International Journal of Biochemistry Research & Review
9(3): 1-9, 2016, Article no.IJBcRR.22547
ISSN: 2231-086X, NLM ID: 101654445

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Carotenoid Levels, Total Phenolic Content and Antioxidant Activity Variations in Varieties of *Citrullus lanatus* under Storage at Room Temperature

J. Y. Jr. Hena[1,2], D. M. Andala[3], H. N. Nyambaka[1] and M. P. Nawiri[1*]

[1]Kenyatta University, Nairobi, Kenya.
[2]University of Liberia, Monrovia, Liberia.
[3]Multimedia University of Kenya, Nairobi, Kenya.

Authors' contributions

This work was carried out in collaboration between all authors. Author HNN designed the study, wrote the protocol and supervised the work. Author JYJH carried out all laboratories work and performed the statistical analysis. Author DMA managed the analyses of the study. Author MPN wrote the first draft of the manuscript. Author JYJH managed the literature searches and edited the manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/IJBCRR/2016/22547
Editor(s):
(1) Carmen Lúcia de Oliveira Petkowicz, Federal University of Parana, Curitiba, Parana, Brazil.
Reviewers:
(1) Anonymous, University of Missouri-St. Louis, USA.
(2) Yongchun Zhu, Shenyang Normal University, China.
Complete Peer review History: http://sciencedomain.org/review-history/12587

Original Research Article

Received 9th October 2015
Accepted 10th November 2015
Published 8th December 2015

ABSTRACT

Introduction: Non-communicable diseases (NCDs) are economically burdening and are globally projected to increase deaths upto 75% by 2030. With the cause partly attributed to unhealthy diets, the major contributing factor is production of reactive oxygen and nitrogen species in biochemical reactions in human cells. Through a dietary approach, this can be countered by carotenoid and phenolic antioxidants in *Citrullus lanatus* (watermelon). The challenge presented is on the effect of room temperature (RT) storage on the levels of β- carotene, lutein and lycopene carotenoids and phenolics in watermelon. Varieties, of watermelon grown in Mwea, Kenya are reported for their carotenoid levels; antioxidant activity (AA) and total phenolic content (TPC) within 14 days storage period at RT.

**Corresponding author: E-mail: nawiri.mildred@ku.ac.ke;*

Methods: The methods employed were HPLC, FCR and DPPH assay.
Results: The ranges of carotenoid levels were; β-carotene; 0.13±0.03- 3.15±0.02 µg/100 g, lutein; 10357.58±62.98-30573.99±434.40 µg/100 g and lycopene 113.42±0.39-522.76±1.36 µg/100 g. The AA ranged between 3.10±0.04-41.35±0.29 and 59.12±0.15- 79.93±0.18% in fresh and dried watermelons, while the TPC ranged between 266.00±5.00-896.67±7.37 mg/100 g GAE. The effect of storage on the nutritional values was shown by a general decrease on levels of β-carotene, lycopene, AA and TPC while lutein levels increased. There were significant differences ($p<0.05$) in measurements of carotenoids, AA and TPC between all sample varieties.
Conclusion: Storing watermelons for upto 14 days has both negative and positive implications on the carotenoid and total phenolic levels but clearly reduces the antioxidant activity and hence the effectiveness on the fight against NCDs.

Keywords: Citrullus lanatus; carotenoids; total phenolics; antioxidant activity.

1. INTRODUCTION

About 60% of global mortality, morbidity and disability cases are caused by non communicable diseases (NCDs) such as cardiovascular diseases (CVDs) and cancer among others [1]. In Kenya, over 1.6 million and nearly 7% of those affected are estimated to die annually from CVDs and cancer [2,3]. The statistics on NCDs are mainly pegged to unhealthy diets, tobacco use, exposure to environmental carcinogens and physical inactivity [1]. Fundamentally, these diseases are due to the presence of reactive oxygen and nitrogen species; superoxide, hydroxyl radical and nitric oxide radical, that are a consequence of aerobic respiration [4]. Medication for NCDs is generally unaffordable especially developing nations and therefore a dietary approach, given its efficacy and affordability would be a sustainable approach. As such, specific attention has been drawn to antioxidants (free radical scavengers) including carotenoid and phenolic nutraceuticals that are found in fruits such as *Citrullus lanatus* [5,6].

