



Research Article

Selection of Salt-Stress Tolerant Chickpea Cultivars and Their Characterization Using High Performance Liquid Chromatography (Hplc-Uv)

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Abstract | Chickpea (*Cicer arietinum* L.) eight released cultivars: KC-98, LAWANGHAR-2000, SHEENGHAR-2000, FAKHR-E-THAL, CHATTAN, KARAK-1 (KK-1), KARAK-2 (KK-2), and KARAK-3 (KK-3) received in 2021-22 from Ahmad Wala Agricultural Research Station, Karak, Khyber Pakhtunkhwa, Pakistan. The Pot experiments were conducted at the greenhouse, University of Swat, to check the growth of cultivars under salt (NaCl) stress conditions. The cultivars were subjected to a biochemical detection (BCD) using HPLC-UV, a technique to find out the genetic change in antioxidants of chickpea un-evaluated cultivars. The identification and quantification of phenolic antioxidants were carried out by a specific peak at a particular retention time (Rt) to obtain a chromatogram of an External Standard Mixture (ESM) at $\lambda = 320$ nm. At various growth stages, later to 28 days of germination, the concentrations of salt, including 0, 50, 100, and 200 Mm, were applied to cultivars. RL (Root Length), RW (Root Weight), NR (Number of Roots), SW (Shoot Weight), and SR (Secondary Root) resulted in poor growth at the increasing level of salt (NaCl) concentration (50mM, 100mM and 200mM) when compared with normal growth conditions. The cultivars KK-2, KK-3, and KC-98 were found to be tolerant to salt stress with promising characteristics. The analysis of phenolic antioxidants, including Malic acid, Galic acid, Phloroglucinol, and Quercetin, using HPLC-UV revealed variation among the selected lines, KK-1, KK-2, KK-3, Chattan, Fakhra-e-Thal, Sheenghar-2000, and KC-98, which exhibited high antioxidant potential, offering prospects for further genetic research and breeding efforts. The identified salt-tolerant cultivars, including KK-2, KK-3, and KC-98, should be prioritized for further investigation and breeding programs to enhance salt tolerance in the chickpea crop.

Received | September 02, 2025; **Accepted** | October 21, 2025; **Published** | January 26, 2026

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Citation | Qayum, U., Z. Ahmad, I. Khan, K. Bibi, A. Hazrat, G. Rahim, S. Nasim and K.U. Rahman. 2026. Selection of salt-stress tolerant chickpea cultivars and their characterization using high performance liquid chromatography (hplc-uv). *Sarhad Journal of Agriculture*, 42(1): 171-178.

DOI | <https://dx.doi.org/10.17582/journal.sja/2026/42.1.171.178>

Keywords | Chickpea, Salt tolerance, BCD, HPLC-UV, Antioxidant potential



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Introduction

Chickpeas are the most cost-effective source of vitamins, minerals, phenolic bioactive compounds, and dietary protein, which may reduce the chances of prolonged diseases, oxidative stresses, and cholesterol-related disorders (Siddique *et al.*, 2000; Hasler, 2000; Han *et al.*, 2007; and Klongklaew *et al.*, 2022). Analyzing the genetic resources of dietary legumes is crucial for breeding new varieties with enhanced traits. Chickpea is the most essential edible legume known globally after the common beans (FAOSTAT, 2014). In 2016, the UN declared it the Year of Pulses, highlighting their role as nutrient-rich seeds for a sustainable future (Cernay *et al.*, 2016). In Pakistan, legumes occupy 1.9% of the arable land, primarily in regions like Punjab's Thal and Potohar divisions, Khyber Pakhtunkhwa, and the Malakand regions. Recently, interest has been resurgent in their potential impact on human health, with chickpeas being considered a "functional food" rich in various beneficial components (Milner, 2000).

