Chapter 3

Comparison of camptothecin production from intact plants and callus culture of Ophiorrhiza mungos
Camptothecin was originally identified in the extracts of *Camptotheca acuminata* during a screening program for anti-tumour agents by the U.S National Cancer Institute (Wall and Wani, 1968).

It exhibited a potent anti-neoplastic activity that is ascribed to its inhibitory action upon DNA topoisomerase I (Redindo et al, 1998). Although due to its cytotoxicity, camptothecin itself was not used clinically, its derivatives, irinotecan and topotecan are used for the treatment of cancer throughout the world.

In 1976, Tafur et al isolated camptothecin from *Ophiidrhiza mungos* Linn (Rubiaceae), which is a small shrub. The alcoholic extract of *O.mungos* leaves, roots and stem showed potent inhibition of Herpes virus. The plant as a whole was used in the treatment of cancer. Since then *Ophiidrhiza* species were screened for the presence of camptothecin. Species like *O.discolor, O.major, O.rugosa, O.pumila* were found to be containing camptothecin. But no attempt has been done yet to enhance the production of camptothecin from *O.mungos* through tissue culture.

The present chapter describes the isolation and quantification of CPT from various plant parts of *O.mungos*. An attempt was made to standardize cultural conditions for the successful establishment of callus and cell suspension culture of *O.mungos* for the production of camptothecin.

### 3.1 Materials and Methods

*Ophiidrhiza mungos* plants were collected from Konni, a southern district of Kerala, (Plate 2, Figure:1) and were authentically identified by Dr. Sasidharan, Taxonomist, Kerala Forest Research Institute, Peechi, Kerala. A voucher specimen was kept in herbarium of Amala Cancer Research Centre (ACRH NO: 98).
3.1.1. Preparation of extract

Freeze dried plant parts were powdered, defatted with light petrol (60-80°C) continuously in a soxhlet apparatus and extracted as described in Materials and Methods (Section 2.17).

3.1.2. Isolation and Characterization of Camptothecin.

Isolation and crystallization of camptothecin was done according to the procedure given in section 2.18 of Materials and Methods. Determination of melting point, UV-visible absorption spectra, Mass spectra of isolated CPT and standard CPT (Sigma chemicals) etc were recorded.

3.1.3. HPLC analysis

The isolated CPT from various plant parts - stem, root, leaf etc. were subjected to HPLC analysis as described in Materials and Methods (Section 2.22).

3.1.4. Topoisomerase inhibition assay

To determine the biological activity of camptothecin, topoisomerase inhibition assay was performed as described in Materials and Methods, Section 2.24.

3.1.5. Establishment of callus cultures

Various explants like nodal segments, leaf segments etc. were used. Surface sterilization was done with 0.1% Bavistin (fungicide) for 5 minutes, followed by 0.1% mercuric chloride (HgCl₂) as described in Materials and Methods (Section 2.4).

MS and B5 media supplemented with various growth regulators like NAA, IAA, 2,4-D etc were tried alone or in combination with cytokinins like BA and Kinetin (as depicted in Materials and Methods, Section 2.6).
Inoculated cultures were kept in a dark room at 25 ± 2°C. Each experiment was repeated 4 times. Observations were done regularly and the responses recorded accordingly. Growth Index was calculated as the dry mass of cells on day of harvest / initial dry mass.

3.1.6. Suspension Cultures

Three weeks old friable callus (≈ 5-10 g of fresh weight) was transferred to 250 ml conical flasks containing 100 ml of MS medium supplemented with NAA (0.1 - 2mg/l) and placed in a rotatory shaker (Certomat S II) at 120 rpm in dark at 25 ± 2°C. Cells were sub cultured into fresh medium of the same composition every two weeks and maintained on a shaker. Suspension cultures were harvested from shaker every 5th day to determine packed cell volume (PCV), fresh weight and dry weight as mentioned in Materials and Methods (Section 2.8, 2.9 and 2.10).

