Antibacterial and Antifungal Activity of *Acalypha wilkesiana.*

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ABSTRACT

We studied the antibacterial and antifungal activities of the leaves of *A. wilkesiana*. The methanolic extract and four derivative fractions from the extract were tested against human pathogenic bacteria namely strains of *Staphylococcus aureus*, *Streptococcus pyogenes*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Escherichia coli* and fungi; *Aspergillus niger*, *A. flavus*, *A. carbonerium*, *Trichophyton metagrophytes* and *Candida albicans*. 200 mg/ml of the methanolic extract and its fractions were tested on the bacteria and fungi using the disc diffusion method. Results showed broad spectrum antimicrobial activity against the Gram-negative and Gram-positive bacteria but same cannot be said about its activity against the fungi. The result further showed that the ethyl acetate fraction was the most potent, closely followed by the aqueous fraction while hexane fraction demonstrated the least antimicrobial activity. The extract and its fractions were active against some of the bacteria which standard antibiotics were not able to inhibit. Methanolic extract of *A. wilkesiana* leaves and its fractions showed a better antibacterial activity than antifungal activity. The demonstration of antimicrobial activity against the test organisms is an indication that there is possibility of sourcing alternative antibiotic substances in this plant for the development of newer antibacterial agents.

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1.0 INTRODUCTION.

Plants have been a source of medicine in the past centuries and today scientists and the general public recognize their value as a source of new or complimentary medicinal products [1]. This plant-based, traditional medicine system continues to play an essential role in health care, with about 80% of the world's inhabitants relying mainly on traditional medicines for their primary health care [2]. Long before mankind discovered the existence of microbes, the idea that certain plants had healing potential and that they contained what we would currently characterize as antimicrobial principles, was well accepted. Since antiquity, man has used plants to treat common infectious diseases and some of these traditional medicines are still included as part of the habitual treatment of various maladies. For example, the use of bear-berry (*Arctostaphylos uvaursi*) and cranberry juice (*Vaccinium macrocarpon*) to treat urinary tract infections is reported in different manuals of phytotherapy, while species such as lemon balm (*Melissa officinalis*), garlic (*Allium sativum*) and tea tree (*Melaleuca alternifolia*) are described as broad-spectrum antimicrobial agents [3].

During the last two decades, there has been a considerable increase in the study and use of medicinal plants all over the world especially in advanced countries. Medicinal plants have been used in Africa before the introduction of antibiotics and other modern drugs [4].

According to World Health Organization, medicinal plants would be the best source to obtain a variety of drugs [5]. Therefore, such plants should be investigated to better understand their properties, safety and efficacy.

The success story of chemotherapy lies in the continuous search for new drugs to counter the challenge posed by resistant strains of microorganisms. The investigation of certain indigenous plants for their antimicrobial properties may yield useful results. Many studies indicate that some plants have substances such as peptides, unsaturated long chain aldehydes, alkaloidal constituents, essential oils, phenols, ethanol, chloroform, methanol and butanol soluble compounds. These plants have emerged as plants with compounds possessing significant therapeutic potential against human pathogens, including bacteria, fungi or virus [6].

Nigeria has a great variety of natural vegetation, which is used in trado-medicine to cure various ailments [7]. Among the plants used for medicinal purpose in Africa, particularly in Nigeria is *Acalypha wilkesiana*.

The genus "Acalypha" comprises about 570 species [8]. *Acalypha wilkesiana* belongs to the family euphorbiaceae and grows as an annual bedding plant [9].This fast growing, evergreen shrub provides a continuous splash of color in the landscape with the bronze red to muted red, 4 to 8 inch long, hear-shaped leaves available in varying mottled combinations of green, purple, yellow, orange, pink or white, depending upon cultivar [10]. Investigation is ongoing on almost all the available cultivars within Nigeria with respect to their phytochemicals and antimicrobial action against medically inclined and agriculturally related pathogens [9,11,12,13,14,15]. Consequently, this plant has been reported to have antibacterial and antifungal properties [13] as the expressed juice or boiled decoction is locally used within Nigeria and some other parts of West Africa for the treatment of malaria, dermatological and gastrointestinal infections [12].

Seeds from *Acalypha wilkensiana* are essential components of a complex plant mixture used by traditional healers in southwest Nigeria in the treatment of breast tumors and inflammation [16].

