

Quantitative analysis of camptothecin, total phenolic, flavonoid content and antioxidant activity of *Nothapodytes nimmoniana* collected from different geographical locations

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Abstract

Nothapodytes nimmoniana is an endangered medicinal tree endemic to Western Ghats, India and it is one of the important sources for large scale isolation of camptothecin (CPT), a potent antitumor compound. Camptothecin is a monoterpenoid indole alkaloid isolated from various plant species such as *Ophiorrhiza mungo*, *Ervatamia hyneana*, *Nothapodytes nimmoniana* etc. The plant extracts has shown various activities such as antimicrobial activity, anti-malarial activity, anti-inflammatory activity, antitumor/cytotoxic activity, anti-oxidant activity, etc. In the present study, *N. nimmoniana* samples were collected from different geographical locations viz., Amboli, Joida, Jamboti, Panhala and Ulvi of Karnataka and Maharashtra regions and evaluated for camptothecin content, quantitative estimation of total phenolic, flavonoid content and their anti-oxidative potential. The leaves and stem samples were dried, powdered and extracted with five different solvents (Petroleum benzene, Ethyl acetate, Methanol, Butanol, and Water) and accessed for quantitative analysis. Samples were also extracted and analyzed for camptothecin content using HPLC. The antioxidant activity of *N. nimmoniana* was also assessed by DPPH, reducing power and phosphomolybdenum assay. Varied results were observed with different solvents and samples collected from different locations. Aqueous extract of leaf sample from Amboli and aqueous extract of stem sample from Ulvi showed highest phenolic content (11.25g GAE/ 100g and 5.20g GAE/100g) respectively. Highest flavonoid content were observed from aqueous extract of leaf and stem samples collected from Joida and Jamboti (2.07g QE/100g and 1.06g QE/100g) respectively. Among the leaf samples collected from different locations, aqueous extract from Joida showed highest radical scavenging activity of 99.86% followed by aqueous extract from amboli (99.36%), and the lowest activity was observed in the ethyl acetate extract from Joida. Among stem samples, Ethyl acetate showed highest activity with 98.69% and lowest being sample from Jamboti with 14.56%. Reducing power of ethyl acetate extract showed highest while methanol extracts being the lowest among leaf samples. Among stem samples, Butanol extract showed highest activity while aqueous extract showed lowest activity. In Phosphomolybdenum method, butanol extract from amboli showed highest activity in both leaf and stem samples. The highest content of camptothecin was obtained from the stem samples collected from Panhala with 749 µg/g DW and lowest being from Amboli with 130µg/g DW. Among leaf samples, camptothecin content was highest from Amboli with 160 µg/g DW and lowest being from Panhala with 50µg/g DW.

Key words

Nothapodytes nimmoniana, Camptothecin, Antioxidant activity, HPLC analysis, Total phenolic content, Total flavonoid content.

Introduction

Nothapodytes nimmoniana (J. Graham) Mabberly (Syn. *Nothapodytes foetida*) belonging to family Icacinaceae, is an important anti-cancer medicinal plant. This plant is known by different names: Durvasanemara, kodsas, hedare (Kannada), ghenara (Hindi), amruta, narkya, kalgur, kalagaura (Marathi), arali, choral, perum pulagi, kal kurinj (Tamil). The plant is distributed in the major forest divisions of Western Ghats in Karnataka, Kerala, Tamilnadu and Maharashtra [1]. Because of destructive harvesting and habitat loss, the tree has been placed in 'Endangered' category [2]. *N. nimmoniana*, a tree crop, has got a 7–8 yr long gestation period. *N. nimmoniana* is a rich source for the potent alkaloid camptothecin (CPT) and 9-methoxy camptothecin. It also contains 3-ketooctadec-cis-15-enoic acid (16.0%), palmitic acid (12.3%), stearic acid (4.2%), oleic acid (16.2%), linoleic acid (11.6%) and linolenic acid (39.7%). Other chemical constituents isolated from this plant are acetylcamptothecin, (+)-1-hydroxypinoresinol, Ω -hydroxypropioquiainone, p-hydroxybenzaldehyde, scopoletin, sitosterol, sitosteryl- β -D-glucoside, trigonelline. Camptothecin (CPT) yield depends on the age of the plant. If the plant age is high, then percentage yield will also be higher. Highest percentage of CPT is produced by root wood, and minimum by the leaves. Different extracts of *N. nimmoniana* showed different activities such as antimicrobial activity, antimalarial activity, anti-inflammatory activity, antitumor/cytotoxic activity, anti-oxidant, anti-anaemic activities, etc [3]. Most of the activities shown by the plant are because of the presence of alkaloids, especially camptothecin.

Camptothecin (CPT) is a monoterpenoid indole alkaloid originally isolated from *Camptotheca acuminata*. Various other plant species such as *Ophiorrhiza mungo*, *Ervatamia hyneana* and *Nothapodytes foetida* are also known sources of CPT. CPT is known for its amazing inhibitory action against tumour cells by blocking the eukaryotic topoisomerase-I and also for its activity against the human immunodeficiency virus (HIV) [4]-[5]. Several derivatives with better activity than the parent compound are developed for the treatment of cancer. Topotecan and Irinotecan are the two major water soluble anti-cancer drugs used for the treatment of cervical, ovarian, lung and colorectal cancers [6]. Since there is no convenient synthetic source for CPT, there is dependence for raw materials from natural

populations. As CPT accumulates in shoot and root of *N. nimmoniana*, whole tree is cut to generate biomass for extraction.

