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STORAGE STABILITY AND THE ANTIOXIDANT ACTIVITIES OF OPTIMISED ALOE VERA–LEMON–ORANGE BLEND

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ABSTRACT
In order to develop a healthy ready to serve beverage from *Aloe vera* gel, lemon juice and orange juice, the Box Behnken design with 15 experimental runs was used to optimise the ingredient composition. *Aloe vera* gel (40≤A≤70%), lemon juice (20≤B≤40%) and orange juice (30≤C≤60%) were used as the independent variables while pH, total soluble solid, titrable acidity and vitamin C were the responses. The optimal blend was evaluated for physico-chemical changes, bioactive compounds, antioxidant activities and sensory characteristics during cold storage. The results showed that actual optimized composition of the blend was in ratio 47:14:39 for *Aloe vera* gel, lemon juice and orange juice, respectively. There were no remarkable changes ($p < 0.5$) in pH, total soluble solids, total titratable acidity and sensory scores of the juice blend during storage while the loss of vitamin C, total phenol content, total flavonoid content, antioxidant activities (ferric reducing antioxidant power and 1,1-dyphenyl-2- picrylhydrazyl) after 60 days storage were 66%, 35%, 48%, 21% and 44%, respectively. The *Aloe vera*-lemon-orange blend had good sensory properties and storage stability.

Keywords: *Aloe vera*, Lemon, Orange, Antioxidant activities, storage studies

INTRODUCTION
Aloe barbadensis Miller commonly called *Aloe vera* is a member of the *Liliacea* family. It is a perennial herb grown throughout Nigeria. The beneficial properties of *Aloe vera* have led to its utilization since ancient times. The therapeutic properties of *Aloe vera* are ascribed to the inner colourless gel contained in the *Aloe vera* leaves (Shubhra *et al*., 2014). The gel has antioxidant, radical scavenging activities, and anti-inflammatory properties due to its vitamins A, C and E. In traditional medicine, it has been used for alleviating pain and treating variety of ailments from burns and lacerations, ulcers, dermatitis, high blood pressure, hair loss and leprosy (Grindlay and Reynolds, 1986; Borrelli and Izzo, 2000). *Aloe vera* has been used in various forms such as fresh gel, juice and other formulations for health and medicinal purposes and also for cosmetic purposes (Shubhra *et al*., 2014). Due to the expanding global interest in the use of plants and fruits for their proposed health benefits, *Aloe vera* drink/beverage is becoming more accepted. The beverage industry is also turning towards hybridization of traditional beverage. These include traditional drinks combined with fruit juices that improve their health-promoting characteristics, flavour and acceptability.

Citrus fruits are essential in influencing the sweetness and sourness of a drink and also improving the general well being of consumers. They contain high level of vitamin C and are extremely rich in antioxidants and phytochemicals (Marlett and Vollendorf, 1994). The antioxidant activities of citrus fruits are attributed to the high level of vitamin C content. Vitamin C obtained from lemon by-products was shown to improve the antioxidant activity of new beverages (Majchrzak *et al*., 2004).

Mixing two or more kinds of fruits can result in a product with more vitamins and minerals and with different sensory, colour and flavour characteristics when compared with the raw materials (Akinwale, 2000; Rodrigo *et al.*, 2003; Jain and Khurdiya, 2004). During preparation of fruit juice mix, the colour, sugar or natural acid, which gives sweetness and sourness, have needs to be balanced to give a desirable fruit juice. Optimization is therefore, a special technique developed to increase the desirable quality parameters by analyzing the various components of individual factors such as sourness, sweetness and dilution, required in relation to sensory evaluation to obtain maximum acceptability. It can be achieved by statistical design using Response Surface Methodology (RSM). RSM is an innovative approach to model a system with the collection of statistical techniques wherein interactions between multiple processes variables can be identified with a fewer experimental trials (Bas *et al*., 2007). It has been highly useful and widely applied for optimum formulation for food product (Singh *et al*., 2004, Wadikar *et al*., 2010).

Considerable amounts of research have been carried out to develop new healthy drinks that trigger an increase in plant juice consumption for a wholesome diet laden with antioxidant and phytochemicals. Therefore, this study evaluated the antioxidant activity, bioactive compounds, sensory characteristics and stability of optimised blend of Aloe vera, lemon and orange juice during 60 days cold storage.

Materials and Methods

***Raw Materials*:** Fresh leaves of *Aloe vera* L. (*Aloe barbadensis* Miller) plants were carefully picked from a local farm in Ilesa, Osun State. The leaves were cleaned and the gel was extracted using the traditional hand filleting method of Ahlawat and Khatkar, (2011). The leaves were cut vertically into two half and the inner transparent, mucilaginous pulp was scooped out with a stainless spoon. The gel was homogenised using a blender (Kenwood), strained and stored at 4°C until further use. Fresh orange and lemon were obtained from a farm in Ilesa, Osun State, cleaned, halved and juiced using an electric citrus extractor (National Inc. model, USA). The freshly squeezed juices were stored at 4°C for further use.

