HERBAL PLANT EXTRACTS OF *Gentianakurroo*AND *Rauwolfia serpentine:* ITS IMPLICATIONS IN-BREAST CANCER

Project report submitted in partial fulfillment of the requirement for the degreeof

BACHELOR OF TECHNOLOGY

IN

BIOTECHNOLOGY

BY

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DECLARATION

I, herebydeclare that the project work entitled "**Effect of herbal plant extracts of *Gentianakurroo and Rauwolfia serpentina*: Its implications in-breast cancer"** submitted to the Department of Biotechnology(BT) and Bioinformatics(BI), Jaypee University Of InformationTechnology Solan(H.P), is an authentic record of original work done by us . The work was carried out the under the guidance of **Dr. Udayabanu**and **Dr**. **Hemant Sood.** This work has not been submitted anywhere else.

Signature

PRAHI GUPTA (161819)

Date:

This is to certify that the above statement made by the candidates is correct to the best of my knowledge.

_____. _____

Dr. Udayabanu **Dr.HemantSood**

Head of the Department/Project Coordinator

_____

SUPERVISOR'S CERTIFICATE

This is to certify that the work titled **"Herbal plant extracts of *Gentianakurroo and Rauwolfia serpentine*: Its implications in-breast cancer "**submitted by **Prahi Gupta(161819)** has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of any degree or appreciation.

Date: 18.07.20

Signature of Supervisor _____

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Designation - **Associate Professor**

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LIST OF ABBREVIATIONS

IBA	Indole-3-butyric acid
KN	Kinetin
GA	Gibberellic acid
TDZ	Thidiazuron
MS	Murashige and Skoog
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
HPLC	High performance liquid chromatography
ATP	Adenosine Triphosphate

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ABSTRACT

The occurrence of cancer has a major effect on the social, physical and mental aspects of human life, and is an critical concern for the century. The second-largest reason of death after cardiovascular disease is Cancer. Breast cancer is currently the most common type of malignant neoplasms in women, with more than a million cases each year. The need for new ways to prevent this illness is progressively developing. Plants have always been a source of conventional medicinal processes and have offered lasting alternatives to the human race for thousands of years.For this research these two plants were chosen because they have exceptional medicinal properties. Rauwolfia curving usually called Indian snakeroot also known as devil pepper may be a flower species within the dicot family. This is native to the subcontinents of India and East Asia. It used to treat a number of diseases because of presence of alkaloids, sugars, flavonoids, phlobatannins,glycosides, phenols, saponins sterols,resins, tannins and terpenes. For centuries, the various components of Rauwolfia, i.e., leaves, roots and rhizomes, are being used in Ayurvedic medicines to treat various diseases like mental stress, high pressure, epilepsy, trauma, anxiety,excitement, eight schizophrenic conditions, psychopathy and sedative sleep disorder. Of the species Rauwolfia, about 80 alkaloids are isolated; among them, reserpine is the most important active constituent. Rauwolfia contains a variety of alkaloids including ajmalimine,rescinnamine,ajmaline, deserpidine,reserpine,ajmalicine,serpentine,indobinine, ,rescinnamidine, reserpilineand yohimbine. In the western and northwestern Himalayas of GentianakurrooRoyle, Rauwolfia Serpentina has been found to have, antimicrobial, antitumor, antidiarheogic,anti-candidal,anti-inflammatory and analgesic properties. Strongly processed with rhizomeand root. Phytochemical screening showed thatplant comprisesof few essential phyto-constituents (xanthones,iridoids, mangiferin C-glucoxanthoneand C-glucoflavones etc.) with a remedial value for several acute as well as for chronic diseases. The medicinal plant uses different treatments such as antioxidant ,antibacterial, anti-inflammatory ,anti-cancer,anti-arthritic, analgesic function.

Chapter – 1 Introduction

Inside a healthy body, natural processes regulate the development , growth and death of cells. Cancer arises when certain processes fail to operate properly. When the cells don't die at normal levels, thecell growthis more than cell death. The overgrowth may cause a tumor to develop. Breast cancer occurs if the cells in breastsgrow and divide without normal control. The tumors in breast appear to develop gradually. At the time when the lump is big enough to feel, it might have grown for up to ten years. A few tumors can beaggressive, andcan grow at much faster rate. Around 50-75% of total breast cancer cases start in milk ducts, approximately 5-15% start in lobules and very few begin in the other breast tissues.[1]

Ductal in situ carcinoma (DCIS) Is a noninvasive breast cancer. DCIS contains the abnormal cells in the milk ducts (channels which carry milk fromlobules into the nipple openings during breastfeeding). This is called "in situ" (meaning "in place") since the cells did not exit the milk ducts to invade tissue nearby. You will also hear the words "pre-invasive" or "pre-cancerous" when discussing DCIS. Because DCIS is known to be non-invasive, it may grow into invasive breast cancer without treatment. [2]

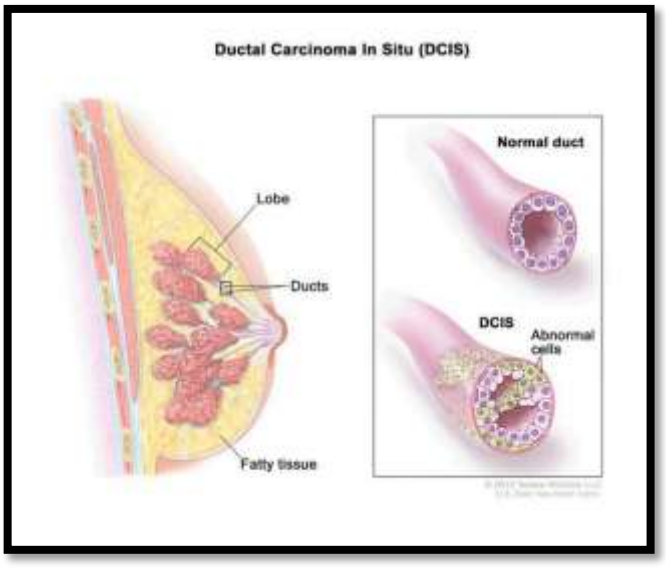


Fig1: Ductal carcinoma in situ (DCIS)[3]

Invasive breast cancer:The Invasive breast cancer develops when irregular cells inside the milk duct or lobule break into surrounding breast tissue. Cancers can spread from breast to the other parts of body via bloodstream or lymphatic system. If the breast cancer spread, lymph nodes inunderarm area (axillary lymph nodes) are mostly the first place to go). [4]

