In vivo screening of Ophiorrhiza species
1.1 Introduction

For centuries, plants have been one of the important sources of fine chemicals that fall in the class called secondary metabolites. Even today, 25% of the entire drugs produced are still derived from plants. But these are of limited distribution in plant kingdom, usually confined to a particular taxa and the production may be seasonal or tissue specific. In plants, secondary metabolites are produced in very low quantities. And as a result, these biologically active compounds form high value low volume products. Demand for such a plant product for use in pharmaceuticals and other industry is on rise now. An analysis of the number of chemotherapeutic agents and their sources indicates that over 60% of approved drugs are derived from natural compounds (Cragg et al., 1997).

As the natural habitat for wild plants is becoming endangered, environmental and geopolitical instabilities make it difficult to acquire certain plant-derived chemicals; it may become critical to develop alternative sources of important natural secondary constituents or product without further sacrifice of the environment.

Nature is an attractive source of new therapeutic compound as tremendous chemical diversity is found in millions of species of plants, animals, marine organism and micro organisms. The development of novel agents from natural sources present obstacles that are not usually met when one deals with synthetic
compounds. For instance, there may be difficulties in accessing the source of samples, identification and isolation of active compound in the sample, and problems in synthesizing the necessary amount of the compound of interest.

Alkaloids are a diverse group of low molecular weight, nitrogen containing molecules found in about 20% of plant species. The potent biological activity of some alkaloids has traditionally been exploited by humans for hunting, execution, warfare and the treatment of diseases. Plant derived alkaloids currently in clinical use include anticancer agents camptothecin, taxol, vincristine and vinblastine, analgesics codeine and morphine, the gout suppressant colchicines, muscle relaxant tubocurarine, antiarrythmic ajmalicine, antimalarial quinine, antiamoebic emetine, antibiotic sanguinarine, sedative scopolamine, and topical analgesic capsicain, to mention some of the most representative examples (Raskin et al., 2002).

Cancer is a growing public problem whose estimated new incidence worldwide is about 6 million cases per year. It is the second major cause of death after cardiovascular diseases (Sreevastava et al., 2005). The alkaloid camptothecin, a quinoline indole alkaloid, added an entirely new dimension to the field of chemotherapy (Sreevastava et al., 2005). It is a naturally occurring Topoisomerase-1 inhibitor. Camptothecin and its derivatives are clinically used as antineoplastic alkaloid that biogenetically belongs to monoterpenoid indole alkaloids. Camptothecin and its close chemical relatives Aminocamptothecin, Camptothecin-11 (Irinotecan), DX8951f, Topotecan, etc., are well known naturally occurring DNA Topoisomerase-1 inhibitors.

Camptothecin is used as a drug in the treatment of Colon, Head, Breast and Bladder cancer (Hsiang et al., 1985). Camptothecin also possesses activity against Retrovirus, Human Immunodeficiency Virus (Priel et al., 1991a, b). The
worldwide market of irinotecan and topotecan has currently reached one thousand million US dollars per year, which represents approximately one ton of CPT in terms of raw material. In addition, water insoluble analogues 9-aminocamptothecin and 9-nitrocamptothecin (rubitican), both of which were reported to possess activity sufficient to merit clinical evolution (Thomas et al., 2004). Rubitican serves as a metabolic precursor to 9-aminocamptothecin and it was submitted to the Food and Drug Administration (FDA) in the United States and in the European Agency for the Evaluation of Medicinal Product (EMEA) as an orally active camptothecin for the treatment of pancreatic cancer. Consequently, the demand of camptothecin will be more expanding in the future.

It is one of the newest chemotherapy drugs and as such, cancer researchers are really interested in it. A recent clinical trial reveals that CPT-11 is very effective in the treatment of cancer, including stomach cancer and non-Hodgkin’s lymphoma. Despite its increasing demand, camptothecin is still supplied exclusively from the intact plants, mainly Camptotheca acuminata and Nothopodytes foetida (Govindachari and Viswanathan, 1972).

1.1.1 Aims and objectives

Wall and Wani first extracted Camptothecin from the stem wood of Camptotheca acuminata “Xi Shu” or Tree of Joy, a deciduous tree native to China and Tibet, which has been extensively used in traditional Chinese medicine. It has also been isolated from Mapia foetida, Ervatamia heyneana, Ophiorrhiza, etc. Nearly six species of Ophiorrhiza have been tested for their glycoalkaloid content in terms of camptothecin. Tissue culture studies with O.prostata, O.pumila and O.rugosa (Vineesh et al., 2005) for the enhanced production of camptothecin have been reported. In O.mungos, Sudo et al. (1991) patented the enhanced production of camptothecin and 10-methoxy camptothecin. A perusal of literature indicates
that there are still many more species of *Ophiorrhiza* yet to be explored for camptothecin production. *O.grandiflora*, *O.barberi*, *O.hirsutila*, *O.incarnata*, *O.caudata*, *O.brunonis*, *O.pectinata*, *O.munnarencis*, *O.nairii*, *O.bykarensis*, *O.radicans*, *O.roxburgiana* and *O.shendurunii* are reported to be present in the Western Ghats. The origin of the present research problem arises from the idea of targeting those plants growing in the Eastern parts of Kerala and the Western parts of Tamilnadu as new sources of camptothecin.

