"ENHANCED POLYPHENOL PRODUCTION IN MEDICINAL PLANTS USING TISSUE CULTURE"

A DISSERTATION

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REQUIREMENTS FOR THE AWARD OF THE DEGREE

OF

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IN

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Submitted by:

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CANDIDATE'S DECLARATION

I, Shruti Gautam, 2K19/MSCBIO/12 hereby certify that the work which I presented in the Major Project entitled 'Enhanced polyphenol production in medicinal plants using tissue culture ' in fulfillment of the requirement for the award of the Degree of Masters of Science in Biotechnology and submitted to the Department of Biotechnology, Delhi Technological University, Delhi is an authentic record of my own, carried out during a period from 7-Jan-2021 to 28-May-2021, under the supervision of Dr. Navneeta Bharadvaja.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other University. The work has been communicated in Scopus indexed journal with the following details:

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CERTIFICATE

I hereby certify that the Project dissertation titled "**Enhanced polyphenol production in medicinal plants using tissue culture**" which is submitted by **Shruti Gautam**, **2K19/MSCBIO/12**, Department of Biotechnology, Delhi Technological University, Delhi in partial fulfillment of the requirement for the award of the degree of Master of Science, is a record for the project work carried out by the student under my supervision. To the best of my knowledge this work has not been submitted in part or full for any Degree or Diploma to this University or elsewhere.

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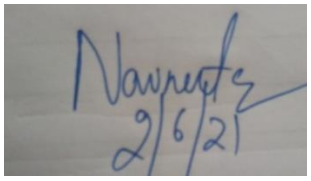

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ABSTRACT

Medicinal plants are an indispensable source of phytochemicals. Polyphenols are one of the most prominent bioactive components produced by medicinal plants. Polyphenols are the plant's secondary metabolites which are secreted in response to any kind of abiotic or biotic stress. The stress can be due to harmful UV rays, pathogen attack, infection, temperature variation, nutrient deficiency or any kind of infection. These polyphenols are responsible for providing medicinal properties to plants. The polyphenols are great therapeutic agents for humans and are widely used for manufacture of herbal drugs. The polyphenols have found to be strong antioxidant properties and protects epidermal layer from various damages. Polyphenols when either supplied in diet or applied topically have found to be protective effects on skin. It is known to have anti-inflammatory, anti-oxidant, anti- carcinogenesis, anti- melanogenesis property. For meeting such a high demand of polyphenols for therapeutic use, sustainable production of polyphenols is necessary as their extraction from natural sources leads to extinction of rare medicinal plant species. For this, the invitro plant tissue culture proves to be an efficient method of propagating rare medicinal plants and optimizing various environmental conditions for maximizing the production of polyphenols.

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ABBREVIATIONS

UV	Ultra violet radiations
ROS	Reactive Oxygen Species
Mg	Magnesium
IAA	Indole acetic acid
EGCG	Epigallocatechin gallate
FC reagent	Folin’s Ciocalteau reagent
HPLC	High Performance Liquid Chroatography
MS media	Murashige and Skoog medium

CHAPTER 1. INTRODUCTION

1.1 General Introduction

The present project focuses on the enhanced polyphenol production in medicinal plants. The role of plant polyphenols in skin protecting effects has also been reviewed. Medicinal plants have ample source of bioactive phtochemicals. These phytochemicals are responsible for plant's medicinal property. Out of these phytochemicals, polyphenols are one of the prominent bioactive compounds found in medicinal plants. Polyphenols are plant's secondary metabolites produced due to different kind of stress condition. The stress can be of external or internal types. External stress is caused due to environmental conditions such as temperature, UV radiations, pathogen attack, any microbial infection etc. Polyphenols contains large number of family of naturally occurring metabolites. They can differ from each other in terms of their structure and properties. They are found in different plant part's ie. stem, leaves, bark, fruits, flower etc., due to the presence of these secondary metabolites there is some kind of color, taste, astringency and bitterness in them. These secondary metabolites are responsible for the plant's medicinal properties. Secondary metabolites protect the plants against harmful UV radiations, plant pathogen and protect them against infections due to microbes [1]. Polyphenols have anticarcinogenesis, antihyperpigmentive, antioxidative, antiaging, antibacterial and anti-inflammatory properties. Various researches have proven that the polyphenols are very good antioxidants that are directly responsible for their neutralizing capacity of free radicals produced as a byproduct of various metabolic processes occurring in body. Grapes, teas, strawberries, artichoke, parsley all contains the highest amount of polyphenols. Many fruits such as apples, grapes and different kinds of berries has approximately 300mg of polyphenols per 100 g of fresh weight [2].

Due to wide medicinal benefits of polyphenols, these are being incorporated in therapeutic sector. They are widely used in industries for manufacture of herbal products, natural therapeutics, nutraceuticals, cosmetic sector etc. The growing trend of using plant based

cosmetics has made polyphenols a top ingredient for research purpose. Skin being the delicate organ of human body continuously gets influenced by the various external and internal factors. The major cause of skin damage has been attributed to the damage caused by harmful UV radiations. This creates oxidative stress to skin causing cutaneous damage. Oxidative stress may be responsible for causing cancer, premature aging, pigmentation, inflammation, redness etc. [3]. Although various chemically derived synthetic cosmetics are available but they pose skin problems and further deteroites the skin condition. This concern has led to the development of plant based products for treating skin problems. Several research findings prove that plant polyphenols have ability to correct various skin diseases. The research for skin care products need not necessarily confined only to improve skin condition but to prevent various skin disorders too.

Plants being the indispensable resource for health, nutrition, medicine, welfare of animals has provided raw material for synthesis of various medicinal products and health additives [4]. Plants are the chief source that provides the active compounds with various medicinal properties. They have never ending opportunities to treat human diseases. According to WHO more than 80% population all over the globe use plant products as a primary treatment for any disease. The contribution of the drugs extracted from medicinal plants is very high. More than 78% drugs belongs to antimicrobial types and anticancer types. The annual turnover of herbal medicines is very high. Due to their wide pharmacological benefits, they can have protective effects on maintaining good health. These bioactive compounds when used appropriately can inhibit several diseases.

These metabolites can be extracted from naturally existing plants but due to environmental and regional limitations the commercial production is highly affected. Conventional natural process of plant growth is time consuming and takes much time for the production of desired secondary metabolites.To overcome this problem, invitro plant tissue culture is an alternative method for production of medicinal plants in nutrient media and extracts the secondary metabolites for commercial use within short time span. Plant tissue culture ensures the bulk propagation of plant species without any seasonal constraints in optimized environmental condition [5]. Plant tissue culture ensures continuous supply of metabolites without any potential harm to the natural species. There are several factors which affects the production or synthesis of secondary

metabolites in plant tissue culture. These factors in turn can be termed as elicitors which are known to enhance the production of polyphenols. Plant tissue culture ensures multiplication of rapid disease free plants. The rare genotypes species can be biotechnologically enhanced through plant tissue culture. Plant tissue culture also prevents habitat destruction and loss of genetic diversity.

Plant tissue culture is an effective method in plant biotechnology to multiply genetically similar plant species in defined nutritive media in optimum controlled sterile environmental conditions. This technology proves to be advantageous than plant growth by conventional methods. The production of plants through plant tissue culture technique totally depends on the totipotency of a particular plant species. The production of plants in controlled environment in conventional methods is a cumbersome process which is easy task in plant tissue culture. The plant tissue culture technique is helpful for rapid clonal propagation, reducing the stress on natural flora by preserving their genetic stability and producing disease free plants.

1.2 Structural features of polyphenols

These are large macromolecules having a weight of approximately 800 Daltons. This allows them to reach intracellular sites of plant cell by rapidly diffusing through cell membranes. It has been found that the conversion of small polyphenols into larger non hydrolysable tannins generally remain undetected in plant matrix. The polyphenols are formed of repeating phenolic entities connected together by esters or C-C bond. These phenolic moieties are resorcinol, pyrocatechol, phloroglucinol and pyrogallol. The green tea polyphenol epicatechin and catechin are formed of monomeric unit proanthocyanidin [6].

