IN VITRO INHIBITORY ACTIVITIES OF *Persea americana* SEED EXTRACTS ON α-AMYLASE AND α-GLUCOSIDASE

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ABSTRACT
Medicinal plants have immense potential for treatment and management of diseases. The present study investigates in vitro effect of aqueous extract of Persea americana seeds (AEPAS) on a-amylase and a-glucosidase activities, key enzymes of carbohydrates digestion. Inhibitor(s) to these key enzymes offers an effective strategy to lower post prandial hyperglycaemia by lowering carbohydrates digestion. The AEPAS was prepared using standard method and reconstituted in phosphate buffer to evaluate its effects on the activities of porcine pancreatic a-amylase and Saccharomyces cerevisiae a- glucosidase. The results of the inhibitory activities show that the extract has higher inhibitory effect on a-glucosidase than on a-amylase activities. The IC_{50} of the extract on a-amylase and a-glucosidase activities are 401.40 and 13.52 µg/mL respectively, while standard drug, acarbose used as positive control show IC_{50} on the a-amylase and a-glucosidase activities of 60.72 and 112.23 µg/ml respectively. Acarbose is more potent on a-glucosidase than on a-amylase activities. The mode of inhibition by the extract is non-competitive for a-amylase and competitive for a-glucosidase, while acarbose exhibits competitive and non-competitive inhibitory effects on a-amylase and a-glucosidase activities respectively. Base on the finding of this study, Perseaamericana seeds may ameliorate diabetes mellitus by affecting glycaemic index of foods via lowering digestibility. Therefore results may support the claim that Persea americana seed extracts possesses hypoglycaemic activities.
Key words: Perseaamericana, a-amylase, a-glucosidase, inhibition

INTRODUCTION
Diabetes mellitus is an endocrine-metabolic disorder characterized by chronic hyperglycaemia (high blood sugar) giving rise to the risk of microvascular (retinopathy, nephropathy, and neuropathy) and macrovascular (ischaemic heart disease, stroke and peripheral vascular disease) damage, with associated reduced life expectancy and diminished quality of life (Neelesh *et al.,* 2010). It could be due to deficiency of insulin or its ineffectiveness (Kumar *et al.,* 2010). It was reported that about 30 million people suffered from diabetes in 1985 and the number increased to more than 171 million in 2000 (WHO, 2012; 2014). It is estimated that the number will increase to over 366 million by 2030 and that large increase will occur in developing countries, especially in people aged between 45 and 64 years (Chinenye and Young, 2011; Ekam *et al.,* 2013; Patel *et al.,* 2013). Among the strategies in used for the management of type 2 diabetes mellitus is oral

hypoglycemic agents, such as biguanidsand sulfonylureas and the inhibition of degradation of dietary starch by glycosidases such as a-amylase and α-glucosidase (Rang*et al.,* 2003), this offers an important mean of regulation glycemic index of diets.
Persea americana, commonly called Avocado pear is one of the plants that have been extensively used in traditional folk-medicine (Brai *et al.,* 2014). It belongs to the family *Lauraceae*. In Nigeria, the plant has various local names such as 'apoka', or 'ewe pia' in Yoruba; 'ube-beke' or 'akwukwoube' in Igbo and 'fiya' in Hausa (Yasir*et al.,* 2010; Nwauzoma and Dappa, 2013). The tree of *Perseaamericana* is cultivated in tropical and subtropical areas and bear a pear-shaped fruit (Brai *et al.,* 2014). It has been reported that seed of *Perseaamericana*has various application in phytomedicine, example, treatment for diarrhoea, dysentery, toothache, intestinal parasites, skin treatment and beautification (Isaac *et al.,* 2014).

A significant decrease (P<0.001) in blood glucose was observed in alloxan-induced diabetic rats treated with aqueous extract of avocado pear seed and a significant increase in blood glucose (p<0.05) was also observed one week after withdrawal of the extract (Alhassan *et al.*, 2012). Phytochemical compounds reported present areterpenoids glycosides, aliphatic acetogenius (alkanols), flavonoids and coumarin (Yasir*et al.*, 2010).

The study is therefore aimed at evaluating *in vitro* effect of AEPAS on porcine pancreatic α-amylase and *Saccharomyces cerevisiae* α-glucosidase.

