ORIGINAL ARTICLE

Aqueous extracts of ripe red pepper and tomato fruits inhibit Fe^{2+}-induced oxidative stress in rat testes and kidney

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Abstract

Objective: Tomatoes (*Lycopersicon esculentum* Mill) and red pepper fruits (*Capsicum annuum* L) are important vegetables usually consumed as vegetable foods, as the spice added to food fresh, dried, refined, ground, and as the principal or incidental ingredient in sauces. This study sought to investigate the inhibitory effect of the aqueous extract of ripe tomato and red pepper fruits on Fe^{2+}-induced lipid peroxidation in rat testes and kidney.

Methods: The aqueous extract of ripe tomatoes and red pepper were prepared. Then, the total phenolic content and the antioxidant activities of the extracts were evaluated using various spectrophotometric methods.

Result: The aqueous extract of tomato had the highest total phenolic, flavonoid and vitamin C contents, as well as 1,1-diphenyl-2-picrylhydrazyl and hydroxyl radical scavenging ability while the Fe^{2+} chelating ability of the two extracts were almost the same. Furthermore, the two extracts caused a significant decrease in the malondialdehyde contents in testes and kidney with aqueous extract of tomato having the highest inhibitory effect on Fe^{2+}-induced lipid peroxidation.

Conclusion: This protective effect of the extracts on Fe^{2+}-induced lipid peroxidation in rat testes and kidney could be attributed to their phenolic compounds and, the possible mechanism may be through their antioxidant activities. Therefore, ripe tomato and red pepper seem to have potential for the management/prevention of Fe^{2+}-induced oxidative stress in the testes and kidney; among both, ripe tomato presented a greater ability than ripe red pepper.

INTRODUCTION

Oxidation of biological molecules has been postulated to induce a variety of pathological events such as cellular injury and aging process [1]. Evidence has shown that these damaging events are caused by free radicals [2]. Oxidative stress results from either a decrease of natural cell antioxidant capacity or an increased amount of reactive oxygen species (ROS) in organisms. It is well established that free radicals are associated with the process that leads to cell degeneration, especially in organs such as liver, kidney and testes [3].

Iron (Fe), an essential metal for normal cellular physiology, can result in cell injury when in excess. This is because it plays a catalytic role in the initiation of free radical reactions. Free iron may catalyze the Haber-Weiss reaction [4] and generate the highly reactive hydroxyl radical which can damage lipids and other biomolecules [5, 6].

High levels of Fe play a crucial role in degenerative diseases by acting catalytically in the production of ROS, which have the potential to damage cellular lipids, nucleic acids, proteins, and carbohydrate resulting in wide-ranging impairment in cellular function and integrity [7]. ROS can directly attack the polyunsaturated fatty acids of the cell membranes and induce lipid peroxidation. Malondialdehyde (MDA) is the end-product of lipid peroxidation, which is a process where ROS degrade polyunsaturated fatty acids. High levels of both copper (Cu) and Fe, with low levels of zinc (Zn) and manganese (Mn) play a crucial role in the progression of several degenerative diseases [8].

Testes, like other organs, are particularly vulnerable to such kind of injury and mostly mediated by ROS in consequence to iron overload. Oxidative damage here can either affect sperm cells or influence spermatogenic process which could change sperm functions [9]. Excessive production of free radicals or ROS can damage sperm and ROS have been extensively studied as one of the mechanisms of infertility [10].

In the kidney, iron has recently been implicated in the pathophysiology of several models of acute renal disease, including hemoglobin- and myoglobin-induced acute renal failure [11] and puromycin nephrotoxicity [12]. It has also been revealed that the accumulation of iron is associated with chronic renal failure [13].

