

## Phytochemical Content and *In Vitro* Antimycelial Efficacy of *Colocasia esculenta* (L), *Manihot esculenta* (Crantz) and *Dioscorea rotundata* (Poir) Leaf Extracts on *Aspergillus niger* and *Botryodiplodia theobromae*

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**Abstract.** The *in vitro* efficacy of aqueous leaf extracts of three common root crops (*Colocasia esculenta*, *Manihot esculenta* and *Dioscorea rotundata*) were investigated against *Aspergillus niger* and *Botryodiplodia theobromae*. The pathogenic fungi were isolated from rotten tubers of yam using antibiotic-amended potatoes dextrose agar. Graded aqueous leaf extract concentrations (25 g/L, 50 g/L and 75 g/L) of each plant were applied against each test fungi on PDA plates and their mycelial growth inhibition were monitored at  $28 \pm 2$  °C for 144 hrs. Camazeb (Alderelm, UK), a commercial fungicide, at concentrations of 5 and 10 g/L was used as positive control agent. The phytochemical constituents of each extract were also analyzed using standard techniques. The antifungal activities of the extracts were concentration dependents. *Colocasia esculenta* extracts (75 g/L) demonstrated the most significant ( $P < 0.05$ ) antifungal activities with mycelial growth inhibition of 92.2 % and 85.9 % against *A. niger* and *B. theobromae*, respectively. These effects were followed by the activities of *D. rotundata* extracts with 82.8 % and 80.9 % inhibitions, respectively. Though, *M. esculenta* leaf extracts generally demonstrated the least antifungal activities against both pathogens, their activities were significant at 75 g/L. The antifungal potency of both *D. rotundata* and *C. esculenta* were comparable to Camabez which demonstrated 100 % inhibition at both 5 and 10 g/L concentrations. Higher concentrations of saponins (5.64 - 6.71 mg/100g), tannins (4.42 - 7.23 mg/100g) and flavonoids (3.64 - 5.25 mg/100g) were detected in the extracts, and could probably be linked to the observed antifungal activities. Findings from this study suggest that *D. esculenta*, *C. esculenta* and *M. esculenta* leaf extracts posses bioactive molecules that could be exploited in the control of phytopathogens of tubers crops, which are the main staple food in most tropical countries.

### Introduction

Plants serve as the major sources of food for man and other animals. One of the vital challenges to food security in developing countries is colossal loss of agricultural produce due to inadequate storage facilities and microbial-induced wastages [1]. Microbes, particularly fungal infections, have been found to be the leading spoilage pathogens of pre- and post-harvest crops because of their abilities to produce diverse hydrolytic enzymes and mycotoxins [1]. Therefore, the control of fungal pathogens of economic crops is crucial for sustainable food production and distribution.

Over the years, conventional cultural control practices and use of fungicides have been exploited by the government and private organizations in the management of phytopathogens. Currently, some potent fungicides are still be used for the management of field and post-harvest rot diseases of crops with great successes [2, 3]. Though relatively effective and quick at actions, the major drawbacks with some of the chemicals agents have been issues of risks of non-biodegradability, bioaccumulation, biomagnifications of the chemical residues along the food chains, making them environmentally unfriendly agents. Besides, the frequent and indiscriminate application of these fungicides has led to the emergence of more resistance strains of crop

pathogens [4]. To address the issues, researchers, have advocated the shift of attention to natural, biodegradable plants materials with potent phytochemical molecules [5]. Natural agents, such as plants and their by-products, are currently being considered as possible future alternative based on the outcomes of recent reports on their potent bioactive constituents against public health pathogens of plants, animals and humans [6-9]. Hence, there is need for environmentalists to intensify the search for more plants with antimicrobial properties against phytopathogens of economic significance.

*Colocasia esculenta* (L.) Schott (Family: Araceae) is a perennial herbaceous plants that is commonly cultivated for its edible starchy roots (corm) and leaves in tropical and sub-tropical countries [10]. Previous reports have shown that the plant possess essential minerals, vitamins and medicinal properties [10-12]. *Manihot esculenta* (Crantz) (Family: Euphorbiaceae) is an annual, woody shrub that is massively grown in crop in tropical and subtropical regions for its edible starchy tuberous root. The leaves also serve as vegetable in most part of the county. Apart from the nutritional benefits of this plant, their leaves, seed oil, tuber peels have been reported to possess medicinal properties against diverse human and animal ailments of microbial origins [13, 14]. *Dioscorea rotundata* (L) [Poir] is an annual climbing herbaceous plant with cylindrical stem and dark glossy green leaves [15]. They belong to the family Dioscoreaceae and are native to African countries, where they serve as major sources of carbohydrate, minerals, vitamins and dietary fibres [16]. Various species of *Dioscorea* plants have been exploited as ancient medicinal plant for the management of diverse human ailments ranging from superficial to deep-seated infections [15, 17]. Considering the profound nutraceutical and pharmacological potentials exhibited by *C. esculenta*, *D. rotundata* and *M. esculenta* plants, it is pertinent to investigate their antimicrobial potentials against common phytopathogenic pathogens of African staple crops.

