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RESEARCH ARTICLE

# PHYTOCHEMICAL SCREENING AND IN VITRO ANTIOXIDANT AND ANTI-DIABETIC POTENTIALS OF *PERSEA AMERICANA* MILL. (*LAURACEAE*) FRUIT EXTRACT

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## Abstract

**Objective:** Diabetes mellitus (DM) is a metabolic disorder characterized by insulin resistance and pancreatic β-cell dysfunction and the management of blood glucose level is an important strategy in the control of the disease and complications associated with it. Therefore, components that cause uptake of glucose from the bloodstream and inhibitors of carbohydrate hydrolyzing enzymes can be useful in treatment of DM and medicinal plants are often used to achieve this aim. *Avocado* fruit is rich in phytochemicals necessary for treatment of DM. The purpose of this study was to investigate the inhibitory effect of *Persea americana* fruit extracts on α-amylase and α-glucosidase enzymes.
**Methods:** The percentage yield, phytochemical screening (both qualitative and quantitative), *in vitro* antioxidant and anti-diabetic assays, and kinetic studies were performed with different solvent extracts of Avocado fruit pulp.
**Results:** Avocado had great and promising potential as pharmaceutical agent, particularly to be developed as anti-diabetic through the inhibition of α-glucosidase and α- amylase enzymes. *In vitro* studies of the antioxidant activity of the fruit extract gave an evidence and strong biochemical rationale of their therapeutic potential.
**Conclusion:** The fruit extract of *P. americana* may play an important role in the development of nutraceuticals and also in the management of oxidative stress induced DM.
**Keywords:** α-amylase, α-glucosidase, antioxidant, glucose, nutraceuticals, phytochemical.

## INTRODUCTION

Medicinal plant is an important part of traditional health care system and a veritable health care source for the vast majority of the world population. It was estimated that 70-80% of people worldwide use herb for management of mild to moderate illnesses[1-5]. Diabetes mellitus (DM) is an endocrine disorder resulting in obstinate elevation of blood glucose under both fasting and postprandial conditions resulting in micro and macro vascular complications[6]. The prevalence of diabetes is increasing globally and is prophesied to increase by twofold from 150 million in the year 2000 to 300 million by the year 2030[7]. The uncharacteristic regulation of glucose metabolism that results from a malfunctioning/scarce insulin secretion is the key pathogenic event in DM. Currently available drugs for hyperglycemia exhibit adverse side effects on prolonged use. Hence the exploration for novel therapeutic drugs continues. Recent focus has been made towards "functional food", a natural source food purported to have a beneficial health effect for the successful treatment of various ailments especially life style diseases like diabetes. The *Avocado* (*Persea americana* Mill.), unflatteringly known in the past as alligator pear, midshipman's butter and vegetable butter. It has traditionally been used due to its antibacterial, antifungal, hypotensive, anti-inflammatory, and immune-enhancing effects[8,9]. Furthermore, *Avocado* juice made from ripe fruit was very popular due to its numerous health benefits. Because of the limited number of reports on the fruits of *Avocado* available in the literature, it was deemed sensible and justified to systematically investigate the fruits of this plant[10,11]. This present study seeks to validate the folkloric use of *Avocado* fruit extract (AFE) in the management of DM and several oxidative stress induced diseases. The study also confined the

kinetics of α-amylase and α-glucosidase inhibitory potentials of *AFE*.

## MATERIALS AND METHODS

**Plant collection, preparation and extraction.**

Fresh fruits of *P. americana* were selectively collected and authenticated in the Tepi Agricultural Research Center, Tepi and the same was authenticated in department of biology, Mizan Tepi University, Ethiopia. (Vide voucher No. MTU-ETARC 102/08/02). The peel was peeled off and the edible part was chopped into thin pieces, dried at 50-60$^0$C, and ground into powder. Known amount of dried amount was exhaustedly extracted by the process of maceration in an aspirator using various solvents as menstruum. AFE with different extracting solvents (ethanol, 50% hydro-ethanol (v/v), decoction (a concentrated liquor resulting from heating or boiling with water) and aqueous) were concentrated under reduced pressure by rotary evaporator to obtain respective thick syrup mass, and stored at 4$^o$C. Working concentration of the extract was made in non-pyrogenic distilled water before use in the experiments.