Watermelon is a member of the cucurbit family and one of the most widely cultivated crops in the world. Its global consumption is greater than that of any other cucurbit. It accounts for 6.8% of the world area devoted to vegetable production and this is expected to increase due to the increasing demand in the European market due to unfavorable weather experienced in those countries [7]. The recommended varieties are Charleston Gray, Congo, Crimson Sweet, Moon and Stars, Orangeglo, Sugar Baby, Sunday Special. In Kenya, the fruit is a good cash crop with very good market opportunities, particularly in urban areas [8]. The levels of nutraceuticals as well as antioxidant activity in fruits vary depending on factors including climatic conditions, part of the plant used (example, the peel, seeds and red/pink fleshy portion),variety or species, storage period and condition [9-11]. The effect on β- carotene, lutein and lycopene carotenoids as well as the total phenolic content and the antioxidant activity with storage of varieties of *Citrullus lanatus* under normal room temperature were investigated.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Acetonitrile, Dichloromethane, Methanol (all HPLC grade), Hexane, Acetone, Ethanol (all analytical grade), All-trans-lycopene and Lutein standards, Gallic acid, Ascorbic acid (AscA), Butylated hydroxytoluene (BHT), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent, Anhydrous Sodium Carbonate (Na_2CO_3), Anhydrous Sodium Sulphate (Na_2SO_4) and Magnesium Carbonate ($MgCO_3$) were sourced from Sigma-Aldrich Chemical Co., USA. All-trans-beta-carotene standard was obtained from Fluka Chemicals Corp., Switzerland. All chemicals were supplied by Kobian Kenya Limited.

2.2 Experimental Procedures

Varieties of *Citrullus lanatus* were obtained from one geographical region, Mwea in Kenya and identified by a taxonomist. Purposive sampling was employed to obtain mature-ripe samples which were then transported to the laboratory for analysis. Samples were shelved open and stored at normal laboratory room temperature and sun light for 14 days as carotenoid levels, total phenolic content and antioxidant activity were measured. During dried samples analysis of antioxidant activity, samples were diced, air dried on sieve and stored in the laboratory under similar conditions as the fresh samples for two months prior to DPPH assay. Storage in

refrigerator at 4°C was not considered as this may be unaffordable by many consumers especially in developing countries.

The procedures were carried out in subdued light within the shortest possible time to prevent isomerization and photo-degradation of the carotenoids and other phytochemicals [10]. Extraction procedures were done according to [10,12] with minimal modifications.

2.3 Extraction and Analysis of Carotenoids

Two clean samples of pata negra, sugar baby and Charleston grey varieties of *Citrullus lanatus* variety were diced quarterly, opposite quarters homogenized by blending using an electric blender. A 10.0 g (±0.05) of homogenized sample with 0.6 g of $MgCO_3$ (acid neutralizing agent) and 0.8 g of BHT (antioxidant) was sonicated for two minutes and mixed for five minutes with 30 mL of extraction solvents mixture (hexane/acetone/ethanol, 2:1:1 v/v) using an orbital shaker on ice. The mixture was vacuum filtered through filter paper using a 250 mL Buchner flask and funnel and the filtrates transferred into a 250 mL separatory funnel. This process was repeated several times using 10 mL of the extraction solvents until no appreciable colour was observed in both filter paper and the solid residue. A 50 mL aliquot of 15% saturated NaCl solution was added to the filtrate and mixed well for two minutes to enhance phase partitioning. Distilled water was added to themixture to completely wash the organic layer from any impurities. The aqueous phase was decanted and the organic phase was recovered and filtered over 8.0 g of anhydrous $NaSO_4$ which was rinsed three times with 3.5 mL of extraction solvent. The filtrate was evaporated to near dryness under nitrogen using a rotatory evaporator attached to a vacuum pump at 40°C and reconstituted in 5 mL methanol. Extracts were filtered under nitrogen gas into dark reagent vials covered with aluminium foil using a 0.45 µm single membrane filter and stored in the fridge prior to HPLC injection. All extractions and analysis were done in triplicates. Fresh mobile phase consisting of ACN:MetOH:DCM (70:10:20 v/v) was prepared each time by measuring 700 mL (ACN), 100 mL (MetOH) and 200 mL (DCM), filtered and degassed (to remove any air bubble) for 30 minutes using an ultrasonic agitator before use.

