

Catalase Activity and Hydrophobicity Test of Bacteria Susceptible to Extracts of *Cleistopholis Patens* and *Piliostigma Reticulatum*

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ABSTRACT

Antibacterial effect of the ethanol, methanol and ethyl acetate extracts of *Piliostigma reticulatum* and *Cleistopholis patens* on ten pathogenic bacteria including *Pseudomonas aeruginosa*, *Salmonella typhi*, *Escherichia coli*, *Proteus vulgaris*, *Streptococcus pyogenes*, *Klebsiella aerogenes*, *Staphylococcus aureus*, *Serratia marscesnes*, *Yersinia enterocolitica* and *Shigella dysenteriae* was investigated. Survival rate after treatment was investigated. Cell surface hydrophobicity after treatment was tested and the catalase activities of the plant extracts were determined. The results revealed that at 100mg/ml of extracts, the ethanol extracts of *C. patens* showed antibacterial activities against *S. typhi*, *P. vulgaris*, *Str. pyogenes*, *K. aerogenes*, *S. aureus* and *Yersinia enterocolitica* with zones of inhibition of 18mm, 14mm, 18mm, 24mm, 14mm and 18mm respectively and the ethyl acetate extract was active against *Sal. typhi*, *P. vulgaris*, *Str. pyogenes*, *K. aerogenes*, *S. aureus* Ser. *marscesnes* and *Y. enterocolitica* with zones of inhibition of 14mm, 22mm, 18mm, 18mm, 14mm, 16mm and 16mm respectively while ethanol extract of *P. reticulatum* showed activity against *P. aeruginosa*, *Sal. typhi*, *E.coli*, *Str. pyogenes*, *K. aerogenes*, *S. aureus*, and *Sh. dysenteriae* with zones of inhibition of 13mm, 12mm, 10mm, 10mm, 8mm, 10mm and 14mm respectively. The ethyl acetate was active against *E.coli*, *Str. pyogenes*, *K. aerogenes*, *S. aureus* and *Sh. dysenteriae* respectively. The methanol extract had no activity against the test organisms. The kill-time curve showed that susceptible cells die after 10 and 20minutes after contact with the extracts of *C. patens* for most of the test bacteria while cell death occurred at 10, 20, 40, 50 and 60 min after contact. The hydrophobicity test showed that test organisms had hydrophobicity of between 47% and 99% in the extracts. Catalase production reduced considerably in some bacteria after treatment with plant extracts. The results obtained presupposes that the plant extracts are effective against some of the test organisms and their mode of activity are is that they interfere with the ability of bacteria to produce catalase, inducing cell death by the production of hydroxyl radical and also by the enhancement of non-opsonic phagocytosis of bacteria by macrophages.

Keywords: Catalase activity, Hydrophobicity, Kill-time kinetics, *Piliostigma reticulatum*.

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I. INTRODUCTION

Microbial adhesion to eukaryotic cells seems to be the first stage in many bacterial infections [1] Cell surface hydrophobicity is often associated with the binding of bacterial cells to specific cells and tissues receptors of the mucosa surface of the infected host. The cell surface hydrophobicity, surface charge, acid- base properties and surface topography can have significant effect on microbial adhesion [2]-[4].

Adhesion of bacterial cells to surface and biofilm formation depend on the properties of bacterial cells, environmental factors influencing their growth and on the properties of the material to which they adhere [5], but mainly governed by the electrostatic, Van der waal, hydrophobic and contact interaction [6].

High cell surface hydrophobicity enables microorganisms to attach to hydrocarbon droplets on the surface of cells and to move from water to organic, hydrocarbon phase where biosurfactants and enzymes decompose water [7]. The rapid change in environmental condition forces adaptive features on microorganisms which enhances its ability to survive. One of this mechanism is the release of outer membrane vesicle (MV) in Gram negative bacteria causing a significant increase in cell surface hydrophobicity and an enhanced tendency to form biofilms [8].

