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A new plant sources of the anticancer alkaloid camptothec in from two genera of Rubiaceae in southern Western Ghats of India

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Abstract
In this study reported the two new plant sources for anticancer alkaloid Camptothecin (CPT) producing plants from southern Western Ghats namely *Mycetia acuminata* and *Neurocalyx calycina* (Rubiaceae). HPTLC profile of the *N. calycina* and *M. acuminata* showed the significant concentration of anticancer alkaloid were found in the plant parts. Roots of *N. calycina* were found to produce the highest content of CPT 0.233% (W/W) and followed by stem (90.152%) and leaves (0.024). Whereas in *M. acuminata* yielded comparatively the lower quantity of alkaloid content in roots (0.012%), stem (0.012%) and leaves (0.001%). Plant derived monoterpene pyrrolidine alkaloid camptothecin possessed to have unique antitumor activity. The compound is naturally distributed in several unrelated botanical groups with about 53 taxa of higher plant species. The market demand of CPT drug is urged to search the alternative sources of plant species and extraction technology. Hence the present study is added two more genera of Rubiaceae and summarized the CPT producing plants with distribution of anticancer drug in various plant taxa.

Keywords: camptothecin, anticancer, new plant sources, HPTLC, Western Ghats

Introduction
The important anticancer active principle is camptothecin (CPT), a monoterpene indole alkaloid which is first isolated from Chinese happy tree *Camptotheca acuminate* [1]. Later the alkaloid derivatives was isolated from several unrelated group of plants like *Ervatamia heyneana* [2], *Merrillio dendronmegacarpum* [3], *Mostuea brunonis* [4], *Nothapodytes nimmoniana* [5], *Chonemorpha fragrans* [6] and also reported from various species of the genus *Ophiorrhiza* [7, 8]. Among the 416 families of higher plants accounted in the worldwide, 7 families and their 17 genera with 53 species are reported to have a CPT and its analogues [9]. So far maximum concentration of CPT has been reported from *Nothapodytes nimmonia* of wild growing plants species in the Western Ghats [10]. Camptothecin is a potent inhibitor for proliferating cancer cells that inhibits DNA and RNA synthesis and induces DNA damage [11, 12]. Later it was proved that CPT inhibit the cell cycle at various stages and finally induce the cell death [13, 14]. Continuous exploration on the action of mechanisms of CPT, researchers have found out the compound inhibit the DNA replication in association of Topoisomerase-I [15, 16].Recently it was much investigated for anticancer activities of CPT on the cancer cells with its various analogues and launched the anticancer drugs in the market for various types of cancers after proper clinical trials [17]. CPT diversity in higher plants still offers a valuable source of novel and potent chemical lead discovery, but rapid identification of the high potent chemical analogue remains a critical factor to ensure that some new tool of drug discovery need to compete with recent developed technologies such as chemical profile library of plant species and high-throughput screening of combinatorial synthetic efforts. So far other biotechnological efforts are also undertaken for production of CPT by plant tissue culture [18, 19] and endophyte cultures [20, 21]. In spite of rapid growth of market demand of CPT raw materials are still procuring from the natural sources such as *Camptotheca* and *Nothapodytes*. Hence the identification of alternative sources is essential for producing potent anticancer natural drug and also fulfil the demand of pharmaceutical market [22]. While conducting the exploration on the CPT yielding plant sources along the southern Western Ghats, the authors have been collected some interesting Rubiaceae plant species and it was screened and reported for positive production of new CPT sources.

Materials and Methods

Plant material

The whole plants of *Mycetia acuminata* and *Neurocalyx calycina* were collected from Agasthiyamalai region of southern Western Ghats in the month of October 2019. The samples were authenticated by the Botanical Survey of India (BSI), Southern Circle, Coimbatore and voucher specimens (SK1928, SK1973) were kept in the Sriganesan Herbarium of the Department of Botany (SGH), The Madura College (Autonomous), Madurai. Plant materials were washed with tape water and shade dried separately for fifteen days. Drug materials were powdered and stored in airtight container at room temperature for further investigations.

