

## ***In Vitro* Evaluation of the Antibacterial Activity of Crude Extract from *Phytophthora palmivora* – Infected Cocoa Pods on selected Antibiotics Resistant Bacteria of Clinical Origin**

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**Abstract:** The antibacterial activities of crude dichloromethane extracts of cocoa pods infected with *Phytophthora palmivora* were screened against antibiotics resistant strains of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus* spp. and *Klebsiella* spp., by paper disc and agar diffusion methods. The crude extract which had been earlier reported to contain phytoalexins showed various levels of activity on different test organisms. The paper disc method showed that *Staphylococcus aureus* had the least susceptibility to the extracts with the diameter of the zone of inhibition that ranged from 2.0mm at 50mg/ml to 8.0mm at 250mg/ml while *Enterococcus* spp and *Klebsiella* spp had the highest diameter of the zone of inhibition of 11.0mm at 250mg/ml. The agar diffusion method showed that *Pseudomonas aeruginosa* was least susceptible with the diameter of the zone of inhibition that ranged from 4.0mm at 50mg/ml to 17.0mm at 250mg/ml. The susceptibility of the test organisms to the crude extract increased with increase in the concentration of the extract. This study showed that the dichloromethanolic crude extract of cocoa infected with *Phytophthora palmivora* may be incorporated into drug formulations for the treatment of antibiotics resistance bacterial related infections.

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### **1. Introduction**

Black pod disease of cocoa (*Theobroma cacao* L) is an economically serious problem in all area of the cocoa producing countries in the world (Ayeni, 2010). The disease is most severe in West Africa especially Nigeria, Ghana and Cameroon. The disease is caused by a complex species of *Phytophthora* namely *Phytophthora palmivora* and *Phytophthora megakarya* (Opeke, 1992). In response to infections, cocoa like other plants defend themselves against pathogens with their structural characteristics that act as physical barriers and biochemical reactions that take place in the cells and tissues of the plant and produce substances that are either toxic to the pathogen or create conditions that inhibit the growth of the pathogen (Agrios, 2005). This leads to formation of defense chemicals which are specifically produced for specific for pathogen. However, the name given to the group of such compounds is called “phytoalexins” (Pedras *et al.*, 2006; Mazarei *et al.*, 2008). This induced compound produced formed the basis for this study.

Antibiotics resistant bacteria dissemination in clinical and environmental settings has led to failure of several antibiotics which were earlier used against them. Moreover, their abundant presence in the clinical settings has made hospital acquired infections a regular case (Pollack, 2010). There have been several reports on the number of deaths resulting from nosocomial infections each year in different countries all over the

world (Gasink *et al.*, 2009). However, the use of antibiotics to treat these infections have posed a serious threat to the environment because of the increasing dissemination of antibiotics resistance genes and the acquisition of antibiotics resistance by commensals hence the need for an alternative (Wellington *et al.*, 2013).

Studies on the antibacterial activities of crude extracts of *Phytophthora palmivora* - infected cocoa pods have been reported. Fagbohun *et al.* (1987) screened two purified compounds extracted from infected tubers of *Dioscorea rotundata* against *Bacillus cereus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* and reported that dihydropinosylvin exhibited stronger antibacterial activity against all the test bacteria than batatasin IV. Moreover, Fagbohun and Lawal (2011) also reported the field trial of the crude extract of *Phytophthora palmivora* – infected cocoa pods to control black pod disease of cocoa in a farm. However, there is lack of information on the potential antibacterial activities of this crude extract on antibiotics resistant bacteria from clinical settings hence, the need for this study.

The aim of this study was to evaluate the antibacterial activity of crude extract from *Phytophthora palmivora* – infected cocoa pods on selected antibiotics resistant bacteria of clinical origin.

## 2. Materials and Method

### Source of cocoa pods

Freshly plucked matured and healthy cocoa pods were obtained from a cocoa plantation in Otun Ekiti, Ekiti State, Nigeria. The pods were washed with distilled water and air dried.

### Sources of test organisms

The *Phytophthora palmivora* strains were obtained from the Cocoa Research Institute of Nigeria, Idi-Ayunre Station in Oyo State, while the antibiotics resistant bacteria isolates used for this study (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus* spp. and *Klebsiella* spp.) were obtained from the stock cultures in the Medical Microbiology laboratory, Ekiti State University Teaching Hospital, Ado Ekiti, Nigeria. They were selected based on their resistance profile to antibiotics commonly used to treat infections associated with the pathogenic bacteria.

