Camptothecin accumulation in *Ophiorrhiza rugosa* var. *prostrata* from northern Western Ghats

Camptothecins (CPT) are one of the most important alkaloids of the 21st century because of their clinical applications against cancer[1,2] and HIV-I[3]. Recently, they have been found to be active against parasitic trypanosomes and *Leishmania*[4], falciparum malaria[5] and also exhibit antibacterial activity[6]. It is primarily a plant-based alkaloid and known to be present in species belonging to orders and families of angiosperms unrelated to Icacinaceae.

CPT has a unique pentacyclic ring structure which gives itself and its derivatives the necessary antitumour activity and such spatial configuration cannot be synthesized artificially[7]. As a consequence, drug manufacturers have to depend on the natural source of CPT. The main sources of this alkaloid are bark and roots of *Camptotheca acuminata*[8] and *Nothapodytes nimmoniana*[9]. The estimated global turnover of CPT derivatives in the year 2004 was one thousand million US dollars which represents approximately one tonne of CPT in terms of raw material[10]. Reported trade of *N. nimmoniana* woodchips in the last decade from Maharashtra was around 400 metric tonnes. In the Indian market, the current demand for biomass (woodchips) of *N. nimmoniana* is around 500–700 metric tonnes; the supply being 50% less than the current demand. Ever-increasing worldwide demand for CPT from pharmaceutical industries and subsequent pressure on the wild populations of *N. nimmoniana* has endangered the plant[11]. Thus there is an urgent need to find alternative sources of CPT so as to cater to the demands of the pharma industry.

So far efforts have been undertaken to analyse CPT content in hairy root culture of *Ophiorrhiza pumila*[10,12]. However, these estimations were mainly confined to *in vitro* studies. *O. rugosa* var. *decumbens* has been studied for clonal propagation[13]. Studies on direct organogenesis from leaf and internode explants of *O. prostrata* on different media using various growth regulators were conducted[14]. However, CPT estimations from natural populations are lacking.

The present study documents the variation in CPT content across plant parts of *O. rugosa* var. *prostrata* from natural habitats in northern Western Ghats; seasonal variation in CPT content and comparative account with other sources.

O. rugosa var. *prostrata* (family Rubiaceae) is a rare herb found in northern Western Ghats of India. It is usually found in semi-evergreen and evergreen forests along the banks of gentle flowing perennial streams or moist soils along water sources. It is an understorey herb and grows up to a height of 20–30 cm.

The sampling sites were located in Koyna Wildlife Sanctuary and Radhanagari Wildlife Sanctuary situated in the south-western part of Maharashtra. Table 1 depicts various sampling sites from northern Western Ghats. To examine CPT variation across plant parts (root, stem, fruit and leaf), samples were collected from the study sites during 2007–08. To assess the seasonal variation in CPT content if any, totally 53 samples (stem and root) were collected from another nearby site, Kusapur of which 34 were collected in summer and 19 in winter of 2008. Samples from at least five adult plants were collected from a particular site and a composite sample for that site was prepared by pooling them.

Plant tissue samples were dried at 60°C in a hot-air oven for 48 h and the dried samples were ground using a pestle. About 0.1 g sample powder was mixed with 5 ml of 61% ethanol in High Performance Liquid Chromatography (HPLC) grade water. Extraction was done in water bath at 55°C for 3 h with

