

Antioxidant Activities of the Leaf Extract and Fractions of *Dryopteris filix-mas* (L.) Schott could be Attributed to The Abundance of Polyphenol Compounds

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Abstract

Dryopteris filix mas (*D filix-mas*) is widely used in ethnomedicine for the management of rheumatoid arthritis, wounds and other diseases. We investigated the anti-oxidant activities of its leaf extract, and chromatographic fractions. The ethanol leaf extract was partitioned into four fractions; n-hexane, ethyl acetate, n-butanol and water. Ferric reducing anti-oxidant power (FRAP), 1, 1-diphenyl-2-picrylhydrazil (DPPH) and nitric oxide (NO) scavenging *in vitro* assays were carried out on the extract and fractions at 6.25, 12.5, 25, 50, 100, 200, 400 and 800 µg/mL. The most active fraction (ethyl acetate fraction) was further purified using chromatographic techniques to isolate its major compound whose structure was elucidated using ID nuclear magnetic resonance (NMR) and mass spectrometry. The ethyl acetate fraction produced the highest free radical scavenging activity among the other fractions. The fraction (VLC-E7) from which the bioactive compound, quercetin-3-O- α L-rhamnopyranoside, was isolated had the best FRAP and DPPH scavenging activities with EC₅₀ and IC₅₀ values of 88.81 ± 3.41 and 26.87 ± 0.24 respectively more than the ethyl acetate fraction. This study revealed that the polyphenol flavonoid, quercetin-3-O- α L-rhamnopyranoside could be responsible for antioxidant activity of ethno-medicinal property of *D filix-mas* leaf.

Keywords: *Dryopteris filix-mas*; wounds; rheumatoid arthritis; quercetin-3-O- α L-rhamnopyranoside; anti-oxidant properties

INTRODUCTION

Free radicals are constantly produced in all normal living cells (Kumar *et al.*, 2014). Imbalance between the production of these radicals and endogenous antioxidants creates a state of oxidative stress with its associated damaging effects on endogenous molecules such as lipids, proteins and nucleic acids (Yan *et al.*, 2015). Unbridled oxidative stress has been reported to be implicated in several chronic and degenerative diseases such as diabetes, rheumatoid arthritis, cancer, neurodegenerative diseases, atherosclerosis, ischemic heart disease, ageing among others (Khatoun *et al.*, 2013).

In attempt to ameliorating oxidative damages caused by free radicals, synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and tert-butylhydroquinone (TBHQ) had been developed. However, these synthetic antioxidants left much to be desired due to their side effects, such as liver

toxicity, carcinogenicity as well as their high cost and inaccessibility (Deepa *et al.*, 2014). These limitations have inspired the search for more effective antioxidant from natural source, especially medicinal plants, which are biodegradable, less toxic, affordable and accessible (Ezeja *et al.*, 2015; Ahlem *et al.*, 2015).

D filix-mas is an evergreen fern belonging to the family of Dryopteridaceae. Male fern, worm fern, aspidium and shield fern are common names assigned to it. It grows between 60-150 cm high and it is habitat to stream and moist environments (Uwumarongi *et al.*, 2016). It is native to Europe, Asia, and North America. Its leaves are bipinnated and consist of 20-35 pinnae on each side of the rachis (Fig 1). Its stalks are covered with orange-brown scales (Bafor *et al.*, 2017). The leaf decoction is ethno medicinally used in rheumatoid arthritis, wounds, bleeding disorders, ulcers, worm infestation and malaria (Tagarelli *et al.*, 2010; Erhirhie *et al.*, 2019). It is also reported as one of the ferns with

useful secondary metabolites against chronic diseases and aging (Valentyna *et al.*, 2017).

Previous studies have found that it possesses antihelmintic activity (Urban *et al.*, 2014), antimicrobial activity (Mandal and Mondal, 2014), anti-diarrheal (Uwumarongi *et al.*, 2016), uterine relaxant (Bafor *et al.*, 2017) and anti-inflammatory (Erhirhie *et al.*, 2019) activities. A preliminary study by Sekender *et al.* (2012) revealed that it possesses anti-oxidant activity. In this present study, we evaluated the antioxidant activity of the leaf extract and fractions. The bioactive compound responsible for its antioxidant activity was isolated and characterized.



