COMPUTATIONAL ANALYSIS IN *PODOPHYLLUM* SPECIES AND SCIENTIFIC VALIDATION OF MEDICINAL AND AROMATIC PLANTS

Thesis submitted in fulfillments of the requirements for the Degree of

Master of Science in Biotechnology

By

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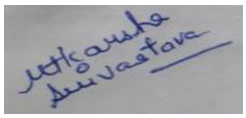
Declaration

I hereby declare that the work reported in the M. Sc Thesis entitled **"Computational analysis for the regulation of podophyllotoxin biosynthesis pathway in *Podophyllum hexandrum* Royale and *Podophyllum peltatum*"** carried out under the supervision of Dr. Hemant Sood from August, 2020 to January, 2021 and "**Scientific validation of medicinal and aromatic plants**" has been carried out as per AYUSH guidelines, under the supervision of **Dr. Daya Nandan Mani**, from February, 2021 to May, 2021, submitted at **Jaypee University of Information Technology, Waknaghat**, Solan, Himachal Pradesh, India, are authentic records of my work. I have not submitted this work elsewhere for any other degree or diploma. I am fully responsible for the contents of my M. Sc Thesis.



(Signature of the Scholar)
Name: Utkarsha Srivastava
Department of Biotechnology and Bioinformatics
Jaypee University of Information Technology, Waknaghat, Solan, Himachal Pradesh, India
Date: 16/05/2021

Certificate

This is to certify that the work reported in the M. Sc Thesis entitled "**Computational analysis for the regulation of podophyllotoxin biosynthesis pathway in *Podophyllum hexandrum* Royale and *Podophyllum peltatum***", submitted by **Utkarsha Srivastava** at **Jaypee University of Information Technology, Waknaghat**, Solan, Himachal Pradesh, India, is a bonafide record of her original work carried out under my supervision during the period from August, 2020 to January, 2021. This work has not been submitted elsewhere for any other degree or diploma.

(Signature of the Supervisor)
Dr. Hemant Sood
Associate Professor
Department of Biotechnology and Bioinformatics
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Waknaghat, Solan, Himachal Pradesh
India

Date: 18/05/2021

केन्द्रीय औषधीय एवं सगंध पौधा संस्थान
CENTRAL INSTITUTE OF MEDICINAL AND AROMATIC PLANTS
Human Resource Development Programme

CERTIFICATE

This certificate is issued to the candidate on successful completion of the M. Sc dissertation work by ***Central Institute of Medicinal and Aromatic Plants, Lucknow.***

Name of the Candidate	: **UTKARSHA SRIVASTAVA**
Institution	: **Jaypee University of Information Technology, Waknaghat, Solan, Himanchal Pradesh**
Category of Training	: **GRADUATE TRAINING**
Training Department	: **Bio-Prospection Product Development [BPD]**
Details of Training	: ***"Scientific* validation of medicinal and aromatic plants as per AYUSH Guidelines".**
Duration of Training	: **Three Months**

The candidate has fulfilled all prescribed requirements of the laboratory work, library consultation and attended the laboratory from **15 February to 14 May 2021.**

The institute wishes the candidate success in her future endeavors.

Supervisor

07/6/21

Dr DN Mani
Senior Principal Scientist
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Acknowledgment

I am extremely privileged to have got this extended support and guidance along the completion of my project. All that I have done is only due to such supervision and assistance that I cannot forget to thank them.

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Chapter I: Computational analysis for the regulation of podophyllotoxin biosynthesis pathway in Podophyllum hexandrum Royale and Podophyllum peltatum

Abstract

Podophyllotoxin is a medicinally important and commercially valued aryltetralignans obtained as a secondary metabolite form the *Podophyllum* species. Due to its high medicinal value, this plant species has been over-exploited extremely and is now enlisted as an endangered species. There is, hence, a need to explore different plant species as potential sources for podophyllotoxin production, the major hurdle being the lack of definite knowledge on the biosynthesis pathway of podophyllotoxin. The current study aims to perform a computational analysis of the pathway and provide an insight on the interconnected networks to understand the pathways governing the biosynthesis of podophyllotoxin. Three important enzymes, secoisolariciresinol dehydrogenase, (rhizome) dirigent protein oxidase and pluviatolide synthase, have been identified for their crucial role in regulating respective biosynthesis pathways independently and therefore studied to understand their role in podophyllotoxin biosynthesis. This study also identifies some other plant species that can be potential substitutes for podophyllotoxin production.

Keywords: Podophyllotoxin, secoisolariciresinol dehydrogenase, dirigent protein oxidase, pluviatolide synthase, *P. hexandrum, P. peltatum*

1.1 Introduction

The plants act as model organisms in the research of transposable elements in heterochromatin and epigenetic control. The current technological advances in the field of plant biology or biotechnology have taken the plant related researches to the next level. The developments of high throughput sequencing techniques have opened up a new world of plant bioinformatics. These advances have given an opportunity to study the plant genetic materials at a molecular level. After the introduction of plant genomic approaches there has been a rapid increase in the genome sequences of various plant species. Plant bioinformatics and genomic researches have made a huge impact on the improvement of economically important plants and the knowledge of plant biology.

The current research focuses on the utilization of computational data for the study of secondary plant metabolite, podophyllotoxin, by utilizing the plant bioinformatics approach. Since there is not much information available on the genomic data about the production of podophyllotoxin by *Podophyllum hexandrum* and *Podophyllum peltatum*, this report might be able give an insight and a better understanding of the metabolite production at the molecular level besides indicating some closely related species by phylogenetic analysis which can be used for the commercial production of podophyllotoxin.

Podophyllotoxin is a medicinally important secondary metabolite obtained from *P. hexandrum* and *P. peltatum* which occurs extensively in the roots and the rhizome of the plant species. The rhizome of the plant contains a resin which is commercially referred to as Indian Podophyllum Resin and is processed to obtain podophyllotoxin or a neurotoxic compound podophyllin. It is the most active naturally occurring cytotoxic product, hence, is used for the preparation of its semi-synthetic derivatives for the treatment of cancer. However, its production depends on a number of factors such as soil pH, rainfall, temperature, humidity, etc. [1]. It has been observed that *P. hexandrum* has more concentration of podophyllotoxin as compared to *P. peltatum* [2]. However, later different compounds have been isolated from podophyllin from both the plants [3].

The chemical structure of podophyllotoxin contains phenylpropane units which are coupled together by β-carbons in their side chain. This belongs to the family of aryltetralignans. The synthesis of this compound involves a complex cycle interlinked with the synthesis of other major secondary metabolites including hinokinin and yatein. The major anti-cancer drugs

obtained from this toxin are etoposide and teniposide. Etoposide is the starting material in the production of drug Vepeside which has been approved by FDA for the treatment of testicular cancer as well as lung cancer. It works by inhibiting replication of cancer cells by preventing the assembly of the microtubule. It is also reported to have certain antiviral activities by interfering with certain vital viral processes [4]. Podophyllotoxin has also shown promising results against a set of human cancer cell lines HL-60, A-549, HeLa, and HCT-8 and also activates the pro-apoptotic endoplasmic reticulum stress signalling pathway [5].

Apart from being an important anti-cancer compound, podophyllotoxin is also found to protect against damages due to radioactivity [6-8]. The enlisting of podophyllotoxin in the World Health Organization's List of Essential Medicines clearly indicates the multi-varied applications and extensive use of this important compound [9]. Due to lack of molecular level information about the synthesis of podophyllotoxin, the utilization of bioreactors for its production has not been evaluated properly.

Many proposed pathways involve the production of the podophyllotoxin via enzyme-controlled reactions that occur during the phenolic oxidative coupling of C6-C3 monomers in the Shikimic Acid pathway [10]. Although complete and conclusive knowledge on this pathway is still not available and research is on to incur more information about the genes and transcription factors that may be involved in the regulation of this pathway, extensive literature survey revealed that substantial linkage can be conclusively determined in the phenylpropanoid pathway [11] and the biosynthesis pathway for podophyllotoxin production [12].

Various other secondary metabolites as hinokinin and yatein are also obtained from the phenylpropanoid pathway and are the interlinks between the phenylpropanoid pathway and podophyllotoxin biosynthesis pathway. The current work is to analyse the role of some major enzymes regulating the biosynthesis of these interlinking compounds, thereby controlling the podophyllotoxin biosynthesis mechanism. This study also reveals some other plant species that are phylogenetically related to *Podophyllum* family with respect to our enzymes of interest, thereby suggesting that these plants may be used as alternative sources for podophyllotoxin production, which would help reduce the burden on *Podophyllum* and may even provide it enough time to regenerate and recover from the massive stress of endangerment.

1.2 Review of literature

1.2.1 Podophyllum species and application of podophyllotoxin

Podophyllum is also known as Mayapple and has three species majorly, namely, *P.hexandrum*, *P.peltatum* and *P.sikkimensis*. *P. hexandrum* is a green herbaceous plant which grows in the Himalayan regions of Asian sub-continent, and is commonly known as the Indian Mayapple. This species has a very peculiar environmental requirement due to which it is mainly found in the temperate and subalpine regions having well drained humus rich soil conditions such as Jammu and Kashmir, Uttarakhand and some areas of North-Eastern India. This plant belongs to family Berberidacea and is listed under the order Ranunculales. Podophyllotoxin is a medicinal metabolite obtained from *P. hexandrum* and *P. peltatum* which occurs extensively in the roots and the rhizome of the plant species, which is commercially referred to as Indian Podophyllum Resin and is processed to obtain podophyllotoxin or a neurotoxic compound podophyllin. It is the most active naturally occurring cytotoxic product, hence is used for the preparation of its semi-synthetic derivatives for the treatment of cancer (Figure1.1). However, its production depends on a number of factors such as soil pH, rainfall, temperature, humidity, etc. [13]. It has been observed that *P.hexandrum* has more concentration of podophyllotoxin as compared to *P. peltatum*. However, different compounds have been isolated from podophyllin from both the plants.[14].

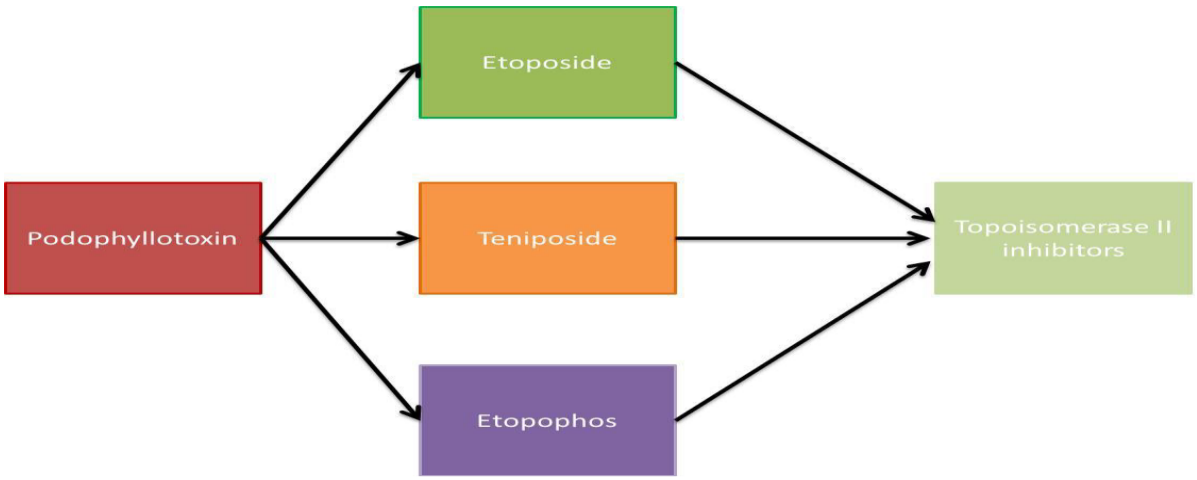


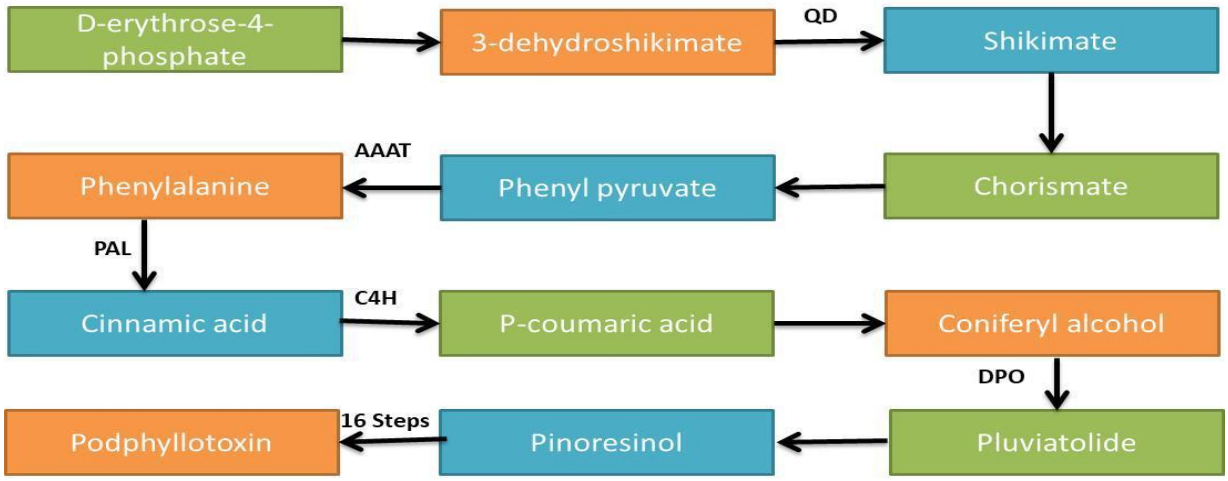
Figure 1.1: Mechanism of action of compounds obtained from podophyllotoxin

1.2.2 Biosynthesis of podophyllotoxin

Being an aryl-tetra lignan, the biosynthesis of podophyllotoxin is a 33-step process which starts with the phenylpropanoid pathway, from which it later diversifies into the synthesis of coniferyl alcohol which is the main precursor of podophyllotoxin [15]. The major difficulty in understanding and determining the podophyllotoxin biosynthesis genes arises due to the scattering of the genes governing the extensive secondary metabolite pathway [16].

Phenylalanine is an aromatic amino acid which is the precursor for the phenylpropanoid pathway (PPP). Figure 1.2 gives an overview of this pathway. The process includes the deamination of phenylalanine to cinnamic acid by phenylalanine ammonia lyase. The product cycles through the PPP and enters the monolignol branch. Important monolignols, namely cinnamyl alcohols, are obtained by two enzymatic reduction steps catalysed independently by cinnamyl-CoA-NADP oxidoreductase and cinnamyl alcohol dehydrogenase. Of the two enzymes, the first one catalyses a reduction reaction while the latter a dehydrogenation reaction. Dirigent mediated coupling of two molecules of coniferyl alcohol produces pinoresinol, which is the precursor of the lignans podophyllotoxin (PTOX) and other lignans as well [17].

A detailed procedure for the synthesis of podophyllotoxin includes the conversion of coniferyl alcohol to pinoresinol catalysed by enzyme coniferyl alcohol dehydrogenase which is mediated by dirigent associated stereoselective coupling [18-23]. The pinoresinol is further reduced to secoisolariciresinol by enzyme pinoresinol-lariciresinol reductase coupled with the utilization of NADPH [24-27]. Secoisolariciresinol dehydrogenase then converts it to matairesinol which is the linking compound for hinokinin and yatein biosynthesis and further on to podophyllotoxin biosynthesis [28]. Figure 1.3 gives an overview of the podophyllotoxin biosynthesis and the reactions catalysed by the three major enzymes that may be considered to play a role in its regulation.



QD: Quinate dehydrogenase; AAAT: Aromatic amino acid transaminase;
PAL: Phenylalanine ammonia lyase; C4H: Cinnamate-4-hydroxylase; and
DPO: Dirigent protein oxidase

Figure 1.2: Biosynthetic pathway for the production of podophyllotoxin

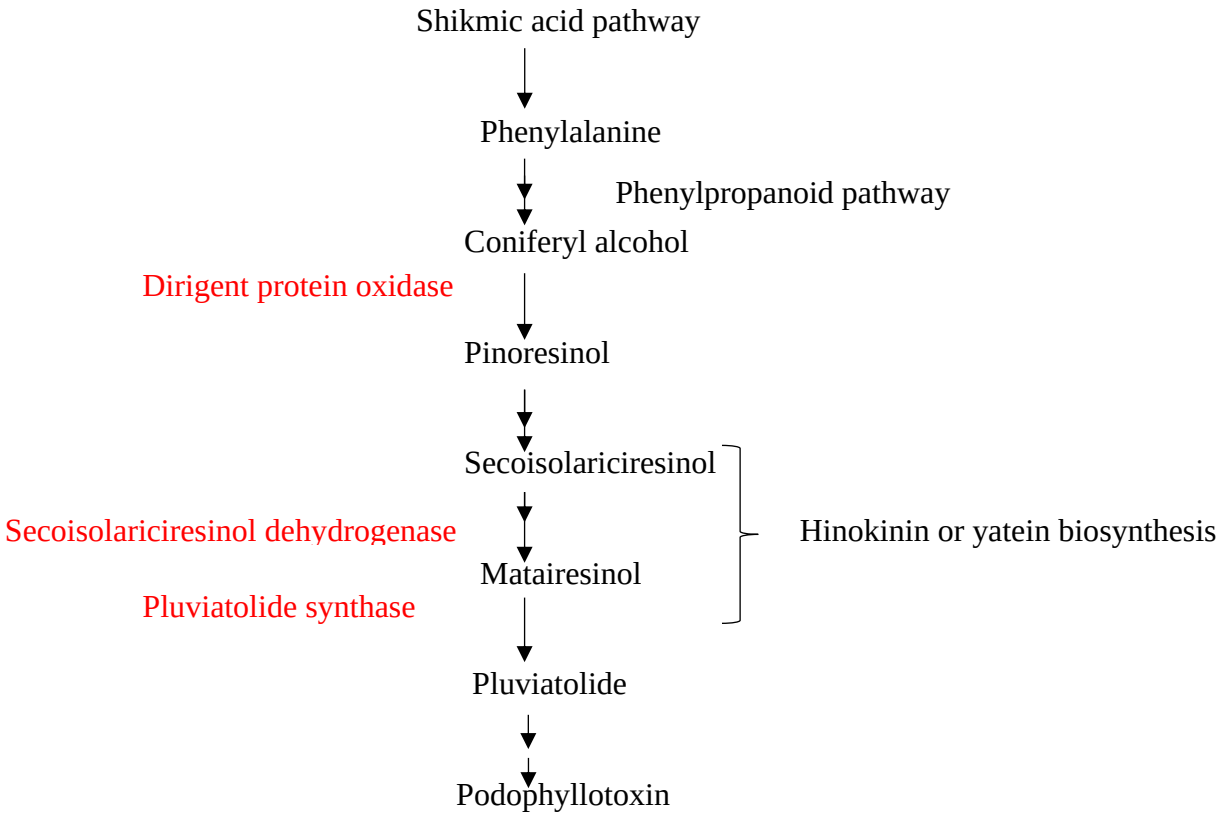


Figure 1.3: A schematic flowchart for the biosynthesis of podophyllotoxin and the intermediate pathways involved in the process. The text in red are our enzymes of interest governing the different steps in the biosynthesis of podophyllotoxin

The BLASTp analysis of secoisolariciresinol dehydrogenase from *P.peltatum*, four variants of the amino acid sequence were obtained. It also revealed that the gene sequence of secoisolariciresinol dehydrogenase also codes for the enzyme that catalyses the formation of matairesinol from secoisolariciresinol in a dehydrogenation reaction. A study by Davin et al revealed dirigent protein oxidase as a unique protein responsible for the enantioselective formation of pinoresinol [29]. The contribution of dirigent protein oxidase is crucial in the formation of (-)-matairesinol since it mediates a stereoselective coupling of two achiral molecules which yield E-coniferyl alcohol followed by (-)-pinoresinol. This biochemical pathway is found not only in *Podophyllum* but also various other plant species [30-33]. Dirigent protein represents a new class of proteins found to be pre-dominantly present in gymnosperms [34], angiosperms that flower [35] and also ferns. Their functions seem to have evolved during the evolution of land plants [36] which can also be attributed to the fact that it requires post-translational modification for appropriate functioning [37].

A study on the structure of secoisolariciresinol dehydrogenase has shown that it is exists as tetramer in both crystal lattice and solution. It is structurally composed of α/β monomers containing seven parallel β strands that are flanked on each side by eight α helices. It is also found to possess a catalytic triad of high efficiency which includes Ser^{153}, Tyr^{167} and Lys^{171}. This triad plays a crucial role in the catalytic conversion of (-)-secoisolariciresinol to (-)-matairesinol which is a central intermediate connecting the biosynthesis of various other lignans including hinokinin, yatein and most importantly podophyllotoxin. An analysis of two enzymes of family cytochrome P450, CYP719A23 and CYP719A24, obtained from *P.hexandrum* and *P.peltatum* respectively, revealed that they can enzymatically convert matairesinol to pluviatolide by catalysing the formation of methylenedioxy bridges. This recent discovery has led to the development of pathway from the conversion of matairesinol to pluviatolide up to the biosynthesis of (-)-4'desmethylepipodophyllotoxin although the formation of podophyllotoxin from this step onwards still remains undiscovered [38-39]. The analysis and study of gene involved in conversion of matairesinol to pluviatolide, CYP719A in-silico, has given some insight into the intermediate steps for podophyllotoxin biosynthesis [40]. A recent study has also identified six different enzymes for the biosynthesis of di-methylepipodophyllotoxin which have proved helpful for the production of podophyllotoxin based drugs [41-42].

Not many natural sources are available for obtaining podophyllotoxin, but of those present the rhizomes of *Podophyllum* species [43] form the major source for the procurement of this

important lignan. Due to extensive demand coupled with the slow growth rate of this important plant [47-50], this species is now endangered, leading to the exploration of other approaches for the chemical synthesis and in-vitro production of this important compound [51-56]. But these methods have been unable to adequately supply to fulfil the demand of podophyllotoxin on commercial level. Therefore, there is an urgent requirement to study the detailed pathway for the biosynthesis of podophyllotoxin and also identify alternative sources for the procurement of the same from species that can substantially be utilised for culturing in-vitro and for production on a commercial scale.

Considering the importance of transcription factors and their role in upgrading the industrial synthesis of podophyllotoxin, the present study worked to identify different enzymes that might be involved in regulating the pathway of podophyllotoxin biosynthesis [57]. The research was conducted in order to identify the Transcription families (TFs) involved in the regulation of podophyllotoxin production. This was done with the help of extensive transcriptome mining. After extensive literature survey, it was observed that four families of TFs (Table 1.1) regulated the phenylpropanoid pathway.

Table 1.1: Transcription families involved in the regulation of phenylpropanoid pathway

Synonyms	**Transcription families**
MYB	Myeloblastosis
bZIP	Basic Leucine Zipper
bHLH	Basic Helix-Loop-Helix
WRKY	NA

Later it was identified that bZIP and MYB were found in the rhizome of P. hexandrum and helped in the production of podophyllotoxin. After quantification and comparative analysis of high and low content of podophyllotoxin accessions, the results showed 0.04 to ~ 16-fold increases in the transcripts of the transcription factors, further supporting the involvement of the identified TFs with the content of podophyllotoxin. The in-silico studies of the promoter region of genes involved in this pathway demonstrated the presences of sequences from MYB and WRKY, thereby suggesting their role in regulating the production of podophyllotoxin.

1.2.3 **Bioinformatics** Approach

1.2.3.1 Next Generation Sequencing

NGS technologies are advancing at a rapid rate owing to the increase in data efficiency and throughput [58]. The most important advantage of NGS and similar technologies is that new genes can be identified in a lesser time frame and thus helps in rapid understanding of the plant metabolism as compared to the traditional approaches like gene cloning labelled precursor administration, enzyme purification and characterization, potential intermediate identification, expressed sequence tag (EST) libraries, etc. A study conducted in 2012 applied NGS for the prediction of gene function in podophyllotoxin biosynthesis and they showed promising results. These stated the *Podophyllum* group has a reduced or low level of alkaloid biosynthesis and also depicted the presence of some of its genes for the podophyllotoxin pathway. Figure 1.4 gives a brief overview of the applications of NGS in plant breeding and genetics.

