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Spectrophotometric Studies of the Antibacterial Activities of Crude Extract from Cocoa Pods Infected with *Phytophthora palmivora*

Emmanuel Dayo Fagbohun[1], Olajide Sunday Faleye[2], Opeyemi Uwangbaoje Lawal[3*] and Oluwadare Akinyemi[4]

[1]Department of Microbiology, Ekiti State University, P.M.B.5363, Ado Ekiti, Ekiti State, Nigeria.
[2]Department of Microbiology, Federal University of Technology, Akure, Nigeria.
[3]Department of Biological Sciences, Evangel University Akaeze, P.M.B. 129, Abakaliki, Ebonyi State, Nigeria.
[4]Department of Mathematics, Ekiti State University, Ado Ekiti, Nigeria.

Authors' contributions

Author EDF initiated, designed and wrote the protocol for the study. Authors OSF and OUL carried out the microbiological analysis, managed the raw data, interpreted the results and managed the literature search. Author OA carried out the statistical analysis. Author OSF wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To study antibacterial activities of the dichloromethane extract of cocoa pods infected with *P. palmivora* on selected bacteria pathogens using paper disc bioassay technique, agar diffusion techniques and spectrophotometric methods.
Place and Duration of Study: Department of Microbiology, Ekiti State University, Ado Ekiti, (formerly University of Ado Ekiti) Nigeria, between January and July, 2010.
Methodology: The crude extract of cocoa pods infected with *Phytophthora palmivora* was extracted using dichloromethane. The extract was concentrated using rotary evaporator at 40°C and stored at 4°C. Selected bacteria isolates pathogenic to plants and human were obtained from

**Corresponding author: E-mail: opeyemil@ymail.com;*

International Institute for Tropical Agriculture (IITA) and Ekiti State University Teaching Hospital, Ado Ekiti, Nigeria respectively. The antibacterial study was carried with different concentrations of the extracts 50, 100, 150 and 200 mg/ml using spectrophometric methods, paper disc and agar diffusion techniques.

Results: The results of the antibacterial activity using paper disc method showed that *Xanthomonas* sp. (Cp) was highly susceptible to the extract with highest zone of inhibition followed by *Bacillus* sp. (R). *E. coli*, *Shigella* sp., *Pseudomonas* sp (R) and *Xanthomonas* sp (R) had no zone of inhibition. The results of the antibacterial activity using agar diffusion method showed that *Xanthomonas* sp. (Cp) and *Bacillus* sp. (R) were highly susceptible to the induced extract with the highest the zone of inhibition followed by *E. coli*, *Shigella* sp., *Pseudomonas* sp. (R) and *Xanthomonas* sp. (R). The result of the antibacterial activity using spectrophotometric method showed that *Xanthomonas* sp (R), *Xanthomonas* sp (Cp), *Pseudomonas* sp. (R), *Bacillus* sp (R), *E. coli* and *Shigella* sp were highly susceptible to the extract at different concentrations.

Conclusion: This present study showed that extract from infected cocoa pods can serve as a good source of antibacterial agent. Further works should be carried out to purify and determine the structures of the active compounds.

Keywords: Spectrophotometry; paper disc method; agar diffusion method; crude extract; infected cocoa pods.

1. INTRODUCTION

Cocoa (*Theobroma cacao* L.) belongs to the genus *Theobroma* in the family Sterculiaceae which include 50 genera and 700 or more species of tropical trees and shrubs. It grows in a limited geographical zone of approximately 20 degree to the North and South of the Equator [1]. Several diseases such as cacao nepovirus causing necrosis, Cacao Swollen Shoot Badnavirus (CSSV) causing swollen shoot, *Moniliophthora pernicosa* causing Moniliophthora pod rot and *Phytophthora palmivora* causing black pod diseases have been reported to affect cocoa tree with the later being the most severe and causing annual crop losses of 30-40% [2-4]. Several attempts to combat these diseases with chemicals have been successful. However, the demerits of convectional fungicides such as its effect on non-target organisms, phytotoxic effect, resistance of pathogens, risk to human life and bioaccumulation in the environment has made the search for alternatives imperative [5].

The concept that induced compounds are formed in plant cells as a response to infection is analogous to the antigen-antibody response in animal tissues. These induced compounds produced plants are called Phytoalexins [6]. Phytoalexins are low molecular weight antimicrobial compounds of various chemical structures which are elicited in plant tissues by either biotic (pathogen challenge) or abiotic (injury, chemicals and irradiations) stress factors [6,7]. The induced mechanisms are associated with local changes at the site of pathogen infection, such as hypersensitive response [6]. Moreover, many of these compounds have been reported to possess antifungal properties [8-11] but few antibacterial properties of this induced compound have been reported [12-15].

