












ORIGINAL RESEARCH ARTICLE

Antibacterial Activity of the Leaf Extract of *Acalypha wilkesiana* against some Human Bacterial Pathogens

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ABSTRACT

Humanity has long been creating traditional treatments based on the knowledge of medicinal plants found worldwide. The medicinal potential of *Acalypha wilkesiana* in treating skin and gastrointestinal problems is highly appreciated. This study aimed to examine the *Acalypha wilkesiana* leaf extract's antibacterial effectiveness against a variety of human bacterial infections. Standard techniques were used to determine whether secondary metabolites were present in methanolic, ethanolic, and hot water extracts. The plant extracts were evaluated for their ability to inhibit the growth of bacteria obtained from Bells University of Technology, including *Staphylococcus aureus*, *Staphylococcus saprophyticus*, *Enterococcus faecalis*, *Escherichia coli*, and *Pseudomonas aeruginosa*. Agar diffusion was used for antibacterial activities, and Mueller Hinton Agar (MHA) minimum inhibitory concentration (MIC) was determined using the agar dilution method. According to the study's findings, the plant sample included flavonoids, tannin, alkaloids, and saponins. With a MIC of 2.5 mg/mL against *Pseudomonas aeruginosa* and *Enterococcus faecalis* and 5.0 mg/mL against *Staphylococcus aureus*, hot water extract exhibited the highest activity level against all tested isolates. As a result, *Acalypha wilkesiana* leaf extract exhibited broad-spectrum antibacterial activity.

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INTRODUCTION

Acalypha wilkesiana, which goes by the names copper leaf, fire dragon, and Joseph's coat, is a species of spurge in the genus *Acalypha* of the Euphorbiaceae family (Makoshi *et al.*, 2016). Although it originated in Fiji and the nearby South Pacific islands, the well-known outdoor plant *Acalypha wilkesiana* has now spread to most of the world, especially the tropics of Africa, America, and Asia. Despite medical technology improvements, millions worldwide continue to seek cures from medicinal plants. This is

especially true in the tropical regions of Africa, America, and Asia. The expressed juice or boiled decoction of *Acalypha wilkesiana* leaf material is used in traditional medicine in Southern Nigeria to cure a range of ailments, such as fungal skin infections, diabetes mellitus, hypertension, and gastrointestinal disorders. A leaf poultice is frequently used to treat headaches, colds, swellings, and malaria (Kumar & Singh, 2022). *Acalypha wilkesiana* has been reported to be used in treating

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gastrointestinal disorders (Gotep *et al.*, 2010) carried out in vitro using plant ethanol extracts, which has necessitated scientific investigation into the biochemical basis of its therapeutic value.

Humanity has long established a practice of employing medicinal plants all around the planet. But since this information is typically transmitted verbally, there's a chance that it will be forgotten in favor of more contemporary medical procedures (Chaplot *et al.*, 2023). However, it allows the local population to receive a low-cost, ad hoc treatment. It is also a potential source of novel and potentially important medicinal molecules. According to estimates from the World Health Organization (W. H. O.), traditional medicine serves over 80% of the world's population's basic medical needs. In areas where the use of plants is still highly regarded, a plethora of information about how to treat different illnesses has accumulated (Cheng *et al.*, 2022). In many parts of Africa, herbal medicine is still used extensively in healthcare, especially in the more remote areas with few clinics and hospitals (Maggassouba *et al.*, 2007). These regions' traditional herbalists use the diversity of plant species to cure various ailments. However, little is known about herbal medicine because of the prevalence of oral tradition in this part of the world and the dearth of knowledge in the scientific literature (Von Gernet, 2022). Medicinal plants have been discovered to be a beneficial source for treating infectious diseases with fewer side effects when compared to commercial drugs. However, issues with synthetic drugs, such as expensive adulteration, increasing side effects, insufficient illness therapy, and medication resistance, are more common in developing countries.