Catalase production by microbes is a mechanism by which these organisms cleave H_2O_2 to produce water (H_2O) and oxygen (O_2), thereby neutralizing the bactericidal effects of antimicrobials [9]. Phagocytes often use oxygen dependent mechanisms to prevent bacterial infections; O^- and

H₂O₂ are some weapons used by phagocytes to kill bacteria [9]. Medicinal plants have been known to use several mechanisms in their killing pattern including disruption of membrane potential, inner membrane permeabilization, blebbing and leakage of cellular content [10]. Several plant derived extracts or active compounds can prevent the attachment of pathogens but little is known about their effects on bacterial adhesion [11].

The main objective of this study is to investigate the antibacterial activity and the effects of extracts of *Piliostigma reticulatum* and *Cleistopholis patens* on the hydrophobicity and the catalase producing ability of ten bacteria.

II. MATERIALS AND METHODS

A. Plant Sampling and Preparation

Stem bark of plants were sampled from private garden of a traditional healer at Ibode in Ibadan, Oyo State. The plants were identified by Mr Omotayo of the Department of Plant Science and Biotechnology, Ekiti State University, Ekiti State. Voucher specimens with voucher numbers WHAE2017/001 and WHAE2017/002 were deposited in the herbarium of the Ekiti State University, Ado- Ekiti.

The ethanol, methanol and ethyl acetate extracts were prepared by dissolving 200gm amount of powdered plant samples in 500mls of each solvent. The solutions were allowed to stand for 5 days (120hr) at room temperature of 27°C ±1°C, after which each solution was filtered using a sterile muslin cloth and a Whatmann no 1 filter paper as described by Fabricant and Fansworth (2001). The collected filtrate was evaporated to dryness under sterile condition at room temperature (27°C).

The extracts were kept in sterile bottles until ready for use.

B. Collection and Maintenance of Microorganisms:

Clinical samples of microorganisms were collected from the Microbiology Department of Obafemi Awolowo University, Ile-Ife, Osun State. The strains were confirmed by biochemical tests and maintained on agar slants.

C. Determination of Antibacterial Activity of Plant Extract:

A five (5) gram portion of the plant solvent (ethanol, methanol and ethyl acetate) extracts was reconstituted in 5mls of 50% Dimethyl sulfoxide (DMSO) to make a stock solution of 100mg/ml of extracts. The agar well diffusion method of Perez, (1990) was adopted for the antibacterial assay. Test organisms were suspended in nutrient broth and incubated for 4 hours to obtain a concentration corresponding to McFarlands constant (0.5 X 10⁸cfu/ml). The inoculum was standardized with barium sulphate. The sterile petri-plates were inoculated by pour plate method. One ml (1ml) of the test organisms was pipetted aseptically into each petri dish and about 20 ml of sterilized nutrient agar was poured into the petri dish containing the inoculum. The agar plates were allowed to set. Wells of 6mm diameter were punched over the agar plates equidistant from each other using sterile cork borer and 0.5ml of each plant extracts of different concentrations as prepared by the serial dilution were added to the wells using a micropipette. The extracts were allowed to diffuse into the agar for about 20 mins after which the plates were incubated for 24 h at 37°C. After incubation the diameter of inhibitory zones formed around each wells were

measured in mm and recorded. Experiments were carried out in triplicates and the average values recorded.

D. Determination of The Survival Rate of Test Organisms in Plant Extracts.

To evaluate the survival rate of test organisms in the extracts, 2 ml of inoculum at a concentration of 0.5 × 10⁸ cfu/ml was added to a test tube containing 100 mg/ml of the extracts and plated on nutrient agar. Colony count (CFU) was determined after 0, 5, 10, 15, 20, 30, 40, 50, 60, 80, 90, 100, and 110 min of incubation at 37°C. At each incubation time, about 0.5 ml of solutions were plated at 37°C for 24hr. After this period, the colonies were counted and the kinetic action curve was constructed.

