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PHYTOCHEMICAL SCREENING AND ANTI-MYCOBACTERIUM ACTIVITIES OF ROOT EXTRACT OF PAVETTA CRASSIPES (K. SCHUM) PLANT

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ABSTRACT

This research aimed at screening the root extract of *Pavetta crassies* (*P. crassies*) plant phytochemically and test for its anti-mycobacterium activities using standard method and agar disc diffusion method respectively, followed by running column chromatography of the crude extract and determining the functional group(s) present. The phytochemical screening shows the presence of flavonoids, saponins, terpenoid, phenols, alkaloids, quinones and tannins. The crude extract inhibits the growth of the following microorganisms (zone of inhibition in mm): *Staphylococcus aureus* at 500 to 6.25 µg/ml is (8–19), *Escherichia coli* (16–23), *Klebsiella pneumoniae* (7–19), *Pseudomonas aeruginosa* (8–12) and *Salmonella typhi* (12–25). The extract was subjected to column chromatography. The fractions obtained were tested for antimycobacterium activity, and the most active fraction was subjected to FTIR analysis. The absorption peaks obtained ranges from 3200–3600 cm^{-1} O-H stretch, O-H stretch ranges from 2500–3300 cm^{-1}, N-H bending range at 1550–1640 cm^{-1}, N-O stretch at 1515–1560 cm^{-1}, C=C stretch at 1400–1600 cm^{-1}, N-O stretch at 1345–1385 cm^{-1}, C -O stretch at 1210–1320 cm^{-1}, and C-O stretch at 1000–1300 cm^{-1} . Therefore, the crude extract of *P. crassipe* possesses metabolites that are active against some pathogenic microorganisms.

Keywords: Antimycobacterium, chromatography, phytochemicals, *Pavetta crassipe*, tannins

INTRODUCTION

Pavetta crassipes K. Schum. (Rubiaceae) is a low shrub of the savannah. The plant is found in the savannah region of west and central Africa, where the leaves are eaten as food or used for the treatment of fever, mental illness, convulsion, pain, hookworms, and various microbial infections [1-3].

In Nigeria, the leaves of this plant are used medicinally in the management of respiratory infections and abdominal disorders. The leaves are also used in Tanzania in the treatment of gonorrhea. In Central Africa, the acid infusion of the leaves is taken as a cough remedy [2-4].

Tuberculosis (TB) is a contagious, infectious disease mainly caused by *Mycobacterium tuberculosis* (MTB) which is an aerobic pathogenic bacterium that establishes its infection usually in the lungs. Progression of TB infection is fundamentally regulated by host's immune system integrity which may succeed through microbial immediate elimination and/or latency conditioning, or fail resulting in development of active disease. Tuberculosis may also occur in the bones, meninges, joints, genito-urinary tract, liver, kidneys, intestines and heart. [4]. Tuberculosis is transmitted between humans through the respiratory route and most commonly affects the lungs, but can damage any tissue. Only about 10 percent of individuals infected with MTB progress to active TB disease within their lifetime; the remainder of persons infected successfully contain their infection. One of the challenges of TB is that the pathogen persists in many infected individuals in a latent state for many years and can be reactivated to cause disease. The risk of progression to TB disease after infection is highest soon after the initial infection and increases dramatically for persons co-infected with HIV/AIDS or other immune-compromising conditions [5].

Therefore, the aim of this research is to carry out phytochemical screening of the root extract of *Pavetta crassies* plant and test for its anti-mycobacterium activities.

EXPERIMENTAL

Collection and Authentication of Plant Sample

The root of *Pavetta crassipes* (Plate 1) used in this study was collected from Shelleng Local Government Area, Adamawa State, Nigeria. The plant sample was identified by the traditional medicine practitioners in different part of Shelleng and authenticated by the Department of Plant Science, Modibbo Adama University, Yola.

Plate1: *Pavetta crassipes* (K. Schum) plant

Sample Preparation

The root of *Pavetta crassipes* was first washed thoroughly with distilled water after collection, and appropriately air dried at room temperature to ensure evaporation of its moisture content. The sample was then grinded to powder using mortar and pestle and the powder was subjected to soxhlet extraction. The extract was then concentrated using water bath after filtration, and the dried sample was stored at room temperature for use.

