Production of somatic seeds of *Nothapodytes nimmoniana* and testing the pharmacological activity of the plant extract

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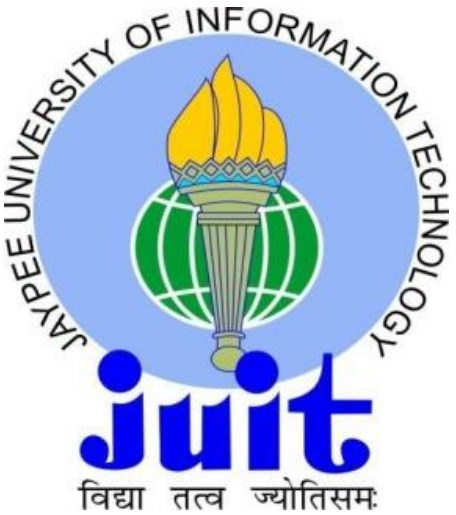

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DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY,

WAKNAGHAT

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CERTIFICATE FROM SUPERVISOR

This is to certify that the work **"Production of somatic seeds of *Nothapodytes nimmoniana* and testing the pharmacological activity of the plant extract"** pursued by Ankita Rajta (133804) in partial fulfilment for the award of degree of B. Tech in Biotechnology from Jaypee University of Information and Technology, Waknaghat has been carried out under my supervision. This work has not been submitted partially or wholly to any other university or institute for the award of any degree or appreciation.

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DATE–

DECLARATION BY THE SCHOLAR

I hereby declare that the work reported in the B-Tech thesis entitled **"Production of somatic seeds of *Nothapodytes nimmoniana* and testing the pharmacological activity of the plant extract"** submitted at Jaypee University of Information Technology, Waknaghat India, is an authentic record of my work carried out under the supervision of **Dr. Hemant Sood (Assistant Professor)**. I have not submitted this work elsewhere for any other degree or diploma.

(Signature)

Name- Ankita Rajta

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I cannot forget to recall with my heartiest feeling, the never ending heartfelt stream of blessings and cooperation of my parents to support me with everything to complete B. Tech in the Dept. of Biotechnology/Bioinformatics JUIT, Solan.

Signature of student

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SUMMARY

The present study was conducted to optimize the culture conditions for the production and germination of artificial seeds through somatic embryogenesis in *Nothapodytes nimmoniana*. The tree belongs to family *Icacinaceae.* The plant is known for important secondary metabolite camptothecin, an important anti-cancer alkaloid. The tree is commonly found in the Western Ghats of Maharashtra (India). Due to overexploitation and lack of organized cultivation, the wild populations of the tree have declined fast. In addition, there are a number of other constraints mentioned in the literature for the propagation and conservation of the tree through seed propagation.

Low seed germination rate and the immense exploitation of the tree for obtaining the high market value anti-cancer alkaloid, camptothecin has resulted in the endangered status of the species. Plant tissue culture offers various effective measures for meeting the demand of camptothecin and rapid clonal propagation for tree conservation. Hence, artificial seed technology can be used for the large-scale production of plantlets which could later be used for the production of camptothecin.

We have carried out a comparative analysis of plant extract prepared from tissue cultured plantlets and field grown shoot and roots on breast cancer cell lines to predict the efficacy of its anticancerous effects. Hydroalcoholic extraction of the samples and their quantification through HPLC has been done which result in (0.03%) camptothecin in in-vitro plantlets, field grown leaves (0.87%) and field grown stem (0.17%). In cell line study, we achieved high efficacy of field grown extract on cancer cell death as compared to in-vitro grown plantlets.

Difference in camptothecin content quantified through HPLC can be attributed to stress conditions that are faced by field grown plants which contributes to secondary metabolite production as compared to the sustained environment provided to in-vitro plants, difference in age group of fields grown plants (5 – 6 years) and in-vitro plantlets (2 – 3 months) old, origin of explants used for culturing in-vitro plants and use of different growth hormones for culturing the in-vitro plants etc. Cytotoxic studies have been performed on HEK 293(Human Embryonic Kidney) to check the effect of plant extract on normal cells. IC_{50} value obtained for all the samples was quiet high which indicates that camptothecin is safe for normal cells similarly anticancerous effects of *Nothapodytes nimmoniana* crude extract have been assessed on MCF-7 and MDA-MB

231 where MDA-MB have shown more sensitivity toward the extract as compared to MCF-7 cells.

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Name – Ankita Rajta	Name – Dr.Hemant Sood
Date -	Date –

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LIST OF ABBREVIATIONS

Abbreviations	Full forms
KN	Kinetin
IBA	Indole-3-butyric acid
GA_3	Gibberellic acid
BAP	6- Benzylaminopurine
NAA	Napthalene acetic acid
2,4-D	2,4 dichlorphenoxyacetic acid
IAA	3- indoleacetic acid
MS	Murashige and skoog
CPT	Camptothecin
HEK	Human embryonic kidney
MDAMB-231	Anderson metastatic breast cancer
MCF-7	Michigan Cancer Foundation-7
DMEM	Dulbecco's Modified Eagles's medium
AO	Acridine orange
L-15	Leibovitz
EtBr	Ethidium bromide

Figure 1: *Nothapodytes nimmoniana*

CHAPTER-1

INTRODUCTION

1. Introduction

Nothapodytes nimmoniana Graham (common name – "Stinking tree, Ghanera") is an endangered medium sized medicinal tree belonging to the family Icacinaceae. It is distributed in the evergreen forest of Western Ghats of India, North-East India, Sri Lanka, Myanmar and China [**1**]. The tree is said to have a slow growth rate, and propagation is mostly achieved by seeds. The tree requires 7–8 years from seedling to reach maturity before start of flower production [**2**]. The seeds are said to be recalcitrant due to their high sensitivity to desiccation, freezing.Seeds have a short shelf life **[3], [4]**). This tree is a rich source of camptothecin, isoquinoline alkaloid. Camptothecin is one of the most promising anti-cancer drug of the twenty-first century **[5], [6]**. CPT is currently being used for treating colorectal and ovarian cancer **[7], [8]**. Worldwide annual sales of the analogs of camptothecin have reached $1,000 million which is about 1 ton of camptothecin raw material representing about 1,000–1,500 tons of *N. nimmoniana* wood chip **[9][2]**. In Western Ghats of India, whole *N. nimmoniana* trees are cut to generate biomass for extraction and export **[2]**. Annual *N. nimmoniana* biomass demand had reached 500–700 metric tons in 2001, wood chips trade volume reached 1,600 tons in 2002. In the absence of synthetic sources, the global demand for this alkaloid is met by natural populations of *N. nimmoniana*. This has led to decrease in the population of this species in the Western Ghats, India, and**,** in fact, due to the extremely high pressure, the species has been declared as endangered **[9]**. Camptothecin has been reported to be isolated at higher yield from *N. nimmoniana* with greater quantity in stem androot of the tree than other natural sources. The recalcitrant seeds produced by the plants may be considered an alternative source of camptothecin when they lose viability **[10]**. Tissue culture techniques can help in its conservation. An efficient method has been developed for the in-vitro mass propagation of *N.nimmoniana* through indirect organogenesis using semisolid and liquid cultures **[11]**. Cryopreservation of embryonic axes is also employed in short term preservation of the species **[12]**. Effects of elicitor concentration and time course changes of camptothecin production after elicitor addition has also studied **[13]**. The tissue cultured plants have also been evaluated for their chemical potency using high performance liquid chromatography (HPLC) for analysis of CPT content **[14]**. All the above techniques hold promise in meeting demand for camptothecin and conservation of *N. nimmoniana* natural populations. This study possesses a robust potential in increasing the germination potential of *N.nimmoniana* by optimizing the culture conditions for the artificial seed generation and germination through somatic embryogenesis as embryo culture helps in decreasing the breeding cycle of the plants.

Not only this, artificial seed generation will help in germplasm conservation, large scale propagation of endangered species, and can be used as an alternative for camptothecin extraction **[15]**.

Anticancer and cytotoxic effects of the alkaloid i.e. camptothecin has been studied where human breast cancer cell lines such as MDA-MB-231 and MCF-7 and normal human kidney cell line (HEK-291) have been have been used for the analysis. We explored the effects of *Nothapodytes nimmoniana* plant extract from field grown shoot and root and tissue cultured plants so as to know how much effective the tissue cultured plant extract is in killing the cancer cells. In future camptothecin can be extracted from in-vitro grown plants so that the natural population of the tree species can be saved from further destruction. All the above factors have been kept in mind and the present study have been carried out with the following objectives:

OBJECTIVES

Nothapodytes nimmoniana is being overexploited because of the secondary metabolite production of anti-cancer compound camptothecin by the tree. The seed dormancy rate being very high in the plant which can be attributed to hard seed coat, physiological conditions like higher phenolic compounds, absisic acid etc leads to another major problem in the mass propagation of the tree. In order to tackle all these problems, we have to develop alternate strategies for the large scale production of the plantlets and also for the production of anti-cancer compound so that it could be further utilized for the anti-cancerous drug formulations by the giant pharmaceuticals.

So, the aim of the present study deals with:

1. Optimization of the culture conditions for the production and germination of artificial seeds of *Nothapodytes nimmoniana*.
2. Effect of the plant herbal extracts from in-vitro grown and field grown plants on human breast cancer cell lines.

CHAPTER-2

REVIEW OF LITERATURE

2.1 Description of plant

Botanical name : *Nothapodytes nimmoniana* (Graham) Mabb**.**

Kingdom : Plantae

Family : Icacinacea

Species : *Nothapodytes nimmoniana*

Common Name : Ghanera, Pinaari, Narkya

Synonyms : *Mappia foetida, Nothapodytes Foetida*

Habitat : Found in peninsular India

Flowering : August

Figure 2.1: ***Nothapodytes nimmoniana***

Nothapodytes nimmoniana is a small tree, 3-8 m tall. It has a brownish, wrinkled branch which is about 5mm thick. It has prominent leaf scars. Leaves are alternately arranged, spiral and clustered at twig ends. Leaf base is often unequal. Plant has long leaf stalks 3-6 cm.

Flowers are bisexual, creamy yellow in colour. Plant has a foul smell which makes the plant easily recognisable. Fruits and seeds of *Nothapodytes nimmoniana* are drupe, purplish red, and smooth, oblong with a single seed. Multiple shoots have been regenerated and development of somatic embryos has been observed on the same medium.

2.2 In vitro studies

2.2.1 In- vitro multiplication

As the plant is endangered it is desirable to have various conservation techniques. According to in-vitro studies a well optimized protocol has been developed for in-vitro multiplication of this species using MS media supplemented with 0.91 μM thidiazuron (TDZ), 3% sucrose, 0.8% agar and pH 5.8 in Standard Culture Conditions. Various explants have been tried but the best response has been shown by isolated seed embryos.

Shoot elongation (2-19 mm) was observed on MS basal medium after duration of one month. Root initiation of in-vitro plants has been ideal on MS medium with 1mg/L IBA. It was also observed that reduced temp and light had a good effect on in-vitro plants. In- vitro plants grew well even after six months of incubation under such conditions **[16]**.

2.2.2 Enhanced in vitro multiplication using semisolid and liquid cultures

Adventitious shoots have been induced from callus derived from nodal explants. Semisolid and liquid MS medium supplemented with 1.0, 2.0, 5.0 and 10.0 μM 6-benzylaminopurine or kinetin or 2-isopentenyl adenine (2-iP).

Liquid medium with (165.9) shoots per explant was found better for adventitious shoot regeneration in comparison to semisolid medium with (41.9) shoots per explants. The highest number of adventitious shoots was regenerated on medium supplemented with 2.0 μM BAP. Shoots were rooted on MS semisolid medium of one-fourth strength containing IBA (2.4 μM) and IAA (5.7 μM) **[17].**

2.2.3 Enhanced in-vitro seedling recovery

One of the most difficult challenges in the establishment of the aseptic in vitro culture in case of *Nothapodytes nimmoniana* the seed germination. Pre-germination treatment was given by soaking in water. Germination capacity was enhanced by seed soaking in water or GA3 for a period of 24 hours. Better response was obtained when seeds were soaked in GA3 (2.6 μM) and germinated on half strength MS medium supplemented with the GA3. Outer, inner seed coat was inoculated on medium amended with or without GA3. Seedling recovery time was reduced to 3-4 weeks. Vigorous growth was shown by seedlings obtained on medium

fortified with GA3 with complete plantlet development than on PGR-free medium. This strategy can be employed in reducing seedling recovery time to within 3-4 weeks compared to 7-10 weeks required for germinating the seeds in vitro **[18]**.

2.3Secondary metabolite

Camptothecin, a monoterpene indole alkaloid, is regarded as one of the most promising anticancer drugs of the twenty-first century. It inhibits the DNA enzyme topoisomerase I .CPT has shown good anti cancerous effects in preliminary clinical trials but also low solubility and adverse drug reaction. Synthetic and medicinal chemists have developed various derivatives to increase the benefits of the chemical, with good results. Two CPT analogues have been approved and are used in cancer therapy. Topotecan and irinotecan are two most widely used synthetic derivatives of camptothecin **[19]**.

