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Original Article

EVALUATION OF FREE RADICAL SCAVENGING ABILITY AND ANTIRADICAL ACTIVITIES OF *XIMENIA CAFFRA* FRUIT EXTRACTS AT DIFFERENT RIPENING STAGES

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ABSTRACT

Objective: To evaluate the free radical scavenging ability and antiradical activities of *Ximenia caffra* fruit extracts in their different ripening stages.

Methods: Using standard procedures, *Ximenia caffra* fruit extracts were determined for ferrous ions chelating ability, nitric oxide and hydroxyl radical scavenging ability. Antiradical activities were assessed using 1,1-diphenyl-2-picrylhydrazyl (DPPH) in which the fruit extracts were evaluated for the number of antiradical units (AU_{515}), the number of antiradical units per 1 mg of extracts (EAU_{515}) and the total number of antiradical units per 1g of raw fruits (TAU_{515}).

Results: The fruit extracts exhibited significant higher ferrous ion chelating and free radical scavenging capacity compared to synthetic antioxidants (standard). Fruit extracts in early ripening stage (ERS) exhibited stronger ion chelation, nitric oxide and hydroxyl radical scavenging ability with low effective fruit extract concentration required to reduce free radicals by 50% (EC_{50}) that were 14, 25 and 30µg/ml respectively than the late ripening stage (LRS) extracts. The fruit extracts also showed high AU_{515}, EAU_{515} and TAU_{515}. The values of AU_{515} ranged from 0.93 to 0.95, while EAU_{515} from 78.30 to 79.34 and TAU_{515} ranged from 19762.46 to 23821.23 of the extracts in LRS and ERS respectively.

Conclusion: Based on these observations *Ximenia caffra* is potentially beneficial to human health due to its strong ability to scavenge free radicals. Its utilization can potentially reduce the risk of degenerative diseases to human beings.

Keywords: Antiradical activities, Iron chelation, Radical scavenging, Ripening stages, *Ximenia caffra*

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INTRODUCTION

Free radicals are constantly generated in the human body during the oxidation process. Oxygen is an essential element, particularly in the production of adenosine triphosphate (ATP) which provides energy for cellular functioning. However, during the process several by-products are generated as free radicals [1]. The free radicals have been implicated in many human diseases including accelerated ageing, cancer, cardiovascular diseases, neurodegenerative disease and inflammation. These deleterious effects result from an imbalanced body process of free radicals production and removal that leads to their excessive accumulation such that the bodily defenses fail to prevent oxidative damage [2, 3]. Consumption of food rich in antioxidants promotes body defense systems by keeping the amount of the free radicals balanced [4]. However the use of synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) that are widely used in the food industry has been associated with harmful effects on human health, like liver damage and carcinogenesis [5, 6]. In consequence, public attention has been focused on natural antioxidants from plant materials as an alternative.

Ximenia caffra is a wild fruit tree of the family *Olacaceae* native to Tanzania, Kenya, Malawi, Mozambique, South Africa, Uganda and Zambia. It grows in dry woodland and wooded grassland, often on rocky hillsides [7, 8]. The fruits from the tree are picked and eaten raw after ripening, and refreshing juice can be prepared by squeezing fruits in water and adding sugar [7, 9]. It has been reported that *X. caffra* has a potential medicinal use by the local communities. The leaves are used as a remedy for malaria, coughs, toothache, stomach-ache, ulcers and hookworm while roots are also used as a cure for infertility, mental illness, bilharzia, scorpion bites, menstrual problems, anaemia, hernia and intestinal worms [9, 10]. Several studies confirmed that large numbers of medicinal plants are also good free radical scavengers [11-13].

Previous research reported that extracts from leaf and seed of X. caffra have a good number of polyphenol compounds including phenolic acid and flavonoids with strong antioxidant capacity [9, 14]. Such information is limited in the fruit extracts and this article aimed to unveil the antioxidant potential of *X. caffra* fruits by evaluating their ability to scavenge free radicals and antiradical capacity. The study also determined the optimum fruit phase with a strong antioxidant capacity between the early and late fruit ripening stages. This is because the antioxidant ability is highly correlated with the concentration of phytonutrients that also change with the fruit ripening process [15-17].

