

Original Research Article

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

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, & 0.168mg/ml) indicated marginal antibacterial activity with range of 17, 20, 14mm zone of inhibition for *S. aureus*, *E. coli* and *C. albican*. While, both basic fraction showed highest activity against *E. coli* and *C. albican* at 15mm& 15mm ; along with acidic and methanolic fraction haven large spectrum against *S. aureus*, *E. coli* and *C. albican* at 13,12, 12mm . Moreover hexane did not showed antimicrobial activity for both *S. aureus* and *C. albican* except for *E. coli* which showed activity at 12mm. The study clearly indicated that basic fraction showed highest antimicrobial activity for selected micro-organisms with lower minimum inhibitory concentration which ranges from 18.75µg/ml to 150µg/ml; followed by wider spectrum of antimicrobial activity for acidic and methanolic fraction against all tested organisms with minimum inhibitory concentration from 75µg/ml to 150µg/ml; while 300µg/ml (MIC) stand for hexane fraction. Thus, *P. tomentosa* L. particularly, the basic fraction (alkaloid) and, both acidic and neutral 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* & *Candida albicans*

28 1. INTRODUCTION

29 Disease and ailments is inseparable companion of life. They strike the urban and rural, rich and poor
30 alike, and are so respecer of status, class or community. The efforts to cure disease and ailments and the
31 desire to attain vitality and longevity prompted early man to explore his natural surroundings and in the
32 process he selected a number of therapeutic agents, mainly from plants. Main while, Resistance to
33 antibiotics and the occurrence of toxicity during prolonged treatment with the present day drugs have
34 been the reasons for extended search for new drugs to treat microbial infections [1].

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

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

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

71

72 **2.MATERIALS AND METHODS**

73 **2.1 Chemicals**

74 1, 1-Diphenyl-2-2picrylhydrazyl (DPPH) and ethanol from Sigma-Aldrich Chemical Co. (St. Louis,
75 USA), Vitamin C, Methanol and all others solvent.(Analytical grade) from Merck Co. (Darmstadt;
76 Germany).

77 **2.2 Sample Collection**

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

83 **2.3 Extraction and Fractionation Procedure**

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

95 **2.4 Phytochemical Screenings of the Ethanolic Extracts**

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

98 **2.5 Antimicrobial Sensitivity Test**

99 Antibacterial activity of the plant extracts and fractions were carried out using agar diffusion method
100 [14].with slight modification. The test organisms used were clinical bacteria isolate of *Staphylococcus*
101 *aureus* and *Escherichia coli* as well as the fungus *Candida albicans*. Pure cultures of the test isolates
102 were obtained from the Microbiology bank of the Department of Biotechnology Advanced Laboratory
103 of Sheda Science and Technology Complex (SHESTCO). Nutrient Agar was used for the repeated sub
104 culturing and preservation of the isolate before being used for the antibacterial assay. It was prepared
105 according to the manufacturer's specification and sterilized inside the autoclave at 121°C for 15 minutes.
106 Mueller Hinton's Agar (MHA) and Sabrobaud Dextrose Agar (SDA) was used for the test. It was
107 poured into sterile petri plates. All flasks and equipment used were sterilized accordingly inside the hot
108 air oven. The hood was sterilized by swabbing with 70% ethanol using cotton wool. The test were

seeded onto the agar surface by streaking. The agar well diffusion method was used for the 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 of 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; a standard drug was used as control: using sterile tips attached to a micropipette. The set up was then incubated for 24 hours at 37 °C in the 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 1×10^6 CFU/ml was add to 4.5 ml of susceptibility test broth containing 0.5 ml serial twofold dilutions of the test samples. All tubes were incubated in air at 37°C for 24 hr before being read. The MIC was considered the lowest concentration of the sample that prevented visible growth. This was validated by measuring the absorbance of the broth against a blank at 600 nm.

