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Screening of Superior Phenotypic and Physicochemical Indices of *Equisetum arvense* L. Using Path Analysis

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ABSTRACT

Horsetail (*Equisetum arvense*) has gained prominence in the herbal products industry, particularly for nail and hair-strengthening formulations, owing to its high silicon (Si) content. Silicon plays a crucial role in the plant's synthesis of key antioxidants, specifically phenolic and flavonoid compounds. Therefore, identifying methods to enhance the Si content in plants is of paramount importance. This study aimed to investigate and attempt to assess how inorganic (nitrogen and silicon) and organic (rice husk) compounds influence the phytochemical and growth properties of *E. arvense*. For this purpose, an RCBD experiment with three replications was conducted at the Rice Research Institute of Iran in 2019. The treatments included organic (rice husk) and inorganic (N and Si) fertilizers at varying proportions. The experiment results showed that the majority of traits were highly correlated with each other. Total flavonoid content exhibited the strongest correlations with total phenolic content (0.709) and rhizome count per plant (0.771), both of which were positive and statistically significant. Four types of models were generated four the stepwise regression analysis phase, in which total phenolic and total flavonoid contents exerted had the most significant influence in determining silicon content. The R^2 values for the models were as follows: Model 1 (22.1%), Model 2 (37.5%), Model 3 (59.6%), and Model 4 (63%). The direct positive effect for total phenolic content had a direct positive effect of 0.470 content was 0.470, while total flavonoid content showed a direct positive effect of 0.866 and a direct negative effect of -0.558. POD enzymatic activity demonstrated direct positive effects (1.524) as well as direct negative effects (-0.822 and -0.691). For stem length, direct positive effects were observed (1.312 and 0.320), along with direct negative effects (-0.558, -0.605, -0.691, and -0.831).

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1. Introduction

Equisetum, which Persian speakers refer to as "Dom-e-Asb," represents the sole surviving genus within Equisetaceae. The family includes 15 species found worldwide, with each species exhibiting alternating gametophyte and sporophyte phases in their development. Among these, Iran's native flora features four Equisetum species: *E. arvense* L., *E. telmateia* Ehrh., *E. palustre* L., and *E. ramosissimum* Desf (Mozaffarian, 2009). *E. arvense*, one of the more widespread species, is characterized by its rhizome-based growth pattern and high silica content. This perennial herb can be found primarily across Iran's northern and northwestern territories (Malekpour Irde-Mousa *et al*., 2021). Throughout history, people have used the above-ground portions of *E. arvense* as a natural remedy for various conditions including bone health issues, tuberculosis, urinary system problems, and hemorrhage control (Luanda *et al*., 2023). The plant contains several bioactive substances, including caffeic acid, tartaric acid, quercetin, p-coumaric acid, and the compound isoquercitrin ($C_{21}H_{20}O_{12}$) (Mimica-

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Dukic *et al*., 2008). Research has documented numerous health benefits of *E. arvense*, spanning from blood sugar regulation and inflammation reduction to anxiety relief and blood health improvement. Studies also indicate its potential to support hair and nail growth, hepatic function, cardiovascular health, and wound recovery (Shiba *et al*., 2021; Badole and Kotwal, 2017; Uslu *et al*., 2019).

Despite its therapeutic potential, *E. arvense* hasn't been fully incorporated into formal agricultural production systems. The plant material used in herbal preparations is primarily sourced from wild populations rather than cultivated sources. The global supply chain for horsetail is dominated by several countries, with Russia being the primary source, followed by Hungary, Poland, and China (Rajpurohit and Jhang, 2015). While the plant has begun entering commercial markets, its presence in manufactured products remains limited. In Iran's herbal medicine sector, new products including tablets and topical preparations made from *E. arvense* aerial parts have recently become available. The plant known as "horsetail" is rich in phenolic compounds, flavonoids, and polyphenols, all of which are recognized as potent antioxidants capable of functioning with reducing agent properties reducing agents (Peyghambarzadeh *et al*., 2023). Analysis shows that *E. arvense*'s sterile stems yield flavonoids in concentrations ranging from 0.3% to 0.9%. The presence of these antioxidant compounds enhances cellular defense against oxidative damage, suggesting promising therapeutic potential (Luanda *et al*., 2023). Among the diverse chemical constituents in plants, phenolic substances represent a major class of bioactive molecules (Mimica-Dukic *et al*., 2008). These compounds exhibit multiple protective mechanisms, functioning as antioxidants, neutralizers of reactive oxygen species, reducing compounds, metal-binding agents, and suppressors of free oxygen (Chew *et al*., 2011). While flavonoids do not provide nutritional value, scientific studies have revealed their beneficial impact on health. Evidence supports their role in preventing cellular mutations and cancer development, making them valuable in the treatment of long-term health conditions (Hazafa *et al*., 2020).

