

Agro-climatic variation in phytochemicals and biological activities of *Portulaca oleracea* L.

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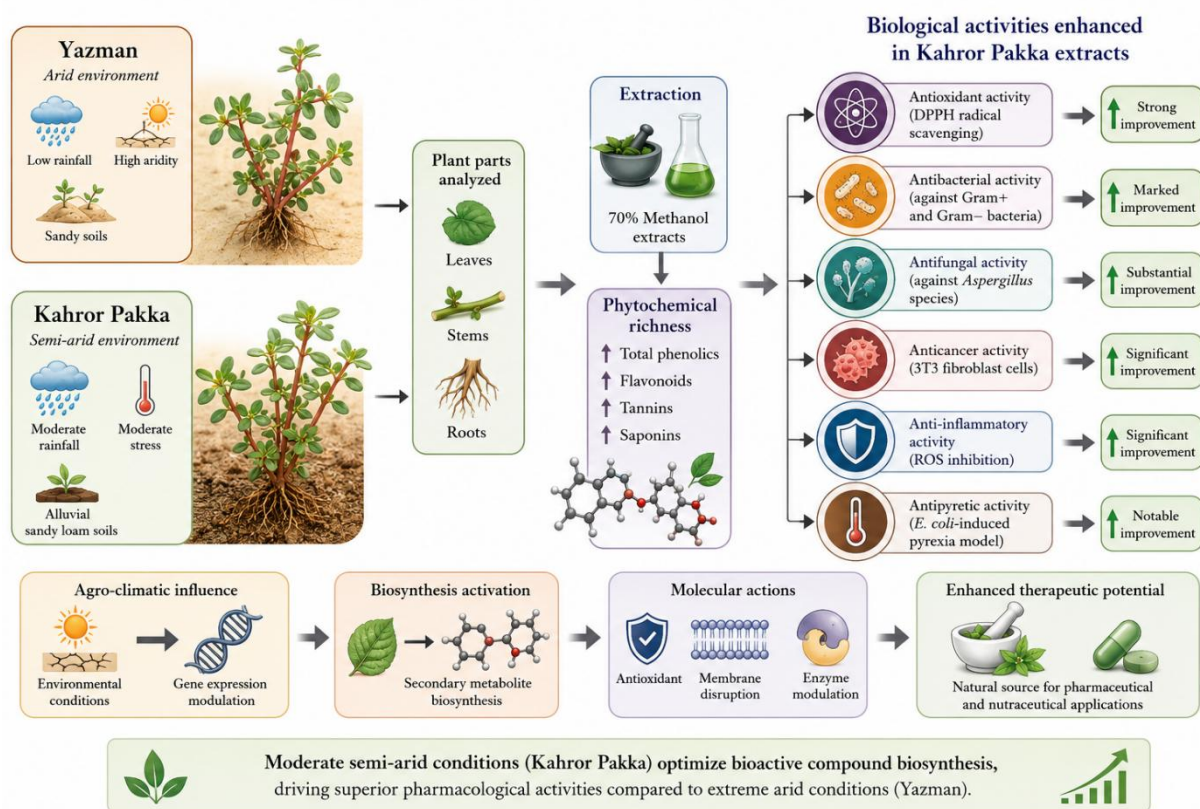
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Abstract

The rising global problem of antimicrobial resistance and chronic diseases requires the discovery of novel therapeutic agents from natural sources. Medicinal plants possessing diverse bioactive compounds offer sustainable alternatives to synthetic pharmaceuticals. This study evaluated the phytochemical composition and biological activities of *Portulaca oleracea* L. collected from two distinct agro-climatic zones (Yazman and Kahrora Pakka) in Southern Punjab, Pakistan during February 2024. The plant parts (leaves, stems and roots) were extracted through cold maceration using methanol. Proximate composition, quantitative phytochemicals (total phenolic, tannin, flavonoid and saponin contents), antioxidant activity (2,2-diphenyl-1-picrylhydrazyl, DPPH, assay), antibacterial efficacy against four pathogenic strains (*Escherichia coli*, *Staphylococcus aureus*, *Streptococcus agalactiae* and *Klebsiella pneumoniae*), antifungal activity against four *Aspergillus* species and pharmacological potentials (*in vitro* cytotoxicity, anti-inflammatory and antipyretic) were assessed using the DPPH radical-scavenging assay (antioxidant), agar-well diffusion (antibacterial and antifungal), the MTT assay on murine 3T3 fibroblasts (cytotoxicity), zymosan-induced ROS chemiluminescence (anti-inflammatory) and an *E. coli*-induced pyrexia rabbit model (antipyretic). Three independent biological replicates were sampled per location and plant part ($n = 3$), each analyzed in analytical triplicate and the resulting plant means were subjected to two-way factorial analysis of variance (ANOVA) testing location, plant part and their interaction, followed by Tukey's honestly significant difference (HSD) test ($p \leq 0.05$). Kahrora Pakka samples exhibited significantly higher crude protein (56.32-64.32%), moisture (6.49-7.21%) and ash (14.19-18.41%) contents, whereas Yazman specimens showed elevated crude fiber (20.43-56.23%) and crude fat (7.21-22.99%). Leaf extracts from Kahrora Pakka demonstrated superior antioxidant activity (75.73% DPPH inhibition), total phenolic content (35.31 mg GAE g⁻¹), total flavonoid content (23.11 mg QE g⁻¹), maximum antibacterial zones of inhibition (11.57-14.57 mm) and enhanced pharmacological activities, including *in vitro* cytotoxicity toward 3T3 fibroblasts (59.40%), anti-inflammatory (61.40%) and antipyretic (34.83%) effects. Pearson correlation analysis revealed highly significant positive relationships ($p \leq 0.001$) between secondary-metabolite contents (total phenolics, flavonoids, tannins and saponins) and the antioxidant, antimicrobial and pharmacological activities. Because only two locations were compared and location is inseparable from the accompanying edaphic and climatic differences, these site contrasts are descriptive and cannot attribute the observed variation to any single agro-climatic factor; within that constraint, leaves consistently showed the highest activity of the three organs at both sites. *Portulaca oleracea* from Kahrora Pakka therefore warrants further investigation as a candidate source of antioxidant and antimicrobial constituents.

Graphical abstract



Keywords: Phytochemicals, Antioxidant, Antimicrobial, Pharmacological, Purslane, Methanol, Therapeutics

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Introduction

Environmental stressors and changes in climate are now one of the most important factors impacting secondary metabolism in plants; therefore, it is necessary to study all medicinal plants from every agro-ecological zone (El Gendy et al., 2023; Hounsou et al., 2024; Mykhailenko et al., 2025; Yeshiwas et al., 2026). Increasing concerns regarding multidrug-resistant infections and the rising prevalence of oxidative stress-related chronic diseases and cancer pose a major threat to current healthcare practices (Acierno et al., 2025; Liu et al., 2025). Accordingly, novel, sustainable and environmentally safe bioactive compounds from botanical sources must be identified for use as an addition or alternative to synthetic medicines (Atanasov et al., 2021). Abiotic stressors in the environment, including extreme dry conditions, exposure to radiation and temperature variations of more than 40 °C, have detrimental effects on plant functions; however, they also induce plants to produce excessive amounts of secondary metabolites to enhance their chances of survival (Jangpangi et al., 2025). The significance of this physiological adaptation lies in the fact that the compounds produced by plants in response to adverse conditions possess pharmacological activity that positively influences human well-being (Harisha, 2023). Hence, searching for medicinal plants native to stress-ridden agro-climatic regions is advantageous for the bioprospecting of therapeutically active molecules (Sarfaraz et al., 2014). Ethnobotanical surveys of the high-temperature districts of Southern Punjab have documented extensive local medicinal use of the native flora, which underlines the pharmacological relevance of this region (Usman et al., 2021b).

