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Activity Guided Fractionation with Antimicrobial Evaluation of *Pergularia tomentosa L. (Asclepiadacea)* Whole Plant

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

This research work aimed to present the activity guided fractionation with antimicrobial evaluation from both crude extract and various fractions obtained from *Pergularia tomentosa L*. whole plant. The *P. tomentosa L*. whole plant was extracted with 95% aqueous ethanol; fractionated into acidic, basic, polar and nonpolar fractions. All fractions with the crude extract were screened for both antimicrobial and minimum inhibitory concentration (MIC) potentialities. For crude extract, all concentrations (1.5, 0.75, 0.35, & 0.168 mg/ml) indicated marginal antibacterial activity with range of 17, 20, 14 mm zone of inhibition for *S. aureus*, *E. coli* and *C. albicans*. While, both basic fraction showed highest activity against *E. coli* and *C. albicans* at 15 mm & 15 mm; along with acidic and methanolic fraction haven large spectrum against *S. aureus*, *E. coli* and *C. albicans* at 13, 12, 12 mm. Moreover hexane did not showed antimicrobial activity for both *S. aureus* and *C. albicans* except for *E. coli* which showed activity at 12 mm. The study clearly indicated that basic fraction showed highest antimicrobial activity for selected micro-organisms with lower minimum inhibitory concentration which ranges from 18.75 µg/ml to 150 µg/ml. Followed by wider spectrum of antimicrobial activity for acidic and methanolic fraction against all tested organisms with minimum inhibitory concentration from 75 µg/ml to 150 µg/ml; while 300 µg/ml (MIC) stand for hexane fraction. Thus, *P. tomentosa* L. particularly, the basic fraction (alkaloid) and, both acidic and neutral

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fractions will be centered areas for further scientific research findings in isolating an active antimicrobial component therein.

Keywords: P. tomentosa L.; phytochemical analysis; bioassay guided fractionation protocol; Staphylococcus aureus and Escherichia coli and Candida albicans.

1. INTRODUCTION

Disease and ailments is inseparable companion of life. They strike the urban and rural, rich and poor alike, and show no respect for status, class or community. The efforts to cure disease and ailments, desire to attain vitality and longevity, prompted early man to explore his natural surroundings; and in the process he selected a number of therapeutic agents, mainly from plants. Meanwhile, resistance to antibiotics and occurrence of toxicity during prolonged treatment with the present day drugs, have been the reasons for extended search of new drugs to treat microbial infections [1].

There is a current shift towards evaluating the activity of phytochemicals using fractionating guided protocol in actualizing most antimicrobial uses for medicinal plant. Herbal medicines derived from plant extracts are being increasingly utilized to treat a wide variety of clinical diseases. Plants provide abundant resources of antimicrobial compounds and have been used for centuries to inhibit microbial growth [2]. This is due to the presence from various kinds of phytochemicals including phenolic compounds, alkaloids, terpenoids and essential oils [3]. The most important of these bioactive constituents from plants are alkaloids, tannins, flavonoids, saponins, glycosides and other phenolic compounds. The phytochemicals isolated are then screened for different types of biological activity [4]. Alternatively, crude plant extracts can be first assayed for particular activities and the active fractions are then analyzed phytochemically [4]. One of the varieties for bioassays guided fractionation protocols are now available for the phytochemist to use in such work [5].

The milk weed family, *Asclepiadeceae*, comprises 200 genera and 2500 species of perennial shrubs and herbs distributed throughout tropics and temperatures areas of the world. *P. tomentosa* L. is reputed for diverse folk uses as an antirheumatic and in treatment of some skin diseases, as laxative, abortive and for treatment of asthma and bronchitis [6]. The plant was reported to have mulluscal activity [7] and persistent hypoglycemic effects [8]; a potential antitumor agent [6]; antifungal effect against *Aspergillus niger* [9]; Anti-insecticidal activity [10] and antidermatophytes [11]. The presence of ghalakinoside, calactin and pergularoside was reported in the roots of *P. tomentosa L.*; three cardenolides, desglucouzarin, coroglaucigenin and uzarigenin from the aerial parts of *P. tomentosa* [6] and two triterpenes of the taraxosterol skeleton were isolated; pergularine A and pergularine B. [12]. Other species and countries are: *P. suaveolens* (R.Br.) Spreng. & *P. viridi flora* (R.Br.)-(Australia); *P. tinctoria* (R.Br.) Spreng. (Sumatra); *P. tomentosa L*. (Middle East, Egypt-Pakistan); *P. adenophylla* Schltr.& K. Schum (Cameroon); *P. brunoniana* (Wight & Arn.) D. Dietr.- (India); *P. calesiana* (Wight) Buch.- Ham.ex Hook.f.-(Himalayas); *P. Clausa* (R. Br.) Spreng-(Jamaica); P*. daemia* (Forssk.) Chiov.–(Africa, S. Asia); *P. exilis* (Colebr.) Spreng.-(Banglandesh); *P. flavescens* (A.Cunn.) Hook.f.ex D. Dietre & P. roylei (Wight) D. Dietr.-(Himachal Pradesh); *P. hamiltonii* (Wight) D. Dietre.- (Uttar Pradesh); & *P. rostrata* (R. Br.) Spreng- (Queensland).

