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RESEARCH PAPER

Preparation and Evaluation of Ready-to-Serve Drink Made from Blend of *Aloe vera*, Sweet Lime, Amla and Ginger

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ABSTRACT

The study was conducted to develop formulations of *Aloe vera* blend by mixing it with ginger, sweet lime and amla. Varied ratio of the *Aloe vera*:ginger:sweetlime:amla in the formulated drinks (40:5:40:15), (50:5:30:15) and (60:5:20:15) were used for F1, F2 and F3 respectively. The products were subjected to standard physico-chemical, sensory and microbial analysis and accordingly, F3 was found to be the most preferred variant with respect to the sensory quality. The *Aloe vera* RTS blends prepared were found to be good source of Vitamin C besides other nutritional parameters. The storage stability studies carried out showed that the physico-chemical and the sensory quality of the RTS blends were acceptable up to 60 days of storage. Microbial analysis of the RTS during storage period up to 60 days revelead that it was free from any spoilage.

Keywords: RTS, *Aloe vera*, ginger, sweet lime

The use of soft drinks due to their thirst quenching potential and taste is well-known all over the world. The demand for soft drinks almost always has an increasing trend and there is a great scope for development of natural nutrient rich beverages owing to negligible synthetic chemical content and also has immense health benefits of these beverages. Therapeutic beverage sector has been reported to be the fastest growing segment (Roberts, 2009) in the soft drink industry sector.

The functional properties and therapeutic benefits of *Aloe vera* are known worldwide. It is a source of active substances including vitamins, minerals, enzymes, sugars, anthraquinones of phenolic compounds, lignin, saponins, sterols, amino acids and salicylic acid. The polysaccharides found in *Aloe vera* have been considered to be the active ingredients for Aloe's anti-inflammation and immune modulation effects (Pugh *et al.,* 2001). Its gel is transparent slippery mucilage containing bioactive polysaccharides, mainly partially acetylated glucomannans. Besides, it is also a good source of vital nutrients (Rodriguez *et al.,* 2010). That is why the Aloe industry is flourishing and the gel is used in many products, such as fresh gel, juice and other formulations for health, medical and cosmetic purposes (Enward and Benward, 2000).

Ginger (*Zingiber officinale* Rosc.) is valued as a spice for ages and is also known for its medicinal properties such as to treat rheumatoid arthritis, ulcer, preventing heart attack and stroke. Ginger is an aromatic tuber crop having volatile oils that account for the aroma of the tubers (Kikuzaki *et al.,* 1991). Not only this, the use of ginger as antiviral, anti-cancer and anti-ulcerogenic drug has been widely accepted

(Denyer *et al.,* 1994); (Katiyar *et al.,* 1996; Yamahara *et al.,* 1988). Aonla (*Emblica officinalis*) is a minor sub-tropical deciduous tree indigenous to Indian sub-continent. It is also known as Indian Gooseberry and has both nutritive and medicinal properties. It is very rich in Vitamin C (500-1500 mg of ascorbic acid per 100 g) (Chauhan *et al.,* 2005). It has also been found to be rich in phenols and tannins such as elegiac acid and gallic acid that prevent the oxidation of vitamin C. The medicinal properties of amla against several ailments like tuberculosis, asthma, bronchitis, scurvy, diabetes, anemia, weakness of memory, cancer, influenza are well known. However, its juice is acidic and astringent in nature which has negative impact on its palatability if consumed fresh (Goyal *et al.,* 2008). The sweet lime fruit is another crop which is processed commercially into various forms mainly juice, frozen concentrates, squash and RTS drinks which provide energy, moderate quantity of vitamin C, potassium, bioflavonoid and folic acid and is considered as an essential item of breakfast. The juice is refreshing, thirst quenching and energizing that improves health and caters to many nutritional requirements (Syed *et al.,* 2011).

The development of novel RTS blends is required for meeting the demands of the consumers and also for growth of the food processing industry. A beverage prepared by blending of fruits, vegetables and products from medicinal plants is an emerging sector in food industry. Owing to its nutraceutical properties *Aloe vera* is being explored as a functional ingredient in numerous health foods and drinks (Ramachandran and Nagarajan, 2014). New RTS blends having *Aloe vera* blended with other fruits have been reported earlier by researchers (Ramachandran and Nagarajan, 2014); (Yadav *et al.,* 2013).