### Induction and extraction of crude extract

The induction was carried out according to the method of Brasier and Griffin (1979) whereby a sterile cork borer of 8 mm was used to cut the healthy matured unripened and uninfected cocoa pod husk. A mycelial disc of 6 mm diameter cut from the advancing edge of a 7 to 10 day old pure culture of *P. palmivora*, was placed into the hole made on the cocoa pods. The husks were replaced appropriately and sealed with sterile Vaseline. The infected pods and uninfected pods (positive control) were placed in a sterile cellophane nylon bag and watered daily for 5 days. After 5 days of infection, the cocoa pods showing symptoms of black pod were separated and chopped into pieces. About 1200g of the pieces was weighed and soaked into 1500 ml of redistilled dichloromethane (BDH Laboratory Supplies Poole, England) for 5 days at room temperature of 28°C. The extract was filtered using sintered funnel and sterilized by filtering, using a sterile 0.4 mm Millipore membrane filter. The filtrate was concentrated using rotatory evaporator at 40°C and stored in the refrigerator at 4°C until when required. The crude extract of uninfected pods was also extracted and stored with the described process.

### Dilution of extracts

About 50, 100, 150, 200 and 250mg of the crude extract obtained from infected pods and 50 and 100 mg of uninfected pods were dissolved in 1 ml of dichloromethane to make varying concentrations of 50, 100, 150, 200 and 250 mg/ml for infected and 50 and 100mg/ml for uninfected cocoa pods.

### Determination of the antibacterial activity of the crude extract

#### Standardization of bacterial cultures

The test bacteria were standardized according to the method described by Clinical Laboratory Standard Institute (CLSI) (2011). A sterile wire loop was used to

pick 3–5 morphologically similar colonies of the organism from a semi-confluent growth of isolates on Mueller Hilton Agar after overnight incubation and suspended in 5ml of sterile normal saline. The turbidity of the suspension was compared with 0.5 McFarland standard. A sterile swab stick was dipped into the normal saline suspension and the inocula were spread evenly over the entire surface of Mueller Hilton Agar plates.

#### Paper Disc Method

The methods of Fagbohun *et al.*, (2004) and CLSI (2011) were used. A sterile pair of forceps was used to load each disc with a prescribed amount of the compound in dichloromethane (50, 100, 150, 200 and 250 mg/ml). The discs, soaked in 2ml of their respective concentrations of the extract were allowed to dry before placing them on dried Mueller Hilton Agar plates. In each plate, there were three control discs, two discs were impregnated with 50 and 100 mg/ml of the extract obtained from uninfected cocoa pods while the third disc was dipped in dichloromethane only. The plates were incubated at 37°C and the diameter of zones of inhibition was measured after 24 hours.

#### Agar Diffusion Method

The methods of Isu (2005) and CLSI (2011) were used. Different concentrations of each compound 50, 100, 150, 200 and 250 mg/ml were introduced into each of the five wells. The sixth and seventh wells (positive control) were filled with 50mg/ml, 100mg/ml of crude extract obtained from uninfected cocoa pods while the eighth well was filled with dichloromethane which served as the negative control. This method was carried out for each of the test bacteria. The plates were then incubated at 37°C for 24 hours after which the zones of inhibition were measured.

The crude extract was classified as active when the diameter of the inhibition was equal to or larger than 8mm (Omar *et al.*, 2000). All the assays were performed in triplicate and expressed as average values.

## 3. Results and Discussion

The result of this study was discussed with reference to closely related works due to few information that exist for the antibacterial activity of induced compounds used against clinical isolates. The antibacterial activity of the dichloromethane extracts of the *Phytophthora palmivora* – infected cocoa pods using the paper disc method are shown in Table 1. The results showed that all the test bacteria were sensitive to the extract at various concentrations. The control plates had little or no zone of inhibition while there was visible zone of inhibition on other plates suggesting the activity of the extracts on the test organisms. The effect of the extracts on *E. coli* increased with as the concentration of the extracts increase from 3.0mm at 50mg/ml to

10.0mg/ml at 250mg/ml. The susceptibility trend of *Pseudomonas aeruginosa* and *Staphylococcus aureus* were similar with the diameter of the zone of inhibition that ranged from 2.0mm at 50mg/ml to 9.0mm at 250mg/ml. *Enterococcus* spp. and *Klebsiella* spp showed the highest susceptibility with the diameter of the zone of inhibition of 11.0mm at 250mg/ml.

In this study, all the test organisms were susceptible at the highest concentration of 250mg/ml of the extracts. The extract was said to be active only on *Escherichia coli*, *Enterococcus* spp and *Klebsiella* spp

at 200mg/ml while it was active on all the test organisms at 250mg/ml. The result of this study is in agreement with the findings of Omar *et al.*, (2000) who reported that extract from plants are said to be active at diameter of zone of inhibition of 8.0mm and above. Moreover, Palombo and Semple (2002) reported a high percentage susceptibility methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant enterococci (VRE) to Australian plants extracts at varying concentration.

