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Research Article

ALTITUDINAL INFLUENCE ON PHYTOCHEMICAL PROFILES AND BIOACTIVITIES OF METHANOL EXTRACTS OF *ARTEMISIA VULGARIS*

Kanhaiya Lal Gupta[1,2], Anup Muni Bajracharya[3], Khaga Raj Sharma[1*]

[1]*Central Department of Chemistry, Tribhuvan University, Kathmandu, Nepal*
[2]*Department of Chemistry, Birendra Multiple Campus, Tribhuvan University, Chitwan, Nepal*
[3]*Department of Microbiology, Balkumari College, Narayangarh, Chitwan, Nepal*
[*]*Correspondence: khaga.sharma@cdc.tu.edu.np*

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ABSTRACT

Artemisia vulgaris is a plant of therapeutic importance distributed across a wide altitudinal range throughout Nepal. This study investigates the influence of altitudinal variations on the phytochemical content and bioactivities of *A. vulgaris,* gathered from three different altitudes of Nepal: Bharatpur (201 m), Lamidada (1396 m), and Daman (2322 m). Aerial parts of the samples were extracted with methanol through cold-percolation and subjected to spectrophotometric evaluation of total flavonoid content (TFC) and total phenolic content (TPC) employing aluminium chloride and Folin-Ciocalteu reagents. Antioxidant potentials were assayed as DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical-scavenging percentage, antibacterial activity via agar well diffusion method (targeting *Staphylococcus aureus, Pseudomonas aeruginosa,* and *Escherichia coli*), and toxicity by brine shrimp lethality assay. The results demonstrated significant variations ($p<0.05$) in the phytochemical contents and bioactivities, increasing with altitude. The highest TFC and TPC were observed in samples from Daman. Correspondingly, the Daman sample showed the strongest antioxidant potency (the lowest $IC_{50} = 58.09 \pm 0.04$ μg/mL). All the extracts demonstrated moderate toxicity ($LC_{50} < 500$ μg/mL), with the sample collected from Bharatpur being the most toxic. Antibacterial activity varied significantly ($p<0.05$), with the Bharatpur and Daman samples showing relatively greater zones of inhibition (ZOI), particularly against *E. coli* and *P. aeruginosa*. These findings indicated that *A. vulgaris* responds to altitudinal stress by altering its bioactive secondary metabolite production, enhancing its antioxidant and antibacterial properties. The study supports the ethnomedicinal relevance of this species and highlights its potential as a natural source of therapeutic agents.

Keywords: Antibacterial, Antioxidant, *Artemisia vulgaris,* Bioactive, Flavonoids, Toxicity

INTRODUCTION

Existing drugs are facing great challenges of multidrug-resistant (MDR) pathogens and adverse side effects. Moreover, exposure to hazardous chemicals and environmental pollution is causing an increase in diseases related to oxidative stress and even cancer (Shetty et al., 2023). Medicaments of herbal origin are emerging as a great hope in this context due to their high efficacy with minimal or zero side effects (Gokhale & Wadhwani, 2015). However, even their use over a long period may lead to the development of resistance to the pathogens, rendering them ineffective against the related diseases. To combat these situations, continuous exploration and development of plant-based drugs containing novel bioactive compounds, non-resistant to pathogens, is a matter of great concern in the scientific community for the development of modern, highly efficient drugs having minimal or no adverse side effects.

The free radicals, generally, reactive oxygen species (ROS) and reactive nitrogen species (RNS), are highly reactive species formed by the metabolic activity of oxygen in the human body (Jomova et al., 2023). Their formation in the body is triggered by stress and pollution (Jomova et al., 2023). The cells and tissues suffer damage by these free radicals through oxidative stress (Liguori et al., 2018; Jomova et al., 2023). Antioxidants such as superoxide dismutase, catalase, alpha-tocopherol, and ascorbic acid are produced in our body to destroy these reactive species and hence, prevent such damage (Meulmeester et al., 2022). However, they are rendered ineffective under the great oxidative stress and lead to various degenerative disorders like cardiovascular diseases, ageing, diabetes, neurodegenerative diseases such as Alzheimer's disease, mutagenesis, and cancer (Brieger et al., 2012; Yang & Lian, 2020). Compounds of plant origin, including flavonoids, polyphenols, tannins, alkaloids, terpenoids, etc.,

possess strong potential for scavenging the free radicals formed to disrupt the chain reaction responsible for oxidative damage. Such compounds, therefore, can delay the rapid aging and degenerative disorders. Besides antioxidants, these compounds also act as anticancer, antibacterial, anti-inflammatory, antifungal, and antiaging agents (Imran et al., 2023). There is a positive correlation between the biological/pharmacological activity and the antioxidant activity (Ouamnina et al., 2024). Hence, interest in the antioxidant potential of medicinal plant extracts and their constituent compounds is increasing for the development of new drug candidates targeting chronic and infectious diseases.