Consequently, the leaf of the three tuberous vegetables (*Colocasia esculenta*, *Manihot esculenta* and *Dioscorea rotundata*) were investigated for their phytochemical contents and antifungal efficacy against two (*Aspergillus niger* and *Botryodiplodia theobromae*) of the most versatile post-harvest spoilage pathogens of economic crops in tropical countries, with a view to providing additional information on potential natural phytochemicals needed for the management of these pathogens.

## Materials and Methods

### Source of plant materials

Fresh leaves of *Colocasia esculenta* (cocoyam), *Manihot esculenta* (cassava) and *Dioscorea rotundata* (white yam) (Fig. 1) were sourced from farm lands within Amai town, Ukwuani Local Government Area of Delta State, Nigeria. Their leaves are manually plucked and transported in black polyethylene bag to the laboratory for further processing.



*Dioscorea rotundata* (Poir)

*Manihot esculenta* (Crantz)

*Colocasia esculenta* (L.)

**Figure 1.** Leaves of the three selected medicinal plants

### Preparation of plant materials

The fresh leaves of each plant were washed, air-dried for two weeks and homogenized mechanically. The leaf extracts prepared were 25 g/L, 50 g/L and 75 g/L using hot water (100 °C) as extracting solvent for 24 hrs. Each extract was filtered using sterile Muslin cloth and concentrated to dryness using a rotary evaporator (Bibby Sterlin Ltd, England, RE. 200) before storing the extracts in refrigerator (4 °C) until further use. Graded concentrations (5 g/L and 10 g/L) of a commercial fungicide, Camabez (Alderelm, UK), were also prepared using sterile distilled water and served as positive reference chemical agents [4].

### Source of the test pathogens (*Aspergillus niger* and *Botryodiplodia theobromae*)

Some yam tubers with symptoms of rots were sourced from Obiaruku Main Markets in Delta State, Nigeria, and transported to the Laboratory for analysis. The spots on the yam with rots were cut into tiny bits and surface sterilized using 2% sodium hypochlorite solution for 60 seconds before repeatedly rinsing them in sterile distilled water. The pre-sterilized yam bits then plated on the sterile antibiotic-amended Potato Dextrose Agar (Oxoid) plates using spreading plated techniques. The cultured plates were incubated at  $28 \pm 2$  °C for 5-10 days. Suspected colonies with characteristics such as black mycelia, dull white fluffy surface mycelia and dark coloured underneath radiating uniformly from the centres of the mycelia were purified, confirmed for dark upright conidiophores with swollen phialides tips and double ovoid-conidia shapes under the microscope. The colonies with cultural and morphological characteristics of *Aspergillus niger* and *Botryodiplodia theobromae* were confirmed using standard reference mycology atlas [18, 19] and used for the study.

### Antifungal activities of plants extracts

The prepared extracts were tested to ascertain their sterility (using sterile PDA plates) prior to antifungal assay. Freshly prepared molten PDA (20 mL) plates were aseptically sprayed with 2 mL each of the plant extracts, mixed thoroughly and allowed to solidify. A 5 mm mycelial disc cut from the actively growing regions (periphery mycelia) of a 7 day old culture, each of *A. niger* and *L. theobromae*, was aseptically placed in an inverted position on the extract-treated PDA plates, before incubating them at  $28 \pm 2$  °C. The negative and positive control plates were treated with 2 mL each of sterile distilled water and Camabez. All the experimental set up were done in triplicates. The effects of each extract and Camabez on the radial mycelial growth were determined periodically at 48, 96 and 144 hrs [12] (20). The percentage mycelial growth inhibitions were determined using the formula below.

$$\text{Mycelial growth inhibition} = \frac{\text{Diameter of Control} - \text{Diameter of treatment}}{\text{Diameter of Control}} \times 100 \% \quad (1)$$