Portulaca oleracea L. (purslane), a highly adaptable succulent characterized by the possession of Crassulacean Acid Metabolism (CAM), a water-conserving photosynthetic pathway in which the stomata open at night to fix CO₂ is recognized for its adaptation to arid and semi-arid environments (Nemzer et al., 2020). Widely used as an effective herbal remedy and a source of functional foods, this plant possesses valuable secondary metabolites, including omega-3 fatty acids, polysaccharides and other bioactive constituents (Agbor et al., 2022). The biochemical nature of the health benefits associated with purslane is inherently tied to its adaptations to environmental stresses such as water scarcity and elevated temperatures through upregulated

phenylpropanoid and terpenoid biosynthesis, thereby scavenging reactive oxygen species (ROS) (Elgamal et al., 2023). These phytochemicals, such as phenolic acids, flavonoids and saponins, have electron-donating capacity that reduces free radicals, disrupts microbial cell walls and modulates mammalian inflammatory responses through their signaling pathways (Georgiev et al., 2017). Biosynthesis of these secondary metabolites greatly depends on the type of environmental stress experienced by the plants during their growing stages. Comparable compilations of the nutritional, medicinal and cosmetic value of bioactive constituents in other widely consumed edible species illustrate how total phenolic and flavonoid pools underpin functional-food value (Usman et al., 2021a). Although many earlier works describe the nutrient composition and identify antioxidants present in *P. oleracea*, information on ecophysiological variability remains limited (Segneanu et al., 2025). Many studies conducted to date have used either one ecotype or plants grown in controlled greenhouses, where ideal conditions are maintained and the impact of the natural environment gradient on the partitioning of phytochemicals was not evaluated (Tzanova et al., 2020). In addition, few studies address the correlation of the tissue-specific distribution of proximal nutrients and secondary metabolites with their pharmacological properties in varying geography (Usman et al., 2022). Most of the earlier studies used an *in vitro* approach to understand the therapeutic effects of *P. oleracea*, leaving a significant gap in the *in vivo* verification of its antipyretic and anti-inflammatory activities. Systematic reviews of the anti-cancer potential of phytochemicals in other plant families report that activity is distributed unevenly among organs and taxa (Usman et al., 2022).

To address these gaps, an integrated analytical approach is required. Such an approach must simultaneously evaluate the interactions among environmental stress, tissue-specific (organ) variation and biological function. Proximate composition analysis (crude protein, moisture, crude fiber, crude fat, ash and dry matter) is the first step in characterizing how a plant adjusts its nutritional and structural composition to the growing environment (Harris and Marshall, 2017). Quantification of total phenolic, tannin, flavonoid and saponin contents then identifies the metabolite classes most likely to underlie a given bioactivity (Singleton et al., 1999). Such phytochemical characterization must be coupled with a broad biological screen comprising antioxidant,

antibacterial (with respect to Gram-positive and Gram-negative bacteria), antifungal, cytotoxicity against mammalian cells and *in vivo* antipyretic assays (Sultana et al., 2015; Veiga et al., 2019; Ijarotimi et al., 2022).

We hypothesized that wild *P. oleracea* growing under the more severe moisture deficit of the hyper-arid Cholistan Desert would preferentially partition assimilates into structural and storage fractions, whereas plants from the less water-limited semi-arid alluvial plain would accumulate higher concentrations of phenolic and flavonoid secondary metabolites and that these metabolite differences would covary with the measured antioxidant, antimicrobial and pharmacological responses. Three objectives followed from this hypothesis. The first was to quantify proximate composition and secondary-metabolite concentrations in roots, stems and leaves of wild *P. oleracea* collected from Yazman (Cholistan Desert) and Kahrora Pakka (alluvial plain) in Southern Punjab, Pakistan. The second was to evaluate the antioxidant, antibacterial, antifungal, cytotoxic, anti-inflammatory and antipyretic responses of the same methanolic extracts. The third was to test whether organ and site differences in metabolite concentration corresponded to differences in biological response. Because the design contrasts only two locations, it is framed here as a descriptive site comparison rather than as a test of any individual climatic or edaphic variable.

Materials and Methods

Climatic conditions of study area and plant material collection

This study was conducted in the Southern Punjab region of Pakistan, and all plant material was collected in a single sampling campaign in February 2024. Leaf, stem and root material of *Portulaca oleracea* L. was collected from two different agro-climatic sites. These sites were Site 1 (S₁): Yazman (Cholistan Desert, Bahawalpur; 29.35440° N, 71.69110° E) and Site 2 (S₂): Kahrora Pakka (Lodhran; 29.53° N, 71.63° E) (Figure 1). Sampling coincided with the peak vegetative stage of the species at both sites. The agro-climatic conditions of Yazman (Cholistan) comprise hot desert to semi-arid conditions under the Köppen-Geiger classification, with mean annual rainfall of 100-250 mm (Sarfaraz et al., 2014). The soil at this site was a sandy clay loam of aeolian origin. The other site selected was Kahrora Pakka (Lodhran), which had hot arid to semi-arid climatic conditions with rainfall in the range of 200-350 mm and alluvial sandy loam soil (Hussain et al., 2020). These sites were selected because of their varied environmental and edaphic characteristics to determine the impact of agro-climatic variations on phytochemical profiles and biological activity. Plants at both sites were wild-growing; no nursery-raised or cultivated material was used. At each site the material for each organ was drawn from three separate replicate units and each replicate unit was treated as one biological replicate for analysis, giving $n = 3$ independent biological replicates per location and organ; the extract prepared from each replicate was subsequently assayed in analytical triplicate.

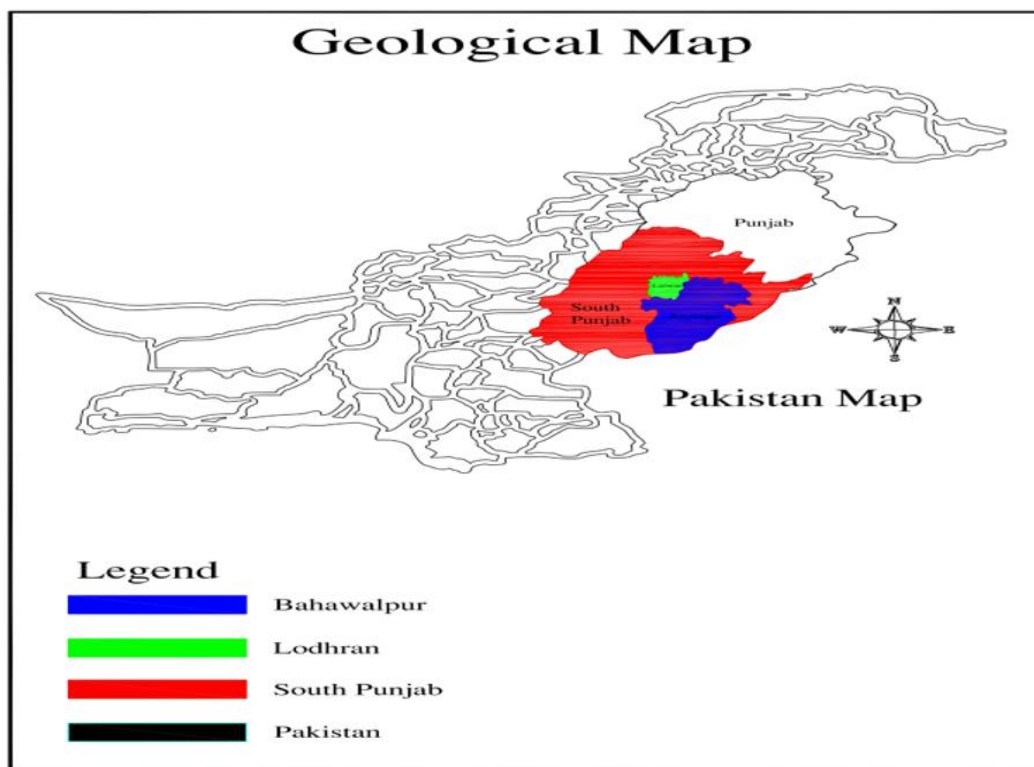


Figure-1. Map of the study area in southern Punjab, Pakistan, showing the sampling locations of *Portulaca oleracea* L. at site 1 (Cholistan Desert, Yazman, Pakistan; 29.35440° N, 71.69110° E) and site 2 (Kahrer Pakka, Lodhran, Pakistan; 29.53° N, 71.63° E).

Botanical identification and authentication

The collected samples of the plant species were identified taxonomically as *Portulaca oleracea* L. (family: Portulacaceae) by morphological examination using standard botanical keys. Macroscopic features, such as growth form (succulent, smooth, prostrate stems), leaves (alternate to subopposite arrangement, obovate to spatulate leaves) and flowers (yellow-colored, pentamerous flowers), were compared with the description given in the Flora of Pakistan. Taxonomical identification was performed by a plant taxonomist at the Department of Botany, The Islamia University of Bahawalpur, Pakistan. Vouchers were prepared using standard procedures and deposited in the herbarium of the Department of Botany, The Islamia University of Bahawalpur, Pakistan (Nemzer et al., 2020).

