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DEVELOPMENT, PHYSICOCHEMICAL CHARACTERIZATION, AND QUALITY EVALUATION OF MORINGA OLEIFERA LEAF EXTRACT-FORTIFIED APPLE JUICE

Farzana Sarwar[1], Muhammad Abdullah[2], Faiza Azmat[3], Azhar Mahmood[*4], Dr Muhammad Ajmal[5], Yasmeen Bano[6], Farah Ahmad[7], Rahat Batool[8], Areej Masood[*9]

[1]Institute of Food Science and Nutrition, University of Sargodha
[2]Department NUR Institute of Food Sciences, University Nur International University
[3]Department of Nutritional Sciences and Environmental Design, Allama Iqbal Open university Islamabad
*[*4]Department of Food Science and Technology, The Superior University Lahore*
[5]Department of Dairy Technology, University of Veterinary and Animal Sciences Lahore
[6]Department of Food Science and Technology, University of Agriculture Faisalabad Sub-Campus, Burewala.
[7]Senior Scientists, Post-Harvest Research Centre, AARI, Faisalabad
[8]Scientific Officer, Mango Research Institute, Multan
*[*9]Department Doctor of Physio Therapy, Government College University Faisalabad (Affiliated with Times University Multan)*

fazimeher30@gmail.com[1], abdullahjaffar817@gmail.com[2], faiza.azmat@aiou.edu.pk[3] azharkasurift@gmail.com[*4], muhammad.ajmal@uvas.edu.pk[5] farahahmadpk@gmail.com[7], rahatbatool08@gmail.com[8], drareej1412@gmail.com[*9]

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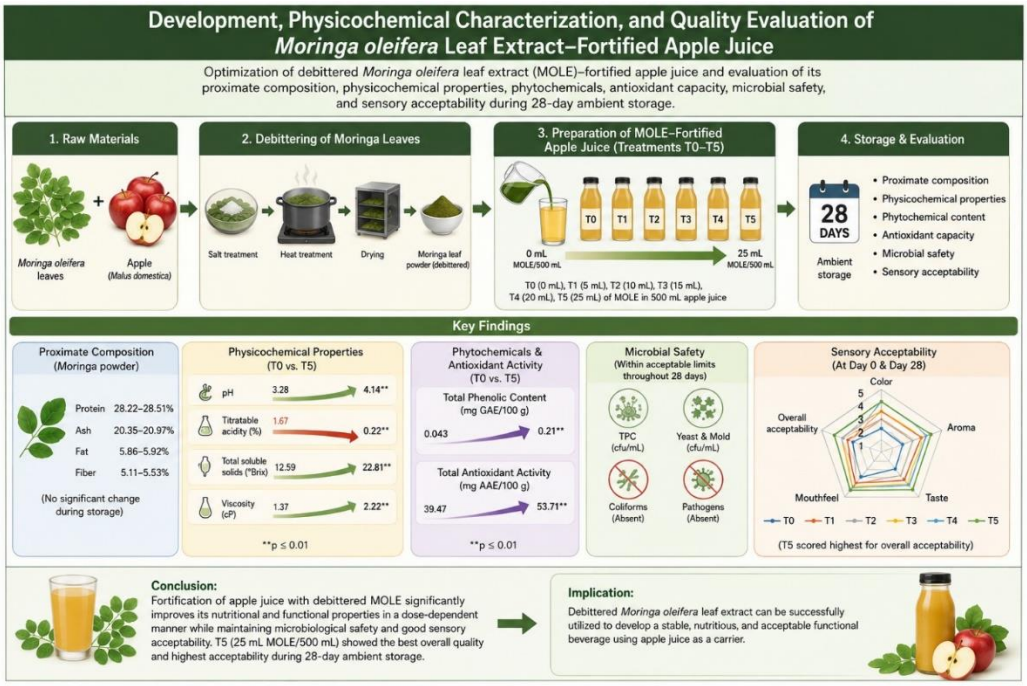
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Azhar Mahmood, Areej Masood

