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Antibiotic Susceptibility of Bacterial Isolates Exposed to *Anacardium occidentale* (Cashew) Ethanol Leaf Extract

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Abstract
The antibiotic susceptibility pattern of some bacterial isolates obtained from Rufus Giwa Polytechnic student hostel environment was evaluated and compared with the ethanol leaf extract from Anacardium occidentale using standard methods. The isolates were identified as *Bacillus subtilis, B. sphaericus, Klebsiella spp*, Coliform, Pseudomonas aeruginosa, Serratia marcescens, Staphylococcus aureus, and Streptococcus pyogenes. The highest total average bacterial count was recorded on Tryptose Soy agar from grey water and surface swab to be 3.28 x 105 and 3.89 x 105 cfu/ml respectively, while isolates on Blood agar samples recorded the lowest average count from grey water and surface swab (3.15 x 102 and 3.15 x 105 cfu/ml). However, bacterial isolates on MacConkney agar, Mannitol Salt Agar, and Pseudomonas agar also recorded significantly high bacterial counts of (4.45 x 105 cfu/ml and 4.56 x 105), (4.65 x 102 and 4.24 x 102) and (4.13 x 103 and 4.02 x 105) cfu/ml from grey water and surface swab respectively. The inhibited sensitivity around the extract ranged around 9.25 and 11.57 mm cleared zone. This inhibitory extract showed a similar antimicrobial activity on Staphylococcus aureus and coliform representing a broad-spectrum effect on gram positive and gram- negative bacteria respectively. Staphylococcus aureus also showed an inhibitory zone ranging from 5.52±0.29 to 12.2±0.53 while coliform ranged from 5.91±0.35 to 12.6±0.45 respectively corresponding to the increase in the concentration. Tin layer chromatography showed retention factor index includes 0.97, 0.91, 0.81, 0.56, 0.23 and 0.12. The obtainability and easy access to Anacardium occidental makes it cheap as an alternative medicine in comparison in term of result to commercially available antibiotics that has develop resistant to most pathogens. Screening and documentation of the potent constituent in the extract is important to further concentrate for better inhibitory effectiveness

Keywords: Coliform, Tin Layer Chromatography, Zone of Inhibition, Anacardium Occidentale, Antibiotic

Introduction

One of the major growth requirement for bacteria are warmth and moisture. These enhance their exponential reproduction per relative time depending on the species and strains of the bacteria. A bacterium could multiply into a million in less than 24 hours. Precise and decisive pathogens identification and detection, is vital for accurate disease diagnosis, disease management, and outbreaks monitoring and tracing. Bacterial identification is used in a wide variety of applications including forensics, criminal investigations, bio-terrorism threats, and environmental studies (Ashelford et al**.,** 2015). The plant *Anacardium occidentale L*. as been identified to be associates of the family of *Anacardiaceae*, indigenously related and found in the tropical regions. The cashew apple has been studied to be rich in carotenoids, tannins, and organic acids, which are all having antimicrobial potentials, insecticidal, and pharmacological activities (Santos & Mello, 2003). The pathogenic bacteria in the formation of a geographical location over a geographical period could inform the endemic and possible epidemic diseases in the lives of people of that area. The resistance and sensitive pattern of such pathogens to antibiotics and aborigine plants with a therapeutic index may correspond to the survival of locals. Hence, it becomes imperative to understudy the types of pathogens humans are prone to by exposure to contaminated environments. Findings from this research will provide useful information on the identity and prevalence rate of bacteria associated with the hostel environment of Rufus Giwa Polytechnic Owo and their sensitivity to antimicrobial agents.

