DETERMINATION OF ANTI-PSEUDOMONAL ACTIVITIES OF COCOA (Theobroma Cacao) POD HUSK USING TIME-KILL-TEST AND AGAR-WELL DIFFUSION TECHNIQUES

ABSTRACT

Aim: To determine the antimicrobial effectiveness of cocoa pod husk against *P. aeruginosa.*

Study design: An experimental research

Place and Duration of Study: Department of Medical Microbiology, Olabisi Onabanjo University Teaching Hospital, Sagamu, Ogun State, Nigeria, between June 2015 and Sept. 2016.

Materials and Method: Seventy-seven (77) isolates of *P. aeruginosa* obtained from three (3) teaching hospitals were involved in this study. Cocoa pod husk was processed into crude extract and its effectiveness against the isolates of *P. aeruginosa* were examined using agar well diffusion, time-kill test techniques and Anova.

Results: The percentage susceptibility of *P. aeruginosa* to Cocoa pod husk at 500mg/mL and 250mg/mL inhibited all the *P. aeruginosa* but only 14.29% were sensitive at a lower concentration of 31.25mg/mL. Moreover, the comparison between the broth micro and macro dilution method of determining the MIC of cocoa pod husk against *P. aeruginosa* highlighted a significant decrease in the mean MIC value of the broth micro dilution method when compared with the broth macro dilution technique ($T=13.519$, $P<0.05$). The time kill test emphasized that *P. aeruginosa* was killed at a lower concentration of 62.5mg/ml at 150mins of introduction in to the Cocoa pod husk suspension.

Conclusion: This study revealed that the Cocoa pod husk possesses antibacterial properties. An increase in the concentration of cocoa pod husk increased its antibacterial activity against *Pseudomonas aeruginosa.* Moreover, the broth micro dilution technique is sensitive for determining the anti-pseudomonal activity.

Keywords: *Pseudomonas aeruginosa, cocoa pod husk, agar well diffusion, time-kill test.*

1. INTRODUCTION

The use of antibiotics has reduced mortality rates which were on the high side due to life-threatening diseases. However, an increase in the misuse and mismanagement of antibiotics which are now the major causes of drug resistance is creating concern in medical practice [1]. *Pseudomonas aeruginosa* is an opportunistic pathogen that causes a wide range of acute and chronic infections in patients who are immunocompromised or have other predisposing conditions. This organism can infect almost all tissues and it is one of the most common causes of nosocomial infections. Chronic infections can last for years or even decades, during which time *P. aeruginosa* populations undergo thousands of generations of growth while facing challenges including antibiotic treatment, iron deprivation, immune system surveillance and interspecies competition [2].

P. aeruginosa is a notoriously difficult organism to control with antibiotics or disinfectants [3]. The organism can tolerate high temperatures as high as 50ºC and it is capable of growing under aerobic as well as anaerobic conditions. Despite possessing a large number of virulence factors [4], *P. aeruginosa* is truly a challenging pathogen in the hospital setting as it is resistant to many antibiotics. Also, it is capable of forming hardy biofilms, both within the body and on the surfaces of most medical instruments. Hence, *P. aeruginosa* continues to be problematic from a treatment perspective [4].

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. There have been several reports on the number of deaths resulting from nosocomial infections each year in different countries all over the world [5]. 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 [5].

The plant kingdom has proven to be effective in the treatment of many diseases and they serve as an important source of all the world's pharmaceuticals [6]. Medicinal plants are used as a medical resource in almost all cultures because they are variety of plants believed to possess medicinal properties. Human societies have been in close contact with their environments since the beginning of their formation and used the ingredients of the environment to obtain food and medicine [7].

Furthermore, the parts of medicinal plants that may be used are different types of seeds, root, leaf, fruit, skin, flowers or even the whole plant. The active compounds in most parts of the medicinal plants have direct or indirect therapeutic effects and are used as medicinal agents. The important bioactive constituents of plants are steroids, terpenoids, carotenoids, flavanoids, alkaloids, tannins and glycosides [6]. The constituents of the plant may interact with each other, and this interaction can be beneficial for both or adverse to either of them or eliminate the harmful effects of both [7].

