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HPLC Analysis, Anti-oxidant and Antiproliferative Evaluation of Methanol Extracts of Leaves and Roots of *Mondia whitei* (Hook. f) Skeels

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ABSTRACT

Mondia whitei (apocynaceae) also known as white ginger is a perennial herbaceous/woody climber that is widely distributed in tropical Africa and used traditionally to manage indigestion, erectile dysfunction, aphrodisiac, pain and depression. The study aims elucidate nature of the phytochemical components of the methanolic extract of the leaf and root of the plant and evaluate the antioxidant and antiproliferative activities. The compositional phytochemical profiling of the leaf and root extracts was done using HPLC analyses with Shimadzu HPLC-UV-DAD model. Rutin and caffeic acid were used as reference compounds. The antioxidant's property was evaluated using reducing capacity and nitric oxide inhibitory assays. Ascorbic acid was used as reference standard.. Total phenolic content was also determined and expressed as gallic acid equivalent. The antiproliferative activity was determined by Sorghum bicolor seed radicle test using methotrexate as reference standard. Results of antioxidant and antiproliferative studies were expressed as the mean ± SEM, Graph pad prism (version 6) was used to determine IC_{50} values, and a two way analysis of variance was used for the antiproliferative study. To test for level of significance, $P<0.001$ was considered to be significant.

The HPLC chromatogram of the leaves gave seven (7) peaks with caffeic acid eluting at 4.77 minutes while the root extract gave fifteen (15) peaks with caffeic acid and rutin eluting at 4.77 and 6.82 minutes respectively. The leaf and root extracts exhibited antioxidant activities against nitric oxide production with IC_{50} values of 6.1 ± 0.5 µg/mL and 173 ± 0.7 µg/mL respectively, which were however not comparable to that of the reference standard drug (ascorbic acid) with IC_{50} value of 3.4 ± 0.2 µg/mL. The extracts also exhibited reducing capacity activity with IC_{50} values of 5.7±0.8 µg/mL and 14.0 ± 1.2 µg/mL; Ascorbic acid had IC_{50} of 7.4 ±0.1 µg/mL. The phenolic content of the leaf and root extracts were 30.9 ± 0.1 and 38.7 ± 0.3 mg/g GAE, respectively. The antiproliferative studies revealed that Mondia whitei leaf and root extracts significantly ($P<0.01$) inhibited the growth of S. bicolor seed over a period of 48-96 h. The dose-dependent inhibition of the seeds by the leaf and root extracts became more evident at 96 h, with the root extract showing a dose-dependent inhibition of 37.9%, 43.4%, 45.5%, 50.5% and 60.8% for seeds treated with 1.00, 2.00, 4.00, 8.00 and 16.00 mg/mL, respectively, while the leaf extract gave a non-dose dependent inhibition of 87.0%, 85.9%, 79.9% 79.4% and 72.6% respectively. The reference drug, methotrexate, gave an inhibition of 51.6% at 96 h. The result of antiproliferative activity of the leaf extract suggests an inverse relationship with the dose after 96 h. The results indicate that Mondia whitei leaf and root extracts have high phenolic content and exhibit good antioxidant and anti-proliferative properties, with the leaf part showing significantly higher antioxidant and antiproliferative activities than the root part. This suggests the presence of antiproliferative chemical components in the extracts which could serve as leads for the development of anti-cancer agent. The HPLC analyses indicated that Mondia whitei contains caffeic acid and rutin, which are known potent antioxidants and antiproliferative/anti-cancer agents.

Keywords: Antioxidants; Antiproliferative; *Mondia whitei;* Total phenolic content

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INTRODUCTION

Cancer is one of the leading causes of death worldwide. Current cancer therapies include surgery, radiation therapy, chemotherapy and hormone therapy among others. The prevalence of cancer is on the increase in conjunction with the existence of cancer therapy-related problems. The occurrence of resistance, the high toxicity and cost of many anticancer agents are some of the disadvantages associated with their use in cancer therapy [1]. Hence the growing need for new anticancer drugs which are effective and less toxic. Medicinal plants and natural products have played a significant role in the prevention and management of cancer through multiple therapeutic effects which include inhibition of cancer activating enzymes and hormones, stimulation of DNA repair mechanisms, enhancing production of protective enzymes, antioxidant and immune boosting activities [2,3].