Compounds such as polyphenols, flavonoids, terpenoids, alkaloids, tannins, etc., are produced by plants as secondary metabolites to defend against diseases and wounds, and to withstand severe climatic conditions (Holopainen et al., 2018). These bioactive compounds render plant-derived medicaments effective in the treatment of various diseases, as practiced traditionally since ancient times (Verpoorte, 1998). Variation in climatic conditions associated with altitude strongly influences secondary metabolite biosynthesis even within a single species. Plants growing at high altitudes have to tolerate low temperatures, heavy snowfall, low oxygen availability, and low atmospheric pressure. Consequently, they synthesize the increased levels of pharmacologically and ecologically active compounds as an adaptive response to such conditions. As a result, the composition and concentration of the secondary metabolites vary in response to environmental gradients (Verma & Shukla, 2015; Li et al., 2020). Such altitude-induced metabolic variation provides a strong scientific basis for comparative phytochemical and bioactivity studies of medicinal plants.

Naturally, Nepal is endowed with unique and magnificent biodiversity, attributed to a wide altitudinal range. It ranges from 59 m in the plains to 8848 m at the summit of Mt. Everest, encompassing eight bioclimatic zones from tropical to nival, harboring a total of 118 ecosystems (Joshi et al., 2017). Medicinal plants growing in the high-altitude regions of Nepal are, therefore, believed to be an abundant reservoir of secondary metabolites and may even contain novel bioactive compounds with potential for the development of future therapeutic agents with enhanced efficacy.

Artemisia vulgaris, commonly called "Mugwort" in English and "Tite pati" in Nepali, is a perennial herb of the Asteraceae (Compositae) family. It is distributed in Nepal across a wide altitudinal range, encompassing tropical, subtropical, and temperate climatic zones (Budhathoki et al., 2020; Pandey et al., 2021; Gautam et al., 2024). Several traditional and folk medicinal practices utilizing the plant species, including other species of the genus Artemisia, have been reported from Nepal and worldwide (Zeb et al., 2019). The plant species have been found to have emmenagogue, stimulant, antispasmodic, anthelmintic, pesticidal, stomachic, purgative, anti-hyperlipidemic, anti-malarial, antifertility, anti-convulsant, and adaptogenic activities (IUCN Nepal, 2000; Budhathoki et al., 2020; Zubair et al., 2020).

Though several earlier works on *A. vulgaris* have been reported, a comparative investigation of the impact of altitudes corresponding to different climatic zones on the plants' bioactivities, along with TPC and TFC, has not yet been systematically investigated. The present study, therefore, examines the effect of altitude on *A. vulgaris* sourced from three geographical locations; Bharatpur (tropical), Lamidada (Sub-tropical), and Daman (temperate); representing major climatic zones of Nepal. Methanol extracts of the samples were analyzed for antioxidant and antibacterial activities, cytotoxicity, and associated phytochemical contents (TPC and TFC). The comparative analysis was designed to elucidate the impact of altitudinal and climatic change on the phytochemical composition and bioactivities of the species.

MATERIALS AND METHODS

Chemicals and reagents

The reagents and chemicals employed in the study were of analytical standard. Methanol, Folin-Ciocalteu reagent (FCR), Dimethyl sulfoxide (DMSO), Na_2CO_3, $AlCl_3$, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, gallic acid (GA), quercetin (QE), and other reagents were used as received.

Plant materials and extractions

Artemisia vulgaris samples were collected from their natural habitat across three locations in Nepal during the mature vegetative stage in February 2022. The collection sites represent different altitudes and climatic zones: Bharatpur, Chitwan (201 m; tropical), Lamidada, Makwanpur (1396 m; sub-tropical), and Daman, Makwanpur (2322 m; temperate). The geographical co-ordinates of the three sites are 27.697777° N & 84.4352777° E, 27.549719° N & 85.054599° E, and 27.610668° N & 85.094075° E, respectively. The Plant material was taxonomically authenticated by Associate Professor Dr. Manoj

Kumar Lal Das, Department of Botany, Birendra Multiple Campus, Bharatpur.

The plants' aerial parts were washed with water, shade-dried, powdered, and stored at 4 °C until extraction. The extraction involved the use of cold-percolation with methanol, soaking a 40 g sample successively in 200 mL solvent, followed by filtration after every 24 hours for three days. From the filtrates, the solvent was evaporated at 45 °C in a rotary evaporator, and the dry extracts were kept in a refrigerator until analysis. The extractive values are recorded in Table 1.

Table 1. Yield percentage of the extracts of *A. vulgaris* growing at different locations

S. N.	Plant materials and location	Wt. of extract (g)	Yield
1	*A. vulgaris* (Bharatpur)	7.471	18.68%
2	*A. vulgaris* (Lamidada)	6.031	15.08%
3	*A. vulgaris* (Daman)	6.475	16.19%

Preliminary phytochemical analysis

The preliminary phytochemical analyses were performed following the established protocol based on the color differentiation method (Harborne, 1973) with slight modification (Alam & Sharma, 2020). The specific color change reactions triggered by the active compounds in the plant extracts upon treatment with the test reagents were used to identify various phytochemical constituents, including alkaloids, flavonoids, terpenoids, sugars, polyphenols, tannins, and saponins.