Medicinal plants are of great importance to the health of individuals and communities. About 30% ingredient of Allopathic medicines and 100% of Ayurvedic, Siddha, Unani and Homeopathic medicine come from plants. In recent years, Allopathy or conventional medicine is becoming more open to Ayurveda or herbal medicine and there are many practitioners who regard the two as complementary. In Ayurveda, the cumulative effect of the whole plant is considered to be more efficacious and research in this field is unfathomable as there are many healing properties of plants yet to be discovered. This work was therefore, designed to evaluate antimicrobial activities of *Pergularia tomentosa* L. whole plant as one of the commonly used plant; in relating to its high potency on different fractions from various solvent extraction properties, polarities and potentialities.

2. MATERIALS AND METHODS

2.1 Chemicals

Ethanol, Mueller Hinton's Agar (MHA), Sabroband Dextrose Agar (SDA) from Sigma-

Aldrich Chemical Co. (St. Louis, USA), Vitamin C, Methanol, Chloroform, Ethyl Acetate, Hydrochloric Acid, Sodium Hydroxide, Hexane and all others solvent (Analytical grade) from Merck Co. (Darmstadt; Germany), and Distilled Water.

2.2 Sample Collection

Fresh plants of *Pergularia tomentosa* L. were collected at Nagazi around Federal College of Education Okene, Kogi State, Nigeria. The plant was identified and confirmed at Ahmad Bello University, Zaria, Kaduna; ABU Herbarium (Botany Unit, Department of Biological Science) by Mr. Muhammad Musa, Voucher no. 645, specimens was deposited in the Herbarium. The plant materials (fresh whole plant) were air dried, pulverized into a fine powder using a commercial blender.

2.3 Extraction and Fractionation Procedure

Extraction and fractionation of the plant extract was carried out by bioassay guided fractionation protocol [5]. The procedure was carried out using ethanol-water (95:5v/v) and different organic solvent in order of polarity (Hexane, chloroform and Methanol) using separatory funnel. These fractions were compared with the crude extract for effective antimicrobial activity respectively. One thousand grams of the powdered plant materials (20 mesh~1 g) were extracted using percolation process in a mixture of 95 ml of distilled ethanol and 5 ml of distilled water at ambient temperature overnight. The extractives was filtered and re-extracted three times. The combined extract were filtered through a Whatman No. 1 paper and then concentrated in vacuo at 40°C using a rotary evaporator, model W2-100 SENCO® @ rpm of 100; Shanghai SENCO technology Co, Ltd Japan. The various extractive concentrates were evaporated to dryness using water bath for some days and residues were obtained in gram for crude extract, basic, acidic, polar and non-polar fraction as 97 g, 7 g, 6 g, 35 g, and 31 g.

2.4 Phytochemical Screenings of the Methanolic Extracts

Qualitative Phytochemical analysis of different Phytochemical compound (flavonoid, tannins, saponins alkaloid, glycosides and steroids were carried out in accordance with the procedure of [13].

