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Full length Research Article

Inhibition of α-Amylase and α-Glucosidase Activities by 2-Hydroxy-1,4-Naphthoquinone

Sulyman, O.A.[1], *Iyanda, A.Y.[2], Aladodo, A.R.[1], and Balogun, A.[3]

[1]*Department of Biochemistry, Faculty of Pure and Applied Sciences, Kwara State University, Malete, Nigeria*
[2]*Department of Environmental Management and Toxicology, Faculty of Pure and Applied Sciences, Kwara State University, Malete. Malete, Nigeria*
[3]*Department of Biological Science (Biochemistry Unit), Faculty of Science, Al-Hikmah University, Ilorin, Nigeria*

Summary: The purpose of the present study was to investigate the α-amylase and α-glucosidase inhibitory potentials of 2-hydroxy-1,4-naphthoquinone (2HNQ). The inhibition of these two carbohydrates metabolizing enzyme was done by varying the concentrations of 2HNQ in the presence of α-amylase, α-glucosidase with starch and p-nitrophenylglucopyranoside (pNPG) respectively as their substrates. The mode of inhibitions of the two enzymes by 2HNQ was determined using double-reciprocal transformation. The result obtained indicated that 2HNQ inhibited α-glucosidase activity with an IC50 0.260 mg/mL lowered than acarbose (1.530 mg/mL). Meanwhile, a moderate inhibitory potential of 2HNQ against α-amylase was observed with an IC_{50} of 1.757 mg/mL compared with acarbose (IC_{50} of 3.600 mg/mL). Furthermore, 2HNQ amazingly mops up reactive oxygen species. The observed inhibitions of α-amylase and α-glucosidase activity as well as radical scavenging potentials of 2HNQ suggest that it may be a potential target for the management of diabetes mellitus.

Keywords*:* *α-amylase; α-glucosidase; inhibition; 2-hydroxy-1,4-naphthoquinone; antioxidants*

**Authors for correspondence: yusuf.iyandaa@kwasu.edu.ng , Tel: +2348067393831*

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INTRODUCTION

Diabetes is a life-threatening ailment caused by the failure of pancrease to produce insulin or the ineffectiveness of the insulin produced by the pancrease (Salehi et al., 2019). This will in turn lead to postprandial hyperglycemia and other various secondary complications like blindness, kidney damage, cardiovascular disease, and lower-limb amputations (Chukwuma et al., 2019). According to Sun et al. (2022), just about 537 million of people between the ages of 20 – 79 yrs have diabetes and this figure has been anticipated to increase to 643 and 783 million by 2030 and 2045 respectively (Ogurtsova et al., 2022). The disease is recognized as a global health challenge and it has translated to a modern-day plague which poses a threat and socioeconomic problems (Mercer et al., 2019). Diabetes mellitus was responsible for the death of about 1.5 million individuals in 2019 according to World Health Organization (WHO). An effective way of managing diabetes mellitus is by the reduction in the postprandial blood glucose and this has been made possible by targeting α-amylase and α-glucosidase inhibitors (Masood et al., 2021). These two enzymes are vital to the regulation of postprandial blood glucose concentration and the inhibitions of these two enzymes can be used to lower the postprandial hyperglycemia thereby preventing or treating type 2 diabetes mellitus (Tacias-Pascacio et al., 2020). To this end, a lot of drugs have been employed in the management of diabetes mellitus and they have shown tremendous and encouraging results. However, despite the wide arrays of positive results shown by those drugs, they are not without shortcomings. Some effects such as nausea, diarrhea, heart failure, drug resistance, cancer, weight gain, hypoglycemia and soon have been reported (ADA, 2003). Diabetes Mellitus been a multifactor life-threatening disease requires a multidimensional therapeutic approach. Therefore, a molecule that is capable to act on more than one mechanism and with a little or no side effect will be an ideal candidate for an antidiabetic drug development. 2-Hydroxy-1,4-naphthoquinone (2HNQ) which is the principal natural compound found in the plant Lawsonia inermis has widely been used as paint and hair dye. Lawsonia inermis Linn. is a traditional plant which has an English name Henna (Kumar et al., 2017). The plant has been reported to have medicinal benefits (Salih et al., 2017). Widyawati et al. (2015) conducted a survey among the diabetes patients in Medan, North Sumatera, Indonesia, and reported that the leaves of Lawsonia inermis control the blood glucose level.