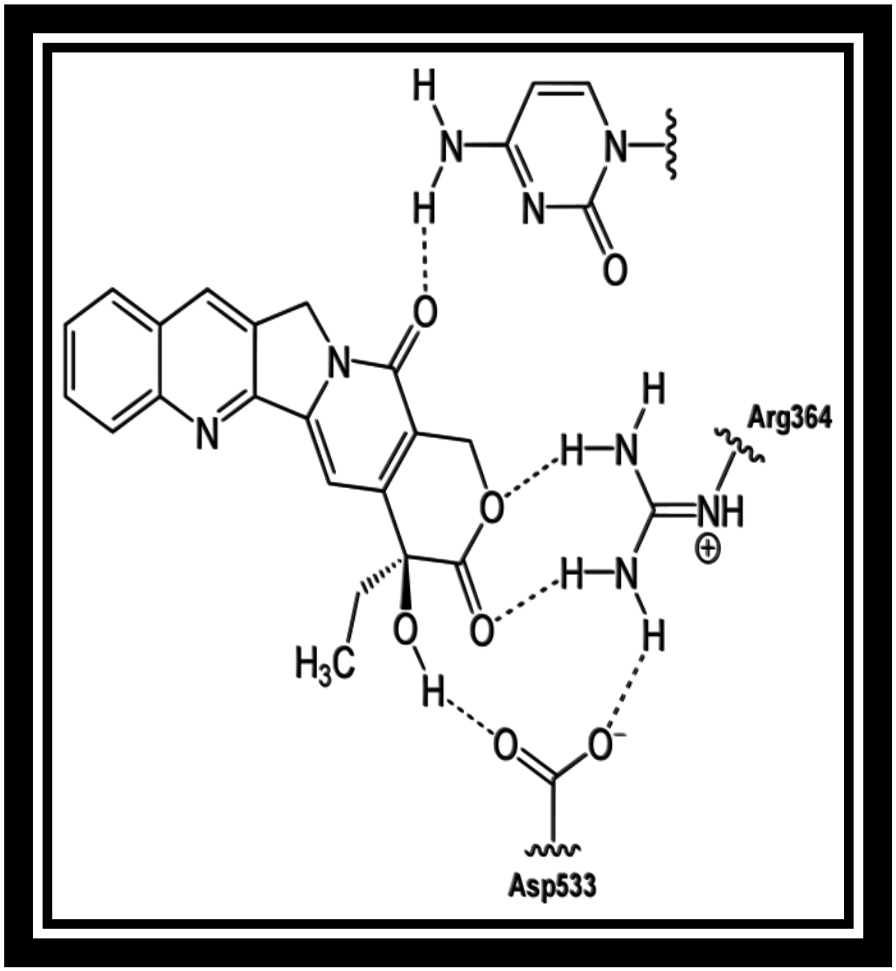


Fig.2.2: Binding of Camptothecin to DNA and Topoisomerase I

Among the various plant sources, the highest yields of the alkaloid are reported from *Nothapodytes nimmoniana* (Icacinaceae). Indiscriminate felling of the plant for camptothecin has resulted in has resulted in endangered status of the plant.

General patterns of accumulation of camptothecin in the tress found in western ghats , across individual plant parts, plant size and sex of plants have been studied **[5]**.The study revealed that there were no differences in CPT content between the sexes. More CPT content was found in younger leaves i.e. tenfold higher content than in older leaves **[6]**. High level of CPT has been reported from bark. Good amounts (0.10%) of the alkaloid are also reported from the seeds **[20]**.

2.3.1 Optimization of culture medium for camptothecin production in cell suspension cultures of *Nothapodytes nimmoniana*

This study was carried out with a view to increase the camptothecin production in *Nothapodytes nimmoniana*. MS medium with 0.5%mM phosphate, nitrogen source feeding ratio of 50/10 mM ammonium to nitrate ratio and 3% sucrose feeding with naphthalene acetic acid and 0.93 μM kinetin. Sucrose was found better than all the other sugars tested for enhancing the amount of camptothecin. The selective medium gave 1.7- 2.3-fold higher intracellular and extracellular camptothecin content over the control culture 29.2- 8.2 μg/g **[21]**.

2.3.2 Camptothecin production by hairy root culture of *Ophiorrhiza pumila*

Hairy root culture of *Ophiorrhiza pumila* was transformed by *Agrobacterium rhizogenes* strain 15834. Camptothecin was produced as a main alkaloid up to 0.1% per dry weight of the cells. Not only the hairy root cells contained camptothecin, even the culture medium also accumulated substantial amounts. Camptothecin amount was increased in the medium by the presence of a polystyrene resin **[22]**.

2.4 HPLC quantification of camptothecin from *Nothapodytes foetida* collected during different time period.

Different plant parts were collected at different time period and methanolic extraction was done to extract camptothecin which was quantified using HPLC. The results of the study revealed that maximum concentration of CPT was found in roots (2.62%)collected during February followed by fruits (January, 1.22%), stem (January, 0.81%) and leaves (February,

0.70%). Fruits showed 2-fold higher concentration of camptothecin when not fully matured in the month of January. Roots had 3-fold higher concentration of CPT than the leaves and stem **[23]**.

2.5 Microwave assisted process intensification extraction of camptothecin

The study revealed the microwave-assisted process intensification for the extraction of camptothecin (CPT) from *Nothapodytes nimmoniana* plant. Various process factors such as stirring, solid to liquid ratio, microwave power, irradiation time and particle size effect on extraction have been studied. The results indicated that stirring, microwave power and the temperature had a significant effect on extraction rate and yields of camptothecin. Extraction yield of 0.41% w/w of CPT have been achieved. Efficiency of about 97% in just 2 min as against 6 h by classical string extraction method has been achieved **[24]**.

2.6Anti-cancerous studies of camptothecin

It has been found we found that nanomolar concentrations of camptothecin (CPT), arrests or delay cell cycle progression during the S and G_2 phases in people with p53mutatations in case of human colon carcinoma HT29 cells **[25]**.

CPT 11 effect as anticancer drug has been studied.16 patients with refractory or relapsed SCLA were entered non-randomised, single institution phase II trial. All 16 patients were treated with cisplatin – based combination chemotherapy.Chemotherapy was carried out for median time of 7.3 months. CPT 11was given at a dose of 100mg/m2 body surface as a ninety minutes' intravenous infusion every week. Seven patients (47%; 95% confidence limits for an overall response rate, 21.4% to 71.9%) responded to CPT-11 with a median duration of response of 58 days. The major toxicities were myelosuppression, diarrhoea, and pulmonary toxicity **[26]**.

Effect of ethanolic extract of *Nothapodytes nimmoniana* barks against Dalton's Ascitic Lymphoma (DAL) in Swiss mice. DAL cells were injected intraperitoneally 1X106cell to the mice. Two days after cell injection animals were treated with ethanolic extract with a dose of 200 mg/kg for 14 days. On day 11, cancer cell number, packed cell volume, decrease in tumour weight of the mice, increase in life span and haematological parameters were evaluated and compared with the same parameters in control. A significant increase in the life

span, tumour weight and a decrease in the cancer cell number were noted in the tumour-induced mice after treatment with the extract. These observations suggested the protective effect of ethanolic extract of heartwood and barks against Dalton's Ascitic Lymphoma (DAL). All the above studies indicated the importance of camptothecin in treating cancer and the emerging needs to find proper alternative strategies for the production of camptothecin as well as conservation of the tree species **[27]**.

CHAPTER-3

MATERIALS AND METHODS

3.1: IN-VITRO PROPAGATION

3.1.1 SELECTION OF PLANT MATERIAL

Nothapodytes nimmoniana plantlets were procured from GKVK, University of Agricultural Sciences Bangalore. The plant was planted and grown in the polyhouse for 5-6 years at the experimental area of the Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology Waknaghat, India.

3.1.2 MEDIA PREPARATION AND CULTURE CONDITIONS

MS medium supplemented with different concentrations and combinations of growth hormones such as TDZ (1.5) mg/L, IBA (1) mg/L and TDZ (1.5) mg/l with sucrose 3% (w/v) have been used. The optimum pH for the medium was adjusted to 5.7 using 0.1N Hydrochloric acid (HCl) and 0.1 N sodium hydroxide (NaOH). Agar-Agar 0.8% (w/v) was added as a gelling agent. The medium was autoclaved at 121°C. The axenic cultures were incubated at 16 hrs light cycle at 25±2°C in plant tissue culture chamber of the department.

3.1.3 SURFACE STERILIZATION OF EXPLANTS

Leaves chosen as explants were washed with distilled water to remove dirt and debris. Explant was surface sterilized with 0.5% bavistin and 0.1% mercuric chloride followed by 4-5 washings with autoclaved distilled water in laminar air flow. Both bavistin and mercuric chloride are known to have antibacterial and antifungal activity respectively. Explants were then cut into small segments using autoclaved blades. Whole procedure was carried out under sterilized conditions in laminar air flow.

3.1.4 CALLUS INDUCTION AND DIRECT REGENERATION

Small incisions were given to the surface sterilized explants. These explants were cultured on MS media comprising of different growth hormone concentrations as stated below in “Table 3.1”. The cultures were kept in the growth chambers for 50-55 days. The callus mass was obtained which was sub-cultured on shooting MS media comprising of different growth hormone concentrations as stated below in “Table 3.2”.

The cultures were incubated at 16hrs light/ 8hrs dark cycle at 25±2°C in growth chamber of the Biotechnology Department of Jaypee University of Information Technology. The callus induced was further subcultured on MS medium containing IBA (1mg/L) +KN (2mg/L) for shoot regeneration.

Table 3.1: MS media supplemented with different concentration of growth hormones for callus induction in *Nothapodytes nimmoniana*

MS MEDIA	IBA(mg/L)	KN(mg/L)	BA(mg/L)	NAA(mg)	TDZ(mg/L)	2,4D(mg/L)
MS1	-	-	-	-	1.5	-
MS2	1	-	-	-	1.5	-
MS3	1.5	-	-	-	2.5	-

Table 3.2: MS media supplemented with different concentrations of growth hormones for shoot regeneration in *Nothapodytes nimmoniana*

MS MEDIA	BAP(mg/L)	NAA(mg/L)	TDZ(mg/L)	IBA(mg/L)	KN(mg/L)
MS 1	2	-	0.05	-	1
MS 2	-	-	-	1	2

3.2 ARTIFICIAL SEED PRODUCTION AND GERMINATION

3.2.1 ENCAPSULATION OF MATURE EMBRYOS

The embryos in the callus obtained after sub culturing was chosen for somatic seed generation. 3% sodium alginate solution was prepared in lukewarm which was later autoclaved. Similarly, Calcium chloride solution was prepared by dissolving 1.10gm of calcium chloride in 100ml of chilled autoclaved water in laminar air flow. Developed somatic embryo obtained by culturing were selected and put into the sodium alginate solution under sterile conditions and then transferred to the chilled calcium chloride solution causing formation of beads. Calcium chloride solution containing the beads was left undisturbed for 30 minutes. The whole process was carried out in laminar air flow.

3.2.2 SOMATIC SEED GERMINATION

MS media supplemented with growth hormones in the ratio IBA+KN+GA3 (1:3:2) and IBA + KN + GA3 + 0.5% activated charcoal as indicated in "Table 3.3" was used as the germination medium for the encapsulated beads formed. The cultures were kept at 25±1C temperature, 16 hrs light to initiate the germination from the somatic beads formed. Data was recorded for the seed germination.

Table 3.3: MS media supplemented with different concentrations of growth hormones for seed germination in *Nothapodytes nimmoniana*

MS MEDIA	IBA(mg/L)	KN(mg/L)	GA3(mg/L)	Activated charcoal
MS 1	1	3	2	-
MS 2	1	3	2	0.5%

3.3 EXTRACTION OF CAMPTOTHECIN BY SOXHLET METHOD

3.3.1 COLLECTION OF PLANT MATERIAL

The plant was collected from the green house of the Department of Biotechnology Jaypee University of Information Technology.

3.3.2 PREPARATION OF PLANT EXTRACT

A) The field grown plant was uprooted and washed thoroughly.
B) The plant parts were dried for 5 days using the sun drying method.
C) Different parts of the plant like root, shoot, leaves were grinded in a mixer to make a fine powder of the plant parts.
D) The in-vitro grown plantlets were washed with tap water to remove the MS medium and dried for some time.
E) The plantlets were crushed using liquid nitrogen to get a fine powder.
F) The respective concentration of Stem powder weighing (25 g), Root powder weighing (25g) and in-vitro grown plantlets (25g) were used for the extraction.
G) Extraction was done using the soxhlet apparatus. The extraction was carried out using the hydroalcoholic solvent (70:30) for more than 24 hours.
H) After the completion of the extraction the extract obtained was concentrated using the Rotary evaporator.
I) A gummy substance obtained was lyophilized for obtaining a fine powder of the extract.

3.4 PHYTOCHEMICAL ANALYSIS

Preliminary tests were performed to get an idea of the compounds present in the plant extract from *Nothapodytes nimmoniana* field grown shoot, root and tissue culture plantlets.

TEST PERFOMED FOR:

(A) Alkaloids
(B) Flavanoids
(C) Glycosides
(D) Tannins

ALKALOIDS

- Dragendorff"s reagent- Alkaloids give reddish brown precipitate with dragendorff"s reagent.
- Tannic acid test- Alkaloids give buff colour precipitate with tannic acid solution.
- Picrolonic acid test- Alkaloids give yellow colour precipitate picrolonic acid.

GLYCOSIDES

- Test for Hydroxy- anthroquinones- Treat the sample with potassium hydroxide solution red colour is produced.
- Saponin glycosides – (Froth formation test) Place 2ml solution of drug in water in a test tube, shake well; stable froth (foam) is formed.

TANINNS

- Ferric chloride test- Treated the extract with ferric chloride solution, blue colour appears if hydrolysable tannins are present and green colour appears if condensed tannins are present

FLAVONOIDS

- Alkaline reagent test- To the test solution add few drops of sodium hydroxide solution intense yellow colour is formed which turns to colorless on addition of few drops of dilute acid indicate presence of flavanoids.
- Zinc hydrochloride test- To the test solution add a mixture of zinc dust and concentrated HCL. It gives red colour after few minute.

3.5CYTOTOXICITY STUDIES FROM PLANT EXTRACT OF FIELD GROWN SHOOT, ROOT AND IN-VITRO GROWN SHOOTS ON HEK-293 CELL LINES.

3.5.1 SUBCULTURING

The cell line was subcultured on DMEM (Dulbecco's Modified Eagles's medium) medium and left for incubation for 24 hours. After 24 hours' cell confluency was checked. Once the cell had obtained 70-80% confluency cells were again subcultured. While subculturing, PBS washing was given to the cell which was followed by trypsinization .The cells were centrifuged for 5 minutes the supernatant was removed and fresh media was added to the cell. Cells were counted using the haemocytometer.

3.5.2 SEEDING

The actively dividing cells were put in the 96 well plates at a density of 1×10^{4} cells per well and incubated for 24 hours.

3.5.3 DOSING

Various dilutions of the extract as shown in "Table 3.4" and "Table 3.5" were prepared both for the root and shoot sample. The old media from the 96 well plates was discarded before the dosing was done. Dosing was done in the serum free media. The root and shoot extract was diluted with different concentration of serum free media. Cells were incubated for 24 hours.

