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ANTIMICROBIAL ACTIVITY OF MORINGA ON EAR, NOSE AND THROAT ASSOCIATED FUNGI, AND VANCOMYCIN RESISTANT COCCI ISOLATED AT AMINU KANO TEACHING HOSPITAL, KANO, NIGERIA

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ABSTRACT

This study was aimed at evaluating the antimicrobial activity of Moringa on ear, nose and throat associated fungi and vancomycin resistant cocci. The plant material was extracted with methanol and petroleum ethe and screened for phytochemical contents. The microbial isolates were obtained from females and males patients (both adults and children) attending ear, nose and throat clinic at Aminu Kano Teaching Hospital. Coccal bacteria and fungi were isolated accordingly. The cocci were screened for vancomycin resistance. The antimicrobial assay was carried out using gradient double (12.5-100mg/mL) assay. The MIC, MBC/MFC and Brine shrimp toxicity test were also conducted. *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Candida albicans* and *Aspergillus fumigatus* were isolated. Up to 21.4% of *S. aureus* were vancomycin resistant, 20% of *S. pneumoniae* isolated were vancomycin resistant and 16.7% *S. pyogenes* were vancomycin resistant. The plant extracts showed zones of inhibition of 08mm-20mm at concentrations ranging from 12.5- 100mg/mL. The most susceptible organism to both extracts was *C. albicans* and the least susceptible was *S. aureus*. The MIC of the methanol extracts ranged from 0.78 to 50mg/mL but MBC/MFC ranged from 6.25 to 200mg/mL. The MIC of the petroleum ether was at 50 to 200mg/mL and the MBC/MFC was from 200 to 800mg/mL. The brine shrimp lethality assay showed LC_{50} value of 93.48µg/mL for Moringa methanol extract while the LC_{50} value for Moringa petroleum ether extract was 3.691µg/mL. Moringa methanol extract (100mg/mL), showed appreciable activity against the fungal isolates and vancomycin resistant cocci associated with Ear, Nose and Throat symptoms while Moringa petroleum ether extract showed activity only on the fungal isolate *C. albicans*. The study demonstrated that Moringa methanol extracts was more active than Moringa petroleum ether extracts. The search for novel cytotoxic ingredient in Moringa should be encouraged.

Keywords: Antimicrobial, Moringa, Ear, Nose, Throat,Fungi,Vancomycin, Resistant, Cocci

L'ACTIVITÉ ANTIMICROBIENNE DE MORINGA SUR L'OREILLE, NEZ ET GORGE CHAMPIGNONS ASSOCIÉS, ET D'ENTÉROCOQUES RÉSISTANTS À LA COCCI ISOLÉS À L'HÔPITAL, AMINU KANO Kano, Nigéria

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RÉSUMÉ

Cette étude avait pour but d'évaluer l'activité antimicrobienne de Moringa sur l'oreille, nez et gorge champignons associés et cocci résistants à la vancomycine. L'usine a été extraite avec du méthanol et du pétrole ela et tamisée pour contenu phytochimique. Les isolats microbiens ont été obtenus à partir de les hommes et les patients (adultes et enfants) fréquentant l'oreille, nez et gorge clinique à l'Hôpital d'enseignement Aminu Kano. Coccal les bactéries et champignons ont été isolés en conséquence. Les coques ont été examinés pour la résistance à la vancomycine. L'antimicrobien a été réalisée à l'aide de double gradient 12.5-100(mg/mL). Le MIC, MBC/MFC et l'essai de toxicité d'artémia ont également eu lieu. Staphylococcus aureus, Streptococcus pyogenes, Streptococcus pneumoniae, Candida albicans et Aspergillus fumigatus étaient isolés. Jusqu'à 21,4 % des S. aureus étaient résistants à la vancomycine, 20 % de S. pneumoniae isolées étaient résistantes à la vancomycine et 16,7 % étaient S. pyogenes résistantes à la vancomycine. Les extraits de plantes ont montré des zones d'inhibition de 08mm-20mm à des concentrations variant de 12,5- 100mg/mL. Les plus sensibles à l'organisme les deux extraits a été C. albicans et le moins sensible était S. aureus. Le MIC de l'extrait au méthanol variait de 0,78 à 50 mg/mL mais MBC/MFC variait de 6,25 à 200mg/mL. Le MIC de l'éther de pétrole était à 50 à 200 mg/mL et le CBM/MFC a été de 200 à 800 mg/mL. La létalité d'artémia ont montré la valeur de LC50 93.48µg/mL pour le Moringa extrait méthanolique tandis que la CL50 pour le Moringa L'éther de pétrole extrait a été

