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Quantification and artificial intelligence-based clustering of phenolic compounds in pearl millet genotypes

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Abstract
Pearl millet, also known as *Pennisetum glaucum*, is a staple food crop in many parts of Africa and Asia. It is a hardy, drought-resistant crop that can grow in poor soil conditions and is well-suited to regions with low rainfall. The analysis of phenolic compounds in pearl millet can provide valuable information on the nutritional and health benefits of this crop. Ultra-high-performance liquid chromatography (UPLC) is a common method used to quantify the phenolic compounds in pearl millet. In this study six different phenolics compounds namely quercetin, epicatechin, cinnamic acid, syringic acid, gallic acid and sinapic acid were quantified in thirteen diverse pearl millet genotypes. In addition to traditional analytical methods, Artificial Intelligence (AI) techniques were used to analyse the data from UPLC and identify any correlations or patterns. This can provide insights into the variability of phenolic compounds in pearl millet depending on the environmental and agronomic conditions. The AI-based analysis presented in the study gave vital information on the phenolic properties of the target genotypes. The result of this analysis is useful in breeding and cultivation practices for pearl millet to improve its nutritional profile and health benefits for human consumption.

Key words: Phenolic compounds, AI, pearl millet, genotype clustering, rankingx

Introduction
Millets are annual grasses having small grains, belongs to various genera, mostly grown for food, feed and fodder in developing countries of Asia and Africa. Moreover, millets are able to grow in harsh environments where other crops have limited scope to grow. These have shorter growing season and good productivity under poor, dry soil and higher temperature. Millets have low carbon and water footprint, can be grown with minimal inputs on relatively poor soil (Das & Rakshit 2016) [6]. Considering these facts, United nations General Assembly accepted India's proposal to declare 2023 as "International year of millets" to increase awareness of millets contribution for nutrition and food security and, to improve quality of millets and its sustainable production. Sorghum and pearl millet are two most grown crops in millets (Crawford & Lee 2003; Poole & Kane-Potaka 2020) [5, 15].
Nonetheless, pearl millet is an under-utilized, dryland coarse cereal crop that provides food and nutritional security in arid and semi-arid regions. Being one of the best crop for climate-resilient agriculture, pearl millet is free from major insect attack and diseases, and it can be grown in harsh areas with drought, high salinity, low fertility, low pH and high temperature where maize and wheat are uncultivable (Goswami *et al*. 2020) [9]. It is also adaptable to harsh temperature conditions caused due to current climate change scenario. In India, it is mainly grown in western Rajasthan, Gujarat and Haryana. Livelihood of millions of third world people still depend on pearl millet. Being rich source of iron and zinc, it can be a low-cost solution for reduction of mineral deficiencies in millet consuming areas (Djanaguiraman *et al.* 2018; Krishnan and Meera 2018) [7, 13]. Pearl millet grains have good functional properties because of its relatively higher fiber content, phytochemical and fatty acid composition. It has anticarcinogenic, antihypertensive, anti-inflammatory and antioxidant characteristics and reduces the risk of atherosclerosis and inflammatory bowel diseases (Vinutha *et al*. 2022) [20]. Phenolic compounds are one of the secondary plant metabolites and their type and content in grain depends on several factors such as the type of cereal, variety,

part of the grain, climatic conditions and cultivation practices (Naczk and Shahidi, 2004) [14]. Phenolic compounds are natural plant secondary metabolites with a wide range of properties that have attracted a lot of attention in recent years. These compounds have several potential health benefits, including their antioxidant properties that protect cells from oxidative damage, and their ability to prevent chronic diseases such as cancer. They also play a role in promoting plant growth and tolerance to environmental stressors (Ali *et al*. 2022) [2]. The bran portion of cereal grains contains phenolic compounds, particularly phenolic acids, in free, soluble conjugated, and insoluble bound forms (Hemery *et al*. 2007) [12]. Polyphenols plays many bioactive roles in human body like it acts as free radical scavengers and also acts as inhibitors of low density lipoprotein but it also has some negative impact like it reduce food digestibility and bioavailability by precipitating digestive enzymes, dietary proteins and carbohydrates (Ram *et al*. 2020) [17].

Therefore, in current study quantification of six different phenolic compounds were performed using UPLC in 13 diverse pearl millet genotypes. In addition, artificial intelligence based analysis was carried out in the presented study for the following tasks:

1. Clustering of the genotypes to identify the genotypes for similar traits.
2. Trait-distance based Dendogram generation for pointing out the similar genotypes.
3. Multiple Ranking of genotypes is done for identifying the best-performing genotypes in different scenarios.
4. PCA-based analysis to identify the most effective attribute for genotype selection.

Materials and Methods

Plant materials: Pearl millets grains of 13 different genotypes were collected from Division of Genetics, Indian Agricultural Research Institute (New Delhi, India). Grains were milled sieved using 0.3 mm mesh sieve and obtained flour was stored in airtight container.

Standards of quercetin, epicatechin, cinnamic acid, syringic acid, gallic acid and sinapic acid were purchased from Sigma Aldrich. All other chemicals, solvents and reagents were either HPLC grade or analytical grade.

Sample preparation

Extraction of phenolic compounds were carried out using method of Emmons *et al*., (1999) [8]. 1 gm fine pearl millet flour was defatted using n-hexane (1:5 w/v, 1 hour) at ambient temperature. After that 0.5 gram dry flour was mixed with 70% methanol and kept for overnight shaking at 300 rpm at 37 °C. Next day sonication of samples was carried out at high frequency (No. 6) for a minute and after that centrifuged at 10,000 rpm for 5 minutes, collected supernatant was filtered through 0.22 micron syringe filter.

