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In vitro alpha-amylase and antioxidant activities of bark extracts of charcoal tree (*Trema orientalis* Linn.)

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Abstract

The objective of the present study is to evaluate the α–amylase inhibitory and antioxidant activities, as well as the total phenolics content of methanol (80:20 v/v) and aqueous bark extracts of *T. orientalis* through *in vitro* analyses. Preliminary phytochemical profiling was performed to identify the bioactive constituents. Toxicity screening was also determined through brine shrimp lethality assay. Phytochemical screening revealed the presence of various chemical compounds with some identified phytoconstituents using GC-MS. Data indicated that methanol extract exhibited high DPPH scavenging ability and α-amylase inhibitory activity with IC_{50} values of 48.40 µg/ml and 127.56 µg/ml, respectively. Furthermore, higher total phenolics content was determined in the methanol extract than the aqueous extract. The results suggest that the bioactivity could be due to the synergistic effect of the active compounds, with emphasis on the phenolic compounds (phenols, flavonoids and tannins). The methanol fraction was also shown to have a weak cytotoxicity against brine shrimp. However, the aqueous extract prepared through decoction revealed to be non-toxic, thus supporting the safety of traditional preparation. The combined effects of α–amylase inhibitory and antioxidant activities of bark extract of *T. orientalis* may indicate a good antidiabetic potential of the plant.

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Introduction

Alpha-amylase is one of the pancreatic carbohydrate hydrolyzing enzymes that initiates starch hydrolysis into smaller oligosaccharides and disaccharides which are further degraded to glucose by the alpha-glucosidase enzyme for absorption into the blood (Kamtekar *et al.*, 2014). This starch digestion proceeds at high rate leading to increase postprandial hyperglycemia (PPHG). Studies have shown that HPA (human pancreatic α-amylase) activity in the small intestine correlates to hyperglycemia, which is the earliest metabolic defect to occur in diabetes mellitus (DM) (Kim *et al.*, 2005; Matsui *et al.*, 1996). Hence, the inhibition of all or some of the pancreatic amylase through alpha-amylase inhibitors is currently one of the therapeutic strategies for DM management as it delays the carbohydrate digestion, thereby, decreases PPHG. However, conventional oral inhibitors have been reported with gastrointestinal side effects like bloating, abdominal pain, diarrhea, and flatulence in diabetic patients (Sudha *et al.*, 2011).

It has been proposed that oxidative stress play a pivotal role in the pathophysiology of diabetic complications. Oxidative stress occurs when there is an imbalance between antioxidants and ROS due to either depletion of antioxidants or accumulation of ROS (Birben *et al.*, 2012). Evidences showed that hyperglycemia induced oxidative stress and increase Advanced Glycation End (AGE) product formation (Lazo-de-la-Vega-Monroy and Fernandez-Mejia, 2013; Perera *et al.*, 2013). This is further exacerbated with the decrease of antioxidant levels in diabetic patients (Johansen *et al.*, 2005).

In view of this background, a drug or therapy that can elicit alpha-amylase inhibitory and antioxidant activities is a good potential candidate for antidiabetic agents. Plants have rich antioxidants and are unexplored source of potential hypoglycemic drugs. Recent studies have shown that secondary metabolites such as polyphenolic compounds can be linked to intestinal α- glucosidase and pancreatic α-amylase inhibitory activities *in vitro*. Geraniin in *Nephelium lappaceum* was found to inhibit α-glucosidase, α-amylase, aldose reductase and AGEs formation (Palanisamy *et al.*, 2011). However, most plant species with high potential are not employed in standard diabetes care due to their unknown mechanism and lack of clinical validation (Trojan-Rodrigues *et al.*, 2011).

Trema orientalis Linn (Ulmaceae) is among the many indigenous plants utilized to treat diabetes and its complications. It is extensively used in African continent due to its medicinal values as treatment for hematuria, dysentery, cardiovascular diseases, malaria, and respiratory illnesses (Adinortey *et al.*, 2013). Pharmacological research has indicated its anti-pyretic, analgesic, anti-microbial, anti-convulsant activity, antibacterial, anti-sickling, and antiplasmodial potential supporting its traditional use (Rout *et al.*, 2012; Mpiana *et al.*, 2011; Abiodun *et al.*, 2011; Panchal *et al.*, 2010; Uddin *et al.*, 2008; Chowdhury and Islam, 2004).

The Manobo tribe in Bukidnon, Philippines traditionally uses the bark decoction of *T. orientalis* as treatment for diabetes. Some phytochemical studies on the bark of *T. orientalis* have reported the presence of secondary metabolites with antidiabetic properties (Wen-Lung *et al.*, 2007; Noungoue *et al.*, 2001).

Previous study on the *in vivo* antidiabetic potential of aqueous stem bark extract of *T. orientalis* concluded that the extract of this plant possessed glucose-lowering property in streptozotocin-induced diabetic rat by mechanism different from sulfonylurea drugs (Dimo *et al.*, 2006).

In view of this background, the main objective of this study is to evaluate the α –amylase inhibitory activity and antioxidant capacity of bark extracts of *T. orientalis* under *in vitro* conditions. Specifically, this study was done to identify the possible inhibitory phytoconstituents present and to assess the toxicity of the extracts to further support the pharmacological assay. The findings of this study may further validate the traditional use of *T. orientalis* as an antidiabetic

agent.

Materials and methods

Plant Sample Collection and Processing

Fresh *Trema orientalis* (Ulmaceae) bark samples were collected from Barangay Panadtalan, Maramag, Bukidnon in January, 2015 and authenticated by Edgardo Aranico, a professor in the Department of Biological Sciences, MSU-IIT. Herbarium specimen of each plant was deposited at the MSU-IIT Natural Science Museum. Bark samples of *T. orientalis* were washed, chopped into small pieces, and air dried for one week. Air-dried samples were powdered using commercial grinder (Osterizer) and were stored in an airtight container for further use.