Figure 1. *D. Filix-mas* photograph.

METHODOLOGY

Materials

Visible Spectrophotometer (721g, Zhejiang, China), Thermostatic water bath (Equitron Mumbai India), Analytical weighing balance (Ohaus Corp. NJ USA), Quercetin (Institute of Pharmaceutical Biology and Biotechnology, Heinerich-Heine University, Dusseldorf, Germany), 2,2-Diphenyl-1-picrylhydrazyl, DPPH (Sigma Aldrich), Ascorbic acid (Sigma- Aldrich).

Procedure

Plant collection, extraction, fractionation and purification of active compound

Plant collection, authentication, extraction, fractionation, chromatographic separation, and structural elucidation techniques were carried out using similar method as

described in our earlier study (Erhirhie *et al.*, 2019). The purification and structural elucidation was based on bioactivity guided antioxidant screening of liquid-liquid fractions (n-hexane, ethyl acetate, butanol, and water fractions), vacuum liquid chromatographic fractions and Sephadex fractions.

DPPH scavenging assay

The test was carried out based on the method described by Ajaghaku *et al* (2017). The reaction mixture contained 0.1 mL of various concentrations (6.25, 12.5, 25, 50, 100, 200, 400 and 800 µg/mL) of sample, 0.1 mL of 0.6 mMol of DPPH and 0.8 mL of methanol. The mixture was incubated in the dark for 30 minutes at room temperature. The absorbance of the sample was measured at 517 nm against blank (methanol) using a spectrophotometer. Ascorbic acid and quercetin were used as standards. A tube containing 0.1 mL of DPPH solution and 0.9 mL of methanol served as control. Experiments were carried out in duplicate. Free radical scavenging activities of each sample were determined as follows:

$$\text{DPPH scavenging activity} = (AC - AS) / AC \times 100$$

AC : Average absorbance of control
AS : Average absorbance of sample

A graph of percentage inhibition against concentration was plotted and the concentration that produced 50% inhibition (IC₅₀) was extrapolated using a regression analyses equation.

Ferric reducing antioxidant power (FRAP) assay

FRAP assay was carried out following the method described by Habibur *et al.*, (2013). Two hundred and fifty microliter (0.25 mL) of various concentrations, 6.25, 12.5, 25, 50, 100, 200, 400 and 800 µg/mL of samples were mixed with 0.625 mL of phosphate buffer and 0.625 mL of 1% potassium ferricyanide [K₃FeCN₆]. The mixtures were heated at 50°C for 20 minutes. Then, 0.625 mL of 10% trichloroacetic acid (TCA) was added and the mixtures were centrifuged at 3000 rpm for 10 minutes. From the upper layer, 0.625 mL was pipetted and mixed with 0.625 mL of distilled water and 0.125 mL of 0.1% (w/v) ferric chloride (FeCl₃) solution. Absorbances of the mixtures were measured at 700 nm against air using a spectrophotometer. Ascorbic acid and quercetin were used as standards. Tests were performed in duplicate and percentage inhibition was calculated using the formula below.

$$\% \text{ Inhibition} = \frac{\text{Average absorbance of sample} - \text{Average absorbance of blank}}{1} \times 100$$

A graph of percentage inhibition against concentration was plotted and the effective concentration (EC₅₀) was extrapolated using a regression analyses equation.

Nitric oxide scavenging activity

This assay was carried out following the method described by Ezeja *et al.*, (2015). Two milliliter of 10 mM sodium nitroprusside prepared with phosphate buffer saline (pH 7.4) was mixed with 0.5 mL of samples at various concentrations (6.25, 12.5, 25, 50, 100, 200, 400 and 800 µg/mL). The mixture was incubated at room temperature for 150 minutes. Thereafter, 0.5 mL of the reaction mixture was withdrawn and mixed with 0.5 mL of Griess reagent (1% sulphanilamide + 0.1% naphthylethylenediamine dichloride + 3% phosphoric acid) was added to each test tube. The absorbance of the pink chromophore formed was measured at 540 nm. Ascorbic acid and quercetin were used as standards. Assays were carried out in duplicates. Percentage inhibition was calculated using the formula below

$$\frac{\text{Abs Control} - \text{Abs Test}}{\text{Abs Control}} \times \frac{100}{1}$$