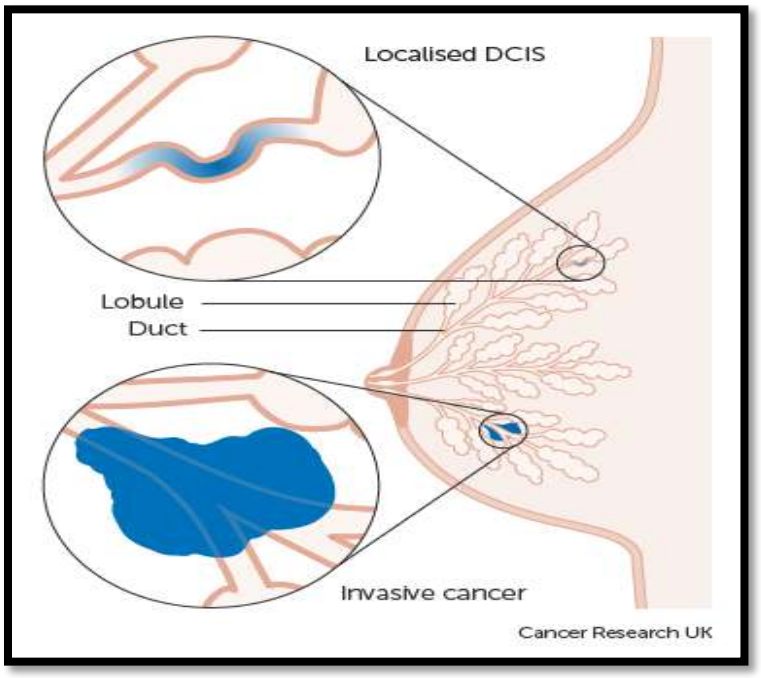


Fig 2: Invasive breast cancer [5]

Metastatic breast cancer: Metastatic breast cancer, known as stage IV or sophisticated breast cancer is an aggressive breast cancer, which has the spread beyond breast & axillary lymph nodes to many other areas of the body (most frequentlylungs , bones,brain or liver). Metastatic breast cancers do not make up a precise type of breast cancer. This is most advanced stage of breast cancer. [6-8]

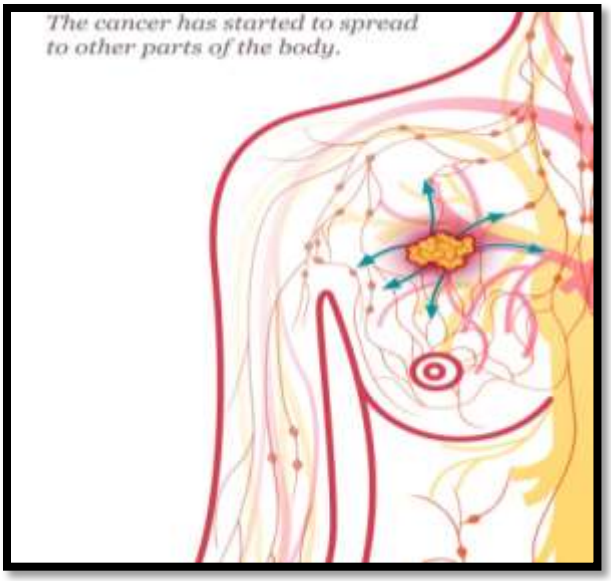


Fig 3: Metastatic breast cancer. [9]

Breast cancer ranks among the most dubious of all exports from our modern world. Once thought of as being a rich man 's disease, it is now a global epidemic.

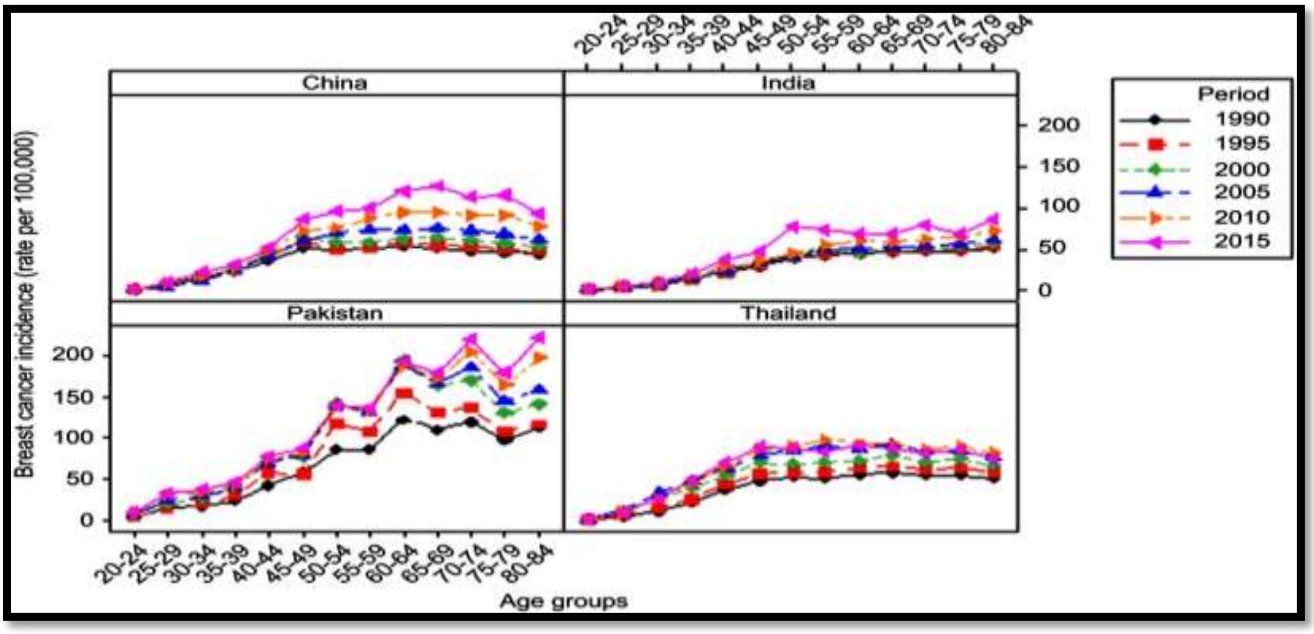


Fig 4:Trends between four Asian countries in the incidence of female breast cancer by specific age groups in different years. [10-14]

Gentianakurroo Royle

The traditional name Gentiana was derived from the word "Gentius," named after the king of Illyria (Europe), and he wasconsidered to discover therapeuticsignificance of the gentian root. Indeed, GentianakurrooRoyle 's specific name derives from the local word for the plant's roots, meaningbitterfor the word “Karu”. G. KurrooRoyle popularly called "Indian Gentian"and Himalayan Gentian, "Karu" in Hindi, "Traayamaana" in Sanskrit, moreover in Himalayas,known as "Nilkanth" but Bakarwals, high-altitude shepherds, of Sinthon top and regions of Daksum, called it in the native language as "Tazakhzand."[15]