Plant Extraction

The methanol extract was prepared using 15.0 g of *Trema orientalis* powdered bark mixed with 80% methanol for 72 hrs in a water bath shaker at 100 rpm, 50°C. The procedure was repeated twice to obtain adequate amount of methanol extract. The combined extract was filtered through a vacuum filter using Whatman No.1 filter paper. The resulting solution was subjected to rotary evaporator (55°C) under reduced pressure to obtain the crude extract. The aqueous extract was obtained by boiling 20.0 g of the powdered bark samples in 80 ml distilled water in a (1:4 w/v) ratio until the volume was reduced to approximately 50 ml. The respective extracts were decanted and vacuum-filtered through Whatman No. 1 filter paper. The filtrates were concentrated under reduced pressure at 50 °C to obtain the crude extracts.

Preliminary Screening of Phytochemical Constituents

The methanol extract of *T. orientalis* bark was subjected to preliminary screening of phytochemical constituents using the protocol of Sermakkani and Thangapandian (2010) and Ayoola *et al.* (2008).

Test for Tannins

Approximately 0.25 g of extract was boiled in 5.0 ml distilled water in a test tube and filtered. A few drops 0.1% ferric chloride were added to the filtrate and observed for brownish green or blue-black coloration.

Test for Steroids

The crude plant extract (0.5 mg) was dissolved in chloroform (5.0 ml), and then added with an equal volume of concentrated H_2SO_4 draining along the sides of the test tube. A red color change in the upper layer in the test tubes into red and a yellow color change with green fluorescence in the lower layer indicated the presence of steroids.

Test for Terpenoids

About 0.25 g each of the extract was mixed with 1 ml of chloroform and then 1.5 ml of $H_2S_0_4$ was carefully added to form a layer. A reddish brown coloration of the interface indicated the presence of terpenoids.

Test for Flavonoids

Approximately 0.25 g of the crude extract was heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was mixed thoroughly with 1 ml of dilute ammonia solution. A yellow coloration indicated the presence of flavonoids.

Test for Saponins

A 1.0 ml volume of the sample stock solution (0.25g/ml) was mixed with 20.0 ml of distilled water and shaken by hand for 15 minutes. Formation of a foam layer on the top of the tube indicated the presence of saponins.

Test for Phenols

One millilitre (ml) of the sample stock solution (0.25g/ml) was added with 2.0 ml of distilled water followed by few drops of 1% ferric chloride solution. Formation of blue or green color indicated the presence of phenols.

Test for Alkaloid

About 0.25g of the crude sample was diluted to 10.0 ml with HCl, warmed and filtered. Two millilitres (ml) of dilute ammonia were added to 5.0 ml of the filtrate followed by 5 ml of chloroform and shaken

gently to extract the alkaloidal base. The chloroform layer was extracted with 10.0 ml of acetic acid. Formation of a cream precipitate with Mayer's reagent indicated the presence of alkaloids.

Determination of total phenolics content

Total phenolics content was determined using the Folin-Ciocalteu reagent method (Formagio *et al.*, 2014) with slight modifications. Initially, the crude extract was dissolved in methanol (1.0 mg/ml). One hundred microliters (100.0 µl) of the solution were mixed with 1.0 ml of distilled water and 0.5 ml of Folin-Ciocalteu's reagent (1:10 v/v). After mixing, 1.5 ml of 2% aqueous sodium bicarbonate was added. The mixture was allowed to stand for 30 min with intermittent shaking. The absorbance of the mixture was measured at 765nm using UV-Vis spectrophotometer (SHIMADZU). Methanol was used as a blank. Each solution was triplicated. Total phenolics content is expressed as gallic acid equivalent in mg per gram of extract. Gallic acid solution with concentrations ranging from 8.0-63.0 µg/ml was used for calibration. A dose response linear regression was generated by using the gallic acid standard absorbance and the levels in the samples were expressed as gallic acid equivalents (mg of gallic acid/g of extract).

Determination of in vitro α-amylase inhibitory activity

This assay was carried out using modified procedure of Das *et al.* (2015). A 500.0 µl aliquot of samples and Acarbose (standard) (200-800 µg/ml) was added to 500.0 µl of 0.20 mM phosphate buffer (pH 6.9) solution containing 0.5mg/ml of α-amylase and incubated at 25°C for 10 min. One percent starch solution (500 µl) in 0.02 M sodium phosphate buffer (pH 6.9) was added to each tube at timed interval. Reaction mixtures were incubated at 25°C for 10 minutes. The reaction was halted with 1.0 ml of 3, 5 dinitrosalicylic acid reagent. The test tubes were incubated in a water bath for 5 min and allowed to cool to room temperature. Reaction mixture was diluted by adding 10 ml distilled water and absorbance was measured at 540 nm using UV-Vis spectrophotometer (SHIMADZU UV mini 1240). The control represented 100% enzyme activity and was prepared by replacing extract with water (aqueous extract) or methanol (methanol extract). The inhibition activity (%) of DPPH was calculated using the following equation:

% inhibition activity = $I_0 - I_S/I_0 \times 100$.

where I_0 is the absorbance of the control and I_S is the absorbance of the tested extract.

Determination of free radical scavenging activity by DPPH assay

DPPH (2,2 diphenyl-2-picrylhydrazyl hydrate) (Sigma) scavenging activity was determined *in vitro* using a spectrophotometric method adopted from a modified method of Perera *et al.* (2013). Freshly prepared DPPH (6.5×10^{-5} M) solution was used for each experiment. Ascorbic acid was used as a reference standard. Briefly, a 2.5 ml aliquot of DPPH solution was mixed with 0.5 ml extract solution of various concentrations (250-2000 µg/ml) at a timed interval. The control was prepared as accordingly replacing the extract with 0.5 ml methanol. The reaction mixture was shaken well and incubated in the dark for 30 minutes. The absorbance was taken at 540 nm using UV-Vis spectrophotometer (SHIMADZU UV mini 1240). All assays were conducted in triplicate. The scavenging activity (%) of DPPH was calculated using the following equation:

% scavenging activity = $A_0 - A_S/A_0 \times 100$.

where A_0 is the absorbance of the control and A_S is the absorbance of the tested extract.

