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Some observations on *Nothapodytes foetida*: An overview

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ABSTRACT

Nothapodytes foetida is a medium size tree distributed in Western Ghats of India, is considered to be an endangered species. Phytochemical studies of the drug revealed the presence of camptothecin, hydroxy camptothecin, acetyl camptothecin, methoxy camptothecin, steroids and fatty acids. The highest concentration of camptothecin (CPT) is found in young immature fruits. Tissue culture studies of the drug show that a lot of hormones (naphthalene acetic acid, benzyl amino purine (BAP), 2, 4-dichloro phenoxy acetic acid (2,4,D), thidiazuron (TDZ), 2, 4, 5-trichlorophenoxy acetic acid (2,4,5 T) and picloram enhance CPT content. The most important combination affect callus induction and CPT content are 2, 4-D with BAP and 2, 4, 5-T with NAA in one month old callus. At concentration (1.36 µM) TDZ simulate shoot formation (46.25%). Camptothecin and 9-methoxycamptothecin have promising anti-cancer activity. The molecular target of camptothecin is inhibiting the nuclear enzymes topoisomerase I DNA complex. Camptothecin inhibits Tat-mediated transactivation of HIV-1 LTR, a potential target for therapy of HIV-1 infection. Camptothecin itself is not used clinically due to its cytotoxicity, but its derivatives (irinotecan and topotecan) are most effective for the treatment of cancer.

KEY WORDS: Camptothecin, *Mappia foetida*, Anticancer, Murashige and skoog medium, Callus.

Abbreviations: CPT, camptothecin; ACPT, O-acetyl-CPT; HCPT, 10-hydroxy CPT; MACPT, 9-methoxy-20-O-acetyl-CPT; MCPT, 9-methoxyCPT; *N. foetida*, *Nothapodytes foetida*; 2,4-D, 2, 4-dichlorophenoxy acetic acid; 2,4,5-T, 2,4,5-tri chlorophenoxy acetic acid; NAA, naphthalene acetic acid; IAA, indole acetic acid; BAP, 6-benzyl amino purine; *C. acuminata, Camptotheca acuminata; N. foetida, Nothapodytes foetida; O. mungos, Ophiorrhiza mungos; O. pumila, Ophiorrhiza pumila; P. klaineana, Pyrenacantha klaineana; M. megacarpum, Merriliodendron megacarpum; M. brunonis, Mostuea brunonis; E. heyneana, Ervatamia heyneana; C. lowreyana, Camptotheca lowreyana; C. yunnanensis, Camptotheca yunnanensis;*

INTRODUCTION

Nothapodytes foetida (also known as *Mappia foetida* or *Nothapodytes nimmoniana*) is a medium size tree belonging to family Icacinaceae. It is distributed in Southern India, North India, Srilanka, Myanmar and Thailand (1). In recent years, because of the enormous demand for the chemical worldwide, there has been an indiscriminate extraction of the trees from many parts of India, especially from the Western Ghats, a megadiversity forest range along the western coast of India (2). It is estimated that over the last decade there has been a 20% decline of the natural population of this species in the Western Ghats (2, 3). Owing to this threat, *N. nimmoniana* has been classified as a 'vulnerable' species. So biotechnological approaches (plant tissue culture) will be the better option because of

1. Conservation of endangered species.
2. Plants often more uniform.
3. Plants grow faster due to high multiplication rates.
4. Woody plants that are difficult to propagate can be micropropagated.
5. It is easier to produce pathogen free clones.
6. Micropropagation tends to rejuvenate or reinvigorate clones, making it easier to propagate these clones.
7. Plants often mature earlier than when propagated by seed.
8. Improved progeny evaluation of breeding experiments.
9. Fast release of newly developed cultivar.

Camptothecin, a monoterpene indole alkaloid, is regarded as one of the most promising anticancer drugs of the twenty first century (4–7). Irinotecan and topotecan, two water soluble derivatives of camptothecin (CPT), have been approved by the food and drug administration (FDA) of the United States of America for treating colorectal and ovarian cancer (8–10). CPT was first isolated from a Chinese deciduous tree *Camptotheca acuminata* (11, 12). Later it was isolated from a variety of plant species including *Merriliodendron megacarpum* (13, 14), *N. foetida* (15), *Ophirrohiza mungos* (16) and *O. pumila* (17), *Ervatamia heyneana* and *Mostuea brunonis*. Among these, the highest concentration of CPT (about 0.3% on a dry weight basis) has been reported from *N. nimmoniana*, formerly known as *N. foetida*. The leaves of *N. nimmoniana,* contained very little CPT, though CPT content in the leaves was as high as that in the wood. Roja and Heble reported about 0.075% CPT in shoots of mature trees (18). Padmanabha *et al* determined CPT content in different parts of *N. foetida* (19).

