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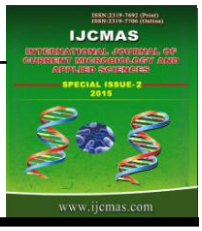

Original Research Article

Phytochemical Characterization and Cells Based Analysis of Bioactive Components of *Nothapodytes nimmoniana* (J. Graham)

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Keywords

Nothapodytes nimmoniana, Camptothecin, Liquid Chromatography Mass Spectrometry, Minimum Inhibitory Concentration, HeLa cells

A B S T R A C T

The main objective of this study is characterization and analysis of *Nothapodytes nimmoniana* by studying its antimicrobial activity, antiglycation activity and also the anticancer effect of its hydro alcoholic extract on HeLa cell lines. For this purpose a plant sample is collected from forests of Mulshi and medicinal plant garden of National Research Institute of Basic Ayurvedic Sciences (NRIBAS, Pune). Extraction of stem, stem bark, leaves of *Nothapodytes nimmoniana* was carried out by using cold decoction and microwave assisted extraction (MAE) methods. To check the presence of an alkaloid, (Camptothecin, mol. wt. 348.352g/mol) in the plant, qualitative assay by using Mayer's reagent was performed and its quantitation by dye based colorimetric analysis was done. Maximum amount of CPT was observed in stem bark of the sample collected from Mulshi which was confirmed by the mass spectrometry (LCMS). Antimicrobial activity was analysed by using Tetrazolium micro plate microbial viability assay against *Pseudomonas aeruginosa* (MIC at 50mg/ml) in leaves and in *Escherichia coli* (MIC at 25mg/ml) and *Pseudomonas aeruginosa* (MIC at 25mg/ml) for stem bark extract. The antiglycation and quenching assay was performed to determine the production of advanced glycation end product, which was reduced in the presence of the stem bark extract (IC_{50} at 10.356µg/ml) and leaves extract (IC_{50} at10.995µg/ml). The cytotoxic effect on HeLa cell lines was determined using MTT assay, where IC_{50} for leaves, stem bark and standard CPT was 178µg/µl, 1540µg/µl, and 29µg/µl respectively.

Introduction

Nothapodytes nimmoniana is a small tree acquiring a height of 4–10 meter, with a spreading crown. This shrubby small tree has broad dark green leaves and flowers (Khan Nazeerullah *et al.*, 2012a,b). This plant is found in Western Ghats of Maharashtra, Tamilnadu, Goa, Kerala, Assam, Jammu and Kashmir where they are

commonly known as Narkya, Kalgur, Ghanera, Amruta (Marathi), Foetid Mappia (English) (Ajay Namdeo *et al.,* 2010), (Khan Nazeerullah *et al.,* 2012a,b). This plant shows many pharmacological activities such as anti-cancer, anti-HIV, antimalarial, antibacterial, antioxidant, and anti-inflammatory, antifungal. It is also useful in curing of anaemia as well as sarcomas such as lungs, breast and uterine cervical cancers (Senthil Rajan Dharmalingam *et al.,* 2014). It belongs to Kingdom: Plantae, Family: Icacinaceae, Genus: *Nothapodytes* and Species: *nimmoniana* (The additional principal chief conservator of forests, Pune, Prioritised plants brochure). This plant is a major source of monoterpene indole alkaloid, named 9-hydroxy camptothecin and mappicin are potent anticancer components. *Nothapodytes nimmoniana* (from India) has been reported to have highest levels of CPT (approx. 0.3% w/w). The empirical formula for CPT is $C_{20}H_{16}N_2O_4$ and formula weight is 348.40 (Fig.1).

The phytochemicals have been found to act as antioxidants by scavenging free radicals, and many have therapeutic potential for free radical associated disorders. It is well known that free radicals are the major cause of various chronic and degenerative diseases such as coronary heart disease, inflammation, diabetes mellitus and cancer. Antioxidant supplements may be used to help the human body in reducing oxidative damage by free radicals and active oxygen. It has been studied that diabetes mellitus is associated with an increased production of free radicals leading to oxidative stress. Thus disturbed balance between radical formation and radical neutralization leads to oxidative damage of cell components such as proteins, lipids and nucleic acids which facilitates formation of advanced glycation end products. The fluorescence quenching assay is the binding of chemical moiety with protein molecule indicates the increased hydrophobic nature on surface of protein, which decreases fluorescence intensity of spectra. Continuous red shift of spectra indicates conformational changes in protein (Kazeem *et al.,* 2012). MTT assay is being performed to access the cytotoxic activity on the cancer cell lines and to identify the IC50 value for the stem bark and leaves extract. The present study is aimed at characterization of *Nothapodytes nimmoniana* using phytochemicals, antioxidants, antimicrobial and cytotoxicity assays for understanding its applicability in human welfare.

Materials and Methods

Collection of plant samples

Plant samples (stem barks and leaves) were collected from forests of Mulshi, Pune and medicinal plants garden of National Institute of Basic Ayurvedic Sciences (NRIBAS), Pune. The plants identity was validated at NRIBAS and specimen was preserved (Fig. 2).

Chemical reagents and standards

Standard camptothecin powder, fetal bovine serum, bromo cresol green, formic acid were obtained from MP Biomedicals (India). Folin's reagent, DPPH reagent, ABTS reagent and HPLC grade acetonitrile, methanol and water that were used for LCMS analysis was of make Sigma-Aldrich. Bovine serum albumin (BSA), 20% Inositol Tetrazolium (INT), minimal essential medium with glutamine and without sodium bicarbonate was used for growing the cells; antibiotics of penicillin and streptomycin, (0.25%) trypsin- EDTA, Dimethyl sulfoxide (DMSO), MTT (3- (4, 5-Dimethyl thiazol-2yl)-2, 5- diphenyl tetrazolium bromide)

was obtained from Hi-Media. 0.04% Trypan blue was obtained from Invitrogen.

Microbial strains, culture medium and inoculum preparation

Pure cultures of five species of microorganisms were obtained from National Research Institute of Basic Ayurvedic Sciences (NRIBAS). The test organisms included *Pseudomonas aeruginosa* NCIM 2036, *Salmonella typhimurium* NCIM 2027, *Escherichia coli* NCIM 2065, *Klebsiella pneumoniae* NCIM 2719, *and Bacillus subtilis* NCIM 64. Each of the organisms was grown in Luria Bertani broth (LB). The flasks were incubated at 37ºC for 24 hours. Optical density (OD) of the medium was determined on biophotometer (Eppendorf).

Tumor cell lines

The HeLa cell line, derived from cervical cancer, was procured from National Centre for Cell Science (NCCS), Pune.

Preparation of extracts

The stem barks and leaves of *Nothapodytes* sp. were dried at room temperature for 24 hours. The bark was removed with the help of the scalpel. Stembark as well as leaves were crushed by using mixer grinder. 22.31gram stem bark and 6.22gram leaves were subjected to extraction. The hydroalcoholic (1:1 v/v ethanol - MilliQ water) solvent was used to maximally yield soluble compounds (Fig. 3).

Cold decoction

The plant materials were soaked in aqueous and alcoholic solvents separately for 48 hours. The plant material was then filtered using Whatmann's filter paper no.1. These aqueous and alcoholic extracts were combined to get a hydro-alcoholic extract.

Microwave Assisted Extraction (MAE)

Extracts of stem bark and leaves were prepared by using microwave assisted extraction. In this method microwave intermittent pulses were given for 3 minutes for three times to the extracts. The extracts obtained were concentrated in rotary evaporator (Lab companion) at 37ºC for 48 hours.

Lyophilization

The hydroalcoholic extracts were kept in deep freezer at -80ºC and were subjected to lyophilisation to obtain concentrated stem bark and leaves extracts (Labconco).