Phytochemical Screening

Test for Alkaloids

About 2 g of root extract was boiled in a water bath with 20 ml of 5% sulphuric acid in 50% ethanol. The mixture was cooled and filtered. A portion was reserved. Another portion of the filtrate was put in 100 ml of separating funnel and the solution was made alkaline by adding two drops of concentrated ammonia solution. Equal volume of chloroform was added and shaken gently to allow the layer to separate. The lower chloroform layer was run off into a second separating funnel. The ammoniacal layer was reserved. The chloroform layer was extracted with two quantities each of 5 ml of dilute sulphuric acid. 1 ml of Mayer's reagent was added drop by drop. Formation of a greenish coloured or cream precipitate indicates the presence of alkaloids [6].

Test for Flavonoids

The test for flavonoid adopted is as reported by [7, 8]. Exactly 0.50 g of root extract was weighed into a beaker containing 30 cm^3 of distilled water for 2 hours and filtered with whatman filter paper. About 5 cm^3 of 1.0 M dilute ammonia solution was added to 10cm^3 of the aqueous filtrate of each plant extract followed by the addition of 5cm^3 of concentrated tetraoxosulphate (VI) acid. Appearance of yellow colorations and disappearance on standing shows the presence of flavonoids.

Test for Phenols

Equal volume of the root extract was added to the equal volume of the ferric chloride. A deep bluish solution indicates the presence of phenol [7, 8].

Test for Saponin

Exactly 0.5 g of the root extracts was placed in a test tube and 10 ml of distilled water was added and shaken vigorously for 30s. It was then allowed to stand for 30 min and observed. Formation of honey comb froth indicates the presence of saponins [7, 8].

Test for Tannin

About 2 g of root extract was mixed with 10 ml of 50% alcohol, It was then filtered and the filtrate was divided into three portions for the following tests. Ferric chloride test: Three drops of diluted solution of $FeCl_3$ was added to the test tube, production of a blue or greenish-black colour that changes to olive green as more ferric chloride is added indicates the presence of tannins [6].

Test for Terpenoid

Few drops of concentrated sulphuric acid were added to the test samples in chloroform, a red colour appears at the lower layer indicating the presence of terpenoids [7, 8].

Evaluation of Antimicrobial Activity

Agar disk-diffusion method was used in testing the antimicrobial activity. In this procedure, agar plates are inoculated with a standardized inoculum of the test microorganism. Then, filter paper discs (about 6mm in diameter), containing the test compound at a desired concentration, are placed on the agar surface. The petri dishes are incubated under suitable conditions. Generally, antimicrobial agent diffuses into the agar and inhibits germination and growth of the test microorganism and then the diameters of inhibition growth zones are measured. The crude extract was dissolved in dimethylsulfoxide (DMSO) to have a solution of 100mg/ml concentration. Exactly 10g of the extract was dissolved in 1ml of DMSO and diluted in 1:100 by adding 50micromill of middle brook broth 7H9 broth to give concentration of 10ml/ml of 100mg/ml concentration and stored at 20°C until use [9].

Micro-organism Preparation

About 250 µl of test organism mycobacterium Bovis (BCG) freshly thawed stock was inoculated into 25 ml of sterile middlebrook 7H9/ADC broth medium and incubated at 30 °C for 5-7 days. The optical density of the resulting cultured was measured using a UV/visible spectrophotometer. The optical density of the resulting culture determined at 650 nm was approximately 0.2% which equals to 109 cfu/ml [9].

Minimum inhibitory concentration (MIC) Determination

The broth micro-dilution method was employed for the determination of MIC of the extracts. Fifty microliters (50 µl) of sterile 7H9 broth are transferred into each of the wells of 96 micro-well plates starting from Well 2 to 12, Using multi-channel pipettor, 100 µl of extract was pipetted into Well 1 from which 50 µl was transferred to Well 2 mixed thoroughly by

pipetting up and down four times and 50 µl was withdrawn and transferred to Well 3 and the process continued to Well 11 from which 50 µl was withdrawn and discarded that is serial dilution. Well 12 served as organism control. Extracts were first dissolved in DMSO and then diluted in the middle brook 7H9 broth, to give a stock concentration which was diluted out across 96 well microliters in a two-fold serial dilution to give final testing concentrations. The same procedure was repeated for the Rifampicin as reference drug. The initial concentration of 25 ml/µl of Rifampicin were diluted to the testing concentrations the plates were then incubated for 5-7 days at 37 °C. The MICs of the extract were recorded as the lowest extract concentration at which no mycobacterial growth was observed [9].