Camptothecin producing plants along with geographical profile

From the distribution of CPT and other secondary metabolites, it has been postulated that the genes encoding enzymes involved in their biosynthesis evolved early during evolution. These genes were presumably not lost during evolution but might have been ''switched off'' during a certain period of time and ''switched on'' again at some later

point. The CPT derivatives, irinotecan and topotecan, are used throughout the world for the treatment of various cancers, and over a dozen more CPT analogues are currently at various stages of clinical development. The worldwide market size of irinotecan/topotecan in 2002 was estimated at about $750 million and at $1 billion by 2003. In spite of the rapid growth of the market, CPT is still harvested by extraction from bark and seeds of *C. acuminata* and *N. foetida*. All parts of *C. acuminata* contain some CPT, although the highest level is found in young leaves (4–5 mg/g dry weight), approximately 50% higher than in seeds and 250% higher than in bark. The development of hairy root cultures of *O. pumila* and *C. acuminata*, and the cloning and characterization of genes encoding key enzymes of the pathway leading to CPT formation in plants has opened new possibilities to propose alternative and more sustainable production systems for this important alkaloid (20).

Phytochemical studies

Extraction methods using stirring extraction, soxhlet extraction, ultrasonic extraction and microwave assisted extraction (MAE) were evaluated for the percentage extraction of CPT and 9-MCPT from *N. foetida*. The extracts were analyzed by high performance liquid chromatography (HPLC). Methanol (90%, v/v) extracted high percentage extraction of CPT and 9-MCPT compared to ethanol (90%, v/v). The results show that the percentage extraction of CPT and 9-MCPT from *N. foetida* by MAE was more efficient in short time followed by soxhlet extraction, ultrasonic and stirring extraction methods. Maximum percentage extraction of CPT (2.67%, w/w) was obtained by MAE technique. MAE has need of 3 min, whereas ultrasonic extraction, soxhlet extraction and stirring extraction techniques require 30, 120 and 30 min, respectively to teach higher percentage extraction of CPT and 9-MCPT. The time taken by the microwave extraction process were 40 times less than the soxhlet extraction for percentage extraction of alkaloids. These results showed that the extraction efficiency and considerable saving of time by MAE was more competent than the other extraction techniques (21).

A new naturally occurring alkaloid, acetylcamptothecin, together with (+)-l-hydroxypinoresinol, ω-hydroxypropioguaiacone, p-hydroxybenzaldehyde, scopoletin, uracil, thymine, sitosterol, sitosteryl-ß-D- glucoside, 3 ß - hydroxy-stigmast-5-en-7-one, stigmast-5-en-3 ß,7α-diol, 6fl-hydroxystigmast-4-en-3-one, sitost-4-en-3-one, linoleic acid, trigonelline, camptothecin, 9-O-MCPT and pumiloside were isolated and characterized from the stem of *N. foetida*. Among them, scopoletin, CPT, 9-O-MCPT and O-ACPT showed significant cytotoxic activity (22).

The naturally occurring alkaloids, camptothecin and mappicine ketone were converted to racemic mappicine acetate which was enantioselectively hydrolyzed to (S) - and (R)-mappicine in high optical purity using baker's yeast. Treatment of the racemic acetate with baker's yeast afforded (S)-mappicine while with lipase yielded (R)-mappicine. 9-MCPT and 9-methoxymappicine ketone underwent similar conversion to (S) - and (R)-9-methoxymappicines (23, 24).

The air dried stems of *N. foetida* were powdered and extracted with a soxhlet apparatus using hexane for 24 h. The defatted plant material was again extracted with acetone using the same soxhlet apparatus for 72 h. The acetone extract was concentrated and subjected to column chromatography over silica gel. The elution of the column with hexane-EtOAc (1:1) afforded 9-methoxy-20-O-ACPT (yield 0.00025%) (25).

Yamazaki *et al* studied metabolite profiling of alkaloids and strictosidine synthase activity in camptothecin producing plants (26). *N. foetida* contains 16.0% of 3-ketooctadec-cis-15-enoic acid. It also contains fatty acids such as palmitic acid (12.3%), stearic acid (4.2%), oleic acid (16.2%), linoleic acid (11.6%) and linolenic acid (39.7%). The identification and characterization was based on ultraviolet spectroscopy, infrared spectroscopy, nuclear magnetic resonance, mass spectroscopy, gas liquid chromatography and chemical degradations (27).