A special feature of higher plants is their capacity to produce number of organic molecules with high structural density called as secondary metabolites. The accumulation of these phytochemicals in plants has gathered knowledge for the production of desired phytoconstituents with the potential to act against multi resistant free radicals. Reactive oxygen species (ROS) such as singlet oxygen, Superoxide ion, Hydroxyl ion and Hydrogen peroxide are highly reactive, toxic molecules which are generated normally in cells during metabolism. However, a higher amount of ROS leads to severe damage to proteins, lipids, enzymes etc. by covalent bonding leading to tissue damage. Natural antioxidants have been much of interest because of their ability to scavenge these free radicals [7]. Free radicals have been involved in the development of number of diseases, such as cancer, neurodegeneration and inflammation [8]-[10], which lead to the study of antioxidants for the prevention and treatment of diseases. The presence of antioxidants such as phenolics, flavonoids, tannins etc. in plants may play a major role in providing protection against number of diseases [11]. Therefore medicinal plants are being investigated for their antioxidant properties and the demand for these plants is increasing [12]. In view of the this, the present study is aimed at quantification of the active metabolites specially, the anticancer compound and natural antioxidants along with their antioxidant potential from *N. nimmoniana* collected from different geographical location.

Materials and methods

Chemical reagents and Standards

Standard CPT (HPLC grade) was obtained from Sigma-Aldrich (India). Aluminium chloride, ammonium molybdate, DMSO, DPPH reagent, FC reagent, ferric chloride, potassium acetate, potassium ferricyanide, sodium carbonate, sodium phosphate, sulfuric acid, Trichloro acetic acid were of analytical grade. Standards of gallic acid, quercetin, and α -tocopherol were obtained from CDH. Acetonitrile, methanol and water were of HPLC grade.

Sample collection

The samples were collected from different geographical regions viz. Amboli, Panhala, Jamboti (Maharashtra), Joida and Ulvi (Karnataka) respectively during the month of August

(Fig 1). The collected plant material was cleaned and dried at room temperature for 2 weeks, once the plant material was dried; it was finely powdered using mixer grinder. 1g of the powdered material was taken in different conical flasks, to that 10ml of different solvents i.e., Methanol, Ethyl acetate, Butanol, Petroleum benzene and Water were added. The mixture was kept on orbital shaker for 24 hours for complete extraction. After 24 hours, the mixture was filtered to get respective extracts.



Figure 1: *N. nimmoniana* samples collected from different geographical locations

Quantitative analysis

Determination of Total Phenolic Contents

The total phenolic contents were determined by the Folin-Ciocalteu (FC) method [13] with some modifications. Distilled water (3.10ml) was mixed with extract (100 μ l) then, 200 μ l of FC reagent was added and incubated at room temperature for 5 minutes. Then, 600 μ l of 15% sodium carbonate was added and mixed well. Again the solutions were incubated at room temperature for about 1 to 2 hours. Absorbance of the developed blue color was determined at 765nm using UV-spectrophotometer (Elico, India). The concentration of the total phenolic content was determined as mg of Gallic acid equivalent by using an equation obtained from gallic acid calibration curve. The estimation of phenolic compounds was carried out in triplicate and the results were averaged.

Determination of Total Flavonoid Contents

Total flavonoid content was determined by using aluminium chloride colorimetric method [13] with some modifications. 100 µl of extract was mixed with 0.1ml of 10% aluminium chloride and 0.1ml of 1M potassium acetate. Then, 4.70ml of distilled water was added and mixed well. The solutions were incubated at room temperature for 30 minutes. After incubation the absorbance was measured at 415 nm using UV-spectrophotometer (Elico, India). The concentration of the total flavonoid content was determined as mg of Quercetin equivalent by using an equation obtained from quercetin calibration curve. The estimation of total flavonoids was carried out in triplicates and the results were averaged.

Antioxidant Activity

Radical scavenging assay

The method of radical scavenging assay was determined as described by Jung *et al.*, [13] with some modifications. The antioxidant activity of the extracts was determined based on the scavenging activity of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical. Briefly 100 µl of the extracts were taken in different test tubes with 4 ml of a 0.006% MeOH solution of DPPH. Water/methanol in place of the extracts was used as control. Absorbance at 517 nm was determined after 40 min of incubation at room temperature. Radical scavenging activity was expressed as the inhibition percentage and was calculated using the formula:

$$\text{Radical scavenging activity (\%)} = [(A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}}] \times 100$$

Where A_{blank} is the absorbance of the control at 40 min reaction (containing all the reagents except the test compound), and A_{sample} is the absorbance of the sample at 40 min.