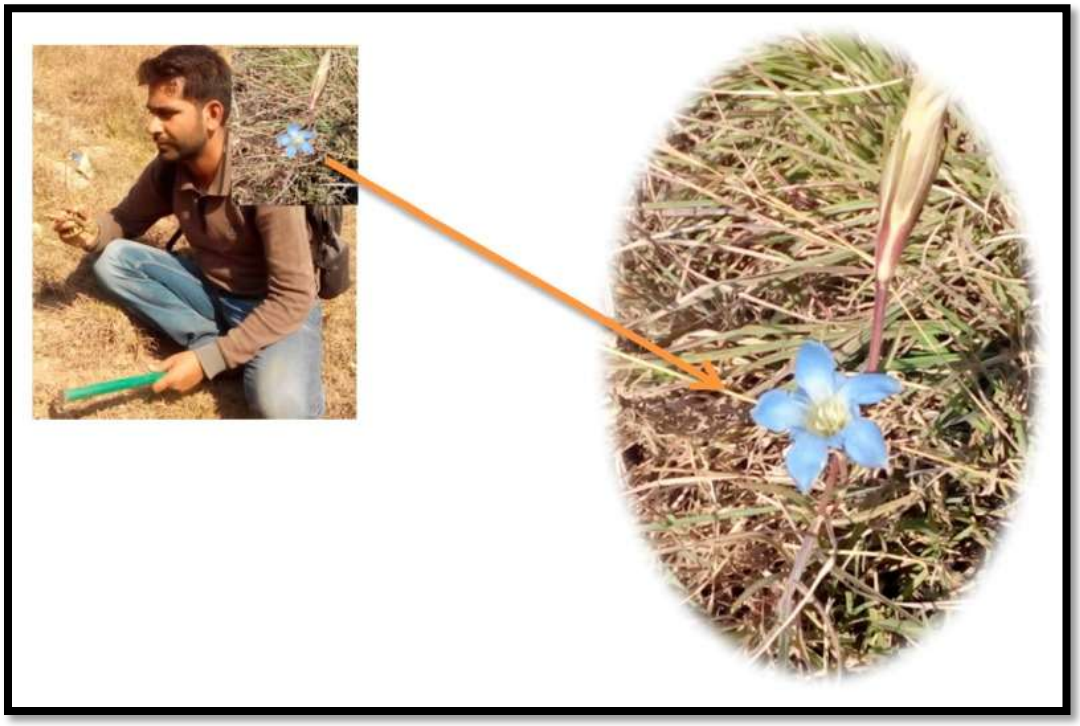


Fig 5: GentianakurrooRoylegathered from the village of Khrew, Pulwama district, J&K.

Gentianakurroo is a member of the Gentianaceae family, as the flowering plant culture. Its critically endangered medicinal plant found in western parts of the Himalayas and to the northwest. It is widely distributed throughout India, Nepal and Pakistan in the Region of the Himalayas. There are more than 90 genera and 1650 species within the gentianaceae family.

Over just a ten year period, due to its high rate for exploitation of its natural habitat, over 80 percent of the species population has occurred in India.

Some essential phyto-constituents have been found in phytochemical screening that includes xanthones,iridoids,C-glucoflavones and C-glucoxanthonemangiferin. Those have therapeutic benefits for a number of acute as well as chronic diseases. This medicinal herb is heavily used for root and rhizome. [16]

The following are some of the important marker compounds found in Gentianakurroo:

1. Amarogentin
2. Swertiamarin
3. Gentiopicroside
4. Sweroside
5. Gentioside

Rauwolfia serpentina

Rauwolfia serpentina, a component of the Apocynaceae family of milkweeds, is native to south as well as subtropical regions of the world such asAustralia, Asia , , Europe, Africa, Cemtral and South America. Widely called as the Sarpagandha or Indian snakeroot. It is widely distributed in sub-Himalyan regions up to 3,300 feet in height in India.

Fig 6: *Rauwolfia serpentina*

It conatins different phytoconstituents including alcohols, tannins, phytosterols, flavanoids, fatty acids, alkaloids, and oleoresins. Indole alkaloids are found in bark of roots at the highest concentration although these are found in all plant parts including stems and leaves. Ajmaline and Ajmalicine are the root-specific indole alkaloids found therein. Ajmaline is effective in antihypertensive and antiarrhythmic activities and Ajmalicine is best known for its circulatory disorder activities. Other indole and indole alkaloids include resperine,coryanthine, serpentine canescine, deserpidine, isoserpiline,neoajmaline,lankanescine, papaverine,raucaffricine, rescinnamine, reserpiline,rauwolfinine, recanescine, serpentinine, thebaine, yohimbinine, isoajmaline,isoserine,raubasine, rauhimbine, and yohimbine. [17]

Chapter – 2Objectives

The aims of this project work are as follows:

2.1 OBJECTIVE

- To carry out the phytochemical screening and moreover to confirm the anti-cancerous potential of***Gentianakuroo*** and ***Rauwolfia serpentina*** extract.

2.2 SPECIFIC OBJECTIVE

- In vitro multiplication of medicinal plants *Gentianakuroo* and *Rauwolfia serpentina*.
- Carrying out phytochemical assay for *Gentianakuroo* and *Rauwolfia serpentine.*
- Extraction of marker compounds from both plants and its quantification by HPLC.
- Testing herbal plant extract on cancer cell lines for anticancer properties.

Chapter – 3Review of Literature

- ## Plant Tissue Culture

In vitro propagation of plants :

Micropropagation provides immense promise through morphogenesis induction, through tissue culture, to generate millions of clonal specimen. The morphogenic reaction of different types varies and respond differently for restoration as per arrangements of different growth regulators. Numerous explants from in vitro-growing cultures can be used for direct regeneration without even callus formation. The explants are grown on MS medium which is further supplemented by several regulators of plant growth such as BA (0.25 mg / l–2.5 mg / l), NAA (0.10 mg / l) and TDZ (0.10 mg / l–1.0 mg / l) .The observations are reported for the average amount of explants exhibiting regeneration as well as for the percentage of shoots regenerated straight from explants on different medium combinations.

Gentianakurroo

GentianaKurroo demonstrates propagation of rhizomes, seeds, somatic embryogenesis, and nodal shoot segment microproliferation by clippings.

Fig 7: In vitro grown***Gentianakurroo***

Phytochemistry

The family of the Gentianaceae has taxonomically valuable forms of compounds, and pharmacological behavior. The numerous compounds were reported, such asxanthones, iridoids, C-glucoflavonesand C-glucoxanthonemangiferin. The iridoids (mostoftenlysecoiridoid glucosides) seem to be found across all the species studied, while of 127 species in 24 genera 90 different iridoid compounds have been identified. Although it is not common to find Xanthones in Gentianaceae, around hundred different factors were found from the 121 species in the 21 genera. The allocation of C-glucoxanthonemangiferin, also reported to be found from 42 species in 7 genera, is more limited than the normal xanthonesand theiridoids. Likewise, exactly nine separate compounds of C-glucoflavonesfrom bube genera have been identified already .The family includes almost all of the bitter compounds; one taste a bit bitter even at 1:58,000,000 dilution, recognized as Amarogentin which is a glycoside has its use in scientific basis of bitterness measurement. Bitter products historically , have been the treatments for feverand also forloss of appetite and even today's world they still used in a number oftonic preparations.[18-21]

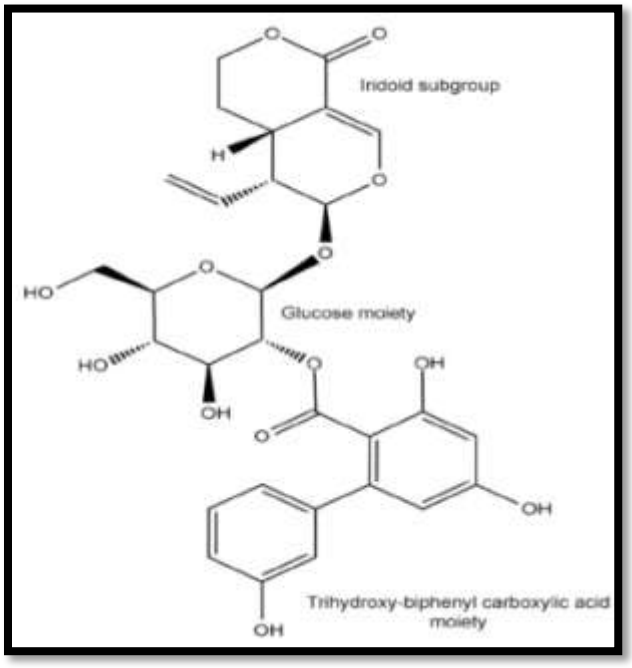


Fig 8: Amarogentine form-a glycoside secoiridoid. Amarogentin comprises of 3 basic subgroups: the glucose moiety, the iridoid group, and the biphenyl-triol rings.