Screening of Samples for Anti-Tuberculosis Activity

The micro broth dilution method was employed for the determination of MIC of isolated compounds. Into each well of 96 microwell plate was transferred 50 µl of sterile 7H9 broth starting from well 2 to 12. To each of the wells was added 100 µl of l0% DMSO, l00 µl of 25 g/ml solution of Rifampiein (control drug which was prepared by dissolving 250mg of rifampicin powder in 10rnl DMSO and diluted 1:1000 by dispensing 25 µl of rifampicin in 25ml 7H9 middlebrook broth) and l00 µl of each isolated compound. Using a multichannel pipettor, 50 µl was carefully removed from well 1 to 2, mixed thoroughly and the process continued to well 11 from which 50 µl of diluted BCO culture and incubated at 30°C for a period of seven days. The results were confirmed by staining the wells with tetrazolium dye after the incubating period. The reduction of tetrazolium salt from colorless or weakly coloured to brightly coloured derivative in the wells is an indication of sample inactivity but if the dye remains colourless that confirms the activity of the sample. The last well of the sample where there was no growth is regarded as the MIC of the sample. Well 12 served as organism viability control (OVC).

Isolation of Bioactive compound (Column Chromatography)

Exactly 5 g of the crude extract was loaded into a column of stationary phase. An organic solvent or a mixture of solvent system (hexane-ethlyacetate, methanol-chloroform 10% step wise increase) flows through down the column. Components of the sample separate from each other by partitioning between the stationary (silica) and the mobile phase (solvent system). Molecules with different polarity partition to different extent, and therefore move through the column at different rates. The eluents are collected in fractions and concentrated.

From the TLC profile of the fractions, those with the same separation were put together to give major fractions [10].

RESULTS AND DISCUSSION

Phytochemical screening of the crude extract of *Pavetta crassipe*

The phytochemical screening of the root exacts of *Pavetta crassipe* (Table 1) shows that alkaloid, flavonoid, phenol, terpenoid, saponin, tannin and quinone are present in the root. These phytochemicals are known to be behind the antimicrobial activities, antifungal, anti-allergenic, antispasmodic and anti-inflammatory properties of the medicinal plants.

Table 1: Qualitative analysis of the methanolic root extracts of *Pavetta crassipes*

Plant	Alkaloid	Flavonoid	Phenol	Terpenoid	Tanin	Saponin	Quinone
Pavettacressipes	+	+	+	+	+	+	+

Keys: + = present; - = absent

Anti-Microbial Activity of Root Extractsof *Pavetta cressipes*

Escherichia coli

The antimicrobial activities of the root of *P. crassipes* against *E.coli* (Figure1) shows that at 500 µg/ml, the zone of inhibition of the methanolic root extract is 23 mm which is higher than that of the control drug (augmentin), at 250 µg/ml, the zone of inhibition is 21 mm which showed the same susceptibility with that of the positive control, likewise, at 125 and 6.25 µg/ml, the root has zone of inhibition 18– 16 mm.

This indicates that the test organism (*E. coli)* is highly susceptible to the methanolic extract of the root of *P. crassipes*