2.5 Antimicrobial Sensitivity Test

Antibacterial activity of the plant extracts and fractions were carried out using agar diffusion method [14] with slight modification. The test organisms used were clinical bacteria isolate of *Staphylococcus aureus and Escherichia coli* as well as the fungus *Candida albicans.* Pure cultures of the test isolates were obtained from Microbiology bank, Department of Biotechnology Advanced Laboratory, Sheda Science and Technology Complex (SHESTCO) Abuja Nigeria. Nutrient Agar was used for repeated sub culturing and preservation of the isolate, before being used for the antibacterial assay. It was prepared according to manufacturer's specification and sterilized inside the autoclave at 121°C for 15 minutes. Mueller Hinton's Agar (MHA) and Sabrobaud Dextrose Agar (SDA) was used for the test. It was poured into sterile petri plates. All flasks and equipment used were sterilized accordingly inside the hot air oven. The hood was sterilized by swabbing with 70% ethanol using cotton wool. The test were seeded onto agar surface by streaking. The agar well diffusion method was used for antimicrobial assay. This was done using a cork borer of 6 mm diameter, which was sterilized after each use by dipping in 70% ethanol and flaming with the bunsen flame. To the test organisms, 100 µl of 50 mg/ml for each fractions (equivalent to 5 mg/well) and a half dilution series of 1.5, 0.75, 0.375 and 0.168 mg/well were then applied to each of the holes labelled 1, 2, 3 and 4 respectively with Gentamycin; as a standard drug used as control, using sterile tips attached to a micropipette. The set up was then incubated for 24 hours at 37°C in incubator. The zone of inhibition were then taken, after the incubation period using a graduated ruler and recorded in millimetre (mm). All tests were carried out in triplicates.

2.6 Minimum Inhibitory Concentration (MIC)

Minimal inhibitory concentration (MIC) was carried out as described [15] with slight modification. The plant extracts were tested for antibacterial activity using the macrobroth dilution method in broth media Mueller-Hinton. In these experiments, 50 µl of a suspension containing 1 x 10^6 CFU/ml was add to 4.5 ml of susceptibility test broth containing 0.5 ml serial two fold dilutions for the test samples. All tubes were incubated in air at 37°C for 24 hr before being read. The minimal inhibitory concentration (MIC)

was considered the lowest concentration of sample that prevented visible growth. This was validated by measuring absorbance of the broth against a blank at 600 nm.

2.7 Percentage Inhibition

Data were analyzed according to Patton et al. (2005). The optical density was determined just prior to incubation and again after 24hrs incubation at 600 nm. The OD for each replicate at T0 was subtracted from the OD for each replicate at T24. The adjusted OD of each control well is then assigned a value of 100% growth. The percent inhibition of growth is thus determined using the formula.

Percent Inhibition = 1 - (OD test well/OD of corresponding control well) × 100.

3. STATISTICAL ANALYSIS

For each crude extract and fractions, samples were analyzed and the assays were carried out in triplicate. The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD test with $P<0.05$ (different letters mean significant differences; this treatment was carried out using Graph Pad Prism version 5.02 for Windows (1992-2009) Graph Pad Software Inc.

4. RESULTS

The Phytochemical analysis for all screened fractions including crude extract was determined. Both crude extract and fractions revealed the presence of all tested Phytochemical compounds. These includes alkaloids, anthraquinones, flavonoids, glycosides, saponins, steroids and tannins; as it is expected for ethanol (solvent used for active component extraction) in Table 1; with their various weights of fractions and crude extract in Table 2.

Phytochemicals are known to possess antimicrobial properties as reported by [16-18]. Findings from this work also agreed with the previous work of [19,20] who reported that the plant contains flavonoids, triterpenoids and others.

Analysis of variance (one way) Anova conducted on the result of zones of inhabitation and minimum inhibitory concentration indicated that there are significant differences between most fractions values ($P\leq0.05$ at 95% confidence level) except for the following bacteria's; No significant difference between *S. aureus* and *E. coli*, *S. aureus* and *C. albicans* in Figs. 1, 2 and 3; No significance difference between *E. coli* and *C. albicans*, also in Fig. 3; no significance difference between *S. aureus* and *C. albicans* in Figs. 4 and 5., More so, there was no significance difference for all tested organisms considering their minimum inhibitory concentration ($P\leq0.05$). However, significance difference exists between all tested fractions, crude extract with Gentamycin. The minimum inhibitory concentration (MIC) of the various extract with crude extract ranges from 300 µg/ml to 18.75 µg/ml on tested bacteria's. Table 3 depicted the minimum inhibitory concentration and mean zone of inhibition of various fractions and crude extract of whole plant of *P. tomentosa L*.

