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Phytochemical and *in-vitro* antioxidant properties of ethyl acetate leave extract of *Dryopteris dilatata* on Wistar rats

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Medicinal plants contain natural bio-active constituents in various parts called photochemical that are of therapeutic importance. This research was undertaken to determine the phytochemical constituents and *in-vitro* antioxidant activities of ethyl acetate extract of *Dryopteris dilatata* leaves (DDL). Phytochemical analysis was determined on the plant samples following established protocols. The antioxidant property of *Dryopteris dilatata* leaves was evaluated using ferric reducing antioxidant power (FRAP), superoxide scavenging radical activity (SSRA) and hydroxyl radical activity (OH) assay. The results of the phytochemical analysis revealed the presence of the following phenols, terpenoid, alkaloids, flavonoids, tannins, cardiac glycosides and saponins, while steroids, carbohydrates, proteins, amino acids and phlobotannins were below detectable levels. DDL leaves produced significant (P<0.05) levels of ferric reducing antioxidant power (FRAP), superoxide scavenging radical activity (SSRA) and hydroxyl radical activity (OH) in a concentration dependent manner compared to the reference antioxidants, ascorbic acid and manitol; therefore, the extract could serve as free radical scavenger, acting as primary antioxidants. Based on the phytoconstituents and antioxidant activities, it could be concluded that *D. dilatata* could be of great value in the management of hyperglycemia, hyperlipidemia, and cancer, among other diseases that could be caused as a result of oxidative stress. This calls for further exploration of its bioactive compound.

Key words: Phytochemicals, *Dryopteris dilatata*, *in-vitro* antioxidant and, oxidative stress.

INTRODUCTION

Since ancient time, herbal remedies have been a natural source of medicines that have been implicated traditionally in the prevention and treatment of a wide range of human diseases in many parts of the world,

where cost and several undesired side effects has limited the prospect of some conventional medicines (Priyanga et al., 2014). These medicinal plants contain natural bioactive constituents in their various parts known as the phytochemical which include carbohydrates, phenols, flavonoids, steroids, tapenoids, alkaloids, tannins compounds. There are endogenous metabolites that can contribute to pharmacological properties of plants (Sowmya et al., 2015). These metabolites are found in abundant quantity in plants. Several of these plants are still used by various over 80% ethnic groups worldwide for the management of several diseases such as dysentery, asthmatic attacks, malaria ,skin diseases (Manandhar, 1998), whose effectiveness have been shown to be relatively nontoxic, free from underlying side effects and safe (Iniaghe et al., 2008). Medicinal plants with numerous active ingredients have bioactive and chemical entities with various pharmacological activities such as anticancer, antibacterial, analgesic, anti-inflammatory, antitumor, antiviral and other activities

Free radicals are produced naturally in cells during stress or respiration and from external sources such as smoking, radiation, alcohol, viral toxins and bacteria. Protection of the living systems against deleterious effect caused by oxidative stress is done by antioxidant defense mechanism (Jayachitra and Krithiga, 2010). Imbalance between reactive species generation, other free radicals and the antioxidant activity of living systems, results in oxidative stress. Excessive oxidative stress results in the loss of the functions of the cells producing a lot of structural damages such as lipid, proteins and DNA (Hiransai et al., 2016), resulting in the pathogenesis of several chronic and metabolic diseases such as cardiovascular diseases, cancer, aging, diabetes and metabolic syndrome (Maehre et al., 2015). Adverse effects such as carcinogenicity leading to restrictions in the use of synthetic antioxidants has increased the search for foods and plants with naturally occurring antioxidants potentials with the capability to eradicate or neutralize the harmful effect of free radicals in cells and tissues (Patel et al., 2010). This has revealed the importance of medicinal plants to prevent and control chronic diseases associated with oxidative stress through their antioxidant potentials.