Mondia whitei (apocynaceae) also known as white ginger or mondia and isirigun" by the Yoruba ethnic group of Nigeria, is a known stimulant believed to have numerous medicinal properties. The plant is a perennial herbaceous/woody climber commonly found in Ghana and Nigeria, and widely distributed in tropical Africa [4,5]. Traditionally, it is effective in the treatment of malaria, erectile dysfunction and loss of appetite, gonorrhoea, paediatric asthma, and gastrointestinal disorder [6,7]. *Mondia whitei* has been reported to have several pharmacological activities such as aphrodisiac [8], antimicrobial [9,10], anti-inflammatory [11], anti-tyrosinase [12], antioxidant [13,10], Antisickling [14] and androgenic properties [15]. Compounds such as 2-hydroxy-4-methoxybenzaldehyde [12,16], isovanillin, coumarinoligan [17] and loliolide [18] have been isolated from the plant. The relationship between constituent phytochemicals and pharmacological activities of extracts/fractions of medicinal plants has well been established by many authors [10,14]. However, quantification of the total phenolic and know antioxidants like rutin, and the comparative study of the root and leaf methanolic extracts have not been reported. This study was designed to evaluate the phytochemical contents as well as the anti-oxidant and anti-proliferative properties of methanol extracts of the root and leaf of *M. whitei* with a view towards establishing any correlation between some of its constituent phytochemicals and pharmacological activities such as antioxidant and antiproliferative activities. Such correlation may provide insight into further development of the plant as lead for anticancer therapy.

MATERIALS AND METHODS

Unless otherwise stated, all chemicals and reagents were of analytical grade. All solvents for chromatographic purpose were HPLC grade, purchased from Zayo Sigma.

Collection and Preparation of Plant Sample

Fresh leaves and roots of *Mondia whitei* were collected in August 2016 from Imimi, Afijio LGA Oyo state Nigeria. The plant was identified by an expert Taxonomist in NIPRD herbarium Abuja. Voucher specimen was also deposited with voucher number NIPRD/H/6885. Samples were air dried at room temperature for two weeks, pulverized, packaged and stored in an airtight container until required for analysis. 100 g of each pulverized plant sample was extracted in 99.9 % methanol using a soxhlet apparatus, and concentrated under reduced pressure at 40°C. The concentrate was heated over a water bath to obtain a solvent free extract and allowed to dry.

Antioxidant Activities

Nitric oxide inhibition

Nitric oxide was generated from sodium nitroprusside and measured by the Greiss reaction as described by Janetia et al. [19]. Sodium nitroprusside (10 mM) in phosphate-buffered saline (pH 7.4) was mixed with different concentrations of the extract (50-800 µg/mL) dissolved in phosphate-buffered saline in a test tube and incubated at 25 °C for 180 min in the dark. 1 mL of the mixture was reacted with 1 mL of Greiss reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% napthylethylenediaminedihydrochloride). The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethylenediamme was read at 550 nm. Inhibition activities of nitrite formation by the plant extracts and the standard antioxidant ascorbic acid were calculated relative to the control. The percentage inhibition was linearized against the concentration of each extract and standard. The IC_{50} which is an inhibitory concentration of each extract required to reduce 50% of the nitric oxide formation was determined.

Reducing power capacity

The determination of the reducing power was conducted according to the method described by Chanola et al [20]. Different concentrations (2.5 mL) of extracts were mixed with 2.5 mL of phosphate buffer (pH 6.6) and 2.5 mL of

30 mM potassium ferricyanide. The mixture was then incubated at 50°C for 20 min. 2.5 mL of 600 mM of trichloroacetic acid was added to the mixture and centrifuged at 3000 rpm for 10 mins. The supernatant (2.5 mL) was mixed with 2.5 mL distilled water and 0.5 mL 0f 6 mM ferric chloride, absorbance was measured at 700 nm with a UV–vis spectrophotometer. Ascorbic acid was used as standard. Results were presented as IC_{50}.