Abstract

Moringa oleifera (MO) is known around the world because of its high nutritive density and medicinal properties, but the development of a pleasant functional beverage using it has not received enough attention so far. The objective of this research is the production and optimization of debittered Moringa oleifera leaf extract (MOLE)-fortified apple juice with six different treatments (T0-T5), where T5 contains 25 mL MOLE/500 mL and to evaluate the effects of proximate composition, physicochemical characteristics, phytochemical content, antioxidant capacity, microbial safety, and sensory acceptability during 28-day ambient storage. Debittered Moringa powder showed very good composition stability; protein content 28.22–28.51%, ash 20.35–20.97%, fat 5.86–5.92%, and fiber 5.11–5.53% (all values non-significant with respect to storage time). In the case of fortified apple juice, the amount of MOLE significantly increased the pH value from 3.28 in T0 to 4.14 in T5 ($p \leq 0.01$), lowered the titratable acidity value (1.67% in T1 to 0.22% in T5), increased total soluble solids (12.59 to 22.81 °Brix), and increased the viscosity (1.37 to 2.22 cP). There was a positive

dose-dependent relationship between total phenolic content (0.043 to 0.21 mg GAE/100g) and total antioxidant activity (39.47 to 53.71 mg AAE/100g.



INTRODUCTION

A lot of development has been observed in the global functional food market owing to the increasing awareness of consumers on the relationship between diet and diseases and need for health products apart from nutritional value. Functional plant beverages have emerged as an interesting area where phytochemicals are added to functional beverages. Traditional medicine systems used by about 80% of the world population (World Health Organization) serve as the basis for the identification of bioactive plants.

Moringa oleifera Lam. Family: Moringaceae, also called Miracle Tree or Tree of Life, belongs to one of the most nutritionally and pharmaceutically versatile plants known. Indigenous to the Indian subcontinent, M. oleifera is widely grown in Pakistan, India and tropical Africa. The leaves of the M. oleifera plant show the presence of high amounts of macronutrients and micronutrients, such as crude protein 26.9-29.5%, calcium 2089.0 mg/100g, potassium 1488.7 mg/100g, and iron 28.8 mg/100g (Chhikara et al., 2021; Ravindra et al., 2019). Flavonoids (quercetin, kaempferol, isorhamnetin), phenolic acids, glucosinolates, and ascorbic acid 826.1 mg/100g fresh and total antioxidant activity of 178.3 mg/100g have been reported in the leaves of M. oleifera. These phytochemicals have been known for their antimicrobial, anti-inflammatory, antioxidant, antihypertensive, and hepatoprotective activity (Redha et al., 2021; Padayachee and Baijnath, 2020). The global moringa market size is expected to reach USD 7,902.9 million by 2025 (Razis et al., 2021).

In addition, apples (Malus domestica) form one of the most consumed fruits in the world, with a global output of 93 million tons recorded in 2021 (FAOSTAT, 2023). Apple juice is a source of quercetin glycosides, chlorogenic acid, catechins, phloretin, and procyanidins, which have cardioprotective, antidiabetic, and antioxidant activities (Boyer and Liu, 2022). The inherent acceptability, popularity, and low cost of apple juice make it the best carrier matrix for functional food. Stevia rebaudiana, 100-300 times as sweet as sucrose without caloric contribution, serves as a suitable natural sweetener that improves the therapeutic value of the formulation (Samuel et al., 2018).

Despite the established pharmacological activities of MO, there are certain technical considerations involved in the formulation of a health beverage using this medicinal extract, such as the inherent

bitterness of MO due to its glucosinolate content, sensory incompatibility at high concentrations, and physicochemical instability upon storage. Salt-heat debittering has been demonstrated to be effective in addressing these limitations while maintaining the integrity of bioactive compounds (Safdar et al., 2021). No studies have reported on a debittered MOLE-apple juice formula optimised in various concentrations, along with a quality and shelf-life analysis.

The purpose of this study is to develop and evaluate six formulations of MOLE-apple juice at different treatment levels (0-25 mL MOLE in 500 mL of apple juice) for their proximate, physicochemical, phytochemical, microbiological and sensory characteristics for 28 days of ambient storage.

