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Development and quality analysis of Bhagwa pomegranate (*Punica granatum*) wine

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Abstract

This research presented the development and evaluation of a Bhagwa variety pomegranate (*Punica granatum*) wine. The study monitored quality parameters and phytochemical composition throughout the winemaking process. The choice of cultivar and winemaking procedures, especially fermentation duration, significantly influenced the final wine quality and composition. The study found that *Saccharomyces cerevisiae* can be effectively used for pomegranate wine development. Glucose and fructose levels decreased from 4.39 and 5.04 to 0.04 and 0.22 g 100mL^{-1}, respectively, which resulted in successful fermentation. Anthocyanin content decreased from 110 to 70 100 mL^{-1}. Gallic acid peaked at 28.65 before dropping to 18.47 g 100mL^{-1}. The antioxidant capacity slightly decreased from 18 to 14 mM Trolox. The research suggests future studies should aim to preserve and enhance these properties during fermentation.

Key words: Bhagwa variety, wine, winemaking, fermentation duration, *Saccharomyces cerevisiae*, anthocyanin, antioxidant capacity

Introduction

Pomegranate (*Punica granatum* L.), a fruit native to western Asia and Mediterranean Europe, is now grown in warm temperature regions of the Americas and other parts of the world (Holland and Bar-Ya'akov, 2018). This fruit is popular not only for its vibrant colour and distinct flavour, but also for its health-promoting properties. Pomegranates are considered a dietary source of bioactive compounds because of their nutritional benefits. They found to improve redox balance and protect against a wide range of diseases, including cardiovascular disease, diabetes, Alzheimer's, and cancer (Pirzadeh *et al.,* 2021; Lavoro *et al.,* 2021). Recent studies have discovered that the antioxidants in pomegranates can help protect the heart. The anti-inflammatory and anticancer abilities of pomegranate have promising implications for cancer treatment and prevention (de Oliveira *et al.,* 2020). Despite the health benefits, the consumption of fresh pomegranate fruit can be inconvenient due to the difficulty of extracting the edible arils. This has led to the development of various pomegranate products such as juices, fruit vinegar, jellies, and grenadine syrup. These products are commercially booming and well-appreciated throughout the world for their desirable taste, aroma, flavour, and interesting nutritional features (Dhumal *et al.,* 2022). However, there is a significant waste of pomegranate fruits, particularly secondary quality and over-ripe fruits. There is also a need to improve the quality of processed products further. To address these issues, new uses and methods for pomegranate processing are being developed.

One innovative approach is the production of pomegranate wine, which enhances health benefits through fermentation. Pomegranate wines have garnered significant attention and been extensively studied. The fermentation process induces notable changes in volatile compounds and flavor development, resulting in a distinctive, aromatic pomegranate product (Ranjha *et al.*, 2021). The presence of bioactive compounds in pomegranate wine has been explored in a few studies, which suggest that fermentation could generate new functional ingredients. However, the real-time evolution of these compounds and the antioxidant properties of the wine during fermentation and aging require further investigation. Gaining insights into these changes could maximize the health benefits of pomegranate wine (Kandylis and Kokkinomagoulos, 2020). Volatile compounds play a crucial role in the flavor quality of pomegranate wine, but their real-time changes and overall flavor characteristics during winemaking are not well understood. Additional research is needed to comprehend these changes and their impact on the final product, potentially leading to techniques that enhance the wine's flavor profile. Effective real-time monitoring of the fermentation process is essential to prevent issues like stuck or sluggish fermentation. Understanding changes in antioxidant capacity, taste, and aroma can improve pomegranate wine quality (Mashitoa *et al.*, 2021). This knowledge could inform best practices for consistent, high-quality pomegranate wine production.

The study was conducted to monitor total phenol content, total anthocyanin content, and DPPH-free radical scavenging activity throughout the winemaking process. Additionally, quality parameters such as pH, titratable acidity, total soluble solids, alcohol content, and organic acid profiles were consistently tracked.

Materials and methods

Chemicals and reagents: The dry yeast, specifically *Saccharomyces cerevisiae*, was sourced from Bioven Ingredients Ltd. (Noida, India). The 2-octanol of GC grade was procured from (Sigma-Aldrich, India). All the chemicals and reagents utilized were of HPLC grade, and were acquired from Loba Chemical Company, Ltd. (Mumbai, India).