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Aim and Objectives of the Study

This study aimed to investigate the antibiotic susceptibility of bacterial isolates obtained from the student hostel environment of Rufus Giwa Polytechnic, Owo, and to evaluate the antibacterial efficacy of ethanol leaf extract of *Anacardium occidentale* against the isolates. The objectives of this study were to:

1. isolate and identify bacterial species present in grey water and surface swab samples collected from the student hostel environment;
2. determine the bacterial load associated with the different culture media used for isolation;
3. evaluate the antibiotic susceptibility patterns of the bacterial isolates using ciprofloxacin as a reference antibiotic;
4. assess the antibacterial activity of ethanol leaf extract of *Anacardium occidentale* against the identified bacterial isolates at different concentrations;
5. compare the inhibitory effects of the plant extract with those of the standard antibiotic;
6. determine the antibacterial spectrum of *Anacardium occidentale* ethanol leaf extract against Gram-positive and Gram-negative bacteria;
7. characterize the bioactive constituents of the ethanol leaf extract using thin-layer chromatography (TLC)

Materials and Methods

Sample collection

A sample numbers of one hundred and seventy- seven (177) isolates were collated around the 8^{th} -9^{th} hour of the morning via a normal saline moistened sterile swab sticks to guarantee firm attachment of microbes to it and sterile pipette. The swab sticks were used to scoop the grey areas, and bathroom floors, while the pipette was used to collect grey waters into sterile sample bottles. This was collected in various locations within hostel environment. All the collected samples were transferred to the lab on ice bag containing coolants.

Culture media preparation

All the culture media used in this study were prepared in accordance with the manufacturer's specifications. These includes Nutrient agar, MacConkey agar, Blood agar, Mannitol salt agar, and Tryptose soy agar. They were separately dissolved and prepared according to the manufacturer's description using distilled water (100ml). The agar solutes were totally dissolved by incubation at a temperature of 50^{o}C over a period of 45 minutes. The conical flask cotton plugged and enclosed with aluminum foil. The media were further autoclaved at 121^{o}C for 15 minutes.

Classification of isolated bacteria

All samples were analysed within an hour of collation. The samples were serially diluted using sterile distilled water to reduce the microbial population before inoculations into the various agar respectively and incubated aerobically at 37°C for 24 to 48 hr. Growth of bacterial colonies were spotted and verified by standard bacteriological techniques as described by Cheesbrough, (2010). Distinct colonies were isolated and stored on agar slants at refrigeration temperature as described by Fawole & Oso, (2007).

Cultural Documentation

Distinct bacterial colonies were characterized based the following morphological appearance; colour, shape, pigmentation, and opacity within a day of incubation. Further classification were documented via microscopic evaluation following their various staining reaction. This include Gram staining procedure (Brooke et al., 2012), Capsule staining procedure and Spore staining, (Cheesbrough, 2010), biochemical tests (Olutiola et al., 2011; Fawole & Oso 2007); and comparison of probable bacterial isolates was classified according to Cowan & Steel (2002) and Cheesbrough, (2010).

Preparation of plant extracts

Anacardium occidentale leaves plant were harvested in the early hours of the day. It was well washed and allow to dried at room temperature, before powdered. A 50g pulverished leave samples was saturated for three days using absolute ethanol for the extraction. The solution was sieved using a muslin cloth (Asoso et al., 2016). The supernatant was evaporated and concentrated using the rotary evaporator. The evaporated concentrates was recorded as 100% stock concentration. It was stored at refrigeration temperature inside an airtight covered universal sample bottle. It was further dissolved and graduated with 30% Tween-20 to obtain 50, 100, 200, and 300 mg/ml respectively and were all sieved via a 0.45µm membrane filter to enable desolation of the crude extracts.

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Antibacterial assay of plants extracts

The antibacterial activity of the extracts was tested on the bacterial isolates using the agar well-diffusion method. The bacterial isolates to be tested were cultured for 18-24h in nutrient broth at 37ºC. The broth cultures were prepared and spread on a freshly prepared plate of Mueller Hinton agar. These plates were left for 40 minutes for the test bacterial isolates to be well embedded and acclimatized in the seeded medium. Four wells of equal depth were bored with the aid of a 6 mm sterile cork borer in each of the agar plates. About 0.5 ml of 50 mg/ml of the reconstituted extracts was introduced to the agar wells. The negative control for the experiment was 30% aqueous Tween-20, while 5mg/ml of Ciprofloxacin was used as the positive control. All the plates were incubated at 37ºC for 24 hr. Each treatment was replicated three times and the sensitivity of the test bacterial isolates to each of the extracts was evident by the appearance of a clear zone of inhibition around each agar well. This procedure was repeated for 25 mg/ml, 50mg/ml, 100 mg/ml, and 200mg/ml concentrations of the extracts. The diameter of each of these zones of inhibition was measured with a transparent ruler and used as an index to ascertain the degree of sensitivity of test bacterial isolates to each of the plant extracts evaluated (Asoso et al., 2016).

