ETHANOL EXTRACT OF *MANIHOT ESCULENTA* LEAF: A POTENTIAL SOURCE OF ANTIOXIDANT, XANTHINE OXIDASE AND LIPASE INHIBITORS

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Received: 15 December 2016 / Revised: 09 January 2017 / Accepted: 01 February 2017 / Published: 4 April 2017

Keywords: Xanthine oxidase, pancreatic lipase inhibition, antioxidant, gout, obesity.
Abstract: This work describes a study carried out to determine the antioxidant properties, xanthine oxidase and pancreatic lipase inhibitory potential of crude ethanol extract of *Manihot esculenta* matured leaf, *in vitro*. Antioxidant potential of the extract was ascertained using the 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging and the nitroblue tetrazolium (NBT) assays. Xanthine oxidase (XO) inhibitory potential of the ethanol extract was assayed spectrophotometrically using a continuous time scan mode at 293 nm under aerobic condition; while porcine lipase was employed for the determination of its lipase inhibitory activity. The extract showed moderate antioxidant activity against DPPH (40.42±0.43%) and promising superoxide scavenging activity against NBT (94.76±0.44%) compared to different standard antioxidants including butylated hydroxylanisole (BHA), uric acid (UA), curcumin (CUR) and ascorbic acid (ASC). This extract showed mild (50.70±0.48%) xanthine oxidase inhibitory activity in comparison to allopurinol (92.1±0.35%), standard inhibitor of XO. Inhibition of porcine pancreatic lipase by *Manihot esculenta* leaf extract was significantly lower than that of the standard drug, Orlistat (67.53±0.22% and 94.81±0.78%, respectively). The results demonstrated that *Manihot esculenta* leaf extract is a promising source of antioxidants, xanthine oxidase and pancreatic lipase inhibitory agents.

INTRODUCTION

Manihot esculenta Crantz, an edible rainforest plant is a food crop that originated from tropical America, particularly from North-eastern and Central Brazil (Allen, 2002; Miller and Gross, 2011). Across tropical continents, cassava tubers are a popular carbohydrate staple for human consumption and animal feed (Lenis *et al*, 2006). The green leaves of the plant are eaten as vegetables in some parts of sub-Saharan Africa, including Nigeria, Uganda, the Democratic Republic of Congo (DRC), and interestingly in some Asian countries such as Indonesia, Philippines and Malaysia (Almazan and Theberge, 1989). The leafy vegetable may be dried; powdered and packed for exportation to nations like Belgium and France (Ngudi *et al*, 2003a) for use in food industries as it is rich in vitamin A and proteins (Ngudi *et al.*, 2003a, b). Apart from this, the crop is also a reliable source of high quality starch for industrial food processing, pharmaceuticals and animal husbandry (Eruvbetine *et al.*, 2003; Adepoju *et al.*, 2010). Over the centuries, plant antioxidants have found usefulness in phytotherapeutic medicines, where they are employed in protection against various diseases (Halliwell, 1996). These natural antioxidants have been reported to possess a wide range of pharmacological properties including anticancer, anti-inflammatory and anti-aging (Okpuzor and Oloyede, 2009). Indeed plants have found usefulness for sophisticated molecular screening in the search of new chemical entities towards elaborate system of drug development (Gurib-Fakim, 2006). In the rich forests of these continents are numerous ethnobotanical resources which offer a vast and relatively unharnessed potential source of new chemical entities and promising drug candidates derivable from the tropical flora. The occurrence of natural products with medicinal properties has been the major thrust for ethnopharmacological and phytotherapeutic research (Idu and Onyibe, 2007).

