***Original Research Paper**

Comparative Assessment of Antibacterial and Antifungal Activity of Dried Leaves of *Acalyphawilkesiana.*

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Abstract

Comparative studies on the antimicrobial activities of the leaves of *A. wilkesiana* were carried out. Methanol was used as the extraction solvent. The crude methanolic extracts and four other derivative fractions were tested against human pathogenic bacteria namely strains of *S.aureus, S.pyogenes, E. faecalis, P. aeruginosa, P.vulgaris*and*E.coli*and fungi;*Aspergillusniger, A. flavus, A. carbonerium, Trichophytonmetagrophytes*and *Candida albicans*. 200mg/ml of each of the extract and the 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 while hexane fraction demonstrated the least antimicrobial activity. The extract and fractions were potent against some of the bacteria which standard antibiotics were not able to inhibit.Methanolic extracts of *A.wilkesiana* leaves showed a better antibacterial activity than antifungal activity. The demostration 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.

Keywords: *Acalyphawilkesiana,* antimicrobial, antibacterial, antifungal, resistance, zone of inhibition.

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. (Premanath, R., Lakshmideri, N. 2010).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 (Owolabi*et al*., 2007). Long before mankind discovered the existence of microbes, the idea that certain plants had healing potential, indeed, 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 (Arctostaphylosuvaursi) and cranberry juice (Vacciniummacrocarpon) 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 (Melaleucaalternifolia) are described as broad-spectrum antimicrobial agents (R´ıo, JL.,Recio, MC. 2005)

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 (Kabir*et al*., 2005)

According to World Health Organization, medicinal plants would be the best source to obtain a variety of drugs. Therefore, such plants should be investigated to better understand their properties, safety and efficacy (Nascimento*et al*., 2000)

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 in some plants there are many substances such as peptides, unsaturated long chain aldehydes, alkaloidal constituents, some essential oils, phenols and water, ethanol, chloroform, methanol and butanol soluble compounds. These plants then emerged as compounds with potentially significant therapeutic application against human pathogens, including bacteria, fungi or virus (El astal*et al*., 2005).

Medicinal plants are used by 80% of the world population as the only available medicines especially in developing countries (EL-Kamali, HH., EL-amir, MY 2010). More importantly in Africa, particularly West Africa, new drugs are often beyond the reach of the poor such that up to 80% of the population use of medicinal plants as remedy against infections and diseases (Kirby,G.C. 1996; Hostettmann, K., and Maston, A. 2002). Nigeria has a great variety of natural vegetation, which is used in trado-medicine to cure various ailments.(Egwaikhinde, P.A., Gimba, P.C.2007). Among plants use for medicinal purpose in Africa, particularly in Nigeria is *Acalyphawilkesiana*.

The genus "Acalypha" comprises about 570 species(Riley,H.P.1963). *Acalyphawilkesiana* belongs to the family euphorbiaceae and grows as an annual bedding plant (Oladunmoye, M.K 2006).This large ,fast growing, evergreen shrub provides a continuous splash of colour 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 (Gilman, E.F 1999). 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 (Adesina*et al*., 2000; Akinde, B.E., Odeyemi, O.O 1987; Alade,P.I.,Irobi, O.N., 1993;Ezekiel *et al*., 2009;Ogbo,E.M.,Oyibo, A.E 2008;Oladunmoye, M.K 2006).Consequently, this plant has been reported to have antibacterial and antifungal properties (Alade, P.I., Irobi, O.N.,1993) 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 (Akinde, B.E., Odeyemi, O.O 1987).

Seeds from *Acalyphawilkensiana* are essential components of a complex plant mixture used by traditional healers in southwest Nigeria in the treatment of breast tumors and inflammation (Udobang*et al*., 2010).

The aim of this study was to compare the antibacterial and antifungal potency of *Acalyphawilkesiana* against bacteria and fungi of medical importance.

MATERIALS AND METHODS.

Collection and Identification of Plant Samples

Healthy and matured fresh plant leaf samples of *Acalyphawilkesiana* 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 2weeks. The dried leaves were grounded into fine texture using an electric blender, then stored in sealed and labeled containers for use.