Estimation of total phenolic content (TPC)

TPC of the extracts was assessed spectrophotometrically depending on the color intensity produced by the reaction with FCR, using standard GA, following the reported protocol with minor modifications (Ainsworth & Gillespie, 2007). Firstly, the absorbance of standard GA solutions (10, 25, 50, and 100 µg/mL) was determined using a UV-Visible spectrophotometer at 760 nm. This was done after mixing 1 mL of each standard solution with 5 mL 10% FCR and 4 mL 7% Na_2CO_3 solution, followed by 30 minutes incubation at 40 °C. For determining TPC, a GA calibration curve was constructed based on the absorbance data. The extract solutions of varying concentrations (1.0 mg/mL, diluted serially in half, up to 0.125 mg/mL) were treated similarly, and the absorbance was subsequently measured. The corresponding concentrations of GA were determined using the calibration curve through linear regression, and TPC was calculated as mg of GA equivalent contained in each gram of dry extract using equation (1) as follows.

$$\boldsymbol{C = \frac{C_1 . 1000}{C_2}} \quad \ldots (1)$$

Where, C = TPC (mg GAE/g)

C_1 = GA concentration (mg/mL) calculated using the calibration curve

C_2 = the plant extract concentration (mg/mL)

Estimation of total flavonoid content (TFC)

The $AlCl_3$-based colorimetric method, with quercetin as the reference, was used to measure TFC, based on the approach by Zhishen and co-workers with slight adjustments (Zhishen et al., 1999). At first, absorbance of 1 mL each of standard QE solutions (2.5, 5, 10, 20, 50 and 100 µg/mL) mixed serially with 4 mL distilled water (DW), 5% $NaNO_2$ and 10% $AlCl_3$ (0.3 mL each at 1 minute), 1 M NaOH (2 mL at 6 minutes) and additional 2.4 mL DW, was recorded at 510 nm. Using the data recorded, the QE calibration curve was plotted to estimate TFC. The absorbance was also measured in a similar manner using varying concentrations of extract solutions (1.0 mg/mL diluted up to 0.125 mg/mL). The corresponding QE concentrations were determined using the calibration curve through linear regression, and TFC, as mg of QE equivalent present in each gram of the dry extract, was calculated using equation (2).

$$\boldsymbol{C = \frac{C_1 . 1000}{C_2}} \quad \ldots (2)$$

Where, C = TFC (mg QE/g)

C_1 = QE concentration (mg/mL) calculated using the calibration curve

C_2 = The extract concentration (mg/mL)

Evaluation of antioxidant activity

DPPH radical scavenging assay was used to estimate the antioxidant potential of the plant extracts, taking ascorbic acid as the standard (Brand-Williams et al., 1995). 20 to 100 µg/mL solutions of ascorbic acid and

plant extracts, in increments of 20, were prepared. Each solution (2 mL) was blended with 2 mL DPPH solution (0.2 mM in methanol). This mixture was kept in the dark for a half-hour period, followed by measurement of absorbance at 517 nm. Thereafter, using equation (3), the radical scavenging percentage was calculated.

$$\text{Percent radical scavenging (RSA)} = \frac{(A_0 - A_s)}{A_0} \boldsymbol{x}\ \mathbf{100}\% \quad \ldots (3)$$

Where A_0 and A_s are the absorbance of the DPPH, with solvent (control) and the sample solution, respectively.

From the plot of RSA vs concentration, the half-maximum inhibition concentration (IC_{50}) for each of the samples and ascorbic acid was determined through non-linear regression.

Evaluation of antibacterial activity

The antibacterial activity of the sample extracts was assessed employing the agar-well diffusion method (Joshi et al., 1970), and the bacteria under assessment were *S. aureus* (ATCC 43200), *P. aeruginosa* (ATCC 24853), and *E. coli* (ATCC 25922). Suspension of each bacterial strain was made in sterile medium (Mueller Hinton Broth (MHB)), maintaining the McFarland turbidity of 0.5 standard, resulting in 1.5 $\times 10^8$ colony-forming units (CFU) of the bacteria per milliliter. The microorganisms were incubated at 37 °C for 24 hours, and the Mueller-Hinton Agar (MHA) plates were inoculated with the developed microbial suspensions in a lawn culture format, and holes of 6 mm diameter were punched for loading the sample. To identify an antibiotic suitable to be chosen as a positive control for the assay, an antibiotic susceptibility test was performed on various antibiotic discs. 50 µL of each extract solution (100 mg/mL) prepared in DMSO was introduced into wells of 6 mm diameter on an MHA plate inoculated with the bacterial strain. Antibiotic disc (Ciprofloxacin (5 µg) selected through the susceptibility test) and DMSO (50 µL) were employed as the positive and negative control, respectively. The plates were incubated overnight (18-24 hours) at 37 °C and the growth-inhibition zones were recorded.

Evaluation of toxicity

The potentials of plant extracts toxicity were estimated through the lethality assay of brine shrimp (*Artemia salina)* larvae (Meyer et al., 1982). The larvae, ten in number, were exposed to 10 mL of each extract solution of 10, 100, and 1000 µg/mL in water with DMSO for about 24 hours under light. The number of dead larvae was counted, and half-lethal concentrations (LC_{50}) were determined through probit regression analysis. Extracts were considered toxic when values of LC_{50} were observed to be less than 1000 µg/mL (Meyer et al., 1982).

