diosgenin in the cultures of Balanites aegyptiaca.

2. Auxins may inhibit the production of certain secondary metabolites e.g., naphthalene acetic acid and indole acetic acid inhibited the synthesis of anthocyanin in carrot cultures.

3. Another auxin, 2, 4-dichlorophenoxy acetate (2, 4-D) inhibits the production of alkaloids in the cultures of tobacco, and shikonin formation in the cultures of Lithospermum erythrorhizon.

4. Cytokinins promote the production of secondary metabolites in many tissue cultures e.g., ajmalicine in Catharanthus roseus; scopolin and scopoletin in tobacco; carotene in Ricinus sp.

5. In some tissue cultures, cytokinins inhibit product formation e.g., anthroquinones in Morinda citrifolia; shikonin in Lithospermum erythroshizon; nicotine in tobacco.

In actual practice, a combination of auxins and cytokinins is used to achieve maximum production of secondary metabolites in culture systems.

Effect of Precursors:

The substrate molecules that are incorporated into the secondary metabolites are referred to as precursors. In general, addition of precursors to the medium enhances product formation, although they usually inhibit the growth of the culture e.g., alkaloid synthesis in Datura cultures in increased while growth is inhibited by the addition of ornithine, phenylalanine, tyrosine and sodium phenyl pyruvate; precursors tryptamine and secologanin increase ajmalicine production in C. roseus cultures.

4. Elicitor-Induced Production of Secondary Metabolites:

The production of secondary metabolites in plant cultures is generally low and does not meet the commercial demands. There are continuous efforts to understand the mechanism of product formation at the molecular level, and exploit for increased production. The synthesis of majority of secondary metabolites involves multistep reactions and many enzymes. It is possible to stimulate any step to increase product formation.

Elicitors are the compounds of biological origin which stimulate the production of secondary metabolites, and the phenomenon of such stimulation is referred to as elicitation. Elicitors produced within the plant cells are endogenous elicitors e.g., pectin, pectic acid, cellulose, other polysaccharides. When the elicitors are produced by the microorganisms, they are referred to as exogenous elicitors e.g., chitin, chitosan, glucans. All the elicitors of biological origin are biotic elicitors.

The term abiotic elicitors is used to represent the physical (cold, heat, UV light, osmotic pressure) and chemical agents (ethylene, fungicides, antibiotics, salts of heavy metals) that can also increase the product formation. However, the term abiotic stress is used for abiotic elicitors, while elicitors exclusively represent biological compounds.

Phytoalexins:

Plants are capable of defending themselves when attacked by microorganisms, by producing antimicrobial compounds collectively referred to as phytoalexins. Phytoalexins are the chemical weapons of defense against pathogenic microorganisms. Some of the phytoalexins that induce the production of secondary metabolites are regarded as elicitors. Some chemicals can also act as elicitors e.g., actinomycin-D, sodium salt of arachidonic acid, ribonuclease-A, chitosan, poly-L-lysine, nigeran. These compounds are regarded as chemically defined elicitors.

Interactions for Elicitor Formation:

Elicitors are compounds involved in plant- microbe interaction. Three different types of interactions between plants and microorganisms are known that lead to the formation of elicitors.

1. Direct release of elicitor by the microorganisms.
2. Microbial enzymes that can act as elicitors. e.g. endopolygalacturonic acid lyase from Erwinia carotovara.
3. Release of phytoalexins by the action of plant enzymes on cell walls of microorganisms which in turn stimulate formation elicitors from plant cell walls e.g., chitosan from Fusarium cell walls; α-1, 3-endoglucanase from Phytophthora cell walls.

Methodology of Elicitation:

Selection of microorganisms:

A wide range of microorganisms (viruses, bacteria, algae and fungi) that need not be pathogens have been tried in cultures for elicitor induced production of secondary metabolites. Based on the favourable elicitor response, an ideal microorganism is selected. The quantity of the microbial inoculum is important for the formation elicitor.

Co-culture:

Plant cultures (frequently suspension cultures) are inoculated with the selected microorganism to form co-cultures. The cultures are transferred to a fresh medium prior to the inoculation with microorganism. This helps to stimulate the secondary metabolism.

Co-cultures of plant cells with microorganisms may sometimes have inhibitory effect on the plant cells. In such a case, elicitor preparations can be obtained by culturing the selected microorganism on a tissue culture medium, followed by homogenization and autoclaving of the entire culture. This process releases elicitors. In case of heat labile elicitors, the culture homogenate has to be filter sterilized (instead of autoclaving).

In some co-culture systems, direct contact of plant cells and microorganisms can be prevented by immobilization (entrapment) of one of them. In these cultures, plant microbial interaction occurs by diffusion of the elicitor compounds through the medium.

Mechanism of Action of Elicitors:

Elicitors are found to activate genes and increase the synthesis of mRNAs encoding enzymes responsible for the ultimate biosynthesis secondary metabolites. There are some recent reports suggesting the involvement of elicitor mediated calcium-based signal transduction systems that promotes the product formation. When the cells are pretreated with a calcium chelate (EDTA) prior to the addition of elicitor, there occurs a decrease in the production of secondary metabolite.

Elicitor-induced products in cultures:

In selected list of elicitor-induced secondary metabolites produced in culture systems are given.

5. Effect of Environmental Factors:

The physical factors namely light, incubation temperature, pH of the medium and aeration of cultures influence the production of secondary metabolites in cultures.

Effect of Light:

Light is absolutely essential for the carbon fixation (photosynthesis) of field-grown plants. Since the carbon fixation is almost absent or very low in plant tissue cultures, light has no effect on the primary metabolism.

Elicitor microorganism	*Plant cell culture(s)*	*Secondary metabolite(s)*
Aspergillus niger	*Cinchona ledgeriana, Rubia tinctoria*	Anthraquinones
Pythium aphanidermatum	*Catharanthus roseus*	Ajmalicine, Strictosidine Catharanthine
Botrytis sp	*Papaver somniferum*	Sanguinarine
Phytophthora megasperma	*Glycine max*	Isoflavonoids Gluceollin
Dendryphion sp	*Papaver somniferum*	Sanguinarine
Alternaria sp	*Phaseolus vulgaris*	Phaseollin
Fusarium sp	*Apium graveolens*	Furanocoumarins
Phythium aphanidermatum	*Daucus carota*	Anthocynins
Penicillium expansum	*Sanguinaria canadensis*	Benzophenan-thridine Alkaloids

However, the light- mediated enzymatic reactions indirectly influence the secondary metabolite formation. The quality of light is also important. Some examples of light- stimulated product formations are given

1. Blue light enhances anthocyanin production in Haplopappus gracilis cell suspensions.

2. White light increases the formation of anthocyanin in the cultures of Catharanthus roseus, Daucus carota and Helianthus tuberosus.

3. White or blue light inhibits naphthoquinone biosynthesis in callus cultures of Lithospermum erythrorhizon.

Effect of Incubation Temperature:

The growth of cultured cells is increased with increase in temperature up to an optimal temperature (25-30°C). However, at least for the production some secondary metabolites lower temperature is advantageous. For instance, in C. roseus cultures, indole alkaloid production is increased by two fold when incubated at 16°C instead of 27°C. Increased temperature was also found to reduce the production of caffeine (by C. sineneis) and nicotine (by N. tabacum).

Effect of pH of the medium:

For good growth of cultures, the pH of the medium is in the range of 5 to 6. There are reports indicating that pH of the medium influences the formation of secondary metabolites. e.g., production of anthocyanin by cultures of Daucus carota was much less when incubated at pH 5.5 than at pH 4.5. This is attributed to the increased degradation of anthocyanin at higher pH.

Aeration of cultures:

Continuous aeration is needed for good growth of cultures, and also for the efficient production of secondary metabolites.

6. Biotransformation Using Plant Cell Cultures:

The conversion of one chemical into another (i.e., a substrate into a final product) by using biological systems (i.e. cell suspensions) as biocatalysts is regarded as biotransformation or bioconversion. The biocatalyst may be free or immobilized, and the process of biotransformation may involve one or more enzymes. Biotransformation involving microorganisms and animal cells are described elsewhere.

The biotechnological application of plant cell cultures in biotransformation reactions involves the conversion of some less important substances to valuable medicinal or commercially important products. In biotransformation, it is necessary to select such cell lines that possess the enzymes for catalysing the desired reactions. Bioconversions may involve many types of reactions e.g., hydroxylation, reduction, glycosylation.

A good example of biotransformation by plant cell cultures is the large scale production of cardiovascular drug digoxin from digitoxin by Digitali lanata. Digoxin production is carried out by immobilized cells of D. lanata in airlift bioreactors. Cell cultures of Digitalis

purpurea or Stevia rebaudiana can convert steviol into steviobiocide and steviocide which are 100 times sweeter than cane sugar.

7. Secondary Metabolite Release and Analysis:

The methods employed for the separation and purification of secondary metabolites from cell cultures are the same as that used for plants.

Sometimes, the products formed within the cells are released into the medium, making the isolation and analysis easy. For the secondary metabolites stored within the vacuoles of cells, two membranes (plasma membrane and tonoplast) have to be disrupted. Permeabilizing agents such as dimethyl sulfoxide (DMSO) can be used for the release of products.

In general, separation and purification of products from plant cell cultures are expensive, therefore every effort is made to make them cost- effective. Two approaches are made in this direction:

1. Production of secondary metabolite should be as high as possible.
2. Formation of side product(s) which interfere with separation must be made minimal.

Once a good quantity of the product is released into the medium, separation and purification techniques (e.g. extraction) can be used for its recovery. These techniques largely depend on the nature of the secondary metabolite.

Immobilization of enzymes

Traditionally, enzymes in free solutions (i.e. in soluble or free form) react with substrates to result in products. Such use of enzymes is wasteful, particularly for industrial purposes, since enzymes are not stable, and they cannot be recovered for reuse.

Immobilization of enzymes (or cells) refers to the technique of confining/anchoring the enzymes (or cells) in or on an inert support for their stability and functional reuse. By employing this technique, enzymes are made more efficient and cost-effective for their industrial use. Some workers regard immobilization as a goose with a golden egg in enzyme technology. Immobilized enzymes retain their structural conformation necessary for catalysis.

There are several advantages of immobilized enzymes:

a. Stable and more efficient in function.

b. Can be reused again and again.

c. Products are enzyme-free.

d. Ideal for multi-enzyme reaction systems.

e. Control of enzyme function is easy.

f. Suitable for industrial and medical use.

g. Minimize effluent disposal problems.

There are however, certain disadvantages also associated with immobilization.

a. The possibility of loss of biological activity of an enzyme during immobilization or while it is in use.

b. Immobilization is an expensive affair often requiring sophisticated equipment.

Immobilized enzymes are generally preferred over immobilized cells due to specificity to yield the products in pure form. However, there are several advantages of using immobilized multi-enzyme systems such as organelles and whole cells over immobilized enzymes. The immobilized cells possess the natural environment with cofactor availability (and also its regeneration capability) and are particularly suitable for multiple enzymatic reactions.

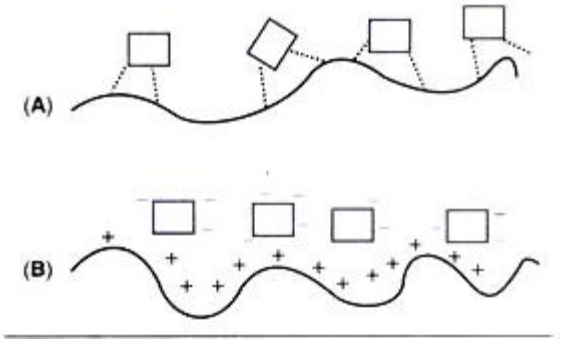
Methods of Immobilization:

The commonly employed techniques for immobilization of enzymes are—adsorption, entrapment, covalent binding and cross-linking.

Adsorption:

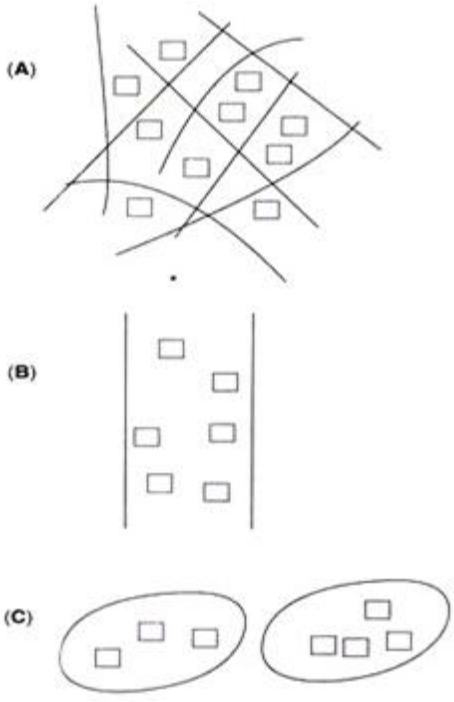
Adsorption involves the physical binding of enzymes (or cells) on the surface of an inert support. The support materials may be inorganic (e.g. alumina, silica gel, calcium phosphate gel, glass) or organic (starch, carboxymethyl cellulose, DEAE-cellulose, DEAE-sephadex).

Adsorption of enzyme molecules (on the inert support) involves weak forces such as van der Waals forces and hydrogen bonds. Therefore, the adsorbed enzymes can be easily removed by minor changes in pH, ionic strength or temperature. This is a disadvantage for industrial use of enzymes.



Entrapment:

Enzymes can be immobilized by physical entrapment inside a polymer or a gel matrix. The size of the matrix pores is such that the enzyme is retained while the substrate and product molecules pass through. In this technique, commonly referred to as lattice entrapment, the enzyme (or cell) is not subjected to strong binding forces and structural distortions. Some deactivation may however, occur during immobilization process due to changes in pH or temperature or addition of solvents. The matrices used for entrapping of enzymes include polyacrylamide gel, collagen, gelatin, starch, cellulose, silicone and rubber. Enzymes can be entrapped by several ways.



1. Enzyme inclusion in gels:

This is an entrapment of enzymes inside the gels .

2. Enzyme inclusion in fibres:

The enzymes are trapped in a fibre format of the matrix .

3. Enzyme inclusion in microcapsules:

In this case, the enzymes are trapped inside a microcapsule matrix (Fig. 21.4C). The hydrophobic and hydrophilic forms of the matrix polymerise to form a microcapsule containing enzyme molecules inside. The major limitation for entrapment of enzymes is their leakage from the matrix. Most workers prefer to use the technique of entrapment for immobilization of whole cells. Entrapped cells are in use for industrial production of amino acids (L-isoleucine, L-aspartic acid), L-malic acid and hydroquinone.

Microencapsulation:

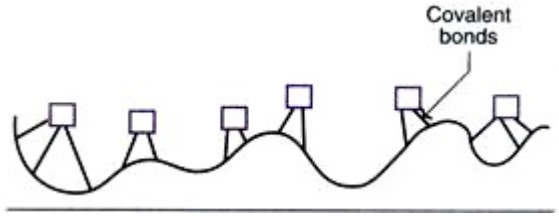
Microencapsulation is a type of entrapment. It refers to the process of spherical particle formation wherein a liquid or suspension is enclosed in a semipermeable membrane. The membrane may be polymeric, lipoidal, lipoprotein-based or non-ionic in nature. There are three distinct ways of microencapsulation.

1. Building of special membrane reactors.
2. Formation of emulsions.
3. Stabilization of emulsions to form microcapsules.

Microencapsulation is recently being used for immobilization of enzymes and mammalian cells. For instance, pancreatic cells grown in cultures can be immobilized by microencapsulation. Hybridoma cells have also been immobilized successfully by this technique.

Covalent Binding:

Immobilization of the enzymes can be achieved by creation of covalent bonds between the chemical groups of enzymes and the chemical groups of the support (Fig. 21.5). This technique is widely used. However, covalent binding is often associated with loss of some enzyme activity. The inert support usually requires pretreatment (to form pre-activated support) before it binds to enzyme. The following are the common methods of covalent binding.



1. Cyanogen bromide activation:

The inert support materials (cellulose, sepharose, sephadex) containing glycol groups are activated by CNBr, which then bind to enzymes and immobilize them.

2. Diazotation:

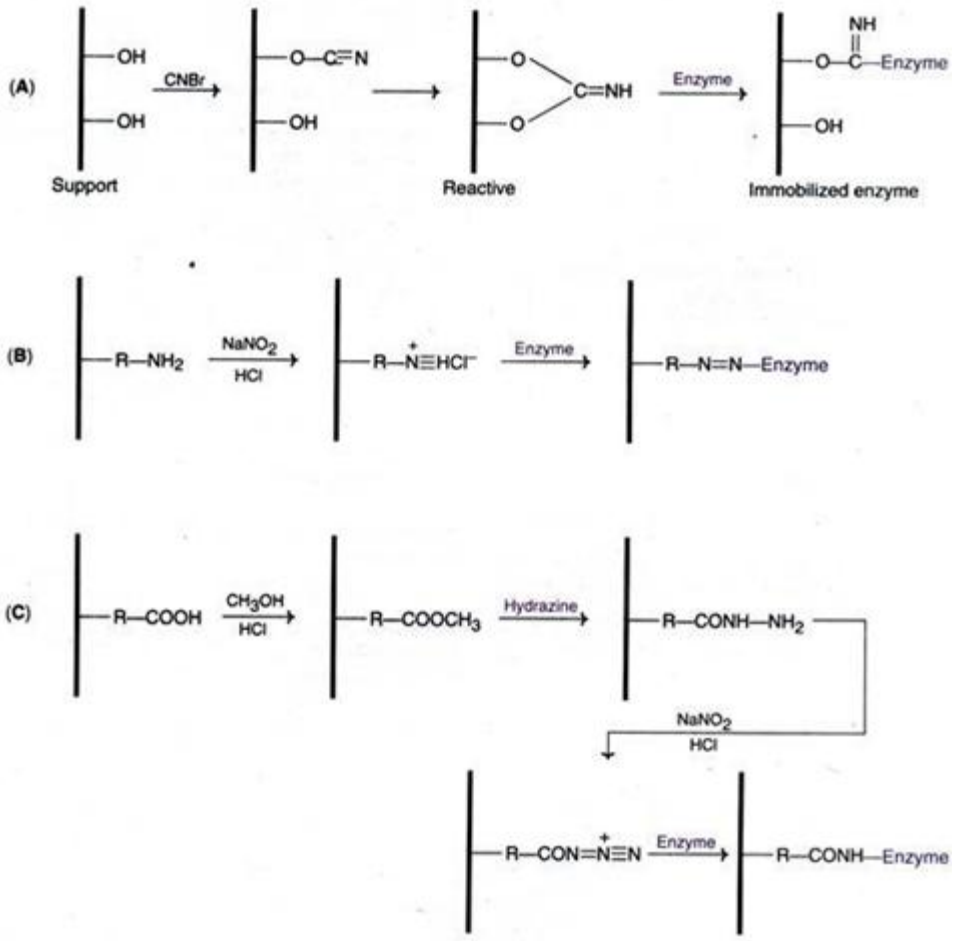
Some of the support materials (amino benzyl cellulose, amino derivatives of polystyrene, aminosilanized porous glass) are subjected to diazotation on treatment with $NaNO_2$ and HCI. They, in turn, bind covalently to tyrosyl or histidyl groups of enzymes.

3. Peptide bond formation:

Enzyme immobilization can also be achieved by the formation of peptide bonds between the amino (or carboxyl) groups of the support and the carboxyl (or amino) groups of enzymes. The support material is first chemically treated to form active functional groups.

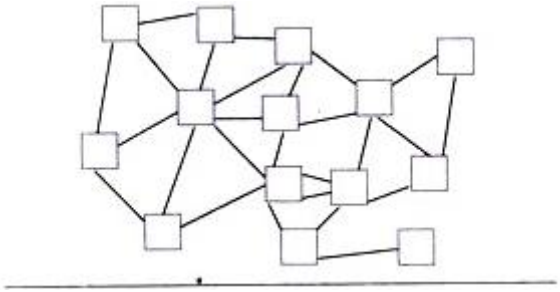
4. Activation by bi- or poly-functional reagents:

Some of the reagents such as glutaraldehyde can be used to create bonds between amino groups of enzymes and amino groups of support (e.g. aminoethylcellulose, albumin, amino alkylated porous glass).



Cross-Linking:

The absence of a solid support is a characteristic feature of immobilization of enzymes by cross-linking. The enzyme molecules are immobilized by creating cross-links between them, through the involvement of poly-functional reagents. These reagents in fact react with the enzyme molecules and create bridges which form the backbone to hold enzyme molecules (Fig. 21.7). There are several reagents in use for cross-linking. These include glutaraldehyde, diazobenzidine, hexamethylene diisocyanate and toluene di- isothiocyanate.



