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HEALTH SCIENCES

An *in vitro* Approach to Protective Effect of Lactoferrin on Acrylamide-induced Oxidative Damage

MAHMUT BODUR, GÜLİZAR AYDOĞDU, AYŞE ÖZFER ÖZÇELİK & ERKAN YILMAZ

Abstract: Acrylamide is a compound that occurs with high temperature during food processing and causes oxidative damage. Recently, the importance of antioxidative components is increasing to prevent oxidative damage. Lactoferrin is an antioxidant protein mainly found in milk. Therefore, the aim of this study is to determine the dose-dependent protective effects of lactoferrin on oxidative damage caused by acrylamide. In this study, HepG2 cell lines were treated with lactoferrin doses (0, 25, 50, 100µM) and half maximal inhibitory concentration of acrylamide. After 24 hours malondialdehyde, superoxide dismutase, catalase and glutathione reductase levels were measured. Acrylamide significantly increased malondialdehyde levels in HepG2 cells compared to the control group; however, catalase, superoxide dismutase and glutathione reductace significantly reduced. On the other hand, added lactoferrin doses (50-100µM) significantly reduced lipid peroxidation levels. Besides, it was found that glutathione reductase, catalase and superoxide dismutase levels significantly increased. As a result, the protective effect of lactoferrin against the oxidative damage caused by acrylamide in HepG2 cells was determined. This effect is thought to be due to the antioxidant capacity of lactoferrin. In this context, it is recommended that more studies are carried out on the mechanism of action of lactoferrin on oxidative stress caused by acrylamide.

Key words: Acrylamide, antioxidant, lactoferrin, oxidative damage.

INTRODUCTION

Acrylamide is a compound that occurs as a result of Maillard reaction which happens with high temperature during processing of foods and is considered to be deleterious on health. Processed potato products, bread, breakfast cereals, biscuits, chocolate, and coffee are reported to contain high amounts of acrylamide (Dybing et al. 2005). Acrylamide also has industrial uses such as laboratory gels, stabilizers, and various cosmetic products (EFSA 2015, Mehri et al. 2015, Shipp et al. 2006). Acrylamide, also known as 2-propenamide, is evaluated by the International Agency for Research on Cancer as "Class 2A" in the class "possible carcinogenic effects on humans" (IARC 1994). Similarly, the National Toxicology Program and the World Health Organization define acrylamide as "reasonably expected to be carcinogenic to human" (WHO 2002, NIEHS 2012). Recent studies indicate that acrylamide has effects on oxidative stress, neurotoxicity, genotoxicity, and carcinogenesis. (Al-Gholam et al. 2016, Catalgol et al. 2009, Mehri et al. 2015).

The frequent and high consumption of foods that contains high acrylamide diet has poses a potential health risk (EFSA 2015, Shipp et al. 2006). In recent years, the increasing consumption of processed foods, is thought to be effective in the development of chronic diseases

(Ludwig 2011). Therefore, reducing the amount of acrylamide taken through diet or using different compounds to prevent acrylamide formation in foods is important for public health.

Acrylamide causes oxidative damage through increasing levels of intracellular reactive oxygen species (ROS) and free radicals, triggering the inflammatory responses (Cao et al. 2008, Catalgol et al. 2009). Oxidative stress takes part in the etiology of chronic diseases. To prevent oxidative damage caused by acrylamide, in cell culture and animal studies have been conducted on different functional compounds that may have antioxidant properties such as ginger (Cao et al. 2008), N-asetyl cysteine (Alturfan et al. 2012), L-carnitine (Zamani et al. 2018). Similarly, lactoferrin, which reduces lipid peroxidation by its iron binding capacity, may also be protective against oxidative damage (Mayeur et al. 2016). However, there have been no studies showing the protective effect of lactoferrin on acrylamide-induced oxidative damage.

Lactoferrin, an important host defense molecule with high biological activity, is a protein with antioxidant, immunomodulating and antiviral effects. Lactoferrin, mainly found in milk, is a 80 kDA molecular weight, glycoprotein attached to iron (Garcia-Montoya et al. 2012). Lactoferrin, produced industrially through recombinant technologies, has been used in various pharmaceutical products and functional foods in recent years (Conesa et al. 2010). Indeed, it is reported that various amounts of lactoferrin enrichment have been made in infant formula, supplements, yogurt and dairy products in Japan (Wakabayashi et al. 2006).

