Summary & Conclusion
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Cancer is a major health problem worldwide and of the most important causes of morbidity and mortality in children and adults. Cancer arises from the uncontrolled proliferation and spread of clones of transformed cells. In a year presently, a little over 10 million new cases of cancer and 6.4 million deaths due to cancer have been estimated to occur globally based on the rates of the year 1990. Even with the assumption of no change in incidence/mortality rates over the decades, the absolute numbers have increased because of the steady increase in the world population and its progressive aging, with major implications for cancer control. The prevention of cancer requires knowledge of its causes. History shows that epidemiological studies have been the key to the control of a wide range of infectious diseases.

Medicinal plants constitute nearly 25% of the prescribed drugs. In recent years screening of such plants for biological activities has resulted in the development of therapeutics used in the treatment of cancer, AIDS and others. A large number of medicinal plants are exploited from the natural flora for the commercial production of the drugs (Paul et al., 1992). Most of the drugs in tree species accumulate either in root or the stem bark of the tree after several years of growth. As a result for the isolation of medicinals these medicinal plants have been indiscriminately exploited from the natural habitat. In order to conserve the natural flora and meet the increasing demand for the plant based drugs certain alternate methods have become more important. The biotechnological approach such as tissue cultures initiated from medicinal plants is a viable method for the production of therapeutic compounds. It is also possible to reduce the entire plant to small cell masses and use them for the synthesis of high value constituents. The present investigation pays attention in isolation and characterization of CPT and gossypin and this work is also an approach to develop a viable protocol for the production of these two active secondary metabolites and a detailed pharmacological activity checking of these compounds.
Isolation of CPT was performed from both intact field grown plant parts like leaves, and roots and which were quantified, compared with in vitro callus, multiple shoots and roots.

It was interested to note that the High Performance Liquid Chromatography (HPLC) analysis of the entire field grown plant gave a CPT yield of 0.056% where as the entire tissue culture plant shows a yield of 0.065% on a dry weight basis. In the plant itself the amount of CPT is much promising in Ophiorrhiza rugosa var. decumbens than the CPT yielding plants ever reported. In Nothapodytes foetida, another plant, which contains CPT, has only 0.05% dry wt. of CPT (Roja and Heble, 1994) and the amount present in O.rugosa var. decumbens was also higher than the amount detected from Ophiorrhiza mungos (Tafur et al., 1976).

When came to tissue cultures in vitro multiple shoot cultures and root cultures are the better sources of CPT. Multiple shoot cultures in solid medium showed a CPT yield of 0.039% in a 45 day old culture with a hormonal combination of NAA, BA 4 mgl⁻¹ and IBA 2 mgl⁻¹ yield a CPT content of 0.03% in Murashige and Skoog (MS) medium. On the same time shoot suspension cultures were found as a viable source for CPT. Half strength medium (MS) with a hormonal combination of BA 5mgl⁻¹ yielded 0.085% of CPT on a dry weight basis. But full strength MS medium with same concentration of BA yield 0.099% of CPT.

As early reports, callus and cell suspension cultures of Ophiorrhiza rugosa var. decumbens produce only traces of CPT. It may be due to the lack of differentiation of cell into well-organized parts. The formation of several indole alkaloids in tissue cultures was shown inseparably connected with morphological differentiation of the cells. (Verpoorte et al., 1991). Root suspension cultures of O. rugosa var. decumbens are found to be the best strategy for the camptothecin production. A suspension root culture (MS + BA 1mg⁻¹ + NAA 1 mg ml⁻¹) yields a promising amount of CPT (0.928%). While in the MS medium with 1AA 2mgl⁻¹ root suspension produce 0.63% of CPT. In view of low concentration of CPT detected in ex vitro plants, tissue culture systems have been studied with great interest as an alternate source of the drug. The efforts were therefore directed to determining the exact mode and
causative factors involved in the synthesis of CPT in in vitro cultures. The results clearly indicated that camptothecin was not synthesized to a promising level in callus and cell suspension cultures of *O. rugosa* var. *decumbens*. However when the shoot or roots initiation takes place from the calli and cells, the CPT production starts. These results indicated that complete differentiation is a prerequisite for CPT synthesis in *O. rugosa* var. *decumbens*. (Fulzele et al., 1991; Akhila et al., 1987).

Hairy root cultures of *O. rugosa* var. *decumbens* can be exploited for quick biomass production thereby enhancing the CPT content. Root cultures are often exhibiting a remarkable ability to synthesize a diversity of plant secondary metabolites (Flores et al., 1999). The hairy root cultures (transformed with the pathogenic soil bacterium *Agrobacterium rhizogenes*) are an attractive option by its rapid growth and subsequent high productivity of secondary metabolite. Fast growing hairy roots of *O.rugosa* var. *decumbens* yield 0.45% of camptothecin and it was a 4.5 fold increase in production reported in *O.Pumilia* hairy roots (Saito et al., 2001).