Reagents and standards: The **following** chemicals were used: 1,1-diphenyl-2-picryl-hydrazyl (DPPH), 2,4,6-tripyridyl-s-triazine (TPTZ), 2,6, dichlorophenolindophenol, L(+)-Ascorbic acid, glacial acetic acid, sodium acetate trihydrate, ferric chloride, aluminium trichloride, Folin-Ciocalteau reagent (FC reagent), gallic acid, hydrochloric acid (HCl), methanol, phenolphthalein indicator, quercetin, sodium bicarbonate, sodium carbonate anhydrous, sodium chloride (NaCl), sodium hydroxide (NaOH) and sodium nitrite. All other chemicals and solvents used in this study were of analytical grade.

Experimental design for optimization of formulation: The ingredient composition of the blend was optimized by RSM (Design expert 8.0). The experiment was designed according to the Box-Behnken design consisting of 15 experimental runs, including 3 centre point replications, which was used for fitting a second-order response surface. The design was used to evaluate the combined effect of 3 independent variables; *Aloe vera* (A), lemon (B), and Orange (C) juice while pH, vitamin C, total soluble solids (TSS) and titratable acidity (TA) were the responses. The experimental ranges of independent variables used with actual and coded values are given in Table 1. The variance for each factor assessed was partitioned into linear and quadratic components and were represented using the second order polynomial function as follows:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i A + \sum_{i=1}^{n} \beta_{ii} B + \sum_{i \neq j=1}^{n} \beta_{ij} C \qquad (1)$$

where, β_o was the value of the fitted response at the centre point of the design (0,0,0), i.e., the intercept; β_i, β_{ii} *and* β_{ij} were the linear coefficient, quadratic coefficient and cross

product (interaction effect) regression terms respectively and 'n' denoted the number of independent variables.

Table 1: **Experimental ranges and levels of independent variables used in RSM in terms of actual and coded factors for *aloe vera*-lemon-orange blend**

Variables (%)	Range of Levels					
	Actual	Coded	Actual	Coded	Actual	Coded
Aloe vera gel juice (A)	40	-1	55	0	70	+1
Lemon juice (B)	20	-1	30	0	40	+1
Orange juice (C)	30	-1	45	0	60	+1

***Processing and formulation of the blend*:** The flow chart for the processing of *Aloe vera* leaves, oranges and lemon into *Aloe vera*-lemon-orange blend is shown in Figure 1. Sodium benzoate was used as preservative.

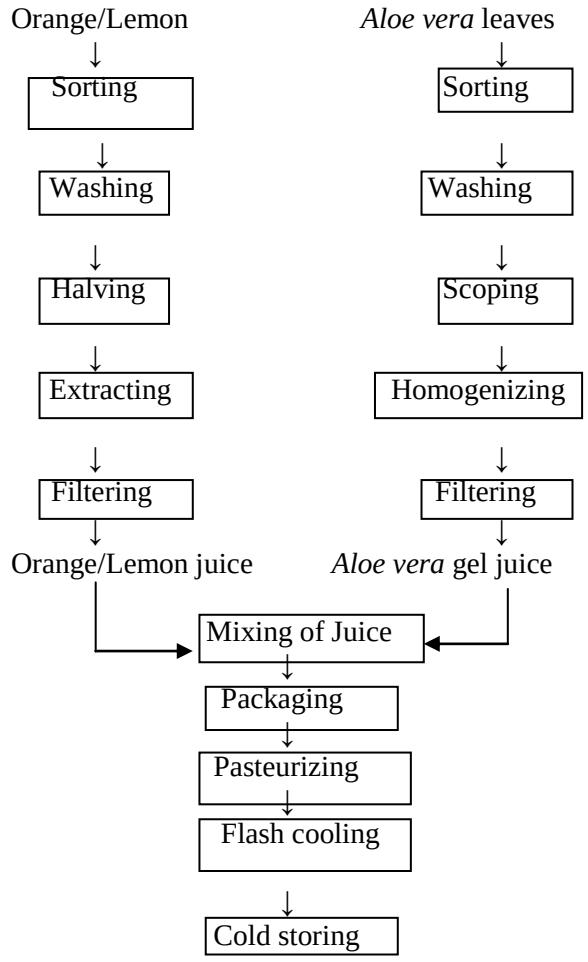


Figure 1: Flow chart for the production of *Aloe vera*-lemon-orange blend

Analytical evaluation

pH, TSS and TA

The pH values were measured using a pH-metre (Hanna Instrument, USA). Total soluble solid (TSS) was measured using a refractometer with values expressed as °Brix. Titratable acidity (TA) and vitamin C were carried out using standard AOAC (2012).