The present study was focused to determine the antibacterial activities of induced extract from cocoa pods infected with *Phytophthora palmivora* against some selected bacterial pathogens of both plants and animal origin.

2. MATERIALS AND METHODS

2.1 Source of Plant Materials

Fresh, green and healthy cocoa pods without injury were collected from a cocoa plantation in Ado Grammar School, Ado-Ekiti, Nigeria.

2.2 Source of Microorganisms

Cultures of *Xanthomonas sp (*R*)*, *Xanthomonas* sp (cowpea: Cp), *Bacillus* sp (rice: R), *Pseudomonas* sp (R) were obtained from International Institute of Tropical Agriculture (IITA), Ibadan, *Escherichia coli*, *Shigella* sp were obtained from Ekiti State University Teaching Hospital, Ado Ekiti, Nigeria while *Phytophthora palmivora* was obtained from the Cocoa Research Institute of Nigeria, Ibadan, Oyo-State, Nigeria.

2.3 Induction and Extraction of Crude Extract

Plugs of cocoa husks were cut from healthy mature cocoa pods using a sterile cork borer of 8 mm diameter. A mycelial disc of 6 mm diameter cut from the advancing edge of 5 day old cultures of *P. palmivora* was introduced into each opening made by the cork borer on the pods. After that husk plugs were replaced and the edges were sealed with sterile vaseline. Each pod was put into a white transparent cellophane bag, and the pods were sprayed daily with distilled water and kept at room temperature of 28°C. After 8 days of infection of the pods, cocoa pods showing symptoms of black pod disease were separated and cut into smaller pieces. About 1200 g of the pieces was soaked into 1500 ml of redistilled dichloromethane for 7 days at a 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 in a rotary evaporator at 40°C and stored at 4°C until required for use.

2.4 Determination of the Antibacterial Activities of the Induced Extract

The antibacterial activity was determined using the paper disc method, agar diffusion method [16] and Spectrophotometric method [17].

2.4.1 Standardization of inoculums

A flamed and cooled wire loop was used to pick inoculum from the stock culture (on slant) and then streaked on sterilized nutrient agar (Peptone 5.0 g/l, beef extract 3.0 g/l, sodium chloride 8.0 g/l, Agar No. 2 12.0 g/l; Oxoid, United Kingdom) plate. The plates were incubated at 37°C for 18 h. After the incubation, about 3 to 5 colonies were picked with a sterile loop from the plate and transferred into test tubes containing sterile nutrient broth (Peptone 5.0 g/l, beef extract 3.0 g/l, sodium chloride 8.0 g/l; Oxoid, United Kingdom). The preparation was left stand for 3 to 5 h until turbidity matched that of freshly prepared Barium Sulphate (0.5 Mcfarland standard).

2.4.2 Agar diffusion method

In this method, 0.1 ml of standardized inoculum of each of the test bacteria was aseptically transferred to solidified nutrient agar plates and spread over the surface with a sterilized glass spreader. The plates were allowed to dry for one hour of pre-diffusion. Using a flamed cork borer (7.0 mm diameter) two wells of equidistance were cut in each of the plates. Different concentrations of the extracts 50, 100, 150 and 200 mg/ml were introduced into one of the two wells in each of the plates while the second well contained the same amount of the extracting solvent only which served as control. The plates were incubated at 30°C for 24 h after which the diameter of the zone of inhibitions were measured with the aid of a pair of caliper (in mm). All plates were made in duplicates.

2.4.3 Paper disc method

About 0.1 ml of standardized inoculum of each of the test bacteria was aseptically transferred to each petri-dishes containing solidified nutrient agar. A sterile glass spreader was used to spread this evenly over the surface of the nutrient agar. The plates were allowed to dry for one hour of pre-diffusion. Sterile filter discs (6.00 mm in diameter) were soaked in various concentrations of the crude extract (50, 100, 150 and 200 mg/ml). Enough time was allowed for the solvents to dry before transferring the discs to the surfaces of the agar plates by a pair of sterile forceps. For control experiment, the paper discs were soaked in extracting solvent. The plates were incubated at 30°C for 24 h after which the zone of inhibition were measured. All plates were made in duplicate.