Determination of Total Phenolic Content

The total phenolic content in the plant extracts was determined using the method described by singleton et al [21]. The reaction mixture was prepared by mixing 0.5 mL of methanolic solution of extract, 2.5 mL of 10% Folin-Ciocalteu's reagent dissolved in water and 2.0 mL of 7.5% $NaHCO_3$. Blank was also prepared containing 0.5 mL methanol, 2.5 mL of 10% Folin-Ciocalteu's reagent dissolved in water and 2.0 mL of 7.5% of $NaHCO_3$. Samples were allowed to stand for 30 mins. The absorbance was determined using spectrophotometer at 765 nm. The samples were prepared in triplicate for each analysis and the mean values of absorbance were obtained. The same procedure was repeated for the standard solution of gallic acid and the calibration line was construed. The content of phenolics was determined from the calibration line and phenolic content in the extracts was expressed in terms of gallic acid equivalent (GAE mg/g of extract).

Anti-proliferative Assessment

Sorghum bicolor seed radicle growth inhibition study was used. The choice of use of *S. bicolor* was borne out of the fact that meristematic cells of seeds just like cancer cells have the tendency to proliferate when exposed to favourable environmental conditions.

Seed Viability Test

Experimental plant, *Sorghum bicolor* seeds, (Guinea corn) was obtained from Karmo market Abuja Nigeria and the seeds were subjected to viability test by submerging in water and observing the ability to remain submerged. The seeds that floated were discarded while the totally submerged seeds were allowed to dry before use.

Determination of Growth Inhibitory Effect

The modified methods of [22-24] was used for this study. 3.2g of *M. whitei* (root and leaf extracts) was weighed and dissolved in 10 mL of methanol and made to 100mL with distilled water to obtain the stock solution of 32 mg/mL. Different concentrations (1, 2, 4, 8 and 16 mg/mL) were prepared from the stock solution by dilution. Methotrexate 0.05 mg/mL was used as standard drug. Petri dishes were layered with cotton wool and calibrated filter paper numbered from1 to 24 (Whatman No.1). Twenty four (24) viable seeds were placed in each of the Petri dishes. The control seeds were treated with 15 mL distilled water. The seeds were treated with each of the prepared concentrations and placed in separated petri dishes. The mean length (mm) of the radicle emerging from the seeds was measured at 48, 72 and 96 hours consecutively using a divider and a meter rule.

The percentage inhibition and percentage growth was calculated as

$$\text{Inhibition (\%)} = \frac{\text{(mean radicle length (control) - mean radicle length (treated)) X 100}}{\text{mean radicle length (control)}}$$

Growth (%) = 100 - percentage inhibition.

High Performance Liquid Chromatography Analysis

The extract of *Mondia whitei* was analysed by high performance liquid chromatography (HPLC). The HPLC consisted of Ultra-Fast LC-20AB equipped with SIL-20AC autosampler; DGU-20A3 degasser; SPD-M20A UV-diode array detector; column oven CTO-20AC, system controller CBM-20Alite and Windows LC solution software (Shimadzu Corporation, Kyoto Japan); column, 5 µm VP-ODS C18 and dimensions (4.6 x 1_{50} mm). The chromatographic conditions included mobile phase: 0.2% v/v formic acid and acetonitrile (20:80); mode: isocratic; flow rate 0.6 mL/min; injection volume 10 µL of 10 mg/mL solution of extract in water; detection UV 254 nm, column oven temperature was programmed at 40°C and total run time was 20 minutes. Flavonoids and phenolic acid standards such as apigenin, rutin, quercetin, caffeic acid and ferullic acid were employed for the identification of the phytoconstituents by comparing the retention time under similar experimental conditions [25].

