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Article

Phytochemical screening and antifungal activity of *Chromolaena odorata* extracts against isolate of *Phytophthora megakarya* using agar-well diffusion method

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Abstract: *In-vitro* study of *Chromolaena odorata* extracts was determined against isolated *Phytophthora megakarya* for the purpose of investigating the antifungal activity of ethanolic, cold-water and warm-water extracts of the medicinal plant against *Phytophthora megakarya*. Their antifungal effects were assessed with agar-well diffusion methods. Phytochemical screening and minimum inhibitory concentrations of the extracts were also determined. A qualitative phytochemical analysis of the extracts showed the presence of biologically active constituents such as flavonoids, phenols, tannins, sterols, amino acid and glycosinulate. Results showed that antifungal effects of *Chromolaena odorata* against *Phytophthora megakarya* were most efficient by ethanolic extracts, followed by extract of cold-water while hot water extract had no visible antifungal effect. Zones of clearance range from 15.00 mm to 32.00 mm using ethanolic extractions with concentrations from 1% to 5% while zones of clearance using cold water extract was from 5.00 mm to 30.00 mm with varying concentrations of 1% to 5%. The negative control using sterile distilled water showed no inhibitory effect on *Phytophthora megakarya* while 41.00 mm zone of clearance was observed in the positive control test using Metalaxyl and cuprous oxide-containing synthetic fungicide. This study provides a scientific confirmation of the use of *Chromolaena odorata* against cocoa pod infections by some local farmers.

Keywords: antifungal; extracts; cocoa pod; agar well; concentrations

1. Introduction

Phytophthora spp. is a cosmopolitan genus of Oomycete obligate plant pathogens containing approximately 60 described species (Erwin and Ribeiro, 1996). *Phytophthora* species attack a wide range of plants and are responsible for some of the world's most destructive plant diseases - examples include the European potato famine of the 19th century caused by *P. infestans* (Bourke, 1964). *Phytophthora* diseases have been well studied in the temperate regions of the world. However, *Phytophthora* diseases are very common throughout the wet tropical regions of the world and cause significant diseases losses in many tropical fruit crops in the form of root rots, collar rots, stem cankers, leaf blights and fruit rot. For example, *P. palmivora* alone causes a myriad of severe diseases on many different crops including: black pod of cocoa; root, stem and fruit rot of pawpaw; root rot and blight of citrus; bud rot in palms; black stripe in rot in durian.

Phytophthora megakarya appears to be confined to West Africa (Zentmeyer, 1987), where it is the most common species of Phytophthora causing black pod disease of cocoa. *Theobroma cacao* (cocoa) is indigenous to the New World and ranges from southern Mexico in the north to Brazil and Bolivia in the south. The centre of origin is considered to be the basin of the Upper Amazon (Wood, 1975). This would indicate that if *P. megakarya* is only present in West Africa, it must have another host that is as yet undetermined and cocoa is a new host. Alternatively, *P. megakarya* may have co-evolved with cocoa in South America, and subsequently been introduced to West Africa. No records appear to exist for *P. megakarya* outside West Africa and the only

host on which it has been recorded is cocoa. The capacity of *P. megakarya* to cause root infections is equivocal. Gregory *et al*. (1984) considered that root infection maintained a reservoir of inoculum, allowing zoospores to be released into the soil surface water. From there, the zoospores were spread up the plant by small splash droplets in convection currents into the leaf canopy. On pods, the first macroscopic sign of infection is observed about 2 days after initial infection, and is manifested as a minute translucent spot on the pod surface. Insects, particularly the small black ant (*Crematogaster striatula*), are responsible for moving inoculum from the soil to the canopy (Evans, 1973). These ants also use old infected pods to construct tents around the pod peduncle, and this can lead to infection from the peduncle region.