Assay of reductive potential

The reductive potential of the *N.nimmoniana* extracts were determined as described by Jung *et al.*, [13] with some modifications. 100 µl of extract was added with 900 µl of distilled water, 2.5ml of phosphate buffer (pH 6.6), and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50⁰C for 20 minutes. Then, 2.5ml of TCA (10%) was added to the mixture and centrifuged at 650 g for 10 min. The upper layer of the solution (2.5 ml) was mixed with 2.5 ml distilled water and 0.5ml of 0.1% FeCl₃, Absorbance was measured at

700nm against a blank. Increased absorbance of the reaction mixture indicates increased reducing power. All analysis were run in triplicates and averaged.

Evaluation of antioxidant activity by Phosphomolybdenum method

The total antioxidant activity of the *N.nimmoniana* extracts was determined as described by Jung *et al.*, [13] with some modifications. 0.1ml of extract was combined with 1ml of reagent solution (0.6M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes were incubated at 95⁰C for 90 minutes. Then the tubes were cooled to room temperature. The absorbance of the solution was measured at 695 nm against blank. Blank contains 1ml of reagent solution and incubated under same conditions. The antioxidant activity was determined as mg of α -tocopherol equivalent by using an equation obtained from α -tocopherol calibration curve. The antioxidant activity was carried out in triplicates and the results were averaged.

Extraction and quantification of camptothecin

Sample preparation: 2g of powdered material was taken and 10ml of methanol was added to it. The mixture was warmed to $60 \pm 2^{\circ}\text{C}$ for 5 minutes, cooled to room temperature and centrifuged at 5000 rpm for 15 minutes to obtain 20% extract. After centrifugation, the samples were dried at room temperature and stored at 4⁰ C for further use. When needed, the samples were dissolved with known volume of methanol for further experimentation [14].

The HPLC analysis was performed on Thermo scientific chromatographic system. Chromatographic separation was achieved on phenomenex column 18 (5 μm , 4.6 \times 250mm). Mobile phase consisting of acetonitrile: water (40:60) with an injection volume of 20 μl . A chromatographic condition of 1ml/min flow rate at 254nm. Camptothecin was accurately weighed and dissolved in few drops (50 μL) of DMSO by warming and the volume was made with methanol to produce a standard stock solution. The stock solution of CPT was serially diluted to obtain working concentrations (10, 20, 30, 40, and 50 $\mu\text{g/ml}$) for plotting calibration curves. The camptothecin concentrations of the samples were calculated based equation obtained from the standard calibration curve.

Results and discussion

Quantitative analysis

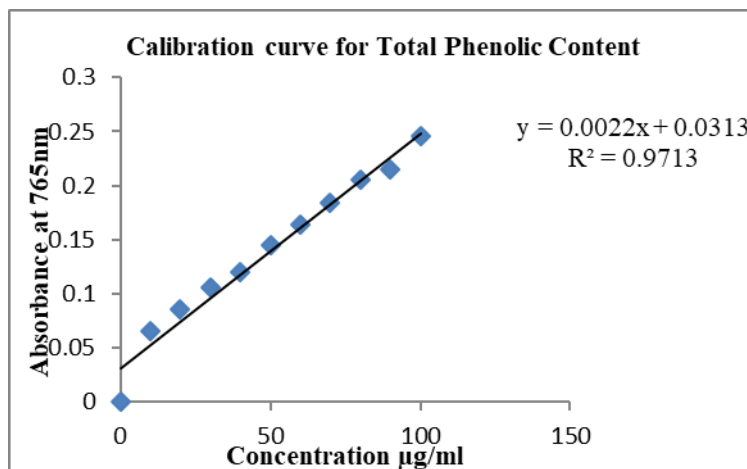
Determination of Total Phenolic Content

The total phenolic content of the different extracts from leaves and stem samples of *N. nimmoniana* collected from different geographical locations was determined by FC method. The results are expressed as equivalents of gallic acid. Among the five extracts from both leaf and stem samples, aqueous extract of leaf samples from Amboli showed highest (11.25 g/ 100g DW) amount of phenolic compounds followed by methanol extract from Joida (6.29 g/100g DW), butanol extract from Jamboti (4.07 g/100g DW), petroleum benzene extract from Panhala (2.16 g/100g DW) and ethyl acetate extract from Joida (1.75 g/100g DW). From stem samples, aqueous extract from Ulvi showed highest (5.20 g/100g DW) followed by methanol extract from Amboli (2.09 g/100g DW), ethyl acetate extract from Joida (1.75 g/100g DW), butanol extract from Jamboti (1.73 g/100g DW) and petroleum benzene extract from Ulvi (0.76 g/100g DW) (Table 1). Phenolic compounds have redox properties, which allow them to act as antioxidants (Soobrattee, 2005). Total phenolic concentration could be used as a basis for rapid screening of antioxidant activity because their activity is mediated by hydroxyl groups. The antioxidant activity of phenolic compounds and their derivatives depends on the number and position of the hydroxyl groups bound to the aromatic ring, the binding site and mutual position of hydroxyl groups in the aromatic ring and the type of substituents [15]-[16]. The Folin-Ciocalteu (FC) reagent is used to estimate the amount of phenolic compounds present in an extract. However, the assay have been non-specific not only to polyphenols but to any other substance that could be oxidized by FC reagent [17]-[18].