Gas Chromatography-Mass Spectrometry to determine semi-volatile phytochemicals in plant materials

The methanol crude extract of *T. orientalis* was diluted with chloroform and subjected to GC-MS analysis using Agilent Technologies 7890A GC system couple with (an Agilent) 5976C Mass Selective detector HP-5MS capillary column (30x 0.25 m). The carrier gas was helium (He). The injector temperature was set at 320°C. The initial oven temperature was

70°C which was programmed to increase to 280°C at the rate of 10 C/ min with a hold time of 4 min at each increment. Injection of 1.0 µl was made in split mode with split ratio of 100:1. The mass spectrometer was operated in the electron ionization mode at 70 eV and electron multiplier voltage at 1859 V. Other MS operating parameters were as follows: ion source temperature 230°C quadruple temperature 150°C, solvent delay system 3 min and scan range 33-50 amu. The compounds were identified by direct comparison of mass spectrum of the analyte at a particular retention time to that of a reference standard found in the National Institute Standard and Technology (NIST) library. Total GC-MS running time was 45 minutes. The protocol was adopted from the study of Chipiti *et al.* (2015)

Brine shrimp lethality assay

Brine shrimp toxicity assay was conducted according to the protocol of Elias *et al.* (2014). Ten nauplii of 24-hour old *Artemia salina* were placed in each tube using a glass pipette. From a 10^4 µg/ml stock solution, 5, 50, 250, and 500 µl of samples were pipetted into the tubes. The volume was adjusted to 5 ml by adding filtered sterile seawater. Alcohol-based extracts were allowed to dry for 2 days prior to the addition of nauplii, then 150 µl of DMSO was added for the concentrations 500 µg/ml and 1000 µg/ml while 75 µl of DMSO was added for the concentrations 10µg/ml and 100µg/ml. The samples were kept under 100-watt illumination and the number of live nauplii was counted and recorded after 6 and 24 hours. Using Probit Analysis, lethal concentration (LC_{50}) was assessed with 95% confidence level. LC_{50} refers to the concentration that is lethal to half of the population of the *A. salina*. Chronic and acute LC_{50} represents the dose that rendered 50% mortality of the test animals after 6-hr and 24-hr exposure, respectively. Percentage mortality (%M) was also calculated using the following equation:

$$\text{Mortality Percentage (\%)} = \frac{\text{Initial live nauplii - Total number of live nauplii}}{\text{Initial live nauplii}} \times 100\%$$

All experiments were performed in triplicates. Data were expressed in Mean ± SD. Linear regression was calculated using SPSS version 20. One-way ANOVA with Tukey's Post hoc test was calculated using in GraphPad Prism 6. Difference at $p< 0.05$ was considered statistically significant.

Results and discussion

Preliminary Screening of Phytochemical Constituents

Qualitative phytochemical analysis of *T. orientalis* bark indicated the presence of phenol, flavonoids, tannins, saponins, alkaloids, and terpenoids (Table 1).

Table 1. Phytochemical constituents of methanol extract of *T. orientalis* bark.

Class Compounds	Methanol extract	Aqueous extract
Phenol	+	+
Tannin	+	+
Saponin	+	+
Flavonoid	+	+
Alkaloid	+	+
Steroids	-	+
Terpenoids	+	+

Present (+); Absent (-).

Quantitative estimation of phenolics content showed that aqueous and methanol extracts contain a significant amount of phenols. Results revealed that methanol extract (80:20 *v/v*) (116.60 mg GA/g dry matter) had higher total phenolics content than aqueous fraction (94.12 mg GA/g dry matter) (Table 2). Solubility of phenolic compounds is governed by the polarity of solvent used, the degree of polymerization of phenols, and interaction between solvent and phenolic compound (Khoddami *et al.*,

2013; Djeridane *et al.*, 2006). Furthermore, methanol-water ratio (80:20 *v/v*) increases the polarity of the methanol making it more suitable for extraction of high polar compounds and a good range of nonpolar compounds (Dai and Mumper, 2010). Hence, the use of hydromethanol (80:20 *v/v*) than pure methanol as an extracting solvent in the present study could be the reason that the alcoholic extract had higher phenolics content than aqueous extract (decoction). This result is similar to an earlier study on selected medicinal plants wherein hdyroalcoholic extracts exhibited higher phenolic contents and antioxidant activity than aqueous extracts (Sultana *et al.*, 2009).

Table 2. Total phenolic content of methanol and aqueous bark extracts of *T. orientalis*.

Extract	Total phenolic content (mg GA/g dry matter)
Methanol extract	116.60
Aqueous extract	94.12

Determination of α-amylase inhibitory activity

One of the current approaches in diabetes management is the reduction of postprandial hyperglycemia (PPHG) through inhibition of either α-amylase or glucosidase enzymes (Kazeem *et al.*, 2013). In an attempt to explore the antidiabetic property of plants, this present study assessed the α-amylase inhibition potential of the methanol and aqueous fractions of *T. orientalis* bark, under *in vitro* conditions. The initial screening of both methanol and aqueous extracts demonstrated a positive α-amylase inhibitory activity with a concentration-dependent increase in amylase inhibition percentage (Fig. 1). In comparison to acarbose percentage inhibitory activity, aqueous extract showed significant difference in all concentrations. However, methanol extract inhibitory activity showed no significant difference at lower (200 µg/ml) and highest concentration (800 µg/ml). However, the half maximal inhibitory concentration (IC_{50}) values of the two tested extracts were significantly higher than the standard compound acarbose (Table 3). The IC_{50} value refers to the concentration required to inhibit 50% of the biological or biochemical function. Generally, lower IC_{50} value means the inhibitor has great potency.