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 = $1 - (\text{OD test well} / \text{OD of corresponding control well}) \times 100$.

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.

5. RESULT

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. Phytochemical contents of 95% aqueous ethanolic extracts of *P. tomentosa* L. whole plant

Phytochemical compounds							
Plant extracts	Alkaloids	Anthraquinones	Flavonoid	Glycoside	Saponins	Steroid	Tannins
<i>P. tomentosa</i> L.	+	+	+	+	+	+	+

Key: - =absent, + =present

Table 3. 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 4. Antibacterial potentialities of the extract and fractions from *P. tomentosa* L. whole plant

Samples	<u>Bacteria strains with mean zone of inhibition (mm)</u>			
	Concentration (µg/ml)	<i>S. aureus</i>	<i>E. coli</i>	<i>C. albican</i>
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	-	15	15
	0.75	-	13	12
	0.35	-	11	11
	0.168	-	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	-	12	-
	0.75	-	12	-
	0.35	-	11	-
	0.168	-	10	-
Gentamycin		34	33	35

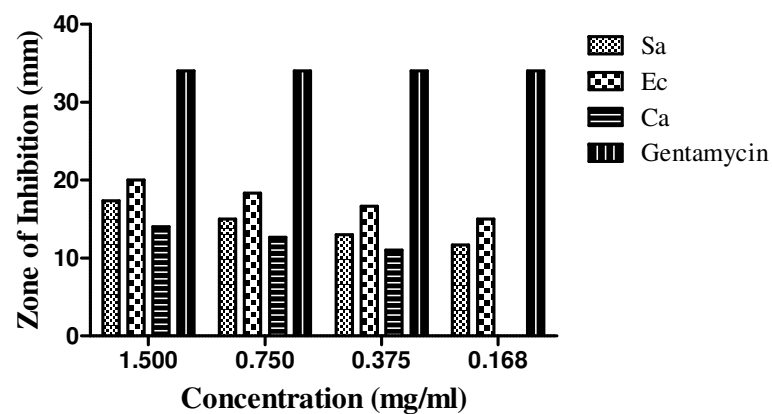


Fig. 1: Comparative Inhibitions of Crude extract of *P.tomentosa* plant against *S. aureus*, *E.coli*, and *Candida* with gentamycin.

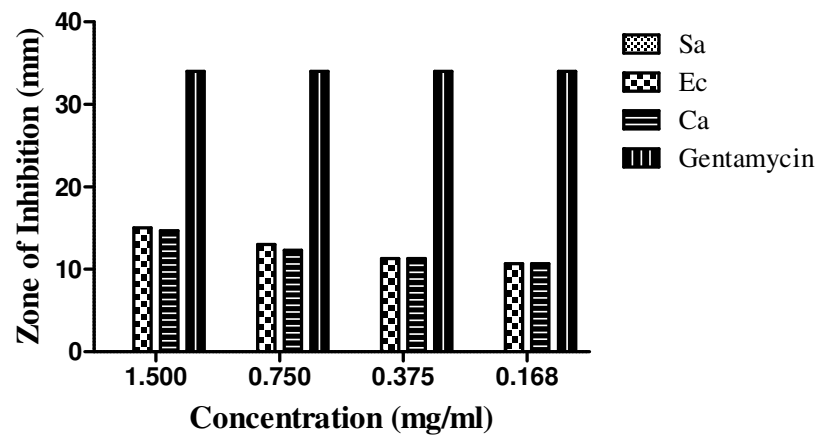


Fig. 2: Comparative Inhibitions of Basic fraction of *P. tomentosa* plant against *S. aureus*, *E.coli*, and *Candida* with gentamycin.

