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Phytochemical Constituents and *In vitro* Antioxidant Activity of Methanolic Extract of *Tetracarpidium conophorum* (African walnut) Seeds

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ABSTRACT: This study evaluated the total phenolics, proanthocyanidins, and flavonoids contents as well as the antioxidant activity of the methanolic extract of *Tetracarpidium conophorum* seeds. The extract of *T. conophorum* was evaluated for its phytochemical constituents such as alkaloids, tannins, flavonoids and steroidal glycosides and its *in vitro* antioxidant activities using 1, 1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging assay and reducing power assay. The total phenolic, proanthocyanidin, flavonol and flavonoid contents were determined using standard methods. The methanolic extract of *T.conophorum* has 50% inhibitory concentration (IC_{50}) value for radical scavenging activity of 0.41mg/mL which was significantly higher than that of vitamin E (0.13mg/mL). The extract has a significantly higher concentration of total phenols, proanthocyanidin and flavonoids compared to their various standards respectively. *T.conophorum* has the potential for use as natural antioxidants against free radical damage.

KEYWORDS: *Tetracarpidium conophorum*, antioxidant capacity, phenolics, flavonoids

1. Introduction

The generation of reactive oxygen species and free radicals in humans has recently been suggested to contribute to a wide range of pathological diseases such as atherosclerosis, renal failure, inflammation, cancer, diabetes and arthritis (Haraguchi *et al*., 1997; Shindo *et al*., 2004). The generation of reactive oxygen species is normally catalysed by transition metals such as Fe^{2+} and Cu^{+} and subsequently leads to lipid peroxidation and ultimately cell death (Smith, 1985). The role of antioxidant may therefore include decreasing the concentrations of reactive oxygen species, interception of singlet oxygen, prevention of the initiation of lipid peroxidation by scavenging primary radicals, binding metal ion catalyst, decomposing primary products to non-radical compounds and chain breaking to prevent continued hygrogen abstraction from lipid substrates (Saha *et el*., 2004).

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Natural products such as polyphenols and flavonoids have been reported to exhibit antioxidant activity (Bork *et al*., 1997; Haraguchi *et al*., 1997; Jodynis-Liebert *et al*., 2000). These products are naturally obtained from food and medicinal plants.

Tetracarpidium conophorum (Mull. Arg) Hutch & Dalziel Syn. (Euphorbiaceae) is one of the prominent plant species used for both medicinal and culinary purposes in Africa. It is a climber found in the wet part of Southern Nigeria and West Africa. It is usually a large tree with the greenish fruits containing four round seeds each. The seed testa is hard whereas the cotyledons are white in colour (Burkill, 1984). The seeds are edible, and can be eaten raw, and or cooked. This plant possesses multiple medicinal properties such as antifertility, antimicrobial and antioxidant (Ajaiyeoba and Fadare, 2006), anticancer (Herbert *et al*., 1998) and antidiabetic (Kaneto *et al*., 1999). As an attempt to further evaluate the ethnomedicinal potential of *T. conophorum*, the methanolic extract of the seeds was screened for its phytochemicals, polyphenolic contents as well as antioxidant activity. This was done with

the aim of further providing information on one of the mechanism among others that confer medicinal properties on *T. conophorum*.

2. Materials and methods

2.1 Plant materials

The seeds of *T. conophorum* used in this study were collected from a private farmland in Edo state, Nigeria. The fresh walnut seeds were identified by a Botanist in the Department of Plant Biology and Biotechnology of the University of Benin, Benin City, Nigeria.

2.2 Preparation of crude extract

The seeds were rinsed properly, de-shelled, cut into pieces, sundried in the open air until fully dried. The dried samples were then pulverized into powder and stored in an air-tight container. *T. conophorum* seed powder (200 g) was extracted in 2000 mL of absolute methanol at room temperature for 48 hours. The samples were filtered with Whatman No. 50 filter paper and the filtrate evaporated to dryness by a rotary evaporator (RE 300, Bibby Scientific, UK) to give 22.86 g corresponding to a percentage yield of 11.43%. The resultant yield was stored in an air-tight container and kept in the refrigerator maintained at 4^{o}C.