Phytochemical screening of G. As shown in Table 1 KurrooRoyle showed diverse essential phytoconstituents. The quantitative calculation (percentage) of chemical components and the G-flower tops flavonoid fraction. Table 2 shows the kurrooRoyle, while table 3 displays the overall flavonoid as well as phenolic content of extract of leaf and root. Even so, root extract contains constituents besides the flavonoid and phenolic content, as shown in Table 4. The roots and rhizome is the origin of Iridoid glycosides-gentiopicrine, gentiamarine, amaroswerin, and gentianine alkaloids. The leaves containseveral essential bitter compounds. Leaves contain glycoside of 2′-(2,3-dihydroxybezoyloxy)-7-ketologanin iridoid, and around 16 volatile compounds. Several of GentianaKurrooRoyle 's main leaf extracts include dimethyl sulphide , 2-ethylfuran , 1.8-cincole , a-terpinyl acetate and methandriol .[22-26]

Roots and Rhizomes
Tannins
Alkaloids
Saponins
Glycosides (Gentiopicrine, Gentianine)
Terpenes
Flavonoids
Phenolics
Carbohydrates
Genianic Acid
Pectin

Flower tops
Flavonoids (Robinetin-0, Luteolin, Apigenin, Kaempferol, Kaempferid)
Glycosides
Free phenols
Terpenes /sterols

Leaves
Iridoid Glycoside
2′-(2,3-Dihydroxybezoyloxy)-7-Ketologanin
Volatile Aroma Compounds Dimethyl Sulphide
2-Ethylfuran 1,8-Cincole
A-Terpinyl Acetate Methandriol
1,3-Propanediol
2-Methyl Sulphide
3-Methyl Butanol
Pentanol
Hexanal
7-Oxabicylo(4,1,0)-Heptanes

Table 1 :Phytoconstituents of *Gentianakurroo* Royle.[27-28]

Flower Tops	Percentage%	Flavonoid Name
Flavonoids	0.31 ± 0.01	Kaempferol &Kaempferid
Phenols	2.91 ± 0.07	Robinetin-0
Sterols	1.36 ± 0.01	Apigenin
Alkaloids	0.33 ± 0.02	Luteolin

Table 2 :Percentage estimation (quantative) of chemical components and name of a fraction of GentianakurrooRoyle flower tops Flavonoids.

Extract	Flavonoid (approx.)	Phenolic (approx.)
Leaf	21	35
Root	42	70

Table 3 :Complete flavonoid (aluminum chloride colorimetric process by and phenolic material by root and leaf extracts of GentianakurrooRoyle (Folin – Ciocalteu reagent system)..[30]

Different Phytoconstituents	Presence
Phenolics	Highly positive
Cardiac glycosides	Highly positive
Flavonoids	Highly positive
Carbohydrates	Positive
Saponins	Positive
Tannins	Highly positive
Terpenes	Highly positive
Alkaloids	Positive

Table 4 :**Phytochemical screening of gentianakurrooRoyle root methanolic extract.[31]**

Rauwolfia serpentina

Evidently, Rauwolfia (*Rauwolfia serpentina*) is a perennial shrub belonging to a group of apocynaceae. And more than 100 species of the genus Rauwolfia reside in the tropical and subtropical regions of the world, including Europe , Africa , Asia, Australia and Central and South America.The *Rauwolfia serpentina* is confined to the moist, deciduous forests in South East Asia. The plant typically rises to 60-90 cm in height and has light green leaves that are 7-10 cm long and 3.5-5.0 cm wide. The plant has several shiny, black, or purple, round fruits, about 0.5 cm wide. The plant has a large tuberous as well as soft taproot, hitting 30 to 50 cm long and 1.2 to 2.5 inches in width. In Indian folk medicine Rauwolfia serpentina was used to treat a wide-ranging variety of disorders involving snake and insect bites, dysentery, febrile symptoms, malaria etc. This was also used as a uterine stimulant, febrifuge for treating insanity. The plant was mentioned in Indian manuscripts for as long as 1000 bc, and is also recognized as "sarpagandha" and "chandrika".[32-36]

Fig 9:In vitro grown ***Rauwolfia serpentina***

Chemical composition

Rauwolfia comprisesof many phytochemicals containing alcohols, fats, phytosterols, oleoresins, tanninsetc. Indole alkaloids are perhaps the most common alkaloids present in the fruit, with more than 50 of those alkaloids isolated in the fruit. The indole alkaloids are anassembly of

nitrogen containing compounds derived from the amino acid tryptophan. They have a common heterocyclic ring structure of 5 and 6 carbons with 1 molecule of nitrogen.[37]

All plant parts, which include twig and leaves, comprise indole alkaloids but are discovered at maximum concentration in the root bark. The identified indole and indole alkaloids include: raucaffricine, rauhimbine, ajmalidine, reserpiline, reserpinine, sarpagine, ajmalin, ajmalin, aricine, canescine, coryanthin, , serpentine, serpentinine, thebaine, yohimbine etc. [38-39]

RESERPINE

Reserpine is one of the plant 's important alkaloids. Reserpine content was found to be highest in the root and bottom of stems and leaves. Scientists believed it was the most prevalent indole alkaloid in the plant; however, this assertion has been challenged by various assays. Reserpine concentration in the plant was found varying from 0.03 percent to 0.14 percent of the plant's dry weight. The same study found that the root's reserpine content in different plants ranged from 0.038 per cent to 0.14 per cent. Reserpine content in one study was 33 mg of 496 mg of total alkaloids per gram of root. Reserpine content in another Rauwolfia root study was 0.955 mg / g.