Extraction

200mg of the dried and powdered *A. wilkesiana* leaves were extracted at room temperature with absolute methanol. The crude methanol extract obtained was redissolved in methanol and made aqueous with distilled water. The aqueous solution was extracted with hexane in a separating funnel to obtain the hexane fraction. The aqueous layer was further partitioned with chloroform to obtain the chloroform layer. Finally, the remaining aqueous layer was partitioned with ethylacetate to obtain ethylacetate fraction and aqueous fraction. A portion of the crude extract was also kept for analysis. All the fractions were concentrated in the rotary evaporator at 45^{o}C and stored at 4^{o}C till use. (Fig.1).

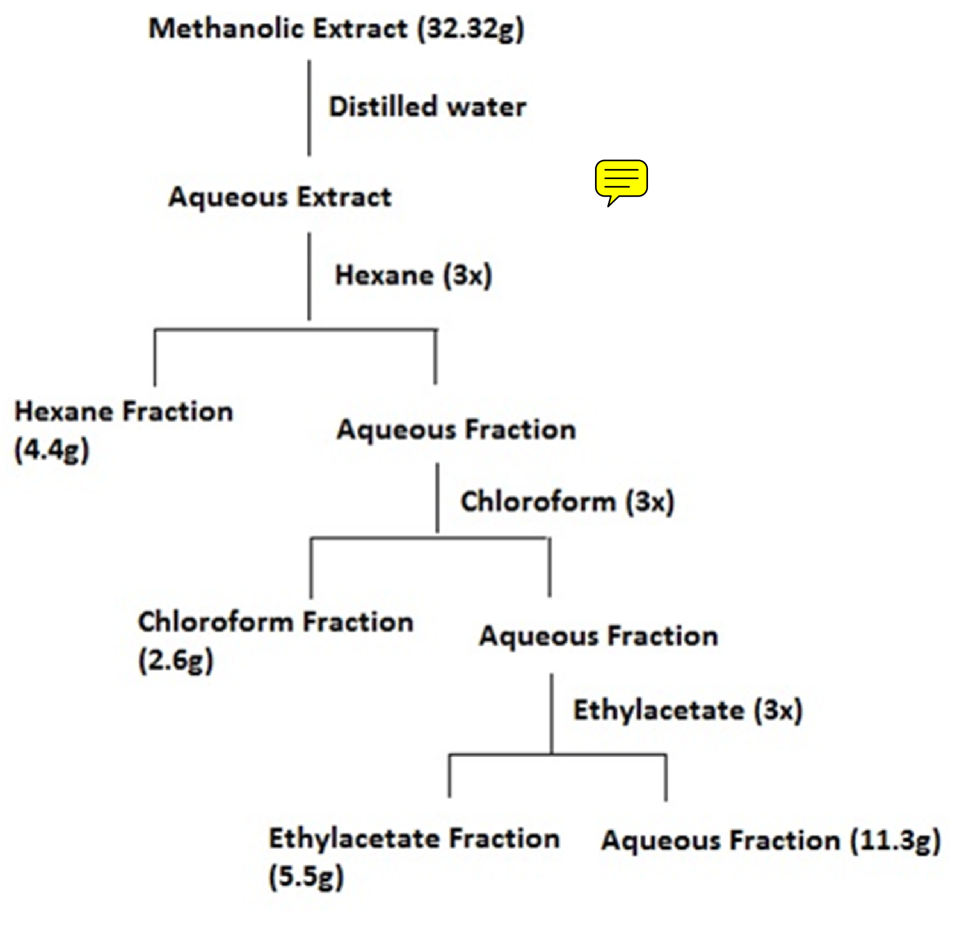

Figure 1: Extraction yield of all the fractions of methanolic extract of A. wilkesiana.

Collection and maintenance 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 stains 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 *Klebsiellapneumoniae*(ATCC 700603),*Pseudomonas aeruginosa* (ATCC 27853) and *Proteus vulgaris* (ATCC 13315). The fungi isolates include: *Aspergillusniger*, *A. flavus*, *A. carbonerius*, *Trichophytonmetagrophytes* and *Candida albicans*. They were obtained from the Department of

Medical Laboratory Sciences, Babcock University, Ilisan-Remo, Ogun state. Biochemical analysis was carried out on each of the test organisms for confirmation.

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 24hours at 37^{o}C. The cultures were diluted with Mueller-Hinton broth to achieve optical densities corresponding to 2.0 x10^{-6} colony forming units (CFU/ml)

Antimicrobial Assay of the Crude metanolic extracts and the various fractions.