Samples and standards (20 µL aliquot) were introduced into the reserved phase HPLC column (Gemini-NX 5uc 18110A, 250 × 4.6 mm id × 5 µm particle size) through an autosampler. The HPLC also consisted of CTO-10ASVP oven and a LC-20AD pump. At a mobile phase flow rate of 0.8 mL/min on a C_{18} column for 18 minutes, the analytes were isocratically eluted. Using a deuterium lamp UV-visible detector (SPD-20A) with sensitivity set at 0.05-absorbance units full scale (aufs), beta-carotene (BC) was detected at 450 nm, lutein at 452 nm while lycopene at 471 nm. The carotenoids peaks were identified by comparing the retention time to that of the standards and by spiking the samples with known concentration of the standards. Quantification was done based on linear calibration curves using peak areas. Concentrations in mg/100g of fresh samples were calculated as shown in Equation (1).

$$C_X\left(mg/100g\right)=\left(\frac{P_X-B}{S}\right)\times D_f\times 100g \quad (1)$$

Where, C_X= concentration of analyte in mg/100 g, P_X = Peak area of analyte, B = intercept, S = slope and D_f= dilution factor

From BC concentration, the amount of retinol equivalents was calculated based on 12:1 conversion for BC/VA (FNB, 2001).

2.4 Extraction of Total Phenolics and Antioxidant Activity

Similar extraction procedure was carried out fort TPC and AA as was done for carotenoids. However, a 3.0 g of homogenized samples with 0.3 g $MgCO_3$ and 0.5 g BHT was sonicated and mixed well with 20 mL of extraction solvents. The mixture was vacuum filtered repeatedely and dried over 5.0 g $NaSO_4$. The filtrate was evaporated to near dryness and redissolved in 5 mL methanol. It was stored in the dark in air tight vials covered with aluminium foil until DPPH and FCR assays.

2.5 Determination of Total Phenolic Content

Total phenolic content of *Citrullus lanatus* extracts was determined by employing the Folin-Ciocalteu reagent (FCR) method with some modifications [13]. A 0.5 mL of extract was mixed with 5 mL of Folin-Ciocalteu regent (diluted 10 times with distilled water) and allowed to stand for 10 minutes at room temperature. A 4 mL of Na_2CO_3 (200 g/L) was added to the mixture, mixed thoroughly and incubated for 60 minutes in

the dark. Triplicate measurements were made and absorbance measured at 765 nm against a blank (containing all the reagents except the sample) and compared to a gallic acid calibration curve. Total phenolics were estimated as gallic acid equivalent (GAE) in milligram per 100 g of extractive (mg of GAE/ 100 g of extractive) using Equation 2.

$$TPC(mg/100gGAE) = \left(\frac{A_E - C}{M}\right) \times D_F \times 100g \quad (2)$$

Where,

TPC_E= total phenolic content of sample extract in mg/100g of Gallic acid equivalent, A_E = absorbance of sample extract, C = intercept, M = slope and D_F = dilution factor.