Quantification of Phenolic compounds

The quantification of phenolic compounds was carried out using Acquity Ultra Performance Liquid Chromatograph (UPLC) with C_{18} column. Mobile phase consisted of two phases, A-acetonitrile: water (20:80), B-acetonitrile: water (80:20) with 0.1% formic acid in each phase. A gradient programme with 0.2 mL/min flow rate, with 0-1 min/ 80% A, 1-6.0 min/ 40% A, 6-12 min/ 20%A, 12-15 min/ 80% A was used. Standard curves of standard compounds were prepared using 5, 10, 50, 100 and 200 ppm and phenolics compounds are quantified using the standard curves.

AI-based analysis

In order to find out the optimal number of clusters that can be formed with the target genotypes, first, the silhouette scores were captured with clusters 2-9. Later, K-means clustering was used with the obtained values of k (Number of clusters) using the phenolic contents of genotypes. For the denodogram generation, the Euclidean distance between the genotypes was taken into consideration to find the distances between genotypes and the graph was formulated. For the PCA analysis, the first three PCAs were obtained and the variation captured by them and the largest components of the PCAs were noted. For the genotype ranking, the TOPSIS method was used with three weightage settings, to obtain different ranks for different scenarios.

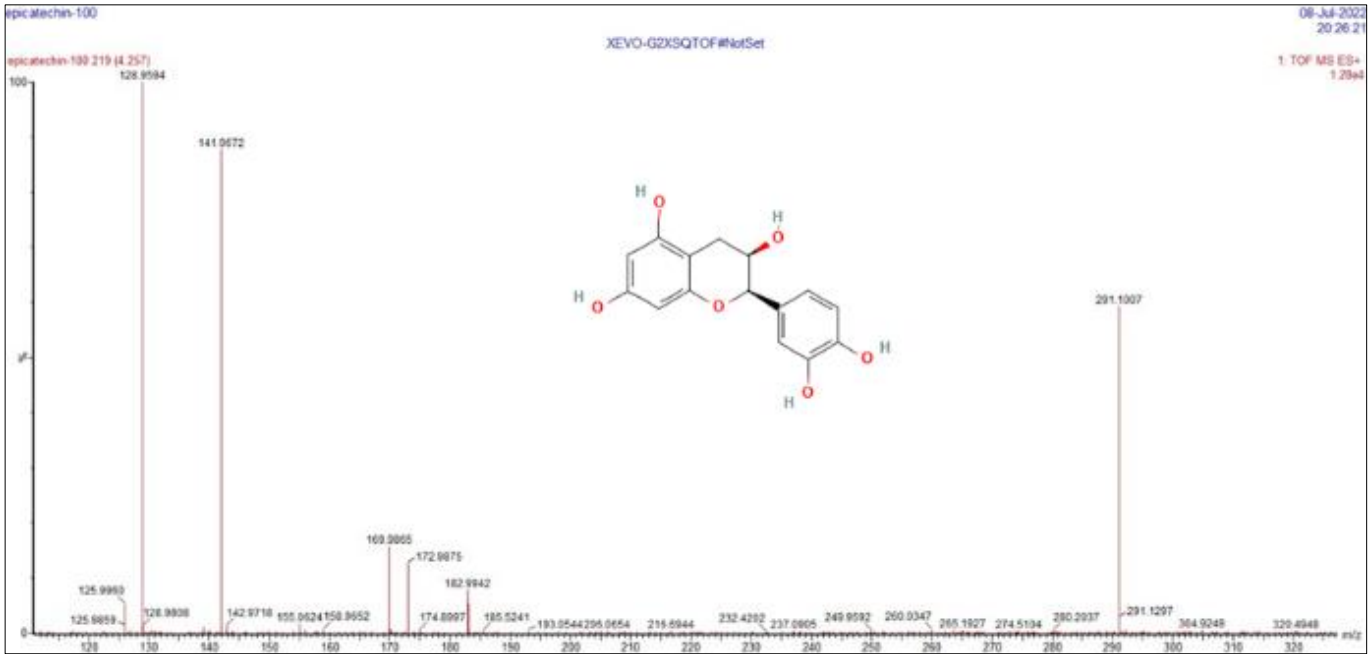
Results and Discussion

Quantification of Phenolic compounds

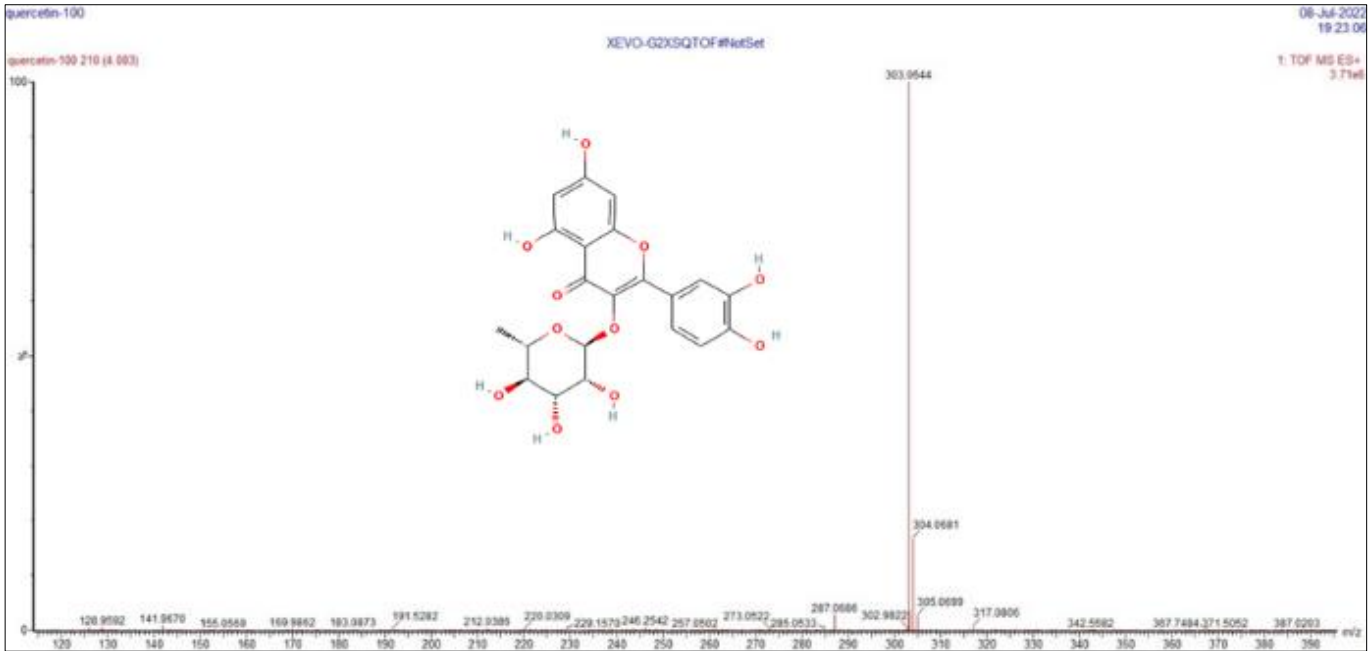
Pearl millet genotypes showed variability in phenolic compounds composition and quantity. Based on the commercial availability, six phenolic compounds, namely cinnamic acid, epicatechin, gallic acid, quercetin, sinapic acid, and syringic acid, were quantified in pearl millet genotypes (Table 1). Mass spectra of these six compounds are shown in Fig. 1. Out