Plant bioactives and biotechnological approaches to enhance their production and bioactivity

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Abstract

Plants considered as an excellent source for the discovery of new products with medicinal significance in drug/functional foods/nutraceutical formulations. Bioactive compounds of plant origin are those secondary metabolites exhibiting health benefit in human. It is obvious that works related to plant bioactives continue to develop in many aspects involving researchers from various scientific disciplines. In the search for alternatives for production and enhancements of desirable bioactives from plants, biotechnological approaches such as fermentation, plant tissue culture, metabolic engineering, genetic transformation, plant cell elicitation, microencapsulation (as delivery mode) are found to have potential in industrial production of bioactive plant metabolites. The present review discusses the different categories of plant bioactives along with an overview of their medicinal applications as antioxidants, anti-inflammatory agents, anti-diabetic and anti-cancer agents. The current trend on applications of recent biotechnological tools in the production of plant bioactives has also been discussed.

Comment [WU2]: I felt this was a very common statement and not suitable to include in the abstract.

1. Introduction

Comment [WU3]: This is very fundamental and please extend the introduction at least up to 600/700 words. It is never clear that the fact to wrote this review article. There are many papers on this aspect and how is your one significant? The introduction does not show any new synthesis findings. Re-write the introduction.

It is a well known fact that ~~the~~ plants with medicinal properties are the most widely used source of life saving drugs for ~~the~~ human beings. In indigenous medicine uses different kind of plants extracts with the bioactive constituents as medicines. Plants produce a variety of organic compounds, the great majority of which do not appear to participate directly in growth and development and these substances are~~,~~ traditionally referred to as secondary metabolites (Hussain et al., 2012). Bioactive compounds of plant origin are those secondary metabolites exhibiting health benefits in humans (Kaur and Das 2011). Recent evidence from various epidemiological and clinical studies has proved that a diet rich in plant foods can reduce the risk of some chronic diseases, such as diabetes, obesity, cardiovascular complications, and cancer. Therefore, bioactives extracted from plants?? are used as pharmaceuticals, agrochemicals, cosmetics and food additives. It is obvious that works related to plant bioactives continue to develop in many aspects involving researchers from various scientific disciplines. In the search for alternatives for

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Comment [WU5]: Old reference? Use up to date references from 2021/2022. Use two more at least.

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production and enhancements of desirable bioactives from plants, biotechnological approaches such as fermentation, plant tissue culture, metabolic engineering, genetic transformation, plant cell elicitation, microencapsulation (as a delivery mode) are found to have potential in the industrial production of bioactive plant metabolites. The present review discusses the different categories of plant bioactives along with an overview of their medicinal applications as antioxidants, anti-inflammatory agents, anti-diabetic and anti-cancer agents. The current trend on applications of recent biotechnological tools in the production of plant bioactives has also been discussed.

Comment [WU7]: Use a figure/chart and clearly outline what you are going to discuss. There should be a logical flow.

Comment [WU8]: Use separate paragraphs to explain this research aim and objectives. This is a review and it's fine. But the aim/ objectives should be clearly mentioned. Also, where is the methodology? Even the review please mention how you achieved the secondary data? Which platforms did you use? How many papers you have used for this paper write up. Do you use grey literature? Did you use any unpublished data? Did you read non-native language published papers? Please mention your methodology very clearly.

2. Plant bioactives and their uses

With the development of analytical techniques including gas chromatography (GC), high-performance liquid chromatography (HPLC), mass spectrometry and nuclear magnetic resonance spectrometry, the isolation, purification, and structure elucidation of bioactives in plant extracts have become available. These biologically active compounds are also known as "phytochemicals.". These phytochemicals including polyphenols, alkaloids, terpenes, saponins, *etc*. have been widely used in nutraceuticals as ingredients to provide a health benefit beyond basic nutrition. Table 1 shows the four most common categories of these phytochemicals such as polyphenols, alkaloids, terpenes, and saponins and their common examples and uses as described in Hussain et al (2012).

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Comment [WU9]: You put as described by Hussain et al (2012), but in the table, it was Zhao et al, 2015, why this contradiction? please clear it out.

Table. 1. Plant Bioactives and their uses (Adopted from Zhao et al, 2015)

Bioactive Category		**Bioactives**	**Uses- common examples**
Polyphenols	Simple Phenolics	hydroxybenzoic acids: gallic acid, salicylic acid,salicylaldehyde, protocatechuic acid hydroxycinnamic acids: coumaric acid, caffeic acid, ferulic acid, and sinapic acid	Hydroxybenzoic acids are potent antioxidants that may help protect the body from free radicals. Gallic acid has been used as an astringent and styptic, and it also possesses antineoplastic, bacteriostatic, and antimelanogenic activities. Salicylic acid exerts anti-inflammatory,

			keratolytic, analgesic, antipyretic, antifungal, and antiseptic properties for several skin conditions (e.g., dandruff and seborrheic dermatitis, acne, ichthyosis, and psoriasis)
	Coumarins	Imperatorin and xanthotoxin	Higher plants such as Rutaceae and Umbelliferae are the richest sources of coumarins. fruits such as cloudberry and bilberry, chicory, and green tea. coumarins and coumarin derivatives, including free radical scavenging activity, anticancer, antiinflammatory, antimicrobial, anticoagulant
	Lignans	Enterodiol, Enterolactone	plant lignans possess powerful antioxidant activity higher than that of vitamin E and may be effective in the treatment of several other diseases such as cardiovascular disease, coronary heart disease, and diabetes
	Flavonoids	Flavones :Apigenin, Luteolin Flavonols: Kaempferol, Quercetin, Myricetin Flavanones: Naringenin, Eriodictyol, Hesperetin Flavonols: Catechins, Gallocatechin	Apigenin exhibit antiinflammatory, hypotensive, and antibacterial effects and diuretic activity and promote smooth muscle relaxation. Quercetin is a powerful antioxidant playing an important role inprotecting the body against reactive oxygen species. It also shows antiatherosclerosis, anti-inflammatory, anticancer, and cholesterol-lowering properties and protects eye health
	Isoflavonoids	Isoflavone, Isoflavanone, Isoflavanol, Isoflavene, Rotenoid, Daidzein, Genistein	Soybeans- The soy isoflavones have structures similar to mammalian estrogens which confer them the capacity of working as phytoestrogens

	Anthocyanins	Pelargonidin, Cyanidin, Peonidin, Delphinidin, Petunidin, Malvidin	Antioxidantactivity, antiedema, decreasing capillary permeability, increasing the regeneration of visual purple, improving vision at dusk, anti-inflammatory activity, and anticarcinogenic activity
	Tannins	1.Condensed tannins (Proanthocyanidins) 2.hydrolysable tannins	cinnamon as an antitumor, anti-inflammatory, antioxidant, antimicrobial, and cholesterol-lowering agent, as a treatment of infectious diseases, and for the prevention of cardiovascular diseases.
	Quinones	*p*-Quinone, Anthraquinone, *o*-Quinone, Naphthoquinone, Naphtodianthrone	Aloe-emodin is a natural anthraquinone from Aloe vera L. leaves that has been reported to exhibit anti-neuroectodermal tumor activity
	Stilbenes	Resveratrol	Resveratrol is a potent antioxidant and thereby may play a role in the chemopreventive effects. Inhibit carcinogenesis at the initiation, promotion, and progression stage
Alkaloids		indole alkaloids, pyrrolidine alkaloids, tropane alkaloids, quinolone alkaloids, isoquinoline alkaloids, and izidine alkaloids	Yohimbine- is an active indole alkaloid with sexual stimulant and aphrodisiac effects found naturally in Pausinystalia yohimbe. Caffeine- a stimulator of wakefulness and to enhance concentration and minimize the sensation of fatigue
Terpenes (isoprenoids)		Classifyterp enes according to their molecular formula monoterpenes, C10; sesquiterpenes, C15; diterpenes, C20; sesterterpenes, C25; triterpenes, C30;	Thymol-antibacterial, antiworms. Lutein -is a xanthophyll belonging to the carotenoid group, which can protect our eyes
Saponins		Chemically, saponin can be divided into two groups, the	1.Centella asiatica is a medicinal herb containing

	triterpenoidal saponins and the steroidal saponins	high amounts of triterpenoid saponins which are the primary active constituents in C. asiatica 2. Ginsenosides are the primary active ingredients of ginseng and belong to triterpene saponins

3. Biotechnological approaches to enhance production ~~)~~ and bioactivity

3.1 Fermentation

Comment [WU10]: There are many evidence of significant positive results of outcomes and failures. So, use as much as contradictions where it is applicable. Then the future researchers can have hints and thereby your work may cite more. Otherwise, this paper becomes another just a review paper. If you found more data, even you can summarise those in a table.

