Chapter 7.
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In the area of cancer research, the rigorous efforts have been continuing for the discovery of novel molecules to combat with cancer. The disease, cancer, is becoming a large public health problem and dreadful cause of death in these days. Due to many unique genetical characteristics of transformed cell, they acquire resistance to many therapeutic agents that are currently in use (Cress AE & Dalton WS, 1996). The secondary tumors are also a block for cancer chemotherapy. In 2006, more than 13 lakhs of new cases of cancer have been reported of which highest number holds the cancer of genital system (Jemal A, et al., 2007). The prevention and effective treatment of cancer can be brought about by better understanding of cause of cancer and characteristic alterations in the genetical and biochemical behavior of transformed cells. The research is ongoing to find novel molecules with anticancer properties that spare normal cells.

Nature has been regarded treasure trove of many wonderful chemical entities that could be possible candidates for cancer chemotherapy. Plants are the reliable source of humans for thousands of years in maintaining health and improving the quality of life. WHO estimated that approx. 80% of earth’s inhabitants rely on traditional medicine for their primary health care needs. Reserpine, which is widely used for the treatment of high blood pressure, was originally extracted from the plant Rauwolfia serpentina, whereas digitalis, used as a heart stimulant, was derived from the foxglove plant (Digitalis purpurea). The Chinese herb ephedra (Ma huang), which contains the active substance ephedrine, was used early on for the treatment of asthma, whereas salicylic acid (a precursor of aspirin) was obtained from willow tree bark (Salix alba) to help relieve fevers. Over-the-counter laxatives commonly contain psyllium, senna, or Cascara
The laxative effect of the latter 2 herbs is due to the presence of anthraquinones, which stimulate peristalsis, whereas the mucilages in psyllium provide a bulking effect (Bruneton J, 1995). The important clinical drugs like taxol, camptothecin, vincristine, vinblastine and lignan podophyllotoxin are derived from plant sources. These important molecules are generally accumulated in their bark or roots.

For the isolation of these compounds, destructive harvesting of plant species usually carried out. Due to natural origin, the market prices of these drugs are quite high: 1kg of vincristine costs about US$ 20,000 and cost of Taxol is estimated to be US$ 5 million/kg. Prices for camptothecin, podophyllotoxin are in range of vincristine (Wink M, et al., 2005). Since the natural supply of these compounds are limited, in order to meet the requirement for these drug leads, alternative sources like in vitro cell, tissue and organ cultures of their respective natural source plant became an option. In the present study, we examined methods for the production and enhancement of high value compound, camptothecin, from the in vivo cultures of O. rugosa. Also, anthraquinone pigments are isolated from the in vitro plants and evaluated the anticancer property experimentally.

We quantified camptothecin of in vivo plant and in vitro plant by HPLC. In vivo plant itself is a very good source of camptothecin. We compared two species of Ophiorrhiza viz., O. rugosa and O. pectinata. The CPT was quantified in leaf, stem, root and inflorescence of both the species. It was surprising to find that O. rugosa produced the highest amount of CPT that has ever been reported. The quantity was 3.732mg/g DW of root. And the other parts also yielded a very good amount of CPT. Inflorescence contained same level of CPT in both the plants. In other plant species like Nothapodytes foetida (0.05% of dry weight, Roja G & Heble MR, 1994) and O. mungos (Tafur S, et al., 1976), the CPT content is significantly very less compared to O. rugosa. The potential of O. rugosa to serve as a viable source of CPT, we
established shoot and root cultures of *O.rugosa* and analyzed the production of CPT in cultures.

Plant growth regulators like NAA & BA alone or in combination were studied for their effect on CPT production in shoot and root cultures. In shoot cultures, the maximum amount of CPT production as 0.311mg/g DW when cultures were supplemented with 6mg/l NAA. This was comparable with the CPT yield from the stems of *in vivo* plant when the CPT yield was 0.233 in stem and 0.57mg/g DW of leaves respectively. Eventhough, we didn’t obtain higher amount of CPT than *in vivo* plant in cultured plants.

The combination of NAA with 6BA in multiple shoot cultures has yielded 0.911mg CPT on per gram dry weight basis.