of six phenolic compounds, three compounds i.e., quercetin, epicatechin and cinnamic acid were present in all 13 pearl millet genotypes. In GKD 3 quercetin (6.34 ppm), sinapic acid (53.05 ppm) and syringic acid (23.84 ppm) were reported in highest content. Epicatechin was found highest in Sulkhaniya Bajra (424.39 ppm). Cinnamic was reported highest in Damodar Bajra (93.92 ppm). Jafrabadi showed maximum amount of gallic acid (741.05 ppm). All these phenolic acids has potential nutraceutical value exhibit antioxidant, antimicrobial property (Abd El-Raouf *et al*. 2015) [1], anticancer (Wang *et al*. 2019) [21], neuroprotective, anti-inflammatory and antidiabetic properties (Guo *et al*., 2019) [10]. Cinnamic acid prevent radical chain reactions by giving electrons to radicals that combine with each other and create stable products (Ugazio *et al*. 2008) [18]. Additionally, it serves as a fragrant component in detergents, flavourings, cosmetics, and toiletries (Yilmaz *et al*. 2018) [22].

Table 1: Phenolics compounds quantification in different pearl millet genotypes

Genotypes	Compounds (ppm)					
	Quercetin	Epicatechin	Cinnamic acid	Syringic acid	Gallic acid	Sinapic acid
Pusa 1201	4.011	377.350	4.566	2.042	0.000	0.000
MPMH 17	4.970	143.553	5.318	11.791	344.675	0.000
Dhanshakti	4.689	135.264	17.976	0.000	0.000	41.564
Pusa Purple	2.898	62.912	5.780	9.222	120.636	0.000
WGI 100	3.319	125.891	4.740	0.000	52.778	0.000
Jafrabadi	3.278	284.549	5.896	9.947	741.052	15.077
Chanana Bajra 2	5.588	135.438	4.508	0.000	0.000	17.016
GKD 3	6.340	178.879	46.240	23.846	467.466	53.050
Dedha Bajra	3.883	13.097	3.584	1.844	0.000	0.000
Sulkaniya Bajra	3.219	424.393	23.814	2.437	20.465	0.000
Chadhi Bajri	2.036	20.194	19.363	0.856	15.080	2.918
Damodar Bajra	4.068	14.837	93.925	2.569	20.465	0.000
Dhodasar Local	1.563	17.422	2.890	1.581	0.000	11.013