It was also noted that elicitation could be utilized for better production of CPT. In the present study, elicited multiple shoot cultures of *O.rugosa* var. *decumbens* irradiated with 100 rads cause a notable hike in CPT content after 72h of the irradiation (0.099 to 0.1%). Hirata et al., 1991; 1992; Eilert, 1987 and Brodelius, 1990, also supported elicitation for plant secondary metabolite production.

The presence of 10-hydroxy CPT was found from Mass spectral analysis. 10-hydroxy CPT was also detected along with CPT from *Camptotheca accuminata* (Wiedenfeld et al., 1997). 10-hydroxy CPT is very important in developing clinically active derivatives of CPT. For eg. The introduction of a stable side chain at the 9th position of the A ring of 10-hydroxy CPT yielded another water soluble drug, topotecan (NSC 609699, SKF 104864, 4-dimethyl-10-hydroxy CPT ) (Kingsbury et al., 1991).

Another major class of compound with well-established anti.tumour potential, isolated from *O. rugosa* var. *decumbens* is an anthraquinone. In contrast to the early report anthraquinones was found only in intact plants but not in cell
cultures or in the medium. From O. pumilla, two anthraquinones were detected but only from cell suspension cultures (Kitajima et al., 1998).

Gossypin was found successfully isolated from H. furcatus and the petals contain 2% gossypin on a dry weight basis. In vitro callus and multiple shoots contain only traces of gossypin.

Investigation of preliminary toxicity studies of gossypin was carried out in male wistar rats. Animals given acute doses of 500, 1 and 2g of gossypin did not produce any toxicity. Animal behavior, food and water intake were normal during the acute toxicity study. Renal dysfunction may be the cause of raised plasma urea, uric acid and creatinine level (Varley, 1964). There is no such signs of renal toxicity was observed. Transaminases (GOT, GPT) and alkaline phosphatase are good indices of liver and kidney damage (Martin et al., 1981). The drug did not induce any damage to liver and kidney, which could be inferred from normal activity of these enzymes. Histopathological observation showed no detrimental changes caused by gossypin administration.

Antioxidant studies were carried out to reveal the free radical scavenging activity of gossypin. The compound effectively scavenge the free radicals like superoxide, hydroxyl radicals, nitric oxides and also inhibits in vitro lipid peroxidation, only 3 μg/ml of the compound is needed to cause 50% inhibition for super oxide radicals generated. For hydroxyl radicals it requires 41 μg/ml and for nitric oxide radicals 12 μg/ml are sufficient for causing 50% inhibition. As gossypin has a flavanoid origin, some of the flavanoids have the anti oxidant activity. It is also assumed that ROS are involved in both initiation and promotion of the development of cancer (Boyd and Guire, 1991; Cerutti, 1994; Toyokuni et.al., 1995).

Hepatoprotective activity of gossypin was studied using CCl₄ induced acute hepatotoxicity model in rats. Acute CCl₄ administration significantly (P<0.001) increased liver and serum lipid peroxides, GOT, GPT and ALP in control animals. Administration of gossypin significantly reduced liver and serum lipid peroxides, GPT, GOT and ALP levels in a dose dependent manner. Histological studies also confirmed the hepatoprotective effect of gossypin (20mg/kg). The histoarchitecture
of CCl₄ treated rat liver sections showed cloudy swelling and fatty degeneration of hepatocytes. Necrosis of the cells was also seen. The drug treatment (20mg/kg) almost normalized these defects in the histoarchitecture of the liver. The hepatoprotective mechanism of gossypin was not known since the treatment reduced the levels of lipid peroxides in liver, may be implicated due to the antioxidant property of gossypin.

Cytotoxicity of Gossypin was determined using different cell lines (DLA, EAC, L-929, HT-29 and K-562) to contemplate the compound as a cytotoxic agent in cancer therapy. DLA and EAC cells were used for the short-term in vitro cytotoxicity assay (trypan blue exclusion method) and the compound produce significant death in 3h assay. In vitro cytotoxicity of the compound using L929, HT-29 and K-562 cells in culture could cause significant death of the cells. For 50% inhibition gossypin needed only 30μM concentration for L-929 cells. It needs 42.5 μM and 45.1 μM of gossypin for 50% inhibition of HT-29 and K-562 cells respectively. Brine shrimp cytotoxicity assay was also checked in order to determine the activity of the compound in 24 h period.

Another important finding in the investigation was the discovery of the inhibitory activity of gossypin on DNA topoisomerases. 250 ppm of gossypin exhibits a zone of inhibition of 8mm for mutant cultures (JN 394, JN394t-1 and JN 394t2-5) of Saccharomyces cerevisiae. A number of polyphenols, flavones and isoflavones have been identified as topo II poisons (Austin et al., 1992). Although flavanoids are the group of polyphenols to have detailed structure activity relationships published for topo II inhibition, other polyphenols such as ellagic acid can also interact with topo I and topo II enzymes (Constantinou, 1995).