The most likely and practical way to fight degenerative diseases is to improve body antioxidant status, which could be achieved by higher consumption of fruits and vegetables. Phenolic compounds are an important group of secondary metabolites, which are synthesized by plants because of plant adaptation to biotic and abiotic stress conditions such as infection, water stress and cold stress [14]. In recent years, phenolic compounds have attracted the interest of researchers because of their antioxidant capacity; they can protect the human body from free radicals, whose formation is associated with the normal natural metabolism of aerobic cells. The antiradical activity of flavonoids and phenolics is principally based on the structural relationship between different parts of their chemical structure [15].

Polyphenols are common constituents of the human diet, present in most foods and beverages of plant origin. They are considered to contribute to the prevention of various degenerative diseases. This assumption originally came from *in vitro* studies, showing the antioxidant properties of several polyphenols and their ability to modulate the activity of various enzymes. Research suggests that many flavonoids are more potent antioxidants than vitamins C and E [16, 17].

Red pepper fruits (*Capsicum annuum* L) are important vegetables used as vegetable foods, as the spice added to food fresh, dried, refined, and ground, and as the principal or incidental ingredient in sauces. The phytochemicals in peppers have been reported to possess many biochemical and pharmacological properties, such as antioxidant, anti-inflammatory, anti-allergic and anti-carcinogenic activities [18, 19]. Ripe red peppers are naturally rich in ascorbic acid (vitamin C) and provitamin A [20, 21], which neutralize free radicals in the human body and, thus, reduce the risk of diseases, such as arthritis, cardiovascular disease [22] and cancer [23, 24], and in addition, delay the aging process [25]. Carotenoids, which are fat-soluble antioxidants found in peppers, have received considerable interest by researchers due to their antioxidant properties [26] and the necessity for human epithelial cellular differentiation [27]. In addition, several studies have demonstrated the antimicrobial activity of peppers [28, 29].

Tomatoes (*Lycopersicon esculentum* Mill), are consumed fresh or as processed products (sauces, juice, ketchup, soup). The consumption of fresh tomatoes and tomato products has been inversely related to the development of some types of cancer [30] and to plasma lipid peroxidation [31]. Tomato contains different classes of substances with antioxidant properties such as carotenoids, ascorbic acid and phenolics. Lycopene is the major carotenoid present in tomato and have been revealed to show strong antioxidant activity both *in vitro* and *in vivo* [32].

Although several works have been reported on the antifungal, antiviral, anti-carcinogenic and antioxidant properties of tomato and red pepper fruits [18, 19, 32], there is however, limited information on their potential in the prevention of oxidative stress in the testes and kidney. Hence, the objective of this study was to investigate the inhibitory effect of water extractible phytochemicals from ripe tomato and red pepper fruits on Fe^{2+}-induced lipid peroxidation in rat testes and kidney.

MATERIALS AND METHODS

Collection and identification of plant samples

Fresh ripe samples of tomato and red pepper fruits were purchased from a local farm around Ado Ekiti, Ekiti State, Nigeria. Ten adult male Wistar strain rats were purchased from the Animal Production and Health Department, Federal University of Technology, Akure, Nigeria, and acclimatized for 2 weeks, during which period they were maintained *ad libitum* on a commercial diet and water. The experimental study was approved by the Institutional Animal Ethical Committee of the Ekiti State University. The handling of animals was carried out in accordance with the recommended international standard (National Research Council, 1988). A UV-visible spectrophotometer (Model 6305; Jenway, Barlo World Scientific, Dunmow, UK) was used to measure absorbance.

Chemicals and reagents

Chemicals and reagents used such as thiobarbituric acid (TBA), 1,10-phenanthroline, deoxyribose, gallic acid, quecertin, ascorbic acid, Folin-Ciocalteau's reagent were procured from Sigma-Aldrich (St. Louis, MO, USA). Trichloroacetic acid (TCA), MDA and 1,1-diphenyl-2- picrylhydrazyl (DPPH) were sourced from Sigma-Aldrich (Steinheim, Germany). Hydrogen peroxide, methanol, acetic acid, and HCl were sourced from BDH Chemicals (Poole, England, UK). Sodium carbonate, $AlCl_3$, potassium acetate, Tris-HCl buffer, sodium dodecyl sulphate (SDS), $FeSO_4$, potassium ferricyanide and ferric chloride were of analytical grade while the water was glass distilled.

