academicJournals

Vol. 13(5), pp. 693-698, 29 January, 2014
DOI: 10.5897/AJB2013.13225
ISSN 1684-5315 ©2014 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Fractionation and determination of total antioxidant capacity, total phenolic and total flavonoids contents of aqueous, ethanol and n-hexane extracts of *Vitex doniana* leaves

Yakubu, O.E.[1,2], Nwodo, O.F.C.[1], Joshua, P.E.[1], Ugwu, M.N.[2], Odu, A.D.[2] and Okwo F.[2]

[1]Department of Biochemistry, Faculty of Biological Sciences, University of Nigeria, Nsukka, Nigeria.
[2]Department of Medical Biochemistry, Cross River University of Technology, Calabar, Nigeria.

Accepted 20 January, 2014

As a result of normal metabolic processes, the human body produces reactive oxygen species capable of oxidizing biomolecules that can damage DNA, cells and also contribute to the development of chronic diseases. The process can be attenuated or perhaps reversed by herbs and diets containing components that can scavenge reactive oxygen species. In this study, the total antioxidant capacity (TAC), total polyphenolic content (TPC) and total flavonoids content (TFC) of aqueous, ethanol, n-Hexane extract as well as ethanol extract fractions of *Vitex doniana* leaves were determined. Ethanol extract showed the highest 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity (69.01±1.13) followed by aqueous extract (66.14±1.12) and n-hexane extract (50.05±2.11). The total flavonoids content is in the order; aqueous (304±4.14) > ethanol (276 ±4.69) > n-Hexane (88±3.45). Hence, the total phenolic content is in a similar order as that of total antioxidant capacity. Chloroform : ethyl acetate fraction has the highest antioxidant capacity (165mg/ml). methanol : H_2O fraction (76mg/ml) and 100% methanol (76mg/ml). Similarly, the total flavonoids content is in the order of fractions; 1>6>4>13>12>2 and others. Total phenolics were in the order of fractions; 1>5>4>12>7>2. There was a strong relationship (R^2 = 0.77) between total antioxidant activity and total flavonoid contents and (R^2 = 0.6517) for total phenolic content of the fractions. The present study demonstrated that *V. doniana* leaves extracts contain high amounts of flavonoids and phenolic compounds so that these compounds are efficient free radical scavengers.

Key words: 1,1-Diphenyl-2-picrylhydrazyl (DPPH), polyphenols, flavonoids, *Vitex doniana*.

INTRODUCTION

Natural products are important sources for biologically active drugs and wild herbs have been investigated for their antioxidant properties (Gazzaneo et al., 2005). Medicinal plants containing active chemical constituents with high antioxidant property play an important role in the prevention of various degenerative diseases and have potential benefit to the society (Lukmanul et al., 2008). Natural antioxidants from plant sources are potent and safe

*Corresponding author. E-mail: oj4real_2007@yahoo.co.uk.

Abbreviations: DPPH, 1,1-Diphenyl-2-picrylhydrazyl; **TAC,** total antioxidant capacity; **TE,** trolox equivalent; **GAE,** gallic acid equivalent; **QE,** quercetin equivalent; **TFC,** total flavonoid content.

due to their harmless nature. A free radical in each molecule is determined as an unpaired electron that occupies an atomic or molecular orbital on its own. This reactive molecule is to another electron to pair, this in step an uncontrolled chain reaction that can damage the natural function of the living cell, resulting in different diseases (Zhishen et al., 1999). Many fruits and vegetables, herbs, cereals, seeds that contain natural antioxidants can abstract the lone electron from free-radical molecules and help humans to keep control on these harmful species. Most of these antioxidants in plants are highly coloured anthocyanines, proanthocyaninidins, flavans, flavonoids, and their glycosides, carotenoids, like β-carotene and lycopene (Matkowski et al., 2009). Isolation of antioxidants from plants depends on the polarity of these compounds. First distribution of antioxidants between a polar (aqueous, hydro ethanol) and a semi-polar solvent (n-butanol, ethyl acetate) can be used to determine the distribution factor of the compounds between phases (Matkowski et al., 2009).