According to the results obtained, the methanol extract has significantly higher α-amylase inhibition percentage per concentration than aqueous extract, with a low IC_{50} which is four-fold higher compared to the IC_{50} value of acarbose. The 50% inhibitory concentration of aqueous extract was 12-fold higher than the standard acarbose. Hence, 80% methanol showed better α-amylase inhibitory activity than aqueous extract (decoction).

The result of this study suggests that α-amylase inhibitory activity of the *T. orientalis* bark tends to favor extraction in polar solvent which is similar to the results of other studies (Hashim *et al.*, 2013). This response could be due to the presence of significant amount of polyphenols in the extract. Phenols and flavonoids are known to be soluble in more polar solvents. Polyphenols are reported to inhibit carbohydrate hydrolyzing enzymes due to their ability to bind with proteins. In addition, barks have high content of tannins and reported to exhibit enzyme inhibition due to their ability to strongly bind to proteins forming insoluble and indigestible complex or their ability to form hydrogen bond with the reactive sites of the enzyme altering their catalytic activity (de Sales *et al.*, 2012; Manaharan *et al.*, 2011).

The significant difference in the activities of the plants extracts and acarbose indicates that *T. orientalis* has mild to moderate inhibitory activity which is beneficial to diabetic patients. It has been reported that excessive inhibition of pancreatic α-amylase results in the abnormal bacterial fermentation of undigested carbohydrates in the colon which causes the gastrointestinal side effects of current oral inhibitors (Bischoff, 1994; Horii *et al.*, 1986). Therefore, mild α-amylase inhibition activity is

desirable in antidiabetic agents. Moreover, the positive α-amylase inhibitory activity of the extracts, as depicted in this present study could support the findings of Dimo *et al.* (2006) on the reduction of glucose absorption as the possible mechanism of the antiglycemic activity of the plant.

Table 3. IC_{50} values of *T. orientalis* 80% methanol and aqueous extracts against α-amylase activity.

Extract/Standard	IC_{50} (µg/ml)
Acarbose (standard)	30.83**
Methanol extract	127.56 [a]**
Aqueous extract	369.69 [b]

*IC_{50} value separated by different letters is significantly different compared to Acarbose at [a] [b] $p<0.0001$; ** extrapolated values based on standard curve.

Determination of free radical scavenging activity by DPPH assay

Results of the present study indicated that the different extracts (aqueous and methanol) of *T. orientalis* have a concentration- dependent increase in scavenging activity of DPPH (Fig. 2). The methanol extract exhibited comparable inhibitory activity to standard ascorbic acid in each concentration. Aqueous extract showed moderate scavenging activity in each concentration. The methanol extract, with an IC_{50} value of 48.40 µg/ml, exhibited a higher antioxidant activity than aqueous extract (IC_{50} value= 55.69 µg/mL) (Table 4).

Table 4. IC_{50} values of *T. orientalis* fractions for DPPH radical scavenging activity.

Extract/Standard	IC_{50} (µg/ml)**
Ascorbic acid (standard)	40.34
Methanol extract	48.40 [a]
Aqueous extract	55.69 [b]

*IC_{50} value separated by different letters is significantly different compared to ascorbic acid at [a] $p<0.005$ and [b] $p<0.001$; ** extrapolated values based on standard curve.

Although the obtained IC_{50} value of the methanol ($p<0.05$) and aqueous ($p<0.0001$) extracts are significantly different compared to the potency of ascorbic acid, results still indicate the potential of the plant extracts as an antioxidant. These results are in concordance to the study of Uddin (2008) where methanol extract of *T. orientalis* leaves exhibited higher scavenging activity compared to aqueous extract, with comparable activity to ascorbic acid. Hence, its scavenging activity can be attributed to high phenolics content and the presence of flavonoids and tannins, as reported earlier.

Table 5. Phytoconstituents identified in the methanolic bark extract of *T. orientalis* by GC-MS.

Compound	Abundance (%)	Percent Similarity (%)
Decane	2.805	90
Decane, 2-methyl	0.878	62
D-limonene	1.674	91
Decane-4-methyl	0.895	64
Nonadecane	0.954	68
Decane, 3-methyl	0.915	78
Undecane	4.213	94

The Diabetes Control and Complications (DCCT) trial revealed that control PPHG can significantly reduce clinical complications. However, this approach could not prevent development of complications. Hence, suggested the use of antioxidants to combat cellular oxidative stress and prevent the development of diabetic complications (Johansen *et al.*, 2005). Various naturally-occurring dietary antioxidants were reported to be exerting beneficial effects against diabetic vascular damage. A phenolic antioxidant, Resveratrol, found in wine has been demonstrated to decrease vascular lesions and vascular endothelial growth factor (VEGF) induction in retinas of diabetic mice, suggesting its mitigating role in diabetic retinopathy (Kim *et al.*, 2012). Hence, the antioxidant activity of *T. orientalis* could be beneficial as a diabetes treatment since it may aid in the prevention of the development of micro- or macrovascular diabetic complications.

Table 6. Percentage mortality of *A. salina* larvae in aqueous and methanol extracts of *T. orientalis* bark after 6 h and 24 h incubation.

Extract	Concentration (ppm or µg/ml)	Mortality Percentage (%)	
		Acute (6 hours)	Chronic (24 hours)
Aqueous extract	10	0	0
	100	3.33	0
	500	16.67	16.67
	1000	16.67	23.33
Methanol extract	10	0	6.67
	100	10	6.67
	500	20	46.67
	1000	33.33	83.33

results are expressed as means, n=3.

Table 7. Acute and chronic LC_{50} values of aqueous and methanol extracts of *T. orientalis* bark against the brine shrimp *A. salina*.