The aim of this study was to compare the antimicrobial activity of *Acalypha wilkesiana's* methanolic extract and its fractions on bacterial and fungal isolates.

2.0 MATERIALS AND METHODS.

2.1 Materials

2.1.1 Collection and Identification of Plant Samples

Healthy and matured fresh leaf samples of *A. wilkesiana* were collected from the horticulture garden of Babcock University, Ilishan Remo, Ogun state in May 2011 and Identified by a botanist from the botanical unit of the same institution. The leaves were thoroughly rinsed twice in running tap water and then in sterile water before being air-dried for 2 weeks. The dried leaves were ground into fine texture using an electric blender, then stored in sealed and labeled sterilized glass container.

The test organisms used were obtained from the Department of Medical Laboratory Sciences, Benjamin Carson's (Snr) College of Medicine, Babcock University, Ilisan-Remo, Ogun state.

2.2 Methods

2.2.1 Extraction

200 g of the dried and powdered *A. wilkesiana* leaves were extracted at room temperature with 2L absolute methanol for 72 hrs. The filtrate obtained was concentrated using rotatory evaporator at 45 ^{o}C. 32g from the methanolic extract obtained was re-dissolved in methanol and distilled water at ratio 1:3 to obtained aqueous methanolic extract solution. The aqueous methanolic extract solution was partitioned with hexane (3 × 200ml) to obtain the hexane fraction. The aqueous solution remaining was further partitioned with chloroform and ethyl acetate (3 × 200ml) respectively to obtain the chloroform and ethyl acetate fractions. The remaining aqueous solution became the aqueous fraction. All the fractions were concentrated in the rotary evaporator at 45 ^{o}C and stored at 4 ^{o}C till use (Fig.1).

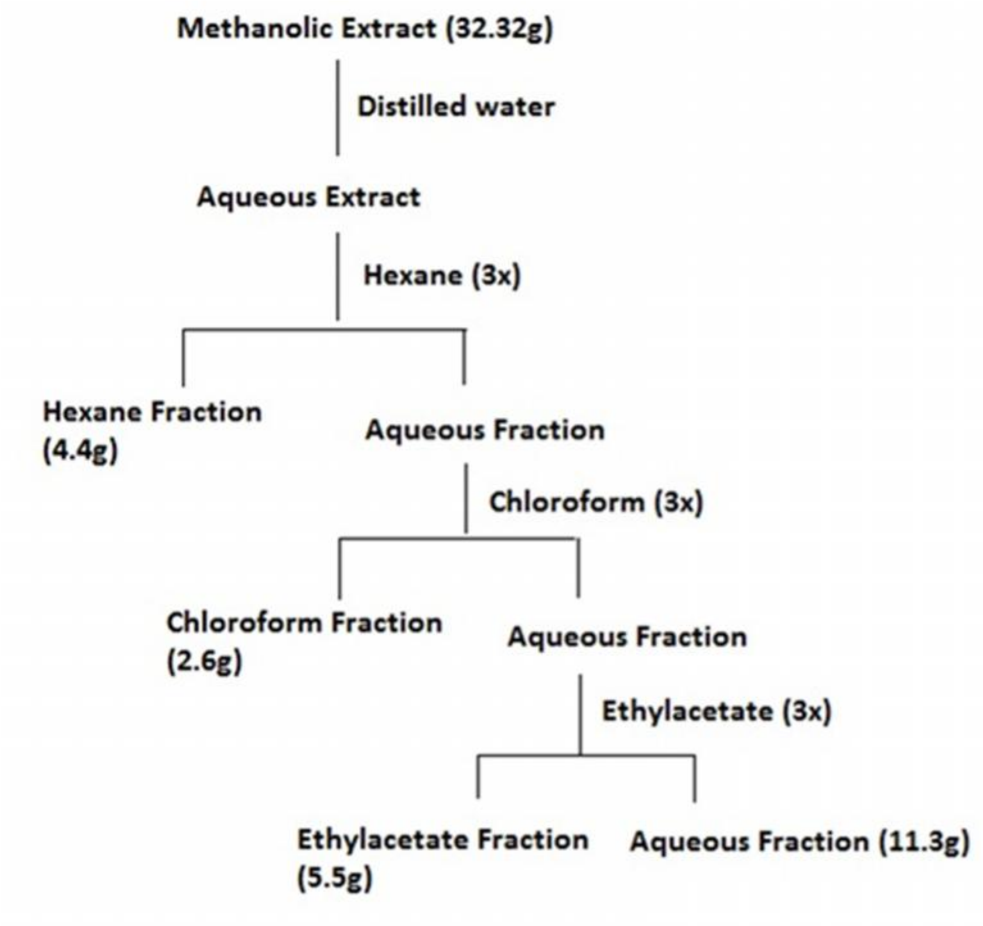

Figure 1: Extraction yield of all the fractions of methanolic extract of *A. wilkesiana*.