2.3 Chemicals and reagents

Methanol, chloroform, 1,1-Diphenyl-2-picrylhydrazine (DPPH), gallic acid, Folin-Ciocalteu phenol reagent and potassium acetate were products of Sigma-Aldrich Chemical Company Ltd (St Louis, U.S.A) while L-ascorbic acid, vitamin E and aluminium chloride hydrate were from BDH Laboratory, England.

2.4 Phytochemical screening
2.1.1 Qualitative determination

Flavonoids, tannins, alkaloids and steroidal glycosides in the methanolic extract of *T. conophorum* were screened qualitatively using the procedures described by Trease and Evans (1983).

2.4.2 Quantitative determination
2.4.2.1 Alkaloids

The alkaloid content of the extract was determined by the procedure described by Harborne (1973). Briefly, the sample (5g) was weighed into a 250 ml beaker, and 200 ml of 10% acetic acid in ethanol was added and left undisturbed for 4 hours. This was filtered using Whatmann No.42 filter paper, concentrated in water bath to ¼(50 ml) of the original volume. Thereafter, concentrated NH_4OH was added drop wise to the extract until the precipitate was complete. The suspension was allowed to settle, washed with NH_4OH and then filtered. The residue was dried and weighed. The concentration of the alkaloid was calculated according to the expression:

$$\%\ Alkaloid = \frac{weight\ of\ residue}{weight\ of\ sample} x \frac{100}{1}$$

2.4.2.2 Tannins

The sample (5g) was shaken constantly for 1 min with 3 ml of methanol in a test tube and then poured into a Buchner funnel with the suction already turned on. The tube was quickly rinsed with an another 3 ml of methanol and the content poured at once into the funnel. The filtrate was mixed with 50 ml of water and analyzed within an hour. About 3 ml of 0.1 ml $FeCl_3$ in 0.1 NH_4Cl was added to 5 ml of the extract and followed immediately by timed addition of 3 ml of 0.008 ml $K_2Fe\ (CN)_6$. Tannic acid was used as standard and its absorbance was read at 720 nm (Onwuka, 2005).

2.4.2.3. Steroidal glycosides

The glycosides content of the extract was determined by dissolving 0.5 g of the extract in 100 ml of 50% H_2SO_4 in test tubes. The mixture was heated in boiling water for 15 minutes, 10 ml of Fehling solution added, and the resultant mixture boiled. A red precipitate in the extract indicates the presence of steroidal glycosides (Harborne, 1973). The percentage glycoside was calculated.

2.4.2.4 Total phenol

Total phenolic (TP) content was determined according to the Folin and Ciocalteau's reagent using gallic acid as standard (Folin and Ciocalteau, 1927). Concentrations of 0.2, 0.4, 0.6, 0.8, and 1 mg/mL of gallic acid were prepared in methanol. Concentrations of 0.1 and 1mg/ml extract of *T. conophorum* were also prepared in methanol. About 0.5 ml of the sample was mixed with 2.5 ml of a ten-fold diluted Folin- Ciocalteau reagent and 2 mL of 7.5% sodium carbonate. The mixture was left undisturbed for 30 minutes at room temperature before the absorbance was read at 760 nm. All the determinations were performed in triplicates. The total phenolic content in the methanolic extract in gallic acid equivalents (GAE) was calculated using the following expression:

$$C = c X \frac{V}{m}$$

where: C is total content of phenolic compounds, mg/g plant extract, in GAE; c is the concentration of gallic acid as obtained from the calibration curve, mg/mL; V is the volume of extract; m is the weight of *T. conophorum* methanolic extract.