Researchers have employed different cultivation strategies, such as supplementing with both natural and synthetic fertilizers, to optimize the growth patterns and biochemical profiles of therapeutic plants across various environmental conditions, particularly during periods of environmental stress. An important agricultural trend, driven by both financial and ecological factors, is the shift toward natural fertilizer options instead of synthetic ones for improving both yield and quality of medicinal crops (Miransari *et al*., 2021; Miransari *et al*., 2022). Rice has emerged as a significant natural source of defensive compounds, supplying essential elements like silicon alongside secondary metabolites including phenolics and flavonoids (Sen *et al*., 2020). A study by Keshavarz Mirzamohammadi et al. (2021) examined how different fertilization methods - specifically urea (inorganic) and vermicompost (organic) - influenced the biochemical composition of peppermint (*Mentha piperita* L.) when grown under water-limited conditions. Considering this plant's economic value, establishing effective cultivation and production methods for *E. arvense* has become crucial to meet growing demand from pharmaceutical, cosmetic, and wellness sectors. This study aimed to characterize Persian *E. arvense* (EAPs) by analyzing its phenotypic and physicochemical characteristics using path analysis. The findings of this research may offer valuable insights for producers, facilitating the effective utilization of horsetail species for enhanced commercial production and applications.

2. Materials and methods

2.1. Treatments

The study utilized *E. arvense* specimens obtained from Baladeh, situated on the Haraz Road in Iran's Mazandaran Province, at an elevation of 2014 meters. The research employed a randomized complete block experimental design, featuring 22 distinct treatments replicated three times, conducted at the Mazandaran Province Rice Research Institute's greenhouse facilities. The soil treatments included: Control group (water only), Rice husk (organic fertilizer) applied at three rates: 10, 15, and 20 g kg^{-1} soil, Rice husk ash incorporated at 5, 7.5, and 10 g kg^{-1} soil, Nitrogen fertilizer (N) added at 2, 5, and 10 g kg^{-1}, Potassium silicate (K_2SiO_4) applied in solutions of 25, 50, and 75 mg L^{-1}, Combined N/K treatments administered at two intervals: one month and one week before planting. The experimental setup utilized 66 pots to accommodate all treatments, with each pot housing two plants with intact rhizome systems. Following initial planting, the aerial portions underwent a drying period, after which new growth emerged over 28 days.

2.2. Measurement of traits

The research team obtained soil specimens and processed them through a 2 mm sieve prior to analytical testing (Nasiri *et al*., 2022). Multiple soil parameters were evaluated: pH readings were taken with a Model 817 pH meter, while salinity assessment was conducted by measuring electrical conductivity (EC). Soil texture classification was determined through hydrometric analysis. The team quantified organic carbon levels using chromic acid titration following the Walkley-Black protocol (Walkley and Black, 1934). Additionally, the concentrations of iron sulfate ammonium and total nitrogen were measured with a Kjeldahl apparatus (Model VIP45S), and the silicon content in both soil and plant samples was analyzed using a spectrophotometer (Table 1).

Table 1. Physicochemical properties of culture medium

Sand (%)	Silt (%)	Clay (%)	Texture	pH	O.C (%)	N	EC (ds^{-1})	Si (mg. Kg^{-1})
40	30	30	Sandy loam	3.7	2.64	0.43	1.1	25.6

Four months after regeneration, researchers harvested and evaluated the specimens, documenting both stem measurements and rhizome quantities. To prepare for biochemical analysis, the harvested material underwent a sequential preparation process: washing three times with distilled water, drying in an 85°C oven for 48 hours, and mechanical grinding to achieve a fine powder consistency (Handa, 2008). To evaluate bioactive compounds, particularly phenolics and flavonoids, researchers employed a wet extraction protocol. The concentration of phenolic compounds was determined spectrophotometrically, using gallic acid as a reference standard to create a calibration curve, with results normalized to milligrams of gallic acid per 100 grams of dried plant matter (Singleton *et al*., 1999). Similarly, flavonoid quantification utilized quercetin as the reference compound, with results expressed as milligrams of quercetin per 100 grams of dried material (Beketov and Liess, 2005).