Characterization of bioactive components of *Anacardium occidentale* Via Thin-Layer Chromatography (TLC)

This method was used to detect the various solute organic compounds present in the plant samples. Cellulose acetate paper was marked with a pencil and ethanol extract of *Anacardium occidentale* was dotted on the cellulose acetate paper. This was further immersed into ethanol solvent to the 1 cm pencil marked line on the paper and capillary action was monitored with the mobile phase of the solvent and the various molecular bands. The cellulose paper acetate was further put in an iodine chamber to enhance the visibility of the bands. The fraction of the distance travelled by solute and Distance travelled by solvent dives (Rf). The result was compared with compound with similar Rf values as described by Igwe et al. (2015) and Bob et al. (2016).

$$Rf = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

Results

Table 1 Types and Loads of Bacterial isolates and Antibiotic sensitivity patterns

All the isolated bacteria exhibited diverse biochemical reactions and were categorised morphologically via microscopy into colour, stains retention, capsular and spore formation, cocci and bacilli. The isolates were identified as *Bacillus subtilis, B. sphaericus, Klebsiella spp, Coliform, Pseudomonas aeruginosa, Serratia marcescens, Staphylococcus aureus*, and, *Streptococcus pyogenes*. (Table. 1). The isolation of bacteria isolates able to form capsule is a significant indication of the virulence of the pathogens the students are been exposed to. This may be a determinant of bacteraemia upon infection. The presence of spore formers may also associated with inhalation and consequently pulmonary infection.

Table 2 reveals the total average counts of bacteria isolated from the Campus hostel in RUGIPO. The highest total average bacterial count was recorded on Tryptose Soy agar from grey water and surface swab to be 3.28 x 10^5 and 3.89 x 10^5 cfu/ml respectively, while isolates on Blood agar samples recorded the lowest average count from grey water and surface swab (3.15 x 10^2 and 3.15 x 10^5 cfu/ml). However, bacterial isolates on MacConkney agar, Mannitol Salt Agar, and Pseudomonas agar also recorded significantly high bacterial counts of (4.45 x 10^5 cfu/ml and 4.56 x 10^5), (4.65 x 10^2 and 4.24 x 10^2) and (4.13 x 10^3 and 4.02 x 10^5) cfu/ml from grey water and surface swab respectively. Tables 3 showed antimicrobial resistant forms of the isolates to ethanol extracts of *Anacardium occidentale at* graduated concentrations. This ethanolic extract showed a similar antimicrobial influence on *Staphylococcus aureus* and *coliform* representing a broad spectrum effect on gram- positive and gram -negative bacteria respectively. *Staphylococcus aureus*, a gram positive bacteria showed sensitivity with zone of inhibitory ranging from 5.52±0.29 [a] to 12.2±0.53 while *coliform* representing the group of gram negative bacteria was ranged from 5.91±0.35 to 12.6±0.45 [b] respectively corresponding to the increase in the concentration. Ciprofloxacin was the most potent antibiotic agent on the isolates. The zone of inhibition ranged from 9.25±0.31 to 11.57±0.30 mm. Plate 1 shows the TLC result of the *Anacardium occidentale.* The tin layer chromatography analysis plate of the solvent system of ethanol in Table 4.4 shows the retention factor by the crude and fractions of each extracting solvents value on a pre-coated thin layer chromatographic paper. The retention factor index includes 0.97, 0.91, 0.81, 0.56, 0.23 and 0.12. The closer the retention value is closer to 1, the higher the retention factors of these extracts justify the inference that the active metabolites present in the plants have different better affinities for the solvents used.