2. Materials and Methods

2.1 Procurement of Raw Materials

The apples, lemons, stevia (Stevia rebaudiana), and chemicals used for preservation were obtained from the fruit market of Sargodha, Pakistan. Moringa oleifera leaves in their fresh form were harvested from the campus garden of Maryum Hall, University of Sargodha and PAF Musaf Base Sargodha. All the above-mentioned materials were taken to the laboratory of IFSN and processed to get rid of damaged/dirty items.

2.2 Debittered Moringa Powder (DMP) Preparation

The freshly collected MO leaves were cleaned and subjected to the debittering process in order to make them less bitter. Leaves were immersed in 15% NaCl solution and heated at 50°C for 30 minutes. The drained leaves were then dried under a fan air current at ambient temperature for 2-3 hours, followed by oven drying at 50°C for 72 hours (Safdar et al., 2021). Dried leaves were powdered using a laboratory grinder. Proximate composition of DMP was performed based on AOAC (2006) methodology: Moisture (oven drying at 105°C), Ash (muffle furnace at 550°C), Crude protein (Kjeldahl method; N×6.25), Crude fat (Soxhlet method using petroleum ether), Crude fiber (acid-base digestion) and Nitrogen Free Extract (NFE) by difference method.

2.3 Apple Juice Preparation

Juice from fresh apples was processed by blending and straining the mixture through double-layered muslin cloth. This juice was standardized to 12° Brix equivalent using Stevia (10%) and citric acid (0.3 g/500 mL) as follows (Table 1).

Table 1. Standard formulation of Moringa oleifera leaf extract-fortified apple juice per 500 mL

Ingredient	Quantity
Apple juice	25 mL
Water	65 mL
Stevia	10%
Food-grade colour	0.1 g
Citric acid	0.3 g
Carboxymethylcellulose (CMC)	0.5 g
Apple flavor	0.05 mL
Lemon juice	1 mL
Moringa oleifera leaf extract (MOLE)	As per treatment (T0–T5)
Potassium metabisulphate (preservative)	Standard concentration

2.4 MOLE Preparation and Treatment Design

MOLE aqueous solution was made by placing 1 g MO powder in 10 mL distilled water and heating it to 50 degrees Celsius for 30 minutes. It was passed twice through muslin cloth. The six treatment levels used

were: T0 (0 mL MOLE, control); T1 (5 mL); T2 (10 mL); T3 (15 mL); T4 (20 mL); and T5 (25 mL). Juice was treated using potassium metabisulphate and kept in room temperature for 28 days.

2.5 Physicochemical Analyses

TSS analysis was carried out using digital refractometer. TA was estimated by titrating 0.1N NaOH to phenolphthalein endpoint, and it was reported as percentage of citric acid equivalents (AOAC, 2011, 942.15). pH measurement was carried out using pH meter. AOAC (2011, 981.12) describes the method. Measurement of viscosity was carried out using Brookfield Viscometer.

2.6 Phytochemical and Antioxidant Analyses

Total phenolic content (TPC) was estimated using Folin-Ciocalteu reagent (Nadeem et al., 2018), gallic acid being the standard; TPC was expressed in mg GAE/100g FW (absorbance at 760 nm). Total flavonoid content (TFC) was assessed using aluminum chloride colorimetry (Malik et al., 2022), catechin being used as a standard; TFC was reported in mg CE/100g FW (absorbance at 510 nm). Total antioxidant activity (TAA) was quantified using the ammonium molybdate phosphomolybdenum assay (Prieto et al., 1999); ascorbic acid was used as a standard; TAA was expressed in mg AAE/100g FW (absorbance at 695 nm). Free radicals scavenging activity (DPPH RSA%) was determined at 517 nm.

2.7 Microbial Analysis

The total plate count was performed based on FDA bacteriological analytical manual procedures (FDA, 2001). The samples were serially diluted with sterile saline solution and then spread on the Plate Count Agar media and incubated at 32°C for 48 hours. Colony forming units (CFU/mL) were counted.

2.8 Sensory Evaluation

Sensory attributes odour, colour, taste, and overall acceptability were evaluated by a panel of semi-trained PhD faculty judges (n=10) using a 9-point hedonic scale (Land and Sphered, 1988), assessed every two weeks over 28 days.