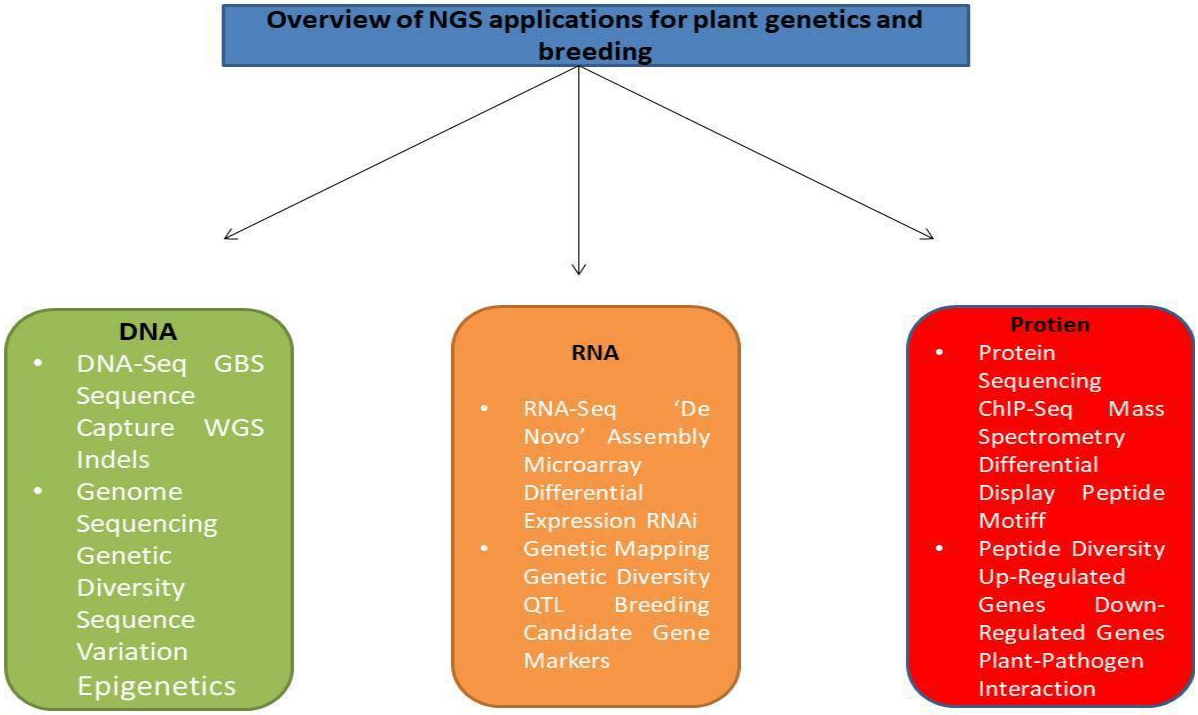


Figure 1.4: Next Generation Sequencing

1.2.3.2 Plant Bioinformatics Databases

i) National Centre for Biotechnology Information (NCBI)

National Centre for Biotechnology Information (NCBI) was established in 1988 by Claude Pepper, as a division of the National Library of Medicine (NLM) at the National Institutes of Health (NIH). It acts as a resource for information on molecular biology and helps to develop new technologies that aid in the better understanding of basic molecular as well as genetic process that are involved in health and diseases. NCBI is a database that stores and analyses the information on various biological fields such as biochemistry, molecular biology and genetics (Figure 1.5). It helps researchers to gather information and also to analyse the structure and function of various important molecules [59].

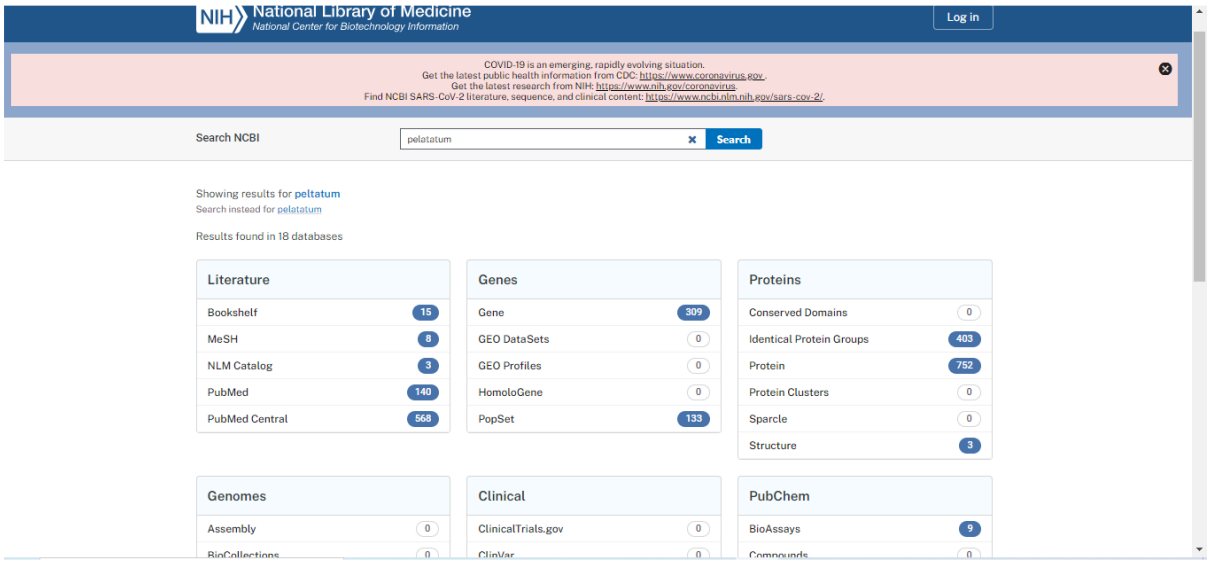


Figure 1.5*:* NCBI Database

ii) MedPITranscriptome

Also known as Europe PMC (Figure 1.6) was developed by EMBL-EBIis a partner of PubMed Central which includes resources from PubMed and PubMed Central (PMC), NCBI. It is also a part of the PMC International (PMCI) network repositories that also

includes PMC Canada. This database helps the researchers to access the publications related to the life sciences [60].

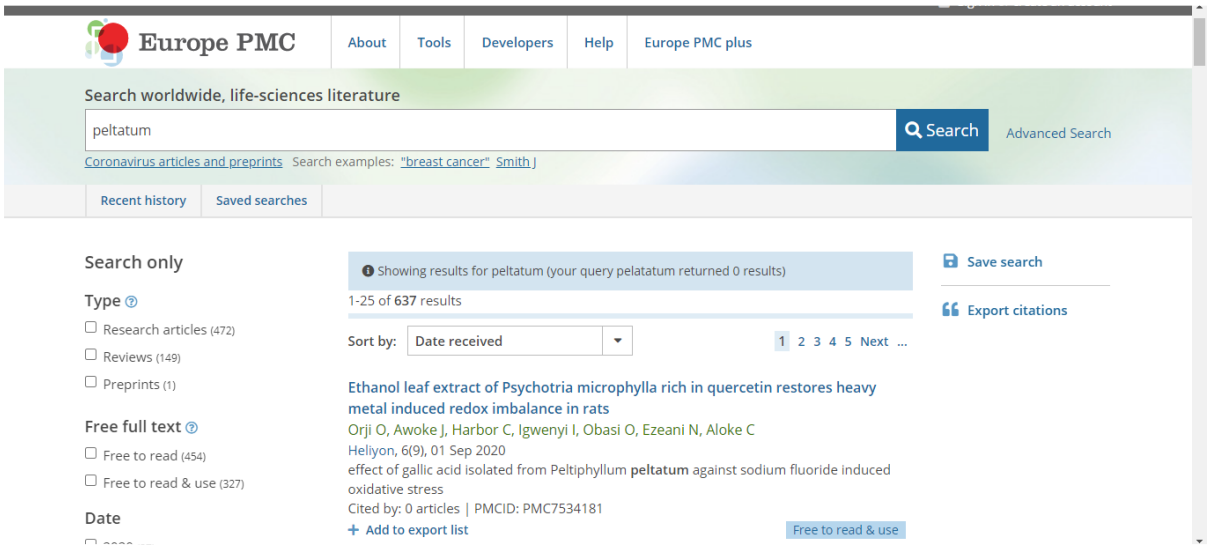


Figure 1.6: Europe PMC

iii) MetaCyc

MetaCyc is a curated database of 2847 pathways found 3161 different organisms. MetaCyc stores the information about pathways involved in both primary and secondary metabolism and their associated metabolites, reactions, enzymes, and genes. This database helps the researchers by the representative sample of each experimentally elucidated pathway [61]. Figure 1.7 gives an overview of applications of MetaCyc.

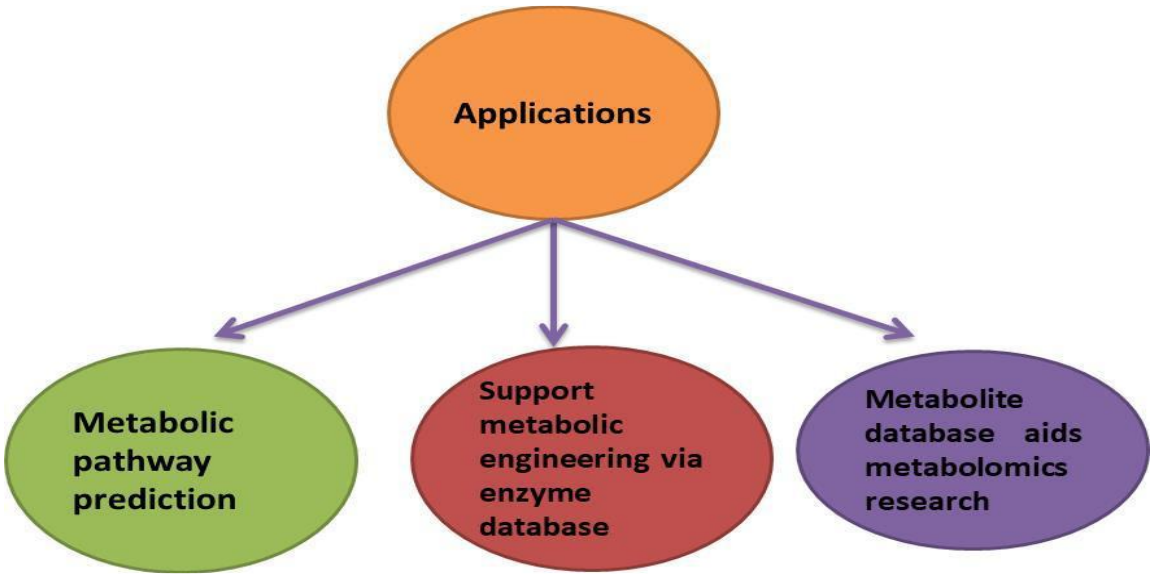


Figure 1.7: Applications of MetaCyc Database

iv) BioCyc Database

BioCyc is a database that stores information of more than 18,000 Pathway/Genome Databases (PGDBs), plus software tools for exploring them [62]. Figure 1.8 enlists various features of this databse.

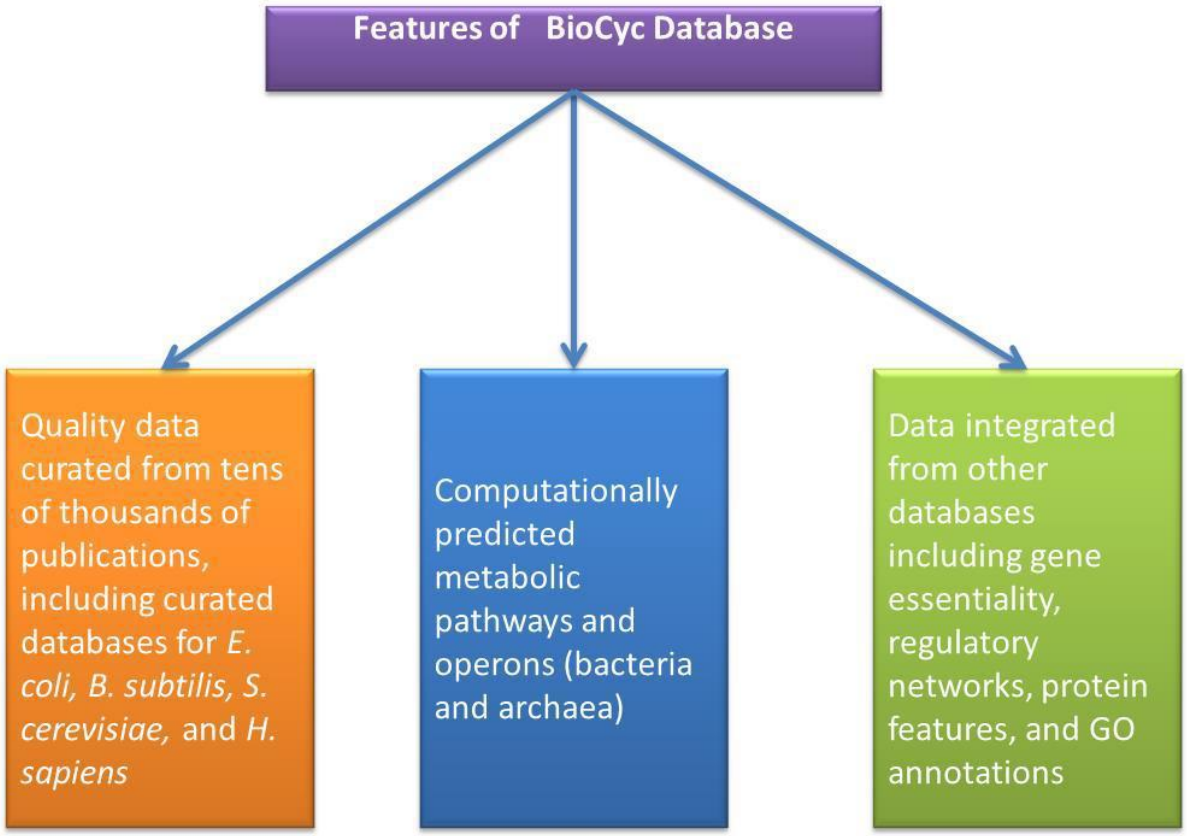


Figure 1.8: Key aspects of BioCyc Database

1.3 Aim and Objectives

The aim of the current study is to perform **Molecular analysis for phytochemical screening of medicinal significance of *Podophyllum hexandrum* Royale and *Podophyllum peltatum*** using bioinformatics approach.

The objectives to be achieved during the course of this study are-

- To perform computational analysis of podophyllotoxin biosynthesis pathway of *P.hexandrum* and *P.peltatum*.
- To perform comparative study for the yield of podophyllotoxin from both the plant species.
- To screen for the medicinally significant metabolites, other than podophyllotoxin in *P.hexandrum* and *P.peltatum*.

1.4 Materials and Methods

1. NCBI: Known as the National Centre for Biotechnology Information, is a part of the National Library of Medicine under the National Institute of Health. It is a globally recognized platform containing various resources and databases including several tools for the curation of information and accessing research in various domains of natural sciences and bioinformatics [63]. Data was obtained from NCBI using Nucleotide and protein databases and three nucleotides were identified from *Podophyllum peltatum* which had potential role in podophyllotoxin biosynthesis governing phenylpropanoid pathway. The proteins associated with these nucleotides were also studied.

2. UnirProtKB: The UniProt Knowledgebase is a part of the UniProt and contains information on proteins including their sequence, function and post-translational modification. It gives information on the organisms in which the protein is found and the metabolic pathway it is associated with [64].

3. BioCyc: It is a collection of databases containing pathway and genome databases specific to different organisms. It, therefore, provides well-structured information on different metabolic pathways and their related genes besides providing input about the enzymes involved in the pathways and the related pathways associated with the one in question. It is maintained by SRI International and has its office in California [65-66].

4. BLAST: The Basic Local Alignment Search Tool, known as BLAST, is used to run a comparative study of the nucleotide or protein sequence(s) under study, to obtain the details of the related sequences from same and different species. It is written in C and C++ language and is critical in bioinformatics studies [67].

An overview of the procedure followed for the current study is depicted in the flowchart of Figure 1.9.

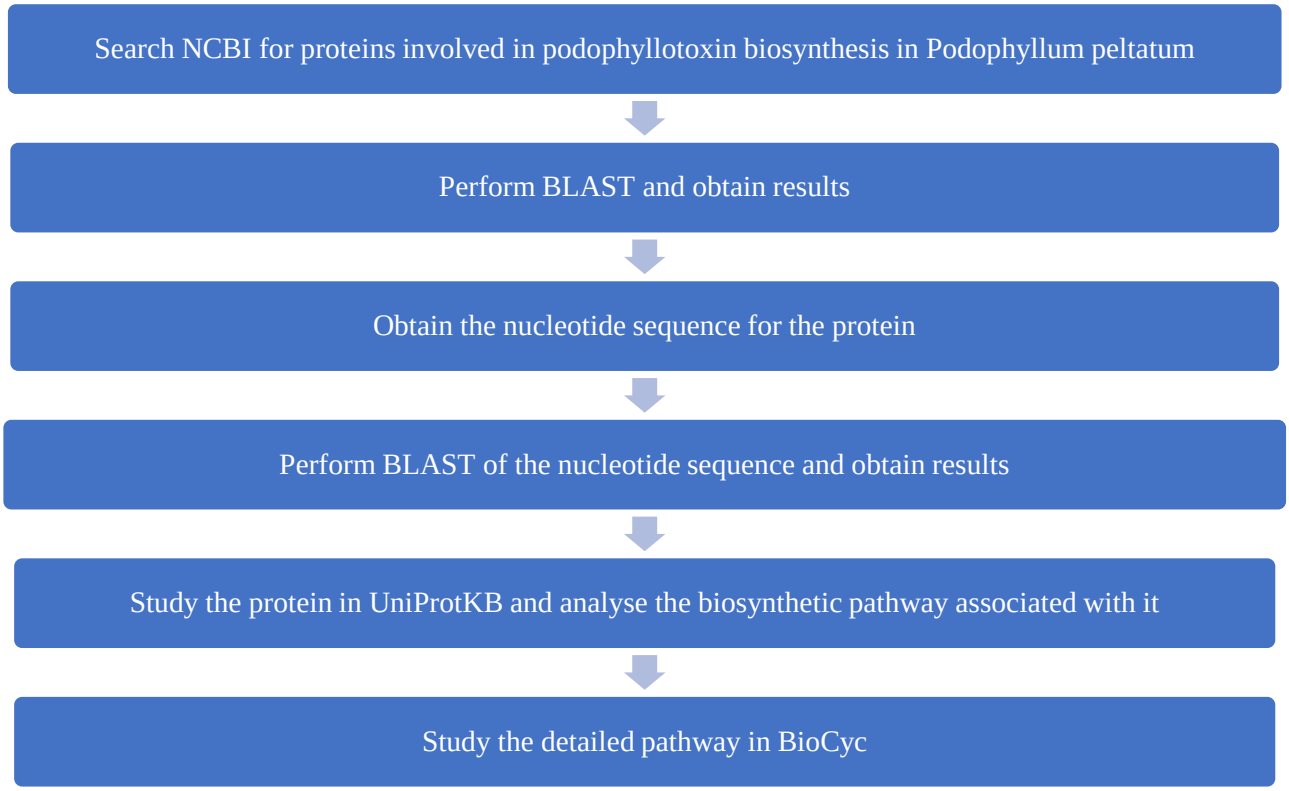


Figure 1.9: Flowchart of the methodology followed

NCBI was searched against the protein database to identify for the key factors involved in podophyllotoxin producing phenylpropanoid pathway in *Podophyllum peltatum* and *Podophyllum hexandrum* [68]. Three protein sequences were identified.

The three enzymes identified to have role in podophyllotoxin biosynthesis pathway:

1. Secoisolariciresinol dehydrogenase [69]
2. (rhizome) Dirigent protein oxidase [70]
3. Pluviatolide synthase

Upon further analysis of these proteins, individually, BLAST (Blastp) was performed. The Blast results were studied further to obtain distance tree results for all the similar sequences and graphics for results from *Podophyllum hexandrum* and *Podophyllum peltatum*.

The nucleotide sequences of these proteins were obtained from NCBI against the nucleotide database identified. BLASTn was performed against the three nucleotide sequences so identified and the

results were analysed to obtain distance tree for all the related sequences and results as graphics for similar sequences obtained from *Podophyllum hexandrum* and *Podophyllum peltatum* as source species.

The proteins were studied separately in UniProtKB database to obtain more detailed information about the same. The metabolic pathways associated with these proteins were also enlisted in the data from UniProtKB. The results of UniProtKB also highlighted the cellular occurrence of the protein and the organisms from which it can be obtained besides giving an insight on the Post Translational Modifications of the protein.

The BioCyc database was simultaneously searched for the metabolic pathway identified from the UniProtKB database. The results from BioCyc gave the details on the metabolic pathways associated with our compound of interest. The results obtained were narrowed down to select for our organisms of interest, which were *Podophyllum hexandrum* and *Podophyllum peltatum*. The data obtained from the analysis was put together to identify a metabolic pathway network interconnected via various other networks, including phenylpropanoid biosynthesis pathway, matairesinol pathway, hinokinin pathway and yatein pathway, to develop a podophyllotoxin biosynthesis pathway.

1.5 Results

Table 1.2 : Nucleotide and protein sequence ID numbers corresponding to our enzymes of interest obtained from NCBI

Enzyme Name	Nucleotide (GenBank ID)		Protein ID	
	P.peltatum	***P.hexandrum***	***P.peltatum***	***P.hexandrum***
(rhizome) dirigent protein oxidase	AF352736.1	KJ595571.1	AAK38666.1	AIA24213.1
Seccoisolariciresinol dehydrogenase	KR779861.1	EF205022.1	ALD51317.1	ABN14311.1
Pluviatolide synthase CYP719A2 (3)/(4)	KC110998.1	KC110997.1	AGC29954.1	AGC29953.1

The GeneBank and Protein ID for the selected enzymes for both *P.hexandrum* and *P.peltatum* was obtained from NCBI (Table 1.2). BLAST analysis for the individual enzymes for both its protein and nucleotide sequences gave results used as form of phylogenetic tress as shown in Figures. 1.11, 1.12, 1.14, 1.15, 1.20 and 1.21. The concerned reactions governed by these enzymes and the biosynthesis pathways for different secondary metabolites obtained from these reactions were also obtained from BioCyc and MetaCyc databases as shown in Figures. 1.10, 1.13, 1.16, 1.17, 1.18 and 1.19.