2.4.4 Spectrophotometric method

Kinetics of the antibacterial activity of the extracts were determined in nutrient broth (LAB) using the methods described by [17]. The different strains of the organisms were exposed to increasing concentrations. About 0.2 ml of the bacterial suspension was inoculated into 9.8 ml of freshly prepared nutrient broth (35°C) containing different concentrations (50, 100, 150 and 200 mg/ml) of the extracts. About 1.0 ml of the suspension was withdrawn immediately and the absorbance (optical density) was read with a Snimadzu spectrophotometer (Models 6400/6405, Jenway, England) to give a control zero time. Absorbance samples were taken at intervals of one hour over 5 h and read with a Shimadzu spectrophotometer at wavelength 540 nm.

2.4.5 Statistical analysis

The data obtained were analysed for mean, standard deviation, standard error and analysis

of variance with SPSS version 17 software. Duncan multiple range test (DMRT) was used to determine the P values at p = 0.05.

3. RESULTS AND DISCUSSION

The results of the antibacterial activities of redistilled dichloromethane extract of cocoa pods infected with *P. palmivora* against the test bacteria using paper disc method are shown in Table 1. *Xanthomonas* sp (Cp) had a zone of inhibition that varied from 6mm at 50.0 mg/ml to 13.0 mm at 200.0 mg/ml, *Bacillus* sp (R) had a zone of inhibition of 3.0 mm at 50 mg/ml to 8.0 mm at 200 mg/ml. Also, *Shigella* sp had a zone of inhibition of 2.0 mm at 50 mg/ml to 8.0 mm at 200 mg/ml while *Xanthomonas* sp (R) and *Pseudomonas* sp (R) had no zone of inhibition at all concentrations of the extract used.

This result revealed that increasing concentrations of the extract increased the zone of inhibition which implies progressive susceptibility of the test bacteria. This result is in agreement with the findings of [13] who reported the susceptibility of the test bacteria (*Enterobacter aerogenes*, *Klebsiella pneumonia*, *Proteus mirabilis*, *Shigella sonnei*) when the concentration of cocoa extract was progressively increased. In addition, [18] reported a progressive increase in the zone of inhibition with increased concentration of ethanolic leaf extract of whole plant *Indonessiiella echioides* on the test bacteria. The induced extract had weak antibacterial activity on *Bacillus* sp (R) (3.0 mm), *E. coli* (3.0 mm) and *Shigella* sp (2.0 mm) at 50 mg/ml while their control was 0.0 mm. It had a moderate antibacterial effect on *Bacillus* sp (R), *E. coli* and *Shigella* sp at 100 mg/ml with zone of inhibition that varied from 4.0 to 5.0 mm while the control was 0.0 mm. It had strong antibacterial activities on *Xanthomonas* sp (Cp) at all concentrations used with zone of inhibition of 6.0 mm (50 mg/ml) to 13.0 mm (200 mg/ml).

The result of the antibacterial activities of induced extracts from cocoa pods on the test bacteria using agar diffusion method are shown in Table 2. *Xanthomonas* sp (Cp) had a zone of inhibition that varied from 2.0 mm (50 mg/ml) to 8.0 mm (200 mg/ml), *Bacillus* sp (R) had a zone of inhibition of 3.0 mm (50 mg/ml) to 7.0 mm (200 mg/ml), *E. coli* had a zone of inhibition of 1.0 mm (50 mg/ml) to 5.0 mm (200 mg/ml). Also, *Shigella* sp had a zone of inhibition of 1.0 mm (50 mg/ml) to 4.0 mm (200 mg/ml) while *Xanthomonas* sp (R) and *Pseudomonas* sp (R) had no zone of inhibition at all concentrations used.

This implied that the test bacteria were inhibited with increasing concentrations of the extract. This is in agreement with the work of [14] who reported increased antibacterial activities of *Artemisia nilagirica* leaf extract on *Erwinia* sp., *Xanthomonas campestis*, *Clavibacter michiganese* and *Pseudomonas syringae* when the concentration was increased. The crude extract exhibited antibacterial effect against *Xanthomonas* sp (Cp) and *Bacillus* sp (Cp) at 50 mg/ml with zones of inhibition of 2.0 mm and 3.0 mm respectively while their control was 0.0 mm. It had a strong antibacterial effect on *Xanthomonas* sp (Cp) and *Bacillus* sp (R) at 150 and 200 mg/ml with zones of inhibition of 6.0 to 8.0 mm and 7.0 to 8.0 mm respectively while the control was 0.0 mm.