Table 1: Total phenolic content of different extracts from leaf and stem samples of *N. nimmoniana*

Sample locations	Total phenolic content (g/100g DW) expressed as gallic acid equivalents (Absorbance at 765nm)									
	Methanol		Petroleum benzene		Ethyl acetate		Butanol		Aqueous extract	
	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem
Jamboti	5.475	1.508	0.855	0.531	1.158	0.530	4.075	1.735	4.312	1.221
Panhala	5.751	0.515	2.165	0.549	1.370	0.521	2.288	0.804	6.612	2.281
Ulvi	5.100	1.000	1.016	0.766	1.270	0.645	2.347	0.598	10.130	5.207
Joida	6.294	1.790	0.625	0.717	1.755	1.755	2.047	0.525	4.309	1.001
Amboli	3.034	2.090	1.039	0.481	1.182	0.363	1.651	0.635	11.251	4.008

collected from different geographical locations



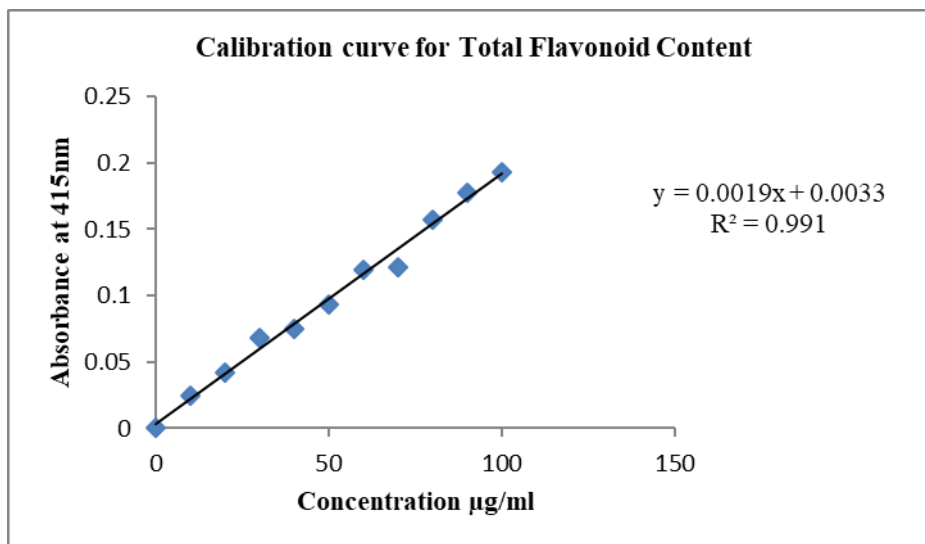
Calibration curve - $Y = 0.0022X + 0.0313$

$R^2 = 0.9713$

Determination of Total Flavonoid content

The total flavonoid content of the different extracts from leaves and stem samples of *N. nimmoniana* collected from different geographical locations was determined by aluminium chloride method. The results are expressed as equivalents of quercetin. Among the five extracts from both leaf and stem samples, aqueous extract of leaf samples from Joida showed highest (2.07 g/100g DW) amount of flavonoid compounds followed by methanol extract from Panhala (1.90 g/100g DW), butanol extract from Jamboti (0.96 g/100g DW), ethyl acetate extract from Ulvi (0.80 g/100g DW) and petroleum benzene from Amboli (0.63 g/100g DW). From stem samples, aqueous extract from Jamboti showed highest (1.06 g/100g DW) amount of flavonoid compounds followed by methanol extract from Jamboti (0.79 g/100g DW), butanol extract from Panhala (0.76 g/100g DW), ethyl acetate extract from Jamboti and Joida (0.75 g/100g DW) (Table 2). Flavonoids including flavones, flavonols and condensed tannins are plant secondary metabolites; their activity depends on the presence of free hydroxyl groups, especially 3- OH groups [19].

Table 2: Total flavonoid content of different extracts from leaf and stem samples of *N. nimmoniana* collected from different geographical locations



**Calibration curve- $Y = 0.0019X + 0.0033$
 $R^2 = 0.991$**

Antioxidant activity

Radical scavenging assay

The free radical scavenging activity of the different extracts of *N. nimmoniana* collected from different geographical locations was tested using DPPH method. DPPH is usually used as a substrate to evaluate antioxidative activity of antioxidants. The method is based on the reduction of methanolic DPPH solution in the presence of a hydrogen donating antioxidant, due to the formation of the non-radical form DPPH-H by the reaction. The extracts were able to reduce the stable radical DPPH to the yellow coloured diphenyl picryl

Sample locations	Total flavonoid content (g/100g DW) expressed as quercetin equivalents (Absorbance at 415nm)									
	Petroleum benzene		Ethyl acetate		Methanol		Butanol		Aqueous extract	
	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem
Jamboti	0.587	0.697	0.686	0.759	1.412	0.791	0.967	0.616	1.970	1.063
Joida	0.573	0.650	0.558	0.759	1.597	0.614	0.947	0.541	2.071	0.986
Amboli	0.636	0.565	0.796	0.492	0.843	0.600	0.884	0.478	2.067	1.013
Ulvi	0.579	0.540	0.804	0.533	1.608	0.667	0.555	0.758	1.923	0.958
Panhala	0.579	0.531	0.579	0.515	1.901	0.588	0.730	0.769	1.914	0.878

hydrazine. Among the leaf samples collected from different locations, aqueous extract from Joida showed highest radical scavenging activity of 99.86% followed by ethyl acetate extract from Panhala (99%), and the

lowest activity was observed in the ethyl acetate extract from Joida (Fig 2). The variations in the antioxidant activity from the stem samples of different locations and different extracts were observed (Fig 3).