Inhibitory concentration, IC₅₀ was estimated from graph of % inhibition against concentration using regression analyses equation

Statistical analysis

Results were presented as mean ± standard error of mean (SEM) using Statistical Package for Social Science (SPSS, version 20). Calculation of fifty percent inhibitory concentration (IC₅₀) of the extracts and fractions was carried out using regression equation in Microsoft Excel, 2010.

RESULTS

DPPH, scavenging activity of extract and fractions

In DPPH scavenging assay, 50% inhibitory concentration (IC₅₀) of 134.12 ± 2.88, 94.97 ± 0.14, 63.91 ± 1.29, 62.09 ± 0.21 and 45.72 ± 0.16 µg/mL were recorded in n-hexane, water fractions, extract, butanol and ethyl acetate fractions respectively. The ethyl acetate fraction produced the lowest IC₅₀ value among other samples, except ascorbic acid and quercetin which showed IC₅₀ values of 12.26 ± 0.20 µg/mL and 10.46 ± 0.15 µg/mL respectively (Table 1).

Inhibition against DPPH at 100 µg/mL by VLC fractions

Ethyl acetate VLC fractions, VLC-E1 (78.68%), VLC-E3 (52.33%), VLC-E7 (72.38%), VLC-E6 (73.84%), VLC-E14 (65.60%), VLC-E8 (73.08%), VLC-E10 (74.81%), VLC-E12 (72.09%), VLC-E16 (69.67%) produced more than 50 % inhibition against DPPH (Table 2).

Table 1. Free radical (DPPH) scavenging activities of extract and fractions of *D filix-mas*.

	DPPH, scavenging activity, IC ₅₀ (ug/ml)
Extract	63.91 ± 1.29
n-hexane fraction	134.12 ± 2.88
Ethyl-acetate fraction	45.72 ± 0.16
Butanol fraction	62.09 ± 0.21
Water fraction	94.97 ± 0.14
Ascorbic acid	12.26 ± 0.20
Quercetin	10.46 ± 0.15

Values are expressed as mean ± Standard error of mean.

Table 2. DPPH scavenging activities of ethyl acetate vacuum liquid chromatographic fractions of *D filix-mas*.

Solvent ratio	Sample code	Inhibition against DPPH (%)
Ethyl acetate fraction		73.26
N(500): E (0)	VLC-E1	78.68
N(450): E (50)	VLC-E2	19.38
N(400): E(100)	VLC-E9	18.12
N(350): E(150)	VLC-E13	23.06
N(300):E(200)	VLC-E4	28.00
N(250): E(250)	VLC-E11	36.34
N(200):E(300)	VLC-E3	52.33
N(150):E(350)	VLC-E15	16.86
N(100):E(400)	VLC-E5	12.79
N(50): E(450)	VLC-E17	37.79
N(0):E(500)	VLC-E7	72.38
D(500):M(0)	VLC-E6	73.84
D(450):M(50)	VLC-E14	65.60
D(350):M(150)	VLC-E8	73.06
D(250):M(250)	VLC-E10	74.81
D(100):M(400)	VLC-E12	72.09
D(0):M(500)	VLC-E16	69.67

N = n-hexane, E = ethyl acetate, M = methanol.

Ferric reducing antioxidant power (FRAP), Nitric oxide (NO) and DPPH scavenging activities of extract selected fractions.