A quantitative analysis using 1H-NMR has been developed by Li *et al* (2005) for the determination of camptothecin derivatives and trigonelline in *N. foetida* root, stems and leaves. In the region of δ value 9.5-5.5, the signals of H-7 of camptothecin, H-10 of 9-methoxycamptothecin, H-19 of pumiloside and H-2 of trigonelline, were well separated from each other in DMSO-d6. The quantity of the compounds was calculated by the ratio of the intensity of each compound to the known amount of internal standard 3, 4, 5-trimethoxy benzaldehyde (28).

Tissue culture studies on Nothapodyte foetida

Roja and Heble reported the production of CPT and 9-MCPT from callus cultures of *N. foetida*. The callus was reported to produce 1 µg per g dry weight of 9-MCPT. Only MS medium with 2 mg picloram/l mg/1, 3% (w/v) sucrose induced callus formation after 3 weeks (Table 2). Thin layer chromatography (TLC) analysis showed for the presence of CPT and 9-MCPT as indicated by spots with the same Rf values as authentic samples and blue fluorescence under UV light. The HPLC analysis further confirmed their presence as these spots had the same retention times (9.03 min and 9.60 min) and the same UV absorption maxima as authentic samples for CPT (254 nm) and 9-methoxy-CPT (267 nm). The content of CPT in callus 9.5 µg g^{-1} per g dry weight along with traces of 9-MCPT (18).

Thengane *et al* studied the effect of 2, 4-dichlorophenoxy acetic acid (2,4-D), 2,4,5-tri chlorophenoxy acetic acid (2,4,5-T), naphthalene acetic acid (NAA) and indole acetic acid (IAA) with 6-benzyl amino purine (BAP) / kinetin on callus induction and camptothecin accumulation in *N. foetida*. Hyper production of camptothecin (1.306% dw) was observed with a combination of 2, 4-dichlorophenoxy acetic acid (2, 4-D) with benzyl amino purine (BAP) and 2, 4, 5-trichlorophenoxy acetic acid (2, 4, 5-T) with NAA in one month old callus shown in Table 3 (29).

Table 1: Correlation of CPT content (% CPT/gdw) between different tissues.

	Wood	Bark	Rwood	Rbark	Leaf
Wood	1.000	0.225	0.183	- 0.116	0.179
Bark		1.00	0.186	0.483	0.051
Rwood			1.00	0.469	0.295
Root bark				1.00	0.011

Table 2: Sites of accumulation of the anti-tumor alkaloid CPT and its natural derivatives in natural sources.

Species	Tissue analyzed	Sample origin	Camptothecinoids content (µg/gram dw)
C. acuminata	Young leaves	Texas, USA	CPT 4000–5000,HCPT 20–30
	Seeds		CPT 3000, HCPT 25
	Bark		CPT 2000, HCPT 2-90
	Root		CPT 400, HCPT 13–20
	Young fruit		CPT 842
	Old fruit		CPT 2362
C. lowreyana	Young leaves	Texas, USA	CPT 3913–5537
	Old leaves		CPT 909–1184
C. yunnanensis	Young leaves	Texas, USA	CPT 2592–4494
	Old leaves		CPT 590
E. heyneana	Bark	India	CPT 1300, MCPT 400
N. foetida	Stem wood	Okinawa, Japan	CPT 1400–2400
	Stem	Taiwan	ACPT 0.24
	Shoot	Mahabaleshwar,India	CPT 750, MCPT 130
	Plantlet culture		MCPT 7
	Callus		MCPT 1
	Stem	Godavari, India	MCPT 2.5
	Callus	Ooty, India	CPT 9.5, MCPT traces
	Cell	Satara, India	CPT 1.1, MCPT 0.81
M.megacarpum	Leaves and stem	Guam	CPT 530, MCPT 170
M. brunonis	Entire plants	Lope, Gabon	CPT-20-O-b-glucoside100
O. mungos	Entire plants	Colombo	CPT 12, MCPT 10.41
O. pumila	Leaves	Japan	CPT 300–400
	Young roots		CPT 1000
P. klaineana	Stem	Ghana	CPT 4.8, MCPT 1.6

Table 3: Different constituents in N. foetida root, stems and leaves (%w/w).