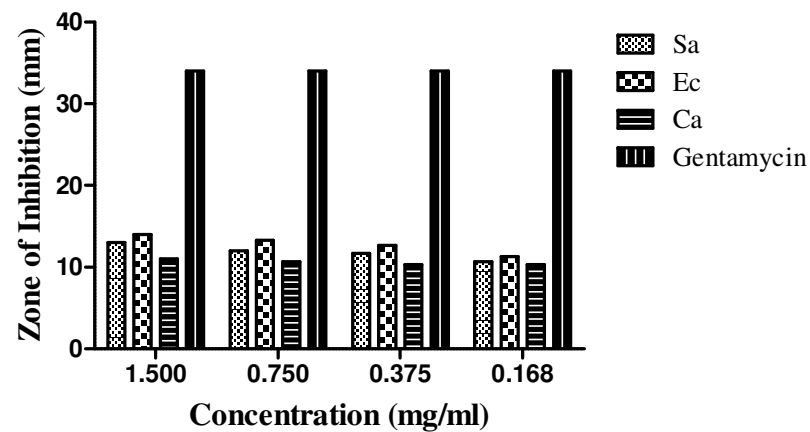


Fig. 3: Comparative Inhibitions of Acidic fraction of *P. tomentosa* plant against *S. aureus*, *E.coli*, and *Candida* with gentamycin.

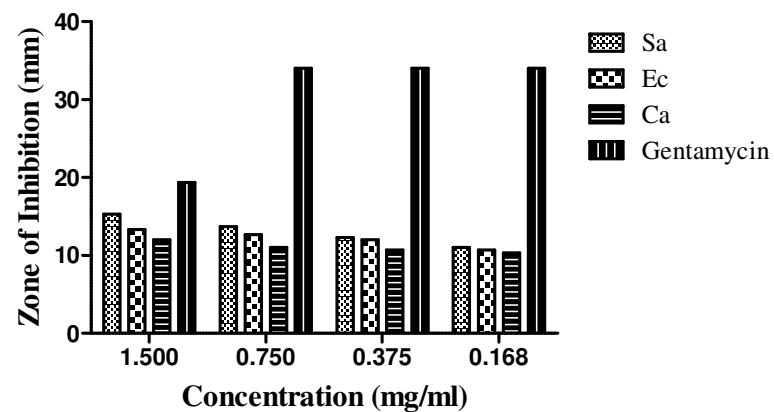


Fig. 4: Comparative Inhibitions of Methanolic fraction of *P. tomentosa* plant against *S. aureus*, *E.coli*, and *Candida* with gentamycin.

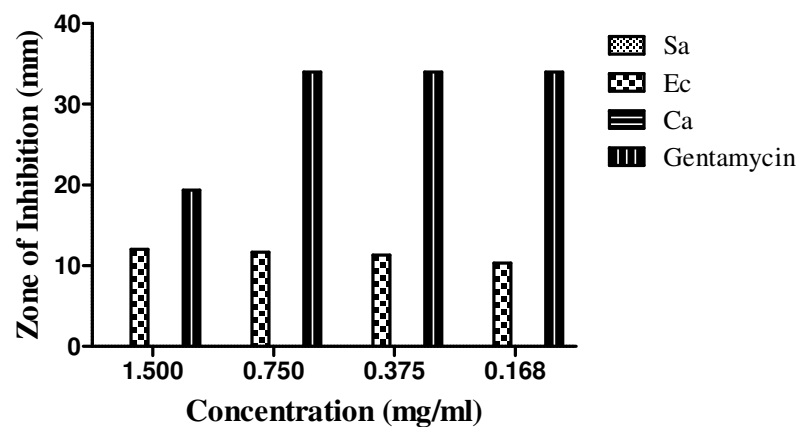


Fig. 5: Comparative Inhibitions of Hexane fraction of *P. tomentosa* plant against *S. aureus*, *E.coli*, and *Candida* with gentamycin.

