Optimization of culture conditions for callus and cell suspensions of *Nothapodytes nimmoniana*

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CERTIFICATE FROM SUPERVISOR

This confirms that the following tittle "**Optimization of culture conditions for callus and cell suspensions of *Nothapodytes nimmoniana*.**" sought after by **Rudrashish Nadir (151828)** in incomplete satisfaction for of level of B. Tech (Biotechnology) from JAYPEE UNIVERSITY OF INFORMATION TECH, Waknaghat has been done under msupervision. This following hasnt been submited to some different college or organization for the honor of any degree.

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DECLARATION BY THE SCHOLAR

I hereby declare that the work reported in the B-Tech thesis entitled" **Optimization of culture conditions for callus and cell suspensions of** ***Nothapodytes nimmoniana*** **"** submitted at JAYPEE INSTITUTE OF INFORMATION TECH, Waknaghat , is an authentic record of work carried out under the guidance of **Dr. Hemant Sood (Associate Professor)**. This work has not submitted elsewhere for any other degree or diploma.

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ABSTRACT

Nothapodytes nimmoniana is respected for significant optional camptothecin, secondary metabolite, a significant enemy of Cancer. The plant is regularly found in the Ghats(Western). Because of overexploitation and absence of composed development, the wild populaces of the tree have declined quick.

It is profoundly abused for acquiring the high market esteem hostile to malignant growth alkaloid, camptothecin has brought about making the species jeopardized.Tissue culture techniques offers different viable techniques for gathering the interest of camptothecin and fast proliferation for tree preservation.

Enhancement of callus cultures was completed on MS and TDZ and IBA(1.5:1) And cell suspensions were created on MS and TDZ and IBA(1.5:1) So in this investigation in vitro approachs were created for the Camptothecin generation .HPLC(High Performance Liquid Chromatography) was done for evaluating Camptothecin content in callus accounting to 0.82% and furthermore in various tissues of Nothapodytes nimmoniana.

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Figure 1: *Nothapodytes nimmoniana*

CHAPTER - 1

INTRODUCTION

1. Introduction

Nothapodytes nimmoniana Graham is an imperiled medium measured restorative tree.. This is appropriated in the evergreen backwoods of Western Ghats of India, North-East India, Sri Lanka, Myanmar and China [**1**]. The trees have a have a moderate development rate, and proliferation is for the most part accomplished by seeds. The plant grows from seedling to achieve development before beginning of blossom generation within 7-8 years [2]. Plant is a rich wellspring of camptothecin. Camptothecin is a promising enemy of malignant growth medication of the 21st Century [5], [6]. Camptothecin is as of now being utilized for treating and ovarian disease [7], [8]. Overall yearly offers of the analogs of camptothecin have come to $1,000 million which is around 1 ton of camptothecin crude material speaking to around 1,000–1,500 tons of wood chip [9][2]. In India, entire N. nimmoniana trees are sliced to create biomass for fare [2]. This has prompted decline in the number of inhabitants of this species in the Ghats(Western), India, and, truth be told, because of the very high weight, it has been seen as jeopardized [9]. Camptothecin has been accounted for to be disconnected at higher yield from N. nimmoniana with more noteworthy amount in stem androot of the tree than other common sources. Tissue culture strategies can aid in its preservation. In-Vitro mass proliferation of N.nimmoniana via circuitous organogenesis utilizing semisolid and fluid societies has been demonstrated to be a proficient strategy [11]. Cryopreservation is additionally utilized in momentary protection of the species [12]. This examination has a potential in expanding the reasonability capability of N.nimmoniana by enhancing the way of life conditions for the phone suspension culture and callusing through substantial embryogenesis as developing life aids in diminishing the rearing cycle of the plants.

Camptothecin is being exploited from In-Vitro plants in order to save these valuable plants from extinction.. All the factor have been pondered upon for the project has been carried out with the bellow mentioned objectives:

OBJECTIVES

N. nimmoniana is being overexploited due to the auxiliary metabolite generation of hostile to malignancy compound camptothecin from the tree. So as to handle every one of these issues, we need to create exchange systems keeping in view the huge scale generation of the plantlets utilizing tissue culture methods and furthermore for the creation of Camptothecin with the goal that it could be additionally used for the counter carcinogenic medication definitions by various leading pharmaceuticals firms.