Aqueous extract preparation

The extract was prepared using a modified method of Hong *et al* [33]. The samples were properly sorted and washed under running water to remove any contaminant, thereafter 20 g of the samples were ground to paste and filtered. The filtrate was centrifuged at 4000g for 10 min, the clear supernatant

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collected and 1 ml of the supernatant was mixed with 20 ml of distilled water and was used fresh while the remaining was kept in the refrigerator at 4ºC throughout the period of the analysis.

Determination of phenolic content

The extractable phenolic content was determined on the two extracts using the method reported by Singleton *et al* [34]. Appropriate dilutions of the extracts were mixed with 2.5 ml of 10% Folin-Ciocalteau's reagent (v/v) and neutralized by 2 ml of 7.5% sodium carbonate. The reaction mixture was incubated for 40 min at 45ºC and the absorbance was measured at 765 nm. The total phenol content was subsequently calculated using gallic acid as standard.

Determination of flavonoid content

The extractable flavonoid contents of the two extracts were determined using a slightly modified method reported by Meda *et al* [35]. Briefly, 0.5 ml of appropriately diluted sample was mixed with 0.5 ml methanol, 50 µl of 10% $AlCl_3$, 50 µl of 1 mol/l potassium acetate and 1.4 ml water, and allowed to incubate at room temperature for 30 min. Thereafter, the absorbance of the reaction mixture was measured at 415 nm. The total flavonoid was calculated using quercetin as standard.

Determination of vitamin C content

The vitamin C content of the sample was determined using the method of Benderitter *et al* [36]. Briefly, 75 μl DNPH (2 g dinitrophenyl hydrazine, 230 mg thiourea and 270 mg $CuSO_4 \bullet 5H_2O$ in 100 ml of 5 M H_2SO_4) were added to 500 μL reaction mixture (300 μl of an appropriate dilution of the extract with 100 μl 13.3% TCA and water). The reaction mixtures were subsequently incubated for 3 h at 37ºC, then 0.5 ml of 65% H_2SO_4 (v/v) was added to the medium; their absorbance was measured at 520 nm and the vitamin C content of the sample was subsequently calculated using ascorbic acid as standard and expressed as mg/g ascorbic acid equivalent.

Preparation of tissue homogenates

The rats were decapitated under mild diethyl ether anesthesia and the testes and kidney tissues were rapidly dissected and placed on ice and weighed. These tissues were subsequently homogenized in cold saline (1:10 w/v) with about 10-up-and-down strokes at approximately 1200 rpm in a Teflon-glass homogenizer. The homogenate was centrifuged for 10 min at 3000g to yield a pellet that was discarded, and a low-speed supernatant (S1) containing mainly water, proteins and lipids (cholesterol, galactolipid, individual phospholipids, gangliosides) was kept for the lipid peroxidation assay [37].

Lipid peroxidation and TBA reactions

The lipid peroxidation assay was carried out using the method of Ohkawa *et al* [38]. Briefly, 100 ml S1 fraction was mixed with a reaction mixture containing 30 µl of 0.1 M Tris-HCl buffer (pH 7.4), extracts of red pepper and tomato (0-100 µl) and 30 µl of 250 mM freshly prepared $FeSO_4$. The volume was made up to 300 µl with water before incubation at 37ºC for 1 h. The color reaction was developed by adding 300 µl 8.1% SDS to the reaction mixture containing S1; this was subsequently followed by the addition of 500 µl of acetic acid/HCl (pH 3.4) and 500 µl 0.8% TBA. This mixture was incubated at 100ºC for 1 h. The absorbance of TBA reactive species (TBARS) produced was measured at 532 nm. MDA produced was expressed as % control. The IC_{50} (extract concentration required to inhibit 50% of MDA produced) values were calculated using nonlinear regression analysis.

