Caspian Journal of Environmental Sciences
Online ISSN: 1735-3866
Print ISSN: 1735-3033

Morphological and phytochemical variation in *Salix* species from Ardabil Province, Northwestern Iran

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Abstract

The genus *Salix* is increasingly recognized for its medicinal compounds, vegetative regeneration, and biomass productivity. Yet, no comprehensive studies in Iran have quantitatively assessed its ecotypic diversity or the combined effects of environmental, genetic, and gender-related factors on key traits. This study evaluated 19 Salix specimens representing 11 species collected from different locations across Ardabil Province using comparative and multivariate approaches. We measured ten quantitative morphological traits, along with total phenolic content, total flavonoid content, and DPPH antioxidant activity in bark and leaves. Principal Component Analysis showed that the first three components explained 71.14% of the observed morphological variation, with PC_1 primarily associated with trunk diameter, bud length, and height, while PC_2 was mainly associated with crown area and leaf weight. The PCA revealed variation in the morphological characteristics of the sampled specimens. S. alba showed relatively high PC_1 scores, whereas *S. acmophylla* female and *S. elbursensis* female from Khalkhal City showed relatively low PC_1 scores. Crown area exceeded tree height in most sampled specimens. Total phenolic content was generally higher in bark than in leaves, whereas flavonoid content was generally higher in leaves than in bark, indicating differences between plant organs. Considerable variation was also observed in DPPH antioxidant activity among the sampled specimens. Since one representative tree was sampled for each species or ecotype, the observed differences should be interpreted as descriptive patterns rather than statistically established effects of species, gender, ecotype, age, or environmental factors. These findings provide baseline information on morphological and phytochemical variation among the sampled Salix specimens in Ardabil Province and support the need for future studies using replicated sampling to investigate species-, gender-, and environment-related effects.

Keywords: DPPH, Morphological characteristics, *Salix* genus, Total flavonoid, Total phenol.

INTRODUCTION

The willow genus, *Salix*, belonging to the family Salicaceae, comprises over 300 identified species worldwide (Talebi *et al.* 2020). Willows play a vital ecological role in natural ecosystems, contributing to vegetative regeneration and biomass production (Moreau *et al.* 2015). In addition to their ecological significance, *Salix* species are recognized for their diverse phytochemical profiles and therapeutic potential (Qin & Sun 2005; Jürgenliemk *et al.* 2007).

The quantity and quality of phytochemicals in willow species are influenced by both genetic and environmental factors (Talebi *et al.* 2020). Among environmental gradients, altitudinal variation offers a natural laboratory for studying plant adaptation, as it encompasses sharp changes in temperature and habitat heterogeneity over short spatial scales (Bargali *et al.* 2018; Pandey *et al.* 2023; Manral *et al.* 2023). Previous research has demonstrated

Caspian J. Environ. Sci.
DOI:

Received:
Article type: Research

that morphological and physiological traits of high-altitude species differ markedly from those of low-altitude populations (Boscutti *et al.* 2018; Fartyal *et al.* 2025).

Climate change further affects phenological, genetic, and biochemical traits in both plant and animal species. Plants respond to environmental shifts through morpho-physiological, biochemical, phenological, and behavioral adaptations (Nock *et al.* 2016; Khatri *et al.* 2023). Phenotypic plasticity enables plants to express variable traits that enhance survival and performance under diverse climatic conditions (Karban 2015; Khatri *et al.* 2024; Negi *et al.* 2025). For instance, Orlandi *et al.* (2020) conducted a phenological study on *Salix acutifolia* and *S. smithiana* across altitudinal gradients in Italy, revealing that *S. acutifolia* exhibited greater sensitivity to lower, warmer elevations during developmental stages, although altitude had minimal impact on flowering time.

Globally, the taxonomic, morphological, and chemical diversity of *Salix* species has been well documented (Froiland 1962; Zhin & Dong 1981). However, in Iran, such studies remain limited. Hemmati *et al.* (2007) conducted one of the few systematic surveys, identifying 11 *Salix* species across various provinces. Despite this, comprehensive investigations linking ecological variation to morphological and phytochemical traits—particularly in Northeastern Iran—are lacking.

Phytochemically, *Salix* species are rich in flavonoids, including flavones, flavanols, flavanones, dihydroflavanols, isoflavones, chalcones, dihydrochalcones, flavan-3-ols, and anthocyanins (Jürgenliemk *et al.* 2007). These compounds are most concentrated in leaves and bark. For example, *S. acutifolia* contains apigenin as a major constituent (Shelyuto & Bondarenko 1985), while *S. cheilophila* is characterized by angeloxiflavone and isoflavones (Shen *et al.* 2008). In *S. babylonica*, chrysoeriol and 7-O-glucuronide have been identified as dominant compounds, whereas kaempferol-7-O-glucoside has also been reported, likely reflecting climatic and regional influences. Studies on *S. matsudana* similarly highlight 7-O-glucoside and kaempferol as key constituents (Han *et al.* 2003; Liu 2012).

In Iran, limited phytochemical studies have been conducted. For instance, *S. aegyptiaca* has shown antioxidant and free radical scavenging activity through the presence of 1,1-diphenyl-2-picrylhydrazyl (DPPH), primarily in bark and male inflorescences (Sonboli *et al.* 2010; Rabbani *et al.* 2010). Mantashloo *et al.* (2017) reported that *S. alba* exhibits the highest total phenol and flavonoid content in young branches and trunk bark, with ethanolic extracts from these tissues demonstrating strong DPPH scavenging activity (0.129 and 0.130 mg mL^{-1}, respectively).

This study examines morphological and phytochemical traits of 19 *Salix* specimens representing 11 species collected in Ardabil Province, Northwestern Iran. We hypothesize that (i) both morphological and phytochemical traits vary among sampled ecotypes; (ii) plant sex may be associated with variation in phytochemical composition and antioxidant activity; and (iii) local environmental conditions may contribute to variation in morphological and phytochemical traits. Through a comparative analysis of key characteristics, this study aims to improve our understanding of the adaptive mechanisms that underlie phenotypic and chemical diversity in *Salix* species in response to ecological pressures.

MATERIALS AND METHODS

Study design and region

In this study, a comparative approach was used to characterize morphological and phytochemical variation among sampled *Salix* specimens in Ardabil Province, Northwestern Iran. Bark and leaf samples were collected from one-year-old branches of *Salix* trees at previously identified sites across the province. Species identification was confirmed by botanists and local experts from Mohaghegh Ardabili University, the Forests and Rangelands Organization, and the Natural Resources Organization of Ardabil Province.

Taxonomic classification was based on key morphological features—including leaf shape, bud structure, bark texture, and catkin characteristics—using standard identification keys from Flora Iranica (Rechinger 1993) and the Salicaceae volume of the Flora of Iran (Mozaffarian 1996). A total of eleven species were recognized: *S. alba*, *S. babylonica*, *S. elbursensis*, *S. pentandra*, *S. triandra f. discolor*, *S. aegyptiaca*, *S. purpurea*, *S. excelsa*, *S. acmophylla*, *S. fragilis*, and *S. pycnostachya*, along with *S. triandra f. concolor*, was treated as a distinct ecotype. Nineteen specimens were sampled, with some species represented by multiple specimens or ecotypes—for example, *S. alba* (5), *S. aegyptiaca* (3) and *S. fragilis* (2).

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One representative tree was selected for each sampled species or ecotype; therefore, the individual tree constituted the biological sampling unit. Multiple measurements performed on extracts from the same tree were treated as technical replicates and were not considered independent biological replicates.

Fig. 1 illustrates the locations and gender of the sampled *Salix* specimens across the province, while Table 1 provides detailed information on species and their collection sites.

Table 1. Information on *Salix* species collected from Ardabil Province, including spatial characteristics such as the name of the sampling area and the latitude and longitude of the samples.