Therefore, this study investigates the α-amylase and α-glucosidase inhibitory potentials of 2-hydroxy-1,4-naphthoquinone (2HNQ).

MATERIALS AND METHODS

Chemical and Reagents: 2-hydroxy-1,4-naphthoquinone (2HNQ), 1,1-diphenyl-2-picryl-hydrazyl (DPPH), ferric chloride ($FeCl_3$), potassium ferricyanide, porcine pancreatic α-amylase, rat intestinal α-glucosidase and *p*-nitrophenyl-α-D-glucopyranoside (pNPG), Potassium persulfate, methanol, DMSO were products of Sigma Chemical Co., St. Louis, Missouri, USA. Starch, dinitrosalicylic acid (DNS), maltose and sucrose were products of J.T. Baker Inc., Phillipsburg, USA, while acarbose, 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) Trichloroacetic acid (TCA) were procured from Santa Cruz Biotechnology, Heidelberg, Germany. Distilled water was obtained from Medical Biochemistry and Pharmacology Department, Kwara State University, Malete, Ilorin, Nigeria. All other chemicals and reagents used were of analytical grade.

In vitro Antidiabetic Assays

Alpha-Amylase Inhibitory Assay: The α-amylase inhibitory assay was done by following a method described by McCue and Shetty (2004) with slight modification. In brief, 250μL of 2HNQ (1.25–10mg/mL) was mixed with 250 μL of α-amylase solution (0.5mg/mL) prepared in 0.02M sodium phosphate buffer (pH 6.9) in test tube. The mixture was pre incubated at 25 ºC for 10 min and after the addition of 250 μL of 1% starch solution, it was incubated again at 25 ºC for 10min. The reaction was quenched by adding 500 μL of quenching (dinitrosalicylic acid (DNS)) reagent. The mixture was boiled for for 5min and cooled to room temperature and 5mL of distilled water was used to dilute the mixtures after which the absorbance was monitored at 540 nm using spectrophotometer. DMSO was used as control replacing 2HNQ and the α-amylase inhibitory potential was expressed as percentage inhibition:

$$\%\text{Inhibition} = \left[\frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{extracts}}}{\text{Abs}_{\text{control}}}\right] \times 100.$$

Concentrations of 2HNQ resulting in 50% inhibition of α-amylase (IC_{50}) were determined graphically.

Mode of α-Amylase Inhibition: The mode of inhibition of α-amylase by 2-hydroxy-1,4-naphthoquinone (2HNQ) was carried out using the concentration of 2HNQ with the lowest IC_{50} according to the modified method described by Ali et al. (2006). Briefly, 250 μL of 2HNQ (5mg/mL) was mixed with 250 μL of α-amylase solution and the mixture was first incubated for 10min at 25°C. Varying concentrations of starch solution (5.0 - 0.3125 mg/mL) were added to the mixture and incubated for another 10min at 25°C. The reaction was stopped by adding 500 μL of DNS solution. The mixture were placed in boiling water for 5min, cooled to room temperature and 5mL of distilled water was added after which the absorbance was monitored at 540 nm using spectrophotometer. The amount of reducing sugars released was determined spectrophotometrically using a maltose standard curve and converted to reaction velocities. A double reciprocal plot (1/V versus 1/(S)) where V is reaction velocity and (S) is substrate concentration was plotted. The type (mode) of inhibition as well as Km and Vmax was determined (Nelson and Cox, 2008).

Alpha-Glucosidase Inhibitory Assay: The procedure described by Kim et al. (2005) with slight modification was adopted for the In vitro α-glucosidase inhibitory potential of 2HNQ. Briefly, 100 μL of α-glucosidase (1.0 U/mL) was mixed in a test tube with 50 μL of the varying concentrations of 2HNQ (1-0.0625mg/ml). the reaction was left to stand for 10min. Then 50 μL of 3.0mM (pNPG) prepared in 20 mM phosphate buffer (pH 6.9) was then added to kick start the reaction. The reaction mixture was incubated at 37°C for 20min and quenched by adding 2mL of 0.1M Na_2CO_3. The yellow-colored paranitrophenol released from pNPG was measured at 405 nm. The results were expressed as percentage of the blank control. Control was set up by replacing 2HNQ with phosphate buffer and acarbose was also used as a positive control. The inhibition percentage (%) was calculated using the formula:

$$\%\text{Inhibition} = \left[\frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{extract}}}{\text{Abs}_{\text{control}}}\right] \times 100.$$

Concentrations of 2HNQ resulting in 50% inhibition of enzyme activity (IC_{50}) were determined graphically.