The results of the antibacterial activities of the induced extract against test bacteria using spectrophotometric method at 540 nm are shown in Figs. 1 to 6. The antibacterial activities of different concentration of the extract against *E. coli* are shown in Fig. 1. At concentration of 50 mg/ml, the optical density decreased from initial reading of 0.29 at zero h to 0.22 at the 5th h. While at higher concentration of 200 mg/ml, the density decreased to 0.19 at the 5th h. The antibacterial activities of different concentration of the extract against *Bacillus* sp (R) are shown in Fig. 2. At concentration of 50 mg/ml, the optical density decreased from initial reading of 0.30 at zero h to 0.25 at the 5th h. While at higher concentration of 200 mg/ml, the density decreased to 0.17 at the 5th h. The antibacterial activities of different concentration of the extract against *Pseudomonas* sp (R) are shown in Fig. 3. At concentration of 50 mg/ml, the optical density decreased from initial reading of 0.30 at zero h to 0.23 at the 5th h. While at higher concentration of 200 mg/ml, the density decreased to 0.17 at the 5th h. The antibacterial activities of different concentration of the extract against *Xanthomonas* sp (R) are shown in Fig. 4. At concentration of 50 mg/ml, the optical density decreased from initial reading of 0.35 at zero h to 0.30 at the 5th h. While at higher concentration of 200 mg/ml, the density decreased to 0.25 at the 5th h. The antibacterial activities of different concentration of the extract against *Xanthomonas* sp (Cp) are shown in Fig. 5. At concentration of 50 mg/ml, the optical density decreased from initial reading of 0.35 at zero h to 0.29 at the 5th h. While at higher concentration of

200 mg/ml, the density decreased to 0.24 at the 5th h. The antibacterial activities of different concentration of the extract against *Shigella* sp are shown in Fig. 6. At concentration of 50 mg/ml, the optical density decreased from initial reading of 0.28 at zero h to 0.20 at the 5th h. While at higher concentration of 200 mg/ml, the density decreased to 0.17 at the 5th h.

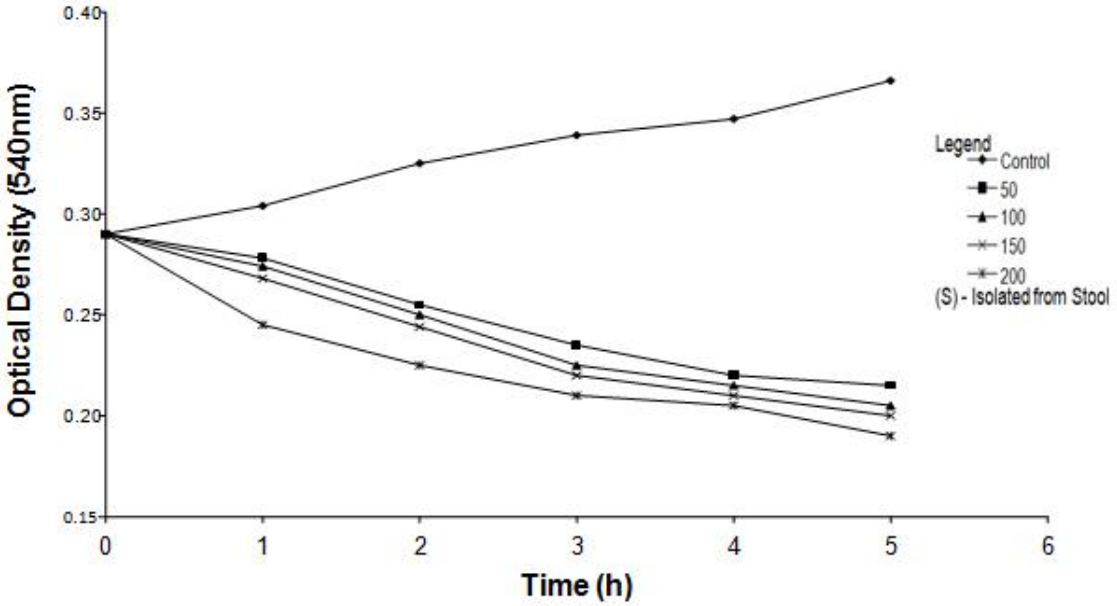

Fig. 1. The effect of crude extract of cocoa pods infected with *P. palmivora* on the growth of *Escherichia coli (S)*