2.2.2 Confirmation of test organisms.

The test organisms used were standard strains of pathogenic bacteria and clinical isolate of fungi. They include five strains of Gram-positive bacteria; which are three strains of *Staphylococcus aureus*; *S. aureus* (ATCC 29213), *S. aureus* (ATCC 55620) and *S. aureus* (ATCC 25923), *Streptococcus pyogenes* (ATCC 8662) and *Enterococcus faecalis* (ATCC 29212). Six strains of Gram-negative bacteria namely; three strains of *Escherichia coli* - *E. coli* (ATCC 23922), *E. coli* (ATCC 25922) and *E. coli* (ATCC 35218) others are *Klebsiella pneumoniae* (ATCC 700603), *Pseudomonas aeruginosa* (ATCC 27853) and *Proteus vulgaris* (ATCC 13315). Biochemical analysis was carried out on each of the test organisms for confirmation as described by MacFaddin [17], Fobres *et al*. [18] and Leboffe and Pierce [19].The Bergey's Manual of Systematic Bacteriology [20] was used for species authentication. The fungi isolates include: *Aspergillus niger*, *A. flavus*, *A. carbonerius*, *Trichophyton metagrophytes* and *Candida albicans*, as identified via macroscopic and microscopic observations as well as germ tube test and chlamydospore production on cornmeal agar fortified with Tween 80 polysorbate for the yeast [21-23].

2.3 Antimicrobial Assay of the methanolic extract and its fractions.

2.3.1 Antimicrobial susceptibility test for Bacteria.

Stock cultures were maintained at 4 °C on slopes of nutrient agar. Active cultures for experiments were prepared by transferring a loopful of cells from the stock cultures to test tubes of Mueller-Hinton broth (MHB) and were incubated without agitation for 24 h at 37 ^{o}C. The cultures were diluted with Mueller-Hinton broth to achieve optical densities corresponding to 2.0 x10^{-6} CFU per ml. The disc diffusion method was used to determine the antibacterial activity of the methanol extract and the other four fractions. *In vitro* antibacterial activity was screened by using Mueller Hinton Agar (MHA) (LAB, United Kingdom). The MHA plates were prepared by pouring 15 ml of molten media into sterile petri dishes. The plates were allowed to solidify for 10 mins and a standard loopful of each of the eleven bacteria strain was streaked uniformly on the different plates and incubated at room temperature for 10 mins after which sterile cork borer of 5 mm diameter was used to make two ditches (wells) on each inoculated plate and filled with 1 ml of the methanol extract of the plant and the same was done for each of the eleven bacteria strain using the other four fractions. These were carried out in triplicate for each bacterium. They were left on the bench for 30 mins to ensure adequate diffusion of the extract and fractions and thereafter were incubated at 37 °C for 24 h and the diameter of all resulting zones of inhibition around the ditches were measured to the nearest millimeter along two axis and the mean of the two measurements was calculated. Each set of culture plates were compared for confirmation.

Antibiotic susceptibility test was carried out on the test bacteria as control. A multi-sensitivity disc bearing different antibiotics of GBMTS-NEG (Lot: NH05/P)(Abtek Biologicals ltd. Liverpool L9 7AR, UK) with their concentrations; Amoxycillin(25µg), Cotrimoxazole(25µg), Notrofurantoin(300µg), Gentamicin(10µg), NalidixicAcid(30µg), Ofloxacin(30µg), 30µg Augmentin (amoxicillin and clavulanate), Tetracycline(30µg) and DT-POS (Lot: JB04/P) with their concentrations; Ampicillin(10µg), Chlorampheicol(10µg), Cloxacillin(5µg), Erythromycin(5µg), Gentamicin(10µg),Penicillin(1 i.u),Streptomycin(10µg), Tetracycline(10µg) were used against each of the test bacteria inoculated on Mueller Hinton agar plates. These were incubated at 37 °C for 24 h. After incubation, the diameter of the zone of inhibition around each ditch was measured to the nearest millimetre along two axes and the mean of the two readings was then calculated.