Total phenolic content (TPC)

TPC was determined using the Folin-Ciocalteu reagent by modifying the method of Singleton and Rossi (1965). About 100 µl of sample was mixed with 50 µl of Folin Ciocalteu reagent (10-fold dilution with distilled water) and allowed to stand for 5 minutes at room temperature. Then, 300 µl of 20 % sodium carbonate was added to the mixture. The solution was vortexed and kept aside for 15 minutes before 1ml of distilled water was added. The blue colour produced was measured against a reagent blank at 725 nm using a UV-visible spectrophotometer (Shimadzu, model UV-1800)). The total phenolic content was determined from a standard curve of gallic acid at 0.02 – 0.1 mg/ml concentrations. Total phenolic content was calculated and expressed as µg per 100 ml of gallic acid equivalent (GAE) of the blend.

Total flavonoid content (TFC)

TFC was obtained from a regression equation of the calibration curve of quercetin, and expressed as quercetin equivalent (QE). The total flavonoid content of the blend was determined using the method of Jia *et al*. (1999). The sample (250 µl) was mixed with 1.25 ml of distilled water

followed by addition of 75 µl of 5% sodium nitrite solution and allowed to react for 5 min. Then 150 µl of 10% aluminium chloride was added and allowed to further react for 6 min before 500 µl of 1 M sodium hydroxide was added. Distilled water was added to bring the final volume of the mixture to 3 ml and the absorbance of the mixture was immediately measured at 510 nm against a prepared blank sample consisting of the sample solution and distilled water.

1,1-dyphenyl-2- picrylhydrazyl (DPPH)

The ability of the extract to scavenge 1,1-dyphenyl-2- picrylhydrazyl (DPPH) free radicals was assessed by the method described by Kumarasamy *et al.* (2007). An aliquot of 1 ml of blend and ascorbic acid at different concentrations (0.02 – 0.1mg/ml) was mixed with 1 ml of 100 µM DPPH (dissolved in 80% ethanol). The mixture was vigorously shaken and left to stand at room temperature for 30 min in a dark room. The absorbance was read at 515 nm against blank (80% ethanol). The scavenging effect was calculated using the following equation:

$$\text{Scavenging effect (\%)} = [1 - (\text{Absorbance sample}/\text{Absorbance control})] \times 100 \quad (2)$$

The EC_{50} was determined from the graph of scavenging activity against the concentrations of the samples, which is defined as total antioxidant necessary to decrease the initial DPPH radical by 50 %.

Ferric reducing antioxidant power (FRAP)

The FRAP assay was assessed according to the method of Benzie and Strain (1999). Approximately 1.5 ml of FRAP reagent (0.1 M acetate buffer: 0.02 M $FeCl_3$: 0.01 M TPTZ = 10:1:1) freshly prepared was mixed with 50 µl of diluted sample and incubated for 30 mins at 37 °C. The absorbance of the mixtures were taken at 593 nm and the FRAP concentration was determined from an ascorbic acid standard curve. The antioxidant potential of the sample was determined against a standard curve of ascorbic acid and the FRAP value was expressed as µM Fe^{2+} equivalents per 100 ml of blend. It is assumed that the higher the measured FRAP value, the higher the content of antioxidants which could reduce the ferric ion to ferrous ion.

Sensory Evaluation

Samples were assessed by 15 semi-trained members for colour, taste, aroma and overall acceptability of the blend. The panellists were asked to rate the samples on a 9-point Hedonic scale where 9 meant "like extremely" and 1 meant "dislike extremely".

Storage Studies

The optimal blend was packaged in plastic bottles and kept under refrigerated storage condition for 60 days. Analyses were carried out at 10 days interval.

Statistical analysis

All analyses were done in triplicates. Analysis of variance and test of significance were carried out using SPSS 16.0 software (Chicago, USA) at 95% confidence level.

Results and Discussion

In the optimisation of *Aloe vera*-lemon-orange blend, acidity and pH were chosen as the responses since the product contains lemon as a stabilizing ingredient. Vitamin C and TSS were also taken as other responses in the study as juice quality determinants. The experimental design with actual levels of independent variables and the responses used in this study are shown in Table 2. The result showed that the pH values ranged between 3.80 – 4.02 while vitamin C content was between 166 - 230 mg/100 ml, the total soluble solid content was 3.4 – 4.2 °Brix and the titratable acidity ranged between 0.12 – 0.14 mg/100ml.