Sample preparation: The Bhagwa variety of pomegranate fruits, characterized by their bright red sweetness, were procured from a local market in Vadodara, Gujarat, India. The arils were manually extracted from the pith of these fruits. The juice was obtained

by subjecting the arils to pressure using a laboratory beating apparatus, followed by filtration through a muslin cloth. The resultant pomegranate juice (TSS content of 15 °Brix, TA of citric acid 0.636 g 100mL^{-1} and a pH value of 3.48) was collected in a volume of approximately 5 L and preserved at -20 ºC for future use. The TSS of the pomegranate juice was adjusted to 20 °Brix within a 2500 mL glass fermentation cylinder. Upon the addition of activated yeast at a rate of 1 g L^{-1}, the fermentation process was initiated, maintaining a consistent temperature of 28±1 °C. Secondary fermentation was conducted by adding pectinase (3 g L^{-1}), sulphurous acid (1 mL L^{-1}), and additional yeast (1 g L^{-1}) on Day 2. The wine was then transferred to a new glass vessel and kept in darkness at room temperature for a period of 10 days to age. Wine samples were collected on days 0, 2, 4, 6, 8, 10, 18, 20, 22, 24, and 26, subsequently sealed and stored at -20 °C until further analysis.

Quality and microscopic parameters of pomegranate wine: Titratable acidity (TA) (expressed as g L^{-1} of citric acid), pH, total soluble solids (TSS) and alcohol were determined as per AOAC, 2019. Microbial analysis was done to determine the growth of *S. cerevisiae.* using a simple gram staining process and microscope.

Organic acids and sugars analysis: The simultaneous determination of organic acids and sugar was carried out as per the methodology outlined by Mena *et al*. (2011). All the samples underwent centrifugation at 10,480 g for 5 min using a SorvalITM ST 8R small benchtop centrifuge (Thermo Scientific). A 1.5 mL aliquot of the supernatant was filtered through a 0.45 µm PVDF membrane, and the phenolic compounds were absorbed onto a C18 SPE cartridge (Cole-Parmer, India). The samples, in 10 µL aliquots, were then injected onto a Supelcogel C610H column (30 cm x 7.8 mm), heated to 30 °C, and protected with a Supelcogel C610H guard column (5 cm x 4.6 mm). An HPLC equipped with a UV–vis detector set at 210 nm and coupled with a refractive index detector was employed. Chromatograms were recorded and the mobile phase was a mixture of water and phosphoric acid (99.9:0.1, v v^{-1}) with a flow rate of 0.5 mL min^{-1}. Various organic acids and sugars were characterized and quantified by comparing the chromatographic profiles with analytical standards.

HPLC analysis, identification and quantification of phenolic: The method as described by Mena *et al*. (2012) was followed for the HPLC analysis. All samples underwent centrifugation for 5 min at 10,480 g in ambient conditions. The supernatant was then filtered through a 0.45 µm nylon membrane prior to HPLC analysis. The HPLC system was equipped with a diode array UV–vis detector, an autosampler, a pump, and an interface. Chromatograms were subsequently recorded and processed. A 20 µL sample was subjected to analysis on a C18 column (25 cm x 0.46 cm, 5 µm particle size) with a security guard C18-ODS (4.0 x 3.0 mm) cartridge system. The mobile phase consisted of water/formic acid (95:5, v v^{-1}) (solvent A) and HPLC-grade methanol (solvent B). The elution was carried out at a flow rate of 1 mL min^{-1}. The linear gradient commenced with 1 % B, maintaining isocratic conditions for 5 min, reaching 20% B at 20 min, 40% B at 30 min, 95% B at 35 min, and returning to 1% B after 41 min. UV chromatograms were recorded at wavelengths of 280, 360, and 520 nm. The various phenolic compounds were characterized by comparing the chromatographic profiles with analytical standards, as per previous reports (Pérez-Vicente *et al.*, 2004) and quantified based on the absorbance of their corresponding peaks. Anthocyanins were quantified as cyanidin 3-glucoside (detected at 520 nm); both punicalagin isomers and ellagic acid (free and glucoside) were quantified as ellagic acid (at 360 nm); and gallic acid was quantified as gallic acid (at 280 nm).

Determination of total phenols by Folin–Ciocalteau's reagent: The Folin–Ciocalteau method, modified for microscale use (González-Molina *et al.,* 2008), was employed to measure the Total Phenols Content (TPC). The procedure involved adding 790 µL of Milli-Q water, 10 µL of a methanol-diluted sample, and 50 µL of Folin–Ciocalteau's reagent to a 1.5 mL Eppendorf microtube, followed by vortexing. After a precise 1 min interval, 150 µL of 200 g L^{-1} sodium carbonate was added, and the solution was vortexed again. The mixture was then left to stand at room temperature in the dark for 120 min. The absorbance was measured at 750 nm, and the results were quantified using gallic acid as a reference standard. The results were reported as milligrams of gallic acid equivalents (GAE) per 100 mL.