### Phytochemical analysis of the medicinal plants

The extracts from each plant were analyzed quantitatively for selected phytochemicals, such as tannins, flavonoids, alkaloids, saponins, steroids, phenols and cardiac glycosides by previously described methods [21-24]

#### Test for tannins content

10.0 cm<sup>3</sup> of the filtrate was weighed from each hot water extract and mixed with 50.0 cm<sup>3</sup> of distilled water before adding 5.0 cm<sup>3</sup> of Folin-Denis reagent and 10 cm<sup>3</sup> of saturated sodium bicarbonate (Na<sub>2</sub>CO<sub>3</sub>) for colour development [24]. The solution was carefully mixed and allowed for reaction on water bath (25 °C) for 30 min. The absorbance of the test solutions were measure at 700 nm using Spectrum Lab23A spectrophotometer. Standard tannic acid solution was prepared along the test solutions absorbance obtained spectrophotometrically were used to prepare a standard curve for analysis of tannins in the test solution. Tannin contents were determined from the standard curves.

### **Determination of alkaloid content**

2.5 g of each leaf powder was extracted using 100 ml of 20% acetic acid in ethanol for a period of 4 hrs. The contents were filtered and concentrated to about 25 mL. Thereafter, concentrated ammonium chloride was added in stepwise manner for precipitation to occur and settle. The precipitates were collected and washed with dilute ammonium hydroxide and filtered again. The final filtrates were discarded and pellets obtained were dried and weighed using electronic weighing balance Model B-218 [21].

### **Determination of total flavonoid content**

The total flavonoid content was determined using the aluminum chloride colorimetric method [22]. This method is based on the formation of a complex flavonoid–aluminium, having the absorbance maximum at 430 nm. 0.5 mL of each plant extract was mixed with 1.5 mL of methanol, 0.1 mL of 10%  $\text{AlCl}_3$ , 0.1 mL of 1 M potassium acetate and 2.8 ml of distilled water. After incubation at room temperature for 15 min, the absorbance of the reaction mixture was measured at 430 nm on UV–visible spectrophotometer [Shimadzu UVPC-1650 (Japan)]. Total flavonoid contents of extract samples were expressed as mg/100 g dry weight of extract through the calibration curve with rutin as standard.

### **Determination of saponin content**

5.0 g of each powdered leaf sample was added to 100 cm<sup>3</sup> of 20 % aqueous ethanol and heated with constant stirring over a water bath (55 °C) for about 4 hrs. After filtering the content, the aqueous ethanol extraction was repeated for 4 hrs at 55 °C with continuous stirring. The pooled extracts were then evaporated using water bath (90 °C) to about 40 cm<sup>3</sup>. The partially concentrated extract was placed in a separating funnel before adding 20 cm<sup>3</sup> of diethyl ether, mixed properly, and allowed to settle into layers. The aqueous layer was recovered while the ether layer was discarded before. further purification using 60 cm<sup>3</sup> of n-butanol and 10 cm<sup>3</sup> of 5% sodium chloride. The sodium chloride layer was later discarded before concentrating the residues over water bath for 30 min and to dryness using oven (Jenway) before determining the saponins content [21-24].

### **Determination of cyanogenic glycoside content**

1.0 g of each powdered plant was sample was weighed into 200 cm<sup>3</sup> distilled water, allowed to autolyse for 2 hrs before complete distillation in flask containing 2.5 % sodium hydroxide and tannic acid as an antifoaming agent. The distillates were mixed with 100 cm<sup>3</sup> of cyanogenic glycosides, 8 cm<sup>3</sup> of ammonium hydroxide and 2 cm<sup>3</sup> of potassium iodide, before titrating the content with 0.02 M silver nitrate against a dark background to a constant turbid end point. The cyanogenic contents of the samples were then calculated [23, 24].

### **Determination of steroid Content**

1.0 g of each powdered plant was weighed into conical flasks containing 20 cm<sup>3</sup> of ethanol and macerated for some hours before filtering using Whatman's No 1 filter paper. Equal volume of the filtrate (2 cm<sup>3</sup>) was added to cholesterol colour reagent before taking the absorbance at 559 nm using Spectrum Lab23A spectrophotometer. The steroid content were then estimated from the standard curve [21, 22].

### **Determination of total phenolic content**

The plant samples were initially defatted using ether. The phenolic contents were then extracted by boiling 0.5 g of each defatted sample in flask containing 50 cm<sup>3</sup> of ether reagent.