Dryopteris dilatata (Broad buckler fern) is a medicinal plant that is found within the *Dryopteridaceae* family and can grow to about 120 cm and 90 cm in height and width respectively. This plant has dark green tripinnate fronds, with the ribs covered in brown scales (Rünk et al., 2012). *D. dilata* is known as Okpomie by the Olomoro ethnic group in Isoko South Local Government Area, Delta State, Nigeria. The plant is distributed throughout tropical region of Nigeria and people from Olomoro ethnic region in Isoko South Local Government Area of Delta State make concoctions of this plant when diabetic and their conditions usually get ameriolated using the plant. It has also been documented by Brown et al., (2011) that the leaves and roots are used for medicinal purposes such as management and treatment of dandruff and worm expeller According to Ajiriogehne et al., (2018), *D. dilatata* leaves contain phytochemicals such as phenols, tannins, flavonoids, saponins, glycosides, steroids, terpenoids, and alkaloids. Hence this study was undertaken to determine the phytochemicals and *in-vitro* antioxidant properties of ethyl acetate extract of *D. dilatata* leaves on Wistar rats.

MATERIALS AND METHODS

Equipment/Instruments

Spectrophotometer 20D, Laboratory Incubator DNP-9082, Centrifuge 80-2. Electric water baths (model DK 420) were products of Techmel and Techmel USA and Electronic weighing balance by Metlar, China. Refrigerator was by Haier Thermocool, Micropipettes (REMI) (100-1000 µl), porcelain mortar and pestle, stop watch, dissecting set, measuring cylinders, beakers, spatula, syringe, filter paper, micropipette, and test-tubes

Chemicals/reagents and manufacturers

Disodium hydrogen phosphate, Sodium dihydrogen phosphate, Adrenalin by Loba Chemie Mumbai, India. Hydrogen chloride, Sodium carbonate, Trichloroacetic acid Thiobarbituric acid, Sodium hydroxide pellet, Sodium chloride. Sodium chloride Alanine aminotransferase Assay kits, Aspartate aminotransferase Assay kits. Alkaline phosphatase Assay kits, Triglyceride Assay kits, Total Cholesterol Assay kits, High Density Lipoprotein Assay kits , Urea kits, Randox laboratory Ltd, UK .Creatinine kits. Randox Laboratory Ltd, UK with cover, rotary evaporator, oven, beaker (20 ml, 50 ml and 500 ml).

Plant collection, identification and authentication

D. dilatata leaves were harvested from a wide growing habitat at Olomoro Community in Isoko South Local Government Area of Delta State, Nigeria. The samples were identified and authenticated at Forestry Research Institute of Nigeria, Ibadan, Oyo State and was assigned a herbarium number: FHI 1100338.

Phytochemical analysis

Qualitative analysis of the phytochemicals

The extract of *D. dilatata* was subjected to different phytochemical analysis using standard methods of Harborne (1973) to investigate

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the availability of bioactive substances as follows.

Test for proteins: This was done using Millon's test as follows: 2 ml of Million's reagent was mixed with one gram of the crude extract and the presence of white precipitate which turned red upon gentle heating confirmed the presence of protein.

Test for carbohydrates: This was also tested using Iodine test procedure as stated: 2 ml of iodine solution was mixed with One gram of crude extract of *D. dilatata* leaves and the presence of a dark blue or purple coloration indicated the presence of the carbohydrate.

Test for phenols and tannins: 2 ml of 2% solution of $FeCl_3$ was mixed with one gram of crude extract of *D. dilatata* leaves and a blue-green coloration indicated the presence of phenols and tannins.

Test for flavonoids: This was an alkaline reagent test which was performed as stated: 2 ml of 2% solution of NaOH was mixed with one gram of crude extract of *D. dilatata* leaves and the formation of an intense yellow color which later turned colorless on addition of few drops of diluted acid indicated the presence of flavonoids.

Test for phytosterol: A solution of alcoholic potassium hydroxide was refluxed with one gram of *D. dilatata* leaves extract till complete saponification occurred. The mixture was extracted after dilution with a solution of ether. The ether layer was evaporated and the residue was tested for the presence of phytosterol, The residue was dissolved in few drops of diluted acetic acid. Later, 3 ml of acetic anhydride was added followed by few drops of concentrated H_2SO_4. Appearance of bluish green coloration indicated the presence of phytosterol.

Test for triterpenoids: Ten milligram (10 mg) of the *D. dilatata* leave extract was dissolved in 1 ml of chloroform, This was followed by the addition of 1 ml of acetic anhydride followed by 2 ml of concentrated H_2SO_4. The formation of reddish violet color indicated the presence of triterpenoids.

