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Patterns of accumulation of berberine alkaloid and chemical profiling of natural populations of *Coscinium fenestratum* (Menispermaceae) in the Central Western Ghats, India

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Berberine and its derivatives are being pursued as a new class of anti-diabetic medication world over. *Coscinium fenestratum*, a dioecious woody liana, is a rich source of berberine. With no other synthetic sources and huge industrial demand, natural populations of *C. fenestratum* are being rampantly harvested from the Western Ghats of India, making the species 'critically endangered'. Prospecting for high berberine yielding individuals from different populations of *C. fenstratum* is a prerequisite to clonally mass-multiply and/or to develop *in vitro* production systems, thereby reducing the pressure on natural populations. Towards this end, the present study was carried out to chemically profile natural populations distributed in the Western Ghats and to determine the pattern of accumulation of berberine with respect to age, tissues, and sex. A total of 90 individual lianas were subjected to the chemical analysis. The concentration of berberine in methanol extract was determined using a C-18 reverse phase column with UV detection at 344 nm. Berberine content varied significantly with respect to the tissue, and sex of the individuals. The average berberine content irrespective of age, sex and tissues ranged between 0.64 to 3.01 %. Out of 45 adult individuals, 18 individuals yielded more than 5 % of berberine in the root samples. Further, the herbivore attack resulted in a significant increase in berberine content of leaves. These results hold an important implications to identify 'chemical hot-spots' of berberine.

Keywords: Berberine, Chemical diversity, *Coscinium fenestratum*, HPLC, Western Ghats.

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Introduction

Berberine, a quaternary ammonium salt from the group of isoquinoline alkaloid (molecular formula $C_{20}H_{19}NO_5$ and molecular weight of 353.36), has a long history of medicinal use both in Ayurvedic and Chinese traditions[1]. The extracts and decoctions containing berberine have demonstrated significant antimicrobial activity against a variety of organisms including bacteria, viruses, fungi, protozoans, helminths, and Chlamydia[2]. Hence various plant species, with berberine as bioactive compound, are widely used in traditional health care systems, either as a single plant remedy or in poly herbal formulation in organized systems of medicine such as Ayurveda, Siddha and Unani[3]. Berberine hydrochloride ($C_{20}H_{18}ClN0_4$, MW 371.817 g/mol), the most useful form of berberine, is being pursued as a new class of anti-diabetic medication[4]. Currently, the predominant clinical uses of berberine include treatment for bacterial diarrhoea, intestinal parasitic infections, and ocular trachoma infections[5] 1 kg of berberine costs around US $3,000 to $9,000[2]. *Coscinium fenestrtaum* is the main source of berberine in southern part of India. It is estimated that about 114 t of woody stem bark of this species is extracted annually from the Western Ghats[6]. In a recent estimation it is reported that 2000 t of extract from *C. fenestratum* was used by Indian pharmaceutical industry in 2011 to prepare a wide range of formulations and the demand for its extract has reached unsustainable levels[7]. Berberine was first isolated by Chevalier and Pelletan in 1826 from golden seal plant, *Hydrastis sp*. Later it was isolated from a number of plant species such as *Berberis sp.*[8-10], *Coptis teeta*[11], *Coptis chinensis*, *Hydrastis canadensis*[12], *Phellodendron amurens*[13], *Tinospora cordifolia*[14], *Mahonia swaseyi*[15], *Arcangelisia flava*[16]. In India, *C. fenestratum,* an endemic species

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of the Western Ghats, is the main source of berberine in southern part which yields up to 5 % berberine on a dry weight basis[17].