DPPH antioxidant capacity assays: All samples underwent centrifugation at 10,480 g for 5 min at room temperature. The DPPH method in aqueous media, as described by Mena *et al*. (2011), was used to determine the free radical-scavenging activity. The antioxidant activity was assessed by observing the change in absorbance at 515 nm after a 50 min reaction period with DPPH. The assays were conducted using 96 well microplates and an Infinite M200 microplate reader. Each reaction was initiated by adding 2 µL of the appropriately diluted sample to the well containing the stock solution (250 µL), resulting in a final assay volume of 252 µL. The results were reported in millimolar Trolox equivalents.

Colour measurement: The colour measurement was carried out as described by Pérez-Vicente *et al*. (2004), with a few modifications. In brief, all samples were centrifuged at 10,480g for 5 min at room temperature. The solutions were then measured in 2 mm path length glass cells (CT-A22) at 520 nm using a tristimulus colour spectrophotometer. This spectrophotometer was equipped with a transmittance adaptor, and measurements were taken under illuminant D65 and a 10° observer, following the CIELAB 76 convention. The data, including CIEL*, CIEa*, CIEb*, chroma, and hue angle, were recorded and analysed using a PC-based colorimetric data system.

Statistical analysis: Analysis of variance (ANOVA) and the multiple range test were carried out using the Design Expert 13 software.

Results and discussion

Quality parameters of Bhagwa pomegranate juice and wine: The pomegranate juice had a pH of 3.48, a total acidity of 6.36 g L^{-1} in terms of citric acid, and total soluble solids measured at 15°Brix. The final wine had a slightly higher pH of 3.53. The total acidity was nearly the same as the juice, at 6.42 g L^{-1} of citric acid, while the volatile acidity was 0.28 g L^{-1}. The final alcohol content was determined to be 7.11 % by volume. Gram staining of *S. cerevisiae* revealed a healthy amount of multiplication of yeast cells contributed to ideal fermentation process from Day 0 to 8 with a healthy amount of growth of the yeast as shown in Fig. 1.

Sugar consumption kinetics: The primary sugar components in pomegranate juices are glucose and fructose, with the concentration of fructose being higher than that of glucose (Fig. 2). The levels of these carbohydrates significantly decreased

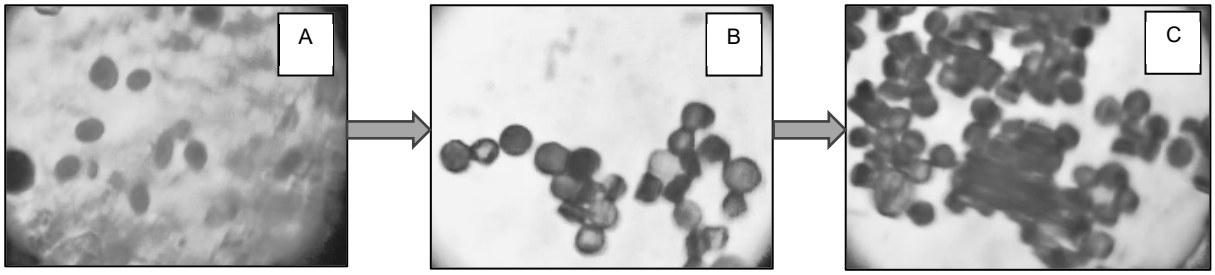

Fig. 1. Microscopic view of *S. cerevisiae* growth during the fermentation process at different stages (A) Day 0, (B) Day 2 and (C) Day 8

during the fermentation process, stabilizing around the 8th day (Fig. 2). The degradation patterns for both sugars were comparable. Beyond the 8th day, the sugar levels remained relatively stable throughout the remaining wine production procedures. However, while the glucose content nearly vanished (0.04 g 100mL^{-1}) from initially 4.39 g 100mL^{-1}, traces of fructose persisted at the conclusion of the winemaking process (0.22 g 100mL^{-1}) from initial 5.04 g 100mL^{-1}, similar to other fruit wines (Joshi *et al.,* 2021; Shehadeh *et al.,* 2020; Kelebek *et al.*, 2009).

