RESEARCH ARTICLE

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Study of Antimicrobial Activity of Leaf of *Musa sapientum* L. and Development and Evaluation of Topical Formulations

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Abstract: The present investigation was design to check antimicrobial activity of leaves *Musa sapientum* L. and development of suitable dosage form. Leaves of *Musa sapientum* were collected, air dried, powdered and extracted with ethanol, 50% ethanol and water by maceration method and subjected for phytochemical analysis. Antimicrobial activity of extracts was carried out by the agar well diffusion method and tube dilution method. The extracts were tested on clinical isolates include aerobic, facultative bacteria namely *Stapyhlococcus aureus* ATCC 25921, *Escherichia coli* ATCC 25922, *Bacillus subtilis* and fungus *Aspergillus niger*. Ethanolic extracts showed greater antimicrobial activity than aqueous and hydroalcoholic extracts. The Minimum inhibitory concentration (MIC) of ethanolic extract varied from species of microorganisms, it was found to be 1.5 to 2.5 mg/ml. Further ethanolic extract was used for preparation of topical formulations such as gel (2.5%) and ointment (2.5%). TLC and HPTLC pattern of extract, gel and ointment was compared which showed no change in R_f and 3D spectrum. Then both the formulations are evaluated for their antimicrobial activity, physical characteristics and stability study.

INTRODUCTION

Since ancient times, plants have been an exemplary source of medicines. India has about 45000 plants species and among them, several thousands have been claimed to possess medicinal properties. [1] The demand for plant remedies results from several factors like effectiveness of plant medicine and the side effects of synthetic drugs. Several important drugs used in modern medicine have come from plant. [2] Natural products, such as plants extract, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug discoveries because of the unmatched availability of chemical diversity. *Musa*, a plant genus has an extraordinary significance to human societies, produces the fourth most important food in the world today. [3] *Musaceae*, the banana family of plants, consisting of 2 genera, *Musa* and *Ensete,* with about 50 species native to Africa, Asia and Australia. Among them *Musa sapientum* (MS) commonly called banana is very important species. The leaf of MS is entire above-ground portion of the plant, not a true woody trunk, as in other trees, but a "false trunk" or "false stem" that consists of leaves and their fused petiole bases, referred to as a pseudostem. The pseudostem supports a canopy consisting of 6–20 (or more) leaves. [4]

Evidently the bioactive component of plants has been used for a long time against a wide variety of microorganisms and curing various infectious diseases. Due to failure of chemically synthesized antibiotic to protect the emergence of multi drug resistant bacteria, so there is need to evaluate the antibacterial activity of the leaf of MS for the purpose of searching out newer and safer antibacterial drug. Considering all views, the present study evaluates the efficiency of antibacterial activity of leaf of MS can be used new tools for novel drug development.

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MATERIALS AND METHODS

Collection of Leaves

Leaves of *Musa sapientum* were procured from local market, Dadar, Mumbai and authenticated at G.N. Khalsa College, Mumbai, India (Authentication Voucher specimen no. #SAC/070312A). Then samples were air-dried at 50°C in a hot air oven. Dried samples were ground to powder using a mechanical grinder and stored in a sealed plastic container.

Extraction

The dried powder of leaves was extracted by maceration using solvents were water, 50% ethanol and ethanol (Herb to menstrum ratio 1:10) (Ethanol Procured from SD Fine Pvt. Ltd.). [5] Dried powder of inflorescence was kept for maceration in stoppered container with frequent agitation for 18 h. The filtrate was separated and solvent was removed by distillation. Brownish colored dried aqueous hydroalcoholic and ethanolic extracts were obtained. All extracts were subjected to phytochemical investigation.

TLC of Extract of Leaves

All extract of leaves prepared in concentration of 100mg/10ml of methanol (SD Fine Pvt. Ltd.). The extract solution was applied on TLC plate (Silica gel 60 $F_{254,}$ E. Merck). Then plate was conditioned for 15 min and placed in presaturated chamber with solvent system Toluene: Ethyl acetate: Formic acid (6:6:1 %v/v/v). The plate was observed under UV Chamber (CAMAG) 254 and 366 nm. [6, 7]

Collection of Microorganisms

Bacterial and fungal cultures of *Escherichia coli* and Gram-positive bacteria Staphylococcus aureus, *Bacillus subtilis* and *Aspergillus niger* were collected from the microbiology laboratory, Bombay College of Pharmacy, Mumbai University.