2.6 Determination of Antioxidant Activity

The free radical scavenging effects (antioxidant activity) was determined at initial storage period (day 2) at extract concentration of 100 µg/mL. This was determined based on the extracts scavenging activity on the stable 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical with slight modifications as described [14,15]. Extract concentration of 100 µg/mL for both fresh and dried samples (100 µg extract in 1 mL of 95 % methanol) was added to 2 mL of 20 ppm DPPH methanolic solution. The reaction mixture was shaken vigorously and incubated for 30 minutes at room temperature in the dark. The absorbance was read at 516 nm against DPPH solution (2 mL DPPH in 2 mL of 95% methanol) as control. The ability of the extracts to scavenge the DPPH radical (antioxidant activity or radical scavenging activity) was calculated as a decrease in the absorbance of DPPH radical using Equation 3.

$$\%RSA = \left(1 - \frac{A_{TS}}{A_C}\right) \times 100 \quad (3)$$

Where, %RSA= percentage radical scavenging activity as a measure of antioxidant activity, A_{TS} = absorbance of test sample, A_C = absorbance of control.

The effective concentration (EC_{50}) of extracts required to decrease the initial DPPH radical concentration by 50% was determined by plotting scavenging activity against concentration of extracts. In similar procedures, the antioxidant activity of two synthetic antioxidants (AscA and BHT) was determined for purpose of comparison.

2.7 Data Analysis

Values obtained for the carotenoids, TPC and antioxidant activity at room temperature storage in the varieties of *Citrullus lanatus* were expressed as means of three replicate measurements ± standard deviation and compared by One-Way ANOVA at 95% using SPSS version 20 for windows. Values of $p<0.05$ were considered significantly different and SNK-test at $\alpha=0.05$ indicated differences among means. Student's T-test was applied for mean comparison of antioxidant activity of fresh and dried samples.

3. RESULTS AND DISCUSSION

3.1 Levels and Trends of Carotenoid and Total Phenolics

The levels of lutein, β-carotene, lycopene, total phenolic content (TPC) and retinol equivalent (RE) determined at day one in the three varieties of *Citrullus lanatus* are presented in Table 1. The trends in level of the carotenoids and total phenolic content during the 14 day storage are presented in Figs. 1-4.

The levels ranged between 10357.58-16287.07 µg/100 g (lutein), 0.49-3.15 µg/100 g (β-carotene), 113.42-522.76 µg/100 g (lycopene) and 0.038-0.242 µg/100 g (RE). The levels of the carotenoids (lutein, β-carotene, lycopene), TPC and RE differred significantly ($p<0.05$) between varieties, which is attributed to among other factors that of variation in the varieties. The highest level of lutein was found in Charleston grey while that of β-carotene, lycopene and RE were found in sugar baby variety. As per Britton and Khachik (2009), the levels of lutein were very high (>2 mg/100 g), whereas β-carotene and lycopene were low (0-0.1 mg/100 g) and moderate (0.1-0.5 mg/100 g) respectively. Calculations show that the daily recommended intake of VA (Retinol equivalent of 1-3 mg/day) can be realized by consuming 10 kg/day of pata negra and sugar baby and 30 kg/day of Charleston grey as this will provide 1.1, 2.42 and 1.14 mg/day RE respectively [16]. The levels of TPC ranged from 266.00 to 896.67 mg/100 g GAE. Charleston grey had the highest TPC level among the three varieties. Although a number of factors not within the scope of this study such as extent of maturity and ripening stage are key, the differences is attributed to be that of differences in the varieties [9,17,18].

