*https://doi.org/ 10.33472/AFJBS.6.5.2024. 8096-8116*

***African Journal of Biological Sciences***


# L-Arginine Supplementation: Its Potential Role As Antiglycation, Antioxidant, And Antihyperlipidemic Agent

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Article History
Volume 6, Issue 5, 2024
Received: 22 May 2024
Accepted: 29 May 2024
doi: ***10.33472/AFJBS.6.5.2024. 8096-8116***

## Abstract

The prevalence of type 2 diabetes (T2D) around the world is undesirably increasing and undeniably creates a negative impact both on human health and the economy. Contributing factors such as insulin resistance, obesity, oxidative stress, and glucolipotoxicity condition expedite the development of T2D. As a major site for glucose disposal, skeletal muscle is a good target tissue for metabolic disorder therapy such as T2D. L-arginine (ARG), primarily contained in muscle-builder supplements, was investigated for its potential as an antiglycation, antioxidant, and antihyperlipidemic effect using some in vitro. Results indicated herein that L-arginine could dose-dependently exhibit an antiglycation effect (68.45 ± 1.32 %; 33.76 ± 1.34 %, respectively) using BSA-glucose and BSA-MGO models. Similarly, it was shown to protect 24.75 ± 0.78 % against potential protein damage using the Congo red binding assay. Antihyperlipidemic effects of L-arginine were also observed as it inhibited pancreatic lipase activity up to 39.62 ± 0.97 %, comparable to the positive control. Antioxidant and radical scavenging activities of L-arginine were also measured and found to inhibit 35.57 ± 1.65 %, 39.30 ± 2.36 %, 34.06 ± 0.21 %, and 93.57 ± 0.33 % formation reactive species using DPPH, FRAP, MDA, and Iron chelation assays, respectively. The preliminary results suggest that L-arginine might offer an alternative treatment strategy for type 2 diabetes.

**Keywords:** Advanced glycation end-products, methylglyoxal, reactive oxygen species, hyperglycemia, hyperlipidemia, insulin resistance.

## Introduction

Diabetes mellitus (DM), a metabolic disease, is considered one of the most severe threats and chronic conditions to human health and causes death (Sun et al., 2022). Per the International Diabetes Federation (IDF), the disease's prevalence is estimated at around half a billion people globally. It is predicted to rise to 12.2% in 2045, anticipating an increase in the total diabetes-related health expenditure (IDF Diabetes Atlas 2021 | IDF Diabetes Atlas, 2021). Most of the diabetic cases have type 2 DM (T2D), around 90-95 %. It is primarily characterized by a combination of insulin resistance (IR) and beta cell malfunction of the pancreas. It occurs when there is an unstable cellular response to insulin in insulin- dependent cells such as adipocytes, skeletal muscle, and liver, which are not able to take up and utilize the glucose from the blood,

and the pancreas poorly functions due to inadequate release of insulin, making the glucose stays in the blood respectively (Sun et al., 2022). If it is not treated, it eventually leads to complications such as kidney damage, heart attack, eye disease, blindness, nerve damage, and amputation (Habib Yaribeygi et al., 2018). Hyperglycemia and hyperlipidemia allow the risk of T2D pathogenesis in the body, wherein high glucose and accumulation of excessive fat may progress from normal glucose tolerance to T2D pathogenesis (Habib Yaribeygi et al., 2018; Muñiz et al., 2018; Cripps et al., 2017 and Lavilla, 2020). The simultaneous occurrence of these factors is also termed glucolipotoxicity (Lavilla, 2020 and Poitout & R. Paul Robertson, 2008). If such conditions prevail, it could result in insulin resistance affecting glucose transport and disruption of a cascade of cellular signaling, increase protein glycation that forms an irreversible advanced glycation end product (AGE's), cause an overproduction of free radicals or reactive oxygen species, which causes oxidative stress (IDF Diabetes Atlas, 2021; Habib Yaribeygi et al., 2018; Muñiz et al. Huang et al., 2018; Starowicz & Henryk Zieliński, 2019; Adjimani & Asare, 2015; Kim et al., 2017; Karuppasamy Balamurugan et al., 2014; Patel et al., 2012 and Sims-Robinson et al., 2015)

Glucose disposal mainly occurs about 70-80% in the skeletal muscle, becoming a critical cellular process for diabetic individuals (Teng & Huang, 2019). With the advent of muscle supplements that contain amino acids, most athletes and individuals who had intense workouts take this to repair damaged muscle during muscle contraction. Conversely, some studies have reported that some amino acids have shown potential as an antidiabetic agent for T2D (Pietro Lucotti et al., 2006; Shatanawi et al., 2020 and Monti et al., 2012). In the case of ARG, some reports show favorable results that it ameliorates insulin sensitivity and metabolism of glucose in patients with T2D (Hu et al., 2017; Miczke et al., 2015; Liang et al., 2017; Wang et al., 2021 and Wu et al., 2013).