Table 2: Design of experiments with actual level of variables in *Aloe vera*-orange-lemon blend on pH, Vitamin C, TSS and TTA

Run Order	Factors			Responses			
	Aloe vera gel	Lemon juice	Orange juice	pH	Vitamin C (mg/100ml)	TSS (°Brix)	TA (mg/100ml)
	%						
1	40	20	45	3.82	191.0	4.0	0.13
2	40	40	45	3.83	198.0	4.2	0.14
3	70	20	45	3.92	178.0	3.8	0.12
4	70	40	45	3.89	193.6	3.4	0.14
5	40	30	30	3.82	208.3	4.0	0.13
6	40	30	60	3.97	230.3	4.4	0.14
7	70	30	30	3.94	166.6	3.6	0.13
8	70	30	60	4.02	196.0	3.8	0.13
9	55	20	30	3.83	181.3	3.6	0.14
10	55	20	60	3.93	208.0	4.0	0.13
11	55	40	30	3.88	198.5	3.4	0.14
12	55	40	60	3.80	220.5	3.6	0.13
13	55	30	45	3.91	205.8	4.0	0.13
14	55	30	45	3.89	196.0	4.2	0.13
15	55	30	45	3.92	193.6	4.0	0.13

The effects of independent variables on the responses are represented in the perturbation graph (Figures 2a-d). Figure 2a revealed that *Aloe vera* gel and orange juice showed an increasing effect with reference to pH while lemon juice levels lowered the pH of the blend. The ascorbic acid content was influenced by the level of *Aloe vera* gel, lemon juice and orange juice as represented in the Figure 2b. Figure 2c shows that the TSS of the blend was significantly affected by the level of *Aloe vera* gel. The titratable acidity decreased with respect to the levels of *Aloe vera* gel and orange juice and increased as lemon juice increased as revealed in Figure 2d. The regression equation obtained for the responses are as follows:

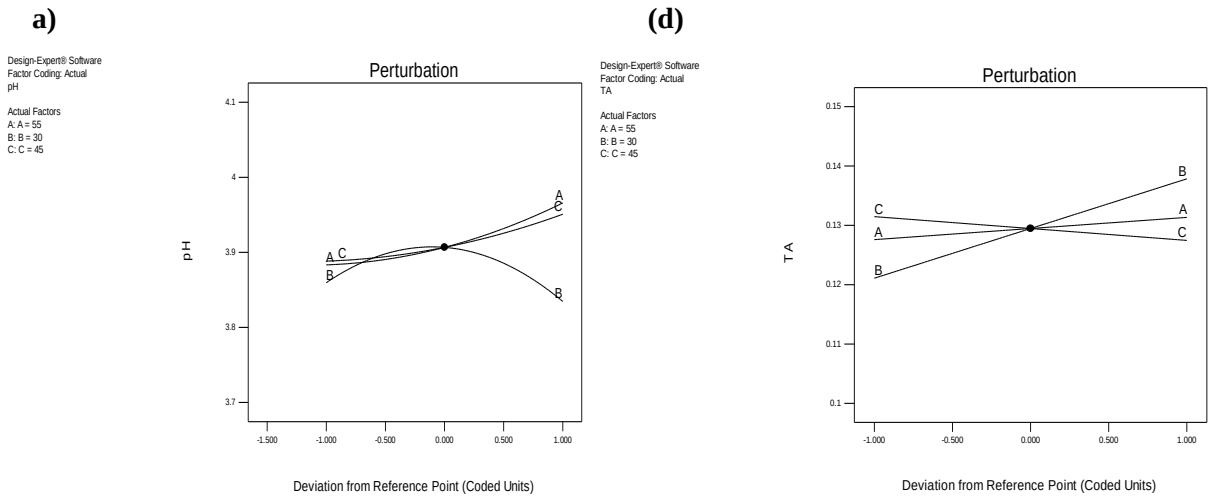
$$\boldsymbol{pH} = +3.91 + 0.041A - 0.0125B - 0.03125C - 0.01AB - 0.0175AC - 0.045BC + 0.0179A^2 - 0.06B^2 + 0.01292C^2$$

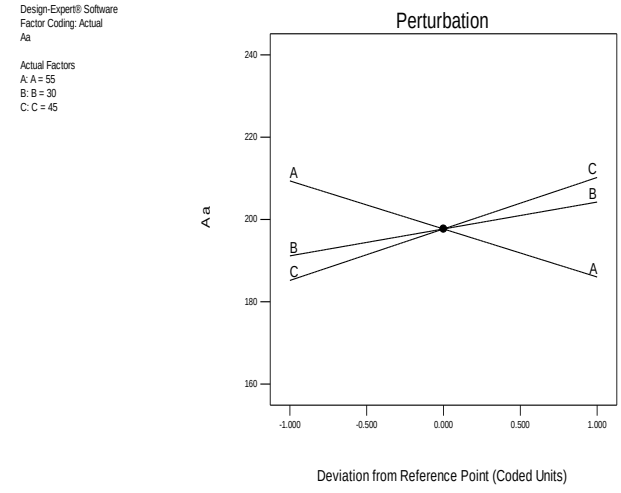
$$\textbf{Vitamin C} = 197.70 - 11.68\text{A} + 6.54\text{B} + 12.51\text{C}$$