1.3 Biosynthesis of Polyphenols

Different types of phenolic acid such as cinnamic acid and gallic acid are the metabolites produced in shikhimic acid pathway. Primary metabolism in plant is responsible for biosynthesis

of complex flavonoids. Flavonoids are produced through mitochondrial and plastid derived intermediates. These intermediates are then exported into cytoplasm from there these get incorporated into different parts of molecule. There are total 3 ring structures in flavonoids in which ring B and chromane ring are formed from phenylalanine amino acid. This phenylalanine is itself a product of shikhimic acid pathway. The ring A originates from 3 units of malonyl-CoA added as a result of decarboxylation- condensation reaction, initiating flavonoids synthesis. In phenylpropanoid pathway the enzyme that performs the conversion of phenylalanine to cinnamic acid is Phenylalanine ammonia lyase. This forms C6-C3 structures. Enzyme chalcone synthase catalyzes the formation of first flavonoids naringenin after the condensation reaction of 3 molecules of malonyl-CoA and 4-coumaroyl-CoA. After that the isomerization of chalcone occurs to form flavanones by chalcone flavanone isomerase. This flavanone formed is an intermediate for the synthesis of all the different classes of flavonoids [7].

1.4 ORGANIZATION OF THESIS

The following thesis title as ‘Enhanced polyphenol production in medicinal plants using tissue culture” is a reviewed information gathered from various research and review article. The thesis focuses on the enhancement of polyphenols through biotechnological approaches. The thesis also contains information on the effects of plant polyphenols in treating various skin diseases.

The chapter1 ie. Introduction of thesis briefs about the plant polyphenols, their structural features, their biosynthesis, their role in therapeutics, role of polyphenols in skin diseases and the brief about plant tissue culture,

The chapter 2 is a review of literature which contains a broad knowledge about the plant polyphenols and its types. It summarizes various plants and their polyphenols being useful for treating skin diseases. It also contains the invitro production of polyphenols through plant tissue culture techniques and the various types of abiotic elicitors that can be used in plant tissue culture for enhancement of polyphenols and future prospects.

The chapter 3 is a proposed methodology for the project and briefs about the extraction process, estimation techniques that can be used for detection of polyphenols.

The chapter 4 contains result, discussion and conclusion part.

CHAPTER 2. REVIEW OF LITERATURE

2.1 Polyphenols

Polyphenols are produced in plants as secondary metabolites due to various kinds of stress. Many biotic and abiotic factors such as UV, temperature, infection, microbial attack etc. create stress condition in plants. These secondary metabolite imparts medicinal and healthy properties to plants. Polyphenols are the prominent bioactive compounds being used for commercial purposes due to their antioxidative, antitumorogenesis, antiviral, antimicrobial properties. Polyphenols can be found in different plant parts such as fruit, flowers, leaves, roots, stem, bark etc. Due to various metabolites the characteristic feature of colour, taste, aroma, astringency and bitterness is imparted to plant part. The two main function of polyphenols in plants are to assist in their physiological functions and other is to act as a defense system [8].

Secondary metabolites are derivative of primary metabolites like carbohydrates and amino acids, derived through various pathways like hydroxylation, methylation and glycosylation. The monomeric unit responsible for different kinds of polyphenols is phenol ring. There are about 10,000 different varieties of polyphenols, each containing one or more aromatic ring attached to hydroxyl group [9]. These different types of polyphenolic entities are derived either from phenylalanine or shikimic acid pathway.

2.2 Types of polyphenols

Polyphenols are classified on the basis of number of phenolic ring present and the attachment of structural element that bind to these rings. They are broadly classified as phenolic acids, flavonoids, stibenes, tannin, lignan and these classes are further subdivided. Flavonoids comprises of approximately half the number of polyphenols.

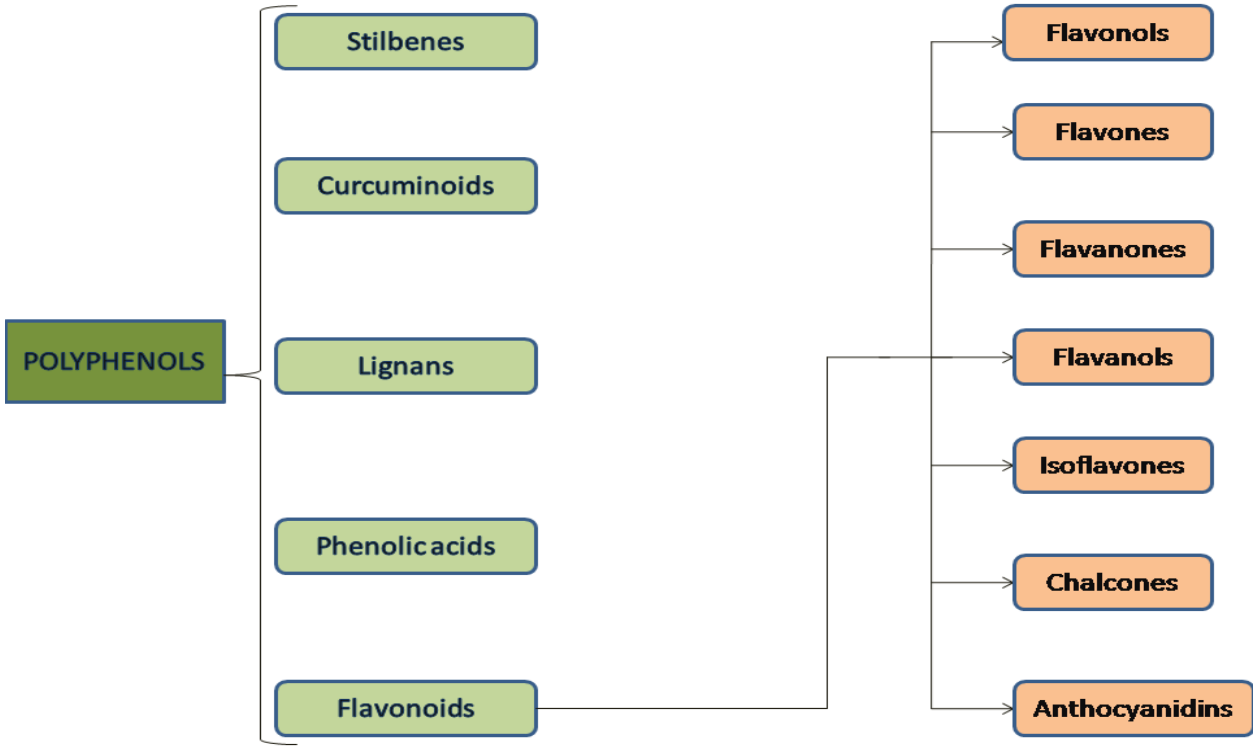

Fig. 1 Broad classification of polyphenols

Flavonoids : Flavonoids are the largest group of secondary metabolites under polyphenols. Till now more than 5000 flavonoids have been isolated and identified. The flavonoids structure similarity is with chalcones, having chemical structure of 1,3-diphenylpropan-1- one [10]. Polyphenols belong to low – molecular weight phenolic compounds prominently found in plants. They are further classified into subclasses flavones, anthocyanin, chalcones, flavonols, flavonones, flavanols, catechin and isoflavones [11].

They are most commonly used compounds in cosmetics for their antioxidant and soothing nature. The non- specific activity flavonoids exhibit are anti-oxidant, anti- inflammatory, absorption of UV, free radical neutralization, metal chelation, microbial growth inhibition etc. [11].

Phenolic acids: Phenolic acids are the most prominent available polyphenol in plant tissue comprises of hydroxycinnamic acids. The subtypes of hydroxycinnamic acids present in plants are ferulic acid, caffeic acid, coumaric acid, chlorogenic acid and sinapic acid. Among all these hydroxycinnamic acids, caffeic acid is most commonly widespread, majorly in coffee, olive, lettuce, potato, spinach, apples, wine, tobacco leaves. The other group of phenolic acids is hydroxybenzoic acids which comprises vanillic acid, syringic acid, p-hydroxybenzoic acid and gallic acid, ellagic acid, gentisic acid [12]. Hydroxycinnamic acids are present as simple esters with glucose or hydroxyl carboxylic acids whereas the hydroxybenzoic acids are mainly present as glucosides. Phenolic acids are potent antioxidants not only in terms of their ability to scavenge free radicals but also in strenghthening the endogenous antioxidant defense system of body.