No	Species	Sex	Area	Longitudes	Latitudes	Altitude (m a.s.l)
1	*S. alba* (2)	Male	Ardabil	38.215827	48.29542	1374.5
2	*S. pentandra*	Male	Owltan	39.59049148	47.73161329	71
3	*S. triandra. f. discolor*	Male	Aslan duz	39.44364555	47.39468912	137
4	*S. aegyptiaca*	Male	Fandoghlou	38.405047	48.505013	1385
5	*S. aegyptiaca*	Female	Hir	38.09185472	48.52135206	1665
6	*S. purpurea*	Female	Hir	38.09121721	48.52005924	1655
7	*S. fragilis*	Male	Hir	38.09121721	48.52005924	1655
8	*S. excelsa*	Male	Kousar	37.686001	48.353858	1330.7
9	*S. elbursensis*	Female	Khalkhal	37.683523	48.40245	1666.8
10	*S. babylonica*	Male	Ardabil	38.230812	48.267675	1367
11	*S. acmophylla*	Female	Shal	37.68334467	48.40253583	1675
12	*S. aegyptiaca*	Female	Fandoghlou	38.405057	48.505013	1385
13	*S. alba*	Female	Fandoghlou	38.4100084	48.498837	1408.2
14	*S. alba*	Female	Agh Ghabagh	39.49459366	47.52857644	122
15	*S. alba*	Male	Ardabil	38.27669352	48.31814602	1333
16	*S. fragilis*	Female	Ardabil	38.219178	48.265934	1372.6
17	*S. alba*	Female	Hir	38.09137342	48.52162833	1648
18	*S. triandra. f. concolor*	Male	Ardabil	38.362227	48.24499	1316
19	*S. pycnostachya*	Female	Lord	37.682194	48.402381	1660

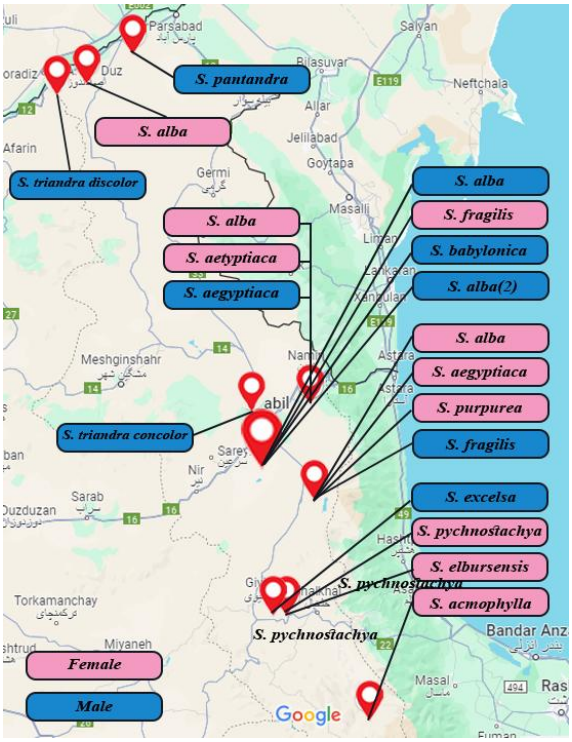

Fig. 1. Locations and genders of *Salix* species collected from Ardabil Province, Northwest Iran.

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Morphological characteristics analysis

Morphological measurements were conducted on one-year-old branches from the collected *Salix* samples. For each recognized species, one representative tree was selected for detailed morphological analysis, due to limited availability of individuals in the study area. These trees were the only known occurrences of their respective species within the sampled sites in Ardabil Province. Measured traits included tree height, crown area (estimated using ellipse and circle formulas), bud length, leaf traits (length, width, fresh and dry weight), petiole length, branch diameter, and trunk diameter at breast height (DBH). For each species, 40 mature and healthy leaves were sampled from multiple orientations of the lower canopy to ensure representative coverage.

Leaf length was measured from the base of the lamina to the apex, and leaf width was recorded at the widest point perpendicular to the midrib using a digital caliper with a precision of ± 0.01 cm. Bud dimensions were measured using the same method. Fresh weights were recorded immediately after sampling, followed by air-drying and oven-drying to determine dry weight. Trunk and branch diameters were measured using a measuring tape and caliper, respectively.

All samples were labelled, dried, and stored under controlled conditions for subsequent analysis. Data were recorded in Microsoft Excel, and statistical analyses and graphical representations were performed using Excel and other relevant analytical software. Sampling was carried out during May and June, which ensured collection during the active growing season of the willow trees.

Extraction and data analysis

After complete drying, the samples were ground to a powder and extracted with 70% ethanol for 72 hours using a shaker set at 150 rpm (Gligorić *et al.* 2019). The extracts were then dried using a rotary evaporator. For subsequent tests measuring total phenol, total flavonoid, and DPPH antioxidant activity, 0.025 g of the dry extract was dissolved in 4 mL distilled water. Phytochemical analyses, including total phenol, total flavonoid, and antioxidant capacity (DPPH), were conducted spectrophotometrically using standard protocols. Each phytochemical assay was performed in triplicate on the same extract solution. These triplicate measurements were treated as technical replicates and were used to assess analytical repeatability; they were not considered independent biological replicates. Since only one representative tree was sampled for each species or ecotype, the individual tree was considered the biological sampling unit. Therefore, inferential statistical comparisons among species or ecotypes were not performed by treating the technical replicates as independent observations. Phytochemical results are presented descriptively as mean values of the technical replicates.

Principal component analysis (PCA)

Principal Component Analysis (PCA) was employed to identify the primary axes of morphological variation across 19 sampled *Salix* specimens. The analysis included 10 quantitative morphological traits standardized to a mean of zero and a standard deviation of one. PCA was performed using the online *statistical platform Stats Kingdom*. Principal components were selected based on the Kaiser criterion, with components having eigenvalues greater than one retained. The proportion of total variance explained by each component was assessed, and results were visualized using a scree plots and two-dimensional biplots of the first two principal components. PCA scores for each specimen were extracted to describe multivariate relationships and patterns of morphological variation. Before PCA, the Kaiser-Meyer-Olkin (KMO) measure and Bartlett's test of sphericity were conducted to assess the suitability of the data for PCA. The KMO value of 0.524, while Bartlett's test of sphericity was significant ($p < 0.001$), indicating correlations among the variables sufficient for conducting PCA.

Total phenol measurement

To measure total phenols, 100 µL of plant extract, 2 mL of 2% sodium carbonate, 2.8 mL of distilled water, and 100 µL of 50% Folin–Ciocalteu reagent were added sequentially. Absorbance was measured at 720 nm after 30 minutes. Gallic acid served as a standard, and results were reported as milligrams of gallic acid equivalent per gram of dry plant weight (Meda *et al.* 2005).

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Total flavonoid measurement

For total flavonoids, 500 µL of each extract was combined with 1.5 mL of 80% methanol, 100 µL of 10% aluminium chloride solution, 100 µL of 1 M sodium acetate solution, and 2.8 mL of distilled water. After 40 minutes, absorbance was measured at 415 nm against a blank containing all components except that 80% methanol replaced the extract. Quercetin was used as the standard, and results were reported as milligrams of quercetin equivalent per gram of dry plant weight (Mita *et al.* 1997).

Antioxidant capacity measurement

Using the DPPH method, 2 mL of 0.004% methanolic DPPH solution was mixed with 2 mL of the extract. The control contained 2 mL of DPPH and 2 mL of methanol. Solutions were kept in the dark at room temperature for 30 minutes, and absorbance was measured at 517 nm against a methanol blank. Percentage of radical scavenging activity (I%) for each extract was calculated (Miliauskas *et al.* 2004).

$$Percent\ of\ Inhibition\ (\%) = \left(\frac{Absorbance\ of\ Control\ (Ac) - Absorbance\ of\ Sample\ (As)}{Absorbance\ of\ Control\ (Ac)}\right) \times 100 \qquad (1)$$

Morphological and phytochemical data were summarized using descriptive statistics in Microsoft Excel. Phytochemical measurements were performed in technical triplicates, and the results are presented as mean values of these technical replicates.