Fermentation is a well-established classic industrial process that has been utilized for the enhancement of shelf life, nutritional and organoleptic properties and for the removal of undesirable compounds from primary food substrates. Recently it has been explored as an economically viable cost effective strategy to enhance the synthesis of secondary metabolites with biological properties to achieve diverse functional benefits. In the concept of biotechnology the term fermentation is used in the broader sense, for the deliberate use of microorganisms to make products useful to humans (biomass, enzymes, primary and secondary metabolites, recombinant products, and products of biotransformation) on an industrial scale. More specifically when focusing on bioactive compounds, fermentation breaks down or converts the undesirable substrates into compatible components under the action of microbial enzymes, thereby improving the substrate properties *via* the production and enrichment of bioactive compounds (Hussain et al., 2016).

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However the outcomes of fermentation can vary as each fermentation system is fundamentally composed of three elements: microorganisms, substrate and environmental conditions. Microorganisms used for fermentation are very diverse. They include bacteria (e.g.,……), yeasts (e.g.,……), and molds (e.g.,……), and thus fermentation is based on their action on the substrate (ref?). Various types of fermentation can occur (lactic, alcoholic, propionic, malolactic, butyric) that share the same basic features of being an anaerobic

catabolism of organic compounds that yields different types of bioactives. Basically fermentation process can be divided into two systems; Submerged fermentation (SmF) and Solid State Fermentation (SSF). SmF is based on the cultivation of microorganisms on a liquid medium which contains nutrients whereas SSF consists of the microbial growth and product formation on solid particles in the absence (or near absence) of water; however, substrate contains the sufficient moisture to allow the microorganism growth and metabolism (Martins et al., 2011). This part of the chapter encompasses the usage of fermentation as a biotechnological approach to enhance the contents of bioactives in different plant food groups such as vegetables, fruits, cereals and legumes. Additionally various bioactivities of these bioactive compounds enhanced through fermentation is also discussed.

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3.1.1 Enrichment of bioactive compounds in plants through fermentation

Bioactive compounds are available in different concentrations in the matrix of plants and their availability is influenced by several factors. Alteration of these factors by fermentation has been an efficient and secured method to enhance the accessibility of these bioactive compounds for beneficial purposes. Influence of fermentation on protein solubility, hydrophobic and hydrophilic domains of the components, bio-transformation and generation of novel metabolites, mobility of bioactives, glycosidic bonds and structural break down of plant cell walls have been attributed as the main causes for the improved bioactive content in post fermentation conditions (Sadh et al., 2018; Sandhu and Punia 2017;Hussain et al., 2016).

3.1.1.1 Fruits

Fruits are one of the main sources of dietary bioactives specially polyphenols which has been widely researched in recent times. They are rich sources of phenolic acids, flavonoids, proanthocyanins and hydrosable tannins, which have focused the attention of researchers to enhance their value by using processing technologies including fermentation. Fermentation process has been applied for whole fruits, fruit juices and other related products to improve

their bioactive content. For example, studies conducted by Lee et al., (2016) on fermenting *Morinda citrofolia* (Noni) fruits using *Pediococcus pentosaceus* BCRC 14053 has significantly increased the Total Phenolic Content (TPC) of the aqueous extracts of the fruits. Also the researcher has revealed that the octanoic acid content of the fruits has increased approximately by 1.5 folds compared to the non-fermented fruits. During the fermentation of *Punica granatum* (Pomegranate) fruits by *Saccharomyces cerevisiae*and juice by lactic acid bacteria (LAB) content of gallic acid and ellagic acid in fruit and juice respectively has increased due to the hydrolysis of ellagitannins and other oxidation reactions -(Gumienna et al., 2016). Fermentation of *Sambucus nigra* (Elder berry) juice with different strains of LAB increased the polyphenolic content by 60%-80% depending on the strains. This study also highlights the bacterial fermentation mechanism of producing phenyllactic acid through amino acid metabolism, modifications in hydro cinnamic acid and increase in flavonoid glycosides by deconjugation process of β-glucosidase activity (Ricci et al., 2019).

3.1.1.2 Vegetables

Consumption of vegetables either in raw or processed forms confer a variety of health benefits such as controlling degenerative diseases. Vegetables contain a number of polyphenol classes, including hydroxycinnamic and hydroxybenzoic acids, flavonols, flavan-3-ols, flavones, flavanones, anthocyanins, phenolic aldehydes, stilbenes, hydrolyzable tannins andproanthocyanidins (Andarwulan et al., 2012). Most commonly LAB fermentation has been employed as one of the technique to efficiently exploit bioactives from vegetables.

Pan et al., (2015) has suggested that fermentation of *Allium chinense* (Chinese jiaotou) bulbs with *Lactobacillus plantarum* ZDY 2013 could be used as a technique to improve the phenolic content in Chinese jiaotou bulbs. Accordingly the investigation has shown a 2.7 fold increase in the TPC and the researcher suggests that in addition to the catalysis of glycosides and release of conjugated phenols, secretion of cellulase and tannase by the bacteria can

break down the plant cell wall and facilitate the release of phenols and contribute to the increased TPC. The investigation of Cai et al., (2019) on enhancing the biotransformation of glucoraphanin into sulforaphane in *Brassica oleracea* (Broccoli) using strains of *Leuconostoc mesenteroides* and *Lactobacillus plantarum* has given a positive result by increasing the yield of sulforaphane in the broccoli puree. ~~Also~~In addition, the formed sulforaphane was stable throughout the storage period indicating that fermentation facilitates the production of safe stable sulforaphane in broccoli puree. Additionally it also has increased the TPC of the puree by approximately 83%.*Cucumis sativus* (Cucumber) fermented with strains of *Lactobacillus pentosus*has been reported to show increased contents of total bioactive peptides. Also the study has identified the synthesis of 05 bioactive peptides: IPP/ LPP, VPP, KP, and RY during the fermentation process suggesting that lactic acid fermentation of cucumber can be used to enhance its bioactive composition (Fideler et al., 2019).

3.1.1.3 Cereals

Cereal grains are considered to be an important source of dietary carbohydrates, proteins, fiber, vitamins and phytochemicals such as phenolics, saponins and phytoestrogens. Fermentation of cereals have been practiced for the production of beverages, bakery products and gruels for a long time. Recent fermentation techniques include SSF of cereals by different strains of fungi (ref).

SSF of *Pennisetum glaucum* (Pearl millet) by *Aspergillus sojae* can be used to improve the phenolic content of finger millet. Accordingly after 06 days of fermentation 5.3 fold of increase in phenolic content was observed. At the same time based on HPLC analysis the content of ascorbic acid, gallic acid and p-coumaric acid was higher in the fermented pearl millet compared to the unfermented pearl millet (Salar et al., 2017). Investigations conducted by Sandhu and Punia (2017) on fermentation of different cultivars of *Hordeum vulgare* (Barley) by *Aspergillus awamorinakazawa* (MTCC-548) has revealed that SSF effectively

increases the TPC and total flavonoid content(TFC) of barley with the maximum content observed at the 5th day of fermentation. Fermentation of *Avenasativa* .L (Oats) by *Monascusanka* has shown to increase the free, bound and conjugated phenolics in different fractions rising up the TPC by 18 folds. During this fermentation a significant increase in chlorogenic acid, caffeic acid, ferulic acid, catechin and rutin was observed (Bei et al., 2017). The sequences of amino acid of fermented *Chenopodium quinoa* (Quinoa) flour analyzed by chromatography shared compositional features which are typical of antioxidant peptides. This study demonstrated the capacity of autochthonous lactic acid bacteria to release peptides with antioxidant activity through proteolysis of native quinoa proteins (Rizzello et al., 2017).

3.1.1.4 Legumes

Legumes play a major role in diet as sources of carbohydrate and protein. Fermentation of legumes has been important in improving the nutritional value of the substrate by increasing the antioxidants, vitamins and other compounds of good digestibility. Additionally it is important to decompose anti-nutrients present in the legumes. *Phaseolus vulgaris*(Kidney beans) subjected to SSF by *Bacillus subtilis* showed a significant increase in the soluble phenolic content with fermentation. This was mainly due to the hydrolysis of β-glucosidic bonds from conjugated phenolics and release of phenolic aglycones by the activity of β-glucosidase produced by the bacteria (Limón et al., 2015). Fermentation of *Glycine max*(Soybean) with *Aspergillus oryzae*, *Rhizopus oryzae*, *Bacillus subtilis* and *L. plantarum* produced an increase in the TPC of soyabean (María Landete et al., 2015). Work by Gan et al., (2017) revealed that natural and LAB-mediated fermentation increased soluble TPC in *Vigna unguiculate* (Mottled cowpea) with a significant increase of soluble catechin, which was associated with the degradation of proanthocyanidins in the bean coat. Natural fermentation of mottled cowpea also increased the contents of bound ferulic and p-coumaric acids.