$$\textbf{TSS} = 4.07 - 0.25\text{A} - 0.10\text{B} + 0.15\text{C} - 0.15\text{AB} - 0.050\text{AC} - 0.050\text{BC} + 0.0417A^2 - 0.258B^2 - 0.158C^2$$

$$\textbf{TA} = 1.31 - 0.025\text{A} + 0.044 - 0.024\text{C}$$

These responses were subjected to model fitting by studying the ANOVA values and using the diagnostics tool available in the Design Expert Software. Table 3 shows the ANOVA and model statistic for the four responses. The quadratic model was fit for the pH and TSS responses with F-value of 4.17 and 14.37 respectively while for vitamin C and TA responses, linear model was found suitable with non significant lack of fit. The results showed that the models for the responses of pH and TSS were highly adequate because they have satisfactory levels of R^2 of more than 80%.





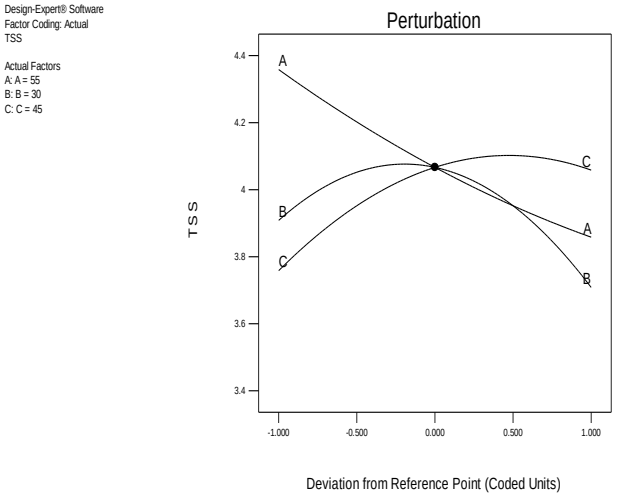


Figure 2: Pertubation graph showing the effect of independent variables on (a) pH (b) ascorbic acid (c) total soluble solids (d) titratabe acidity

Optimisation of blend formulation

The optimization criterion was to maximise the pH and keeping the other responses (TA, TSS, and Vitamin C) in range along the fitted polynomial models. The best combination with a suitable fit model was chosen as the optimized composition. The optimised ingredient compositions of the independent variables with the predicted responses are given in Figure 3 with the desirability (0.972) plot with respect to *Aloe vera* and orange juice in Figure 4.

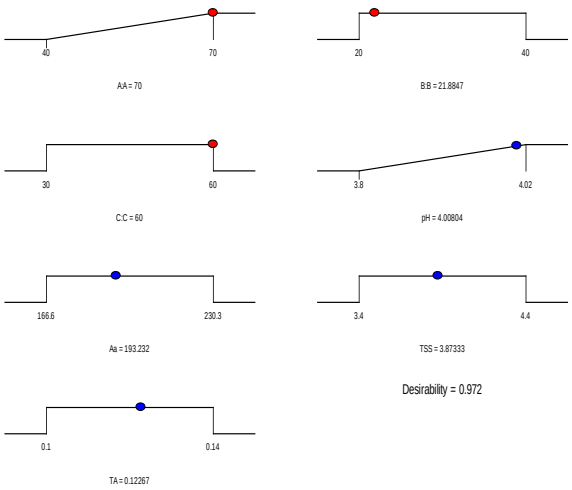


Figure 3: Optimised ingredient level and the predicted response for *Aloe vera*, Lemon and Orange

Table 3: ANOVA and Model Statistic for *Aloe Vera*-Lemon-Orange Blend

Term	Response			
	pH	**Vitamin C**	**TSS**	**TA**
Model	Quadratic	Linear	Quadratic	Linear
F – Value	4.17	11.05	14.37	2.02
P > F	0.0652	0.0012	0.0045	0.1702
Lack of fit	8.54	2.15	0.50	0.77
Mean	3.89	197.73	3.87	0.13
S.D.	0.036	9.00	0.097	0.00538
C.V. %	0.92	4.55	2.50	4.05
R squared	0.8824	0.7511	0.9628	0.3547
Adjusted R squared	0.6707	0.6832	0.8957	0.1787
Predicted R squared	-0.7645	0.4900	0.6968	-0.3389
Adequate precision	6.696	10.408	13.311	4.500