A) Qualitative and Quantitative assay:

Equal volume of Mayer's reagent was added in the sample and centrifuged to get a precipitate of alkaloids. Supernatant was discarded and the pellet was diluted with chloroform.

Dye based colorimetric quantitation

Equal volume of dye was added in the diluted sample and absorbance was taken at 470nm on ELISA plate reader (EPOX) (Mulla *et al.,* 2012).

Liquid Chromatography Mass Spectrometry

The original extract samples were subjected to Liquid Chromatography Mass Spectrometry (Agilent Technologies) to determine the actual content (ng/ml) of the alkaloid compound camptothecin in the extracts of *Nothapodytes nimmoniana*. After setting up various parameters such as from LC section types of solvents, their %

mixing, flow rate, type of column, run time, injection volume, column temperature, pre-processing of samples – and from MS section mass range, ion polarity mode, capillary voltage, fragmentor voltage, gas temperature, gas flow rate, data storage mode, selection of reference masses, preferred chromatograms, collision energy to be applied the LCMS analysis was carried out.

B) Antimicrobial assay:

Preparation of plant sample

100 mg of stembark and leaves powder of *Nothapodytes nimmoniana* was dissolved in 1ml of Milli Q water and was used for analysis.

Tetrazolium microplate microbial viability assay

The minimum inhibitory concentration (MIC) of test microorganisms was determined by using tetrazolium microplate assay. This assay was performed using flat bottom 96-well clear microtitre plates. 150µl of bacterial suspension of respected organisms were added to the wells from column A and H of each row. 100µl working solution of plant extracts (stem bark and leaves) was serially diluted from column A to G. The column H was kept as drug free control. The rows 11 and 12 were kept as a media control (without organisms). The culture plate was sealed with parafilm and kept in incubator at 37ºC for 24 hour.

The MIC of samples was detected following addition (50µl) of 0.2mg/ml of p-iodonitrotetrazolium chloride in all the wells and incubated at 37°C for 30 min. Microbial growth were determined by observing the change of colour p-iodonitrotetrazolium chloride (INT) in the microplate wells (pinkish-red formazan when there is growth and clear solution when there is no growth). MIC was defined as the lowest sample concentration showing no colour change (clear) and exhibited complete inhibition of bacterial growth (Shanmugapriya Perumal *et al.,* 2012).

C) Phytochemical assays:

Determination of Proteins

The proteins are first treated with copper ion in alkali solution, and then aromatic amino acids in the treated sample reduce the phosphomolybdate phosphotungstic acid present in the Folin reagent. The end product of this reaction has a blue colour. The amount of proteins in stembark and leaves extracts was determined according to the method described by Lowry *et al.* (1951). The amount of proteins was calculated by taking the absorbance at 700nm against the standard solution of 50% of bovine serum albumin (BSA).

Determination of Carbohydrates

In hot acidic medium glucose is dehydrated to form hydroxymethyl furfural. This forms a green coloured product with phenol. The carbohydrates content in plant samples was determined according to the method given by Krishnaveni *et al.* (1984). Absorbance was measured at 490 nm. Readings were compared against standard curve made using different concentrations of 25 mg% glucose.

Determination of phenolics

Phenols react with phosphomolybdenic acid in Folin Ciocalteau reagent in alkaline medium and produce blue colour complex (molybdenum blue).

The Phenolics amount in plant samples was calculated by method of Malick and Singh

(1980). Absorbance was read at 650 nm against blank. A standard curve was prepared using different concentrations of 100 mg% catechol.

Determination of glycosides

Glycosides react with Baljet's reagent and develop an orange-red color with (picric acid in alkaline medium).

The glycosidic content of the plant extracts was determined according to the method given by Mosa Qasheesh. Absorbance of color produced is read at 495nm which is proportional to the concentration of the glycosides. The intensity of the color obtained was read against the blank at 495nm by ELISA reader.

Determination of flavonoids

When flavonoids react with aluminium chloride it forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols. Total Flavonoids content in plant extracts was determined according to the method described by Elija Khatiwora *et al.* (2010).

The absorbance is measured at 415nm against the standard quarcetin. Various concentrations of standard quarcetin solution are prepared to make a standard calibration curve. The concentration of flavonoid in the test sample was calculated from the calibration plot.

Determination of Tannins

The tannins in the extract react with potassium ferrycyanide ion and oxidized while the Fe $(CN)_6^{3-}$ is reduced to ferrycyanide ion Fe $(CN)_6^{3-}$. Then this reacts with ferric ion to form ferric ferrycyanide. Tannins in the plant extract were calculated as per method given by Graham (1992). Absorbance was measured at 700 nm against blank. Standard curve was plotted using various concentrations of 0.001M gallic acid.

Determination of lipids

Total lipids content in the plant extracts was determined according to the method described by Ganai *et al.* (2010). This mixture was incubated for 30 min. Absorbance was read at 540nm under ELISA reader.

D) Antioxidant assays

Reducing power/ FRAP (Ferric reducing antioxidant potential assay)

FRAP assay depends upon ferric tripyridyltriazine [Fe- III (TPTZ)] to the ferrous tripyridyltriazine [Fe- II (TPTZ)] by a reductant at low pH [Fe- II (TPTZ)] has an intense blue colour which can be measured at 700nm. The change in absorbance directly related to combined or total reducing power of electron to donate antioxidants present in the extracts. The reducing power of *Nothapodytes nimmoniana* stem bark and leaves extracts was determined according to the method of Oyaizu (1986). The absorbance was measured at 700 nm against Butylated Hydroxy Toluene (BHT) which was taken as a standard.

Inhibition of DPPH radical

The free radical scavenging activity of *N. nimmoniana* extracts was measured by 1; 1-diphenyl-2-picryl-hydrazil (DPPH) as a method described by (Blois, 1958). This assay measures the capacity of the extract to donate hydrogen or to scavenge free radicals. DPPH radical is stable free radical and on reacting with an antioxidant compound which can donate hydrogen, it is

reduced to diphenyl picryl hydrazine (DPPH). The change in colour from deep-violet to light yellow) was measured spectrophotometrically at 517 nm for various concentrations against the standard Butylated Hydroxy Anisole (BHA).

ABTS radical cation decolourisation assay

The assay was performed according to the methods described by Re *et al.* (1999). When an antioxidant scavengers the free radicals by hydrogen donation, the colours in the ABTS•+ assay solution becomes lighter.

In this assay, the oxidant is generated by persulfate oxidation of 2, 2,-azinobis (3-ethylbenzoline-6-sulfonic acid)-($ABTS^2$). The absorbance was read at 745nm and the percentage inhibition was calculated.

Thiobarbituric acid reactive species (TBARS) assay

Oxidative stress in the cellular environment results in the formation of highly reactive and unstable lipid hydroperoxides.

Decomposition of the unstable peroxides derived from polyunsaturated fatty acids results in the formation of malondialdehyde (MDA), which can be quantified colorimetrically at 532nm following its controlled reaction with thiobarbituric acid. TBARS assay was according to the method given by Aazza *et al.* (2011).

E) Glycation assay

Concentrations of Stem bark and leaves extracts (0-15µg/µl) made from a stock of 1 mg/ml of the extracts with 50mg/ml of BSA, 0.1M glucose for one reaction and incubated at 37ºC for 7 days. The absorbance was taken at excitation 375nm and emission scan at 410–500nm wavelength.

Quenching assay

Increasing concentration of extracts from 2 - 10 µl (from stock of 50 mg/ml) was titrated with 20mg/ml of Bovine serum albumin. Fluorescence was taken at excitation 295 nm and emission scan at 300–400 nm in Varioskan fluroscent plate reader (Thermo Scientific). The Analysis was carried out in ORIGIN.61, a tool especially used for quenching assays.