FRAP activity

In FRAP assay, quercetin and ascorbic acid produced the lowest EC₅₀ values followed by VLC-E7, ethyl acetate fraction, extract and butanol fraction. In nitric oxide scavenging assay, ascorbic acid exhibited the lowest IC₅₀ value (14.74 ± 0.11 µg/ml) followed by ethyl acetate fractions (15.38 ± 3.65 µg/ml) and butanol fractions (15.45 ± 2.72 µg/ml), followed by VLC-E7 fraction (16.66 ± 0.48 µg/ml), quercetin (18.14 ± 2.57 µg/ml) and extract (1854.60 ± 200.25 µg/ml). In DPPH assay, quercetin and ascorbic acid produced the lowest IC₅₀ values (11.50 ± 0.08 and 14.19 ± 0.43 µg/mL) followed by VLC-E7 (26.87 ± 0.24 µg/mL), ethyl acetate fraction (50.25 ± 0.40 µg/mL), butanol fraction (62.09 ± 0.21 µg/mL) and crude extract (63.91 ± 1.29 µg/mL) (Table 3).

DPPH, scavenging activity of Sephadex fractions

DPPH scavenging activities of samples tested decreased in the following order; Quercetin ($10.46 \pm 0.15 \mu\text{g/mL}$) > Ascorbic acid ($12.26 \pm 0.20 \mu\text{g/mL}$) > SPH-E3 ($26.35 \pm 1.38 \mu\text{g/mL}$) > VLC-E7 ($26.87 \pm 0.24 \mu\text{g/mL}$) > SPH-E6 ($33.44 \pm 0.38 \mu\text{g/mL}$) > SPH-E5 ($37.30 \pm 0.30 \mu\text{g/mL}$) > SPH-E7 ($46.95 \pm 0.61 \mu\text{g/mL}$) > SPH-E4 ($86.10 \pm 7.69 \mu\text{g/mL}$) (Table 4).

Table 3. Ferric reducing antioxidant power (FRAP), Nitric oxide (NO) and DPPH scavenging activities of extract and fractions of *D filix-mas*.

Sample	FRAP, EC ₅₀ ($\mu\text{g/mL}$)	Nitric oxide, IC ₅₀ ($\mu\text{g/mL}$)	DPPH, IC ₅₀ ($\mu\text{g/mL}$)
Extract	1854.60 ± 200.25	38.51 ± 2.23	63.91 ± 1.29
Ethyl acetate fraction	461.75 ± 22.91	15.38 ± 3.65	50.25 ± 0.40
Butanol fraction	1911.03 ± 137.44	15.45 ± 2.72	62.09 ± 0.21
VLC-E7	88.81 ± 3.41	16.66 ± 0.48	26.87 ± 0.24
Ascorbic acid	69.34 ± 2.41	14.74 ± 0.11	14.19 ± 0.43
Quercetin	30.81 ± 0.86	18.14 ± 2.57	11.50 ± 0.08

FRAP: Ferric reducing antioxidant power. Values are expressed as mean \pm Standard error of mean.

Table 4. DPPH scavenging activity, of Sephadex (SPH) fractions of *D filix-mas*.

	DPPH, scavenging activity, IC ₅₀ ($\mu\text{g/mL}$)
VLC-E7	26.87 ± 0.24
SPH-E1	-
SPH-E2	22.10 ± 0.62
SPH-E3	26.35 ± 1.38
SPH-E4	86.10 ± 7.69
SPH-E5	37.30 ± 0.30
SPH-E6	33.44 ± 0.38
SPH-E7	46.95 ± 0.61
Ascorbic acid	12.26 ± 0.20
Quercetin	10.46 ± 0.15

Values are expressed as mean \pm Standard error of mean.

DISCUSSION

It is well established that by-products of oxygen metabolism produce free radicals such as reactive oxygen and nitrogen species which results to cellular damage and pathogenesis of several diseases such as cancer, cardiovascular diseases, diabetes, neurodegenerative diseases among others (Li *et al.*, 2016).

There is a revitalization of interest in the search for natural anti-oxidant that could ameliorate reactive oxygen species which are implicated in numerous disease conditions (Ahlem *et al.*, 2015; Deepa *et al.* 2014).

In this study, we investigated the antioxidant properties of the extract and fractions of *D filix-mas* and also elucidated the major compound responsible for its antioxidant activity using *in vitro* bioassay guided isolation approach.

DPPH test is a widely acceptable and reproducible assay for screening potential antioxidants (Patel *et al.*, 2010). This method involves the ability of anti-oxidants to donate electrons to DPPH thereby causing discoloration of the reaction mixture from deep violet color to yellow color (Patel *et al.*, 2010). The yellow color change observed in this study substantiates the ability of the extract and fractions of *D filix-mas* to scavenge free radicals generation by DPPH. This further suggests that the constituents present in the extract and fractions could ameliorate oxidative damages by free radicals.

