Official publication of Pakistan Phytopathological Society
Pakistan Journal of Phytopathology
ISSN: 1019-763X (Print), 2305-0284 (Online)
http://www.pakps.com

SENSITIVITY ASSESSMENT OF SOIL-BORNE PATHOGENS TO ETHANOLIC AND METHANOLIC LEAF EXTRACT OF *CHROMOLAENA ODORATA* (L.) USING DILUTION AND DIFFUSION ASSAY METHODS

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A B S T R A C T

The sensitivity of pure isolates of *Phytophthora colocasiae* and *Fusarium oxysporum* to methanolic and ethanolic leaf extracts of *C. odorata* was evaluated using diffusion(agar and disc diffusion) and dilution(broth and agar dilution) assays. Basic quantitative and qualitative analyses of the extracts were determined. The experiment was laid out as factorial in Completely Randomized Design (CRD) with three replications. Data collected were adequately analyzed using Univariate approach and Least Significance Difference (LSD) test to separate significant at $P \leq 0.05$. Observations from the study revealed that antimicrobial activities of the plant extracts were dependent upon the assay methods used which varied significantly. Ethanolic leaf extracts of *C. odorata* significantly increased the inhibition zone diameter (IZD) against *F. oxysporum* when compared to methanolic leaf extracts of same plant. However, significant differences were not observed in IZD between ethanolic and methanolic leaf extracts against *P. colocasiae*(12.67 ± 0.79 and 12.56 ± 0.74). Ethanolic extracts of *C. odorata* were found to possess higher inhibitory effects shown by the wider inhibition zone against the pathogens using disc diffusion assay. There were significant and non- significant reductions respectively in IZD of *F. oxysporum* and *P. colocasiae* with increased concentrations of the leaf extracts. The methanolic and ethanolic extracts of *C. odorata* showed significant higher inhibitory activities against the pathogens at MICof 6.25 and25 mg/ml respectively, inhibiting the growth of the pathogens completely.

Keywords: Antimicrobial; Medicinal plants; Phytochemistry; Assay; *Phytophthora colocasiae*; *Fusarium oxysporum*.

INTRODUCTION

Fungi are a very useful class of microorganisms which mostly includes saprophytes, which live on dead organic (material) (Joshi *et al*., 2013). Many diseases caused by fungi in plants are responsible for yield losses in numerous economically important crops (Gawai, 2015). Soil-borne diseases are caused by a diverse group of fungi and related organisms which persist in the soil matrix and in residues on the soil surface. The most important soil borne pathogens genera include *Pythium*, *Phytophthora*, *Rhizoctonia* and *Fusarium*. *Phytophthora* and *Fusarium* are among the predominant soil borne pathogens (Rani and Sudini, 2013).

Soil-borne diseases are difficult to control because they are caused by pathogens which can survive for long periods in the absence of the normal crop host, and often have a wide host range including weeds (Rani and Sudini, 2013). Generally, phytopathogenic fungi are controlled by synthetic fungicides. However, these chemical fungicides are not readily biodegradable, they tend to persist for years in the environment and a few fungi have developed resistance to them (Ramaiah and Garampalli, 2015).

Plants contain thousands of constituents which are valuable sources of new and biologically active molecules possessing antimicrobial property (Gurjar *et al*., 2012). A precise evaluation of fungal sensitivity to plant extract is pivotal for the successful management of fungal diseases and to the relative analysis of antimicrobial agents. The lack of standardized *in vitro* methods for testing antimicrobial activities of plant crude extracts has led to variations in

Submitted: April 04, 2020
Revised: June 04, 2020
Accepted for Publication: June 06,2020
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results between research groups. Many problems associated with this research area lies on the various methods used in the extraction of plants and antimicrobial assessment (Othman *et al*., 2011). Owing to the rapid increase in fungal infections and the new attraction to the properties of new antimicrobial products, it is important to develop rapid and accurate methods of antifungal susceptibility assay for screening and quantifying the antimicrobial effect of an extract for its applications in human health; to increase safety of the food to the populace, agriculture and the environment (Balouiri *et al*., 2016). These antimicrobial activities of various plant extracts are scientifically proved (Sousa *et al*., 2012). In the present study, agar dilution, broth dilution, agar well diffusion and disc diffusion assays were evaluated for susceptibility of fungi (*Phytophthora colocasiae* and *Fusarium oxysporum*) to *C. odorata*. The efficacy of these methods was compared in order to determine the assay method with the most significant effect and to obtain a simple test to evaluate antimicrobials from leaf (extract).