Cultivation of Microorganism [8]

5 days old cultures of *E-coli* ATCC 25922*, Bacillus subtilis, Staphylococcus aureus* (ATCC 25921) cultivated on Nutrient Agar medium were transferred to Nutrient broth and allowed to grow for 2 days at 37±2°C. In case of *A. niger*, 2

Table 1: Test Tubes Dilutions

S. No.	Test Tubes	Broth (ml)	Culture (ml)	Standard (ml)	Extracts (ml)	Water (ml)
1	Blank (-ve control)	9	___	___	___	1
2	Blank (Standard)	9	___	0.1	___	0.9
3	Blank (Extract)	9	___	___	0.1	0.9
4	Negative control	9	0.9	___	___	0.1
5	Standard	9	0.9	0.1	___	___
6	Extracts	9	0.9	___	0.1	___

Table 2: Composition of Gel (2.5%w/w)

S. No.	Ingredients	Quantity
1	Ethanolic extract of leaves	2.5 gm
2	Carbopol 974	1 gm
3	Benzalkonium chloride	(0.01% w/v) 0.01 ml
4	Triethanolamine (q. s)	1.2 ml
5	Distilled water	Up to 100 ml

Table 3: Evaluation of Topical Gel of *Musa sapientum* Leaf Extract (0 Month)

S. No.	Parameters	Observation
1	Color	Greenish
2	Appearance	Homogeneous
3	pH	6.98
4	Spreadibility	20 (dyn.s/cm^2)
5	Viscosity (at 20 RPM, Spindle#7)	41000 to 44000 cps

Table 4: Stability Testing at 0°C±2°C (1 Month) of Topical Gel of *Musa sapientum* Leaf Extract

Color	Appearance	Spreadibility (g.cm/sec)	pH	Viscosity (cps)
Greenish	Homogeneous	20.38	7	41500 to 44000

Table 5: Stability Testing at 25°C±2°C (1 month) of Topical Gel of *Musa sapientum* Leaf Extract

Color	Appearance	Spreadibility (g.cm/sec)	pH	Viscosity (cps)
Greenish	Homogeneous	21.65	6.98	41000 to 45000

days old culture were transferred to Sabraud dextrose broth and allowed to grow for 2 days at 25°C.

Preparation of Culture Medium

Broth contains peptone, NaCl and yeast extract was prepared as below:

1. Nutrient broth (NB): To 1000 ml of distilled water, 13 gm of nutrient broth was suspended. [8]
2. Nutrient agar: To 1000 ml of distilled water, 13 gm of nutrient broth was suspended. To this nutrient broth 20gm agar was added. It is then sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.
3. Sabraud dextrose broth (SD): To 1000 ml distilled water 30 g of Sabraud dextrose broth was suspended and heated to dissolve in the medium completely. It is then sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.
4. Sabraud dextrose agar: To 1000 ml distilled water 65 g of Sabraud dextrose agar was suspended and heated to dissolve in the medium completely. It is then sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.
   (Nutrient broth, agar, Sabraud dextrose broth and Sabraud dextrose agar Procure from HiMedia)
5. Preparation of 0.5 McFarland Standard: 9.95 ml of 1% H_2SO_4 was added to 0.05 ml of 1% $BaCl_2$ to obtain McFarland's No. 0.5 solution. A 0.5 Mc Farland standard is comparable to a bacterial suspention of 10^8 to 10^6cfu/ml.

Preparation of Inoculum

The stock cultures of microorganisms used in this study were maintained on Agar slants at +4°C. The bacterial culture medium was prepared as following; nutrient broth and soft agar medium adjusted to pH 6.6 and autoclaved at 121°C for 20min. Cell suspensions were prepared by inoculation of loop full bacteria into 10 ml of Nutrient Broth (NB Hi media). Incubation was performed at 37°C for 24 h. After optimum growth was observed, the Cultures of *E. coli, Bacillus subtilis, Staphylococcus aureus* from Nutrient broth (NB) agar medium were transferred to nutrient broth and allowed to grow for 2 days, at 37±2°C. In the same way cultures of a fungus *Aspergillus niger* from Sabraud dextrose (SD) agar medium were transferred to Sabraud dextrose broth and allowed to grow for 2 days, at 25±2°C. After growth, the culture solutions of bacteria and fungus adjusted to a turbidity equivalent to 0.5 McFarland with sterile water. [9]