Lactoferrin is defined as safe (GRAS) by the American Food and Drug Administration (FDA). While the consumption of lactoferrin in rats at a dose of 2 mg / kg / day does not cause any toxicity (Yamauchi et al. 2000), it has been found that the consumption of lactoferrin in individuals with chronic hepatitis C did not cause any adverse effects in human studies (Okada et al. 2002). In addition, lactoferrin remains its antioxidant and antibacterial properties against applied heat treatments (Wakabayashi et al. 2006). According to a study, it was reported that the iron binding capacity of lactoferrin, which was exposed to 70 °C for 3 minutes after ultra high temperature (UHT) (130 °C, 2 second) at pH 4, decreased by only 3% compared to unheated samples. Similarly, the antigenic activity of lactoferrin is only 5% loss by boiling at 90 °C (Wakabayashi et al. 2006). These results suggest that lactoferrin can be used to prevent harmful effects of components resulting from high temperature in food processing. However, there is no studies on the protective effects of lactoferrin on oxidative damage caused by acrylamide. In the light of these data, the aim of this study is to determine IC50 values of acrylamide in HepG2 cell line and the protective effects of lactoferrin on oxidative damage caused by acrylamide.

MATERIALS AND METHODS

Cell culture and reagents

In this study, HepG2 cells were purchased from the American Type Culture Collection (ATCC) and cultured in high glucose (4.5 mg/ml) Dulbecco's modified eagle medium (DMEM) (Sigma D6429) containing 10% fetal bovine serum (FBS) (Bio-04-127-1A), supplemented with 100 U/ml penicillin, 100 µg/ml streptomisin (Sigma P4333) in a humidified atmosphere with 5% CO_2 at 37 °C.

In this study, human lactoferrin (Sigma, USA), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Biovision Catalog # K229-1000), glutathione (GSH) assay kit (Biovision Catalog # K261-100), catalase assay kit (Biovision Catalog # K773-100), lipid hydroperoxide (MDA) assay kit (Biovision

Catalog # K739-100) and superoxide dismutase (SOD) assay kit (Biovision Catalog # K335-100) were used.

Determination of acrylamide cytotoxicity and lactoferrin intervention

In this study, colorimetric MTT test was applied to determine the cytotoxicity of acrylamide (Biorad Catalog # K229-1000). This method is briefly based on measuring the dehydrogenase enzyme activity, which can convert the MTT compound into a blue, insoluble formazan compound. This method analyzes changes in mitochondria, so indirectly cytotoxicity.

HepG2 cells (10^5 / ml) are treated with acrylamide at different doses of 0, 0.5, 1, 2, 5, 10, 15, 20 mM within 24 hours. MTT reagent was added and the cells were incubated at 37 °C for 3 hours. 150 µL MTT solvent was added and incubated in the shaker for 15 minutes in room. Then the absorbance values at 590 nm and the IC50 dose for acrylamide were determined.

After the determination IC50 dose of acrylamide, HepG2 cells were treated for 24 hours with acrylamide and lactoferrin doses (0, 25, 50, 100 µg / ml) each sample in triplicate.

Acrylamide was prepared as a stock solution in DMEM, passed through a 0.22 µm filter (Millex-GP-SLGP033RS) and stored at -20 °C before using. Similarly, lactoferrin was prepared as a stock solution in DMEM and passed through a 0.22 µm filter (Millex-GP-SLGP033RS) and stored at -20 °C before using. Acrylamide at a concentration of 7 mM; lactoferrin was prepared at doses of 25, 50 and 100 µg / ml (Safaeian et al. 2015). During the experiment, stock solutions of lactoferrin and acrylamide were diluted with cell culture at desired concentrations.

Determination of lipid peroxidation

In this study, colorimetric MDA kit was used to determine lipid peroxidation (Biovision Catalog # K739-100). Determination of lipid peroxidation was carried out according to the manufacturer's procedure. This method is based on the principle that MDA, a lipid peroxidation product, reacts with thiobarbituric acid to form a TBA-MDA composition.