Fe^{2+} chelation assay

The Fe^{2+} chelating ability of the two extracts was determined using the method of Puntel *et al* [39]. Freshly prepared 500 mmol/l $FeSO_4$ (150 µl) was added to a reaction mixture containing 168 µl of 0.1 mol/l Tris-HCl (pH 7.4), 218 µl saline and the extracts (0-120 µl). The reaction mixture was incubated for 5 min, before the addition of 13 µl of 0.25% 1,10-phenanthroline (w/v). The absorbance was subsequently measured at 510 nm. The Fe^{2+} chelating ability was subsequently calculated using following formula:

$$\mathbf{[(Abs_{ref} - Abs_{sample}) / Abs_{ref}] \times 100} \quad (1)$$

-$\mathbf{Abs_{ref}}$ = absorbance of reference (reacting mixture without test sample)

-$\mathbf{Abs_{sample}}$ = absorbance of reacting mixture with the test sample

Degradation of deoxyribose (Fenton's reaction)

The ability of the two aqueous extracts to prevent Fe^{2+}/H_2O_2-induced decomposition of deoxyribose was carried out using the method of Halliwell and Gutteridge [40]. Briefly, freshly prepared aqueous extract (0-100 µl) was added to a reaction mixture containing 120 µl 20 mM deoxyribose, 400 µl 0.1 M phosphate buffer, 40 µl 20 mM hydrogen peroxide and 40 µl 500 mM $FeSO_4$, and the volume made with 800 µl with distilled water. The reaction mixture was incubated at 37ºC for 30 min, and the reaction was stopped by the addition of 0.5 ml of 2.8% TCA, this was followed by the addition of 0.4 ml of 0.6% TBA solution. The tubes were subsequently incubated in boiling water for 20 min. The absorbance was measured at 532 nm and calculated using formula 1 (see above).

1,1-DPPH free radical scavenging ability

The free radical scavenging ability of the two extracts was evaluated as described by Gyamfi *et al* [41]. Briefly, appropriate dilution of the extracts (1 ml) was mixed with 1 ml 0.4 mM methanol

solution containing DPPH radicals, the mixture was left in the dark for 30 min and the absorbance was taken at 516 nm. The DPPH free radical scavenging ability of the extracts was subsequently calculated (formula 1).

Determination of IC_{50}

In order to determine the IC_{50} (the concentration of extracts required to inhibit 50% of the enzyme activity) values, the percentage of enzyme inhibition of the leaf extract was plotted against the extracts at various concentrations. The IC_{50} was then calculated using nonlinear regression curve.

Data analysis

The result of three replicate experiments were pooled and expressed as mean ± standard deviation [42]. The statistical analysis was carried out using ANOVA and Student t-test where appropriate. Significance level was set at $P < 0.05$.

RESULTS

The total phenolic, flavonoid and vitamin C contents of tomato and red pepper fruits are presented in Table 1. The result revealed that tomato had the highest total phenolic content while red pepper had the least; also tomato had the highest flavonoid content while red pepper had the least (5.1 mg QUE/g), while there was a significant difference ($P < 0.05$) between the vitamin C content of tomato (5.82 mg/g) and red pepper (2.79 mg/g).

The aqueous extracts of tomato and red pepper caused a dose dependent significant inhibition in the MDA production in the testes and kidney (Fig.1). Considering the IC_{50} values shown in Table 2, aqueous extract of tomato had higher inhibitory effects on MDA production in the testes and kidney than red pepper.

Furthermore, as presented in Fig.2, the two extracts scavenged DPPH radicals in a dose-dependent pattern (0-4.17 mg/ml). Among both, the aqueous extract of tomato had the higher DPPH radical scavenging ability (16.67-41.89%).