2.3.2 Antimicrobial susceptibility test for Fungi.

Stock fungi were maintained at room temperature on Potato Dextrose Agar (Oxoid, UK). Active fungi for experiments were prepared by seeding a loopful of fungi into Potatoes dextrose broth and incubated without agitation for 48 h at 25 ^{o}C. The broth was diluted with Potatoes dextrose broth to achieve optical densities corresponding to 2.0 x 10^{-5} spore/ml for the fungal strains

The disc diffusion method was also used to screen for antifungal properties. *In vitro* antifungal activity was screened by using Potato Dextrose Agar (PDA). The PDA plates were prepared by pouring 15 ml of molten media into sterile petri plates. The plates were allowed to solidify for 10 mins and 1 ml of the test culture was introduced into agar and allowed to spread while the excess was drained off. The plate was incubated at room temperature for 10 mins. A sterile cork borer of 5 mm diameter was used to make two ditches (wells) on each plate and filled with 1ml of the methanol extract. The same was repeated for each fungus strain using the different fractions of the extract. These were carried out in triplicate for each fungus. The plates were incubated at 25

^{o}C for 96 h and the resulting zone of inhibition around the ditches were measured to the nearest millimeter along two axis and the mean of the two measurements was calculated. Each set of seeded plates were compared for confirmation. Control test was carried out using 10mg/ml of Fluconazole

3. RESULTS AND DISCUSSION

3.1 Result

The result of this study revealed the *in vitro* susceptibility of some bacteria to the methanolic extract of *A. wilkesiana* and its fractions. Table 1 shows the mean ±standard deviation of the inhibition zone in the various agar plates of bacteria exposed to the extract fractions. It was noticed that all the fractions and the extract used inhibited the growth of *S. aureus* (ATCC 25923). However, the methanolic extract, aqueous, ethyl acetate and hexane fractions inhibited S*. aureus* (ATCC 29213), while methanolic extract, aqueous and ethyl acetate fractions inhibited *S. aureus* (ATCC 55620). This study showed that all the *S. aureus* strains were the only organisms susceptible to the crude methanol extract while aqueous and ethyl acetate fractions were the only fractions that inhibited *P. vulgaris* (ATCC 13315), *P. aeruginosa* (ATCC 27853) and *S. pyogenes* (ATCC 8662). The *E. coli* strains and *Enterococcus faecalis* were resistant to the crude extract and two of the fractions except ethyl acetate and chloroform which inhibited *E. coli* (ATCC 35218) and *E. faecalis* (ATCC 29212) respectively. *Klebsiella pneumoniae* (ATCC 15380), *E. coli* (ATCC 25922) and *E. coli* (ATCC 23922) were not susceptible to any of the fractions used in this study.

The aqueous fraction against *S. pyogenes* (ATCC 8662) yielded the highest inhibition value while ethyl acetate fraction gave the greatest number of inhibition, i.e. more test bacteria were susceptible to ethyl acetate fraction.

Table 1: The mean± S.D (mm) of zone of inhibition observed on bacteria cultured plates of isolates exposed to methanolic extract and different fractions of *A. wilkisiana*.

Organisms	Methanolic	Aqueous	Ethyl acetate	Hexane	Chloroform
S. aureus (ATCC 25923)	5.0±0.0	6.5±0.3	7.50±2.9	5.0±0.0	6.0±0.0
S. aureus (ATCC 29213)	7.5±0.9	5.0±0.0	7.0±0.4	3.0±0.0	0.0
S. aureus (ATCC 55620)	7.5±0.3	7.25±0.5	9.5±2.3	0.0	0.0
P. aeriginosa (ATCC 27853)	0.0	5.0±0.0	8.0±0.4	0.0	0.0
P. vulgaris (ATCC 13325)	0.0	7.0±0.0	6.5±0.3	0.0	0.0
S. pyogenes (ATCC 8662)	0.0	10.0±0.0	8.75±0.5	0.0	0.0
E. faecalis (ATCC 29212)	0.0	0.0	0.0	0.0	6.5±0.3
E. coli (ATCC 35218)	0.0	0.0	9.5±0.3	0.0	0.0
E. coli (ATCC 23922)	0.0	0.0	0.0	0.0	0.0
E. coli (ATCC 25922)	0.0	0.0	0.0	0.0	0.0
K. pneumonia (ATCC 15380)	0.0	0.0	0.0	0.0	0.0

The susceptibility of the clinical fungi isolates used is shown in Table 2, which revealed that four of the fungi were completely resistant to all fractions of the extract. *A. niger* was susceptible to the ethyl acetate fraction while *C. albicans* was susceptible to the aqueous, ethyl acetate and chloroform fractions, with the plate treated with aqueous fraction producing the highest zone of inhibition observed.