Statistical Analysis

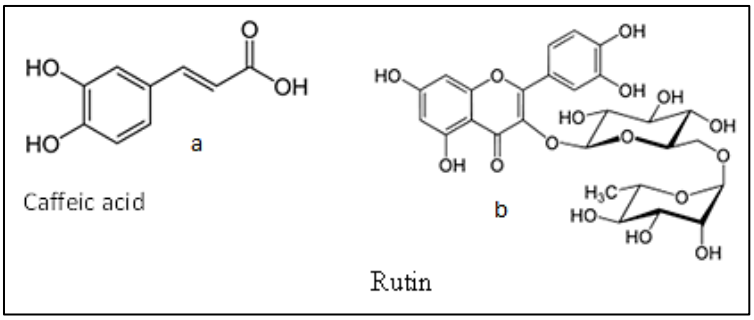
The data obtained were expressed as mean ± standard error mean. IC_{50} was determined using Graph pad prism (version 6). Two ways analysis of variance was used for the antiproliferative study to test for significance <0.001 was considered to be significant.

RESULT AND DISCUSSION

HPLC Analysis

The HPLC spectrum of *Mondia whitei* leaves (Figure 1) gave seven (7) peaks with caffeic acid eluting at 4.77 minutes while the root extract (Figure 2) gave fifteen (15) peaks with caffeic acid and rutin eluting at 4.77 and 6.82 minutes respectively. Rutin was not detected in the leaf extract. Apigenin, quercetin and ferullic acid were absent in both extracts. An earlier report had previously revealed the presence of phenols (6.40 g), flavonoids (2.99 g) and tannins (0.073 g) in the root ethanolic extract of the plant [13]. Caffeic acid is a phenolic acid and has been reported to have anticancer activity [26]. Rutin, quercetin, ferullic acid and many other phenolics have been reported to have antioxidant activity [27], cytoprotective activity [28], antiproliferative activity [29] and anticancer activity [30].

Rutin ($C_{27}H_{30}O_{16}$) or quercetin-3-O-rutinoside is glycoside of quercetin which belongs to a class of secondary metabolites known as flavonoids. Quercetin is known to be widely distributed in many plants with antioxidants property (Scheme 1). Flavonoids play role vital in the maintenance of membrane integrity through their scavenging or chelating abilities [31]. Phenolics manifest their activity by decreasing oxygen concentration and intercepting singlet oxygen, hence preventing the formation of free radicals [32].