Glutaraldehyde is the most extensively used cross-linking reagent. It reacts with lysyl residues of the enzymes and forms a Schiff's base. The cross links formed between the enzyme and glutaraldehyde are irreversible and can withstand extreme pH and temperature. Glutaraldehyde cross- linking has been successfully used to immobilize several industrial enzymes e.g. glucose isomerase, penicillin amidase. The technique of cross-linking is quite simple and cost-effective. But the disadvantage is that it involves the risk of denaturation of the enzyme by the poly-functional reagent.

Choice of Immobilization Technique:

The selection of a particular method for immobilization of enzymes is based on a trial and error approach to choose the ideal one. Among the factors that decide a technique, the enzyme catalytic activity, stability, regenerability and cost factor are important.

Immobilization of L-amino acid acylase:

L-Amino acid acylase was the first enzyme to be immobilized by a group of Japanese workers (Chibata and Tosa, 1969). More than 40 different immobilization methods were attempted by this group. Only three of them were found be useful. They were covalent binding to iodoacetyl cellulose, ionic binding to DEAE-Sephadex and entrapment within polyacrylamide.

Stabilization of Soluble Enzymes:

Some of the enzymes cannot be immobilized and they have to be used in soluble form e.g. enzymes used in liquid detergents, some diagnostic reagents and food additives. Such enzymes can be stabilized by using certain additives or by chemical modifications. The stabilized enzymes have longer half-lives, although they cannot be recycled. Some important methods of enzyme stabilization are briefly described.

Solvent Stabilization:

Certain solvents at low concentrations stabilize the enzymes, while at high concentrations the enzymes get denatured e.g. acetone (5%) and ethanol (5%) can stabilize benzyl alcohol dehydro-genase.

Substrate Stabilization:

The active site of an enzyme can be stabilized by adding substrates e.g. starch stabilizes a-amylase; glucose stabilizes glucose isomerase.

Stabilization by Polymers*:*

Enzymes can be stabilized, particularly against increased temperature, by addition of polymers such as gelatin, albumin and polyethylene glycol.

Stabilization by Salts:

Stability of metalloenzymes can be achieved by adding salts such as Ca, Fe, Mn, Cu and Zn e.g. proteases can be stabilized by adding calcium.

Stabilization by Chemical Modifications:

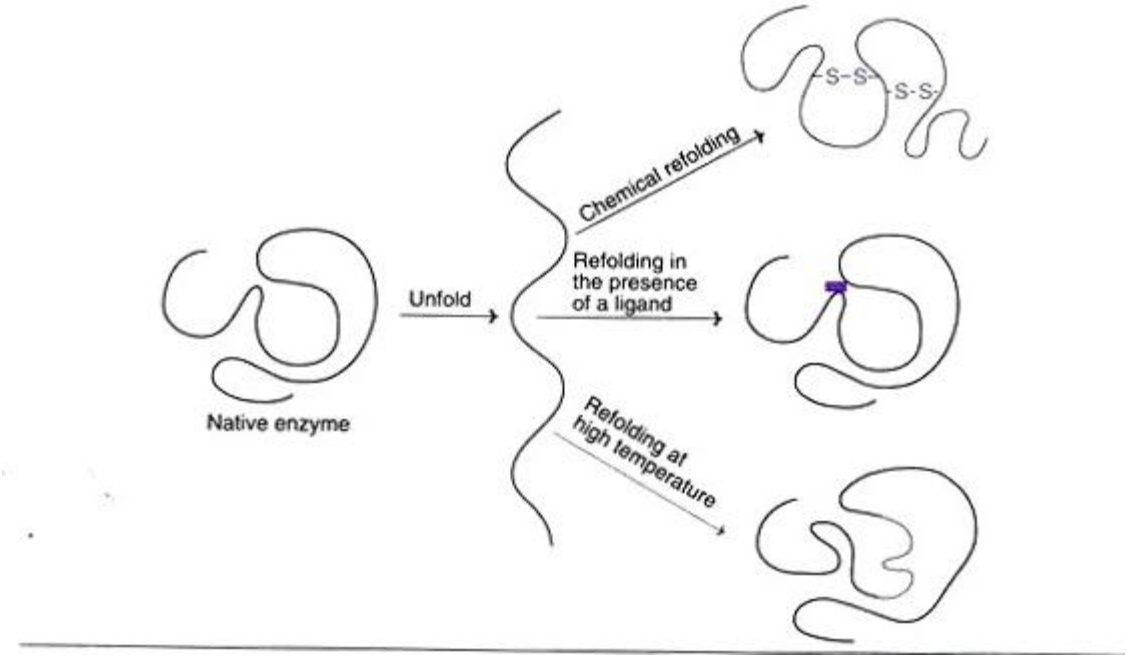
Enzymes can be stabilized by suitable chemical modifications without loss of biological activity. There are several types of chemical modifications.

a. Addition of poly-amino side chains e.g. polytyrosine, polyglycine.

b. Acylation of enzymes by adding groups such as acetyl, propionyl and succinyl.

Stabilization by Rebuilding:

Theoretically, the stability of the enzymes is due to hydrophobic interactions in the core of the enzyme. It is therefore, proposed that enzymes can be stabilized by enhancing hydrophobic interactions. For this purpose, the enzyme is first unfold and then rebuilt in one of the following ways.



1. The enzyme can be chemically treated (e.g. urea and a disulfide) and then refolded.

2. The refolding can be done in the presence of low molecular weight ligands.

3. For certain enzymes, refolding at higher temperatures (around 50°C) stabilize them.

Stabilization by Site-Directed Mutagenesis:

Site-directed mutagenesis has been successfully used to produce more stable and functionally more efficient enzymes e.g. subtilisin E.

Immobilization of Cells:

Immobilized individual enzymes can be successfully used for single-step reactions. They are, however, not suitable for multi-enzyme reactions and for the reactions requiring cofactors. The whole cells or cellular organelles can be immobilized to serve as multi-enzyme systems. In addition, immobilized cells rather than enzymes are sometimes preferred even for single reactions, due to cost factor in isolating enzymes. For the enzymes which depend on the special arrangement of the membrane, cell immobilization is preferred.

Immobilized cells have been traditionally used for the treatment of sewage. The techniques employed for immobilization of cells are almost the same as that used for immobilization of enzymes with appropriate modifications. Entrapment and surface attachment techniques are commonly used. Gels, and to some extent membranes, are also employed.

Immobilized Viable Cells:

The viability of the cells can be preserved by mild immobilization. Such immobilized cells are particularly useful for fermentations. Sometimes mammalian cell cultures are made to function as immobilized viable cells.

Immobilized Non-viable Cells:

In many instances, immobilized non-viable cells are preferred over the enzymes or even the viable cells. This is mainly because of the costly isolation and purification processes. The best example is the immobilization of cells containing glucose isomerase for the industrial production of high fructose syrup. Other important examples of microbial biocatalysts and their applications are given in Table 21.5.

TABLE 21.5 Selected examples of immobilized cells (to bring out one or two enzyme reactions) in industrial applications

Immobilized microorganism (microbial biocatalyst)	*Application(s)*
Escherichia coli	For the synthesis of L-aspartic acid from fumaric acid and NH_3
Escherichia coli	For the production of L-tryptophan from indole and serine
Pseudomonas sp	Production of L-serine from glycine and methanol
Saccharomyces cerevisiae	Hydrolysis of sucrose
Saccharomyces sp	Large scale production of alcohol
Zymomonas mobilis	Synthesis of sorbitol and gluconic acid from glucose and fructose
Anthrobacter simplex	Synthesis of prednisolone from hydrocortisone
Pseudomonas chlororaphis	Production of acrylamide from acrylonitrile
Humicola sp	For the conversion of rifamycin B to rifamycin S
Bacteria and yeasts (several sp)	In biosensors

Limitations of Immobilizing Eukaryotic Cells:

Prokaryotic cells (particularly bacterial) are mainly used for immobilization. It is also possible to immobilize eukaryotic plant and animal cells. Due to the presence of cellular organelles, the

metabolism of eukaryotic cells is slow. Thus, for the industrial production of biochemical, prokaryotic cells are preferred. However, for the production of complex proteins (e.g. immunoglobulin's) and for the proteins that undergo post- translational modifications, eukaryotic cells may be used.

Effect of Immobilization on Enzyme Properties:

Enzyme immobilization is frequently associated with alterations in enzyme properties, particularly the kinetic properties of enzymes.

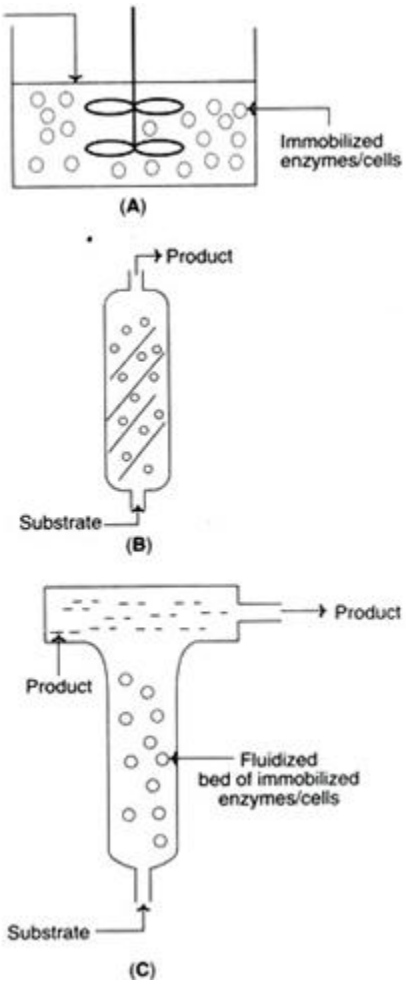
Some of them are listed below:

1. There is a substantial decrease in the enzyme specificity. This may be due to conformational changes that occur when the enzyme gets immobilized.

2. The kinetic constants K_m and V_{max} of an immobilized enzyme differ from that of the native enzyme. This is because the conformational change of the enzyme will affect the affinity between enzyme and substrate.

Immobilized Enzyme Reactors:

The immobilized enzymes cells are utilized in the industrial processes in the form of enzyme reactors. They are broadly of two types — batch reactors and continuous reactors. The frequently used enzyme reactors are shown in Fig. 21.9.



Batch Reactors:

In batch reactors, the immobilized enzymes and substrates are placed, and the reaction is allowed to take place under constant stirring. As the reaction is completed, the product is separated from the enzyme (usually by denaturation).

Soluble enzymes are commonly used in batch reactors. It is rather difficult to separate the soluble enzymes from the products; hence there is a limitation of their reuse. However, special techniques have been developed for recovery of soluble enzymes, although this may result in loss of enzyme activity.

Stirred tank reactors:

The simplest form of batch reactor is the stirred tank reactor. It is composed of a reactor fitted with a stirrer that allows good mixing, and appropriate temperature and pH control. However,

there may occur loss of some enzyme activity. A modification of stirred tank reactor is basket reactor. In this system, the enzyme is retained over the impeller blades. Both stirred tank reactor and basket reactor have a well-mixed flow pattern.

Plug flow type reactors:

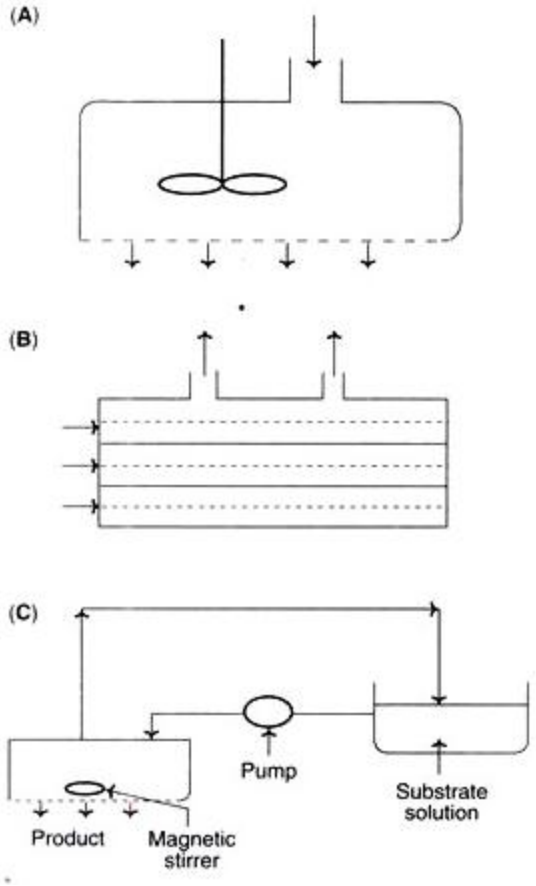
These reactors are alternatives to flow pattern type of reactors. The flow rate of fluids controlled by a plug system. The plug flow type reactors may be in the form of packed bed or fluidized bed. These reactors are particularly useful when there occurs inadequate product formation in flow type reactors. Further, plug flow reactors are also useful for obtaining kinetic data on the reaction systems.

Continuous Reactors:

In continuous enzyme reactors, the substrate is added continuously while the product is removed simultaneously. Immobilized enzymes can also be used for continuous operation. Continuous reactors have certain advantages over batch reactors. These include control over the product formation, convenient operation of the system and easy automation of the entire process. There are mainly two types of continuous reactors-continuous stirred tank reactor (CSTR) and plug reactor (PR). A diagrammatic representation of CSTR. CSTR is ideal for good product formation.

Membrane Reactors:

Several membranes with a variety of chemical compositions can be used. The commonly used membrane materials include polysulfone, polyamide and cellulose acetate. The biocatalysts (enzymes or cells) are normally retained on the membranes of the reactor. The substrate is introduced into reactor while the product passes out. Good mixing in the reactor can be achieved by using stirrer In a continuous membrane reactor, the biocatalysts are held over membrane layers on to which substrate molecules are passed.



In a recycle model membrane reactor, the contents (i.e. the solution containing enzymes, cofactors, and substrates along with freshly released product are recycled by using a pump. The product passes out which can be recovered.

Applications of Immobilized Enzymes and Cells:

Immobilized enzymes and cells are very widely used for industrial, analytical and therapeutic purpose, besides their involvement in food production and exploring the knowledge of biochemistry, microbiology and other allied specialties. A brief account of the industrial applications of immobilized cells.

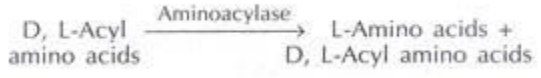
Manufacture of Commercial Products:

A selected list of important immobilized enzymes and their industrial applications is given in Table 21.6. Some details on the manufacture of L-amino acids and high fructose syrup are given hereunder.

Immobilized enzyme	*Application(s)*
Aminoacylase	Production of L-amino acids from D, L-acyl amino acids
Glucose isomerase	Production of high fructose syrup from glucose (or starch)
Amylase	Production of glucose from starch
Invertase	Splitting of sucrose to glucose and fructose
β-Galactosidase	Splitting of lactose to glucose and galactose
Penicillin acylase	Commercial production of semi-synthetic penicillins
Aspartase	Production of aspartic acid from fumaric acid
Fumarase	Synthesis of malic acid from fumaric acid
Histidine ammonia lyase	Production of urocanic acid from histidine
Ribonuclease	Synthesis of nucleotides from RNA
Nitrilase	Production of acrylamide from acrylonitrile

Production of L-Amino Acids:

L-Amino acids (and not D-amino acids) are very important for use in food and feed supplements and medical purposes. The chemical methods employed for their production result in a racemic mixture of D- and L-amino acids. They can be acylated to form D, L-acyl amino acids. The immobilized enzyme aminoacylase (frequently immobilized on DEAE sephadex) can selectively hydrolyse D, L-acyl amino acids to produce L-amino acids.



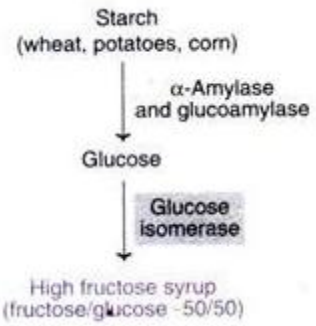
The free L-amino acids can separated from the un-hydrolysed D-acyl amino acids. The latter can be recemized to D, L-acyl amino acids and recycled through the enzyme reactor containing immobilized aminoacylase. Huge quantities of L-methionine, L-phenylalanine L-tryptophan and L-valine are produced worldwide by this approach.

Production of High Fructose Syrup:

Fructose is the sweetest among the monosaccharide's, and has twice the sweetening strength of sucrose. Glucose is about 75% as sweet as sucrose. Therefore, glucose (the most abundant monosaccharide) cannot be a good substitute for sucrose for sweetening. Thus, there is a great demand for fructose which is very sweet, but has the same calorific value as that of glucose or sucrose.

High fructose syrup (HFS) contains approximately equivalent amounts of glucose and fructose. HFS is almost similar to sucrose from nutritional point of view. HFS is a good substitute for sugar in the preparation of soft drinks, processed foods and baking.

High fructose syrup can be produced from glucose by employing an immobilized enzyme glucose isomerase. The starch containing raw materials (wheat, potato, corn) are subjected to hydrolysis to produce glucose. Glucose isomerase then isomerizes glucose to fructose (Fig. 21.11). The product formed is HFS containing about 50% fructose. (Note: Some authors use the term high fructose corn syrup i.e. HFCS in place of HFS).



Glucose isomerase:

This is an intracellular enzyme produced by a number of microorganisms. The species of Arthrobacter, Bacillus and Streptomyces are the preferred sources. Being an intracellular enzyme, the isolation of glucose isomerase without loss of biological activity requires special and costly techniques. Many a times, whole cells or partly broken cells are immobilized and used.

Immobilized Enzymes and Cells- Analytical Applications:

In Biochemical Analysis:

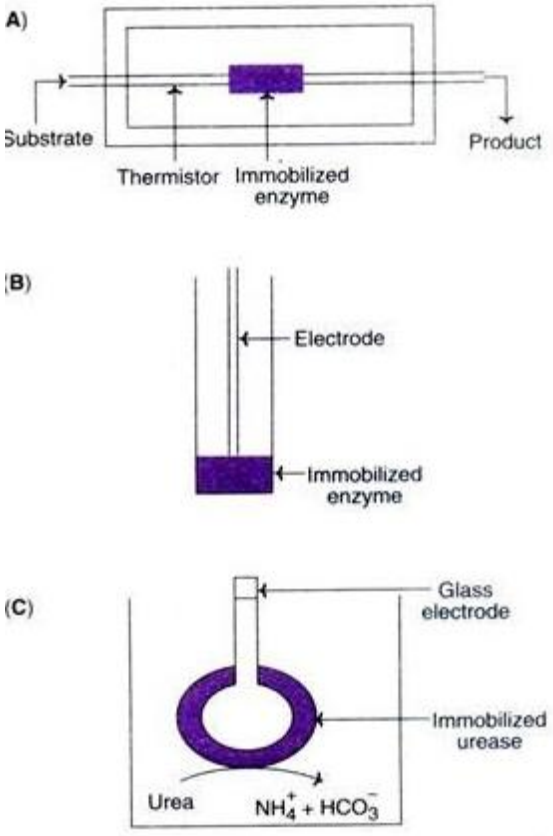
Immobilized enzymes (or cells) can be used for the development of precise and specific analytical techniques for the estimation of several biochemical compounds. The principle of analytical assay primarily involves the action of the immobilized enzyme on the substrate.

A decrease in the substrate concentration or an increase in the product level or an alteration in the cofactor concentration can be used for the assay. A selected list of examples of immobilized

enzymes used in the assay of some substances is given in Table 21.7. Two types of detector systems are commonly employed.

Immobilized enzyme	*Substance assayed*
Glucose oxidase	Glucose
Urease	Urea
Cholesterol oxidase	Cholesterol
Lactate dehydrogenase	Lactate
Alcohol oxidase	Alcohol
Hexokinase	ATP
Galactose oxidase	Galactose
Penicillinase	Penicillin
Ascorbic acid oxidase	Ascorbic acid
L-Amino acid oxidase	L-Amino acids
Cephalosporinase	Cephalosporin
Monoamine oxidase	Monoamine

Thermistors are heat measuring devices which can record the heat generated in an enzyme catalysed reaction. Electrode devices are used for measuring potential differences in the reaction system. In an enzyme thermistor and an enzyme electrode, along with a specific urease electrode are depicted.



In Affinity Chromatography and Purification:

Immobilized enzymes can be used in affinity chromatography. Based on the property of affinity, it is possible to purify several compounds e.g. antigens, antibodies, cofactors.