3.691µg/mL. Le moringa extrait au méthanol (100mg/mL), a montré une activité appréciable contre les isolats fongiques et d'entérocoques résistants à la cocci associés à l'oreille, nez et gorge symptômes, tandis que l'éther de pétrole extrait de Moringa a montré que l'activité sur l'isolat fongique C. albicans. L'étude a démontré que les extraits de Moringa le méthanol a été plus active que l'éther de pétrole extrait de Moringa. La recherche de nouveaux ingrédients dans le Moringa cytotoxiques doivent être encouragés.

INTRODUCTION

Medicinal plants have been used from ancient time for their medicinal values as well as to impact flavor to food. Nowadays, the crude extracts and dry powder samples from medicinal and aromatic plants species are used for the development and preparation of alternative medicine and food additives (1) . ***Moringa***, native to parts of Africa and Asia, is the sole genus in the flowering plant family Moringaceae. Important medicinal properties of the plant include antipyretic, antiepileptic, antiinflammatory, antiulcerative (2) , antihypertensive (3) cholesterol lowering (4) antioxidant (5) anti diabetic, hepatoprotective (6) , (antibacterial and antifungal activities (5). Vancomycin is an antibiotic used to treat a number of bacterial infections (7). It is recommended intravenously as a first-line treatment for complicated skin infections, bloodstream infections, endocarditis, bone and joint infections, and meningitis caused by methicillin-resistant *Staphylococcus aureus* (8). In addition to natural circumstances, misuse of vancomycin has led to vancomycin resistance. The reasons for clinical failure of vancomycin are many and have been hypothesized to include poor penetration of the drug to certain tissues (9, 10).Wide varieties of Ear, Nose and Throat diseases are usually presented to the Otorhinologist (head and neck surgeons) (11). The pattern of these diseases may vary from community to community or hospital to hospital based on the availability of specialist personnel or facilities for the management of such diseases which are either congenital or acquired in origin. Ear, nose and throat diseases are serious public health problems with universal distribution affecting all age groups (12).

One of the research problems facing chemotherapy today is that microorganisms are now gaining resistance to vancomycin, which has been considered to be the reference standard for the treatment of bacterial infection. In Nigeria today, ear, nose and throat- related conditions constitute a major burden of infections. Yet the majority of the citizens are ignorant of ENT treatment options. Disease of the ear, nose and throat (ENT) affect the functioning of adults as well as children, often with significant impairment of the daily life of affected patients (13). Due to the emergence of vancomycin resistance which is the last resort antibiotic where other antibiotics have failed and ignorance of the severity of ear, nose and throat infections, there is need for an easy, effective and affordable means to cure infections of the ear, nose and throat (ENT). ***Moringa*** is known for its numerous medicinal properties one of which is its antimicrobial activity (14). It is very common worldwide to find people consuming this plant in combination or alone as remedies against symptoms believed to be associated with the selected microorganisms targeted in this work. There is a need to find out if this plant has potent antimicrobial activity against ENT fungi and vancomycin resistant cocci. This plant is easy to afford. Moringa can be included in our foods and drinks e.g. tea and soups (15).

MATERIAL AND METHODS

Collection and Identification of Plant Materials

The Moringa leaves were collected from Naibawa in Kano state. It was identified and compared to voucher specimen with voucher number (Moringa BUKHAN 0011) at the department of Plant Biology Bayero University, Kano Herbarium with the assistance of Baha'uddeen Said Adam(16).

Processing and Extraction of Plant Materials

The ***Moringa*** leaves were thoroughly washed with distilled water and air dried in a shady environment for two weeks and made into powdered form using a clean pestle and mortar, then it was sieved through a mesh to obtain fine powder of approximately 20µm particle size. The powder was stored at room temperature in sealed container until required for use as demonstrated(17).[Accordingly, one hundred grams (100g) of the powdered plant material was extracted separately with methanol and petroleum ether using soxhlet apparatus as demonstrated (18).