Scheme 1: a) Caffeic acid, b) Rutin

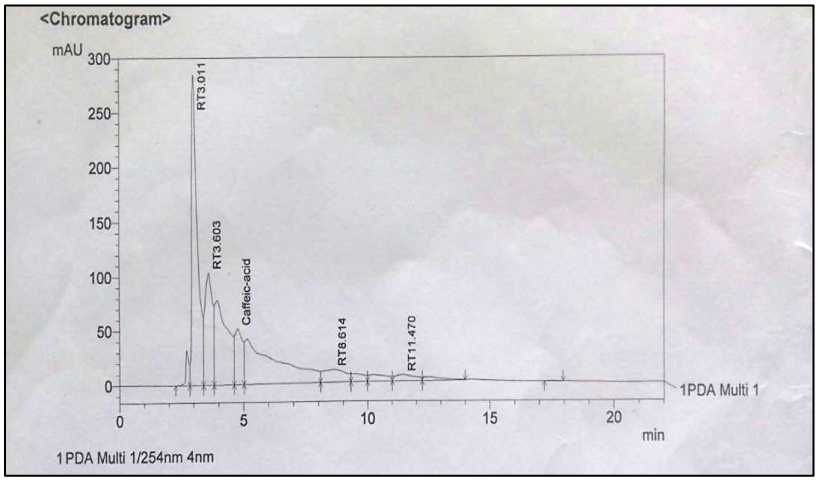


Figure 1: HPLC spectrum of leaf methanol extract of *Mondia whitei*

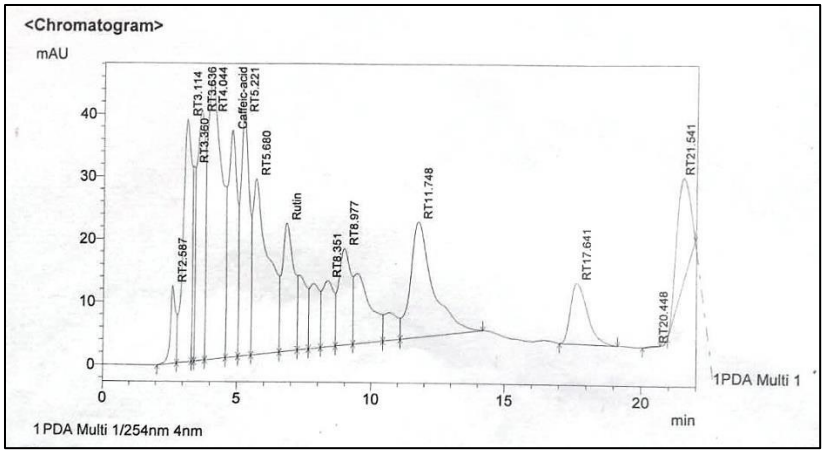


Figure 2: HPLC spectrum of root methanol extract of *Mondia whitei*

Table 1: Antioxidant activity of methanol extract of *M. whitei* leaves and roots

Extracts	Nitric oxide inhibition IC_{50} (µg/mL)	Reducing capacity IC_{50} (µg/mL)	Total phenol content (mg/g GAE)
M. whitei (leaves)	6.1 ± 0.5	14 ± 1.2	30.9 ± 0.1
M.whitei (Roots)	173 ± 0.7	5.7 ± 0.8	38.7 ± 0.3
Ascorbic acid	3.4 ± 0.2	7.4 ± 0.5	-

Table 2: Percentage inhibition and percentage growth for *S. bicolor* seeds treated with methanol extract of *M. whitei* leaves

Treatment (mg/mL)	Percentage inhibition			Percentage growth		
	48 hr	72 hr	96 hr	48 hr	72 hr	96 hr
1.00	81.10	85.90	87.00	18.90	14.00	12.90
2.00	83.80	87.80	85.90	16.20	12.20	14.10
4.00	88.80	82.00	79.80	17.20	18.00	20.10
8.00	77.80	83.70	79.00	22.20	16.80	21.00
16.00l	73.40	77.50	72.60	26.60	22.50	27.40
Control	0.00	0.00	0.00	100.00	100.00	100.00
Methotrexate	65.50	57.60	51.60	34.50	48.40	48.40

The extract significantly ($P < 0.001$) inhibited *S. bicolor* seed growth compared with the control.

Table 3: Percentage inhibition and percentage growth for *S. bicolor* seeds treated with *M. whitei* root methanol extract

Treatment (mg/mL)	Percenta Percentage inhibition*			Percentage growth		
	48 hr	72 hr	96 hr	48 hr	72 hr	96 hr
1.00	18.4	37.7	37.9	81.6	62.3	88.3
2.00	25.1	42.9	43.4	74.9	74.9	43.4
4.00	41.9	44.8	45.5	58.1	55.2	54.5
8.00	66.9	58.3	50.5	33.1	41.7	49.5
16.00l	68.8	68.6	60.8	31.2	31.4	39.3
Control	0.0	0.0	0.0	100	100	100
Methotrexate	65.5	57.6	51.6	34.5	48.4	48.4

The extract significantly ($P < 0.001$) inhibited *S. bicolor* seed growth compared with the control.