Compound	Root	Stem batch 1	Stem batch 2
Camptothecin	4.85	3.86	3.65
Methoxy CPT	4.73	2.96	2.95
Pumiloside	8.68	2.96	2.95
Trigonelline	6.68	3.25	8.16

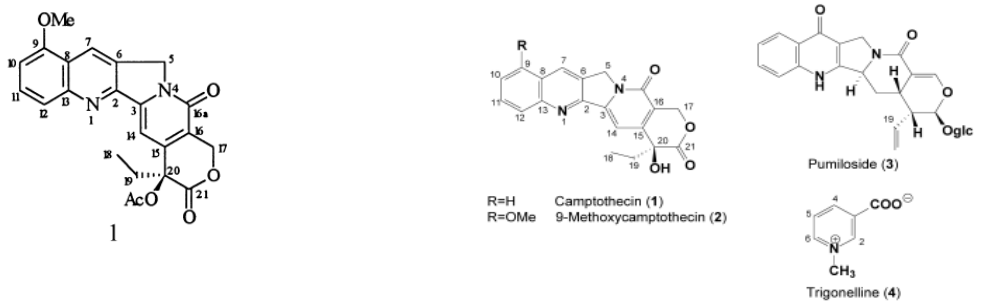

Fig. 2: Structure of major constituents in N. foetida

Table 4: Effect of different hormones on callus initiation in N. foetida.

Medium	Phytohormone added	Response
MS[a]	2,4-D (2 mg/ l) + Kn (1 mg/l)	-
MS	Picloram (2 mg/ l)	+++
MS	2,4-D (2 mg/ l)	+
MS	2,4-D (2 mg/ l) + BA (0.2 mg/ l)	-
MS	2,4-D (1 mg/ l) + BA (0.2 mg/ l)	-
MS	Kn (1 mg/l) + NAA (0.2 mg/ l)	+++
B5 [b]	Picloram (2 mg/ l)	+
B5	Dicamba (2 mg/ l) + Kn (1 mg/l)	-
0.5 B5	Dicamba (2 mg/ l) + Kn (1 mg/l)	-

Murashige & Skoog (1962). b. Gamborg et al. (1968). c. Callus formation from embryos + poor, ++ good, +++ very good, – = no response.

Table 5: Effect of plant hormones on callus induction and alkaloid production in N. foetida.

2,4-D	2,4,5-T	NAA	BAP	Kinetin	% Callus response
0.90	-		2.22	-	26.88
2.26	-		2.22	-	72.97
4.52	-		2.22	-	34.37
13.57	-		2.22	-	33.33
0.90	-		-	2.32	18.00
2.26	-		-	2.32	22.95
4.52	-		-	2.32	26.61
-	0.78		2.22	-	24.39
-	1.96		2.22	-	36.58
-	3.91		2.22	-	35.86
-	11.74		2.22	-	35.36
-	-	1.07	2.22	-	53.65
-	-	2.69	2.22	-	41.60
-	-	5.37	2.22	-	40.62
-	-	16.11	2.22	-	65.55

Table 6: Effect of culture period on production of CPT and MCPT with growth index.

Days	CPT	9-OMCPT	Growth index (gm)
15	0.02	0.09	1.71±1.1
30	0.04	0.11	3.62 ±0.8
45	0.71	0.61	4.19 ±1.0
60	0.70	0.59	5.89 ± 2.8

Table 7: Effect of increasing concentration of growth regulators on CPT and 9-OMCPT production in N. foetida.

2,4-D	2,4,5-T	NAA	BAP	% Callus response
4.52	-	-	4.44	34.37
9.05	-	-	4.44	42.85
13.57	-	-	4.44	35.29
18.10	-	-	6.66	16.00
-	3.91	-	4.44	51.92
-	11.74	-	6.66	66.17
-	15.66	-	6.66	70.45
-	19.57	-	6.66	46.96
-	-	5.37	4.44	37.83
-	-	10.74	4.44	37.69
-	-	16.11	4.44	35.63
-	-	21.48	4.44	37.77

All concentrations were expressed in (µM). Thengane et al studied the effect of thidiazuron on adventitious shoot regeneration from seedling explants of N. foetida (30).

Table 8: Effect of thidiazuron on adventitious shoot regeneration from seedling explants of N. foetida.

Plant parts	TDZ (µM)	Explant forming shoots (%)
Leaf	0.45	-
	0.91	40.30 ±1.20
	1.36	46.25 ± 2.27
	1.82	21.20 ±2.15
	2.27	26.00 ±1.57
	4.54	21.29 ±1.19
Hypocotyl	0.45	6.50 ±0.60
	0.91	11.99 ±1.10
	1.36	10.20 ±2.50
	1.82	16.20 ±2.22
	2.27	28.90 ±1.90
	4.54	20.50 ±1.85
Cotyledons	0.45	-
	0.91	-
	1.36	5.71 ±1.70
	1.82	9.75 ±2.65
	2.27	11.20 ±1.91
	4.54	17.15 ±2.05

Table 9: Effect of BA and IBA on elongation of shoots produced from leaf,hypocotyls and cotyledons of N. foetida.