Sample preparation and extraction

The collected plant material samples were first washed using running tap water to remove soil and dust particles and then rinsed with distilled water. These

plant samples were dried in the laboratory under shade at room temperature (25-30 °C) for 25 days, providing sufficient air circulation to prevent microbial growth and enzymatic degradation of plant constituents (Tzanova et al., 2020). The dried materials were ground into a fine, homogeneous powder using an electric mill (Model: KF-100, China) and stored in airtight glass containers under dry conditions. For extraction, 100 g of powdered plant material from each sample was subjected to cold maceration with 500 mL of analytical-grade methanol (99.9%, Merck, Germany) for seven days with intermittent shaking. The mixture was filtered through Whatman No. 1 filter paper, and the extraction process was repeated twice. The combined filtrates were concentrated under reduced pressure at 40-45 °C using a rotary evaporator (Model: RE-300, Bibby Sterling, UK) to obtain crude methanolic extracts. The dried extracts were stored in amber-colored glass containers at 4 °C until further analysis (Usman et al., 2022). The percentage extraction yield of each crude methanolic extract was

calculated as the mass of dried extract recovered per 100 g of powdered plant material.

Proximate composition analysis

Moisture and dry matter contents were determined by oven-drying fresh samples at 105 °C to a constant weight (Harris and Marshall, 2017). Crude protein was quantified using the Kjeldahl method, converting total nitrogen with a factor of 6.25 (Latimer and Horwitz, 2019). Crude fiber content was estimated through sequential digestion with 1.25% H₂SO₄ and 1.25% NaOH, followed by ashing at 600 °C to calculate the weight loss on ignition (Latimer and Horwitz, 2019). Crude fat was extracted using petroleum ether (40-60 °C) in a Soxhlet apparatus (Latimer and Horwitz, 2019). The total ash content was determined by incinerating samples in a muffle furnace at 650 °C for 3 hours to ensure complete combustion of organic matter (Harris and Marshall, 2017).

Quantitative phytochemical analysis

The total phenolic content (TPC) and total tannin content (TTC) were quantified spectrophotometrically using Folin-Ciocalteu and Folin-Denis reagents, respectively. The values were obtained at 765 and 700 nm and were expressed in mg GAE and TAE per gram (Price et al., 1978, Singleton et al., 1999). Total flavonoid content (TFC) was calculated using the aluminum chloride test at 430 nm and expressed as mg QE per gram (Zhishen et al., 1999). Total saponin content (TSC) was obtained using the vanillin-sulfuric acid test at 560 nm, with aescin as the standard (mg AE per gram) (Hiai et al., 1976). Calibration curves were constructed for every assay using the corresponding reference standard: gallic acid for TPC, tannic acid for TTC, quercetin for TFC and aescin for TSC.

Evaluation of biological activities

Antioxidant activity was analyzed using the DPPH radical-scavenging test, which is based on the reduction in the absorbance value at 517 nm after incubating for 60 minutes, where ascorbic acid was used as the control substance (Ijarotimi et al., 2022). Inhibition of bacterial pathogens, namely *E. coli*, *S. aureus*, *S. agalactiae* and *K. pneumoniae*, was determined using an agar well diffusion test, with ciprofloxacin as the control antibiotic (Veiga et al., 2019). Antifungal activity against four different *Aspergillus* strains (*A. fumigatus*, *A. ustus*, *A. flavus*

and *A. niger*) was measured using the agar-well diffusion method on YPD agar plates (Khursheed and Jain, 2021). In vitro cytotoxicity was evaluated in non-malignant mouse fibroblasts (3T3 line), where the cells were exposed to the test extract for 48 hours before measuring the MTT dye formazan absorbance value at 540 nm, using doxorubicin as the control drug (Segneanu et al., 2025). Anti-inflammatory activity was estimated using a luminol-dependent chemiluminescence reaction to measure ROS generation in zymosan-activated whole blood, with indomethacin as the reference drug (Georgiev et al., 2017). For *in vivo* antipyretic and pyrexia induction assays, approved by the Institutional Animal Ethics Committee (CUVAS/IAEC/2024/015), fever was induced in rabbits via intravenous *E. coli* administration. The maximum rectal temperature rise (°C) and percentage reduction following oral extract treatment (50-150 mg kg⁻¹) were monitored over 4 hours, with paracetamol as the reference drug (Sultana et al., 2015; Zayed et al., 2023). For both antimicrobial assays, bacterial and fungal inocula were standardized before plating, wells of uniform diameter were loaded with a fixed mass of extract and methanol was applied as the solvent control alongside the antibiotic or antifungal reference.

Statistical analysis

All assays were performed on three independent biological replicates per location and plant part, each analyzed in analytical triplicate. Analytical replicates were averaged within replicate plants so that the plant and not the analytical reading, served as the experimental unit ($n = 3$ per location $\times$ plant part combination; 18 experimental units in total), which avoids pseudoreplication in both the analysis of variance and the correlation analysis (Steel and Torrie, 1960). The 23 response variables were analyzed by two-way factorial ANOVA partitioning variation into location (df = 1), plant part (df = 2) and their interaction (df = 2) against an error term with 12 df, using Statistix 8.1 software (Batič et al., 2012). Residual normality was assessed by the Shapiro-Wilk test ($W = 0.77-0.98$), which indicated departures from normality for 14 of the 23 variables; for these variables the conclusions were confirmed by Kruskal-Wallis tests across the six locations $\times$ plant part combinations (all $p \leq 0.008$). No transformation was retained, because log₁₀ transformation did not restore residual normality for most of the affected variables. Homogeneity of variance was confirmed for all 23

variables by Levene's test (minimum $p = 0.56$). Means were separated by Tukey's Honestly Significant Difference (HSD) test at $\alpha = 0.05$, with significance reported at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ (Wester, 2018). Effect sizes are expressed as the proportion of the total sum of squares attributable to each model term. Pearson correlation coefficients were computed on the 18 plant means rather than on individual analytical readings. All graphs were prepared using OriginPro 2021 (Origin Lab Corporation, Northampton, MA, USA) (Edwards, 2002).

Results

Proximate composition parameters

The proximate composition parameters of *Portulaca oleracea* L. collected from the distinct agro-climatic zones of Yazman and Kahrir Pakka were evaluated (Figure 2). Crude protein differed between locations, with Kahrir Pakka samples containing higher concentrations than those of the Yazman plants (root: 56.32% vs. 47.30%; stem: 60.32% vs. 51.31%; leaf:

64.32% vs. 55.32%). Moisture content was higher in the Kahrir Pakka plants (root: 6.49%; stem: 6.79%; leaf: 7.21%) than for the Yazman samples (root: 2.42%; stem: 2.25%; leaf: 2.47%; $p \leq 0.05$). Conversely, crude fiber concentration was higher for the Yazman samples (root: 20.43%; stem: 43.38%; leaf: 56.23%) than for the Kahrir Pakka samples (root: 16.79%; stem: 18.35%; leaf: 22.30%). Crude fat was likewise higher in the Yazman samples (root: 7.21%; stem: 19.07%; leaf: 22.99%) than in the Kahrir Pakka samples (root: 4.81%; stem: 5.12%; leaf: 6.40%). Ash content, which reflects total mineral content (e.g., in Kahrir Pakka, root = 14.19%, stem = 15.58%, leaf = 18.41% vs. Yazman, root = 11.66%, stem = 8.46%, leaf = 7.39%) indicated that overall mineral content was higher in Kahrir Pakka than Yazman samples. The dry matter percentages from Yazman were higher (i.e., root = 97.56%, stem = 98.71%, leaf = 97.53%) than those of the Kahrir Pakka samples (i.e., root = 93.49%, stem = 93.20%, leaf = 92.78%). This indicates lower water content in the arid Cholistan region.

