In vitro Cultures for the Production of Some Anticancer Agents (Review Article)

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Abstract: Organ, tissue and cell culture and other biotechnological techniques are useful ways to obtain biologically active constituents those play an important roles in our life (They have pharmaceutical, medicinal and economical importance in our life). These ways (*in vitro*) of obtaining secondary metabolites are better than the classical methods since: They are natural in origin, so they are of little toxic side effects compared with synthetic drugs. They are safe sources and do not cause any environmental pollution as like as those occurred by applications of pesticides and insecticides to farm lands. They are performed under controlled conditions (since the yield can be increased, with increasing replicates number and by using elicitors, fomenters and bioreactors in a large application scale), they are performed under aseptic conditions (This means that, they are system free of contaminating microbes " fungi and bacteria", consequently they are clean sources of drugs). Using these techniques we can direct the culture for producing the organ that contains the highest amounts of the needed product (for example, root culture can be produced using *Agrobacterium rizogenesis* to obtain substances that produced in root cells only). Production cycle is smaller than that of normal culture in land (it takes little time), since callus cultures with short life cycle may be a good source for production of needed phytochemicals. Using these methods we can conserve our natural resources (wild plants) instead of over-collection by herbalists. Finally, the cost can be decreased if done on a large scale (instead of Fedens language "large areas" we use jars in small place = higher productivity of secondary metabolites). This review will discuss with some detailed examples the *in vitro* production of some anticancer agents.
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1. Introduction:

The culture of microorganisms (*Penicillium, Streptomyces* etc.,) has long been used industrially to obtain substances such as penicillin, streptomycin etc., which are important pharmaceutically. Higher plants are also an important source of all types of substances, especially medicines (flavonoids, alkaloids, saponins, anthraquinones, cardiac glycosides, ethereal oils, steroids etc.,)

Traditionally, the medicinal plants have been grown and then active components extracted and this is likely to remain the normal procedure. However the production of medicinal plants can present problems, which have led to the search of other ways to produce naturally occurring substances:

1- Production in the field is strongly dependent on season, weather, climate, diseases and pests etc.,.

2- Naturally ocuring sources, especially in the tropics and subtropical zones are becoming limited and some medicinal plants are extremely scarce.

3- There may be technical and economic problems in production.

4- Production is labor intensive and therefore costs are high.

5- There may be political instability in the country where the plants are available resulting in an interrupted supply (Pierik, 1987).

The biotechnological production of valuable secondary metabolites in plant cell, tissue or organ cultures is an attractive alternative source to the extraction of whole plant material (Alam, 2012 $_{a,b}$, 2013).

Field of the plant biotechnology is focusing on the production of plant secondary metabolites. For most compounds of interest, e.g., morphine, quinine, vinblastine, atropine, scopolamine and digoxin, one has so far not been able to come to a commercially feasible process (Kumar *et al.,* 2012).

Several strategies are being followed to improve yields of secondary metabolites in plant cell cultures. First of all the screening and selection of high producing cell lines and the optimization of growth and production media can be mentioned as common approaches. In the case of shikonin and berberine with success, in many others with limited success.

In the past years new approaches have been developed: the culturing of differentiated cells (e.g., shoots, roots and hairy roots), induction by elicitors and metabolic engineering. With the culture of differentiated cells one has in most cases been able to get the production of the desired compounds in levels comparable to that of the plant; however the culture of such differentiated tissues on a large scale in bioreactors is a major constraint. For studies of

the biosynthesis, such systems are very useful. The second approach mentioned, the use of elicitors has been successful in several cases. However, it remains limited to a certain types of compounds for each plant, compounds which almost likely act as phytoalexins in these plants. Therefore attention is more and more focused on metabolic engineering which includes increasing activity of enzymes which are limiting in a pathway; include expression of regulatory genes; block competitive pathways; block catabolism.

Plant cell cultures provide an excellent system for studying the biosynthesis of secondary metabolites. For the large scale production of these compounds in most cases the production is too low for a commercialization. To improve yields metabolic engineering offers promising prospective, but requires understanding the regulation of the secondary metabolite pathways involved on the levels of products, enzymes and genes, including aspects as transport and compartmentation. Unraveling the regulation requires a joint effort of several disciplines, e.g., phytochemistry, plant physiology, cell biology and molecular biology (Purohlt, 2007).

The present review aimed at discussing some successful examples of i*n vitro* production of active pharmaceutical products against cancer.

2. Materials and Methods:

In this review *in vitro* cultures were used as great sources of active pharmaceutical products against cancer as following:

A. Production of carotenoids from *in vitro* cultures of tomato cell suspension cultures (*Lycopersicon esculentum*) as anticancer agents (Campbell *et al.*, 2006, Lu *et al.,* 2008 and Engelmann *et al.,* 2010)

B. *In vitro* cultures of *Rheum palmatum* L. for the production of anthracene derivatives as anticancer agents (Kasparová *et al.,* 2003).

C. *In vitro* cultures of *Polygonum hydropiper* for the production of some anticancer agents **(**Ono *et al.,* 1998).

D. *In vitro* cultures of *Rumex alpinus* L. for the production of some anthraquinones as anticancer agents (Van and Labadie, 1981).

E. Quercetin production from *in vitro* cultures of *Pluchea lanceolata* Oliverr and Hiern as an anticancer agent (Arya *et al.,* 2008).

F. Terpenoids and flavonoids production from *in vitro* cultures of *Azadirachta indica* as anticancer agents (Babu *et al.,* 2008).

G. Tropane alkaloids production from *in vitro* cultures of *Brugmansia suaveolens* as anticancer agents (Zayed and Winka, 2004).

H. Anthraquinones production from *in vitro* cultures of *Cinchona robusta* as anticancer agents (Schripsema *et al.,* 1999).

I. *In vitro* cultures of *Fagopyrum esculentum* for the production of some flavonoids as anticancer agents (Trotin *et al.,* 1993).

J. Podophyllotoxin production from *in vitro* cultures of *Podophyllum* (Sauerwein *et al.*, 1992, Bcnisrll *et al.,* 2004 and Bhattacharyya *et al.,* 2012).

K. Paclitaxel from *in vitro* cultures of *Taxus (*Kurz, G.W. and Constabel, F. . *National Research Council of Canada publication* no. 38467, Hara *et al.,* 1991 and 1993, Baebler *et al.,* and Bcnisrll *et al.,* 2004).

L. Terpenoids from *in vitro* cultures of *Fossombronia* (Sauerwein *et al.*, 1992 and Kurz, G.W. and Constabel, F. . *National Research Council of Canada publication* no. 38467).

M. Indole Alkaloids: Vinblastine derivatives from *in vitro* cultures of *Catharanthus* (Kurz, G.W. and Constabel, F. *National Research Council of Canada publication* no. 38467).

N. Camoptothecin derivatives from *in vitro* cultures of *Camptotheca* (Watase *et al*., 2004 and Bcnisrll *et al.,* 2004).

O. Antitumor activity of *in vitro* cultures of green tea seed (*Camellia sinensis* L.) (Choi *et al.,* 1989, Alam, 2012 [a,b], 2013).).

P. Production of shikonin derivatives from *in vitro* cultures of *Arnebia* (Royle) Johnst (Ruanzicao) as an antitumor agent (Xiong *et al.,* 2009).

Q. Anticancer activity of *in vitro* cultures of rice (Deshpande A *et al.,* 2011).

3. Results and Discussion:

Some detailed examples of i*n vitro* cultures producing anticancer agents:

A- Production of carotenoids from *in vitro* cultures of tomato cell suspension cultures (*Lycopersicon esculentum*) as anticancer agents:

An abundance of epidemiological evidence suggests that, a diet rich in fruits and vegetables is associated with a reduced risk of several forms of cancer. Phytochemicals present in fruits and vegetables have been proposed to provide protection from carcinogenesis through various biological actions. With respect to tomatoes, increased consumption of tomatoes and tomato products has been significantly associated with a reduced risk of prostate cancer in several epidemiological studies.

Carotenoids are natural yellow, orange, and red pigments present in tomatoes and possess a wide range of proposed biological functions, including antioxidant and anticarcinogen properties and immunoprotection. Lycopene, the most abundant tomato carotenoid, has been the primary focus of

both *in vitro* and *in vivo* studies examining the relationship between increased intake of tomatoes and reduced risk of prostate cancer. However, recent studies suggested that, other tomato phytochemicals may also modulate prostate cancer.

