Phytochemical analysis in callus cultures of

Podophyllum hexandrum

Dissertation submitted in partial fulfillment of the requirement of

Masters of Science

In

Biotechnology

By

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Under the supervision of

Dr. Hemant Sood

2023

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Candidate's Declaration

I hereby declare that the work presented in this report entitled **"Phytochemical analysis in callus cultures of *Podophyllum hexandrum*"** in partial fulfillment of the requirements for the award of the degree of Master's in Biotechnology submitted in the Department of Biotechnology & Bioinformatics, Jaypee University of Information Technology, Waknaghat is an authentic record of my own work carried out over a period from July 2022 to May 2023 under the supervision of Dr. Hemant Sood (Associate Professor, Department of Biotechnology & Bioinformatics).

(Student Signature)

Parul Katoch, 217808

This is to certify that the above statement made by the candidate is true to the best of my knowledge.

(Supervisor Signature)

Dr. Hemant Sood

Associate Professor

Department of Biotechnology & Bioinformatics

Dated: 12 May, 2023

SUPERVISOR'S CERTIFICATE

This is to certify that the project work titled "**Phytochemical Analysis in callus cultures of *Podophyllum hexandrum*"** by **Parul Katoch** during their end semester in fulfillment for the award of degree of Masters of Science in Biotechnology from Jaypee University of Information Technology, Solan has been carried out under my supervision. This work has not been submitted partially to any other University or Institute for the award of any degree or appreciation.

Signature of Supervisor

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LIST OF ABBREVIATIONS

PTOX - Podophyllotoxin

MS - Murashige and Skoog

IAA - Indole-3-acetic acid

IBA - Indole-3-butyric acid

BAP - 6-Benzyl Amino Purine

KN - Kinetin

2, 4-D - 2, 4-Dichlorophenoxyacetic Acid

TDZ - Thidizuron

GA3- Gibberellic acid

HPV - Human Papilloma Virus

DPPH- 2, 2-Diphenyl-2-picrylhydrazyl

CE- Callus Extract

TPC- Total Phenolic Content

TFC- Total Flavonoid Content

MIC- Minimum Inhibitory Concentration

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ABSTRACT

The endangered medicinal plant *Podophyllum hexandrum* is a member of the Berberidaceae family. The rhizome contains a lot of *P. hexandrum's* lignans, which have anticancer properties. The most powerful cytotoxic substance that occurs naturally is podophyllotoxin.
The anticancer medications etoposide and teniposide are made from it as a basic material.

The formation of microtubules is prevented by podophyllotoxin. From the callus of Podophyllum hexandrum that were aseptically cultivated, callus cultures were created. A fully defined MS medium supplemented with several PGRs was efficient for both the beginning and continuation of callus tissue formation. Plant growth regulators significantly impacted the relative proportion of callus. Elicitors were used for the enhancement of biomass and podophyllotoxin production.
Flavonoids, terpenes, saponins, and glycoside chemicals were detected in the phytochemical screening of a variety of methanolic extracts. The methanolic extract of red and greenlight treated callus produced the best results in terms of total phenol, total glycoside, anti-oxidant activity, MIC, and well diffusion technique.

Keywords: *Podophyllotoxin, Callus extract, Total Flavonoid Content, Total Phenolic Content, Protein Estimation, MIC, Well Diffusion Method*

CHAPTER - 1

INTRODUCTION

1.1 The name "podophyllum" is derived from a prehistoric Greek term that meant foot leaf. The plant is also known as mayapple due to the fact that its fruit ripens in spring. The Podophyllum genus comprises three species, namely P. peltatum, P. sikkimensis, and P. hexandrum, which are found worldwide. P. hexandrum, also known as Indian mayapple, is a tree that grows in the Himalayan Mountains of Asia, while P. peltatum, also known as American mayapple, is widely distributed in Atlantic North America. P. hexandrum is found growing naturally in sub-Himalayan regions of India, Bhutan, Pakistan, Afghanistan, China, and Taiwan, particularly in Uttarakhand, Jammu and Kashmir, and some parts of northeastern India. Podophyllum is a herbaceous green plant in the family Berberidaceae of the order Ranunculales. Indian Podophyllum thrives in temperate and subalpine regions with welldrained, humus-rich soil conditions. In Ayurvedic medicine, it is referred to as Vanyakarkati. The plant's stem is succulent and features a perennial rhizome. The stem's petiole has a pair of umbrella-shaped leaves that droop down, and it reaches a height of about 30 cm before the leaves reach their full size, which is usually around 25 cm in width. The plant blooms in May, with pinkish-white flowers, and its fruit, which is about the size of a lemon, ripens in August or September and turns bright orange in color.

FIG 1.1: *Podophyllum hexandrum* leaves & flower, *Podophyllum hexandrum* fruit

Kingdom: Plantae

Subkingdom: Tracheiobionta

Superdivision: Spermatophyta

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Magnolide

Order: Ranunculales

Family: Berberidaceae

Genus: *Podophyllum* L.

Species: *Podophyllum hexandrum* Royale

FIG1.2: Taxonomic Classification of *Podophyllum hexandrum*.

Chemotherapy is a major cancer treatment method that affects a large number of people worldwide, but its use is limited due to severe side effects caused by currently available anticancer drugs. To overcome this problem, researchers are looking for new anticancer therapies that are selectively toxic to cancer cells and have minimal side effects. Natural resources are being explored to create compounds that have the best pharmacological effects against cancer and other diseases. Podophyllotoxin (PTOX), a well-known naturally occurring aryltetralinlignane extracted from Podophyllum peltatum, is used as a chemotherapy treatment for various malignancies. Existing PTOX compounds like etoposide (VP-16) and teniposide (VM-26) are used in clinical settings for small cell lung cancer treatment, but their low absorption and drug resistance call for the development of new PTOX-based anticancer drugs.

Objective:

1. Sub culturing and regeneration of callus of *Podophyllum hexandrum*.
2. Elicitor mediated enhancement of callus biomass and Podophyllotoxin production.
3. Phytochemical analysis in callus cultures of *Podophyllum hexandrum*.

CHAPTER-2

REVIEW OF LITERATURE

2.1 PODOPHYLLOTOXIN (PTOX):

The aryltetralin lignan, podophyllotoxin, has been discovered in *Podophyllum* species, with *Sinopodophyllum hexandrum* and *Podophyllum peltatum* being the most commonly used sources. These perennial herbs are widely found in Western China and the Himalayan region. Podophyllotoxin has been used in traditional medicine for various medical conditions, including gonorrhea, tuberculosis, psoriasis, and venereal warts. The podophyllotoxin extract has been found to possess several therapeutic properties, including mitotoxic, antibacterial, anti-inflammatory, and antispasmogenic actions. The compound's effectiveness in treating cancer cells has been extensively studied, but its clinical applicability has been limited due to its adverse effects, such as hair loss, bone marrow suppression, and gastrointestinal toxicity. As a result, researchers have developed less toxic derivatives or equivalents of podophyllotoxin to create novel medicinal medicines. The podophyllotoxin extract has a history of usage for laxative purposes and as a treatment for a number of medical conditions, including gonorrhea, tuberculosis, menstruation problems, psoriasis, dropsy, cough, syphilis, and venereal warts. It is currently known that the podophyllotoxinfamily elicits a variety of therapeutic qualities, including mitotoxic, neurotoxin, insecticidal, antibacterial, antiinflammatory, antispasmogenic, hypolipidemic, immunosuppressive, antioxidative, analgesic, and cathartic actions. The effectiveness of its antimitotic properties attracted more attention when initial investigations revealed its strong action against cancer cells. However, due to its unfavorable side effects, including hair loss, bone marrow suppression, neurotoxicity and gastrointestinal toxicity etc. podophyllotoxin's clinical applicability was significantly hindered which led to the discovery of less toxic derivatives or equivalents. Since then, the creation of novel medicinal medicines has used these compounds as a structural foundation. **[2]**

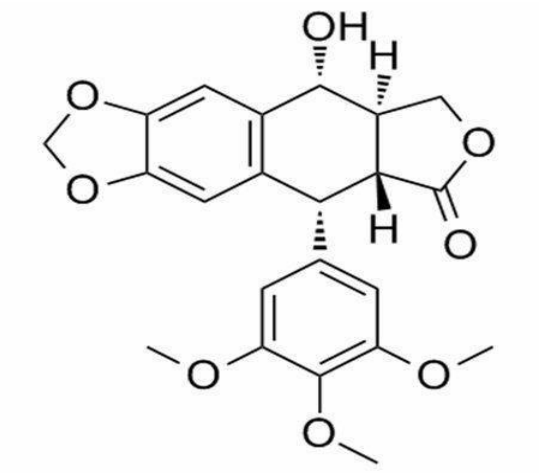

FIG 2.1: Structure of podophyllotoxin.