2.3. Statistical analysis

The research team performed data analysis by systematically reducing complex datasets into interpretable formats, followed by more detailed statistical examination. Relationships between variables were examined through Pearson's correlation analysis using IBM SPSS software (Ver. 26, IBM Corporation). The team implemented stepwise regression ($p<0.01$) to identify significant predictors of silicon content in *E. arvense*. To understand both direct and indirect influences of model parameters, the researchers applied path analysis to map variable relationships. Statistical processing encompassed several approaches: tests for normal distribution, correlation analysis, stepwise regression modeling, and path analysis, all executed in SPSS Ver. 26. The team utilized AMOS Ver. 24 to evaluate causal relationships between variables.

3. Results and discussion

3.1. Descriptive analysis

The analysis generated comprehensive statistical metrics for each measured trait, including central tendency measures (mean), variability indicators (standard deviation, range), extreme values (minimum, maximum), and the phenotypic coefficient of variance (PCV). Among the descriptive statistics, the PCV for total flavonoid content (13.88%) exhibited the highest value, while the PCV for chlorophyll b index (2.82%) exhibited the lowest value. Table 2 reveals that the traits SOD, POD, and total phenolic content displayed the highest range of variation, as well as the greatest standard deviation. The results indicate significant physicochemical diversity among the traits evaluated in this study (Table 2).

Table 2. Descriptive statistics related to different traits in the physicochemical indices of *E. arvense*

Trait	Mean ± SE	PCV (%)	Minimum	Maximum	Range
Total flavonoids	74.45±0.66	13.88	66.29	83.74	17.45
Total phenol	115.79±1.86	7.70	80.02	135.02	55
Silica content	43.84±1.11	4.86	25.03	54.06	29.03
Number of rhizomes in plant	5.36±0.18	3.56	3	9	6
Stem length	26.01±0.56	5.66	18.75	35.9	17.15
Chlorophyll a	0.72±0.019	4.52	0.39	0.92	0.53
Chlorophyll b	0.37±0.016	2.82	0.15	0.55	0.39
Total chlorophyll	1.09±0.029	4.62	0.58	1.44	0.86
Carotenoid	0.18±0.004	4.98	0.11	0.25	0.14
SOD	386.10±15.4	3.10	190	577	387
POD	166.73±3.46	5.96	100	200	100
Anthocyanin	15.30±0.28	6.74	10	19	9

SOD: Superoxide dismutase; POD: Peroxidase.

3.2. Correlation coefficients results

The correlation coefficient matrix indicated that most traits were highly correlated with each other. Total flavonoid content exhibited the highest correlation with total phenolic content (r = 0.709) and rhizome count per plant (r = 0.771), both of which were positive and statistically significant (Fig. 1). This suggests that an increase in these traits contributes to a rise in flavonoid yield. A negative and significant correlation was observed between the rhizome count per plant and the chlorophyll b (r = -0.056) as well as stem length (r = -0.090) (Fig. 1). This indicates that an increase or decrease in any of these traits results in a corresponding decrease or increase in the others.