3.5.4 MTT ASSAY

MTT (40µl) was added to the cells and further incubated at 37 degrees for 1-4 hours. Viable cells could reduce the MTT dye to insoluble formazan. 50-100 µl of DMSO or ethanol was added to convert the insoluble formazan crystals to soluble formazan. Absorbance was taken at two wavelengths 595 and 630nm.

The cell cytotoxicity was calculated using the formula:

% cell cytotoxicity = Absorbance of test/ Absorbance of control * 100

Table 3.4: *Nothapodytes nimmoniana* field grown root extracts dilutions for HEK-293 cell line

Root extract	Serum free media
750 micro litre	250 micro litre
500 micro litre	500 micro litre
250 micro litre	750 micro litre
125 micro litre	875 micro litre

Table 3.5: *Nothapodytes nimmoniana* field grown shoot extracts dilutions for HEK-293 cell line

Root extract	Serum free media
750 micro litre	250 micro litre
500 micro litre	500 micro litre
250 micro litre	750 micro litre
125 micro litre	875 micro litre

3.6 ANTICANCEROUS STUDY

3.6.1 SUBCULTURING

MCF-7 was subcultured on DMEM (Dulbecco's Modified Eagles' medium) and MDA-MB 231 cells were cultured on L-15 (Leibovitz) media and left for incubation at 37 degrees for 24 hours. Cell confluency was checked. Once cells had obtained 70-80% confluency cells were again subcultured. While subculturing PBS washing was given to the cells which were followed by trypsinization. The cells were centrifuged for 5 minutes. The supernatant was removed and fresh media was added to the cell. Cells were counted using the haemocytometer.

3.6.2 SEEDING

The actively dividing cells from both the cell lines were put in the 96 well plates at a density of 1×10^4cells per well and incubated for 24 hours.

3.6.3 DOSING

Various dilutions of the extract (125µl, 250µl, 500µl, 750µl were prepared for the field grown root, shoot sample as well as in- vitro grown plantlets .The old media from the 96 well plates was discarded before the dosing was done. Dosing was done in the serum free media. The field grown root, shoot and in- vitro grown plantlets extract was diluted with different concentration of serum free media. Cells were incubated for 24 hours.

3.6.4 MTT ASSAY

MTT (40µl) was added to cells and further incubated at 37 degrees for 1- 4 hours. Viable cells could reduce the MTT dye to insoluble formazan. 50-100 µl of DMSO or ethanol was added to convert the insoluble formazan crystals to soluble formazan. Absorbance was taken at two wavelengths 595 and 630nm. IC50 values for each cell were obtained.

3.6.5 ACRIDINE ORANGE (AO) AND ETHIDIUM BROMIDE (ETBR) STAINING

IC_{50} concentration was analyzed for its cell morphological changes in MCF-7 and MDA-MB-231 cell line by dual AO and ETBR staining. Cells were seeded at a density of 2×10^5 cells/well in 6-well plate comprising 1 ml of growth medium. To obtain the confluency around 70-80%, cells were incubated at 37°C in CO2 incubator. After desired period, the cells were harvested, washed using ice-cold phosphate-buffered saline (PBS), and fixed with 4% of paraformaldehyde for 30 minutes. Supernatant was discarded, and cells were treated with 1% triton ×-100 for 15 minutes followed by washing of cells with PBS. Later cells were stained with dye mixture; comprising 5 µl of AO (1 mg/ml) and 5 µl of ETBR (1 mg/ml) for 5 minutes and then washed thrice with PBS. The stained cell with morphological changes was observed by fluorescence microscope.

3.7 THIN LAYER CHROMATOGRAPHY

- TLC is used to separate components from a mixture.
- Performed on sheet of glass, plastic or aluminium foil, which is coated with a thin layer of adsorbent, usually silica gel.
- This layer of adsorbent is known as stationary phase.
- Mobile phase is drawn up the plate via capillary action.
- After the experiment, the spots are visualised simply by projecting UV light on the silica gel plate.

- Stock solution was prepared for TLC .2mg plant extract was dissolved in 200µl methanol for all the three samples i.e. standard, In-vitro and field grown plantlet
- Thin layer chromatography was carried out for the detection of marker compound i.e.camptothecin present in all the three different plant extracts obtained from *Nothapodytes nimmoniana.*

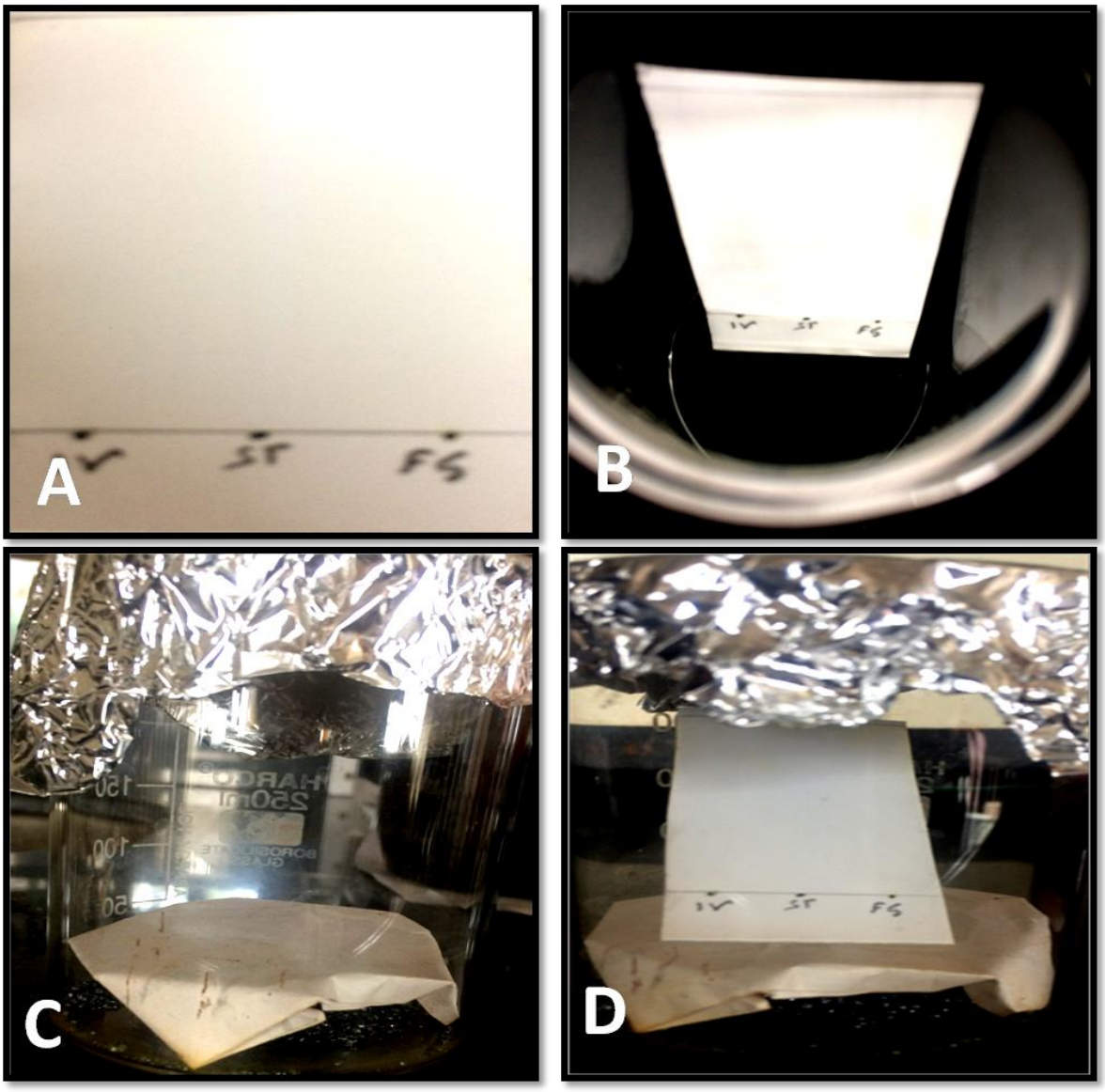


Figure 3.1: Thin layer chromatography for camptothecin detection in plant extract.
(A) Silica gel plate with samples spotted on it. **(B)** Silica gel plate dipped inside the mobile phase, **(C)** Iodine chamber. **(D)** Iodine chamber containing the silica gel plate.

3.8 QUANTIFICATION OF CAMPTOTHECIN BY HIGH PERFOMANCE LIQUID CHROMATOGRAPHY

3.8.1 SAMPLE PREPARATION

- Collected the plant parts and washed them thoroughly.
- Dried the plant parts i.e. leave, stems and in-vitro grown plantlets.
- Using liquid nitrogen, the plant parts were crushed to make fine powder.
- 1g of powder was dissolved in 10ml of methanol and 1mg of standard was used.
- Centrifugation of each sample was done for 3-5 min. Followed by Sonication for 5 minutes.
- The process was repeated thrice for complete extraction of CPT.
- Samples were left for overnight incubation at room temp.
- Samples were filtered under sterilised conditions using syringe filters.
- 3ml of samples were used for HPLC quantification and rest was stored in refrigerator for future use.

3.8.2 HPLC PROCEDURE

- 20µl of extract solution in the concentration range of 10 µg/ml was applied in triplicate to the column.
- The peak was recorded at the wavelength of 360nm.
- Quantification of CPT in the samples was calculated using the linear regression equation derived from the calibration curves.

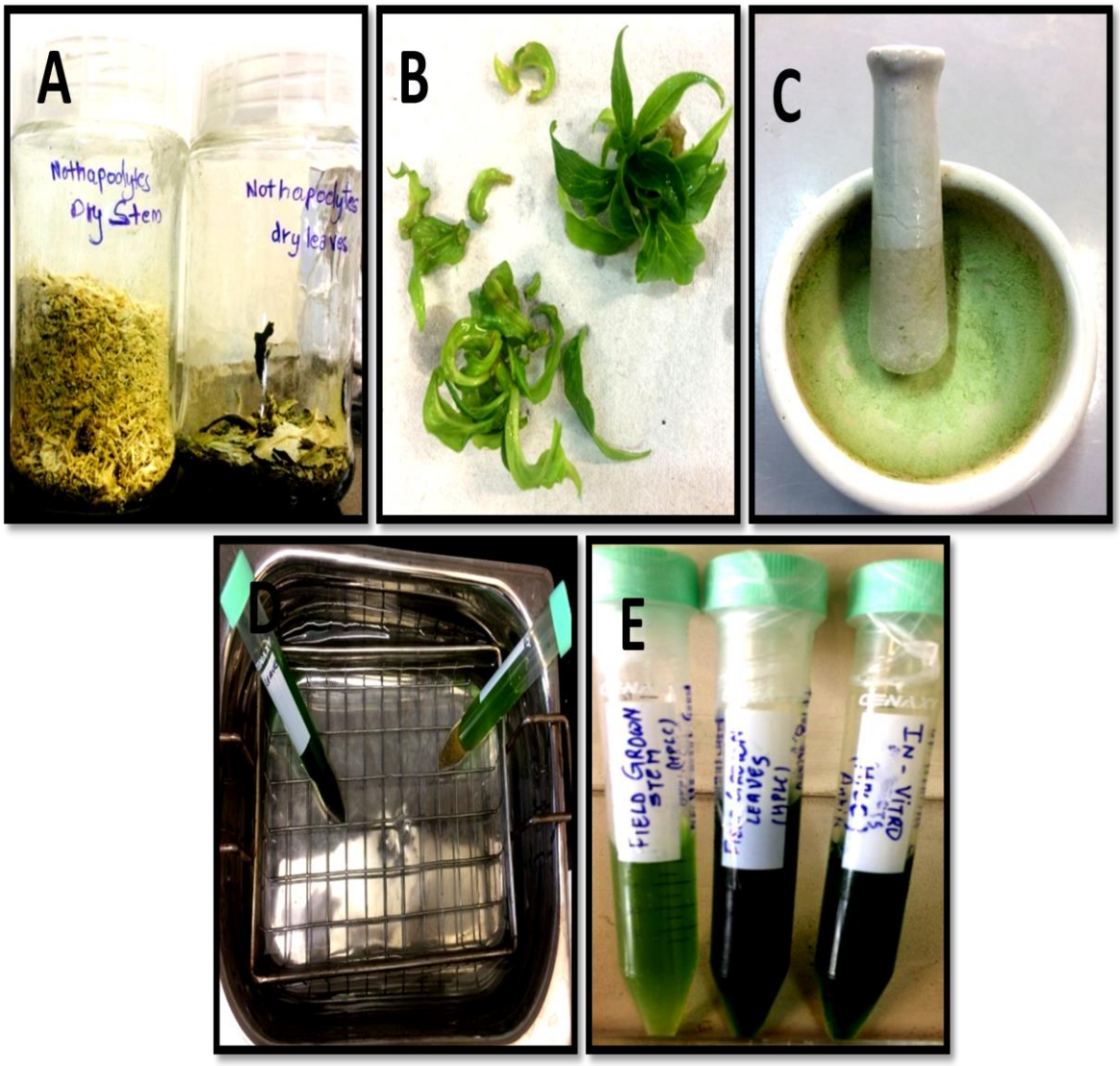


Figure 3.2: Preparation of samples for HPLC quantification.

(A) Sun dried parts of stem and leaves. **(B)** Thoroughly washed and dried in-vitro plantlets. **(C)** Crushing of plant parts with liquid nitrogen to make a fine powder. **(D)** Sonication of the samples. **(E)** Samples (i.e. field grown stems, leaves and in-vitro plantlets) ready for HPLC quantification after filtration using syringe filters**.**

CHAPTER-4

RESULTS AND DISCUSSIONS

4.1. CALLUSING AND SHOOT REGENERATION

Best results for callus induction was seen in MS medium supplemented with growth hormones with ratio of IBA (1mg/L) and TDZ (1.5mg/L) as shown in "Fig.4.1". 90% callusing was observed within 50±55 days on the media mentioned above. The callus obtained was sub cultured on shooting media where the best results for shoot regeneration i.e. 5-6 shoot primodia was obtained on MS medium containing IBA(1 mg/L) and KN (2 mg/L) within 10-15 days as shown in "Fig.4.2".