Test for phlobatannins: 2 ml of 1% HCl was mixed with one gram of the powdered *D. dilatata* leave and the mixture was boiled. The formation of a red precipitate was an indication for the presence of phlobatannins.

Test for saponins: 5 ml of distilled water was mixed with one gram of the crude extract of *D. dilatata* leaves in a test tube, and this was shaken vigorously. The formation of stable foam showed the presence of saponins.

Test for glycosides: Keller-Kilani test for glycosides was performed as stated: 2 ml of glacial acetic acid containing 1-2 drops of 2% solution of $FeCl_3$. was mixed with one gram of the crude extract of *D. dilatata* and the mixture was poured into another test tube containing 2 ml of concentrated H_2SO_4. The appearance of a brown ring at the interphase indicated the presence of cardiac glycosides.

Test for steroid: 2 ml of chloroform concentrated with H_2SO_4 was mixed with one gram of the crude extract of *D. dilatata*. A red coloration that formed in the lower chloroform layer indicated the presence of steroids. Another test was performed by mixing the crude extract with 2 ml of chloroform. Then, 2 ml of each of concentrated H_2SO_4 and acetic acid was poured into the mixture. The development of a greenish coloration indicated the presence of steroids.

Test for terpenoids: 2 ml of chloroform was mixed with one gram of the crude extract of *D. dilatata* leaves and was evaporated to dryness. Then, 2 ml of concentrated H_2SO_4 was added and heated for 2 min and a greyish coloration indicated the presence of terpenoid.

Test for alkaloids: 2 ml of 1% HCl was mixed with one gram of the crude extract of *D. dilatata* leaves and heated gently, after which Mayers and Wagner's reagents were added to the mixture. Turbidity of the resulting precipitate indicated the presence of alkaloids (Harborne, 1973).

In vitro antioxidant activities of *D. dilatata* leaves

Assay hydroxyl radical scavenging assay

The assay was performed using standard methods (Salah et al., 1995). The fundamental principle that governs the assay deals with the 2-deoxyribose degradation product quantification by condensation with TBA. Furthermore, the Fe^{3+}-ascorbate-EDTA-H_2O_2 system, also known as the Fenton reaction has helped to generate the hydroxyl radical.

The reaction mixture is contained in a final volume of 1 ml, 2-deoxy-2-ribose (2.8 mM); KH_2PO_4-KOH buffer (20 mM, pH 7.4); $FeCl_3$ (100 µM); EDTA (100 µM); H_2O_2 (1.0 mM); ascorbic acid (100 µM) and various concentrations (0–200 µg/ml) of the plant extract. The reaction mixture remained incubated at 37°C for 1 h; post incubation, 0.5 ml of the reaction mixture was mixed with 1 ml 2.8% TCA in addition to 1 ml of 1% aqueous TBA. The solution was subjected to incubation for 15 min at 90°C to produce coloration. Thereafter, the solution was allowed to cool and the absorbance was determined at 532 nm against a suitable blank solution. All tests were conducted six times. As a classical •OH scavenger, Mannitol served as a positive control. Evaluation of inhibition percent was done using the ensuing equation:

$$\% \text{ of inhibition} = [(A_0 - A_1) / A_0] \times 100$$

Where A_0 = control absorbance and A_1 = absorbance in the presence of the samples and standard.

Superoxide radical scavenging assay

Based on NBT reduction, this experiment was conducted in line with a previously reported technique (Salah et al., 1995). After the non-enzymatic PMS/NADH system produces superoxide radicals, nitro blue tetrazolium are then reduced into a purple-colored formazan by these radicals. The 1 ml reaction mixture contained phosphate buffer (20 mM, pH 7.4), NADH (73 µM), NBT (50 µM), PMS (15 µM) and different sample solution concentrations (0–50 µg/ml). The reaction mixture was subjected to incubation for 5 min at room temperature; thereafter, the absorbance was taken at 562 nm against an appropriate blank solution. All tests were conducted six times with quercetin used as positive control. Further, percent inhibition of superoxide anion generation was estimated with the aid of the ensuing formula:

$$\% \text{ of inhibition} = [(A_0 - A_1) / A_0] \times 100$$

Where A_0 = Absorbance of the control; A_1 = Absorbance in the presence of the samples and standard.