Table 2: The mean± S.D (mm) of zone of inhibition observed on fungi seeded plates of isolates exposed to methanolic extract and different fractions of *A. wilkisiena*.

Organisms	Methanolic	Aqueous	Ethyl acetate	Hexane	Chloroform
Aspergillus niger	0.0	0.0	6.5±0.3	0.0	0.0
Aspergillus flavus	0.0	0.0	0.0	0.0	0.0
Aspergillus carbonerium	0.0	0.0	0.0	0.0	0.0
Candida albicans	0.0	7.5± 0.3	7.0 ±0.4	0.0	5.0± 0.0
Trichophyton metagrophytes	0.0	0.0	0.0	0.0	0.0

All test organisms expressed various resistant pattern as shown in table 3 for bacteria which were tested against known commercially prepared antibiotics while, table 4 shows the resistant pattern of the fungi to Fluconazole used.

Table 3: Antibiotic Resistant Pattern of the Test Bacteria

Bacteria	**Antibiotic Resistance**	**Antibiotic Susceptibility**
Staphylococcus aureus (ATCC 55620)	AMP, CHL,CXC,ERY,GEN,PEN,STR and TET	OFL, AUG, NIT, AMX, COT, NAL and PEN
S. aureus (ATCC 29213)	Resistant to none	AMP, CHL,CXC,ERY,GEN,PEN,STR, TET, OFL, AUG, NIT, AMX, COT, NAL and PEN
S. aureus (ATCC 25923)	AMP,CXC,ERY,GEN,PEN and STR	CHL, TET, OFL, AUG, NIT, AMX, COT, NAL and PEN
Streptococcus pyogenes (ATCC 8662)	AMP,CHL,CXC,ERY,GEN,PEN,STR and TET	OFL, AUG, NIT, AMX, COT, NAL and PEN
Enterococcus faecalis (ATCC 29212)	AMP, CHL, CXC, ERY, PEN,STR and TET	GEN, OFL, AUG, NIT, AMX, COT, NAL and PEN
Pseudomonas aeruginosa (ATCC 27853)	AMX, COT,NIT,GEN, NAL,AUG and TET	AMP, CHL, CXC, ERY, PEN, STR, OFL and PEN
Proteus vulgaris (ATCC 13315)	AMX, COT,NIT, NAL and AUG	AMP, CHL, CXC, ERY, GEN, PEN, STR, TET, OFL and PEN
Escherichia coli (ATCC 35218)	AMX, COT,NAL, and AUG	AMP, CHL, CXC, ERY, GEN, PEN, STR, TET, OFL, NIT and PEN
E. coli (ATCC 23922)	AMX and AUG	AMP, CHL ,CXC, ERY, GEN, PEN, STR, TET, OFL, NIT, COT, NAL and PEN
E. coli (ATCC 25922)	AMX and AUG	AMP, CHL, CXC, ERY, GEN, PEN, STR, TET, OFL, NIT, COT, NAL and PEN
Klebsiella pneumonia (ATCC 700603)	AMX,COT,NIT,NAL and AUG	AMP, CHL,CXC,ERY,GEN,PEN,STR, TET, OFL and PEN

Key:

OFL = Ofloxacin	GEN = Gentamicin	STR= Streptomycin
TET = Tetracyclin	AUG = Augumentin	
NIT = Nitrofurantoin	AMX = Amoxicillin	
COT = Cotrimoxazole	CHL = Chloramphenicol	
NAL = Nalidixic acid	ERY = Erythromycin	
AMP= Ampicillin	CXC= Cloxacillin	
GEN= Gentamicin	PEN= Penicillin	

Table 4: Antifungal Susceptibility pattern to Fluconazole

Fungi	Susceptibility pattern
Aspergillus niger	Resistant
A. flavus	Resistant
A. carbonerium	Resistant
C. albicans	Susceptible
Trichophyton metagrophytes	Susceptible

3.2 Discussion.

Many studies have established the usefulness of medicinal plants as a great source for the isolation of active principles for drug formulation [24-26].