2.9 Statistical Analysis

The data were statistically analysed using two way ANOVA, with treatment × storage being the factors. Mean differences were compared using Duncan's Multiple Range Test (DMRT) at a 5% level of significance (Steel et al., 1997). Data are presented in Means ± SD form. Levels of significance: *p≤0.05, **p≤0.01, NS=non-significant.

3. Results and Discussion

3.1 Proximate Composition of Debittered Moringa Powder (DMP)

Table 2 highlights the proximate composition analysis of debittered MO powder on a 28-day period of ambient storage. There were observed variations of moisture contents on a daily basis (F=8.83*, p≤0.05) from 24.11±0.64% on day 1 to 27.13±0.74% on day 28. The continuous increase in moisture content is attributed to non-hermetic storage that allowed water vapor entry from the atmosphere. According to Emelike and Ebere (2016), lower moisture content contributes to increased shelf life of dried products. The current values (24-27%) are higher than those observed in hermetically sealed MO powder (2.56-5.93% as reported by Olabode et al., 2015), showing the necessity for vacuum packaging in the industry. Fat content (5.86-5.92%) had no variations on a daily basis (F=0.07NS) due to the low fat content in MO leaves (~2.3%) and hermetic packaging protecting the sample from atmospheric oxidants (Mashau et al., 2021; Akhtar et al., 2005). Similarly, crude fiber (5.11-5.53%, F=1.74NS), protein (28.22-28.51%, F=0.55NS), ash (20.35-20.97%, F=3.01NS), and NFE (15.09-15.24%, F=1.33NS) all did not vary significantly during storage and thus exhibited good compositional stability. The high protein content (≈28.3%) is comparable to that of egg and milk protein.

Table 2. Proximate composition of debittered Moringa oleifera powder during 28-day storage (Means ± SD)

Day	Moisture (%)	Fat (%)	Fiber (%)	Protein (%)	Ash (%)	NFE (%)
1	24.11±0.64b	5.86±0.35a	5.36±0.26a	28.27±0.079a	20.50±0.49a	15.19±0.12a
7	24.83±0.70b	5.92±0.02a	5.21±0.22a	28.47±0.152a	20.66±0.152a	15.09±0.03a
14	25.11±0.84b	5.92±0.03a	5.23±0.28a	28.35±0.075a	20.97±0.08a	15.14±0.03a
21	26.63±0.74a	5.89±0.03a	5.11±0.10a	28.51±0.489a	20.90±0.138a	15.10±0.09a
28	27.13±0.74a	5.91±0.01a	5.53±0.15a	28.22±0.380a	20.35±0.19a	15.24±0.144a
F-value	8.83*	0.07NS	1.74NS	0.55NS	3.01NS	1.33NS

Mean values in a column with different superscript letters differ significantly (p≤0.05). *Significant; NS=Non-significant.

3.2 pH of MOLE-Fortified Apple Juice

The effect of treatment (F = 73.97**) and storage day (F = 10.33**) was highly significant on pH level while the interaction of treatment and storage day had a non-significant effect (F = 0.53NS) (Table 3). The concentration of MOLE showed a dose-dependent increase in pH: T0 (3.28 ± 0.25) < T1 (3.44 ± 0.12) < T2 (3.61 ± 0.12) ≈ T3 (3.68 ± 0.04) < T4 (3.94 ± 0.03) < T5 (4.14 ± 0.03), all values being statistically different (p ≤ 0.01). The reason for this pH increment lies in alkaline mineral components such as calcium, magnesium, and potassium of MO, which neutralize malic and citric acids of apple juice, as confirmed by Kumar et al. (2024) in dairy products fortified by Moringa. During the process of storage, the pH levels were decreased gradually from 3.80 ± 0.09 (day 0) to 3.56 ± 0.57 (day 28) due to organic acids accumulation as a result of enzymatic reactions and insufficient microbial fermentation (Sharma and Patel, 2024). However, all treatments kept pH below 4.