The artificial synthesis of berberine is not possible due to the key role of a berberine bridge enzyme, in the crucial step of biosynthetic pathway[18]. Hence, most of the extractions depend on the natural source. Today, in the absence of a synthetic source, the huge demand for berberine by pharmaceutical/ayurvedic sectors is being met by the extraction of naturally existing populations of *C. fenestratum* from the Western Ghats, India[19]. Consequently in the last few decades, it is feared that over 70 % of the population of the species has been lost from the Western Ghats[6]. Indiscriminate extraction coupled with certain inherent properties of the liana such as high habitat specificity, dioecious nature, poor seed set and lack of regeneration had made the species critically endangered[19, 20]. However, despite of its importance, there are scanty attempts being made to explore natural populations to identify high yielding individuals among different populations to be used for clonal multiplication. Recently, such attempts made with respect to an endangered plant *Nothapodytes nimmoniana,* an important anticancer drug (Camptothecin) yielding species of India, have been successful in identifying high yielding individuals among populations[21-22]. These high yielding lines of *N. nimmoniana* have been deployed in agro-forestry systems for the sustainable production of camptothecin[23]. Further, the concentration of secondary metabolites in plant species is known to largely vary across habitats, geographical locations, seasons, and parts of the plant[24]. Rojsanga, 2006 has shown that berberine content vary across different populations of *C. fenestratum* in Thailand[25]. However, there are no reports that focus on chemical characterization of remnant populations of *C fenestratum* of the Western Ghats[26]. These studies have emphasized the need to characterize the pattern of berberine accumulation with respect to age and sex of the plant, and also to chemically profile natural populations such that high yielding sources are identified. Further, it is believed that secondary metabolite accumulation in plant tissues is an evolutionary response of the plants against herbivore predation. In natural populations, secondary metabolites accumulation is seasonally triggered by the herbivore attack. With this background, the present study has focused on assessing inter and intra population variation in berberine accumulation in seven distinct natural populations of *C. fenestratum* in the central Western Ghats. Further, variation for berberine content with respect to (i) different plant tissues such as root, stem, leaf and fruits, (ii) male and female individuals, (iii) different size classes of liana, and (vi) predated and non-predated individuals was also assessed.

Materials and Methods

Study site

The present study was carried out in the central Western Ghats region of Karnataka, India. Reconnaissance survey of natural populations of *C. fenestratum* was undertaken in 35 forest ranges, covering approximately 30 % area of the central Western Ghats. Samples for chemical profiling were collected from the individuals from seven distinct natural populations of the central Western Ghats as shown in the Plate 1. The geo-coordinates and elevation of all natural populations were recorded using Global Positioning System to the nearest 20 m[19].

Collection of samples

Since the species is a critically endangered, the number of individuals in a population are limited. Hence from each population, a total of 5 adult lianas and 5 regenerating individuals were sampled randomly for the chemical analysis. The geo coordinates and DBH (diameter at breast height) of the each adult individual were recorded. From each individual, samples of root, stem, leaf and fruits of *C. fenestratum* were collected to screen the metabolite variation in different plant parts of the liana, individuals, size class and variation across the populations. A total of 90 individuals (adults and regenerates) were drawn from the 7 natural populations. The collected samples were identified by taxonomist Dr, Srikanth Gunaga and the

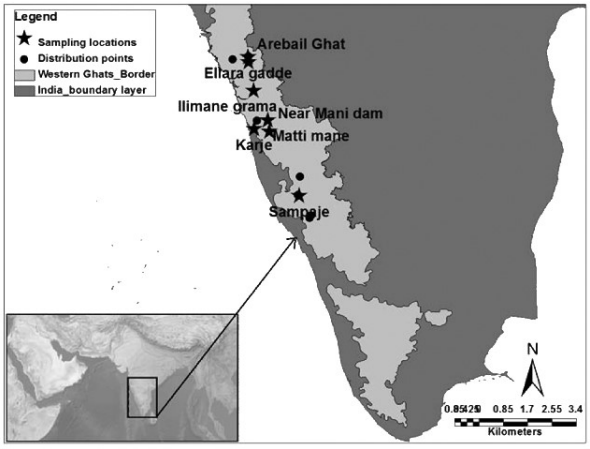

Plate 1 — Distribution of *C. fenestratum* populations across the central Western Ghats region, India.

voucher specimen (collection number- COFH872) was deposited in the Plant resource herbarium, College of Forestry, Sirsi. Perhaps this is the largest sample size, till date, subjected to chemical profiling representing distinct populations of *C. fenestratum*.