Vitex doniana sweet, (family *Verbanaceae*) is a perennial shrub widely distributed in tropical West Africa, and some East African countries including Uganda, Kenya and Tanzania and high rainfall areas. It is found in the middle belt of Nigeria particularly Kogi, Benue, and parts of the savannah regions of Kaduna, Sokoto and Kano states (Etta, 1984). It is variously called *vitex* (English), *dinya* (Hausa), *dinchi* (Gbagyi), *uchakoro* (Igbo), *oriri* (Yoruba) *ejiji* (Igala) and *olih* (Etsako) (Burkill, 2000). *V. doniana* is employed in the treatment of a variety of diseases. Hot aqueous extracts of the leaves are used in the treatment of stomach and rheumatic pains, inflammatory disorders, diarrhoea dysentery and diabetes (Irvine, 1961; Etta, 1984). Yakubu et al. (2012; 2013) reported the antidiabetic properties of the leaves. The roots and leaves are used for nausea, colic and epilepsy (Bouquet et al., 1971; Iwu, 1993). In North-Central and eastern parts of Nigeria, the young leaves are used as vegetables or sauces and porridge for meals, especially for diabetic patients.

MATERIALS AND METHODS

Collection and preparation of plant materials

Fresh leaves of *V. doniana* were collected from its natural habitat in Ankpa, Kogi State. It was identified and authenticated by the Ethnobotanist in the Department of Medicinal Plant Research and Traditional Medicine of the National Institute for Pharmaceutical Research and Development (NIPRD) Abuja, Nigeria. A voucher specimen number NIPRD/H/6415 was deposited at the herbarium of the department. The plant material was dried in the laboratory at room temperature and pulverized using laboratory mortar and pestle.

Aqueous extraction

About 400 g of the pulverized sample was soaked in 2 l of distilled water (1:5 W/V) and was allowed to stand for 24 h at room temperature according to the study of Iwueke and Nwodo (2008). The extract was filtered and the filtrate was concentrated using rotary evaporator under reduced pressure. It was allowed to dry at room temperature and stored in refrigerator prior to usage.

Ethanol / n-hexane extraction

About 400 g of the pulverized sample was soaked in 2 l (1:5 w/v) of ethanol/n-Hexane (2:1 v/v) for 24 h. The extract was filtered under reduced pressure using filter paper, membrane filter and vacuum pump. Ethanol extract was separated from the n-hexane extract using separatory funnel and the filtrates were concentrated using rotary evaporator under reduced pressure, respectively.

Fractionation

The ethanol extract was subjected to column chromatograph to separate the extract into its component fractions. Silica gel was used in packing the column while varying solvent combinations of increasing polarity were used as the mobile phase.

Packing of column

In the packing of the column, the lower part of the glass column was stocked with glass wool with the aid of glass rod. 75 g of silica gel (G_{60-200} mesh size) was dissolved in 180 ml of absolute chloroform to make the slurry. The chromatographic column (30mm diameter by 40 mm height) was packed with silica gel and was allowed free flow of the solvent into a conical flask below. The set up was seen to be in order when the solvent drained freely without carrying either the silica gel or glass wool into the tap. At the end of the packing process, the tap was locked and the column was allowed 24 h to stabilize after which, the clear solvent at the top of the silica gel was allowed to drain down the silica gel meniscus

Elution

The ethanol extract (2 g) was dissolved in 2 ml absolute methanol and the solution was applied unto a chromatographic column (30 mm diameter by 400 mm height). Elution of the extract was done with solvent system of gradually increasing polarity, beginning from chloroform, ethyl acetate, methanol and finally water. The following ratios of solvent combinations were sequentially used in the elution process: Chloroform : ethyl acetate 100:0, 80:20, 60:40, 40:60, 20:80, 0:100; ethyl acetate : methanol 80:20, 60:40, 40:60, 20:80, 0;100; methanol : water 50:50 and 0:100. A measured volume (400 ml) of each solvent combination was poured into the column each time using separator funnel. The eluted fractions were collected in aliquots of 10 ml in test tubes.

Total antioxidant capacity

The scavenging action of the plant extracts and the resulting fractions from ethanol extract on 1,1-diphenyl-2-picrylhydrazyl (DPPH) was determined colorimetrically at 517 nm using Trolox as standard according to the method described by Singleton et al. (2002). About 1.0 ml tris HCl buffer was added to test tube containing 1.0 ml absolute ethanol, 2.0 ml DPPH (0.1 mM) solution was added and the solution was thoroughly mixed. The absorbance was measured within 30 s after addition of sample at 517 nm. The absorbance was measured in triplicate for each extract/fraction. Total antioxidant capacity (TAC) was calculated as mg/ml of trolox equivalent (TE) using the regression equation from calibration curve.