F) MTT Assay:

Maintenance of HeLa cell lines

HeLa cell lines were grown as monolayer in MEM medium with 10% FBS and 2% antibiotics. Stock cultures were sub-cultured every 4th day after harvesting the cells with trypsin-EDTA and then seeding them in T25 culture flask to maintain in exponential phase. The cells were observed regularly under inverted microscope (Nikon eclipse TS100), for its confluence and any contamination.

Assay procedure

The cell suspension was mixed gently and an aliquot was added to the Trypan blue solution (100 µL cell suspension; 100 µL dye) and was then counted in haemocytometer and Countess. MTT, 3- (4, 5-Dimethyl thiazol-2yl)-2, 5- diphenyl tetrazolium bromide) is cleaved by mitochondrial dehydrogenase of viable cells, yielding a measurable purple Formosan product. This Formosan production is proportionate to the viable cell number and inversely proportional to the degree of cytotoxicity (Mosmann, 1983). HeLa cell lines were grown in 96 well microtiter plate (6000 cells/well) for 24 hours after seeding.

The plates were incubated with leaf and bark extracts at different concentrations ranging from 50 to 500 µg and without extracts for 24 and 48 hours respectively (triplicates for each concentration for leaf and bark extracts). The medium was refreshed and 20 µl of MTT (5 µg/ml) was added. The plates were incubated for 4 hours in 5% CO_2 incubator (New Brunswick Galaxy 170S). The Formosan crystals developed were solubilized with 100 µl of DMSO per well and the plate was kept in the dark for another 5 to 10 min. The colour developed was measured in a Varioskan reader (Thermo Scientific) at wavelength 570 nm.

Results and Discussion

Qualitative and quantitative assay

Dye based quantitation

The standard graph of camptothecin was obtained using ELISA based protocol by bromocresol green dye (A_{252}) (Fig 4). The highest amount of camptothecin was observed in *N. nimmoniana* stembark samples (303.81µg/ml) as compared to leaves leaves (31.75 µg/ml) (Fig 5).

Liquid Chromatography Mass Spectrometry based quantitation

To utilize sensitivity and selectivity of LCMS technology the comparative quantitative study was repeated on LCMS with ELISA. based assays Liquid Chromatography Mass Spectrometry (LCMS) was carried out in +ESI mode which showed that molecular weight of alkaloid of interest, camptothecin was 349.1743 m/z with major product ions 231.0965 and 160.0946 m/z that exactly matched with standard CPT (mol. wt. 348.352 g/mol). As every component has a unique fragmentation pattern of its own the quantitation carried out on LCMS is always considered as flawless as there is no scope for getting any false-positive or negative values. High correlation value was obtained for this standard plot (r^2=0.99). The quantitative analysis of CPT in various *N. nimmoniana* extracts showed parallel trend as of the ELISA based method (Fig. 6 and 7). A negligible retention time shift was observed in quantitative LCMS analysis of CPT, for 21 min run time the CPT was throughout getting eluted at 16.418 min (RT) (Fig. 8). Through qualitative mode of operation a number of still unidentified plant metabolites have been found out, they have been separated into groups and their characterization is in progress. In a comparative chromatogram of representation of relative CPT contents in stem bark samples (red), leaves sample (green) and water blank (black), the relative higher CPT content in stem bark samples have been reiterated (Fig 9).

Antioxidant assays: Antioxidant assays were carried out to judge the free radical scavenging potential of *N. nimmoniana* extracts. The standard plots were generated for ABTS assay, DPPH assay, FRAP assay and TBARS assay using suggested standard compounds and protocols and these were used further for calculating the IC_{50} values of extracts (Fig.10). The antioxidant potential of *N. nimmoniana* extracts by ABTS, DPPH and TBARS assays were expressed in terms of IC_{50} value (µg/ml) (Table 1). In ABTS and TBARS assays the stem extract showed comparatively higher antioxidant potential than leaf extracts indicated by lower $IC_{50.}$ For FRAP assay 336.04 and 409.03 µg BHT equivalent/1000 µg of extracts values were obtained for *N. nimmoniana* stembark and leaves extracts.

Phytochemical assays: Tannins, flavonoids, glycosides, carbohydrates, proteins, lipids

and total phenolics are the highly vital classes of phytochemical components; they are directly responsible for imparting medicinal values into plants. Their assessment hence is very much necessary as a part of basic characterization of any plant with medicinal properties.

Phytochemicals quantitation of 7 components was carried out using standard plots and respective quantities were calculated (µg/ml) and arranged in tabular format, except lipids all other 6 components were higher in stem bark extract samples (Table 2).

Antimicrobial assay

The MIC value was determined by observing the pink colour that was generated after addition of 20% cold INT, indicated bacterial growth (+) and colourlessness that indicated inhibition of bacterial growth (-) (Fig. 11a) and b).

N. nimmoniana leaves extract showed antimicrobial activity against *Pseudomonas aeruginosa* (MIC at 50mg/ml) while stem bark showed antimicrobial activity against *Escherichia coli* (MIC at 25mg/ml) and *Pseudomonas aeruginosa* (MIC at 25mg/ml) (Table 3).

Glycation

In BSA-glucose antiglycation assay, as the concentration of extract increased the glycation activity was observed to be decreased. The observed IC_{50} values of extracts of stem bark and leaves are at 10.356µg/ml and 10.995µg/ml respectively (Fig.12).

Quenching assay

In this assay, there is a continuous reduction with concentration of extract in absorbance at 320nm and excitation at 295nm which shows the binding of extract components with the proteins. (Fig. 13)

MTT Assay

The formula used for IC50 calculation was as follows:

Inhibitory ratio = $(A_0 - A_1) \times 100 / A_0$

Where, A_0 is the absorbance of control, $A_{1\ is}$ the absorbance with addition of test sample. Linear regression analysis was used to calculate IC_{50} values (Pratt, 1992). IC_{50} values calculated for leaves, stem bark and standard CPT were 178µg/µl, 1540µg/µl, and 29µg/µl respectively. Leaves showed lesser IC_{50} as compared to stem barks extracts that directly relates to its efficiency as drug in terms of killing of cancerous cells. The morphological changes in HeLa cell lines post MTT assay were quite differentiable.

Methanol extracts of *Nothapodytes nimmoniana* prepared by maceration and vortex mixer showed highest concentration of CPT (Surabhi Sharma *et al.*, 2013). Seasonal variation, geographical and climatic conditions influence the content of camptothecin in *Nothapodytes nimmoniana* (Ajay Namdeo *et al.*, 2010). There were no consistent differences in the CPT content between the sexes (Padmanabha *et al.*, 2006). According to Padmanabha *et al.* (2006) the roots have the highest content of CPT but as the plant have become endangered, to use roots for the purpose of extraction would not be a recommended approach, the quantitative assay and LC-MS analysis suggests the CPT content was found higher in stem bark than in leaves of *Nothapodytes nimmoniana* which correlate with the results obtained in earlier studies (Suhas *et al.*, 2007). Though leaves have lesser CPT content, the IC_{50} values got for

them under antioxidant assays and MTT cell cytotoxicity assay are quite impressive, suggesting probably presence of some non-CPT, still unidentified plant compounds which are in synchronization are giving the effective anticancer-oxidative stress reducer performance than the stem bark extract.

The stem bark and leaves extracts showed presence of various phytochemicals such as flavonoids, lipids, proteins, carbohydrates, tannins, phenolics and glycosides which resembles the study carried out by Khan Nazeerullah *et al.* (2012a,b), Ajay Sharma *et al.* (2008) and Surabhi Sharma *et al.* (2012). Quantitative analysis of these phytochemicals showed that abundance of lipids in leaves extract and high amounts of glycosides in stem bark extract. These results give preliminary insight about various compounds present in *N. nimmoniana* from which biological activities of the compounds can be studied. In comparison to stem bark leaves have only higher lipid content but still they have a comparatively better cancerous cells killing so here there is scope for study of anticancer phytolipids from leaves. It is very well known that alkaloids, tannins and phenols occupy more than 60–80% space of plant derived drugs but if we provide equal attention towards phytolipids then we may come across some useful future drugs from there also. This study is minor lead in that direction.