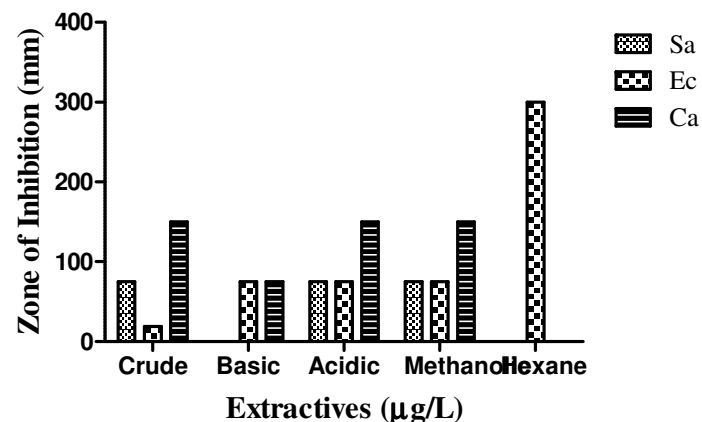


Fig. 6: Comparative of minimum inhibitory concentration (MIC μg/mL) of crude extract, basic fraction, acidic fraction, methanolic fraction and hexane fraction of *P. tomentosa* plant against *S. aureus*, *E.coli*, and *Candida*.

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 as it is expected for ethanolic extraction. Phytochemicals are known to possess antimicrobial properties as reported by [16, 17, 18]. Findings from this work also agreed with the previous work of [19, 20] who reported that plant containing flavonoids, triterpenoids and others. However, the result from this work has clearly shown that antimicrobial activities are contained in basic, acidic and methanol fraction majorly apart from crude extract.

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 ($P \leq 0.05$ at 95% confidence level) except for the following bacteria's; No significant difference between *S. aureus* and *E. coli*, *S. aureus* and *C. albican* in Fig 1, 2 and 3; No significance difference between *E. coli* and *C. albican*, also in Fig 3; no significance difference between *S. aureus* and *C. albican* in Fig. 4 and 5., More so, there was no significance difference for all tested organisms considering their minimum inhibitory concentration ($P \leq 0.05$). However, significance difference exists between all tested fractions, crude extract with gentamycin.

Table 5. Minimum Inhibitory Concentration of crude extract and their various fractions from *P. tomentosa* L. whole plant

MIC values (µg/ml) whole plant								
Sample	Organisms	600	300	150	75	37.5	18.75	
Crude extract	<i>S. aureus</i>	-	-	-	-	+	+	
	<i>E. coli</i>	-	-	-	-	-	-	
	<i>C. albican</i>	-	-	-	+	+	+	
Basic extract	<i>S. aureus</i>	-	-	-	-	-	-	
	<i>E. coli</i>	-	-	-	-	+	+	
	<i>C. albican</i>	-	-	-	-	+	+	
Acidic extract	<i>S. aureus</i>	-	-	-	-	+	+	
	<i>E. coli</i>	-	-	-	-	+	+	
	<i>C. albican</i>	-	-	-	+	+	+	
Methanolic extract	<i>S. aureus</i>	-	-	-	-	+	+	
	<i>E. coli</i>	-	-	-	-	+	+	
	<i>C. albican</i>	-	-	-	+	+	+	
Hexane extracts	<i>S. aureus</i>	-	-	-	-	-	-	
	<i>E. coli</i>	-	-	+	+	+	+	
	<i>C. albican</i>	-	-	-	-	-	-	

Key: - =absent, + =present

228 6. DISCUSSION

229 Basic fractions of *P. tomentosa* L. whole plant showed good antimicrobial activity depending on the
 230 concentration for its zone of inhibition in line with [10] as proved that alkaloids of aerial part of *P.*
 231 *tomentosa* L. showed considerable toxicity and growth inhibitory activities. The higher concentration
 232 used, the higher antimicrobial activity effect expressed in percentage inhibition compared to gentamycin
 233 are shown in Fig 1, 2, 3, 4, & 5 for all verified studies including Fig. 6 for comparison (MIC) analysis.
 234 Thus, increased intake of *P. tomentosa* L. whole plant extracts as used by traditional practitioner will be
 235 supported only within normal concentration.