Wet, showery conditions are essential for infection and spread. Wood (1974) has shown that long periods of relative humidity at saturation point are required for the rapid spread of disease. The theory that relative humidity is the most important climatic factor helps to explain the higher incidence in Nigeria than in Ghana, and the almost complete absence of black pod disease in Malaysia.Black pod disease of cocoa in West Africa, caused almost exclusively by P. megakarya, still remains one of the most serious constraints on cocoa production. Surveys during the 1978 and 1979 harvest season in Togo revealed losses of up to 80%, when no control measures were taken (Djiekpor *et al*., 1981). Erwin and Ribeiro (1996) estimated a 20-30% loss of the world's cocoa crop to black pod, and in some areas they estimated that 90-95% of the crop is rendered unusable.

Symptoms in cocoa caused by *P. megakarya* can be easily confused with those caused by the three other species of Phytophthora which also cause black pod and include: *P. palmivora, P. capsici* and *P. citrophthora*. *P. palmivora* is the dominant species on cocoa in Nigeria and Cameroon (Gregory and Maddison, 1981; Zentmeyer, 1987).

Black pod caused by *P. megakarya* and *P. palmivora* can be distinguished because P. *megakarya* produces lesions with irregular edges on the fruit whereas lesions caused by P. *palmivora* have regular borders and are generally smaller (Erwin and Ribeiro, 1996). Pods are susceptible at all stages of development and may be infected at any place on the surface. The first symptom is a brown to black spot on the pod, which spreads rapidly in all directions and eventually covers the whole pod. The beans become infected internally about 15 days after the initial infection and are soon of no commercial value.

Generally, pods closest to the ground are first infected, with the disease rapidly spreading to affect fruit on the entire tree. *P. megakarya* can also cause seedling blight and trunk cankers (Zentmeyer, 1987), but its capacity to cause root rot is equivocal. Luz and Mitchell (1994) reported that even at high inoculum levels *P. megakarya* caused little damage to roots and no seedling mortality. Despreaux *et al*. (1987) also indicated that *P. megakarya* is not pathogenic to cocoa roots. Gregory *et al*. (1984), however, stated that *P. megakarya* is primarily a root-infecting pathogen.

Most synthetic fungicides used for the management and control of cocoa black pod infections contaminate stored foods commodity, leaving behind harmful residues, especially when application dosages are not properly followed (Dennis *et al*., 1994). Some exhibit phytotoxic effects on cocoa trees while in some, antimicrobial resistance may occur swiftly following repeated use. Bio magnification of some fungicides has also been reported by some workers. These shortcomings have led to loss of economic value on the produce as well as less acceptance from partner nations of the produce (Evans *et al.*, 1971). In order to circumvent these constraints, farmer must resort to more proactive, sustainable and environmentally friendly means of controlling and managing the disease caused by *Phytophthora megakarya.* The potency of *Chromolaena odorata* (Akintola) to control *Phytophthora megakarya* infection of cocoa pods as claimed by some local farmers is being investigated as an alternative environmentally acceptable biological control method.

The study covers the isolation of *Phytophthora megakarya* from the lesions of infected cocoa pod with concomitant determination of its susceptibility (using agar-well diffusion method) to varying amounts of extracts of *Chromolaena odorata* (Akintola) in comparison with a selected synthetic fungicide as the positive control. The specific objective of this study are to; isolate *Phytophthora megakarya* from the lesions of infected cocoa pods; determine its susceptibility to varying extracts of *Chromolaena odorata* (Akintola); compare the antifungal potencies of these extracts with a selected synthetic fungicide and determine the minimum inhibitory concentration (MIC) of the various extracts at various concentrations.

2. Materials and Methods

2.1. Media preparation

Medium used during the process of experiment was Difco product of Potato Dextrose Agar supplemented with streptomycin (PDA for selective isolation of *phytophthora megakarya*), the medium for isolation of Phytophthora was prepared by strictly following the manufacturer instructions and all the steps taken during the process of medium preparation were subjected to aseptic condition (sterile environment).