The motive of the present study is:

1. Optimization of the culture for callus induction and multiplication and subculturing.
2. Optimization of culture condtions for cell suspension of *Nothapodytes nimmoniana.*

CHAPTER -2

LITERATURE REVIEW

2.1 PLANT DESCRIPTIONS

Botanical name	**:**	*Nothapodytes nimmoniana* (Graham).
Kingdom	**:**	Plantae
Family	**:**	Icacinacea
Species	**:**	*Nothapodytes nimmoniana*
Common Name	**:**	Ghanera, Pinaari, Narkya
Synonyms	**:**	*Mappia foetida, Nothapodytes Foetida*
Habitat	**:**	Peninsular India
Flowering	**:**	August

Figure 2.1: ***Nothapodytes nimmoniana***

N. nimmoniana is quite small of little tree, 3-8 m tall. It has an earthy, branchs which are about 5mm stout. It has unmistakable leaves scars. Leaves are then again arranged,clustered, winding and at finishes. The leaf's base is frequently unequal. Plant has long leafy stalks 3-6 cm.

Blossoms are promiscuous, smooth yellow in shading. Plant is foul smelling which makes the plant effectively unmistakable. Leafy foods of the plant are purplish red, and elliptical with smooth surface and a solitary precursor seed.

2.2 Studies of In-Vitro Origin

2.2.1 In- vitro multiplication

Th plant being as of now on skirt of annihilation requires tissue refined procedures. As indicated by in-vitro considers in-vitro duplication of this specie utilizing MS media enhanced with 0.91µM (TDZ), 0.8% Agar, sucrose 3% and a 8.5pH in Standard conditions.

Callus Induction was seen in MS media post one month. Fundamental inception of In-vitro plants has been perfect on MS mediu with IBA(1mg/l). It was additionally seen that diminished temperature and light goodly affected In-Vitro plants. In-vitro plants developed well even following a half year of hatching under comparative processes [16].

2.2. Enhanced In -Vitro multiplication using liquid and semi- solid cultures

Extrinsic entities were instigated from the Callus gotten from nodes explants. The semisolid and Liquid MS medium enhanced with 1.5, 2.5, 550 µM 6-BAP & kinetin &(2-iP).

Fluid media with HIGH TDZ per media was discovered better for callus recovery in contrast with semisolid medium with less callus in this arrangement..High calus development giving society is likewise a decent subculturing media[17].

2.3Metabolite of Interest

Camptothecin, is a monoterpene alkaloid, and viewed as a standout amongst the most encouraging anti-cancer medications of the 21st century. It restrains the DNA protein Topioisomerase I

.Camptothecin has been demonstrated to be great enemy of destructive medication in starter clinical preliminaries yet additionally low dissolvability and unfavorable medication response. Manufactured scientific experts have created different subordinates to build the advantages of the compound, with great outcomes. Two CPT analogs have been endorsed and are utilized in malignancy treatment. Irinotecan and Topotecan are two generally utilized engineered subordinates of CPT [19].