Table 3. Effect of MOLE treatments and storage duration on pH of fortified apple juice (Means ± SD)

Treatment	Day 0	Day 7	Day 14	Day 21	Day 28	Treatment Mean
T0 (0 mL)	3.48±0.22	3.36±0.23	3.36±0.02	3.06±0.49	3.15±0.08	3.28±0.25 E
T1 (5 mL)	3.58±0.22	3.46±0.18	3.48±0.04	3.38±0.13	3.29±0.07	3.44±0.12 D
T2 (10 mL)	3.69±0.16	3.70±0.05	3.60±0.15	3.54±0.01	3.55±0.14	3.61±0.12 C
T3 (15 mL)	3.77±0.04	3.80±0.09	3.68±0.05	3.60±0.08	3.55±0.03	3.68±0.04 C
T4 (20 mL)	4.10±0.03	4.07±0.07	3.97±0.08	3.77±0.08	3.77±0.09	3.94±0.03 B
T5 (25 mL)	4.21±0.02	4.15±0.03	4.16±0.07	4.12±0.01	4.06±0.04	4.14±0.03 A
Day Mean	3.80±0.09 A	3.76±0.11 AB	3.71±0.08 B	3.58±0.16 C	3.56±0.57 C	

Means with different capital letters in the Treatment Mean column or the Day Mean row differ significantly (DMRT, p≤0.05).

3.3 Titratable Acidity

Titratable acidity (TA) was significantly affected by treatment (F=1659.69**) and storage (F=56.52**), but interaction was non-significant. A reciprocal dose-dependent effect of MOLE concentration was noted in regard to acidity: T5 recorded the lowest TA (0.22±0.20%), whereas T1 recorded the highest TA (1.67±0.02%), with T0 (control) having an intermediate value of 0.63±0.05% (Table 4). The high acidity of T1 compared to that of the control probably indicates that acidic phenolic compounds are extracted

by low MOLE concentrations, while alkaline mineral buffering starts to prevail in high MOLE concentrations (T4, T5). Increasing mean acidity due to storage from 0.77±0.04% on day 0 to 0.99±0.03% on day 28 (+28.6%) could be attributed to bacterial synthesis of lactic and acetic acids, and oxidative degradation of phenolic compounds into organic acids (Martinez and Rodriguez, 2024). Arif et al. (2022) have reported a similar pattern of decreasing acidity with increasing MO extracts in blended beverages, indicating.

Table 4. Effect of MOLE treatments and storage on titratable acidity (%) of fortified apple juice (Means ± SD)

Treatment	Day 0	Day 7	Day 14	Day 21	Day 28	Treatment Mean
T0	0.50±0.04	0.62±0.01	0.59±0.08	0.69±0.01	0.74±0.03	0.63±0.05 D
T1	1.51±0.11	1.60±0.02	1.69±0.03	1.79±0.14	1.76±0.02	1.67±0.02 A
T2	1.20±0.01	1.27±0.05	1.30±0.01	1.34±0.002	1.37±0.04	1.30±0.03 B
T3	0.83±0.01	0.86±0.02	0.94±0.01	1.04±0.12	1.04±0.02	0.94±0.02 C
T4	0.45±0.03	0.53±0.02	0.54±0.06	0.62±0.02	0.69±0.04	0.56±0.03 E
T5	0.11±0.02	0.18±0.01	0.21±0.01	0.28±0.01	0.32±0.03	0.22±0.20 F
Day Mean	0.77±0.04 C	0.84±0.03 B	0.88±0.02 B	0.96±0.03 A	0.99±0.03 A	

3.4 Total Soluble Solids (TSS) and Viscosity

The TSS showed a highly significant effect due to the treatment (F=99.47**) while the storage showed no effect (F=0.19NS). A dose-dependent increase in TSS was shown from 12.59±1.15 °Brix (T0) to 22.81±1.12 °Brix (T5) (Table 5). The increase is due to the dissolved solids provided by MO leaf extract (carbohydrates, minerals, and proteins), plus the possibility of sucrose inversion in high phytochemical concentration due to enzymatic activity (Akande and Ojekemi, 2013). The lack of effect due to storage shows that there is good sugar-solid stability of the formulation in 28 days.