The structure of the selective cyclolignan podophyllotoxin (C22H22O8) was initially determined in the 1930s. Etiposide, teniposide, and etopophos are three therapeutically effective anticancer medications that were developed following extensive structural alterations of podophyllotoxin but it took twenty years for their action mechanism to be fully recognised. To exert their antimitotic actions, podophyllotoxins were known to interact with DNA and the replication process. For instance, etiposide stops DNA topoisomerase II and results in S-phase cell cycle arrest. By preventing tubulin from polymerizing PTOX is also known to restrict cell development and control the arrangement of mitotic spindles. **[3]**

2.2 MECHANISM OF ACTION:

Inhibition of Tubulin:

PTOX has demonstrated powerful anticancer action since it has been shown to have pharmacological properties that prevent microtubule assembly by obstructing the colchicine binding site. This results in an inhibition of microtubule protein polymerization and causes G2/M blockage.

FIG 2.2: Inhibition of Tubulin.

Inhibitor of DNA Topoisomerase II

PTOX's usage in cancer treatment has been constrained due to its significant side effects, including high toxicity and gastrointestinal distress. Numerous structural changes to PTOX have led to the development of more potent and less toxic anticancer drugs like etoposide and teniposide, which are frequently used in cancer treatment. Inhibiting topoisomerase II degradation and ultimately leading to the accumulation of chromosome breakage that lead to cell death, they stabilize the cleavage complex produced between the enzyme and its DNA substrate. In addition to being a promising class of anticancer targets topoisomerases is crucial for DNA replication and transcription. The new PTOX analogue 4acrylamidopodophyllotoxin congeners were created as a possible anticancer agent. The bulky motif at the C-4 position of PTOX may be responsible for the suppression of topo II, and -H2AX is a typical marker of double-stranded DNA breaks after DNA damage. **[4]**

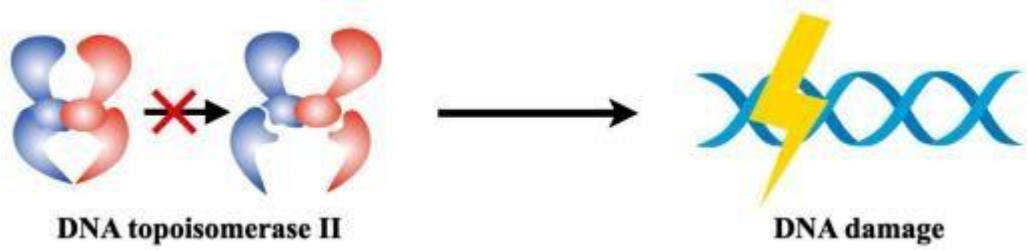

FIG 2.3: Inhibition of DNA Topoisomerase II

2.3 Use of PTOX:

Out all the hundreds of naturally occurring plant lignans, podophyllotoxin is the most wellknown. For more than 250 years, compounds of podophyllin resin have been utilised as medicines. Podophyllotoxin was regarded by Native Americans as a potent remedy for snakebites. Since then, it has been employed as a poison, a local antifungal, and an antihelminthic. In his natural history of Carolina, Florida, and the Bahamas Islands, the medical literature was published by Catesby in the first report in 1731. In early 19th-century western medicine, *Podophyllum* was originally employed as a laxative for medical purposes.

Since 1940, warts and condylomas have all been treated topically with *Podophyllum* resin. The first mention of podophyllotoxin as a topical treatment for venereal warts (condyloma acuminata), a condition brought on by a papilloma virus, was made by Kaplan in 1942. Firstline treatment for condyloma acuminata podophyllotoxin is still used now in all parts of the world and also for various herbal cathartic medicines. Additionally, it has been used to treat vaginal infections caused by non-cervical human papilloma virus.

To get minimally extensive external condylomas necrosis during the course of treating external condylomas, podophyllotoxin is standardized and dissolved in alcohol. 0.5-2.0% podophyllin, which the patient applies themselves, is a safe way to treat penile warts for a lot less money than podophyllotoxin that is sold commercially. For nations with few healthcare resources, Podophyllin extract has a minimum three-month shelf life. Rheumatoid arthritis has long been treated with a combination of natural and semi-synthetic glycosides from *Podophyllum hexandrum*; however this medication has gastrointestinal side effects. Oral hairy leukoplakia linked to HIV is a symptomless lesion that can be treated effectively, affordably, and with little side effects by using a 25% solution of *Podophyllum* resin. At subtoxic, well-tolerated, consecutive dosages of *Podophyllum hexandrum* aqueous extract, Goel et al. (1998) demonstrated a strong antitumor activity. Additionally, they reported *P. hexandrum*'s radio protective qualities, which are 19 equivalents to those of man-made radio protectors such diltizem and other similar substances. Etoposide (VP-16-213) and teniposide are chemically synthesized using podophyllotoxin as a starting material (VM-26). **[5]**

2.4 Medicinal value of PTOX:

Podophyllotoxin or podophyllin, a neurotoxin, can be extracted from the plant's rhizome by processing a resin found there called both marketed and known under the generic name Indian Podophyllum Resin. The primary lignan found in the resin, podophyllotoxin, is a byproduct of the phenylpropanoid pathway that has dimerized. Etoposide (vepeside), an FDA-approved anticancer medication, is made from podophyllotoxin as its primary component and has been used to treat lung and testicular cancer by preventing the growth of cancer cells. Leukemia, lung and testicular cancer, as well as skin conditions like warts, psoriasis, and rheumatoid arthritis, can all be treated with podophyllotoxin, which is used as a precursor for semi-synthetic topoisomerase inhibitors. Because of its ability to scavenge free radicals, it has a wide range of uses in contemporary medicine.

Another in vitro investigation identified as a potential cytotoxin, podophyllotoxin for use against a number of human cancer cell lines, including HL-60, A-549, HeLa, and HCT-8. PTOX was also discovered to stimulate the endoplasmic reticulum stress that causes proapoptosis signaling pathway. The several cancer-fighting medications made from podophyllotoxin include etoposide, teniposide, and etopophos.

Podophyllotoxin is discovered to have numerous other significant therapeutic qualities in addition to being a significant anticancer agent, some of which include:

a. Protection from radioactivity: Several studies have shown that different podophyllotoxin extracts, such as chloroform, methanolic, and hydro-alcoholic extracts, gave 70-95% protection from radioactivity.
b. *Aspergillus niger* and *Candida albicans*-specific antifungal activities.
c. This substance's dichloromethane extract is being tested for its potential to be insecticidal.
d. Native Americans have traditionally used this substance as an antihelminthic. The roots' aqueous preparations have been used in India as both a cathartic and a treatment for ophthalmia. **[1]**

2.5 Biosynthetic pathway of Podophyllotoxin

Comparatively speaking to the microbiological pathways that have been characterised so far, the understanding of plant pathways is absolute. Unknown plant genes limit their transfer to heterologous hosts for industrial production. These genes are involved in the metabolic pathways of many therapeutic medicines obtained from plants. Only recently in 2015, did the full biosynthesis pathway for podophyllotoxin become clear, making it easier to obtain the clinically relevant derivatives that are naturally occurring and processed but otherwise challenging to manufacture on a large scale.

Coniferyl alcohol serves as the precursor in the 33 steps necessary for plants to produce the toxin podophyllotoxin. The system is known as the phenylpropanoid pathway. Coniferyl alcohol is created from phenylalanine in the first nine steps of the production of podophyllotoxin. Coniferyl alcohol then experiences a unique and site-selective enantiodimerization to produce (+)-pinoresinol. When pinoresinol is converted to (-)secoisolariciresinol, a dehydrogenase catalyses the conversion to (-)-matairesinol. The subsequent intermediate is (-)-pluviatolide, which is methylated to generate (-)-5′desmethoxy-yatein, which is then transformed into yatein. Podophyllotoxin is the final product, whereas yatein is the intermediate. **[4]**