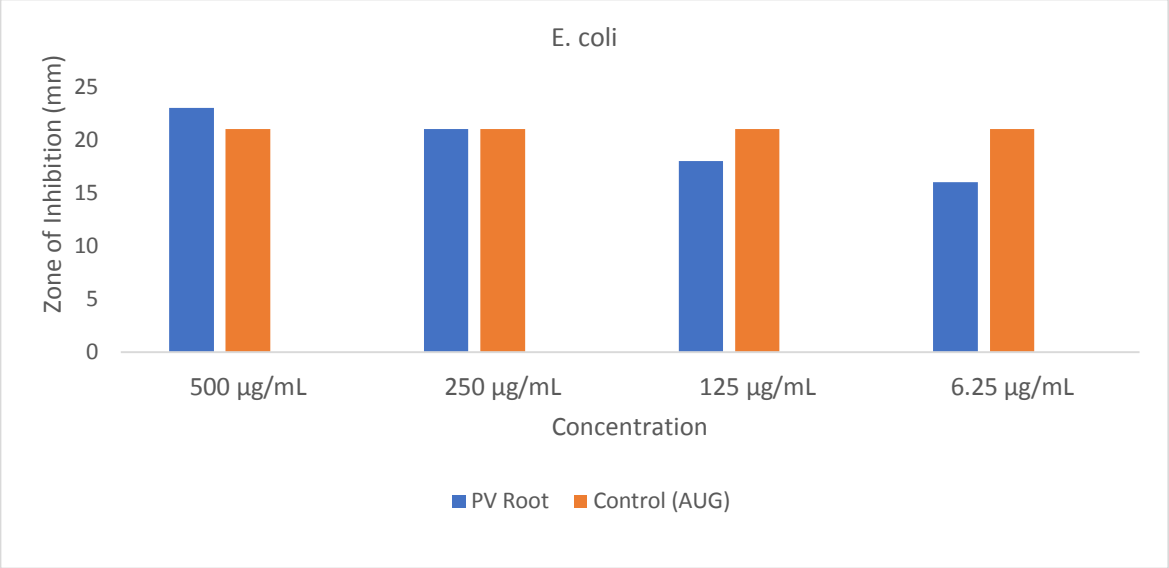

Figure 1: Antimicrobial activities of the root extract of *P. crassipes* against *E. Coli*.

Staphylococcus aureus

The antimicrobacterial activities of the root extracts of *P. crassipes* against *Staphylococcus aureus* (Figure 2) shows that at different concentration of methanolic extract of the root of *P. crassipes*, there is zone of inhibition. This indicates that the test organism is susceptible to the root extract, indicating that the test organism is slightly susceptible at 6.25 µg/ml at 8mm of the root extract.

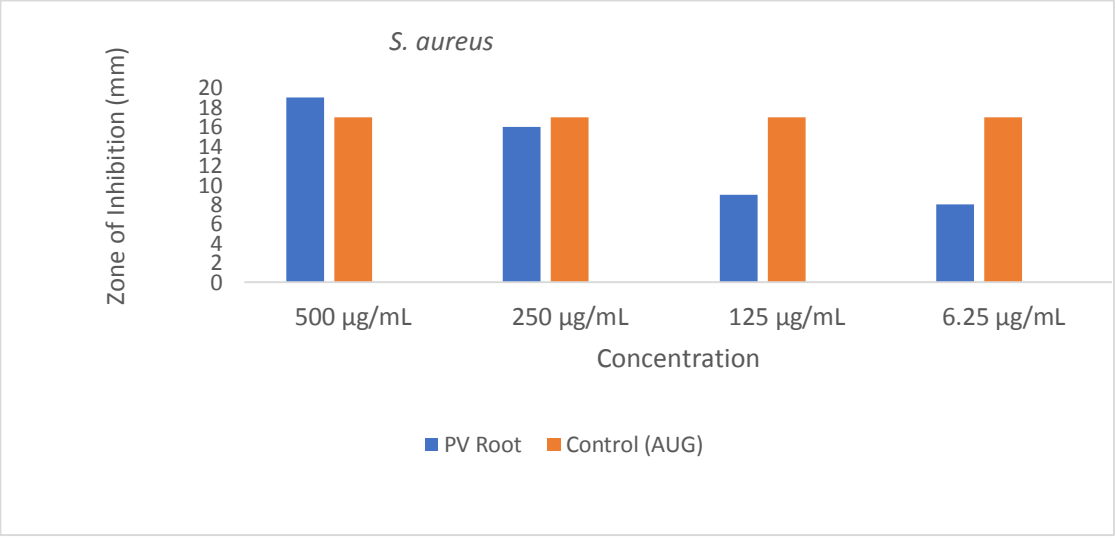

Figure 2: Antimicrobial activities of the root extract of *P. crassipes* against *Staphylococcus aureus*

Klebsiella pneumonia

The antimicrobial activities of the root extract of *P. crassipes* against *K. pneumonia* (Figure 3) shows that the tested organism at the lowest concentration (6.25 µg/ml) shows the least amount of the zone of inhibition at 7 mm of the root. Whereas, at the highest concentration (500 µg/ml) of the plant extract, the root gives the highest zone of inhibition than the control (AUG).