Table 6: Stability Testing at 40˚C±2˚C (1 Month) of Topical Gel of *Musa sapientum* Leaf Extract

Color	Appearance	Spreadibility (g.cm/sec)	pH	Viscosity (cps)
Greenish	Homogeneous	20.22	7.2	43200 to 43900

Table 7: Composition of Ointment (2.5% w/w)

S. No.	Ingredients	Quantity
1	Ethanolic extract of leaves	250 mg
2	Cetosteryl alcohol	500 mg
3	Hard paraffin	500 mg
4	Lanolin	250 mg
5	White soft paraffin	8.5 g
6	Lemon oil (fragrance)	Q.S

Table 8: Evaluation of Physicochemical Parameters of Formulation

S. No.	Formulation properties	Observations
1	Appearance	Smooth
2	Color	Green
3	Odor	Lemon oil
4	Appearance	Homogeneous

Screening of Antimicrobial Activity of Extracts

Aqueous, hydroalcoholic and ethanolic extracts of leaves were evaluated for antimicrobial activity against *Escherichia coli, Bacillus subtilis, Staphylococcus aureus* and fungus *Aspergillus niger* by agar well diffusion assay and broth dilution method.

1. Agar Well Diffusion Assay

The *in-vitro* inhibition assay of extract and standard were carried out by agar well diffusion assay**.** NB agar and SD agar were used as a medium and bore well method was used for studies. Sterile NB agar and SD agar 500 ml was seeded with 25 µl of microbial culture and poured on petri plates. Well was made with the help of borer after cooling NB and SD agar. Aqueous, Hydroalcoholic and ethanolic solution of extracts (4000 µg/100 µl, 3000 µg/100 µl, 2000 µg/100 µl, 1000µg /100 µl) were prepared and Standard Amoxicillin (500 µg/100µl) and Cotrimazole (500 µg/100 µl) used as standard (positive control)for bacteria and fungus. Each set of microorganism contained standard, negative control, drug extract and sterility control and incubated bacteria for 24 h at 37±2°C and fungus for 24 h at 25±2°C. After incubation period, the zone of inhibition was measured as a diameter of well for both extract and standard. The results for aqueous, hydroalcoholic and ethanolic were compared. [9]

2. Broth Dilution Method

Cultures of *E. coli, Bacillus subtilis, Staphylococcus aureus* from Nutrient broth (NB) were diluted with sterile water to 10 µl/ml and transferred to test tubes containing NB. In the same way culture of a fungus *Aspergillus niger* from Sabraud dextrose (SD) broth was diluted with sterile water to 10 µl/ml and transferred to test tubes containing SD broth. Aqueous, Hydroalcoholic and ethanolic extracts of concentrations 40000 µg/1000 µl, 3000 µg/1000 µl, 2000 µg/1000 µl and 1000 µg/1000 µl were added in test tubes. Along with this, standard Amoxicillin (500 µg/1000µl), negative control and sterility broth were Prepared as shown in Table 1. Bacteria allowed growing for 24 h at 37±2°C and fungus for 24 h at 25 ± 2°C. [8] Estimation of growth and inhibition of microorganism and fungus were performed by viable count method. [8]

a. Viable Count Method

5 µl of above NB and SD broth in each test tube were diluted to 100 µl with sterile water and added in petri plates containing NB and SD agar. It was kept for incubation for 24 h at 37±2°C and 25±2°C respectively. Numbers of colonies of bacteria and fungus were calculated and results of different concentrations of extracts were compared. [8]

Formulation Topical Gel

The attempt was made to formulate leaf extract into gel with different polymers. The extract of leaves of *Musa sapientum* (2.5%) was formulated into gel with polymers (Sodium CMC, Carbopol 974) using various formulae. The combination with Carbopol 974 resulted in the best gel formulation, which was smooth and stable. [10]

1. Procedure

1 g of Carbopol 934 was dispersed in 50 ml of distilled water with continuous stirring. 5 ml of distilled water was taken and required quantity of Benzalkonium chloride was dissolved in water. The solution was cooled and further required quantity ofethanolic extract of leaves was mixed to the above mixture and volume was made up to 100 ml with distilled water. Finally ingredients were mixed with Carbopol 934 gel by continuous stirring and triethanolamine was added drop wise to the formulation for adjustment of required skin pH (6.8-7). The composition of gel is given in Table 2 and to obtain the gel at required consistency. The same method was followed for preparation of control sample without adding extract. [10]