Reserpine and breast cancer

In recent years intense interest has been shown in the function ofProlactin in breast cancer, mainly because of the important portion of prolactin in the development of mammary tumors in rodents. Hypophyseal isographers, hypothalamic lesions and drugs that enhance the secretion of prolactin all increase the incidence of mammalian tumors in rats and mice. Both prolactin and ovarian hormones are required to achieve optimum conditions for chemical breast epithelium transformation and for the growth of developed or transplanted mammary tumors in rodents.[40]

Specific medications, compromising reserpine, phenothiazine, methyldopa, and tricyclic antidepressants, are having knowledge to increase human prolactin secretion. Short-term reserpine use significantly increases serum prolactin levels to the lactation-typical range, and this effect continues for weeks after reserpine use is stopped. These broad prolactin elevations in rodents will contribute to substantially higher levels of mammalian cancer. At least 11Case-

control studies on reserpine and breast cancer clearly showed that these significant increases are occurring **in breast cancer risk do not occur in women taking reserpine.**

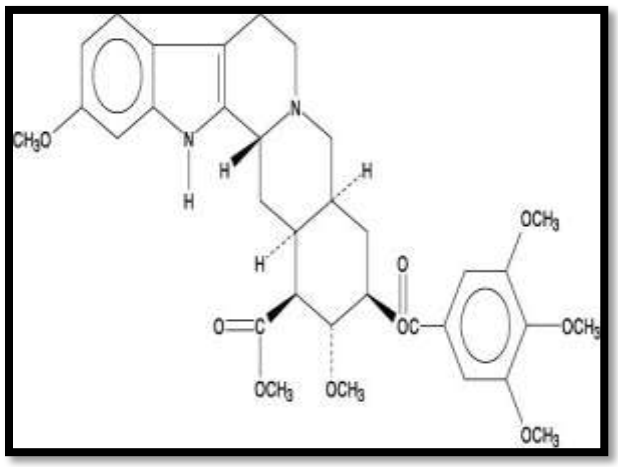


Fig10: Structure of reserpine

Reserpine was found to increase levels of prolactin. In 15 females who used reserpine for at least 5 years, prolactin levels were compared to 15 females who used nonreserpine antihypertensive. Mean prolactin levels were 50 percent higher in females who used reserpine relative to those who did not. The researchers concluded that such an increase in postmenopausal women would likely only cause a small rise in breast cancer, as epidemiological studies have shown.

DIVERSE TYPES OF EXTRACTION TECHNIQUES:

1. **1. Homogenization of tissues:** throughout this technique of extraction, the plant has been either dried or fresh, in any state, shredded in fine powder and immersed in solvent for 24hrs or continuously stirred for atleast for 10 minutes**.**It is left to dry after filtrate is

collected by using rotary evaporator to gain the crude extract , further is diluted with the solvent in order to discover the concentration. Technique is extensively employed by technologists.[41]

2. **Exhaustive extraction :**Another frequently utilized method to collect the extract. Throughout this phase the air dried sample is consecutively immersed with the higher polarity in use in different solvents than the previous solvent. This method is used to make sure the compound is obtainable from a wide variety of polarities.[41]
3. **Extraction of soxhlet:** This method should solitary be used if a specific compound isn't completely soluble ,when the impurities within this particular solvent are completely insoluble. This method is chosen simply because it uses less solvent .If the solvent compound is soluble in water then simple filtration method can be applied. None the less, it is just not suggested for the thermolabile constituents because of it's long heating time, as it may drop the quality of compound. [42]
4. **Decoction:** The driven plant sample must be immersed in the water for a period of 2-4 hours and afterwards kept for boiling for approximately 15-20 min to develop a drug identical uniformity to which a satisfactory quantity of cold water is added to the mixture to generate the texture as needed. This approach is suitable for compounds which are heat stable and water-soluble.[42]
5. **Infusion**: This method is exploited for the completely soluble constituents which are taken from crushed plant sample. It can be made fresh by distilling the powder in cold or hot boiling water for a short time.[43]
6. **Digestion:** It will be the other method of maceration in which some gentle heat was applied to the sample mixture soaked in water to increase the solvent production. This technique is used only as temperatures increase marginally.[43]
7. **Percolation:** This process is used to remove a substance. The percolator tool is used to receive the conical liquid extract from both of the opening ends. Then the fine powder was immersed in the wanted quantity of solvent and left untouched for 4 hours later which the soot was loaded into the percolator. This solvent is sprinkled over the excess to create a thin solvent layer. The mixture was shuttered inside the percolator and permitted to soakfor 24 hours. Afterwards , the gathered liquid is permitted to gradually trickle into the container. The solvent was enriched to the mass again before the gathered liquid from

the percolator measured amount of the needednecessary substance by three quarters. The appropriate quantity of solvent is supplemented to make up the essential volume & ultimately liquid is cleaned bythe process of decanting or simply filtration. [43]

8. **Sonication :**The particular method is advantageous in just a few cases e.g. Because of its greater price it is not utilized to collect extract from its root rauwolfia. This helps to rise the penetration capacity of the cell walls & helps create vacuum in the arrangement as it utilizes ultrasonic waves .But the crucial drawback of the strategy is need for a greater energy ultrasonic wave, which can harm bioactive compounds derived from therapeutic plants by creating free radicals and thus undesirably substituting the drug molecule.[44]

Significance of bioactive constituents:

TANNIS: The active ingredient fits to a family of astringent, a polyphenolic biomolecules related to numerous chemical compounds similar to amino acids , alkaloids, etc. The most important groups are hydrolyzable tannins, phlorotannins&phlobatannins.

i. Hydrolyzable tannins: As heated along with hydrochloric acids, these are the tannins that produce ellagic or gallic acids. Plants are the key source of tannins and the base unit is gallic acid.

ii. Phloroglucinol:The tannin source often present in brown algae, or stay usually oligomers of the compound phloroglucinol, that would be the base product of these tannin groups.

iii. Phlobatannins: Those are the water-insoluble tannins, although they are solvable in alcohol moreover are predominantly located in plants. For such forms of tannins the basic unit is "the flavan-3-ol scaffold."

Tannins, that are mainly polyphenols although few of them are also antioxidant, play an essentialfunction in our body as they have many curative properties described below:

• Antioxidants are those which lower the number of free radicals inside the bodies while protecting us against heart disease.

• An irregular cell division that causes body tissue destruction is the main cause of cancer but prohibits cell damage because of its antioxidant properties.

• Due to its simple use, catechin and resveratrol present in grapes and black or green tea has numerous health remunerations.

FLAVANOIDS: This active ingredient refers to a category of secondary fungus and plant metabolites. Such active compounds have been then broken down into three main bioflavonoid, isoflavonoid&neoflavonoid classes. Flavonoids play the key role in supplying pigmentation in crops. The dark vegetable coloured, seeds , leaves and many more, is directly proportionate to the antioxidant properties of plant. The presence of flavonoids brings many health benefits, such as:

• Anti-inflammatory mediators eg-procyanidins.

• The ways for delays in getting older.

• Helps to lower blood pressure, hence lowering the vary impact of hypertension.

• Different flavonoids showsantibacterial activity in contradiction to bacterial infection.

• Free radical scavenging processes inhibit cancer

ALKALOIDS: The active component fitsinto the family of organic compounds containing nitrogen atoms. Those substances are auxiliary divided in the five key groups:

i. Real alkaloids-Alkloids are derived primarily from proteins and contain heterocyclic nitrogen atom.

ii .Protoalkaloids –Alike to natural alkaloids .

iii. Polyamine alkaloids- Such alkloids are variants of sperm, putrescine and spermine.

iv. Peptide alkaloids and cyclopeptides.

v. Pseudoalkaloids-do not derive from such amino acids.