E. Determination of Hydrophobicity of Test Organisms After Treatment With Plant Extracts.

A slightly modified method as described by Rosenberg *et al.*, [13] was adopted in this test. A 2ml portion of bacteria culture was centrifuged at 5000rpm for 4 minutes then washed with phosphate buffer saline (PBS). The cells were resuspended in 15ml of PBS and the absorbance (Ao) measured at 620nm using spectrophotometer (T 70 UV-VIS Spectrometer, PG Instruments Ltd.). A 0.5ml portion of xylene was added to 3.5ml of bacterial suspension in PBS and the mixture agitated on a vortex at 2500 twiddles/min for 20min. The optical density (A) of the aqueous phase was measured and recorded. Percentage bacterial cells in the xylene layer was calculated as percentage hydrophobicity using the equation

$$\% \text{ hydrophobicity} = \frac{(1-A)}{A} \times 100$$

Ao

Where A is the absorbance of bacterial cells in PBS and Ao is the absorbance of cells in PBS after treatment with xylene. After treatment with plant extracts, Ao is the absorbance of cells in PBS after treatment with plant extracts and the results compared with the result of cells in xylene. Statistical analysis using the T- test was carried out on the result.

F. Evaluation of The Specific Catalase Production of Test Organism in Plant Extract.

Catalase production was measured by the time required for culture-soaked filter paper disks to float to the surface of a 1% hydrogen peroxide solution, based on the protocol used by Hsieh *et al* [14] and Chan *et al* [15]. A 1% (v/v) hydrogen peroxide solution was prepared from the 30% stock solution. 16 µl of test culture was absorbed onto 5 mm disks of 3 mm filter paper (#3 Whatman) for 10sec before being dropped into 50 ml of 1% (v/v) H₂O₂, which was approximately 3 cm of liquid height in a 100 ml beaker. Catalase activity was determined as the inverse of the time taken for the disk to float to the top. Disks that sank to the bottom or clung to the walls of the beaker were disregarded and the samples repeated. Also, turbidity measurements of the original culture were used to normalize the data so that it reflected specific catalase activity to account for differences in catalase production. The same procedure was repeated with culture treated with plants' extracts and the results recorded.

III. RESULTS

Cleistopholis patens showed antibacterial activities against some of the test organisms at 100mg/ml with the ethanol extract showing a zone of inhibition of 18mm, 14mm, 18mm, 24mm 14mm and 16mm against *S. typhi*, *P. vulgaris*, *S. pyogenes*, *K. aerogenes*, *S. aureus* and *Y. enterocolitica* respectively. The ethyl acetate extracts showed activity against *S. typhi*, *P. vulgaris*, *S. pyogenes*, *K. aerogenes*, *S. aureus*, *S. marscences*, and *Y. enetrocolitica* with zone of inhibition of 14mm, 22mm, 18mm, 18mm, 14mm, 16mm and 16mm respectively.

Comparatively, the ethanol extract of *Piliostigma reticulatum* showed antibacterial activities against *P. aeruginosa*, *S. typhi*, *S. pyogenes*, *K. aerogenes*, *S. aureus*, and *S. dysenteriae*. With zones of inhibition of 13mm, 12mm, 10mm, 10mm, 8mm, 10mm, and 14mm respectively while the ethyl acetate extract showed zones of inhibition of 16mm, 18mm, 10mm, 16mm and 20mm against *E.coli*, *S.pyogenes*, *K. areogenes*, *S. aureus*, and *S. dysenteriae* respectively. Methanol extracts of the two plants showed no antibacterial activity against the test organisms (Table I).

A. Survival Rate of Test Bacteria

Fig (1a) demonstrates that the number of viable cells decreased in proportion to the exposure time of microorganisms to the ethanol extract of *Piliostigma reticulatum*. At first contact (0 min) with the extract, the cell population was high, but as more time elapsed, there was a corresponding decrease in bacteria population, which reduced the logarithmic cycle and highlights the potent bactericidal action of this compound. Interestingly, the times required for the total kill of the bacterial cells were not significantly different among the two plant extract.