Xanthine oxidase (XO) is a regulatory enzyme involved in purine metabolism under normal physiological condition; whereas in the hyperactive state, it is the main causative agent for the pathogenesis of gout (Torres *et al.*, 2011). The enzyme is also involved in several conditions associated with metabolic syndrome, adipogenesis, vascular disorders in diabetes, ischemia reperfusion injury, cancer and in various cardiovascular disorders (Agarwal *et al*., 2011). A type of inflammatory arthritis known as gout, is triggered by the crystallization of uric acid. The crystals, which in turn become localized within the joints, are formed often in association with hyperuricemia (Insel, 1996). The occurrence of gout in the United States between 1988 – 1994 and 2007 – 2010 is very high, and continues to be a substantially increasing burden, which sometimes may be self-reported or physician-diagnosed. However, women and men, regardless of race, experience it (Juraschek *et al*., 2013).

Postprandial hyperglycemia remains an early symptom of diabetes mellitus type 2. This is due to impaired secretion of after-meal insulin. In turn, hyperglycemia increases reactive oxygen species and free radical generation. This preponderance

culminates in oxidative tissue damage and diabetic complications including retinopathy, nephropathy and neuropathy as well as memory impairment (Maritim *et al.*, 2003). The inherent ability of medicinal plants comprises various biological activities such as antiinflammation (Bralley *et al.*, 2008), cardiovascular protection (Yim *et al.*, 2000), neuroprotection (Wang *et al.*, 2009), and mitigation of biochemical events characteristic of age-related neurodegenerative disorders such as Parkinson's (Li *et al.*, 2005) and Alzheimer's disease (Um *et al.*, 2006). Lipase inhibitors such as Orlistat are capable of reducing fat absorption by about 30% in adults as it is not absorbed but rather localized within the intestine. Therefore any drug that gives inhibition less than 40 % *in vitro* would be irrelevant (Ballinger and Peikin, 2012).

MATERIALS AND METHODS

Plant Material

Manihot esculenta matured leaves were harvested from the University of Ado-Ekiti cassava plantation, Ekiti State, Nigeria and identified at the Plant Biology Department of the University of Ilorin, Nigeria (Voucher Number: UIH002/1094). The leaves were air-dried for 3 weeks and pulverized to powder. 100 g dry powder of which was soaked in 1L 95 % ethanol for 48 h, sieved using a filter paper and the filtrate volume was reduced by rotary evaporator. Crude ethanol extract was screened for antioxidant properties, xanthine oxidase and porcine pancreatic lipase inhibitory activities.

DPPH free radical scavenging assay

This method is widely accepted to calculate the ability of a plant extract to scavenge free radicals (Nabavi *et al*., 2008). Antioxidant activity of the extract was evaluated by DPPH free radical scavenging assay by Zhao *et al* (2006) with slight modifications. Briefly, 0.5 ml of 5 mg/ml extract in DMSO was added to 1 ml of 100 µg/ ml DPPH prepared in DMSO and thoroughly mixed and left in the dark for 30 minutes at room temperature until it reaches a steady state. 1 mg/ml each of butylated hydroxyanisole, curcumin and ascorbic acid was used as standard. Decolourisation of DPPH was read as decreases in absorbance at 517 nm, and the DPPH radical scavenging effect was calculated as follows:

% scavenging rate = $[1- (A_1 – A_2)/ A_0] \times 100$

Where A_0 is the absorbance of the control

A_1 is the absorbance of extract

A_2 is the absorbance of extract without DPPH

NBT assay: Superoxide dismutase (SOD) Inhibitory Activity

The NBT scavenging activity was determined following the method of Okamura *et al* (1993). The reduction of NBT was measured as corresponding to the rate of removal of xanthine/xanthine oxidase-generated superoxide. Briefly, 5 mg/ml extract was added to 1 ml potassium phosphate buffer (50 mM, pH 7.5) containing EDTA (0.05 mM), xanthine (0.1 mM) and NBT (0.2 mM). Xanthine oxidase (0.1 ml) (Sigma, USA; 0.8 unit/mL) diluted in 50 mM phosphate buffer (pH 7.5) was added, and the resulting mixture was incubated at 37°C for 20 min. The reaction was terminated upon addition of 2 ml of 2.5 N HCl, which resulted in increased coloration of NBT, which was measured at 540 nm. The SOD scavenging activity of the extract was compared to those of 1 mg/ml each of uric acid, curcumin and ascorbic acid. The percentage reduction (removal rate) of NBT by sample was calculated relative to the control as follows:

% Reduction = $[(A_o – A_1) / A_o] \times 100$

Where A_o = Absorbance of control

A_1 = Absorbance of extract and/ or standards.