Extraction

Shade dried, powdered plant materials of roots, stems and leaves were extracted separately with Soxhlet's apparatus using 200 ml EtOH for 6 h continuous reflux. After extraction, solventswere fully removed using a rotary evaporator and yields of extracts
(w/w) were calculated for each part of the plant samples.

HPTLC analysis

Quantification of CPTin selected plant part extracts was carried out using HPTLC (CAMAG, Switzerland) made up of TLC Scanner 3 and WinCATS Software 4.03 [23]. Ethanolic extracts of plant parts (50µl or 200 µg each) were applied onto silica gel HPTLCplates (60F-254, E. Merck, Germany, 20 x 10 cm, 0.2 mm thickness) as 6 mm wide bands with the automatic Linomat V sample applicator fitted with a micro syringe in N2 flow (application rate - 150 nl/s, space between two bands - 11 mm, slit dimension - 6 x 0.45 mm, scanning speed - 20 mm/s). CPT standard (Sigma Aldrich, India) was also applied along with the sample plant extracts. Plates were developed up to 80 mm in 20 ml of EtOAc:CHCl3:MeOH (5:4.5:0.5, v/v) mobile phase in the twin trough glass chamber, under saturated conditions (30 min). These plates were scanned densitometrically at 544 nm (tungsten lamp) using TLC Scanner 3 and the data were analyzed using WinCATS Software 4.03.CPTcontents of plant extracts were determined by means of the calibration plot, y = (2.705 x 0.294 + 06) x + 67.12, R2 = 0.999, made of standard CPT (at 0.001 µg/µl in EtOH). Linearity of CPT calibration curve in the range 0.0001-0.004 µg was ensured. Specificity was tested by repeated application of standard CPT. Integrity and purity of CPT peak were also tested by spectral analysis [24].

Results and Discussion

The present investigation resulted that the two plant species of Rubiaceaenamely *Mycetia acuminata* and *Neurocalyx calycinus* are reported to have a CPT producing plants of southern Western Ghats and it is added a new source of natural CPT. The phytocompound is well known cancer treating potential and several of its analogues in pharmaceutical markets. Over 60% of drugs employed in current cancer chemotherapy are derived from plant sources and microogranisms. Any potential anticancer compound may act as antiproliferative due to alter the cell signalling pathways [25]. HPTLC studies indicated that the positive presence of CPT by sharp peak of standard CPT compared with sample extracts (Fig. 2).

The maximum CPT concentration was observed in the roots of *N. calycina* (0.233 µg/g) followed by stem (0.152 µg/g) and leaf (0.024µg/g) extracts. Whereas in *M. acuminata* showed the least amount of CPT when compared to *Neurocalyx* (Fig.1). Both the plant samples were observed that root parts accumulated maximum concentration of CPT than the aerial parts. The chromatogram for root, stem and leaves of selected samples are depicted in Fig. 2 (A-G). The specificity of standard and analysed extracts coincide the absorption maxima at 224 nm as shown in Fig. 2H. The calibration curve of standard CPT was found to be 2 - 80 µg/g and the regression line correlation coefficient (R^2) was calculated and found to be 0.9994 which is indicative of good linearity of calibration curve intercept with y=2.705. Concerned results confirmed the presence of CPT in selected plant extracts with considerable amount in *M. acuminata* and *N. calycina* (Fig.1). The present investigation reported that the two new Rubiaceaeplant sources for CPT producing plants from southern Western Ghats.