Table 4.1: Effect of different growth hormones in callus induction of *Nothapodytes nimmoniana*

MS MEDIUM	REGENERATION DAYS	%OF CALLUS INDUCTION
MS+TDZ+IBA (2.5:1.5) mg/l	30 -32days	30-40%
MS+TDZ (1.5) mg/l	30 -32days	50-65%
MS+TDZ+IBA (1.5:1) mg/l	30-32 days	65-90%

Table 4.2: Effect of different growth hormones in shoot regeneration of *Nothapodytes nimmoniana*

MS MEDIUM	REGENERATION DAYS	NO. OF SHOOTS FORMED AFTER REGENERATION
MS+BAP+KN+TDZ(2:1:0.05)mg/l	20-25 days	3-4
MS+IBA+KN(1:2)mg/l	20-25 days	7-8

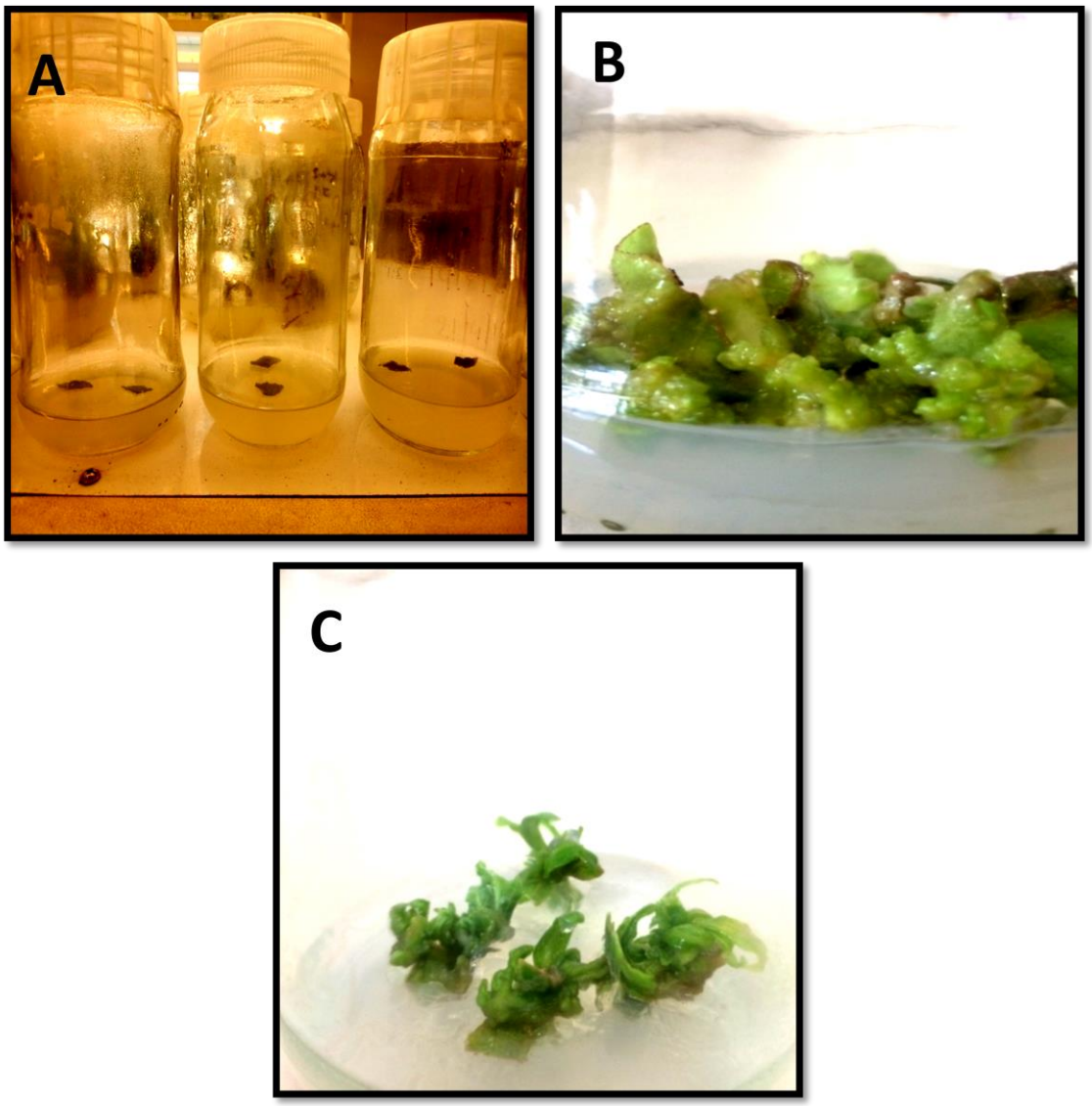


Figure 4.1: Callus and shoot regeneration in *Nothapodytes nimmoniana*

(A) Leaf explants cultured higher auxin media for callus induction, **(B)** Callus induction after 5-6 weeks of incubation, **(C)**Shoot regeneration observed from callus after 20±5 days of incubation.

4.1.2 ARTIFICIAL SEED GERMINATION OF *NOTHAPODYTES NIMMON IANA*

Mature embryos form the callus were taken and used for the artificial seed formation using 3% sodium alginate and 100mM calcium chloride which was best for the seed formation. The seeds so formed were germinated using MS medium containing growth hormones (IBA+KN+GA3) in the ratio (1:3:2) mg/L and (IBA+KN+GA3) in the ratio (1:3:2) mg/L and 0.5% activated charcoal **[15].** Although the germination was observation both the medium but

the best suited growth was in medium IBA+KN +GA3 (1:3:2) mg/L without activated charcoal. In 10-13 days, the seeds were successfully germinated as shown in “Fig.4.2”.

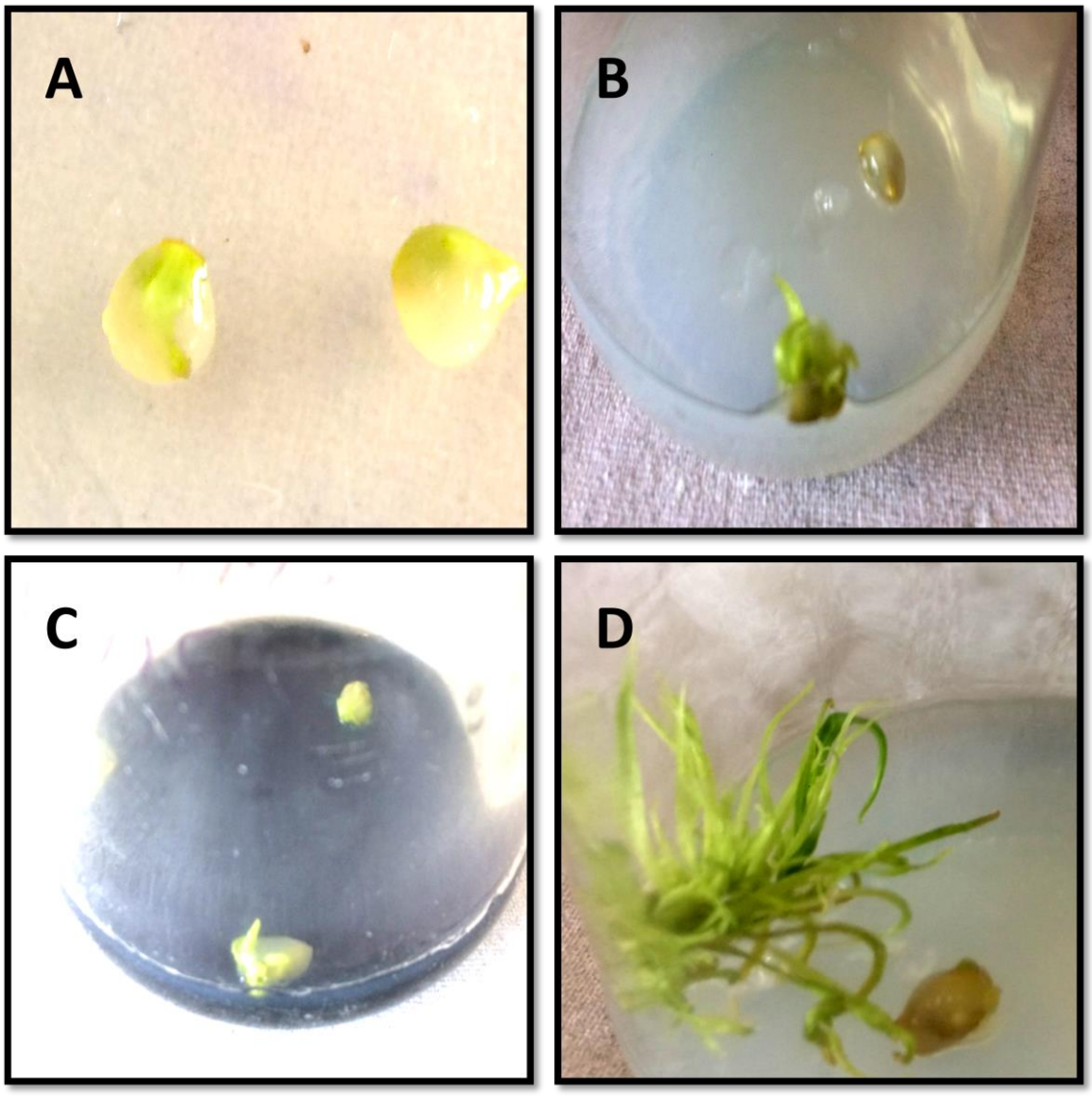


Figure 4.2: Artificial seed production and germination.

(A) Somatic seeds of *Nothapodytes nimmoniana*. **(B), (C)**In-vitro germination of seeds seen in 10-13 days. **(D)**Seedlings formed after 30-35 days of germination.

4.2 HYDROALCOHOLIC EXTRACTION

The plant extract has been collected as shown in "Fig.4.3" by doing hydroalcoholic extraction (70:30) of *Nothapodytes nimmoniana* plant samples by using25g of plant material for field grown root, shoot and tissue cultured plantlets.

Figure 4.3: Hydroalcoholic extraction for camptothecin in *Nothapodytes nimmoniana*

(A) Hydroalcoholic extraction using soxhlet apparatus**. (B)** Separation of solvent from extract using rotary evaporator.

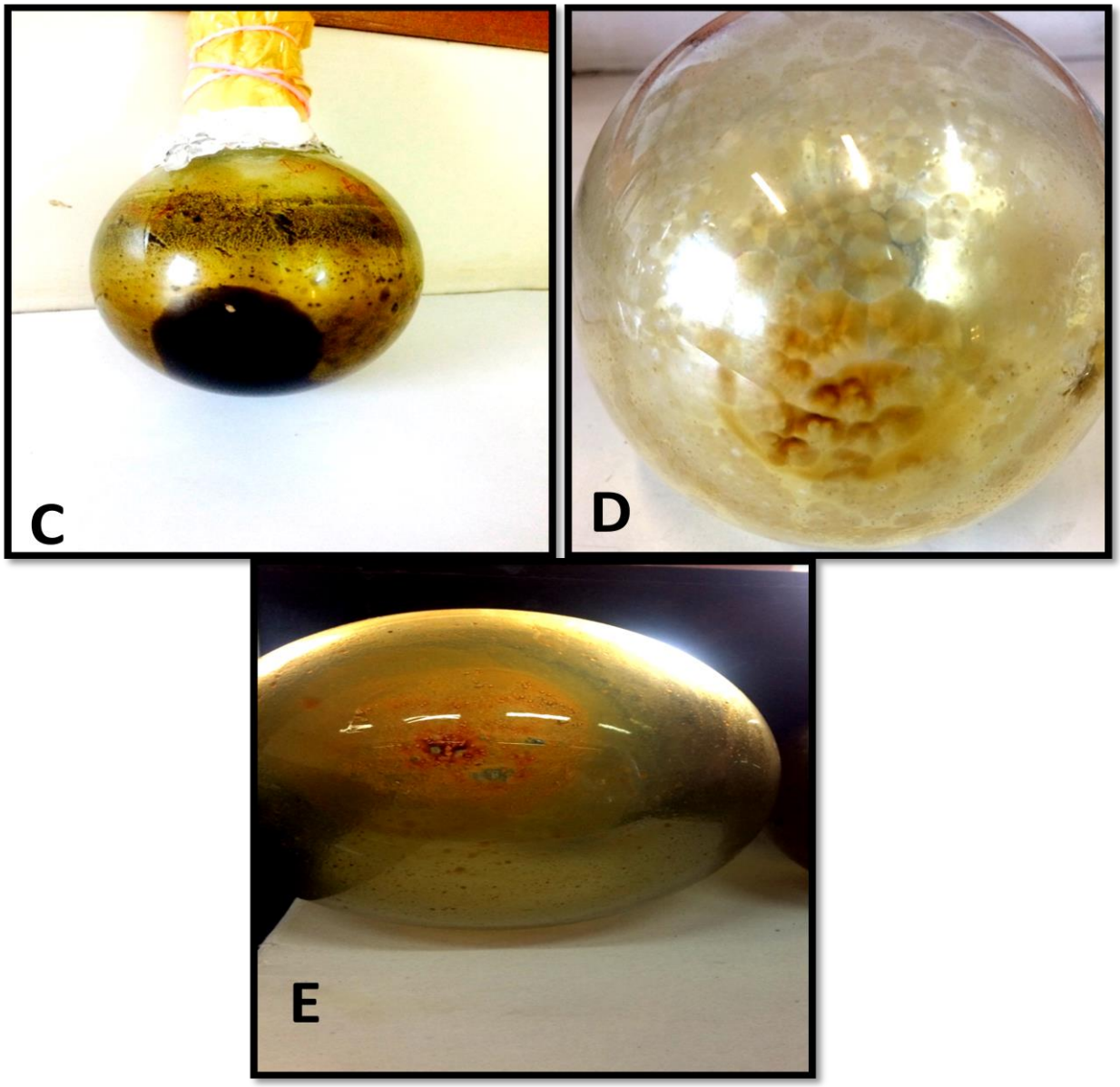


Figure 4.3: (C, D, and E) extract obtained from field grown shoot, root and tissue cultured plantlets from *Nothapodytes nimmoniana*.