The carotenoids and total phenolic content had varying trends during the storage period in the different varieties of *Citrullus lanatus*. Lutein (Fig. 1) showed a regular and significant increase in levels ($p<0.05$), an increase that may be attributed to the conversion of other carotenoids to lutein or to the presence of ε-cyclase and ε-hydroxylase enzymes in *Citrullus lanatus* [6]. Beta-carotene and lycopene (Figs. 2 & 3) did not show a general trend, however a significant change ($p<0.05$) in content was observed with storage period possibly due to the presence and activation of β-cyclase and hydroxylase enzymes, while its reduction in pata negra and in sugar baby may be due to said enzymes inactivation [6]. An identical trend of total phenolic content was shown in the three varieties over the 14 days of storage period, with the levels comparatively high at day 4 (Fig. 4). An extreme decrease in levels was observed between days 4 and 6 while day 6-14 remained relatively low and statistically unchanged ($p>0.05$) in each variety. The degradation of TPC is attributed to the presence of phenolic acids that decrease with storage and ripening of fruits [17]. However, its increase at day 4 could be due to the synthesis of more anthocyanins and other phenolic compounds at said day, which later declined perhaps owing to physiological changes [17].

Table 1. Levels of carotenoids, total phenolic content and retinol equivalent in varieties of *Citrullus lanatus*

	Concentration (Mean±SD; n=3)				
Variety	**Lutein (µg/100 g)**	**β-carotene (µg/100 g)**	**Lycopene (µg/100 g)**	**TPC (mg/100 g GAE)**	**RE (µg/100 g)**
Pata negra	10357.58±62.98^{a}	1.43±0.02^{b}	113.42±0.39^{a}	266.00±5.00^{a}	0.110±0.01^{b}
Sugar baby	15972.27±155.14^{b}	3.15±0.02^{c}	522.76±1.36^{c}	693.00±6.56^{b}	0.242±0.03^{c}
Charleston grey	16287.07±157.81^{c}	0.49±0.01^{a}	248.70±0.70^{b}	896.67±7.37^{c}	0.038±0.01^{a}
p-value	<0.001	<0.001	<0.001	<0.001	<0.001

Mean values with different small letters within the same column are significantly different ($p<0.05$, SNK-test)

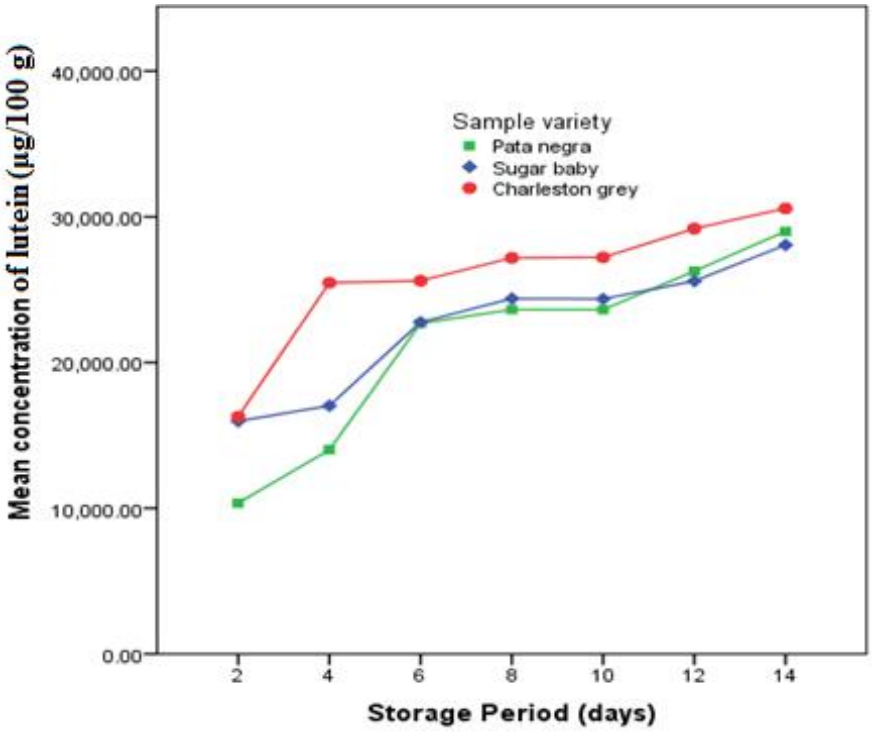

Fig. 1. Trends of levels of lutein in varieties of *Citrullus lanatus* stored at room temperature for 14 days