When evaluating the survival rate of test bacteria to plant extract against *S. pyogenes* and *S. aureus*, (Figs 1 and 2), it was observed that cell death occurred in only 30 min after contact of *S. pyogenes* with *C. patens* while cell death occurred at 40min after exposure for *S. aureus*. When the cells were treated at 40 and 50 min, respectively, the same bactericidal effect was experienced. The extracts from *C. patens* plants were able to significantly reduce the number of cells just after 30min, while in the control, cells did not stop growing. After 50mins of contact with *C. patens*, no growth was recorded for *Staphylococcus aureus*, *Streptococcus pyogenes*, *Salmonella typhi* and *Klebsiella aerogenes*. For *Piliostigma reticulatum*, the same survival was recorded as that found with *Cleistopholis patens*. For most of the test organisms, reduction in microbial viability started at about 20 minutes after contact, however, for *Salmonella typhi*, *Klebsiella aerogenes* and *Shigella dysenteriae*, cell death occurred at 40 minutes after contact with the extract. The log value of time-kill values showed cell death to have occurred at 20min after exposure for *Staphylococcus aureus* and *Shigella dysenteriae*.

However, there was no decrease in density of *Shigella dysenteriae*, *E.coli Pr. vulgaris* and *Ps. aeruginosa* in *C. patens* and *Pr. vulgaris* and *Ps. aeruginosa* in *P. reticulatum*. Interestingly, the times required for the total kill of the bacterial cells were significantly different among the two extracts evaluated.

B. Catalase Production of Test Organisms

Y. enterocolitica and *Serratia merscences* were catalase negative while the other test organisms were catalase positive. When these organisms were subjected to plant extracts, some of them lost their catalase status and the catalase positive organisms became catalase negative

TABLE I: Antibacterial activity of plant extracts against test organisms (in mm)

	<i>Cleistopholis patens</i>			<i>Piliostigma reticulatum</i>		
	Ethanol	Methanol	E.acetate	Ethanol	Methanol	E..acetate
<i>P. aeruginosa</i>	-	-	-	13	-	-
<i>S. typhi</i>	19	-	14	12	-	-
<i>E.coli</i>	-	-	-	10	-	16
<i>P. vulgaris</i>	14	-	22	-	-	-
<i>S. pyogenes</i>	18	-	18	10	-	18
<i>K. aerogenes</i>	24	-	18	8	-	10
<i>S. aureus</i>	14	-	14	10	-	16
<i>S. merscences</i>	-	-	16	-	-	-
<i>Y. enterocolitica</i>	18	-	16	-	-	-
<i>S. dysenteriae</i>	-	-	-	14	-	20

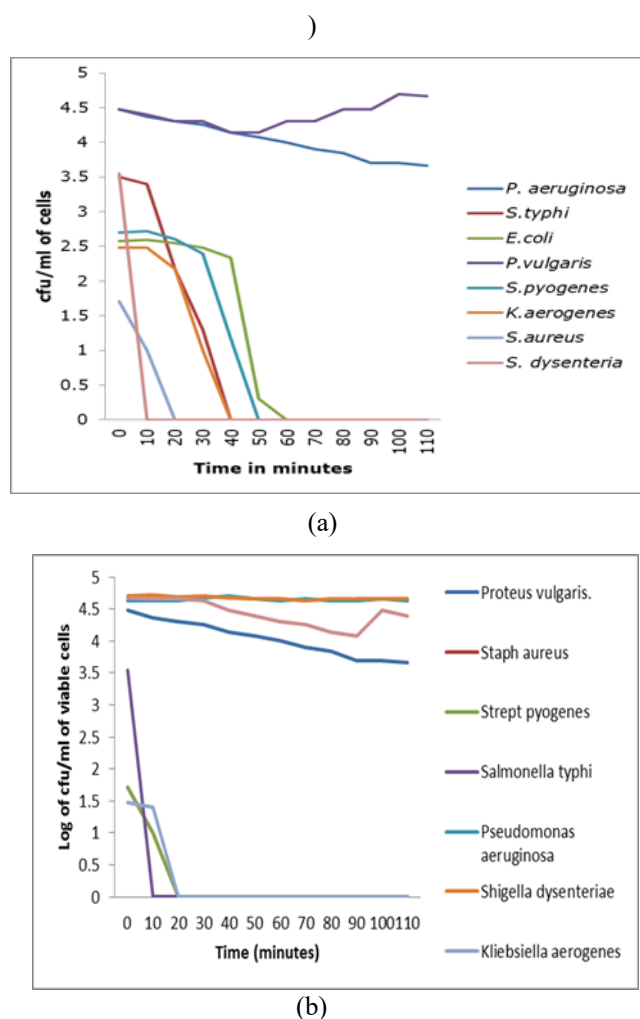


Fig. 1. (a) Survival curve of bacteria subjected to ethyl acetate extract of *Piliostigma reticulatum*, (b) shows Survival curve of bacteria subjected to ethyl acetate extract of *Cleistopholis patens*

while the catalase negative organisms retained their negative status. *Pseudomonas aeruginosa*, *Shigella dysenteriae* and *Escherichia coli* retained their ability to produce catalase after treatment.