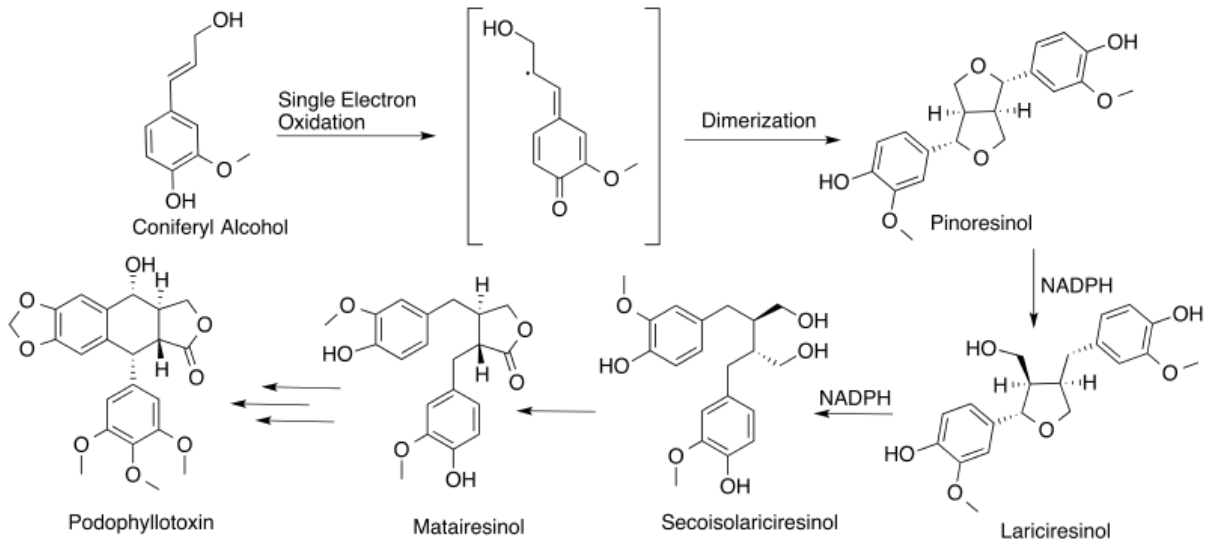

FIG 2.4: Schematic representation of podophyllotoxin biosynthetic pathway

2.6 PTOX AND ITS DERIVATIVES:

A subclass of lignans, or a class of secondary metabolites found in plants, podophyllotoxin (PTOX) is a compound found in the *Podophyllum* resin of *Podophyllum* plants. A typical lignan structure, the PTOX structure is made up of a dimeric backbone that is created by a 'beta-beta'' connection between two phenyl propane units. Moreover, PTOX contains each functional group found in aryl naphthalene lignans. It is made up 5 rings in total, each having aryl-tetrahydrofuran-type backbone, a trans-lactone, and four chiral centres. PTOX as well as its offshoots hence possess enormous possibility as the best using the chemical weapons to exploit anticancer medicines.

More PTOXs are being created and developed as a result of the emergence of simple synthesis techniques & improvements within analytical procedures like crystallography using X-rays.

The most fundamental chemicals are employed to modify create better antitumor medicines are PTOX, a chemotherapeutic drug, and its beta-configuration derivative, epipodophyllotoxin.

C-4 of the ring C location is frequently employed to be conjugated with another molecule. Combining molecular combinations with additional pharmacologically active compounds may lessen the recognized negative the effects of separate substances while improving their anticancer effectiveness. When PTOX is hybridized with (PEG) groups i.e. polyethylene glycol for instance, its low water solubility can be enhanced. Additionally, peptides or amino acids that are frequently used as hybrid molecules might significantly enhance a drug's capacity to pass through tumor cell membranes.

Higher treatment effectiveness against tumors is also obtained by combining PTOX with another anticancer agent, which is the most used hybridization technique. In this review, we emphasize how each compound's structural diagram shows how the hybrid molecules significantly increase PTOX.

Hybridization is a great method for the development of new anticancer medications since such substances can increase the therapy specificity for tumors, lessen adverse effects, and overcome drug resistance. **[6]**

Etoposide and teniposide, the cytostatic agents (antimitotic) glucosides, were therefore introduced into clinical practice as a result of research efforts to make several alterations on the basic podophyllotoxin skeleton in order to avoid several side-effects.

Both are distereoisomer-4-glucopyranosyl derivatives of podophyllotoxin, also known as epipodophyllotoxin. These semi-synthetic podophyllotoxin derivatives are crucial in the treatment of lung cancer, several types of leukemia and other solid tumors.

The effective conversion of etoposide and teniposide has incorporated podophyllotoxin as a sparked interest in structural optimization to create novel derivatives with an improved pharmacological profile and more varied medicinal applications. Etopophose is a brand-new etoposide phosphate created to get over the problems brought on by etoposide's poor solubility. A benefit of the treatment is that etoposide can be given intravenously in greater doses and is quickly converted to etoposide by the plasma enzyme phosphatase. **[7]** In Europe, a novel derivative called **CPH 82**, a combination of benzylidinated podophyllotoxin glycosides, is being evaluated for the treatment of rheumatoid arthritis.

Other derivatives are used to treat psoriasis and malaria.

Ones in the initial stages either of clinical studies or pre-clinical research include **NK611, TOP 53, and GL 311** as some of the most promising podophyllotoxin derivatives. Based on the chemical makeup of podophyllotoxin, **azatoxin** has proven to be the most effective synthetic anticancer substance. It is currently undergoing clinical studies. **[8]**

ETOPOSIDE:

Etoposide, also known as Demethylepipodophyllotoxin-ethylideneglucopyranoside (Demethylepipodophyllotoxin-ethylideneglucopyranoside, EPE, epipodophyllotoxin), is marketed by Bristol-Myers Squibb under the brand name Vepesid, sometimes known as VP16. Several chemotherapy regimens frequently prescribe it. It is a well-known and very effective anti-cancer drug. It is utilised largely in combination treatments for leukemia and testicular tumours and is effective against a wide variety of tumour types. This is the most effective single to treat small cell lung cancer. The medication can be taken orally in the form of liquid capsules or as a liquid that can be injected for intravenous infusion.

Nk-611:

NK 611 is a brand-new derivative of podophyllotoxin. The sugar ring underwent increased activity alteration as a result of replacing a glycosylated moiety with acrylamines.
Contrasted with etoposide The D-glucose moiety of NK 611 has a dimethylamino group. NK 611 was shown to have an antitumor activity that was on par with or better than etoposide.

USES:

It is primarily used to treat a variety of cancers, including acute lymphocytic leukaemia, acute myelogenous leukaemia, germ cell tumours, Hodgkin disease, ovarian cancer,
Rhabdomysarcoma, and glioblastoma that was just discovered multiforme. For histiocytosis, kaposis sarcoma, wings sarcoma, and brain tumours, and neuroblastoma it is used less commonly. Treatment for juvenile patients with primary cerebral yolk sac tumors and embryonal cancer involves an etoposide in conjunction with chemotherapy cisplatin and concurrent radiotherapy, followed by the excision of the tumour. An efficient autologous transplantation preparative regimen for lymphoma with minimal toxicity uses etoposide in combination with other medicines.
Hair loss, nausea, anorexia, diarrhoea, low leucocyte counts and platelet levels, and diarrhoea are among the more severe adverse effects. Women who are pregnant or nursing should not use etoposide as it is known to cause birth abnormalities and foetal harm. **[5]**

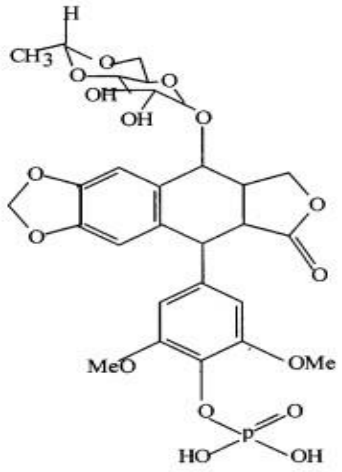

FIG 2.5: Structure of Etoposide.

TENIPOSIDE:

Teniposide and a replacement acute refractory leukaemia lymphomas, as well as brain and bladder malignancies, are all treated with VM-26. It is a single-drug therapy option for remission induction. The excipient increases the likelihood of an initial allergic reaction with severe respiratory distress in addition to the haematological toxicity. It is utilised less frequently than etoposide.

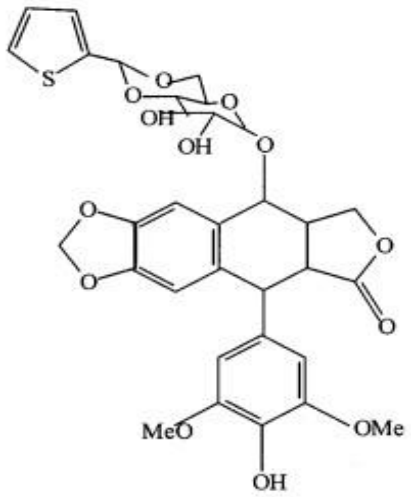

FIG 2.6: Structure of Teniposide.

2.7 PTOX DERIVATIVES AS ANTI-CANCEROUS DRUGS:

PTOX compounds offer strong anticancer activity, which has been extensively studied. Numerous new derivatives have been used and their effectiveness has been confirmed in a variety of neoplasms.

We concentrate on the tumours that have been the subject of the most research, including colorectal, breast, lung, and liver cancers.

1. BREAST CANCER:

According to the most recent information on Cancer from (IARC), breast cancer is now the most prevalent malignancy in the world, posing a severe threat to women's health. the breast cancer kind that is most aggressive, **triple-negative breast cancer (TNBC)**, which accounts for 15–20% of all cases of breast cancer detected, is characterised by a high rate of recurrence and metastasis. PTOX and its derivatives are attractive selective therapies because they can prevent breast cancer from proliferating, migrating, and invading both in vivo and in vitro. On the BT-549 human breast cancer cell line, acetylpodophyllotoxin showed specific inhibitory action. The human breast cancer (MCF-7) cell line was similarly damaged by PTOX piperazine acetate derivatives, which caused G2/M blockage and breakdown of the microtubules, though no harm was seen in non-cancerous cells.

