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Altitudinal gradients influence the accumulation of pharmaceutically important phenolic compounds in the leaves of *Lobelia nicotianifolia* Roth. and regulates its antioxidant and anticancer property

Ganesh D Mankar[1], Akshay B Gulave[2], Kailas D Datkhile[3] & Saurabha B Zimare[4]*

[1]Modern College of Arts, Science and Commerce (Autonomous) Shivajinagar, Pune – 411 005, Maharashtra, India

[2]Tuljaram Chaturchand College of Arts, Science and Commerce, Pune-413 102, Maharashtra, India

[3]Department of Molecular Biology & Genetics, Krishna Institute of Medical Sciences University, Pune-415 539, Maharashtra, India

[4]Naoroji Godrej Center for plant research, Pune-412 801, Maharashtra, India

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This study intended to investigate the effects of altitudinal gradients on the accumulation of phenolic compounds and bioactivity among different populations of *Lobelia nicotianifolia* Roth. from Northern Western Ghats of India. High-performance liquid chromatography (HPLC) revealed a maximum content of embelin (16.36 µg/g DW), gallic acid (53.47 µg/g DW), and quercetin (18.93 µg/g DW) in Kas population (1148 m m.s.l.). *L. nicotianifolia* from Kas region has higher radical scavenging activity in 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and 2,2 -azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays and also exhibited higher cytotoxicity against two breast cancer cell lines (MCF-7 and MDA-MB231). A correlation analysis indicates a significant relationship between altitudinal gradients of *L. nicotianifolia* population and accumulation of phenolic compounds and bioactivity. Principle component analysis (PCA) and hierarchical cluster analysis (HCA) grouped *L. nicotianifolia* populations into different clusters based on their altitudes. *L. nicotianifolia* from Kas region (1148 m) is selected as an elite population because of its potential to accumulate higher phenolic compounds and subsequent bioactivity.

Keywords: Breast cancer, Elite population, Environmental factors, *Lobelia nicotianifolia* Roth., Phenolics, Wild tobacco

Antioxidants are health-promoting substances which lower the oxidative stress and reduce the chances of formation of degenerative diseases[1]. Cancer is the second largest death-causing degenerative diseases created massive social and economic burden[2]. Different medicinal plants are major source of anticancer agents and have fewer side effects than synthetic drugs[3]. Plants are known to produce a wide array of bioactive secondary metabolites (BSMs) of which phenolic compounds are accepted as antioxidant and anticancer agents[4-6]. The quality and quantity of phenolic compounds are mainly influenced by the different environmental factors such as altitudinal gradient which further regulates the bioactivity of those plants[7-9]. Till date, more than 8000 phenolic compounds have been reported from the plant kingdom and some of them like embelin, gallic acid, and quercetin are potent bioactive agents[10-13]. Recent studies have proved the effectivity of embelin, gallic acid and quercetin as potent antioxidant and anticancer agents[11-16]. Likewise, different phenolic compounds chemical synthesis of embelin, gallic acid, and quercetin is difficult hence are mainly obtained from different medicinal plants.

Lobelia (Wild tobacco) is a cosmopolitan genus of Campanulaceae family known for its BSMs and different bioactivities[17]. Different wild and cultivated species of *Lobelia* are native to India of which plant parts of *Lobelia nicotianifolia* Roth. is commonly employed in the treatment of more than a few diseases and disorders[18,19]. Characterization of *L. nicotianifolia* leaves using liquid chromatography-high resolution mass spectrometry (LC-HRMS) revealed the presence of embelin, gallic acid, and quercetin (data not revealed). Considering the pharmaceutical potential of these phenolic compounds the effect of altitudinal gradients on their accumulation in the leaves of *L. nicotianifolia* is need to be addressed.

In this light, the leaves of *L. nicotianifolia* were collected from northern Western Ghats (NWGs) of

*Correspondence:
E-mail: saurabhabot@gmail.com

India and were screened for the accumulation of embelin, gallic acid, and quercetin. Further, the antioxidant potential of the collected population was assessed using DPPH and ABTS assay. Whereas, the anticancer activity was studied against two breast cancer cell lines *viz*. MCF-7 and MDA-MB231. Further, an elite population of *L. nicotianifolia* was scored for the higher accumulation of phenolic compounds and bioactivity using different statistical programs.