2.2. Collection of samples for the isolation of *Phytophthora megakarya*

Samples of infected cocoa pods were ramdomly selected from a cocoa plantation in Igodan community, Okitipupa local government area of Ondo state, and transported to Ondo State University of Science and Technology (Osustech) Microbiology Laboratory under aseptic condition. Scraps of lesions was aseptically and directly plated onto already prepared solid PDA.

2.3. Media and antibiotics for the isolation of *Phytophthora* from diseased plant tissue and soil

The Oomycetes are not true fungi, and therefore special techniques are required for their isolation. Most species of *Phytophthora* grow rather slowly *in vitro* compared with saprophytic fungi and bacteria. In addition, bacterial populations need to be kept low because they may suppress the growth of *Phytophthora* by direct competition, and antagonism caused by antibiotic production, or by direct parasitism. The use of selective media usually overcomes these problems. Antibiotics are added to isolation media in order to suppress the growth of bacteria.

2.4. Antibiotic stock solution preparation

Stock solution of the antibiotic was firstly prepared, by dissolving 500 mg of streptomycin powder in 10ml of sterile injection water, and then allowed to dissolve appropriately. The unused stock solution for antibiotic was stored at -4^{o}C.

2.5. Preparation of plant extracts and inoculum

Chromolaena odorata leaves were harvested at OSUSTECH farm, they were carefully washed in sterile distilled water, exactly 150 g was weighed using weighing balance and subsequently blended with the use of electric blender, the resulting mash was then steeped in 1litre of the three solvents used for the extraction (i.e ethanol, cold-water and warm –water) and then allowed to stand at room temperature for 24 hours. The resulting solution was subsequently diluted and was then subjected to different concentrations. A 48 h fungal cultures (isolated from infected cocoa pods) grown on selective PDA at room temperature were used for the test.

2.6. Antifungal activity screening test

The antifungal activity of aqeous ethanol extracts, cold-water and warm-water extract of *Chromolaena odorata* against the fungal isolate was screened by using the agar-well diffusion method. Approximately 3 ml of the antibiotic (streptomycin) prepared as described above was aseptically dispensed into PDA medium after it has been allowed to cool to 55^{o}C, the liquid medium was then poured onto sterile petri dishes and allowed to gel. Isolated pure culture of *Phytophthora megakarya* was used for the inoculation of the gelled medium. Sterile cork borer was used to make a well on each of the plates. 1%, 2%' 3%, 4% and 5% of each extracts was introduced into each well made on the medium, the plates were allowed to stand for 15minutes in order to allow for proper diffusion of the extract into the medium before incubation. The plates were incubated at room temperature for about three days and the resulting inhibition zones were measure and recorded in millimeters (mm).

2.7. Determination of minimum inhibitory concentration

The initial concentration of the plant extract (100 mg/ml from 150 g into 1000 ml) was diluted using double fold serial dilution by transferring 5 ml of the sterile plant extract (stock solution) into 5 ml of sterile Nutrient broth to obtain 50 mg/ml concentration. The above process was repeated several times to obtain other dilutions: 25, 12.5, 6.25, and 3.125 mg/ml (Rasooli and Abyaneh, 2004). Having obtained the different concentrations of the extracts, each concentration was inoculated with 0.1 ml of the standardized spore suspension and incubation was done at 30°C for 72 h. The growth of the inoculum in the broth is indicated by turbidity or cloudiness of the broth and the lowest concentration of the extract which inhibited the growth of the test organism were taken as the MIC (Jeff-Agboola and Onifade, 2016).

2.8. Phytochemical screening

Phytochemical examinations were carried out for all the extracts according to standard methods (Harborne, 1973; Odebiyi and Sofowora, 1978; Onwukeame, Ikuegbvweha and Asonye, 2007; Sofowora, 1982).

2.8.1. Detection of tannins

To the extract, 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of tannins.

2.8.2. Detection of flavonoids
Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids.

2.8.3. Detection of glycosides
Extract was mixed with 2ml of glacial acetic acid containing 2 drops of 2% FeCl3. The m ixture was poured into another tube containing 2ml of concentrated sulfuric acid. A brown ring at the interphase indicates the presence of glycosides.