Organic acids profile: The profile and concentration of organic acids in pomegranate juice were consistent with a previous study (Samson and Singh, 2017; Mena, *et al.*, 2012). Citric acid maintained similar levels in both the juice and wine,

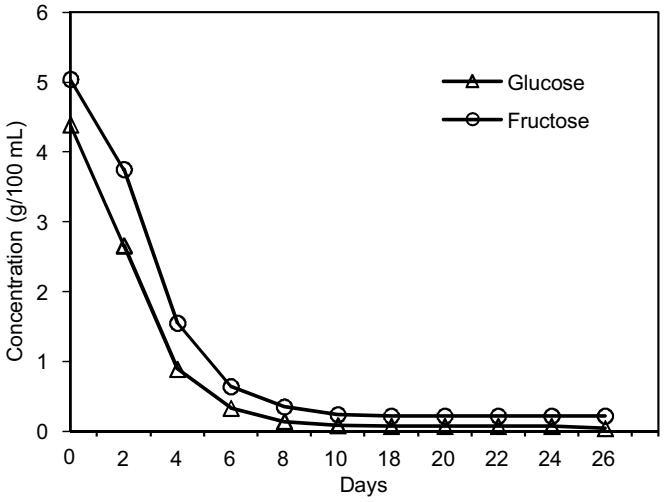

Fig. 2. Evolution of sugars in wines made from pomegranate

remaining relatively constant throughout the various stages of wine production despite some fluctuations (Fig. 3A). As a result, the citric acid content in pomegranate wine was close to that in the juice (1.03 g 100mL^{-1}). Malic acid showed significant changes during the winemaking process. The initial amounts in all varietal juices were around 0.34 g 100mL^{-1}. Still, malic acid rapidly decreased to 0.12 mg 100mL^{-1} during fermentation (Fig. 3A), stabilizing after the clarification and racking stages (Fig. 3A). Regarding minor organic acids in pomegranate, tartaric acid showed some variations (Fig. 3B). In contrast, acetic acid significantly increased ($p < 0.01$) in pomegranate wine (Fig. 3B). Interestingly, pomegranate juices initially lacked acetic acid, which only appeared during fermentation.

A significant increase in acetic acid, up to 0.004 g 100mL^{-1}, was observed in the fermented pomegranate juice. The peak formation of acetic acid occurred between the 6th and 18th days of winemaking. Following this, a decrease in its content was noted (Fig. 3B, Day 18 - 22), with the levels of acetic acid stabilizing after the wine underwent racking and clarification