2. LUNG CANCER:

Continued to be the most common cancer is lung cancer causes greatest worldwide cancerrelated death factor, coming in second only to breast cancer in terms of incidence rate. PTOX and its derivatives have been proven in numerous studies to be able to suppress lung cancer cells in humans are expanding as well as to cause apoptosis and G2/M cell cycle blockage & the formation of ROS. Inhibition of activity of c-MET kinase was revealed to be a factor in PTOX-induced cell death by Oh et al. (2021), who investigated human lung cancer cells generated by PTOX, apoptosis occurs.

Ching001, a derivative of podophyllotoxin that they created, is selectively fatal to several different types of cell lines of human lung cancer, while posing no substantial cytotoxicity to lines of human lung cells in good health. In contrast to the original PTOX, formononetin hybrids, and PTOX showed improved suppression of The A549) cell line from human lung cancer exhibits invasion and migration. Additionally, these PTOX compounds mostly employed the route to elicit apoptosis in A549 cells of caspase against both small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) cell lines; 40-demethylepigallocatechin, which comes from tertiary amines, adducts demonstrated high cytotoxicity.

3. COLORECTAL CANCER:

The second most common cause of cancer-related death is colorectal cancer (CRC), which is one of the most prevalent malignant diseases in affluent nations. Cell lines of human CRC are frequently employed in PTOX pharmacological studies. The Triazol-1-yl-4-[(4-substituted)1, 2, 3] derivatives of PTOX were created, and it was discovered that they were cytotoxic to all seven cancer cell lines from people, with a notable impact of chemotherapy on HCT-15 and 502713 human CRC cell lines in particular. In order to stop the growth of the human CRC (HCT116) cell line, created the aroyl-thiourea analogue of podophyllotoxin known as HY-1 (4b-[benzoylthiourea]-4-deoxypodophyllin) (4b-[benzoylthiourea]-4deoxypodophyllin).

4. HEPATOCELLULAR CARCINOMA:

The majority of liver cancers, or about 80% of all liver cancer types, is caused by hepatocellular carcinoma (HCC), which is a kind of cancer. The therapeutic PTOX and its derivatives' effects on HCC have likewise been quite good. PTOX conjugates were created by Zhang et al. (2017a) using non-steroidal anti-inflammatory medications (NSAIDs), which demonstrated specific toxicity towards cells resistant to 5-FU. Their network of microtubules was broken down by PTOX-NSAIDs, which successfully induced apoptosis and the G2/M blockage. **[9]**

2.8 PHARMACOLOGICAL SIGNIFICANCE OF PTOX:

A wide range of pharmacological possibilities exists for podophyllotoxin and its derivatives. The podophyllotoxin pharmacophore's extensive usefulness as a therapeutic molecule is a result of the possibility of broad chemical alterations. Outstanding among all of its clinical characteristics is antineoplastic activity. The anticancer properties of GL331, NK-611, TOP53, and NPF as well as other podophyllotoxin compounds such etopophos, teniposide, etoposide, and etoposide phosphate have been supported by numerous researches.

Numerous clinical studies have demonstrated the effectiveness of these chemicals in treating a variety of cancers, including non-lymphocytic leukaemia, non-Hodgkin lymphoma, glioblastoma lymphoma in several forms & lung cancer. Additionally, podophyllotoxin has demonstrated effectiveness against several (resistant to several drugs) tumour cells. As an illustration, Hu & colleagues reported a derivative of the toxin 4-anilino-podophyllotoxin

possible anticancer treatment against the VCR/KB cells in both the in vitro and in vivo settings. Podophyllotoxin analogues are the main focus of current research for the creation of novel anticancer medicines. Additionally, it has been determined that peltatins, deoxypodophyllotoxin, and picropodophyllotoxin are all effective antiviral substances. The cytomegalovirus and Sindbis has both been the target of activity. After the virus has been absorbed into cells, they either reduce the contaminated cell's potential for virus spread or stop these viruses from replicating at a crucial early stage. In addition to this, podophyllotoxin can be used to treat additional venereal and perianal warts as well as Condyloma acuminatum, a condition that is typically brought on by the HPV (papilloma virus). Cocktail therapies, which mix other approved chemotherapeutic drugs with other proven methods of battling cancer and other viral diseases, are currently being used with the aim of improving therapeutic effectiveness. **[10]**

In addition to their notable both cancer and antiviral properties, PTOX & the derivatives it is derived from have also demonstrated a number of other intriguing properties, including antitrypanosomal, receptor for antimelanocortin-4 (MC4R), antioxidant, anti-inflammatory, emetic, laxative, hypolipidemic, antispasmodic, and immunosuppressive properties. Additionally, other experiments using podophyllotoxins were conducted during the course of the previous century in an effort to treat conditions like cough, gonorrhoea, dropsy, gout, psoriasis, tuberculosis, tumours, irregular menstruation, and venereal warts. The genus Podophyllum, which has been used for therapeutic purposes as both antihelminthic and cathartic drugs from ancient times, is particularly rich in podophyllotoxin. For the treatment of genital warts, podophyllotoxin has been shown to be an effective and generally safe medication. It is also a registered active ingredient in Condylox liquids and Wartec ointments. **[11]**

Activity	PTOX derivative	Mechanism
Antibacterial	The bactericidal activity of newly synthesized podophyllotoxin precursors was tested.	*Escherchia coli, Shigella sonnei, Salmonella typhi,* and activity were all monitored**.**
Antitumour	(NSC-141540) VP 16-213	Activity on N/D mice, was examined against tumour ascites L1210**.**
Antineoplastic	Cyclolignans	Activity was tested against HT-29 colon cancer, murine leukaemia P-388, and tA human lung carcinoma 549.
Cytotoxic	Cleistantoxin	Cancer cell lines MCF-7, MCF-7R, KB, and HT29 were tested for activity.

Table 2.1: Pharmacological activities of Podophyllotoxin.

CHAPTER - 3

MATERIALS AND METHODS

3.1 Growing Media i.e., MS-Media Composition

Stock solution and components	For MS medium (g/l)
STOCK SOLUTION A (10X)	
KNO3	19.00
KH_2PO_4	1.70
$MgSO_4.7H_2O$	3.7
STOCK SOLUTION B (20X)	
NH_4NO_3	33.0
STOCK SOLUTION C (100X)	
$CaCl_2.2H_2O$	44
STOCK SOLUTION D (100X)	
$FeSO_4.7H_2O$	2.785
Na_2 EDTA	3.726
STOCK SOLUTION E (100X)	
KI	0.083
STOCK SOLUTION F (100X)	
$COCl_2.6H_2O$	0.0025
H_3BO_3	0.62
$ZnSO_4.7H_2O$	0.86
$MnSO_4.4H_2O$	2.23
$Na_2MoO_4.2H_2O$	0.025
STOCK SOLUTION G (100X)	
Glycine	0.20
Myo-Inositol	10.00
STOCK SOLUTION H (100X)	
Nicotinic acid	0.050

Pyridoxine HCl	0.050
Thiamine-HCl	0.010

TABLE 3.1: Composition of MS-media.

Growing media: Plants grow in materials referred to as growing media. Growing media is created expressly to aid plant growth. To develop a growth media, different elements are combined. A variety of plants are grown using various growing media. It is also referred to as substrate, culture medium, or grows media. [12]

MS-MEDIA: Plant cell cultures are grown in laboratories using a media for plant development called **MS** medium. It's frequently used in suspension culture, callus culture, and organ culture. Inorganic salts, vitamins, and amino acids are combined in the formulation to provide nutrients. The most popular tissue culture medium, of which numerous variants have been made, it provides all the necessary macro- and micro-elements as mentioned in **Table 3.1.**

PLANT SOURCE: The (mother plant) or callus samples were procured from Forest protection division, HFRI, Shimla.

3.2 Preparation of Media:

MS media was prepared using the previously prepared stock solutions:

STOCK A: 100 ml/L

STOCK B: 50 ml/L

STOCK C: 10 ml/L

STOCK D: 10 ml/L

STOCK E: 10 ml/L

STOCK F: 10 ml/L

STOCK G: 10 ml/L

STOCK H: 10 ml/L

Different compositions MS media with different PGR's were used:

Experiment 1: Preparation of Media 1 and sub-culturing of calli.

Methodology:

Step1: Media Preparation [Total volume: 1L)

In MS media, **Stocks A-H** are added i.e. stock- A=100ml/L, stock-B=50ml/L and stocks CH= 10ml/L.

[MS media + PGR's: IBA (3mg/L) + KN (1mg/L)]

Added 30g/lit Sucrose & 9g/lit Agar-agar, Set pH between 5.6-5.7

Experiment 2: Preparation of Media 2 and sub-culturing of calli.

