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ORIGINAL ARTICLE

Antioxidant Activities and Estimation of Phenol and Flavonoid Contents in The Extracts Of *Trema Orientalis* Linn Blume

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SUMMARY

Phytochemical analysis is important in the evaluation of bioactive compounds from plants. Oxidative stress has been implicated in the pathology of many diseases such as atherosclerosis, rheumatoid arthritis, cancer, cataract, diabetes, cardiovascular diseases, chronic inflammatory conditions, and stroke. The aim of this study was to evaluate polyphenolic content and antioxidant activity of *Trema orientalis*. Antioxidant activity was estimated spectrophotometrically using 2,2–diphenyl–1–picrylhydrazyl radical scavenging method. The total polyphenolic and flavonoid contents of the *Trema orientalis* extracts were determined using standard methods. Independent Sample T–test was used for Data analyses. Phytochemical screening revealed the presence of saponins, tannins, steroids, cardiac glycosides, alkaloids, triterpenes, flavonoids and phenolic compounds. Total phenolic contents were found to be 260.96±2.31 mg GAE/g and 134.08±0.56 mg GAE/g in the ethanol and aqueous extracts respectively. Similarly, total flavonoid contents were between 32.71±0.89 and 4.70±0.23 mg GAE/g. The radical scavenging effect was observed in ethanol extract with IC_{50} = 9.27 µg/mL. The abundance of polyphenolic compounds and antioxidant activities of the *T. orientalis* could confirm their good therapeutic potentials in ethnobotany.

Keywords: *Trema orientalis*, Phenols, Flavonoids, Antioxidant activities

INTRODUCTION

Phenolic compounds are classes of chemicals consisting of a hydroxyl group (–OH) bonded directly to an aromatic hydrocarbon group. They comprise of flavonoids, phenolic acids, tannins, lignans, coumarins, xanthones, among others (Kuete, 2013; Ngameni *et al.*, 2013; Shahat and Marzouk, 2013; Tsopmo *et al.*, 2013). Free

radicals in living systems, drugs and food, are produced primarily through oxidative process (Pourmorad *et al.,* 2006). Antioxidants fight against free radicals. The electron donation ability of natural products can be measured by 2,2–diphenyl–1–picrylhydrazyl (DPPH) purple–colored solution bleaching (Nunes *et al.,* 2008). About 80% of world's population rely on traditional medicines most of which involve the use of extracts of plants with *Trema orientalis* Linn. Blume among the most used plants (Sandhya *et al.,* 2006; Panchal *et al.,* 2010; Abiodun *et al.,* 2011; Adinortey *et al.,* 2013).

Trema orientalis is an evergreen flowering plant of the hemp family, Ulmaceae (Adinortey *et al.,* 2013). The leaves vary from 0.012 – 0.072 m wide and 0.020 – 0.20 m long, and taper from the base to apex (FAOSTAT, 2008). Flowers are small, greenish and inconspicuous, carried in short, and dense bunches. The fruits are small (3 – 5 mm long), round, and dark green or purple drupes and change to black when ripe; carried on very short stalks (Wagner *et al.,* 1999; FAOSTAT, 2008). The common names of the plant include *charcoal–tree* (English), *telemukwu* (Igbo), *afefe* (Yoruba), *menarong* (Malay) and *chikan* (Hindi) (Malan and Notten, 2005; GRIN, 2007; Orwa *et al.,* 2009). In Africa and some parts of Asia, livestock farmers use various parts of *T. orientalis* for fodder as feed to cattle, buffaloes and goats because of its high crude protein content and palatability (Motooka *et al.,* 2003; Orwa *et al.,* 2009; Holmström, 2013). The present study aims to quantitatively estimate total phenols, flavonoids and determine the antioxidant potential of *T. orientalis*.