The present study limits only one amino acid, L-arginine, mostly contained in supplements. The objective is to investigate the potential of L-arginine as a muscle-based supplement through in vitro investigation that is closely similar to the T2D condition. Aside from the present health benefits of L-arginine, exploring its significant function in lessening the occurrence of insulin resistance and other contributing factors might be an effective treatment strategy in T2D.

## Materials And Methods

Selected in vitro assays designed for T2D, such as antiglycation, antihyperlipidemic, and antioxidants, were conducted as modeled methods in the study, investigating ARG using an

analytical reagent grade (99.0 % assay, LOBA CHEMIE PVT. LTD.). The procedure and the assay concentrations were based on the cited references with few modifications. Either the absorbance or fluorescence was measured using a microplate reader, and the obtained measurements were expressed as a percentage change or inhibition relative to the control using the equation below.

$$\%\ Inhibition\ wrt\ control = \frac{Absorbance\ or\ Fluorescence\ (ARG)}{Absorbance\ or\ Fluorescense (Control)}\ x\ 100$$

**Advanced Glycation End Products (AGEs) Assay**

In the AGEs assay, the sample's inhibitory effect was determined by the two model systems: BSA-glucose (final stage) and BSA-MGO (early stage). The sample was monitored against glycation using fluorescence spectroscopy relative to the control (Starowicz & Henryk Zieliński, 2019). For BSA-glucose, 10 mg $mL^{-1}$ of BSA and 90 mg $mL^{-1}$ glucose reaction solution was prepared separately in phosphate buffer (pH 7.4). The reaction occurs in a test tube containing 0.5 mL of each reaction solution with 0.5 mL of the sample, ARG. Blank was also prepared without the sample. For positive control, 0.5 mL of 1 M aminoguanidine solution was added with 0.5 mL of each reaction solution. All test mixtures were added with 100 µL of 3 nM $NaN_3$ solution to prevent microbial contamination. These were then incubated in a dark, temperature-controlled incubator for 7 days at 37 $^{O}$C. The resulting mixture was then measured for its AGEs fluorescence using a microplate reader at excitation, and the emission wavelengths 360 nm and 420 nm, respectively. While BSA-MGO model, has the same preparation as the BSA-glucose model except the BSA concentration was 2 mg $mL^{-1}$, and instead of glucose 400 mg $mL^{-1}$ MGO was prepared separately in phosphate buffer (pH 7.4). The test solutions were incubated for 14 days at 37 $^{O}$C. The AGEs fluorescence was measured at 420 nm emission and 360 nm excitation wavelengths. (Starowicz & Henryk Zieliński, 2019).

**Congo Red Assay**

In glycated condition, a test tube containing 50 mg $mL^{-1}$ BSA and 36 mg $mL^{-1}$ glucose were prepared as a general reaction mixture. Separately, 25 ppm and 100 ppm of ARG were added, respectively. In another set, 10 mg $mL^{-1}$ aminoguanidine was added for positive control and PBS for negative control. Each was prepared in 0.2 M phosphate buffer saline (pH 7.4) and added with 3 mM $NaN_3$. The resulting mixture was incubated for 24 hours at 60 $^{O}$C. The non-

glycated condition was prepared in the same manner with negative control, except that it was not incubated. Each test solution was divided into half, one with 0.7 mL of 75 µM congo red solution (prepared in phosphate buffer saline–ethanol 10% (v/v)), and the other half was set aside for blank. Absorbances were then measured at 530 nm using (Wu et al., 2013)

**Pancreatic Lipase Inhibition Assay**

In a test tube containing 1 mg $mL^{-1}$ PPL in 0.1 mM phosphate buffer saline (pH 7.4) and test concentrations (25 and 100 ppm) of ARG and Orlistat as a positive control. Then, 0.1% tryton in 0.1 mM phosphate buffer saline (pH 7.4) was added and pre-incubated for 1 hour at 30 °C. The reaction mixtures were added with 2.5 mM p-nitrophenyl butyrate (p-NPB) and then incubated at 30 °C for 5 minutes. A negative control was also prepared with the same procedure without the sample. The p-nitrophenol level released in the reaction was measured at 405 nm (Roh & Jung, 2012)

**DPPH Radical Scavenging Assay**

Various concentrations of ARG were prepared (25, 50, 75, & 100 mg $L^{-1}$), and 1 mL each was added to 1 mL of 0.1 mM DPPH in methanol solution. Methanol is the negative control, while ascorbic acid is the positive control. The mixture was then incubated for 30 minutes in the dark incubator and the absorbance was measured at 517 nm (Irina Georgiana Munteanu & Constantin Apetrei, 2021).