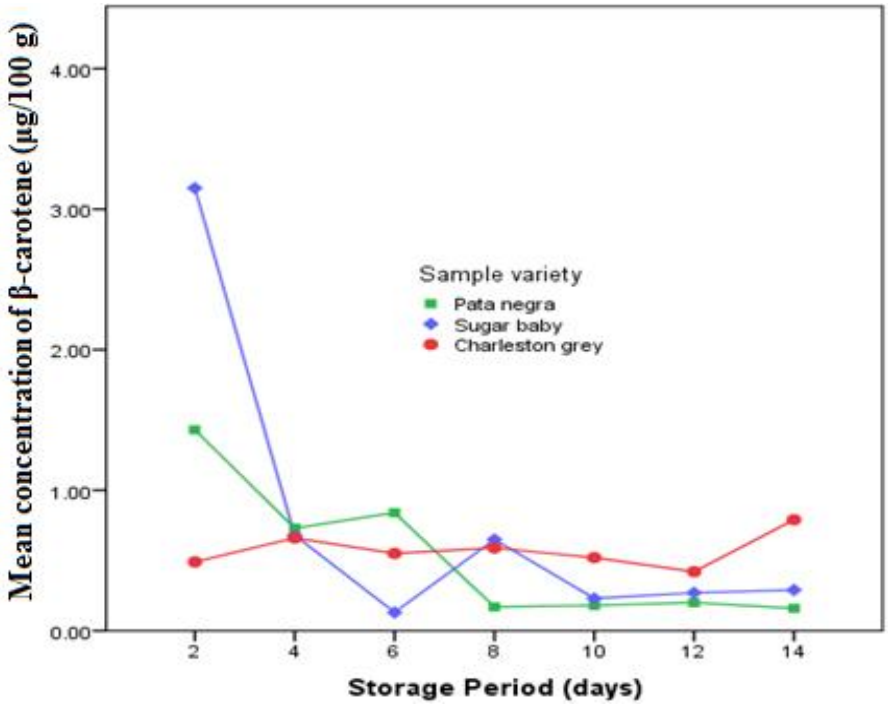

Fig. 2. Trends of levels of β-carotene in varieties of *Citrullus lanatus* stored at room temperature for 14 days

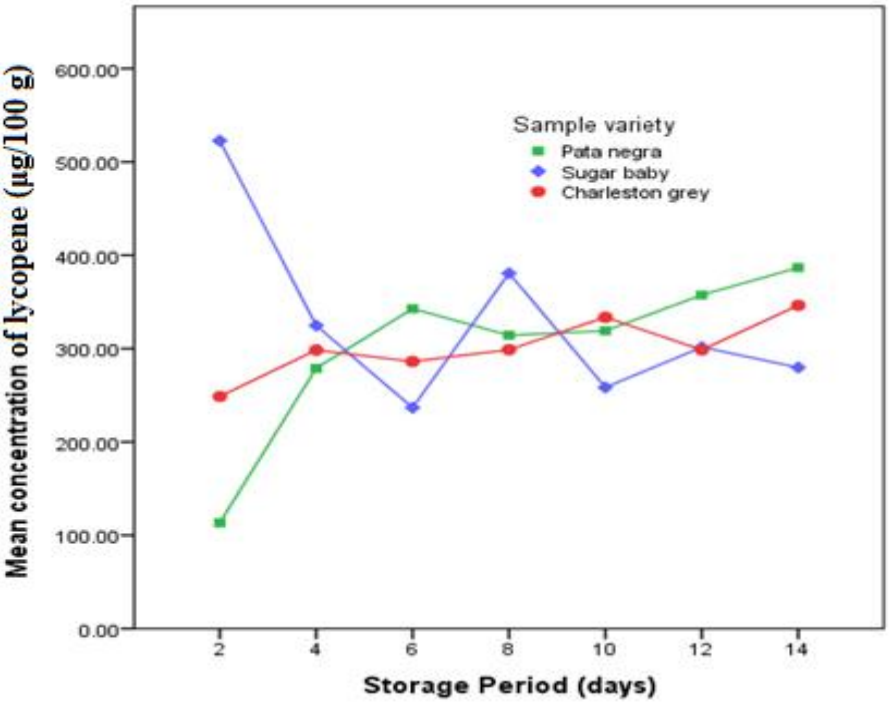

Fig. 3. Trends of levels of lycopene in varieties of *Citrullus lanatus* stored at room temperature for 14 days