One of the goals of producing these blends also include the reduction of unpalatable taste of the ingredients. The blending of two or more juices helps in utilization of astringent and acidic fruits like amla. Blending of *Aloe vera* based RTS drinks with other medicinal plants like ginger and amla may be an excellent way to deliver these therapeutic benefits of *Aloe vera* to the consumers.

Keeping these facts in view, the present investigation was undertaken to develop an *Aloe vera* based RTS blended with juice of amla, ginger and sweet lime.

MATERIALS AND METHODS

Raw materials

The research work was carried out in the Food Processing Unit of the College of Home Science Tura, Meghalaya. Undamaged, mould/rot free and mature *Aloe vera* leaves were procured from local farmers and were made into pulp was taken out according to the traditional hand filleting method (Yadav et al. 2013). In brief, the lower 1 inch of the leaf base, the tapering point (2-4inch) of the leaf top and the short, sharp spines located along the leaf margins were removed with a sharp knife then, the knife was introduced into the mucilage layer below the green rind, avoiding the vascular bundles, and the top rind was removed. The bottom rind was also similarly removed and the rind parts, to which a significant amount of mucilage remained attached was discarded. The filleting process was completed within 36 hours of harvesting the leaves. The pulp was heated to 60-65^{0}C for 10 min and was mashed with the hand beater. The mashed pulp was strained with muslin cloth to retrieve the *Aloe vera* juice which was stored under refrigerated conditions for future use.

Other ingredients like ginger, sweet lime, amla, sugar, citric acid and preservative (KMS) was purchased from local market and their respective juices were extracted with the help of a laboratory blender followed by filtering through muslin cloth and stored separately under refrigerated conditions for future use.

Formulation of the RTS

The *Aloe vera* based RTS blended with ginger, amla and sweet lime juice was prepared as per the flow chart given in Fig. 1. The RTS was prepared in four different variations coded as F1, F2 and F3. The calculated amount of sugar syrup was added so as to maintain a constant 13^{0}brix (given in Fig. 1). Citric acid @ 0.2% per kg of pulp was added to

avoid browning reactions and KMS @ 100 ppm was added and pasteurized at 72°C and kept at ambient temperature for 60 days for further analysis.

Physio-chemical analysis

The pH values were determined with the help of a digital pH meter), TSS was measured with the help of a hand refractometer PR-101 and values were expressed as ^{0}Brix. Acidity of various samples was determined by titrating against 0.1 N NaOH according to AOAC (1995) method. Reducing and total sugars were determined by the method recommended by (Ranganna 2001). Ascorbic acid content was determined by the titration method using 2,6-dichlorophenol endophenol dye as recommended by (Ranganna 2001).