Mode of α-glucosidase Inhibition: The mode of inhibition of α-glucosidase by 2-hydroxy-1, 4-naphthoquinone was determined using the concentration with the lowest IC_{50} according to the modified method described by Ali et al. (2006). Briefly, 50 μL of the (1mg/mL) 2HNQ was added to 100 μL of α- glucosidase solution in a test tube and incubated for 10 min at 25°C. Then, varying concentrations of 50 μL of pNPG (5–0.3125mg/mL) was added to the mixture to kick start the reaction. The mixture was then incubated for 10min at 25°C. The reaction was terminated by adding 500 μL of Na_2CO_3. The amount of reducing sugars released was determined spectrophotometrically using a paranitrophenol standard curve and converted to reaction velocities. A double reciprocal plot (1/V versus 1/[S]) where V is reaction velocity and [S] is substrate concentration was plotted. A double reciprocal plot (1/V versus 1/(S)) where V is reaction velocity and (S) is substrate concentration was plotted. The type (mode) of inhibition as well as Km and Vmax was determined.

In vitro Antioxidant Assays:

Reducing Power Assay: The reducing power of 2HNQ was evaluated by adopting the method of Belkacem et al. (2017) with slight modification. Varying concentrations of 2HNQ (1000 – 5000 µg/mL) were added to 2.5 mL of 0.2M phosphate buffer (pH6.6) and 2.5mL of 1% potassium ferricyanide ($K_3Fe(CN)_6$). The mixture was incubated at 50°C for 20 min prior to addition of 2.5mL of trichloroacetic acid (TCA). The solution was centrifuged at 3000 rpm for 10min and 2.5mL of the supernatant was mixed with an equal amount of distilled water and 0.5mL of 0.1% $FeCl_3$. The absorbance of the resulting solution was then read at 700nm.

DPPH Radical Scavenging Assay: The ability of 2HNQ to bleach the purple-coloured ethanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was done by following the

procedure described by Turkoglu et al. (2007). Briefly, 1mL of various concentrations (1000 – 5000 µg/mL) of 2HNQ were mixed with 1mL of a 0.2 mmol/L sample of DPPH in methanol and were left to stand for 30 minutes at room temperature. The absorbance was then read at against blank at 516 nm. Inhibition rate (I%) on the DPPH radical was calculated using the expression:

$$\text{Radical scavenging } (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where $A_{control}$ is the absorbance of the control, A_{sample} is the absorbance of the compound. Using standard calibration curve, the concentration of 2HNQ causing 50% inhibition (IC_{50}) of DPPH radical was estimated

ABTS Radical Scavenging Assay: The ABTS radical cation method described by Nicoletta (1999) and was modified to evaluate the free radical-scavenging effect of 2HNQ. The ABTS reagent was prepared by mixing 5mL of 7mM ABTS with 88 μL of 140mM potassium persulfate. The mixture was then kept in the dark at room temperature for 16 h to allow free radical generation and was then diluted with water (1: 44, v/v). To determine the scavenging activity, 100 μL ABTS reagent was mixed with 100 μL of sample in a test tube and was incubated at room temperature for 6 min. After incubation, spectrophotometer was used to measure the absorbance at 734 nm. The control used was 100 % methanol. The ABTS scavenging effect was measured using the following formula:

$$\text{Radical scavenging } (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Statistical Analysis of Data: Free radical and reactive oxygen species (ROS) scavenging activities were expressed in percentage while other data were expressed as the mean ± standard error of mean (SEM) of triplicate determinations. One way analysis of variance with Dunnett's post hoc test using GraphPad prism version 5.02. Values were considered statistically significant at the 95% confidence level.