3.1.2 Enhancing bioactivity of plants through fermentation

In addition to the enrichment of bioactive compounds several lines of evidence indicate that fermentation also improves the pharmacological properties of plants by enhancing their bioactivities and disease preventing effects.

3.1.2.1 Antioxidant property

Bioactive substances present in plant matrix exert antioxidant activity by controlling oxidative stress through several different mechanisms including reducing activity, free radical-scavenging, potential complexing of pro-oxidant metals and quenching of singlet oxygen. Fermentation also has had various effects on antioxidant activities.

In Oats fermented by *Monascusanka* and Quinoa flour dough fermented by LAB the DPPH and ABTS radical scavenging activities of free, bound and conjugated phenolics have significantly increased (Bei et al., 2017; Rizello et al., 2017). Considering legumes, FRAP values of naturally fermented mottled cowpea and speckled kidney beans have significantly increased indicating an increase in the reducing power of the tested grains (Gan et al., 2016). Investigations by Bhat et al., (2016) has revealed that FRAP activities of Guava fermented with *Lactobacillus planatarum* has increased and reached the highest level at the 16^{th} hour of fermentation.

3.1.2.2 Anti-inflammatory properties

Plant foods are good sources of anti-inflammatory agents due to the presence of potential bioactive compounds, which exert a protective role in the inflammatory process thorough reduction of pro inflammatory enzymes and by activating prostacyclin. Enrichment of bioactives by fermentation has supported improved anti-inflammatory process through diverse mechanisms. Studies by Puupponen-Pimiä et al., (2016) has indicated that fermentation of *Rubuschamaemorus* (Cloud berry) by *Pediococcus pentosaceus* VTT E-072742 has increased inhibitory effect on production of NO and IL6 in macrophages

activated by exposure to bacterial endotoxin LPS. This indicates the enhanced anti-inflammatory of cloudberries after fermentation. Mixture of 18 different vegetables fermented by *Lactobacillus planatarum* has shown a significant reduction in the secretion of IL 6 and TNF-α levels in RAW 264.7 cells indicating an increased anti-inflammatory property after fermentation. This outcome was directly related to the increased production of metabolites specially organic acids such as lactate, indole-3-lactate, β-hydroxybutyrate, γ-aminobutyrate and glycerol(Kim et al., 2019).The results obtained in the work of Gabriele et al., (2018) indicate that sourdough fermentation is a useful biotechnology to enrich the protective effect of *Triticum aestivum* (Wheat) flour against HT-29 alterations induced by TNF-α exposure. Particularly, fermented wheat flour (FW) pretreatments exerted a similar inhibitory effect on IL-8 and COX-2 up-regulation, greater than unfermented wheat flour (UFW) inhibition at comparable doses. Moreover, compared to UFW effects, FW better normalized the proinflammatory response via NF-κB signaling pathway modulation and HO-1 up-regulation, not only upon exposure to TNF-α but also at basal conditions.

3.1.2.3 Anti-hypertensive properties

Secondary metabolites as well as functional peptides of plants have shown to exhibit anti-hypertensive properties. Various clinical trialson angiotensin converting enzyme inhibitory (ACE-I) property of fermented products has provided scientific evidence for the application of peptides derived from fermentation for the prevention and treatment of hypertension. ACE plays a key physiological role in controlling blood pressure by the rennin angiotensin pathway thus ACE inhibitory activity is used as an *in-vitro* indicator of anti-hypertensive properties of functional foods. Bioactive peptides released by the action of microbial proteases after SSF by LAB in lentils has shown to increase the ACE inhibitory activity by 93% (Torino et al., 2013). ACE inhibitory activity in Lupin and Quinoa fermented with *Bifidobacterium breve* and *Bifidobacterium longum* has increased significantly after 24h of

fermentation. This was primarily due to the changes that has occurred in proteolysis during fermentation (Ayyash et al., 2018). Investigations by Ahrén et al., (2015) has shown that freeze-dried *Lactobacillius plantarum* fermented blueberries significantly lowered the L-NAME induced blood pressure increase in rats within two weeks of treatment. It has been emphasized that the novel phenolic acids such as phenyl actic acid, hydroxyphenyl lactic acid, 3,4-hydroxyphenyl-propionic acid generated during the fermentation process could have contributed to the increase in anti-hypertensive properties of blueberries.

3.1.2.4 Anticancer property

A number of bioactive compounds from plants have been reported to combat cancer efficiently through diverse mechanisms. Anticancer agents derived from plants have shown to inhibit angiogenesis, suppress cell proliferation, reverse tumor development and also have expressed general antioncogenic effects. Fermentation has been an efficient strategy to improve the bio efficacy of polyphenolic compounds from plants and utilize them as anticancer agents.

In the study conducted on blueberries fermented with *Lactobacillus plantarum* the fermented blueberry extract showed greater capability than raw blueberry extract at inhibiting the proliferation of HeLa cells, with very low cytotoxicity to normal fibroblast cells. This suggests that *L. plantarum* fermentation bio-transformed blueberry polyphenols into active phenol metabolites with strong antiproliferative activities (Ryu et al., 2019). Investigation on *Brassica oleracea*(Curly kale) which is a member of *Brassicaceae* family fermented with LAB showed a statistically confirmed antitumor activity. Salicylic acid and gentisic acid produced during this fermentation process was proved to control the solid tumors in both *in vitro* and *in vivo* conditions by reducing the number of HT29 and SW620 cells (Michalak et al., 2020).Hydrophilic extracts of *Ipomea batatas* (Sweet potato) fermented with *Lactobacillus acidophilus* exhibited an increased level of dose dependent inhibition mode on

PC-12 cell proliferation compared to the raw sweet potato extracts. The researcher suggests that this may be due to the higher levels of phenolics such as caffeic acid, p-coumaric acid and ferulic acid generated during the fermentation process (Shen et al., 2018).

3.1.2.5 Neuro protective properties

Naturally occurring bioactives are recently being investigated for the treatment of several neurodegenerative diseases. Neuro protective effects have been assessed based on attenuation of impaired learning and memory induced by cerebral ventricle administration of Aβ-42 peptides, alleviation of oxidative stress in neurons and mitochondria of rat brains mediated by Aβ-42 peptides, control of singlet-oxygen-induced cerebral stroke, familial amyotrophic lateral sclerosis and ischemia-reperfusion injury.

Studies conducted on a Cheonggukjang (CGK), a fermented soy bean product produced by the mixture of *Bacillus subtilis* and *Lactobacillus sakei*has shown to exert protective effects against alterations inneuronal cell death, acetylcholinesterase (Ach) activity, nerve growth factor secretion and signaling pathway in trimethyltin induced cognitive defects in mice (Go et al., 2016). Administration of ginger fermented (FG) with *Schizosaccharomyces pombe* improved recognition memory, impaired by scopolamine injection, than that of non-fermented ginger. In addition, FG ameliorated memory impairment in amyloid beta (Aβ) plaque-injected mice by protecting neuronal cells in of the mouse hippocampus. Also FG restored the pre and postsynaptic protein levels decreased by a β plaque-toxicity (Huh et al., 2018).

3.2.Tissue culture technology

Plant cell culture and tissue culture hold great promise for controlled production of useful secondary metabolites (Hussain et al., 2012). Based on the recent research, cell culture techniques which are capable of producing specific medicinal compounds or molecules at a rate similar or superior to that of intact plants have accelerated in the last few years. In the

aims of obtaining higher yields suitable for commercial exploitation, many efforts have been focused on isolating the biosynthetic activities of cultured cells, achieved by optimizing the cultural conditions, selecting high-producing strains and employing precursor feeding, transformation methods, and immobilization techniques (Hussain et al., 2012).