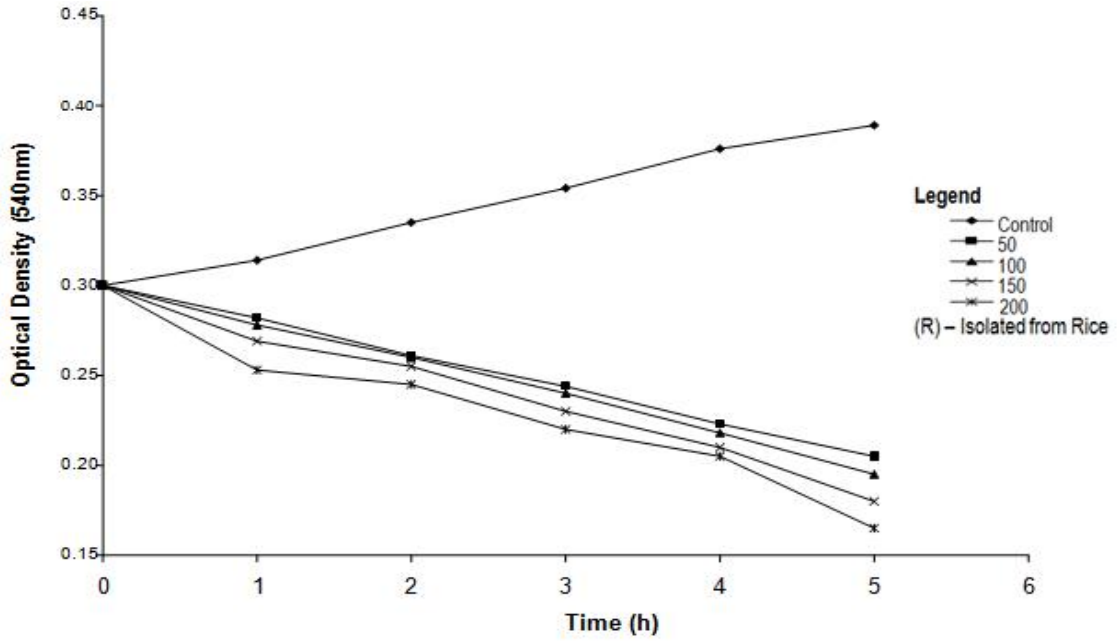

Fig. 2. The effect of crude extract of cocoa pods infected with *P. palmivora* on the growth of *Bacillus* sp (R)