**Chemicals and reagents**

Porcine pancreatic α-amylase, rat intestinal α-glucosidase, 1, 1-diphenyl-2-picrylhydrazyl, gallic acid, acarbose and p-nitrophenyl-glucopyranoside were products of Sigma-Adrich, South Africa. Other chemicals and reagents were of analytical grade and the water used was glass distilled.

**Measurement of percentage yield**

The percentage yield of the extract was calculated as-

$$\% \text{ Yield} = \frac{c-b}{a} X100$$

Where a = weight of sample; b = weight of beaker and c= weight of beaker + sample.

**Phytochemical Screening**

**1. Qualitative Phytochemical screening**

Using described procedure[12], the AFE was subjected to qualitative phytochemical screening with different extracting solvents.

**2. Quantitative Phytochemical Analysis**

**a. Assessment of Total Phenolic Content (TPC)**

The quantification of TPC with different solvents of *AFE* was carried out using the prescribed procedure reported by Wolfe K *et al.*, using Folin Ciocalteu reagent[13]. Gallic acid was used as standard. TPC was expressed as mg/g gallic acid equivalent using the equation obtained from a calibration curve of gallic acid.

**b. Determination of Total Flavonoid Content (TFC)**

The TFC with different solvents' extracts were determined using the method employed by Swanny[14]. TFC was calculated as quercetin (mg/g) equivalent using the equation obtained from a calibration curve of quercetin.

***In vitro* Antioxidant Assays**

All experiments were conducted in triplicates and all the negative control (blank) was prepared using the same procedure replacing the AFE with distilled water.The free radical scavenging activity of the AFE were evaluated with various solvents based on its scavenging activities on the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical according to the method described by Braca A *et al.*,[15]. Determination hydroxyl radical scavenging potential of AFE with various solvents to prevent $Fe^{2+}/H_2O_2$ induced decomposition of deoxyribose was carried out using the modified method of Mathew and Abraham[16]. Determination of superoxide anion radical scavenging potential of AFE with various solvents were achieved according to the method employed by Liu and Chang[17]. The chelating of $Fe^{2+}$ by AFE with various solvents was estimated as described by Dinis *et al.*,[18]. Ferric ions reducing power of the with various solvents' extracts and standards were determined according to the method adopted by Müller *et al.*,[19]. The ability of *AFE* to scavenge 2, 2-Azino-bis(3-ethylbenzothiazo - line-6-sulphonic acid) (ABTS) cation chromophore obtained from the oxidation of ABTS solution and potassium persulphate with various solvents was determined according to the method of Re *et al.*,[20]. To these above said antioxidant assays, the percentage inhibitory/scavenging activity of the AFE/standard was calculated using following equation-

$$AFE = \frac{A0-A1}{A0} X100$$

Where $A_0$ is the absorbance of the control, and $A_1$ is the absorbance of the AFE / standard. The half maximal inhibitory concentration ($IC_{50}$) value were calculated from the linear regression equation using following equation-

$$y = m x + c,$$

Where; y is the percentage activity and equals 50, m is the slope, c is the intercept and x is the $IC_{50}$ value.

***In vitro* Anti-diabetic Assays**

The α-amylase and α-glucosidase inhibitory assays were carried out using the procedure of *Apostolidis E et al.*,[21]. The 50% inhibition of enzyme activity ($IC_{50}$) of these enzymes was expressed as % inhibition using the expression:

$$\% \text{ Inhibition} = \frac{\text{Acontrol} - \text{AAFE}}{\text{Acontrol}} X100$$

Where $A_{control}$ and $A_{AFE}$ are the absorbance's of the control and AFE respectively. Concentrations of AFE /standard resulting in 50% inhibition of enzyme activity ($IC_{50}$) were determined graphically using the linear regression equation-

$$y = mx + c$$

Where y is the percentage activity and equals 50, m is the slope, c is the intercept and x is the $IC_{50}$ value.