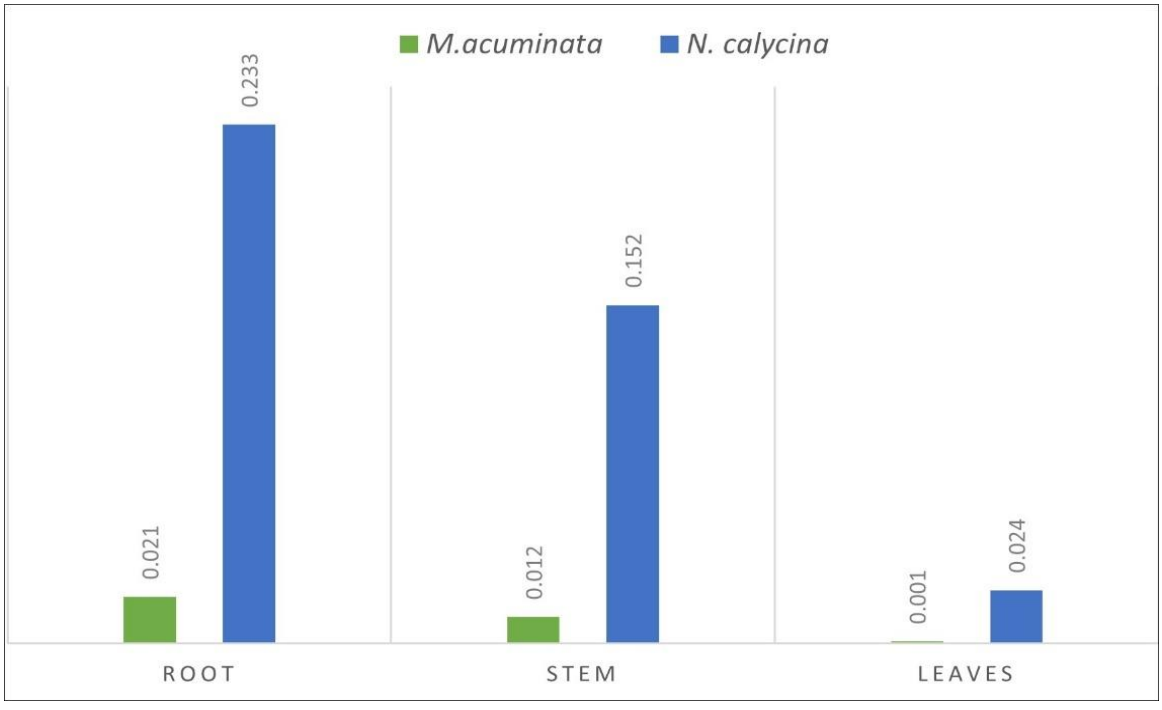

Fig 1: Camptothecin contents (% w/w) in various parts of *Mycetia acuminata* and *Neurocalyx calycina* collected from southern Western Ghats.