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 24hours at 37^{o}C. The cultures were diluted with Mueller-Hinton broth to achieve optical densities corresponding to 2.0 x10^{-6} colony forming units (CFU/ml). The disc diffusion method was used to determine the antibacterial activity of the crude methanol extracts 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 minutes and a standard loopfulof each of the eleven bacteria strain was streaked uniformly on the different plates and incubated at room temperature for 10mins after which sterile cork borer of 5mm diameter was used to make two ditches (wells) on each inoculated plate and filled with 200 mg/ml of the crude 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 duplicate for each organism. They were left on the bench for 30 minutes to ensure adequate diffusion of the fractions of extract and thereafter were incubated at 37°C for 24 hours 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 measurement was calculated. The duplicate cultures were used 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)(AbtekBiologicals ltd. Liverpool L9 7AR, UK) with their concentrations; Amoxycillin(25µg), Cotrimoxazole(25µg), Notrofurantoin(300µg),Gentamicin(10µg),NalidixicAcid(30µg),Ofloxacin(30µg),Augmentin(30 µg),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 24hours. After incubation, the diameter of the zone of inhibition around each ditch was measured to the nearest millimetre along two axis and the mean of the two readings was then calculated.

Antimicrobial susceptibility test for Fungi

Stock fungi were maintained at room temperature on Potatoes 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 hours 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 was screened by using Potatoes Dextrose Agar (PDA). The PDA plates were prepared by pouring 15ml of molten media into sterile petri plates. The plates were allowed to solidify for 10minutes and 1ml 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 minutes. A sterile cork borer of 5mm diameter was used to make two ditches (wells) on each plate and filled with 200mg/ml of the crude methanol extract. The same was repeated for each fungus strain using the different fractions of the extract. The plates were incubated at 25^{o}C for 96hrs and the resulting zone of inhibition around the ditches were measured to the nearest millimeter along two axis and the mean of the two measurement was calculated. The duplicate seeded agar were used for confirmation. Control test was carried out using 10mg/ml of Fluconazole

Determination of Minimum Inhibitory Concentration (MIC) :

In determining the antimicrobial activity of *Acalyphawilkesiana* , the minimum bacterial growth inhibition was accessed using the crude methanol extract and other fractions used in this study.

2mls of nutrient broth was prepared into test tubes for the crude methanol extract and the four fractions of the extract. 0.5ml of 25g/ml, 50g/ml, and 100g/ml of each extract fraction was added to different test tubes containing the nutrient broth. This was prepared for each organism and done in duplicate. A colony of 24hrs cultured organism was inoculated into test tube containing 1ml of normal saline to form a turbidity of 0.5 McFarland standard and was thereafter dispense into the test tube containing the suspension of nutrient broth and the various fractions of the extract . This was done for all the organisms at the varying concentrations. All test tubes were properly corked and incubated at 37^{o}C for 24hrs and at 25^{o}C for 96hrs for bacteria and fungi respectively . After which they were observed for absence or present of visible growth . The lowest concentration without visible growth (turbidity) of organisms was regarded as the Minimum inhibitory concentration (MIC). It was further standardized in terms of absorbance at 600 nm in a visible spectrophotometer. Positive and negative controls were set up alongside this experiment.

RESULT

Susceptibility of the test bacteria to crude and fractions of A.wilkesiana extract.

The result of this study revealed the in vitro susceptibility of some bacteria to the crude extracts and other fractions of *A. wilkesiana*. Table 1 shows the mean ±standard deviation of the zone of inhibition in the various agar plates of bacteria exposed to the extract fractions. It was noticed that all the fractions of *A.wilkisiena* extract used inhibited the growth of *S.aureus* (ATCC 25923). However, the crude methanol extract, aqueous, ethyl acetate and hexane fractions inhibited*S.aureus* (ATCC 29213),. while crude, 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. *Klebsiellapneumoniae* (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 different fractions of *A.wilkisiena* extract