Extract	LC_{50} values in µg/ml	
	Acute (6 hours)	Chronic (24 hours)
Aqueous extract	1741.4**	1272.51**
Methanol extract	1457.38**	592.34

** extrapolated values based on standard curve.

Although the presence of flavonoids and polyphenols could contribute to hypoglycemic and scavenging activities of the extracts, the specific phytoconstituents which may be responsible for these activities were identified through GC-MS analysis. Preliminary screening on methanol extract detected 51 peaks, however, compounds were in too low concentrations to be correctly identified (<60% similarity index) (Fig. 3). Majority of the compounds detected are hydrocarbons which coincide with the earlier studies (Table 5) (Wen-Lung *et al.*, 2007). In addition, high abundance of d-limonene was also identified in bark extracts of *T. orientalis* methanolic extract.

This monoterpene has been reported to have antidiabetic, anti-inflammatory and antioxidant activity (Murali *et al.*, 2013; Murali and Saravanan, 2012). However, no previous studies reported the presence of d-limonene in *T. orientalis*.

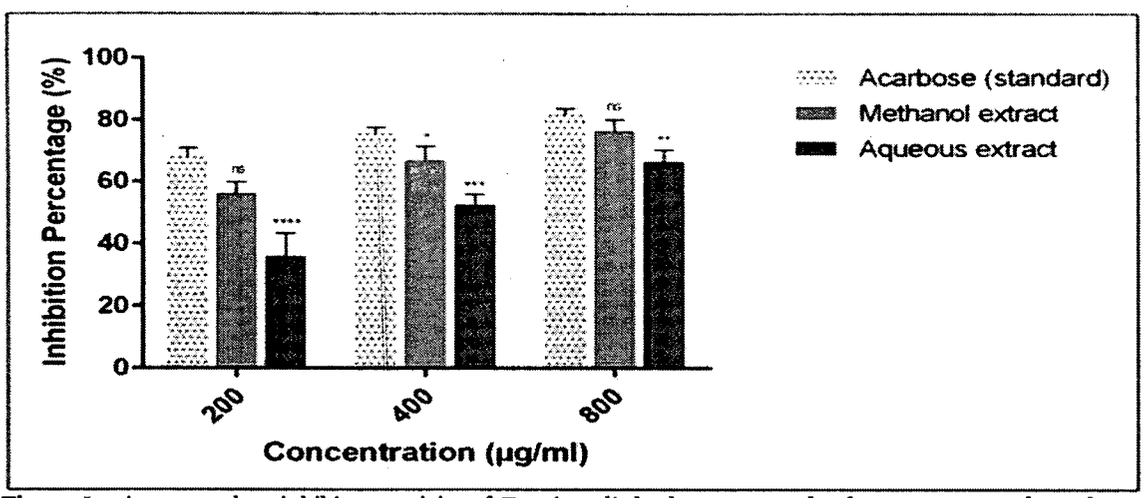

Fig. 1. *In vitro* α-amylase inhibitory activity of *T. orientalis* bark extracts and reference compound Acarbose. Data expressed in mean ± SD, n = 3. ****$p<0.0001$, ***$p<0.001$, **$p<0.005$, *$p<0.05$, nonsignificant (ns) compared. to acarbose.

Earlier studies on chemical constituent of *T. orientalis* bark reported the presence of methylswertianin, decussatin, glycosides of decussatin, sweroside, scopoletin, (-)-epicatechin, lupeol, *p*-hydroxybenzoic acid, adian-5-en-3-one, 2α, 3ß-dihydroxyurs-12-en-28-oic acid, (+)-catechin, (+)-syringaresinol, *N*-(*trans-p*-coumaroyl) tyramine, N-(*trans-p*-coumaroyl) octopamin, *trans*-4-hydroxycinnamic acid, and 3,5-dimethoxy-4-hydroxyphenyl-1-O-ß-D-glucoside (Wen-Lung *et al.*, 2007; Dijoux-Franca *et al.*, 2001; Noungoue *et al.*, 2001). However, these compounds were not identified in the present GC-MS analysis. This could be due to the difference in extraction protocol and spectroscopic analysis employed.

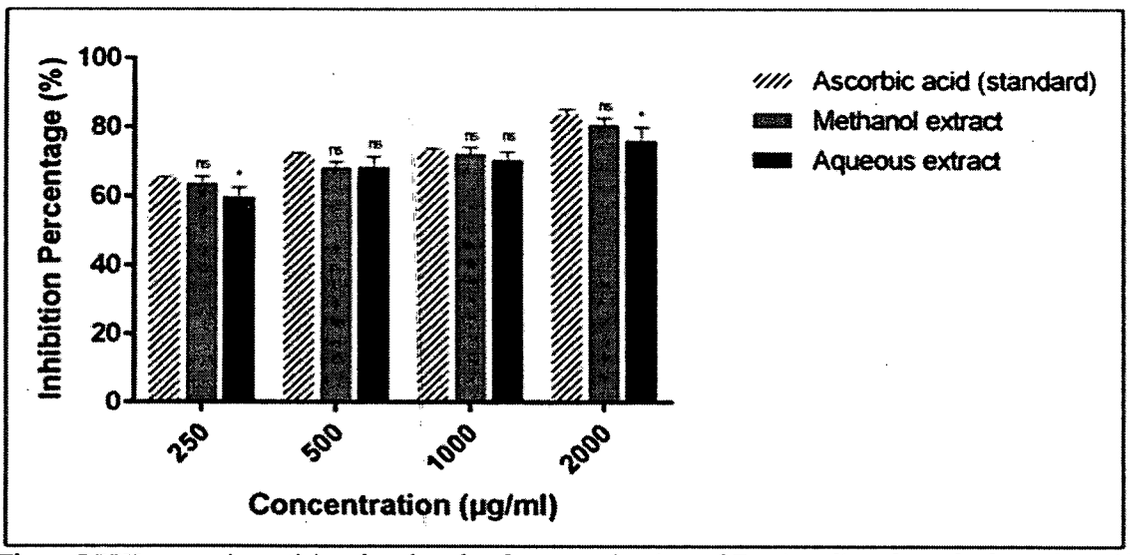

Fig. 2. DPPH scavenging activity of methanol and aqueous fractions of *T. orientalis* and standard ascorbic acid. Data expressed in mean ± SD, n = 3. *$p<0.05$, nonsignificant (ns) compared to ascorbic acid.