Confirmation of the Bioactive Components of the Plants

Phytochemical screening was carried out to confirm the bioactive components of the plant as follows:

Test for Alkaloids

This was carried out qualitatively as demonstrated (19). Using a pipette, 1.0 ml of the extracts was placed in two separate test tubes. Using a dropper, three drops of Meyer's reagent was added separately. A white precipitate with Meyer's reagent indicated the presence of alkaloids.

Test for Saponins

This was carried out as demonstrated by the method reported (20). 0.5g each of the extracts was placed in

a test-tube, 5.0ml of sterile distilled water was added to the extract in the test-tubes and shaken vigorously. A froth that persisted for 15 minutes was an indication of the presence of saponins.

Test for Steroids
This was carried out as demonstrated by the method (19). 2g of each the extracts was placed in a test tube and evaporated to dryness. The extract was then dissolved in acetic anhydride followed by the addition of chloroform and then concentrated sulphuric acid was added by the side of the test tube. Appearance of a brown ring at the interface of the two liquids and the appearance of violet colour in the supernatant layer indicated the presence of steroids in the extract.

Test for Reducing Sugar
This was carried out as demonstrated by the method (20). Here, 1g each of the extracts was weighed and introduced into separate test tubes. The extracts were diluted with 2.0ml each of dimethyl sulphoxide (DMSO) and sterile distilled water respectively. Fehling's solution was added to the solution obtained, and then the mixture was warmed. A brick-red precipitate at the bottom of the test tubes indicated the presence of reducing sugar.

Test for Tannins
2ml of each of the plant was diluted with distilled water in separate test tubes and 2-3 drops of 5% ferric chloride ($fecl_3$) were added. A green-black or blue-black colouration indicated the presence of tannin as demonstrated (19).

Test for Flavonoids
This was carried out as demonstrated (21). A 4mg weight of the extracts and a piece of magnesium ribbon were added together followed by concentrated HCL drop-wise. A colour change from crimson to magenta indicated the presence of flavonoids in the extracts.

Test for Terpenoids
0.5ml of the extracts was added to 2ml of chloroform, 3ml of concentrated H_2S0_4 was added to form a layer. A reddish brown colouration at the interface indicates the presence of terpenoids as demonstrated by the method (21) .

Test for Anthraquinone
0.5ml of the extract was taken into a dry test-tube and 5ml of chloroform was added and shaken for 5mins. The extract was filtered and drops of ammonia solution were added. A pink violet or red colour in the ammonical layer (lower layer) indicates positive results. This is as demonstrated by the method (21).

Tests for Phenol
Few drops (0.5%) of dilute ferric chloride solution was added 0.5 ml of each of the extracts, the formation of a dark green colour shows the presence of phenol according to the method (22).

Collection and Identification of Test Isolates
The isolation was carried out in Aminu Kano Teaching Hospital after ethical clearance has been approved. The isolation was carried out under the supervision of a medical laboratory technician. Thirty three specimens were collected from patients attending ENT clinic in any age group. The organisms were isolated from the ear, nose and throat swabs. The specimen was cultured on Sabouraud Dextrose Agar (Manufacturing date, 2016; Expiring date, 2018) for the isolation of fungi. After 3-7days of incubation the fungi isolates were identified macroscopically and microscopically with the help of scheme (23).

The specimens were cultured on Chocolate agar for the isolation of cocci bacteria. The cocci were identified macroscopically in the culture plates after 24 hours of incubation, after which gram staining was carried out. This was followed by catalase and coagulase test to confirm the species of Staphylococci. Optochin, bacitracin disc and bile solubility test were used to further confirm the species of streptococci. The identified cocci were subjected to vancomycin sensitivity disc (30µg) and the cocci that were found to be resistant to the vancomycin were used for this study as demonstrated (24).

BIOASSAY

Preparation of Extracts Concentrations
This was carried out according to the method described by the method (25). Stock solution of moringa, methanol and petroleum ether crude extracts were prepared by dissolving 0.6g of each of the plant extracts in 6mL of dimetyhylsulphoxide (DMSO) in glass vial bottles. Therefore, each stock solution had concentration of 100000µg/mL (100mg/mL). The stock solution was double-diluted to get three varied extracts concentrations in addition to it to make them four different concentrations of 100mg/mL, 50mg/mL, 25mg/mL and 12.5mg/mL (26).