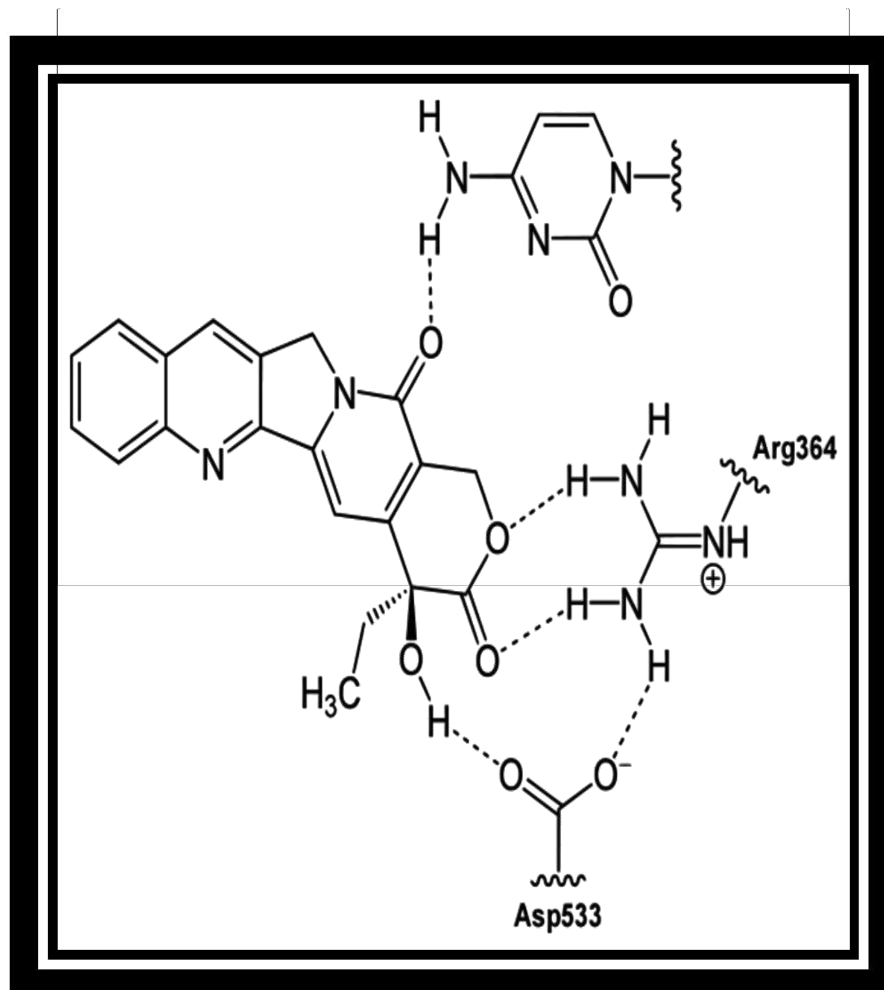

Fig.2: Camptothecin binding with DNA and Toipoisomerase I

From different plant sources, the most noteworthy yields of Camptothecin is accounted for by ideals of Nothapodytes nimmoniana . Unpredictable deforestation for camptothecin has brought t about the plant ending up exceptionally imperiled.

General examples of aggregation of camptothecin is in the tress of Ghats , crosswise over plant parts as individual, size of the plant and sexual orientation have been considered [5].The thought about uncovered that there lie no distinctions in CPT content between the genders. More CPT was seen in more youthful leaves for example ten times higher substance than in more established leaves [6]. Abnormal state of CPT has been accounted for from bark [20].

2.2. Culture Optimisation vehicle for CPT generation in Suspension Cultures of N. nimmoniana

The experimentation was completed so as to build the camptothecin generation in THE PLANT. MS media (0.5% mM phosphate), nitrogen source encouraging proportion of 50/100 mM ammonium nitrate proportion and sucrose amounting to 3% bolstering with 0.94 µM of kinetin. Sucrosee was built up betterr than various sugars tried for eproducing the measure of CPT. The specific medium gave 2-2.5 occasions higher camptothecin.

2.3 Generation of Camptothecin by bushy root culture of Ophiorrhiza pumila

Bushy culture of root was changed utilizing *Agrobacterium rhizogenes* . CPT was created as a primary alkaloid up to 0.1% per load of oells. Not just the shaggy cells contained camptothecin, even the medium collected generous measures of the compound. Camptothecin sum was expanded in mediA by the nearness of the polystyrene pitch [22].

2.4 HPLC evaluation of CPT from *Nothapodytes foetida* gathered amid various timespan.

Distinctive plant parts were gathered at various timespan and extraction was done to extricate Metabolite. which was evaluated utilizing HPLC. The consequences of the examination uncovered that the most astounding centralization of CPT estimated in roots (2.61%) collected amid February pursued by organic products (Jan, 1.24 %), stem (Jan, 0.82%) and leaves (Feb**,**

0.71%). Fruits displayed 3-times greater conc of CPT when immature in the month of Jan. Roots had 3-times greater conc of CPT than other parts.