Materials and Methods

Chemicals

Organic solvents (HPLC grade), tannic acid, ascorbic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), Folin–Ciocalteu phenol reagent, and sodium carbonate were procured from Himedia, India. Embelin, gallic acid, and quercetin were obtained from Sigma, United States of America.

Plant material

The leaves of well-grown mature individuals of *L. nicotianifolia* were collected from ten populations from NWGs of Maharashtra from altitude ranging from 616–1148 m (m.s.l.) (Table 1 & Fig. 1). The collected plant materials were identified using the Flora of Maharashtra State[20] and all the herbarium specimens were deposited to the Naoroji Godrej Centre for Plant Research (NGCPR 1901-1910).

Table 1 — Details of the collection sites and the variability in the accumulation of embelin, gallic acid, and quercetin in the leaves of *L. nicotianifolia* from Northern Western Ghats of India. Mean values followed by different letter in a column are significantly different ($P \leq 0.05$)

Population	Codes	Latitude	Longitude	Altitude (m)	Accession	Phenolic compounds (µg/g DW)		
						Embelin	Gallic acid	Quercetin
Brahmagiri	LN-1	19°55’07” N	73°31’20” E	1085	NGCPR–1910	13.21 ± 0.16^{c}	45.12 ± 0.41^{c}	15.01 ± 0.96^{b}
Ratangad	LN-2	19°31’34” N	73°43’21” E	746	NGCPR–1911	11.41 ± 0.11^{d}	27.32 ± 0.43^{f}	9.11 ± 0.57^{d}
Bhimashankar	LN-3	19°04’40” N	73°32’31” E	1011	NGCPR–1912	14.57 ± 0.29^{b}	47.23 ± 0.55^{b}	15.21 ± 0.62^{b}
Ingalunghat	LN-4	18°55’11” N	73°33’51” E	713	NGCPR–1909	10.45 ± 0.30^{e}	25.09 ± 0.29^{g}	8.78 ± 0.59^{d}
Tamhini	LN-5	18°28’16” N	73°33’51” E	632	NGCPR–1914	7.75 ± 0.22^{g}	12.27 ± 0.41^{i}	6.23 ± 0.62^{f}
Varanda	LN-6	18°06’52” N	73°38’19” E	850	NGCPR–1902	13.01 ± 0.29^{c}	34.12 ± 0.61^{d}	13.37 ± 0.97^{c}
Kas	LN-7	17°43’24” N	73°48’47” E	1148	NGCPR–1901	16.36 ± 0.22^{a}	53.47 ± 0.30^{a}	18.93 ± 0.42^{a}
Kumbharli	LN-8	17°23’27” N	73°40’21” E	697	NGCPR–1904	8.37 ± 0.23^{f}	14.12 ± 0.43^{h}	7.31 ± 0.59^{e}
Gagan Bawada	LN-9	16°32’34” N	73°50’39” E	618	NGCPR–1905	6.71 ± 0.48^{h}	10.17 ± 0.70^{j}	4.23 ± 0.15^{g}
Amboli	LN-10	15°57’21” N	74°01’32” E	834	NGCPR–1906	12.78 ± 0.33^{c}	32.01 ± 0.53^{e}	13.09 ± 0.17^{c}