S.D. – standard deviation

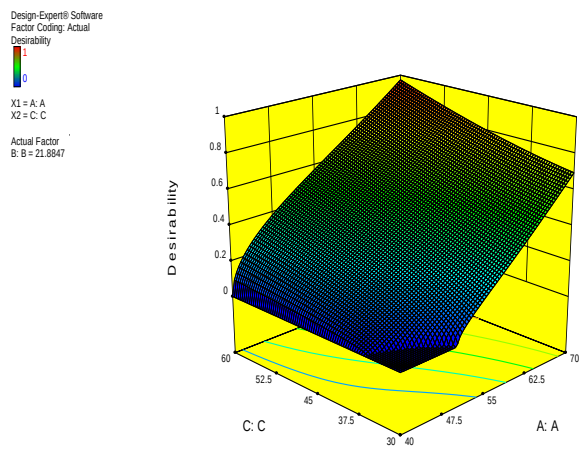


Figure 4: Desirability plot for *Aloe vera*-lemon-orange blend

The optimised levels of *Aloe vera* gel juice, lemon juice and orange juice were 46.5 ml, 14.4 ml and 39.1 ml with predicted values for pH, ascorbic acid, TA and TSS of 4.01, 193.2 mg/100 ml, 0.12 % and 3.87 °Brix respectively. The blend was prepared using the optimized ingredient compositions and was evaluated for the responses to validate the prediction strength of the models. The actual responses of the blend made from the optimised level were 4.01, 147.9 mg/100 ml, 0.12 % and 3.88 °Brix for pH, ascorbic acid, TA and TSS respectively. The predicted and the actual values were in close agreement with each other thereby validating the robustness of the models. Thus the final ingredient composition of the optimised blend was *Aloe vera* gel juice 47 ml, lemon juice 14 ml, and orange juice 39 ml.

Storage stability

The storage stability studies (Table 4) of the blend revealed that the pH values of the blend changed from 4.01 – 3.90 while the TA of the blend increased from 0.12 to 0.14 mg citric acid/100 ml during storage. The TSS value of the blend changed from 3.88 to 5.08 °Brix. The vitamin C value (147.9±4.35 mg/100 ml) decrease throughout storage.

A loss of 66% ascorbic acid was noted after 60 days of cold storage. Oszmiański *et al*., (2007) reported losses in vitamin C as 58 %, 7-19 % and 26 % in apple, apple juice with blackcurrant and blackcurrant juices respectively during 6-month storage at 4 °C. This result is also similar to the report of Burdulu *et al*., (2006), who reported high ascorbic acid destruction in lemon and orange juice concentrate and established that ascorbic acid decomposes easily in acidic solution.

Table 4: Effects of Storage on the Physico-chemical Properties, Bioactive Compounds and Antioxidant Properties of *Aloe vera*-Lemon-Orange Blend

Parameters	Storage time (days)						
	0	10	20	30	40	50	60
TSS (°Brix)	3.88±0.00^{a}	4.88±0.02^{b}	4.94±0.12^{bc}	4.88±0.01^{b}	5.08±0.01^{c}	5.08±0.01^{c}	5.08±0.01^{c}
pH	4.01±0.00^{d}	3.97±0.00^{c}	3.94±0.00^{b}	3.94±0.00^{b}	3.90±0.00^{a}	3.94±0.00^{b}	3.97±0.00^{c}
TA (mg citric acid/100ml)	0.12±0.00^{a}	0.13±0.00^{b}	0.14±0.00^{c}	0.14±0.00^{c}	0.14±0.00^{c}	0.14±0.00^{c}	0.14±0.00^{c}
Vitamin C (mg/100ml)	147.9±0.60^{g}	136.3±2.30^{f}	121.8±1.76^{c}	108.8±0.92^{d}	88.5±0.00^{c}	75.4±1.08^{b}	50.2±2.92^{a}
Phenol (µg GAE/ml)	9333±0.00^{g}	8813±23.09^{f}	8685±33.49^{e}	8027±47.92^{d}	7529±58.50^{c}	6652±23.67^{b}	6098.67±21.36^{a}
Flavonoids (µg QE/ml)	1851±28.87^{f}	1633.3±27.83^{e}	1444.5±38.49^{d}	1083±28.87^{c}	977.7±38.49^{b}	822.2±38.49^{a}	960±0.00^{b}
FRAP (µg AAE/ml)	6.6±0.09^{e}	6.4±0.06^{e}	6.2±0.02^{d}	5.9±0.03^{c}	5.7±0.07^{c}	5.4±0.04^{b}	5.2±0.02^{a}
DPPH (EC_{50}) (µl/100ml)	3.52±0.40^{a}	3.69±0.33^{ab}	4.31±0.08^{bc}	4.40±0.13^{c}	5.86±0.25^{d}	6.21±0.08^{d}	6.34±0.12^{d}

(TSS - total soluble solid; TA – titratable acidity)
Means followed by the same letter in a row are not significantly different.