Total polyphenol content (TPC)

Total polyphenol component was estimated colorimetrically at 765 nm as described by Lachman et al. (2000), using Follin-Ciocalteu reagent and expressed as gallic acid equivalent (GAE). Exactly 0.25 ml sample was added to test tube containing 2.50 ml Follin reagent. Sodium carbonate solution (2.0 ml) was added and was allowed to stand for 15-20 min at room temperature. The reactions were conducted in triplicates and absorbance of the sample was measured against the reagent blank. The results were expressed as GAE.

Estimation of total flavonoids content (TFC)

Flavonoids were determined using the aluminum chloride colorimetric method of Chang et al. (2002). Quercetin was used for derivation of the calibration curve. Exactly 0.5 ml of the diluted sample was added into test tube containing 1.5 ml methanol. 0.1 ml of 10% aluminum chloride ($AlCl_3$) solution and 0.1 ml potassium acetate (CH_3COOK) were added. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm and the concentration of flavonoids in the sample was estimated from the calibration curve. The amount of 10% aluminum chloride was substituted by the same amount of distilled water in blank. Total flavonoids were expressed as mg/ml quercetin equivalent (QE).

RESULTS AND DISCUSSION

Total antioxidant capacity, total flavonoids and total polyphenolic contents of aqueous, ethanol and n-Hexane extracts of *Vitex doniana* leaves

Ethanol extract showed the highest antioxidant activity (69.01±1.13) followed by aqueous extract (66.14±1.12) and n-hexane extract (50.05±2.11). The total flavonoids content is in the order; aqueous (304±4.14) > ethanol (276±4.69) > n-hexane (88±3.45). Hence, the total phenolic content is in a similar order as that of total antioxidant capacity.

Total antioxidant capacity, total flavonoids and total polyphenolic contents of different fractions obtained from ethanol extract of *Vitex doniana* leaves

Table 2 unveils the total antioxidant capacity, total flavonoids and total phenolic contents of different fractions obtained from the ethanol extract of *V. doniana* leaves. Chloroform: ethyl acetate fraction has the highest antioxidant capacity (165 mg/ml). Methanol: H_2O fraction (76 mg/ml) and 100% methanol (76 mg/ml). Similarly, the total flavonoids content is in the order of fractions; 1>6>4>13>12>2 and others. Total phenolics is in the order of fractions; 1>5>4>12>7>2.

Correlation between total antioxidant capacity and total flavonoids content of different fractions obtained from ethanol extract of *V. doniana* leaves

Strong positive correlation (R^2 = 0.77) between total antioxidant capacity and total flavonoids content of ethanol extract fractions of *V. doniana* leaves was observed (Figure 1).

Correlation between total antioxidant capacity and total polyphenolic content of different fractions obtained from ethanol extract of *V. doniana* leaves

Linear correlation between total antioxidant capacity and total polyphenolic content of fractions obtained from ethanol extract of *V. doniana* leaves showed positive correlation (R^2 = 0.6517) (Figure 2).

Correlation between total polyphenolic and total flavonoids contents of different fractions obtained from ethanol extract of *V. doniana* leaves

There was a strong positive correlation (R^2 = 0.8825) between total flavonoids and total polyphenolic contents of fractions obtained from *V. doniana* ethanol extract of leaves (Figure 3).

DISCUSSION

Flavonoids as antioxidant compounds in our study reportted in range of 88-304 mg/ml QE (Table 1) and 100-390 mg/ml QE dry weight of fractions (Table 2). The TFC for ethanol extract was 304±4.14 mg/ml QE. After partial purification, TFC of the fractions was found within the range 100-390 mg/ml QE which is higher than the TFC of the whole extract. This implies that the extract contains a lot of phytochemicals other than flavonoids. Furthermore, total phenolic content of the aqueous extract was 460±2.24 and 380±1.97 mg/mlGAE for ethanol extract while TFC for aqueous extract was 276.69±mg//mQE and 304±4.14mg/mlQE for ethanol extract. It means aqueous extract contained higher concentration of phenolics than ethanol extract, but lower concentration of flavonoids than ethanol extract. This is an indication that flavonoids take 80% of total phenolic content of ethanol extract and 60% of aqueous extract.