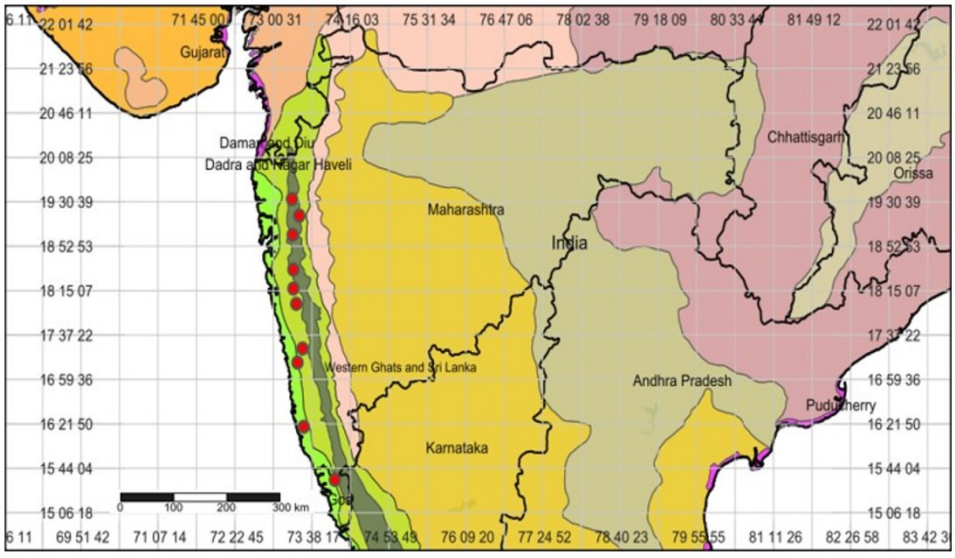

Fig. 1 — Geographic locations of the collection sites of *L. nicotianifolia* populations from northern Western Ghats of India. *Populations of *L. nicotianifolia* from north to south are Brahmagiri (1085 m m.s.l), Ratangad (746 m m.s.l), Bhimashankar (1011 m m.s.l), Ingalunghat (713 m m.s.l), Tamhini (632 m m.s.l), Varanda (850 m m.s.l), Kas (1148 m m.s.l), Kumbharli (697 m m.s.l), Gagan Bawada (618 m m.s.l), and Amboli (834 m m.s.l)

Extraction of plant material

The leaves of *L. nicotianifolia* were washed thoroughly under tap water to remove dust and dirt particles. The shade dried pulverized leaves were ultrasonicated at 33 kHz for 15 min at 45°C with ethanol. Further, the extracts were filtered through a Whatman filter paper no. 1 and concentrated on a rotary vacuum evaporator to dryness. Dried extracts were stored at 4°C until the next analysis.

Quantification of embelin, gallic acid, and quercetin

Embelin, gallic acid, and quercetin were quantified in the leaves of *L. nicotianifolia* using Agilent-1100 high-performance liquid chromatography (HPLC) by following a method described by Kolap *et al.*[21].

Antioxidant assays

DPPH assay

In DPPH assay percent radical scavenging activity (%RSA) of the leaves was evaluated using the method of Brand-Williams *et al.*[22] with some modifications. The absorbance of this solution was measured at 518 nm, and the ability of the extract to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH \%RSA} = \frac{\text{AC} - \text{AS}}{\text{AC}} \times 100 \quad \ldots (1)$$

ABTS assay

The %RSA of *L. nicotianifolia* was evaluated according to Re *et al.*[23] with minor changes. The absorbance of the reaction mixture was recorded spectrophotometrically at 734 nm, and the %RSA of extracts was determined by using the following equation:

$$\text{ABTS}^{\bullet+}\ \text{\%RSA} = \frac{\text{AC} - \text{AS}}{\text{AC}} \times 100 \quad \ldots (2)$$

In equation 1 and 2, AC is the absorbance of the negative control/blank (DPPH/ ABTS•$^{+}$ solution + methanol), and AS is the absorbance of the extract (DPPH/ ABTS•$^{+}$ solution + extract/ AA).

Anticancer activity

Cell lines, cultures, and proliferation assay (MTT Assay)

The *in vitro* cytotoxicity of *L. nicotianifolia* leaves extract (200 µg/mL) for all populations were studied against breast cancer cell lines (MCF7 and MDA-MB231). The cultures without plant extracts were treated as control. The MCF7 and MDA-MB231 cell lines were maintained in T-25 flasks with MEM media with 10% heat-inactivated FBS and 100 U/mL and 100 µg/mL penicillin and streptomycin, respectively. These cell lines were maintained under an atmosphere of CO_2 (5%) and humidity (95%) at 37°C. Further, a MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) for MCF7 and MDA-MB231 cell lines were studied as described by Abd Razak *et al*[24]. The percent inhibition (%) for this study was calculated by using the following equation:

$$\text{Percentage inhibition} = 100 - \frac{\text{AS}}{\text{AC}} \times 100 \quad \ldots (3)$$

In Equation 3, AS is the absorbance of treated cells and AC is the absorbance of control cells at 560 nm wavelength.

Statistical analysis

All the experiments were performed in triplicates (n=3). The statistical analysis of phenolic compounds and antioxidant activity were performed by using one-way ANOVA in SPSS (V20). Paleontological Statistics software (PAST version 4.03) (PCA and HCA) was used to identify an elite population of *L. nicotianifolia*.