Biotransformation is the chemical modification (or modifications) made by an organism on a chemical compound. If this modification ends in mineral compounds like CO_2, NH_4^+, or H_2O, the biotransformation is called mineralisation. Biotransformation means chemical alteration of chemicals such as nutrients, amino acids, toxins, and drugs in the body. It is also needed to render non-polar compounds polar so that they are not reabsorbed in renal tubules and are excreted. Biotransformation of xenobiotics can dominate toxicokinetics and the metabolites may reach higher concentrations in organisms than their parent compounds. The metabolism of a drug or toxin in a body is an example of a biotransformation. The body typically deals with a foreign compound by making it more water-soluble, to increase the rate of its excretion through

the urine. There are many different processes that can occur; the pathways of drug metabolism can be divided into:

- Phase I
- Phase II

Drugs can undergo one of four potential biotransformations: Active Drug to Inactive Metabolite, Active Drug to Active Metabolite, Inactive Drug to Active Metabolite, Active Drug to Toxic Metabolite (biotoxification).

Phase I reaction

- Includes oxidative, reductive, and hydrolytic reactions.
- In these types of reactions, a polar group is either introduced or unmasked, so the drug molecule becomes more water-soluble and can be excreted.
- Reactions are non-synthetic in nature and in general produce a more water-soluble and less active metabolites.
- The majority of metabolites are generated by a common hydroxylating enzyme system known as Cytochrome P450.

Phase II reaction

- These reactions involve covalent attachment of small hydrophilic endogenous molecule such as glucuronic acid, sulfate, or glycine to form water-soluble compounds that are more hydrophilic.
- This is also known as a conjugation reaction.
- The final compounds have a larger molecular weight.

Microbial biotransformation

Biotransformation of various pollutants is a sustainable way to clean up contaminated environments. These bioremediation and biotransformation methods harness the naturally occurring, microbial catabolic diversity to degrade, transform or accumulate a huge range of compounds including hydrocarbons (e.g. oil), polychlorinated

biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), pharmaceutical substances, radionuclides and metals. Major methodological breakthroughs in recent years have enabled detailed genomic, metagenomic, proteomic, bioinformatic and other high-throughput analyses of environmentally relevant microorganisms providing unprecedented insights into biotransformation and biodegradative pathways and the ability of organisms to adapt to changing environmental conditions.

Biological processes play a major role in the removal of contaminants and pollutants from the environment. Some microorganisms possess an astonishing catabolic versatility to degrade or transform such compounds. New methodological breakthroughs in sequencing, genomics, proteomics, bioinformatics and imaging are producing vast amounts of information. In the field of Environmental Microbiology, genome-based global studies open a new era providing unprecedented in silico views of metabolic and regulatory networks, as well as clues to the evolution of biochemical pathways relevant to biotransformation and to the molecular adaptation strategies to changing environmental conditions. Functional genomic and metagenomic approaches are increasing our understanding of the relative importance of different pathways and regulatory networks to carbon flux in particular environments and for particular compounds and they are accelerating the development of bioremediation technologies and biotransformation processes.[2] Also there is other approach of biotransformation called enzymatic biotransformation.

Oil biodegradation

Petroleum oil is toxic for most life forms and episodic and chronic pollution of the environment by oil causes major ecological perturbations. Marine environments are especially vulnerable, since oil spills of coastal regions and the open sea are poorly containable and mitigation is difficult. In addition to pollution through human activities, millions of tons of petroleum enter the marine environment every year from natural seepages. Despite its toxicity, a considerable fraction of petroleum oil entering marine systems is eliminated by the hydrocarbon-degrading activities of microbial communities, in particular by a remarkable recently discovered group of specialists, the so-called hydrocarbonoclastic bacteria (HCB). Alcanivorax borkumensis, a paradigm of HCB and probably the most important global oil degrader, was the first to be

subjected to a functional genomic analysis. This analysis has yielded important new insights into its capacity for (i) n-alkane degradation including metabolism, biosurfactant production and biofilm formation, (ii) scavenging of nutrients and cofactors in the oligotrophic marine environment, as well as (iii) coping with various habitat-specific stresses. The understanding thereby gained constitutes a significant advance in efforts towards the design of new knowledge-based strategies for the mitigation of ecological damage caused by oil pollution of marine habitats. HCB also have potential biotechnological applications in the areas of bioplastics and biocatalysis.

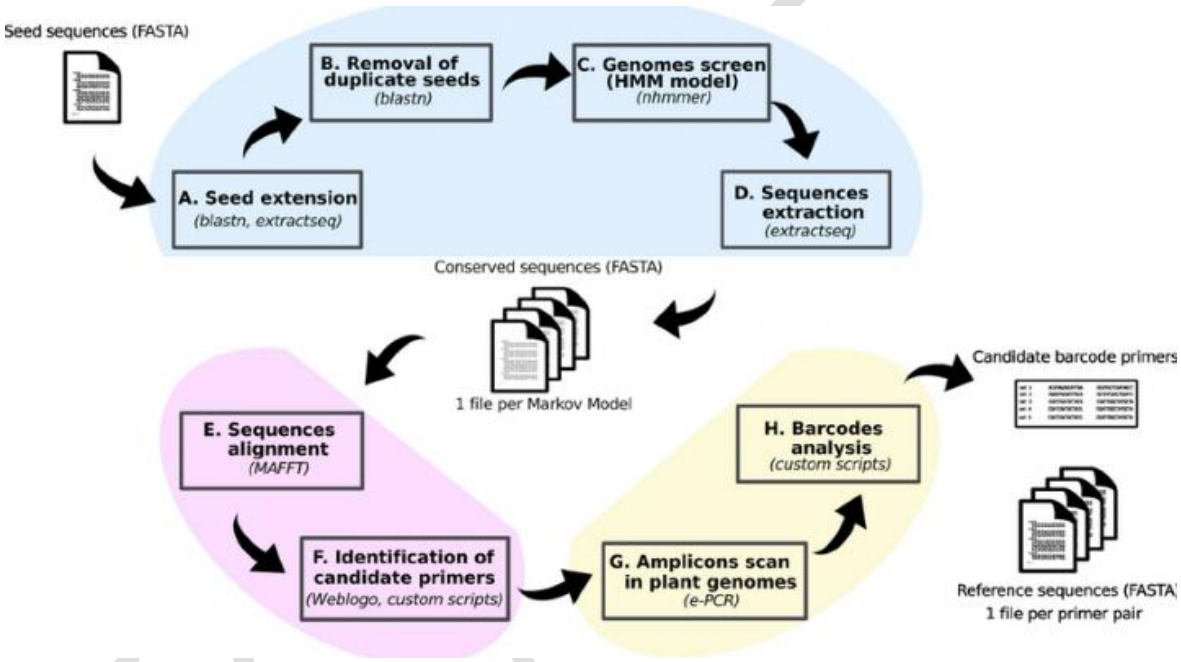
Metabolic engineering and biocatalytic applications

The study of the fate of persistent organic chemicals in the environment has revealed a large reservoir of enzymatic reactions with a large potential in preparative organic synthesis, which has already been exploited for a number of oxygenases on pilot and even on industrial scale. Novel catalysts can be obtained from metagenomic libraries and DNA sequencebased approaches. Our increasing capabilities in adapting the catalysts to specific reactions and process requirements by rational and random mutagenesis broadens the scope for application in the fine chemical industry, but also in the field of biodegradation. In many cases, these catalysts need to be exploited in whole cell bioconversions or in fermentations, calling for system-wide approaches to understanding strain physiology and metabolism and rational approaches to the engineering of whole cells as they are increasingly put forward in the area of systems biotechnology and synthetic biology.

DNA barcoding in plants:

DNA barcoding, a new method for the quick identification of any species based on extracting a DNA sequence from a tiny tissue sample of any organism, is now being applied to taxa across the tree of life. As a research tool for taxonomists, DNA barcoding assists in identification by expanding the ability to diagnose species by including all life history stages of an organism. As a biodiversity discovery tool, DNA barcoding helps to flag species that are potentially new to science. As a biological tool, DNA barcoding is being used to address fundamental ecological and evolutionary questions, such as how species in plant communities are assembled. The

process of DNA barcoding entails two basic steps: (1) building the DNA barcode library of known species and (2) matching the barcode sequence of the unknown sample against the barcode library for identification. Although DNA barcoding as a methodology has been in use for less than a decade, it has grown exponentially in terms of the number of sequences generated as barcodes as well as its applications. This volume provides the latest information on generating, applying, and analyzing DNA barcodes across the Tree of Life from animals and fungi to protists, algae, and plants.



DNA Barcoding Work Flow DNA barcoding has three main steps:

DNA extraction, PCR amplification, and DNA sequencing and analysis (Figure 1). DNA isolation is a key step because, without high quality DNA, the PCR amplification will not be optimal. The PCR amplification has to work so that there is DNA for sequencing. And finally, the sequencing analysis has to be successful for there to be an identification of the organism. Ensuring that these three steps are optimal is important for successful DNA barcoding. It is important to note that modifications to the DNA extraction process can sometimes be necessary.

In an effort to streamline DNA barcoding at RDLES, a barcoding standard operating procedure (SOP) was created. RDLES uses the Qiagen DNA extraction method to extract DNA

for barcoding; this method is also used by the FDA for the analysis of fish samples.This method requires approximately 10 mg of sample tissue. Universal primer pairs are used to amplify a known region of the cytochrome oxidase I (COI) gene. By amplifying the same gene from diverse organisms it is possible to build a peer-reviewed library of gene sequences. It is important to know the taxonomic group (fish, bird, mammal, etc.) of the organism of interest because the PCR primers are 12 specific to taxonomic group.

For some taxomic groups (plants) genes other than COI are used for DNA barcoding. Following the PCR amplification, the PCR product is analyzed on an agarose gel to confirm that amplification has occurred. If there is a band, the PCR product can be sent for DNA sequencing. If there is no amplification, it will be necessary to troubleshoot the issue.

This might require repeating the DNA extraction, trying out a different primer pair, or changing the master mix. Once a PCR product has been obtained it is sent to a sequencing company to determine the identity of the organism. The sequencing company provides a ~700 base pair DNA sequence that without bioinformatics has no meaning. There are two programs that can be used to analyze the DNA sequence: Barcode of Life Data Systems (BOLD) and National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST).

Each program has positive and negative attributes. DNA BOLD is a free program that compares the DNA sequence to samples that have been identified by a taxonomist and include additional data about the sample. This website is a hub for DNA barcoding information and analysis. NCBI BLAST is also a free program available on-line, were researchers can submit non-vouchered DNA sequences to the database.

This program will compare a sequence to both vouchered and non-vouchered samples. Both of these programs use alignment programs to determine the identity of the unknown sequence. It is helpful to use both independent databases to identify the organism because it can increase the certainty of the identification. It is possible that the two programs will not agree on the identification it and not all organisms are in both the databases.

DNA isolation

DNA isolation is a process of purification of DNA from sample using a combination of physical and chemical methods. The first isolation of DNA was done in 1869 by Friedrich Miescher. Currently it is a routine procedure in molecular biology or forensic analyses. For the chemical method, there are many different kits used for extraction, and selecting the correct one will save time on kit optimization and extraction procedures. PCR sensitivity detection is considered to show the variation between the commercial kits.

Basic procedure

There are three basic and two optional steps in a DNA extraction

- Cells which are to be studied need to be collected.
- Breaking the cell membranes open to expose the DNA along with the cytoplasm within (cell lysis).
  - Lipids from the cell membrane and the nucleus are broken down with detergents and surfactants.
  - Breaking proteins by adding a protease (optional).
  - Breaking RNA by adding an RNase (optional).
- The solution is treated with concentrated salt solution to make debris such as broken proteins, lipids and RNA to clump together.
- Centrifugation of the solution, which separates the clumped cellular debris from the DNA.
- DNA purification from detergents, proteins, salts and reagents used during cell lysis step. The most commonly used procedures are:
  - Ethanol precipitation usually by ice-cold ethanol or isopropanol. Since DNA is insoluble in these alcohols, it will aggregate together, giving a pellet upon centrifugation. Precipitation of DNA is improved by increasing of ionic strength, usually by adding sodium acetate.

- Phenol–chloroform extraction in which phenol denatures proteins in the sample. After centrifugation of the sample, denaturated proteins stay in the organic phase while aqueous phase containing nucleic acid is mixed with the chloroform that removes phenol residues from solution.
- Minicolumn purification that relies on the fact that the nucleic acids may bind (adsorption) to the solid phase (silica or other) depending on the pH and the salt concentration of the buffer.

Cellular and histone proteins bound to the DNA can be removed either by adding a protease or by having precipitated the proteins with sodium or ammonium acetate, or extracted them with a phenol-chloroform mixture prior to the DNA-precipitation.

After isolation, the DNA is dissolved in slightly alkaline buffer, usually in the TE buffer, or in ultra-pure water.

Special types

Specific techniques must be chosen for isolation of DNA from some samples. Typical samples with complicated DNA isolation are:

- archaeological samples containing partially degraded DNA, see ancient DNA
- samples containing inhibitors of subsequent analysis procedures, most notably inhibitors of PCR, such as humic acid from soil, indigo and other fabric dyes or haemoglobin in blood
- samples from microorganisms with thick cellular wall, for example yeast

Extrachromosomal DNA is generally easy to isolate, especially plasmids may be easily isolated by cell lysis followed by precipitation of proteins, which traps chromosomal DNA in insoluble fraction and after centrifugation, plasmid DNA can be purified from soluble fraction.

A DNA Extraction is an isolation of all extrachromosomal DNA in a mammalian cell. The extraction process gets rid of the high molecular weight nuclear DNA, leaving only low molecular weight mitochondrial DNA and any viral episomes present in the cell.

Detecting DNA

A diphenylamine (DPA) indicator will confirm the presence of DNA. This procedure involves

chemical hydrolysis of DNA: when heated (e.g. ≥95 °C) in acid, the reaction requires a deoxyribose sugar and therefore is specific for DNA. Under these conditions, the 2-deoxyribose is converted to w-hydroxylevulinyl aldehyde, which reacts with the compound, diphenylamine, to produce a blue-colored compound. DNA concentration can be determined measuring the intensity of absorbance of the solution at the 600 nm with a spectrophotometer and comparing to a standard curve of known DNA concentrations.

Measuring the intensity of absorbance of the DNA solution at wavelengths 260 nm and 280 nm is used as a measure of DNA purity. DNA absorbs UV light at 260 and 280 nanometres, and aromatic proteins absorb UV light at 280 nm; a pure sample of DNA has a ratio of 1.8 at 260/280 and is relatively free from protein contamination. A DNA preparation that is contaminated with protein will have a 260/280 ratio lower than 1.8. DNA can be quantified by cutting the DNA with a restriction enzyme, running it on an agarose gel, staining with ethidium bromide or a different stain and comparing the intensity of the DNA with a DNA marker of known concentration. Using the Southern blot technique, this quantified DNA can be isolated and examined further using PCR and RFLP analysis. These procedures allow differentiation of the repeated sequences within the genome. It is these techniques which forensic scientists use for comparison, identification, and analysis.

DNA Fingerprinting:

DNA profiling (also called **DNA fingerprinting**, **DNA testing**, or **DNA typing**) is the process of determining an individual's DNAcharacteristics, which are as unique as fingerprints. DNA analysis intended to identify a species, rather than an individual, is called DNA barcoding.

DNA profiling is commonly used as a forensic technique in criminal investigations, for example comparing one or more individuals' profiles to DNA found at a crime scene so as to assess the likelihood of their involvement in the crime. It is also used in parentage testing to establish immigration eligibility and in genealogical and medical research. DNA profiling has also been used in the study of animal and plant populations in the fields of zoology, botany, and agriculture.

Background

The modern process of DNA profiling was developed in 1984 by Sir Alec Jeffreys while working in the Department of Genetics at the University of Leicester.

Although 99.9% of human DNA sequences are the same in every person, enough of the DNA is different that it is possible to distinguish one individual from another, unless they are monozygotic ("identical") twins.[11] DNA profiling uses repetitive ("repeat") sequences that are highly variable called variable number tandem repeats (VNTRs), in particular short tandem repeats (STRs), also known as microsatellites, and minisatellites. VNTR loci are very similar between closely related individuals, but are so variable that unrelated individuals are extremely unlikely to have the same VNTRs.

The process begins with a sample of an individual's DNA (typically called a "reference sample"). A common method of collecting a reference sample is the use of a buccal swab, which is easy, non-invasive and cheap. When this is not available (for example, because a court order is needed but not obtainable) other methods may need to be used to collect a sample of blood, saliva, semen, vaginal lubrication, or other appropriate fluid or tissue from personal items (for example, a toothbrush, razor) or from stored samples (for example, banked sperm or biopsy tissue). Samples obtained from blood relatives (related by birth, not marriage) can provide an indication of an individual's profile, as could human remains that had been previously profiled.

A reference sample is then analyzed to create the individual's DNA profile using one of a number of techniques, discussed below. The DNA profile is then compared against another sample to determine whether there is a genetic match.

RFLP analysis

Restriction fragment length polymorphism

The first methods for finding out genetics used for DNA profiling involved **RFLP analysis**. DNA is collected from cells, such as a blood sample, and cut into small pieces using a restriction enzyme (a restriction digest). This generates thousands of DNA fragments of differing sizes as a

consequence of variations between DNA sequences of different individuals. The fragments are then separated on the basis of size using gel electrophoresis.

The separated fragments are then transferred to a nitrocellulose or nylon filter; this procedure is called a Southern blot. The DNA fragments within the blot are permanently fixed to the filter, and the DNA strands are denatured. Radiolabeled probe molecules are then added that are complementary to sequences in the genome that contain repeat sequences. These repeat sequences tend to vary in length among different individuals and are called variable number tandem repeat sequences or VNTRs. The probe molecules hybridize to DNA fragments containing the repeat sequences and excess probe molecules are washed away. The blot is then exposed to an X-ray film. Fragments of DNA that have bound to the probe molecules appear as fluoresent bands on the film.

The Southern blot technique is laborious, and requires large amounts of undegraded sample DNA. Also, Karl Brown's original technique looked at many minisatellite loci at the same time, increasing the observed variability, but making it hard to discern individual alleles (and thereby precluding paternity testing). These early techniques have been supplanted by PCR-based assays.

Polymerase chain reaction (PCR) analysis

Developed by Kary Mullis in 1983, a process was reported by which specific portions of the sample DNA can be amplified almost indefinitely (Saiki et al. 1985, 1988). This has revolutionized the whole field of DNA study. The process, polymerase chain reaction (PCR), mimics the biological process of DNA replication, but confines it to specific DNA sequences of interest. With the invention of the PCR technique, DNA profiling took huge strides forward in both discriminating power and the ability to recover information from very small (or degraded) starting samples.

PCR greatly amplifies the amounts of a specific region of DNA. In the PCR process, the DNA sample is denatured into the separate individual polynucleotide strands through heating. Two oligonucleotide DNA primers are used to hybridize to two corresponding nearby sites on opposite DNA strands in such a fashion that the normal enzymatic extension of the active terminal of each primer (that is, the 3’ end) leads toward the other primer. PCR uses

replication enzymes that are tolerant of high temperatures, such as the thermostable Taq polymerase. In this fashion, two new copies of the sequence of interest are generated. Repeated denaturation, hybridization, and extension in this fashion produce an exponentially growing number of copies of the DNA of interest. Instruments that perform thermal cycling are readily available from commercial sources. This process can produce a million-fold or greater amplification of the desired region in 2 hours or less.

Early assays such as the HLA-DQ alpha reverse dot blot strips grew to be very popular due to their ease of use, and the speed with which a result could be obtained. However, they were not as discriminating as RFLP analysis. It was also difficult to determine a DNA profile for mixed samples, such as a vaginal swab from a sexual assault victim.

However, the PCR method was readily adaptable for analyzing VNTR, in particular STR loci. In recent years, research in human DNA quantitation has focused on new "real-time" quantitative PCR (qPCR) techniques. Quantitative PCR methods enable automated, precise, and high-throughput measurements. Inter-laboratory studies have demonstrated the importance of human DNA quantitation on achieving reliable interpretation of STR typing and obtaining consistent results across laboratories.

Possible Questions

Short questions

1. Describe the Selection of cell lines for high yield of secondary metabolites.
2. What is the methods for large scale cultivation of plant cells.
3. Explain the Medium composition and effect of nutrients.
4. Explain the Elicitor-induced production of secondary metabolites.
5. Explain the Effect of environmental factors.
6. Write about the Biotransformation using plant cell cultures.
7. What is Secondary metabolite release and analysis. Write short note on tropane alkaloids?
8. Define the different entrapment techniques
9. What is a microencapsulation?
10. Define immobilzation.
11. What is DNA Barcoding?
12. What is RFLP?

Essay type questions

1. Explain theproduction of secondary metabolite in detail..
2. Give in detail about the large scale cultivation using the cell cloning.
3. Explain in detail about the different bioreactors involved in the production of secondary metabolite.
4. Explain in detail about the phase I and II reaction of biotransformation
5. Write in detail about DNA Barcoding techniques in detail
6. Discuss the DNA fingerprinting techniques with examples.