Stilbene : Another subclass of non-flavonoids compounds is stilbene. Stilbene comprises of resveratrol, piceatannol, isorhapontigenin, pterostilbene, rhapontigenin. Stilbenes contain two phenyl moieties which are linked by methylene bridge connecting two carbon molecule [13].Stilbene compounds are majorly found in species of grape wine, sorgum, peanut and other tree species like pine and picea. Stilbenes posses potent antimicrobial and antioxidant properties [14].

Tannins : Another subtype of non- flavonoid group is tannins, which is widely distributed in plants. Tannins are found to have astringent, bitter properties that are known to precipitate or shrink proteins. Tannins can be found in leaf, roots, stem, bud, seed tissue. Tannins are present in *Camellia sinensis* ,nuts, wines etc. Tannins have anti-inflammatory, antimicrobial and anticarcinogenic properties [15]. Tannins are polyphenolic compounds containing sufficient hydroxyl and carboxyl groups which are known to form strong complexes with macromolecular entities and proteins. These have molecular weight ranging from 500 to 3000. Tannins are abundantly found in bark of trees acting as a barrier to protect them from attack of microorganisms. Condensed tannins are known as proanthocyanidins. Proanthocyanidins are widely distributed in red wine, pine bark and grape seeds [16]. Proanthocyanidins also functions as reducing agent in chelating metal ions and scavange free radicals. The condensed tannin present in apple is approximately ten times higher than that that of unripe apple. These proanthocyanidins posses variety of physiological functions and the most common one is their antioxidant properties.

Lignan: Lignans also constitute the non– flavonoid group of polyphenols which are diphenols containing 2,3- dibenzylbutane structure which is formed due to process of dimerization of two residue of cinnamic acid. They are precursor of phytoestrogens and are mainly produced as an antifeedants in seeds and plants against herbivores. These are found in various seeds, fruits, vegetables and the high lignan source are flax seeds and sesame seed. The major lignan possessing various medicinal properties are sesamin, artigenin, (-) secoisolariciresinol, (+) pinoresinol, enterolactone, (+)- lariciresinol etc. These are known to provide several bioactive properties like anti-estrogenic, anti- inflammatory, anti- carcinogenic and antioxidant [17].

Curcuminoids : In curcuminoids, two phenol rings are joined together by linear diarylheptanoid. The curcuminoids present in plants give them yellow colour. The curcuminoids due to its structure is less absorbable and has poor solubility. Among different curcuminoids, curcumin is the most prominent natural compound found in plants. It is used as a herbal supplements for treatment of various diseases [8].

2.3 Role of polyphenols in skin diseases

Being high in pro- oxidants, these polyphenols have skin protective effects and it has been proved by various researches. Given below are the evidences which suggest that polyphenols have skin protecting effects.

2.3.1 Photo protective effects

Polyphenols acts as excellent photo protective agents. They do so by screening and absorbing harmful UV radiations. They have UV absorbing properties due to the different pigments present in them such as yellow, red and purple. It has been found that the topical use of specific polyphenols blocks harmful UV B rays to pass through the stratum cornuem. They have antioxidant, anti- inflammatory, free radical scavenging properties. Polyphenols of green tea such as EGCG, silymarin, anthocyanin can prevent inflammation, DNA damage etc. They are involved in blocking the signaling molecules associated with inflammatory pathways [18].

2.3.2 Anti- oxidant

The natural anti- oxidant system of skin gets continuously affected and damaged from environmental stresses. The UV rays exposure leads to free radical generation leading to oxidative stress and cause damages leading to various skin disorders and formation of tumors. Polyphenols has been found to have very high oxidants level which upon topical application or oral use enhances the endogenous antioxidant defense system. They enhance suppression of lipid peroxidation, and decrease the level of NO and H2O2. Many studies have been found which states that polyphenols interact with the enzymes for modulating redox status of cell. The cell's ability to survive is generally enhanced [19].

2.3.3 Anti aging

Polyphenols are commercially exploited for the manufacture of antiaging cosmetics. Premature skin aging is due to various kinds of extrinsic and intrinsic factors. Intrinsic aging can be due to diseased health such as excessive production of free radicals, physiological changes, nutritional deficiency while extrinsic aging is due to pollution, harmful UV exposure, smoking etc. [20]. It has been found that green tea polyphenols inhibits COX2 expression induced due to UV exposure and inhibits skin carcinoma. EGCG, GTPs, Silymarin etc. polyphenols protect skin from UV induced skin damages [21].

2.3.4 Anti- melanogenesis

The polyphenols are widely used for their anti- tyrosinase activities. Due to excessive exposure to UV rays there may be formation of reactive oxygen species. Due to this there is excessive generation of melanin pigments in the skin. It has been found that the quercetin polyphenol has effective anti- melanogenesis property. These polyphenols block the melanogenesis pathways. Bearberry leaves is rich in arbutin which is an effective inhibitor of melanosomal tyrosinase pathway. Several plant polyphenols can block the proliferation of melanocytes.

2.3.5 Anti- inflammatory properties

The UV exposure in skin initiates the pathways associated with inflammatory responses leading to erythema and sin redness. The COX-2 enzyme expression and enhancement in proteins

relating to inflammation, cutaneous edema, presence of inflammatory cytokines all are characteristic feature for inflammatory responses. The plant polyphenols being anti-inflammatory helps in suppressing inflammation. The EGCG present in green tea is a great anti-inflammatory polyphenols. It reduces the myeloperoxidase activity and proinflammatory cytokines induced due to UV irradiation. Another polyphenol resveratrol inhibits skin thickness induced due to UV irradiation [16].

2.4 Polyphenol rich plants for skin diseases

Plants have adequate source of indispensable bioactive phytochemicals. These bioactive components inturn proves to be useful to humans. There has been considerable growth in industrial sector to manufacture plant based products due to their minimal or no side effects and due to their natural origin. Medicinal plants have polyphenolic components that are being used to manufacture herbal medicines, medicinal products and are widely used across the globe for treatment of various diseases. These phenolic components provide plant with antioxidant, anti-tumorogenesis, anti-depressant, anti- inflammatory, anti- aging etc. features. Given below in table1 are some medicinal plants with their polyphenolic components.

Table1 : Plants with polypenolic content having skin protective effects

Plant species	**Main polyphenols present**	**Properties**	**Reference**
Acacia nilotica	epigallocatechin- 3-gallate, flavonoids and phenolic compounds	anti tumor ,anti inflammatory properties, anti- aging, radical scavenging	[22] [23]

Achillea spp *Achillea sivasica*	1,8-cinneole	Anti- tyrosinase	[24]
Achillea alpine *A. biebersteinii*	3,5- dicaffeoyl quinic acid, Isovitexin 5- caffeoylquinic acid, santin, apigenin pentoside, luteolin derivatives, cirsimaritin, camphor	Antioxidant , Anti- melanogenic anti- pollution, Photoprotectant, anti-carcinogenesis and anti-melanocyte,	[25] [26]
Agastache rugosa kuntze	Phenylpropanoids , terpenoids, rosmarinic acid, tilianin, acacetin, agastachoside, methyl flavones, agastinol agastenol, apigenin and quercetin	Anti-aging, Antioxidant, Anti- elastase and anti-hyaluronidase	[27] [28] [29]
Aspalathus linearis	Aspalathin	UV protectant ,anti-inflammatory anti-aging	[30] [31]
Benincasa hispida	triterpenoids, flavonoids, carotene, glycosides, β sitosterin, saccharides and uronic acids	anti- aging	[32]
Calendula officinalis	Quercetin, terpenoids, triterpenes, saponins, carotenoids, flavonoids,	anti- aging effects skin whitening properties, anti-inflammatory,	[33] [34]