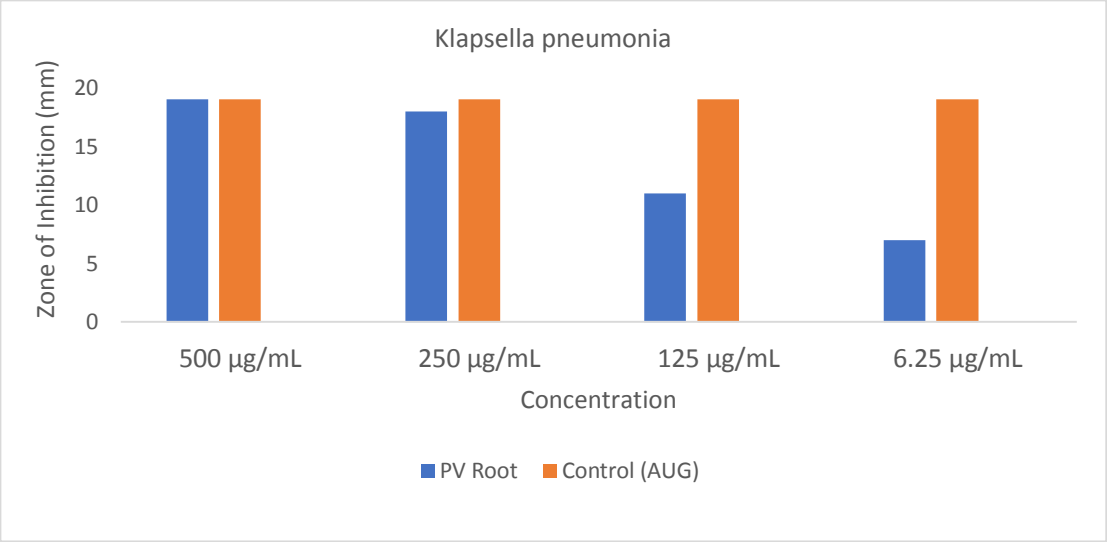

Figure 3: Antimicrobial activities of the root extract of *P. crassipes* against *K. Pneumonia.*

Pseudomonas aeruginosa

The antimicrobial activities of the root extract of *P. crassipes* against *P. aeruginosa*

(Figure 4) shows that the root extract at the highest concentration (500 µg/ml) have slightly higher zone of inhibition compared to that of the control at the concentration (30 µg). Likewise, at the lowest concentration (6.25 µg/ml), the root extract, shows the highest zone of inhibition. This indicates that the test organism (*P. aeruginosa*) is susceptible to the methanolic leaf extract of *P. crassipes*. This is a change in the antimicrobial activities against the bioactive or secondary metabolite of the plant. Unlike the other tested organisms that shows more susceptibility to the ethanolic root extract than the *P. aeruginosa.*