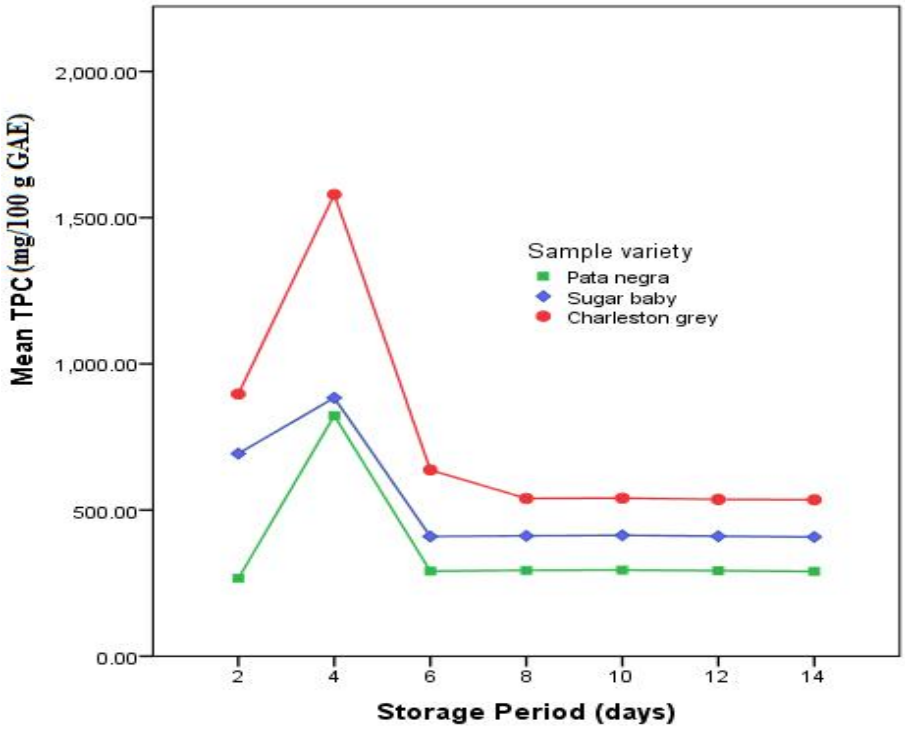

Fig. 4. Trends of levels of total phenolic content in varieties of *Citrullus lanatus* stored at room temperature for 14 days

3.2 Levels and Trends of Antioxidant Activity

The levels, expressed as percentage radical scavenging activity (%RSA) and the effective concentration (IC_{50}) of fresh and dried samples of *Citrullus lanatus* varieties and synthetic antioxidants; ascorbic acid (AscA) and BHT are presented in Table 2. The trend in levels of antioxidant activity during stored at room temperature for 14 days is shown in Fig. 5.

The % RSA of fresh and dried samples of *Citrullus lanatus* ranged from 17.10±0.16-28.78±0.17% and 59.12±0.15-79.93±0.18%, whereas IC_{50} ranged between 14.96-25.70 µg/mL (low % RSA) and 2.38-4.73 µg/mL (high % RSA), respectively. There were significant variations ($p<0.05$) among varieties due to the different levels of phytochemicals among other factors mentioned for differences in carotenoids and TPC [6,9,17,18]. Charleston grey and sugar baby varieties had the lowest and highest % RSA respectively. This was in both fresh and dried samples, where dried samples were comparatively high, perhaps due to pre-concentration/drying of samples. The radical scavenging efficiencies (IC_{50}) of the dried samples were similar to the synthetic antioxidants. The differences between these results and from other authors can be explained due to the use of different extraction solvents and cultivars among other factors [9,18].