RESULTS

In vitro Inhibitory Effect of 2HNQ on α –amylase and α-glucosidase: The potential of 2HNQ to inhibit α -amylase was determined (Figure 1). There was no significant difference in the inhibition of α- amylase by 2HNQ at concentrations between 0.63– 1.25 mg/mL but at higher concentrations between 2.5–5mg/mL, a significant increase in the percentage inhibition was observed ($P < 0.05$). The effectiveness of α-amylase was extrapolated from a dose response curve and this revealed that 2HNQ has a lower IC_{50} (1.757 mg/mL) when compared with acarbose (3.6 mg/mL) (Table 1). The mode of inhibition of 2HNQ showed that it displayed an uncompetitive mode of inhibition (Figure 2).

The ability of 2HNQ to inhibit α-glucosidase was displayed in Figure 3. 2HNQ inhibited α-glucosidase strongly in that it has an IC50 of 0.26 mg/mL compared to acarbose (1.53 mg/mL). The double-reciprocal transformation plot used to determine the mode of inhibition of the2HNQ revealed that 2HNQ displayed an uncompetitive mode of inhibition (Figure 4).

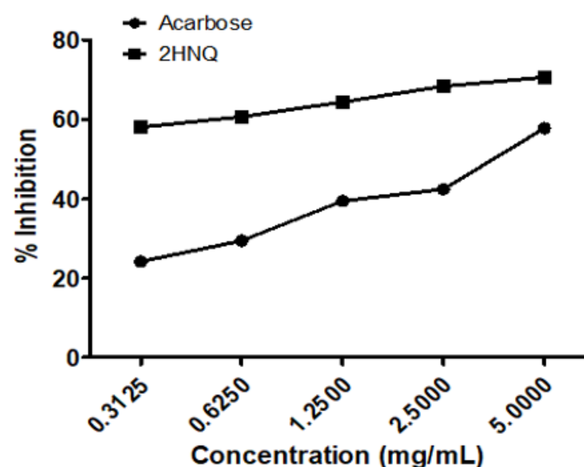

Figure 1:
Inhibitory potential of 2HNQ against α-amylase activity. The values are expressed as means ± SEM of triplicate determinations. Means not sharing a common letter at the same concentration were significantly different ($p < 0.05$).

Table 1:
IC_{50} values for α-amylase and α-glucosidase inhibitory potential of 2HNQ.

Compound	IC_{50} (mg/mL)	
	α -amylase	α -glucosidase
2HNQ	1.757	0.260
Acarbose	3.600	1.530

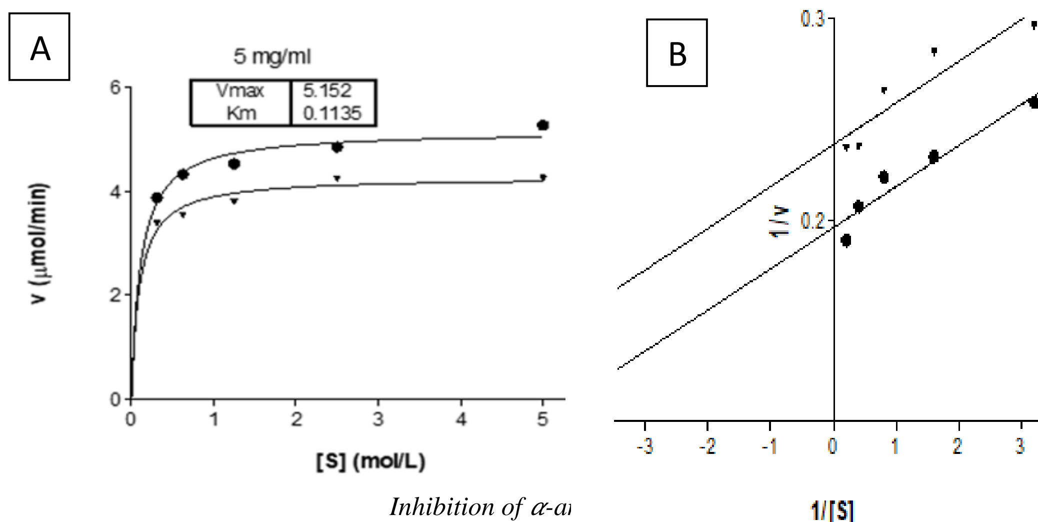

Figure 2:
Mode of inhibition of α-amylase by 2HNQ. (a) Michaelis-Menten plot and (b) Lineweaver-Burk plot