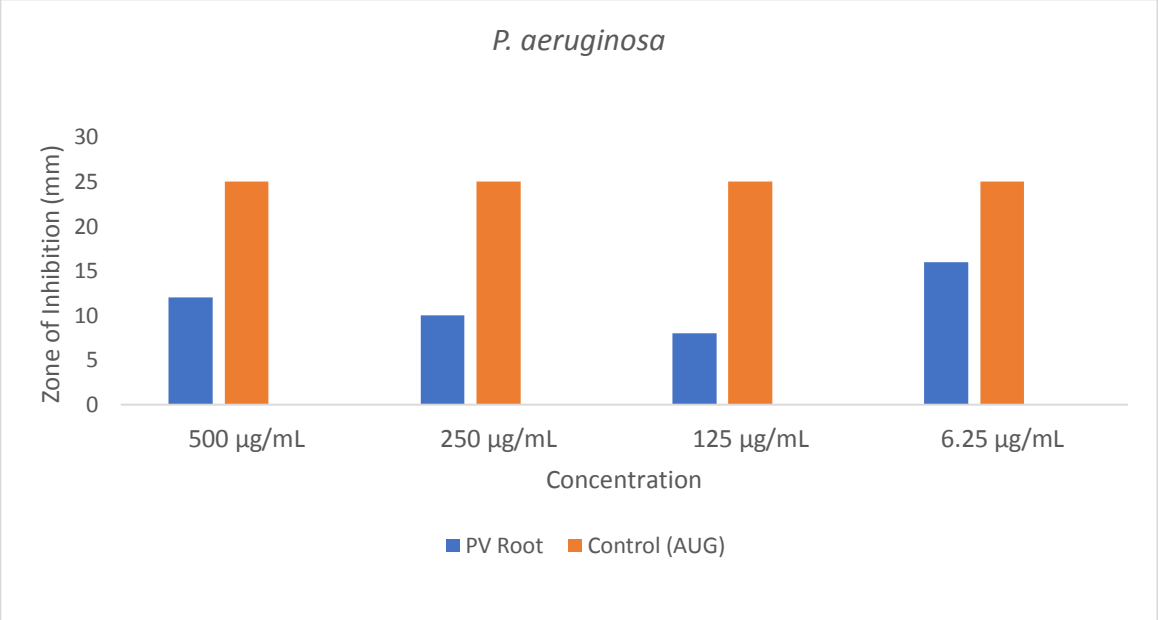

Figure 4: Antimicrobial activities of the root extract of *P. crassipe* against *pseudomonas aeruginosa.*

Salmonella typhi

The antimicrobial activities of the methanolic extract of the root of *P. crassipes* against *Salmonella typhi* (Figure 5) shows that the root extract is very potent against the test organism. The root extract shows a higher zone of inhibition even more than the control drug (augmentin). The organism is highly susceptible to the ethanolic root extract.

The antibacterial activity of the *P. crassipes* is strongly linked with the presence of bioactive compounds especially flavonoid, which is one of the principal phytochemical components of the plant [11].