GLYCOSIDE: Sugar molecule striding the former functional group that is connected by the glycosidic bond. Inactivated glycosides, which plant species process, could be triggered by using hydrolysis enzyme. The hydrolysis process splits the sugar portionand tends to make the chemical likely to use. Such types of compounds are used for medicines. Example: Salicin was Aspirin's previously recognized origin & is also chemically related to it.

ANIMAL TISSUE CULTURE

Mechanism of Apoptosis commenced by phytochemicals

Apoptosis pathways are significant for treatments linked to cancer. Indeed, several phytochemicals were originallyexpended for the anti-inflammatory or can be anti-viral reagents, their anti-tumor functions are known and used, like as pointing apoptosis mechanisms in cancer, while recognizing the cancer process deepens.

Apoptosis ,the conditioned procedure of cell death which might take place in multicellular organisms. This procedure involves blebbing, shortening of cell, and nuclear device fragmentation. Poor apoptosis in the cancer relates to unregulated cell proliferation. Apoptosis cycle includes multiple signal transduction pathways. Proteins can form membrane pores and stimulate mitochondrial swelling, andraise the permeability and leakage of apoptotic effectors to mitochondrial membrane. Mini mitochondrial caspase activator (SMACs) which is released from the mitochondria into cytosol, binds to apoptosis protein inhibitors (APIs), disables IAPs and prevents arrestprocess of cell apoptosis.As the mitochondrial apoptosis-induced channel (MAC) becomes developed inside the outer membrane, the cytochrome c is freed from mitochondria and interrelatesthru apoptotic protease-activating factor-1 (Apaf-1) and ATP, which further attaches to pro-caspase-9 to build a fusion protein apoptosome and cuts pro-caspase and discharge the active arrangement of caspase-9,thenactivates the effector caspase-9. Bcl-2 family proteins control MAC and Mitochondrial Outer Membrane Permeabilization Pore (MOMPP) in which

pro-apoptotic Bax and/or Bakbuild up the pore, but anti-apoptotic Bcl-2, Bcl-xL or Mcl-1 prevents pore conformation.[45-46]

Tumor necrosis factor (TNF), primarily formed by activated macrophages, the principal mediator of the binary hipaloptic apoptosis. This initiates cell proliferation and inflammatory reactions as TNF interacts with its receptor. Fas ligand (FasL) which is a common protein which is present in transmembrane TNF. FasL and Fas receptor interactions (Apo-1 or CD95) shape the Death-inducing Signaling Complex (DISC) comprising Fas-associated Death Domain Protein (FADD), Caspase-8, and Caspase-10.

The Bcl-2 family creates and retains a equilibrium in the mammalian cells amid pro-apoptotic and anti-apoptotic proteins. Caspase activator like cytochrome c and SMAC may be freed from mitochondrial membrane and then the membrane becomes permeable due to the creation of the pro-apoptotic homodimers in the outer membrane of the mitochondrion. The Inhibitor caspases like caspase 2 , 9 , 8, 10necessitate directly binds to particular oligomeric adaptor proteins; effector caspases including certain caspase 6 , 7 ,3 are activated via proteolytic cleavage by a active initiator caspase and deterioration of a host of the intracellular proteins t start the cell death cycle.[47-48]

HPLC(High performance liquid chromatography)

Principle: The high-performance liquid chromatography operates on the premise that certain components of a mixture take longer than others to move through the chromatographic column. The length of time the atoms take depends on the orientation of the molecule to the mobile (liquid or gas) and the stationary (solid or liquid) stages. Those with the most stationary phase affinity will take longer to go through the column, and vice versa.

Overview of the HPLC

HPLC is an excellent method for analyzing chemical compounds, which include biopolymersand polymers etc. Within that technique a sample is suspended to first develop a solution. The liquid

is afterward poured into a resin-containing "bar," which intermingles with the sample. Hence, it would gradually slow down the passage from which sample passes into "pipe," and when sample appears out from the other side of the column, that gets identified. The previous stepspermits you to know both when the sample is coming out and the intensity of the sample.

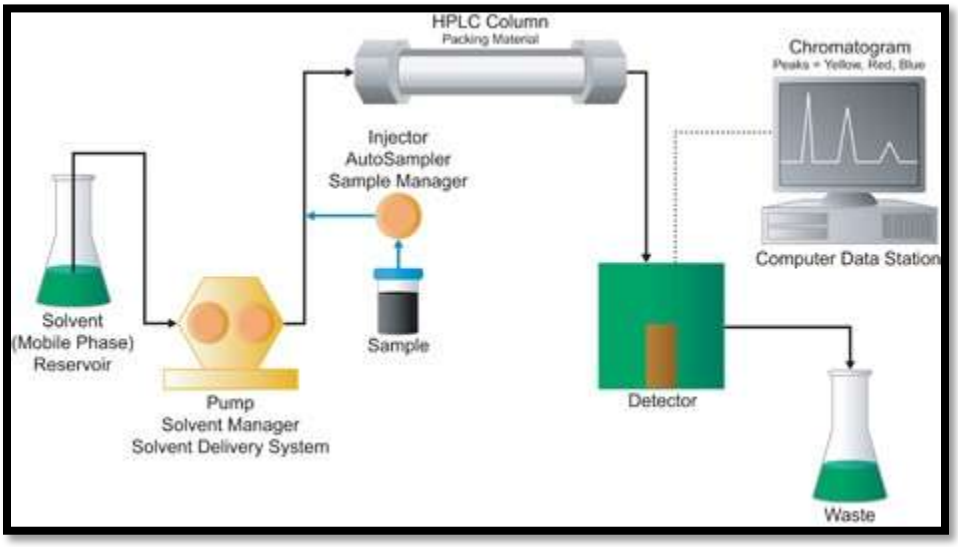


Fig 11: schematic explaination of HPLC

And when some buffer runs via the tube constantly, sample is also injected and realize the diverse molecules of the sample coming out at distinct "retention intervals." Column end detector could be any detector and the most communal types are refractive index (RI), ultraviolet (DAD) and fluorescence (FLD). Each of them can detect different molecular propertiescoming out from the column and show a chromatogram.

MCF-7 Breast Cancer Cell Line

HISTORY

In 1973, Dr. Soule with his associates at the Michigan Cancer Foundations, MCF-7 cells obtained their name, were obtained from 69-year-old woman who was having metastatic illness pleural effusion. For just benign tumor, the female endured a mastectomy of her one side of breast 7 years before commencement of principal cell culture then, consequent radical

mastectomy was done of her another side of breast for the malignant adenocarcinoma . Local recurrences were monitored for time period of 3 years by hormone therapy and radiotherapy.

In the days prior totamoxifen,patient was presumably administered alongside heavy doses moreover the cancer was managed longer than expected showing that the tumor was responding to hormone. June 1970, following widespread nodular recurrences, samples were collected from pleural effusion for tests. A key breast cancer breakthrough was the identification of ER in the MCF-7 cells. In 1975, anti-estrogen tamoxifen was found to decrease the development of MCF-7 cells.