4.3 PHYTOCHEMICAL ANALYSIS

Results of phytochemical analysis showed that tannins and flavanoids are present in the *Nothapodytes nimmoniana* plant extract apart from commonly found alkaloids. Phytochemical tests are qualitative in nature so HPLC quantification of all the samples was done.

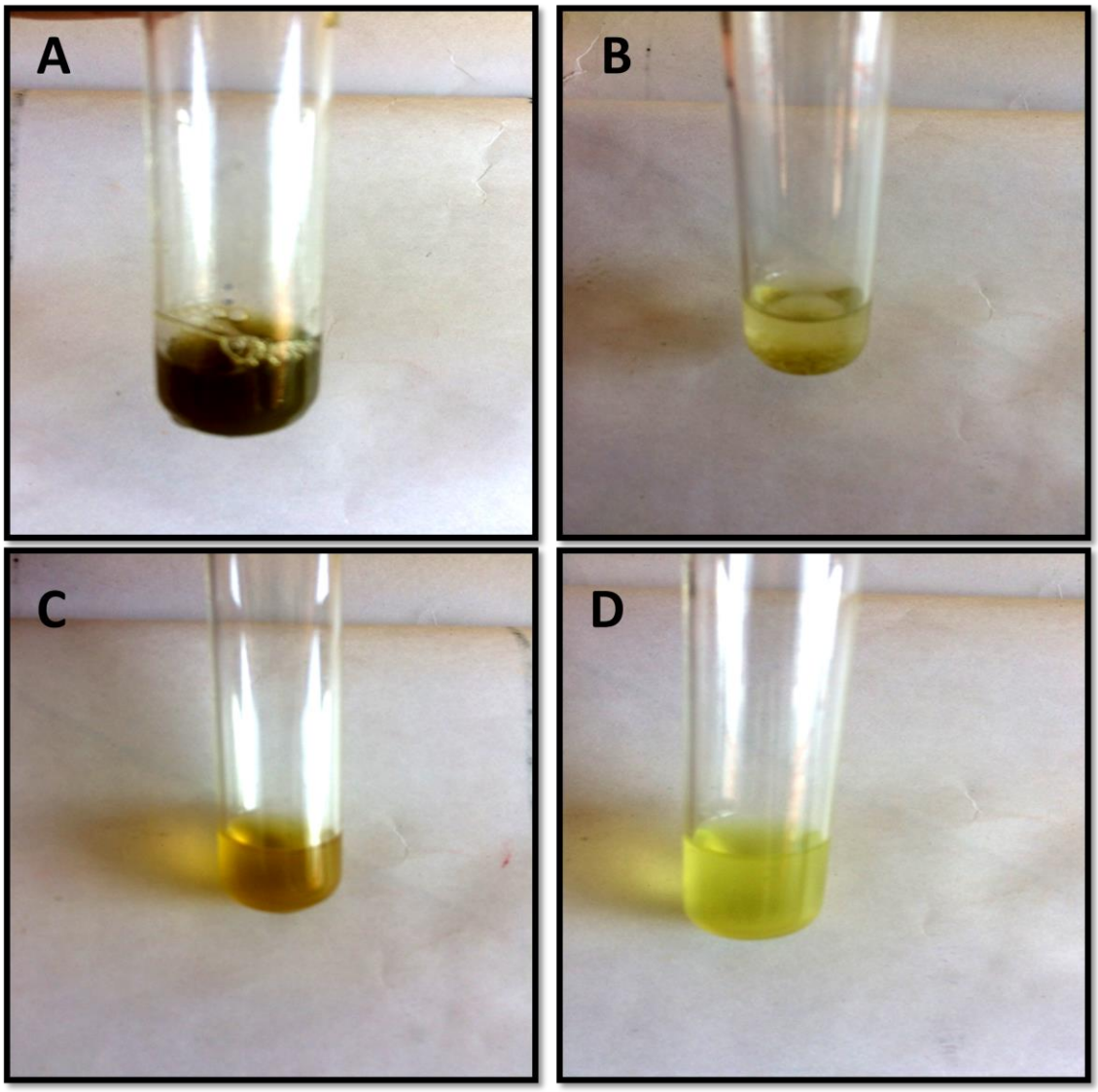


Figure 4.4: Phytochemical screening for different compounds in plant extract

(A) Ferric chloride test (dirty green colour seen) indicating the presence of tannins. **(B)** Tannic acid test (buff colour precipitate seen) indicating the presence of alkaloids. **(C, D)** Alkaline reagent test (dark yellow colour changes to light yellow) indicating the presence of flavanoids.

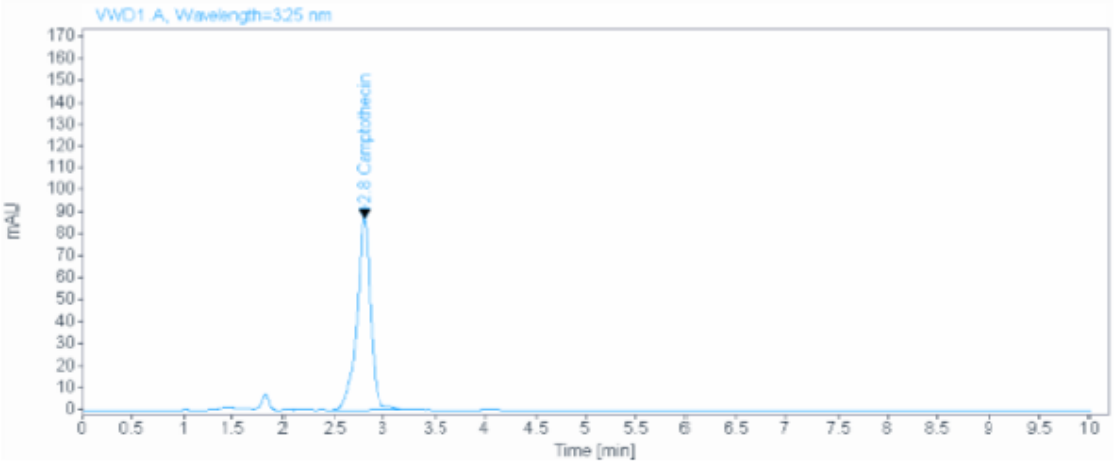
4.4 QUANTIFICATION OF CAMPTOTHECIN BY HPLC

The procedure followed by **[28]** where determination of CPT was done by using the column C18 (250MM 4.6mm, 50µ). CPT was determined using acetonitrile:water (45:55v/v) as the optimised mobile phase. The peak area was obtained at the wavelength of 360nm. Chromatograms obtained in our study have been developed on the same line. Retention time for standard camptothecin was 2.8 min covering an area of 833.834. The other sample i.e. field grown leaves had a retention time of 2.797as shown in "Figure 4.7", total camptothecin quantified in this sample was 0.87%.

The amount of camptothecin quantified in field grown stem was 0.17% with retention time 2.791 as shown in "Figure 4.6".

In-vitro plantlets have very little camptothecin quantified (0.03%) as shown in "Figure 4.8" with a retention time of 2.805 minutes.

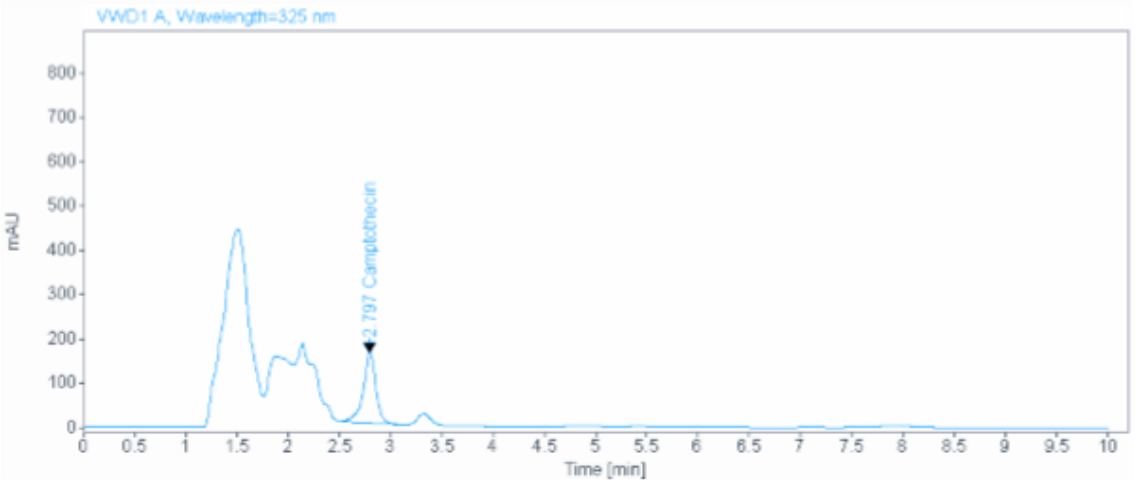
From the HPLC quantification we can make out that more quantity of camptothecin is found in field grown leaves followed by field grown stems. Very less or negligible amount of camptothecin has been quantified in in-vitro grown plantlets. This can be attributed to various factors such as different explants used for culture establishment which has been later been used for HPLC, different growth hormones used can also have an effect on camptothecin production, Field grown plant used was 5-6 years old whereas the in-vitro grown plantlets were just one and a half month old so age of plant used can also be one major factor for different concentration of camptothecin present in the samples. Field grown plants are in open environment which faces more stress conditions therefore more secondary metabolite is produced whereas in case of in- vitro plants they are given sustained environment which leads to less stress conditions and therefore less secondary metabolite production.



Signal: VWD1 A, Wavelength=325 nm

Name	RT[min]	Area	Area%	Height	Height %	Compound Amount	Compound Amount Unit
Camptothecin	2.800	833.834	100.00	86.840	100.00	100.222	ng/ul

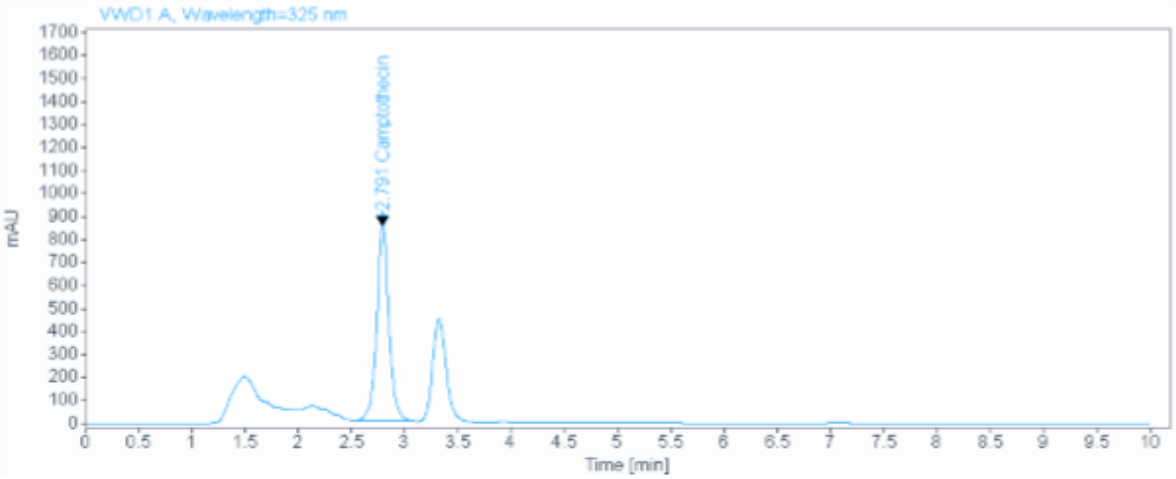
Figure 4.5: HPLC chromatogram recorded for standard camptothecin



Signal: VWD1 A, Wavelength=325 nm

Name	RT[min]	Area	Area%	Height	Height %	Compound Amount	Compound Amount Unit
Camptothecin	2.797	1346.847	100.00	158.524	100.00	161.883	ng/ul

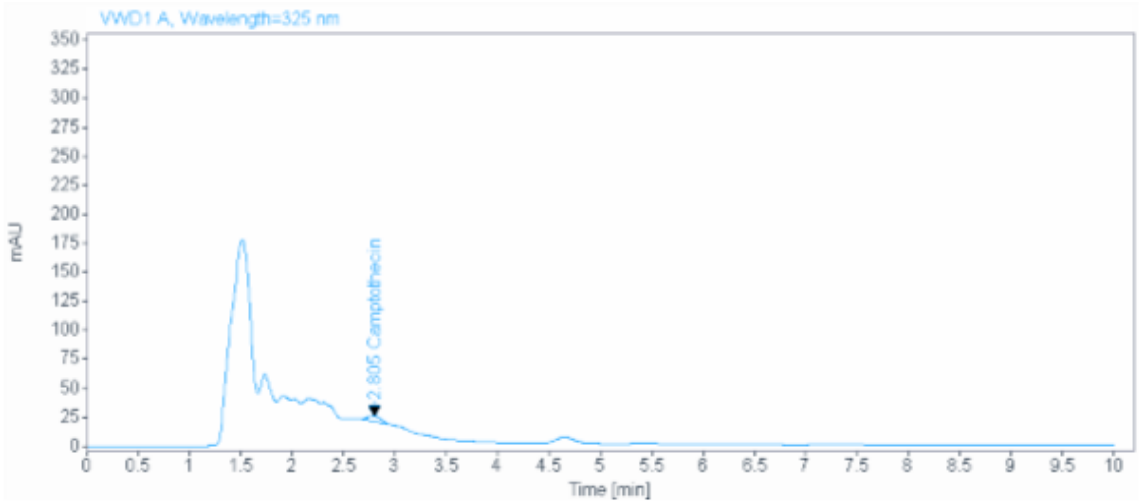
Figure 4.6: HPLC Chromatogram recorded for field grown shoots



Signal: VWD1 A, Wavelength=325 nm

Name	RT[min]	Area	Area%	Height	Height %	Compound Amount	Compound Amount Unit
Camptothecin	2.791	6745.598	100.00	847.364	100.00	810.781	ng/ul

Figure 4.7: HPLC chromatogram recorded for field grown leaves



Signal: VWD1 A, Wavelength=325 nm

Name	RT[min]	Area	Area%	Height	Height %	Compound Amount	Compound Amount Unit
Camptothecin	2.805	40.030	100.00	4.575	100.00	4.811	ng/ul

Figure 4.8: HPLC chromatogram recorded for in-vitro plantlets

Table 4.3: HPLC quantification of *Nothapodytes nimmoniana* plant extract

Plant sample	HPLC quantification
Field grown leaves	0.87%
Field grown stems	0.17%
In-vitro plantlets	0.03%

4.5 CYTOTOXICITY AND ANTICANCEROUS STUDY

The results obtained for cytotoxicity and anticancerous study are as follows:

In "Table 4.4" optical density (OD) has been calculated for HEK-293 cells after treatment with field grown root sample. "Graph1" shows the percentage viability for the HEK-293 cells at varying concentrations of the plant extract. Similarly, "Table 4.5" and "Table 4.6" represent the OD calculated for HEK-293 cell after treating cells with field grown shoot and in-vitro plant extract. Graph no. "2 "and "3" shows the percentage viability of HEK-293 cells for the respective extracts.