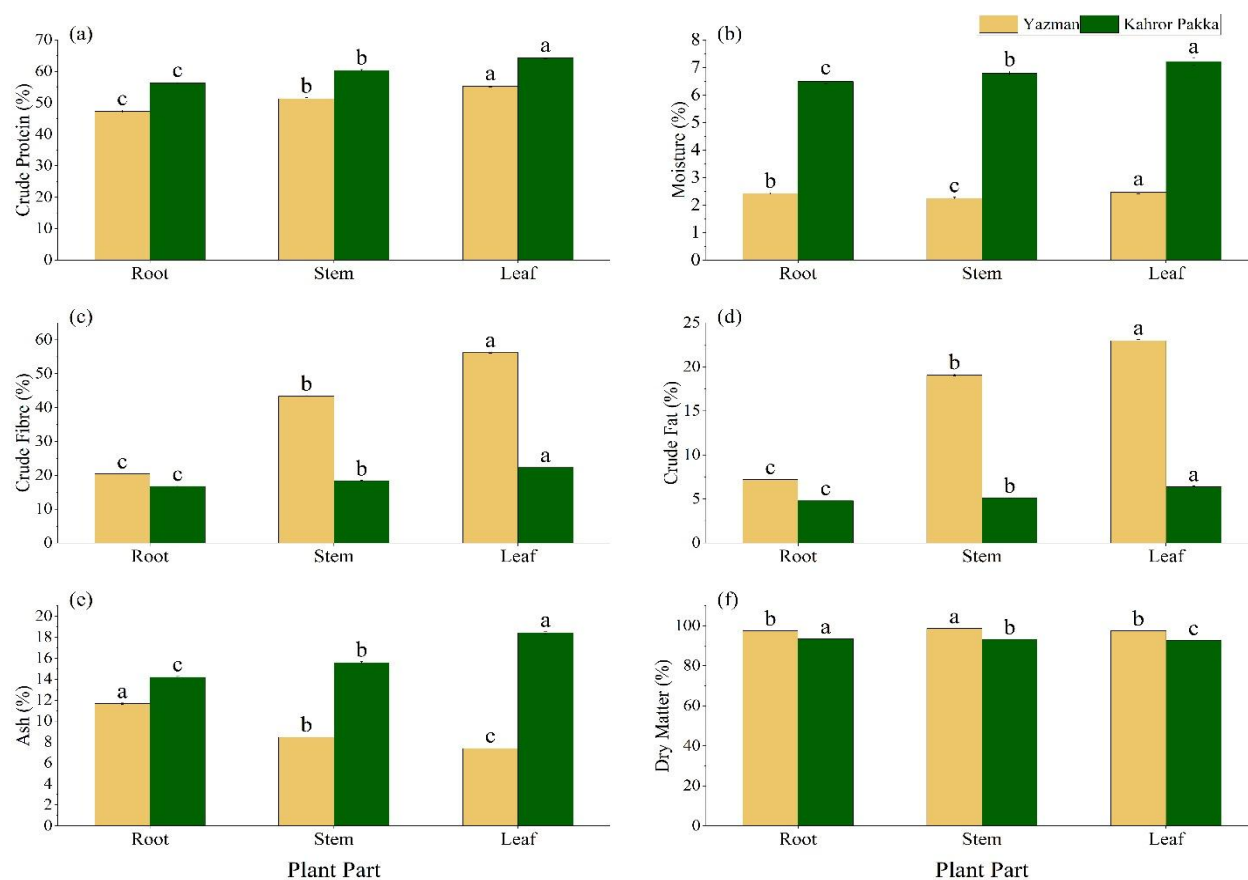


Figure-2. Comparative study on the parameters of proximate composition in various parts (root, stem and leaf) of *Portulaca oleracea* L. grown in Yazman and Kahrir Pakka. Data are presented as mean $\pm$ SD ($n = 3$).

Significance was determined by performing two-way factorial ANOVA (location $\times$ plant part) and then applying the Tukey HSD post-hoc test at $p \leq 0.05$. Lowercase letters compare plant parts within a single sampling site; within each site, bars not sharing a letter differ at $p \leq 0.05$ by Tukey's HSD. The letters do not compare the two sites with each other, and between-site differences were tested separately by the location term of the two-way factorial ANOVA.

Antioxidant activity (% DPPH inhibition)

Marked variation was recorded in the antioxidant efficacy of *P. oleracea* L. extracts based on the radical-scavenging activity test (Figure 3). Leaves exhibited the highest radical-scavenging activity, with a mean inhibition percentage of 70.63%, followed by stems (65.03%) and roots (60.19%). Among the two

sampling sites studied, samples from the Kahrar Pakka location showed higher antioxidant activity, with respective percentages of 65.18%, 70.04% and 75.73% for the roots, stems and leaves compared with 55.19%, 60.02% and 65.53% for the same plant parts from Yazman. Across both sites, leaf extracts exhibited significantly higher antioxidant activity than the stem and root extracts ($p \leq 0.05$, Tukey's HSD).

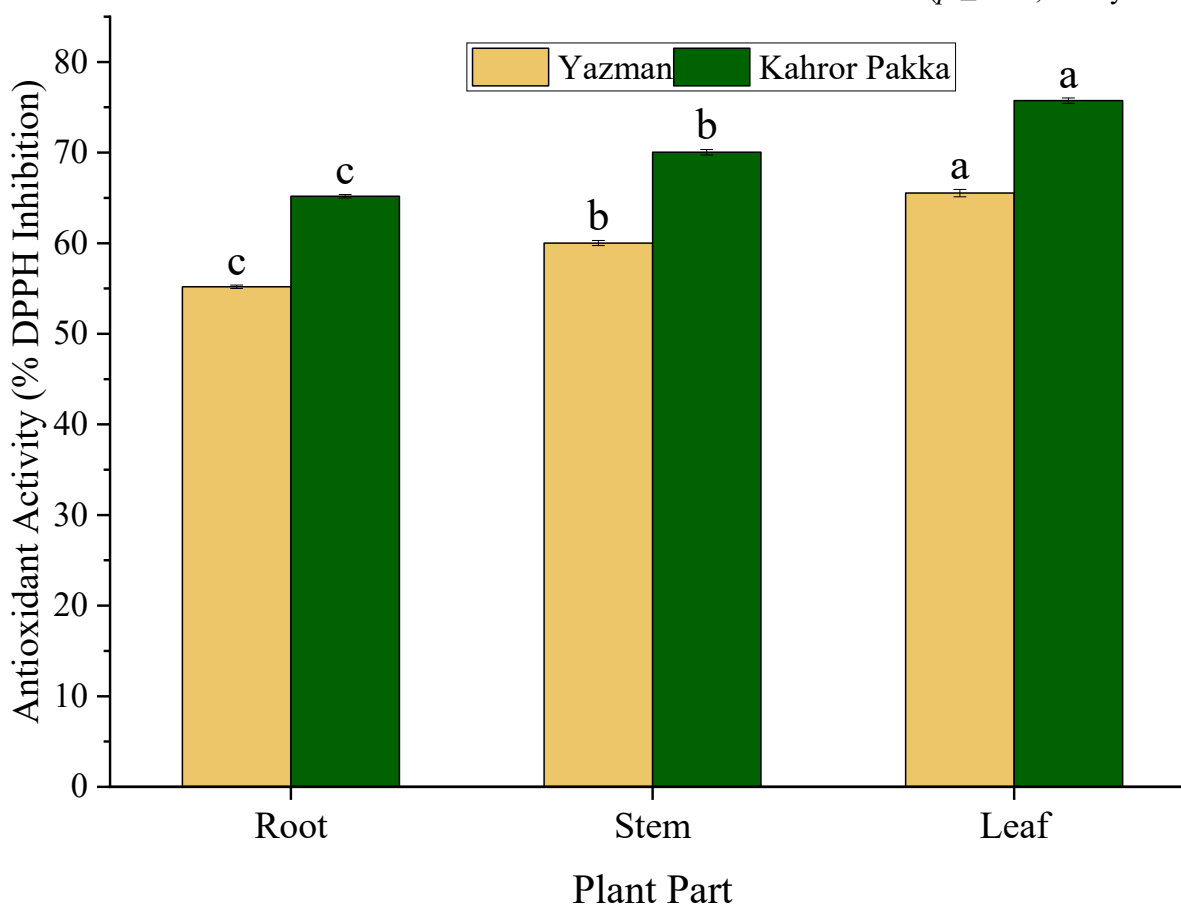


Figure-3. Antioxidant activities (% of DPPH scavenging ability) of different parts of *Portulaca oleracea* L. methanolic extracts obtained from Yazman and Kahrar Pakka.

Results are reported as mean $\pm$ standard deviation ($n = 3$). Two-way factorial ANOVA (location $\times$ plant part) was performed, with Tukey's HSD post-hoc test at significance level $p \leq 0.05$. Lowercase letters compare plant parts within a single sampling site; within each site, bars not sharing a letter differ at $p \leq 0.05$ by Tukey's HSD. The letters do not compare the two sites with each other, and between-site differences were tested separately by the location term of the two-way factorial ANOVA.