DESCRIPTION

MCF-7 is a widely used line of breast cancer cells, which has been propagated by many groups for many years. This appears to be an appropriate model cell line in addition to worldwide breast cancer studies including those related to anticancer drugs. MCF-7 has, over time, created more realistic data on patient care awareness than any other line of breast cancer cells.It is an ER-positive and progesterone (PR)-positive receptor and is part of the subtype A of luminal molecules. MCF-7 is a cell line that is highly reactive and non-invasive, generally known to have low metastatic potential.

Although often regarded as a single cell, the human breast line MCF-7 comprises of a vast total of individual phenotypes, each of which comprise a limited percentage of the total population. The phenotypes differ by gene expression profile, receptor activity, and signaling pathway. Throughout the progressive line group, a mixture of multiple phenotypes is often maintained in terms of differences in the rate of proliferation of individual phenotypes, probably through some sort of signaling cooperation.With sufficient selective criteria, the minimal sub-lines present in the parental line can be extended. The time frame for the in vitro selection process (6 months and more) is successful in attempting to develop resistance to anti-estrogen therapy and likely aromatase inhibitors for both the clinically occurring long duration of patients with breast

cancer.However, a crucial concern about therapy will be how the changing sub-line reflects modified receptors and associated signaling pathways.

Studies on clonal variations for the production of MCF-7 cells were released fairly early in the literature. Depending on the type studied, MCF-7 cells show substantial aneuploidy, with major differences in chromosome numbers from 60 to 140. There were several cytogenetic differences that included the presence or lack of particular chromosome markers. Existing evidence revealed elevated levels of genetic instability in cells with MCF-7. Due to different cultural conditions, the karyotypical variations may represent changes in the selective pressure. MCF-7 cells have a portion of the stem cells that may bring in clonal heterogeneity.

It was suggested as an reason for this cell line's heterogeneity, and as a mechanism for heterogeneity in the breast tumor. Diverse forms of MCF-7 exhibit differences in genomic and RNA expression. MCF-7 cells are used for tests of ER-positive breast cancer cell lines, with other experiments using them with proven anti-estrogen drug resistance. MCF-7 cells are well suited for tolerance tests for anti-hormone therapy, as they are rapidly grown and retain ER expression when treated with such selective therapy. Populations of MCF-7 cells adapted to different anti-hormone conditions were developed to examine the properties of the breast cancer cells that have been acquired with anti-hormone resistance.

In vitro, and modified to in vivo models that mimic clinical treatment more closely, the MCF-7 models gradually progressed a step further into clinical practice. In vivo models offer a new standard for determining the importance of cancer cell function, angiogenesis, cell proliferation and respiration, processes that in cell culture can not be accurately assessed.[49]

Cell viability analysis through MTT assay

Among all the suitability assays which focus on cell lines to convert substrates into chromogenic products, The MTT assay remains one of its most flexible and usable assays. The MTT assay includes either the transformation of the water-soluble yellow dye MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] into an insoluble violet formazan by mitochondrial reductase.Formazan is hydrolysed, and the amounts at 570 nm are determined by optical pressure. The result is a robust analysis with impressive linearity of up to 106 cells per file. As with the Alamar Blue assay, in the absence of overt cell death, small variations in metabolic activity will lead to major changes in MTT, allowing cell stress to be observed when exposed to a toxic agent.The test for adherent or nonadherent cells, established in many wells, has been standardized. The technique allows use of a 96-well plate as usual. It may also be adjusted to fit a particular type of flower. Place 500-10,000 cells onto a 96-well plate per line. Up to 106 cells have strong linearity on the assay.

CHAPTER -4MATERIALS AND METHODS

- **Collection of plant material**

  Gentianakurroo and *Rauwolfia serpentina* were obtained as a source of explants from previously maintained cultures from the culture room of Department of Biotechnology and Bioinformatics JUIT Waknaghat.

- **Media preparation and culture conditions**

  Murashige and Skoog liquid media and plant growth hormone concentrations of IBA and KN (3:1 respectively) were produced from seed apices and micro seed multiplication in-vitro to create axenic cultures. Sucrose (30 g / l) was applied to plant growth hormone-containing liquid MS media and pH was set between 5.6-5.8. Agar-Agar has been added to 9g / l.It was then heated on a heating plate until it obtained a clear solution. Next, media was autoclaved at 121 ° C and 15 psi for 20 minutes. Cultures were incubated in a plant growth chamber held at 25 ± 1 ° C within a 16 h day/8 h night photoperiod of 3 klux white light strength illumination, of 60-70% of relative humidity.

- **Selection and culture of explants**

  Shoot apices from both mother plants were used as explants and cultured in solid MS media in Laminar air flow in 10 jars. This was done twice to obtain the large amount of in vitro grown cultures.

- **Extract preparation for HPLC:**

- In vitro grown plants were taken and media was removed from the roots followed by washing. With the help of liquid N_2 the plants were crushed in mortar pestle until powder form is obtained.
  - 1gm sample of each plant was taken and kept overnight in 100 ml 80% methanol. After 24h , sonication was done at 2 sec pulse with 30% amplitude for 10 min.
  - Centrifugation at done at 10,000 rpm for 10 min. Pellet was discarded and supernatant was filtered using 0.22µM syringe filter.

- Extract obtained was kept in centrifuge tubes at 4ºC.

- **Extract preparation for performing the phytochemical Assay**
  - 2gm extract in powder form of *Rauwolfia serpentina*was dissolved in 100 ml of methanol. It was then kept for shaking for 48 hrs.
  - Then the solution was poured in petriplates and left for evaporation.
  - Finally the extract was scratched off with blade and collected in eppendorf.
  - 7 mg of extract was dissolved in 35ml DMSO for carrying out phytochemical assay.

PHYTOCHEMICALSCREENING STANDARD METHODOLOGY:

Test compounds	Procedure
Coumarins	0.5 ml of plant extract is combined with 0.25 ml ethanol and 0.25 ml with 0.5 N KOH. Yellow precipitate formation suggests the existence of coumarins.
Flavanoids	0.5ml of plant extract shall be mixed with 2 M NaOH 0.5ml. Then a handful of Dil drops. Added HCL. The transition of solution color from yellow to colourless suggests the presence of flavanoids in the extract of the fruit.
Saponins	0.5ml of purified water is applied to 0.5ml of extract and shake vigorously. Froth formation indicates saponins are present in plant extract.
Proteins	A few drops of conc per 1ml of plant extract. HNO3 has been added from the test pipe sides. The pink-colored coloring suggests the presence of proteins in the sample.
Steroids	1ml of plant extract is mixed with 1.5ml of chloroform and filtered the solution with the help of whatman filter paper no. 1. To the filtrate , conc. H_2SO_4

	isadded . a reddish brown color ring indicates the presence of steroids in plant extract.
Phenols	0.5ml of extract from plants, a few drops of conc. HNO3 was added to the test tube from the sides. The appearance of pink or violet color indicates that the extract contains phenols.
Terpenoids	1ml of plant extract is mixed with 0.5ml chloroform in a test tube. Then ,H_2SO_4 is dropwise added. A reddish brown ring at the interphase of the two layers indicates the presence of terpenoids.
Tannins	0.5ml of plant extract is mixed with few drops 10% lead acetate. Precipitates at the bottom of the test tube indicates the presence of tannins in plant extract.