Theobroma cacao is a plant from which cocoa was derived. Cocoa as a food ingredient is fast becoming very popular in the food and confection industry worldwide. It is available in a wide variety of forms, colors and flavors and is used in numerous applications [8]. The cultivation of cocoa is of economic importance for several countries such as Ghana, Ivory Coast, Nigeria, Indonesia, Malaysia, and Brazil [9].

Abundant of waste from cocoa areas can be turned into value-added end products. It was estimated that only 25% from total weight of fresh cocoa fruits were used to produce cocoa beans for cocoa and chocolate processing. Other parts including the pods and shells are usually discarded. Despite of being thrown away, cocoa pods can also be used as sources of fertilizer or animal feed and activated carbon production. Other potential use of cocoa pods are in producing gum [10], potash in soap-making as well as food colorant. Cocoa pod ash is a commercial ingredient used as a component of African Black Soap that in turn is used in environmentally-free conditioning cleansing cream [11]. On the other hand, these cacao husks contain a range of biologically active compounds and, therefore, may represent a valuable source for the isolation of substances of economic value [9].

2. MATERIALS AND METHODS

2.1 Sample collection

Isolates of Pseudomonas species isolated from different samples were obtained from the routine bacteriological laboratories of Olabisi Onabanjo University Teaching Hospital (OOUTH) Sagamu, University College Hospital (UCH) Ibadan, and Federal Medical Centre (FMC) Abeokuta (all in Southwest, Nigeria). A total of 77 isolates were used for this study and re-identification of *P. aeruginosa* was achieved by the procedures of oxidase test and growth on cetrimide agar.

2.2 Processing of Cocoa pod husk

The Cocoa pod husks (CPH) were purchased from a cocoa farmer, sundried and burnt into ashes. The ash was soaked in sterile water and the filtrate obtained was evaporated to dryness in a rotary evaporator. The powder obtained was weighed and put in a sterile container with a small pack of dessicant (to absorb moisture) until it was used.

2.3 Identification of *Pseudomonas aeruginosa*

2.3.1 Growth on cetrimide agar

Cetrimide agar (Lab M, Lancashire, Uk) plates were prepared according to manufacturer's instructions and all samples collected from different teaching hospitals were subcultured on the cetrimide agar plates and incubated at 37^0C for about 24hrs. Isolates with greenish or blue-green colouration were accepted as positive.

2.3.2 Oxidase test

On a filter paper, few drops of oxidase reagent (Dalynn, Alberta, Canada) were dropped. An applicator stick charged with colonies of identified organisms (*P. aeruginosa*) was rubbed on the surface of the filter paper and a change in color was observed. A purple coloration of the rubbed organism indicated a positive reaction.

2.4 Antimicrobial susceptibility testing using agar well diffusion techniques

0.5 McFarland standard broth culture of *P. aeruginosa* was flooded on a prepared Mueller Hinton agar plates and five (5) wells were created at equidistance on each plate with the aid of a sterile 6mm cork borer. 100µl of corresponding labeled concentrations of cocoa pod husk extract were carefully dispensed into respective wells and incubated at 37^0C for 18-24hrs, after which the diameter zones of inhibition were read.

2.5 Minimum Inhibitory Concentration (MIC) by microtube dilution

Into a sterile microtitre plate, 100µl of sterile nutrient broth and 100µl of known antibiotic concentration were dispensed in to it to make serial doubling dilution. Equal volume of 100µl of overnight bacteria broth culture of 0.5 McFarland standard was dispensed in to all the dilutions and incubated at 37^0C for 24hrs.

2.6 Minimum inhibitory concentration (MIC) by tube dilution

1ml of sterile 1% peptone water and 1ml of known antibiotic concentration were dispensed In to sterile test tubes to make serial dilution. 1ml of 0.5 McFarland standard of overnight bacteria broth culture was introduced to all tubes and all the tubes were incubated overnight at 37^0C.

2.7 Minimum Bactericidal Concentration (MBC)

Contents from the last tube showing no turbidity, to the previous four tubes, were cultured on prepared nutrient agar plates. The plates were incubated overnight at 37^0C for 18-24hrs.