The search is still on for new and so far unexplored species, which could yield higher quantities of camptothecin than those are already reported. The species-wise and organ-wise screening of *Ophiorrhiza* species in terms of Camptothecin content is not yet done. Moreover, previous analysis was based on different extraction and quantification procedures. This provides a major obstacle for acquiring reliable information on the camptothecin content of *Ophiorrhiza* species for initiating research work aimed to further improve camptothecin content in *In vitro* conditions.

Many of the above mentioned *Ophiorrhiza* species are rare, endangered or even extinct category (Deb and Mondal, 1997) (Red data book of Indian Plants). Therefore, these plants are to be conserved to maintain biodiversity.

In this background, the objectives of the present study are:

- To collect rare and endemic species of *Ophiorrhiza* to conduct *in vivo* phytochemical screening.

- To assess and compare species-wise and organ-wise distribution of camptothecin.
To bring into light, if possible, new and unexplored species as new source of camptothecin.

1.1.2 Source of plant

The family Rubiaceae is tree, shrubs or infrequent herbs comprising about 450 genera and 6500 species. *Ophiorrhiza* is one of the important genus in Rubiaceae with about 340 species, sub species, varieties and forms. The term ‘*OPHIORRHIZA*’ is derived from the Greek terms ‘OPHIS’, a snake and ‘RHIZA’, a root alluded to healing properties in snake-bite in Indian subcontinent particularly *O.mungos* and *O.japanica* BI (Done, 1834). Linnaeus described the genus *Ophiorrhiza* in Flora Zeylanica (Linnaeus, 1747) and included in Materia Medica(1749).

The species of *Ophiorrhiza* vary in habit from creeping herbs to small shrubs up to 3 meter high, growing in humid regions at varying altitude. Most of them are erect suffrutescent, some are prostate and a few creeping.

In India, about 50 species reported, are distributed in peninsular India mainly in the Western Ghat (Deb and Mondal, 1997).

1.1.2.1 *Ophiorrhiza mungos* L. var. *mungos*. (Figure 1)
Ver.name: Eng- mongoos plant, Hindi-sarahati, Sans-sarpakshi, Mal-avilpori

The plant is found in Nepal and Sikkim in the North; Nagaland, Manipur in the East; Malaya, Sumatra in the South East and Tamilnadu, Kerala and Sri Lanka in the South. It grows in cool shady places on the clayeys loam with fairly good amount of moisture. It is usually a herb annual, 8-100 cm tall, sometimes under shrubs; Stem erect, branching, somewhat fleshy; Leaves elliptic, acuminate at
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apex; Flowers 3.5-18 mm long, white. This species shows considerable variation in size of leaves, flowers and style. (Deb & Mondal, 1997). The propagation is through numerous seeds that are girt by the calyx limb and occasionally the seeds found germinated in the persistent calyx cup, which is a characteristic feature of Ophiorrhiza species. (Binoy et al., 2004)

Traditionally the roots of O.mungos are used against snake-bite (Done, 1834; Drury, 1864; Baillon, 1881). The roots are said to be useful in the treatment of cancer. A decoction of roots, leaves and bark is given as stomach ache. The leaves are used for dressing ulcers. The flowers are prescribed as a stimulant and cardiac tonic in rheumatism and diseases of the heart. The roots are used in Punjab, Himalaya and Trans-Indus region as a red dye for wool. In Nepal, the roots are boiled with oil and used as a dye for the hair (Watt, 1891). Roots contain starch, a light brown resin and small amount of a bitter amorphous alkaloid (Nadkarni, 1976).

1.1.2.2 Ophiorrhiza pectinata Arn. (Figure 2)

The plant is found in India and Sri Lanka mostly in Tamil Nadu and Kerala. It grows on damp shady places near streams at 910-150 m above m.s.l. It is a herb, 15-50 cm tall, stem erect, branching, internodes 3-8 cm long; Leaves 4-17.5x2-5.5cm, elliptic, lanceolate, caudate acuminate at apex, tapering at base, glabrous; Inflorescence axillary, and terminal capitately cymes, 0.8-3.75 cm across, some time pubescent; Flowers heterostylos, 6-11.5 mm long, pink, bract and bracteoles similar, persistent. Raphides present in stem, calyxlobes, ovary and capsule.

In the present study, the plants were collected from Cheeyappara, Idukki District of Kerala State.
1.1.2.3 *Ophiorrhiza barberi* Gamble. (Figure 3)

The plant is found growing wild in Tamilnadu and Kerala especially in Munnar Ghat. The plant grows on cool shady places at 1025-1350 m above m. s. l.

It is a herb, 35-50 cm tall, stem erect, branching, glabrous; Leaves 4-16 x2-5 cm, elliptic, caudate-acuminate at apex; Inflorescence axillary scorpiod cyme, branches spreading; Flowers 6-8 mm long, white; Seeds 0.35-0.4x03-0.4 mm, 4-8 angular, brown.

In the present study, the plants were collected from Kallaar, Idukki District of Kerala State.

1.1.2.4 *O.munnarensis* Fischer. (Figure 4)

The plant is found in Munnar Ghat, Idukki, Kerala on cool shady places at an elevation above 1200 – 1500 m. It is an annual herb, 5-16 cm tall; stem erect or procumbent, rooting below, branched, brown pubescent. Leaves 2.5-6.5 x1-2.4 cm, elliptic lanceolate, acute, acuminate at apex. Petioles 0.3-1.4 cm long, slender, puberulous; stipules 2.5-5 mm long. Inflorescence terminal capitate cyme, 1-1.4 cm across. Flowers 8.5-10.5 mm long.