The herbicide norflurazon was added to establish cell suspension cultures of tomato (*Lycopersicon esculentum* cv. VFNT cherry), to induce the biosynthesis and accumulation of the lycopene precursors, phytoene and phytofluene, in their natural isomeric forms (15-cis-phytoene and two cis-phytofluene isomers). Norflurazon concentrations, solvent carrier type and concentration, and duration of the culture exposure to norflurazon were screened to optimize phytoene and phytofluene synthesis. Maximum yields of both phytoene and phytofluene were achieved after 7 days of treatment with 0.03 mg norflurazon/40 mL fresh medium, provided in 0.07 % solvent carrier. Introduction of ^{14}C-sucrose to the tomato cell culture medium enabled the production of ^{14}C-labeled phytoene for subsequent prostate tumor cell uptake studies. In DU 145 prostate tumor cells, it was determined that, 15-cis-phytoene and an oxidized product of phytoene were taken up and partially metabolized by the cells.

The ability to biosynthesize, radiolabel and isolate these carotenoids from tomato cell cultures is a novel, valuable methodology for further *in vitro* and *in vivo* investigations into the roles of phytoene and phytofluene in cancer chemoprevention.

The carotenoid biosynthetic enzyme, phytoene desaturase, causes two consecutive desaturation reactions to convert phytoene to α-carotene, with phytofluene as an intermediate product. Norflurazon is a known inhibitor of phytoene desaturase.

Therefore, it was hypothesized that, through the provision of norflurazon to this tomato cell suspension culture system, this enzyme would be primarily blocked at the first desaturation step, thus ultimately eliciting phytoene production within the tomato cells. Subsequently, phytofluene was expected to accumulate to a lesser extent than phytoene in the tomato cells following norflurazon addition.

In preliminary trials, experiments were conducted to determine whether various concentrations of norflurazon and duration of culture exposure to norflurazon affected tomato cell yield of phytoene. Tomato cells were treated with norflurazon (0.3, 0.03 and 0.003 mg norflurazon/40 mL fresh media, with final media concentration of 0.29 % ethanol) for 3, 5, 7, or 9 days. Results indicated that, a norflurazon treatment of 0.03 mg norflurazon/40 mL fresh media for 7 days provided the highest phytoene yield (170.3 μg phytoene/L tomato cell suspension), as compared to the other treatments. The addition of norflurazon to the tomato cell cultures was effective in the inhibition of phytoene desaturase, as no quantities of *ú*-carotene or lycopene were detected in the treated cells.

Data from these preliminary trials were utilized to conduct further experiments in this tomato cell suspension culture system. A novel approach has successfully developed to produce naturally accumulating tomato isomers of phytoene and phytofluene in tomato cell suspension cultures through the incorporation of a phytoene desaturase inhibitor to the culture medium.

Moreover, ^{14}C-labeled and 15-*cis*-phytoene have been biosynthesized and isolated through this culture system. Preliminary data from a human prostate tumor cell type suggested that, indeed 15-*cis*-phytoene is taken up by cells, as well as, a potential oxidized product of 15-*cis*-phytoene, and are apparently metabolized to unidentified polar phytoene metabolites. Through biosynthesizing naturally accumulating isomers of radiolabeled phytoene and phytofluene through *in vitro* plant techniques, further *in vitro* and subsequent *in vivo* bioavailability and metabolism experiments can be conducted. Only then can studies begin to adequately evaluate the potential roles of phytoene, phytofluene, and/or their metabolites in health, including prostate cancer prevention.

Isotopically labeled tomato carotenoids, phytoene, phytofluene and lycopene, are needed for mammalian bioavailability and metabolism research but are currently commercially unavailable. Multiple *in vitro* tomato cell lines were established and screened for carotenoid production, test the best producers with or without the bleaching herbicides, norflurazon and 2-(4-chlorophenyl-thio)triethylamine (CPTA) and to use the greatest carotenoid accumulator for *in vitro* ^{13}C-labeling. Different *Solanum lycopersicum* allelic variants for high lycopene and varying herbicide treatments were compared for carotenoid accumulation in callus and cell suspension cultures of the *hp*-1 line were chosen for isotopic labeling. When grown with [U]-^{13}C-glucose and treated with CPTA, *hp-1* suspensions yielded highly enriched ^{13}C-lycopene with 45% of lycopene in the M+40 form and 88 % in the M+35 to M+40 isotopomer range.

Response surface methodology (RSM) was employed, which combines fractional factorial design and a second-degree polynomial model. Tomato cells were homogenized with ethanol, saponified by KOH, and extracted with hexane, and the lycopene content was analyzed by HPLC-PDA. Five factors were varied at five levels: ethanol volume (1.33–4 mL/g); homogenization period (0–

40 s/g); saturated KOH solution volume (0–0.67 mL/g); hexane volume (1.67–3 mL/g); and vortex period (5–25 s/g). Ridge analysis by SAS suggested that, the optimal extraction procedure to extract 1 g of tomato cells was at 1.56 mL of ethanol, 28 s homogenization, 0.29 mL of KOH, 2.49 mL of hexane, and 17.5 s vortex. These optimal conditions predicted by RSM were confirmed to enhance lycopene yield from standardized tomato cell cultures by more than 3-fold.

B- *In vitro* cultures of *Rheum palmatum* L. for the production of anthracene derivatives as anticancer agents:

Kasparová *et al.,* 2003 examined the effect of four concentrations of calcium ions on the production of anthracene derivatives by means of the callus and suspension cultures of *Rheum palmatum* L. (Polygonaceae), which was elicited by an abiotic elicitor "20 μM solution of lead dichloride". The culture was cultivated on a Murashige-Skoog medium with an addition of 10 mg/l of alpha-naphthylacetic acid. It follows from the results that, the maximal content of anthracene derivatives, determined by the photometric determination according to PhBs4, was demonstrated in the suspension culture after 24 hours administration of the elicitor and 10 mM calcium chloride solution. In comparison with the control and elicited groups, the production was stimulated by 44 and 17%, respectively. After an addition of calcium ions to the elicited callus culture, no positive influence on the production was observed. It is well known that anthracene derivatives can play an important role as anticancer agents.

C- *In vitro* cultures of *Polygonum hydropiper* for the production of some anticancer agents:

Callus and suspension-cultured cells were induced from hypocotyls of *Polygonum hydropiper* seedlings. The callus and suspension-cultured cells produced mainly (+)-catechin accompanied by (−)-epicatechin and (−)-epicatechin-3-*O*-gallate. The (+)-catechin production of suspension-cultured cells increased with cell growth and reached the maximal value (29.0 mg/g dry wt) after 6 days from the start of subculture. This is the highest value of (+)-catechin content among reports on catechin production *in vitro* so far published. The amount of (−)-epicatechin was in the range of 1.1–7.7 mg/g dry wt and that of (−)-epicatechin-3-*O*-gallate was in the range of 2.6–6.4 mg/g dry wt for a culture period of 15 days, respectively. Comparing with plant parts in regard to (+)-catechin, the amount of suspension-cultured cells was about 1.5 times as much as callus cells, about 9 times that of leaves (3.2 mg/g dry wt) and about 7 times that of stems (4.0 mg/g dry wt). The maximal yield of total catechins in suspension-cultured cells was 4.3% dry wt. It is well known that (+)-catechin, (−)-epicatechin and (−)-epicatechin-3-*O*-gallate can play an important role as anticancer agents.

D- *In vitro* cultures of *Rumex alpinus* L. for the production of some anthraquinones as anticancer agents:

Tissue cultures set up from roots of *Rumex alpinus* L. were grown on Murashige and Skoog medium with kinetin and either 2,4-D or naphthaleneacetic acid (NAA) as growth factors. The 2, 4-D cultures produced a broader spectrum of hydroxyanthracene, hydroxynaphthalene and hydroxybenzene derivatives than the NAA cultures. In the 2,4-D cultures the anthraquinones chrysophanol, physcion and emodin, the dianthrones of chrysophanol and physcion and their heterodianthrone and the monoglucoside of chrysophanol were identified. The naphthalene-1,8-diols nepodin, nepodinmonoglucoside and methoxynepodin were identified. The occurrence of 2-acetylorcinol and its monoglucoside was established. In the NAA cultures accumulation of only chrysophanol, physcion and 2-acetylorcinol were proved. The yields of these secondary metabolites produced in the NAA cultures were much lower than those produced by the 2,4-D cultures. It is well known that anthraquinone derivatives (emodin, chrysophanol and physcion) can play an important role as anticancer agents.