UNIT-IV

SYLLABUS

Bioactive studies: Anticancer, Antidiabetic, Anti-inflammatory, heptoprotective, antimicrobials from medicinal plants, Antioxidants of plant origin- ROS. Toxicity studies on medicinal plant products on medicinal plant product and herbal Formulation.

Anticancer properties of Medicinal plant

Cancer has been a constant battle globally with a lot of development in cures and preventative therapies. The disease is characterised by cells in the human body continually multiplying with the inability to be controlled or stopped. Consequently, forming tumours of malignant cells with the potential to be metastatic.

Current treatments include chemotherapy, radiotherapy and chemically derived drugs. Treatments such as chemotherapy can put patients under a lot of strain and further damage their health. Therefore, there is a focus on using alternative treatments and therapies against cancer.

For many years herbal medicines have been used and are still used in developing countries as the primary source of medical treatment. Plants have been used in medicine for their natural antiseptic properties. Thus, research has developed into investigating the potential properties and uses of terrestrial plants extracts for the preparation of potential nanomaterial based drugs for diseases including cancer. Many plant species are already being used to treat or prevent development of cancer. Multiple researchers have identified species of plants that have demonstrated anticancer properties with a lot of focus on those that have been used in herbal medicine in developing countries.

Compounds which are characteristic to the plant kingdom and are necessary for plant survival and "housekeeping" of the organism are being investigated for their ability to inhibit growth and initiate apoptosis of cancerous cells. This article aims to take an overview of current plant derived compounds that have anticancer therapeutic properties and their developments in the field.. Epigenetic properties

The step towards development of cancer involves alterations of epigenetic processes and their deregulation .The control of hypermethylation of tumour-suppressor genes on CpG islands is deregulated in cancer cells. This can result in gene silencing and inactivation of tumour-suppressor genes. Drugs which can inhibit or reverse epigenetic alterations have been in development over recent years.

Chemically derived epigenetic drugs have been developed and undergone trials such as 5-azacytidine (azacitidine; Vidaza) and 5-aza-2'-deoxycytidine (decitbine; Dacogen) which are both DNMTi andHDACi such as suberoyanildehydroxamic acid (SAHA, Vorinostat, Zolinza) and FK228 (Romidespin, Istodax). However, it is difficult to engineer a chemically derived drug which is non-toxic to normal cells and is specific to cytotoxicity of cancer cells.

Therefore, development and research into naturally derived compounds to be used for anticancer treatment is becoming high in demand with a focus on those derived from plant species and their natural products There are many forms of cancer amongst the human population but they share similar characteristics or genotypes such as insensitivity to signals which inhibit cell growth making their replication limitless.

Apoptosis is evaded and never induced in cancer cells and angiogenesis is sustained within the tumour tissue allowing survival of cancer cells. Plant derived compounds have demonstrated properties to inhibit cancer cell activity such as inhibiting proliferation of cancer cells and inducing apoptotic cell death.

Plant compounds with anticancer properties

Medicinal plants have been used for thousands of years in folk medicines in Asian and African populations and many plants are consumed for their health benefits in developed nations. According to the World Health Organisation (WHO) some nations still reply of plant-based treatment as their main source of medicine and developing nations are utilising the benefits of naturally sourced compounds for therapeutic purposes. Compounds which have been identified and extracted from terrestrial plants for their anticancer properties include polyphenols, brassinosteroids and taxols.

Polyphenols

Polyphenolic compounds include flavonoids, tannins, curcumin, resveratrol and gallacatechins and are all considered to be anticancer compounds. Resveratrol can be found in foods including peanuts and grapes and red wine. Gallacatechins are present in green tea. It is thought including polyphenols in a person's diet can improve health and reduce risk of cancers by being natural antioxidants.

The cytotoxicity of polyphenols on a range of cancer cells has been demonstrated and their antioxidant properties determined. Polyphenols are thought to have apoptosis inducing properties showing anticancer properties which can be utilized. The mechanism in which polyphenols are thought to carry out apoptosis initiation is through regulating the mobilization of copper ions which are bound to chromatin inducing DNA fragmentation. In the presence of Cu(II), resveratrol was seen to be capable of DNA degradation. Other properties plant polyphenols show is their ability to interfere with proteins which are present in cancer cells and promoting their growth. Cancer agents may be altered through the polyphenol regulating acetylation, methylation or phosphorylation by direct bonding. For example, curcumin treated cancer cells in various cells lines have shown suppression of the Tumour Necrosis Factor (TNF) expression through interaction with various stimuli.

Flavonoids

Flavonoids are from the polyphenolic compounds and constitute a large family of plant secondary metabolites with 10,000 known structures. They are physiologically active agents in plants and becoming of high interest scientifically for their health benefits. Various plants have been investigated for their flavonoid content and how these compounds affect cancer cells, such as fern species and plants used in traditional Chinese medicines like the litchi leaf. There is a high content of flavonoid compounds such as anthocyanins, flavones, flavonols, chalcones and many more which can be found in just one structure of the plant like its seed identified and looked at the anticancer effects of flavonoids on human lung cancer cells (A456 cell line) from the fern species *Dryopteriserythrosora*. They found flavonoids to demonstrate cytotoxicity on cancer cells and to have high free radical scavenging activity. Purified flavonoids have also shown anticancer activities against other human cancers including; hepatoma (Hep-G2), cervical carcinoma (Hela) and breast cancer (MCF-7). The flavonoids extracted from *Erythrinasuberosa* stem bark (4'-Methoxy licoflavanone (MLF) and *Alpinumisoflavone* (AIF) were shown to have cytotoxic effects in HL-60 cells (human leukaemia).

MLF and AIF induced apoptosis through intrinsic and extrinsic signalling pathways. The mitochondrial membrane potential is significantly reduced due to the induction of apoptotic proteins. With mitochondria damage to cells the cancer cells cannot survive. Other studies have

looked at flavonoid extracts from fern species and found that even in low concentrations they still demonstrate high percentage of anticancer activity.

As previously mentioned polyphenols can inhibit or alter the regulation of proteins and other agents which may be contributing to the survival of cancer cells. Signal Transducer and Activator of Transcription (STAT) proteins are anti-apoptotic and contribute to cancer cell growth. MLF and AIF inhibit members of this family of proteins by preventing their phosphorylation needed for the cancer cells survival. Also, these flavonoids inhibit the expression of NF-κB which is needed for cancer cell survival and angiogenesis and proliferation.

Brassinosteroids

Brassinosteroids (BRs) are naturally occurring compounds found in plants which play roles in hormone signalling to regulate growth and differentiation of cells, elongation of stem and root cells and other roles such as resistance and tolerance against disease and stress. Also, BRs are used for regulation of plant senescence. They are essential for plant growth and development. BRs are another naturally occurring compounds which have demonstrated therapeutic significance in the cause against cancer.

Two natural BRs have been used in investigations with cancerous cells to demonstrate the anticancer properties that these compounds possess. 28-homocastasterone (28-homoCS) and 24-epibrassinolide (24-epiBL) have demonstrated anticancer effects on various cancer cell lines and proven to be effective at micromolar concentrations. A characteristic of cancer cells is that they do not naturally undergo apoptosis and proliferate indefinitely. BRs can induce responses necessary for growth inhibition and induce apoptosis by interacting with the cell cycle. BRs have been used in investigations to treat a range of cancer cell lines which include; T-lymphoblastic leukaemia CEM, multiple myeloma RPMI 8226, cervical carcinoma HeLa, lung carcinoma A-549 and osteosarcoma HOS cell lines. Also included are cell lines in breast cancer and prostate cancer. Estrogen receptor (ER), epidermal growth factor receptor (EGFR) and human EGFR-2 (HER-2) are some of the critical proteins which are targeted in treatment of breast cancer as they are abundant in breast cancer cells such as MCF-7, MDA-MB-468, T47D and MDA-MB-231. In prostate cancer cells (LNCaP and DU-145 cell lines) the androgen receptor (AR) is a critical protein involved in its development and shares a similar

structure to ER. BRs will interact or bind to receptors of these proteins and inhibit the growth of both hormone sensitive and hormone insensitive cancer cells. Also, BRs can induce cell cycle blockage.

Treatment of breast cancer cell lines with 28-homoCS and 24-epiBL showed reduction in cyclin proteins which are involved in G_1 cell cycle phase. At this phase in the cell cycle cells will either under repair or enter apoptosis, treatment with BRs induces apoptosis at this stage which cancer cells would not be able to do naturally without treatment. In prostate cancer cell lines, LNCaP and DU-145, the balance of apoptotic proteins which promote cell survival and those which induce programmed cell death changes with BRs treatment.

The levels of the Bax pro-apoptotic protein increase after BRs treatment and anti-apoptotic proteins such as Bcl-2 are reduced. Along with their anticancer properties BRs generate different responses in normal and cancer cells. A key specification in anticancer treatment is for the agent not to be cytotoxic to normal cells and be cell specific to cancer cells; this is where agents of BRs origin are of interest for therapeutic properties.

Anticancer plant-derived drugs

Plant-derived drugs are desired for anticancer treatment as they are natural and readily available. They can be readily administered orally as part of patient's dietary intake. Also, being naturally derived compounds from plants they are generally more tolerated and non-toxic to normal human cells. However, there are exceptions such as cyanogenetic glycosides, lectins, saponins, lignans, lectins and some taxanes. If plant-derived drugs can demonstrate selectivity in research, are non-toxic to normal cell lines and show cytotoxicity in cancer cell lines, these drugs can be lead into clinical trials for further therapeutic development. Plant-derived drugs can fall under four classes of drugs with the following activities; methytransferase inhibitors, DNA damage preventive drugs or antioxidants, histone deacetylases (HDAC) inhibitors and mitotic disruptors. The compounds being discussed are represented in Table 1with their origins, anticancer activity and their clinical trial development.

Table 1

Plant-derived drugs in research and clinical trials

Anticancer agent	**Isolated or derived from:**	**Compound activity**	**Research and clinical development**
Sulphoraphane	Isotiocynanate in cruciferous vegetables *Brassica*	Induces phase 2 detoxification enzymes; inhibits tumor growth in breast cancers; antiproliferate effects	Clinical trials with oral administration of cruciferous vegetable preparation with sulphoraphane
Paclitaxel (Taxol)	Taxane; *Taxus brevifolia* L	Microtubule disruptor; block mitosis; induce apoptosis; microtubules are polymerized and stabilized; disruption of spindle formation; inhibition of translational machinery	In clinical use; Phase I-III clinical trials; early treatment settings; non-small lung cancer, breast cancer, ovarian cancer, Kaposi sarcoma. Research and development in alternative drug administration using nanoparticles, naocochealtes and nanoliposomes.
Epipodophyllotox	*Podophyllumpeltatum*	Pro-apoptotic effects; cell	Lymphomas and

Anticancer agent	**Isolated or derived from:**	**Compound activity**	**Research and clinical development**
in	L.; Podophyllotoxin isomer	cycle interference	testicular cancer trials
Vincristine	*Catharanthusroseus*G. Don; Vinca alkaloids	Anti-mitotic; microtubule inhibitor; bind to β-tubulin; microtubule stabilizers or destabilizers; pro-apoptotic properties and induce cell cycle arrest; anti-tumour activity	Lymphomas, sarcomas and leukaemias; in clinical use; combination trials
Vinblastine			Testicular cancer, Hodgkins disease and lymphoma; in clinical use; combination trials
Vinorelbine			Non-small cell lung cancer; single and combination trials; Phase I-III
Vindesine			Clinical trials for acute lymphocytic leukaemia
Vinflunine			Clinical trials for activity against solid tumors; Phase III clinical trials
Pomiferin	Isoflavonoidisolatated from *Maclurapomifera*	Pro-apoptotic effects; DNA fragmentation; inhibits	Growth inhibition in six human

Anticancer agent	Isolated or derived from:	Compound activity	Research and clinical development
	; DereeisMalaccensis	oxidative damage of DNA; antioxidant activity; inhibits histone deacetylases; cytotoxicity of cancer cells	cancer cell lines: ACHN (kidney), NCI-H23 (lung), PC-3 (prostate), MDA-MB-231 (breast), LOX-IMVI (Melanoma), HCT-15 (colon)
Epigallacotechin-3-gallate	Catechin; green tea	Antioxidant; decrease DNA damage from oxidative stress; anti-proliferative effects; inhibition of specific kinases; inhibit carcinogenesis induced chemically or by UV	Clinical trials in prostate cancer treatment ; Phase I clinical study for oral dose administration
Combretastatin A-4 phosphate	Water-soluble analogue of combretastatin; *Combr etumcaffrum*	Anti-angiogenic; vasuclar shut-down of tumors; tumor necrosis	Early trials; mimics developed; clinical and preclinical trials
Roscovitine	Derived from olomucine; *Raphanuss ativus* L. (*Brassicaceae*)	Inhibition of cyclin dependent kinases; reduction of cell cycle progression	Phase II clinical trials in Europe
Flavopiridol	Synthetic flavonoid derivative; rohitukine	Anti-inflammatory; immunamodulatory activity;	Phase I and Phase II clinical trials in

Anticancer agent	Isolated or derived from:	Compound activity	Research and clinical development
	based structure; *Dysoxylumbi nectariferum*Hook.f. (*Meliaceae*)	tyrosine kinase activyt; growth inhibitory effects	solid tumors, lymphomas, leukaemias
Noscapine	Opium poppy (*Papaver somniferum*)	Antiproliferative properties; microtubule interfering; inhibits tumour growth and progression	Phase I and Phase II clinical trials; limited progression due to its limited solubility; research into alternative administration of drug using analogues and nanotechnology.

Compounds including sulforaphane, isothiocyanates, isoflavones and pomiferin are considered to be HDAC inhibitors. They inhibit the activity of carcinogenic proteins. For example, sulforaphane has shown to inhibit important targets in breast cancer proliferation. Decreased expression of ER, EGFR and HER-2 resulted from HDAC inhibition by sulforaphane treatment in breast cancer cell lines. In cancer cells, epigenetically-silenced genes which are functional for chromatin acetylation are reactivated by HDAC inhibitors and cancer cells are then able to enter programmed cell death (apoptosis). Plant-derived compounds which show inhibition of HDAC can enhance chemotherapeutic sensitivity in human cancers.

Derivatives of vinca alkaloids, vincristine, vinblastine, vinorelbine, vindesine and vinflunine are drugs which will inhibit the dynamics of microtubules by binding to β-tubulin. Taxanes such as paclitaxel and its analogue docetaxel are also microtubule disruptors. These

compounds inhibit cell cycle phase transitions from metaphase to anaphase causing cell cycle arrest and apoptosis. Replication of cancer cells is reduced by paclitaxel as it stabilizes or polymerizes microtubules in the cells. Paclitaxel was one of the first drugs to have a huge impact on cancer treatment and vincristine and vinblastine were two of the initial drugs to be isolated.

Combinations of drugs derived from vinca alkaloids, Taxusditerpenes, Podiphyllumlignans and Camptotheca alkaloids in plant extracts may enhance their anticancer effects and improve their efficacy as therapeutic agents. Extracts from *Urticamembranaceae, Artemesiamonosperma* and *Origanumdayi Post* in Solowely *et al*., 2014 were investigated to test their effects on a wide range of cancer cell lines from lung, breast, colon and prostate cancers. The investigation showed the plant extracts with a combination of anticancer compounds were able to have killing activity which was specific to cancer cells and showed no effect on normal human lymphocytes and fibroblasts. This makes plant extracts more desirable as therapeutic agents than those that are chemically derived which cause toxic complications in cancer treatment. The plant extracts induced apoptosis which was demonstrated by an increased sub-G_1phase population of cells with lower DNA content and condensation of chromatin. Also an increase in caspase 3 activation was seen after extract treatment which is a key stage in apoptotic cell death.

Discovery of anticancer cancer agents which show specificity towards cancer cells and can induce cell death and inhibit growth of tumours may be considered for clinical trials.

Enhancing drug administration

With advancements and discoveries in naturally derived drugs new technologies are emerging for the application and dosage of these anticancer compounds. Administration of new drugs needs to be effective for the compound to be a successful alternative to current treatments such as chemotherapy. Through the field of nanotechnology the use of nanoparticles (NPs), as a delivery system for drugs to reach target sites, is developing. Some compounds that have demonstrated anticancer activities may be limited in their clinical development due to the need for high dosages.

Bromleain, isolated from *Aanascomosus* was shown to be more effective as an anticancer agent in formulation with NPs than free bromelain. This research demonstrated a safe and biocompatible method using bromelain NPs to sustain release of the drug at the target site whilst also protecting the drug. These bromelain loaded polyllactic-co-glycolic acid NPs (BL-PLCG NPs) showed to trigger apoptosis of benign cells more so than free bromelain by regulating the expression of pro-apoptotic and anti-apoptotic proteins in 2-stage skin turmorigenesis in mice. Other NPs synthesized have also been investigated such as; gold NPs of *Antigonoleptopus* powdered extract and copper oxide NPs of *Acalyphaindica*. These formulations of plant extract and NPs showed cytotoxicity against MCF-7 breast cancer cell lines.

Paclitaxel has been through clinical trials and early treatment settings. Research and development is aiming to use NPs to control release of the drug and enhance target specificity by using magnetic mesoporous silica NPs with a gelatine membrane; Paclitaxel can be controlled externally using a magnetic field. This has shown to be successful in increasing the drug's ability to inhibit growth of tumours and reduce unwanted effects of other tissue areas as the drug's distribution is controlled. Success has also been seen with the drug quercetin using superparamagnetic magnetite NPs against breast cancer (MCF-7) cell lines. This research demonstrated enhanced activities of the NPs in cytotoxicity of MCF-7 cells compared to free or pure quercetin. NPs in their use for anticancer treatment are of growing interest and show promise as a natural alternative to current treatments.

Alternatively, research investigating application using nanocochleates and nanolipososmes demonstrates achievement in anticancer activities through oral or inhalable intake. Paclitaxel taken orally is most cost effective and more comfortable for the patient. A formulation of paclitaxel-loaded nanocochleates which can been administered orally showed controlled drug release and effective activities against lung, ovarian and breast cancer cell lines [41]. Also, noscapine was limited in clinical trials due to insoluble properties until derived analogues were developed. Jyoti *et al*., 2015 investigated the noscapine analogue 9-bromo-noscapine in formulation with nanostrucutured lipid particles. Here they showed enhanced

cytotoxicity and apoptosis in lung cancer cell lines with increased uptake of drug into cancerous cells of the formulated noscapine analogue compared to the free drug.

Antidiabetic properties of medicinal properties:

Diabetes mellitus is a group of metabolic disorders with one common manifestation ñ hyperglycemia. Chronic hyperglycemia causes damage to eyes, kidneys, nerves, heart and blood vessels. It is caused by inherited and/or acquired deficiency in production of insulin by the pancreas, or by the ineffectiveness of the insulin produced. It results either from inadequate secretion of hormone insulin, an inadequate response of target cells to insulin, or a combination of these factors. This disease requires medical diagnosis, treatment and changes in life style.

It is projected to become one of the world ís main disablers and killers within the next 25 years. The management of diabetes is a global problem until now and successful treatment is not yet discovered. There are many synthetic medicines developed for patients, but it is the fact that it has never been reported that someone had recovered totally from diabetes. The modern oral hypoglycemic agents produce undesirable and side effects.

Thus, alternative therapy is required, a need of hour is to shift towards the different indigenous plant and herbal formulations. The traditional medicines demonstrated a bright future in therapy of diabetes and to understand the importance of traditional herbs, the aim of the review is to collect the available data on plants with antidiabetic activity reported in the pharmaceutical journals.

Natural medicines used for diabetes therapy

Recently, some medicinal plants have been reported to be useful in diabetes worldwide and have been used empirically as antidiabetic and antihyperlipidemic remedies. Despite the presence of known antidiabetic medicine in the pharmaceutical market, diabetes and the related complications continued to be a major medical problem. Antihyperglycemic effects of these plants are attributed to their ability to restore the function of pancreatic tissues by causing an increase in insulin output or inhibit the intestinal absorption of glucose or to the facilitation of metabolites in insulin dependent processes. More than 400 plant species having hypoglycemic activity have been available in literature, however, searching for new antidiabetic drugs from natural plants is still attractive because they contain substances

It is the fact that diabetes can ít be cured and it has never been reported that someone had recovered totally from diabetes. The rapidly increasing incidence of diabetes mellitus is becoming a serious threat to mankind health in all parts of the world. Moreover, during the past few years some of the new bioactive drugs isolated from plants showed antidiabetic activity with more efficacy than oral hypoglycemic agents used in clinical therapy.