For the viscosity, a highly significant effect is also seen with treatment (F=634.76**) while storage showed no effect (F=0.36NS). T5 showed the highest viscosity of 2.22±0.04 cP compared with T0 having 1.37±0.08 cP. This is due to the dissolved polysaccharides, proteins, and pectins in the MO extract providing rheological property.

Table 5. Treatment means for TSS (°Brix) and viscosity (cP) of MOLE-fortified apple juice

Treatment	MOLE Vol.	TSS Mean (°Brix)	Viscosity Mean (cP)
T0	0 mL	12.59±1.15 F	1.37±0.08 F
T1	5 mL	14.49±1.65 E	1.60±0.03 E
T2	10 mL	16.80±1.88 D	1.73±0.03 D
T3	15 mL	18.65±1.42 C	1.89±0.02 C
T4	20 mL	20.70±1.11 B	2.06±0.04 B
T5	25 mL	22.81±1.12 A	2.22±0.04 A
F-value (Treatment)	—	99.47**	634.76**
F-value (Days)	—	0.19 NS	0.36 NS

3.5 Total Phenolics, Flavonoids, and Antioxidant Activity

3.5.1 Total Phenolic Content (TPC)

TPC was greatly affected by both the treatment (F = 527.27**) and storage (F = 31.18**). At T1, TPC values were lower (0.014±0.004 mg GAE/100g) than the control (0.043±0.04) but gradually increased as the amount of MOLE inclusion increased in subsequent treatments: T2 (0.106±0.003) < T3 (0.140±0.040) < T4 (0.193±0.045) < T5 (0.210±0.030 mg GAE/100g). This drop in value at T1 was probably due to dilution effect where the lower MOLE content failed to compensate for the displacement of apple juice with sufficient phenolic compounds from MOLE; however, beyond this level, inclusion of MOLE phenols was greater than dilution, resulting in increasing values from T1 to T5 (Table 6). TPC gradually reduced during storage from 0.22±0.05 (Mena et al., 2013; Ali et al., 2015).

3.5.2 Total Flavonoid Content (TFC)

There was a strong significance of TFC between treatments (F=378.35**), as well as good stability in storage (F=1.41NS). The highest TFC was found for T0 (2.30±0.03 mg CE/100g), followed by a sharp decrease for T1 (0.18±0.02), after which it progressively increased as more MOLE was added: T2 (0.46±0.02) < T3 (0.76±0.03) < T4 (1.20±0.03) < T5 (1.63±0.03 mg CE/100g). This trend is similar to that noted in TPC analysis: the own flavonoids in apple juice (quercetin glycosides, catechins, epicatechins, phloretin) get diluted in lower MOLE volumes quicker than they get replenished by MOLE.

3.5.3 Total Antioxidant Activity (TAA)

TAA was highly significantly treatment-dependent (F=710.91**) and storage-stable (F=0.17NS). Among MOLE treatments, TAA increased dose-dependently from 39.47±2.35 mg AAE/100g (T1) to 53.71±2.17 (T5), a 36% enhancement. The T0 control showed the highest TAA (82.40±1.99), reflecting apple juice's inherently high antioxidant capacity. This pattern aligns with Ogundele et al. (2016), who showed improved radical-scavenging with increasing plant extract quantities. The non-significant storage effect on TAA (52.83 to 52.33 mg AAE/100g across days) indicates that antioxidant compounds in MOLE are stable under ambient storage conditions.

Table 6. Phytochemical and antioxidant parameters by MOLE treatment level (Treatment Means ± SD)

Treatment	TPC (mg GAE/100g)	TFC (mg CE/100g)	TAA (mg AAE/100g)
T0 (Control)	0.043±0.04 A	2.30±0.03 A	82.40±1.99 A
T1 (5 mL)	0.014±0.004 F	0.18±0.02 F	39.47±2.35 F
T2 (10 mL)	0.106±0.003 E	0.46±0.02 E	43.77±2.24 E
T3 (15 mL)	0.140±0.040 D	0.76±0.03 D	45.86±2.41 D
T4 (20 mL)	0.193±0.045 C	1.20±0.03 C	49.67±2.13 C
T5 (25 mL)	0.210±0.030 B	1.63±0.03 B	53.71±2.17 B
F-value (Treatment)	527.27**	378.35**	710.91**
F-value (Days)	31.18**	1.41 NS	0.17 NS