According to Giri and Narasu (2000), transgenic hairy root cultures have revolutionized the role of plant tissue culture in secondary metabolite production. In terms of their genetic and biosynthetic stability, easily maintain and faster in growth, transgenic hairy root cultures are considered as more unique. Using this tissue culture technique, a wide range of chemical compounds has been synthesized. Advances in tissue culture, combined with improvement in genetic engineering of pharmaceuticals, nutraceuticals, and other beneficial substances are helpful for the production of bioactives. Recent advances in the molecular biology, enzymology, and fermentation technology of plant cell cultures suggest that these systems will become a viable source of important secondary metabolites or bioactives (Abdin, 2007). Genome manipulation is resulting in relatively large amounts of desired compounds produced by plants infected with an engineered virus, whereas transgenic plants can maintain constant levels of production of proteins without additional intervention (Abdin and Kamaluddin, 2007). Large-scale plant tissue culture is found to be an attractive alternative approach to traditional methods of plantation as it offers controlled supply of biochemical's independent of plant availability.

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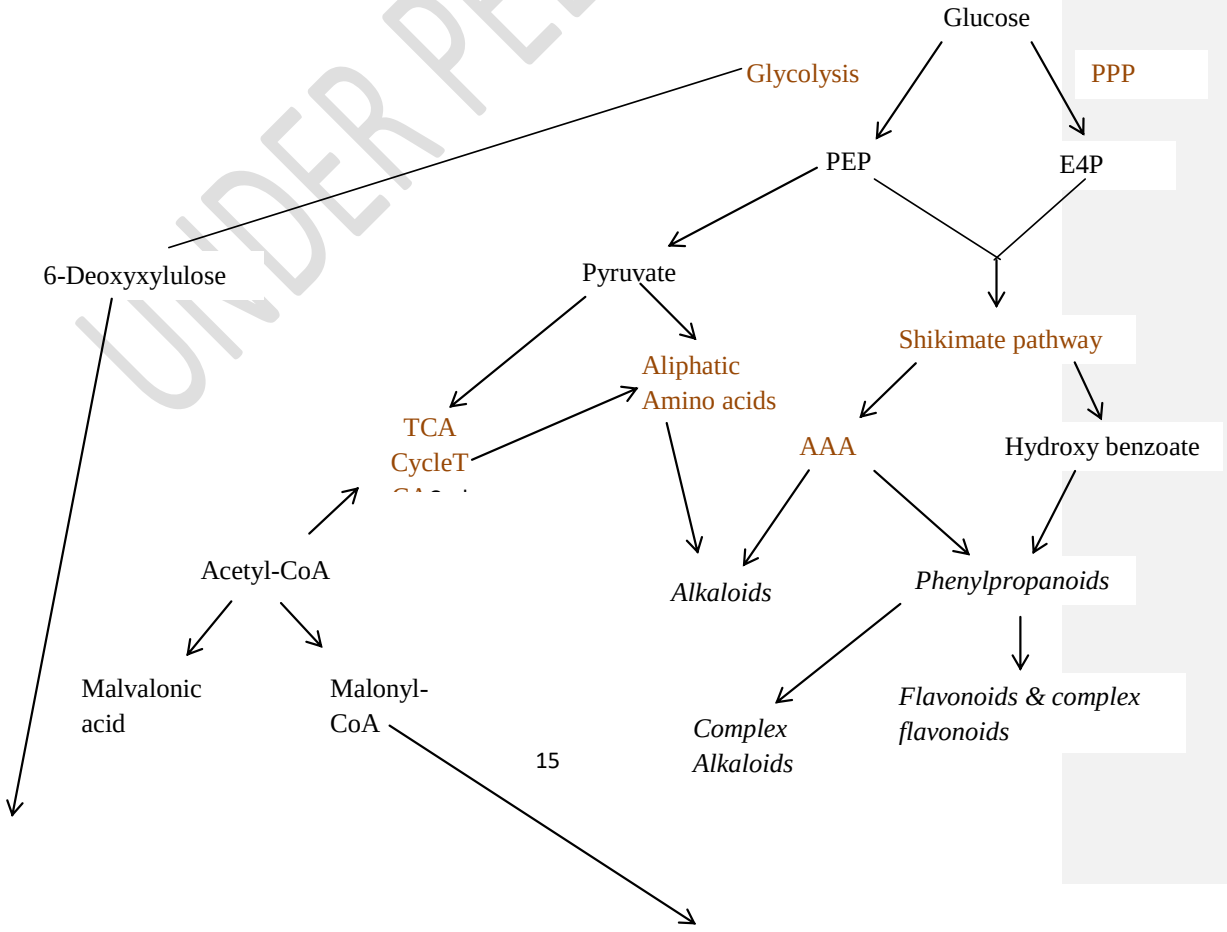
3.3. Metabolic engineering and production of bioactives.

The metabolic network of plants is by far more comprehensive than in most other organisms. Apart from synthesizing primary metabolites, plants also produce an enormous range of secondary metabolites(bioactive compounds) and more than 200,-000 such structures have been investigated (Aharoni and Galili, 2011). The definition of primary and secondary metabolites is not entirely clear. However, primary metabolites include universal and

essential building blocks of sugars, amino acids, nucleotides, lipids, and energy sources. Unlike primary metabolites, secondary metabolites do not appear to participate directly in growth and development. These are play an important role in plant defense against herbivory and other interspecies defenses while from ancient time, humans use bioavtive compounds as medicines, flavorings, and recreational drugs (Tiwari and Rana, 2015). The major bioactives synthesized by plants can be categorized under three main groups as phenolic compounds, terpenoids/isoprenoids and nitrogen or sulfur containing compounds such as alkaloids and glucosinolates. The three major classes of bioactive compounds are produced from pathways of different primary metabolites, including glycolysis, the TCA cycle, aliphatic amino acids, pentosephosphate pathway, shikimate pathway and notably thearomatic amino acids (Aharoni and Galili, 2011).



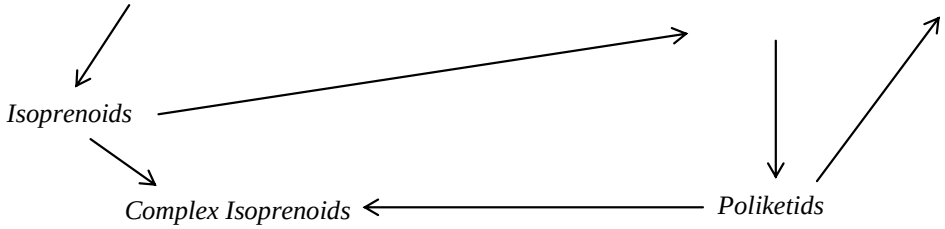


Figure 1.The production of secondary metabolites is tightly associated with pathways of central metabolism, such as glycolysis, shikimate pathwayand the production of AAA and aliphatic amino acids. Modified from Aharoni and Galili, 2011. Note: AAA; Aromatic Amino Acids, PEP; Phosphoenol pyruvate, E4P; Erythrose-4-phosphate, PPP; Pentose phosphate pathway.

People have being using plant bioactives since past thousand years for multiple purposes, such as dyes, flavors, fragrances, stimulants, insecticides, human poisons as well as most importantly therapeutic agents. Hence, bioactive compounds have become to an interested research field in recent years since many investigations with related to human nutrition pointed out that modest, long-term intakes of certain bio active compounds will have possibility of preventing the occurrence of many chronic diseases (Enfissi *et al.*, 2010). However, the extraction of these bioactives from plant sources does not result in large quantities that enough to meet the increasing market demands. The extraction of natural products using conventional extraction processes, appears to be limited due to the low concentration levels of these phytochemicals in plants, time-consuming, expensive, wasteful in regard to natural resources and, sometimes, environmentally unsafe due to the usage of solvents during the isolation and purification procedures. Taking all of the above unfavorable conditions into consideration, metabolic engineering for stimulating the levels of these active molecules in plants is nowadays an emerging area for plant biotechnology. Metabolic engineering can provide various strategies to improve productivity, such as increasing the number of producing cells, increasing the carbon flux through a biosynthetic pathway by overexpression of genes, codify for rate-limiting enzymes or blocking the mechanism of

feedback inhibition and competitive pathways and decrease catabolism (Hussain *et al.*, 2012). Recent advances in microbial biotechnology have significantly supported the expression of partial or entire metabolic pathways, allowing the biosynthesis of high value end-products. Current metabolic engineering approaches to achieving remarkable concentrations of natural bioactive compounds in microorganisms, with potential application in the industry.

3.3.1. Metabolic engineering of microorganisms for the production of common plant secondary metabolites/bioactives

3.3.1.1. Flavonoids

Flavonoids are known to be produced by all terrestrial plants. They include a large group of natural compounds deriving from the phenyl propanoid metabolism, which has evolved in plants to produce a large number of associated flavonoid structures (Trantas *et al.*, 2015).Currently, the most utilized biological platforms metabolically engineered for the heterologous production of flavonoids are the *Escherichia coli* and the yeast *S. cerevisiae.* Examples for producing certain bioactives from plats following metabolic engineering are shown in Table 2.