In the present study, *O.munnarensis* was collected from Neryamangalam of Idukki District.

1.1.2.5 *O.rugosa* Wall. var. *prostata*. (Figure 5)

The plant is found in Nepal, Bhutan, Bihar, and Orissa to Malaya through Myanmar and Maharashtra, Goa, Tamilnadu and Kerala to Sri Lanka. It is a herb, 7-46 cm long: stem prostrate, rooting at lower nodes, glabrous, Leaves 1.5-5 x1.2 cm, broadly ovate, glabrous, lateral nerves 5-7 on either side, petioles 0.5-1 cm
In the present study specimen was collected from Pampakuda, Ernakulam district.

1.1.2.6 *Ophiorrhiza caudata* Fischer. (Figure 6)

Annual herb, 25-50 cm high and glabrous throughout. Stem subterete, glabrous; internodes 2-9 cm long. Leaf blades petiolate, elliptic-lanceolate, 4.5-11 cm long, 1.5-3.5 cm wide, at apex narrowly acuminate to caudate with tips 1-1.5 cm long, tapered towards base, at base attenuate, lamina often unequal, margins entire, dark green above and pale yellowish-green beneath, glabrous or sparsely puberulent on the principal nerves beneath; secondary nerves 6-8 pairs, broadly curved, ascending, usually extending to the margin; petioles 0.5-1.5 cm long, glabrous; stipules interpetiolar, caducous, ovate-triangular, 3-6 mm long, acuminate at apex, glabrous. *Inflorescence* terminal subcapitate cymes, 1-2 cm across, erect, glabrous; peduncles 1.5-3 cm long, slender, green, glabrous. Peduncles 1.5-3 cm long, slender, green, glabrous. *Flowers* 4-6 mm long, white, bracts and bracteoles similar, persistent, 4-5 x 1.5-4 mm, ovate-lanceolate, midvein prominent, oblique, acute, glabrous; pedicels 1-1.5 mm long, glabrous. *Hypanthium* 1 x 0.5 mm, cupular, glabrous. *Calyx lobes* 5, ovate-lanceolate, 1-1.5 x 0.3-0.4 mm, acute, shortly keeled at back, glabrous. *Corolla lobes* 5, white, ovate-acute, 3 x 3 mm, glabrous, tube 4 mm long, glabrous outside, densely hispid on throat within. *Stamens* 5, inserted above the middle of the corolla tube; anthers oblong, 1 mm long, yellow, dehiscence longitudinal, sessile or stalked, filaments 0.5 mm long (for long-styled flowers) to 3 mm long (for short-styled flowers). *Ovary* 0.5-1 mm, long, obovoid, glabrous; style 3 mm long, lobes subrotund, minutely papillae. Capsules not seen.
This is a species reported as possibly extinct by many authors (Nayar & Sastry, 1987; Deb & Mondal, 1997). This species was originally described by C.E.C. Fisher in 1938, based on a collection made by E. Barnes (Barnes1560, K Image) in 1937 from “Travancore High range, Kalaar”. Despite intensive botanical exploration in this region during the last several years, this species seems to be escaped the attention of botanists.

In the present investigation, *O.caudata* was collected from Kallar, cardamom Hills of Idukki District. After critical studies (Drawing 1) and comparison with type specimen (E. Barnes 1560, K Image), it was revealed to be a rediscovery and a research paper was published in Rheedia Journal (Ginu and Joy, 2009).
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Drawing 1. *Ophiorrhiza caudata* C.E.C.Fish. – A. Habit; B. Flower bud; C. Bract; D. Flower; E. Long styled flower: Corolla opened showing androecium and pistil; F. Short styled flower: corolla opened showing androecium and pistil; G. Pistil with hypanthium. (All from *Ginu Joseph* 86952).
1.2 Review of Literature

The following topics carry a brief description of the quintessence of an expansive literature survey conducted. These topics are intended to present a summarized state of knowledge regarding camptothecin and camptothecin carrying plants.

1.2.1 Camptothecin and its derivatives-Chemical aspects.

Camptothecin \( (C_{20}H_{16}N_2O) \) is a quinoline indole alkaloid with a pentacyclic ring consisting of an indole moiety and quinoline moiety. In addition, it has a lactone ring at one end. Although camptothecin is structurally grouped in the quinoline alkaloid; it is biogenetically modified monoterpenoid indole alkaloid. In biosynthetic pathway, it is formed from the common intermediate Strictosidine via strictosamide that was actually proved by Hutchinson (Hutchinson \(^{a,b}\)). Strictosidine is formed by the condensation of tryptamine with the iridoid glucoside, secologanin. The condensation is catalyzed by the enzyme Stricosidine synthase (Yamasaki \textit{et al.}, 2003). The discovery that the primary cellular target of camptothecin is type 1 DNA topoisomerase (Topo 1) was the breakthrough that received interest in the drug in mid-1980s (Hsiang \textit{et al.}, 1985). Advances in the medicinal chemistry of camptothecin during the late 1980s and early 1990s resulted in semi-synthetic, water soluble analogous, which are used clinically, and over a dozen of new derivatives are currently under clinical development at various stages.
Camptothecin biosynthesis may occur in limited parts where expression of mRNA and enzyme activity (stricosidine synthase) takes place and then camptothecin is transported to other parts in spite of its poor solubility (Yamasaki et al., 2003).