"Table 4.7", "Table 4.8" and "Table 4.9" represents the OD calculated for MCF-7 cells when treated with *Nothapodytes nimmoniana* field grown root, shoot and in-vitro extract subsequently. Percentage viability of cells for MCF-7 has been represented in "Graph 4", "Graph 5" and "Graph 6" respectively.

Similar to the above results, OD for MDA-MB-231 cells have been calculated using field grown shoot, root and in-vitro plantlets as shown in "Table 4.10", "Table 4.11" and "Table 4.12" respectively. Percentage viability of MDA-MB-231 cells has been shown in "Graph 7", "Graph 8" and Graph 9" correspondingly. "Figure 4.9", "Figure 4.10" and "Figure 4.11" shows the cells which have been treated with AO and EtBr. AO and EtBr are used as fluorescent tags. Cells treated with AO (Acridine orange) represents both dead and live cells whereas EtBr (Ethidium Bromide) treated cells as represented in red colour in all the above-mentioned figures, are the dead cells. From these figures, we can make out the number of dead and live cells for all the cell lines when treated with *Nothapodytes nimmoniana* plant extract. From the graphs and IC_{50} values, we found that extract of in-vitro plantlets is required

in higher dose to cause death in cancer cells when compared to field grown shoot and root extract.

The plant extract of all the three samples were more effective on MDA-MB-231 cells as compared to MCF-7 cells, as we observed IC_{50} values for MDA-MB-231was less as compared to MCF-7. This is possibly because; MDA-MB-231 cell line is more sensitive in comparison to MCF-7 cell line, as reported in literature previously.

Cytotoxic results revealed that, the plant extract is safe for normal HEK-293 cells as the IC_{50} values obtained are quite high. Pattern of IC_{50} value was seen in following order; in-vitro plant extract (3333.3µg/L)> shoot (1742.21µg/L) >root (1209.72µg/L) respectively.

Table 4.4: OD calculated for the root sample in HEK-293 cell line

Concentration (µg/L)	OD-1	OD-2	Average	% viability
Control	1.103	1.096	1.0995	100
750	0.785	0.764	0.7745	70.44111
500	0.821	0.796	0.8085	73.533
250	0.847	0.827	0.837	76.125
150	0.987	0.964	0.9755	88.722

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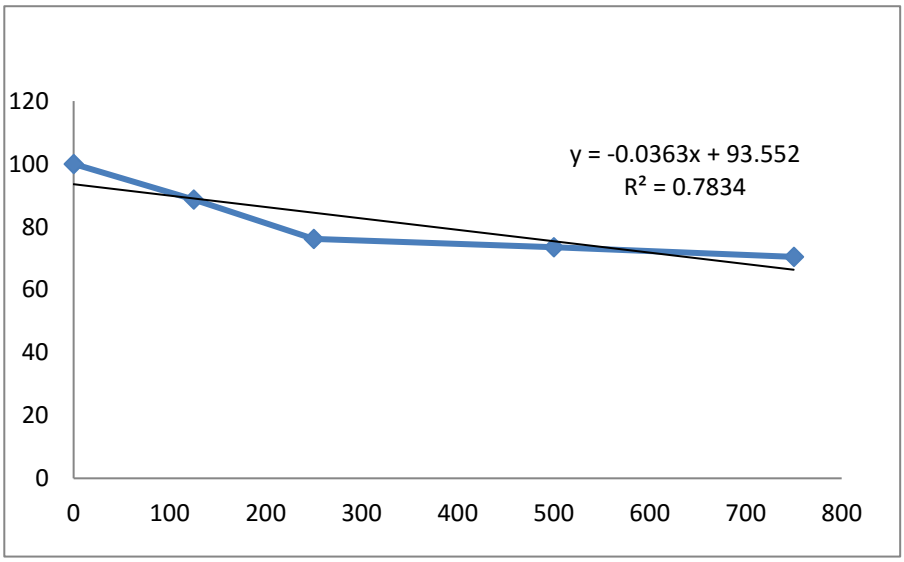
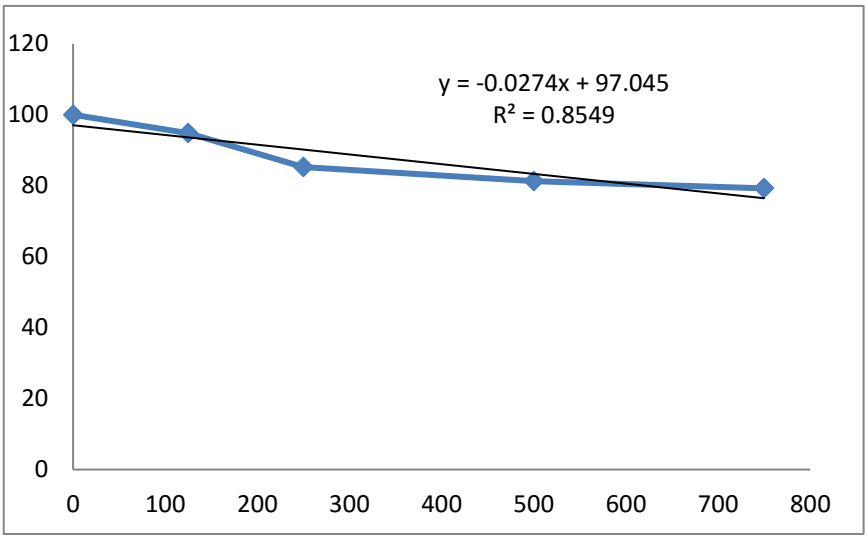


Graph 1: IC_{50} value of the root was calculated.

IC_{50} –1209.72µg/l

Table 4.5: OD calculated for shoot extract in HEK-293 cell line

Concentration µg/L	OD-1	OD-2	Average	% viability
Control	1.103	1.0960	1.0995	100
750	0.837	0.906	0.8715	79.263
500	0.894	0.356	0.894	81.309
250	0.972	0.903	0.9375	85.26603
125	1.097	0.933	1.0425	94.815

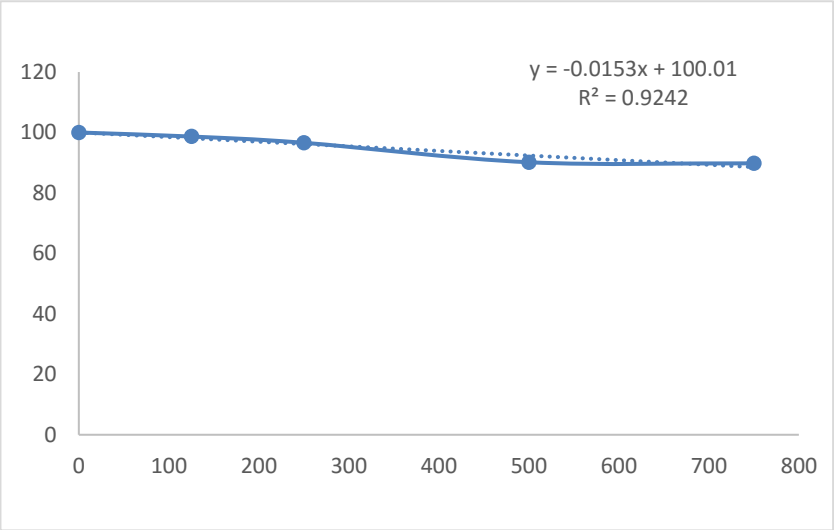


Graph 2: IC_{50} value of the shoot extract was calculated.

IC_{50}- 1742.22µg/l

Table 4.6 OD calculated for HEK-293when treated with in-vitro grown plantlets

Concentration µg/L	OD-1	OD-2	Average	% viability
Control	0.984	0.957	0.9705	100
125	0.954	0.961	0.9575	98.660
250	0.972	0.903	0.9375	96.599
500	0.849	0.856	0.857	90.159
750	0.837	0.906	0.8715	89.799

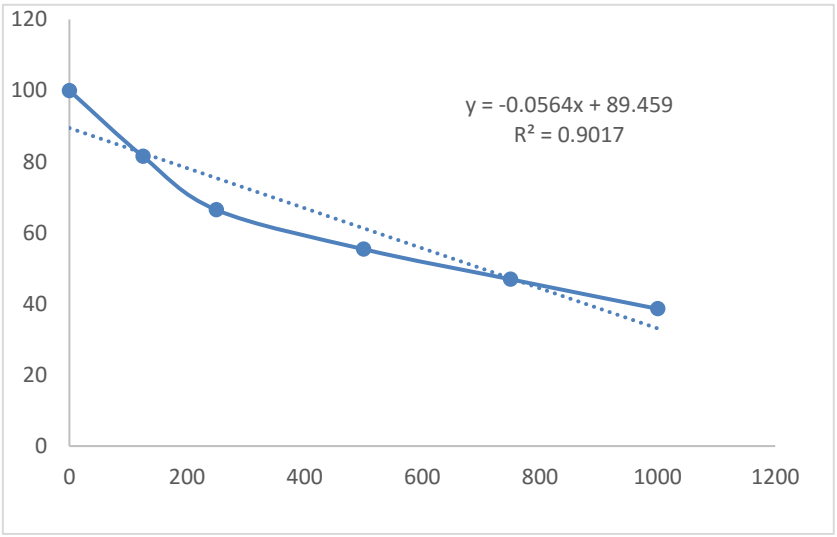


Graph 3: IC_{50} value of the in-vitro extract was calculated.

IC_{50}- 3333.3 µg/l

Table 4.7: OD calculated for MCF-7 cells when treated with root extract

Concentration µg/L	OD-1	OD-2	OD-3	Average	% viability
Control	0.956	0.982	0.976	0.97133	100
125	0.80	0.794	0.772	0.79133	81.469
250	0.648	0.624	0.664	0.64533	66.438
500	0.514	0.5480	0.552	0.538	55.387
750	0.425	0.478	0.464	0.45566	46.911

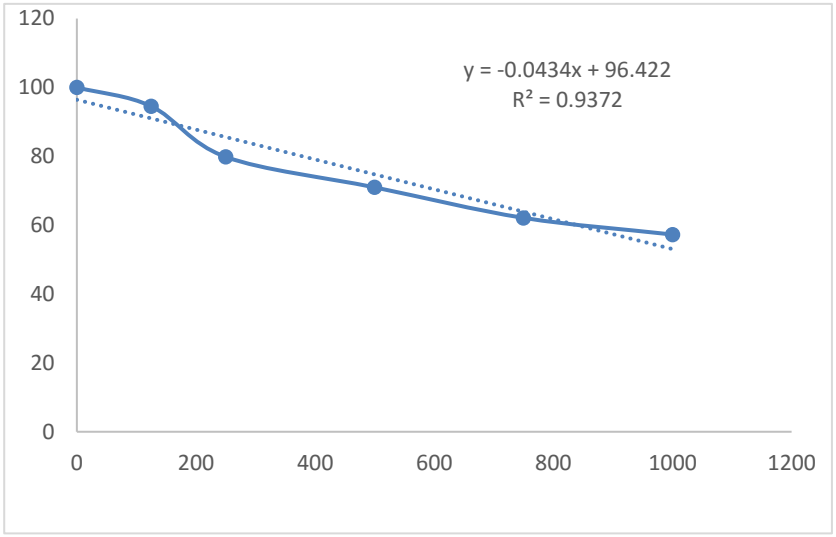


Graph 4: IC_{50} value of the root extract was calculated

IC_{50}- 704.464µg/l

Table 4.8: OD calculated for MCF-7 cells when treated with shoot sample

Concentration µg/L	OD-1	OD-2	OD-3	Average	% viability
Control	0.956	0.982	0.976	0.9713	100
125	0.923	0.917	0.914	0.918	94.509
250	0.785	0.743	0.798	0.775	79.821
500	0.694	0.659	0.715	0.689	70.968
750	0.587	0.599	0.624	0.603	62.114

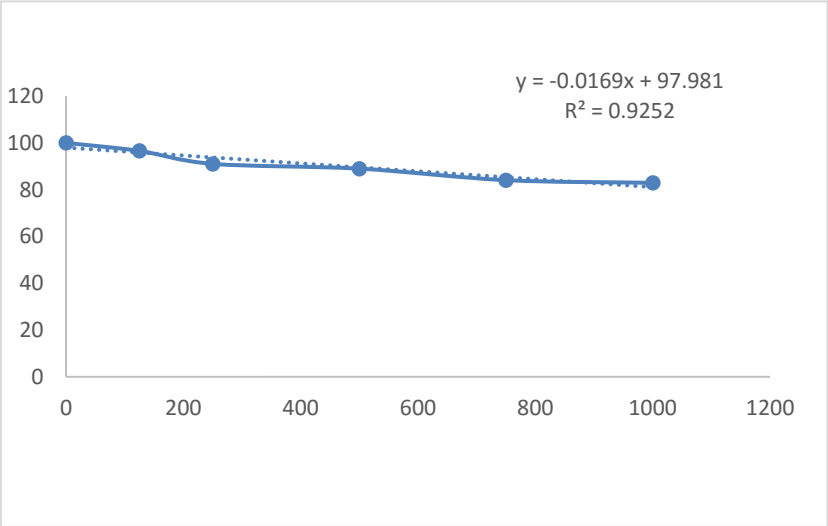


Graph 5: IC_{50} value of the shoot extract was calculated

IC_{50}- 1079.53µg/l

Table 4.9: OD calculated for MCF-7 cells when treated with in-vitro sample

Concentration µg/l	OD-1	OD-2	OD-3	Average	% viability
Control	0.956	0.982	0.976	0.971	100
125	0.949	0.938	0.927	0.938	96.5686
250	0.897	0.88	0.876	0.884	91.043
500	0.847	0.874	0.872	0.864	88.984
750	0.817	0.827	0.806	0.816	74.077