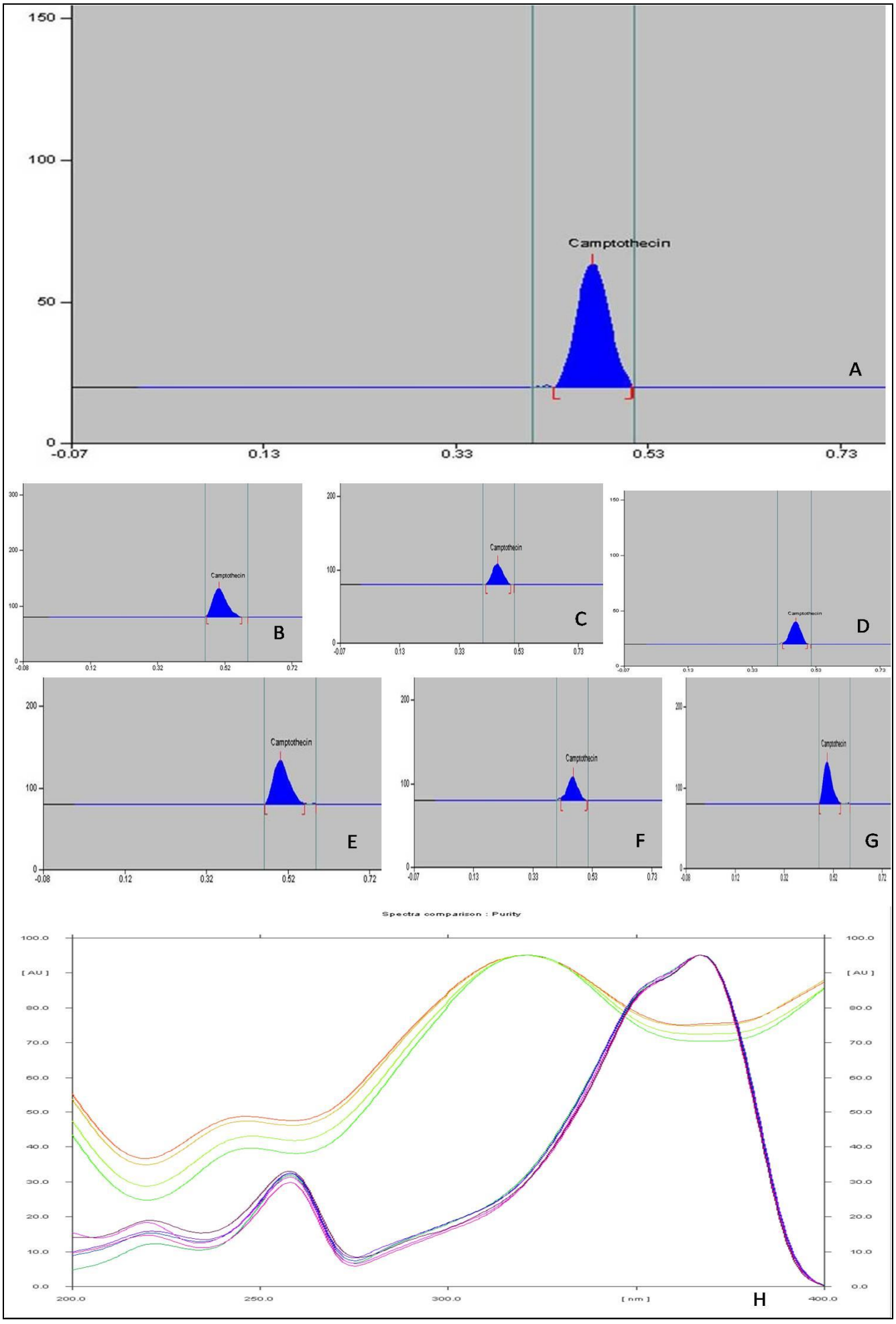

Fig 2: HPTLC chromatogram of standard camptothecin, ethanolic extract of *Mycetia acuminata* and *Neurocalyx calycina* root, stem and leaves.

The comparative account of CPT concentration reported so far summarized in table 1. It indicated the presence of CPT in *Apodytes dimidiata* (<0.01% in stem), *Codiocarpus andamanicus* (<0.01% in fruit), *Gomphandra comosa* (<0.01%in fruit), *Gomphandra coriacea* (<0.01% in leaf, twig and fruit), *Gomphandra polymorpha* (<0.01% in leaf), *Gomphandra tetrandra* (<0.01% in leaf and stem), *Iodescirrhosa*(<0.01% in fruit), *Iodeshookeriana* (0.009% in fruit), *M. dentata* (1.418% in Cotyledons), *M. kleinii* (0.153% in fruit), *Nastiatum. herpeticum* (0.026%in fruit), *Pyernacanta volubilis* (0.488% in fruits) and *Sarcostigmakleinii* (0.018% in leaf) (Table 1). Up to date two other genera of the family Rubiaceae, namely *Ixora coccinia* [26] and *Ophiorrhiza* species [63, 76] have been reported to contain CPT. The highest content of CPT was reported from *Nothapodytes nimmoniana* (0.3% by dry weight of stem bark) [5] and the other species had very low levels of CPT in *Pyrenacanta klaineana* (0.00004%) and *Merilliodendron megacarpum* (0.05%) [3, 80]. CPT accumulation in *Miquelia dentata* is depending on the various developmental stages of fruits. Immature fruits yielded 0.008–0.011% (w/w), whereas in mature fruits about 0.63–0.80% of CPT. In fruit coat is 0.199%, seed coat is 0.054%, in cotyledons 1.418% and in root 0.153%. Leaves and twigs consists of very less amount of CPT [27]. HPLC analysis of various geographically isolated genetic population of *Pyrenacantha volubilis* fruits revealed that maximum CPT content (1.06% w/w) yielded in Puthupattu population, followed by Pazhaiyasivaram (1.02% w/w), M. Kottai (0.91% w/w), Villiampakkam (0.85% w/w), Otteri (0.66% w/w), Vallathirakottai (0.66% w/w), Mangalam (0.51% w/w),and Kizhoor (0.48% w/w) [28]. This study proved that the CPT accumulation is dependent on genotypein *P. volubilis*. Similar genotype-dependent variations in CPT accumulation havealready been reported in *Camptotheca* [29, 30] and *Nothapodytes nimmoniana* [31]. The present investigation added the two more genera of Rubiaceae are CPT producing plants from the southern Western Ghats and its needed for further exploration and characterization of new sources of CPT producing species to meet out the pharmaceutical demand.