A



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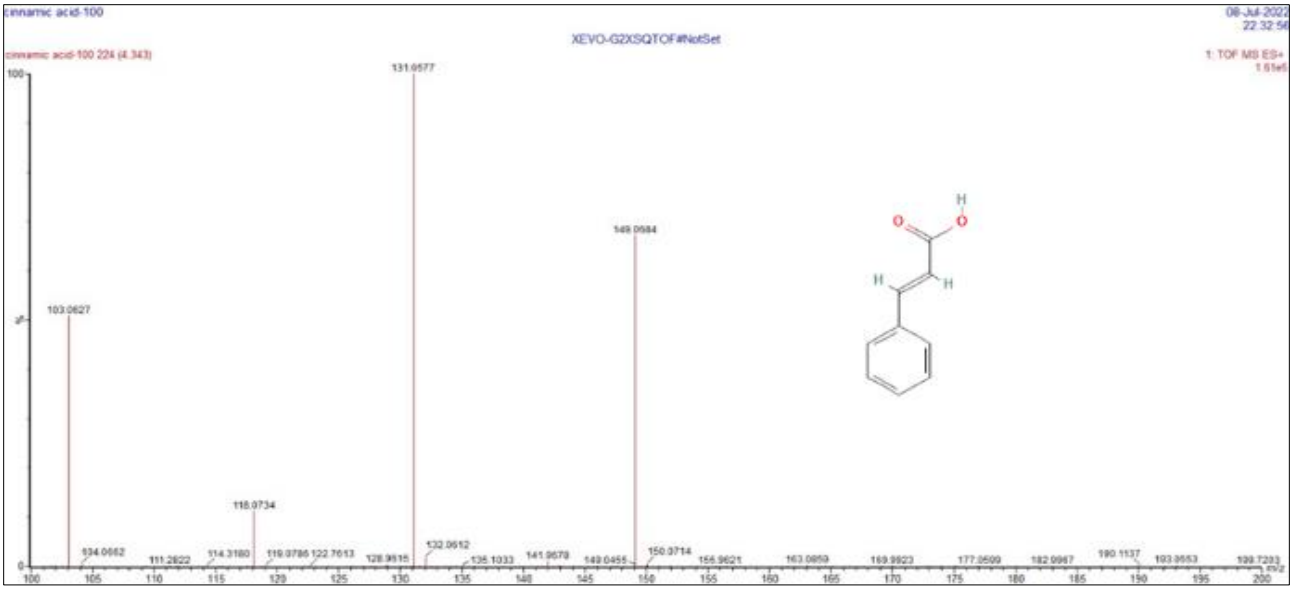
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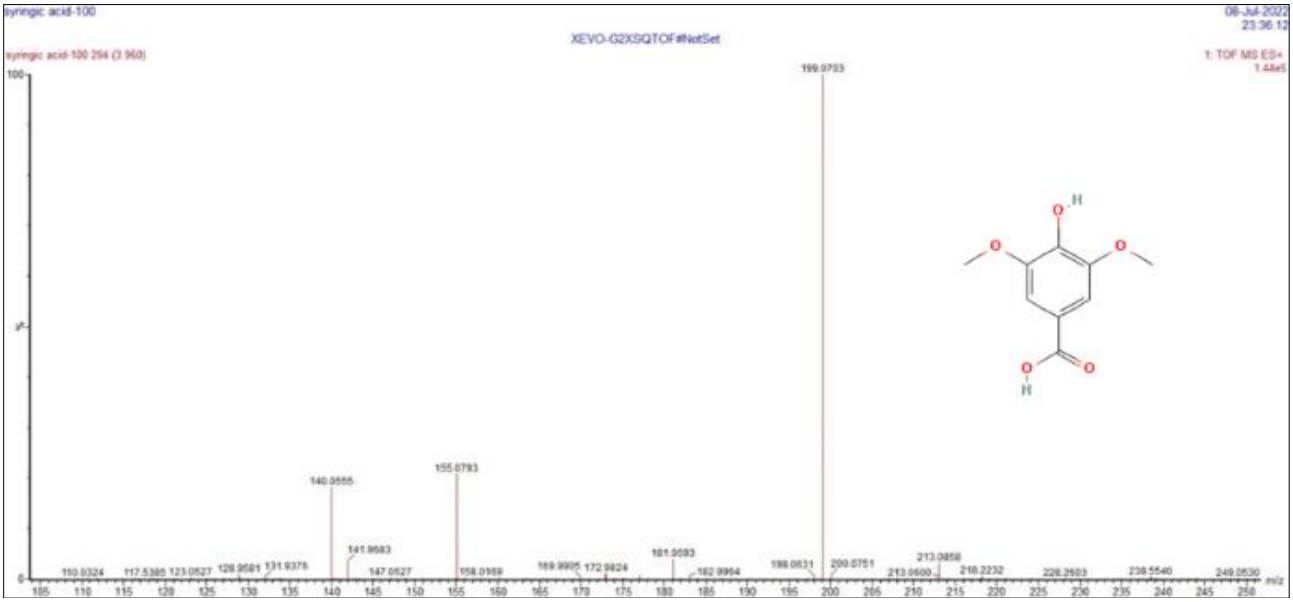
149.0084
103.0627
118.0734

C


syringic acid-100
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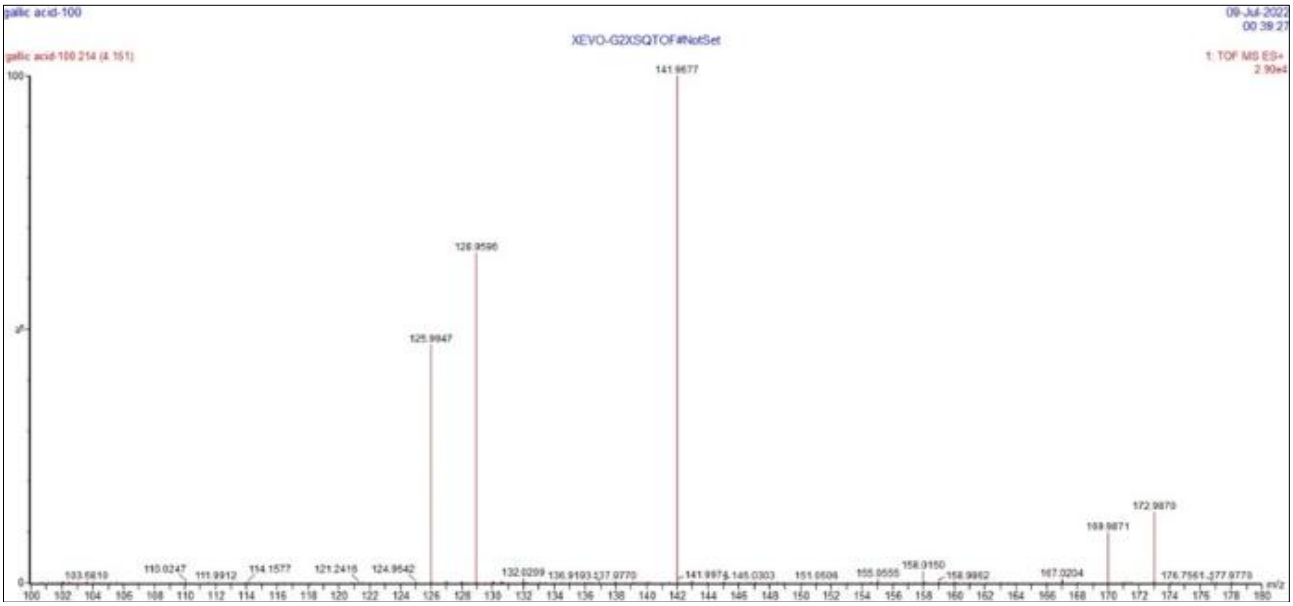
155.0793
140.0555
141.9683