2.8 Time kill test

Twelve (12) sterile test tubes (per number of organisms) were arranged on a test tube rack. 1ml of cocoa pod husk extract and 1ml of Nutrient broth were introduced into the test tubes to make serial doubling dilution. 100µl of organism was inoculated in to the test tubes then start timing. Each test tube content was inoculated on prepared Mueller Hinton agar plates at every 30 minutes and the plates were incubated at 37^0C overnight.

3. RESULTS

Table 1: Comparison of Mean Diameter Zone of Various Concentration of Cocoa pod extract against Pseudomonas aeruginosa

Various Concentration of Cocoa Pod extract	N	Diameter Zone of inhibition (mm) Cocoa pod extract Mean ± SD
500 mg/ml	77	20.732 ± 2.952
250 mg/ml	77	16.098 ± 2.483
125 mg/ml	77	1.810 ± 2.248
62.5 mg/ml	77	8.565 ± 1.002
31.25 mg/ml	77	8.455 ± 1.968
F-ratio		16.783
P- value		<0.05

N – Number tested

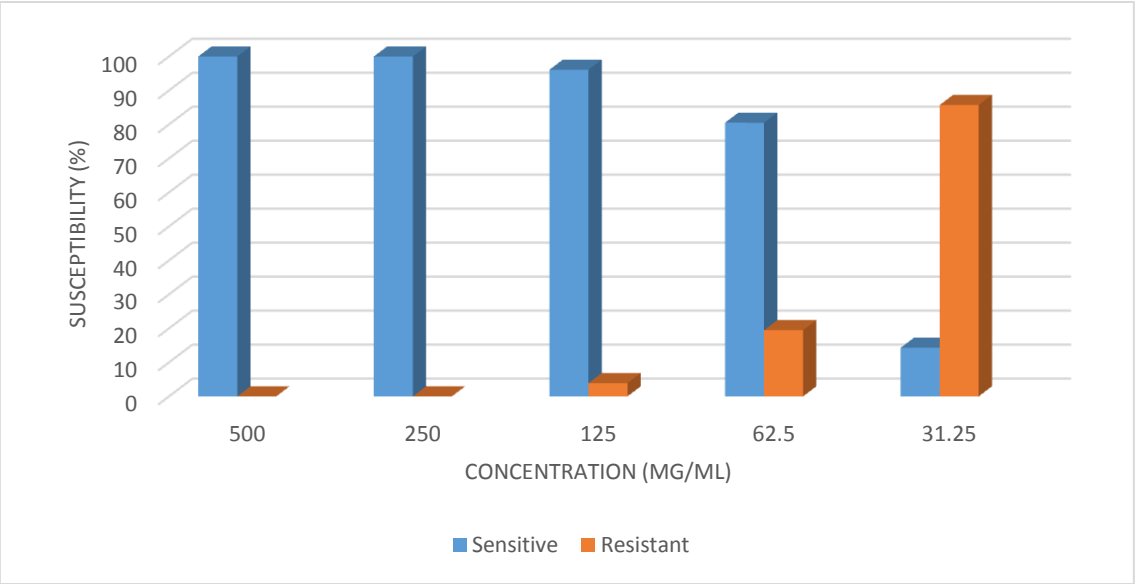

Fig.1: Antibacterial activities of cocoa pod husk against *Pseudomonas aeruginosa*

Table 2: Time taken for various concentration of cocoa pod husk to kill the isolate of *Pseudomonas aeruginosa*

Cocoa pod husk concentration (mg/mL)	Time (Minutes)															
	30	60	90	120	150	180	210	240	270	300	330	360	390	420	450	480
500	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
250	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
125	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
62.5	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
31.25	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
15.625	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

(-) = No growth, (+) = Growth

Table 3: Comparison between Mean MIC and MBC of Cocoa pod husk against *Pseudomonas aeruginosa*

Antibacterial Agent	N	Cocoa pod extract (mg/mL) Mean ± SEM
MIC	10	40.119 ± 14.060
MBC	10	55.832 ± 11.87
T-test		2.341
P-value		<0.05

N- Number tested

Table 4: Comparison between broth micro and broth macro dilution methods of determining the MIC of cocoa pod husk against *Pseudomonas aeruginosas*