**Kinetic Studies**

The kinetics on inhibition of α-amylase and α-glucosidase activity by AFE with various solvents was conducted using modified methods of Ali *et al.*,[22] and Nagmoti and Juvekar[23] respectively. The amount of reducing sugars released was determined spectrophotometrically using maltose standard curve for α-amylase and p-nitrophenol standard curve for α-glucosidase. A double reciprocal (Lineweaver–Burk) plot (1/v versus 1/[S]) where v is reaction velocity and [S] is substrate concentration was plotted to determine the mode of inhibition. Thus, reaction rates (v) were calculated and double reciprocal plots of enzyme

kinetics $K_m$ and $V_{max}$ values were also calculated from Lineweaver-Burkplot (1/v versus 1/[S])[24].

**Statistical Analysis**

Statistical analysis was performed using a Graph Pad Prism 5 statistical package (Graph Pad Software, San Diego, MA, USA). Data were expressed as means of replicate determinations±SD, for all assays and was subjected to one-way analysis of variance (and nonparametric) followed by Bonferroni: compare all pair of column. Statistical significance was considered at $p<0.05$.

## RESULTS

The percentage yield of AFE with different extracting solvents is shared out in Table 1.

**Table 1: The percentage yield from different extracting solvents used in AFE.**

| AFE | Ethanol | 50% Hydro-ethanol | Decoction | Aqueous |
|---|---|---|---|---|
| Yield (%) | 11.12 | 29.71 | 8.05 | 18.55 |

Values are mean and standard deviation (SD) of triplicate determination. n=3; ($p<0.05$).

**Phytochemicals (PC)**

The qualitative analyses of the AFE with different extracting solvents are presented in Table 2. Phenols, alkaloid, tannins and triterpenes were detected at varying degree in all the tested extracts while flavonoid was found in trace amount in all solvent extracts besides anthraquinone and phytosterol were found in trace amount in the ethanol and 50% hydro-ethanol extracts. The results of the quantitative phytochemical screening (TFC and TPC) of AFE with different extracting solvents are depicted in Table 3.

**Antioxidant activity**

The *in vitro* antioxidant potentials of the AFE with different extracting solvents are shown in Figure 1 to Figure 6. The extracts scavenged/inhibited/chelated the generated radicals/ions/metals in all assays were evaluated. Ethanolic extracts showed better capability to scavenge DPPH and hydroxyl radicals in a dose dependent manner (0.125-1.00 mg/ml) (Figure 1 and Figure 2). Its corresponding $IC_{50}$ value is 0.52 and 0.59 µg/ml which is lower and significantly different ($p<0.05$) from the standard (silymarin) $IC_{50}$: 1.09 and 1.12 µg/ml as seen in Table 4. However, hydro-ethanol showed remarkable capability in scavenging superoxide anion radical (Figure 3), its $IC_{50}$ value is 0.49 µg/ml which is comparable to silymarin with $IC_{50}$ value 1.12 µg/ml. AFE also showed significant metal chelating potential against ferrous ion (Figure 4) and the respective IC50 value when compared with the standard (citrate) is presented in Table 4.

**Table 2: Phytochemical constituents of the AFE with different extracting solvents.**

| Phytochemicals | Ethanol | 50% Hydro-ethanol | Decoction | Aqueous |
|---|---|---|---|---|
| Alkaloids | +++ | +++ | ++ | ++ |
| Phenols | +++ | ++++ | +++ | ++ |
| Flavonoids | + | + | - | - |
| Anthraquinones | ++ | ++ | +++ | +++ |
| Tannins | ++++ | ++++ | ++ | ++ |
| Triterpenes | - | ++ | +++ | ++++ |
| Phytosterol | - | ++ | +++ | +++ |

+: detected; +++: degree of intensity; -: not detected or in trace amount.