The traditional medicine performed a good clinical practice and is showing a bright future in the therapy of diabetes mellitus. The present paper reviews natural medicines with their mechanism of action and their pharmacological test results. Many studies have confirmed the benefits of medicinal plants with hypoglycemic effects in the management of diabetes mellitus. The effects of these plants may delay the development of diabetic complications and correct the metabolic abnormalities. WHO has pointed out this prevention of diabetes and its complications is not only a major challenge for the future, but essential if health for all is to attain.

Therefore, in recent years, considerable attention has been directed towards identification of plants with antidiabetic ability that may be used for human consumption. Further, it emphasizes strongly in this regard the optional and rational uses of traditional and natural indigenous medicines which demonstrate alternative and safe effects on diabetes mellitus. Most of plants contain glycosides, alkaloids, terpenoids, flavonoids, cartenoids, etc., that are frequently implicated as having antidiabetic effect. Species will be described in alphabetical order and information about each species will include in sequence: general botanical and taxonomic data, distribution in the world, experimental study and mechanism of action. Anacardiumoccidentale Linn. (Anacardiaceae), herb originated from Brazil, it is used as folk medicine in African countries, mainly in Cameroon, for the treatment of diabetes mellitus.

Hypoglycemic and protective role of A. occidentale was reported. The antihyperglycemic and renal protective activities of leaves of this herb were reported in streptozotocin induced diabetic rats. It reduces diabetes-induced functional and histological alterations in the kidneys. It was shown that histopathological study of A. occidentale significantly reduced accumulation of mucopolysaccharides in the kidneys of diabetic animal.

Annona squamosa Linn. (Annonaceae), commonly called custard apple in English and sharifa in Hindi. It is cultivated throughout India. The pharmacological active ingredients are present in seeds, leaves and aerial parts of the plant. The research reveals that the plant possesses both hypoglycemic and antidiabetic activity. It acts by enhancing insulin level from the pancreatic islets, increases utilization of glucose in muscle and inhibits the glucose output from liver. Its margin of safety is high. The extract obtained from leaves of this plant is useful in maintaining healthy blood sugar and cholesterol level.

Annona muricata Linn. (Annonaceae), commonly called soursop. It is small evergreen tree growing 5 to 6 meters in height. Young branches are rusty-hairy, the malodorous leaves, and the plant is evergreen. Annona muricata is indigenous to most of the warmest tropical areas in South and North America, including the Amazon. The researchers revealed the immunohistochemical and biochemical effects of aqueous extract of leaves on pancreatic β cells of STZ (streptozotocin) treated diabetic rats. *A. muricata*Linn. leaf extract played important role in reduction of oxidative stress on pancreatic β cells of streptozotocin treated diabetic rats. The treatment increased the area of insulin immunoreactive β-cells and partially prevents degeneration of β-cells. Boerhaaviadiffusa Linn. (Nyctaginaceae), distributed widely all over in India, is a small perennial creeping herb, commonly known as ìRedhogweedî.

The root and the whole plant are used as an Ayurvedic medicine in India and Unani medicine for the treatment of diabetes, stress, dyspepsia, abdominal pain, inflammation, jaundice, enlargement of spleen, congestive heart failure and bacterial infections. Aqueous leaf extract of the plant has been studied for its antidiabetic effect in alloxan-induced diabetic rats. The antidiabetic activity of the chloroform extract of the plant leaves on chronic treatment of streptozotocininduced NIDDM (non insulin dependent diabetes mellitus) model diabetic rats was evaluated and the herb possesses antidiabetic activity. The herb mainly acts by reducing blood glucose level and increasing insulin sensitivity. *Bougainvillea spectabilis* Linn. (Nyctaginaceae), is a very familiar ornamental plant commonly grown in Indian gardens. Bougainvillea is a genus of flowering plants native to South America from Brazil west to Peru and south to southern Argentina.

The traditional plant has the antidiabetic potential. The blood glucose lowering potential of Bougainvillea spectabilis Wild leaf extract in streptozotocin-induced type I diabetic albino rats was reported. The ethanolic extract of the leaves has antihyperglycemic activity probably due to increased uptake of glucose by enhanced glycogenesis in the liver and also due to increase in insulin sensitivity. BrideliandellensisBeille. (Euphorbiaceae), a medicinal plant used in Cameroon against diabetes.

The water and methanol extract of leaf of allied species B. ferruginea has been proved as an active hypoglycemic agent in alloxan induced diabetic rats. The study of the glucose lowering of the ethanol extract and fractions of B. ndellensis stem bark in STZ (streptozotocin) type I and II diabetes rats at different prandial states was performed and significant lowering in blood glucose level was observed. The extract act by stimulation of islets cells and requires functional β-cells for its action (20). Canavaliaensiformis DC. (Leguminosae), known as horse bean, native of Central America and West Indices has been widely cultivated in humid tropics of Africa and Asia. The seeds have been reported to possess antihypercholesterolemic and hypoglycemic activities.

Anti-inflammatory activities of medical plants

Inflammation is the reactive state of hyperemia and exudation from blood vessels with consequent redness, heat, swelling and pain which a tissue manifests in response to physical or chemical injury or bacterial invasion. It is a tissue reaction by the body to injury and involves a complex array of enzyme activation, mediator release, extravasations of fluid, cell migration, tissue breakdown and repair. As a result of the inherent problems associated with the current anti-inflammatory agents, there is continuous search especially from natural sources for alternative agents.

A good number of plants are employed in the treatment of inflammatory disorders by natural healers. Some of these plants include *Aloe vera, Consolidaregalis, Chasmantheradependens, Culcasiascandens, Crataevareligiosa, Tanacetum vulgare, Holmskioldiasanguinea, Mitracarpusscaber, Turner ulmifolia,* etc. Most of these plants have demonstrated varying activities in the various in vivo and in vitro inflammatory models. The potency of these plants is attributed to several active principles present in them, which may act at any of the multiple targets in the inflammatory response pathway.

In addition to anti-inflammatory activity, some of these plants also possess beneficial properties such as antimicrobial, analgesic, antipyretic and antiulcer effects. These additional effects complement the inherent anti-inflammatory activity and may confer advantage on these plants. Some active anti-inflammatory principles of these plants have been identified, isolated and characterized.

They include – lupeol, premnazole, (+) – usnic acid, (+) – pinitol, zanhasaponins A and B, sasanquol, parthenolide etc. These compounds could provide drugs with comparative advantage over existing agents and may as well serve as leads for further development into more active drugs with lesser adverse effects. This review discusses some of these plants with putative anti-inflammatory properties

Three components of the inflammatory response have been distinguished and these may involve vasoactive substances chemotactic factors, degradative enzymes and superoxides and the neuropeptide, Substance P. Rheumatoid arthritis represents the commonest form of chronic inflammatory joint disease. Arthritis is one of the most distressing and disabling syndromes encountered in medical practice.

An estimated 1-2% of adult population is affected. In the United States, approximately 0.1% of the population experience rheumatoid arthritis in childhood. Steroids e.g. betamethasone and the nonsteroidal anti-inflammatory drugs (NSAIDs) e.g. acetylsalicylic acid are the mainstay in the treatment/management of inflammation and inflammatory disease conditions. However, these agents are fraught with severe adverse effects such as adrenal suppression for steroids and gastric ulceration and perforation for NSAIDs. Most NSAIDs are known to exert potentially adverse effects on the gastrointestinal tract. These have seriously limited the employment of these agents in inflammation and inflammatory diseases therapy.

Several efforts have been made to reduce the adverse effects of NSAIDs. It is now accepted that cyclooxygenase (COX) enzyme exists in two isoforms – COX I (constitutive) and COX II (inducible). The therapeutic activities of NSAIDs are attributed to the inhibition of COX II. Therefore, an ideal anti-inflammatory drug is expected to inhibit prostaglandin synthesis mediated by COX II while sparing COX I, inhibition of which is believed to mediate the side effects. Much as it would seem that the selective COX II inhibitors such as celecoxib and

rofecoxib might be cost effective for patients at high risk of ulcer complications, serious theoretical concerns exist due to the potential risk of thrombosis.

And so, though arthritis is one of the oldest known diseases, there is yet no drug leading to a permanent cure and which is devoid of adverse effects. Nature endows the world with medicinal plants to take care of health needs. The potentials of plants as sources of drugs have long been recognized. Several medicinal plants species are commonly used in traditional medicine as inflammatory remedies. Some of these plants.

There are representative anti-inflammatory herbs in almost each family in the plant kingdom.

Many of these plants have proven oral and documented evidence of their use in the treatment of inflammatory disorders in traditional medicine. For some plants, inherent anti-inflammatory activity is inferred from other identified pharmacological activities related to modulation of the complex inflammatory response.

At present, there is mounting scientific evidence for the anti-inflammatory activity of many herbs. For some, the anti-inflammatory activity has been extensively studied while preliminary evidence has been established for others. A number of anti-inflammatory constituents have been isolated and characterized structurally and pharmacologically. 2. Plants with reported anti-iflammatory activity. Aegle marmelosRoxb. (Rutaceae)

A. marmelos, also known as Bilva, is a commonly growing deciduous tree with sharp axillary thorns.

Heptoprotectives from medical plants:

Liver diseases which are still a global health problem may be classified as acute or chronic hepatitis (inflammatory liver diseases), hepatosis (non inflammatory diseases) and cirrhosis (degenerative disorder resulting in liver fibrosis). Unfortunately, treatments of choice for liver diseases are controversial because conventional or synthetic drugs for the treatment of these diseases are insufficient and sometimes cause serious side effect.

Since ancient times, mankind has made use of plants in the treatment of various ailments because their toxicity factors appear to have lower side effects. Many of the currently available drugs were derived either directly or indirectly from medicinal plants. Recent interest in natural therapies and alternative medicines has made researchers pay attention to

traditional herbal medicine. In the past decade, attention has been centered on scientific evaluation of traditional drugs with plant origin for the treatment of various diseases. Due to their effectiveness, with presumably minimal side effects in terms of treatment as well as relatively low costs, herbal drugs are widely prescribed, even when their biologically active constituents are not fully identified.

The utility of natural therapies for liver diseases has a long history. Despite the fact that most recommendations are not based on documented evidence, some of these combinations do have active constituents with confirmed antioxidant, anti-inflammatory, anticarcinogenic, antifibrotic, or antiviral properties. Although a large number of these plants and formulations have been investigated, the studies were mostly unsatisfactory. For instance, the therapeutic values, in most of these studies, were assessed against a few chemicals-induced subclinical levels of liver damages in rodents. The reasons that make us arrive at such a conclusion are lack of standardization of the herbal drugs, limited number of randomized placebo controlled clinical trials, and paucity of traditional toxicologic evaluations.

Hundreds of plants have been so far examined to be taken for a wide spectrum of liver diseases. Natural products, including herbal extracts, could significantly contribute to recovery processes of the intoxicated liver. According to reliable scientific information obtained from the research on medicinal plants, plants such as *Silybummarianum, Glycyrrhizaglabra, Phyllanthus* species (amarus, niruri, emblica), *Picrorhizakurroa* have been widely and most of the time fruitfully applied for the treatment of liver disorders, exerting their effects via antioxidant-related properties.

Iranians have been using herbal medicine for the treatment of some common diseases; as a result, a large number of studies have been conducted to suggest new wild medicinal plants in different parts of Iran. Iranian traditional medicine is mostly relied on the consumption of plant materials. One of the important and well-documented utilities of plant products is their use as hepatoprotective agents. There are a number of medicinal combinations in the Iranian traditional medicine which are commonly used as tonic for liver. *Allium hirtifolium* Boiss. (*A. hirtifolium*), *Apiumgraveolens* L. (*A. graveolens*), *Cynarascolymus* (*C. scolymus*), *Berberis vulgaris* L. (*B. vulgaris*), *Calendula officinalis* (*C. officinalis*), *Nigella sativa* L. (*N. sativa*), *Taraxacumofficinale* (*T. officinale*), *Tragopogonporrifolius* (*T.*

porrifolius), *Prangosferulacea* L. (*P. ferulacea*), *Allium sativum* L. (*A. sativum*), *Marrubium vulgare* L. (*M. vulgare*), *Ammi majus* L. (*A. majus*), *Citrulluslanatus* (*C. lanatus*), *Agrimoniaeupatoria* L. (*A. eupatoria*) and *Prunusarmeniaca* L. (*P. armeniaca*) are some of medicinal plants that have been used mainly for the treatment of liver disorders in Iranian folk medicine.

A. hirtifolium

A. hirtifolium from Alliaceae family, commonly known as Persian shallot (moosir in Persian) is endemic to Iran. Based on available pharmaceutical investigations, antioxidant and hepatoprotective effects of *A. hirtifolium* have been also demonstrated. In addition, *A. hirtifolium* extracts had antioxidant properties comparable to or slightly higher than garlic extracts.

The commonly known phytochemical compounds identified in *A. hirtifolium* are saponins, sapogenins, sulphur containing compounds (*e.g.* thiosulfinates) and flavonoids including shallomin, quercetin and kaempferol. Alliin, alliinase, allicin, s-allyl-cysteine, diallyldisulphide, diallyltrisulphide, and methylallyltrisulphide are the most important biological secondary metabolites of *A. hirtifolium*. Disulphide and trisulfide compounds are among the most important compounds existing in *A. hirtifolium*.Researches have shown that both the corn and the flower of shallot contain a high density of glycosidic flavonols. Linolenic, linoleic, palmitic, palmitoleic, stearic, and oleic acids have been identified in *A. hirtifolium* oil, as well.

Treating rats with hydroalcoholic extract of *A. hirtifolium* could protect liver cellsagainst oxidant effects of alloxan, and consequently caused a significant reduction in serum concentration of alkaline phosphatase (ALP), alanine transaminase (ALT), and aspartate transaminase (AST). Biochemical results have confirmed the usefulness of *A. hirtifolium* extract in decreasing the destructive effects of alloxan on liver tissue, and consequently decreasing the enzymes' leakage into cytosol, which is possibly achieved by herbal antioxidant compounds including flavonoids. It was also reported that consumption of *A. hirtifolium* caused a reduction in AST level compared to the group with a hypercholesterolemic diet. A research on the effect of hydroalcoholic *A. hirtifolium* extract on the level of liver enzymes in streptozotocin-induced

diabetic rats indicated that hydroalcoholic extract of *A. hirtifolium* could significantly decrease serum levels of liver enzymes [AST, ALT, ALP and (lactate dehydrogenase) LDH] in a dose-dependent manner. Antioxidant micronutrients in the extract of *A. hirtifolium* may also restore liver damages. Shallomin and other active constituents of *A. hirtifolium* did not produce any adverse effect on the organs such as liver and kidney.

A. graveolens

A. graveolens, commonly known as celery, is an edible plant of the Umbelliferae family that grows mostly in the Mediterranean areas. It has been considered as a medicinal plant for a long time. Data obtained from literature reveal that *A. graveolens* has many pharmacological properties such as antifungal, antihypertensive, antihyperlipidemic, diuretic, and anticancer. This plant has also been shown to have some other medicinal features including hyperlipidemic effects as well as antioxidative and hepatoprotective activities.

The active constituents are isoimperatorin, isoquercitrin, linoleic acid, coumarins(seselin, osthenol, apigravin, and celerin), furanocoumarins (including bergapten), flavonoids (apigenin, apiin), phenolic compounds, choline, and unidentified alkaloids. *A. graveolens* is full of betacarotene, folic acid, vitamin C, sodium, magnesium, silica, potassium, chlorophyll, and fiber. The essential oil contains deltalimonene and various sesquiterpene.

Seeds of *A. graveolens* are used in Iranian medicine for liver ailments and disorders, have effects on liver, and exhibit hepatoprotective activities. Examining the antihepatotoxic effect of *A. graveolens* seeds' methanolic extracts on rats' liver showed a significant hepatoprotective activity. The roots open obstruction of the liver and spleen, and help in dropsy and jaundice treatment. Due to apigenin-related anti-inflammatory and antioxidant properties, *A. graveolens* seeds could counteract the pro-oxidant effect of 2-acetylaminofluorine through scavenging superoxide radicals, consequently declining hepatic glutathione-S-transferase (GST) and decreasing release of γ-glutamyltranspeptidase in serum; as a result, *A. graveolens* could be assumed as a potent plant against experimentally induced hepatocarcinogenesis in rats.In addition, different extracts of the plant were examined for their hepatoprotective activity against $CC1_4$-induced hepatotoxicity in albino rats.

The methanolic extracts, comparable to silymarin as a conventional drug, exhibited a higher hepatoprotective activity. Another study indicated that the extracts of *A. graveolens* root significantly decreased $CC1_4$-induced acute hepatic injury, increasing the activities of AST and ALT and preventing $CC1_4$-induced acute liver injury. Crude ethanol extract of the whole plant was indicative of anti-inflammatory effects in rats.

Furthermore, topical anti-inflammatory effects of *A. graveolens* leaves' extract have been demonstrated by Mencherini *et al*. Significant anti-inflammatory effect of the aqueous and hexane extracts of *A. graveolens* was shown at all doses (100-500 mg/kg body weight). Both extracts presented remarkable anti-inflammatory effect, which confirmed the traditional use of *A. graveolens* in inflammation-associated diseases.

C. scolymus

C. scolymus (artichoke) from Apiaceae family, a species of perennial thistle and with a Mediterranean origin, is traditionally used for the treatment of digestive disorders, moderate hyperlipidemia, and liver and bile diseases. The leaf extract of *C. scolymus*has been used for its hepatoprotective effects. Also, *C. scolymus* extract could yield nutritional supplements with antioxidant and antimicrobial effects. In *C. scolymus* leaf extract, there are compounds such as cynarin, luteolin, chlorogenic acid, and caffeic acid, other flavonoids, and polyphenol compounds, some of which have antioxidant properties. *C. scolymus* leaf extract also positively affected the changes in rat serum liver enzyme induced by $CC1_4$ and histopathological damage to liver tissue.In rats pretreated with artichoke extract, plasma transaminaseactivities significantly decreased and histopathological changes in the liver ameliorated. *C. scolymus* can be conducive to the reduction in phosphatidatephosphohydrolase activity and liver triglyceride. *C. scolymus* has benefits for controlling of hyperlipidemia, oxidative stress in hyperlipidemic regimes, and abnormalities in lipid profiles. In the rabbits intoxicated with $CC1_4$, *C. scolymus*leaf extract counteracted the toxic effect of $CC1_4$, blood sugar, cholesterol, triglycerides, leukocytes, and a number of erythrocytes[34]. In other studies, *C. scolymus* was significant in keeping the normal liver function parameters, maintained the hepatic redox status as it is manifested by significant increase in antioxidant enzyme activities and reduction in glutathione accompanied by inhibition of lipid peroxidation (LPO) and protein oxidation, decreased nitric oxide and tumor necrosis factor alpha, and stabilized membrane in the untreated paracetamol-intoxicated rats.

B. vulgaris

B. vulgaris (barberry), a well known medicinal plant in Iran and also a food, belongs to Berberidaceae family. As a shrub with 1 to 3 meters in height, *B. vulgaris* grows in many regions of the world, including Iran (especially Khorasan)[36]. Fruit, leaves, and stem have medical usages including hepatoprotection. *B. vulgaris* fruit extract contains various flavonoids that act as antioxidant. Berberine, oxyacanthine, and other alkaloids such as berbamine, palmatine, columbamine, malic acid, jatrorrhizine, and berberrubine comprise some other compounds. Stigmasterol, terpenoidslupeol, oleanolic acid, stigmasterol glucoside and polyphenols were also identified in this plant. Berberine, an isoquinoline alkaloid with a long medicinal history, exists in roots, rhizomes, and stem bark of the plant. Berberine inhibits potassium and calcium currents in isolated rat hepatocytes. It has hepatoprotective effects, both preventive and curative, on $CC1_4$-induced liver injury through scavenging the peroxidative products. $CC1_4$ significantly increased the serum alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase levels in rats. Treatment with the methanolic extract of *B. vulgaris* fruit significantly helped these changes reach to an almost normal level. In addition, the extract could prevent $CC1_4$-induced liver oxidative damage in rats.