Methodology:

Step1: Media Preparation

In MS media, **Stocks A-H** are added i.e. stock- A=100ml/L, stock-B=50ml/L and stocks CH= 10ml/L.

[MS media+ BAP (1mg/L) + PGR's: 2, 4-D (1mg/lit) +GA3 (0.5mg/lit)]

Added 30g/lit Sucrose & 9g/lit Agar-agar, Set pH between 5.6-5.7

Experiment 3: Preparation of Media 3 and sub-culturing of calli.

Methodology:

Step1: Media Preparation

In MS media, Stocks A-H are added i.e. stock- A=100ml/L, stock-B=50ml/L and stocks CH= 10ml/L.

[MS media + PGR's: 2, 4-D (1mg/lit) + IAA (2mg/L) +BAP (1mg/lit)]

Added 30g/L Sucrose and 9g/L Agar-agar, Set pH between 5.6-5.7

Experiment 4: Preparation of Media 4 and re-generation of calli.

Methodology:

Step1: Media Preparation

In MS media, Stocks A-H are added i.e. stock- A=100ml/L, stock-B=50ml/L and stocks CH= 10ml/L.
[MS media + PGR's: KN (1mg/L) + GA3 (0.5mg/L) +BAP (1mg/L)]

Added 30g/L Sucrose and 9g/L Agar-agar, Set pH between 5.6-5.7

Experiment 5: Preparation of Media 5 and re-generation of calli.

Methodology:

Step1: Media Preparation

In MS media, **Stocks A-H** are added i.e. stock- A=100ml/L, stock-B=50ml/L and stocks CH= 10ml/L.
[MS media + PGR's: TDZ (1mg/L) + BAP (1mg/L) +GA3 (0.5mg/lit)]

Added 30g/lit Sucrose & 9g/L Agar-agar, Set pH between 5.6-5.7

3.3 CALLUS SUB-CULTURING AND REGENRATION:

The term "callus culture" refers to clusters of disorganized parenchyma tissue that form under cultured conditions from aggressive division of cells from any type of explants with no polarity. Callus production can involve any kind of solid yet living tissue, including phloem cells, parenchyma, cortical tissue, collenchyma, pith cells, meristematic tissue and cambium cells. The action either exogenous or endogenous growth chemicals lead to the rejuvenation of the permanent tissue cells and the stimulation of cell division.

Any sort of tissue explant's ability to develop into a proliferative callus mass is largely dependent plant's genotype, the origin of explant of the tissue's physiological state in culture. A fresh medium is used to further transfer the callus after the callus tissue has developed from the explants. Subculturing describes this method of transmission. On agar media, callus culture

will display a sigmoid or S-phase development pattern with each passage when sub-cultured often. [14]

3.4 PROCESS OF SUB-CULTURING:

1) Placed the objects (petriplates, scalpel, forceps, media jars, surface sterilizers) required in the procedure in Laminar Air Flow.

2) Switched ON the UV light of the LAF 15-20 before starting to work in it.

3) Turned off the UV light of the LAF and transferred the callus jars in LAF.

4) With the help of forceps and scalpel callus was transferred.

5) Cut the callus in various pieces.

6) Then callus was placed in the sub-culturing media jars with the help of forceps.

7) The jars were labelled properly.

8) Then the jars were moved to the culture room and also under uv-light to observe the growth of the callus.

FIG 3.1: Sub-cultured jars

3.5 Elicitator mediated enhancement of Biomass and Podophyllotoxin production:

1. Sub-cultured calluses were placed under different lights.

Effect of LED's on growth and podophyllotoxin accumulation:

Spectral Light Treatment: **Red>Green >Yellow>White**

As physical elicitors treatments of different LEDs, were used, including:

Red LEDs (660 nm, 24h), Green LEDs (460 nm, 24 h), Yellow LEDs (400-700 nm, 24h), white light (24 hrs.). (ODTech, Ltd., Korea) **[20]**

2. Sub-cultured calluses were placed under cold storage.

FIG 3.2: Effect of LED's on growth and podophyllotoxin accumulation

3.6 Plant Extract Preparation:

The callus plant material was subjected to four different light treatments: red, green, yellow, and a combination of red and green. After drying with liquid N2 and grinding into a fine powder by a mortar-pestle, it was freeze-dried using a lyophilizer. The resulting powder was then dissolved in 80% methanol, and a shaker was used to extract the powder with methanol. The extracts obtained were rotary vacuum evaporator-concentrated and kept at 4 °C for future use. [16].

3.7 Preliminary Phytochemical Screening:

Phytochemical testing involves both qualitative and quantitative analyses. Qualitative analysis focuses on determining the presence or absence of specific phytochemicals in a plant sample, while quantitative analysis measures the concentration or amount of a particular phytochemical present. Preliminary phytochemical screening is typically the first step in this process before conducting a quantitative analysis. Standard methods, as outlined by Sofowora (1993), Trease and Evans (1989), and Herborne (1973), were utilized to perform both quantitative & qualitative analyses of various phytoconstituents in Podophyllum hexandrum methanolic extracts [19].

3.7.1 Preliminary qualitative test:

i. Alkaloid test or Mayer's test:

To detect the presence of alkaloids, add a small amount of plant sample extract to a test tube, and then add 2 drops of Mayer's reagent carefully along the sides of the tube. If a white or creamy precipitate is formed, it indicates the presence of alkaloids [22].

ii. Carbohydrate test or Molisch Test :

To test for the presence of carbohydrates in the plant extract solution, two to three drops of the Molisch reagent and 1 ml of the plant extract at a concentration of 10 mg/ml were mixed in a test tube. Then, roughly 2-3 drops of sulphuric acid were added. The appearance of a violet ring indicates the presence of carbohydrates [21].

iii. Protein Test or Biuret Test:

Add 4-6 drops of copper sulfate solution and 2 ml of sodium hydroxide to the plant extract, mix the contents well by gently shaking the test tube, and allow the mixture to rest for about 4-5 minutes. A bluish-violet color indicates the presence of protein [21].

iv. Amino acid test or Ninhydrin test:

After adding one milliliter of plant extract with a concentration of 10 mg/ml to a test tube, a few drops of ninhydrin reagent were introduced. The test tube was then heated to 40°C and incubated for seven to ten minutes. The appearance of a green or purple color indicates the presence of amino acids in the extract solution [21].

v. Saponin Test or Foam test:

A plant extract with a concentration of 10 mg/ml was placed in a test tube, followed by vigorous shaking and the addition of a few drops of double-distilled water. The formation of foam indicates the presence of saponin [23].

vi. Terpenes test:

After adding 1 ml of plant extract at a concentration of 10mg/ml to a test tube, a small amount of FeCl3 solution was introduced. If a deep or dark green color appears, this indicates the presence of tannins [21].

vii. Glycoside Test or Salkowski test:

To detect the presence of glycosides in a plant extract solution, 1 ml of the extract at a concentration of 10mg/ml was mixed with 2 ml of H2SO4 and 2 ml of chloroform in a test tube. The solution was thoroughly mixed, and a reddish-brown color ring occasionally appeared, indicating the presence of glycosides [23].

viii. Phenolic Test or Ferric Chloride Test:

To detect the presence of phenols in a plant extract, the extract is first dissolved in water or a mixture of water and ethanol. Then, a few drops of neutral ferric chloride solution (FeCl3) prepared in deionized water are added to the mixture. Sodium hydroxide is then added until a permanent brown precipitate is formed. The appearance of a red, green, green or purple color is indicative of the presence of phenols in the extract [23].

ix. Flavanoid Test or Alkaline Reagent Test:

When an aqueous plant crude extract was mixed with a 2.0% NaOH solution, a deep yellow color was observed, which indicated the presence of flavonoids. The addition of a few drops of dilute acid neutralized the color, confirming the existence of flavonoids in the extract [23].

3.7.2 Methods for Quantitative Analysis

I. General procedure for estimation for carbohydrates:

Glucose solutions of different concentrations (0, 2.0, 4.0, 6.0, 8.0, and 10.0 mg/ml) were prepared from a stock solution of 10 mg/ml. A plant extract with a concentration of 10 milligrams per milliliter was also prepared. One milliliter of the test material, either the plant extract or glucose solution at different concentrations, was mixed with four milliliters of anthrone reagent. The test tube was then heated at 100 °C for 8-10 minutes. The absorbance was measured at 630 nm using a spectrophotometer [21].

II. General procedure for estimation of protein:

A solution of Reagent 1 was prepared by combining 48 ml of NaCO3 with 0.1 ml of 0.1 N NaOH, 1 ml of KNaC4H4O64H2O, and 1 ml of CuSO4. Reagent 2 was prepared by mixing Folin reagent and distilled water in a 1:1 ratio. Various concentrations of BSA (0, 2.0, 4.0, 6.0, 8.0, and 10.0 milligrams per milliliter) were prepared. The plant extract at a concentration of 10 mg/ml was added to a test tube. 1 ml of the test sample was mixed with 4 ml of Reagent A and incubated at 30°C for 10 minutes. Then, 0.50 milliliters of B reagent were added to the test tube, which was then incubated in the dark for 20 minutes. The absorbance at 750nm was measured using a spectrophotometer [21].

