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 illnesses14,15. Diabetes mellitus (DM) is an endocrine disorder resulting in obsolete elevation of blood glucose under both fasting and postprandial conditions resulting in micro and macro vascular complications7. 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 20307. 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 lifestyle 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 effects8,9. 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 plant9,10,11. 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°C, and ground into powder. Known amount of dried amount was exhaustively extracted by the process of maceration in an aspirator using various solvents as menstrum. 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°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-picylhydrazyl, gallic acid, ascorbolic and p-nitrophenyl-glucopyranoside were products of Sigma-Adrich, South Africa. Other chemicals and reagents were of analytical grade and were obtained from the methods employed by Mathew and Abraham. Determination of superoxide anion radical scavenging potential of AFE with various solvents was achieved according to the method method employed by Liu and Chang. The chelating of Fe²⁺ by AFE with various solvents was estimated as described by Dinis et al., and ferric ions reducing power of the with various solvents’ extracts and standards were determined according to the method adopted by Muller et al., The ability of AFE to scavenge 2, 2-Azino-bis-(3-ethylbenzthiazolo - 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.. To these above said antioxidant assays, the percentage inhibitory / scavenging activity of the AFE / standard was calculated using following equation-

\[
\text{AFE} = \frac{A_0 - A}{A_0} \times 100
\]

Where \( A_0 \) is the absorbance of the control, and \( A \) is the absorbance of the AFE / standard. The half maximal inhibitory concentration (IC₅₀) value were calculated from the linear regression equation using following 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 IC₅₀ value.

**In vitro Anti-diabetic Assays**

The α-amylase and α-glucosidase inhibitory assays were carried out using the procedure of Apostolidis *et al.*. The 50% inhibition of enzyme activity (IC₅₀) of these enzymes was expressed as % inhibition using the expression:

\[
\text{Inhibition} = \frac{A_{control} - A_{AFE}}{A_{control}} \times 100
\]

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 (IC₅₀) 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 IC₅₀ 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.*, and Nagmoti and Juvkar 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 $1/[S]$).

**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>
<thead>
<tr>
<th>Solvent</th>
<th>Yield (%)</th>
<th>50% Hydro-ethanol</th>
<th>Decoction</th>
<th>Aqueous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>11.12</td>
<td>29.71</td>
<td>8.05</td>
<td>18.55</td>
</tr>
</tbody>
</table>

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

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. 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).

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

<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th>Ethanol</th>
<th>50% Hydro-ethanol</th>
<th>Decoction</th>
<th>Aqueous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Phenols</td>
<td>+++</td>
<td>+++++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Anthraquinones</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Tannins</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Triterpenes</td>
<td>-</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Phytosterol</td>
<td>-</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

+: detected; +++: degree of intensity; -: not detected or in trace amount.
Table 3: The result of the quantitative phytochemical screening of AFE with different extracting solvents.

<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th>Ethanol</th>
<th>50% Hydro-ethanol</th>
<th>Decoction</th>
<th>Aqueous</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFC (mg quercetin in g⁻¹)</td>
<td>0.36</td>
<td>1.10</td>
<td>0.61</td>
<td>0.30</td>
</tr>
<tr>
<td>TPC (mg gallic acid g⁻¹)</td>
<td>8.35</td>
<td>10.29</td>
<td>10.79</td>
<td>10.41</td>
</tr>
</tbody>
</table>

Table 4: The IC₅₀ values of the free radical scavenging/chelating capabilities of different extracts of P. americana fruit.

<table>
<thead>
<tr>
<th>Samples</th>
<th>DPPH IC₅₀ (µg/mL)</th>
<th>ABTS IC₅₀ (µg/mL)</th>
<th>Hydroxyl IC₅₀ (µg/mL)</th>
<th>Superoxide IC₅₀ (µg/mL)</th>
<th>Metal Chelating</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silymarin</td>
<td>1.09±0.02</td>
<td>0.39±0.05</td>
<td>1.12±0.02</td>
<td>1.12±0.01</td>
<td>-</td>
</tr>
<tr>
<td>Citrate</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.51± 0.01</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.52±0.05</td>
<td>0.38±0.02</td>
<td>0.59±0.01</td>
<td>0.63±0.10</td>
<td>0.39± 0.01</td>
</tr>
<tr>
<td>Hydro-ethanol</td>
<td>1.15±0.03</td>
<td>0.30±0.02</td>
<td>0.94±0.01</td>
<td>0.49±0.00</td>
<td>0.41± 0.05</td>
</tr>
<tr>
<td>Decoction</td>
<td>1.78±0.01</td>
<td>0.49±0.02</td>
<td>1.03±0.01</td>
<td>0.57±0.01</td>
<td>1.73± 0.02</td>
</tr>
<tr>
<td>Aqueous</td>
<td>1.05±0.01</td>
<td>0.49±0.05</td>
<td>1.76±0.01</td>
<td>0.60±0.01</td>
<td>0.67± 0.01</td>
</tr>
</tbody>
</table>

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.