Statistical analysis

Three independent replicates were used for all measurements, and the outcomes were presented as the mean ± standard deviation. The raw data were initially compiled in Microsoft Excel for descriptive statistical analysis. Inferential statistical analyses were subsequently performed using RStudio and GraphPad Prism (Version 10.0). Relationships between geographic and phytochemical variables were evaluated using linear regression and Pearson's correlation coefficient (R^2). Half-maximal inhibitory concentrations (IC_{50}) for DPPH radical scavenging were calculated via non-linear regression analysis, while half-maximal lethal concentrations (LC_{50}) for brine shrimp larval mortality were determined using linear regression models. Results were regarded statistically significant for p-value below 0.05 as evaluated by one-way ANOVA using RStudio (R Core Team, 2025).

RESULTS

Preliminary phytochemical analysis

Preliminary phytochemical tests showed that the extracts of all three samples contain alkaloids, flavonoids, polyphenols, tannins, saponins, quinones, terpenoids, and glycosides.

Total phenolic and flavonoid content

The total phenolic and flavonoid contents, estimated using the GA and QE calibration curves (Figure 1, 2) respectively, through linear regression, are summarized in Table 2. The linear regression for the GA calibration curve showed y = 0.114x with a Pearson's correlation coefficient (R2) value of 0.9947. Similar treatment of the standard QE calibration curve revealed y = 0.0068 + 0.0101 and R2 = 0.9997

Table 2. TPC and TFC of methanol extracts of *A. vulgaris* growing at different locations

Location (Altitude)	TPC (mg GAE/g)	TFC (mg QE/g)
Bharatpur (201 m)	80.59 ± 15.40	56.415 ± 6.271
Lamidada (1396 m)	111.75 ± 12.46	67.592 ± 3.273
Daman (2322 m)	128.42 ± 10.61	84.540 ± 3.736

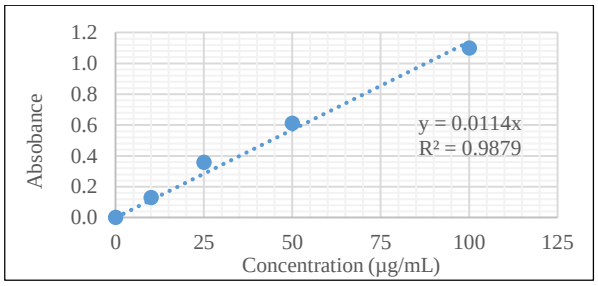

Figure 1. Gallic acid calibration curve

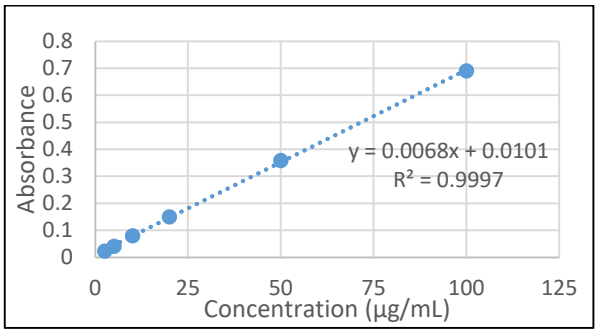

Figure 2. Quercetin calibration curve

The results showed that the extract of *A. vulgaris* collected from Daman (2322 m) contained the highest TPC and TFC values of 128.426 ± 10.611 mg GAE/g and 84.540 ± 3.736 mg QE/g, respectively. The extract of *A. vulgaris* collected from Bharatpur (201 m) was found to contain the least TPC and TFC values of 80.592 ± 15.405 mg GAE/g and 56.415 ± 6.271 mg QE/g, respectively. The plant extract from Lamidada (1396 m) was found to contain the intermediate TPC and TFC values of 111.754 ± 12.463 mg GAE/g and 67.592 ± 3.273 mg QE/g, respectively. Comparisons of TPC and TFC are presented in Figure 3 and 4, respectively.