Chickpea productivity can be hindered by both biotic stressors like diseases, insects, pests, and plant-parasitic nematodes, as well as abiotic stressors, including salinity, drought, flooding and high temperatures (Doupis *et al.*, 2011). Soil salinity, in particular, is a significant challenge, affecting plant growth and nutrient absorption. Salinity can lead to nutrient imbalances and hinder plant growth (Grattan and Grieses, 2004). Chickpeas are particularly sensitive to salinity stress (Lauter and Munns, 1986). To address these environmental stresses, research is crucial for enhancing chickpea resilience (Singh *et al.*, 2004). Genetic diversity is important in breeding for salt tolerance, and various methods, including genetic markers and DNA-based indicators, are used to measure diversity (Ahmad *et al.*, 2012). Additionally, high-performance liquid chromatography (HPLC) can be employed to assess the phenolic antioxidants in chickpea, providing insights into their bioactive molecules (Gharibi *et al.*, 2019). The current research was thus based on identifying salt-tolerant and genetically variable in antioxidant cultivars from KARAK through morphological characterization using significant yield-related traits and HPLC-UV, a biochemical detection technique to recommend elite genotypes for chickpea sustainable production.

Materials and Methods

The chickpea genotypes, KC-98, LAWANGHAR-2000, SHEENGHAR-2000, CHATTAN, and FAKHR-E-THAL were sown in 2021-2022 at the greenhouse University of Swat in 96 Pots with replicates to apply them salt (NaCl) at 200mM, 100mM, and 50mM concentration and compare the data with the positive control. The Salt strain was applied 4 weeks later of germination. Four seeds were taken from each genotype for sowing in pots, added with the same amount of humus and soil. When the seeds germinated, 3 samples of each line were treated with 200mM solution (11.72g/1000 ml), 100mM (5.86g/1000ml) and 50mM (2.93g/1000ml), respectively. The 3 samples of the control group were treated with 0mM salt concentration. After 28 days to germination, the salt stress was applied and recorded different morphological traits. The valuable traits like, Plant height (PH), Secondary branches (SB), Plant biomass (PBM), Leaflets per Leaf (L/L), Internode Length (IL), Number, length and weight of roots (NR, RL, WR), Secondary Roots (SR) and Secondary Shoot weight (SW) were observed of each sample to follow the procedure represented by chickpea descriptor (ICRISAT, ICARDA and IBPGR, 1993) with minor editing.

Extracts preparation for HPLC-UV

The method described by Zeb (2015) was used for HPLC-UV quantification and description. For extract preparation, one gram of powder of each cultivar was added in water & methanol, keeping the ratio 20mL; 1:1. Then, heat up the mixture for an hour under 70°C temperature in a water bath, following the next step of centrifugation for 10 minutes (4000 rpm). The PTFE filter was used to filter the extract and 2mL sample was poured into HPLC vials. The system of HPLC with UV detector were used to identify the phytochemicals. The Agilent Zorbax Eclipse XDBC18 column separated the phenolic compounds. The gradient system consisted of deionized water, solvent-A (methanol: acetic acid:100:20:180, v/v volume), and solvent-B (methanol: Acetic acid: deionized water, 900:20:80, v/v). The temperature of the column was set at 25°C with 10µ injection 280nm UV-detection for the analysis of phenolic compounds. The isolated phytochemicals were identified by the difference in retention time (Rt) through a standard reference and peak positions by the following one-point calibration formula:

$$C_x = \frac{A_x \times C_s \mu\text{g mL} \times V(\text{mL})}{A_s \times \text{Sample wt (gm)}} (1)$$

Where, C_x = Sample concentration; A_s = Standard peak area; A_x = Sample peak area; and C_s = Standard concentration (0.09 $\mu\text{g/g}$).

The morphological data for each cultivar were taped in triplicate to obtain the mean value of each sample for statistical analysis after repeating the experiment. The results had been analyzed through version 22, SPSS, MS-Excel, 2016, and Graph Pad Prism version 5 for one-way and Two-way Analysis of Variance (ANOVA) to check the significance.

Results and Discussion

Impact of salt stress on chickpea cultivars

Chickpea *Cicer arietinum* L. cultivars were subjected to different levels (0, 50, 100 and 200mM) of salt stress after 28 days of germination. Various morphological traits, e.g., Plant height (PH), Secondary branches (SB), Plant biomass (PBM), Leaflets per Leaf (L/L), Internode Length (IL), Number of roots (NR), Root length (RL), Root weight (RW), Secondary Roots (SR) and Secondary Shoot weight (SW) were carefully observed and recorded.