Sampling of male and female individuals

In order to study the variation of berberine content with respect to sex of the individual, the adult lianas in the reproductive stage were marked during flowering season (August to November) in Ellara Gadde population (P2). Root, stem, leaf and fruit samples were collected during fruiting season (December to March) from three male and female individuals for the analysis. Further, the fruit samples were separated as outer rind, inner rind and the pulp (embryo and endosperm) for the analysis.

Sampling of predated and non-predated leaves

Leaves of *C. fenestratum* are often predated by an unidentified larva during the spring season. In order to study the variation of metabolite content due to herbivore attack, young and matured leaves which are healthy and predated were collected during April to June from three individuals of the nursery grown plants of *C. fenestratum* maintained at College of Forestry, Sirsi (14.6171° N, 74.8449° E), which is situated in the heart of the Western Ghats.

Berberine extraction

Collected plant tissues were separated and dried in a hot air oven at 60 °C for 5-6 days. Dried samples were finely grounded for the chemical analysis. About 50 mg of fine tissue powder was extracted in 5 mL of methanol at 50 °C for 1 h in a shaking water bath. After cooling to room temperature, 5 mL of extract was centrifuged at 10,000 rpm for 10 min at 10 °C. The supernatant was filtered using 0.2 µ filter and analysed for berberine content using HPLC[19].

Berberine chloride analysis and quantification

Berberine was analysed by reverse phase HPLC (Supelco 516, LC-10AS, and Shimadzu, Japan) on a C18 column (250x4.6 mm, 5 µm). The mass of the samples was analysed and confirmed using LC-ESI-MS (LCMS-2020, Shimadzu, Japan). The HPLC conditions were: 344 nm as the detector wavelength, 0.8 mL/min flow rate and 10 µL sample loop. The mobile phase was adjusted as follows: 50 % acetonitrile and 50 % 0.1 % Trifluro-acetic acid (TFA) in an isocratic mode[27]. A berberine chloride (Sigma, HPLC purified) standard sample was procured from Sigma Chemicals and standard was prepared using methanol. For every five runs, the HPLC was re-standardized using the berberine standard. Berberine levels were quantified in the plant samples by using the regression of peak areas against the standard berberine and expressed as % dry weight of tissue. The appropriate statistical analyses of the data were conducted using the Statistica version 4.0 software.

Results and Discussion

Intra and inter-population variation for berberine accumulation

The % berberine in different samples of *C. fenestratum* was analysed by using Reverse Phase HPLC at 4.5 min elution. A total of 45 adult individuals and 45 regenerating individuals from 7 populations of the central Western Ghats (Plate 1) were chemically profiled for berberine content. Large and significant inter-population variation in the overall percentage of berberine content was found, although the intra-population variations were not statistically significant (Table 1). The overall percentage of berberine content pooled over all the tissues analysed from the populations ranged from 2.59 to 3.45 % in Arebail Ghat (P1), 0.66 to 3.11 % in Ellara gadde (P2), 2.34 to 3.27 % in Ilimane grama

Table 1 — Analysis of variance (ANOVA) for per cent berberine content (w/w) in a) root, b) stem and c) leaf tissues of *C. fenestratum* populations

	Sources of Variation	df	Sums ofsquares	Mean sums ofsquares	F-ratio	P-value
Root tissue	Among populations	6	156.326	26.05	11.37	<0.001
	Within populations	38	86.99	2.29		
	Total	44	243.315			
Stem tissue	Among populations	6	24.426	4.071	3.23	0.02
	Within populations	38	47.834	1.26		
	Total	44	72.26			
Leaf tissue	Among populations	6	5.668	0.95	1.27	NS
	Within populations	38	27.901	0.74		
	Total	44	35.569			

(P3), 1.05 to 4.73 % in Mani dam (P4), 1.59 to 3.11 % in Mattimane (P5), 2.38 to 4.79 % in Sampaje (P6) and 0.02 to 0.81 % in Karje (P7) populations. Least intra-population variation was observed in Karje population, while largest intra-population variation was in Ellara Gadde. The overall mean % of berberine content, pooled over all tissues, was highest in Sampaje (3.44±3.64 %) and Mani dam (3.21±2.56 %) populations and the least in the Karje (0.39±0.56 %) population (Fig. 1).