**Lipid Peroxidation Radical Scavenging Activity (TBARS)**

Thiobarbituric acid reactive species (TBA) was the radical molecule used in this assay. A 0.5 mL of 10% v/v egg homogenate was added to 1 mL of various ARG concentrations (50, 100 & 500 mg $L^{-1}$). The mixture was added with 1 mL distilled water and 5 µL of 0.07 M $FeSO_4$, then incubated for 30 minutes to induce lipid peroxidation. Then 1.5 mL of 20% acetic acid and 1.5 mL of 0.8% (w/v) TBA were added in 1.1% SDS and 0.5 mL 20% TCA, then heated at 95 $^{O}C$ for 60 minutes. Another set of samples was prepared without TBA. The resulting mixture was added with 5 mL of butanol and centrifuged at 5000 rpm for 10 minutes. The absorbance of the formed organic layer in the reaction solution was measured at 532 nm (Sharma Mridula et al., 2021)

**Ferric Reducing Antioxidant Power (FRAP) Assay**

FRAP reagent constitutes 20 mM ferric 2,4,6-tripyridyl-s-triazine (TPTZ), and 20 mM $FeCl_3 6H_2O$ in 0.3 M sodium acetate buffer (pH 3.6) of a total ratio of 10:1:1. This was pre-warmed at 37 $^{O}$C for 10 to 15 minutes. Various concentrations (25, 50, 75, & 100 mg $L^{-1}$) of the ARG test solution were prepared. Ascorbic acid and buffer were used as positive and negative controls, respectively. In a test tube containing 0.2 mL of each sample, 1.8 mL of FRAP reagent was mixed thoroughly. This was left in a dark area and incubated for 10 minutes at room temperature. The resulting solution measured its absorbance at 593 nm (Cao et al., 2022).

**Metal chelating activity determination**

The reagents used were 0.1 mM $FeSO_4$ and 0.25 mM ferrozine. Taken 0.2 mL and 0.4 mL, respectively, and subsequently added to 0.2 mL of sample concentrations (25, 50, 75, & 100 mg $L^{-1}$). The same concentrations were used with positive control using EDTA. It was incubated at room temperature for 10 minutes, and then the absorbance of the reaction mixture was determined at 562 nm (Wong, 2014).

**Statistical Tool**

All assays performed were done in 3 trials, and the gathered results were expressed as mean ± standard error of the mean. Statistical analysis of the obtained results was calculated using GraphPad Prism Software. Data involving more than two conditions was performed with a simple one-way analysis of variance (ANOVA) test and results that turned out to be significant, $P<0.05$, was used post hoc test (Tukey's test) using critical values at $\alpha = 0.05$, and results with $P<0.05$ values are considered significant (Statistics and Curve Fitting Resources - GraphPad, 2024).

## Results And Discussion

**Results**

Antiglycation test was done using AGEs assay and Congo red assay. These assays have investigated the sample ARG as an inhibitor of the AGEs formation and the conformation change in protein structure. In BSA-glucose and BSA-MGO, the obtained fluorescence result is the rate of the AGEs after treatment of ARG with respect to the glycated, as presented in Figure 1.