D


gallic acid-100
XEVO-G2XSQTOF#NotSet
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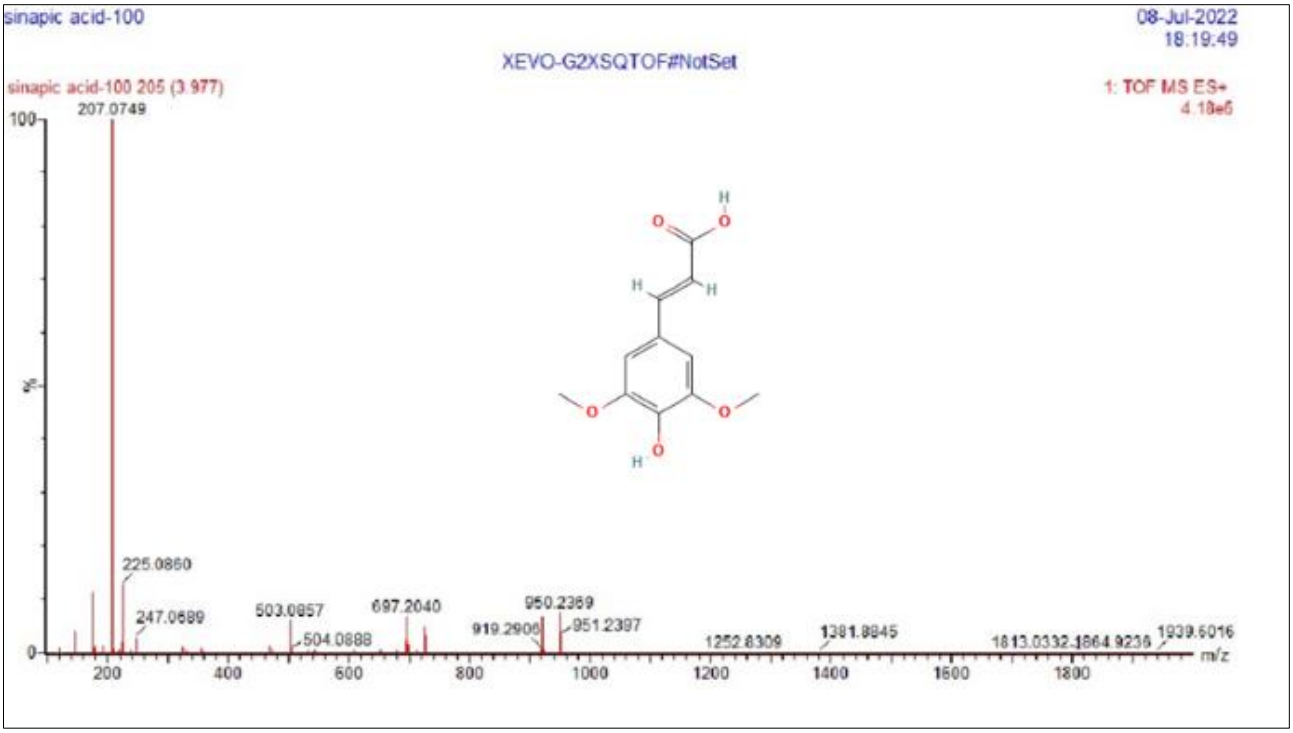
126.9596
125.9947
172.9879
169.9871

E



F

Fig 1: Mass spectra and chemical structure of (a) Quercetin (b) Epicatechin (c) Cinnamic acid (d) Syringic acid (e) Gallic acid (f) Sinapic acid

Clustering: Pearl millet genotypes were classified in different clusters using K-means algorithm based on phenolic acid content. First the number of identified clusters was ranged between 2-9 based on Silhouette score. Cluster count 3 and 4 showed the maximum Silhouette score, so for maximizing the clusters and Silhouette score corresponding to 4-clusters was selected (Fig. 2). The obtained four clusters were named as A, B, C and D for the convenience (Table 2). Cluster A consist of 8 genotypes having on an average same amount of each phenolic compound along with having same amount of sum of all phenolic compounds. Cluster B has two genotypes GKD-3 and MPMH-17 due to similar content of quercetin, epicatechin, syringic acid and gallic acid. Cluster C also has two genotypes PUSA 1201 and Sulkaniya Bajra both has similar content of quercetin, epicatechin and syringic acid while gallic acid was absent in both the genotypes. Cluster D has only Jafrabadi due to significantly highest content of gallic acid.

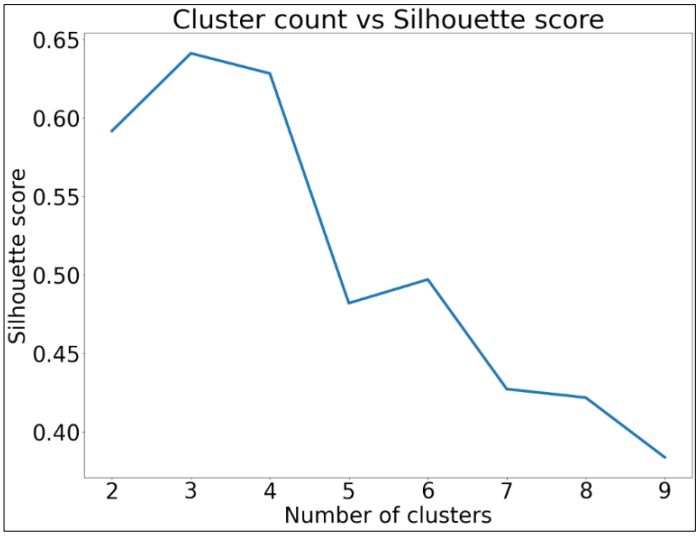


Fig 2: Cluster count vs Silhouette score

Table 2: Clustering of pearl millet genotype based on Cluster count vs Silhouette score

Clusters			
A	**B**	**C**	**D**
Dhanshakti, Pusa Purple, WGI 100, Chanana Bajra 2, Dedha Bajra, Chadhi Bajri, Damodar Bajra, Dhodasar Local	MPMH 17, GKD 3	Pusa 1201, Sulkaniya Bajra	Jafrabadi

Dendrogram

Dendrogram shows the closeness of different genotypes with respect to phenolic content (Fig. 3). It showed that Dedha Bajra and Dhodasar Local are most closely related, it was due to similar content of quercetin, epicatechin, cinnamic acid, syringic acid and due to absence of gallic acid in both the genotypes. Chadhi Bajri showed relatedness with both Dedha Bajra and Dhodasar Local due to similar content quercetin, epicatechin, syringic acid and similar amount of sum of all phenolic compounds. After above genotypes Dhanshakti and Chanana Bajra 2 were closely related due to similar content of quercetin, epicatechin and both showed absence of syringic acid and gallic acid.