Table 2.Levels of natural flavonoid products produced either during heterologous biosynthesis in various hosts (*Escherichia coli* or *Sacharomyces cerevisiae*),

Flavonoid target	Plant source Common name *Scientific name*	Health benefit/s	Metabolically engineered host	Externally fed precursor	Titer (mg/ L)	References
		Flavonones				
Eriodictyol	Mexican oregano *Lippiagraveolens*	Anti-inflammatory and antioxidant	*E. coli*	L-tyrosine	107	Zhu *et al.*, 2014
Pinocembrin	Mexican oregano *Lippiagraveolens*	Reduce cerebral ischemia/reperfusion and blood–brain injury, anti-oxidative and anti-apoptotic effects	*E. coli*	Glucose	40.02	Wu *et al.*, 2013
Naringenin	Tomato *Solanumlycopersicum*	Antioxidant, anti-inflammatory, blood lipid and cholesterol lowering effect	*S. cerevisiae*	Glucose	105.9	Koopman *et al.*,2012; Kanaze *et al.*, 2007
		Flavones				
Hypolaetin	-	Anti-inflammatory activity	*E. coli*	Luteolin	88	Lee *et al.*, 2014; Barros *et al.*, 2016
Apigeninglucosides	Mexican oregano *Lippiagraveolens*	Antidepressant, anti-inflammatory, hepatoprotective, antithrombotic, anticancer, antioxidant and estrogenic effects	*E. coli*	Apigenin	4.67	Choi *et al.*, 2012; Tang *et al.*, 2017
		Flavonols				

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3-O-Xylosyl quercetin	-	Lowering of blood pressure, anti-proliferative and apoptotic effects, against allergy, asthma, cardiovascular disease, diabetes, hypertension, inflammation,prostatitis, arthritis, and obesity	*E. coli*	Quercetin	23.78	Pandey *et al.*, 2013
Kaempferol 3-O-glucoside (Astragalin)	Bean *Phaseolusvulgaris*	Anti-oxidative, anti-allergic, anti-inflammatory, analgesic, anti-hepatotoxic properties	*E. coli*	Narigenin	109.3	Malla *et al.*, 2013
Quercetin-3-Orhamnoside	Lingonberry *Vacciniumvitis-idaea*	Anti-diabetic, anti-proliferative activity	*E. coli*	Quercetin	200	Kim *et al*.,2012

3.3.1.2. Isoprenoids

Metabolic engineering of isoprenoid compound pathways, including synthesizing many important industrial and pharmaceutical products such as terpenes and carotenoids, provide excellent examples of the utility of this approach in secondary metabolite production. Two main metabolic routes are known forisoprenoid biosynthesis, knowingly mevalonate and non mevalonate pathways and considerable effort has been focused on manipulating these pathways for improved precursor supply in heterologous hosts such as *Escherichia coli* (Mijts and Dannert, 2003). The non-mevalonate pathway is widespread amongst bacteria and proceeds from glyceraldehyde-3-phosphate and pyruvate precursors through a series of catalytic steps to the common isoprenoid precursor isopentenyl diphosphate.Overexpression of the dxr (1-deoxy-D-xylulose-5-phosphate reductoisomerase) and dxs(1-deoxy-Dxylulose-5-phosphate synthase) genes, individually and in combination used to improvethe yield of carotenoids when combined with engineered carotenoid biosynthetic pathways in *E. coli*. Furthermore, lycopene production in *E. coli* was also enhanced by altering metabolic flux to generate a more balanced supply of thenon-mevalonate pathway precursors (Farmer and Liao, 2001) and metabolic engineering of the non-mevalonate pathway in*E. coli*has also been used to improve production ofisoprenoid compounds other than carotenoids, such as taxadiene (Huang *et al.,* 2001). There are also examples of the mevalonateisoprenoid biosynthesis pathway being utilized for isoprenoid metabolic engineering such as engineering of the fungus *Neurosporacrassa* by overexpressing *Saccharomyces cerevisiae* HMG-CoA reductase to increase flux towards mevalonate improved production of the native carotenoids lycopene and neurosporaxanthin sixfold and twofold, respectively (Wang and Keasling, 2002). In addition to improving production levels by

manipulating precursor supply, engineering approaches have been utilized to control downstream isoprenoid biosynthetic pathways and produce new isoprenoid compounds.

3.3.1.3. Alkaloids

With over 10,000 structurally identified members, plant alkaloids are important privileged compounds with many pharmacological activities (Leonard *et al.*, 2009). Today, numerous alkaloids are pharmacologically well characterized and are used as clinical drugs, ranging from cancer chemotherapeutics to analgesic agents. A study has shown that, expressed genes coding for strictosidine synthase and strictosidineglucosidase enzymes from medicinal plant *C. roseus* in *S. cerevisiae* and successfully produced cathenamine from tryptamine and secologanin by functionally expressing those two enzymes in yeast (Geerlings *et al.*, 2001). Along with the increased knowledge on biosynthetic pathways of many plant bioactive compounds, utilities of different yeast species should be investigated in future for the efficient microbial production of such compounds. Overcoming rate-limiting steps, reducing flux through competitive pathways, reducing catabolism, and over expression of regulatory genes are some of the strategies that can be used for increased secondary metabolites production through metabolic engineering.

3.4. Genetic transformation to manipulate the quality and production of bioactive natural compounds

Genetic transformation is a process by which the genetic material carried by an individual cell is altered by the incorporation of foreign (exogenous) DNA into its genome(Zhou *et al*., 2011). That technique has been implemented to manipulate the quality and production of bioactive compounds. Mainly, genetic manipulation is done in hairy root culture for the production of bioactives are useful as pharmaceuticals, cosmetics, and food additives (Georgiev *et al*.,2007; Srivastava *etal*., 2007). The hairy root system based on inoculation with *Agrobacterium rhizogenes*, a gram-negative soil bacterium has become popular as a method of producing secondary metabolites synthesized in plant roots (Karuppusamy, 2009).In detail, bio active compounds synthesized by hairy roots arising from the infection of plant material by *A. rhizogenes* are the same as those usually metabolized in intact parent roots, with similar or higher yields (Sevón and Caldentey, 2002). Further, other T-DNA sequences are not required for the transfer with the exception of the border sequences. Hence, the rest of the T-DNA can be replaced with the foreign DNA and introduced into cells from which whole plants can be regenerated (Hussain *et al.*, 2012). There are several studies have been conducted regarding genetic transformation on production of bioactives, for an example, Gong et al induced hairy roots of *Solanum photeinocarpum*. grew rapidly and the dry weight contents of total glycoalkloid and saponin were 31 and 107 times higher than those of the original ones, respectively (Gong *et al*., 2001). Another investigation revealed the content of total saponin extracted from *Phytolacca esculenta* hairy roots was about 1.54 times higher than as that of the natural roots (Shi *et al*., 2003). Further, induced hairy roots from *Taxus brevifolia* produced bioactive compound called taxol and *Polygonum multiforum* produced chrysophanol in hairy root by genetic transformation

(Wang *et al.*, 2006). Another example of this approach is the heterologous expression of *Vitreoscilla* hemoglobin in plastids using the pVHb-RecA construct, leading to increased production of hyoscyamine and scopolamine in *Hyoscyamusniger* L. in vitro transgenic plant cultures (Guo *et al.*, 2018). Therefore, it was indicated that secondary metabolites, which had the same even higher content as the original plants, could be determined in most of the transformed hairy roots. Particularly, the content of some medicinal ingredient exceeded that of the original plants so enormously that it could be used in industrialized production.

Calli cultures and cell suspension cultures are also relate with genetic transformation engaging with the production of bioactive compounds. Calli cultures are often used successfully to produce secondary metabolites of medical significance, many of which may be used in treating human diseases (Mittal and Sharma, 2017; Rady *et al.*, 2018). An example would be to induce overexpression of three stilbene synthase (STS) genes of *Piceajezoensis* Carr, *viz. PjSTS1a*, *PjSTS2* and *PjSTS3*, in calli cultures of *Vitisamurensis* resulting in an increase in the content of stilbene (Suprun *et al.*, 2019). Another good source of secondary metabolites is by the use of transgenic cell suspension cultures, as an example the stable transformation of *Silybum marianum* (L.) Gaertn. cell suspension cultures with the *Vitisvinifera* L. stilbene synthase gene, allowing increased synthesis of t-resveratrol (Hidalgo *et al.*, 2017).