Table 1. Sampling details and ecological parameters across sites

Sampling site and forest type	Geographic coordinates: latitude/ longitude	Altitude (m above msl)	Average annual rainfall (mm)	Sample size (*n*)	Month of sampling	Phenological phase
Valvan (Koyna WLS; predominantly moist deciduous forests)	17°44′17.6″N 73°34′49.2″E	715	2500–5000	Stem-14 Root-8	December	Fruiting
Kusapur (Koyna WLS; predominantly moist deciduous forests)	17°41′34.5″N 73°42′9.5″E	769	2500–5000	Stem-27 Root-26	December May	Fruiting Fruiting
Kandvan (Amba Ghat; predominantly moist deciduous forests)	17°02′47.3″N 73°51′23.4″E	812	1500–1700	Stem-10 Root-6	December	Fruiting
Godambyacha dang (Amba Ghat; predominantly moist deciduous forests)	16°49′55.9″N 73°49′20.4″E	640	1500–1700	Stem-4 Root-4	December	Fruiting
Anapmaal (Radhanagari WLS; predominantly moist deciduous forests)	16°13′35.5″N 73°57′22.3″E	922	200–2500	Stem-4 Root-4	December	Flowering
Amboli (Reserve Forest; semi-evergreen forests)	15°56′59.9″N 74°00′0.29″E	857	5500–6000	Stem-10 Root-9	December	Fruiting

intermittent shaking and then cooled overnight. The extracts were centrifuged at 13,000 rpm for 20 min and the centrifugate was filtered with nitrocellulose membrane filters (φ 25 mm, pore size 0.2 µm). Filtered extracts were analysed using RP-HPLC (Perkin Elmer SR 200, Column configuration: C18) with 20 µl sample loop using 40% acetonitrile in water as mobile phase at a flow of 1.6 ml/min, λ = 254 nm (deuterium lamp)[15].

Standard solution of CPT (Standard from Sigma Aldrich) was prepared in 1 : 50 DMSO : methanol following Padmanabha *et al.*[15].

Significantly higher levels of CPT were found in the roots (0.16 ± 0.06%) than stem (0.08 ± 0.04%), fruits (0.01 ± 0.005%) and old leaves (0.002%) (Figure 1) of *O. rugosa* (*F* test, $p < 0.05$). This pattern of CPT accumulation was consistent for all the sampled localities. Significant seasonal variation was also noticed in CPT content of root (Student's *t* test; $p < 0.05$) and stem in Kusapur sample (Student's *t* test; $p < 0.05$). Samples collected in winter season showed significantly relatively higher CPT content than those collected in summer (Figure 2). CPT content in stem was approximately three times more in winter than in samples collected during summer. Similar trend was observed in root samples where CPT content was 1.5 times more during winter than in summer. To test whether the CPT accumulation differs across localities, ANOVA (analysis of variance) was performed for CPT values of root and stem samples separately. Difference in CPT content in stem across localities was statistically significant (*F* test, $p = 0.02$) whereas CPT content in root samples did not differ significantly (*F* test, $p = 0.09$) (Figure 3). The CPT content in the roots ranged from 0.01% to 0.32% whereas that in the stem ranged from 0.01% to 0.19%. The mean CPT content (stem and roots) was highest in Kusapur followed by Kandvan. To assess whether *Ophiorrhiza* can be a potential candidate for CPT harvest on large scale, we compared its CPT content with other species known in the literature (Table 2). *N. nimmoniana* is found to produce CPT in the range of 0.03–1% or even 2.5% with a mean of 0.3%[16]. Whereas, *O. rugosa* var. *prostrata* contains an average of 0.2% CPT. Current method of extraction of CPT from wood chips and seeds of *N. nimmoniana* exposes difficulties in purification due to presence of woody tissues and oils respectively. *O. rugosa*, being a herbaceous plant, is a promising candidate as an additional source of CPT. It can be used for experimentation in the field of tissue culture techniques and for further domestication. Further studies on the natural population of this plant in different habitats, across seasons, across the physical parameters, under different stress conditions can be done to know more about the *in situ* CPT production. Besides

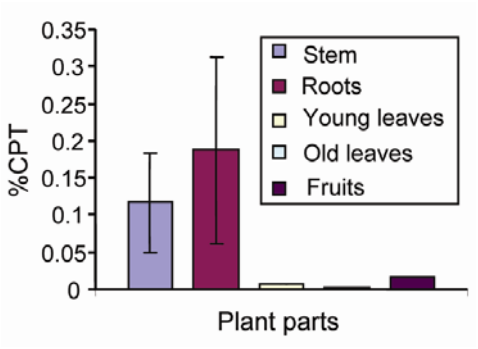

Figure 1. CPT content in *O. rugosa* var. *prostrata* across plant parts. Error bars indicate standard deviation values.