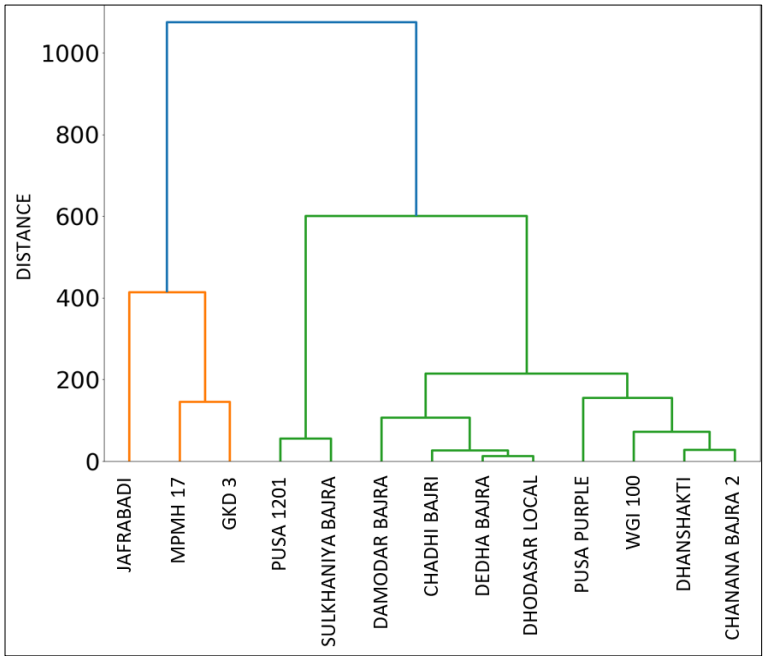


Fig 3: Dendrogram of different pearl millet genotypes based on phenol content.

Feature wise plot

Feature wise plot shows the feature wise variation, here scatteredness of plot shows range of chemical content of each phenolic compounds with respect to various genotypes. Feature wise variation observed maximum in case epicatechin and gallic acid because both compounds had shown wide range in occurrence, epicatechin was observed from 13.097 to 424.393 ppm whereas gallic acid was observed from nil in some genotypes to 741.052 ppm.

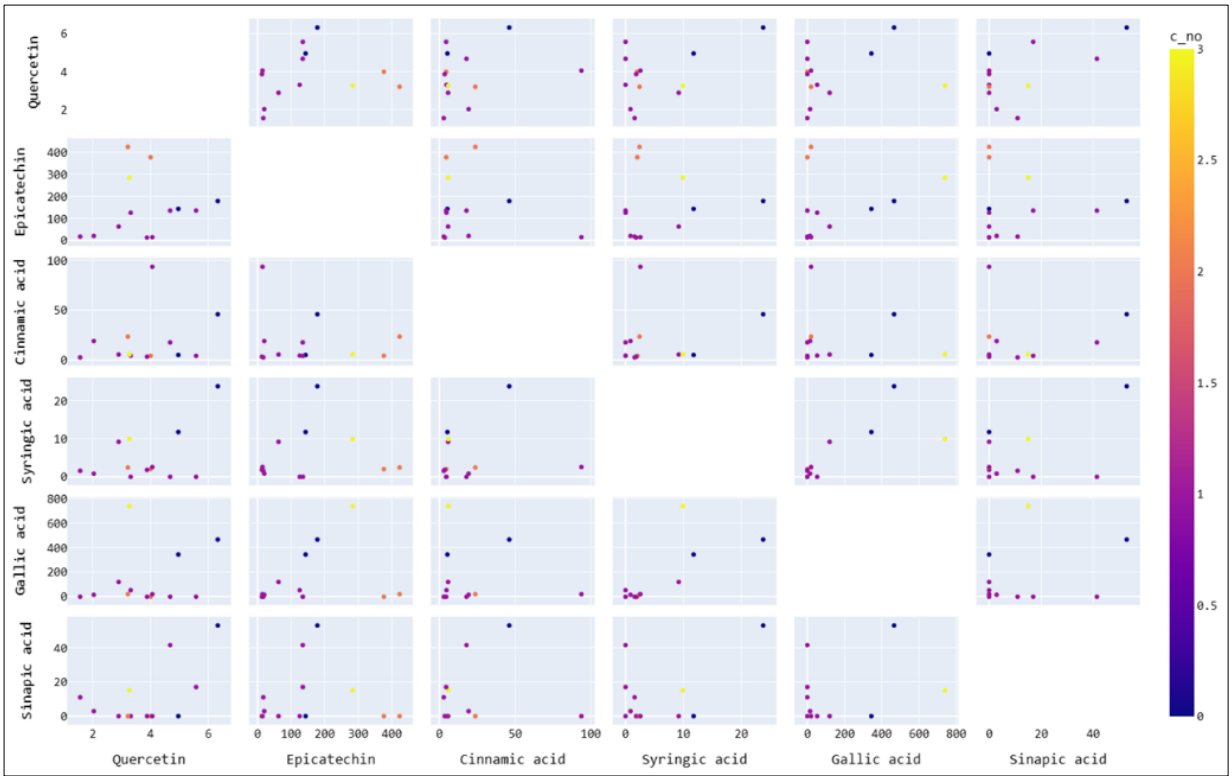


Fig 4: Feature wise plot of each phenolic compounds with respect to various genotype

Principal component analysis

PCA helps in simplifying computational complexity, eliminating noise from data, and enabling the visualization of high-dimensional data. PCA analysis showed that the pearl millet genotypes can be differentiated mainly based on 3 phenolic compounds i.e., syringic acid, cinnamic acid and epicatechin (Table 3). Data can be easily visualised using 3-D PCA plot which covers 99.63% variance (Fig. 5), similarly 2-D PCA plot with 2 phenolic compounds cover 98.7% variance (Fig. 6). Percentage of variance covered with addition of each component of PCA can be seen in Fig 7.