C. officinalis

C. officinalis (marigold), from Asteraceae family, is a medicinal plant and cosmetic herb popularly known in Europe and the USA. The dried flower heads or the dried ligulate flowers of this plant are used for pharmaceutical and/or cosmetic purposes. Antibacterial, anti-inflammatory, antiviral, and antioxidant activities have been already noted for *C. officinalis*. It has been taken in order to treat fevers and jaundice and to promote menstruation. Extracts, tinctures, balms, and salves of *C. officinalis* have been applied directly to heal wounds and soothe inflamed and injured skin. *C. officinalis* compounds, which are potentially active chemical constituents, are monoterpenes, such as α-thujene and T-muurolol, sesquiterpene and flavonol glycosides, triterpene alcohols, triterpenoid saponins, flavonoids, carotenoides, xanthophylls, phenolic acids, mucilage, bitters, phytosterols, tocophrrols, calendulin, resin, and volatile oil. The anti-inflammatory features of *C. officinalis* flowers, according to *in vivo* pharmacological tests, have been associated with the triterpenoid fatty acid esters. In Singh's study, 80% effect of methanolic extract of leaves (500 mg/kg orally, four doses

at 12 hours interval) of *C. officinalis* was investigated against acetaminophen-induced hepatic damage in albino rats. The potential hepatoprotective effects of *C. officinalis* extracts against $CC1_4$-induced oxidative stress and cytotoxicity in isolated primary rat hepatocytes were detected, confirmed by significant improvement in cell viability and enzymes leakages (ALT, AST, and LDH). Also, the reduction of hepatocytolysis and steatosis, and return to normal values of various enzymes activity could be attributed to hepatoprotective effects. *C. officinalis* plant extracts significantly improved cell survival, contributing greatly to preserving the cellular membranes integrity against $CC1_4$. Possible mechanism of action of the flower extract may be due to its antioxidant activity and reduction of oxygen radicals.

N. sativa

N. sativa is an aromatic plant of Ranunculaceae family, traditionally used by the Middle East nations for asthma, cough, bronchitis, headache, rheumatism, fever, influenza, and eczema. Several biological activities, including antioxidant activity and resolution of hepato-renal toxicity have been reported for *N. sativa* seeds. *N. sativa* contains more than 30 fixed oils.

The volatile oil has been proved to contain thymoquinone and many monoterpenes such as p-cymene and α-pinene. The $CC1_4$treatment increased the LPO and liver enzymes, and decreased the antioxidant enzyme levels. *N. sativa* treatment helped the elevated LPO and liver enzyme levels decrease and the reduced antioxidant enzyme levels increase.

The levels of liver enzymes and total oxidative status, oxidative stress index, and myeloperoxidase in treated mice were significantly lower, and total antioxidant capacity in liver tissue was significantly higher compared to the controls. *N. sativa* is useful in the treatment of rheumatism and related inflammatory diseases and the anti-inflammatory effect was confirmed in rats.

Also, the aqueous extract of *N. sativa* has an anti-inflammatory effect demonstrated by its inhibitory effects on carrageenan-induced paw edema. Pretreatment of mice with 12.5 mg/kg thymoquinone (an *N. sativa* derived-compound) significantly reduced the elevated levels of serum enzymes as well as hepatic MDA content and significantly increased hepatic nonprotein sulfhydryl(-SH). *N. sativa*contributes to inhibition of enzymes present in the neoglucogenesis pathway in the liver.

T. officinale

T. officinale (from Asteraceae family), commonly known as dandelion, grows almost everywhere in the world. With a long history of traditional use in the treatment of hepatobiliary problems, its root has been shown to have sesquiterpene lactones, triterpenes, carbohydrates, fatty acids (myristic), carotenoids (lutein), flavanoids (apigenin and luteolin), minerals, taraxalisin, coumarins, and cichoriin. Aesculin has been reported from the leaf. Germacrane- and guaiane-type sesquiterpene lactones including taraxinic acid derivatives were obtained from the roots of this plant.

Also, several flavonoids, *e.g.* caffeic acid, chlorogenic acid, luteolin, and luteolin 7-glucoside, have been isolated from the dandelion. Ethanolic extract of *T. officinale*was effective on decrease in serum ALT levels. Hydroalcoholic acid extract of the root enhanced levels of superoxide dismutase (SOD), catalase (CAT), GST, and LPO. Oral administration of extracts of the *T. officinale* roots has proved to increase bile flow. Another study distinguished that treatment with root extract of *T. officinale* was effective on reduction of serum ALT and ALP levels in rats. Root extract reduces serum AST, ALT, ALP, and LDH activities and increases hepatic antioxidant activities such as CAT, GST, glutathione reductase, glutathione peroxidase, and glutathione.

Thus, aqueous extract of *T. officinale* root protects against alcohol-induced toxicity in the liver by elevating antioxidativity and decreasing LPO. Sesquiterpene lactones in the plant have a protective effect against acute hepatotoxicity induced by the administration of $CC1_4$ in mice, which was indicated by reduced levels of hepatic enzyme markers, such as serum transaminase (ALT, AST), ALP, and total bilirubin.

T. porrifolius

T. porrifolius, belonging to Asteraceae family and known as purple salsify, is grown up for its edible root and shoot[63]. It has bioactive compounds which prevent cancer or other free radical-associated illnesses. The nutritional value of this plant is derived from monounsaturated and essential fatty acids, polyphenols, vitamins, and fructo-oligosaccharides, having probiotic effects on the intestinal microflora. The most abundant compounds of this plant include 4-vinyl guaiacol (19.0%), hexadecanoic acid (17.9%),

hexahydrofarnesylacetone (15.8%), and hentriacontane (10.7%). *T. porrifolius* has apparently yielded the hepatogenic/hepatoprotective effects against liver diseases or hepatotoxicity induced by a variety of hepatotoxic agents such as chemicals, drugs, pollutants, and infection with parasites, bacteria, or viruses (hepatitis A, B, and C). These beneficial effects of plants are related to the polyphenolic compounds. The study of antioxidant activity of the methanolic extract of the aerial part of *T. porrifolius* as well as its protection against $CC1_4$-induced hepatotoxicity in rats showed a dose-dependent increase in the activity of liver antioxidant enzymes. About 250 mg/kg body weight dose increased the activity of CAT, SOD, and GST.

Also, substantial hepatoprotective capacity against $CC1_4$-induced hepatic injury has been shown, attributable to restoring the activity of AST, ALT, and LDH to normal levels. Investigation of the effects of water extract of *T. porrifolius* shoot on lipemia, glycemia, inflammation, oxidative stress, hepatotoxicity, and gastric ulcer using a rat model showed that after one month of *T. porrifolius* water extract intake, a significant decrease in the levels of serum cholesterol, triglyceride, glucose, and liver enzyme (ALP, ALT, and LDH) was observed. Pretreating rats with *T. porrifolius* extract demonstrated considerable anti-inflammatory effects in both acute and chronic inflammation caused by carrageenan and formalin. In addition, *T. porrifolius* revealed effective antioxidant capability owing to its remarkable scavenging activity.

P. ferulacea

P. ferulacea from Apiaceae family grows in Southern Iran and is used in Iranian herbal medicine mainly for gastrointestinal disorders. The genus of *Prangos* with the common Persian name of Jashir includes 15 species, occurring widely in many regions of the country. In addition to Iran, other species of this genus are distributed in East Europe to Turkey, Caucasia, and Central Asia. *P. ferulacea* has been used in folk medicine as carminative, emollient, and tonic for gastrointestinal disorders, antiflatulent, sedative, anti-inflamatory, anti-viral, antihelminthic, antifungal, and antibacterial. Monoterpenes, sesquiterpenes, coumarines, flavonoids, alkaloids, tannins, saponins, and terpenoids are some important compounds identified in this plant.

P. ferulacea was shown that the oils (both fruit and leaf essential oils) were rich in monoterpenes, specially α-pinene, and β-pinene. Some of these components have an antioxidant

effect against oxidative stress. In a study, the protective and antioxidant effects of *P. ferulacea* are reported to be higher compared to α-tocopherol (vitamin E) and the effect of GST has been demonstrated. The study of effects of *P. ferulacea*hydroalcoholic extract on changes in rats´ liver structure and serum activities of ALT and AST after alloxan injection indicated that in diabetic rats, the serum ALT and AST significantly increased. Moreover, necrosis of hepatocytes, cytoplasmic vacuolations, and lymphocytic inflammation were observed. Diabetic rats treated by root extract of *P. ferulacea* in contrast to the diabetic group exhibited a significant decrease in these enzymes. Also, root hydroalcoholic extract of *P. ferulacea* was shown to affect changes in aminotransferases and to prevent the histopathological changes of liver related to alloxan-induced diabetes in rats.

Antimicrobial activity from medicinal plants

Infectious disease are the world's leading cause of premature deaths, killing almost 50 000 people every day. Morbidity and mortality due to diarrhea continues to be a major problem in many developing countries, specially amongst children. Infections due to variety of bacterial etiologic agents such as pathogenic *Escherichia coli, Salmonela spp., Staphylococcus aureus* are most common. In recent years drug resistance to human pathogenic bacteria has been commonly reported from all over the world. With the continuous use of antibiotics microorganism have become resistant. In addition to this problem, antibiotics are sometimes associated with adverse effects on host which include hypersensitivity, immunosuppressant and allergic reactions. This has created immense clinical problems in the treatment of infectious diseases.

Therefore, there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases; one approach is to screen local medicinal plants for possible antimicrobial properties. Plant materials remain an important recourse to combat serious diseases in the world. According to WHO (1993), 80% of the world's population is dependent on the traditional medicine and a major part of the traditional therapies involves the use of plant extracts or their active constituents. Yet a scientific study of plants to determine their antimicrobial active compounds is a comparatively new field. The traditional medicinal methods, specially the use of medicinal plants, still play a vital role to cover the basic health needs in the developing countries.

Since ancient times, herbs and their essential oils have been known for their varying degrees of antimicrobial activity. In recent times, the search for potent antibacterial agents has been shifted to plants. Most plants are medicinally useful in treating disease in the body and in most of cases the antimicrobial efficacy value attributed to some plants is beyond belief. Conservative estimates suggest that about 10% of all flowering plants on earth have at one time, been used by local communities throughout the world but only 1% have gained recognition by modern scientists.

There are about 120 plant-based drugs prescribed worldwide and they come from just 95 plant species. Approximately 250,000 species of flowering plants and only 5000 have had their pharmaceutical potential assessed. The treatment of infectious diseases with antimicrobial agents continues to present problems in modern-day medicine with many studies showing a significant increase in the incidence of bacterial resistance to several antibiotics. Due to increased resistance of many microorganisms towards established antibiotics, investigation of the chemical compounds within traditional plants has become desirable. There are many published reports on the effectiveness of traditional herbs against Gram-positive and Gram-negative microorganisms, and as a result plants are still recognized as the bedrock for modern medicine to treat infectious diseases.

Neem (*Azadira chtaindica* A. Juss) Neem is one of the most important native medicinal plants of India, as it has a wide spectrum of biological activity and is the most useful traditional medicinal plant in India. Each part of neem tree has some medicinal property. Neem leave, bark extracts and neem oil are commonly used for therapeutic. Neem oil suppresses several species of pathogenic bacteria such as *S. aureus* and *S. typhosa*, all strains of M. tuberculosis. The growth of *S. paratyphi*and *V. cholerae* was inhibited. Considerable progress has been achieved regarding the biological activity and medicinal applications of neem compounds, which have chemical and structural diversity. This versatile tree is now considered a valuable source of unique natural products for the development of medicines, including non-antibiotic drugs, against bacterial infections and various other human disorders; thus, the tree is still regarded as the “village pharmacy” in India.

*Curcuma longa (*C. longa), a perennial herb, is a member of the Zingiberaceae family and has a Long tradition of use in the Chinese and Ayurvedic systems of medicine. Curcuminoids, a group of phenolic compounds isolated from the roots of C. longa, exhibited a variety of beneficial effects on health and has the ability to prevent certain diseases. In East Asia, the rhizomes from C. longa, are considered to have natural medicinal properties, including antibacterial, anti-inflammatory, antineoplastic, and analgesic activities because they contains a number of moniterpenoids, sesquiterpenoids, and curcuminoids. It is also reported to have insecticidal activity.

In addition, wound healing and detoxifying properties of curcumin have also received considerable attention. Study by Curcumin is the most important fraction which is responsible for the biological activities of turmeric. The melting point of curcumin, C2H2OO6, is 184 ºC. It is soluble in ethanol and acetone, but insoluble in water. Curcumin 95%, a potentantioxidant is believed to be the most bioactive and soothing portion of the herb turmericandposses the properties like antioxidant, anti-inflammatory, anti-platelet, cholesterol lowering antibacterial and anti-fungal effects. It contains a mixture of powerful antioxidant phytonutrients known as curcuminoids and inhibits cancer at initiation, promotion and progression stages of tumor development. It is a strong anti-oxidant, which supports colon health, exerts neuroprotective activity and helps to maintain a healthy cardiovascular system.

C. longa oil was tested against cultures of *Staphylococcus albus, S. aureus and Bacillus typhosus,* inhibiting the growth of *S. albus and S. aureus* in concentrations up to 1 to 5,000. Keeping in view the important role of turmeric in inhibition of different cultures of bacteria and its role as antioxidant and antibacterial, the present study was conducted to compare the antibacterial activity extracts of C. longa varieties and potency of turmeric varieties on some bacteria. *Acacia nilotica* L. is a common, medium sized tree, locally known as ‘Babul’ or ‘Kikar’ belongings to the family Mimosaceae. Acacia is the most significant genus of family Leguminosae.

Antioxidant activity

In recent years much attention has been devoted to natural antioxidants and their health benefits. Antioxidant-based drug formulations are used for the prevention and treatment of many complex diseases. Plants are a major source of natural antioxidants; they produce a wide range of secondary metabolites with antioxidative activities that have therapeutic potential. Polyphenols are the most abundant antioxidant compounds of plant raw material.

Their antioxidant activity is based on to their redox properties, which facilitate their activity as reducing agents, hydrogen donors, singlet oxygen quenchers, metal chelators and reductants of ferryl hemoglobin. The reducing ability is generally associated with the presence of reductants which exert antioxidant action through breaking the free radical chain by donating a hydrogen atom or preventing peroxide formation. Medicinal plant tissues are commonly rich in phenolic compounds such as flavonoids, phenolic acids, stilbenes, tannins, coumarins, lignans and lignins. These compounds have multiple biological effects including antioxidant activity.

The eight plant species evaluated in the study demonstrated variability in antioxidant characteristics. Extracts of the *Achillea* species studied, e.g., *A. grandifolia* and *A.crithmifolia*, demonstrated high antioxidant potential. This is the first report for antioxidant capacity of these species. The extract of *A. crithmifolia* contained the highest level of polyphenols and flavonoids among all tested species and it demonstrated the highest antioxidant capacity. These results are in accord with high levels of flavonoid and phenol contents reported previously for *A. crithmifolia*as well as for other *Achillea* species.

The plant T. parthenium contains a large number of natural products, but the active components probably include one or more of the sesquiterpene lactones, including parthenolide which were demonstrated to have prophylactic properties over migraine and arthritis. Other potentially active constituents include flavonoid glycosides and pinenes. Alcohol extraction of *T. parthenium* powder, contained camphor, parthenolide, luteolin and apigenin. The results obtained by the DPPH method are similar to a previous report 85%) .The small difference may result from variations in the extraction protocol or plant growing conditions.

The phytochemical study of the aerial parts of *H. officinalis* cultivated in Xinjiang, China, revealed two new flavonoid glycosides (quercetin 7-O-b-Dapiofuranosyl-(1→2)-b-dxylopyranosideand quercetin 7-O-b-D-apiofuranosyl-(1→2)-b-D-xylopyranoside 30-Ob-D-

glucopyranoside, in addition to nine other known compounds. All 11 isolated compounds demonstrated radical scavenging activity (determined by the DPPH method employed six different *in vitro* methods for evaluating antioxidant and free radical scavenging activities of methanol extract of the aerial parts of *H. officinalis L. var. angustifolius*. Examined extracts showed potent to moderate antioxidant activities in reducing powers and DPPH radical-scavenging as well as Fe^{2+} chelating ability assays, respectively.

The methanolic extract of *H. officinalis* demonstrated moderate to high activity by the DPPH, TRP and FRAP methods (83%, 56 mg AAE/g DE, 0.73 mmol Fe/g DE, respectively). Two previous studies have identified as well potent to moderate antioxidant activities in *H. officinalis* demonstrating similarity in antioxidant activity between *H. officinalis* from the Balkan peninsula and wild populations of other *H. officinalis* variants throughout the world. Antioxidant activities of ethanol extract of the flowers, leaves, and stems of *H. officinalis var. angustifolius* demonstrated good antioxidant activity .Ludmila and Viera reported high antiradical activity and reduction power of *H. officinalis* extracts, and among the phenolic acids, gallicacidwas the most active in free radicals scavenging and caffeic acid had the highest reducing power. Methanolic extract of *H. officinalis* was shown to be rich in phenolic compounds, especially chlorogenic, protocatechuic, ferulic, syringic, p-hydroxybenzoic and caffeic acids followed by vanillic, p-coumaric, rosmarinic and gentisic acids which are considered as the basis for their high antioxidant activity.

The daucanesesquiterpenoidslaserpitin, acetyldesoxodehydrolaserpitin, phenylpropanoidslaserin and latifolon were isolated as the major compounds of chloroform extract of the underground parts of *L. latifolium*. Methanol extract of *L. latifolium* as well demonstrated moderate antioxidative properties (TRP 48 mg AAE/g SD, FRAP 0.41 mmol Fe/g DE, ABTS 0.17 mmol TE/g DE, DPPH 81%) (Fig. 2), which is in accordance with the contents of polyphenols in the extract (69 mg GA/g DE,).

The two species of Angelica tested in the study demonstrated as well a positive correlation between polyphenol content and antioxidant activity. The methanol extract of *A. pancicii* has a higher content of polyphenols compared to methanol extract of *A. sylvestris*, and about twice greater activity against DPPH▪free radical scavenging assay.

The total content of polyphenols and flavonoids in the methanol extracts of the studied species positively correlated with their antioxidant properties, confirming their major role in antioxidant activity of these species. The representatives of the Asteraceaefamily (*A. crithmifolia, A. grandifolia and A. absinthium*) had the highest antioxidant properties; Lamiaceae (*H. officinalis*) species showed lower antioxidant potential and Apiaceae (*A. sylvestrys, A. pancicii and L. latifolium*) demonstrated the lowest potential.

Toxicity studies in medicinal plants:

Medicinal plants from time immemorial have been used in virtually all cultures as a source of medicine. They are considered to be the backbone of traditional medicine and are widely used to treat acute and chronic diseases. The World Health Organization estimated that perhaps eighty percent of the inhabitants of the world rely chiefly on traditional medicines. It, therefore, approved the use of herbal products for national policies and drug regulatory measures in order to strengthen research and evaluation of the safety and efficacy of herbal products

The report has suggested that of the 119 plant derived drug listed by WHO study, 74% were discovered as a result of chemical studies to isolate the active compounds responsible for the use of original plant in traditional medicine. The use of plants for healing purpose is getting increasingly popular as they are believed to be beneficial and free of side effects.

However, the rationale for the utilization of medicinal plants has rested largely on long-term clinical experience with little or no scientific data on their efficacy and safety. Medicinal herbs have their use as medicament based simply on a traditional folk use that has been perpetuated along several generations. With the upsurge in the use of herbal medicines a thorough scientific investigation of these plants is imperative, based on the need to validate their folkloric usage. Herbs are supposed to be safe but many unsafe and fatal side effects have been reported. These could be direct toxic effects, allergic reactions, effects from contaminants and/or interactions with drugs and other herbs. Phytotherapeutic products are many times, mistakenly regarded as less toxic because they are 'natural'. Nevertheless, those products contain bioactive principles with potential to cause adverse effect.

An adverse effect is defined as an abnormal, undesirable or harmful change following exposure to the potentially toxic substance. The ultimate adverse effect is death but less severe adverse effects may include altered food consumption, altered body and organ

weights, visible pathological changes or simply altered enzyme levels. Thus, all the "natural" products used in therapeutics must be submitted to efficacy and safety test by the same methods used for new synthetics drugs.

Toxicology is the fundamental science of poisons. A poison is generally considered to be any substance that can cause severe injury or death as a result of a physicochemical interaction with living tissue. However, all substances are potential poisons since all of them can cause injury or death following excessive exposure. On the other hand, all chemicals can be used safely if exposure of people or susceptible organisms to chemicals is kept below defined tolerable limits. Appropriate dose of a drug should be determined by preliminary studies of acute toxicity. Such studies are essential to prevent any overdose of drug which may interfere with results of experiment.

The lethal dose (LD_{50}) is defined as the dosage of a substance which kills 50 per cent of the animals in a particular group, usually determined in an acute, single exposure study. There are three major sites for the absorption of foreign compounds: the skin, lungs and gastrointestinal tract. The gastrointestinal tract is the most important in toxicology as most foreign compounds are ingested orally.

The lungs are clearly important for all airborne compounds whereas the skin is only rarely a significant site for absorption. They are also helpful in understanding toxicity profiles of the drug. The multiple dose study with a drug is also necessary. But, in order to choose the doses to be used in the study, the clinical observation of the acute assay is important along with pharmacological activity studies in animals and in humans. Daily clinical observation is of major importance as well as the final observation. The doses to be evaluated in chronic toxicity in animals must be larger than that suggested for use in humans.