A plant used in traditional African medicine that includes alkaloids and other secondary metabolites has been found to cause resistance in various bacteria (Bano *et al.*, 2022). Because traditional medicine is more affordable, more broadly accepted in society, and more successful than modern medicine in treating certain ailments, communities continue to use it extensively. Furthermore, it is believed that plant medicine is an important branch of medicine that mostly uses readily available therapeutic herbs in the area (Bhat *et al.*, 2023). The testing of plant-based medications against a variety of opportunistic diseases has yielded encouraging findings. While there are benefits to using antimicrobial chemicals from medicinal plants, which are preferred in combination therapy because they have fewer side effects, improved patient tolerance, are relatively inexpensive, have a long history of use, and are renewable (Shehu *et al.*, 2022), antibiotics can occasionally cause negative effects (Tan *et al.*, 2022). These figures highlight the need for creative substitute drug regimens.

The need for sustainable management of traditional medicinal plant resources stems from their potential as a source of novel drugs and the fact that traditional medicinal plants are still used for medical purposes (Cunningham *et al.*, 1993). Since it has been demonstrated

that plant extracts and essential oils obtained from a wide variety of plants exhibit biological activity both in vitro and in vivo, supporting research on traditional medicine, ethnobotanical research is both important and urgent.

Traditional medicinal herbs are utilized for medical purposes and are a potential source of innovative medications; therefore, it is imperative to manage their resources sustainably (Ellis *et al.*, 2023). The interest in and significance of ethnobotanical research are thus made clear by the numerous plants from which plant extracts and essential oils have been shown to have biological activity both in *vitro* and in *vivo*, supporting research on traditional medicine.

This research aims to identify the Antibacterial Activity of the Leaf Extract of *A. wilkesiana* against some Human Bacterial Pathogens

MATERIALS AND METHODS

Sample Collection and preparation

Fresh leaves of *Acalypha wilkesiana* were collected from the Bells University of Technology environment and specimen samples were deposited at the herbarium of the Department of Botany, Faculty of Science, University of Lagos. The plant was thus authenticated as *Acalypha wilkesiana* LUH 7536 (Copper leaf).

Fresh *Acalypha wilkesiana* leaves were thoroughly cleaned and rinsed in sterile distilled water before being allowed to air dry for two weeks at room temperature. An electric blender was used to grind up the dried materials. Until needed, the powdered sample was kept at room temperature in an airtight container.

Sample Extraction

Ethanol, methanol, and hot water were used to extract the powdered material. In this investigation, the extraction process was carried out in accordance with Afrid *et al.* (2023). 50 g of dried *Acalypha wilkesiana* leaf powder was obtained and placed in a different container for each of the three solvents. The extraction solvent was then added in an amount of 250 mL. After six hours of mechanical shaking every thirty minutes, the mixture was left to stand for roughly twenty-four hours throughout the extraction process. The filtrate was then collected after **filtering the solution through the** Whatman No. 1 filter paper (Bhopi *et al.*, 2022). The process was carried out three times using new extraction solvent volumes, and the filtrate was combined. Extract Processing

The resulting extract was then evaporated in a water bath at 100 °C to concentrate it until it was completely dry. The acquired crude extracts, methanolic and ethanolic, were reconstituted for the hot water extract using 100% Dimethyl Sulfoxide (DMSO) and distilled water. Amber bottles served as storage for the extracts.

Phytochemical Screening

Preliminary phytochemical screening for detecting various plant constituents was described by (Trease *et al.*, 1989; Adewale *et al.*, 2023).

Test for Alkaloids

In a steam bath, 0.5 g of the extract was mixed with 3 mL of 1% aqueous hydrochloric acid and filtered. One milliliter of the filtrate was subjected to a small amount of Mayer's, Wagner's, and Draggendorf's reagents. The presence of alkaloids was first suspected based on precipitation (Cream, Reddish-brown, Orange, or Reddish-brown) with either of these reagents.

Test for tannins

After combining 0.5 g of the extract with 1 mL of distilled water, it was filtered. The filtrate was mixed with a solution of ferric chloride. A green, blue-green, or blue-black precipitate was considered proof positive of the presence of tannins.

Test for resins

5 mL of boiling ethanol was used to dissolve 2g of the extract. These were passed through Whatman No. 1 filter paper, and 4 milliliters of 1% aqueous HCl were used to dilute the filtrate. There were resins present because a resinous precipitate formed.

Test for steroids

Two milliliters of chloroform were used to dissolve around 0.1 grams of the extract, and sulphuric acid was carefully added to generate a lower layer. When a steroidal ring is present, the interphase will appear reddish-brown in hue.