MATERIALS AND METHODS

Collection of samples: The leaves of *C. odorata* were collected from Botanic Garden of the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka and confirmed at the Herbarium of the Department of Plant Science and Biotechnology. This study was carried out in Plant Pathology Laboratory, Department in the same Department.

Preparation of sample: Fresh leaves of *C. odorata* collected were washed with tap water and rinsed with sterile distilled water. Then kept dry under room temperature for two weeks, after which they were ground into fine powder and kept in sealed containers for extraction.

Extraction and Phytochemical analysis procedure: The extraction was carried out using modified soaking methods as described by Doherty *et al*. (2010).The ethanolic and methanolic extract of the plants were prepared by soaking 100 g of the ground plant samples in 1,000 ml of absolute ethanol and methanol respectively. The suspension was left overnight for 24 hours before filtering with No. 1 Whatman filter paper and concentrated using a rotary evaporator leaving behind the crude extracts. The crude extracts were collected in a sterile 1000 ml round bottom flask and stored in a refrigerator at 12°C until required.

The presence of biological active ingredients (alkaloid, saponin, tannins, flavonoid, terpenoids, tannins, steroids, hydrogen cyanides, phenols and glycoside) in the leaf extracts was investigated using different standard methods as described by (Anukworji *et* al., 2016 and Doherty *et al*. 2010)

Reconstitution of the Extract: The method described by Eze and Ezejiofor (2014) was used to reconstitute the Extract by dissolving the extract in 20% concentration of Dimethyl Sulphoxide (DMSO) (JHD, China) in the ratio of 1:10 (1g of crude extract dissolved in 10ml of DMSO) to give a concentration of 100mg/ml. Other concentrations of 50 mg/ml, 25 mg/ml, 12.5mg/ml and 6.25 mg/ml were made from the stock concentration (100mg/ml). Control plates containing 0.1 ml of 32 mg/l Fluconazole served as positive control while 1 ml of 20 % DMSO served as negative control.

Preparation of culture media: Potato Dextrose Agar (PDA) (Titam biotech, India) was used for both culturing and sub-culturing of fungi to obtain pure cultures while Muller Hinton Agar ((MHA) Titam biotech, India) was used for Disc diffusion, Agar well diffusion and Dilution assay. Following the modified method of Kalpana *et al*. (2013), MHA was prepared by dissolving 39 g of the commercially available Muller Hinton Agar in 1000ml of distilled water. The dissolved powder was autoclaved at 103KNM^{-2}for 15 minutes at 121°C.The autoclaved medium was mixed thoroughly and poured into sterile Petri dishes approximately 25ml and allowed to set at ambient temperature until when required. Potato Dextrose Agar (PDA) was dissolved in 500ml of water by boiling at the same time. The filtrate of potato broth was poured into the agar and dextrose was added and the volume restored to 1000ml with sterile distilled water. The medium were poured into two 500 ml conical flasks and test tubes plugged with cotton wool and sterilized by autoclaving at 103 KNM^{-2} pressures for 20 minutes at 121 ^{0}C. The medium was allowed to cool and 200 µg of Chloramphenicol was added. The medium were poured aseptically into sterile Petri dishes and allowed to solidify.

Sources and isolation of test organisms: Diseasedtaro leaves for the isolation of *Phytophthora colocasiae*was collected and confirmed at the herbarium unit of the Federal University of Agriculture, Umudike, while *Fusarium oxysporum* was collected from Plant Pathology Laboratory, Department of Plant Science and Biotechnology, University of Nigeria, Nsukka.The organisms were maintained on Potato Dextrose Agar (PDA) at 12°C and constantly revived on fresh PDA plates.

The isolation technique described by Chiejina (2008) was used for the fungal isolation while identification of fungal

isolates was based on their macroscopic and microscopic features, then confirmed with the aid of standard mycological identification texts by Agrios (2005) and Dugan (2017). Inoculum preparation was performed according to the method of Ohikhena *et al*. (2017).