Brine Shrimp Lethality Assay

The toxicity and antitumor potential of plant extracts were also assessed using brine shrimp assay. Results of the present study expressed a directly proportional relationship between the extract concentrations and time exposure to mortality percentage of *A. salina* nauplii (Table 6). Highest mortality was observed at 1000 µg/ml in methanolic extract with 83.33% at 24

hours and lowest mortality was recorded at 100 µg/ml with 0% mortality for both methanolic and aqueous extracts. Mortality percentage was significantly lower in aqueous extracts in all tested concentrations than in methanolic extracts. However, in both extracts mortality percentage increased upon chronic exposure (24 hours) to the extracts.

An LC_{50} value < 1000 µg/ml is considered as bioactive in standard brine shrimp lethality bioassay for toxicity evaluation of plant extracts (Meyer *et al.*, 1982).

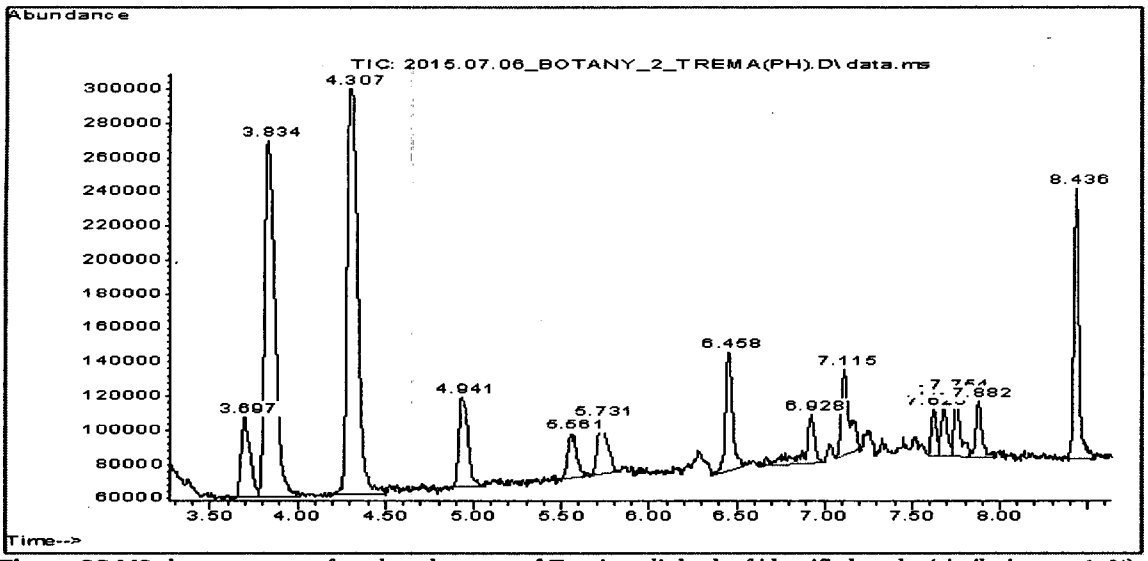

Fig. 3. GC-MS chromatogram of methanol extract of *T. orientalis* bark of identified peaks (similarity at ≥60%).

Specifically, cytotoxic activity is considered weak or low within 500-1000 µg/ml LC_{50} values, moderate within 100-500 µg/ml LC_{50} values, strong when the LC_{50} ranges from 0 to 100 µg/ml (Clarkson *et al.*, 2004), and defined as non-toxic when the LC_{50} value is greater than 1000 µg/ml (Meyer *et al.*, 1982). Based on this benchmark, the methanol extract is considered to have a weak cytotoxic activity upon chronic exposure (LC_{50}:1457.38 µg/ml at 6 h and LC_{50}: 592.34 µg/ml at 24 h) (Table 7).

This may indicate that *T. orientalis* may contain potent bioactive compounds. As identified by GC-MS, the presence of undecane may have contributed to its weak toxicity. Undecane is an antimicrobial agent and used as a carcinogen (Krishnamoorthy and Subramaniam, 2014).

On the other hand, aqueous extracts were revealed to have LC_{50} value >1000 µg/ml, thus were inactive suggesting that decoction preparation possessed no acute or chronic toxicity and may support the safety of traditional preparation methods.

Conclusion

The present study demonstrated a positive antioxidant and α-amylase inhibitory activities of *T. orientalis* bark. Positive bioactivity demonstrated by extracts could be due to the synergistic effect of the active compounds, with emphasis on the phenolic compounds (phenols, flavonoids and tannins). Higher total phenolics content was also exhibited in methanol extract than aqueous extract.

However, only hydrocarbons and d-limonene were properly identified by GC-MS in high concentrations while other phytoconstituents were detected in too low concentrations with ≤60% similarity index.

In addition, traditional preparation (decoction) is revealed to be non-toxic and methanol extract exhibited weak toxicity against brine shrimp assay. Thus, the result of this study provides pharmacological evidence on the antidiabetic potential of *T. orientalis* bark and its traditional use as a diabetes treatment.

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References

Abiodun O, Gbotosho G, Ajaiyeoba E, Happi T, Falade M, Wittlin S, Sowunmi A, Brun R, Oduola A. 2011. *In vitro* antiplasmodial activity and toxicity assessment of some plants from Nigerian ethnomedicine. Pharmaceutical Biology **49(1)**, 9–14. http://dx.doi.org/10.3109/13880209.2010.490224.