3.1.7. Extraction of camptothecin from callus

30 gm of callus was weighed and freeze-dried and the dry weight was determined. The dried callus was powdered and extracted with chloroform. The extract was evaporated to dryness, dissolved in acetonitrile: water (40:60 v/v) and 20 µl of the sample was injected into HPLC system (Materials and Methods, section 2.22).

3.1.8 Extraction of camptothecin from suspension culture.

The cells were separated from the culture medium by centrifuging the suspension at 200 × g for 5 minutes. Volume of the medium was measured. From the medium camptothecin was extracted by partitioning between chloroform and water in a separating funnel.

3.2 Results

Nodal segments, leaf segments etc. were taken as explants for culture establishment. Of these, nodal segments showed a higher frequency of callusing response (78.2%) compared to leaf segments.
Rate of contamination was higher in leaf explants, which showed a survival rate of only 42.9%, whereas the survival rate of stem explants was 62.4%.

Callusing response was obtained in both MS and B5 medium. For initial callus induction, a combination of auxin and cytokinin was necessary. Once induced, the calli exhibited growth response in media containing either type of hormones.

Good callusing response, highest growth rate (1.2%) and growth index (5.987±0.7) were obtained in MS medium supplemented with 4 mg/l BA and 1 mg/l NAA (Plate 2- Fig:2). But the callus was compact even after several subcultures and could not be used for suspension culture studies (Table 3.1).

For studying the effects of varying concentrations of hormones on callus growth, friable callus developed in MS medium supplemented with 1-3 mg/l of NAA (Plate 2 - Fig:3) was transferred to liquid medium of the same composition. The fresh weight and PCV of cells were determined at 5 days interval. Of the three concentrations of NAA tried, 3 mg/l NAA exhibited maximum growth rate (0.61 %). (Fig: 3.3).

Column chromatography of chloroform extracts yielded CPT in chloroform : methanol fraction (24:1 v/v). TLC analysis of purified fraction on silica plates using chloroform: methanol (24:1 v/v) as mobile phase showed an $R_f$ value of 0.5, same as that of standard camptothecin under similar conditions. The crystallized CPT showed melting point at 278°C. The UV -visible spectra of isolated CPT showed $\lambda$ max at 256, 290 and 363 nm (Fig: 3.2.1 &Fig: 3.2.2). The values were similar to that of standard CPT. The HPLC analysis of plant extracts showed chromatographically homogenous peaks with base line and the same retention time (5.7 minutes) as the standard CPT.
Topoisomerase inhibition assay was performed to determine the biological activity of camptothecin. Inhibition zones (8mm) were obtained for both JN394 and J394-5 clearly indicating Topoisomerase I inhibition.

The camptothecin profile of different plant parts, like leaves, stem, roots etc were analyzed (Table 3.2). The stem of O.mungos had the highest CPT content (0.009 ± 0.002 % of dry weight) compared to roots (0.0027 ± 0.0002 % of dw) and leaves (0.0005 ± 0.0001 % of dw).

For comparing the influence of different hormones on CPT production, callus was transferred to MS medium supplemented with 1 mg/l concentration of NAA, IBA, IAA and 2,4-D. Of these IBA showed maximum amount of CPT production (0.025% of dw) at the given concentration. The amount of CPT was lowest in NAA (0.00082% of dw) containing medium. Both IAA (0.0099 % of dw) and 2,4-D (0.00285 % of dw) exhibited moderate levels of CPT production. There was no detectable amounts of CPT production in the basal medium.

To study the effect of different concentrations of auxins on CPT production from callus, 1-3 mg/l of NAA, IAA and IBA was added to basal MS medium. From the results it is evident that (Fig;3.4) 2mg/l concentration was optimum for CPT production in the case of IBA (0.0488% of dw) and NAA (0.011% of dw). 2 mg/l IBA produced 0.048% of dw of CPT which was approximately double the amount produced by 1 mg/l IBA (0.025% of dw). But higher concentrations of IBA failed to keep up this trend (3 mg/l IBA produced only 0.0058% of dw of CPT). At low concentrations (1-2mg/l), IBA induced root formation from the callus (Plate 3- Fig: 1 & 2). This type of organogenesis might have enhanced the CPT production. But at higher concentrations (3 - 5 mg/l) rhizogenesis was absent, favouring fast cell
division to form unorganized callus resulting in lower level of CPT production.