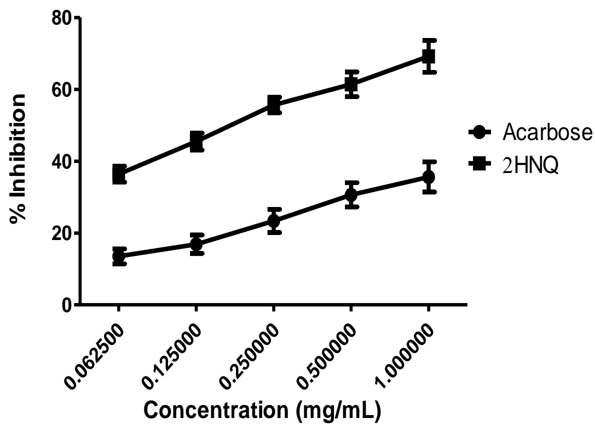

Figure 3:
Inhibitory potential of 2HNQ against α- glucosidase activity. Values are expressed as means ± SEM of triplicate determinations. Means not sharing a common letter at the same concentration were significantly different ($p < 0.05$).

Antioxidant Activity: The reducing power of potential of 2HNQ was shown in figure 5. The results showed a dose dependent increased in the reducing power ability of 2HNQ and these were significantly higher than that of silymarin (Figure 5). There was a dose dependent increase in the DPPH radical scavenging potential of 2HNQ in a somewhat similar manner to that of the silymarin (Figure 6). The IC50 of 4.52µg/mL was obtained for 2HNQ as compared to silymarin (4.33µg/mL) (Table 2). The potential of 2HNQ to scavenge ABTS was shown in figure 7. The compound (2HNQ) scavenged ABTS in a dose dependent manner in a similar way to the reference silymarin (Figure 7). The IC50 of 2HNQ was 1.34µg/mL as compared to silymarin which has an IC50 of 3.67µg/mL (Table 2).