Antibacterial activity (Zone of inhibition, mm)

The antibacterial properties of *Portulaca oleracea* L. methanolic extracts were assessed against four

bacterial strains using the agar well diffusion technique, with the methanolic extract loaded per well at 50 mg per well and ciprofloxacin as the positive

control; the findings were presented in terms of zones of inhibition in millimeters (Figure 4). The extracts exhibited varying activity against all tested bacterial strains, with Kahrora Pakka exhibiting higher zones of inhibition than Yazman samples across all parts of the plant. The inhibition zones observed in *Escherichia coli* were maximal in leaf extracts (14.57 mm for Kahrora Pakka and 12.57 mm for Yazman), followed by stems (13.57 mm for Kahrora Pakka and 11.57 mm for Yazman), while minimal zones were recorded in roots (12.57 mm for Kahrora Pakka and 10.57 mm for Yazman). On *Staphylococcus aureus*, the zones of inhibition on leaf extracts were 13.57 mm and 11.57 mm for Kahrora Pakka and Yazman, respectively, with reduced inhibition zones recorded on stems and roots. The antibacterial effect against *Streptococcus agalactiae* showed the same pattern, where the leaf

extract gave a zone of inhibition of 12.57 mm (Kahrora Pakka) and 10.57 mm (Yazman), whereas the stem and root extracts had lower activity. Regarding *Klebsiella pneumoniae*, which showed the lowest susceptibility among all the strains used, leaf extract from Kahrora Pakka provided a zone of inhibition of 11.57 mm compared to 9.57 mm observed in Yazman samples. The leaf extract showed higher antibacterial activity than the stem and root extracts against all bacterial strains tested, thus implying the presence of more antimicrobial compounds in leaves than other plant tissues. Extracts collected from Kahrora Pakka showed significantly higher activity than Yazman samples ($p \leq 0.05$, Tukey's HSD), indicating that both edaphic and environmental factors affected the production of antibacterial compounds in the plant.

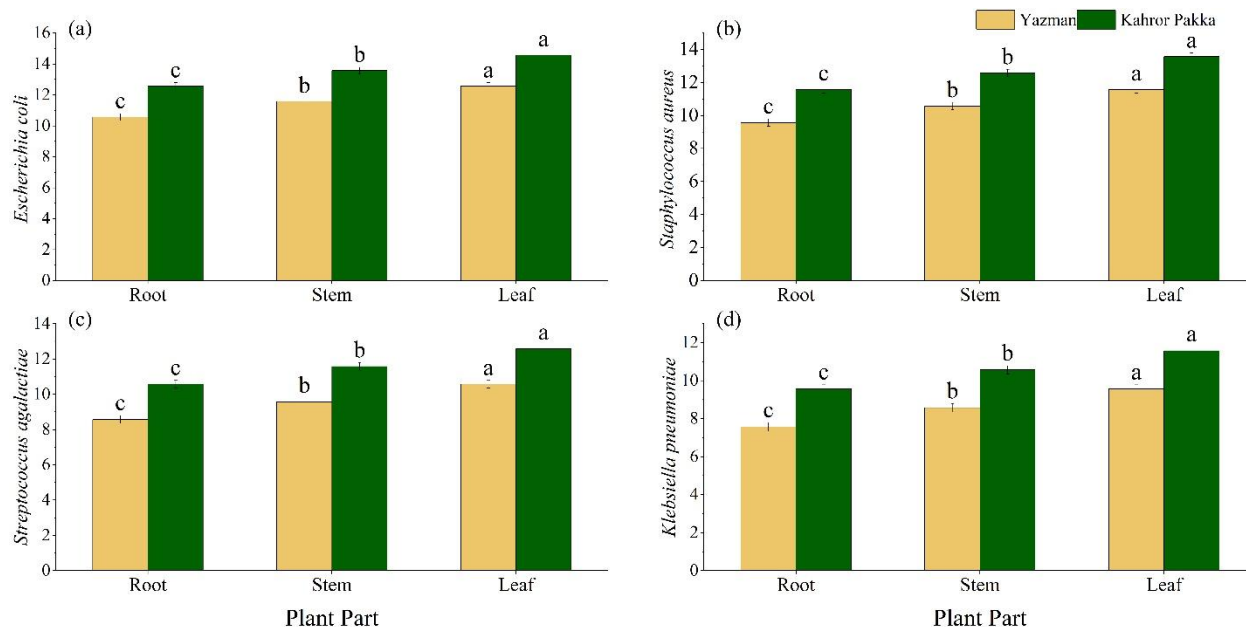


Figure-4. Comparative evaluation of the antibacterial effect in terms of zones of inhibition (mm) of methanol extracts of *P. oleracea* L. against four bacterial strains, including (a) *E. coli*, (b) *S. aureus*, (c) *S. agalactiae* and (d) *K. pneumoniae*. Efficiency was studied for different parts of the plant, such as the root, stem and leaf of *P. oleracea* grown in two locations, Yazman and Kahrora Pakka.

Values are expressed as mean $\pm$ SD ($n = 3$). Statistical analysis was performed using two-way factorial ANOVA (location $\times$ plant part), followed by Tukey's HSD test. Lowercase letters compare plant parts within a single sampling site; within each site, bars not sharing a letter differ at $p \leq 0.05$ by Tukey's HSD. The letters do not compare the two sites with each other, and between-site differences were tested separately by the location term of the two-way factorial ANOVA.

Pharmacological and biological activities

The pharmacological and biological potentials of *P. oleracea* L. extracts were evaluated using *in vitro* cytotoxicity, *in vitro* anti-inflammatory and *in vivo*

antipyretic assays, along with the assessment of pyrexia induction across different plant parts and collection sites (Figure 5). Test extracts were applied as methanolic solutions at concentrations ranging from

1 to 30 $\mu\text{g mL}^{-1}$ and 1, 10 and 100 $\mu\text{g mL}^{-1}$, respectively, for the *in vitro* cytotoxicity and anti-inflammatory assays, while the *in vivo* antipyretic and pyrexia assays were administered orally at 50, 100 and 150 mg kg^{-1} , with paracetamol as the reference standard. Cytotoxicity toward 3T3 fibroblasts (% inhibition) differed by tissue type and location, with the highest % inhibition occurring in leaf extracts from plants (Kahrur Pakka: 59.40%; Yazman: 49.40%), with stem and root extracts exhibiting lower % inhibition (stem extracts: Kahrur Pakka: 54.40%; Yazman: 45.41%; root extracts: Kahrur Pakka: 51.41%; Yazman: 41.40%). The *in vitro* anti-inflammatory potential (% inhibition) followed a similar trend to the cytotoxicity data, with leaf extracts showing the highest % inhibition (Kahrur Pakka: 61.40%; Yazman: 53.40%), followed by stem (Kahrur Pakka: 57.40%; Yazman: 49.41%) and root extracts (Kahrur Pakka: 53.41%; Yazman: 45.41%). Similarly, fever reduction (as % of original fever) as an indicator of antipyretic efficacy showed the same trend as both

previous indications, with leaf extracts exhibiting the most significant fever reductions (Kahrur Pakka: 34.83%; Yazman: 28.83%); the respective reductions for stem and root extracts were as stated before (Kahrur Pakka: 29.19% and 25.20%; Yazman: 25.23% and 21.18%, respectively). When measuring the increase in temperature expressed in $^{\circ}\text{C}$, the leaves had higher pyrexia-inducing activity than the root and stem tissues. For example, Kahrur Pakka had 2.23 $^{\circ}\text{C}$ for the leaves versus 1.96 $^{\circ}\text{C}$ for the stem and 1.63 $^{\circ}\text{C}$ for the root and Yazman had 1.83 $^{\circ}\text{C}$ for the leaves versus 1.53 $^{\circ}\text{C}$ for the stem and 1.23 $^{\circ}\text{C}$ for the root. At all other pharmacological parameters evaluated, the Kahrur Pakka extracts demonstrated significantly higher bioactivity than those from Yazman and leaf extracts showed higher bioactivity than both stem and root extracts ($p \leq 0.05$, Tukey's HSD). Hence, both the environmental conditions and plant organ specificity contribute significantly to the pharmacological activity of *P. oleracea*.