The TAC of the extracts are given as follow; ethanol > aqueous > n-Hexane extract (Table 1). This difference may be attributed to differences in extraction or hydrolysis time (Ismail et al., 2004; Andarwulan et al., 2010). In our study, flavonoids content were correlated with antioxidant activity in the DPPH. It is known that flavonoids (Wojdyło et al., 2007) have the strongest radical-scavenging power among all natural phenolic compounds. Moreover, it is a potent antioxidant against lipid peroxidation in mitochondrion and microsome (Wang et al., 2010); therefore, absolute ethanol, as a polar solvent, was the better extraction solvent for antioxidant capacity and TFC in this study.

Moore and Adler (2001) reported that apolar solvents are among the most employed solvents for removing polyphenols from water (Moure et al., 2001; Anjaneyulu and Chopra 2004). Several studies have reported on the relationships between phenolic content and antioxidant

Table 1. Total antioxidant capacity, total flavonoids and total polyphenolic contents of aqueous, ethanol and n-hexane extracts of *Vitex doniana* leaves.

Extracts	TAC (mg/ml TE)	TFC (mg/ml QE)	TPC (mg/ml GAE)
Aqueous	66±1.12	276±4.69	460±2.24
Ethanol	69±1.23	304±4.14	380±1.97
Hexane	50±2.11	83. ±3.45	202±1.11

Data are mean SD of triplicate determinations.

Table 2. Total antioxidant capacity, total flavonoids and total polyphenolic contents of different fractions obtained from ethanol extract of *Vitex doniana* leaves.

Fraction	Solvent combination	Ratio	TAC (mg/ml TE)	TFC (mg/ml QE)	TPC (mg/ml GAE)
1	Chloroform : ethyl acetate	10:00	165	390	680
2	Chloroform : ethyl acetate	08:02	64	200	300
3	Chloroform : ethyl acetate	06:04	53	87	75
4	Chloroform : ethyl acetate	04:06	66	265	430
5	Chloroform : ethyl acetate	02:08	71	140	180
6	Chloroform : ethyl acetate	00:10	72	287	475
7	Ethyl acetate : methanol	08:02	71	105	320
8	Ethyl acetate : methanol	06:04	71	100	100
9	Ethyl acetate : methanol	04:06	74	140	180
10	Ethyl acetate : methanol	02:08	73	107	115
11	Ethyl acetate : methanol	00:10	76	107	115
12	Methanol : H_{2O}	05:05	76	212	325
13	Methanol : H_2O	00:10	76	240	380

Data are mean of triplicate determinations.

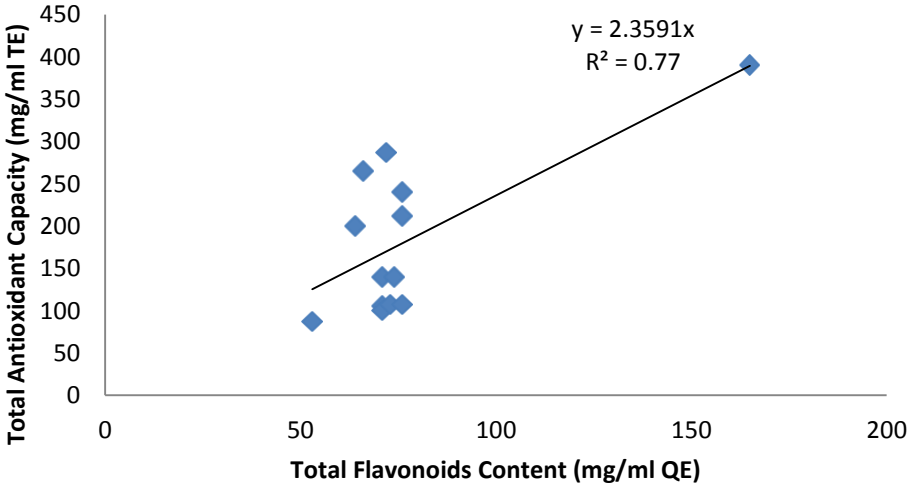

Figure 1. Linear correlation between total antioxidant capacity and total flavonoids content of fractions obtained from ethanol extract of *Vitex doniana* leaves.

activity (Ismail et al., 2004). Velioglu et al. (1998) reported a strong relationship between total phenolic content and antioxidant activity in selected fruits and vegetables.