In roots of *in vivo* plant produced 3.732mg CPT /g DW. This is the highest amount ever found in *Ophiorrhiza species*. In *C. acuminata*, young leaves were found to produce 3-5mg CPT/g DW(Lopez-Mayer *et al*., 1994). In root cultures, the combination of 0.5NAA with 2BA yielded 0.65mg CPT /g DW. It was found that modulation of phytohormones induced variation in secondary metabolite production.

We studied different elicitors to enhance the CPT content in cultures. The initiation of a plant defense response of production of secondary metabolites (Côté and Hahn, 1994) requires the perception of pathogen-derived (exogenous) or plant derived (endogenous) signal molecules, collectively referred to as elicitors (Boller T, 1995). Chitin actually decreased the CPT yield compared to control cultures. The increase of CPT by 1mg/l chitin on 7th day was statistically not significant (p>0.05). But chitosan (5mg/l) increased CPT content twofold from control cultures on 7th day. Chitosan is an effective elicitor that utilizes octadecanoid pathway of jasmonic acid to activate plant defense genes (Doeres SH, *et al*., 1995). Chitosan is found to have a positive effect on anthraquinone production in *Rubia tinctorum* cultures (Vasconsuelo A, *et al*., 2004). The elicitation with signal
molecule methyl jasmonate (MJ) increased CPT yield to 0.83 mg/g DW on 7th day of culture treated with 5 mM MJ. Tryptophan decarboxylase (TDC) and strictosidine synthase (STR) are two enzymes involved in the biosynthesis of monoterpenoid indole alkaloid such as CPT. MJ itself induced Tdc and Str gene expression when added exogenously. The exudation of CPT into medium by MJ was very low. It was almost equal to control cultures. Cultures extract of *Saccharomyces cerevisiae* enhanced CPT yields to a level of 0.98 mg in cultures and exudated amount of CPT into medium was 0.017 mg/l. This amount was higher than induced by methyl jasmonate. It was found that nicotine and other alkaloids are produced by the increase of endogenous jasmonate pools when plant cells are treated with an elicitor prepared from yeast cell walls. In turn, jasmonates are known to induce accumulation of secondary metabolites in cell culture (Ketchum RE, *et al.*, 1999). In *C. roseus* cells, the involvement of octadecanoid pathway in elicitor induced transcriptional activation of genes of the terpenoid indole alkaloid (TIA) biosynthetic pathway is already established (Menke FLH *et al.*, 1999). Surfactants like Tween 20 used to permeabilize CPT into medium. It enhanced CPT yield to 0.9 mg/g DW of cultures but the permeabilization was poor compared to control cultures. It was found that elicitation could enhance CPT yield compared to control cultures. Even though, the yield of CPT in the roots of *in vivo* plant was found superior to cultures.

These concentrations of tryptophan (Trp), viz, 200 mM, 300 mM and 400 mM, were studied for CPT production. 400 mM tryptophan produced 0.42 mg CPT/g DW of culture. Tryptophan biosynthetic in plants in addition to contributing to protein biosynthesis, also supplies precursors for the biosynthesis of plant growth regulating like auxin and indole alkaloids including camptothecin (Lu H & McKnight TD, 1999). Tryptophan provides the indole moiety for monoterpenoid indole alkaloid biosynthesis. Trp is decarboxylated by TDC to produce tryptamine. Tryptamine is then conjugated to the terpenoid
secologanin, to form the key intermediate strictosidine. Strictosidine is a precursor to more than 1800 alkaloids, including camptothecin (Kutchan, 1996). The *C.acuminata* genome encodes two TDC genes that are differentially expressed. TDC1 expression is correlated with the sites and times of camptothecin accumulation.

The spontaneous origin of albino plants in cultures treated with higher concentrations of BA, opened new possibilities to enhance the CPT production. The amount of CPT production in albino plants was higher than compared to their normal green counterparts.

The effect of sucrose at different concentrations was found 3% of sucrose was optimal for CPT yield. The yield of CPT from cultures was never achieved the level produced by *in vivo* roots of *O.rugosa*. Eventhough, the *in vitro* culture can be exploited for CPT production without disturbing the natural flora.