Test for anthraquinones

A dry test tube containing 0.5 g of extract, 5 mL chloroform, and five minutes of shaking were required. After filtering, an equivalent volume of 100% ammonia solution was shaken with the filtrate. **Free anthraquinones** are indicated by the ammoniacal layer's (lower layer) pink, violet, or red color.

Test for saponins

About 0.5 g of the extract was shaken with water in a test tube. Frothing which persists on warming was taken as preliminary evidence for the presence of saponins.

Test for flavonoids

Using acetone, a 2.0 g powdered sample was held. To ensure that all traces of acetone were eliminated, the sample was submerged in hot water. The sample that was

detained was added to boiling distilled water. Filtration was done on the heated mixture. After cooling the filtrate, 5 mL of 20% sodium hydroxide was added to **equalize the filtrate's volume**. A **yellow solution** suggests the presence of flavonoids.

Collection of the Bacterial Isolates

The following isolates were used: *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus saprophyticus*, and *Enterococcus* species. These were obtained from Bells University of Technology Microbiology Laboratory.

Preparation of Bacterial Suspension

The preparation of the bacterial suspension followed the 0.5 McFarland guidelines. A culture from Mueller Hinton agar plates that was 24 hours old was employed to prepare the bacterial solution. The organism was suspended in sterile distilled water, with the turbidity adjusted to contain roughly 1×10^8 cells per milliliter. It was attained by bringing the bacterial suspension's optical density up to 0.5 McFarland turbidity standards. Using a sterile wire loop, isolated colonies of the organisms on Mueller Hinton Agar plates were picked and emulsified in 9ml of sterile distilled water in McCartney bottles. The mixture was then homogenized and thus referred to as the stock solution.

Preparation of 0.5 Mcfarland Standard

To make a 1% V/v solution of sulphuric acid, one milliliter (1 mL) of concentrated sulphuric acid was added to 99 mL of distilled water, and the mixture was well mixed. Next, 50 mL of distilled water was mixed with 0.5 g of dehydrated barium chloride ($\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$) to create 1% W/v of barium chloride. Subsequently, 99.5 mL of the sulphuric acid solution was combined with 0.5 mL of the barium chloride solution and well mixed. The turbid solution was poured into a McCartney bottle of the same kind used to make the test inocula in a volume of nine milliliters (9 mL).

Antibiotic susceptibility test

In compliance with the CLSI guidelines, antimicrobial susceptibility tests were conducted utilizing the disk diffusion method, as advised by the Kirby-Bauer method (Tsukatani *et al.*, 2012). This technique is good for organisms that develop quickly at 35–37°C overnight. After absorbing moisture from the agar, the antibiotic (specified concentration) impregnated disc diffuses into the agar medium. The diffusion rate is less than the pace at which the antibiotic is extracted from the disc. A logarithmic decrease in the antibiotic concentration occurs with increasing distance from the disc. The susceptibility is evaluated by measuring the zone of inhibition of bacterial growth surrounding each disc. Muller Hinton Agar was the medium employed.

The antibiotics tested are: Gram-negative disc (Oxoid); Ceftazidime (30 µg), Cefuroxime (30 µg), Ampicillin (10 µg), Nitrofurantoin (300 µg), Gentamycin (10 µg), Ciprofloxacin (5 µg), Ofloxacin (5 µg); Gram-positive disc (Oxoid); Ceftazidime (30 µg), Cloxicillin (5 µg), Erythromycin (30 µg), Caftriaxone (30 µg), Gentamycin (10 µg), Cefuroxime (30 µg), Ofloxacin (5 µg), Amoxycillin (30 µg) for bacterial isolates.

The test organism's stock solution was used to collect inoculums using a sterile swab stick, and any excess was removed by pressing the stick against the test tube's interior above the suspension level. After evenly swabbing the whole surface of the Mueller Hinton agar (MHA) plates with the test organism inoculums, the plates were left to dry for three to five minutes. Using sterile forceps, the right antibiotic discs were carefully selected and positioned in the middle of the inoculated Petri plates with the relevant labels. After inverting the plates and incubating them for the entire night at 37 °C, the zones of inhibition were quantified and interpreted in accordance with the Clinical Laboratory Standard Institute (CLSI, 2009) standards.

