Phytochemical Screening and *In Vitro* Antioxidant and Anti-Diabetic Potentials of *Persea americana Mill*. (Lauraceae) Fruit Extract.

ABSTRACT

Backgound: Diabetes mellitus (DM) is a metabolic disorder and 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. Literature review: *Avocado* fruit is rich in phytochemicals necessary for treatment of DM. Objective: The purpose of this study was to investigate the effect of Persea americana fruit extracts' inhibitory effect on α-amylase and α-glucosidase. Methods: The of percentage yield, phytochemical screening (both qualitative and quantitative), *in vitro* antioxidant and antidiabetic assays, and kinetic studies were performed with different solvent extracts of Avocado fruit pulp. Results: Avocado has greater and promising potential as pharmaceutical agent, particularly to be developed as antidiabetics through the inhibition of α-glucosidase and α- amylase enzymes. *In vitro* tests of the antioxidant activity of the fruit extract gives evidence and strong biochemical rationale of their therapeutic potential. Conclusion: 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.

Keywords: glucose; Phytochemical; α-Glucosidase; α-Amylase; Antioxidant; Nutraceutical.

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 normoglycemia 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, vegetable butter. It has traditionally been used due to its antibacterial, antifungal, hypotensive, anti-inflammatory, and immune-enhancing effect[8,9]. Furthermore, Avocadojuice made from ripe fruit was very popular due toits numerous health benefits. Because of the limitednumber of reports on the fruits of Avocado availablein the literature, it was deemed sensible and justifiedto 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, MizanTepi University, Ethiopia. (Vide voucher No. MTU-ETARC 102/08/02). The skin 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, hydro-ethanol, decoction and aqueous) were concentrated under reduced pressure by rotary evaporator to obtain respective thick syrup mass, and stored at 4^{0}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 ((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 TPCwith 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 Contents (TFC)

The TFCwith different solvents' extracts were determined using the method employed by Swanny [14]. TFC were 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 byBraca Aet al,[15].Determination hydroxyl radical scavengingpotentialof 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 AFEwith 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 Mülleret al, [19].The ability of *AFE* to scavenge 2,2-Azino-bis(3-ethylbenzothiazoline-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 $[(A_0–A_1)/A_0] \times 100$, 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 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* Antidiabetic Assays**

Theα-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 enzymeswas expressed as % inhibition using the expression:

%Inhibition= $[(A_{control}-A_{AFE})/A_{control}]\times100$, 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 = 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.

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, reactionrates (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, forall 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.

Phytochemicals (PC)

The qualitative analyses of the AFEwith different extracting solvents are presented in table 2. Saponins, phenols, flavonoids, anthraquinones, alkaloid, tannins, triterpenes and phytosterols were detected at varying degree in all the tested extracts while anthraquinone and phytosterol were found in trace amount in the ethanol and hydro-ethanol extracts. The results of the quantitative phytochemical screening (TFC and TPC) of AFE with different extracting solvents are depicted in table 3.