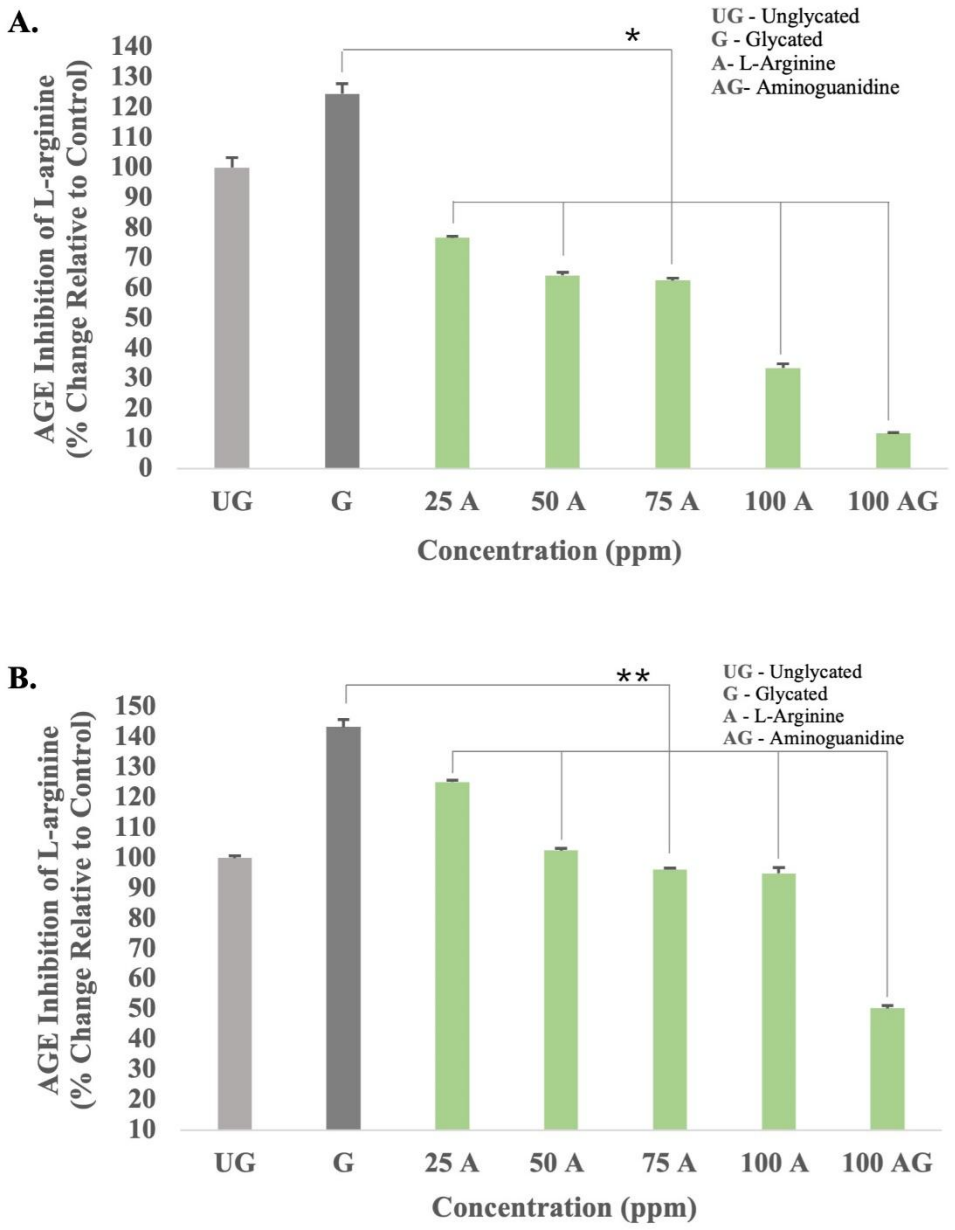
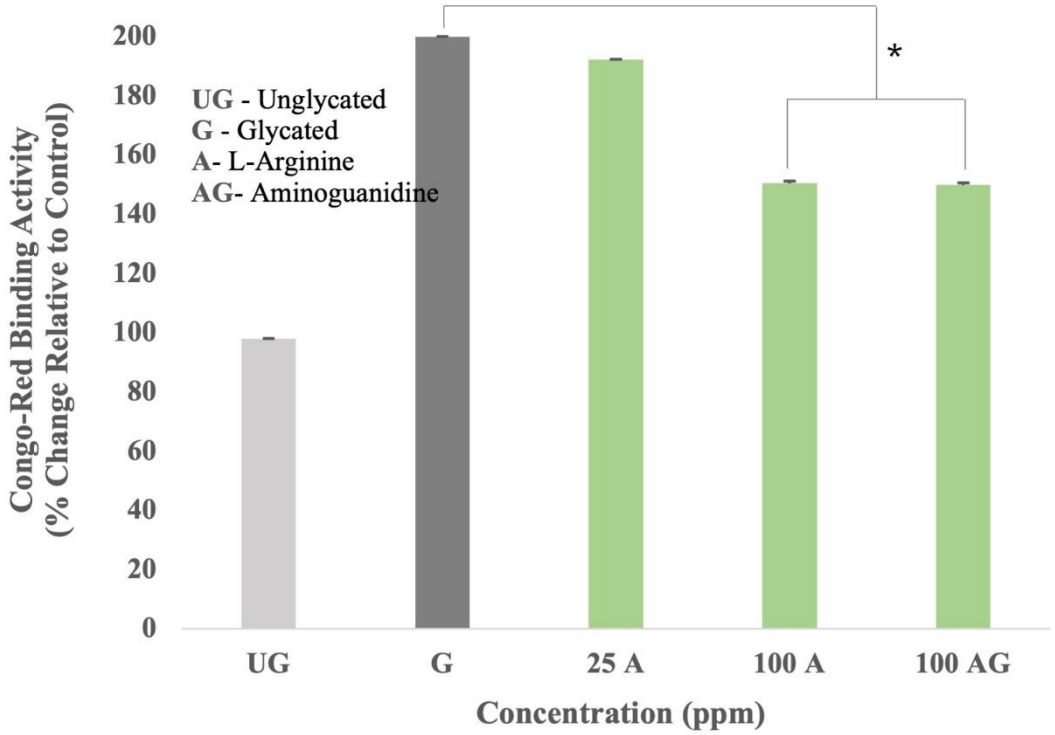



**Figure 1. Inhibitory action of L-arginine against potential AGE formation. (A) BSA - glucose and (B) BSA - MGO model system.** *(Results are expressed in terms of percentage change relative to glycated control. Values are the mean ± SEM of n ≥ 3 measurements where * & ** have statistically significant difference at α = 0.05 with a p < 0.0001 using Tukey's test.)*

It shows that the aminoguanidine (AG) in both BSA- glucose/MGO setups is an effective inhibiting agent with the lowest AGEs level. As for the ARG, it shows a decreasing effect as the concentration increases. The percent difference of inhibition ranges from 27.72 ± 0.46 % to 68.45 ± 1.32 % compared to AG which has 88.89 ± 0.25 % for BSA-glucose while 12.68 ± 0.48% to 33.76 ± 1.34% compared to AG which has 64.91 ± 0.7% for BSA-MGO. As for the

congo red assay, Figure 2 shows the absorbance of the prevented structure alteration at 100 ppm ARG with 24.75 ± 0.78 %, comparable to 100 ppm AG with 25.07 ± 0.70%. Significant differences among the means inferred that ARG has a protective structural effect in congo red staining. However, the conformation of the protein structure cannot be located since it is just the change of the measured absorbance.