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Table 1: Morphological and biochemical characteristics of the bacterial isolates

Media	Colour morphology	GS	SP	CS	CP	OX	CG	CT	L	S	G	Man	M	Gal	Probable Organism
BA	non hemolytic	-	-	Rod	+	-	-	+	+	-	+	-	+	-	*Klebsiella sp*
MA	Redish pink	-	-	Rod	+	-	-	+	-	-	+	+	+	+	*Coliform*
BA	Greyish white β haemolytic	+	-	Cc	+	-	-	+	+	+	+	+	+	+	*Streptococcus spp*
MA	Dark Red	+	-	Rod	+	-	-	+	+	+	+	+	+	+	*Serrattia marcescens*
NA	Greenish discoloration	-	-	Rod	+	+	-	+	-	-	-	-	-	-	*Pseudomonas aeruginosa*
TSA	White-creamy circular shape, flat, irregular margin	+	+	Cc	-	-	-	+	-	-	+	+	-	-	*Bacillus subtilis*
MSA	Yellow colonies / discoloration	+	-	Rod	+	-	+	+	+	+	+	+	+	+	*Staphylococcus aureus*
BA	Grey α haemolytic smooth, circular	+	+	Rod	+	+	-	+	-	+	+	-	-	-	*Bacillus sphaericus*

KEY: -:BA: Blood Agar, M.A.: MacConkey Agar, NA: Nutrient Agar, TSA: Tryptose Soy Agar, MSA: Mannitol Salt Agar, Negative to the test, +: positive to the test, Cc- cocci shape, GS-gram staining, SP- spore staining, CS-cell shape, OX- oxidase test, CG- coagulase test, CT- catalase test, L- lactose, S- sucrose, G-glucose, Man- mannitol, M- maltose, Gal- Galactose

Table 2: Total bacterial counts of samples collected from Campus hostel in RUGIPO

Media	Average Bacterial counts from grey water (cfu/ml)	Average Bacterial counts from surface swab (cfu/ml)
Tryptose Soy agar	3.28×10^5	3.89×10^5
Blood agar	3.15×10^2	3.15×10^5
MacConkney agar	4.45×10^2	4.56×10^5
Mannitol Salt Agar	4.65×10^2	4.24×10^5
Pseudomonas agar	4.13×10^3	4.02×10^5

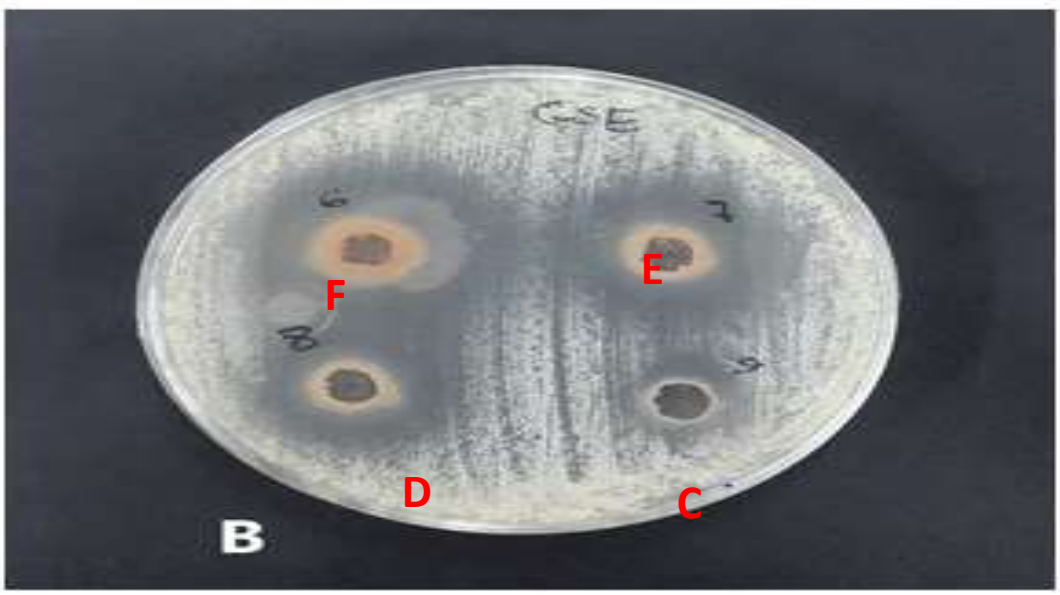

Plate 1: *In-vitro* sensitivity test of bacterial isolates treated with varying concentrations of *Anacardium occidentale* ethanol leaf extract (x10)