The HepG2 (10^5 / ml) cell line was centrifuged at 13000 x g for 10 minutes after homogenizing on ice with MDA lysis solution. TBA solution was added to the supernatants obtained and incubated at 95 °C for 60 minutes. The resulting TBA-MDA mixture was determined spectrophotometrically at 532 nm and the amount of MDA was expressed in ng/mmol.

Determination of superoxide dismutase

Within the scope of the study, colorimetric SOD kit was used to determine the amount of superoxide dismutase (Biovision Catalog # K335-100). The determination of superoxide dismutase was carried out according to the manufacturer's procedure. Briefly, this method of reduction of superoxide anion linearly based on the principle of inhibition of the enzyme xanthine oxidase which increased by SOD.

$$SOD\ activity\ (inhibiton\ rate\ \%) = \frac{(\text{Blank1} - \text{Blank3}) - (\text{Sample} - \text{Blank2})}{(\text{Blank1} - \text{Blank3})} * 100$$

HepG2 cells were centrifuged at 4 °C 14000 x g for 5 minutes and the supernatant collected. The obtained samples were placed on a 96-well plate. WST solution included in the kit was added and absorbance values were determined at 450 nm after 20 minutes' incubation at 37 °C. Superoxide dismutase activity was determined according to the formula below. The values shown in the formula represent the absorbance amounts.

Determination of glutathione reductase

Within the scope of the study, colorimetric GSH kit was used to determine the reduced glutathione amount (Biovision Catalog # K261-100). Reduced glutathione determination was carried out according to the manufacturer's procedure.

Briefly, HepG2 (10^5 / ml) cells were washed with 0.5 ml PBS, then centrifuged at 700 x g for 5 minutes at 4 °C. Then the supernatant was discarded and the lysed cells were incubated for 10 minutes with 80 µL cold glutathione solution. The sulfosalicylic acid solution was added and the cells were centrifuged at 8000 x g for 10 minutes, the supernatant was taken into a new tube and the samples were prepared, added to the prepared samples from the reaction mixture, incubated for 10 minutes at room temperature. Incubated for 10 minutes at room temperature, then substrate solution was added and absorbance values were determined with a microplate reader at 405 nm after 10 minutes' incubation at room temperature

Determination of catalase

Colorimetric CAT kit was used to determine the amount of catalase in the study (Biovision Catalog # K773-100).

Catalase determination was carried out in accordance with the manufacturer's procedure. HepG2 (10^5 / ml) cell samples and H_2O_2 were added to the high controls (HC) to begin the catalase assay. After the stop solution was added at the end of the 30-minute incubation period, the developer mix (assay buffer, OxiRed probe and HRP solution) was added and incubated at 25 °C for 10 minutes. At the last stage, absorbance values of the samples were determined at 570 nm.

The amount of catalase in the samples was determined by the formulation below, and the results are expressed in mU / ml.

$$Catalase\ activity = \frac{\mathrm{B}}{\mathrm{V} * 30} * \text{Dilution factor}$$

B is the decomposed H_2O_2 amount from H_2O_2 standard curve (in nmol/ml). V is the pretreated sample volume added into the well (in ml) and 30 is the reaction time in minutes.

Statistical analysis

Statistical analysis and graphical representations were made with the Graphpad Prism program. Results are presented as mean ± standard deviation. Assays were performed in triplicate. Differences between groups were determined using the one-way analysis of variance (ANOVA) test followed by the post-hoc Tukey test. Statistical significance level was accepted as $p < 0.05$.

RESULTS

As a result of the MTT test, the IC50 value for acrylamide was found to be 7.37 µM (95% CI 5.72-10.26 µm) (Figure 1).

Malondialdehyde levels were measured to determine lipid peroxidation. As seen in Figure 2, MDA levels increased significantly because of acrylamide in HepG2 cells ($p < 0.05$). In

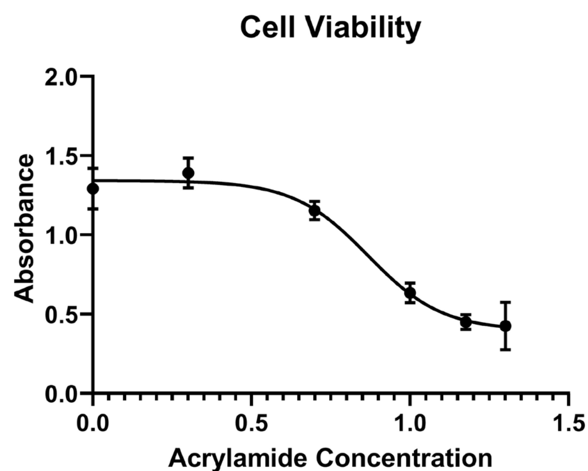

Figure 1. The MTT results of the acrylamide concentration.