Statistical analysis

Since one representative tree was sampled for each of the 19 *Salix* specimens, the individual tree was considered the biological sampling unit. Phytochemical measurements (total phenol, total flavonoid, and DPPH activity) were performed in triplicate for each extract; these measurements represented technical replicates rather than independent biological replicates and were used to assess analytical repeatability. Morphological and phytochemical data were summarized using descriptive statistics, and phytochemical results are presented as mean values of the technical replicates. No inferential statistical comparisons among species, ecotypes, or genders were performed, since independent biological replication was not available.

RESULTS

Morphological characteristics

As part of a broader investigation into morphological and phytochemical diversity, morphological traits were descriptively analyzed to document variation among ecotypes; no statistical tests were applied, and data were reported as observed values.

Fig. 2 illustrates tree height and crown area, revealing that in most species, crown area exceeded tree height, with the exception of *S. purpurea* (female, Hir). The largest crown area was observed in *S. fragilis* (female, Ardabil), followed by *S. babylonica* (male, Ardabil), *S. pentandra* (male, Owltan), and *S. alba* (female, Fandoghlou). The smallest crown area was recorded in *S. acmophylla* (female, Shal).

Fig. 3 presents leaf length and width data. Leaf width showed no statistically significant variation across ecotypes, whereas leaf length differed substantially. The longest leaves (11.3–12.5 cm) were found in *S. babylonica* (male), *S. elbursensis* (male, Ardabil), and *S. excelsa* (male, Kosar). The shortest leaves (6.3–7.3 cm) were observed in both forms of *S. triandra* and in *S. aegyptiaca* (female, Hir).

Fig. 4 compares fresh and dry leaf weights. The lowest fresh weights (0.08–0.12 g) were recorded in *S. triandra f. concolor* (male, Ardabil), *S. purpurea* (female, Hir), and *S. excelsa* (male, Kosar). The highest values (0.18–0.21 g) were observed in *S. alba* (female, Fandoghlou), *S. pentandra* (male, Owltan), *S. elbursensis* (male, Ardabil), *S. aegyptiaca* (female, Fandoghlou), and *S. alba* (male, Ardabil).

Fig. 5 illustrates trunk diameter measured at breast height (1.3 m). The smallest diameter was found in *S. acmophylla* (female, Shal), while the largest was recorded in *S. triandra* f. *discolor* (male, Aslandoz). Most ecotypes exhibited trunk diameters of at least 10 cm.

Table 2 summarizes bud length, petiole length, and young stem diameter. Bud length ranged from 0.5 mm to 10 mm, with the longest buds observed in *S. triandra* f. *discolor*, *S. pentandra*, and *S. fragilis*. Petiole length varied between 0.2 and 1 cm, with the shortest values recorded in *S. alba* (female, Hir), *S. acmophylla* (female, Shal), and *S. purpurea* (female, Hir). Young stem diameter was 1 cm in 11 ecotypes, while others measured 8, 7, or 5

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mm. The smallest diameter (0.5 cm) was observed in *S. elbursensis* (male, Ardabil), *S. aegyptiaca* (female, Hir), *S. babylonica* (male, Ardabil), and *S. elbursensis* (female, Khalkhal).

Table 2. Values of bud length, petiole length, and young stem diameter of *Salix* species.

Species	Area	Bud Length (cm)	Petiole Length (cm)	Diameter of young Stem (cm)
S. alba (Female)	Fandoghlou	1	1	1
S. alba (Male)	Ardabil	1	1	1
S. aegyptiaca (Male)	Fandoghlou	0.5	1	0.7
S. pentandra (Male)	Owltan	1	1	1
S. aegyptica (Female)	Fandoghlou	0.5	1	0.7
S. elbursensis (Female)	Khalkhal	0.5	1	0.5
S. triandra f. discolor (Male)	Aslan duz	1	0.8	1
S. babylonica (Male)	Ardabil	0.5	0.7	0.5
S. excelsa (Male)	Kousar	1	0.7	1
S. triandra f. concolor (Male)	Ardabil	0.5	0.7	1
S. alba (Female)	Agh Ghabagh	0.7	0.6	1
S. alba (2) (Male)	Ardabil	0.5	0.5	0.5
S. fragilis (Male)	Hir	1	0.5	1
S. purpurea (Female)	Hir	0.5	0.5	0.8
S. pycnostachya (Female)	Lord	0.5	0.5	1
S. fragilis (Female)	Ardabil	1	0.5	0.8
S. aegyptiaca (Female)	Hir	1	0.5	0.5
S. acmophylla (Female)	Shal	0.5	0.3	1
S. alba (Female)	Hir	0.7	0.2	1

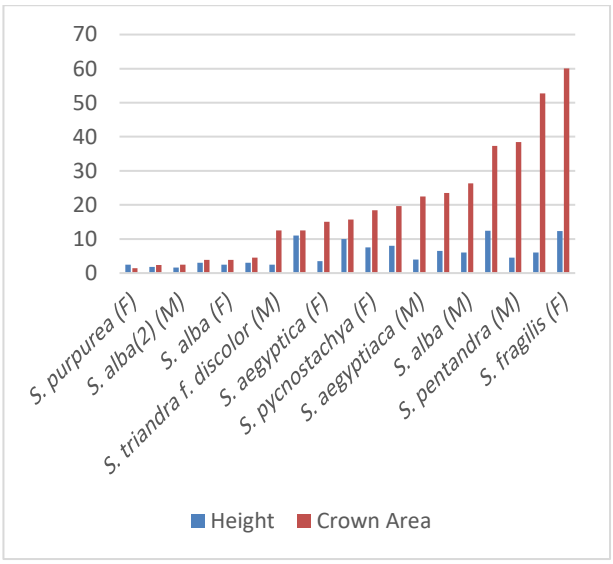

Fig. 2. Comparison of height (m) and crown area (m^2) of *Salix* species samples from Ardabil Province.

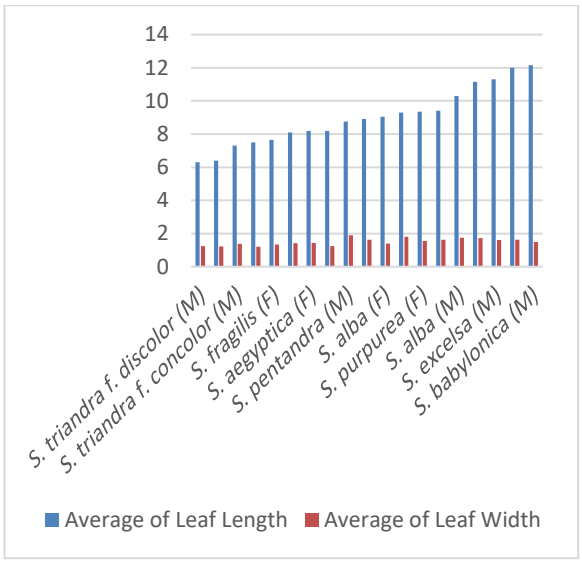

Fig. 3. Comparison of average leaf length (cm) of one-year-old branch and Average of leaf width (cm) of one-year-old branch of *Salix* species samples from Ardabil Province.