E- Quercetin production from *in vitro* cultures of *Pluchea lanceolata* Oliverr and Hiern as an anticancer agent:

Arya *et al.,* 2008 found that, maximum quercetin content (0.23 mg/g dry weight of tissues) was obtained in 6 weeks old callus tissues derived from leaf explants of *Pluchea lanceolata* Oliverr and Hiern. It is well known that quercetin can play an important role as anticancer agents.

F- Terpenoids and flavonoids production from *in vitro* cultures of *Azadirachta indica* as anticancer agents*:*

Babu *et al.,* 2008 found that, the most significant enhancement of terpenoid and flavonoids productivity can be obtained by feeding sucrose to the cell suspension cultures media of *Azadirachta indica* (A. Juss), sucrose added to the media (liquid MS medium containing 5% sucrose, 1% mannitol and 0.1 mg/l IBA) prolonged the stationary phase and sustain viability of cells in cultures without subculturing process for 60 days, thus accumulates higher amounts of secondary metabolites including three terpenoid compounds (azadirachtin, mimbin and salamin) and two flavonoids (quercetin and kaempferol). It is well known that flavonoids

(especially quercetin) and terpenoids can play an important role as anticancer agents.

G- Tropane alkaloids production from *in vitro* cultures of *Brugmansia suaveolens* as anticancer agents:

Hairy root cultures of *Brugmansia suaveolens* were set up by infection of root tips with *Agrobacterium rhizogenes*. The successful transformation was confirmed by analyzing rolC and virC genes using polymerase chain reaction (PCR). Hairy root cultures were employed to study the formation of tropane alkaloids, such as hyoscyamine. The transformed cultures were incubated with potential elicitors, such as methyljasmonate, quercetin and salicylic acid in order to stimulate the biosynthesis of tropane alkaloids. Profile and amounts of tropane alkaloids were analyzed using capillary GLC-MS. At least 18 different tropane alkaloids could be identified. Treatment of the cultures with 200 μm methyljasmonate increased the alkaloid accumulation 25-fold up to a level of 1 mg/g fresh weight as compared to untreated controls. Quercetin enhanced the alkaloid production 10 fold (0.4 mg/g fresh weight) within 24 hours. In contrast 100 μm salicylic acid decreased alkaloids to a level of 1 μg/g fresh weight. It is well known that tropane alkaloids can play an important role as anticancer agents.

H- Anthraquinones production from *in vitro* cultures of *Cinchona robusta* as anticancer agents:

A suspension culture of *Cinchona robusta* which under normal culture conditions does not produce anthraquinones but produces a range of these anthraquinones after elicitation [Eight new anthraquinones were identified as robustaquinones, in addition to two known anthraquinones 0-2-7 trihydroxy-1-methoxyanthraquinone and copareolatin 5-methyl ether]. Their oxygenation pattern characterized by a 5-6 disubstitution in the A ring is rare and raises questions about their biogenetic origin]. They might be derived from phenylpropanoids and not from o-succinyl-benzoic acid. It is well known that anthraquinone derivatives can play an important role as anticancer agents.

***I- In vitro* cultures of *Fagopyrum esculentum* for the production of some flavonoids as anticancer agents:**

Hairy roots culture of *Fagopyrum esculentum* was established by infection with *Agrobacterium rhizogenes* strain 15834. Faster growth (four-fold higher than normal roots culture) in B5 liquid medium was observed and the synthesis of five flavanols obtained : (+)-catechin, (-)-epicatechin, (-)-epicatechin-3-0-gallate, procyanidin B2 and procyanidin BZ3'-0-gallate also present in normal roots culture with a common prominency of epicatechin-3-0- gallate synthesis and inversion in the contents rates of (+)-catechin and (-) tepicatechin. The highest content of procyanidin B2-3'-0-gallate was obtained in hairy roots which can be considered as a better production source for flavanols, especially of the two galloylated derivatives. It is well known that flavonoids (catechin and epicatechin derivatives) can play an important role as anticancer agents.

J- Podophyllotoxin production from *in vitro* cultures of *Podophyllum*:

Podophyllotoxin (PTOX), the precursor for semi-synthesis of cancer therapeutics like etoposide, teniposide and etophos, is primarily obtained from an endangered medicinal herb, *Podophyllum hexandrum* Royle. PTOX, a lignan is biosynthetically derived from the phenylpropanoid pathway.

This compound is extracted from two *Podophyllum* species, *P. hexandrum* and *P. peltatum*. The former contains up to 4 % dry wt. of podophyllotoxin in its roots. As the natural sources of podophyllotoxin are very restricted, the attempt has been made to produce it by tissue cultures. The callus culture of *P. hexandrum* was established from roots of the *in vitro* cultures on a modified Gamborg B_5 medium. Suspension cultures were derived from the calli in the same liquid medium. The callus and cell suspension cultures showed a wide variety in producing podophyllotoxin (up to 0.1 % dry wt.). High yielding cell lines were selected by their dark brown color. The highest content which was obtained in callus and cell suspension cultures was 0. 3 % dry wt., produced by calli grown in the dark on modified Gamborg B_5 medium. Precursor feeding (coniferyl alcohol and coniferin) increased the productivity of the low producing cell suspension cultures, but not over a maximal yield of 0. 3 % dry wt. The production of podophyllotoxin derivatives has been reported from root and cell suspension cultures of *Linum flavum*.The highest amount of methypodophyllotoxin which was produced in these culture systems was ***1. 3*** % dry wt. in organized roots and 0. *2 %* dry wt. in cell suspension.

Since 5-methoxypodophyllotoxin, an analogue of podophyllotoxin, has strong cytostatic activity, many researchers have tried to improve its yield through tissue cultures.

The HPLC analysis showed approximately 7-8 fold change in accumulation of PTOX, in the 12d old cell suspension culture (i.e., after 9 days of elicitation) elicited with 100 uM MeJA as compared to the control. The phenylpropanoid and monolignol pathway enzymes were identified, amongst these, chalcone synthase, polyphenol oxidase, caffeoyl

CoA 3-O-methyltransferase, S-adenosyl-L-methionine-dependent methyltransferases, caffeic acid-O-methyl transferase etc. are noted as important. Elicitor-induced PTOX accumulation in *P. hexandrum* cell cultures provides a responsive model system to profile modulations in proteins related to phenylpropanoid/monolignol biosynthesis and other defense responses. Present findings form a baseline for future investigation on a non-sequenced medicinal herb *P. hexandrum* at a molecular level.

K- Paclitaxel from *in vitro* cultures of *Taxus:*

(Taxol; paclitaxel) and taxanes are complex oxygenated diterpenes, initially isolated from the stem bark of Pacific yew *(Taxus brevifolia* Nutt.) by Wani *et al.,* [1971]. More recently, these compounds have also been demonstrated to occur in needles of various *Taxus spp.* and in the mycelium of *Taxomyces andreanae* an endophytic fungus of Pacific yew. Little is known about the biosynthesis of paclitaxel and the possibility of engineering its production remains frustratingly remote.

For commercial use, paclitaxel was first extracted from the bark of the yew *T. brevifolia*, which has brought this species to the verge of extinction. Later, paclitaxel was produced semi-synthetically from precursors (baccatin III and 10-deacetyl baccatin III) isolated from needles of other yew species.

Taxus tree, is one of the most promising anticancer agents known due to its unique mode of action on the micro tubular cell system. At present, production of taxol by various *Taxus* species cells in cultures has been one of the most extensively explored areas of plant cell cultures in recent years owing to the enormous commercial value of taxol, the scarcity of the *Taxus* tree, and the costly synthetic process.

In 1989, Christen *et al.,* reported for the first time the production of taxol (placlitaxel) by *Taxus* cell cultures.

Fett-Neto *et al.*, (1995) have studied the effect of nutrients and other factors on paclitaxel production by *T. cuspidata* cell cultures (0.02% yield on dry weight basis). Srinivasan *et al.,* (1995) have studied the kinetics of biomass accumulation and paclitaxel production by *T. baccata* cell suspension cultures.