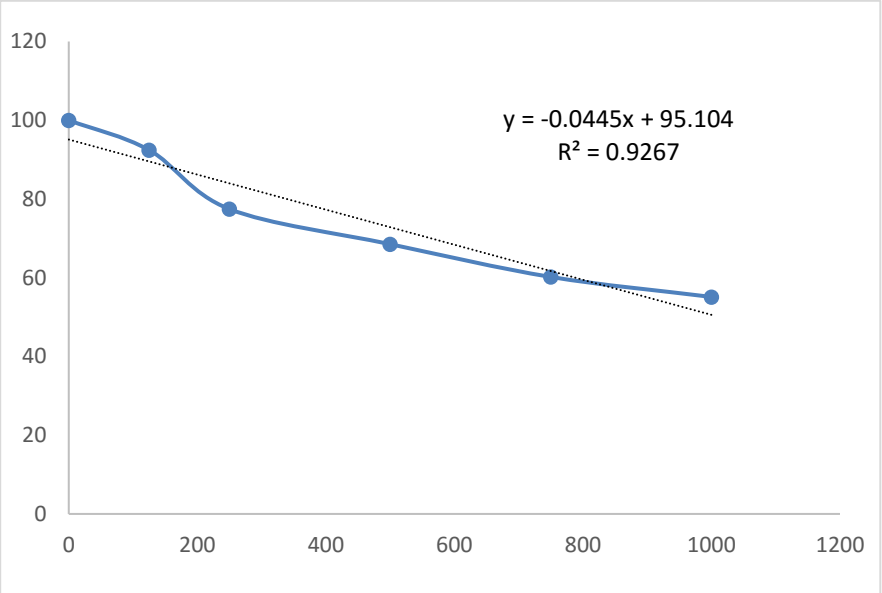


Graph 6: IC_{50} value of the in-vitro plant extract was calculated

IC_{50}- 2998.75µg/l

Table 4.10: OD calculated for MDAMB-231 cells when treated with shoot sample

Concentration µg/L	OD-1	OD-2	OD-3	Average	% viability
Control	1.278	1.228	1.218	1.241	100
125	1.141	1.16	1.141	1.147	92.427
250	0.948	0.963	0.973	0.961	77.443
500	0.846	0.867	0.839	0.850	68.528
750	0.741	0.573	0.748	0.747	60.204

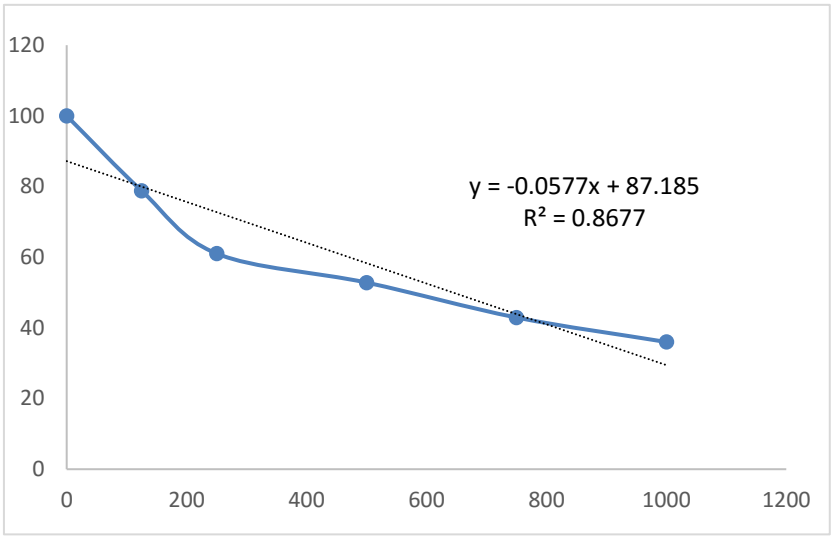


Graph 7: IC_{50} value of the shoot extract was calculated

IC_{50}- 1025µg/l

Table 4.11: OD calculated for MDAMB-231 cells when treated with root sample

Concentration µg/L	OD-1	OD-2	OD-3	Average	% viability
Control	1.278	1.228	1.218	1.241	100
125	0.987	0.989	0.958	0.978	78.786
250	0.741	0.754	0.778	0.757	61.036
500	0.668	0.657	0.642	0.655	52.819
750	0.541	0.514	0.544	0.533	42.937

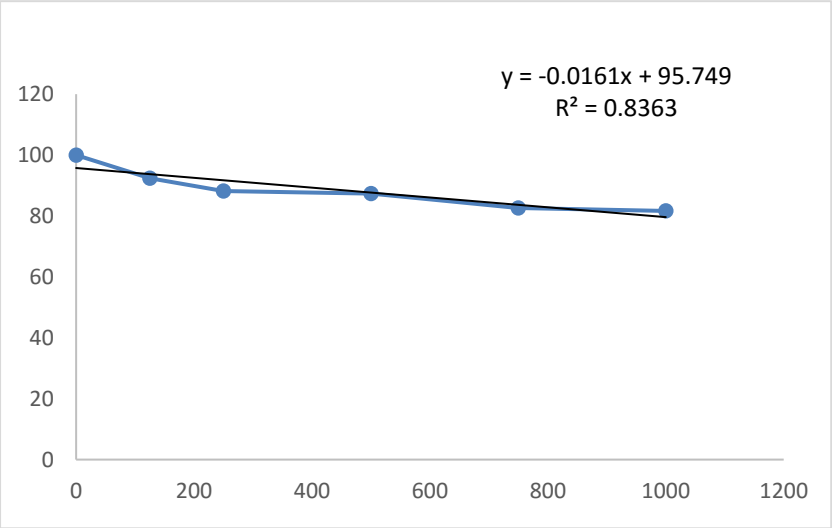


Graph 8: IC_{50} value of the root extract was calculated

IC_{50}- 652.28µg/l

Table 4.12: OD calculated for MDAMB-231 cells when treated with in-vitro sample

Concentration µg/L	OD-1	OD-2	OD-3	Average	% viability
Control	1.278	1.228	1.218	1.241	100
125	1.145	1.156	1.141	1.147	92.4275
250	1.174	0.965	1.144	1.094	88.171
500	1.125	0.958	1.145	1.084	87.325
750	1.121	0.983	0.971	1.025	81.659

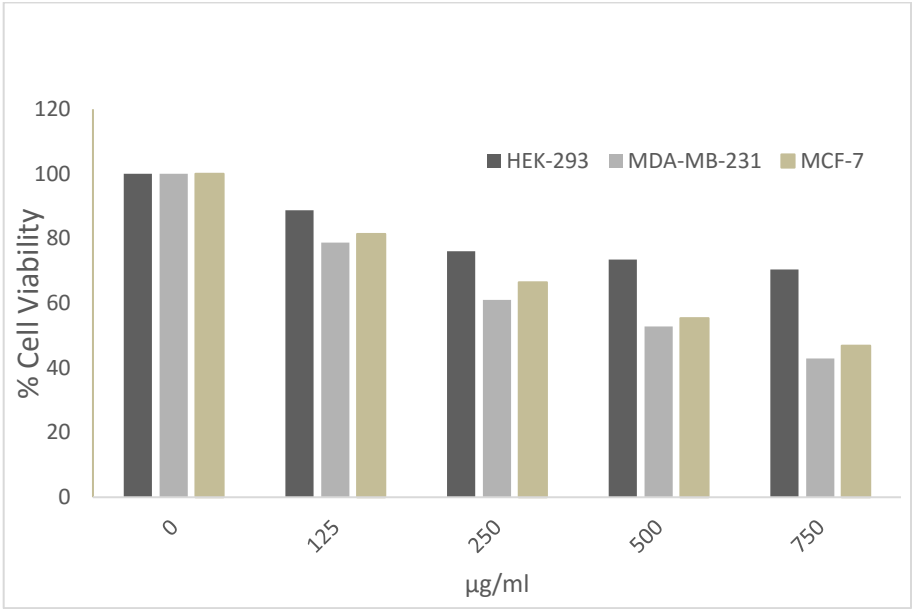


Graph 9: IC_{50} value of the in-vitro plant extract was calculated

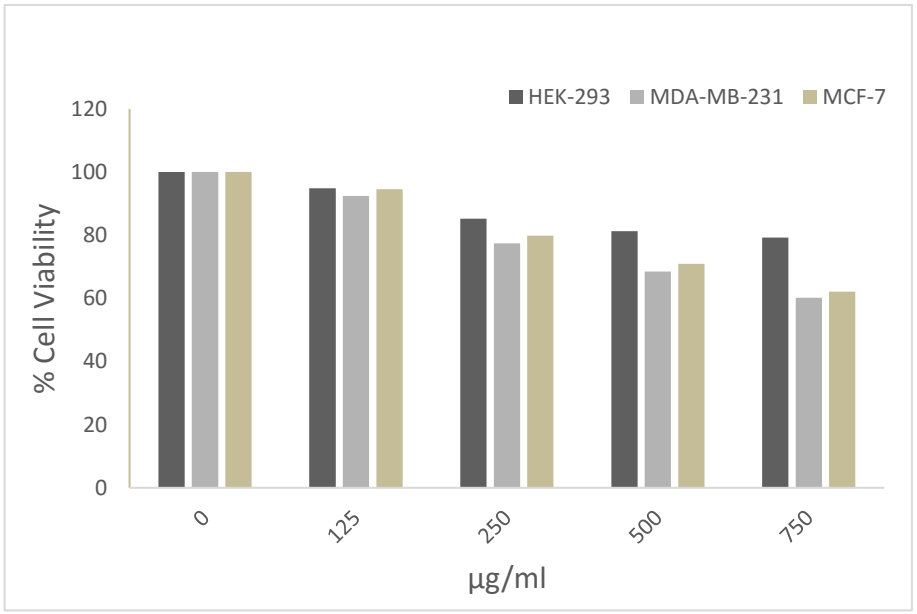
IC_{50}- 2858.75µg

Table 4.13: IC_{50} of all the cell line

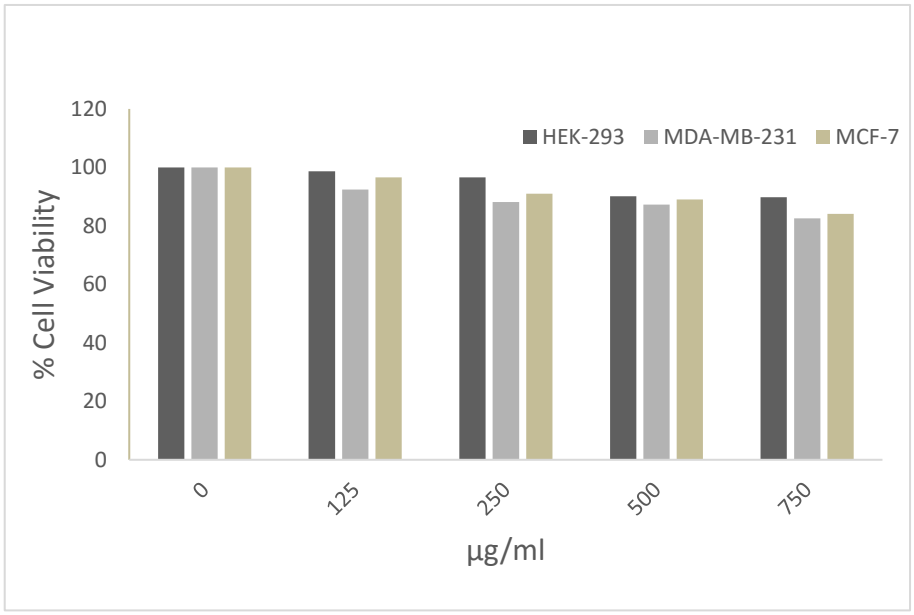
IC_{50}	Root	Shoot	In-vitro plantlets
HEK-293	1209.72	1742.21	3333.33
MDAMB-231	652.28	1025	2858.75
MCF-7	704.464	1079.53	2997.75



Graph 10: Comparison of effect of field grown roots on HEK-293, MDAMB-231 and MCF



Graph 11: Comparison of effect of field grown shoots on HEK-293, MDAMB-231 and MCF-7



Graph 12: Comparison of effect of In-vitro grown plantlets on HEK-293, MDAMB-231 and MCF-7