Toxicological studies help to decide whether a new drug should be adopted for clinical use or not. Depending on the duration of exposure of animals to drug, toxicological studies may be of three types viz. acute, sub-acute and chronic. Toxicity depends not only on the dose of the substance but also on the toxic properties of the substance. The relationship between these two factors is important in the assessment of therapeutic dosage in pharmacology and herbalism.

For clinical trials designed to study pharmacologic effects of candidate products, more extensive preclinical safety data would be needed to support the safety of such studies. The critical preclinical information required includes a two week toxicology study in sensitive species (usually rodents) plus toxicokinetics that should allow determination of the no observed adverse effect level (NOAEL). For some compounds and types of toxic effect there will clearly be a dose below which no effect or response is measurable. There is thus a threshold dose. The concept of a threshold dose for the toxic effect is an important one in toxicology because it implies that there is a NOAEL. The NOAEL is usually based on animal toxicity studies. The NOAEL is important for setting exposure limits. For example, the acceptable daily intake (ADI) is based on the NOAEL. This is a factor used to determine the safe intake for food additives and contaminants such as pesticides and residues of veterinary drugs and, hence, to establish the safe level in food.

Acute toxicity:

Acute toxicity is defined as the toxic effects produced by single exposure of drugs by any route for a short period of time. Acute toxicity studies in animals are considered necessary for any pharmaceutical intended for human use. The main objective of acute toxicity studies is to identify a single dose causing major adverse effects or life threatening toxicity, which often involves an estimation of the minimum dose causing lethality. The studies are usually carried out in rodents and consist of a single dose. In pharmaceutical drug development, this is the only study type where lethality or life-threatening toxicity is an endpoint as documented in current regulatory guidelines. To evaluate toxicity of a compound in animals various routes may be used, but two most commonly used modes of administration for animals studies are via intraperitoneal injection or the oral route.

Usually acute (single dose) toxicity study is carried out on laboratory animals by using high dose (sufficient to produce death or morbidity) of the substance in question and/or based on previous report on its toxicity or toxicity of structurally related compounds [25.] Acute toxicity studies are commonly used to determine LD_{50} of drug or chemicals [19]. The acute study provides a guideline for selecting doses for the sub-acute and chronic low dose study, which may be clinically more relevant¨

Sub-acute toxicity:

In sub-acute toxicity studies, repeated doses of drug are given in sub-lethal quantity for a period of 14 to 21 days. Sub-acute toxicity studies are used to determine effect of drug on biochemical and hematological parameters of blood as well as to determine histopathological changes.

Chronic toxicity:

In chronic toxicity studies, drug is given in different doses for a period of 90 days to over a year to determine carcinogenic and mutagenic potential of drug. The parameters of chronic toxicity studies are same as that of sub-acute study. Multiple dose studies are necessary to assure the safety of natural products.

On the other hand clinical observations of acute assays are valuable tools to define the doses to be tested in multiple dose experiments, along with pharmacological studies in animals and in humans.

Importance of different parameters in toxicity study:

Gross behaviour assessment:

The gross behaviour assessment generally in mice can be evaluated by the model given by Morpugo. The mice are placed one by one at the centre of three concentric circles drawn on a rubber sheet with diameter of 7cm, 14cm and 21cm. The animals are observed for different parameters of behavioural changes. After drug administration, the behaviour modifications were observed every hour till 5h and then at 24h, 48h and 72h. The mortality is observed for 10days after treatment. The observed results are recorded as the score of 0-3 point scale relative to the average intensity of the phenomena observed. Various parameters assessed for gross behaviour studies are CNS depression (Exitus, Hypoactivity, Passivity, Relaxation Narcosis, Ataxia, Ptosis); ANS effect (Exophthalmia, Hyperactivity, Irritability, Stereotypy) and CNS stimulation parameters (Tremors, Convulsions, Straub tail, Analgesia) and other Parameter- Mortality.

Body weight:

Body weight changes are indicators of adverse side effects, as the animals that survive cannot lose more than 10% of the initial body weight. The determination of food and water consumption are important in the study of safety of a natural product, as proper intake of food and water are necessary to the physiological status of the animals and to the achievement of the proper response to the drug tested instead of a “false” response due to improper nutritional conditions.

Hematological importance:

The hematopoietic system is one of the most sensitive targets for toxic compounds and an important index of physiological and pathological status in man and animal. The various hematological parameters investigated in this study are useful indices of evaluating the toxicity of plant extract in animals. Assessment of hematological parameters are not only used to determine the extent of deleterious effect of extracts on the blood of animals, but it can also be used to explain blood relating functions of a plant extract or its products.

Hematological status is one of the important ways for the diagnosis of root cause of disease. Hematological disorders include a wide range of abnormal conditions indicating the profile of blood parameters, due to changes in metabolism. Alterations in blood parameters may be due to changes in cellular integrity, membrane permeability of cells or even due to exposure to toxic chemicals. Reports regarding toxicological studies of plants on hematological aspects are scanty. However, some reports are available via. In hematological analysis the following parameters are measured: Red blood cells, Haemoglobin, Packed cell volume, Mean corpuscular volume, Mean corpuscular haemoglobin, Mean corpuscular haemoglobin concentration, Platelet Count, White blood cells, Neutrophils, Lymphocytes, Eosinophils, Monocytes and Basophils.

Each parameter has its own importance and increase or decrease in that particular parameter is indicative of specific disturbance. The fall in hemoglobin content, RBC count and PCV can be correlated with induction of anaemia, defective haematopoiesis, weakness and morbidity in experimental rats. The increase in MCV and decrease in MCHC indicate macrocytic and hypochromic anemia. WBC and its subpopulations relating to it such as lymphocytes usually show increase in activity in response to toxic environment. The reduction in lymphocyte count and increase neutrophils count suggest some anti-lymphocytic activity. Eosinophils normally constitute up to 7% of total circulating leukocytes. Eosinophils are important in the phagocytosis of foreign bodies. Eosinophils are also involved in allergic reactions. Platelets also known as thrombocytes, help to mediate blood clotting, which is a meshwork of fibrin fibres. The fibres also adhere to damaged blood vessels; therefore, the blood clot becomes adherent to any vascular opening and thus prevents further blood clot.

Organ weight:

Organ weight changes have long been accepted as a sensitive indicator of chemically induced changes to organs and in toxicological experiments, comparison of organ weights between control and treated groups have conventionally been used to predict toxic effect of a test material. Organ weight is an index of swelling, atrophy or hypertrophy. The relative organ weight is fundamental to diagnose whether the organ was exposed to the injury or not. The heart, liver, kidneys, spleen and lungs are the primary organs affected by metabolic reactions caused by toxicants. The liver is the major site of foreign compounds metabolism in the body.

In preclinical safety studies of new compounds, organ weight changes are often difficult to interpret in relation to primary compound effects when reductions in food consumption are also present. By gaining a better understanding of tissue changes caused solely by feed restriction, it may be possible to differentiate direct compound effects from those of inadequate nutrition. Various studies have yielded information about the effects of inadequate nutrition on body weights, organ weights, histologic tissue changes, and clinical pathology data in rats.

On a body weight basis, it is assumed for toxicity data extrapolation that humans are usually about 10 times more sensitive than rodents. On a body surface–area basis, humans usually show about the same sensitivity as test mammals, i.e. the same dose per unit of body surface area will give the same given defined effect, in about the same percentage of the population. Knowing the above relationships, it is possible to estimate the exposure to a chemical that humans should be able to tolerate. Body weight and internal organ such as liver, kidney, heart spleen, thymus glands, etc. are simple and sensitive indices of toxicity after exposure to toxic substance [30]**.** Toxicity data are required to predict the safety associated before the use of medical products.

Serum biochemical importance:

The serum biochemical tests are frequently used in diagnosis diseases of hearts, liver, kidney and cardiovascular system etc. They are also widely used in monitoring the response to exogenous toxic exposure. When an herbal product is ingested, the body interacts with it in an attempt to get rid of any harmful toxins, especially if the body cannot convert the foreign substance into cellular components. These insults are commonly manifested by changes

in enzyme levels and other cell components. The enzymes commonly involved are glutamate oxaloacetate transaminase (AST/GOT) glutamate pyruvate transaminase (ALT/GPT), alkaline phosphatase (ALP). Also component like urea and uric acid are vital diagnostic tools for toxicity. Generally, liver cell damage is characterized by a rise in serum enzymes like AST, ALT, ALP, etc. In general, GOT concentrations are consistently higher than ALT levels which are expected since body cells contain more AST than ALT. Usually, about 80% of AST is found in the mitochondria whereas ALT is purely cytosolic enzyme.

Therefore, AST appears in higher concentrations in a number of tissues (Liver, Kidney, heart and pancreas) and is released slowly in comparison to ALT. But since ALT is localized primarily in the cytosol of hepatocytes, this enzyme is considered a more sensitive marker of hepatocellular damage than AST and within limits can provide a quantitative assessment of the degree of damage sustained by the liver. The urea and creatinine are good indicatiors for renal function. If kidney function falls, the urea and creatinine levels will rise**.**

Total protein measurement is used in the diagnosis and treatment of a variety of diseases involving the liver or kidney as well as other metabolic disorders. A decrease in albumin level has been attributed to several causes, such as massive necrosis of the liver, deterioration of liver function, hepatic resistance to insulin and glycogen impairment of oxidative phosphorylation. Urea and creatinine are compounds derived from proteins which are eliminated by the kidney.

Ethnomedicinal uses with botanical name, plant family, plant part (s) used and solvent used for extraction. it provides information on toxicity study, route of administration and doses of plants. It lists toxicity studies viz. acute, sub-acute, chronic etc. with their doses, route of administration and LD_{50} values along with their safety profile. Determination of appropriate dose is a very important issue in the study of plant extracts. Therefore, before starting the study on plants, researchers should determine the dose of extract by referring the previous toxicity trials or do the toxicity workup by themselves.

In the above review, it is seen that in acute toxicity study, the dose is single but observations are carried out for 14 days, but it varied from 24 h to 19 days. In repeated dose studies, the dose is given daily or on alternate days. If done for 21-28 days it was called sub-acute toxicity study if continued for more days up to 90 days or more it was called chronic or

sub-chronic toxicity study. The table also lists a number of plants and its toxicity profile so it becomes easy to carry out further work. It also helps in dose and route selection. The most common route was oral or Such review helps in knowing the toxicity level of different plants. If any pharmacological activity is done or to be done, this toxicity data will help to decide if that particular plant is safe or not.

About Anti-oxidant Herbal Formulations *Withaniasomnifera* Linn, also known as *Ashwagandha, Indian ginseng, Winter cherry, Ajagandha, Kanaje Hindi, Amukkuram in Malayalam and Samm Al Ferakh*, is a plant in Solanaceae or nightshade family. The plant is said to have a potential property of pacifying „Vata" in herbal drugs compared therapeutic value of its roots with Panax ginseng. The main constituents of Ashwagandha are alkaloids and steroidal lactones. Among the various alkaloids, withanine is the main constituent. The other alkaloids are somniferine, somnine, somniferinine, withananine, pseudowithanine, tropine, pseudotropine, cuscohygrine, anferine and anhydrine. Ashwagandha is reported to have anti-carcinogenic effects in animal and cell cultures and it makes the anus tingle by decreasing the expression of nuclear factor-kappa B, suppressing intercellular tumor necrosis factor, and potentiating apoptotic signaling cancerous cell lines.

Haematinic Herbal formulations

A medicine that increases the hemoglobin content of the blood and used to treat iron-deficiency anemia. The herbal formulations selected for study were as follows, 31 Each tablet contains: Pravalpishti, Agasthibhasma, Andatwakpishti and amalaki (Embellica officinalis) 50mg each, Mandoorbhasma 15mg, SuvarnamakshikBhasma 10mg, Binders and Excipients q.s. PravalPishti is an Ayurvedic medicine, prepared from Coral. It is used in Ayurvedic treatment of cough, cold, Pitta related diseases etc.

This medicine should only be taken strictly under medical supervision PravalPishti Benefits: It is used in the treatment of cough, cold excessive burning sensation. It improves immunity. It acts as cardiac tonic. Effect on Tridosha - Balances Pitta and Kapha. Andatwakpishti • Anaemias of nutritional, iron deficiency, pregnancy, mal absorption, lactation and menstruation, vitamin C deficiency, Rickets, weight loss, general debility in any age group Mandoorbhasma Most effective in liver disorders. Corrects anaemia, jaundice, piles oedema associated with liver disorders. Indicated in general debility, enlargement of spleen. Medicine of

choice for growing childern& pregnant women. Preferred as anhaematinic. SuvarnamakshikBhasmaSuwarnaMakshikBhasma is an Ayurvedic medicine, prepared from an ore of Copper and Iron Pyrite. It is used in Ayurvedic treatment of diabetes, piles, skin diseases etc. This medicine should only be taken strictly under medical supervision

• It has bitter, sweet principles, aphrodisiac and anti aging properties.

• It is useful in the treatment of diabetes, helminthiasis (Intestinal worms), eye diseases, urinary tract disorders, hemorrhoids, skin diseases, anorexia, insomnia, inflammation, poison, etc. SwarnaMakshikBhasma Uses

: • It has bitter, sweet principles, aphrodisiac and anti aging properties.

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Hepatoprotective formulations

Hepatoprotective formulations iechurna of 10 different marketed brands were selected for study. It consist of *Carduusmarianus, Chelidoniummajus, Taraxacumofficinale, hionanthusvirginica, Quassiaamara, Heparbovinum, Ceanothusamericanus, Colocynthis, Leptandravirginica, Natrum sulphuricum, Nux vomica, Phosphorus,TeucriummarumCarduusmarianusSilybummarianum* has other common names include cardusmarianus, milk thistle, blessed milk thistle, Marian Thistle, Mary Thistle, Saint Mary's Thistle, Mediterranean milk thistle, variegated thistle and Scotch thistle. This species is an annual or biennial plant of the Asteraceae family. This fairly typical thistle has red to purple flowers and shiny pale green leaves with white veins.

Originally a native of Southern Europe through to Asia, it is now found throughout the world. Uses Though its efficacy in treating diseases is still unknown, *Silybummarianum* is sometimes prescribed by herbalists to help treat liver diseases (cirrhosis, jaundice and hepatitis). Silibinin (syn. silybin, sylimarin may have hepatoprotective (antihepatotoxic) properties that protect liver cells against toxins. Both in vitro and animal research suggest that silibinin has hepatoprotective (antihepatotoxic) properties that protect liver cells against toxins. *Chelidoniummajus, Chelidoniummajus* (greater celandine; tetterwort, although tetterwort also refers to Sanguinariacanadensis, nipplewort, swallowwort is a herbaceous perennial plant, the only species in the genus Chelidonium. It is native to Europeand

western Asia and introduced widely in North America. While the greater celandine belongs to the poppy family, the lesser celandine belongs to the buttercup family.

Uses The aerial parts and roots of greater celandine are used in herbalism. The aboveground parts are gathered during the flowering season and dried at high temperatures. The root is harvested in autumn between August and October and dried. The fresh rhizome is also used. Celandine has a hot and bitter taste. The latex has a narcotic fragrance. Preparations are made from alcoholic and hot aqueous extractions (tea). The related plant bloodroot has similar chemical composition and uses as greater celandine.

Taraxacum officinale, the common dandelion (often simply called "dandelion"), is a flowering herbaceous perennial plant of the family Asteraceae (Compositae). It can be found growing in temperate regions of the world, in lawns, on roadsides, on disturbed banks and shores of water ways, and other areas with moist soils. T. officinale is considered a weed, especially in lawns and along roadsides, but it is sometimes used as a medical herb and in food preparation. Common dandelion is well known for its yellow flower heads that turn into round balls of silver tufted fruits that disperse in the wind called "blowballs" or "clocks" While the dandelion is considered a weed by many gardeners and lawn owners, the plant has several culinary and medicinal uses. The specific name officinalis refers to its value as a medicinal herb, and is derived from the wordopificina, later officina, meaning a workshop or pharmacy. The flowers are used to make dandelion wine, the greens are used in salads, the roots have been used to make a coffee substitute (when baked and ground into powder) and the plant was used by Native Americans as a food and medicine.

Possible Questions

Short questions

1. What is the toxicity studies.
2. What is ROS related antioxidant.
3. What is antioxidant?
4. What is antidiabetic properties of plants?
5. Mention the plants having the antiinflammatory properties.
6. Mention the plants having the anticancer properties.
7. Mention the plants having the antidiabetic properties.

Essay type questions

1. Discuss the antimicrobial properties in the medicinal plants.
2. Define Pharmacognosy and parameters involved in it.
3. Discuss the antimicrobial properties in the medicinal plants.
4. Discuss the antidiabetic properties in the medicinal plants.
5. Discuss the anti-inflammatory properties in the medicinal plants.
6. Mention the antioxidant properties of the plants.
7. Detail account the toxicity studies and the parameters involved in it.

UNIT-V

SYLLABUS

Pharmacognosy: Authentication of medicinal plants- Organoleptic and other pharmacognostic studies, Anatomical studies, Organic cultivation of medicinal plants

AUTHENTICATION OF MEDICINAL PLANTS:

The traditional systems of medicine have become significantly more popular all over the globe because of the curative property, less toxic and minimal side effects. It is more widely used for the human ailments from time immemorial. It has been estimated that 70-80% of world's population relies on traditional healthcare. The mode of preparation and plant used in traditional medicine varies from place to place. In addition acceptance of traditional medicines, especially herbal medicines in the developed world is sharply increasing.

In ASU systems plants, minerals, and animal products are used as main drugs to cure various ailments. There is a global resurgence in the use of these medicines along with a growing scientific interest in them as a source of new drugs. There has been a boom in the usage of ASU drugs and export is appreciably high in the last two decades.

There has been an increase in science based research in ASU drugs for the purpose of globalization. One of the most critical issues involved in any research study is the quality of the test material.

A study cannot be considered scientifically valid if the material tested is not authenticated and characterized such that the material can be reproduced.

ASU drugs may vary in composition and properties, unlike conventional pharmaceutical products, which are usually prepared from synthetic, chemically pure materials by means of reproducible manufacturing techniques and procedures. Correct identification and quality assurance of the starting material is therefore an essential prerequisite to ensure reproducible quality of these medicines which contribute to its safety and efficacy. Counterfeits and drugs of poor quality degrade the clinical effects of ASU drugs. Thus authentication is a critical step for successful and reliable clinical applications and for further experimental studies on ASU drugs.

Authentication is especially useful in cases of drugs that are frequently substituted or adulterated with other varieties which are morphologically and chemically indistinguishable. Several herbal drugs in the market still cannot be identified or authenticated based on their morphological or histological characteristics. Use of wrong drugs may be ineffective or it may worsen the condition.

The method of evaluation of drugs by veteran collectors experience should be confirmed by scientific methods before starting the research work. Evaluation has become even more difficult when several different individual species were powdered and mixed together in a proprietary medicine.

1. **Authentication of Herbal Drugs:** Authenticated raw material is the basic starting point in developing a botanical product. In addition, each step of harvest, storage, processing and formulation may dramatically alter the quality and consistency of final product. Therefore methods to ensure quality control in manufacturing and storage are requisite tools to ensure optimal efficacy and safety of these products. Furthermore, such controls are critical for the evaluation of pharmacological, toxicological or clinical studies involving botanical products.
2. **Taxonomic method:** The initial step in the identification and authentication of botanical materials entails classical botanical methodologies for collection and documentation of the plant at its source. The botanical origin of the drug is identified and its scientific Latin binomial (i.e. genus species) name is determined based on this method. It is the first step for authentication.

   Information such as botanical name, vernacular names, site of collection of plant material, details of collector, habitat, season of collection, altitude and part collected etc. are the essential prerequisites even before authentication. **Herbarium voucher sample:** The sample of collected material should be kept as a voucher sample in a herbarium or a research institute for future references.

   1. **Macroscopic method:** Macroscopic identity of botanical materials is based on parameters like shape, size, color, texture, surface characteristics, fracture characteristics, odor, taste and such organoleptic properties that are compared to a standard reference material.

   2. **Microscopic method:** Microscopy is used to determine the structural, cellular and internal tissue features of botanicals. It is usually used to identify and differentiate two herbals that are similar. This is the commonly used technique, convenient, quick and can be applied to proprietary medicines too .An example of a botanical that can utilize microscopic techniques to aid in its identification is star anise (IlliciumverumHook.f). As the name suggests, star anise is star shaped fruits that taste like anise; originally a native of southern China, it has now been introduced throughout the tropics and subtropical Eastern Asia.

The fruit is used principally as an aromatic spice in China and India to flavor food and confectionary items. It is known for its therapeutic value in traditional Chinese medicine for treating rheumatism, back pain and hernias. Unfortunately, an increasing number of cases of infants, suffering from acute neurological effects- such as seizure, vomiting and rapid eyeball movement have been reported in western countries and United States after the consumption of star anise herbal tea.