Caspian J. Environ. Sci.
DOI:

Received:
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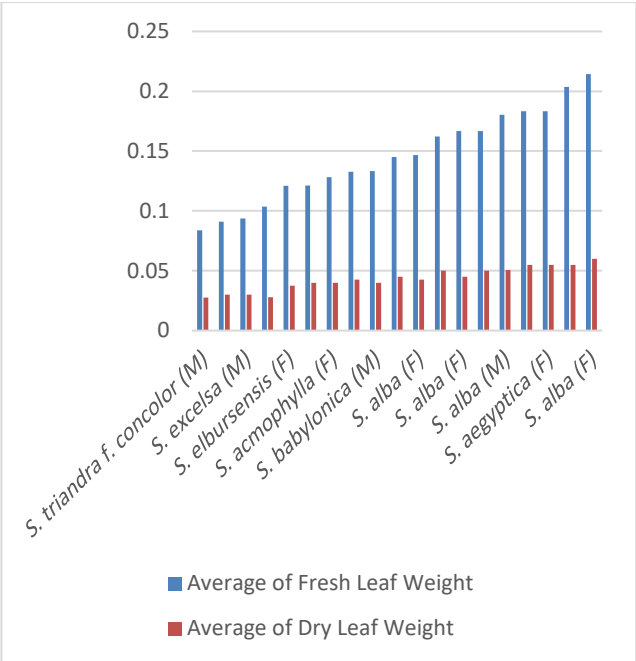

Fig. 4. Comparison of average of fresh leaf weight (g) and average of dry leaf weight (g) of *Salix* species samples from Ardabil Province.

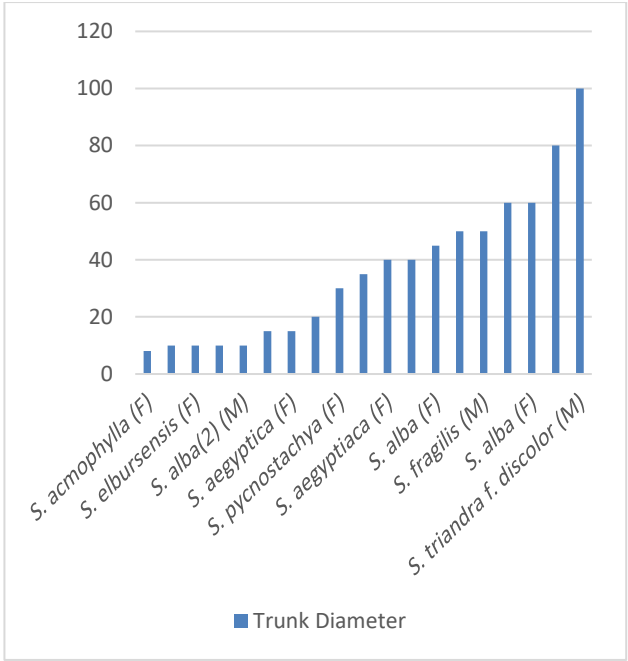

Fig. 5. Comparison of average trunk diameter (cm) at 1.3 m of *Salix* species samples from Ardabil Province.

PCA of morphological traits

Principal Component Analysis (PCA) based on ten morphological traits in diverse *Salix* species collected from different regions of Ardabil Province revealed that the first three components (PC_1, PC_2, and PC_3) together explained 71.14% of the total variance. PC_1 accounted for 36.09% and PC_2 for 23.68% of the total variance (Table 3, Fig. 7). The scree plot showed a marked decrease in eigenvalues after the third component, supporting the retention of the first three components for interpretation (Fig. 7). In the scatter plot based on PC_1 and PC_2, variation in the positions of the sampled specimens was observed (Fig. 6). *S. alba* (female from Fandoghlou and male from Ardabil) exhibited relatively high positive scores along PC_1, corresponding to relatively high values for traits such as height, trunk diameter, and bud length (Table 4). In contrast, *S. acmophylla* (female from Khalkhal) and *S. elbursensis* (female from Khalkhal) showed relatively low PC_1 scores. Along PC_2, *S. excelsa* (male from Kousar) and *S. triandra* f. *discolor* (male from Aslan Duz) showed relatively high scores, with crown area and leaf weight contributing substantially to this component (Table 4). Analysis of the variable vectors in the biplot showed that PC_1 was most strongly associated with trunk diameter at 1.3 m ($r = 0.74$), bud length ($r = 0.62$), and height ($r = 0.59$), whereas PC_2 was primarily associated with crown area ($r = 0.46$), leaf weight (approximately $r = 0.47$), and leaf width ($r = 0.26$; Fig. 6).

Table 3. Eigenvalues and explained variance of principal components.

Parameter	PC_1	PC_2	PC_3	PC_4	PC_5	PC_6	PC_7	PC_8	PC_9	PC_{10}
Eigenvalue	3.6092	2.3683	1.1367	0.9492	0.8956	0.3853	0.3271	0.2207	0.1003	0.007
% of Variance	36.0923	23.6828	11.3667	9.4922	8.9564	3.8529	3.2708	2.2073	1.0025	0.076
Cumulative (%)	36.0923	59.7751	71.1418	80.634	89.5904	93.4433	96.714	98.9213	99.9238	100

Eigenvalues, percentage of variance explained by each principal component, and their cumulative contribution to total variance in *Salix* morphological data.

Caspian J. Environ. Sci.
DOI:

Received:
Article type: Research

Table 4. PCA scores of *Salix* samples for each principal component.

Specie	PC_1	PC_2	PC_3	PC_4	PC_5	PC_6	PC_7	PC_8	PC_9
S. alba (2)(M)Ardabil	-0.3475	-3.2476	-0.4834	1.0043	-0.638	-0.2903	-1.0582	-0.5504	-0.2824
S. pentandra (M)Owltan	2.7185	-0.8153	1.1172	-1.2175	0.2439	0.2535	-0.3102	0.9209	0.1923
S. triandera f. discolor (M)Aslan duz	-1.1192	3.0859	0.3736	-1.1975	1.2515	0.7303	-1.2029	-0.714	-0.2585
S. aegyptica (M)Fandoghlou	0.3936	-1.7272	-0.3719	-0.7319	0.7343	0.1828	0.3253	0.1744	-0.2281
S. aegyptica (F) Hir	-0.8102	1.6797	-1.9516	-0.4484	-0.3794	-1.6231	-0.281	0.3991	0.05616
S. purpurea (F) Hir	-2.4357	-0.4999	0.6482	1.0305	0.5166	-0.09494	0.01622	0.5693	-0.1256
S. fragilis (M)Hir	0.862	0.8436	0.8518	0.4257	-0.5525	-0.1062	-0.5421	0.328	0.0633
S. excelsa (M)Kousar	0.3954	1.8603	0.9979	1.9734	1.0314	-0.7959	0.376	-0.4134	0.1544
S. elbursensis (F) Khalkhal	-1.7531	-1.2001	-0.9709	-0.6411	1.5399	-0.6068	0.1545	0.03512	0.07696
S. babylonica (M)Ardabil	0.7134	-0.6096	-1.9381	1.9426	0.8657	1.3148	-0.1281	0.1811	0.2831
S. acmophylla (F) Shal	-2.6976	-0.06667	0.4139	-0.4937	-1.5018	0.3974	0.1331	-0.1994	0.2505
S. aegyptica (F) Fandoghlou	0.0576	-1.8465	-0.688	-1.548	0.1306	-0.01771	0.2953	-0.4722	0.0322
S. alba (F) Fandoghlou	4.0062	-0.08488	0.2783	0.01824	-0.2273	-0.3681	0.5645	-0.7975	0.06183
S. alba (F) Agh Ghabagh	1.0666	-0.08327	0.994	0.4161	-0.3429	-0.05246	0.2445	0.3403	-0.9875
S. alba (M)Ardabil	2.1643	-0.4285	1.1053	-0.3218	0.5287	-0.1402	-0.1389	-0.06121	0.4964
S. fragilis (F) Ardabil	2.1604	2.3532	-1.8846	-0.1545	-1.1402	0.4784	0.1261	0.3832	-0.1055
S. alba (F) Hir	-1.5863	-0.315	0.9479	0.2864	-1.7433	-0.01476	-0.4712	0.05657	0.3514
S. triandera f. concolor (M)Ardabil	-2.7503	0.5395	0.7862	-0.272	0.7183	0.3238	0.8389	0.2983	0.06141
s. pycnostachya (F) Lord	-1.0381	0.5623	-0.2259	-0.07053	-1.0355	0.4295	1.0581	-0.4781	-0.09216

Principal component scores (PC_1, PC_2, and PC_3) of different *Salix* genotypes collected from various regions of Ardabil Province, based on ten morphological traits.