3.6 Sensory Evaluation

All sensory parameters (odour, colour, taste, overall acceptability) are shown in Table 7. There were no significant treatment effects nor storage effects (F=0.76NS; F=0.32NS) for overall acceptability, indicating consumer acceptability for all levels of MOLE. T5 had the highest score of overall acceptability (6.93±0.34/9.0), slightly higher than T0 (6.86±0.34). This implies the effectiveness of the debittering process in removing the bitterness of MO, in accordance with Johnson and Williams (2023), who successfully removed the bitterness of MO through a salt-heat debittering protocol.

Odour was significantly treatment-dependent (F=2.87*): T1 got the highest score (7.37±1.02) while T5 had the lowest score (6.77±1.04), implying that MOLE at the highest level produced a slight grassy or herbal odour. No storage effect was observed on the odour parameter (F=2.12NS): the mean daily odour scores ranged from 6.74 to 7.24. The colour scores were entirely non-significant (F=0.56NS for treatments; F=0.22NS for days): T0 received the highest score (7.39±0.48), followed by T1 with the lowest score.

Table 7. Sensory evaluation scores (9-point hedonic scale) by MOLE treatment (Treatment Means ± SD, n=28-day average)

Treatment	Odour	Colour	Taste	Overall Acceptability
T0 (Control)	7.05±0.17 AB	7.39±0.48 A	6.82±0.15 A	6.86±0.34 A
T1 (5 mL)	7.37±1.02 A	7.20±0.34 A	6.83±0.14 A	6.86±0.18 A
T2 (10 mL)	6.83±0.04 B	7.33±0.38 A	6.90±0.21 A	6.81±0.18 A
T3 (15 mL)	6.80±0.02 B	7.42±0.34 A	6.91±0.16 A	6.80±0.15 A
T4 (20 mL)	7.08±0.02 AB	7.38±0.57 A	6.88±0.28 A	6.92±0.35 A
T5 (25 mL)	6.77±1.04 B	7.32±0.42 A	6.88±0.28 A	6.93±0.34 A
F-value (Treatment)	2.87*	0.56 NS	0.44 NS	0.76 NS
F-value (Days)	2.12 NS	0.22 NS	0.17 NS	0.32 NS

3.7 Optimal Treatment Selection

When combined, the results showed that T5 (25 mL MOLE per 500 mL juice) is the best choice. T0 had higher natural antioxidant capacity because of the polyphenolic content of apple juice itself, but T5 was clearly the best choice for MOLE-fortified treatment: it contained the highest total phenolic content due to MOLE addition (0.21 mg GAE/100g) and had the highest antioxidant capacity (53.71 mg AAE/100g); the highest TSS (22.81 °Brix) and viscosity (2.22 cP), the best overall acceptability (6.93/9.0), and the best pH values throughout the storage period of 28 days without crossing the limit <4.2 due to its good buffering capacity. This result is in agreement with the dose-dependent bioactivity trend of MO-fortified functional food, which shows better nutrition enrichment without affecting the sensory properties of the product if debittering is done (Ali et al., 2015; Emelike et al., 2015).

4. Conclusion

It was successfully developed and optimized an apple juice fortified with debittered Moringa oleifera leaf extract (MOLE) was successfully developed and optimized at six concentrations levels. Debittered MO powder showed good compositional stability during 28 days. The increase in pH, total soluble solids (TSS), viscosity, and total phenolic content was observed in apple juice depending on the MOLE concentration, while titratable acidity was decreased. Significant enhancement in total antioxidant capacity was observed in MOLE fortified samples. The sensory analysis showed high consumer acceptability at all concentrations; however, the T5 treatment (25 mL MOLE) showed the best results concerning fortification, bioactivity, and acceptability (6.93 out of 9.0). All formulations showed microbiological safety (pH<4.2) during 28 days of ambient storage. T5 is suggested as the optimal formula for further clinical testing.

Declaration of Competing Interests

The authors declare no conflict of interest.

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