**Figure 2. Inhibitory effect of L-arginine against protein structural change using Congo Red binding assay.** *(Results are expressed in terms of percentage change relative to non-glycated control. Values are the mean ± SEM of n = 3 measurements where * have a significant difference at α = 0.05 with a p < 0.0001 interpreted using Tukey's test.)*




A pancreatic lipase inhibition assay was conducted to determine if ARG could inhibit dietary triglyceride absorption as antihyperlipidemic. The result of ARG inhibits the lipase enzyme in the modeled reaction, as shown in Figure 3. The decrease of p-nitrophenol level against control indicates inhibition of hydrolysis driven by the pancreatic lipase. Its decrease significantly displayed the effectivity of Orlistat and ARG as inhibitors. Significantly, ARG is comparable with orlistat with an inhibited percentage ranging from 35.07 ± 4.31 % to 39.62 ± 0.97 % and 46.20 ± 0.26 % - 55.65 ± 1.54 % respectively.

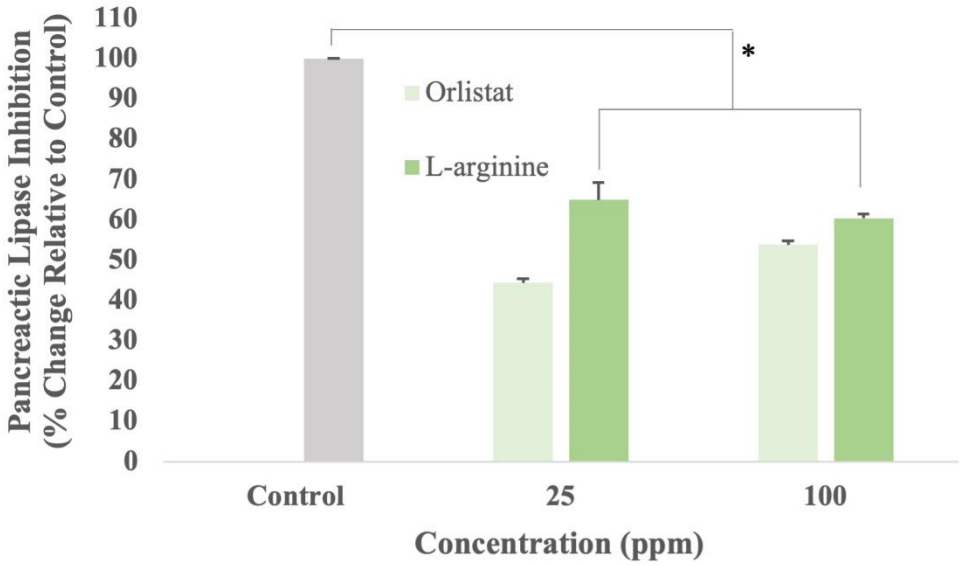



**Figure 3. Pancreatic lipase inhibition activity influence of L-arginine and orlistat.** *(Results are expressed in terms of percentage change relative to control. Values are the mean ± SEM of n ≥ 3 measurements where * have significant difference with respect to control at α = 0.05 with a p < 0.0001 interpreted using Tukey's test.)*

Scavenging activity tests of sample ARG using different types of stable free radicals were employed in methods such as DPPH assay, TBARS in lipid peroxidation assay, NO, and $H_2O_2$ scavenging assays. Based on the result, ARG produced a notable scavenging activity, as shown in Figure 4. ARG scavenges in percent change of DPPH level with respect to control, ranging from 23.34 ± 8.26 % to 35.57 ± 1.65 %, not as high compared to ascorbic acid from 94.09 ± 1.16 % to 98.66 ± 0.24 %, suggesting that L-arginine scavenges reactive radical DPPH.

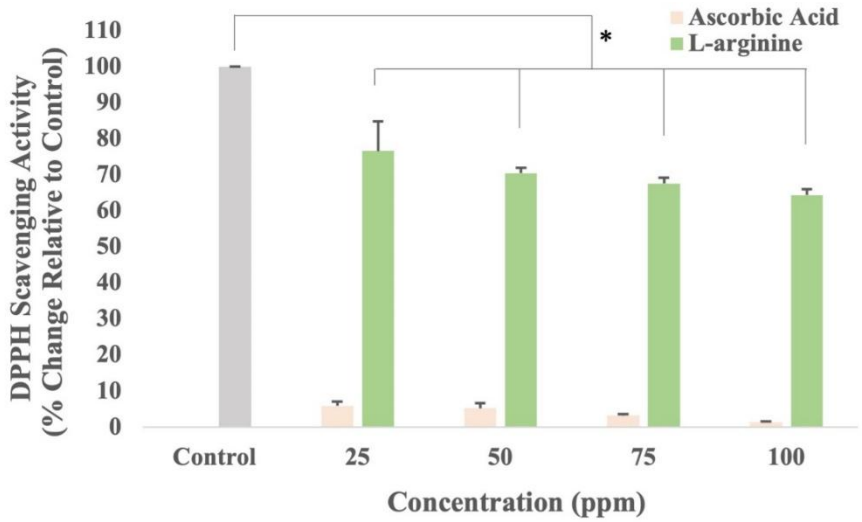



**Figure 4. Scavenged DPPH as a potential antioxidant of L-arginine and ascorbic acid.**

*(Results are expressed in terms of percentage change relative to control. Values are the mean ± SEM of n = 3 measurements where * have a significant difference at α = 0.05 with a p < 0.0001 interpreted using Tukey's test.)*