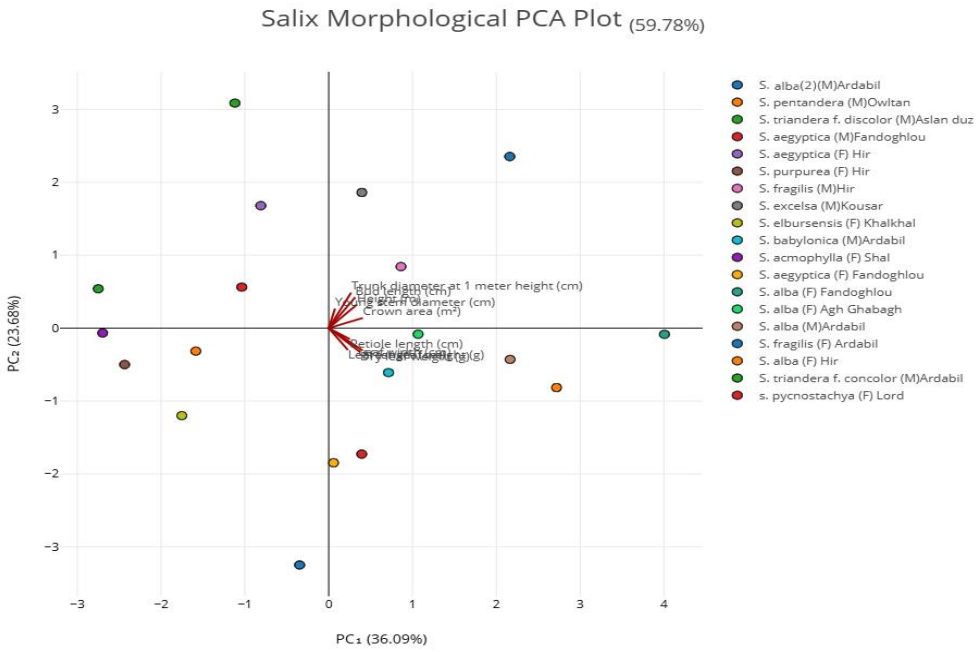

Fig. 6. PCA biplot of *Salix* samples based on PC_1 and PC_2 with variable vectors. [The plot shows the distribution of *Salix* genotypes across the first two principal components (PC_1 and PC_2). Arrows represent the contribution of each morphological trait to the components].

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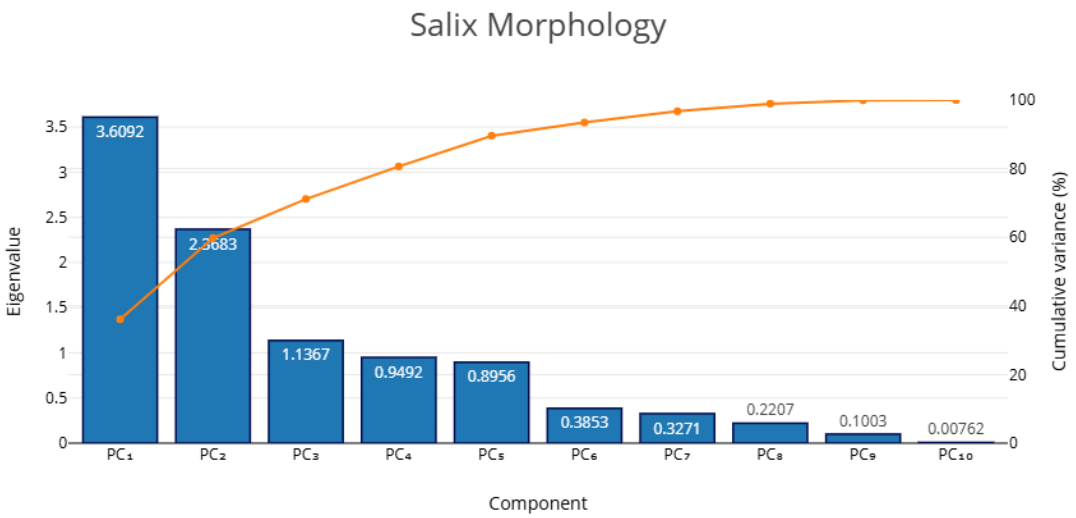

Fig. 7. Scree plot showing eigenvalues and cumulative variance of PCs. (The scree plot illustrates the variance explained by each principal component. A sharp drop after PC_3 justifies the selection of the first three components for further analysis.)

Phytochemical properties

Total phenolic content of leaves

The total phenolic content of leaf extracts varied across the sampled *Salix* specimens. The highest value was observed in *S. alba* female from Hir (75.18 mg GAE/g DW), whereas the lowest value was observed in *S. aegyptiaca* male from Fandoghlou (25.61 mg GAE/g DW; Table 5). *S. aegyptiaca* female from Hir, *S. excelsa* male from Kousar, and *S. pycnostachya* female from Lord showed relatively high phenolic contents (59.21, 57.01, and 55.18 mg GAE/g DW, respectively; Table 5). Overall, the descriptive values indicated variation in total phenolic content among the sampled specimens, with *S. alba* female from Hir showing the highest value and *S. aegyptiaca* male from Fandoghlou showing the lowest value.

Total flavonoid content of leaves

Flavonoid content varied across the sampled *Salix* specimens (Table 5). *S. alba* female from Hir exhibited the highest flavonoid content (28.99 mg QE/g DW), whereas *S. aegyptiaca* female from Fandoghlou displayed the lowest value (6.85 mg QE/g DW). *S. purpurea* female from Hir also showed a relatively high flavonoid content (27.39 mg QE/g DW). *S. fragilis* male from Hir and *S. triandra* male from Aslan Duz showed relatively high flavonoid contents (19.67 and 19.71 mg QE/g DW, respectively). Conversely, *S. aegyptiaca* male and female from Fandoghlou showed relatively low values (8.39 and 6.85 mg QE/g DW, respectively). Overall, the descriptive values indicated variation in flavonoid content among the sampled specimens, with *S. alba* female from Hir showing the highest value, while *S. aegyptiaca* female from Fandoghlou the lowest.

DPPH antioxidant activity of leaves

DPPH antioxidant activity varied across the sampled *Salix* specimens (Table 5). *S. babylonica* male from Ardabil exhibited the lowest DPPH value (43.54%), whereas *S. aegyptiaca* female from Fandoghlou had the highest (90.38%). Relatively high antioxidant activity was also observed in *S. elbursensis* female from Khalkhal (88.22%), *S. alba* female from Fandoghlou (86.99%), and *S. acmophylla* female from Shal (80.05%). In contrast, relatively low values were observed in *S. aegyptiaca* male from Fandoghlou (43.67%), *S. alba* male from Ardabil (46.21%), and *S. triandra* f. *concolor* male from Ardabil (47.98%). Overall, the descriptive values showed variation in DPPH antioxidant activity among the sampled specimens. The observed distribution of values between female and male specimens should be interpreted cautiously, since sex was not independently replicated across species and sampling locations.

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Table 5. Duncan grouping of total phenol, total flavonoid, and DPPH antioxidant of leaf samples from *Salix* species in Ardabil Province.