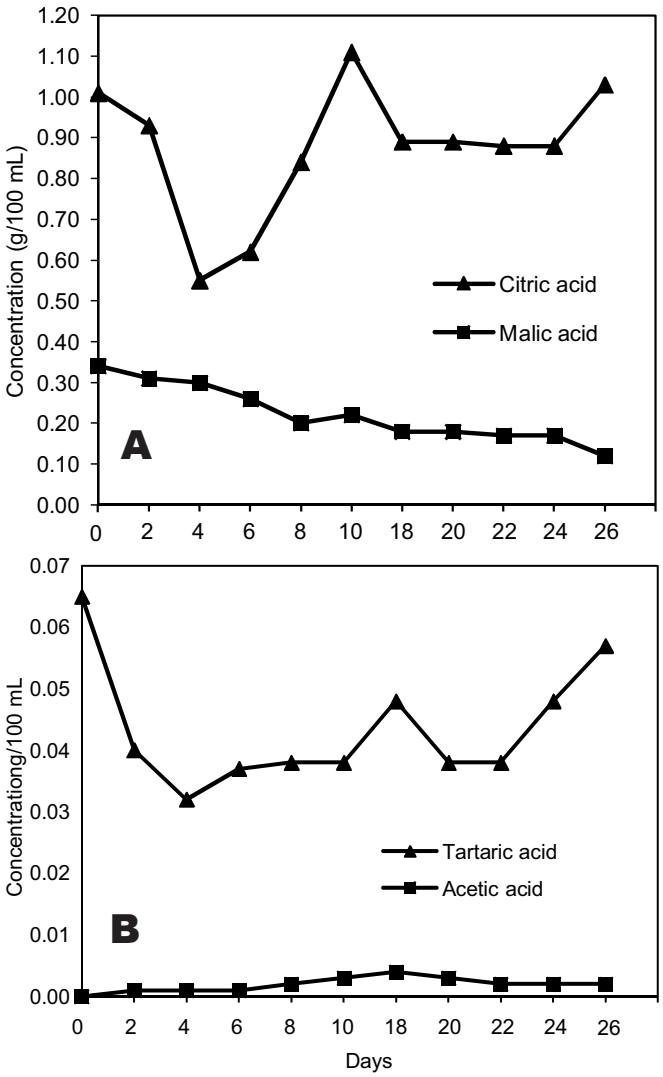

Fig. 3. Evolution of organic acids in wines during the winemaking

(Fig. 3B, Day 22 - 26). Overall, while the levels of citric and tartaric acids remained relatively constant, malic acid decreased due to compositional changes during the production of fruit wine (Vion *et al.,* 2023; Morata *et al.*, 2020; Kim *et al.*, 2008; Main *et al.*, 2007). As a result of the degradation of malic acid, its levels, initially highest in the fruit, fell below those of citric acid in the wine, where citric acid was consistently the predominant organic acid. Furthermore, the type of cultivar used in winemaking played a significant role in the formation of acetic acid, as has been recently reported for other fruit wines (Vavříník *et al.,* 2022; Zhu *et al.,* 2022).

Anthocyanins of pomegranate wines: Anthocyanins, the bioactive phenolics that give pomegranate juices their red colour, were found to have a concentration of 110 mg 100mL^{-1} in the juice, aligning with previous findings by Mena *et al*. (2012).

However, these compounds experienced a significant reduction of 46 % ($p < 0.01$) during the winemaking process (Fig. 4). The majority of this loss occurred during the initial fermentation stage (Fig. 4, Day 0 - 6), with levels remaining relatively stable for the remainder of the wine production (Fig. 4, Day 6 - 26). In addition to the impact of the processing, the type of grape variety used also influenced the degradation of anthocyanins, as observed in other grape and pomegranate wines (Xie *et al.*, 2021; Bar-Ya'akov *et al.,* 2019; Wojdyło *et al.*, 2019; Szalóki-Dorkó *et al.*, 2015). Regarding structural stability, diglucosides proved to be more stable than monoglucosides (Fig. 4, Day 6 - 26). The most significant depletion among individual anthocyanins was seen in cyanidin 3-glucoside, which decreased by 81 %, while delphinidin-3,5-diglucoside and cyanidin-3,5-diglucoside were reduced by only 29 % and 36 %, respectively. Pelargonidin glycosides saw a drastic reduction on days 10 and 18, maintaining minimal levels throughout the rest of the winemaking process. This trend is consistent with other fruit wines (Li *et al.,* 2021; Castillo-Munoz *et al.,* 2009; Garzon and Wrolstad, 2002). The changes in anthocyanin levels could be attributed to several processes during vinification *i.e.,* direct oxidation of anthocyanin with O_2 and polymerization reactions involving the condensation of anthocyanins with acetaldehyde, the presence of anthocyanin-degrading enzymes such as microbial β-glucosidases and (3), produced by yeast metabolic activity (Mena *et al.,* 2012).

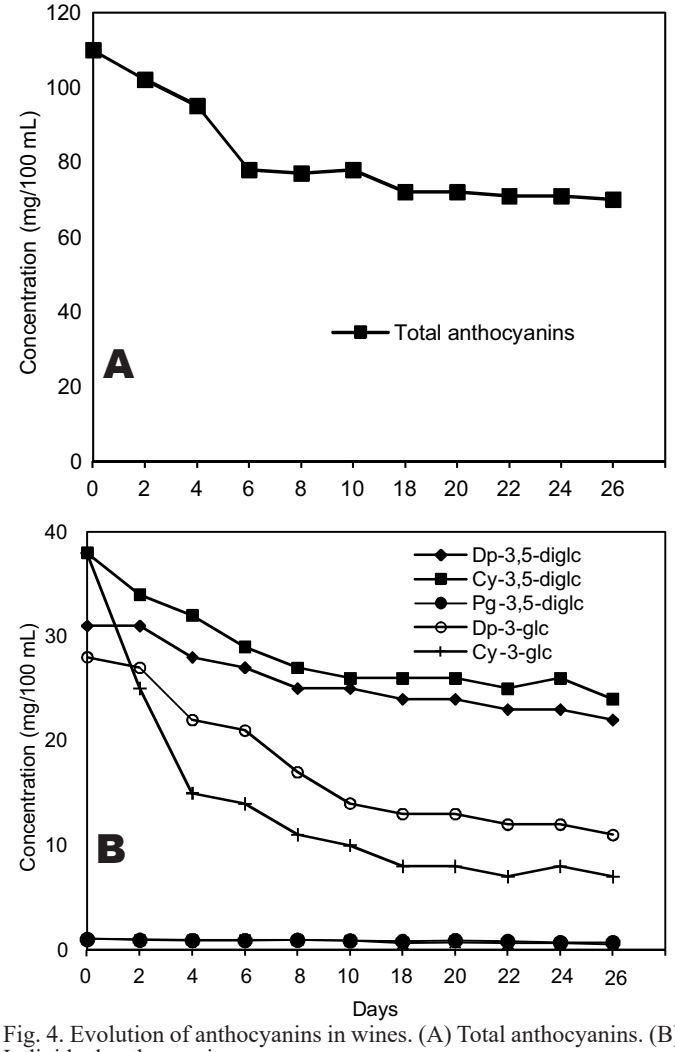

Fig. 4. Evolution of anthocyanins in wines. (A) Total anthocyanins. (B) Individual anthocyanins