The antimicrobial assay of extracts showed significant inhibition against *Pseudomonas aeruginosa* and *E. coli* indicating that the plant contains presence of various antibacterial metabolic toxins. The antimicrobial study was previously carried out by Uma *et al.* (2013a,b) and Devi Prasad *et al.* (2013). The quenching graphs suggest that the extract has bound to the proteins in the BSA and has undergone a conformational change affecting the red shift in the graphs, the glycation assay indicates that this binding of protein and the extract has led to reduction in the production of advanced glycation end products. The reduction in glycation activity highlights its benefits in reducing diabetic and cancerous activities in cells. Some important compounds such as phenolics, oligosaccharides and polysaccharides, carotenoids, unsaturated fatty acids and many others have been reported to possess antiglycation activity (Perera *et al.,* 2013). Both synthetic and natural products have been evaluated as inhibitors against the formation of Advanced Glycation End products (AGE). Many plant derived products have been shown to possess hypoglycaemic, hypolipidemic as well as antioxidant properties (Vasu *et al.,* 2005) (Jayashree *et al.,* 2012). The IC_{50} of the leaf, bark and root extract against MDA-MB-231 cells are 450 and 500 µg/ml respectively (Premalakshmi *et al.,* 2012). MTT results for HeLa cell lines show IC_{50} of leaves extract lower than in stem bark extract.

Through this research work we are trying to link up the ayurvedic knowledge to modern sciences. It is known since ages that *Nothapodytes* has beneficial effects but there is necessity of in depth characterization of various phytochemicals present in this plant, as these are the active components by virtue of which plant has got medicinal values and by now we only know handful of active components from this endangered plant. Except CPT very little pharmacological work has been done on *Nothapodytes* components. The variation in plant components in response to various geographical as well as laboratory based parameters is also need to be understand so as to properly govern the medicinal value of the drug to be generated.

Table.1 Antioxidant analysis of *N. nimmoniana*

Sr. no	Constituents	Stembark of *N. nimmoniana* IC_{50} (µg/ml)	Leaves of *N. nimmoniana* IC_{50} (µg/ml)
1	ABTS assay	17.01	21.93
2	DPPH assay	18.82	16.74
3	FRAP assay (µg BHT/1000 µg of extracts)	336.04	409.03
4	TBARS assay	18.56	34.72

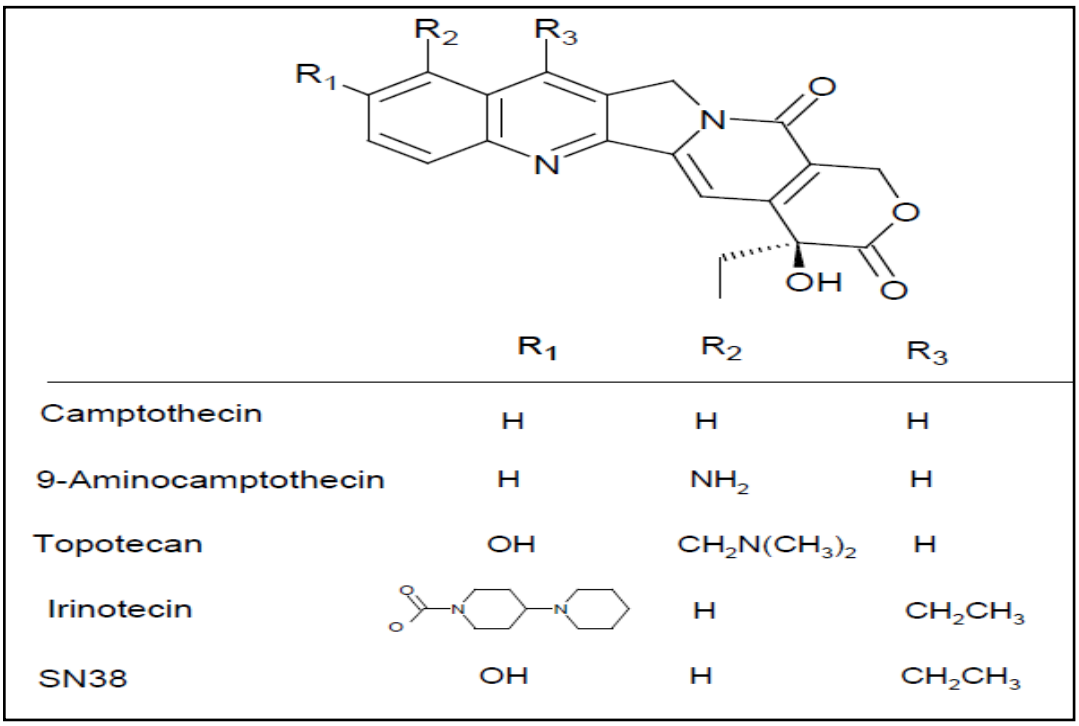
Table.2 Quantitative analyses of various phytochemicals present in *N. nimmoniana* plant parts

Sr no.	Constituents	Stembark of *N. nimmoniana* (µg/ml)	Leaves of *N. nimmoniana* (µg/ml)
1	Tannins	298	203.01
2	Flavonoids	34.89	27.62
3	Glycosides	1325.8	851.7
4	Carbohydrates	148.5	105.42
5	Proteins	7.88	3.87
6	Lipids	466	568.18
7	Phenolics	7.31	5.37

Table.3 Layout and observations made in antimicrobial assay carried out 96 microwell titre plate (SB: stem bark extract; L: leaf extract)

Bacteria		*Escherichia coli*		*Bacillus subtilis*		*Klebsiella pneumonae*		*Salmonella typhimurium*		*Pseudomonas aeroginosa*		Drug free Control	
Concentration		1 SB	2 L	3 SB	4 L	5 SB	6 L	7 SB	8 L	9 SB	10 L	11	12
200mg/ml	A	+	+	+	+	+	+	+	+	+	+	+	+
100mg/ml	B	+	+	+	+	+	+	+	+	+	+	+	+
50 mg/ml	C	+	+	+	+	+	+	+	+	+	-	+	+
25 mg/ml	D	-	+	+	+	+	+	+	+	-	-	+	+
12.5 mg/ml	E	-	+	+	+	+	+	+	+	-	-	+	+
6.25 mg/ml	F	-	+	+	+	+	+	+	+	-	-	+	+
3.125 mg/ml	G	-	+	+	+	+	+	+	+	-	-	+	+
1.56 mg/ml	H	-	+	+	+	+	+	+	+	-	-	+	+

Figure.1 Major chemical constituents reported from *N. nimmoniana* (Nazeerullah Khan *et al.*, 2012a,b)



	R_1	R_2	R_3
Camptothecin	H	H	H
9-Aminocamptothecin	H	NH_2	H
Topotecan	OH	$CH_2N(CH_3)_2$	H
Irinotecin		H	CH_2CH_3
SN38	OH	H	CH_2CH_3

Figure.2 Leaves and stembark of *N. nimmoniana* collected for extraction and preparation of drug are being shed dried

Figure.3 Hydroalcoholic extracts of leaves and stembark of *N. nimmoniana* after the filtration step and prior to freeze-drying (lyophilization)