Determination of *F. oxysporum* and *P. colocasiae* sensitivity to methanolic and ethanolic extracts of *C. odorata* using dilution and diffusion methods: Four assay methods were employed for the antifungal screening of the plant extracts. The Disc diffusion assay and Agar well diffusion assay were used to measure the inhibition zone diameter of the extracts (Bukar *et al*., 2010; Doherty *et al*., 2010; Pachkore *et al*., 2011) while agar dilution and broth dilution assays, according to the methods of Ohikhena *et al*. (2017) were used to determine the MICs of the extracts. The minimum extract concentration that inhibited the growth of the pathogens will be taken to be the minimum inhibitory concentration.

The experiment was conducted as a 2×2×6 factorial laid out in a completely randomized design (CRD) and data collected from the study were subjected to univariate analysis using IBM SPSS Statistics software version 23, and significant means were separated using least significance difference (LSD) at P ≤ 0.05.

RESULTS

The qualitative screening of the phytochemical constituents of the plant extracts showed that all the phytochemicals tested (terpenoids, tannins, alkaloids, saponins, steroids, hydrogen cyanides, flavonoids, phenols and glycosides) were present in different proportions as shown in Table 1 below. However, there was no observable significant difference in the phytochemical content between the ethanolic and methanolic extracts. Quantitatively, methanolic extract of *C. odorata* recorded significantly higher phytochemical content as compared to ethanolic extract except the hydrogen cyanide (HCN) which was not significantly different (Table 1).

Table 1. Phytochemical constituent of ethanolic and methanolic extracts of *Chromolaena odorata*

Phytochemical (mg/100 g)	Qualitative Screening		Quantitative Screening		t-value
	Ethanolic extract	Methanolic extract	Ethanolic extract	Methanolic extract	
Terpenoids	+	+	884.45 ± 0.22	904.72 ± 3.15	-6.42**
Tannins	+++	+++	2205.27 ± 0.03	2414.34 ± 0.00	-7174.25***
Alkaloids	++	++	331.01 ± 0.38	332.78 ± 0.01	-4.71**
Saponins	+	+	1.22 ± 0.00	1.89 ± 0.00	-144.500***
Steroids	+	+	0.67 ± 0.02	1.13 ± 0.00	-21.042**
HCN	+	+	2.11 ± 0.06	2.12 ± 0.00	-.049^{NS}
Flavonoids	++	++	532.60 ± 0.04	646.93 ± 0.01	-2996.72***
Phenols	++	++	1094.17 ± 0.50	1217.19 ± 0.01	-2446.29***
Glycosides	++	++	605.51 ± 0.11	613.63 ± 0.01	-73.81***

+ = present; ++ = highly present; +++ = very highly present

* = significantly higher at P ≤ 0.05; * = significantly higher at P ≤ 0.01; * = significantly higher at P ≤ 0.001; ^{NS} = not significantly different at P ≤ 0.05

The macroscopic and microscopic examinations of *P. colocasiae* on PDA plate showed no definite pattern. Young mycelia were fluffy and appeared as a pinkish white mass on the surface and reddish at the reverse side of the plate. The microscopic examination of the slide revealed the presence of ovoid to ellipsoidal sporangia borne on irregularly branched sporangiophores with hyphal swellings at branch points. Sporangia were semi-papillate and deciduous. Both hyphae and sporangiophores were non-septate (Plates 1 and 2).

White cottony mycelia of *F. oxysporum* were seen on PDA plates. The aerial mycelia were white and purple at the base. The colony grew rapidly, covering the entire plates within 5 days. The conidiophores were short. Macro conidia were fusiform, slightly curved, pointed at the tips, mostly monoseptate and scattered. Micro conidia were abundant, and ellipsoidal to cylindrical, as well as straight or curved (Plates 3 and 4).

The results of the minimum inhibitory concentration (MIC) of plant extracts against *P. colocasiae* and *F. oxysporum* using broth and agar dilution assay were summarized in Tables 2. The MIC values against *P. colocasiae* and *F. oxysporum* ranged from 6.25 mg/ml to 50 mg/ml across both assay methods and extracts. The methanolic extract recorded the lowest MIC value (6.25 mg/ml) on *F. oxysporum* using broth dilution assay method while ethanolic extract MIC value of (6.25 mg/ml) on *F. oxysporum* using broth dilution assay was low (Table 2).