Basal media	BA (µM)	IBA (µM)	Response (%)
Full length	-	-	Nil
	0.88-2.22	0.49	< 1
3/4 Strength	-	-	Nil
	0.88	0.49	4.0-5.5
	1.33	0.49	7.8-9.5
	2.22	0.49	10.0-12.0
1/2 Strength	-	-	Nil
	0.88	0.49	0.5
	1.33	0.49	1.7
	2.22	0.49	3.0

Table 10: Effect of modified MS medium on camptothecin content in N. foetida.

MS medium with hormones	Dry weight (mg)	CPT (% dw)	9-MCPT (% dw)
NAA (71.36µM)+ BA (8.87µM)	338.6±0.2	0.0107±0.2	0.00164±0.4
NAA (35.68µM)+ BA (8.87µM)	265.5±0.2	0.00024±0.1	0.000055±0.01
NAA (71.36µM)+ BA (9.29µM)	201.5±0.2	0.00016±0.03	0.000038±0.03
NAA (35.68µM)+ BA (9.29µM)	198.5±0.1	0.00017±0.1	0.000058±0.02

Table 11: Tissue culture production of camptohecinoids among different species(20).

Species	Tissue analyzed	Camptothecinoids content (µg/g dw)
C. acuminata	Hairy root	CPT 1000, HCPT 150
	Callus culture	CPT 2040-2360, HCPT 80-100
	Cell culture	CPT 2.5-4
N. foetida	Plantlet culture	MCPT 7
	Callus	MCPT 1
	Stem	MCPT 2.5
	Callus	CPT 9.5, MCPT traces
	Cell	CPT 1.1, MCPT 0.81
O. pumila	Hairy roots	CPT 1000
	Cell culture	None

Fulzele *et al* developed untransformed root culture of *N. foetida* from immature embryo on MS medium supplemented with different concentration of growth hormones. Basal medium containing NAA and BA achieved maximum number of elongated shoots. Maximum concentration of CPT (0.01% dw) and 9-MCPT (0.0016% dw) were obtained with MS medium supplemented with NAA (71.36µM) and BA (8.86µM) (31).

Fulzele *et al* studied the effect of hormones on camptothecin production by cell suspension culture of *N. foetida*. They found that cell biomass was higher in presence of medium supplemented with NAA (10.74µM) and BA (2.22µM) and attained 31.3g/l DW during 20 days of cultivation in shaking flasks. In presence of NAA maximum concentration of CPT (0.035mg/l) and 9-MCPT (0.026mg/l) were found (32).

Pharmacological studies

Biological screening have recognized that camptothecin and 9-methoxycamptothecin have promising anti-cancer activity. The molecular target of camptothecin is inhibiting the nuclear enzymes topoisomerase I DNA complex. Camptothecin inhibits Tat-mediated transactivation of HIV-1 LTR and this important result offered a potential target for therapy of HIV-1 infection. Camptothecin itself is not used clinically due to its cytotoxicity, but its derivatives are most effective for the treatment of cancer. Interest in camptothecin congeners was renewed when it was reported that 9-aminocamptothecin exhibits curative activity against human colon adenocarcinoma. Camptothecin and its derivatives inhibit the growth of human breast carcinoma cell in vitro and induce complete regression of breast tumors. This stimulated the development of water soluble analogues of camptothecin and two analogues, irinotecan (CPT-11) and topotecan, have been of major interest in the present decade and approved for the treatment of cancer (33). Successive petroleum ether, chloroform and methanol extract of *N. foetida* leaves and stem were tested for their antibacterial activity. The methanol fractions were found to be most effective against *Salmonella typhi*, *E. coli* and *Aeromonas hygrophila* (34).

CONCLUSION

N. foetida contains camptothecin, regarded as one of the most promising anticancer drugs of the twenty first century. Other anticancer principles of the drug are acetylcamptothecin, methoxy camptothecin, hydroxy camptothecin, scopoletin, ß-sitosterol, sitostery l-ß-D-glucoside, trigonelline and pumiloside. The molecular target of camptothecin is inhibiting the nuclear enzymes topoisomerase I DNA complex. Camptothecin inhibits Tat-mediated transactivation of HIV-1 LTR, a potential target for therapy of HIV-1 infection. It is one of the future drugs for cancer, AIDS, herpes, malaria and other protozoal diseases. Being a vulnerable species, so biotechnological approaches (plant tissue culture) will be the better option for enhancing secondary metabolite production, conservation of species by micropropagation and maintain genetic uniformity of species.

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