Plant height (PH) at the control salt level was calculated to be 15.01cm, which significantly decreased with higher salt concentrations, ranging

from 10.22cm to 1.62cm. The leaflets' percentage per leaf (LF) was highest under normal conditions (7.92%), and it gradually declined as salt stress increased: 4.59% at 50mM, 2.03% at 100mM, and 0.67% at 200mM. The length of internodes (IL) also decreased as salt stress intensified, with measurements decreasing from 4.40cm to 3.60cm at 50mM, 1.14cm at 100mM, and 0.35cm at 200mM. The appearance of secondary branches (SB) dropped significantly with the application of higher salt concentrations, particularly at 100mM and 200mM. The Plant biomass (PBM) experienced a substantial decrease as salt stress increased, ranging from 1.47g to 0.19g across the various salt levels. Interestingly, plant biomass at 50mM salt concentration was similar to that of the control. The number of roots (NR) increased expressively by 100mM salt treatment, reaching a maximum value of 1.36%, along with an increase in root weight to 1.78g. The control group showed 1.16% and 0.60g, respectively. The maximum root length (22.96cm) was observed under control conditions, followed by 14.79cm at 50mM and a minimum length of 3.13cm at 200mM salt concentration. The percentage of secondary roots (SR) decreased significantly as salt stress intensified, ranging from 15.06% at 0mM to 1.15% at 200mM, with an observation of 8.54% at 50mM. Shoot weight (SW) increased at 50mM and 100mM NaCl levels compared to the control. However, it decreased significantly at the highest level of salt stress, 200mM NaCl (Table 1, Table 2).

Table 1: Effect of salt (NaCl) stress on yield associated morphological traits

Treat-ment	PH (cm)	LF (%)	Internode L (cm)	SB (%)	PBM (g)	NR (%)	RW (g)	RL (cm)	SR (%)	SW (g)
Control	15.01±3.12	7.92±4.16	4.40±2.72	1.36±0.90	1.47±0.47	1.16±1.05	0.60±0.14	22.96±12.40	15.06±5.07	1.59±2.19
50Mm	10.22±3.86	4.59±0.92	3.60±3.13	0.91±0.58	1.36±0.55	1.12±0.95	0.65±0.28	14.79±8.91	8.54±4.18	2.40±1.48
100Mm	3.91±2.56	2.03±1.28	1.14±0.53	0.06±0.13	0.92±0.34	1.36±1.42	1.78±0.82	12.43±7.28	5.79±6.71	2.76±2.50
200Mm	1.62±1.78	0.67±0.26	0.35±0.62	0.28±0.79	0.19±0.29	0.21±0.31	0.06±0.09	3.13±0.25	1.15±1.84	0.09±0.14

Abbreviations: PH= Plant height, LF= Leaflets, SB= Secondary branches, PBM= Plant biomass, NR= Number of root, RW= Root weight, RL= Root length, SR= Secondary root, SW= shoot weight

Table 2: Mean square of studied morphological traits

Source	DF	PH	LF	IL	SB	PBM	NR	RW	RL	SR	SW
Variety	5	2547.18***	566.45***	14465.2***	9.30***	407.13***	690.86***	1.09NS	882.50***	125.48***	20.11***
Treatment	3	28595.6***	8017.90***	24022.8***	49.30***	3903.05***	352.10***	2.58NS	154964***	407.27***	6.88***
Variety+ treat-ment	15	1042.30***	865.52***	2425.04***	9.27***	394.86***	114.84***	0.94NS	163.03***	62.18***	4.03***
Error	46	46	46	46	46	46	46	46	46	46	46
Total	71	71	71	71	71	71	71	71	71	71	71

P-value; 0.01; *, 0.001; *** and 0.0001; *** respectively.

The analysis of variance (ANOVA) results revealed a highly significant relationship between the salt stress treatments and the various chickpea varieties for all the observed traits, including; PBM, PH, SB, LF, NR, RL, RW, SR, and SW. This highlights the significant impact of salt stress on these morphological characteristics in the different chickpea cultivars (Table, 2).