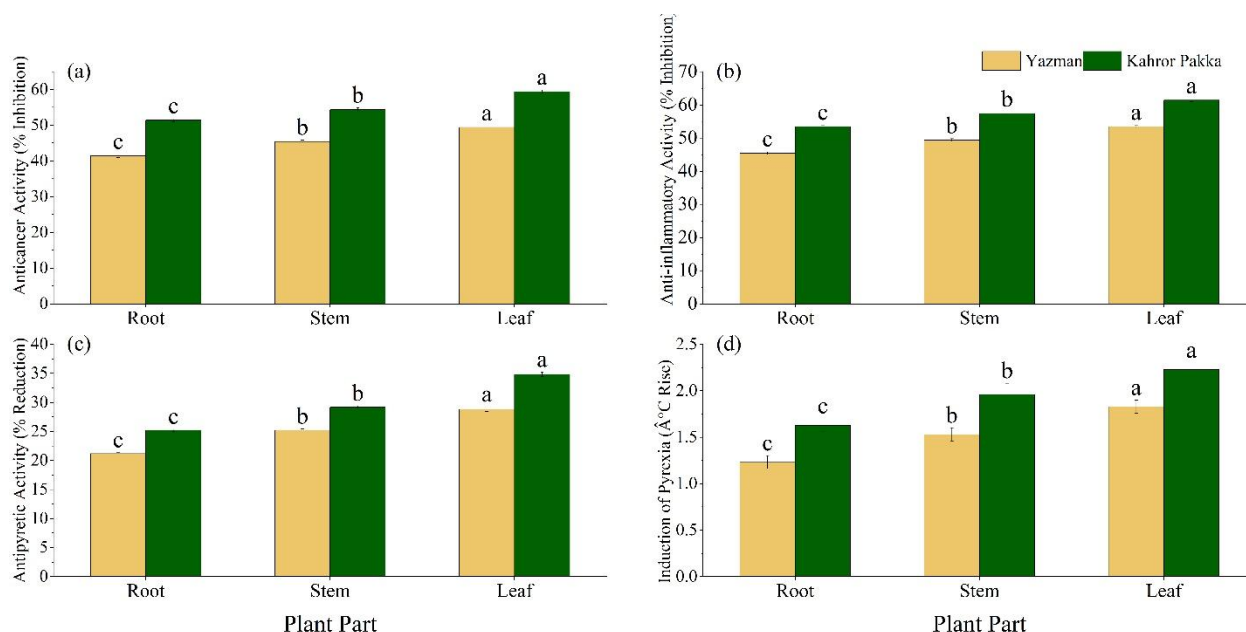


Figure-5. Pharmacological responses of *Portulaca oleracea* L. methanolic extracts from the roots, stems and leaves collected at Yazman and Kahrur Pakka, expressed as % inhibition of 3T3 fibroblast viability (cytotoxicity), % inhibition of anti-inflammatory activity, % reduction of antihyperthermic activity and an increase in temperature ($^{\circ}\text{C}$) during pyrexia induction.

Values are means $\pm$ standard deviation of three independent biological replicates ($n = 3$) and were compared by two-way factorial ANOVA (location $\times$ plant part; $p \leq 0.05$) followed by Tukey's HSD post-hoc test. Lowercase letters compare plant parts within a single sampling site; within each site, bars not sharing a letter differ at $p \leq 0.05$ by Tukey's HSD. The letters do not compare the two sites with each other, and between-site differences were tested separately by the location term of the two-way factorial ANOVA.

Phytochemical content of secondary metabolites

The methanolic extracts from *P. oleracea* L. were quantitatively analyzed for their secondary metabolites. A summary of the results is presented in Figure 6. Significant differences were observed in the total phenolic content (TPC) (tissue and location dependent). The leaves had the highest TPC (35.31 mg GAE g⁻¹ for Kahrora Pakka and 28.64 mg GAE g⁻¹ for Yazman), followed by the stems (30.11 mg GAE g⁻¹ for Kahrora Pakka and 24.31 mg GAE g⁻¹ for Yazman) and then roots (25.11 mg GAE g⁻¹ for Kahrora Pakka and 19.57 mg GAE g⁻¹ for Yazman). The same result was observed for total tannin content (TTC) in the leaves (16.57 mg TAE g⁻¹ for Kahrora Pakka and 12.57 mg TAE g⁻¹ for Yazman), stem (13.11 mg TAE g⁻¹ for Kahrora Pakka and 9.57 mg TAE g⁻¹ for Yazman) and roots (9.11 mg TAE g⁻¹ for Kahrora Pakka and 6.57 mg TAE g⁻¹ for Yazman). The same trend was observed in the total flavonoid content (TFC). The leaves had the

highest TFC (23.11 mg QE g⁻¹ for Kahrora Pakka, 18.58 mg QE g⁻¹ for Yazman), followed by the stems (19.57 mg QE g⁻¹ for Kahrora Pakka, 15.10 mg QE g⁻¹ for Yazman) and then the roots (15.11 mg QE g⁻¹ for Kahrora Pakka, 11.57 mg QE g⁻¹ for Yazman). The highest total saponin content (TSC) (9.11 mg g⁻¹ in the leaves of Kahrora Pakka (KP) and 6.57 mg g⁻¹ in the leaves of Yazman (Y)) was recorded in the leaf extracts at both sites. Saponin concentrations were lower in the stems (6.57 mg g⁻¹ in KP and 4.57 mg g⁻¹ in Y) and in the roots (4.57 mg g⁻¹ in KP and 3.11 mg g⁻¹ in Y). For the evaluated secondary metabolites, KP had significantly higher phytochemical concentrations than Y ($p \leq 0.05$, Tukey's HSD) and the leaf tissues consistently exhibited higher concentrations of phytochemicals than the stem and root tissues; these data demonstrate that there are significant effects on the biosynthesis and accumulation of bioactive secondary metabolites in *P. oleracea* from both environmental factors and from differences between plant organs.

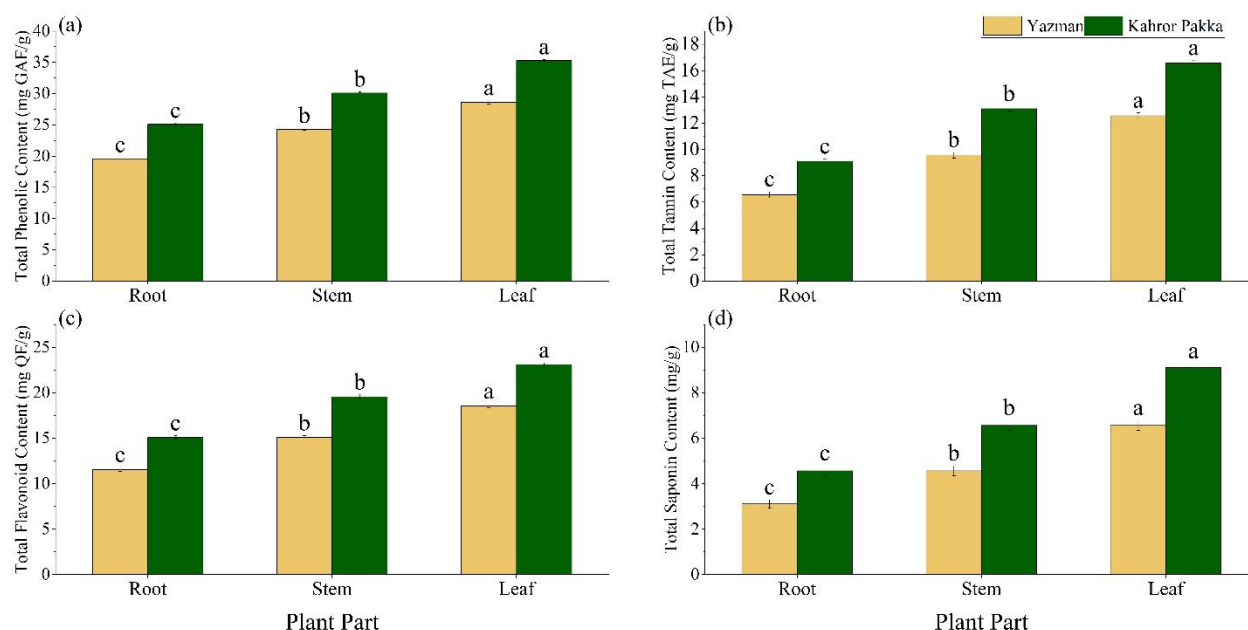


Figure-6. A comparative assessment of secondary metabolite concentrations in *Portulaca oleracea* L. methanolic extracts was made for four classes of phytochemicals. The three plant parts included root, stem and leaf and were obtained from plants that were growing in two different locations (Yazman and Kahrora Pakka).

Data are reported as mean $\pm$ SD ($n = 3$). The statistical significance of any difference in plant part or location was analyzed using two-way factorial ANOVA (location $\times$ plant part) with Tukey's HSD post-hoc test at $\alpha = 0.05$. Lowercase letters compare plant parts within a single sampling site; within each site, bars not sharing a letter differ at $p \leq 0.05$ by Tukey's HSD. The letters do not compare the two sites with each other, and between-site differences were tested separately by the location term of the two-way factorial ANOVA.

Pearson correlation analysis of phytochemical and biological attributes

Pearson correlation analysis was performed on the 18 plant means (three independent biological replicates $\times$ two locations $\times$ three plant parts), so that each observation represents an independent biological sample rather than a repeated analytical reading (Figure 7). Total phenolic, tannin, flavonoid and saponin contents were positively correlated with every measured biological activity, with coefficients ranging from $r = 0.90$ to $r = 1.00$ ($p \leq 0.001$, $n = 18$); total phenolic content was most closely associated with antipyretic activity ($r = 1.00$), antioxidant activity ($r = 0.98$) and anti-inflammatory activity ($r = 0.97$). Dry matter was negatively correlated with metabolite concentrations and with biological activity (total phenolic content: $r = -0.65$; antioxidant activity: $r =$

-0.79 ; cytotoxicity: $r = -0.85$). Moisture content was positively correlated with crude protein ($r = 0.85$), ash ($r = 0.91$), total phenolic content ($r = 0.66$) and antioxidant activity ($r = 0.81$), whereas crude fiber and crude fat were negatively correlated with moisture ($r = -0.69$ and $r = -0.75$, respectively). Crude protein was positively correlated with total phenolic content ($r = 0.96$) and antioxidant activity ($r = 1.00$) and ash showed a weaker positive association with total phenolic content ($r = 0.56$). Positive correlations among all antimicrobial assays indicate that these assays respond to a common pool of extract constituents. These coefficients describe covariation only; because no individual metabolite was isolated, depleted or added back, they cannot establish that the quantified metabolites caused the observed biological activities.