Organisms	Crude	Aqueous	Ethyl acetate	Hexane	Chloroform
S. aureus (ATCC 25923)	5.0±0.00	6.5±0.29	7.50±2.88	5.0±0.00	6.0±0.0
S. aureus (ATCC 29213)	7.5±0.86	5.0±0.0	7.0±0.41	3.0±0.0	0.00
S. aureus (ATCC 55620)	7.5±0.28	7.25±0.5	9.5±2.28	0.00	0.00
P. aeriginosa (ATCC 27853)	0.00	5.0±0.0	8.0±0.41	0.00	0.00
P. vulgaris (ATCC 13325)	0.00	7.0±0.0	6.5±0.29	0.00	0.00
S. pyogenes (ATCC 8662)	0.00	10.0±0.0	8.75±0.49	0.00	0.00
E. faecalis (ATCC 29212)	0.00	0.00	0.00	0.00	6.5±0.29
E. coli (ATCC 35218)	0.00	0.00	9.5±0.29	0.00	0.00
E. coli (ATCC 23922)	0.00	0.00	0.00	0.00	0.00
E. coli (ATCC 25922)	0.00	0.00	0.00	0.00	0.00
K. pneumonia (ATCC 15380)	0.00	0.00	0.00	0.00	0.00

Susceptibility of the test fungi to crude and fractions of A.wilkesiana extract.

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 of zone of inhibition observed.

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

Organisms	Crude	Aqueous	Ethyl acetate	Hexane	Chloroform
Aspergillusniger	0.00	0.00	6.5±0.29	0.00	0.00
A.flavus	0.00	0.00	0.00	0.00	0.00
A. carbonerius	0.00	0.00	0.00	0.00	0.00
C. albicans	0.00	7.5± 0.28	7.0 ±0.41	0.00	5.0± 0.00
Trichophytonmetagrophytes	0.00	0.00	0.00	0.00	0.00

Antibiotics sensitive test

Antibiotics sensitivity test were carried out on all the test orgsnisms. All test organisms expressed various resistant pattern as shown in table 3 for bacteria 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
Staphylococcus aureus (ATCC 55620)	AMP, CHL,CXC,ERY,GEN,PEN,STR and TET
S. aureus (ATCC 29213)	Resistant to none
S. aureus (ATCC 25923)	AMP,CXC,ERY,GEN,PEN and STR
Streptococcus pyogenes (ATCC 8662)	AMP,CHL,CXC,ERY,GEN,PEN,STR and TET
Enterococcus faecalis (ATCC 29212)	AMP, CHL, CXC, ERY, PEN,STR and TET
Pseudomonas aeruginosa (ATCC 27853)	AMX, COT,NIT,GEN, NAL,AUG and TET
Proteus vulgaris (ATCC 13315)	AMX, COT,NIT, NAL and AUG
Escherichia coli (ATCC 35218)	AMX, COT,NAL, and AUG
E. coli (ATCC 23922)	AMX and AUG
E. coli (ATCC 25922)	AMX and AUG
Klebsiella pneumonia (ATCC 700603)	AMX,COT,NIT,NAL and AUG

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
Aspergillusniger	Resistant
A.flavus	Resistant
A.carbonerius	Resistant
C.albicans	Susceptible
Trichophytonmetagrophytes	Susceptible

Discussion

Many studies (Banso, A., Mann, A 2006; El-Mahmood, A.M., Ameh, J.M 2007; Falodun*et al.*, 2006) have established the usefulness of medicinal plants as a great source for the isolation of active principles for drug formulation.

Several species of the genus Acalypha has been studied and it has been demonstrated that they present antioxidant, wound healing, post-coital antifertility, neutralization of venom, antibacterial, antifungal andantitrypanosomal activities (Perez Gutierrez,R.M.,Vargas,S.R. 2006; Marwah*et al.*, 2007;Shirwaikar*et al.*,2004). 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*)

However the effectiveness of its antimicrobial potency seems to be more of antibacteria than antifungi. This study revealed that only *A.niger* and *C.albicans* were inhibited among the fungi used which support the work of Onocha and Olusanya 2010 which showed that the methanolic extracts of *A.wilkesiana* inhibited only *A.niger* and *C.albicans*. Also to the report of Oladunmoye 2006 which revealed that *A.niger* was inhibited by methanolic extracts. 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 food to the fungi instead of inhibiting their growth after they have been rendered non toxic due to degradation (Tortora*et al.*,2002).

The result also showed thatthe extract and its fraction was potent 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 (D.Gatsing*et al*.,2010) This demonstration of activity againstsuch test bacteria may form the scientific bases for the local dependent on this plant in the treatment of various ailments.

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

In conclusion,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 and preferential potential. They can serve the desired purpose with lesser side effects that are often associated with synthetic antimicrobialagents.

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