The maximum critical and extensively produced Rabi legume crop in Pakistan is chickpea. even though the chickpea production capability is excessive, it has not been completely found out thanks to some abiotic stresses, which includes salinity and drought (Jha *et al.*, 2014; Kashiwagi *et al.*, 2015). Chickpea is extremely sensitive to salinity at all phases of life cycle resulting in poor production of crop in chickpea flourishing areas (Klongklaew *et al.*, 2022). Since Screening of crops is imperative to develop tolerant germplasm for the best usage of salinity-affected land. We therefore have tested, five levels of salinity after four weeks of germination of seeds to explore the level of tolerance among eight novel cultivars of chickpea from Karak Research Center by observing especially their yield related morphological traits *i.e.*, Plant height (PH), Primary and Secondary branches (PB & SB), Plant Biomass (PBM), Number of Roots (NR), Root length (RL), Root and Shoot weight (RW & SW). The calculated mean value of Plant Height (PH), ranged from 10.33 to 498cm in KK-1 and KC-98, respectively, showed that salt strain had a notable effect on the height of the plant in recent study. Such readings suggested the highest salt endurance ability of KK-1 and the sensitivity of KK-2 and KC-98. In terms of plant height, the cultivars KK-3, Chattan, and Fakhr-e-thal were differentiated by moderate endurance to salt. The findings of Manchanda and Sharma (1989), are complemented by our observation that tolerance to chloride depends on genotypes, and a high salt stress badly affected the agronomic characters in chickpea.

The PBM was notably decreased at the increased level of salt uptake (100, 150, and 200mM). The Maximal PBM was listed for KK-2 and minimal for KK-1, while the rest of the genotypes revealed average plant biomass agreed with the results of Hossain *et al.*, 2015 and Khan *et al.*, 2015 regarding the salinity effect on total biomass of a plant. Contrary to this, high salt concentration has not affected the root biomass according to the report of Machado and Serralheiro (2017).

In the current work number of Primary and secondary roots drastically declined with the increasing level (200mM) of salt concentration, ranging from 1.12 to 0.21% along with root weight. The cultivar KK-3 showed the maximum, and “chattan” exhibited the minimum number of roots. The maximum root weight was recorded for KK-2 and Chattan, less in Fukhr-e-thal and KK-1. Similarly, the higher root length was noted in KC-98 and KK-2, minimal in Chattan, Fukhr-e-thal, KK-3 and KK-1. Such scoring of results is harmonized with Munns and Tester in 2008, where they resulted in mild salinity stress to raise the growth of root and a higher amount of salt application showed an adverse effect on the same trait.

Salt stress thus significantly affected the morphological traits of chickpea cultivars. These findings highlight the importance of cultivating salt-tolerant chickpea varieties and further research to minimize the severity of the issue.

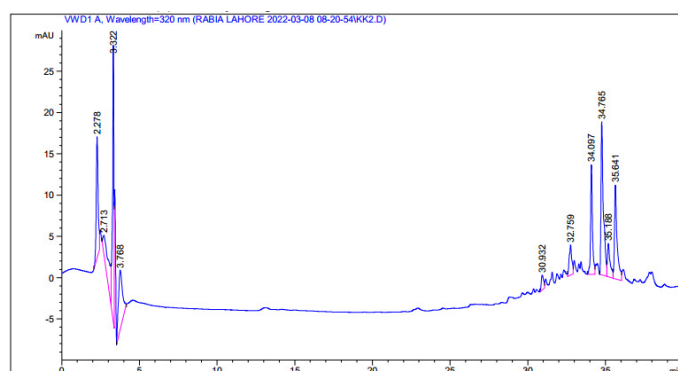


Figure 1: HPLC-UV chromatogram of phytochemicals in KK-2

Biochemical analysis of chickpea antioxidants

The reliable technique of HPLC-UV was used to identify the Phenolic compounds contained by the chickpea cultivars from their chromatogram peaks detection. In KK-2, five distinct compounds, namely Malic acid, Galic acid, Caffeic acid, Mandellic acid, and Phloroglucinol, were successfully identified through HPLC-UV analysis. These compounds were confirmed using reference standards illustrates the precise quantification and identification of each phenolic compound, along with their specific peak positions and retention times (Rt), as revealed in the chromatogram. The HPLC analysis at $\lambda = 320$ nm indicated that KK-2 had the highest concentration of Malic acid, resulting in a significant peak area of 122.87, followed by Phloroglucinol constituting