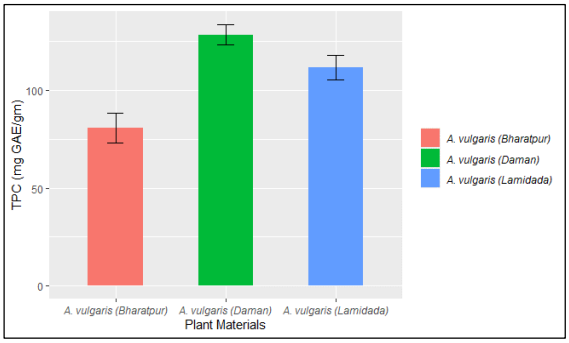

Figure 3. Total phenolic content (TPC) of different *A. vulgaris* extract

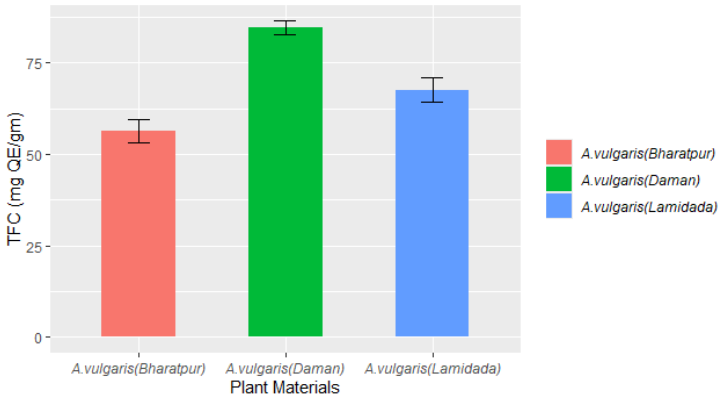

Figure 4. Total flavonoid content (TFC) of different *A. vulgaris* extract

Antioxidant activity

The percent DPPH radical scavenging activity (RSA), calculated based on the absorbance measurement, was found to increase with the concentration. The variation of the RSA of ascorbic acid and *A. vulgaris* extracts with the different concentration is shown in Figure 5 and 6, respectively, from which IC_{50} values were determined for each of the individual curves through non-linear regression analysis using GraphPad Prism (Version 10.0).

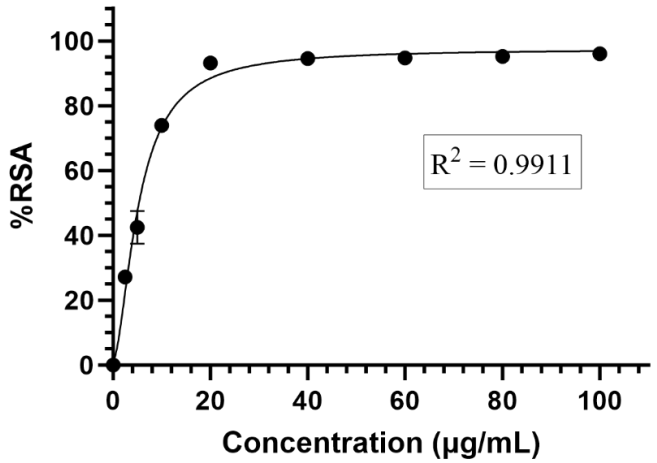

Figure 5. Percentage radical scavenging response to different concentrations of ascorbic acid

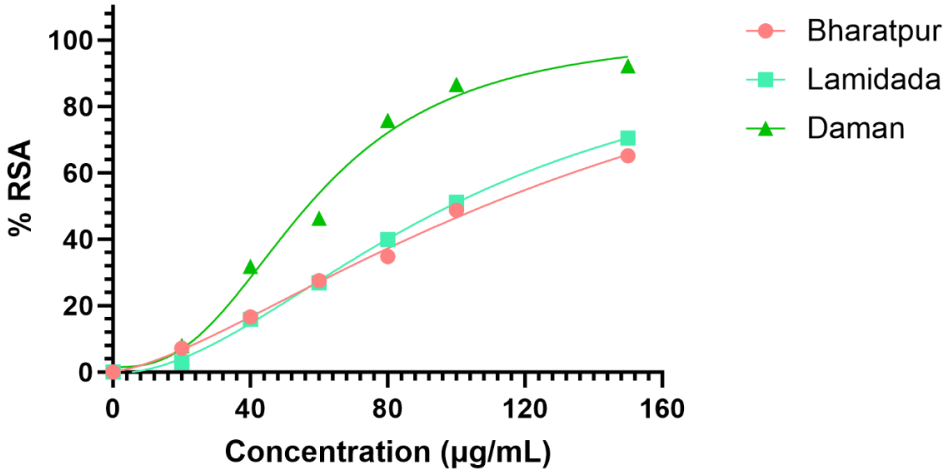

Figure 6. Percentage radical scavenging activity response to the concentration of the plant samples from different locations

The IC50 values presented in Table 3 were highest for A. vulgaris collected from Bharatpur followed by Lamidada and Daman.

Table 3. IC_{50} values of *A. vulgaris* methanol extracts and standard ascorbic acid

S. N.	Sample	IC_{50} (µg/mL)
1	Standard ascorbic acid	5.308 ± 0.004
2	*A. vulgaris* (Bharatpur)	154.30 ± 3.46
3	*A. vulgaris* (Lamidada)	102.367 ± 0.603
4	*A. vulgaris* (Daman)	58.407 ± 0.093

The result showed that the extract of *A. vulgaris* from Daman (2322 m) has the highest potential of radical scavenging with IC_{50} value of 58.407 ± 0.093 µg/mL (lowest value among the extracts) followed by that of Lamidada with the IC_{50} value of 102.367 ± 0.603 µg/mL while the extract of the plant from Bharatpur (201 m) has the lowest antioxidant/radical scavenging potential with the IC_{50} value of 154.30 ± 3.46 µg/mL. The IC_{50} values differed significantly among extracts ($p<0.05$). The results showed methanol extracts of the plant, even from Daman, have relatively lower RSA compared to ascorbic acid (IC_{50} = 5.308 ± 0.004 µg/mL).

Antibacterial potential

The ZOI for bacterial growth about each of the antibiotics taken in the antibiotic susceptibility test is summarized in Table 4.