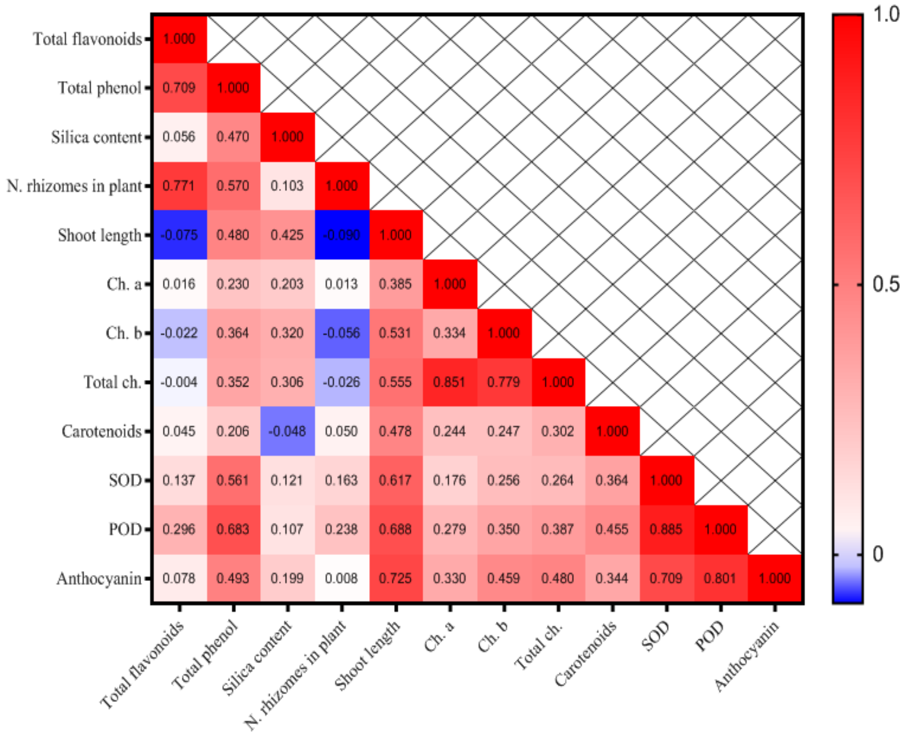

Figure 1. Heat map of mutual relations of variables in correlation coefficient for studied traits

3.3. Path analysis

Causal mediation analysis was conducted to thoroughly examine the relationships between traits. This analysis dissects the relationship between independent and dependent variables into direct and indirect effects, aiding researchers in accurately interpreting results. High correlations between traits can lead to multicollinearity. In conventional Causal mediation analysis, multicollinearity can distort the model, resulting in unrealistic variable selection and inflated error rates. Sequential Causal mediation analysis, used in this study, is one approach to reduce multicollinearity and simplify the relationships among traits. The direct effects and the overall effects of studied traits on dry mass are presented in Table 5. Results revealed that 4 types of models have been generated in the Stepwise regression analysis phase, in which total phenolic and flavonoid contents exerted the most significant influence in determining silicon content (Table 3). The R^2 values were 22.1% for the first model, 37.5% for the second, 59.6% for the third, and 63% for the fourth model.

Table 3. Stepwise regression analysis for total phenol and flavonoid, antioxidant enzyme POD and stem length of *E. arvense* plant

Variables entered to model	Step			
	1	2	3	4
Intercept	11.405	54.940	79.579	57.732
Total phenol	0.279	0.514	0.905	0.780
Total flavonoids	-	-0.951	-1.397	-1.030
POD	-	-	-0.220	-0.265
Stem length	-	-	-	0.635
R^2 (%)	22.1	37.5	59.6	63

The direct positive effect for total phenolic content was 0.470, while total flavonoid content showed a direct positive effect of 0.866 and a direct negative effect of -0.558. POD enzymatic activity demonstrated direct positive effects (1.524) as well as direct negative effects (-0.822 and -0.691). For stem length, direct positive effects were observed (1.312 and 0.320), along with direct negative effects (-0.605 and -0.831) (Table 4).

Table 4. Direct effects of studied traits on silicon content of *E. arvense*

Trait	Total phenolic	Total flavonoids	POD	Stem length	Silicon content
Phenolic content	0.470	0.866	1.524	1.312	0.056
Total flavonoids	-	-0.558	-0.820	-0.605	0.470
POD	-	-	-0.691	-0.831	0.107
Stem length	-	-	-	0.320	0.425

In Indirect causal mediation analysis, total phenolic and flavonoid content were identified as influencing factors on POD enzymatic activity and stem length, with a direct positive effect on Si content. As shown in Fig. 2, the correlation of total phenolic and flavonoid content with Si content is indirect, mediated by other traits. The indirect causal mediation analysis indicates that all four measured traits affect Si content. Among these traits, stem length only affects Si content, while total phenolic and flavonoid content positively affects both POD enzymatic activity and Si levels. These findings highlight the importance of these traits. Therefore, to gain more broad insights in this field, it is essential to focus on enhancing yield. Consequently, farm management practices, in terms of agronomy and nutrition, should focus on enhancing the above-mentioned traits.