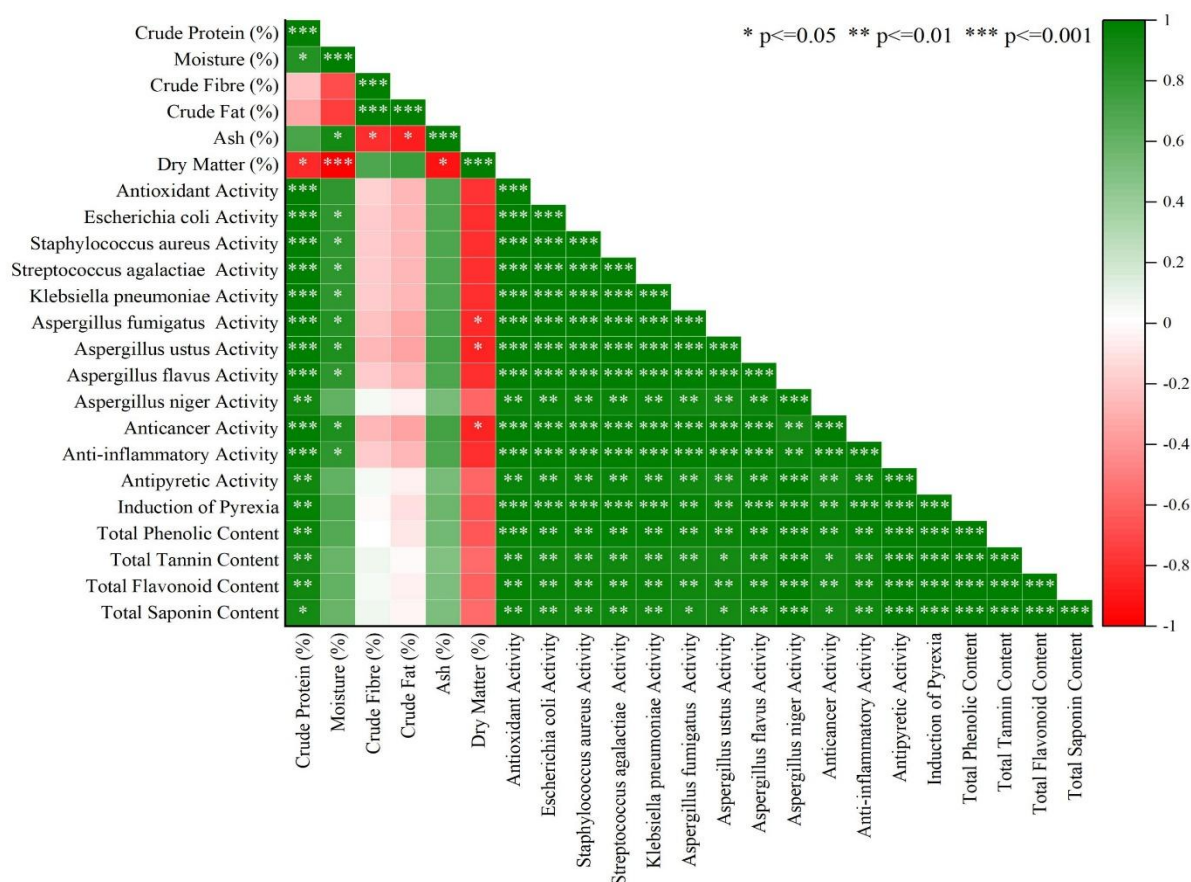


Figure-7. Pearson correlation matrix depicting the relationships between proximate compositions, secondary metabolite compositions and biological activities of *P. oleracea* L. specimens collected from Yazman and Kahrur Pakka.

Scale depicting the correlation coefficients (r) from -1 (strongest negative correlation; red) to +1 (strongest positive correlation; green). Significance levels are indicated by asterisks: * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

Discussion

The purpose of this study was to determine how different environmental conditions can affect the proximate composition, phytochemical profile and biological activities of the medicinal plant *Portulaca oleracea* L. (Purslane), which was obtained from arid and semi-arid areas of southern Punjab, Pakistan.

The current study suggests substantial differences between the two sampling locations in terms of proximate composition parameters. The Kahrur Pakka purslane samples contained significantly higher crude protein than the Yazman samples (approximately 16-19% higher across tissues; $p \leq 0.05$), which corresponds with previous reports of increased protein accumulation under environmental stress (Nemzer et al., 2020) in purslane plants. The higher protein concentrations in the Kahrur Pakka samples are attributable to greater nitrogen availability in the alluvial sandy loam soil and to moderate water deficit stimulating amino acid biosynthesis (Hussain et al., 2020; Butt et al., 2021). Conversely, while Yazman samples had significantly higher crude fiber (~22-152% higher across tissues; $p \leq 0.05$) and crude fat, which represent an adaptive response by the plants to arid conditions, a pattern consistent with greater assimilate allocation to structural and storage fractions, although neither lignin nor individual lipid classes were quantified in this study, which are consistent with other findings that show that structural carbohydrates and lipids have been produced at increased rates when exposed to drought conditions (Tzanova et al., 2020; Usman et al., 2022). Moisture content was markedly higher in Kahrur Pakka (approximately 2.6- to 2.9-fold; $p \leq 0.05$) and is consistent with the differing precipitation regimes and soil water-holding capacities of the two regions, although rainfall cannot be separated from the other climatic and edaphic variables that also differ between sites, as described by Sarfaraz et al. (2014) and Qureshi et al. (2023) assessing climate characteristics for southern Punjab.

The phytochemical analysis showed uniformly higher concentrations in plants from Kahrur Pakka, comprising of total phenolic concentration (25.11-35.31 mg GAE g⁻¹), total flavonoid concentration (15.10-23.11 mg QE g⁻¹), total tannin concentration (9.11-16.57 mg TAE g⁻¹) and total saponin concentration (4.57-9.11 mg g⁻¹) compared to their corresponding plants from Yazman. One possible explanation, which the present data cannot test

because no transcript or enzyme-activity measurements were made, is greater flux through the phenylpropanoid pathway supplying phenolic and flavonoid biosynthesis; such stress-responsive induction has been reported by Singleton et al. (1999) and Zhishen et al. (1999). The increased concentration of bioactive compounds in Kahrur Pakka compared to Yazman is associated with moderate water stress (200-350 mm annual rainfall), which may induce protective secondary metabolism without severely restricting carbon fixation, although neither photosynthetic rate nor pathway flux was measured here. On the other hand, Yazman's very low moisture (100-250 mm rainfall) may restrict overall metabolic activity; this suggestion is supported by both Haider et al. (2025) and Jangpangi et al. (2025).

The DPPH assay revealed a strong positive correlation between the amount of phytochemicals and antioxidant ability. Kahrur Pakka demonstrated the highest antioxidant capacity (75.73%), followed by Yazman (65.53%). This finding indicates that the amount of phenolic compounds and flavonoids affects the amount of free radicals quenched (i.e., DPPH). Ijarotimi et al. (2022) and Price et al. (1978) explained that hydrogen atom transfer and electron transfer reactions are the underlying mechanisms for this antioxidant activity in these extracts. Hydroxyl groups from phenolic compounds contribute to DPPH stabilization by donating electrons. Antioxidant activity varied among plant organs. Leaf tissues had higher antioxidant activity than stem and root tissues at both locations. This is attributable to higher rates of photosynthesis and secondary metabolite biosynthesis in photosynthetically active tissues (Nemzer et al., 2020; Segneau et al., 2025). The antioxidant activity found in this study was higher than that of many commonly used medicinal plants; therefore, *P. oleracea* has potential as a natural source of antioxidants for use in pharmaceuticals and nutraceuticals.