3.5. RNA interference (RNAi) technology to enhance the production of bioactives

The RNAi approach is used to enhance the accumulation of the bioactives by down-regulating the gene expression of the succeeding gene in the metabolic pathway. RNAi technology provides an alternative whenever the use of antisense RNA and co-suppression approaches have failed to block the activity of an enzyme that is coded by multigenes. RNAi is a process of double-strand RNA (dsRNA)-mediated gene silencing in which only the mRNA associated with dsRNA is

specifically degraded. This type of RNAi-mediated gene silencing is also referred to as co-suppression or posttranscriptional gene silencing in plants (Bindu *et al*.,2018).

In the case of *Eschscholzi acalifornica*, RNAi-mediated suppression of berberine bridge-forming enzyme resulted in accumulation of reticuline which is the precursor of isoquinoline alkaloids like morphine, codeine, and beberine. Sanguinarine, an end-product of this pathway, was considerably reduced. However, laudanine, a methylated derivative of berberine, accumulated in the transgenic plants (Fujii *et al.*, 2007). Further, RNAi technology showed its utility in enhancing β-carotene and lutein contents in potato by downregulating the expression of β-carotene hydroxylase that converts β-carotene to zeaxanthin (Van Eck *et al.*, 2007; Amar *et al.*, 2016). Examples for RNAi-mediated gene silencing for bioactive products in medicinal plants are presented in Table 3.

Table 3. RNAi-Mediated Gene Silencing in Medicinal Plants for synthesizing bioactive compounds

Plant	Therapeutic effect of the bioactive compound	Product	Enzyme	Reference
1. *Salvia miltiorrhiza*	Antiviral, antimicrobial, and anti-inflammatory activities	Rosmarinic acid	Phenylalanine ammonia-lyase	Song and Wang, 2011
2. *Withaniasomnifera*	Immune system modulation, antistress, antihyperglycemic, anti-inflammatory, antioxidant, and anti-cancer effects	Withanalides	Cycloartenol synthase	Mishra *et al.*, 2016
3. *Papaversomniferum*		Codeine and morphine	Codeinonereductase (COR)	Allen *et al.*, 2004
		Morphinan alkaloids	Salutaridinol	Allen *et al.*, 2008
			7-O-acetyltransferase	Kempe *et al.*, 2009

	Morphine, codeine, sanguinarine	Berberine bridge enzyme and N-methyl coclaurine 3′-hydroxylase	Frick *et al.*, 2004
4. *Artemisia annua*	Salicylic acid and artemisinin	Cinnamate-4-hydroxylase	Kumar *et al.*, 2016

3.6. Production of bioactive compounds by sponges

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Consistency and sufficient supply means of bioactive compounds for pharmaceutical and nutraceutical production are much sought after by the technological world nowadays. Production of bioactive compounds using Sponges has been experimented since decades where its approaches have engrossed the industrial community. Sponges are multi-cellular invertebrates of Phylum *Porifera* meaning pore bearers. They don't have true tissues, organs or organ systems in their porous body structure which are diverse in colours and shapes. The small pores called ostia allow water flow into the sponge making them filter feeders. Attached to solid surfaces in both shallow and deep ocean beds, from one end of their body, the other end which is open to the environment is called osculum. Most live in marine environment while some can survive in fresh and brackish water. Sponges feed on microorganisms such as bacteria and algae in water pumped through their body and with the help of flagella in cell ends, the oxygen and nutrients are taken in while excreting waste and carbon dioxide. Sponges can produce toxic substances, chemicals such as antiviral compounds (e.g., nucleoside analogues), antimicrobial compounds (e.g., polyketides), and cytostatic compounds (e.g., avarol), as a means of defense from their predators (Muller et al., 2004). The sponge *Callipelta* sp has been identified to produce two new cytotoxic peptides; callipeltin B and C (Auria et al., 1996) and bioactive polyketides-Spiculoic acid

isolated from *Plakortis Zyggompha*has shown anti-tumoral and anti-microbial activity (Berrue et al., 2007). The sponge extract of *Tedania (Tedaniopsis) oxeata* was selectively cytotoxic against the cancer cell lines (Berne et al., 2016). Two new brominated phenolic compounds, subereaphenols B and C, isolated from Red Sea sponge *Subereamollis* showed significant antioxidant activities (Abou-shoer et al., 2008). Many such compounds have been identified as beneficial to human in terms of their bioactivity like antibiotic activity, anti-tumor, anti-inflammatory and with respiratory, cardiovascular and gastrointestinal tract. Sponges, by providing home for numerous types of bacteria and algae within their pores, are having a symbiotic relationship for food and protection. It is reported in Muller et al (2004), that bacteria and microorganisms coevolved with sponges, acquiring a complex common metabolism. It is suggested that (at least) some of the bioactive secondary metabolites isolated from sponges are produced by functional enzyme clusters, which originated from the sponges and their associated microorganisms (Muller et al., 2004). The microorganisms that can resist the sponge digestive process and immune response inhabit as symbionts to enhance potency of Sponge to synthesize bioactive compounds (Lee et al., 2001).

Methods of producing bioactive compounds by sponges?????

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Manufacturing drugs using Sponge bioactive compounds has many obstacles to overcome. Secondary metabolites of interest are produced only in trace amounts and the majority of promising compounds have very complex structures economical not accessible by chemical synthesis (Brummer & Nickel, 2003). Laboratory bioassays on bioactivity and small-scale cell biology investigations have to rely on limited wild harvest. Other experiments like clinical and pre-clinical trials will need enormous amounts of biomass which is not possible to harvest from natural population, under clauses stated in sustainable use of natural resources given by Agenda

21 of the Earth Summit in Rio de Janeiro (UN 1992). Cultivating sponges is also hurdled by the limited availability of larger quantities of defined sponge material. So far, few methods have been identified as feasible for obtaining large quantities of secondary metabolites from sponges.

In Situ Cultivation of Bath Sponges is done by picking up the sponges using a drag net and cutting the sponges by means of a very fine saw into fragments, measuring 26 mm longitudinally with as much ectosome as possible. The fragments were threaded on bamboo sticks and replaced in shallow waters with a certain current present and protected against rays of light (Brummer & Nickel, 2003).

Sponge Farming used two techniques: vertical rope technique used in open sea and horizontal structures moored close to the sea bottom in lagoon areas. Sponge cuttings were fixed in either ways and highest growth and survival were obtained in sheltered locations and the ratio of body size to wound size and the overall size of the wounds are very important, greatly influencing survival (Brummer & Nickel, 2003).Farming of *Callyspongia (Euplacella) biru* sponge using horizontal structures moored close to the sea bottom in lagoon areas was done by de Voogd (2007).

Ex Situ Maintenance of Sponges in Aquaria is an in vivo cultivation system based on an airlift bioreactor containing artificial seawater. All the ecological parameters like salinity, temperature, light conditions, the substrate sponges grow on, currents and soluble organic matter are kept under control. *Crambecrambe,* a Mediterranean marine sponge producing guanidine alkaloids with cytotoxic and antiviral activity which may be used as compounds for potential anticancer drugs were cultured using this method (Bondu et al., 2012, Perez-Lopez et al., 2014).

In Vitro Cultivation of Sponges or sponge cell cultures is an appropriate way to produce not only sponge biomass, but also the desired bioactive compounds. It is done in three major steps; the dissociation of the sponge tissue, a selective enrichment using density gradient centrifugation and the application of antibiotics (Brummer & Nickel, 2003). The contamination problem and the difficulties in identifying sponge cells are some obstacles that should be overcome by using regular cell-type verification either by the use of DNA sequences or by the use of sponge-specific antibodies. The primmorph culture system - three-dimensional multicellular aggregates consisting proliferating and differentiating cellswas developed to overcome the rapid loss of telomerase activity in single cell suspensions after dissociation which indicated the need for cell-cell contact for the sponge cells to proliferate (Brummer & Nickel, 2003).