The Fe^{2+} chelating ability of the aqueous extracts from tomato and red pepper are to see in Fig.3. Both of the extracts were able to chelate Fe^{2+} in a dose dependent manner (0-1.2 mg/ml) representing almost the same value. At lower concentration (0.4 mg/ml),

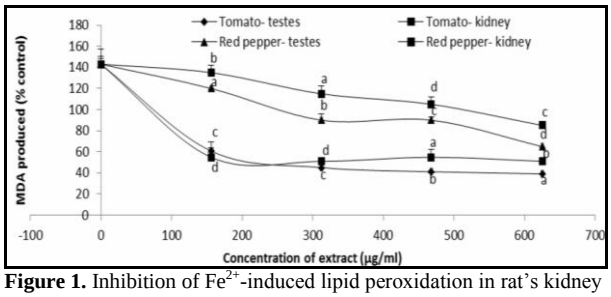

Figure 1. Inhibition of Fe^{2+}-induced lipid peroxidation in rat's kidney and testes by aqueous extract of tomato (*Lycopersicon esculentum* Mill) and red pepper (*Capsicum annuum* L). Values with different superscript letters are significantly ($P < 0.05$) different.

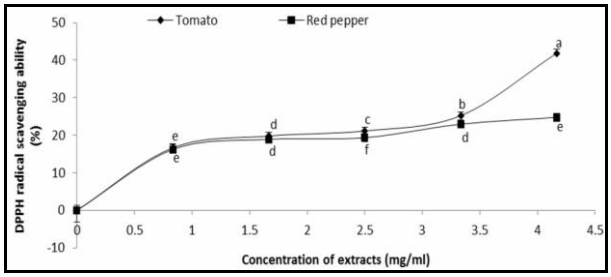

Figure 2. DDPH free radical scavenging ability of aqueous extract of tomato and red pepper. Values with different superscript letters are significantly ($P < 0.05$) different.

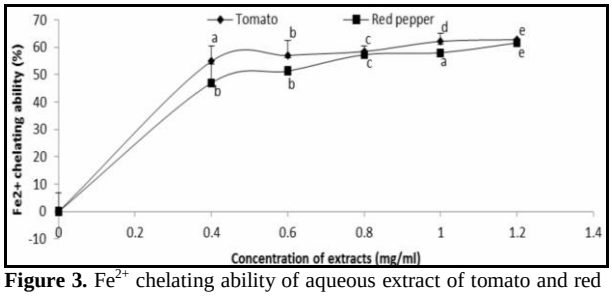

Figure 3. Fe^{2+} chelating ability of aqueous extract of tomato and red pepper. Values with different superscript letters are significantly ($P < 0.05$) different.

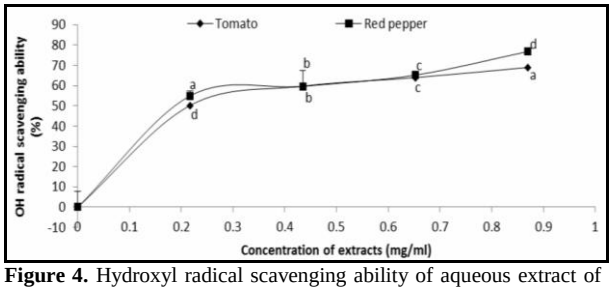

Figure 4. Hydroxyl radical scavenging ability of aqueous extract of tomato and red pepper. Values with different superscript letters are significantly ($P < 0.05$) different.

Table 1. Total phenolic, flavonoid and vitamin C contents of aqueous extract of tomato and red pepper

	TP (mg GAE/g)	TF (mg QUE/g)	Vitamin C (mg AAE/g)
Tomato (*Lycopersicon esculentum* Mill)	21.89 ± 1.09^{a}	7.29 ± 0^{c}	5.82 ± 2.29^{a}
Red pepper (*Capsicum annuum* L)	17.06 ± 0.62^{b}	5.1 ± 0.73^{d}	2.79 ± 1.64^{b}

AAE, ascorbic acid equivalent; GAE, gallic acid equivalent; QUE, quercetin equivalent; TP, total phenolic content; TF, total flavonoid content. Values represent mean ± standard deviation of triplicate readings. Values with the same superscript along the column are not significantly ($P < 0.05$) different.