Table 5: Standard methodology for the phytochemical screening.

Maintaining the MCF-7 cell lines :

▪ The culture flasks were removed from the incubator and placed in laminar flow hood . The flask was tip up onto its corner to remove the media .
▪ The flasks were rinsed twice with sterilized 3ml of 1x PBS for T25 flask. The PBS removed the left over media from the cell.
▪ 1.5ml of trypsin was added in T25 flask to loosen the cells from bottom of flask .
▪ The flasks were placed in incubator for 5 minutes to allow trypsin to act . when trypsin was added , it was initially pink in color but after incubation it was more of orange/rust color .
▪ The flasks were removed from the CO_2 incubator and gently tilt them back and forward once inside the hood . This was done so that all cells are loosened and none are left behind. The flask was turned onto its corner again remove the trypsin solution.
▪ 4ml of DMEM media was poured in the T25 flasks and then cells were transferred in microcentrifuge tube.

▪ The cells were placed in microcentrifuge to allow centrifugation for 10 mins at 1200rpm.
▪ The tubes were removed from the centrifuge and placed into laminar air flow .
▪ 1.5ml DMEM media was added to cell clump and transferred to the T25 flask and then vortexing was done to resuspend the cells.
▪ 3.5 ml DMEM media was further added in the T25 flask and was kept in CO_2 incubator .

Table 6: protocol for maintaining the MCF-7 cell lines

Chapter - 5

RESULTS AND CONCLUSIONS

- ✓ Optimization of media was carried out for the best growth of plants.
  - The best growth of both plants was obtained in MS media supplemented with growth hormones in the ratio IBA:KN (3:1)

MS MEDIA	Different concentrations of hormones		
	IBA	**KN**	**TDZ**
	1	0.5	0.5
	0.5	1	0.5
	0.5	2	0.5
	1	1	0.5
	3	1	0

Table 7: Optimization of media

- ✓ In vitro propagation of *Gentianakuroo* and *Rauwolfia serpentina*has been performed successfully on MS media.

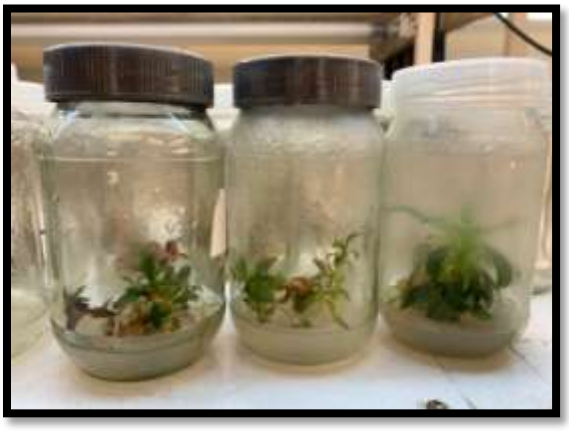


Fig 12: In-vitro grown *Gentianakurroo*

Fig 13: In vitro grown***Rauwolfia serpentina***

- ✓ After culturing on below MS media data was collected for number of shoots,number of roots and biomass.

	No. of roots	**No.of shoots**	**Biomass(gm)**
Rauwolfia serpentina	5	10	2.87
	6	12	2.96
	4	7	2.63
	10	8	3.02

Table 8 (a): Growth parameters of *Rauwolfia serpentina*

	No. of roots	**No.of shoots**	**Biomass(gm)**
Gentianakurroo	4	8	2.40
	6	10	2.63
	7	11	2.87
	2	8	2.38

Table 8 (b): Growth parameters of *Gentianakurroo*

- ✓ Extract preparation of both plants has been carried for quantification by HPLC.

Fig 14(a):Extract Preparation of ***Rauwolfia serpentina*** after crushing in liquid N_2

Fig 14(b) : Extract of ***Rauwolfia serpentina*** after filtration

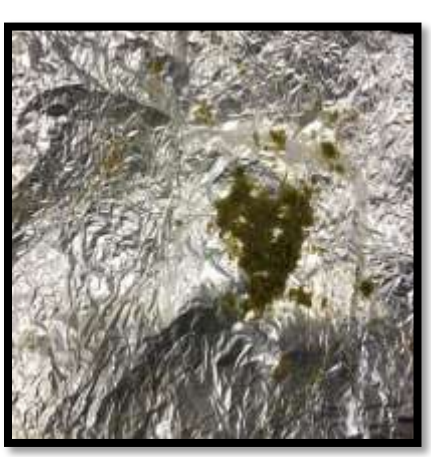


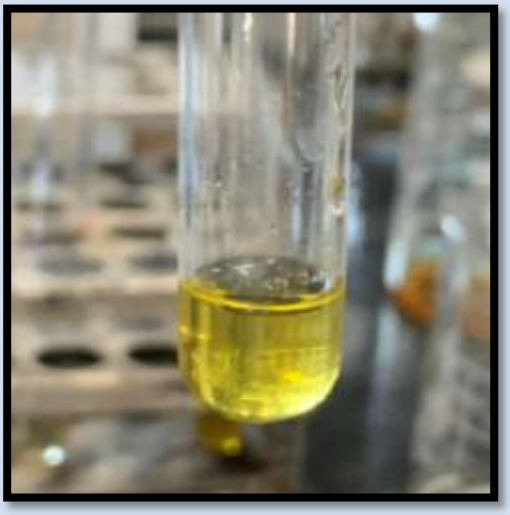
Fig 15(a) : Extract Preparation of ***Gentianakurroo*** after crushing in liquid N_2.

Fig 15(b) : Extract of ***Gentianakurroo*** after filtration.

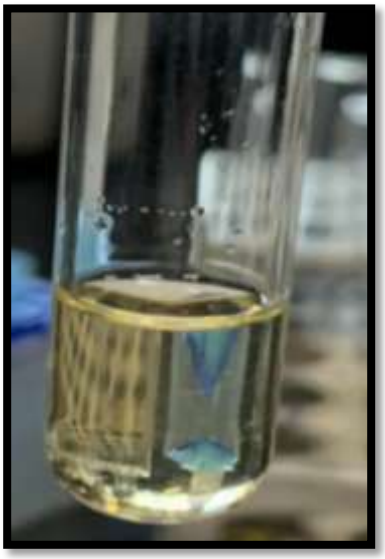
Fig 16(a) : Extract preparation of ***Rauwolfia serpentina*** for phytochemical assay.

Fig 16(b) : Extract of **Rauwolfia serpentina** after dissolving in DMSO

PHYTOCHEMICAL SCREENIN G OF SECONDARY METABOLITES

Test compounds	Inference	Results
Coumarins	**No precipitate formed** 	**Absence of compound**

Flavanoids	**Turns green solution to colorless.** 	**Presence of compound**
Saponins	**Froth formed** 	**Presence of compound**

Proteins	**No pink color observed** 	**Absence of compound**
Steroids	**Red / brown ring formed** 	**Presence of compound**

Phenols	**Dark green colored ring formed** 	**Presence of compound**
Terpenoids	**No interface ring formed** 	**Absence of compound**
Tannins	**Precipitate formed** 	**Presence of compound**

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