MATERIALS AND METHODS

Chemicals and reagents

Methanol, ferric chloride, ferrozine, ethylene diamine tetraacetic acid (EDTA) sodium, nitroprusside, sulfanilamide, naphthylethylene-diamine dihydrochloride, hydrogen peroxide, potassium phosphate, potassium hydroxide, 1, 1-diphenly-2-picrylhdrazyl (DPPH) ascorbic acid, catechin, and ethyl acetate procured from Sigma–Aldrich Co. (Germany).

Collection and preparation of fruit samples

Fresh fruits of *X. Caffra* were collected from miombo woodlands of theTunduru District (Latitude: 10.932125 °N and Longitude: 37.238597 °E and Altitude: 686m) of Ruvuma Region Tanzania in February 2017. The plant was botanically authenticated by Curator Mr. Frank Mbago of Botany Department Herbarium, University of Dar es Salaam and voucher specimen N0: 20 dated 23/02/2017 was deposited in the Herbarium Department of Botany, University of Dar es Salaam, Tanzania for future reference. Ripe fruits were picked directly from the trees and stored as two different batches. The first batch was for those fruits which were at their early ripening stage (ERS) determined by their bright pink color and second batch were those with deep pink color observed at the late ripening stage (LRS). Samples were then packed in a cooler facility and transported to the Botany Department Laboratory University of Dar es Salaam. In the laboratory, each batch was stored as a dry, pulverized sample and frozen fresh until further analysis.

Determination of ferrous ion chelating ability

Ferrous ion chelating ability was determined following the procedure described by Denis TC *et al.,* 1994 [18]. Ten milligrams of fruit extracts were dissolved in 1 ml of distilled water and then 3.7 ml of methanol and 0.1 mmol ferrous chlorides were added. To initiate the reaction 0.2 ml of 5 mmol ferrozine was also added and the mixture was kept at room temperature for 10 min before determining the absorbance at 562 nm against a blank. The effective concentration of the fruit extracts in which ferrous ion was chelated by 50 % (EC_{50}) was obtained by plotting the percentage chelation against the concentration of fruit extract. Ethylenediamine tetraacetic acid (EDTA) that was prepared at a concentration similar to that of fruit extracts (0.01 to 0.07 mg/ml) was used as a standard.

Nitric oxide radical scavenging activity

Nitric oxide was determined by generating it from sodium nitroprusside and measured by the Griess reaction as described by Marcoci L *et al.,* 1994 [19]. The procedure is based on the principle that, sodium nitroprusside in aqueous solution at physiological *p*H spontaneously generates nitric oxide radicals, which interacts with oxygen to produce nitrite ions that can be estimated by the use of Griess reagent (1% sulfanilamide, 2% H_3PO_4, and 0.1% naphthylethylenediamine dihydrochloride). Nitric oxide scavengers compete with oxygen leading to reduced production of nitric oxide radicals. In this study 5 mmol sodium nitroprusside in a standard phosphate buffer solution (0.025 mmol, pH 7.4) was mixed with a different concentration of fruit extracts (0.01-0.07 mg) and incubated at 25 °C for 60 min. After the incubation time, 0.5 ml of the solution was diluted with 0.5 ml of Griess reagent. The absorbance of the chromophore formed during the diazotization of nitrite with sulfanilamide and its subsequent coupling with naphthylethylene-diamine dihydrochloride was read at 546 nm, relative to the absorbance of standard solutions treated in the same way with Greiss reagent. The EC_{50} was obtained by plotting the percentage nitric oxide radicals reduced against the concentration of fruit sample extract.

Hydroxyl radical scavenging activity

Hydroxyl radical was determined using deoxyribose sugar, which is degraded by exposure to hydroxyl radicals generated by Fenton reaction (Fe^{2+}-ascorbate-EDTA-H_2O_2) as described by Halliwell B *et al.,* 1987 [20]. A mixture (1 ml) contained deoxyribose (2.8 mmol), KH_2PO_4–KOH (20 mmol; pH 7.4), $FeCl_3$ (100 mmol), EDTA (104 µ*M*), H_2O_2 (1 mmol) and ascorbate (100 µ*M*) were added with different concentration of fruit extracts ranging from 0.01 to 0.07 mg and incubated at 37 °C for 1 h to develope a pink chromogen which was measured at 535 nm. Catechin which was prepared at a concentration similar to that of fruit extracts was used as a standard. The EC_{50} was obtained by plotting the percentage hydroxyl radical reduced against the concentration of extract tested.