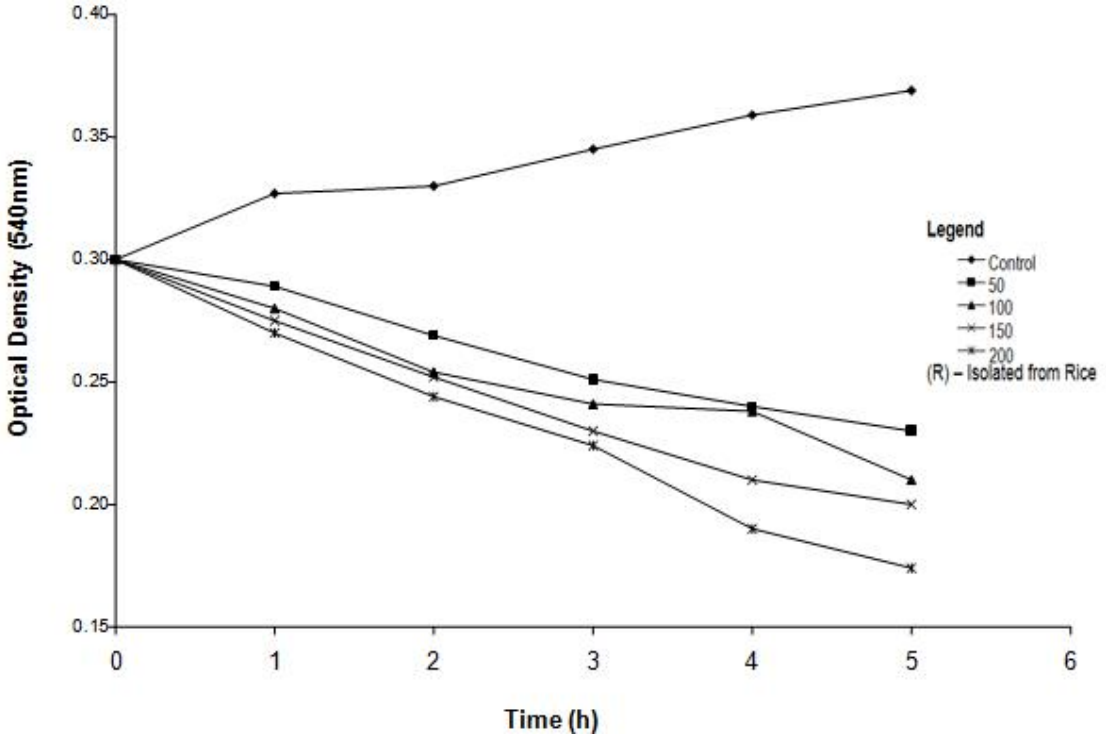

Fig. 3. The effect of crude extract of cocoa pods infected with *P. palmivora* on the growth of *Pseudomonas* sp (R)

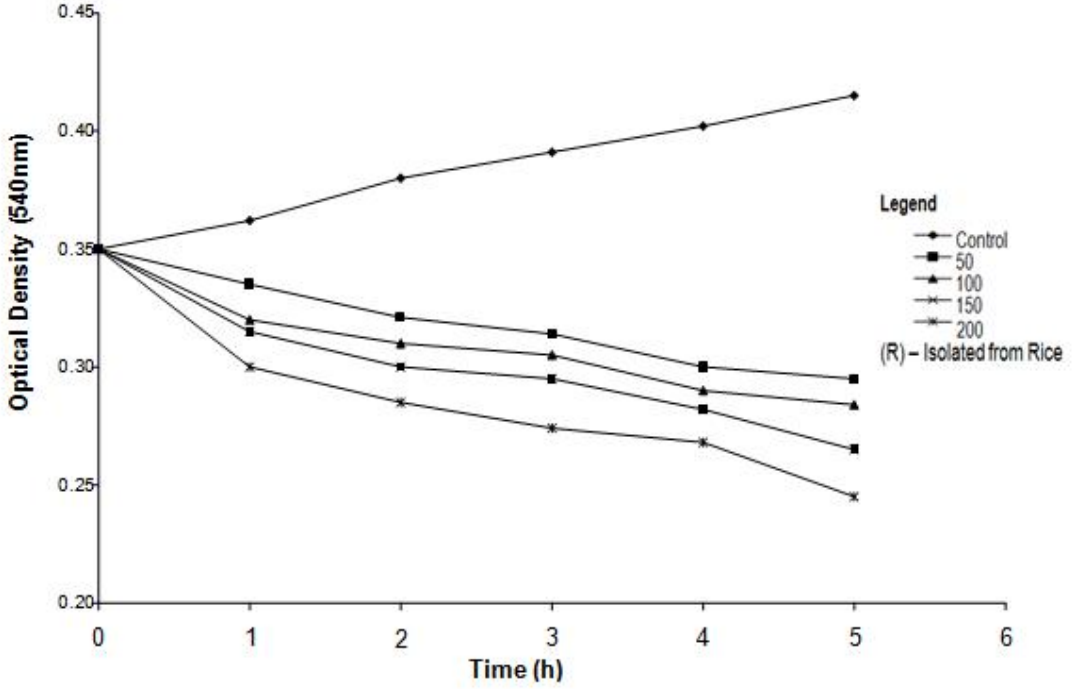

Fig. 4. The effect of crude extract of cocoa pods infected with *P. palmivora* on the growth of *Xanthomonas* sp (R)