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 IC₅₀ (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 IC₅₀ value of 0.57 and 0.62 μg/mL which is higher and significantly different (p<0.05) from acarbose and hydro-ethanol (IC₅₀;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 IC₅₀ values for different extracts of P. americana fruit on specific activities of α-amylase and α-glucosidase enzymes.

<table>
<thead>
<tr>
<th>Samples</th>
<th>IC₅₀ (µg/mL) α-Glucosidase</th>
<th>IC₅₀ (µg/mL) α-Amylase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acarbose</td>
<td>0.52±0.04</td>
<td>0.47±0.01</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.15±0.00</td>
<td>0.57±0.01</td>
</tr>
<tr>
<td>Hydro-ethanol</td>
<td>0.39±0.00</td>
<td>0.42±0.05</td>
</tr>
<tr>
<td>Decoction</td>
<td>0.46±0.01</td>
<td>0.62±0.03</td>
</tr>
<tr>
<td>Aqueous</td>
<td>0.45±0.04</td>
<td>0.53±0.08</td>
</tr>
</tbody>
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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 and herbal medicine is still a major part of habitual treatment of different diseases. 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 distill 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 30g 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.

In this experiment, the use of plant extracts at different concentration (0.125-1 μg/mL) and the percentage inhibition as indicated in Figure 7 and Figure 8 respectively. Ethanolic extract has the lowest IC₅₀ which is significantly different (p<0.05) from all other extracts and acarbose. Ethanol and decoction extracts show milder inhibition of α-amylase with their respective IC₅₀ value of 0.57 and 0.62 μg/mL which is higher and significantly different (p<0.05) from acarbose and hydro-ethanol (IC₅₀;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.

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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 vitro study, because a lot of factors like absorption barrier of the PC in the gastrointestinal tract (GIT), the effects of different enzymes such as the glucosidase, esterase, oxidase and hydrolases originating from the host and the mycobacteria which may inhibit PC activity in the GIT. PC are known to possess varying antioxidant activities 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. 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 IC₅₀ 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. 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. Saponins, triterpenes and phytosterol have been demonstrated to scavenge superoxide anion.
Flavonoids are currently receiving attention as a potential protector against various human diseases. Major flavonoids have been shown to have neutralizing effects on free radical and ROS like hydroxyl radical, superoxide radical, and hydrogen peroxide.

Marked postprandial hyperglycaemia is important in the pathogenesis of T2DM. It induces mitochondrial superoxide overproduction which potently inhibits the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase, thus diverting upstream metabolites from the glycolytic pathway to the pathway of glucose overutilization resulting in the formation of diacylglycerol from dihydroxyacetone phosphate (DHAP), a potent activator of protein kinase C (PKC) which ultimately causes β-cell destruction and insulin resistance.

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 IC₅₀ values. Mild inhibition of α-amylase and strong inhibition of α-glucosidase enzymes is targeted as a way of reducing postprandial hyperglycaemia and eliminating the unwanted effect like gastrointestinal discomfort, flatulence, and diarrhoea associated with the use of acarbose. In this study, ethanol and decoction extracts mildly inhibit α-amylase with their respective IC₅₀ values of 0.57 and 0.62 µg/ml, which is higher and significantly different (p<0.05) from acarbose with lower IC₅₀ (0.47 µg/mL). The result of the inhibitory potentials of the extracts on α-glucosidase showed ethanol and decoction extracts have potent inhibition of the enzyme activity. Thus, it may be employed in the management of postprandial hyperglycaemia. This finding is consistent with findings of many authors 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 responsible for glucose toxicity observed in T2DM.
The ethanolic extract which possesses the highest IC$_{50}$ for $\alpha$-amylase enzyme and lowest IC$_{50}$ for $\alpha$-glucosidase compared to acarbose and other tested extracts of *Avocado* fruit was used to determine the mode of inhibition of $\alpha$-amylase and $\alpha$-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$^{2-5}$. Nevertheless, our past research on *Avocado*’s antihyperglycemic, antiadipogenic, 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 $\alpha$-amylase enzyme showed that the ethanolic AFE is competitively inhibiting the breakdown of disaccharides and oligosaccharides which are substrate for $\alpha$-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$^{-2}$ M$^{-1}$ for reaction pathway without inhibition to 1.44x10$^{-2}$ M$^{-1}$ with inhibitor. Decreased $K_m$ value signifies increase affinity. This result proposed competitive mode of inhibition. However, the mode of inhibition of $\alpha$-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$^{-2}$ M$^{-1}$ to 4.69x10$^{-2}$ M$^{-1}$ without inhibitor and with inhibitor respectively and also a decrease in $V_{max}$ from 19.76 $\mu$M/min without inhibition to 14.66 $\mu$M/min with inhibition which suggests a 39.74% decrease in overall activity of $\alpha$-glucosidase enzyme in the presence of ethanolic extract of fruit of *Persea americana* Mill.

**CONCLUSION**

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 $\alpha$-glucosidase and $\alpha$-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.

**COMPETING INTERESTS**

Authors have declared that no competing interests exist.

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19. Müller L, Fröhlich L, Böhm V. Comparative antioxidant activities of carotenoids measured by ferric reducing