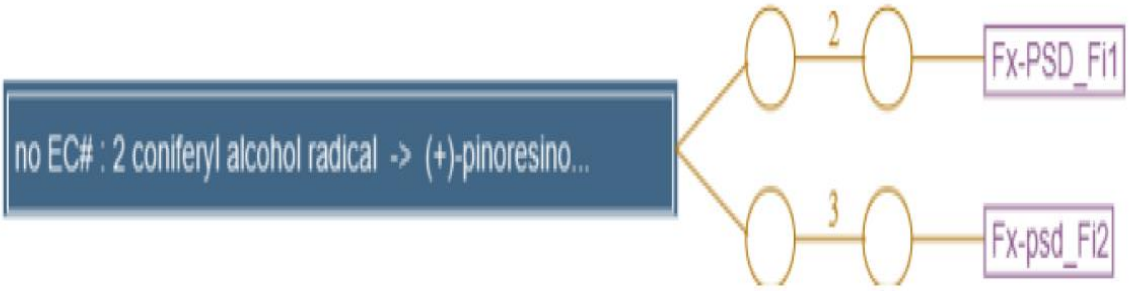


Figure 1.10: Schematic gene reaction for enzyme dirigent protein

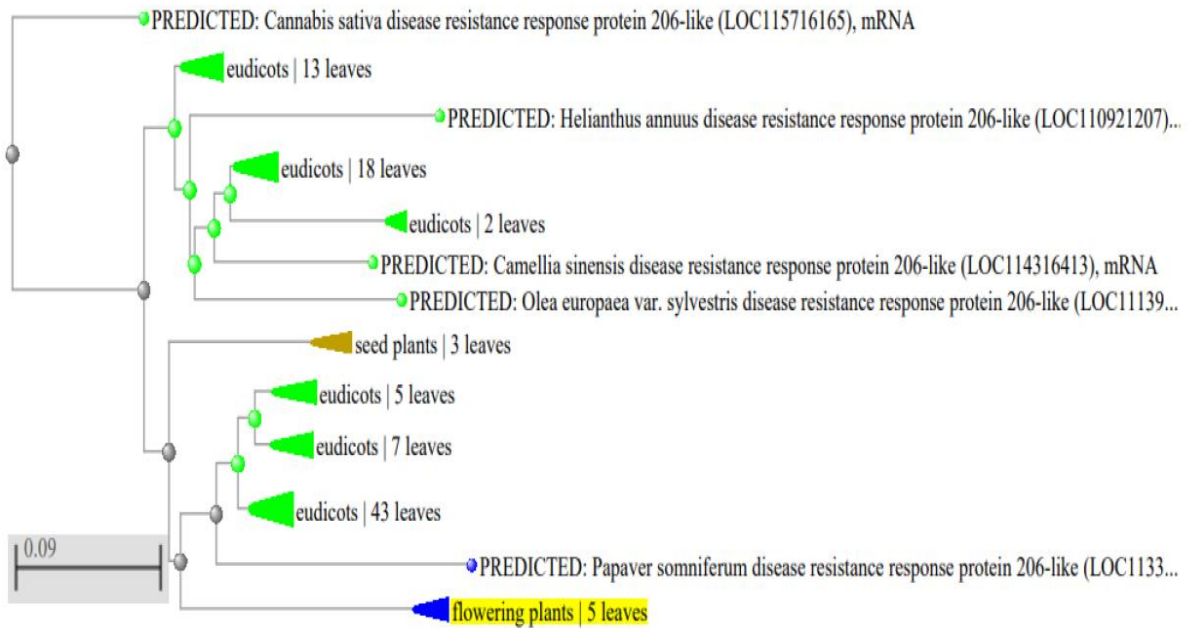


Figure 1.11: Distance tree obtained for nucleotide BLAST of nucleotide of ID AF352736.1

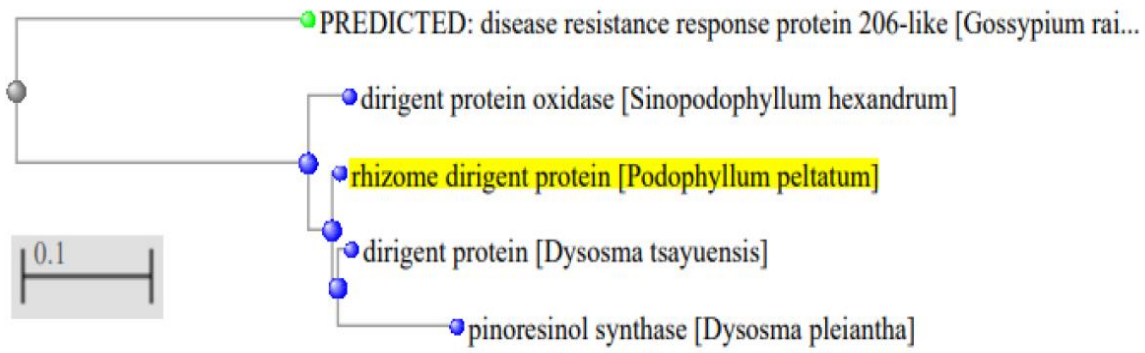


Figure 1.12 Distance tree obtained for protein BLAST of protein AAK38666.1

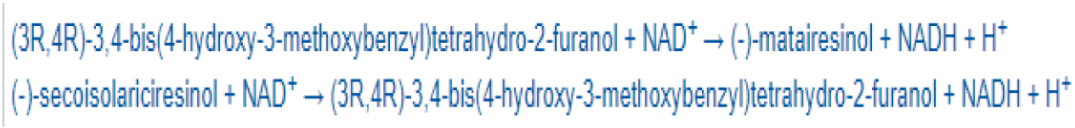


Figure 1.13: Reaction catalysed by secoisolariciresinol dehydrogenase in *P.peltatum*

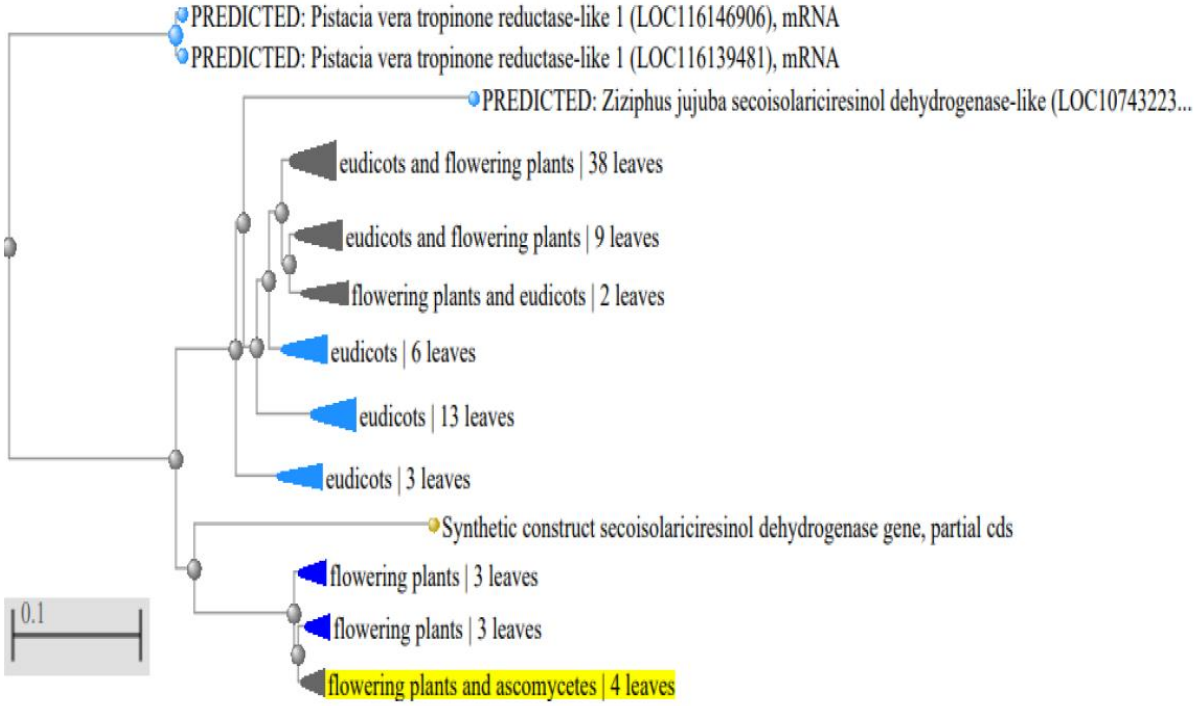


Figure 1.14: Distance tree obtained for nucleotide BLAST of nucleotide ID KR779861.1

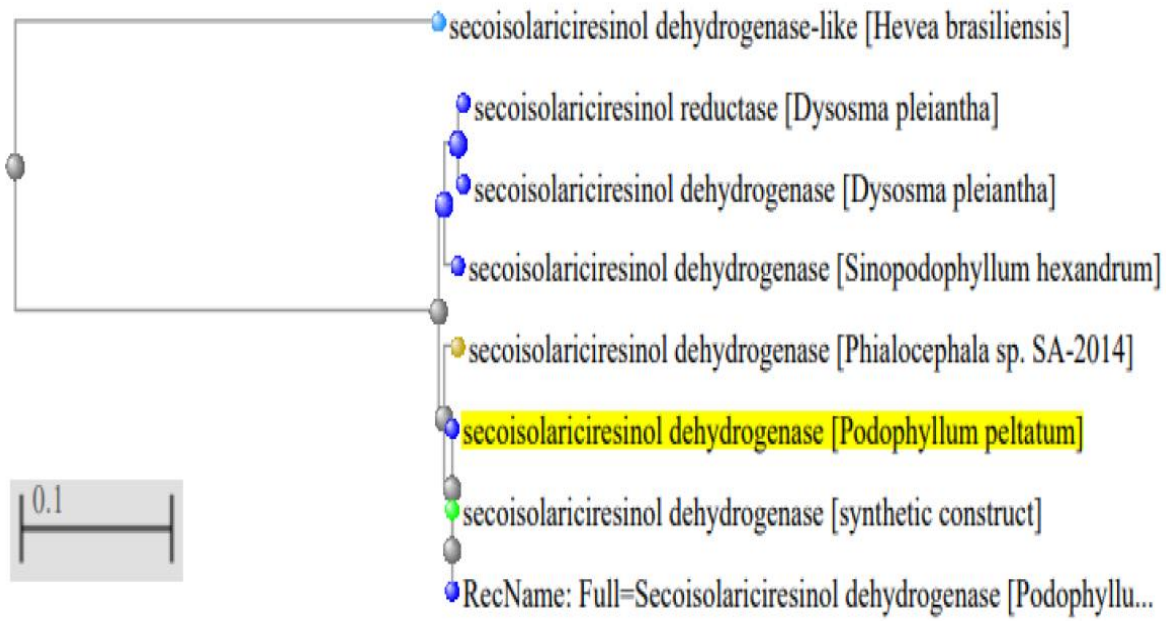


Figure 1.15: Distance tree obtained for protein BLAST of ALD51317.1

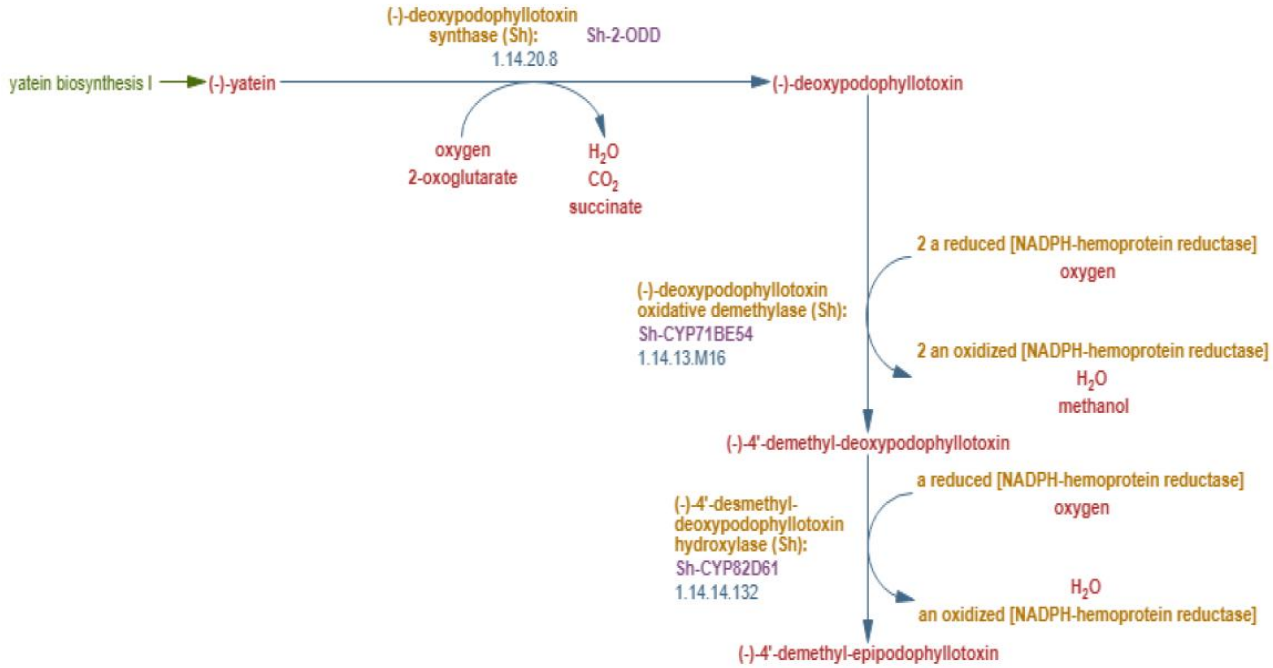


Figure 16: Pathway for (-)-4'-demethyl-epipodophyllotoxin

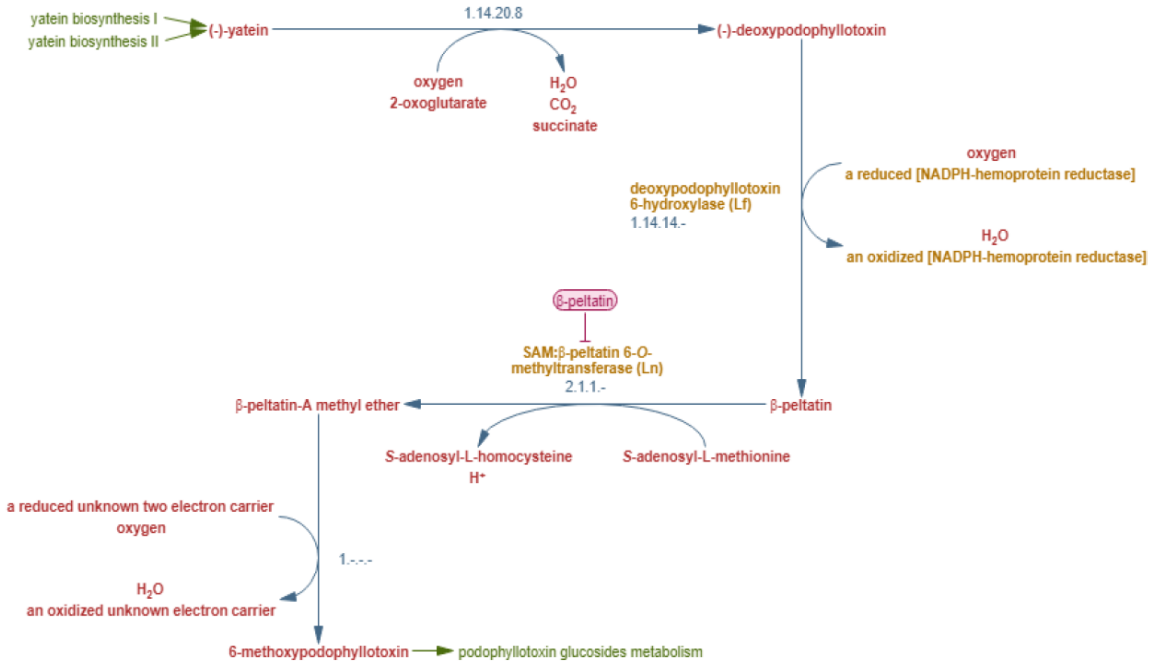


Figure 1.17: Pathway for 6-methylpodophyllotoxin

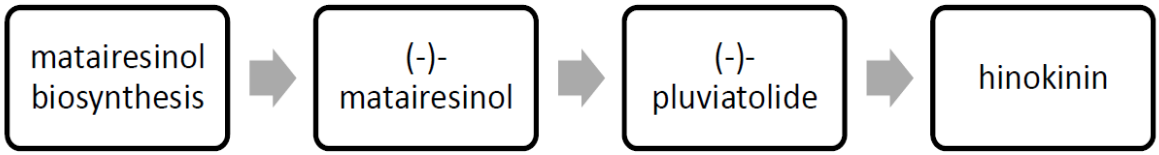


Figure 18: Matairesinol pathway linked to hinokinin biosynthesis involving pluviatolide synthase

Figure 1.19: Schematic gene reaction for enzyme pluviatolide synthase

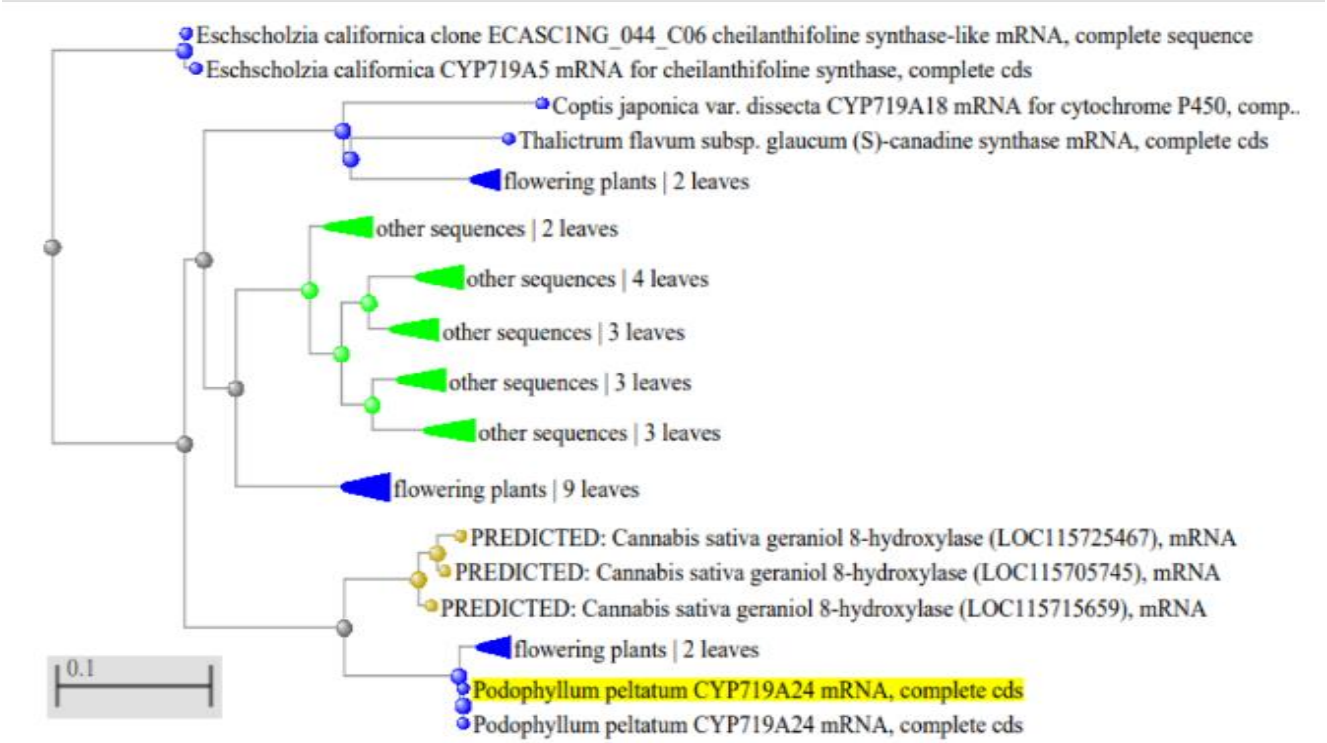


Figure 1.20: Distance tree obtained for nucleotide BLAST of ID KC110998.1

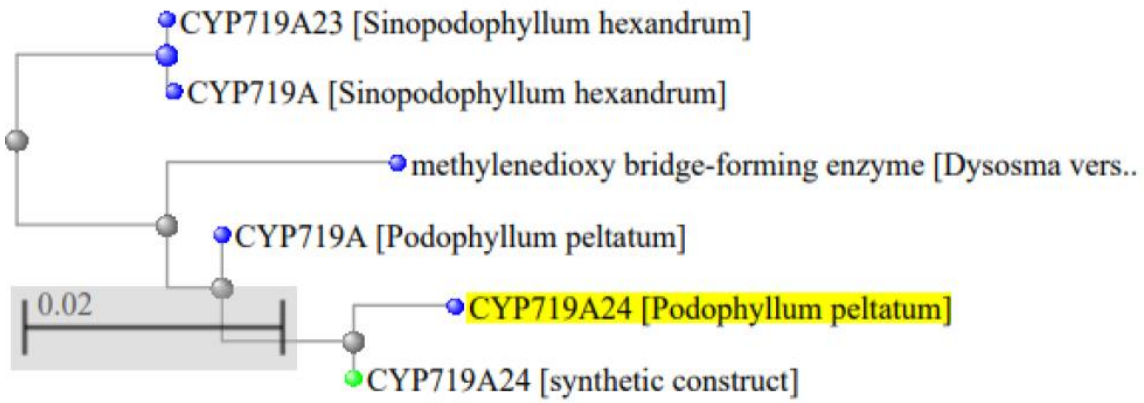


Figure 1.21: Distance tree obtained for protein BLAST of ID AGC29954.1

1.6 Discussion

BLAST analysis of the nucleotides and protein sequences of these three enzymes provided an insight into the phylogenetic relationship between *P.hexandrum* and *P.peltatum*. The distance tree results revealed that, although, *Podophyllum hexandrum* and *Podophyllum peltatum* belonged to the same genus and therefore the same family of plant species, Berberidacea, they are not highly similar in their nucleotide sequences for the enzymes in question. This dissimilarity can be identified as a possible reason for the difference in the yields of podophyllotoxin with higher yields being obtained from *P.hexandrum* compared to *P.peltatum* [71].

Further examination, revealed an interconnecting network of pathways that were involved in the production of different secondary metabolites in the plant species, as matairesinol, hinokinin, yatein, being the important ones here. In the presence of dirigent protein oxidase E-coniferyl alcohol is converted to pinoresinol which is then reduced to (+)-lariciresinol and further to secoisolariciresinol. Secoisolariciresinol is dehydrogenated to yield matairesinol. Matairesinol is used for the production of important compounds as epipodophyllotoxin, β-peltatin,4'-demethylpodophyllotoxin and α-peltatin in *Podophyllum* [72], establishing that matairesinol is a connecting compound which is a common precursor for both the persistent groups of lignans in *Podophyllum* species [73]. Matairesinol is converted to yatein and its derivatives as 4’-demethylyatein, which is then converted to podophyllotoxin and its derivatives.

The detailed metabolic biosynthesis pathway was then developed as given in figure 1.22.

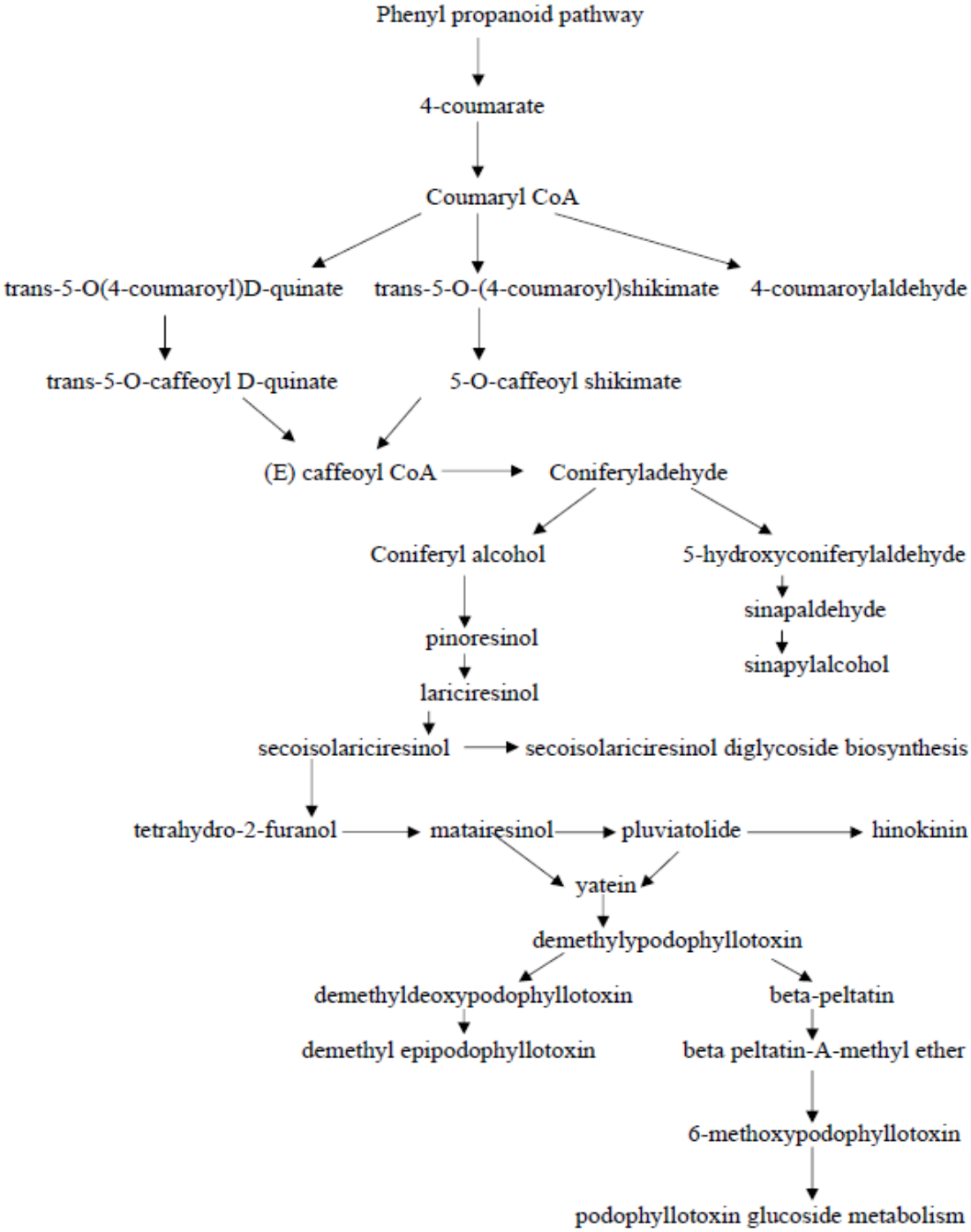


Figure 1.22: The interlinking pathway for podophyllotoxin biosynthesis as developed after analysis of the metabolic pathways governed by the three enzymes of interest

This detailed pathway brought to light the different substrates that are precursors for the biosynthesis of different secondary metabolites but are essentially required for podophyllotoxin biosynthesis as well. The biosynthetic pathway for the production of podophyllotoxin, therefore, cannot be studied independently and must be studied via these

interlinking networks to obtain better insight into the pathway for the production of this important lignan.