Determination of Xanthine Oxidase Inhibitory Activity

Xanthine oxidase (XO) is ubiquitous especially in the various tissues of mammals (Harrison, 2002). It occurs majorly as a dehydrogenase, *in vivo* but can be transformed to an oxygen-dependent oxidase by various conditions (Nomura *et al*., 2013). The ability of the ethanol extract of *M. esculenta* leaves to inhibit xanthine oxidase was ascertained spectrophotometrically according to Tamta *et al*. (2005) using allopurinol as standard. Briefly, 100 µl of 5 % Xanthine oxidase was added to 2.75 ml of KH_2/K_2HPO_4 buffer, pH 7.6 and incubated with various concentrations of extract for 10 minutes at 37 ºC. Then xanthine (33.33 µM) was added and absorbance read at 293 nm by continuous time scan mode for 10 minutes under aerobic condition.

Determination of Lipase Inhibitory Activity

Using the method of Lee *et al* (1993), freshly prepared pNPP as a 10 mM solution of p-nitrophenol palmitate (pNPP) in acetonitrile/ MtOH (1:1) was used to make 3.33mM pNPP; 100 µL of 3.33 mM pPNP was then added to 1.63 ml of 75 mM Tris-HCl buffer (pH 8.5). 150 µl of extract and 120 µl of enzyme solution (5 mg/ml Type II crude porcine pancreatic lipase; centrifuged for supernatant) were added and the mixture was incubated at 37°C for 25 minutes. Absorbance was read at 405 nm. One unit of enzyme activity is that amount of enzyme that liberates 1.0 µmol/ min of p-nitrophenol under standard

assay conditions. The ability of the extract to inhibit porcine pancreatic lipase was reported as difference between enzyme activity in Orlistat and enzyme activity in reaction mixture and expressed as a percentage of the enzyme activity of Orlistat.

$$\% \text{ Inhibition} = \frac{\text{Enzyme Activity}_{\text{Orlistat}} - \text{Enzyme Activity}_{\text{Extract}}}{\text{Enzyme Activity}_{\text{Orlistat}}} \times 100$$

Statistical Analysis

Three determinations were made, and data presented as Mean ± Standard Deviation.

RESULTS

The ability of the extract to scanvenge DPPH (40.42±0.43%) was significantly higher than those of BHA (27.86±0.20%) and ascorbic acid (30.05±0.61%), but lower compared to curcumin (50.69±0.27%). The extract exhibited anti-superoxide activity against NBT (94.76±0.44%) compared to other standard antioxidants, *viz*: uric acid (11.61±0.10%), curcumin (69.59±0.52%) and ascorbic acid (22.29±0.62%). Allopurinol almost completely inhibited XO activity (92.1±0.35 %), while the extract had 50.7±0.48% inhibition. The crude ethanol extract of *M. esculenta* (5mg/ml) had 67.53±0.22% pancreatic lipase inhibition, while Orlistat (0.5mg/ml), a standard obesity drug known for irreversible lipase inhibition exhibited strong lipase inhibitory activity (94.81±0.78%). Orlistat decreases lipid absorption, *in vivo*, to about 30%; therefore any drug that gives inhibition less than 40 % *in vitro* would be irrelevant.