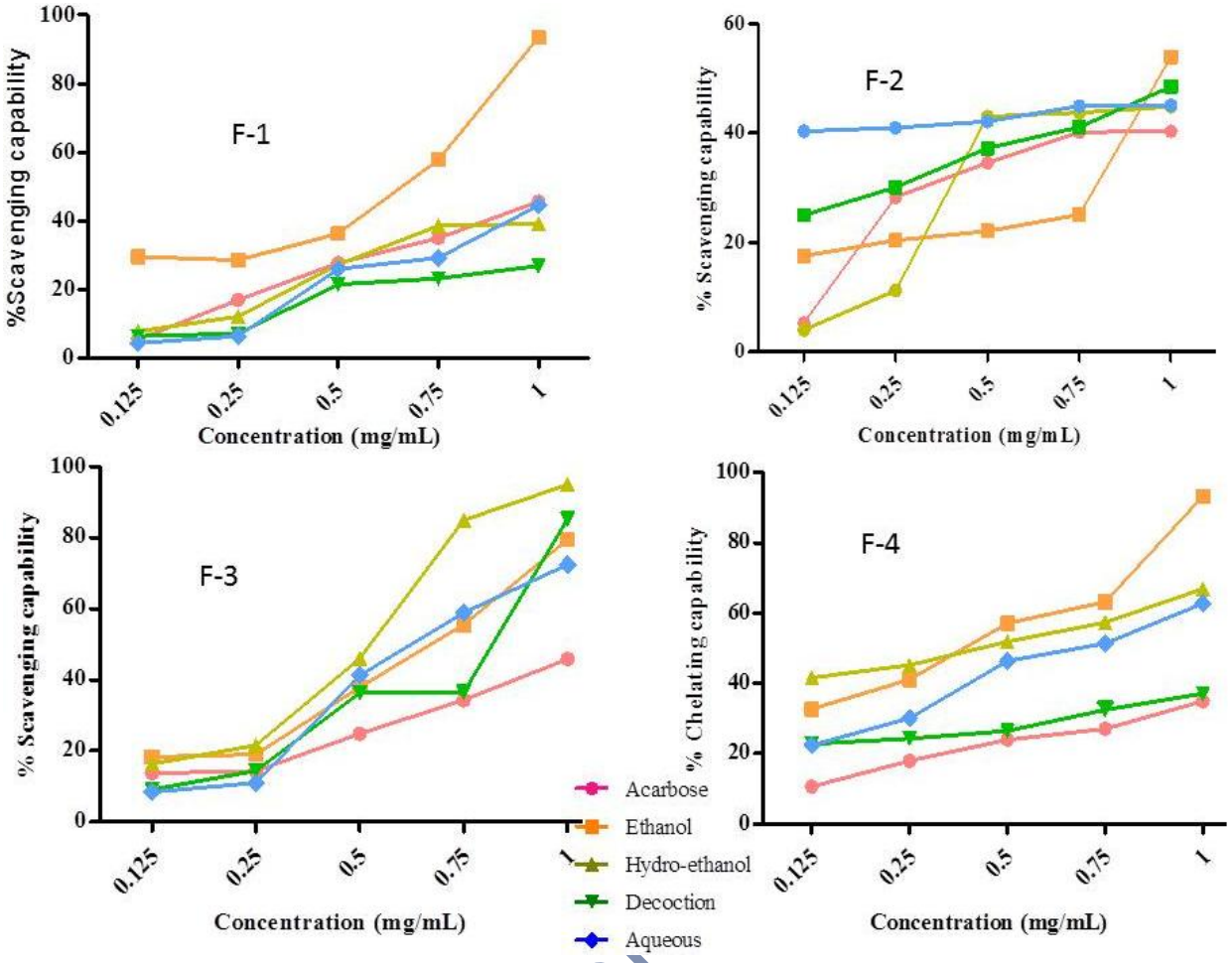
Antioxidant activity

The *in vitro* antioxidant potentials of the AFEwith different extracting solventsare shown in figures 1-6. 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) (figures 1 and 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 IC50 value is 0.49 µg/mL which is comparable to silymarin with IC50 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).

***In vitro* antidiabetic 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 figures 7 and 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.