Plate 1. Morphology of *P. colocasiae*

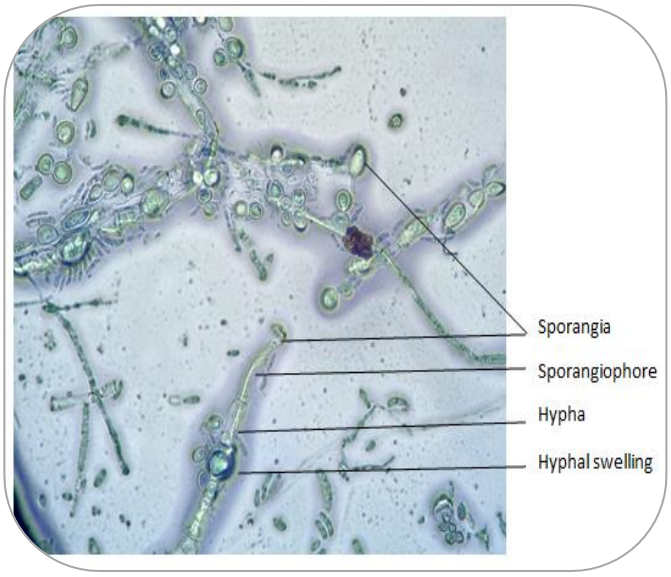

Plate 2. Photomicrograph of *P. colocasiae* (x400)

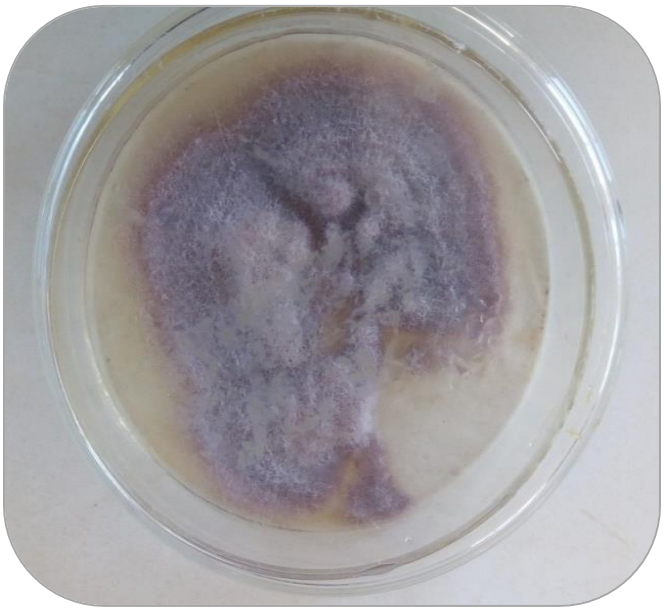

Plate 3. Morphology of *F. oxysporum*

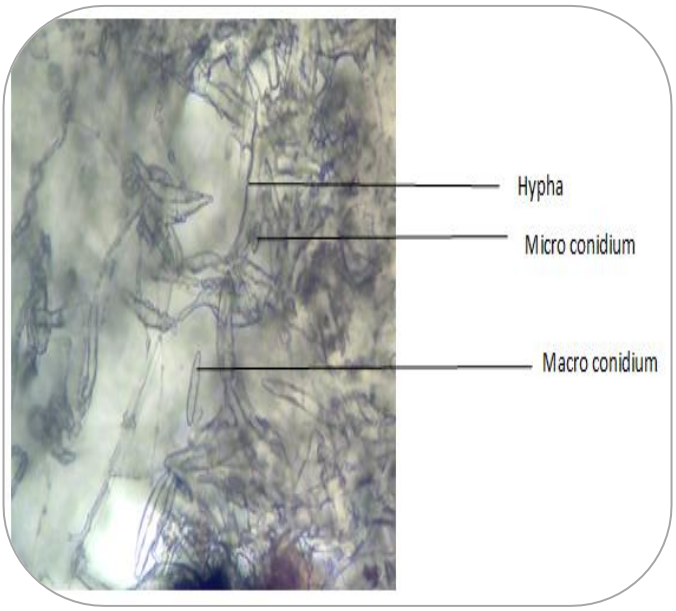

Plate 4. Photomicrograph of *F. oxysporum*(x400)

Table 2. Minimum inhibitory concentration (MIC) of *Chromolaena odarata* leaf extracts against *Phytophthora colocasiae* and *Fusarium oxysporum* using broth and agar dilution assay