Table 4. Antibiotic susceptibility screening of selected bacterial strains

Antibiotics	Zone of Inhibition (ZOI)		
	E. coli	***S. aureus***	***P. aeruginosa***
AMX (10 µg)	+ (12 mm)	+ (33 mm)	-
AZM (15 µg)	+ (50 mm)	+ (26 mm)	+ (15 mm)
CIP (5 µg)	+ (30 mm)	+ (28 mm)	+ (29 mm)
COT (25 µg)	+ (30 mm)	+ (29 mm)	-
DOX (30 µg)	+ (40 mm)	+ (27 mm)	-
GEN (10 µg)	+ (40 mm)	+ (28 mm)	+ (25 mm)

'+' = susceptible to the antibiotic, '-' = resistant to the antibiotic, AMX = Amoxicillin, AZM = Azithromycin, CIP = Ciprofloxacin, COT = Cotrimoxazole, DOX = Doxycycline, GEN = Gentamycin

The results showed that *E. coli* and *S. aureus* were susceptible to each of the antibiotics under study. However, *P. aeruginosa* was found to be resistant to amoxicillin (AMX (10 µg)), cotrimoxazole (COT (25 µg)), and doxycycline (DOX (30 µg)). Hence, the antibacterial assay discarded them to employ as the positive control. Among the antibiotics effective against all selected bacteria, CIP (5 µg) was chosen as the positive control, as it is almost equally effective against each of them.

For the plant extracts as well as positive and negative controls, the evaluation of antibacterial properties involved measuring the zone of inhibition (ZOI) for each bacterial strain, as summarized in Table 5.

Table 5. Antibacterial activity of *A. vulgaris* extracts growing at different locations

Bacteria	Zone of Inhibition (ZOI) mm			
	***A. vulgaris* (Bharatpur)**	***A. vulgaris* (Lamidada)**	***A. vulgaris* (Daman)**	**Positive control (CIP 5µg)**
E. coli	18.3 ± 0.6	15.7 ± 0.6	18.3 ± 0.6	40.0 ± 1.0
P. aeruginosa	16.0 ± 1.0	11.7 ± 0.6	18.3 ± 0.6	30.3 ± 0.6
S. aureus	20.3 ± 0.6	20.0 ± 1.0	19.3 ± 0.6	29.7 ± 0.6

The results showed that all samples of *A. vulgaris* possess antibacterial activity potential against each of the chosen bacterial strains, including *P. aeruginosa*, which was found to be resistant to antibiotics such as amoxicillin, cotrimoxazole, and doxycycline as revealed by the antibiotic susceptibility test. The methanol extracts of *A. vulgaris* from Daman and Bharatpur had almost equal antibacterial potential against *E. coli*, with the ZOI of 18.3 ± 0.6 mm higher than that of 15.7 ± 0.6 mm found for the plant extract from Lamidada. Against *P. aeruginosa*, the potential activity of the extract from Daman was found to be the highest, with an inhibition zone of 18.3 ± 0.6 mm, followed by that of the extract from Bharatpur with a ZOI of 16.0 ± 1.0 mm. The extract from Lamidada showed the lowest activity with 11.7 ± 0.6 mm of inhibition zone. The antibacterial activity of extracts of *A. vulgaris* from Daman and Bharatpur against *S. aureus* was found to be almost equal, with the ZOI of 20.3 ± 0.6 mm and 20.0 ± 1.0 mm, respectively, which are relatively higher than that of 19.3 ± 0.6 mm found for the plant extract from Lamidada. The results showed that the plant extracts from Daman and Bharatpur have relatively higher antibacterial activity potential compared to those from Lamidada. The zones of inhibition (ZOI) observed during the antibacterial assay are shown in 7.

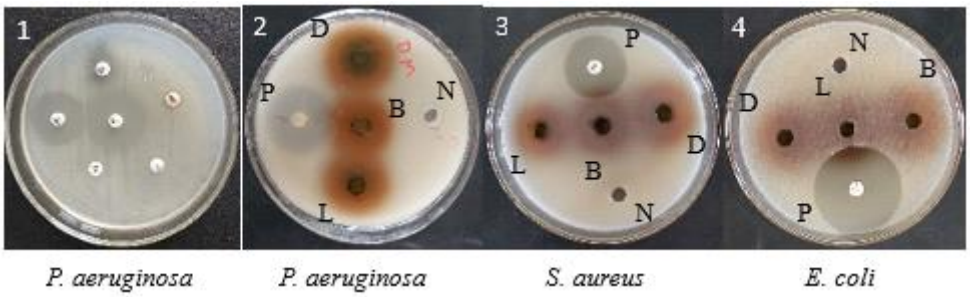

Figure 7. Images illustrating the zones of inhibition (ZOI) during antibacterial tests

(1= Antibiotic susceptibility test, 2-4 = antibacterial tests; P = Positive control, N = Negative control, D = sample from Daman, B = Bharatpur, and L = Lamidada**)**

Toxic potential

The lethal concentrations that kill 50% of the exposed population of *A. salina*, denoted as LC_{50} values in µg/mL, were calculated for the plant extracts using the probit regression analysis (Agresti, 2007; Pohan et al., 2023).