MATERIALS AND METHODS

Chemicals

Solvents and reagents used in the current study were of analytical grade. Ethanol (95%), tetraoxosulphate (VI) acid (H_2SO_4), Iron (III) chloride ($FeCl_3$) and dimethylsulfoxide (DMSO) were obtained from R&M Chemicals Co., Essex, UK. Standards acids (garlic acid, GA), Quercetin (98% HPLC) and DPPH were obtained from Sigma-Aidrich Chemicals Co., St Louis, USA. Folin–Ceocalteur's (FC's) phenol reagent, $NaHCO_3$, $AlCl_3$, Water (HPLC Gradient grade) was purchased from Loughborough, Leics, UK.

Collection, Identification and Preparation of Leaf Sample

Wild *Trema orientalis* leaves ware collected in November, 2014 in Pasir Akar, Besut Terengganu, Malaysia. The leaf sample was authenticated by Nashriyah Binti Mat, a Professor of botany at the School of Agricultural Sciences and Biotechnology, Faculty of Bioresources and Food Industry (FBIM), University Sultan Zainal Abidin (UniSZA), Tembila Campus, Besut Terengganu, Malaysia. A voucher specimen was deposited at the Herbarium of School of FBIM, UniSZA, with Voucher No.: 00267. The sample was gently pre–washed using tap water to remove impurities and air dried at ambient temperature until a constant weight was obtained. The dried leaves were then crushed and pulverized to obtain fine homogeneous powder using a Laboratory blender (HGB550, USA). This improved the kinetics of analytic extraction and increased the contact of sample surface with the solvent system. Proper actions were taken to ensure that potential active constituents were not lost, distorted or destroyed during the preparation of the extract.

Determination of Percentage Loss on Drying

Percentage loss on drying (% LOD) was determined gravimetrically according to Geneva, (1998). Five grams of accurately weighed air–dried leaf sample was placed in a previously dried and tared flat weighing bottle. The sample was dried in an oven (Memmert UN 110, Germany) at 105 °C until a constant weight was obtained. The % LOD was calculated using the equation below;

$$\% \text{ LOD} = \left(\frac{\text{Loss in Weight}}{\text{Weight of Dried Sample}}\right) \times 100$$

Determination of Ethanol Soluble Extractive Value

The extraction was conducted according to the Quality Control Methods for Medicinal Plants Materials Jain and Argal, (2013). Five grams of oven–dried leaf powder of *T. orientalis* was transferred to a conical flask. One hundred mL of 95 % ethanol was added, and the flask was covered with aluminium foil. It was then placed on an Orbital shaker (2 Tier, 722–2T, Malaysia) during the first 6 hours and allowed to stand for 18 hours separately. After that, it was filtered rapidly taking precaution to minimize the loss of ethanol. Twenty-five mL of the filtrate was collected and transferred to a weighed thin porcelain. It was evaporated to dryness on a water bath and dried completely in an oven at 90 ^{o}C until a constant weight was reached. It was kept in a desiccator to cool and the percentage of alcohol soluble extractive yield was calculated with reference to oven–dried leaf accoding to Sharma and Sharma, 2013; Upreti, *et al.,* 2013 using the following equation;

$$\text{Percentage Extractive Value} = \left(\frac{\text{Initial Weight} - \text{Final Weight}}{\text{Initial Weight}}\right) \times 100$$

Preparation of Crude Ethanol and Aqueous Leaf Extracts

Approximately 500 g of powdered leaf sample of *T. orientalis* was placed in a 5 L conical flask, completely soaked with 3.5 L of 95 % ethanol, and then covered with aluminium foil. The mixture was allowed to stand at ambient temperature (25^{o}C ± 2) for 72 hours with frequent agitation in order to facilitate dissolution of the soluble matter. The mixture was strained using muslin cloth to remove solid material. The extraction was repeated to ensure maximum yield by soaking the solid material using 1.5 L of the ethanol. Similar procedure was carried out for aqueous extract preparation except for the extraction period which 12 hours. The strained liquids were clarified by filtration by gravitation using Smith filter paper. The filtrate was then concentrated to solid extract under reduced pressure (180 m/bar) at 40^{o}C using Rotary evaporator (BUCHI Rotavapor R–210, Switzerland). The ethanol leaf extract of *T. orientalis* (ELETO) obtained was dark–green in colour.