The distance tree results also helped us to identify the other plant species that can potentially be used for the production of podophyllotoxin. The *Dysosma* and *Linum* species were found to be close relatives of *Podophyllum* in evolutionary context and possessed nearly similar sequences for the enzymes under investigation. The *Dysosma* species was particularly closely related and should be analysed further as being a potential sustainable source of podophyllotoxin. Other secondary metabolites including matairesinol, hinokinin [74] and thujaplicatin were also found to be of significant medicinal importance and can be studied further in association with podophyllotoxin biosynthesis. These metabolites are found to possess anti-oxidant and anti-radical properties thereby making them a suitable candidate as natural nutraceutical [75].

1.7 Conclusion

The biosynthesis pathway for podophyllotoxin production in *P.hexandrum* and *P.peltatum* is closely related but not exactly similar and therefore needs to studied individually in detail. The two plant species might belong to the same family but are phylogenetically different which can be accounted for the difference in their yields of podophyllotoxin.

Their pathway for podophyllotoxin synthesis is extremely complex and dependant on a number of secondary metabolites that act as precursors in the reaction. These secondary metabolites have a potential significance of their own and some of them have been identified to possess medicinal properties as well.

We, therefore, suggest to study the podophyllotoxin biosynthesis pathway as an interlinked network of different secondary metabolites so as to obtain a wholesome detail on the different enzymes and transcription factors governing the same.

Also, to counter the over-exploitation of a particular species as *Podophyllum* for podophyllotoxin, we propose to explore the potentials of different plants species as *Dysosma* and *Linum* varieties, to obtain better yields and sustainable production without endangering a particular variety.

Further detailed analysis needs to be conducted to study the Transcription factors important in the regulation of this interconnected pathway and hence the podophyllotoxin biosynthesis pathway.

Chapter II: Scientific Validation of Medicinal and Aromatic Plants from CSIR-CIMAP as per AYUSH Guidelines

Abstract

With extensive and exponential advancements of modern science since the past century and mostly in the last five decades, the trust of human kind on the ancient medical system for the treatment of various diseases, has dwindled at an appalling rate. A more recent review of traditional knowledge in the light of current scientific technologies and proven principles, has revived hope in the classical medical system, for providing leads and information on potent plants and drugs for the cure of many diseases. The current study aims to standardize five well known and medicinally important aromatic plants by scientifically validating them as per the guidelines and test parameters mentioned by the Ministry of AYUSH, Government of India. This would further provide an understanding of the selected plant species. It will also help in evaluating its role in the treatment of the diseases as mentioned in Ancient literature. It would also develop a standardized procedure for the preparation of plant extract for various pharmaceutical formulations.

Keywords: AYUSH, Standardization, Cold maceration, Phytochemicals

2.1 Introduction

Plants have been a constant source for treatment of many diseases since the beginning of civilization. Even today, plants are source for various important drugs including morphine, reserpine, teniposide, etophos, and most recently paclitaxel [1-2]. With the advancement in the understanding of scientific knowledge, the plants have been used as a constant source of various medicinally important compounds and secondary metabolites [3]. Medicinal plants are very important to the health of individuals and communities. They have been studied individually to characterize for the presence of important phytochemicals that are thought to have pharmacological action on the human body [4]. The reduced availability of medicinal plants with constantly decreasing availability of the same in the market has affected the herbal market. A lack of proper post-harvest management and increased cost of handling may also contribute to decreased availability of herbal drugs. It has led to a break in the demand and supply chain, which has consequentially resulted in sale of adulterated and low-quality material [5]. Therefore, there is an extensive need to evaluate the selected raw material for its quality before using it for the preparation of various herbal products and phytomedicines [6-7].

Traditional knowledge has been documented to be used by the tribes and in different cultures globally. Therefore, the need to regulate the use of this knowledge on basis of substantial and scientific evidence is essential to ensure a proper regulated and well acknowledged system of medicine for cure of diseases [8]. Scientific validation of this knowledge and quality control of the products thus obtained, is therefore, essential. It ensures the safety of these products for the consumer besides guaranteeing its therapeutic efficacy. India has been the land of knowledge since the ancient times with scholars as Aryabhatt and Charak, whose discoveries are considered a milestone for the development of science and technology. The Indian System of Medicine includes various branches of therapeutics and treatment including Yoga, Ayurveda, Siddha, Homeopathy and Unani. Today, this knowledge is regulated and evaluated by a dedicated department under the government of India, the Ministry of AYUSH (Ayurveda, Yoga, Siddha, Unani, Homeopathy). This Ministry also regulates the education and practice of the traditional knowledge besides ensuring its appropriate usage in the development of medicines and therapeutic remedies [9].

Quality control and standardization of traditional knowledge is extremely crucial. Therefore, the World Health Organization (WHO) laid down guidelines for the same. The WHO, has

stated a set of guidelines and test parameters as part of its Good Manufacturing Practices in 1975, which also ensure the quality of the product obtained via traditional system. Many countries as India, China and the European nations, have taken into account their classical system of medicine and treatment to develop procedures and protocols for the standardization and quality specifications of the same.

The parameters set forth for the validation of this knowledge include evaluation for color, size, taste, texture, collectively known as organoleptic evaluation, test for pH, foreign matter, macroscopic and microscopic studies. The phytochemical screening is also performed to check for the qualitative and quantitative amounts of active constituents. The detailed study of chemical constituents is also done through TLC, HPLC and UPTLC. Depending on the type of raw material, the presence of volatile oils is also evaluated.

Apart from screening for the active constituents, the tests are also extensively performed for the quality control of the material. These tests are inclusive of the presence of pesticides, aflatoxin and microbial screening, also the screening for the presence any specific micro-organism. The tests for evaluating the shelf life of the product are also conducted.

The herbal products have major chances of contamination and confusion with other similar raw plant materials and products. This may be due to lack of proper post-harvest management. Their scientific validation is necessary to authenticate not only the raw material but also the finished product. It also ensures the reproducibility of both the quality and the quantity of the product as per a definite protocol. Lack of such validation may be thought to possess serious medical implications as decreased efficacy or lack of efficacy and side-effects. Therefore, it is extremely crucial, to ensure the raw material and the drug are properly evaluated for their efficacy, their treatment, dosage and even toxic effects and allergies [10].

The current study aims to standardize five selected raw plant material, used extensively in various formulations of the Indian classical system of medicine. They are known for their therapeutic values and are evaluated as per the accepted guidelines and as mentioned by the Ministry of AYUSH, Government of India.

2.2 Review of literature

2.2.1 Early *Ayurveda*

The basic fundamentals of *Ayurveda* have proved to be true for all times and have undergone little or no change. These fundamentals are governed by intrinsic causes and regulated by the human understanding and advancing knowledge. The definitive origin of *Ayurveda* as a system of medicine can be dated back to the period of *Atharva Veda*. The book contains a description of various diseases with their treatments. The period from the 6th century B.C. to the 7th century B.C. witnessed a systematic development of scientific knowledge and is referred to as the *Saṁhitā* period. It was during this period, that various authors wrote about a system of organized medical care accounting to the current classical literature. The *Charaka Saṁhitā*, written during this period, is a compilation of the information dealing with various medical aspects as etiology, symptoms, treatment and cure of a disease. Similar work includes the *Sushrut Saṁhitā,* written on the surgical aspect of treatments at that time [11].

The *Saṁhitā* period witnessed a rise in the indigenous science of ancient times to its glory. Most of the 'classical' modes of pharmaceuticals were now being addressed and acknowledged in this period. There was a systematic rise and development of Traditional system of medicine. It can be noticed in the various compiled literatures of the *Saṁhitā* period. These texts are a storehouse of complex pharmacy procedures and drug formulations from a multi-ingredient source. These books also hold information on the compatibility between ingredients with focus on their incompatibility as well. It also gives tips on formulations and developing a systematic classification for various preparations.

The Indian traditional system of medicine considers three basic sources for drug formulation. They may be herbal, mineral or of animal origin. Two basic types of drugs are classical drugs and proprietary drugs. The drugs composed using the traditional Indian system of medicine may be administered in any of the 18 studied dosage forms. These may be either *Arista, Avaleha, Kvatha, Churna, Bhasma, Arka, Gritha, Lepa,* and *Vati,* among others. The herbal based drugs are prepared from the extract of the plant material [12-13]. The five basic and ancient modes of plant extract preparations are technically known as "*Kaṣāya Kalpanā*". These include preparations as juice (*Svarasa*), paste (*Kalka*), decoction (*Kvātha*), cold infusion (*Śīta Kaṣāya*) and hot infusion (*Phāṇṭa*). The *Kaṣāya Kalpanā*" preparations have a very short shelf life. Therefore, they are prepared as per requirements without the compulsion

for long-term storage for future use. Apart from these five need-based preparations, other preparations having longer shelf life are also mentioned in the classical literature. These, include medicated fatty preparations (oils, *Ghrita*, etc.), jelly or semi solid preparations (*Avaleha*), fermented products (*Ariṣta, Āsava* etc.) & pills (*Guṭikā*) [14].

2.2.2 Traditional system of medicine in Modern times

The global acceptance rate, for the herbal products and phytomedicines, has witnessed a multifold increase. There has been nearly 380% increase in sales of these products in the United States during the last decade of the 20th century. The sales of herbal products including herbal medicines had already crossed over USD 10 during this period [15]. It was estimated to experience a greater increase in the next two decades [16]. The graph represented in the Figure 2.1 below, shows a cumulative information, during a period of 5 years, on the sales of herbal products and herbal medicines from three major herbal companies, Patanjali, Himalaya and Emami, in India.

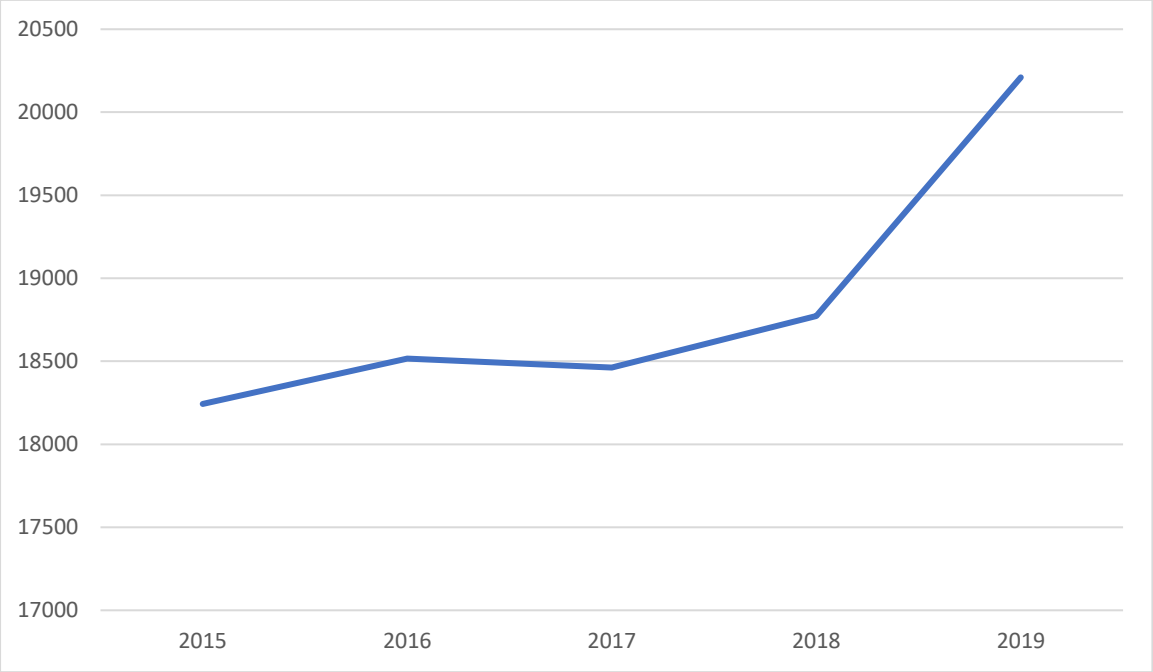


Figure 2.1 The graph shows an increase in the sales (in Cr INR) of herbal products including phytomedicines over a period of 5 years [17-19].

The WHO, having realized the unprecedented role and extreme importance of Traditional Medicine, has developed an action plan for the period of 2014-2023, in accordance with the World Health Assembly [20]. Its sole objective was to support the member countries in developing influential and strong policies for implementing the action plan. The action plan

was to intensify and accelerate the role and research of Traditional Medicines in the upkeeping of the health of the population [21-22].

2.2.3 Ayurvedic pharmacopeia of India and Ayurvedic Formulary of India

The Ayurvedic Pharmacopeia of India (API) holds a legal binding as entrusted by the Government of India. It gives a detailed description of drugs manufactured, licensed and sold in the entire country, including their purity, strength and quality. The information is forwarded and checked by the Pharmacopeia Commission of Indian Medicine and Homeopathy (PCIM&H), initially known as the Pharmacopeia Commission of Indian Medicine (PCIM), since 2010. The PCIM&H is the highest authority of the Ministry of AYUSH, Government of India. It guides the Ayurvedic Pharmacopeia Committee (APC) on the Ayurvedic drugs regarding these standards. Till 2017, the body has released 540 monographs on plant, animal and minerals origin. It has also documented nearly 976 compound formulations from traditional knowledge including the legal status of the same. This ensures their quality without compromising on their standards [23].

The API is presented in two parts. The Ayurvedic Formulary of India (AFI) is the first part. It contains information on selected formulations of natural origin including monographs on the same. The second part contains information in the form of monographs on single drugs that are obtained from the schedule-1 books mentioned under the Drugs and Cosmetics Act, 1940, storing information regarding the classics of different time periods [24]. The first edition of Indian Pharmacopeia (IP) was published in 1946, by the Government of India Press of Calcutta, with the title “Indian Pharmacopeial List 1946” [25].

The First edition of the AFI was published by Government of India, Ministry of Health and Family Planning, Department of Health, in 1978 [26]. The monograph published under AFI consists of literature regarding the formulations as mentioned in classical literature. It also contains the Sanskrit name and the reference on the classics. Further, the book gives information regarding the dosage, proportion and important therapeutic usage of the drug. It also enlists the origin of the drug including plant, animal or mineral origin and the type used in specific formulation [27]. The second revised English edition of AFI was published in the year 2003 under Department of Indian Systems of Medicine and Homeopathy, Ministry of Health and Family Welfare, Government of India, [28]. Part II of the API is in parts, of which the Vol-I contains information on formulations. Its first edition was published in 2007. It

includes standardization of 50 most frequently used formulations. The literature also mentions the contribution of different sources for drug formulations with 80% being that of plant as source followed by 12% from animal source while mineral source contributes to 8% in medicinal usage [29-30].

2.2.4 Scientific validation of traditional knowledge of *Ayurveda*

Ayurveda, the Indian system of traditional medicine was developed and has evolved over time as a system of sound rationale and logical upbuilding. Although, this system has proved efficient in treating various ailments since the ancient times, but it has experienced major criticism. It is due to the lack of systemic upgradation with scientifically proven facts in the light of current understanding of human knowledge and the progress of science over centuries. The fact that classical knowledge has undergone little change and remained scientifically untouched over a course of time, have also been considered as a major point of its drawback and lack of huge acceptance [31].

Medicinal plants have had a crucial role in the treatment of diseases since time immemorial. With the advancement of modern science, discoveries of modern medicines from natural products, has in fact, given evidence that the lead for such major discoveries was provided by the traditional knowledge prevalent in some country or culture [32]. The western system of medicine has adopted many of these drugs more recently in the 1950s. A major example is the use of reserpine obtained from *Rauwolfia serpentina,* known as *Sarpgandha*, in *Ayurveda.* Its current usage is that of a tranquilizer, since 1954 as explained by Nathan Kline and its first scientific description was reported as early as 1931 [33]. The application of this plant for the treatment of hypertension, insanity, insomnia and other such related disorders has been explained in the *Ayurveda*, dating back to almost 3000 years [34]. The medicinally important alkaloid was isolated from this plant in 1952 [35] while its structure was established in 1954 [36].

Another substantial example is that of the “Podophyllum” resin which has been used by Native Americans for the treatment of cancer [37]. Currently, the medicinally important resin, podophyllotoxin, is the precursor for the synthesis of anticancer drugs as etoposide and teniposide [38]. Therefore, the study of ethno-therapeutics is now being seen as a potentially important branch of science. It is being considered as a successful source of providing information for the discovery of clinically efficacious products [39].

In the light of the latest trends in herbal pharmaceutical sector, the Government of India has considered to conveniently use the Drugs and Cosmetics Act of 1940 to regulate and control the utilization and manufacturing of drugs from *Ayurveda, Siddha* and *Unani* knowledge, by means of amending this Act. It is worth consideration here, that the modes of different formulations and compound mixtures, known as *Yogas*, have developed gradually over time with advancement over the centuries from the pre-*Vedic* to the *Vedic* and on to the *Saṁhitā*, and the *Saṅgraha* periods and continue to do so even today.

To scientifically validate the existing knowledge from classical literature, standardization of this data is essential to ensure the quality and efficacy of these herbal products [40]. The process of standardization of herbal products, with focus prominently on herbal drugs, includes the process of prescribing a definitive set of standards. It includes constant parameters and qualitative and quantitative data to ensure the quality, identity, efficacy and reproducibility of these compounds. On a whole, the process of standardization includes the confirmation of the identity, quality and purity of the formulations throughout the process of its manufacture. This development of standardization and scientific test parameters and validation data involves the evaluation of physical, chemical and biological aspects of analytics.

The 'Evaluation' of a drug includes the affirmation of the identity of the drug and confirmation of the purity of the same [41]. It includes different parameters as the physical, chemical and biological and also includes the analytical methods [42]-

1. Physical Evaluation- It includes data regarding the physical characterization of the drug or plant material or formulation. It gives the detailed description on the same with available highly illustrative and well documented images. A description of the macroscopic and microscopic evaluation is also included besides the report on impurities obtained after the screening of the sample. It includes macro and microscopic analysis.
2. Chemical Evaluation-The chemical screening provides information regarding the active constituents which helps in determining the potency of the drug, besides helping determine the identity of unknown compounds or samples. It includes screening for the phytochemicals by use of different reagents.
3. Biological Evaluation- The pharmacological activity of drugs is evaluated by testing them on biological specimens as living animals or organs which helps in determining not only the efficacy and dosage of the drug but also provides a parameter of

standardization for the drug. It includes testing the dosage and efficacy on animal models.

4. Analytical Methods- Simple analytical methods for standardization help in determining the identity, quality and potency of the sample. It includes techniques as chromatography, TLC, HPLC, GC-MS, NMR and the like.

The quality control and standardization of herbal products is primarily required for the following reasons [43]-

1. A proper system of standardization was lacking.

2. Differences in the vernacular names and identification characteristics in the ancient times, may have led to mis-interpretation of traditional knowledge today. It may also have caused uncertainties over the identification of the same plant species from different locations.

3. Due to extensive commercialization and the global challenge of sufficient marketing, the supply and procurement of authentic raw material for development is very challenging.

The process of standardization involves phytochemical screening. It includes preliminary testing to identify the presence of different groups of phytochemicals, followed by the quantification of the compounds and chemicals of our interest and then the establishment of fingerprint profiles of the compounds [44].

BPD-01/Feb/21 is a plant found commonly growing in Northern India but is distributed widely in the Indian peninsula including Burma, Bangladesh, Thailand and the region of Indo-China. It belongs to family Rutaceae [45]. It is a deciduous tree with alternate trifoliate leaves and globular fruits [46]. Constitutively, this plant contains several phytochemicals as marmenol, marmin, psoralen, alloimperatorin, betulinic acid and marmesin. It contains a variety of organic acids, the important ones being ascorbic, tartaric, oxalic and malic acids [47], apart from a range of phenolics present in it including chlorogenic acid, ellagic acid, gallic acid and ferulic acid being the most prominent [48]. This species has always held an important place as an efficient phytomedicine and recent pharmacological evaluation of this plant has revealed its important pharmacological activities including antipyretic, anticancer, chemoprotective, antifertility, anti-inflammatory and ulcer healing properties [49].

BPD-02/Feb/21 is a tree belonging to the Lauraceae family, of which this species has been extremely exploited for various purposes since the ancient times. In fact, the Egyptians around 3000 BC were known to use this species for its flavor and fragrance and also for

mummifying the dead [50]. The plant may be classified as a tree with a height of 50 m. It is found to possess a characteristic smell owing to its phytochemicals, specifically cinnamic aldehyde. The leaves are hairy and opposite with scalariform intercostal venation and may be glabrous. Bisexual flowers are present with superior ovary. Fruit is ellipsoidal to ovoid with waxy epicarp and one seed per fruit which is smooth and glabrous and lacks endosperm [51].

BPD-03/Feb/21 belongs to the Indian sub-continent including the region of Malaya, China and Sri Lanka. The medicinally important part of the plant is the fruit which is extensively used in various Preparations of *Ayurveda* and helps in increasing longevity by enhancing the health [52]. It is the richest known source of Vitamin C and also possesses anti-oxidant properties and several active tannoid principles which have contributed to its potential for greater health benefits [53-54]. The fruit has also been reported to have antibacterial, hypoglycemic and even purgative properties [55-56] besides hypolipidemic and hepatoprotective activity [57]. The aqueous extract has been reported to have anti-pyretic laxative, tonic properties and also showed antibacterial activity [58]. The fresh fruit contains a high amount of ascorbic acid, which is reported to be the highest in all fruits evaluated so far and seconds only to Barbados cherry [59]. The important phytochemical constituent of its fruit is rich content of tannins which may be present as two varieties Emblicanin A and B, and the antioxidant properties are attributed to this phytochemical and predominantly give ellagic acid and glucose on hydrolysis [60-62].

BPD-04/Feb/21 is also a native on the Indian subcontinent and is even worshipped as sacred in India. It is found to possess great health benefits owing to its plethora of phytochemicals and secondary metabolites which include primarily volatile oils constituting nearly 0.7% of the whole of which 71 % is eugenol while 20% is methyl eugenol. The other constituents are carvacrol, linalool, limmatrol, sesquiterpine hydrocarbon, caryophyllene, cirsilineol, circimaritin, isothymusin, and apigenin. In addition, the leaves also contain orientin, vicenin, ursolic acid, apigenin, luteolin, apigenin-7-O-glucuronide, luteolin-7-O glucuronide, molludistin, and sesquiterpenes and monoterpenes like bornyl acetate, α-elemeneneral, myrtenal, α- and β-pinenes, camphene, campesterol, stigmasterol, and β-sitostero [63-64]. Extensive studies on this plant species have revealed its pharmacognostic effects as antidiabetic, antifungal, antibacterial, hepatoprotective and even immunomodulatory and cardioprotective activities [65-69].

BPD-05/Feb/21 belongs to family Solanaceae and is found throughout the Indian subcontinent [70]. It is a prickly perennial herb. It has berry fruit with glabrous seed [71].