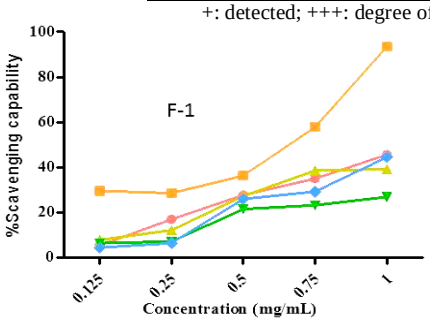


**Figure 1: DPPH scavenging effect of AFE with different extracting solvents.**

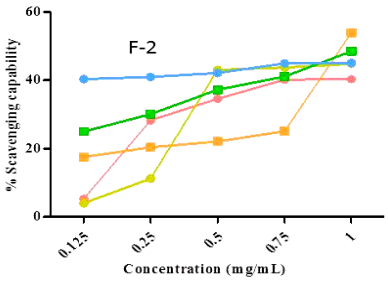


**Figure 2: Scavenging effect of AFE with different extracting solvents on hydroxyl radical.**

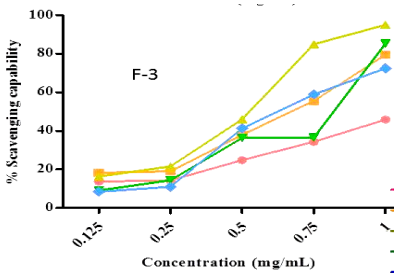


**Figure 3: Scavenging effect of AFE with different extracting solvents on superoxide anion radical.**

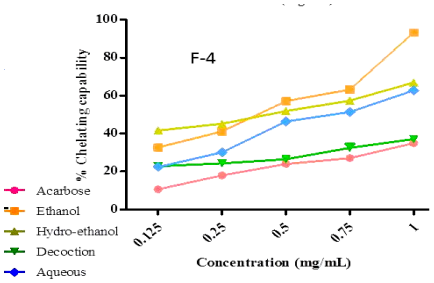


**Figure 4: Metal chelating capability of AFE with different extracting solvents.**

**Table 3: The result of the quantitative phytochemical screening of AFE with different extracting solvents.**

| Phytochemicals | Ethanol | 50% Hydro-ethanol | Decoction | Aqueous |
|---|---|---|---|---|
| TFC (mg quercetin in $g^{-1}$) | 0.36 | 1.10 | 0.61 | 0.30 |
| TPC (mg gallic acid $g^{-1}$) | 8.35 | 10.29 | 10.79 | 10.41 |

**Table 4: The $IC_{50}$ values of the free radical scavenging/chelating capabilities of different extracts of *P. americana* fruit.**

| Samples | $IC_{50}$ (µg/mL) | | | | |
|---|---|---|---|---|---|
| | DPPH | ABTS | Hydroxyl | Superoxide | Metal Chelating |
| Silymarin | 1.09±0.02 | 0.39±0.05 | 1.12±0.02 | 1.12±0.01 | - |
| Citrate | - | - | - | - | 1.51± 0.01 |
| Ethanol | 0.52±0.05 | 0.38±0.02 | 0.59±0.01 | 0.63±0.10 | 0.39± 0.01 |
| Hydro-ethanol | 1.15±0.03 | 0.30±0.02 | 0.94±0.01 | 0.49±0.00 | 0.41±0 .05 |
| Decoction | 1.78±0.01 | 0.49±0.02 | 1.03±0.01 | 0.57±0.01 | 1.73± 0.02 |
| Aqueous | 1.05±0.01 | 0.49±0.05 | 1.76±0.01 | 0.60±0.01 | 0.67± 0.01 |

The values are expressed as mean±standard deviation (SD) of triplicate determination. ($p$<0.05). Silymarin is the standard antioxidant agent for all the antioxidant assays except metal chelating that has citrate as the standard.

The reducing power (Figure 5) and ABTS cation scavenging capability (Figure 6) of the extracts competed well with silymarin in a dose dependent manner (0.125-1 µg/mL) with the highest dose of 1µg/ml showing the best activity (Table 4).

***In vitro* anti-diabetic assays**

The inhibitory potentials of AFE on both α-amylase and α- glucosidase enzymes is dose dependent (0.125-1 µg/mL), and the percentage inhibition is presented in Figure 7 and Figure 8 respectively. Ethanolic extract has the lowest $IC_{50}$ (0.15 µg/mL) which is significantly different ($p$<0.05) from all other extracts and acarbose (Table 5). Ethanol and decoction extracts show milder inhibition of α-amylase with their respective $IC_{50}$ value of 0.57 and 0.62 µg/ml which is higher and significantly different ($p$<0.05) from acarbose and hydro-ethanol ($IC_{50}$:0.47 and 0.42 µg/mL) respectively. Lineweaver-Burk plot of ethanolic extract of Avocado fruit eliciting competitive and uncompetitive inhibition on α-amylase (Figure 9) and α-glucosidase activity (Figure 10) respectively.