Variation in berberine content among different plant tissues

Large variations were found for the accumulation of berberine in root, stem, leaf, and fruit tissues of *C. fenestratum*. Overall, the range in % berberine content in different plant parts was 0.005 to 10.89 %. Pooled over all the populations, the root tissues yielded the highest berberine content with a mean of 4.62±2.65 %, followed by that in the stem (1.86±1.45 %). Leaf and fruit samples contained the least amount of berberine at 0.90±0.99 % and 0.03±0.01 %, respectively (Fig. 2). The percentage of berberine in root tissue samples ranged from 0.05 to 10.89 %, while the range was narrower in stem tissue samples (0.01 to 5 %) and leaf tissue samples (0.01 to 3 %). Berberine content was also estimated in different parts of the fruits. Outer rind and inner rind tissues of the fruits showed almost identical content of berberine (0.036±0.002 % in outer rind vs. 0.034±0.006 % in the inner rind); however, the fruit pulp showed 0.021±0.004 % of berberine.

There was significant variation in the root berberine content among the populations (p <0.01; Table 1). The mean berberine content in root tissue was highest (7.67±2.6 %) in Sampaje population (P_7), followed by that in Arebail Ghat (P_1; 6.92±0.68 %) and Maani dam (P_4; 5.17±2.6) populations. The lowest content was recovered from Karje population (P_7; 0.79±0.7 %). Interestingly, out of 45 adult individuals sampled, 18 individuals yielded more than 5 % of berberine content from the root samples. However, the patterns of accumulation in stem and leaf samples were not identical with respect to geographic locations. The highest berberine content in the stem tissue was recorded in Maani dam population (3.59±1.96 %) and lowest in Karje population (0.36±0.48 %).

To examine the allometric relation among the various tissues for the accumulation of berberine, correlation of berberine content among different tissues was computed using Pearson's co-efficient of correlation. Berberine content of root tissue was significant and it positively correlated with the beberine in stem tissue (r = 0.44, p <0.02; Fig. 3) and the association of berberine content of stem tissue with that of leaf tissue was positive but non-significant (r = 0.19, p >0.05; Fig. 4).

Variation in berberine content between adults and regenerating individuals

Pooled over stem and root tissues, slightly higher berberine content was found among the adults than that of the regenerates (2.59±1.0 % vs 2.36±0.79 %). This pattern was true in Arebail Ghat (P_1), Ellara gadde (P_2), Maani dam (P_4) and Mattimane (P_5) populations (Fig. 5). However, only in Karje (P_7) population, the pattern appeared to be reversed and significant. No

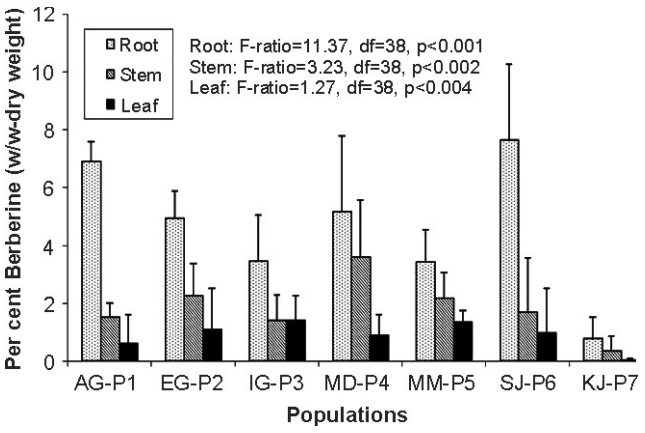

Fig. 1 — Mean per cent berberine content (on dry weight basis) in root, stem and leaf tissues from the seven different populations of *C. fenestratum* from the central Western Ghats, India. The error bars indicate +1 standard deviation. The F-test results and P levels are also indicated.