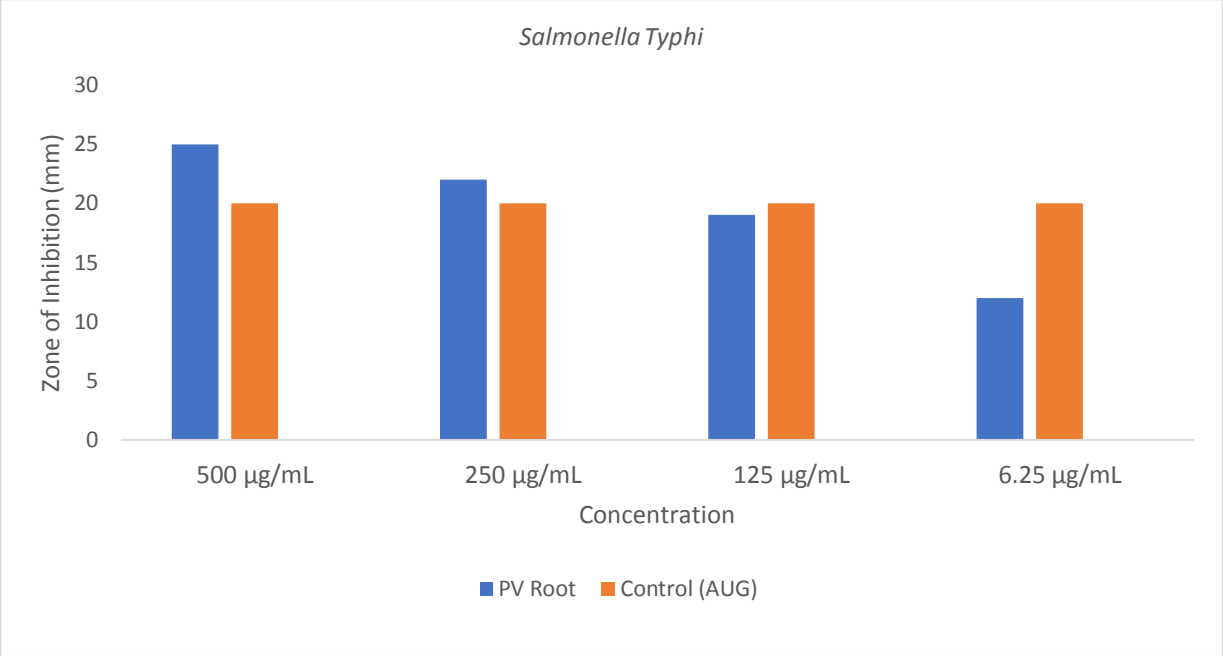

Figure 5: Antimicrobial activities of the root extract of *P.crassipe* against *salmonella typhi*.

Anti-mycobacterium Activity the Root Extracts of *Pavetta crassipes.*

The result of the anti-mycobacterium activities of root extracts of *Pavetta crassipes* shows that the zone of inhibition (mm) of bacteria growth at different concentration of methanol extract of *Pavetta crassipes* (Table 2) shows that the root extract inhibited all the isolates with zone diameter ranging from 11-26 mm at 500 µg/ml respectively. Many researches have shown that *Pavetta crassipes* contains many bioactive compounds that are active against the causative organism of tuberculosis. *Pavetta crassipes* has different species that contains secondary metabolites that are effective against microbial infections. *P. crassipes* has antimicrobacterial activity against *klebsiella pneumonia, Proteus species, Pseudomonas aeruginosa and Staphylococcus aureus* [12, 13].

Table 2: Anti-mycobacterium analysis of root extracts of *Pavetta crassipes* zone of inhibition in (mm) at different concentrations in (µg)

	Root extract of *Pavetta crassipes* plant				Control (Streptomycin)
Concentrations	500	250	125	6.25	30
Zone of inhibition	26	18	15	11	28

Bioactive compounds obtained from column chromatography

The separation of bioactive compounds from the plant sample was obtained using the following procedures: The aqueous extract of the plant (37g) was extracted with hexane, ethyl acetate and butanol to produce 6.1, 9.5 and 17.9 extracts respectively. The butanol extract was used for the isolation and then separated by column chromatography using gradient elution techniques as follows:

4 fractions (100% Hexane), 7 fractions (EtOAc =100), 8 fractions (EtOAc: Butanol = 90:10), 6 fractions (EtOAc: Butanol = 80 :20), 11 fractions (EtOAc : Butanol =70:30), 3 fractions (EtOAc : Butanol = 60:40), 11 fractions (EtOAc : Butanol = 50:50), 6 fractions (EtOAc : Butanol = 40:60), 9 fractions (Butanol: EtOAc =30 :70), 4 fractions (EtOAc : Butanol = 20:80), 13 fractions (EtOAc : Butanol = 10: 90), 9 fractions (Butanol = 100%). Out of the 11 fractions obtained from the column chromatography, 5 fractions were used to test for the antimycobacterium activity. From the result obtained, the RF2 (root fraction) which is 70:30 produces the highest zone of inhibition compared to other root fractions at the same concentration of 6.25µg/ml. The RF2 which was the most active and at 0.52 RF (retention factor) was used to determine the IR spectrum.

Fourier Transform Infrared (FTIR)

In this research work, the FTIR was used to determine the chemical bond (functional group) present in the plant which is shown in Table 3. Fourier transformation infrared spectrum of the methanolic root extract of *Pavetta crassipes* gives the data on the peak values and probable functional groups present in the methanolic root extract of *Pavetta crassipes*

The methanolic extract of the root of *Pavetta crassipes* shows the following characteristic absorption bands: at 3200-3600 cm^{-1} (for OH stretch), 2500-3300 cm^{-1} (for OH stretch), 1550-1640 cm^{-1} (for N-H bending), 1515-1560 cm^{-1} (for N-O stretch), 1400 – 1600 cm^{-1} (for C=C stretch), 1210-1320 cm^{-1} (C-O stretch), 1000-1300 cm^{-1} (for C-O stretch).