There is a decrease in the formation of MDA as the concentration of ARG increases as reflected in Figure 5. It exhibits a percent difference in scavenging activity ranging from 8 ± 0.27 % to 34.06 ± 0.21 % compared to ascorbic acid, from 29.22 ±0.48 % to 77.41 ± 0.23 % at 50 ppm to 500 ppm.

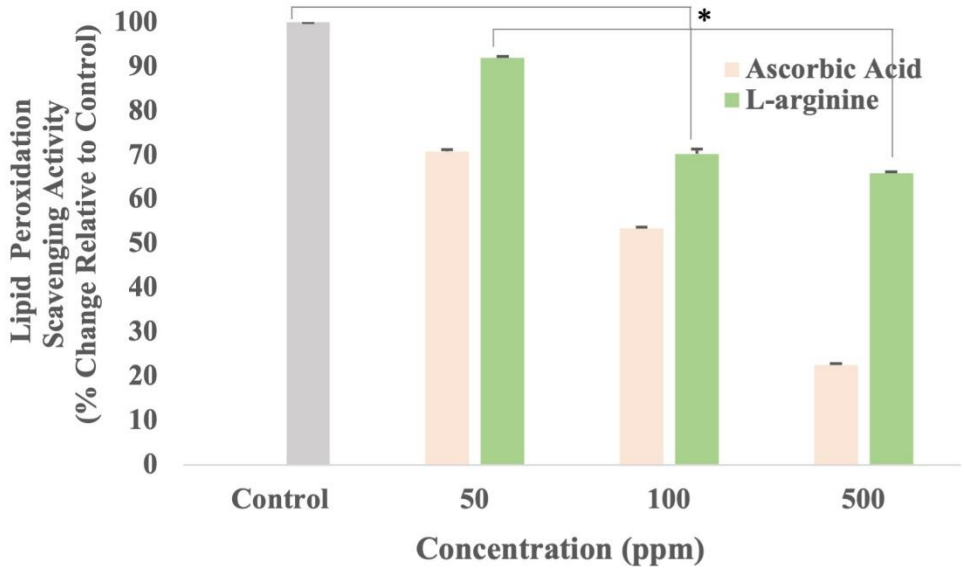



**Figure 5. Lipid peroxidation radical scavenging capacity L-arginine and ascorbic acid.** *(Results are expressed in terms of percentage change relative to control. Values are the mean ± SEM of n = 3 measurements where * have a significant difference at α = 0.05 with a p < 0.0001 interpreted using Tukey's test.)*

Reducing power and chelating ability of ARG were investigated using the formation of complexes from the respective source of iron. Figure 6 shows the percentage inhibition of the blue-colored ferrous ion-TPTZ complex in FRAP assay. It shows a reducing power effect, obtaining a percent inhibition difference ranging from 37.41 ± 4.31 % to 39.30 ± 2.36 %, slightly higher than the result obtained in the first reducing assay. Ascorbic acid exhibits a much lower formation of ferrous ion-TPTZ complex with 87.80 ± 0.06 % to 95.64 ± 0.02 % inhibition.

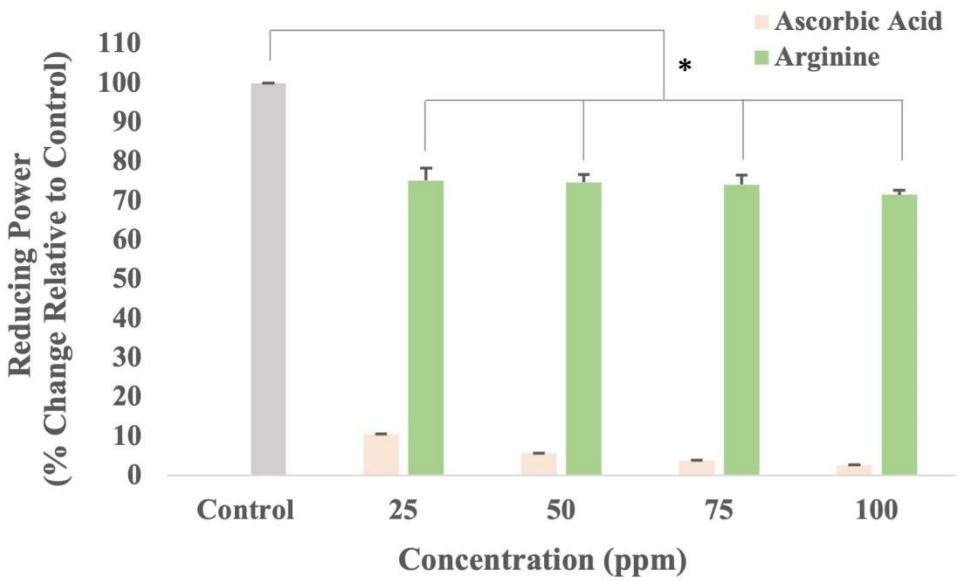



**Figure 6. Ferric reducing power determination of L-arginine.** *(Results are expressed in terms of percentage change relative to control. Values are the mean ± SEM of n = 3 measurements where * have a significant difference with respect to control at α = 0.05 with p < 0.0001 analyzed using Tukey's test.)*