Table 3: *Phytochemical concentration profile across multiple cultivars*

HPLC-UV chromatogram of phytochemicals in KK-2				
Rt (min)	Compound	λ_{max} (nm)	Peak value	Id Reference
2.278	Malic acid	320	122.87	Standard
2.713	Galic acid	320	97.56	Standard
30.932	Caffiec acid	320	18.57	Standard
32.759	Mandelic acid	320	42.17	Standard
34.097	Phloroglucinol	320	111.44	Standard
HPLC-UV chromatogram of phytochemicals in Sheenghar				
2.31	Malic acid	320	111.79936	Standard
3.73	Galic acid	320	169.08817	Standard
32.866	Mandelic acid	320	40.94134	Standard
34.919	Phloroglucinol	320	149.29156	Standard
35.858	Hydroxy benzoic acid	320	117.49722	Standard
HPLC-UV chromatogram of phytochemicals in KC-98				
2.3	Malic acid	320	161.5818	Fischer <i>et al.</i> , 2011
3.717	Galic acid	320	176.90091	Standard
34.745	Phloroglucinol	320	194.06607	Standard
35.612	Hydroxy benzoic acid	320	129.58879	Standard
HPLC-UV chromatogram of phytochemicals in Chattan				
2.277	Malic acid	320	34.34886	Standard
3.737	Galic acid	320	175.71449	Standard
30.856	Caffiec acid	320	17.07375	Standard
34.046	Phloroglucinol	320	102.33392	Standard
35.858	Hydroxy benzoic acid	320	117.49722	Standard
HPLC-UV chromatogram of phytochemicals in KK-1				
2.264	Malic acid	320	77.67879	Ref. Standard
3.762	Quercetin	320	160.72971	Ref. Standard
32.92	Mandelic acid	320	78.87707	Ref. Standard
36.034	Hydroxyl benzoic acid	320	123.03258	Ref. Standard
HPLC-UV chromatogram of phytochemicals in KK-33				
2.294	Malic acid	320	30.90547	Ref. Standard
3.766	Quercetin	320	244.80597	Ref. Stand
32.927	Mandelic acid	320	46.64917	Ref. Stand
35.522	Phloroglucinol	320	26.05812	Ref. Stand
HPLC-UV Identification of phytochemicals in Fakhar-e-Thal				
2.375	Malic acid	320	45.72987	Ref. Stand
3.7205	Quercetin	320	115.26289	Ref. Stand
32.874	Caffeic acid	320	41.88222	Ref. Stand

111.44% of the peak area. Similarly in Fakhr-e-Thal plant, Phloroglucinol exhibited the highest concentration with a peak area of 118.02675 (Figure

1, Table 3).

The Galic acid exhibited the highest concentration

in Sheenghar, with a peak area of 169.08817. Phloroglucinol was the next most abundant compound, with a peak area of 149.29156 (Figure 2, Table 3). Nevertheless, Hydroxy benzoic acid found in this sample instead of caffeic acid recorded in KK-2.