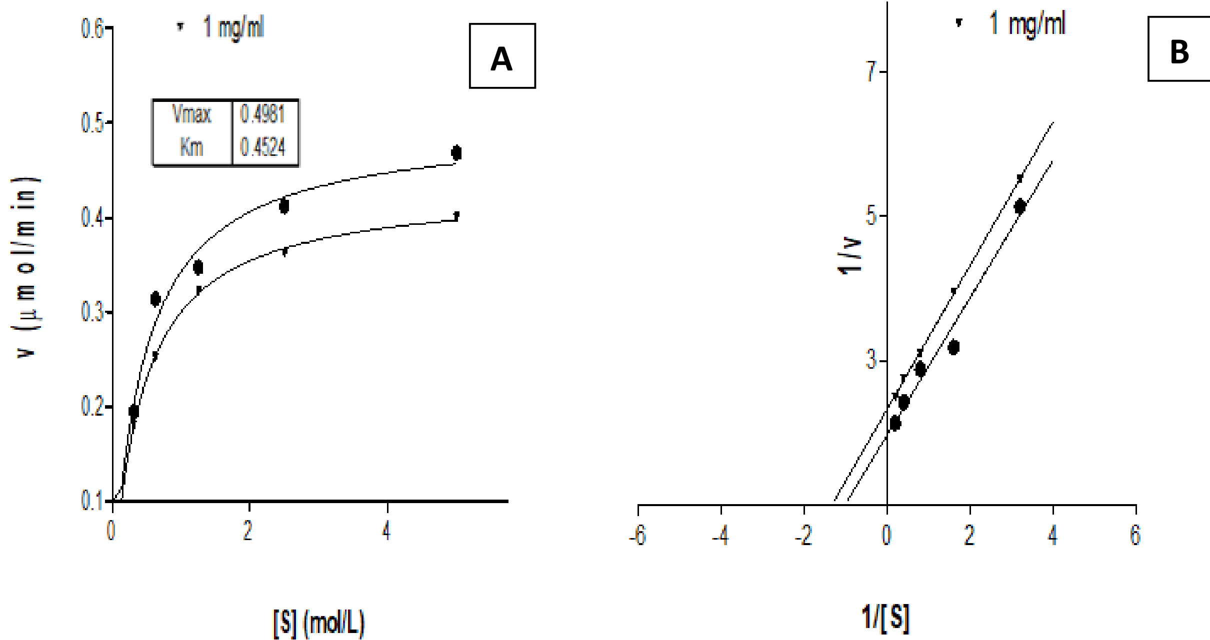

Figure 4:
Mode of inhibition of α-glucosidase by 2HNQ. (a) Michaelis-Menten plot and (b) Lineweaver-Burk plot.

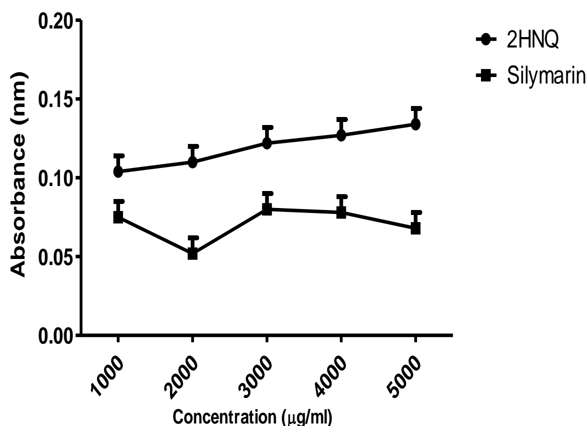

Figure 5:
Reducing power potential of 2HNQ. Values are expressed as means ± SEM of triplicate determinations. Means not sharing a common letter at the same concentration were significantly different ($p < 0.05$).

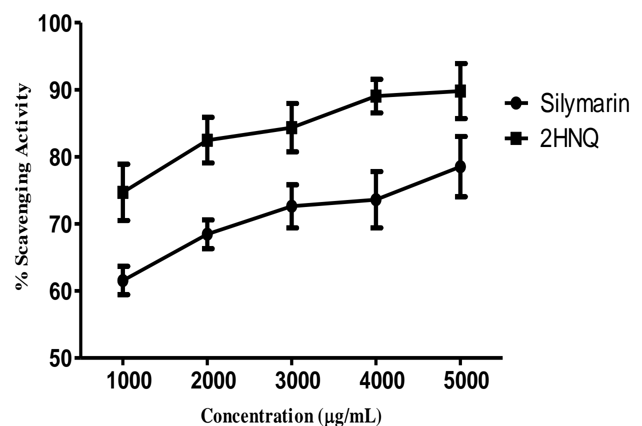

Figure 6:
DPPH scavenging effect of 2HNQ. Values are expressed as means ± SEM of triplicate determinations. Means not sharing a common letter at the same concentration were significantly different ($p < 0.05$).

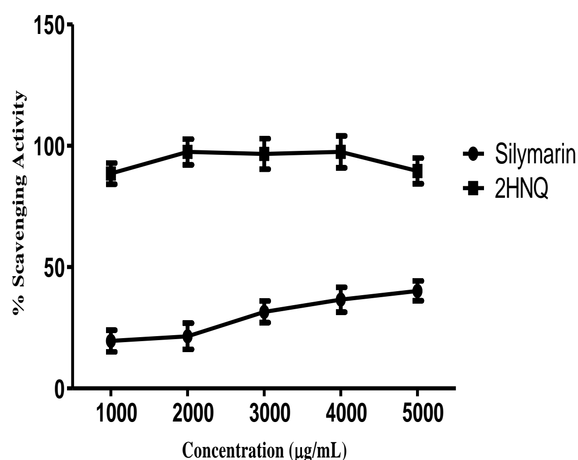

Figure 7:
ABTS scavenging effect of 2HNQ. Values are expressed as means ± SEM of triplicate determinations. Means not sharing a common letter at the same concentration were significantly different ($p < 0.05$).