**Table 5: The $IC_{50}$ values for different extracts of *P. americana* fruit on specific activities of α-amylase and α-glucosidase enzymes.**

| Samples | $IC_{50}$ (µg/mL) | |
|---|---|---|
| | α- Glucosidase | α-Amylase |
| Acarbose | 0.52±0.04 | 0.47±0.01 |
| Ethanol | 0.15±0.00 | 0.57±0.01 |
| 50% Hydro-ethanol | 0.39±0.00 | 0.42±0.05 |
| Decoction | 0.46±0.01 | 0.62±0.03 |
| Aqueous | 0.45±0.04 | 0.53±0.08 |

The values are expressed as mean±standard deviation (SD) of triplicate determination. Means down vertical column not sharing a common superscript are significantly different ($p$<0.05) from each other.

## DISCUSSION

The use of plants in treating diseases is as old as civilization[25] and herbal medicine is still a major part of habitual treatment of different diseases[26]. The process in the preparation of herbs like pulverization, extraction and solvents deployed in the extraction of raw material for drugs affects the percentage yield of the biologically active compound present in the extracts. In this experiment, local solvents (ethanol, 50% hydro-ethanol, decoction and distil water) were used in Avocado fruit extract preparation. The percentage yield indicated that 50% hydro-ethanol (v/v) has the highest yield of 29.71% from the 30g dry weight of the fruit sample extracted while decoction extract yield 8.05% of the 30 gm dry weight of the sample. It is worthy of note that the traditional healer use decoction (boil the dry fruit pulp) as their method of extracting the biologically active component of the plant. It may be suggested that this method of extraction accounted for low yield of extract which may be lesser efficacious.

Result of the quantitative phytochemical assays indicated the concentration of the different quantity of the PC found in AFE though, its bioavailability is unpredictable in the *in vivo* study, because a lot of factors like absorption barrier of the PC in the gastro intestinal tract (GIT), the effects of different enzymes such as the glucosidase, esterase, oxidase and hydrolases originating from the host and the mycobiota which may inhibit PC activity in the GIT[27]. PC are known to possess varying antioxidant activities[28-32]. Antioxidant activity of a medicinal plant cannot be concluded based on a single antioxidant test model[28] as such several *in vitro* antioxidant tests were conducted on the extracts using silymarin as positive control for all assays except metal chelating assay where citrate was used as the standard. The free radical scavenging capability of fruit of *Avocado* on the molecules of DPPH radicals, ABTS cations radical, the reducing power, superoxide anion radicals were determined; nevertheless, also assayed the hydroxyl radical which is one of the most potent ROS in the biological system that reacts with polyunsaturated fatty acid moieties of cell membrane phospholipid causing cellular damage[33]. The result of the assay showed that ethanolic *AFE* has better performance in antioxidant activity compared to the standard and other extracts tested for DPPH, hydroxyl radical and metal chelating activities while hydro ethanol showed superior activity compared to the standard and other extracts tested in ABTS, superoxide anion and reducing power. All these predictions are based on the standard curve of percentage

inhibition/scavenging effect and $IC_{50}$ value of the tested extract which revealed a decrease in concentration of the ROS which may be due to the scavenging ability of *AFE*. Similar findings have been documented for the antioxidant and anti-inflammatory properties of Avocado fruit[34]. It is noteworthy that the tested extract demonstrated the ability to neutralize the ROS at different degree which may be because of the presence of PC like polyphenols which has capability to directly scavenge superoxide and other ROS like hydroxyl and peroxyl radicals[35-37]. Saponins, triterpenes and phytosterol have been demonstrated to scavenge superoxide anion[38-40].

Flavonoid are currently receiving attention as a potential protector against variety of human disease, major flavonoid has been shown to have neutralizing effect on free radical and ROS like hydroxyl radical, superoxide radical, hydrogen peroxides[28,39,41-43,].