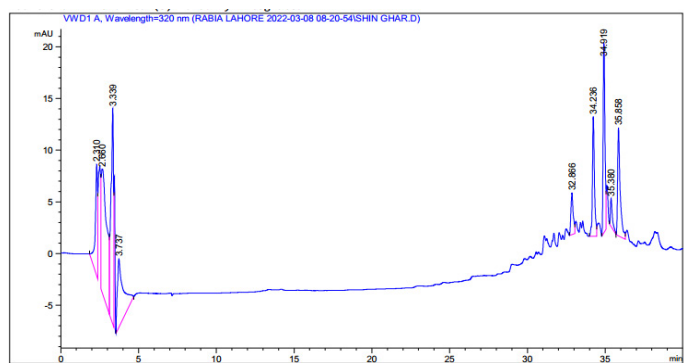


Figure 2: HPLC-UV chromatogram of phytochemicals in Sheenghar-2000

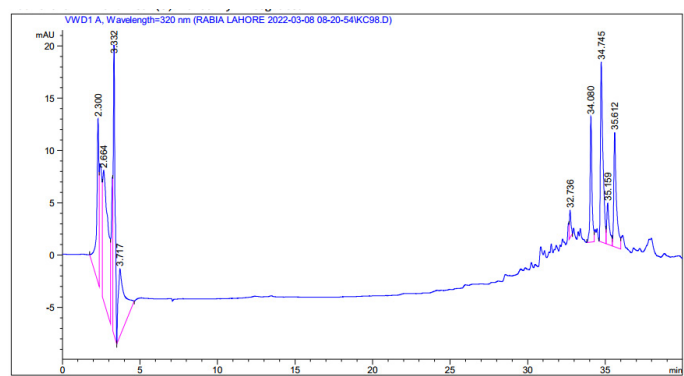


Figure 3: HPLC-UV chromatogram of phytochemicals in KC-98

The Phloroglucinol was the most concentrated compound in KC-98, with a high peak area of 194.07. The next highest compound detected by HPLC was Galic acid, with a peak area of 176.90 (Figure 3, Table 3). Where in Chattan identified Galic acid in the highest concentration (175.71449) and peak position. Caffeic acid exhibited the lowest percentage (17.07375) as shown in Figure 4 and Table 4.

Table 4: HPLC-UV of phytochemicals in chattan

2.277	Malic acid	320	34.34886	Standard
3.737	Galic acid	320	175.71449	Standard
30.856	Caffiec acid	320	17.07375	Standard
34.046	Phloroglucinol	320	102.33392	Standard
35.106	Hydroxy benzoic acid	320	48.34124	Standard

In KK-1 and KK-3 unlike other cultivars the primary phenolic compound was detected Quercetin, with a

high peak area concentration calculated, 160.72971 and 244.80597 respectively (Figure 5, Figure 6 and Table 3).

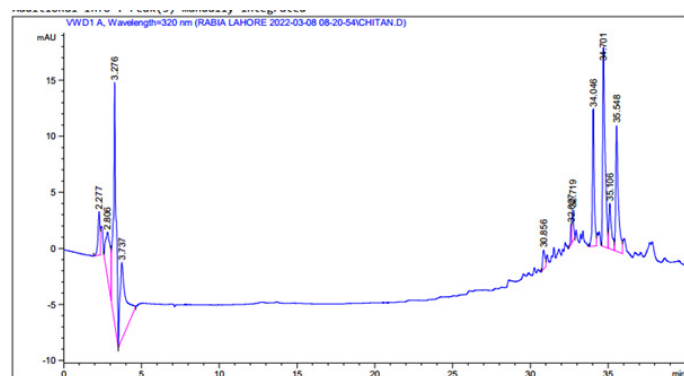


Figure 4: HPLC-UV chromatogram of phytochemicals in Chattan

The analysis of phenolic antioxidants including Malic acid, Galic acid, Phloroglucinol, and Quercetin using HPLC –UV revealed genetic variation among the cultivars, KK-1, KK-2, KK-3, Chattan, Fakhar-e-Thal, Sheenghar-2000 and KC-98, exhibited high antioxidant potential, offering prospects for further genetic research and breeding efforts.