Results and Discussion

Accumulation of embelin, gallic acid, and quercetin

The effect of altitudinal gradients on the accumulation of embelin, gallic acid, and quercetin across all populations of *L. nicotianifolia* is represented in (Table 1). The quantity of embelin, gallic acid, and quercetin was ranged in between 10.17 to 53.47, 6.71 to 16.36, and 4.23 to 18.93 µg/g DW, respectively. The HPLC analysis revealed that the higher concentration of embelin, gallic acid, and quercetin was observed in LN-7 population (Fig. 2) which collected from the highest altitude (1148 m) among all populations (Table 2). Whereas, the lower amount of these phenolic compounds was reported in LN-9 population (618 m) (Table 1). This analysis has revealed that altitudinal gradients played a crucial role in the accumulation of embelin, gallic acid, and quercetin in the leaves of *L. nicotianifolia* which was in agreement with previous studies for different plant species[25-27]. It is documented that the quantity of phenolic compounds is not only governed by the genetic makeup but is also significantly influenced by the abiotic factors one of which is altitude[28,29]. Altitudinal gradients are associated with the differences in environmental factors mainly the solar radiation which elicits the accumulation of BSMs.

Table 2 — Details of geographical coordinates, altitude and accession numbers of *L. nicotianifolia* populations collected from Northern Western Ghats of India

	DPPH	ABTS	MCF-7#	MCF-7##	MDA-MB231#	MDA-MB231##
belin	.942**	.938**	.980**	.984**	.980**	.981**
lic acid	.987**	.985**	.980**	.971**	.977**	.976**
ercetin	.952**	.950**	.989**	.983**	.989**	.989**

#Anticancer activity at 24 h; ##Anticancer activity at 48 h; **Correlation is significant at the 0.01 level (2-tailed)

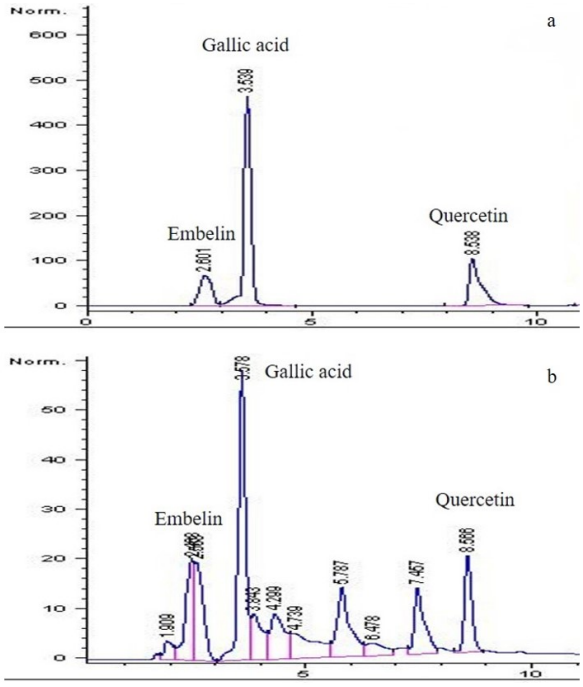

Fig. 2 — HPLC chromatogram of (A) standards; and (B) leaves extract of *L. nicotianifolia* population of Kas region (LN-7) indicating the presence of embelin, gallic acid, and quercetin

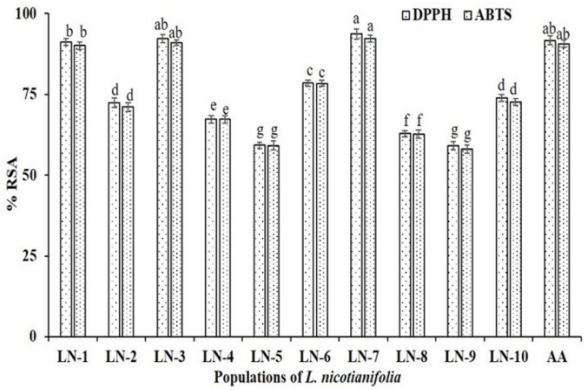

Fig. 3— Representative histogram showing radical scavenging activity of *L. nicotianifolia* populations in DPPH and ABTS assays at 1 mg/mL The results represent the means of three independent experiments, and error bars represent the standard error of the means. Mean values followed by different letters are significantly different ($P< 0.05$)

It is noted that the intensities of solar radiations like UV-B vary with altitudinal gradients which further correlate with the alteration in the phenolic compounds[30,31]. In the present study, higher phenolic compounds were observed in the LN-7 population which was procured from the highest altitude (1148 m) of all the ten populations (Table 1 & Fig. 1). At a higher altitude, UV-B radiation generates photo-oxidative and free radical stress which is countered by an accumulation of higher quantity of phenolic compounds[9,32-34]. The intensities of the UV radiations are confined to the altitude[35] could be the probable reason for the higher accumulation of the phenolic compound in the Kas population (LN-7). Plants phenolics are mainly synthesized through a shikimate or phenylpropanoid pathway and the genes involved in the pathways are upregulated by different abiotic stress leading to the accumulation of various phenolic compounds[34,36,37].