Paclitaxel was found to accumulate at high yields (1.5 mg/L) exclusively in the second phase of growth. Kim *et al.*, (1995) established a similar level of paclitaxel from *T. brevifolia* cell suspension cultures following 10 days in culture with optimized medium containing 6% fructose.

Ketchum and Gibson, (1996) reported that, the addition of carbohydrate during the growth cycle increased the production rate of paclitaxel, which accumulated in the culture medium (14.78 mg/l). In addition to paclitaxel, several other taxoids have been identified in both cell and culture medium of *Taxus* cultures. Parc *et al.*, (2002) reported that, the production of taxoids by callus cultures from selected *Taxus* genotypes.

In order to increase the taxoid production in these cultures, the addition of different amino acids to the culture medium were studied and phenylalanine was found to assist in maximum taxol production in *T. cuspidata* cultures.

The influence of biotic and abiotic elicitors was also studied to improve the production and accumulation of taxol through tissue cultures.

To enhance the productivity of *Taxus* cell cultures, biogenetic precursors, Nutrient feeding, *in situ* extraction, elicitors, a combination of inducing factors and jasmonic acid, have all been used. On the other hand, production of paclitaxel by immobilized cell cultures of *Taxus sp.* with a bioreactor has been reported before. The amount of paclitaxel released into the medium ranged from about 0.4 to 4 mg/L. The production could be enhanced by adding an elicitor or biogenetic precursor. However, the productivity was not as high as that in free cell cultures.

Paclitaxel produced after the addition of elicitors, chito-oligosaccharides and chito-heptaose, the addition of a biogenetic precursor, phenylalanine and the addition of chito-heptaose or jasmonic acid and another biogenetic precursor, phenylisoserine, with a combination of two elicitors and with a combination of one elicitor and the biogenetic precursor, in cell suspension cultures. The amount of paclitaxel produced was increased by supplying air to the cell suspension cultures, though productivity depended on the amount of air supplied. Furthermore, some of the paclitaxel produced in the cultures was released into the medium when cells from *T. cuspidata* var. nana were immobilized with calcium alginate.

For the production of taxol (paclitaxel) by immobilized cells cultured in a bioreactor, cells prepared from *Taxus cuspidata* var. nana were entrapped with calcium alginate and the amount of paclitaxel released into F4G4 liquid medium was investigated. Immobilized cell cultures were developed using various calcium alginate gel concentrations (2, 2.5 and 4% w/v) and cell concentrations (3, 5 and 10% w/v). Some of the paclitaxel produced in the immobilized cells was found to be released into F4G4 liquid medium. The amount released depended on the gel and cell concentrations used. The free cells in suspension cultures accumulated paclitaxel in intracellular compartments, while the cells immobilized with the

calcium alginate gel released some paclitaxel into the medium. The gel forced some of the paclitaxel produced in the cells to be released into the medium. When the cultures using immobilized cells entrapped at a 3% cell concentration and 2.5% gel concentration with an air-lift bioreactor were conducted for 30 days, the amount of paclitaxel released into the medium increased with time on the addition of an elicitor, chito-heptaose.

The release of paclitaxel was enhanced to 2.4 mg/L of culture medium after the 30 days of incubation. This is the first report that the release of paclitaxel into the medium by immobilized cell cultures of *Taxus* sp. was enhanced by the addition of an elicitor. Eight five percent of the paclitaxel produced in the cells was released by the immobilized cell cultures in 30 days. The results obtained in this study, with immobilized *T. cuspidata* var. nana cells in a 2.5 % calcium alginate gel, may pave the way for the production of paclitaxel by immobilized cell cultures in a bioreactor.

The production of taxol from nodule cultures containing cohesive multicultural units displaying a high degree of differentiation has been achieved from cultured needles of seven *Taxus* cultivars.

Factors influencing stability and recovery of paclitaxel from suspension cultures and the media have been studied in detail by Nguyen *et al.*, (2001). The effects of rare earth elements and gas concentrations on taxol production have been reported.

Results in Table. 1 are significant inasmuch as they show paclitaxel synthesis and accumulation in cells cultured *in vitro*. One may, thus, assume that, paclitaxel occurs in parenchyma cells, likely in older cells and according to one observation, in cells that tend to be rich in phenolics, for they easily turn brown over time. Stimulation of pacli taxel production may well be achieved by permutation of concentrations of various media components, by application of two-phase systems, or by application of biotic and abiotic stress agents (elicitors). Phyton Catalytic (New York) is said to have increased paclitaxel yields to 1-3mg/L of supernatant using elicitors. The idea of a cocultivation, as a mixture or separated by a membrane, of *Taxus* cells and fungus *(Taxomyces andreanae)* is intriguing.

Table. 1: Production of paclitaxel by *Taxus* spp. trees and cells cultured *in vitro*.

Species	Material	Medium	% DW	Ref.
T. brevifolia	Bark		0.015	Wickremesinhe and Arteca, 1993
	Roots	—	0.0004	
	Needles	—	0.015	
	Needles		0.006	Wickremesinhe and Arteca, 1994
T. brevifolia	Cell suspension	B_5[a]	—	Gibson *et al.,* 1993
	Gall[b] cells	$_{WP}$[b]	Trace	Han *et al.,* 1994
T. x *media*	Callus	B_5[C]	0.001	Wickremesinhe and Arteca, 1993
	Cell suspension	B_5[d]	Trace	Wickremesinhe and Arteca, 1994
T. cuspidata	Callus	B_5[e]	0.02	Fett-Neto *et al.,* 1992
	Immobilized cells	B_5[e]	0.012	Fett-Neto *et al.,* 1992
	Callus	B_5[f]	0.04	Fett-Neto *et al.,* 1993
T. andreanae	Mycelium		50 ng/L	Strobel *et al.,* 1993

[a]Medium after Gamborg *et al.,* (1968) with I mg/L 2,4-D and 0.2% casamino acids. [b]*Agrobacterium tumefaciens* gall-derived cells. Woody plant medium after Lloyd and McCown, (1980) without hormones. [c] Habituated callus. Medium after Gamborg *et al.*, (1968) with 2 x B_5 vitamins, without hormones. [d]Medium as for c with 1 mg 2,4-D and 1 mg/L kinetin plus 20 g/L sucrose + 2.5 g/L glucose + 2.5 g/L fructose. [e]Medium after Gamborg *et al.*, (1968) with 1 mg/L 2,4-D and 1 mg/L kinetin, plus 1.5% polyvinylpyrrolidone. [f]Medium after Gamborg *et al.*, (1968) as for e plus 0.1 mg/L phenylalanine.

For the bioengineering of paclitaxel production, prospects of success are slim, at the moment. For one, its biosynthetic pathway is undefined. The likely precursor is geranylger- anylpyrophosphate. Isolation and partial purification of an enzyme that catalyzes cyclization of this precursor has been reported. Second, transgenic conifers have been demonstrated. Still, a workable concept on how to genetically manipulate paclitaxel synthesis and accumulation has not been devised. ESGA genetics (California) may succeed in using a by-way by employing hairy root cultures for improved production. As it stands, 1 kg paclitaxel would require the harvest of 500-1000 bioreactors of 500 L each filled with a heavy suspension of *Taxus* cells.

Recently, commercial production of paclitaxel by *Taxus* cell suspension cultures was established. Yew cell suspension culture is used as an alternative source of paclitaxel (Taxolw), an effective anti-cancer drug (for advanced breast, ovarian and other cancers).

To optimize the initiation protocol, highly dormant yew seeds (*Taxus* X *mediarehd*) were germinated *in vitro* and the seedlings used to establish callus culture. The best sources of explant for callus initiation and growth were seedling stems and roots, and the most successful medium was modified B_5 medium containing 2,4-dichlorophenoxyacetic acid and kinetin. Calluses were friable and suitable for establishing cell suspension cultures, which were maintained for over 3 years.

Flow cytometric analysis of nuclear DNA content revealed that, 2 years old cell suspension cultures consisted predominantly of putative euploid and aneuploid cells coexisting as sub-populations. Additional measurements performed 3 and 7 mo. later revealed further genomic instability, with a tendency towards a higher proportion of cells with elevated nuclear DNA content. In a selected cell line, which showed significant taxane production, the addition of 100 mM jasmonic acid strongly enhanced total taxane production and slightly inhibited growth while no effect on nuclear DNA content was noted.