Table 9: Stability Testing After 1 Month of Topical Ointment of *Musa sapientum* Leaf Extract

S. No.	Appearance	Color	Appearance
0°C ± 2°C	Smooth	Green	Homogeneous
25°C ± 2°C	Smooth	Green	Homogeneous
40°C ± 2°C	Smooth	Green	Homogeneous

Table 10: Zone of Inhibition of Gel, Ointment and Standard against Microorganism

S. No.	Formulation	*B.subtilis*	*E.coli*	*S.aureus*	*A.niger*
1	Ointment (100 mg)	16.5± 3 mm	17.5±1 mm	20±1 mm	Not found
2	Gel (100 mg)	19± 2.5 mm	20.75±2.2 mm	21.75±0.25 mm	13± 1.4 mm
3	Standard (500 µg)	26.25±1.2 mm	25± 0.9 mm	35.25± 1.4	36.4±0.9 mm

Table 11: Viable Count for 5 µl of broth Containing Microbes with Ointment, Standard and Control Sample

S. No.	Formulation	*B.subtilis*	*E.coli*	*S.aureus*	*A.niger*
1	Ointment (100 mg)	178	321	359	8
2	Gel (100 mg)	139	115	217	3
3	Standard (500 µg)	24	09	78	2
4	Control	362	415	374	9

Evaluation of Topical Gel [11, 12]

Gel was evaluated for Physical characteristics, pH, spreadibility, viscosity and stability given in Table 3 to Table 6.

Formulation of Ointment [13]

The ointment from ethanolic extract of leaf (2.5% w/w) was prepared by fusion method and composition given in Table 7.

1. Procedure

The weighed quantity of Cetosteryl alcohol, hard paraffin, lanolin and white soft paraffin were melted together at 45-50°C. Then the extract was accurately weighed and added to the melted base and stirred properly to get uniform ointment. [13]

2. Evaluation of Physicochemical Properties

The ointment was evaluated for physicochemical properties and stability. Observations are recorded in Table 8 and 9. [12]

HPTLC Analysis of Ethanolic Extract, Gel and Ointment

The 10 µl of 10000 ppm solution of ethanolic extract, gel and ointment were applied to the plate as bands 6.00 mm wide and 15 mm from the bottom edge of the HPTLC plate (Silica gel $60F_{254}$) by the use of Camag Linomat V sample applicator equipped with 100 ul syringe. Ascending development to a distance of 85 mm was performed at room temperature (25±2°C) in the Camag twin trough chamber previously saturated for 15 minutes with mobile phase Toluene: Ethyl Acetate: Formic acid (6:6:1 v/v/v). After development plates were scanned at 254 nm.

Evaluation of Antimicrobial Activity of Formulations

The *in-vitro* inhibition assay of gel, ointment and standards were carried out agar well diffusion assay**.** NB agar and SD agar were used as a culture growth medium and bore well method was used for studies. Sterile NB agar and SD agar 500 ml was seeded with 25 µl of culture and poured on petriplates. Well was made after cooling NB and SD agar. In agar well 100 mg of gel and ointment were added (100 mg gel contain 2500 µg of ethanolic of extracts leaf) and Amoxicillin capsule (500 µg/100 µl) used as a standard. In case of fungus Fluconazole (500 µg/100µl) used as a standard. Each set of microorganism contained standard, negative control, drug extract and sterility control and incubated bacteria for 24 h at 37±2°C and fungus for 24 h at 25±2°C.[9] After incubation period, the zone of inhibition was measured as a diameter of well for formulations and standards. The results of gel, ointment and standard given in Table 10.