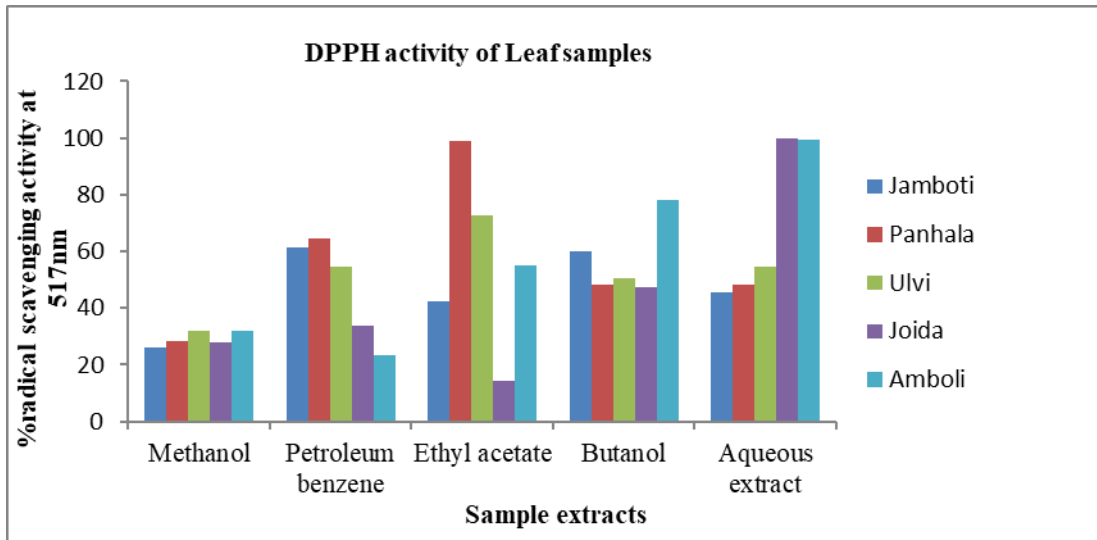


Figure 2: Free radical scavenging activity of the leaves extracts from *N. nimmoniana* collected from different geographical locations by DPPH method. Each sample was assayed in triplicate and the results are expressed as means of three parallel measurements

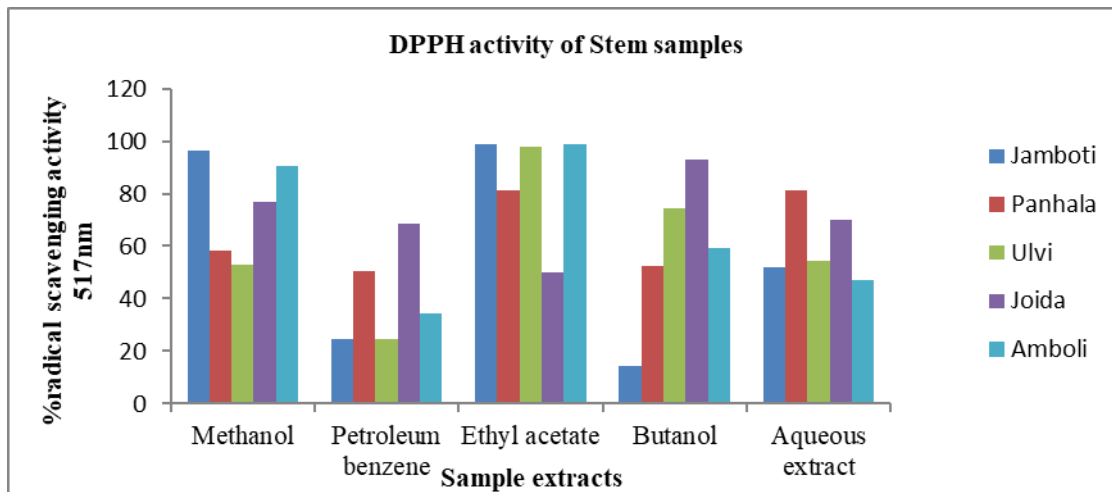


Figure 3: Free radical scavenging activity of the stem extracts from *N. nimmoniana* collected from different geographical locations by DPPH method. Each sample was assayed in triplicate and the results are means of three parallel measurements

Assay of reduction potential

The reducing power of the different extracts of leaf samples are in the order Ethyl acetate extract < Petroleum benzene extract < Butanol extract < Aqueous extract < Methanol extract (Table 3). The reducing power of the different extracts of stem samples are in the order Butanol extract < Ethyl acetate extract < Methanol extract < Petroleum benzene extract < Aqueous extract (Table 4). Various mechanisms, including reducing capacity, prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction and radical scavenging have been claimed to explain the antioxidant activities [20]. The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom [21].