The chelating activity of ARG is shown in Figure 7 which displays the percentage inhibition relative to the control. There is a decreasing effect of ARG on inhibiting the complex formation as the concentration of L-arginine increases, the same with EDTA as the positive control. There is no significant difference between the working concentrations of 25 ppm EDTA and 75 ppm ARG. Significantly, it implies that at 75 ppm, ARG has an antioxidant effect by inhibiting the ferrous ion-ferrozine complex. The chelating activity of ARG with 93.57 ± 0.33 % at 100 ppm is within the EDTA's result, obtaining 82.37 ± 1.31 % to 98.00 ± 1.17 %.

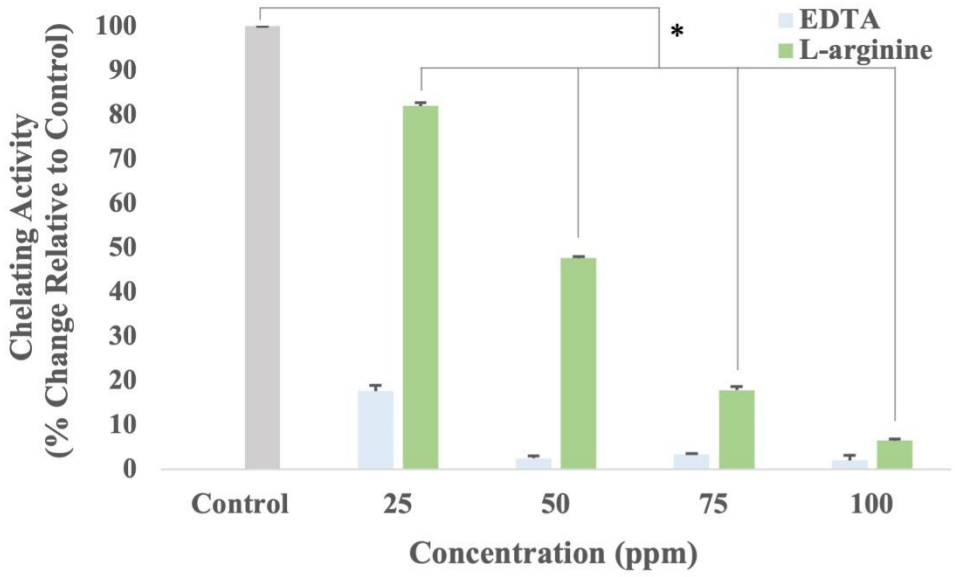


**Figure 7. Iron chelating capacity of L-arginine**. *(Results are expressed in terms of percentage change relative to control. Values are the mean ± SEM of $n \geq 3$ measurements where * have a significant difference at $\alpha = 0.05$ with a $p < 0.0001$ interpreted using Tukey's test.)*

**Discussion**

The present study has investigated the biological action and therapeutic effects of ARG through in vitro-based assays designed for T2D. ARG is an amino acid that participates in cellular processes to maintain the homeostasis state of the body. On the other way, ARG has been used as a main component in muscle-building supplements since the body uses amino acids to repair damaged muscle tissues due to intense exercise. Skeletal muscle becomes a crucial site for diabetic individuals being the major glucose disposal through the insulin-stimulated process, thus the availability of glucose as well as lipid concentration may change when metabolic disorder persists (Zhao & Keating, 2007).

High levels of advanced glycation end products (AGEs) are usually associated with diabetes. It forms non-enzymatically during consequent protein glycation processes involving sugars through the Maillard reaction followed by Amadori rearrangement or non-oxidative rearrangement of methylglyoxal (MGO). The high-rate formation of AGEs is known to be active in an oxidative stress condition. Consequently, when accompanied by high glucose availability, the rate level of AGEs increases, which becomes a key indicator in most diabetic patients (Muñiz et al., 2018; Starowicz & Henryk Zieliński, 2019 and Adjimani & Asare, 2015). In the study, ARG shows a significant antiglycation effect. Two model systems were employed, BSA-glucose and BSA-MGO, and it was observed that ARG could inhibit more in the final stage of AGEs formation than in the middle stage respectively. Congo red assay was performed to evaluate the influence of ARG on protein structures. Conformational protein or protein misfolding changes have been reported in its participation in T2D pathogenesis (Mukherjee et al., 2015 and Mukherjee et al., 2015). To some degree, congo red can detect secondary structural changes of BSA protein through specific binding at β-pleated sheet structure with dye (Klunk et al., 1989 and Elmira I. Yakupova et al., 2019). Based on the results there is a change in absorbance indicating the conformation in the structure of the BSA protein. However, to accurately determine the structural modification of the protein can be done using circular dichroism spectroscopy.