Assay method	Organisms	Ethanol extract (mg/ml)	Methanol extract (mg/ml)
Broth dilution	*Phytophthora colocasiae*	25	50
	F. oxysporum	50	6.25
Agar dilution	*Phytophthora colocasiae*	12.5	50
	F. oxysporum	6.25	25

The result as presented in Table 3 reveals that the inhibition zone diameter (IZD) in *P. colocasiae* plate was significantly wider ($P < 0.05$) using disc assay method than the well method. On the other hand, ethanol extract significantly ($P<0.05$) inhibited *F. oxysporum* as compared to methanol extract. The different concentrations of *C. odorata* did not show significant difference ($P < 0.05$) in the IZD of *P. colocasiae* but the IZD of *F. oxysporum* were significantly ($P<0.05$) with lower concentration of *C. odorata* leaves extracts (6.25 mg/ml) than 50 and 100 mg/ml (Table 3). The combined effect of the extract and assay method reveals that combining ethanol extract of *C. odorata* with disc assay will effectively inhibit both *P. colocasiae* and *F. oxysporum* (Table 4).

Table 3. Mean effect of Assay method, Extract and concentration of *Chromolaena odarata* on Inhibition Zone Diameter of *Phytophthora colocasiae* and *Fusarium oxysporum*

	Phytophthora colocasiae	*Fusarium oxysporum*
Assay method		
Disc	16.78 ± 1.09*	16.22 ± 1.38
Well	13.03 ± 1.40	16.12 ± 1.33
Extract		
Methanol	14.03 ± 1.31	13.95 ± 1.41
Ethanol	15.78 ± 1.26	18.39 ± 1.17*
Concentration		
Control	30.08 ± 0.50^{a}	30.00 ± 0.49^{a}
100 mg/ml	10.11 ± 1.09^{b}	10.23 ± 1.18^{c}
50 mg/ml	12.48 ± 1.35^{b}	10.13 ± 0.98^{c}
25 mg/ml	12.00 ± 1.10^{b}	14.50 ± 2.27^{bc}
12.5 mg/ml	13.08 ± 1.29^{b}	14.75 ± 0.88^{bc}
6.25 mg/ml	11.67 ± 0.53^{b}	17.42 ± 1.35^{b}
LSD		

*-significant higher mean; means with different alphabets along each vertical array represents significant difference

Table 4. Combine mean effect of extract and assay method on Inhibition Zone Diameter of *Phytophthora colocasiae* and *Fusarium oxysporum*

		Phytophthora colocasiae	*Fusarium oxysporum*
Methanol	Disc	15.67 ± 1.60^{ab}	12.78 ± 1.94^{b}
	Well	12.40 ± 2.05^{b}	15.13 ± 2.08^{ab}
Ethanol	Disc	17.89 ± 1.49^{a}	19.67 ± 1.63^{a}
	Well	13.67 ± 1.95^{ab}	17.11 ± 1.67^{ab}

means with different alphabets along each vertical array represents significant difference

The combined effects between extract concentration and assay method are presented in Table 5. From the result, there was no significant difference in the IZD of *P. colocasiae* between the controlled concentration and the two extracts and assay methods used. Similarly, 12.5 mg/ml of ethanol extract recorded the widest inhibition zone of 15.33 ± 2.01 which was not significantly higher than other concentration induced IZDs. However, 25 mg/ml ethanol extract induced significantly ($P<0.05$) wider IZD on *F. oxysporum* than 50 and 100 mg/ml methanol and ethanol extracts concentrations. Furthermore, 6.25 mg/ml with well assay and 25 mg/ml with disc assay induced wider IZD of 19.83 ± 1.45 and 17.83 ± 3.95 respectively (Table 5). Generally, there was a decrease in IZD across all concentrations of the extract as compared to the control. However, 6.25 mg/ml methanol extract in well assay method inhibited *F. oxysporum* more after the control with a diameter of 20.67 ± 0.67 (Table 6). While 50 mg/ml ethanol extract in disc assay method inhibited *P. colocasiae* more after the control with a diameter of 19.00 ± 0.00.