Similar observations were obtained in the case of IAA also. In the case of NAA even though the growth index was higher, the amount of CPT produced was comparatively low.

Higher concentrations of 2,4 -D (> 1mg/l) was deleterious to cells resulting in sponginess and cell death due to cell lysis. So these concentrations were omitted from the studies.

For comparing the effect of two different basal media on CPT production, calli were inoculated to basal MS and B5 media. But no detectable amount of CPT production was obtained. Hence three different hormone combinations were used for supplementing the basal media. The result showed that in all the 3 combinations, CPT production was higher in MS medium compared to that of medium B5. (Fig: 3.5).

To study the effect of cytokinins on callus growth and CPT production, calli were inoculated to MS media containing BA (1-3mg/l) and Kin (1 mg/l). The result showed that with increasing concentrations of BA, CPT production also increased (Fig3.6).

The effect of sucrose concentration on CPT production from callus was studied. Of all the concentrations tried, 5% sucrose was found to be better. Concentrations higher than 5% were not favourable for cell growth. Higher concentrations of sucrose resulted in osmotic cell lysis (Table 3.4).

The medium with highest callus growth index (MS+ 4mg/l BA +1mg/l NAA) was supplemented with 1-3 mg/l of GA to study the effect of gibberrellic acid. The results showed that the optimum concentration of GA was 2 mg/l for CPT production (0.0106% of dw) compared to that of control (0.0016 % of dw) (Table 3.4).
Even though growth was good, only traces of CPT was obtained in cell suspension cultures. One of the major problems associated with cell suspension cultures was the production of phenolics, which eventually led to cell death.

Production of CPT was influenced by the growth phase of culture. Maximum production was obtained during late log phase (15-20 days) (Fig:3.7). At the end of stationary phase, CPT production was found to be decreasing. It might have been degraded into some other by products.
Plate 2- Figure :1 *Ophiorrhiza mungos* mother plant

Plate 2- Figure :2 *Ophiorrhiza mungos* compact callus

Plate 2- Figure :3 *Ophiorrhiza mungos* friable callus
Plate 3- Figure 1 *Ophiorrhiza mungos* rhizogenesis

Plate 3- Figure 2 *Ophiorrhiza mungos* rooted callus
Mass Spectra of Standard Camptothecin
SAMPLE-1-STD, DR JOSE PADIKKALA
2NV089A 1 (0.589) Cn (Top, 4, Ht); Sb (5, 33.33); Sm (SG, 2x0.70)

100, 129

15-Nov-2002
Scan ES+
10.00e6
Mass spectra of isolated camptothecin
Fig. 3.2.1 UV absorption spectrum of standard camptothecin

Fig. 3.2.2 UV absorption spectra of isolated camptothecin
Table 3. Effect of hormones on callus production from *O. mungos*

<table>
<thead>
<tr>
<th>NAA</th>
<th>IBA</th>
<th>IAA</th>
<th>2,4-D</th>
<th>BA</th>
<th>KIN</th>
<th>Response</th>
<th>Growth Index</th>
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<td>-</td>
<td>-</td>
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<td>1.21±0.1</td>
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<td>-</td>
<td>-</td>
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<td>1</td>
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<td>-</td>
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<td>3.95±0.34</td>
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<td>+++</td>
<td>4.62±0.54</td>
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<td>1.95±0.34</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
<td>++</td>
<td>2.65±0.34</td>
</tr>
<tr>
<td>-</td>
<td>0.5</td>
<td>-</td>
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<td>-</td>
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<td>3.75±0.64</td>
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<td>1</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>2</td>
<td>++</td>
<td>2.62±0.35</td>
</tr>
</tbody>
</table>