This plant has been used in the *Ayurveda* since the ancient times. It is found to possess anti-inflammatory, anti-infertility and anti-allergic properties [72-73]. The decoction of the plant has been used as an anti-diuretic and also in the treatment of gonorrhea. The seed is efficacious against cough, fever and asthma. It also has cytotoxic, anti-spasmodic and anti-tumor activities [74-76]. The major phytochemical constituents present include steroidal glycoalkoloids and caffeic acid coumarins besides saponins [77-79].

2.2.5 Test Parameters as mentioned by AYUSH

The Ministry of AYUSH has stated various test parameters, as shown in Table 2.1, depending on the type of herbal or ayurvedic material to be standardized.

Table 2.1 Test parameters for raw plant material and plant extract as per AYUSH guidelines [80].

S.No	Test Parameter for Raw Plant Material	Test Parameter for Plant Extract
01.	Passport data, Botanical description, Adulteration and substitution (as per literature)	Passport data, Botanical description, Adulteration and substitution (as per literature)
02.	Foreign matter	Foreign matter
03.	Organoleptic character	Organoleptic character
04.	Macroscopic and Microscopic character including Powder microscopy	Moisture content calculated as loss on drying at 105ºC
05.	Moisture content calculated as loss on drying at 105ºC	pH value for 10% aqueous extract
06.	pH value for 10% aqueous extract	Total ash
07.	Total ash	Acid-insoluble ash
08.	Acid-insoluble ash	Water-soluble extractive
09.	Water-soluble extractive	Alcohol-soluble extractive
10.	Alcohol-soluble extractive	Volatile oil for oil bearing plants generally of family Lamiaceae and Asteraceae

11.	Volatile oil for oil bearing plants generally of family Lamiaceae and Asteraceae	Chromatographic studies (TLC/HPLC/HPTLC/GLC) as per requirement
12.	Chromatographic studies (TLC/HPLC/HPTLC/GLC) as per requirement	Assay for active constituents (total alkaloids/ total flavonoids, etc.)
13.	Assay for active constituents (total alkaloids/ total flavonoids, etc.)	Heavy/ toxic metal analysis
14.	Heavy/ toxic metal analysis	Pesticide residue analysis
15.	Pesticide residue analysis	Test for microbial contamination
16.	Test for microbial contamination	Test for specific pathogen
17.	Test for specific pathogen	Aflatoxins
18.	Aflatoxins	Shelf life
19.	Shelf life	

Adulteration literally means substituting the concerned product with a substance of inferior quality possessing similar morphological features so that it is difficult to differentiate between the two. This, most often, results in profit in terms of money to the seller while it may have serious implications on the consumer, both monetarily and even medically [81].

Evaluation of organoleptic characters is done by simple physical and non-chemical methods as taste, smell, color, size and even the structure of the sample. This analysis helps to establish and evaluate the identity and purity of the sample rapidly. Similarly, the macroscopic and microscopic characteristics of the sample is also established by proper evaluation which helps in conforming the identity of the sample [82].

The ash value of the sample also gives an estimate of the purity of the sample and may help in identifying impurities as carbonate, oxalate and silicate. The ash value may be determined as water soluble ash or acid in-soluble ash. The assessment of water-soluble ash gives an overview of the quantity of inorganic components present in the sample while the acid in-soluble ash gives an overview on the presence of earthy materials, as silica, in the sample. The moisture content estimation is done at 105℃ and also known as loss on drying at 105℃. The value of loss on drying, helps to analyze the amount of moisture in a sample. This affects the shelf life of the sample, as greater the moisture content, the more will be the chance of early contamination by bacteria or fungi and the lesser will be the shelf life. Although, the

preparation of extract is a necessary step for the preparation of formulations, but the evaluation of extractive yield under different solvent systems helps in determining the quantity of active constituents in a fixed quantity of sample or raw plant material. The solvent system used for extraction also influences the type and quantity of phytochemicals extracted from a sample [83].

2.3 Aim and Objectives

The aim of the present study is **Scientific Validation of Medicinal And Aromatic Plants From CSIR-CIMAP As Per AYUSH Guidelines.**

The objectives are:

- Collection and authentication of plant material used in various formulations according to traditional knowledge.
- Standardization of the raw plant material to the selected test parameters as per AYSUH guidelines.
- Preparation of extract and standardization of extraction procedure of the procured plant material.
- Standardization of plant extract to the selected test parameters as per the AYUSH guidelines

2.4 Materials and Methods

The general procedure for the standardization of the five plant species selected is summarized in the Figure 2.2.

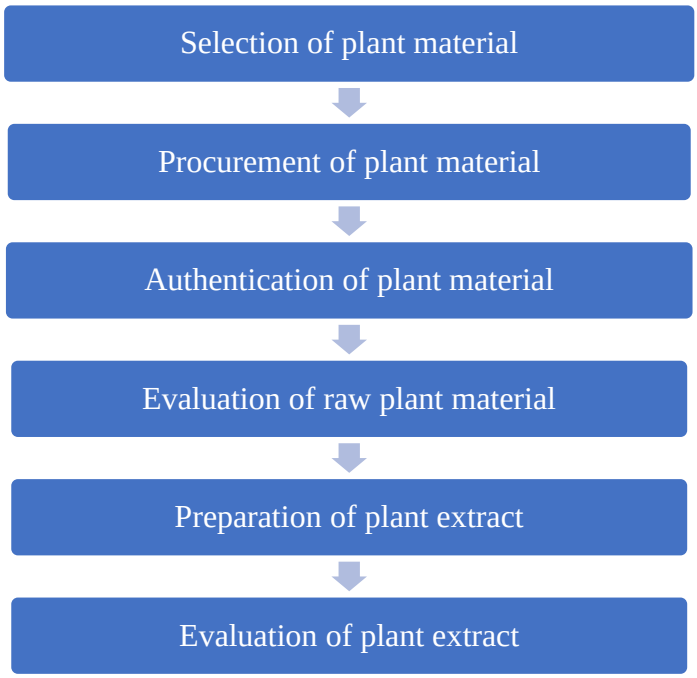


Figure 2.2 General procedure for the standardization of raw plant material.

The selection of plant material was done as per their importance and lack of previous data of standardization. They were given specific code names as shown in Table 2.2. These were standardized independently as per the protocols obtained after extensive literature survey.

Table 2.2 Plant codes as obtained and the place of procurement of the plant material.

S.No	Plant Code	Place of Procurement
1.	BPD-01/Feb/21	CSIR-CIMAP, Lucknow
2.	BPD-02/Feb/21	Market
3.	BPD-03/Feb/21	CSIR-CIMAP, Lucknow
4.	BPD-04/Feb/21	CSIR-CIMAP, Lucknow
5.	BPD-05/Feb/21	Market

The raw plant material was shade dried initially. A sample of the selected plant materials was submitted to the Department of Botany, CSIR-CIMAP, Lucknow, for authentication.

Determination of organoleptic character and foreign matter:

The evaluation of organoleptic character was performed by assessing the size, shape, color, texture, odor and taste of the sample [84]. The procedure followed was as shown in Figure 2.3.

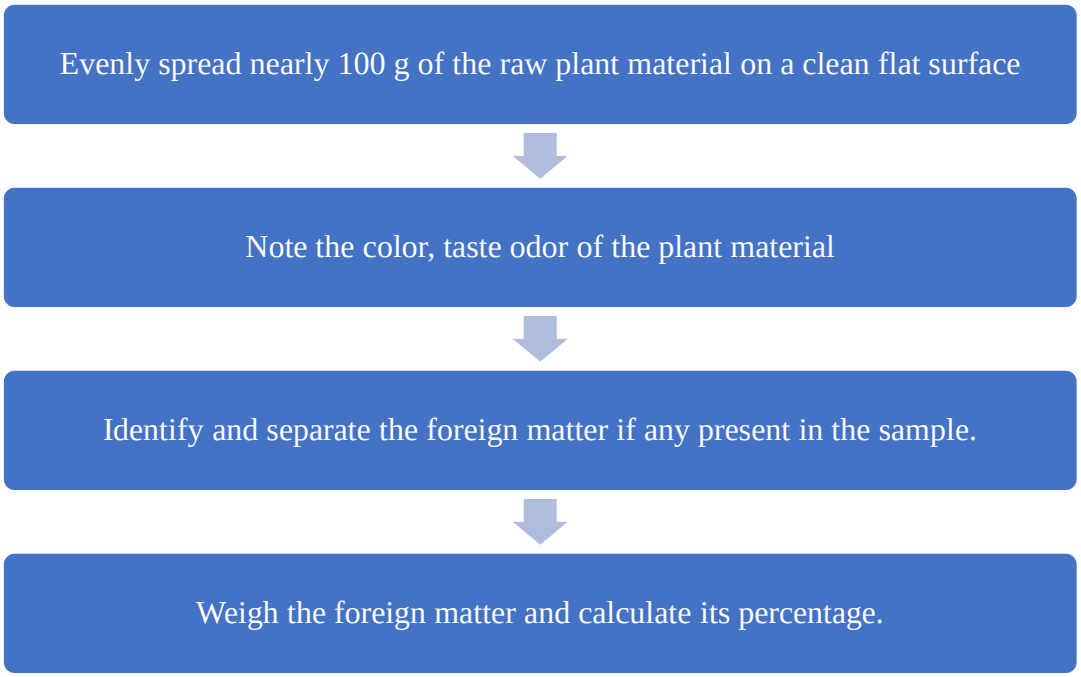


Figure 2.3 Procedure for the assessment of organoleptic character and foreign matter if any is present in the sample.

The foreign matter was also checked in the sample and its percentage calculated. 5g of the raw plant material was taken. It was spread on a plane sheet and checked manually for the presence of any foreign material. The foreign matter may be the unwanted soil particles or small stones and parts of plant other than the plant part taken. The foreign material was collected and weighed and percentage of foreign matter was thus calculated [85].

The percentage of foreign matter was calculated using the formula-

$$Percentage\ of\ foreign\ matter = \frac{Weight\ of\ foreign\ matter}{Weight\ of\ coarse\ powder\ taken} \times 100$$

Estimation of moisture content in the raw plant material

The loss on drying or the moisture content of the plant material was measured at 105ºC. The protocol followed for estimation of moisture content is as mentioned in Figure 2.4.

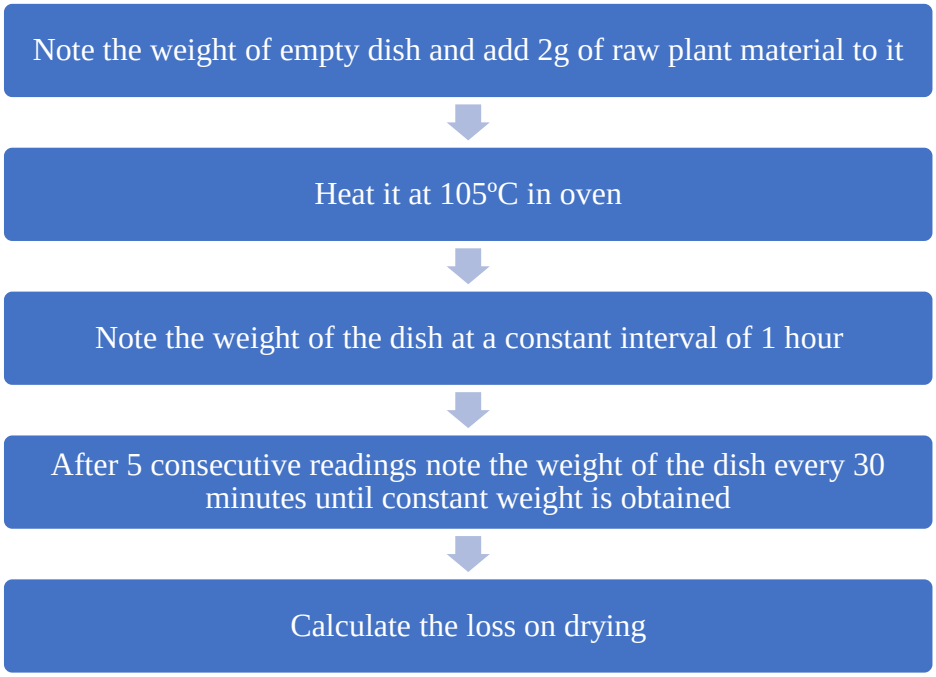


Figure 2.4 The procedure for measuring the loss on drying at 105ºC for the raw plant material.

The moisture content is calculated using the formula-

$$Moisture\ content = \frac{W_0 - W_f}{W} \times 100$$

Where, W is the weight of the raw plant material taken initially

W_0 is the initial weight of the dish with the plant material

W_f is the final weight of the dish with the plant material

Preparation of plant extract and calculation of extractive yield

Technique of cold maceration (Figure 2.5) was used for the preparation of plant extract.

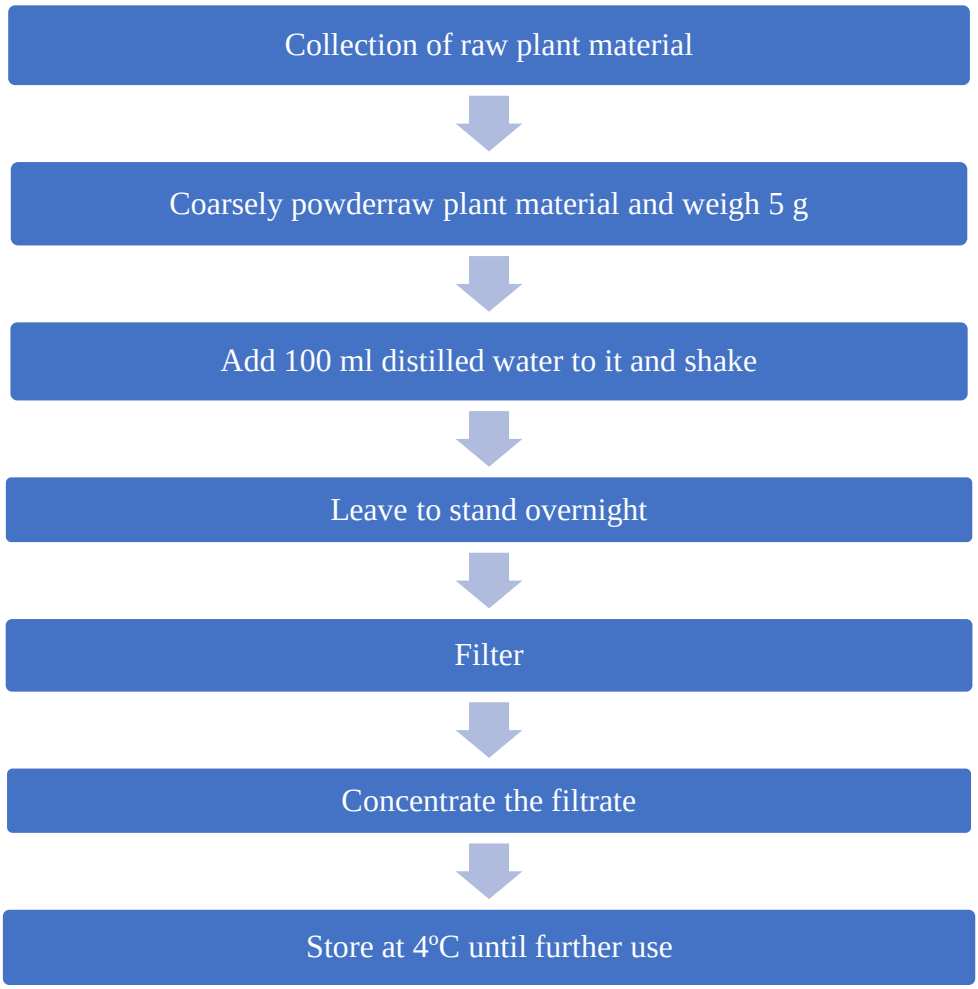


Figure 2.5 Protocol for the preparation of plant extract [86-87].

The extractive yield was calculated by the formula [88]-

$$Percentage\ of\ extractive\ yield = \frac{Weight\ of\ the\ concentrated\ plant\ extract}{Weight\ of\ the\ raw\ plant\ material\ taken} \times 100$$

Estimation of pH for the raw plant material and plant extract

The pH of 10% aqueous solution of raw plant material and 10% aqueous solution of plant extract is prepared (Figure 2.6).

10% w/v aqueous solution for raw plant material is prepared by taking 10 gm of raw plant material and adding 100 ml of distilled water to it.

10 % w/v aqueous solution of plant extract is prepared by taking 1 mg of plant extract and adding 10 ml of distilled water to it.

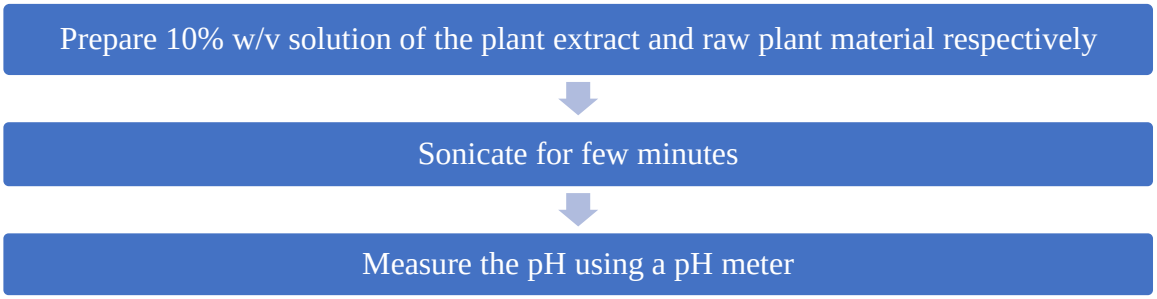


Figure 2.6 Procedure for measurement of pH.

Phytochemical analysis for the raw plant material

Table 2.3 gives a comprehensive overview of the phytochemical tests conducted for the selected plant materials.

Table 2.3 A comprehensive table enlisting the tests used for the qualitative assessment of different phytochemicals and their expected observations [89].

Phytochemical	Test name	Observation
Saponins	Frothing test	Presence of Froth
Tannins	Braymer's test (Ferric chloride test)	Greenish yellow or blue-black color obtained
Flavonoids	Alkaline reagent test	Yellow color to discoloration

1. Phytochemical screening for the presence of saponins:

   Saponins have a soap like texture and therefore show frothing when mixed with water. The defined protocol followed for saponin testing is depicted in Figure 2.7.

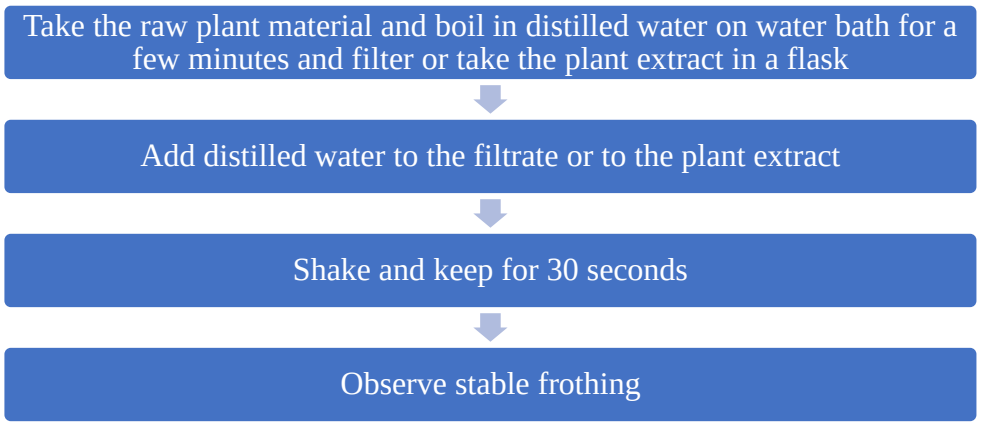


Figure 2.7 Procedure for qualitative determination of saponins [90].

2. Phytochemical screening for the presence of tannins:

The ferric chloride test (Figure 2.8) is used for checking the presence of tannins in a sample.Ferric chloride solution is freshly prepared by adding anhydrous ferric chloride and distilled water in the ratio of 1:3.

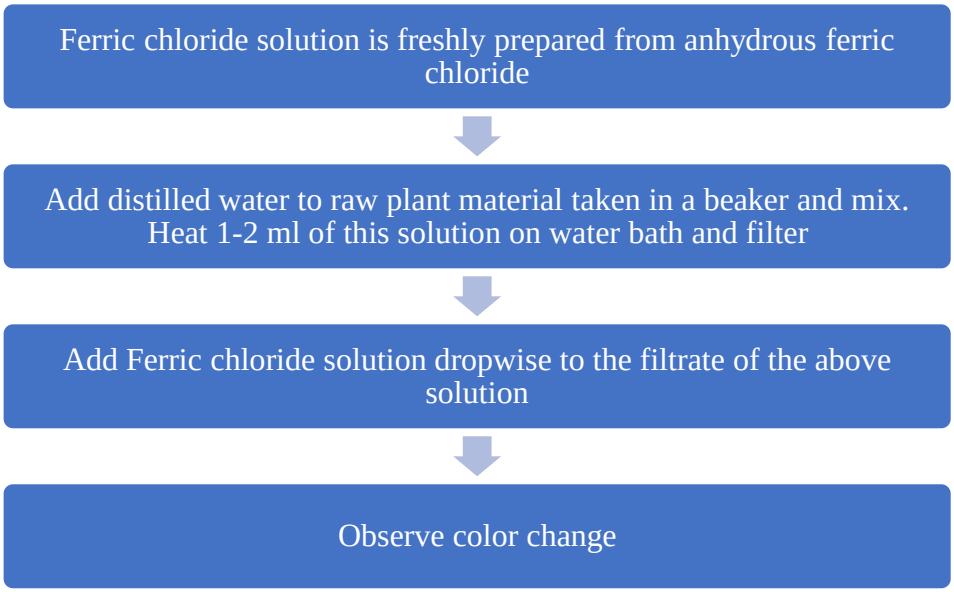


Figure 2.8 The procedure for qualitative analysis of tannins in a sample [91].

Two types of color change may be observed, brownish green or bluish black depending on the type on tannin dominantly present.

3. Phytochemical screening for the presence of flavonoids:
The flavonoids were tested by alkaline reagent test (Figure 2.9).

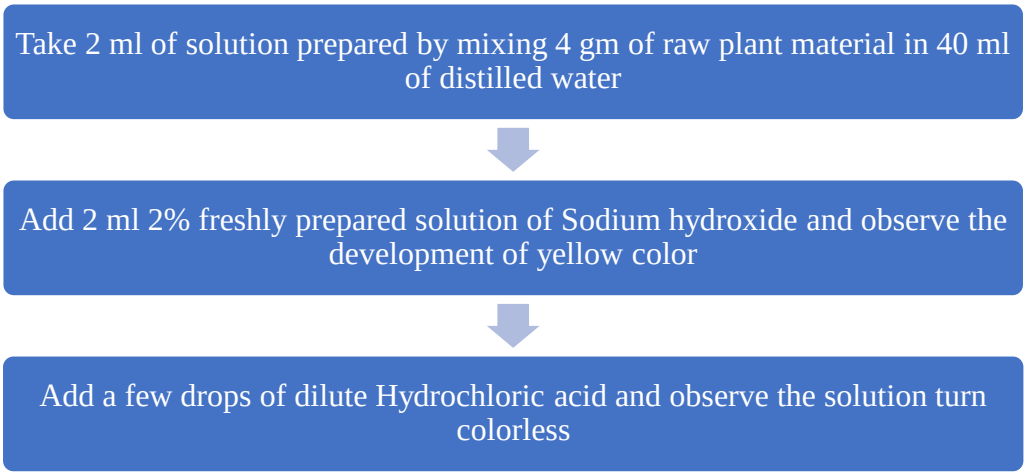


Figure 2.9 Procedure for qualitative estimation flavonoids in test sample [92].

2.5 Results

The passport data of the raw plant material (Table 2.4) is inclusive of the sample type under consideration and the presence of adulterant in the sample. It also mentions the date of collection of the sample which also gives an estimate on the shelf life of the sample.

Table 2.4 The passport data of the raw plant material.

Plant	Date of collection	Dried plant part used	Adulterant (if any)
BPD-01/Feb/21	February 2021	Fruit	Diseased and old samples
BPD-02/Feb/21	February 2021	Bark	Other woody plant parts
BPD-03/Feb/21	February 2021	Fruit	None reported
BPD-04/Feb/21	February 2021	Whole plant	Similar plant parts from other sources
BPD-05/Feb/21	February 2021	Whole plant	Morphologically similar stem parts

Botanical description (Table 2.5) gives an overview of the morphology of the plant and its place of occurrence. This may also help in analyzing the type of important secondary metabolites that may be present in this species.