Measurement of ferric reducing power

This was done according the method of Oyaizu with slight medication to determine the ferric reducing power of ethyl acetate extract of *D. dilatata* leaf. Various concentrations (0–1.0 mg/ml) of

Table 1. Qualitative phytochemical screening.

Phytochemicals	Ethyl acetate extract mg/100 ml
Phenols	++
Steroids	-
Tarpenoids	+
Carbohydrates	-
Proteins	-
Amino acids	-
Alkaloids	+++
Phlobotannins	-
Cardiac glycosides	+
Flavonoids	++
Tannins	+++

-Absent, + trace, ++ moderately present, +++abundantly present.

Table 2. Result of the ferric reducing antioxidant power.

Concentration (ug/ml)	Ethyl acetate	Ascorbic acid
250	61.18±0.07	70.05±0.07
125	51.35±0.33	60.22±0.33
63.5	45.81±0.58	54.68±0.58
31.5	36.29±0.06	45.16±0.06
15.25	19.52±0.56	28.38±0.55
7.625	2.68±.17	4.55±0.17
3.825	0.09±.07	1.62±0.27

Values are expressed as mean ± SEM. ANOVA followed by Post Hoc (LSD) multiple range tests. Values not sharing a common superscript differ significantly at P<0.05.

extract (0.5 ml) were mixed with 0.5-ml phosphate buffer (pH 6.6) and 0.5 ml 0.1% potassium hexacyanoferrate. The solution was subjected to incubation for 20 min in a water bath at 50°C; thereafter 0.5 ml of trichloroacetic acid (10%) was added to end the reaction. The upper portion of the solution (1 ml) was mixed with 1 ml distilled water plus a further 0.1 ml $FeCl_3$ solution (0.01%). After leaving the reaction mixture at room temperature for 10 min, absorbance will be determined at 700 nm against an appropriate blank solution. All tests were conducted six times. A higher absorbance of the reaction mixture is an indication of greater reducing power. In this approach, butylated hydroxyltoluene (BHT) was used as a positive control.

Statistical analysis

Data collected were presented as Mean ± SEM (standard error of mean). Results were analysed using one-way analysis of variance (ANOVA), followed by post Hoc Fisher's test (LSD) for multiple comparison and $P < 0.05$ were considered statistically significant.

RESULTS

The preliminary phytochemical analysis (Table 1) revealed the presence of phenols, terpenoids, alkaloid, cardiac glycoside, flavonoids, tannins and saponins in ethyl acetate extract of the leaves of *D. dilatata*, and the absence of phlobotannins, amino acids, steroids, carbohydrates and proteins.

Ferric reducing power of ethyl acetate leaf extract of *D. dilatata* showed the ferric reducing capacity of the plant extract. The reducing power of ethyl acetate extract was seen in a concentration-dependent manner at 250 ug/ml where there is maximum value, and decreases as the concentration reduces for ethyl acetate which has significant ferric reducing power compared to the reference compound ascorbic acid (Table 2).

Hydroxyl radical scavenging capacity of ethyl acetate leaf extract of *D. dilatata* leaves was seen in concentration-dependent manner with the maximum inhibition at 125 ug/ml and declined as the concentration reduces compared to the reference antioxidants manitol (Table 3).

Superoxide scavenging radical capacity of ethyl acetate leaf extract of *D. dilatata* was seen in concentration-dependent manner with maximum scavenging activity

Table 3. Result of the hydroxyl radical.

Concentration (ug/ml)	Ethyl acetate	Manitol
250	4.22±0.09	9.20±0.24
125	68.51±0.29	86.92±0.38
62.5	43.05±0.75	68.80±.42
31.25	33.95±0.53	54.26±0.89
15.625	28.91±0.49	38.38±0.29
7.825	20.49±0.07	26.61±0.18
3.906	14.17±0.14	14.97±0.50

Values are expressed as mean ±SEM. ANOVA followed by PostHoc (LSD) multiple range tests. Values not sharing a common superscript differ significantly at P<0.05.

Table 4. Superoxide scavenging radical activity.