Adinortey MB, Galyuon IK, Asamoah NO. 2013. *Trema orientalis* Linn. Blume: A potential for prospecting for drugs for various uses. Pharmacognosy Reviews **7(13)**, 67–72. http://doi.org/10.4103/0973-7847.112852

Ayoola GA, Coker HAB, Adesegun SA, Adepoju-Bello AA, Obaweya K, Ezennia EC, Atangbayila TO. 2008. Phytochemical Screening and Antioxidant Activities of Some Selected Medicinal Plants Used for Malaria Therapy in Southwestern Nigeria. Tropical Journal of Pharmaceutical Research **7(3)**, 1019-1024. http://dx.doi.org/10.4314/tjpr.v7i3.14686

Birben E, Sahiner UM, Sackesen C, Erzurum S, Kalayci O. 2012. Oxidative Stress and Antioxidant. Defense. The World Allergy Organization Journal **5(1)**, 9-19. http://dx.doi.org/10.1097/WOX.0b013e3182439613

Bischoff H. 1994. Pharmacology of Glucosidase Inhibitor. European Journal of Clinical Investigation **24**, 3–10.

Chipiti T, Ibrahim MA, Koorbanally NA, Islam S. 2015. *In vitro* Antioxidant Activity and GC-MS Analysis of the Ethanol and Aqueous Extracts of *Cissus cornifolia* (Baker) Splanch (Vitaceae) Parts. Acta Poloniae Pharmaceutica-Drug Research **72(1)**, 119-127.

Chowdhury A, Islam MS. 2004. Antibacterial activity of *Trema orientalis.* Dhaka University Journal of Pharmaceutical Sciences **3(1-2)**, 115–7. http://dx.doi.org/10.3329/dujps.v3i1.186

Clarkson C, Vineshm JM, Neil RC, Olwen MG, Pamisha P, Motlalepula GM, Niresh B, Peters JS, Peter IF. 2004. *In vitro* antiplasmodial activity of medicinal plants native to or naturalized in South Africa. Journal of Ethnopharmacology **92**, 177-191.

Dai J, Mumper RJ. 2010. Plant Phenolics: Extraction, Analysis and Their Antioxidant and Anticancer Properties. Molecules **15**, 7313-7352. http://dx.doi.org/10.3390/molecules15107313.

Das MP, Yasmine Y, Devi PV. 2015. Evaluation of Qualitative Phytochemical Constituents and *In Vitro* Anti-Diabetic Activity of *Cardiospermum halicacabum* L. International Journal of Pharma and Bio Sciences **6(3)**, 711 – 719.

De Sales PM, de Souza PM, Simeoni LA, Magalhães PDO, Silveira D. 2012. Alpha-amylase inhibitors: A review of raw material and isolated compounds from plant source. Journal of Pharmacy and Pharmaceutical Sciences **15(1)**, 141–183.

Dijoux-Franca MG, Noungoué-Tchamo D, Cherel B, Cussac M, Tsamo E, Mariotte AM. 2001. New dihydrophenanthrene and phenyldihydroisocoumarin constituents of *Trema orientalis.* Journal of Natural Products **64**, 832–835. http://dx.doi.org/10.1021/np000275s

Dimo T, Ngueguim FT, Kamtchouing P, Dongo E, Tan PV. 2006. Glucose lowering efficacy of the aqueous stem bark extract of *Trema orientalis* (Linn.) Blume in normal and streptozotocin diabetic

rats. Pharmazie **61(3)**, 233–6.

Djeridane A, Yousfi M, Nadjemi B, Boutassouna D, Stocker P, Vidal N. 2006. Antioxidant activity of some Algerian medicinal plants extracts containing phenolic compounds. Food Chemistry **9**, 654–660.

Elias NU, Nuñeza OM, Uy MM. 2014. Evaluating the Potential Cytotoxic Activity of *Acmella grandiflora* Flower and Whole Plant using Brine Shrimp Lethality Test. International Research Journal of Biological Sciences **3(10)**, 90-92.

Formagio ASN, Volobuff CRF, Santiago M, Cardoso CAL, Vieira MC, Valdevina Pereira Z. 2014. Evaluation of Antioxidant Activity, Total Flavonoids, Tannins and Phenolic Compounds in *Psychotria* Leaf Extracts. Antioxidants **3(4)**, 745-757. http://dx.doi.org/10.3390/antiox3040745

Hashim A, Khan MS, Khan MS, Baig MH, Ahmad S. 2013. Antioxidant and α-Amylase Inhibitory Property of *Phyllanthus virgatus* L.: An *In Vitro* and Molecular Interaction Study. BioMed Research International **2013**, 1-12. http://dx.doi.org/10.1155/2013/729393

Horii S, Fukase H, Matsuo T, Kameda Y, Asano N, Matsui K. 1986. Synthesis and α-D-glucosidase inhibitory activity of N-substituted valiolamine derivatives as potent oral antidiabetic agents. Journal of Medicinal Chemistry **29(6)**, 1038–1046

Johansen JS, Harris AK, Rychly DJ, Ergul A. 2005. Oxidative stress and the use of antioxidants in diabetes: Linking basic science to clinical practice. Cardiovascular Diabetology **4**, 5. http://dx.doi.org/10.1186/1475-2840-4-5

Kamtekar S, Keer V, Patil V. 2014. Estimation of Phenolic content, Flavonoid content, Antioxidant and Alpha amylase Inhibitory Activity of Marketed Polyherbal Formulation. Journal of Applied Pharmaceutical Science **4(09)**, 061-065. http://dx.doi.org/10.7324/JAPS.2014.40911

Kazeem MI, Adamson JO, Ogunwande IA. 2013. Modes of Inhibition of α-Amylase and α-Glucosidase by Aqueous Extract of *Morinda lucida* Benth Leaf. BioMed Research International **2013**, 1-6. http://dx.doi.org/10.1155/2013/527570.

Khoddami A, Wilkes M, Roberts TH. 2013. Techniques for Analysis of Plant Phenolic Compounds. Molecules **18**, 2328-2375. http://dx.doi.org/10.3390/molecules18022328

Kim YH, Kim YS, Roh GS, Choi WS, Cho GJ. 2012. Resveratrol blocks diabetes-induced early vascular lesions and vascular endothelial growth factor induction in mouse retinas. Acta Ophthalmologica **90**, 31-37.