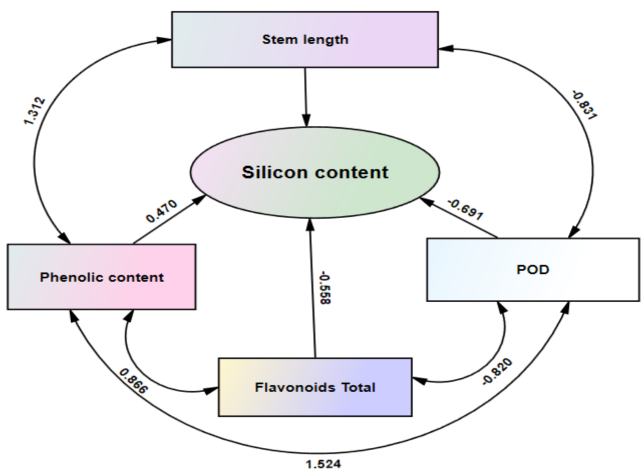

Figure 2. Sequential path model showing interrelationships between silicon content with total phenolic and flavonoid index, POD and stem length for *E. arvense* plant

Correlation and regression analyses play a vital role in understanding relationships between variables in agronomic research (Salehi Sardoei *et al*., 2024c). When studying citrus cultivars, Salehi Sardoei et al. (2023) have found these statistical approaches particularly useful for examining connections between growth parameters and physiochemical characteristics. While correlation analysis helps plant scientists identify stress-related traits indirectly through more easily measurable characteristics (Salehi Sardoei *et al*., 2024a), it has limitations when applied to physiological traits alone, as it may not fully capture the complex interactions between different plant characteristics (Babarabi *et al*., 2024). To overcome these limitations, researchers employ stepwise regression, which helps prioritize the most influential variables among multiple measured traits when describing treatment effects (Salehi Sardoei *et al*., 2024c). This methodological approach effectively filters out less significant variables, focusing attention on traits that demonstrate meaningful changes (Salehi Sardoei *et al*., 2024b). The application of path analysis provides additional depth to the investigation, offering insights into how different traits interact and influence silicon content in plants.

A stepwise regression approach was utilized to determine which traits had the strongest influence on silicon content. This analysis helped eliminate variables with minimal impact before conducting path analysis. The statistical models showed increasing explanatory power, with R^2 values ranging from 22.1% to 63% across four successive models. The final analysis identified four key variables that best-explained silicon content variation: total phenolic content, total flavonoid content, POD enzymatic activity, and stem length. To gain deeper insights beyond the initial correlation and stepwise regression results, these variables were further examined through path analysis. The findings revealed that total phenolic content had a substantial positive direct effect (0.470), while total flavonoid content showed a strong negative direct effect (-0.558). The correlation coefficients (0.056 and 0.470 respectively) indicated these components directly influenced silicon content, with minimal indirect effects through other characteristics. These findings suggest that both phenolic and flavonoid content could serve as effective selection criteria in *E. arvense* breeding programs.

4. Conclusion

knowledge of various physiological phenomena assists researchers in selecting the best utilization and breeding strategies. The present study was an applied investigation aimed at facilitating optimal trait selection for breeders. Overall, the results obtained from Pearson correlation, stepwise regression, and causal mediation analysis were mutually supportive. For instance, The Pearson correlation coefficients revealed that total phenolic content (0.709) and the rhizome count per plant (0.771) had the most significant and positive correlations. Decomposition of coefficients of correlation with other traits into direct and indirect effects through causal mediation analysis also demonstrated that traits such as phenols, total flavonoid content, POD antioxidant enzyme, and stem length have a strong positive direct effect on Si content. In the stepwise regression analysis, these traits also accounted for the highest coefficients of determination in the multivariable regression model. POD exhibited the highest positive direct effect on biochemical traits. Therefore, treatments incorporating higher values for these traits will result in higher Si content.

Conflict of interests

All authors declare no conflict of interest.

Ethics approval and consent to participate

No humans or animals were used in the present research. The authors have adhered to ethical standards, including avoiding plagiarism, data fabrication, and double publication.

Consent for publications

All authors read and approved the final manuscript for publication.

Availability of data and material

All the data are embedded in the manuscript.

Authors' contributions

All authors had an equal role in study design, work, statistical analysis and manuscript writing.

Informed consent

The authors declare not to use any patients in this research.

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