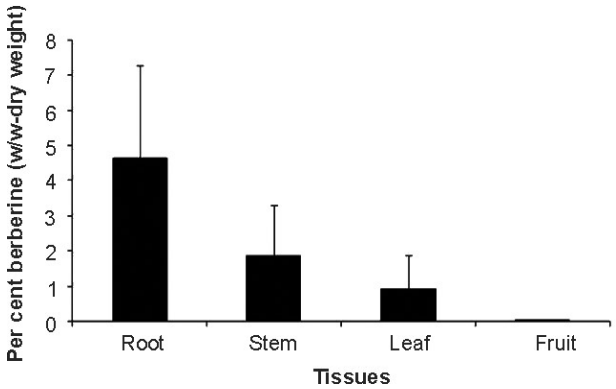

Fig. 2 — Mean per cent berberine content (on dry weight basis) in different tissues of *C. fenestratum* sampled pooled over all the seven populations. The error bars indicate +1 standard deviation. The Student's t-test, a) between stem and root tissues p <0.0001 at df = 44; b) between stem and leaf tissues p <0.01 at df = 44; c) between root and leaf tissues p <0.0001 at df = 44. The n values for the fruit tissue was low, hence test not done.

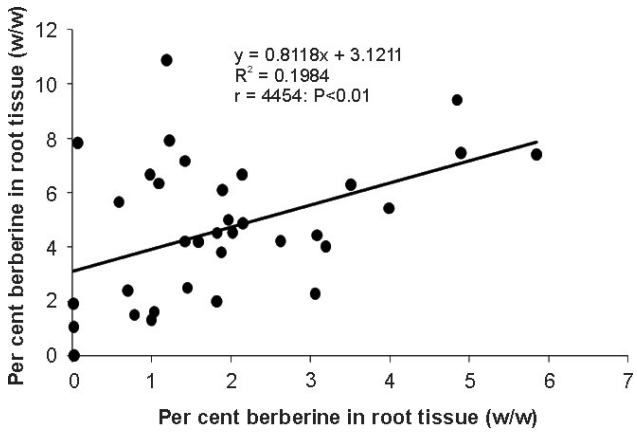

Fig. 3 — Association between per cent berberine content (on dry weight basis) in stem tissues with that of root tissues in *C. fenestratum* sampled from the central Western Ghats, India. The Pearson's correlation co-efficient (r) = 0.4454; p <0.01.

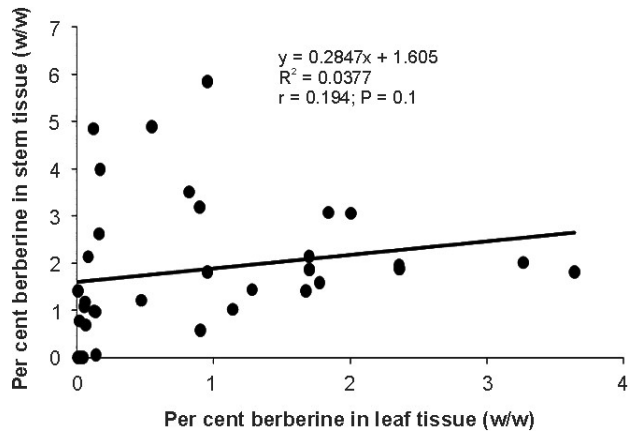

Fig.4 — Association between per cent berberine content (on dry weight basis) in leaf tissues with that of stem tissues in *C. fenestratum* sampled from the central Western Ghats, India. The Pearson's correlation co-efficient (r) = 0.194; p = 0.1 (non-significant).

significant variation was observed between adult and regenerates in Ilimane grama (P_5) and Sampaje populations (P_6). Further, no significant correlation was also observed between the stem diameters of the liana and stem berberine content. This suggests that the age of the adults may not significantly influence the concentration of the berberine.