CHAPTER-3

MATERIALS AND METHODS

ANNEXURES

Requisites for Murashige and Skoog basal medium (MS MEDIA)

STOCKS	CHEMICALS	BASE STRENGTH(mg/l)	STOCKS(g/l)	FINAL VOLUMES
A - 10X	KNO_3	1800	18	
	$MgSO_4.7H_20$	360	3.6	100 ml/l
	KH_2PO_4	160	1.60	
B - 20X	NH_4NO_3	1600	33	150 ml/l
C - 100X	$CaCl_2.2H_2O$	440	44	10 ml/l
D-100X	Na $_2$EDTA	37.26	3.72	10 ml/l
	$FeSO_4.7H_2O$	22.85	2.28	
E-100X	KI	0.82	0.082	100 ml/l
F-100X	H_3BO_3	6.1	0.61	
	$CoCl_2.6H_2O$	0.025	0.0025	
	$ZnSO_4.7H_2O$	8.5	0.85	10 ml/l
	$CuSO_4.5H_2O$	0.025	0.0025	
	$MnSO_4.4H_2O$	22.4	2.24	
	$Na_2MbO_4.2H_2O$	0.25	0.025	
G-100X	m-INOSITOL	100	10	10 ml/l
	GLYCINE	2	0.2	
H-100X	PYRIDOXINE-HCL	0.5	0.05	
	NICOTINE ACID	0.5	0.05	10 ml/l
	THIAMINE	0.1	0.01	

3.1: IN-VITRO PROPAGATION OF CALLUS

3.1.a SELECTING PLANT MATERIAL

N.nimmoniana plantlet were procured from Jaypee University of Information technology. The plant was planted and harvested experimentally for recent years at the labs of Department of Biotech&Bioinfo, JUIT ,Waknaghat,

3.1.b: CULTURE PARAMETERS AND MEDIA PREPERATION

MS medium enhanced with variable compositions of development hormones, for example, TDz, IBA with 3% sucrose were utilized. The ideal pH of medium was 5.8 utilizing (HCl) and (NaOH). Agar 0.8% was the gelling operator. The media was autoclaved at 15PSI &121°C. The samples were brooded for 16 hours cycle at "25±3°C" in PTC labs.

3.1.c STERILIZATION OF EXPLANT SURFACE

Explants were cleaned with autoclaved water to get rid of dust and debris. It was then surface disinfected with 0.10% Mercuric Chloride and 0.50% Bavistin pursued by rehashed washing with refined water in LAF. Bavistin and Mercuric Chloride act as "Antibacterial" and "Antifungal" coumpound separately. Explants cut into little pieces utilizing autoclaved surgical tool. Entire strategy was completed under sanitized conditions in "laminar air flow".

3.1.d INDUCING CALLUS AND DIRECTED REGENERATION

Minute cuts are made to the explants surface. They were then cultured on "MS media" with variable growth hormones concentration as mentioned in "Table 3.1". The observational duration for the callus in growth chamber was 52-56 days.

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The cultures had an 8 hours night/16 hours day brooding cycle at “25±3°C” in the labs of Department of Biotech&Bioinfo ,JUIT. The callus actuated was then Sub-Cultured using MS media g of TDZ+ IBA (1mg/l)

“MS MEDIA”	“IBA(mg/l)”	“KN(mg/l)”	“BA(mg/l)”	“NAA(mg)”	“TDZ(mg/l)”	“2,4D(mg/l)’

Table 3-a: Different Media compositions for Callus Induction in N.nimmonianna.

“MS MEDIA”	“IBA(mg/l)”	“KN(mg/l)”	“BA(mg/l)”	“NAA(mg)”	“TDZ(mg/l)”	“2,4D(mg/l)’
MS A	-	-	-	-	1.5	-
MS B	1	-	-	-	1.5	-
MS C	1.5	-	-	-	2.5	-
MS D	-	-	0.5	-	1.5	-
MS E	-	-	-	-	2	1

3.1.5 CELL SUSPENSION CULTURE

MS medium with variable hormone fixation and without agar was utilized for cell suspension societies for further development from the callus and foundation of cell suspension societies. Best development was watched cups where friable callus was taken to start suspension cultures."Table 3.2" demonstrates the different medias utilized for cell suspension refined. Best outcomes were gotten in MS+ TDZ(1.5mg/l)+IBA).