Determination of antiradical activity

Fruit sample for determination of antiradical activity was extracted using three different methods that provide exhaustive extraction of organic compounds as they have different solubility among solvents [21, 22]. In the first method (*M I*) samples were extracted using methanol as the first solvent in order to extract a larger quantity of flavonoid and phenolic acid which are soluble in methanol. Five grams of the powdered dry sample was extracted using 200 ml of methanol at 50 °C for three days. The sample was filtered, and the methanol was evaporated from the filtrate under reduced pressure. The resulted dry residue was dissolved in 50 ml of hot water and stored at 4 °C for three days. The solution was filtered and the precipitate obtained was marked as sample A (*SA*) while the remaining solution was extracted five times with 50 ml of ethyl acetate. The resulted solution was then separated by separating funnel, the ethyl acetate extract was evaporated under reduced pressure to obtain an extract marked as sample B (*SB*) while the remaining aqueous solution was concentrated to dryness under reduced pressure to obtain an extract marked sample C (*SC*).

In the second method (*M II*) a mixture of methanol and water in a ratio of 1:1 was the first solvent and it was targeted to include large quantities of tannin in the sample extract because tannin is soluble in a mixture of methanol and water in a ratio of 1:1 but is insoluble in methanol. Five grams of fruit powder was treated with 200 ml solution containing methanol and water for two days at 50 °C. Twenty percent of the total volume of the mixture was measured and evaporated under reduced pressure to dryness to obtain an extract marked *SA*. Methanol from the remaining 80 percent of the solution was evaporated under reduced pressure while the resulted aqueous solution was stored at 4 °C for two days. The solution was filtered and the precipitate was discarded while the filtrate was extracted with ethyl acetate. The solution was then separated as ethyl acetate extract that was further evaporated under reduced pressure to dryness to obtain the extract marked *SB* while the remaining aqueous solution was concentrated to *SC*.

The third method (*M III*) was aimed to determine the antiradical activities of fresh fruit juice. In this method, samples were obtained by concentrating the squeezed fruit juice, whereby 200 g of *X. caffra* fruits were blended to obtain the fruit juice. Then 60 ml of juice was obtained and concentrated to dryness to obtain an extract marked as juice sample (*SJ*).

The antiradical activities were measured using stable radical 1, 1-diphenly-2-picrylhdrazyl (DPPH), as described by Brand-Williams W *et al.,* 1995 [23]. DPPH has characteristic absorbance at 515 nm which disappears after its reduction by an antiradical compound. The reduction of DPPH can be monitored using spectrophotometer by measuring the decrease in its absorbance during the reaction. At room temperature, 40 µl of fruit sample solution prepared by dissolving the extracted sample in methanol was added to 1460 µl of a radical solution prepared by adding 2 mg of DPPH in 54 ml of methanol. The absorbance was then measured at time 0 and after 1 min. The antiradical activities were then calculated as the number of antiradical units (AU_{515}), the number of antiradical units per 1 mg of extracts (EAU_{515}) and the total number of antiradical units per 1g of raw fruits (TAU_{515}) according to the equations below.

Percentage yield was calculated for each sample extraction method (I, II and III) according to equation 1.

$$Y\,\% = \frac{Cle}{W_R}\ 100 \qquad \text{Equation 1.}$$

Where; Cle = Total amount of extract (g).

W_R = Weight of fruit material used for extraction

The antiradical activity unit (AU_{515}) was calculated as

$$AU_{515} = (A_0 - A_1) - (A_{0C} - A_{1C}) \qquad \text{Equation 2.}$$

Where; AU_{515} = Antiradical activity of the extract,

A_0 = Absorbance of the sample at the beginning of the reaction (0 min),

A_1 = Absorbance of the sample after 1 min of the reaction,

A_{0C} = Absorbance of the control sample at the beginning of the reaction,

A_{1C} = Absorbance of the control sample after 1 min of the reaction.