III. General Procedure for Total Flavonoid Content:

A standard curve was created by preparing various concentrations of gallic acid in methanol, while quercetin was dissolved in 100.0 mL of methanol to produce a final concentration of 1% (10 mg/mL) quercetin. Then, 150.0 microliters of Al2Cl3 was added, and the mixture was incubated for 5 minutes before adding 200 microliters of NaOH. The absorbance at 510 nm was measured, and the flavonoid content was estimated in terms of quercetin equivalents [23].

IV. General Procedure for Total Phenolic Content:

In accordance with Ainsworth et al. (2007), the total phenolic content was determined through a spectroscopic method. A reaction mixture was created by combining 1 mL of plant extracts (1 mg/mL), 1 mL of 10% Folin-Ciocalteu's reagent, and 5 mL of 7% Na2CO3 solution in 13 mL of deionized water. The mixture was allowed to react for two hours at room temperature in the dark, after which the absorbance was measured at 760 nm using a spectrometer. The mean absorbance value was obtained after three repetitions of the experiment, and the total phenolic content was calculated using a calibration curve created with a gallic acid solution. The total phenolic content was reported as gallic acid equivalent (mg GAE) per gram of the dried sample used as the unit of measurement [23].

3.7.3 Method to check anti-oxidant activity of *Podophyllum hexandrum*:

DPPH Assay: A fresh solution of 0.002% (2,2-diphenyl-1-picryl-hydrazyl-hydrate) was created by dissolving it in the solvent methanol. The absorbance of this solution was recorded at 515.0 nanometers to establish a standard value. Gallic acid was dissolved in methanol to produce solutions of varying concentrations (0, 2, 4, 6, 8, 10). To test for antioxidant activity, 50 microliters of plant extract (at a concentration of 1.0 mg per milliliter) was added to 3 ml of 2,2-diphenyl-1-picryl-hydrazyl-hydrate, followed by a 15-minute incubation period at room temperature. The absorption was then recorded at 515 nanometers to determine the antioxidant capacity of the plant extract.

Percentage inhibition of (2,2- diphenyl-1-picryl-hydrazyl-hydrate) calculated the by formula:

$$\% \text{ Inhibition} = (A-B) \div A*100$$

Where, A= (2,2-diphenyl-1-picryl-hydrazyl-hydrate) in pure form absorbance (oxidized), B = sample absorbance measured after a 15-minute incubation period.

To determine the IC50 value of the extracts, which is the concentration required to scavenge 50% of 2,2-diphenyl 1-picryl-hydrazyl-hydrate, a calibration curve was generated. The curve was constructed using varying concentrations of gallic acid and the corresponding percentage of inhibition. Each experiment was performed in triplicate [24].

3.7.4 Method for anti-microbial activity of *Podophyllum hexandrum*:

The methanolic extract was retrieved by using a vacuum rotary evaporator and lyophilizer to evaporate the solvent. To obtain the crude extracts, the fractions were filtered using Whatmann filter paper and then evaporated using a rotary evaporator under reduced pressure. This process was repeated three times to ensure maximum extraction. After evaporating the solvent, all crude extracts were weighed and stored in capped vials at 4°C for later use [24].

Preparation of Inoculum

The stock cultures were kept at a temperature of 4°C on nutrient agar slopes. For the experiments, active cultures were created by transferring a small amount of cells from the stock cultures to test tubes containing Mueller-Hinton broth (MHB) and then incubating them at 37°C or 25°C without any movement for 24 hours. A volume of 0.2ml of the culture was then inoculated into 5ml of MHB and left to incubate until it reached the same level of cloudiness as the standard 0.5 McFarland solution at 600nm, which is equivalent to a concentration of 106-108 CFU/ml [24].

I. Well Diffusion Method:

The purpose of this study was to determine whether extracts from plants or microbes possess antibacterial properties. The first step involved culturing bacteria in Muller Hilton broth and adjusting the turbidity to meet the 0.5 McFarland criterion, which meant that the broth contained 106 cells. Next, the bacteria were spread on Muller Hilton agar plates, which were

left to dry, and then sterile cork borers were used to puncture wells. To test for antibacterial activity, different concentrations of plant extracts (in 50 µl amounts) were added to each well, while a positive control consisting of an antibiotic and a blank consisting of DMSO were also used. Finally, the plates were incubated in an incubator at 37°C for 24 hours, after which the zones of inhibition for several microorganisms were measured [24].

II. MIC (Minimum Inhibitory Concentration):

The minimum inhibitory concentration (MIC) is the smallest quantity of any antimicrobial agent required to halt the growth of the tested microorganism. In this experiment, 100 µl of Muller Hilton broth was added to each of the 96 wells in a plate, and 3 * 106 bacterial cells and 100 µl of plant extract were added to each well. The plate was then incubated for 24 hours at 37°C. Resazurin dye was added to each well, and the color transition from green to pink was observed. This was used to determine the MIC for the plant extract against the microorganism being tested [24].

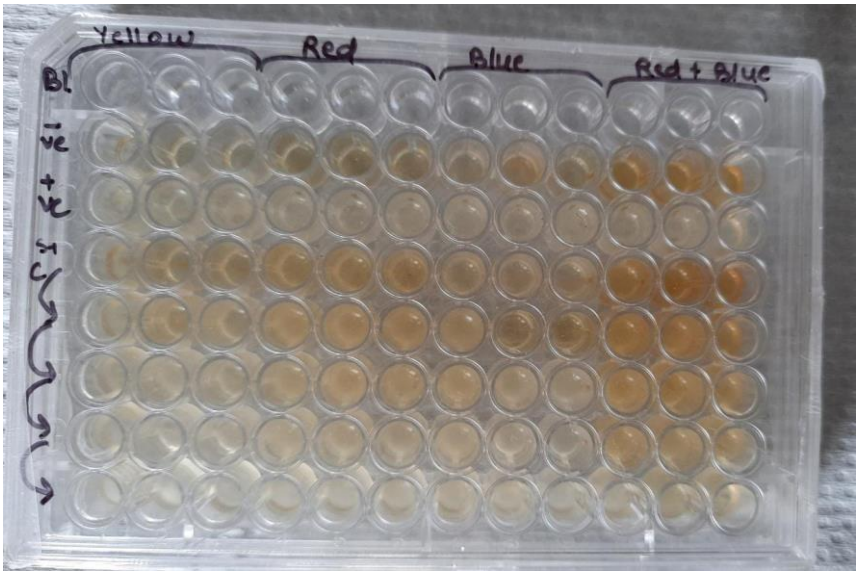

FIG 3.3: 96 wells plate method

CHAPTER-4

RESULTS AND DISCUSSION

4.1 Results obtained after 2-3 months of sub-culturing the callus of *Podophyllum hexandrum.*

TABLE 4.1: Results of sub-culturing of callus.

Experiment	Media Composition	Elicitor	Results
Exp.1	MS media + PGR's IBA(3mg/L) + KN(1mg/L)	White Light	Dark brown color is obtained.
Exp.2	MS media + PGR's : 2,4D(1mg/L) + BAP(1mg/L) +GA3 (0.5mg/L)	White Light and in darkness	Brown color is obtained.
Exp.3	MS media + PGR's : 2,4D(1mg/L) + IAA(2mg/L) +BAP (1mg/L)	Red Light	Yellow Green color is obtained.
Exp.4	MS media + PGR's : BAP(1mg/L) + GA3(0.5mg/L) + KN(1mg/L)	Green Light	Creamish white to Yellow color is obtained.
Exp.5	MS media + PGR's : BAP(1mg/L) + GA3(0.5mg/L) +	Yellow Light	Light Brown to Pale

	TDZ(1mg/L)		Yellow color is obtained.

i. An effect of LED's on callus growth:

The use of red LEDs resulted in the highest levels of phenolic content, total flavonoid, and antioxidant activity, while green light significantly increased the amount of cyanidinmonoglucosides in the sample. A change in callus morphology was caused by the culture of callus under various wavelengths, with dark, green, white & red LEDs resulting in brown, light-green, and creamish white-brown callus, respectively. The effect of light spectra on callus phenotype was observed as paler or brown in the absence of light compared to green or red callus grown under light. The use of LED lights was found to increase the biomass, total phenolic content, and antioxidant activity total glycoside content, total flavonoid content in callus [20].