(± mild response, ++ moderate response, +++ good response)
### Table 3.2. Camptothecin content of different plant parts of *O. mungos*

<table>
<thead>
<tr>
<th>Plant part</th>
<th>CPT content (%) of DW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stem</td>
<td>0.009 ± 0.002</td>
</tr>
<tr>
<td>Leaves</td>
<td>0.0005 ± 0.0001</td>
</tr>
<tr>
<td>Root</td>
<td>0.0027 ± 0.0002</td>
</tr>
<tr>
<td>Whole plant</td>
<td>0.004 ± 0.0002</td>
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</tbody>
</table>

### Table 3.3. Influence of Sucrose and Gibberellic Acid on Camptothecin production from *O. mungos* callus

<table>
<thead>
<tr>
<th>Media Combination</th>
<th>CPT yield (%) of DW</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS + BA (4mg/l) + NAA (1mg/l) *</td>
<td>0.0016 ± 0.00025</td>
</tr>
<tr>
<td>MS + BA (4mg/l) + NAA (1mg/l) + GA (1mg/l)*</td>
<td>0.0083 ± 0.0005</td>
</tr>
<tr>
<td>MS + BA (4mg/l) + NAA (1mg/l) + GA (2mg/l) *</td>
<td>0.0106 ± 0.00037</td>
</tr>
<tr>
<td>MS + BA (4mg/l) + NAA (1mg/l) + GA (3mg/l) *</td>
<td>0.001 ± 0.00025</td>
</tr>
<tr>
<td>MS + BA (4mg/l) + NAA (1mg/l) + Sucrose (30 g/l) **</td>
<td>0.0016 ± 0.00025</td>
</tr>
<tr>
<td>MS + BA (4mg/l) + NAA (1mg/l) + Sucrose (40 g/l) **</td>
<td>0.00083 ± 0.000819</td>
</tr>
<tr>
<td>MS + BA (4mg/l) + NAA (1mg/l) + Sucrose (50 g/l) **</td>
<td>0.002 ± 0.000153</td>
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</table>

**f > f_{0.05} * f < f_{0.05}**
Fig: 3.3 Influence of Plant hormones on Callus growth of O. mungos

Fig: 3.4 Influence of Auxins on Camptothecin production from O. mungos
Fig. 3.5 Influence of basal medium on CPT production from callus

Fig. 3.6 Influence of N\textsuperscript{6} Benzyl adenine on CPT production from \textit{O. mungos} Callus
Fig 3.7 Influence of callus age on CPT production

Dry weight (mg)

Time (days)

CPT (% of DW)

Dry weight (mg/l)

Cpt (% of DW)
HPLC Profiles of Selected Samples
HPLC profiles of samples

Sample ID : MS+NAA (1 mg/ml)

Sample ID : MS + NAA(2mg/ml)

Sample ID : MS + NAA (3 mg/ml)

Sample ID : MS+1 IAA (1mg/ml)
Sample ID: MS+ BA(4mg/l)+NAA(1 mg/l)+ 3% Sucrose

Sample ID: MS+ BA(4mg/l)+NAA(1 mg/l)+ 4% Sucrose

Sample ID: MS+ BA(4mg/l)+NAA(1 mg/l)+ 5% Sucrose
Sample ID: MS+ BA(4 mg/l) + (NAA 1 mg/l) + GA (1 mg/l)

Sample ID: MS+ BA(4 mg/l) + (NAA 1 mg/l) + GA (2 mg/l)
Sample ID: *O. mungos* root

Sample ID: *O. mungos* stem

Sample ID: *O. mungos* leaf
Discussion

The plant cells possess totipotency i.e. each cell of a plant possess the capacity to regenerate an entire plant. In addition, the genetic make up of all cells in a plant is similar. Hence, the cells in a callus should have the ability to do all the functions done by cells in its mother plant. If a plant is capable of producing a particular secondary metabolite, the callus developed from it should also produce the secondary metabolite. However, in certain cases, the callus does not produce the secondary metabolite or it may produce it in lesser quantities. This means that in an unorganized state, the genes in a callus do not express themselves as they do in a well-organized plant. For example, even though *Ophiophriza pumila* plants produced CPT, callus cultures derived from it failed to produce camptothecin (Kitajima et al., 1998).