In conclusion, chromosomal rearrangements causing major genome size variation occur regularly in long-term yew cell suspensions. Such major genomic changes could affect the metabolic activity of cells, which is of major importance when they are used for paclitaxel production. The structure and stability of nuclear DNA content should be carefully monitored in large-scale suspension cultures.

L- Terpenoids from *in vitro* cultures of *Fossombronia:*

Liverworts (bryophyta) are known to produce a large variety of terpenes. Some of them exert interesting biological activities such as insecticidal, molluscicidal or tumor inhibitory activity. It is quite difficult to investigate secondary metabolites in *Fossombronia pusilla,* a small liverwort, since there is difficulty collecting a sufficient amount in the wild. Therefore, the *in vitro* culture of *F. pusilla* was established in order to analyze its secondary metabolites (sesqui- and diterpenes). The plantlets grew well on Gamborg B_5 solid medium, but when they were transferred to Gamborg B_5 liquid medium the cultures died. On the other hand, the addition of vitamin B_{i2} to the liquid culture of *F. pusilla* stimulated its growth and the production of the terpenes. After 32 days of culture the 5-fold amount of terpenes was produced compared to the solid medium. The main products were the diterpenedialdehydes perrottetianal A, B and 8-hydroxyperrottetianal A. Santonin, which was reported for the first time as constituents of a bryophyte, was produced in a lower amount. The *in vitro* culture of *F. pusilla* made it possible to obtain the material for the first phytochemical analysis of this liverwort.

M- Indole Alkaloids: Vinblastine derivatives from *in vitro* cultures of *Catharanthus* :

Vinblastine, a dimeric compound composed of catharanthine (1) and vindoline (2), two monoterpene indole alkaloids, is found in *Catharanthus roseus* (L.) G. Don (*Apocynaceae*), Madagascar periwinkle. The biosynthetic pathway has been elucidated to the extent that initial steps from tryptophan and mevalonic acid to strictosidine and catharanthine and from tabersonine to vindoline have been well analyzed and described. Natural dimerization to vinblastine includes 3'4'-anhydrovinbIastine. Chemical dimerization has been accomplished by way of the Polonovsky reaction.

In plants, vinblastine occurs at very low concentrations (5 g/2 tons FW). Owing to its cytotoxic effect, it may well function as a phytoalexin, a chemical defense agent. Vinblastine may occur in laticifers and idioblasts of *Catharanthus,* it accumulates in parenchyma throughout the plant. Of the two monomers, catharanthine has the highest concentrations in youngest, fully expanded leaves; the vindoline concentration was highest in leaves 5 and 7 from the apex.

Ever since 1958 when vinblastine and its analogue vincristine were recognized as anticancer agents and established as important drugs in the chemotherapeutic treatment of Hodgkin's disease and acute leukemia, its supply has been of great concern. Plantations of *C. roseus* in various parts of the world could match the demand, were it not for the extreme cost associated with extraction and purification. Alternative sources of higher value (i.e., rich in vinblastine and unaccompanied by a host of related alkaloids) are the goals targeted by R&D. Not surprisingly, the development of cell cultures of *C. roseus* began as soon as *in vitro* culture had been established as a method in plant physiology, with callus, crown-gall, and habituated tissue cultures. More recently, these efforts have been complemented by DNA technologies.

Despite many years of effort and remarkable *success* in increasing the production of catharanthine in cell suspensions by treatment with biotic and abiotic elicitors (Table. 2).

Table. 2: Production of catharanthine, vindoline, and vinblastine in plant tissues derived from *Cathararuhus roseus* and cultured *in vitro.*

Material	Medium Catharanthine	%DW	Ref.
Leaf 5	—	0.2	Balsevich and Bishop, 1989
Leaf 3	—	0.3	Balsevich and Bishop, 1989
Cell suspension	B_5[a]	0.001	Eilert *et al.*. 1986
Cell suspension	MS[b]	0.15	Smith *et al.,* 1988
Hairy roots	SH[C]	0.3	Jung *et al.,* 1994
Cell suspension	B_5[d] Vindoline	6.8	Fujita *et al.,* 1990
Leaf 5	—	0.3	Balsevich and Bishop, 1989
Leaf 3	—	0.4	Balsevich and Bishop, 1989
Hairy roots	B5[e]	Traces	Parr *et al.,* 1988
Shoot cultures	MS[f]	0.0015	Miura *et al.,* 1988
Regenerated shoots	—	0.01	Hirata *et al.,* 1993
Vinblastine/anhydrovinblastine			
Leaf 5	—	0.3	Balsevich and Bishop, 1989
Leaf 9	—	0.4	Balsevich and Bishop, 1989
Hairy roots	B_5[e]	Traces	Parr *et al.,* 1988
Shoot cultures	MS[f]	0.0015	Miura *et al.,* 1988
Regenerated shoots	—	0.01	Mirata *et al.,* 1993

[a]Medium after Gamborg *et al*., (1968) with *Pythium aphamdermatum* homogenate. [b]Medium after Murashige and Skoog, (1962) with 0.1 mg/L NAA, 0.1 mgA Idnetine, plus SO mg/L vanadylsulfate (selected cell line JOH). [c]Medium after Schenk and Hildebrandt, (1972), two-phase culture with inorganic salts x 1/3 in second phase. [d]Medium after Gamborg *et al*., (1968) using a high-yielding cell line at high inoculation density. [e]Medium after Gamborg *et al.,* (1968) without hormones. [f]Medium after Murashige and Skoog, (1962) with 1 mg/L benzyladenine.

Vinblastine has eluded all laboratories. The problem is a lack of vindoline synthesis and accumulation in cell suspension cultures. Still, vindoline recurs in shoots regenerated from callus and accumulates in multiple shoot cultures. This observation underpinned the notion that vindoline synthesis in material cultured *in vitro* requires prior differentiation of cells. Incidentally, differentiation of material as expressed in shoot cultures also stimulated the formation of vinblastine. Also, vindoline and catharanthine production in shoot cultures was influenced by phytohormone levels in the medium and treatment with near-ultraviolet light. Evidence for vindoline synthesis as a function of differentiation may be deduced from seedling analyses that show that enzymes involved in the transformation of tabersonine to vindoline are localized in the chloroplast, the endoplasmic reticulum, a putative alkaloid synthesizing vesicle, and cytoplasm, and are phytochrome-dependent, respectively. It remains to be seen whether photoautotrophic cells of *C. roseus* would produce sizable amounts of vinblastine. So far, photoautotrophic cell cultures have been incapable of synthesizing vindoline.

Recently, vindoline-producing cell cultures of *C. roseus* have been presented. These cultures had been treated with *A. tumefaciens* and characterized as habituated (i.e., growing in medium without hormones under constant fluorescent light of 2500 lux). Hairy root cultures generated by employing A *rhizogenes* have shown remarkable levels of catharanthine and traces of vinblastine.

Enhanced production of catharanthine and vindoline in *planta* and *in vitro is* expected to be effected by genetic manipulation of their biosynthesis or by genetically directed changes of the cytoenvironment of alkaloid synthesis and accumulation. Early work has shown that, elicitors would induce tryptophan decarboxylase and strictosidine activities in cell cultures of *C. roseus.* Since then, initial steps have been completed with the isolation and characterization of tryptophan decarboxylase cDNA and the isolation and heterologous expression of cDNA and genes of strictosidine synthase from *R. serpentina* and from *C. roseus*.

Finally, given sufficient yields of catharanthine from cell cultures and vindoline from shoot cultures or plants, vinblastine can be obtained through dimerization catalyzed by cell-free extracts of cells cultured in vitro, by enzymes of such cultures and by horseradish peroxidase with 3' 4' -anhydrovinblastine as an intermediate. Following a procedure by Vucovic, a chemical reaction involving Fe^{3+} as a catalyst may result in coupling of the two precursors to anhydro vinblastine at a yield of 90 % and in a

second step in the formation of vinblastine at a yield of 50 %.

The latter would appear to be the most promising technology for vinblastine production, to date.

Cryopreservation of *C. roseus* cells has been successfully demonstrated.