Keys:

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A= Ciprofloxacin (5mg/ml)
B= Sterile water
C= Concentration of *Anacardium occidentale* ethanolic extract (25 mg/ml)
D= Concentration of *Anacardium occidentale* ethanolic extract (50 mg/ml)
E= Concentration of *Anacardium occidentale* ethanolic extract (100 mg/ml)
F= Concentration of *Anacardium occidentale* ethanolic extract (200 mg/ml)

Table 3: Sensitivity patterns of bacterial isolates to Ethanol leaf extracts of *Anacardium occidentale leaves*

	25 mg/ml	50 mg/ml	100 mg/ml	200 mg/ml	Positive control	Sterile distil water
Coliform	5.91±0.35 [a]	7.39±0.53 [a]	10.99±0.45 [b]	12.6±0.45 [b]	10.33±0.24[d]	0.00
Staphylococcus aureus	5.52±0.29 [a]	7.25±0.28 [a]	10.11±0.59 [b]	12.2±0.53 [b]	11.50±0.21[c]	0.00

KEY: Positive control= Ciprofloxacin (5mg/ml)

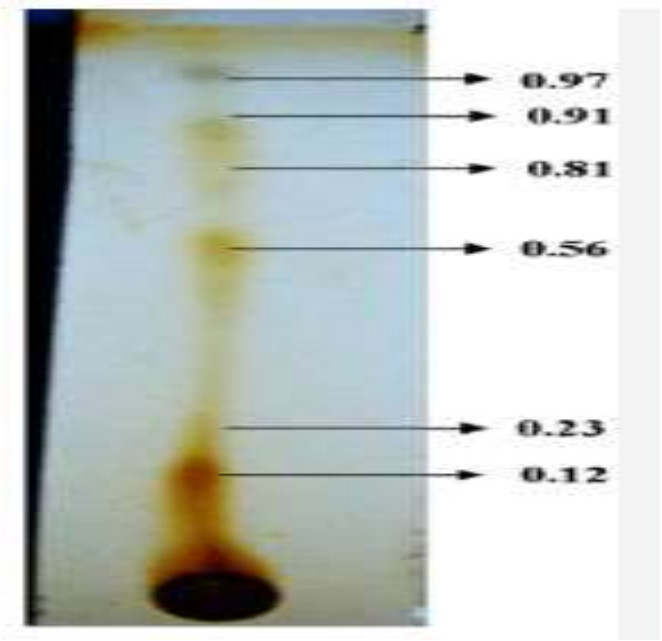

Plate 2: Thin-Layered Chromatography of *Anacardium occidentale* ethanol leaf **extracts**

Table 4 Retention factor (RF) values of ethanol leaf extract of *Anacardium occidentale*

S/N	Retention factor values
1	0.97
2	0.91
3	0.81
4	0.56
5	0.23
6	0.12

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Discussion

The number of pathogens isolated and identified can be very virulent on infections with entrance into the blood streams. The high bacterial count and variations in the bacterial count recorded in this study shows how contaminated the hostels are and how prone to infection the students are. The variations in microbial load from different locations may be attributed to the variances in ecological extrinsic factors i.e. water activity, pH, redox potential, and nutrient content.

Grey water are known to harbour human pathogens, culture and molecular-based work has shown that it is a reservoir for several pathogens, this research reveals that several pathogenic bacteria were associated with Campus hostel in RUGIPO such as; *Serratia marcescens, Pseudomonas aeruginosa, Bacillus subtilis, B. sphaericus, Coliform, Klebsiella spp, Staphylococcus aureus* and, *Streptococcus sp,*. This data corroborates the findings of Nkengfack et al. (2016) who reported variations in the number and species of these bacteria isolated from different hostels across different sample locations. Hence, the implication of location on the variation of the bacterial isolates cannot be overemphasised. The increased occurrence of *Bacillus sphaericus, Bacillus subtilis, Coliform, Klebsiella spp, Pseudomonas aeruginosa, Serratia marcescens, Staphylococcus aureus,* and, *Streptococcus sp,* observed in this study suggest that this family of bacteria are pathogens naturally existing in the grey water.