Bioactive potential of *L. nicotianifolia* populations

The collected populations of *L. nicotianifolia* were also screened for their bioactive potential (antioxidant and anticancer). The leaves of the LN-7 population (1148 m m.s.l.) exhibited significantly higher % RSA in DPPH (93.63%) and ABTS (92.23%) assays. Similarly, *L. nicotianifolia* populations collected from Kas (LN-7) committed for the higher inhibition against MCF-7 (58.16%) and MDA-MB231 (57.96%) cell lines at 48 h (Fig. 3). The lower antioxidant and anticancer were observed in LN-9 population which was collected from a lower altitude. These observations revealed that the studied bioactivity of *L. nicotianifolia* is influenced by altitudinal gradients, the concentration of extracts, and treatment duration (Figs. 3 & 4 and Table 1). The morphological changes in the studied cell lines for Kas population (LN-7) at 48 h are illustrated in (Figs. 5 & 6). The DPPH and ABTS are well-accepted antioxidant assays which are mainly used to score the antioxidant potential of plants[38]. The ability of plant extracts to quench free radicals are mainly associated with the quantity and array of phenolic compounds in the extracts, which donate hydrogen ions and works as chain breakers[39].

However, breast cancer was chosen for this study because of its mortality rate around the world[40] and the efficacy of embelin, gallic acid, and quercetin against this cancer type[41-43]. Anticancer effect of phenolics is mainly because of its ability to restricts the initiation and progression of cancers cells by modulating genes associated with cancer[44] which has been clinically proven at omics level[41-43]. Higher antioxidant and anticancer activity of LN-7 population might be confined to the higher accumulation of embelin, gallic acid, and quercetin (Table 1). The Pearson correlation analysis validates our observations where the accumulation of embelin, gallic acid, and quercetin of different populations were significantly correlated with antioxidant and anticancer activity at 24 and 48 h (Table 2). Furthermore, the LN-7 population was from a higher altitude perceives more UV radiations to produce higher phenolics and associated antioxidant and anticancer activity which is in agreement with previous studies in different plant species[7,45].

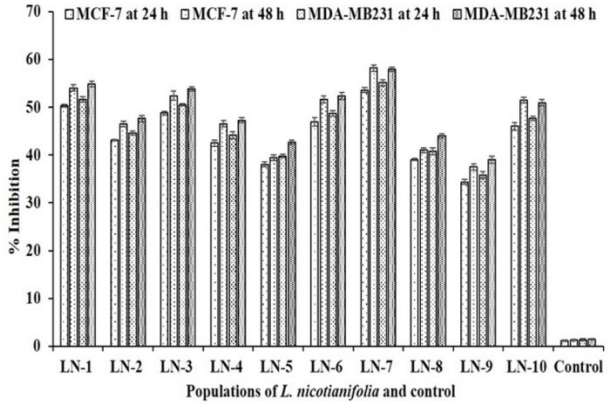

Fig. 4 —Representative histogram showing percentage cell growth inhibition MCF-7 and MDA-MB-232 cells exposed to 200 µg/mL concentration of *L. nicotianifolia* leaves extract for 24 and 48 h. The results represent the means of three independent experiments, and error bars represent the standard error of the mean

Identification of the elite population

PCA analysis discriminated the *L. nicotianifolia* populations based on their quantitative analysis of phenolics (embelin, gallic acid, and quercetin) and its bioactivities (antioxidant and anticancer) (Fig. 7A