Species	Area	Total phenol	Total flavonoid	DPPH
S. acmophylla (Female)	Shal	36.91^{gh}	19.11^{d}	80.05^{d}
S. aegyptiaca (Female)	Hir	59.21^{b}	9.42^{m}	79.16^{e}
S. aegyptiaca (Female)	Fandoghlou	34.18^{hi}	6.85^{q}	90.38^{a}
S. aegyptiaca (Male)	Fandoghlou	25.61^{j}	8.39^{p}	43.67^{o}
S. alba (Female)	Hir	75.18^{a}	28.99^{a}	74.16^{f}
S. alba (Female)	Fandoghlou	52.11^{cb}	16.71^{f}	86.99^{c}
S. alba (Female)	Agh Ghabagh	51.31^{cb}	11.66^{j}	72.51^{g}
S. alba (Male)	Ardabil	44.08^{ef}	16.21^{g}	46.21^{n}
S. babylonica (Male)	Ardabil	43.21^{ef}	15.01^{h}	43.54^{o}
S. elbursensis (Female)	Khalkhal	31.34^{i}	9.21^{o}	88.22^{b}
S. alba (2) (Male)	Ardabil	43.24^{ef}	9.31^{n}	53.75^{j}
S. excelsa (Male)	Kousar	57.01^{b}	16.70^{f}	51.50^{l}
S. fragilis (Female)	Ardabil	39.68^{fg}	14.30^{i}	64.84^{h}
S. fragilis (Male)	Hir	47.41^{de}	19.67^{c}	52.69^{k}
S. pentandra (Male)	Owltan	47.21^{de}	10.78^{l}	48.20^{m}
S. purpurea (Female)	Hir	30.18^{i}	27.39^{b}	71.75^{g}
S. pycnostachya (Female)	Lord	55.18^{b}	11.08^{k}	73.70^{f}
S. triandra (Male)	Ardabil	49.08^{cd}	17.52^{e}	47.99^{m}
S. triandra (Male)	Aslan duz	36.21^{gh}	19.71^{c}	63.87^{i}

Total phenol of bark

Total phenolic content varied across the sampled *Salix* specimens (Table 6). The highest value was observed in *S. alba* female from Agh Ghabagh (107.08 mg GAE/g DW), whereas the lowest value was in *S. alba* (2) male from Ardabil (56.35 mg GAE/g DW). Relatively high phenolic contents were also observed in *S. pycnostachya* female from Lord (104.11 mg GAE/g DW), *S. alba* male from Ardabil (103.11 mg GAE/g DW), and *S. excelsa* male from Kousar (102.18 mg GAE/g DW). Relatively low values were observed in *S. babylonica* male from Ardabil (62.35 mg GAE/g DW), *S. fragilis* female from Ardabil (67.01 mg GAE/g DW), and *S. triandra* f. *discolor* male from Aslan Duz (77.31 mg GAE/g DW). Overall, the descriptive values indicated considerable variation in total phenolic content among the sampled specimens.

Total flavonoids of bark

Flavonoid content varied across the sampled *Salix* specimens (Table 6). The highest value was observed in *S. purpurea* female from Hir (8.73 mg QE/g DW), whereas the lowest was in *S. aegyptiaca* female from Fandoghlou (0.93 mg QE/g DW). Other specimens with relatively high flavonoid contents included *S. excelsa* male from Kousar (4.24 mg QE/g DW), *S. alba* (2) male from Ardabil (4.21 mg QE/g DW), and *S. alba* female from Hir (4.21 mg QE/g DW). Relatively low flavonoid contents were also observed in *S. acmophylla* female from Shal (1.14 mg QE/g DW), *S. fragilis* female from Ardabil (1.35 mg QE/g DW), and *S. aegyptiaca* female from Hir (1.41 mg QE/g DW). Overall, the descriptive values indicated substantial variation in flavonoid content among the sampled specimens.

Antioxidant of bark

Antioxidant activity varied across the sampled *Salix* specimens (Table 6). The highest antioxidant activity was observed in *S. alba* female from Hir (89.03%), whereas the lowest was in *S. acmophylla* female from Shal (65.18%). Relatively high antioxidant activities were also observed in *S. fragilis* female from Ardabil (88.06%), *S. purpurea* female from Hir (86.83%), and *S. aegyptiaca* male from Fandoghlou (86.62%). Relatively low values were observed in *S. pentandra* male from Owltan (66.71%), *S. alba* female from Agh Ghabagh (68.78%), and *S. aegyptiaca* female from Hir (75.05%). Overall, the descriptive values indicated variation in antioxidant activity among the sampled specimens.

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Table 6. Duncan grouping of total phenol, total flavonoid, and DPPH antioxidant of bark samples from *Salix* species in Ardabil Province.

Species	**Area**	**Total Phenol**	**Total Flavonoid**	**DPPH**
S. acmophylla (Female)	Shal	92.08	1.14	65.18
S. aegyptiaca (Female)	Hir	101.11	1.41	75.05
S. aegyptiaca (Female)	Fandoghlou	84.55	0.93	80.39
S. aegyptiaca (Male)	Fandoghlou	91.11	2	86.62
S. alba (Female)	Agh Ghabagh	107.08	1.81	68.78
S. alba (Female)	Fandoghlou	94.75	2.02	82
S. alba (Female)	Hir	92.48	4.21	89.03
S. alba (Male)	Ardabil	103.11	2.66	77.51
S. babylonica (Male)	Ardabil	62.35	3.31	85.3
S. elbursensis (Female)	Khalkhal	95.65	3.33	84.92
S. alba (2) (Male)	Ardabil	56.35	4.21	75.48
S. excelsa (Male)	Kousar	102.18	4.24	83.06
S. fragilis (Female)	Ardabil	67.01	1.35	88.06
S. fragilis (Male)	Hir	95.18	1.6	76.28
S. pentandra (Male)	Owltan	94.21	2.3	66.71
S. purpurea (Female)	Hir	88.51	8.73	86.83
S. pycnostachya (Female)	Lord	104.11	3.79	79.37
S. triandra (Male)	Ardabil	101.81	2.69	77.89
S. triandra (Male)	Aslan Duz	77.31	2.19	78.1