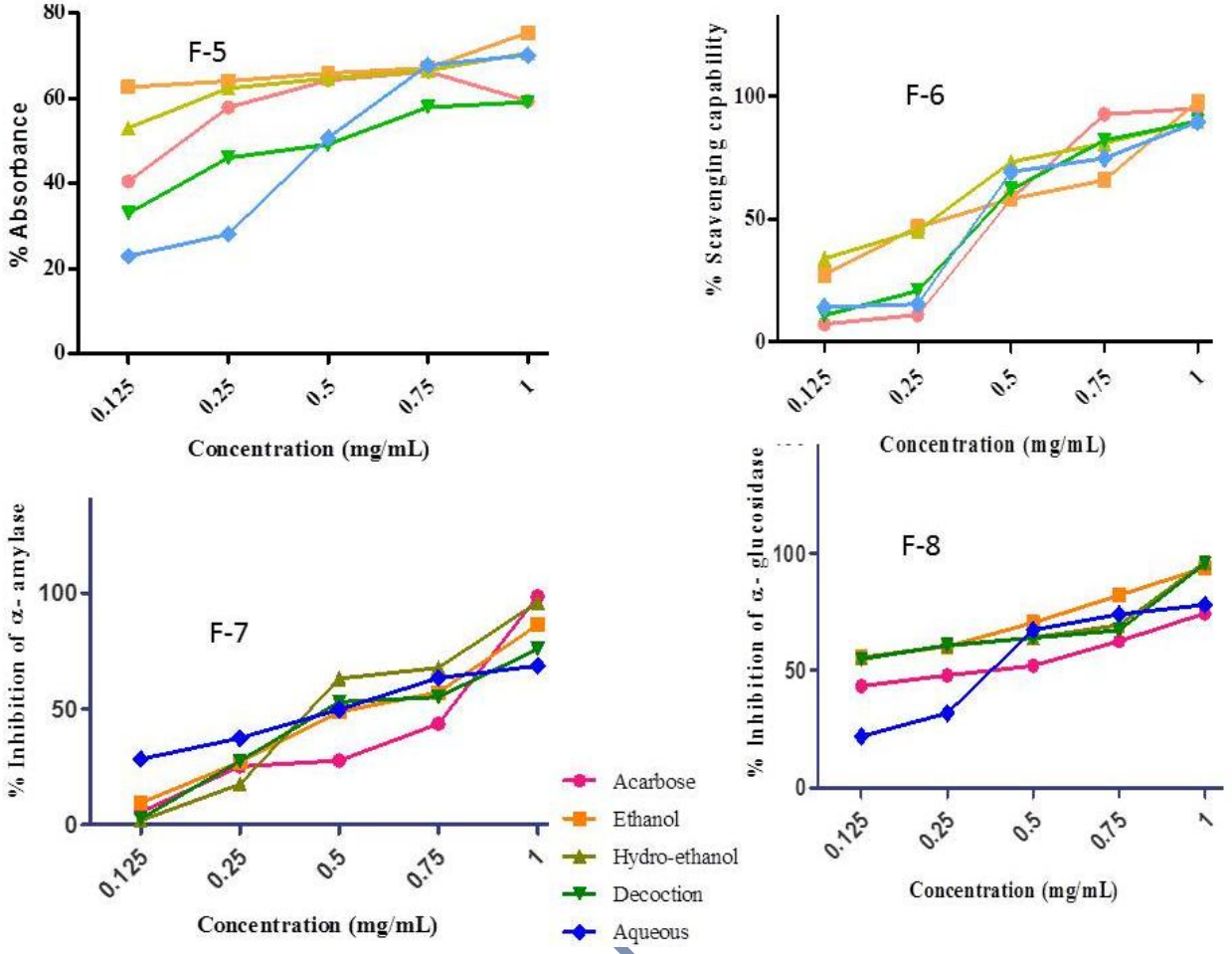


F-1: DPPH scavenging effect of AFE with different extracting solvents.