2.2.5 Total flavonoids

Total flavonoid content was determined using the method of Miliauskas *et al.* (2004). To 2 mL of the sample was added 2 mL of 2% $AlCl_3$ in ethanol. The absorbance was read at 420 nm after 1 hour at room temperature. Concentration of 1 mg/mL of the extract in methanol was used, while rutin concentrations of 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL were used to obtain the calibration curve. The solutions were prepared in methanol. The total flavonoid content of the extract in rutin equivalents (RE) was calculated from the following formula:

$X = (A.m_o \times 10)/(Ao \times M)$,

where X is flavonoid content, mg/g methanolic extract of *T. conophorum* seed in RE; A is the absorption of the extract solution; Ao is the absorption of standard rutin solution; m-the weight (g) of the seed extract; m_o-the weight (g) of rutin in the solution.

2.2.6 Total flavonol

The total flavonol content of the extract was determined by the method of Yermakov *et al* (1987). The rutin calibration curve was prepared by mixing 2 ml of 0.2, 0.4, 0.6, 0.8, 1.0 mg/mL rutin methanolic solutions with 2 ml (20 g/L) aluminium trichloride and 6 ml (50 g/L) sodium acetate. The absorbance at 440 nm was read after 2.5 hour at 20^o C. The same procedure was repeated with 2 ml of the sample (50 g/L) instead of rutin solution. All determinations were carried out in triplicates. The total flavonol content, in rutin equivalent (RE) was calculated by the following formula:

$$X = C X \frac{V}{m},$$

where; X is flavonol content, mg/g seed extract in RE; C is the concentration (mg/ml) of rutin solution, obtained from the calibration curve; v and m are the volume (ml) and the weight (g) of the seed extract.

2.2.7 Proanthocyanidin

The determination of proanthocyanidin was based on the procedure described by Sun *et al* (1998). A volume of 0.5 ml of 1.0 mg/mL of the extract preparation was mixed with 1.5 mL of 4 % methanol solution and 0.75 mL concentrated hydrochloric acid. The mixture was left undisturbed for 15 minutes after which the absorbance was read at 500 nm. The extract was evaluated at a final concentration of 1 mg/mL. The absorbance of catechin was read under the same conditions. Standard catechin solutions were prepared from 0.05 g catechin. Total proanthocyanidin contents (mg/g) were expressed as catechin equivalents (CE) using the following equation based on the calibration curve: $y= 0.541x$, $R^2 = 0.979$.

2.3 Determination of antioxidant activity

2.3.1 DPPH's Radical Scavenging Activity

The radical scavenging activity of the extract against 1, 1-diphenyl-1-picryl-hydrazyl radical

was determined by reading the absorbance at 517 nm. Radical scavenging activity was done by a slightly modified method of Brand-Williams *et al* (1995). The following concentrations of extract were prepared: 0.2, 0.4, 0.6, 0.8, and 1 mg/mL. α-Tocopherol was used as standard, and the same concentrations were prepared as the test solution. All the solutions were prepared with methanol. 2 mL each of the prepared concentrations were placed into test tubes, and 0.5 mL of 1mM DPPH solution in methanol was added. The experiments were carried out in triplicates. The test tubes were incubated for 15 minutes at room temperature, and the absorbance read at 517 nm. A blank solution containing the same amount of methanol and DPPH was prepared and the absorbance read. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The radical scavenging activity was calculated using the following formula:

DPPH radical scavenging activity (%) = $[(A_0 - A_1)/ (A_0)] \times 100$,

where A_0 was the absorbance of DPPH radical + methanol; A_1 was the absorbance of DPPH radical + sample extract or standard. The 50% inhibitory concentration value (IC_{50}) is indicated as the effective concentration of the sample that is required to scavenge 50% of the DPPH free radical.