MATERIAL AND METHODS
Reagents
Porcine pancreatic α-amylase, *Saccharomyces cerevisiae* α-glucosidase and other chemicals were purchased from Sigma-Aldrich USA.
Sample collection and Preparation
Fifty gram (50g of the powder was soaked in 500ml of distilled water and allowed to stand for 24 hours with intermittent shaking and thenfiltered with cheese cloth. The filtrate was centrifuged at 8000 rpm for 10 minutes and filtered using no 1 Whatman filter papers. The crude extract was subsequently freeze-dried and reconstituted in 20mM sodium phosphate buffer (pH 6.9) to 10, 30, 60 and 80µg/ml.

In vitro effects of the extract
IC_{50}determinationon α-Glucosidase
Procedure:
Each concentration of the extractwasseparately incubated with 50µL α-glucosidase enzyme at 37°C for 10 minutes. Fifty mircoliters (50µL) of 10mM p-Nitrophenyl Glucopyranoside (pNPG) was then added to start the reaction and this was also incubated for another 10 minutes at 37°C. The reactions were quenched by adding 2mL of Na_2CO_3 solution. The absorbance was then read at 405nm. Control was also run with this experiment but buffer was substituted for extract.

The experiment was run in triplicate and average absorbance determined. The absorbance was converted to the amount of p-nitrophenol released using a p-nitrophenol standard curve and then enzyme activities were determined by using the formula;

$$\text{A unit of } \alpha\text{-glucosidase activities} = \frac{\text{mg of p-nitrophenol released x Dilution factor}}{\text{Time of incubation x mg of enzyme in the reaction mixture}}$$

Percentage of enzyme activities was determined from the formula:

$$\text{Where:-}\quad \% \text{ Enzyme activities} = \frac{\text{Abs1 - Absx}}{\text{Abs1}} \text{ x } 100$$

Abs1 = Absorbance of the control
Absx = Absorbance of the test at different concentration of the extract
A plot of percentage enzyme activities against the concentrations of the extracts was made and the concentration of the extract that inhibits 50% enzyme activities (IC_{50}) determined graphically.

Mode of α-Glucosidase Inhibition
The mode of inhibition of the extract was conducted according to the modified method of Ali *et al* (2006). All the experiments were run in triplicate and average values were determined.Exactly 50µL of the extract (5mg/mL) was pre-incubated with 100µL of α-glucosidase solution for 10 mins at 37°Cin a set of tubes. In another set of tubes α-glucosidase was pre-incubated with 50µL of 20mM sodium phosphate buffer (pH 6.9). fifty micro liters (50µL) of pNPG at increasing concentrations 10, 30 and50µg/mL was added to both sets of reaction mixtures to start the reaction and incubated for 10 mins at 37°C, then 2mL of Na_2CO_3 was added to stop the reaction. The amount of reducing sugars released was determined spectrophotometrically using a 4-nitrophenol standard curve and converted to reaction velocities. The type (mode) of inhibition by theaqueous extract on α-glucosidase activity was determined by analysis of the double reciprocal (Lineweaver-Burk) plot.

IC_{50} determination on α-Amylase
This assay was carried out using a procedure of Kazeem *et al* (2013) with modification. Various concentrations, 80, 160, 240, 320 and 400µg/mL of extracts were prepared in 20mM sodium phosphate buffer, pH 6.9. Each concentration was incubated with 250µL α-amylase enzyme at 25°C for 10 minutes. Then 250µL of 1% starch solution was added to start the reaction and this was also incubated for another 10 minutes at 25°C. The reactions were quenched by adding 500µL of dinitrosalicylic acid (DNSA) solution. The mixture was boiled for 5 minutes and cooled to room temperature (25°C).The absorbance was then read at 540nm. Control was also run with this experiment but buffer was substituted for the extract.

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The experiment was run in triplicate and average absorbance determined. The absorbance was converted to the amount of maltose released using a maltose standard curve and then enzyme activities were determined by using the formula;

$$A \text{ unit of } \alpha\text{-amylase activities} = \frac{\text{mg of maltose released x Dilution factor}}{\text{Time of incubation x mg of enzyme in the reaction mixture}}$$

Percentage of enzyme activities was determined from the formula:

$$\% \text{ Enzyme activities} = \frac{\text{Abs1 - Absx}}{\text{Abs1}} \times 100$$

Where Abs1 = Absorbance of the control test without the extract

Absx = Absorbance of the test when the concentration of the extract is 80, 160, 240, 320 and 400µg/mL respectively.