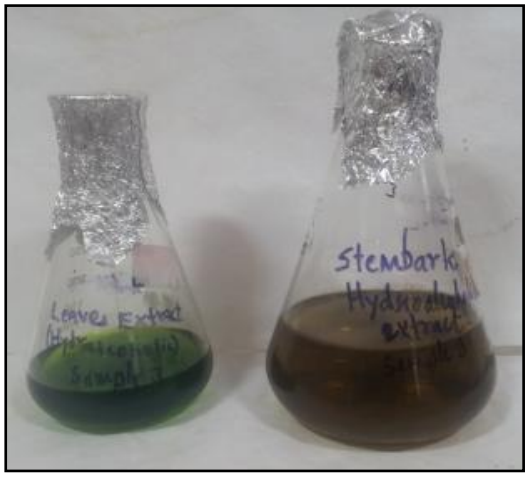


Figure.4 Standard graph camptothecin (CPT) obtained through BCG reagent method on ELISA plate reader platform (A252)

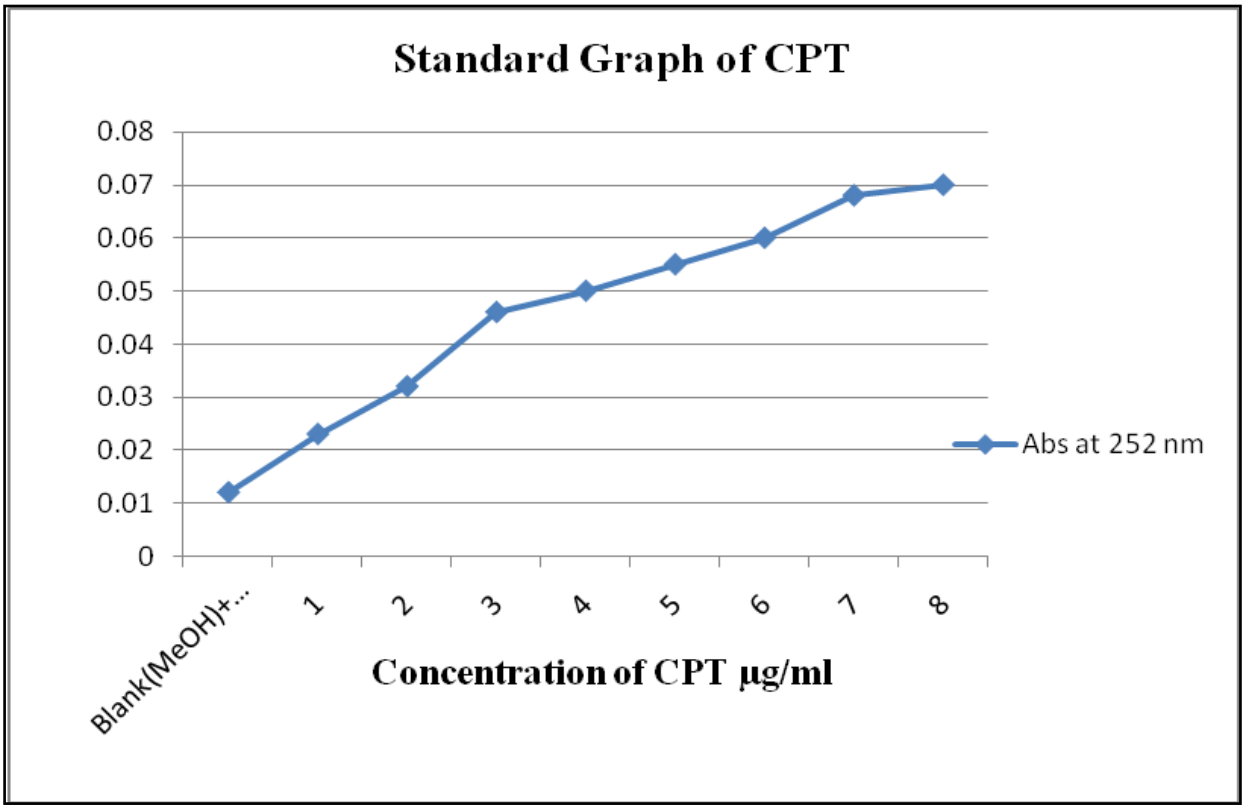


Figure.5 Comparison of CPT content (µg/ml) in various *N. nimmoniana* plant stem bark samples collected from various regions

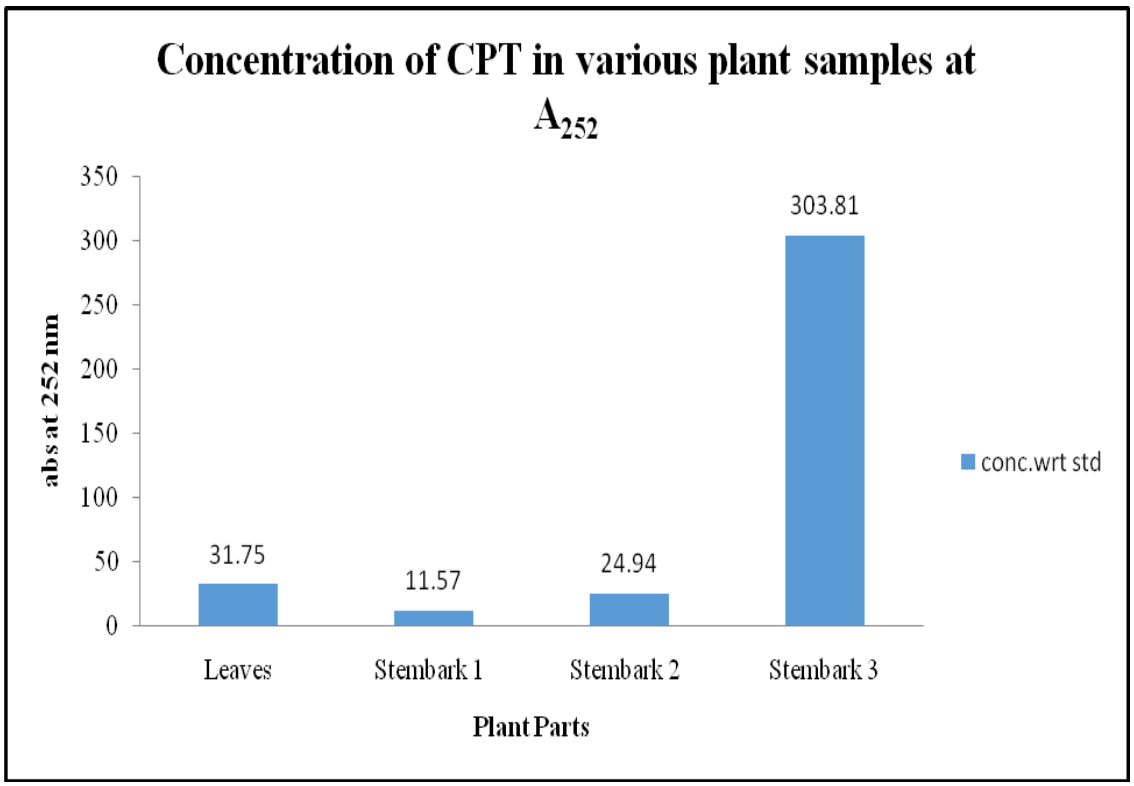


Figure.6 Standard plot of CPT (349.1743 m/z) obtained through LCMS in +ESI mode by working on a range of conc. of CPT 1.00-100.00 ng/ml