Table 6. Toxicity (LC_{50}) values for different samples of *A. vulgaris* extracts

S.N.	Plant Sample/location	Altitude (m)	LC_{50} (µg/mL)
1.	*A. vulgaris* (Bharatpur)	201	162.33 ± 93.08
2.	*A. vulgaris* (Lamidada)	1396	193.57 ± 107.03
3.	*A. vulgaris* (Daman)	2322	303.60 ± 192.22

LC_{50} values (<1000 µg/mL) indicate moderate cytotoxicity and notable bioactivity of the extracts. With the lowest value of LC_{50} (162.33 ± 93.08 µg/mL), the extract of *A. vulgaris* from Bharatpur was observed to have the highest toxicity potential, while the extract of the plant from Daman was found to be the least toxic with the highest value of LC_{50} (303.60 ± 192.22 µg/mL (Table 6).

DISCUSSION

The present study evaluated the phytochemical composition and biological activities of methanol extracts of *A. vulgaris* collected from three distinct altitudes: Bharatpur (201 m), Lamidada (1396 m), and Daman (2322 m), belonging to three climatic regions of Nepal: tropical, sub-tropical, and temperate zones, respectively. The findings revealed the altitudinal differences of statistical significance ($p<0.05$) in TPC, TFC, antioxidant activity, and cytotoxicity, while antibacterial responses showed the strain-dependent variation.

The presence of the major secondary metabolites, as confirmed by the phytochemical screening, is consistent with the previous reports (Ekiert et al., 2020; Zubair et al., 2020) and provides the chemical basis for the broad-spectrum bioactivities and potential pharmacological applications of *A. vulgaris*.

However, quantitative phytochemical assays revealed significant altitudinal variations across the geographical locations. Total phenolic and flavonoid contents were found to increase significantly with altitude; the extract from Daman had the highest TPC and TFC, followed by that from Lamidada and Bharatpur. Similar variations in TPC and TFC with altitude were also reported by Sharma and co-worker (Sharma & Adhikari, 2023); however, the values (TPC = 26.04 to 66.38 mg GAE/g and TFC = 31.54 to 71.15 mg QE/g) were lower than those found in the present study. The findings are supported by the fact that plants growing at higher altitudes need to synthesize a rich source of bioactive secondary metabolites to survive in severe climatic conditions. For *A. vulgaris*, which can thrive in tropical to temperate climatic zones, it is expected that the cold climate of the temperate zone, being quite harsh for its growth, may have promoted enhanced biosynthesis of bioactive compounds, including phenols and flavonoids (He et al., 2023). Nevertheless, genetic variability and soil chemistry may also contribute to the observed differences (Pandey et al., 2017; Khalil et al., 2020).

The antioxidant activity assay revealed a significant ($p<0.05$) increase in the antioxidant potential with the altitude. Among the samples tested, the extract from Daman showed the highest antioxidant potential, while that of Bharatpur exhibited the least antioxidant potential, and the plant extract of Lamidada showed intermediate antioxidant potential. The strong inverse correlation between IC_{50} values and TPC/ TFC further validates the contribution of TPC and TFC to the antioxidant potential of *A. vulgaris*, which is also supported by a previous study by Vinson and coworkers (Vinson et al., 1998). These correlations also align with recent findings reported by Sharma and Adhikari (Sharma & Adhikari, 2023). The comparatively stronger antioxidant activity observed for Daman extract is attributed to the higher bioactive phenolic and flavonoid contents relative to the extracts of Lamidada and Bharatpur (the least antioxidant activity, TPC and TFC). Our observation of elevated antioxidant activity at higher altitudes aligns with a recent study by Gautam et al. (2024). They reported an IC_{50} value of 19.00 ± 0.81 µg/mL for the methanol extract of *A. vulgaris* collected from Khaptad (2907 m), which indicates notably stronger antioxidant activity than that observed in our Daman sample (IC_{50} = 58.407 ± 0.093 µg/mL). The altitude-dependent increase in antioxidant potential observed here aligns with the positive correlation between altitude and anti-inflammatory activity reported by Pandey et al. (2021). This parallel trend is biologically expected, as antioxidant mechanisms frequently underlie and mitigate inflammatory pathways. The need for tolerance of the plant to the extreme climatic conditions of the temperate climatic zone can be expected to promote the synthesis of higher levels of phenolic and flavonoid compounds in the samples from Daman in the present study. Hence, the plants from Daman have higher antioxidant potential compared to those from Lamidada and Bharatpur. However, the activity recorded in the present study was lower than that reported by Pandey et al. (2017) for the methanol extract from Dhulikhel (IC_{50} = 48.77 ± 0.11 µg/mL) and, it is even lower than that reported by Temraz and El-Tantawy (2008) for the aqueous extract (IC_{50} = 11.4 µg/mL) of the plant from Cairo, Egypt (Temraz & El-Tantawy, 2008) though the corresponding values of TPC and TFC were found much higher. The extremely high antioxidant potential of the aqueous extract (IC_{50} = 11.4 µg/mL) with relatively lower TPC and TFC values: 19 ± 0.16 mg GAE and 0.76 ± 0.0 mg RE (rutin equivalent)/g, respectively, has been reported in the previous study (Temraz & El-Tantawy, 2008). This discrepancy may arise from differences between the extraction solvent, phytochemical composition, or assay conditions and warrants further comparative investigation.