*Ophiiorrhiza* species contain many anthraquinones as reported by Kitajima M, et al(1998) from *O.pumila* and Chan HH, et al(2005) from *O.hayatana* Ohwi. The efficacy of anthraquinones as antineoplastic agent has been well enumerated so far. The very structure, hydroxyl and other substitutions in the anthraquinone skeleton make them potent candidate for anticancer drug discovery. We studied the quantity of total AQs *in vivo* plant and different cultured plants and found more quantity of anthraquinone pigments is produced by *in vitro* culture systems than *in vivo* system. Chromatographic analysis showed that a few pigments, which are present *in vivo*, are absent in *in vitro* and vice versa. Since the quantity of AQs from *in vivo* plants is very low, we isolated anthraquinones from *in vitro* cultures of *O.rugosa*. This was done parallel with the studies on CPT production.

The anthraquinone fraction(AQf) from *O.rugosa* cultures were studied for antioxidant, antitumor and anti-inflammatory activities. The antioxidant assays revealed that AQf has the efficacy to scavenge OH´ radicals and prevent lipid peroxidation. It showed no activity to
scavenge NO\(^{1-}\) radicals. To our surprise AQf generated O2\(^{1-}\) radicals instead of scavenging them. Anthraquinones in aqueous medium by acting as an electron carrier, produce superoxide radicals. AQf showed good antitumor activity. It decreased the volume of solid tumor and increased the life span of ascites tumor bearing animals compared to control groups. A good antiinflammtory activity was found for AQf in experimental animals.

The study on the mechanism of action of antitumor activity of AQf, cytotoxic effect AQf and anthraquinones was carried out. AQf effectively killed tumor cells in vitro as evaluated by trypan blue exclusion method. In 12h cultures of DLA and EAC cells, apoptotic death was induced by more efficiently by two anthraquinones. These anthraquinones induced apoptosis by generation of O2\(^{1-}\) radicals and intracellular H2O2 as detected by NBT assay and DCFH-DA staining respectively. It was also found that AQ1 induce apoptosis by the activation of caspase-3. ROS has been known to play an important role in apoptosis. Emodin, a natural dihydroxy anthraquinone is reported to elevate ROS levels on HeLa cells. Increased ROS levels in turn promoted mitochondrial transmembrane potential collapse; inhibited the activation of transcription factors NF-\(\kappa\)B. Thus anthraquinones like emodin induces apoptosis by inhibiting survival signaling as well as eliciting apoptotic-signaling pathway(Yi J, et al., 2004). Emodin was also found to activate caspase-3 and release cytochrome c in human lung adenocarcinoma A549 cells. These events are accompanied by inactivation of ERK and AKT, generation of ROS, disruption of mitochondrial membrane potential, decrease in Bcl-2 and increase of Bax in mitochondria(Su YT, et al., 2005). Emodin by increasing the intracellular ROS level sensitizes EC/CUHK1 cells, a cell derived from eosophageal carcinoma, to arsenic and on a nude mice model(Yang J, et al., 2004). This can be concluded that AQ treatments lead to apoptosis through generation of O2\(^{1-}\) and H2O2 and subsequent activation of caspase-3 enzyme.
The following inferences can be drawn from the present study.

# In vivo plant of *O.rugosa* is a better source of CPT over CPT producing plants available in India such as *Nothapodytes foetida*, *O.mungos* and *Ervetamia heyneana*.

# Since harvesting of roots from *in vivo* plants is destructive to them, *in vitro* cultures of *O.rugosa* can be exploited as an alternative source.

# Elicitation provides better opportunity to enhance the yield of CPT in comparably low time schedule.

# Fungal culture extract are good inducers of CPT production

# Albino plants, due to the lack of chlorophyll pigment are a good source of CPT compared to normal counterparts.

# The anthraquinone production is higher in *in vitro* plants than *in vivo* plants

# Tissue cultures of *O.rugosa* are a viable source of more antineoplastic secondary metabolites as shown by AQs.

# AQs from *O.rugosa* showed good efficacy against solid tumor and ascites tumor. It also showed anti-inflammatory activity.

# It can be concluded that AQs of *O.rugosa* are potent inducers of apoptosis via reactive oxygen species and subsequent caspase-3 activation.