These organisms were resistant to the solvent extracts of *Cleistopholis patens* as recorded in table 1. Figure 2(a) presents the catalase production abilities of the test organism before and after treatment with the extracts of *Cleistopholis patens* at 100mg/ml of extract. It shows the inverse values of the time it took each paper disk to float on the surface of the 1% of hydrogen peroxide (H_2O_2).

For the solvent extracts of *Piliostigma reticulatum* (Fig 2b), *Staph aureus*, *Sal. typhi*, *Str. pyogenes*, *K. aerogenes*, *Yer. enterocolitica*, and *Pr. vulgaris* produced catalase in ethanol and methanol extracts, but not in ethyl acetate extract, while *Staph aureus*, *Salmonella typhi*, *Str. pyogenes*, *K. aerogenes*, *Yersinia enterocolitica* and *P. vulgaris* were unable to produce catalase in the ethyl acetate extract.

The result of the statistical analysis carried out using the T-test showed that the catalase activity between the control (culture without extract treatment) and culture in ethanol extracts of *Cleistopholis patens* showed significant difference at 0.05% level of confidence (≤ 0.05). The control versus the methanol extract treatment showed no significant difference at 0.05% level of confidence ($P \geq 0.05$) while the ethyl acetate extract showed a significant difference at 0.05% level of confidence (≤ 0.05).

The result of the statistical analysis carried out using the T-test showed that the catalase activity between the control (culture without extract treatment) and culture in ethanol extracts of *Cleistopholis patens* showed significant difference at 0.05% level of confidence (≤ 0.05).

The control versus the methanol extract treatment showed no significant difference at 0.05% level of confidence ($P \geq 0.05$) while the ethyl acetate extract showed a significant difference at 0.05% level of confidence (≤ 0.05).

A. Percentage Hydrophobicity of Test organisms before And After Treatment With Plant Extracts.

The hydrophobicity of test organisms are presented in table II and III. The higher the percentage of hydrophobicity, the lower the water affinity. *Ser. marscesces*, *S. aureus* and *Yer. enterocolitica* in xylene showed hydrophobicity of respectively 96%, 98% and 99% while the same organisms in ethyl acetate extract of *C. patens* had hydrophobicity of 79%, 93% and 90% respectively.

Other test organisms showed hydrophobicity of between 47% and 77% in xylene while in ethyl acetate extract of *C. patens* hydrophobicity ranged from 68% to 91%. The test organisms in ethanol extract showed hydrophobicity of between 71% and 99% with *Shigella dysenteriae* having the highest percentage hydrophobicity. Test organisms on the other hand showed hydrophobicity of between 67% and 96% in ethyl acetate extract of *Piliostigma reticulatum* with *E. coli* showing the highest percentage hydrophobicity of 96%.