“MS MEDIA”	“IBA(mg/l)”	“KN(mg/l)”	“BA(mg/l)”	“NAA(mg)”	“TDZ(mg/l)”	“2,4D(mg/l)’
MS A	-	-	-	-	1.5	-
MS B	1	-	-	-	1.5	-
MS C	1.5	-	-	-	2.5	-
MS D	-	-	0.5	-	1.5	-
MS E	-	-	-	-	2	1

Table 3-b: Different Media compositions for Cell suspension culture in N.nimmonianna.

Fig.3-a Cell Suspension culture of *Nothapodytes nimmoniana*

3.1.6 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF THE CALLUS.

3.1.6.1- Preparation of the Sample-

- Collected the callus and washed it thoroughly.
- Liquid nitrogen was used to crush the callus to make it into a finer.
- In about 10ml of Methanol, 1grm of callus powder
- .The sample was then subjected to sonication.
- Centrifugation was done at 10,000RPM for 10 min
- The Supernatant was collected whereas the pellet was discarded.

- In sterlised conditions, syringe filters(0.22mm) were used to filter the sample.
- Rest of the sample was stored under refrigeration.

3.1.6.2- Procedure for High Performance Liquid Chromatography-

- Extract amounting to 20ml was experimented upon using HPLC column..
- Peak of the graph was observed at a wavelength of 360nm.
- Linear Regression Equation was obtained by virtue of Calibration curves and was used for Camptothecin Quantification in samples.

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CHAPTER-4

RESULTS

4.1: CALLUS INDUCING AND SUBCULTURING

Supreme outcomes of callus enlistment was found in MS media enhanced with development growth factor with proportion of TdZ (1.5mg/L) and IBA (1mg/L) as appeared in "Table.4.1.1". 90% callus was seen inside 51-56 days referenced previously

Table 4.1.a: Implications of various growth factors with respect to callus formation in *N. nimmoniana*

MEDIA	DAYS REQUIRED	CALLUS-INDUCTION(%)
"TDZ++MS+IBA" (2.5:1.5) mg/l	30days	30-40%
"TDZ+MS (1.5) mg/l"	30days	50-65%
"TDZ+MS+IBA (1.5:1) mg/l"	30days	65-90%

4.1.2 CELL SUSPENSION CULTURE

Best results for the cell suspension culture viability was observed in Murashige and Scoog augmented with growth factors "TDZ (1.5mg/l)" and "IBA (1mg/l)" but this media was devoid of agar as it's a liquid media.

4.1.3 HPLC ANANLYSIS-

HPLC analysis shows that the callus contains 0.82% camptothecin content other types of plant parts may have variable amounts.