Preliminary antimicrobial screening of crude extracts

To get the necessary extract dilutions, which ranged from 12.5 to 100 mg/mL, the plant extract was reconstituted in sterile distilled water for the hot water extraction and concentrated DMSO for the methanol and ethanol extracts. The agar well diffusion method was used to test the crude extracts for antibacterial activity.

Standardization of microbial cell suspension

Mueller-Hinton agar (about 20 mL) was produced, sanitized, and aseptically put into sterile dishes before being let to set. To create a 1 in 100 (10⁻²) dilution, 0.1 mL of the overnight suspension was added to 9.9 mL of sterile distilled water. A sterile spreader applied 0.2 mL of this dilution to the set agar and evenly distributed across its surface. After that, the plates were left to dry. Equidistant wells were drilled into the agar plates using a sterile cork borer with an 8 mm diameter, and the wells were labeled appropriately.

Using a micropipette, approximately 100 µL of the reconstituted extracts at the various dilutions (100 to 12.5 mg/mL) were dropped into each well, filling them to fullness.

In every instance, the controls were concentrated DMSO and sterile distilled water. The plates were incubated upright at 37 °C for 24 hours after being left to stand on the bench for an hour to allow the extracts to properly diffuse into the medium. The nearest millimeter (mm) was

used to measure the inhibition zones. Every experiment was carried out in three copies.

Determination of minimum inhibitory concentration (mic) of crude extraction against selected pathogens

Using a series of doubling extract concentrations, the standard agar dilution method procedures, as outlined by Lawal *et al.* (2014), were used to determine the minimum inhibitory concentrations (MIC). Various dilutions were made to get 50, 25, 100, 12.5, and 6.25 mg/mL concentrations. Final concentrations of 10, 5, 2.5, and 1.25 mg/mL were obtained by combining approximately 1 mL of each extract dilution with 19 mL of melted Mueller-Hinton agar (1 in 20 dilution), then poured into Petri dishes and let to set. After letting the set agar plate surfaces dry, the chosen isolates' overnight broth culture was streaked over them, and the plates were then properly incubated. Next, the presence or lack of development on the plates was assessed. The minimum inhibitory concentration was defined as the lowest extract concentration that prevented each organism on the agar plate from growing visibly. Every experiment was carried out three times.

RESULTS

This study examined the plant to see whether it contained any secondary metabolites. The phytochemicals found in the study plant sample, as indicated in Table 1, included tannins, alkaloids, flavonoids, saponins, terpenoids, and sterols, according to the study's findings.

The studied pathogens/isolates' antibiotic susceptibility pattern was also investigated, as shown in Tables 2a & b, where inhibition zones are expressed in millimeters (mm). All examined Gram-negative bacterial isolates were completely resistant to ampicillin, cefuroxime, and augmentine, according to an analysis of the isolates' sensitivity and resistance patterns to several antibiotics. However, among the Gram-positive bacterial isolates, *Enterococcus faecalis* was susceptible to amoxycillin, *Staphylococcus aureus* showed susceptibility to ofloxacin and gentamycin, and *Staphylococcus saprophyticus* demonstrated resistance to all antibiotics.

Investigations into the ethno-pharmacological claims made by the plants also demonstrated the effectiveness and potency of the plant extracts against specific microorganisms; zones of inhibition were identified and are thus shown in Table 3 below. Dimethylsulphoxide (DMSO) and distilled water were the two distinct solvents used to reconstitute the plant extracts, and each examined isolate responded differently to these dilutions (concentrations).

Table 4 lists additional investigated parameters, such as the extracts' minimum inhibitory concentration against particular isolates, expressed in milligrams per milliliter (mg/mL). The minimum inhibitory concentration (MIC) for *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and hot water extract varied from 2.5 mg/mL to 5.0 mg/mL, and for the methanolic extract, it was 10.0 mg/mL for all

tested isolates. However, no MIC was found for the ethanol extract.

Many studies have established the usefulness of medicinal plants as a great source for isolating active principles for drug formulation (Banso & Mann, 2000; El-Mahmud, 2007; Falodun *et al.*, 2006).

Table 1: the phytochemical constituents of *Acalypha wilkesiana*

COMPONENT	RESULT
Flavonoids	+
Tannins	+
Resins	-
Steroids	-
Anthraquinones	-
Saponins	+
Alkaloids	+

+ = Present, - = Absent

Table 2a: Antimicrobial susceptibility testing of the Gram-negative isolates.