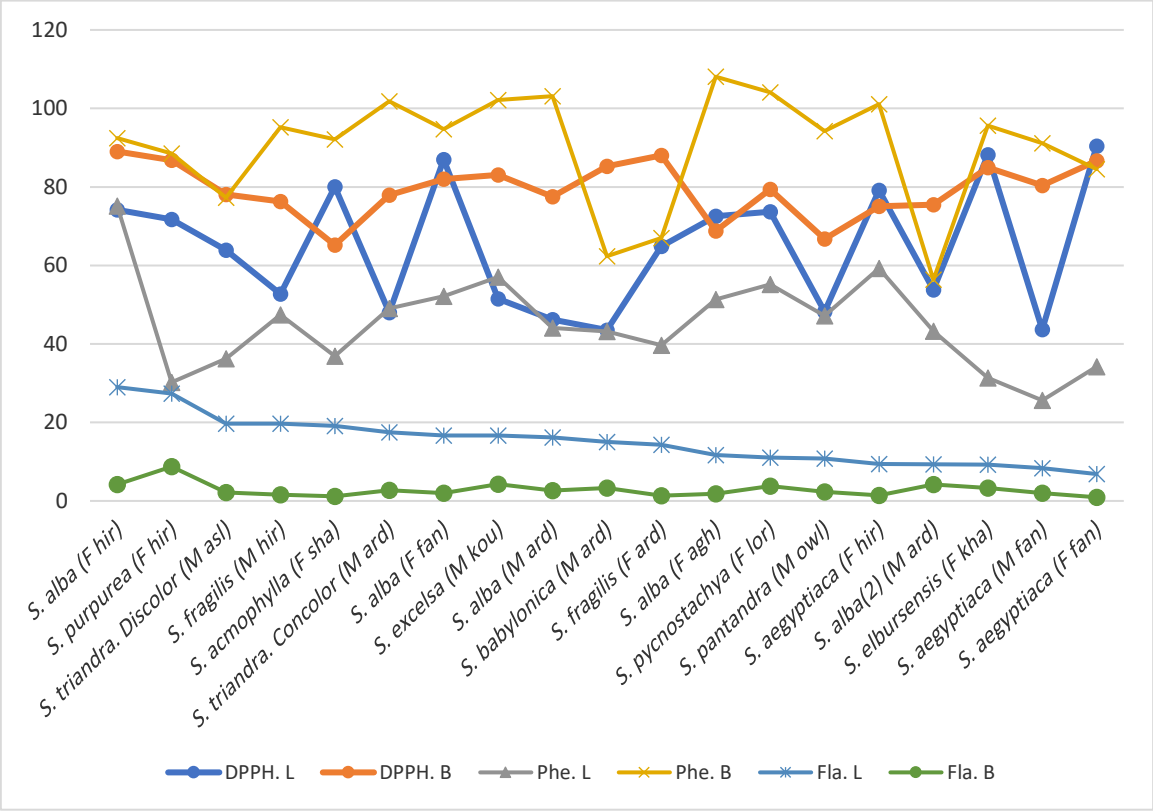

Fig. 8. Comparison of total phenol content (mg GAE/g DW), total flavonoid content (mg QE/g DW), and DPPH radical scavenging activity (% inhibition) in leaves (gray, light blue, blue) and bark (yellow, green, orange) of *Salix* specimens from Ardabil Province.

DISCUSSION

Morphological changes

Salix species have attracted the attention of many researchers due to their morphological diversity and rich phytochemical compositions. This study investigates the morphological and phytochemical variation among

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different *Salix* specimens in Ardabil Province, Iran. Because the sampling design included one representative tree for each species or ecotype, the observed morphological differences should be interpreted as descriptive patterns rather than as statistically established effects of species, ecotype, or gender. A comparative examination of tree height and crown area of the studied samples revealed that crown area exceeded tree height in most specimens. This may be related to the growth form of willow trees, including the development of relatively extensive crowns. However, as shown in Fig. 2, some specimens, such as *S. alba* female from Fandoghlou and *S. fragilis* female from Ardabil, had considerably larger crown areas relative to tree height. Differences in tree age, local growing conditions, and resource availability may contribute to these patterns; however, these factors were not directly measured in the present study and therefore should be considered possible explanations rather than confirmed causes. For example, *S. alba* females from Fandoghlou and Agh Ghabagh exhibited crown-to-height ratios of 3.0 (66.6%) and 2.4 (58.3%), respectively, despite comparable age and genetic background. Similarly, in *S. triandra* (excluding *discolor* and *concolor forms*), the specimens near Aslan Duz displayed a ratio of 5.0 (height: 2.5 m; crown area: 12.56 m²), whereas the specimen from Ardabil showed a ratio of 1.5 (height = 3 m; crown area = 4.5 m²) (Fig. 2).

Bud length showed relatively limited variation among the studied specimens, although differences in tree age and vigor may contribute to variation in bud development (Table 2). Leaf length on one-year-old branches also varied among the sampled specimens. Species-specific characteristics and differences in light exposure may contribute to this variation. For example, *S. triandra f. discolor* male (Ardabil and Aslan Duz), *S. aegyptiaca* female (Hir), *S. acmophylla* female (Shal), and *S. fragilis* female (Ardabil), which grew under relatively high-light conditions, had shorter leaves than *S. babylonica* male (Ardabil) and *S. alba* (2) male (Ardabil), which were located in more shaded conditions (Figure 3). However, because light exposure was not experimentally controlled or quantitatively measured, its contribution to leaf-length variation should be considered a possible explanation rather than a demonstrated causal factor. Differences in fresh and dry leaf weight were also observed among the sampled specimens. The association between smaller leaf size and younger plants may reflect developmental differences; however, the present data do not allow conclusions regarding nutrient storage mechanisms in the *Salix* genus. Further physiological studies would be required to evaluate this possibility (Figure 4).

Petiole length varied among the sampled specimens, with *S. alba* showing both the minimum (0.2 cm; female Hir) and maximum (1 cm; male and female Fandoghlou) recorded values. The observed variation may be associated with differences in age, genotype, and local growing conditions. For example, young *S. elbursensis* females (Khalkhal) displayed long petioles (1 cm), similar to *S. aegyptiaca*, *S. alba* males (Ardabil), and *S. pentandra* males (Owltan). In contrast, older *S. fragilis* rootstocks (Ardabil/Hir) showed shorter petioles than younger specimens such as *S. triandra f. discolor* male from Aslan Duz, *S. babylonica* male from Ardabil, and *S. excelsa* male from Kousar. These observations suggest possible associations with developmental stage and local conditions, but the relative effects of age, species, gender, and environment cannot be distinguished with the present sampling design.

Differences in young stem diameter were observed between the sampled male and female specimens; however, these observations should not be interpreted as evidence of a general gender effect because gender was not independently replicated across species and locations. Most of the smaller stem diameters were observed in female specimens, whereas some of the larger diameters (approximately 1 cm) occurred in male specimens (Table 2). Exceptions included *S. alba* (2) male from Ardabil and the *S. aegyptiaca* population at Fandoghlou, where both male and female individuals were represented. These patterns may also reflect differences in species identity, tree age, and local growing conditions. Therefore, the present observations do not permit separation of gender effects from these potentially confounding factors.

Another important index measured in this study was the diameter of the trunk at a height of 1.3 m above the ground, a commonly used parameter in forest evaluation and management. This measurement is useful for characterizing tree size and estimating biomass and volume (Paramonov et al. 2020).

Trunk diameter is known to be influenced by multiple factors, including tree age, resource availability, competition, and climatic and edaphic conditions (Paramonov et al. 2020; Sumida et al. 2013). Previous studies have reported effects of moisture availability and climatic conditions on radial growth in *Salix* species. However, these factors were not directly measured in the present study and therefore cannot be identified as causes of the observed differences among the sampled specimens. The present observations should consequently be interpreted as descriptive variation rather than evidence of specific environmental effects. In particular, the absence of

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statistical differences reported in the original analysis should not be used to infer the absence or presence of gender, species, or regional effects, given the lack of independent biological replication. Future comparative studies should include multiple trees per species and location, with standardized tree age and quantitative measurements of environmental variables such as water availability, soil properties, and light conditions, to distinguish species-, gender-, and environment-related effects on trunk development.

PCA-Based Assessment

The results of Principal Component Analysis (PCA) revealed morphological variation among the sampled *Salix* specimens in the Ardabil region. The first principal component (PC_1) was primarily associated with traits such as height and stem diameter, while the second principal component (PC_2) was mainly associated with crown architecture and foliar morphological traits (Figure 6; Table 4). The strong contribution of traits such as trunk diameter at 1 m, bud length, and height to PC_1, and crown area, leaf weight, and leaf width to PC_2, indicates that these morphological characteristics contributed substantially to the observed multivariate variation. Specimens such as *S. triandra f. discolor* and *S. excelsa* showed relatively high PC_2 scores, whereas samples from different collection sites, including Fandoghlou, Ardabil, Khalkhal, and Hir, showed distinct positions within the PCA space. These patterns may reflect the combined influence of species characteristics and local growing conditions; however, the present sampling design does not allow these effects to be separated. Similarly, the predominantly negative PC_1 values observed in Khalkhal samples may be associated with differences in local environmental conditions, but altitude, precipitation, and soil properties were not directly measured in this study and therefore cannot be identified as causal factors. Because each species or ecotype was represented by a single sampled tree, the PCA should be interpreted as a descriptive visualization of morphological variation rather than evidence of genetic differentiation or adaptation. Overall, the PCA highlights morphological heterogeneity among the sampled *Salix* specimens and identifies the traits contributing most strongly to this variation, providing a basis for further studies using replicated sampling across species, populations, and environmental conditions.