236 The minimum inhibitory concentration (MIC) of the various extract with crude extract ranges from
 237 300µg/ml to 18.75µg/ml on tested bacteria's. Table 4 was depicted the minimum inhibitory
 238 concentration and mean zone of inhibition of various fractions and crude extract of whole plant of *P.*
 239 *tomentosa* L. The maximum antibacterial activity against *E. coli* and *C. albican* was basic fraction while
 240 the large spectrum antibacterial activity was acidic and methanolic fraction as shown in Table 3. Hexane
 241 has lower antibacterial activity against *E. coli* only; this lower antibacterial activity of hexane has been
 242 reported earlier by [21] rather than Lupeol acetate isolated from it; while it also showed
 243 antidermatophytic activities [15] before further fractionation. The significant antibacterial activity of
 244 basic fraction was found at 15mg/ml zone of inhibition with minimum inhibitory concentration of
 245 18.75µg/ml on *S. aureus* and 75µg/ml on both *E. coli* and *C. albican*. But, for both acidic and methanolic
 246 fractions, large spectrum zone of 14mg/ml and 15mg/ml corresponding to minimum inhibitory
 247 concentration of 75µg/ml and 150µg/ml. 300µg/ml was for hexane fraction as shown in Table 4.

248 Looking at minimum inhibitory concentration relationship; both 75µg/ml and 150µg/ml stand for crude
 249 extract, acidic fraction and methanolic fractions against *S. aureus* and *C. albican*; 75µg/ml relate for
 250 basic, acidic and methanolic against *E. coli*. Hexane has no relationship with other fraction at 300µg/ml.
 251 this observation, therefore, support the notion that, in general, the Gram negative bacteria are more
 252 resistance than the Gram- positive one [22] Thus minimum inhibitory concentration at 75µg/ml against
 253 all tested microbes in this study indicated the high level of potency for *P. tomentosa* L. whole plant in
 254 the management of diseases or ailments caused by these bacterial such as; minor skin infections, such as
 255 pimples, impetigo, boils (furuncles), cellulitis folliculitis, carbuncles, scalded skin syndrome, and
 256 abscesses, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic
 257 shock syndrome (TSS), bacteremia, and sepsis. Its incidence ranges from skin, soft tissue, respiratory,
 258 bone, joint, endovascular to wound infections which is caused by *S. aureus*; gastroenteritis, urinary tract
 259 infections, and neonatal meningitis caused by *E. coli* virulent strains; and morbidity and mortality in
 260 immunocompromised patients (e.g., AIDS, cancer chemotherapy, organ or bone marrow transplantation)
 261 caused by *C. albicans*. Thus, the *P. tomentosa* L. whole plant can be harnessed for medicinal uses in
 262 pharmaceutical industries and the likes.

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267 7. CONCLUSION

268 In spite of the previous studies reported on the various activities of *P. tomentosa* L. plant, our results has
 269 confirmed and confined such documented activities to the basic fraction (alkaloid) particularly; which
 270 showed higher antimicrobial activity alongside with large antimicrobial spectrum coverage for both
 271 acidic and neutral fractions, compared to the total antimicrobial potency analyzed from whole plant of *P.*
 272 *tomentosa* L. Well, alkaloid has been reported as the most important group of natural substance [23].
 273 More so, it has been suggested that they constitute part of the plant defenses against phytophagous
 274 animals [24] together with terpenoids, phenols, flavonoid, and steroid. *P. tomentosa* L. As such, the
 275 basic fraction (alkaloid) and both acidic and neutral fractions will be centered areas for further scientific
 276 research findings in isolating an active antimicrobial component therein. Thus, pharmacological
 277 screening of medicinal plant remains important to provide a scientific basis for the continued traditional
 278 use of plants and to provide society with potential sources of new, effective and safe drugs.

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