Table 5. Combine mean effects between concentrations with extract and assay method on Inhibition Zone Diameter of *Phytophthora colocasiae* and *Fusarium oxysporum*

	Phytophthora colocasiae		*Fusarium oxysporum*	
	Extract			
	Methanol	Ethanol	Methanol	Ethanol
Control	30.33 ± 0.80^{a}	29.83 ± 0.65^{a}	30.00 ± 0.73^{a}	30.00 ± 0.73^{a}
100 mg/ml	10.23 ± 1.95^{b}	10.00 ± 1.21^{b}	7.13 ± 0.28^{e}	13.33 ± 1.50^{d}
50 mg/ml	10.47 ± 1.22^{b}	14.50 ± 2.22^{b}	7.92 ± 0.66^{e}	12.33 ± 1.36^{d}
25 mg/ml	10.33 ± 0.80^{b}	13.67 ± 1.89^{b}	8.50 ± 0.56^{e}	20.50 ± 2.84^{b}
12.5 mg/ml	10.83 ± 1.11^{b}	15.33 ± 2.01^{b}	14.33 ± 1.71^{cd}	15.17 ± 0.65^{bcd}
6.25 mg/ml	12.00 ± 0.37^{b}	11.33 ± 1.02^{b}	15.83 ± 2.24^{bcd}	19.00 ± 1.39^{bc}
	Assay method			
	Disc	Well	Disc	Well
Control	30.00 ± 0.73^{a}	30.17 ± 0.75^{a}	30.00 ± 0.73^{a}	30.00 ± 0.73^{a}
100 mg/ml	13.50 ± 0.76^{b}	6.73 ± 0.31^{b}	9.92 ± 1.28^{ef}	10.55 ± 2.12^{def}
50 mg/ml	15.83 ± 1.47^{b}	9.13 ± 1.18^{b}	10.42 ± 1.67^{def}	9.83 ± 1.19^{f}
25 mg/ml	12.67 ± 0.71^{b}	11.33 ± 2.16^{b}	17.83 ± 3.95^{bc}	11.17 ± 1.64^{d}
12.5 mg/ml	15.83 ± 1.54^{b}	10.33 ± 1.38^{b}	14.17 ± 1.05^{cdef}	15.33 ± 1.48^{bcd}
6.25 mg/ml	12.83 ± 0.40^{b}	10.50 ± 0.72^{b}	15.00 ± 1.88^{bcde}	19.83 ± 1.45^{b}

means with different alphabets along each vertical and horizontal array for each organism represents significant difference

Table 6. Effects of concentration, extract and assay method combination on inhibition Zone Diameter of *Phytophthora colocasiae* and *Fusarium oxysporum*

		Phytophthora colocasiae		*Fusarium oxysporum*	
		Disc	Well	Disc	Well
Methanol	Control	30.00 ± 1.15^{a}	30.67 ± 1.33^{a}	30.00 ± 1.15^{a}	30.00 ± 1.15^{a}
	100 mg/ml	14.33 ± 1.45^{bc}	6.12 ± 0.06^{h}	7.50 ± 0.50^{ghi}	6.77 ± 0.15^{i}
	50 mg/ml	12.67 ± 0.88^{cdef}	8.27 ± 1.37^{fgh}	6.83 ± 0.17^{hi}	9.00 ± 1.00^{fghi}
	25 mg/ml	11.67 ± 0.33^{cdefg}	9.00 ± 1.15^{defgh}	9.33 ± 0.67^{fghi}	7.67 ± 0.67^{ghi}
	12.5 mg/ml	13.00 ± 1.00^{cdef}	8.67 ± 0.67^{efgh}	12.00 ± 0.58^{defgh}	16.67 ± 2.96^{bc}
	6.25 mg/ml	12.33 ± 0.33^{cdef}	11.67 ± 0.67^{cdefg}	11.00 ± 1.15^{efghi}	20.67 ± 0.67^{b}
Ethanol	Control	30.00 ± 1.15^{a}	29.67 ± 0.88^{a}	30.00 ± 1.15^{a}	30.00 ± 1.15^{a}
	100 mg/ml	12.67 ± 0.33^{cdef}	7.33 ± 0.33^{g}	12.33 ± 1.45^{defg}	14.33 ± 2.85^{cde}
	50 mg/ml	19.00 ± 0.00^{b}	10.00 ± 2.08^{cdefgh}	14.00 ± 1.00^{cdef}	10.67 ± 2.33^{e}
	25 mg/ml	13.67 ± 1.20^{cd}	13.67 ± 4.06^{cd}	26.33 ± 2.33^{a}	14.67 ± 0.88^{cde}
	12.5 mg/ml	18.67 ± 1.67^{b}	12.00 ± 2.52^{cdefg}	16.33 ± 0.67^{bcd}	14.00 ± 0.58^{cdef}
	6.25 mg/ml	13.33 ± 0.67^{cde}	9.33 ± 0.88^{defgh}	19.00 ± 0.58^{bc}	19.00 ± 3.06^{bc}

means with different alphabets along each vertical and horizontal array for each organism represents significant difference