Phytochemical Evaluation

Phytochemical screening was conducted to detect the presence of metabolites according to Godghate *et al.* (2012) method. Total flavonoid content (TFC), total phenolic content (TPC) and antioxidant activity (AA) were evaluated according to a modified version of Quettier *et al.* (2000), Kumarasamy *et al.* (2007) and Stanković, (2011) respectively. Stock solution was prepared at 50 µg/mL.

Determination of Total Phenolic Content

The total phenolic content in both aqueous and ELETO was determined using FC's phenol reagent. The reaction mixture contained 250 µL (1 mg/mL) of diluted crude ELETO, 1.25 mL of freshly prepared diluted 10 % (v/v) FC's phenol reagent and 1 ml of 7.5 % (w/v) $NaHCO_3$. The final mixture was diluted to 7 mL with deionized water. The mixture was kept in dark at ambient temperature for 1h to complete the reaction. The sample was prepared in triplicate, and the absorbance was measured at 760 nm using a spectrophotometer (Shimadzu UVmini–1240, Japan). The same procedure was repeated for each sample, and the standard solution of gallic acid, (GA) (Sigma–Aidrich Chemicals, USA) prepared at 150 µg/mL. Control was concomitantly prepared using a mixture containing 1.25 mL 10 % FC's reagent dissolved in water and 1 mL of 7.5 % $NaHCO_3$. The calibration line was construed using the GA with a concentration of 4.70, 9.40, 18.80, 37.50, 75.00, and 150.00 µg/mL. Based on the measured absorbance, the concentration of phenols was read (µg/mL) from the calibration line. The TPC in extracts was expressed as gallic acid equivalent (GAE) in mg/g of extract.

Determination of Total Flavonoid Content

Total flavonoid content (TFC) in both aqueous and ethanol *T. orientalis* leaf extracts were determined using aluminium chloride ($AlCl_3$) method. The reaction mixture contained 250 µL of sample at 1mg/mL concentration, 50 µL potassium acetate, and 50 µL of 10% $AlCl_3$ solution dissolved in 95% ethanol and 2.50 mL of ethanol. The mixture was incubated at ambient temperature for 1 h. The sample was prepared in triplicate, and the absorbance was measured at 415 nm using a spectrophotometer (Shimadzu UVmini–1240, Japan). The same procedure was repeated for each sample and the standard solution of quercetin at 160 µg/mL. The control was prepared to contain an equal amount of all the reagents used except the sample extract. The calibration line was construed at 5, 10, 20, 40, 80, and 160 µg/mL. Based on the measured absorbance, the concentration of flavonoids was read (µg/mL) on the calibration line. The TFC in extracts was expressed as quercetin equivalent (QE) in mg/g of extract.

Evaluation of Antioxidant Activity

The DPPH free radicals scavenging activity of the *T. orientalis* was assessed by the standard method with suitable modifications (Kumarasamy *et al.,* 2007) in both aqueous and ethanol the leaf extracts. The test samples and the standard (quercetin) solution were prepared in ethanol at 1 mg/mL each. Dilution was made to obtain a concentration of 500.00, 250.00, 125.00, 62.50, 31.25, and 15.62 µg/mL in each sample. Diluted solution (60 µL each) was mixed with a 200 µL solution of DPPH dissolved in 1% dimethylsulfoxide (DMSO) and kept as 0.1 mM concentration. Control sample contained all the reagents except the extract and quercetin in the case of the standard. After 45 min incubation in darkness at ambient temperature (25 ^{o}C ± 1), the absorbance was recorded at 517 nm using Multifunctional Microplate Reader (Tecan Infinite M200PRO, Australia). The percent radical scavenging activity of the samples was determined in comparison with ethanol–treated control groups, whereas half-maximal inhibitory concentration (IC_{50}) values were estimated using linear regression graph. The percentage inhibition (%I) was calculated using the following equation;

$$\%I = 100 - \left(\frac{\text{Absorption of Sample}}{\text{Absorption of Control}}\right) \times 100$$

Data analyses

Total phenolic and total flavonoid contents were analyzed by Independent Sample T–test. Pearson correlation between the TPC, TFC and AA values were evaluated using SPSS version 20. Results are expressed as mean ± standard deviation (SD) of three determinants. *P* values <0.05 were considered significant.