Dependence of plants for natural products is essential because some compounds are difficult to synthesize due to their structural complexity. Secondary metabolite can be applied as the starting compound for further chemical modification. Camptothecin was first isolated by Wall and Wani from *Camptotheca acuminata* (Tree of Joy) in 1958. The research on camptothecin was interrupted for about twenty years because of its intolerable toxicity and insolubility in water.

The discovery that the primary cellular target of camptothecin is DNA Topoisomerase-1, an enzyme involved in DNA replication, was the breakthrough that revived interest in the drug in the mid-1980s (Hsiang et al., 1985). In 1902, after Irinotecan or CPT-11 was found to be less toxic yet with retained antitumor activity (Masuda et al., 1992), a whole array of camptothecin derivatives were developed for the replacement of camptothecin as an anticancer agent. A number of camptothecin derivatives like Topotecan, Amino camptothecin, DX89518, Methoxy camptothecin were found effective for the treatment of colon, breast, head, and bladder cancer (Zhang et al., 2007). Camptothecin also possesses activity against retrovirus, Human Immuno deficiency virus (HIV) (Priel et al., 1991a, b). Most notably two camptothecin derivatives were approved by the US food and drug administration in 1996 for clinical treatment of ovarian cancer (Hycamtin®, Smithkline Beecham Pharmaceuticals) and colon cancer (Camptosar®, Pharmacia and Upjohn, Inc.) (Zhang et al., 2007). Camptothecin is regarded as the most promising anticancer drug of the twenty first century (Lorence and Craig, 2004). Moreover, the molecular and cytotoxic effects of
camptothecin on *Plasmodium falciparam* have proven that it is an interesting target for new anti-malarial drug development (Bodley *et al*., 1998).

It is important to note that the structural features of camptothecin that are essential for activity, include the 20(S) hydroxy (Wang *et al*., 1999), the pyridine moiety of the D-ring, the lactone moiety of the E-ring and the planarity of the five membered ring system. Hence, the C, D, E rings of camptothecin cannot be altered without severely affecting its activity. Modification of the 9, 10 and 11 positions of the A-ring and the 7 position of the B-ring are generally well tolerated and may enhance the potency of camptothecin analogues (Lorence and Nessler, 2004).

### 1.2.2 Mechanism of action

The primary cellular target of camptothecin is DNA Topoisomerase-1. DNA Topoisomerase-1 is an enzyme which facilitates DNA unwinding during DNA replication. Here, camptothecin inhibits DNA Topoisomerase-1 and that causes stop signal for DNA replication and that in turn stops cell division (Srivastava *et al*., 2005). Camptothecin is selectively cytotoxic to S-phase cells, arrests cells in G2 phase, and induces fragmentation of chromosomal DNA. With increasing concentration and exposure duration camptothecin irreversibly inhibits DNA (Ulukan, 2002).

### 1.2.3 *In vivo* sources of camptothecin

Camptothecin is originally isolated from the wood and bark of Chinese tree *Camptotheca acuminate* (Nyssaceae) (Wall *et al*., 1966). Later camptothecin was reported from the following unrelated orders.
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Order—Celastrales:

*Nothopodytes foetida* (Icacinaeeae) (Aiyama *et al*., 1988)

*Pyrenacantha klaineana* (Icacinaeeae) (Zhou *et al*., 2000)

*Merrilliodendrum megacarpam* (Icacinaeeae) (Arisawa *et al*., 1981)

Order—Gentianales:

*Ervatamia heyneana* (Apocynaceae) (Gunasekara *et al*., 1979)

*Mostuea brunonis* (Gelsemiaceae) (Dai *et al*., 1999)

*Chonemorpha grandiflora* (Apocynaceae) (Kulkarni *et al*., 2010)

Table 1 summarizes the information generated by multiple research teams regarding the sites of accumulation of camptothecin and its concentration in multiple natural sources.

With increasing demand for CPT, search for new CPT source was started and that led to a promising genus *Ophiorrhiza* in the family Rubiaceae. CPT is reported from many species of *Ophiorrhiza*. *O.pumila* (Yamasaki *et al*., 2003), *O.eriantha* (Fijesh *et al*., 2006), *O.prostata* (Martin *et al*., 2007), *O.rugosa* (Babu *et al*., 2001), *O.mungos* (Binoy *et al*., 2004) and *O.filistipula* (Arbain *et al*., 1993).
<table>
<thead>
<tr>
<th>Species</th>
<th>Tissue analyzed</th>
<th>CPT content µg/g. dw.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Camptotheca accuminata</em></td>
<td>Young leaves</td>
<td>4000-5000</td>
<td>Lopez-mayer et al., (1997)</td>
</tr>
<tr>
<td></td>
<td>Seeds</td>
<td>3000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bark</td>
<td>1800-2000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Roots</td>
<td>400</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Young leaves</td>
<td>2421-3022</td>
<td>Li et al., (2002)</td>
</tr>
<tr>
<td></td>
<td>Old leaves</td>
<td>482</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Young fruit</td>
<td>842</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Old fruit</td>
<td>2362</td>
<td></td>
</tr>
<tr>
<td><em>C. a lowreyana</em></td>
<td>Young leaves</td>
<td>3913-5537</td>
<td>Li et al., (2002)</td>
</tr>
<tr>
<td></td>
<td>Old leaves</td>
<td>909-1184</td>
<td></td>
</tr>
<tr>
<td><em>C. yunnanensis</em></td>
<td>Young leaves</td>
<td>2592-4494</td>
<td>Li et al., (2002)</td>
</tr>
<tr>
<td></td>
<td>Old leaves</td>
<td>590</td>
<td></td>
</tr>
<tr>
<td><em>Ervatamia heyneana</em></td>
<td>Wood and bark</td>
<td>1300</td>
<td>Gunasekara et al., (1979)</td>
</tr>
<tr>
<td><em>Nothopodytes foetida</em></td>
<td>Stem</td>
<td>1400-2400</td>
<td>Aiyama et al., (1988)</td>
</tr>
<tr>
<td></td>
<td>Shoot</td>
<td>750</td>
<td>Roga and Heble, (1994)</td>
</tr>
<tr>
<td><em>Merriliodendrum megacarpam</em></td>
<td>Leaves and Stem</td>
<td>530</td>
<td>Arisawa et al., (1981)</td>
</tr>
<tr>
<td><em>Ophiorrhiza mungos</em></td>
<td>Entire plant</td>
<td>12</td>
<td>Tufer et al., (1976)</td>
</tr>
<tr>
<td><em>O. pumila</em></td>
<td>Leaves</td>
<td>300-400</td>
<td>Saito et al., (2001)</td>
</tr>
<tr>
<td></td>
<td>Young root</td>
<td>1000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Entire plant</td>
<td>300-510</td>
<td>Yamasaki et al., (2003a)</td>
</tr>
<tr>
<td><em>Chonemorpha grandiflora</em></td>
<td>Stem</td>
<td>13</td>
<td>Kulkarni et al., (2010)</td>
</tr>
</tbody>
</table>