At the end of storage there was a 35 % loss with respect to the initial value of 9333 µg/ml in total phenolic content. The decline in phenolic content was probably due to polyphenol oxidases activities that degrades phenolics in fruit or probably due to precipitation of flavanones, a major compound found in orange and lemon. Ryszard *et al*. (2009) reported that a dramatic loss in juice phenolics (35 %) during freezing due to storage and thawing.

Flavonoid content decreased significantly during the storage period with a percentage loss of 48%. Ryszard *et al*. (2009) monitored orange juices and reported about 20 % loss of flavones during freezing after one month storage.

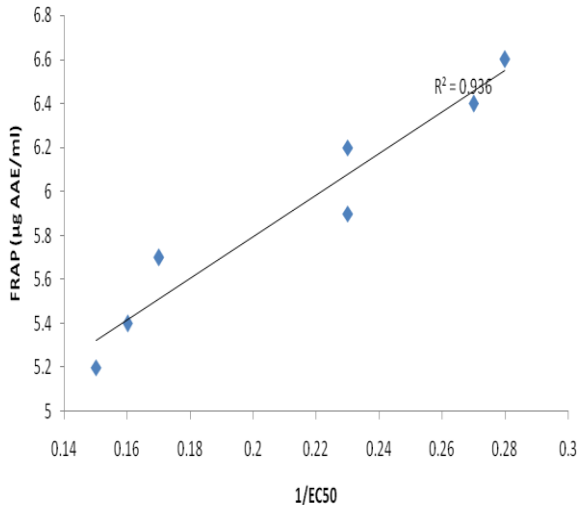
The antioxidant capacity of the blend was analysed by using free radical scavenging (DPPH) assay and ferric reducing antioxidant power (FRAP). DPPH assay measures the ability of the fruit extract to donate hydrogen to the DPPH radical resulting in bleaching of the DPPH solution. The greater the bleaching action, the higher the radical scavenging, and this is reflected in the EC_{50} value. FRAP measures the reducing potential of the extract to react with ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex and produce blue colour of ferrous form (Fe^{2+}TPTZ) (Benzie and Strain, 1996). The higher the FRAP value, the greater is the antioxidant activity. The antioxidant capacity evaluated by DPPH radical scavenging (EC_{50}) ranged from 3.52 to 6.34. The lower the EC_{50} value, the higher the antioxidant capacity. The antioxidant activity of blend analysed, decreased by 44 % after 60 days of storage. The FRAP value declined upon storage, at the end of storage; the value was reduced by 21 %. Klimczak *et al*. (2007), Plaza *et al*. (2006) and Yang *et al*. (2007) reported that a decline of FRAP values and radical scavenging activity (DPPH) of tropical fruit juice during storage may be related to destruction of ascorbic acid.

Correlation of Antioxidant Activities and Bioactive Compounds

Figure 5a and 5b shows a linear correlation between $1/EC_{50}$ and FRAP (R^2 = 0.936) and DPPH and FRAP (R^2 = 0.9302) assays, respectively. A strong correlation which indicated that the blend possessed antioxidant activities by the mechanisms of free radical scavenging and the reducing power was observed. The reducing power of bioactive compounds is responsible for the antioxidant activity (Yildirim *et al*., 2001). Figures 6a and 6b shows the relationship between total phenolics content and total flavonoid content with FRAP and DPPH values respectively. The R^2 of TP versus FRAP and FC versus DPPH

were 0.983 and 0.828 respectively. These suggest that the presence of the phenolic and flavonoid compound largely accounted for the antioxidant capacity measured with DPPH and FRAP. Several reports also showed a close relationship between total phenolic content and high antioxidant activity (Prasad *et al*., 2005; Amin *et al*., 2006; Li *et al*., 2009).