Organism	Zones of inhibition produced by each antibiotic (Mean ± SD)							
	Cef	Cex	Gen	Cip	Ofl	Amg	Nif	Amp
Pa	-	-	12±0.4	15±0.6	15±1.2	-	10±0.1	-
Ec	-	-	18±1.5	11±0.5	11±0.4	-	21±0.2	-

NOTE: All readings are average of triplicate experiments; zones of inhibition are represented in millimeters (mm). Cef = Cefotaxime 30µg, Cex = Cefuroxime 30µg, Amp = Ampicillin 10µg, Nif= Nitrofurantoin 300µg, Gen = Gentamycin 10µg, Cip = Ciprofloxacin 5µg, Ofl = Ofloxacin 5µg, Amg = Amoxicillin 30µg, Pa = *Pseudomonas aeruginosa*, Ec = *Escherichia coli*. - = resistant

Table 2b: Antimicrobial susceptibility testing of the Gram-positive isolates.

Organism	Antibiotics (± SD)							
	Ctr	Ery	Cxc	Ofl	Aug	Cef	Cex	Gmn
Ef	-	-	-	-	-	17±0.1	19±0.3	-
Sa	-	-	-	24±0.3	-	-	-	15±0.5
Ss	-	-	-	-	-	-	-	-

All readings are average of triplicate experiments; zones of inhibition are represented in millimeter (mm). Cef = Cefotaxime 30µg, Cex = Cefuroxime 30µg, Amp = Ampicillin 10µg, Ntn= Nitrofurantoin 300µg, Gmn = Gentamycin 10µg, Cpx = Ciprofloxacin 5µg, Ofl = Ofloxacin 5µg, Aug = Amoxicillin 30µg, Sa = *Staphylococcus aureus*, Ss = *Staphylococcus saprophyticus*, Ef = *Enterococcus faecalis*. - = resistant,

Table 3: Antimicrobial susceptibility of test isolates to crude extracts

Org	Zones of Inhibition produced by the various concentrations of the extracts												Control		
	Ethanol				Methanol				Hot water				E	D.S	
	100 (mg/m L)	50 (mg/m L)	25 (mg/m L)	12.5 (mg/m L)	100 (mg/m L)	50 (mg/m L)	25 (mg/m L)	12.5 (mg/m L)	100 (mg/m L)	50 (mg/m L)	25 (mg/m L)	12.5 (mg/m L)			
Pa	22±0.0	17±0.5	-	-	-	-	-	-	-	-	-	-	-	-	-
Ef	-	-	-	-	23±0.9	-	-	-	23±0.6	-	-	-	-	-	-
Ec	25±0.3	15±0.4	13±0.9	13±0.3	18±0.2	-	-	-	23±0.5	17±0.3	13±0.0	15±0.4	-	-	
Sa	27±0.4	17±0.5	17±0.3	15±0.1	22±1.2	17±0.5	15±0.0	15±0.2	22±0.2	21±0.0	16±0.2	15±0.5	-	-	
Ss	13±0.3	-	-	-	-	-	-	-	-	-	-	-	-	-	

All readings are average of triplicate experiments and are represented in millimeter (mm). Pa = *Pseudomonas aeruginosa*, Ef = *Enterococcus faecalis*, Ec = *Escherichia coli*, Sa = *Staphylococcus aureus*, Ss = *Staphylococcus saprophyticus*, -= resistant, E = Ethanol and DS = Distilled water

Table 4: The Minimum Inhibitory Concentration (MIC) of methanolic, ethanolic and hot water extracts of *A. wilkesiana* leaves against selected isolates

Organism	Concentration (mg/mL)				MIC
	10.0	5.0	2.5	1.25	
Ethanol					
Pa	+	+	+	+	ND
Ef	+	+	+	+	ND
Ec	+	+	+	+	ND
Sa	+	+	+	+	ND
Ss	+	+	+	+	ND
Methanol					
Pa	-	+	+	+	10.0
Ef	-	+	+	+	10.0
Ec	-	+	+	+	10.0
Sa	-	+	+	+	10.0
Ss	-	+	+	+	10.0
Hot water					
Pa	-	-	-	+	2.5
Ef	-	-	-	+	2.5
Ec	+	+	+	+	ND
Sa	-	-	+	+	5.0
Ss	+	+	+	+	ND

All readings are average of triplicate experiments, ND = Not detected.