Table 3: Fourier Transform Infrared Spectral peak values and Functional groups present in the root of *Pavetta crassipes.*

Peak Value (cm^{-1})	**Functional Group**	**Type of vibration**	**Characteristic Absorption (cm^{-1})**	**Intensity**
3421.1	O-H	Stretch, (H-bonded)	200-3600	Strong, broad
2941.1	O-H	Stretch	2500-3300	Strong, very broad
1617.3	N-H	Bending	1550-1640	Medium weak
1521.7	N-O	Stretch	1515-1560 & 1345-1385	Strong, two bands
1445.3	C=C	Stretch	1400-1600	Medium-weak, multiple bands
1371.7	N-O	Stretch	1515-1560 & 1345-1385	Strong, two bands
1273.7	C-O	Stretch	1210-1320	Strong
1107.7	C-O	Stretch	1000-1300	Two bands or more

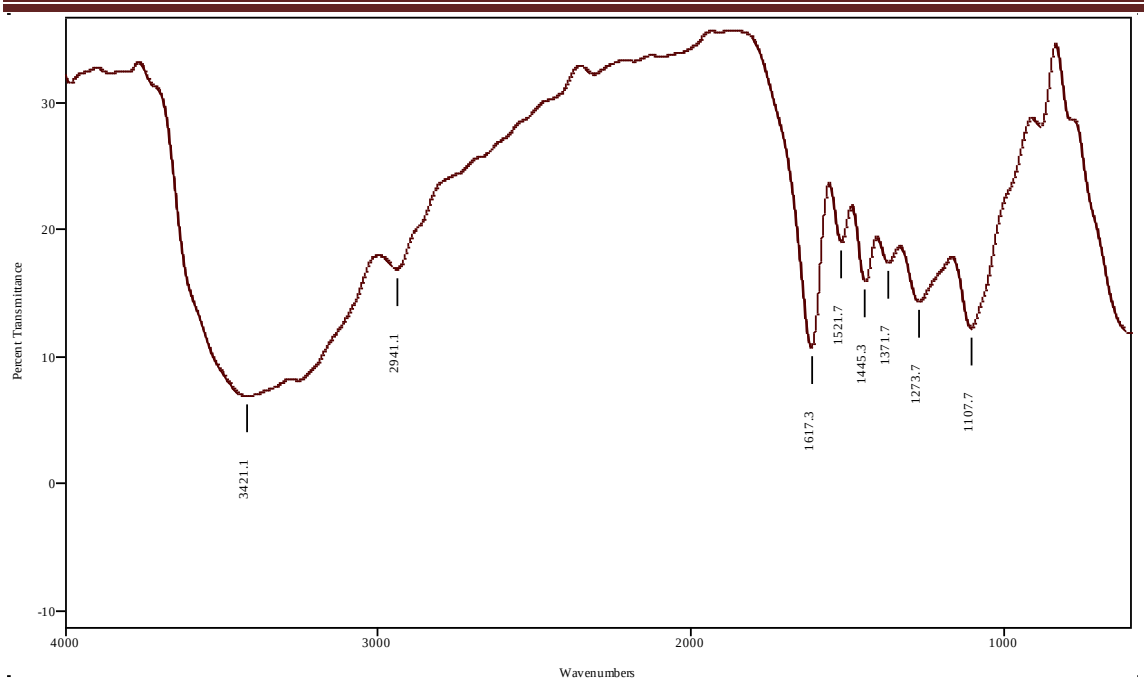

Figure 6: FTIR spectrum of active fraction obtained from the root extract of *Pavetta crassipe* plant

CONCLUSION

Based on the result obtained from this research work, the phytochemical screening of the root extract of *Pavetta crassipe* shows the presence of flavonoids, saponins, terpenoid, phenols, alkaloids, quinones and tannins and also the extract shown that it can inhibit the growth of some microorganisms. It is recommended that further research should be carried out on the root of the plant using different solvents in order to determine more bioactive secondary metabolites that could be present in the root of *Pavetta crassipes* which makes it more active against different pathogenic microorganism.

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