But there are certain cases where a callus culture in an appropriate media generate comparable amounts of secondary metabolites to that produced in its mother plant. For example, Shikonin, a red pigment isolated from *Lythospermum erythrorhizon* is obtained from callus cultures in higher amounts than that of the intact plant (Fujita et al., 1988). From this it can be conferred that by providing suitable signals, the genes in a callus can be induced to express themselves in a constructive way. These signals include various growth hormones, various physical and chemical signals (e.g.: variation in temperature, light, precursors) etc.

So far, numerous research activities were carried out in this field and some of them were successful to a certain extend. Peterson et al. (1994) reported increased amount of rosmarinic acid in *Coleus blumei* cultures with 4% sucrose. Similarly, the maximum yield of rosmarinic acid produced by cell suspension cultures of *Salvia officinalis* was 0.7
g/l in the medium containing 3% sucrose. But it increased to 3.5 g/l when 5% sucrose was used (Whittaker et al, 1984). Stimulation of taxol production at elevated levels of sucrose in nodule culture of Taxus was reported by Ellis et al (1996). In the present study, increased sucrose concentration was found to be enhancing camptothecin production (Table 3.4).

Sakato and Misawa (1974) reported that addition of gibberrellin to MS medium enhanced camptothecin production from callus cultures of C.acuminata. Van Hengel et al (1992) obtained 0.998 mg CPT/l of MS medium containing 4 mg/l NAA. They stated that auxins like NAA or 2,4-D are important for induction of callus from C.acuminata plants. Roja et al (1987) reported that shoot cultures of Rauwolfia serpentina raised on MS liquid medium with BA and NAA produced significantly higher amounts of alkaloid. Kodja et al (1989) observed that addition of BA increased the levels of ajmalicine in cell suspension cultures of C.roseus. Widenfeild et al (1997) proposed that highest growth rate of C.acuminata was observed on media with 4mg/l NAA and 2 mg/l BA.

Cytokinins were found to stimulate alkaloid biosynthesis in callus cell lines of C.roseus (Kodja I, 1989). The effect observed was dependent on cell line, the nature and the concentration of the cytokinin and the growth phase at which the cells were treated (Decendit et al, 1994). In the given study also, various auxins and cytokinins had considerable influence on CPT production from callus.

According to Rech et al (1998) , the higher concentrations of alkaloid present in the callus compared to that in the suspension culture was probably due to better cell-cell contact, ageing and a limited differentiation of the cells in callus culture. They found that the total alkaloid content in Rauwolfia selloi callus was 2-3 % of dw where
as that of suspension culture were only 1.28%. In *C.acuminata* plants CPT production in suspension cultures was 100 times less than that in the original plant material (Van Hengel *et al*, 1992). Here the camptothecin content varied with time and was dependent on the growth medium used and production maximum did not always occur at the same time as the maximum CDW.

In the present study also, there was variation in CPT content with time. The maximum production was obtained in the late log phase (15-20 days after inoculation). Later the amount was found to be decreasing towards the end of growth phase. Another interesting feature observed was that the phenolic exudation increased at the end of growth phase and resulted in cell death. There can be a relationship between inhibition of CPT production and enhancement of phenolics. Camptothecin might have been converted into some other products at the end of growth phase. Camptothecin production was decreased considerably at the end of growth phase of *O.pumila* cultures (Sudo *et al*, 2002). *C.acuminata* cell suspension cultures broke down camptothecin when growth stops because of sucrose limitation (Van Hengel *et al*, 1992). In cell suspension cultures of *O.mungos*, only trace amounts of CPT was obtained in the present study.