A plot of percentage enzyme activities against the concentrations of the extracts was made and the concentration of the extract that inhibits 50% enzyme activities (IC$_{50}$) determined graphically.

Mode of α-Amylase Inhibition

The mode of inhibition of the extract was conducted according to the modified method of Ali *et al* (2006). All the experiments were run in triplicate and average values were determined.Briefly, 200µL of the extract(1mg/mL) was pre-incubated with 200µL of α-amylase solution for 10 minutes at 25ºC in one set of tubes. In another set of tubes α-amylase was pre-incubated with 200µL of 20mM sodium phosphate buffer (pH 6.9). 200µL of starch at increasing concentrations (0.5,1.0 and 2%w/v) was added to both sets of reaction mixtures to start the reaction. The mixture was then incubated for another 10 minutes at 25˚C, and 400µL of DNSA was added to stop the reaction. The reaction mixture was then boiled for 5 minutes. The amount of maltose 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 of the aqueous extract on α-amylase activity was determined by analysis of the double reciprocal (Lineweaver-Burk) plot using Michaelis-Menten kinetics.

Statistics: the experiments were performed in multiples, n= 5 and results are expressed as mean ±SEM (standard error of the mean). Statistical analysis was performed for ANOVA (analysis of variance) followed by Bonforoni test, a package of SPSS version 20.0. Values of pwhich were = 0.05 were considered as significant.

RESULTS AND DISCUSSION

α-Amylase Activity Inhibition

This study shows that the activity of α-amylase enzyme reduces as the concentration of the aqueous extract increases. The concentration of *AEPAS* that inhibits 50% α-amylase enzyme activities (IC$_{50}$) was found to be 401.40µg/mL. Figure 1(a) below depicts a plot of percentage enzyme activities against the concentrations of *Persea americana* and the concentration of *Persea americana* that corresponds to 50% enzyme activities was shown. When compared with the IC$_{50}$ of the standard drug (acarbose) fig 1c, the AEPAS show significantly (P<0.05) less inhibitory effect against Alpha Amylase Activities. The finding is similar to that of Carene *et al* (2014) on native plant of Mauritius.
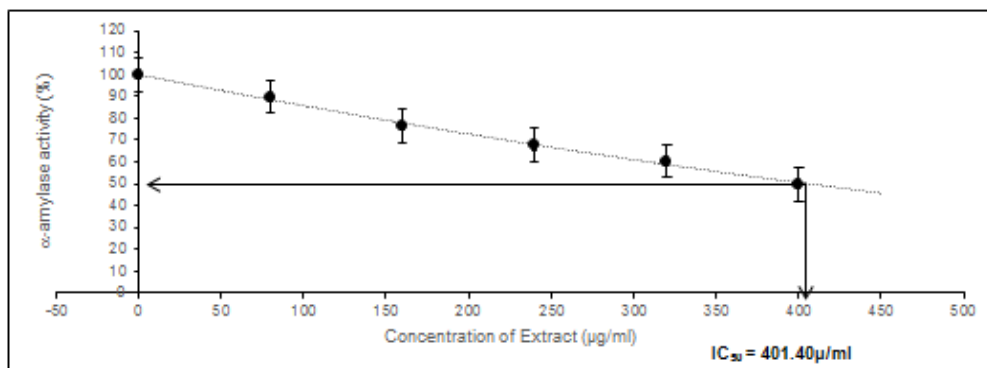

Figure 1(a): the *in vitro* percentage porcine alpha amylase inhibition (%) at varying AEPAS concentration
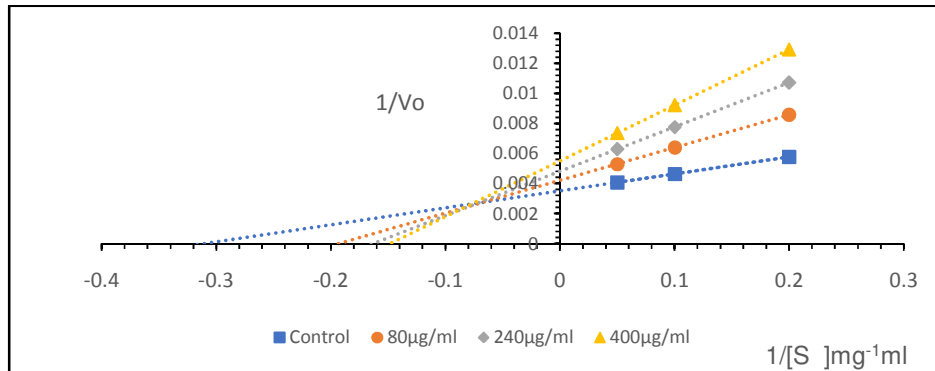The data is presented as mean ± SE, n= 5, p <0.05