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Table 2. IC_{50} values of aqueous extract of tomato and red pepper on Fe^{2+}-induced lipid peroxidation in rat's testes and kidney

	Testes	Kidney
Tomato	422.07 µg/ml	493.16 µg/ml
Red pepper	745.38 µg/ml	1029.03 µg/ml

the aqueous extract of tomato had the higher Fe^{2+} chelating ability while at higher concentration (1.2 mg/ml) there was no significant difference ($P > 0.05$) between the two extracts.

The hydroxyl radical scavenging ability of the aqueous extracts of ripe tomato and red pepper is presented in Fig.4. The results revealed that the two extracts were able to scavenge •OH produced from the decomposition of deoxyribose in the Fenton reaction in a dose dependent manner (0-0.89 mg/ml). At higher concentration (0.89 mg/ml), tomato presented higher •OH scavenging ability.

DISCUSSION

Phenolic compounds are potent enough to protect the human body against free radicals, whose formation is associated with the normal metabolism of aerobic cells. They are strong antioxidants with the ability of removing free radicals, chelating metal catalysts, activating antioxidant enzymes, reducing α-tocopherol radicals and inhibiting oxidases [43]. With regard to the phenolic content measured in the present study (see Table 1), the values were higher than reported for pepper fruits [44], ripe and unripe *Capsicum pubescens* [14] and some commonly consumed fruits [45, 46].

Furthermore, the actually measured flavonoid contents (Table 1) were also higher than reported for tropical clove bud [47] and some tropical green leafy vegetables [48]. Flavonoids are a class of widely distributed phytochemicals with antioxidant and biological activity. They are constituents of plant foods that have been implicated in the reduction of cancer risk [49]; moreover, numerous studies have conclusively shown that the majority of the antioxidant activity may be from compounds such as flavonoids, isoflavones, flavones, anthocyanins, catechin and isocatechin rather than from vitamins C, E and β-carotene [50]. Polyphenols are considered to be strong antioxidants due to the redox properties of their hydroxyl groups [51].

Vitamin C has been reported to contribute to the antioxidant activities of plant food. Ascorbic acid is a good reducing agent and exhibits its antioxidant activities of electron donation [16]. It helps the immune system to fight off infections and tumor cells, supports the cardiovascular system by facilitating fat metabolism and protecting tissues from free radical damage, and it assists the nervous system by converting certain amino acids into neurotransmitters. The results of the present study (Table 1)revealed that there is a significant difference between the vitamin C content of tomato and red pepper reflecting higher values in tomato.

High levels of iron have some side effects; it has been established that it plays a crucial role in degenerative diseases by acting catalytically in the production of ROS, which have the potential to damage cellular lipids, nucleic acids, proteins, and carbohydrate resulting in wide-ranging impairment in cellular function and integrity [7]. Therefore, the ability of aqueous extract of tomato and red pepper to prevent Fe^{2+}-induced lipid peroxidation in testes and kidney are highlighted in this study. The aqueous extract of tomato and red pepper caused a dose dependent significant inhibition in the MDA production in the testes and kidney (Fig.1). This inhibition of MDA production may be due to the fruits' the rich content of biologically active phytochemicals with antioxidant activities such as phenolics, flavonoids, isoflavones, flavones, anthocyanins, catechin and isocatechin [52, 53]. Aqueous extract of tomato had higher inhibitory effects on MDA production in the testes and kidney than red pepper (Table 2) which could be due to its significantly higher total phenolic, flavonoid and vitamin C content (Table 1). The mode of inhibition of Fe^{2+}-induced lipid peroxidation cannot be categorically stated. However, there is possibility that the water extractable phytochemicals could have formed complexes with the Fe^{2+}, thereby preventing them from catalyzing the initiation of lipid peroxidation and/or the possibility that the phytochemicals could have scavenged the free radical produced by the Fe^{2+}-catalyzed reaction [54]. Furthermore, it is worth noting that both aqueous extracts of tomato and red pepper, protected the testes and kidney from lipid peroxidation (Fig.1, Table 2).