Table 3: Assay of reductive potential of leaf samples collected from different geographical locations of *N. nimmoniana*

Sample locations	Petroleum benzene	Ethyl acetate	Methanol	Butanol	Aqueous extract
	Stem	Stem	Stem	Stem	Stem
Jamboti	0.3048±0.001	0.2356±0.001	0.2830±0.001	0.7958±0.002	0.7828±0.002
Joida	0.3102±0.001	0.2152±0.001	0.4130±0.005	1.0418±0.021	0.4482±0.002
Amboli	0.4090±0.006	0.3542±0.003	0.4516±0.003	0.5550±0.002	2.2718±0.004
Ulvi	1.4944±0.004	1.2366±0.001	0.4378±0.001	1.1530±0.002	1.2256±0.001
Panhala	0.3868±0.005	0.2916±0.003	1.3218±0.001	0.5516±0.007	1.0936±0.005

Table 4: Assay of reductive potential of stem samples collected from different geographical locations of *N. nimmoniana*

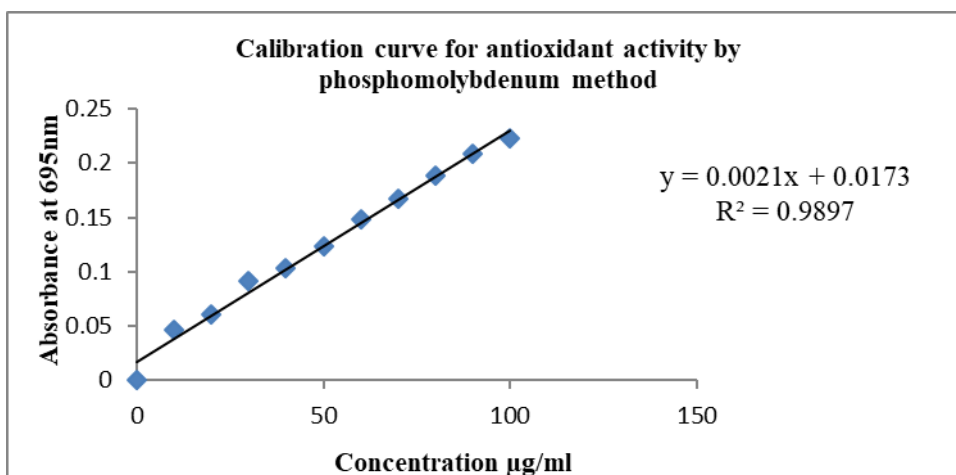
Antioxidant activity by phosphomolybdenum method

Antioxidant activity of different extracts of both leaf and stem samples of *N. nimmoniana* collected from different locations was determined by phosphomolybdenum method, which is based on the reduction of Mo (IV) to Mo (V) by the sample and the subsequent formation of green phosphate/Mo (V) compounds with a maximum

Sample locations	Petroleum benzene	Ethyl acetate	Methanol	Butanol	Aqueous extract
	Leaf	Leaf	Leaf	Leaf	Leaf
Jamboti	0.4892±0.005	0.2342±0.047	0.3542±0.002	0.3768±0.001	0.2772±0.002
Joida	0.2154±0.001	0.3182±0.001	0.3912±0.004	0.7370±0.001	0.3498±0.005
Amboli	0.3890±0.006	0.2294±0.005	0.2730±0.002	0.5168±0.001	1.1102±0.002
Ulvi	0.2710±0.004	0.3884±0.006	1.0348±0.002	0.3552±0.001	0.5842±0.010
Panhala	2.4822±0.002	0.3748±0.001	1.4478±0.007	0.9300±0.001	0.3036±0.003

absorption at 695 nm. The results are expressed as equivalents of α -tocopherol. Among the extracts of both leaf and stem samples, butanol extract from Jamboti showed highest activity (Table 5).

Table 5: Antioxidant activity of *N. nimmoniana* leaf and stem extracts collected from different geographical locations by phosphomolybdenum method



Sample locations	Antioxidant activity [as equivalent to α -tocopherol (mg/100g)] (Absorbance at 695nm)									
	Petroleum benzene		Ethyl acetate		Methanol		Butanol		Aqueous extract	
	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem
Jamboti	53.85	50.52	63.00	72.14	50.14	55.28	142.90	127.00	61.00	61.28
Joida	55.76	59.57	56.71	56.23	63.57	54.80	72.71	81.19	79.00	73.09
Amboli	44.04	80.14	63.00	69.47	53.47	51.66	159.76	150.42	69.28	69.57
Ulvi	44.80	52.71	45.28	63.47	56.71	55.28	112.71	107.76	71.09	45.66
Panhala	93.47	56.90	75.28	50.61	51.47	52.23	71.19	55.76	80.90	50.23

Calibration curve - $Y = 0.0021x + 0.0173$

$R^2 = 0.9897$

Quantification of camptothecin using HPLC:

Quantification of camptothecin was performed using HPLC. The HPLC data revealed that leaf sample from Amboli showed highest amount of camptothecin and sample from Panhala showed lowest quantity (Fig4). Among the stem samples analyzed using HPLC, the data revealed that the sample from Panhala showed highest amount of camptothecin whereas sample from amboli showed lowest amount of camptothecin (Fig 5). The HPLC profile of all samples is given in fig.6. Among many camptothecin producing plants, *N.nimmoniana* has the highest CPT accumulation [22]. Many authors have reported that camptothecin content is high in stem

followed by leaves. Geographical locations and climatic conditions also play a vital role in content of CPT accumulation in *N.nimmoniana* [14], [23].