Antitumour studies were carried out using ascites and solid tumour models in mice. Among different treatment modalities adopted intralesion mode of administration was found much effective in reducing the tumour burden in solid tumour harboring animals. Simultaneous mode of treatment was also found noteworthy than prophylactic and 10 days after treatment in both solid tumour and ascites tumour bearing animals. In ascites tumour bearing animals, gossypin
20mg/kg b.wt significantly increase the life span (P<0.001) compared with control animals.

Investigation of the synergistic action of gossypin was also performed. It was looked into the synergistic action of gossypin with other modalities of treatments such as chemotherapeutic agent like cyclophosphamide and radiation to find out whether it can act as an adjuvant response modifier. When gossypin 20mg/kg was administered intraperitoneally along with cyclophosphamide, reduces the tumour volume effectively (P< 0.001) than when cyclophosphamide or gossypin administered individually. The same phenomenon was observed when radiation and gossypin was administered together. A synergistic reduction in tumour volume was noticed in the above group.

Anticarcinogenic activity of gossypin was determined using DMBA induced two-stage mouse skin papilloma. Gossypin delayed the formation of papilloma in a dose dependent manner (P< 0.001). The percentage incidence of papilloma formation in gossypin (20 mg/kg) treated animals was reduced by 40% than control group of animals (100%) after 12 weeks of experimental period. Number of papilloma per mouse was also found decreased from 10 in control groups (including small papillomas) to 5-6 in treated group. The histoarchitecture of the skin of control animals found enlarged and hyperchromatic in layers of epidermis associated with hyperplasia of basal cells. The invasive tumour cells exhibit enlarged nuclei and finally progression to full thickness atypia heralds the development of the squamous carcinoma in situ. All of these symptoms were quiet reduced in gossypin treated group of animals. It is well established that free radicals play an important role in carcinogenesis (Halliwell and Gutteridge, 1984). It was noted that naturally antioxidants could inhibit the DMBA/Croton oil induced Carcinogenesis. Voluminous reports illustrated that several antioxidants, flavanoids isolated from plants such as querecetin, rutin etc. effectively inhibit the DMBA/croton oil induced skin carcinogenesis in experimental animals (Meishiang et al., 1997).

The efficiency of gossypin in preventing the inflammation was studied using carrageenan, formalin, dextran and croton oil induced models in mice and rats. Oral
administration of the drug (10 and 20mg/kg) decreased the paw inflammation dose dependently in mice. The efficiency of gossypin (20mg/kg) was comparable \((P<0.001)\) with that of diclofenac (10mg/kg). Gossypin inhibited the formalin induced chronic inflammation significantly. In formalin induced pedal oedema 6 consecutive days of gossypin treatment significantly \((P< 0.001)\) inhibited the oedema. The compound also dose dependently decrease the oedema in dextran induced paw inflammation. In croton induced mouse skin oedema 20mg/kg gossypin decrease the weight of the skin punch as compared to control animals. The active ingredient of croton oil is nothing but TPA. It is well established that TPA is a tumour promoter (Razazadeh and Athar, 1987).

Gastroprotective studies of gossypin were carried out in order to determine the anti-ulcer activity of the compound. In ethanol induced gastric injury, a significant protective effect ranged between 49 and 78\% was attained. It is interesting to note that the combined administration of gossypin and ranitidine (5 mg/kg) showed an inhibition of 93\%.

In aspirin induced gastric ulcer model, gossypin significantly inhibited the ulceration. Gossypin 20 mg/kg lowered the incidence of ulceration in aspirin induced ulcer by 78\% compared with the untreated control animals. A drug that possesses both anti ulcer and anti-inflammatory activities is of great therapeutic importance as most of the anti-inflammatory drugs used in modern medicine are ulcerogenic (Surender, 1999). Previous studies revealed the free radical scavenging activity of gossypin. Studies in rats showed that oxygen derived free radicals (ODFR) are directly implicated in the mechanism of acute and chronic gastro duodenal ulceration and that scavenging then stimulated the healing of ulceration (Yang et al, 1991).

The following conclusions could be drawn from this investigation.

2. Among biotechnological production strategies root suspension cultures are better source for camptothecin. 
Agrobacterium rhizogenes mediated transgenic roots is an attractive alternative for biomass production with in a short period of time.

3. Gossypin, a bioactive secondary metabolite was isolated from petals of Hibiscus furcatus and compared with callus and multiple shoot content.

4. Gossypin was found non-toxic in acute toxicity study. The compound was found cytotoxic towards L 929, HT 29 and K 562 cells in tissue culture.

5. Gossypin exhibited significant reduction of solid tumours and increase the life span of ascites tumour harboring animals. Also gossypin act as an adjuvant response modifier along with radiation and cyclophosphamide and the compound delayed the onset of papilloma formation in mice.

6. Gossypin effectively scavenging the reactive oxygen species and inhibits lipid peroxidation. The compound showed a wide array of pharmacological properties like hepatoprotective, anti-inflammatory, and anti-ulcer activity.