	coumarines	antioxidant properties, photo protective, reduced skin erythema	 [35]
Camellia sinensis	(-)-epicatechin-3-gallate (ECG), (-)-epicatechin(EC), and EGCG	anti- aging anti- carcinogenic anti- inflammatory	[36]. [37]. [38]
Centella asiatica	ursane- and oleanane-type pentacyclic triterpenid, madecasosside, madecassic acid, asiaticoside and Asiatic acid	skin aging anti- hyaluronidase activity, anti-elastase , inhibits H_2O_2 induced antioxidant and anti inflammatory property,	[39] [40] [41]
Coffee Arabica	chlorogenic acid, quinic acid, ferulic acid, hydroxycinnamic acid, cafestol and kahweol	wound healing, antioxidant UV protectant, anti-photo carcinogenesis, anti-inflammatory	[42] [43] [44]
Coriandrum sativum	quercetin, kaempferol, acacetin, p- coumaric acid, vanillic acid, cis and trans form of ferulic acid	Upregulates oxidative defense system. protect from UVB induced skin damage,	[45] [46]
Crataegus pinnatifida Bge.	oligomeric procyanidins and their glycosides, chlorogenic acid,	antioxidant, antiphotoaging collagenase inhibitory	[47] [48]

	rutin, quercetin, isoquercetin, epicatechin, gallic acid	activity, antiphotoaging and anti-inflammatory,	[49] [50]
Curcuma longa	Curcumin Terpenoids, phenolic compounds	anti- aging agent Antioxidant anti- psoriasis agent	[51] [52]
Cyclopia spp.	Xanthones, flavonones, heperidin, mangiferin	antioxidant and anti-inflammatory, reduced signs of skin peeling, sunburn, reduced erythema, edema, skin hardening, modulated epidermal hyperplasia	[53] [31]
Emblica officinalis	flavonoids, phenolic acids like ellagic and gallic acid, tannins like puniglucoin, pedunclagin and emblicanin	Broad – spectrum antioxidant Antitumor Anti-wrinkle Anti-tyrosinase Anti-inflammatory	[54] [55] [56]
Foeniculum vulgare	linolenic, oleic and linoleic acid	Anti-photoaging	[57] [58] [59]
Fragaria vesca L.	flavonoids, catechins, phenolic acids, ellagitannins, proanthocyanidins, ellagic acid	anti- melanogenic, antioxidant, photoprotectant	[60] [61]

Glycine max	genistein and daidzen 7, 3',4'-trihydroxyisoflavone, o-dihydroxyisoflavone, 7, 8, 4'-trihydroxyisoflavone	UV protectant,antiaging Anti-photoaging Anti-inflammatory, Anti-tyrosinase	[62] [63]. [64] [65]
Hippophae rhamnoides	Casuarinin, β- carotene and tocopherol quercetin, kaempferol, isorhamnetin, catechin, flavonoids, quercetin, oleic acid and linoleic acids, procyanidins, quercetin, kaempferol, myricetin, isorhamnetin, kaempferol-3-rutinoside,	antimelanogenic properties, anti- aging effects reduced skin melanin and helped melasma patients, anti inflammatory, UV-induced skin anti aging regulating skin hydration, antioxidant, photoprotectant,	[66] [67] [68] [69] [70] [71]
Hydrangea serrata (Thunb.) Ser	hydrangenol	Photprotectant, Antioxidant,	[72]

		Moisturizing properties, antiaging	[73]
Ixora parviflora	chrysin 5-O-β-D-xylopyranoside, chlorogenic acid	anti photoaging properties, antioxidant photo protective effects	[74] [75]
Mangifera indica	Mangiferin	antioxidant properties, anti- elastase , anti-collagenase, improves the overall cellular response to heat stress, antiaging agent and photoprotectant	[76] [77] [78] [79]
Momordica charantia	charatin, flavonoids, normordin	Cytoprotective, Antioxidative, tissue remodeling, hydrating, moisturizing and antipigmentation properties	[80]
Morus alba	gallic acid, tannins, anthocyanin, flavonoids	Anti-photoaging, Anti- melanogenesis	[59] [81]
Myristica fragrans Houtt	macelignan	Antimelanogenic, inhibition of UVB induced inflammation and photoaging of skin	[82] [83]
Ocimum basilicum	quercetin, gallic acid, caffeic acid, epicatechin, rutin, reseveratrol,	improvement in retaining skin moisture, antiaging effects , antihyperpigmentive,	[58] [59] [84]

	kaempferol, coumaric acid, rosmarinic acid, protocatechuic acid,	antioxidant, anti-photoaging,	[85]
Panax ginseng	chlorogenic acid, syringic acid, kaempferol, quercetin, resveratrol, naringenin, gentisic acid, rutin, catechin, n- and p- coumaric acid , vanillic acid flavonoids	anti- skin aging, photo protective effect, Antimelanogenic, anti-tyrosinase activity	[86] [87] [88] [89] [90]
Patrinia villosa	kaempferol	anti- melanogenic	[91]
Penthorum Chinese pursh	Quercetin	Antioxidant,antiaging agent, UVB protectant	[92]
Polypodium leucotomos	ferulic , caffeic acids , cinnamic acid and chlorogenic acids	Antioxidant, Antiphotoaging, antiphotocarcinogenesis	[93]
Punica granatum	ellagitannins, ellagic acid, punicalagins, anthocyanins	Anti-photoaging, Anti- metastatic, Anti-proliferative, Antioxidant, Photoprotection	[94] [95]
Rhus coriaria L.	tannins, myricetin derivatives, quercetin and gallic acid flavonoids, phenolic acid and gallotannins	antioxidant, anticarcinogenic, antifibrogenic, genoprotective effect	[96] [97]
Silybum marianum	Silibinin	Antitumor,	[98]

	ocadaic acid	Anti- photo carcinogenesis, Antioxidant	[99] [100]
Spatholobus suberectus	Formononetin, butin	anti- tyrosinase activity, inhibiting UVB induced ROS production,	[101] [102]
Terminalia catappa	catechin, ellagic acid, gallic acid, chlorogenic acid, quercetin, isoquercetin, kaempferol, rutin, isoquercetin, kaempferol, rutin, punicalin, ursolic acid, chebulagic acid, isovitexin	antioxidant, immunomodulatory, anti inflammatory properties, metal chelating activity,	[103] [104] [105]
Theobroma cacao L.	monomeric (-) epicatechin and (+) catechin, proanthocyanidin, flavonols	Antioxidant, anti-inflammatory, immunomodulatory, photoprotection	[106]

2.5 Plant tissue culture technique

The extending utilization of these secondary metabolites for industrial manufacture has initiated a need to look for more sustainable production of these secondary metabolites than to pose a threat to natural flora. There has been an increasing pressure upon industries for the manufacture of natural products due to people's changing notion of using natural products. Due to this there

has been an increasing exploitation to natural flora leading to their extinction and a bottle neck effect has been created. To this, plant tissue culture is an emerging technique for enhancement and production of secondary metabolites. Plant tissue culture ensures sustainable, economical and bulk production of metabolites without any geographical dependence or seasonal constraints. It allows uniform production of metabolites.

Plant tissue culture is the technique for propagation of aseptic cells, tissue or organ of plants under defined nutrient media in controlled environmental condition. There are several methods that can be employed for tissue culture but the two main methods are organogenesis and callogenesis. In the process of organogenesis there is development of plant organs ie. root or shoot directly from the meristem or through indirect process of callus formation. These culture then can be used to produce plants through micropropagation or the growth of particular organs, for eg. hairy root culture.

The tissue culture technique needs some steps to be followed which are mentioned below. These are the basic steps that need to be followed:

a Selection of plant of interest based on the study; most preferable a healthy plant should be taken.
b The next step is the culturing of plants invitro, for which the explants is taken out from the parent plant or seeds of that plant can also be used.
c Explants or seeds are then surface sterilized with chemicals. Most commonly used sterilization chemicals are sodium hypochlorite, ethanol, autoclaved distilled water, mercuric chloride etc.
d The sterilized explants are then cultured in defined nutrient media and incubated for some small period of time.
e Screening is done and contaminated explants are discarded, while the healthy cultures are taken for next step and are allowed to grow under defined environmental conditions.