FIG 4.1: Effects of LEDs on callus growth

ii. Chilling effect on callus growth:

Changes in temperature of the growth medium can affect the cellular functions and metabolic pathways of cultured cells, leading to changes in growth and secondary metabolite production. Typically, callus tissues are initiated and cultured at temperatures ranging from 4 to 10 degrees Celsius. While exposure to heat or cold stress can stimulate the synthesis of secondary metabolites, it can also negatively impact plant growth and trigger the onset of senescence [18].

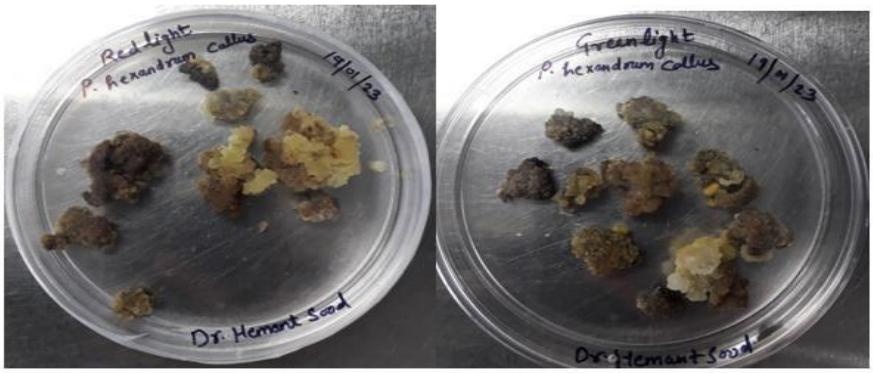

FIG 4.2: Effect of low temperature on callus growth

4.2 Parametric Analysis of Podophyllotoxin Biosynthesis:

Table 4.2: The influence of varying parameters on yield of plant-biosynthesized podophyllotoxin.

Parameters	General Effect	Sub-Parameter	Podophyllotoxin Yield
Light	Light can increase or decrease the biosynthesis of podophyllotoxin.	Red light	Substantial increase
		Blue light	Slight increase
		White light	Decrease
Chilling Temperature	Chilling temperature can increase or decrease the biosynthesis of podophyllotoxin	4 °C	5-folds increase
		10 °C	3.33-folds increase

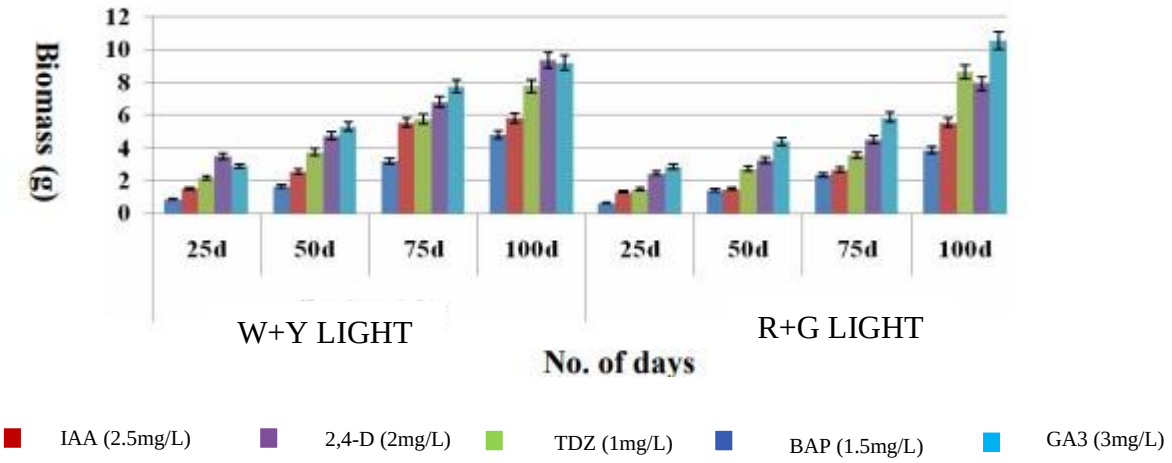

FIG 4.3: Influence of Growth Regulator (2,4-D+BAP+IAA+GA3+TDZ) on the Fresh and Dry Biomass of *Podophyllum hexandrum* Callus.

4.3 Result of Qualitative Test:

Qualitative test done for the *Podophyllum hexandrum* plant extract and the results obtained for Proteins, Phenols, Terpenes, Saponins, Flavonoids and Glycosides was positive and negative results were obtained for Carbohydrates, Amino acids, tannins and alkaloids.

TABLE 4.3 Result of Phytochemical analysis.

Phytochemical Test	**Result**	**Observance**
Protein (Biuret Test)	Present **(++)**	Bluish violet color was observed.
Phenol (Ferric chloride test)	Present **(++)**	Red, green, green or purple color was observed.
Terpenes Test	Present **(++)**	Reddish brown color interface was observed.
Saponin (Foam Test)	Present **(++)**	Foam formation was observed.
Flavonoids (Alkaline reagent Test)	Present **(++)**	Greenish Yellow Precipitates were observed.
Glycoside (Salkowski test)	Present **(++)**	Reddish color was observed.

4.4 Results for Protein Estimation:

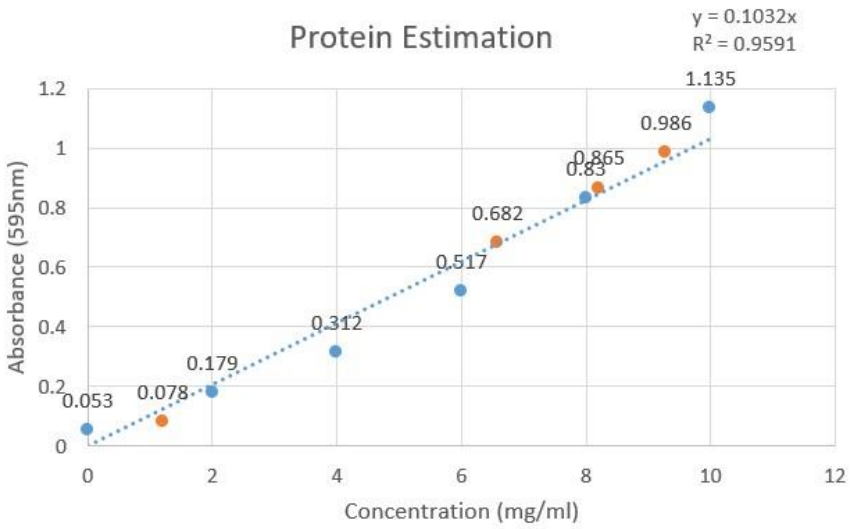

FIG 4.4: Estimation of Protein

4.5 Results for Anti-oxidant Activity:

On performing Anti-oxidant activity on plant *Podophyllum hexandrum*, the results were obtained by performing DPPH assay.

DPPH scavenging activity of methanolic extracts of *Podophyllum hexandrum.*

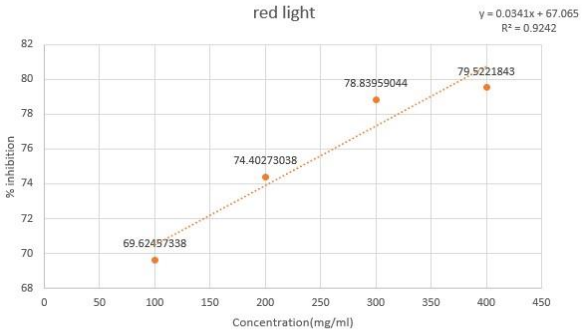

FIG 4.5: From this IC50 value was calculated equation, (IC50=MX+C), where, IC50= 0.0341x+67.065 and the obtained is 500.439 mg

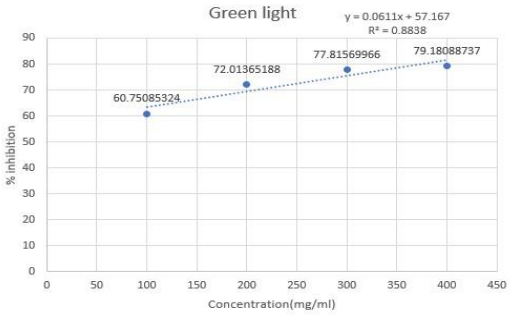

FIG 4.6: From this IC50 value was calculated from from equation, (IC=MX+C), where, IC50= 0.0611x+57.167 and the Value value obtained is 117.299 mg

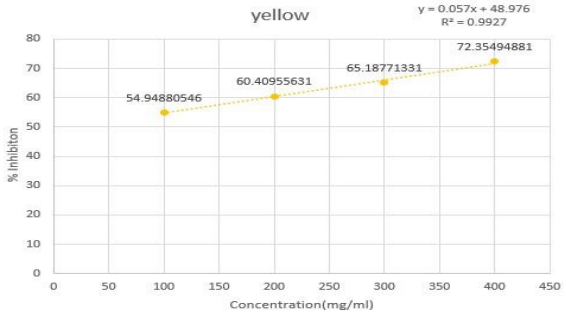

FIG 4.7: From this IC50 value was calculated from equation (IC50=MX+C), where, IC50=0.057x+48.976 and the is 17.964 mg