Table 1. Site of accumulation of camptothecin in different plant species
1.3 Materials and methods

1.3.1 Materials

1.3.1.1 Collection and maintenance of source plant.

Most of the Ophiorrhiza species are endangered or even belong to possibly extinct category. So, a thorough field investigation was made in search of Ophiorrhiza species. The source plants were collected from different locations of Kerala. Ophiorrhiza mungos and O. rugosa were collected from Ernakulam district and O. pectinata from Cheyappara, Idukki district. O. munnarensis was collected from Neryamangalam and O. caudata from Kallar, Idukki District, Kerala.

O. mungos and O. rugosa were established and maintained in home garden under regular watering. Other species were collected directly from their natural habitat for experiments as they are highly endemic and sensitive to different environmental conditions.

The taxonomical features were critically studied and identified initially with the help of ‘Taxonomic Revision of the Genus Ophiorrhiza in Indian Subcontinent (Deb and Mondal, 1997) and The Flora of the Presidency of Madras. For confirmation of identification, the specimen was matched with those of the authenticated ones at the Herbaria of Botanical Survey of India, Coimbatore and a voucher specimen was deposited at Botanical Survey of India Herbaria,

1.3.1.2 Preparation of plant material for chemical analysis.

Stem, root, leaves and floral parts were thoroughly washed, coarsely chopped and dried separately soon after collection. Excessively woody portions of stem and roots were excluded.

The plant materials were quickly dried in hot – air oven at 60 °c. to constant dry weight and then ground to fine powder using mortar and pestle. The powder was stored in airtight, opaque plastic container. Analysis was carried out within two months of drying. These precautions were necessary to prevent enzymatic degradation of camptothecin during drying and storage.

1.3.1.3 Chemicals

Camptothecin with 99 percent purity was obtained from Sigma Chemical Co., USA. Solvents used for chromatography were of HPLC grade (Merck, Germany). Whatman No.1 filter paper-42,90mm. Precoated Silica gel $^{60}$ F $^{254}$ aluminum sheets (Merck, Germany) was used for TLC analysis. Reagents used for sample processing and other purposes were of AR grade (Merck, Germany) or equivalent.
1.3.1.4 Glassware

Screw capped culture tubes (5 ml), test tubes, separating funnel, Measuring jar, glass pipette, beakers, and conical flasks were the glasswares used.

1.3.1.5 Instruments.

Electronic weighing balance (Sartorius, Germany), water bath, Soxhelt apparatus, magnetic stirrer, HPLC (SHIMADZU, JAPAN), HPTLC (CAMAG), refrigerator were used in experiments and analysis of samples.

1.3.2 Methods

The analysis was done in two phases. The first phase was aimed to find out the suitable extraction procedure for maximum camptothecin recovery. In vivo screening of camptothecin was done in the second phase.

1.3.2.1 Comparison of techniques for maximum camptothecin recovery.

Mainly two extraction methods were tried for maximum camptothecin recovery. Here the plant sample used was dried and powdered *O.mungos* leaf.

1.3.2.1.1 Stirring Extraction

To find out the appropriate solvent either alone or in combination and temperature conditions, five experiments, as mentioned in table 2 were performed. Two hundred mg Plant sample was taken in a 100 ml beaker and 50 ml solvent was added. The beaker was then placed on a platform of the Magnetic Stirrer. The stirring speed was adjusted to 150 g. When heat was required, the hot plate was
switched on. After extraction, the extract was filtered using Whatman no.1 filter paper to get the supernatant. Supernatant was evaporated to 5 ml. Quantification of camptothecin recovered as percentage was done using HPLC method. Details of sample type, solvent type, time of extraction, temperature, and agitation speed of different methods are listed in Table 2.