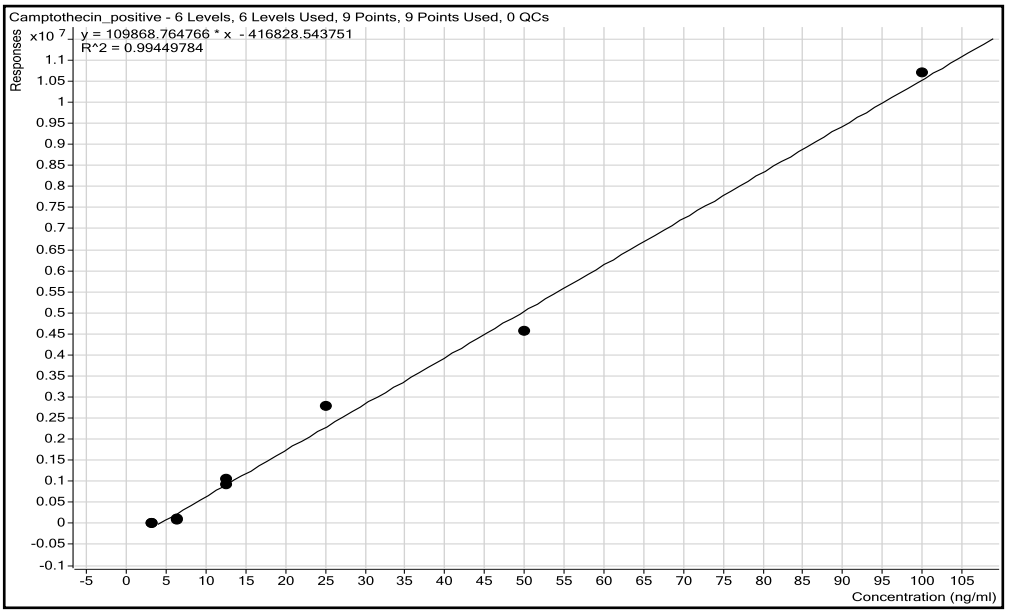


Figure.7 The major product ion of CPT was observed as 305.1283 m/z. The parent daughter ion pair becomes 349.1743 305.1283 m/z. In above product ion spectra the diminished parent CPT ion was indicated in blue dot and on its left all the products ion are lined

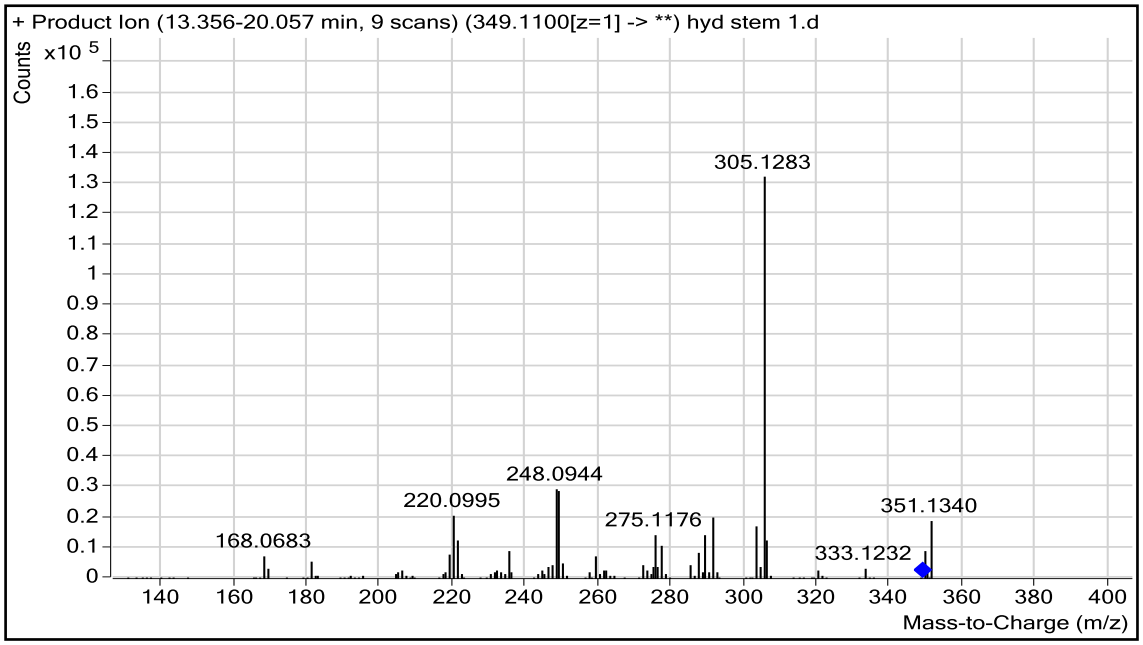


Figure.8 A highly non-significant retention time (RT) variations/shifts were observed in LCMS runs depicting accuracy and consistency achieved in the method developed for analysis of CPT standard and various samples of *N. nimmoniana*

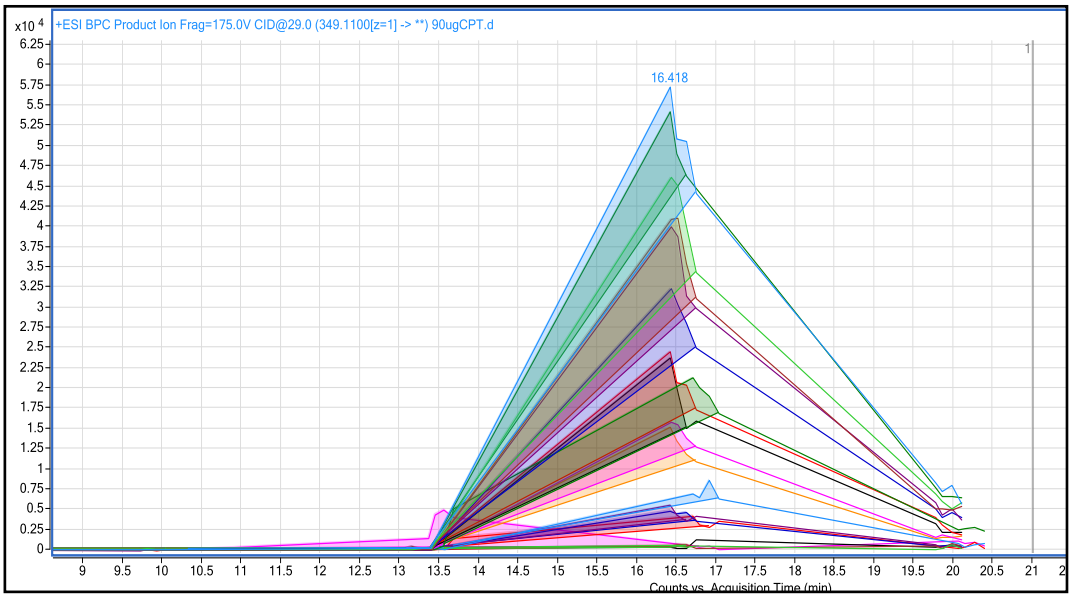


Figure.9 A comparative chromatogram of representation of relative CPT contents in stem bark samples (red), leaves sample (green), and water blank (black)