5. DISCUSSION

Basic fractions of *P. tomentosa L.* whole plant showed good antimicrobial activity depending on the concentration for its zone of inhibition in line with [10] as proved that alkaloids of aerial part from *P. tomentosa L*. showed considerable toxicity and growth inhibitory activities. The higher concentration used, the higher antimicrobial activity effect expressed in percentage inhibition compared to Gentamycin are shown in Figs. 1-5 for all verified studied. Thus, increased intake of *P. tomentosa L.* whole plant extracts as used by traditional practitioner will be supported only within normal concentration.

The maximum antibacterial activity against *E. coli and C. albicans* was basic fraction while the large spectrum antibacterial activity was acidic and methanolic fraction as shown in Table 4. Hexane has lower antibacterial activity against *E. coli* only; this lower antibacterial activity of hexane has been reported earlier by [21] rather than Lupeol acetate isolated from it; while it also showed antidernmatophytic activities [15] before further fractionation.

The significant antibacterial activity for basic fraction was found at 15 mg/ml zone of inhibition with minimum inhibitory concentration of 18.75 µg/ml on *S. aureus* and 75 µg/ml on both *E. coli* and *C. albicans*. But, for both acidic and methanolic fractions, large spectrum zone of 14 mg/ml and 15 mg/ml corresponding to minimum inhibitory concentration of 75 µg/ml and150 µg/ml. 300 µg/ml was for hexane fraction as shown in Fig. 6 (MIC).

Table 1. Solvents used for active component extraction

Water	Ethanol	Methanol	Chloroform	Ether	Acetone
Anthocyanins, Starch, Tannins, Terpenoid, Polypeptide, Lectins.	Tannins, Polyphenols, Polyacetylenes, Flavonol, Terpenoids, Steroid. alkaloids.	Anthocyanins, Terpenoids, saponins, Tannins, Totarol, Xanthoxyllines, Lactone, , Quassinoids, Flavones, Henones & Polyphenols	Terpenoid, Flavonoids.	Alkaloids, Terpenoids, Coumarin, Fatty Acids	Phenol, Flavonol

Table 2. Amount of residues obtained after extraction and fractionation from *P. tomentosa L.* whole plant extract

Plant fraction	Crude extract	Basic extract	Acidic extract	Methanolic extract	Hexane extract
Weight (g)	97	7	6	35	31

Table 3. Minimum inhibitory concentration of crude extract and their various fractions from *P. tomentosa L*. whole plant

		MIC values (µg/ml) whole plant			
Sample	**Organisms**	**150**	**75**	**37.5**	
Crude extract	*S. aureus*	00	00	75	75
	E. coli	00	00	00	00
	C. albicans	00	150	150	150
Basic extract	*S. aureus*	00	00	00	00
	E. coli	00	00	75	75
	C. albicans	00	00	75	75
Acidic extract	*S. aureus*	00	00	75	75
	E. coli	00	00	75	75
	C. albicans	00	150	150	150
Methanolic extract	*S. aureus*	00	00	75	75
	E. coli	00	00	75	75
	C. albicans	00	150	150	150
Hexane extract	*S. aureus*	00	00	00	00
	E. coli	300	300	300	300
	C. albican	00	00	00	00

Key: 00=absent

Table 4. Antibacterial potentialities for the extracts and fractions from *P. tomentosa L.* whole plant

Samples	Concentration (µg/ml)	Bacteria strains with mean zone of inhibition (mm)		
		S. aureus	*E. coli*	*C. albicans*
Crude extract	1.5	17	20	14
	0.75	15	18	13
	0.35	13	17	11
	0.168	12	15	11
Basic fraction	1.5	00	15	15
	0.75	00	13	12
	0.35	00	11	11
	0.168	00	11	11
Acidic fraction	1.5	13	14	11
	0.75	12	13	11
	0.35	12	13	10
	0.168	11	11	10
Methanolic fraction	1.5	15	13	12
	0.75	14	13	11
	0.35	12	12	11
	0.168	11	11	10
Hexane fraction	1.5	00	12	00
	0.75	00	12	00
	0.35	00	11	00
	0.168	00	10	00
Gentamycin		34	33	35