2.3.2 Total reducing power

The reducing power of the extract was determined using the ferricyanide trichloroacetic acid as described by Lai *et al* (2001). The extract (1mg/mL) was mixed with phosphate buffer (2.5 mL, 2 M, pH 6.6), 1% potassium ferricyanide (2.5 mL) and incubated at 50^{o}C for 20 minutes. Trichloroacetic acid (10%, 2.5 mL) was added and the mixture was centrifuged at 1000 *g* for 10 minutes. The supernatant was mixed with distilled water (2.5 mL) and 0.1% ferric chloride (0.5 mL) after which the absorbance and that of ascorbic acid was read at 700 nm against the blank. Standard ascorbic acid solutions were prepared from 0.05 g ascorbic acid.

2.4 Statistical analysis

The data were expressed as mean ± SEM of three replicates. The data were subjected to one-way analysis of variance (ANOVA), and differences between means were determined by Duncan's multiple range test using the Statistical Analysis System (SPSS Statistics 17.0) where applicable. P values ≤ 0.05 were regarded as significant

3. Results

The results of the qualitative analysis of the phytochemicals of methanolic extract of *T. conophorum* seed are presented in Table 1. The screening revealed the presence of alkaloids, tannins and flavonoids whereas steroidal glycosides were not detected (Table 1). The results of the quantitative analysis of the phytochemicals in the methanolic extract of *T. conophorum* seed revealed that the extract has a reasonable amount of alkaloid while the polyphenolic compounds are present in the order: proanthocyanidins > total phenol > total flavonol > total flavonoids (Table 1). The IC50 value for the methanolic extract of *T. conophorum* seeds (0.41 ± 0.30) was significantly higher than that of the reference drug, vitamin E (0.13 ± 0.00). The DPPH radical scavenging activity of the extract was comparable with the reference drug, vitamin E and was also concentration and time dependent (Figure 1). The reducing activity of the extract also compared well with that of the reference vitamin C (Figure 2).

4. Discussion

The phytochemical screening and quantitative estimation of the percentage crude yield of phytochemical constituents of the methanolic extract of the seeds of *Tetracarpidium conophorum* showed that the seeds are rich in alkaloids, flavonoids and tannins. Flavonoids are the most common group of polyphenolic compounds in the human diet and are found ubiquitously in plants where they exhibit antioxidant activity. They modify the body's reactions to allergens, viruses, and carcinogens. They show anti-allergic, anti-inflammatory, anti-microbial and anti-cancer

activities (Balch and Balch, 2000). The presence of flavonoid in the methanolic extract of *T. conophorum* seed confers it with antioxidant actvity, and has been suggested to be responsible for many of the pharmacological effects of the seed extract. Alkaloids are organic compounds that contain nitrogen, and are physiologically active with sedative and analgesic properties. They are used in relieving pains, anxiety and depression (Jisika *et al.,* 1992). Alkaloids are toxic due to their stimulatory effects, leading to excitation of cells and neurological dysfunction (Obochi, 2006). The presence of tannins in the seed of *T. conophorum* can support its strong

Table 1: Some phytochemicals in the methanolic extract of *T. conophorum* seeds

Phytochemical	**Concentration**
Alkaloids (%)	31.00± 0.21
Tannins (%)	0.06± 0.01
Flavonoids	
-Total Phenols (mg GAE/g)	0.65± 0.00
-Total Flavonoids (mg RE/g)	0.19± 0.00
-Total Flavonols (mg RE/g)	0.29± 0.00
-Proanthocyanindins (mg CE/g)	0.87± 0.00
Steroidal Glycoside	Not detected

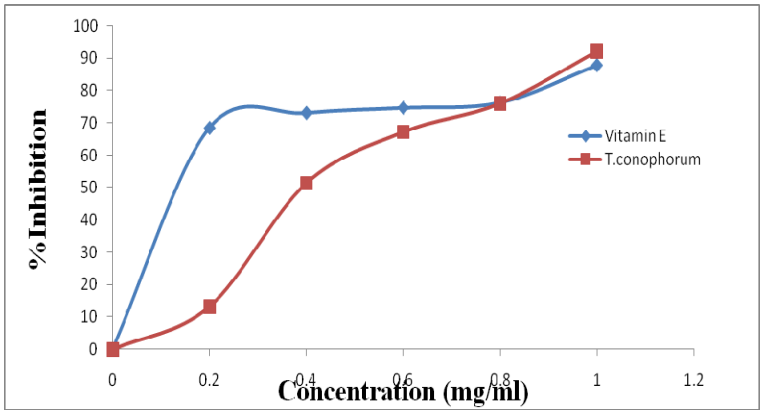

Figure 1: DPPH radical scavenging activity of methanolic extract of *T. conophorum* seeds