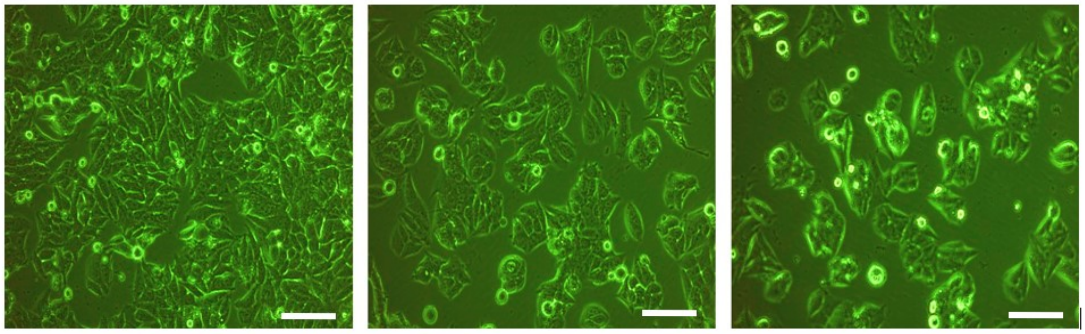

Fig. 5 — Cell morphology of MCF-7 cells exposed to 200 µg/mL concentration of *L. nicotianifolia* leaves extract from Kas population (A) control (without extract); after (B) 24; and (C) 48 h treatment. All images are taken at 20X magnification with Carl Zeiss phase-contrast microscope where a scale bar is 100 µM

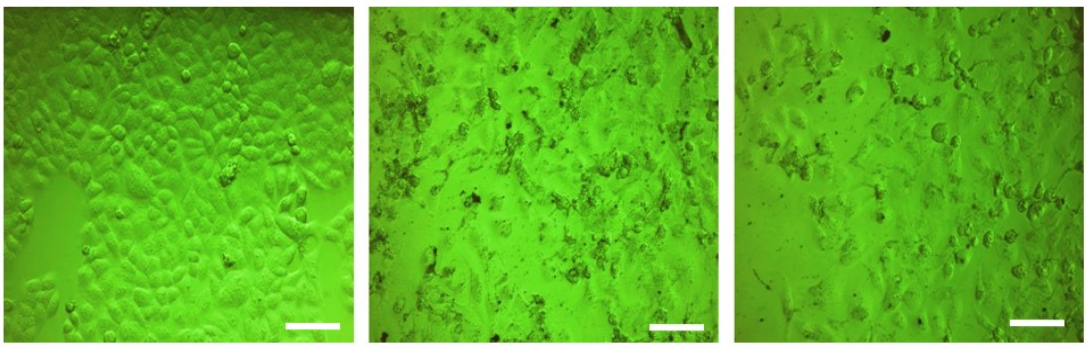

Fig. 6 — Cell morphology of MDA-MB-231 cells exposed to 200 µg/mL concentration of *L. nicotianifolia* leaves extract from Kas population (A) control (without extract); after (B) 24; and (C) 48 h treatment. All images are taken at 20X magnification with Carl Zeiss phase-contrast microscope where a scale bar is 100 µM