2.5.1Role of media composition in tissue culture

The growth of explants in tissue culture is based on defined nutritive media. The most widely used media in different culture system is MS media ie. Murashige and Skoog. The incorporation

of various inorganic nutrients, carbon source, organic nutrients, nitrogen source and various plant phytohormones significantly affects the plant growth and differentiation in Plant tissue culture.

2.5.1.1 Inorganic nutrients

The plant life is supported due to various mineral elements. Different types of minerals affects plants in different ways. The inorganic nutrients such as calcium, nitrogen, magnesium, molybdenum, iron, zinc, carbon, hydrogen etc. all affects plant growth in some ways. The calcium is a major component in the synthesis of cell wall. Similarly nitrogen in media is responsible for synthesis of proteins, vitamins, nucleic acids and amino acids. Chrolophyll has the presence of magnesium. Various types of enzymes in plants are part of mineral elements like molybdenum, iron and zinc. Carbon, hydrogen and nitrogen are the major structural unit forming in plants. For maximizing the growth of plants in tissue culture these nutrients are meant to be supplied in defined optimum value. Upon addition of different inorganic nutrients in distilled water there is a process of dissociation and ionization. These ions acts as the active factor in the medium supplied to plants. Therefore these nutrients must be added in media at their optimum value.

2.5.1.2 Vitamins

The plants found in their natural habitat are able to synthezise different types of vitamins by themselves for their growth and development. But in tissue culture the vitamins are supplemented to media from outside. Vitamins like nicotinic acid, inositol, thiamine, pyridoxine are supplemented to media at a defined concentration.

2.5.1.3 Carbon source

The sucrose is the most widely carbon source in culturing. But variety of carbon sources can be utilized for different plant species. In tissue culture we can use different carbon source ie. sucrose, fructose, maltose, galactose and starch. These carbohydrates acts as a energy source in plant tissue culture because the plants are not capable of photosynthesizing, therefore carbon source is added from outside. The sucrose is most preffered over other sugar sources as it readily

breaks down into glucose and fructose. Generally, 2-5% sucrose concentration is recommended for plant growth in tissue culture.

2.5.1.4 Growth regulators

Growth regulators in other terms are plant growth hormones. These are organic compounds which plants synthesize on their own in natural environmental condition. In order to synthesize these hormones in tissue culture there are synthetically derived plant growth regulators which work similarly to the natural hormones present in plants. Plant growth hormones are responsible for stimulating signals for stress response. It has been found that the endogenous regulation of growth hormones can majorly affects the acclimatization against stress. There has been studies that indicates their exogenous application can make plants stress tolerance against heavy metals. The plant response is severely affected when any kind of stress is applied but the use of hormones can help plant to tolerate different kind of stresses. There are major five types of plant growth hormones available that are auxins, cytokinins, gibberellins, abscisic acid, and ethylene. The naturally occurring plant growth are isopentenyladenine, Indole Acetic acid, cytokinin, zeatin. But the two main class of plant growth hormone are Auxin and cytokinins.

Auxin

The plant hormone auxin is responsible for inducing cell elongation and its division. They are the major hormone for inducing callus formation. Auxins are responsible for root generation in plants. They are rhizogenic in nature. It has been found that the auxin supply also inhibits axillary and adventitious shoot formation. The effect of auxin on plant growth depends on its concentration supplemented in tissue culturing. It is seen that the high concentration of auxin is responsible for callus formation, whereas auxin in its low concentration enables adventitious root development. The most commonly used auxin are naphthalene acetic acid, p- chlorophenoxy acetic acid, indole butyric acid etc.

Cytokinins

They are derived from adenine. Their basic role is to induce shoot in plants. They are responsible for cell division in plants. Cytokinins are responsible for plant growth and development. Their

basic role is to promote organogenesis in plants. They stimulate bud formation. they function antagonistically to auxins. As at high concentration, cytokinins are known to produce adventitious shoot and inhibits root formation. They decrease apical dominance and promotes axillary shoot formations. The naturally occurring cytokinins are zeatin and 2- isopentyladenine. Kinetins, 6- Benzyladenine are the synthetically derived one. The most commonly used cytokinin are 6- Benzyladenine and 6- Benzyl aminopurine.

Gibberellins

These are most prominently found in roots, young leaves, root tips, active buds and seeds. Their application on whole plant is responsible for stimulating growth of main stem. They play a major role in plant regeneration. During meristem culturing gibberellins are preferred as they induce their growth. The internode elongation in tissue culture is induced by gibberellins. Gibberellins acts as antagonistic to organogenesis. They inhibits shoot and adventitious root formation. They inhibits the process of dedifferentiation.

Abscisic acid

They induce embryogenesis and it is a growth inhibitor. It is produced during the stress in plants. It is also known as dormancy hormones.

Ethylene

Ethylene is a gaseous growth hormone. This is produced by cultured plants in plant tissue culture. The exact role of ethylene is still not known during cell and organ culture. This is usually applied as a powdered form enhances its penetration through plant tissue and liberates ethylene.

2.5.1.5 Gelling agent

Agar is the most widely used solidifying agent in media preparation. Agar has high molecular weight. The concentration of agar decides its binding with water. If the concentration of agar is higher then its binding increases with water. The optimum concentration of agar is very

important in media formation. The high concentration of agar in media makes the media hard and it blocks the penetration of nutrients into tissue.

The synthetic gelling agents are preferred as they are generally clear in appearance at low concentration. This clear gel makes possible the detection of contamination of any type in media.

2.5.1.6 pH

The optimum pH is responsible for plant's specific biological activity. It has been found that the pH ranging from 5.0 to 6.0 is suitable for plants invitro. pH exceeding the pH value 7.0 and lower than 4.5 hampers the plant growth.

2.6 Biotechnological approaches for secondary metabolites production

2.6.1 Micropropagation

It is an invitro technique for production of homogenous genetically similar plants in short period of time. It is a successful technique for enhancement of secondary metabolites in medicinal plants. Optimizing growth and secondary metabolite production can be possible by variating different media composition, altering the nutrient quantity in the media, introduction of heavy metals in the media, abiotic environmental stresses such as change in pH, temperature, light can be applied to plantlets in culture. The invitro shoot culture growth is highly influenced by the growth regulators used. It has been found that the use of thidiazuron and kinetin in micropropagation of *Linum usitatissimum* resulted in enhanced quantity of flavonoids, lignans, polyphenols and antioxidant enzymes in shoot culture [107].

2.6.2 Hairy root culture

In hairy root the explants is infected with bacteria *Agrobacterium rhizogenes*. The bacteria inserts its T-DNA through Ri plasmid and genetically modifies the plant tissue. After some days the hairy root will be induced. The decontaminated roots are subcultured on another liquid media. The infection leads to neoplastic growth of roots in culture media without any hormone.

This is because the production of auxins which is root promoting plant hormone is endogenously produced by the genes present in T-DNA. This ensures high productivity of desired polyphenolic stable products. They can produce metabolites that are produced in roots as well as in aerial parts of plant [108]. Further, the hairy root cultures are manipulated in order to produce volatiles by inserting desired transgene into T-DNA.

2.6.3 Callus culture

The other process of callus culture is an indirect process in which the undifferentiated mass of cells is produced in response to various growth regulators. These undifferentiated mass of cells are then transferred into liquid media and continuously agiatated for cell suspension culture. Further, this callus can regenerate whole plant for the production of industrially important secondary metabolites in bulk [109]. Although, cell suspension culture is less feasible because of many limitations associated with it such as instability of cell lines, slow growth, lower yield of metabolites compared to other methods and problems in scaling up. The elicitation highly affects the synthesis of secondary metabolites in callus culture or cell suspension culture.

The use of cell suspension culture is generally preferred for studying the secondary metabolites synthesis. The cultivation of cells has potential benefits in secondary metabolites production which are:

- The environmental factors such as climate, microbes, pests etc. do not interfere in polyphenols synthesis.
- Any cell of the plant can be made to produce any specific secondary metabolite and these cells can be further multiplied.
- A homogenous same product can be synthesized using a particular characterized plant cell line.
- The metabolic processes occurring in plants and the cell growth can be regulated depending upon the production of metabolites.
- Biotransformation of specific substrate can be possible in cell culture and this biotransformation can lead to production of valuable products due to altering in functioning of enzymatic activity.