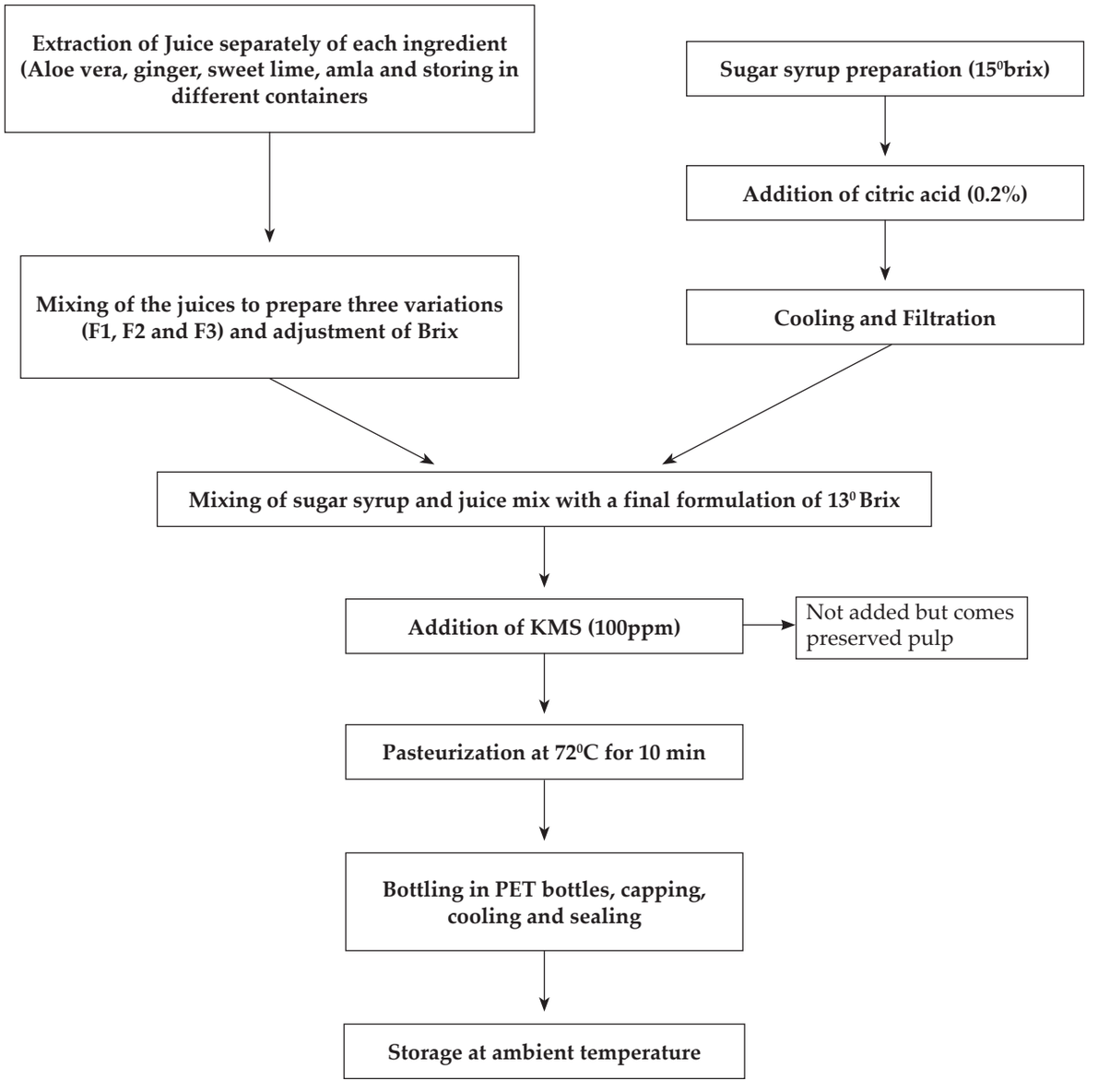

Fig. 1: Flow chart describing the preparation of Aloe vera based RTS formulations

Microbiological analysis

The prepared beverage formulations were studied for microbial load. The total microbial load was calculated by standard plate count (SPC) method. The SPC was done according to the method described earlier (APHA, 1967).

Sensory evaluation

The fresh and stored beverage samples prepared with varying levels of *Aloe vera*, ginger, sweet lime and amla were served chilled for sensory evaluation which was carried out by semi-trained panel of twenty judges on a 9.0 point Hedonic scale. These were served in different chambers. (Amerine *et al.*, 1965).

Statistical Analysis

Data obtained for the various parameters were expressed as mean values ± standard deviations of three replications. Statistical analyses were performed using GraphPad 6.07 software. Data were analyzed using One Way ANOVA to check the impact of storage period on the physico-chemical and organoleptic attributes of the formulations developed. Statistical difference between the means was determined using Tukey's test with the confidence limits set at $P < 0.01$ (99%).

RESULTS AND DISCUSSION

The raw ingredients used for the preparation of the *Aloe vera* based RTS formulations were analysed for important physico-chemical characteristics (Table 1). It is apparent that amla juice had the highest vitamin c content (885.6mg/100g), the total soluble solids expressed as ^{0}Brix was the highest in the sweet lime juice (14.5), while the pH was recorded highest in the *Aloe vera* juice (4.6). The total sugar content was the highest in sweet lime juice (10.4) whereas other ingredients were low in the total sugar content. Highest acidity was recorded in amla juice (2.4%) but was lowest in *Aloe vera* juice (0.06). The selection of sweet lime with high ^{0}Brix and total sugar in this study was done to mitigate the off taste and bitterness of the *Aloe vera* based RTS formulations while that of amla contributed to the taste and the purpose of ginger was to have flavour. Similar efforts to alleviate the bitter taste of the *aloe vera* juice have been made earlier also (Ramachandran and Nagarajan, 2014; Yadav *et al.*, 2013).