Table 1: A comparative account of camptothecin content in different plant parts and tissues.

Plant species	Plant part(s) analysed	CPT content (% dry weight w/w)	Methods of analysis	References
Alnus nepalensis	Leaves	0.192	HPLC	[32]
Apodytes dimidiate	Leaves	0.1	HPTLC	[33]
	Stem bark	0.0051	HPLC	[27]
Camptotheca acuminata	Young leaves Seeds Bark	0.4-0.5 0.3 0.18 - 0.2	HPLC	[34, 35, 30]
	Young leaves	0.24 - 0.30	HPLC	[29, 30]
	Leaves	0.04 – 0.07	LC-ESI-MS/MS	[36, 37]
	Whole parts	0.1 – 0.4	HPLC	[38]
	Dried shoots	0.042	HPLC	[39]
	Dried root	0.051	HPLC	[39]
	Roots	1.0 – 0.89	HPLC	[40]
	Hydroponic culture	0.55 – 0.83	HPLC	[44]
	Stem and leaf callus	0.1 – 0.25	HPLC	[41]
	Hairy roots	0.1	HPLC	[45]
		1.0 – 0.15	HPLC	[42]
	Callus	0.20 - 0.23	HPLC	[43]
Camptothecalowreyana	Young leaves	0.39-0.55	HPLC	[29]
	Old leaves	0.09 -0.11	HPLC	[29]
Camptothecaynnanensis	Young leaves	0.25 - 0.44	HPLC	[29]
	Old leaves	0.059	HPLC	[29]
Codiocarpus andamanicus	Fruit	0.0003	HPLC	[27]
Chonemorpha grandiflora	Stem callus	0.013	HPLC	[46]
	Bark callus	0.003	HPLC	[46]
Chonemorpha fragrans	Seeds	0.0057	HPLC	[47]
	Hairy root	0.1	HPLC	[48]
	Root	0.026	HPLC	[6]
	Stem bark	0.014	HPLC	[6]
	In vitro shoots	0.012	HPLC	[6]
Dysoxylum binectariferum	Stem bark	0.122	LC-MS	[49]
Ervatamia heyneana	Wood and stem bark	0.13	HPLC	[2]
	Stem	0.0014	HPTLC	[50]
Gomphandra coriacea	Twigs	0.01	HPLC	[51]
	Leaves	0.0031	HPLC	[27]
	Seed coat	0.0005	HPLC	[27]
	Root	0.0286	HPLC	[27]
Gomphandra comosa	Fruit	0.00031	HPLC	[27]
Gomphandra polymorpha	Fruit	0.011	HPLC	[27]
Gomphandra tetrandra	Leaves Fruits	0.00045 0.006	HPLC	[27]
Iodes cirrhosa	Fruits	0.01	HPLC	[27]
Iodes hookeriana	Leaves Fruits	0.0001 0.0098	HPLC	[27]
Ixora coccinia	Young leaves	0.4146	RP-HPLC	[26]