RESULTS

Percentage Loss on Drying

Mean percentage loss on drying (% LOD) value of *T. orientalis* leaf was found to be 59.80±0.53 with a minimum value of 59.27 and a maximum 60.43 (Table I).

TABLE I: Percentage loss on drying of *Trema orientalis* leaf

Fresh weight (g)	Dry weight (g)	Loss in weight (g)	% Loss in weight	Mean % LOD±SD (n = 5)
5.0	1.97	3.03	60.60	59.80±0.53
5.0	2.00	3.00	60.00	
5.0	2.02	2.98	59.60	
5.0	2.02	2.98	59.60	
5.0	2.04	2.96	59.20	

Percentage loss on drying: % LOD, Standard deviation: SD, Sample size: n

Ethanol Soluble Extractive Value

The mean ethanol–soluble extractive value of *T. orientalis* leaves was found to be 14.33±0.32 percentage with a minimum value of 13.90 and a maximum 14.6 (Table II).

Results of preliminary phytochemical screening on ELETO showed presence of saponins, steroid, cardiac glycoside, alkaloids, triterpenes, flavonoids and phenolic compounds (Table III).

The amount of TPC was found significantly (P<0.05) higher in ELETO (260.96±2.31 mgGAE/g) compared to the aqueous crude extract (134.08±0.56 mgGAE/g) (Table IV, Figure I). Similarly, TFC was also above in ethanol extract (32.71±0.90 mgQE/g) compared to the aqueous extract (4.70±0.24 mgQE/g) (Table IV, Figure II). Ethanol extract exhibited higher values of antioxidants compared to aqueous extract (Figure III). There was a significant positive correlation between the AA and phenolic content of the solvent extracts of the plant (r = 0.798, P < 0.05).

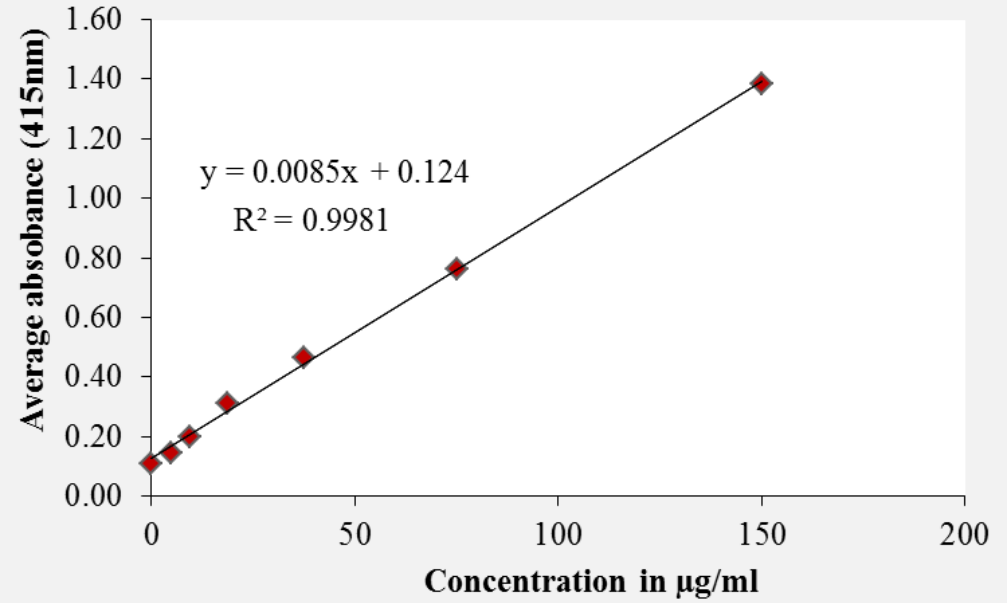

Figure I: Showing the standard calibration curve for the quantification of total phenolic content in *Trema orientalis* leaf extract