Reduction of ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions is a documented method of determining antioxidants with reducing power, capable of breaking free radical chain progression in lipid peroxidation (Habibur *et al.*, 2013). From this study, reduction of iron (III) complex to the ferrous form, iron (II) could be a possible antioxidant mechanism as a result of the presence of reducing agents in the extract and fractions of *D filix-mas*. Phenolic compounds such as quercetin has been reported to prevent free radicals by forming complex and chelating metal ions such as iron and copper, thereby preventing auto-oxidative damage to the living system (Nimse and Pal, 2015).

Nitric oxide generated following incubation of sodium nitroprusside with phosphate buffer in the presence of oxygen is known to cause toxicity to biomolecules (Deepa *et al.*, 2013). The scavenging capacities of the extract and fractions against nitric oxide generation suggest that *D filix-mas* could prevent nitric oxide generation in various disease conditions. Polyphenols such as quercetin has been reported to interact with nitric oxide synthase resulting in modulation of nitric oxide production (Hussain *et al.*, 2016).

The roles of phenolic compounds from medicinal plants as supplement in the treatment of oxidative stress and free radicals mediated tissue damage cannot be overemphasized (Li *et al.*, 2016). Flavonoids and phenolic acids from polyphenols have been reported to play a significant role in scavenging free radicals and prevention of oxidative damage to cells. Phenolic compounds have capacity to neutralize several forms of oxidizing free radicals due to their electron donating abilities (Habibur *et al.*, 2013; Ajaghaku *et al.*, 2017). Ojo *et al.*, (2013) reported that flavonoids inhibit lipid peroxidation in various biological systems. Presence of hydroxyl groups in flavonoids allows them to disconnect free radical chain reactions via the formation of intramolecular hydrogen bonds (Monika *et al.*, 2011).

From the forgoing, antioxidant properties elicited by the extract and fractions of *D filix-mas* could be attributed to presence of flavonoids. This is substantiated by presence of the flavonoid, quercitrin - as the major compound in the HPLC chromatogram of the extract and fractions of *D filix-mas* as documented in our earlier study (Erhirhie *et al.*, 2019).

The ethyl acetate VLC fraction (VLC-E7), where the compound, quercetin-3-O- α L-rhamnopyranoside was isolated exhibited the highest anti-oxidant activity compared to the extract and other fractions. In line with this finding, quercetin-3-O- α L-rhamnopyranoside isolated from other medicinal plants was found to elicit significant antioxidant activity (Zhang *et al.*, 2014). It is possible that possible that quercetin-3-O- α L-rhamnopyranoside may be the key antioxidant compound of *D filix-mas*.

Quercetin, the most widely distributed flavonoid in nature, existing mostly in its glycoside form quercitrin has been reported to possess various properties such as antioxidant, anti-inflammatory, neuroprotective, antiviral, anticancer, hepatoprotective, cardioprotective, antimicrobial and anti-obesity properties (Maalik *et al.*, 2014). Mir, *et al.*, (2017) reported that flavonoids from *Tridax procumbens* possess antioxidant properties. Phenolic compounds, catechin, epicatechin, dihydroquercetin isolated from *Alchornea floribunda* leaf were also found to elicit antioxidant properties (Ajaghaku *et al.*, 2017).

In this study, the ability of extract and fractions of *D filix-mas* to scavenge generation of free radicals from DPPH, nitric oxide as well as its chelating capacity could be attributed to the phenolic compound, quercetin-3-O- α L-rhamnopyranoside, which was postulated to be responsible for its anti-inflammatory activity as earlier reported (Erhirhie *et al.*, 2019).

CONCLUSION

The isolated flavonoid, quercetin-3-O- α L-rhamnopyranoside from ethyl acetate chromatographed fraction of *D filix-mas* could be responsible for its antioxidant activity, which validates its folkloric use in the management of rheumatoid arthritis and other free radical mediated diseases.

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