Antioxidant Activity

The extracts showed reducing capacity activity with IC_{50} values of 5.7 ± 0.8 and 14 ± 1.2 µg/mL for root and leaf extracts respectively (Table 1). The root extract had better reducing capacity activity with IC_{50} values of 5.7 ± 0.8 µg/mL compared to the leaf extract. However, the leaf extract exhibited a better NO inhibitory activity with IC_{50} of 6.1 ± 0.5 µg/mL than the root, which gave an IC_{50} of 173 ± 0.7 µg/mL, although the NO activities of two extracts were not comparable to that of the reference compound, ascorbic acid, which had an IC_{50} of 3.4 ± 0.2 µg/mL (Table 1). The occurrence of antioxidants (reductants) in extracts causes reduction of Fe (III) to Fe (II) complexes which can be determined by the formation of perusian blue colour. This reduction may be as a result of donation of hydrogen atom from phenolic compounds [33] which also relates to the presence of reducing agent. The number and position of the hydroxyl group of phenolic compounds is also imperative to their antioxidant activity [(34)]. Thus, reducing power may be used as a pointer to potential antioxidants [35].

Again, several studies have confirmed the connection between antioxidant activities, nitric oxide inhibition and reducing power in plant extracts [36]. Nitric oxide (NO) is a significant chemical mediator generated by endothelial cells, macrophages, neurons, etc. Excess concentration of NO is associated with several diseases [37]. Compounds that have the ability to show reducing capacity and also inhibit NO are said to be antioxidants [38-40]. The NO inhibition activity could be due to the presence of phenolic compounds, as revealed by the HPLC analyses, and other compounds such as terpenes, reducing sugars and alkaloids [8, 41,10] in the plant. The roots showed a significantly higher TPC (38.7 mg/g GAE) than the leaves (30.9 mg/g GAE) (Table 1). Phenolics belong to a wide and heterogeneous group of chemical components that have one or more benzene rings with a conjugated aromatic system and one or more hydroxyl groups. They donate an electron or a hydrogen atom to a free radical and as such are important as free radical scavengers. Phenolics therefore, have relevant *in vitro* and *in vivo* antioxidant activities. They also occur in free and conjugated forms with sugars, acids, and other biomolecules as water-soluble compounds (phenolic acids e.g. caffeic acid, ellargic acid, gallic acid flavonoids, flavonols (quercetin, kaempferol, myricetin), flavanols (catechins and epicatechin) and quinones) or water-insoluble compounds (condensed tannins) and hydrolysable tannins, such as ellagitannins [42]. The result of our antioxidant study agrees with a report of an earlier study by [10]. The results also support the positive correlation between phenolic content and reducing capacity or antioxidant activities of plant extracts as previous reported by several authors [40,43,44].

Antiproliferative Activity

The antiproliferative studies revealed that *Mondia whitei* leaf and root extracts significantly ($P<0.001$) inhibited the growth of *S. bicolor* seed over a period of 48-96 h (Tables 2,3). At 96 h, the root extract showed a dose-dependent inhibition of 37.9%, 43.4%, 45.5%, 50.5% and 60.8% for seeds treated with 1.00, 2.00, 4.00, 8.00 and 16.00 mg/mL, respectively, while the leaf extract gave a non-dose dependent inhibition of 87.0%, 85.9%, 79.9% 79.4% and 72.6% respectively. The reference drug, methotrexate, gave an inhibition of 51.6% at 96 h. The extracts significantly ($P<0.01$) inhibited the growth of the *S. bicolor* seeds at the applied concentrations more than the positive control used. It is important to note that the leaf extract exhibited good antiproliferative property even at a low concentration of 1 mg/mL from 48 to 96 h.

CONCLUSION

M. whitei roots extract exhibited a higher total phenolic content, better reducing capacity but lesser nitric oxide inhibitory activities compared to the leaves. However the leaves exhibited higher antiproliferative activities than its roots. The reducing capacity of the root extracts, possibly due to the presence of rutin and caffeic acid, was comparable to that of the well-known antioxidant, ascorbic acid, and could be developed as a substitute nutraceutical product. The anti-proliferative activity of the extracts suggests that the plant has potentials and could be a lead in the development of anti-cancer therapy.

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