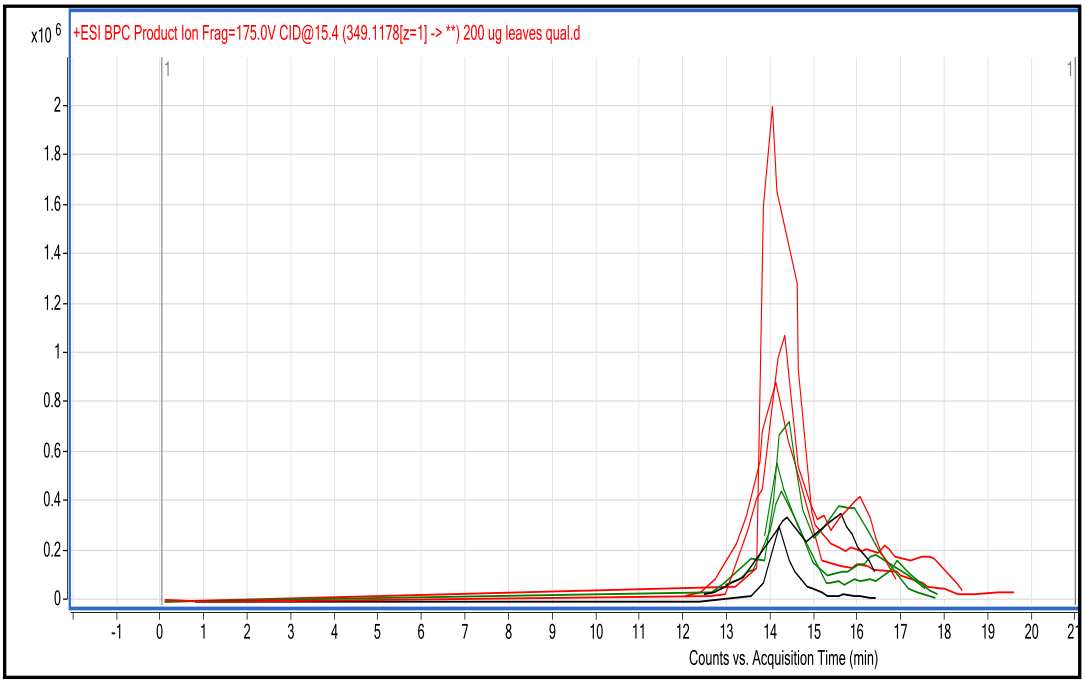


Figure.10 Standard plots of antioxidant assays generated and utilized in phytochemical characterization of *N. nimmoniana* extracts

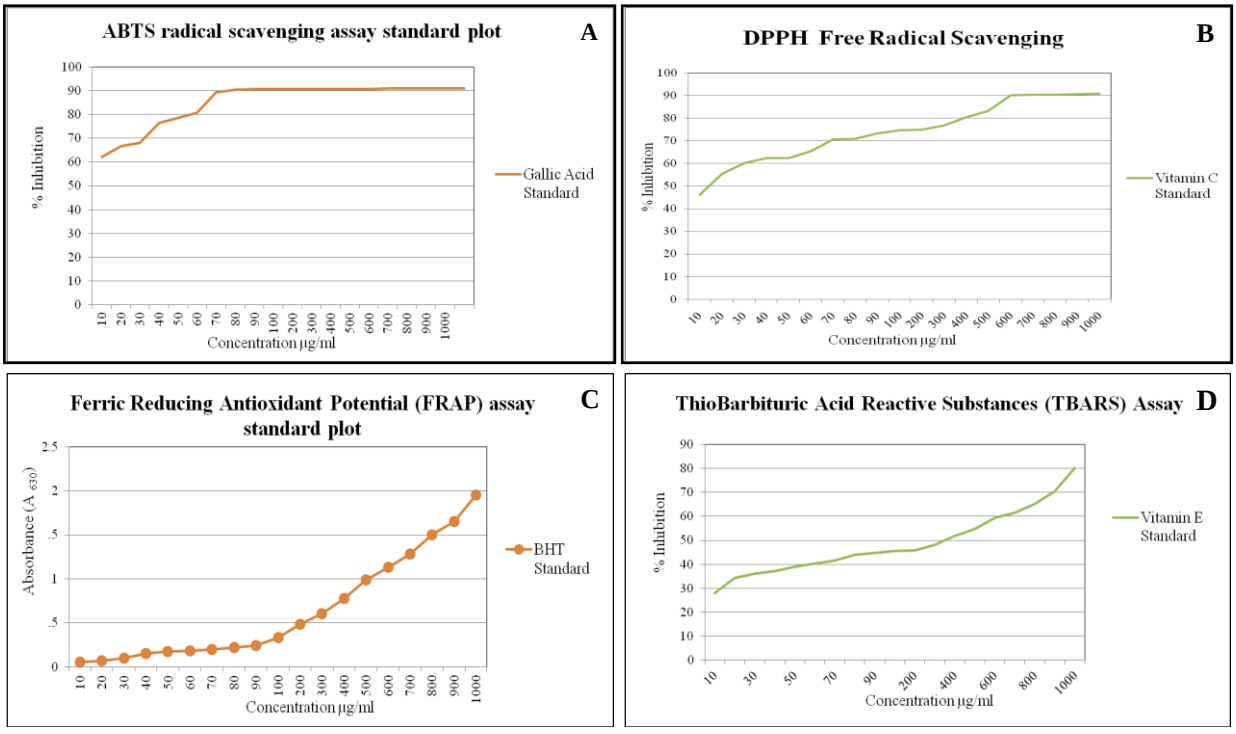


Figure.11a) Tetrazolium based antimicrobial plate assay, the media containing grown pathogens and *N. nimmoniana* extracts were incubated together

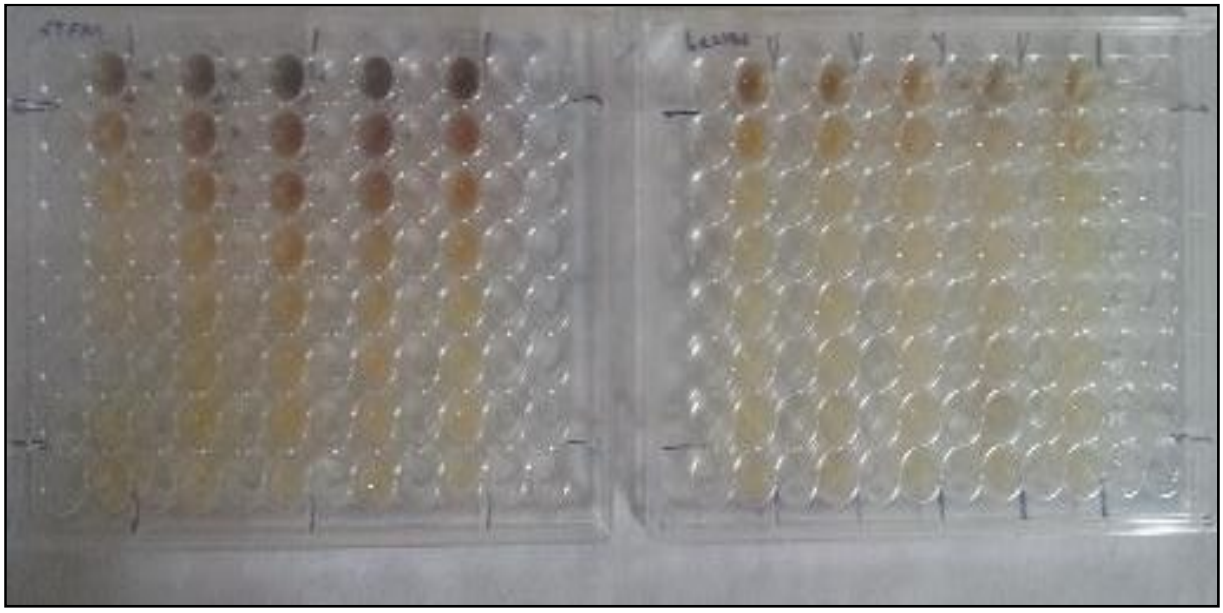


Figure.11b) After overnight incubation, cold 20% tetrazolium salt 2-(4-iodophenyl)-3-(4-nitrophenyl)-5- phenyltetrazolium chloride (INT) was added and changes in colour were recorded