DISCUSSION

These results obtained from the phytochemical analyses of ethanolic and methanolic extracts of *C. odorata* revealed the presence of alkaloids, saponins, tannins, phenols, flavonoids terpenoids, steroids, hydrogen cyanide and glycosides. Similar bioactive constituents have been reported in the leaf extracts of different plants (Okwu and Joshia, 2006; Eze and Ezejiofor, 2014). The result showed that the methanolic extract had the highest production of phytochemicals than the ethanolic extracts except for hydrogen cyanide. According to Moonmun *et al*. (2017), the likely contributing factor to differences in phytoconstituents could be as a result of a change in solvent, method of extraction and influence of geographical region from which the plant was collected. The presence of these bioactive substances has been reported to confer resistance to plants against bacteria, fungi and pests (Piasecka *et al*., 2015) Thus, the antifungal property of *C. odorata* are primarily due to the presence of these phytochemicals, hence this therefore explains the demonstration of the antifungal activities by plants used in this study.

C. odorata contain fungitoxic compounds since they were able to inhibit the growth diameter of the test pathogens. This agrees with earlier reports of several workers on different fungal organisms (Mandal*et al*., 2007; Witayapan and Sombat, 2007; Okigbo *et al*., 2009; Bukar *et al*., 2010; Ijato *et al*., 2010). The ethanol and methanol extracts inhibition against *F. oxysporum* varied significantly. According to Abayhne and Chauhan, (2016), some plants may have different antimicrobial activity in different solvents. The possible reason may be that each plant contains different components in the form of secondary metabolites that have a different characteristic effect in various solvents which varies on the basis of physical and chemical properties. These properties have different modes of action on different microbes and may results in variable results This agrees with earlier reports of several workers on different solvents (Sen and Batra, 2012; Bassey *et al*., 2013 and Kalpana *et al*., 2013).

The findings showed that the antimicrobial activity of *C. odorata* extracts were dependent upon the assay method used. Both assay methods tested were effective in determining the antimicrobial activity of the plant extracts under study. In comparing these results, the inhibition zones for the disc diffusion assay were greater against *P. colocasiae* while well diffusion assay were greater against *F. oxysporum*. Generally, the well diffusion assay had lower inhibition zones for each of the plant extracts tested. This agrees with the work of King and Dykes (2008) that revealed that the well diffusion assay hindered diffusion of each of their test agents used to a larger extent, probably because the agents are not initially in direct contact with the organism but must first diffuse into the agar to exert an antimicrobial effect. Dimethyle sulphoxide (DMSO) was utilized as a negative control because it was used as a solvent in dissolving the extracts. DMSO showed no significant effect on the growth of *F. oxysporum* and *P. colocasiae.* Fluconazole were used as the positive control and recorded the highest inhibition zone diameter against *F. oxysporum* and *P. colocasiae*.

Conclusively, this study has shown that agar disc

diffusion assay proved to be more sensitive than well diffusion assay. Since disc diffusion method is easy and rapid to perform, it can be adapted for the routine antifungal susceptibility testing of fungi in clinical laboratories. The variability shown when different susceptibility testing methods were used suggests the need to apply multiple methods when conducting *in vitro* antimicrobial testing of plant extracts since a single assay method may result in misleading conclusions. This study demonstrated that when a diffusion method is used, multiple concentrations of the agent must be assayed to ensure that a relationship exists between the concentration of the agent and the inhibition zone size. When a relationship does not exist, antimicrobial activity should be determined by a quantitative dilution technique.

ACKNOWLEDGEMENT

The authors wish to thank the services of Mr. Eugene Ojua for the statistical analysis and contributions in developing this manuscript.

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Augusta Okoro	:	Conduct research and write manuscript and data analysis
Chiemeka Onaebi	:	Reviewed manuscript and help in conducting research