1.3.2.1.2 Soxhelt Extraction

To standardize the soxhlet extraction procedure and to find out the appropriate solvent, three experiments, as mentioned in Table 3 were performed. Five hundred mg of sample was put in a thimble and placed in the thimble holder. Hundred ml of solvent was added to the bottom flask. The whole apparatus was then placed on a heater. The temperature was maintained to 50°C. The extract was filtered and evaporated to 5 ml. Quantification of camptothecin recovered as percentage was done using HPLC method. Different solvents used, time, Temperature, etc., are listed in Table 3.

1.3.2.2 Further extraction and quantification of camptothecin in Ophiorrhiza species.

One gm. of plant sample was taken in a 100 ml beaker. Fifty ml of 70 percentage methanol was added. The mixture was stirred at 150 gm for 2 hours. After two hours stirring, it was stopped to collect the supernatant and fresh solvent was added. This process was repeated for three times. The collected supernatant at each step was mixed and filtered using Whatman No.1 filter paper. The filtrate was then partitioned with equal volume of chloroform. The chloroform extraction was repeated three times. The chloroform fractions was pooled, evaporated to dryness, dissolved in 5 ml methanol (HPLC grade), clarified using Millipore filters (0.22µm) and subjected to HPLC analysis.
1.3.2.3 Thin Layer Chromatography (TLC)

For qualitative confirmation of camptothecin, Thin Layer Chromatography (TLC) of selected samples was done. Precoated Silica gel 60 F 254 aluminum sheets were used. For confirmation standard camptothecin was also run along with the samples. Saturated chamber with chloroform: ethanol in the ratio 24:1 was used for separation. After separation, the chromatogram was scanned using CAMAG TLC scanner at 360 nm.

1.3.2.4 High Performance Liquid Chromatography (HPLC)

HPLC of each extract was done for the quantitative estimation of camptothecin. HPLC analysis was carried out on a LC-2010 system (Shimadzu, Japan) using a reverse phase C-18 phenomenex column of 250 mm length and 4 mm diameter. The samples were eluted with methanol: water (6:4 v/v) at a flow rate of 1 ml/min. Detection was done at 254 nm by UV detector, quantified by calibration with standard camptothecin.
1.4 Results

1.4.1 Comparison of techniques for maximum camptothecin recovery.

The results of experiments A – E, as mentioned in 1.3.2.1.1 are presented in table 2. For camptothecin recovery, as the percentage shows, three times stirring with 70 percent methanol for two hours at room temperature and subsequent extraction with pure chloroform, gives better recovery (0.09 %) (Figure 9). Seventy percent methanol has more extractive power than 100 percent methanol. It was also evident that chloroform alone cannot extract camptothecin. Stirring at room temperature was more efficient than stirring at 70 °C. temperatures

Table 2. Different stirring extraction methods for CPT recovery in *O.mungos* leaf

<table>
<thead>
<tr>
<th>Experiment</th>
<th><strong>Solvent - 1</strong></th>
<th>Time of extraction</th>
<th>Temperature</th>
<th>Agitation</th>
<th><strong>Solvent - 2</strong></th>
<th>CPT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>70% MeOH</td>
<td>2 hrs, x 3</td>
<td>70 °C</td>
<td>150 g</td>
<td>Nil</td>
<td>0.05 ± 0.007</td>
</tr>
<tr>
<td>B</td>
<td>70% MeOH</td>
<td>2 hrs, x 3</td>
<td>RT</td>
<td>150 g</td>
<td>Nil</td>
<td>0.08 ± 0.008</td>
</tr>
<tr>
<td>C</td>
<td>70% MeOH</td>
<td>2 hrs, x 3</td>
<td>RT</td>
<td>150 g</td>
<td>Three times</td>
<td>0.09 ± 0.006</td>
</tr>
<tr>
<td>D</td>
<td>100% MeOH</td>
<td>2 hrs, x 3</td>
<td>RT</td>
<td>150 g</td>
<td>Three times</td>
<td>0.01 ± 0.002</td>
</tr>
<tr>
<td>E</td>
<td>CHCl₃</td>
<td>2 hrs, x 3</td>
<td>RT</td>
<td>150 g</td>
<td>Nil</td>
<td>Not detected</td>
</tr>
</tbody>
</table>

Values represented are means of three replicates ± standard deviation.
RT-Room temperature.
When different Soxhlet extraction experiments (F - H) as mentioned in 1.3.2.1.2 were compared, extraction with solvents in the successive order petroleum ether, seventy percent methanol, chloroform was found better (0.08) (Table 3) (Figure 10). This also indicated that defatting with petroleum ether has a promoting effect in the extraction. Repeated extraction with 70 percent methanol also favoured better camptothecin recovery.

Table 3. Different soxhlet extraction methods for CPT recovery in *O.mungos* leaf

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Solvent - 1</th>
<th>Time of extraction</th>
<th>Solvent - 2</th>
<th>Time of extraction</th>
<th>Solvent - 3</th>
<th>CPT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>Petroleum ether</td>
<td>Nil</td>
<td>70% Methanol</td>
<td>one time</td>
<td>Chloroform</td>
<td>0.04 ± 0.007</td>
</tr>
<tr>
<td>G</td>
<td>One time</td>
<td>5 hrs.</td>
<td>One time</td>
<td>8 hrs.</td>
<td>Three times</td>
<td>0.05 ± 0.008</td>
</tr>
<tr>
<td>H</td>
<td>One time</td>
<td>5 hrs.</td>
<td>Three times</td>
<td>8 hrs.</td>
<td>Three times</td>
<td>0.08 ± 0.003</td>
</tr>
</tbody>
</table>