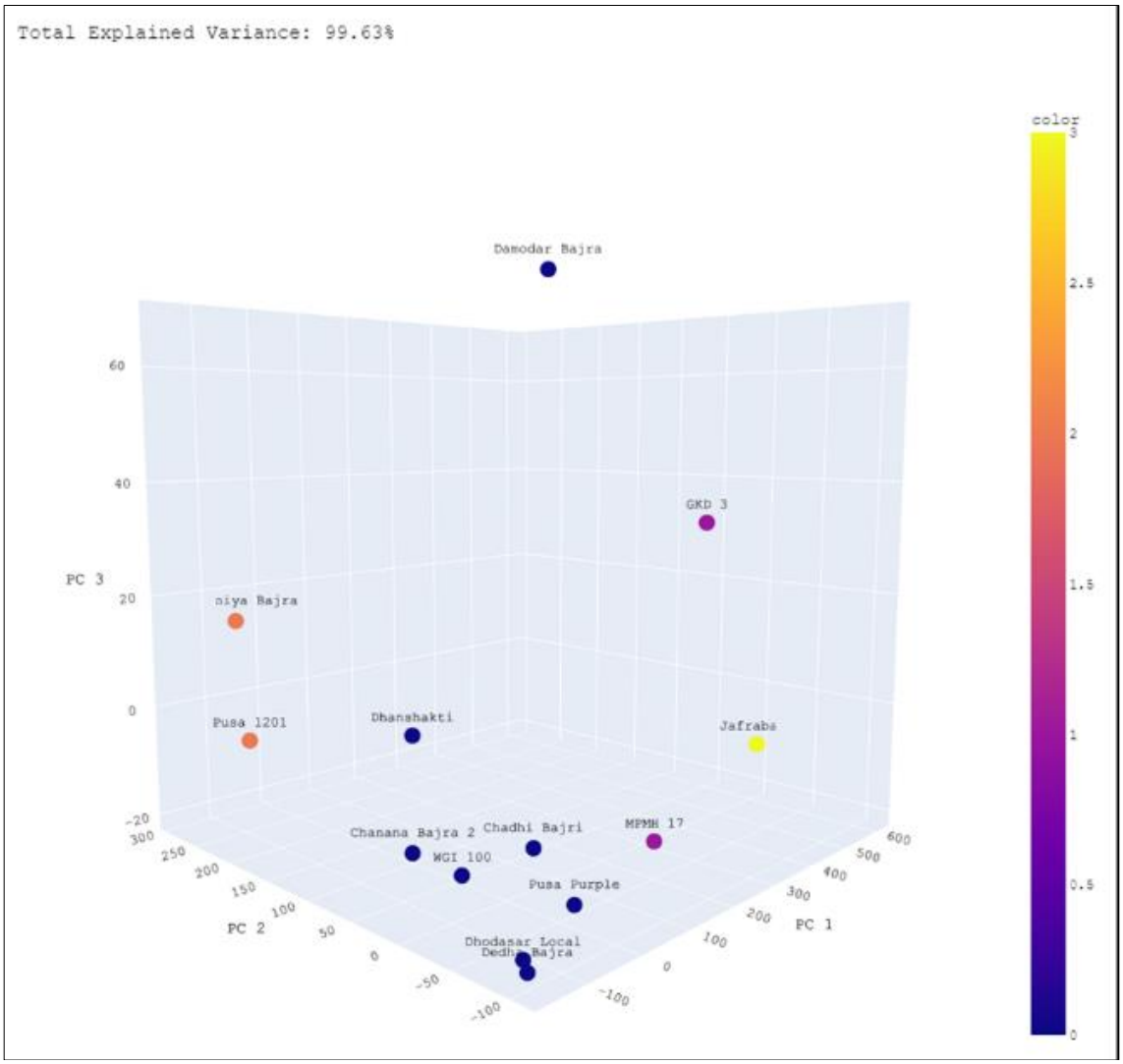


Fig 5: Principal component analysis 3-D plot

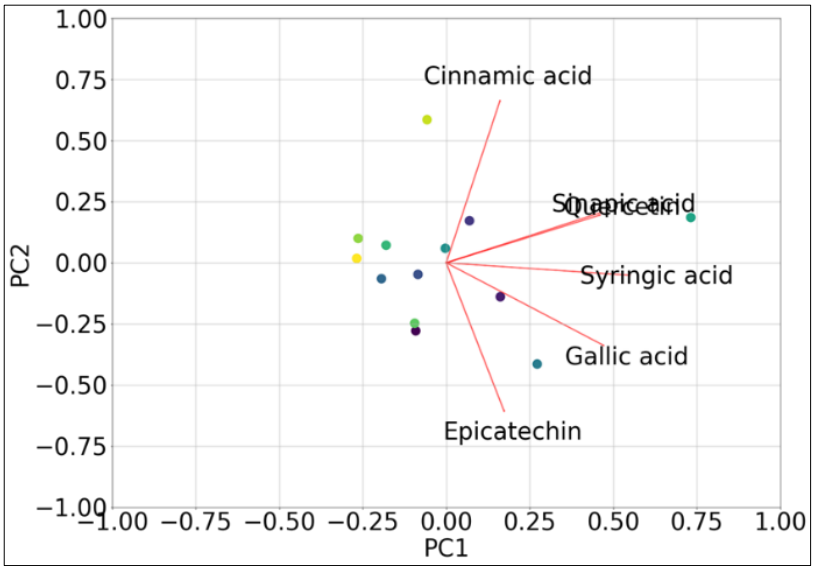


Fig 6: Principal component analysis 2-D plot

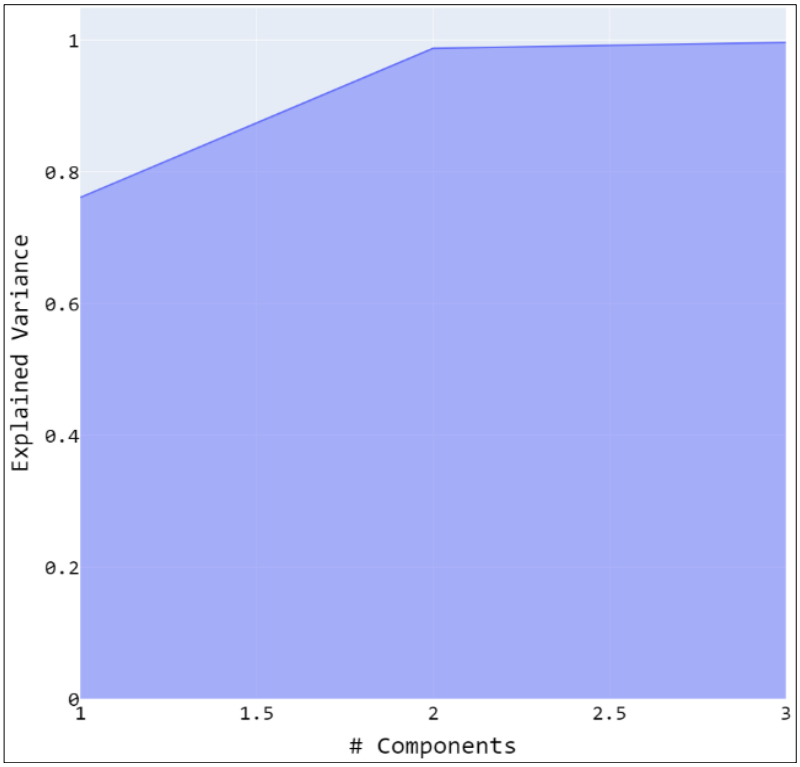


Fig 7: Explained variance with number of components.

Table 3: Most contributing compounds in each PCA.

Principal component	Phenolic compound
PCA 1	Syringic acid
PCA 2	Cinnamic acid
PCA 3	Epicatechin

Ranking of genotypes

Even after having many phenolic compounds in millet grains all cannot contribute significantly to total phenolic content due to its lesser amount. Hence genotypes were ranked using the TOPSIS algorithm, the advantage of using this algorithm is that the users can provide the weightages according to their requirements. In the presented study, three rankings are generated using three different weightage schemes (Table 4). Ranking obtained based on the various weightages are indicated in table 5. Genotypes under study were ranked based on giving more weightage to two major phenolic compounds i.e., gallic acid and epicatechin whose content are relatively higher than other compounds. Jarfrabadi ranked first and GKD 3 ranked second when gallic acid were given highest weightage, similarly Sulkhaniya bajra and PUSA 1201 ranked first and second when epicatechin were given highest weightage. On giving equal weightage to gallic acid & epicatechin and equal to rest of the acids, Jafrabadi ranked first and GKD 3 ranked second.

Table 4: Weightages given to different compounds for ranking.

		Weightage allotment					
Ranking	Description	Quercetin	Epicatechin	Cinnamic acid	Syringic acid	Gallic acid	Sinapic acid
R1	More weightage to Gallic acid	0.10	0.10	0.10	0.10	0.50	0.10
R2	More weightage to Epicatechin	0.10	0.50	0.10	0.10	0.10	0.10