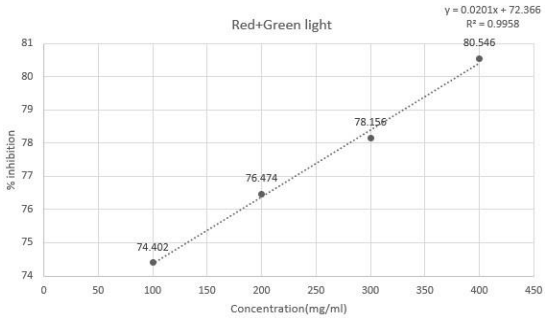

FIG 4.8: From this IC50 value was calculated from equation (IC50=MX+C), where, IC50=0.0201x+72.366 and the value obtained value obtained is 1112.73 mg

4.6 Results for Total Flavonoid Content (TFC):

The total flavonoids present in the various methanolic plant extracts were assessed in the current investigation using the Hossain et al. method and given as quercetin equivalents. The red plus green light treated methanol extract of callus from *P. hexandrum* had the most flavonoids of any of the four methanolic extracts from the plant. The results of earlier investigations on the contents of flavonoids have been published in a similar manner. The diverse extraction techniques and solvent polarity could be to blame for the inconsistent outcomes. Some volatile active chemicals may be lost or evaporated from the samples during processing and drying.[23] as shown in figure 4.9

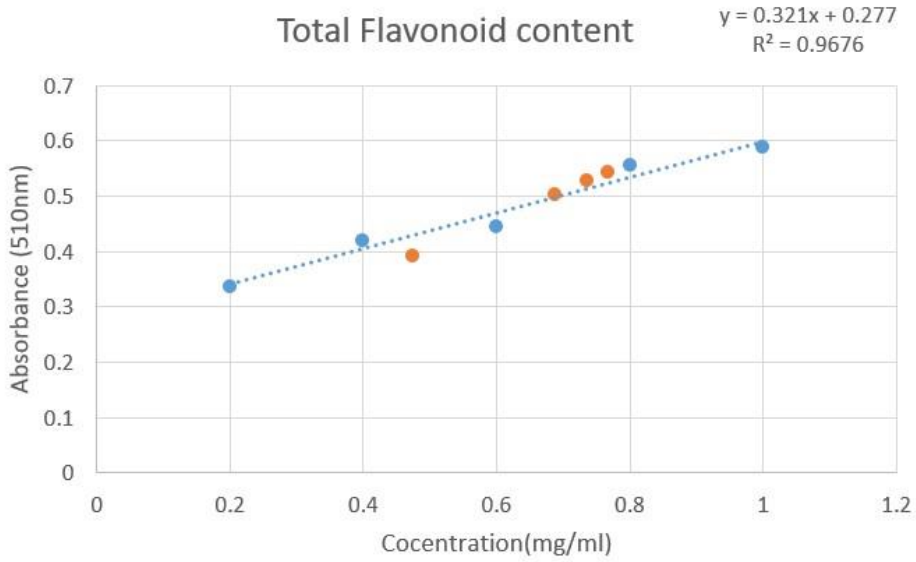

FIG 4.9: Total Flavonoid Content (TFC)

4.7 Results for Total Phenolic Content (TPC):

Gallic acid equivalents are used to report the total phenol content of the methanolic extracts as assessed by a recognized method. The most phenolic chemicals were found in the red plus green light treated methanolic extract, which was one of the four methanolic extracts. The results of the literature search indicate that there has never been any research on the total phenol content of this Podophyllum hexandrum. Comparing our findings to other reported findings on total phenol reveals significant differences.[23] as shown in figure 4.10

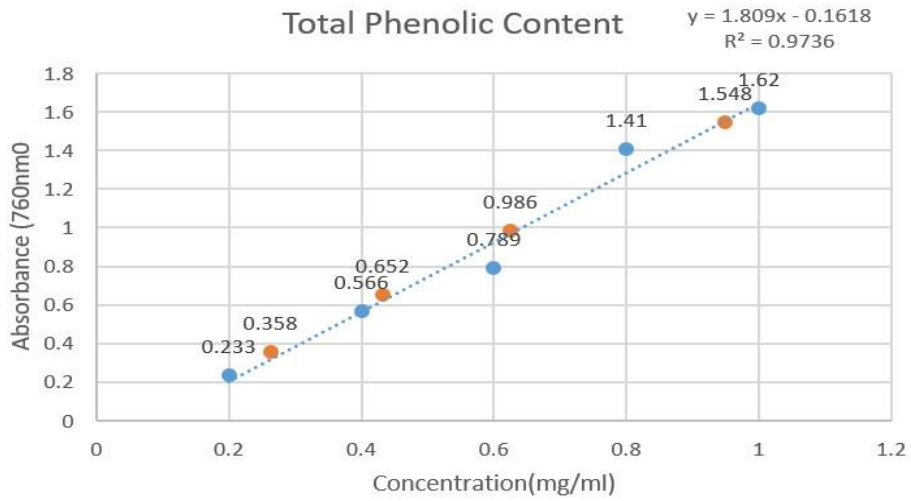

FIG 4.10: Total Phenolic Content (TPC)

4.8 Results for Well Diffusion Method:

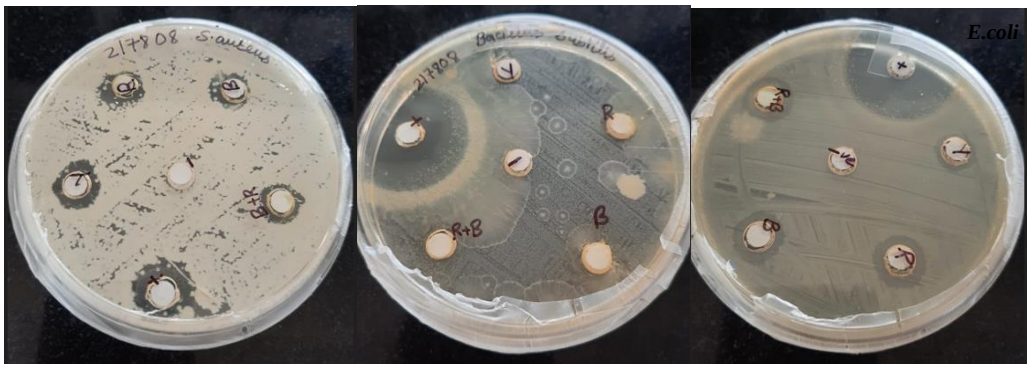

FIG 4.11: i. Zone of Inhibition shown by *S. aureus*

ii. Zone of Inhibition shown by *Bacillus subtilis*

iii. Zone of Inhibition shown by *E. coli*

Sample	*S. aureus*	*Bacillus subtilis*	*E. coli*
Antibiotic	6 mm	10 mm	26 mm
Red Light	4 mm	4 mm	6 mm
Green Light	4 mm	6 mm	6 mm
Yellow Light	2 mm	4 mm	4 mm
Red+Green Light	8 mm	8 mm	8 mm

Table 4.4: Zone of Inhibition shown by *S. aureus, Bacillus subtilis, E. coli*

4.9 Results for MIC:

When MIC was calculated for different bacterial strains, the results obtained were:

Table 4.5: Minimum inhibitory concentration by *Bacillus subtilis:*

Sample	**Concentration (µg/ml)**
Podophyllum hexandrum	6.25
Antibiotic	0.78

Table 4.6: Minimum inhibitory concentration by *S. aureus:*

Sample	**Concentration (µg/ml)**
Podophyllum hexandrum	6.25
Antibiotic	0.39

Table 4.7: Minimum inhibitory concentration by *E. coli:*

Sample	**Concentration (µg/ml)**
Podophyllum hexandrum	3.12
Antibiotic	0.390

The minimum inhibitory concentration, or MIC, of an antibiotic is the concentration at which bacterial growth is fully prevented. The value found here is expressed in µg/ml, which is 1000 times smaller than mg/ml, proving that this was a successful study. The Podophyllotoxin efficiency as a strong inhibitory agent is demonstrated by the MIC with the lowest concentration [24].

CHAPTER-5

CONCLUSION:

In this thesis research work, i worked on plant *Podophyllum hexandrum* callus extracts and find different qualitative and quantitative analysis in which protein, tannin, flavonoid, saponin and glycosides were found. This plant has shown anti-oxidant activity, which was proven by assays like DPPH method and antimicrobial activity, which was proven by MIC and well diffusion method. Tissue culture techniques using plant material i.e., callus from different populations can help ensure high Podophyllotoxin content. To decrease pressure on the natural population, a sustained effort is needed, including callus sub-culturing and regeneration (in vitro) culture and estimation. The study assessed the podophyllotoxin concentration of callus, standardised medium for tissue culture, callus initiation, and generation, and raised *Podophyllum hexandrum* extract.

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