T2D disease is also associated with obesity and usually progresses to hyperlipidemia. This is when elevated levels of lipids and cholesterol could lead to T2D if not taken with

antilipidemic drugs (Wang et al., 2021; Wu et al., 2014 and Marella et al., 2015. A pancreatic lipase inhibition assay was conducted to determine if ARG could inhibit dietary triglyceride absorption since pancreatic lipase hydrolyzes most of the triglycerides to fatty acids and glycerol, approximately 50 to 70% of total dietary fat (Zielinska-Blizniewska et al., 2019 and Brobst, 1980). Results show that ARG has a potential antihyperlipidemic action through the inhibition of pancreatic lipase, preventing lipid accumulation in the modeled system.

Compounds with antioxidant properties can eliminate free radicals that cause oxidative stress by inhibiting the chain reaction of oxidation (Ali et al., 2023 and Roh & Jung, 2012). Free radicals are reactive species that chemically react with cells and participate in cellular pathways, when in excess it reverts the normal physiological function and generates more unstable or reactive radicals (Roh & Jung, 2012 and Qing Chong Xiu et al., 2022) Results on the scavenged radical species such as DPPH and MDA have observed a significant rate upon supplementation of ARG. In DPPH assay, DPPH is a nitrogen-based radical that uses its odd electron to react with the sample of interest (Qing Chong Xiu et al., 2022 and Sharma et al., 2021). The violet color of the solution is due to the delocalization of spare electrons, which makes it stable and can be disrupted by any antioxidant agent by donating a proton, which counteracts the radical resulting in discoloration of the violet solution (Irina Georgiana Munteanu & Constantin Apetrei, 2021; Kedare & Singh, 2011 and Al-Amiery et al., 2015). While in lipid peroxidation assay the basis of the inhibition is by measuring MDA as the indicator of antioxidant agents represses the formation of complex compounds. Free radicals, such as reactive oxygen species (ROS), have a deleterious effect on lipid membranes, leading to a cascade of radical chain reactions (Swartz et al., 2005; Luca Valgimigli, 2023 and Behl et al., 2022). Oxidatively, TBARS reacts with malondialdehyde (MDA), a secondary by-product of oxidation that forms the MDA-TBA2 complex, resulting in a pink solution (Sung Won Hwang et al., 2016). Results have found that the ability of ARG to counteract some types of such as DPPH and MDA levels were scavenged in a dose-dependent response depicting that at higher concentrations ARG could scavenge more radicals.

Metal ions such as iron participate in the oxidation reaction. Iron chelating & reducing power of ARG was conducted to test its potential as an antioxidant agent. Iron is highly reactive and attributes redox properties as an integral part of biological reactions, but when overproduced, it catalyzes the propagation of extremely unstable free radicals, such as ROS, which mainly result in oxidative stress and eventually lead to T2D (Leon & Borges, 2020 and Iris F.F. Benzie & Strain, 1996). FRAP assay measures the ability of ARG to reduce the $[Fe^{3+}\text{-}(TPTZ)_2]^{3+}$ to

$[Fe^{3+}$- $(TPTZ)_2]^{2+}$ that produces an intense blue-colored complex (Cao et al., 2022). The increase in color intensity also increases absorbance value, which demonstrates more ferrous ion-TPTZ complexes were formed (Cao et al., 2022 and Dimitrios Galaris et al., 2019). In the chelating assay, ferrous sulfate and ferrozine quantitatively form complexes, resulting in a red color and (Adjimani & Asare, 2015 and Wong, 2014). Absorbance measurement of the color reduction indicates the disruption of the complex formation or the binding ability of the chelator. The higher the absorbance, the weaker the ferrous iron binding strength of the chelator (Adjimani & Asare, 2015 and Wong, 2014). Based on the results ARG can inhibit iron chelation and slightly on the reducing power.

Collectively, the results show some significant observations that would assist that ARG could be a potential alternative treatment strategy for T2D. The antiglycation, antioxidant, and antihyperlipidemic effects of ARG that have been observed in the study might contribute to arresting the occurrence of insulin resistance.

## Conclusion

The present study conducted a preliminary analysis on the effect of ARG, a muscle builder component, through selected application of antiglycation, antioxidant, and antihyperlipidemic in vitro assays. Based on the gathered results, it shows potential in its ability to inhibit glycated model condition, scavenge some radicals, bind & reduce the iron, and inhibit pancreatic lipase action. These combined observations amend the contributing risk factors that facilitate the development of T2D.

## Acknowledgments

The authors thank the Mindanao State University-Iligan Institute of Technology – Biochemistry Lab and University College Sedaya International – Biotechnology Lab for access to conduct experiments in their respective laboratory facilities. The study was funded by the Philippines' DOST-ASTHRDP graduate scholarship and DOST Research Enrichment Program.

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