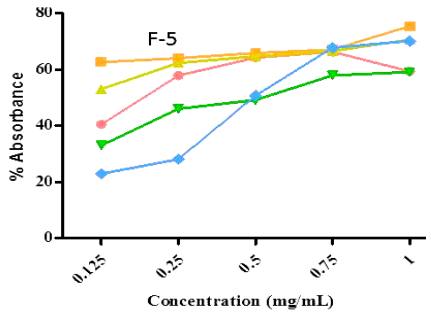


**Figure 5: Reducing potentials of AFE with different extracting solvents.**

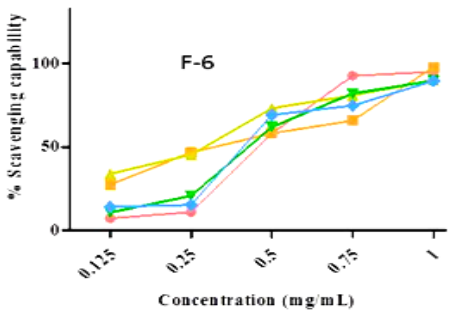


**Figure 6: ABTS scavenging effect of AFE with different extracting solvents.**

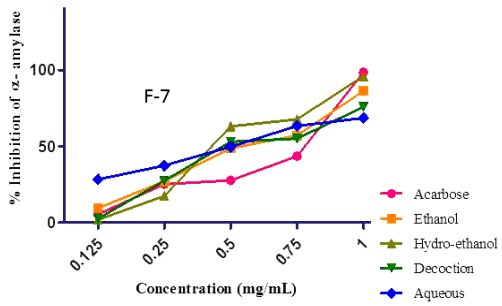


**Figure 7: The inhibitory potentials of AFE with different extracting solvents on α-amylase activity.**

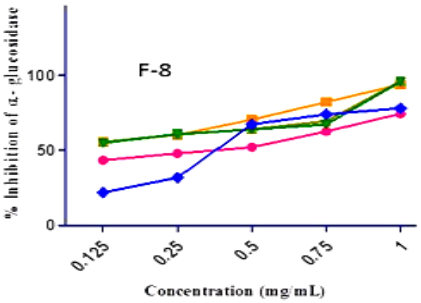


**Figure 8: The inhibitory potentials of AFE with different extracting solvents on α-glucosidase activity.**

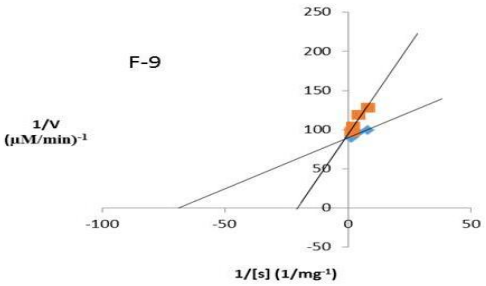


**Figure 9: Lineweaver-Burk plot of ethanolic extract of *Avocado* fruit eliciting competitive inhibition on. α- amylase activity.**

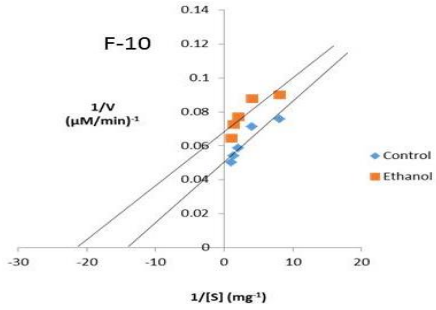


**Figure 10: Lineweaver-Burk plot of ethanolic extract of *Avocado* fruit eliciting uncompetitive inhibition on α-glucosidase activity.**

Marked postprandial hyperglycaemia is important in the pathogenesis of T2DM. It induces mitochondrial superoxide overproduction which potently inhibits the glycolytic enzyme glyceraldehyde-3-phosphate thus, diverting upstream metabolites from glycolytic pathway into pathway of glucose overutilization resulting in formation of diacyl glycerol from dihydroxyl acetone phosphate (DHAP) a potent activator of protein kinase C (PKC) which ultimately causes β-cells destruction and insulin resistance[44-46]. The unregulated hydrolysis of starch by α- amylase and α-glucosidase which catalyze the rate limiting step in the conversion of oligosaccharides and disaccharides into monosaccharide's is responsible for the elevated blood glucose seen in T2DM. Therefore, controlling hyperglycaemia via inhibition of carbohydrate hydrolysing enzymes is an important strategy in the management of T2DM[47-49]. *In vitro* evaluation of the inhibitory effects of the AFE on α-glucosidase and pancreatic α-amylase enzymes was carried out using acarbose as the standard to determine its percentage inhibition and their respective $IC_{50}$ value. Mild inhibition of α-amylase and strong inhibition of α-glucosidase enzymes is targeted as a way of reducing postprandial hyperglycaemia, and elimination of the unwanted effect like gastrointestinal discomfort