Another important study reported that the periwinkle, *Catharanthus roseus* L. (G.) Don, produces several commercially valuable secondary metabolites including the anticancer agent, vinblastine, vincristine and the hypertension drugs, ajmalicine and serpentine.

Periwinkle seeds, after sterilization were cultured on MS medium. Petiole segments of seedlings (4 days old) were subcultured to medium containing various concentrations of NAA accompanied with Kinetin and subcultured to regenerate the callus and root. Callus and roots were obtained from petioles in some of treatments. The extracts of callus and roots from different treatments were analyzed by spectrophotometer, TLC and HPLC with respect to the indole alkaloids producing capacity. Alkaloids were produced from callus and roots from petiole of *C. roseus* in the presence of 0.1, 5, 10 and 20 mg/L Kinetin and NAA. MS with 0.1 mg/L NAA + 0.1 mg/L Kinetin had the highest vindoline, catharanthine, vincristine and root organogenesis capacity. But the level of these alkaloids and ajmalicine were very low compared to that in petiole of intact plant, and the level of serpentine was similar. New roots, callus roots, and callus from MS medium containing 0.1 mg/L NAA + 0.1 mg/L Kinetin were subcultured in hormone-free and 0.1 mg/L NAA + 0.1 mg/L Kinetin media and for organogenesis and growth.

The most alkaloids amount was produced in new roots and callus roots. The indole alkaloid levels of new roots in new media were higher than in petioles of intact plants. 10-fold catharanthine, 125-fold serpentine, 0.5-fold vindoline and 0.34-fold ajmalicine were produced by new roots. The most interesting result was presentation of two important ant-cancer dimeric alkaloids, vinblastine and vincristine with amounts of 20-fold vinblastine and 6-fold vincristine to compare that in the petioles of intact plants.

N- Camoptothecin derivatives from *in vitro* cultures of *Camptotheca:*

Camptothecin, a potent antitumor alkaloid was isolated from *Camptotheca acuminata.* Sakato and Misawa, (1974) induced *C. acuminata* callus on MS medium containing 0.2 mg/L 2,4-D and 1 mg/L kinetin and developed liquid cultures in the presence of gibberellin, L-tryptophan, and conditioned medium, which yielded camptothecin at about 0.0025 % on a dry weight basis. When the cultures were grown on MS medium containing 4 mg/L NAA, accumulation of camptothecin reached 0.998 mg/L. 10-Hydroxycamptothecin, a promising derivative of camptothecin is in clinical trials in the US.

Elicitor-induced accumulation of secondary metabolites in plant cell culture has received increasing attention. This is due to, in part, the fact that elicitation can improve the efficiency of secondary metabolite accumulation in culture systems where product formation appears near or after the late growth phase. Elicitation of secondary metabolite production is a function of the source material used for elicitor preparation. Some elicitors can cause induction and accumulation of secondary metabolites, whereas others may result in no response. In some cases, different elicitors may also result in unique responses within the same cell culture. The elicitor concentration is a factor that strongly affects the intensity of the response. The accumulation rate is highly affected by elicitor concentration at low elicitor concentration, but virtually unaffected at high elicitor concentration. Inhibition by overdosed elicitor can reduce the accumulation of secondary metabolites as well as cell growth. Camptothecin production was increased with elicitors, methyl jasmonate, jasmonic acid, yeast extract elicitor, and ferulic acid in suspension cultures of *Camptotheca acuminata.* Jasmonic acid was found to be the most efficient elicitor. Camptothecin production increased 11 times by using the optimum dosing concentration of jasmonic acid which was 50 t~ M. The kinetics of camptothecin accumulation in response to the treatment with jasmonic acid showed that, the camptothecin accumulation reached the maximum value at 4 days after jasmonic acid dosing and then a rapid decrease in camptothecin accumulation was observed (Song and Byun, 1998).

O- Antitumor activity of *in vitro* cultures of green tea seed (*Camellia sinensis* L.):

The antitumor activity of suspension cultures of tea callus cells grown in the presence of different concentrations of the growth regulator 2,4-dichlorophenoxy acetic acid (2,4-D) with or without light irradiation. The methanol and ethanol extracts of precipitated cells (MEP, EEP) exhibited stronger inhibitory effects on the growth of tumor cell lines than the water extract of precipitated cells (WEP) or the supernatant.

Compared to culture under dark conditions, exposure to light irradiation led to significantly higher antitumor activity. The MEP from light irradiated cells at 250 μg/mL with 2.0 mg/L 2,4-D displayed more than 64 % growth inhibition of HEP-2 cells, whereas normal cells showed less than 25 % growth inhibition. The some fractions of MEP obtained from Diaion HP-20 column chromatography displayed the

majority of inhibitory activity against the HEP-2 cell line. These results showed that, 2,4-D, and light stimulated the synthesis of antitumor compounds.

P- Production of shikonin derivatives from *in vitro* cultures of *Arnebia* (Royle) Johnst (Ruanzicao) as an antitumor agent:

Shikonin derivatives have cytotoxic and antitumor effects. The antitumor effects of acetylshikonin isolated from a Chinese medicinal herb *Arnebia euchroma (Royle) Johnst* were investigated. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the *in vitro* antitumor effects of acetylshikonin on {human lung adenocarcinoma cell line A549, human hepatocellular carcinoma cell line Bel-7402, human breast adenocarcinoma cell line MCF-7} and mouse Lewis lung carcinoma (LLC) cell line. C_{57}BL/6 mice with LLC model were used to study the *in vivo* antitumor effects of acetylshikonin. The expression of bax, bcl-2 and caspase-3 proteins in LLC tissue was determined with immunohistochemical staining. In A549, Bel-7402, MCF-7 and LLC cell lines, acetylshikonin inhibited cell growth in a dose-dependent manner. IC_{50} (means ± SD) were 5.6 ± 0.86 μg/mL, 6.82 ± 1.5 μg/mL, 3.04 ± 0.44 μg/mL and 2.72 ± 0.38 μg/mL, respectively. Acetylshikonin suppressed tumor growth in C57BL/6 mice with LLC. The inhibition rate of acetylshikonin (2 mg/kg) was 42.85 %.

Immunohistochemical staining revealed that, in the acetylshikonin groups the expression of bax and caspase-3 increased, whereas the expression of bcl-2 decreased, suggesting that, acetylshikonin induced tumor cell apoptosis through activating the pro-apoptotic bcl-2 family and caspase-3. Acetylshikonin isolated from *Arnebia euchroma (Royle) Johnst* cell suspension cultures exhibits specific *in vivo* and *in vitro* antitumor effects.

Q- Anticancer activity of *in vitro* cultures of rice:

A multitude of natural products from plant extracts have been tested for their ability to inhibit the progression of several diseases including cancer. A novel approach of evaluating plant (rice) callus suspension cultures for anticancer activity is reported.

The ability of different dilutions of rice callus suspension cultures to inhibit growth of two human cancer cell lines was tested employing varying cell numbers and different incubation times. A crystal violet assay was performed to assess cell viability of the cancer cell lines. Furthermore, microscopic analysis was carried out to determine the effect of the rice callus culture on the morphology of the cancer cells. Rice callus suspension cultures significantly inhibited the growth of human cancer and renal cell lines at densities of 5000 and 10000 cells/mL when incubated for 72 and 96 hours.

Rice callus suspension culture was more efficient than paclitaxel (Taxol®) and etoposide in selectively killing human colon and renal cancer cell lines compared with a control cell line (human lung fibroblasts). The use of plant callus suspension cultures is a novel approach for inhibiting the growth of cancer cells, which will lead to the development of new agents for selectively killing cancer cells.

It could be concluded that, *in vitro* cultures of some plants (*Lycopersicon esculentum, Rheum palmatum* L., *Polygonum hydropiper, Rumex alpinus* L., *Pluchea lanceolata* Oliverr and Hiern, *Azadirachta indica, Brugmansia suaveolens, Cinchona robusta, Fagopyrum esculentum, Podophyllum, Taxus, Fossombronia, Catharanthus, Camptotheca, Camellia sinensis* L., *Arnebia* (Royle) Johnst (Ruanzicao) and rice) can be considered rich sources of anticancer agents (alkaloids, flavonoids, anthraquinones, etc.,).