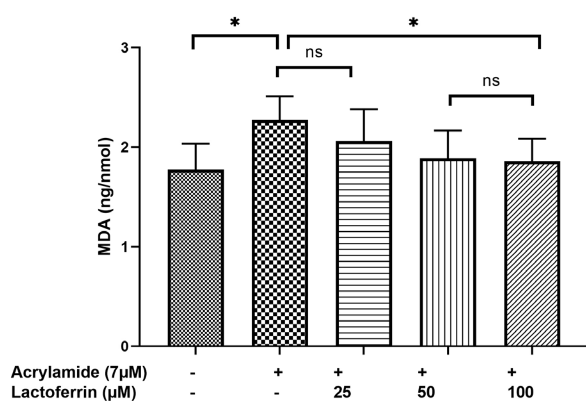

Figure 2. Protective effect of lactoferrin on lipid peroxidation against acrylamide-induced oxidative stress in HepG2 cells. The results were shown with mean±standard deviation. ns: not significant * p < 0.05.

acrylamide-induced lipid peroxidation, 50-100 µM lactoferrin decreased significantly in treated cells ($p < 0.05$, for both) and decreased to similar levels with the control group ($p > 0.05$).

In the case of acrylamide-induced oxidative damage, the effect of lactoferrin applied at different doses in HepG2 cells on catalase is shown in Figure 3. Catalase levels decreased significantly in the HepG2 cell line as a result of acrylamide ($p < 0.001$). It was found that catalase levels increased with lactoferrin application depending on the dose ($p < 0.05$). It was determined that application of 50 and 100 µM lactoferrin significantly increased catalase levels ($p < 0.05$ and $p < 0.001$, respectively). However, although 25 µM lactoferrin application increased catalase levels, it was not statistically significant ($p > 0.05$).

The effect of lactoferrin applied on different doses in the case of oxidative stress induced by acrylamide on superoxide dismutase enzyme activity in HepG2 cells is shown in Figure 4. SOD activity levels decreased significantly due to acrylamide in the HepG2 cell line ($p < 0.05$). SOD activity increases with lactoferrin application depending on the dose. Application of 100 µM lactoferrin significantly increases SOD levels ($p < 0.05$). However, although 25 and 50 µM lactoferrin application increased SOD activity levels, this increase was not statistically significant ($p > 0.05$).

The effect of lactoferrin applied in different doses in the case of oxidative stress induced with acrylamide on glutathione reductase (GSH) in HepG2 cells is shown in Figure 5. GSH activity levels decreased significantly as a result of acrylamide in the HepG2 cell line ($p < 0.001$). GSH levels increase with lactoferrin application depending on the dose. Application of 100 µM lactoferrin significantly increases GSH levels ($p < 0.001$). However, although 25 and 50 µM lactoferrin application increased GSH levels, this increase was not statistically significant ($p > 0.05$).

DISCUSSION

According to the results, the protective effects of dose-dependent lactoferrin addition in the HepG2 cell line on acrylamide-induced oxidative damage were found. Acrylamide causes oxidative damage by increasing lipid peroxidation in the HepG2 cell line. However, lactoferrin added to

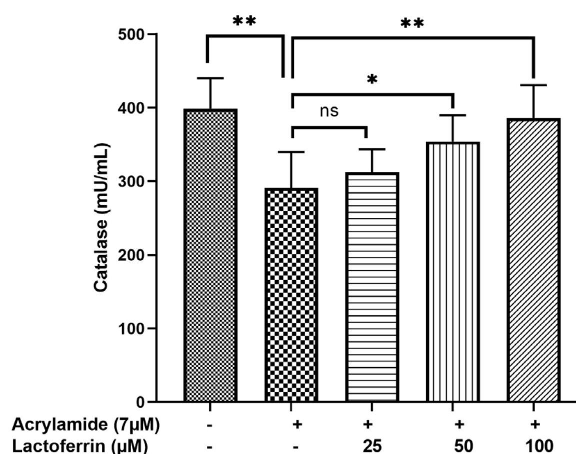

Figure 3. Protective effect of lactoferrin on catalase against acrylamide-induced oxidative stress in HepG2 cells. The results were shown with mean±standard deviation. ns: not significant * p < 0.05, ** p < 0.001.

the HepG2 cell line can reduce oxidative damage caused by acrylamide depending on the dose.