Variation in berberine content between male and female individuals

Considering overall berberine content pooled over all the tissues, the % berberine accumulation did not differ significantly between male and female plants. However, stem tissues from female lianas had significantly higher berberine content than that

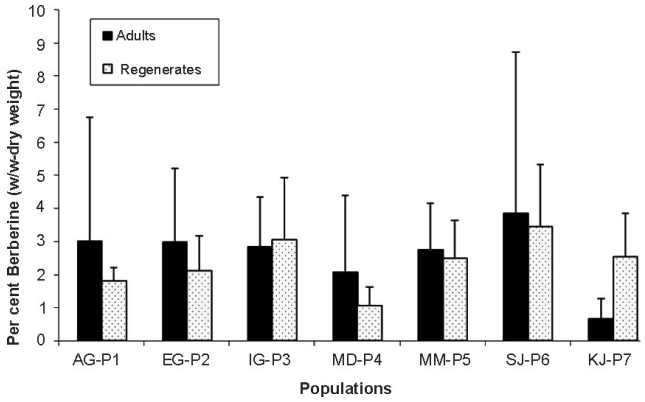

Fig. 5 — Mean per cent berberine content (on dry weight basis) in adults and regenerating individuals of *C. fenestratum* collected from seven populations from central Western Ghats, India. Data pooled over stem and root tissues. The error bars indicate +1 standard deviation.

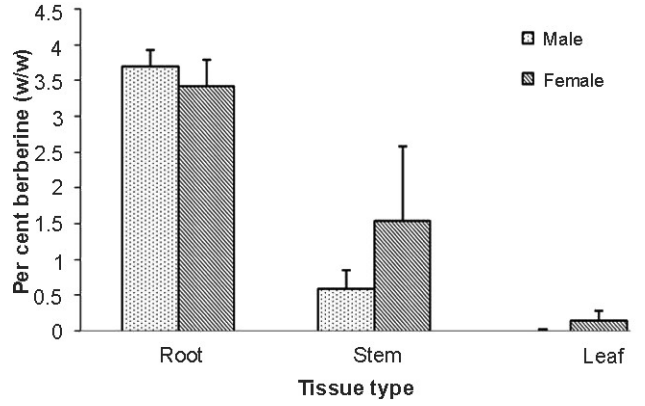

Fig. 6 — Mean per cent berberine content (on dry weight basis) in root, stem and leaf tissues of male and female individuals of *C. fenestratum* sampled from the central Western Ghats, India. The error bars indicate +1 standard deviation.

of the male individuals (0.60±0.26 % in males and 1.54±1.05 % in females; Fig. 6). The difference between sexes was not significant with respect to root (3.69±0.23 % in male and 3.42±0.36 % in females; Fig. 6) and leaf tissues (0.05±0.001 % in male and 0.13±0.002 % in female; Fig. 6).

Variation in berberine content between predated and non-predated leaves

In *C. fenestratum*, predated leaves clearly showed higher accumulation of berberine than the un-predated leaves. Predated-mature leaves had about 80 % higher berberine than the un-predatedmature leaves (0.145±0.002 % vs. 0.08±0.05 %, respectively; Fig. 7). The % berberine content among young predated leaves was also slightly higher than that in un-predated (0.019±0.01 % vs. 0.015±0.003 % respectively, Fig.7).

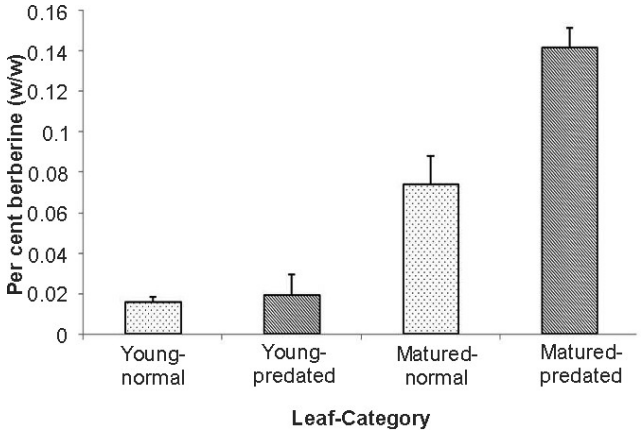

Fig. 7 — Mean per cent berberine content (on dry weight basis) among predated and un-predated (young and matured) leaves. The error bars indicate +1 standard deviation.