DISCUSSION

Stable DPPH free radical scavenging is a widely accepted method to establish the ability of plant extracts to scanvenge free radicals (Nabavi *et al.*, 2008). The assay is important as it serves to reveal if test samples possess the inherent property of known primary antioxidants, which is the ability to donate protons in order to qualify to serve as free radical inhibitors. DPPH accepts an electron or hydrogen radical to become a stable diamagnetic molecule. In the presence of an antioxidant, the dark colour of the DPPH radical solution turns lighter. The extent of discoloration correlates to the scavenging potential of the antioxidant tested (Hasnat *et al*., 2013). Here we report that the ability of the extract to scavenge stable DPPH radical was significantly lower than that of curcumin (50.69±0.27%). Nevertheless, the extract showed some proton-donating ability compared to BHA (27.86±0.22%) and ascorbic acid (30.05±0.61%), which act as primary antioxidants. The moderate DPPH antioxidant activity of the *M. esculenta* leaf extract may be due to less number of hydrogen donors among the bioactive compounds of the extract and thus decreased reduction reaction between DPPH and these molecules (Lim *et al.*, 2007).

Quantitative evaluation of the extract of *M. esculenta* by NBT assays revealed a significantly higher activity in comparison to three other renowned antioxidants: NBT (94.76±0.44%), curcumin (69.59±0.52%), ascorbic acid (22.29±0.62%) and uric acid (11.61±0.10%) – *M. esculenta* >> Curcumin >>> Ascorbic acid >> Uric acid. Its inhibition percentage was very high compared to these standard compounds.

Allopurinol almost completely inhibited XO activity (92.1±0.35%), while the extracts had 50.7±0.48% inhibition.

Hyperaccumulation of fatty adipose tissue predisposes to obesity, an important member of the metabolic syndrome (Rasouli *et al.*, 2007). Orlistat, a potent hydrogenated inhibitor of the different classes of lipase has become a first-line drug indicated for human obesity (Ballinger and Peikin, 2002). The findings of this research showed that the ethanol extract of *M. esculenta* leaf possess

potent pancreatic lipase inhibitor that can be useful in managing obesity by significantly contributing to weight management and reduction efforts to avert obesity.

CONCLUSION

Oxidative stress is a key player in the pathogenesis of metabolic syndrome. The ethanol extract showed moderate and significant antioxidant activities in both DPPH and NBT assays, respectively compared to butylated hydroxyanisole, curcumin and ascorbic acid which were used standards. The ability of the extract to serve as antioxidant was in accord with its total phenol content. The DPPH and NBT assay results reveal a linear relationship (284 mg TAE/ g DW; $r^2 = 0.999$) between this ability to serve as antioxidant and total phenolic content (Ajayi *et al*., 2015). The ability of *M. esculenta* extract to scavenge stable DPPH radical and reduce NBT were thus confirmed. The results provide evidence that ethanol extract of *M. esculenta* leaf can indeed be a promising source of antioxidants, xanthine oxidase and lipase inhibitors.

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Acknowledgement
The authors acknowledge the DBT-TWAS for granting 2009 DBT-TWAS Sandwich Postgraduate Fellowship to do part of his PhD research in India.
Conflict of Interest
The authors declare no potential conflicts of interest.