Exactly, 5 cm<sup>3</sup> of the ether extract was added into conical flask containing mixture of 10 cm<sup>3</sup> of distilled water, 2 cm<sup>3</sup> of 0.1 N ammonium hydroxide and 5 cm<sup>3</sup> of amyl alcohol. The content was mixed thoroughly for 30 min. [24]. The total phenol contents were then determined by comparing the optical density (505 nm) with the tannic acid standard solutions prepared along with the samples [21, 24].

## Statistical analysis

The data obtained were statistically analysed using SAS software packages and the means obtained were determined for significance differences at 5% probability level using Duncan Multiple Range Test (DMRT)

## Results and Discussion

Analysis of the *in vitro* antifungal activities of each extract concentration against the test pathogens revealed significant findings when compared with those of the chemical fungicides. The effects of the extracts were concentration dependent. Generally, the best mean percentage mycelial growth inhibitions effects were observed at each of the highest extract concentration under study. At this concentration (75 g/L), the percentage mycelial growth inhibition recorded for *C. esculenta*, *D. rotundata* and *M. esculenta* extracts were 92.2 %, 82.8 %, 60.2 % against *A. niger* and 85.9 %, 80.9 %, 68.2 % against *B. theobromae*, respectively, after 144 hrs of incubation (Table 1). This suggests that *C. esculenta* extracts were generally the most potent antifungal agent in this study. The leaf extracts of *C. esculenta* have been reported to significantly control the spore germination and mycelial growth of *Fusarium* species [10]. Similarly, their stem bark, leaf and root extracts have been reported demonstrate potent antimicrobial activities against public health significant pathogens [25-29]. Thus, finding from this study further laid credence to the antimicrobial potency of this medicinal plant.

The results in also revealed that the effects of *D. rotundata* extracts were significant against both pathogens. The mean percentage inhibitions ranged from 63.8 – 82.8% against *B. theobromae* and 61.9 – 80.9% against *A. niger* (Table 1). The observed antifungal activities of *D. rotundata* were better than the antifungal effects reported earlier for *Dioscorea wallichii*, where only the acetone extracts was effective, while the methanol and ethyl acetate extracts recoded zero effects against the fungus, *Candida albicans* [30]. Similarly, very poor antimicrobial activities were reported for the aqueous tuber and peel extracts of *Dioscorea bulbifera* [31, 32]. However, the tuber mucilage of *D. esculenta* was reported to possess potent antibacterial activity against some human pathogens [33]. The observed difference could be attributed to the differences in the extracting solvents and nature of the pathogen. Most studies in the past have reported the use of different plant extracts in the control of *B. theobromae* rot pathogens of *Dioscorea rotundata* [4, 34-36], but our study has shown that the hot aqueous leaf extract of the same plant possesses potent biological control properties against their own pathogens.

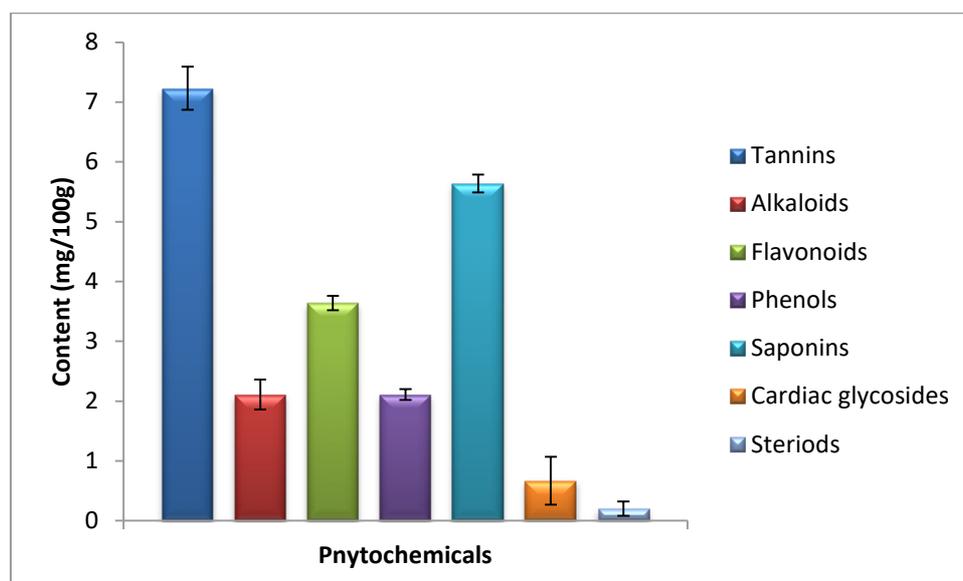
**Table 1.** Antifungal activities of plant extracts against *A. niger* and *B. theobromae* after 144 hrs of incubation