Dissociation of Sponge cells are done by transferring Sponge tissue samples to CMFSW-E (Ca^{2+}- and Mg^{2+} -free artificial seawater containing EDTA) and shaking. After washing the cells are transferred to Ca^{2+} - and Mg^{2+} -containing seawater, supplemented with antibiotics (100 IE/ml of penicillin and 100 Ilg/ml of streptomycin), and kept at 16°C (Schroder et al., 2003). Immediately after transferring to the Ca^{2+} - and Mg^{2+} -containing seawater and during shaking, the single cells form small aggregates containing - 20 cells, which grow in size during the subsequent 3 days to 10 mm large cell clumps. After (usually) 5 days primmorphs are formed. As the basal medium the natural seawater is enriched to 0.2 % with RPMI1640-medium. The primmorphs are marked by the presence of proliferating cells and a characteristic histology(Schroder et al., 2003). BrdU {5-bromo-2' -deoxy-uridine)-labeling and detection assay have been used to demonstrate that the cells organized in the primmorphs regain the capacity to proliferate. The growth conditions could be optimized by supplementing the natural seawater/0.2 % RPMl1640-medium with silicate. The optimal concentration of silicate for cell proliferation

and spicule formation is 60 jlM. Iron plays an essential role, since it occurs usually in seawater at concentrations of <1 nM and for optimal growth conditions of primmorphs, Fe^{3+} concentration should be increased to 30 jlM. A bioreactor was developed which allows the cultivation of primmorphs at stronger current, since it is required for growth and canal formation in the primmorphs (Schroder et al., 2003).The first successful production of a sponge secondary metabolite in a bioreactor was achieved with primmorphs from *Dysideaavara* that produced avarol which has a potential antiviral, antitumor and anti-inflammatory activity (Muller et al., 2000, Schroder et al., 2003). The structure elucidation and identification of such extracted bioactive compounds have been done using High Performance Liquid Chromatography, Mass Spectrometry and Nuclear Magnetic Resonance techniques. However, it is still controversial whether these secondary metabolites are of sponge cell or of bacterial origin. Therefore, cloning the genes that encode enzyme clusters required for the synthesis of low molecular weight bioactive compounds has been experimented using sponges *Suberites domuncula* and *Geodia cydonium*, to isolate polyketide synthases (Muller et al., 2004). Future prospects to improve the sponge cell culture include the elucidation of those genes which control the proliferation phase and the morphogenesis phase, two developmental phases which the cells in primmorphs undergo. Nevertheless, the immortalization of sponge cells by transfection with genomic DNA appears to be a promising way (Schroder et al., 2003).

3.7. Plant Cell Elicitation

Plant secondary metabolites, which are of pharmaceutical importance include alkaloids, glycosides, flavonoids, volatile oils, tannins, resins etc. They are commonly known as phyto-pharmaceuticals and mostly isolated from wild and cultivated plants. The yield of these compounds from plants is often low and largely depends on plant physiology. The chemical

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synthesis of most compounds is not successful since it is extremely difficult and infeasible to generate the same efficacy and pharmacological specificity. Although the biotechnological production of secondary metabolites in plant cell or organ cultures appeared to be an alternative method, due to lack of knowledge about the synthesizing pathway of these metabolites, it had only limited success (Namdeo, 2007). Since compounds can be produced without affected by soil conditions and climatic changes, free of microbes and insects, with reduced labor cost and using automated control of cell growth to yield specific metabolites, biotechnological production of secondary metabolites using plant cell and organ cultures would be worth experimenting further (Varma, 2010).

Among many biotechnological strategies experimented for enhancing the secondary metabolite production, 'elicitation' is one of the key strategies. Elicitation is a process that induces or enhances the synthesis of secondary metabolites by the plants and/or plant cells in-vitro, as they respond physiologically and morphologically to microbial, physical or chemical factors which are known as 'elicitors' (Mejía-Tenienteet al., 2010). Secondary metabolites may be the products of chemical adaptions to environmental stress by the plant cells, which act as defensive, protective or offensive chemicals against microorganisms, insects and higher herbivorous predators (Namdeo, 2007). Stress factors like UV-irradiation, osmotic shock, fatty acids, inorganic salts and heavy metal ions induce the synthesis of secondary metabolites in plants. Therefore, an 'elicitor' may be defined as a substance which, when introduced in small concentrations to a living cell system, initiates or improves the biosynthesis of specific compounds (Namdeo, 2007).

3.7.1 ???Classification of elicitors

Elicitors can be classified in two ways; based on their 'nature' or based on their 'origin'.

When classified according to their 'nature' two types of elicitors can be identified as abiotic elicitors and biotic elicitors. Substances which are of non-biological origin, mostly physical factors acting as elicitors like metal ions (Cu and Cd ions, Ca^{2+}) and high pH are called abiotic elicitors. Substances with biological origin such as polysaccharides derived from plant cell walls (pectin or cellulose) and micro-organisms (fungi, bacteria or herbivores, plant cell wall fragments) and glycoproteins or G-protein or intracellular proteins whose functions are coupled to receptors and act by activating or inactivating a number of enzymes or ion channels are known as biotic elicitors (Namdeo, 2007).

When classified according to their 'origin' two types of elicitors can be identified as exogenous elicitors and endogenous elicitors. Substances originated outside the cell, such as polysaccharides, polyamines and fatty acids are termed as exogenous elicitors. Substances originated inside the cell, such as galacturonide or hepta-β-glucosides etc. are known as endogenous elicitors (Namdeo, 2007).

3..7.2.????? Mechanisms of elicitation

Treating plants with elicitors causes a series of defense reactions that result in accumulation of defensive secondary metabolites in plants or plant cell cultures. However, the exact mechanism of elicitation through biotic and abiotic elicitors on the production of secondary metabolites is poorly defined. Some of the mechanisms hypothesized in this regard are messenger Ca^{+2}, factors affecting cell membrane integrity, inhibition/ activation of intracellular pathways and changes in osmotic stress (Namdeo, 2007).

Namdeo (2007) reported the general mechanism of biochemical responses performed by plant or plant cells when challenged by the elicitor. The elicitor binds to the plasma membrane receptor for elicitation process. The Ca^{2+} influx to the cytoplasm from the extracellular environment and

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intracellular Ca^{2+} reservoirs. As a mechanism of elicitation, rapid changes occur in protein phosphorylation patterns and protein kinase activation. Mitogen-activated protein kinase (MAPK) stimulation and G-protein activation happens and cytoplasm acidification is caused by H^{+}-ATPase inactivation, whereas the decrease in membrane polarization, extracellular increase of pH has been reported in elicitor treated plant tissues. Bioactive fatty acid derivatives and reactive oxygen species like superoxide anion and H_2O_2which are having direct antimicrobial effect, are produced. H_2O_2 act as a secondary messenger and may be involved in the transcriptional activation of defence genes.

Another hypothesis relates about the accumulation of defence-related proteins, pathogenesis related proteins such as chitinases and glucanases, endopolygalacturonases that contribute to the release of signalling pectic oligomers (endogenous elicitors), hydroxyproline-rich glycoproteins, and protease inhibitors (Namdeo, 2007).Some groups reported the Hypersensitive response to cell death at the infection site (Namdeo, 2007).

However, the exact mechanism of elicitation is the study of these events and their interconnection and intercorrelation between them is highly complex and is still under investigation. All elicitors do not follow the same sequence of events but varies with their origin, specificity, concentration, physiochemical environment, stage of their growth cycle, nutritional uptake (Namdeo, 2007).

3.7.4.????Characteristics of Elicitors

There are several characteristics of elicitors such as elicitor concentration and selectivity, duration of elicitor exposure, age of culture, cell line, growth regulation, nutrient composition, quality of cell wall materials, substantial enhancement of product accumulation etc. If the elicitor concentration is high, higher amount of bioactive compounds will be produced. High dosage of

elicitor has been reported to induce hypersensitive response leading to cell death, whereas, an optimum level was required for induction (Namdeo, 2007).With high exposure time, higher production was observed, but further increasing exposure time resulted in decrease of production. Age of subculture plays is an important parameter in production of bioactive compounds by elicitation. Nutrient composition of medium or selection of medium also played a vital role for elicitation process. Apart from these characteristics, the efficiency of elicitation also depends on elicitor specificity, cell line or clones of microbial elicitor used, presence of growth regulators and the environmental conditions.

3.7.5./???Applications of Plant cell elicitation

The exact molecular structures of elicitors like polysaccharides, oligosaccharides, proteins, glycoproteins, and fatty acids have been elucidated in recent studies (Angelova et al., 2006). Many carbohydrate elicitors have been used on different cultures to produce secondary metabolites. Chitosan has been used on N. tabacum, E. californica cultures to produces metabolites like phytoalexins.β-1,6-1,3-Glucans have been used upon Glycine maxcultures to produce isoflavonoids. Chitin, alginate, pectin, guar gum rhamsan and xanthan have been used as elicitors to produce Anthraquinones from Morinda citrifolia cultures (Radman et al., 2003, Angelova et al., 2006).