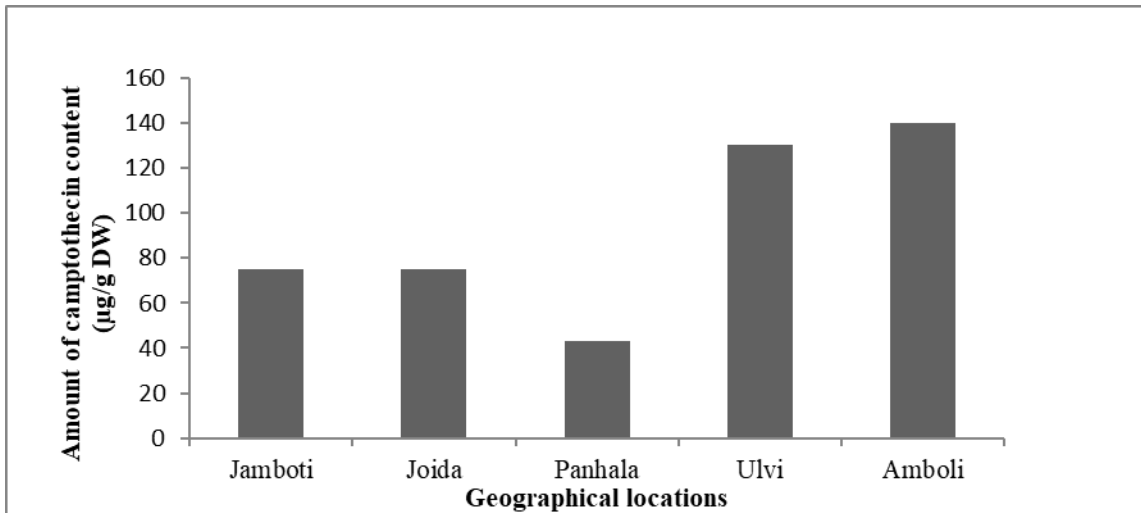


Figure 4: Quantification of camptothecin from leaf samples of *N. nimmoniana* collected from different geographical locations