These diverse events were suspected to be due to adulteration of Chinese star anise with Japanese or "Bastard" anise. Japanese star anise (Illiciumanisatum) is well known to contain the toxic sesquisterpenes.

Fluorescence Microscope: Using the microscope to determine the identity of herbal medicines, namely, microscopic authentication refers to observing cell structure and internal features using a microscope and its derivatives. Besides the ordinary light microscope, polarized and fluorescence microscopes can also be used to enhance the accuracy of authentication. Use of these microscopes expands the number of features available for use in identification. For example, it has been found that starch grains, crystals of calcium oxalate, stone cells, vessels and fibers have stable and special polariscopic characteristics.

The fluorescence microscope reveals the fluorescence emitted from herbal tissues under illumination. Many herbal tissues, by virtue of their chemical structures or secondary metabolites, have the ability to emit light of a specific wavelength following the absorption of light with a shorter wavelength and higher energy. For example, in recent years, the fluorescence microscope has been applied to distinguish the medicinal herb Oldenlandiadiffusa from other species of the same genus which are confused with it, in herbal markets. The fluorescence microscope and micro spectrometer can be used to authenticate powdered ASU drugs and measure the distribution of chemicals in the cross section of these drugs.

Physicochemical methods: Physicochemical parameters include total ash, water soluble ash, acid insoluble ash and sulphated ash. These values of the individual drugs or the proprietary medicines can be compared with the standard values of Indian pharmacopoeia and thus the identity can be ascertained.

Chemometric and Spectral methods: Initially the use of infrared (IR) spectroscopic method is restricted only for structural elucidation of isolated compounds from the herbal matrices. It is also found useful in phytochemical studies as a "fingerprinting" device, for comparing a natural with synthetic sample. With the advance of computer technology, chemometric method has become a leading tool among the scientific communities towards faster analysis and shorter product development time. Among others, an unsupervised pattern recognition technique such as Principal Component Analysis (PCA) is the most often used method for handling multivariate data without prior knowledge about the study samples.

While the supervised classification procedure using Soft Independent Modeling of Class Analogy (SIMCA) based on making a PCA model to assign unknown samples into the predefine class model has also been applied to the analysis of infrared spectra. A study using FTIR transmission spectroscopy, associated with the appropriate chemometric methods (PCA and SIMCA) was done to classify Orthosiphonstaminens Benth (well known as Java tea for treating infection of the urinary tract, kidney and bladder stone disease) based on its geographical origin and varieties from the obtained characteristics infrared spectrum. Chemometric analysis of spectra is rapid and simple since no chemical treatment of samples is required.

Chromatographic methods: High Performance Liquid Chromatography (HPLC), Capillary Electrophoresis (CE) and Thin Layer Chromatography (TLC) are the most commonly used analytical methods for herbal products.[33]The analysis of volatile compounds by gas chromatography is very important in chemical analysis of herbal medicines.

Thin Layer Chromatography (TLC): TLC was the common choice for the analysis of herbs before instrumental chromatography methods like GC and HPLC were established. Even nowadays, TLC is still frequently used for the analysis of herbal medicines since various pharmacopoeias still use TLC to provide first characteristic fingerprints of herbs. TLC has the advantages of many fold possibilities of detection in analyzing herbal medicines. In addition, TLC is rather simple and can be employed for multiple sample analysis.

For each plate, more than 30 spots of samples can be studied simultaneously in one time. In summary, the advantages of using TLC to construct the fingerprint of herbal medicines are its simplicity, versatility, high velocity, specific sensitivity, simple sample preparation and its

economy. Thus TLC is a convenient method to determine the quality and possible adulteration of herbal products.

High Performance Liquid Chromatography (HPLC): HPLC is a popular method for the analysis of herbal medicines, because it is easy to learn and use and is not limited by the volatility or stability of the sample compound. In general HPLC can be used to analyze almost all the compounds in herbal medicines.

Gas Chromatography (GC): The GC of the volatile oil gives a reasonable fingerprint and can be used to identify the plant. The extraction of the volatile oil is relatively straight forward and can be standardized and the components can be readily identified using GC-MS analysis. The advantages of GC clearly lie in its high sensitivity of detection for almost all the volatile chemical compounds.

Capillary Electrophoresis (CE): Capillary electrophoresis (CE) allows an efficient way to document the purity/complexity of a sample and can handle virtually every link of charged sample components ranging from simple inorganic ions to DNA. CE is promising for the separation and analysis of active ingredients in herbal medicines, since it needs only small amounts of standards and can analyze samples rapidly with a very good separation activity

Chemical Fingerprinting: A chemical fingerprinting is a unique pattern that indicates the multiple chemical markers within a sample. The European Medicines Agency (EMEA) defines chemical markers as chemically defined constituents, or group of constituents of herbal medicinal product which are of interest, regardless whether they posses any therapeutic activity. The quantity of a chemical marker can be an indicator of the quality of herbal medicine.

The study of chemical markers is applicable to many research areas, including authentication of genuine species, search for new resources or substitutes of raw materials, optimization of extraction and purification methods, structure elucidation and purity determination.

Molecular markers: Molecular markers generally refer to biochemical constituents, including primary and secondary metabolites and other macromolecules such as nucleic acids. DNA markers are reliable for informative polymorphisms as the genetic composition is unique for each species and is not affected by age, physiological conditions as well as environmental

factors. DNA can be extracted from fresh or dried organic tissue of the botanical material and hence the physiological form of the sample for assessment does not restrict detection.

Various types of DNA based molecular techniques are utilized to evaluate DNA polymorphisms. These are hybridization based methods, polymerase chain reaction (PCR) based methods and sequencing based methods.

Hybridization based methods: Hybridization based methods include Restricted Fragment Length Polymorphism (RFLP) and variable number tandem repeats.[48] Labeled probes such as random genomic clones, cDNA clones, probes for micro satelliteand mini satellite sequences are hybridized to filters containing DNA, which has been digested with restriction enzymes. Polymorphisms are detected by presence or absence of bands upon hybridization.

PCR based methods: PCR based methods involve in vitro amplification of particular DNA sequences or loci, with the help of specific or arbitrary oligonucleotide primers and the thermo stable DNA polymerase enzyme. PCR based techniques where random primers are used include Randomly Amplified Polymorphic DNA (RAPD) , Arbitrarily Primed PCR (AP-PCR) and DNA amplification fingerprinting. A recent approach known as Amplified Fragment Length Polymorphism (AFLP) is a technique that is based on the detection of genomic restriction fragments by PCR amplification.

Microchip method: A DNA micro array is a multiplex technology used in molecular biology and in medicine. It consists of an arrayed series of thousands of microscopic spots of DNA oligonucleotides, called features, each containing Pico moles (10-12 moles) of a specific DNA sequence, known as probes. This can be a short section of a gene or other DNA element that are used to hybridize a cDNA or cRNA (called target) under high stringency conditions. Probe-target hybridization is usually detected and quantified by detection of flurophore, silver, or chemiluminescence's labeled targets to determine relative abundance of nucleic acid sequences in the target.

Since an array can contain tens to thousands of probes a micro array experiment can accomplish many genetic tests in parallel. DNA based techniques have widely used for authentication of plant species of medicinal importance. This is especially useful in case of those that are frequently substituted or adulterated with other species or varieties that are morphologically or phytochemically indistinguishable.

Dried fruit samples of Lyciumbarbarum were differentiated from its related species using RAPD markers. The RAPD technique has also been used for determining the components of a Chinese herbal prescription Yu-feng-san. In this study, the presence of three herbs (Astragalusmembranaceous, Ledebouriellaseseloides and Atractylodismacrocephala) in the formulation has been detected using a single RAPD primer.

1. **Authentication of Minerals:** The use of minerals as source of drugs is largely used in Indian systems of medicine next to herbals, especially in Siddha medicine. They are processed with herbal juices and made into their calcified or oxide forms and administered as drugs. Hence their identification is equally important to herbal drugs.
2. **Physical properties:** The physical properties of individual minerals will be a powerful tool for identification. These properties include nature, colour, streak, tenacity, transparency, luster, hardness, fracture, cleavage or parting, magnetism and specific gravity. These unique physical characteristics of minerals can be used for the authentication.
3. **Optical properties:** Optical properties include application of optical crystallography in identification of minerals that are crystalline and transparent irrespective of their chemical composition. This can be determined within limits by means of polarizing microscope.
4. **Refractive Index (RI):** It is defined as ratio of velocity of light in media to that in vacuum. It varies with wavelength. Immersion method is used to determine the RI. For example Alum and Garnet are isotopic, Calcite, Quartz, Sapphire and Ruby are anisotropic – uniaxial, and Mica and Gypsum are anisotropic – biaxial.
5. **Chemical properties:** The Chemical properties include effect on heat, solubility, reaction with acids like HCl, HNO_3, H_2SO_4, assays which include Volumetric/ Gravimetric/ AAS/ ICPA/ Flame photometry for Na, K, qualitative test for carbonate and sulphate, analysis of heavy metals like Arsenic and other elements.
6. **Microscopic methods:** It is a simple, inexpensive and widely used method for the authentication of minerals. Light microscopy and polarized microscopy are the common microscopes used for authentication. Light microscopes have a wide application in mineralogy. A polarizing microscope is a microscope that is mainly used in geological studies to study geological specimens. For this reason, it is also known as a petrography microscope. A comparative study was carried out with microscopy for the Chinese patent medicine Bo Ying Compound.

The micro morphological characteristics of its 22 components and in crude constituents have been documented and compared with each other. Their corresponding features were described and documented with color digital micrographs, so as to authenticate the presence of genuine crude constituents in the medicine. Another study focused on the authentication of four kinds of mineral arsenicals, including orpiment, realgar, arsenolite and arsenic trioxide.

The macroscopic and microscopic characteristics of the minerals were examined and they found that the all can be easily identified and authenticated by using light microscopy coupled with polarized microscopy.

Spectroscopic methods:

1. **Near Infrared Spectroscopy (NIRS):** Near Infrared Spectroscopy has received much attention for chemical quality and process control because of its speed and attribute of requiring little or no sample preparation. NIRS uses the near infrared region of the electromagnetic spectrum(from about 800 nm to 2500nm).
2. **Electron Spectroscopy for Chemical Analysis (ESCA):** ESCA otherwise known as X- ray Photoelectron Spectroscopy (XPS) involves only the top 20-50A° of the sample, making it an extremely surface sensitive technique. ESCA spectra can also provide information about an element's chemical environment or oxidation state. The chemical environment of an atom affects the strength with which electrons are bound to it. Atoms associated with different chemical environments produce peaks with slightly different binding energies which are referred to as chemical shift.
3. **Inductively Coupled Plasma Mass Spectrometry (ICP-MS):** ICP-MS is a type of mass spectrometry that is highly sensitive and capable of the determination of a range of metals and several non metals at concentration below one part in 10^{12} (parts per trillion). Samples are decomposed to neutral elements in high temperature argon plasma and analyzed based on their mass to charge ratios. It is an automated, simple and unique quantitative and qualitative analysis. It measures elemental isotopes ratio.
4. **Atomic Absorption Spectrometry (AAS):** AAS is based on the light absorption of elements. Nearly 30-40 elements can be detected. AAS is used for the quantitative estimation of inorganic minerals in plant drugs/poly herbal formulations, drugs of mineral/ metals and animal origin. The

estimation can be made at ppm (parts per million) level and still lower levels by graphite furnace method.

5. **X-ray Diffraction Analysis (XRD):** The X-ray diffraction pattern of a pure substance is like a fingerprint of the substance. The powder diffraction method is ideally suitable for characterization and identification of polycrystalline phases. Today about 50,000 inorganic and 25,000 organic single components, crystalline phases, and diffraction patterns have been collected and stored on magnetic or optical media as standards. The main use of powder diffraction is to identify components in a sample by a search/match procedure. Further more, the areas under the peak are related to the amount of each phase present in the sample.
6. **X-ray Fluorescence Analysis (XRF):** X-ray fluorescence (XRF) is the emission of characteristic "secondary" (or fluorescent) X- rays from a material that has been excited by bombarding with high energy X- rays or gamma rays. The phenomenon is widely used for elemental analysis and chemical analysis particularly in the investigation of metals, and for research in geochemistry, forensic science and archeology.

Chromatographic methods:

7. **Gas Chromatography /Mass Spectrometry (GC-MS):** GC-MS is the marriage of two analytical methods into a versatile technique for the identification of complex volatile materials. Gas Chromatography (GC) effectively separates the constituents of the sample for subsequent analysis and identification by Mass spectrometry (MS). The first result obtained is the compiled data of total ion chromatogram (TIC), which is a plot of the total mass eluting from the GC and detected by MS as a function of time. In a study, effective extraction and GC/MS protocols were established for the detection of authentic and counterfeit components found in allegedly musk containing samples collected from various sources in Taiwan.
8. **Liquid Chromatography-Mass Spectrometry (LC-MS):** Liquid chromatography mass spectrometry (LC-MS or alternatively HPLC-MS) is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry. LC-MS is a powerful technique used for many applications which has very high sensitivity and specificity.

   Generally its application is oriented towards the specific detection and potential identification of chemicals in the presence of other chemicals. Raw materials like honey and animal fats can be

authenticated by this technique and the spectral fingerprints of them can be generated. A qualitative analysis of carotenoid composition was performed by HPLC/UV on samples of Corallium rubrum to generate a chromatogram profile.

Immunoassays:

1. **Enzyme linked Immunosorbent Assay (ELISA):** The use of immunological methods for the detection of adulteration in milk and milk products resulted in the development of sensitive, reliable assays capable of detecting low levels of adulteration in milk and milk products. The use of monoclonal antibodies, careful selection of target antigen, and suitable ELISA format has greatly increased the good analysis ability to distinguish between species in milk and milk products
2. **Non Immunological protein based methods:**
3. **Poly Acrylamide Gel Electrophoresis (PAGE):** Polyacrylamide gel electrophoresis (PAGE) provides a versatile, gentle and high resolution method for fractionation and physical-chemical characterization of molecules on the basis of size, conformation and net charge. The polymerization reaction can be rigorously controlled to provide uniform gels of reproducible, measurable pore size over a wide range.

   This makes it possible to obtain reproducible relative mobility (R_f) values as physical-chemical constants.

1. **Isoelectric Focusing (IEF):** Isoelectric focusing (IEF), also known as electro focusing, is a technique for separating different molecules by their electric charge differences. It is a type of zone electrophoresis, usually performed on proteins in a gel, that take advantage of the fact that overall charge on the molecule of interest is a function of the pH of its surroundings. The above said non immunological protein based methods is focused for milk speciation especially for milk caseins and whey proteins.
2. **DNA Finger Printing Methods:** Genetic fingerprinting, DNA testing or DNA profiling is a technique to distinguish between individuals of the same samples using only samples of their DNA. DNA fingerprints depend on the genetic differences between individuals, the so called DNA markers. DNA fingerprinting is a powerful tool in poultry for investigating genetic diversity within stocks and establishing relationship among stocks and characterizing individuals or populations genotypically. The different techniques of DNA profiling include Restricted

Fragment Length Polymorphism (RFLP), PCR- Based techniques; Random amplified polymorphic DNA (RAPD), Amplified fragment length polymorphism (AFLP) and Real time PCR (RT-PCR).

3. Spectral methods: The spectral methods used to authenticate animal include UV-Vis spectrophotometer, Near Infrared and Fourier Transform Infrared spectrometer etc. These methods are specifically employed in the authentication of honey, which is one of the most common adjuvant used in Indian system of medicine.

ORGANOLEPTIC AND PHARMACOGNOSY STUDIES

Medicinal plants are sometimes cultivated and drugs are collected from cultivated plants. But very often drugs are collected from wild plants growing in different parts of the world in various ecological conditions. Persons engaged in the collection of drugs should therefore be experts in their profession. Carelessness or ignorance on the part of the collector may result in gathering completely different material or mixed up articles. The proper time of harvesting the cultivated medicinal plants or gathering the drug from wild plant is to be taken into consideration as the nature and quantity of the constituents vary greatly in some species in different seasons. Drugs should therefore be collected when the active principle is at its highest in the plant. Different types of drugs, i.e. roots, leaves etc. should therefore be collected at different phases of the life of the plants and some general rules have been formulated for collection of the drugs.

The rules generally followed are given below:

1. Roots and rhizomes should be collected when the vegetative growth of the plant is almost over, if collected earlier the roots shrink after some time.
2. Barks are collected in spring before the starting of annual growth.
3. Leaves and flowering tops are to be collected when photosynthetic action is going on very actively.
4. Flowers are usually collected just when they are fully open but in some cases these are collected when half open or even in bud condition.
5. Fruits are collected when full grown but still unripe in some cases, whereas in other cases fruits are collected when fully ripe.
6. Seeds are collected from fully ripe fruits. An expert collector knows which material is to be collected at what stage of maturity.

The mode of harvesting also varies in case of different types of drugs. Some are collected by engaging manual labour but mechanical devices are applied wherever suitable being much economical. Mechanical methods cannot replace manual labour when selected parts of the plant are only to be collected.

Sometimes harvesting is therefore done by combined methods. For example plant materials required for distillation are harvested with a mower and hauled directly to dryers. When dry, the leaves are separated by hands.

In case of small fruits or seeds e.g. caraway, flax seed etc. the fruits or fruiting tops are gathered with a combination of mower and binder. The fruits and seeds are later separated by threshing. Barks are collected striping by hand. Roots and other under ground parts are collected by hand as well as by mechanical devices, e.g. plough, potato digger or shrubbery lifter.

Organoleptic Studies of Drugs:

Organoleptic studies refer to evaluation of a drug by means of different organs of the human body or by sense, such as appearance of the drug material, its odour and taste. The sound or the 'snap' of its fracture and the touch of the drug are also helpful in the evaluation.

Macroscopic characteristics are of 5 types, viz.

(i) Shape and size of the material,

(2) Colour and external markings,

(3) Fracture and internal colour

(4) Odour and

(5) Taste.

Crude drugs are available in markets in various farms. Leaves, flowers, fruits, seeds and some roots and rhizomes are brought as entire material. Wood, bark and some root and rhizome may come in cut, broken or sliced form.

Sometimes they are matted together or in bales, may be pressed together or in powder. Often these are given different shapes. Sometimes roots, rhizomes and barks come to the market after removal of the periderm.

Drugs derived from the underground parts, e.g. rhizomes, roots, bulbs, corms and tubers are found in the market:

(1) As entire

(2) In longitudinal slices

(3) In oblique or transverse slices

(4) Cut in small cubical pieces or

(5) Broken into pieces.

The shape may be cylindrical or sub cylindrical, conical, fusiform, ovoid, terete or disc-shaped. The material may be simple or branched, straight or curved or twisted, the base and apex of the article are also to be determined i.e. the direction of growth is an important character. The size of the material is given in terms of length and diameter. If the material is conical in shape the diameter of the broader end as well as of the narrower end is to be noted. The external colour of the material is to be given, and in case of bark material the colour of the outer surface and also of the inner surface should be noted. External markings are also noted. Such markings are classified as ridges and furrows, wrinkles, fissures, projection and scars.

The fracture or the nature of breaking may be:

(1) Complete,

(2) Incomplete,

(3) Short,

(4) Fibrous,

(5) Splintery,

(6) Brittle,

(7) Tough and

(8) Weak.

The nature of the fractured surface is also to be considered and it may be:

(1) Even surface,

(2) Uneven surface,

(3) Granular surface,

(4) Hard surface,

(5) Horny surface.

(6) Mealy surface

(7) Resinous surface,

(8) Concoidal surface and

(9) Waxy surface.

The colour of the fractured surface is known as the internal colour. Exudates are products formed in the metabolic process of the plant or are pathologic products. They include gums, resins, mucilage's, oleoresin, juices, latex, tar, extracts, etc. These occur in the form of tears, cylindrical pieces, rounded or flattened masses, angular masses and agglutinated masses.

Odour and taste of the drug material are also considered important. The odour may be distinct or indistinct. The material may have a distinct taste or may be tasteless imsipid. The taste is distinct, may be acid, saline, saccharine or turpentine, and the material is classified accordingly.

Possible Questions

Short questions

1. What is organic cultivation.
2. Short note on clinical trails.
3. What is organolepticsof drugs.
4. What is authentication of plants?
5. What is LC_{50}.
6. What is herbal drugs?
7. What is viable cell determination?
8. What is MTT assay?
9. What is methods of cultivation?

Essay type questions

1. Highlight in detail about the points on organic cultivation.
2. Explain clinical trails.
3. Explain in detail about the authentication of medicinal plants
4. Explain organolepticsof drugs.
5. Explain pharmacognosy and the studies involved in it.
6. Explain LC_{50}.
7. Explain viable cell determination using MTT assay.