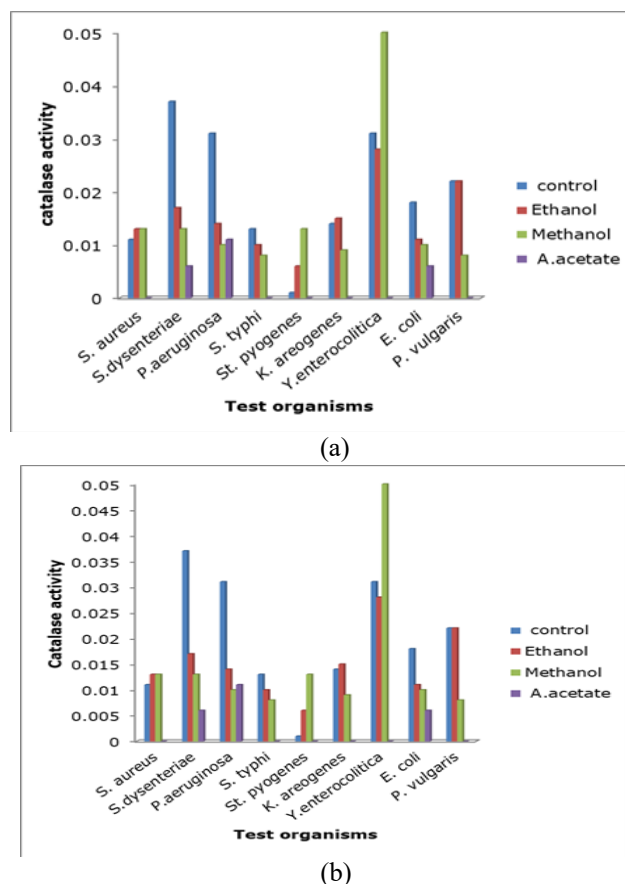


Fig. 2. (a) shows catalase activity bacteria subjected to ethyl acetate extract of *Cleistopholis patens*, (b) shows the catalase of bacteria subjected to ethyl acetate extract of *Piliostigma reticulatum*

The test organisms in ethanol extract of *Piliostigma reticulatum* showed percentage hydrophobicity of between 67 and 98 with *S. aureus* and *Ps. aeruginosa* showing the highest affinity of 98 % each (Table III.)

Statistically, there was a significant difference between the hydrophobicity of test organisms in xylene and the hydrophobicity of test organisms in plant extracts at 0.05% level of significance ($P \leq 0.05$). The hydrophobicity in PBS versus test organisms in ethyl acetate extracts of *C. patens* (Table II) showed a significant difference at 0.05% level of confidence ($P \leq 0.05$). Similarly, the hydrophobic effect of test organisms in PBS was significantly different from the hydrophobic effect of test organisms in ethanol extract at 0.05% level of confidence ($P \leq 0.05$).

The Hydrophobic effect of test organisms in PBS against the hydrophobic effect of test organisms in the ethyl acetate extract of *P. reticulatum* showed a significant level of difference at 0.05% level of confidence ($P \leq 0.05$) (Table III). In the same vein, the hydrophobic effects of test organisms in PBS and organisms showed in ethanol extracts showed a significant difference at 0.05% ($P \leq 0.05$)

TABLE II: PERCENTAGE HYDROPHOBICITY OF ETHANOL AND ETHYL ACETATE EXTRACT OF *C. PATENS*

Test organisms	xylene	ethanol	ethyl acetate
<i>S. aureus</i>	99	98	86
<i>S. dysente</i>	51	67	67
<i>P. aerugin</i>	56	98	83
<i>S. typhi</i>	62	97	94
<i>S. pyogenes</i>	77	79	80
<i>K. areogenes</i>	47	84	75
<i>Y. entero</i>	98	90	94
<i>E. coli</i>	69	91	96
<i>P. vulgaris</i>	67	88	89
<i>S. marscenc</i>	96	84	89

TABLE III: PERCENTAGE HYDROPHOBICITY OF ETHANOL AND ETHYL ACETATE EXTRACT OF *P. RETICULATUM*

Test organisms	xylene	ethanol	ethyl acetate
<i>S. aureus</i>	99	98	86
<i>S. dysente</i>	51	67	67
<i>P. aerugin</i>	56	98	83
<i>S. typhi</i>	62	97	94
<i>S. pyogenes</i>	77	79	80
<i>K. areogenes</i>	47	84	75
<i>Y. entero</i>	98	90	94
<i>E. coli</i>	69	91	96
<i>P. vulgaris</i>	67	88	89
<i>S. marscenc</i>	96	84	89

IV. DISCUSSION

Plants have been known from time immemorial to have antibacterial activities. This is evidenced in this study as the two plants used; *C. patens* and *P. reticulatum* both have broad spectrum antibacterial activities. Several authors have reported the antibacterial [16]- [17] anti-candidal activity [18] of *C. patens*. *P. reticulatum* has been shown to possess antimicrobial and anti-inflammatory [16], Antispasmodic [19], antioxidant and anti-diarrheal [20] properties. The report of this study confirms the status of the two plants as potent antibacterial as used by traditional medicine practitioners.