The finding that Fe^{2+} caused a significant increase in the MDA content of the testes and kidney agreed with earlier report where Fe^{2+} was shown to be a potent initiator of lipid peroxidation [54]. The increased lipid peroxidation in the presence of Fe^{2+} could be attributed to the fact that Fe^{2+} can catalyze one-electron transfer reactions that generate ROS, such as the highly reactive •OH, which is formed from H_2O_2 through the Fenton reaction [4]. Iron also decomposes lipid peroxides, thus generating peroxyl and alkoxyl radicals, which favors the propagation of lipid oxidation [55]. In the testes, Fe^{2+}-induced lipid peroxidation destroys the structure of the lipid matrix in the membranes of spermatozoa and is associated with loss of motility and impairment of spermatogenesis [56]. Also, the accumulation of iron in the kidney has been associated with chronic renal failure [13]. Therefore, possible depletion of iron could decrease oxidative stress throughout the whole body [56].

The ability of plant phytochemicals to chelate and deactivate transition metals, prevent such metals from participating in the initiation of lipid peroxidation and oxidative stress through metal catalyzed reaction is considered an important antioxidant mechanism of action [57]. The mechanism through which the aqueous extracts from tomato and red pepper prevent Fe^{2+}-induced MDA production was subsequently evaluated by determining the two extracts' iron chelating ability, DPPH radical scavenging ability, and the ability to inhibit Fe^{2+}/H_2O_2-induced decomposition of deoxyribose.

Antioxidants carry out their protective role on cells, either by preventing the production of free radicals or by neutralizing/scavenging free radicals produced in the body [43, 54, 58]. In the current study, the result of the DPPH radical scavenging ability revealed that the two extracts scavenged DPPH radicals in a dose-dependent pattern (Fig.2); the aqueous extract of tomato had higher DPPH radical scavenging ability than red pepper. Also, the Fe^{2+} chelating ability of the aqueous extracts from tomato and red pepper showed that the extracts were able to chelate Fe^{2+} in a dose dependent manner (Fig.3); the Fe^{2+} chelating ability was almost the same in the two extracts. This result, however, is in agreement with the Fe^{2+}-induced lipid peroxidation (Fig.1), phenolic content (Table 1) and antioxidant activity of the extracts, suggesting that Fe^{2+} chelation may be one of the possible mechanisms through which antioxidant phytochemicals from tomato and red pepper extracts prevent lipid peroxidation in tissue by forming a complex with Fe^{2+}, thus preventing the initiation of lipid peroxidation [58].

The hydroxyl radical scavenging ability of the aqueous extracts of ripe tomato and red pepper revealed that the two extracts were able to scavenge •OH produced from the decomposition of deoxyribose in the Fenton reaction in a dose dependent manner (Fig.4). At higher concentration, tomato had higher •OH scavenging ability than red pepper. This ability of tomato fruits may have accounted for its higher inhibition of MDA production in the testes and kidney. The trend in the •OH scavenging ability agrees with that of the total phenolic content; therefore, the higher •OH scavenging of the tomato, as possibly dictated by higher phenolic content, could have contributed immensely to its highest ability to inhibit the MDA production in the testes and kidney.

In conclusion, the aqueous extract of ripe tomato (*Lycopersicon esculentum* Mill) and red pepper (*Capsicum annuum* L) fruits were able to protect the testes and kidney from Fe^{2+}-induced lipid peroxidation; among both, aqueous extract of ripe tomato seem to possess higher protective effect than red pepper. Therefore, the protection of testes and kidney tissues from Fe^{2+}-induced lipid peroxidation by the extract of ripe tomato and red pepper could be attributed to their phenolic compounds and the mechanism through which they possibly do this depend on their Fe^{2+}-chelating, •OH and DPPH radical scavenging abilities. So, ripe tomato and red pepper have potential for the management/prevention of Fe^{2+}-induced oxidative stress in the testes and kidney.

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