4. References:

1. Alam, A. Eman. *In Vitro* Studies on *Rumex vesicarius* L. (Polygonaceae) for improving Some Active Constituents (Ph.D), Botany Department, Faculty of Science, Helwan University, Egypt (2012 a).
2. Alam, A. Eman. Initiation of Pharmaceutical Factories depending on more Application of Biotechnology on some Medicinal Plants Review Article (*In Vitro* Production of some Antioxidant, Analgesic, Antibacterial, Antidiabetic agents). *Research Journal of Recent Sciences*. 1(ISC-2011): 398-404 (2012 b).
3. Alam, A. Eman. Plant Biotechnologies and Pharmaceutical Products. (Published in many Publishers in Different Countries, Al-Ahram Publisher, Osiris Publisher, The Anglo Bookshop (Egypt) and Marsland Press (United States of America) and Innovare Academic Sciences (India), (First Edition 2013).
4. Anand, S. Various approaches for secondary metabolite production through plant tissue culture. *Pharmacia*.1 (1):1-7 (2010).
5. Arya, D.; Patni, V. and Kant, U. (2008). *In vitro* propagation and quercetin quantification in callus cultures of Rasna (*Pluchea anceolata* Oliver& Hiern.). Indian Journal of Biotechnology, 7: 383-387.
6. Babu, V.S.; Narasimhan, S.; Nair, G.M. (2008). Enhanced accumulation of terpenoids and flavonoids in cell suspension cultures of *Azadirachta indica* (A. Juss) with an extended stationary phase. Indian Journal of Biotechnology, 7: 270-272.
7. Baebler, S., Hren, M., Camloh, M., Ravnikar, M., Bohanec, B., Plaper, I., Vcman, R. and Zel, J. Establishment of Cell Suspension Cultures of

Yew (*Taxus X media Rehd.*) and Assessment of Their Genomic Stability. In vitro Cell. Dev.Biol-Plant. 41:338-343.
8. Balsevich, J. and G. Bishop. Distribution of catharanthine, vindoline, and 3'4'-anhydrovinblastine in the aerial parts of some *Catharanthus roseus* plants and the significance thereof in relation of alkaloid production in cultured cells. *Primary and Secondary Metabolism of Plant Cell Cultures,* (Kurz, W. G. W. ed.). Springer Verlag, Berlin, 1989, pp 149-153.
9. Bcnisrll, M., Lee, C.Y, Lo, S.F., Nclcwcdl, S.M., Lin, C.Y. and Tscy, H.S.. *Bot. Bull. Acad.Sin.* 45:1-22 (2004).
10. Bertoli, A., Ruffoni, B., Pistelli, L. and Pistelli, L., Analytical methods for the extraction and identification of secondary metabolite production in *'in vitro'* plant cell cultures. *Adv Exp Med Biol.* 698:250-266 (2010).
11. Bhattacharyya, D., Sinha, R., Ghanta, S., Chakraborty, A., Hazra, S. and Chattopadhyay, S. . Proteins differentially expressed in elicited cell suspension culture of *Podophyllum hexandrum* with enhanced Podophyllotoxin content. *Proteome Science.* 2012: 10-34 (2012).
12. Campbell JK, Rogers RB, Lila MA and Erdman JW Jr . Biosynthesis of ^{14}C-phytoene from tomato cell suspension cultures (*Lycopersicon esculentum*) for utilization in prostate cancer cell culture studies. *Journal of Agricultural and Food Chemistry* . 54(3): 747-755 (2006).
13. Choi, K.T., D.C. Yang and J.C. Park. Characterization of cell line of ginseng (*Panax ginseng* C.A. Mayer) transformed by *Agrobacterium tumefaciens. In* S. Lyams, and G. Takeds (eds.), Proc. Of the 6th Internatl. Congr. of SABRAO, Tokyo, 1989, pp. 519-522.
14. Choi, K.T., I.O. Ahn and J.C. Park. Production of ginseng saponin in tissue culture of ginseng (*Panax ginseng* C. Mayer). *Russian J. Plant Physiol*. 41: 784-788 (1994).
15. Christen, A.A., J. Bland and G.M. Gibson. Cell cultures as a means to produce taxol. *Proc. Am. Assoc. Cancer Res.* 30: 566 (1989).
16. Deshpande A, Dhadi SR, Hager EJ and Ramakrishna W. Anticancer activity of rice callus suspension culture. *Phytother Res.* 26 (7): 1075-1081 (2011).
17. Eilert, U., F. Constabel and W. G. W. Kurz. Elicitor mediated induction of tryptophan decarboxylase and strictosidine synthase in cell suspension cultures of *Catharanthus roseus. Arch. Biochem. Biophys.* 254:491-497 (1987).
18. Elena Cequier-Sánchez, Covadonga Rodríguez, Roberto Dorta-Guerra, Ángel G Ravelo and Rafael Zárate. *Echium acanthocarpum* hairy root cultures, a suitable system for polyunsaturated fatty acid studies and production. Cequier-Sánchez et al. BMC Biotechnology 2011, 11:42 (2011).
19. Engelmann, N.J. J, Campbell , J.K.,Rogers, R.B., Rupassara, S.I., Garlick, P.J., Lila, M.A. and Erdman , J.W. .Screening and Selection of High Carotenoid Producing in Vitro Tomato Cell Culture Lines for [^{13}C]-Carotenoid Production .*J. Agric. Food Chem.*58 (18): 9979–9987 (2010).
20. Fett-Neto A, G., J.J. Pennington and F. DiCosmo. Effect of white light on taxol and baccatin III. Accumulation in cell cultures of *Taxus cuspidata* Sieb and Zucc. *J. Plant. Physiol*. 146: 584-590 (1995).
21. Fett-Neto, A. G., F. DiCosmo, W. F. Reynolds and K. Sakata. Cell culture of *Taxus* as a source of the antineoplastic drug Taxol and related taxanes. *Biotechnology*. 10:1572-1575 (1992).
22. Fett-Neto, A. G., S J. Nlelanson, K. Sakata, and F. DiCosmo. Improved growth and Taxol yield in developing calli of *Taxus cuspidate* by medium composition modification. *Biotechnology :*11.731-734 (1993).
23. Fett-Neto, A.G., J.M. Stewart, S.A. Nicholson, J.J. Pennington and F. DiCosmo. Improved taxol yield by aromatic carboxylic acid and amino acid feeding to cell cultures of *T. cuspidata. Biotechnol. Bioeng*. 44: 967-971 (1994).
24. Fujita, Y., Y. Hara, T. Morimoto and M. Misawa. Semisynthetic production of vinblastine involving cell cultures of *Catharanthus roseus* and chemical reaction. *Progress and Plant Cellular and Molecular Biology* (H. J. J. Nijkarnp, L. H. W. van der Plass, and J. van Aartrijk, eds.), Kluwer Academic Dordrecht, 1990, pp. 738-743.
25. Gamborg, O.L., R.A. Miller and K. Ojima. Nutrient requirements of suspension cultures of soyabean root cells. *Exp. Cell Res*. 50: 151-158 (1968).
26. Gibson, D. M., R. E. B. Ketchum, N. C. Vance and A. A. Christen. Initiation and growth of cell lines of *Taxus brevifolia* (Pacific yew). *Plant Cell Rep.*72:479-482 (1993).
27. Han, K. H., P. Fleming, K. Walker, M. Loper, W. S. Chilton, U. Mocek, M. P. Gordon and H. G. Floss. Genetic transformation of mature *Taxus.* An approach to genetically control the *in vitro* production of the anticancer drug, Taxol. *Plant Sci.* 95:187-196 (1994).