- Cells could be manipulated in order to enhance the route of synthesis of metabolites. This can lead to discovery of novel products that are generally not found in plants.

The production of polyphenols ie. secondary metabolites in plant tissue culture is highly affected by several factors such as explants selection, nutrient concentration provided to plant, type of culture media used, growth regulators, salt strength in media provided and various kinds of biotic and abiotic elicitors.

2.7 Role of abiotic elicitors in polyphenol enhancement

There are many environmental stresses that causes damage to plant and are harmful to them such as change in temperature, UV radiation, pH variation, carbon concentration, nitrogen concentration, nutrient concentration, heavy metals etc. the main function of these elicitors is basically to create stress condition in plants under plant tissue culture for secondary metabolite production.

2.7.1 Temperature

The exposure of plant with high or low temperature results in formation of ROS which causes cellular damages in plant. The free radical produced disturbs the structure of protein, DNA and lipid membrane causing cellular damage. To protect itself plant has a number of enzymatic and non- enzymatic mechanisms which lead to production of secondary metabolites. Varying incubating temperature of the culture affects the plants metabolic process and ontogeny. *Eleutherococcus senticosus* somatic embryo when grown in different temperature condition showed variation in growth and production of secondary metabolites. Embryos grown at 12 and 30 degree showed inhibited growth.the chlorogenic acid content was also found to reduce at decreasing temperature. It has also been found that the phenolic content decreased at 12, 18 and 30 degree but increased at 24 degree [110]. There has been studies which suggests that both light and temperature can have synergistic effect on the growth and secondary metabolites production. The callus culture of *Helicteres Isora* L when subjected to varying light and temperature source was found to varying effects on growth and phenolics production. It has been found that the calli

which are irradiated with white light for 28 days showed maximum total phenolic content and total flavonoids content. Similarly, when the calli were exposued to temperature ranging 28 degree celcius showed increase callus growth with increment in phenolic and flavonoids content but reduction in the level of betulinic acid. Betulinic acid content increment to 200% was seen when the calli were incubated at low temperature (13degree celcius) for 28 days [111].

2.7.2 Salt stress

High pH condition means high salt condition; this stress causes cellular dehydration leading to removal of cytoplasmic water resulting in overall decrease in volume of cytoplasm and vacuole. This kind of stress may either lead to accumulation of metabolites or decrease in metabolite production as this stress causes both osmotic and ionic stress in cultured plant [112]. It has been found that the high salt concentration in soyabean is found to increase the production of various secondary metabolites ie. flavonols, saponin, flavones, alkaloids, saponin and flavonoids [113] . Another study on *Spinacia oleracea* suggests production of industrially important secondary metabolite named as 20E [114]. Generally the amount of anthocyanins increases upon salt stress but it is repressed in salt sensitive species [115].

2.7.3 Nutrient stress

Nutrient media is a source of carbon, macronutrients, micronutrients and growth hormone for plant growing in tissue culture. Any change in concentration, form or proportion of nutrient media can severely affect the growth pattern of culture plant and also affects the production of secondary metabolites. Various nutrient availability can affect differently to plants in vitro. It has been seen that the phosphorus stress could lead to decreases plant growth and can be helpful for accumulation of anthocyanin. Magnesium playing the role in chlorophyll formation, ATP synthesis, CO2 synthesis, photosynthetic products assimilation in plant. The stress caused due to Mg has been linked to generation of reaction oxygen species that further affects the production of carotenoids. The rutin production in *Morus albus* is highly affected by the ammonium nitrate concentration . It has been seen that low NH_4^+/NO_3 ratio resulted in higher rutin production that the one containing high ratios in root culture of Morus albus [116].

2.7.4 Carbon

Carbohydrates being the necessary source of carbon for invitro plants also affect the production of secondary metabolites. Sucrose and glucose are two most commonly used sugars added in a basal media at a concentration of 2 to 4%. But, maltose , fructose and other sugars can also be used to check the growth of different plant cells. The choice of a particular sugar totally depends upon the type of parent plant and products to be extracted. In a study, the flavonoids content increased to 72.3g/L from 18.8mg/L upon increasing the amount of sucrose from 10g/L to 50g/L in cell suspension culture of *Glycyrrhiza inflate* [117]. In another study it has been found that the culture of *Vitis vinifera* when supplemented with sucrose or mannitol enhanced the accumulation of anthocyanin upto 1.5 times due to enhanced osmotic pressure due to sugar used [118]. In *Coleus blumei* the yield of rosamarinic acid increased to 3.3g/L from 0.8g/L upon changing the concentration of sucrose in nutrient media to 7.5% to2.5%.

2.7.5 Nitrogen

The nitrate and ammonium ions are the source of nitrogen in culture media such as MS, B5 or LS. In *Vitis Bailey Alicante* cell suspension the anthocyanin production increased upon reducing the nitrate ion concentration and increasing the ammonium ion in turn in culture media. The flavonoids content in *Glycyrrhiza inflate* increased upto 73.1mg/L from 36.4mg/L when the nitrogen concentration was raised from 10 mmol/L to 120 mmol/L in MS medium used for cell suspension culture.

2.7.6 Light

Light is a major limiting factor affecting the growth. It also affects the production of secondary metabolites in plants either growing in nature or under controlled environmental condition. Different plants respond differently to the type of light, intensity of light, exposure duration in their physiological response and production of secondary metabolites. The flavonoid rutin concentration also gets affected when the light variation has been provided to plant Hyptis *marrubioides* Epling. The seedlings cultured in invitro were provided with different variations of light like blue, green, white, yellow and red and has been found that the plants which were irradiated with red light leads to high biomass production and high number of leaves.

The cultures irradiated with white and blue light promoted the increased rutin concentration [119]. Invitro cell suspension culture of *Artemisia absinthium was evaluated for differential light intensity for the secondary metabolite production and biomass growth. The results suggested that light and dark grown cultures significantly affected the biomass production. The total phenolic content, total phenolic production also increased in cultures that were light grown during the log phase [120]*. *The secondary metabolite named as* 6-gingerol and zingiberene was increased in light treated callus culture of *Zingiber officinale* [121]. Panax ginseng cell culture was found to have increased phenolic content upon the combined stress by UV and ultrasonic [117].

These all stresses significantly affects the metabolism of plant along with the synthesis of secondary metabolites. Given below is the list which includes increment in polyphenols in medicinal plants through various stress conditions.

Table 2: Various elicitors for polyphenol enhancement

Type of stress	Plant species	Polyphenol increased	References
Nutrient stress	*Ocimum basilicum*	Rosmarinic acid	[122]
Light stress	*Orthosiphon aristatus*	Total phenolic content, flavonoids	[117]
Light stress	*Betula pendula* and *B. resinifera*	condensed tannins, hydroxycinnamic acids	[123]
UV-B stress	*Kalanchoe pinnata*	flavonoid	[124]
Nutrient stress	*Melissa officinalis* L.	flavonoids	[125]

Heavy metal	*Panax ginseng*	phenolics and flavonoids	[126]
Heavy metal	*Camellia sinensis L.*	flavanoids	
Low pH and temperature	*Silybum marianum*	flavolignans	[116]
Boron stress	*Olea europaea*	phenolic compounds	

2.8 Future prospects

Medicinal plants have gained a lot of importance in alternative medicines and for extraction of health promoting bioactive compounds. The proper knowledge of bioactive chemical present in plant species and its effect on humans is of high importance. Proper identification of plant, its harvesting and post harvesting are important. The phytochemical profile of bioactive compound and its specific activity is greatly affected by the environmental factors and genetics of plants. Hence, it is of utmost importance to check and identify the particular plant species before culturing. The plant species not capable of producing enough polyphenols may be a rare and endangered species. Hence its must be propagated through biotechnological ways of tissue culture. The production of plant species in controlled environmental condition allows the study of pathways of the phytochemicals produced by a particular plant. Plant cell culture holds potential for manufacturing economically and valuably important bioactive compounds for therapeutics.

CHAPTER3. METHODOLOGY

Aim: The main purpose behind this project was to enhance the polyphenol concentration in plants upon providing various stress conditions and to check the effect of carbon and nitrogen sources on plant growth and polyphenol production.