Oxidative stress is one of the mechanisms involved in pathophysiological conditions caused by the chemicals exposed. Oxidative stress is seen as a result of the imbalance between the increased reactive oxygen species in cells and tissues and the antioxidant defense system (Rahman et al. 2012). Reactive oxygen species are oxygen derivatives such as superoxide, hydroxyl radical, hydrogen peroxide, nitric oxide. Increased reactive oxygen species in plasma and tissues can lead to oxidation of lipids, proteins and DNA (Zhao & Shen 2005). However, under normal conditions, antioxidant enzymes such as catalase, superoxide dismutase and glutathione reductase prevent lipid peroxidation in tissues and protect against oxidative damage. Antioxidant enzymes are responsible for the elimination or conversion of reactive oxygen species into harmless components. Superoxide dismutase is involved in catalysis of the superoxide radical while protecting the cell from damage of the superoxide radical; catalase plays a role in the degradation of hydrogen peroxide to oxygen and water (Rahman et al. 2012). Similarly, glutathione is also an important intracellular antioxidant tripeptide that detoxifies reactive oxygen species and free radicals in cells (Couto et al. 2016).

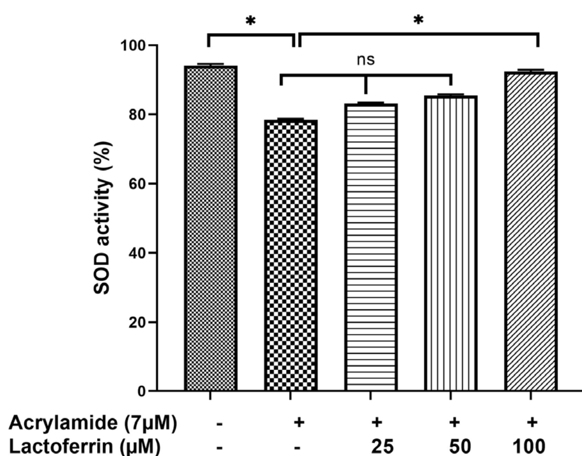

Figure 4. Protective effect of lactoferrin on superoxide dismutase activity rate against acrylamide-induced oxidative stress in HepG2 cells. The results were shown with mean±standard deviation. ns: not significant * $p < 0.05$.

Toxic components that occur during heat treatment applied in foods may play a role in the emergence of chronic diseases by causing various dysfunctions in tissues and organs (Koszucka & Nowak 2019). Previous studies have shown that acrylamide and its active metabolite, glisidamide, have effects such as neurotoxicity, oxidative stress, carcinogenesis, and these effects may have harmful effects on human health (Cao et al. 2008, Catalgol et al. 2009).

Many different mechanisms are proposed for acrylamide toxicity. Acrylamide is thought to cause oxidative damage in cells by increasing reactive oxygen species, lipid peroxidation and glutathione oxidation due to the breakdown of the cellular redox chain (Cao et al. 2008, Catalgol et al. 2009).

The increase of lipid peroxidation products is a marker of oxidative damage and DNA damage at an early stage. In vitro studies, acrylamide has been reported to cause oxidative damage by increasing lipid peroxidation (Cao et al. 2008, Zamani et al. 2018). Similarly, in this study, acrylamide increases lipid peroxidation levels in HepG2 cells, but lactoferrin reduces acrylamide-induced lipid peroxidation products (Figure 2). Levels of antioxidant enzymes such as catalase, superoxide dismutase, and glutathione reductase indicate levels of endogenous antioxidants. However, oxidant stress source components such as acrylamide are known to reduce levels of antioxidant enzymes through various mechanisms. Dixit et al. (1984), reported that acrylamide reacts with GSH S-transferase of glutathione reductase in cells, causing oxidative damage and reducing the level of glutathione reductase in the cell. In this study, acrylamide