Plant extracts	Conc. (g/L)	Mean diameter of mycelial growth inhibition (%)	
		<i>A. niger</i>	<i>B. theobromae</i>
<i>C. esculenta</i>	25.0	54.9 ± 3.44 <sup>c</sup>	67.2 ± 1.51 <sup>c</sup>
	50.0	77.1 ± 2.21 <sup>b</sup>	80.3 ± 3.33 <sup>b</sup>
	75.0	92.2 ± 1.91 <sup>a</sup>	85.9 ± 4.03 <sup>b</sup>
<i>M. esculenta</i>	25.0	28.9 ± 4.06 <sup>f</sup>	35.5 ± 3.22 <sup>f</sup>
	50.0	33.5 ± 1.54 <sup>f</sup>	59.7 ± 1.22 <sup>d</sup>
	75.0	60.2 ± 0.95 <sup>d</sup>	68.2 ± 2.34 <sup>c</sup>
<i>D. rotundata</i>	25.0	63.8 ± 2.72 <sup>d</sup>	61.9 ± 3.16 <sup>d</sup>
	50.0	75.2 ± 0.82 <sup>c</sup>	68.4 ± 2.11 <sup>c</sup>
	75.0	82.8 ± 5.22 <sup>b</sup>	80.9 ± 4.08 <sup>b</sup>
Camazeb	5	100 ± 0.00 <sup>a</sup>	100 ± 0.00 <sup>a</sup>
	10	100 ± 0.00 <sup>a</sup>	100 ± 0.00 <sup>a</sup>

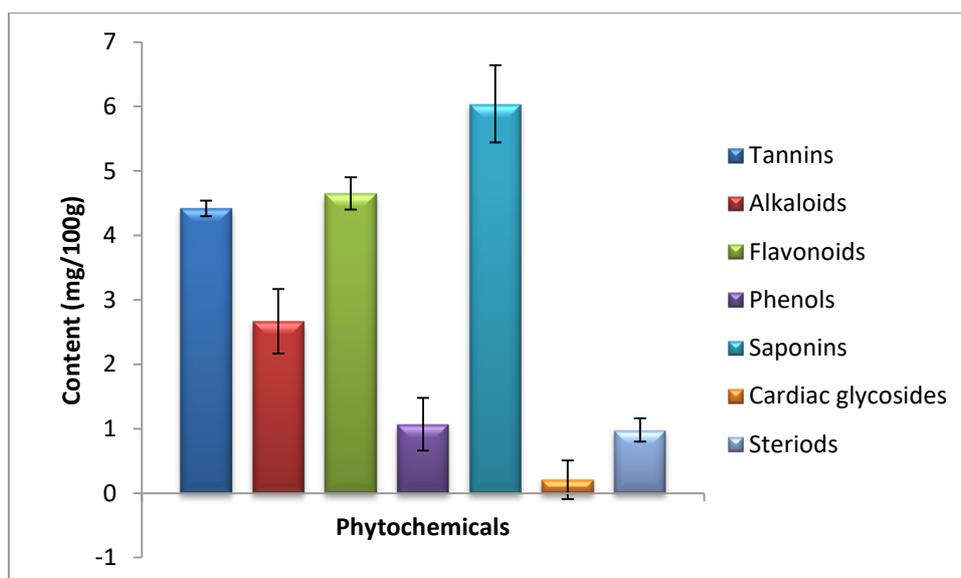
\*Values for means in the same column for each concentrations with dissimilar alphabet (superscript) are statistically significant at P<0.05.

Our results further revealed that *M. esculenta* leaf extracts generally demonstrated the least antifungal activities against the test pathogens, but were significant at 75 g/L, with percentage inhibition of 60.2 % and 68.2 % against *A. niger* and *B. theobromae*, respectively (Table 1). This finding probably suggests that *M. esculenta* leaf extracts possess antifungal activities, but at relatively high concentrations, unlike *C. esculenta* and *D. rotundata* extracts. This observation is in consonance with the recent report on the antibacterial activity *M. esculenta* and *Tamarindus indica* extracts against antibiotic-resistant bacteria [37]. Other studies have also reported the antimicrobial activities of the leaf, seed oil and tuber peels of *M. esculenta* [13, 38-39] and are in agreement with our findings. The significant antifungal activities expressed by the three medicinal plants against *A. niger* and *B. theobromae* in this present study suggest that they possess bioactive secondary metabolites fungal pathogens.