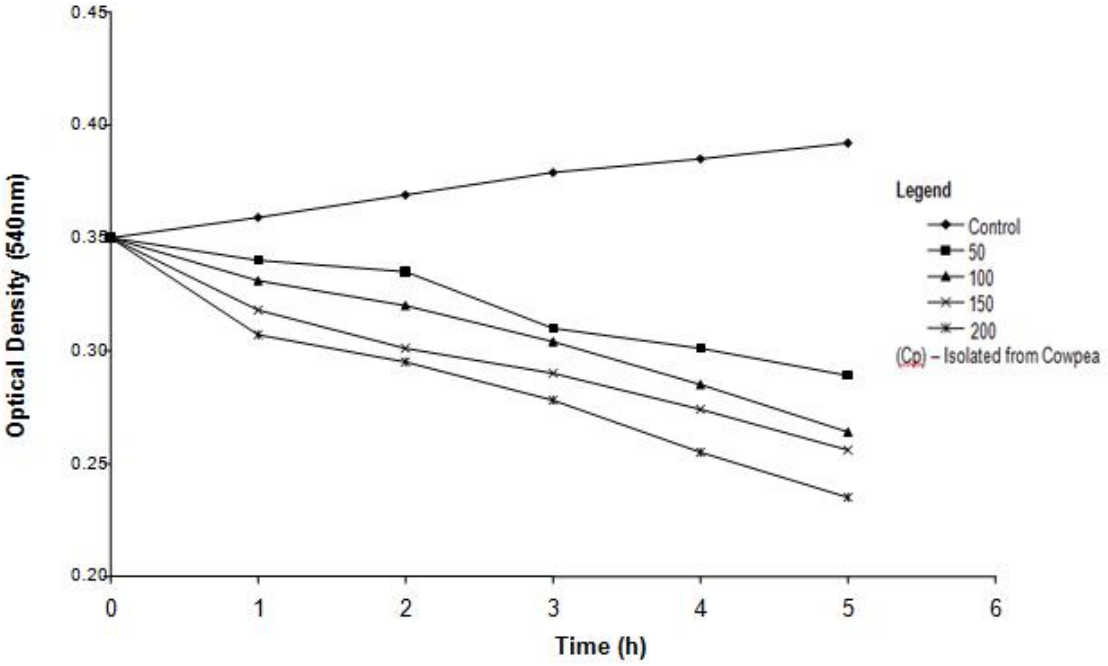

Fig. 5. The effect of crude extract of cocoa pods infected with *P. palmivora* on the growth of *Xanthomonas* sp (Cp)

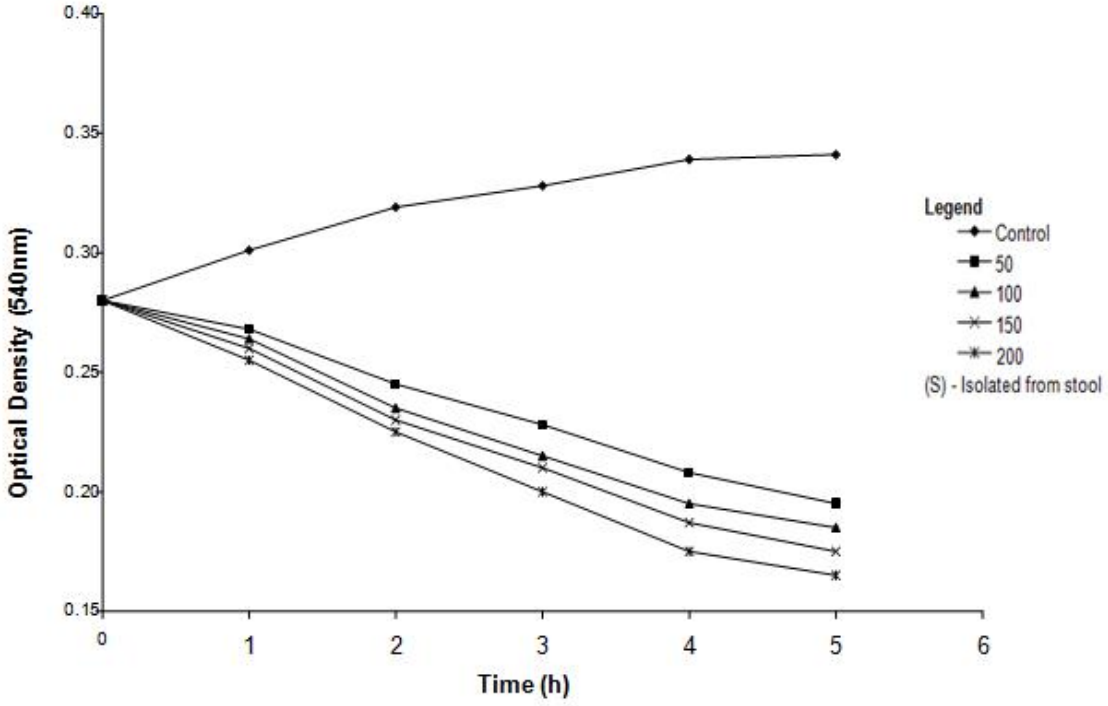

Fig. 6. The effect of crude extract of cocoa pods infected with *P. palmivora* on the growth of *Shigella* sp (S)