The number of antiradical activity units per 1 mg of each fruit extract (EAU_{515}) was calculated according to equation 3.

$$EAU_{515} = \frac{AU_{515}}{Ie} \qquad \text{Equation 3.}$$

Where; EAU_{515} = number of antiradical activity per 1 mg of fruit extract,

AU_{515} =antiradical activity of the extract,

Ie = amount of extract in the sample (mg).

The total number of antiradical activity units (PAU_{515}) was calculated separately for each sample extract (*SA, SB, SC* and *SJ*) according to equation 4.

$$PAU_{515} = \frac{Cle}{Ie}\ AU_{515} \qquad \text{Equation 4.}$$

Where; Cle = total amount of extract (mg),

Ie = amount of extract in the sample (mg).

Then the total number of activity unit obtained from 1 g of raw fruit (TAU_{515}) in *M I* and *M II* was calculated as per equation 5.

$$TAU_{515} = \frac{PAU_{515}(A) + PAU_{515}(B) + PAU_{515}(C)}{W_R} \quad \text{Equation 5.}$$

Where; PAU_{515} (*A*) = PAU_{515} calculated from extract *SA*,

PAU_{515} (*B*) = PAU_{515} calculated from extract *SB*,

PAU_{515} (*C*) = PAU_{515} calculated from extract *SC*,

W_R = weight of a fruits taken for sample extraction (g)

The total number of activity units (TAU_{515}) obtained per 1 g of raw fruits under method *M III* was calculated as per equation 6.

$$TAU_{515} = \frac{PAU_{515}(J)}{W_R} \quad \text{Equation 6.}$$

Where; PAU_{515} (*J*) = PAU_{515} calculated from extract *SJ*,

W_R = weight of fruits used to make juice (g)

Statistical analysis

Statistical analysis was performed using ANOVA. Significant differences between means of extract samples and standards were determined using the Turkey multiple comparison tests as applicable and considered significant when $P<0.05$

RESULTS AND DISCUSSION

Ferrous ion chelating ability

Chelating ability of the *X. caffra* fruit extracts increased with concentration (fig. 1). The fruit extract in ERS had shown significant stronger chelating capacity, thereby at a concentration of 0.05 mg/ml had 94.61 % than in LRS that had 90.17 % (LSD = 4.447, q = 8.262, $P<0.01$). However, both ERS and LRS extracts showed a stronger chelating capacity than the synthetic metal chelator EDTA (70.75 %) that was used as standard (LSD = 23.860, q = 44.332, $P<0.001$) and (LSD = 19.413 q = 36.070, $P<0.001$) respectively. The EC_{50} was significantly low (14 µg/ml) in the fruit extract from ERS that signify their strong chelating capacity. While the EC_{50} from LRS fruit extract and that of EDTA were 17 and 14 µg/ml respectively (F = 555.76, $P<0.0001$) as shown in table 1. Ferrous ions are highly reactive and are considered the most effective pro-oxidant among various species of metal ions. It accelerates lipid oxidation by breaking down hydrogen and lipid peroxidase to reactive free radicals through the Fenton reaction [1]. Therefore chelating activity reduces the concentration of the catalyzing transition metal ions in lipid peroxidation and regards as an antioxidant mechanism [24]. The findings of this study showed *X. caffra* fruit extracts can reduce the concentration of metal ions in the human body better than the synthetic metal chelator EDTA and could be an alternate to synthetic antioxidants available in the market that might be healthy unsafe.