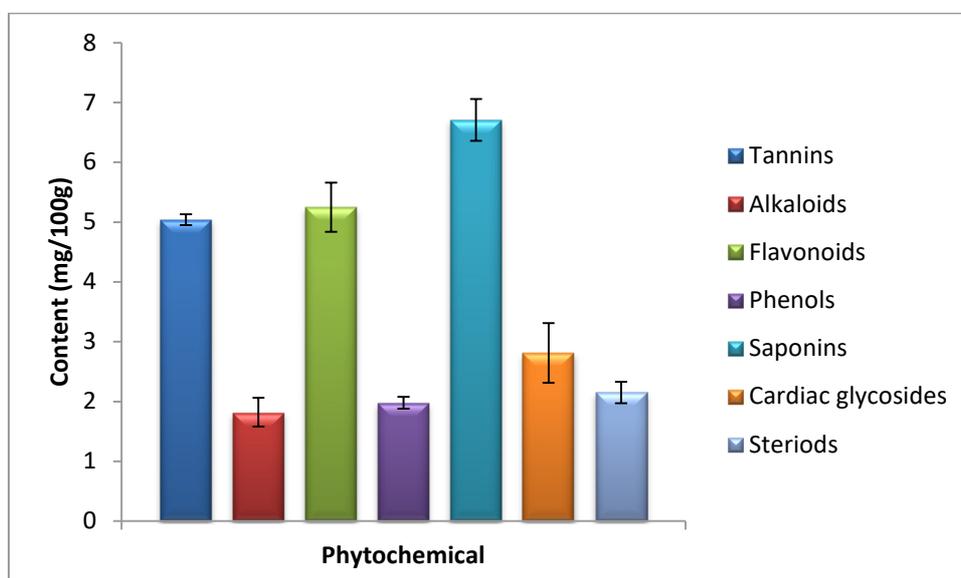
Analysis of the phytochemical constituents of each extract confirmed the presence of tannins, flavonoids, alkaloids, saponins, steroids, phenols and cardiac glycosides at various concentrations as shown in Fig. 2, 3 and 4. From the results, it was observed that significantly ( $P < 0.05$ ) higher concentrations of saponins (5.64 - 6.71 mg/100g), tannins (4.42 - 7.23 mg/100g) and flavonoids (3.64 - 5.25 mg/100g) were detected all the extracts (Fig. 2). They could probably be the bioactive ingredients responsible for observed antifungal activities, considering the reports of previous workers [4, 10, 29, 31, 39, 40]. Saponins have been reported to possess significant antifungal activities [41]. Flavonoids and Tannins extracts have also been reported to possess antioxidants and antimicrobial properties [42]. Hence, the outstanding antifungal activities of *D. rotundata* and *C. esculenta* in this study could be linked to the relatively high concentrations of saponins, tannins and flavonoids detected in their hot aqueous leaf extracts. It is also important to point out that the presence of the remaining phytochemicals, though in moderate amount, has been reported in antimicrobial activities. Alkaloids present in most stem barks, leaves and roots of plants have long been reported to possess potent antimicrobial properties [43]. Apart from their antioxidants properties, phenolic compounds, cardiac glycosides and steroids have also been documented in most medicinal plants as active antimicrobial compounds against diverse pathogens of plants and animals [31, 44-46]. Therefore, the nature of antifungal properties of the studied plants could be attributed to the presence and quantity of the bioactive compounds detected in each leaf extract.



**Figure 2.** Quantitative phytochemical contents of the *D. rotundata* leaf extract



**Figure 3.** Quantitative phytochemical contents of the *M. esculenta* leaf extract



**Figure 4.** Quantitative phytochemical contents of *C. esculenta* leaf extract

## Conclusion

This study has revealed that the hot aqueous leaf extracts of *D. rotundata*, *C. esculenta*, and *M. esculenta* possess significant *in vitro* antifungal activities against *A. niger* and *B. theobromae* at concentration of 75 g/L. The antifungal effects were comparable to the excellent activities of Camabez used as reference chemotherapeutic agent. The antifungal effects of the extracts were linked to the saponins, flavonoids and tannins, which were found in relatively high concentration in the extracts. Therefore, the phytochemical properties of the leaves of the three edible crops possess antifungal activities against *A. niger* and *B. theobromae* *in vitro*. *In vivo* studies of the bioactive molecules are thus recommended with a view to exploiting them as bio-control agents of these versatile phytopathogens of tubers crops, which are the main staple food in most tropical countries.

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