Table 1: Analysis of raw ingredients used in *Aloe vera* based RTS formulations

Physico-chemical characteristics	*Aloe vera* juice	Ginger juice	Sweet Lime juice	Amla juice
^{0}Brix	0.09	2.8	14.5	2.9
Acidity (%)	0.06	0.8	1.7	2.4
pH	4.6	4.2	2.76	2.9
Total sugar (%)	1.90	Not Detected	10.4	3.23
Vitamin content (mg/100g)	1.68	3.4	23.16	885.6

Physico-chemical attributes of the *Aloe vera* based RTS formulations

The data obtained (Table 2) clearly indicate that the pH of the *Aloe vera* formulations decreased during the storage period from 0 to 60 days after storage. The minimum decrease in pH was, however observed in the F2 formulation (4.56 to 4.25) and maximum in F3 formulation (4.80 to 4.40). The pH of the formulations was also impacted by varying the ingredients to prepare the four formulations of the *Aloe vera* RTS. But pH increased for the treatments F1 to F3 and the same trend was observed during the storage period. The decrease in pH during the storage period may be correlated with the increasing acidity. Similar findings have been reported (Tandon et al. 1983; Sandhu et al. 2001) earlier also. The decreasing trend has been reported earlier by (Hamaran and Amutha 2007) in the case of banana and sapota RTS.

The data pertaining to titratable acidity (Table 2) showed that there was significant increase in titratable acidity of all the formulations of the *Aloe vera* based RTS with storage time. The increase in the titratable acidity was almost similar in all the formulations. The formulations F1 and F3 exhibited an increase of 0.11 % and the formulations F2 had an increase of 0.10 % increase in titratable acidity. A strong correlation between the increase in acidity and decrease in vitamin c content has been reported earlier (Simsek 2011). The degradation of polyphenols in ginger and amla and rapid conversion of proteins to amino acids in *Aloe vera* RTS are also the reasons for increase in the titratable acidity of the Aloe vera RTS blends. The findings are in accordance with the findings reported earlier (Yadav *et al.,* 2013).

The total soluble solids in RTS formulations (Table 2) are expressed as ^{0}Brix. It is an indicator of sugar content in the RTS blends and it primarily results due to the sucrose, glucose and fructose components. In the present investigation the TSS for all the variations was maintained at 13^{0}Brix initially. There was an increasing trend in the TSS from 0 to 60 days after storage for all the formulations. The maximum increase took place in F3 formulation (13.0 to 14.9). The increase in the reducing sugar due to hydrolysis of sugars by acid, increase in hydrolysis of starch component of the *Aloe vera* ingredient into simple sugars and formation of invert sugar from sucrose may have led to the increase in the TSS value of the RTS formulations during the storage period. Similar trends have been reported in RTS developed from guava by (Kalra *et al.,* 1991), bitter gourd based RTS (Barwal *et al.,* 2005) and *Aloe vera* – papaya based RTS (Ramachandran and Nagarajan, 2014). The ratio of the TSS and titratable acidity contribute to the flavour and taste of the fruits and juices. The TSS is directly proportional to sweetness index (ratio of TSS and titratable acidity) (Sadler & Murphy, 2010). The increased TSS values during the storage period have a great role in conserving the taste and flavour of the RTS blends during the storage period. Similar findings have been reported in the case of lime blended amla squash (Reddy and Chikkasubbanna 2008).

Table 2: Effect of storage period on physic-chemical parameters of *Aloe vera* based RTS formulations