Major phenolics compounds of pomegranate wine: Phenolic compounds significantly influence the sensory characteristics of beverages, impacting colour, astringency, and aroma (Sam *et al.*, 2021). The total phenolic content of pomegranate juice was measured at 334 mgGAE 100mL^{-1} (Fig. 5). Following the winemaking process; there was a 35 % reduction in phenolic content, which is relatively minor compared to other pomegranate wines with lower phenolic content, where the decrease ranged between 14 % and 55 % post-fermentation (Duan *et al.*, 2021). It's also worth noting that the production of red wine typically results in a 25% reduction in phenolic content and up to 50 % for orange wine (Filipe-Ribeiro *et al.*, 2018; Czyzowska and Pogorzelski, 2002; Kelebek *et al.*, 2009).

The decrease in phenolic content in pomegranate wines could be attributed to significant changes during fermentation, where anthocyanins and ellagitannins saw a drastic reduction. A strong correlation was found between total phenolic content and anthocyanins ($r = 0.94$, $p < 0.01$). Various factors could be responsible for these changes in the phenolic profile of wines, including condensation and polymerization reactions, enzymatic activity, hydrolysis, oxidative processes, and adsorption onto yeast cell walls (Gutiérrez-Escobar *et al.,* 2021; Czyzowska and Pogorzelski, 2002). The higher phenolic content of the pomegranate wine compared to other berry and fruit wines, where concentrations typically range from 9 to 300 mgGAE 100mL^{-1} (Zhang *et al.*, 2021; Kalaycıoğlu and Erim, 2017).

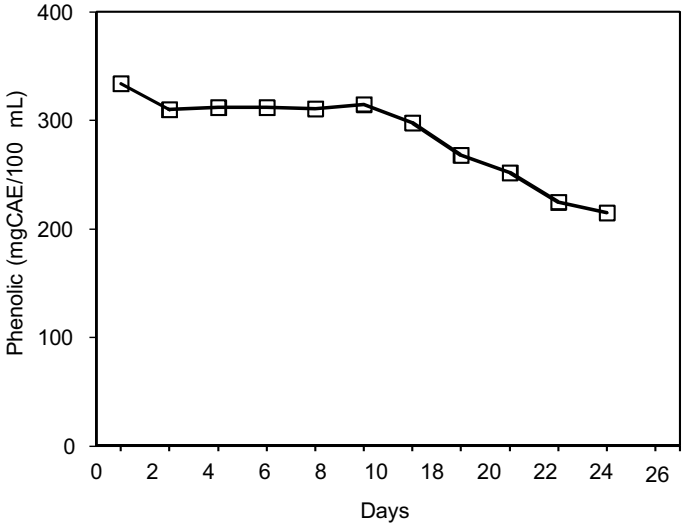

Fig. 5. Evolution of total phenolics in wines made from pomegranate Bhagwa variety during the winemaking

Punicalagins, which are the primary hydrolysable tannins found in pomegranate juices, have a concentration of 1.13 mg /100mL in the Bhagwa variety of pomegranate (Fig. 6A). When it comes to the transformation of punicalagins during the process of wine production, it was found that these compounds undergo significant degradation in pomegranate wine following the introduction of yeast, dwindling to barely detectable levels by the third day (Fig. 6A). The swift and/or early breakdown of these ellagitannins could potentially be attributed to a high fermentation rate or the particular effectiveness of microbial enzymes on this type of compound, as depicted in Fig. 1B.

In this study, we tracked the levels of ellagic acid throughout the wine production process. The concentration of ellagic acid

in the juice was found to be 2.73 mg 100mL^{-1} (Fig. 6A). The fermentation process caused a swift decrease in the content of ellagic acid (Fig. 6A, Day 0 - 4), after which the levels of this compound remained low until the completion of the wine production process (Fig. 6A, Day 8 - 26). The reduction in ellagic acid in pomegranate wine could be due to oxidation and/or the formation of insoluble sediments (Talcott and Lee, 2002). Interestingly, derivatives of ellagic acid might remain unaffected by the fermentation process, as observed in other berry wines (Pérez-Gregorio *et al.*, 2011).