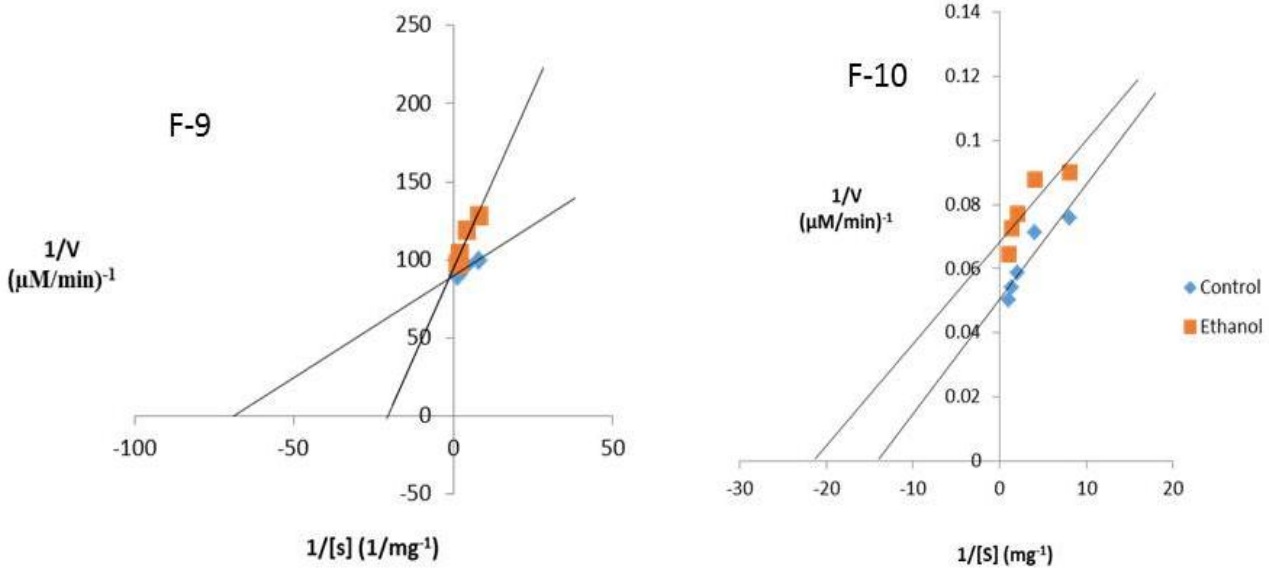
F-2: Scavenging effect of AFE with different extracting solvents on hydroxyl radical.

F-3: Scavenging effect of AFE with different extracting solvents on superoxide anion radical.

F-4: Metal chelating capability of AFE with different extracting solvents.



F-5: Reducing potentials of AFE with different extracting solvents.
F-6: ABTS scavenging effect of AFE with different extracting solvents.
F-7: The inhibitory potentials of *AFE* with different extracting solventson α-amylase activity.
F-8: The inhibitory potentials of *AFE* with different extracting solventson α-glucosidase activity.



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

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

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

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

AFE	**Ethanol**	**Hydro-ethanol**	**Decoction**	**Aqueous**
Percentage yield (%)	11.12	29.71	8.05	18.55

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

Phytochemicals	**Ethanol**	**Hydro-ethanol**	**Decoction**	**Aqueous**
Alkaloids	+++	+++	++	++
Phenols	+++	++++	+++	++
Flavonoids	+	+	-	-
Anthraquinones	++	++	+++	+++
Tannins	++++	++++	++	++
Triterpenes	-	++	+++	++++
Phytosterol	-	++	+++	+++

Key: +: 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.

Phytochemicals	**Ethanol**	**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± .02
Aqueous	1.05 ± 0.01	0.49 ± 0.05	1.76 ± 0.01	0.60 ± 0.01	0.67± .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.

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
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, hydro-ethanol, decoction and distil water)were used in Avocado fruit extract preparation.

The percentage yield indicated that hydro-ethanol 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 lesserefficacious.

Result of the quantitative phytochemical assays indicated the concentration of the different quantity of the PC found in AFEthough, its bioavailability is unpredictable in *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 assayedthehydroxyl radical which is one of the most potent ROS in the biological system that react 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 is 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].

Marked postprandial hyperglycaemia is important in the pathogenesis of T2DM, it induces mitochondrial superoxide overproduction which potently inhibit the glycolytic enzyme glyceraldehyde-3-phosphate thus, diverting upstream metabolites from glycolytic pathway into pathway of glucose overutilization resulting in formation of diacylglycerol from dihydroxylacetone 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 AFEon 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 [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 and 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 possess the highest IC_{50} for α- amylase enzyme and lowest IC_{50} for α- glucosidase compared to acarbose and other tested extracts of *Avocado fruit*was 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*.

From this work, it has been conjectured that fruit of Avocado has great and promising potential as pharmaceutical agent, particularly to be developed as antidiabetics through the inhibition of α-glucosidase and α- amylase enzymes. This natural approach is thought to be safer and cost 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. americanamay play an important role in the development of nutraceuticals and also in the management of oxidative stress induced DM.

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