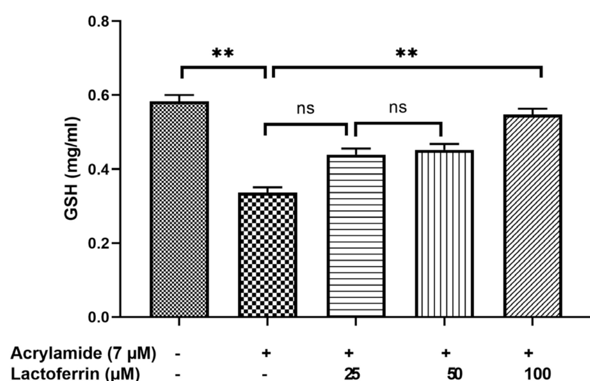

Figure 5. Protective effect of lactoferrin on glutathione reductase against acrylamide-induced oxidative stress in HepG2 cells. The results were shown with mean±standard deviation. ns: not significant, ** p < 0.001.

administration reduced the level of glutathione reductase in HepG2 cells. Lactoferrin, on the other hand, increases glutathione reductase levels depending on the dose (Figure 5).

Lactoferrin, with its antioxidant capacity, is thought to be effective in preventing oxidative stress (Wang et al. 2008). Within the scope of this study, the reduction of lactoferrin lipid peroxidation is due to the antioxidant properties of lactoferrin. Lactoferrin has an antioxidant capacity due to its high iron binding capacity (Mayeur et al. 2016). It is also noted that lactoferrin is an important regulation of free iron levels in body fluids (Baker & Baker 2004). Excessive free iron can lead to oxidative stress by increasing the production of cytotoxic hydroxyl radicals through Fenton reactions. Iron is also an important component of enzymes such as cytochrome, oxygen-binding molecules, and SOD, CAT. Thus, lactoferrin is thought to be able to increase levels of enzymes such as SOD and CAT by sequestration of iron. It was determined that SOD and CAT levels decreased in cells as a result of acrylamide exposure (Figure 3-4). However, lactoferrin dose has significant effects on catalase (100 μM) and SOD (50-100 μM) levels (Figure 3 and 4).

As a result, lactoferrin, which is from the transferrin family, is thought to be an important functional component in preventing oxidative damage caused by acrylamide through binding pro-oxidative iron molecules.

Strength and limitations

This study has some limitations and strengths. To our knowledge, although the antioxidant capacity of lactoferrin is known, this study is the first to demonstrate the protective effects of lactoferrin against acrylamine-induced oxidative damage. The use of HepG2 cells is one of the strengths of this study, since acrylamide is mainly metabolized in the liver. However, the limitations of this study are that it was an in vitro study, and worked with a single cell line.

CONCLUSION

The results of this study suggest that lactoferrin may have a dose-dependent protective effect on oxidative damage caused by acrylamide in HepG2 cells. Acrylamide increases lipid peroxidation products in HepG2 cells while reducing levels of glutathione reductase, catalase, and superoxide dismutase enzymes. However, with the addition of lactoferrin, protective effects can be seen. Given the results obtained in this study, it is thought that lactoferrin may be a novel functional component useful against pathological conditions that acrylamide induced. More comprehensive animal and human studies are required in this regard.

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MAHMUT BODUR[1]
https://orcid.org/0000-0002-2105-8485

GÜLIZAR AYDOĞDU[2]
https://orcid.org/0000-0001-8390-1477

AYŞE ÖZFER ÖZÇELİK[1]
https://orcid.org/0000-0002-9087-2042

ERKAN YILMAZ[3]
https://orcid.org/0000-0001-7355-3878

[1]Ankara University, Faculty of Health Sciences, Department of Nutrition and Dietetics, 06290, Ankara, Turkey

[2]Ordu University, Faculty of Science and Literature, Department of Molecular Biology and Genetics, 52200, Ordu, Turkey

[3]Ankara University, Biotechnology Institute, 06135, Ankara, Turkey

Correspondence to: **Mahmut Bodur**
E-mail: mahmutbodur@ankara.edu.tr

Author contributions

MB and GA designed the study, carried out data collection and analysis and contributed to the draft of the manuscript. AOO and EY finalized the manuscript. All the authors read and approved the final manuscript.