Table 1: Antibacterial activity of the crude *Phytophthora palmivora* – infected cocoa pods extract using the paper disc method (diameter of the zone of inhibition measured in mm)

Test organisms	*Negative Control	**Positive control (mg/ml)		Concentration of extract in mg/ml				
		50	100	50	100	150	200	250
<i>Escherichia coli</i>	0.0	0.0	0.0	3.0	5.0	7.0	8.0	10.0
<i>Pseudomonas aeruginosa</i>	0.0	0.0	0.0	2.0	3.0	5.0	6.0	9.0
<i>Staphylococcus aureus</i>	0.0	1.0	1.0	2.0	3.0	5.0	6.0	8.0
<i>Enterococcus</i> spp.	0.0	0.0	0.0	3.0	4.0	6.0	9.0	11.0
<i>Klebsiella</i> spp.	0.0	0.0	0.0	2.0	5.0	7.0	9.0	11.0

\*Negative control (paper disc dipped in dichloromethane only)

\*\* Positive control (paper disc dipped in 2ml of varying concentrations of crude extract from uninfected cocoa pods)

Table 2: Antibacterial activity of the crude *Phytophthora palmivora* – infected cocoa pods extract using the agar diffusion method (diameter of the zone of inhibition measured in the mm)

Test organisms	*Negative Control	**Positive control (mg/ml)		Concentration of extract in mg/ml				
		50	100	50	100	150	200	250
<i>Escherichia coli</i>	0.0	1.0	1.0	6.0	8.0	11.0	14.0	18.0
<i>Pseudomonas aeruginosa</i>	0.0	1.0	1.0	4.0	7.0	10.0	13.0	17.0
<i>Staphylococcus aureus</i>	0.0	0.0	0.0	5.0	7.0	12.0	15.0	18.0
<i>Enterococcus</i> spp.	0.0	2.0	3.0	6.0	9.0	12.0	16.0	21.0
<i>Klebsiella</i> spp.	0.0	0.0	1.0	4.0	7.0	11.0	15.0	20.0

\*Negative control (well filled with dichloromethane only)

\*\* Positive control (well filled with crude extract from uninfected cocoa pods)

The antibacterial activity of the dichloromethane extracts of the *Phytophthora palmivora* – infected cocoa pods using the agar diffusion method are shown in Table 2. The results showed that all the test bacteria were sensitive to the extract at various concentrations. The control plate had no zone of inhibition while there was visible zone of inhibition on other plates suggesting the activity of the extracts on the test organisms. The extract was active only on *Escherichia coli* and *Enterococcus* spp at 150mg/ml while all the test organisms were active at 200mg/ml and 250mg/ml.

The effect of the extracts on all the test organisms increased with the increase in concentration of the extracts with *Pseudomonas aeruginosa* having the least diameter of the zone of inhibition of 17.0mm at 250mg/ml while *Enterococcus* spp. has the highest diameter of zone of inhibition with 21.0mm at 250mg/ml. The result of the study is similar to the work of Duyilemi and Fagbohun (2007) who reported

an increase in the diameter of the zone of inhibition of *Klebsiella* spp. and some other pathogenic bacteria when the concentration of the crude extract from cocoa pods infected with *P. palmivora* was increased. Similarly, Reddy and Jose (2010) also reported that the methanolic extract of test plants exhibited marked activity against *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella* spp. and *Pseudomonas aeruginosa*.

The agar diffusion method showed more antibacterial activity than the paper disc method. This may be due to the absorptive ability of the disc and the diffusion ability of the extract in the medium. It is worthy of note that the crude extract obtained from uninfected cocoa pods when compared with the extract obtained infected cocoa pods suggest that the extract from infected cocoa pods contains some active compounds which was present as a result of the induction by the infecting pathogen (*Phytophthora palmivora*) as reported by Pedras *et al.*, (2006). Also,

the activity of the crude extracts may be attributed to the presence of 7, 8, 9, 10-tetrahydro-5, 8-dihydroxynaphthalene and ester of glycerol as reported by Fagbohun (2012).

#### 4. Conclusion

This study showed that the dichloromethanolic extract of cocoa infected with *Phytophthora palmivora* contains active compounds which exhibited antibacterial activity against all the test organisms. These active compounds when extracted and purified may be incorporated into drug formulations for the treatment of antibiotics resistant bacteria related infections. In addition, the extract can be further investigated for their *in vivo* antibacterial activity on experimental animals.

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