3.1 Process of Culturing :

3.1.1 Composition of MS media for 1L

Macronutrients	g/L
$NaNO_3$	1.65
KNO_3	1.9
$CaCl_2.2H_2O$	0.44
$MgSO_4.7H_2O$	0.37
KH_2PO_4	0.17
Micronutrients	mg/L
KI	0.83
H_3B0_3	6.20
$MnSO_4.7H_2O$	8.6
$ZnSO_4.7H2O$	8.6
$Na2MoO_4.2H2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025

Na_2EDTA	37.3
$FeSO_4.7H_2O$	27.8
Vitamins	mg/L
Inositol	100
Glycine	2
Thiamine HCl	0.1
Pyridoxine HCl	0.5
Nicotinic acid	0.5

After these nutrients, 30g sucrose and 8 gram of Agar is added.

3.1.2 Stock solution preparation

To avoid the tedious process of weighing individual salts each time, it is best to prepare concentrated solution of desired chemicals. These are called as stock solutions. They are made in distilled water and after use stored at 4^0C in refrigerator.

We prepared the stock solution 1mg/ml of micronutrients and vitamins except for inositol. After weighing 40 mg of each micronutrients in falcon tube, 40 ml of distilled water is added and the solution is dissolved properly. For Inositol stock we dissolved 1g inositol in 10ml of dissolved water.

3.1.3 Preparation of MS Media

For 1L media preparation, a 1000L conical flask is used. Initially all the macronutrients were weighed and dissolved properly in 50 ml of distilled water. After that the micronutrients are pipette out from 1mg/1ml stock solution prepared. The salts are mixed properly so that homogenous solution can be obtained. After that the desired concentration of vitamins are pipetted out from stock solution prepared in conical flask. After all the mixing has been done,

30g of sucrose is added and volume is make up to 1000ml with distilled water. Then, the pH is checked and maintained at 5.8. The pH is balanced by either 1M HCl or 1 N NaOH solution. After the pH is balanced 8 grams of agar is added and mixed slightly. The basal media is then autoclaved for 20 min at 15psi at 121^0C. Keep all the required apparatus such as petridish, scalpels, foreceps, culture tubes, measuring cylinder, distilled water, test tube stand for autoclaving to ensure aseptic culturing process.

3.1.4 Pouring of MS Media

The laminar air flow hood is sterilized using 70% ethanol and on the UV switch of laminar flow hood for 15 minutes before starting the procedure of pouring. The autoclaved basal media is made to slightly cooled down and then plant growth hormones 175mg/L IAA and 215mg/L kinetin are added. The MS media is then poured in autoclaved culture tubes and made to solidify. The culture tubes are then incubated at room temperature for 36 hours to check if any tube is contaminated.

3.1.5 Plant material collection

From the plants reviewed for skin problems we choose plants for further experimentation. The medicinal plant *Kalanchoe pinnata* was collected from the nursery of Delhi Technological University. Various types of explants like leaf, root, axillary shoot, node were collected from the parent plant in 1% saline solution.

The seeds of *Melissa officinalis* were purchased from Allthatgrows.

3.1.6 Sterilization of explants

Explants of *Kalanchoe pinnata* were removed from 1% saline solution and dipped in 70% ethanol for approx 2 minutes. the explants were properly trimmed using scalpels. The explants were surface sterilized using 1% mercuric chloride solution for approx 30 seconds followed by washing twice with autoclaved distilled H_2O.

3.1.7 Inoculation of explants

The MS media poured in culture tubes were screened for any kind of contaminated. The decontamined culture tubes are used for inoculation of explants in culture tubes. For inoculation, the sterilized explants are transferred carefully into the culture tubes using sterilized forceps.

3.1.8 Incubation of cultured plantlets

The inoculated tubes are sealed with parafilm and tubes are incubated at 25^0C and 16 hr photoperiod in tissue culture rack.

3.1.9 Subculturing of cultured plantlets

Subculturing was done on media containing different variation of carbon (glucose, fructose and sucrose) and nitrogen (potassium nitrate, sodium nitrate) for optimizing secondary metabolite production in cultured plantlets.

3.2 Extraction of polyphenols

The different types of extraction methods can be used for extraction of polyphenols. There are many techniques for extraction. Some of them are ultrasonic extraction, microwave assisted extraction, enzyme assisted extraction.

3.2.1 Microwave assisted extraction:

This process uses the energy of microwave to heat the solvent present in contact with the sample. The heat produced in turn increases the diffusibility of solvent towards the sample. This in turn extracts and diffuses polyphenols out of the matrix. The hydrogen bonds are denatured due to the dipole rotation of molecules. Due to which solvent penetrates into the matrix and allows the dissolution of metabolites into the liquid matrices [127]. Monomeric polyphenols are generally extracted by this method.

3.2.2 Ultrasound Assisted extraction:

The micro- sized bubbles are made to cause mechanical disruption of tissues allowing the release of phytochemical into the solvent. The ultrasound with frequency 20 to 2000 kHz is used to produce cavitation which enhances permeability of cell walls. Polyphenols from leaves, stem, bark, stem are extracted using this technique.

3.2.3 Enzyme Assisted Extraction:

Different types of enzymes such as cellulase, pectinase, hemicellulase and other such enzymes are made to hydrolyze the component of cell walls which enhance the extraction of polyphenolic component from the cell [127].

3.3 Determination of total phenolic content and flavonoids content

The biochemical assays that can be performed for determining the quantity of total phenolics and flavonoids content in plants.

- For total phenolic content

Folin ciocalteau (FC) assay is used for determination of total phenolic content in plant extract. For this the plant extract is made after extraction with methanol. Further the test sample approx 0.2 ml is taken and 0.6ml distilled water is added to it along with 0.2 ml of F-C reagent. After 5 min of incubation, 1ml sodium carbonate (8% w/v in water) is mixed in mixture. Then further volume makeup can be done by adding 3ml of distilled water. Incubation of mixture is further carried out in dark for 30 minutes. Finally after centrifugating the absorbance of blue colour sample is carried at 765nm taking gallic acid as standard [128].

- For total flavonoids content

Aluminium chloride calorimetric method is used for the estimation of flavonoids in test sample. For this plant extract is made. The standard curve is made by taking quercetin as standard. Stock of quercetin is made in methanol by dissolving 5mg quercetin in1ml methanol. Further serial dilution is done using methanol for the standard solution of quercetin. Then 0.6ml of 2%

aluminium chloride is mixed with plant extract and mixed it properly. Further incubate the mixture for 60 minutes. The absorbance is taken at 420 nm against blank at 420nm [128].

For separation, identification and qualification of polyphenols classical techniques like gas chromatography, HPLC, capillary electrophoresis and thin layer chromatography can be used as a detection tool for polyphenolic profiles [129].

CHAPTER 4 RESULT

The *Kalanchoe pinnata* is a medicinal plant that belongs to Crassulaceae family. It is highly rich in polyphenols and that's why it is used for its biological activities such as antihypertensive, antibacterial, skin hydrating, wound healing and antileishmanial properties. Although the plant is high in polyphenols but its leaves are highly rich in flavonoids. The major flavonoid isolated from leaves of *Kalanchoe pinnata* is quercetin 3-O-α-L-arabinopyranosyl-(1→2)-α-L-rhamnopyranoside. This polyphenol has potential of wound healing and is also used to treat inflammation [130]. Quercitrin is also a polyphenol majorly present in *Kalanchoe pinnata.* Several studies has found the effect of abiotic stresses in enhancing polyphenol content in *Kalanchoe pinnata.* In a study the UV-B has been used as an abiotic stress to enhance the polyphenols content and results proved that supplementation of UVB radiation enhances the flavonoids content in leaves without disturbing the antioxidant property of plant. The plants supplemented with white and UVB contained high amount of quercitrin [124]. In another study the effect of blue light and UVA was seen. It was found from the results that blue light when supplemented to plant increased their antioxidant properties. The blue light also changed the phenolic profile of the plant and increased the flavonoids content of plant [131].