In our study, there was strong relationship (R^2 = 0.77) between antioxidant activity and total flavonoid contents and (R^2 = 0.6517) for total phenolic content of the fractions. It could be deduced however that the antioxidant capacity of the fractions is majorly dependent on its flavonoids content although there is a wide grade of variation between different phenolic compounds in their effec-

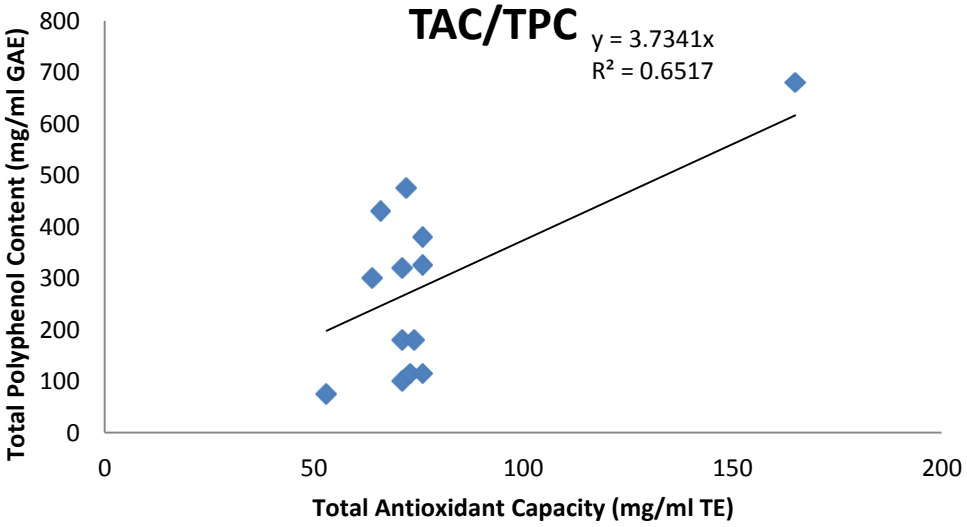

Figure 2. Linear correlation between total antioxidant capacity and total polyphenolic content of fractions obtained from ethanol extract of *Vitex doniana* leaves.

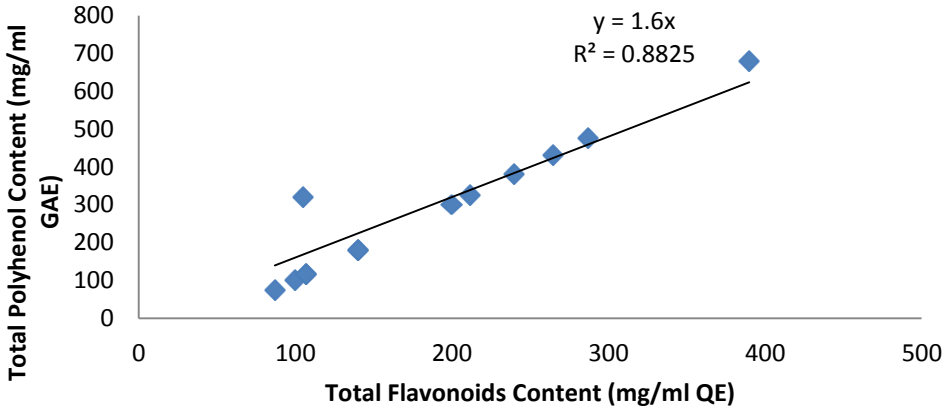

Figure 3. Linear correlation between total flavonoids content and total polyphenolic content of fractions obtained from ethanol extract of *Vitex doniana* leaves.

tiveness as antioxidant (Robards et al., 1999; Bjelakovic et al., 2007). Hence, concentration and pH can also play role in the antioxidant activity of phenolics (Bouayed et al., 2011). In addition, the chemical structure of phenolics play a role in the free radical scavenging activity, mainly depending on the number and position of hydrogen donating hydroxyl groups on the aromatic rings of the phenolic molecules (Bouayed et al., 2011).

The temperature during drying and extraction, affects the compound stability due to chemical and enzymatic degradation, casualties by volatilization or thermal analysis, these latter have been suggested to be the main mechanism causing the reduction in polyphenol content (Moure et al., 2001). Also, for synthetic antioxidants, evaporation and analysis were the main mechanisms for the loss of activity. Of course, the temperature during extraction can affect the extractable compounds differently: boiling and static increased the total phenol content on the other hand, proanthocyanidin content decreased. The antioxidant activity depends on the extract concentration. The results of the present study showed that *V. doniana* leaves are rich in flavonoids and phenolic constituents and demonstrated good antioxidant activity. This plant, rich in flavonoids and phenolic acids could be a good source of natural antioxidant.

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