2.8.4. Detection of saponins
Extracts were diluted with distilled water to 20 ml and this was shaken in a graduated cylinder for 15 min. Formation of 1 cm layer of foam indicates the presence of saponins.

3. Results
The results of the antifungal assay of the plant extract indicated that this plant exhibits antifungal activity against *Phytophthora megakarya* at different time interval. The result obtained in antifungal testing of the plant extract shows that the effectiveness of the plant extract varies with time and concentration of extracts. The results obtained from Agar-well diffusion method for warm-water extract showed that, warm water extract of this plant exhibited no inhibitory effect (0.00 mm) on the test fungus (*Phytophthora megakarya)* while the phytochemical analysis of warm-water extract revealed the absence of saponin and amino acid. In contrast phenol was tested positive with very high level, flavonoid and Glucosinulate were also tested positive but with low level, tannin was also detected at extremely low level for warm-water extract of *Chromolaena odorata*.
The results (Table 1) obtained from Agar-well diffusion method for ethanolic, warm and cold water extracts showed inhibitory effect at 1%, 2%, 3%, 4%, and 5% concentration with diammeter zones of clearance of 15.00 mm, 16.00 mm, 18.00 mm, 21.00 mm and 32.00 mm respectively for ethanolic extract and 5.00, 15.00, 18.00, 20.00 and 30.00 respectively for cold water while warm water has no zone of clearance for all concentrations. The negative control using sterile distilled water showed no inhibitory effect on *Phytophthora megakarya* while 41.00 mm zone of clearance was observed in the positive control test using Metalaxyl and cuprous oxide-containing synthetic fungicide as shown in Table 2.

Table 1. Potency of *Chromolaena odorata* extracts against *P. megakarya.*

Concentrations (%)	Diameter of zones of clearance (MM)		
	Ethanolic extract	Cold water extract	Warm water extract
1%	15.00[a]	5.00[b]	0.00
2%	16.00[a]	15.00[a]	0.00
3%	18.00[a]	18.00[a]	0.00
4%	21.00[a]	20.00[a]	0.00
5%	32.00[b]	30.00[a]	0.00

[ab] Values are means of three replicates determinations; SEM = Standard error of mean. Means with different superscripts in a row are significantly different ($P < 0.05$)

Table 2. Results for control test.

Parameters	Concentrations	Diameter of zone of clearance (mm)
Negative Control (70%)	0.2 ml sterile distilled water	0.00
Positive control (synthetic fungicide/ red-force)	5 mg	41.00

Table 3. Result for minimum inhibitory concentration.

Concentrations (mg/ml)	Cold-water extract	Ethanolic extract	Warm-water extract
50.00	-	-	+
25.00	-	-	+
12.50	-	+	+
6.25	+	+	+
3.123	+	+	+

(+) turbidity, (-) no turbidity

Table 4. Active ingredients tested in extracts of *Chromolaena odorata.*

Active agents	Cold-water extract	Warm-water extract	Ethanolic extract
Saponin	+	-	+
Tannin	+	+	+
Phenol	+	+	+
Flavonoid	+	+	+
Glucosinolate	+	+	+
Amino acid test	+	-	-

(+) = Positive (-) =Negative

Phytochemical screening revealed the presence of saponin, tannin, phenol, flavonoid and glucosinulate, while amino acid was tested negative for aqeous ethanol extract of *Chromolaena odorata*. Results obtained from Agar-well diffusion for cold-water extract showed high degree of antifungal activity at various concentrations used. All the biologically active agents were tested positive for cold-water extract which include saponin, phenol, glucosinulate, flavonoid, and amino acid. The concentrations used were prepared from the initial stock solution of extracts containing 150 g/l of distilled water. The results obtained on the minimum inhibitory concentration (Table 3) show that 50 mg/ml and 25 mg/ml of cold water extract of *Chromolaena odorata* had fungicidal effect against *Phytophthorame gakarya*. The results indicated that among the tested concentrations of cold water extract, there was a significant difference. On the other hand, extract of warm-water had neither fungicidal nor fungistatic effect against the test organism. Ethanolic extract also showed significant level of fungicidal activity at 50 mg/ml and 25 mg/ml.