Levels of gallic acid were also measured to check its potential as an indicator of the degradation of hydrolysable tannins. The initial concentration was 9.74 mg 100mL^{-1} for Bhagwa pomegranate juice, and this increased consistently throughout the winemaking

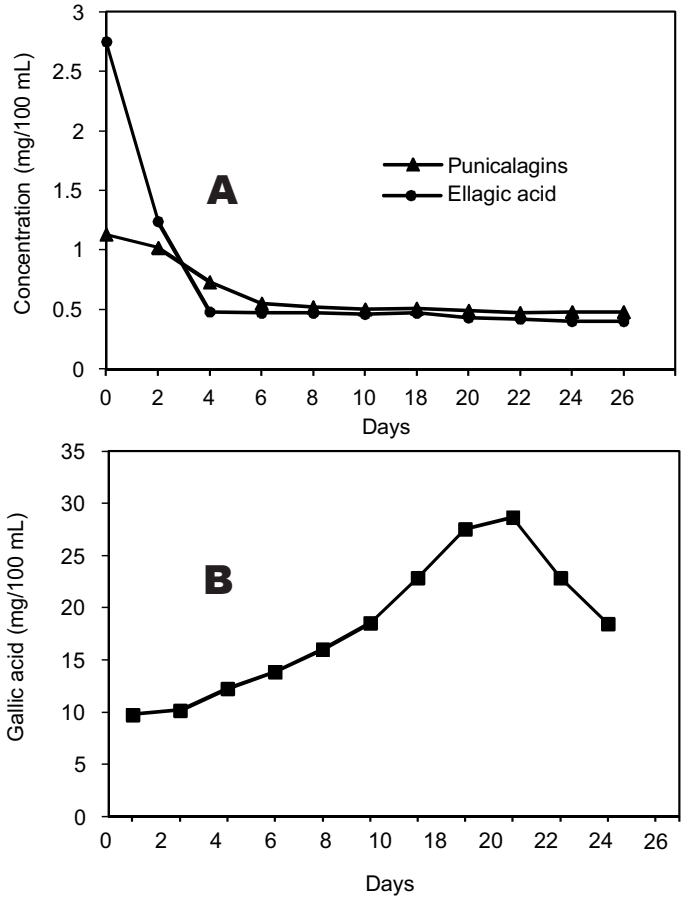

Fig. 6. Evolution of hydrolysable tannins derivatives in wine made from Bhagwa pomegranate variety during the winemaking. (A) Punicalagins, ellagic acid. (B) Gallic acid

process (Fig. 6B). The maximum concentration of gallic acid recorded was 28.65 mg 100mL^{-1}. Interestingly, unlike other phenolics in pomegranate that stabilize after fermentation (such as anthocyanins, punicalagins, and ellagic acid), the levels of gallic acid continued to rise even after the fermentation phase had ended. This post-fermentation increase in gallic acid suggests that the hydrolysis of ellagitannins, which releases this molecule (Sójka *et al.,* 2019; Garcia-Villalba *et al.,* 2015; Czyzowska and Pogorzelski, 2004), may not be solely due to yeast activity and could be influenced by other oxidative mechanisms during wine production. Furthermore, given its high concentrations, Gallic acid could be considered one of the most significant antioxidants in pomegranate wines.

Antioxidant activity of pomegranate wines: Fermented pomegranate juice has demonstrated a robust antioxidant activity, surpassing that of red wine and comparable to green tea (Rios-Corripio and Guerrero-Beltrán, 2019; Pontonio *et al.,* 2019). However, a comprehensive assessment of antioxidant capacity should be performed using DPPH *In vitro* assays. This method was used to measure the antioxidant capacity of varietal juices and wines (Fig. 7). The Bhagwa variety, in both juice and wine forms, exhibited a higher antioxidant potential. The significant influence of the cultivar on the antioxidant activity of pomegranate wines and other fermented juices has been noted by several authors and is attributed to their phytochemical composition. Moreover, the winemaking process can significantly alter the antioxidant activity of fruit wines (Cendrowski *et al.,* 2021; Tarko *et al.,* 2020; Jiang and Sun, 2018; Wang *et al.,* 2015). Reductions in antioxidant tests were observed throughout the wine processing, particularly marked in the first three days (Fig. 7), but less significant once the fermentation period concluded (Fig. 7, Day 8 - 26). The antioxidant capacity was found to decrease by 22 % for DPPH (Figs. 7). A strong correlation was also observed between total phenolic contents and DPPH data ($r = 0.85$; $p < 0.01$), and anthocyanins were positively correlated ($p < 0.01$). This suggests that anthocyanins contribute to the decrease in the antioxidant capacity of pomegranate wines during fermentation. Despite these reductions, the pomegranate wine maintained high antioxidant values, indicating the promising potential of these new fruit wines.