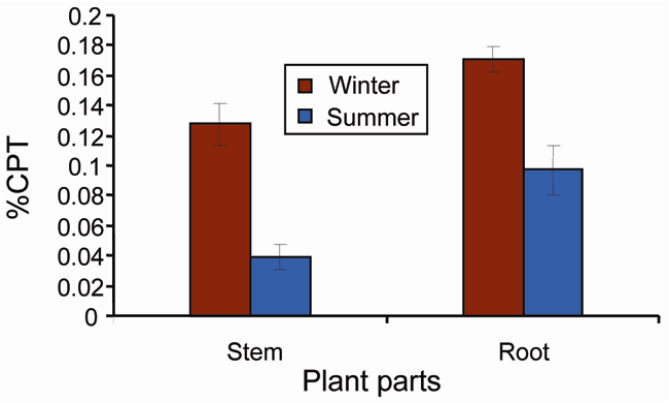

Figure 2. CPT content in *O. rugosa* var. *prostrata* across seasons. Error bars indicate standard deviation values.

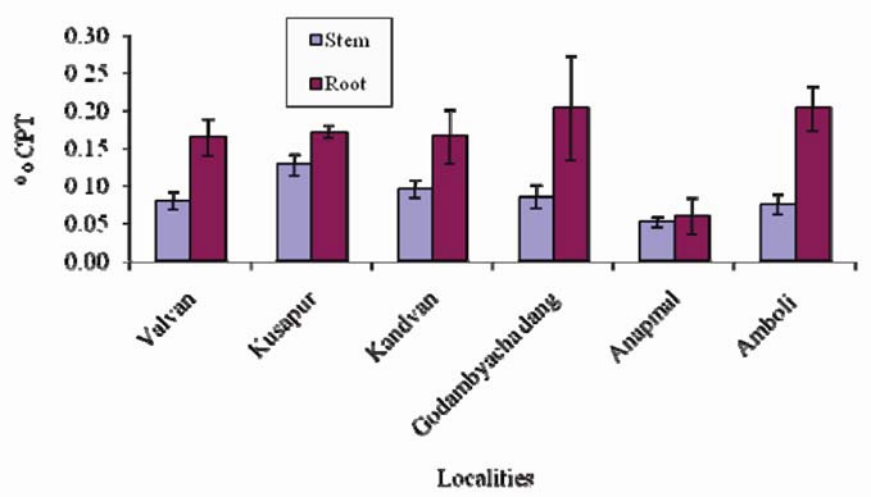

Figure 3. Variation in CPT content in stem and root samples across localities. Error bars indicate standard deviation values.

Table 2. Comparative account of CPT content in different species

Species	Plant part	Sample origin	CPT*	Reference
N. nimmoniana (family Icacinaceae)	Stem bark	Uttara Kannada, India	0.23%	Padmanabha *et al.*[15]
	Root bark		0.33%	
	Stem bark	Okinawa, Japan	1400–2400	Aiyama *et al.*[9]
	Shoot	Mahabaleshwar, India	750	Roja and Heble[17]
C. acuminata (family Nyssaceae)	Bark	Texas, USA	1800–2000	López-Meyer *et al.*[18]
	Roots		400	
	Hairy roots		1000	
	Young leaves		4000–5000	
	Seeds		3000	
O. rugosa var. *prostrata* (family Rubiaceae)	Stem	Northern Western Ghats	0.08%	Present study
	Root		0.16%	
	Young leaves		0.0062%	
	Old leaves		0.0022%	
	Fruits		0.0165%	

*CPT content is expressed as μg/g dry weight unless otherwise mentioned.

these, developing cultivation strategy could be the focus of future studies. Optimizing the growing conditions to increase the biological yield as well as CPT would result in consistent supply of high quality raw material to the industries. Domestication and cultivation also helps to solve problems that are inherent to herbal medicines such as misidentification, instability in the supply of material, toxic components and contaminants.

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