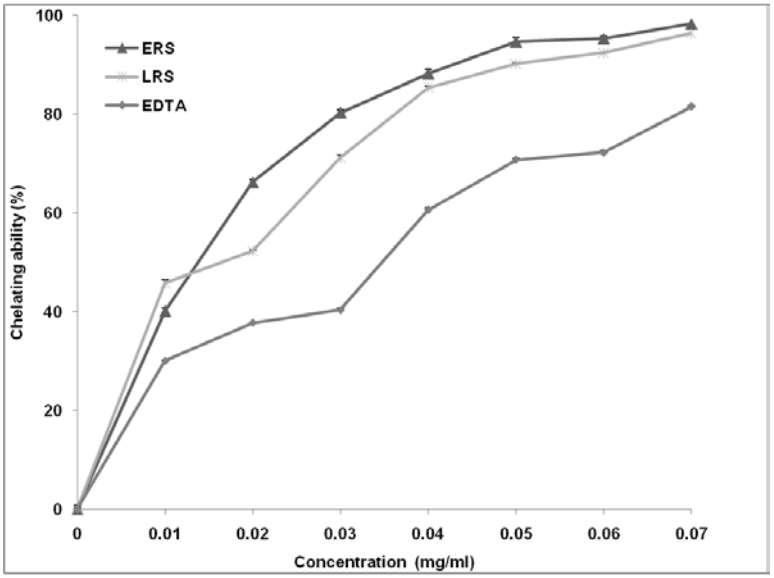

Fig. 1: Ferrous ion chelating ability of *X. caffra* fruit extracts in their early ripening stage (ERS) and late ripening stage (LRS) that were compared with the standard Ethylene diamine tetra acetic acid (EDTA), values are means±SE (n=3)

Nitric oxide radical scavenging activity

Ximenia caffra fruit extracts showed a dose-dependent ability on scavenging nitric oxide radical (fig. 2). At 0.04 mg/ml concentration of dry fruit extracts and ascorbic acid, a synthetic nitric oxide scavenger, ERS showed the highest scavenging ability of 75.21 %, followed by extracts from the LRS having 73.16 % and the lowest ability of 30.03 % was recorded in the ascorbic acid. The differences in nitric oxide scavenging ability among fruit extracts and the standard ascorbic acid were statistically significant based on one-way analysis of variance (F = 978.83, $P<0.0001$). EC_{50} of ascorbic acid was achieved at significantly higher concentrations of 50 µg/ml than that of fruit extracts in ERS (LSD = 25.00, q = 56.084, $P<0.001$) and LRS (LSD = 23.000, q = 51.598, $P<0.00$) as shown in table 1. Although nitric oxide is an essential bioregulatory molecule responsible for a number of physiological processes like neural signal transmission, immune response, vasodilation and control of blood pressure [25], but excessive accumulation may result in several deleterious body conditions such as cancer. Food of plant origin may counteract this effect since they are natural and safe sources of antioxidant than the synthetic antioxidant [26, 27]. In this study, the extracts of *X. caffra* strongly inhibited the formation of the toxic form of nitric oxide when it was generated from sodium nitroprusside. This finding implies that *X. caffra* fruit had effective nitric oxide scavenging ability and can be used to a significant reduction of nitric oxide harmful effect in the body than the synthesized antioxidant.

Hydroxyl radical scavenging activity

The ability of *X. caffra* fruit extracts to scavenge hydroxyl radicals was measured by studying competition between deoxyribose and test compounds for hydroxyl radical generated by Fenton reaction. *Ximenia caffra* fruit extracts showed the strong and concentration dependent capacity of inhibiting deoxyribose oxidation by scavenging the hydroxyl radicals (fig. 3). The fruit extracts in ERS showed a higher

hydroxyl radical scavenging ability of 80.26 % than in LRS that had 63.75 % and the standard catechin having 42.16 % under 0.05 mg/ml concentration. Turkey`s test showed that the scavenging ability of the standard catechin was significantly lower than that of fruit extract in ERS (LSD = 38.093, q = 45.092, P<0.001 and LRS (LSD = 21.583, q = 25.549, P<0.00). The low ability of catechin on hydroxyl radical scavenging was also supported by the recorded higher EC_{50} value of 57 µg/ml while those of fruit extracts were 30 and 36 µg/ml in ERS and LRS fruit extracts respectively (F = 904.50, P<0.0001). The hydroxyl radical is known as the most powerful radical since it induces severe damage to adjacent bimolecules in the body and its scavenging activity is considered to be one of the most important antioxidant mechanisms in the body [28-30]. Generally, in a concentration range from 0.01 to 0.07 mg/ml, *X. caffra* fruit extracts had significant stronger scavenging capacity than catechin. These results support that *X. caffra* fruit extracts are better hydroxyl radical scavengers and can potentially reduce the oxidative damage of human body cells.