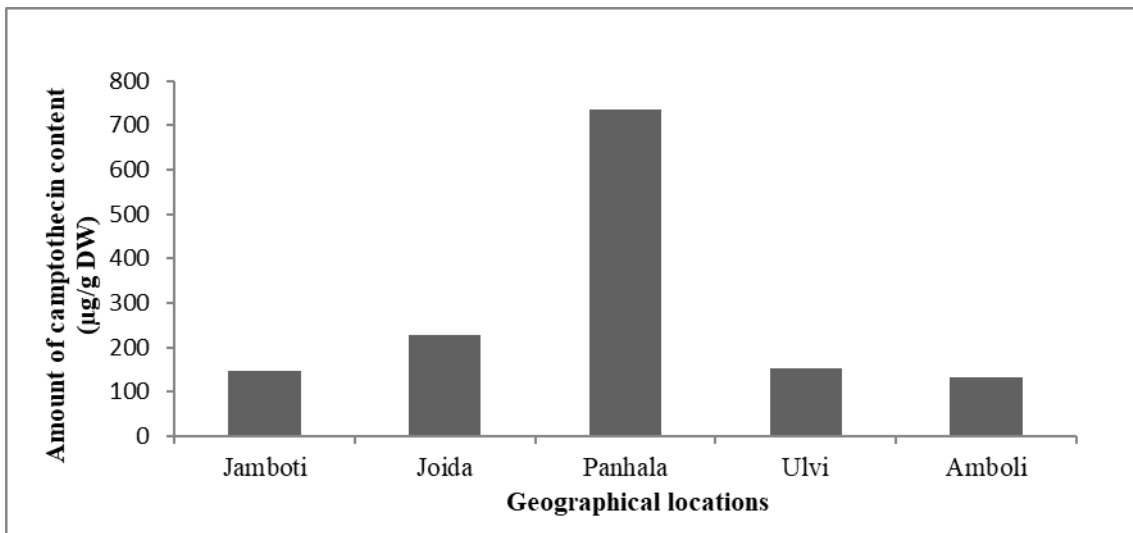
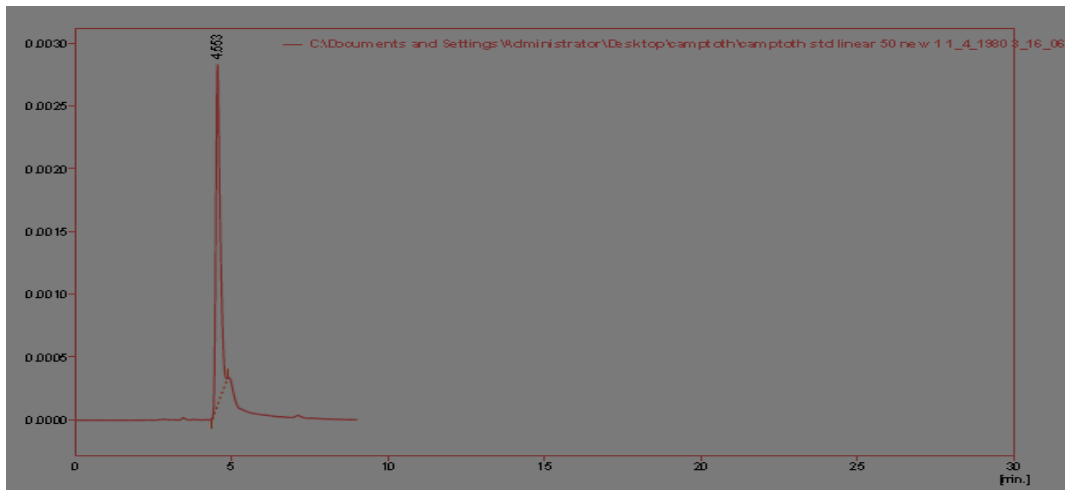
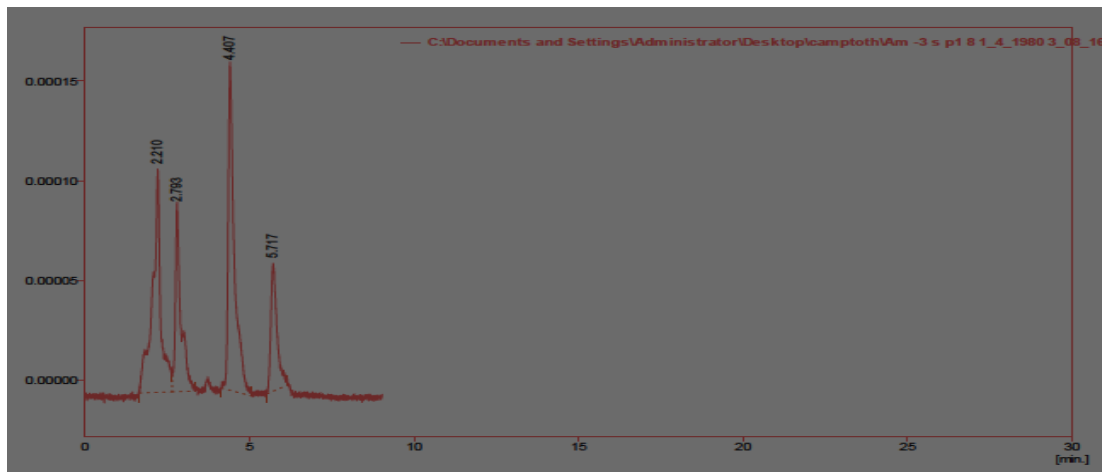


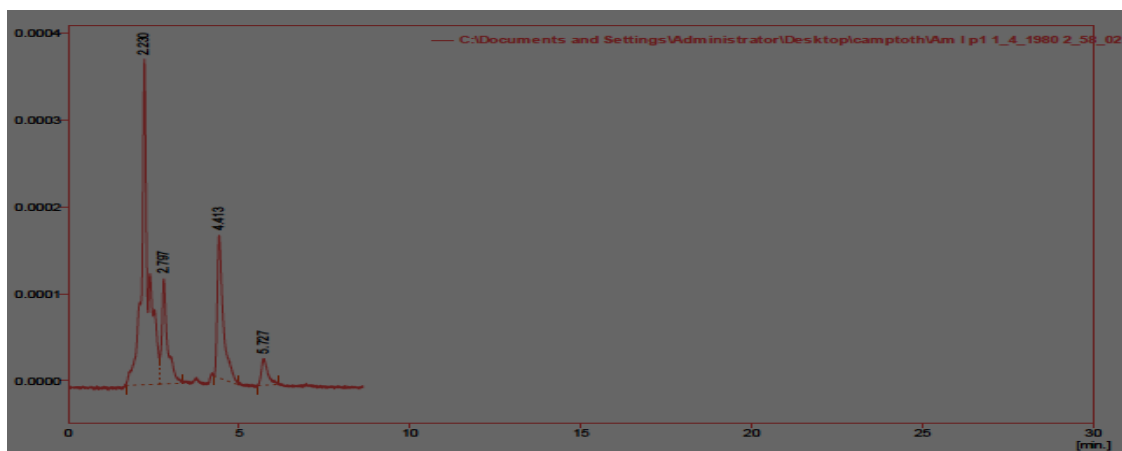
Figure 5: Quantification of camptothecin from stem samples of *N. nimmoniana* collected from different geographical locations



(A)



(B)



(C)

Figure 6: HPLC profile of methanol extracts of samples collected from different geographical locations.

(A) - Camptothecin (50 µg/ml)

(B) - Amboli stem

(C) - Amboli leaf

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