1. Broth Dilution Method

Cultures of *E.coli, Bacillus subtilis, Staphylococcus aureus* from Nutrient broth (NB) were diluted with sterile water to 10^{-2} and transferred to test tubes containing NB. In the same way culture of a fungus *Aspergillus niger* from Sabraud dextrose (SD) broth was diluted with sterile water to 10^{-2} and transferred to test tubes containing SD broth. In each test tube 100 mg of gel and ointment were added (100 mg gel contain 2500 µg of ethanolic of extracts leaf) and Amoxicillin capsule (500 µg/100µl) used as a standard. In case of fungus Fluconazole (500 µg/100µl) used as a standard. Along with this, negative control and sterility broth were maintained and allowed to grow bacteria for 24 h at 37±2°C and fungus for 24 h at 25±2°C. (Ointment mixed with small amount of methanol before addition in to test tubes). [8]

2. Viable Count Method

5 µl of above NB and SD broth in each test tube were diluted to 100 µl with sterile water and added in petri plates containing NB and SD agar. It was kept for incubation for 24 h at 37±2°C and 25±2°C respectively. Numbers of colonies of bacteria and fungus were calculated and results of different extract concentrations were compared as given in Table 11.

Table 12: Zone of Inhibition by Ethanolic Extract of Leaves by Well Diffusion Method

S. No.	Ethanolic Extract of Leaves	*B.subtilis*	*E.coli*	*S.aureus*	*A.niger*
1	1000 µg	Not found	12±1mm	Not found	15±1mm
2	2000 µg	14±2mm	14±1.5mm	14±3mm	23±2mm
3	2500 µg	16±3mm	14±1mm	22±3mm	34±2mm
4	3000 µg	16±1mm	16±3.5mm	22±1mm	34±3mm
5	3500 µg	18±1mm	19±1mm	29±1mm	35±2mm
6	4000 µg	18±2mm	20±2mm	31±2mm	35±2mm
7	Standard(500ug)	26.25±1.2mm	25± 0.9mm	35.25± 1.4	36.4±0.9mm

Table 13: Comparison of % Growth Inhibition of Ethanolic Extract Broth Dilution Method

S. No.	Concentration of Extract/Standard	*E.coli*	*B. subtilis*	*S. aureus*	*A. niger*
1	1000 µg	26.58	28.32	3.505	63.77
2	1500 µg	45.39	41.39	15.02	68.59
3	2000 µg	78.52	46.18	29.71	75.75
4	2500 µg	89.36	69.71	49.24	78.51
5	3500 µg	92.84	79.73	70.95	80.85
6	500 µg Std Amoxycilin	96.11	86.49	91.81	95.45

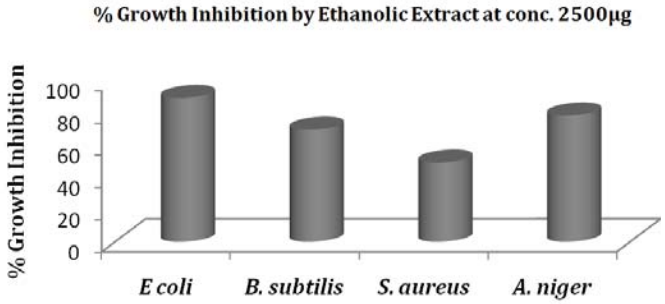


Figure 1: % Growth inhibition by ethanolic extract at concentration 2500 µg (broth dilution method)