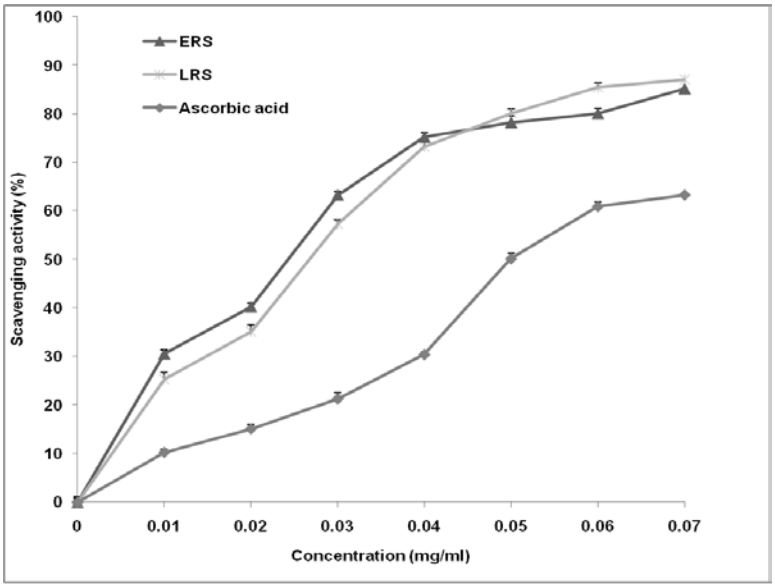

Fig. 2: Nitric oxide radical scavenging activity of *X. caffra* fruit extracts in their early ripening stage (ERS) and late ripening stage (LRS) that were compared with the standard ascorbic acid, values are means±SE (n=3)

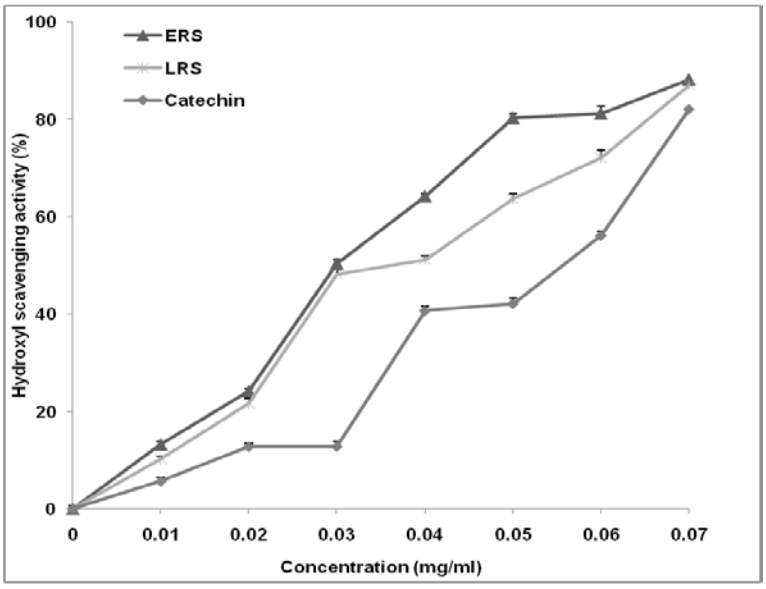

Fig. 3: Hydroxyl radical scavenging activity of *X. caffra* fruit extracts in their early ripening stage (ERS) and late ripening stage (LRS) that were compared with the standard catechin, values are means±SE (n=3)

Table 1: EC_{50} values of *X. caffra* fruit extracts in their early ripening stage (ERS) and late ripening stage (LRS) with those of standards

	EC_{50} (µg/ml)		
	Iron chelating activity	**NO scavenging activity**	**OH scavenging activity**
ERS	14±0.6[a]	25±0.6[a]	30±0.5[a]
LRS	17±0.4[b]	27±0.1[b]	36±0.6[b]
Standard	35±0.4[c]	50±0.5[c]	57±0.3[c]

Values are means±SE (n=3). Different letters in superscript indicate significant differences (P<0.05) within the same column