Figure 1(b): primary plot of 1/V0 against 1/[S$_0$] at varying AEPAS concentration, indicating non-classical non-competitive inhibition against alpha Amylase **and the data is presented as mean ± SE, n= 5, p <0.05.**

Figure 1(b)depicts the Lineweaver-Burk plot from which the nature of inhibition was determined to be non-classica lNon-competitive inhibition

When compared with the *AEPAS*, the drug, significantly reduces α-amylase activity (p <0.05)with increase in concentration of standard drug (acarbose). The concentration of acarbose that inhibit 50% α-amylase enzyme activities (IC$_{50}$) was found to be 60.72μg/mL which is significantly lower compared to 401.40μg/mL for the AEPAS. Figure 1(c) below depicts a plot of percentage enzyme activities against the concentrations of acarbose and the concentration of acarbose that correspond to 50% enzyme activities was shown.

Unlike for PEPAS the mode of inhibition by acarbose against alpha amylase activities was found to be competitive as depicted on figure 1d below.
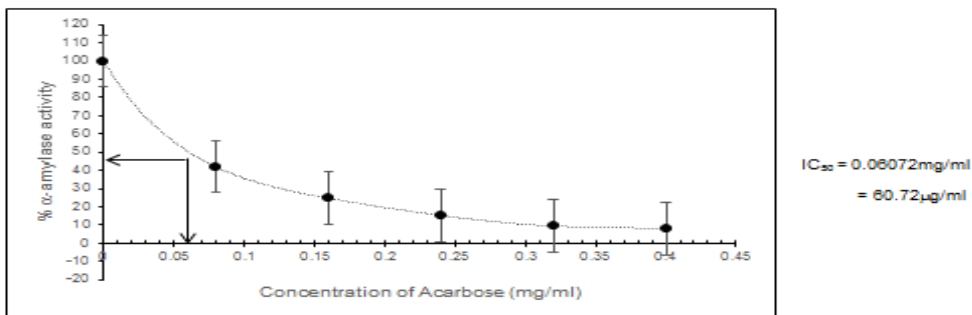

Figure 1(c): the *in vitro* percentage porcine alpha amylase inhibition (%) at varying acarbose concentration The data is presented as mean ± SE, n= 5, p <0.05
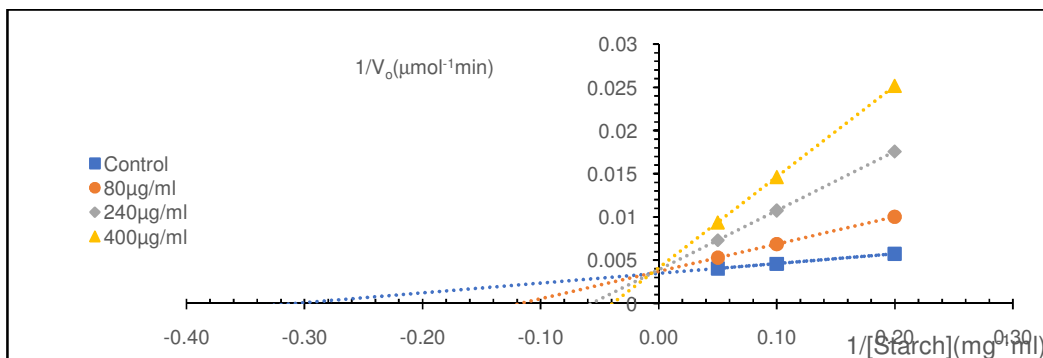

Figure 1(d): primary plot of 1/V$_0$ against 1/[S$_0$] at varying acarbose concentration, indicating competitive inhibition against alpha Amylase, **the data is presented as mean ± SE, n= 5, p <0.05.**