flatulence, diarrhoea associated with the use of acarbose[6,49,50]. In this study, ethanol and decoction extracts mildly inhibit α- amylase with their respective $IC_{50}$values of 0.57 and 0.62 µg/ml which is higher and significantly different ($p$<0.05) from acarbose with lower $IC_{50}$ (0.47 µg/mL). The result of the inhibitory potentials of the extracts on α-glucosidase showed ethanol and decoction extracts has potent inhibition of the enzyme activity. Thus, it may be employed in the management of postprandial hyperglycemia. This finding is consistent with findings of many authors[47,48,51] who described moderate inhibition of α-amylase and strong inhibition of α-glucosidase as a better therapeutic approach to be deployed in the delay and regulation of carbohydrate hydrolysis in the intestine which is responsible for glucose toxicity observed in T2DM.

The ethanolic extract which possesses the highest $IC_{50}$ for α-amylase enzyme and lowest $IC_{50}$ for α-glucosidase compared to acarbose and other tested extracts of *Avocado* fruitwas used to determine the mode of inhibition of α- amylase and α- glucosidase enzymes in other to investigate its enzyme inhibition kinetics. Similar findings were observed by our previous study on *Morinda citrifolia* and its secondary metabolite scopoletin[52-54]. Nevertheless, our past research on Avocado's antihyperglycemic, antidiabetic dyslipidemic and antioxidant potentials with different studies in *in vivo* models well line up with the present findings[55-57]. Result for the mode of inhibition of α-amylase enzyme showed that the ethanolic AFE is competitively inhibiting the breakdown of disaccharides and oligosaccharides which are substrate for α- amylase. The $V_{max}$ values obtained with inhibitor and without inhibitor in the reaction pathway is the same, the $K_m$ values decreased from $4.85x10^{-2}$ $\mu M^{-1}$ for reaction pathway without inhibition to $1.44x10^{-2}$ $\mu M^{-1}$ with inhibitor. Decreased $K_m$ value signifies increase affinity. This result proposed competitive mode of inhibition. However, the mode of inhibition of α-glucosidase by ethanolic AFE is by uncompetitive inhibition. The propose model is the binding of the AFE (inhibitor) to a site other than the active site and only when the substrate is binding to ES complex thereby inhibiting the formation of product. The kinetic further shows that there is a decrease in $K_m$ from $7.10x10^{-2}$ $\mu M^{-1}$ to $4.69x10^{-2}$ $\mu M^{-1}$without inhibitor and with inhibitor respectively and also a decrease in $V_{max}$ from 19.76 µM/min without inhibition to 14.66 µM/min with inhibition which suggests a 39.74% decrease in overall activity of α- glucosidase enzyme in the presence of ethanolic extract of fruit of *Persea americana* Mill.

## CONCLUSIONS

From this work, it has been conjectured that the fruit of *Avocado* has great and promising potential as pharmaceutical agent, particularly to be developed as anti-diabetic agents through the inhibition of α-glucosidase and α-amylase enzymes. This natural approach is thought to be safer and more effective compared to its synthetic version (e.g., acarbose and voglibose). Added to this, demonstrated the *in vitro* tests of the antioxidant activity of the fruit extract, which gives evidence and strong biochemical rationale of their therapeutic potential. Therefore, the promising results shall be carried forward to *in vivo* test as well as clinical trial to further validate the activity. Besides, data generated from these studies further promote the traditional use of plants in medicine. Therefore the fruit extract of *P. americana* may play an important role in the development of nutraceuticals and also in the management of oxidative stress induced DM.

## ACKNOWLEDGEMENTS

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## AUTHOR'S CONTRIBUTION

**Mahadeva Rao US:** Writing original draft, review, methodology, data curation, literature survey, editing.

## DATA AVAILABILITY

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## CONFLICT OF INTEREST

None to declare.

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