	Mature leaves	5.061		
Miquelia dentata	Leaf callus	0.8 – 1.2	HPLC	[52]
	Twig	0.003	HPLC	[27]
	Leaves	0.024	HPLC	[27]
	Seed coat	0.054	HPLC	[27]
	Root	1.418	HPLC	[27]
	Fruit	1.22	HPLC	[27]
Miquelia kleinii	Fruit	0.153	HPLC	[27]
Merillodendron megacarpum	Leaves and stem	0.053	HPLC	[3]
Mosteua brunonis	Whole plant	0.030	HPLC	[4]
Natsiatumherpticum	Twig Fruits	0.0026 0.026	HPLC	[27]
Nothapodytes foetida	Stem wood	0.14-0.24	HPLC	[53, 54]
	Shoot	0.075	HPLC	[55, 10]
	Stem bark	0.23	HPLC	[56]
	Root	1.973	HPLC	[57]
	Twig	0.910	HPLC	[57]
	In vitro callus	1.3	HPLC	[58]
	Leaves	0.08	HPLC	[59]
	Fruits	1.22	HPLC	[56]
	Plant	0.048	HPLC-DAD-LC-MS	[60]
Nothapodytes nimmoniana	Stem bark	0.3	UV, IR, NMR, MS	[72, 73]
	Stem	0.01	UFLC-PDA	[74]
	Leaves	0.081	HPLC	[31]
	Stem bark	0.081	HPLC	[31]
	Root bark	0.236	HPLC	[31, 73]
	Stem wood	0.14	HPLC	[31]
	Root wood	0.18	HPLC	[31]
	Seed	0.16	HPLC	[47]
	Stem bark	0.07	HPLC	[24]
	Root	1.87	HPLC	[24]
	Senescent leaves	0.07	HPLC	[24]
	Stem	0.107	HPLC	[24]
Nothapodytes pittosporoides	Roots	0.172	HPLC	[61]
Ophiorrhiza alata	Whole plant	0.0083 – 0.038	HPLC	[62]
Ophiorrhiza eriantha	Whole plant	0.3	HPLC	[63]
Ophiorrhiza grandiflora	Plant	1.07	HPLC	[63]
Ophiorrhiza hirsutula	Whole plant	0.17	HPTLC	[63]
Ophiorrhiza japonica	Whole plant	0.0073	HPLC	[64]
Ophiorrhizaliukiuensis	*In vitro* plantlets	0.1	HPLC	[65]
Ophiorrhiza kuroiwai	*In vitro* plantlets	0.12	HPLC	[65]
Ophiorrhiza pumila	Young shoots	0.1	HPLC	[66]
	Hairy roots	0.1	HPLC	[66, 67]
Ophiorrhiza mungos	Whole plant	0.0012	HPLC	[8, 68]
	Whole plant	0.039	HPTLC	[54, 63]
	In vitro shoots	0.126	HPLC	[69]
	In vitro shoots	0.063	HPLC	[70]
	Hairy roots	0.1 – 0.3	HPLC	[71]
Ophiorrhiza prostrata	Adventitious roots	0.16	HPLC	[75]
Ophiorrhiza shendurunii	Whole plant	0.05	HPLC	[76]
Ophiorrhiza rugosa	Albino plants	0.1	HPLC	[8, 77]
	Normal plant	0.03	HPLC	[77]
	Stem	0.08	HPLC	[7]
	Root	0.16	HPLC	[7]
	Old leaves	0.002	HPLC	[7]
	Fruits	0.016	HPLC	[7]
	Whole plant	0.018	HPTLC	[63]
	Leaf callus	0.04	HPLC	[78, 79]
Ophiorrhiza trichocarpon	Whole plant	0.20	HPLC	[76]
Mostuea brunonis	Whole plant	0.01	HPLC	[4]
Pyrenacantha klaineana	Stem	0.0048	HPLC	[80]
Pyrenacantha volubilis	Fruits	0.488	HPLC	[27]
	Seed powder	1.06	HPLC	[28]
	Plant	1.35	HPLC	[81]
Sarcostigma kleinii	Leaves Stem bark Fruits	0.0042 0.0037 0.0003	HPLC	[27]

Conclusion

The demand of CPT in pharmaceutical market is ever increasing due to its anticancer potential and with their pharmacological efficacy of diverse natural chemical derivatives. It is urged the identification of alternative source to meet out the pharmaceutical demand and conservation of high valued plant species. A total of 53 different species of higher plants have already been reported for the bioavaliability of CPT apart from the microbial and other biotechnological sources. In this investigation added two more Rubiaceae genera from the southern Western Ghats are new bioresources for CPT extraction. This data can be used to extract the CPT from the alternative sources for effective anticancer drug development.

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