α-Glucosidase Activity Inhibition

Activity of α-glucosidase reduces significantly (p<0.05) as the concentration of the AEPAS increases. Figure 2(a) shows a plot of percentage α-glucosidase activities against *Persea Americana* extract concentration. The concentration of *Persea americana* that reduces the activities of the enzyme to 50% (IC$_{50}$) was determined to be 13.52μg/mL.
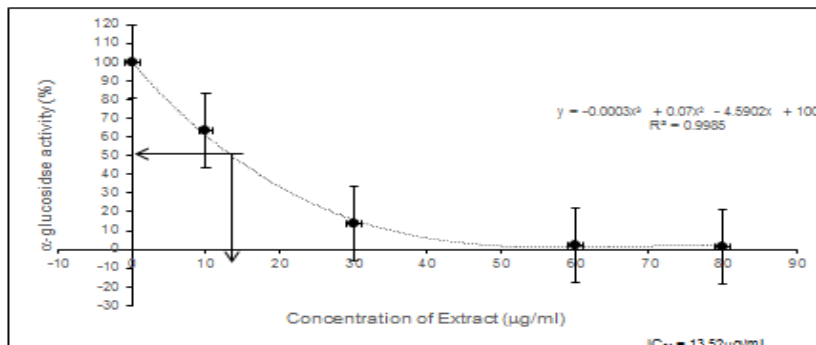

Figure 2(a): the *in vitro* percentage α-Glucosidase Activities inhibition (%) at varying AEPAS concentration. The data is presented as mean ± SE, n= 5, p <0.05.

The mode of inhibition of *AEPAS* against α-glucosidase was found to be competitive inhibition as depicted on figure 2(b)
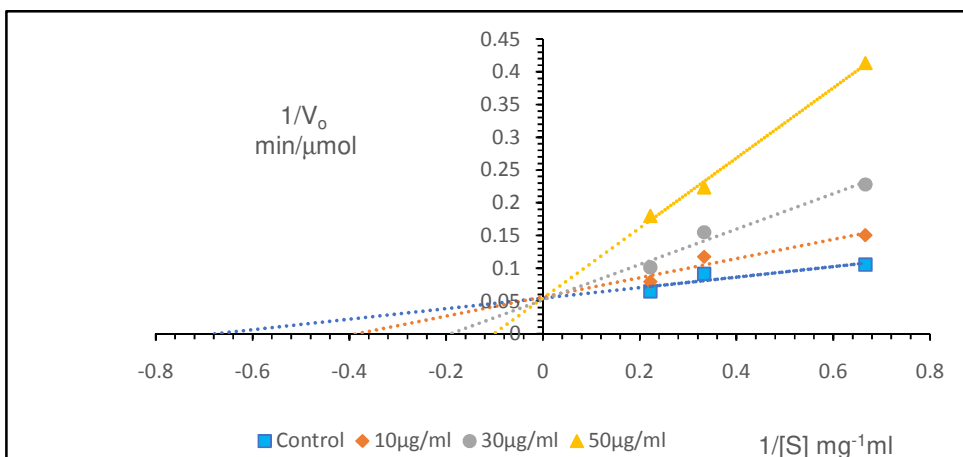

Figure 2(b): primary plot of 1/V0 against 1/[S$_0$] at varying AEPAS concentration, indicating non - classical non-competitive inhibition against α-Glucosidase and the data is presented as mean ± SE, n= 5, p <0.05.

In comparison the effect of AEPAS with the standard drug (acarbose), activity of α-glucosidase slightly reduces as the concentration of acarbose increases Figure 2(c).The concentration of acarbose that reduces the activity of the enzyme to 50% (IC$_{50}$) was calculated from the curve equation on the graph. The IC$_{50}$ is 112.23μg/mL, which greater than the IC 50 for the AEPAS. Therefore the AEPAS could effectively reduce the activity of α-glucosidase, hence reducing the glycaemic of foods.