28. Hara, M., S. Tanaka and M. Tabata. Induction of a specific methyltransferase activity regulating berberine biosynthesis by cytokinin in *Thalictrum minus* cell cultures. *Phytochemistry*. 36:327-332 (1994).
29. Hara, M., T. Kitamura, H. Fukui and M. Tabata. Induction of berberine biosynthesis by cytokinins in *Thalictrwn minus* cell suspension cultures. *Plant Cell Rep*. 12:10-73 (1993).
30. Hara, M., Y. Kobayashi, H. Fukui and M. Tabata. Enhancement of berberine production by spermidine in *Thalictrum minus* cell suspension cultures. *Plant Cell Rep*. 10: 494-497 (1991).
31. Hara, Y., T. Laugel, T. Morimoto and Y. Yamada. Effect of gibberellic acid on berberine and tyrosine accumulation in *Coptis japonica*. *Phytochemistry*. 36:643-646 (1994).
32. Hirata, K., M. Asada, E. Yatani, K. Miyamoto and K. Miura. Effects of near-ultraviolet light on alkaloid production in *Catharanthus roseaus* plants. *Planta Med*. 59:46-50 (1993).
33. Jung. K.-H., S. S. Kwak. C. Y. Choi and J. R. Liu. Development of a two-stage culture process by optimization of inorganic salts for improving catharanthine production in hairy root cultures of *Catharanthus roseus*. *J. Ferment. Bioeng*. 77:57-61 (1994).
34. Karuppusamy, S. A review on trends in production of secondary metabolites from higher plants by *in vitro* tissue, organ and cell cultures. *Journal of Medicinal Plants Research*. 3(13):1222-1239 (2009).
35. Kasparová, M.; Siatka, T. and Dusek, J. (2003). Effect of abiotic elicitation and calcium ions on production of explant cultures of *Rheum palmatum* L. Ceska Slov Farm, 52 (5): 248-251.
36. Ketchum, R.E.B. and D.M. Gibson. Paclitaxel production in suspension cell cultures of *Taxus*. *Plant Cell Tiss. Org. Cult*. 46: 9-16 (1996).
37. Kim, J.H., J.H. Yun, Y.S. Hwang, S.Y. Byun and D. I. Kim. Production of taxol and related taxanes in *Taxus brevifolia* cell cultures: Effect of sugar. *Biotechnol. Lett*. 17(1): 101-106 (1995).
38. Kumar, S.; Mishra, S. and Mishra, A.P. *Plant Tissue Culture (Theory and Techniques)*. India. Scientific Publishers, 2012.
39. Kurz, G.W. and Constabel, F. . *National Research Council of Canada publication* no. 38467.
40. Lauren E. Conlon, Ryan D. King , Nancy E. Moran and John W. Erdman , Jr. Coconut Oil Enhances Tomato Carotenoid Tissue Accumulation Compared to Safflower Oil in the Mongolian Gerbil (*Meriones unguiculatus*). *J. Agric. Food Chem.*60 (34): 8386-8394.
41. Lloyd, G. B. and B. H. McCown. Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia,* by use of shoot-tip culture. *Proc. Int. Plant Prop. Soc*. 30:421-427 (1980).
42. Lu, C.H.,. Engelmann, N.J., Lila, L.M. and Erdman, J.W. . Optimization of Lycopene Extraction from Tomato Cell Suspension Culture by Response Surface Methodology. J Agric Food Chem. 56(17): 7710–7714 (2008).
43. Matkowski, A. Plant *in vitro* culture for the production of antioxidants – A review. *Biotechnol. Adv*. 26: 548- 560 (2008).
44. Miura, Y., K. Hirata, N. Kurano, K. Miyamoto and K. Uchida. Formation of vinblastine in multiple shoot culture of *Catharanthus roseus*. *Planta Med*. 54:18-20 (1988).
45. Murashige, T. and F. Skoog. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant*. 75:473-497 (1962).
46. Nguyen, T., J. Eshraghi, G. Gonyea, R. Ream and R. Smith. Studies on factors influencing stability and recovery of paclitaxel from suspension media and cultures of *Taxus cuspidata* cv Densiformis by high-performance liquid chromatography. *J. Chromatogr*. A. 911: 55-61 (2001).
47. Ono, K.; Nakao, M.; Toyota, M.; Terashi, Y.; Yamada, M.; Tetsuya, Y.; Kohno, M. and Asakawa, Y. (1998). Catechin production in cultured *Polygonum hydropiper* cells. Phytochemistry, 49 (5): 1935-1939.
48. Parc, G., A. Canaguier, P. Landre, R. Hocquemiller, D. Chriqui and M. Meyer. Production of taxoids with biological activity by plants and callus cultures from selected *Taxus* genotypes. *Phytochemistry*. 59: 725-730 (2002).
49. Parr, A. J., C. J. Peerless, J. D. Hamill, N. J. Walton, R. J. Robins and M. J. C. Rhodes. Alkaloid production by transformed root cultures of *Catharanthus roseus*. *Plant Cell Rep*. 7:309-312 (1988).
50. Pierik, R.L.M. *In vitro* Culture of Higher Plants. Netherlands. Martinus Nijhoff Publishers, 1987.
51. Purohlt, S.S. *Plant Biotechnology*. India. Student Edition Publisher, 2007.
52. Sasson, A. *Biotechnologies and Development*. Paris, UNESCO/CTA, 1988, pp. 361.
53. Sasson, A. Production of useful biochemicals by higher-plant cell cultures: biotechnological and economic aspects. *Options Mediterraneenes*. 14: 59-74 (1991).
54. Sauerwein, M.; Yoshimatsu, K. and Koichiro, S. Further Approaches in the Production of Secondary Metabolites by Plant Tissue Cultures. *Plant Tissue Culture Letters*. 9 (1):1-9 (1992).
55. Schenk, R. U. and A. C. Hildebrandt. Medium and techniques for induction and growth of monocotyle- donous and dicotyledonous plant cell cultures. *Can. J. Bot*. 50:199-204 (1972).

56. Schripsema, J.; Ramos, V.A. and Verpoorte, R. (1999). Robustaquinones\ novel anthraquinones from an elicited *Cinchona robusta* suspension culture. Phytochemistry, 51: 55-60.
57. Smith, J.I., N.J. Smart, M. Misawa, W.G.W. Kurz, S.G. Tallevi and F. DiCosmo. Increased accumulation of indole alkaloids by some cell lines of *Catharanthus roseus* in response to addition of vanadyl sulphate. *Plant Cell Rep*. 6: 142-145 (1987).
58. Song, S.H. and Byun, S.Y. (1998). Elicitation of camptothecin production in cell cultures of *Camptotheca acuminata*. Biotechnological Bioprocess Engineering, 3: 91-95.
59. Srinivasan, V., L. Pestchanker, S. Moser, T. Hirasuma, R.A. Taticek and M.L. Shuler. Taxol production in bioreactors; kinetics of biomass accumulation, nutrient uptake and taxol production by cell suspensions of *Taxus baccatus*. *Biotechnol. Bioeng*. 47: 666-676 (1995).
60. Strobel, G.A., A. Stierle and J.G.M. Van Kuijk. Factors influencing the *in vitro* production of radiolabelled taxol by Pacific yew, *Taxus brevifolia*. *Plant Sci*. 84: 65-74 (1992).
61. Trotin, F.; Moumou, Y. and Vasseur, J. (1993). Flavanol production by *Fagopyrum esculentum* hairy and normal root cultures. Phytochemistry, 32 (4): 929-931.
62. Van and Labadie (1981). The production of acetate derived hydroxy anthraquinones hydroxy dianthrones hydroxy naphthalenes and hydroxy benzenes in tissue cultures from *Rumex alpinus*. Planta Medica, 41 (2): 169-173.
63. Wani, M.C., H.L. Taylor, M.E. Wall, P. Coggon and A.T. McPhail. Plant antitumor agents VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. *J. Am. Chem. Soc*. 93: 2325-2327 (1971).
64. Watase, I., Sudo, H., Yamazaki, M. and Saito, K. . Regeneration of transformed *Ophiorrhiza pumila* plants producing camptothecin. *Plant Biotechnology*. 21(5): 337–342 (2004).
65. Wickremesinhe, E.R.M. and R.N. Arteca. *Taxus* callus cultures: Initiation, growth optimization, characterization and taxol production. *Plant Cell Tiss. Org. Cult*. 35: 181-193 (1993).
66. Wickremesinhe, E.R.M. and R.N. Arteca. *Taxus* cell suspension cultures: optimizing growth and production of taxol. *J. Plant Physiol*. 144: 183-188 (1994).
67. Xiong, W., Luo, G., Zhou, L., Zeng, Y. and Yang, W.. *In vitro* and *in vivo* antitumor effects of acetylshikonin isolated from *Arnebia euchroma (Royle) Johnst* (*Ruanzicao*) cell suspension cultures *Chinese Medicine*. 2009: 4-14 (2009).
68. Zayed, R. and Winka, M. (2004). Induction of tropane alkaloid formation in transformed root cultures of *Brugmansia suaveolens* (Solanaceae). Z. Naturforsch, 59: 863-867.

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