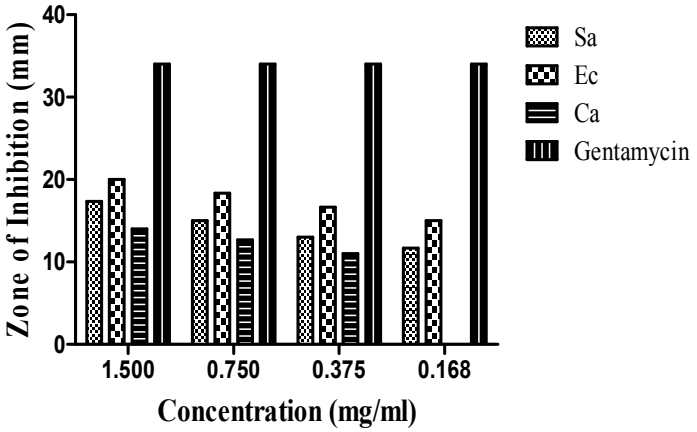

Fig. 1: Comparative Inhibitions of Crude extract of *P.tomentosa* plant against *S. aureus, E.coli, and Candida with gentamycin.*

Looking at minimum inhibitory concentration relationship; both 75 µg/ml and 150 µg/ml stand for crude extract, acidic fraction and methanolic fractions against *S. aureus* and *C. albicans*; 75 µg/ml relate to basic, acidic and methanolic against *E. coli*. Hexane has no relationship with other fraction at 300 µg/ml. this observation, therefore, support the notion that, in general, the Gram negative bacteria are more resistance than the Gram- positive one [22]. Thus, minimum

inhibitory concentration at 75 µg/ml against all tested microbes in this study, indicated high level potency for *P. tomentosa L.* whole plant in the management of diseases or ailment caused by these bacterial such as; minor skin infections, such as pimples, impetigo, boils (furuncles), cellulitis folliculitis, carbuncles, scalded skin syndrome, and abscesses, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome (TSS), bacteremia, and sepsis. Its incidence ranges from skin, soft tissue, respiratory, bone, joint, endovascular to wound infections which is cause *by S. aureus*; gastroenteritis, urinary tract infections, and neonatal meningitis *cause by E. coli* virulent strains; and morbidity and mortality in immunocompromised patients (e.g., AIDS, cancer chemotherapy, organ or bone marrow transplantation) cause by *C. albicans.* Thus, the *P. tomentosa L.* whole plant can be harnessed for medicinal uses in pharmaceutical industries and the likes.

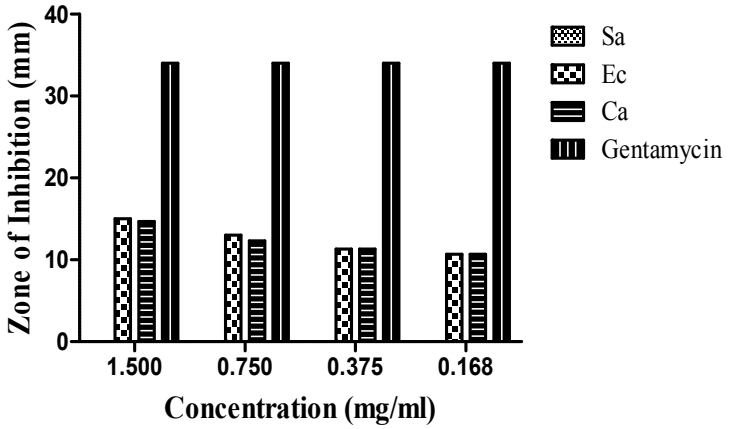

Fig. 2: Comparative Inhibitions of Basic fraction of *P. tomentosa* plant against *S. aureus, E.coli, and Candida with gentamycin.*

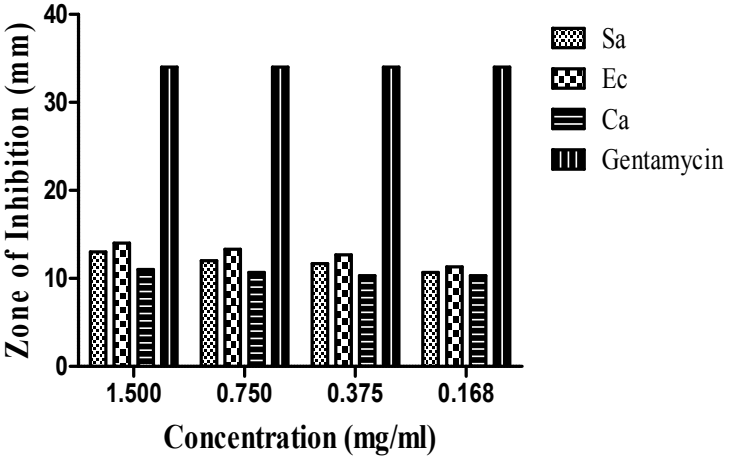

Fig. 3: Comparative Inhibitions of Acidic fraction of *P. tomentosa* plant against *S. aureus, E.coli, and Candida with gentamycin.*