Table 2.5 Botanical description of the selected plant species.

Plant	Habit and habitat	External morphology
BPD-01/Feb/21	Tree, grows in dry forests in both hills and plains	Aromatic deciduous plant 5-9 meters heigh with bisexual flowers. Pulpy fruit globose in structure, gray or yellow in color.
BPD-02/Feb/21	Tree, grows in sub-tropical or tropical areas especially the tropical rainforests	Evergreen. Leaves are smooth 10- 16 cm in length. Berry fruit black in color. Strongly aromatic with the medicinal properties contained mostly in bark.

BPD-03/Feb/21	Grows in semi-arid regions of India including the plains	Deciduous plant of medium height (1-9 m). Greenish yellow flowers. Spherical fruit greenish-yellow in color with sour-bitter taste.
BPD-04/Feb/21	Shrub found naturally growing in tropical and sub-tropical regions	Shrub with simple aromatic leaves 12-24 inch in height. Flowers are elongated raceme. Grows abundantly in south Asian countries including India.
BPD-05/Feb/21	Semi-tropical plant found growing naturally in tropical regions	Herb ranging from 2-6 cm in height. Numerous needle-like structures present on stem. Obtuse leaves 5-10 in number. Fruit is berry enclosed in an enlarged calyx.

The amount and percentage of foreign matter in a sample gives an estimate of the purity of the sample. If the amount of foreign matter falls in range, the sample is considered to be pure and safe for use as shown in Table 2.6.

Table 2.6 The type and amount of foreign matter in raw plant material.

Plant	Foreign matter	% Foreign matter	Expected % of foreign matter
BPD-01/Feb/21	Fibers and stones	1.00	Not more than 2.0 %
BPD-02/Feb/21	Fibers up to 2 cm in length, soil particles	0.80	Not more than 2.0 %
BPD-03/Feb/21	Fibers and stones	1.56	Not more than 3.0 %
BPD-04/Feb/21	Small parts of stem and branches	0.74	Not more than 2.0 %
BPD-05/Feb/21	Soil particles	1.84	Not more than 2.0 %

The evaluation of organoleptic characters of the plant as the color, taste and texture, help in conforming the identity of the sample as shown in Table 2.7 and Figure 2.10.

Table 2.7 The organoleptic properties of the raw plant material.

Plant	Color	Taste	Odor
BPD-01/Feb/21	Reddish brown	Tangy bitter	Strong
BPD-02/Feb/21	Dark brown	Sweet to bitter	Very strong
BPD-03/Feb/21	Greenish brown to greyish black	Tangy	Strong
BPD-04/Feb/21	Dark green	Bitter	Very strong
BPD-05/Feb/21	Creamy light brown	No taste	No odour

BPD-01/Feb/21

BPD-02/Feb/21

BPD-03/Feb/21

BPD-04/Feb/21

BPD-05/Feb/21

Figure 2.10 The evaluation for organoleptic character of color as seen in the raw plant material.

The percentage of moisture content in a sample (Table 2.8) may help in analyzing the shelf of the sample.

Table 2.8 The percentage of moisture content or loss on drying of the raw plant material at 105ºC.

Plant	% Moisture content
BPD-01/Feb/21	9.85 %
BPD-02/Feb/21	10.45 %
BPD-03/Feb/21	13.57 %
BPD-04/Feb/21	10.82 %
BPD-05/Feb/21	10.80 %

The percentage of extractive yield (Table 2.9) gives an estimate of the quantity of the active constituents that may be obtained from a specific amount of raw plant.

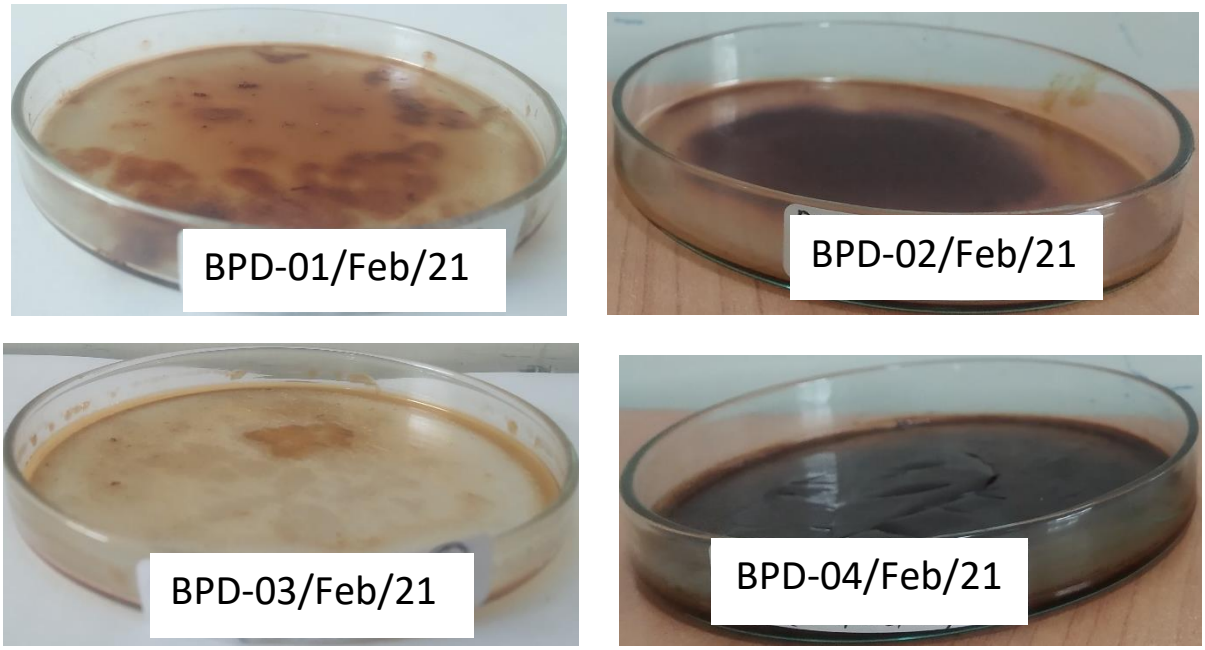
Table 2.9 The percentage of extractive yield as obtained after cold maceration.

Plant	Extractive Yield	Expected Extractive Yield
BPD-01/Feb/21	33.896 %	Not less than 30 %
BPD-02/Feb/21	6.248%	Not less than 3 %
BPD-03/Feb/21	30.704 %	Not less than 30 %
BPD-04/Feb/21	8.19 %	Not less than 8 %
BPD-05/Feb/21	9.248%	Not less than 16 %

The organoleptic evaluation of the plant extracts (Figure 2.11 and Table 2.10) is also done, similar to the organoleptic evaluation for the raw plant material.

Table 2.10 The organoleptic characters as observable for the prepared plant extracts.

Plant Name	Description	Color	Odor	Taste
BPD-01/Feb/21	Light slightly sticky mass	Reddish brown	Sweet strong aroma	Subtle slightly woody
BPD-02/Feb/21	Light towards the border and concentrated in the center	Brownish black	Pleasant smell	Woody
BPD-03/Feb/21	Powdery mass evenly concentrated	Light brown	Stingy sweet aroma	Bitter and sour
BPD-04/Feb/21	Paper like shiny extract which breaks on slight touching	Blackish brown	Very strong pleasant aroma	Bitter clove like
BPD-05/Feb/21	Evenly concentrated	Dark brown	Slight aroma	No prominent taste



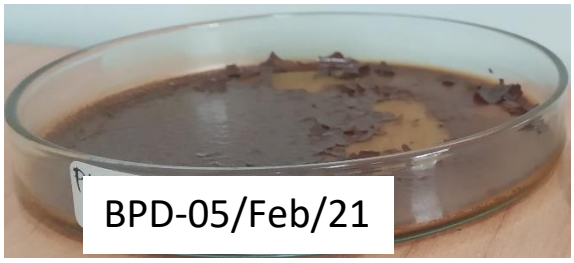


Figure 2.11 The plant extracts observed for their organoleptic characters.

The pH of the raw plant material and the plant extract, as in Table 2.11, may give an idea of the chemical nature of the phytochemicals present. The data may be used in future to confirm the purity of a sample.

Table 2.11 pH of the 10 % w/v aqueous solution of raw plant material and 10 % w/v aqueous solution of plant extract.

Plant	pH	
	For raw plant material	For plant extract
BPD-01/Feb/21	3.1	2.9
BPD-02/Feb/21	5.4	5.2
BPD-03/Feb/21	4.2	3.9
BPD-04/Feb/21	6.5	6.8
BPD-05/Feb/21	6.8	6.5

The assessment for saponins is by a simple frothing test. The froth is observed as shown in Figure 2.11 while the stability and quantity of froth is represented by different symbols, like +++ meaning highly stable and high amount of froth, ++ comparatively less stable and less amount of froth, + means very less amount of froth that disappears very early. This is represented in Table 2.12.

Table 2.12 The results for the frothing test to check for the presence of saponins in the raw plant material.

Plant Name	Saponin presence
BPD-01/Feb/21	+
BPD-02/Feb/21	+++
BPD-03/Feb/21	+
BPD-04/Feb/21	+++
BPD-05/Feb/21	++

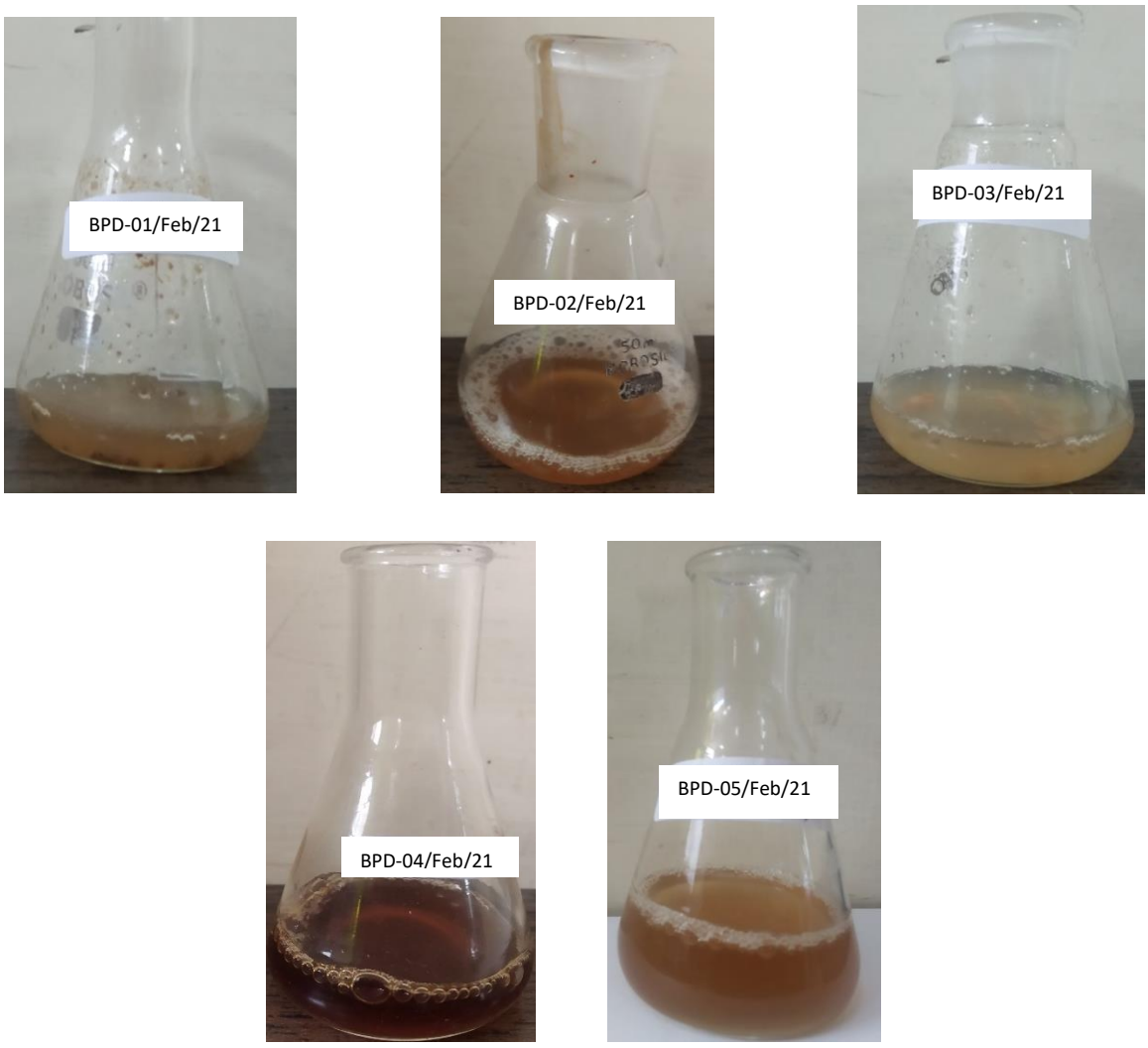


Figure 2.12 The presence of saponins as observed after frothing test in the raw plant material.

The ferric chloride test for the presence of tannins may give either bluish-black or greenish-yellow color change depending on the type of tannins. No color change is observed when tannins are absent. The results are represented in Table 2.13 and Figure 2.13.

Table 2.13 The presence of tannins as evaluated by Braymer's ferric chloride test in the raw plant material.

Plant name	Tannins	Color Observed
BPD-01/Feb/21	+	Bluish Black
BPD-02/Feb/21	+	Greenish Yellow
BPD-03/Feb/21	+	Greenish Yellow
BPD-04/Feb/21	+	Greenish Yellow
BPD-05/Feb/21	-	No color change

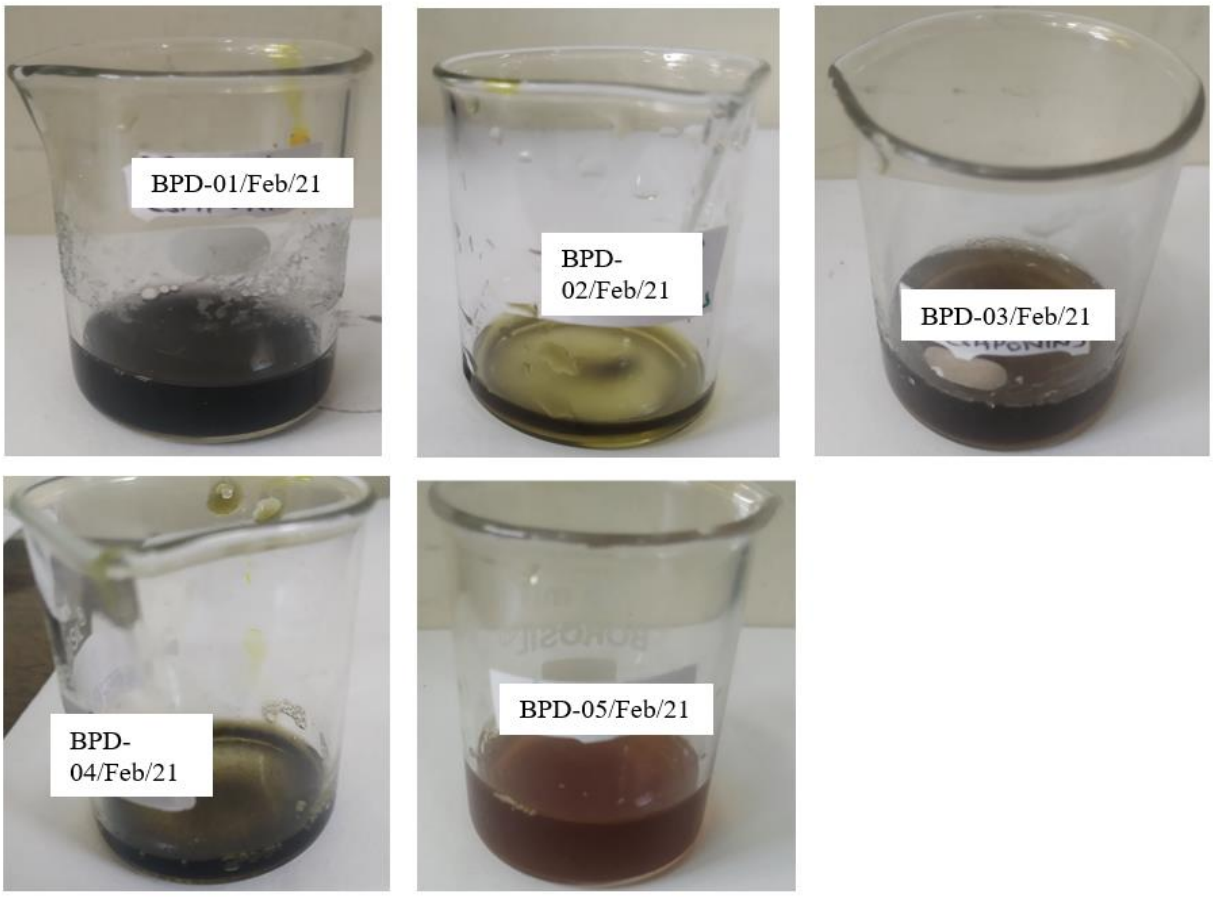


Figure 2.13 Ferric chloride test to check for the presence of tannins in the raw plant material.

The alkaline reagent test for flavonoids shows the development of yellow color upon addition of alkali which disappears on reaction with hydrochloric acid. The results are represented in Table 2.14 while the observations in color change upon addition of alkali (before treatment) followed by hydrochloric acid (after treatment) is shown in Figure 2.14.

Table 2.14 Results for flavonoid presence in the raw plant material.

Plant name	Flavonoid presence
BPD-01/Feb/21	+
BPD-02/Feb/21	+
BPD-03/Feb/21	+
BPD-04/Feb/21	+
BPD-05/Feb/21	+

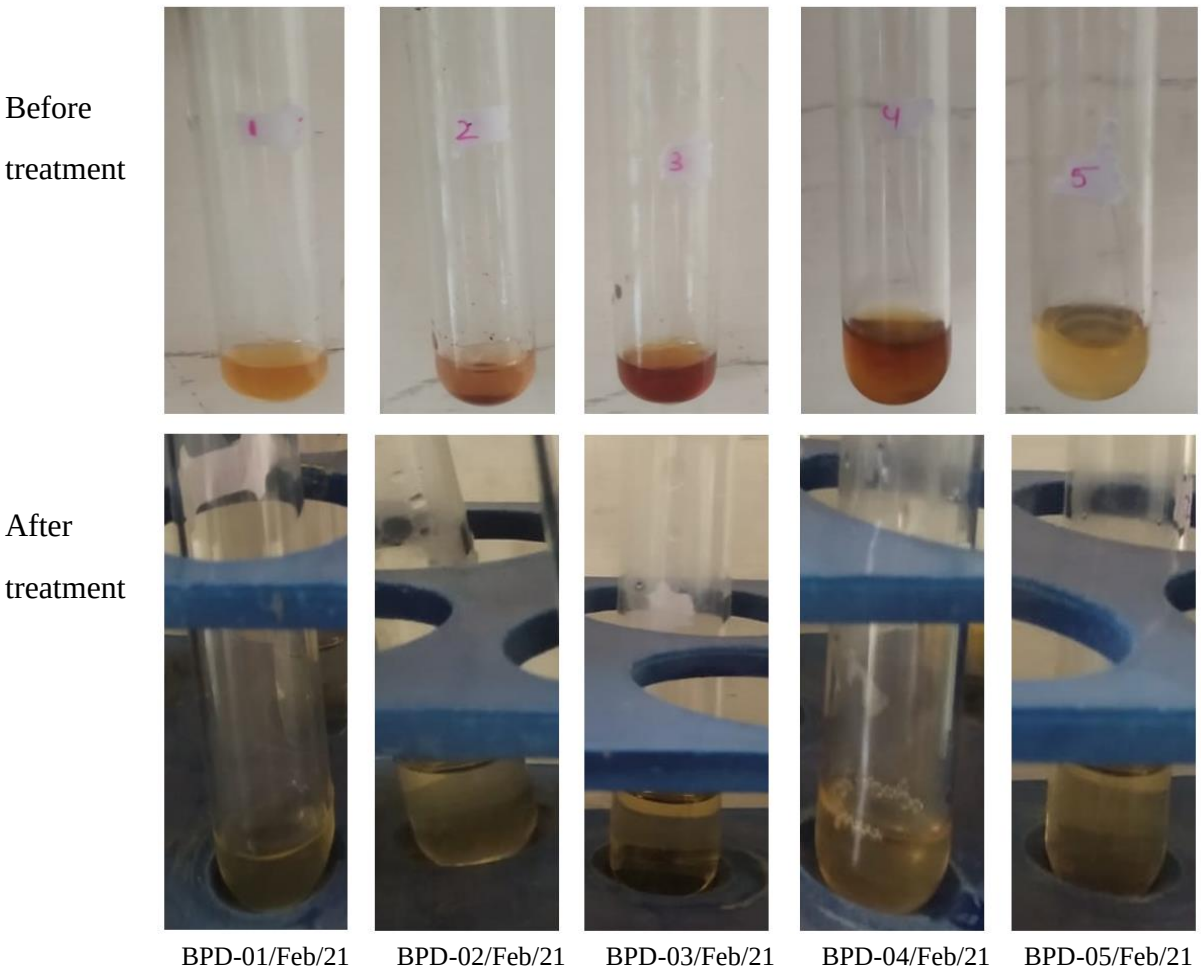


Figure 2.14 Result for phytochemical screening for the presence of flavonoids in the raw plant material.

2.6 Discussion

Different plant parts were used for assessment of their pharmacological properties and tested on the parameters as per the AYUSH guidelines. Dried fruits were used as parts of interest for BPD-01/Feb/21 and BPD-03/Feb/21. The dried bark was used in case of BPD-02/Feb/21. Whole plant was used for BPD-04/Feb/21 and BPD-05/Feb/21. The passport data of the plant material is the first test parameter which includes the description of the date of collection of the plant material. The botanical description of the plant material is also an essential test parameter. The collected samples were evaluated for the presence of foreign matter which includes materials as, soil particles, small stones, fibers or plant parts other than the part under consideration and adulterants from other plants. For example, in case of dried fruits, the foreign matter may be inclusive of parts of stem or branches from the same plant and other plants as well. The amount of foreign matter in a sample gives an approximate value on the purity of the obtained sample. In general, a sample is deemed fit for use if the foreign matter present in it is not more than 4-5 % but the expected range differs for different plant and parts used.

Before use, the raw plant material is shade-dried to remove excess moisture. The heating of raw material at 105℃ ensures that the extra moisture content is lost from the material. Higher percentage of moisture content gives an estimate of shelf life and the microbial load in the sample. The optimum range of moisture content for sun dried plant samples is between 8-10%. [93]. A higher moisture content indicates improper drying or increased chances of early contamination of the sample. The extracts were prepared for the collected plant materials. The amount of extract obtained was converted to percentage and compared to the expected range of extractive yields [94-97]. The appropriate amount of extractive yield for a given plant material differs according to the solvent for extraction as well as the part of plant used. It may also differ with the difference in the collection time and season of the concerned plant part. The assessment of pH of both the raw plant material and the plant extract gives an estimation of the purity of the sample besides giving a brief overview for the appropriate condition for the action of the plant material. An optimum range of pH ensures the kind of microbial contamination and the condition at which it will be least.

The saponins are high molecular weight glycosides. They have a bitter taste, therefore, protect the plant against microbes and grazing animals. They show hemolytic and cholesterol-binding activities. They form a soapy froth upon agitation with water. [98]. The

froth test, is therefore, a simple test to check the presence of saponin in a plant sample. Tannins are water-soluble secondary metabolites that are derivatives of phenol. In plants they have role in wound healing and prevent fungal spore formation and germination on plants. This compound has extensively been used in the tanning and leather industry [99]. Two color changes observed for the ferric chloride test which indicate the presence of tannin, the bluish-black coloration and the greenish-yellow coloration. The difference in color change may be attributed to the presence of different types of tannins. The hydrolysable tannins, as gallitannins and eligallitannins, are 3-16 gallic acid residue derivatives, which are easily hydrolysable by a weak acid or base and give bluish black color. The condensed tannins give a greenish-yellow to greenish-black color change [100-101]. Flavonoids are polyphenols of low molecular weight and are important secondary metabolites in plants. They have role in the development of aroma and color in plants which help in pollination by attracting pollinating agents. They also protect the plant from various abiotic and biotic stress. They also act as anti-microbial agents [102]. Flavonoids show the development of intense yellow color upon reacting with sodium hydroxide which is later visible as a prominent discoloration upon addition of few drops of dilute hydrochloric acid.