Values represented are means of three replicates ± standard deviation

1.4.2 Camptothecin content in *Ophiorrhiza* species

The camptothecin content in different plant samples was quantified using HPLC, but presentation of chromatogram of all the samples is cumbersome and therefore only a few selected samples were depicted (Figure 13-18). Among the six species analyzed, *O.mungos*, *O.rugosa*, *O.pectinata* and *O.barberi* showed the presence of camptothecin. *O.mungos* and *O.rugosa* showed camptothecin in leaf, stem, root and flower unlike *O.barberi* that showed camptothecin only in its roots. *O.pectinata* showed camptothecin in its flower, stem and root only. The extractable camptothecin ranged from 0.013 to the 0.26 percent (Table 4). Among the six species screened *O.mungos* showed highest level of camptothecin content (0.26%) in flowers. The organ wise comparative evaluation of camptothecin in *O.mungos*, *O.rugosa*, *O.pectinata* and *O.barberi* is presented in Figure 11.
Table 4. Comparative distribution of camptothecin in *Ophiorrhiza* species

<table>
<thead>
<tr>
<th>Plant</th>
<th>Camptothecin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Leaf</td>
</tr>
<tr>
<td><em>O.mungos</em></td>
<td>0.082 ± 0.004</td>
</tr>
<tr>
<td><em>O.rugosa</em></td>
<td>0.08 ± 0.066</td>
</tr>
<tr>
<td><em>O.pectinata</em></td>
<td>ND</td>
</tr>
<tr>
<td><em>O.barbata</em></td>
<td>ND</td>
</tr>
<tr>
<td><em>O.caudata</em></td>
<td>ND</td>
</tr>
<tr>
<td><em>O.mununarensis</em></td>
<td>ND</td>
</tr>
</tbody>
</table>

ND-Not Detected. Values represented are means of six replicates ± standard deviation

1.4.3 Distribution of camptothecin in various plant organs.

Among the six species analyzed, the plant part that accumulates maximum camptothecin was found to be flowers in two species *viz.*, *O.mungos* and *O.rugosa*. In *O.pectinata*, root showed maximum camptothecin accumulation. Next to the flower, the root showed comparatively high levels of accumulation in *O.mungos* and in *O.rugosa*, it was leaf.

When plant parts of different species were compared, maximum camptothecin in stem was found in *O.mungos* (0.074%) and the minimum in *O.pectinata* (0.0013%). In root, the maximum quantity was recorded in *O.mungos* (0.26%) and the minimum in *O.pectinata* (0.007%). Among the leaves of different species, *O.mungos* and *O.rugosa* showed almost equal amount of camptothecin (0.08%). Among the flowers *O.mungos* also showed the maximum (0.26%). In
O. barberi camptothecin was detected only in root. However, it was higher than camptothecin present in O. pectinata and O. rugosa root.

On analyzing the data based on individual species, O. mungos showed the distribution of camptothecin in different parts in the order, flower > root > leaf > stem. In O. rugosa, it was flower > leaf > root > stem. In O. pectinata, the distribution showed in the order, root > flower > stem. O. barberi showed the presence of CPT in its root only. The relative contribution of each part of the net content is depicted in figure 12.
1.5 Discussion

By comparing various extraction methods for camptothecin from *O.mungos* leaf, stirring extraction with 70 percent methanol followed by subsequent chloroform extraction was more efficient than other time-consuming techniques. Methanol as an extraction solvent was much more efficient in recovering camptothecin than chloroform. Chloroform alone showed no camptothecin recovery. This revealed that the polar nature of the solvents and the compounds to be separates were an important factor in solvent extraction techniques. The strength of the water in methanol played a key role in maximally extracting camptothecin (Zhang *et al*., 2007). Seventy percent methanol has more extractive power than 100 percent methanol. Very high polar nature of water molecule may increase the extractive power of diluted methanol. The extraction temperature was also found to be an important factor. Fulzele *et al*., (2005), when working with *Notopodytes foetida* at a temperature between 40 -70, reported percentage of extraction increases with increase in temperature .The present results showed that room temperature is ideal for maximum camptothecin recovery.

Comparing stirring and Soxhlet extraction, the camptothecin recovery was almost the same in both the cases, but the later method was time consuming and it needed bulk amount of solvent. Moreover, as reported by Fulzele *et al*., (2005), prolonged Soxhelt extraction decreases camptothecin recovery due to partial decomposition of the compound. Stirring extraction needed only 6 hours. On the
other hand soxhlet extraction needed 29 hours for complete camptothecin recovery. Therefore, stirring extraction is a suitable alternative for camptothecin recovery from dry powder as it is less time consuming and needs only little amount of solvent.

Among the six species, tested three species, *O. mungos*, *O. rugosa* and *O. barberi* emerged as the promising sources for possible exploitation as row material for this important alkaloid. *O. barberi* and *O. pectinata*, *O. munnarensis* and *O. caudata* were not yet screened in terms of camptothecin content. It is for the first time, that appreciable levels of camptothecin content are being reported from *O. barberi*. *O. pectinata* showed the presence of camptothecin but the quantity was not in exploitable level. *O. caudata* and *O. munnarensis* do not show the presence of camptothecin even in minute quantity.