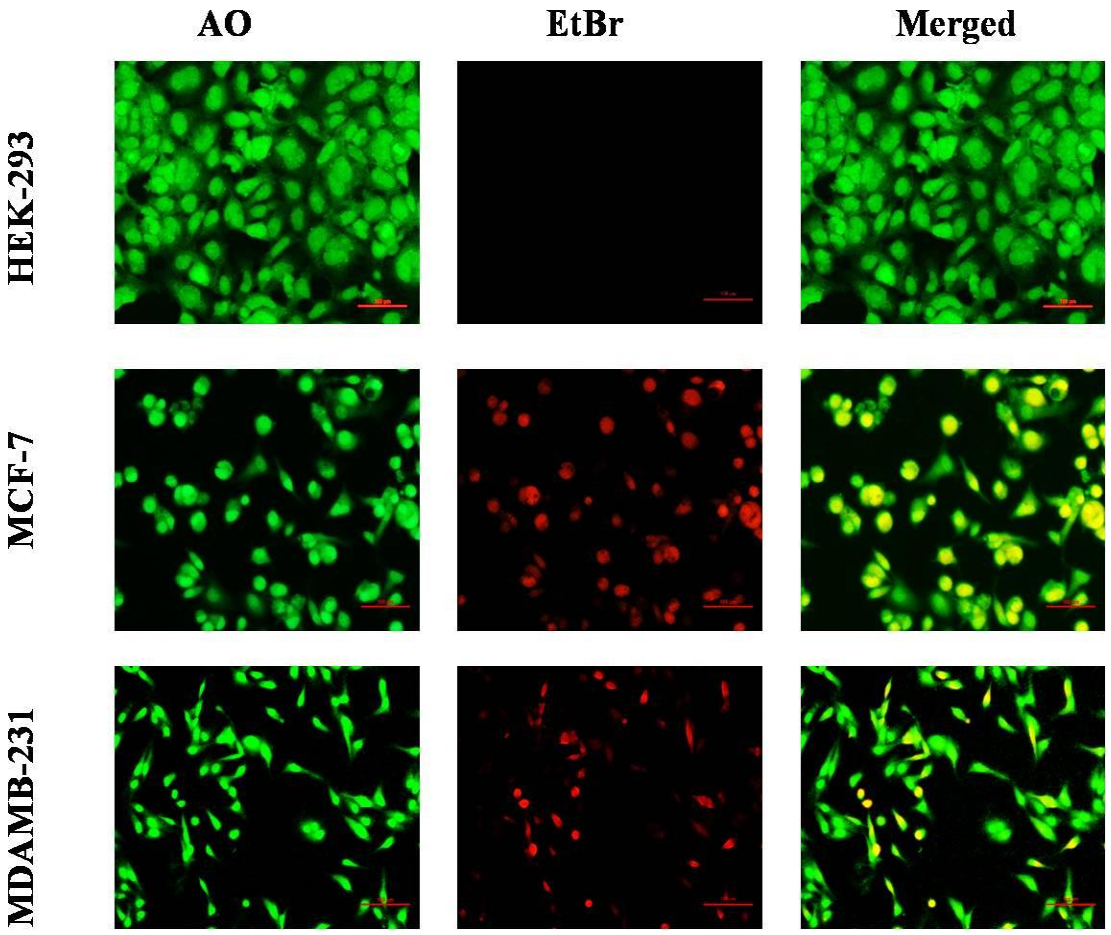


Figure 4.9: Cells treated with field grown root extract and stained by AO and EtBr

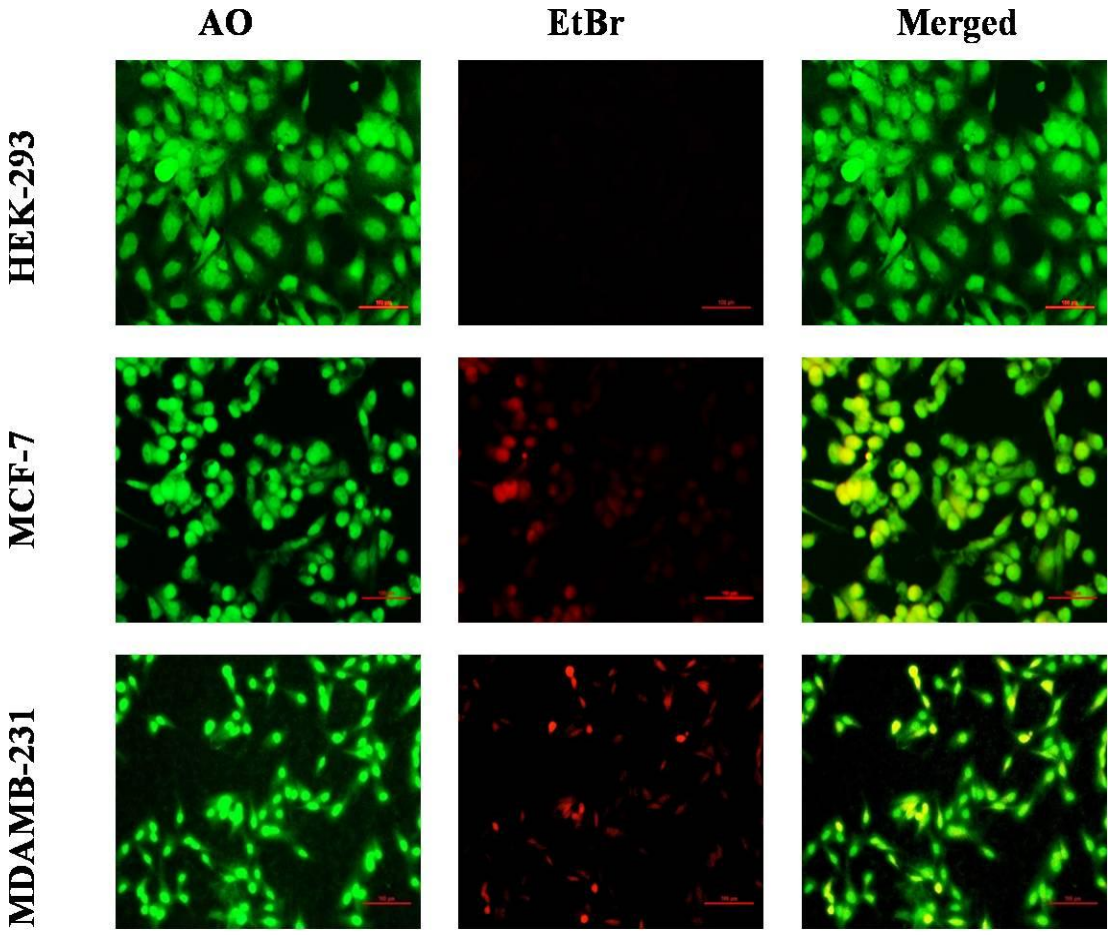


Figure 4.10: Cells treated by field grown shoot and stained by AO and EtBr

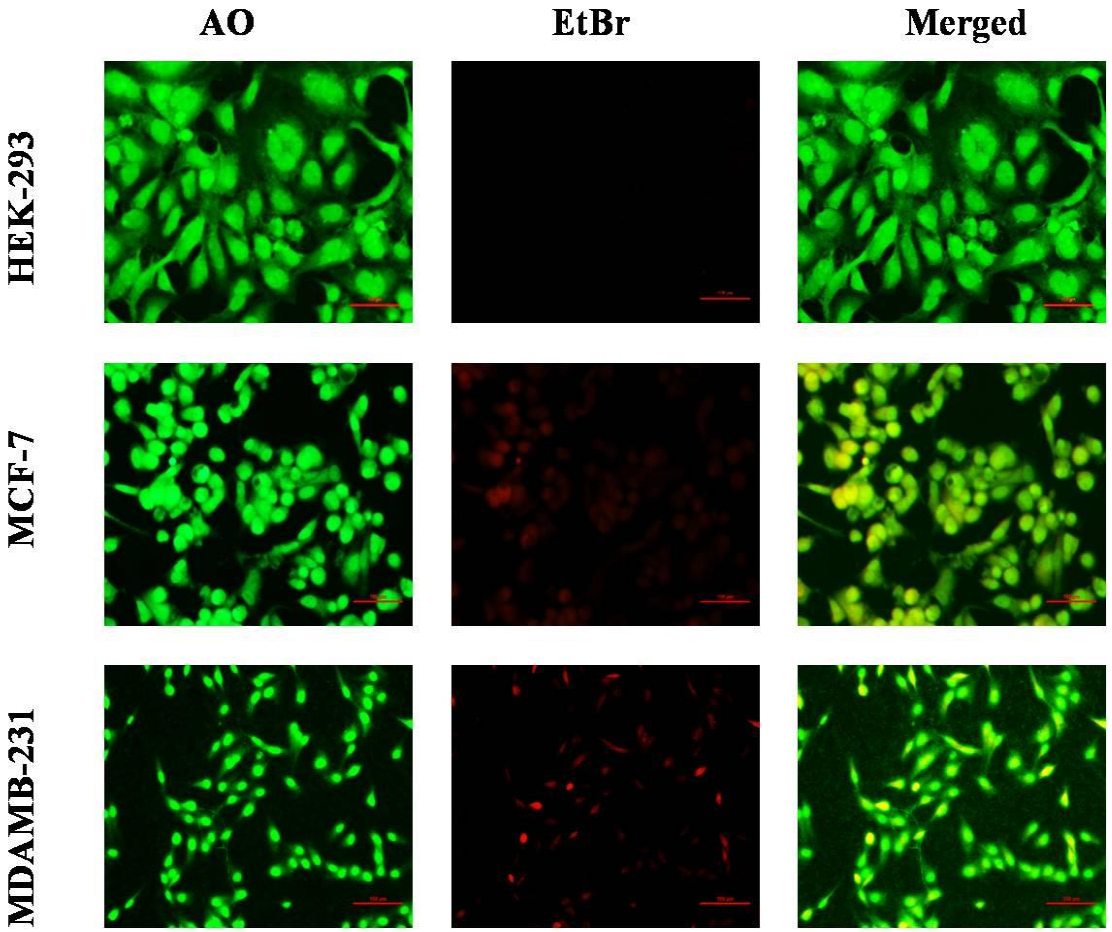


Figure 4.11: Cells treated by in-vitro plantlets and stained by AO and EtBr

CHAPTER-6

DISCUSSION

It has been found out that high concentration of auxin results in good callusing in explants as reported by **[29]**, **[30]** so we carried out our study on the very same lines. Callusing in *Nothapodytes h*ave been reported in the present study where the best results have been achieved using MS medium concentrated with TDZ(1.5mg/L) and IBA (1 mg/L).

Somatic embryogenesis and direct shoot regeneration has been achieved successfully in *Nothapodytes nimmoniana* using growth hormones IBA and KN in the ratio (2:1mg/L). In the same study, we have achieved artificial seed production and germination in *Nothapodytes nimmoniana* plants by using (IBA+KN+GA3) in the ratio (1:3:2) mg/L and (IBA+KN+GA3) in the ratio (1:3:2) mg/L and 0.5% activated charcoal which was in alignment with **[15]**. Quantification of camptothecin from both field grown and tissue cultured plants of *Nothapodytes nimmoniana* was done where highest content of camptothecin was found in field grown leaves (0.87%) and very less amount of camptothecin was quantified in tissue cultured plantlets because of the age difference in field grown and tissue cultured plants.. Secondary metabolites of medicinally important plant posse's anticancer properties as reported by **[31]** where anticancer effects of disogenin found in *Trillium govanianum* (Nag chhatri) have been reported. In this work plant extract of *Trillium govanianum* has been extracted by hydroalcoholic extraction and the effect of the plant extract has been studied on three human cancer cell lines such as MDA-MB-231, MCF-7 and MDCK for its cytotoxicity using MTT assay. Cell viability and morphological changes have been studied for these cell lines. On the very same line our study has been carried out where we have tested the crude plant extract of *Nothapodytes nimmoniana* on MCF-7 and MDA-MB-231 i.e. human breast cancer cell lines as well as cytotoxic effect of crude extract containing camptothecin has been tested on HEK-239 cell line using MTT assay.

Potential anticancer effects of *Nothapodytes nimmoniana* extract was studies on two human breast cancer cell lines (MDA-MB-231 and MCF-7 cells) and subsequently the effect of extract was also analysed on normal epithelial human origin HEK-293. We found that field grown samples showed higher killing efficacy towards cancerous cells as compared to tissue cultured samples because of the difference in camptothecin concentration in both these samples which can be due to the difference in age of field grown (5-6 years) and tissue cultured plants (approximately 2 months) which leads to less efficacy of tissue cultured plants in killing of cancer cells.

CHAPTER 5

CONCLUSION

In various medicinal plants, poor seed germination has contributed towards their reduced cultivation making the plants endangered or extinct. This can be attributed to various unfavourable climatic conditions, seasonal dormancy, heterozygosity of seed, minute seed size, and presence of reduced endosperm etc.

The overall objective of the current study was to develop an in vitro system for optimizing culture conditions for artificial seed formation of *Nothapodytes nimmoniana*. To the best of our knowledge, this is the first report on artificial seed production and germination in *Nothapodytes nimmoniana*. Artificial seeds have been prepared successfully through somatic embryogenesis using sodium alginate beads and calcium chloride solution. The production of artificial seeds and its germination protocol can now be utilized as a raw material for the production of camptothecin. The developed protocol can also be used for large scale production of seeds which can be further cryopreserved and used as a handy raw material.

The tree has gained a lot of attention from giant pharmaceuticals owing to its secondary metabolite camptothecin which has anti cancerous properties. Along with anticancerous study, cytotoxicity assay was carried out for getting an insight into the properties of camptothecin as an effective anticancer agent. A comparative study of the extract obtained from field grown plant and in-vitro grown plantlets have been tested on breast cancer cell lines MCF-7 and MDA-MB-231 and the effects of camptothecin on normal cells was also tested on HEK-293 cell lines. Results showed that field grown plants have higher efficacy in causing cancer cell death as compared to in-vitro grown plantlets. MDA-MB-231 cell line was more sensitive toward the field grown extract in comparison to MCF-7 cell line. Overall the root extract was more effective in killing cancer cells as compared to shoot and in-vitro plantlets.

LIST OF PUBLICATIONS

[1.] Rajta A, Popli D, Kotvi P, Sood H, "Optimization of culture conditions for the production and germination of artificial seed in an important medicinal plant, *Nothapodytes nimmoniana*", International Journal of Innovative Research in science and Engineering", vol. 3, (2017).

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ANNEXURE

Composition of Murashige and Skoog basal medium (MS MEDIUM)

STOCKS	CHEMICALS	ORIGINAL STRENGTH(mg/l)	STOCK(g/l)	FINAL VOLUME
A-10X	KNO_3	1900	19	
	$MgSO_4.7H_20$	370	3.7	100 ml/l
	KH_2PO_4	170	1.70	
B-20X	NH_4NO_3	1650	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
	$FeSO_4.7H_2O$	27.85	2.28	
E-100X	KI	0.83	0.083	100 ml/l
F-100X	H_3BO_3	6.2	0.62	
	$CoCl_2.6H_2O$	0.025	0.0025	
	$ZnSO_4.7H_2O$	8.6	0.86	10 ml/l
	$CuSO_4.5H_2O$	0.025	0.0025	
	$MnSO_4.4H_2O$	22.3	2.23	
	$Na_2MbO_4.2H_2O$	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	