Phytochemical changes

The phytochemical characteristics of the sampled *Salix* specimens showed variation in total phenolic content, total flavonoid content, and DPPH antioxidant activity in both leaves and bark (Figure 8; Tables 5 and 6). Overall, bark samples generally showed higher descriptive values of total phenolic content than leaf samples. However, some leaf samples exhibited higher values than particular bark samples. For example, the leaves of female *S. alba* from Hir had a total phenolic content of 75.18 mg GAE/g DW, compared with 67.01, 56.35, and 62.35 mg GAE/g DW in the bark of female *S. fragilis*, male *S. alba (2)*, and male *S. babylonica*, respectively. These observations indicate variation among specimens and plant tissues, but the relative effects of species, gender, and geographic origin cannot be separated because each species or ecotype was represented by a single tree.

In the leaf samples, female *S. alba* from Hir showed the highest descriptive total phenolic content (75.18 mg GAE/g DW), whereas other relatively high values were observed in female *S. aegyptiaca* from Hir, male *S. excelsa* from Kousar, and female *S. pycnostachya* from Lord. In the bark samples, female *S. alba* from Agh Ghabagh showed the highest descriptive value (107.08 mg GAE/g DW). These patterns suggest considerable variation among the sampled specimens; however, they should not be interpreted as evidence that *S. alba* has inherently superior phenolic content across regions. Differences in tree age, local growing conditions, water availability, and nutritional status may contribute to the observed variation, but these variables were not quantitatively measured in the present study.

Previous studies have indicated that phenolic accumulation can vary with tree age and developmental stage. Jahanban-Esfahlan et al. (2019) reported differences in phenolic content among walnut trees of different ages, while Castro et al. (2015) found higher phenolic content in young *Acrocarpus fraxinifolius* trees than in mature trees. These findings support the importance of considering developmental stage when interpreting variation in phenolic content. Environmental stress may also influence phenolic biosynthesis; however, because site-specific environmental variables were not measured in the present study, environmental stress should be considered a possible explanation rather than a demonstrated cause of the observed differences.

Flavonoid content was generally higher in leaves than in bark among the sampled specimens (Figure 8; Tables 5 and 6). An exception was female *S. purpurea* from Hir, whose bark flavonoid content (8.73 mg QE/g DW) exceeded the leaf value observed in female *S. aegyptiaca* from Fandoghlou (6.85 mg QE/g DW). The highest

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descriptive leaf flavonoid value was observed in female *S. alba* from Hir (28.99 mg QE/g DW), whereas relatively high values were also observed in female *S. purpurea* from Hir and male *S. triandra* from Aslan Duz. These differences indicate variation among the sampled specimens but do not establish a general effect of species or gender.

Differences in local water availability and environmental stress may potentially contribute to variation in flavonoid content. Several specimens with relatively high flavonoid values were collected from locations that may differ in their proximity to water sources, whereas some specimens with lower values were collected from areas with apparently greater water availability. For example, female *S. aegyptiaca* from Hir was located on a river-facing slope, while other specimens were collected under different local growing conditions. Nevertheless, water availability and stress were not directly measured in this study; therefore, these observations should be regarded as hypotheses requiring further investigation. Species-specific characteristics may also contribute to the observed variation. The bark of female *S. purpurea* from Hir showed the highest descriptive flavonoid content among the bark samples. Differences in bark age, developmental stage, light exposure, temperature, moisture, and other environmental conditions may also influence flavonoid accumulation, However, the individual contribution of these factors cannot be determined from the present data because they were not independently measured.

The general pattern observed in this study is consistent with the findings of Gligorić et al. (2023), who reported higher total phenolic content in bark than in leaves and higher total flavonoid content in leaves than in bark across the examined *Salix* species. In contrast, Enayat and Banerjee (2009), working with *S. aegyptiaca* extracts prepared using different solvents and plant tissues, reported particularly high phenolic, flavonoid, and antioxidant values in bark extracts. Similarly, Wahab et al. (2018) reported high phenolic content and antioxidant activity in methanolic bark extracts of *S. babylonica*. Differences among these studies may be related to species, tissue type, extraction solvent, and experimental conditions.

DPPH antioxidant activity also varied among the sampled *Salix* specimens (Figure 8; Tables 5 and 6). In leaf extracts, female *S. aegyptiaca* from Fandoghlou showed the highest descriptive value (90.38%), whereas *S. babylonica* male from Ardabil showed the lowest value (43.54%). Other relatively high values were observed in female *S. elbursensis* from Khalkhal, female *S. alba* from Fandoghlou, and female *S. acmophylla* from Shal. In bark extracts, female *S. alba* from Hir showed the highest antioxidant activity (89.03%), whereas female *S. acmophylla* from Shal showed the lowest value (65.18%). These observations demonstrate variation in antioxidant activity among the sampled specimens but do not provide sufficient evidence to establish species- or gender-specific effects.

Some comparisons between male and female specimens collected from the same or nearby locations showed differences in their descriptive antioxidant values. For example, in *S. aegyptiaca* from Fandoghlou, female specimens showed higher descriptive leaf and bark antioxidant values than male specimens. Similar differences were observed among some specimens from Aslan Duz, Owltan, and Agh Ghabagh. However, these observations cannot be interpreted as evidence that gender is a more influential determinant than species identity because gender, species, location, and potentially tree age are confounded in the present sampling design. In particular, the presence of both sexes within a single location provides useful descriptive observations, but replication across multiple trees and populations would be required to test a general gender effect.

Another potentially informative comparison involves the two male specimens of *S. triandra* collected from Aslan Duz and Ardabil, which originated from locations differing substantially in elevation and environmental conditions. Their differences in antioxidant activity illustrate the potential importance of local growing conditions, but because environmental variables were not directly measured and biological replication was absent, no causal relationship can be established.

The findings of Enayat and Banerjee (2009) showed that antioxidant activity in *S. aegyptiaca* varied among plant tissues and extraction solvents, with ethanolic bark extracts showing high antioxidant activity. Gligorić et al. (2023) likewise reported tissue- and species-dependent differences in antioxidant activity among *Salix* species. For example, bark showed higher activity than leaves in *S. fragilis*, whereas leaves showed higher activity than bark in several other species. These findings, together with the present observations, indicate that antioxidant activity can vary substantially according to species, tissue, and extraction conditions.

Finally, some specimens with relatively high leaf flavonoid contents also showed relatively high bark antioxidant activity. For example, female *S. alba* and female *S. purpurea* from Hir showed relatively high values for these characteristics. This co-occurrence may indicate a possible relationship between secondary metabolite profiles

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and antioxidant activity, but such an association cannot be established from the present descriptive dataset and requires targeted analysis with independent biological replication.

Conclusions

In conclusion, the morphological and phytochemical characteristics of the sampled *Salix* specimens in Ardabil Province showed considerable variation among species, ecotypes, sampling locations, and individual trees. Because the study included one representative tree for each species or ecotype, these differences should be interpreted as descriptive patterns rather than statistically established effects of species, gender, ecotype, age, or environmental factors. The results showed variation in crown architecture, leaf and stem characteristics, and phytochemical profiles, including total phenolic content, total flavonoid content, and DPPH antioxidant activity. Differences in water availability and other environmental conditions may contribute to the observed variation, but these factors were not directly measured and therefore cannot be considered confirmed determinants. The PCA further identified the morphological traits contributing most to the observed multivariate variation. Overall, this study provides descriptive information on morphological and phytochemical variation among the sampled *Salix* specimens in Ardabil Province and provides a basis for future studies using replicated sampling and direct measurements of environmental variables to evaluate species-, gender-, and environment-related effects.

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Caspian J. Environ. Sci.
DOI:

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Article type: Research