Standardization of Inoculum
The isolates were adjusted to 0.5 McFarland standard (1.5×10^8 CFU/mL) turbidity for bacteria isolates and 1×10^6spores/mL for the fungi isolates by adding sterile normal saline. McFarland standards were used as a reference to adjust the turbidity of microbial suspension so that the number of microorganisms

will be within a given range. For the preparation of the 0.5 McFarland standard, 0.05mL of barium chloride ($BaCl_2$) (1.17% w/v $BaCl_2.2H_2O$) was added to 9.95mL of 0.18M H_2SO_4 (1.0% w/v) with constant stirring. To aid comparison the standard was compared against a white background with a contrasting black line (27).

Preparation of Antibiotic Dilution

The antibiotic ciprofloxacin and fluconazole were purchased from Lamco pharmacy Kano State, Nigeria and was reconstituted by dissolving 3g of the ciprofloxacin and fluconazole powder in 100ml of distilled water so as to get a concentration of 30mg/mL. The prepared dilution of the antibiotics was used for subsequent antimicrobial test as positive control (21).[21]

Antimicrobial Assay

The bioassay was carried out using the agar well diffusion method described by cheesbrough (2006). 0.1mL of the standardized inoculums (1.5 x 10^8 CFU/ml) of *Staphylococcus aureus* was inoculated onto sterile prepared Mueller Hinton Agar and was spread with a sterile swab while *Streptococcus pneumoniae* was inoculated on chocolate agar and *Streptococcus pyogenes* was inoculated on sterile blood agar plates.

Aspergillus fumigatus and *Candida albicans* were inoculated on sterile Sabouraud dextrose agar plates. Six wells were made with a 6mm sterile cork borer into the agar plates containing the bacterial and fungal inoculums and 0.lmL of the four different concentrations from the stock solution of the extracts at concentrations (100, 50, 25, and 12.5mg/mL) were introduced into their respective wells. 0.1mL of DMSO was introduced into the fifth well to serve as negative control while 0.1mL of 30mg/mL of ciprofloxacin was introduced into the sixth well to serve as a positive control for the bacterial isolates and fluconazole was used for the fungal isolates. The inoculated plates were left to stand for about 30 minutes to allow diffusion of extract before incubating at 37◦C for 24 hours for the bacterial isolates and the fungal isolates were incubated at 37◦C for 3 days. The zones of clearance produced around the wells after incubation was observed and measured using a vernier caliper and recorded (in mm). Each of the experiment was conducted thrice and mean results were taken for the test organisms (28).

Determination of Minimum Inhibitory Concentration, Minimum Bactericidal and Fungicidal Concentration

The minimum inhibitory concentration (MIC) is defined as the lowest concentration of the antimicrobial agent that inhibited visible growth of microorganisms after overnight incubation (Andrews, 2002). The doubling macro dilution broth method was used to determine the MIC. Two (2) mL of the reconstituted crude extract at a concentration of 100000µg/ml was added to 2mL sterile Mueller Hinton broth for the bacterial isolates, 2mL of the reconstituted crude extract was added to 2mL of Sabouraud dextrose broth for the fungal isolates. Two (2) mL of this extract concentration was transferred to another test-tube and this dilution continued until the 12th test-tube was reached, giving extract concentrations ranging from 800-0.39mg/mL in different test tubes. 0.1mL of an 18h culture of bacteria and 3 days culture of fungi previously adjusted to 0.5 MacFarland standard was inoculated into each of the test tubes and the contents were thoroughly mixed. A test tube containing the broth and extract was used as positive control while a test tube containing the broth and bacteria/fungal inoculum was used as negative control. The inoculated culture tubes were incubated at 37◦C and were observed for growth after 24 hours for the bacterial isolates and 3days for the fungal isolates. The lowest concentration of extract showing no visible growth when compared with the control was considered as the MIC as demonstrated by the method (27).