		Cocoa pod extract MIC (mg/mL)				
Isolate	**N**	**Broth Micro Dilution Mean ± SEM**	**N**	**Broth Macro dilution Mean ± SEM**	**T-test**	**P-value**
Pseudomonas aeruginosa	**10**	**40.118±14.06**	**10**	**65.07 ± 9.035**	**13.519**	**<0.05**

N- Number tested

Table 1 shows the comparison of the mean diameter zone of inhibition of various concentration of cocoa pod husk extract against *P. aeruginosa.* The mean diameter zone of inhibition ranged between 20.732mm and 8.455mm. Consequently, the susceptibility percentage of *P. aeruginosa* to Cocoa pod husk extract (Fig.1) explained that the Cocoa pod husk at 500mg/ml and 250mg/ml inhibited all the *P. aeruginosa* 77 (100%), followed by 125mg/ml and 62.5mg/ml at 74 (96.10%) and 62 (80.52%) respectively. But only 11(14.29%) were sensitive at a lower concentration of 31.25mg/ml.

Table 2 revealed the time taken for various concentration of cocoa pod husk extract to terminate the isolate of *P. aeruginosa.* At concentrations of 500mg/ml, 250mg/ml and 125mg/ml respectively, no growth of organism was observed; however, *P. aeruginosa* was eliminated at 150mins in 62.5mg/ml concentration of the extract.

There is a significant increase in the mean MBC of Cocoa pod husk extract (T=2.341, $P<0.05$) against the isolates of *P. aeruginosa* (Table 3). Furthermore, the broth micro dilution technique exhibited a significant decrease in the mean MIC value (T=13.519, $P<0.05$) when compared with the mean MIC of the broth macro dilution technique (Table 4). Fig.2 displays the diameter zones of inhibition of cocoa pod husk at different concentrations against an isolate of *Pseudomonas aeruginosa* using agar well diffusion method.

Fig. 2: Agar well diffusion method showing the diameter zones of inhibition of cocoa pod husk at different concentrations against an isolate of *Pseudomonas aeruginosa*

4. DISCUSSION

The comparison of the mean diameter zone of inhibition of various concentration of cocoa pod husk extract against *P. aeruginosa* statistically revealed a significant increase in the mean diameter zone of inhibition of cocoa pod husk extract against *P. aeruginosa* at 500mg/mL and 250mg/ml respectively when compared with other concentrations. This study agrees with a previous study carried out in Ado-Ekiti, Nigeria, where it was shown that *P. aeruginosa* was susceptible with the diameter zone of inhibition of 17.0mm at 250mg/ml. The susceptibility of the test organisms to the crude extract increased with increase in the concentration of the extract [5].

Consequently, the time taken for various concentration of cocoa pod husk extract to kill the isolate of *P. aeruginosa* was observed. The extract was able to kill the organism at 30mins in concentrations of 500mg/ml, 250mg/ml and 125mg/ml but at 62.5mg/ml, the death of the *P. aeruginosa* occurred at 150mins. The time required to kill the organism was determined after 2hrs of introduction of organism in to the Cocoa pod husk extract suspension at 62.5mg/ml.

Thus, the antipseudomonal effect of the cocoa pod husk extract against the organism was experienced at 150mins after the introduction of *P. aeruginosa* into the extract suspension of 62.5mg/ml concentration. This indicates that, the lower the concentration of Cocoa pod husk extract, the longer the time taken for elimination to occur.

Furthermore, the broth micro dilution technique was more effective in determining the MIC of the Cocoa pod husk extract when compared with the broth macro dilution technique, as it was observed that the time kill test of the extract against *P. aeruginosa* was achieved at a lower concentration that falls within the MIC range.

CONCLUSION

From this study, the extract of cocoa pod husk is said to possess antibacterial properties against *P. aeruginosa*. It is therefore recommended that the crude extract should undergo further separation techniques for the identification of active components which should be subjected to further antipseudomonal activities.

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COMPETING INTEREST DISCLAIMER

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly used products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of

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Authors Contributions

Authors A and B designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors C, D and E managed the analyses of the study. Authors F and G managed the literature searches. All authors read and approved the final manuscript.

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