BPD-01/Feb/21 was obtained from the CSIR-CIMAP, Lucknow farm. It was light brown in color. It had a strong aroma and tangy taste. It has a rich resin content. It is a deciduous plant with alternate leaves 4-10 cm long. The plant bears fragrant flowers in April-May which have fleshy petals. Mature fruit is oval 5-20 cm in diameter and yellow in color. The pulp of the fruit stores 10-15 seeds which have woolly hairs and transparent mucilage [103]. The extractive yield of the sample in aqueous solvent was nearly 34% which was considered appropriate in comparison to earlier reported results. The moisture content of the raw plant material at 105ºC is 9.85 %. The pH of 10% w/v aqueous solution of raw plant material was 3.1 while the 10% w/v aqueous solution of this plant extract had pH 2.9. This implied that this plant material had acidic fruit, which could be citrus in nature. It gave a positive test for the presence of saponins with the development of very less and mildly stable froth. The ferric chloride test for the presence of tannins gave a bluish-black color. This indicated that BPD-01/Feb/21 had more quantity of hydrolysable tannins. The alkaline reagent test for the presence of flavonoids gave a positive result. Development of intense yellow color was observed upon addition of alkaline reagent. It disappeared upon addition of few drops of hydrochloric acid.

BPD-02/Feb/21 was procured from the market. It was reddish brown in color. It had a very strong aroma. It had a woody texture with sweet and gritty taste. It is an evergreen tree with yellow flowers and a strong odor. The mature fruit is a fleshy berry, black in color. The bark is dark brown on the outside while yellow on the inside [104]. Soil particles as small stones were an adulterant for this plant material. The extractive yield of the sample in aqueous solvent was nearly 6%. This could be due to the type of plant part used. The bark is made of dead tissue of the plant; therefore, the extractive yield may be low. The moisture content of the raw plant material is 10.45% indicating the sample may not have been properly shade-dried. The pH of raw plant material was 5.4 while the 10% w/v aqueous solution of this plant extract had pH 5.2. This plant material could be considered to have high saponin content since it showed the development of very intense and highly stable froth. The ferric chloride test for the presence of tannins gave a greenish-yellow color, indicating the presence of condensed tannins. It showed the presence of rich flavonoid content by alkaline reagent test. It showed the development of deep yellow color upon addition of alkali, which instantly disappeared on addition of hydrochloric acid. This may indicate the presence of high concentration of flavonoid in the sample.

BPD-03/Feb/21 was obtained from the CSIR-CIMAP, Lucknow farm. It was green and greyish black in color. It had a strong aroma and tangy slightly acidic taste. The plant is deciduous 1-8 m tall with long simple leaves. It bears greenish-yellow flowers and fruits. The fruit is spherical in shape and rich in pulp. The visible adulterants include the leaves and stem of the plant. The extractive yield of the sample in aqueous solvent was nearly 31% which was considered to be appropriate. The moisture content of the raw plant material is 13.6 %. This was the highest among all the five samples under study. It may be since the fruit is rich in pulp. The pH of raw plant material was 4.2 while of the plant extract was 3.9. This may imply that the fruit is citrus and a rich source of Vitamin C. It gave positive test for the presence of saponins but the froth formed was very less as compared to other samples and disappeared sooner. The ferric chloride test gave an indication for the presence of condensed tannins in the sample. The alkaline reagent test for flavonoids showed the development of intense yellow color. The yellow color had greater intensity than BPD-01/Feb/21 and BPD-02/Feb/21. This may imply that this plant has more amount of flavonoid than BPD-01/Feb/21 and BPD-02/Feb/21.

Whole dried plant was standardized for BPD-04/Feb/2. It was procured from the market. It had dark green color and strong aroma. It had a strong slightly bitter taste. It is a strongly

aromatic shrub 30-60 cm tall. It possesses simple opposite leaves green or purple in color. The flower is purple in color with elongated racemes and verticillaster inflorescence [105]. The extractive yield of the sample in aqueous solvent was 8% which is low. This might indicate improper procedure of extraction or the presence of considerable amount of foreign matter in the sample. It may also indicate the need of a different solvent system for extraction by cold maceration. The moisture content of the raw plant material is 11 % which is considerable high. The plant may be considered slightly acidic since the pH of raw plant material was 6.5 while the plant extract had pH 6.8. It gave a positive test for the presence of saponins. The froth formed was extremely stable and intense in comparison to all the selected samples. This implies the presence of large amounts of saponin in the plant. The ferric chloride test for the presence of tannins gave a greenish-yellow color conforming the presence of condensed tannins. The alkaline reagent test for the presence of flavonoids gave a positive result. This plant showed the maximum intensity of color development among all the samples by extremely concentrated brownish-yellow color formation upon addition of alkali. It may be inferred that this plant species contains the maximum quantity of flavonoids.

BPD-05/Feb/21 was procured from the farm of CSIR-CIMAP, Lucknow. It did not have a strong aroma and was creamy to light brown color. It was strongly fibrous and has various filaments. It is a prickly perennial herb with numerous branches. Leaves are sub-pinnate and 2-6 cm in length. It has berry fruit yellow in color and 1-2 cm in diameter. Seeds are glabrous [106]. The adulterant included plant material which had a different color. Weeds may also be present extensively in the raw plant material. The extractive yield of the sample in aqueous solvent was nearly 9%. It was very less than its minimum expected yield of 16%. This variation in extractive yield could be due to more amount of foreign matter as compared to the other plant samples. The moisture content of the raw plant material was 10.8%. The pH was 6.8 for raw plant while that of the plant extract was 6.5. It gave a positive test for the presence of saponins with development of stable but less amount of froth in comparison to other samples. The ferric chloride test for the presence of tannins gave no color change. This indicated that it did not have a considerable quantity of tannins. The alkaline reagent test for the presence of flavonoids gave a positive result with the development of yellow color of least intensity. This may be attributed to the presence of very less quantity of flavonoids in the plant.

The current study gives a comprehensive analysis of the five selected plant materials. The parameters of physical evaluation as organoleptic properties, pH and moisture content, help

in determining the purity of the raw plant material. The results for these test parameters give an estimate of the characteristic feature of the plant material, its identification and authentication. This may help in identifying and characterizing the most prominent active constituent in the plant. This can be seen in case of samples BPD-01/Feb/21 and BPD-03/Feb/21. Both these samples have a tangy slightly acidic taste. It may be assumed that these plants could be a good source for Vitamin C. Hence, they can be used in treatment of cold, cough, allergies and enhancement of immunity in general. The results of this study also reveal the different types of phytochemicals present in these plants. The phytochemical screening also gives an insight into the type of specific phytochemical present in the plant. This can be observed by Braymer's ferric chloride test which helps to identify the type of tannin present as per the color observed.

The present work also standardizes the procedure for preparation of plant extract from the raw plant material with distilled water as the solvent system for extraction by cold maceration. This will help to eliminate the requirement for a specific solvent system for extraction as chloroform: water or alcohol. A comparative study may also be performed to analyze the change in properties of the plant extract with the change in the solvent system for extraction.

Further study on identifying the active constituents in these plants needs to be performed using techniques of chromatography as TLC, HPLC. Spectroscopic studies using NMR and GC-MS techniques will also help in conforming the results. This will help in defining the application of the plant and the diseases against which it can be used.

2.7 Conclusion

The ancient system of medicine has been widely followed globally, since the beginning of civilization. It has been prevalent not only in countries as India and Egypt, which are known for their supremacy of traditional knowledge but also by the natives of America and Europe, which are considered to be more scientifically advanced than the rest of the world. Traditional knowledge has provided information on various common and rare diseases, both in terms of their known pharmacological facts and probable medical cure. This wealth is being extensively exploited by many people to develop and provide "novel" means of understanding and curing a disease, but has even led to biopiracy and non-ethical usage of traditional information.

The formation of committees to protect this ancient knowledge and even develop it further by developing means and parameters to scientifically validate it, is now considered a necessity. The WHO, and in India, the Ministry of AYUSH, Government of India, have developed and shared guidelines on the usage of the ancient system of medicine and validation of its knowledge. These guidelines contain a set of test parameters that need to be checked for validating this traditional knowledge.

The five plants considered in the current study, have been extensively used since time immemorial. Their applications include treatment of simple common diseases as cough and cold, besides preparation of complex formulations for advanced disorders. These plants even find applications in the daily kitchen for their aromas and taste. They hold important therapeutic and pharmacological properties including anti-allergic, anti-fungal and anti-biotic. The scientific validation of these plants as per the AYUSH guidelines, not only provides a substantiated knowledge on these plants and their active constituents but also gives an insight into the vast plethora of phytochemicals they possess and which may have a role in conferring these plants their pharmacological properties.

A more detailed insight into the phytochemical screening of these plants and their extracts needs to be performed to identify the active constituent responsible for its various therapeutic properties. Their role on a chemical and biological level, in the treatment of the various diseases, for which they are extensively used, also need to be assessed.

References

CHAPTER I:

1-J. L. Hartwell, “α-Peltatin a new compound isolated from Podophyllum peltatum.” Journal of the American Chemical Society, vol. 69, no. 11, pp. 2918-2918, 1947,

2-D. E. Jackson and P. M. Dewick, “Aryltetralin lignans from Podophyllum hexandrum and Podophyllum peltatum.” Phytochemistry, vol. 23, no. 5, pp. 1147-1152, 1984

3-J. L. Hartwell and A. W. Schrecker, “The Chemistry of Podophyllum.” Fortschritte der Chemie organischer Naturstoffe / Progress in the Chemistry of Organic Natural Products / Progrès dans la Chimie des Substances Organiques Naturelles, pp. 83-166, 1958

4-A. Giri and M. Lakshmi Narasu, "Production of podophyllotoxin from Podophyllum hexandrum: a potential natural product for clinically useful anticancer drugs", Cytotechnology, vol. 34, no. 12, pp. 17-26, 2000.

5-Y. You, "Podophyllotoxin Derivatives: Current Synthetic Approaches for New Anticancer Agents", Current Pharmaceutical Design, vol. 11, no. 13, pp. 1695-1717, 2005.

6-H.C Goel, R Arora, J. A Prasad Process for Preparation of a Radioprotective Herbal Formulation.Indian patent filed, Patent Office, New Delhi, India; 2001

7-H. Goel, H. Prakash, A. Ali and M. Bala, "Podophyllum hexandrum modulates gamma radiation-induced immunosuppression in Balb/c mice: Implications in radioprotection", Molecular and Cellular Biochemistry, vol. 295, no. 1-2, pp. 93-103, 2006.

8-S. Wani, K. Shah and M. Ahmad, "Antifungal activities of methanolic extracts of Podophyllum hexandrum and Rheum emodi against human pathogenic fungal strains", International Journal of Pharmaceutical Sciences Review and Research, vol. 19, no. 2, pp. 56-59, 2013.

9-“WHO model list of essential medicines, 20th list (March 2017 ..” https://apps.who.int/iris/handle/10665/273826 (accessed: Apr. 28, 2021).

10-P. Kumar, T. Pal, N. Sharma, V. Kumar, H. Sood and R. Chauhan, "Expression analysis of biosynthetic pathway genes vis-à-vis podophyllotoxin content in Podophyllum hexandrum Royle", Protoplasma, vol. 252, no. 5, pp. 1253-1262, 2015.

11-T. Vogt, "Phenylpropanoid Biosynthesis", Molecular Plant, vol. 3, no. 1, pp. 2-20, 2010.

12-H. Stähelin and A. von Wartburg, "The chemical and biological route from podophyllotoxin glucoside to etoposide: ninth Cain memorial Award lecture.", Cancer Res., vol. 51, no. 1, pp. 5-15, 1991.

13-J. Hartwell, "α-Peltatin, A New Compound Isolated From Podophyllum peltatum", Journal of the American Chemical Society, vol. 69, no. 11, pp. 2918-2918, 1947

14-J. Hartwell and A. Schrecker, "The Chemistry of Podophyllum", Fortschritte der Chemie organischer Naturstoffe / Progress in the Chemistry of Organic Natural Products / Progrès dans la Chimie des Substances Organiques Naturelles, pp. 83-166, 1958.

15-A. Nag, “Spatial transcriptional dynamics of geographically separated genotypes revealed key regulators of podophyllotoxin biosynthesis in Podophyllum hexandrum.” Industrial Crops and Products, vol. 147, p. 112247, 2020,

16-S.L.B. Arneaud and J. R. Porter, “Investigation and Expression of the Secoisolariciresinol Dehydrogenase Gene Involved in Podophyllotoxin Biosynthesis.” Molecular Biotechnology, vol. 57, no. 11, pp. 961-973, 2015

17-D. Bhattacharyya, R. Sinha, S. Ghanta, A. Chakraborty, S. Hazra, and S. Chattopadhyay, “Proteins differentially expressed in elicited cell suspension culture of Podophyllum hexandrum with enhanced podophyllotoxin content.” Proteome Science, vol. 10, no. 1, p. 34, 2012

18-L. B. Davin, “Stereoselective Bimolecular Phenoxy Radical Coupling by an Auxiliary (Dirigent) Protein Without an Active Center.” Science, vol. 275, no. 5298, pp. 362-367, 1997

19-D. R. Gang, “Regiochemical control of monolignol radical coupling: A new paradigm for lignin and lignan biosynthesis.” Chemistry & Biology, vol. 6, no. 3, pp. 143-151, 1999

20-Z.-Q. Xia, M. A. Costa, J. Proctor, L. B. Davin, and N. G. Lewis, “Dirigent-mediated podophyllotoxin biosynthesis in Linum flavum and Podophyllum peltatum.” Phytochemistry, vol. 55, no. 6, pp. 537-549, 2000

21-S. C. Halls and N. G. Lewis, “Secondary and Quaternary Structures of the (+)-Pinoresinol-Forming Dirigent Protein†.” Biochemistry, vol. 41, no. 30, pp. 9455-9461, 2002

22-L. B. Davin and N. G. Lewis, “An historical perspective on lignan biosynthesis: Monolignol, allylphenol and hydroxycinnamic acid coupling and downstream metabolism.” Phytochemistry Reviews, vol. 2, no. 3, pp. 257-288, 2003,

23-S. C. Halls, L. B. Davin, D. M. Kramer, and N. G. Lewis, “Kinetic Study of Coniferyl Alcohol Radical Binding to the (+)-Pinoresinol Forming Dirigent Protein†.” Biochemistry, vol. 43, no. 9, pp. 2587-2595, 2004

24-A. Chu, A. Dinkova, L. B. Davin, D. L. Bedgar, and N. G. Lewis, “Stereospecificity of (+)-pinoresinol and (+)-lariciresinol reductases from Forsythia intermedia.” Journal of Biological Chemistry, vol. 268, no. 36, pp. 27026-27033, 1993

25-A. T. Dinkova-Kostova, D. R. Gang, L. B. Davin, D. L. Bedgar, A. Chu, and N. G. Lewis, “(+)-Pinoresinol/(+)-Lariciresinol Reductase from Forsythia intermedia.” Journal of Biological Chemistry, vol. 271, no. 46, pp. 29473-29482, 1996

26-M. Fujita, D. R. Gang, L. B. Davin, and N. G. Lewis, “Recombinant Pinoresinol-Lariciresinol Reductases from Western Red Cedar (Thuja plicata) Catalyze Opposite

Enantiospecific Conversions.” Journal of Biological Chemistry, vol. 274, no. 2, pp. 618-627, 1999

27-T. Min, “Crystal Structures of Pinoresinol-Lariciresinol and Phenylcoumaran Benzylic Ether Reductases and Their Relationship to Isoflavone Reductases.” Journal of Biological Chemistry, vol. 278, no. 50, pp. 50714-50723, 2003

28-A. Nag, “Spatial transcriptional dynamics of geographically separated genotypes revealed key regulators of podophyllotoxin biosynthesis in Podophyllum hexandrum.” Industrial Crops and Products, vol. 147, p. 112247, 2020, doi: 10.1016/j.indcrop.2020.112247.

29-C.Y. Chen, “Candidate genes involved in the biosynthesis of lignan in Schisandra chinensis fruit based on transcriptome and metabolomes analysis.” Chinese Journal of Natural Medicines, vol. 18, no. 9, pp. 684-695, 2020, doi: 10.1016/s1875-5364(20)60007-3.

30-Davin, L. B., Wang, H. B., Crowell, A. L., Bedgar, D. L., Martin, D. M., Sarkanen, S., & Lewis, N. G. (1997). Stereoselective bimolecular phenoxy radical coupling by an auxiliary (dirigent) protein without an active center. Science, 275(5298), 362-367.

31-D. Gang et al., "Regiochemical control of monolignol radical coupling: A new paradigm for lignin and lignan biosynthesis", Chemistry & Biology, vol. 6, no. 3, pp. 143-151, 1999. Available: 10.1016/s1074-5521(99)89006-1

32-S. Halls and N. Lewis, "Secondary and Quaternary Structures of the (+)-Pinoresinol-Forming Dirigent Protein†", Biochemistry, vol. 41, no. 30, pp. 9455-9461, 2002. Available: 10.1021/bi0259709

33-S. Halls, L. Davin, D. Kramer and N. Lewis, "Kinetic Study of Coniferyl Alcohol Radical Binding to the (+)-Pinoresinol Forming Dirigent Protein†", Biochemistry, vol. 43, no. 9, pp. 2587-2595, 2004. Available: 10.1021/bi035959o

34-M. Kim, J. Jeon, L. Davin and N. Lewis, "Monolignol radical–radical coupling networks in western red cedar and Arabidopsis and their evolutionary implications", Phytochemistry, vol. 61, no. 3, pp. 311-322, 2002. Available: 10.1016/s0031-9422(02)00261-3.

35-V. Burlat, M. Kwon, L. Davin and N. Lewis, "Dirigent proteins and dirigent sites in lignifying tissues", Phytochemistry, vol. 57, no. 6, pp. 883-897, 2001. Available: 10.1016/s0031-9422(01)00117-0.

36-L. Davin and N. Lewis, "Dirigent Proteins and Dirigent Sites Explain the Mystery of Specificity of Radical Precursor Coupling in Lignan and Lignin Biosynthesis", Plant Physiology, vol. 123, no. 2, pp. 453-462, 2000. Available: 10.1104/pp.123.2.453

37-C.Kazenwadel, J. Klebensberger, S.Richter, J.Pfannstiel, U.Gerken, B.Pickel, et al. “Optimized expression of the dirigent protein AtDIR6 in Pichia pastoris and impact of glycosylation on protein structure and function.” Applied Microbiology and Biotechnology, 97, 7215–7227, 2013

38-B. Youn, S. Moinuddin, L. Davin, N. Lewis and C. Kang, "Crystal Structures of Apo-form and Binary/Ternary Complexes of Podophyllum Secoisolariciresinol Dehydrogenase, an Enzyme Involved in Formation of Health-protecting and Plant Defense Lignans", Journal of Biological Chemistry, vol. 280, no. 13, pp. 12917-12926, 2005. Available: 10.1074/jbc.m413266200.

39-J. Marques et al., "Next Generation Sequencing in Predicting Gene Function in Podophyllotoxin Biosynthesis", Journal of Biological Chemistry, vol. 288, no. 1, pp. 466-479, 2013. Available: 10.1074/jbc.m112.400689

40-C. Canel, R. Moraes, F. Dayan and D. Ferreira, "Podophyllotoxin", Phytochemistry, vol. 54, no. 2, pp. 115-120, 2000. Available: 10.1016/s0031-9422(00)00094-7

41-W.Lau, E.S.Sattely, “Six enzymes from mayapple that complete the biosynthetic pathway to the etoposide aglycone.” Science 349, 1224–1228, 2015

42-S. Hazra and S. Chattopadhyay, "An overview of lignans with special reference to podophyllotoxin, a cytotoxic lignan", Chemical Biology Letters, vol. 3, no. 1, pp. 1-8, 2016

43-R. Moraes, E. Bedir, H. Barrett, C. Burandt Jr, C. Canel and I. Khan, "Evaluation of Podophyllum peltatum Accessions for Podophyllotoxin Production", Planta Medica, vol. 68, no. 4, pp. 341-344, 2002. Available: 10.1055/s-2002-26740

44- R. Moraes, C. Burandt, M. Ganzera, X. LI, I. Khan and C. Canel, "The American mayapple revisited—podophyllum peltatum—still a potential cash crop?", Economic Botany, vol. 54, no. 4, pp. 471-476, 2000. Available: 10.1007/bf02866546.

45-R. Rust and R. Roth, "Seed Production and Seedling Establishment in the Mayapple, Podophyllum peltatum L.", American Midland Naturalist, vol. 105, no. 1, p. 51, 1981. Available: 10.2307/2425009

46-H. Xu, M. Lv and X. Tian, "A Review on Hemisynthesis, Biosynthesis, Biological Activities, Mode of Action, and Structure-Activity Relationship of Podophyllotoxins: 2003-2007", Current Medicinal Chemistry, vol. 16, no. 3, pp. 327-349, 2009. Available: 10.2174/092986709787002682..

47- C. Canel, R. Moraes, F. Dayan and D. Ferreira, "Podophyllotoxin", Phytochemistry, vol. 54, no. 2, pp. 115-120, 2000. Available: 10.1016/s0031-9422(00)00094-7.

48-V. Anbazhagan, C. Ahn, E. Harada, Y. Kim and Y. Choi, "Podophyllotoxin production via cell and adventitious root cultures of Podophyllum peltatum", In Vitro Cellular & Developmental Biology - Plant, vol. 44, no. 6, pp. 494-501, 2008. Available: 10.1007/s11627-008-9134-1

49-F. Mingoia, M. Vitale, D. Madec, G. Prestat and G. Poli, "Pseudo-domino palladium-catalyzed allylic alkylation/Mizoroki–Heck coupling reaction: a key sequence toward (±)-podophyllotoxin", Tetrahedron Letters, vol. 49, no. 5, pp. 760-763, 2008. Available: 10.1016/j.tetlet.2007.11.202.

52- R. Ward, "Asymmetric synthesis of lignans", Tetrahedron, vol. 46, no. 15, pp. 5029-5041, 1990. Available: 10.1016/s0040-4020(01)87810-8.

53- Y. Wu, H. Zhang, Y. Zhao, J. Zhao, J. Chen and L. Li, "A New and Efficient Strategy for the Synthesis of Podophyllotoxin and Its Analogues", Organic Letters, vol. 9, no. 7, pp. 1199-1202, 2007. Available: 10.1021/ol0630954.

54- Y. Wu, J. Zhao, J. Chen, C. Pan, L. Li and H. Zhang, "Enantioselective Sequential Conjugate Addition−Allylation Reactions: A Concise Total Synthesis of (+)-Podophyllotoxin", Organic Letters, vol. 11, no. 3, pp. 597-600, 2008. Available: 10.1021/ol8026208

56- S. Arneaud and J. Porter, "Investigation and Expression of the Secoisolariciresinol Dehydrogenase Gene Involved in Podophyllotoxin Biosynthesis", Molecular Biotechnology, vol. 57, no. 11-12, pp. 961-973, 2015. Available: 10.1007/s12033-015-9888-8.

57-P. Kumar, V. Jaiswal, T. Pal, J. Singh and R. Chauhan, "Comparative whole-transcriptome analysis in Podophyllum species identifies key transcription factors contributing to biosynthesis of podophyllotoxin in P. hexandrum", Protoplasma, vol. 254, no. 1, pp. 217-228, 2016.

58- E. Mardis, "The impact of next-generation sequencing technology on genetics", Trends in Genetics, vol. 24, no. 3, pp. 133-141, 2008.

59- "National Center for Biotechnology Information", Ncbi.nlm.nih.gov, 1998. [Online]. Available: https://www.ncbi.nlm.nih.gov/.