TABLE II: Ethanol extractive value of the *Trema orientalis* leaf

Weight of dried leaves (g)	Final weight (g)	Weight of extract (g)	% extract value	% mean extractive value ± SD (n=5)
5.00	4.27	0.73	14.60	14.33±0.32
5.00	4.27	0.73	14.69	
5.00	4.25	0.72	14.30	
5.00	4.29	0.71	14.18	
5.00	4.31	0.70	13.90	

Percentage: %, Standard deviation: SD, Sample size: n

TABLE III: Results of phytochemical screening of *Trema orientalis*

Chemical groups	Saponins	Tanins	alkaloids	steroid	Cardiac glycoside	Triterpenes	flavonoids	phenolic compounds
Ethanol extract	++	++	++	+	++	++	++	+++
Aqueous extract	+++	+	-	+	+	+	+	+

Strong positive: +++, moderately positive: ++, Low positive: +, negative test: –

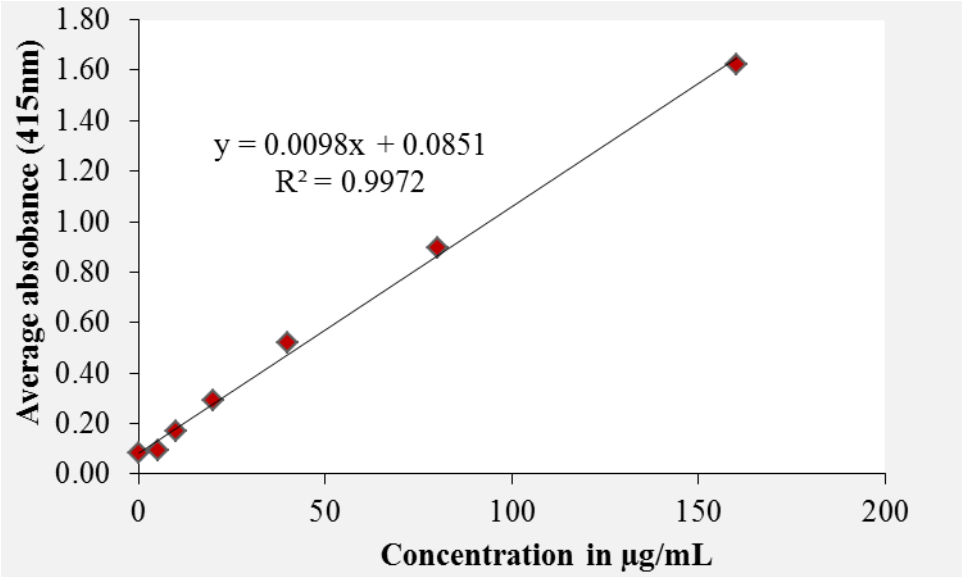

Figure II: Showing standard calibration curve for the quantification of total flavonoid content in *Trema orientalis* leaf extract

TABLE IV: Total phenolic content, total flavonoid content, and antioxidant activity of ethanol and aqueous extracts of *Trema orientalis* leaf (Mean ± SD, n = 5)

Solvent	TPC (mg GAE/g)	TFC (mg QE/g)	% Inhibition	IC_{50} (µg/mL)
Ethanol	260.96±2.31[a]	32.71±0.89[b]	89.11±0.26	9.27
Aqueous	134.08±0.56[b]	4.70±0.23[a]	40.26±0.47	–

Means with different superscripts (a,b) indicate significant differences (P<0.05), total phenolic content: TPC, Total flavonoid content: TFC, Gallic acid equivalent: GAE, quercetin equivalent: **QE**