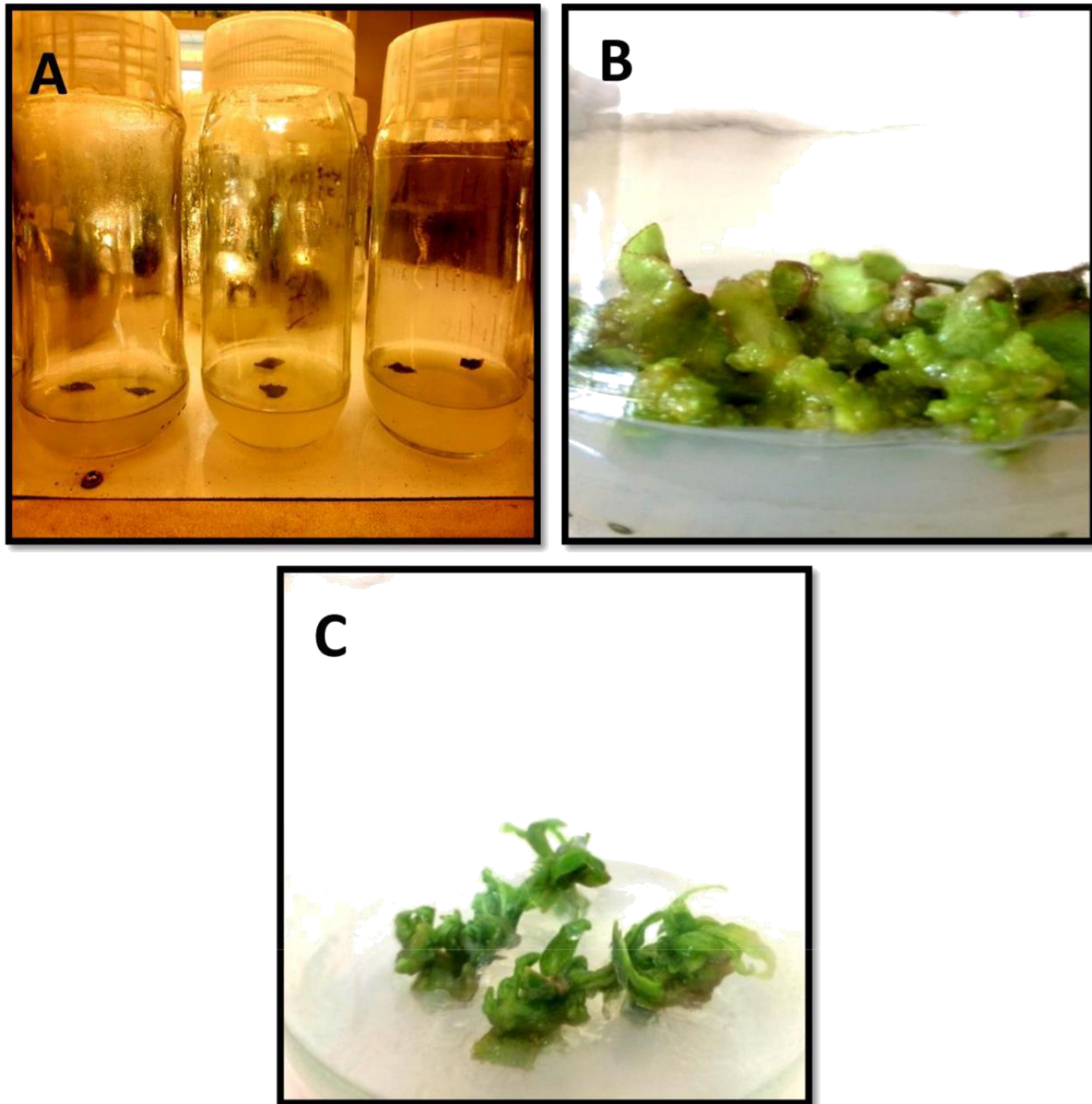

Figure 4.1-a: Callusing in *Nothapodytes nimmoniana*

(AExplants cutured on Auxin rich media,
(B) Calluses formed after few weeks of incubation.
(C) Plantlet **of *Nothapodyte nimmoniana***

CHAPTER 5

DISCUSSION

A higher Auxin content is basically responsible for friable callusing in the explants so the study was planned on same guidelines. Callus in *Nothapodytes* are ellucidated in this project where optimum outcomes are displayed by virtue of MS media augmented with **IBA(1 mg/L**) and **TDZ (1.5 mg/l)[4].**

Cell Suspension Culturing techniques have been modeled successfully in *Nothapodytes nimmoniana* using growth factors **IBA(1 mg/L)** and **TDZ (1.5 mg/l**) with proper shaking,where as no detail reports are available in this species regarding this.

HPLC estimation shows that the callus has a concentration of about **0.82%** of Camptothecin. Camptothecin production and estimatation however has a different approach with respect to different kind of research articles.Heavy research is underway and we might find some ground breaking potential technology soon.[5]

CHAPTER 6

CONCLUSION

In an assortment of medicinal plants, decreased stone germnation, species variety and other hereditary elements have played a crucial role in diminished development of the plants leading to the getting jeopardized or wiped out. Can behighly likewise be contributed by negative climatic conditions, regular lethargy, heterozygosis and seed size, and nearness of decreased endosperm and so forth.

The general goal of the present investigation was to build up an in vitro framework for streamlining society conditions for cell suspension of Nothapodytes nimmoniana so commercialization of restorative mixes can be completed with more experimentation and verification.

The tree having restorative significance has likewise picked up a great deal of consideration from mammoth pharmaceuticals attributable to auxiliary metabolite CPT that has hostility to dangerous characteristics. Anyway other pertinent research is in progress toward improvement of in vitro frameworks for the metabolite generation.

CHAPTER 7

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