Several species of the genus Acalypha have been studied and it has been demonstrated that they present antioxidant, wound healing, post-coital antifertility, neutralization of venom, antibacterial, antifungal and antitrypanosomal activities [27-29]. The result of this study support the antibacterial and antifungal activities of *A. wilkesiana* as a broad spectrum antimicrobial agent since it inhibited the growth of gram positive (*S. aureus*, *S. pyogenes*, *E. faecalis*) and gram negative bacteria (*E. coli*, *P. aeruginosa, P. vulgaris*) as well as some fungi (*A. niger*, *C. albicans*).

The fact that the methanolic extract of *A. wilkesiana* and its fractions showed activity against most of the test organisms is a major breakthrough in appreciating the medicinal potential of the plant especially in the management of both community acquired and nosocomial associated infections.

Also that some organisms were not susceptible to its activity, corroborated the fact that resistance to antimicrobial agents cannot be eliminated but curtailed since some organisms are intrinsically resistant as stated by Oluremi *et al*. [30]

However the effectiveness of its antimicrobial potency seems to be more of antibacterial than antifungal. This study revealed that only *A. niger* and *C. albicans* were inhibited among the fungi used which support the work of Onocha and Olusanya [31] which showed that the methanolic extracts of *A. wilkesiana* inhibited only *A. niger* and *C. albicans*. Also support the report of Oladunmoye [9] which revealed that *A. niger* was inhibited by methanolic extract of this plant. It is note worthy to see that *A. niger* which was resistant to the fluconazole was susceptible to the ethyl acetate fraction. The resistance of fungi to the tested extract may be due to the presence of more complex cell wall with rigidity than the thin cell membrane of bacteria. Also, this may be due to their ability to produce extracellular enzymes that helps them to degrade and metabolize substrate such that the extract becomes a source of food to the fungi instead of inhibiting their growth after they have been rendered nontoxic due to degradation [32].

The result also showed that the methanolic extract and its fractions were active against *S. aureus* (ATCC 29213) and *Streptococcus pyogenes* (ATCC 8662) which were resistant to standard antibiotics. The disparity between the activities of the extract and the standard antimicrobial drug may be due to the mixtures of bioactive compounds present in the extract compared to the pure compound contained in the standard antibiotics [33]. This demonstration of activity against such test bacteria may form the scientific bases for the local dependencet on this plant in the treatment of various ailments.

This present study also revealed that the ethyl acetate fraction of the extract was the most potent of all the fractions used. It was the only fraction which inhibited the highest number of bacteria and fungi. The only exception to this was *E. faecalis* which was only susceptible to the Chloroform fraction.

Several strains of pathogenic *S. aureus* and *E. coli* used in this study revealed that the methanolic extract inhibited all strains of *S. aureus* but did not inhibit any strain of *E. coli* and the other Gram negative bacteria, this may suggest that the methanolic extract is mainly active against *S. aureus*. Though the methanolic extract did not inhibit any Gram negative bacteria, the ethyl acetate and aqueous fractions did and this may be due to the partial purification of the methanolic extract which enabled the bioactive compounds to act. Gallic acid, corilagin and geraniin have been reported to be the active compounds responsible for the antimicrobial activity of *A. wilkesiana* [11], however, that study was limited to bacteria isolates. Further purification of the ethyl acetate and aqueous fractions of methanolic extract of *A. wilkesiana* will give more insight into the bioactive compounds responsible for the antibacterial and antifungal properties of this plant.

4. CONCLUSION.

The demonstration of activity against both gram-negative and gram-positive bacteria and fungi is an indication that the plant can be a source of bioactive substances that could be of broad spectrum of activity. The fact that the plant was active against both clinical and laboratory isolates is also an indication that it can be a source of very potent antibiotic substances that can be used against drug resistant microorganisms. The search for new drugs to counter the challenge posed by resistant strains of bacteria and some fungi might have started yielding results as the investigation of this plant has demonstrated enormous therapeutic potential. It can serve the desired purpose with lesser side effects that are often associated with synthetic antimicrobial agents.

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