The mode of inhibition of acarbose against α-Glucosidase was determined from Lineweaver-to be non-classical non-competitive inhibition (Figure 2d)
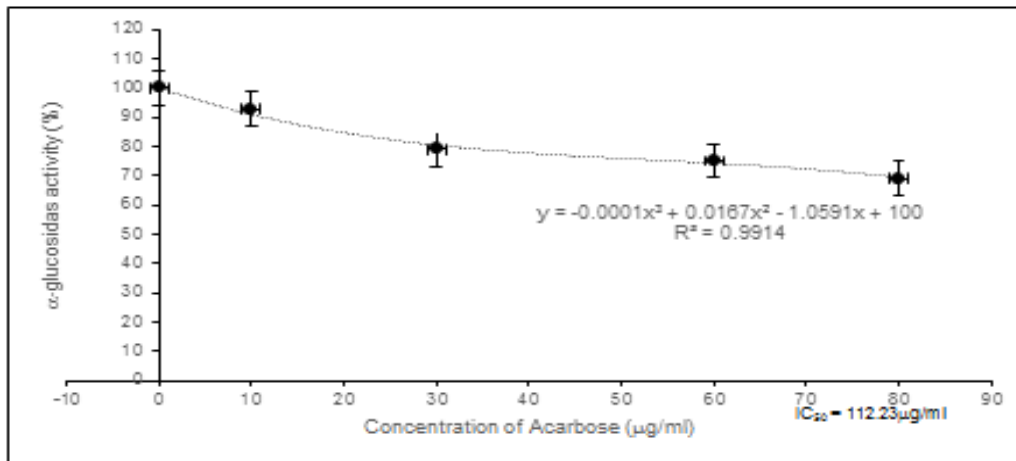
Figure 2(c): the *in vitro* percentage α-Glucosidase Activities inhibition (%) at varying acarbose concentration. The data is presented as mean ± SE, n= 5, p <0.05.
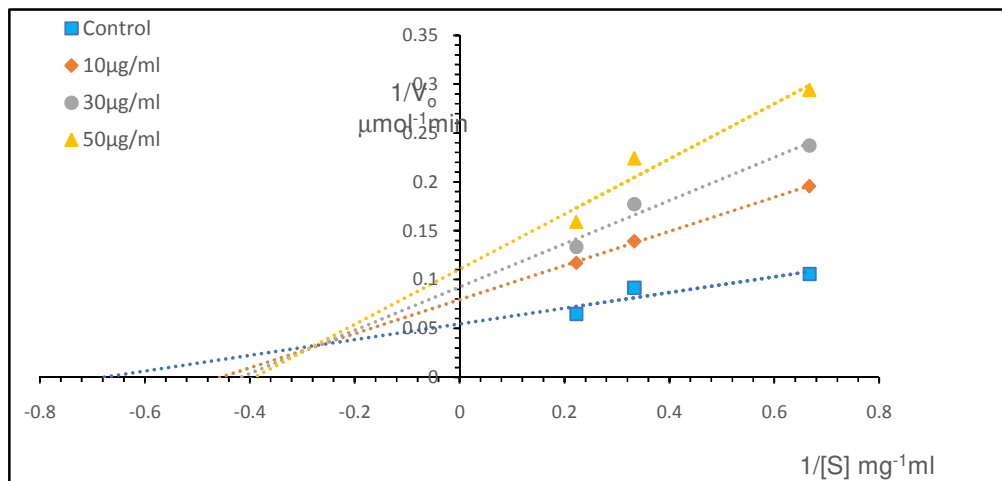

Figure 2(d): primary plot of $1/V_0$ against $1/[S_0]$ at varying acarbose concentration, indicating Non classical competitive inhibition against alpha Amylase**, the data is presented as mean ± SE, n= 5, p<0.05.**

Several studies had indicated that inhibiting key digestive enzymes that liberates glucose from carbohydrates could slow down carbohydrate digestion, reducing glucose absorption rate, consequently preventing postprandial glucose increase (Carene *et al.*, 2014). The present study shows that *AEPAS* is more potent (inhibitory effect) on α-glucosidase than on the α-amylase as depicted on Figure 1(a) and Figure 2(a). Also, when comparing with acarbose, the standard drug, *Persea americana* exhibits lower inhibitory effect on α-amylase compared to acarbose (figure 1a and 1c),whereas *Persea americana* exhibits higher inhibitory effect on α-glucosidase than acarbose (figure 2a and 2c).These observations may attract attention on the use of *Persea Americana* seed in controlling glucose liberation from carbohydrates as therapeutic modality in the management of diabetic mellitus.

CONCLUSION
The study show that AEPAS exhibits different degree of inhibitory activities against key enzymes of carbohydrates digestion. The nature of inhibition, *Persea americana* exhibits non classical non-competitive mode of inhibition on α-amylase and competitive inhibition on α-glucosidase activities (figure 1b and 2b). Whereas acarbose exhibit competitive mode of inhibition on α-amylase and non - classical non-competitive mode of inhibition on α-glucosidase activities (Figure 1d and 2d).

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Concomitant use of the standard drug and AEPAS could be double barrel therapy for management of hyperglycaemia. This study supports the claims by other researchers that aqueous extract of *Persea americana* has potent hypoglycaemic activities, which is used to treat or manage diabetes mellitus.

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