There were significant variations ($p<0.05$) in the antioxidant activity with storage period for each sample variety, with the degradation trends being identical in pata negra and sugar baby varieties ($p<0.05$).The antioxidant activity levels were high at day 4 for pata negra and sugar baby and at day 2 for Charleston grey, which possibly could be due to favorable physiological changes and increase in the synthesis of some phytochemicals at said days, but thereafter declined with progression of storage period [17,19]. The extent of degradation in antioxidant activity was reasonably high in Charleston grey, resulting to a loss of 55.0%, followed by 47.6% in sugar baby, while 19.8% in pata negra had the least reduction. Geometric isomerization of carotenoids, different content of antioxidants and the physiological changes that accompany the phytochemicals during fruit storage may be responsible for the degradation in the antioxidant activity [6,19].

Table 2. Levels of % RSA and IC_{50} of *Citrullus lanatus* varieties and synthetic antioxidants

Variety	% RSA (Mean±SD), (n = 3)		IC50 (in mg/mL)		Synthetic antioxidant
	Fresh samples	Dried samples	Fresh samples	Dried samples	
Patanegra	20.67±0.16[b]	61.90±0.07[b]	23.34	3.96	AscA: %RSA = 70.00±0.17 IC_{50}= 5.22
Sugar baby	28.78±0.15[c]	79.93±0.18[c]	14.96	2.38	BHT: %RSA =
Charleston grey	17.10±0.16[a]	59.12±0.15[a]	25.70	4.73	77.47±0.10
P-value	<0.001	<0.001			IC_{50} = 2.60

Mean values with different small letters within the same column are significantly different (p<0.05, SNK-test)

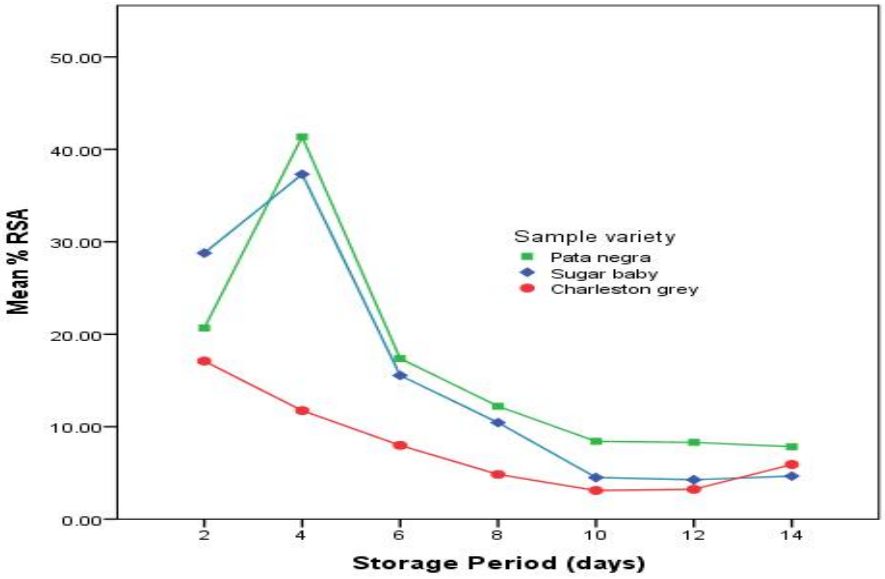

Fig. 5. Trends of levels of antioxidant activity in varieties of *Citrullus lanatus* stored at room temperature for 14 days

4. CONCLUSION

Storing watermelons for upto 14 days has both negative and positive implications on the carotenoid and total phenolic content levels but clearly reduces the antioxidant activity and hence the effectiveness on the fight against NCDs.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
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