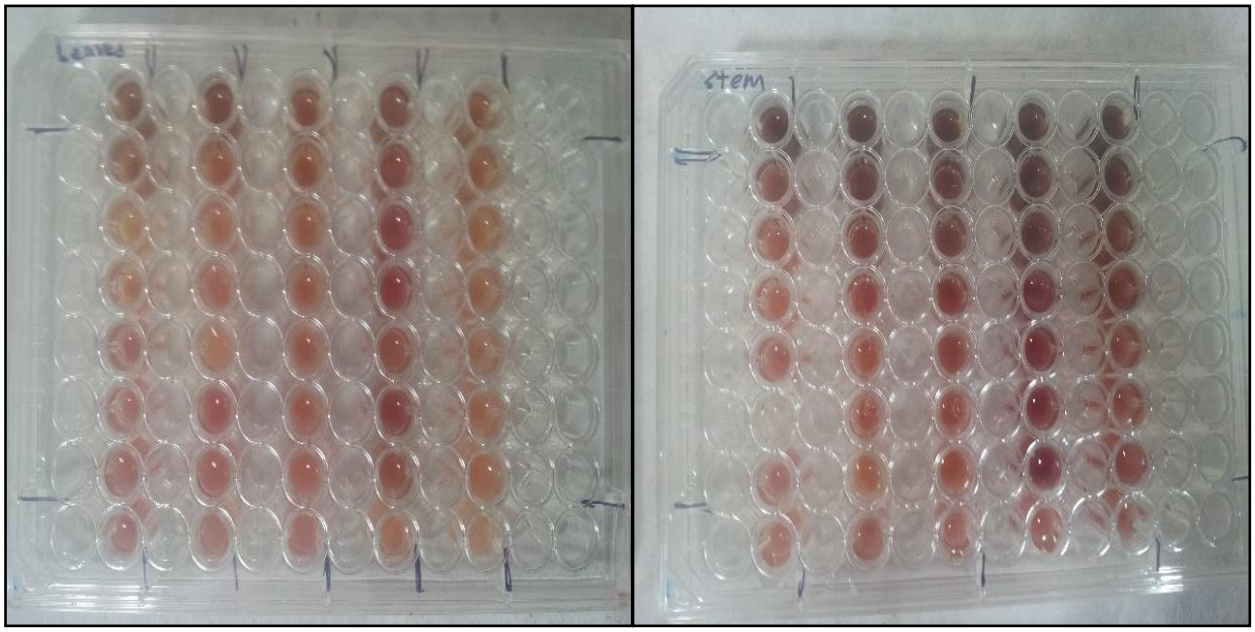


Figure.12 Comparison of antiglycation activity with leaves and stem bark extracts of *Nothapodytes nimmoniana*

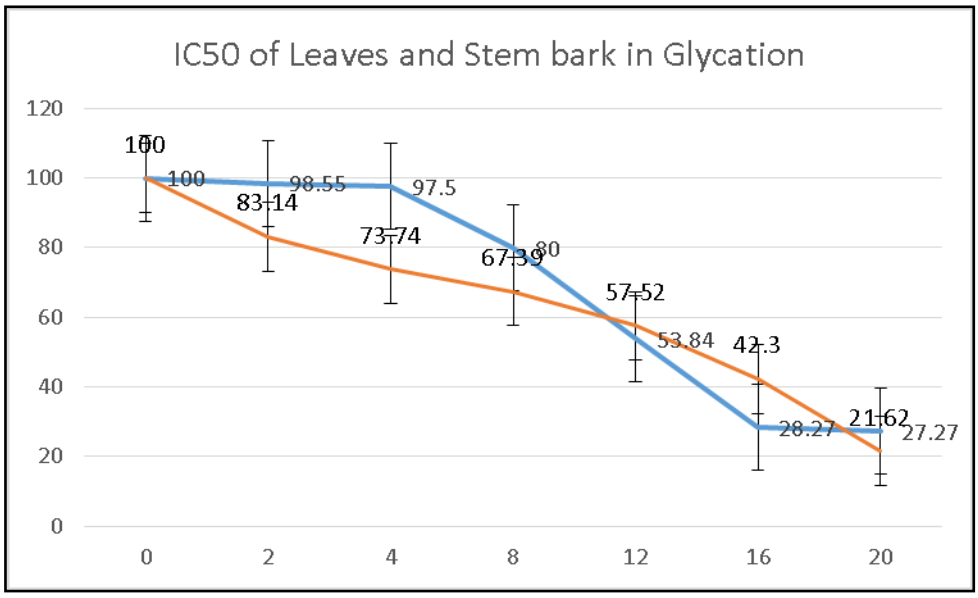
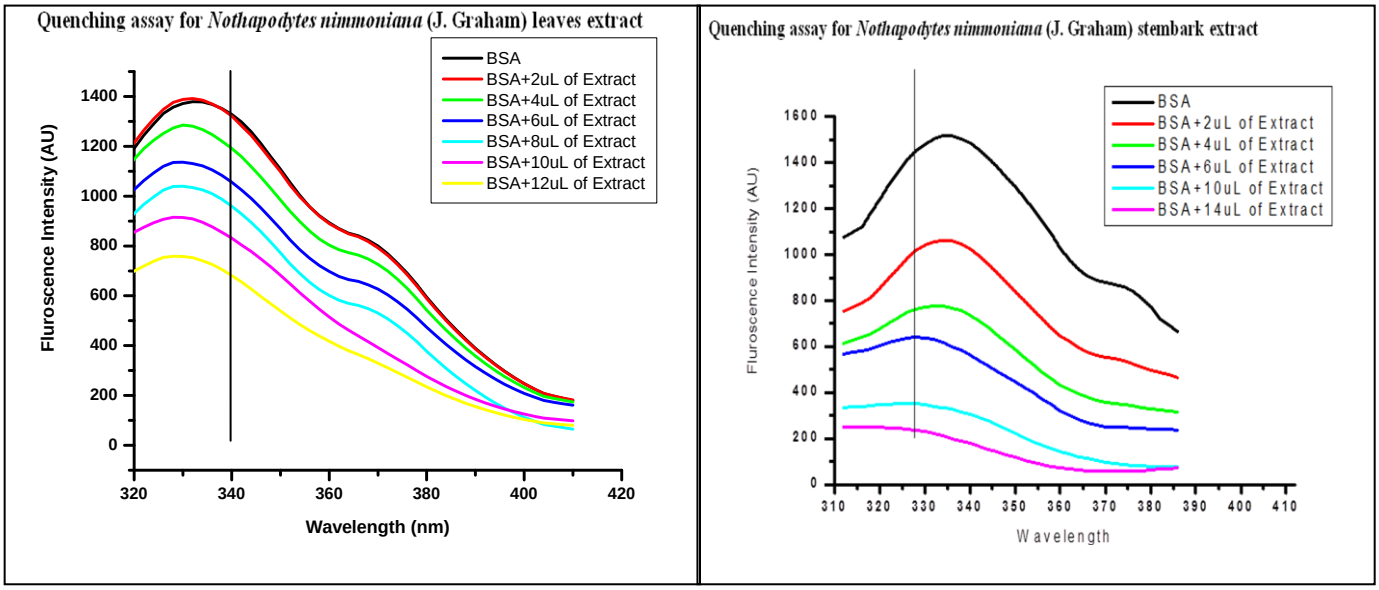


Figure.13 a) and b): Quenching Assay for various concentration of Leaves extract and Stem bark extract



This is an attempt to redefine the beneficial effects of *Nothapodytes* extract in molecular terms, molecular terms involve phytochemical as well as HeLa components. Out of these two, here we dealt with plant components mostly. Several unknown phytochemicals obtained through qualitative analysis of plant extracts are actually offering us various new opportunities for getting a more effective-less harmful anticancer drug leads. Through this characterization of drug value of *Nothapodytes* we are trying to make a basic platform for further studies into its mode of action in cancerous cells. Except LCMS based analysis all the techniques and reagents we used here are simple, easy to obtain/handle or repeat so by combining all these techniques together we are trying to create a workflow for future researchers following which they could characterize their drug maximally and will come to know the "seen-unseen" useful properties of plants. To combine cost effective approaches and to use traditional i.e. ELISA based and modern technologies like LCMS in tandem is a need of the hour to get the maximum information about a medicinal plant so as to better understand its strengths to an extent, and a better understanding will help us to better utilize its potentials against cancer which is now enhancing at alarming rate throughout the world, with minimum harsh effects on host which is the indigenous beauty of most of ayurvedically prescribed medicines.

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