Antiradical activities

Antiradical activity characterizes the ability of compounds to react with free radicals in a single reaction [31]. Antiradical activity of *X. caffra* fruit extracts were recorded as AU_{515}, EAU_{515}, and TAU_{515} as summarized in table 2. The results indicated that different *X. caffra* fruit extracts have strong antiradical activity AU_{515}, EAU_{515}, and TAU_{515}. The antiradical activity AU_{515} and EAU_{515} were higher in *SJ* fruit extracts followed by *SA, SB* and lowest activity was found in *SC* among the extracts. The antiradical activity TAU_{515} was stronger in the extract obtained using *M III* with activity values ranging from 19762.46±1.4 to 23821.32±2.3 followed by *M I* that had 3594.37±1.93 to 4828.96±1.7, the lowest activity of 1671.60±1.9 to 2369.67±2.7 was obtained in *M II* under LRS and ERS fruit extracts respectively. Extracts from LRS fruits had stronger antiradical activity AU_{515}, EAU_{515}, and TAU_{515} compared to that of ERS fruit. This observation could imply a higher concentration of the antiradical exhibiting compound in the LRS compared to the ERS fruits. This is similar to the observation made by Addai ZR *et al.,* 2014 [15] in paw paw, Ding p and Syazwani S 2016 [32] in MD-2 pineapple, Xie G *et al.,* 2016 [17] in wild *Rosa laevigata* that phenol compounds increase significantly with increasing ripening process. This is because significant physiological, biochemical and structural changes in fruits such as degradation of chlorophylls and synthesis of phytochemical contents occur during the ripening stages [33). This study also revealed that *X. caffra* had strong antiradical activities than most other plant materials reported in the literature. Several studies have reported the presence of higher antiradical activities in different tea species than other plant materials [34-37]. However the quantities reported are lower than those of *X. caffra* observed in this study. Therefore fruits of *X. caffra* should be regarded as a potential source of natural antioxidant and its consumption could be effective in the prevention of oxidative damage. Juice from LRS fruits had strong antiradical activity, hence should be a suitable stage for the harvesting of the fruits so as to obtain the best result based on antiradical capacity.

Table 2: Antiradical activity of *X. caffra* fruit extracts in their early ripening stage (ERS) and late ripening stage (LRS)

Methods	Sample code	Yield %		AU_{515}		EAU_{515}		TAU_{515}	
		ERS	LRS	ERS	LRS	ERS	LRS	ERS	LRS
	SA	44.86±0.9	47.57±0.7	0.66±0.0	0.83±0.0	54.81±0.1	74.96±0.3		
M I	*SB*	4.78±0.1	3.35±0.2	0.42±0.0	0.54±0.0	35.16±0.0	44.48±0.1	3594.37±1.93	4828.96±1.7
	SC	1.61±0.0	0.29±0.1	0.18±0.0	0.23±0.0	15.49±0.0	17.86±0.0		
	SA	18.42±0.1	20.47±0.2	0.73±0.0	0.91±0.0	61.22±0.2	77.59±0.6		
M II	*SB*	1.42±0.0	1.39±0.1	0.28±0.0	0.36±0.0	23.16±0.1	30.14±0.0	1671.60±1.9	2369.67±2.7
	SC	11.28±0.0	10.17±0.1	0.17±0.0	0.15±0.0	10.65±0.3	12.09±0.1		
M III	*SJ*	4.28±0.1	5.13±0.1	0.93±0.0	0.95±0.0	78.30±0.0	79.34±0.1	19762.46±1.4	23821.32±2.3

Antiradical activity unit (AU_{515}), total number of antiradical activity unit per 1 mg of extract (EAU_{515}) obtained in different sample codes (*SA, SB, SC* and *SJ*) and total number of antiradical activity unit per 1g of raw fruit material (TAU_{515}) calculated in each method of sample extraction (MI, MII, MIII), values are means±SE (n = 3).

CONCLUSION

This is the first report on the free radical scavenging ability and antiradical activities in *X. caffra* fruits of Tanzania. The results indicated that X. *caffra* extracts exhibit excellent antiradical activities and free radical scavenging ability in all assays employed. Extracts from the early stage of fruit ripening are more active radical scavengers while those of the late stage of fruit ripening was more active in antiradical activities. Overall, *X. caffra* fruits are a promising source of natural antioxidants and can be recommended to be included as supplements to human diets so as to minimize cell damage caused by the over-accumulation of free radicals in human bodies.

AUTHORS CONTRIBUTIONS

Pensia Mapunda designed the experimental study and analysis of the data. Cosmas Mligo and Herbert Lyaruu coordinated manuscript preparation and revision. All authors approved the final version submitted.

CONFLICTS OF INTERESTS

Declared none

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