In the present study, of the 45 adult individuals assayed, 18 lianas yielded more than 5 % berberine, one individual showed over 10 % berberine on dry weight basis. These estimates are nearly two to five folds more than what has been reported hitherto in the literature. Rojsanga and Gritsanapan have reported that berberine content in *C. fenestratum* grown in Thailand ranges from 1.17±0.25 to 2.88±0.21 % on dry weight basis[1]. Rojsanga *et al*. have also reported far below berberine yield in other populations of Thialand[25]. Through a hot methanolic extraction procedure, Arawwawala and Wickramaarachchi have reported highest value of 2.00±0.1 % of berberine on dry weight basis from the Sri Lankan populations[28]. The high yields of berberine observed in the present study could not be attributed to their developmental stages since there was no significant association between berberine and dbh of these lianas. In a study conducted on *D. Binectariferum,* Mohan Kumar has also shown a non-significant correlation (r= 0.004) between the girth of the tree and % Rohitukine content[29]. Suhas *et al*. have also shown that mean CPT content was not significantly correlated with the age in *N. nimmoniana*[22].

Further, tissue specific accumulation of berberine has been demonstrated wherein highest % of berberine was found in roots followed by that in stem and leaves. The least content was found in fruits (Fig. 2). Higher levels of berberine in roots indicate that the synthesized berberine in leaves might be sequestered and stored in stem and in root tissues.

Interestingly, adult plants with insect-pest infestations showed about 70 % higher accumulation of berberine than the un-infested ones. This points to the fact that berberine could be largely accumulated to guard against insect pests. It is well known that plants adopt an array of chemicals against insect pests in order to increase their fitness. Such co-evolved systems wherein a chemical arms race have been well documented in tropical trees[30]. Increased accumulation of berberine in infested leaves may suggest the possibility of increasing the berberine content through artificial clipping of the leaves in order to sustainably harvest berberine from leaves. However, more focused work is necessary on these issues. Ramesh *et al*. have reported an intriguing observation of chrysomelid beetles (*Kanarella unicolor* Jacobby) feeding on the leaves of *N. nimmoniana* without any apparent adverse effect[31]. LC-MS/MS analysis of the beetles indicated that 54.9 % of the ingested camptothecin (CPT') was recovered from the wings, followed by lesser amounts in the head and abdomen. They have also conjectured that the accumulation of CPT may have an adoptive advantage to beetles against their predators.

The differences among the sites could not be attributed to either the geographical location (latitude) or the altitudinal differences. It is likely that the differences in the levels of berberine are a function of both the genetic and environmental backgrounds of the population. Few individuals yielded more than 7 % of berberine which is much higher compared to the earlier reports estimated through HPLC as it is evident by consistent estimates by the independent sampling and estimations. Further, the yields of berberine were largely unaffected by the size class of liana.

While substantial population level variations in berberine content and its lack of correlation with age of the liana point to the genetic basis of these differences, more focused studies are required to critically examine these differences. It would be interesting to study the parent-offspring regression in the accumulation patterns of berberine. This would reveal the exact genetic basis. It would also be interesting to investigate the proximate causes of very high accumulation of berberine in certain individuals. Subject to further confirmation, these 'elite' lianas and populations could be focused on conservation and judicious utilization through clonal multiplication. Tissue samples could also be used as a source for *in vitro* production system, as was done for several other systems such as taxane from *Taxus walichiana*[32] and for podophyllotoxin from *Podophyllum peltatum*[33]. Clonally multiplied elite lines could be deployed as a

profitable perennial liana component in suitable agroforestry systems from which berberine could be extracted on sustainable basis without disturbing the natural populations.

The results presented here form the first data set on a comprehensive screening of *C. fenestratum* populations for berberine. The study has demonstrated significant population level variation in berberine content. Our studies clearly indicate the promising potentiality of exploring the natural variability in berberine content among individuals across a wider geographical range and identifying high-yielding lines. These results hold important implications for chemically profiling the populations of the species in the larger landscape of the Western Ghats including all three states (*viz*. Karnataka, Kerala and Tamil Nadu) in search of 'chemical hot-spots' of berberine. Individuals with high content could be a potential source for raising plantations of high berberine yielding lianas as a part of agroforestry systems without disturbing the natural populations.

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