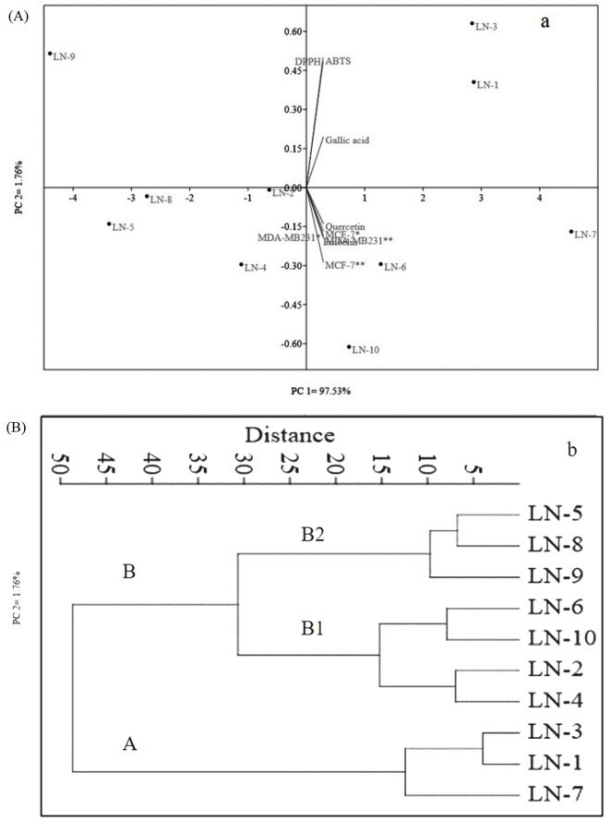

Fig. 7 — *DPPH- 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), ABTS- 2,2-diphenyl-1-picrylhydrazyl, MCF-7*- cytotoxicity against MCF cell line at 24 h, MCF-7**- cytotoxicity against MCF cell line at 48 h, MDA-MB231*- cytotoxicity against MDA-MB231cell line at 24 h, MDA-MB231**- cytotoxicity against MDA-MB231cell line at 48 h. *LN-1Brahmagiri (1085 m), LN-2 Ratangad (746 m), LN-3 Bhimashankar (1011 m), LN-4 Ingalunghat (713 m), LN-5 Tamhini (632 m), LN-6 Varanda (850 m), LN-7 Kas (1148 m), LN-8 Kumbharli (697 m), LN-9 Gagan Bawada (618 m), and LN-10 Amboli (834 m)

and B). The identification of elite population can be employed for mass multiplication to meet the needs for commercial demand[29]. The use of multivariant analysis (PCA and HCA) for identification of elite populations has been practised in *Valeriana jatamansi* and *Coleus forskohlii*[29,45]. In the present study, PCA was performed on the normalized data set to evaluate the effect of altitudinal gradients on the phenolic compounds, antioxidant and anticancer activity in *L. nicotianifolia*. PCA of the entire dataset evolved into seven PCs (PC1 to PC7). Usually, the first two principal components *viz*. PC1 and PC2 are adequate to explain the maximum variations of the entire data. In the present study, the two-dimensional score plot is defined by PC1 and PC2 which represents a total of 99.49% of the total variance (Fig. 7A). PC1 was highly positively correlated more than 97% with phenolic compounds (embelin, gallic acid, quercetin), antioxidant (DPPH and ABTS), and anticancer (MCF-7 and MDA-MB231) activity. *Lobelia nicotianifolia* populations collected from higher altitude (1011-1148 m) namely LN-1, LN-3 and LN-7 clustered as group I which showed highest accumulation of phenolic compounds and bioactivities (antioxidant and anticancer). Moreover, LN-7 population exhibited highest phenolic compound contents, antioxidant, and anticancer activity amongst the group I (Fig. 7A). Group II included the populations from the 834–850 m altitudes (LN-6 and LN-10) which is present in the right lower quadrant. Group III included populations from the 713-746 m altitudes (LN-2 and LN-4) which is present in right lower left quadrant. Group II had lower and higher phenolic contents, antioxidant and anticancer activity as compared to group I and group III, respectively. Group IV included populations from 618-697 m altitude (LN-5, LN-8 and LN-9) represents the lowest accumulation of phenolic compounds and subsequent bioactivity. Further HCA was also performed to validate this data and to prioritize and identify the elite populations of *L. nicotianifolia* based on the accumulation of phenolic compounds and bioactive potential.

In the HCA, the ten *L. nicotianifolia* populations were separated into two main clusters A and B (Fig. 7B). The cluster A comprised of LN-1, LN-3, and LN-7 which belonged to the altitude higher than 1000 m. However, cluster B was subdivided into B1 and B2, where cluster B1 represents LN-2, LN-4, LN-6, and LN-10 which are from 713-834 m. The cluster B2 comprised of three populations *viz*. LN-5, LN-8, and LN-9, which was collected from the lowest altitude of 618-697 m. Cluster A comprised of populations from higher altitudes showed a correlation with the data analysed using PCA for accumulation of higher phenolic compounds, antioxidant and anticancer activity.

Conclusion

In conclusion, the present work is the first study performed on the influence of the altitudinal gradients on the accumulation of phenolic compounds and bioactivity in the leaves of *Lobelia nicotianifolia*. The higher accumulation of embelin, gallic acid, and quercetin and bioactivity were seen for Kas population among all studied populations from northern Western Ghats of India. Further, the Pearson correlation analysis supports our observations that

altitudinal gradients are an important parameter to perceive significant results in *L. nicotianifolia*. The statistical tools like PCA and HCA categories the studied populations in different clusters based on their altitude and their response to an accumulation of phenolic compounds and bioactivity. The results conclude that the different plant populations need to be screened to score the influence of different environmental factors instead of haphazardly collection for the pharmaceutical industries.

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Conflict of interest

All authors declare no conflict of interests.

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