Table 2:
IC_{50} of ABTS and DPPH scavenging capabilities of 2HNQ

ABTS	Silymarin	2HNQ
IC_{50}	3.67	1.34
R^2	0.9602	0.0062
Regression equation	y = 0.0056x + 12.96	y = 0.0002x + 93.302
DPPH		
IC_{50}	4.33	4.52
R^2	0.9448	0.9136
Regression equation	y = 0.0039x + 59.23	y = 0.0037x + 73.039

DISCUSSION

Generally, the reduction in the plasma blood glucose towards normal range can be achieved through the applications of oral hypoglycaemic agents and insulin. Nevertheless, these oral hypoglycaemic agents have some significant metabolic side effects (Campbell et al., 1996), and have encouraged scientists to work on the alternatives therapy with less toxicity for the management of this ailment. The upsurge in the blood glucose observed in diabetic patient is a consequence of an uncontrolled conversion of polysaccharides to an oligosaccharide by pancreatic α- amylase and the subsequent utilization of monosaccharides usually glucose by by intestinal α-glucosidases (Sabiu et al., 2016). An efficient way to handle this ailment is to sturdily prevent α-glucosidases from acting on the oligosaccharides as well as stylishly inhibit pancreatic α-amylase activity, which in turn regulate the availability of glucose in the blood (Kerru et al., 2018). A tactic to be employed in bringing down the blood glucose is to thwart carbohydrates absorption. Complex polymers need to be broken down respective monomeric form before they can be mobilized for enzyme production. This hydrolysis can be achieved by enzymes like α-amylase and α-glucosidases. Therefore, inhibition of these two enzymes prevents or stops the hydrolysis of disaccharides to glucose. Natural compound from plant origin with outstanding antioxidant ability present pretty substitute in this regard (Kumavat et al., 2012).

The compound (2HNQ) displayed a very strong inhibition of α-amylase activity. This finding agreed with Kwon et al. (2007) who reported that a mild inhibition of α-amylase activity is desirable because undue inhibition of pancreatic α- amylase could lead to an abnormal bacterial fermentation of undigested carbohydrates in the colon. The double reciprocal transformation of the data obtained revealed that 2HNQ inhibited α-amylase in an uncompetitive manner. This implies that this compound (2HNQ) binds to the complex formed between the α-amylase and substrate thereby decreasing both the Kcat and Km. It has been reported that this mode of inhibition is desirable for drug designs as the inhibitor binds to the enzyme target only when the target is active and in the presence of substrate.

The 2HNQ showed potent inhibition against α-glucosidase activity and this agreed with the finding of Kwon et al. (2007) who reported that natural compounds from plant have been reported to inhibit α-glucosidase and therefore can effectively be used as therapy for postprandial hyperglycemia with no side effect. The uncompetitive inhibition types obtained from double reciprocal transformation of data revealed that 2HNQ did not compete with enzyme for substrate binding but rather bind to a complex formed between the enzyme and the substrate which led to a decreased in both the Km and Vmax of the enzyme. The binding of 2HNQ to the complex formed between enzyme and substrate will inhibit the conversion of disaccharides to monosaccharides (Mogale et al., 2011).

Oxidative stress has been broadly reported as a contributor in the progress and evolution of diabetes (Rolo et al., 2006). Strangely high levels of reactive oxygen species and spontaneous reduction in the antioxidant defense mechanisms could result to dent of cellular organelles and enzymes, upsurge in lipid peroxidation, and progression of insulin resistance (Weyer et al., 2001). Ajiboye et al. (2013) reported that the antiradical potentials of any compound or extract can be used to test for the DPPH and ABTS scavenging effect as wells as reducing power potential. Since oxidative stress has been implicated in the pathogenesis of diabetes mellitus, it is thought that the disorders in this disease may be because of an increase in the oxidative stress (Modak et al., 2007).

The results obtained from this study revealed that 2HNQ scavenged free radical comparable to the silymarin used as a reference drug. The reduction in the IC50 values of 2HNQ is a pointer to the radical scavenging potential of the compound. This is supported by the closeness of the R2 values to 1.0.

Overall, the present study elucidates the mechanisms of α- amylase and α-glucosidase inhibitory potential of 2HNQ and apart from the fact that the compound inhibited α-amylase and α-glucosidase, it also scavenged free radicals resulted from increased oxidative stresses.

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