3.8. Microencapsulation for the improved delivery of bioactive compounds into foods/medicine

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Phytochemicals or plant bioactive compounds are recognized as beneficial products for chronic diseases in human. Many studies (Ref 1, Ref 2, Ref3, Ref4, Ref 5), focused on developing functional food products and nutraceuticals using these phytochemicals encounter difficulties in

maintaining their stability until delivery to target site. Since they are more prone to degradation during processing and storage, there must be a way to increase their stability within food matrix and external environment to protect against degradation. Many natural bioactive compounds are unstable and readily oxidized with exposure to light, heat and pH (Corrêa-Filho et al., 2019). Food industries mostly look for functional products having more shelf life and designed to release at the target site during desired time and increased bioavailability after release. Microencapsulation is a process that can enhance stability of bioactive compounds which are sensitive to adverse environmental conditions by trapping the compounds within a protective polymeric material. This application is widely used as a preservation and stabilization method for functional compounds in food and pharmaceuticals.

Microencapsulation is a physiochemical or mechanical process that in which small particles or droplets of liquid(of an active agent) are wrapped in a shell or coated with a continuous film of polymeric material to produce small particles ranging from a few nanometers to a few millimeters, which are called microcapsules or microspheres, for protection and/or later release (Corrêa-Filho et al., 2019). Microcapsules have been widely used in the pharmaceutical sector and currently it is highly recommended to the food industries to enhance their product quality with high thermal stability, controlled release of compounds, texture/sense improvement and sheltered from being volatile (Yang et al., 2020).

Microcapsules are different from microspheres from their internal structure and morphology. Microcapsules are hollow and possess an internal reservoir system while microspheres are dense matrix systems (Corrêa-Filho et al., 2019). Microencapsulation process allows the active ingredient or the core material, to be protected from adverse external environmental conditions by the coating, which is called the encapsulating agent or wall material. Core materials are

microencapsulated in monolayer or multilayer's of wall materials with a variety of molecular interactions, including electrostatic attraction, van der Waals forces, and hydrogen bonding or ionic interaction (Yang et al., 2020). The wall material protects the core material from adverse conditions until it passes through human digestive tract where the outer layers of microcapsules get dissolved in gastric acid at lower pH and then the core substances, released are readily absorbed in the small intestine. Microcapsule preparation is a low cost activity which requires a smaller production cost and simple equipment. And also the wall and core materials should meet food safety standards (Yang et al., 2020) and made out of food grade edible polymers like maltodextrin, inulin and arabic gum.There are many reports regarding the microencapsulation of natural bioactive compounds such as phenolic compounds, carotenoids, other organic compounds and essential oils (Corrêa-Filho et al., 2019).

3.8.1.????Microencapsulation methods

There are many important techniques for microencapsulation like pan coating, polymerization, salting out, air suspension coating, hot-melt (Tarun and Murthy, 2011), spray drying, coacervation, freeze drying, electrospraying, ionic gelation, and fluidized bed coating(Corrêa-Filho et al., 2019), emulsification coacervation, layer-by-layer, extrusion, supercritical, solvent evaporation, and nanocapsule preparation (Yang et al., 2020).

Spray drying microencapsulation atomizes the emulsion of wall material and core material in the dry and high-temperature environment and moisture is evaporated via heat exchange between droplets and drying medium which solidifies the shell of the droplet quickly wrapping the core material. This is widely used for mass production with only few materials like Arabic gum and modified starch which are having good water solubility, low viscosity, and good fluidity (Corrêa-Filho et al., 2019, Yang et al., 2020).

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Emulsification technique is a chemical embedding method where the mixture of core material and wall material (dispersed phase) is added into a large number of vegetable oil (continuous phase), containing the emulsifier to form a stable emulsion and microencapsulate under the action of cross-linking agent. The production cost of this method is usually very high and therefore, this is not feasible (Yang et al., 2020).

Freeze-drying is a method that sublimates the ice into vapor under high-vacuum condition after quick freeze. Due to ice sublimation, the process is kept cool and safe for biological samples. The economic feasibility of this method is low due to high energy cost and long processing time (Corrêa-Filho et al., 2019, Yang et al., 2020).

In Coacervation technique, the core material is emulsified in the solution of wall material and another substance or solvent is added to reduce the solubility of the wall material, which is evenly aggregated and surrounded by the core material to form microcapsules. This is a commonly used technique as it does not need any special equipment, has mild processing conditions and does less damage to the core material quality (Yang et al., 2020).

In layer-by-layer assembly technique, layers are spontaneously attached with each other to form the stable molecular aggregates. This technique is used to produce multilayer microcapsules. However, product quality for some products may be poor due to weak interactions between layers (Yang et al., 2020).

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Extrusion technique is a physical embedding method for forming microcapsules by squeezing core material and colloid mixture into the hardening bath in the form of the liquid drops through the needle tube under pressure. The cost will be more than twice of the spray-drying technique but commonly used for embedding all kinds of volatile, vitamin, and pigment compounds or other heat-sensitive materials (Yang et al., 2020, Bamidele and Emmambux, 2020).

Supercritical technique is used for nonvolatile substances which dissolve in the supercritical fluid which could a very short time when decompressed through the pore capillary, so that the solute oversaturates and a large number of fine particles are formed. Hollow microcapsules are separated and precipitated under controlled conditions. The supercritical CO_2 is mostly used due to low critical temperature, low viscosity, high solvent, high dispersion, high mass transfer, and nontoxicity (Yang et al., 2020).

Electrospray technique decomposes the polymer fluid transported by the conductive capillary pump into the fine droplets through a high-voltage electric field. The polymer particles are generated and collected to obtain microcapsules after evaporating the solvent. This technique has a great potential in the encapsulation of bioactive substances, volatile compounds, sustained-release preservatives, and functional foods in food industry (Yang et al., 2020).

Fluidized Bed Coating is a tool used for producing microparticles, based on the additional coating of powder particles in a batch processor or a continuous setup. The particles intended for coating should be spherical (the lowest surface area) with a narrow particle size distribution and good flowability. At the beginning, the particles are suspended by an air stream, at a predefined temperature, and then sprayed with coating material. The evaporation of water is controlled by several factors like spraying rate, water content, air flow, humidity of the air inlet, and temperature (Đorđević et al., 2014).

Nano capsulation is a technique that produces Nano sized capsules which can penetrate through capillaries, penetrate into tissues be absorbed by cells, thus enabling more precise targeting of the core materials. Nanocapsules need additional special treatments, usually high energy, based on the preparation of ordinary capsules, such as ultrasound, high pressure, or intense mechanical agitation(Gómez et al., 2018).

??????????Polymers used in microencapsulation

According to Yang et al (2020), there are many natural food grade materials that are used for microencapsulation process with several different properties in each. Cellulose and its esterified or etherified derivatives like methyl cellulose (MC), carboxymethyl cellulose (CMC), hydroxypropyl cellulose (HPC), and hydroxypropyl methyl cellulose (HPMC) possess improved solubility and thermoplasticity. Chitosan is another material that has many unique properties such as biodegradability, biocompatibility, and osmotic enhancement effects and widely used in fresh fruit, vegetable and food additives. Alginate is famous as a coating material due to its thickening, flocculability, film-forming property, stability, chelation, and biocompatibility, as well as mild reaction conditions, nontoxic and harmless characteristics, simple gelatinization process, and low cost. Starch and its hydrolysates are also used as wall materials owing to their properties like easiness of retrogradation and dehydration. Pectin which is a partially methylated α-1,4-D-polygalacturonic acid has a good gelatinization and emulsification stability, widely applied in the food industry, such as ice cream, jam, and fruit juice gelatinization. Protein widely holds processing properties such as solubility and emulsification, as well as the physiological activities of oxidation resistance, good biocompatibility and biodegradability. In addition, the susceptibility to pH rendered the protein in a great role in strict pH-controlled release conditions (Yang et al., 2020). Many above discussed materials are added together in compatible ratios to prepare composite wall materials with enhanced properties.

??????????Application of microencapsulation in food/medicine for improved delivery of bioactive compounds

Many studies have been conducted to find the real scenario behind the microencapsulated bioactive compound after being incorporated into different food matrices. A study reported the

incorporation of microencapsulated carotenoids from sea buckthorn extract by coacervation using whey protein isolate and Arabic gum as wall materials, into muffins and 55 % stability of the bioactive compounds after 21 days' storage at 25°C (Ursache et al., 2018). Green tea extract rich in polyphenols was microencapsulated in maltodextrin by spray-drying andfreeze-drying and then added to bread (Pasrijaet al., 2015). The study evaluated the impact of microcapsules on bread quality and polyphenol content after baking. The amount of polyphenols present in the baked bread was 33% lower than in the dough before baking (Corrêa-Filho et al., 2019). However, through optimization of the above discussed microencapsulation techniques thus formed microparticles containing phytochemicals can be incorporated into food matrices to enhance the delivery of therapeutic effects to humans.

Conclusion??

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