Kim YM, Jeong YK, Wang MH, Lee WY, Rhee HI. 2005. Inhibitory effect of pine extract on alpha-glucosidase activity and postprandial hyperglycemia. Nutrition **21(6)**, 756-61.

Krishnamoorthy K, Subramaniam P. 2014. Phytochemical Profiling of Leaf, Stem, and Tuber Parts of *Solena amplexicaulis* (Lam.) Gandhi Using GC-MS. International Scholarly Research Notices **2014**, 1-13. http://dx.doi.org/10.1155/2014/567409

Lazo-de-la-Vega-Monroy ML, Fernández-Mejía C. 2013. Chapter 9: Oxidative Stress in Diabetes Mellitus and the Role of Vitamins with Antioxidant Actions. In: Morales-Gonzalez JA, ED. Oxidative Stress and Chronic Degenerative Diseases - A Role for Antioxidants. InTech. http://dx.doi.org/10.5772/51788.

Manaharan T, Teng LL, Appleton D, Ming CH, Theanmalar M, Palanisamy UD. 2011. Antioxidant and antiglycemic potential of Peltophorum pterocarpum plant parts. Food

Chemistry **129(4),** 1355-1361. http://dx.doi.org/10.1016/j.foodchem.2011.05.041

Matsui T, Yoshimoto C, Osajima K, Oki T, Osajima Y. 1996. In vitro survey of alpha-glucosidase inhibitory food components. Bioscience, Biotechnology, and Biochemistry **60(12),** 2019-2022.

Meyer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DE, McLaughlin JL. 1982. Brine shrimp: A convenient general bioassay for active plant constituents. Planta Medica **45(5),** 31-34.

Mpiana PT, Ngbolua KN, Mudogo V, Tshibangu DS, Atibu EK, Tshilanda DD, Misengabu NM. 2011. Anti sickle erythrocytes haemolysis properties and inhibitory effect of anthocyanins extracts of *Trema orientalis* (Ulmaceae) on the aggregation of human deoxyhemoglobin S in vitro. Journal of Medical Sciences **11(3),** 129–137. http://dx.doi.org/10.3923/jms.2011.129.137

Murali R, Karthikeyan A, Saravanan R. 2013. Protective Effects of D-Limonene on Lipid Peroxidation and Antioxidant Enzymes in Streptozotocin-Induced Diabetic Rats. Basic & Clinical Pharmacology & Toxicology **112,** 175–181. http://dx.doi.org/10.1111/bcpt.12010

Murali R, Saravanan R. 2012. Antidiabetic effect of D-limonene, a monoterpene in streptozotocin-induced diabetic rats. Biomedicine and Preventive Nutrition **2(4),** 269–275. http://dx.doi.org/10.1016/j.bionut.2012.08.008

Noungoue TD, Cartier G, Dijoux –Franca MG, Tsamo AM. 2001. Xanthones and others constituents of *Trema orientalis*. Pharmaceutical Biology **39,** 202–205.

Palanisamy UD, Ling LT, Manaharan T, Appleton D. 2011. Rapid isolation of geraniin from *Nephelium lappaceum* rind waste and its anti-hyperglycemic activity. Food Chemistry **127(1),** 21–27. http://dx.doi.org/10.1016/j.foodchem.2010.12.070

Panchal HS, Master SM, Shah UD, Saluja AK, Dholwani KK. 2010. Anti-convulsion activity of leaf of Trema orientalis. International Journal of Pharmaceutical Research **2,** 53–55.

Perera PRD, Ekanayake S, Ranaweera KKDS. 2013. *In vitro* study on antiglycation activity, antioxidant activity and phenolic content of *Osbeckia octandra* L. leaf decoction. Journal of Pharmacognosy and Phytochemistry **2(4),** 198-201.

Rout J, Sajem AL, Nath M, Sengupta M. 2012. Antibacterial Efficacy of Bark extracts of an Ethnomedicinal plant *Trema orientalis* Blume. Current Trends in Biotechnology and Pharmacy **6(4),** 464-471.

Sermakkani M, Thangapandian V. 2010. Phytochemical Screening for Active Compounds in *Pedalium murex* L. Recent Research in Science and Technology **2(1),** 110-114.

Sudha P, Zinjarde SS, Bhargava SY, Kumar AR. 2011. Potent a-amylase inhibitory activity of Indian Ayurvedic medicinal plants. BMC Complementary and Alternative Medicine **11,** 5. http://dx.doi.org/10.1186/1472-6882-11-5

Sultana B, Anwar F, Ashraf M. 2009. Effect of Extraction Solvent/Technique on the Antioxidant Activity of Selected Medicinal Plant Extracts. Molecules **14(6),** 2167-2180. http://dx.doi.org/10.3390/molecules14062167

Trojan-Rodrigues M, Alves TLS, Soares GLG, Ritter MR. 2011. Plants used as antidiabetics in popular medicine in Rio Grande do Sul, Southern Brazil. Journal of Ethnopharmacology **139(1),** 155-163. http://dx.doi.org/10.1016/j.jep.2011.10.034.

Uddin SN. 2008. Antioxidant and antibacterial activities of *Trema orientalis* Linn: an indigenous medicinal plant of indian subcontinent. Oriental Pharmacy and Experimental Medicine **8(4),** 395-

399.
http://dx.doi.org/10.3742/OPEM.2008.8.4.395

Uddin SN, Uddin KM, Ahmed F. 2008. Analgesic and antidiarrhoeal activities of *Trema orientalis* Linn in mice. Oriental Pharmacy and Experimental Medicine **8(2),** 187-191.
http://dx.doi.org/10.3742/OPEM.2008.8.2.187

Wen-Lung K, Yu-Ling H, Shr-Ting W, Ching-Li N, Bor-Jinn S, Chien-Chih C. 2007. Chemical constituents of Trema orientalis. Journal of Chinese Medicine **18,** 27–36.