The evaluation of antibacterial activity showed that inhibition zone diameters were organ-specific. Inhibition zones were measured against both Gram-positive (e.g., *S. aureus*, *S. agalactiae*) and Gram-negative (e.g., *E. coli*, *K. pneumoniae*) bacteria. For each organism, Kahrur Pakka leaf extracts produced significantly larger inhibition zones than Yazman extracts (approximately 16-21% greater across strains; $p \leq 0.05$) and were compared against the ciprofloxacin standard (25.0 mm), which was approximately 42% higher than the most active plant extract (Figure 4).

The greater antibacterial activity against Gram-positive organisms (11.57-13.57 mm) compared with that against Gram-negative organisms (9.57-14.57 mm) may be a result of differences in the structure of the two cell walls; for example, the peptidoglycan layer in Gram-positive organisms is thick and therefore more susceptible than an outer lipopolysaccharide membrane of a Gram-negative organism to the penetration of phenolic compounds (Veiga et al., 2019; Khursheed and Jain, 2021). The mechanism of action is believed to be multifaceted, involving the ability of phenolic compounds/flavonoids to disrupt membranes, inhibit enzymes, or interfere with the synthesis of nucleic acids, which ultimately leads to the degradation of the integrity and metabolic function of the bacteria. The superior activity of the Kahrro Pakka extracts was associated with their higher phytochemical concentrations, which supports the hypothesis that phenolic acids/flavonoids work in a synergistic manner to produce bactericidal effects (Hiai et al., 1976; Georgiev et al., 2017).

Pharmacological assays showed measurable effects of *P. oleracea* extracts on cell viability (51.41-59.40% inhibition of 3T3 fibroblast growth), inflammation (53.41-61.40% inhibition of ROS formation) and fever (25.20-34.83% inhibition). Cytotoxicity was determined by the MTT method on 3T3 mouse fibroblasts, which are non-malignant cells; the assay therefore measures general cytotoxicity rather than selective anticancer activity and no cancer cell line or selectivity index was tested. Effects reported elsewhere include apoptosis induction, cell cycle arrest and angiogenesis inhibition attributed to phenolic compounds and flavonoids, as described by Segneanu et al. (2025) and Zayed et al. (2023). Anti-inflammatory activity, assessed using ROS generation inhibition in zymosan-stimulated whole blood, significantly correlated with the total amount of phenolic compounds and has been attributed in the literature to effects on inflammatory signaling involving nuclear factor kappa-B and cyclooxygenase-2, neither of which was measured here (Georgiev et al., 2017; Moslemi et al., 2021). The antipyretic effect of *P. oleracea*, detected during *E. coli*-induced pyrexia of rabbits, manifested as a dose-related inhibition of fever, a response for which hypothalamic prostaglandin E₂ suppression and cytokine modulation have been proposed, although neither was measured in this study, in accordance with Sultana et al. (2015) and Zayed et al. (2023).

Statistical evidence of such relationships was obtained using Pearson's correlation matrix analysis, where highly statistically significant positive correlations ($p \leq 0.001$) between the concentration of total phenolics, flavonoids, tannins and saponins and the biological activities of the studied plant extract were observed, indicating that the accumulation of secondary metabolites was the key factor responsible for pharmacological activity. Correlations between various antibacterial tests pointed out that there was a unified mechanism of action mediated by phenolic substances, while negative correlations between dry matter percentage and biological activities demonstrated that metabolic activity and accumulation of active substances were inversely proportional to the formation of structural biomass of the plant, as described by Hussain et al. (2020) and Jangpangi et al. (2025). A correlation between crude protein/ash content and biological activities implies the involvement of mineral nutrition and the availability of nitrogen in secondary metabolite biosynthesis via the regulation of enzyme activities and precursors, as suggested by Haider et al. (2025) and Harisha (2023). An extensive analysis of agro-climatically diverse regions revealed that a relatively moderate level of environmental stress along with sufficient nutrition is ideal for the synthesis of bioactive secondary metabolites, consistent with the study's objective of relating site-specific agro-climatic conditions to phytochemical and pharmacological potential. The higher levels of phytochemicals and biological activities of samples from Kahrro Pakka indicate that a combination of moderate water deficit (200-350 mm rainfall per year), alluvial sandy loam soil type and climate are associated with higher metabolite concentrations, a pattern that has been attributed elsewhere to phenylpropanoid and terpenoid pathway induction but that was not tested here. These results have practical relevance for medicinal plant cultivation and standardization, highlighting the importance of selecting sites with suitable agro-climatic conditions to improve the medicinal properties of herbals. High correlation between phytochemicals and biological activities of *P. oleracea* supports the use of total phenolic and flavonoid contents as markers for its standardization.

Conclusion

Wild *P. oleracea* from the hyper-arid Yazman site contained higher concentrations of structural and

storage fractions, whereas material from the semi-arid Kahrur Pakka site contained higher concentrations of phenolics, tannins, flavonoids and saponins. At both sites, leaf extracts showed the highest antioxidant, antibacterial and anti-inflammatory activity and the highest cytotoxicity toward 3T3 fibroblasts and these responses covaried closely with the measured metabolite concentrations. Because the comparison involves only two locations and location cannot be separated from the climatic and edaphic variables that differ alongside it, these results describe the responses observed at these two sites and do not identify optimal growing conditions for the species. The cytotoxicity data were obtained on non-malignant fibroblasts and therefore indicate general cytotoxicity rather than anticancer selectivity. Within these limits, the study shows that plant organ and provenance both materially affect the phytochemical and biological profile of wild purslane and it identifies leaf material from the semi-arid site as the most promising starting point for the compound isolation, selectivity testing and minimum inhibitory concentration work that would be required before any pharmaceutical application could be considered.

Novelty and implications

The novelty of this work lies in the simultaneous profiling of proximate composition, four secondary-metabolite classes and multiple biological activities across three organs of wild *P. oleracea* from two contrasting agro-climatic settings, combined with *in vivo* antipyretic testing that most previous purslane studies have not included. Reporting organ-resolved rather than whole-plant values identifies the leaf as the tissue in which both metabolite concentration and several measured biological responses are highest at both sites, which carries direct practical consequence for harvest timing, for the choice of plant part in extract preparation and for the selection of marker compounds in standardization protocols.

Limitations

Several limitations constrain the interpretation of these results. First, only two locations were compared and location is completely confounded with the accompanying climatic and edaphic differences, so the study cannot attribute the observed variation to rainfall, temperature, soil texture or nutrient status individually; a multi-site design spanning a continuous aridity gradient, with measured soil physicochemical data at every site, would be required for that inference.

Second, sampling was restricted to a single season, so interannual variation was not captured. Third, the cytotoxicity assay used non-malignant 3T3 fibroblasts, so the data describes general cytotoxicity and non-anticancer selectivity. Fourth, no molecular, enzymatic or compound-isolation work was undertaken, so the mechanisms discussed above remain hypotheses drawn from the literature rather than findings of this study. Fifth, the correlation analysis establishes covariation among measured variables and cannot establish causation. Sixth, the quantitative antifungal inhibition-zone dataset requires verification because the values reported in the previous version contained apparent transcription/carry-over errors; therefore, antifungal numerical results are not interpreted in the present manuscript.

Future research directions

Future work should extend sampling to multiple sites along a continuous aridity gradient over two or more seasons, with paired soil and microclimate measurements, so that individual environmental drivers can be separated from one another. Bioassay-guided fractionation with chromatographic identification of the active constituents would replace the total-content proxies used here and screening against a panel of human cancer cell lines alongside a normal cell line would permit a selectivity index to be calculated. Targeted transcript or enzyme-activity measurement of phenylpropanoid and terpenoid pathway steps would test the biosynthetic explanations proposed above and determination of minimum inhibitory concentrations would place the antimicrobial results on a quantitative footing comparable with clinical reference agents. The antifungal component should also be reconstructed from verified laboratory records or repeated using documented agar-diffusion assays, with raw measurements and representative plate photographs retained for quality control.

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Conflict of Interest: None.

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Ethical Approval Statement

This study did not involve human subjects. The *in vivo* antipyretic experiment used rabbits and was approved

by the Institutional Animal Ethics Committee under approval number CUVAS/IAEC/2024/015. All procedures complied with institutional and national guidelines for the care and use of laboratory animals and are reported in accordance with the ARRIVE guidelines.

Data Availability Statement

All the raw data in this research can be obtained from the corresponding authors upon reasonable request.

Contribution of Authors

Muhammad RB, Hussain T & Saeed A: Conceived the study idea and designed research methodology

Muhammad RB: Completed the experiments and wrote the original draft.

Hussain T & Rafay M: Analysed the data and performed visualizations and statistical data analysis.

Mehmood H & Huseynova A: Reviewed and edited the manuscript.

Huseynova A: Reviewed the manuscript and provided funds.

All authors made valuable revisions, edited, read and approved the final draft of the manuscript.

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