DISCUSSION

The % LOD and ethanol soluble extractive value of *T. orientalis* leaf were determined in the present study in order to estimate the amount of fresh and dried leaf that gives a particular amount of solid ELETO; since moisture content is of direct economic importance to the processor and the consumer. The value obtained is slightly higher compared % LOD of *Geranium ocellatum* leaf with the yield value of 51.7 % (Joseph and George, 2011). The ethanol soluble extractive value obtained is similar to that of *Aritochia indica* Linn., *Zanthoxylum armatum* D.C., , and *Gynura segetum* Lour with value 15.53%, 14.66% and 14.13% respectively (Mridula *et al.*, 2011; Devi and Divakar, 2012; Seow *et al.*, 2013; Upreti, *et al.*, 2013). Tuo *et al.* (2015) reported significant higher extractive value (20%) of ELETO. Other plants with higher ethanol extractive value include *Prosopis cineraria* Linn. and *Catunaregum spinosa* Thunb. with value 19.0% and 20.93%, respectively (Shrivastava and Leelavathi, 2010; Ravichandra and Paarakh, 2011; Kumawat *et al.*, 2012; Singh *et al.*, 2013; Komlaga *et al.*, 2014). However, several plants were reported to have lower ethanol extractive yield, including *Ficus microcarpa* L. (8.0%), *Solanum macrocarpon* (8.66%) and *Balanites aegyptiaca* L. (8.79%). Extraction of any plant with a particular solvent yields a solution containing different phyto–constituents. Extractive values are primarily useful for the determination of exhausted or adulterated drugs, evaluation of chemical constituents present and estimation of specific constituents soluble in that particular solvent used for extraction.

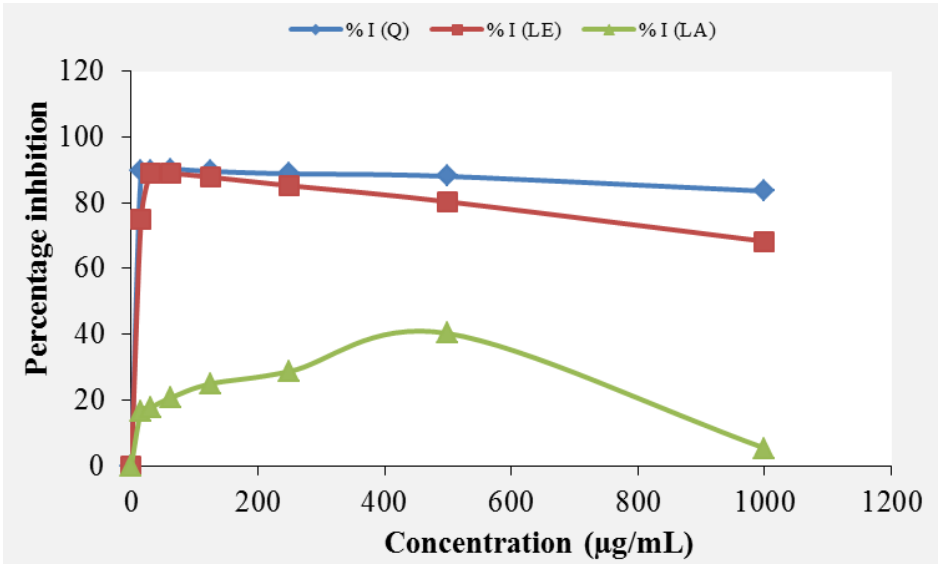

Figure III: Showing free radical scavenging activities of *Trema orientalis* leaf extract

The preliminary phytochemical screening result obtained in the current study agrees with findings of Tchamo *et al.* (2000); Gbadamosi *et al.* (2012) and Adinortey *et al.* (2013); who reported the presence of saponins, tannins, flavonoids, including cardiacglycoside, alkaloids, triterpenes in *T. orientalis leaves extract*. However, Ayoade *et al.* (2014) reported that *T. orientalis* leaf extract was found to be more abundant in steroids, flavonoids, and alkaloids, while saponins, tannins, glycosides, and terpenoids were present. According to Tuo *et al.* (2015), *T. orientalis* leaf extract is devoid of steroids. Several studies of other plants species have shown that saponin and tanins causes inappetance, mass loss, hepatotoxicity, icterus, tachycardia, ruminal stasis, dyspnoea, anaemia, necrosis recumbency and death in animals (Cornick *et al.*, 1988; Shumaik *et al.*, 1988; Miles s*et al.*, 1993; Adedapo and Abatan, 2005). According to Graydon *et al.* (1991), toxic effect of the steroidal saponins is related to their normal metabolism in the rumen. The toxic effects of steroidal saponins are mediated by hydrolysis in the rumen, leading to release of their corresponding sugars and sapogenins (aglycones). The sapogenins are absorbed and