Our study also aimed at optimizing the polyphenol content in *Kalanchoe pinnata.* After approximately 30 days the cultured plantlets were seen proliferating. Out of the different explants inoculated in MS media only axillary shoot proliferated indicating that the *Kalanchoe pinnata* can be propagated by using axillary shoot. After about 40 days the cultured plantlets can be subcultured in MS media with different carbon and nitrogen sources and different stress can be induced such as pH, temperature, light. Further for total polyphenolic content Folin-ciocalteau test is done and for flavonoids content aluminium chloride calorimetric assay can be performed. The carbon source acts as a major framework for plant species. The process of photosynthesis is for the carbon synthesis in plants and this is stored in the form of energy source. Carbon is the main source that affects the growth of plantlets in culture media. Normally, the best source recommended for explants growth is 2-4% sucrose. Nitrogen in turn is responsible for protein synthesis in plants. The change in nitrogen source can affect the plant

growth of different species. Our study aims to see the impact of carbon and nitrogen source on plant growth and polyphenol production.

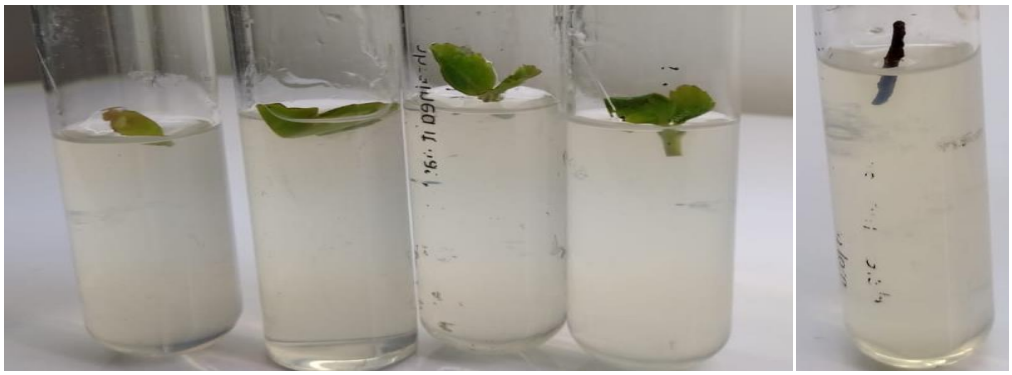

Fig2. *Kalanchoe pinnata* cultured plantlets

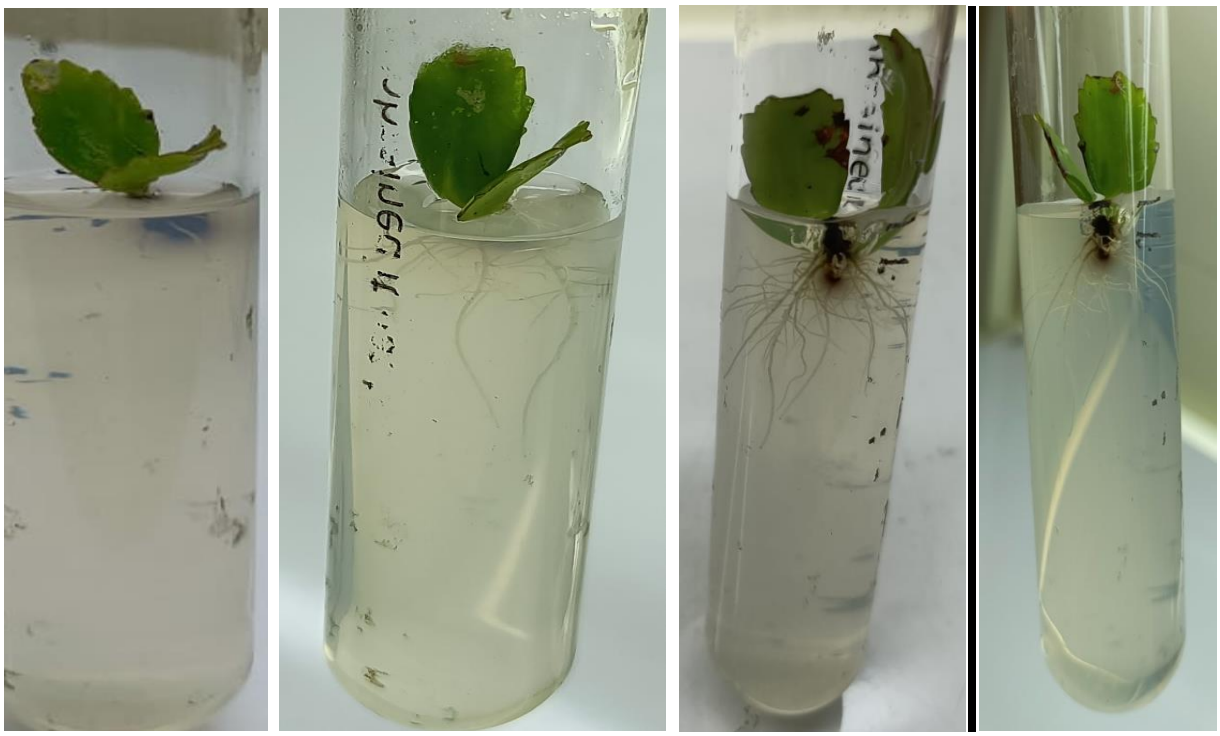

Fig3. *Kalanchoe pinnata* cultured plantlets after 40 days

4.1 Discussion

Secondary metabolites are of immense importance for both plants and humans. The plants in their natural environmental condition are capable of producing secondary metabolites for protecting themselves from any kind of environmental stress. These secondary metabolites have become a new source for drug discovery. According to WHO more than 70% of population is dependent on herbal products for treating any disease at primary level. To meet such a high demand for secondary metabolite for manufacturing herbal products, the biotechnological ways such as plant tissue culture is a major breakthrough in its production. The technique of plant culture ensures continuous production of medicinal plants whole year without any seasonal or geographical constraints. It also prevents the ecologically important plant species by preventing their excessive harvesting. The mass cloning through plant tissue culture provides the sustainable and cost- effective method for secondary metabolite production. Organ culture, plant cell suspension culture, hairy root culture are suitable option for production of secondary metabolites. The benefit of metabolite production through tissue culture lies on the fact that these can be manually manipulated in order to trigger the defense response in plant tissues grown invitro. These stresses can be triggered by the use of various elicitors for enhancement of secondary metabolites. The scaling up of invitro culturing make continuous production of high- valued stable secondary metabolites. Plant suspension culture ensures process of scaling convenient for metabolite synthesis because of the shorter cycle and simpler bioreactor construction. The hairy root cultures is a breakthrough for synthesis of metabolites in aromatic and medicinal plants because of the feasibility of plant metabolic engineering process in them. However their optimization is a prerequisite for their maximum production as it has been found that the plant cultures are able to produce 10 folds more amount of phytochemicals when optimized properly.

4.2 Conclusion

The biotechnological ways for enhancement of plant secondary metabolites through invitro tissue culture method has potential for meeting increased demand of natural bioactive compounds for commercial purposes. The plant tissue culture technique has made possible to enhance the research of active components for improved understanding of the pathways involved in plants for production of secondary metabolites. The secondary metabolites in turn has found to be an emerging source of herbal medicines. It has been found from many researches conducted that the polyphenols have skin protective effects. Being natural in origin and having very less side effects they had gain enormous popularity among consumers as natural cosmetics. But for meeting the increasing demand of industries for herbal products many natural plant species are in danger of extinction. Therefore plant tissue culture has become a boom for enhanced production of polyphenols. It has been found that various abiotic or biotic stress can be given to plants for enhanced polyphenol production in tissue culture. The use of invitro techniques has made it possible to know about the biosynthetic pathways for enhancement of polyphenols that can be employed in therapeutics related to skin diseases. However, due to insufficient data on safe use of plant polyphenols, the concerns are still there. This wrong perception among people that the plant products are always safe and without any side effects is wrong. A lot of toxic compounds may be found in different plant species. To overcome this problem, a detailed knowledge of the plant bioactive components and their associated pathways is very important. This somewhat simplifies the process of targeted extraction and production of plant metabolites through biotechnological approaches.

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