Concentration (ug/ml)	Ethyl acetate	Ascorbic acid
250	59.7651±.34073	72.53±0.34
125	45.1510±.55675	67.94±0.56
62.5	38.4635±.82301	61.23±0.82
31.25	25.8133±.51445	48.58±0.51
15.825	14.29301±.09923	37.06±0.09
7.825	9.3670±.14411	19.37±0.14
3.906	6.5899±.10600	6.59±0.11

Values are expressed as mean ± SEM. ANOVA followed by PostHoc (LSD) multiple range tests. Values not sharing a common superscript differ significantly at P<0.05

observed at 250 ug/ml concentration which declined as the concentration reduces compared to the reference antioxidant ascorbic acid (Table 4).

DISCUSSION

Bioactive substance found in plants provides biological systems the ability to defend against degenerative and life threatening disorders (Tripoli et al., 2007). The phytochemical investigation of ethyl acetate extract of *D. dilatata* extract revealed the presence of phenols, terpenoid, alkaloid, cardiac glycoside, flavonoids, tannins and saponins in ethyl acetate extract of the of *D. dilatata* leaves and the absence of phlobotannins, amino acids, steroids, carbohydrates and proteins. Terpenoid and cardiac glycosides were present in trace, phenols, flavonoids and saponins were moderately present while alkaloids and tannins were abundantly present.

The findings of these phytochemicals are similar to the findings of Mordi et al. (2016) and Ajirioghene et al., (2018). Some of these phytochemical constituents are known to produce a wide range of pharmacological activities, as medicinal plants contain different phytoconstituents possessing biological activities with a wide range of therapeutic index (Nagaraj et al., 2016); such bioactive compounds that are found in this study like phenols, terpenoids, alkaloid, cardiac glycoside, flavonoids, tannins and saponins are associated with several biological activity such as antioxidant and lipid lowering capacity (Chimaobi et al., 2019). Ability of the ethyl acetate extract of *D. dilatata* leaf to reduces Fe^{3+} to Fe^{2+} suggest that it donates electron which signifies it reducing power. This shows the antioxidant activity of plant extract as reported in other medicinal plants studies (Sahreen et al., 2010). In the present study, ethyl acetate extracts of *D. dilatata* compared to the standard compound reveals its electron donating capacity, showing its antioxidant potential as a medicinal plant against free radicals (Reenu et al*.,* 2015).

The highly reactivity of hydroxyl radical that is being continuously formed in a process of reduction of oxygen to water which causes lipid peroxidation is evident in its deleterious effect in cells and organs of the body (Dizdaroglu and Jaruga, 2012). Ethyl acetate extracts of *D. dilatata* inhibited the generation of reactive species of hydroxyl radical at different inhibition values in different concentrations as reported in other medicinal plant studies (Treml and ˇSmejkal, 2016). In the present study, the ethyl acetate extract derived from *D. dilatata* leaves

showed a significant superoxide scavenging radical activity. Superoxide radical is one of the strongest reactive oxygen species among the free radicals (Gabriele et al., 2017). It is generated in living systems through incomplete metabolism of oxygen which causes damage of cells components and organs (Pizzino et al., 2017). This harmful effect to cellular components can be prevented by removing superoxide radicals (Ighodaro and Akinloye 2018). The result from the present study indicates that the scavenging activity of superoxide of extract compared to the reference antioxidant increased as the concentration increased which reveals the potency of the extract to scavenge for superoxide radical and reverse its deleterious effect to cells and organs of living systems. This indicates that *D. dilatata* could be efficacious (Lipinsk, 2011), and could be useful in ailments such as chronic pain, inflammation and Crohn's disease, cancer (murine and cell line research models).

Conclusion

The results of this study have shown that the ethyl acetate leaf extract of *D. dilatata* contains considerable amounts of bioactive compounds, with phenols, alkaloids, flavonoids, and tannins abundantly present, which possess high antioxidant and free radicals scavenging activities. The *in vitro* antioxidants activity of the *D. dilatata* leaf showed that the plant contain significant quantity of hydroxyl radical, super oxide and ferric reducing power activity compared to the reference antioxidant compound ascorbic acid and manitol and could be a source of natural antioxidants that ameliorate the induction and progression of oxidative stress.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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