transported to the liver where they form conjugates of epsimilagenin with glucuronic acid and excreted in the bile. Once in the bile, it crystallizes by forming insoluble calcium salts of sapogenin glucuronate precipitate crystals block inside and around the biliary ducts leading to the toxicity signs above mentioned above. Sofowora (1993) reported that saponins precipitate proteins, bind cholesterol, and haemolyse RBC whereas, hydrolysable tannins (astringents) bind plasma and organ proteins causing coagulation and necrosis (Spier *et al.,* 1987).

Higher values of TPC, TFC and AA in ethanol fraction than the aqueous obtained in this study and the strong positive correlation between AA and, TPC and TFC were also reported in several previous studies (Yang *et al.,* 2002; Maksimović *et al.,* 2005; Stratil *et al.,* 2006; Kratchanova *et al.,* 2010; Thoo *et al.,* 2010; Sah *et al.,* 2012). According to Wong *et al.* (2006), low correlation between TPC and AA occurs because of an error introduced in the assays. However, there are also reports of no such correlation (Bajpa *et al.,* 2005). Assays that were based on the measurement of an end product, one could be measuring the AA of the reaction by–products, rather than the compounds present in the original mixture (Halliwell, 2009). An *in vitro* technique has been used to determine the AA in order to allow easy screening of drugs since substances that have low AA *in vitro,* will probably show little activity *in vivo* (Nunes *et al.,* 2008). A large decrease in the absorbance of the reaction mixture indicates significant free radical scavenging activity of the tested compound under test (Krishnaiah and Sarbatly, 2011). Usually, extracts that contain high amount of polyphenols also show high AA (Wong *et al.,* 2006). High scavenging activity on DPPH radicals could be due to the low molecular weight phenolic compounds in the samples studied. Paixão *et al.* (2007) reported that DPPH is known to react specifically with low molecular weight phenolic compounds. Factors influencing the recovery of antioxidant of specific sample were antioxidants concentration, extraction medium (time and polarity), pH of medium, temperature, chemical structures and position in the molecule (Prior *et al.,* 2005; Zhang *et al.,* 2007; Thoo *et al.,* 2010). Hence, high yield of individual phenolic compounds may not exhibit a high AA as it is dependent on the synergistic effects of the extracted phenolic compounds. Extraction conditions can significantly influence AA in plants.

Almost every part of *T. orientalis* is used as traditional medicine in different disease conditions such as pneumonia, pleurisy, tooth ache, hematuria, blood stasis, laxative, hyperglycemia, anticonvulsant, antiplasmodial and helminthiasis (Yanes, 2007; Orwa *et al.,* 2009; N′guessan, 2009; Panchal *et al.,* 2010; Abiodun *et al.,* 2011; Adinortey *et al.,* 2013). These effects may be attributed to its important biologically active compounds such as polyphenols (Adinortey *et al.,* 2013). However, Matuschek and Svanberg (2002) reported that naturally occurring polyphenols bind with non–heme iron *in vitro* in model systems, possibly reducing its absorption thereby leading to anaemia. The antioxidant activities observed could be ascribed both to mechanisms exerted by phenolic compounds and to synergistic effects of different phyto compounds. The identification of phytochemical constituent defined in this study will facilitate the evaluation of the *in vitro* models for predicting farm animal toxicity.

CONCLUSION

Both ethanol and aqueous extracts of *T. orientalis* contain saponins, tannins, steroids, cardiac glycosides, triterpenes, flavonoids and phenolic compounds. The TPC and TFC are significantly higher in ethanol extract compared to aqueous. The radical scavenging effect with IC_{50} value of 9.27 µg/mL proved that ELETO has abundance polyphenolic compounds capable of donating hydrogen to free radical to scavenge a potential damage. Hence, *T. orientalis* has good therapeutic potentials in ethnobotany.

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