Ethanolic crude extract obtained from the pulverized leaves of *Anacardium occidentale* retains significant antimicrobial activity against bacterial contaminants, this is corroborated by the findings of Agedah et al. (2010). These zones of inhibition showed clearer zone in *Staphylococcus aureus* plate in comparison with coliform plates. However**,** the plant extract exhibits a broad spectrum inhibition since it inhibited both the coliforms and *S. aureus*, alike in terms of zone of inhibition. The more the concentrates of the ethanolic extract, the better and more distinct the zone of inhibition in comparison with the commercial antibiotic, ciprofloxacin as the positive control. At 25 mg/ml, 50 mg/ml, 100 mg/ml, and 200 mg/ml, ethanol extracts of the *Anacardium occidentale* gave visible zones of inhibition against both gram positive and negative bacteria. Also, the zones of inhibition produced by the extract varies from one isolate to another as observed by Morton, (2018). The variation in the zone of inhibition observed among the isolates has been reported in the work of Ayinla et al. (2011) in which a lower concentration of leaf extracts was used and derived smaller zones of inhibition. The variations in the zone of inhibition of the extracts on the isolates can be directly associated with the organism's susceptibility to the antibacterial components present in the extracts. This justifies the assertion that antimicrobial agent's activity are more distinct at higher concentrates than at lower concentrates (Ayinla et al., 2011). The antimicrobial effect of the various crude concentration extracts were similar to those of standard antibiotics.

The retention factor in Plate 2 showed six bands which indicates six compounds with different at different mobile capillary rate. Some of these compounds will be responsible as the active ingredient responsible for the antimicrobial properties. Each of this compound can be isolated, purified and tested independently and synergistically to know the compounds or group of compounds responsible for the antimicrobial effects. The distinct zone of inhibition against the test bacteria suggests the possibility of alternative antibiotic substances of plants origin (leaves of *Anacardium occidentale*) for the advancement of newer antibacterial agents. In general, the leaves, roots, barks, and seeds of plants has been studies to have an enormous range of indigenous therapeutic applications, from antipyretic, laxative, analgesic, antifungal, antibacterial, to the non-inflammatory potentials. Also, this research provides some scientific justification for the utilization of extract from leaves to treat contagious diseases. However, it is important to note that these crude extracts need to be further purified through some quality control techniques and antibacterial active ingredients of the leaves needs to be fractionated in order to screen, isolate and identify the compounds responsible for antibacterial activity.

Conclusion

This study suggests that grey water in the gully system and those that litters our environments play a major role in the harbouring of viable potential disease causing bacteria. Almost all the isolated bacteria showed sensitivity to both the conventional antibiotics and crude ethanolic filtrate from leaves of *Anacardium occidentale*. In addition, this research also reveal the common bacterial pathogen often in contact with students in campus living environment. *Staphylococcus aureus* was the most profound in the environment and can cause notable food borne infections and intoxication which can lead to bacteraemia and toxigenicity. Other isolates such as *Bacillus spp, Coliform, Klebsiella spp, Salmonella spp, and Pseudomonas spp,* are also indicator index for infection, endemic and possible epidemic if not properly managed.

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Recommendations

1. Results from this study have shown the presence of pathogenic bacteria that can be found in the student hostels vicinity, suggesting a looming health hazard. It is therefore necessary to put more emphasis on the hygienic practices.
2. The surveillance of potential pathogenic organisms in the campus hostels environment should also be given more attention; this can be achieved by the regular monitoring of the public health personnel sanitation procedures. Negligence of these facts can cause serious illnesses, and endemic and epidemic of diseases among students.
3. The results of the antimicrobial activity of *Anacardium occidentale* indicate the antibiotics potential of the leaf extract as a panacea for drug development from plant origin in the management of diseases caused by antibiotic resistance pathogenic strains.
4. The obtainability and accessibility of *Anacardium occidental* makes a cheap substitute indigenous medicine in the absence of expensive conventional antibiotics that bacterial strains now showed resistance towards.
5. Additional screening and refining of the fractionated filtrate and documentation of the potent component is essential to boost better efficacy.
6.

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