Duration of Storage (Days)	Formulations		
	F1	F2	F3
	pH		
0 DAS	4.48 ^{b} ± 0.02	4.56 ^{c} ± 0.02	4.80 ^{e} ± 0.01
15 DAS	4.40 ^{ab} ± 0.05	4.60 ^{cd} ± 0.02	4.75 ^{ef} ± 0.03
30 DAS	4.32 ^{a} ± 0.02	4.35 ^{d} ± 0.02	4.60 ^{f} ± 0.03
45 DAS	4.21 ^{a} ± 0.04	4.30 ^{d} ± 0.02	4.45 ^{f} ± 0.03
60 DAS	4.15 ^{a} ± 0.03	4.25 ^{d} ± 0.04	4.40 ^{f} ± 0.04
	Titratable acidity (%)		
0 DAS	0.24^{a} ± 0.02	0.26 ^{d} ± 0.02	0.27 ^{c} ± 0.02
15 DAS	0.26 ^{ab} ± 0.01	0.29 de ± 0.01	0.28 ^{cd} ± 0.03
30 DAS	0.29 ^{b} ± 0.01	0.31 ^{e} ± 0.02	0.33 ^{d} ± 0.02
45 DAS	0.32 ^{b} ± 0.01	0.33 ^{e} ± 0.02	0.35 ^{d} ± 0.03
60 DAS	0.35 ^{c} ± 0.02	0.36 ^{f} ± 0.02	0.38 ^{e} ± 0.03
	Total Soluble Solids (^{0}Brix)		
0 DAS	13.00^{a} ± 0.00	13.00^{c} ± 0.00	13.00 ^{e} ± 0.00
15 DAS	13.50 ^{a} ± 0.10	13.75 ^{c} ± 0.05	13.70 ^{e} ± 0.05
30 DAS	13.70^{ab} ± 0.05	14.10^{cd} ± 0.05	14.30^{ef} ± 0.05
45 DAS	14.01 ^{a} ± 0.07	14.50 ^{c} ± 0.05	14.80 ^{e} ± 0.05
60 DAS	14.60 ^{a} ± 0.05	14.80 ^{c} ± 0.05	14.90 ^{e} ± 0.05
	Total Soluble Sugar (%)		
0 DAS	14.50 ^{a} ± 0.04	14.70^{c} ± 0.07	16.58^{e} ± 0.08
15 DAS	14.35 ^{ab} ± 0.03	14.56^{cd} ± 0.02	16.25^{ef} ± 0.03
30 DAS	13.93 ^{ab} ± 0.04	14.31^{cd} ± 0.15	15.86^{ef} ± 0.04
45 DAS	13.72 ^{b} ± 0.02	13.85 ^{d} ± 0.05	15.56 ^{f} ± 0.03
60 DAS	13.56 ^{a} ± 0.01	13.70 ^{c} ± 0.05	15.20 ^{e} ± 0.02
	Reducing Sugar Content (%)		
0 DAS	8.45^{b} ± 0.01	8.50 ^{c} ± 0.06	9.23^{e} ± 0.03
15 DAS	8.59 ^{ab} ± 0.02	8.64 ^{cd} ± 0.04	9.38 ^{ef} ± 0.03
30 DAS	8.70 ^{ab} ± 0.01	8.85 ^{cd} ± 0.04	9.65 ^{ef} ± 0.03
45 DAS	8.86 ^{a} ± 0.02	8.90^{d} ± 0.04	9.78^{f} ± 0.01
60 DAS	8.98 ^{a} ± 0.01	9.12 ^{d} ± 0.03	9.89 ^{f} ± 0.02
	Vitamin C Content (mg/100g)		
0 DAS	11.30^{a} ± 0.02	11.45^{c} ± 0.02	11.65^{e} ± 0.02
15 DAS	11.15 ^{ab} ± 0.03	11.21 ^{cd} ± 0.02	11.40^{ef} ± 0.02
30 DAS	10.83 ^{b} ± 0.02	11.05^{d} ± 0.02	11.32^{f} ± 0.02
45 DAS	10.65 ^{b} ± 0.03	10.86 ^{d} ± 0.02	11.16 ^{f} ± 0.02
60 DAS	10.42 ^{b} ± 0.02	10.70 ^{d} ± 0.03	11.02 ^{f} ± 0.01

NOTE:

Each value is the mean of three independent samples. Mean values ± SD

Means followed by same letters within each column were not significantly different. (P<0.01 - Tukey test).

The ratio of the *Aloe vera*:ginger:sweet lime:amla in the four variations were (40:5:40:15), (50:5:30:15) and (60:5:20:15) for F1, F2 and F3 respectively.