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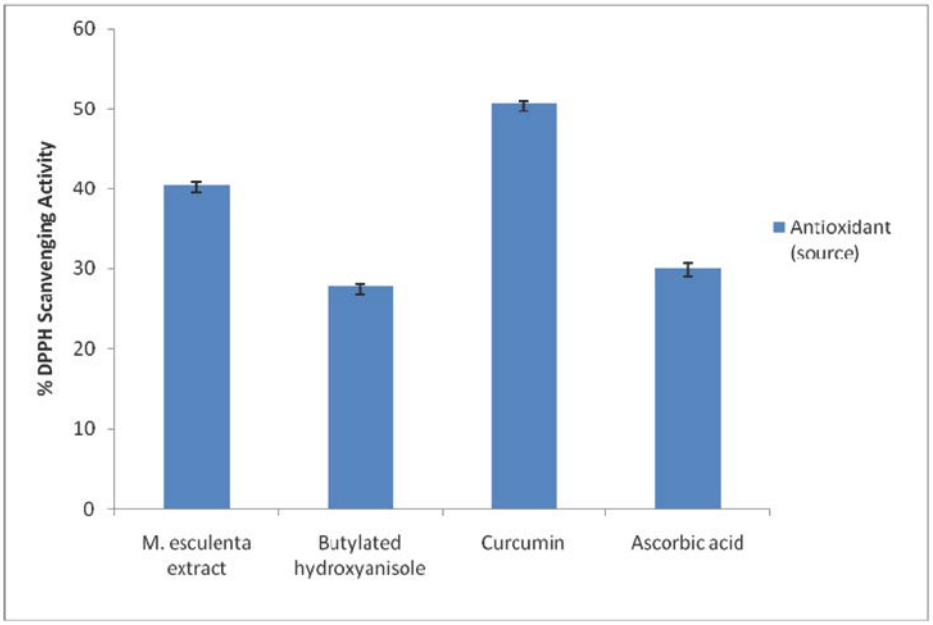

Figure 1: DPPH free radical antioxidant activity of 5 mg/ml *M. esculenta* leaf extract

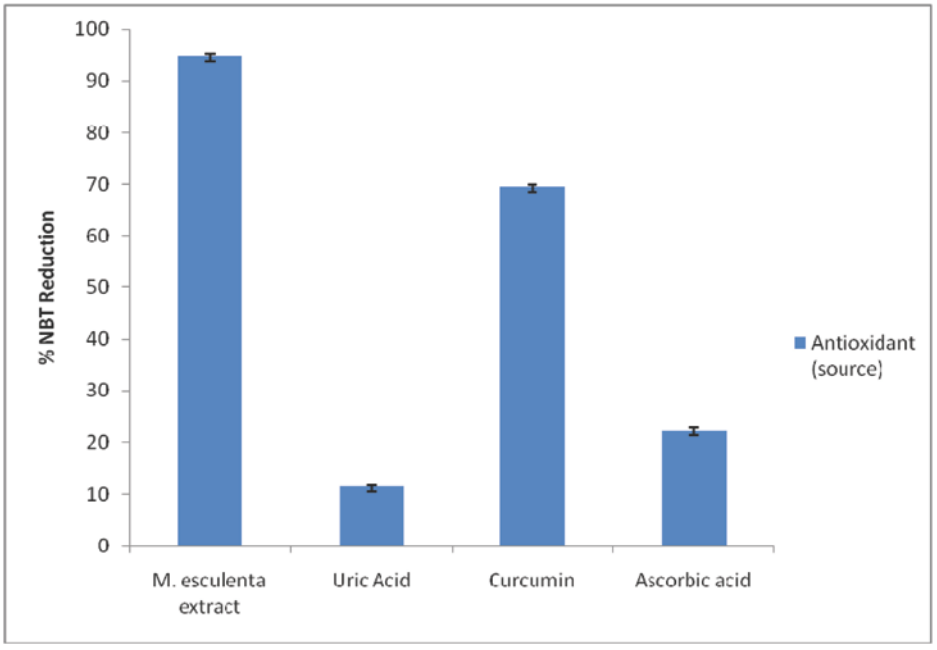

Figure 2: Superoxide dismutase (SOD) antioxidant activity of 5 mg/ml *M. esculenta* leaf extract

Table 1: The xanthine oxidase and lipase inhibitory activities of *M. esculenta* leaf extract

Inhibition Assay	**Inhibitory Activity (%)**
Inhibition of xanthine oxidase	
Manihot esculenta leaf extract	50.7 ± 0.48
Allopurinol	92.1 ± 0.35*
Inhibition of pancreatic lipase	
Manihot esculenta leaf extract	67.53 ± 0.22
Orlistat	94.81 ± 0.78*

All inhibitory parameters were recorded as representation of 3 determinations (Mean±SD, n = 3); *$p < 0.05$