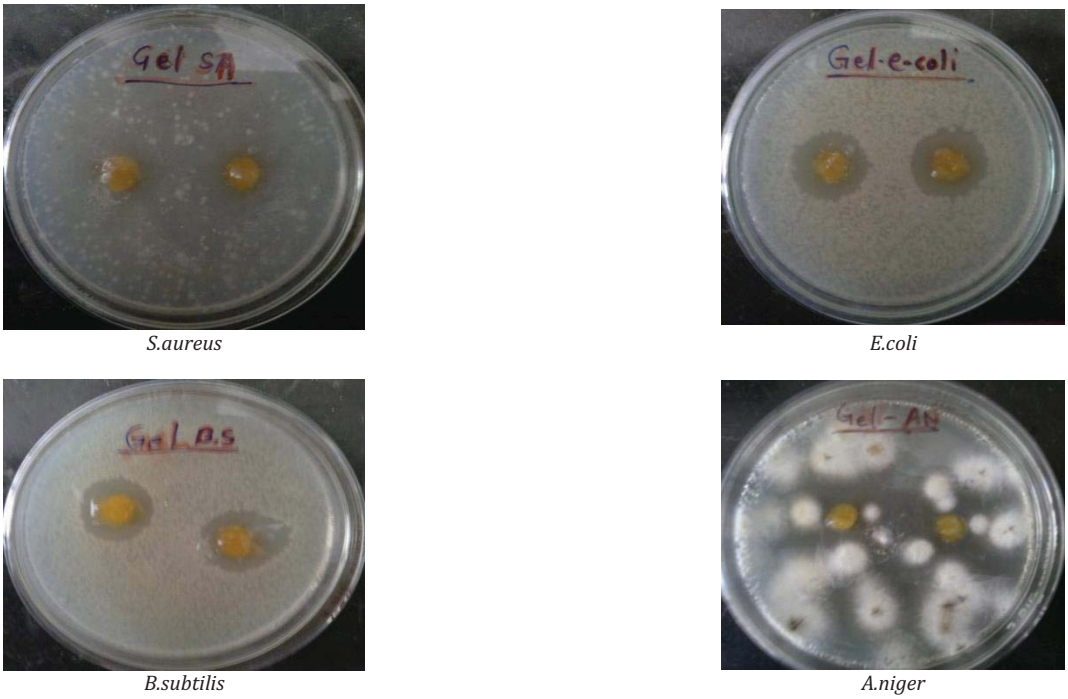
RESULTS AND DISCUSSION

The yield of aqueous, hydroalcoholic and ethanolic extracts of leaves was around 12%, 14% and 2.6% w/w respectively. The preliminary phytochemical analysis of aqueous, hydroalcoholic and ethanolic extracts of leaves have showed the presence of tannins and phenolic compounds, in addition to this aqueous and hydroalcoholic extracts of leaves showed the presence of carbohydrate and mucilage. On the TLC plate, three spots were observed in the ethanolic extract and one spot in gel and ointment. The spot of chlorophyll of green orange color was observed at the solvent front.

All the extracts of leaves were subjected to *in-vitro* antimicrobial studies by pour plate method and broth dilution method. The aqueous extract of leaves has not shown significant antimicrobial activity in pour plate method probably because of more carbohydrate present in the extracts and lack of antimicrobial ingredients, observed during phytochemical screening. The ethanolic extract showed more promising antimicrobial activity as compared to hydroalcoholic extract. In pour plate method, the ethanolic extract of leaves has showed significant bactericidal activity against *Escherichia coli, Bacillus subtilis, Staphylococcus aureus* and fungistatic against *Aspergillus niger* at concentration 2.5 mg/ml given in Table 12. The further comparison of antimicrobial studies of ethanolic and hydroalcoholic extract was done by broth dilution method. In broth dilution method ethanolic extract showed better activity against microbes as compared to hydroalcoholic extract. The minimum inhibitory concentration of ethanolic extract was found to be 1.5 to 2.5 mg/ml. So, the ethanolic extract was subjected to TLC analysis and topical formulations were prepared from the ethanolic extract of leaves. The solvent system Toluene: Ethyl Acetate: Formic acid (6:6:1 %v/v/v) gave good resolution of spots. In the ethanolic extract of leaves, orange green spot of chlorophyll was found just below the solvent front; along with this two different spots were found in the ethanolic extracts. The ethanolic extract of leaf was formulated into 2.5% w/w Carbopol gel and 2.5% w/w simple ointment IP. The excipients compatibility with extracts was checked by HPTLC analysis. The R_f and 3-D view showed the extract was compatible with excipients of gel and ointment at different temperature conditions. The gel and ointment was characterized for physicochemical stability at different temperature conditions. The formulations prepared were further tested for antimicrobial activity studies by well diffusion assay and broth dilution method. The results showed that the both formulations retained antimicrobial activity of the extract. The gel has shown more promising activity than ointment.

CONCLUSION

The antimicrobial activity was probably due to the presence of some important inimical secondary metabolites. Therefore, this plant can be used to discover



S.aureus *E.coli*

B.subtilis *A.niger*

Figure 2: Petri plates photographs for zone of inhibition by 2.5% gel (100mg) against *bacillus subtilis, staphylococcus aureus, escherichia coli* and *Aspergillus niger*

natural bioactive products that potentially serve as leads in the development of novel pharmaceutical research activities and suggesting for using as potential antibacterial agent to cure bacterial infection.

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Abbrevations

MS: *Musa sapientum*
NB: Nutrient broth
SD: Sabraud Dextrose broth
MIC: Minimum Inhibitory Concentration.

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