The data presented in Table 2 establish that there was a decreasing trend in the total sugar content from 0-60 DAS in all the formulations of the *Aloe vera* based RTS blend. The minimum decrease took place in the variation F1 (14.50 to 13.56 per cent). The decrease in the Total Sugar Content (TSC) was statistically significant during the storage time of 60 days. The hydrolysis of the complex sugars into simple sugars due to higher acidity may be the cause of declining total sugar content in the present investigation. Studies reported (Verma and Gehlot, 2007) in bael (*Aegle marmelos*) based RTS, (Ramachadran and Nagarajan, 2014) are contrary to the findings of this study.

The results pertaining to the reducing sugar content (in %) depicted in Table 2 reveal that there was an increasing trend in the reducing sugar content with storage period from 0 to 60 days after storage in all the formulations developed. The reducing sugars are very much instrumental in determining the taste of any fruit or juice sample. The conversion of the complex sugars including the disaccharides (non reducing sugars) which are important constituents of the total sugar into reducing sugar under acidic conditions is an important reason for the increasing trend seen in this investigation during the storage period from 0 to 60 DAS. The trend was evident in all the formulations and is in accordance with the findings reported earlier (Narayanan et al. 2002; Yadav et al. 2010).

A cursory glance on the data in Table 2 clearly indicates a decreasing trend in the Vitamin C content during the storage period from 0 to 60 days after storage in the formulations. The results exhibited statistically significant variations during the duration of the storage period (60 days). The decrease in the Vitamin C content can be attributed to the oxidation sensitive nature of the vitamin and its solubility in water. The maximum decrease in the vitamin c content was observed in the formulation F1 (11.30 to 10.42 mg/100g) and the minimum decrease was observed in the F3 formulation (11.65 to 11.02mg/100g). Ramachandran and Nagarajan, (2014) have reported that the decrease in vitamin c content on spiced papaya beverage was upto 74% and 79% during 90 DAS and 150 DAS respectively. The decrease in the present investigation ranged from 5.4% to 7.8% only. The decline may be due to high antioxidative properties of *Aloe vera* (Hu et al. 2003) which may have had a protective impact and reduced the oxidation of Vitamin C in the RTS formulations prepared in this study. Similar results have been reported by Yadav et al. (2013).

Sensory attributes of the *Aloe vera* based RTS formulations.

The results (Table 3) clearly indicate that treatment F3 scored significantly higher scores with respect to all the sensory attributes like appearance, mouth feel, taste, flavour and overall acceptability also. One of the limitations of the *Aloe vera* juice/beverages was its bitter and off taste. The increase in the sweet lime juice concentration up to 20 percent had a positive impact on all the sensory attributes of the RTS formulations. The addition of sugar syrup as per FSSAI specification (2006) also led to a refreshing taste of the RTS variations prepared. Successful attempts to prepare refreshing RTS blends from *Aloe vera* have been reported by Yadav *et al.* (2013) using mint and ginger as ingredients. Ramachandran and Nagarajan (2014) also reported the development of spiced papaya and *aloe vera* based RTS blend. This study is unique as it has utilized sweet lime to develop an *Aloe vera* based RTS blend. The results clearly indicate that the variation F3 was the best formulation with respect to all the sensory attributes. The decline in the sensory attributes during storage period may have occurred due to increase in acidity in the samples during storage. The development of bitterness due to increase in non-volatile compounds like gingerol in ginger during storage may also have contributed to the declining sensory attributes in all the formulations. The loss of volatile aromatic

substances may also have resulted in decreased flavour of the RTS formulations. Similar findings have been reported by Thakur and Barwal (1998); Jain *et al.* (2011).

Table 3: Effect of storage period on sensory of *Aloe vera* based RTS formulations