The assessment of brine shrimp lethality revealed that the extracts of all three plant samples have moderate cytotoxic activity (Clarkson et al., 2004). LC_{50} values showed an increasing trend with altitude, suggesting that the extract of *A.* vulgaris from low altitudes may contain higher amounts of potentially toxic compounds than that from high altitudes. The elevated toxicity observed in the low-altitude *A. vulgaris* extracts from Bharatpur may be attributed to the bioaccumulation of environmental pollutants, given the site's proximity to an urban center. Furthermore, literature suggests that plants at lower elevations often synthesize higher levels of toxic secondary metabolites, such as alkaloids, as a defense mechanism against intense grazing pressure (Lahiri & Krishna, 2024). Nevertheless, comprehensive in vivo toxicological evaluations remain necessary to validate these mechanisms.

The extracts of *A. vulgaris* from all three locations were found to have antibacterial activity against all three bacterial strains selected, including the notorious *P. aeruginosa*, which was found to be resistant against the antibiotics COT, AMX, and DOX. Earlier work by Sharma and co-worker (Sharma & Adhikari, 2023), however, has reported no antibacterial activity of the methanol extract of the leaves of *A. vulgaris* from Chitwan (208 m), Gorkha (862 m) and Kathmandu (1324 m), though they along with Pandey and co-

workers (Pandey et al., 2017) have reported antibacterial activity of the essential oil increasing with the altitude. The antibacterial activity assay suggested the plant is a reliable source of bioactive compounds, which need to be isolated, characterized, and identified for developing future drug formulations against various infectious diseases. The results also justified the effectiveness of the plant in ethnomedicinal practice against a variety of diseases, including gastrointestinal issues, indigestion, and inflammation, etc. The higher value of ZOI for the extracts of *A. vulgaris* from Bharatpur and Daman (both equal to 18.3 ± 0.6) compared to that of Lamidada against *E. coli* can be interpreted as a result of the two extreme climatic conditions at Bharatpur (tropical climate) and Daman (temperate climate) that have to be tolerated by the plants growing at these regions compared to the relatively moderate sub-tropical climatic conditions at Lamidada. So, *A. vulgaris* growing at the two extreme climatic conditions of Bharatpur and Daman has a greater potential source of bioactive compounds compared to that of Lamidada. A similar interpretation is plausible for the antibiotic activity against *P. aeruginosa*. Here, the Daman extract showed greater ZOI compared to that of Bharatpur and Lamidada, suggesting that the bioactive compounds formed to tolerate the extreme cold climatic conditions of the temperate zone of Daman are more abundant and effective against *P. aeruginosa*. However, against *S. aureus*, the ZOI was found to be the highest for the plant extract from Bharatpur, followed by that of Lamidada and Daman. These results suggest that *A. vulgaris* growing under the pollution-induced stress conditions of Bharatpur may synthesize alternative bioactive metabolites. Although this sample exhibited lower TPC and TFC, these distinct stress-induced compounds appear to possess superior antibacterial efficacy against *S.* aureus compared to those from Lamidada and Daman. While this hypothesis is supported by recent literature (Lahiri & Krishna, 2024), further rigorous research is required for validation. Future investigations should focus on the isolation, characterization, and definitive identification of specific bioactive compounds within *A. Vulgaris* populations across these regions.

CONCLUSION

The study demonstrates that *A. vulgaris* growing at different altitudes exhibits significant variation in TPC, TFC, antioxidant activity, cytotoxicity, and antibacterial responses. The extract from the highest altitude (Daman, 2322 m) had TPC and TFC in the greatest quantity, and exhibited the strongest antioxidant activity. All samples showed moderate cytotoxicity and antibacterial activity, particularly against *S. aureus, P. aeruginosa,* and *E. coli.* Notably, the extracts from Bharatpur (tropical) and Daman (temperate) had relatively higher antibacterial potential compared to that from Lamidada (sub-tropical). This trend may be associated with the environmental stress conditions at both low and high altitudes, which are known to influence secondary metabolite biosynthesis.

Overall, these findings support the ethnopharmacological application of *A. vulgaris* and highlight it as a potential reservoir of novel antioxidant and bioactive compounds. The significant positive correlations ($p<0.05$) of the TPC, TFC, and antioxidant activity of the extract of *A. vulgaris* with the altitude demonstrate that the therapeutic efficacy of *A. vulgaris* extracts, particularly against oxidative stress-related diseases, enhances at higher elevations. Further studies focusing on the isolation, purification, and characterization of the bioactive constituents, along with mechanistic and *in vivo* evaluations, are warranted to explore their potential application in drug discovery pipelines.

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AUTHORS CONTRIBUTION

Conceptualization: KLG; Methodology: KLG, AMB; Validation: KRS, KLG; Investigation: KLG; Data Analysis: KLG; Writing-original draft: KLG, Writing-review & editing: KRS; Supervision: KRS; Funding acquisition: KLG

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ORCIDs

Kanhaiya Lal Gupta:
https://orcid.org/0009-0001-5461-1160
Anup Muni Bhajracharya:
https://orcid.org/0009-0008-2495-0322
Khaga Raj Sharma:
https://orcid.org/0000-0002-1555-0887

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this article.

ETHICAL STATEMENT

The work presented is original and is neither published nor under review for publication elsewhere.

DATA AVAILABILITY STATEMENT

All relevant data are available upon reasonable request.

SUPPLEMENTARY INFORMATION

None.

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