The survival curve of the test organisms after challenge with the plant extracts is a function of the ability of some microorganisms to tolerate antimicrobials. However, tolerance is seldom taken into consideration explicitly in healthcare as compared to resistance [21]. The success of antimicrobial therapy is determined by the complex interaction between an administered drug, a host and the infective agent. The time-kill curve reveals a time dependent antimicrobial effect of test plants. For the test bacteria, a short lag phase was experienced. A fast decline was observed for all the susceptible bacteria strains used reaching a very low phase only after 110min after contact with test substance. In contrast, there was no significant difference in the population density of resistant bacteria like *S. dysenteriae*, *E. coli*, and *P. aeruginosa* in ethanol extract of *C. patens* as compared with the control. This is an indication that the plant could be bacteriostatic to the test bacteria since there was no significant increase in the density of bacteria cells.

However, in *P. reticulatum*, there was a considerable increase in the population of *P. vulgaris* while the population of *P. aeruginosa* remains constant. This study provides a fundamental information about the pharmacodynamics relationship between the antibacterial and the test bacteria. Ferro *et al.* [22] in their work were able to also establish the pharmacodynamic relationship between several antibiotics and *Mycobacteria*. Shami and Almasri [23] demonstrated from the time-kill curve obtained in their work that antimicrobial effect of antimicrobials is time and concentration dependent. They also posited that time-kill test is used to determine the bactericidal effect of antimicrobials. The result of this study shows that at 100mg/ml of plant extract, the effect on susceptible bacteria is bactericidal.

Microorganisms have various fascinating ways of resisting harmful antimicrobials. Genetic alterations in bacteria can cause resistance in one of four ways; i) Target molecules are structurally altered to prevent antibiotic binding; ii) antibiotics are excluded from cell entry; iii) They are inactivated through enzymatic degradation and ;iv) they are pumped out (efflux) [24]. Factors produced by microorganisms and that evoke diseases are known as the virulence factors. This include toxin production, surface coats that inhibit phagocytosis and surface receptors that bind to host cells. Hydrophobicity is the ability of organisms to repel water and attach to surfaces and it's a measure of bacterial virulence/ pathogenicity [25]. A significant increase in hydrophobicity is an adaptive reaction against antimicrobial agent [26]. However, high hydrophobicity of test organisms results in the enhancement of non-opsonic phagocytosis of bacteria by macrophages.

The treatment of bacteria with antibiotics induce increase in hydrophobicity and a decrease in negative charge of bacterial surface. Nomura *et al.*, [27] suggested that an increase in hydrophobicity and a decrease in surface charge

results in the non-opsonic phagocytosis of bacteria cells by macrophages which is observed in this study, an increase in hydrophobicity was observed in the susceptible bacteria when compared with hydrophobicity in xylene as presented in table 2. This shows that non-opsonic phagocytosis could be a mode of action of the test plants used in this study.

The ability of bacteria to produce catalase contributes to their pathogenicity by detoxifying the oxygen-dependent microbicidal products of phagocytic cells. Low level of reactive oxygen species (ROS) such as hydrogen peroxide (H₂O₂) are produced as a consequence of aerobic respiration and metabolism. Large concentration of H₂O₂ may overwhelm an organism's defenses and may prove fatal to microorganisms. Antimicrobials produce their killing effect by inducing the production of radicals within bacteria cells [28]. Research has also shown that antibiotic greatly induce oxidative stress in microbial cells [29]. So the loss of the ability to produce catalase by the test bacteria indicates the inability of bacteria to deactivate antimicrobial by catalase production. This is evidenced in this study with the correlation between catalase production and sensitivity of test organisms to plant extracts as presented in table 1.

V. CONCLUSION

Conclusively, the plants *C. patens* and *P. reticulatum* have broad spectrum antimicrobial activities and they justify their uses in traditional treatment of microbial infections and as such can be considered as lead candidates in antimicrobial drug production.

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