DISCUSSION

According to studies conducted by Marwah et al. (2007) and Shurwalker et al. (2004), a number of *Acalypha* species have been discovered to have antibacterial, antifungal, antitrypanosomal, wound-healing, antioxidant, and post-coital antifertility characteristics. The results of the study support *A. wilkesiana*'s antibacterial activities as a broad-spectrum antimicrobial agent since the plant inhibited the growth of both gram-positive (*S. aureus*, *S. saprophyticus*, and *E. faecalis*) and gram-negative (*E. coli* and *P. aeruginosa*) bacteria (Marwah et al., 2007 and Shurwalker et al., 2004).

A. wilkesiana leaf extracts in methanolic, ethanolic, and hot water extracts showed activity against most test organisms, which is a major step forward in our understanding of the plant's potential medicinal uses, especially for nosocomial-associated infections and community-acquired infections. Furthermore, the finding that some organisms were resistant to its action supported Oluremi et al. (2010) assertion that resistance to antimicrobial drugs cannot be completely eradicated but can only be reduced.

This finding corroborates the findings of Onocha & Olusanya (2010), who demonstrated that only *S. aureus* and *E. coli* were inhibited by the methanolic, ethanolic, and hot water extracts of *A. wilkesiana*. The investigation also revealed that only *S. aureus* and *E. faecalis* were inhibited from all the tested bacteria. This correspond with Oladunmoye's (2006) results, which demonstrated that *S. aureus* was suppressed by the methanolic extract of this plant. Notably, despite *S. aureus* resistance to cefuroxime, amoxicillin, erythromycin, ceftriaxone, and ceftazidime, the ethanolic extract was effective against the bacterium.

While the hot water extract had the lowest minimum inhibitory concentration (MIC) value of 2.5 mg/mL on two of the five test bacteria, the methanolic extract showed the same value of 10 mg/mL for all tested isolates, according to the current investigation. This implies that the aqueous fraction, or hot water, had a greater effect in destroying the germs. The remarkably low minimum inhibitory concentration (MIC) of 5.0 mg/mL for the hot water extract for *S. aureus* indicates an exceptionally high resistance level. Additionally, this resistance characteristic was shown by the organism's response to the test antibiotics. Furthermore, this may be due to their ability to produce extracellular enzymes that facilitate the breakdown and metabolism of the substrate, allowing the extract to function as a food source for the bacteria instead of inhibiting their growth after degradation has made the material nontoxic (Tortora et al., 2002). The results also showed a difference between the methanolic, ethanolic, and hot water extracts and the standard antibiotics; the latter were able to inhibit the growth of organisms (*Pseudomonas aeruginosa* and *Enterococcus faecalis*), whereas the former was not. The extracts' or its fractions'

mixtures of bioactive components, as opposed to the pure compounds found in the standard antibiotics, may be the reason for the variations in the extracts' activity compared to the standard antimicrobial treatment (Gatsing et al., 2010). The scientific underpinnings for the local reliance on this plant for the treatment of various illnesses may come from its demonstration of activity against such test germs.

Of the fractions used in this experiment, the hot water fraction of the extract was found to be the most beneficial. The fraction exhibited the highest level of bacterial inhibition out of all the others. These guidelines were only broken by *Staphylococcus saprophyticus* and *Escherichia coli*.

CONCLUSION

The plant's ability to exhibit action against both Gram-positive and Gram-negative bacteria suggests that it may contain a variety of bioactive chemicals with a broad range of potential uses. The plant may be a source of exceptionally potent antibiotic chemicals that can be utilized to treat drug-resistant infections, as evidenced by its potency against laboratory isolates. Research on this plant has shown great therapeutic promise, so perhaps the hunt for new drugs to combat the issues caused by bacterial strains with increased resistance has started to pay off. Compared to synthetic antibacterial medications, it can provide the intended result with fewer side effects. Plants, the sleeping giants of the pharmaceutical industry, provide an unlimited supply of natural medications for treating ailments and annoyances caused by microbial attacks.

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