Duration of Storage (Days)	Formulations		
	F1	F2	F3
	Appearance and Color		
0 DAS	6.88 ^{a} ± 0.02	7.22 ^{b} ± 0.02	7.44 ^{d} ± 0.04
15 DAS	6.50 ^{a} ± 0.02	7.10 ^{b} ± 0.02	7.35 ^{d} ± 0.02
30 DAS	6.40 ^{a} ± 0.02	7.00 ^{b} ± 0.02	7.25 ^{d} ± 0.02
45 DAS	6.30 ^{ab} ± 0.02	6.90 ^{bc} ± 0.01	7.10 ^{de} ± 0.02
60 DAS	6.20 ^{a} ± 0.02	6.50 ^{b} ± 0.02	6.90 ^{d} ± 0.01
	Mouth Feel		
0 DAS	6.55^{a} ± 0.02	6.22^{b} ± 0.03	7.11^{c} ± 0.05
15 DAS	6.40 ^{a} ± 0.02	6.10 ^{b} ± 0.03	7.05 ^{c} ± 0.03
30 DAS	6.35 ^{a} ± 0.02	6.00 ^{b} ± 0.01	6.85 ^{c} ± 0.02
45 DAS	6.30 ^{a} ± 0.02	5.80 ^{b} ± 0.03	6.70 ^{c} ± 0.02
60 DAS	6.24 ^{a} ± 0.02	5.70 ^{b} ± 0.05	6.60 ^{c} ± 0.02
	Taste		
0 DAS	6.55 ^{a} ± 0.01	5.85 ^{c} ± 0.02	6.85 ^{e} ± 0.01
15 DAS	6.40 ^{ab} ± 0.02	5.70 ^{cd} ± 0.01	6.70 ^{e} ± 0.02
30 DAS	6.30 ^{a} ± 0.02	5.60 ^{c} ± 0.03	6.40 ^{ef} ± 0.05
45 DAS	6.25 ^{a} ± 0.02	5.40 ^{c} ± 0.03	6.30 ^{e} ± 0.03
60 DAS	6.10 ^{a} ± 0.02	5.30 ^{c} ± 0.04	6.10 ^{e} ± 0.03
	Flavor		
0 DAS	6.55 ^{a} ± 0.01	5.85 ^{c} ± 0.02	6.85 ^{e} ± 0.04
15 DAS	6.25 ^{a} ± 0.01	5.70 ^{c} ± 0.07	6.70 ^{e} ± 0.04
30 DAS	6.10 ^{ab} ± 0.04	5.60 ^{cd} ± 0.02	6.40 ^{ef} ± 0.02
45 DAS	5.90 ^{ab} ± 0.04	5.40 ^{cd} ± 0.03	6.30 ^{ef} ± 0.04
60 DAS	5.70 ^{b} ± 0.03	5.30 ^{d} ± 0.04	6.10 ^{f} ± 0.08
	Overall Acceptability		
0 DAS	6.35^{c} ± 0.02	5.27 ^{b} ± 0.02	6.95 ^{e} ± 0.02
15 DAS	6.20 ^{c} ± 0.02	5.22 ^{b} ± 0.05	6.75 ^{e} ± 0.03
30 DAS	6.10 ^{bc} ± 0.02	5.11 ^{ab} ± 0.07	6.60 ^{de} ± 0.05
45 DAS	5.90 ^{b} ± 0.05	5.00 ^{a} ± 0.06	6.50 ^{d} ± 0.05
60 DAS	5.70 ^{b} ± 0.03	4.90 ^{b} ± 0.06	6.40 ^{d} ± 0.03

Each value is the mean of three independent samples.
Mean values ± SD
Means followed by the same letters within each column were not significantly different. (P<0.01 - Tukey test).

Microbiological analysis

The microbial analysis of the *Aloe vera* based RTS formulations were performed at 15, 30, 45 and 60 days after storage. Treatments from any microbial growth till the end of storage period in terms of bacterial plate count. This indicates that the RTS formulations can be stored without any microbial damage till 60 days after preparation. The absence of the microbial count could be because of many reasons particularly use of citric acid and KMS as preservative and pasteurization. The results are in accordance with the findings of (Ramachandran and Nagarajan 2014).

CONCLUSION

The results of the present investigation provide an effective way of delivering the bioactive benefits of *Aloe vera* juice in a tasty and refreshing way to the consumers. The study revealed that the all the variation were suitable for storage up to 60 DAS as revealed by physic-chemical and sensory attributes analysed. The storage studies performed indicate that the *Aloe vera* based RTS formulations can be stored up to a period of 60 days at ambient temperature without any spoilage and deterioration in desirable characteristics.

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Contribution of the authors

1. Senior Research Fellow for the Project (1st Author): Routine research study and data recording.
2. Co-PI of the Project (2nd Author): Helped in product development and Manuscript preparation

3. PI of the Project (3rd Author): Product analysis, manuscript preparation and data analysis.

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