(a)



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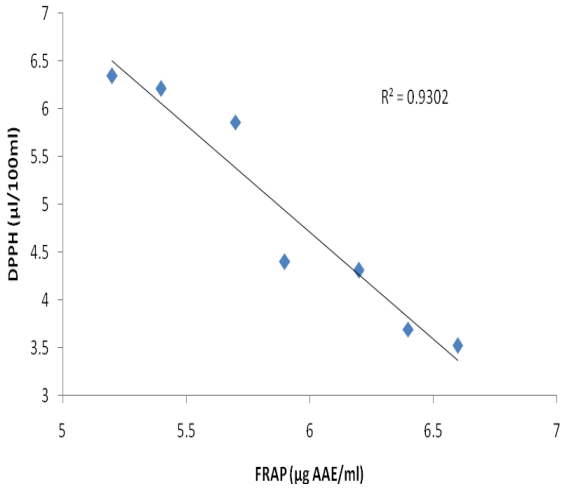
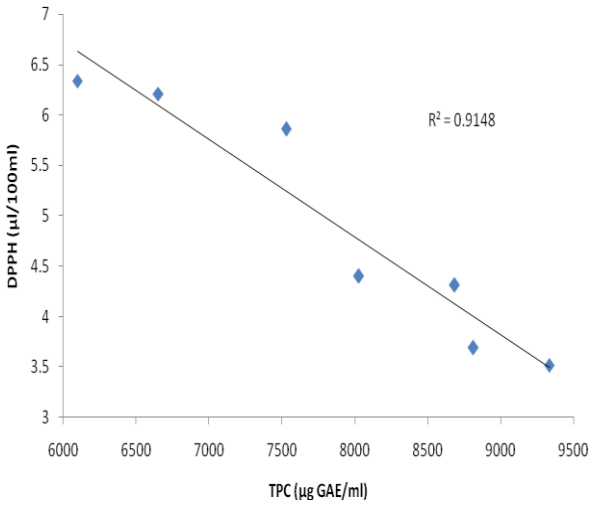


Figure 5: Correlation between (a) FRAP and $1/EC_{50}$ (b) DPPH and FRAP

(a)



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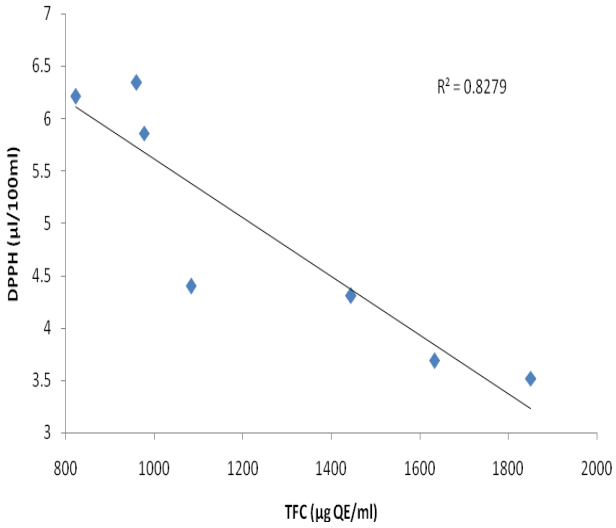

Figure 6: Correlation between (a) reducing power and TPC (b) free radical scavenging activities and TFC during storage.

Sensory Evaluation

Sensory scores (Table 5) for colour, aroma, taste and overall acceptability were ranged from 7.6 to 7.8, 4.6 to 4.8, 6.9 to 7.2 and 5.2 to 5.6 respectively. Colour and aroma was relatively good as shown by the high sensory scores recorded during its evaluation. The overall acceptability was determined by the taste of the blend. There was no significant ($p<0.05$) change in the sensory quality of the blend.

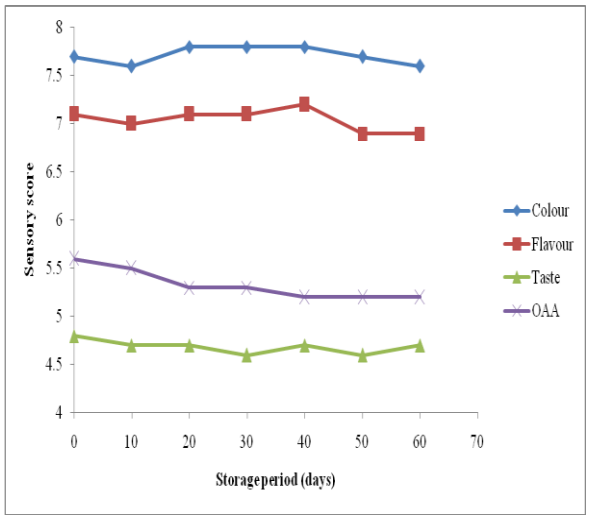

Figure 4.9: Sensory analysis (1: dislike extremely,2: dislike very much,3: dislike moderately, 4: dislike slightly, 5: neither like nor dislike, 6: like slightly, 7: like moderately, 8: like very much, 9: like extremely)

Conclusion

The investigation in the production of *Aloe vera*-lemon-orange blend using RSM indicated that Box-Behnken design was effective in optimising the ingredient composition. The phytochemicals and antioxidant activities of the blend reduced during 60 days refrigeration storage while changes in the physico-chemical properties were negligible. Maintaining the antioxidant activities and sensory attributes of the blend can improve its function.

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