The results obtained from phytochemical screening show that, tannin, phenol, flavonoid and glucosinulate were present in all the extracts irrespective of the method of extraction adopted. Amino acid was absent for both ethanolic and warm-water extract, whereas all the five biologically active agents screened tested positive for cold water extract. The warm-water extraction method might have influenced the absence of saponin as this active agent was also tested negative for in warm-water extracts.

4. Discussion

Table 1 clearly show that, *Phytophthora megakarya* exhibits varying degree of susceptibility to extract of *Chromolaena odorata* at various concentrations using cold water and ethanol as the extract compared to warm water extract in which no susceptibility was seen. Investigators in the past had also clearly shown that ethanolic extracts and cold-water extracts were more effective than warm-water extract (Bakht *et al.,* 2011). They have attributed this observation to the high volatility of ethanol which tends to extract more active compound from the sample than warm-water, which tends to have negative effect on amino acid properties and saponin. Hence, these studies followed similar trends. The efficiency of the antifungal activity of the extracts was enhanced by increase in the concentration of the extracts. This finding agrees with the report of Mares *et al*. (2004) that higher concentration of antimicrobial substance showed appreciation in growth inhibition.

The minimum inhibitory concentration values (Table 3) of the plant extracts against the test organism showed that fungi vary widely in the degree of their susceptibility to antifungal agents. Several reports stated that the extracts of medicinal plants play an important role in controlling many phytopathogenic fungi (Abd-El-Khair and Haggag, 2007; Choi *et al*., 2004; Lin, Zon, Lin and Tan, 2001; Okemo, Bias and Vivanco, 2003; Perez-Sanchez *et al.,* 2007). Results obtained from phytochemical screening of the extract (Table 4) showed that, the inhibitory effect of *Chromolaena odorata* plant might be due to the presence of steroids, terpenoids, alkaloids, flavonoids, glucosinulate, amino acid, tannins, phenolic compounds and saponins. In recent years much research has been conducted in the field of antifungal effects of different plants. In the present, investigation, the antifungal activity of the plant extracts was assayed against *Phytophthora megakarya* at different time interval and with different concentrations of extracts to understand the most effective concentration. Several workers have made similar observations by testing the antifungal activity of the extract or complex mixture from higher plants against some filamentous fungi, unicellular fungi and moulds (Gonzalez-Lamothe *et al.,* 2009). Some medicinal plants have higher antifungal properties and higher diffusion power (Gonzalez-Lamothe *et al.,* 2009).

The fact that the results of this study showed that plants extracts exhibited antifungal properties justifies their traditional use as medicinal plants. This may be due to the presence of active agents in the plant materials. Plants generally produce many secondary metabolites which constitute an important source of microbicides, pesticides and many pharmaceutical drugs (de Billerbeck *et al.,* 2001). Plant products still remain the principal source of pharmaceutical agents used in orthodox medicine.

5. Conclusions

These findings suggest a new pathway in elucidating a potent antifungal agent from plant like *Chromolaena odorata.* This study indicates that the plant contains antifungal compound that can be further developed as phytomedicine for the therapy of infection (Jeff-Agboola *et al.,* 2012). Such screening of various natural organic compounds and identification of active agents is the need of the hour because, this can effectively address the residual effects seen with the use of synthetic fungicides for the control and management of infections caused by the test fungus. In conclusion, the action of extracts upon the antifungal models justified its usefulness in herbal formulation. In conclusion, the results obtained from this study show that the plants extracts used in this study exhibit antifungal activities against *Phytophthora megakarya*. Extracts of the plant used in this study could be useful in the treatment of fungal infections caused by *Phytophthora megakarya*.

Conflict of interest

None to declare.

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