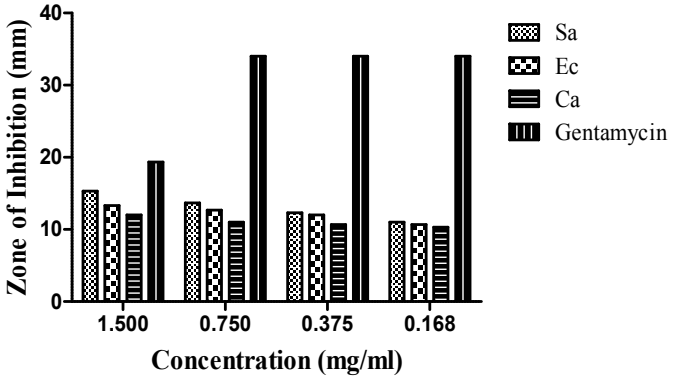

Fig. 4: Comparative Inhibitions of Methanolic fraction of *P. tomentosa* plant against *S. aureus, E.coli, and Candida with gentamycin.*

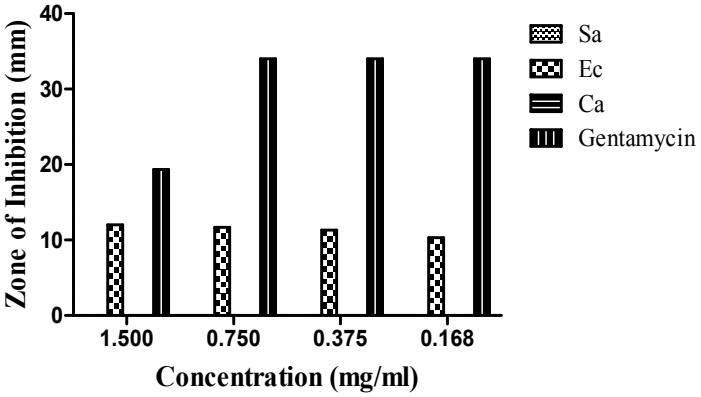

Fig. 5: Comparative Inhibitions of Hexane fraction of *P. tomentosa* plant against *S. aureus, E.coli, and Candida with gentamycin.*

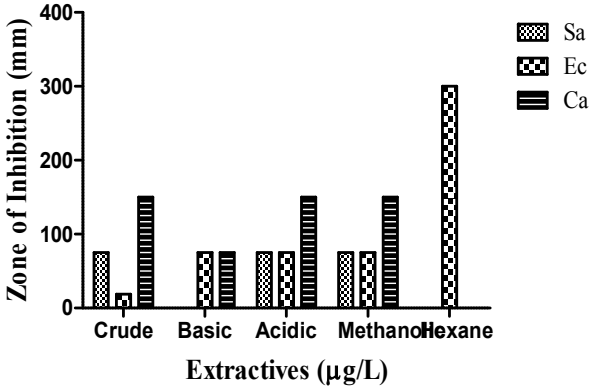

Fig. 6: Comparative of minimun inhibitory concentrtion (MIC µg/mL) of crude extract, basic fraction, acidic fraction, methanlic fraction and hexane fraction of *P. tomentosa* plant against *S. aureus, E.coli, and Candida.*

6. CONCLUSION

The result of this study on P. *tomentosa* L. whole plant showed large antimicrobial spectrum coverage for both acidic and methanolic fractions, compared to the total antimicrobial potency analyzed from whole plant of *P. tomentosa L.* However, basic fraction (alkaloid) possessed highest antimicrobial activity in this study. Well, alkaloid has been reported as the most important group of natural substance [23]. More so, it has been suggested that they constitute part of the plant defenses against phytophagous animals [24] together with terpenoids, phenols, flavonoids, and steroid. *P. tomentosa L*. as such, particularly, basic (alkaloid) and acidic fractions will be centered areas for further scientific research findings in isolating an active antimicrobial component therein. Thus, pharmacological screening of medicinal plant remains important to provide a scientific basis for the continued traditional use of plants and to provide society with potential sources of new, effective and safe drugs.

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COMPETING INTEREST

Authors have declared that no competing interests exist.

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