Table 1. Zone of inhibition of dichloromethane extract of infected cocoa pods against the test bacteria using paper disc method (mm)

Conc. of extract (mg/ml)	*Xanthomonas* sp. (Cp)			*Xanthomonas* sp. (R)			*Pseudomonas* sp. (R)			*Bacillus* sp. (R)			*E. coli* (S)			*Shigella* sp. (S)		
	M	SD	SE	M	SD	SE	M	SD	SE	M	SD	SE	M	SD	SE	M	SD	SE
50	6.00^{a}	0.91	0.46	0.00	0.00	0.00	0.00	0.00	0.00	3.00^{a}	0.91	0.46	3.00^{a}	0.41	0.20	2.00^{a}	0.41	0.20
100	10.00^{b}	2.04	1.02	0.00	0.00	0.0	0.00	0.00	0.00	4.00^{b}	0.82	0.41	5.00^{b}	0.91	0.46	4.00^{b}	0.41	0.20
150	11.50^{bc}	1.08	0.54	0.00	0.00	0.00	0.00	0.00	0.00	6.00^{b}	0.91	0.46	7.00^{b}	0.41	0.20	6.00^{b}	0.58	0.20
200	13.00^{c}	0.41	0.20	0.00	0.00	0.00	0.00	0.00	0.00	8.00^{c}	0.41	0.20	8.00^{c}	0.71	0.35	8.00^{d}	0.71	0.35

Legend: Cp: Cowpea, R: Rice, S: Stool, M: Mean, SD: Standard Deviation, SE: Standard Error
**Mean not followed by the same letter in the same column are significantly different*

Table 2. Zone of inhibition of dichloromethane extract of infected cocoa pods against the test bacteria using agar diffusion method (mm)

Conc. of extract (mg/ml)	*Xanthomonas* sp. (Cp)			*Xanthomonas* sp. (R)			*Pseudomonas* sp. (R)			*Bacillus* sp. (R)			*E. coli* (S)			*Shigella* sp. (S)		
	M	SD	SE	M	SD	SE	M	SD	SE	M	SD	SE	M	SD	SE	M	SD	SE
50	2.00^{a}	0.41	0.20	0.00	0.00	0.00	0.00	0.00	0.00	3.00^{a}	0.00	0.00	0.50^{a}	0.58	0.29	1.00^{a}	0.41	0.20
100	4.00^{b}	0.41	0.20	0.00	0.00	0.0	0.00	0.00	0.00	5.25^{b}	1.19	0.60	2.00^{b}	0.41	0.20	2.00^{ab}	0.71	0.35
150	6.00^{c}	0.41	0.20	0.00	0.00	0.00	0.00	0.00	0.00	6.00^{bc}	0.41	0.20	4.00^{c}	0.41	0.20	3.00^{bc}	0.41	0.20
200	8.00^{d}	0.41	0.20	0.00	0.00	0.00	0.00	0.00	0.00	7.00^{c}	0.41	0.20	5.00^{d}	0.41	0.35	4.00^{c}	0.82	0.41

Legend: Cp: Cowpea, R: Rice, S: Stool, M: Mean, SD: Standard Deviation, SE: Standard Error
**Mean not followed by the same letter in the same column are significantly different*

The result of this spectrophotometric examination of the crude extract against the test bacteria showed that as concentrations of the extract increased, the optical density was found to decrease, hence the bacteria population decreased. [17] reported that the optical density of a sample is a measure of turbidity. The optical density has been shown to increase in a linear fashion with increase in cell number as reflected in the control experiments. However, the addition of different concentrations of the crude extract in this study inhibited the growth of test bacterial plant pathogen, hence, the decrease in optical density observed. The result of this study is in agreement with the findings of [15] who reported that *Xanthomonas campestris* showed inhibitory responses after exposure to different concentrations of lettucenin. Similarly, [19] reported the antibacterial activities of momilatones A (M_A) and B (M_B) which are phytoalexins derived from the rice plant (*Oryza sativa*) against *P. ovalis, B. cereus, B. pumilus* and *E. coli but* momilatone B (M_B) exerted significant stronger antibacterial activities than momilatone A (M_A) against the test bacteria. The antibacterial property exhibited on some pathogens in this study might be attributed to the presence of active components in the induced extract.

4. CONCLUSION

This finding showed that induced extract from cocoa pods can serve as a good source of antibacterial agent since it had great inhibitory effects on some of the test plant bacterial pathogens.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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