Generally, the concentration of the active constituent present in the herbs may vary due to many factors like time and period of collection, geographical and climatic conditions. Different species assessed in different countries or regions possesses widely varying quantities of camptothecin. Plants growing equally well in different localities may or may not produce the active constituents in the same amounts. Many times, even the absence of active constituent may be observed with the same plant collected from different regions, which was difficult to assess earlier. Recent developments in analytical techniques made this risk easier to identify and quantify the active constituents in the herbs (Bilia, 2002; Houghton, 1998; Marcus, 2002).

Several factors such as worldwide changes in seasonal patterns, weather events, temperature changes, biotic and abiotic stress may affect the production of secondary metabolite in plants (Dixon and Paiva, 1995; Cavaliere, 2009; Evans, 1996). It has also been reported that camptothecin content increases when grown
Immature leaves serve as a major sinks of photosynthates and sites of production for some phytohormones. They are nutrient rich and tender in physical structure, features that make them attractive to herbivory and pathogens. Many species defend their juvenile development stages metabolically. It is likely that 5-6 fold of the camptothecin content in young *C.acuminata* leaves compared to mature ones and the peak of camptothecin production in young seedlings (Lopez Mayer *et al.*, 1997; Lu and MC knight, 1999), represent a chemical defense mechanism deployed by young leaves and seedlings to deter attacks by herbivores, pathogens or both. Although the role of camptothecin as a defense chemical has not been directly tested, there are indirect lines of evidence indicating its involvement in plant defense. Liu *et al.* (1998) observed lack of any insect or pathogenic damage to *C.acuminata* plantations in USA.

Camptothecin has spatial variations within a plant and its accumulation varies with organ. The simultaneous analysis of the four major organs of the plant gives a clear picture about the relative distribution and accumulation of camptothecin in the whole plant. The plant part that accumulates maximum camptothecin was found to be flower in two species *viz.*, *O.mungos* and *O.rugosa*. In *O.pectinata*, root showed maximum camptothecin accumulation. Next to the flower, the root showed comparatively high levels of accumulation in *O.mungos*, and in *O.rugosa* it was leaf.

In *O.pumila*, Yamasaki *et al.* (2003) reported camptothecin content is highest in flower followed by young leaves, stems and roots. The authors observed that old leaves contained lower levels of camptothecin. When compared to per leaf basis, the top leaves contain 2- fold camptothecin to the old leaves. In a study
involving eight *Camptotheca* seed sources, seeds were shown to contain 0.09-0.16% camptothecin on a dry weight basis, significantly higher than the leaves of 0.04-0.13 % (Liu and Adams, 1998). Tissue-wise, camptothecin tends to accumulate more in the root bark than root wood, possibly due to the high activities in the form of an enzyme, tryptophan synthase, responsible for the key intermediate tryptophan in the camptothecin biosynthesis pathway (Liu et al., 1998; Lu and McKnight, 1999).

The results of the present study showed that the quantity of camptothecin in the stem was consistently low compared to other organs except in *O. mungos*. The normally low levels are understandable since the stem is seldom a major site of synthesis and accumulation of secondary metabolites. Nevertheless, the meager presence of alkaloids is indicative of their role in the transition of alkaloids. The photosynthetic tissues with its active metabolism, may also contribute to local alkaloid concentration through limited synthesis. Another possibility for apparent diminution of alkaloids in the stem could be the presence of metabolically inactive xylem tissues which occupy the bulk of the stem. In a stem, parenchyma cells, even though expressing normal synthetic activity, are comparatively few and restricted to the outer regions. This would lead to a low yield on a dry weight basis in a sample where the whole of the stem is taken.

For the analysis of raw herbal materials and herbal preparations, HPLC is superior to other instrumental analytical techniques because it is a simple, fast, sensitive and automated technique with high degree of resolution. Therefore, HPLC technique is suggested for the determination of camptothecin in *Ophiorrhiza* species. The HPLC chromatogram of different samples shown similar retention time for the compound (6.8). The method was validated and was found to be satisfactory for the quantification assay.
1.6 Illustrations
Figure 1-6: Different *Ophiorrhiza* species collected. 1) *O.mungos*, 2) *O.pectinata*, 3) *O.barberi*, 4) *O.munnarensis*, 5) *O.rugosa*, 6) *O.caudata*.
Figure 7. HPTLC chromatogram showing camptothecin from different 
*Ophiiorrhiza* species. 1-2) *O.mungos*, 1) leaf, 2) flower; 3-5) *O.pectinata*, 3) root, 4) flower 5) leaf; 6-9) *O.barberi*, 6) root, 7) flower, 8) stem, 9) leaf; 10) *O.mungos* leaf chloroform residue, 11) *O.mungos* leaf methanolic extract, 12-16) camptothecin standard 1-5 microgram.

Figure 8. 3 D plot of HPTLC anologue curves of samples (Track 1 to 11) and standard camptothecin (Last 5 tracks)
Figure 9: Camptothecin recovery in stirring extraction experiments

Figure 10: Camptothecin recovery in soxhlet extraction experiments

Figure 11: Relative distribution of camptothecin in *Ophiorrhiza* species
Figure 12. Distribution of camptothecin in different parts of various *Ophiorrhiza* species.
Figure 13. Sample: *O. mungos* leaf

Figure 14. Sample: *O. mungos* root

Figure 15. Sample: *O. rugosa* leaf

Figure 13-15: HPLC profile of some selected samples
Figure 16. Sample: *O. rugosa* root

Figure 17. Sample: *O. rugosa* stem

Figure 18. Sample: *O. barperi* root

Figure 16-18. HPLC profile of some selected samples