R3	More weightage to both compounds	0.10	0.30	0.10	0.10	0.30	0.10

Table 5: Ranks assigned to genotypes based on TOPSIS score.

Genotypes	Ranking		
	R1	R2	R3
Pusa 1201	8	2	5
MPMH 17	3	5	4
Dhanshakti	6	6	6
Pusa Purple	5	10	9
WGI 100	10	8	10
Jafrabadi	1	3	1
Chanana Bajra 2	9	7	8
GKD 3	2	4	2
Dedha Bajra	12	12	12
Sulkaniya Bajra	7	1	3
Chadhi Bajri	11	11	11
Damodar Bajra	4	9	7
Dhodasar Local	13	13	13

Summary and Conclusion

Pearl millet is a rich source of phenolic compounds, which have been shown to have a wide range of health-promoting properties, including antioxidant, anti-inflammatory, and anti-cancer activities. In this study quantification of six different phenolic compounds were done with help of UPLC. Gallic acid and epicatechin were found in relatively higher content as compared to other four phenolic compounds. Moreover, the study uses Artificial Intelligence techniques for clustering of the genotypes, which helps in grouping the undertaken genotypes according to their phenolic traits. Furthermore, using the PCA, the study identifies the similar genotypes. From the analysis it was inferred that Dedha Bajra and Dhodhasar Local have similar phenolic traits. In addition, the study utilizes TOPSIS technique for multiple-ranking of genotypes for different scenarios. The study results show that the ranking of genotypes in decreasing order with Gallic acid is: Jafrabadi > GKD 3 > MPMH 17 > Damodar Bajra > Pusa Purple > Dhanshakti > Sulkaniya Bajra > Pusa 1201 > Chanana Bajra 2 > WGI 100 > Chadhi Bajri > Dedha Bajra > Dhodasar Local. In future scope, the authors intend to investigate more number of genotypes along with a wider range of phenolic compounds in the study.

Conflicts of interest

The authors have no conflict of interest to declare.

Acknowledgments

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