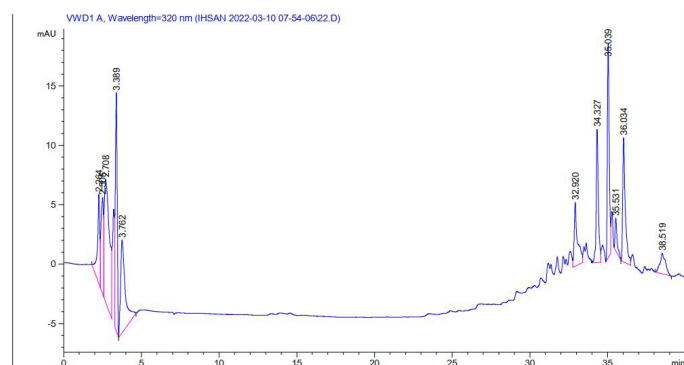


Figure 5: HPLC-UV chromatogram of phytochemicals in KK-1

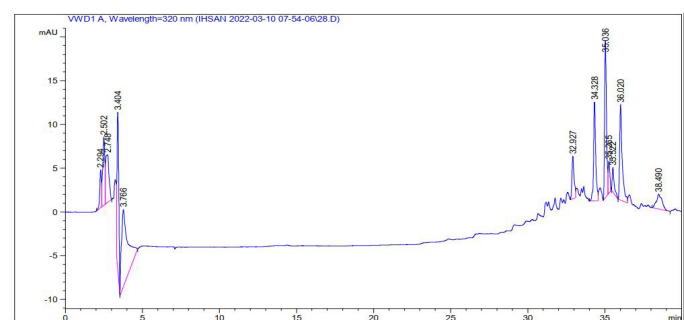


Figure 6: HPLC-UV chromatogram of phytochemicals in KK-3

Conclusions and Recommendations

The increase in yield of chickpea cultivars as an

approach to combat salt stress proves effective, with KK-1, KK-2, and KK-3 showing higher growth at 50mM salt application, even as Chattan, Sheenghar-2000, Fakhare-e-Thal, and KC-98 exhibit reduced growth at higher salt concentration.

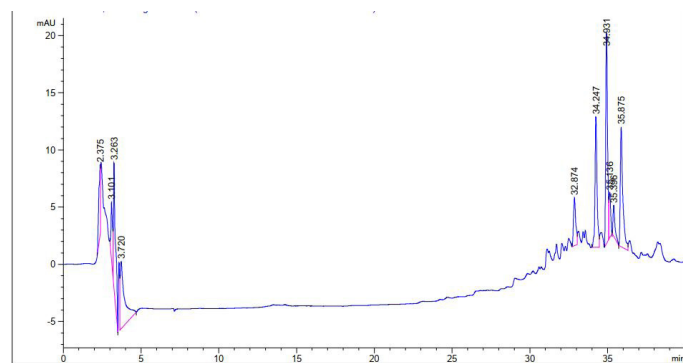


Figure 7: HPLC-UV chromatogram of phytochemicals in Fakhare-e-Thal

Conversely, root-related traits like number of roots, root length, root weight, secondary root, and shoot weight have shown a maximum growth at 100mM salt uptake for Chattan, Fakhare-e-Thal, Sheenghar-2000, and KC-98, at the same time as KK-1, KK-2, and KK-3 scored a minimum value in the development at 100mM NaCl concentration. HPLC-UV analysis unveiled variations in phenolic and antioxidant compounds in the various lines of *Cicer arietinum*, underscoring the capability of salt-tolerant cultivars in safeguarding critical phytochemicals from salt-triggered oxidative harm. Each cultivar gives a precise compound profile, in addition to endorsing its promise for future applications.

The contemporary research recommended the use of salt-tolerant genotypes, KK-1, KK-2, and KK-3 for improving crop production. Further, the excessive antioxidant potential makes them more promising lines along with Chattan, Fakhare-e-Thal, Sheenghar-2000, and KC-98 for genetic consistency.

Acknowledgements

The authors are thankful to the Agricultural Research Center Ahmad Wala Karak and Center for Plant Sciences and Biodiversity, University of Swat, for facilitating this Research work.

Novelty Statement

The selected eight cultivars have not been evaluated

for salt stress tolerance and Biochemical analysis using the HPLC-UV technique before the current study in Pakistan.

Author's Contribution

Uzma Qayum: Field work and result collection
Zakia Ahmad: Helped in the Title and objective configuration and the overall Research study
Israr Ahmad: Statistical analysis of the Results using software.
Ali Hazrat: Compilation
Gul Rahim: Help with lab work
Sahar Nasim: Paper writing and grammar setting
Kashif ur Rahman: Overall paper setting and references.

Generative AI or AI assisted technology statement

The authors declare that no generative AI was used to conceive, analyze, or interpret the research data.

Conflict of interest

The authors have no conflict of interest.

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