The minimum bactericidal/fungicidal concentration is the lowest concentration of antimicrobial agent that prevented the growth of an organism. 0.1mL aliquot from the tubes that showed no visible bacterial/fungal growth from the determination of minimum inhibitory concentration was inoculated on a sterile Mueller Hinton Agar for 24 hours at 37◦C for the bacterial isolate while the fungal isolates were inoculated on sterile Sabouraud dextrose agar at 37◦C. The lowest concentration in which no growth occurred was taken as the minimum bactericidal concentration (MBC/MFC) as demonstrated (27).

Assay for LC_{50} of the Plant Extracts by Brine Shrimp Lethality Test

The eggs were hatched in a hatching chamber containing ocean sea salt water (75ml). Natural light was allowed to penetrate into the hatching chamber for 48 hours so that the eggs will hatch into the shrimp larvae. Twenty milligram (20mg) of the extracts were separately dissolved in 2ml of methanol and equally a positive control of which 20mg of the extracts was dissolved in 2ml of distilled water. 500, 50 and 5ml of the solution equivalent to 1000, 100 and 10 µg/mL respectively was transferred into vials. A negative control which is simply the solvent (methanol) without the test extracts was also prepared. Each concentration were tested in triplicate,

therefore each extracts had 9 test tubes. The methanol in the test tubes containing the extracts were allowed activity against Brine shrimp larvae (*Artemia salina*). To each test sample vial, sea water was added and a drop of DMSO solvent was added in order to facilitate the solubility of each test samples in the sea water. Ten (10) shrimps were transferred using a Pasteur pipette and natural sea water was added to make a total volume of 5ml. After 24 hours, the number of surviving shrimps at each concentration was counted and recorded. Lc50 values were determined at 95% confidence intervals by analyzing the data on a computer loaded with a "Finney Programme." The Lc50 values of the brine shrimps obtained for extracts of the plants studied were recorded (29).

RESULTS AND DISCUSSION

Phytochemical *Moringa* Plant Extracts

Some Phytochemical components of ***Moringa*** plant extracts are presented in Table 1. The data showed that, phenol, steroids, reducing sugar, flavonoid, terpenoids, tannins anthraquinone were present in both Moringa methanol and petroleum ether extracts. Alkaloid and saponin were present in Moringa methanol but absent in moringa petroleum ether extracts.

Inhibitory Activity of the *Moringa* Extracts on ENT Associated Fungi and Vancomycin-Resistant Cocci

The inhibitory activity of Moringa on the test organisms is presented on Table 2. Moringa methanol extract showed zones of inhibition ranging from 8 – 20mm at concentrations ranging from 12.5 – 100mg/mL on the test organisms. However Moringa petroleum ether extract was not active on all the organisms except *Candida albicans* and at a concentration of 100mg/mL with a 09mm zone of inhibition.

MIC and MBC/MFC of the *Moringa* Extracts on the Test Organisms

The MIC and MBC/MFC of the methanol and petroleum ether extracts on the test organisms is presented in Table 3. From the data presented, the MIC for the test organism ranged from 0.78 to 50mg/mL while the MBC/MFC ranged from 6.25 to 200mg/mL. The MIC of the petroleum ether extracts ranged from 50 to 200mg/mL while the MBC/MFC ranged from 200 to 800mg/mL.

to evaporate to dryness for about 48 hours at room temperature and were subjected to test for their

Assay for the LC_{50} of Moringa extracts by Brine Shrimp Lethality Test

Brine shrimp lethality toxicity assay of the plant extracts is presented in table 4. The brine shrimp results in this study are interpreted as follows: LC_{50} <1.0 µg/mL – highly toxic; LC_{50} 1.0-10.0 µg/mL – toxic; LC_{50} 10.0-30.0 µg/mL – moderately toxic; LC_{50} >30 <100µg/mL – mildly toxic, and > 100µg/ml as non-toxic (Moshi *et al*., 2010). From the data presented in table 4, the LC_{50} for MME is 93.48µg/mL while the LC_{50} MPE is 3.691µg/mL, From this result, MPE is toxic while MME is mildly toxic.