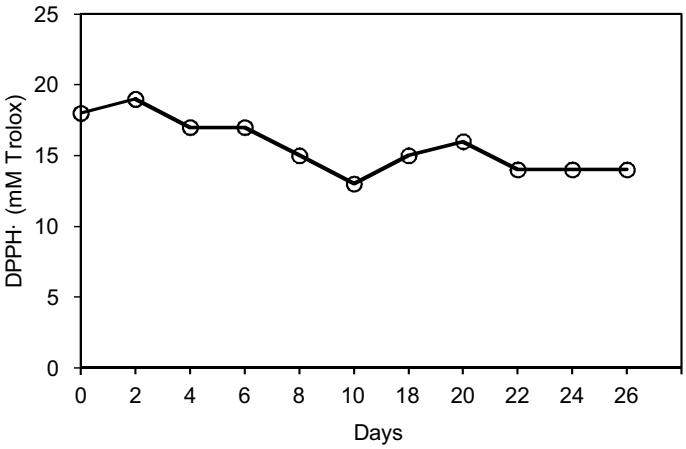

Fig. 7. Antioxidant capacity by DPPH· in wine made from Bhagwa pomegranate variety during the winemaking

Changes in colour: Colour plays a crucial role in the quality of beverages, influencing purchasing decisions. The desirable and characteristic colour of certain pomegranate juices is particularly important. During the fermentation process, the lightness of pomegranate wine, represented by CIEL* values, increased and remained stable throughout the remaining winemaking stages (Table 1). However, a significant effect was noticed on both CIEa* and CIEb* colour parameters (Table 1), with a reduction in CIEa* and CIEb* values of pomegranate wine. Chroma values generally decreased (Table 1), regardless of the cultivar tested. The hue values of pomegranate wine decreased, indicating that the cultivar more influences changes in colour intensity of pomegranate wine.

It was observed that all colour variations reported here occurred exclusively during the fermentation stage, with colour remaining almost unchanged afterward. These variations could be linked

Table 1. Changes in colour parameters of pomegranate wine.

Days	L*	a*	b*	Chroma	Hue angle
0	38.14	59.27	55.37	86.5	38.94
8	46.33	62.58	21.55	62.58	18.12
10	43.45	65.22	21.63	64.66	16.25
26	45.5	64.86	10.44	65.02	15.20

to the degradation of anthocyanins recorded at the initial period of wine production (Fig. 3A, Day 0 - 8). Significant correlations were found between total phenolic compounds and colour parameters (0.98) and fructose (0.94). Interestingly, no significant relationship was observed for the hue angle. However, the relationship of anthocyanins to both CIEa* and CIEb* should be carefully considered due to the noticeable varietal effect on the evolution pattern obtained for these colour parameters. Overall, this information supports the impact of phytochemicals on the final quality of fruit wines (Linda *et al.,* 2022; Bayoï *et al.,* 2021; Gomes *et al.,* 2020; Tchabo *et al.,* 2017).

A Bhagwa variety pomegranate wine was developed and evaluated. Important quality parameters and phytochemical composition were monitored throughout the winemaking process. This led to the identification of a wide array of unique characteristics that could influence consumer acceptance and preference, highlighting the promising potential of these bioactive-rich beverages. The choice of cultivar and the winemaking procedures, particularly the duration of fermentation, played a significant role in determining the final quality and composition of the wine. *S. cerevisiae* can be effectively used for the development of pomegranate wine. Glucose and fructose content reduced up to 0.04 g $100mL^{-1}$ and 0.22 g $100mL^{-1}$, respectively. Citric acid content was maintained throughout the fermentation process on an average of 1.03 g $100mL^{-1}$. Malic acid content was found to be decreased from 0.34 g $100mL^{-1}$ to 0.12 mg $100mL^{-1}$. Anthocyanins showed a 46 % decrease during the winemaking process, which was found to be the result of individual anthocyanins reduction. Total phenolics were also reduced by 35 % during the winemaking. However, antioxidant activity was found to be reduced by 22 % in DPPH analysis. Future research should aim to preserve and enhance these desirable wine properties during the fermentation stage, with the goal of producing varietal pomegranate wines with superior characteristics.

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