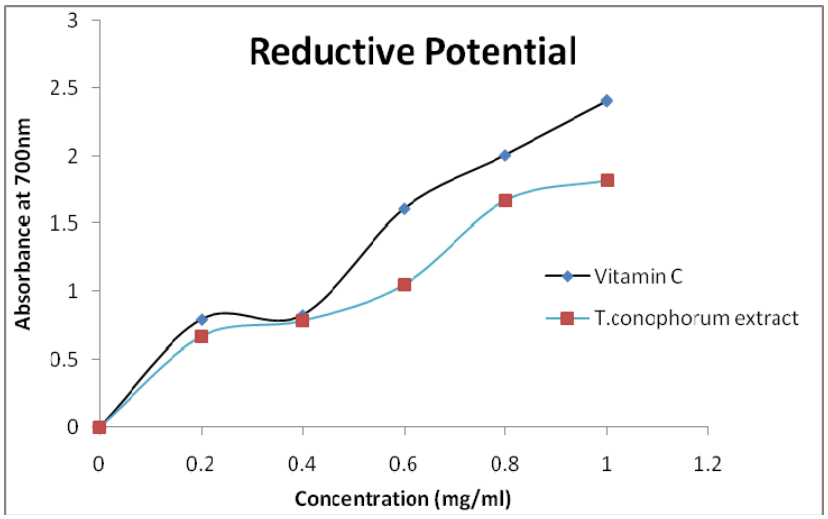

Figure 2: Reducing power of methanolic extract of *T. conophorum* seeds

use for the healing of haemorrhoids, frost bite and varicose ulcers in herbal medicine (Igboko, 1983). The interest in searching for natural antioxidants has increased considerably over the last years. Polyphenolic compounds such as proanthocynaidins have been reported to posses strong antioxidant effects and thus responsible for reducing the risk of diseases associated with oxidative stress (Meyer *et al.,* 1998; Lu and Foo, 2000; Sanchez-Moreno *et al*., 2000). Therefore, the presence of these substances in the methanolic extract of the seed of *T. conophorum* may be responsible for the *in vitro* antioxidant activity of the extract.

The result of the DPPH radical scavenging assay showed that *Tetracarpidium conophorum* extract had appreciable DPPH radical scavenging effect at all concentrations when compared to vitamin E, the standard. The radical scavenging activity of the sample exhibited a concentration and time dependent reaction trend. Increasing the concentration of the assay sample increased the radical scavenging effect. As reported in this study, the higher 50% inhibitory concentration (IC_{50}) in the extract was higher than the IC_{50} of vitamin E. These results are similar to the finding of Amaeze *et al* (2011) who also reported that walnut leaves exhibited potent DPPH scavenging capacities.

The reducing potential of the extract compared to vitamin C, the standard was manifested through their reducing power as shown in Figure 2. In this assay, the Fe^{3+} to Fe^{2+} transformation was established as reducing potential. Vitamin C exhibited a superior reducing power at all concentrations compared to the methanolic extract. The reducing capacity of the sample, increased with increasing concentration of the sample which implied that the samples are electron donors.

The result of the study demonstrated that methanolic extract of *T. conophorum* seed exhibited appreciable antioxidant activities as manifested through their reducing potential, free radical scavenging activities, high amount of polyphenols and bioflavonoids indicates that the plant is a source of natural antioxidants or nutraceuticals with potential application to reduce oxidative stress and consequent health benefits.

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