In this study Moringa methanol was found to be more active than moringa petroleum ether extract. Moringa methanol inhibited the growth of all the organisms it was tested on. Moringa petroleum ether extract on the other hand inhibited only the growth of *Candida albicans* at a concentration of 100mg/mL which gave a 09mm zone of inhibition. However when the concentration was increased from 200-800mg/mL it was found to inhibit the growth of the organisms and it also had bactericidal and fungicidal effect. A similar study was conducted and moringa leaf petroleum ether extract was found to be active at this same concentration as reported (26). It was also revealed in this study that moringa methanol extract possessed all the phytochemicals it was screened for. Alkaloids and saponins were absent in moringa petroleum ether extracts, this could be the reason for its poor activity. However, a similar research carried out (30), reported the presence of Alkaloid and Saponin but in low amount. Alkaloids are naturally occurring chemical compounds containing basic nitrogen atoms. They often have pharmacological effects and are used as medications and recreational drugs (31). Saponins cause hemolysis of red blood cells (32).

TABLE 1: PHYTOCHEMICAL COMPONENTS OF MORINGA IDENTIFIED

Test	MME	MPE
Phenols	+	+
Alkaloids	+	-
Saponins	+	-
Steroids	+	+
Reducing Sugar	+	+
Flavonoids	+	+
Tannins	+	+
Terpenoids	+	+
Anthraquinone	+	+

KEY:, MME= *Moringa* Methanolic Extract, MPE= *Moringa* Petroleum Ether Extract; + = Presence of Secondary Metabolite, - = Absence of Secondary Metabolite

TABLE 2: INHIBITORY ACTIVITY OF MORINGA (*MORINGA OLEIFERA*) EXTRACTS ON ENT FUNGI AND VANCOMYCIN RESISTANT COCCI

Organisms	Zones of inhibition (mm) MME				MPE					
	100	50	25	12.5	100	50	25	12.5	DMSO	Cip/Flu
S. aureus	10	08	00	00	00	00	00	00	00	30
S. pyogenes	14	11	08	08	00	00	00	00	00	25
S. pneumonia	10	08	08	00	00	00	00	00	00	28
A. fumigates	19	12	12	10	00	00	00	00	00	00
C. albicans	20	16	10	00	09	00	00	00	00	10

KEY: DMSO= Dimethyl sulfoxide, Cip= Ciprofloxacin, Flu= Fluconazole, MME = Moringa methanol extract, MPE = Moringa petroleum ether extra

TABLE 3: MIC AND MBC/MFC OF THE METHANOL AND PETROLEUM ETHER EXTRACTS ON ENT FUNGI AND VANCOMYCIN RESISTANT COCCI

Organisms	MME MIC	MBC/MFC	MPE MIC	MBC/MFC
S. aureus	50	100	200	800
S. pyogenes	50	200	100	200
S. pneumoniae	6.25	50	100	400
A. fumigatus	6.25	25	100	200
C. albicans	0.78	6.25	50	200

KEY: MIC= Minimum Inhibitory Concentration, MBC/MFC = Minimum Bactericidal Concentration/Minimum Fungicidal Concentration

TABLE 4: BRINE SHRIMP LETHALITY TOXICITY TEST

Extracts	Concentrations (µg/mL)	Total Survival	% Mortality	LC_{50}
MME	1000	11	26.7	93.48
	100	22	6.7	
	10	28	53.3	
MPE	1000	14	46.7	3.691
	100	16	26.7	
	10	22	26.7	

KEY: MME = Moringa methanol extract, MPE = Moringa petroleum ether extra

RECOMMENDATIONS

1. The study demonstrated that Moringa extracts was active, therefore, the search for the novel cytotoxic ingredients in Moringa should be encouraged.
2. Individual compounds of the plants should be isolated, purified, characterized and tested.
3. Other solvents and extraction method should be used for the extraction of the plant material to see if the performance of the extracts will be better.
4. The plant extracts should be evaluated *in vitro* to ascertain their activity on ear, nose and throat fungi and vancomycin resistant cocci and also to evaluate their effect on vital organs of the body.
5. Combination of Moringa with plants with lower toxicity should be encouraged.

CONCLUSION

The present study deduced that, Moringa methanol extract was more active than Moringa petroleum ether extracts, although Moringa petroleum ether extract was experimentally more active on *C. albicans* than on vancomycin resistant coccal bacteria. *Aspergillus fumigatus* was the most predominant fungus while *Staphylococcus aureus* was the most predominant cocci associated with ENT at the time of this study. Moringa methanol extract was more active on *S. aureus*, *S. pyogenes, S. pneumoniae, Aspergillus fumigatus* and *C. albicans*.

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