60- E. PMC, "Europe PMC", Europepmc.org, 2007. [Online]. Available: https://europepmc.org/

61- "MetaCyc: Metabolic Pathways From all Domains of Life", Metacyc.org, 1997. [Online]. Available: https://metacyc.org/

62-R. Caspi and P. D. Karp, “Using the MetaCyc Pathway Database and the BioCyc Database Collection.” Current Protocols in Bioinformatics, vol. 20, no. 1, 2007, doi: 10.1002/0471250953.bi0117s20.

63- E. Sayers et al., "Database resources of the National Center for Biotechnology Information", Nucleic Acids Research, vol. 48, no. 1, pp. D9-D16, 2019. Available: 10.1093/nar/gkz899

64- The UniProt Consortium, UniProt: a worldwide hub of protein knowledge, Nucleic Acids Research, Volume 47, Issue D1, 08 January 2019, Pages D506–D515, https://doi.org/10.1093/nar/gky1049

65-"BioCyc Pathway/GenomeDatabase Collection", Biocyc.org, 1997. [Online]. Available: https://biocyc.org/

66-R. Caspi et al., "The MetaCyc Database of metabolic pathways and enzymes and the BioCyc collection of Pathway/Genome Databases", Nucleic Acids Research, vol. 36, no., pp. D623-D631, 2007. Available: 10.1093/nar/gkm900

67-T. Madden, "The BLAST Sequence Analysis Tool", in The NCBI Handbook, 2nd ed., US: National Center for Biotechnology Information, 2013

68-J. Marques et al., "Next Generation Sequencing in Predicting Gene Function in Podophyllotoxin Biosynthesis", Journal of Biological Chemistry, vol. 288, no. 1, pp. 466-479, 2012.

69-S. Arneaud and J. Porter, "Investigation and Expression of the Secoisolariciresinol Dehydrogenase Gene Involved in Podophyllotoxin Biosynthesis", Molecular Biotechnology, vol. 57, no. 11-12, pp. 961-973, 2015.

70-Z. Xia, M. Costa, J. Proctor, L. Davin and N. Lewis, "Dirigent-mediated podophyllotoxin biosynthesis in Linum flavum and Podophyllum peltatum", Phytochemistry, vol. 55, no. 6, pp. 537-549, 2000.

71-D. Jackson and P. Dewick, "Aryltetralin lignans from Podophyllum hexandrum and Podophyllum peltatum", Phytochemistry, vol. 23, no. 5, pp. 1147-1152, 1984.

72-C. Canel, R. M. Moraes, F. E. Dayan, and D. Ferreira, “Podophyllotoxin.” Phytochemistry, vol. 54, no. 2, pp. 115-120, 2000, doi: 10.1016/s0031-9422(00)00094-7.

73-A. Broomhead, M. Rahman, P. Dewick, D. Jackson and J. Lucas, "Matairesinol as precursor of Podophyllum lignans", Phytochemistry, vol. 30, no. 5, pp. 1489-1492, 1991. Available: 10.1016/0031-9422(91)84194-w

74- M. C. Marcotullio, A. Pelosi, and M. Curini. “Hinokinin, an Emerging Bioactive Lignan.” Sep. 17, 2014. https://www.mdpi.com/1420-3049/19/9/14862 (accessed: Apr. 28, 2021).

75-P. Yen et al., "Antioxidative lignans from phytochemical extract of Calocedrusformosana florin”, Bioresources, vol. 7,no.3,pp.4122-4131,2012.

CHAPTER II:

1-R. Misra, “Modern drug development from traditional medicinal plants using radioligand receptor-binding assays.” Medicinal Research Reviews, vol.18, no.6, pp.383-402, 1998

2-N. R. Farnsworth, “The Role of Ethnopharmacology in Drug Development.” Ciba Foundation Symposium 154 - Bioactive Compounds from Plants, pp.2-21, 2007

3-A. Nostro, M. Germano, V. D’Angelo, A. Marino, and M. Cannatelli, “Extraction methods and bioautography for evaluation of medicinal plant antimicrobial activity.” Letters in Applied Microbiology, vol.30, no.5, pp.379-384, 2000

4-P.Garg, R.Garg, “Phytochemical screening and quantitative estimation of total flavonoids of Ocimum sanctum in different solvent extract.” TPI, vol.8, no.2, pp.16-21, 2019

5-N. G. Bisset, and M. Wichtl, “Herbal Drugs and Phytopharmaceuticals.” Medpharm GmbH Scientific Publishers, 1994.

6-S.Garg, “Substitute and adulterant plants. Delhi, India.” Periodical Experts Book Agency, 1992

7-N.Tewari, “ Some crude drugs, source, substitute and adulterant with special reference to KTM crude drug market.” Sachitra Ayurved, vol.44, no.4, pp.284–290, 1990

8-P. K. Mukherjee, “Evaluation of Indian Traditional Medicine.” Drug Information Journal, vol.35, no.2, pp.623-632, 2001

9-P. K. Mukherjee, R. K. Harwansh, S. Bahadur, S. Banerjee, and A. Kar, “Evidence-Based Validation of Indian Traditional Medicine: Way Forward.” From Ayurveda to Chinese Medicine, pp.137-167, 2017

10-A. D. Vaidya and T. P. Devasagayam, “Current Status of Herbal Drugs in India: An Overview.” Journal of Clinical Biochemistry and Nutrition, vol.41, no.1, pp.1-11, 2007

11-V. Narayanaswamy, “Origin and development of ayurveda:(a brief history)”. Ancient science of life, vol.1, no.1, pp.1,1981

12-R. Jain and P. Venkatasubramanian “Proposed correlation of modern processing principles for Ayurvedic herbal drug manufacturing: A systematic review.” Ancient science of life, vol.34, no.1, pp.8, 2014

13-A. Gupta, M. Jaiswal and K.P. Prajapati, “Shelf life of Ayurvedic dosage forms-Traditional view, current status and prospective need.”. Indian Journal of Traditional Knowledge, vol. 10, no. 4, pp-672-677, 2011

14-“Ayurvedic Pharmacopeia of India Part I Vol. IX”, Pharmacopeial Commission for Indian Medicine and Homeopathy, Ministry of AYUSH, Government of India, 2016

15-Commission for European Communities “The Rules Governing Medicinal Products in the European Community.” Office for Official Publications of the European Communities, Luxembourg, III, 31,1989

16-T. Y. K. Chan, J. C. N. Chan, B. Tomlinson, J. A. J. H. Critchley, “Chinese Herbal Medicines Revisited: A Hong Kong Perspective.” The Lancet vol.342, 1532,1993

17-“Annual Report 2019-20.” https://www.dabur.com/amp/in/en-us/investor/financial-information/reports/annual-reports (accessed: May 03, 2021).

18-“Disclosures Under Companies Act-Patanjali Ayurved Limited.” https://patanjaliayurved.org/Disclosures-Under-Companies-Act/ (accessed: May 03, 2021)

19-“Annual report.” http://www.emamiltd.in/images/annualreportpdf/20200825100100small460.pdf (accessed: May 03, 2021)

20-Z. Qi, Z., “Who traditional medicine strategy. 2014-2023.” Geneva: World Health Organization, 2013

21-Anonymous, “National policy on traditional medicine and regulation of herbal medicines, Report of a WHO global survey.” World Health Organization, 2005

22-Anonymous, “WHO Traditional medicine Strategy 2014-2023.” World Health Organization, Geneva,2013

23-V. K. Joshi, A. Joshi, and K. S. Dhiman, “The Ayurvedic Pharmacopoeia of India, development and perspectives.” Journal of Ethnopharmacology, vol.197, pp.32-38, 2017

24-V. Malik, “Drugs and Cosmetic Act, 1940”. Eastern Book Company, Lucknow 2003

25-B. Mukerji, “The First Indian Pharmacopoeia.” pp.495-496, 1956

26-A. Singh “Introduction to Ayurvedic Pharmacopoeia of India.” Regulatory and Pharmacological Basis of Ayurvedic Formulations, CRC press, pp.8-13, 2017

27-Anonymous, “The Ayurvedic Formulary of India Part-I,” first English ed. Government of India, Ministry of Health and Family Planning, Department of Health, New Delhi, 1978

28-Anonymous, “The Ayurvedic Formulary of India Part-I”, second revised English ed. Government of India, Ministry of Health and Family Welfare, Department of Indian Systems of Medicine & Homeopathy, New Delhi,2003

29-Agnivesa, “Charaka Samhita by Agnivesa revised by Charaka and Dridhabala with the Ayurveda”, Dipika Commentary of Chakrapanidatta, Edited by Vaidya Jadavji Trikamji Acharya. Chaukhambha Sanskrit Sansthan, Varanasi, 1992

30-Susruta, “Susruta Samhita of Susruta, with the Nibandhasangraha Commentary of Sri Dlhanacharya”, Edited by Vaidya Jadavji Trikamji Acharya. Chaukhambha Orientalia, Varanasi, 1992.

31-D.Frawley and S. Ranade, “Ayurveda, nature's medicine”. Motilal Banarsidass Publishers, 2004

32-S. M. Kupchan, “Drugs from Natural Products—Plant Sources.” Drug Discovery, pp.1-13, 1971

33-G. Sen and K. Bose, “Indian Med.” World, vol.2, 1931

34-N. S. Kline, “Use of Rauwolfia Serpentina Benth. In neuropsychiatric conditions.” Annals of the New York Academy of Sciences, vol.59, no.1, pp.107-132, 1954

35-J. M. Müller, E. Schlittler, and H. J. Bein, “Reserpin, der sedative Wirkstoff ausRauwolfia serpentina Benth.” Experientia, vol.8, no.9, pp.338-338, 1952

36-L. Dorfman, “Die Konstitution des Reserpins. 8. Mitteilung über Rauwolfia-Alkaloide.” Helvetica Chimica Acta, vol.37, no.1, pp.59-75, 1954

37-J. L. Hartwell and A. W. Schrecker, “The Chemistry of Podophyllum.” Fortschritte der Chemie organischer Naturstoffe / Progress in the Chemistry of Organic Natural Products / Progrès dans la Chimie des Substances Organiques Naturelles, pp.83-166, 1958

38-C. Canel, R. M. Moraes, F. E. Dayan, and D. Ferreira, “Podophyllotoxin.” Phytochemistry, vol.54, no.2, pp.115-120, 2000

39-S. Dev, “Ancient-modern concordance in Ayurvedic plants: some examples.” Environmental Health Perspectives, vol.107, no.10, pp.783-789, 1999

40-P. Patel, N. Patel, and R. Goyal, “Quality control of herbal products.” Quality control of herbal products, vol.5, no.45, pp.26-30, 2006.

41-C. Kokate, A. Purohit, and S. Gokhale, “Pharmacognosy.” Nirali Prakashan, pp.6-19, 2005.

42-R. Kumari and M. Kotecha, “A review on the Standardization of herbal medicines.” Int J Pharm Sci Res, vol.7, no.2, pp.97-106, 2016

43-B.Patwardhan, “Ayurveda: The designer medicine.” Indian drugs, vol.37, no.5, pp.215-227, 2000.

44-J. Calixto and J. Barz, “Efficacy, safety, quality control, marketing and regulatory guidelines for herbal medicines (phytotherapeutic agents).” Brazilian Journal of Medical and Biological Research, vol.33, no.2, pp.179-189, 2000

45-S. Brijesh, P. Daswani, P. Tetali, N. Antia, and T. Birdi, “Studies on the antidiarrhoeal activity of Aegle marmelos unripe fruit: Validating its traditional usage.” BMC Complementary and Alternative Medicine, vol.9, no.1, 2009

46-C. Roy and S. Das, “The protective role of Aegle marmelos on aspirin-induced gastro-duodenal ulceration in albino rat model: A possible involvement of antioxidants.” Saudi Journal of Gastroenterology, vol.18, no.3, pp.188, 2012

47-Y. Bansal and G. Bansal, “Analytical methods for standardization of Aegle marmelos: A review.” Journal of Pharmaceutical Education and Research, vol.2, no.2, pp.37, 2011

48-D. Prakash, G. Upadhyay, P. Pushpangadan, and C. Gupta, “Antioxidant and Free Radical Scavenging Activities of Some Fruits.” Journal of Complementary and Integrative Medicine, vol.8, no.1, 2011

49-S. Rahman and R. Parvin, “Therapeutic potential of Aegle marmelos (L.)-An overview.” Asian Pacific Journal of Tropical Disease, vol.4, no.1, pp.71-77, 2014

50- T. P. J. Solomon, A. K. Blannin, “Effects of short-term cinnamon ingestion on in vivo glucose tolerance.” Diabetes, Obesity and Metabolism, vol.9, no.6, pp.895-901, 2007

51-S. Wuu-Kuang, “Taxonomic revision of Cinnamomum (Lauraceae) in Borneo.” Blumea - Biodiversity, Evolution and Biogeography of Plants, vol.56, no.3, pp.241-264, 2011

52-N. Rajeshkumar, M. Therese, and R. Kuttan, “Emblica officinalis Fruits Afford Protection Against Experimental Gastric Ulcers in Rats.” Pharmaceutical Biology, vol.39, no.5, pp.375-380, 2001

53-T. Rao, K. Kumari, B. Netaji, and P. Subhokta, “Ayurveda siddha.” J Res, vol. 6, pp. 213-224, 1985

54-R. P. Rastogi, B. Mehrotra, and R. Pastogi, “Compendium of Indian Medicinal plants. Central drug research institute.” Lucknow and Publications \& Information Directorate, New Delhi, India, vol.2, pp.10, 1993

55-S. Jayshri, and C. Jolly, “Phytochemical antibacterial and pharmacological investigations on Monordica chiranlia and Emblica officinalis.” Ind. J. Pharm. Sci, vol.1, pp.6-13, 1993.

56-K. Jamwal, I. Sharma, and L. Chopra, “Pharmacological investigations on the fruits of Emblica Officinalis.” J. Sci. Ind. Res, vol.18, pp.180-181, 1959.

57-C. Thakur, and K. Mandal, “Effect of Emblica officinalis on cholesterol-induced atherosclerosis in rabbits.” The Indian journal of medical research, vol.79, pp.142-146, 1984

58-T. Vinayagamoothy, “Antibacterial activity of some medicinal plants of Srilanka Ceylon.” J. Sci. Biol, vol.11, pp.50-55, 1982.

59-G. Shanker, “Aonla for your daily requirement of vitamin C.” Indian Hort, 1969

60-Wealth of Asia, CD-ROM, NISCOM, New Delhi, 1998

61-S. Ghosal, “Active constituents of Emblica officinalis: Part I. The chemistry and antioxidative effects of two new hydrolysable tannins, Emblicanin A and B.” Indian J. Chem, vol.35, pp.941-948, 1996.

62-Dictionary of Indian Medicinal plants, CIMAP, Lucknow, pp.340,1988

63-A. K. Pandey, P. Singh, and N. N. Tripathi, “Chemistry and bioactivities of essential oils of some Ocimum species: an overview.” Asian Pacific Journal of Tropical Biomedicine, vol.4, no.9, pp.682-694, 2014

64-D. Singh and P. K. Chaudhuri, “A review on phytochemical and pharmacological properties of Holy basil (Ocimum sanctum L.).” Industrial Crops and Products, vol.118, pp.367-382, 2018

65-M. S. Baliga, “Ocimum SanctumL (Holy Basil or Tulsi) and Its Phytochemicals in the Prevention and Treatment of Cancer.” Nutrition and Cancer, vol.65, pp.26-35, 2013

66-N. Mahajan, S. Rawal, M. Verma, M. Poddar, and S. Alok, “A phytopharmacological overview on Ocimum species with special emphasis on Ocimum sanctum.” Biomedicine & Preventive Nutrition, vol.3, no.2, pp.185-192, 2013

67-S. Balakumar, S. Rajan, T. Thirunalasundari, and S. Jeeva, “Antifungal activity of Ocimum sanctum Linn. (Lamiaceae) on clinically isolated dermatophytic fungi.” Asian Pacific Journal of Tropical Medicine, vol.4, no.8, pp.654-657, 2011

68-S. S. Choudhury, L. Bashyam, N. Manthapuram, P. Bitla, P. Kollipara, and S. D. Tetali, “Ocimum sanctum leaf extracts attenuate human monocytic (THP-1) cell activation.” Journal of Ethnopharmacology, vol.154, no.1, pp.148-155, 2014

69-M. Cohen, “Tulsi - Ocimum sanctum: A herb for all reasons.” Journal of Ayurveda and Integrative Medicine, vol.5, no.4, pp.251, 2014

70-S. Kumar and A. K. Pandey, “Medicinal attributes of Solanum xanthocarpum fruit consumed by several tribal communities as food: an in vitro antioxidant, anticancer and anti-HIV perspective.” BMC Complementary and Alternative Medicine, vol.14, no.1, 2014

71- S. Parmar, A. Gangwal, and N. Sheth, “Solanum xanthocarpum (yellow berried night shade): a review.” Der Pharm Lett, vol.2, no.4, pp.373-383, 2010

72-O. M. Singh and T. P. Singh, “Phytochemistry of Solanum xanthocarpum: an amazing traditional healer.” NISCAIR-CSIR, India, 2010.

73-S. Kumar, U. Sharma, A. Sharma, and A. Pandey, “Protective efficacy of Solanum xanthocarpum root extracts against free radical damage: phytochemical analysis and antioxidant effect.” Cellular and Molecular Biology, vol.58, no.1, pp.174-181, 2012

74-G.P. Vadnere, R.S. Gaud, A.K. Singhai. Vadnere, “Evaluation of anti-asthmatic property of Solanum xanthocarpum flower extracts.” Pharmacologyonline, vol.1, pp.513-522, 2008

75-A. Ghani, “Medicinal plants of Bangladesh - chemical constituents and uses”. Asiatic Society of Bangladesh, Dhaka, 1998

76-S. S. Gupta, M. Rai and N. K. Gupta, “Histamine releasing effects of a few Indian medicinal plants used in bronchial asthma.” Current Science, vol.36, no.2, pp.42-43, 1967

77-I.Z. Saiyed, D.D. Kanga, “Chemische Untersuchung der Früchte von Solanum xanthocarpum. In.” Proc. Indian Acad. Sci., Sect. A, vol.4, pp.255-260, 1936

78-S. Tupkari, A. Saoji, and V. Deshmukh, “Phytochemical study of Solanum xanthocarpum.” Planta medica, vol.22, no.6, pp.184-187, 1972

79-P. C. Josekutty, “Selection and characterization of Solanum xanthocarpum cell line with augmented steroid metabolism.” South African journal of botany, vol. 64, no.4, pp.238-243, 1998

80-“General guidelines for drug development of ayurvedic formulations, Vol-I”, Central council for research in ayurvedic sciences, ministry of AYUSH, govt. of India, New Delhi, 2018

81-Y.-Z. Liang, P. Xie, and K. Chan, “Quality control of herbal medicines.” Journal of chromatography B, vol.812, no.1-2, pp.53-70, 2004

82-C. Sumitra, “Importance of pharmacognostic study of medicinal plants: An overview.” Journal of Pharmacognosy and Phytochemistry, vol.2, no.5, 2014

83-A. Tatiya, S. Surana, S. Bhavsar, D. Patil, Y. Patil, “Pharmacognostic and preliminary phytochemical investigation of Eulophia herbacea Lindl. Tubers (Orchidaceae).” Asian Pac J Trop Disease, vol.812, no.1-2, pp.53-70, 2012

84-R. Kaur, B. Singh, and S. Kaur, “Pharmacognostic studies on leaves of Ageratum conyzoides Linn.” Journal of Pharmacognosy and Phytochemistry, vol.7, no.3, pp.3181-3185, 2018.

85-V. K. Dixit, R. Gupta, N. Singh, R. Arjariya, and A. Irchhaiya, “Standardization Parameters for Herbal Formulation, Avipattikar Churnam.” Journal of Drug Delivery and Therapeutics, vol.9, no.4, pp.579-584, 2019

86-S. Rashid, “Authentication of herbal drug Tukhm-e-balango (Lallemantia royleana Benth.) using microscopic, pharmacognostic, and phytochemical characterization.” Microscopy Research and Technique, vol.82, no.6, pp.731-740, 2019

87-D. D. Sarvade, “Quantification of total alkaloid, tannin, flavonoid, phenolic, and chlorogenic acid contents of Leea macrophylla Roxb. Ex Hornem.” International Journal of Green Pharmacy, vol.14, no.2, 2020

88-R. Naz, “Antimicrobial activity, toxicity and anti-inflammatory potential of methanolic extracts of four ethnomedicinal plant species from Punjab, Pakistan.” BMC Complementary and Alternative Medicine, vol.17, no.1, 2017

89-R. Y. M. Ryan, A. Fernandez, Y. Wong, J. J. Miles, and I. E. Cock, “The medicinal plant Tabebuia impetiginosa potently reduces pro-inflammatory cytokine responses in primary human lymphocytes.” Scientific Reports, vol.11, no.1, 2021

90-I. Cock and L. Kukkonen, “An examination of the medicinal potential of Scaevola spinescens: Toxicity, antibacterial, and antiviral activities.” Pharmacognosy Research, vol.3, no.2, pp.85, 2011

91-G. S. Kumar, K. N. Jayaveera, C. K. Kumar, U. P. Sanjay, B. M. Swamy, and D. V. Kumar, “Antimicrobial effects of Indian medicinal plants against acne-inducing bacteria.” Tropical Journal of Pharmaceutical Research, vol.6, no.2, 2007

92-Y. Kunatsa, C. Chidewe, and C. J. Zvidzai, “Phytochemical and anti-nutrient composite from selected marginalized Zimbabwean edible insects and vegetables.” Journal of Agriculture and Food Research, vol. 2, pp.100027, 2020

93-R. Rao, B.R.. (2015). Re: How high moisture content (%) is acceptable for the plant powder used for Soxhlet extraction?. Retrieved from: https://www.researchgate.net/post/How-high-moisture-content-is-acceptable-for-the-plant-powder-used-for-Soxhlet-extraction/55388325d4c11852078b4580/citation/download

94-S. Pareek and L. Kitinoja, “Aonla (Emblica officinalis Gaertn.).” Postharvest Biology and Technology of Tropical and Subtropical Fruits, pp.65, 2011

95-G. Alam and A. K. Mishra, “Traditional and Modern Approaches for Standardization of Herbal Drugs: A Review.” Acta Biomedica Scientia, vol.2384, 2017

96-S. Rashid, “Authentication of herbal drug Tukhm-e-balango (Lallemantia royleana Benth.) using microscopic, pharmacognostic, and phytochemical characterization.” Microscopy Research and Technique, vol.82, no.6, pp.731-740, 2019

97- J. Thomas and K. Kuruvilla, “Cinnamon.” Handbook of Herbs and Spices, pp.182-196, 2012

98- K.R. Price, I.T. Johnson, G.R. Fenwick and M.R. Malinow, “The chemistry and biological significance of saponins in foods and feeding stuffs”. Critical Reviews in Food Science & Nutrition, vol.26, no.1, pp.27-135, 1987

99-T. White, “Tannins—their occurrence and significance.” Journal of the Science of Food and Agriculture, vol. 8, no.7, pp. 377-385, 1957

100-G. Trease and W. Evans, “Pharmacognosy.” English Language Book Society, Bailliere Tindall, Britain, 1989

101-M. A. Bharudin, S. Zakaria, and C. H. Chia, “Condensed tannins from acacia mangium bark: Characterization by spot tests and FTIR.” AIP Conference Proceedings, vol.1571, no.1, pp.153-157, 2013

102-A. Samanta, G. Das and S. Das, “Roles of flavonoids in Plants.” International Journal of pharmaceutical science and technology, vol.6, pp.12-35, 2011

103-S.S. Mali, R.L. Dhumal, V.D. Havaldar, S.S. Shinde, N.Y. Jadhav and B.S. Gaikwad, “A systematic review on Aegle marmelos (Bael).” Research Journal of Pharmacognosy and Phytochemistry, vol.12, no.1, pp.31-36, 2020

104-I. Rawat, N. Verma and K. Joshi, “Cinnamon (Cinnamomum zeylanicum).” Jaya Publishing House, pp.164-177, 2019

105-P. Pattanayak, P. Behera, D. Das, and S.K. Panda, “Ocimum sanctum Linn. A reservoir plant for therapeutic applications: An overview.” Pharmacognosy reviews, vol. 4, no.7, pp.95, 2010

106-S. Parmar, A. Gangwal, and N. Sheth, “Solanum xanthocarpum (yellow berried night shade): a review.” Der Pharm Lett, vol.2, no.4, pp.373-383, 2010